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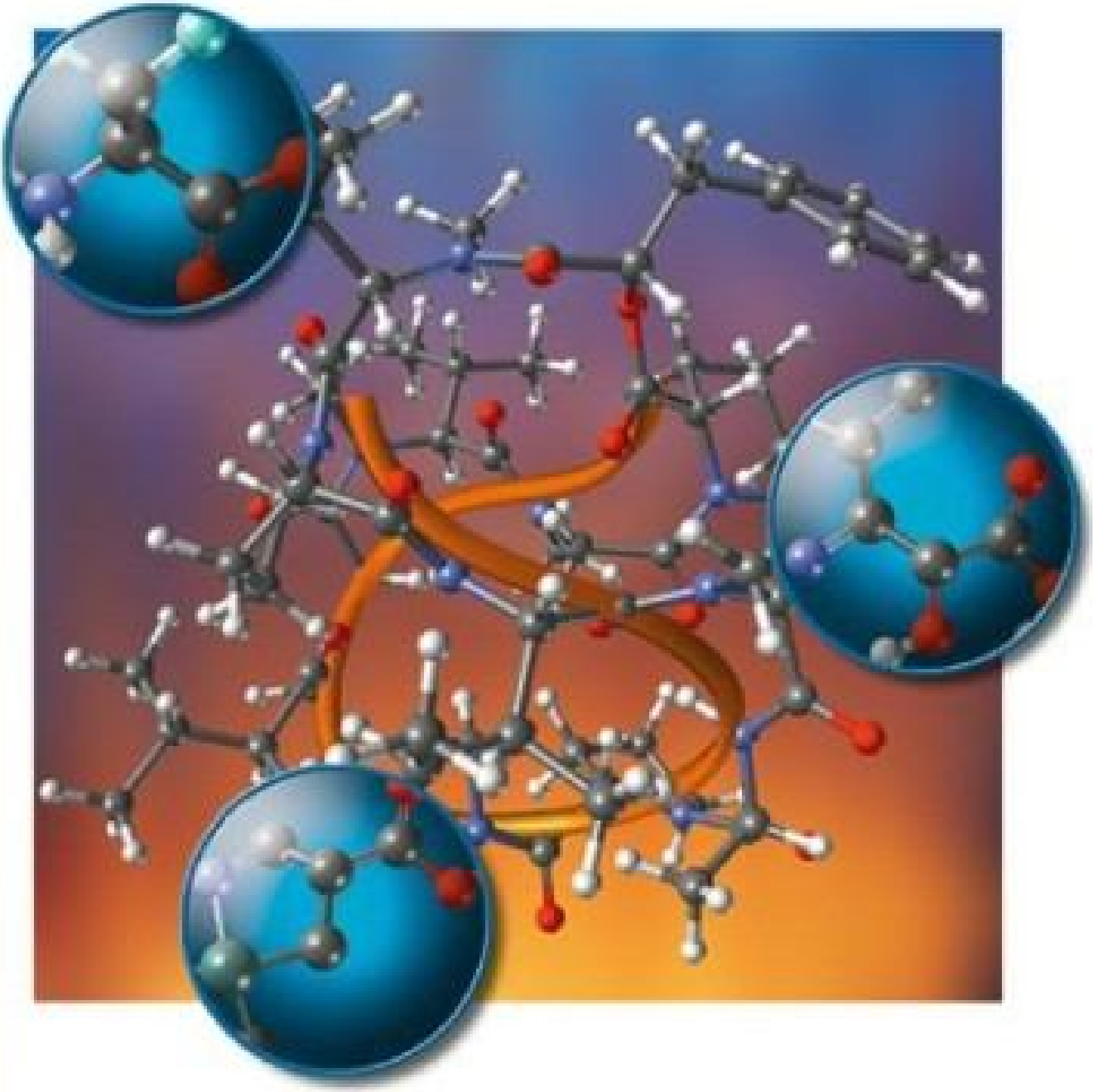
Amino Acids, Peptides and Proteins in Organic Chemistry

Volume 4
Protection Reactions, Medicinal Chemistry, Combinatorial Synthesis



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1
Protection Reactions

Vommina V. Sureshbabu and Narasimhamurthy Narendra

1.1
General Considerations

Peptides, polypeptides, and proteins are the universal constituents of the biosphere. They are responsible for the structural and functional integrity of cells. They form the chemical basis of cellular functions that are based on highly specific molecular recognition and binding, and are involved as key participants in cellular processes. A peptide or a protein is a copolymer of α-amino acids that are covalently linked through a secondary amide bond (called a peptide bond). They differ from one another by the number and sequence of the constituent amino acids. Generally, a molecule comprised of few amino acids is called an oligopeptide and that with many amino acids is a polypeptide (molecular weight below 10 000). Proteins contain a large number of amino acids. Due to the vitality of their role for the function as well as survival of cells, peptides and proteins are continuously synthesized. Biosynthesis of proteins is genetically controlled. A protein molecule is synthesized by stepwise linking of *unprotected* amino acids through the cellular machinery comprised of enzymes and nucleic acids, and functioning based on precise molecular interactions and thermodynamic control. Thousands of proteins/peptides are assembled through the combination of only 20 amino acids (referred to as coded or proteinogenic amino acids). Post-translational modifications (after assembly on ribosomes) such as attachment of nonpeptide fragments, functionalization of amino acid side-chains and the peptide backbone, and cyclization reactions confer further structural diversity on peptides.

The production of peptides via isolation from biological sources or recombinant DNA technology is associated with certain limitations *per se*. A minor variation in the sequence of a therapeutically active peptide isolated from a microbial or animal source relative to that of the human homolog is sufficient to cause hypersensitivity in some recipients. Further, the active drug component is often not a native peptide but a synthetic analog, which may have been reduced in size or may contain additional functional groups and non-native linkages. The development of a drug from a lead peptide involves the synthesis (both by conventional and combinatorial methods) and screening of a large number of analogs. Consequently, the major proportion of the

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demand for peptides is still met by chemical synthesis. Chemical synthesis is also crucial for synthesizing peptides with unnatural amino acids as well as peptide mimics, which by virtue of the presence of non-native linkages are inaccessible through ribosomal synthesis.

Synthetic peptides have to be chemically as well as optically homogenous to be able to exhibit the expected biological activity. This is typically addressed by using reactions that furnish high yields, give no or minimum side-products, and do not cause stereomutation. In addition, the peptide of interest has to be scrupulously purified after synthesis to achieve the expected level of homogeneity. The general approach to synthesize a peptide is stepwise linking of amino acids until the desired sequence is reached. However, the actual synthesis is not as simple as the approach appears to be due to the multifunctional nature of the amino acids. Typically, a proteinogenic amino acid (except Gly) contains a chiral carbon atom to which is attached the amino (α-amino), carboxy, and alkyl group (referred to as the side-chain). Gly lacks the alkyl substitution at the α-carbon atom. Also, the side-chains of many of the amino acids are functionalized.

A straightforward approach to prepare a dipeptide A–B would be to couple the carboxy-activated amino acid A with another amino acid B. However, this reaction will yield not only the expected dipeptide A–B, but also an A–A (through self-acylation) due to the competing amino group of A. The so-formed dipeptides can further react with A since they bear free amino groups and form oligopeptides A–A–B, A–A–A, or A–A–A–A, and the reaction proceeds uncontrollably to generate a mixture of self-condensation products (homopolymers) and oligopeptides of the type A_nB. The process becomes even more complicated when reactive functional groups are present in the side-chains of the reacting amino acid(s). The uncontrolled reactivity of multiple groups leads to the formation of a complex mixture from which it becomes a Sisyphean task to isolate the desired product, which would have been formed, mostly, in low yield. The solution to carry out peptide synthesis in a chemoselective way is to mask the reactivity of the groups on amino acids that will not be the components of the peptide bond prior to peptide coupling step. This is done by converting the intervening functional group into an unreactive (or less reactive) form by attaching to it a new segment, referred to as a protecting group (or protection or protective function). The chemical reactions used for this purpose are known as protection reactions. The protecting groups are solely of synthetic interest and are removed whenever the functional group has to be regenerated. In other words, the protection is *reversible*. In the light of the concept of protection, the steps involved in the synthesis of the above dipeptide A–B are depicted in Figure 1.1.

Protections are employed for α-amino, carboxy, and side-chain functional groups (Figure 1.2). Since peptide synthesis is a multistep and repetitive process, the longevity of different protecting groups on the peptide under synthesis varies. In the present and widely followed approach of assembling peptides, wherein the peptide chain extension is from the carboxy- to amino-terminus (C → N direction), the α-amino protection is removed after each peptide coupling step to obtain a free amino group for subsequent acylation and, hence, this protection is temporary. The carboxy and side-chain protections are generally retained until the entire sequence

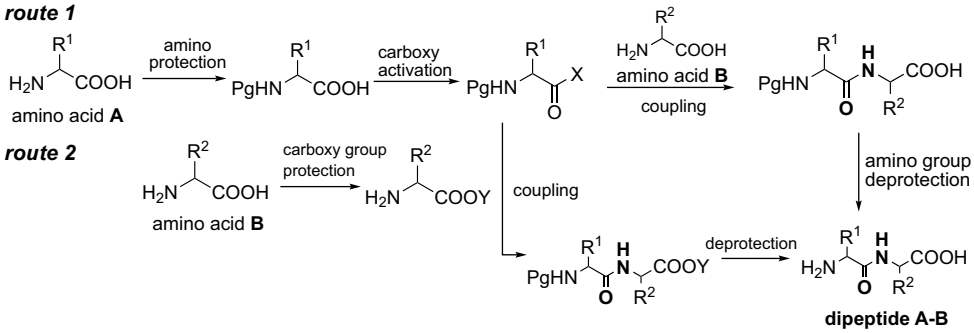


Figure 1.1 Illustration of synthesis of a dipeptide using α-amino and carboxy protections.

is assembled, and are removed simultaneously in a single step at the end of the synthesis. Hence, they can be regarded as semipermanent groups. The transient α-amino protection should be removed using reagents/conditions that do not affect the stability of semipermanent groups and, importantly, the newly assembled peptide bond(s). Consequently, it should be orthogonal to semipermanent groups with respect to its susceptibility to a particular cleavage reaction. Sometimes it may be required to remove only the carboxy protection or a particular side-chain protection in order to obtain a N^{α}-protected peptide acid or to regenerate a side-chain functional group (for site-selective peptide modification). In such cases, the α-amino and semipermanent groups have to be orthogonal to one another.

In practice, the orthogonality among protecting groups is achieved by either differential reactivities or different rates of reaction of protective units towards a particular cleavage reagent. The compulsion for the requirement of semipermanent groups can be lifted especially with respect to the protection of side-chain functionalities if there is no possibility of an undesired reaction from the unprotected group during coupling or deprotection of the α-amino group. Hence, the degree of protection can widely vary (from maximum to minimum) depending upon the synthetic design and the choice of chemistry.

An ideal protecting group should be quantitatively introduced and removed (desirably using mild reagents/conditions), should leave no residue nor form a byproduct that is difficult to separate from the product, should not be prematurely deblocked or modified during synthesis, and should not cause side-reactions including stereomutation. In addition, it should not influence the reactivity of the adjacent groups or, if it does, it should be in predictable ways.

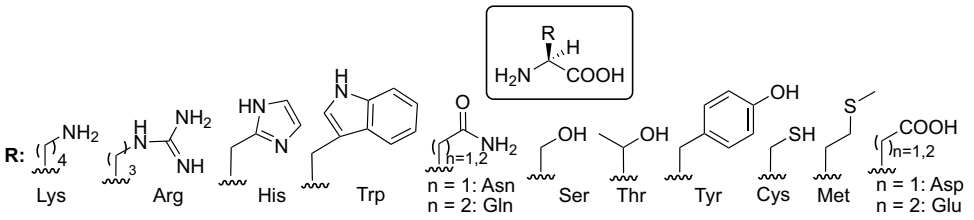


Figure 1.2 Side-chain functional groups of amino acids that entail protection.

In this chapter, various α-amino, carboxy, and side-chain protecting groups are presented. The general features of each type of protecting groups, methods of introduction and removal, and improved analogs are discussed. Typical and widely used preparative methods are mentioned under each category of protecting groups. The reader may refer to many earlier works for accounts on the development of protecting groups and for detailed discussions on different aspects of protecting group chemistry in peptide synthesis [1].

1.2 α-Amino Protection (N^{α} Protection)

The α-amino group is protected to reduce its nucleophilicity. In addition to the general properties of a protecting group, an ideal α-amino protection is expected to possess more properties unique to itself. Deblocking of the N^{α} protection should take place with a high degree of selectivity so that there will be no progressive loss of the semipermanent groups with repetitive deblocking steps as the peptide chain is elongated. The N^{α} protection should not sterically or electronically disfavor the reactions at the carboxy group by virtue of its proximity. It should not be involved or promote side-reactions, including those that lead to stereomutation. Further, it should form stable and crystallizable amino acid derivatives. Indeed, due to such stringent requirements for a α-amino protecting group, the success in the development of a good N^{α} protection has always been critical to progress in the development of efficient coupling methods and, in turn, to the overall growth of the field of peptide synthesis.

The α-amino protections are of different types and they can be categorized using different approaches. However, based on the criteria of the magnitude of the present utility of each type, the groups can be classified into non-urethane- and urethane-type *N* protections. Presently, the latter are the extensively used N^{α}-protecting groups for both solution and solid-phase peptide synthesis (SPPS) due to reasons that will be discussed later. The extent of the utility of the non-urethane-type amino protectors in peptide synthesis is currently comparatively lesser. Only a few groups of this category have been demonstrated to be efficient as N^{α}-protectors for general applications. Nonetheless, they are useful as protecting groups for side-chain functions as well as for the protection of the α-amino group for the synthesis of peptide mimics and unnatural amino acids. Their importance in peptidomimetic synthesis owes much to the vast diversity in chemistry required for accomplishing a wide range of backbone modifications of peptides leading to novel nonpeptidic molecules.

1.2.1 Non-Urethanes

1.2.1.1 Acyl Type

Reaction of amino acids with alkyl or aryl carboxylic acid derivatives yields *N*-acyl amines or amides. Acyl groups were the first generation of N^{α}-protecting groups used for peptide synthesis. The necessity for the protection of the α-amino group for

successful peptide synthesis was identified as early as 1900s by the two distinguished chemists of the time, Emil Fischer and Theodor Curtius, who mostly employed formyl (For), acetyl (Ac), and benzoyl (Bz) groups for this purpose. However, it was soon realized that the selective removal of these protections from peptides was not successful. The acyl groups present two synthetic difficulties in general – difficulty in the removal of the group without destroying the meticulously assembled peptide bonds and a high degree of racemization of N^{α}-acyl-protected amino acid derivatives. The only mode of deprotection of an acyl-protected amine is the fission of the acyl-nitrogen (-CO−NH-) bond. However, since the peptide bonds (secondary amides) are chemically similar to the amide bond (of the protective function), they are often simultaneously cleaved. Although selective removal of the N^{α}-acyl group has been attempted through special methods such as the enzymatic and CNBr-mediated cleavage of N-terminal Z-Arg and Met peptides, respectively, these protocols have not found widespread application. However, if the N^{α}-acyl group contains an electron-withdrawing substitution (e.g., CF_3CO-, trifluoroacetyl (Tfa) group), then the amide carbonyl of the protective function becomes more susceptible to nucleophilic substitution relative to the peptide carbonyl and thus the amino group can be selectively deprotected under acceptable conditions. Selectivity can also be achieved by using groups that can be modified (postcoupling) into units, which can be eliminated through processes such as lactam formation. Barring these examples, simple acyl groups do not find established applications as α-amino protections for conventional peptide synthesis. Nonetheless, the For protection can be attributed with a unique application. The N^{α}-formyl group of protected amino acid esters/amides and peptide esters **1** can be readily dehydrated into the isocyano group and the resulting α-isocyano esters/amides **2** can be used as key components to synthesize peptides and peptide libraries through multicomponent reactions (MCRs). MCRs have been shown to be particularly useful to assemble peptides linked by sterically hindered amino acids such as α,α-dialkylamino acids. For instance, an extremely difficult sequence **4** with three successive α,α-diphenylglycine (Dph) units has been assembled through a modified Ugi reaction of isonitrile **3** with Z-Dph-OH and diphenylmethanimine (Figure 1.3) [2]. Mild and racemization free conversion of N^{α}-For-protected amino acid and peptide derivatives into isonitriles can be carried out by the treatment with triphosgene in dichloromethane (DCM) at −75 to −30 °C (Figure 1.3) or Burgess reagent [3].

1.2.1.1.1 Monoacyl Groups

Trifluoroacetyl (Tfa) Group Tfa is of special interest as a monoacyl-type protecting group. Due to the negative inductive effect of the $-CF_3$ substitution, the trifluoroacetamides readily undergo hydrolysis in mild alkaline conditions to which peptide bonds and most carboxy esters are largely stable, not withstanding methyl and ethyl esters (which are susceptible to saponification). Optically pure N^{α}-Tfa-amino acids are prepared by treating amino acids with trifluoroacetic anhydride (TFAA) in anhydrous trifluoroacetic acid (TFA) solvent at −10 to + 10 °C [4]. The method can also be successfully used to obtain N^{α}-Tfa-Lys/Orn from Lys/Orn. The acidity of the

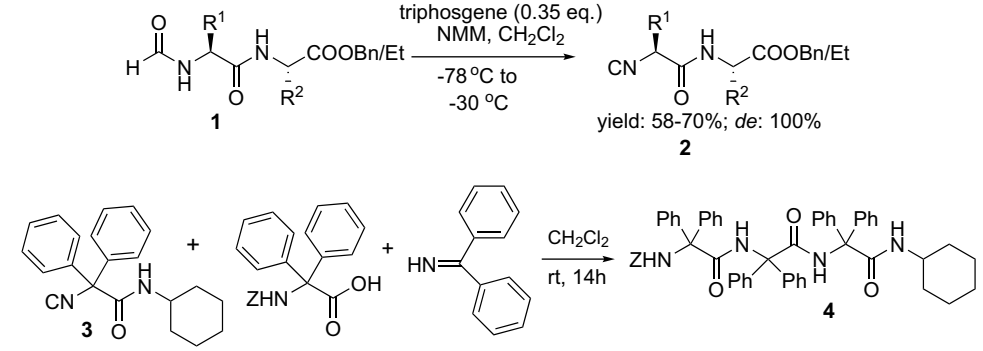


Figure 1.3 Synthesis of isocyanato peptides from *N*-For-protected peptide esters and the Ugi four-component reaction of α-isocyanato esters.

medium protonates the more basic ω-amino group of Lys/Orn into the ammonium form, which do not undergo acylation. However, the strong acidic condition is disadvantageous in the case of preparation of Tfa-Ser-OH and Tfa-Thr-OH as these hydroxy amino acids are dehydrated into unsaturated amino acids. Trifluoroacetylation can also be carried out using ethyl thioesters and phenyl/alkyl esters of TFA such as ethyl trifluoroacetate [4] or reagents such as 1-(trifluoroacetyl)imidazole. The N^{α}-Tfa group is cleaved by the action of 0.2 N NaOH [5] or $Ba(OH)_2$ or by dilute NH_3 solution. Piperidine [6] and $NaBH_4$ in EtOH can also be employed. The group is resistant to acids except for Tfa-Ser/Thr derivatives in which it is cleaved by mild acidic reagents. However, strong acidic conditions such as boiling methanolic HCl can cleave the group.

1.2.1.1.2 **Groups Cleavable via Lactam Formation** 2-(4,5-Dimethyl-2-nitrophenoxy)-2-methylpropionyl group **5a** and its phenyldiazenyl analog **5b** are introduced by the reaction of the corresponding acid chlorides with amino acids. Cleavage is accomplished in two steps (Figure 1.4). The first step is the reduction of the nitro group into an amino group by catalytic hydrogenation or catalytic transfer hydrogenation (CTH). Step 2 is the cyclization of the resulting amino compound **6** into a lactam **7** at neutral pH with concomitant elimination of the protected amine [7]. A similar process also cleaves **5b** [8]. Nevertheless, incomplete reduction and cyclization steps have been the major concerns for a broad application of these groups in spite of selective and acceptable cleavage conditions.

Racemization The high degree of racemization of N^{α}-acyl-protected amino acids has been attributed to the facile formation of optically labile azlactone intermediates

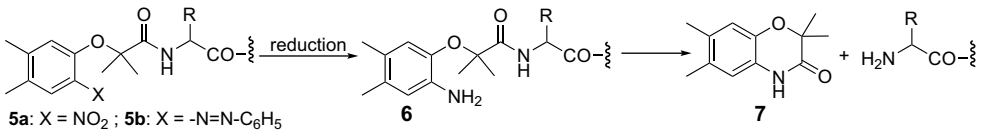


Figure 1.4 Cleavage of N^{α} protection via lactam formation.

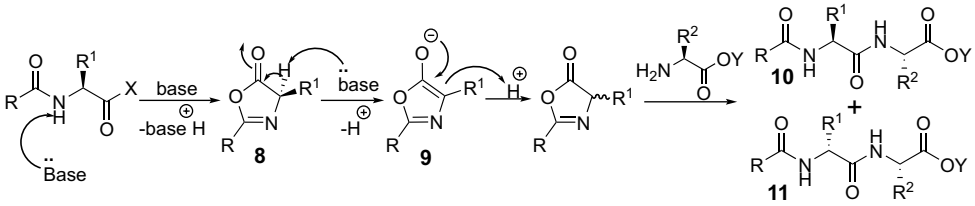


Figure 1.5 Racemization of N^{α}-acyl-α-amino acid derivatives.

8. Activated N^{α}-acyl amino acids readily undergo base-catalyzed ring closure to azlactones (2,4-disubstituted oxazol-5(4*H*)-ones). Enolization of the latter to oxazol-5-ol **9** in the presence of a base results in the loss of chirality at the α-carbon atom. Azlactones can acylate amines, but the resulting product will be a mixture of epimeric peptides (**10** and **11**, Figure 1.5). In the case of *N*-methyl-α-amino acids (NMAs), the oxazolium intermediate can be formed even in the absence of base, due to the electron-releasing effect of the *N*-alkyl substitution. Hence, N^{α}-acyl, N^{α}-alkylated amino acid derivatives are extremely sensitive to racemization during coupling. Base-catalyzed enolization of the activated amino acid derivatives with the abstraction of the α-proton also contributes to racemization.

Racemization can also take place during the introduction of N^{α}-acyl protection because of the *in situ* activation of the carboxy group by acid anhydrides and acid chlorides (used as reagents for acylation of α-amino group) followed by cyclization to azlactones. For instance, the N^{α}-Tfa-amino acids prepared by the treatment of amino acids with an excess of TFAA in the absence of TFA have been found to be contaminated with the D-isomer. This is due to the activation of Tfa-amino acids by TFAA to unsymmetrical or symmetrical anhydrides, which rearrange with racemization to the corresponding Tfa-azlactones.

1.2.1.1.3 **Diacyl Groups** Reaction of amino acids with 1,2-dicarboxylic acid derivatives yields imides that are stable to acids and also to hydrogenolysis, thus making the diacyl-type protection suitable for usage in diverse synthetic conditions. These groups are cleaved by nucleophilic substitution by hydrazine or thiols. The aromatic 1,2-dicarboxylic acid, phthalic acid, is employed for N^{α} protection, whereas the alkyl counterpart *N*-maleoyl group has been replaced by the dithiasuccinoyl (Dts) group.

Phthaloyl (Phth) Group N^{α}-Protected Phth-amino acids **12** are prepared under mild and racemization-free conditions by using phthaloylating reagents (Figures 1.6 and 1.7) such as *N*-(ethoxycarbonyl)phthalimide **13**, monoethyl phthalate **14** [9], and

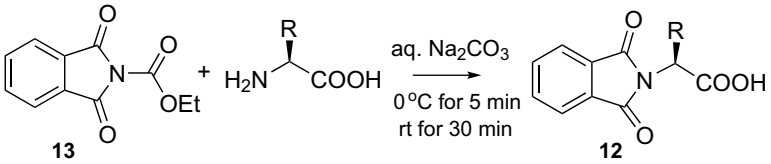


Figure 1.6 Preparation of N^{α}-Phth-amino acids.

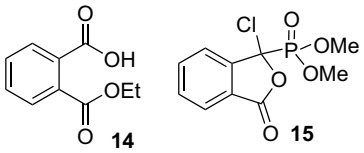


Figure 1.7 Phthaloylating reagents.

3-chloro-3-(dimethoxyphosphoryl)isobenzofuran-1(3*H*)-one **15** [10]. *N*-Phthaloylation by these reagents has almost completely replaced the original and harsh route of fusing amino acids with phthalic anhydride, which invariably caused racemization. An improvement in the method was achieved by using solvents such as benzene, dioxane, and so on, but could not overcome the racemization problem completely.

The N^{α}-Phth group is normally removed by means of hydrazinolysis by treatment with hydrazine hydrate in refluxing MeOH or EtOH [11]. Alternatively, a two-step procedure, which involves a reductive ring opening, followed by an acid-catalyzed lactonization of the resulting hydroxy compound (**17**) with concomitant fission of acyl-nitrogen bond, has also been developed (Figure 1.8) [12]. Interestingly, Phth protection cannot be removed by treatment with alkali. The alkali opens the five membered ring to a monoacyl amide of phthalic acid **18** (*O*-carboxybenzoyl amide) which is stable to hydrazine and to bases, thus representing an irreversible protection. Hence, saponification cannot be used as a method to cleave esters of N^{α}-Phth-protected peptide acids. On the other hand, treatment with $SOCl_2$ or methanolic HCl converts **18** back to phthalimide. In fact, this cyclization has been used as the basis for the development of a mild protocol for preparation of phthalimides. Tetrachlorophthaloyl group is an improved analog of Phth and can be removed under mild conditions by treatment with 15% hydrazine in *N*,*N*′-dimethylformamide (DMF) for 1 h at room temperature [13].

Groups Removed by Reductive Cleavage Dithiasuccinoyl (Dts) imides are stable to acids and to photolysis, and are cleaved by reductive thiolysis. N^{α}-Dts-amino acids **19** are prepared through a multistep route, which involves the reaction of the *tert*-butyl esters of amino acids with alkyldithiocarbonate or trithiodicarbonate to form

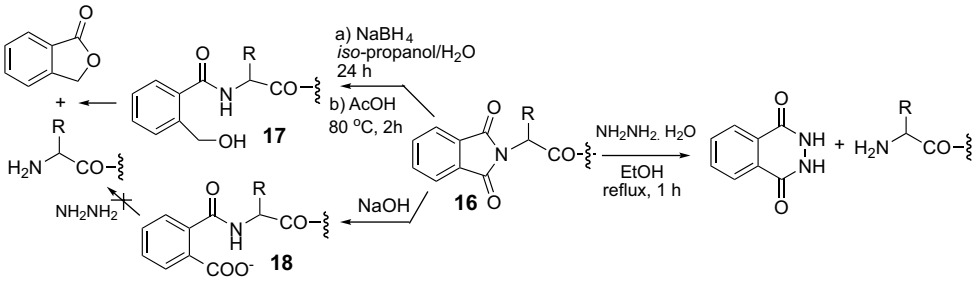


Figure 1.8 Cleavage of the N^{α}-Phth group.

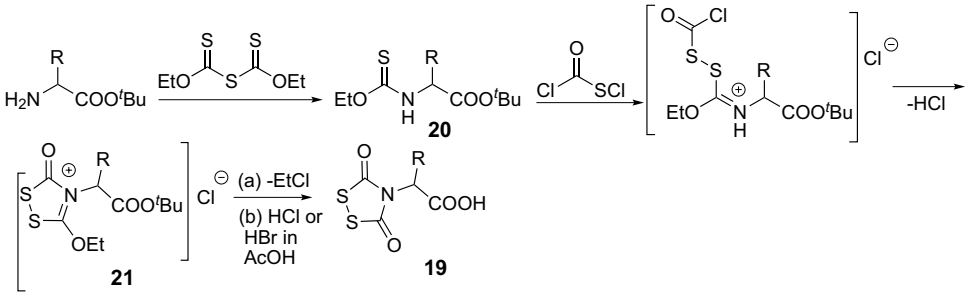


Figure 1.9 Preparation of N^{α}-Dts-protected amino acids.

N^{α}-ethoxythiocarbonyl amino acid esters **20**. The latter, upon treatment with chlorocarbonylsulfanyl chloride (Cl−CO−SCl), forms a cyclic intermediate **21**, which eliminates chloroethane to yield *N*-Dts-imides (Figure 1.9). The final step is the acidolytic cleavage of the *tert*-butyl ester [14]. Alternatively, a one-pot procedure based on the treatment of amino acids with polymeric poly(ethylene glycol) (PEG)-xanthane ($PEG\text{-}OCSSCH_2CONH_2$) has been developed [15], which also circumvents the generation of carbamate impurities by reaction with Cl−CO−SCl in the former method. Dts protection is cleaved within minutes by mercaptoethanol in DCM in the presence of triethylamine (TEA) or diisopropylethylamine (DIPEA) [16] (Figure 1.10).

The group can also be removed by using *N*-(methylsulfanyl)acetamide, trialkylphosphines and hydride donors. It is noteworthy that the Dts group is deblocked through a process initiated by nucleophilic attack on the sulfur atom adjacent to the amide carbonyl unlike the other acyl-type protections wherein the cleavage is due to nucleophilic attack at the amide carbonyl. Hence, in the case of this acyl-type protection, a cleavage reagent can selectively act at the protection unit and not at peptide bonds.

The N^{α}-(alkyldisulfanyl)carbonyl groups **22** and **23** are cleaved similarly by thiols and trialkylphosphines (Figure 1.11) [17]. Hence, these groups represent useful monoacyl-type protections.

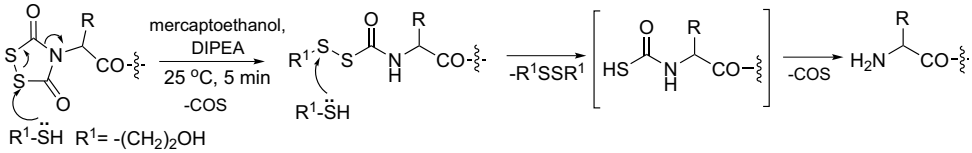


Figure 1.10 Thiolytic cleavage of N^{α}-Dts protection.

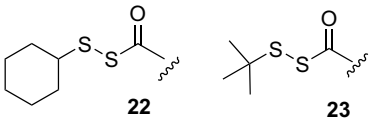


Figure 1.11 Monoacyl protections cleaved by thiolysis.

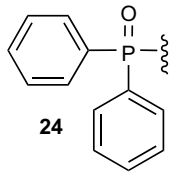


Figure 1.12 Dpp group.

1.2.1.2 **Phosphine-Type Groups**

Phosphine groups such as the diphenylphosphine (Dpp) group **24** (Figure 1.12) are stable to bases and catalytic hydrogenation, and sensitive to acids. They differ from the other acid-labile protections (e.g., triphenylmethyl (trityl or Trt), *tert*-butoxycarbonyl (Boc)) in that the acidolytic cleavage of the group does not result in the formation of carbocations which can cause undesired alkylations (see below). The Dpp group has been successfully employed in peptide synthesis. N^{α}-Dpp-protected amino acids are prepared by treating amino acid methyl esters with diphenylphosphinic acid chloride (Dpp-Cl) followed by alkaline hydrolysis of the ester. The protection is removed by treatment with 2 equiv. of 4-toluenesulfonic acid (TsOH) in MeOH (1–6 h) or 6 equiv. of HCl in MeOH (2–3 h) [18].

1.2.1.3 **Sulfonyl-Type Groups**

Reaction of amino acids with aryl/alkylsulfonic acid derivatives yields the corresponding sulfonamides. The 4-toluenesulfonyl (tosyl or Ts) group **25** is the first example of this type, which was described by Emil Fischer. However, its application to peptide synthesis has been constrained due to the difficulties such as cumbersome removal conditions (the only method of cleavage is reduction with sodium in liquid NH_3), high reactivity of the sulfonamide nitrogen (source of a number of side-reactions such as N^{α}-alkylation), and rapid hydrolysis of Ts-Gly peptides. Hence, the Ts group has been replaced by the more efficient 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) **26** [19], 2-nitrobenzenesulfonyl (Nbs) **27** [20], 4-nitrobenzenesulfonyl (nosyl) **28** [21], 2-(trimethylsilyl)ethanesulfonyl (SES) **29** [22], and *tert*-butylsulfonyl (Bus) **30** groups (Figure 1.13) [23].

N^{α}-Protected Pbf amino acids are prepared by the action of Pbf-Cl on amino acids under Schotten–Baumann conditions. The group is stable towards bases and catalytic hydrogenation, and cleaved by 10% dimethyl sulfide (DMS) in TFA. Nbs and nosyl groups are typically deblocked by 5% thiophenol in DMF and mercaptoacetic acid/

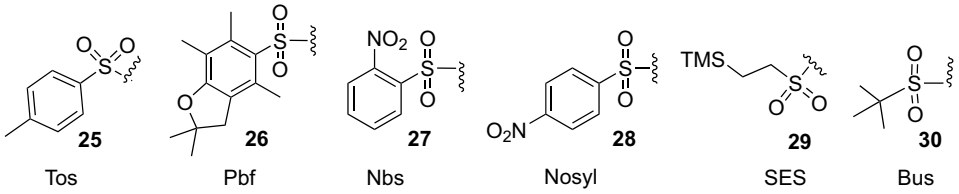


Figure 1.13 Sulfonyl-type α-amino protections.

sodium methoxide in CH_3CN, respectively. The nosyl group on *N*-methylated α-amino groups is deblocked much more readily than that on unsubstituted α-amino groups. SES groups derived from alkylsulfonic acids are stable even towards strong acidic conditions (boiling TFA, 6 M HCl in refluxing tetrahydrofuran (THF), $BF_3{\cdot}O(C_2H_5)_2$) as well as to alkali. The group is cleaved by treatment with cesium fluoride. Notably, the C–Si bond of the group is stable to desilylating reagents, which cleave other silyl protections, particularly the *O*-silyl groups. Hence, SES protection can be used in combination with silyl ethers. The N^{α}-Bus group is introduced by treatment of amino acids with *tert*-butylsulfanyl chloride followed by oxidation using *m*-chloroperbenzoic acid (mCPBA). It is removed using 0.2 N TfOH in DCM in the presence of anisole at 0 °C. N^{α}-Sulfonyl-protected amino acid derivatives are not able to rearrange to oxazol-5-ones even when the α-carboxy moiety is highly activated (e.g., as acid chlorides), thus precluding the possibility of racemization. Also, the N^{α}-sulfonyl-protected amino acid halides are more reactive compared to their carbamoyl counterparts due to the increased inductive effect of the sulfonyl unit. Hence, extremely difficult coupling of sterically hindered amino acids (e.g., MeAib to MeAib for aminoisobutyric acid) has been satisfactorily accomplished with good yields using Pbf-MeAib-Cl [19].

1.2.1.4 **Alkyl-Type Groups**

Alkylation increases the nucleophilicity of amines, in contrast to the primary requirement of a protection to diminish it. Consequently, monoalkylated amines with simple aliphatic *N*-substitutions are seldom protected. Nonetheless, bulky *N*-alkyl groups suppress the reactivity of the amine through steric hindrance. Hence, the α-amino group can be protected by placing crowded groups like Trt and benzhydryl on it. This type of protection is advantageous since the activated N^{α}-alkyl-amino acids do not racemize under standard peptide coupling conditions, as the bulkiness of the protection prevents the abstraction of the α-proton by a base. However, an innate limitation of the method is that the bulkiness of the N^{α} protection can sterically disfavor reactions at the carboxy end, thereby making incorporation of N^{α}-alkyl-amino acids into peptides a difficult task.

1.2.1.4.1 **Triphenylmethyl (Trityl or Trt) Group** N^{α}-Trt-amino acids **31** can be prepared by treating amino acid methyl esters with Trt-Cl followed by alkaline hydrolysis of the ester. Hydrolysis is rather sluggish due to steric hindrance by the Trt group. Alternatively, the amino acids can be directly treated with Trt-Cl (or a more efficient Trt-Br) followed by methanolysis of the *N*,*O*-bis-Trt intermediate (Trt ester) [24]. Formation of Trt esters can be circumvented by using *N*,*O*-bis-trimethylsilyl (TMS) amino acids (Me_3Si-NH-CHR-COO$SiMe_3$), and trisilyl derivatives of Ser, Thr, and Tyr as substrates for tritylation (Figure 1.14) [25]. N^{α}-Trt-amino acids are isolated as stable diethylammonium salts. The Trt group is stable to bases. It is cleaved by mild acids such as 1% TFA or 3% trichloroacetic acid (TCA) in DCM, 0.1 M 1-hydroxy-1*H*-benzotriazole (HOBt) in trifluoroethanol (TFE), or moist 0.2% TFA in DCM [26–28]. The latter two conditions are compatible with acid-labile linkers in SPPS. The group can be preferentially cleaved in the presence of other acid sensitive groups like

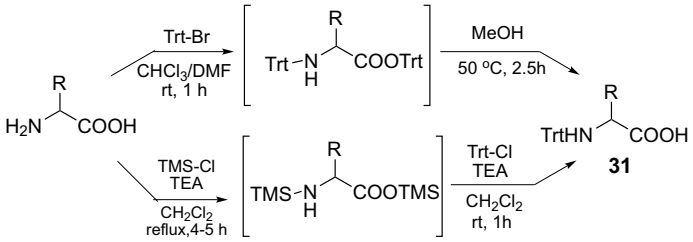


Figure 1.14 Preparation of N^{α}-Trt-protected amino acids.

2-(biphenyl-4-yl)prop-2-yloxycarbonyl (Bpoc) and Boc by pH-controlled titration with HCl in aqueous TFE [29]. Realkylation of the amine during cleavage is prevented due to protonation of the amine in the acidic medium and most effectively by the use of reducing silanes like triethylsilane or even MeOH and TFE. Catalytic hydrogenation and reduction with Na/liquid NH_3 also remove the group.

1.2.1.4.2 **Benzhydryl Groups** The N^{α}-benzhydryl groups (e.g., dibenzosuberyl (Sub) group (Figure 1.15) **32**) are more stable to acids than the Trt group and in addition offer lesser steric hindrance to peptide coupling [30].

1.2.1.4.3 ***N,N*-Bis-Benzyl Protection** *N,N*-bis-Benzyl amino acids are typically prepared by treating amino acids with benzyl chloride in the presence of K_2CO_3. The major product is the *N,N*-bis-benzyl amino acid benzyl ester, which is subjected to alkaline hydrolysis to obtain the free acid. *N,N*-bis-Benzyl protection is preferred to *N*-urethane protections in diastereoselective addition reactions of N^{α}-protected amino aldehydes due to the high rate of racemization in *N*-urethane protected versions [31].

1.2.1.4.4 **Vinyl Groups** 1,3-Diketones (R-CO-CH_2-CO-CH_3) such as acetylacetone, benzoylacetone, and acetoacetic acid ester or the cyclic diketone, 5,5-dimethylhexa-1,3-dione (dimedone) condense with amino acids to give the corresponding *N*-enamine derivatives (R-CO-CH=C(CH_3)-NH-CHR^1-CO-Y). The 1-methyl-3-oxo-3-phenylprop-1-enyl (Mbv) group (R = Ph), can be introduced by condensing amino acids with benzoylacetone in methanolic KOH. The products are isolated as potassium or dicyclohexylamine (DCHA) salts [32]. Acidification of the salts to generate free carboxylic acids is difficult due to high acid sensitivity of the group. However, the Tfa analogs (R = CF_3) can be isolated as free acids by acidification [33]. The N^{α}-Mbv group can be removed by treatment with dilute AcOH or 0.4 M HCl in THF or 0.1 M

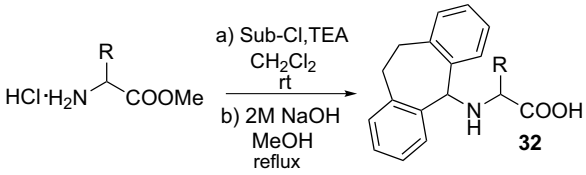


Figure 1.15 Preparation of N^{α}-Sub-amino acids.

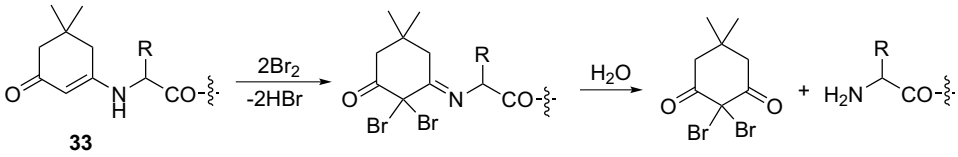


Figure 1.16 Cleavage of the N^{α}-Dim group by bromination.

TosOH in THF [34]. The N^{α}-vinyl derivatives are not prone to racemization. An additional advantage of vinyl-type protections is that the acid-catalyzed hydrolytic cleavage of the protection regenerates the 1,3-dioxo compound that can be recovered and reused. In contrast to the above acid-labile *N*-vinyl groups, the 5,5-dimethyl-3-oxocyclohexen-1-yl (Dim) group **33** is stable to acids and also to hydrogenolysis. It is removed by treatment with bromine water (Figure 1.16) or nitrous acid in AcOH [35]. These conditions can cause bromination and nitrosation of Tyr residues. 1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde) groups are the other vinyl-type protections which are more useful for the protection of ω-NH_2 of Lys.

1.2.1.5 **Sulfanyl-Type Groups**

The N^{α}-sulfanyl groups such as the 2-nitrophenylsulfanyl (Nps) group provide protection against racemization and also do not offer a disfavorable steric effect for peptide coupling. N^{α}-Nps-amino acids **34** are prepared by the reaction of Nps-Cl **35** with amino acids in the presence of a base [36]. Alternatively, Nps-Cl can be converted into a more stable Nps-SCN **36** (by treatment with NaSCN) and then it is used for introducing the group (Figure 1.17) [37]. Crystalline solids of Nps-amino acids are obtained as DCHA salts. The extreme acid stability of the group necessitates special precautions for handling of the free carboxylic acids. Nps groups can be selectively cleaved in the presence of acid-labile *tert*-butyl-based groups by using HCl or HBr in alcohol [38] or in aprotic solvents such as EtOAc or DMF [39]. The acidolytic fission of the sulfenamide bond gives rise to the free amine as well as Nps-Cl, which can cause reattachment of the group. In alcoholic solvents, this is prevented by the conversion of Nps-Cl intermediate into a sulfenic acid ester liberating 1.0 equiv. of HCl that protonates the deblocked amine. However, a similar kind of deactivation of Nps-Cl is

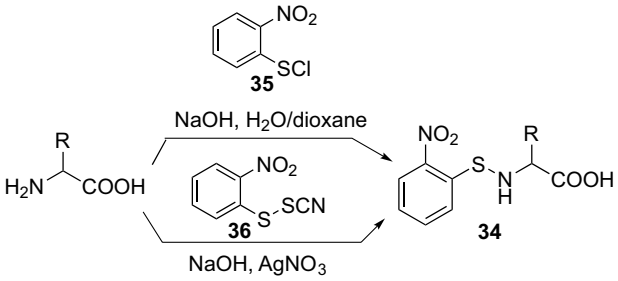


Figure 1.17 Preparation of N^{α}-Nps-protected amino acids.

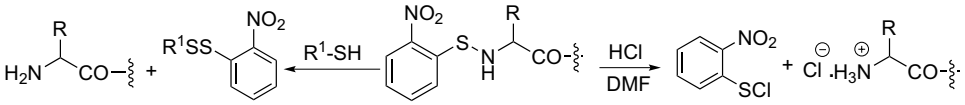


Figure 1.18 Cleavage of the N^{α}-Nps group.

not possible in aprotic solvent. In this case an additional equivalent of HCl is added to protect the deblocked amine as its hydrochloride salt. Scavengers such as 2-methyl indole and 1-acetyl tryptophan are added to decrease the activity of the hydrogen halide in alcohol, and thus protect other acid-labile groups. Alternatively, the group can be cleaved without the risk of formation of Nps-Cl intermediate through thiolysis (Figure 1.18).

1.2.2
Urethanes (Carbamates or Alkyloxycarbonyl Groups)

In 1932, Bergmann and Zervas introduced the benzyloxycarbonyl (Cbz or Z) group as a new amino protecting group [40]. This event not only led to a new epoch in the history of peptide synthesis, but also introduced a new perspective to the conception of protecting group chemistry in organic synthesis as a whole. It spark-started the era of modern peptide synthesis. Until then the practice of peptide synthesis largely relied on the use of acyl groups for α-amino protection, whose selective removal without hydrolyzing the painstakingly assembled peptides was not always possible. In order to circumvent the problem of instability of peptide bonds in harsh deblocking conditions of N^{α}-acyl groups, an approach of peptide chain extension from the amino-terminus (N → C direction) was inevitably followed. Although, repetitive deblocking of N^{α} protection could be avoided, the strategy offered several synthetic difficulties *per se*. Consequently, it was not possible to extend the peptide chain beyond a few amino acid units. The new group (Z group) of Bergmann and Zervas was a urethane-type protection that could be removed, similar to benzyl esters, by catalytic hydrogenation, against which the peptide bonds and alkyl esters were completely stable. It was stable to most of the coupling methods. Later, it was found that the group could be selectively and quantitatively removed by acidolysis too. Addition of these new dimensions of N^{α}-deprotection provided the much-needed stimulus to step up the practice of peptide synthesis to the extent of successfully synthesizing polypeptides. It was also established that the N^{α}-urethane-protected amino acids were less prone to racemization than were the acyl-protected counterparts. The impact of the introduction of this new type of protection on peptide synthesis was so enormous that in only a few years a large number of biologically active peptides as well as several hundreds of their analogs were synthesized. It also initiated studies on the discovery of new urethane protections principally orthogonal to the Z group. Currently, a plethora of urethane protections and a large number of deprotection methods are available.

Urethanes **37** can be regarded as esters of carbamic acids (although the latter are not stable), and the urethane linkage as a hybrid of ester and amide bonds. Due to this

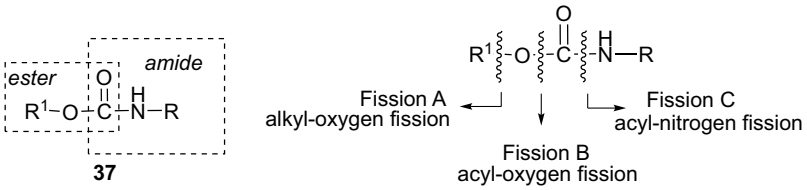


Figure 1.19 Urethane protection: structure and modes of fission.

structure, unlike simple *N*-acyl amines, there exists more than one bond whose fission can result in the deprotection of the amine (Figure 1.19).

Fissions B and C are less likely due to the low reactivity of urethane carbonyl to nucleophiles. Fission A (alkyl-oxygen fission) is the most probable pathway leading to deprotection of the urethane protected amine. It generates the carbamic acid

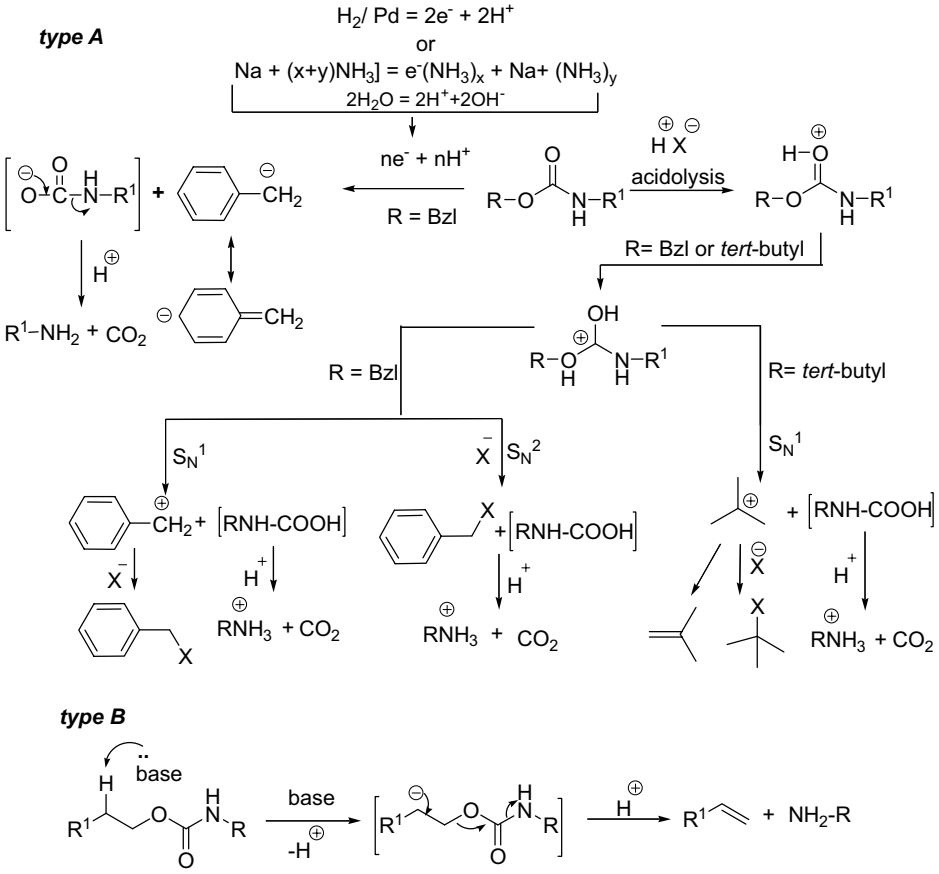


Figure 1.20 Reaction conditions for cleavage of urethane protections. Type A: benzyl and *tert*-butyl urethanes. Type B: urethanes cleaved via β-elimination. (Compiled from [1d].)

that spontaneously decomposes to the amine liberating CO_2. This fragmentation is facilitated by the formation of inductively or resonance-stabilized carbocations or carbanions of the ruptured alkyl fragments (e.g., benzyl- and *tert*-butyl-based urethanes). The important reactions that bring about alkyl-oxygen fission are shown in Figure 1.20 (type B). The reaction mechanism and the type of intermediates formed are also depicted. Alkyl-oxygen fission is also possible via a β-elimination pathway (E_1C_B mechanism) when an acidic methylene group is present β to the oxycarbonyl unit (Figure 1.20, type B). Abstraction of a proton by a base generates a resonance-stabilized carbanion that undergoes an electron shift to form a double bond with the elimination of the oxycarbonyl group, which further loses CO_2 to release the amine (e.g., 9-fluorenylmethyl- and 2-sulfonylethyl-based urethanes).

1.2.2.1 Formation of the Urethane Bond

Urethanes can be considered as the product of a reaction between the components shown in Figure 1.21. The order of incorporation of the components can be different. It can be through an initial formation of a chloroformate followed by its aminolysis (route 1) or through the formation of an isocyanate followed by its alcoholysis (route 2).

In either of the modes, the variable component is only the alcohol. Therefore, a wide variety of urethanes can be prepared by changing the alcohol component. Indeed, the properties of N^{α}-urethane-protected amino acids, such as stability, solubility, methods of cleavage, and reactivity, depend on the nature of the alcohol component of the urethane segment. Hence, in this treatise, the important urethane-type protections are presented according to the structure of the alcohol component.

1.2.2.2 Urethanes Derived from Primary Alcohols

1.2.2.2.1 Benzyloxycarbonyl (Cbz or Z) Group Since its introduction, the Z group has been the most widely employed N^{α} protection for peptide synthesis preferably for solution-phase synthesis. The stability of N^{α}-Z-amino acids, facile introduction and removal conditions (with formation of easily removable cleavage products), and minimum side-reactions of the Z-protected amino acid derivatives have contributed to the widespread utility of this group. The Z group has retained its popularity even to date and it continues to be the protection of choice for peptide synthesis.

Preparation Z-Amino acids **38** can be prepared by acylation of amino acids with benzyl chloroformate **40** (or Z-Cl). The reaction is carried out in the presence of

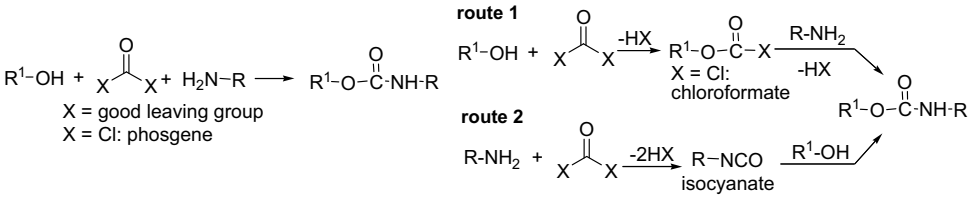


Figure 1.21 Routes to urethane bond formation.

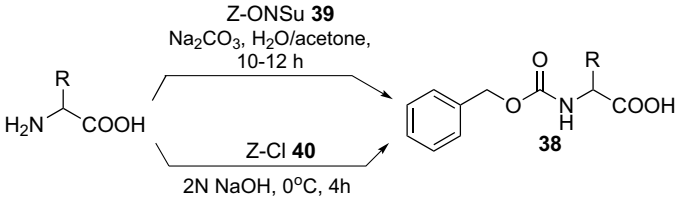


Figure 1.22 Preparation of N^{α}-Z-amino acids.

Na_2CO_3 or NaOH in an aqueous–organic mixture (Schotten–Baumann conditions) (Figure 1.22) or in the presence of tertiary amines in organic solvents [41]. Z-Cl is commercially available and can also be prepared by the treatment of benzyl alcohol with phosgene (*caution*: phosgene is a highly poisonous gas and should be handled with extreme caution). However, the formation of Z-protected dipeptides as side-products and acylation of the hydroxy group of Ser and Thr and the phenolic function of Tyr (Z-Tyr-OH is obtained by alkaline hydrolysis of the corresponding bis-Z derivative) are the disadvantages of this highly active reagent. Hence, the moderately reactive mixed carbonate, Z-succinimido carbonate (Z-OSu) **39** is increasingly used in the preparation of Z-amino acids. Z-OSu furnishes good yields of Z-amino acids and also minimizes the formation of peptide impurities [42]. The reagent is commercially available and can also be prepared by the treatment of *N*-hydroxysuccinimide with Z-Cl. It is stable and can be stored without decomposition for a long time at low temperature with the exclusion of moisture. Most of the Z-amino acids are obtained as crystalline solids. The oily Z-amino acids can be crystallized as DCHA salts. Benzyl benzotriazolyl carbonate (Z-OBt) **41** and dibenzyldicarbonate (Z_2O or benzyl pyrocarbonate or Z-anhydride) **42** are the other Z-donors (Figure 1.23) proposed for the preparation of Z-amino acids [43, 44].

Cleavage The favored methods for the removal of N^{α}-Z groups are catalytic hydrogenation and acidolyis. Reagents and conditions, and common side-reactions encountered under each method of deblocking the Z group are furnished in Table 1.1.

The acid lability of Z group can be modulated by placing electron-withdrawing or -donating groups on the phenyl ring (Figure 1.24). When $X = NO_2$ (**43**), Cl (**44**), or Ph-N=N (**45**) (electron-withdrawing substituents), the acid stability of the groups increases due to the destabilization of the benzyl cation produced during acidolytic

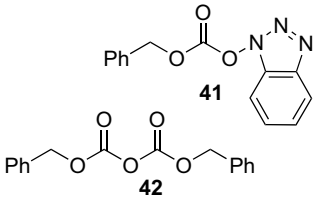
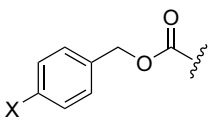


Figure 1.23 Z-donors.

Table 1.1 Reagents and conditions for the removal of N^{α}-Z protection.

Reaction with reagent and conditions	Notes
Catalytic hydrogenation employing H_2 in the presence of catalysts like Pd/C or Pd/$BaSO_4$ in MeOH, EtOH, or AcOH [45] (promoted by the addition of small amount of acids)	*N*-alkylated peptides can be formed due to a series of side-reactions starting from palladium-catalyzed oxidation of the alcohol (solvent) to aldehyde. The aldehyde forms Schiff's base with the deprotected amine, which in turn undergoes reduction to *N*-alkylated products. This can be avoided by carrying out hydrogenation in completely oxygen-free medium or with the addition of small amount of water (to suppress oxidation). Alcohols such as *iso*-propanol, which are resistant to oxidation, can be used as solvent.
	Not compatible with sulfur-containing amino acids due to catalytic poisoning
Catalytic hydrogenation employing Pd-$BaSO_4$ catalyst [45b]	Compatible with Met-containing peptides, but not with peptides with *S*-alkyl-Cys residues.
Catalytic hydrogenation in liquid ammonia solvent at −33 °C [46]	Compatible with sulfur-containing amino acids including *S*-alkyl-Cys.
Silyl hydrides such as triethylsilane or *tert*-butyldimethylsilane in the presence of $PdCl_2$ [47]	
Sodium in liquid ammonia (Birch reduction)	
CTH with hydrogen donors such as 85% HCOOH, $HCOONH_4$, cyclohexenes, and hexadienes in the presence of Pd/C catalyst; $HCOONH_4$ and Pd/C under microwave irradiation in *iso*-propanol solvent [48]	The C^{α}–C^{β} double bond of didehydroalanine (ΔAla) residues is stable to CTH.
Acidolysis using anhydrous liquid HF, HBr-AcOH, pyridinium polyhydrogen fluoride (30% pyridine/70% HF), sulfonic acids such as methane sulfonic acids, fluoro- or trifluorosulfonic acid in DCM or TFA [49–51]	Benzylation of Tyr and Trp and *S*-benzylation of Met due to the formation of benzyl cation. This can be controlled by the addition of anisole or thioanisole as scavenger.



43: X=NO_2; **44**: X=Cl; **45**: X=Ph-N=N;
46: X=OMe; **47**: X=Me

Figure 1.24 Substituted Z groups.

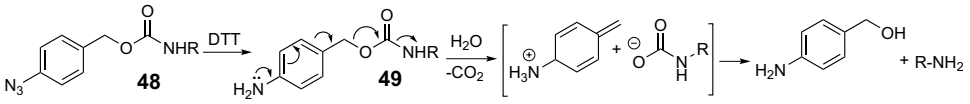


Figure 1.25 Cleavage of the Z(N_3) group via reduction of the azide.

cleavage [52–54]. The mechanism of acidolytic cleavage is shifted towards the S_N2 pathway by these substituents. The electron-releasing substituents (X = OMe (**46**) or Me (**47**)) increase the acid lability of the groups by offering higher resonance stabilization to the benzyl cation formed during cleavage [55, 56]. Deblocking preferentially occurs through an S_N1 pathway. In terms of hydrogenolytic cleavage of **43**, **45**, and **48**, a deviation from the standard mode of alkyl-oxygen bond fragmentation is observed. When X = N_3 (**48**), the dithiothreitol (DTT)-mediated reduction of the azido group results in the formation of 4-aminobenzyloxycarbonyl derivative **49** which undergoes a 1,6-electron electron shift to liberate the amine (Figure 1.25) [57]. Similarly, **43** and **45** undergo rapid hydrogenolysis even in neutral solution due to the formation of the same intermediate **49**. The substitutions also impart favorable properties such as a higher tendency to crystallization (**46**) and coloration (**44**) to the protecting group. The presence of a chromophore is helpful for monitoring reactions through spectrophotometric methods.

1.2.2.2.2 Urethanes Cleaved by β-Elimination

9-Fluorenylmethoxycarbonyl (Fmoc) Group [58, 59] The Fmoc group was introduced for peptide synthesis in the 1970s. The group is completely stable to acids and to a large extent to catalytic hydrogenation, although prolonged catalytic hydrogenation can cleave the group (this surprising reactivity of Fmoc to catalytic hydrogenation has been attributed to the β-phenylethyloxy skeleton that can be fragmented through hydrogenolysis, although much less readily than arylmethyloxy system). It is base-labile and removed by treatment with alkyl amines such as piperidine and diethylamine (DEA). The base-labile property of the Fmoc group introduced a "third dimension" to the then existing deprotection reactions, which mainly consisted of hydrogenolytic and acidolytic cleavage of benzyl- and *tert*-butyl-based protections, respectively. Further, the acid stability of the Fmoc group made possible the preparation of stable and highly active N^{α}-urethane-protected amino acid chlorides (the same are not accessible with Z- and Boc-protected amino acids) for rapid and difficult peptide couplings. Presently, Fmoc is a well-established and an extensively used α-amino protector for peptide synthesis, in general, and for solid-phase synthesis, in particular. A few prominent advantages of the group that have led to its popularity are:

i) The protection strategy based on the combination of Fmoc group (for N^{α} protection) and *tert*-butyl-based groups (carbamates, esters and ethers, for side-chain protection) – the Fmoc/*tert*-butyl approach – is superior to the traditional Boc/benzyl approach since the repetitive deblocking of the Fmoc group by base treatment does not cause a progressive loss of side-chain protections. It also enables the use of acid-labile linkers and resins for SPPS

from which peptides can be obtained as free acids directly. In contrast, the Boc/benzyl approach suffers from the partial loss of side-chain protections with every cycle of acidolytic removal of N^{α}-Boc group. It also precludes the use of acid-labile solid supports.

ii) The Fmoc group can be cleaved using mild reagents such as piperidine.

iii) With Fmoc as protective function, the progress of reactions can be monitored through UV (λ_{max}: 267, 290, 301 nm) and fluorescence spectrometry – a property that can be used to advantage in fully automated peptide synthesis.

- **Preparation:** 9-Fluorenylmethyl chloroformate (Fmoc-Cl) **51** and Fmoc-OSu **52** are the favored reagents for introducing the Fmoc group. Fmoc-Cl is commercially available (also prepared by the action of phosgene on 9-fluorenylmethanol) and storable for a long time under anhydrous conditions at low temperature. Acylation of amino acids with Fmoc-Cl is carried out under Schotten–Baumann conditions at 0 °C (Figure 1.26) [59]. Formation of detectable amounts of Fmoc protected peptide impurities is a major concern of this reagent, but this has been considerably overcome with modifications in the reaction conditions (see above). Fmoc-OSu is the reagent of choice for the preparation of Fmoc-amino acids [60]. It is commercially available, storable, and furnishes impressive yields without causing the formation of dipeptide side-products. The other reagents that have been proposed as Fmoc donors are the azido formate, Fmoc-N_3, and 9-fluorenylmethyl-1-chloroalkyl carbonate [61, 62].
- **Cleavage:** The Fmoc group is deblocked by nonhydrolytic base treatment by a variety of organic amines, but most efficiently by unhindered cyclic amines such as piperidine and morpholine in polar solvents such as DMF. The group is cleaved through an E_1C_B mechanism as shown in the Figure 1.21 (type B). The rate-limiting step is the abstraction of the proton from the bulky 9-fluorenylmethyl ring, and hence deblocking by sterically crowded amines is sluggish and often incomplete. The possibility of premature deblocking of the group during coupling due to the basicity of the free amino group of amino acid ester or peptide ester has been studied, and found to be at unalarming levels due to a huge difference in the rates of coupling and deblocking. However, when couplings are slow, as in the case of the synthesis of difficult sequences such as polyproline, deblocking of Fmoc can take place to a higher extent (also Pro is a secondary amine that can deblock Fmoc more effectively). Precautions such as slow addition of a solution

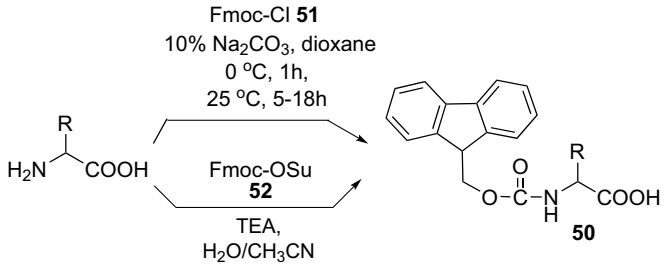


Figure 1.26 Preparation of N^{α}-Fmoc-protected amino acids.

of the free amino compound to a cold solution of activated Fmoc-amino acid (to create a high dilution of amine), and addition of acidic reagents such as HOBt and certain phenol derivatives (to bring down the basicity of the medium) can suppress a low level of deblocking that could happen during coupling. However, in SPPS the premature deblocking is unlikely due to spatial separation of the resin bound amines. Generally, the cleavage of a urethane-type protection (Boc and Z groups) results in the formation of byproducts (volatile or water-soluble compounds) that are easily separable On the other hand, fragmentation of Fmoc gives rise to dibenzofulvene (DBF), a reactive electrophilic reagent (Michael acceptor), as the byproduct. The presence of DBF is undesirable since it tends to polymerize, forming an insoluble mass or gels. It can also form an irreversible addition product with the deblocked amine. However, these potential problems are alleviated since the DBF is trapped by the deblocking amine, which is typically used in excess, through a facile formation of an adduct (a tertiary amine) that can be separated from the desired products. The adduct formation is dependent on factors such as the intrinsic basicity of the deblocking amine. For instance, amines such as piperidine, morpholine, and piperazine form adducts, while DEA does not. Further, the adduct formation is a reversible reaction and the position of equilibrium depends on the nature of the amine. Based on the criteria of the removal of unreacted deblocking amine, formation of DBF adduct, and the mode of its separation from products, different approaches have been developed for the selection of reagents for solid- and solution-phase cleavage of the Fmoc group. Such distinction is normally not made in the case of the other commonly used urethane protections (e.g., Boc group) where the reagents for solid- and solution-phase cleavage are essentially the same except that an excess amount is used on solid supports.

- **Deprotection in Solid Phase**: In SPPS, the process is straightforward without the need for special consideration for the removal of unreacted deblocking amine and the DBF adducts since these can be washed off the resin. The most widely used combinations are 20–50% piperidine in DMF or *N*-methyl-2-pyrrolidone (NMP) and 60% DEA in DMF [63–65].
- **Deprotection in Solution Phase**: The two main considerations are the separation of DBF adducts from the products and the complete elimination of the unreacted deblocking amine. These have been addressed by employing 4-(aminomethyl) piperidine (4-AMP) as deblocking as well as scavenging reagent [66]. 4-AMP forms the adduct which is soluble in phosphate buffer (pH 5.5) and hence can be removed by extraction with the buffer. The unreacted 4-AMP is removed prior to buffer wash by extraction with saturated NaCl solution. However, separation of the deblocked amine from 4-AMP-DBF adduct can sometimes be inefficient due to the formation of emulsions during extraction. This limitation has been overcome by replacing 4-AMP with tris-(2-aminoethyl)amine (TAEA) [67]. Both the amines have been employed in the Fmoc/polyamine approach to rapid peptide synthesis of several peptides including [Leu^5]enkephalin and substance P. A different approach to separate the adduct is to use polymer-supported amines such as polystyrene-bound or silica gel-bound piperazine as deblocking and scavenging reagents [68, 69]. The resin-bound DBF adduct and the unreacted

polymer-supported amine can be removed through filtration. However, complete scavenging has not been possible with these reagents with about 10–15% of residual DBF in solution. In this regard, it has been found that polymer-supported thiols are more efficient than polymer-supported amines as scavengers [70]. Alternatively, complete scavenging by polymer-supported amines can be achieved by substitution of indene based 3-indenylmethyloxycarbonyl (Imoc) and 2-chloro-3*H*-indenylmethyloxycarbonyl (Climoc) groups for Fmoc group as base-labile N^{α}-protectors [58]. Fmoc can also be deblocked using volatile amines such as DEA or dimethylamine (DMA), which can be completely removed by evaporation after the reaction [71, 72]. Deblocking has also been accomplished by using catalytic quantities (3 mol%) of the non-nucleophilic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in combination with a large excess of 1-ocatane-thiol in THF or polystyrene-supported *N*-(2-mercaptoethyl)amine in THF [70]. The thiols function as scavengers of DBF. Other bases employed for deblocking are hydrazine, hydroxylamine, and Tesser's base (30: 9: 1 dioxane/MeOH/4 N NaOH) [59]. Deblocking can be carried out in nonbasic conditions by using fluorine reagents such as 0.02 M tetrabutylammonium fluoride (TBAF·$3H_2O$)/DMF in MeOH and KF/18-crown-6 in the presence of scavengers (thiols) [73–75]. However, *O*-silyl protections are sensitive to fluoride ion treatment.

- **Side-Reactions During Cleavage:** The base used for deblocking Fmoc can catalyze intramolecular aminolysis reactions leading to the formation of side-products such as diketopiperazine (or 2,5-dioxopiperazine or piperazine-2,5-dione or DKP) and aspartimide (Figure 1.27). Free amino dipeptide alkyl esters **53**, particularly those which contain Pro or NMA at the N-terminus (since the configuration of peptide bond is *cis*), readily cyclize to DKPs **54** in basic media. Formation of DKPs can be minimized by adopting methods like fragment condensation with Fmoc-dipeptide acids or using esters such as benzyl or *tert*-butyl esters that have lower reactivity to aminolysis for protection of the carboxy terminus of peptides. The latter approach has been employed for the successful solution-phase synthesis of cyclosporine O – a peptide with several NMAs using Fmoc chemistry [76]. Employing fluoride reagents for deblocking Fmoc can also prevent DKP formation.

  Fmoc Analogs: The low solubility of Fmoc-protected derivatives in organic solvents is a limitation of the group. This has been addressed by the development of Fmoc analogs such as 2,7-di-*tert*-butyl-Fmoc group **55** whose derivatives are about 2 times more soluble due to hydrophobic alkyl substitutions on the fluorenyl ring [77] However, deblocking of **55** is about 4 times slower than Fmoc. The Sulfmoc group **56** is useful to improve the purity of synthetic peptides [78].

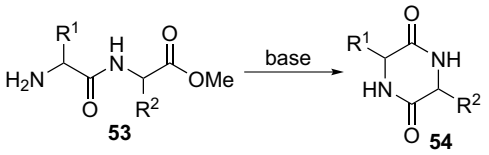


Figure 1.27 Base-catalyzed formation of DKP.

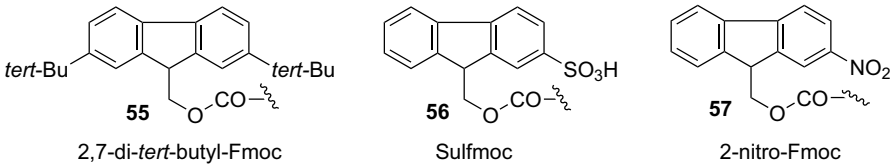


Figure 1.28 Fmoc analogs.

The strongly acidic Sulfmoc group can be introduced at the end of the synthesis to the growing peptide chain and the Sulfmoc-protected peptides can be efficiently separated from the rest of the non-growing and terminated peptides through chromatography. The 2-nitro-Fmoc group **57** is a photocleavable and optically active analog of Fmoc (Figure 1.28) [79].

Sulfonylethoxycarbonyl Groups The prototype 2-tosylethoxycarbonyl group (Tec) was introduced in 1964 [80] and since then a number of groups of the kind have been described. Presently, 2-(methylsulfonyl)ethoxycarbonyl (Msc) **58** and 2-(4-nitrophenylsulfonyl)ethoxycarbonyl (Nsc) **59** groups (Figure 1.29) are the important examples in the series. N^{α}-Msc-amino acids are prepared by using mixed carbonates Msc-OSu or Msc-ONp [81]. The group is stable to acids and to catalytic hydrogenation (an advantage over Fmoc) and does not inactivate the hydrogenation catalysts. Therefore, catalytic hydrogenation can be employed as a method of deblocking protecting groups from Msc-protected peptides. The group is cleaved within minutes in NaOH solution of pH 10–12 at 0 °C in the presence of MeOH, whose role is to trap the byproduct vinyl sulfone [82]. The reaction mixture should be acidified to decompose the stable carbamate intermediate, which may not be compatible with highly acid-sensitive groups. The Msc group is more hydrophilic and is favored when high solubility in aqueous media is desired. N^{α}-Nsc amino acids **60** are prepared through acylation of *N,O*-bis-TMS-amino acids with Nsc-Cl (Figure 1.30) [83]. The group can be an efficient substitute to Fmoc group due to: a higher tendency to crystallize, a

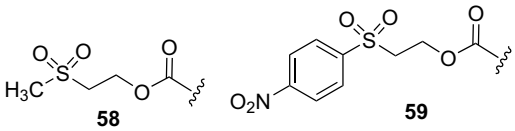


Figure 1.29 N^{α}-Sulfonylethoxycarbonyl groups.

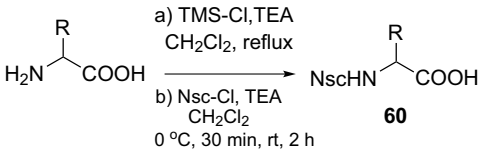


Figure 1.30 Preparation of N^{α}-Nsc-amino acids.

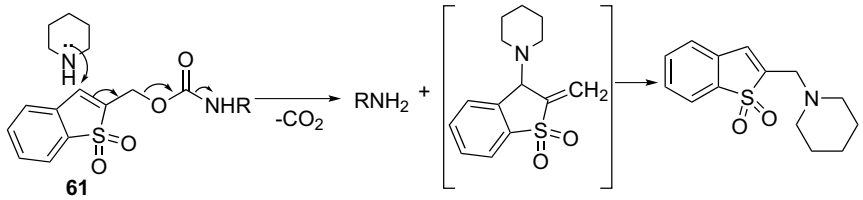


Figure 1.31 Mechanism of N^{α}-Bsmoc cleavage.

lower rate of cleavage (which leads to increased stability in DMF solvent and also avoids premature deblocking during coupling), the nonpolymerizing property of the vinyl sulfone byproduct, and less steric hindrance and lower rates of racemization of N^{α}-Nsc-protected His, Lys, and Ser.

1.2.2.2.3 **Urethanes Cleaved via Michael-Type Addition** A major concern with Fmoc is the reversible formation of DBF adduct and incomplete scavenging of DBF, particularly by polymer-supported amines. Consequently, groups such as 1,1-dioxobenzo[*b*]thiophen-2-ylmethoxycarbonyl (Bsmoc) **61**, 2-(*tert*-butylsulfonyl)-2-propenyloxycarbonyl (Bspoc), and 2-(methylsulfonyl)-3-phenyl-2-propenyloxycarbonyl (Mspoc) that are cleaved by a process in which the deblocking event (Michael-type addition) is simultaneously a scavenging event have been developed (Figure 1.31) [84]. The Bsmoc group additionally contains an alkyl substituent at the β-position of the Michael unit, which prevents premature deblocking of the group. The Bsmoc group is several times more sensitive to cyclic amines like piperidine than Fmoc group. It can be deblocked by piperidine solutions of lower concentration (2% v/v in DMF for 5 min as against 20% v/v in DMF for about 30 min for Fmoc removal), thus leading to minimum or no formation of side-products such as aspartimide that arise due to base-catalyzed reactions. In addition, the Bsmoc group can also be selectively cleaved over Fmoc group by using 2% TAEA in DCM. Further, when TAEA is used to deblock the Bsmoc group, a water-soluble adduct is formed that can be removed by extraction with water without the prerequisite for acidic phosphate buffer (as for Fmoc-TAEA adduct removal), which may cause partial loss of the deblocked amine. These advantages have amounted to the application of Bsmoc group in improved synthesis of several peptides including cyclosporine O [85].

A different category of base-labile *N*-protecting groups (e.g., 5-benzisoxazolylmethoxycarbonyl (Bic) group **62**) in which the deblocking is due to base-induced opening of the benzisoxazole ring followed by a 1,6-electron shift have also been proposed (Figure 1.32) [86].

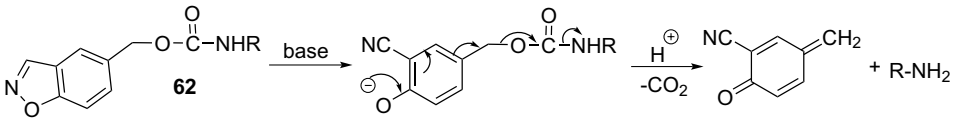


Figure 1.32 β-Elimination via isoxazole ring opening.

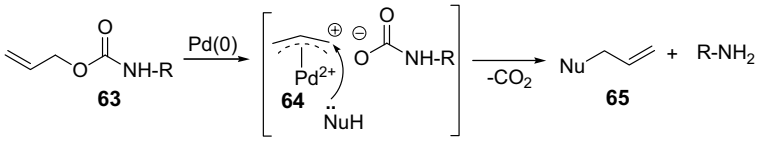


Figure 1.33 Pd(0)-mediated cleavage of allyl urethanes.

1.2.2.2.4 **Allyloxycarbonyl (Aloc) Group** Allyl-based protections were not paid much attention until 1987, when it was demonstrated that they could be selectively removed via a Pd(0)-catalyzed allyl transfer reaction (Tsuji–Trost reaction) under mild conditions [87]. Indeed, the discovery of this new method introduced what is now referred to as a "fourth dimension" to the protection schemes due to its compatibility with benzyl-based (removed by hydrogenolysis), *tert*-butyl-based (removed by acids), and 9-fluorenylmethyl-based (removed by bases) protections.

The Aloc group **63** is introduced using allyl chloroformate (Aloc-Cl) or diallyl dicarbonate $(Aloc)_2O$ [88, 89].

Cleavage The Aloc group is fragmented in the presence of a Pd(0) catalyst through the formation of η^3-allyl palladium complex **64**, which is subsequently decomposed by the transfer of the allyl group to a nucleophile (Figure 1.33). Allyl transfer to the deblocked amine is avoided by the addition of scavengers, which preferentially accept the allyl group from the palladium complex.

As catalyst, the commercially available *tetrakis*(triphenylphosphine)palladium(0) $[Pd(PPh_3)_4]$ is extensively used although palladium complexes with differentially substituted ligands have also been proposed. The reagents that can be employed as scavengers are summarized in Table 1.2.

1.2.2.3 Urethane Groups Derived from Secondary Alcohols

These groups (Figure 1.34) do not exhibit any unique properties complementary to the other routinely used N^{α}-amino protecting groups. Nonetheless, groups such as cyclopentyloxycarbonyl (Cpoc) **66**, 2-adamantyloxycarbonyl (Adoc) **67**, and isobornyloxycarbonyl (Iboc) **68** [94–96], which exhibit cleavage characteristics similar to the Boc group, have been used with limited scope. The di-2-pyridinylmethyloxycarbonyl (Dpoc) **69** group is fragmented by Zn-AcOH treatment similar to the Z group [97].

1.2.2.4 Urethanes Derived from Tertiary Alcohols

1.2.2.4.1 ***tert*-Butoxycarbonyl (Boc) Group** The Boc group was introduced in 1957 for peptide synthesis [98]. It was the first successful urethane-type protection that was orthogonal to the Z group, which was the only extensively used urethane protection then, due to its stability to catalytic hydrogenation (to which Z is labile) and its lability to mild acidic conditions (to which Z is stable). The group is resistant to alkali and to reduction by Na-liquid NH_3. Soon, it became an important amino protecting group to be used along with Z group. The introduction of the Boc group also made it possible to devise a general protection scheme based on *tert*-butyl/benzyl protections (for N^{α}

Table 1.2 Reagents and conditions for cleavage of N^{α}-Aloc protection.

Scavengers	Notes
Secondary amines Piperidine, DEA, morpholine;[a] 4-methylmorpholine in DCM/AcOH [90]	Excess nucleophile is required to displace the equilibrium of the allyl transfer reaction towards deprotection.
1,3-Dicarbonyl compounds as nucleophiles;[b] Dimedone, *N,N'*-dimethylbarbituric acid [91]	The liberated amine abstracts a proton from the acidic dicarbonyl compound, thus forming an ammonium derivative that is not allylated.
Hydride donors LAH, $NaBH_4$, phenylsilane ($PhSiH_3$), tributyltin hydride (Bu_3SnH), $BH_3{\cdot}NH_3$, $BH_3{\cdot}NHMe_2$ [92, 93]	The fragmentation results in a stable pseudometallic carbamate that is decomposed to the free amine through hydrolysis or acidolysis.
	If deprotection is carried out in the presence of an acylating agent, a transacylation can take place between the pseudometallic carbamate and the acylating agent leading a new amide bond. Hence, a tandem deprotection–coupling (with acid fluorides or active esters) of N^{α}-Aloc amino acids gives rise to peptides under almost neutral conditions.

a) Not compatible with Fmoc.
b) Compatible with Fmoc.

and side-chain functions), which was a major breakthrough for the expansion of SPPS methodology. Presently, the Boc group is the most favored N^{α}-protecting group (similar to Z and Fmoc) for both solution-phase synthesis and SPPS.

Preparation di-*tert*-Butyldicarbonate (di-*tert*-butylpyrocarbonate or $(Boc)_2O$) **71** and 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON) **72** are the widely employed reagents for the preparation of Boc-amino acids **70** (Figure 1.35). $(Boc)_2O$ furnishes impressive yields in short duration and even at low temperature. In addition, the byproducts, CO_2 and *tert*-butanol, can be removed easily. The reagent is commercially available, stable, and can be stored for a long time at low temperature and under anhydrous conditions. It has also been used to prepare several *N*-Boc-protected unnatural amino acids [99]. Boc-ON provides good yields of Boc-amino acids [100] and is more soluble in organic solvents. However, the oxime byproduct is water insoluble and is removed by extraction with Et_2O. It is commer-

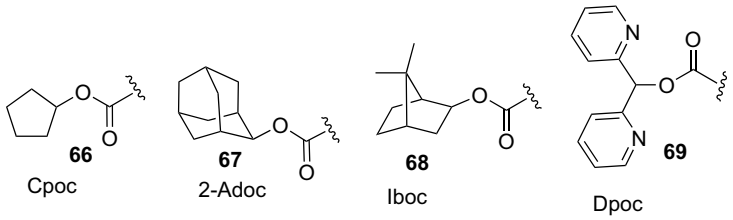


Figure 1.34 Urethane protections derived from secondary alcohols.

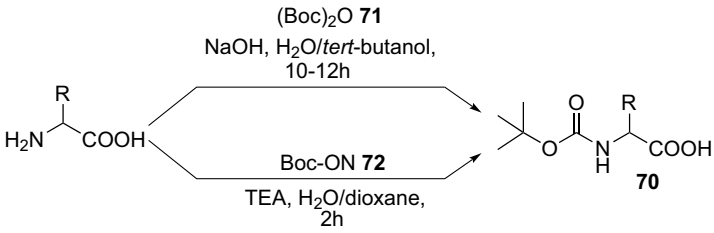


Figure 1.35 Preparation of N^{α}-Boc-amino acids.

cially available as well as stable for long duration. Acidification of the alkali salt of Boc-amino acids (and work up of *N*-Boc-protected compounds) is best done using 10% citric acid solution. Acids like HCl are avoided for this purpose due to the risk of partial cleavage of the group. The other important Boc donors proposed are Boc-F, Boc-N_3, and the mixed carbonates (*tert*-butoxycarbonyl succinimido carbonate (Boc-OSu) and *tert*-butoxycarbonyl-4-nitrophenyl carbonate (Boc-ONp)), and *N*-Boc-substituted heterocycles like Boc-imidazole [101]. With Boc-N_3 (prepared freshly from Boc-NH-NH_2), best yields are obtained by conducting the reaction in a pH stat.

Deprotection A wide range of reagents encompassing protic acids, Lewis acids, organosilanes, and even hot water (deionized water, Millipore grade) [102] have been proposed for deblocking the Boc group.

Protic Acids HCl in organic solvents and TFA are the most frequently employed reagents for deblocking N^{α}-Boc function. Presently, TFA is being increasingly favored. With both reagents, the cleavage proceeds through a S_N1 pathway with the formation of the *tert*-butyl cation, a strong alkylating agent that can cause the attachment of the *tert*-butyl group to nucleophilic sites of His, Met, Tyr, and Cys. The fate of the cation depends on the strength of the conjugate base of the deblocking acid. Since Cl^- is a weaker nucleophile the cation is trapped slowly into *tert*-butyl chloride and, in the process, a portion of the cation rearranges into unreactive isobutene. With TFA, the cation is completely trapped by the more nucleophilic trifluoroacetate ion to form *tert*-butyl trifluoroacetate, which is also a good alkylating agent. Also, since TFA is not strong enough to protonate nucleophilic centers of Met, Tyr, and Cys, these sites are exposed to alkylation. The addition of scavengers such as anisole or thioanisole or thiols can minimize the undesired alkylations. When thioanisole is added, deprotection takes place through a S_N2 mechanism with direct formation of the *tert*-butyl derivative of thioanisole. Hence no potential alkylating agents are formed. Deblocking in solution as well as on solid supports is carried out by using neat TFA or TFA in CH_2Cl_2 in the presence of a variety of scavengers [103]. A mixture of TFA and phenol/*p*-cresol and TFA in H_2O/DCM/AcOH can selectively remove the Boc group in the presence of acid sensitive *O*-silyl protections such as *tert*-butyldimethylsilyl (TBDMS) [104]. Trifluoromethane sulfonic acid (TfOH), methanesulfonic acid, anhydrous liquid HF, HBr in AcOH, and 98% formic acid are the other reagents used for cleavage. Anhydrous TsOH (2.0 M) in dioxane solution removes the Boc group without decomposition of Trp.

Organosilanes Progressive loss of benzyl-based protections and the peptide chains anchored to resins through benzyl ester linkages with each acidic deprotection cycle of TFA-mediated Boc cleavage is a major concern in the synthesis of long peptides through the Boc/benzyl protection strategy in SPPS. Cleavage of peptides from resin has been found to be about 0.7–2% per cycle. The loss of protections can be minimized by the use of organosilicon reagents such as TMS-Cl as highly selective reagents for Boc removal. A combination of 1 M TMS-Cl and 3 M phenol in DCM provides excellent selectivity along with quantitative cleavage [105]. With this reagent the loss of peptide chains has been drastically brought down to nearly 0.0004%, and that of benzyl ester, ether, and carbonate groups to about 0.01 to 0.17% for each per hour deprotection cycle. A variation of this method in which the silicon reagent is $SiCl_4$ has also been proposed [106]. Trimethylsilyl trifluoromethanesulfonate (TMS-triflate or TMS-OTf)/2,6-lutidine can selectively remove the Boc group on TFA-sensitive resins [107].

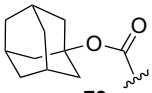
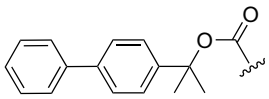
Lewis Acids Nonprotic acids such as $AlCl_3$, $BF_3{\cdot}OEt_2$ [108, 109], montmorillonite (acidic clay), celite, silica gel, and ion-exchange resins like Amberlyst 15 can also be used for deblocking the Boc group [110]. The last example can be used for simultaneous deprotection and purification of Boc-protected peptides. $AlCl_3$-mediated cleavage can be carried out in short duration under microwave irradiation [111]. When nonprotic acids are used for cleavage, the free amino peptide can be converted into its salt by the addition of HCl or HBr solutions in anhydrous Et_2O/EtOAc/dioxane to facilitate isolation, given there is no other acid-labile group.

Oxidizing Agent Excess ceric ammonium nitrate (CAN) in refluxing acetonitrile removes the Boc group [112]. The method is compatible with acid-sensitive TBDMS protections since the reaction is carried out under neutral conditions.

Removal of Boc Groups in the presence of tert-Butyl Esters Selective removal of Boc groups in the presence of acid-labile *tert*-butyl esters is of enormous practical utility since the *tert*-butyl esters are extensively used carboxy protections for peptide synthesis in solution. This has been accomplished using HCl/dioxane or 1.0 M HCl in EtOAc or concentrated H_2SO_4 (1.5–3.0 equiv.) in *tert*-butyl acetate or methanesulfonic acid (1.5–3.0 equiv.) in *tert*-butyl acetate/DCM (4: 1, v/v) [113]. The latter combination is based on the observation that *tert*-butyl carbamates are cleaved through an irreversible process with permanent loss of CO_2, while the cleavage of *tert*-butyl esters is reversible. Hence, addition of *tert*-butyl acetate as a source of *tert*-butyl cation shifts the equilibrium in the direction of acid-catalyzed esterification. TsOH (1.0 equiv.) in toluene [113] and Zeo-Carb 225/H^+ ion exchange resin have also been used to remove Boc groups with retention of *tert*-butyl-esters [114].

1.2.2.4.2 **Boc Analogs** Selected Boc analogs (**73**, **74**) with distinctive properties are listed in Table 1.3.

Table 1.3 Boc analogs.

Analogs of Boc	Notes
Adamantyl-1-oxycarbonyl (Adoc)	Cleavage sensitivity is similar to Boc; higher solubility and stability than Boc due to bulky hydrophobic group [115]
 **73**	
2-(Biphenyl-4-yl)-2-propoxycarbonyl (Bpoc)	Highly acid sensitive; removed under very mild acidic conditions with selectivity over Boc, Trt, acid-sensitive resin, and even extremely acid-sensitive thioxopeptides [116]
 **74**	Free acids are not stable to storage due to autocatalyzed acidolysis; storable as stable DCHA or CHA salts as well as stable active esters

1.2.2.5 Other Aspects of Urethane Protectors

1.2.2.5.1 Formation of Dipeptide Impurities during the Introduction of Urethanes and Protocols to Overcome It Formation of detectable amounts of *N*-protected dipeptide acids (and even tripeptide acids) has been observed (a solvent system of toluene/AcOH (10: 1) efficiently differentiates Fmoc-peptide acids from Fmoc-amino acids on thin-layer chromatography (TLC)) when chloroformates such as Fmoc-Cl and Z-Cl are employed for the preparation of N^{α}-urethane-protected amino acids. This has been explained based on the activation of the carboxy group of amino acids by chloroformate to mixed anhydrides followed by their aminolysis by the amino acids (Figure 1.36). The mixed anhydride intermediates are sufficiently stable in aqueous/organic reaction mixture and give rise to peptides even under Schotten–Baumann conditions. The peptide acids are difficult to separate from the products through work-up or crystallization or column purification. As a result, their presence decreases the homogeneity of *N*-protected amino acids, which are the building blocks for the synthesis of products (peptides) that are expected to be of highest purity. This side-reaction is also a major concern for large-scale and industrial production of *N*-protected amino acids.

Certain modifications in the reaction conditions have been proposed for dipeptide-free synthesis when Fmoc-Cl is used for the introduction of Fmoc group. A combination of 20–25% excess of amino acid over Fmoc-Cl, a 4-fold excess of Na_2CO_3 over amino acid, and 1: 10 (v/v) mixture of dioxane and water as the solvent system has been recommended to be suitable [117]. However, the method is not appealing in the case of sterically hindered amino acids, Val and Leu. Consequently, performing the acylation in neutral conditions has been demonstrated to be an

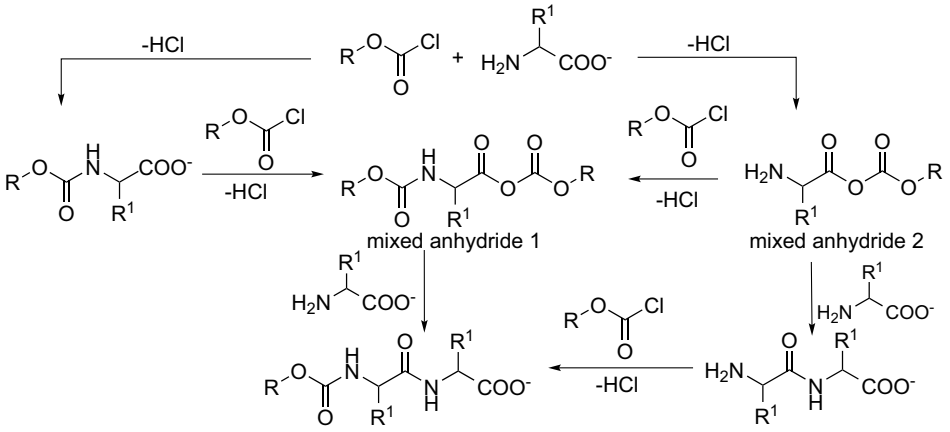
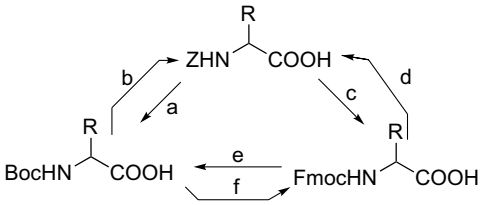


Figure 1.36 Reaction mechanism for the formation of oligopeptides during introduction of N^{α}-urethane protections.

efficient alternative. In a basic medium, the carboxy group exists as the carboxylate ion, which reacts with the chloroformate and forms a mixed anhydride. The usage of nonbasic reagents like zinc dust to eliminate HCl (liberated during acylation of the α-amino group by Fmoc-Cl) without raising the pH of the medium, prevents ionization of the carboxy group and in turn, the formation of mixed anhydrides. Thus, the possibility of the formation of a dipeptide is eliminated [118]. Acylation of *N,O*-bis-TMS-amino acids also avoids the formation of mixed anhydrides since the carboxy group in these compounds is protected as a silyl ester [119].

Dipeptide formation can also be avoided by substituting moderately reactive Fmoc-OSu and Fmoc-N_3 for highly active Fmoc-Cl as Fmoc donors. In these cases, only the α-amino group, which is more nucleophilic than the carboxy group, reacts with the reagents. However, the acylation can be comparatively sluggish and lower yielding particularly with Fmoc-N_3. Fmoc-OSu has been the reagent of choice and the best alternative to Fmoc-Cl for the preparation of N^{α}-Fmoc-amino acids for a long period. Rigorous high-performance liquid chromatography (HPLC) analysis has recently shown that the peptides synthesized using Fmoc-amino acids are contaminated with Fmoc-β-Ala-OH and Fmoc-β-Ala–Xaa-OH to the extent of 0.1–0.4%. The origin of these impurities has been explained based on a process that involves nucleophilic ring opening of the HOSu moiety of Fmoc-OSu and Lossen rearrangement of the resultant *O*-acyl hydroxamate intermediate as key reactions [120]. Consequently, Fmoc-2-mercaptobenzothiazole (MBT) has been proposed as an alternative to Fmoc-OSu [120]. This new Fmoc donor has shown to cause no formation of dipeptide as well as β-Ala impurities. The reagent can be prepared by the treatment of the DCHA salt of MBT with Fmoc-Cl in chloroform.

1.2.2.5.2 **Introduction of Urethanes via Transprotection** Interchange of N^{α} protections is relevant when a switch over of the chemoselectivity of protecting groups is required due to a change in the synthetic methods. In this regard, benzyl,



(a) cyclohexadiene, 10% Pd/C, $(Boc)_2O$, EtOH, rt; (b) TFA, DCM, 1h, 0 °C, Z-Cl, aq. Na_2CO_3, Dioxane; (c) H_2, 10% Pd/C, 2, 2′-dipyridyl, Fmoc-OSu, MeOH; (d) KF, TEA, DMF, benzyl- 5-norbornene-2,3-dicarboximido carbonate (Z-ONdc), rt; (e) KF, TEA, $(Boc)_2O$; (f) aqueous HCl, Fmoc-OSu, NaOH, rt, 6 h

Figure 1.37 Interconversion of N^{α}-urethane protections.

9-fluorenylmethyl and *tert*-butyl carbamates have been successfully interconverted (Figure 1.37) [121].

1.2.2.5.3 **Protection of the Nitrogen of α-Amino Acid *N*-Carboxy Anhydrides (NCAs) 122** *N*-Carboxy anhydrides (NCAs) **75** (Leuchs anhydrides or internal anhydrides of amino acids) represent simultaneously protected and activated amino acid derivatives. However, polymerization and rapid hydrolysis properties of NCAs make acylations involving them difficult. Nonetheless, protection of the nitrogen of NCAs converts them into efficient peptide coupling agents. Urethane-protected NCAs **76–78** (urethane-protected *N*-carboxy anhydrideUNCAs) are particularly advantageous since they retain the reactivity of NCAs, but exclude the possibility of oligomerization. In addition, they are stable, and can be crystallized and stored. Fmoc- and Z-protected NCAs are prepared by treating NCAs with the corresponding chloroformates (Figure 1.38). *N*-Methylmorpholine (NMM) is specifically used as base in this reaction since unlike most of the tertiary amines, it does not catalyze polymerization of the NCAs. Boc-NCAs **78** are prepared by using Boc_2O. On the other hand, Trt-protected NCAs **79** can be obtained directly from N^{α}-Trt-amino acids by treatment with triphosgene [123].

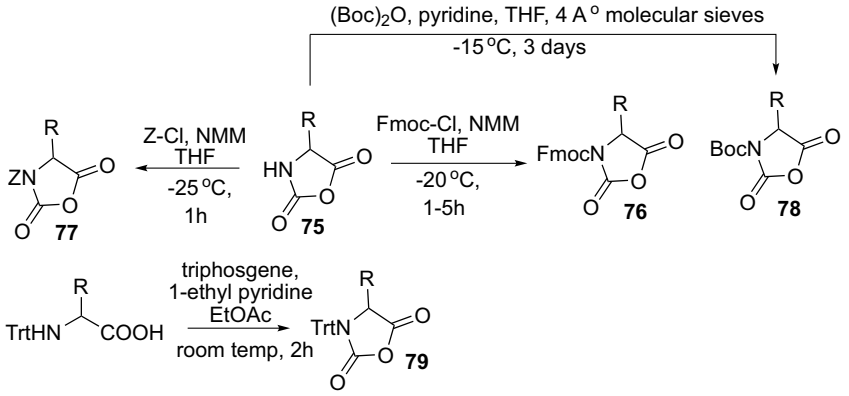
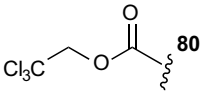


Figure 1.38 Protection of NCAs.

1.2.2.5.4 **N^{α},N^{α}-bis-Protected Amino Acids** Double protection of the nitrogen of the α-amino group is occasionally required to avoid side-reactions arising from the acidic -NH proton of the urethane linkage of N^{α}-protected amino acids/peptides. The N^{α},N^{α}-bis-Boc protection is introduced by the treatment of benzyl esters of Boc-amino acids with $(Boc)_2O$ in the presence of 4-(dimethylamino)pyridine (DMAP) in CH_3CN, followed by catalytic hydrogenation [124, 125]. The product is purified from the possible *N*-mono-Boc-contaminants by recrystallization in petroleum ether. N^{α}-Z,N^{α}-Boc-amino acids are prepared by treatment of 9-fluorenylmethyl esters of Z-amino acids with $(Boc)_2O$ in the presence of DMAP followed by cleavage of the ester with piperidine [125]. The N^{α},N^{α}-bis protected derivatives can be synthetically valuable compounds. The N^{α},N^{α}-bis-Boc-amino acids as well as N^{α},N^{α}-Boc, Z-amino acids can be converted into Boc- and Z-protected NCAs, respectively by the action of $SOCl_2$ in DMF [126]. This route presents an alternative to the preparation of UNCAs since it obviates the requirement of a NCA precursor. The bis-Boc derivatives can be transformed to mono Boc derivatives by selective elimination of one Boc moiety by treatment with hydrazine or LiOH or 20% magnesium perchlorate [127]. Under standard conditions of peptide coupling, the activated N^{α},N^{α}-bis-alkyloxycarbonyl-amino acids do not racemize via the formation of oxazol-5-one intermediates. However, enolization through base-catalyzed α-proton exchange (leading to racemization) can be promoted by the presence of an additional electron-withdrawing substituent on the α-amino group. This can be minimized through rapid couplings by using highly active Boc_2-amino acid fluorides as acylating agents [126, 128]. However, N^{α},N^{α}-bis-Boc protected dipeptides with C-terminal Gly show an increased tendency to form hydantoins rather than the corresponding Boc-dipeptides.

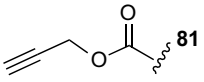
1.2.3
Other N^{α}-Protecting Groups

2,2,2-Trichloro-ethyloxycarbonyl (Troc)



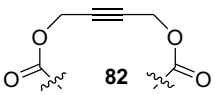
Cleaved using zinc or cadmium or their couples. Fission of alkyl-oxygen bond is thorough Grob fragmentation [129].

Propargyloxycarbonyl (Poc)



Stable to acids and bases. Removed using tetrathiomolybdate salt [130].

Bis-but-2-ynyloxycarbonyl (Bbc)



C_2 symmetric protecting group, introduced by treating amino acid methyl esters (2 equiv.) with Bbc-Cl followed by ester hydrolysis. The group is stable to acids (6 N HCl, 12 h) and bases (4 N NaOH, 12 h), removed using tetrathiomolybdate salt as well as resin-bound tetrathiomolybdate [131].

1.2.3.1 α-Azido Acids as α-Amino Acid Precursors

The conversion of the α-amino group into an azido group eliminates the problem of competing nucleophilicity. This transformation can be easily reversed to regenerate the α-amino group through reduction of the azide. α-Azido acids can be synthesized by azidolysis of α-bromo acids (synthesized by diazotization of amino acids in the presence of KBr or α-bromination of alkyl acids by *N*-bromosuccinimide (NBS)) with NaN_3 [132]. Alternatively, optically pure α-azido acids can be obtained in a single step by treating amino acids with triflyl azide [133]. α-Azido acids can be converted into α-azido acid bromides and chlorides (acid bromides of N^{α}-urethane-protected amino acids are not accessible; but N^{α}-Phth- and Nbs-amino acids can be converted to acid bromides), which are particularly useful for difficult couplings. They are very reactive, do not racemize (since they are unable to form oxazol-5-ones), and offer no steric hindrance to coupling [134]. Catalytic hydrogenation or treatment with phosphine followed by hydrolysis coverts the α-azido group into an α-amino group [135]. The azido compounds can be directly converted into a carbamate through reduction in the presence of a urethane donor [136]. *In situ* condensation into peptides is also possible through treatment with *tert*-butylphosphine in the presence of phenyl diselenide and an N^{α}-protected amino acid [137].

1.2.3.2 One-Pot N^{α} Protection and C^{α} Activation

The two key reactions in peptide synthesis, N^{α} protection and α-carboxy activation, can be executed in a single pot when pentafluorophenyl carbonates such as 9-fluorenylmethylpentafluorophenylcarboante (Fmoc-OPfp) are employed as urethane donors. N^{α}-Fmoc-amino acid pentafluorophenyl esters can be directly obtained by *in situ* esterification of the pentafluorophenol (liberated during acylation of the α-amino group by Fmoc-OPfp) by the addition of *N,N′*-dicyclohexylcarbodiimide (DCC) to the reaction mixture after the formation of N^{α}-Fmoc-amino acids [138]. This approach has been extended to prepare a variety of *N*-urethane-protected amino acid Pfp esters by treating amino acids with 2 equiv. of pentafluorophenyl carbonates (which display a dual role as urethane donors as well as carboxy activators). A series of Pg-Xaa-OPfp, where Pg = Aloc, Z, Boc, or Troc, has been synthesized via this method [139].

1.2.3.3 Effect of N^{α}-Protecting Groups in the Synthesis of NMAs

The two important approaches to synthesize NMAs are methylation of N^{α}-protected amino acids/esters and the reductive ring opening of oxazolidin-5-ones derived from *N*-protected amino acids [140]. The feasibility of the former approach is strongly dependent on the nature of N^{α} protection. N^{α}-Boc- and Z-amino acids are treated with a strong base such as NaH or KOH (finely powdered solid) to remove the NH proton of the carbamate followed by methylation using MeI or Me_2SO_4. The strongly basic condition is not compatible with substrates bearing the Fmoc moiety. An alternative and mild method is to use Ag_2O/MeI, but the product obtained will be in the form of a methyl ester, which is readily racemized during alkaline hydrolysis *en route* to its conversion into a free acid. On the other hand, N^{α}-sulfonamide-protected amino acids can be methylated using mild and racemization free conditions without the requirement of a strong base due to the increased acidity of the sulfonamide

nitrogen. N^{α}-Nosyl- as well as N^{α}-Nbs-protected amino acids are treated with methylating agents such as methyl *p*-nitrobenzyl sulfate in the presence of bases like 7-methyl-1,5,7-triaza-bicyclo[4.4.0]dec-5-ene (MTBD) or K_2CO_3 to obtain the corresponding NMAs. An even milder method is to use diazomethane as the methylating agent under neutral conditions. Site-selective methylation of the sulfonamide nitrogen of N^{α}-sulfonyl-protected peptide or an orthogonally protected amino acid derivative is also possible due to the substantial difference in the acidity of NH protons of the sulfonamide unit and peptide or carbamate bonds. Reduction of oxazolidin-5-ones obtained from N^{α}-protected amino acids by silyl hydrides in presence of TFA furnishes good yields of NMAs and is compatible with most of the urethane protections. It is especially useful for the synthesis of N^{α}-Fmoc-protected NMAs. The catalytic hydrogenation of oxazolidin-5-ones of Z-amino acids has been shown to directly give free amino NMAs [140b]. However, the usefulness of this route as an efficient means to access NMAs has not been undoubtedly established (elimination of formyl carbon from oxazolidinone upon removal of the *N* protection, thereby giving rise to amino acids has been observed in several cases).

1.3 Carboxy Protection

Protection of the carboxy group is not mandatory if carboxy activation and peptide couplings are carried out in separate steps (e.g., active ester coupling method). α-Amino acids can be acylated exclusively at the amino group since it is a much stronger nucleophile than the carboxy group. However, for couplings carried out via *in situ* activation of the carboxy group in the presence of the amino component (e.g., carbodiimide-mediated couplings), the carboxy group of the amino acid that is acylated has to be protected to avoid its self-condensation. However, irrespective of the kind of chemistry used for peptide bond formation, it has become a common practice to protect the carboxy group as part of a global protection strategy. The advantages associated with using carboxy-protected substrates for synthesis are: improved solubility in organic solvents, ease of purification and product isolation, suppression of the acidity of substrates, and prevention of side-reactions including racemization arising from the reactivity of the free carboxy group.

Since the carboxy protection is a semipermanent protection, it is expected to survive the conditions of repetitive deblocking of α-amino protections. The most important and extensively used method for carboxy group protection is esterification. Conversion into amides is another possibility, but this is not adopted as the amides represent irreversible protection due to the lack of methods for their selective hydrolysis in the presence of the peptide bond. Esters are cleaved via the fission of acyl-oxygen or alkyl-oxygen bonds. The cleavage parameters are determined by the structure of the alcohol component of the ester. Esters and carbamates which are derived from the same alcohol are cleaved via a fundamentally similar mechanism. Hence, there exists a colinear relationship between an ester–carbamate pair with respect to cleavage conditions. Boc and *tert*-butyl esters (cleaved by acids), Z and

benzyl esters (cleaved by hydrogenolysis), Fmoc and Fm esters (cleaved by base), and Aloc group and allyl esters (cleaved by Pd(0)-catalyzed allyl transfer) are the important examples for such a relationship.

1.3.1 Methyl and Ethyl Esters

There is essentially no difference between the unsubstituted methyl and ethyl esters in terms of methods of introduction as well as cleavage, although the latter are more resistant to attack by base and nucleophile. Methyl and ethyl esters are prepared by acid-mediated esterification of amino acids with the corresponding alcohol. Originally, anhydrous HCl was used in the preparation. A more efficient and milder protocol is to use $SOCl_2$ wherein the reaction can be carried out at 0 °C and the gaseous byproducts, HCl and SO_2, can be easily removed [141]. Amino acid methyl esters are isolated and stored as HCl salts, but have to be deprotonated to the free amino methyl esters for their utilization as amino components in coupling. The salts are generally deprotonated *in situ* by the addition of an equimolar quantity of tertiary amine followed by treatment with the acid component for peptide coupling. Nonetheless, this regular practice is associated with shortcomings such as difficulty in the addition of a stoichiometric amount of the tertiary amine, which may result in transfer of excess of base. The presence of excess base can promote side-reactions such as O-acylations and aspartimide formation, and racemization of the carboxy-activated residues. The rates of these reactions are influenced by the basicity of the tertiary amine. Using NMM as a preferred base for deprotonation can minimize these side-reactions. On the other hand, amino acid methyl esters can be obtained (whenever needed) by treating the suspension of the hydrochloride salt in organic solvent with aqueous $NaHCO_3$ or an equivalent amount of a tertiary amine (usually TEA or NMM) followed by extraction into EtOAc or Et_2O. The use of aqueous $NaHCO_3$ for deprotonation, although it eliminates side-reactions, can lead to reduction in the yields of amino acid methyl esters during extraction into the organic layer. Alternatively, deprotonation can be carried out in a base-free medium by using activated zinc dust as the proton scavenger. After deprotonation, the excess zinc and $ZnCl_2$ are removed by filtration, and the organic solvent is evaporated to obtain amino acid methyl esters in quantitative yields [142]. The acidic condition of preparation may render peptide and acid-sensitive protections unstable. Hence, methyl esters of N^{α}-protected (and side-chain protected) amino acids and peptides are prepared by using diazomethane (ethereal solution at 0 °C) – a mild and efficient methylating agent [143]. Diazo(trimethylsilyl)methane is a safer alternative to diazomethane (toxic and explosive).

Methyl as well as ethyl esters are stable to acidolysis (HBr in AcOH, TFA), catalytic hydrogenation, and to nucleophiles such as thiols and amines. They are cleaved by saponification with methanolic or ethanolic KOH or NaOH or LiOH in a wide range of solvents such as DMF, dimethyl sulfoxide (DMSO), pyridine, and aqueous mixtures of alcohol, acetone, EtOAc, and dioxane [144]. Methyl esters of small peptides are hydrolyzed within 0.5–1.5 h at room temperature, but hydrolysis

becomes increasingly difficult with the length of the peptide chain. Methyl esters of long-chain peptides require excess alkali and longer reaction times and sometimes elevated temperatures for completion of cleavage. The Fmoc group is not stable under the standard conditions of methyl ester cleavage. Nonetheless, addition of 0.8 mmol of $CaCl_2$ has been shown to increase the lifetime of Fmoc protections in alkaline hydrolytic conditions (1.2 equiv. of NaOH in *iso*-propanol/water mixture at 20 °C) [145]. Two equivalents of LiOH in THF at 0 °C has also been found to cause selective cleavage of methyl ester in the presence of N^{α}-Fmoc function [146]. The base used for methyl ester cleavage can promote racemization (either by base-catalyzed α-hydrogen exchange or 5(4*H*)-oxazolone formation) of the C-terminal amino acid (which is more pronounced in the case of peptides containing NMAs and Cys(Bzl) residues), DKP formation, and hydantoins from Z-Xaa–Gly-OMe peptides. Alkaline treatment can also promote the formation of ΔAla from hydroxy protected Ser residues. In this regard, tetrabutylammonium hydroxide (TBAH) has been proposed as the reagent that cleaves methyl esters best without causing racemization [147]. Trimethylsilyliodide (TMS-I) and bis-(tributyltin) oxide (BBTO) can be used as nonbasic reagents to circumvent many of the base-catalyzed side-reactions [148].

1.3.1.1 Substituted Methyl and Ethyl Esters

These esters (**83–90**) are cleaved under different conditions than the simple methyl or alkyl esters. Selected examples are shown in Table 1.4.

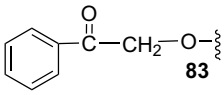
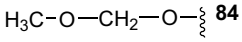
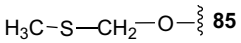
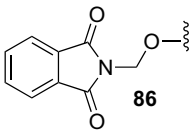
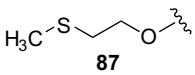
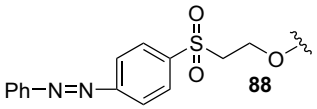
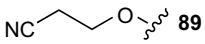
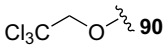
1.3.2 Benzyl Ester

Benzyl esters were introduced to peptide synthesis [157] along with the Z group, both of which are cleaved by catalytic hydrogenation and acidolysis, although the ester is comparatively less reactive to the latter reaction. Benzyl esters are stable to moderate basic conditions and to nucleophiles. Amino acid benzyl esters are typically prepared by esterification with benzyl alcohol in the presence of TosOH in benzene or toluene under azeotropic removal of water in a Dean–Stark apparatus [158]. This esterification has been efficiently carried out within minutes under microwave irradiation [159]. Dibenzyl esters of Asp and Glu can also be prepared by the same protocol and the ω-benzyl ester can be selectively cleaved by HI to obtain α-carboxy monobenzyl ester [160]. Benzyl esters of amino acids are obtained as TosOH salts which can be stored under anhydrous conditions. They are deprotonated using procedures similar to that of methyl esters. N^{α}-Protected amino acid benzyl esters can be prepared by the treatment of cesium salt of the corresponding amino acids with benzyl bromide [161] or by DCC-mediated esterification with benzyl alcohol in the presence of DMAP catalyst [162].

1.3.2.1 Cleavage

Quantitative cleavage is accomplished through catalytic hydrogenation in solvents such as alcohols and dioxane usually within 1 h for small peptides, but prolonged duration is required for long-chain peptides. Benzyl esters of sulfur-containing

Table 1.4 Substituted methyl and ethyl esters.

Ester	Cleavage conditions
Phenacyl (Pac)[a)]  **83**	Zn/AcOH at room temperature, NaSPh in DMF, TBAF in DMF at room temperature (30 min) [149]
Methoxymethyl (Mom)  **84**	$MgBr_2$ in Et_2O at room temperature (several hours) [150]
(Methylsulfanyl)methyl (Mtm)  **85**	Oxidation of sulfide into sulfonyl by H_2O_2 or ammonium molybdate or CH_3I followed by alkali hydrolysis [151]
Phthalimidomethyl  **86**	Zn/AcOH, HBr in AcOH, NaSPh, NH_2-NH_2 [152]
2-(Methylsulfanyl)ethyl[b),c)]  **87**	Oxidation of the sulfide moiety into sulfonyl group by H_2O_2/ammonium molybdate followed by alkali hydrolysis (12–24 h); alkylation to sulfonium salt by CH_3I followed by rapid hydrolysis at pH 10 (10-15 min) [153]
2-(4-Phenylazophenyl sulfonyl)ethyl  **88**	NaOH, pH 10–11, H_2O/dioxane [154]
2-Cyanoethyl (Cne)  **89**	10% aqueous K_2CO_3 [155]
2,2,2-Trichloroethyl (Tce)  **90**	Zinc in 90% AcOH (cleavage similar to Troc group) [156]

a) Catalytic hydrogenation reduces the oxo group to form the 2-phenylethyl ester that is stable to reductive cleavage.
b) Oxidation or alkylation generates an electron-withdrawing group β to the ester, treatment with base then cleaves the ester through fragmentation via β-elimination.
c) Acidolytic cleavage of protections can cause alkylation of the sulfur atom.

peptides can be cleaved without poisoning of the catalyst by carrying out catalytic hydrogenation in liquid NH_3 solvent at $-33\,^{\circ}C$ (boiling point of NH_3) for 16–18 h [163]. Benzyl esters are cleaved through CTH (e.g., Pd/C, 85% HCOOH) within minutes at 25 °C [47]. Anhydrous liquid HF treatment as well as by Birch reduction (Na-liquid NH_3) and saponification can also be employed to cleave the ester quantitatively [164]. Benzyl esters can be converted to TBDMS esters by treatment with Pd(II)acetate in the presence of TBDMS-Cl.

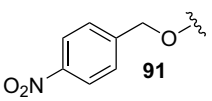
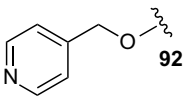
1.3.3
Substituted Benzyl Esters

Although benzyl esters offer excellent protection of the α-carboxy group, they are not completely stable to repetitive acidolytic steps used for deblocking N^{α}-Boc group (in Boc/benzyl protection strategy). To combat this limitation, substituted benzyl esters, which contain an electron-withdrawing group on the *para* position of the phenyl ring (e.g., 4-nitrobenzyl **91** and 4-pyridylmethyl **92**), have been introduced (Table 1.5). The substitution destabilizes the benzyl cation, which is a key intermediate during acidolytic cleavage, thus increasing the resistance of the ester to acids. However, the substitution increases the lability of the ester to alkaline hydrolysis.

1.3.4
tert-Butyl Ester

tert-Butyl esters are the widely used α-carboxy-protecting groups due to their remarkable stability to bases and nucleophiles as well as to catalytic hydrogenation, which make them suitable to be used in combination with Fmoc and Z groups [167]. They possess cleavage characteristics similar to Boc group. They are stable to weak acidic conditions that cleave extremely acid sensitive groups like Bpoc, Nps, and Trt. Stability of *tert*-butyl ester to nucleophiles when compared to *n*-alkyl esters can be attributed to

Table 1.5 Substituted benzyl esters.

Substituted benzyl ester	Preparation	Cleavage conditions
4-Nitrobenzyl (Nbz) [165]  **91**	Azeotropic distillation of amino acids with *p*-nitrobenzyl alcohol in toluene	Catalytic hydrogenation, reduction with Zn/AcOH, $SnCl_2$/DMF/AcOH, alkali hydrolysis
4-Pyridylmethyl (Pic) [166]  **92**	Treatment of N^{α}-protected amino acids with (4-chloromethyl) pyridine in the presence of TEA DCC-mediated esterification with pyridine-4-methanol	Catalytic hydrogenation, Na/liquid NH_3, 1 N NaOH in dioxane

the bulkiness of the group, which offers resistance to substitution at the ester carbonyl. However, exposure to high concentration of base for a long time can hydrolyze the ester.

tert-Butyl esters of amino acids as well as N^{α}-protected amino acids are prepared by treatment with isobutene in organic solvent in the presence of H_2SO_4 or HCl or TosOH [168]. Transesterification with commercially available *tert*-butyl acetate is also an efficient method of preparation [169]. Both these methods cause *O*- and *S*-alkylations. Hence, the hydoxy groups of N^{α}-Z-Thr/Ser are protected (with acetoacetyl group removable by treatment with hydrazine in EtOH) before esterification. Treatment of silver salts of N^{α}-protected amino acids with *tert*-butyl iodide is a mild method of preparation [170]. A combination of Boc-F and DMAP in CH_2Cl_2-*tert*-butanol (room temperature, 4 h) [171] and *tert*-butyl bromide in the presence of K_2CO_3 [172] also produce good yields of *tert*-butyl esters of Z-amino acids. Direct esterification of N^{α}-protected amino acids with *tert*-butanol can be done in the presence of $MgSO_4$ and H_2SO_4 [173] or $(Boc)_2O$ and catalytic amount of DMAP [174]. Alcoholysis of the mixed anhydride intermediate (generated by treatment of N^{α}-protected amino acid with 2,4,6-trichlorobenzoyl chloride in the presence of TEA) with *tert*-butanol also yields the esters [175]. Amino acid *tert*-butyl esters (distillable liquids) are stable and can be stored for months. Further, the free amino dipeptide esters do not undergo ring closure to DKP. Essentially, the reagents used for Boc cleavage can be used to cleave *tert*-butyl esters. Similar to the cleavage of Boc group, *tert*-butyl cation or *tert*-butyl trifluoroacetate formed during acidolysis can alkylate nucleophilic sites, which is suppressed by the addition of scavengers. Selective removal of the Boc group in the presence of *tert*-butyl ester can also be carried out (see Section 1.2.2.4.1).

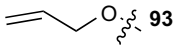
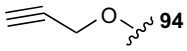
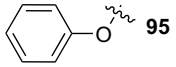
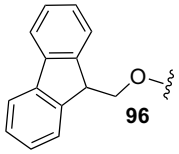
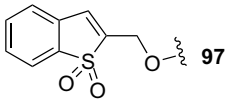
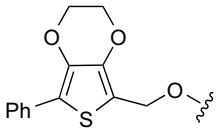
1.3.5
Other Acid-Labile Esters

Many acid-labile alkyl esters have been developed. In the order of increasing resistance to acidolysis, these esters can be arranged as: trityl (Trt) < diphenylmethyl (Dpm) = *tert*-butyl (*t*Bu) = 4-methoxybenzyl (Mob) = pentamethylbenzyl (Pmb) = trimethylbenzyl (Tmb) = phthalimidomethyl (Ptm) = 9-anthrylmethyl (Am) < benzyl [1]. 1-Adamantyl (1-Ada) esters have acid sensitivity similar to the Boc group, but are stable to treatment with 4 M HCl in anhydrous dioxane at room temperature for 25 min, a condition that removes the Boc group. Other important carboxy protections are summarized in Table 1.6.

1.3.6
Temporary α-Carboxy Protection

Temporary or transient α-carboxy protections are employed to suppress the activation of the carboxy group generally during the synthesis of short peptide acids or introduction of N^{α}-urethane protections. TMS esters are the commonly used temporary carboxy protections. They are quite stable in a nonaqueous medium but are instantaneously hydrolyzed upon exposure to very mild acid or base, or only to water. Hence, the ester can be quantitatively cleaved during aqueous work-up itself.

Table 1.6 Selected carboxy protections.

Protection	Cleavage conditions
Allyl  **93**	Similar to Aloc group Pd(0)-catalyzed allyl transfer in the presence of nucleophile [176]
Propargyl  **94**	Formation of alkyne–cobalt complex by reaction with complexes such as $Co_2(CO)_8$ followed by acidolysis with TFA [177]
Phenyl  **95**	Rapid saponification by treatment with alkali (these esters show weak acylating activity) [178]
9-Fluorenylmethyl (Fm)  **96**	Similar to Fmoc group[a)]
1,1,-Dioxobenzo[*b*]thiophene-2-yl (Bsm)  **97**	Similar to Bsmoc group[a)]
Phenyl-3,4-ethylenedioxythiophene (EDOT) 	0.01–0.5% TFA in DCM (the ester can be selectively removed over Boc) [179]
Amides α-carboxamides	Enzymatic hydrolysis through peptide amidase isolated from flavedo of oranges (optimum pH range: 6.8–8.4)
Tertiary amides	Potassium *tert*-butoxide (in water) in Et_2O selectively acts on tertiary amides without hydrolyzing peptide bonds (secondary amides) [180]

a) The combination of the Fm ester-Bsmoc group and Bsm ester-Fmoc group permits selective modification of the protections (β-elimination versus Michael acceptor units) by appropriate choice of the base; 2% TAEA and DBU remove only Bsmoc and Fmoc units, respectively.

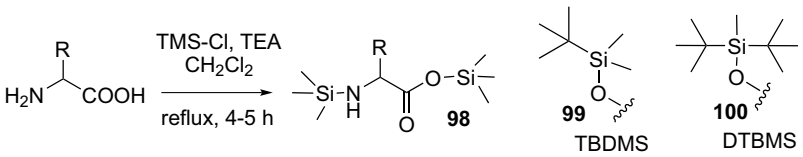


Figure 1.39 Silyl ester protection.

TMS esters of amino acids are obtained as *N,O*-bis-silyl derivatives **98** by the treatment of amino acids with TMS-Cl in the presence of TEA in refluxing DCM for 4–5 h (Figure 1.39) [181]. With Ser, Thr, and Tyr, the trisilylated derivative is formed. The *N,O*-bis-silyl amino acids are soluble in organic solvents, but decompose gradually upon standing. They are used as such for reactions without isolation. TBDMS **99** and di-*tert*-butylmethylsilyl (DTBMS) **100** esters (Figure 1.39) are comparatively more resistant to hydrolysis due to the presence of bulkier substitutions on the silicon atom [182, 183].

1.3.7
α-Carboxy Protectors as Precursors to Useful Amino Acid Derivatives: Formation of Acid Hydrazides

The esters of N^{α}-amino acids and peptides, particularly methyl and ethyl esters, can be directly converted into synthetically useful amino acid derivatives such as amino acid hydrazides **101**, which in turn can be converted to acid azides **102** or diimides (Figure 1.40) that are effective peptide coupling agents [184]. Diazotization of hydrazides with an N^{α}-Boc residue is also possible, although the reaction is carried out in the presence of HCl due to the very low temperature ($-30\,^{\circ}C$) employed.

1.4
Side-Chain Protection

1.4.1
ω-Amino Group of Diamino Acids

The ω-amino group of diamino acids, Lys and the nonproteinogenic Orn, 1,3-diaminobutyric acid (Dab), and 1,2-diaminopropionic acid (Dap), is strongly nucleophilic and entails compulsory protection irrespective of the method of peptide bond

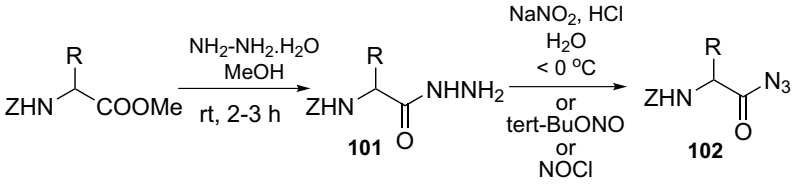


Figure 1.40 Preparation of Z-amino acid azides from methyl esters.

formation. In fact, the ω-amino group is more nucleophilic than the α-amino group due to the absence of a geminal electron-withdrawing group as in case of the latter. Fundamentally, all the groups employed as N^{α} protection can be used as N^{ω} protection. However, the stability and sometimes even the chemoselectivity of the N^{ω} and N^{α} protections differ from each other. Since the ω-amino group is a stronger nucleophile, the protection on it is more stable (to acids and bases), except in the case of the comparatively less nucleophilic β-amino group of Dap. Nonetheless, there is more freedom in the choice of a protecting group for the ω-amino group than that for the α-amino group. Since the vicinity of the ω-amino group is devoid of a sensitive chiral center or an activated carboxy moiety, the N^{ω} protection generally has a lower tendency to cause racemization or offer steric hindrance to coupling. Hence, acyl-type and bulkier groups, which are less useful for N^{α} protections, pose no difficulty as N^{ω} protectors.

Symmetrically protected diamino acids with identical N^{α},N^{ω} protection (e.g., Fmoc-Lys(Fmoc)-OH) can be synthesized in a straightforward manner by using 2 equiv. of an acylating agent. However, these compounds are not very useful for peptide synthesis since selective removal of N^{α} protection from them for subsequent acylation is not always successful (a rudimentary way to address this is to exploit the difference in the stabilities of N^{ω} and N^{α} protections, and deblock the latter with mild reagents with an acceptable level of selectivity). Therefore, only diamino acids that contain orthogonal N^{α},N^{ω} protections, and thus allow for selective removal of the N^{α} protection and in turn for chain extension in a linear fashion, are employed for peptide synthesis. The widely adapted approach to introduce differential N^{α},N^{ω} protections is the complexation method (Figure 1.41). Here, the α-amino group is made unreactive by trapping it in a Cu(II) complex and the free ω-amino group is acylated to introduce the N^{ω} protection. The complex is later decomposed by treatment with H_2S, Na_2S, thioacetamide or ethylenediaminetetraacetic acid (EDTA) or 8-quinolinol to liberate the α-amino group, which can then be acylated with the second reagent to introduce the N^{α} protection [185]. When acid stable N^{ω} protections are present, 6 N HCl can be used for decomposition of the copper complex. This method can also be executed with $BF_3{\cdot}Et_2O$ as the complexating agent. However, the approach is not successful in the case of Dap since it can form a six-membered

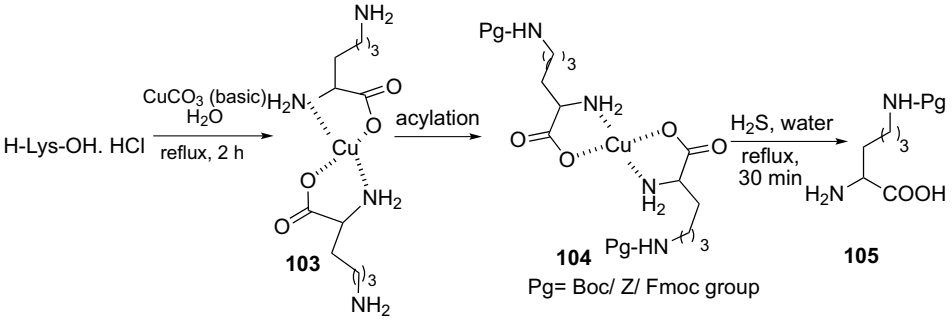


Figure 1.41 N^{ω}-Protection of Lys through complexation.

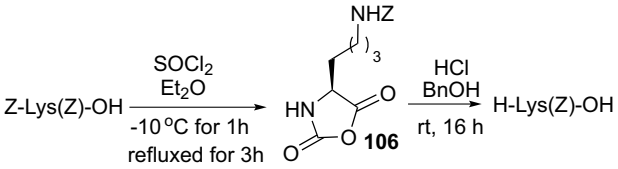


Figure 1.42 Preparation of H-Lys(Z)-OH from Z-Lys(Z)-OH.

chelation complex (in which the ω-amino group is trapped). Another approach is to make use of the greater nucleophilicity of the ω-amino group and selectively acylate it with moderately active reagents. This has been applied to introduce N^{ω}-For, Tfa, Boc, and Z groups by using *p*-nitrophenylformate (For-ONp), thioethyl trifluoroacetate, *tert*-butyloxycarbonyl-4-nitrobenzoate, and benzyloxycarbonyl phenolate, respectively [186]. In some cases, symmetrically substituted diamino acids such as Z-Lys(Z)-OH can be converted into the corresponding NCAs (Lys(Z)-NCA **106**) with the loss of only the N^{α} protection (Figure 1.42). The NCA can be hydrolyzed later to obtain ω-amino-protected diamino acids [187]. The widely used protections of the ω-amino group are summarized in Table 1.7.

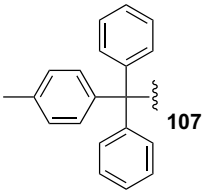
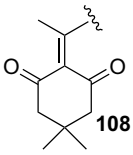
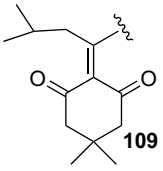
1.4.2 Guanidino Group of Arg

Ideally, all the three nitrogens (N^{δ}, N^{ω}, and $N^{\omega\prime}$) of the guanidino group in Arg have to be protected to completely suppress the nucleophilicity of the group. The unprotected δ, ω′, and ω-nitrogens can be involved in the formation of δ-lactams (**110**), cycloheptane derivatives (**111**), and deguanidilation of Arg to Orn (**112**), respectively (Figure 1.43). However, an ideal protection represented by N^{δ},N^{ω},$N^{\omega\prime}$-*per* derivatized guanidino group has not yet been devised (except Trt-Arg(Trt)$_3$-OMe, but its utility in peptide synthesis is yet to be established). Hence, in practice, N^{ω}- or $N^{\omega\prime}$-monoprotected and N^{δ},N^{ω}- or N^{ω},$N^{\omega\prime}$-bis-protected Arg derivatives are employed. The effectiveness of the protection is determined by the electron-withdrawing property or bulkiness (or by a combination of both these factors) of the protecting group(s), and on factors like synthetic strategy and activation method.

1.4.2.1 Protection Through Protonation

The strongly basic guanidino group ($pK_a = 12.5$) exists in its protonated form for most of the time in peptide coupling and hence is protected against side-reactions. Protonation is carried out with HCl, perchlorate pyridinium salt, and even with weakly acidic HOBt, but most effectively by HBr (due to the counter-ion effect) [196]. However, the N^{ω}-protonated Arg can be partially deprotonated during coupling by moderately basic components, even by H-Pro-*t*Bu, in the reaction mixture. In addition, the C-terminal residue is likely to cyclize to give a δ-lactam even in the protonated form.

Table 1.7 ω-Amino protections of diamino acids.

Protections	Stability	Cleavage
For		Methanolic HCl (room temperature) [188]
Tfa	Acids	Alkali, 1 M piperidine in water [189]
Mtt	Bases and nucleophiles	1% TFA in DCM [190]
 **107**		
Boc	Catalytic hydrogenation, bases and nucleophiles	Neat TFA or 25–50% TFA in DCM [191]
Z(2-Cl)	Bases, nucleophiles, mild acids	Catalytic hydrogenation, anhydrous liquid HF [192]
Dde[a)]	Acids, piperidine, DBU	2% Hydrazine hydrate in DCM [193]
 **108**		
ivDde (or Ddiv)[a)]	Acids, piperidine, DBU	2% Hydrazine hydrate in DCM [194]
 **109**		
Nbs	Acids, bases	β-Mercaptoethanol, DBU in DMF [195]

a) Migration of the Dde moiety on to the ω-amino group of neighboring unprotected diamino acid residues takes place during piperidine-mediated Fmoc removal. This migration is suppressed with the ivDde group.

1.4.2.2 Nitration

$Arg(NO_2)$ is commercially available and can also be prepared by the action of a nitrating mixture on Arg (Figure 1.44). The α-amino group of $Arg(NO_2)$ can be acylated in the usual manner to obtain N^{α}-protected $Arg(NO_2)$ [197]. The strong

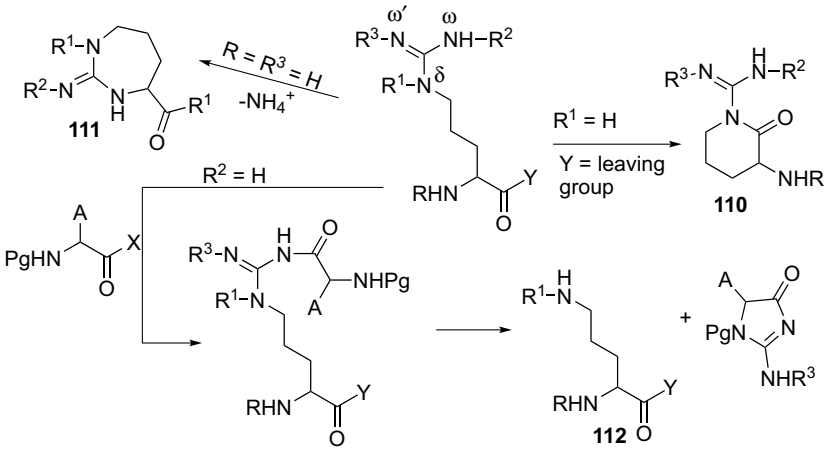


Figure 1.43 Side-reactions of unprotected Arg residues.

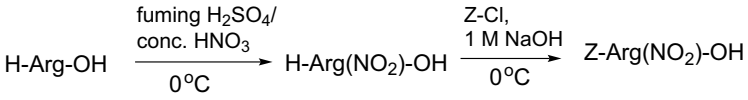


Figure 1.44 Preparation of Arg(NO_2) and N^{α}-protected Arg(NO_2).

electron-withdrawing property of the NO_2 group satisfactorily suppresses δ-lactam formation and N^{ω}-acylation, although a few exceptions have been found [197b,c]. The group is stable to mild acids and bases and is removed by catalytic hydrogenation, CTH (Trp can also undergo hydrogenation) and reduction by $SnCl_2$ [198]. On-resin removal of the group can be done via anhydrous liquid HF treatment. Other common Arg protectors are summarized in Table 1.8.

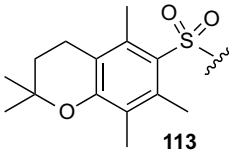
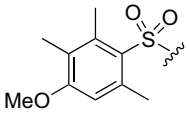
1.4.2.3 **Arg Precursors**

An Orn residue in peptides can be converted to an Arg residue through guanidilation of the δ-amino group by reagents such as *N,N*^{1}-bis(alkoxycarbonyl)thiourea **115**, *N,N*^{1}-bis(alkoxycarbonyl)-2-triflylguanidine **116**, or *N,N*^{1}-bis(alkoxycarbonyl)pyrazole-1-carboximidamide **117** (Figure 1.45) [206]. However, site-specific guanidilation in the presence of multiple diamino acid residues requires an additional level orthogonality in the protection of ω-amino groups. Also, guanidilation of multiple Orn residues is difficult.

1.4.3 **Imidazole Group of His**

Unprotected C-terminal His residues are racemized (e.g., His acid azide **118**) either by an autocatalyzed α-proton abstraction (by the π-nitrogen of the imidazole ring acting as a base) followed by enolization or by the formation of optically labile

Table 1.8 Arg protections (selected examples).

Protection	Cleavage conditions
N^{ω}-Ts[a]	HF/anisole at 0 °C, fluorosulfonic acid/anisole, Na/liquid NH_3) [199]
N^{ω}-2,2,5,7,8-Pentamethylchroman-6-sulfonyl (Pmc)[b]	TFA with several combinations of scavengers (i.e., anisole, PhSMe, Ph-OH, iPr$_3$SiH, and ethane-1,2-dithiol) [200]
 **113**	
N^{ω}-Pbf[b]	TFA/phenol in the presence of iPr$_3$SiH [19]
N^{ω}-4-Methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr)	95% TFA/thioanisole; TFA/thioanisole in the presence of phenol and thiols [201]
 **114**	
N^{ω},$N^{\omega'}$-bis-Boc	TFA in the presence of scavengers [202]
N^{ω}-Boc	TFA in the presence of scavengers [203]
N^{δ},N^{ω}-bis-Adoc[c]	Neat TFA [204]
N^{δ},N^{ω}-bis-Z	Catalytic hydrogenation [205]

a) Deprotection of amino group of Arg(Ts) can lead to Orn formation, Na/liquid NH_3 treatment may cause desulfurization, racemization, and several decomposition reactions.
b) Pbf causes lower level of sulfonylation of Trp than Pmc.
c) α-amino free Arg(Adoc)$_2$ residues cyclize to cycloheptane derivatives in MeOH.

imidazolides **120** (Figure 1.46). In addition, the imidazole group of His undergoes acylation, in which case active acyl imidazoles that can cause undesired acyl transfers are formed.

In solution the His derivatives exist as a mixture of rapidly interconverting (through proton exchange) tautomers. Upon reaction with an electrophile, mono-

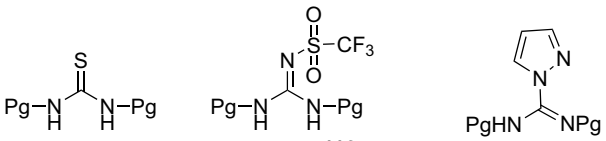


Figure 1.45 Guanidilating agents for conversion of Orn to Arg.

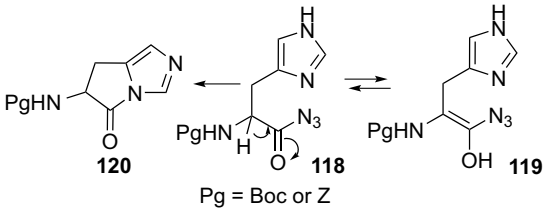


Figure 1.46 Racemization of unprotected His.

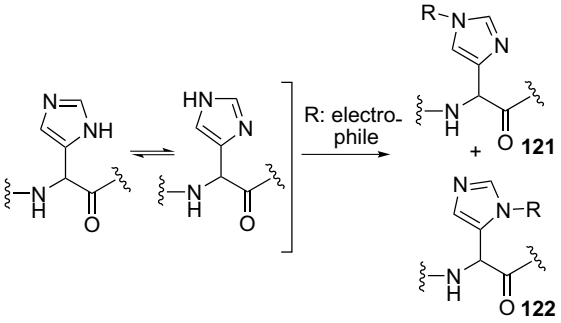


Figure 1.47 Electrophilic substitution of the indole ring of unprotected His.

substituted products are formed predominantly over N^{τ},N^{π}-disubstituted products. The product is typically a 3 : 1 mixture of stable N^{τ}-His **121** and N^{π}-His **122** (Figure 1.47). They are generally inseparable, with few exceptions such as the separation of the N^{τ} isomer (the major product) from the mixture of N^{α},N^{τ}-bis-Boc-His-OMe and N^{α},N^{π}-bis-Boc-His-OMe [207]. The proportion of the regioisomers formed can vary depending on the nature of the electrophilic reagent, structure of the substrate and reaction conditions, but in majority of the cases, the N^{τ} derivative predominates. Access to single N^{τ} isomers is advantageous as they can be used as substrates for the preparation of N^{π} derivatives that are usually not directly obtainable by electrophilic substitution of unprotected His. Preparation of N^{α}-Boc,N^{π}-Bom-His-OH **125** by employing N^{α},N^{τ}-bis-Boc-His-OMe **123** as starting material is shown in Figure 1.48 [207]. Formation of N^{τ},N^{π}-disubstituted products is usually rare, unless an excess amount of electrophilic reagent or unhindered reagents are used.

The presence of an electron-withdrawing group on the N^{π} position prevents autocatalyzed racemization. For instance, it was observed that the activated N^{α}-Z,N^{π}-

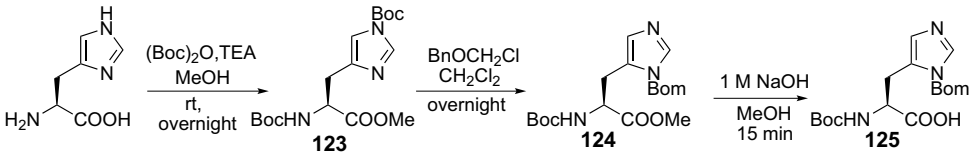
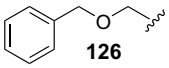


Figure 1.48 Preparation of N^{π}-Bom-substituted His.

Table 1.9 His protections.

Group	Stable to	Cleavage Conditions
N^{τ}-Trt	Bases and nucleophiles	Anhydrous liquid HF, HBr/AcOH [25, 209]
N^{π}-Benzyloxymethyl (Bom)	Bases and nucleophiles and TFA	Hydrogenolysis, acidolyis (addition of scavenger required to trap the byproduct HCHO formed during cleavage) [207]
 **126**		
N^{π}-Boc	Catalytic hydrogenation, bases and nucleophiles	TFA in the presence of scavengers [210]
N^{π}-Pac	Acids	Zinc in AcOH [208]

Pac-His-OH did not racemize, while the corresponding N^{τ} isomer did [208]. Nonetheless, even an N^{τ} substitution can be helpful in minimizing racemization since it decreases the nucleophilicity of the N^{π}-nitrogen by its electron-withdrawing effect. However, this is not always guaranteed as the level of suppression can still depend upon factors like the activation method. The widely used His-protecting groups are summarized in Table 1.9.

1.4.4 Indole Group of Trp

The unprotected indole group of Trp can undergo alkylation, sulfonylation, oxidation (to produce oxyindolyl derivative **127**), and dimerization (due to self-acylation by protonated Trp) during acidolytic cleavage of protections (Figure 1.49). *tert*-Butylation and nitrosation of the indolyl moiety is observed during Boc group removal and diazotization of acid hydrazides of Trp, respectively. Addition of scavengers during cleavage can significantly bring down the extent of these side-reactions. The

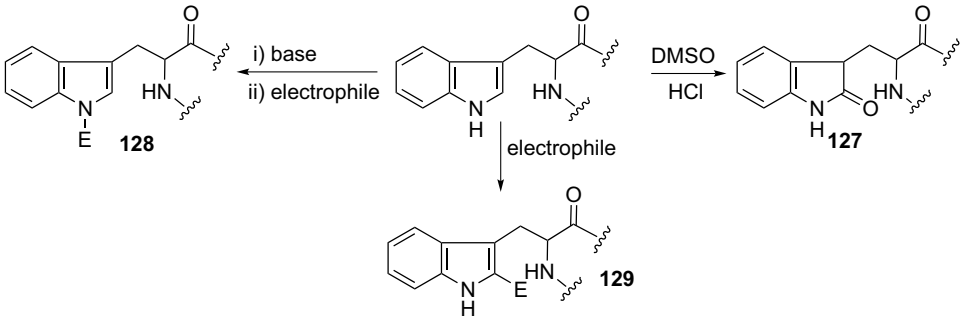


Figure 1.49 Side-reactions of unprotected Trp.

expulsion of air and oxidants and pretreatment of the solution of Trp with indole can avoid oxidation. Both 1- (in strongly basic medium **128**) and 2-positions **129** of the indole residue are susceptible to electrophilic substitution. However, in the proposed protection strategy, an electron-withdrawing group is placed on the ring nitrogen (1-position) – a method that also ensures that the electron availability in the heteroaromatic ring is decreased, thereby protecting the 2-position simultaneously.

The N^{in}-For group is stable to mild acids, and cleaved by treatment with hydrazine, piperidine in DMF, and anhydrous liquid HF-thiols [211]. The N^{in}-Boc group is stable to bases and catalytic hydrogenation, and is removed by treatment with TFA [212]. However, it is more stable to acidolysis and, further, the cleavage results in a stable carbamic acid intermediate, which undergoes decarboxylation slowly. The process can be accelerated by exposure to aqueous acids, which is usually at the work-up stage. In any case, the presence of an electron-withdrawing carboxy group on the *in*-nitrogen provides extended protection through the synthesis. The cyclohexyloxycarbonyl (Hoc) group is remarkably stable to bases and is removed by anhydrous liquid HF/*p*-cresol. The N^{in}-Aloc group is stable to acids and is removed by a Pd(0)-catalyzed allyl transfer reaction [213]. The group is labile to piperidine but stable to DBU. Hence, in the presence of this group, Fmoc is deblocked using DBU and not piperidine.

1.4.5
ω-Amido Group of Asn and Gln

The ω-amido group of C-terminal Asn and Gln (to lesser extent) is converted to a cyano group forming nitrile **130**, when the activation is carried out with carbodiimides or uronium-type reagents, or, to some extent, even with mixed anhydrides (Figure 1.50). However, the ω-amido group of N-terminal and as well as the *endo* Asn/Gln residues (as well as α-carboxamides) do not undergo dehydration. This suggests that dehydration occurs via a cyclic intermediate (an isoimide) formed by the intramolecular attack of the ω-amide oxygen on an activated carboxy group. Activation of Asn and Gln also leads to the formation of stable imides. The aspartimides **131** can be aminolyzed to Asn-containing peptides, but in the process β-aspartyl peptides are also formed. The imides can also be optically labile leading to racemization. The unprotected α-amino free Gln residues undergo cyclization to pyroglutamic acid **132** in acidic media (Figure 1.51). These side-reactions can be minimized by protecting the amido group of Asn and Gln. Also, the ω-amido protections increase the solubility of glutamine peptides, which are known to aggregate otherwise due to intermolecular

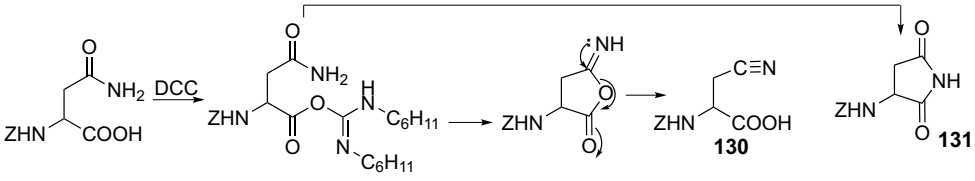


Figure 1.50 Formation of nitrile and aspartimide from unprotected Asn.

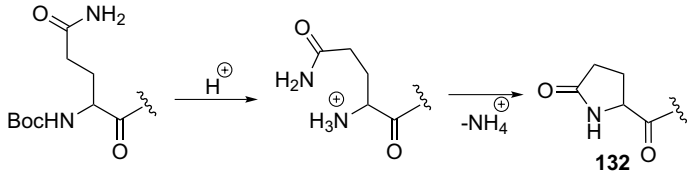


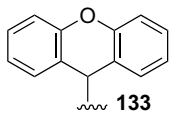
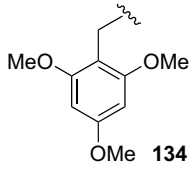
Figure 1.51 Acid-mediated formation of pGlu.

hydrogen bonding involving the unprotected ω-amido group. The widely used Asn and Gln protections are shown in Table 1.10.

1.4.6
β-Thiol Group of Cys

The thiol group of Cys is vulnerable to attack by electrophiles. Acylation of the thiol function of Cys during peptide coupling is not a serious concern since the *S*-acyl Cys (or any thioesters *per se*) are too reactive to nucleophiles and hence the transformation can be easily reversed. When *S*-acylation of N-terminal Cys takes place, the resulting thioester readily rearranges to a Cys peptide via an $S \rightarrow N$ acyl migration. In fact, this acyl transfer reaction has formed the basis for the development of native chemical ligation, a technique useful for linking two unprotected peptide fragments [217]. Self-

Table 1.10 ω-Amide protections (selected examples).

Protection	Stable to	Cleavage conditions
Trt	Catalytic hydrogenation, alkali, bases, and nucleophiles, dilute acids	95% TFA (10 min); when α--amino group is free, detritylation takes longer duration [214]
9H-Xanthen-9-yl (Xan)  **133**	Catalytic hydrogenation, bases	TFA/anisole (72 °C, 30 min), HBr/AcOH (room temperature, 1–2 h) [215]
2,4,6-Trimethoxy-benzyl (Tmb)  **134**	Catalytic hydrogenation, bases	TFA/anisole (room temperature, 5 min), HBr/AcOH (room temperature, 1–2 h), [216]

acylation of activated Cys can be prevented via a temporary and internal protection through conversion into thiolactones. The latter can be opened up by amines to yield a peptide incorporating Cys [218]. The unprotected thiol function can be irreversibly alkylated by electrophiles generated during the acidolytic removal of protections (Boc and Z groups). Peptides that contain N-terminal Cys can condense with HCHO released during the HF-mediated removal of the N^{π}-Bom group and form thiazolidine carboxylic acids (Thz peptides). The unprotected thiol moieties are also prone to oxidative formation of disulfide. In the case of the synthesis of peptides containing cystine (a dimer of Cys cross-linked by a -S–S- linkage), the regioselective formation of the disulfide bond is an essential step. Nevertheless, unprotected Cys(s) do not always guarantee regioselective disulfide formation. Consequently, the thiol group of Cys is compulsorily protected during synthesis. The important thiol protections are summarized in Table 1.11.

1.4.6.1 **Common Side-Reactions with S-Protected Cys Derivatives**

1.4.6.1.1 **Racemization** The carboxy-activated *S*-protected Cys and Cys residues anchored to resin by an ester linkage **139** (and not Cys α-carboxamides and *endo* residues) are racemized even during rapid peptide couplings due to high rates of base-catalyzed enolization. This can be minimized by base-free couplings and by the selection of suitable *S*-protectors. The rate of epimerization during SPPS using N^{α}-Fmoc protection has been found to vary with different thiol protections. The minimum rate was found with *S-tert*-butyl protection and the maximum with the *S–S-tert*-butyl group.

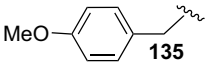
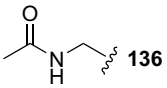
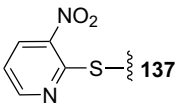
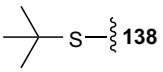
1.4.6.1.2 **β-Elimination** Protected Cys are converted to ΔAla residues **140** by the action of strong bases or strong acids through a β-elimination process involving the α-proton and the protected thiol function. The resultant ΔAla residues are reactive to nucleophiles and can form adducts such as β-(1-piperidyl)Ala **141** (Figure 1.54).

1.4.6.1.3 **Oxidation** Sulfide, disulfide as well as thioacetal-based protections are oxidized to the corresponding sulfoxides in the presence of peroxides. The process is also feasible by air oxidation.

1.4.6.2 **Synthesis of Peptides Using Cystine as "Self-Protected" Cys**

Symmetrical cystine derivatives can be employed for the synthesis of peptide dimers cross-linked by a disulfide bond and peptides containing a single Cys residue. In the case of the latter, the disulfide bond is reduced (using β-mercaptoethanol) to liberate two molecules of the Cys peptide. An application of this method can be seen in the synthesis of "H peptide" [227]. The method can also be extended to make asymmetric Cys peptides and also cyclic cystine "loops" by using unsymmetrical disulfides of Cys. The method is less successful for SPPS since acylation of resin bound amines with activated *N,N′*-bis-protected cystine which is often incomplete leading to the formation of mono-cystine peptides, which upon subsequent acylation form asymmetric Cys peptides. The major limitation of the method is the high degree of sensitivity of

Table 1.11 Selected β-thiol protections of Cys.

Protection Alkyl Type	Stable to	Cleavage[a] conditions
4-Methoxybenzyl (Mob)  **135**	TFA, bases,	$Hg(OAc)_2$ in TFA, anhydrous liquid HF, TFA/scavengers, I_2 [219]
Xanthyl	Bases and nucleophiles	Hg(II), anhydrous liquid HF (20 °C), $Ph_2SO/MeSiCl_3$ [220]
Trt	Bases and nucleophiles	TFA/H_2O or $DCM/isoPr_3SiH$, $AgNO_3$, I_2 [221]
Acetamidomethyl (Acm, Figure 1.52)  **136**	Bases, TFA, liquid HF (0 °C)	I_2, heavy metal ions, R-SCl, $Ph_2SO/MeSiCl_3$ [222]
3-Nitro-2-pyridylsulfanyl (NPys)  **137**	Anhydrous liquid HF	Thiols including free Cys [223]
tert-Butylsulfanyl[b]  **138**	TFA, bases, RSCl	Thiols, TFE [224]

a) Cys protections can be removed with concomitant formation of a disulfide linkage via I_2-mediated oxidation. This procedure (Figure 1.53), first developed for the cleavage of Acm group [225], has been successfully extended to other protections as well and further to selective removal of thiol protections (e.g., Trt) in the presence of the Acm group. Thallium(III) acetate can also be used for the removal of the Acm group with disulfide formation. An illustrative application of this procedure is the total synthesis of human insulin by the stepwise disulfide formation approach [226].

b) When the Acm group is present, thiols and thioethers are avoided as scavengers as they can cause disulfide exchange.

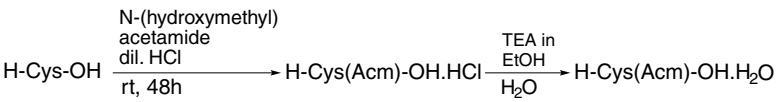


Figure 1.52 Preparation of *S*-Acm protected Cys.

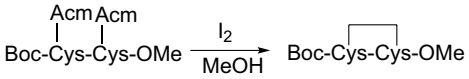


Figure 1.53 Oxidative removal of *S*-Acm protection of Cys with concomitant disulfide formation.

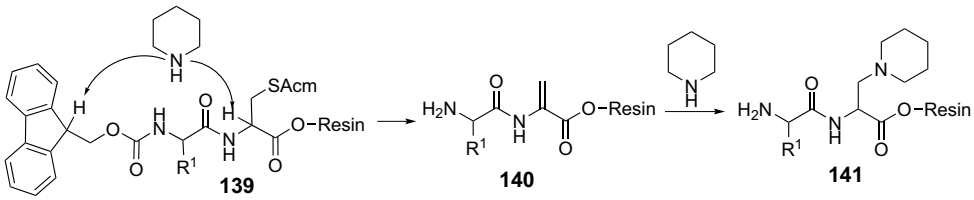


Figure 1.54 Racemization and β-elimination in *S*-protected Cys residues.

disulfide bonds to regular synthetic conditions. The -S–S- linkages are sensitive to oxidation and reduction. Disulfide exchange can also take place with scrambling of the -S–S- linkages in the case of asymmetric cystine peptides. This reaction is catalyzed by acids or bases or by a small amount of free thiols. Hence, exposure to conditions, which promote these side-reactions, should be avoided when working with cystine derivatives.

1.4.7
Thioether Group of Met

The methyl thioether moiety of Met is susceptible to oxidation and alkylation. The *S*-alkylated function can be internally displaced by the carboxy group to form a homoserine lactone. The favored method of protection of Met is oxidation of the thioether group to sulfoxide **142**. Oxidation of Met is associated with the formation of sulfone byproducts and a mixture of (2*S*,*R*)- and (2*S*,*S*)-sulfoxides due to asymmetric induction. Several reagents have been proposed for stereospecific and sulfone-free oxidation. Sodium perborate is efficient in terms of preventing sulfone formation but gives a mixture of epimers (Figure 1.55). The required *S*-Met(O) is isolated as the picrate salt by recrystallization. Met can also be oxidized in excellent yields by H_2O_2-mediated oxidation, but the reaction gives rise to varying amounts of epimeric sulfoxides [228]. The thioether moiety is regenerated by reduction with mercaptoacetic

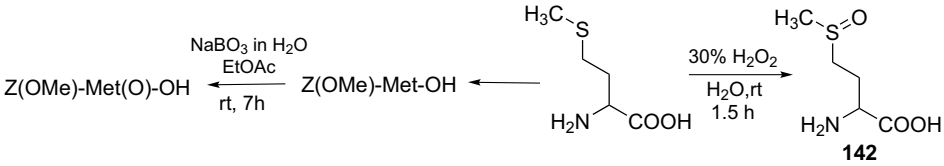


Figure 1.55 Protection of Met through oxidation.

acid [229]. TMS-SPh, *N*-methyl sulfanylacetamide, selenophenols, TMS-OTf, and NH_4I/TFA are the other reagents proposed for the reduction of the sulfoxide of Met.

1.4.8
Hydroxy Group of Ser, Thr, and the Phenolic Group of Tyr

The β-hydroxy group of Ser and Thr (to lesser extent) and the phenolic function of Tyr are acylated in the presence of excess of acylating agents, which is typically the case in SPPS and during difficult couplings. N-Terminal Ser can undergo double acylation by carboxy-activated aspartic acid ω-esters to give an eight-membered macrocycle containing amide and ester linkages. *O*-Acylation of Ser has been found to be promoted by the catalytic activity of the imidazole moiety of unprotected His. In addition, the hydroxy function of Ser/Thr undergoes acid-catalyzed dehydration to ΔAla derivatives. An intramolecular $N \rightarrow O$ acyl shift can take place in the presence of strong acids such as liquid HF and TfOH. However, this isomerization can be easily reversed by brief exposure to mild alkali (the principle has formed the basis for a peptide ligation route called the "click peptide" method). Problems associated with the usage of unprotected Ser as an acylating agent include self-acylation (which is more pronounced under strong activation conditions and long hours of standing) and formation of oxazolidin-2-ones. The problem of *O*-acylation is more serious in the case of Tyr due to the formation of the strongly nucleophilic phenoxide ion, which is also accessible to acylating agents without steric constraints. In addition, the phenolic function activates the aromatic ring to electrophilic substitution at the 3-benzyl position. Hence, scavengers are invariably used during acidolytic removal of protecting groups. The common protections of Ser and Thr are summarized in Table 1.12, and those of Tyr in Table 1.13.

Table 1.12 Protectors of the hydroxy group of Ser/Thr.

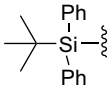
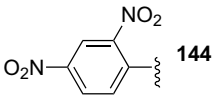
Protection	Stable to	Cleavage conditions
Alkyl type		
Benzyl	Acids, bases	Catalytic hydrogenation, liquid HF, TfOH [230]
tert-Butyl	Catalytic hydrogenation, bases	90% TFA in DCM, neat TFA [231]
Trt	Catalytic hydrogenation, bases	1% TFA in DCM [232]
Silyl ethers		
TBDMS	Bases, catalytic hydrogenation	TFA, TBAF [233]
TBDPS	Bases, catalytic hydrogenation, acids	TBAF, alkali [234]
 **143**		

Table 1.13 Protectors of the phenolic function of Tyr.

Group	Cleavage conditions
Alkyl ether	
Benzyl, Bzl	Catalytic hydrogenation, anhydrous liquid HF, TfOH, [235]
tert-Butyl, Trt	Mild acids
Aryl Ether	
2,4-Dinitrophenyl (Dnp)	Thiolysis, piperidine, DBU [236]
 **144**	
Silyl ether	
TBDMS	TBAF, mild acids [233]
Acyl esters	
Z(2-Br)	Super acid, piperidine [237]
Propargyloxycarboxyl	Benzyltriethylammonium tetrathiomolybdate in acetonitrile [238]

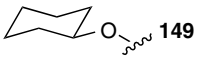
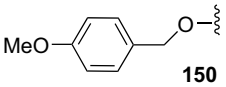
1.4.9 ω-Carboxy Group of Asp and Glu

Protection of the ω-carboxy group of Asp and Glu is required to avoid its activation during coupling (which would lead to branching of the peptide chain at Asp and Glu residues), and to minimize the formation of imides as well as pGlu derivatives. There are different synthetic routes to access ω-esters of Asp/Glu. These esters can be prepared in good yields by acid-mediated esterification of Asp and Glu [239]. Protonation of the α-amino group in acidic media has a deactivating effect on the adjacent α-carboxy group because the presence of a positive charge on the amino group prevents protonation (the key step in acid-catalyzed esterification) of the latter. Treatment of Asp and Glu with excess isobutene and catalytic TsOH in dioxane yields the monoesters (55–60% yield), with a ω/α ester ratio of 65 : 35 for Glu and 60 : 40 for Asp. The monoesters can be derivatized with N^{α}-Fmoc group and ω-*tert*-butyl esters of Fmoc-Asp/Glu can be obtained by crystallization from CH_2Cl_2/petroleum ether [240]. ω-Esters can also be synthesized by using Cu^{2+} complex of Asp and Glu or oxazolidin-5-ones of N^{α}-protected Asp/Glu in which the α-carboxy group is trapped through bonding [241, 242]. Hydrolysis of diesters of Asp and Glu in the presence of Cu^{2+} salts gives access directly to the ω-esters [243]. The most widely used ω-carboxy protections are listed in Table 1.14.

1.4.9.1 Aspartimide Formation

A persistent side-reaction during peptide synthesis is the base or acid-catalyzed cyclization of the ω-protected Asp residues into aspartimide. The homologous glutarimide is formed to a lesser extent. The extent of aspartimide formation is

Table 1.14 ω-Esters of Asp and Glu (selected examples).

ω-Esters	Cleavage
Benzyl	Catalytic hydrogenation, anhydrous liquid HF, saponification [244]
4-Nitrobenzyl	More stable to acidolytic cleavage than benzyl ester; cleaved by catalytic hydrogenation; TBAF; Na_2S [245]
Cyclohexyl (cHx)	Anhydrous liquid HF or MsOH [246]
 **149** *tert*-Butyl	
tert-Butyl	TFA in DCM, HCl in dioxane [247]
4-Methoxybenzyl	1% TFA in DCM [248]
 **150**	
Fm	20% Piperidine in DMF or 15% DEA in DMF [249]
Allyl	Similar to Aloc group [250]

high in syntheses employing Fmoc for N^{α} protection due to the high frequency of exposure of peptides to bases. Aspartimides are reactive to nucleophiles, and undergo hydrolysis to a mixture of α- and β-peptides **145** and **146**. When the nucleophile is piperidine, the corresponding α- and β-piperidine amides **147** and **148** are formed (Figure 1.56). An ideal ω protection is expected to prevent or at least minimize aspartimide formation. Generally, the ω-esters of primary alcohols are more prone to

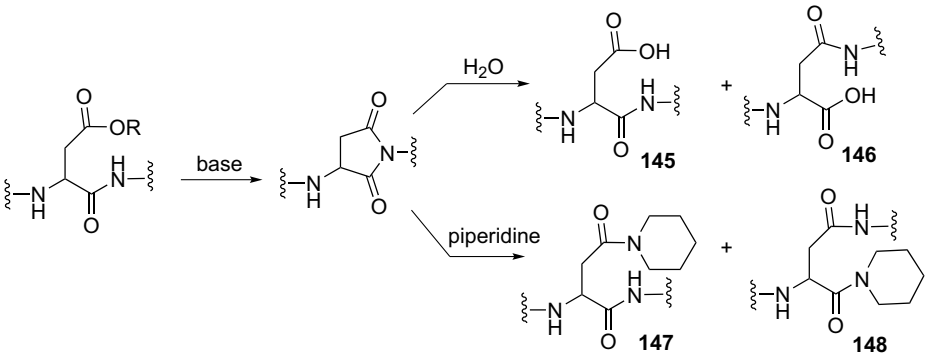
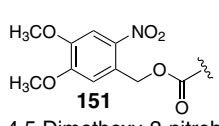


Figure 1.56 Formation of aspartimide and β-peptide and amide.

cyclization than that of secondary and tertiary alcohols. Hence, methyl and ethyl esters, and to some extent benzyl esters, are not suitable to prevent aspartimide formation. However, sterically hindered esters such as cycloalkyl esters (cyclopentyl, hexyl, heptyl, octyl, and dodecyl esters), menthyl esters, adamantyl esters, *tert*-butyl esters, Fm esters as well as allyl esters are resistant to aspartimide formation. Consequently, the classical alkyl esters are increasingly replaced by the more efficient cyclohexyl esters.

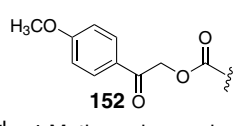
1.5
Photocleavable Protections

Groups that are fragmented by absorption of electromagnetic radiation have been developed as protective functions for peptide synthesis. The major advantages of using photocleavable protectors are their higher degree of orthogonality with other protections when compared to the conventional acid- or base-labile protecting groups, and the prospect of carrying out the deprotection reaction in neutral conditions, which is ideal to minimize a number of side-reactions. Photocleavable carboxamides **154** and **155** are attractive units as carboxy protections since they can be cleaved with excellent levels of selectivity [251]. The suitability of a photocleavable group is determined by the quantum efficiency and the degree of selectivity of the cleavage reaction, and the nature of the photoproducts formed during cleavage, which is expected to be inert. Typically, photocleavable groups are designed to be cleaved with irradiation with ultraviolet radiation with wavelength around 320 nm. Radiations of higher energy cause multiple fissions leading to a decrease in the selectivity level and those with lower energy (e.g., visible light) lead to photodegradation of the protecting group. Important photocleavable groups employed as α-amino protectors are shown in Figure 1.57. Photocleavable carboxy protectors are the esters derived from the alcohol component of the alkoxycarbonyl derivatives in Figure 1.57 [252–254].



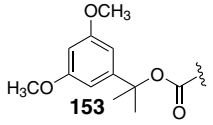
151

4,5,Dimethoxy-2-nitrobenzyl-oxycarbonyl (Nvoc)



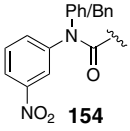
152

4-Methoxyphenacyloxy-carboxyl (Phenoc)

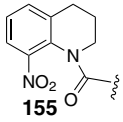


153

2-(3,5-Dimethoxyphenyl)prop-2-yloxycarbonyl (Ddz)



154



155

Figure 1.57

1.6 Conclusions

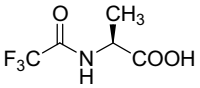
Since Emil Fischer's disclosure of the significance of the N^{α}-protecting group for the successful chemical assembly of a dipeptide, the protection of the functional groups of α-amino acids has been the topic of intense research. The breakthroughs achieved in the development of a new protecting group or the discovery of a new cleavage reagent/condition have eventually resulted in stretching the limits to new dimensions of the achievable size, level of purity, and quantity of synthetic peptides through solution-phase synthesis as well as SPPS. Investigations continue to be undertaken in the direction of describing new protecting groups and improved analogs, and efficient deprotection conditions. The important aspects are the development of new methods/reagents for the introduction of protections, increasing the homogeneity of protected amino acids, addressing solubility issues, and minimization of side-reactions during peptide coupling and cleavage steps. Several nonconventional methods such as microwave and ultrasonication-mediated synthesis, photochemistry and enzyme catalysis are being tried as new routes for introduction as well as removal of protectors. Progress is also being made in the standardization of methods with respect to meeting the principles of "green" synthesis through innovations such as development of water-soluble protectors, aqueous-phase reactions, and atom-economical synthetic methods. Protectors are also being optimized for their suitability in rapid peptide synthesis via microwave-SPPS.

A vast diversity of N^{α}, carboxy, and side-chain protections and cleavage reagents are currently available. It is not possible to identify an ideal protecting group or a combination. The choice of an appropriate protection/protection scheme depends on factors such as the sequence of interest, mode of synthesis (solution-phase or SPPS), protection strategy (global versus minimum protection), desired purity levels of peptides, scale, time, and cost. Nonetheless, since their inception, the urethanes have dominated as N^{α}-protecting groups for peptide synthesis and the trend appears to not change in the near future. The Fmoc/*tert*-butyl protection strategy is currently the most widely employed protocol for the synthesis of peptides through SPPS. However, the non-urethane-type protections retain importance in many cases. An example is the coupling of extremely sterically crowded amino acids, in which case the hindrance offered by the urethane appears to be the limiting factor for peptide coupling [19b]. Further, with the advent and popularization of peptidomimetics, the groups, which have not found appreciable level of utility as protective functions for conventional peptide synthesis, are being explored due to the vast diversity in reactions being carried out using amino acids as substrates. Also, there are indications that the protecting groups may not necessarily be viewed as inevitable extra appendages in substrates, but as units with synthetic value. Postsynthetic modifications wherein the protective function, instead of being removed, is directly converted to functional groups is being carried out [255].

1.7
Experimental Procedures

1.7.1
Protection Reactions

1.7.1.1 General Procedure for the Preparation of Tfa-Arg-OH [4]

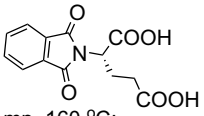


mp. 70 °C;
$[\alpha]^{16}_D$ = -60.7 (*c* 1, H_2O)

TEA (3.1 ml, 22.0 mmol) was added to a solution of Ala (2.0 g, 22.0 mmol) in MeOH (11 ml). After 5 min, ethyl trifluoroacetate (3.3 ml, 28.0 mmol) was added and the reaction was stirred for 24 h. The solvent was evaporated and the residue was dissolved in H_2O (35 ml) and acidified with concentrated HCl (approximately 4 ml). After stirring for 15 min, the mixture was extracted with EtOAC (30 ml × 4) and the combined organic layers were washed with brine (25 ml), dried ($MgSO_4$), filtered, and concentrated to leave a clear oil that upon subjecting to high vacuum for 24 h solidified into a hygroscopic solid. Yield: 87%.

Note: N^{α}-Tfa-amino acids, except Tfa-Ser and Tfa-Thr, are prepared efficiently by treating amino acids with TFAA in TFA solvent at –10 °C [4].

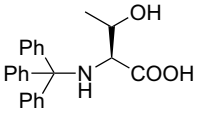
1.7.1.2 General Procedure for the Preparation of N^{α}-Phthaloyl Amino Acids using *N*-(Ethoxycarbonyl)phthalimide [256]



mp. 160 °C;
$[\alpha]^{25}_D$ = -48 (*c* 1, dioxane)

Finely powdered *N*-(ethoxycarbonyl)phthalimide (5.0 g, 22.8 mmol) was added to a suspension (0 °C) of Glu (2.4 g, 16.3 mmol) and Na_2CO_3 (4.2 g, 40.0 mmol) in H_2O (20 ml), and stirred at 0 °C for 5 min. The mixture was acidified to pH 2.5 with 6 M HCl and the resulting oil crystallized upon cooling. The product was filtered, washed with cold H_2O, and dried. Yield: 80%.

1.7.1.3 General Procedure for the Preparation of N^{α}-Trt-Amino Acids [25]



mp. 165 °C;
$[\alpha]^{25}_D$ = -6.7 (*c* 2, MeOH)

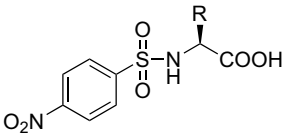
i) Preparation of TMS-amino acid: TMS-C1 (17.75 ml, 140.0 mmol) was added to a suspension of Thr (4.76 g, 40.0 mmol) in DCM (70 ml) and the mixture was refluxed for 20 min. Et_3N (19.51 ml, 140.0 mmol) in DCM (40 ml) was added after cooling to room temperature and refluxed again for 45 min. The TMS-amino acid was used directly for the next step.

ii) The mixture, obtained from the above step, containing TMS-amino acid, was cooled to 0 °C, anhydrous MeOH (2.43 ml, 60.0 mmol) in DCM (10 ml) was added dropwise, and the mixture was allowed to attain room temperature. Et_3N (5.58 ml, 40.0 mmol) was added followed by Trt-Cl (11.25 g, 40.0 mmol) in two portions over a 15 min period. The mixture was stirred for 5 h and then excess MeOH was added. The residue obtained after evaporation of the solvent was partitioned between EtOAc (50 ml) and a precooled 5% citric acid solution (50 ml). The organic layer was extracted with 1 N NaOH (20 ml × 2) and H_2O (20 ml × 2). The combined aqueous layers were washed with EtOAc (20 ml), cooled to 0 °C, and neutralized with glacial AcOH. The precipitated product was extracted with EtOAc (30 ml × 2), and the combined organic layers were washed with H_2O, dried over $MgSO_4$, and concentrated. The resulting residue was dissolved in EtOAc (20 ml) followed by the addition of Et_2NH (1 ml, 10.0 mmol) to obtain the diethylammonium salt. Yield: 67%.

Note:

- In the case of amino acids other than Ser, Thr, and Tyr, the corresponding *N,O*-bis-TMS amino acids can be analogously prepared using 2 equiv. of TMS-Cl.
- Trt-amino acids can also be prepared using Me_2SiCl_2 and Ph_2SiCl_2 in place of Me_3SiCl [25].

1.7.1.4 General Procedure for the Preparation of N^{α}-Ns-Amino Acids [257]



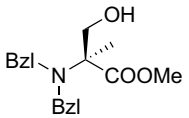
p-Nitrobenzenesulfonyl chloride (1.5 g, 1.6 mmol) was added slowly to a solution of the amino acid (1.0 mmol) in 1 N NaOH (0 °C) and the mixture, maintained at pH 9, was stirred for 2–3 h (TLC: $CHCl_3$/MeOH 90 : 10, v/v). The mixture was extracted with EtOAc (10 ml × 3). The aqueous phase was acidified to pH 2.0 with 1 N HCl and extracted with EtOAc. The organic layer was washed with H_2O and brine, dried over Na_2SO_4, and evaporated to afford the product.

Note:

- The side-chain-protected amino acids (i.e., Ns-Lys(Boc)-OH, Ns-Asp(O*t*Bu)-OH, Ns-Cys(Trt)-OH, and Ns-Asn(Trt)-OH) have been prepared using *p*-nitrobenzenesulfonyl chloride in presence of TEA in dioxane-H_2O [21].
- N^{α}-Nbs-amino acids are prepared through the treatment of the amino acid with 1.5 equiv. of Nbs-Cl in a mixture of 1 : 1 dioxane and 1 M NaOH. This is a modified

procedure originally employed for the preparation of benzylsulfonyl amino acids [258].

Bzl$_2$-α-Me-Ser-OMe [259]

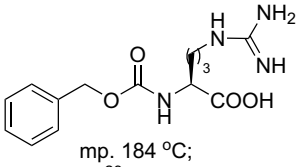


$[\alpha]^{23}_D$ = -34.3 (*c* 1.17, $CHCl_3$)

H-α-Me-Ser-OMe·HCl (2.96 g, 17.5 mmol) was dissolved in THF/DMSO (80 ml/20 ml), and $NaHCO_3$ (8.82 g, 105.0 mmol) and BzlBr (8.36 ml, 70.3 mmol) were added. The solution was refluxed overnight and H_2O (50 ml) was added, and the mixture was extracted with EtOAc (100 ml). The organic layer was dried over $MgSO_4$ and evaporated. The resulting residue was purified via flash chromatography (hexane/EtOAc (9 : 1) to hexane/EtOAc (6 : 4)). Yield: 64%. IR: 3455, 2952, 1728, 1454 cm^{-1}.

Note: The methyl ester can be hydrolyzed following the procedure described for closely related substrates by refluxing with LiI and NaCN in pyridine under N_2 atmosphere [259].

1.7.1.5 General Procedure for the Preparation of N^{α}-Z-Amino Acids



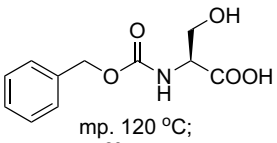
mp. 184 °C;
$[\alpha]^{23}_D$ = -9.3 (*c* 2, 1N HCl)

1.7.1.5.1 **Method A: Using Z-Cl [41a]** To a solution of Arg·HCl (21.3 g, 100 mmol) in 1 N NaOH (100 ml) maintained at 0 °C, Z-Cl (22.1 g, 130.0 mmol) and 2 N NaOH (55 ml) were added in portions, alternatively. The pH of the mixture was kept between 9.0 and 10.0. Stirring was continued for a further 2 h during which the pH drops to 7.0–7.5. The resulting precipitate was filtered, washed with cold H_2O (50 ml), and recrystallized from boiling H_2O (about 130 ml). Crystallization was completed in the cold (ice-H_2O bath). The product was air-dried, and then the powder was suspended in acetone (50 ml) and filtered, and washed with acetone (20 ml) and ether (50 ml). The purified material was dried *in vacuo* at 50 °C. Yield: 89.5%.

Note: Z-Cl is prepared by treating benzyl alcohol with phosgene (gaseous or in toluene solution (commercially available)) at −20 °C and continuing stirring for several hours and allowing the mixture to attain room temperature. The product is isolated after work-up [260].

Caution: Phosgene is a highly toxic gas and should only be used following strict safety measures.

1.7.1.5.2 Method B: Using Z-OSu [42]



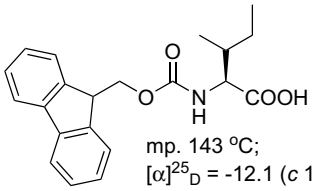
mp. 120 °C;
$[\alpha]^{26}_D$ = +5.7 (*c* 6, AcOH)

Z-OSu (1.24 g, 5.0 mmol) was added to a solution of Ser (525 mg, 5.0 mmol) and $NaHCO_3$ (420 mg, 5.0 mmol) in a mixture of H_2O (7 ml) and acetone (7 ml), and the whole was stirred overnight. The acetone was then evaporated and the solution was washed with DCM (3 ml × 2). The aqueous layer was acidified to pH 2.5 with concentrated HCl and extracted with EtOAc (5 ml × 3). The combined organic layers were washed with H_2O, dried, concentrated, and the residual product was recrystallized. Yield: 78%.

Note: Z-OSu is prepared by treating the DCHA salt of *N*-hydroxysuccinimide with Z-Cl for 12 h at room temperature [42].

1.7.1.6 General Procedures for the Preparation of N^{α}-Fmoc-Amino Acids

1.7.1.6.1 Method A: Using Fmoc-OSu [117]

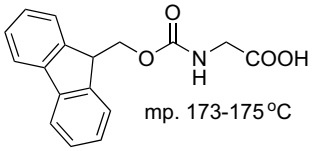


mp. 143 °C;
$[\alpha]^{25}_D$ = -12.1 (*c* 1, DMF)

Fmoc-OSu (165.0 g, 0.49 mol) was added to a solution of Ile (66.0 g, 0.50 mol) and Na_2CO_3 (53.0 g, 0.50 mol) in H_2O/acetone (1:1, 1.3 l) over a period of 1 h while the pH was kept at 9.0–10.0 by the addition of 1 M Na_2CO_3 solution. Stirring was continued overnight, EtOAc (2 l) was added and the mixture was acidified with 6 M HCl. The organic layer was washed with H_2O (1.5 l in four portions), dried, and concentrated to approximately 500 ml. On addition of petroleum ether, the product crystallized. Yield: 93%.

Note: Preparation of Fmoc-OSu: To a chilled solution of Fmoc-Cl (102.8 g, 0.40 mol) and *N*-hydroxysuccinimide in dry dioxane (700 ml) was added TEA (55.75 ml, 0.40 mol), and the mixture was stirred for 4 h. The TEA·HCl was filtered, washed with dioxane, and the combined filtrates were concentrated. The product was crystallized by the addition of petroleum ether. Melting point 148–149 °C [117].

1.7.1.6.2 Method B: Using Fmoc-Cl and *N*,*O*-bis-TMS-Amino Acids [119]

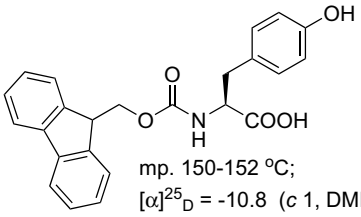


mp. 173-175 °C

TMS-Cl (9.52 ml, 75.0 mmol) was added to finely ground Gly (2.81 g, 37.5 mmol) suspended in DCM (87.5 ml) and the mixture was refluxed for 1 h and then cooled in an ice bath. Then DIPEA (11.3 ml, 65.0 mmol) and Fmoc-Cl (6.47 g, 25.0 mmol) were added. The solution was stirred with cooling for 20 min and warmed to room temperature for 1.5 h. The mixture was concentrated and partitioned between Et_2O (200 ml) and 2.5% $NaHCO_3$ (250 ml). The aqueous layer was washed with Et_2O (50 ml × 2) and the Et_2O layers were back-extracted with H_2O (25 ml × 2). The combined aqueous layers were acidified to pH 2.0 with 1 N HCl and extracted with EtOAc (75 ml × 3). The combined EtOAc layers were dried and concentrated to obtain the product that was recystallized using EtOH/H_2O. Yield: 88%.

Note: For the preparation of Fmoc-Cl, 9-fluorenylmethanol (12.8 g) was added slowly to a chilled solution of phosgene (7.12 g) in DCM (75 ml), and the mixture was stirred for 1 h in an ice bath and then allowed to stand for 4 h at ice-bath temperature. The solvent and excess phosgene were removed *in vacuo* to leave an oil which crystallized after several hours. The resulting product was recrystallized twice from ether. Yield: 86%; melting point 61.5–63 °C [261].

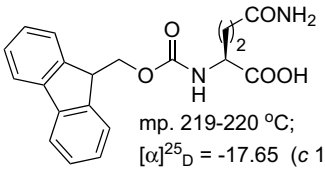
1.7.1.6.3 Method C: Using Fmoc-Cl in the Presence of Zinc Dust [118]



mp. 150-152 °C;
$[\alpha]^{25}_D$ = -10.8 (*c* 1, DMF)

Tyr (1.81 g, 10.0 mmol) was dissolved in a minimum quantity of 1 N HCl in MeCN followed by the addition of activated zinc dust (prewashed with dil. HCl and dried) in small portions until the reaction mixture attained neutral pH. A solution of Fmoc-Cl (2.7 g, 10.0 mmol) in MeCN (10 ml) and zinc powder (650 mg, 10.0 mmol) were added and stirred at room temperature for about 20 min. The mixture was filtered, washed with MeCN and the filtrate was evaporated. The residue was dissolved in 5% Na_2CO_3 (20 ml) and washed with ether (10 ml × 3). The aqueous layer was acidified to pH 2.0 with 6 N HCl and the precipitate was extracted into EtOAc (20 ml × 3). The combined organic extracts were washed with 0.1 N HCl, H_2O, dried over Na_2SO_4, and concentrated to give a residue that was recrystallized using a suitable solvent. Yield: 84%.

1.7.1.6.4 Method D: Using Fmoc-N_3 [61a]



mp. 219-220 °C;
$[\alpha]^{25}_D$ = -17.65 (*c* 1, DMF)

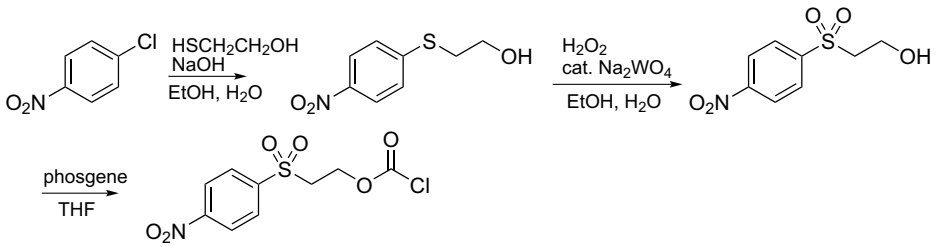
NaN_3 (0.78 g, 12.0 mmol) in H_2O (4 ml) was added to a solution of Fmoc-Cl (2.58 g, 10.0 mmol) in dioxane (5 ml) and the mixture was stirred at room temperature until completion (about 1 h, HPLC) while no precipitate appeared. Gln (1.6 g, 11.0 mmol), dissolved in 1% aqueous Na_2CO_3/dioxane (60 ml), was added to this mixture and it was stirred at room temperature for 48 h, during which the pH was kept between 8.0 and 10.0 by the addition of 10% aqueous Na_2CO_3 solution. Once the reaction was complete (HPLC), the mixture was poured into H_2O (100 ml) (keeping a basic pH) and extracted four times with *tert*-butyl methyl ether. The aqueous layer was cooled to 4 °C and acidified to pH 2.0 with 2 N HCl. The white precipitate was filtered, washed with ice H_2O (made slightly acidic with dilute HCl, pH 4–5), and dried overnight under vacuum. Yield: 71%.

Note: Fmoc-N_3 can also be prepared as an isolable solid (melting point 83–85 °C) through the treatment of Fmoc-Cl with NaN_3 in a water/acetone system and then used to introduce the Fmoc group [58b].

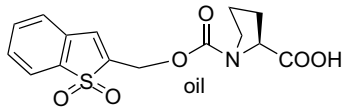
1.7.1.7 General Procedure for the Preparation of N^{α}-Nsc-Amino Acids [83]

To a solution of the amino acid (20.0 mmol) in DCM (40 ml) was added TEA (5.56 ml, 40.0 mmol) and TMS-Cl (5.27 ml, 40.0 mmol) dropwise with stirring. The mixture was refluxed for 1.5 h, cooled (0 °C) and Nsc-Cl (17 mmol) was added. After stirring at 0 °C for 30 min and at room temperature for 2 h, the solution was concentrated, and the residue was partitioned between Et_2O and 5% $NaHCO_3$ solution. The aqueous layer was acidified to pH 2.0–2.5 with 1 M H_2SO_4, extracted with EtOAc, dried (Na_2SO_4), and concentrated. The residue was recrystallized from EtOAc/petroleum ether.

Note: The synthesis of the reagent Nsc-Cl is outlined in the following scheme [262].



1.7.1.8 General Procedure for the Preparation of N^{α}-Bsmoc-Amino Acids [84a]



A solution of benzothiophenesulfone-2-methyl *N*-succinimidyl carbonate (0.5 g, 1.48 mmol) in acetone (10 ml) was added to the solution of Pro (0.17 g, 1.48 mmol) and $NaHCO_3$ (0.25 g, 2.96 mmol) in H_2O (10 ml), and the mixture was stirred at room temperature overnight. The mixture was diluted with H_2O and extracted twice with DCM. The aqueous layer was cooled and acidified to pH 2.0 with concentrated HCl.

The resulting white precipitate or oil was extracted with EtOAc (25 ml × 3), and the combined organic layers were washed with H_2O (30 ml), brine (30 ml), and dried over $MgSO_4$. The solvent was evaporated and the crude product was recrystallized. Yield: 90%.

1.7.1.9 General Procedure for the Preparation of N^{α}-Aloc-Amino Acids [88]

Aloc-Cl (10.6 ml, 0.1 mol) and 4 M NaOH (25 ml) were added alternatively within approximately 30 min to a solution of the amino acid (0.1 mol) in 4 M NaOH (25 ml) at 0 °C and the mixture was stirred for an additional 15 min at room temperature. The mixture was extracted with Et_2O and the aqueous layer was acidified with concentrated HCl. After cooling for several hours, the product was collected, dried, and recrystallized.

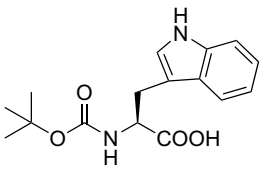
The DCHA salts were obtained by adding DCHA (1 equiv.) to a solution of the Aloc-protected amino acid derivatives in EtOH or EtOAc and precipitation with Et_2O or Et_2O/petroleum ether [92].

1.7.1.10 General Procedures for the Preparation of N^{α}-Boc-Amino Acids

1.7.1.10.1 Method A: Using $(Boc)_2O$ [99] To an ice-cold solution of the amino acid (10.0 mmol) in a mixture of dioxane (20 ml), H_2O (10 ml) and 1 N NaOH was added $(Boc)_2O$ (2.4 g, 11.0 mmol), and the mixture was stirred at room temperature for 30 min. The mixture was concentrated *in vacuo* to about 10–15 ml, cooled, covered with a layer of EtOAc (30 ml), and acidified to pH 2.0–3.0 with a dilute solution of $KHSO_4$ (to Congo paper red). The aqueous phase was extracted with EtOAc (15 ml × 2). The organic layers were combined, washed with H_2O (30 ml × 2), dried over Na_2SO_4, and evaporated. The residue was recrystallized with a suitable solvent.

Note: $(Boc)_2O$ is prepared by treating *tert*-butyl potassium carbonate (potassium *tert* butoxide and CO_2) with phosgene and then the resulting tricarbonate with diazabicyclo[2.2.2]octane [99a].

1.7.1.10.2 Method B: Using Boc-ON [100]



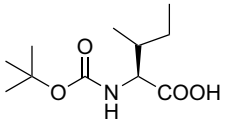
mp. 137-138 °C;
$[\alpha]^{25}_D$ = -20 (*c* 1, DMF)

Boc-ON (2.71 g, 11.0 mmol) and dioxane (6 ml) were added to a solution of Trp (2.05 g, 10.0 mmol) and TEA (2.1 ml, 15.0 mmol) in H_2O (6 ml) at room temperature, and stirred for an additional 2 h. After addition of H_2O (15 ml) and EtOAc (20 ml), the aqueous layer was separated, washed with EtOAc (20 ml), acidified with 5% citric acid solution, and extracted with EtOAc. The organic extracts were combined and concentrated. Yield: 99%.

Note: Preparation of Boc-ON:

- BzlCN and methyl nitrite are reacted in the presence of NaOH in MeOH.
- The resulting oxime is treated with phosgene in benzene and further with *tert*-butanol [100].

1.7.1.10.3 Method C: Using Boc-N_3 [263–265]



mp. 65-68 °C;
$[\alpha]^{20}_D$ = +3.8 (*c* 10, 80% AcOH)

i) Generation of *tert*-butyl azidoformate (Boc-N_3). To an ice-cold solution of Boc-$NHNH_2$ (477.0 g, 3.6 mmol) in dioxane (2 l) and H_2O (500 ml) was added slowly 5 M HCl (720 ml, 3.6 mmol) followed by a solution of $NaNO_2$ (255.0 g, 3.6 mmol) in H_2O (750 ml) and the mixture was stirred for 1–3 h at room temperature.

ii) Ile (393.0 g, 3.0 mol) was suspended in the above solution containing Boc-N_3, and dioxane (500 ml) was added and titrated at pH 10 with 4 M NaOH (1.5 l) over a period of 12 h. The mixture was neutralized to pH 7.0 with 1 M H_2SO_4 and concentrated. It was then acidified to pH 3–3.5 and extracted with EtOAc. The organic phase was washed with H_2O, dried (Na_2SO_4), and concentrated to an oily residue, which solidified under petroleum ether. Yield: 93%.

Caution: Warning Boc-N_3 is sensitive to heat and shock.

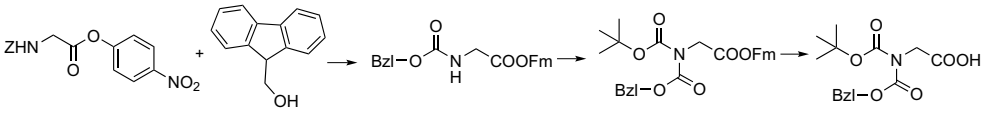
1.7.1.11 General Procedure for the Preparation of *N*,*N*′-di-Boc-Amino Acids [125]

i) $(Boc)_2O$ in MeCN (0.5 ml, 1.1 mmol) was added to a solution of the Boc-Gly-OBzl (0.26 g, 1.0 mmol) and DMAP (0.012 g, 0.1 mmol) in MeCN (1–2 ml), and the reaction was monitored by TLC (2: 1 toluene/MeCN or DCM). Upon completion of the reaction (If the starting material remained after 4–6 h, more $(Boc)_2O$ (approximately 0.5 equiv.) was added; if necessary, this procedure is repeated until all starting material is consumed.) the resulting mixture was concentrated (in some cases, excess $(Boc)_2O$ was removed under high vacuum overnight) and the residue was dissolved in EtOAc, washed with H_2O and dried. The solvent was evaporated and the product obtained was recrystallized from light petroleum. Yield: 99%; melting point 31–31.5 °C.

ii) The Boc_2-amino acid benzyl ester was hydrogenated for 1–2 h with 5% (w/w) of Pd/C in MeOH. The catalyst was filtered off and the filtrate was concentrated to afford a residue, which crystallized spontaneously when allowed to stand. The product was recrystallized from ether/light petroleum or EtOAc/light petroleum.

Note: *N*,*N*′-di-Boc-amino acids are also prepared starting from Boc-amino acid allyl esters [125].

N,N.Boc, Z-Gly-OH [125]



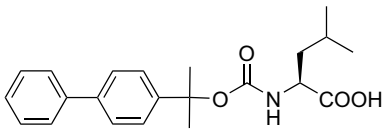
i) To a suspension of 9-fluorenylmethanol (2.15 g, 11.0 mmol) and imidazole (1.22 g, 18.0 mmol) in toluene, was added *o*-nitrophenyl ester of Z-Gly (2.09 g, 10.0 mmol) and the reaction was stirred at room temperature overnight. The solvent was removed, and the residue was taken up in EtOAc and washed with 1 M $KHSO_4$, 1 M Na_2CO_3, and saturated NaCl solutions. After drying, evaporation gave an oil that crystallized from EtOAc/light petroleum to obtain pure Z-Gly-OFm as an oil.

ii) Z-Gly-OFm (1.95 g, 5.0 mmol) and DMAP (61 mg, 0.5 mmol) were suspended in MeCN and Boc_2O (1.2 g, 6.0 mmol) was added, and the mixture was stirred for 3 h at room temperature. The solution was evaporated and the residue was dissolved in EtOAc. The organic phase was washed with H_2O and brine, dried, and evaporated to afford an oil that was chromatographed on a short column eluting first with toluene, then with toluene/MeCN (4: 1). Yield: 83%.

iii) *N,N'*-Boc, Z-Gly-OFm (1.0 g, 2.1 mmol) was treated with a 15% solution of piperidine in DMF (10 ml) for 2 h. The mixture was partitioned between EtOAc (100 ml) and 1 M $KHSO_4$ (30 ml). The organic phase was washed with further portions of 1 M $KHSO_4$ (30 ml × 2) and then extracted with 1 M $NaHCO_3$ (30 ml × 3). The combined aqueous extracts were acidified to pH 3.0 with solid $KHSO_4$ and extracted with EtOAc (30 ml × 3). The organic extract was washed with brine and dried ($MgSO_4$), and evaporated to afford a colorless oil. Yield: 64%.

Note: The above procedure gives good yields particularly for Gly. For other amino acids, acceptable yields can be obtained using allyl esters instead of Fm esters.

1.7.1.12 General Procedure for the Preparation of N^{α}-Bpoc-Amino Acids [266]



mp. 227-230 °C;
$[\alpha]^{22}_D$ = -12.2 (*c* 1, MeOH)

Leu (13.1 g, 100 mmol) was dissolved in a 2.5 *N*-benzyltrimethylammonium hydroxide in MeOH (40 ml, 100.0 mmol) and the solvent was removed *in vacuo*. The residue was dissolved in DMF (30 ml), the solution was evaporated, and the addition and evaporation of DMF was repeated. The dry salt was redissolved in DMF (40 ml), warmed to 50 °C, 2-(biphenyl-4-yl)-2-propyl-phenyl carbonate (33.3 g, 100 mmol) was added, and the mixture was stirred at 50 °C for 3 h and then cooled. The mixture was diluted with H_2O (200 ml) and ether (200 ml) was added. The

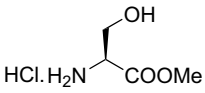
aqueous phase was acidified to pH 2.0–3.0 with a 1 M citric acid solution and re-extracted with ether (100 ml × 2). The combined ether extracts were washed with H_2O (100 ml × 2), dried over Na_2SO_4, and evaporated at 30 °C. The crystalline residue was triturated with ether (15 ml) and petroleum ether (boiling point 40–60 °C) and the insoluble product was collected by filtration. Yield: 70%.

Note: 2-Biphenyl-4-yl-2-propyl-phenyl carbonate is prepared by treating *p*-biphenylyl-dimethyl-carbinol with phenyl chlorocarbonate in DCM in the presence of pyridine [266].

1.7.1.13 General Procedures for the Preparation of Amino Acid Methyl Esters

1.7.1.13.1 Preparation of Amino Acid Methyl Ester Hydrochloride Salts

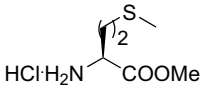
Method A: Using $SOCl_2$ [141]



mp. 168 °C;
$[\alpha]^{25}_D$ = -2.5 (*c* 1.8, DMF)

Freshly distilled $SOCl_2$ (26 ml) was added (over 10 min) to absolute MeOH (20 ml) maintained at −10 °C followed by the addition of Ser (10.5 g, 10.0 mmol). The suspension was stirred at room temperature until complete dissolution. After 24 h, the solvent was removed and the residue was recrystallized from MeOH/Et_2O. Yield: 99%.

Method B: Using 2,2-Dimethoxypropane and Aqueous HCl [267]

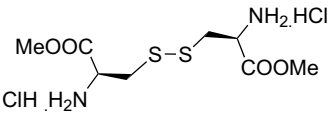


mp. 147-150 °C;
$[\alpha]^{26}_D$ = +25.2 (*c* 5.1, H_2O)

A solution of 36% HCl (1 ml) was added to a suspension of Met (0.199 g, 1.0 mmol) and 2,2-dimethoxypropane (10–15 ml), and the reaction mixture was allowed to stand at room temperature for 18 h. The mixture that darkened considerably after standing was concentrated and the residue was dissolved in a minimum amount of absolute MeOH. Addition of about 25 ml of Et_2O resulted in solidification of the product, which was recrystallized from MeOH-ether. Yield: 95%.

Note: In the case of Lys and of Glu, addition of 3–4 ml of MeOH and refluxing for 2–5 h is recommended.

Method C: Using TMS-Cl [268]

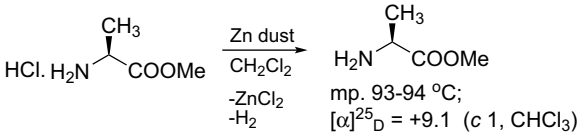


mp. 173 °C dec;
$[\alpha]^{26}_D$ = +37 (*c* 0.013, MeOH)

Freshly distilled TMS-Cl (26.0 ml, 0.2 mol) was added slowly to cystine (24 g, 0.1 mol) with stirring. MeOH (100 ml) was added and stirring was continued at room temperature. The mixture was concentrated to obtain the product. Yield: 98%.

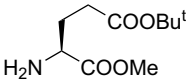
Note: Amino acid methyl ester TsOH salts are prepared by refluxing the mixture of amino acid and TsOH in MeOH for 24 h [269].

1.7.1.13.2 Isolation of Amino Acid Methyl Esters: Deprotonation of the Hydrochloride Salt Using Zinc Dust [142]



Activated zinc dust (100 mg) was added to a suspension of H-Ala-OMe·HCl (0.103 g, 1.0 mmol) in DCM or THF (10 ml) and the mixture was stirred for 5 min at room temperature. The mixture was filtered and the filtrate was evaporated. The product was precipitated using Et_2O. Yield: quantitative.

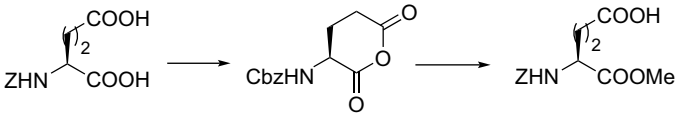
1.7.1.13.3 Glutamic Acid α-Methyl, γ-*tert*-Butyl Diester Using Diazomethane [270]



mp. 135-136 °C dec;
$[\alpha]^{20}_D$ = +17 (*c* 0.03, MeOH)

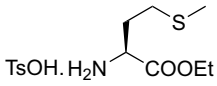
A solution of CH_2N_2 in ether was added to a solution of Glu(O*t*Bu) (5.0 g, 24.6 mmol) in MeOH (100 ml) at 0 ° to + 5 °C to give a stable yellow-colored solution. The mixture was kept for 6 h at 20–22 °C, several drops of glacial AcOH were added, and the solution was evaporated to dryness. The residue was dissolved in EtOAc (50 ml), and the organic phase was washed with 1 N $NaHCO_3$ (50 ml × 3), H_2O, dried ($MgSO_4$), and evaporated to obtain the residue that was recrystallized from EtOAc/hexane. Yield: 91%.

1.7.1.13.4 Z-Glu-OMe via Methanolysis of Cyclic Anhydride [271]



Z-Glu was self-condensed in the presence of Ac_2O at 20 °C and the resulting anhydride was treated with MeOH/DCHA/Et_2O at 20 °C for 12 h. Yield: 55%.

1.7.1.14 General Procedure for the Preparation of Amino Acid Ethyl Esters ([1f], p. 30)

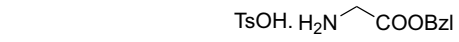


mp. 124-126 °C;
$[\alpha]^{22}_D$ = +13 (*c* 3.3, 95% EtOH)

TsOH (monohydrate, 3.8 g, 20.0 mmol) was added to a suspension of Met (1.49 g, 10.0 mmol) in absolute EtOH (50 ml) and the mixture was refluxed for 24 h. The solvent was evaporated and the residue was triturated with dry Et_2O (100 ml). The product was washed with ether (100 ml) and dried *in vacuo* over P_2O_5. Yield: 98%.

Note: The procedure is a modification of the method reported for the synthesis of amino acid benzyl esters [272].

1.7.1.15 General Procedure for the Preparation of Amino Acid Benzyl Ester *p*-Toluenesulfonate Salts [158]

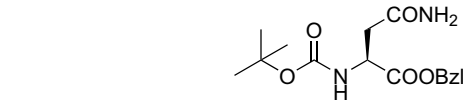


mp. 132-134 °C

Gly (18.8 g, 250.0 mmol) and TsOH (monohydrate, 48.5 g, 255 mmol) were added to a mixture of freshly distilled benzyl alcohol (100 ml) and C_6H_6 (50 ml), and the mixture was refluxed in a Dean–Stark apparatus. When no more H_2O appeared in the distillate the mixture was allowed to cool to room temperature, diluted with ether (500 ml), and cooled for an additional 2 h. The crystals formed were collected, washed with ether (200 ml), and dried in the air. The product was recrystallized from MeOH-ether. Yield: 90%.

1.7.1.15.1 **Preparation of Amino Acid Benzyl Ester *p*-Toluenesulfonate Salts Under Microwave Irradiation [159]** A mixture of the amino acid (10.0 mmol), benzyl alcohol (3 ml), and TsOH (2.09 g, 11.0 mmol) in a 100 ml glass beaker and exposed to microwaves (domestic microwave oven; LG Little Chef model 194A; 2450 MHz) operating at its 40% power for 50–60 s. After the completion of reaction, the mixture was cooled to room temperature, ether was added, and the precipitate was collected and washed with ether (25 ml × 2). Yield: H-GlyOBzl·HCl: 92%; H-AlaOBzl·HCl: 93%; H-ValOBzl·HCl: 94%; H-PheOBzl·HCl: 95%.

Boc-Asn-OBzl through Benzylation of Cs Salt [161]



mp. 120-122 °C;
$[\alpha]^{25}_D$ = -17.3 (*c* 1, DMF)

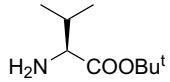
i) A solution of Boc-Asn (23.2 g, 100 mmol), H_2O (40 ml), and MeOH (400 ml) was neutralized with 20% Cs_2CO_3 in H_2O, and then evaporated to dryness. DMF (250 ml) was added and removed *in vacuo* at 45 °C. Addition and evaporation of DMF was repeated.

ii) The cesium salt of Boc-Asn obtained from the above step was treated with DMF (250 ml) and BzlBr (18.8 g, 110 mmol) at room temperature for 6 h, evaporated to dryness, and the residue was triturated with H_2O (1 l). The solid was dissolved in EtOAc (300 ml). The organic layer was washed with H_2O

(150 ml), dried over Na_2SO_4, and evaporated. The product was recrystallized from EtOAc/hexane. Yield: 90%.

Note: The procedure yields methyl, Trt, and phenacyl esters also [161].

1.7.1.16 General Procedure for the Preparation of tert-Butyl Esters of N^{α}-Unprotected Amino Acids Using Isobutene [168]

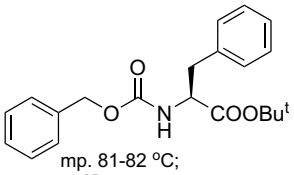


mp. 147-149 °C;
$[\alpha]^{25}_D$ = +20.5 (*c* 2, EtOH)

Liquid isobutene (25 ml) was added to a solution of Val (1.52 g, 0.013 mol) in dioxane (25 ml) and concentrated H_2SO_4 (2.5 ml) in a 500 ml pressure bottle, and the mixture was stirred at room temperature for 4 h. The solution was poured immediately into a cold mixture of ether (200 ml) and 1 N NaOH (125 ml), and the aqueous phase was washed with ether. The ether solution was dried over sodium sulfate and concentrated to about 5 ml. This was diluted with ether (25 ml). Addition of dry HCl gave the crystalline hydrochloride, which was recrystallized from EtOAc. Yield: 65%.

Note: *tert*-Butyl esters of N^{α}-unprotected amino acids can also be accessed via a two-step procedure in which Z-amino acids are esterified with isobutene followed by catalytic hydrogenation of the Z group [161].

1.7.1.16.1 Preparation of Z-Phe-O*t*Bu by the Silver Salt Method [170]

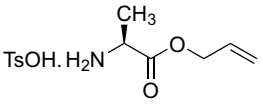


mp. 81-82 °C;
$[\alpha]^{25}_D$ = -4.4 (c 2, EtOH)

i) Z-Phe (54.5 g, 182 mmol) was dissolved in an excess of concentrated $(NH_4)OH$, and the solution was concentrated *in vacuo* to near dryness. *iso*-Propanol was added and the concentrating process was repeated. H_2O (200 ml) was added and the resulting solution was combined with a solution of $AgNO_3$ (30.9 g in 200 ml of H_2O). The resulting precipitate of silver Z-phenylalaninate was collected and dried. Yield: 88%; melting point 183–188 °C dec.

ii) *tert*-Butyl iodide was slowly added to a suspension of silver Z-phenylalaninate in ether (500 ml). The precipitate of AgI was removed and washed with dry ether. Evaporation of the combined ether portions in an open dish left as a crystalline solid that was recrystallized by making a solution of it in warm MeOH (100 ml) and adding this to H_2O (100 ml) containing sodium bisulfite (2.0 g). Yield: 33% from the silver salt.

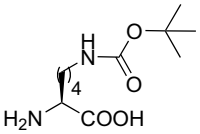
H-Ala-OAl·TsOH[176c]



mp. 84-85 °C;
$[\alpha]^{25}_D$ = -1.6 (*c* 1, MeOH)

A mixture of Ala (2.2 g, 25.0 mmol), allyl alcohol (15.0 g, 0.25 mmol), and $TsOH·H_2O$ (5.7 g, 30.0 mmol) in benzene (200 ml) was refluxed in a Dean–Stark apparatus until the requisite amount of H_2O was trapped. The benzene was evaporated and the product was precipitated by the addition of Et_2O. Yield: 98%.

Lys(Boc) [185, 273]



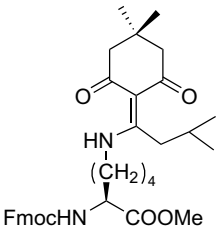
mp. 63-64.5 °C;
$[\alpha]^{20}_D$ = -6.97 (*c* 1, MeOH)

i) A solution of $CuSO_4·5H_2O$ (3.12 g, 12.5 mmol) in H_2O (25 ml) was added to a stirred solution of Lys·HCl (4.56 g, 25.0 mmol) in 2 M aqueous $NaHCO_3$ (25 ml). Then $NaHCO_3$ (2.1 g, 50.0 mmol) and 96% $(Boc)_2O$ (7.37 g, 32.5 mmol) in acetone (30 ml) were added. The mixture was stirred for 24 h, MeOH (6.5 ml) was added, and the solution was stirred for a further 12 h. H_2O (25 ml) and EtOAc (25 ml) were added, and the precipitate was filtered off. The precipitate was suspended in H_2O (62.5 ml) and filtered. These operations were repeated twice and a light-blue solid was obtained that was air-dried. Yield: 94%.

ii) To a suspension of $[Lys(Boc)]_2Cu$ (13.85 g, 25.0 mmol) in H_2O (500 ml), 8-quinolinol (9.45 g, 65.0 mmol) was added and the mixture was stirred for 5 h. Cu(II)8-quinolinolate was filtered off and washed with H_2O (25 ml). The precipitate was suspended in H_2O (250 ml) and kept for several hours. It was filtered off and washed with H_2O. All filtrates and washings were extracted with EtOAc (150 ml × 2), and the organic phase was evaporated. Yield: 96%.

Note: Decomposition of the copper complex is carried out with H_2S, EDTA, and Na_2S also [274].

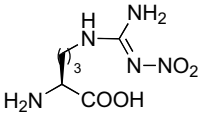
Lys(ivDde) [194a, 275]



mp. 69-71 °C

TFA (0.19 ml, 2.5 mmol) was added to a suspension of Fmoc-Lys-OH (9.2 g, 25.0 mmol) and 2-(1-hydroxy-3-methylbutylidene)-5,5-dimethylcyclo-hexane-1,3-dione (11.2 g, 50.0 mmol) in EtOH (192 ml) at room temperature and the mixture was refluxed for 60 h. The solvent was evaporated and the residue was dissolved in EtOAc (650 ml). The organic phase was washed with 1 M $KHSO_4$ (50 ml × 2), dried ($MgSO_4$), and concentrated to afford a yellow oil which was triturated with hexane and crystallized from EtOAc/hexane. Yield: 64%.

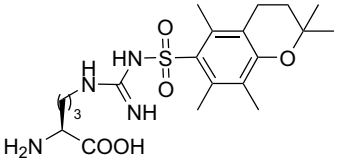
Arg(NO_2) [276]



mp. 251-252 °C;
$[\alpha]^{25}_D$ = +24.3 (*c* 4.12, 2 N HCl)

Arg free base (4.3 g, 25.0 mmol) was added slowly with stirring to a mixture of fuming HNO_3 (5.75 ml) and fuming H_2SO_4 (3.75 ml, containing 30% SO_3) maintained in an ice salt-bath. The mixture was stirred for 1 h with cooling and then poured onto crushed ice (about 50 g). The pH was adjusted to 8.0–9.0 with concentrated NH_4OH and then readjusted to pH 6.0 with glacial AcOH, and the solution was kept in a refrigerator for 4 h. The precipitate was collected, and recrystallized from hot H_2O, washed with 95% EtOH and ether, and dried. Yield: 82%.

Arg(Pbf) [19, 277]

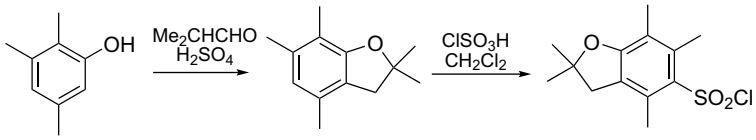


Pbf-Cl (11.45 g, 40.0 mmol) in acetone (55 ml) was added to a solution (at 0 °C) of Z-Arg-OH (7.5 g, 25.0 mmol) in 3.2 M NaOH (32.5 ml) and acetone (88.5 ml), and stirred at 0 °C for 2 h and for a further 2 h at room temperature. After acidification to pH 6.5 with saturated citric acid solution, the acetone was evaporated and the remaining solution was further acidified to pH 3 with saturated citric acid solution, diluted with H_2O (110 ml), and extracted with EtOAc (110 ml × 3). The combined extracts were filtered, washed with H_2O (150 ml × 2) and brine (150 ml × 2), and dried over $MgSO_4$. The solution was then concentrated, cooled in an ice bath, and cyclohexylamine (CHA; 2.85 ml, 25 mmol) was added. Addition of anhydrous ether gave a thick white gum, which solidified on standing overnight at 4 °C. Recrystallization from MeOH/Et_2O afforded the product.

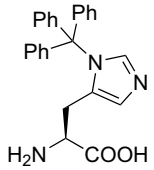
Z-Arg(Pbf)-OH·CHA was converted to its free acid and then subjected to hydrogenation via catalytic hydrogenation with 10% palladium on charcoal in MeOH under H_2 atmosphere [277].

Note:

- The above procedure is a modification of the procedure employed for the synthesis of Arg(Pmc). The Pmc-Cl in the latter is replaced by Pbf-Cl [277b].
- The preparation of Pmc-Cl is outlined in the following scheme. Step 1 is a modified procedure for the synthesis of the benzofuran nucleus [277c].



H-His(N^{τ}-Trt)-OH [25]

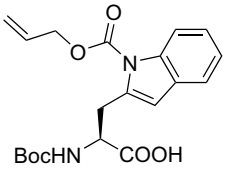


mp. 220-222 °C;
$[\alpha]^{25}_{D}$ = -2.1 (1:1 THF:H_2O)

Me_2SiCl_2 (1.21 ml, 10.0 mmol) was added to a suspension of His (1.55 g, 10.0 mmol) in DCM (15 ml) and the mixture was refluxed for 4 h. Then, Et_3N (2.79 ml, 20.0 mmol) was added and reflux was continued for an additional 15 min. This was followed by the addition of Et_3N (1.39 ml, 10.0 mmol) and a solution of Trt-Cl (2.79 g, 10.0 mmol) in DCM (10 ml) under stirring at room temperature. After 2 h, an excess of MeOH was added and the solvent was evaporated. H_2O was added to the residue and the pH was adjusted to 8.0–8.5 by dropwise addition of Et_3N. The resulting slurry was shaken well with $CHC1_3$ and the insoluble material was collected. Further washing with H_2O and Et_2O provided the product that was recrystallized from THF/H_2O (1:l). Yield: 97%. IR: 3550, 2200, 1650, 1560, 750, 700 cm^{-1}.

Note: The N^{α},N^{τ}-Trt$_2$-His (melting point 198 °C; $[\alpha]_D{}^{25} = +3.6$ (*c* 5, pyridine)) can be analogously prepared using 2 equiv. of Trt-Cl [25].

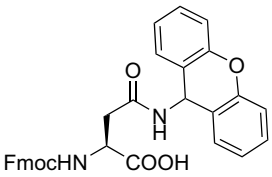
Boc-Trp(Aloc)-OH [213]



mp. 123-124 °C dec;
$[\alpha]^{25}_{D}$ = -16.7 (*c* 1, DMF)

Aloc-Cl (2.75 ml, 26.0 mmol), DBU (3.87 ml, 26.0 mmol), and DMAP (244 mg, 2.0 mmol) were added to a solution of Boc-Trp-O*t*Bu (7.95 g, 22.0 mmol) in MeCN (50 ml) and the mixture was stirred at 25 °C for 5 h. The solvent was evaporated and the residue was dissolved in EtOAc. The organic phase was washed with H_2O, saturated $NaHCO_3$, 5% $KHSO_4$/10% K_2SO_4, brine, dried over Na_2SO_4, and concentrated to afford Boc-Trp(Aloc)-O*t*Bu, which was recrystallized from Et_2O/hexane. Yield: 85%. The ester from the above step was dissolved in a mixture of TFA (6.0 ml) and H_2O (0.12 ml), and the solution was stirred for 30 min under N_2. The solvent was removed and the residue was concentrated 3 times from toluene, and the residue was suspended in a mixture of dioxane (12 ml) and H_2O (12 ml), and then treated with $(Boc)_2O$ (0.75 g, 3.4 mmol) in the presence of $NaHCO_3$ (1.2 g, 13.6 mmol) at 25 °C for 18 h. The resulting solution was diluted with EtOAc and acidified to pH 2 with 1 M HCl. The organic phase was washed with 5% $KHSO_4$/10% K_2SO_4, brine, dried over Na_2SO_4, and concentrated. The product was crystallized from Et_2O/hexane. Yield: 82%.

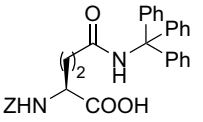
Fmoc-Asn(Xan)-OH [278]



mp. 206-209 °C

To a solution of Fmoc-Asn-OH (8.85 g, 25.0 mmol) and 9*H*-xanthen-9-ol (6.2 g, 37.5 mmol) in DMF (175 ml) was added TFA (8.75 ml, 111.5 mmol) in small portions, and the mixture was stirred at room temperature for 4 h, then poured into ice-H_2O (2 l). The precipitate was collected and washed with H_2O, ice-cold MeOH, and Et_2O. Yield: 99%.

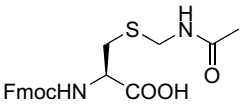
Z-Gln-(Trt)-OH [214]



mp. 161-162 °C;
$[\alpha]^{25}_D$ = -45 (MeOH)

A suspension of Z-Gln-OH (6.97 g, 25.0 mmol), Trt-OH (12.99 g, 50.0 mmol), Ac_2O (4.72 ml, 50.0 mmol), and concentrated H_2SO_4 (0.12 ml, 1.12 mmol) in AcOH (75 ml) was stirred for 1 h at 50 °C. The solution was then slowly added to cold H_2O (750 ml), and precipitate was collected and dissolved in EtOAc (50 ml). The organic layer was washed with H_2O, dried, and concentrated to leave a residue, which was crystallized from EtOAc/hexane. Yield: 67%.

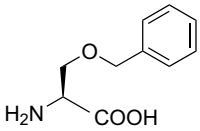
Fmoc-Cys(Acm)-OH [279]



mp. 147-148 °C;
$[\alpha]^{25}_D$ = -44 (*c* 1, DMF)

To a solution of Cys (3.95 g, 25.0 mmol) and *N*-(hydroxymethyl)acetamide (3.11 g, 30.2 mmol) in H_2O (5.82 ml) at 0 °C was added a mixture of TfOH/TFA (1: 19, 37 ml) and the mixture was stirred for 90 min under argon. The solvent was removed and the residue was reconcentrated from Et_2O (20 ml × 5). The resulting oil was dissolved in 10% aqueous Na_2CO_3 (50 ml) and the pH was adjusted to 10.0 with 10% aqueous Na_2CO_3. To this, Fmoc-N_3 (6.02 g, 22 mmol) in dioxane (65 ml) was added, and kept in an ice bath for 2 h and at room temperature for 48 h. The mixture was diluted with H_2O and extracted with Et_2O. The aqueous phase was acidified and extracted with EtOAc, and the organic layer was dried with $MgSO_4$ and concentrated. The product was crystallized from DCM/hexane. Yield: 62%.

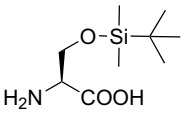
Ser(Bzl) [230]



mp. 221-222 °C;
$[\alpha]^{25}_D$ = +6.0 (*c* 1, 3M HCl)

To H-Ser-OLi (0.81 g, 10.0 mmol) suspended in THF (15 ml) was added $BF_3 \cdot OEt_2$ (6.0 ml) and the solution was stirred at room temperature for 6 h, and then at 40–45 °C for an additional 2 h to give 2,2-difluoro-4-(hydroxymethyl)-1,2,3-oxazaborolidin-5-one in quantitative yield. The THF was evaporated and the residue was dissolved in dioxane (30 ml), and then treated with benzyl trichloroacetimidate (2.15 ml, 11.5 mmol). After 2 h, the mixture was treated with anhydrous MeOH (5 ml), stirred for 10 min, and then heated with 1 M NaOH (30 ml); after stirring for 30 min and concentration at reduced pressure, the residue was dissolved in H_2O (200 ml) and the resulting solution was washed with Et_2O (15 ml × 3). The aqueous phase was adjusted to pH 6.0 and applied to an Amberlite XAD-4 resin column. The column was washed with H_2O and then with 50% EtOH. The latter eluant was collected and concentrated. Yield: 95%.

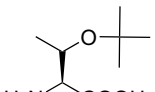
H-Ser(TBDMS)-OH [280]



Imidazole (13.6 g, 2.0 equiv.) and TBDMS-Cl (20.46 ml, 1.1 equiv.) were added to Ser (10.6 g, 0.1 mmol) in DMF (100 ml) and the mixture was stirred at room temperature for 20 h. The DMF was evaporated to give an oily residue which was stirred with H_2O/hexane (1: 1) for 4 h to obtain a white solid. The latter was collected, washed with hexane, and air-dried. Yield: 91%.

Note: Z-Ser(TBDMS)-OH is prepared analogously starting from Z-Ser [276].

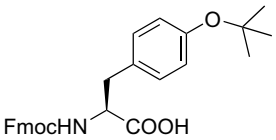
Thr(tBu) [281]



mp. 259-260 °C;
$[\alpha]^{20}_D$ = -42.1 (*c* 2, MeOH)

To a solution of *p*-nitrobenzyl ester of Z-Thr (38.8 g, 100 mmol) in DCM (400 ml) kept in an ice-water bath, isobutene (350 ml) and concentrated H_2SO_4 (5 ml) were added with caution, and left at room temperature for 4 days. The mixture was cooled again to about 0 °C, washed with an ice-cold 5% solution of Na_2CO_3 in H_2O (200 ml × 3), the aqueous phases were re-extracted with DCM (100 ml × 2), and the organic phases were combined washed with H_2O until the washes were neutral. The solution was dried over P_2O_5 and evaporated *in vacuo*. The yellow, crystalline residue was dissolved in a small volume of EtOAc and diluted with hexane until some dark oil separates. The clear solution was decanted and further diluted with hexane. The *tert*-butyl ether separated as needles. Yield: 80%. melting point 55–56.5 °C. The latter (22.3 g, 50.0 mmol) was dissolved in MeOH (150 ml), the solution was diluted with H_2O (50 ml) and AcOH (4 ml), the air was displaced with N_2, a 10% palladium on charcoal catalyst (4.5 g) was added, and the mixture was hydrogenated at room temperature. After removal of the catalyst by filtration and the solvent by evaporation *in vacuo*, the residue was triturated with EtOH and recrystallized from EtOH/acetone. Yield: 85%.

Fmoc-Tyr (tBu)-OH [282]



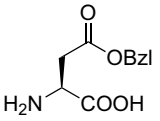
mp. 150-151 °C;
$[\alpha]^{20}_D$ = -28.0 (*c* 1, DMF)

i) Fmoc-Tyr-OMe (5.0 g, 12.0 mmol), concentrated H_2SO_4 (0.33 ml, 6.0 mmol), and DCM (100 ml) were stirred under isobutene gas (260 Torr) for 6 h at room temperature. The solution was washed with cold 10% $NaHCO_3$ (100 ml × 2) and brine (100 ml), dried (Na_2SO_4), and concentrated. The residue was dissolved in MeOH/CCl_4 (1: 1, 400 ml), washed with H_2O (300 ml), MeOH/H_2O (1: 1, 200 ml × 2), dried (Na_2SO_4), and the solvent was evaporated to give a

white solid that was recrystallized (DCM/hexane). Yield: 83%; melting point 90–92 °C; $[\alpha]_D^{20} = -22.1$ (*c* 1, DMF).

ii) A mixture of Fmoc-Tyr(*t*Bu)-OMe (2.0 g, 4.22 mmol) in MeCN (250 ml) and 3% Na_2CO_3 (375 ml) was stirred for 15 h, then washed with hexane (500 ml × 3), acidified with 2 M HCl to pH 3–4, and extracted with $CHCl_3$ (600 ml × 2). The combined $CHCl_3$ fractions were washed with brine (500 ml), dried (Na_2SO_4), and the solvent was removed to provide an oil that gave white crystals from EtOAc/hexane. Yield: 74%.

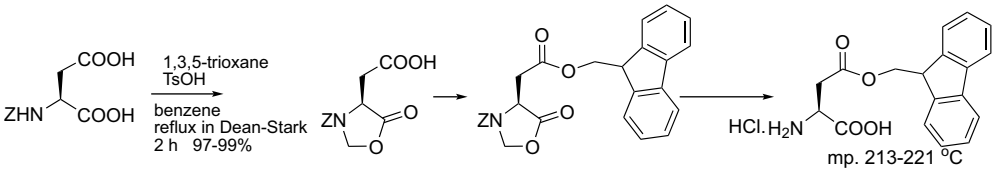
Asp(OBzl) [239]



mp. 218-220 °C;
$[\alpha]^{25}_D$ = +28 (*c* 1, 1 N HCl)

Freshly distilled benzyl alcohol (100 ml) was added to a mixture of dry Et_2O (100 ml) and concentrated H_2SO_4 (10 ml). The ether was evaporated and finely ground Asp (13.4 g, 100 mmol) was added in small portions with stirring. The resulting solution was kept at room temperature for about 1 day, diluted with 95% EtOH (200 ml), and neutralized by the dropwise addition of pyridine (50 ml) under stirring. The mixture was stored in the refrigerator and washed by trituration on the filter with ether. The product was recrystallized from hot H_2O containing a few drops of pyridine. Yield: 40%.

Asp(Fm) [242b]

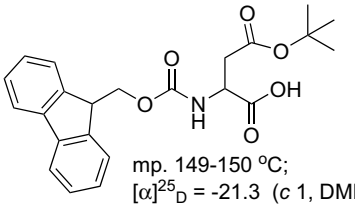


i) Esterification of carboxy group of oxazolidin-5-one of Z-Asp. The title oxazolidin-5-one (2.06 g, 10.0 mmol) and DCC (2.06 g, 10 mmol) were dissolved in dry THF (150 ml) and stirred at room temperature. A second solution, made by dissolving 9-fluorenylmethanol (1.37 g, 7.0 mmol) and DMAP (0.12 g, 1.0 mmol) in dry THF (20 ml), was added dropwise to the first solution over a period of 20–25 min. The mixture was stirred at room temperature for 1 h (TLC; $CHCl_3$/MeOH/AcOH 94: 5: 1 or $CHCl_3$/MeOH/AcOH 80: 18: 2). Additional DCC (approx. 2.0 mmol) was added every 30 min until all the 9-fluorenylmethanol was consumed. The solution was filtered, and

the THF was evaporated and replaced with an equal volume of EtOAc. The solution was placed in the freezer for about 1 h and any insoluble dicyclohexylurea (DCU) found was filtered off. This step was repeated with a fresh aliquot of EtOAc. The EtOAc solution was washed with H_2O (twice), 5% $NaHCO_3$, H_2O, 5% HCl, and H_2O. The organic phase was dried over anhydrous $MgSO_4$ and concentrated to afford a pale yellow, viscous oil. Yield: 84–87%.

ii) Concomitant removal of N^{α}-Z group and ring opening of 5-oxazolidinone. The above product (4.57 g, 10.0 mmol) was dissolved in neat TFA (50-60 ml). HBr gas was bubbled through this stirred solution for 10–15 min and then it was stirred at room temperature for another 30 min. Upon completion of reaction (TLC), HBr/TFA was removed *in vacuo* to afford a deep-blue oil. The oil was dissolved in glacial AcOH (50–60 ml) and lyophilized to complete dryness. This residue was dispersed in EtOAc (150 ml) and HCl gas was bubbled through the slurry with stirring in order to generate the less-soluble HCl salt of the amino acid. The pale yellow precipitate that formed was filtered, washed several times with EtOAc and dried under vacuum. Yield: 72–78%.

Fmoc-Asp (OtBu)-OH [240]



mp. 149-150 °C;
$[\alpha]^{25}_D$ = -21.3 (*c* 1, DMF)

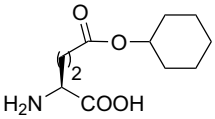
A mixture of Asp (3.9 g, 29.0 mmol) and TsOH (11.1 g, 58.0 mmol) in dioxane (140 ml) was stirred under isobutene gas (350 mbar) for 3 days at room temperature. To this was added aqueous Na_2CO_3 (175 ml, 10%, w/v) followed by dropwise addition of Fmoc-OSu (9.9 g, 29.0 mmol) in dioxane (50 ml) at 0 °C. After stirring overnight at room temperature, the mixture was poured into ice-H_2O (300 ml) and washed with Et_2O (300 ml × 3) to remove excess Fmoc-OSu and Fmoc-di-*tert*-butyl-Asp. The aqueous phase was chilled (0 °C), acidified to pH 5.5 with 1 N HCl, and extracted with EtOAc (300 ml × 3). The combined EtOAc fractions were washed with brine, dried ($MgSO_4$) and concentrated to yield a white solid. This is a mixture of mono-*tert*-butyl esters (Fmoc-Asp(O*t*Bu)-OH and Fmoc-Asp-O*t*Bu) that was dissolved in DCM/petroleum ether (1:1, boiling point 30–60 °C) and chilled (0 °C) overnight. The product was obtained as crystals which were filtered and washed with petroleum ether. Overall yield 35% (HPLC purity 99.5%, C_{18} column, 60% MeOH in 0.1% TFA).

Note:

- The α-*tert*-butyl ester can be crystallized as its DCHA salt from a DCM solution.
- Fmoc-Glu(O*t*Bu)-OH is prepared analogously. Once the mixture of mono-*tert*-butyl esters are obtained, it is dissolved in Et_2O and the desired product was crystallized as the DCHA salt by the addition of DCHA (2.5 ml, 0.6 equiv). HPLC

was conducted via the same solvent system. Overall yield: 39%; melting point 78–80 °C; $[\alpha]_D^{25} = -15.7$ (*c* 1, DMF).

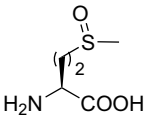
H-Glu(OCγ)-OH [283]



mp. 195-198 °C

Cyclohexanol (250 ml, 2.5 mol) and Glu (36.7 g, 0.25 mol) were added under stirring to chilled Et_2O (250 ml) containing H_2SO_4 (25 ml, 0.5 mmol), and the suspension was heated in a rotary evaporator at 70 °C for 2 h under reduced pressure. The bulk of the solvent was removed during this procedure. The resulting oil was partitioned between EtOAc (250 ml) and 5% aqueous $KHCO_3$ (300 ml). The pH was adjusted to 7.0 with 4 M NaOH and the aqueous layer was concentrated until precipitation occurred. The resulting suspension was chilled overnight and filtered. Yield: 49%.

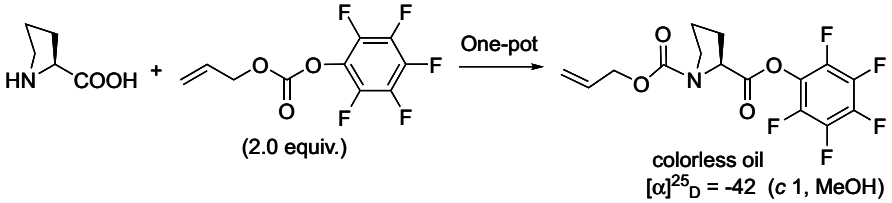
Methionine Sulfoxide [229]



mp. 253 °C;
$[\alpha]^{22}_D$ = +41.5 (*c* 2, 1N HCl)

A suspension of Met (3.0 g, 20.0 mmol) in H_2O (10 ml) was maintained at room temperature with stirring. A solution of H_2O_2 (30%, 2.2 ml) was added in small portions over a period of 30 min. A clear solution was formed. After a further hour at room temperature, absolute EtOH (100 ml) was added. Two hours later, the crystals were collected and washed with 95% EtOH (100 ml). Yield: 97%.

1.7.1.17 General Procedure for Concomitant Protection and Activation of Amino Acids Using Pentafluorophenyl Carbonate [139]



colorless oil
$[\alpha]^{25}_D$ = -42 (*c* 1, MeOH)

To a suspension of Pro (0.126 g, 1.1 mmol) and Aloc-OPfp (0.563 g, 2.0 mmol) in DMF (2 ml) at 0 °C was added pyridine (0.177 ml, 2.2 mmol) dropwise, and the mixture was allowed to attain 28 °C. The stirring was continued until the disappearance of the starting material. The mixture was then diluted with DCM (30 ml), washed with saturated citric acid solution (10 ml), H_2O (10 ml × 2) and brine (10 ml), dried over Na_2SO_4, and concentrated. The resulting product was purified by column chromatography (silica gel, 100–200 mesh) eluting with 5–15% EtOAc/hexane. Yield: 84%.

Note: The one-pot synthesis of Fmoc-amino acid pentafluorophenyl esters has been reported earlier. In this method, amino acid (1.1 equiv.) is treated with Fmoc-OPfp (1.0 equiv.) in a mixture of aqueous Na_2CO_3 and acetone at room temperature and after completion of the reaction (TLC), the mixture was acidified to pH 3 with concentrated HCl and extracted with EtOAc. To the organic layer, DCC (1.1 equiv.) was added at 0 °C and the mixture was stirred for 2 h. The DCU formed was filtered off and the filtrate was concentrated to afford Fmoc-amino acid pentafluorophenyl esters [138].

1.7.2 Deprotection Reactions

1.7.2.1 Removal of the Phth Group by Hydrazinolysis [284]

Phth-Gly–Gly-OH to H-Gly–Gly-OH The starting peptide (2.62 g, 10.0 mmol) was added to a 1 M solution of hydrazine hydrate in absolute EtOH (10 ml). The mixture was diluted with EtOH (30 ml) and refluxed for 1 h. The alcohol was evaporated and the residue was treated with 2 N HCl (25 ml) at 50 °C for 10 min, and then kept at room temperature for 30 min. The insoluble phthalylhydrazine was filtered off and the filtrate was evaporated. The residue was recrystallized from boiling EtOH to afford the purified hydrochloride salt (monohydrate, 1.73 g) that was then dissolved in H_2O (20 ml) and treated with the anion-exchange resin Amberlite IR4B until a drop of the solution gave no positive reaction for chloride ion with $AgNO_3/HNO_3$. The resin was filtered off, washed with H_2O, and the filtrate was concentrated to about 20 ml. The solution was heated on a steam bath, diluted with absolute EtOH until crystallization starts, and then allowed to cool to room temperature. The crystals were collected, washed with 95% EtOH, and dried. Yield: 81%.

1.7.2.2 Removal of the Nps Group

Nps-Lys(Boc)–Leu–Phe–Lys(Boc)–Lys(Boc)-OH to H-Lys(Boc)–Leu–Phe–Lys(Boc)–Lys (Boc)-OH [285] To a solution of ammonium rhodanide (0.16 g, 2.0 mmol) and 2-methyl-indole (0.26 g, 2.1 mmol) in MeOH (6 ml) and AcOH (18 ml), the starting peptide (1.12 g, 1.0 mmol) was added and allowed to stand at room temperature for 3 h. The solvent was evaporated, the residue was washed with warm (about 50 °C)

distilled H_2O (10 ml × 2), with 2 N NH_4OH (10 ml × 2), triturated with ether, and transferred to a filter. Trituration and washing with ether were continued until a white product was obtained that was dried *in vacuo* over P_2O_5 and KOH pellets. Yield: 90%; melting point 217 °C dec.; $[\alpha]_D^{20} = -4.2$ (*c* 1.2, AcOH).

1.7.2.3 Removal of the Z Group

1.7.2.3.1 Protocol A: Employing CH [45]

Z-Pro–Leu–Gly-NH$_2$ to H-Pro–Leu–Gly-NH$_2$ H_2 gas was bubbled through a solution of the starting peptide (3.2 g, 7.6 mmol) in EtOH (30 ml) containing aqueous HCl (1 equiv.) in presence of 5% Pd/C until completion of the reaction (TLC). The catalyst was filtered off, the filtrate was concentrated, and the residue was treated with a slight excess of NH_3 in $CHCl_3$. After NH_4Cl was filtered off, the filtrate was concentrated and the residue was recrystallized from H_2O. Yield: 80%.

Removal of Z through CH from Met-Containing Peptides [286]

Z-Trp–Met–Asp(OtBu)–Phe-NH2 to H-Trp–Met–Asp(OtBu)–Phe-NH$_2$ [46b] A solution of the starting peptide (7.87 g, 10.0 mmol) was dissolved in DMF (450 ml) followed by the addition of distilled H_2O (110 ml) and DIPEA (16 ml), and then 10% Pd-on-$BaSO_4$ (0.80 g). The mixture was stirred in an atmosphere of H_2 until no more gas was absorbed. The catalyst was filtered off and the filtrate was concentrated at 30 °C.

Note:

- Completion of the deprotection is determined by diluting the residue with DMF to about 175 ml followed by the addition of *p*-nitrophenyl ester of Z-Gly (3.64 g, 11.0 mmol). A day later, a spot test with ninhydrin (development of no purple color) indicates complete acylation of the amino group that was set free in the process of catalytic hydrogenation.
- The method cannot be applied when the peptides contain *S*-benzyl-Cys. Alternatively, Z group-protected peptides containing *S*-alkyl-Cys can be removed through catalytic hydrogenation in liquid ammonia solvent particularly in the presence of dimethylacetamide and TEA [46a].

1.7.2.3.2 Protocol B: Employing Silylhydride

Z-Tyr-OMe to Tyr-OMe [287] Neat triethylsilane (39.90 ml, 250.0 mmol) was added dropwise under an argon atmosphere to a stirred solution of Z-Tyr-OMe (8.2 g, 25.0 mmol) and Pd/C (1.23 g, 20% by weight) in 10: 1 MeOH/$CHCl_3$ (75 ml), and the mixture was stirred for 10 min. Upon completion of the reaction (TLC), the mixture was filtered through celite, the solvent was evaporated, and the product was purified on a short silica gel column. The yield was found to be quantitative.

Note: The same procedure has been used to cleave the Aloc group from Aloc-Leu-OMe in 5 min in quantitative yield [287].

1.7.2.3.3 Protocol C: Through CTH using 1,4-Cyclohexadiene as Hydrogen Donor [48b]

Z-Lys(Boc)-Thr(tBu)-OMe to H-Lys(Boc)-Thr(tBu)-OMe A solution of the starting peptide (5.52 g, 10 mmol) in absolute EtOH (50 ml) was maintained at 25 °C. A slow stream of N_2 was led above the surface of the solution and 10% Pd-on-charcoal (5.5 g) was added and the mixture was stirred, followed by the addition of 1,4-cyclohexadiene (8.0 g = 9.4 ml, 100.0 mmol). Upon completion of the reaction (TLC), the catalyst was filtered off and the filtrate was evaporated to leave a residue which was dried *in vacuo*. Yield: 98%.

Note:

- CTH is faster in glacial AcOH than in EtOH or in MeOH. In the case of the latter solvent, the rate is further reduced. With the former solvent, Met-containing peptides can also be used as substrates.
- For the removal of the $N^{\omega\prime}$-NO_2 group of Arg and N^{im}-benzyl group of His, more active Pd-black is used as the catalyst.
- CTH can also be carried out using cyclohexene, hydrazine, ammonium formate and 98–100% formic acid as hydrogen donors. With the last reagent as the hydrogen source in CTH, the Boc group as well as benzyl ethers have been removed concomitantly [48a].

1.7.2.4 Cleavage of the Fmoc Group

1.7.2.4.1 Method A: Using TAEA [67]

Fmoc-Gly–Gly–Phe–Leu-OBn to H-Gly–Gly–Phe–Leu-OBn Tris(2-aminoethyl)amine (7.5 ml, 50 equiv.) was added to a DCM solution containing the starting peptide and stirred for 30 min. During this time a white precipitate separated which dissolved readily in saturated NaCl solution. The reaction mixture was extracted with brine (10 ml × 3) and thrice (15 ml each time) with phosphate buffer (prepared by dissolving 90 g of $NaH_2PO_4 \cdot H_2O$ and 32.7 g of Na_2HPO_4 in 500 ml of H_2O, pH 5.5). There was no interference by either an emulsion or a precipitate. Additional DCM was used for back extraction. The clear organic layer was concentrated. The product can be directly used for coupling.

1.7.2.4.2 Method B: Using DEA: Simultaneous Removal of the Fmoc Group and 9-Fluorenylmethyl Ester [288]

Fmoc-Leu–Phe-OFm to H-Leu–Phe-OH The starting peptide (0.68 g, 1.0 mmol) was dissolved in DMF (9 ml) and DEA (1 ml), and the solution was allowed to stand at room temperature for 2 h. DEA and the solvent were evaporated at a temperature below 30 °C. The residue was triturated with a mixture of Et_2O (3 ml) and hexane (12 ml), and the solid product was collected and washed with a mixture of ether (5 ml) and hexane (5 ml). Yield: 94%; melting point 235–255 °C dec.; $[\alpha]_D^{23} = +37$ (*c* 1.4, AcOH); R_f 0.49 (*n*-butanol/AcOH/H_2O (4: 1: 1)).

1.7.2.5 Cleavage of the Boc Group

1.7.2.5.1 Protocol A: Removal of the Boc group with TFA in the Presence of Scavengers [103]

Boc-Leu–Leu–Gln–Gly–Leu–Val-NH_2 to H-Leu–Leu–Gln–Gly–Leu–Val-NH_2·TFA To a suspension of the starting peptide (0.37 g, 0.0.50 mmol) in 1: 1 PhOH/*p*-cresol (3 ml), TFA (2 ml) was added and the resulting solution was kept at room temperature for 1 h. TFA was evaporated and Et_2O (25 ml) was added to result in a precipitate. The latter was collected by centrifugation, washed with Et_2O (20 ml $\times$ 8), and dried in air under reduced pressure over P_4O_{10} to obtain the TFA salt of the peptide.

1.7.2.5.2 Protocol B: Cleavage of Boc Group with TMS/Phenol [105]

Boc-peptide resin was treated with 15 ml (per 1 g resin) of the 1 M Me_3SiCl/3 M phenol/DCM reagent for 5 min and then with a second 15 ml portion for 15 min. The washing protocol was as follows. The filtered resin was washed with DCM (15 ml), shaken for 3 min with 4% H_2O/DMF (15 ml), filtered, shaken for 3 min with 10% DIPEA/DMF, filtered, and washed 3 $\times$ 1 min with DCM (15 ml $\times$ 3). The resulting hydrochloride of the peptide-resin was neutralized with 10% DIPEA/DMF and washed with DCM in preparation for the next coupling cycle.

1.7.2.6 Transprotection of N^{α}-Protecting Groups: Fmoc-Met-OH to Boc-Met-OH [121e]

To a stirred solution of Fmoc-Met-OH (0.14 mmol) in DMF (1.2 ml) was added, under N_2 atmosphere, KF (0.98 mmol) followed by Et_3N (0.30 mmol). The solution was kept at room temperature until completion and Boc_2O (0.18 mmol) was then added. After stirring for several hours, the reaction was diluted with EtOAc, and washed with H_2O and cold 5% HCl, 5% $NaHC0_3$, and brine. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The resulting crude product was purified by column chromatography to afford the product.

1.7.2.7 Selective Methyl Ester Hydrolysis in the Presence of the N^{α}-Fmoc Group

Fmoc-Gly–Phe–Pro-OMe to Fmoc-Gly–Phe–Pro-OH [145] The starting peptide was treated for 7 h with NaOH (1.2 equiv.) added to 0.8 M $CaC1_2$ in *i*PrOH/H_2O (7: 3, 10.6 ml) at room temperature. The hydrolyzate was neutralized with 1 M AcOH, evaporated, and the solid residue was dissolved in MeOH. Addition of H_2O resulted in a precipitate, which was filtered and extensively washed with H_2O. The crude peptide was recovered from the filter by dissolution into MeOH and evaporation of the solvent (purity greater than 80% by HPLC). The product was purified on silica gel (eluant: CH_2Cl_2/MeOH/AcOH, 5–10% MeOH, 1% AcOH); residual AcOH was eliminated by dissolution of the product in MeOH followed by precipitation and washing with H_2O. Yield: 85%. The product displayed high purity by HPLC and gave the expected fast atom bombardment mass spectrometry spectrum.

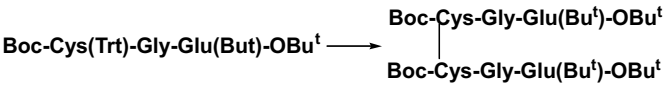
1.7.2.8 Cleavage of tert-Butyl Ester Using $BF_3 \cdot Et_2O$ [289]

Z-Cys–Gly–Gly–Gly–Cys(Bh)–Gly-OtBu to Z-Cys–Gly–Gly–Gly–Cys(Bh)–Gly-OH To a solution of the starting peptide (8.09 g, 0.01 mol) in AcOH (100 ml) was added ethyl mercaptan (5 ml, 0.0675 mol) and $BF_3 \cdot Et_2O$ complex (15 ml, 0.118 mol). The flask was swept with dry N_2 and kept at room temperature for 15 min. The product was precipitated by the addition of excess ether and reprecipitated from DMF. Yield: 95%.

1.7.2.9 Selective Cleavage of Phenacyl Ester in the Presence of the N^{α}-Nosyl Group [290]

Sodium benzenethiolate (5 mmol) was added cautiously to a stirred solution of N^{α}-Me-N^{α}-nosyl amino acid phenacyl ester (1 mmol) in DMF and the mixture was stirred at room temperature for 30 min under an inert atmosphere (N_2) with frequent monitoring (TLC, Et_2O/petroleum ether 70: 30 (v/v)). The solvent was evaporated and the residue was treated with 1 N NaOH and extracted with $CHCl_3$ (3–10 ml). The aqueous solution was acidified with 1 N HCl and then extracted with $CHCl_3$ (3–10 ml). The combined organic extracts were washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford the product. Yield: oil, 70-87%.

1.7.2.10 Removal of the Trt Group (Iodolysis) [291]



The starting peptide (1.53 g, 3.0 mmol) and iodine (508 mg, 2.0 mmol) were dissolved in MeOH (25 ml), and stirred at room temperature for 1 h. The mixture was cooled in an ice-H_2O bath and decolorized by the dropwise addition of 1 M sodium thiosulfate in H_2O, diluted with H_2O (50 ml), and the precipitated material was filtered and dried. It was extracted with petroleum ether (10 ml × 3). The remaining solid was crystallized from EtOAc/hexane. Yield: 91%; melting point 150–152 °C.

1.7.2.11 Deprotection of the Pbf Group from Z-Arg(Pbf)-OH [19]

A sample of Z-Arg(Pbf)-OH (16 mg) was treated with TFA/H_2O (80: 20) at 37 °C. At regular intervals aliquots were diluted 4-fold with H_2O and analyzed by HPLC (mobile phase: H_2O/CH_3CN (A/B) containing 0.1% TFA. gradient: 13% B to 50% B, 15 min; 50% B, 13 min; flow rate: 3 ml min^{-1}). The average of two runs was used for comparison. A preparative run led to the isolation of product in 89% yield.

1.7.2.12 Removal of the Phenoc Group through Photolysis [253]

A solution of Phenoc-Thr-OH (3 mmol) in EtOH (100 ml) was irradiated in an atmosphere of argon at 0 °C in a Raynonet RPR 208 photochemical reactor with a 300-nm lamp After 4 h, a small amount of the starting material remained (TLC). After 14 h, the solvent was evaporated and the resulting solid residue was triturated with Et_2O. Yield: 75%.

1.7.2.13 Conversion of the DCHA Salt of N^{α}-Protected Amino Acids into Free Acids [292]

Nps-Thr(tBu)-OH·DCHA to Nps-Thr(tBu)-OH Finely powdered Nps-Thr(*t*Bu)-OH·DCHA (5.1 g, 10.0 mmol) was added to a two-phase system of EtOAc (40 ml) and a solution of $KHSO_4$ (2 g, 15.0 mmol) in H_2O (40 ml), and shaken by hand until the DCHA salt was completely dissolved. The aqueous layer was extracted with EtOAc (10 ml × 2) and the combined organic layers were washed with H_2O until they were free of sulfate ions, dried over Na_2SO_4, evaporated to dryness *in vacuo*, and the residue was dried over P_2O_5 for several hours.

Note: In the case of highly acid-sensitive protection, the free acid was converted into an active ester without isolation through carbodiimide-mediated esterification to prevent autocatalytic removal of the protection due to the acidity of the free carboxylic acid group.

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Part One
Amino Acid-Based Peptidomimetics

Amino Acids, Peptides and Proteins in Organic Chemistry.
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2
Huisgen Cycloaddition in Peptidomimetic Chemistry

Daniel Sejer Pedersen and Andrew David Abell

2.1
Introduction

Peptides provide important leads for drug discovery because of their diversity of structure and function, and also because of their key role in many fundamental processes associated with life. However, their potential as therapeutics is somewhat limited by an inherent instability towards proteolytic cleavage and a general lack of oral bioavailability [1–3]. Despite this, peptides have found important uses as drugs (e.g., liraglutide in the treatment of diabetes and cyclosporin as an immunosuppressant) [4]. A more general approach to therapeutics is provided by peptidomimetics, where a peptide structure is modified to introduce appropriate biological and chemical properties associated with effective drug action. For example, the metabolic stability of a peptide can be improved by modifying its constituent peptide bonds and/or backbone structure [5].

A heterocycle can be incorporated into a peptide to define conformation, metabolic properties, and/or chemical and biochemical reactivity. For example, replacing a peptide bond with a tetrazole provides a mimic of a *cis*-peptide bond [6]. Triazoles are also a particularly interesting class of hetrocycle in this context. These structures present similar electronic properties, planar geometry, and hydrogen bonding capability to a peptide bond, whilst being hydrolytically and metabolically stable. The recent discovery of the copper catalyzed [2 + 3] cycloaddition by Meldal *et al*. [7] provides the general methodology required for incorporating a triazole into a peptide to give important new peptidomimetics that have found wide application as discussed in the following Sections.

The triazole unit represents an isostere of a *trans*-peptide bond with the advantage of being stable to hydrolytic and proteolytic cleavage [8–11]. While it closely mimics the geometry and hydrogen-bonding capability of a peptide bond, it does alter the separation of the adjacent side-chains – being longer for a 1,4-triazole and shorter for a 1,5-triazole (Figure 2.1). In addition, the dipole of triazole unit is stronger than that of a peptide bond [11].

As an example, Ghadiri *et al*. incorporated a triazole dipeptide analog into a peptide mimicking a protein epitope, the X-ray crystal structure analysis of which shows that

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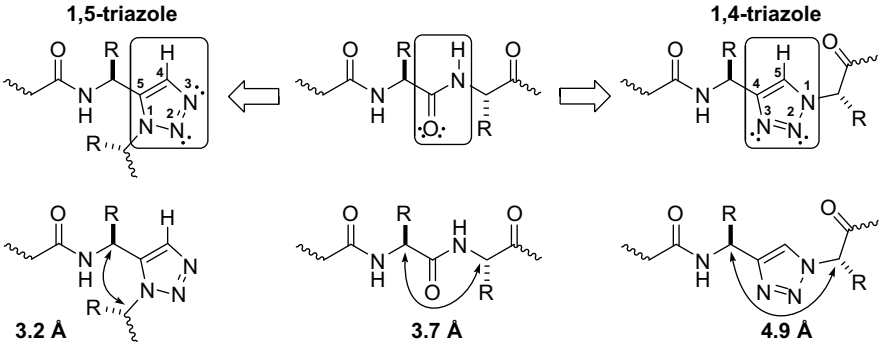


Figure 2.1 Triazoles as peptide bond isosteres.

the secondary and quaternary structure of the parent protein epitope have been retained. Hydrogen bonding of the triazole N2 and C5-hydrogens to an amide NH and an amide carbonyl, respectively was observed [8].

2.2
Huisgen [2 + 3] Cycloaddition Between Azides and Acetylenes

The thermal [2 + 3] cycloaddition between acetylenes and azides, first reported by Michael in 1893 [12], was later studied in great detail by R. Huisgen [13] with his seminal contribution in the area of [2 + 3] dipolar cycloadditions [14, 15]. This reaction, commonly referred to as the Huisgen cycloaddition [13, 16], is of limited synthetic utility because it produces mixtures of 1,4- and 1,5-triazoles, Figure 2.2 (with the exception of some highly electron deficient terminal alkynes and intramolecular cyclizations) [10].

The subsequent discovery and development of the copper-catalyzed Huisgen cycloaddition (Cu-Huisgen) by Meldal *et al.* [7, 17] provided a milestone in contemporary organic chemistry by allowing the selective synthesis of 1,4-triazoles. Copper (I) catalysis also provides enhanced reaction rates (up to 10^7 times) to allow these

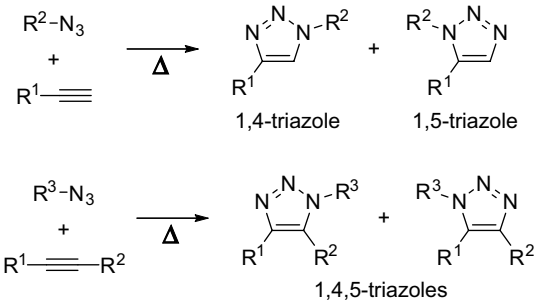


Figure 2.2 The thermal Huisgen cycloaddition.

reactions to be run at ambient temperature. This advance has had a tremendous impact on many areas of chemistry and biology [18]. The Sharpless group later introduced the concept of "click" chemistry [19], with the Cu-Huisgen cycloaddition reaction now being one of the most powerful and widely employed reactions of this type.

The component azides and alkynes are inert to most biological and organic environments, and tolerate most common reaction conditions, including molecular oxygen and water [20]. As a consequence, the Cu-Huisgen reaction has found widespread application in biology and bioorganic chemistry. For example, it has been used to modify bacterial and viral cell surfaces [21–24], to profile proteins *in vitro* and *in vivo* [25], and is sometimes referred to as a bio-orthogonal reaction. One of the most impressive applications of Cu-Huisgen is in the area of nonsense suppression [26], which allows the incorporation of unnatural acetylene and azido amino acids into functional proteins for site-specific surface functionalization [27–29].

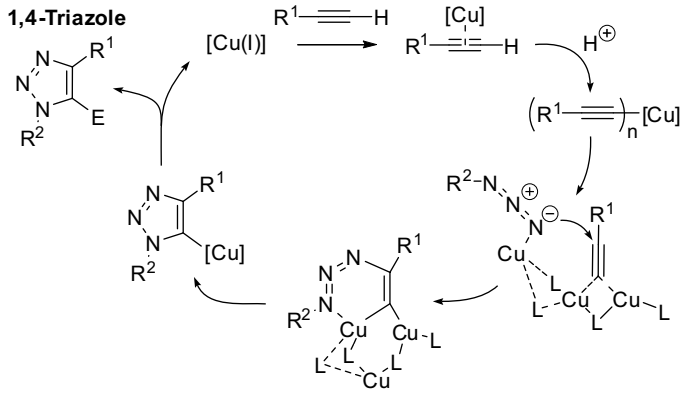
A ruthenium-catalyzed Huisgen cycloaddition (Ru-Huisgen) has also recently been reported, that unlike Cu-Huisgen reaction, allows the selective synthesis of 1,5-triazoles [30]. This cycloaddition reaction is not a click process, but it does represent a very important contribution to organic chemistry by allowing the synthesis of isomeric triazoles with different spatial arrangement of substituents.

The Cu-Huisgen cycloaddition has been reviewed extensively, covering a wide range of chemistry disciplines [10, 18, 31–46], including its use in peptide chemistry [9, 47]. By contrast, the Ru-Huisgen reaction is in its infancy and has only briefly been covered in a review by Tornøe and Meldal [47]. Here, we present an overview of Cu-Huisgen and Ru-Huisgen cycloaddition within the area of amino acid and peptidomimetic chemistry, with general experimental details for the synthesis of 1,4- and 1,5-triazoles. Moreover, the synthesis of acetylene and azido amino acids and ruthenium-based catalysts required for the synthesis of these peptidomimetics is covered. This is not intended to be an exhaustive review, but rather introduces and summarizes possibilities offered by the Cu- and Ru-Huisgen reactions within amino acid and peptidomimetic chemistry. We have selected key examples that showcase different aspects of the chemistry with the main emphasis on backbone-modified peptidomimetics.

2.3
Mechanistic Consideration for the Cu-Huisgen and Ru-Huisgen Cycloadditions

The Cu-Huisgen cycloaddition is catalyzed by Cu(I). It can be carried out by adding a Cu(I) source or alternatively by generation of Cu(I) *in situ* by reduction of a Cu(II) salt or by comproportionation of Cu(II) and Cu(0). A vast variety of copper sources, under a wide range of reaction conditions have been successfully employed as recently reviewed by Tornøe and Meldal [18].

The mechanism for Cu-Huisgen has been reviewed and analyzed by Bock *et al.* [10], and more recently Meldal and Tornøe [18], but it remains to be fully elucidated. Scheme 2.1 presents the mechanistic suggestions proposed by Meldal



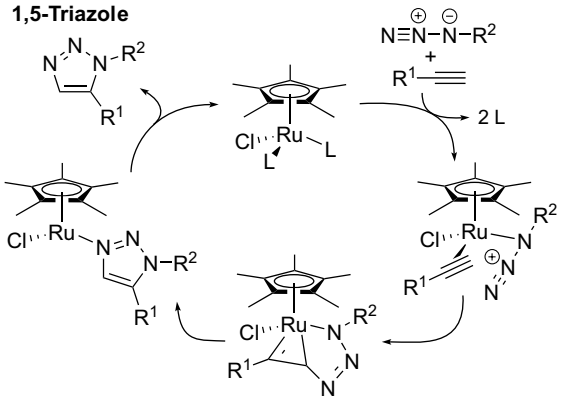
Scheme 2.1 Mechanism for Cu-Huisgen cycloaddition suggested by Meldal and Tornøe [18]. E = trapped electrophile (e.g., H).

and Tornøe, and takes into account all mechanistic details available in late 2007. The Cu-Huisgen cycloaddition, unlike the thermal Huisgen cycloaddition, only takes place with terminal acetylenes since the mechanism proceeds via a copper acetylide. In addition, it should be noted that the Cu-Huisgen cycloaddition works well in a variety of organic solvents, including, acetonitrile, *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), diisopropylethylamine, tetrahydrofuran (THF), dichloromethane, as well as biphasic conditions, most commonly *tert*-butanol/water [48].

There does not appear to be a general correlation between the method employed to promote a Cu-Huisgen reaction and the product yield [18], with $CuSO_4$ often being preferred because of its ease of handling. However, in some cases Cu(I) halides are used since they are known to provide a significant increase in reaction rate. Moreover, CuI is partially soluble in various organic solvents suitable for solid-phase peptide synthesis. Ligands such as tris-[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine (TBTA) [49] are sometimes added to increase the reaction rate [49–51]. It is also important to note for peptide-based applications that histidine residues within a substrate can increase the rate of the Cu-Huisgen cycloaddition. As such, the presence or absence of histidines in a peptide sequence can influence the reaction outcome [52].

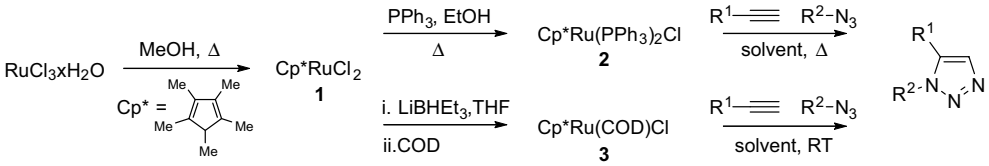
The Cu-Huisgen reaction is generally a highly predictable and efficient reaction. However, alkyne homocoupling [10, 53] can be a significant side-reaction in cases where there is a high density of alkyne or if triazole formation is very sluggish. A side-reaction involving the unnatural amino acid propargyl glycine, giving enol lactones under standard Cu-Huisgen conditions, has also been reported [54].

The Ru-Huisgen cycloaddition is by contrast more specialized, requiring the use of nontrivial ruthenium catalysts, organic solvents, heating in some cases, and the protection of certain functional groups within the substrate [30, 55–57]. The proposed mechanism for the ruthenium-catalyzed reaction of a terminal acetylene is shown in Scheme 2.2 [55]. The fact that both terminal and disubstituted acetylenes participate in this reaction suggests that ruthenium acetylides are not involved.



Scheme 2.2 Mechanism for the Ru-Huisgen cycloaddition suggested by Boren *et al.* [55].

The Ru-catalysts **2** and **3**, containing a pentamethylcyclopentadiene ligand (Cp*) (Scheme 2.3), are commonly employed to promote Ru-Huisgen cycloaddition reactions. These catalysts give the 1,5-triazole in high yield and with excellent regioselectivity for terminal acetylenes. The regioselectivity observed for disubstituted acetylenes can be influenced by directing groups on the acetylene (e.g., propargylic alcohols and amines) [55, 57]. In the absence of directing groups the regiochemical outcome of the reaction is primarily controlled by electronic properties of the alkyne and to a lesser extent steric effects. Moreover, it should be noted that secondary azides require long reaction times and give low yields of product, and tertiary azides generally do not react at all [55].



Scheme 2.3 Synthesis of Ru-catalysts **2** and **3** for the Ru-Huisgen cycloaddition.

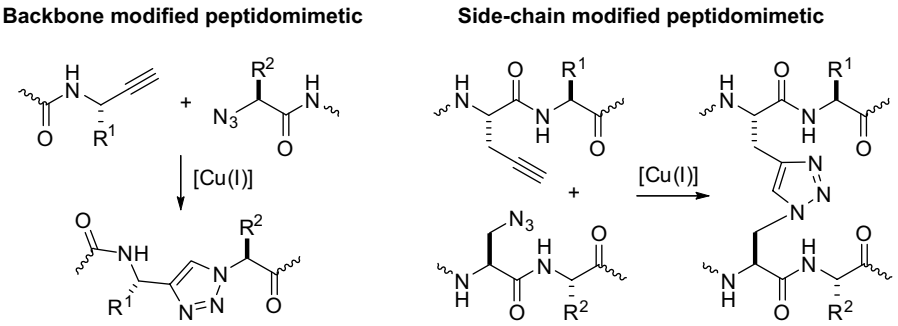
The cyclooctadiene (COD) ligand of catalyst **3** is much more labile than the phosphine ligand of catalyst **2**, as such catalyst **3** is more active and functions at room temperature. This enhanced reactivity is particularly useful for promoting reactions of less reactive disubstituted alkynes and aryl azides. It should be noted that catalyst **3** is rapidly deactivated on heating and thus poor yields of the triazole are observed under these conditions [55]. By contrast, reactions involving catalyst **2** generally require heating, with microwave conditions working particularly well to give good yields and very short reaction times [56, 58]. For ease of preparation we tend to employ catalyst **2**, but it should be noted that catalyst **3** tends to give cleaner reactions and higher yields of the 1,5-triazole [55]. The precursor **1** for making catalysts **2** and **3** is easily synthesized from ruthenium trichloride [59] with the main drawback being the

relatively high cost of Cp*. Detailed experimental procedures for the synthesis of ruthenium complexes **1** [60], **2** [55, 59], and **3** [55] have been published, and we have found compounds **1**, **2**, and **3** to be stable when stored at −20 °C. Catalysts **2** and **3** are also commercially available, but we are unable to comment on the quality of these products.

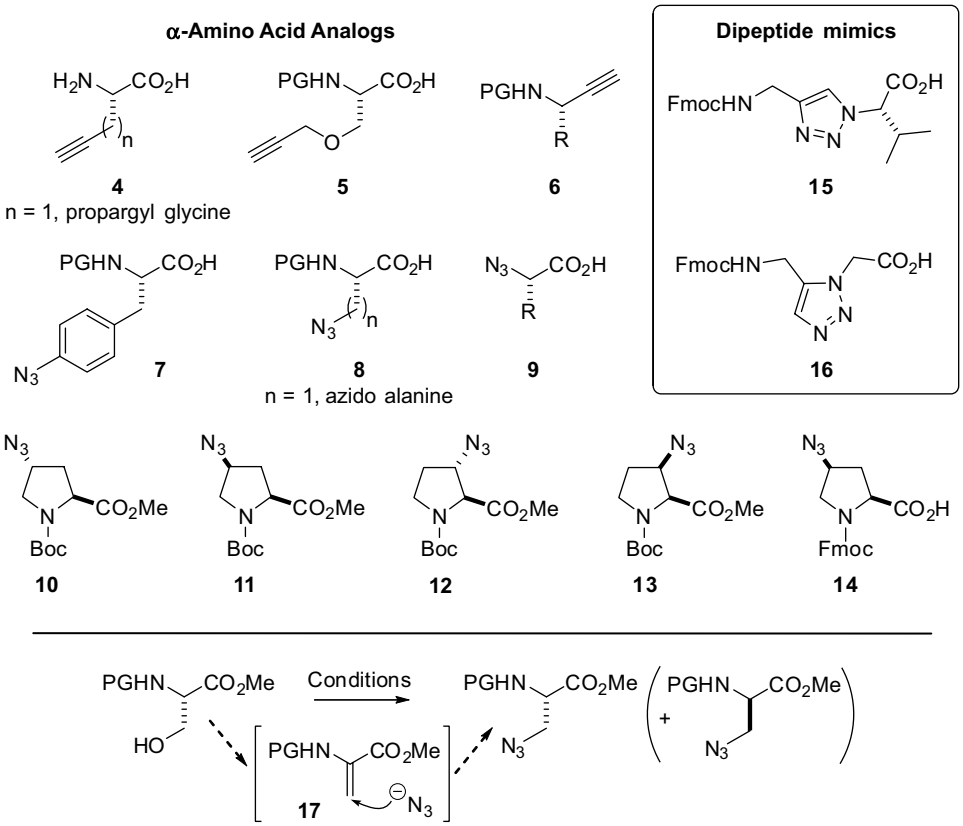
Ru-Huisgen cycloadditions are best carried out using a catalyst loading of approximately 2 mol% in a wide variety of solvents, with some preference for aprotic solvents such as 1,2-dichloroethane, dioxane, toluene, THF, and DMF. The reaction also proceeds in protic solvents such as methanol and 2-propanol; however, side-products are often observed under these conditions, resulting in decreased yield of the triazole. In general, the reactions appear to be quite insensitive to the presence of trace amounts of water and atmospheric oxygen. In addition, many functional groups within the substrates are tolerated [55], with the notable exception of Boc protection within an amino acid [58]. (When Boc-protected amino acids were exposed to standard Ru-Huisgen microwave conditions complex mixtures were obtained. Protection of the carboxylic acid as a methyl ester was sufficient to resolve the problem. It is unclear if the observed result is due to unfavorable interaction of the carboxylic acid with the catalyst. It is known that Boc-protected amino acids in some cases can be Boc-deprotected under microwave conditions [61], which could be another reason for the poor outcome.)

2.4 Building Blocks for the Synthesis of Triazole-Modified Peptidomimetics

Cu- or Ru-Huisgen chemistry has been used to prepare peptidomimetics that have modified peptide backbones, side-chains, or backbone to side-chain modification as shown in Scheme 2.4. This chapter is primarily focused on those examples that give rise to modification of the backbone. Structures of this type are often prepared from the basic building blocks shown in Scheme 2.5, which are themselves derived from optically active α-amino acids (e.g., **4**–**14**, Scheme 2.5) or from dipeptide mimics such



Scheme 2.4 Examples of backbone and side-chain modified peptidomimetics, using Cu-Huisgen chemistry. Backbone to side-chain modified peptidomimetics not shown.

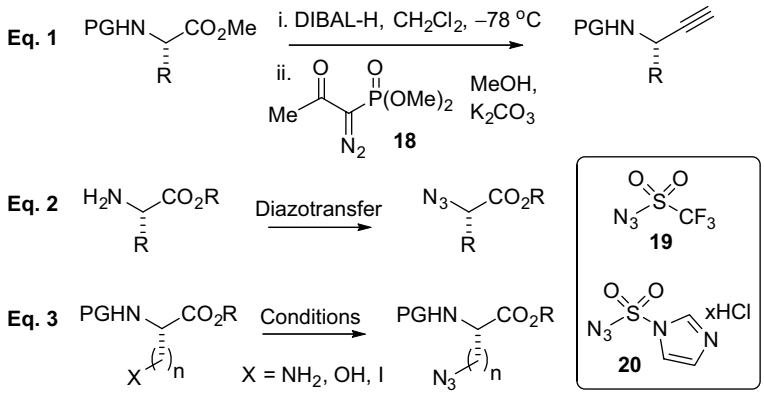


Scheme 2.5 (Top) Building blocks commonly used for making triazole-peptidomimetics. (Bottom) Synthesis of azido alanine and possible route to stereochemical leakage. PG = protection group.

as **15** and **16**. Some of these building blocks are commercially available (e.g., **4**, **7**, **8**, $n = 1$) generally at high cost.

The synthesis of building blocks **4** ($n = 1$ [62] and $n = 2$–4 [63]) is not trivial and involves an asymmetric alkylation of glycine derivatives. By comparison, the side-chain modified serine **5** is easily prepared by simple propargylation of *N*-protected serine [64]. Acetylene **6**, necessary for the synthesis of backbone modified peptidomimetics, is synthesized from an amino acid methyl ester according to the method of Dickson *et al.* employing the Bestmann–Ohira reagent **18** (Eq. 1, Scheme 2.6) [65].

Azides are generally readily accessible from the corresponding amine (when available) by diazotransfer using conditions developed by Alper *et al.* (Eqs. 2 and 3, Scheme 2.6) [66]. The amino acid derivatives **7**–**9** (Scheme 2.5) have been prepared in this way using trifluoromethylsulfonyl azide **19**, itself prepared under biphasic water/dichloromethane conditions using sodium azide and a large excess of triflic anhydride due to competing hydrolysis [67]. An alternative procedure that is compatible with anhydrous reaction conditions has been reported [68]. Goddard-



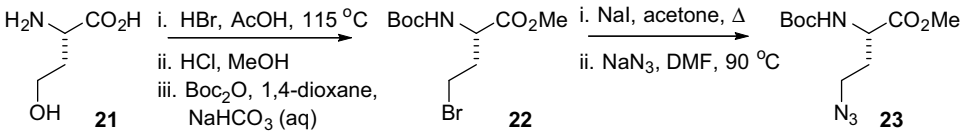
Scheme 2.6 General routes to acetylene and azido-modified amino acid building blocks. R = α-amino acid side-chain.

Borger and Stick recently introduced a new solid and shelf-stable diazotransfer reagent (imidazole-1-sulfonyl azide hydrochloride **20**) that promises to further improve the diazotransfer chemistry of amino acids [69].

Azido alanine **8** ($n = 1$), frequently employed for making side-chain modifications (see below), has been synthesized from both serine and (*S*)-1,3-diaminopropionic acid. However, in our experience particular care should be taken with the synthesis of azido alanine since it is susceptible to racemization as shown in Scheme 2.5. Activation of the serine hydroxyl by conversion to a leaving group, followed by nucleophilic substitution, has been attempted, but this generally results in elimination to give dehydroalanine **17** [70]. Zou *et al*. have reported the synthesis of azido alanine via activation of serine as the phenylmethylsulfonate [71]. However, in our hands dehydroalanine is formed almost quantitatively, with similar results using mesylates and tosylates [70]. Panda and Rao reported the synthesis of azido alanine by nucleophilic displacement of mesylated Weinreb amide derivatives [72]. However, this method is unattractive as it requires two additional synthetic steps to install and remove the Weinreb amide. The two most common methods for making azido alanine employ a Mitsunobu reaction of protected serine using hydrazoic acid [70, 73] or by diazotransfer on (*S*)-1,3-diaminopropionic acid [74]. Diphenylphosphoroyl azide can be used as a replacement for the explosive and toxic hydrazoic acid in the Mitsunobu reactions [75]. The synthesis of azido alanine by diazotransfer is generally carried out on Boc-protected amino acids due to the basic reaction conditions. However, Roice *et al*. have reported the successful synthesis of Fmoc-protected azido alanine under buffered diazotransfer conditions [76]. Diazotransfer can also be carried out on solid phase [77–79].

Longer chain azides, such as azido ornithine **8** ($n = 3$, Scheme 2.5) and azido lysine **8** ($n = 4$), are readily synthesized by diazotransfer without racemization [63] and the azide **23** has been prepared from homoserine **21** (Scheme 2.7) [63].

Efficient procedures for the synthesis of azido prolines **10–13** from commercially available *trans*-3-hydroxy and *trans*-4-hydroxy proline has been reported by Gómez-

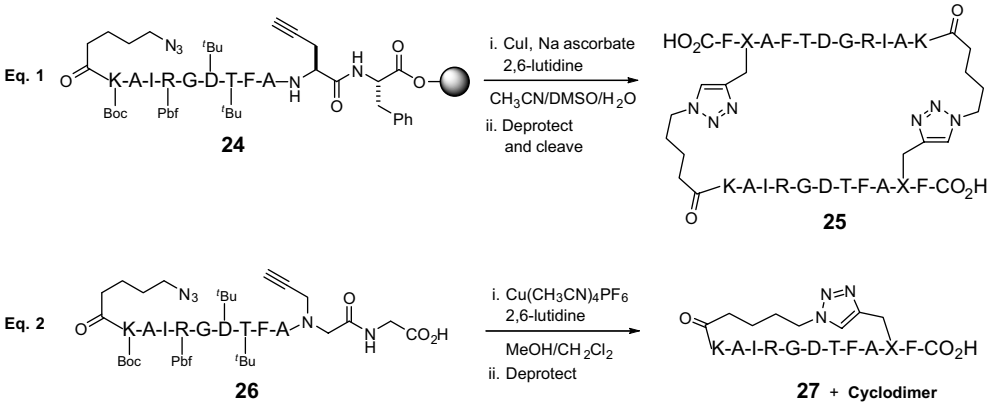


Scheme 2.7 Synthesis of methyl (*S*)-Boc-δ-azido-norvalinate **23** in five steps with no intermediate purification and no loss of stereochemistry [63].

Vidal and Silverman [80]. The synthesis of both enantiomeric series was reported for 4-azido proline (**10** and **11**) but only the two diastereoisomers **12** and **13** of 3-azido proline were published. These building blocks allow the synthesis of wide variety of side-chain modified peptidomimetics and due to the easy access to six azido proline isomers the effect of different spatial arrangement of substituents can be explored in detail [81–83]. Gómez-Vidal and Silverman synthesized all six azido prolines using Mitsunobu methodology employing the reagent diphenylphosphoroyl azide as a substitute for the highly explosive and toxic reagent hydrazoic acid. Klein *et al*. has reported the synthesis of Fmoc-protected 4-azido proline **14** by mesylation and nucleophilic displacement with azide, which gives easy access to this stereoisomer [75]. Another isomer of 4-azido proline has similarly been synthesized by Madalengoitia *et al*. [84]. However, the approach employed by Klein and Madalengoitia is limited to the accessibility of the corresponding hydroxy prolines, some of which are costly or require lengthy syntheses.

2.5
Cyclic Triazole Peptidomimetics

Finn *et al*. reported the formation of cyclodimerization peptidomimetic **25** as the sole product from the Cu-Huisgen cyclization of a solid supported peptide sequence (**24**) using CuI and sodium ascorbate (Eq. 1, Scheme 2.8) [85]. This led to a more detailed



Scheme 2.8 Monocyclization versus cyclodimerization; 10–20% cyclodimerized product was also obtained upon monocyclization of peptidomimetic **26**, Eq. 2.

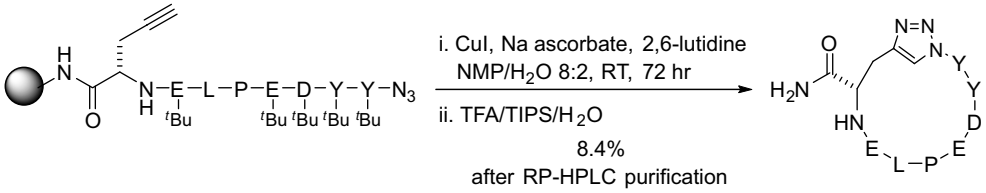
study on the use of the Cu-Huisgen reaction of fully protected α-, β-, and γ-amino acids, as well as mixed β,γ-amino acid sequences [86]. All on-resin cyclization studies were performed on fully protected peptides with an *N*-propargylated glycine residue closest to the resin surface. In most cases the azide coupling partner was attached at the N-terminus as 5-azidopropanoic acid. These studies have shown that the amino acid sequence does not influence the efficiency of cyclodimerization. Cyclodimerization generally worked well for peptide sequences consisting of more than six α- or β-amino acids and most reliably when the acetylene reaction partner was in close proximity to the solid phase.

Monocyclization has also been achieved selectively on-resin (or in solution) using a solvent (such as DMSO) that disrupts peptide–peptide interstrand hydrogen bonding. As has been observed for cyclodimerization, monocyclization appears to be sequence independent with short peptide sequences (less than six residues) particularly favoring monocyclization. It is interesting to note that peptoids (*N*-substituted glycine derivatives) very reliably only return monocyclized product, presumably because peptide–peptide interstrand hydrogen bonding is not possible. Similar peptide sequences give exclusive formation of cyclodimerized product on-resin [85], but predominantly monocyclic peptidomimetics in solution (Eq. 1 versus Eq. 2, Scheme 2.8) [86]. This systematic study strongly suggests that on-resin peptide–peptide interstrand hydrogen bonding brings the acetylenes and azides into close proximity, facilitating the formation of cyclodimeric peptidomimetics.

In 2004, Meldal *et al*. published the synthesis of a side-chain cyclized peptidomimetic that mimics a fragment of the peptide hormone α-melanocyte-stimulating hormone [7, 76]. Incorporation of a propargyl glycine and an azido alanine residue, followed by Cu-Huisgen cyclization on solid support using CuI and $(i\text{Pr})_2\text{NEt}$ in THF, gave high yields of the cyclized product for both the fully protected and deprotected peptidomimetic (respectively, 79 and 76% yield) [76]. This result is somewhat surprising given the study of Finn *et al*. (see above [86]), where it was concluded that on-resin cyclization of sequences six residues or longer favors the formation of cyclodimers. However, it should be noted that there are several differences between the work of Meldal *et al*. and Finn *et al*., most notably the use of a special poly(ethylene glycol)-based amino polymer solid support by Meldal *et al*. Moreover, THF was employed for the on-resin cyclization which would disrupt peptide–peptide interstrand hydrogen bonding.

Goncalves *et al*. designed and synthesized a series of cyclic triazole peptidomimetics consisting of eight amino acid residues (eight examples) and one peptidomimetic consisting of 11 residues as potential ligands for vascular endothelial growth factor receptor 1 (VEGFR1) [87]. These peptides were synthesized using standard N^{α}-Fmoc chemistry on a Rink amide 4-methylbenzhydrylamine (MBHA) resin. Cu-Huisgen cyclization of peptides was accomplished (in low yield after purification by reverse-phase high-performance liquid chromatography (HPLC)) on-resin using a combination of CuI, sodium ascorbate, and 2,6-lutidine (Scheme 2.9).

All the cyclizations gave the desired monomeric peptidomimetics; however, extended reaction times were required for complete consumption of starting material (48–96 h). Cyclodimers were not observed. This is surprising given the study by



Scheme 2.9 Synthesis of potential VEGFR1 antagonists by Cu-Huisgen on-resin cycloaddition.

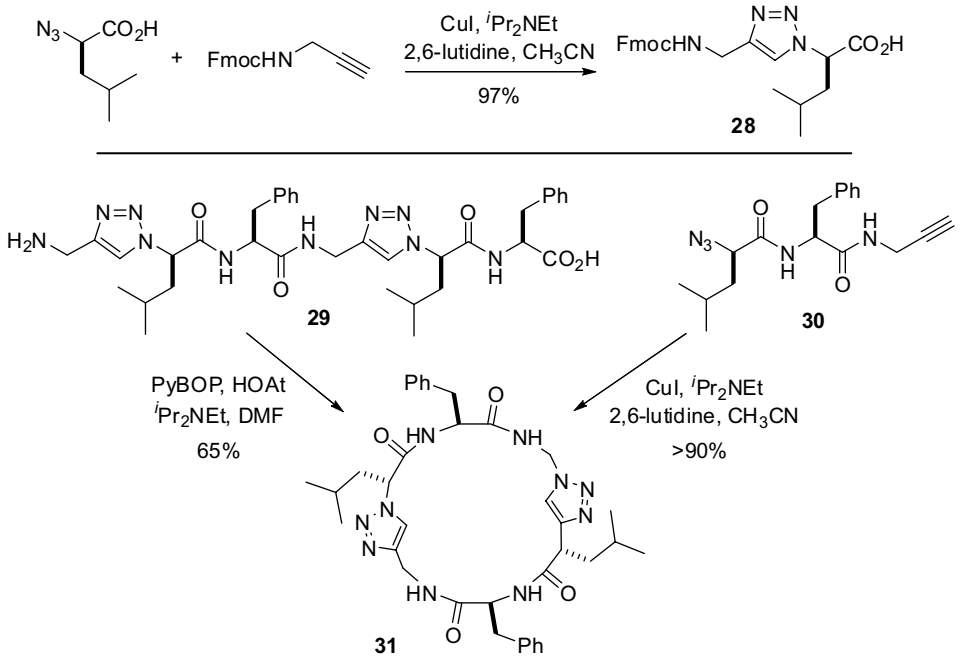
Finn *et al*. (see above) [86] that showed that cyclodimerization is encouraged on-resin when the acetylene is in close proximity to the resin and the peptide sequence is more than residues long. Goncalves *et al*. employed a mixture of *N*-methyl-2-pyrrolidone (NMP) and water, which could account for the observed selectivity for cyclomonomeric peptidomimetics since peptide–peptide interstrand hydrogen bonding would be disrupted under these conditions to favor formation of cyclomonomeric product.

In all cases the acyclic peptidomimetics were equipotent or more potent than the cyclic peptidomimetics. Goncalves *et al*. speculated that the poor affinity of the cyclic peptidomimetics could be due to poor sequence alignment with the receptor surface due to limited backbone flexibility.

Ghadiri *et al*. reported studies on the use of 1,4-triazoles as peptide backbone replacements in the synthesis of self-assembling peptide nanotubes [88, 89]. Self-assembly of the cyclic peptides requires the amino acid residues to have complementary hydrogen bond donor and acceptor pairs on each face of the cyclic peptide. This is achieved using a flat conformation resulting from having an even number of amino acids, with alternating stereochemistry at the α-carbon, presenting the side-chains on the exterior of the peptidomimetic with the amide backbone perpendicular to the plane of the ring. The acyclic peptidomimetic **29** was initially synthesized on solid phase using the amino acid triazole building block **28**, with the final macrocyclization being accomplished in solution under standard peptide coupling conditions (Scheme 2.10) [88]. However, Ghadiri *et al*. realized that there would be a distinct advantage in forming these C2 symmetric peptidomimetics (e.g., **31**) if Cu-Huisgen cycloaddition chemistry was employed for the final macrocyclization utilizing building blocks such as **30** [89]. This approach is highly amenable to analog synthesis as demonstrated by the efficient synthesis of five macrocyclic peptidomimetics with varying side-chain functionalization.

Only small amounts (4–8%) of trimers and larger oligomers were observed. It was suggested that an alternating α-carbon chirality of amino acids kinetically favors formation of the cyclic peptidomimetic [89].

Head-to-tail cyclic peptides occur in a wide variety of natural products that display an impressive range of biological activities [90]. These cyclic peptides generally have improved metabolic stability and can have enhanced potency due to geometric preorganization into an appropriate conformation for binding. As such, these structures are appealing for the design of small protein secondary structure mimics. Efficient head-to-tail cyclization of peptides is, however, quite difficult to achieve using conventional techniques, with tetrapeptides being notoriously difficult



Scheme 2.10 Self-assembling backbone modified peptidomimetics.

to ring-close. Bock *et al*. have published a synthesis of three highly strained cyclic tetrapeptide analogs (e.g., **33**, Figure 2.3) of a naturally occurring cyclic tetrapeptide **32** using Cu-Huisgen chemistry [91, 92].

Attempts to prepare **33** by lactamization of a linear peptide were unsuccessful (Scheme 2.11); however, an alternative Cu-Huisgen strategy, using CuBr and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in refluxing toluene, gave the cyclic product in 70% yield clearly illustrating that peptides too strained for ring closure via lactamization can be obtained using Cu-Huisgen methodology. The Cu-Huisgen reaction did, however, require considerable optimization. Standard $CuSO_4$/sodium ascorbate conditions completely failed to give cyclization even after prolonged reaction time.

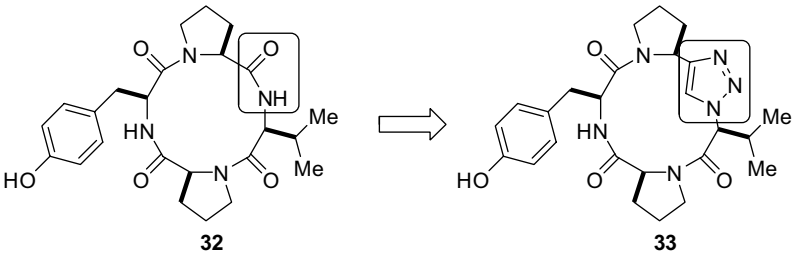
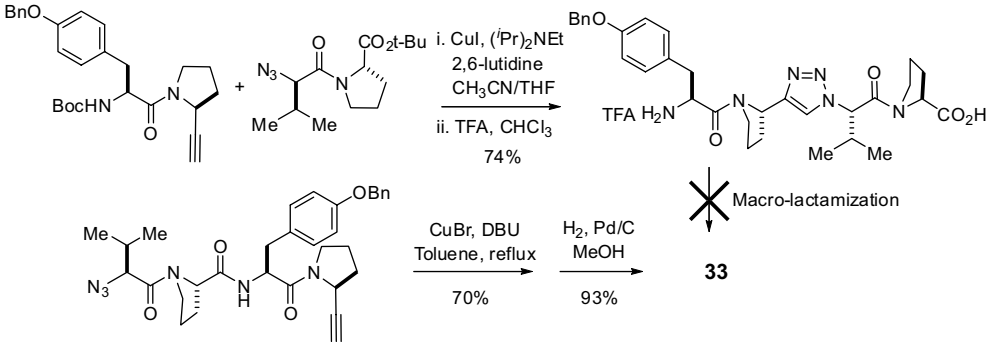


Figure 2.3 Substitution of *trans*-amide bond in the natural product **32** for a 1,4-triazole.



Scheme 2.11 Successful cyclization by Cu-Huisgen cycloaddition, where lactamization fails, to give the triazole peptidomimetic **33**.

The use of CuI gave 33% product, but the 5-iodo-triazole-macrocycle was obtained as a major side-product.

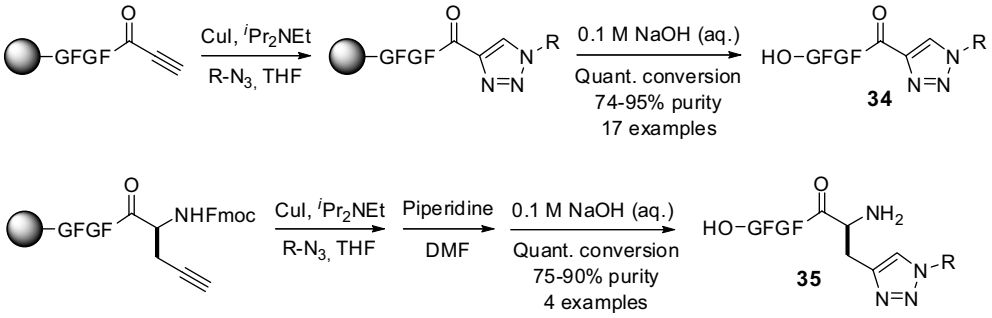
The three synthesized peptidomimetics were approximately 3-fold more potent against mushroom tyrosinase as compared to the natural cyclic peptide. This demonstrates that triazoles can indeed be employed as *trans*-amide bond surrogates.

Waldmann *et al.* have also employed Cu-Huisgen cyclization for the synthesis of head-to-tail cyclic peptidomimetics. They published a synthesis of a series of analogs of the natural product Jasplakinolide, a 19-membered cyclodepsipetide with potent cytotoxicity and antitumor activity [93]. Two macrocyclization strategies were employed to replace an *E*-configured double bond with a 1,4-triazole using either macro-lactonization or Cu-Huisgen cyclization. While product was obtained by the macro-lactonization strategy (28–59%) the yields of cyclic peptidomimetic were significantly improved (65–92%) by performing a Cu-Huisgen cycloaddition with CuI, $(i\text{Pr})_2\text{NEt}$, and 2,6-lutidine in CH_3CN/THF. One of the Jasplakinolide analogs obtained in a 70% yield by Cu-Huisgen reaction was only obtained in trace amounts via the macro-lactonization strategy demonstrating the utility of the Cu-Huisgen cyclization. The biological activity of the Jasplakinolide analogs was not reported.

2.6
Acyclic Triazole Peptidomimetics

A detailed study was published by the Meldal group in 2002 on the use of the Cu-Huisgen cycloaddition for solid- and solution-phase synthesis of peptidomimetics. Both backbone (**34**, Scheme 2.12) and side-chain (**35**) modified peptides were studied [7]. Some representative examples from this seminal paper are outlined in Scheme 2.12.

The reaction was shown to be fully compatible with both $PEGA_{800}$ and $SPOCC_{1500}$ resin. Importantly, it was discovered that the alkyne coupling partner must be immobilized on the solid phase and not vice versa, presumably due to competing alkyne cross-coupling in solution. For example, experiments with PEGA resin,



Scheme 2.12 Solid-phase Cu-Huisgen backbone and side-chain cycloaddition.

acylated with 2-azido-2-methylpropionic acid, failed to undergo Cu-Huisgen cycloaddition even under forcing conditions using a large excess of alkyne.

An early example of peptide bond substitution with a 1,4-triazole was reported by Brik *et al.* in a synthesis of HIV-1 protease inhibitors [94, 95]. A diversity-oriented synthesis, in microtiter plates, efficiently generated a library of 100 peptidomimetics that had one amide bond replaced by a 1,4-triazole. The key Cu-Huisgen cycloaddition was carried out by the addition of $CuSO_4$ and a piece of copper turning to each vial to give quantitative formation of the desired peptidomimetics after 48 h at room temperature. In this manner two potent inhibitors (**36** and **37**, Figure 2.4) were identified and studied by cocrystallization with the protease [96].

These studies strongly suggest that the triazole C5-hydrogen takes the place of the amide bond NH and participates in hydrogen bonding (3.8 Å) with an active- site carbonyl oxygen atom. Moreover, the triazole N2 atom takes the place of the amide carbonyl oxygen atom and hydrogen bonds to a water molecule.

In another study Ghadiri *et al.* examined if it is possible to replace dipeptide **38** within an α-helical coiled coil with a triazole ε^2-amino acid **39** (Figure 2.5), whilst retaining the secondary and quaternary structure of the parent protein epitope [8].

Despite the dipeptide mimic being one atom longer than the native dipeptide, the three peptidomimetics all showed circular dichroism (CD) spectra characteristic of an α-helical structure. Thermal denaturing experiments revealed some marked differences between the three peptides, with only peptidomimetic **42** having a thermal denaturation temperature similar to the parent peptide pLI-GCN4

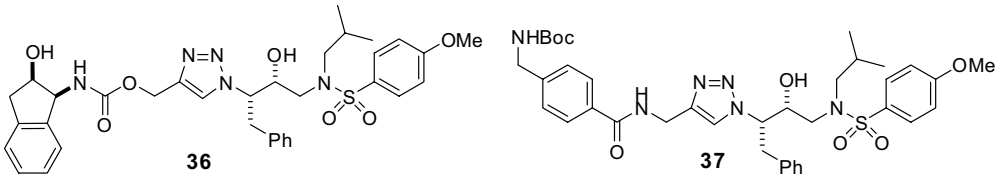
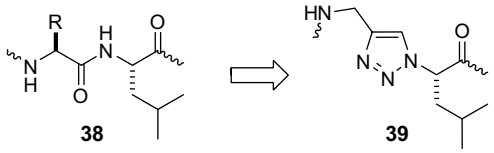


Figure 2.4 Potent peptidomimetic inhibitors of HIV-1 protease. Peptidomimetic **36**: $IC_{50} = 6$ nM, $K_i = 1.7$ nM; Peptidomimetic **37**: $IC_{50} = 13$ nM, $K_i = 4$ nM.

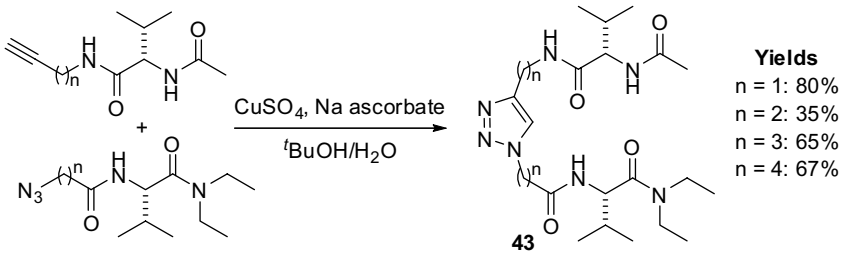


pLI-GCN4: Ac-RMKQIEDKLEEILSKLYHIENELARIKKLLGER-OH T_m = >96 C
40: Ac-RMKQIED **X**EEILSKLYHIENELARIKKLLGER-OH T_m = >96 C
41: Ac-RMKQIEDKLEEILS **X**YHIENELARIKKLLGER-OH T_m = 36 C
42: Ac-RMKQIEDKLEEILSKLYHIEN **X**ARIKKLLGER-OH T_m = 61 C

Figure 2.5 (Top) Exchange of a dipeptide fragment with a triazole dipeptide mimic. (Bottom) Replacement of the native pLI-GCN4 amino acids K_8L_9 (**40**), $K_{15}L_{16}$ (**41**), and $E_{22}L_{23}$ (**42**) with the L-leucine-derived dipeptide mimic **39**. Thermal denaturation temperatures shown.

(Figure 2.5). This suggests that peptidomimetics **40**–**42** retain much of the native α-helical structure, but that the thermodynamic stability of the peptidomimetic is highly dependent on the site of triazole substitution. An X-ray crystal structure of peptidomimetics **40**–**42** (at 2.2 Å resolution) revealed that all three adopted the same parallel tetrameric coiled coil structure observed for the parent peptide pLI-GCN4. The crystal structure of peptidomimetic **41** is similar to that of the parent peptide, with both triazole ε^2-amino acid residues participating in the α-helical backbone hydrogen bonding. Moreover, as observed for the parent sequence, the hydrophobic side-chain of the triazole ε^2-amino acid residues project towards the bundle core. The triazole N2 is observed to hydrogen bond to the Ile12 amide NH and the triazole C5 hydrogen appears to hydrogen bond to the Ile12 amide carbonyl. These and other structural features, give rise to an increased helical pitch of approximately 1.8 Å in the region of the triazole ε^2-amino acid residues. This increase creates a shallow pocket adjacent to the triazole, with a water molecule providing additional hydrogen bonding that may be beneficial for the stability of the helical structure.

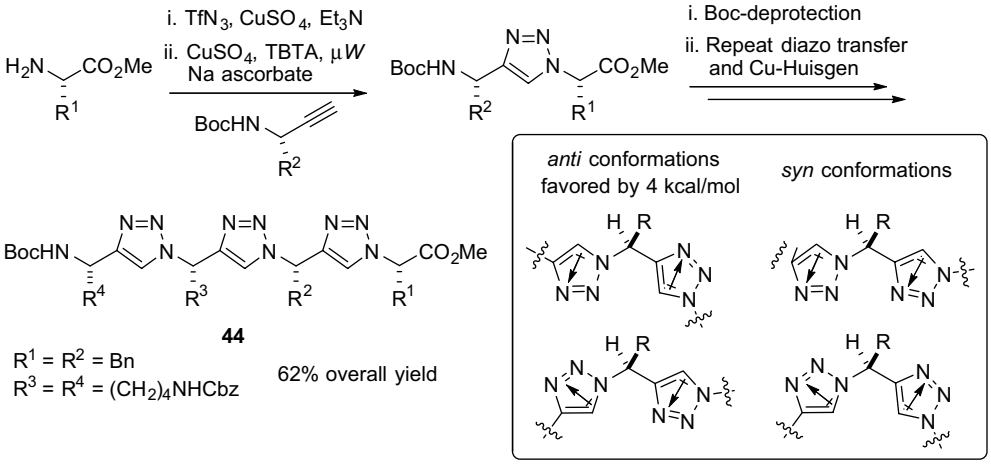
Turn structures are important in defining the secondary structure of proteins and, as such, considerable effort has been devoted to the synthesis peptidomimetics containing these elements. Oh and Guan investigated the use of 1,4-triazoles as turn inducers in peptides [97]. Molecular modeling suggests that the peptidomimetics **43** (Scheme 2.13) possess the appropriate geometry to adopt a β-turn, but that the ability to form intramolecular amide–amide hydrogen bonds is highly dependent on the linker length. A series of tetrapeptides, with varying linker lengths, was synthesized



Scheme 2.13 β-turn-inducing 1,4-triazole peptidomimetics.

and studied by nuclear magnetic resonance (NMR) and Fourier transform-IR in order to evaluate this class of potentially turn inducing peptidomimetics. These studies clearly showed that a three-carbon linker length is optimal for formation of a well defined β-turn in solution.

The use of triazoles in backbone modified peptidomimetics has generally been limited to the replacement of a few peptide bonds with either a 1,4- or 1,5-triazole. However, Angelo and Arora reported the synthesis and study of completely backbone modified peptidomimetics as foldamers [98, 99]. Standard solution-phase Boc chemistry, diazotransfer, and Cu-Huisgen conditions gave triazole trimers in 6–12% overall yield (e.g., **44**, Scheme 2.14) [98]. Subsequent optimization studies improved the yields significantly and revealed that the use of $CuSO_4$, both for diazotransfer and cycloaddition, worked well in solution phase. However, solid-phase synthesis was best achieved using a combination of $ZnCl_2$ for the diazotransfer and $CuSO_4$ for the Cu-Huisgen step [99].

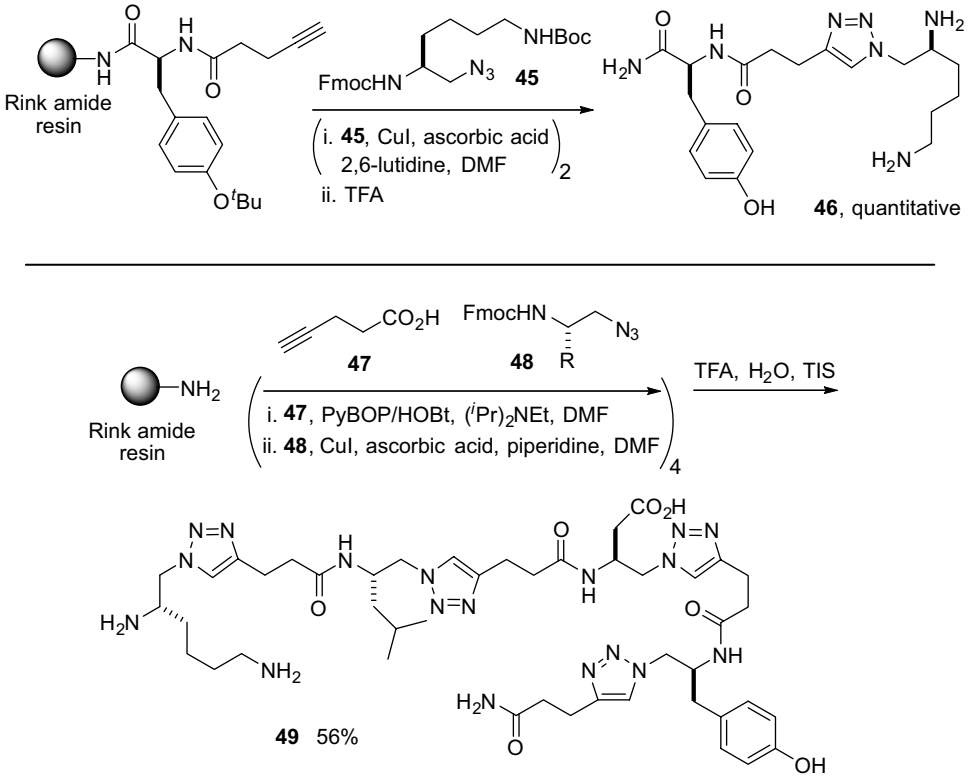


Scheme 2.14 Solution-phase synthesis of triazole trimer. Possible *syn* and *anti* conformations for two adjacent triazoles in a triazole oligomer.

Even short sequences of triazole oligomers adopt distinct conformational preferences, presumably due to the significantly stronger dipole of a triazole, compared to an amide bond. Molecular mechanics and *ab initio* studies suggest that the *anti* conformations (Box, Scheme 2.14) are significantly more stable (approximately 4 kcal/mol) than the corresponding *syn* conformations. Detailed NMR studies, in both DMSO and acetone, revealed that triazole trimers do indeed adopt an *anti* conformation to give a zig-zag structure very similar to that observed in a β-strand [98].

The solid-phase synthesis (Rink amide resin) of peptidomimetics containing alternating triazole and amide bond linkages between residues has also been investigated [100]. Careful optimization of the Cu-Huisgen reaction conditions revealed the best combination of reagents to be CuI, ascorbic acid, DMF, and 2,

6-lutidine. This gave a quantitative yield of **46** after repeating the Cu-Huisgen cycloaddition step twice (top, Scheme 2.15). An alternative method, using piperidine in place of 2,6-lutidine, allowed one-pot Cu-Huisgen and Fmoc deprotection in quantitative yield.

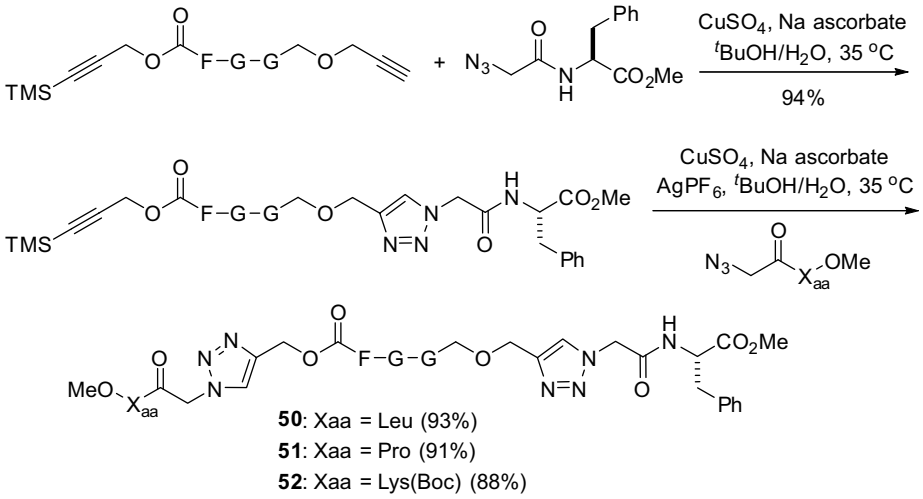


Scheme 2.15 Synthesis of a triazole peptidomimetic with alternating amide bonds and 1,4-triazoles.

The peptidomimetic **49** (bottom, Scheme 2.15) containing hydrophilic, hydrophobic, basic, and acidic residues was successfully synthesized by this method in an isolated yield of 56% after purification.

Peptidomimetics containing two triazole moieties in the backbone [101] have also been prepared in a unique approach using two acetylenes to sequentially introduce the triazole rings. Regioselectivity was achieved by trimethylsilyl (TMS) protection of one acetylene allowing selective Cu-Huisgen reaction of the terminal, unprotected acetylene (Scheme 2.16). The second triazole was introduced by a one-pot silver catalyzed TMS-deprotection and Cu-Huisgen cycloaddition procedure.

Three pseudo-nona-peptidomimetics (**50**–**52**) were prepared in excellent yield using this methodology with only a trace amount (0–4%) of undesired product resulting from incomplete Cu-Huisgen reaction during formation of the first

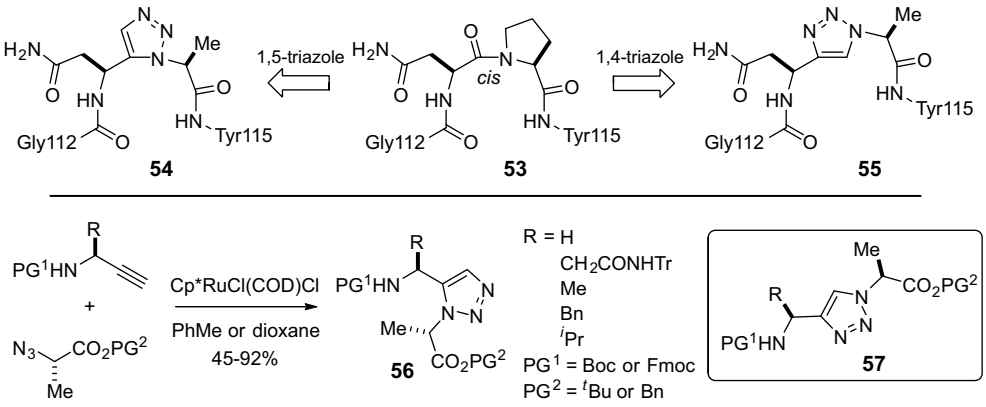


Scheme 2.16 Regioselective, sequential Cu-Huisgen synthesis of pseudo-nona-peptidomimetics.

triazole. Standard $CuSO_4$/sodium ascorbate conditions proved effective for the triazole formation, with gentle heating to 35 °C to increase the reaction rate.

Agnew *et al*. reported a novel method for selective protein capture using molecules comprised of several peptide sequences connected by 1,4- or 1,5-triazoles [102]. This has some distinct advantages over existing methods of protein detection in that no prior knowledge of affinity agents against the target protein is required and also it is relatively simple to identify highly selective capture agents through iterative coupling of diverse peptide fragments. The methodology was tested against human and bovine carbonic anhydrase II with the identification of high affinity ligands (45 and 64 nM, respectively) composed of tripeptide sequences ligated by two triazole moieties. All the peptide sequences employed were composed of D-amino acids, with the triazole linkages formed between terminal D-propargyl glycine residues and a racemic α-amino acid containing an azido-butyl or azido-octyl side-chain with a terminal azide substituent. The required azide building blocks were synthesized using traditional acetamido malonate chemistry. The assembly of the peptidomimetic ligands relies on two peptide fragments, one containing an azide and the other an acetylene, to independently bind to the surface of the target protein to allow a Huisgen cycloaddition to occur. This allows formation of either the 1,4- or 1,5-isomer depending on relative positioning of the two components on the protein surface. The synthesis and subsequent screening was carried out on TentaGel™ beads using one-bead–one-compound libraries with the hit beads displaying an extremely high sequence homology. The peptidomimetics identified in this study did not interfere with the activity of the enzyme – a highly desirable feature commonly seen in natural antibodies raised against protein targets.

The group of Raines has reported the use of a 1,5-substituted triazole as a mimic of a *cis*-peptide bond [103]. More specifically they explored if it is possible to replace a *cis*-prolyl peptide bond, such as Asn–Pro (**53**, Scheme 2.17) in a functional protein, with a



Scheme 2.17 (Top) 1,4- and 1,5-Triazole dipeptide replacements in the native protein (**55** and **54**, respectively). (Bottom) Synthesis of 1,5-triazole building blocks **56** using Ru-Huisgen methodology. (Box) Building block **57** prepared using Cu-Huisgen conditions.

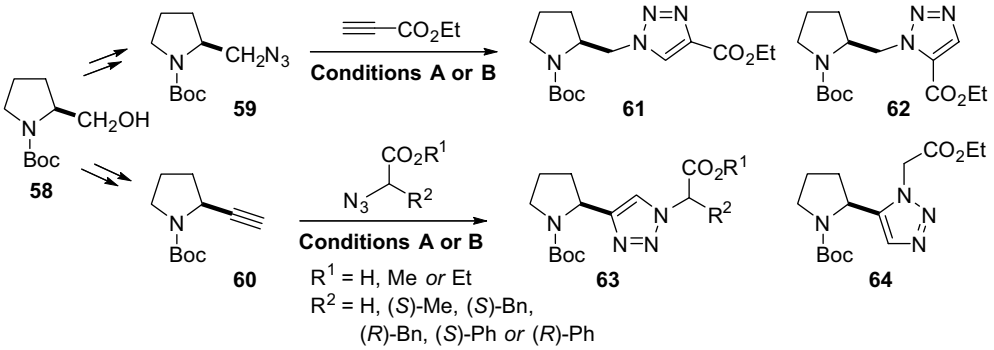
1,5-triazole mimic whilst retaining the protein's catalytic activity. Bovine pancreatic ribonuclease (RNase A) was selected as the model protein and four semisynthetic proteins were designed containing either 1,5-triazole or 1,4-triazole replacements (**54** and **55**, respectively). In all four semisynthetic proteins the dipeptide segment Asn113–Pro114 (**53**), known to adopt a *cis* conformation, was targeted for triazole replacement.

The precursor dipeptide mimics were assembled from azides and acetylenes derived from naturally occurring α-amino acids (See Section 2.4) using either standard Cu-Huisgen conditions (1,4-triazoles) or a ruthenium catalyst developed by Sharpless *et al.* (1,5-triazoles, see Section 2.3). The synthesis of all dipeptide mimics was uneventful with all compounds being obtained in moderate to good yields. Importantly, Raines *et al.* noted than racemization of the triazole building blocks did not occur during solid phase peptide synthesis.

The two triazole dipeptide mimics **56** and **57** ($R = CH_2CONH_2$) were incorporated into RNase A in place of the Asn113–Pro114 unit, and dipeptide mimics **56** and **57** ($R = Me$) into the RNase A variant N113A as a substitute for Ala113–Pro114. As anticipated all four RNase A analogs retained full catalytic activity, with CD spectra indicating retention of the secondary structure found in the native proteins. Both 1,5-triazole peptidomimetics had thermal denaturation temperatures (T_m) comparable to the native proteins; however, the 1,4-triazole peptidomimetics had a significantly lower T_m compared to the native protein. Thus, as anticipated by Raines *et al.*, the 1,5-triazole dipeptides appear better at mimicking the *cis*-prolyl bond seen in the wild-type protein.

The ability to control the *cis*/*trans* amide ratio in proline residues is of considerable interest (e.g., in the activation and deactivation of receptors). In this context, Paul *et al.* investigated the effect of substituting an amide bond, in a Pro–Gly dipeptide, with a 1,4- or 1,5-triazole in an attempt to increase the population of the *cis* or *trans* conformations [104]. The required acetylene and azide building blocks (**59** and **60**,

Scheme 2.18) were synthesized from *N*-Boc-protected prolinol **58**, and triazole formation was achieved either by Cu-Huisgen cycloaddition to give the desired 1,4-triazoles **61** and **63** (36–92%) or by thermal Huisgen cycloaddition to give mixtures of 1,4- and 1,5-triazoles in a ratio of approximately 3 : 1 (78 and 90% combined yield). In most cases the Cu-Huisgen reaction was carried out using standard $CuSO_4$/sodium ascorbate in *t*BuOH/water (in a few instances water was replaced with methanol for solubility reasons) at room temperature or at 40 °C. For triazoles **63** ($R^2 = Ph$) it was necessary to conduct the cycloaddition at room temperature and stop the reaction after 20 h to suppress racemization of the phenyl glycine fragment.



Scheme 2.18 Condition A: $CuSO_4$, Na ascorbate, *t*BuOH/H_2O, room temperature or 40 °C. Condition B: PhMe or EtOAc, reflux.

The conformational properties of the synthesized dipeptide mimetics (**65–68**) were examined by NMR spectroscopy and the results were compared to those for the natural dipeptide derivative, *N*-acetylprolyl-glycine methyl ester **69** (Figure 2.6). Two of the synthesized peptidomimetics (**66** and **68**) did show an enhanced preference for

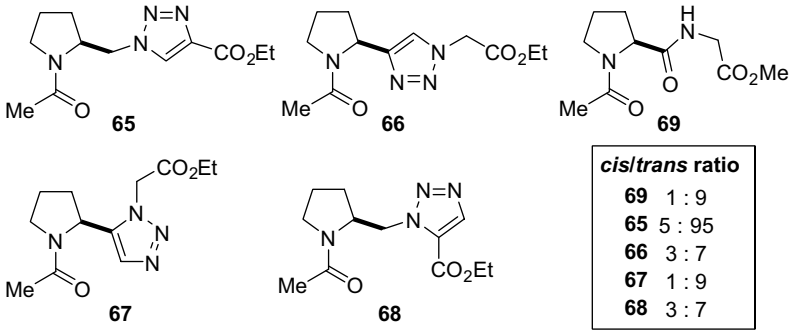


Figure 2.6 *Cis*-amide bond peptidomimetics. Only Peptidomimetics **66** and **68** showed an increased population of the *cis* conformation when compared to the natural dipeptide derivative **69**.

the *cis* conformation (3 : 7 *cis*/*trans* ratio). The reason for the increased population of the *cis* conformation in peptidomimetics **66** and **68** was not determined, but the authors speculate that the effect could be due to attractive and repulsive dipolar interactions between the triazole moiety and the carbonyl group in the *N*-acetyl substituent.

2.7
Useful Experimental Procedures

2.7.1
Monitoring Huisgen Cycloadditions and Characterizing Triazoles

Triazole formation is easily detected by ^{1}H-NMR showing a characteristic triazole C−H resonance in the aromatic region. We have generally run our NMR samples in $CDCl_3$ or CD_3OD and for Boc-protected side-chain modified triazole peptidomimetics (e.g., **70** and **71**, Figure 2.7) a slightly broadened singlet is observed in the 7–8 ppm chemical shift region.

The triazole proton in 1,4-triazoles is shifted downfield compared to the corresponding 1,5-triazole. Tornøe *et al.* have reported the same trend for backbone modified triazole peptidomimetics (**72** and **73**) albeit further downfield [7]. However, in their case the triazole was conjugated with an amide and the spectra were recorded in DMSO-d_6.

When dealing with intermolecular Huisgen cycloadditions in large molecules or systems with many overlapping peaks in the aromatic region it can be difficult to determine if the reaction has occurred. As the starting material and the product have the same mass it can be difficult to conclusively prove that cycloaddition has occurred by mass spectrometric methods. A qualitative assessment of the product composition can be performed by IR spectroscopy looking for the very distinct and strong azide stretch at around 2100 cm^{-1} [77]. However, because the azide stretching frequency is very strong, IR can be misleading in situations where a small quantity of azide is still

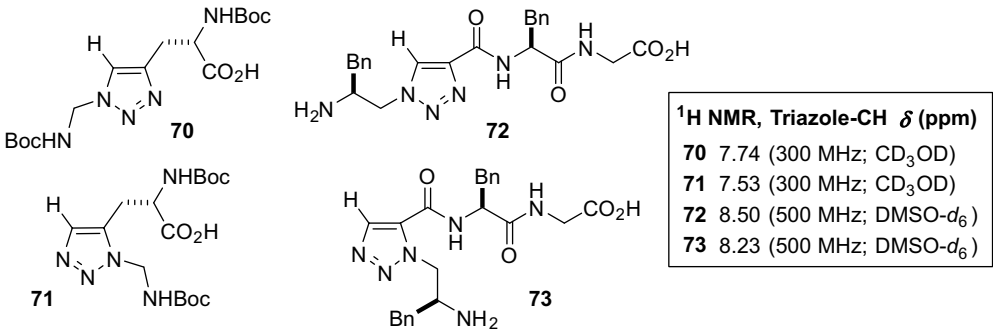


Figure 2.7 ^{1}H-triazole-CH NMR resonances for backbone and side-chain modified peptidomimetics.

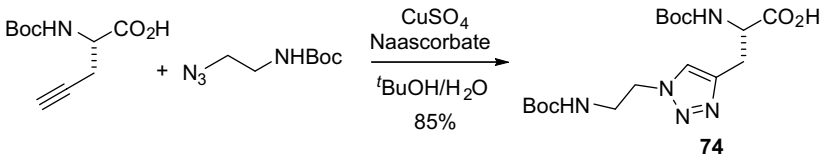
present in the sample. For qualitative assessment the product sample can be subjected to Staudinger reduction conditions, reducing any excess azide to the amine, resulting in a significant mass difference (26 mass units) between triazole product and uncyclized starting material allowing mass spectrometric analysis. The Staudinger reduction approach was employed by Sako *et al.* in their synthesis of triazole bridged peptides [105]. Sako *et al.* performed the Staudinger reduction with tris(carboxyethyl)phosphine on peptides prior to cyclization and after Cu-Huisgen cycloaddition. By analysis of the reduced linear peptides and their "reduced" cyclized counterparts by matrix-assisted laser desorption/ionization-time of flight mass spectrometry they could show that there was no uncyclized peptide in the product.

Punna and Finn have reported a modified Kaiser test for qualitative assessment of azides [106]. The method simply employs the standard Kaiser test, but with a small amount of triphenylphosphine added to convert any present azides to the free amines via the Staudinger reaction. The method works both in solution and with solid supported compounds to give the distinct blue color characteristic for the Kaiser test. Thin-layer chromatography (TLC) plates can also be analyzed using the same principle by including a small quantity of triphenylphosphine in the TLC eluent followed by treatment with a standard ninhydrin TLC stain.

HPLC analysis of products versus original or reduced starting material can also be a very effective way to determine the composition of the obtained product.

2.7.2
General Procedure for the Synthesis of 1,4-Triazoles Using Cu-Huisgen Cycloaddition

(*S*)-2-(*tert*-Butoxycarbonylamino)-3-{1-[2-(*tert*-butoxycarbonylamino)ethyl]-1*H*-1,2,3-triazol-4-yl}propanoic acid 74



74

(*S*)-2-(*tert*-Butoxycarbonylamino)-pent-4-ynoic acid (0.14 g, 0.66 mmol) and *tert*-butyl 2-azidoethylcarbamate [107] (0.13 g, 0.69 mmol) were suspended in a mixture of *tert*-butanol (3 ml) and water (3 ml). $CuSO_4 \cdot 5H_2O$ (5 mg, 5 mol%) and sodium ascorbate (26 mg, 20 mol%) was added. The reaction mixture was stirred vigorously overnight to give a green emulsion that was transferred to a separatory funnel with ethyl acetate (25 ml) and sulfate buffer (25 ml), and the aqueous phase was extracted with ethyl acetate (2×25 ml). The combined organic phases were dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give a clear colorless gum. Purification by dry column vacuum chromatography [108] ($RP18254_S$ silica gel; diameter 2 cm × height 6 cm; 10-ml fractions; $2 \times H_2O$; 10–50% CH_3CN in H_2O, v/v; 10%

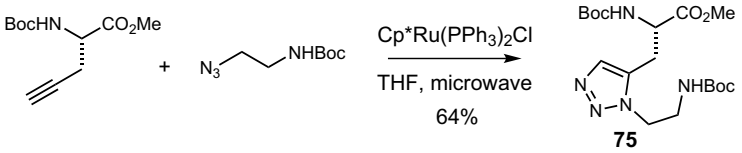
increments; 3 × each solvent mixture) gave 1,4-triazole **74** (0.22 g, 85%) as a white amorphous solid.

- R_f 0.25 (1% AcOH in EtOAc, v/v).
- *m/z* (+ESI) found: MNa^+, 422.2049. ($C_{17}H_{29}N_5O_6Na$ requires *M*, 422.2049).
- ^{1}H-NMR (300 MHz; CD_3OD) δ *7.65* (1H, s, triazole-CH), 4.36 (2H, br t, *J* 6.0, C*H*$_2$CH$_2$N), 4.18 (1H, br t, *J* 5.5, CHCO$_2$H), 3.43 (2H, br t, *J* 6.0, CH$_2$C*H*$_2$N), 3.16 (1H, dd, *J* 14.5 and 5.0, C*H*$_A$H$_B$CHCO$_2$H), 3.06 (1H, dd, *J* 14.5 and 6.5, CH$_A$*H*$_B$CHCO$_2$H), 1.36 (18H, s, 2 × *t*Bu).
- ^{13}C-NMR (75 MHz; CD_3OD) δ 179.1 (CO_2H), 158.0, 157.3 (2 × NHCO), 145.1 (triazole-C4), 124.7 (triazole-C5), 80.3 (2 × *C*Me$_3$), 56.6 (C*H*CO$_2$H), 51.0 (*C*H$_2$CH$_2$N), 41.5 (CH$_2$*C*H$_2$N), 28.9, 28.8 (2 × C*Me*$_3$).

2.7.3

General Procedure for the Synthesis of 1,5-Triazoles Using Ru-Huisgen Cycloaddition

(*S*)-Methyl 2-(*tert*-butoxycarbonylamino)-3-{1-[2-(*tert*-butoxycarbonylamino)ethyl]-1*H*-1,2,3-triazol-5-yl}propanoate 75



75

(*S*)-Methyl 2-(*tert*-butoxycarbonylamino)-pent-4-ynoate (0.12 g, 0.53 mmol) and *tert*-butyl 2-azidoethylcarbamate (0.13 g, 0.69 mmol) were dissolved in anhydrous THF (3 ml) and transferred to a 5-ml microwave vial containing $Cp^*Ru(PPh_3)_2Cl$ (9 mg, 2 mol%) to give a dark-red solution. The solution was purged with nitrogen for 2 min, sealed, and heated in a microwave oven at 120 °C for 20 min. The reaction mixture was transferred to a round-bottom flask and concentrated *in vacuo* to give a dark-brown gum. Purification by dry column vacuum chromatography (internal diameter 4 cm; 20-ml fractions; 4 × *n*-heptane, 5–100% EtOAc in *n*-heptane, v/v −5% increments, 6 × EtOAc) gave 1,5-triazole **75** (0.14 g, 64%) as a slightly yellow film.

- R_f 0.45 (EtOAc).
- *m/z* (+ESI) found: MNa^+, 436.2195. ($C_{18}H_{31}N_5O_6Na$ requires *M*, 436.2172).
- ^{1}H-NMR (300 MHz; $CDCl_3$) δ *7.47* (1H, br s, triazole-CH), 5.27 (1H, br d, *J* 6.5, NH), 5.12 (1H, br s, NH), 4.60 (1H, br dd, *J* 12.5 and 6.5, C*H*CO$_2$CH$_3$), 4.37 (2H, br t, *J* 5.5, C*H*$_2$CH$_2$N), 3.78 (3H, s, OMe), 3.67 (2H, br q, *J* 6.0, CH$_2$C*H*$_2$NH), 3.28 (1H, dd, *J* 15.5 and 5.5, C*H*$_A$H$_B$CHCO$_2$H), 3.13 (1H, dd, *J* 15.5 and 6.5, CH$_A$*H*$_B$CHCO$_2$H), 1.43 (18H, s, 2 × *t*Bu).
- ^{13}C-NMR (75 MHz; $CDCl_3$) δ 170.9 (CO_2H), 155.7, 154.9 (2 × NHCO), 147.5 (triazole-C5), 132.7 (triazole-C4), 80.4, 79.7 (2 × *C*Me$_3$), 52.8 (OMe), 52.4 (C*H*CO$_2$H), 47.5 (*C*H$_2$CH$_2$N), 40.1 (CH$_2$*C*H$_2$N), 28.3, 28.2 (2 × C*Me*$_3$).

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3
Recent Advances in β-Strand Mimetics

Wendy A. Loughlin and David P. Fairlie

3.1
Introduction

3.1.1
β-Strands

The simplest peptide structural element is the peptide β-strand. The β-strand is a linear or saw-toothed arrangement of amino acids with amide bonds being almost coplanar, side-chains alternating above and below the plane of the peptide backbone (Figure 3.1), and there are no intramolecular hydrogen bonds between component amino acid residues. In an antiparallel arrangement, the torsional angles of an idealized β-strand would be ϕ -139°, ψ 135°, and τ -177° with $d = 8.0$ Å [1]. The dipole moments of the peptide bonds alternate along the strands, which imparts stability to the formation of β-sheets from β-strands.

β-Strands are usually found hydrogen-bonded in pairs or arrays that form β-sheet structures within proteins. Previously considered a random structure rather than a discrete element of protein secondary structure, the peptide β-strand is now known to be a crucial structural element recognized on its own by many classes of proteins, a few examples being proteolytic enzymes [2, 3], major histocompatibility complex proteins [4], and transferases [5]. The β-strand must now be viewed as a fundamental structural element that is specifically recognized by biomolecular receptors.

In the last 20 years small-molecule β-strand mimetics have been increasingly reported [6–9]. Small molecules that mimic β-strands could be useful enzyme inhibitors or protein antagonists with important applications in medicine. While there are relatively few examples of monomeric β-strand mimetics identified as such [9], probably due to their high tendency to aggregate or simply a poor realization of what they are [10], this field is beginning to grow. Illustrating the importance of this field, there are now examples of human therapeutics that are β-strand mimetics, notably inhibitors of proteases [11–13].

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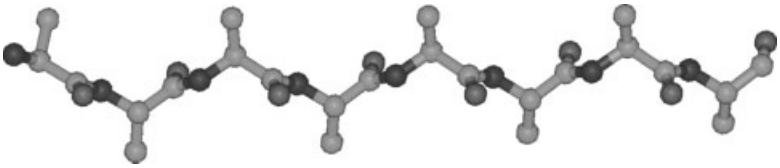


Figure 3.1 Idealized β-strand composed of Ala residues.

3.1.2
β-Sheets

In addition to such specific recognition of discrete β-strands, combinations of two or more strands to form β-sheets not only act as important scaffolding elements to stabilize protein structure, but are sometimes key recognition motifs that bind to other proteins or DNA. The β-sheet secondary structure accounts for over 30% of all protein structure. It consists of two or more paired β-strands arranged in either parallel, antiparallel, or mixed alignments held together through interstrand hydrogen bonds. A more detailed discussion of β-strands is reported elsewhere [14].

Although β-sheet mimetics are more prevalent than structurally validated β-strand mimetics, with a variety of structurally diverse examples having been reported [15, 16], there are to our knowledge no human therapeutics that involve β-sheet mimicry. Most β-sheet mimetics are unlikely to be therapeutically useful, but can provide useful information for the design of improved β-strand mimetics. β-Strand peptidomimetics could thus find important roles as competitive ligands for receptors/enzymes that typically bind to β-sheets or as inhibitors of β-sheet formation when β-sheets themselves are undesirable.

3.1.3
Differences in Strand/Sheet/Turn/Helix Recognition

In order to design and develop molecules that selectively mimic the β-strand, one also needs to be cognizant of the differences between size, shape, and composition of the β-strand versus sheets. The extended β-strand presents contiguous residues on alternating sides of the strand, so that their side-chains are separated by the maximum possible distance. For example, the i and $i + 4$ residues in an extended β-strand are 14.5 Å apart (Figure 3.2a). This separation minimizes steric clashes between side-chains, which also have the maximum possible exposure to solvent (or receptor) in this structure. A single β-strand permits maximum exposure of main-chain atoms for hydrogen bonding to a receptor.

A β-strand within a β-sheet has i and $i + 4$ residues 13.2 Å apart, but the extensive interstrand hydrogen-bonding network also protects all main-chain atoms from solvent/ligand interactions (Figure 3.2b). Parallel and antiparallel β-sheet arrangements present side-chains in two opposing directions. The strands on the edges of the sheets still have half of their main-chain atoms available for hydrogen bonding to a receptor or solvent. The side-chains are exposed for potential enzyme interactions on

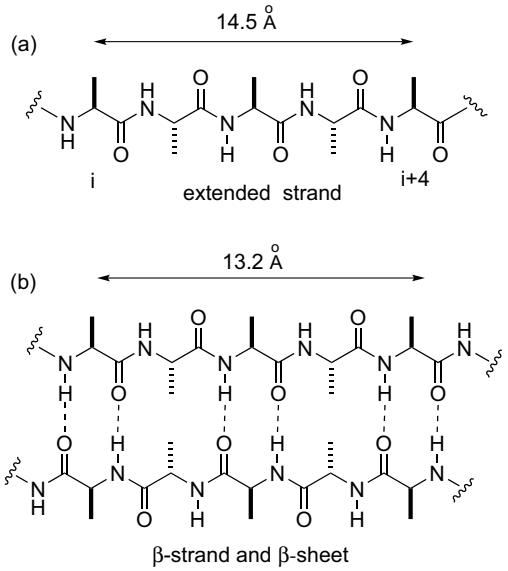


Figure 3.2 Comparative sizes (Å) for (a) extended β-strand ($\Phi = \Psi = 180°$) and (b) antiparallel β-sheet ($\Phi = -139°$, $\Psi = -135°$). Hydrogen bonds are indicated by dashed lines.

both the top and bottom surfaces. This presents a more three-dimensional recognition site, with i and $i + 4$ residues being 13.2 Å apart on the same strand and the αC–αC interstrand distance of adjacent residues on adjacent strands being approximately 4.5–5.5 Å apart (distances were measured from a DNA-binding antiparallel β-pleated sheet complex) [17]. The corresponding hydrogen-bonding distance from amide N to carbonyl O is approximately 3.0 Å. The strand thus offers three different recognition sites – either via the top or bottom faces, or via hydrogen bonding with main amide atoms on each side of the sheet.

3.1.4
Towards β-Strand Mimetics

Mimicking β-strands to antagonize β-sheet formation or recognition may represent a viable therapeutic strategy towards the prevention or treatment of diseases associated with β-sheet structures. Drug design often involves the examination of protein–protein interactions associated with disease, followed by the design of small molecules that can mimic or bind to one of the interacting proteins [11–13, 18–21]. Often the bioactivity of proteins stems from only a small localized region or regions of a protein surface created by folded elements of secondary structure (such as α-helices, β- or γ- turns, or β-strands/sheets), so small molecules based on these structures are at least in principle feasible as antagonists.

The simplest approach to interfering with β-strand/sheet recognition would be to use short peptides corresponding to strand/sheet regions of proteins recognized by other proteins or DNA. However, short peptides suffer from a number of

disadvantages that compromise their use as drugs [13, 22, 23]. They are conformationally flexible, existing mainly as random structures in aqueous solution [21, 24–26]. Peptides are also susceptible to degradation through peptide bond cleavage by peptidases, have low bioavailability, and exhibit poor pharmacological profiles due to a combination of these factors, other forms of metabolism, rapid clearance rates, and poor membrane permeability. Thus, while biomolecular interactions with peptides provide very useful clues for drug design, changes need to be made to create more pharmacologically acceptable drug candidates.

With respect to conformational stability, the arrangement of peptides into β-strands for recognition by biomolecular targets is either a chance event in which the receptor captures the small percentage of peptide present in a β-strand conformation or else the receptor plays an active role in contorting the peptide into the preferred strand shape. A central principle in medicinal chemistry is that molecules, which are conformationally preorganized or fixed into a shape that is recognized by a receptor, can have higher affinity for that receptor due to the reduced entropy penalty for adopting the receptor-binding shape. It is therefore surprising that, unlike the case for turns and helices, there are relatively few conformationally restricted, surrogates for the β-strand reported.

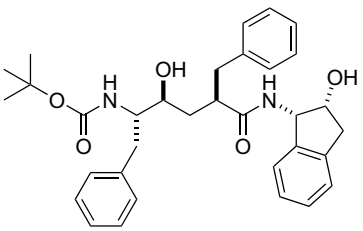
One minimalist approach to conformational stability involves restricting peptide freedom though cyclization [27], either via side-chain to side-chain, side-chain to main-chain, or main-chain to main-chain linkages, to form macrocycles. Nature frequently uses cyclization to force peptides into bioactive conformations [19]. Cyclic peptides also have the advantages over linear peptides of being more resistant to amide bond cleavage by proteolytic enzymes and of being more conformationally restrained or less flexible. Examples are given in the next section of synthetic strand peptidomimetics created through macrocyclization (generally $C_i^{\alpha} \rightarrow C_{i+1}^{\alpha}$, C_{i+2}^{α}, or C_{i+3}^{α}), inter- or intraresidue cyclization, and ring fusion.

Alternatively, conformational constraints can be incorporated in peptide sequences. Nature frequently moulds peptides into turn shapes by replacing amino acid components with a wide variety of constraints like disulfides, double bonds, *N*-methyl amino acids, D-amino acids, aromatic, and heterocyclic rings, often in conjunction with cyclization [19, 21]. Similar conformational constraints could be used to synthetically mimic the peptide β-strand. For example, one or more amino acid residues could be replaced in a peptide sequence by one or more rigid organic units. Such replacements can produce peptidomimetics with drug-like components, examples of which are presented ahead.

Conformationally and metabolically stable peptidomimetics or nonpeptides are the goals of β-strand mimicry. While significant advances have been made in the synthesis of conformationally constrained β-sheet mimetics [28–31], there is a pressing need for small molecules that mimic the β-strand, and for generic rigid scaffolds as components of strands and libraries of strands. Recently, a comprehensive review on nonpeptidic β-strand scaffolds provided an overview of progress in this area between 1990 and 2003, and also included are a few examples from broader reviews on peptide secondary structure [15, 32–41]. Inhibitors reported in crystallographic structures of protease–ligand complexes were included; this information

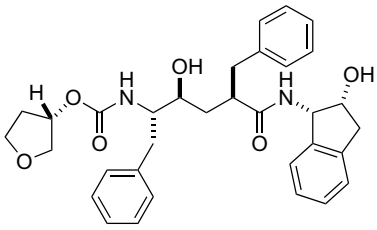
being available in the Protein Data Bank for more than 1500 structures. Scaffolds that were structurally validated in solution by nuclear magnetic resonance (NMR) spectroscopy also are included. Further general reviews that contain references to β-strand mimetics have also been published [42–46].

An integrated approach has often been adopted in the design of β-strand mimetics, thus this chapter focuses on a structure-based coverage of the principal strand mimetic types. In terms of features of the β-strand that are being mimicked, primarily these are replacements for the peptide backbone, main-chain hydrogen bonds, side-chain substituents, or combinations of these. Typically this has been achieved using compounds where the scaffold binds a specific enzyme subsite(s) to help fix the β-strand conformation, or the scaffold merely projects pendant substituents to binding locations, or a combination of both. A scaffold can be classified by amino acid surrogate components, by heterocycles or in other ways. Illustrative of this are 1(*S*)-amino-2-(*R*)-hydroxyindanyl and 3(*S*)-hydroxytetrahydrofuran ligands employed within tripeptide β-strand mimetics as HIV-1 protease inhibitors **1** [47] and **2** [48], respectively.



1

IC_{50} (HIV-1 protease) = 0.3 nM



2

IC_{50} (HIV-1 protease) = <0.03 nM

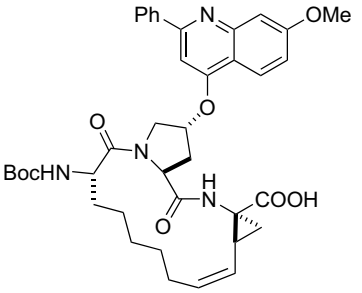
In this chapter, scaffolds will be classified principally on the basis of their heterocyclic subunits, even though they may be perceived as amino acid surrogates elsewhere. An encyclopedia-style organization has been adopted, according to compound class, to facilitate finding compounds, identification of structural similarities and differences, and particularly to compare different chemical types of beta strand mimetics. Here, the focus will be on β-strand mimetics reported between 2004 and mid 2009, placed in the context of the previous comprehensive review in the area [14] to which the reader is referred.

3.2
Macrocyclic Peptidomimetics

Macrocycles formed through condensing peptide side-chains to the main-chain have been shown to be highly constrained structural mimics of tri- and tetrapeptide components of a linear peptide substrate or inhibitor. These peptidomimetics fix the receptor-bound conformation of bioactive peptides in an extended conformation, which translates into functional mimicry. Typically, macrocycles are restrained to a

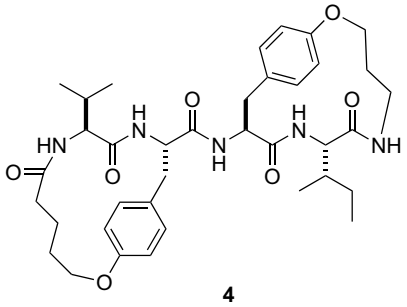
preorganized β-strand conformation for protease binding [49]. The approach of restraining the macrocycle, together with side-chain to side-chain cyclization, has produced a variety of macrocyclic peptidomimetics [14]. For example, (*E*)-**3** is a potent inhibitor of the hepatitis C virus NS3 protease [50].

Recently, further work has been carried out on macrocyclic tripeptides constrained to mimic a β-strand conformation, linked by a scissile peptide bond [51–53]. For example, bicyclic compound **4** is actually a substrate for a protease. It has been cocrystallized with catalytically active synthetic HIV-1 protease and an inactive isosteric (D25N) mutant [51, 52]. The 1.60 Å resolution structure of the inactive HIVPR(D25N)/substrate complex showed a single orientation that preserved a conventional protease-recognizing β-strand structure in **4**. The β-strand mimicry of **4** *in situ* offers an entropic advantage for binding over the acyclic hexapeptide analog Ac-LVFFIV-NH_2, leading to a higher affinity of the bicyclic substrate **4** for enzyme [51, 52].



(*E*)-3

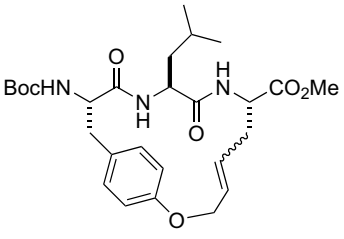
IC_{50} (HCV NS3 protease) = 0.011 μM



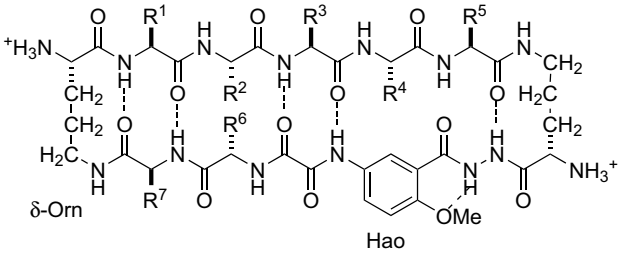
4

Synthetic efforts to create macrocyclic β-strand templates include oxidative coupling of two cysteine thiols to form a disulfide bridge within the macrocycle (for human antibody Fc-binding peptidomimetics) [54] and via ring-closing metathesis of dienes [55–57]. For example, the crystal structure of (*E*)-**5** revealed β-strand geometry for the peptide backbone as defined by P2(Leu) Φ and Ψ torsion angles within the ranges $-160^\circ < \Phi < -100^\circ$ and $90^\circ < \Psi < 160^\circ$. The β-strand conformation of (*E*)-**5** was essential for derivatives of (E)-**5** to bind to proteases [56].

Other macrocyclic peptidomimetics are reviewed elsewhere [14] and typically include macrocycles with *trans* amide bonds, *para*-substituted aromatic rings, 14–17 membered in size or derived from natural products. Constricting peptide sequences into macrocycles in such a way, makes the amide bonds less recognizable to other proteases. Thus, such macrocycles are not only resistant to peptidases of the gut, bloodstream, or cells, but in some cases can also penetrate cell membranes and exhibit potent antiviral activity in cell culture [58–60]. At the same time, macrocycles are preorganized in structure, which contrasts with short peptide segments (3–10 amino acids) that rarely adopt defined structures in solution and the unconstrained β-strand peptide conformation which is not a stable structure in water [24, 61].



5
E:Z 9:1



6
Hydrogen bonds are indicated by dashed lines - - - - - -

Recently, macrocycles of increased size (of up to a 42-membered ring) that form a cyclic modular β-sheet have been reported. Macrocycles, such as **6**, contain a pentapeptide in the upper strand and the amino acid Hao as a relatively rigid tripeptide β-strand mimic. Two δ-linked ornithine residues form flexible chains and allow Hao to form a template for the organization of the upper strand whilst blocking the lower strand to minimize edge-to-edge aggregation [62, 63]. Many macrocycles of this type form tetramers composed of two edge-to-edge β-sheet dimers [63].

3.3
Acyclic Compounds

Acyclic β-strand molecules typically have involved design and optimization of dipeptide isosteres flanked by peptidic or nonpeptidic appendages. This has been inspired by the fact that some proteases and other proteins are oligomers (e.g., HIV-1 protease is a homodimer with a 2-fold axis of symmetry extending through the active site). Compounds that take advantage of this C2 symmetry and project substituents into the S1/S1′ and S2/S2′ binding sites of the protease have been reviewed elsewhere [64–69]. In acyclic protease inhibitors, the central linking unit between side-chains (typically phenyl) has been varied to include a range of linker units, as reviewed elsewhere [14] with recent work being carried out using hexadecanodioic acid [70, 71] and a butylene linker [72].

A popular approach in the design of alicyclic β-stand molecules has been the replacement of the carbonyl group with a suitable nucleophilic trapping moiety, such as a carbon–carbon double bond, or other moieties that allow for maximum preservation of structural features for binding in an extended conformation [14]. Using this concept, a simple replacement that has recently been used in sequence with other amino acids is α-aminoisobutyric acid. Notably, isomeric tripeptides inclusive of α-aminoisobutyric acid show totally different conformational preferences depending on the position of the backbone amino acids within the tripeptides. Tripeptide Boc-Phe–Leu–Aib-OMe adopts a β-strand like conformation, whereas the isomer tripeptide Boc-Phe–Aib–Leu-OMe forms a type II β-turn. Cooperativity of steric interactions between the amino acid residues is thought to account for this observation [73].

3.4
Aliphatic and Aromatic Carbocycles

Planar aromatic spacers, such as the benzene, 1,5- or 1,6-naphthalene, biphenyl, and isocoumarin scaffolds, have also been incorporated into inhibitor design as extended dipeptide mimics [14]. Appropriate substitution of the aromatic spacer has led to 5-amino-2-methoxy benzamide and 5-amino-2-methoxy benzoic hydrazides (**7**) [74] being incorporated into novel β-strand mimetics to induce artificial β-sheet formation (Figure 3.3). These mimetics duplicate the hydrogen-bonding functionality of a dipeptide and tripeptide, respectively. The β-strand mimetic has been located either along one edge (upper or lower) [75–81], or on the edge [82], or in the middle of a three stranded artificial β-sheet [74] and mixed artificial β-sheets [83]. Further aggregation to antiparallel β-sheet dimers also has been observed [76, 77, 84].

Modification of the 5-amino-2-methoxy benzoic acid unit led to development of a range of β-strand mimetics [14]. Recently, the introduction of polar and hydrophobic

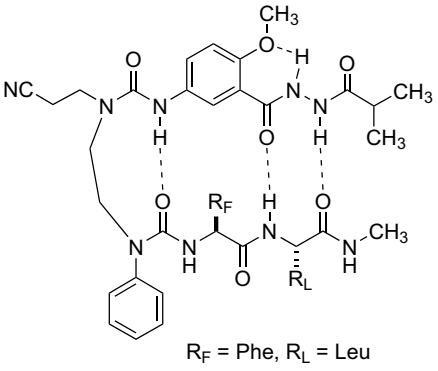


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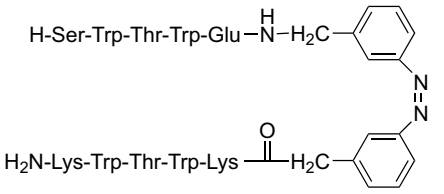
Figure 3.3 Schematic representation of hydrogen bonding in **7**. Hydrogen bonds are indicated by dashed lines. (Adapted from [74].)

variants, by replacing the methyl side-chain of the 5-amino-2-methoxy benzoic acid unit with acidic, basic, and hydrophobic groups, has imparted improved solubility and additional side-chain interactions to peptides containing these types of β-strand templates [85]. Aromatic ligands have also been used as rigid, structure-directing templates for peptidic receptor arms, including a calixarene scaffold that generates a β-sheet arrangement of four peptidic arms [86]. In another example, inclusion of an azobenzene chromophore sets up the peptidic arms to form a β-hairpin peptide **8**. The hairpin structure is controlled by the azobenzene photoswitch through a photo-induced folding or unfolding transition based on the *cis* or *trans* geometry of the N=N bond geometry [87].

Other carbocyclic conformational constraints, which restrict the ϕ angle to approximate the idealized β-strand, include 1,2,3-trisubstituted cyclopropanes, where the side-chain is rigidly fixed to χ angles corresponding to ±60°, a polycyclic cage which contains cyclobutane, and steroid structures as well as less constrained aliphatic carbocycles, such as cyclohexane and cycloheptanone [14]. Similar to the cyclohexane scaffolds, enantiomeric *cis*-oxo-β-norbornene amino acid oligomers **9** were recently shown by NMR and molecular dynamics studies to display bent β-strand secondary structures [88]. In an analogous example, stereo-specific oligomers of bicyclic *exo-cis*-β-amino acids derived from norbornadiene, have been developed. Rotating-frame Overhauser spectroscopy (ROESY) data of **9** revealed NH_i–$C\beta H_{i+1}$ and NH_i–$C\beta H_{i+1}$ backbone nuclear Overhauser effects that confirmed a β-strand-like secondary structure [89].



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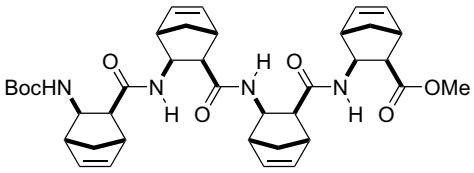


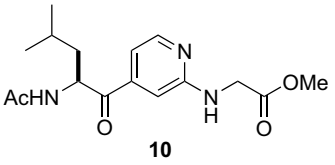
9

3.5
Ligands Containing One Ring with One Heteroatom (N)

Whereas carbocycles, particularly aromatic scaffolds, feature in interesting examples of rigid β-strand mimetics, ligands with one ring and one heteroatom (nitrogen)

represent the largest general class of β-strand mimetics. This class ranges from the three-membered ring of aziridine to the seven-membered ring of azepanone, and includes azetidines, prolines, pyrrolidines, piperidines, the seven-membered analog of piperidines, pyrroles, pyridines, pyrrolinones, pyrrolidinones, lactams, pyridones, and 1,2-dihydro-3(6*H*)-pyridinones (or azacyclohexenone units and termed Ach or @), and is comprehensively reviewed elsewhere [14]. A notable feature of these scaffolds is incorporation of a ketone into the ring, such as pyrrolinones (five-membered) and pyridones (six-membered), which has a dramatic impact on the hydrogen-bonding capacity of the scaffold. Likewise, the ring size and functionality of the heterocyclic ring can influence the positioning of substituents on the heterocyclic ring into an extended conformation, replace the hydrolyzable backbone of bioactive peptides, mimic the positions and orientations of the backbone carbonyls and side-chains, and maintain key side-chain interactions and some of the intermolecular hydrogen bonds made with the enzyme. Further recent representative examples are presented here.

The rigidity, planarity, and solubility of pyridines have made them useful as scaffolds to position side-chains in a β-strand conformation. β-Strand mimetic **10**, a tripeptidomimetic of Leu–Gly–Gly, lacks the central amide bonds of the peptide fragment, but retains some of the hydrogen-bonding capacity [90]. A further variation has included the use of Ach as a β-strand mimetic [91–93]. Alternating oligomers which contain the cyclic amino acid replacement were termed @-tides. NMR studies of the penta-@-tide **11** [92] supported a β-sheet model of dimerization that indicated the ability of **11** to mimic a β-strand. The synthesis of the @-tide unit [92, 94] and solution- and solid-phase methods for its incorporation into @-tide oligomers with a variety of amino acids and with lengths up to 13 units [95] as well as @-tide units alternating with peptide units have been devised [92, 96]. Circular dichroism studies have been able to quantify amino side-chain–side-chain interactions associated with β-sheet stability [97, 98].



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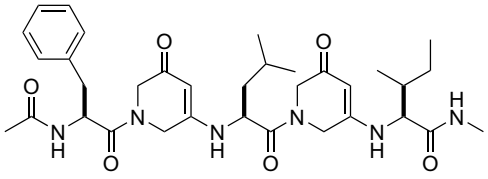


11

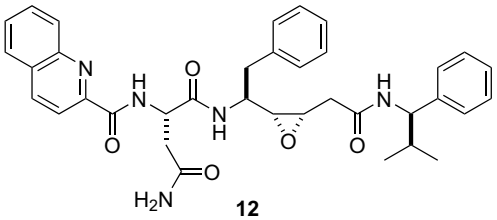
@-tide

3.6
Ligands Containing One or Multiple Rings with One Heteroatom (O, S)

Ligands containing one or multiple rings with one oxygen or sulfur heteroatom include epoxides, coumarins, pyran, dibenzofurans and a seven-membered cyclic sulfone, and are reviewed in detail elsewhere [14]. Little work has been done recently using these types of heterocycles. The main focus of past studies has been on *cis*-epoxides and coumarins. Epoxides, such as **12**, which are used as amide isosteres for

the scissile peptide bond [99], are contained within pseudo C2-symmetric inhibitors [100]. NMR studies of inhibitors containing a *cis*-epoxide, such as **12**, have identified the conformational preference of the inhibitors in solution [101]. Epoxide **12** adopts an extended conformation similar to the β-strand.

Coumarins have been the used to project hydrophobic substituents into the appropriate P2, P1, P1′, and P2′ positions for interaction with the enzyme. Although these compounds do not mimic all the protease-binding hydrogen-bond donors and acceptors of peptide strands, they can still be considered as constrained peptidomimetic scaffolds in which the ring substituents do occupy the same spaces within the protease active site as amino acid side-chains of linear peptide β-strands. The idea is embodied in coumarin-derived inhibitor **13** of HIV-1 protease [102].



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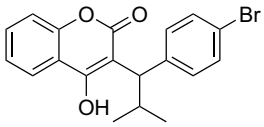


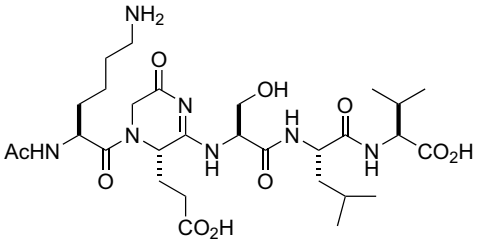
13

IC_{50} (HIV-1 protease) = 10-50 nM

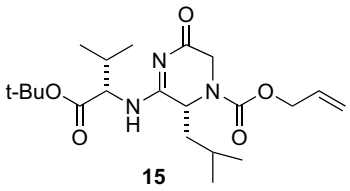
3.7
Ligands Containing One Ring with Two Heteroatoms (N,N)

Given the significant amount of work in the area of one ring with one nitrogen heteroatom, a logical extension has been to scaffolds with one ring and two nitrogen heteroatoms. β-Strand mimetics in this category include pyrimidones, hexahydropyrimidines, pyrimidine triones, 1,3-piperazines, 1,4-piperazines, aminopyrazoles, and cyclic ureas as discussed elsewhere [14].

Recently the main focus has been on dihydropyrazinones, which have been developed as aza-@-units. Incorporation of the aza-@-unit into peptiodiomimetic structures, such as AcK@SLV **14**, which binds to the PDZ domain, reproduces the hydrogen-bonding pattern and side-chain functionality of a β-strand mimetic [103–105]. Diazacyclohexanones, such as **15**, also display an improved ability to assume a β-strand conformation and to enter into β-sheet-like interactions [105].



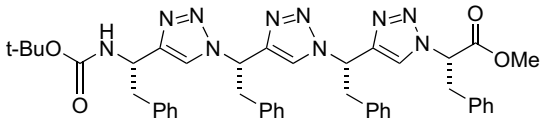
14
Ac-K@^{E}SLV



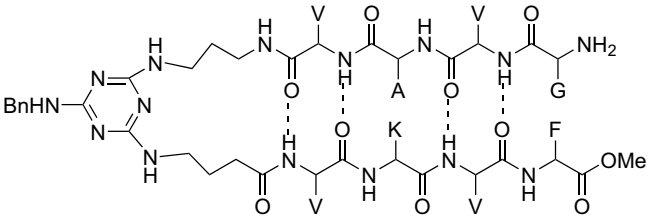
15

3.8
Ligands Containing One Ring with Two Heteroatoms (N,S) or Three Heteroatoms (N,N,S or N,N,N)

Alternative heterocyclic rings used, for example, as proline replacements and as β-strand mimetics include thiazoles, thiazolines, thiazolidines, their corresponding oxazoles and oxazolines, their condensation products with other amino acids to form dipeptide surrogates, as well as 1,3,5-triazines. β-Strand mimetics have also been reported with expanded rings such as a six- or seven-membered ring observed in thiazines or thiazepines, cyclic sulfonamides, azacyclic ureas, and 1,3,4-thiadiazines, and are reviewed elsewhere [14]. Common themes to this range of heterocycles are a ring that is planar and rigid providing a different directional scaffold for side-chains to those previously discussed and unsaturation of the ring to give a heterocycle with a degree of conformational flexibility. It is this category of β-strand mimetics that has attracted the development of new scaffolds in recent years. For example, variations with three nitrogen trimers and tetramers of 1,2,3-triazolylenes, such as **16**, adopt zig-zag conformations reminiscent of peptide β-strands [106, 107]. An unsymmetrical triamino 1,3,5-triazine bearing two alkyl chains, **17**, has been designed, assisted by molecular modeling, as a mimic of the backbone of the $i + 1$ and $i + 2$ residues of a β-turn [108]. The triazine scaffold thus induces extended conformations of the peptidic strands and acts as a template to induce antiparallel β-sheet structure.



16



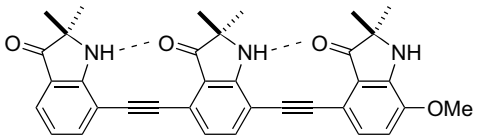
17
V = Val, A = Ala, K = Lys, F = Phe, G = Gly
Hydrogen bonds are indicated by dashed lines

3.9
Ligands Containing Two Rings with One Heteroatom (N or O)

Heterocyclic scaffolds containing two or more rings offer increased opportunities for control of hydrogen bonding and substituent projection, thus promoting β-strand

conformations for mimetic design. The use of conformationally constrained scaffolds to induce a β-strand has been explored predominantly with fused bicycles containing more than one heteroatom and a number of bicyclic scaffolds have now been developed to mimic the β-strand [14]. These include indoles, isatins, 6-amino-5-oxotetrahydroindolizines, 1,2,3,4-tetrahydro-3-isoquinolines, 3,6-diamino quinolones, bicyclic azepinones, and octahydroindoles.

An innovation in this category has been the work on 2,2-disubstituted indolin-3-one **18**, which displays intramolecular hydrogen bonding that promotes a conformation that mimics the residues of a β-strand. The spacing of the indolinone groups allows formation of an intramolecular hydrogen bond between the carbonyl of one indolinone and the proximal NH of another subunit, enforcing the desired conformation [109].



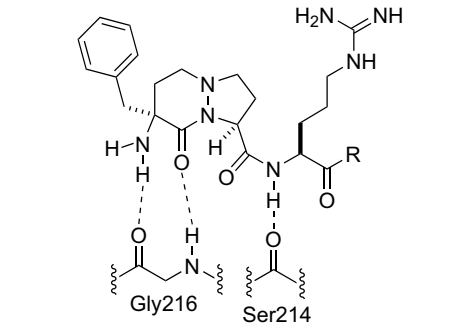
18

Hydrogen bonds are indicated by dashed lines

3.10 Ligands Containing Two Rings with Two or Three Heteroatoms (N,N or N,S or N,N,N)

Introduction of a further heteroatom into scaffolds with two rings has been considered, including diazabicyclo[4.3.0]nonanes, pyrazinones, triazolopyridazines, pyridopyrimidines, benzamidazoles, polycyclic heterocyclic rings such as epindolidiones, and diketopiperazines as discussed elsewhere [14]. Extremely potent enzyme inhibitors have been realized with these scaffolds, which act as a mimetic of the D-Phe–Pro orientation of PPACK (D-Phe–Pro–ArgCH_2Cl). This thrombin inhibitor reached phase II clinical trials [110], and reproduces the three-dimensional orientation of the substrate and its hydrogen-bonding patterns. Numerous inhibitors based on the sequence of PPACK have been reported [111], and include compounds such as **19**, **20**, and **21** (Figure 3.4) [112].

Analogous to the hydrogen bonding observed for **19**, other structures such as **22** are also conformationally constrained by hydrogen-bond interactions and are compounds that mimic the secondary structure of β-strands [113]. Final anomalous examples of a scaffold that nucleates attached polypeptide chains to form β-strands with a parallel orientation are the ferrocenyl-peptide **23** [114–116], and an analogous *cis*-[$PtCl_2$(triarylphosphine)$_2$]-peptide [117].



19 MOL-126 R =

Ki (thrombin) 70 pM

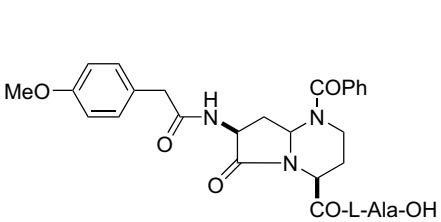
R =

20

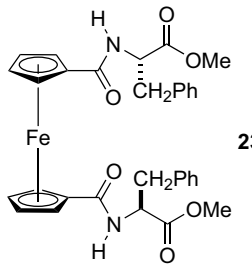
R =

21

Figure 3.4 Schematic representation of the hydrogen bonding pattern between MOL-126 **19**, **20**, or **21** and thrombin. Hydrogen bonds are represented by dashed lines.



22



23

3.11 Conclusions

It has only been relatively recently that the extended peptide β-strand has been recognized as a common recognition motif for certain classes of biomolecular receptors. Compounds that have been moulded into β-strand-mimicking structures have been shown to have high affinity for biomolecular receptors, higher than for unconstrained peptides. Efforts should be made in future to discover whether designed molecules actually do structurally mimic the β-strand well, as relatively few cases exist where rigid constraints have been specifically demonstrated to be

β-strand mimetics. The issues of the extent of conformational flexibility in solution versus the organized interactions within a crystal structure of an inhibitor-enzyme complex need to be approached carefully when designing β-strand mimics. In particular, the $^{3}J_{NHCH\alpha}$ coupling constant in ^{1}H-NMR spectra can be particularly diagnostic (around 9 Hz) for β-strands of peptidomimetics in solution [118].

The trend in drug discovery today is to synthetically elaborate such scaffolds with sufficient foliage to engender high selectivity for the target receptor. Getting the balance right between using common β-strand-mimicking scaffolds and appending sufficient appropriate substituents to create selectivity in receptor binding is clearly a big challenge in this field. Likewise, there is a fine balance needed between using structural constraints (e.g., a bicyclic heterocycle ring), steric effects, and hydrogen bonds to organize the geometry and conformation of a peptide into a β-strand, and considerably more research needs to be done to elucidate the relative importance of these influences in dictating both strand structure and receptor affinity.

β-Strand mimetics are also expected to find emerging uses in coming years in the design of compounds that can interfere with peptide/protein β-strand aggregation. There are already over 30 known "amyloid diseases" in which proteins are thought to misfold into β-strands that aggregate into β-sheet structures [119–128], the toxic agents being thought to be small aggregates. β-Strand mimetics may offer opportunities to build druggable agents that prevent or reverse such β-sheet formation and aggregation. In another context, β-sheets represent some 30% of protein structure, so β-strand mimetics could therefore conceivably have many promising uses in destabilizing protein structures and mimicking or antagonizing protein–protein, protein–DNA, or protein-RNA interactions that are mediated by β-sheets. We encourage further studies into creating newly designed β-strand mimetics, identifying their structures in complex with biological macromolecules, and increasing knowledge of the importance of the β-strand structure in new examples of protein recognition.

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Part Two
Medicinal Chemistry of Amino Acids

Amino Acids, Peptides and Proteins in Organic Chemistry.
Vol.4 – Protection Reactions, Medicinal Chemistry, Combinatorial Synthesis. Edited by Andrew B. Hughes

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4
Medicinal Chemistry of α-Amino Acids

Lennart Bunch and Povl Krogsgaard-Larsen

4.1
Introduction

α-Amino acids constitute the building blocks of peptides and proteins. and thus the basis for all aspects of life. However, when in the free monomeric form α-amino acids may interact directly with specific biological targets such as receptors, transporters, and enzymes. To date, receptors identified that are specifically activated by α-amino acid monomers are limited to the (*S*)-glutamate (Glu) and glycine (Gly) receptors (GluR and GlyR, respectively). Both these receptors and their respective transporters are linked to neurotransmission. However, due to the immense medicinal chemistry efforts within the glutamate research area this chapter will focus on this particular α-amino acid and how medicinal chemistry strategies have been applied.

4.2
Glutamic Acid

In the central nervous system (CNS), Glu functions as the major excitatory neurotransmitter by activating the plethora of GluRs [1, 2], In broad lines, the GluRs are divided into two major classes: the ionotropic Glu receptors (GluRs) and the metabotropic Glu receptors (GluRs) (Figure 4.1). The iGluRs are ligand-gated ion channels and thus mediate fast excitatory response. On the basis of ligand-affinity studies they have been divided further into three groups: the 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptors (comprising the subtypes iGluR1–4), the (−)-(α)-kainic acid (KA) receptors (comprising subtypes iGluR5–7, and KA1 and 2) [3, 4], and the *N*-methyl-D-aspartic acid (NMDA) receptors (comprising subtypes NR1, 2A–D, and 3A–B). The mGluRs belong to the class of G-protein-coupled receptors and therefore produce a much slower signal transduction through second messenger systems. Currently, eight subtypes, mGluR1–8, have been identified, which are clustered into three groups on the basis of the second messenger system involved, pharmacology, and molecular biology (group I: subtypes

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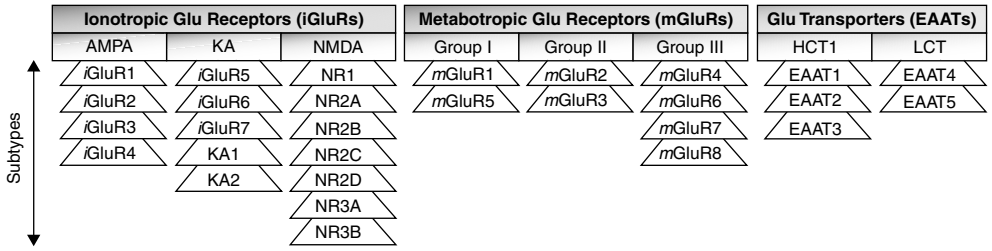


Figure 4.1 Receptor and transporter subtypes in the glutamatergic neurotransmitter system. HCT = high-capacity transport; LCT = low-capacity transport.

mGluR1 and 5; group II: subtypes mGluR2 and 3; group III: subtypes mGluR4 and 6–8) [5]. Termination of the excitatory signal by removal of Glu from the synaptic cleft, is mediated by the excitatory amino acid transporters (EAATs, also referred to earlier as Glu transporters) [6, 7]. At present, five EAAT subtypes have been identified of which EAAT1–4 are present in the CNS, whereas EAAT5 is found exclusively in the retina. The detailed functions of the five transporters differ significantly as EAAT1–3 exhibit high capacity for transporting Glu across the membrane, whereas EAAT4 and EAAT5 function predominantly as chloride channels.

The glutamatergic neurotransmitter system is believed to be involved in important neurophysiological processes such as memory and learning, control of motor functions, and neural plasticity and development. Thus, psychiatric diseases or disorders such as depression [8], anxiety [9], addiction [10, 11], migraine [12], and schizophrenia [13] may be directly related to disordered glutamatergic neurotransmission [6, 14, 15]. Moreover, excessive Glu signaling is neurotoxic and will consequently cause neuronal death [16]. On this basis it has been suggested that neurodegenerative diseases such as Alzheimer's, Huntington's, amyotrophic lateral sclerosis, cerebral stroke [13], and epilepsy [17] may be associated with malfunctioning glutamatergic neurotransmitter system.

To a great extent, the medicinal chemistry research efforts related to glutamatergic neurotransmission have been driven by the desire to understand the function of a specific GluR subtype in basic neurological processes, as well as in pathophysiological states. To allow such studies, the development of subtype selective – or, ideally, subtype specific – ligands is essential. This includes direct-acting (*ortho*-steric) agonists and antagonists for receptors, and substrates and inhibitors for the transporters. Over time, naturally occurring Glu analogs have served as inspiration for such work. Examples of Glu analogs that have been isolated from various natural sources include (Figure 4.2) the cyclic Glu analog, KA, which was first isolated in 1953 from the red marine alga *Digenea simplex* [18]. This alga is found in tropical and subtropical waters, and for centuries it has been used as an anthelmintic (an agent which acts to eradicate parasitic intestinal worms). Despite the complex chemical structure of KA, which holds a 2,3-*trans*,3,4-*cis*-trisubstituted pyrrolidine skeleton, several synthetic strategies have been developed over the years [4]. Compounds that comprise the 2-carboxypyrrolidine-3-acetic acid parental skeleton are often referred

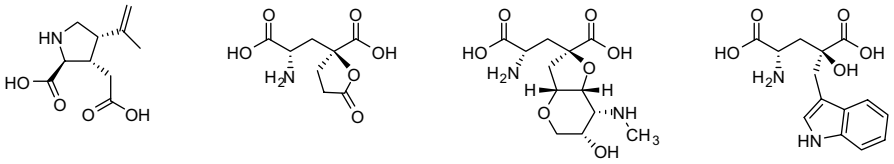


Figure 4.2 Naturally occurring Glu analogs KA, lycoperdic acid, DH, and monatin.

to as kainoids. In 1979, lycoperdic acid [19] was isolated that contains a ring structure in the 4-position of Glu and later, in 2001, the structurally complex Glu analog dysiherbaine (DH) was isolated from the marine sponge *Dysidea herbacea* [20]. In 1992, the Glu analog monatin was isolated from the roots of *Schlerochiton ilicifolius* – a spiny-leaved hardwood shrub that grows in Africa [21]. It is quite interesting that this particular Glu analog possesses an unusual characteristic as it is a high-intensity sweetener.

4.3 Conformational Restriction

The 20 common α-amino acids are flexible molecules. Consequently, upon binding to the biological target loss of entropic energy ΔS contributes negatively to the overall affinity. This correlation is straightforwardly explained from the Gibbs free energy equation:

$$\Delta G = \Delta H - T\Delta S$$

To overcome this dilemma, a frequently used strategy is to design and synthesize conformationally restricted analogs. An important aspect in the design process is whether the target binding pocket may accommodate the atoms added and that the conformation as a result of the restriction process is comparable to the bioactive conformation.

Glu is a highly flexible molecule, and may adapt nine different staggered conformers by rotation of the C(2)–C(3) and C(3)–C(4) bonds (Figure 4.3) [22]. Early on, it

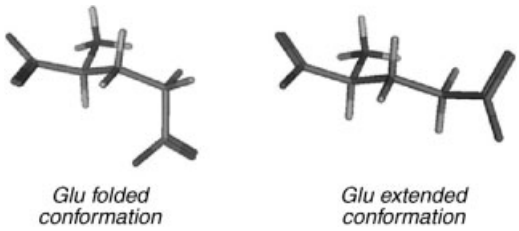


Figure 4.3 Two low-energy conformations of Glu – the folded conformation and the extended conformation.

was hypothesized that the Glu agonist binding mode(s) to the GluRs was (or were) likely found among these nine local energy minima. This is because binding of Glu in an eclipsed conformation would automatically induce an energy penalty (negative ΔH value), resulting in lower binding affinity [23]. Several conformationally restricted Glu analogs have been designed as mimics of local energy minimum conformations. Examples of successfully designed conformationally restricted KA/AMPA receptor agonists are CIP-AS [24] and L-CCG-IV [25], which lock the Glu scaffold in its folded conformation (Figures 4.2 and 4.4). However, rationally designed potential iGluR agonists bicyclooctane-2-aza-dicarboxylate (BOAD) [26] and (±)-2-azanorbornane-3-*exo*,5-*endo*-dicarboxylic acid (DCAN) [27], which also restrict the Glu scaffold to the folded conformation, show low affinity for the iGluRs. In detailed *in silico* studies of DCAN, the dicarboxy-2-azanorbonane skeleton predicted ideal spatial positioning of the ammonium and two carboxylate groups, in comparison with the binding conformation of KA (folded conformation). The unexpected finding that DCAN is a low-affinity ligand at all iGluRs led the authors to conclude that this highly rigid amino acid is acutely sensitive toward disfavored Van der Waals interactions [27]. Detailed *in silico* studies of KA receptor ligands such as KA [28] have also predicted a folded Glu conformation as the agonist binding mode, which has furthermore been supported by several X-ray crystallographic studies (Table 4.2) [29]. Today it is well accepted that KA and AMPA receptor agonists adapt conformations reflecting a folded Glu conformation (Figure 4.3). On the other hand, Glu adopts an extended conformation when acting as an agonist at the mGluRs (Figure 4.3) [30].

During the past years, several conformationally restricted Glu analogs have been designed and synthesized. Together, these compounds have provided important information about the agonist/antagonist binding mode of Glu at the iGluRs and mGluRs, and substrate/inhibitor binding conformation for the EAATs. The Glu analogs that have made a notable contribution towards understanding the structure–activity relationships of iGluR, mGluR, and EAAT ligands are shown in Figure 4.4.

In detail, L-CCG-IV is a full agonist at iGluRs, whereas DCAN is without affinity for this class of receptor even though the two carboxylate groups and the ammonium group are positioned in a similar spatial area. Such observations underline the drawback of applying conformational restriction for increasing agonist potency since the atoms introduced must be fully accommodated by the biological target. In particular, for receptor agonists this is problematic as the mechanism underlying GluR agonism has been shown to involve a definite closing of the ligand-binding domain (LBD) occluding the ligand and inducing a conformational change of the extracellular and transmembrane domains responsible for receptor activation (ion flux) (Figure 4.5).

4.3.1
Synthesis – General Considerations

The synthesis of any conformationally restricted α-amino acid is a major challenge. Generalized synthetic strategies are often impassable due to shifting hetero- or carboskeletons, chiral centers, as well as the introduction of specific functionalities.

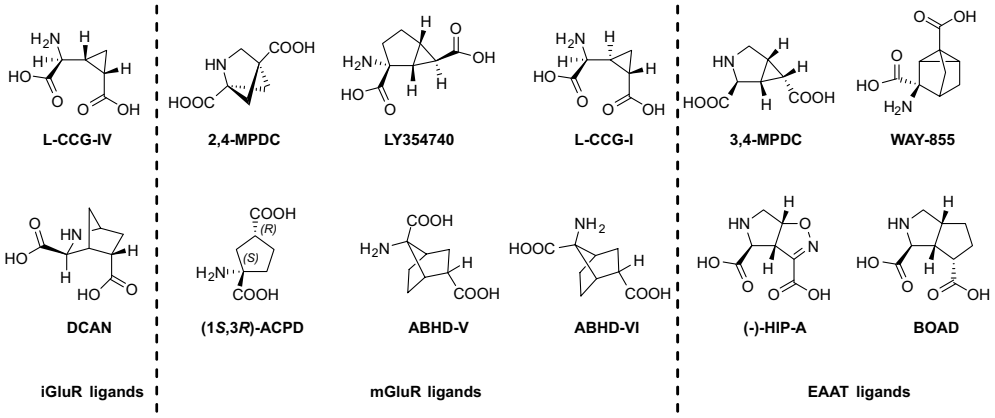


Figure 4.4 Conformationally restricted Glu analogs: L-CCG-IV [31], DCAN [27], 2,4-MPDC [32], LY354740 [33], L-CCG-I [31], (1*S*,3*R*)-ACPD [34], ABHD-V [35], ABHD-VI [35], 3,4-MPDC [32], WAY-855 [36], (–)-HIP-A [37], and BOAD [26].

Thus, for every new analog of interest a retro-synthetic analysis must be made drawing on previous experience with structurally similar compounds, but most importantly with the use of a creative mind. From the examples of the synthesis of conformationally restricted Glu analogs to follow, this is clearly affirmed. Despite the fact that the target compounds are all conformationally restricted Glu analogs, the respective synthetic strategies are quite distinct, which points out the incredible diversity within organic chemistry.

4.3.2
Case Study: Synthesis of DCAN

The conformationally restricted Glu analog DCAN [27] comprises the 2-azanorbornane skeleton that allows for perfect mimicry of the Glu folded conformation

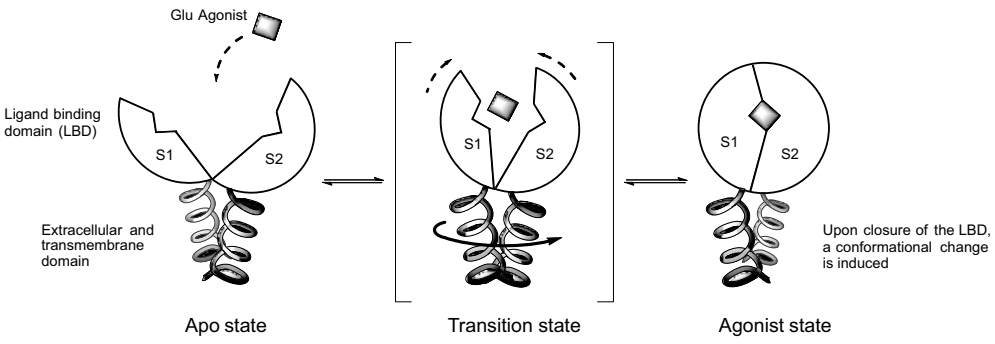
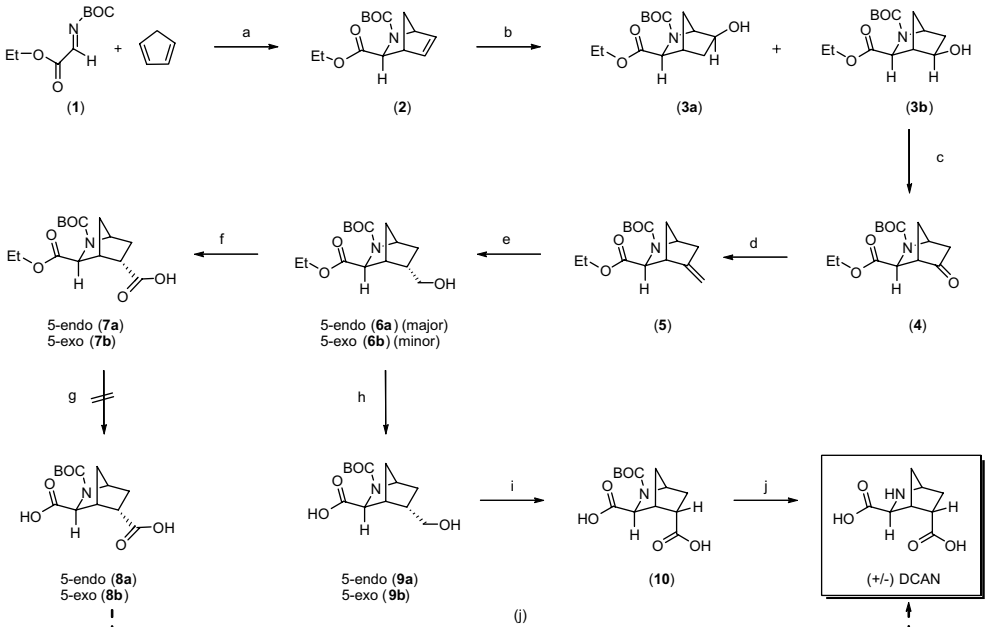


Figure 4.5 Mechanism of iGluR activation: Apo state = free, open state; agonist state = closed, ligand bound. Upon closure of the LBD (transition state) a conformational change of the extracellular and transmembrane domains is induced, triggering receptor activation.

Figure 4.6 Three-point superimposition (ammonium and carboxylate groups) of energy-minimized DCAN (purple), X-ray of KA (green) when bound in iGluR2, and X-ray of Glu (type code) when bound in iGluR2. All three molecules are adapted to the Glu folded conformation.

(Figures 4.3, 4.4 and 4.6). Previously, this specific heterocycle has been synthesized using various strategies, such as a hetero Diels–Alder reaction [38, 39], or by diverse intramolecular strategies: an alkylation reaction [40, 41], an acylation reaction [42, 43], electrophilic addition to an *in situ* formed iminium ion [44], nucleophilic addition to an *in situ* formed bromonium ion [45], or a carbolithiation reaction [46]. Furthermore, 1- and 7-azanorbornanes have also been prepared, but the key steps used here are quite distinct due to altered intramolecular reactions of the amino group.

Since DCAN contains a 3-*exo*,5-*endo*-dicarboxy substitution pattern, the retro-synthetic analysis suggested a hetero Diels–Alder as the optimal strategy for a first synthesis of racemic DCAN (Scheme 4.1). The first step, reacting imine **1** with



Scheme 4.1 Synthesis of DCAN.

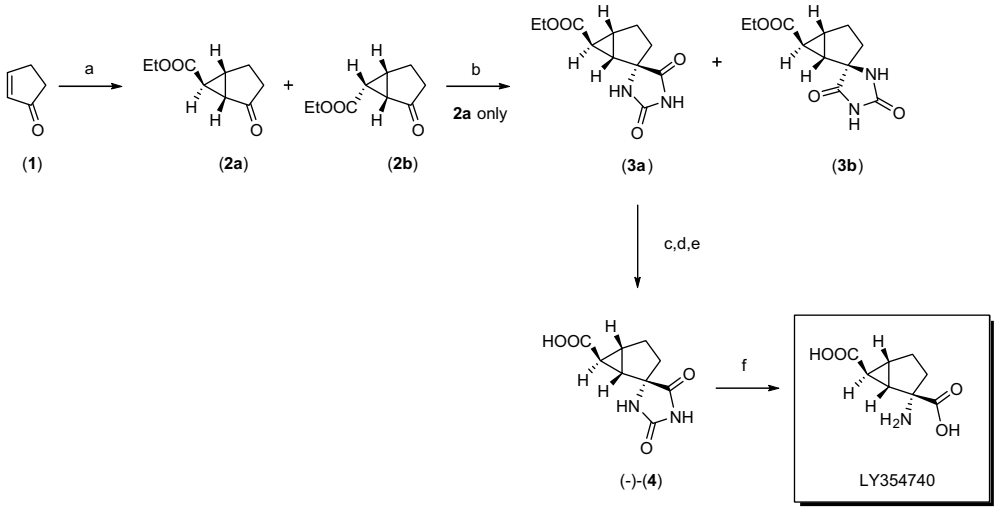
cyclopentadiene to give Diels–Alder adduct **2**, secures the desired *exo*-stereochemistry of the 3-position. Subsequent hydroboration of **2** gave a 1: 1 mixture of the *exo*-alcohols **3a** and **3b**, which were separated using flash chromatography. Conversion of **3a** to **5** under standard conditions made it ready for establishment of the desired relative *endo*-stereochemistry at the 4-position. However, facial selective hydroboration of **5** to give 5-*endo* methylene alcohol **6a** proved to be a major challenge. Extensive experimentation eventually revealed that the catalytic hydroboration methodology, using $Rh(Cl)(PPh_3)_3$/catecholborane (CatBH), gave 95% of the desired 5-*endo* product **6a**. Subsequent oxidation of the diastereomeric alcohols **6a**,**b** to the corresponding carboxylic acids **7a**,**b** was carried out using the improved Sharpless procedure ($RuCl_3/NaIO_4$) [47]. However, not to be foreseen, hydrolysis (LiOH/tetrahydrofuran (THF)/H_2O) of the ethyl ester functionality of **7a**,**b** to give 3,5-diacid **8a**,**b** resulted in the formation of significant amounts of retro-1,4-addition products and further hydrolysis products thereof. The problem was traced back to the susceptible 4,6 bond and the synthetic plan was revised to firstly hydrolyze the ethyl ester of **6a**,**b** followed by oxidation of the alcohol moiety using the improved Sharpless procedure that converted **9a**,**b** smoothly into the 3,5-diacid **10a**,**b**. Finally, the Boc group was removed by treatment with trifluoroacetic acid (TFA) at room temperature, to give distereomerically pure (±)-DCAN, in 96% yield.

- *Reagents and conditions*: (a) room temperature, 18 h (40–60%); (b) BH_3/THF, −78 °C to room temperature, 1 h, then H_2O_2/NaOH (85%); (c) **3b** only: Dess–Martin, room temperature, 8 h (91%); (d) 1.0 equiv. Tebbe's reagent, −40 °C to room temperature (71%); (e) $Rh(Cl)(PPh_3)_3$/CatBH, room temperature, 2 h, then $H_2O_2/NaHCO_3$ (83%); (f) $RuCl_3/NaIO_4$, room temperature, 2 h (70%); (g) LiOH, THF/H_2O, room temperature (10–15%); (h) LiOH, THF/H_2O, room temperature, 24 h (94%); (i) $RuCl_3/NaIO_4$, room temperature, 2 h (76%); (j) TFA, room temperature, 30 min (96%).

4.3.3
Case Study: Synthesis of LY354740

At first glance, the mGluR group II-selective agonist LY354740 [33] (Figure 4.4) appears to be a challenge to synthesize, given its [3.1.0] carbobicyclic skeleton with four chiral centers, one of which is a quaternary carbon. However, a short and efficient strategy (Scheme 4.2) was developed taking advantage of readily available starting material and reagents (steps a–c) followed by salt formation as the key step (steps d and e) to obtain enantiomerically pure (−)-**4**. Eventually, basic hydrolysis of the amino acid synthon (step f) provides LY354740 in excellent overall yield (14%).

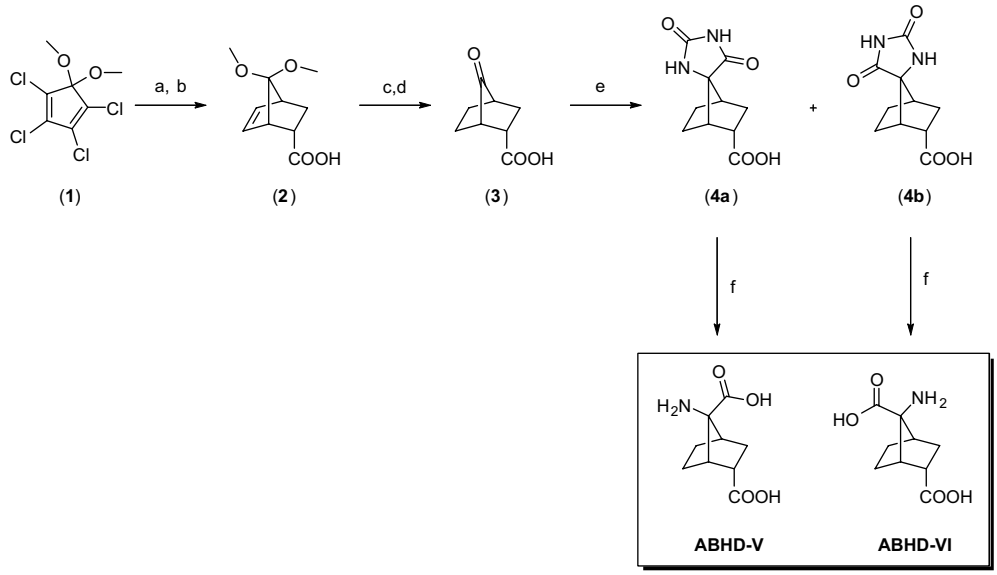
- *Reagents and conditions*: (a) ethyl (dimethylsulfonium)acetate bromide, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), room temperature (68%, 98 : 2 ratio of **2a**: **2b**); (b) $(NH_4)_2CO_3$, KCN, EtOH, H_2O, 35 °C (73%, 87 : 13 ratio of **3a** : **3b**); (c) NaOH, H_2O, room temperature (95%); (d) cocrystallization with (*R*)-phenylethylamine (46%); (e) HCl, H_2O (76%, (−)-enantiomer only); (f) NaOH, H_2O, reflux (86%).



Scheme 4.2 Synthesis of LY354740.

4.3.4
Case Study: Synthesis of ABHD-V and ABHD-VI

The Glu analogs ABHD-V and ABHD-VI (Figure 4.4) both have a highly rigid bicyclic carbon skeleton. The synthesis (Scheme 4.3) of the challenging 3,7-substitution



Scheme 4.3 Synthesis of ABHD-V and ABHD-VI.

pattern of the [2.2.1] carbobicyclic skeleton was solved by taking advantage of a 4 + 2 cycloaddition reaction (step a) of hexasubstituted cyclopentene **1** with methylacrylate followed by a free metal reduction (step b) of the four chlorine atoms. This provides **2** with the desired 2-*endo*-stereochemistry and acetal functionality at position 7, for subsequent functional group transformation into the amino acid moiety (steps c–f). In summary, ABHD-V and ABHD-VI were synthesized in only six steps with a high overall yield. However, the major drawback from the strategy is the lack of enantiopurity of the two target molecules.

- *Reagents and conditions*: (a) methylacrylate, KOH, MeOH; (b) Na, EtOH, (c) Pd-C, H_2, room temperature; (d) 5% H_2SO_4, room temperature; (e) $(NH_4)_2CO_3$, KCN, EtOH, H_2O, 55 °C (1 : 1 ratio of **4a** : **4b**); (f) BaO, H_2O, 120 °C, sealed tube.

4.4 Bioisosterism

The term "bioisosterism" is defined as functional groups which may substitute for a different bioactive group while maintaining the biological properties [48].

With respect to α-amino acids, the amino acid moiety is not easily replaced by bioisosteric units due to its intrinsic zwitterionic character. However, several of the distal functional groups of the 20 common α-amino acids have been replaced successfully and some have even been isolated from natural sources. Examples of naturally occurring Glu analogs which contain a γ-carboxylic acid bioisostere (Figure 4.7) include ibotenic acid (IBO), first isolated from the toxic mushrooms *Amanita muscaria* [49], *Amanita strobiliformis* [50, 51], and *Amanita pantherina* [52], and its chemical structure was found to comprise the 3-hydroxyisoxazole heterocycle. IBO, which racemizes readily, has been shown to be a potent agonist at mGluRs and a weak agonist at the iGluRs (NMDA subtypes). (*S*)-Quisqualic acid (QUIS) has been isolated from the seeds of *Quisqualis indica*. Its chemical structure contains an (*S*)-alanine moiety attached to a five-membered heterocycle as determined by chemical methods [53, 54], synthesis [55], and X-ray crystallographic studies [56]. In binding studies at native iGluRs, QUIS has been shown to be a nonselective high-affinity ligand [57] and, furthermore, in functional assays QUIS has been shown to be a full agonist [58]. Moreover, QUIS is a potent agonist at the metabotropic receptors

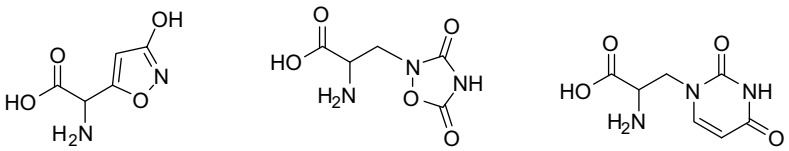


Figure 4.7 Naturally occurring Glu analogs that contain a γ-carboxylic acid bioisoster: IBO, QUIS, and WILLA.

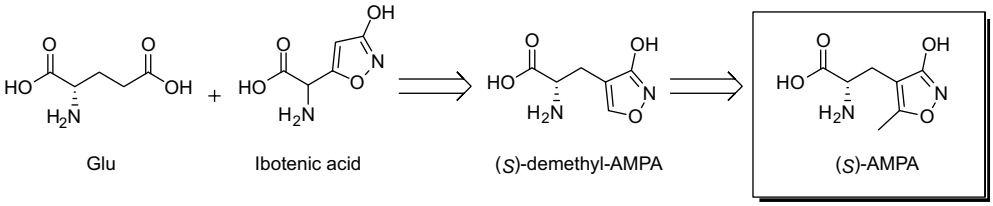


Figure 4.8 Design of AMPA as a hybrid structure of Glu and the natural product ibotenic acid.

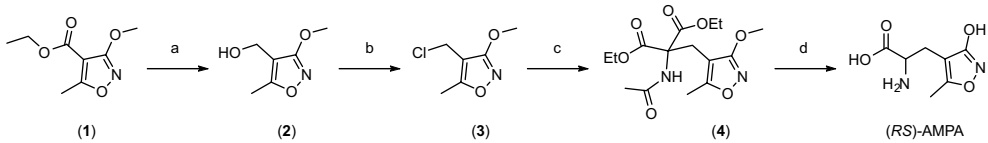
(mGluR5: $EC_{50} = 55$ nM) [59]. An X-ray study of QUIS bound to the iGluR6 subtype shows that the ring nitrogen is sp^3-hybridized and that this hybridization state is critical for QUIS being able to adapt to the folded Glu conformation. The third compound, (*S*)-willardiine (WILLA),was originally isolated from the rose *Acacia willardiana* (Mimosaceae) in 1959 [60]. Its structure was unambiguously determined by chemical synthesis in 1961 [61] and, as for QUIS, it comprises an (*S*)-alanine moiety, however, attached to the six-membered heterocycle uracil. WILLA has been shown to be a partial agonist [62, 63] with low affinity for the KA receptors and a medium range affinity ligand at the AMPA receptors.

4.4.1
Case Study: Design and Synthesis of AMPA

In the late 1970s, the compound AMPA was designed as a hybrid structure of Glu and the naturally occurring Glu analog IBO (Figure 4.8) [64]. Although the compound demethyl-AMPA would be the more logical target structure, the inclusion of the 5-methyl group of the 3-hydroxyisoxazole ring was necessary due to the synthetic strategy used. Later it became evident that this 5-methyl group is indeed crucial for AMPA's observed receptor subtype selectivity profile.

The synthesis of AMPA (Scheme 4.4) [65] commenced with the chemoselective reduction of the ester functionality of *O*-methyl-protected 3-hydroxyisoxazole **1** to give alcohol **2**. Upon treatment with thionyl chloride the corresponding chloride **3** was obtained. Introduction of the amino acid moiety by nucleophilic substitution with the amino acid synthon, diethyl acetamidomalonate, provided **4**, which under strongly acid conditions gave target compound AMPA in its racemic form.

- *Reagents and conditions*: (a) $LiAlH_4$(75%); (b) $SOCl_2$ (80%); (c) $AcNHCH(COOEt)_2$, EtONa (45%); (d) HBr (aqueous) then EN_3N (69%).



Scheme 4.4 First synthesis of AMPA [65].

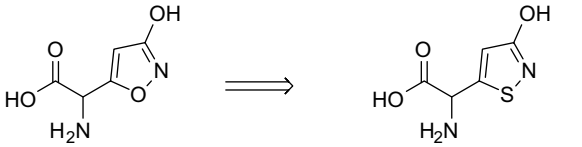
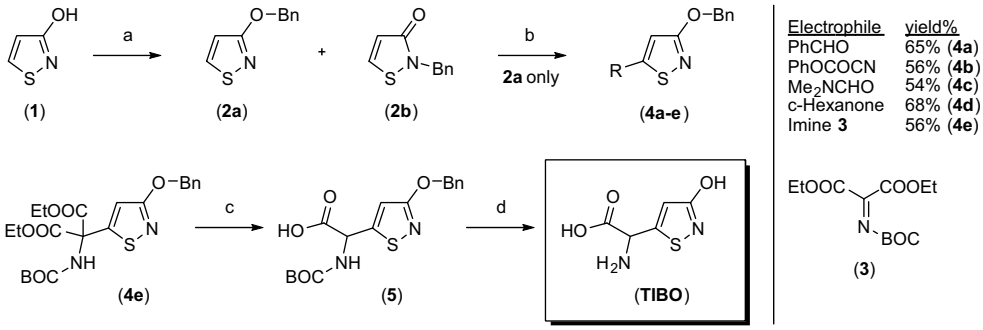


Figure 4.9 Chemical structures of IBO and TIBO.

4.4.2
Case Study: Design and Synthesis of Thioibotenic Acid

Substitution of sulfur for the ring oxygen in 3-hydroxyisoxazole provides the parental system 3-hydroxyisothiazole, which is less acidic ($pK_a = 7$ versus $pK_a = 5$), larger in size, and more lipophilic [66]. On that basis, the synthesis and pharmacological evaluation of thioibotenic acid (TIBO) became of interest (Figure 4.9) [67].

The strategy for the synthesis of TIBO relies on the in-time development of new methodology for the direct functionalization of the 5-position of the 3-hydroxyisothiazole ring (Scheme 4.5). By investigating a number of strong bases, solvents, and temperature, the selective deprotonation of the 5-position and subsequent controlled nucleophilic addition to a number of chemically distinct electrophiles was achieved (yields, 54–68%). With this new methodology in hand, TIBO was prepared in only four steps from 3-hydroxyisothiazole (**1**).



Scheme 4.5 Synthesis of TIBO.

- *Reagents and conditions*: (a) BnBr, K_2CO_3, DMF, room temperature (90%, 2 : 1 ratio of **2a** : **2b**); (b) 1.1 equiv. of LDA, −78 °C, Et_2O, then 1.1 equiv. of E, e.g., imine **3** (56%); (c) LiOH, H_2O/THF, room temperature, 4 h (76%); (d) 10% HBr/AcOH, room temperature, 2 h (60%).

Pharmacological characterization (Table 4.1) of TIBO revealed that it is a potent agonist at mGluR subtypes representing groups I, II, and III. This is in contrast to IBO, which only activates mGluR subtypes within groups I and II [68]. A subsequent detailed modeling study suggests that the origin of this notable difference between

Table 4.1 Agonist potencies of IBO and TIBO at cloned rat mGluR subtypes (EC_{50} in μM) [68].

	Group I		Group II	Group III	
	mGluR1	**mGluR5**	**mGluR2**	**mGluR4**	**mGluR6**
IBO	43	17	110	>1000	>1000
TIBO	12	5.4	52	2.6	0.96

the pharmacological profiles of IBO and TIBO may be attributed to disparities of internal energies at optimal binding modes for mGluR group III subtypes [68].

4.5
Structure–Activity Studies

The ultimate goal for any medicinal chemistry study is to enlighten and eventually understand the detailed relationship between the chemical structure of a ligand and its induced biological activity upon interacting with its biological target. Often this is referred to as a structure–activity relationship. Such complex studies require close collaboration between scientists within the distinct scientific disciplines of organic chemistry, molecular pharmacology, *in vitro*/*in vivo* pharmacology, protein crystallography, and computer modeling.

4.5.1
Case Study: AMPA Analogs

Since the discovery of AMPA in 1980,as a selective agonist for iGluR1–4 [64], about 70 analogs have been synthesized. The vast majority of the synthesized analogs focus on variations of the substitution in the 5′-position of isoxazole ring, but also conformationally restricted analogs such as (*S*)-2-amino-3-(3-hydroxy-7,8-dihydro-6*H*-cyclohepta[1,2-*d*]isoxazol-4-yl)propionic acid (4-AHCP) have been prepared. In summary (Figure 4.10), the extensive work has lead to the conclusion that the

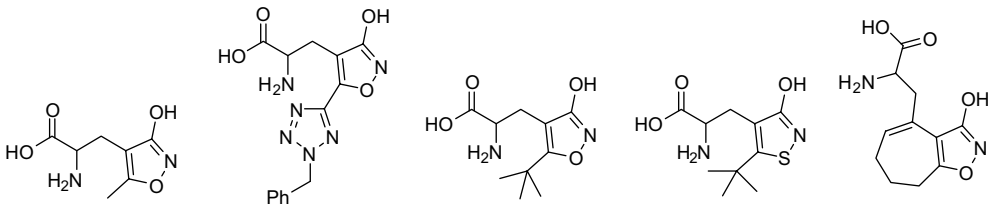


Figure 4.10 Chemical structures of AMPA, 2-Bn-Tet-AMPA, ATPA, Thio-ATPA, and 4-AHCP.

Table 4.2 Binding affinities of AMPA and analogs thereof at native iGluRs (rat brain synaptosomes) (IC_{50} in nM).

Reference	Ligand	[^{3}H]AMPA	[^{3}H]KA	NMDA[a)]
[70, 71]	AMPA[b)]	40	>100 000	>100 000
[72]	2-Bn-Tet-AMPA[b)]	2700	>100 000	87 000
[5, 7]	(*S*)-ATPA	1800	23 000	>100 000
[73]	(*S*)-thio-ATPA	270	14 000	>100 000

a) Radioligand: [^{3}H]CCP.
b) Racemic mixture of compound was used.
For clarity, values are rounded off. –: No data available.

3-hydroxyisoxazole moiety favors the Glu folded conformation which has underlying iGluR agonism. Furthermore, the discovery of (*S*)-2-amino-3-(3-hydroxy-5-*tert*-butyl-4-isoxazolyl)propionic acid (ATPA) – an analog that contains a *tert*-butyl group in the 5′-position – was made. In binding assays at the iGluR subtypes, ATPA showed a significant selectivity the iGluR5 subtype (Tables 4.2 and 4.3). This observation was further investigated in a functional study (Table 4.4), in which ATPA was shown to be a full agonist displaying a 16- to 40-fold selectivity for iGluR5 over AMPA subtypes iGluR1,3, and 4.

Based on the conception that activation (agonism) at the iGluR receptor requires domain closure occluding the ligand, the finding that 2-Bn-Tet-AMPA is a full agonist at the iGluR4 subtype is quite interesting (Table 4.4). This discovery underlines the importance of comprehending receptor proteins as highly flexible molecules that may adapt significantly more bulky ligands than that suggested from judging an X-ray crystal structure of the endogenous agonist, Glu [69].

4.5.2
Case Study: 4-Substituted Glu analogs

To date, some 200 analogs of Glu that contain a substituent in the 4-position, have been synthesized and evaluated as potential ligands for the GluRs and EAATs [76–79].

Table 4.3 Binding affinities of AMPA and analogs thereof at cloned homomeric iGluR1–7 (K_i in nM).

Ref.	Ligand	iGluR1	iGluR2	iGluR3	iGluR4	iGluR5	iGluR6	iGluR7
[70, 71]	AMPA	22	17	21	40	2000	>100 000	>100 000
[72]	2-Bn-Tet-AMPA	7700	750	390	200	58 000	39 000	—
[71, 73]	(*S*)-ATPA	—	—	—	—	4[a)]	>100 000	~10 000
[73]	(*S*)-thio-ATPA	—	—	—	—	—	—	—

a) Racemate
For clarity, values are rounded off. –: No data available.

Table 4.4 Functional characterization of ATPA and analogues at homomeric iGluR1 and 3–7, and heteromeric KA2/iGluR6 subtypes expressed in oocytes (EC_{50} in μM).

Ref.	Ligand	iGluR1	iGluR3	iGluR4	iGluR5	iGluR6	KA2/iGluR6
[69]	2-Bn-Tet-AMPA[a)]	~3000 (0.4)	~1000 (0.6)	75 (1.1)	—	—	—
[74]	(*S*)-ATPA[b)]	22 (0.3)	8 (0.05)	8 (0.7)	0.5 (1.0)	NR	60 (0.03)
[73]	(*S*)-thio-ATPA[b)]	5 (0.8)	32 (1.2)	20 (1.7)	0.1 (1.0)	NR	5 (0.4)
[75]	(*S*)-4-AHCP[b)]	5 (0.7)	7 (1.0)	15 (1.6)	0.1 (1.6)	NR	6 (0.5)

a) Efficacy (I_{max}) relative to Glu.
b) Efficacy (I_{max}) relative to KA for iGluR1 and 3–6, and to AMPA at KA2/GluR6.
For clarity, values are rounded off. –: No data available; NR: no response at 600 μM.

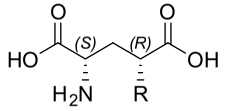
In summary, this extensive work has provided valuable insight into the structure–activity relationship, and resulted in subtype-selective ligands useful for detailed pharmacological characterization of GluRs and EAATs.

The first two analogs to be synthesized were the diastereomeric 4-methyl Glu analogs (entries 1 and 2, Table 4.5). An nuclear magnetic resonance study at diverse pH values and a subsequent *in silico* study led to the conclusion that the diastereomeric methyl substituents favor distinct low-energy conformations of the Glu skeleton [80, 81]. In iGluR binding assays at native receptors, the L-2,4-*syn* diastereomer was a high-affinity ligand for the KA and NMDA receptors, and a low-affinity ligand at native AMPA receptors. In subsequent studies at homomeric iGluR5 clones, it was shown to be a high-affinity ligand at iGluR5 with 25-fold selectivity over iGluR6 and 10-fold selectivity over iGluR7. In contrast, the L-2,4-*anti* diastereomer, is a medium range affinity ligand at native KA receptors, and a low-affinity ligand at native AMPA and NMDA receptors. Building on this observation, a vast number of L-2,4-*syn*-4-arylidenealkyl Glu analogs were synthesized and their potential as selective KA receptor ligands was investigated. The most noteworthy analog (entry 3), which holds a naphthylideneethyl group, shows high affinity for iGluR5 with a high degree of selectivity over iGluR6 (1000-fold) and iGluR7 (40-fold) [79, 82].

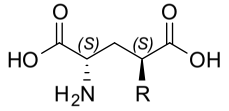
A series of 10 Glu analogs with functionalized substituents in the L-2,4-*syn* position were synthesized using a chemoenzymatic strategy. Upon pharmacological evaluation at the iGluRs, an unexpected and quite intriguing difference of affinity for the methylester analog (entry 4) versus the methylamide analog (entry 5) was found. Whereas the methylester was selective for iGluR5 over iGluR6 and 7 the methylamide analog was a high-affinity ligand at iGluR5–7. While the structural origins for these intriguing observations were addressed *in silico* by the authors, it remains to be established whether a difference in ligand functionality (agonism versus antagonism) may play a role.

Introduction of an alkylidene substituent in the 4-position of Glu also generally favors the folded conformation. The first analog to be synthesized and investigated pharmacologically was 4-methylidene Glu (entry 1, Table 4.6), at native iGluRs and cloned homomeric iGluR5–7 subtypes. In contrast to its close structural analog, L-2,4-*syn*-4-methyl Glu (entry 1, Table 4.5), it has a less discriminating pharmacological profile being a medium range affinity ligand at all targets investigated. Its

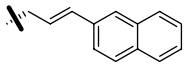
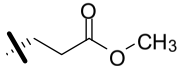
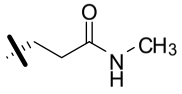
Table 4.5 Binding affinities of 4-alkyl Glu analogs at native iGluRs (rat brain synaptosomes) (IC_{50} in nM), and at cloned homomeric iGluR5–7 (K_i in nM) and KA2 subtypes.



L-2,4-*syn* -Glu

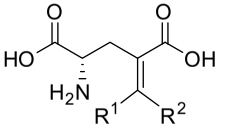


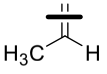
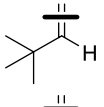
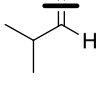
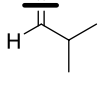
L-2,4-*anti*-Glu

Entry	R group	[^{3}H]AMPA	[^{3}H]KA	NMDA[a]	iGluR5[b]	iGluR6[b]	iGluR7[b]	KA2/iGluR6
1 [78, 83–85]		26600	32	5900	0.7	17	6	—
2 [83]		14600	330	9600	—	—	—	—
3 [79, 82]		>1000[d]	—	—	15[c]	15400[c]	620	>10 000
4 [78]		31000	7900	>100000	43	6700	1000	—
5 [78]		32000	29000	>100000	4	120	57	—

a) Radioligand: [^{3}H]CGP-39653.
b) Rat clone and radioligand: [^{3}H](2*S*,4*R*)-4-Me Glu (SYM-2081).
c) Human clone and radioligand: [^{3}H]KA.
d) Binding at homomeric iGluR1, 2, and 4 subtypes.
For clarity, values are rounded off. –: No data available.

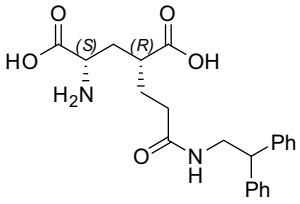
Table 4.6 Binding affinities of 4-alkylidene Glu analogs at native iGluRs (rat brain synaptosomes) (IC_{50} in nM), and at cloned homomeric iGluR5–7 (K_i in nM) and KA2 subtypes.



Entry	R^1, R^2 groups	[^{3}H]AMPA	[^{3}H]KA	NMDA[a]	iGluR5[b]	iGluR6[b]	iGluR7[b]	KA2/iGluR6
1 [82, 84]		150	230	1200	270	450	—	—
2 [2]	(E) 	—	—	—	2200	2500	—	—
3 [86]	(E) 	2000	>100000	>100000	24	>100000	—	—
4 [82]	(E) 	70[c] 3700 5600	—	—	54	83000	520	7500
5 [82]	(Z) 	113000[c]	—	—	94	>500000	10500	82 200

		44900 10300						
6 [82]		>100000[c] 280900 4700	—	—	240	207000	12400	21 400
7 [82]		1100[c] 3700 530	—	—	33	>100000	5700	10 000

a) Radioligand: [^{3}H]CCP.
b) Radioligand: [^{3}H]KA.
c) Binding at homomeric iGluR1, 2, and 4 subtypes, respectively.
For clarity, values are rounded off. –: No data available.



(DPAG)

Figure 4.11 Chemical structure of DPAG – a selective inhibitor of the EAAT2 subtype.

conformation in water has been investigated in detail and it was concluded that the folded Glu conformation found is the predominant conformation at neutral pH [80, 81]. Nevertheless, it has served as a lead structure for the synthesis of several alkylidene [82, 86] and heteroarylidene [87] Glu analogs. Most notable is entry 3, which contains an *E-tert*-butylidene substituent. This analog was designed as a structural hybrid of ATPA (Figure 4.10) and Glu, and showed a pharmacological profile comparable with that of ATPA, being a selective high-affinity iGluR5 ligand. Entries 4 and 5, Table 4.6, are remarkable in the sense that they are *E*/*Z*-stereoisomers and both are high affinity ligands at the iGluR5 subtype. Moreover, the *Z*-stereoisomer displays a higher degree of selectivity – a trend which is also seen for the disubstituted analogs (entries 6 and 7, Table 4.6).

In summary, several X-ray crystallographic and modeling studies have shown that introduction of an alkylidene substituent or an alkyl substitution in the 4-position of Glu (the latter only for the 2,4-*syn* stereochemical relationship) favor the Glu folded conformation. In consensus, this conformation is the Glu agonist conformation at the iGluRs [4] and the competitive inhibitor conformation at the EAATs [7]. The series of 4-substituted Glu analogs has also been investigated at the EAATs and has led to new insight into the structure–activity relationship for EAAT substrate–inhibitor differentiation [88] as well as the discovery of Glu analog (2*S*,4*R*)-2-amino-4-(3-(2,2-diphenylethylamino)-3-oxopropyl)pentanedioic acid (DPAG) [78] (Figure 4.11) – a selective inhibitor for the EAAT2 subtype.

4.6 Conclusions

In conclusion, the α-amino acid neurotransmitter Glu has been investigated in great detail and thus well represents efforts within the topic of medicinal chemistry of α-amino acids. This chapter has illustrated and discussed fundamental aspects and challenges of medicinal chemistry, such as ligand active conformation, the importance of ligand low-energy conformations, ligand conformational restriction, bioisoterism, and structure–activity relationship studies. Selected references to the primary literature are provided and the reader is encouraged to consult these scientific articles for further information.

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5
Medicinal Chemistry of Alicyclic β-Amino Acids

Nils Griebenow

5.1
Introduction

Compared with their open-chain congeners, alicyclic β-amino acids are less prominent in the literature. However, over the past years the interest in alicyclic β-amino acids of the general formula **1** (Figure 5.1) has steadily increased, and these have become an intriguing topic in synthetic and medicinal chemistry [1–3].

In detail, alicyclic β-amino acids frequently occur in pharmacologically important compounds and additionally serve as important structure-forming elements in β-peptides. Herein, we give a comprehensive review on the synthesis and pharmacological profile of five- and six-membered alicyclic β-amino acids with an impact on medicinal chemistry.

5.2
Five-Membered Alicyclic β-Amino Acids

Among the β-amino acid derivatives of cycloalkanes, (1*R*,2*S*)-2-amino-cyclopentanecarboxylic acid (cispentacin, **2**) has received most attention since it shows intriguing biological properties and is the only naturally occurring alicyclic β-amino acid (Figure 5.2). It is a fragment of amipurimycin (**3**), and has been isolated in its free form from the fermentation broth of *Bacillus cereus* L450-B2 [4] and *Streptomyces setonii* 7562 [5].

The structure was confirmed by X-ray [6] and spectroscopic analysis as well as several total syntheses [2, 7]. Cispentacin exhibits only weak *in vitro* activity against *Candida albicans* and *Candida neoformans*, but demonstrated good therapeutic efficacy against a systemic *Candida* infection in mice by both parenteral and *per os* administration. The mode of action is due to an active transport of cispentacin via proline and other amino acid permeases into the fungal cells. After accumulation in the fungal cells, it inhibits the prolyl-tRNA synthase and therefore protein biosynthesis [8]. Kawabata *et al*. investigated the antifungal activity of cispentacin and its stereoisomers against *C. albicans* and *Candida topicalis* (Table 5.1) [6]. Only the

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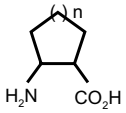


Figure 5.1 General formula.

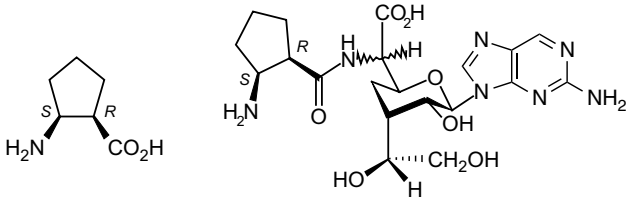
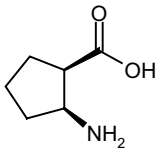
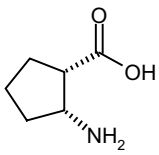
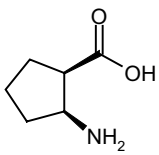
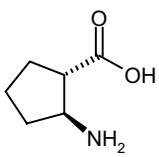


Figure 5.2 Cispentacin (**2**) and amipurimycin (**3**).

Table 5.1 Antifungal activity of cispentacin and its stereoisomers against *C. albicans* and *C. tropicalis*.

Compound	Structure	MIC (μg/ml) *C. albicans* FP578[b)]	MIC (μg/ml) *C. tropicalis* FP 583[b)]
2		6.25	6.25
3		>100	>100
4[a)]		12.5	12.5
5[a)]		>100	>100

a) Racemic.

b) MICs (minimum inhibitory concentrations) were determined by the agar dilution method using minimum essential medium (MEM) agar after incubation at 37 °C for 18 h with an inoculum size of about 10^6 cfu/ml.

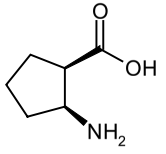
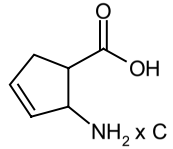
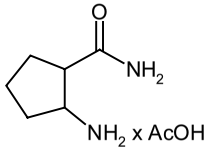
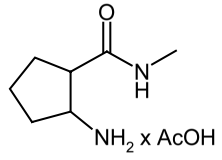
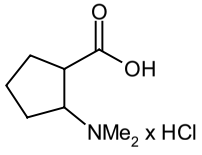
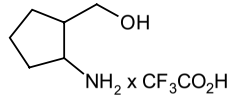
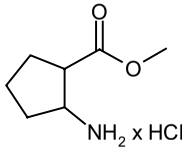
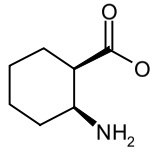
(*S*,*R*)-isomer cispentacin (**2**) showed antifungal activity. The biological activity has led to high interest in the structural optimization of this lead structure [9, 10] that aimed at derivatives with superior (oral) efficacy and good tolerability for the treatment of yeast infections.

To explore the structure–activity relationship of cispentacin. Ohki *et al*. synthesized several congeners and described their antifungal *in vitro* activity against *C. albicans* (Table 5.2) [10]. Some of the derivatives were prepared by [2 + 2] cycloaddition of the corresponding vinylic compounds with chlorosulfonyl isocyanate and hydrolysis of the intermediate β-lactams (Scheme 5.2). Derivatives modified at the carboxyl group such as amides **6** and **10**, primary alcohol **11**, and methyl ester **8** were inactive. The *N*-dimethylamino carboxylic acid **7** and the ring-expanded 2-aminohexane carboxylic acid **12** were also inactive. Only the cyclopentene derivative **9** showed reasonable activity. Thus, Ohki *et al*. concluded that both functional groups, the carboxyclic acid and the primary amino group, were crucial for potent antifungal activity.

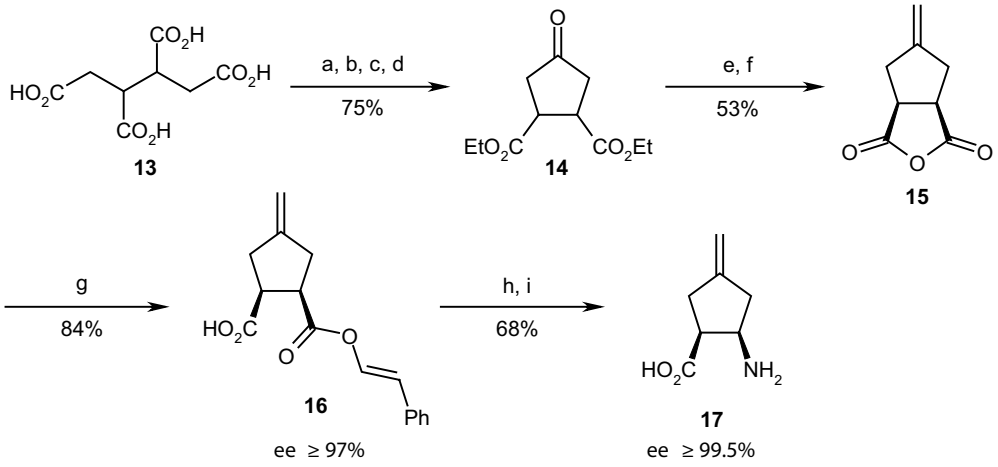
Independently, scientists at Bayer AG discovered that the 2-aminohexene carboxylic acid **18**, which was originally designed as a pyridoxal phosphate suicide inhibitor, exhibits strong antifungal activity against *C. albicans* (Table 5.3) [11]. However, in toxicological studies cyclohexene β-amino acid **18** showed a less favorable profile. These results along with the reported antifungal activity of cispentacin stimulated Mittendorf *et al*. to investigate further derivatives of the lead structures **2** and **18** in order to optimize the efficacy and tolerability profile [9]. A variety of β-amino acids listed in Table 5.3 were synthesized and evaluated for their antifungal *in vitro* activity against *C. albicans*. Many of these β-amino acids were prepared following known methods. However, for several β-amino acids a short and efficient asymmetric synthesis via asymmetric desymmetrization of *meso*-anhydrides was established. In detail, the *exo*-methylene β-amino acid **17** (Table 5.3) was prepared by a highly enantioselective, quinidine-mediated alcoholysis of the *meso*-anhydride **15** to provide the cinnamyl ester **16** with an enantiomeric excess of 97% or greater (Scheme 5.1). Subsequent Curtius rearrangement and palladium-catalyzed removal of the cinnamyl protecting groups afforded **17** with an enantiomeric excess of 99.5% or greater. This procedure was successfully used to produce 5 kg of β-amino acid **17**. The absolute configuration of **17** was assigned by X-ray crystallography. Cispentacin (**2**) was prepared in a similar manner.

Regarding inhibitory activity against *C. albicans*, cispentacin (**2**) and the β-amino cyclohexene carboxylic acid **18** both demonstrated strong *in vitro* activity (IC_{50} 0.13 and 0.03 mg/l, respectively). Whereas dehydro-cispentacin **9** showed slightly lower potency (IC_{50} 0.5 mg/l). Transposition or hydrogenation of the double bond in **26** resulted in a significant loss of activity (e.g., **21** and **22**). Also, insertion of heteroatoms (e.g., **20**) and reduction of ring size **19** hampered antifungal activity. Structure–activity relationship investigations were performed at the 4-position of the cyclopentane ring. Among these congeners, only the introduction of an *exo*-methylene group resulted in reasonable antifungal activity, since derivative **17** ($IC_{50} = 0.13$ mg/l) was equipotent to cispentacin. Overall, a narrow structure–activity relationship was observed, resulting in significant loss of potency when the double bound of **17** was

Table 5.2 Structure–activity relationship studies by Ohki *et al.* [10].

Compound	Structure	MIC[b] (μg/ml) *C. albicans* Yu-1200	Compound	Structure	MIC[b] (μg/ml) *C. albicans* Yu-1200
2		3.13	**9**		25
6		>100	**10**		>100
7[a]		>100	**11**[a]		>100
8[a]		100	**12**[a]		>100

a) Racemic.
b) MICs were determined by Eagle's MEM agar (Nissui), 10^5 cfu/ml. Streak method, 30 °C, 24 h.



Scheme 5.1 (a) EtOH, H_2SO_4; (b) NaOMe, MeOH; (c) HCl, H_2O; (d) EtOH H_2SO_4, 75%; (e) $Ph_3PMe^+Br^-$, KO*t*Bu, tetrahydrofuran (THF), then KOH, THF, H_2O, 71%; (f) $(EtCO)_2O$, 135 °C, 75%; (g) 1.0 equiv. quinine, 1.5 equiv. (2*E*)-3-phenyl-2-propane-1-ol, toluene, −15 °C, 4 h, 84%; (h) $(PhO)_2PON_3$, NEt_3, toluene, 90 °C, then 3-phenyl-2-propane-1-ol, toluene, reflux, 80%; (i) 0.05 mol% Pd $(OAc)_2$, PPh_3, morpholine, EtOH, 85%.

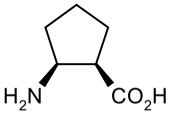
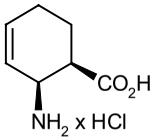
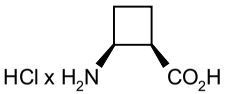
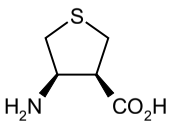
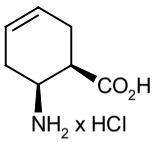
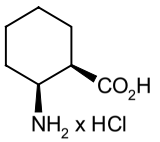
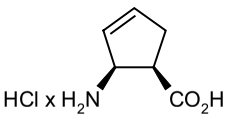
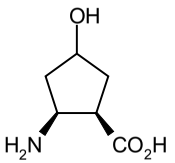
shifted to different *endo* positions and small substituents were introduced (examples **31**–**33**). Also, a shift of the *exo*-methylene group from the 4- to the 3-position of the cyclopentane ring led to a significant loss of potency (e.g., **34**).

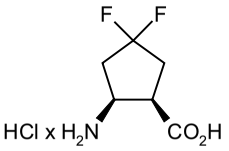
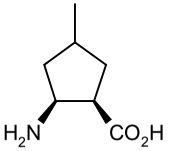
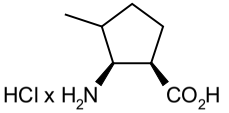
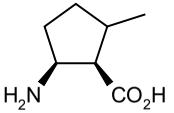
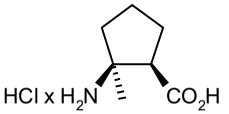
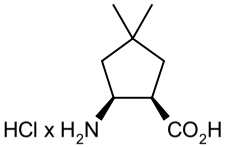
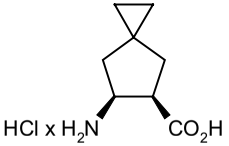
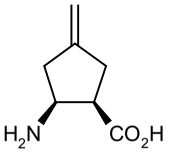
Interestingly, the additional *exo*-methylene group of **17** caused a dual target shift compared to cispentacin. Here, the isoleucyl-tRNA is specifically inhibited and the active transport into the cells is carried out by permeases specific for branched-chain α-amino acids [12].

β-Amino acid **17** showed the most favorable activity–tolerability profile of all β-amino acids so far prepared in this series and was therefore selected for further development.

In 2000, the Croatian pharmaceutical company PLIVA licensed the β-amino acid **17** (BAY 10-8888) from Bayer, where it is now named icofungipen (PDL-118). PLIVA was to take it to phase I or II trials, after which it would seek to establish a global partnership for further development and marketing [13]. Efficacy studies with PLD-118 showed dose-dependent antifungal activity and nonlinear plasma pharmacokinetics in the treatment of experimental fluconazole-resistant oropharyngeal and esophageal candidiasis in immunocompromised rabbits. Efficacy was also achieved in the treatment of experimental disseminated candidiasis, including central nervous system infection. In a phase II study of icofungipen 150 mg versus fluconazole 100 mg for the treatment of oropharyngeal candidiasis in 48 HIV-positive patients, icofungipen demonstrated a good clinical response, but low mycologic eradication rates. Icofungipen was well tolerated and safe, which should allow dose escalation in further studies [14]. By November 2003, a phase IIb trial had been

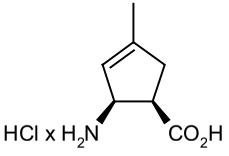
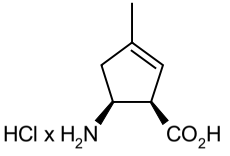
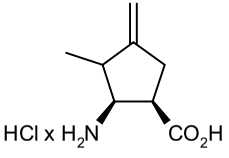
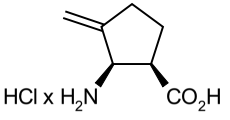
Table 5.3 *In vitro* activity of different cispentacin derivatives against *C. albicans*.

Compound	Structure	IC_{50} (mg/l)[c] *C. albicans*	Compound	Structure	IC_{50} (mg/l)[c] *C. albicans*
2		0.13	**18**		0.03
19		128	**20**		64
21[a]		128	**22**[a]		128
9[a]		0.5	**23**[a] (3: 1 mixture of diastereomers)		128

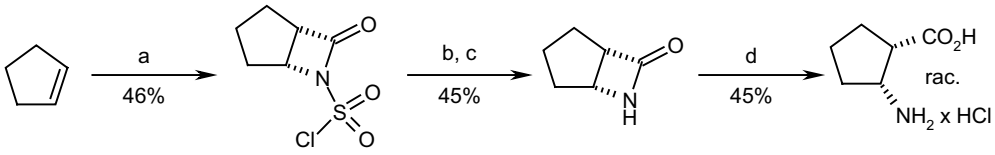
24[a]		>256	**25** (5: 1 mixture of diastereomers)[b]		4
26[a] (4: 1 mixture of diastereomers)[b]		128	**27**[a]		diastereomer A 8[b] diastereomer B 16[b]
28[a]		64	**29**[a]		256
30		32	**17**		0.13

(*Continued*)

Table 5.3 (*Continued*)

Compound	Structure	IC_{50} (mg/l)[c] *C. albicans*	Compound	Structure	IC_{50} (mg/l)[c] *C. albicans*
31		8	**32**		64
33[a] (single diastereomer)[b]		64	**34**[a]		32

a) Racemic.
b) Configuration not known.
c) IC_{50} values were determined after incubation with *C. albicans* AtCC 36082 YNB medium (yeast nitrogen base powder). Read-out was performed photo/nephelometrically on a Spectra III ELISA reader at 360 nm.



Scheme 5.2 Synthesis of racemic β-amino acid **2** via [2 + 2] cycloaddition. (a) $ClSO_2NCO$, −78 to 0 °C, 8 h then room temperature overnight; (b) KI, $NahSO_4$; (c) NaOH, pH 7; (d) HCl, 0 °C, 3 h.

initiated in 84 patients with oropharyngeal candidiasis, using a higher-dose regimen (200–500 mg twice-daily for 2 weeks), in comparison with fluconazole (100 mg/day for 2 weeks). No data are currently available. However, in May 2005 PLIVA exited the proprietary business to focus on generics. By July 2005, the company was seeking to out-license the agent for further development.

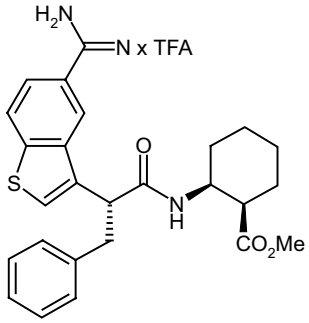
5.3
Six-Membered Alicyclic β-Amino Acids

A literature survey on six-membered alicyclic β-amino acids in medicinal chemistry revealed several hits that are shown in Scheme 5.3. Among the above-mentioned **18**, best known is the synthetic derivative tilidine or *trans*-ethyl-2-(dimethylamino)-1-phenylcyclohex-3-en-1-carboxylate (**36**), which is a mixed μ/κ-agonist/antagonist and is used as a potent analgesic in the therapy to control moderate to severe pain. Tilidine (**36**) was prepared by Diels–Alder cycloaddition of *trans*-1-(dimethylamino)-1,3-butadiene and ethyl atropate. Tilidine (**36**) was the minor stereoisomer formed from the cycloaddition (**36**/**40** = 1: 3, Scheme 5.4). The advantages of tilidine over other opioids are rapid oral efficacy and lack of respiratory depression, of spasmogenic effects on the gastrointestinal and urinary tract, and of tolerance development even after years of application. It is, therefore, especially well suited for the therapy of chronic pain.

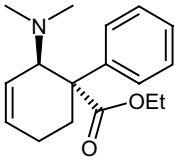
Researchers at Bristol-Myers Squibb synthesized a series of 5-amidinobenzo[*b*] thiophenes, and several compounds were identified as dual Factor IXa and Factor Xa inhibitors for the potential treatment of thrombosis [15].

Thromboembolic events, such as deep vein thrombosis, pulmonary embolism, myocardial infarction, and thromboembolic stroke, are the major causes of morbidity and mortality in the industrialized world. Currently available anticoagulants such as heparin or warfarin are limited by modest therapeutic benefits, narrow clinical applications, increased bleeding risk, and drug-induced thrombophilia. To improve the benefit-to-risk ratio of antithrombotic drugs, small-molecule direct thrombin inhibitors and selective Factor Xa inhibitors are being extensively investigated.

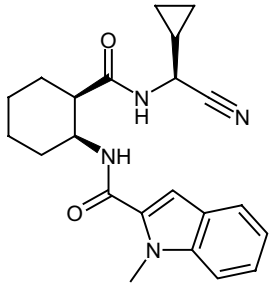
The coagulation cascade is a sequence of proteolytic events, involving several serine proteases, leading to overall maintenance of hemostasis. The cascade consists of three systems: the intrinsic and extrinsic pathways that provide alternative routes for the generation of Factor Xa, and a final common pathway that leads to thrombin



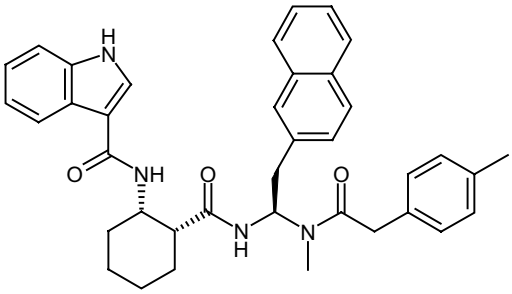
35
5-amidinobenzo[b]thiophene



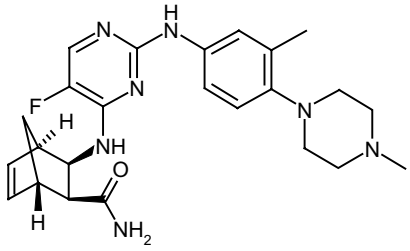
36[a]
Tilidine



37
RO-4383315



38
MEN-11149

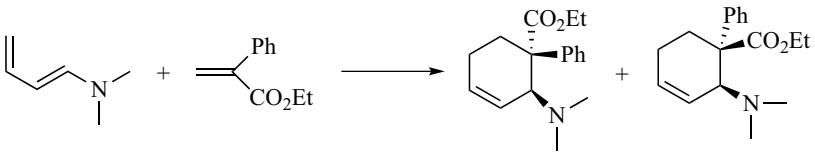


39
3-aminobicyclo[2.2.1]hept-5-ene-2-carboxamide

Scheme 5.3 [a]Racemic.

formation. Factor IXa in the presence of cofactor Factor VIIIa and Ca^{2+} activates Factor X to Factor Xa on the surface of platelets or endothelial cells via the intrinsic pathway. Factor IXa seems to be essential for the amplification of coagulation, as indicated by the high bleeding tendency of hemophilia B patients (Factor IX deficiency). Inhibition of Factor IXa may provide "upstream" inhibition of intrinsic coagulation and thrombus propagation, while leaving hemostasis intact via the Factor VIIa/tissue factor extrinsic pathway. *In vivo* studies with active-site blocked Factor IXa or anti-Factor IXa antibodies have demonstrated that good efficacy in animal models of arterial and venous thrombosis can be achieved via this mechanism.

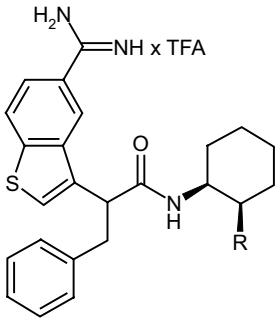
One of the compounds (**35**) exhibited K_i values of 0.14 and 0.12 μM for Factor IXa and Factor Xa, respectively. Modifications at the methyl ester group of **35** did not



40

Scheme 5.4 Synthesis of tilidine **36**.

Table 5.4 Structure–activity relationship at the methyl ester group of **35**.

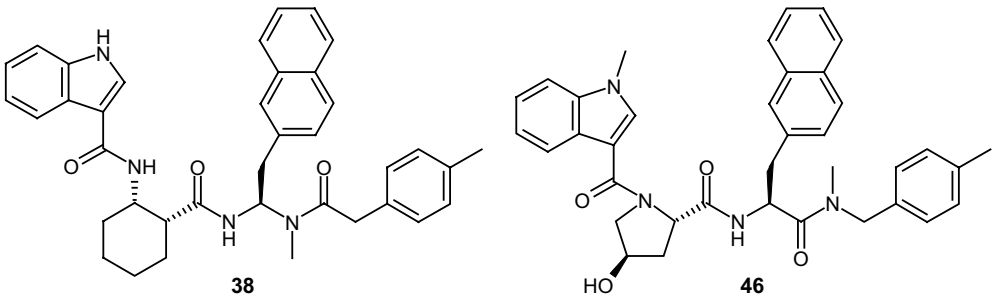


Compound	R[a)]	Factor IXa K_i (μM)	Factor Xa K_i (μM)	Factor IXa/ Factor Xa
35	COOMe	0.14	0.12	1.2
41	COOEt	0.12	0.18	0.7
42	CONHEt	0.43	0.13	3.3
43	$CONHCH_2CONH_2$	0.28	0.065	4.3
44	$CONHCH_2Ph$	>32	>9	—
45	COOH	3.50	0.20	18

a) Stereochemistry of two substituents on the cyclohexyl is *cis*-racemic.

improve Factor IXa potency or selectivity (Table 5.4). Substitution of the methyl ester by a larger group such as benzylamide or hydrolysis of the ester to the acid yielded compounds with decreased Factor IXa potency (**44**, **45**). However, smaller substituents such as the ethyl ester, the ethyl amide and the glycine amide were tolerated (**41**–**43**).

Menarini was investigating MEN-11149 (**38**) for the potential treatment of inflammatory and neurological diseases. MEN-11149 is the prototype of a new class of partially retro-inverse pseudopeptides related to FK888 (**46**) (Scheme 5.5). The main modification is the introduction of a β-amino cyclohexyl carboxylic acid residue, aimed at protection of the analog from enzymatic degradation by alteration of the



Scheme 5.5 Tachykinin NK1 receptor antagonists MEN-11149 and FK888.

peptide backbone, preserving the correct three-dimensional presentation of the aromatic rings of the FK888 molecule – a key requisite for its biological activity. MEN-11149 shows a potent, selective and specific antagonism of human tachykinin NK1 receptors, with an activity in the nanomolar range ($pK_i = 8.5 \pm 0.1$). This antagonism is insurmountable, and its *in vitro* and *in vivo* inhibitory effects are long-lasting and with a very slow rate of disappearance [16]. Further, MEN-11149 is a potent antagonist of tachykinin NK1-mediated motor and inflammatory responses in guinea-pig airways, and this action is evident and prolonged even after oral administration.

Roche was investigating RO-4383315 (**37**), a *N*-methylindole-2-carboxyl-capped dipeptide nitrile cathepsin K inhibitor, for the potential treatment of osteoporosis. RO-4383315 inhibits human cathepsin K with an IC_{50} of 1.8 nM and has a selectivity profile over other closely related cathepsins S, L, and B of 143-, 299-, and 253-fold, respectively [17]. In addition, the compound was evaluated in nonovariectomized monkeys, and showed a promising pharmacokinetic profile with a dose-dependent inhibition of bone resorption between 5 and 20 mg/kg. Thus, the compound was submitted for preclinical evaluation.

Researchers at Rigel Pharmaceuticals filed a patent on 3-aminobicyclo[2.2.1]hept-5-ene-2-carboxamide derivatives as Aurora kinase inhibitors. Compound **39** is the only structure that was specifically claimed and showed significant reduction in tumor growth rate when tested against A549 or Colo205 tumors in a standard xenograft therapeutic model in severe combined immunodeficiency (SCID) mice, and when tested against Colo205 and MiaPaCa tumors in a standard xenograft regression model in SCID mice [18].

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6
Medicinal Chemistry of α-Hydroxy-β-Amino Acids

Zyta Ziora, Mariusz Skwarczynski, and Yoshiaki Kiso

6.1
Introduction

α-Hydroxy-β-amino acids are an important subclass of β-amino acids that have received considerable attention in medicinal chemistry due to the number of applications as building blocks in peptidomimetics. They are substrates for the synthesis of a wide variety of peptide isosteres and they are also constituents of several natural products exhibiting potent biological activity. α-Hydroxy-β-amino acids are similar to α-amino acids in that they contain an amino-terminus and a carboxyl-terminus, but these functional termini are separated by two carbon atoms – C2 (α) and C3 (β), respectively. The C2-α-hydroxy substitution existing as the *R* or *S* isomer and with other than β-amino substitution at C3 existing as the *R* or *S* as well. The total number of possible isomers is therefore 4 (Figure 6.1). From the stereochemical point of view, α-hydroxy-β-amino acids provide enormous scope for molecular design, thanks to their flexibility allowing the generation of a range of stereo- and regioisomers. Many approaches to the synthesis of these compounds have been published, resulting in a great diversity of α-hydroxy-β-amino acids that can be utilized.

This chapter reviews the design and synthesis of α-hydroxy-β-amino acids themselves or as bioisosteres, the cores of peptidase and protease inhibitors, or as biologically active antibiotics, or antitumor agents, with a view on their application in medicinal chemistry.

6.2
α-Hydroxy-β-Amino Acids

6.2.1
α-Hydroxy-β-Amino Acids Occurring in Natural Products

Natural products, isolated from microorganisms, plants, or animals, quite often serve as lead molecules whose activity can be enhanced by manipulation through

Amino Acids, Peptides and Proteins in Organic Chemistry.
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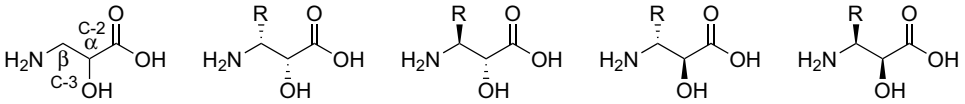


Figure 6.1 Structure of α-hydroxy-β-amino acids and the side-chain stereochemistry of four possible isomers.

combinatorial and synthetic chemistry. Figure 6.2(a) presents naturally occurring α-hydroxy-β-amino acids contained in bioactive compounds. Full structures of those biologically active molecules, isolated from various bacterial strains or plants, are disclosed in the following with data of their potential activities [1, 2]. There are also a

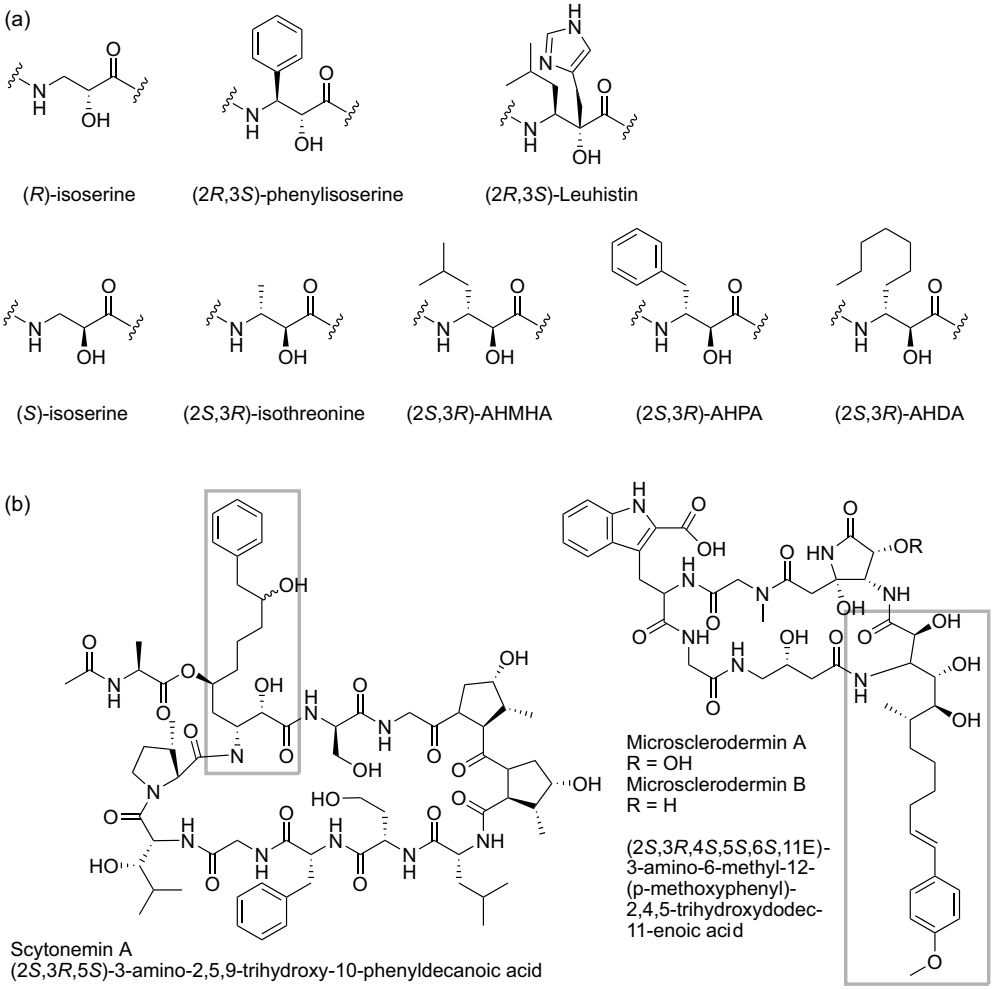


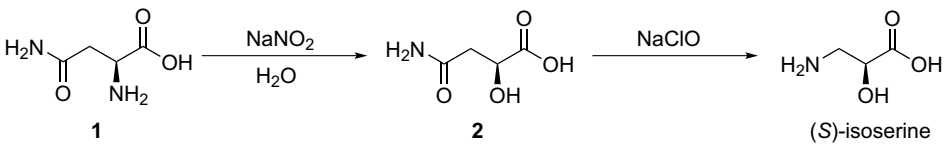
Figure 6.2 (a) Naturally occurring α-hydroxy-β-amino acids contained in bioactive compounds. (b) Cyclic peptides from marine organisms with a novel α-hydroxy-β-amino acid.

few examples of natural compounds isolated from the kingdom of marine organisms, but their application in medicinal chemistry has been not explored yet: scytonemin A, a cyclic peptide with (2*S*,3*R*,5*S*)-3-amino-2,5,9-trihydroxy-10-phenyldecanoic acid, possessing potent calcium antagonistic properties from the blue-green sponge alga *Scytonema* sp., and microsclerodermins, cyclic hexapeptides containing (2*S*,3*R*,4*S*,5*S*,6*S*,11*E*)-3-amino-6-methyl-12-(*p*-methoxyphenyl)-2,4,5-trihydroxydodec-11-enoic acid, from *Miscroscleroderma*, a deep water sponge (Figure 6.2b) [3].

6.2.2
Synthesis of α-Hydroxy-β-Amino Acids

6.2.2.1 Isoserine

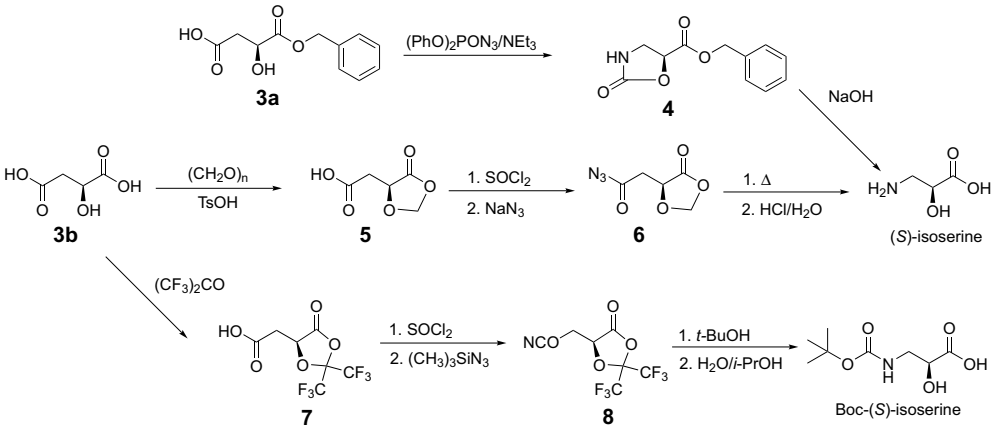
The first synthesis of isoserine was reported by Miyazawa *et al.* [4]. They started from L-asparagine (**1**). Reaction with $NaNO_2/H_2O$ resulted in the (*S*)-β-malamidic acid (**2**), which they then converted into (*S*)-isoserine via a Hoffman rearrangement (Scheme 6.1). This procedure was further modified by Andruszkiewicz *et al.* [5]. The most popular strategy for the synthesis of (*S*)- and (*R*)-isoserine is the application of (*S*)- and (*R*)-malic acids or their derivatives. Maeda *et al.* esterified (*S*)-malic acid to get 1-benzyl ester (**3a**) at first, and then they converted it into the isocyanate by adding diphenyl phosphorazidate (DPPA) and triethylamine. The isocyanate underwent internal cyclization to the benzyl (*S*)-2-oxooxazolidine-5-carboxylate (**4**). Hydrolysis with sodium hydroxide afforded (*S*)-isoserine [6]. Milewska and Polonski transformed (*S*)-malic acid (**3b**) into 4-oxo-1,3-dioxolan-5-acetic acid (**5**) by acid-catalyzed reaction with paraformaldehyde, which enabled protection of the carboxy and hydroxy groups simultaneously. Then, a primary amine was generated from the β-carboxy group by the Curtius procedure via azide **6** and isocyanate [7]. Burger *et al.* applied hexafluoroacetone as a protecting and activating reagent in the reaction with (*S*)-malic acid (**3b**) [8]. To obtain the isocyanate **8** they treated the acid chloride of **7** with trimethylsilyl azide. To provide Boc (*tert*-butoxycarbonyl) or Cbz (benzyloxycarbonyl) protection of the amine group, they utilized *tert*-butyl or benzyl alcohol before deprotection of carboxyl and hydroxyl moieties, finally affording protected (*S*)-isoserine (Scheme 6.2).



1 **2** (*S*)-isoserine

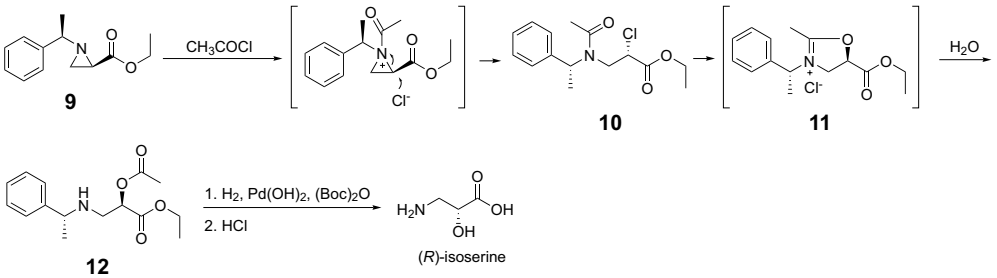
Scheme 6.1

There are other interesting approaches to the synthesis of both isoserine enantiomers described by Andruszkiewicz in his review: in the multistep procedure described by Dureault *et al.*, D-mannitol was employed as a starting material [9]; Pons *et al.* used Sharpless asymmetric epoxidation of allyl alcohol [10]; Lu *et al.* presented a total synthesis through kinetic resolution of aminoalcohol diacetates in



Scheme 6.2

organic solvents using *Candida cylindracea* lipases (CCL) [11]; Nozaki *et al*. synthesized L- and D-isoserine enantioselectively via asymmetric hydrogenation of 3-amino-2-oxopropanoate catalyzed by [RuCl(BINAP)benzene]Cl; [12] and many others [2]. Recently, another strategy has been described – the ring-opening of 2-acylaziridines (**9**) by acid chlorides was applied for a (*R*)-isoserine synthesis (Scheme 6.3). This type of reaction is based on the dual role of an acid chloride as an activator of the aziridine ring and a provider of the nucleophile for the aziridine ring-opening, and results in acyclic β-amino-α-chlorocarbonyl product **10**. Intermediate **10** is readily cyclized due to internal oxygen attack and replacement of the chloride (**11**). Compound **11**, in the presence of a small amount of water or moisture in the air, hydrolyzes to yield **12**. Hydrogenation and the subsequent hydrolysis provided (+)-(*R*)-isoserine [13].



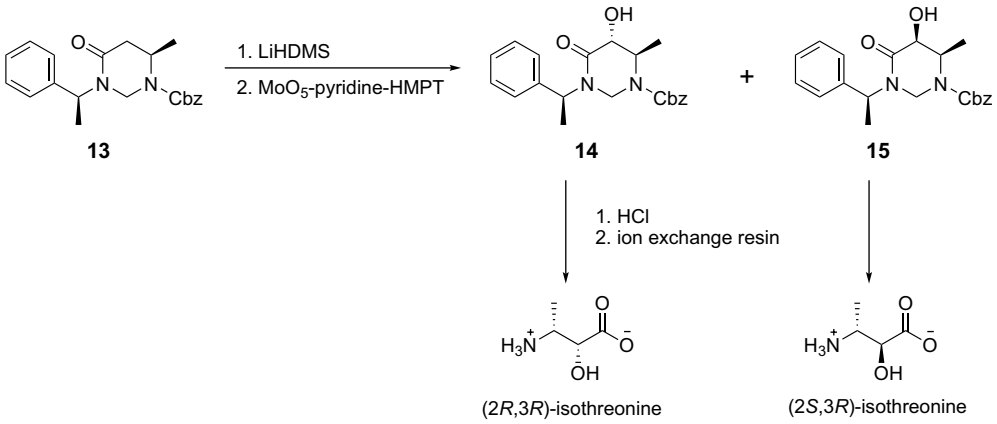
Scheme 6.3

α-Substituted α-hydroxy-β-amino acids are attractive constructs since the presence of an alkyl substituent at the α-position favors folded conformations in the β-peptides [14, 15]. A simple modification of the method in Scheme 6.1 provides a diastereoselective four-step method for the synthesis of (*R*)- and (*S*)-α-alkyl isoserines starting from D- and L-malic acid. This method featured stereocontrolled alkylation of dioxolanone acids through a methylcarbamate via a Curtius

rearrangement [16]. The groups of Avenoza and Peregrina achieved similar derivatives by applying another convenient enantioselective synthesis. By introducing a catalytic Sharpless asymmetric dihydroxylation reaction and using the five-membered ring of sulfates or sulfamidates, they obtained (*R*)- and (*S*)-α-methyl isoserine and (*R*)- and (*S*)-α-trifluoromethyl isoserine [17–20].

6.2.2.2 **Isothreonine**

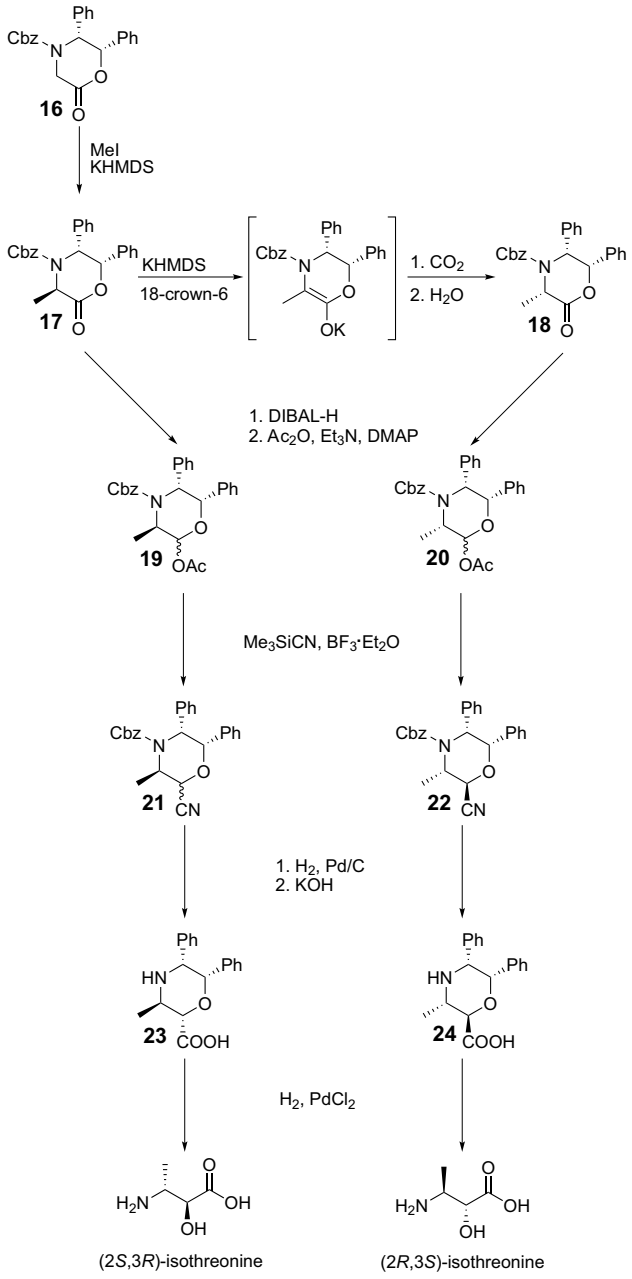
Cardillo *et al*. described the synthesis of enantiomerically pure *syn*- and *anti*-α-hydroxy-β-amino acids through hydroxylation of enolates of perhydropyrimidin-4-ones (Scheme 6.4) [21]. Compound **13** was treated with lithium hexamethyldisilyl azide (LiHMDS) and then the complex MoO_5/pyridine/hexamethylphosphoramide (HMPT). After separation of diastereomers, **14** and **15** were hydrolyzed and purified on a cation-exchange resin to give the corresponding D-*allo*-isothreonine and L-isothreonine.



Scheme 6.4

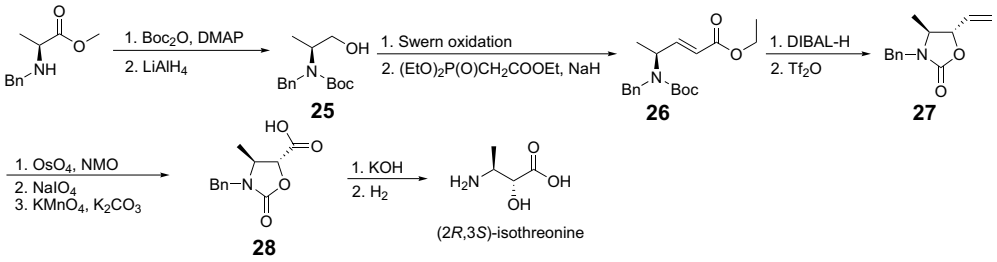
The Williams group utilized commercially available glycine templates as precursors for the synthesis of amino acids and peptide isosteres. They started from oxazinone **16**, which was alkylated at C3 with iodomethane in the presence of potassium hexamethyldisilyl amide (KHMDS) to afford *anti*-3-methyl-oxazinone **17** (Scheme 6.5) [22, 23]. Treatment of **17** with KHMDS followed by addition of dry, gaseous CO_2 and then quenching with water led to the *syn* isomer **18**. *Syn*- and *anti*-3-methyl-oxazinones (**17** and **18**) were converted into the corresponding acetoxy hemiacetals **19** and **20** by treatment with diisobutylaluminum hydride (DIBAL-H) followed by acetylation of the intermediate lactol with acetic anhydride. Reaction of **19** and **20** with trimethylsilylcyanide in the presence of $BF_3 \cdot Et_2O$ proceeded in the case of product **22** with high diastereoselectivity.

Hydrogenolysis of **21** and **22** followed by hydrolysis of the nitrile, and in the case of **23** yielding excellent diastereoselectivity of the carboxylic acid, provided (2*S*,3*R*)- and (2*R*,3*S*)-isothreonine. The same methodology was applied to synthesize


16
MeI
KHMDS
17
KHMDS
18-crown-6
1. CO2
2. H2O
18
1. DIBAL-H
2. Ac2O, Et3N, DMAP
19
20
Me3SiCN, BF3·Et2O
21
22
1. H2, Pd/C
2. KOH
23
24
H2, PdCl2
(2S,3R)-isothreonine
(2R,3S)-isothreonine

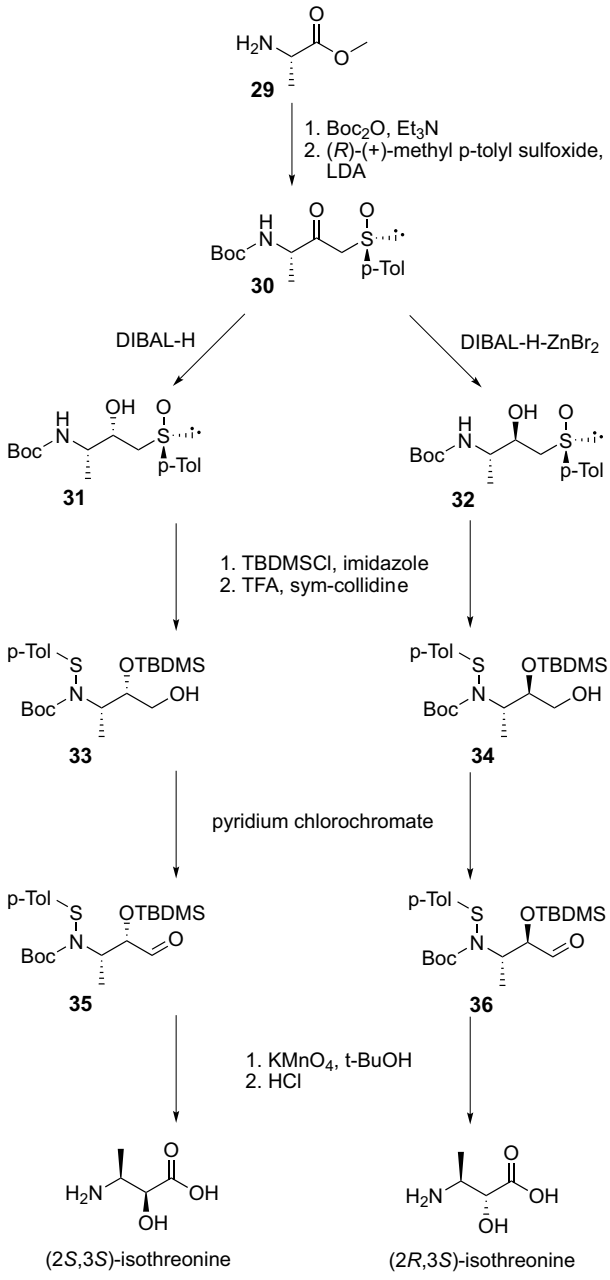
Scheme 6.5

norcyclohexylstatine (instead of β-CH_3 it was β-$CH_2C_6H_{11}$) [24]. Application of L-amino acids, alanine or valine, for the synthesis of the corresponding isothreonine or its derivative was recently reported. This methodology was based on the conversion of α-amino acids into oxazolidin-2-ones through cyclization of (4-hydroxybutenyl) carbamates [25]. The synthesis began from chemoselective reduction of *N*-Bn-alanine methyl ester with lithium aluminum hydride ($LiAlH_4$) resulting in alcohol **25**. Then **25** was transformed into the aldehyde and underwent an *E*-selective Horner–Wadsworth–Emmons reaction to afford the allylic product **26** (Scheme 6.6). Another chemoselective reduction with DIBAL-H generated the allylic alcohol, which was the precursor for the oxazolidinone ring of **27**, obtained after triflation/cyclization. Dihydroxylation of the vinyl substituent on **27** was accomplished with osmium tetroxide followed by cleavage in the presence of periodate and subsequent oxidation with potassium permanganate to yield the acid **28**. The oxazolidinone ring of **28** was easily opened (reflux in aqueous potassium hydroxide) and the (2*R*,3*S*)-product was obtained after deprotection of the amino group. The same group of researchers reported the synthesis of (2*R*,3*S*)-isothreonine and (2*R*,3*S*)-2-hydroxy-3-amino isopentanoic acids by a similar approach [26]. They also applied D-gulonic acid γ-lactone and D-glucono-δ-lactone for the synthesis of all four stereoisomers of isothreonine via a series of selective transformations [27].



Scheme 6.6

Alanine as a common precursor of isothreonine is quite often considered as the starting compound in the synthesis of α-amino-β-amino acids. Another interesting enantioselective synthesis of isothreonine mediated by sulfoxides is presented in Scheme 6.7. The methyl ester of alanine hydrochloride (**29**) reacts with di-*tert*-butyl dicarbonate resulting in the carbamate, and then it was transformed into the ketosulfoxide **30** by reaction performed with (*R*)-(+)-methyl *p*-tolyl sulfoxide and lithium diisopropylamide [28]. Compound **30** was reduced with DIBAL-H or DIBAL-H/$ZnBr_2$ to give diastereoisomerically pure alcohols **31** or **32**, respectively; then they were converted to the corresponding *tert*-butyldimethylsilyl ethers, and after reaction with TFA and *sym*-collidine, sulfenamides **33** and **34** were isolated. Aldehydes formed from **33** and **34** with pyridinium chlorochromate were oxidized to the corresponding acids **35** and **36** using $KMnO_4$ in the presence of *tert*-butyl alcohol. The desired (2*S*,3*S*)- and (2*R*,3*S*)-isothreonines were generated after deprotection of the amine group. The same strategy was applied to synthesize norstatines (Nsts) [29].


29
1. Boc2O, Et3N
2. (R)-(+)-methyl p-tolyl sulfoxide, LDA
30
DIBAL-H
DIBAL-H-ZnBr2
31
32
1. TBDMSCl, imidazole
2. TFA, sym-collidine
33
34
pyridium chlorochromate
35
36
1. KMnO4, t-BuOH
2. HCl
(2S,3S)-isothreonine
(2R,3S)-isothreonine

Scheme 6.7

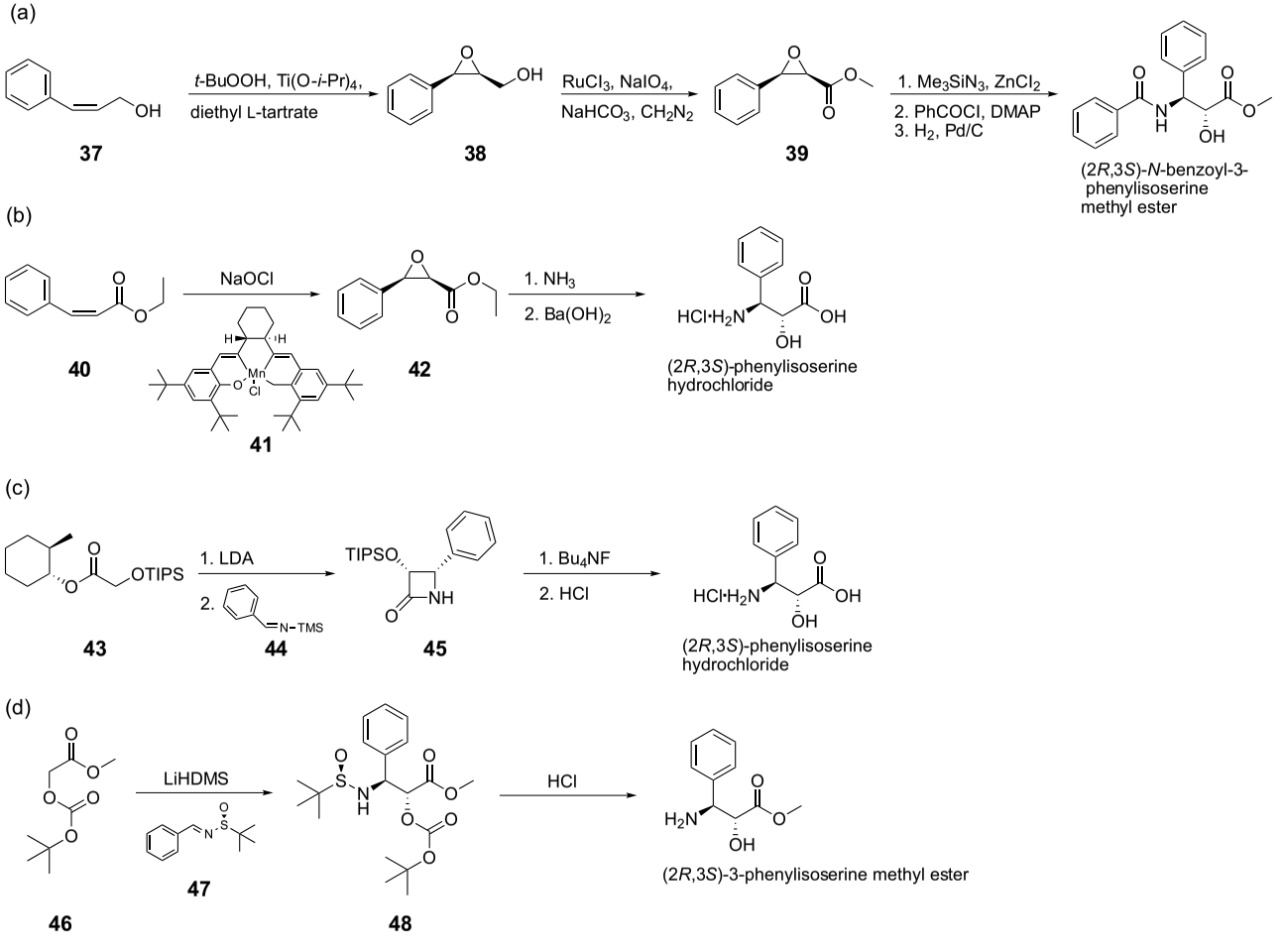
6.2.2.3 **Phenylisoserine**

Synthesis of (2*R*,3*S*)-phenylisoserine has been extensively studied and several synthetic routes have been reported. Synthesis of a mixture of unnatural phenylisoserine diastereoisomers was initially achieved by Umezawa *et al.* [30]; however, the first enantioselective synthesis of (2*R*,3S)-phenylisoserine was reported by Denis *et al.* [31]. In this method, *cis*-cinnamyl alcohol **37** was converted to epoxide **38** via Sharpless asymmetric epoxidation [32, 33], followed by alcohol oxidation and esterification prior to the epoxide cleavage (Scheme 6.8a). Regioselective ring-opening of **39** was achieved by treatment with azidotrimethylsilane. (2*R*,3*S*)-*N*-Benzoyl-3-phenylisoserine methyl ester was obtained by subsequent benzoylation, reduction of azide and spontaneous $O \rightarrow N$ acyl migration of the benzoyl group. Another interesting approach has been proposed by Deng and Jacobsen [34]. Epoxidation of *cis*-ethyl cinnamate **40** in the presence of catalyst **41** afforded the corresponding *cis*-epoxide **42** (Scheme 6.8b). After epoxide ring-opening with ammonia and subsequent hydrolysis of the amide, the target (2*R*,3*S*)-phenylisoserine was achieved. Also, an epoxide ring-opening reaction was applied by Gou *et al.* for the synthesis of this α-hydroxyl-β-amino acid; however, the starting chiral epoxide was obtained by enantioselective transesterification by lipases [35]. Other methods that include oxirane ring regio- and stereoselective opening have also been reported [36–38].

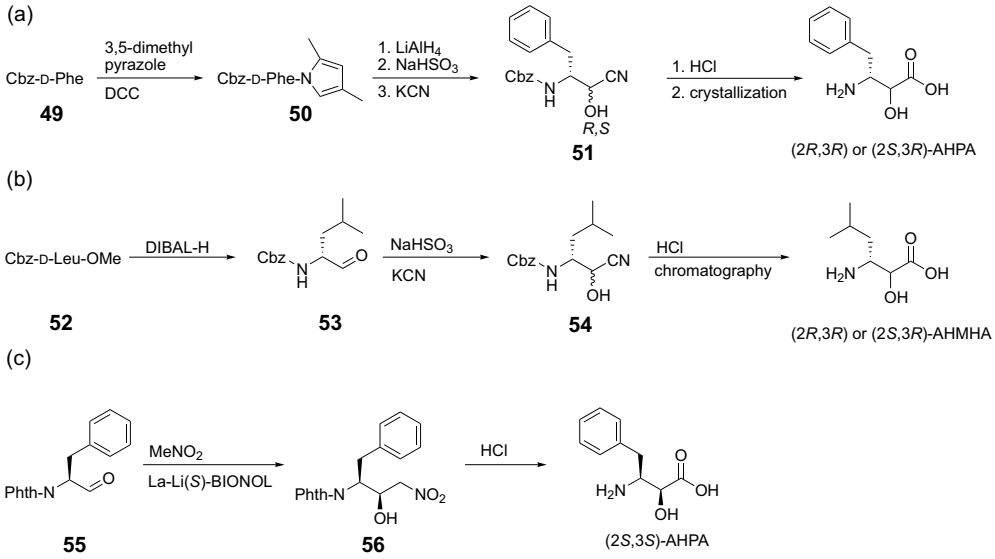
The β-lactam synthon method, known also as the Ojima–Holton β-lactam coupling method, is one the most popular and has been used for the industrial semisynthesis of paclitaxel (see also Section 6.6). Starting from sililoxyacetate **43**, bearing (–)-*trans*-2-phenyl-1-cyclohexanol as a chiral auxiliary, and *N*-(*p*-methoxyphenyl)aldimine **44**, stereoselective synthesis of β-lactam **45** was achieved. This compound was then hydrolyzed to (2*R*,3*S*)-phenylisoserine (Scheme 6.8c) [39, 40]. This method can be easily modified to afford other α-hydroxyl-β-amino acids [41]. In a similar manner, Bourzat and Commercon utilized the β-lactam method with (*S*)-(α-methylbenzyl) amine as a chiral auxiliary [42]. More recently, optically pure (2*R*,3*S*)-phenylisoserine was synthesized through a lithium enolate addition of *O*-Boc-α-hydroxyacetate benzyl ester **46** to benzylidene (S_R)-*tert*-butanesulfinamide **47** (Scheme 6.8d) [43]. Another method includes reaction of a sulfur ylide with *trans*-β-nitrostyrene. The resulting *anti*-5-benzoyl-4-phenyloxazolidin-2-one can be easily converted to phenylisoserine or its analogs, although not to its optically active forms [44]. Several other methods have been examined for the synthesis of this α-hydroxyl-β-amino acid, including synthesis of all unnatural isomers [45–54]. Finally, it is worth mention that nowadays (*2R,3S*)-phenylisoserine is widely commercially available.

6.2.2.4 **Norstatines**

Nst, known also as 3-amino-2-hydroxymethylhexanoic acid (AHMHA), and its derivative phenylnorstatine (Pns; 3-amino-2-hydroxyphenylbutanoic acid (AHPA), have been synthesized using several approaches. Quite often the starting materials were natural amino acids. AHPA was synthesized first by the Takita and Umezawa group applying D-phenylalanine. *N*-Cbz-D-phenylalanine (**49**, Scheme 6.9a) was coupled to 3,5-dimethylpyrazole with dicyclohexylcarbodiimide (DCC). The product


(a)
t-BuOOH, Ti(O-i-Pr)4,
diethyl L-tartrate
RuCl3, NaIO4,
NaHCO3, CH2N2
1. Me3SiN3, ZnCl2
2. PhCOCl, DMAP
3. H2, Pd/C
37
38
39
(2R,3S)-N-benzoyl-3-phenylisoserine methyl ester
(b)
NaOCl
1. NH3
2. Ba(OH)2
40
41
42
(2R,3S)-phenylisoserine hydrochloride
(c)
1. LDA
2.
TIPSO
1. Bu4NF
2. HCl
43
44
45
(2R,3S)-phenylisoserine hydrochloride
(d)
LiHDMS
HCl
46
47
48
(2R,3S)-3-phenylisoserine methyl ester

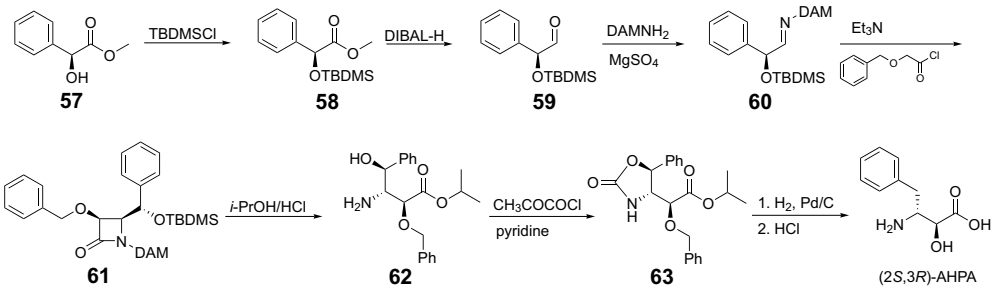
Scheme 6.8



Scheme 6.9

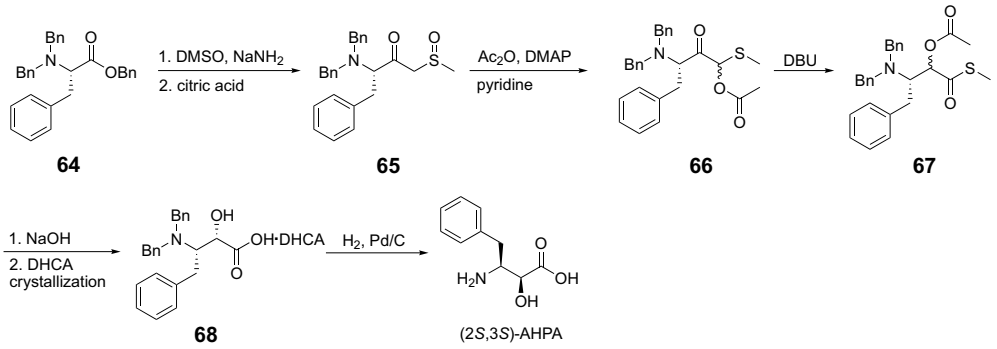
50 of coupling was reduced with lithium aluminum hydride, then it was treated with aqueous sodium hydrogen sulfite and then transformed to the cyanohydrin **51** by reaction with potassium cyanide. Compound **51** was hydrolyzed with HCl yielding a mixture of (2*R*,3*R*)- and (2*S*,3*R*)-AHPA. Resolution of the diastereoisomeric mixture of AHPA was achieved by fractional crystallization. A mixture of (2*R*,3*S*)- and (2*S*,3*S*)-AHPA was prepared from L-phenylalanine. Starting from D-alanine, D-leucine, and D-phenylglycine, other analogs of AHPA were prepared in the same way [30]. Researchers also synthesized a *p*-hydroxyphenyl analog of AHPA (3-amino-2-hydroxy-4-*p*-hydroxyphenylbutanoic acid) applying a similar procedure with D-tyrosine as a precursor [55]. Rich *et al.* modified the initial step of the previously presented method by converting the methyl ester of Cbz-D-leucine (**52**, Scheme 6.9b) to Cbz-D-leucinal (**53**). The mixture of diastereomers (2*R*,3*R*)- and (2*S*,3*R*)-AHMHA was readily separated by chromatography over silica gel [56]. Johnson followed a similar method to synthesize (2*R*,3*S*)- and (2*S*,3*S*)-AHMHA. He prepared their dicyclohexylammonium salts and separated the diastereomers by precipitation [57]. Sasai *et al.* utilized L-phenylalanine to obtain the nitroaldol adduct (**56**, Scheme 6.9c) from freshly prepared (*S*)-3-phenyl-2-phthaloylamino-1-propanal (**55**). Nitroaldol **56** was formed from the catalytic reaction of aldehyde **55** with nitromethane in the presence of La–Li(*S*)–BINOL (1,1′-bi-2-naphthol) complex as catalyst. Hydrolysis gave one enantiomer, (2*S*,3*S*)-AHPA [58]. Herranz *et al.* described a one-pot procedure for the stereoselective synthesis of (2*R*,3*R*)- and (2*S*,3*R*)-AHPA starting also from a protected amino aldehyde and after formation of cyanohydrins the mixture of isomers was created, which was then transformed into 2-hydroxyester. The esters were separated by flash chromatography and finally saponified to furnish both *N*-protected esters [59].

The Park group optimized a route to yield enantiopure AHPA from phenylalanine and subsequent manipulation of oxazolidin-2-ones [60]. Aspartic acid was another amino acid applied to the synthesis of AHPA and other 4-alkyl-3-amino-2-hydroxybutyric acids. Jefford *et al.* described the synthesis of a series of Nst derivatives with the (2*S*,3*R*) configuration based on diastereoselective hydroxylation of 3*S*-(*N*-tosylamino)butyrolactone as a key step. Lactone ring-opening proceeded by treatment with trimethylsilyliodide and gave homoisoserine, which was then alkylated with lithium organocuprates [61]. Seki and Nakao reported a method involving highly stereoselective hydroxylation of an oxazoline-4-acetate to synthesize (2*S*,3*R*)-AHPA [62]. There are other than natural amino acid precursors of Nsts. Kobayashi *et al.* presented the application of methyl (*S*)-mandelate (**57**, Scheme 6.10). Protection of an alcohol group as *tert*-butyldimethylsilyl (TBDMS) ether and then reduction of the protected ester **58** with DIBAL-H generated aldehyde **59**, which was condensed with di-*p*-anisylmethylamine ($DAMNH_2$) in the presence of $MgSO_4$ to give chiral imine **60**. The [2 + 2] cycloaddition reaction of **60** with benzyloxyketene, generated *in situ* from benzyloxyacetyl chloride in the presence of Et_3N, provided azetidinone **61**. Alcoholysis with acidic isopropanol and removal of the TBDMS and DAM protecting groups afforded ester **62**, which was converted to oxazolidone **63**. Subsequent hydrogenolysis and hydrolysis gave (2*S*,3*R*)-AHPA [63].



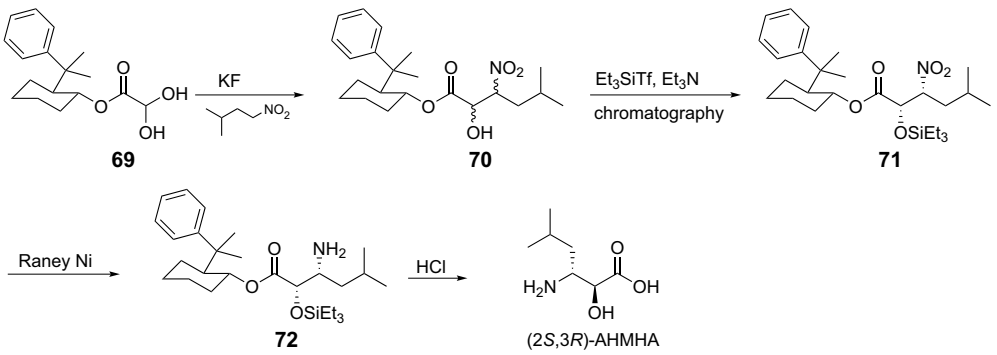
Scheme 6.10

Palomo *et al.* also used a [2 + 2] cycloaddition reaction of an imine with a ketene for a β-lactam formation as a key step in the synthesis of (2*S*,3*R*)-AHPA [64]. (2*S*,3*R*)-AHPA and (2*S*,3*R*)-AHMHA were synthesized from 2-olefinic acids via regiospecific ring-opening of 2,3-epoxy acids by ammonia [65]. Acidic epoxides were also utilized in the one-pot copper-catalyzed synthesis of Nsts [66]. Recently, Sleebs and Hughes described the synthesis of Nst by utilizing 4-substituted-1,3-oxazinan-6-ones [67]. There are quite a few more routes optimized for the synthesis of Nsts, utilizing various precursors and rearrangements. 1,2-Asymmetric induction of iodocyclocarbamation in acyclic allylamines was applied to the synthesis (2*S*,3*R*)-AHPA [68]. Another synthesis of (2*S*,3*R*)-AHPA was initiated from a chiral aziridine obtained by enzymatic transesterification of the corresponding *meso*-diacetate [69]. (2*S*,3*S*)-AHPA was synthesized in a tandem addition–hydroxylation reaction, starting from phenylacetaldehyde and a phosphorane, and then with lithium amide derived from



Scheme 6.11

(*S*)-(α-methylbenzyl)benzylamine and (+)-(camphorsulfonyl)-oxaziridine [70]. All four stereoisomers of AHPA were also obtained from acetals treated with trimethylsilyl cyanide in the presence of boron trifluoride etherate, and then oxidized with pyridinum chlorochromate (PCC) [71]. Norman and Morris have shown that diethyl (*S*)-malate could be a useful intermediate for the synthesis of (2*S*,3*R*)-AHPA through an alkylation with benzyl bromide and LiHMDS [72]. Pummerer rearrangement of ketosulfoxides was involved in an efficient synthetic method beginning from L-phenylalanine [73]. The β-letosulfide (**65**, Scheme 6.11) was prepared by the conventional nucleophilic addition of a dimsyl anion to *N*,*N*-Cbz-L-phenylalanine benzyl ester (**64**). In a one-pot rearrangement/acetylation reaction **65** was easily converted to **66** and treatment of this with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) gave thioester **67**. The authors claimed that the conversion of **66** into **67** occurred with acyl migration. They proposed a possible mechanism where the α-acetoxy sulfide migrated in the presence of DBU, stereoselectively forming the (2*S*,3*S*)-configuration in producing the hydroxyl thioester. Hydrolysis with sodium hydroxide and crystallization of the dicyclohexylamine (DCHA) salt produced pure (2*S*,3*S*)-AHPA. Solladie-Cavallo and Khiar utilized a condensation of 1-nitro-3-methylbutane on (–)-8-phenylmenthol glyoxylate hydrate (**69**, Scheme 6.12) using KF as a mild base [74].

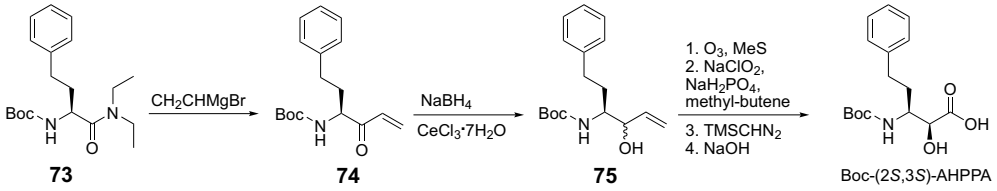


Scheme 6.12

Condensation product **70** was a mixture of mainly two diastereomers. After protection of the alcohol group performed with triethylsilyl triflate in the presence of Et_3N, the resulting mixture of diastereomers was separated by flash chromatography on silica gel to get only one isomer **71**. Reduction with Raney nickel, then deprotection and hydrolysis with HCl, finally afforded (2*S*,3*R*)-AHMHA.

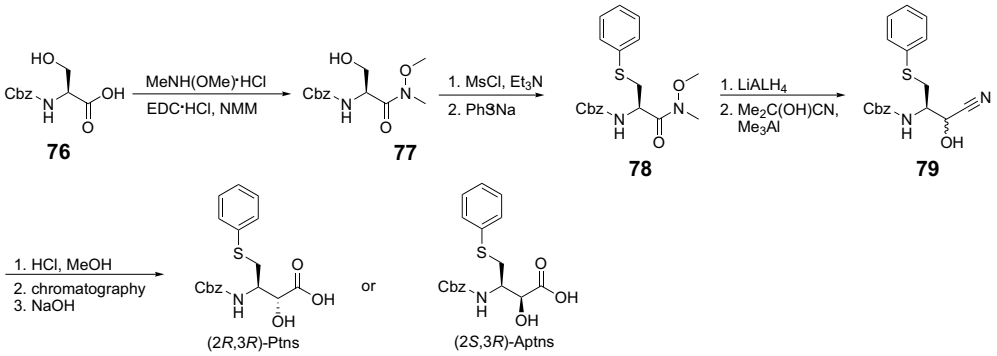
AHMHA has its unnatural counterpart, isonorstatine, (2*R*,3*S*,4*S*)-3-amino-2-hydroxy-4-methylhexanoic acid, which was synthesized for the first time by the Kiso group. The method was based on a cyanide addition to leucinal, easily prepared from (2*S*,3*S*)-isoleucine, and then separation of the diastereomeric product mixture [75]. Recently, Li *et al*. have synthesized isonorstatine starting from L-tartaric acid, which was converted to a threose imine and then introduced to a highly stereoselective addition of 1-butene-3-yl [76].

Homophenylalanine was applied in the synthesis of a non-naturally occurring Nst derivative. *N*,*N*-Diethyl-amide **73** was converted into the corresponding vinyl ketone **74** and then reduced to give a mixture of diastereomers **75** (Scheme 6.13). Ozonolysis of **75** followed by reduction with methylsulfide yielded an aldehyde, which was oxidized to give an acid. To afford diastereomerically pure *N*-Boc-(2*S*,3*S*)-3-amino-2-hydroxy-5-phenylpentanoic acid (AHPPA), the acid was first converted to its methyl ester and the diastereomers were separated by preparative thin-layer chromatogrpahy (TLC), and then hydrolyzed under alkaline conditions [77].



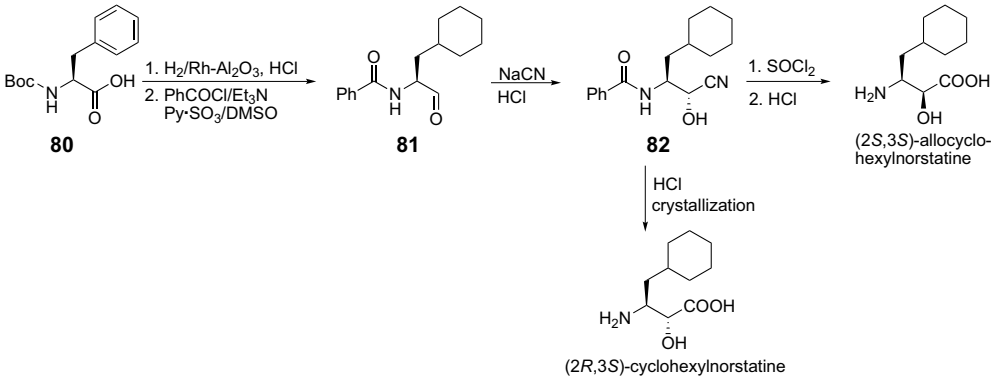
Scheme 6.13

Another unnatural derivative of Nst was synthesized from L-serine. The carboxylic group of *N*-Cbz-L-serine (**76**, Scheme 6.14) was protected as the Weinreb amide **77**, and then it was transformed into its mesylate and treated with sodium thiophenolate to give phenylsulfide **78**. Reduction of **78** with lithium aluminum hydride resulted in aldehyde, which was converted to cyanohydrin **79** obtained by reaction with acetone cyanohydrin and trimethylaluminum. The methyl esters were generated from **79** by treatment with dry methanolic hydrogen chloride, and then they were separated by flash chromatography and saponified to provide corresponding acids: (2*R*,3*R*)-3-amino-2-hydroxy-4-(phenylthio)butyric acid (phenylthionorstatine (Ptns)) or the (2*S*,3*R*)-isomer (allophenylthionorstatine (Aptns)) [78].



Scheme 6.14

The last unnatural Nst derivatives synthesized by several researchers were cyclohexylnorstatine and allocyclohexylnorstatine. The initial amino acid used in one of the reported methods was L-phenylalanine. Kiso *et al*. converted Boc-L-phenylalaninol (**80**, Scheme 6.15) to *N*-benzoylcyclohexylalaninal (**81**) by hydrogenation, Boc deprotection, and benzoylation of the amino group followed by oxidation of alcohol. The aldehyde **81** was hydrocyanated with NaCN and HCl to obtain the nitrile derivative **82**. Hydrolysis of the cyanohydrin provided (2*R*,3*S*)-cyclohexylnorstatine. Treatment of **82** with $SOCl_2$ finalized the oxazoline ring formation and then the oxazoline was hydrolyzed to pure (2*S*,3*S*)-*allo*-cyclohexylnorstatine [79]. Matsumoto *et al*. employed unnatural (2*S*,3*S*)-tartaric acid in the addition reaction of a Grignard reagent to an imine in the presence of cerium chloride. They started from 4-*O*-benzyl-2,3-isopropyliden-D-threose and the cyclohexylnorstatine was obtained through oxazolidinone formation [80]. Dugger *et al*. alkylated diisopropyl (*R*)-malate to finally gain pure cyclohexylnorstatine [81]. Ojima *et al*. synthesized Nst and its analogs using the β-lactam

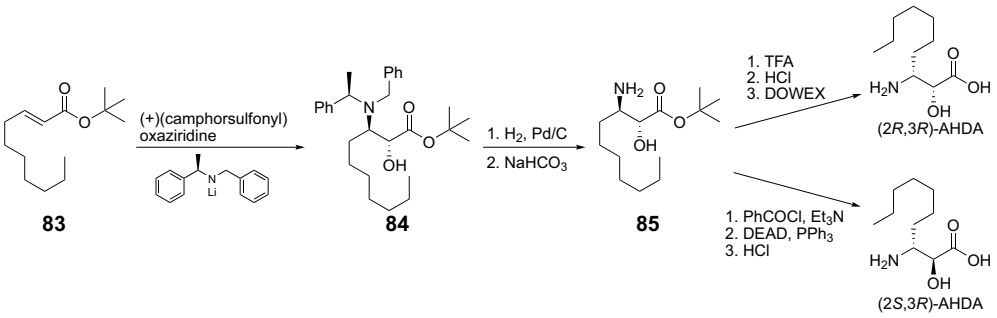


Scheme 6.15

method, via a 3-silyloxy-β-lactam easily available from chiral enolate–imine cyclocondensation [82]. [2 + 2]-Cycloaddition was again employed, this time to synthesize cyclohexylnorstatine [83]. Righi *et al.*, explored the application of metal halide-mediated regioselective opening of a *trans*-epoxide as a key reaction in the synthesis of cyclohexylnorstatine [36].

6.2.2.5 3-Amino-2-Hydroxydecanoic Acid and its Analogs

There are several approaches to the synthesis of 3-amino-2-hydroxydecanoic acid (AHDA). In most cases, functionalization of alkenes via either asymmetric epoxidation or asymmetric dihydroxylation is the common strategy. The first asymmetric synthesis of (2*S*,3*R*)- and (2*R*,3*R*)-AHDA was reported by Bunnage *et al.* [84]. Addition of lithium (*R*)-(α-methylbenzyl)benzylamide to (*E*)-*tert*-butyl-2-decenoate (**83**, Scheme 6.16), followed by *in situ* hydroxylation with (+)-(camphorsulfonyl) oxaziridine provided derivative **84**, which was debenzylated and hydrolyzed to give (2*R*,3*R*)-AHDA. A second isomer, (2*S*,3*R*)-AHDA, was obtained through the intramolecular cyclization of an amide under Mitsunobu conditions to give the *trans*-oxazoline with inversion of configuration, then hydrolysis gave the required AHDA [84].



83 **84** **85**

Scheme 6.16

L-Tartaric acid has been quite often employed in the synthesis of natural products. Shirode *et al.* have investigated the usefulness of the formylazetidin-2-one synthon, prepared from L-diethyl tartrate, in the synthesis of (2*R*,3*S*)- and (2*S*,3*R*)-AHDA isomers. They engaged the Wittig olefination reaction and then catalytic hydrogenation to form alkyl β-lactams [85]. D-Isoascorbic acid was also a precursor of AHDA [86]. Epoxide ring-opening by metal halide found application in another synthesis of AHDA [87].

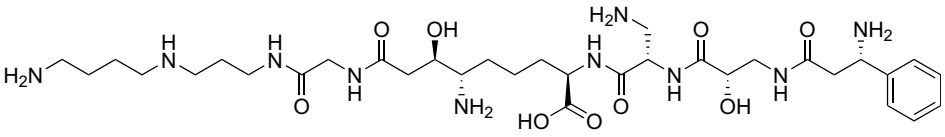
There are other known AHDA derivatives. The method optimized by Jefford *et al.* for the synthesis of a series of AHDA analogs starting from (*S*)-aspartic acid has already been mentioned [61]. Letgers *et al.* chose aliphatic oxirane-2-carboxylic acids for the synthesis of (2*R*,3*S*)- and (2*S*,3*R*)-isomers of alkyl-AHDA based on the conversion of epoxides into oxazoline-5-carboxylic esters [88].

6.2.2.6 Synthetic Demands

Optically active β-amino-α-hydroxy acids are well known as useful intermediates for various drugs and drug candidates. Ubenimex (bestatin) [73], an anticancer agent, launched in Japan, is a peptide mimetic composed of AHPA and L-leucine. The side-chain of paclitaxel (Taxol®) similarly consists of (2*R*,3*S*)-3-amino-2-hydroxy-3-phenyl propionic acid. The final stereochemical configuration of these residues is essential for their contribution in the bioactivity of the construct into which they are built. For example, the opposite relative configuration, such as (2*S*,3*S*)-AHPA, is used as the core building block of HIV-1 protease inhibitors and they were found to be very potent. Thus, there is a huge demand for the technologies producing those intermediates economically on an industrial scale. Regarding AHPA, one efficient synthetic approach is the transformation of L-phenylalanine to the corresponding *N*,*N*-dibenzylamino aldehyde followed by the diastereoselective nucleophilic addition of trimethylsilyl cyanide to produce cyanohydrins [89, 91]. This strategy has gained wide recognition and several different modifications have been reported [58, 66, 91–96]. Although some of those methods seemed to be promising, practical industrial processes still required more investigations. From the commercial manufacturing point of view, one-carbon homologation directly from amino acids or their esters has been of great interest.

6.3 Antibacterial Agents

α-Hydroxy-β-amino acids represent and important class of compounds being constituents of biologically active peptide antibiotics. For example, edeines (Figure 6.3), a broad spectrum of strongly basic pentapeptides produced by *Bacillus brevis* Vm_4, contain (*S*)-isoserine [1]. Edeines, a group of closely related antibiotic oligopeptides, are specific, reversible inhibitors of DNA synthesis in intact prokaryotic microbial cells and specific inhibitors of protein synthesis in isolated ribosomes of prokaryotic and eukaryotic origin; they have been applied as biochemical reagents in bacterial genetic investigations; and have been found to be useful tools for studying various aspects of ribosome function and protein synthesis. Edeines, similar to a series of peptides and derivatives of α,β-diaminopropionic acid [97, 98], undergo reversible



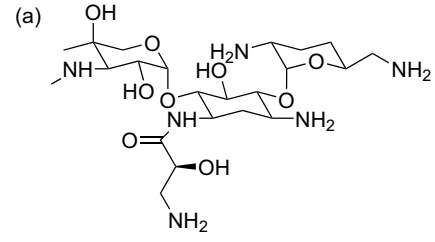
antimicrobial activity of synthetic edeine D (IC_{50}; μg/mL)

Saccharomyces cerevisiae ATCC976	12
Escherichia coli K-12	36
Bacillus subtilis BS-1060	41
Serratia marcescens ATCC13880	3

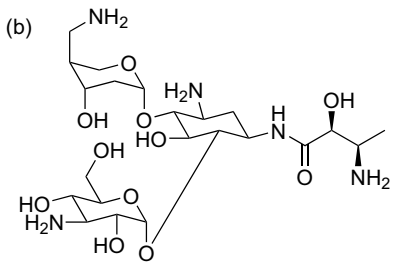
Figure 6.3 Structure of edeine D containing (*S*)-isoserine.

intramolecular aminolysis in water solutions [99]. Such a reaction occurs during isolation and storage of antibiotics. Thus, each of the edeines exists as a mixture of two isomeric peptides differing from one another by the mode of linkage of the α,β-diaminopropionic acid functional groups. The compounds in which isoserine is linked with an α-amino group (α-isomer) were described as active, while β-isomers have been shown to be biologically inactive. To prove this theory and to demonstrate which isomer is the active one, as well as to confirm the postulated structure, both edeine D isomers were synthesized. It was necessary to find out whether isomerization of the synthetic peptides occurred during the deprotection and isolation process. Dansylation of edeine D isomers followed by acid hydrolysis and TLC separation of the dansyl derivatives of α,β-diaminopropionic acid showed that the isomers were not contaminated by one another [100]. The biological activity of synthetic isomers of edeine D tested on model prokaryotic and eukaryotic organisms revealed that only the α-isomer was active. Physicochemical characteristics of synthetic edeine D confirmed the structural analysis of the natural one [1]. Analogs of natural edeines were also synthesized [101–103].

(*S*)-Isoserine has also been applied in semisynthetic aminoglycoside antibiotic derivatives like gentamicin and butirosin in order to enhance antimicrobial activity of these compounds [2]. In the case of gentamicin, the (*S*)-isoserine was introduced by employing *N*-acylation of the free base of the antibiotic. Methods for selective acylation at the C-1 amino group of aminoglycoside–aminocyclitol antibiotics have assumed great importance since those derivatives of aminoglycoside antibiotics were effective against many bacteria resistant to the parent compounds. After optimizing the conditions under which the C-1 amino group was the most reactive in the molecule towards acylating agents, several derivatives of gentamicin have been prepared [104]. One 1-*N*-acyl analog of the gentamicin sisomicin class is presented in Figure 6.4a. This derivative, containing (*S*)-isoserine, exhibited reduced potency compared with the parent antibiotic versus sensitive organisms, but increased



antimicrobial activity	(IC_{50}; μg/mL)
Pseudomonas aeruginosa Stone 20	0.3
Escherichia coli F14-BK	3.0
Klebsiella pneumoniae Georgetown 3694	3.0
Staphylococcus aureus Wood	0.075
Bacillus subtilis 6623	<0.01



antimicrobial activity	(IC_{50}; μg/mL)
Staphylococcus aureus Smith	0.78
Bacillus subtilis PCI 219	0.78
Escherichia coli K-12	3.12

Figure 6.4 (a) Gentamicin-sisomicin containing (*S*)-isoserine. (b) Structure of 1-*N*-(2*S*,3*R*)-3-amino-2-hydroxybutanoyl-2′,3′-dideoxykanamycin A.

activity against sisomicin-resistant organisms. Isepamicin is another aminoglycoside construct of the gentamicin B building block and a peptide-type bonded (*S*)-isoserine antibiotic [105, 106]. Synthesis of isepamicin employing chemoselective *N* protection of both components, based on mercaptobenzothiazol for introduction of urethane-type *N*-protective groups, was reported recently [107].

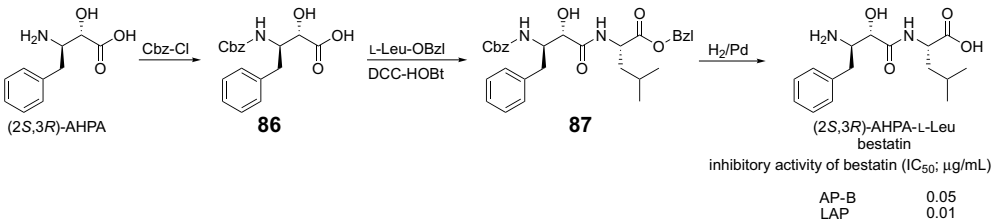
(2*S*,3*R*)-Isothreonine is another example of α-hydroxy-β-amino acids improving the antimicrobial activity in the semisynthetic antibiotic, dideoxykanamycin A. Kanamycin was isolated from the broth filtrate of *Streptomyces kanamyceticus* and then the structure was established [108, 109]. A study by the Umezawa group on the mechanisms of inactivation of aminoglycosidic antibiotics by enzymes of resistant organisms showed that the 1- or 3-amino group of the 2-deoxystreptamine moiety of kanamycin was involved in the binding of the antibiotic with 3′-*O*-phosphotransferases and the modification of one of these amino groups could produce active derivatives against resistant bacteria [110]. A number of kanamycin derivatives have been prepared and among them there were constructs with isothreonine as a pure (2*S*,3*R*)-isomer (Figure 6.4b) or as a racemic mixture of (2*S*,3*S*) and (2*R*,3*R*) [111, 112]. Antibacterial activities of these compounds were enhanced in comparison to the activity of 2′,3′-dideoxykanamycin A.

6.4 Inhibitors of Aminopeptidases

The use of α-hydroxy-β-amino acids in peptidomimetics with biological activity that are also resistant to proteolysis has emerged in recent years with a significant number of applications. It is now well established that proteolytic reactions play an important role in many cellular events such as apoptosis, blood clotting, or hormonal regulation, and enzymes like peptidases or proteases are known to be involved in these various inter- and intracellular processes. Thus, inhibitors of peptidases could serve as potential therapeutic agents for several human diseases. In spite of the advances and successes in enzyme inhibitor design, new approaches to the development of inhibitors against enzymes from different classes are required, particularly for the design of molecules that can specifically inhibit one of a family of very closely related enzymes [113, 114].

Umezawa *et al*. were pioneers in the study of the aminopeptidases family. They demonstrated that activities of these enzymes were located on the surface of various types of animal cells. In order to elucidate the biological role of aminopeptidases in cell functions, they searched for inhibitors against these enzymes, and found several potent inhibitors in the culture filtrates of Actinomycetes and broths of soil *Streptomyces olivoreticuli*. One, named bestatin, was determined to be [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine [115], a compound that has been extensively studied since. To confirm the structure of bestatin, the Umezawa group synthesized it starting from amino-protected *N*-Cbz-AHPA. Coupling with L-leucine benzyl ester proceeded with DCC in the presence of 1-hydroxybenzotriazole (HOBt, Scheme 6.17) [116]. Synthetic bestatin exhibited the same physicochemical proper-

ties and biological activities as the natural material. Bestatin inhibited aminopeptidase B (AP-B; EC 3.4.11.6), a Zn^{2+}-dependent exopeptidase, which selectively removes Arg or Lys residues from the N-terminus of several peptide substrates, and leucine aminopeptidase (LAP; EC 3.4.11.1), a cytosolic exopeptidase catalyzing the hydrolysis of amino acids from the N-terminus, preferentially leucine; both enzymes belong to the M1 family of metallopeptidases. Bestatin, however, showed no biological activity against aminopeptidase A (AP-A; EC 3.4.11.7), an exopeptidase hydrolyzing an N-terminal peptide bond containing glutamic or aspartic acid and belonging to the same M1 family. Researchers fully investigated the inhibition of AP-B and LAP through structure–activity relationships (SARs). Among synthesized derivatives of bestatin, any modification of the stereochemistry of (2*S*,3*R*)-AHPA or L-leucine reduced the activity of inhibitors. Regarding inhibition of AP-B, replacing Leu by other amino acids did not improve potency, except the case for Ile, which retained activity. Modification of the AHPA moiety gave more promising results, as *para* substitution of benzyl group in AHPA increased the inhibitory potency of *p*-nitrobestatin (0.01 μg/ml), *p*-methylbestatin (0.01 μg/ml, but as a diastereoisomeric mixture), and most significantly *p*-hydroxybestatin (0.007 μg/ml) [30, 55].



Scheme 6.17 Synthesis of bestatin.

Umezawa proposed the first probable mechanism of LAP inhibition by bestatin [30] and recently another group suggested a slightly modified inhibition mechanism of AP-B [117]. Burley *et al.* described the three-dimensional structure of the LAP–bestatin complex and suggested that binding in complex might be similar to a tetrahedral intermediate thought to form during bond hydrolysis [118]. Other research groups discussed crystal complexes between bestatin and aminopeptidase N (AP-N; EC 3.4.11.2) [119, 120]. Finally, the general model of bestatin interactions in the active site of metalloaminopeptidases (MAPs) was disclosed (Figure 6.5a). Although the MAP superfamily is quite large and divergent, MAPs utilize a common catalytic mechanism by the coordination of one or two zinc atoms in the active site to activate water for nucleophilic attack on a peptide or protein substrate. Bestatin resembles a Phe–Leu dipeptide substrate. The α-hydroxy group and neighboring carbonyl coordinated the catalytic zinc atom, resulting in a competitive active site-directed inhibitor [121]. Bestatin as a well-defined tight-binding inhibitor, with the S1 and S1′ active-site pocket interactions, has served for the development of novel

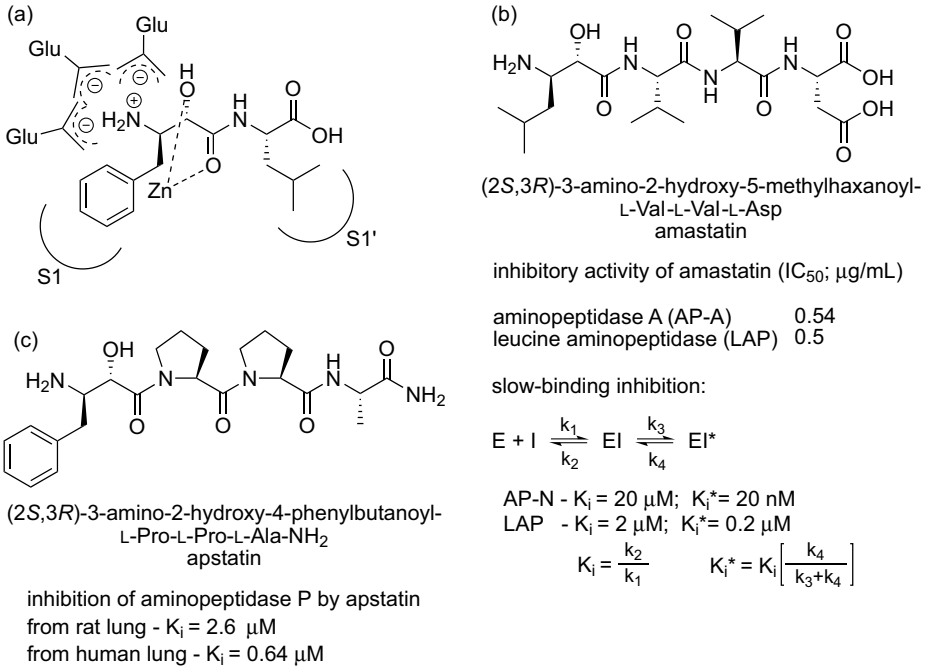


Figure 6.5 (a) General model of bestatin interactions in the active site of MAPs. (b) Structure of amastatin. (c) Structure of apstatin.

activity-based probes, with specificity for MAPs. This class of probes could be a valuable tool for the future characterization of MAP activity in a wide variety of biological systems [121].

Bestatin, known also as ubenimex, was tested as a therapeutic agent against cancer [122] and the clinical use of this product was permitted in Japan. Ubenimex can be administered orally and has extremely weak toxicity (LD_{50} in mice is 4.0 g/kg *per os*). It was recently reported that ubenimex enhanced radiation sensitivity in human cervical cancer, which could represent a new approach for improving the therapeutic efficacy of radiotherapy for uterine cervical cancer [123].

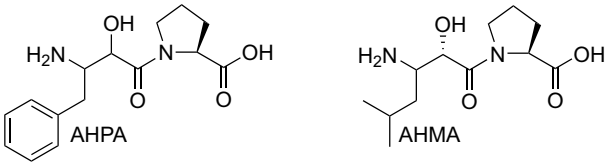
Bestatin was inactive against AP-A, thus Umezawa *et al.* searched for an inhibitor and found one, also from culture filtrates of actinomycetes, named amastatin (Figure 6.5b). The structure of amastatin was determined to be (2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl-L-valyl-L-valyl-L-aspartic acid. Amastatin exhibited inhibitory activity against AP-A and LAP, with low toxicity, and it was inactive against AP-B [124]. Rich *et al.* investigated the bioactivity of amastatin against AP-N, an aminopeptidase like AP-A and belonging to the same M1 family. They found amastatin to be a strong competitive AP-N inhibitor exhibiting a slow-binding inhibition mechanism of AP-N and LAP [125].

Another inhibitor of aminopeptidase containing α-hydroxy-β-amino acids was synthetic apstatin, *N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-prolyl-L-prolyl-

L-alaninamide (Figure 6.5c). Apstatin was designed to inhibit aminopeptidase P (AP-P; EC 3.4.11.9), a membrane-bound or cytosolic form exopeptidase removing the N-terminal amino acid from peptides possessing a proline residue in the second position. AP-P was thought to inactivate bradykinin, a potent vasoactive and cardioprotective peptide hormone, by cleaving the Arg–Pro bond. To test this hypothesis, apstatin was synthesized by standard solid-phase peptide synthesis (i.e., on *p*-methylbenzhydrylamine resin) [126]. Results of bioactivity studies confirmed that apstatin was a potent, stable, and reasonably specific inhibitor of membrane-bound AP-P, which played a significant role in the pulmonary degradation of bradykinin in the rat. The same research group synthesized apstatin with AHMHA instead of AHPA, preserving the same stereochemistry. The substitution of the *N*-terminal benzyl with an isobutyl side-chain resulted in10-fold increased potency ($IC_{50,\ human} = 0.23\ \mu M$ for AHMHA-apstatin compared to $IC_{50,\ human} = 2.9\ \mu M$ for AHPA-apstatin). These results indicated that apstatins as AP-P inhibitors could be efficacious in the treatment of various cardiovascular disorders by increasing levels of endogenously formed bradykinin [127].

The slow-binding inhibition mechanism was observed also for AP-P inactivated by dipeptidic inhibitors consisting of AHPA or AHMHA, respectively (Figure 6.6) [128]. Design of these compounds was based on the structure of bestatin and on the crystal structure of *Escherichia coli* AP-P in complex with apstatin. The slow-binding inhibitors confirmed the preference of both AP-Ps for the (2*S*,3*R*) configuration, but not a great preference for the AHPA- or AHMHA-containing dipeptide with regard to the K_i^* value. AHPA-Pro was slightly more potent against *E. coli* AP-P while AHMHA inhibited rat AP-P more efficiently.

There are other naturally occurring inhibitors of aminopeptidases, mostly generated by *Streptomyces*. AHPA–Val and two closely related derivatives, AHPA–Val–Pro–Hyp and AHPA–Val–Pro–Pro, were extracted from *Streptomyces neyagawaensis* SL-387 (Figure 6.7) [129–131]. Phebestin, a tripeptide with the structure (2*S*,3*R*)-3-



slow-binding inhibition of aminopeptidase P
from E. coli
(2*S*,3*R*)-AHPA-Pro - K_i = 14 μM, K_i^* = 0.17 μM
(2*R*,3*S*)-AHPA-Pro - K_i = 16 μM, K_i^* = 0.74 μM
(2*S*,3*R*)-AHMHA-Pro - K_i = 14 μM, K_i^* = 0.37 μM
from rat
(2*S*,3*R*)-AHPA-Pro - K_i = 32 μM, K_i^* = 2.1 μM
(2*R*,3*S*)-AHPA-Pro - K_i = 120 μM, K_i^* = 11 μM
(2*S*,3*R*)-AHMHA-Pro - K_i = 110 μM, K_i^* = 1.1 μM

Figure 6.6 AP-P inhibitors.

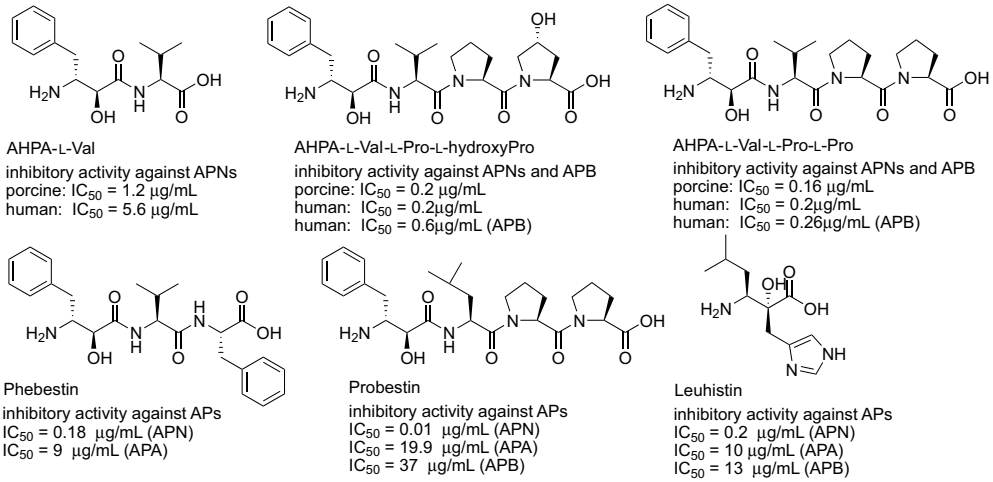


Figure 6.7 Naturally occurring AP-N/CDC13 inhibitors.

amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-phenylalanine was produced by *Streptomyces* sp. MJ716-m3 [132]. Some stereoselective syntheses of phebestin have been reported [133–135]. Probestin, a tetrapeptide with the structure (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucyl-L-prolyl-L-proline, was extracted from *Streptomyces azureus* MH663-2F6 [136, 137]. Total syntheses of probestin have been reported [133–135]. Leuhistin, which has the structure (2*R*,3*S*)-3-amino-2-hydroxy-2-1*H*-(imidazol-4-ylmethyl)-5-methylhexanoic acid, was isolated by the Takeuchi group from the culture broth of a bacterium belonging to the phylum Firmicutes: *Bacillus laterosporus* BM156-14F1. The structure and its absolute configuration were ascertained by the same group [138–140].

To summarize aminopeptidase inhibitor activity, it is important to point out that most of them lack tight specificity by inhibiting other membrane-bound metalloproteases. Bestatin interacts with LAP, AP-B, and aminopeptidase W (AP-W; EC 3.4.11.16), which suggests that some of the observed chemotherapeutic actions of bestatin may be due to inhibition of other cell surface peptidases (in first clinical trials, bestatin has been used to treat patients with acute and chronic myeloid leukemias, and lymphomas). Amastatin and probestatin inhibited AP-A and AP-W in the low micromolar range (1.5–20 μM). Leuhistin inhibited AP-N, AP-A, and AP-B [141]. Thus, the reported data emphasize the need for more specific and targeted aminopeptidase inhibitors.

6.5
Aspartyl Proteases Inhibitors

Aspartyl proteases, also known as acid proteases or aspartyl proteinases, are a widely distributed subfamily of proteolytic enzymes belonging to the endonuclease

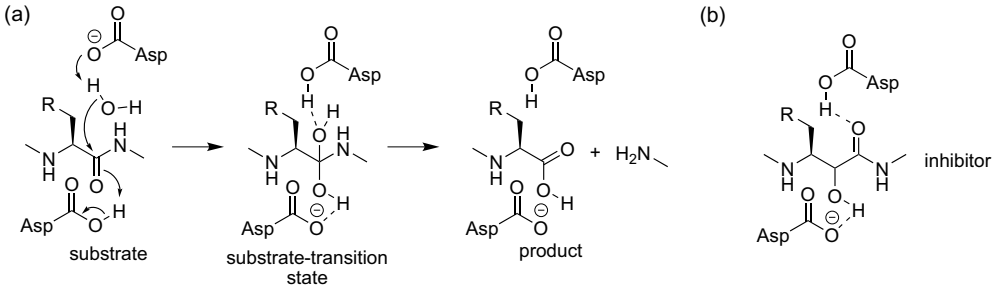


Figure 6.8 (a) Catalytic mechanism for substrate hydrolysis by aspartic proteases. (b) Aspartic protease inhibition by Nst derivatives.

enzyme family. Aspartyl proteases exist in vertebrates, plants, viruses, and retroviruses. The subfamily of aspartic proteases is characterized by having in their primary sequence two different Asp–Thr–Gly sequences. Their apostructure shows these two chains running in opposite directions with a water molecule bound between two aspartates. Aspartic proteases are highly specific proteolytic enzymes cleaving dipeptide bonds that have hydrophobic residues by employing an enzyme-bound activated water molecule as the nucleophile, which attacks the amide carbonyl of the scissile bond. They generally bind 6–10 amino acid regions of their polypeptide substrate that are typically processed with the aid of two catalytic aspartic acid residues in the active site [142]. The possibility of a "hydrophobic binding site" was first mentioned by Tang [143], and Hartsuck and Tang also first proposed the transitional state in the catalysis of pepsin, one of the aspartyl proteases [144]. The general acid–base mechanism that is considered most likely for polypeptide hydrolysis catalyzed by aspartic proteases is depicted in Figure 6.8(a). One catalytic aspartate acts as an acid catalyst to protonate the carbonyl oxygen and the other one acts as a base to pull a proton from water. This permits direct nucleophilic attack by the oxygen from water to the carbonyl carbon to form an amide dihydrate intermediate [145]. Aspartyl proteases play an important role in several aspects of our overall health and physiology, including regulation of blood pressure (renin) and digestion (pepsin), maturation of HIV and human T-cell leukemia virus (HTLV) type I, and other serious diseases causing public health problems (malaria and Alzheimer's disease in the global aging population).

6.5.1 Renin Inhibitors

Renin is a highly specific aspartic protease that is involved in the rate-limiting first step of the renin–angiotensin system (RAS). Renin selectively cleaves α_2-globulin angiotensinogen (Figure 6.9b) to release a decapeptide, angiotensin I. Angiotensin I is converted by angiotensin-converting enzyme (ACE), a dipeptidylcarboxypeptidase, to an octapeptide angiotensin II, which is a potent vasoconstricter, a promoter of

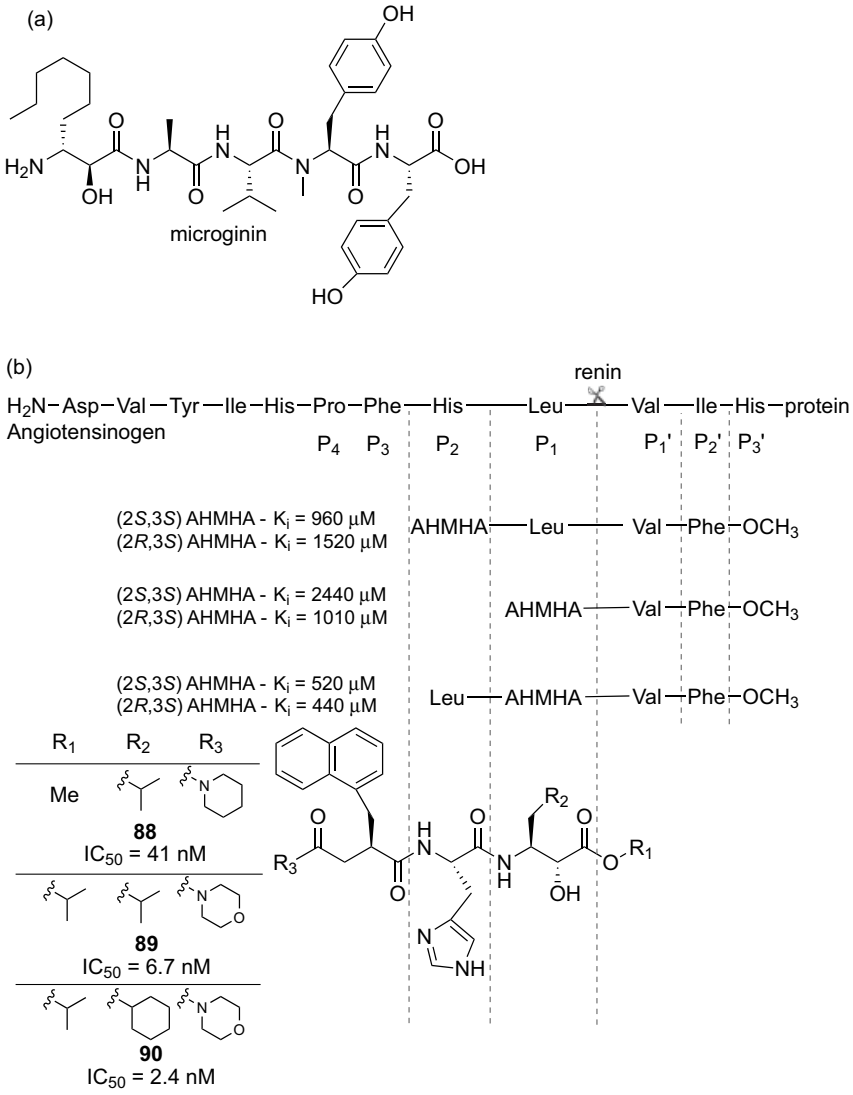


Figure 6.9 (a) Structure of microginin, a naturally occurring ACE inhibitor. (b) Design, structure, and activity of renin inhibitors.

aldosterone release (and thus sodium retention), and a trigger for a variety of other effects on the kidney, brain, and pituitary.

The whole cascade of RAS is an important area for the study of the regulation of blood pressure and electrolyte homeostasis [146]. One of the ACE naturally occurring inhibitors is microginin, a linear pentapeptide containing AHDA (Figure 6.9a). Microginin was isolated by Murakami *et al.* from the freshwater blue-green alga *Microcystis aeruginosa* and was found to possess inhibitory activity against ACE with

IC_{50} of 7.0 μg/ml, while being inactive against papain, trypsin, chymotrypsin, and elastase [147].

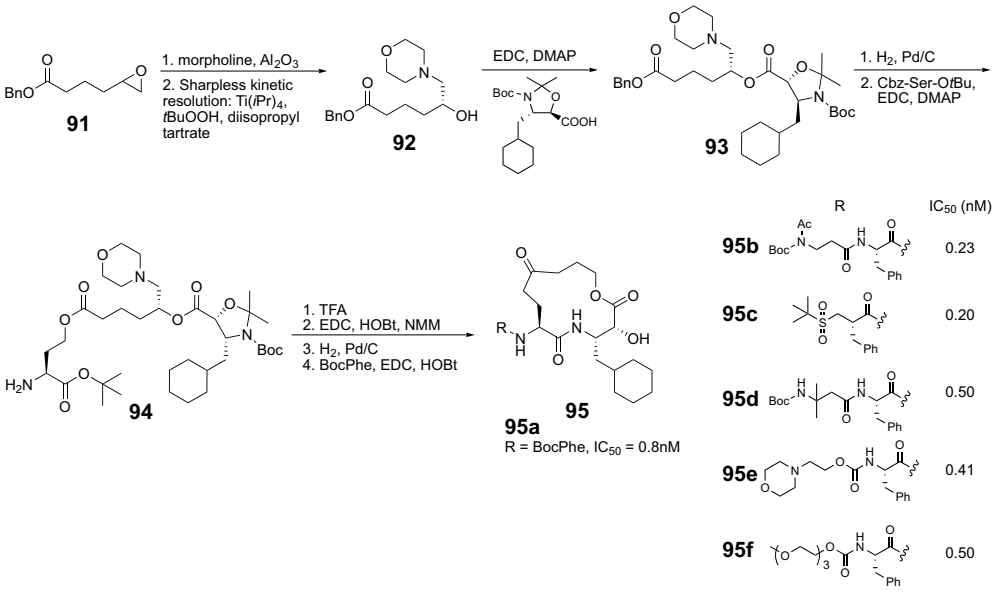
Naturally occurring inhibitors often suffer from problems such as poor oral absorption, short duration of action, proteolytic instability, and rapid biliary excretion. For this reason, new synthetic compounds have been developed as promising antihypertensive agents. Treatment of hypertension and cardiac failure by means of ACE inhibitors has been well studied, but the renin inhibitors provide a more direct probe of the RAS due to the renin specificity towards its substrate, angiotensinogen, that is cleaved between P_1 Leu and P_1' Val (Figure 6.9b). Johnson designed and synthesized renin inhibitors that looked fairly similar to the P_2–P_3' angiotensinogen segment with first replacement of the *N*-His residue with AHMHA, then *N*-Leu was replaced by AHMHA. These compounds were tested *in vitro* for their ability to inhibit human amniotic renin [57]. The most potent in this short series was the analog Leu–(2*R*,3*S*)-AHMHA–Val–PheOCH$_3$.

Most reported renin inhibitors have been peptidomimetics that retain significant peptidic character, which confers low stability and poor oral bioavailability in humans. Thus, the Kiso group designed the inhibitor **88** based on minimizing the number of natural peptide bonds for a long-lived inhibitor, and the presence of a large hydrophobic residue in the P_1 and P_3 positions, and with P_4 enlarged to a piperidinyl moiety to better occupy the S_4 renin binding site. A succinic acid residue, having a P_3–P_4 retro-inverso amide bond, was introduced to reduce the likelihood of premature degradation by other proteases. The P_3 Phe side-chain was replaced by a 1-methylnaphthyl moiety to avoid recognition and proteolytic degradation by chymotrypsin, and also to promote a tighter fit in the S_3 subsite.

For the P_1 inhibitory residue the (2*R*,3*S*)-AHMHA, Nst, was also chosen as its (2*S*,3*S*)-counterpart, allonorstatine, was less potent [148]. The P_4 residue was then further optimized to a morpholinyl moiety. The methyl fragment in the P_1-cap was replaced by an isopropyl one to resemble angiotensinogen's P_1' Val residue, resulting in a more potent inhibitor **89**. Modification of the P_1 residue by introducing other Nst derivatives, Pns, (2*R*,3*S*)-APHA, or its analog, allophenylnorstatine (Apns), (2*S*,3*S*)-AHPA, led to the lower inhibitory potency. When cyclohexylnorstatine, its (2*R*,3*S*)-isomer, was at P_1 instead of Nst, the inhibitor **90** exhibited highly selective renin inhibitory activity *in vitro* while being stable against monkey liver homogenates, human plasma, and chymotrypsin. Oral administration of 10 mg/kg to salt-depleted monkeys resulted in a fall of 10–20 mmHg of mean blood pressure and reduced plasma renin activity for a 5-h period [90]. Thus, compound **90** (KRI-1314) was an orally bioavailable, effective, and long-lasting antihypertensive drug. Kiso *et al.* proposed a model for the interactions of **89** and **90** with human renin where the P_1 hydroxy group of Nst or cyclonorstatine interacted with both Asp38 and Asp226, which is in agreement with the generally accepted mechanism of aspartyl protease inhibition (Figure 6.8b).

(2*R*,3*S*)-Cyclohexylnorstatine (norACHPA) was also incorporated into macrocyclic renin inhibitors synthesized by researchers from Merck. They examined a computational model of the human renin active site developed by Merck with a focus at the P_2 and P_1' side-chains (His and Val). Modeling results suggested that P_2 and P_1' side-

chains of angiotensinogen occupy a common binding pocket within the enzyme, and that these side-chains could be linked to provide a viable design for macrocyclic renin inhibitors [149]. They designed a series of P_2 and P_1'-linked macrocyclic inhibitors, starting the synthesis from epoxide **91** converted to the (*R*)-amino alcohol **92** after Sharpless kinetic resolution with L-tartrate (Scheme 6.18) [150]. Alcohol **92** was coupled with Boc-norACHPA acetonide using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC)/4-dimethylaminopyridine (DMAP). Intermediate **93** was treated with H_2 and Pd/C, and then coupled to *N*-Cbz-serine *tert*-butyl ester (Cbz-Ser-O*t*Bu) with EDC/DMAP to give macrocycle precursor **94**, which was then treated with trifluoroacetic acid (TFA) followed by macrocyclization of the resultant amino acid employing EDC and HOBt to give the macrocycle **95**. Removal of the Cbz protecting group followed by coupling to Boc-Phe gave inhibitor **95a** [151]. The "Boc-Phe" component was replaced with a variety of substituents, some were polar, water-solubilizing groups, leading to highly potent, subnanomolar derivatives **95b–95f**. The *in vivo* activity of inhibitor **95c** was examined in sodium-depleted rhesus monkeys, by monitoring blood pressure. An oral dose of 15 mg/kg of **95c** produced a decrease in blood pressure, which lasted over 4 h.



Scheme 6.18 Synthesis of macrocyclic human renin inhibitors.

For a renin inhibitor to be a commercially successful drug a number of requirements must be achieved. The inhibitor should be able to lower renin blood pressure in hypertensive patients; be orally bioavailable to compete in the market place with ACE inhibitors; advantageous relative to other antihypertensive agents; potent; specific for renin versus pepsin; nontoxic; and metabolically stable. Progress

toward achieving most of these requirements has been substantial and systematic structural modifications lead to molecules with significantly improved properties such as the development of orally bioavailable inhibitors ready for preclinical and clinical trials [152].

6.5.2 HIV-1 Protease Inhibitors

HIV-1 protease is a unique aspartyl protease existing as a dimer of two identical subunits. This enzyme is essential for the life-cycle of HIV, the retrovirus that causes AIDS. HIV-1 protease cleaves polyprotein precursors into polyproteins, which are then further processed, assembled, and developed into a mature virion leading to the pathogenesis of AIDS. Without effective HIV protease, HIV virions remain uninfectious. Thus, mutation of HIV protease's active site or inhibition of its activity disrupts HIV's ability to replicate and infect additional cells. HIV protease inhibition is the subject of much pharmaceutical research and this strategy has been validated by the approval of several HIV protease inhibitors for clinical use [153]. A basis for rational design of selective inhibition against nonhuman proteases is that proteases of the HIV type and other retroviruses recognize the Xaa–Pro sequence as the cleavage site, whereas mammalian aspartic proteases do not. Two well-known cleavage site regions of HIV-1 polyproteins, transframe/protease (TF/PR), and matrix/capsid (MA/CA), share a similar P_1–P_1' Xaa–apro sequence (Figure 6.10) [154]. The Kiso group first synthesized inhibitor **96** containing P_4–P_1 residues similar to TF/TR, while P_1'–P_3' residues were similar to MA/CA, and the critical P_1 isostere residue, Apns, (2*S*,3*S*)-AHPA moiety, provided more potent inhibition than its (2*R*,3*S*)-isomer [155]. To obtain smaller inhibitors, the P_4–P_3 and P_2'–P_3' residues were excluded from the scaffold. A Boc protection group was optimized as a P_2-cap of the former P_3 Phe residue, while a P_1'-cap *tert*-butylamine was optimized to replace the bulky P_2' Ile residue [156]. The resulting tripeptidic inhibitor **97** was less potent. Thus, to increase the fit in the large hydrophobic S_3 subsite, the 1-naphthyloxyacetyl moiety was introduced as a P_2-cap moiety in inhibitor **98** and the P_2 natural amino acid, Asn, was replaced by non-natural amino acid L-methanesulfonylalanine in inhibitor **99**. Considering the subtle balance of lipophilicity–hydrophobicity and molecular size with regard to penetration across the cell membrane and nonspecific adsorption in blood, the P_2-cap moiety was replaced by a 5-isoquinolinyloxyacetyl fragment and the P_1' Pro residue was exchanged for a non-natural amino acid, L-thiazolidine-4-carboxylic acid in kynostatin (KNI)-272 or L-5,5-dimethylthiazolidine-4-carboxylic acid in KNI-227 [157]. Both KNI-272 and KNI-227 were potent and highly selective HIV-1 protease inhibitors with excellent antiviral activity in cells [158]. KNI-272 also had high antiviral activity against a wide spectrum of HIV strains and low cytotoxicity profile against noninfected cells ($LC_{50} > 20\,\mu M$). X-ray diffraction crystallographic data, NMR data (for the first time NMR studies of an HIV-1 protease–inhibitor complex revealed a catalytic mechanism of aspartic protease involving a hydrogen network), and docking simulation studies of both these inhibitors in complex with HIV-1 protease have

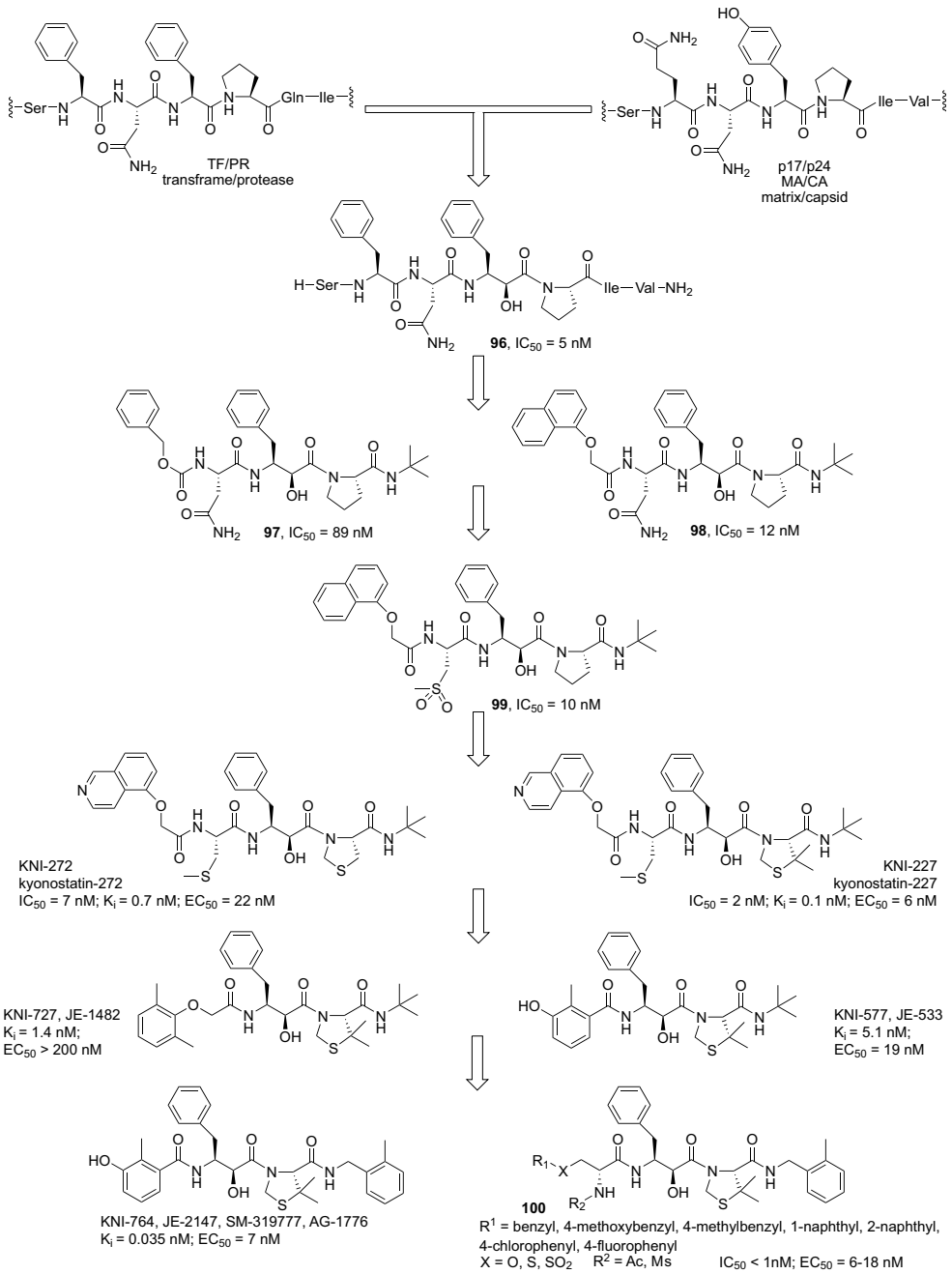


Figure 6.10 Design and structure of HIV-1 protease inhibitors.

been published [159–167]. Although KNI-272 showed good oral bioavailability in a human clinical trial, the plasma half-life was very short, which suggested that the inhibitor should be further optimized for a more desirable pharmacokinetic profile [168, 169]. From the SAR study, very potent KNI-727 and KNI-577 were designed. The cellular antiviral activity of KNI-727 was low, while KNI-577 exhibited potent antiviral activities, low cytotoxicity, and good pharmacokinetic properties after intraduodenal administration. Thus, KNI-577 was further modified at the P_2' position to take into consideration the symmetrical nature of the homodimeric protease; an aromatic moiety was introduced as the P_1'-cap in KNI-764 to better accommodate the S_2' pocket. Dipeptidic KNI-764 was a highly potent inhibitor of HIV-1 protease, and was also effective against all HIV-1 and HIV-2 strains along with clinical HIV-1-resistant variants due to its flexibility and adaptability to the mutated HIV proteases.

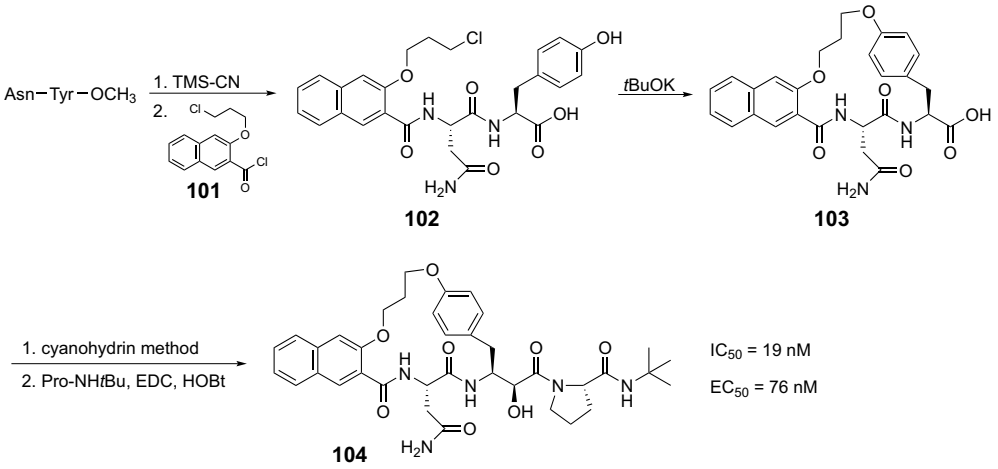
KNI-764 exerted cellular antiviral activity with a relatively longer plasma half-life, and had moderate oral bioavailability and duodenal absorption as well as a low cytotoxicity profile [170, 171]. Then, the next target in the structure optimization was to replace the P_1-cap with D-amino acid residues as an alternative for the P_2 position that resulted in the general structure of inhibitor **100** and its analogs. This modification improved cellular antiviral activity and selective cytotoxicity. Some of the derivatives of **100** possessed potent inhibitory activity against wild-type or against resistant HIVs [172, 173]. Current research in the Kiso group focuses on the development of the next generation of HIV protease inhibitors being potent against all resistant HIVs with prospective profiles of cytotoxicity and α_1-acid glycoprotein binding [174].

Two other groups of researchers have developed Nst-based inhibitors of HIV protease. Researchers from Syntex Research Canada independently synthesized compounds **97** and **98**, and their derivatives, with similar inhibitory activities [175]. They also designed macrocyclic hydroxyamide peptidic inhibitors. The synthesis began from attaching acid chloride **101** to dipeptide Asn–Tyr-OMe, and by the activation of hydroxyl group of **102** in the presence of *t*BuOK, the P_3–P_1 macrocycle **103** was assembled (Scheme 6.19). Hydroxyamide functionality was generated from the macrocycle through the cyanohydrin according to the standard procedure described in the Section 6.2.2.4.

Diastereomer **104** with (2*S*,3*S*)-AHPA was a weaker inhibitor ($IC_{50} = 19$ nM) than its acyclic analog ($IC_{50} = 1.1$ nM), but had better antiviral activity, suggesting improved cell penetration properties and/or resistance to cellular enzymes for the macrocyclic form [176].

Researchers from Dainippon Sumitomo Pharma have been working on optimization of the JE-2147 (known also as a KNI-764, SM-319777, and AG-1776) structure by substitution of Apns (its phenyl moiety) [177, 178].

Low water solubility and insufficient bioavailability are serious problems of protease inhibitors. Since catalytic domain of HIV-1 protease is hydrophobic, HIV-1 inhibitors usually have highly lipophilic properties. Such properties are also required to facilitate inhibitors penetration into the infected cells or virions. This confers an undesirable sparse water solubility on the inhibitors. To overcome this low



Scheme 6.19 Synthesis of a Nst-based macrocyclic, (2*S*,3*S*)-AHPA, HIV protease inhibitor.

water solubility while retaining high lipophilicity, one effective strategy was to convert the water-insoluble parent drugs into hydrophilic prodrugs possessing self-cleavable linkers and solubilizing moieties. The release of parent drugs occurred via a spontaneous imide formation by nucleophilic reaction ($O \rightarrow N$ intramolecular cyclization) under physiological conditions (Figure 6.11) [179–181].

Additionally, released auxiliary moieties could cause some undesired side-effects, and design of prodrugs avoiding such auxiliary units would be an advantage in toxicology and general pharmacology, while limiting the cost for evaluation during drug candidate development. Considering these features, in the Kiso group a new class of prodrugs has been reported [182]. The concept was based on $O \rightarrow N$

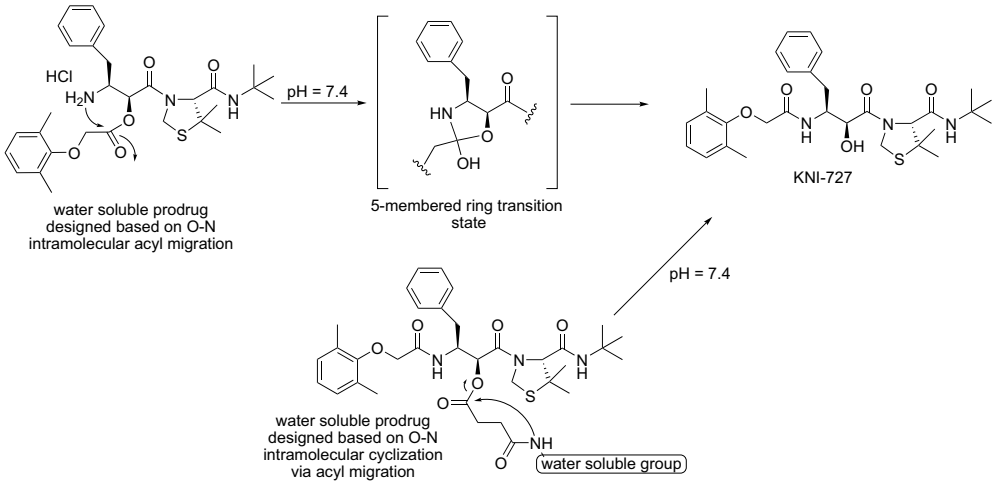


Figure 6.11 Water-soluble prodrugs of HIV-1 protease inhibitors.

intramolecular acyl migration. Prodrugs possessing a hydrophilic amine moiety in an *O*-acyl structure could spontaneously release parent drugs via a five-membered ring transition state. Parent drug release was clearly pH-dependent and migration proceeded slower in acidic pH, and the prodrugs were generally stable under strong acidic pH similar to gastric juice. Therefore, they could potentially be stable in the stomach and then converted under intestinal neutral conditions to the hydrophobic parent drugs suitable for absorption by the intestinal brush border membrane, suggesting that they can be useful when orally administered as water-soluble agents [183–186].

The concept of $O \rightarrow N$ intramolecular acyl migration was applied to the synthetic strategies for the peptides containing difficult sequences as a useful tool for molecular/pathological studies of Alzheimer's disease using chemical/biology technology [187, 188].

6.5.3 HTLV-I Inhibitors

HTLV-I is a retrovirus that is clinically associated with adult T-cell leukemia and myelopathy/tropical spastic paraparesis. HTLV-I encodes a virus-specific aspartic protease responsible for processing the *gag* and *gag–pro–pol* polyproteins, and is required for proliferation of the retrovirus. Therefore, HTLV-I protease is a major target for the development of specific anti-HTLV-I agents [153, 154].

Similar to the development of HIV-1 protease inhibitors, the design for HTLV-I protease inhibitors began with substrate-based structures mimicking the p19/p24 sequence containing the Leu–Pro cleavage site shown in Figure 6.12. From the SAR studies of synthesized compounds it was found that dimethylthioproline (Dmt) was required at the P_1' position for higher inhibitory activity. When the inhibitory KNI-10162 P_1 residue was substituted with Apns, better accommodation by the HTLV-I protease active site was observed, although allonorstatine present in KNI-10160 was structurally closer to the P_1-Leu originally occupying that position in the substrate. Inhibition potencies of KNI-10160 and KNI-10162 were quite similar: both showed 100% of inhibition at 100 μM concentration of HTLV-I, and 63 and 66% at 5 μM concentration of HTLV-I, respectively [189]. KNI-10162 was used as a lead compound for further modifications. A truncation study on the octapeptidic KNI-10162, commencing from N- and C-termini, led to a less potent hexapeptidic analog. With the knowledge that HTLV-I protease cleaves different substrates at many cleavage sites of various precursor polyproteins, in the next design, the P_3–P_2 and the P_3'–P_2' residues of potential inhibitors were substituted with the residues from a small library of natural amino acids. The resulting KNI-10166, composed of a fair number of Ile residues, exhibited high potency against HTLV-I [190]. The next modification in the inhibitory structure was substitution with non-natural amino acid residues to prevent premature proteolysis. During the optimization process, it became apparent that the P_3' replacement of the nonpolar Met residue by a polar amino acid, Gln, maintained high inhibitory activity and subsequently removing the P_3' residue gave moderately potent KNI-10247 [191].

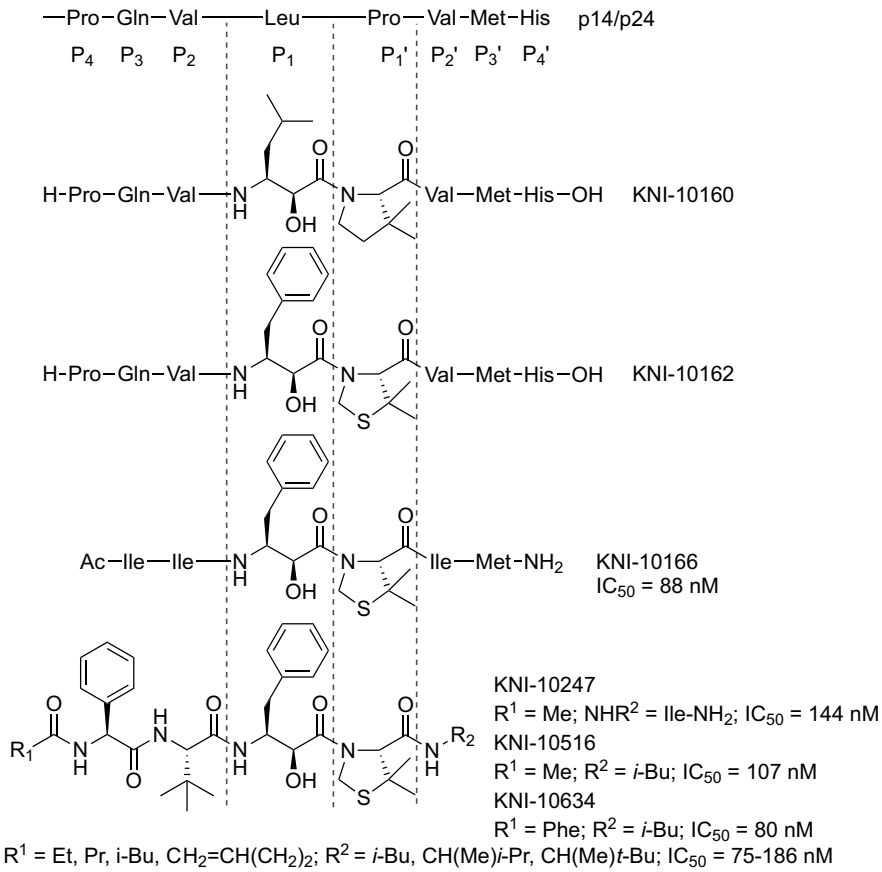


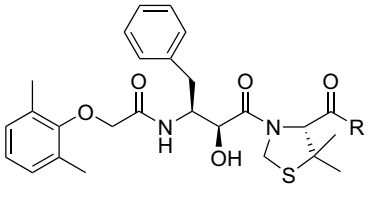
Figure 6.12 Design and structures of HTLV-I protease inhibitors.

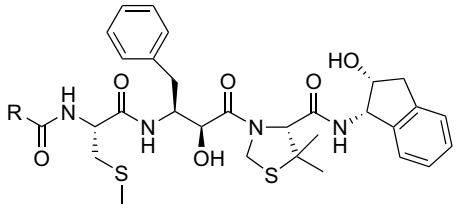
The preliminary optimization study suggested that increased bulk around the β-carbon of the P_1'-cap moiety favored the inhibitory activity, as exemplified in KNI-10516 [192]. Computer-assisted docking experiments suggested that, in the symmetrical homodimeric HTLV-I protease, the P_1' and P_2'-cap moiety resided in the respective mirror pocket of the S_1 and S_2 where the corresponding P_1 and P_2 side-chains fit [193]. In their present research, Kiso *et al.* are exploring the P_2-cap moiety and other P_1'-capping groups to maximize inhibitory activity against HTLV-I protease.

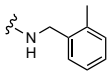
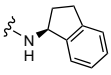
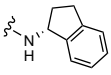
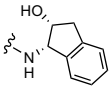
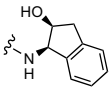
Although HIV and HTLV-I are both from the Retroviridae family, HTLV-I protease had a higher specificity that HIV protease and, consequently, the potent HTLV-I protease inhibitors often exhibited potent inhibition against HIV-I protease. However, potent HIV-1 protease inhibitors did not necessarily possess potent inhibitory activity against HTLV-I protease. Thus, there is a chance for HTLV-I protease inhibitors to be further developed as anti-AIDS drugs [154].

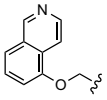
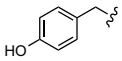
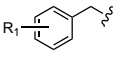
6.5.4
Plasmepsin II Inhibitors

Malaria is a disease caused by a *Plasmodium falciparum* that feeds on the hemoglobin of an infected victim. The parasite produces a family of proteases, called plasmepsins, degrading the host's hemoglobin, and consequently leading to the symptoms of malaria and death of the host. Four plasmepsins, plasmepsin I, II, and IV, as well as histo-aspartyl protease (HAP), have been implicated in hemoglobin degradation. Among them, plasmepsins I and II are known to readily cleave hemoglobin between Phe33 and Leu34 of the α-globin subunit. Between these two, the structure and active site specificity of plasmepsin II is better known [197]. Since the Phe–Leu dipeptide was similar to the Apns-containing scaffold, Kiso *et al*. have hypothesized that their previously prepared HIV-1 inhibitors might also be effective against plasmepsin II and they evaluated the plasmepsin II inhibitory activity of selected Apns-containing HIV protease inhibitors [195]. As predicted, some of these compounds were able to inhibit plasmepsin II potently (KNI-727, Figure 6.13). KNI-727 was effective in killing the malaria parasite, with an EC_{50} value of 10 μM and was used as a lead compound in





	K_i (nM)	R
KNI-727	70	
KNI-840 (EC_{50} = 10.7μM)	20	
KNI-10026	15	
KNI-1269	99	
KNI-10006 (EC_{50} = 6.8 μM)	0.5	
KNI-10007	71	

R	K_i (nM)	
	3	KNI-10033 (EC_{50} = 3.1μM)
	0.2	KNI-10232 (EC_{50} = 4.7 μM)
	< 0.1	KNI-10343
	0.1-18	

R_1 = Me, NH_2, OMe

Figure 6.13 Structure and inhibitory activity of KNI compounds against plasmepsin II.

a further SAR study [196]. That research resulted in a series of extremely potent inhibitors, with KNI-10006 markedly inhibiting plasmepsin II, and being effective against plasmepsin I, II, and HAP [197]. Among several P_1'-cap moieties, (1*S*,2*R*)-1-amino-2-indanol in KNI-10006 was strongly accommodated by the active site of plasmepsin II as a hydrophobic indane structure and its spatially arranged hydroxyl group allowed for proper hydrogen bond interactions with the enzyme that greatly contributed to the high potency of inhibitor [198].

The P_2-cap moiety was elaborated to benzyl-carboxylate derivatives, based on inhibitory potency against plasmepsin II and computer-assisted docking experiments. In the design of plasmepsin inhibitors, plasmepsin IV is critical from the aspect of amino acid sequence homology among the four plasmepsins and optimization for plasmepsin IV might lead to the inhibition of all of them. The crystal structure of plasmepsin IV complexed with KNI-764 was revealed and an unexpected orientation was observed from that complex (Figure 6.14) [199]. Apns occupied the S_1'

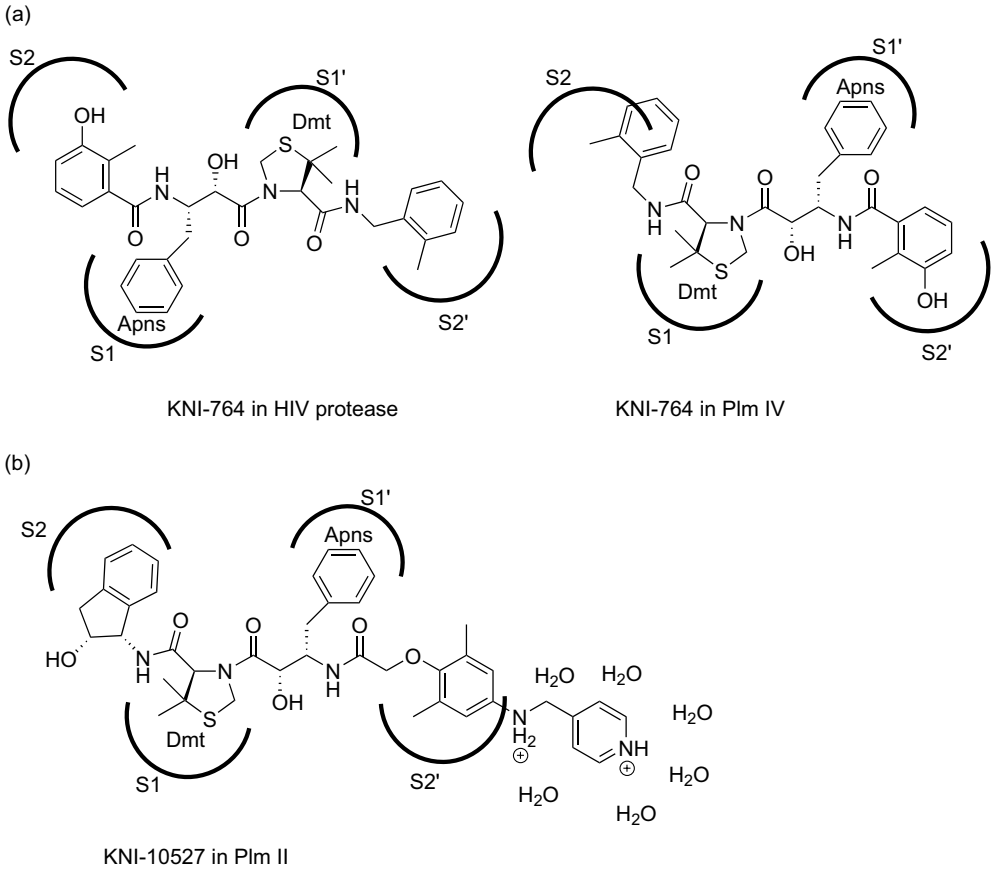


Figure 6.14 (a) Binding directions of KNI-764 in HIV protease and plasmepsin IV. (b) Binding of KNI-10527 in plasmepsin II.

pocket and the P_2 2-methylbenzyl group was found out of the S_2 pocket. These results suggested that potent KNI-10006 would bind to other plasmepsins in a similar manner to that of KNI-764 in plasmepsin IV. Therefore, it was expected that the dimethylphenoxyacetyl group could bind to the S_2' pocket of any plasmepsin. The series of KNI-10006 analogs has been synthesized with specific substitutions on the phenoxyacetyl moiety and among them pyridinylmethyl derivatives (like KNI-10527) had antimalarial activities maintained at the submicromolar level.

To explore the moderate attenuation in plasmepsin II inhibitory activity of KNI-10527 compared to KNI-10006, a binding model of KNI-10527 with plasmepsin II was constructed. The X-ray crystallographic data of KNI-764 in plasmepsin IV was used to generate the initial docking conformation of KNI-10527 in plasmepsin II. After several energy minimizations, a molecular dynamics simulation was performed on the binding direction of KNI-10527, being the same as that of KNI-764. The difference was found at the phenoxyacetyl moiety. Further extension of the pyridine-4-yl-methyl moiety from the *p*-amino group was favored to be surrounded mostly by water outside of the S_2' site. The cytotoxicity of these derivatives was in the acceptable range for clinical inhibitors, which suggests that they are promising for the development of new antimalarial agents [200].

6.5.5
BACE-1 Inhibitors

The accumulation of β-amyloid peptide in the brain is a major factor in the pathogenesis of Alzheimer's disease. β-Amyloid peptide is formed by the initial cleavage of β-amyloid precursor protein (APP) by β-secretase to form a membrane-bound C-terminal fragment, which is then proteolyzed by γ-secretase to produce two major forms: β-amyloid (1–40) peptide and β-amyloid (1–42) peptide. β-Secretase has been identified as a novel membrane-bound aspartyl protease known as β-secretaseBACE-1, memapsin 2, or Asp2 [201–204], and the crystal structure of its catalytic domain has been determined [205]. BACE-1 plays a critical role in the progression of Alzheimer's disease, since the cleavage of APP by BACE is the first step in β-amyloid peptide formation. Therefore, the development of BACE-1 inhibitors is valuable in the elucidation of Alzheimer's disease pathology.

In the Kiso group, the initial goal in designing inhibitors was to make relatively small peptide inhibitors through substrate transition-state mimetic studies. Since BACE-1 is an aspartyl protease, prior experience with related enzymes, especially HIV protease and renin, facilitated the design of inhibitors with a variety of binding motifs. Along the same lines, researchers focused on the substrate sequence deduced from the proffered cleavage of Swedish mutant APP, which showed a mutation at the P_2–P_1 positions (from Lys–Met to Asn–Leu in Figure 6.15) [206]. Initially, they synthesized an octapeptide containing Nst, (2*R*,3*S*)-AHMHA, at the P_1 position. As the BACE-1-inhibitory activity was very low, the P_1 core was changed from Nst to Pns, (2*R*,3*S*)-AHPA, and through SAR studies of the P_3–P_3' positions of the octapeptide series, the inhibitor KMI-008 was selected as the most potent against BACE-1 and was

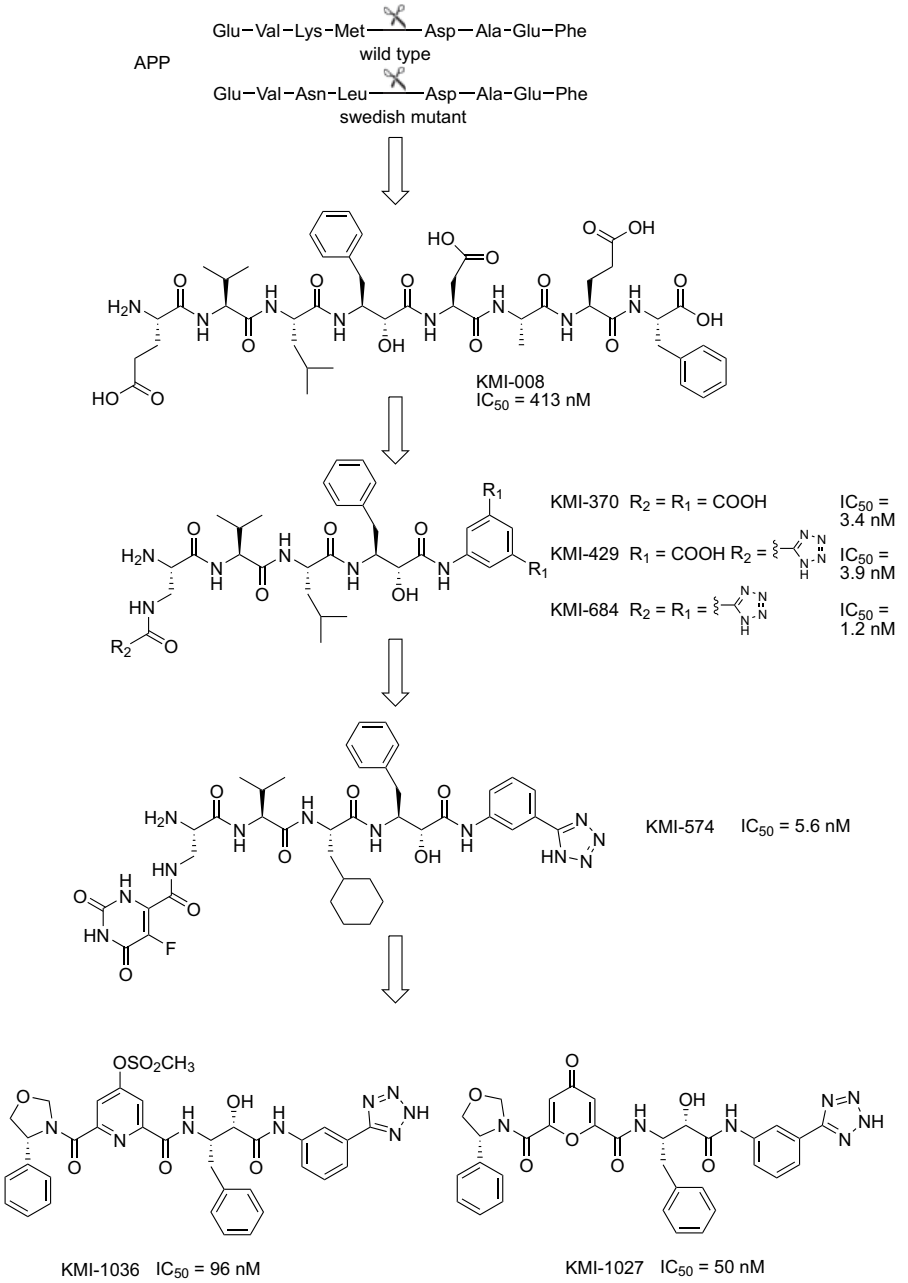


Figure 6.15 Design and structures of BACE-1 inhibitors.

also effective in inhibition of the sAPPβ (a soluble form of APP generated by the action of β-secretase) secretion from COS-7 cells cotransfected with both APP and BACE-1 (Figure 6.15) [207]. Since Pns, a phenylalanine mimetic, had a larger side-chain than Nst, a leucine mimetic, the contribution of the hydrophobic interaction at the S_1 subsite increased BACE-1 inhibition. KMI-008 was not suitable as an Alzheimer's disease therapeutic agent due to its long peptide chain. It was therefore necessary to minimize the number of natural peptide bonds and the molecular weight. In order to determine the smallest structure having BACE-1-inhibitory activity, peptide ladders were designed by gradually removing amino acids from KMI-008. Compounds lacking the P_4–P_2 positions displayed no inhibitory activity, that actually suggested the importance of N-terminal residues interactions, while those of the C-terminus were not as significant. SAR studies focusing on the P_4 and P_1' positions led to optimization of the structure of pentapeptidic KMI-370. KMI-370 had a relatively low molecular weight and was a very potent inhibitor effective on β-secretase activity in HEK-293 (human embryonic kidney) cells transfected with BACE-1 [208]. The *N*-β-oxalyl-L-2,3-diaminopropionic (*N*-β-oxalyl-L-DAP) group in KMI-370 was important for enhancing BACE-1 inhibition, but KMI-370 isomerized in solvent to much less potent *N*-α-oxalyl-L-DAP derivative (as it is known that *N*-β-oxalyl-L-DAP thermally isomerizes to an equilibrium mixture with *N*-α-oxalyl-L-DAP and this was also mentioned in the section regarding antibacterial agents, edeines). Hence, a tetrazole moiety was used as a bioisostere of the free carboxylic acid instead of oxalyl group to improve chemical stability (KMI-429) [209]. KMI-429 was studied *in vivo* and its intrahippocampal injection in wild-type mice markedly reduced production of amyloid-β peptides in both soluble and insoluble fractions [210].

At the same time another series of BACE-1 inhibitors with Ptns was synthesized. The design was based on the sequence of the wild-type APP where β-secretase recognizes Met at the S_1 side. Attempts to replace Pns by its thio derivative in octapeptide type structures did not give any more potent inhibitors. Then, when the size of the inhibitors was reduced to pentapeptides, some of the Ptns-containing compounds possessed inhibitory potency similar to their Pns counterparts [78]. These results showed the possibility of further design of BACE-1 inhibitors with Ptns at the P_1 position as well as the study of other aspartic proteases, and the application of compounds with Ptns or its diastereomer Aptns, as inhibitors.

The next step in the modification was to replace two carboxylic acid groups from the P_1' residue by tetrazole rings and the synthetic KMI-684 exhibited slightly more potent inhibitory activity [211]. KMI-684 was too polar to efficiently penetrate membranes due to the presence of three tetrazole moieties [212]. Consequently, substitution studies of the P_4 tetrazole group with hydrogen-bond acceptor groups resulted in the structure of KMI-574 with a 5-fluoroorotyl fragment at P_4 and with cyclohexylalanine at the P_2 position. Cyclohexylalanine, being a non-natural amino acid, could avoid recognition and premature metabolism by other proteases. KMI-574 was a potent BACE-1 inhibitor and even more effective than KMI-684 against sAPPβ production in HEK293 cells. To improve inhibitory activity in cultured cells, the acidic moieties at the P_1' were replaced with nonacidic and low-molecular sized

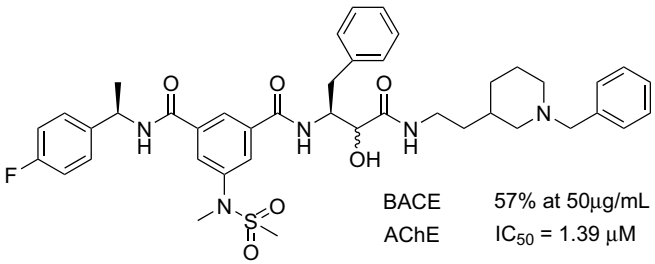


Figure 6.16 Inhibitor against BACE and AChE.

groups [213]. In order to improve *in vivo* enzymatic stability and permeability across the blood–brain barrier, nonpeptidic and small-sized KMI-1027 and KMI-1036 with Apns at P_1, (2*S*,3*S*)-AHPA, were afforded. Pns possessing nonpeptidic and small-sized derivatives exhibited no BACE-1 inhibitory activity. KMI-1027 and KMI-1036 with a heterocyclic ring at the P_2 position and a five-membered ring at the P_3 position are expected to be practical anti-Alzheimer's disease drug candidates [214, 215].

Despite the promising results, the actual effects of BACE-1 inhibitors for Alzheimer's disease patients need to be further evaluated. It is still important to explore novel drug candidates. Considering the complexity of Alzheimer's disease, the classic "one molecule, one target" solution may not be effective enough. To explore novel effective drugs for the Alzheimer's disease treatment, a series of dual inhibitors of acetylcholinesterase (AChE) and BACE-1 have been designed based on the multitarget-directed ligand strategy. The strategy of AChE and BACE-1 dual inhibitors might be a promising direction for developing novel drugs for Alzheimer's disease patients. To explore this concept, one example of employing α-hydroxy-β-amino acid as a hydroxymethylcarbonyl (HMC) scaffold was reported [216]. In a preliminary study, the hydroxyl of HMC in AHPA was not synthesized stereospecifically (Figure 6.16). AHPA was combined with an isophthalamide moiety and *N*-benzylpiperidine groups at the N- and C-termini, respectively. The HMC derivative showed greatly increased AChE activity at the expense of a dramatic loss of BACE-1 potency.

Researchers from academia and the pharmaceutical industry have contributed to the development of BACE-1 inhibitors, which may eventually lead to a practical Alzheimer's disease therapy [217]. In recognizing the potential difficulty in discovering such inhibitors, many research programs have begun to divulge data indicating that additional factors are now being considered in an effort to accelerate the optimization of potential drug candidates [218, 219]. The field has matured greatly and a diverse array of inhibitors has now been identified that have not previously been described as aspartyl protease inhibitors. These novel inhibitors will hopefully address the problems associated with central nervous system penetration and pharmacokinetics that have plagued early inhibitor series, and will move the design closer to the goal of a disease-modifying treatment for this devastating disorder [220].

6.6
Paclitaxel and its Derivatives

Among natural compounds including α-hydroxyl-β-amino acids, probably the most famous and the most intensively studied is paclitaxel (Taxol; Figure 6.17). Thus, this short section provide only a brief overview of the medicinal chemistry of paclitaxel with particular examples demonstrating the general tendency in the development of paclitaxel analogs with improved efficacy. Paclitaxel was discovered during US National Cancer Institute screening of plants for anticancer activity in 1971 [221]. This diterpenoid natural product represents a new class of anticancer agents having a specific mechanism of action. It promotes tubulin polymerization, leading to abnormally stable and nonfunctional microtubules, and resulting in apoptotic cell death [222]. The early development of paclitaxel was hampered by limited supplies of the drug isolated from the original source, the bark of the Pacific yew *Taxus brevifolia*. The problem was solved by the development of semisynthetic approaches to paclitaxel from (*2R,3S*)-phenylisoserine and a naturally occurring compound, 10-deacetylbaccatin III (DAB; Figure 6.17) isolated from a renewable source: leaves of the common yew (*Taxus baccata*) [223]. Several total syntheses of paclitaxel have been reported [224–232]. Despite the fact they are state-of-the-art organic chemistry, they are highly laborious, time-consuming and cost-ineffective, and hence completely unattractive as a competitive source of this antitumor agent. Paclitaxel is in clinical use for treating a variety of malignancies, including ovarian, breast, non-small-cell lung cancers, and AIDS-related Kaposi's sarcoma [233]. The therapeutic potential for paclitaxel against Alzheimer's disease and tuberculosis has also been suggested [234, 235]. The early search for taxoids led also to the development of docetaxel (Taxotere®; Figure 6.17), a semisynthetic analog of paclitaxel with a 3′-benzoyl group replaced by Boc (a group used commonly in peptide synthesis) and a lack of the C-10 acetyl group. Docetaxel is slightly more water soluble than paclitaxel and more potent in the tubulin polymerization process; however, the clinical efficacy of both taxoids is similar. Docetaxel is in clinical use for treating breast, lung, and prostate cancers [236]. Although paclitaxel exhibits superior antitumor activity, it suffers from drawbacks

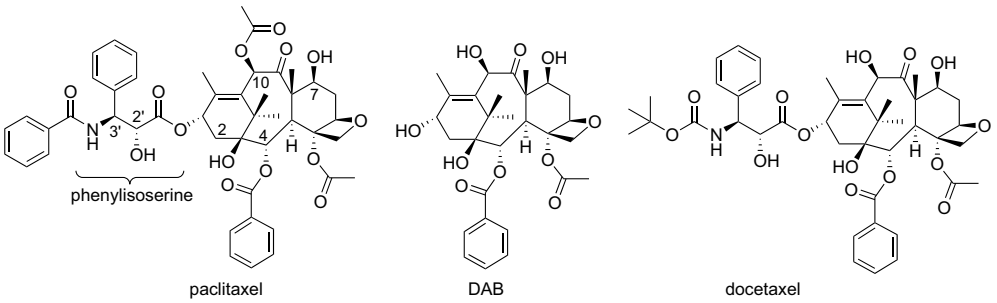
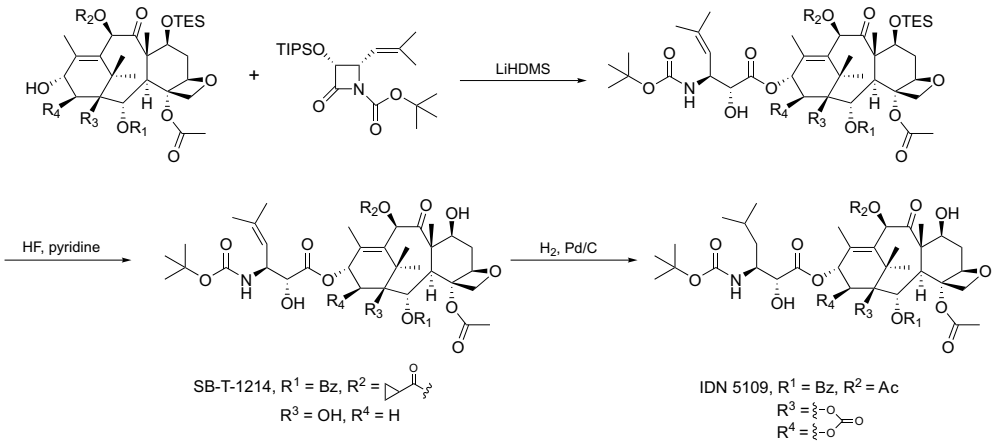


Figure 6.17 The structure of paclitaxel, DAB, and docetaxel.

related to drug-resistant cells of certain cancer types as well as dose-limiting toxicity corresponding to paclitaxel's side-effects [237, 238].

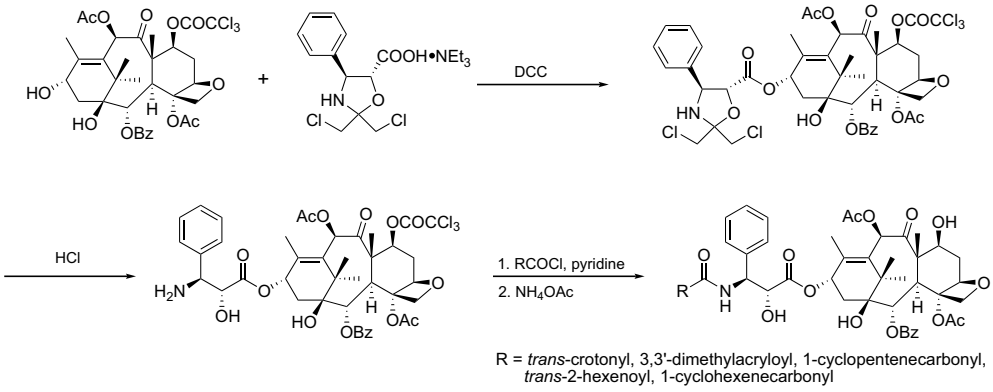
Additionally, paclitaxel has low water solubility and thus is solubilized with a detergent, Cremophor EL. Severe allergic reactions to Cremophor EL have been observed and premedication is often required [239, 240]. These shortcomings have resulted in extensive studies toward the development of new taxoid anticancer agents that would have fewer side-effects, enhanced activity against drug-resistant human tumors, and superior pharmacological properties.

A vast number of paclitaxel analogs have been synthesized [241–250]. Both the amino acid moiety and taxane ring have been extensively modified. However, almost immediately it was realized that not all positions could be modified without reducing paclitaxel antitumor potency. In general, not only the taxane ring is necessary for activity, but also the *N*-acylated α-hydroxy-β-amino acid moiety [251, 252]. Based on their SAR study, Ojima *et al.* indicated that the C-3′ phenyl group and 3′-*N* group can be replaced with other similar groups, the C-10 position can be modified with some acyl groups, and the C-2 position can be substituted [241]. They created large libraries of paclitaxel analogs using the Ojima–Holton coupling of triethylsilyl-protected baccatin III analogs with appropriate β-lactams as a precursor of the α-hydroxyl-β-amino acid (Scheme 6.20). This method is one of the most popular for the synthesis of paclitaxel analogs. Most of these compounds exhibit excellent antitumor activity with high potency against multidrug-resistant (MDR) cell lines [241, 253]. IDN 5109 entered clinical trials [254], although development of this compound has now been discontinued. Recently reported, the most promising candidate for clinical trials was its close analog, SB-T-1214 [255], which exhibited potent *in vivo* efficacy against highly drug-resistant pancreatic as well as human colon tumor xenografts in mice [256].



Scheme 6.20 Representative example of a synthetic route for paclitaxel analogs. Synthesis of IDN 5109 and SB-T-1214.

Several studies have also been performed on natural and semisynthetic 3′-*N*- and/or C-10 modified paclitaxel analogs, demonstrating that exchange of acyl moieties has a strong influence on taxoid activity against MDR tumors [257–262]. Kingston *et al.*, using a similar synthetic approach demonstrated in Scheme 6.20, synthesized a series of analogs of paclitaxel having modified 3′-*N*, C-2, and C-10 positions, and concluded that simultaneous modification of these positions produced effects on the bioactivity of paclitaxel that were not simply the sum of the effects of individual modifications [260]. Song *et al.* synthesized a series of 3′-*N*-acyl-paclitaxel analogs using a different synthetic route, with the key step including coupling of an oxazolidine-type side-chain precursor of (*2R,3S*)-phenylisoserine with 7-(trichloroacetyl)-baccatin III (Scheme 6.21) [261]. Simple esterification of baccatin III with phenylisoserine was not efficient due to the very low reactivity of the C-1 hydroxyl group. It was demonstrated that without multiple modifications of the taxane ring and α-hydroxyl-β-amino acid moiety, the 3′-*N*-position analogs could exhibit higher potency in both sensitive and resistant tumor cell lines.



Scheme 6.21 Synthesis of 3′-*N*-acyl-paclitaxel analogs.

The pharmaceutical companies have also been highly interested in novel paclitaxel analogs. In particular, Bristol-Meyers Squibb, who introduced paclitaxel to the market. Also, Sanofi Aventis, the "father" of docetaxel, put many efforts toward development of new taxoids [252]. Some of the paclitaxel derivatives introduced by them have reached clinical trials, such as the compounds presented in Figure 6.18 [263–266]. Generally, these modifications were based on the substitution of particular groups in paclitaxel by other moieties with similar size but yielding compounds with improved efficacy and/or activity against MDR tumors.

Another approach to improve paclitaxel efficacy while reducing side-effects corresponding to paclitaxel therapy is converting this drug to a prodrug form [242, 244, 245]. Paclitaxel prodrugs are usually designed by introducing a masking moiety to the C-2′ position as this hydroxyl group seems to be required for cytotoxic effects [251, 252]. Esters at this position can be synthesized selectively without protecting of the less reactive C-7 hydroxyl group.

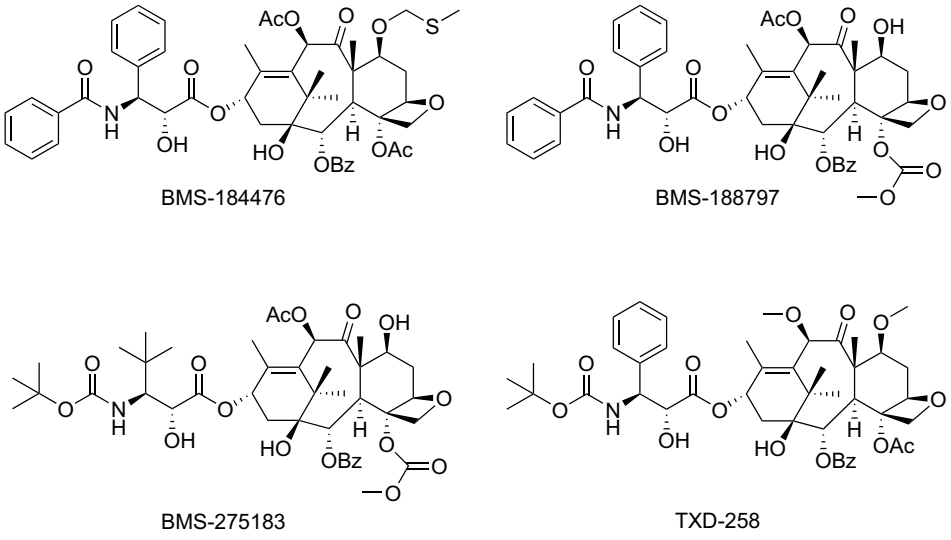
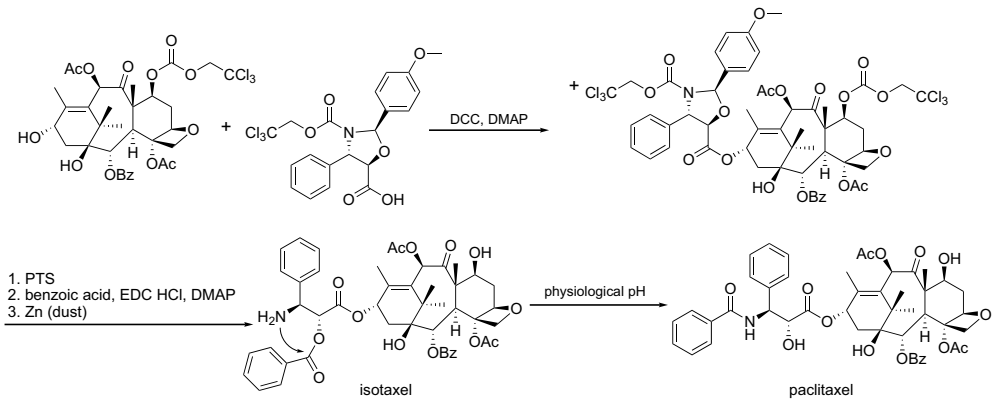


Figure 6.18 Examples of paclitaxel analogs that reached clinical trials.

As the water solubility of paclitaxel is very low (0.00025 mg/ml) [267], many prodrug designs were focused on improving its water solubility to avoid the use of detergents. Thus, the C-2′ position was substituted with variety of carboxylic esters [268, 269], phosphate esters [270], amino acids [271], sugars [272], and other hydrophilic groups [273]. One interesting example of a water-soluble prodrug is presented in Scheme 6.22 [267, 274–276]. Kiso *et al.* designed isotaxel, an isoform of paclitaxel. This prodrug released the parent drug via a method well-known in peptide chemistry, an *O*–*N* intramolecular acyl migration reaction [182], without any side-product formation under physiological conditions (pH 7.4). In contrast, under acid conditions or in solid state isotaxel was stable. Thus, potential undesired side-effects



Scheme 6.22 Synthesis of isotaxel and the release mechanism of paclitaxel.

corresponding to the release of the water-soluble moiety were omitted. This prodrug was synthesized from commercially available (*2R,3S*)-phenylisoserine with the key step including coupling of a 1,3-oxazolidine derivative with 7-Troc-baccatin III (Scheme 6.22). Although all these prodrugs were much more the water-soluble than the parent drug, advantages coming only from improvement of water solubility of paclitaxel seem to be insufficient for clinical development of these compounds [245].

Improvement of paclitaxel water solubility is an important issue in the development of safer anticancer therapy. However, prodrugs designed to be specifically delivered to tumor tissue need much more attention. A successful targeting strategy may overcome most paclitaxel side-effects while improve efficacy of the drug. One strategy comprises conjugation of paclitaxel to polymers. Such prodrugs can accumulate in tumor tissues because of enhanced permeability and retention (EPR) effect [277]. It is important that the polymer utilized fulfils the following criteria: possesses molecular weight above 40 kDa to allow improved entrapment of the prodrug in tumor tissues [278], and is water-soluble, nonimmunogenic, biocompatible, and non-toxic. Several polymers have been applied for EPR-based delivery of paclitaxel, including poly(ethylene glycol) [279], hyaluronic acid [280], *N*-(2-hydroxypropyl)methacrylamide copolymer [281], and carboxymethylcyclodextran [282]. Poliglumex (CT-2103, Opaxio™, formerly known as Xyotax, Figure 6.19) [283] is considered as one of the most successful prodrugs, and has reached advanced clinical trials against ovarian, non-small-cell lung and breast cancer [245]. This prodrug has been synthesized via simple coupling of paclitaxel molecules at the C-2′ position to carboxylic groups of poly(L-glutamic acid). Other prodrugs that may accumulate in tumor tissue via the EPR effect include paclitaxel conjugated to dendrimers [284, 285], protein [286], and nanoparticles [287].

There are several other possible targeting points for selective delivery of anticancer agents [288]. In tumor cells, membrane receptors are often highly overexpressed and thus they can be used for the design of tumor-targeting prodrugs [289]. Bombebsin/gastrin-releasing peptide receptor has been found to be associated with tumor growth and used for the design of a targeting prodrug of paclitaxel. Two copies of a peptide recognizing this receptor were conjugated via glutamic acid and a labile succinate

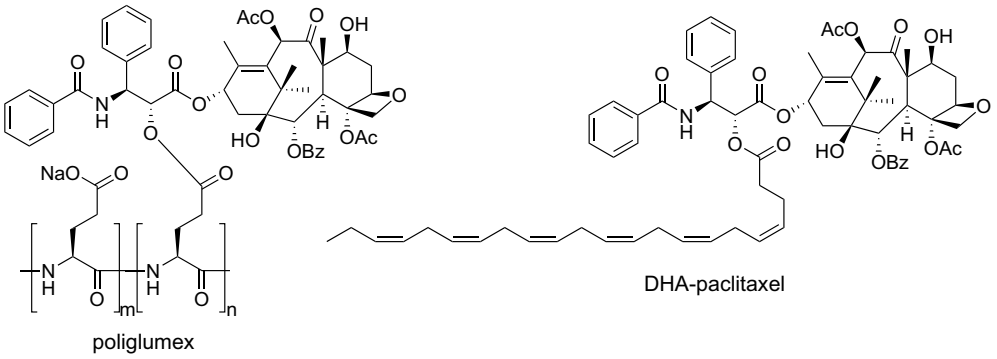
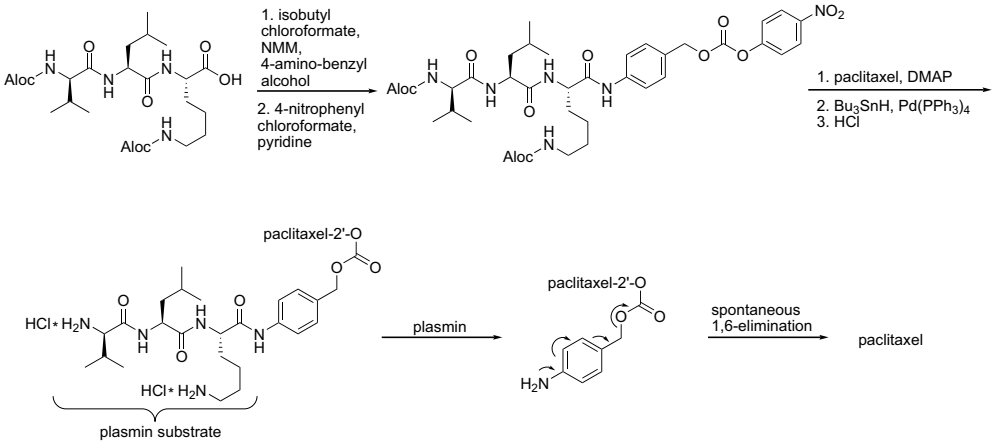


Figure 6.19 Prodrugs of paclitaxel in advanced clinical trials.

linker to a hydroxyl group of paclitaxel α-hydroxy-β-amino acid. The conjugate showed improved cytotoxicity over paclitaxel and over the prodrug with a single copy of the peptide in several human cancer cell lines possessing the targeted receptor [290]. Chen *et al.* developed a prodrug targeting the α-integrin receptor, which is highly overexpressed in metastatic cancer cells [291, 292]. Two copies of the cyclic peptide targeting receptor were conjugated to paclitaxel in the same manner as in the approach above and showed preferential cytotoxicity to integrin-expressing tumor cells. Several other prodrugs targeting somatostatin receptor [293], ErbB2 (receptor tyrosine kinases) [294], folate receptor [295], and luteinizing hormone-releasing hormone have been synthesized [296]. Receptors can be also targeted by monoclonal antibodies. For example, targeting a tumor by conjugation of paclitaxel derivatives to monoclonal antibodies has been studied by Ojima *et al.* [297]. Particular enzymes overexpressed by tumor tissue have also been used for targeted delivery of paclitaxel. For instance, plasmin, which is often overproduced at the surface of tumors cells, was able to cause selective release of paclitaxel from a prodrug developed by Scheeren's group [298]. Synthesis of the prodrug and the mechanism of parent drug release is presented in Scheme 6.23.



Scheme 6.23 Synthesis and release mechanism of a paclitaxel plasmin-sensitive prodrug.

The phenomenon of very low oxygen concentration in solid tumor tissue (hypoxia) [299] was chosen as a target for another group of prodrugs. These prodrugs have been designed to transform to the parent drug under reductive conditions. For example, Vrudhula *et al.* introduced a disulfide bond into the prodrug as cleavage of this bond is a typical biochemical transformation under a reductive environment [300]. Prodrugs that can be activated by selective light delivery to tumor tissues have been designed by Kiso *et al.* [301, 302]. These prodrugs include coumarin derivatives that can be easily cleaved under visible and UV light irradiation. An interesting prodrug was developed by simple esterification of paclitaxel with docosahexaenoic acid (DHA; Figure 6.19) [303]. It has been found that DHA accumulates

in tumors apparently to be used as biochemical precursor and energy source. This prodrug, known as a Taxoprexin®, reached advanced clinical trials against a wide range of tumors [245] and alongside poliglumex they are the only paclitaxel prodrugs that might be accepted in the clinic in the near future.

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7
Peptide Drugs

Chiara Falciani, Alessandro Pini, and Luisa Bracci

7.1
Lights and Shades of Peptide and Protein Drugs

The basis for life resides essentially in the variability and plasticity allowed by the combination of amino acids and by the ability of their different side-chains to interact with each other, either within the same molecule or with reacting groups of other biological molecules. While covalent bonds within amino acids allow construction of the primary structure of peptides and proteins, weak noncovalent bonds among amino acid side-chains allow protein folding into precise and unique three-dimensional structures as well as interaction of proteins and peptides with other biological molecules. Although the physical chemical laws governing interactions among amino acids are known, complexity and variability of their molecular "talking" is such that we are unable to predict on the basis of the simple string of amino acid sequence either how or with whom they can interact.

Thanks to their almost infinite ways of combining into different structures and interactions with each other and with other biological partners, peptides and proteins are the main agents in any biological mechanism, acting as enzymes, receptors, mediators of cell signaling, and structural cell components. No other molecule has comparable ability to fulfill almost any biological necessity.

The versatility of proteins and peptides, and their main role in any biological event, has raised great expectations for their pharmacological use [1]. To date, three types of molecules have been developed as drugs for the treatment of human diseases [2]: (i) small molecules, (ii) proteins, and (iii) peptides.

We should define the difference between proteins and peptides, given that they are both made by amino acids linked by covalent peptide bonds. In a generally accepted assumption, peptides are short linear strings of amino acids, normally not organized in a stable three-dimensional structure, at least before their binding to targets. The length limit between a peptide and a protein is arbitrarily fixed as 50 amino acids.

The common goal of all drugs, whether small molecules, peptides, or proteins, is a highly specific binding to a target protein that plays a key role in a disease. Peptides and proteins have potential as therapeutic molecules not only because of their

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versatility for target recognition, but also because they can engage a larger portion of the target protein, compared to small molecules, thus providing greater specificity.

Nonetheless, the large majority of the therapeutic molecules developed and marketed so far are small molecules. What have been the problems that made peptides and proteins lose in the market, the game for the ideal drug, compared to small molecules?

Some possible limits of either protein or peptides can be identified, which might have accounted for their delay as potential drugs with respect to small molecules. Some important drawbacks are common to peptides and proteins: (i) both have important limits for oral delivery, being degraded by digestive enzymes; (ii) both are not able to diffuse through cell plasma membrane (with a few exceptions for peptides); and (iii) both are generally not able to cross the blood–brain barrier. Apart from these common limits, there are others more typical of each class.

In the case of proteins drugs, the first reason accounting for the gap in their development and marketing in respect of small molecules resides in the higher level of complexity of proteins, which require more advanced technical approaches that became available much later than organic chemical synthesis, with the advent of molecular biology and recombinant DNA. The chemistry for the synthesis of small molecules and for their modification is much easier and also much cheaper than that necessary for developing protein drugs. Nonetheless, we should remember that a protein, insulin, was discovered in 1921 and marketed not much later, in its primary form, purified from animal sources. Several decades later, insulin was the first protein obtained by recombinant DNA technology to be marketed as a drug and still remains the leading protein drug, in a number of different chemically modified forms and delivery formulations. In fact, insulin is sometimes reported as a peptide rather than a protein, because its length (51 amino acids) is right at the edge of the theoretical limit of proteins. Actually, insulin in its active processed form is made of two chains linked by disulfide bonds and with a stable three-dimensional structure, which definitely makes it a protein.

The definition of peptides is helpful for understanding their strengths and limits as potential drugs, with respect to either small molecules or proteins.

The process for peptide synthesis and development is closer to that for the synthesis of small molecules than to the production of proteins. Costs for the development of peptides are variable, depending on peptide length and sequence, but are generally much lower than those required for the production of proteins by molecular biology technologies.

Although processes for the development of small-molecule and peptide drugs are similar, the latter offer a number of drug development challenges, which essentially are linked to their natural short half-life in body fluids. Peptides are much more labile in body fluids than the majority of proteins since they are readily degraded by circulating peptidases and proteases. The quick degradation of natural peptides is essential for their biological functions in that it is the primary natural strategy for modulating their actions. The switch-off of essentially all the biological actions of natural peptides is mediated by their degradation by peptidases that selectively recognize short amino acid sequences devoid of a stable three-dimensional structure.

The low biostability of peptides generates the necessity to add several steps for their manufacturing, including different chemical modifications. Moreover, their short half-life brings tough delivery and the necessity of high doses. The result is a higher manufacturing cost.

Despite the limitations of peptide molecules described above, several factors have largely increased expectations for the development of new peptide drugs in the last few years. These include: (i) progress in technologies and instrumentation for chemical peptide synthesis, allowing construction of peptide libraries of high chemical diversity; (ii) development of high-throughput methods for peptide selection; and, last but not least, (iii) the great progress of technologies for drug delivery.

We describe the state of the art of peptides as drugs, by summarizing peptide therapeutic molecules presently available for different clinical applications. We then describe available peptide drugs and possible perspectives in the two clinical applications with higher activity in peptide therapeutic development: cancer and infection, both billion dollar markets.

7.2
Peptide Drugs Available on the Market

About 40 peptide drugs are presently available on the market for several different clinical indications [1]. Approved agents include natural peptide sequences, such as natriuretic peptide, oxytocin, vasopressin, exendin-4, parathyroid hormone (PTH), calcitonin, and cyclosporine, as well as synthetic or functionalized peptides or peptide analogs, such as DDAVP®, Integrilin®, Lupron®, Symlin®, Fuzeon®, and Sandostatin®.

7.2.1
Natriuretic Peptide (Nesiritide)

Nesiritide (Natrecor®) is the recombinant form of human B-type natriuretic peptide (BNP). BNP is a 32-amino-acid peptide stabilized by a disulfide bond and it is normally produced by the ventricular myocardium. Nesiritide works to facilitate cardiovascular fluid homeostasis and is used to treat acutely decompensated congestive heart failure. It promotes vasodilation, natriuresis, and diuresis [3, 4]. Nesiritide is only administered intravenously.

7.2.2
Oxytocin

Oxytocin (Figure 7.1) is a natural cyclic peptide of nine amino acids. It is synthesized by neurosecretory hypothalamic neurons and then released into the blood from the posterior lobe of the pituitary gland, and it works both as a hormone and as a neurotransmitter in the brain. Oxytocin is best known for its roles in female reproduction by facilitating birth and breastfeeding. The structure of oxytocin is

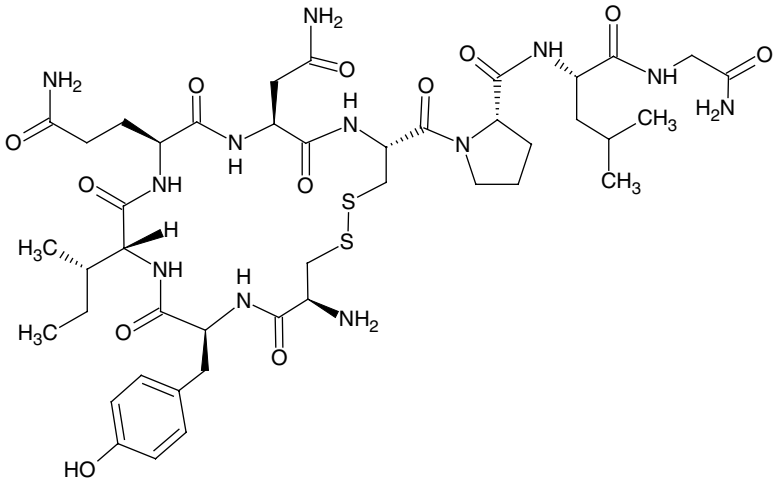


Figure 7.1 Oxytocin.

very similar to that of vasopressin, also a nonapeptide with a sulfur bridge, whose sequence differs from oxytocin at only two positions. Oxytocin and vasopressin were isolated and synthesized by Vincent du Vigneaud in 1953, work for which he received the Nobel Prize in Chemistry in 1955.

Synthetic oxytocin is administered either by injection or as nasal spray. Oxytocin has a half-life of typically about 3 min in the blood. Injected oxytocin analogs are used to induce labor and to increase uterine tone in acute postpartum hemorrhage [5, 6]. Due to oxytocin's role in the induction of labor, antagonists of oxytocin receptors have been designed for the prevention of preterm births, although with much lower success. The tocolytic agent atosiban (Tractocile) acts as an antagonist of oxytocin receptors and it is registered in many countries to suppress premature labor [6]. It has fewer side-effects than different drugs used for analogous indications, but some systematic studies indicated that it may have much lower activity [7].

7.2.3
Vasopressin

Vasopressin is an antidiuretic hormone secreted by hypothalamic neurosecretory neurons, like oxytocin. It binds to receptors in the distal or collecting tubules of the kidney and promotes reabsorbtion of water into the circulation. Due to the similarity of their sequence, there is some cross-reactivity between oxytocin and vasopressin: oxytocin has a slight antidiuretic function and high levels of vasopressin can cause uterine contractions. Vasopressin agonists are used for the treatment of enuresis, polyuria, diabetes insipidus, polydipsia, esophageal varices with bleeding, and in extreme cases of bedwetting by children. Terlipressin and related vasopressin analogs are used as vasoconstrictors in the management of hypotension [6].

7.2.4
Desmopressin

Desmopressin (DDAVP, Stimate®, Minirin®) is a synthetic analog of vasopressin, which is deaminated at its first residue and has D-Arg at position 8. Desmopressin is degraded more slowly than recombinant vasopressin, it can be given by either oral or intranasal administration, and requires less-frequent doses [8]. In addition, differently to vasopressin, it has little effect on blood pressure. Desmopressin can be also used to promote the release of von Willebrand factor and Factor VIII in patients with coagulation disorders such as type I von Willebrand disease, Factor VIII deficiency, and thrombocytopenia [9].

7.2.5
Blood Coagulation Inhibitors

Two different peptides have a major role in the treatment of acute coronary syndrome and ischemic complications of percutaneous coronary intervention, by acting on different steps of the formation of blood clots, as anticoagulants or inhibitors of platelet aggregation [10].

7.2.5.1 Bivalirudin

Bivalirudin (Angiomax®) is a 20-amino-acid peptide that belongs to the anticoagulant class and acts as a direct thrombin inhibitor. It is a synthetic analog of the naturally occurring drug hirudin (found in the saliva of the medicinal leech *Hirudo medicinalis*). Both bivalirudin and hirudin directly inhibit thrombin – a serine protease that plays a central role in the thrombotic process. Bivalirudin is thus indicated to reduce the risk of acute ischemic complications [11]. Hirudin is an irreversible inhibitor of thrombin, whereas bivalirudin is a reversible one. This leads to a small rate of severe bleedings under bivalirudin treatment. The half-life of bivalirudin is 25 min. In Europe bivalirudin is sold under the brand name Angiox®, and in other countries, including the United States, under Angiomax® (the licensing year is 2005 in most countries).

7.2.5.2 Integrilin (Eptifibatide)

Eptifibatide (Figure 7.2) is a cyclic heptapeptide, which is produced by solution-phase peptide synthesis. Eptifibatide works by preventing the binding of fibrinogen, von Willebrand factor, and other blood clotting factors to the glycoprotein IIb/IIIa protein. This leads to inhibition of platelet aggregation – a crucial step in the development of blood clots [12].

7.2.6
Gonadotropin-Releasing Hormone Agonists and Antagonists

7.2.6.1 Gonadorelin

Gonadorelin (Figure 7.3) is a synthetic gonadotropin-releasing hormone (GnRH). It is a synthetic decapeptide prepared using solid-phase peptide synthesis. GnRH is

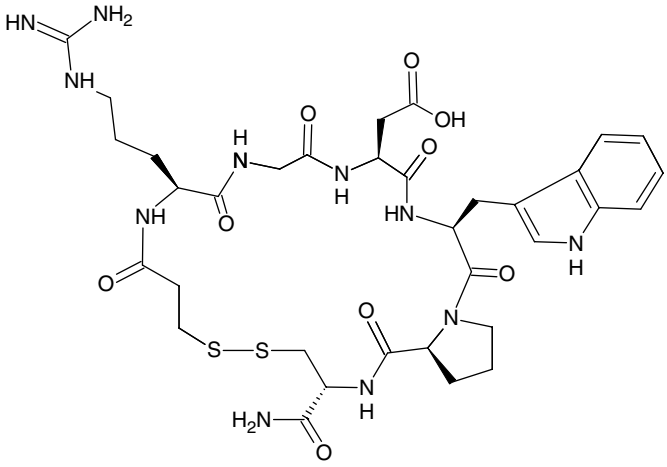


Figure 7.2 Eptifibatide.

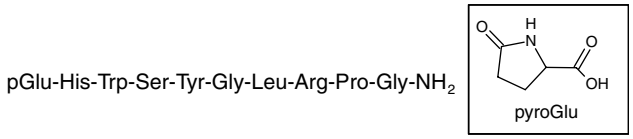


Figure 7.3 Gonadorelin.

responsible for the release of follicle stimulating hormone (FSH) and leuteinizing hormone (LH) from the anterior pituitary. Gonadorelin is used to simulate the physiologic release of GnRH in the treatment of delayed puberty, infertility, and induction of ovulation in women with hypothalamic amenorrhea. This results in increased levels of LH and FSH gonadotropins, which subsequently stimulate the gonads to produce reproductive steroids.

7.2.6.2 **Lupron(Leuprolide)**

Leuprorelin (or leuprolide acetate in the United States; Figure 7.4) is a nine-residue synthetic peptide analog of GnRH (GnRH agonist). By causing constant stimulation of GnRH receptors, leuprolide initially causes stimulation, but thereafter decreases, by receptor downregulation, pituitary secretion of LH and FSH. Like other GnRH agonists, leuprolide may be used in the treatment of hormone-responsive cancers such as prostate cancer or breast cancer as well as estrogen-dependent benign disorders like endometriosis [13] or uterine fibroids, to treat precocious puberty [14] and to control ovarian stimulation in *in vitro* fertilization.

pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt

Figure 7.4 Leuprolide.

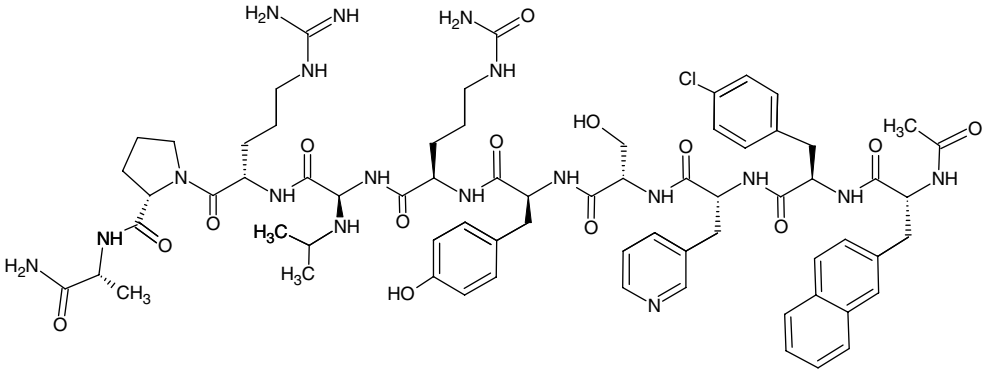


Figure 7.5 Cetrorelix.

7.2.6.3 **Cetrorelix**

Cetrorelix (Cetrotide®) (Figure 7.5) is a synthetic decapeptide that blocks the effects of GnRH (GnRH antagonist). It has essentially identical indications as leuprolide [15]. Cetrorelix competes with natural GnRH for binding to receptors and acts as a potent inhibitor of gonadotropin secretion.

7.2.6.4 **Degarelix**

On 24 December 2008, the US Food and Drug Administration (FDA) approved degarelix (Figure 7.6) for the treatment of patients with advanced prostate cancer in the United States. It was subsequently approved by the European Medicines Agency on 17 February 2009 for the same indication. Degarelix is a synthetic peptide derivative of the natural GnRH decapeptide, and it is a novel GnRH receptor blocker that provides immediate, profound, and sustained testosterone reduction, without an initial surge, which is the main drawback of GnRH agonists [16, 17].

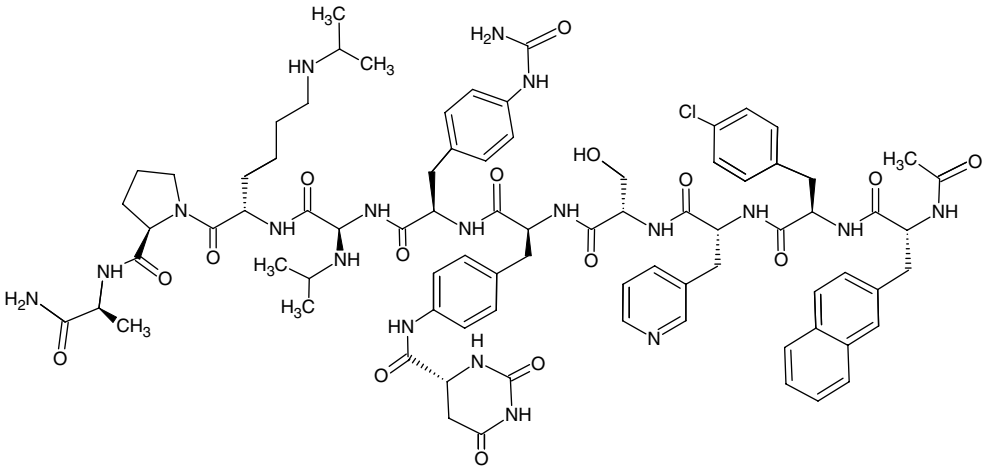


Figure 7.6 Degarelix.

7.2.7
Antihyperglycemics

Starting from the historical use of insulin as a drug, diabetes and glucose control have been a successful training field for the development of peptide drugs. Glucose control depends on two hormones, insulin and amylin, both produced in the β-cells of the pancreas. In patients with type I diabetes and often with type II, β-cells have been damaged or destroyed, resulting in insulin and amylin deficiencies.

Two peptides, pramlintide and exendin-4, are currently used in the clinic for glucose control [18]. A third one, liraglutide, is a lipopeptide that has obtained approval by the European Medicines Agency, in 2009 and by FDA on January 25, 2010. See Figure 7.7.

7.2.7.1 **Symlin (Pramlintide)**

Symlin is a antihyperglycemic agent. It is designed to mimic the activity of the naturally occurring hormone amylin, which is secreted together with insulin and is involved in postprandial glucose control. As a synthetic version of amylin, Symlin functions similarly to the naturally occurring hormone, working in partnership with insulin to improve glucose control.

Since native human amylin is highly amyloidogenic and potentially toxic, the strategy for designing pramlintide was to substitute residues from rat amylin, which is not amyloidogenic.

Symlin is specifically indicated for the meal-time treatment of type I and type II diabetes in combination with standard insulin therapy, in patients who have failed to achieve adequate glucose control on insulin monotherapy. Clinical studies showed that Symlin injection before a meal helps to lower blood glucose, leading to less fluctuation during the day and better long-term glucose control compared to patients taking insulin alone [19].

7.2.7.2 **Exendin-4**

Exenatide (Byetta®), also known as synthetic exendin-4, is a new agent for the treatment of type II diabetes. Exenatide, derived from a compound found in the saliva

1 KCNTATCATQRLANFLVHSSNNFGPILPPTNVSNTY-NH2

2 HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH2

3

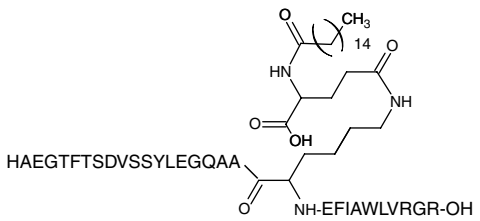


Figure 7.7 Pramlimtide (**1**), exendin-4 (**2**), and liraglutide (**3**).

of the lizard known as the Gila monster, is a 39-amino-acid peptide analog of glucagon-like peptide-1 (GLP-1).

The effects on glucose control obtained with exenatide treatment are thought to be due to several properties that are similar to those of the naturally occurring GLP-1. These actions include stimulating insulin release in response to glucose and preventing glucagon release after meals.

Animal studies have shown that exenatide helps preserve and form new β-cells, the insulin-producing cells in the pancreas, whose activity is impaired in type II diabetes [20]. Exenatide also appears to suppress appetite and favors weight loss.

7.2.7.3 **Liraglutide**

Liraglutide is a GLP-1 derivative for the treatment of type II diabetes. Liraglutide has a half-life after subcutaneous injection of 11–15 h, making it suitable for once-daily dosing (in contrast to the twice-daily dosage of exenatide) [21]. The prolonged half-life of Liraglutide is obtained by addition of a fatty acid molecule to the GLP-1 sequence.

7.2.8
Icatibant

Icatibant (Firazyr®) (Figure 7.8) is a synthetic decapeptide, containing several unnatural amino acids. It is derived from the sequence of bradykinin, and it is a selective and specific antagonist of bradykinin B2 receptors. Bradykinin is a peptide hormone that is formed locally in tissues, very often in response to a trauma. It increases vessel permeability, dilates blood vessels, and causes smooth muscle cells to contract, and it is responsible for the typical symptoms of inflammation, such as swelling, redness, overheating, and pain. These symptoms are mediated by activation of bradykinin B2 receptors.

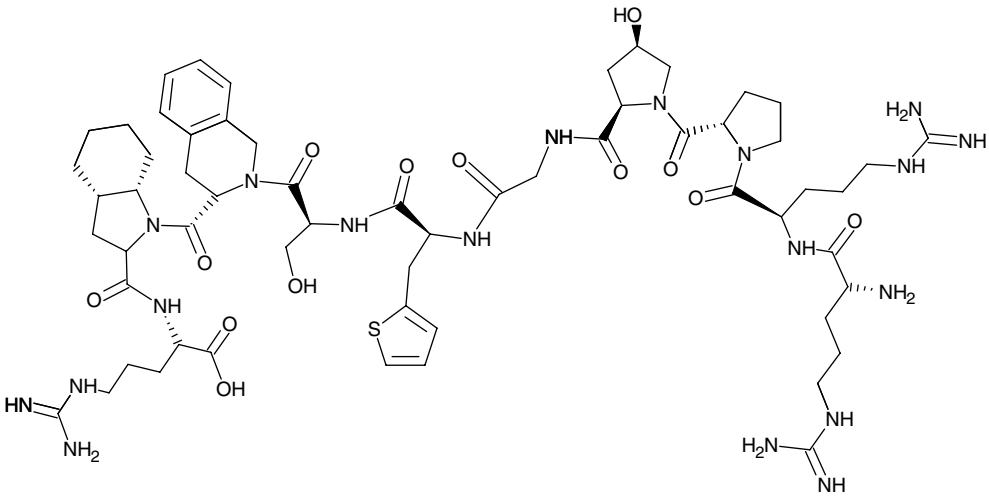


Figure 7.8 Icatibant.

Icatibant has been approved by the European Medicines Agency in 2008 for the symptomatic treatment of acute attacks of hereditary angioedema in adults [22], making it the first drug for the treatment of this rare hereditary disease.

7.2.9
Sermorelin

Sermorelin acetate is the acetate salt of an amidated synthetic 29-amino-acid peptide (Geref®) that corresponds to the amino-terminal segment of the naturally occurring 44-amino-acid human growth hormone-releasing hormone. Geref is similar to the full-length native hormone in its ability to stimulate growth hormone secretion by the pituitary gland, thus increasing plasma growth hormone concentration. Sermorelin is used in the treatment of children with growth hormone deficiency or growth failure [23].

7.2.10
Calcitonin

Calcitonin (Figure 7.9) is a 32-amino-acid linear polypeptide hormone that is produced in humans primarily by the parafollicular cells of the thyroid. It acts to reduce blood calcium, opposing the effects of PTH. Calcitonin inhibits bone removal by osteoclasts and promotes bone formation by osteoblasts. Fish calcitonin, particularly that from salmon, is similar to human calcitonin and more active. It is used for treating postmenopausal osteoporosis, hypercalcemia, and Paget's disease – a bone disease characterized by an abnormally accelerated remodeling of the bones, which become weak and painful. Treatment of osteoporosis with calcitonin was approved by the FDA in 1985. At present, calcitonins for medical use are produced either by recombinant DNA technology or by chemical synthesis. The FDA approved calcitonin intranasal spray in March 1991.

7.2.11
Parathyroid Hormone

Teriparatide (Forteo®; Figure 7.10) is a recombinant form of PTH, used in the treatment of some forms of osteoporosis [24, 25]. Teriparatide reproduces the 1–34 sequence of the 84-amino-acid human PTH. Endogenous PTH is the primary regulator of calcium and phosphate metabolism in bone and kidney. PTH increases serum calcium, partially accomplishing this by increasing bone resorption. Chronic elevation of the PTH level induces bone resorption, whereas intermittent pulses of PTH activate osteoblasts more than osteoclasts. Injections of teriparatide once a day have a net effect of stimulating new bone formation.

CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-OH

Figure 7.9 Human calcitonin.

MIPAKDMAKVMIVMLAICFLTKSDGKSVKKRSVS-OH

Figure 7.10 Teriparatide.

Teriparatide was approved by the FDA in 2002 for the treatment of osteoporosis in men and postmenopausal women.

7.2.12 Cyclosporine

Cyclosporine A (Figure 7.11) is a cyclic 11-amino-acid peptide, containing a single D-amino acid, naturally produced by the fungus *Beauveria nivea*. Cyclosporine is a potent immunosuppressant drug and was approved in 1983 for clinical use to prevent graft rejection in transplantation. Due to its potent immunosuppressive activity, cyclosporine has been investigated for use in several different autoimmune diseases, and it is used in psoriasis, severe atopic dermatitis, pyoderma gangrenosum, and rheumatoid arthritis and related diseases.

7.2.13 Fuzeon

Enfuvirtide (T-20; Fuzeon) is a HIV fusion inhibitor, the first of a novel class of antiretroviral drugs used in combination therapy for the treatment of HIV-1 infection.

T20 was rationally designed to mimic a region of HIV-1 gp41 in order to interfere with conformational changes that are required for the gp41-mediated fusion of the viral and cellular membrane. HIV fusion inhibitors work by preventing virus entry

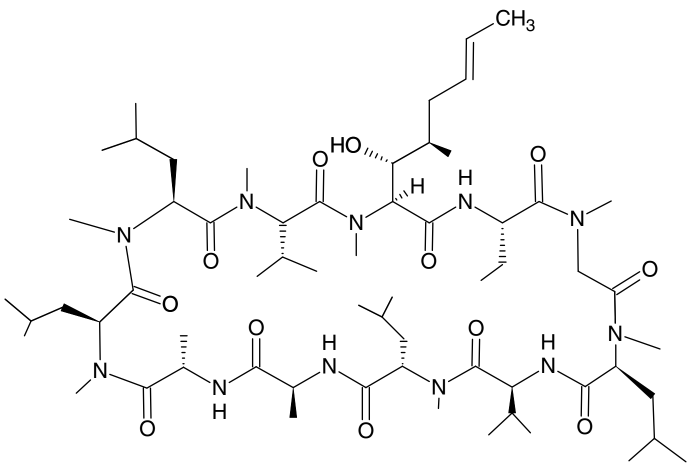


Figure 7.11 Cyclosporine A.

inside target cells. Fuzeon must be administered by injections, generally twice a day [26].

The synthesis of enfuvirtide is complex and expensive. This, together with the high doses that must be administered daily to achieve drug effectiveness, accounts for the high price of Fuzeon therapy, whose estimated cost is US$25 000 per year in the United States [27].

7.3 Approved Peptides in Oncology

Although Paul Ehrlich's concept of "magic bullet" was introduced for the selective killing of infective agents and it is applicable to almost any "ideal drug," it is probably in the field of cancer chemotherapy (another term that was introduced by Ehrlich) that magic bullet drugs have been most sought. The goal of modern cancer therapy is in fact the selective killing of tumor cells, in order to overcome nonspecific toxicity of most less-selective anticancer drugs. The selective killing of cancer cells by molecules that might work as magic bullets requires identification of tumor-specific targets, which are not so easily found, considering that cancer cells, as opposed to microbes, are genetically identical to any healthy cells. Nonetheless, a number of tumor-associated antigens, selectively expressed or overexpressed by tumor cells, have been described. Some of these can already be targeted by agents available on the market and many others are under investigation.

Most tumor-targeting agents presently in use in the clinic are either antibodies or small molecules. Due to their chemical/physical features, antibodies are targeted to membrane and extracellular tumor antigens, whereas most small molecules have cytoplasmic targets, which are usually enzymes. The high specificity of antibody binding is an undoubted advantage in the use of proteins as tumor-targeting agents. Nonetheless, antibodies are not so good as bullets, since they are seldom effective in cancer cell killing, unless they are conjugated to drugs or to other proteins or enzymes in order to produce direct or indirect cell toxicity.

Chemical conjugation of antibodies generally implies nonhomogeneous modification of the protein. This, together with other molecular features of antibodies, like their relevant size, which might limit their penetration into solid tumors, represent limits in the use of antibodies for tumor-targeted therapy.

Peptides may offer some advantages over antibodies as tumor-targeting agents: (i) thanks to their smaller size, they can more easily penetrate solid tumors; (ii) they can be easily synthesized and can be conjugated to functional units, giving chemically defined molecules; and (iii) they are stable to harsh conditions, often necessary for the coupling to functional units.

Due to their physical/chemical features, which limit their penetration inside the cells, peptides are more suitable for membrane and extracellular tumor targets. Nonetheless, a few peptide drugs have been developed against intracellular targets, like the modified tripeptide bortezomib, which is specifically targets the 26S proteasome and the DNA-binding peptide actinomycin D [28–31].

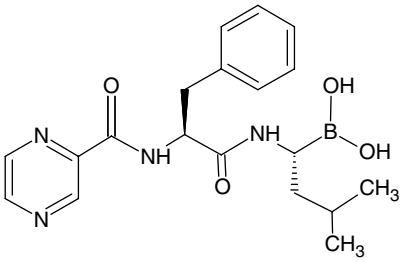


Figure 7.12 Velcade.

7.3.1
Bortezomib

Bortezomib (Velcade®; Figure 7.12) has been filed as a small molecule and was approved by the FDA in 2003 for the treatment of multiple myeloma. It is a pseudo-dipeptide, with a half-life from 9 to 15 h. Bortezomib is a reversible inhibitor of the chymotrypsin-like activity of the 26S proteasome in mammalian cells, and it is effective against dividing multiple myeloma and leukemic cells. In addition, bortezomib appears to increase the sensitivity of cancer cells to traditional anticancer agents (e.g., gemcitabine, cisplatin, paclitaxel, irinotecan, and radiation). It is approved, though due its cardiotoxicity, only for treatment of multiple myeloma in patients who have not been successfully treated with at least two previous therapies.

7.3.2
Actinomycin D

Actinomycin D (Dactinomycin®; Figure 7.13) is a chromopeptide antineoplastic antibiotic isolated from the bacterium *Streptomyces parvulus*. The use of actinomycin D as an antibiotic is precluded by its very high toxicity, but it is one of the older chemotherapy drugs. Actinomycin D is composed of two cyclic peptides attached to

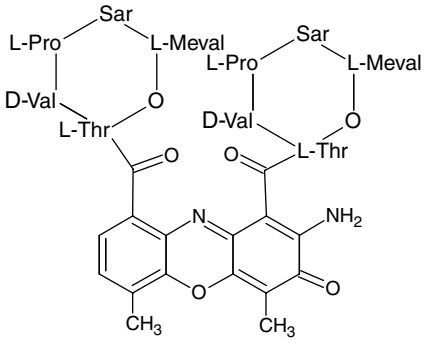


Figure 7.13 Actinomycin D.

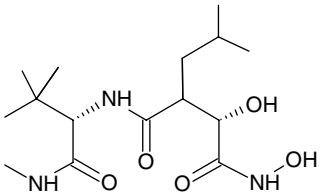


Figure 7.14 Marimastat.

a phenoxazine; it has a half-life of 36 h. It is an approved drug (filed as a small molecule) for the following indications: treatment of Wilms' tumor, childhood rhabdomyosarcoma, Ewing's sarcoma, and metastatic, nonseminomatous testicular cancer as part of a combination chemotherapy and/or multimodality treatment regimen. Actinomycin D binds to DNA and inhibits transcription [32, 33].

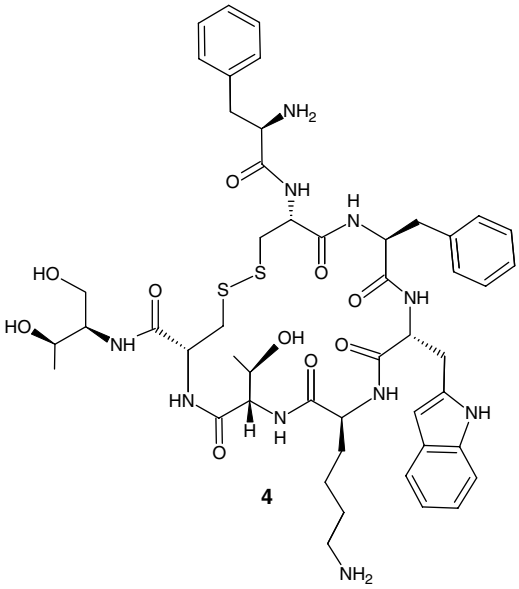
7.3.3
Marimastat

Marimastat (Figure 7.14) is approved for the treatment of cancer; it is an angiogenesis and metastasis inhibitor. It is a small molecule, a pseudo-tripeptide, and belongs to the category of antineoplastic agents and enzyme inhibitors. Marimastat binds to and inhibits matrix metalloprotease-2 and -9, thereby preventing the degradation of the basement membrane by these proteases. This antiprotease action prevents the migration of endothelial cells needed to form new blood vessels. Inhibition of matrix metalloproteases also prevents the entry and exit of tumor cells from existing blood vessels, thereby preventing metastasis [34–36].

7.3.4
Octreotide

The finding that receptors for different endogenous peptides are overexpressed in several primary and metastatic human tumors [37–39] has further increased the number of possible tumor antigens to be targeted by peptide agents. The peptide receptor tumor-targeting strategy offers the advantage of contemporarily providing a possible tumor target and its candidate peptide-targeting agent. Presently, approved peptide drugs for cancer therapy, which are based on the peptide receptor targeting strategy, essentially comprise peptide analogs of the endogenous peptide somatostatin, although many other peptide candidate are under investigation or in clinical trials [40, 41].

Octreotide (Atrigel®, Longastatin®, Sandostatin, Sandostatin LAR®), first synthesized in 1979, is a cyclic octapeptide that mimics the natural peptide somatostatin (Figure 7.15). Octreotide **4** was first approved by the FDA in 1988 for the treatment of carcinoid tumors and vasoactive intestinal peptide (VIP)-secreting adenomas. In June 1995, it also received FDA approval for the treatment of acromegaly.



AGCKNFFWKTFTSC-OH

5

Figure 7.15 Octreotide (**4**) and somatostatin (**5**).

In November 1998, the FDA approved a long-acting dosage form of octreotide, Sandostatin LAR Depot, for the treatment of acromegaly and to control the symptoms of carcinoid tumors and VIP adenomas. There are presently many different octreotide drug forms, produced by different companies, which have received FDA approval.

Octreotide exerts pharmacologic actions similar to the natural hormone, somatostatin. Somatostatin **5** is produce by neuroendocrine neurons in the hypothalamus, and in the periphery it is secreted in different locations in the gastrointestinal tract and by δ-cells in the pancreas. It is an inhibitory cyclic peptide, which regulates neurotransmission and inhibits cell proliferation. Octreotide has also been used to treat the symptoms associated with metastatic carcinoid tumors [42–45].

7.3.5
Vapreotide

Vapreotide (Sanvar®, Sanvar IR®; Figure 7.16) is an analog of octreotide. It is a synthetic octapeptide (registered as a small molecule) somatostatin analog. It was approved for the treatment of esophageal variceal bleeding in patients with cirrhotic liver disease and has also shown efficacy in the treatment of patients with AIDS-related diarrhea. It inhibits the release of peptides and vasoactive compounds from neuroendocrine tumors. It shows a half-life of 30 min [46].

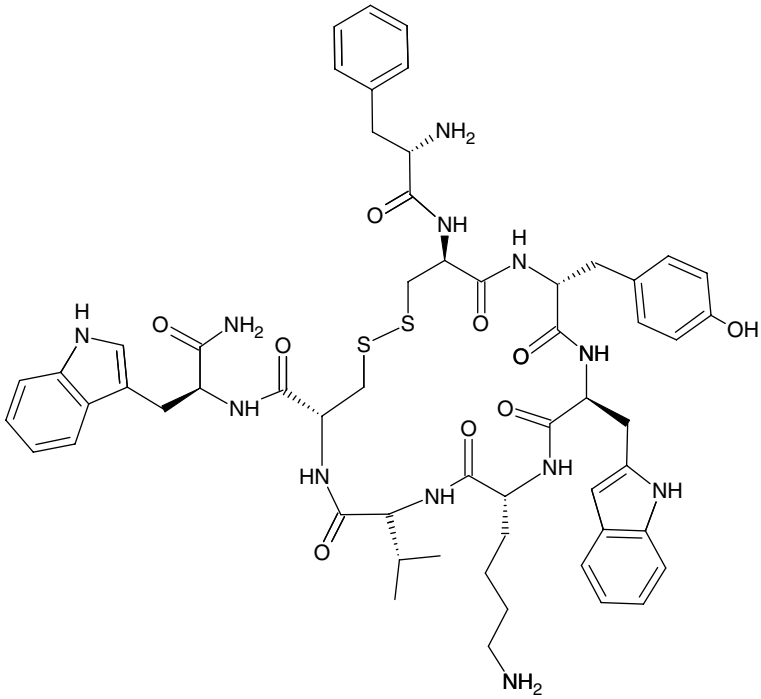


Figure 7.16 Vapreotide.

7.3.6
Octreoscan

Octroscan (Figure 7.17) is octreotide bearing ^{111}In chelated by the chelating agent diethylene triamine pentaacetic acid. It is used to detect gastro-entero-pancreatic neuroendocrine tumors in patients with high clinical suspicion.

It is worth noting that peptides that have been used in oncology so far are either very short molecules (two or three residues) or cyclic peptides. This ensures higher metabolic stability and, when peptides have endocellular targets, like in the case of bortezomib, the possibility to enter the cell. These two limits, length and forced cyclic structure, have certainly limited development of many different peptide sequences, which might have showed promising *in vitro* activity against tumor cells. Moreover, in the particular case of peptide receptor tumor targeting, peptide-targeting agents should be coupled to cytotoxic units, in that, with the notable exception of the cell proliferation inhibitor somatostatin, regulatory peptides that have overexpressed receptors on cancer cells are generally not cytotoxic *per se*. Different chemical modifications, which can be introduced to obtain stabilized analogs, may deeply modify peptide affinity or specificity and coupling of peptides to effector units for tumor imaging or therapy may also interfere with peptide biological activity.

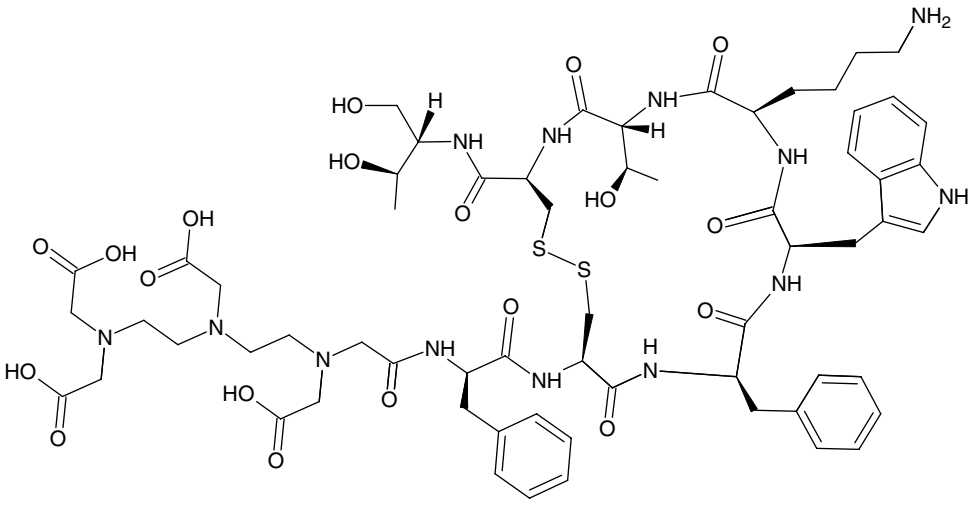


Figure 7.17 Octreoscan.

7.4 Antimicrobial peptides

Today, a number of pathogens like *Staphylococcus aureus*, *Mycobacterium tuberculosis*, some enterococci, *Pseudomonas aeruginosa*, and many other Gram-negative bacteria have developed resistance against most of the traditional antibiotics as well as against those of the new generation. It has therefore become increasingly important to develop new antibiotics. Antimicrobial peptides are considered one of the best alternatives to traditional antibiotics which generally cause the selection of resistant bacteria [47]. Antimicrobial peptides generally consist of 6–50 amino acids and have a positive net charge. Generally, cationic peptides selectively interact with anionic bacterial membranes and with other negatively charged structures such as lipopolysaccharide and DNA. Eukaryotic membranes, in their external layer, are generally less negative than bacterial membranes and they also possess cholesterol molecules that stabilize them. These differences are the basis for cationic peptides' selectivity. Their mechanism of action is consequently due to the specific binding of peptides to bacterial membranes, thus provoking cell permeation and, in some cases, metabolic pathway inhibition. The specific mechanism of action depends on the peptide structure, but generally they are thought to transit the outer membrane via self-promoted uptake. After insertion into the membrane, antimicrobial peptides act by either disrupting the physical integrity of the bilayer, via membrane thinning, transient poration and/or disruption of the barrier function, or translocate across the membrane and act on internal targets. Several models describe these events, including the reorientation of peptide molecules perpendicular to the membrane to form either barrel–stave or toroidal channels, the breakdown of membrane integrity as a result of the swamping of membrane charge by a "carpet" of peptides at the interface, the detergent-like dissolution of patches of membrane, and the formation

of peptide–lipid aggregates within the bilayer. Short cationic amphiphilic peptides are present in virtually every life form as natural antibiotics [47]. In mammals, these peptides often have rather weak antimicrobial activity under physiological conditions and their ability to modulate the immune response through a variety of mechanisms may be more important [48, 49]. Cationic peptides are being considered as a new generation of antibiotics, as well as innate immune modulators.

Studies have been aimed at the identification and characterization of antimicrobial peptide sequences by studying their mechanism of action, their toxicity for eukaryotic cells, and their therapeutic efficacy when administered topically or systemically. Unfortunately, two main problems have hindered the development of antimicrobial peptide drugs. The first is that selectivity of natural antimicrobial peptides for bacteria is generally too low and they appear to be very toxic for eukaryotic cells, particularly erythrocytes, generating a high degree of hemolysis. The second is linked to the generally short half-life of peptides *in vivo*. These are the main reasons why only few antimicrobial peptides have reached the market in the last 10 years. Polymyxin and daptomycin are two successful examples.

Initially, the starting point for new antimicrobial peptide development was the identification of natural antimicrobial peptides followed by their modification and optimization. Companies such as Magainin Pharmaceuticals (renamed Genaera; www.genaera.com) and Micrologix (Migenix; www.migenix.com) designed therapeutic peptides that differed from natural progenitor antimicrobial sequences by only a few amino acids. One of these "first-generation" antimicrobial peptides was pexiganan (MSI-78), a synthetic 22-amino-acid variant of the amphibian peptide magainin 2 (Magainin Pharmaceuticals) (Figure 7.18). Even though phase III clinical studies proved pexiganan to be efficient in wound healing, with few reports of toxicity, the FDA rejected this potential new drug in 1999 as it did not offer any great advantage over the current standard of care. Similarly, IB367, an analog of the pig endogenous peptides, protegrins, failed to achieve efficacy against polymicrobic infections in oral mucositis. Xoma (www.xoma.com) developed an injectable formulation of the cationic bactericidal/permeability-increasing protein fragment rBPI21 (NEUPREX1®), which showed only a trend towards efficacy in a phase III trial for endotoxin-mediated complications of meningococcal disease.

Currently, only one anti-infective with topical application has shown efficacy in phase III clinical studies (MX-226; also known as CPI-226, now termed Omigard™).

Omigard (omiganan pentahydrochloride 1% gel; Migenix; Figure 7.19), a peptide based on the sequence of the bovine endogenous antimicrobial peptide indolicidin, was developed for the prevention of contamination of central venous catheters. In a

6 GIGKFLKKAKKFGKAFVKILKK

7 GIGKFLHSAKKFGKAFVGEIMNS

8 RGGLCYCRGRFCVCVGR

Figure 7.18 Pexiganan (**6**), magainin 2 (**7**), and IB367 (**8**).

ILRWPWWPWRRK-NH_2

Figure 7.19 Omigard.

completed phase III study, Omigard demonstrated a statistically significant 49% reduction of local catheter site infections, as well as a 21% reduction of catheter colonization (http://www.migenix.com/prod_226.html). Cadence (www.cadence-pharm.com) is currently conducting a confirmatory phase IIIb study of Omigard for the prevention of local catheter site infections.

Other current trials involve successfully completed phase II clinical trials against mild to moderate acne (indolicidin-based MX594AN; Migenix) and completed phase I trials for the prevention of infections in patients undergoing allogeneic stem cell transplantation (human lactoferricin-based hLF1-11; AM Pharma; www.am-pharma.com).

7.4.1
Polymyxin

Polymyxins are a group of nonribosomal cationic peptides with a cyclic structure consisting of five different compounds (polymyxin A–E). Only polymyxins B and E have been used in clinical practice. The group of polymyxin E is called colistin. Polymyxins are strongly antimicrobial, and also bind and neutralize bacterial endotoxins. Polymyxin B (Figure 7.20) differs by only one amino acid from colistin. Colistin and polymyxin B are produced from *Bacillus* spp. They were first used in Europe during the 1950s and in the United States since 1959. The intravenous formulations of colistin and polymyxin B were used for approximately two decades,

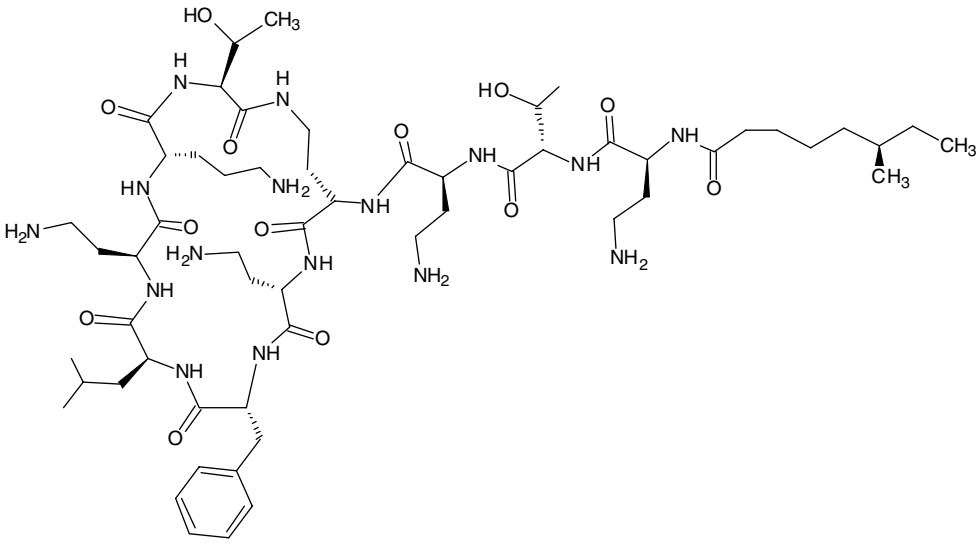


Figure 7.20 Polymyxin B1.

but were gradually abandoned worldwide in the early 1970s because of the reported severe toxicities [50]. During the past two decades, intravenous administration of colistin was mainly restricted for the treatment of respiratory tract infections caused by multidrug-resistant (MDR) Gram-negative pathogens in patients with cystic fibrosis [50]. On the other hand, polymyxins have been used worldwide in topical and ophthalmic solutions for decades. In the recent literature, encouraging findings regarding the reuse of colistin for the treatment of infections caused by MDR pathogens such as *P. aeruginosa* and *Acinetobacter baumannii* were reported [51].

7.4.2
Daptomycin

Daptomycin (Cubist; www.cubist.com; Figure 7.21) is an anionic lipopeptide antibiotic with bactericidal activity against Gram-positive microorganisms. In September 2003, the FDA approved daptomycin for the treatment of complicated skin infections caused by susceptible strains of *S. aureus* (including methicillin-resistant strains), *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subspecies *equisimilis*, and *Enterococcus faecalis* (vancomycin-susceptible strains only) (Cubicin®: package insert; Cubist Pharmaceuticals, Lexington MA; 2003). Intriguingly, daptomycin is Ca^{2+}-dependent and, in the presence of Ca^{2+}, it has a similar action on membranes to the cationic antimicrobial peptides.

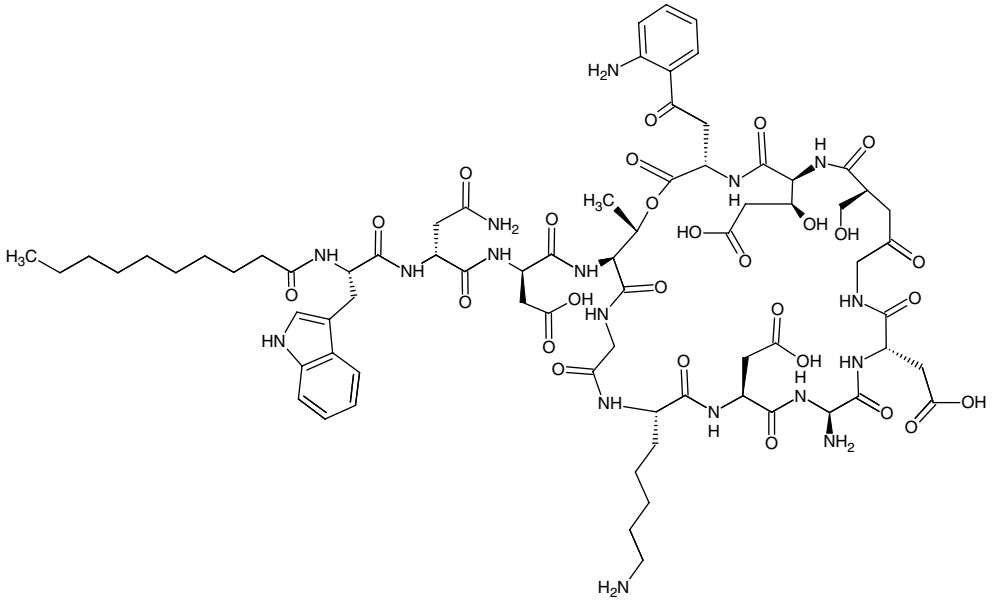


Figure 7.21 Daptomycin.

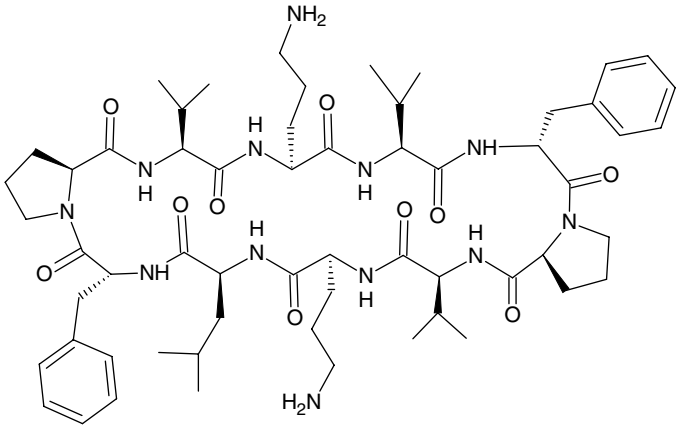


Figure 7.22 Gramicidin S.

7.4.3
Gramicidin S

Gramicidin S (Figure 7.22) is an antibiotic effective against some Gram-positive and Gram-negative bacteria as well as some fungi. It is a derivative of gramicidin, produced by the Gram positive bacterium *Bacillus brevis*. Gramicidin S is a cyclodecapeptide, constructed as two identical pentapeptides joined head to tail, formally written as cyclo(-Val–Orn–Leu–D-Phe–Pro-). Gramicidin S has historically been employed as a topical antibiotic for the treatment of infections from superficial wounds. Gramicidin S causes hemolysis at low concentrations and thus is not an effective drug for the treatment of systemic infections.

7.5
Perspectives

As extensively explained in the previous sections, physiological degradation by peptidases and proteases is still the bottleneck for the development of peptides as drugs.

In 2003, Bracci *et al*. demonstrated that peptides synthesized in an oligo-branched form can retain biological activity – or even increase it through multivalent binding – and become very resistant to circulating peptidases. Protease-resistant oligo-branched peptides can thus escape most general difficulties related to development of peptide drugs.

The solid-phase synthesis of branched peptides was first described by Tam [52] and is based on the elegant concept of using reactive groups on the side-chains of trifunctional amino acids to construct branched peptide-based molecules. In standard solid-phase synthesis, which proceeds from the C- to N-terminus, amino acids

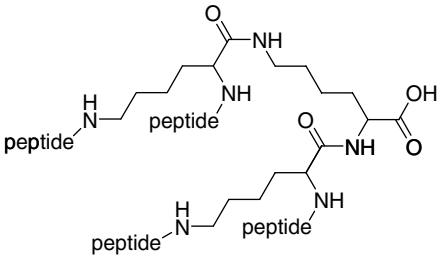


Figure 7.23 Tetra-branched peptide.

with side-chain amino groups, typically lysine, are usually preferred. One lysine will allow synthesis of a two-branched peptide, three lysines will give rise to a tetra-branched peptide (Figure 7.23), and so on. These branched peptide molecules were given the name of multiple antigen peptides, because they were introduced as potential synthetic peptide immunogens, although different applications were subsequently concerned [53, 54]. A generally accepted advantage of branched peptides is that they enable multivalent molecules with a defined, unambiguous chemical structure to be constructed.

A remarkably higher *in vivo* efficiency of the oligo-branched form with respect to the monomeric peptide was reported, and was demonstrated as due to acquired resistance to protease and peptidase activity [55, 56]. Biological stability of branched peptides was demonstrated with peptides of different sequence, length, and origin, and for either *de novo* generated sequences or endogenous peptides [55–60].

7.5.1
Branched Peptides as Tumor-Targeting Agents

Oligo-branched peptides can be novel tumor-targeting drugs that combine the advantages of antibodies and small molecules, which are the two alternative classes of specific tumor-targeting agents. Oligo-branched peptides allow multimeric binding, despite a much lower molecular weight compared to antibodies, and can be easily synthesized and chemically modified, like the small molecules. In fact, some examples of branched peptides in cancer are reported in the literature. Oligo-branched peptides containing the RGD sequence, which specifically targets $\alpha_v\beta_3$ integrin, were reported for imaging [61] and possible therapy [62] in different tumor models. In a few cases, different sequences were combined in the same molecule to construct heterobivalent peptides able to cross-link multiple surface receptors. Branched bombesin–RGD peptide heterodimers were reported for the imaging of different tumors [63, 64].

Branched peptides can also be obtained using nonpeptidic scaffolds able to produce polymeric structures, generally reported as dendrimers [65]. With a few exception, dendrimers result in a highly branched structure, often using commer-

cially available modified polyamidoamine scaffolds, which produce molecules with a much higher molecular weight and very different properties with respect to oligo-branched peptides. Dendrimers were tested in a number of possible clinical applications, including drug delivery and cancer treatment.

A proof of concept for the use of oligo-branched peptides as selective tumor-targeting agents has been obtained with peptides reproducing the sequence of the endogenous peptide neurotensin.

Neurotensin is a 13-amino-acid peptide originally isolated from calf hypothalamus [66]. Like somatostatin and many other human regulatory peptides, neurotensin has the dual function of a neurotransmitter or neuromodulator in the nervous system and a local hormone in the periphery. Neurotensin receptors are overexpressed in severe malignancies like small cell lung cancer, colon, pancreas, and prostate carcinomas [67–69].

Neurotensin stabilized analogs were proposed for tumor therapy [70–78], and neurotensin is still considered the best possible candidate for peptide-based therapy of exocrine pancreatic carcinomas in consideration of the high incidence and density of neurotensin receptors in these tumors [68, 79]. The main drawback in the use of neurotensin, like any other peptide, as a drug is its extremely short half-life in human plasma due to very rapid cleavage by different peptidases. To circumvent this problem various neurotensin analogs were synthesized, including linear peptides [70, 72, 80], cyclic peptides [81], and nonpeptide molecules [82], but affinity of most neurotensin stabilized analogs reported in the literature is lower than that of native neurotensin.

In 2003, it was found that tetra-branched forms of neurotensin and its short functional fragment neurotensin(8–13) are stable for 24 h in human plasma and serum, and their receptor affinity is even higher than that of the corresponding monomeric peptides. The protease-resistant oligo-branched form of neurotensin appeared as a promising alternative to stabilized neurotensin monomeric sequences [55, 56] for tumor targeting). On this basis, different peptide-based branched molecules were synthesized and were coupled during the solid-phase synthesis to different functional units, allowing either visualizing of tumor cells that express neurotensin receptors or delivering cytotoxic moieties to the same tumor cells. A general tetra-branched modulatable molecular scaffold was set up, which allows conjugating of neurotensin sequences to effector units without affecting receptor binding. The biological activity of these tetra-branched neurotensin peptides conjugated to effector units was characterized *in vitro* and *in vivo* [83]. See Figure 7.24.

Oligo-branched modular peptides allow: (i) increasing receptor binding by multivalent interaction and (ii) retaining peptide biological activity by keeping the tumor-targeting peptide sequence far apart from the effector moiety, which is linked to the branched core rather than directly to the peptide.

Cytotoxicity experiments performed on HT-29 human colon adenocarcinoma cells [83] demonstrated that conjugation of different chemotherapy drugs to tetra-branched neurotensin produces a sort of prodrug, which can no longer be

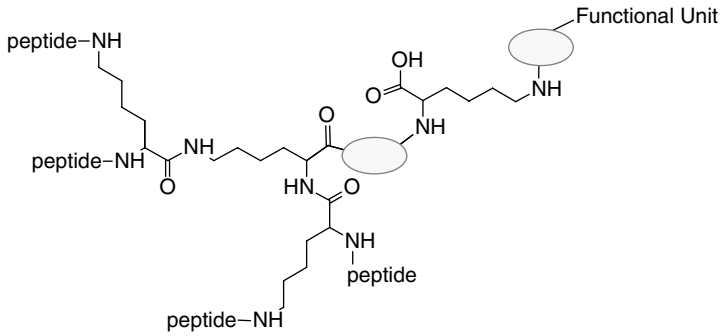


Figure 7.24 General structure of modulatable tetra-branched neurotensin peptides. The functional unit can be a tracer (i.e., fluorophore or radionuclide) or a cytotoxic unit.

transported across plasma membranes by the mechanism of the corresponding free drug and can only be "activated" via peptide receptor binding, profoundly decreasing nonspecific drug toxicity.

Drug-conjugated tetra-branched neurotensin peptides went through the proof-of-concept phase *in vivo* giving 60% reduction of tumor weight in xenografted mice, compared with animals treated with the same amount of unconjugated chemotherapeutic.

Results obtained with tetra-branched neurotensin peptides conjugated to different functional units for tumor imaging and therapy indicate that branched peptides are promising novel multifunctional targeting molecules, which might allow a personalized oncology where cancer detection, screening, diagnosis, and therapy are obtained by means of the same molecule, with no modification in target binding, but simple "exchange" of functional units.

7.5.2
Branched Peptides as Antimicrobials

The oligo-branched structure can be very useful also for antimicrobial peptides as we demonstrated for a panel of synthetic peptides discovered in the author's laboratory and presently in the early stage of development. These peptides derived from the selection of a large phage peptide library, and were modified for stability and efficacy improvement [59, 84]. The best of these peptides proved to be highly potent against many multiresistant clinical isolates of *P. aeruginosa*, *A. baumanii*, *Klebsiella pneumoniae*, and other Gram-negative bacteria with minimal inhibitory concentrations comparable with commercial polymyxin B. Recent results showed that it also neutralizes lipopolysaccharide, blocking the most severe symptoms of sepsis in animals infected with bacteria of clinical interest. A full preclinical study is presently under development.

The oligo-branched structure of short peptides might allow increasing efficacy of many cationic peptides whose efficacy in monomeric form is too low to be considered as candidates for the development of new drugs.

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8
Oral Bioavailability of Peptide and Peptidomimetic Drugs

Arik Dahan, Yasuhiro Tsume, Jing Sun, Jonathan M. Miller, and Gordon L. Amidon

8.1
Introduction

Peptide oral drug delivery has long been a challenging interest. Remarkable advances in recombinant DNA technology and synthetic chemistry, together with the development of receptor-based screening procedures, allow for the large-scale production of structurally diverse peptides exhibiting a broad spectrum of pharmacological effects. However, the development of these peptides/peptide-like compounds into effective drugs, and especially orally administered drugs, has been restricted due to various limitations, such as transport barriers, intestinal and hepatic metabolism, chemical instability, and hepatic elimination mechanisms. Development of an effective oral delivery system for this class of compounds requires a thorough understanding of the various physicochemical, physiological, and dosage form-related factors governing the rate and extent of absorption following oral administration. In this chapter, we review the various factors influencing the oral systemic availability of peptide and peptidomimetic compounds, the barriers that compromise their systemic absorption, and the different approaches that have been utilized to overcome these barriers.

8.2
Fundamental Considerations of Intestinal Absorption

Oral bioavailability (F) is mainly dependent on three general processes: the fraction of dose absorbed (F_a), the fraction of drug that escapes from metabolism in the gut wall (F_g), and the fraction of drug that escapes from hepatic metabolism (F_h), thus $F = F_a \times F_g \times F_h$ [1–3]. F_a is the upper limit of systemic availability and thus of primary interest in the development of a new oral therapeutic agent. F_a is very complex and affected by many factors. These include physicochemical factors (e.g., pK_a, solubility, stability, diffusivity, lipophilicity, polar–nonpolar surface area, presence of hydrogen-bonding functionalities, particle size, and crystal form),

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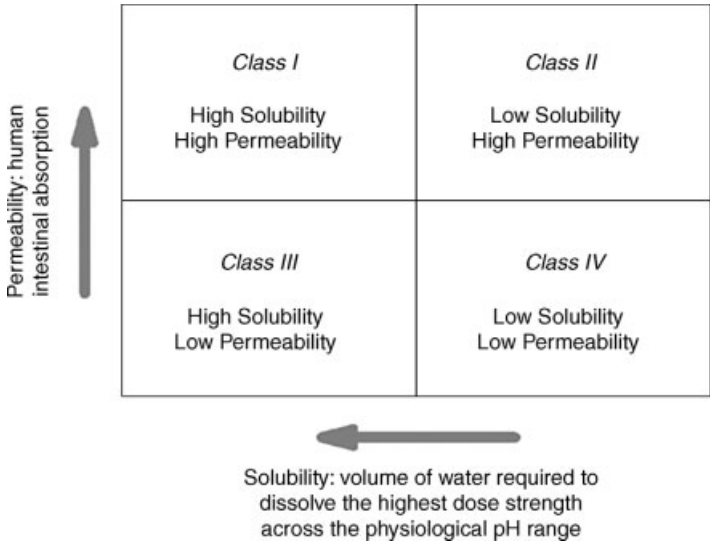

Figure 8.1 BCS as defined by Amidon *et al.* [7]. The BCS classifies drugs by their solubility and permeability properties, in order to stand for the most fundamental view of the drug intestinal absorption process following oral administration.

physiological factors (e.g., gastrointestinal (GI) pH, GI blood flow, gastric emptying, small intestinal transit time, colonic transit time, and absorption mechanisms), and factors related to the dosage form (e.g., tablet, capsule, solution, suspension, emulsion, and gel) [3–6]. Despite this complexity, the work of Amidon *et al.* [7] revealed that the fundamental events controlling F_a are the permeability of the drug through the GI membrane and the solubility/dissolution of the administered dose of the drug in the GI milieu. Based on this analysis, drug substances have been classified into one of four categories according to the Biopharmaceutics Classification System (BCS) proposed by Amidon *et al.* (Figure 8.1) [1, 2, 4, 7]. Hence, the principal factors determining oral drug absorption are mucosal cell permeability, solubility/dissolution rate, and enzymatic/chemical instability.

The mucosal cell permeability (P_w) determines the rate of drug entry into the mucosa across the brush-border membrane. This is the most fundamental step necessary to achieve oral absorption and systemic availability. The intestinal permeability can be mediated by one or more of the potential pathways, schematically illustrated in Figure 8.2. The permeation process can be passive or carrier-mediated and paracellular or transcellular. In general, lipophilic compounds are usually absorbed by passive diffusion through the intestinal epithelium. Many hydrophilic compounds are absorbed through a carrier-mediated process, while some small hydrophilic compounds may be transported through the paracellular junction. Efflux systems that actively transport drugs from the enterocyte back into the GI lumen may also play a role as a barrier for permeability [8–12]. Extensive human intestinal permeability studies on a wide range of compounds have been carried out over the

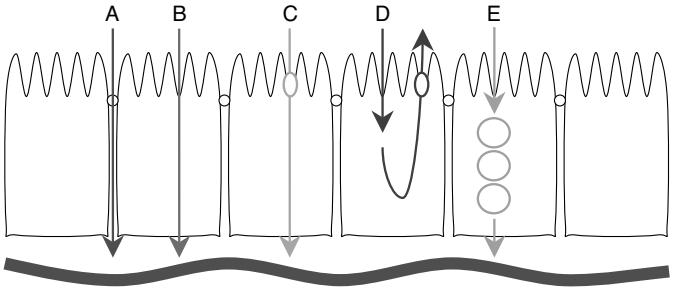

Figure 8.2 Intestinal drug permeability mechanisms: (A) paracellular pathway through tight junctions; (B) transcellular passive diffusion; (C) carrier-mediated transcellular transport; (D) carrier-mediated efflux transport; (E) transcellular vesicular transport (specific receptor-mediated transcytosis or nonspecific fluid-phase endocytosis).

last 20 years. These studies have shown that human permeability of above around 2×10^{-4} cm/s is needed to achieve good absorption [5, 7, 13–16].

The solubility/dissolution of the drug in the GI milieu is a critical factor in the intestinal absorption process following oral administration, because this determines the concentration of the drug at the membrane (C_w) consequently available for permeability (in a well-mixed fluid phase). The equilibrium solubility of the drug must be viewed in light of the dose, as will be discussed in Section 8.3. When applying Fick's first law to a membrane, the overall flux (J_w) of a drug across the GI mucosal surface under sink conditions can be written as $J_w = P_w \times C_w$ [7]. This is a local law, pertaining to each point along the intestinal membrane, and limited to the residence time of the GI tract ($t_{res} \sim 3$ h in humans [17, 18]). The additional principal factor determining drug lost from the GI lumen, extremely relevant to peptide/protein-based drug compounds, is chemical/enzymatic instability, as will be further discussed in Section 8.3.

8.3
Barriers Limiting Oral Peptide/Peptidomimetic Drug Bioavailability

Metabolism, mainly by peptidases, but also phase I and II enzymes, is perhaps the most significant challenge to peptide and peptidomimetic drug oral delivery [19, 20]. Instability may also be the result of chemical degradation, especially in an acidic environment; however, the enzymatic barrier is generally the major obstacle. Proteolytic enzymes are ubiquitous, so peptides/peptidomimetics are susceptible to degradation at multiple sites. Additionally, it is likely that more than one linkage within the peptide backbone will be susceptible to enzymatic degradation [21, 22]. This enzymatic degradation in the GI lumen is the human body's physiological way to break down dietary proteins. However, while in the case of dietary peptides/proteins the absorption of the amino acids produced by the enzymatic degradation is beneficial, it is essential that peptide/protein-based drugs be absorbed intact in order to exert their therapeutic actions [23]. Hence, the hostile environment of the GI tract

to peptide/peptidomimetic compounds represents a major barrier in the oral delivery of such substances.

The gut wall presents both physical and biochemical barriers to the absorption of peptide/protein-based compounds following oral administration [24, 25]. In general, since peptides and proteins are often characterized by large size, charge, and hydrophilic nature, passive transcellular partitioning into the lipidic GI wall, as well as paracellular transport through tight junctions, is minimal [23, 26]. This represents the physical barrier for the absorption of peptide/protein-based drug candidates. The biochemical barrier refers to proteins (enzymes, transporters) associated with the mucosal cell membrane. In this regard, it has been found in several species that the regional distribution and activity of certain intestinal peptidases follows a trend, increasing distally and reaching a peak level in the ileum [27–29]. The lowest activity was measured in the ileo-cecal junction, indicating that colonic delivery of peptide/protein-based drugs may result in decreased enzymatic degradation [30–34]. Site-specific delivery as a strategy to improve peptide/peptidomimetic compounds oral availability will be further discussed later (Section 8.4.2). An additional biochemical barrier may be presented by efflux transporters, for example, P-glycoprotein (P-gp), that actively transport drugs from the enterocyte back to the GI lumen. P-gp has been shown to limit the intestinal absorption of many compounds, including peptide/protein-based substances [35–37]. Interestingly, the expression of P-gp in the small intestine was found to follow a similar pattern to peptidases, increasing from the duodenum to the ileum [10, 38, 39].

It should be noted that solubility/dissolution may or may not affect the oral delivery of peptide-based drugs, depending on the amino acid composition of the peptide [26]. The basic parameter in defining whether or not solubility will play a role in the oral absorption process is the dose number (D_0). D_0 is the ratio of dose to dissolved drug $D_0 = (M_0/V_0)/C_S$, where C_S is the equilibrium solubility of the compound, M_0 is the dose, and V_0 is the volume of water taken with the dose, which is generally set to be 250 ml [1, 2, 4, 7]. This volume was selected based on a typical bioequivalence study that administers an 8-oz (240-ml) glass of water with the oral dosage form. Thus, 250 ml, allowing a small GI residual volume, represents the initial GI volume to which an oral dosage form is exposed in the fasted state. $D_0 \leq 1$ points out that solubility will not be a limiting factor in the intestinal absorption process, while $D_0 > 1$ indicates that solubility may limit the absorption following oral administration.

8.4
Strategies to Improve Oral Bioavailability of Peptide-Based Drugs

8.4.1
Chemical Modifications

8.4.1.1 Prodrug Approach

The prodrug approach has been successfully utilized to overcome undesirable physicochemical properties and pharmacokinetic/pharmacodynamic barriers of

many drugs [40]. Classical prodrug designs use the non-specific approach of covalently modifying the drug molecule of interest by attaching hydrophilic (e.g., phosphate) moieties to increase aqueous solubility or lipophilic (e.g., ester) functionalities to increase passive permeability. Over the last 20 years, more sophisticated prodrug strategies have emerged in which pro-moieties are covalently attached to the molecule of interest to selectively target certain membrane transporters and enzymes [26, 41–43]. These prodrug strategies offer tremendous potential for improving drug bioavailability and selectivity. However, a prodrug is indeed a new molecular entity, thus the impact on other important parameters must be considered when applying this approach (e.g., potency, efficacy, distribution, metabolism, excretion, and toxicity). Moreover, activation of the prodrug after absorption is an important attribute that must be balanced with transporter affinity and specificity in order to achieve optimal prodrug performance *in vivo*.

Recent advances in biochemistry and molecular biology have provided an enormous body of information on the molecular functions and expression levels of biological proteins, enzymes, and transporters. Various of transporters are expressed on the apical side of the small intestine that may be selectively targeted, including the organic anion transporter (OATP), organic cation transporter (OCTN), sodium dependent bile acid transporter (ASBT), sodium-dependent glucose transporter (SGLT), monocarboxylate transporter (MCT), and oligopeptide transporter (PEPT1) (Figure 8.3) [44, 45]. These transporters have been extensively characterized, and have been shown to play important roles in the absorption of certain nutrients and drugs [46, 47]. Since the substrate specificity of these transporters has been identified, many drug molecules were modified to improve oral drug absorption via targeting these transporters. Of all the known intestinal membrane transporters, PEPT1 has captured the most attention as a drug absorption pathway. Thus, peptide transporter-associated prodrug therapy might be a most promising strategy for drug delivery. PEPT1 is predominantly expressed in the small intestine, and accepts dipeptides, tripeptides, and peptidomimetic drugs such as β-lactam antibiotics and angiotensin-converting enzyme inhibitors [40, 47, 48]. Moreover, amino acid ester prodrugs have also been found to be substrates for PEPT1, and thus a variety of amino acid, dipeptide, and tripeptide prodrugs have been investigated for their suitability as substrates for the PEPT1 transporter [49–51]. These studies have revealed that mono amino acid and dipeptide ester prodrugs generally provide enhanced PEPT1-mediated transport, and, as a result, improved oral absorption and bioavailability [43, 49–52]. For instance, the valyl ester prodrug of acyclovir significantly enhances the oral bioavailability of acyclovir and several reports have suggested that oligopeptide transporters are responsible for this absorption enhancement [42, 52, 53] (Figure 8.4). Hence, the antiviral drugs valacyclovir and valganciclovir are examples of the clinical and commercial successes of amino acid ester prodrug strategy.

8.4.1.2 **Structural Modifications**

Structural modifications can improve the physicochemical properties of peptide/peptidomimetic drugs, leading to enhanced lipophilicity, enzymatic stability,

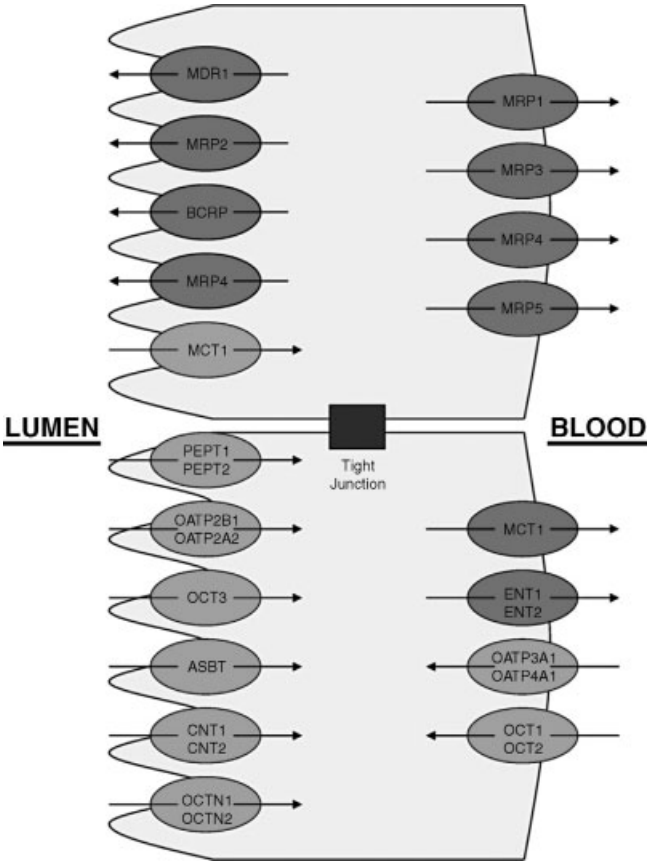

Figure 8.3 Illustration of the various transporters, both efflux and absorptive, that are expressed in the GI tract [44].

and membrane permeability. However, the chemical features that optimize physicochemical properties may not be beneficial for receptor site binding and specificity. Therefore, when chemical modifications are made, it is important to also retain the potency of the drug molecule at the site of action. Chemical modifications include backbone cyclization, *N*-methylation, alternation of side-chains, terminal groups or L/D configuration, covalent attachment to another macromolecule, and so on. For glycoproteins, modifications may also be made to the carbohydrate domains; however, these types of changes may be particularly liable to alter biological activity.

Backbone cyclization can significantly enhance the membrane permeability and metabolic stability of peptide drugs, and, at the same time, keep the biologically important side-chains intact [54, 55]. Hess *et al.* showed that the backbone cyclic peptidomimetic derivatives of hydrophilic hexapeptides resulted in a 5-fold permeability enhancement in comparison to the linear analogs [56]. In order to find an orally available lead to treat obesity, a similar strategy was applied to a potent tetrapeptide melanocortin-4 receptor agonist [57]. In mice, a single oral dose (0.5 mg/kg) of the

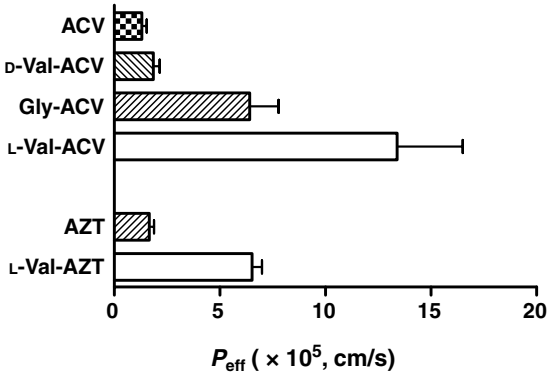

Figure 8.4 Rat intestinal membrane permeabilities of acyclovir (ACV) and zidovudine (AZT), compared with the permeability of their amino acid ester prodrugs [52].

selected candidate led to reduced food consumption and repetitive once-daily oral dosing (0.5 mg/kg/day) for 12 days reduced weight gain.

N-Methylation is another promising structural modification strategy to increase peptide drug stability and intestinal permeation [58–60]. A library of multiple *N*-methylation of Veber–Hirschmann cyclic hexapeptide cyclo(-PFwKTF-) was prepared by Biron *et al.*, achieving up to 10% oral bioavailability of one of the *N*-methylated analogs, while the Veber–Hirschmann peptide was not orally available (Figure 8.5) [61].

Insulin is rapidly degraded in the GI tract, hence the oral delivery of insulin has been a continuous interest for scientists in order to replace inconvenient subcutaneous injections. NOBEX Corp. has developed an orally available form of insulin, hexyl-insulin monoconjugate 2 (HIM2), in which a single amphiphilic oligomer is covalently linked to the free amino group on the Lys-β29 residue of recombinant human insulin via an amide bond [62]. This conjugated insulin leads to 2- to 8-fold

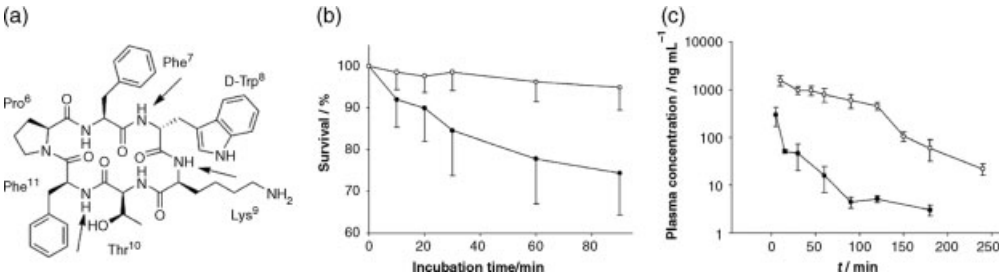

Figure 8.5 (a) Veber–Hirschmann peptide cyclo(-PFwKTF-), **1**. One of the active analogs, peptide **8**, resulted from the *N*-methylation of the amide bonds indicated by arrows. (b) Stability of peptides **1** (•) and **8** (○) in brush-border membrane vesicles. (c) Plasma concentration–time profiles of peptide **8** (10 and 1 mg/kg, respectively) following oral (○) and intravenous (•) administration [61].

higher water solubility and 2- to 10-fold slower degradation by GI enzymes, while maintaining about 70% of the activity of native insulin in receptor binding and cell-based receptor activation assays [62]. Various studies have demonstrated that oral HIM2 is rapidly absorbed from the GI tract, resulting in substantial glucose-lowering effects in normal volunteers, and both type I and II diabetic patients [63–65].

8.4.2 Formulation Technologies

8.4.2.1 Absorption Enhancers

Absorption enhancers have been extensively investigated as an approach for increasing the intestinal membrane permeability of peptide/peptidomimetic drugs. Absorption enhancers are formulation components that temporarily disrupt the intestinal membrane to improve drug permeation [21, 33]. Possible mechanisms of action include a change in membrane fluidity, a decrease in mucus viscosity, leakage of proteins through membranes, and opening of tight junctions. While absorption enhancers may be successful in increasing the intestinal absorption of peptide/peptidomimetic drugs, the major drawbacks with their widespread use include the risk of acute epithelial damage and permeability enhancement of undesirable molecules.

Chitosan has been investigated as a promising nontoxic, biocompatible, and biodegradable absorption enhancer to deliver peptide/peptidomimetic drugs. Protonated chitosan (apparent $pK_a = 5.5$) is able to induce a transient opening of the tight junctions by ionic interactions with the cell membrane, therefore increasing the paracellular permeability of peptide drugs across mucosal epithelia [66]. However, the use of chitosan is limited to the proximal part of intestine due to its insolubility at neutral pH. To solve this problem, many derivatives of chitosan were synthesized and studied, and proved to be more soluble in neutral and basic conditions. Therefore, they can be used to increase drug absorption in the jejunal and ileal segments of the small intestine. Sadeghi *et al.* compared four different chitosan derivatives (trimethyl chitosan (TMC), dimethylethyl chitosan (DMEC), diethylmethyl chitosan (DEMC), and triethyl chitosan (TEC)) for their ability to reduce the transepithelial resistance (TEER) and increase insulin transport in Caco-2 cells, showing their potential as absorption enhancers (Figure 8.6) [67]. Thanou *et al.*

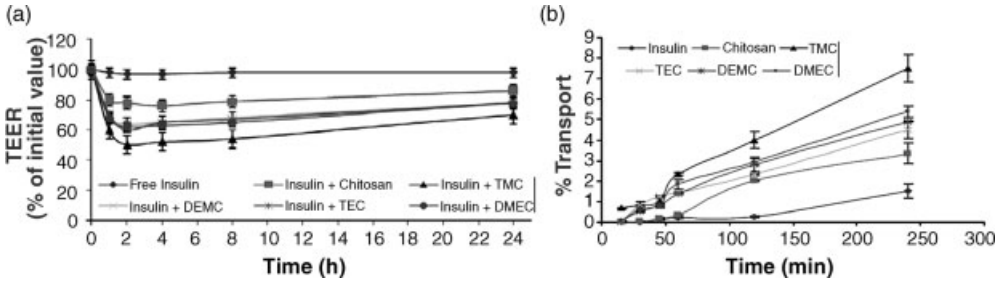

Figure 8.6 The effects of free-soluble polymers on the TEER (a, 240 min incubation) and on the transepithelial transport of insulin (b) across Caco-2 cell monolayers [67].

used TMC chloride as an absorption enhancer for octreotide acetate in rats. Intrajejunal coadministration of 1.0% (w/v) TMC solution resulted in a 5-fold increase of octreotide bioavailability as compared to administration of octreotide alone [68].

8.4.2.2 **Coadministration with Protease Inhibitors**

Coadministration with protease inhibitors helps peptide/peptidomimetic drugs to circumvent enzymatic degradation in the GI tract. For example, the bioavailability of [D-Ala, D-Leu]-enkephalin (YdAGFdL) following ileal administration in the presence of peptidase inhibitor amastatin was 22-fold higher than oral administration and 5-fold higher than ileal administration without inhibitor [69]. Similarly, coadministration of the α-chymotrypsin inhibitor, 4-(4-isopropylpiperadinocarbonyl)phenyl l,2,3,4-tetrahydro-l-naphthoate methanesulfonate (FK-448), with insulin significantly decreased the blood glucose level of healthy volunteers [70].

Although protease inhibitors have shown great promise to increase the oral bioavailability of peptide drugs, their long-term use also has some major potential drawbacks, such as toxicity, increased risk for drug–drug interactions, and intestinal mucosal damage. Other concerns include reduction of nutritive protein digestion and stimulation of protease secretion by feedback regulation [71].

8.4.2.3 **Formulation Vehicles**

Association of drugs with polymetric systems, such as emulsions, lipid suspensions, liposomes, and polymeric nano- and microparticles [72], has been investigated to overcome the absorption barriers of peptides/peptidomimetics. The purposes of using these carrier systems include protection of the drug from degradation, controlling the drug release, targeting the drug to specific intestinal sites, and increasing intestinal residence time.

Lowman *et al.* investigated pH-responsive, poly(methacrylic-*g*-ethylene glycol) hydrogels as oral delivery vehicles for insulin [73]. Insulin was protected from proteolytic degradation in the acidic environment of the stomach and released in the small intestine. These insulin-loaded polymer microparticles have been applied to healthy and type I and II diabetic rats, and have shown significant hypoglycemic effects (Figure 8.7) [74]. Therefore, a delivery system can be a very promising strategy to enable orally administered insulin.

Bioadhesive systems have been developed to extend the contact of drugs with absorptive GI cells, and facilitate drug absorption before dilution and degradation in the GI tract. By grafting short lipophilic, poly(lactic-*co*-glycolic acid) (PLGA) chains onto charge-containing, hydrophilic backbones, namely sulfobutylated poly(vinyl alcohol) (PVAL), novel biodegradable comb polyesters have been employed as novel biodegradable and bioadhesive protein carriers [72]. The negative surface charge leads to significant bioadhesion on the mucosa of the small intestine, which facilitates absorption. The human serum albumin-loaded SB(43)-PVAL-*g*-PLGA nanoparticles successfully localized the protein inside Caco-2 cells and at the cell surface. Similarly, antigen-loaded SB(43)-

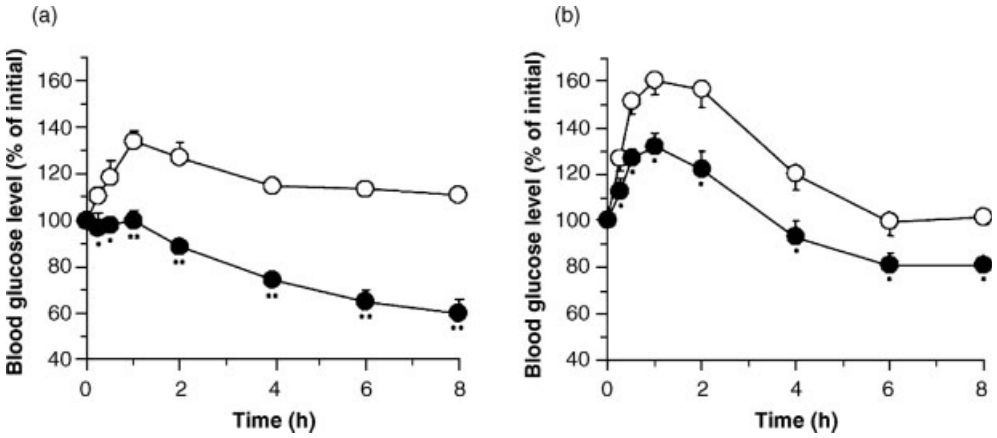

Figure 8.7 Changes in blood glucose levels versus time following oral administration of insulin-loaded microparticles (•) or insulin solution (○) in (a) type I and (b) type II diabetic rats [74].

PVAL-*g*-PLGA nanoparticles induced an immune response after oral administration in a mouse model [72].

8.4.2.4 Site-Specific Delivery

Peptide drug absorption varies significantly at different sites along the GI tract. This difference can be due to many factors, such as absorption mechanism (e.g., carrier mediated versus para- or transcellular) and physiological features of each GI segment (length, surface area, thickness of mucus layer, pH, protease activity, leakiness, etc.). Targeting peptide drugs to specific regions of the GI tract may also help to avoid enzymatic degradation and thus maximize absorption following oral administration.

Micro- and nanoparticles smaller than 10 μm are able to be taken up by the lymphoid aggregates of the gut-associated lymphoid tissue, specifically the Peyer's patches [75]. Therefore, drug delivery to the Peyer's patches can be an interesting approach to improve oral absorption of peptide drugs. Coppi *et al*. used calcium alginate/chitosan microparticles to orally administer a peptide antibiotic, polymyxin B [76]. van der Lubben *et al*. showed that ovalbumin-loaded microparticles were taken up by the Peyer's patches after intragastric feeding to mice [77].

Delivery of peptide drugs to the colon is an attractive strategy due to near-neutral pH, reduced peptidase activity, prolonged residence time, and also greater responsiveness to absorption enhancers, protease inhibitors, and novel bioadhesive and biodegradable polymers [30, 34]. To achieve colon-specific drug delivery following oral administration, the drug needs to be protected from degradation in the upper GI tract. Time- and pH-dependent systems have been investigated for colon-specific delivery. However, these methods are not very specific due to variation of gastric retention time, and lack of significantly different pH between the small intestine and the colon [30, 78].

Many more specific and reliable drug delivery systems have been developed to target peptide drugs to the colon, such as intestinal pressure-controlled capsules,

azo polymers, CODES™ technology, pectin and galactomannan coatings, and so on [30, 34, 78]. Polymers cross-linked with azoaromatic groups (azo polymers) protect peptide drugs from digestion in the stomach and small intestine. In the colon, the azoaromatic bonds are reductively cleaved by indigenous microflora and the drug is released [79, 80]. Saffran *et al*. tested the azo-cross-linked polymers with vasopressin and insulin in rats, and showed the biological responses antidiuresis, and hypoglycemia, respectively [79].

8.5 Conclusions

Improving the bioavailability of orally administered peptide/peptidomimetic drugs has been an ongoing topic of interest for many years. Successful oral delivery of peptide/protein-based compounds would undoubtedly revolutionize the therapeutic management of many diseases. However, as presented in this chapter, this task is extremely challenging. Indeed, the various strategies employed thus far to improve the oral absorption of peptide/peptidomimetic compounds in humans have been met with only marginal success. Clearly, a great deal of research is still needed to enable sufficient absorption of peptides/proteins following oral administration; however, in light of the significant progress that has already been made, this is certainly an achievable goal.

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9
Asymmetric Synthesis of β-Lactams via the Staudinger Reaction

Monika I. Konaklieva and Balbina J. Plotkin

9.1
Introduction

The β-lactam class of antibiotics originated in the 1940s as natural products isolated from soil-colonizing bacteria and fungi. These drugs, which include penicillins and cephalosporins, are acylating agents. They are capable of acylating nucleophilic residues in a diverse range of viral, bacterial, and mammalian enzymes. To date, enzymes capable of being acylated by β-lactams are those that contain serine as an active-site nucleophile. However, the fine-tuning of the β-lactam scaffold has allowed for development of not only serine-based enzyme inhibitors, but also inhibitors of cysteine-based and metalloenzymes. In both areas of β-lactam functionality (antibiotic/enzyme inhibitor) there is an exponentially growing need for new compounds due to the rapidly eroding effectiveness of this class of compounds as the result of the ever-evolving microbial development of β-lactamases. Here, we review asymmetric synthesis of the β-lactams via the most versatile method – the Staudinger reaction, which stands as a powerful method for the successful development of β-lactams as inhibitors for enzymes of bacterial origin (transpeptidases) or for use as inhibitors of mammalian enzymes associated with diseases including cystic fibrosis and Alzheimer's [1, 2].

9.2
Staudinger Reaction

The discovery by Staudinger [3, 4] more than a century ago of a reaction between an imine and a ketene prepared *in situ* remains the most general and useful method for the synthesis of β-lactams (Scheme 9.1) [3–5]. This reaction is a thermal [2 + 2] cycloaddition reaction that allows for the generation of two chiral centers in one step. Staudinger reactions using α,β-unsaturated imines or ketenes can lead to both [2 + 2] (β-lactams) and to the competitive [4 + 2] (δ-lactams) cycloadditions. The mechanism of the Staudinger reaction has been analyzed and revised over the years. The initial understanding was that the reaction goes through a concerted

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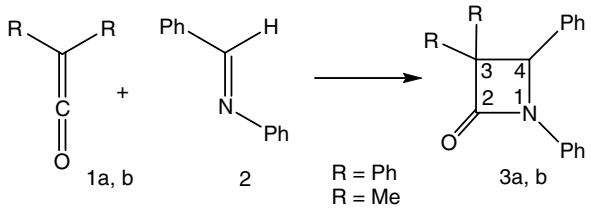
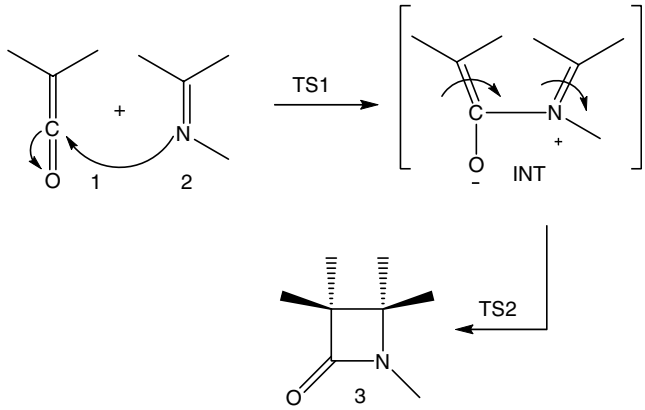


Scheme 9.1 Reaction between ketenes **1a** and **b** and *N*-phenyl benzylideneamine **2**, leading to formation of β-lactams **3a** and **b** as reported by Staudinger in 1907.

mechanism [6, 7]. However, the current mechanistic understanding, which is supported by both theoretical [8–10] and experimental [4, 11, 12] evidence, indicates that the [2 + 2] reaction in solution is actually a two-step reaction (Scheme 9.2). The first step is the nucleophilic attack of the imine-N-atom onto the sp-hybridized carbon of the ketene, which leads to formation of a zwitterionic intermediate. The second step could be viewed as a four-electron conrotatory electrocyclization of the zwitterionic intermediate producing the β-lactam product.

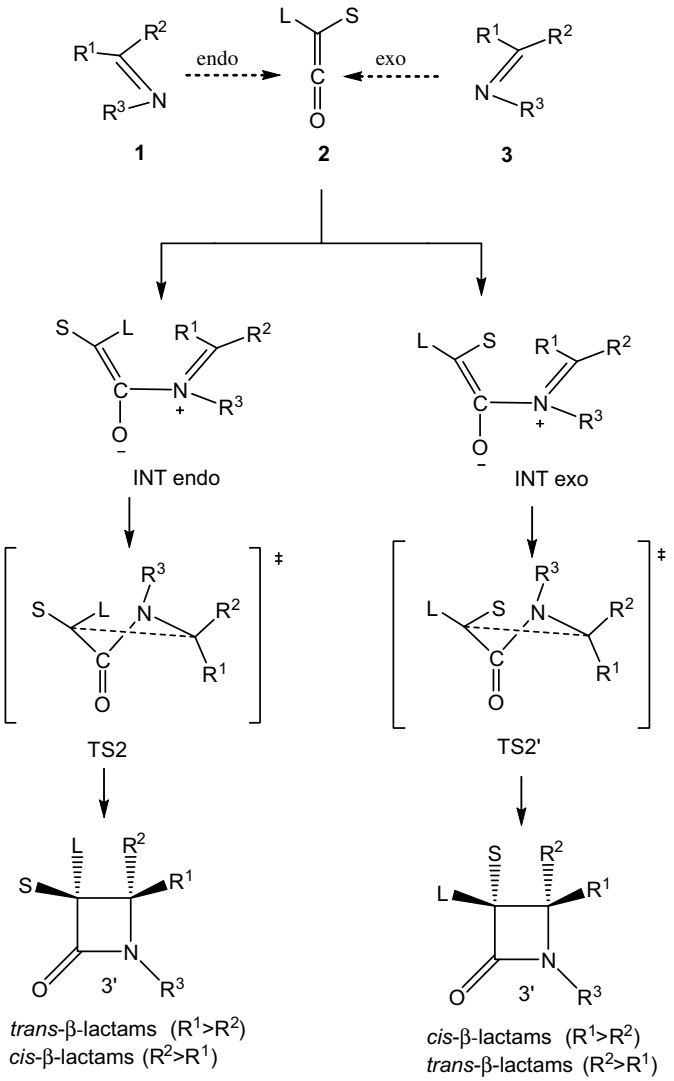


Scheme 9.2 The stepwise mechanism of the Staudinger reaction between ketenes **1** and imines **2**.

9.3
Influence of the Geometry of the Imine on Stereoselectivity in the Reaction

From a stereoselective point of view, it is generally accepted that the initial attack of the imine-N-atom occurs from the less-hindered side of the ketene. The *cis* or *trans* selectivity of the reaction generally depends on the geometry of the imine. As a general rule (*E*)-imines form *cis*-β-lactams and (*Z*)-imines form *trans*-β-lactams. Therefore, the cyclic imines having a "locked" *Z*-configuration lead to formation of *trans*-β-lactams and the *E* isomers of acyclic imines lead to formation of *cis*-cycloadducts as the major isomers. Recently, it has been shown that isomerization paths in

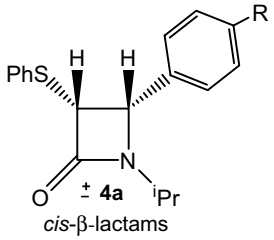
the imine or zwitterionic intermediate may determine the stereoselectivity of the reaction. For example, if the rotation about the N1–C4 bond of the zwitterionic intermediate is faster than the ring cyclization, the formation of *trans*-β-lactams from (*E*)-imines is favored. The isomerization of the (*E*)-imine to a (*Z*)-imine prior to cyclization could also lead to the formation of a *trans*-β-lactam (Scheme 9.3) [9, 13].



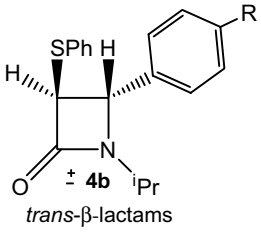
Scheme 9.3 General stereochemistry of the Staudinger reaction.

9.4
Influence of the Polarity of the Solvent on Stereoselectivity of the Reaction

To determine the solvent effect on the Staudinger reaction, a series of β-lactam cycloadditions were performed in solvents with differing polarity while maintaining constant temperature [14]. While the *cis*/*trans* ratio correlates with the Hammett constant, the polarity of the solvent did not increase stereoselectivity [14]. For example, the stereoselectivity of the Staudinger reaction in acetonitrile as a typical polar solvent is similar to the reaction performed in toluene [14]. However, it is interesting that the amount of the *cis* product increased with decreased solvent polarity (e.g., cyclohexane and *n*-octane). Polar solvents favor formation of *trans*-β-lactams **4a** (Scheme 9.4), whereas nonpolar solvents lead to higher amounts of *cis*-β-lactams **4b** (Scheme 9.4). These results indicate that the nonpolar solvents cannot stabilize the zwitterionic intermediates, facilitating the ring closure to form *cis*-β-lactams, while the polar solvents have the ability to stabilize the zwitterionic intermediates, allowing for an increase of the half-life of the latter, which leads to increasing isomerization of the imine moiety to generate *trans* products [14]. It should be noted that for Staudinger reactions with *trans*-β-lactams as major products, solvent polarity enhances diastereomeric excess; for Staudinger reactions with *cis*-β-lactams as major products, solvent polarity decreases their diastereomeric excess.



cis-β-lactams



trans-β-lactams

R = CF_3
in toluene 4a:4b = 42:58
in acetonitrile 4a:4b = 40:60

R = NO_2
in toluene 4a:4b = 73:27
in acetonitrile 4a:4b = 71:29

R = OMe
in toluene 4a:4b = 4:96
in acetonitrile 4a:4b = 3:97

R = Me
in toluene 4a:4b = 7:93
in acetonitrile 4a:4b = 7:93

R = H
in toluene 4a:4b = 12:88
in acetonitrile 4a:4b = 11:89

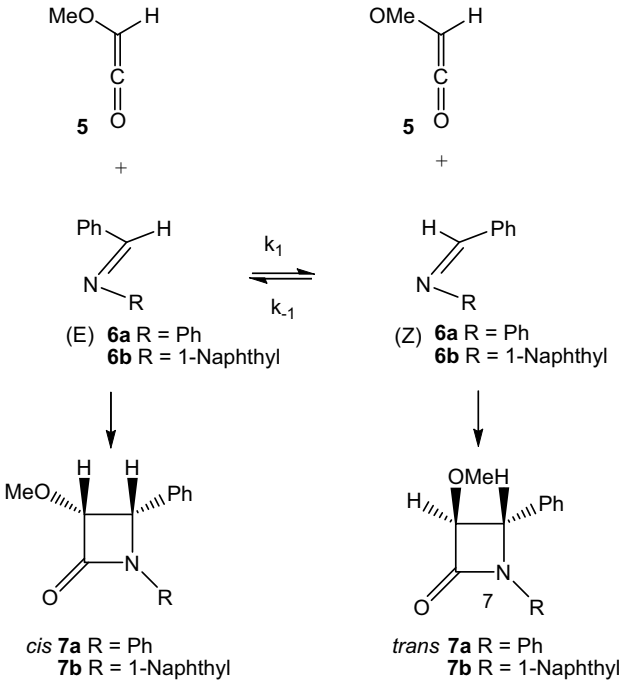
R = Cl
in toluene 4a:4b = 17:83
in acetonitrile 4a:4b = 15:85

Scheme 9.4 Solvent influence on the diastereoisomeric excess of the Staudinger reaction.

9.5
Influence of the Isomerization of the Imine Prior to its Nucleophilic Attack onto the Ketene Stereoselectivity in the Reaction

Selectivity differences (i.e., *cis* versus *trans*) in β-lactam formation have been observed when a methoxyacyl chloride is reacted with an imine with a phenyl or a 1-naphthyl group at the imine nitrogen, respectively. A *cis*-3-methoxy β-lactam is formed with an

N-phenyl imine and a *trans*-3-methoxy β-lactam is formed using *N*-1-naphthyl imine [13, 15]. These experimental findings, which take into account solvent effects, have led to the recognition that the rate of the imine isomerization prior to cyclization stages could be a factor in the *cis* versus *trans* stereoselectivity of the Staudinger reaction. When the substituent on the imine nitrogen is phenyl, the nucleophilic attack on the ketene is faster than *E*/*Z* isomerization of the imine, which leads to a *cis*-β-lactam. In the case of 1-naphthyl substituent on the imine nitrogen, the isomerization is faster than the nucleophilic attack on the ketene, leading to only *trans*-β-lactam (Scheme 9.5).



Scheme 9.5 Isomerization of the imine prior to cycloaddition influences the stereochemistry of the β-lactam.

9.6
Influence of the Order of Addition of the Reactants to the Reaction

Two different addition modes are generally used in the Staudinger reaction between acyl chlorides and imines: (i) the acyl chloride is added dropwise to a solution of the imine and the base tertiary amine (designated here as addition mode A), and (ii) the tertiary amine is added dropwise to a mixture of the imine and the acyl chloride (designated here as addition mode B). The different order of reactant addition affects reaction stereoselectivity because of the different reaction pathways invoked. When

mode A is used, *cis*-β-lactam is formed as the major, or exclusive, stereoisomer due to generation of a ketene prior to cycloaddition. In mode B, *trans*-β-lactam is the major, or exclusive stereoisomer, formed due to the nucleophilic attack of the imine onto the acyl chloride, which leads to formation of a chloroamide, followed by the intramolecular S_N2 displacement, which determines the final *trans* stereoselectivity [14].

9.7
Influence of Chiral Substituents on the Stereoselectivity of the Reaction

Enantiomerically enriched β-lactams require the presence of at least one chiral substituent. In the absence of chiral alkyl substituents (R) either on the ketene or on the imine, the possible zwitterionic intermediates are enantiomeric pairs of conformers which may interconvert via rotation about the N1–C2 bond (Scheme 9.3). When all of the substituents are achiral, the two possible transition states are isoenergetic, resulting in the formation of the racemic pair of β-lactams. However, if one R group is chiral and its effect on the energy of one of the two possible transition states (Scheme 9.3) is large enough, the presence of the chiral R group will lead to predominant, or exclusive, formation of one of the possible *cis*-enantiomeric β-lactams. Enantiomerically enriched β-lactams have been obtained by placing chiral auxiliaries in all of the available positions on the imine or ketene (Scheme 9.3).

9.8
Asymmetric Induction from the Imine Component

Chiral imines can be prepared from achiral aldehydes and chiral amines, and vice versa, from chiral aldehydes and achiral amines. In the former case (achiral aldehydes and chiral amines), the diastereoselectivity of the β-lactams produced is inferior, as compared to the use of imines produced from the latter preparation (chiral aldehydes and achiral amines). The most common examples of imines from chiral aldehydes include: α-oxy-aldehyde-derived imines **8**, sugar aldehyde-derived imines **9** and **10**, and α,β-epoxyimines **11** (Figure 9.1) [16, 17]. The β-lactams obtained using

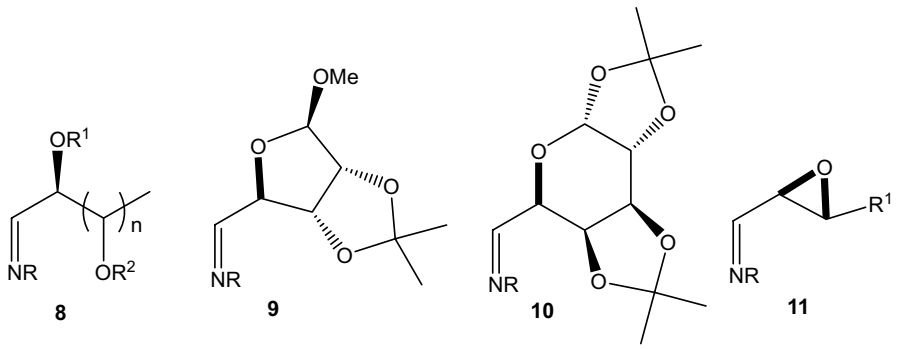
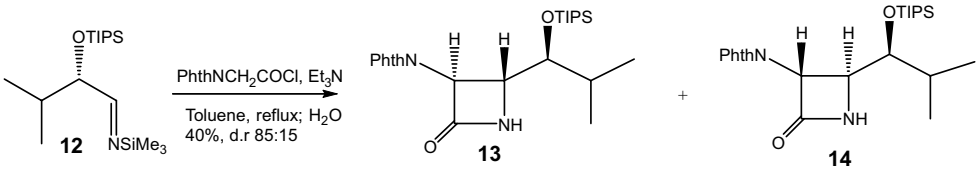


Figure 9.1 Imines from chiral aldehydes.

these imines are usually *cis* configured, and both *cis* diastereomers are obtained often in 90 : 10 ratios. *Trans*-β-lactams **13** and **14** can be prepared when *N*-trimethylsilylimines **12** are used (Scheme 9.6) [26, 27].



TIPS = Si^iPr_3,
Phth = Phthalimido group

Scheme 9.6 *N*-Silylimines leading to unusual *trans* stereochemistry in β-lactams.

The formation of *N*-acyl-imine *O*-trimethylsilyl enolates from these imines in the course of the reaction has been proposed to be responsible for the atypical β-lactam stereochemistry [18].

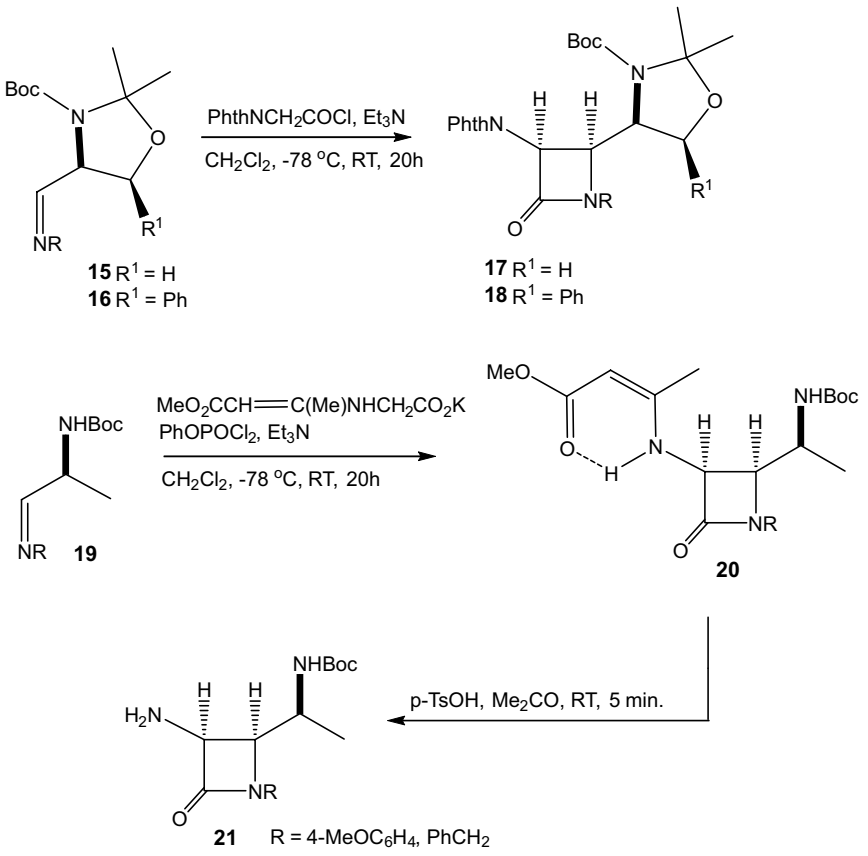
A wide variety of *N*-Boc-α-aminoimines can be prepared and used for the preparation of β-lactams with high diastereoselectivity, as precursors of monobactams and other heterocycles [19]; some examples are shown in Scheme 9.7.

Simple C2-symmetric chiral groups are superior chiral substituents on the imine nitrogen, which lead to excellent diastereoselectivity of the α-hydroxy/(alkoxy)-β-lactam (Scheme 9.8) [20], while other chiral substituents lead to fair selectivity.

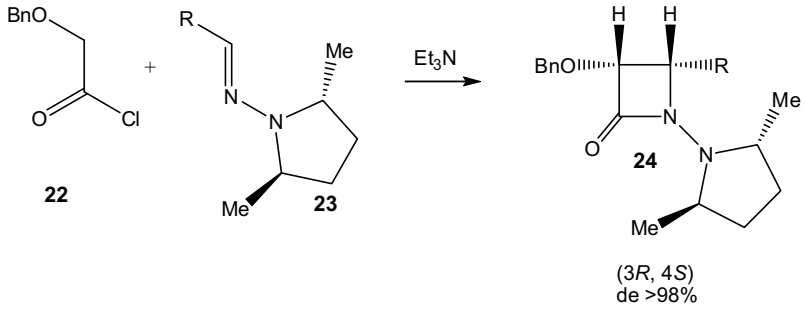
A nearly complete diastereoselectivity is achieved in the reaction of ketenes from acid chloride **22** with hydrazones, such as **23** to yield (3*R*,4*S*)-β-lactams. The other methods usually include reacting achiral alkoxy ketenes with imines from both α-oxy-aldehydes [21, 22] and chiral α-amino-aldehydes [23, 24], where the β-lactams are obtained with good to excellent diastereoselectivity (Scheme 9.9). For example, β-lactams **24** and **26** are obtained as single diastereomers from the imines **23** and **25** with acetoxyacetyl chloride and benzyloxy acetyl chloride. However, in other instances a mixture of β-lactams, such as **28** and **29**, in a ratio of 80 : 20 and 85 : 15 (Scheme 9.9) has been achieved [25] by reacting the imine **27** with the acetoxy- and benzyloxyketene, respectively.

Tricarbonyl(η6-arene)chromium(0) imines, such as **30** and **32** (Scheme 9.10), have also been utilized as chiral reactants in the Staudinger reaction. A mild decomplexation under air and sunlight of the initially formed C4-tricarbonyl(η6-arene)chromium(0)azetidinones leads to the β-lactam **31** in greater than 98% enantiomeric excess and **33**/**34** (72% yield) in 78: 22 ratio, respectively. The lower stereoselectivity of the latter reaction is most likely due to the remote position of the stereogenic arylchromium moiety in the cinnamyl imine **32**; however, the reason for the reversal of the diastereomeric ratio remains unclear.

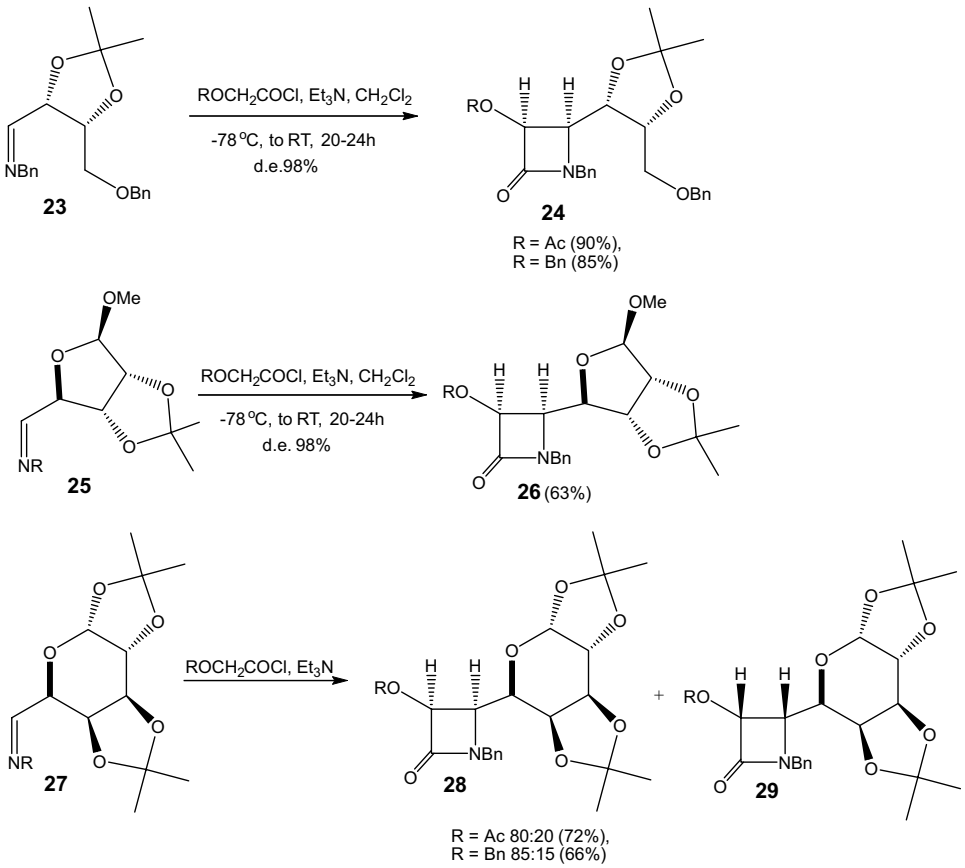
As stated earlier, the diastereoselectivity of the Staudinger reaction involving chiral imines obtained from achiral aldehydes and chiral amines is often low. Examples include β-lactams **35** [28], **36** [29], and **37** [30] (Figure 9.2).



Scheme 9.7 *N*-Boc-α-aminoimines leading to highly stereoselective β-lactam formation.



Scheme 9.8 C2-symmetric chiral groups on the imine nitrogen lead to excellent diastereoselectivity.

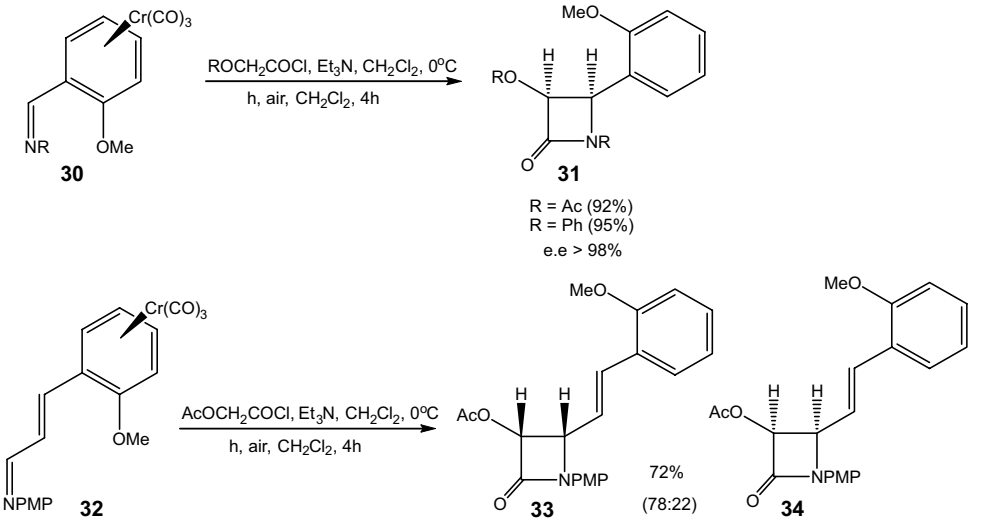


Scheme 9.9 β-Lactams from ketenes and imines derived from chiral aldehydes and amines.

An exception remains the reaction between imine **38**, derived from benzaldehyde and L-threonine with a bulky *O*-silyl protecting group (Scheme 9.11) [31], leading to formation of β-lactams **39**/**40** in ratio 1 : 11.5. When chiral imines, such as **42** (Scheme 9.12), are prepared from chiral aldehydes and chiral amines, which are in a matched relationship, only one diastereomer of the β-lactam **43** is produced.

As a stable class of enolizable imines, the *N*,*N*-dialkylhydrazones, such as **44** (Scheme 9.13), when reacted with benzyloxy ketene by the Staudinger reaction lead to a mixture of *cis*-β-lactams **45** and **46** in a good diastereotopic ratio of 87 : 13 [32, 33].

A [2 + 2] cycloaddition between a ketene and imine is also possible when the ketene has an electron-withdrawing group other than oxygen and nitrogen (e.g., a halogen). An example is the synthesis of β-lactam **48** (Scheme 9.14) as a single diastereomer in 68% yield [34] from fluoroacetyl chloride with imine **47** derived from *p*-anisidine and glyceraldehyde acetonide.



Scheme 9.10 β-Lactams from arenechromium imines.

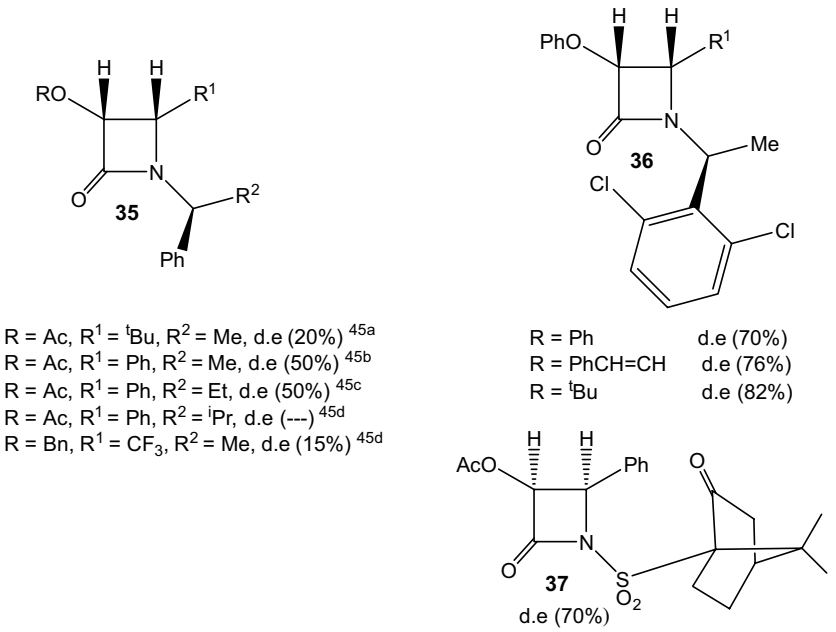
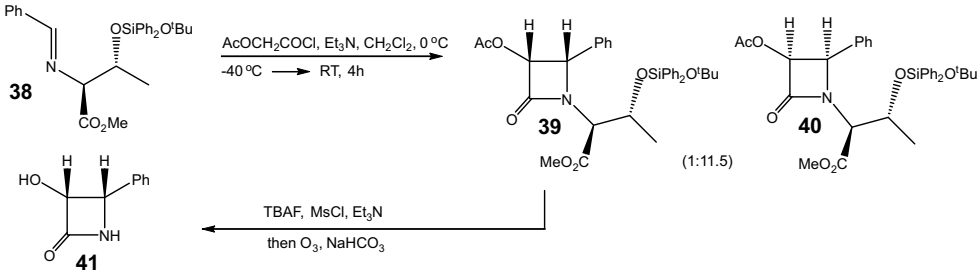
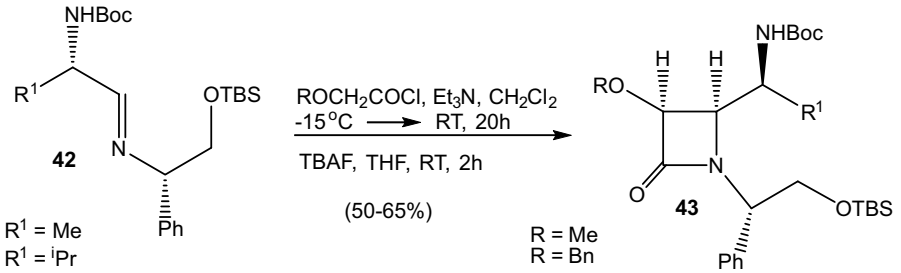


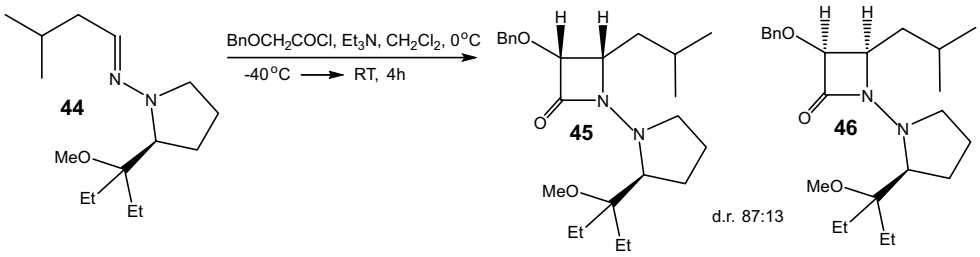
Figure 9.2 β-Lactams obtained from chiral imines, prepared by reaction of achiral aldehydes and chiral amines.



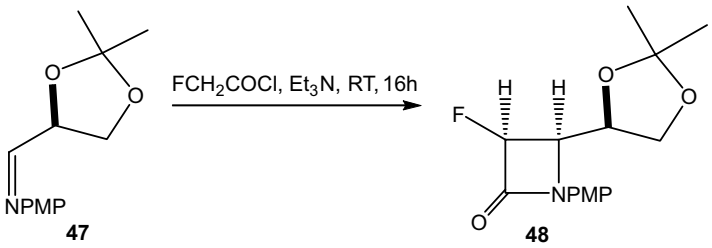
Scheme 9.11 β-Lactams from imine derived from benzaldehyde and L-threonine with an *O*-silyl protecting group.



Scheme 9.12 β-Lactams from imines derived from chiral aldehydes and chiral amines, which are in a matched relationship.

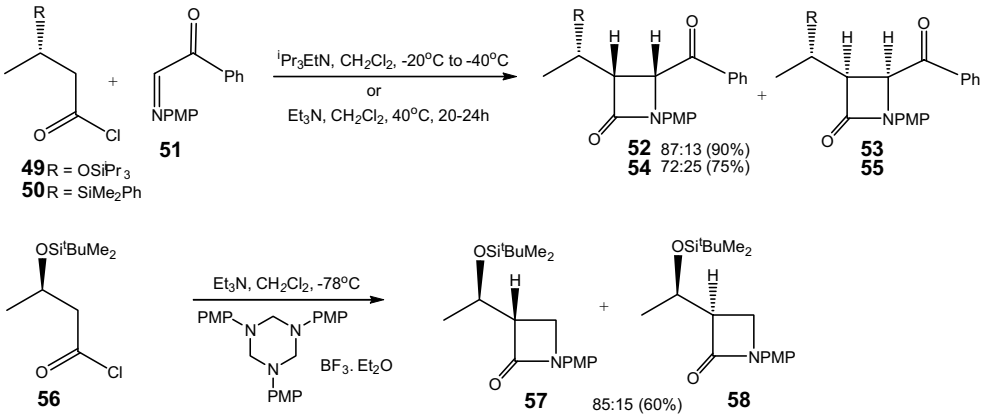


Scheme 9.13 β-Lactams from stable enolizable imines.



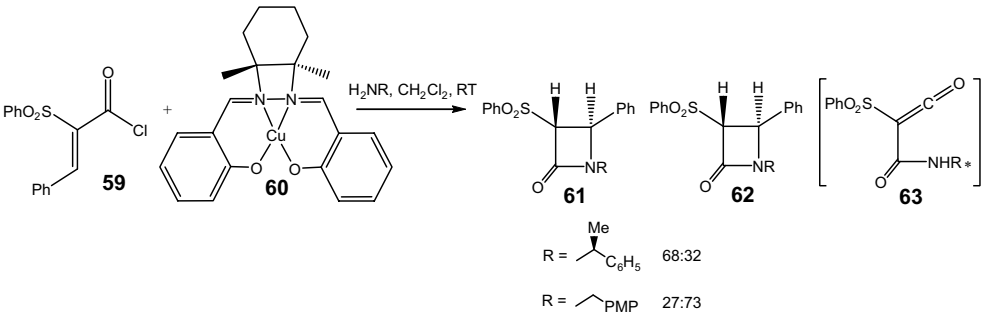
Scheme 9.14 β-Lactams from ketenes with electron-withdrawing group.

Probably the most general access to α-alkyl-β-lactams via the Staudinger reaction involves utilization of imines derived from glyoxylates, pyruvates, phenylglyoxal [33–35], or formaldimine trimers, activated by boron trifluoride etherate [36]. The most suitable ketenes used in these reactions are β-silyloxy- or β-(silyl)-butanoyl chlorides, even though the corresponding β-lactams are produced with moderate stereoselectivity. For example, β-lactams **52** and **57** (Scheme 9.15), having opposite relative configuration at C3–C1, are obtained as the major isomers when the ketenes derived from (*S*)-(triisopropylsilyloxy)-butanoyl chloride **49** and (*R*)-3-(*tert*-butyldimethylsilyloxy)-butanoyl chloride **56** are reacted with the phenylglyoxal imine [35] and *N,N,N-p*-methoxyhexahydro-1,3,5-triazine [36].



Scheme 9.15 β-Lactams from ketenes derived from β-silyloxy- or β-(silyl)-butanoyl chlorides.

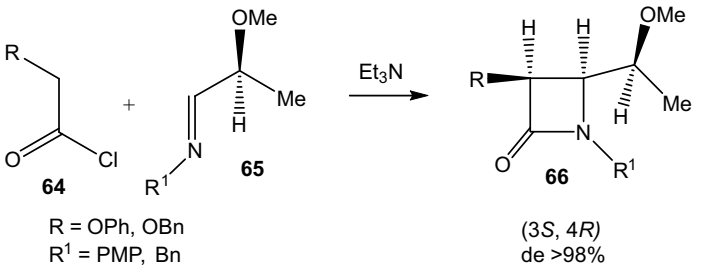
α-Alkenyl β-lactams can be prepared by the Staudinger reaction of imines and α,β-unsaturated ketenes, although asymmetric compounds appear to be difficult to obtain [37]. When chiral amines, such as α-methylbenzylamine, or the complex of 4-methoxybenzyl amine and the copper imine **60** (Scheme 9.16) react with (*Z*)-3-phenyl-2-(arylsulfonyl)-propenoyl chloride **59** via a tandem Michael



Scheme 9.16 β-Lactams from imines prepared from chiral amines and copper catalyst.

addition–cyclization process, they produce *trans*-β-lactams **61**/**62** with low diastereoselectivity. This reaction is believed to occur through the ketene intermediate **63** [38].

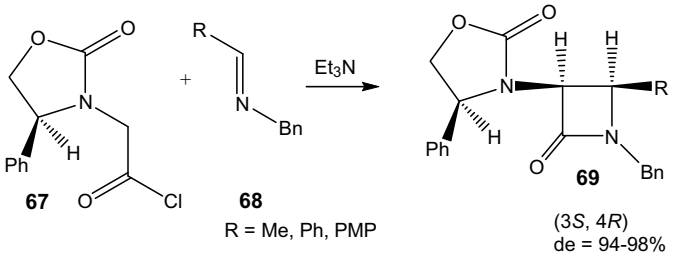
There are several examples of highly diastereoselective [2 + 2] reactions in which chirality is achieved by introducing a chiral auxiliary at the imine carbon. In general, imines derived from enantiopure aldehydes having C–O or C–N bonds (good-attractors) at the α-position are especially efficient in achieving chirality in these reactions. Such imines are those derived from (*S*)-2-methoxypropanal, **65** (Scheme 9.17), that upon reacting with acyl chlorides leads to formation of (3*S*,4*R*)-**66***cis*-β-lactams with high diastereoselectivity [39].



Scheme 9.17 Chiral groups at the imine α-carbon secure high diastereoselectivity of the β-lactam.

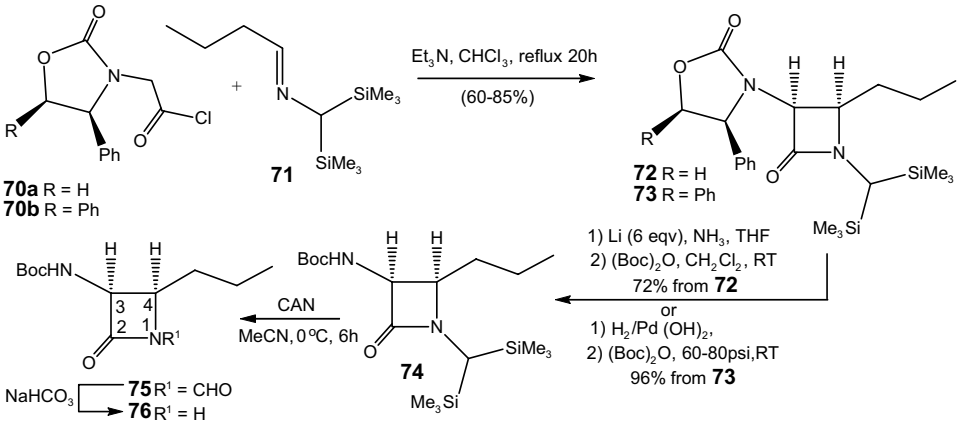
9.9
Asymmetric Induction from the Ketene Component

It has been demonstrated [40] that ketenes derived from chiral acyl chlorides, such as 2-(*S*)-2-oxo-4-phenyloxazolidin-3-yl)acetyl chloride **67** (Evans–Sjögren ketenes) [41, 42] (Scheme 9.18), can react with imines to yield *cis*-β-lactams (3*R*,4*R*)-**69** with excellent control of diastereoselectivity. This strategy has also been employed with very good yields (70–90%) in the preparation of β-lactams by solid-phase synthesis [43].



Scheme 9.18 Ketenes from chiral acyl chlorides lead to high diastereoselectivity.

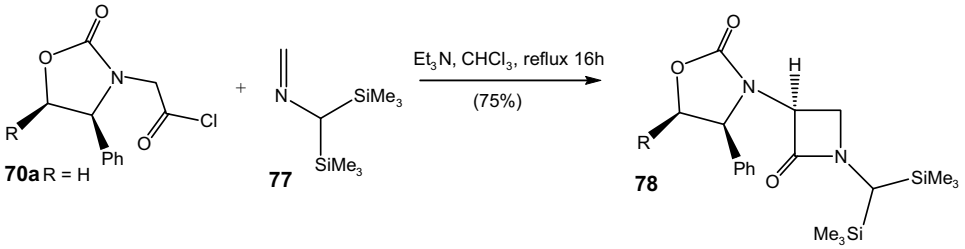
The majority of examples producing 3-amino-β-lactams from chiral ketenes are associated with nonenolizable aldehyde-derived imines. Enolizable aldehyde-derived imines often produce low yields in these reactions due to their facile isomerization to enamines. Recently, it has been found that utilizing *N*-bis(trimethylsilyl)methyl imines **71** (Scheme 9.19) allows for preparation of predominantly *cis*-β-lactams with high diastereoselectivity (e.g., **72**, 90 : 10, where the *trans* isomer is the epimer at C4, Scheme 9.19) and overall very good yield (e.g., **72** in 75%) [44].



Scheme 9.19 β-Lactams from *N*-bis(trimethylsilyl)methyl imines.

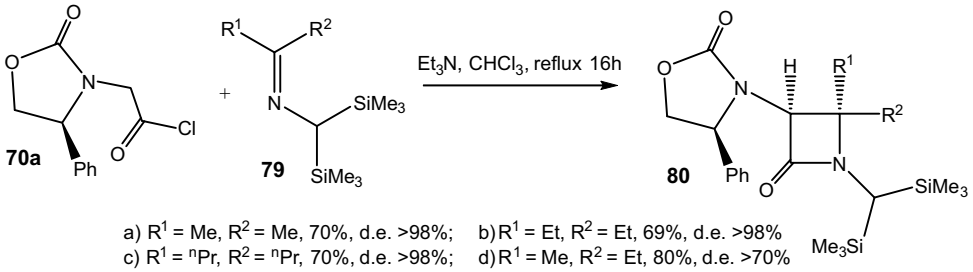
The major isomer **72** (Scheme 9.19) can be transformed to the *N*-Boc derivative **74** by removal of the oxazolidinone moiety following Evans' procedure and subsequent introduction of the Boc group. Alternatively, better yields are obtainable from β-lactam **73**, as a result of the reaction of the aminoketene precursor **70** with **71** [45, 46]. In this example, exposure of **73** to hydrogen over Perlman's catalyst in the presence of di-*tert*-butyl dicarbonate leads to β-lactam **74** in 96% yield. Removal of the *N*-bis(trimethylsilyl)methyl is achieved by treatment of lactam **74** with Ce(IV) ammonium nitrate, with the subsequent deformylation of the intermediate **75** leading to formation of lactam **76** with an 88% yield. In general, a wide variety of *N*-alkylidene *C,C*-bis(trimethylsilyl) amines can be utilized in the preparation of the corresponding imines for the Staudinger reaction. The resulting β-lactams are obtained in diastereomeric *cis*/*trans* ratios ranging from 70 : 30 to 98 : 2; the only exception being glyoxylate imines, which lead to very low diastereoselectivity of the β-lactams [33, 46]. However, the high thermal stability of the glyoxylate imines allows for the direct preparation of C4 unsubstituted β-lactams via reacting the stable, isolable methanimine **77** with ketenes. Using ketenes derived from **70** (Scheme 9.20) leads to formation of **78** in 75% yield and excellent *S*-asymmetric induction at C3 [47].

The chiral acylchloride strategy can be used for the preparation of β-lactams with quaternary stereogenic centers at C4 via reaction with ketimines [48]. Use of symmetrical ketimines, such as **79a–c** (Scheme 9.21), results in the formation of



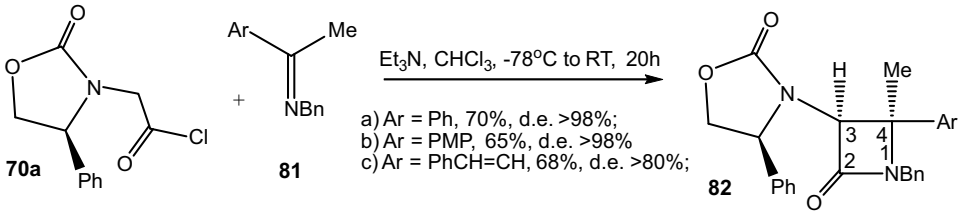
Scheme 9.20 β-Lactams from unsubstituted imines.

β-lactams **80a–c** with almost complete diastereoselectivity. In contrast, use of unsymmetrical ketimines, such as **79d** and **e** (Scheme 9.21), leads to formation of epimeric β-lactam **80d** and **e** mixtures with low diastereoselectivity.



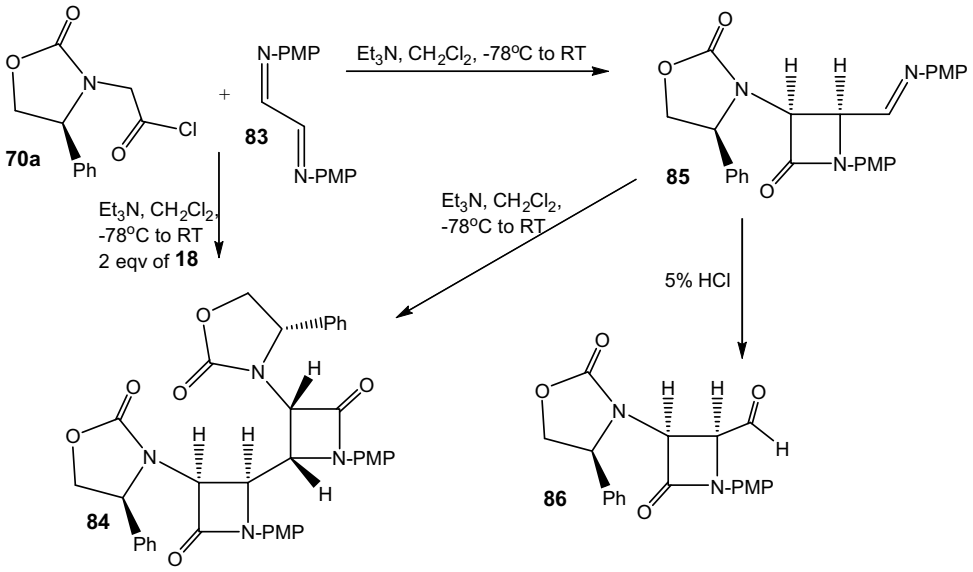
Scheme 9.21 β-Lactams from symmetrical and unsymmetrical ketimines.

Use of aralkyl ketone-derived imines, such as **81a** and **b** in reaction with **70** (Scheme 9.22), results in formation of β-lactams in good yields (65–70%) with essentially complete diastereoselectivity at the newly created stereogenic centers. However, ketimine **81c** under the same conditions produces both lactam **82c** and its C4 epimer.



Scheme 9.22 β-Lactams from ketone-derived imines.

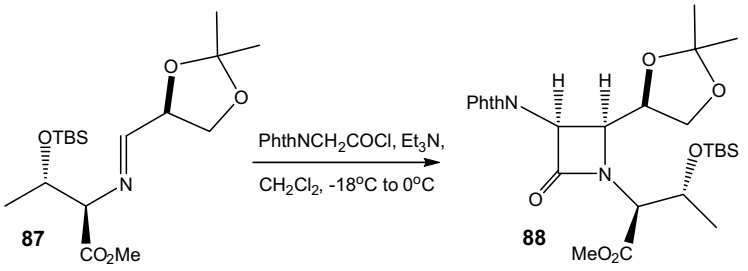
Bis-aldimines **83** [17, 44] can also be used in the synthesis of 3-amino-β-lactams, such as **84** and **85**, which in turn can be easily converted to 4-formylazetidinone **86** (Scheme 9.23).



Scheme 9.23 β-Lactams from bis-aldimines.

9.10
Double Asymmetric Cycloinduction

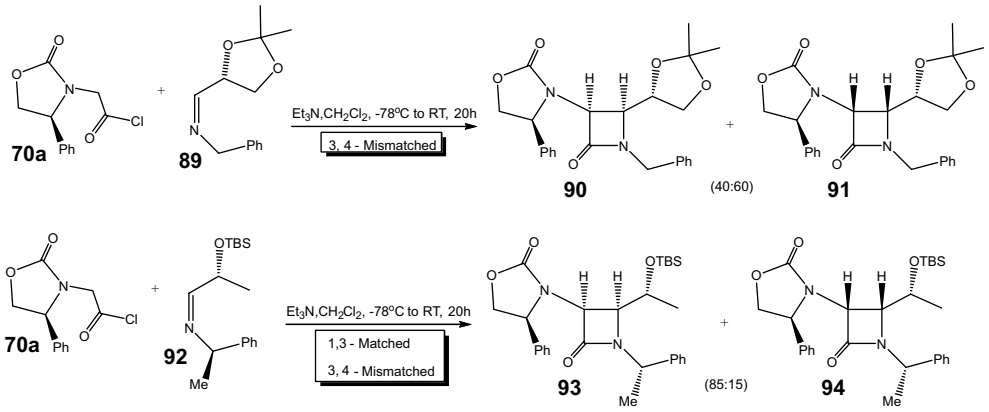
The use of double stereo-differentiating cycloadditions has been applied to the Staudinger reaction with variable success. When matched aldehyde and amine units are used in a chiral imine, such as in imine **87** (Scheme 9.24), and reacted with phthalimidoketenes, β-lactam **88** is formed in 90% yield [49]. This result is a significant improvement over related reactions using threonine imines derived from achiral aldehydes.



Scheme 9.24 β-Lactams from chiral imines derived from matched aldehyde and amine units.

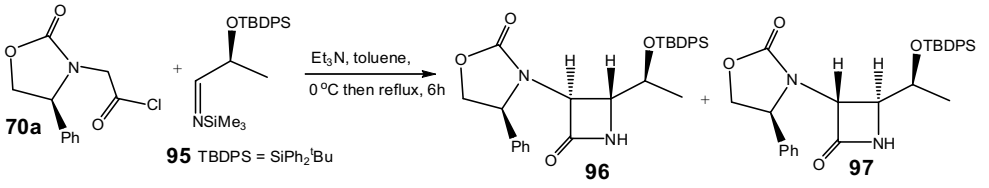
High levels of diastereoselectivity of β-lactams have been observed with Evans–Sjögren ketenes derived from (*R*)- and (*S*)-α-amino acid esters, as well as from the reaction between chiral aldehyde-derived imines [50]. An example of

a reaction between an Evans–Sjögren ketene derived from acylchloride **70a** and imine **89** is shown in Scheme 9.25, where the two reactants have a mismatched relationship leading to formation of β-lactams **90** and **91** in a 40 : 60 ratio. In addition, the reaction of imine **92** and the ketene derived from the acetylchloride **70a** leads to β-lactams **93** and **94** in an 85 : 15 ratio (Scheme 9.25).



Scheme 9.25 β-Lactams from Evans–Sjögren ketenes.

Trans-β-lactams **96** and **97** can be prepared by using the imine **95** with the ketene derived from **69** (Scheme 9.26) in a diastereomeric ratio of 90 : 10 [25, 26, 27].

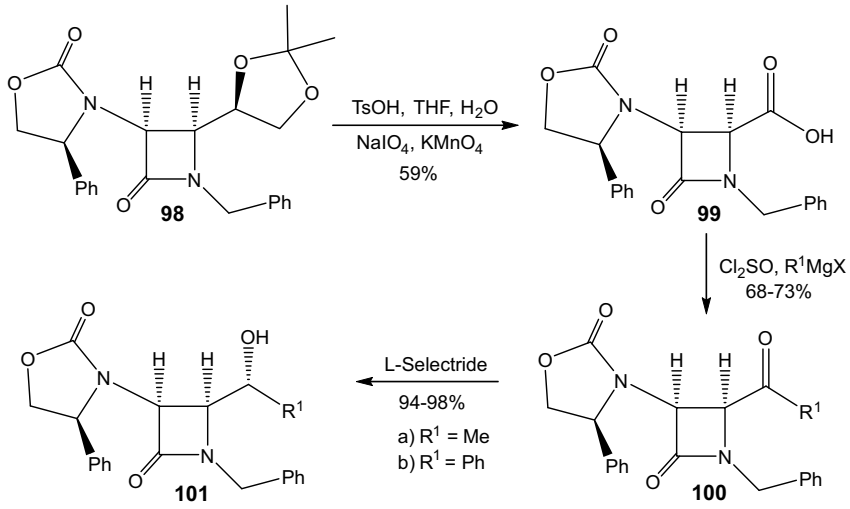


Scheme 9.26 *Trans*-β-lactams from Evans–Sjögren ketenes.

A practical approach to obtaining β-lactams **101** essentially as single isomers is through use of the acylchloride of lactam **99** and Grignard reagents (Scheme 9.27). Using β-lactams such as **98** and/or its enantiomer it is possible to obtain the remaining *cis* diastereomers, which are otherwise not directly accessible from simple or double stereodifferentiating cycloadditions [50].

9.11
Influence of Catalysts on the Stereoselectivity of the Reaction

Catalytic approaches can also be used in achieving diastereoselectivity of the β-lactam ring. One such approach uses chiral amines in the [2 + 2] reaction of ketenes and

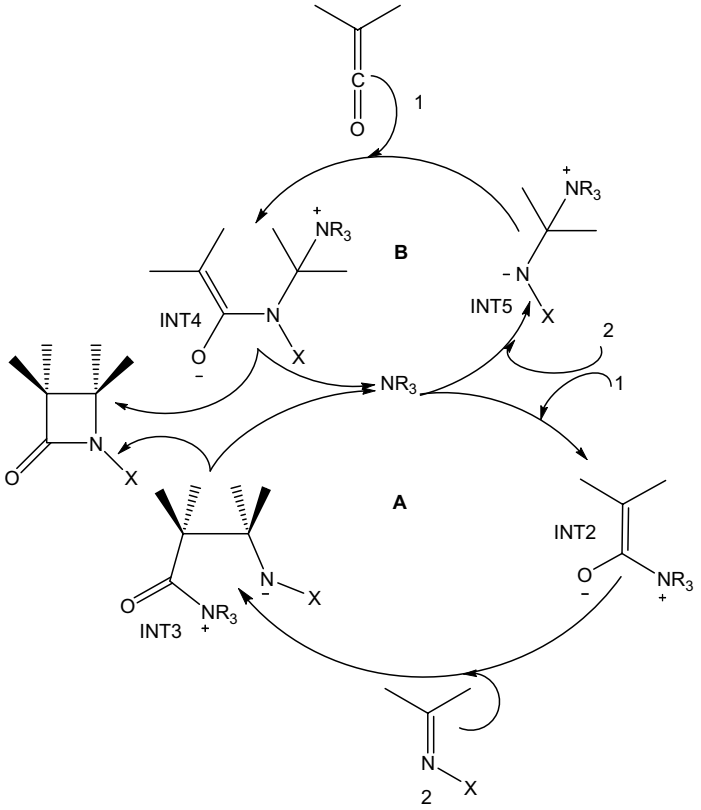


Scheme 9.27 β-Lactams with configurations through double stereodifferentiating cycloadditions.

imines [51, 52]. The latter requires strongly electrophilic imines (e.g., imines that have an *N*-tosyl or alkoxycarbonyl groups) to produce satisfactory diastereoselectivity. The mechanism is believed to involve nucleophilic attack of the catalytic amine onto the sp C-atom of the ketene leading to formation of an enolate (zwitterion). The enolate then attacks the imine via Mannich-type reaction which leads to transferral of the chirality of the amine to the newly formed C–C bond (Scheme 9.28).

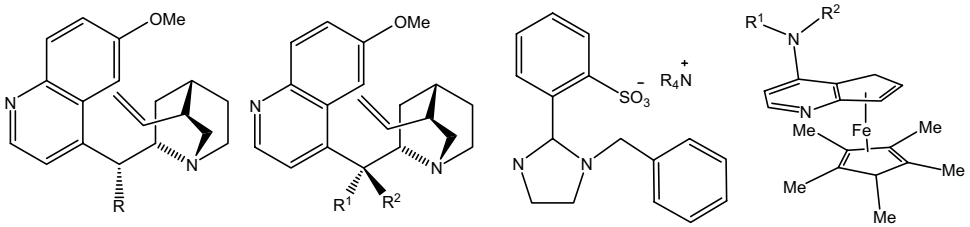
Finally, the cyclization to the β-lactam ring leads to regeneration of the catalyst. Good-to-excellent enantioselectivities for *cis*-β-lactams have been obtained with the amines **102a–e** shown in Figure 9.3; this is most likely due to formation of *Z*-enolates. If the *trans* configuration of the β-lactam is desired, it could be produced using amines such as **102f** (Figure 9.3) [53]. Formation of *E*-enolates has been suggested to explain the change in the stereoselectivity [53]. Efficient catalysts of the Staudinger reaction have been reported to be **102g** and **h** (Figure 9.3). For catalysts **102g** and **h** two mechanisms are proposed that depend upon the nature of the imine used. For *N*-Ts imines (Scheme 9.28), Cycle A mechanism is the one that operates. For *N*-Tf imines leading to *trans*-β-lactams the type B mechanism, which involves activation of the imine (not the ketene) by the catalyst, is most likely to function. In summary, the catalytic version of the Staudinger reaction probably proceeds via a stepwise mechanism in which either the ketene or the imine can be activated by the addition of a catalyst.

The method using the catalysts **102a–11e**, especially **102d** (benzoyl quinine (BQ)) and **102e** (benzoyl quinidine (BQd)) [53, 54], is based on use of a pair of Brønsted bases: a strong base, which forms thermodynamically stable hydrochloride salts, but cannot directly deprotonate acyl chlorides at low temperatures, and a nucleophilic tertiary base (e.g., benzoyl quinine) that β-dehydrochlorinates acyl chlorides under kinetic conditions. The resulting ketenes form intermediate zwitterionic chiral



Scheme 9.28 Proposed mechanism for catalytic Staudinger reaction in the presence of tertiary amines.

enolates, such as **121** (Scheme 9.29), which add to electrophilic imines, such as **122**, to provide β-lactam **123**. Although the reaction yields of the β-lactams are not high (between 35 and 69%), the *cis* product enantiomeric excesses are very high and consistent. In order to obtain the opposite stereochemistry on the β-lactam ring,



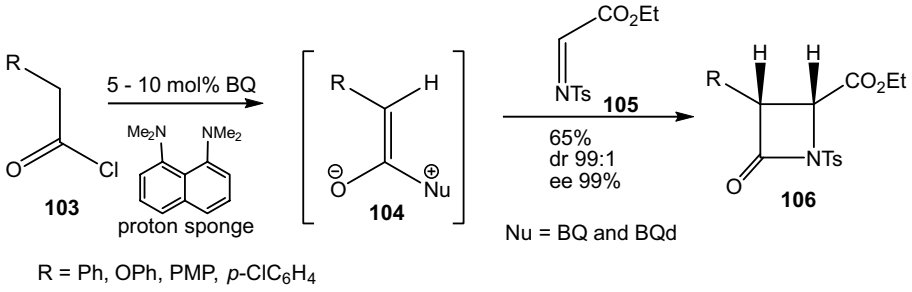
102a R= $PhCO_2$
102b R = H
102c R= $PhCO_2NH$

102d R^1 = $PhCO_2$, R^2 = H BQ
102e R^1 = H' R^2 = $PhCO_2$ BQd

102f R = n-Heptyl

102g R^1 = R^2 = Me
102h R^1 = R^2 = $(CH_2)_4$

Figure 9.3 Chiral catalysts for the Staudinger reaction.



Scheme 9.29 β-Lactams synthesized with chiral catalysts.

switching to the use of BQd is sufficient; β-lactam **123** can then be smoothly *N*-detosylted by using SmI_2. The further development of the synthetic methodology using the two chiral cinchona-based catalysts BQ and BQd ("chiral nucleophiles") also includes an addition of Lewis acid catalysts, thus forming a bifunctional catalytic system that leads to production of β-lactams in high chemical yield, diastereoselectivity, and enantioselectivity [55].

9.11.1
General Procedure for β-Lactams 106 with Proton Sponge

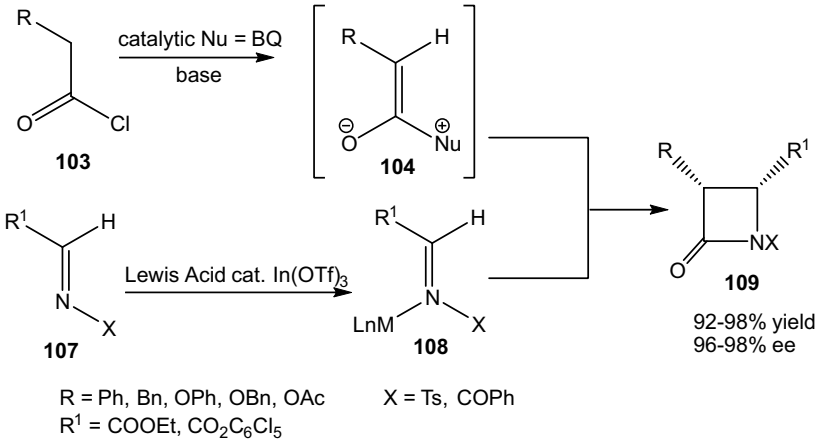
To a solution of phenylacetyl chloride **103** (20 mg, 0.13 mmol) in toluene (0.5 ml) at −78 °C is added proton sponge **3b** (31 mg, 0.14 mmol) in toluene (0.5 ml) immediately followed by benzoylquinine **102e** (6 mg, 0.013 mmol) and *R*-imino ester **105** (33 mg, 0.13 mmol). The reaction was allowed to stir for 5 h as it slowly warms to room temperature. The solvent was removed under reduced pressure and the crude mixture was subjected to column chromatography (15% EtOAc/hexanes) on a plug of silica gel to yield **106** (33 mg, 65% yield).

The two base catalysts BQ and BQd work best with Lewis acids based on Al(III), Zn (II), Sc(III), and most notably In(III) [56]. Mechanistic evidence leads to the conclusion that the chiral nucleophiles form zwitterionic enolates that react with metal coordinated imines (Scheme 9.30).

When the bifunctional catalysts having $In(OTf)_3$ as Lewis acid, are employed, generally the enantiomeric excesses are maintained in the high 90s and the yields are increased by a factor of 1.5–2.0 [56], as compared to the Staudinger reaction catalyzed by the cinchona alkaloids in the absence of In(III) salts (Figure 9.4).

9.11.2
General Procedure for the Tandem Nucleophile/Lewis Acid-Promoted Synthesis of β-Lactams 110

To a suspension of $In(OTf)_3$ (3 mg, 0.013 mmol), benzoylquinine **102d** (5.6 mg, 0.013 mmol), and proton sponge (28 mg, 0.13 mmol) in toluene (7.5 ml) at −78 °C was added dropwise phenylacetyl chloride (**103**) (20 mg, 0.13 mmol) in toluene



Scheme 9.30 Tandem Lewis acid/nucleophile synthesis of β-lactams.

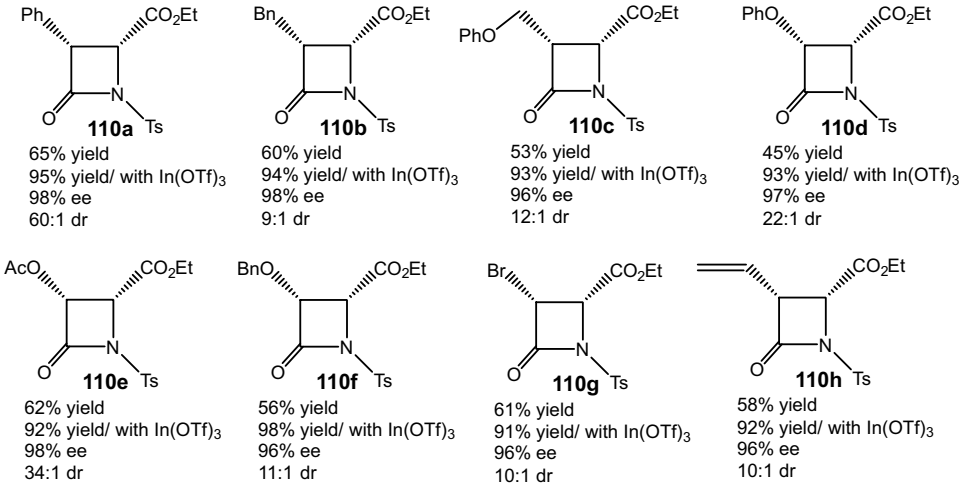
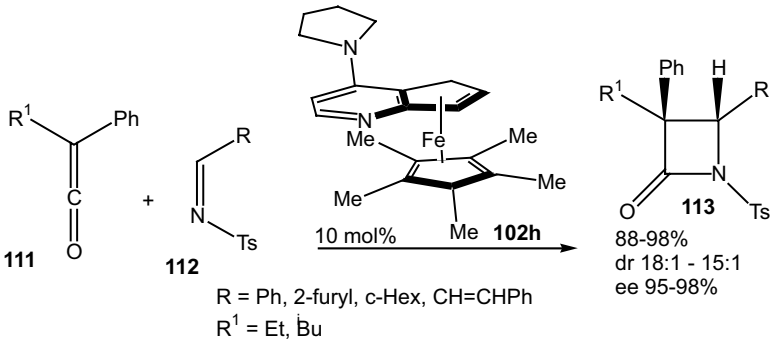


Figure 9.4 β-Lactams synthesized by employing BQ and $In(OTf)_3$ cocatalyst.

(0.5 ml). A solution of imine **105** (32 mg, 0.13 mmol) in toluene (1 ml) was then added via a syringe pump over 1 h. The reaction was allowed to warm to room temperature over 16 h before it was quenched with 1 M HCl (3 ml). The aqueous layer was then extracted twice with CH_2Cl_2, and the combined organic layers were dried over $MgSO_4$ and filtered through Celite. Adsorption onto silica gel followed by column chromatography (10% EtOAc/hexanes) afforded product **110a** (46 mg, 95% yield) and 98% enantiomeric excess (diastereomeric ratio 60 : 1).

Utilizing the same principle as described for the cinchona chiral nucleophiles BQ and BQd, another catalyst type, planar-chiral azaferrocenes **102g** and **h** (Figure 9.3), efficiently promotes the reaction between disubstituted ketenes and a variety of

imines including those that are derived from enolizable aldehydes. The β-lactams produced using the azaferrocene catalysts are obtained in high yield and high enantioselectivities [57] (Scheme 9.31). When unsymmetrical disubstituted ketenes are employed, good *cis* selectivities are observed, such as β-lactam **113**.



Scheme 9.31 β-lactams produced using azaferrocene catalyst.

9.11.3
General Procedure for Catalytic Asymmetric Synthesis of *Trans*-β-Lactams 113 [57]

The two solutions (1.0 equiv. of ketene; 1.0 equiv. of *N*-triflyl imine and 0.10 equiv. of (−)-**102h**) were prepared in a glove box. The rest of the procedure was conducted outside of the glove box under a nitrogen atmosphere.

A solution of 1,1,1-trifluoro-*N*-(phenylmethylene)methanesulfonamide (56.4 mg, 0.24 mmol) and (−)-**1** (8.9 mg, 24 mmol) in toluene (11 ml) was cooled to −78 °C. To this solution phenyl ethyl ketene (34.8 mg, 0.24 mmol) in toluene (1.2 ml) was added over approximately 1 min; the syringe was rinsed with toluene (0.3 ml). The resulting solution was allowed to warm to room temperature with stirring (12 h). After solvent removal by evaporation, the residue was purified by column chromatography (5 → 10% EtOAc/hexanes). The purified β-lactam was a colorless oil (54.3 mg, 60%; mixture of diastereomers). The major diastereomer, was also a colorless oil, and was isolated by column chromatography (2.5% EtOAc/hexanes), yield: (36.6 mg, 40%). High-performance liquid chromatography (HPLC): 65% enantiomeric excesses (Daicel Chiralcel AD-H column; 0.5 ml/min; solvent system: 1.0% isopropanol/hexanes; retention times: 10.7 min (major), 9.92 min (minor)). Second run (same scale): yield: 54.0 mg, 59%. Yield of the major diastereomer: 33.0 mg, 36%, 60% enantiomeric excesses (Scheme 9.31).

The Kinugasa reaction has also been utilized to produce chiral β-lactams [58–60]. First reported in its asymmetric and catalytic version with limited success, utilizing the chiral ligands **114a–c** (Figure 9.5) the Kinugasa reaction [61] is a reaction between terminal alkynes and nitrones promoted by CuI or CuCl (Scheme 9.32) [62]. Using 10 mol% CuI and 20 mol% of bisoxazoline **114a** in the presence of K_2CO_3, the

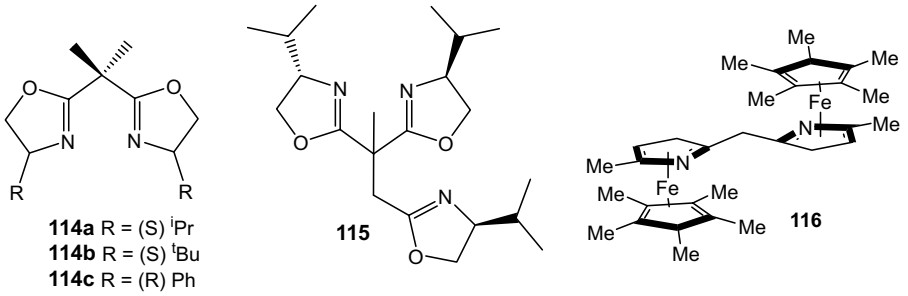
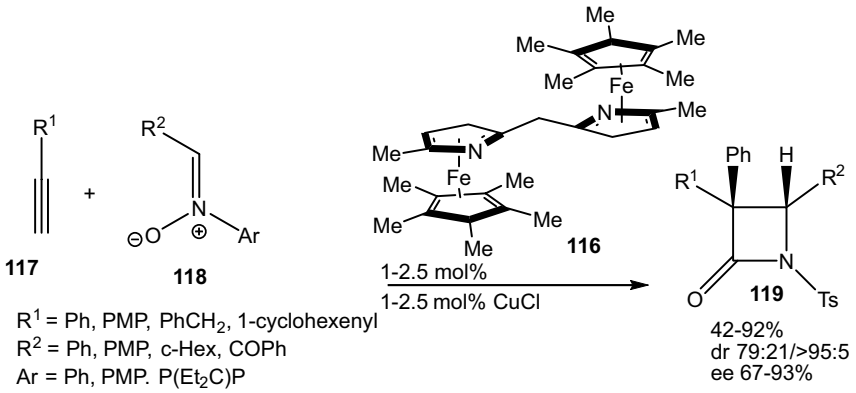


Figure 9.5 Catalysts for the asymmetrical Kinugasa reaction.

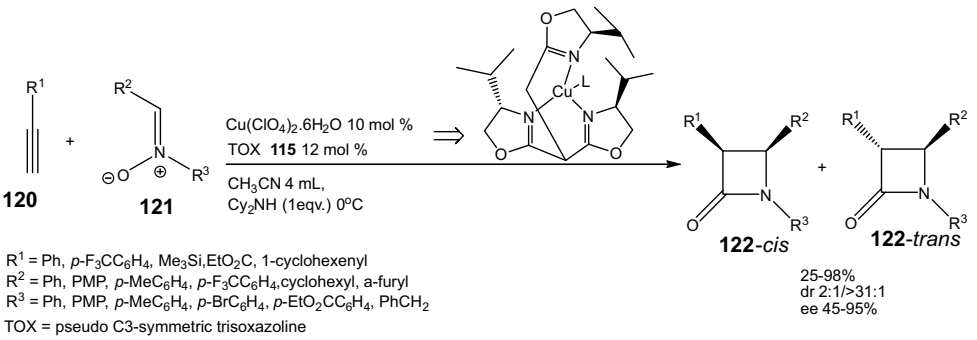
reaction provided β-lactams in 57% enantiomeric excess and in 30% diastereomeric excess (Scheme 9.32) [62].



Scheme 9.32 β-Lactams from the Kinugasa reaction with bis-azaferrocene as catalyst.

Although neither the chemical yields, nor the *cis*/*trans* ratio of the product are impressive, the enantiomeric excess values of the major *cis* diastereoisomer **119** are very high when the planar bis-azaferrocene **116** is used as a catalyst (Scheme 9.32). In addition, 1 mol% bis(azaferrocenes) **116**/CuCl can also catalyze intermolecular Kinugasa reaction affording products with good to high enantioselectivities (up to 93% enantiomeric excess) and with high *cis* diastereoselections [62]. By a similar strategy, these researchers demonstrated that an intramolecular Kinugasa reaction can be used to construct fused tricyclic ring systems efficiently with good enantioselectivities [63].

The reactions of nitrones with terminal alkynes, catalyzed by chiral *i*Pr-trisoxazoline **115**/Cu(ClO_4)2·6H_2O, not requiring inert atmosphere, leads to formation of β-lactams in moderate to good yields with up to 85% enantiomeric excess (Scheme 9.33) [64]. Diastereoselectivity depends on the alkyne used. Propiolate gives the *trans* isomer as a major product, while the other alkynes afford predominantly *cis*-disubstituted lactams. For the first time in the Kinugasa reaction a Cu(II)



Scheme 9.33 β-Lactams from the Kinugasa reaction with chiral *i*Pr-trisoxazoline as catalyst.

salt has proven to be an efficient catalyst precursor, making the reaction more practical. An appropriate base in this reaction is essential to control both diastereoselectivity and enantioselectivity. Compared with primary and tertiary amines, secondary amines give higher enantioselectivities.

9.11.4
Example for Kinugasa Reaction with Cu (II) Catalyst

9.11.4.1 General Procedure for Catalytic Asymmetric Synthesis of β-Lactams 122

A mixture of $Cu(ClO_4)_2{\cdot}6H_2O$ (9.3 mg, 0.025 mmol) and (*S*)-isopropyl trisoxazoline **115** (11.3 mg, 0.03 mmol) in CH_3CN (4 ml) was stirred under an air atmosphere (15 °C, 2 h). The solution was then cooled to 0 °C and Cy_2NH (50 ml, 0.25 mmol) was added, followed after 10 min by addition of the alkyne (0.375 mmol). When the resulting mixture color became light yellow, the nitrone (0.25 mmol) was added to the solution. After completion of the reaction (monitored by thin-layer chromatography), the mixture was passed through a short silica gel column eluting with CH_2Cl_2. The filtrate was then concentrated, and the residue was purified by flash chromatography (petroleum ether/CH_2Cl_2) to afford the product **122**. The diastereoselectivity was determined by ^1H nuclear magnetic resonance spectroscopic analysis of crude product. The determination of enantioselective excess of the *cis* isomer was performed by chiral HPLC with a Daicel Chiralcel OD-H column (eluent: hexane/*i*PrOH 80/20, flow rate: 0.7 ml/min).

9.12
Conclusions

Synthetic methods for the development of the β-lactam ring have been driven traditionally by their functionality as antibiotics with excellent pharmacodynamics and pharmacokinetics. Although the underlying impetus for the development of newer generations of β-lactam antibiotics remains antibiotic resistance, in recent years, the expanding understanding of the spectrum of β-lactam bioactivity as

prokaryotic and eukaryotic enzyme inhibitors has driven the development of β--lactam asymmetric syntheses. The Staudinger reaction remains a versatile reaction, providing the opportunity for the preparation of diversely functionalized β-lactams. In most cases, β-lactams with the *cis* configuration are the major, or sole, reaction product; however, reactions leading to *trans* selectivity are also known. Many diastereoselective methods have been described, with unsolved problems of reaction warranting further investigation. Some of these have been solved by the discovery of the first reliable catalytic methods. The development of asymmetric synthetic methods based on the Staudinger reaction are expected to continue to advance β-lactam development in the future, especially since β-lactams can serve as synthons in organic synthesis for the construction of more complex biologically active molecules.

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10
Advances in *N*- and *O*-Glycopeptide Synthesis – A Tool to Study Glycosylation and Develop New Therapeutics

Ulrika Westerlind and Horst Kunz

10.1
Introduction

Many proteins are co- or post-translationally modified by mono- or oligosaccharides. It has become evident that these glycoproteins play important roles in diverse biochemical processes. The saccharides contribute with physiochemical properties, which include conformational effects on the protein, stability to proteolysis, and lubrication of cells. In addition, the glycans are involved in cell–cell recognition and cell–external agent interactions. These interactions induce biological events, which include cell growth and differentiation, cell proliferation, cell adhesion, binding of pathogens, fertilization, and immune responses [1–6]. The glycans assist in protein folding and transport, and they are involved in pathogenic processes like chronic inflammation [5], viral and bacterial infections [7–10], tumor growth and metastasis [11, 12], and autoimmune disorders [13, 14]. The variety of events affected by protein glycosylation is not surprising. Carbohydrates are unique in their structural complexity and, in contrast to oligonucleotides and proteins, they can be connected in more than one form: connected via different configural positions of the glycan residue and also via an anomeric α- or β-glycosidic linkage. The different modes of connection can lead to long linear or highly branched saccharide structures. By combining different types of glycans like galactose, glucose, *N*-acetyl galactosamine (GalNAc), *N*-acetyl glucosamine (GlcNAc), fucose, mannose, sialic acid, and others, an enormous amount of combinations can be formed. Nature only uses a small portion of these possible combinations. The biosyntheses of different oligosaccharides are dependent of glycosyltransferases, which are gene-encoded. Each type of connection and glycan residue needs a specific enzyme, and for practical reasons this restricts the number of combinations. Nature also limits the number of final products that can be made by having common core structures. The saccharides can be linked to the protein backbone via an *O*-glycosidic bond or via amide bond formation in *N*-glycoproteins. In the most common type of *O*-glycoproteins, the mucin type, a GalNAc residue is connected to the protein backbone via an *O*-glycosidic linkage to serine or threonine [15, 16]. The GalNAc residue can be further extended with

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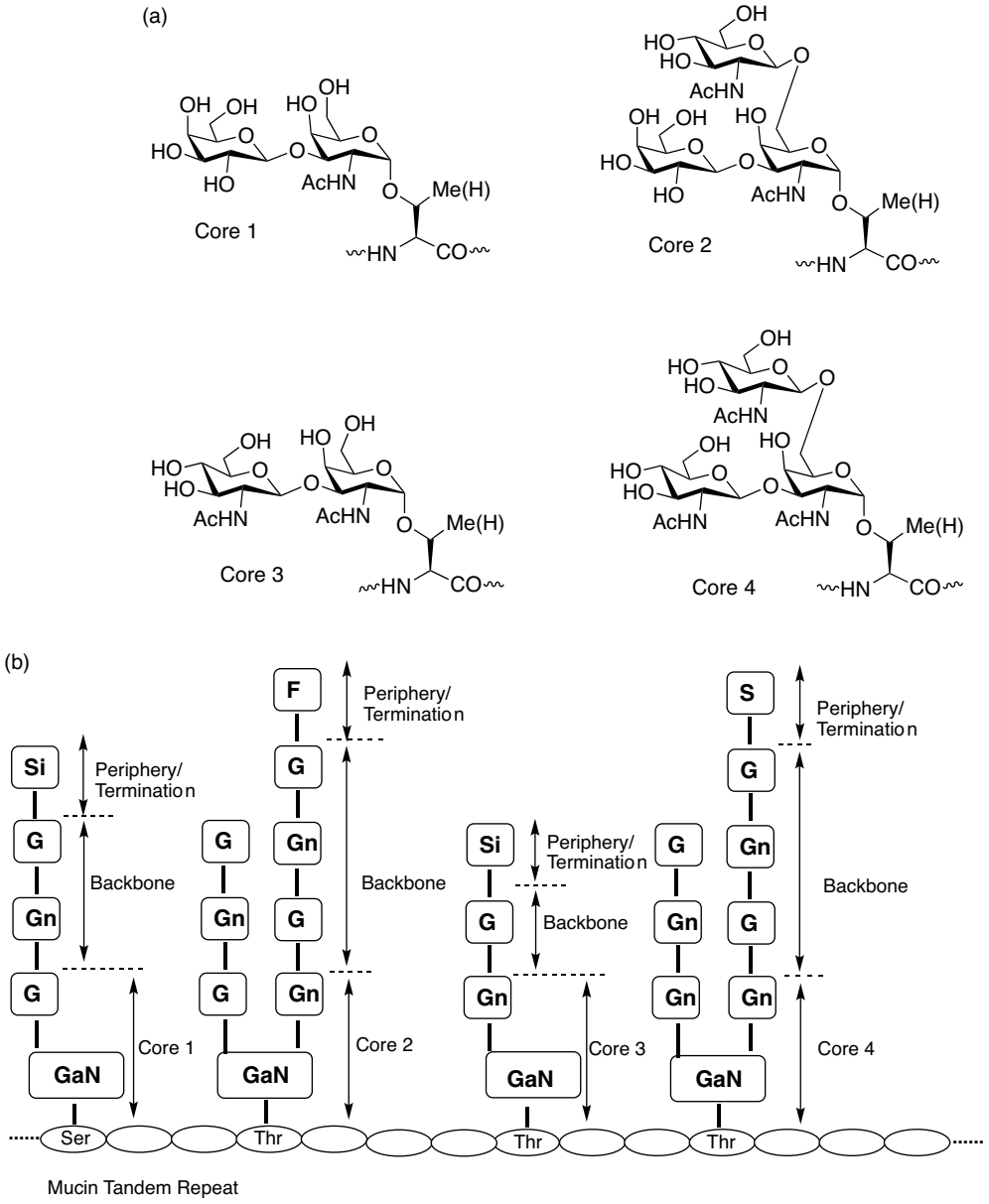


Figure 10.1 Structure of (a) core 1–4 of mucin-type *O*-glycosylation, (b) extended glycosylation of core 1–4, (c) *N*-glycan pentasaccharide core, and (d) extended glycosylation of pentasaccharide core, high mannose, hybrid, and complex type.

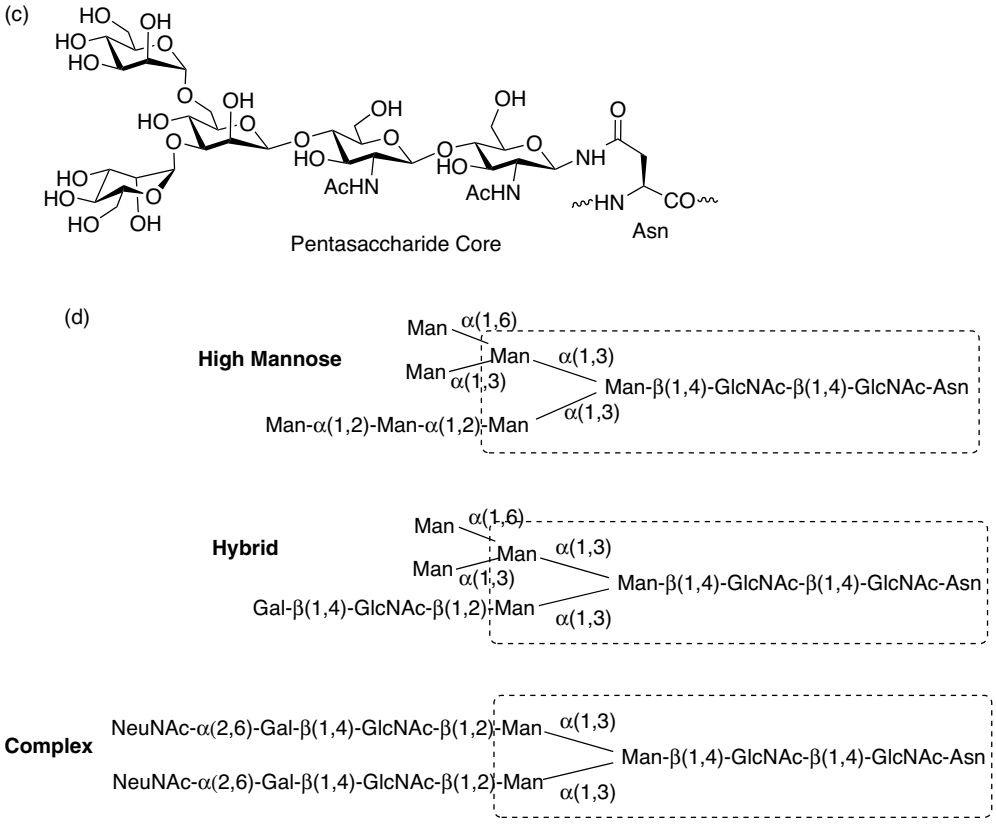


Figure 10.1 (*Continued*)

additional Gal, GlcNAc, or GalNAc residues forming the core structures 1–8. These core structures can then be extended with one or more repeated type 1 (Gal-β1–3-GlcNAc) or type 2 (Gal-β1–4-GlcNAc) glycosylations, and terminated with fucose, sialic acid, and/or GalNAc residues, leading to complex saccharides (Figure 10.1a and b). The majority of *N*-glycoproteins have a common pentasaccharide core consisting of three mannoses and two GlcNAc residues. One of the GlcNAc residues is linked to the protein core via an amide bond to the asparagine side-chain. The pentasaccharide core structure can be additionally extended forming high mannose-type, hybrid-type, or complex-type *N*-glycoproteins [17] (Figure 10.1c and d). In spite of the restrictions used by nature when forming complex oligosaccharide structures, a large number of combinations can be formed and since the majority of glycans are attached to other molecules like proteins or lipids, an additional dimension of complexity results. To understand the function and relevance of saccharides, it is important to not exclude the backbone to which it is connected. In the case of glycoproteins, it is often the effect of glycosylation on the protein backbone that is interesting to study in terms of conformational or proteolytic stability, structure recognition/binding effects, or

multimeric effects. Since glycans are not direct gene products, they are formed in an environment of competing glycosyltransferases and glycosidases with variations in substrate and donor levels. This leads to microheterogeneity of the glycoproteins. Therefore, it is problematic to study glycosylation only by gene expression studies on enzyme levels or by use of isolated proteins. It can also be difficult to isolate pure glycoproteins in large quantities. Using synthetic methods, glycopeptides with a defined structure can be produced. These peptides can be employed to study conformational effects of glycosylation, binding events to cell adhesion molecules or pathogens, or for the production of vaccines or the production of inhibitors mainly through glycopeptide mimics. Synthetic glycopeptides can be ligated to proteins forming homogeneous glycosylated neoglycoproteins, which might be very useful to study glycosylation and for use in therapy.

10.2
Synthesis of *O*-Glycopeptides

The synthesis of glycopeptides can be realized by different strategies. Glycopeptides can be prepared by direct attachment of saccharides to the completed target peptide (convergent synthesis) or by the use of glycosylated amino acid building blocks incorporated by stepwise peptide assembly. The use of the convergent approach is limited since it suffers from low yields of glycosylations due to reactivity problems. Problems with α and β selectivity are more pronounced when larger glycan structures are coupled to the serine/threonine residue. Synthesis of *O*-glycopeptides by Fmoc solid-phase peptide synthesis (SPPS) employing glycosylated amino acids is therefore the standard approach [18–21]. During peptide backbone assembly, the glycosylated amino acid building blocks are normally protected. This is because of the lability of the *O*-glycosidic bond to strong acid treatment, and under treatment with a strong base *O*-glycopeptides can undergo β-elimination of the *O*-linked glycans or epimerization of the stereogenic centers [22]. Due to this labile character the glycosylated amino acid building blocks are normally protected during the peptide backbone assembly. After cleavage from resin of the synthesized peptide, the glycan protecting groups need to be removed under mild conditions. *O*-Acetyl protecting groups are commonly used for the saccharide portion, which can be easily removed using mild conditions like dilute sodium methoxide in methanol [23, 24] or hydrazine hydrate in methanol [25, 26]. Glycopeptides can also be prepared employing an enzymatic approach [27]. However, if multiple glycosylation sites are available, specific attachment of the glycan direct to the peptide backbone is problematic. For example, in mucin-type glycosylation 20 GalNAc transferases are available with different specificity and some have lectin domains, which require preglycosylated peptides in a certain conformation for activity. It can therefore be difficult to predict the result employing this approach. Starting with a peptide glycosylated in defined positions, further extension and termination with glycosyltransferases is therefore preferable. The enzymatic approach is useful when the peptide backbone is more protein-like in size and thereby too difficult to synthesize by standard peptide synthesis or if the glycan structures are complex and therefore very demanding to

synthesize as protected glycosylated amino acid building blocks. The synthetic approach employing amino acid building blocks is favorable in order to form glycopeptides with a defined glycosylation pattern and to secure exact control over the positions that should be glycosylated.

10.2.1 Synthesis of Mucin-Type Glycopeptides

The mucin-type glycoproteins have in common that a GalNAc residue is connected to serine or threonine via a α-glycosidic bond. As mentioned above (Figure 10.1a and b) the branching of the GalNAc residue in the 3- or 6-position with Gal, GlcNAc, or GalNAc gives rise to different core structures (1–8), which can be repeatedly extended with Gal and GlcNAc residues, and terminated with fucose, sialic acid, sulfation, and/or GalNAc. Mucin glycoproteins commonly contain dense clusters of threonine and serine residues modified by α-GalNAc-based glycans. However, mucin-type glycosylations are not only restricted to mucin proteins. The mucin-type *O*-glycoproteins play important roles in many cell–cell and cell–external agent interactions. They carry blood group antigens, and serve as ligands involved in cell adhesion events related to the innate immune system, inflammation, and cancer metastasis. In this section, examples of the synthesis of tumor-associated glycopeptide antigens and total synthesis of P-selectin glycopeptide ligands will be described.

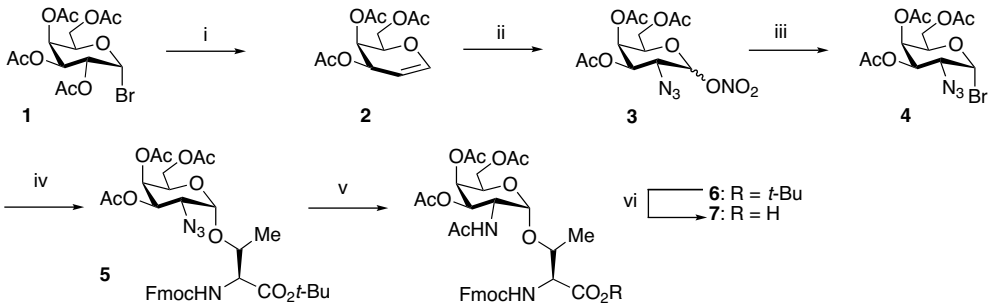
10.2.1.1 Synthesis of Tumor-Associated Glycopeptides and Glycopeptide Vaccines

Mucins are a class of extensively glycosylated proteins expressed on the surface of epithelial cells or secreted to function in mucus. They normally have complex and highly branched *O*-linked carbohydrate structures that obscure the protein core. The membrane-bound glycoprotein mucin 1 (MUC1) is the most intensively studied mucin protein with regard to cancer immunotherapy [12, 28]. The extracellular domain of MUC1 consists of several tandem repeats comprising 20 amino acids of the sequence HGVTSAPDTRPAPGSTAPPA [29], including five potential *O*-glycosylation sites (underlined). MUC1 is extensively overexpressed on epithelial tumor cells. Concomitant downregulation of glycosyl transferases, in particular the core 2 enzyme β-1,6-*N*-acetylglucosaminyltransferase, and upregulation of sialyltransferases results in short saccharides with premature sialylation on the extracellular surface [30–34]. Furthermore, the underglycosylation of the mucin extracellular domain also results in exposure of the peptide backbone. Tumor-associated epitopes consisting of both the saccharide and the peptide structures are formed. Efforts have therefore been made to synthesize MUC1 tandem repeat glycopeptides that mimic the cell surface. These peptides are of interest to develop synthetic vaccines and for use in cancer diagnostics. The tumor-selective MUC1 glycopeptides are only moderately immunogenic and additional stimulation is necessary to get a humoral immune response using carrier proteins or immunostimulating T cell peptide epitopes [35, 36].

10.2.1.1.1 Synthesis of Tn, T, Sialyl-Tn, and Sialyl-T Glycosylated Amino Acid Building Blocks

Chemical synthesis of glycosylated Fmoc-serine and -threonine building blocks for SPPS is a demanding task. Most such building blocks are back integrated to

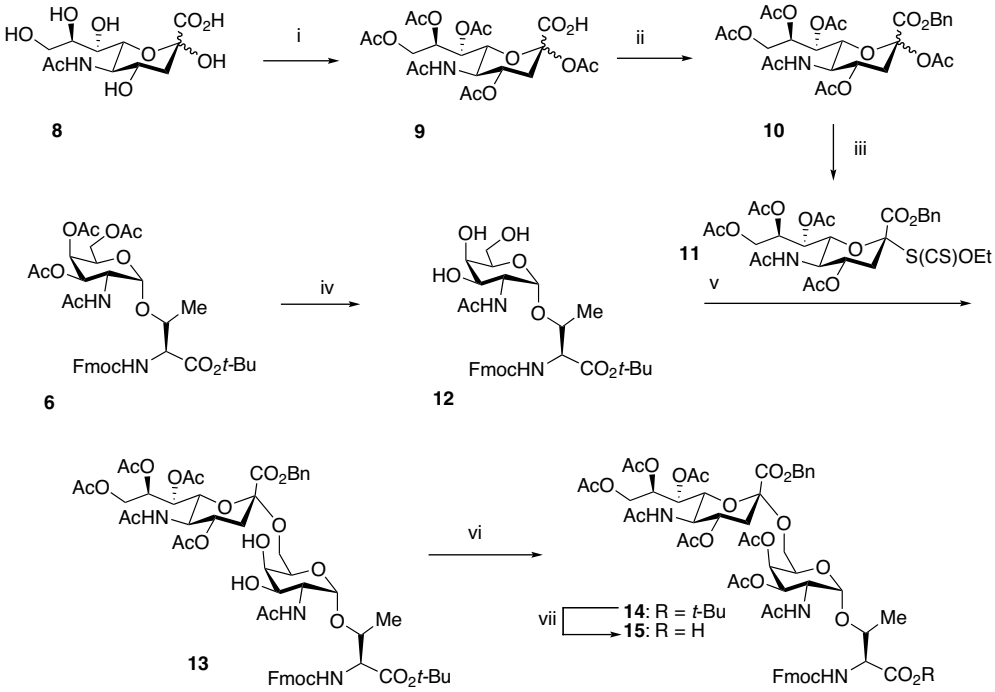
Tn antigen intermediate **12**. By making use of the high reactivity of the 6- and 3-positions of Gal in combination with proper protective groups, highly selective glycosylations can be performed. Tn antigen is synthesized from acetobromogalactose **1** [37] (Scheme 10.1) over the corresponding galactal **2** [38] by azidonitration [39] yielding an anomeric mixture of azidonitrate **3**, which is subsequently converted to the corresponding α-bromide **4** by treatment with LiBr in acetonitrile [39]. Fmoc-threonine *t*Bu-ester [40], or its serine counterpart (not shown), is then glycosylated with **4** under Koenigs–Knorr conditions [41–44] providing **5**. The azido function is then converted to the corresponding acetamide **6** by thioacetic acid [45, 46] and the *t*Bu-ester is cleaved by trifluoroacetic acid (TFA) in the presence of anisole as a scavenger, providing the fully protected Tn antigen building block **7** for further SPPS (Scheme 10.1).



Scheme 10.1 Reagents and conditions: (i) Zn dust, $CuSO_4$, H_2O, HOAc, 85%; (ii) CAN, NaN_3, MeCN, 45%; (iii) LiBr, MeCN 92%; (iv) Fmoc-Thr-O*t*Bu, Ag_2CO_3, $AgClO_4$ 80%; (v) AcSH, 75%; (vi) TFA, anisole, 90%.

Synthesis of 2,6-sialyl-Tn antigen [43] starts with the preparation of sialyl donor **11** (Scheme 10.2). It has been proven that glycosylation reactions with xanthate **11** in the presence of a thiophilic activator are superior to other sialic acid donors [47]. Peracetylation of sialic acid **8** gives the fully acetylated intermediate **9**, which is benzylated with BnBr/cesium carbonate to give **10** as an anomeric mixture [48]. Treatment of **10** with acetyl chloride followed by reaction *in situ* with KO(CS)OEt gives rise to xanthate **11** under thermodynamically controlled conditions as a single anomer [47]. After deacetylation of Tn antigen–Fmoc-threonine **6** [44, 49, 50], the resulting product **12** is sialylated selectively in the 6-position by a combination of xanthate **11** and methylsulfenyl triflate (MeSOTf) [51] at low temperature. At low temperature (−65 °C), only the kinetically favored equatorial product is formed due to assistance of the nitrile solvent [52–54]. Further transformations include peracetylation of **13** and cleavage of the *t*Bu-ester **14** to give finally the Fmoc-SPPS building block **15** (Scheme 10.2) [43].

Tn intermediate **12** can also be converted into the T antigen building block **19** by a simple procedure. Treatment of **12** with benzaldehyde dimethyl acetal under acid catalysis gives the corresponding 4,6-benzylidene acetal **16**, which undergoes subsequent Helferich glycosylation [55] with acetobromogalactose **1**, to give disaccharide

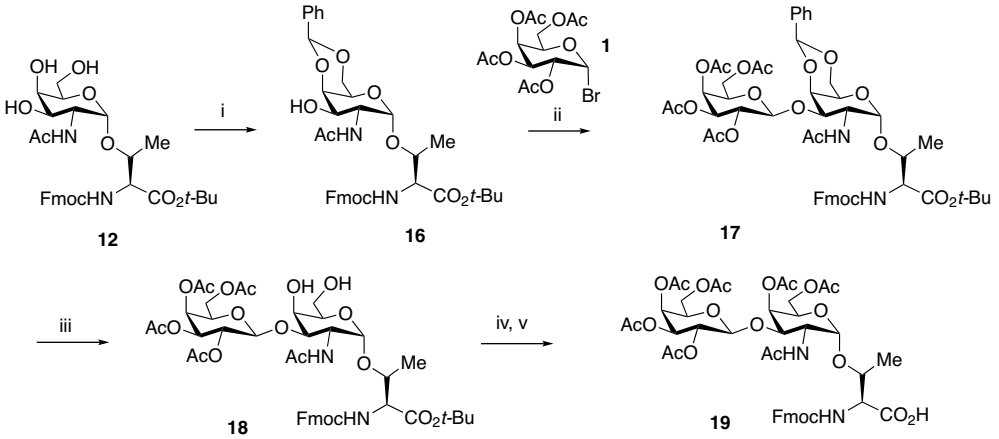


Scheme 10.2 Reagents and conditions: (i) Ac_2O, pyridine, quantitative; (ii) Cs_2CO_3, BnBr, 77%; (iii) AcCl, H_2O, KS(CS)OEt, 64%; (iv) NaOMe, MeOH; (v) MeSOTf, MeCN, CH_2Cl_2 59%; (vi) Ac_2O, pyridine, 90%; (vii) TFA, anisole, 94%.

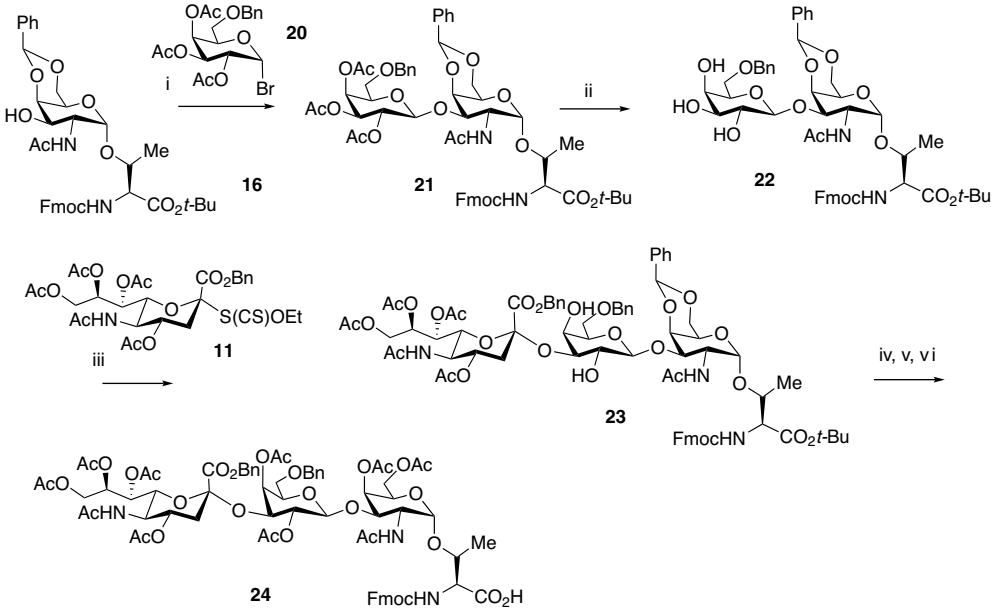
intermediate **17**. The benzylidene acetal can be hydrolyzed under mild acidic conditions [56], providing **18**. Peracetylation of **18** with acetic anhydride and pyridine, followed by removal of the *t*Bu-ester affords the fully protected T antigen Fmoc-SPPS building block **19** (Scheme 10.3) [55].

Sialyl-T antigen is best prepared from intermediate **16**. Compound **16** is glycosylated with 6-OBn-acetobromogalactose **20** [57] and the product **21** is subsequently deacetylated. Sialylation of the more reactive equatorial 3′-position with sialic xanthate **11** proceeds smoothly, thus providing **23**. The benzylidene acetal of **23** is removed under mild acidic [56] conditions and then acetylated under standard conditions. The synthetic sequence ends with removal of the *t*Bu-ester, providing Fmoc-SPPS sialyl-T building block **24** in good overall yield (Scheme 10.4) [58].

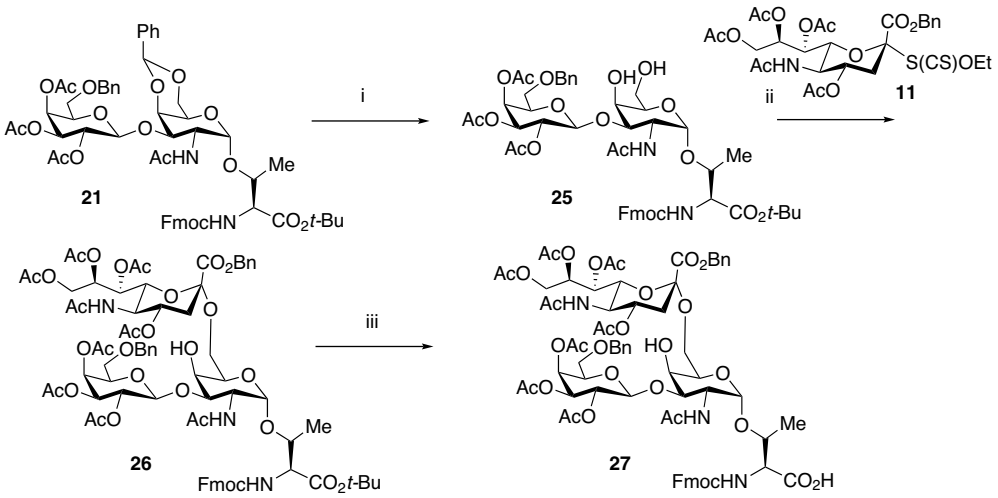
Previous T intermediate **21** can also be transformed to the 6-sialyl-T antigen [55] by selective glycosylation in the 6-position of **25**. Solvolytic removal of the benzylidene acetal in **21** yields the acceptor **25** with free OH groups in the 4- and 6-positions. Due to the intrinsic low reactivity of the axial 4-position OH of galactose, a highly selective sialylation reaction takes place, again employing xanthate **11** as the donor, providing **26**. Removal of the *t*Bu ester gives the Fmoc-SPPS 6-sialyl-T antigen building block **27**. Note that the OH group in 4-position has been left unprotected. Due to the steric



Scheme 10.3 Reagents and conditions: (i) $PhCH(OMe)_2$, TsOH, 75%; (ii) $Hg(CN)_2$, 68%; (iii) 80% aqueous HOAc, 87%; (iv) Ac_2O, pyridine, 92%; (v) TFA, anisole, 95%.



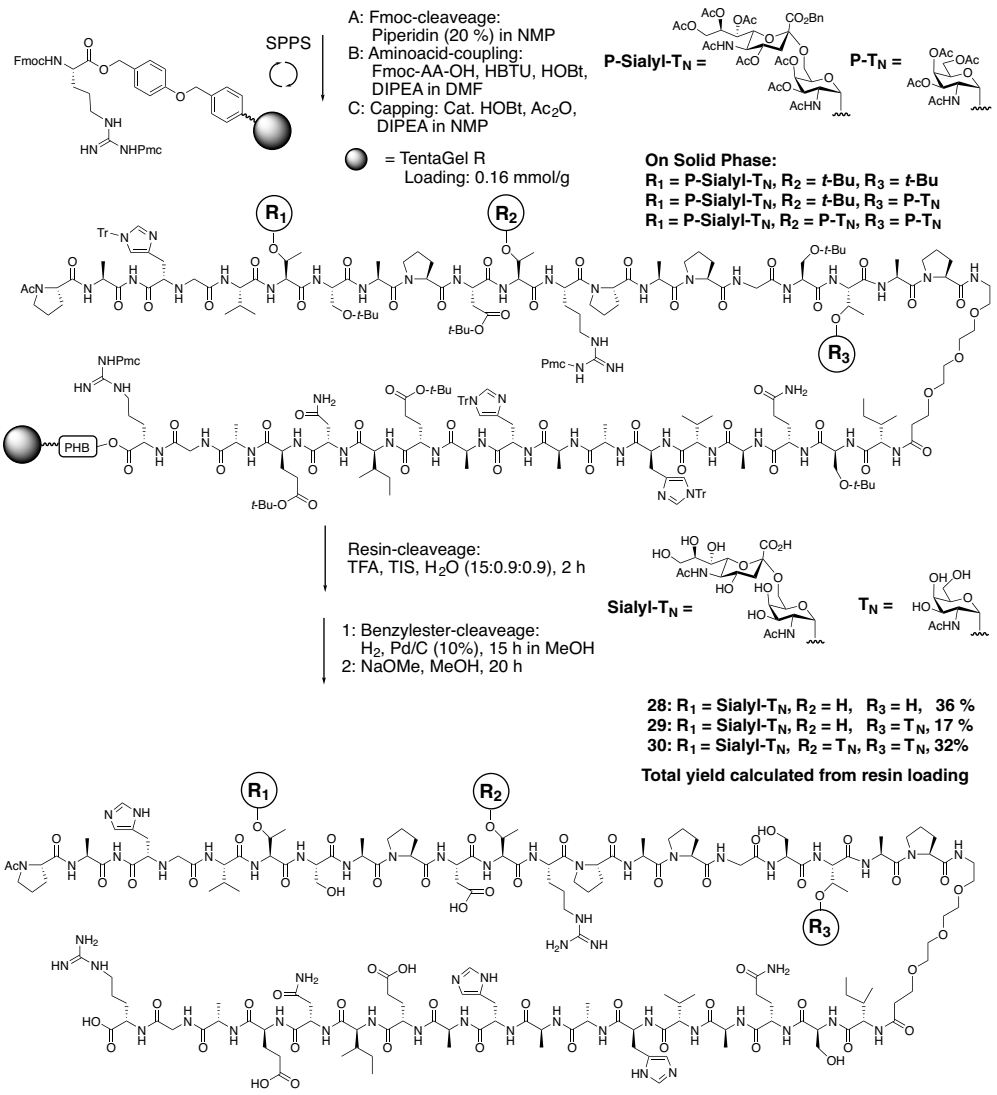
Scheme 10.4 Reagents and conditions: (i) $Hg(CN)_2$, 93%; (ii) cat. NaOMe, pH 8.5, 34%; (iii) MeSOTf, 58%; (iv) 80% aqueous HOAc; (v) Ac_2O, pyridine, cat. 4-dimethylaminopyridine (DMAP), 80% over two steps; (vi) TFA, anisole, quantitative.



Scheme 10.5 Reagents and conditions: (i) 80% aqueous HOAc; (ii) MeSOTf; (iii) TFA, anisole, 98%.

hindrance of the sialic acid moiety, it is not necessary to protect the OH group in the 4-position (Scheme 10.5) [55].

10.2.1.1.2 **Synthesis of Tn, T, Sialyl-Tn, and Sialyl-T Glycopeptides and Vaccines** MUC1 tandem repeat peptides are normally synthesized by Fmoc-SPPS in a stepwise fashion, starting with a suitable resin preloaded with the first C-terminal amino acid. The choice of resin depends on whether the peptide will be cleaved with the amino acid side-chain protecting groups still on using milder cleavage conditions or if the amino acid side-chain protective groups should be cleaved simultaneously with the cleavage from the resin. For milder cleavage conditions the 2-chloro-trityl resin cleavable by 0.1% TFA could be used. When the side-chain protective groups should simultaneously be removed from the peptide during cleavage, Wang resin [59] is normally used together with a 95% TFA cleavage mixture. The choice of resin is also dependent on if the peptide sequence starts with amino acids prone to diketopiperazine formation, such as proline and alanine. In such a case, a bulky resin is required, such as the trityl resin [60]. The glycosylated amino acid building blocks are often coupled manually using 1.5–2 equiv. of the glycosyl amino acid building block, which is activated with the more reactive 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt) [61] coupling reagent, while the other Fmoc-amino acids are normally coupled automatically on a peptide synthesizer using 5–20 equiv. and the standard 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) [62] reagent. Recently, sialyl-Tn, and Tn mono-, di-, and triglycosylated 38-amino-acid MUC1 tandem repeat peptides connected to an ovalbumin $(OVA)_{323-339}$ T cell peptide epitope via a nonimmunogenic spacer (**28**–**30**) (Scheme 10.6) were synthesized [63]. The synthesis started with Wang resin

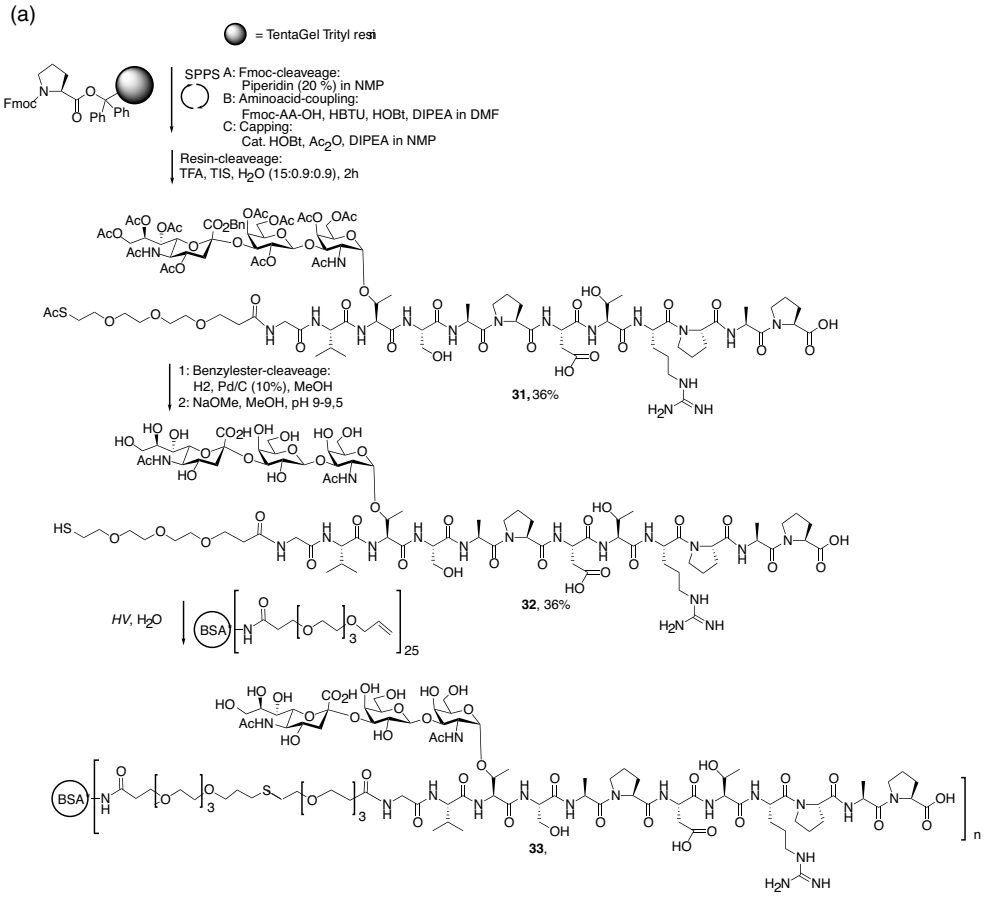


Scheme 10.6 Synthesis of MUC1–OVA glycopeptide vaccines.

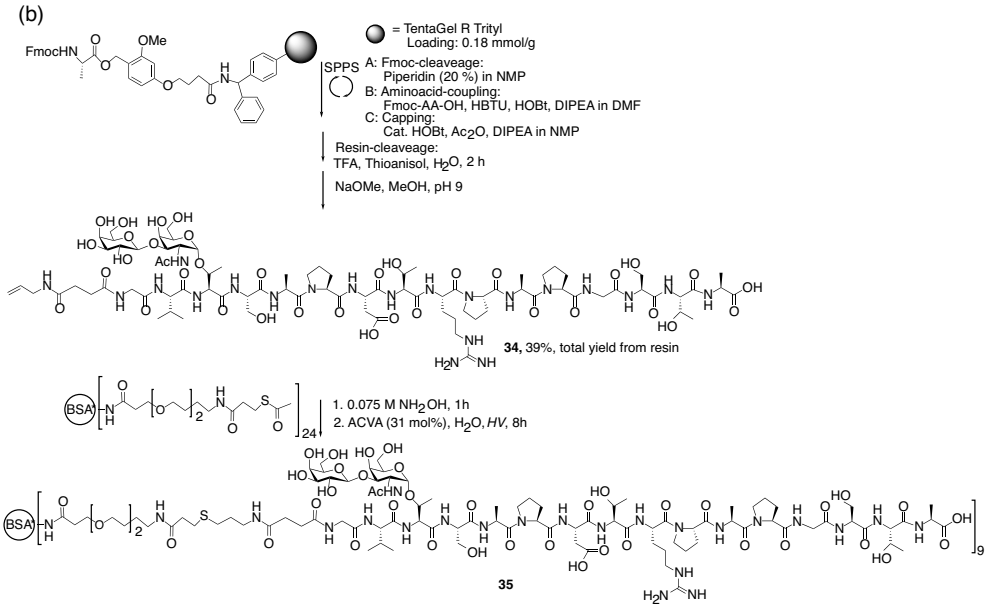
preloaded with Fmoc-arginine according to above outlined strategy. The glycosylated sialyl-Tn (**15**) and Tn-amino (**7**) acid building blocks were used in 2-fold excess and coupled with HATU/HOAt. The other amino acids were coupled according to standard protocols (20 equiv. amino acid and HBTU/HOBt) on a peptide synthesizer. After cleavage from resin and preparative reversed-phase high-performance liquid chromatography (HPLC) purification, the removal of the protective groups of the glycans was performed by first removing the sialic acid benzyl ester by heterogeneous catalytic hydrogenation, followed by removal of the *O*-acetyl protective groups by

transesterification in methanol with catalytic amounts of NaOMe at pH 9–9.5. After completing the global deprotection, the glycopeptide–OVA–vaccine constructs were purified again on preparative reversed-phase HPLC, and isolated in overall yields (calculated from resin) of 36% (**28**), 17% (**29**), and 32% (**30**) (Scheme 10.6) [63]. The vaccine constructs were used for immunization of mice. Highly specific humoral immune responses were generated [63].

MUC1 sialyl-Tand Tglycopeptide-based vaccines have also been prepared. In one example these glycopeptides were connected to a bovine serum albumin (BSA) as the carrier protein via a thioether linkage and a nonimmunogenic spacer [64]. Formation of thioethers via a radical-induced addition of thiols to alkenes should be favorable in comparison to the more common peptide–protein conjugation techniques employing squarates [65] or maleimido [66, 67] linkers, which have potential to be immunogenic themselves. A 2,3-sialyl-T MUC1 glycopeptide with a thiol



Scheme 10.7 Synthesis of (a) sialyl-T antigen–BSA conjugate and (b) T antigen–BSA conjugate through alkene–thiol reaction.



Scheme 10.7 (*Continued*)

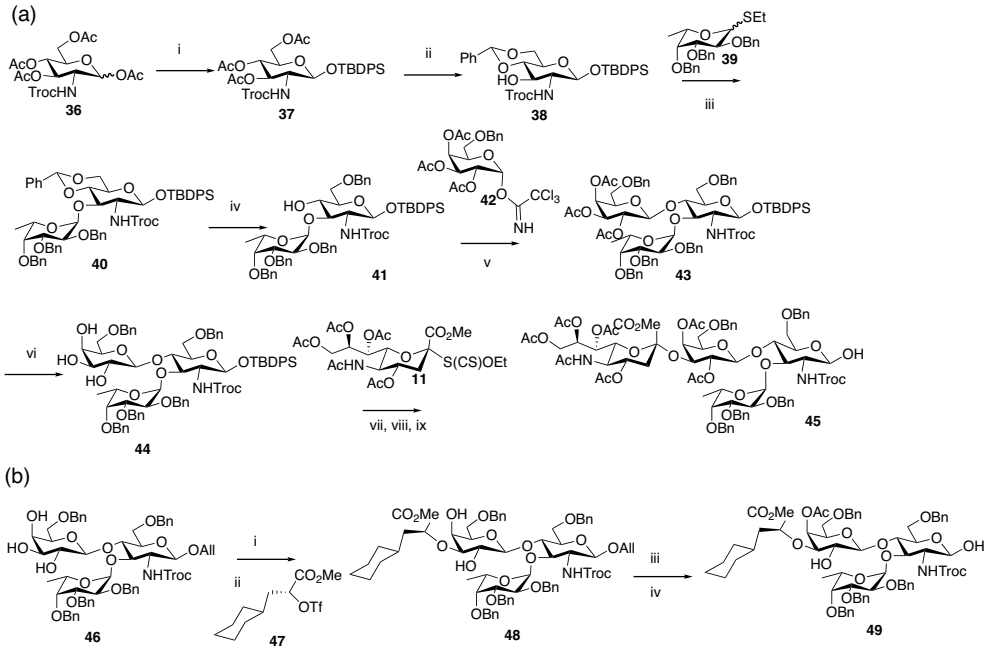
amino acid spacer at the N-terminus was prepared starting from trityl resin preloaded with proline. The peptide backbone assembly and cleavage from resin according to the standard procedure described above gave the partly protected glycopeptide **31** in 36% yield (Scheme 10.7). Removal of the protective groups was performed to give the thiol glycopeptide **32**, which was coupled to olefin-modified BSA in water employing UV radiation. After dialysis and lyophilization the pure antigen–BSA conjugate **33** was obtained (Scheme 10.7a) [63]. The reverse thioether linkage was also constructed by the synthesis of an allylamide-functionalized T antigen MUC1 tandem repeat peptide (**34**) (39% overall yield) and photochemical coupling to BSA carrying a thiol spacer gave **35** (Scheme 10.7b) [63]. A number of other glycopeptide vaccines have been prepared conjugated to different immunostimulants for optimization of the immune response and antibody specificity. In addition to the examples mentioned above, conjugation of MUC1 glycopeptides to tetanus toxoid protein [68], keyhole limpet hemocyanin [69, 70], and Pam_3Cys [71] have been described.

10.2.1.2 Synthesis of Glycopeptide Recognition Domain of P-Selectin Glycoprotein Ligand-1

During acute and chronic inflammation leukocytes are recruited to endothelial cells of the damaged/inflamed tissue. This adhesion process involves binding of E- and P-proteins on the endothelium to E- and P-selectin ligands on the surface of leukocytes [72–75]. Both the E- and P-selectin ligands contain clusters of the terminal sialyl-Lewis X (sLe^x) tetrasaccharide determinant. Migrating tumor cells also express sLe^x

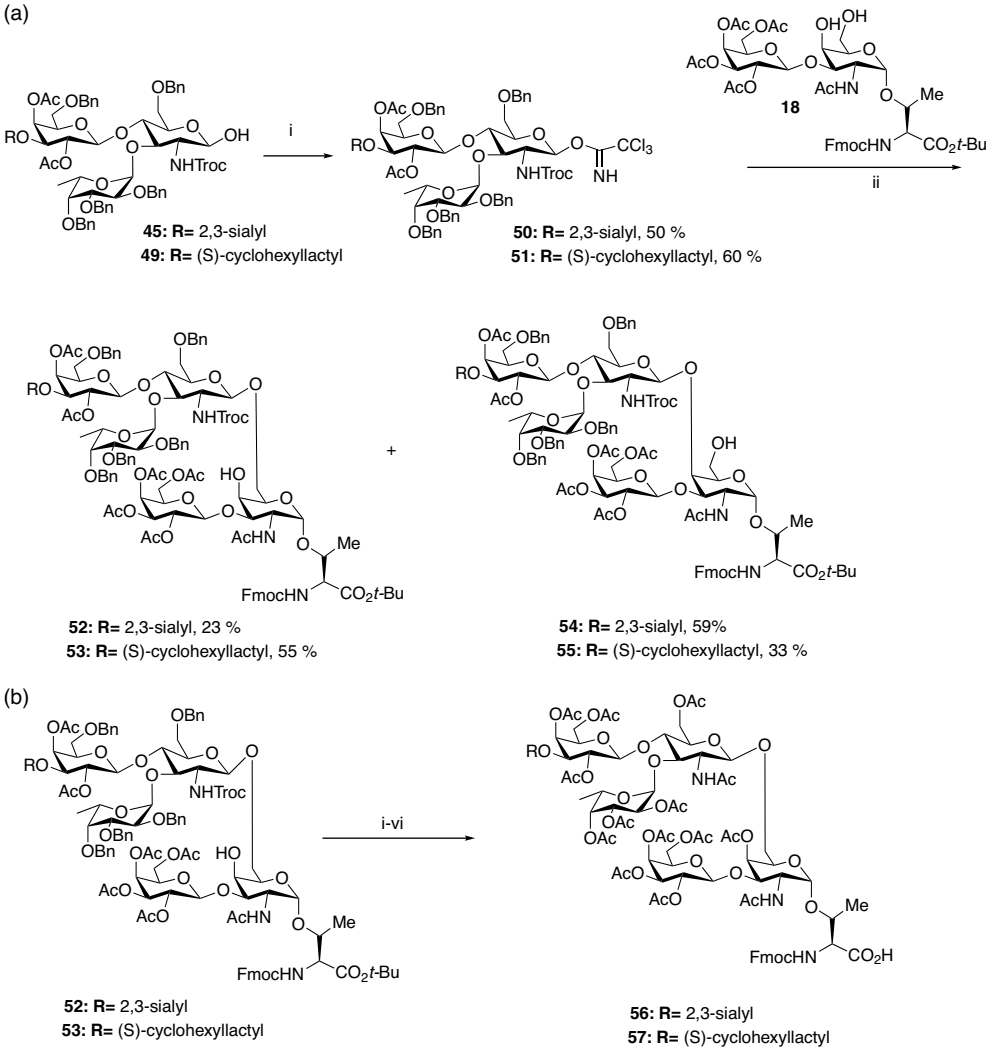
structures to mimic these ligands in order to facilitate attachment to the endothelial cell surface, resulting in tumor metastasis [76]. Synthesis of E- and P-selectin ligands and selectin inhibitors mimicking those are therefore interesting to better understand the adhesion processes and also for potential use as anti-inflammatory agents or for the treatment of cancer. The synthesis of the glycopeptide recognition domain of P-selectin glycoprotein ligand-1 (PSGL-1) [77, 78] will be illustrated here [79–81]. P-selectin requires the N-terminal part of PSGL-1 for binding [77, 78]. This part contains a core 2 *O*-linked glycan with terminal sLe^x at Thr57, and at least one of the tyrosine residues Tyr46, Tyr48, and Tyr51 sulfated.

10.2.1.2.1 **Synthesis of a Core 2 sLe^x Amino Acid Building Block Including a sLe^x Mimic** A sLe^x-T antigen threonine amino acid building block was prepared from a sLe^x glycosyl donor fragment **50** and use of the T antigen acceptor **18** with free hydroxyls in the 4- and 6-positions also described above for synthesis of the Fmoc-SPPS hydroxy groups T antigen building block **19**. For β-selective glycosylation of T



Scheme 10.8 (a) Reagents and conditions: (i) 1, NH_2-NH_2, AcOH; 2, TBDPS-Cl, imidazole, DMAP, DMF, 74%; (ii) 1, NaOMe/MeOH pH 8.5; 2, $PhCH(OMe)_2$, *p*-TsOH, CH_3CN, 40 °C, 86%; (iii) $CuBr_2$, Bu_4NBr, CH_2Cl_2/DMF, 79%; (iv) Et_3SiH, TfOH, CH_2Cl_2, −78 °C, 77%; (v) TMSOTf, CH_2Cl_2, −20 °C, 63%; (vi) NaOMe/MeOH pH 8.5, 85%; (vii) AgOTf, MeSBr, $CH_3CN/CHCl_2$, −50 to −25 °C; (viii) Ac_2O, DMAP, pyridine, 70% over two steps; (ix) TBAF, AcOH, THF, 0 °C to room temperature, 69%. (b) Reagents and conditions: (i) $(nBu_2SnO)_2$, MeOH; (ii) CsF, 1,2-dimethoxyethane (DME), 71% over two steps; (iii) Ac_2O, DMAP, pyridine, 88%; (iv) 1, $[Ir(cod)(PMePh_2)_2]PF_6$, H_2, THF; 2, $NaHCO_3$, H_2O, I_2, THF, 0 °C to room temperature, 86%.

antigen amino acid **18** to the sLe^x glycosyl donor fragment **50**, the *N*-protecting group of the glucosamine in the donor was protected with a directing 2,2,2-trichloroethoxycarbonyl (Troc) [82] group. The anomeric center contained a trichloroacetimidate group, which could be activated with trimethylsilyl triflate (TMSOTf).

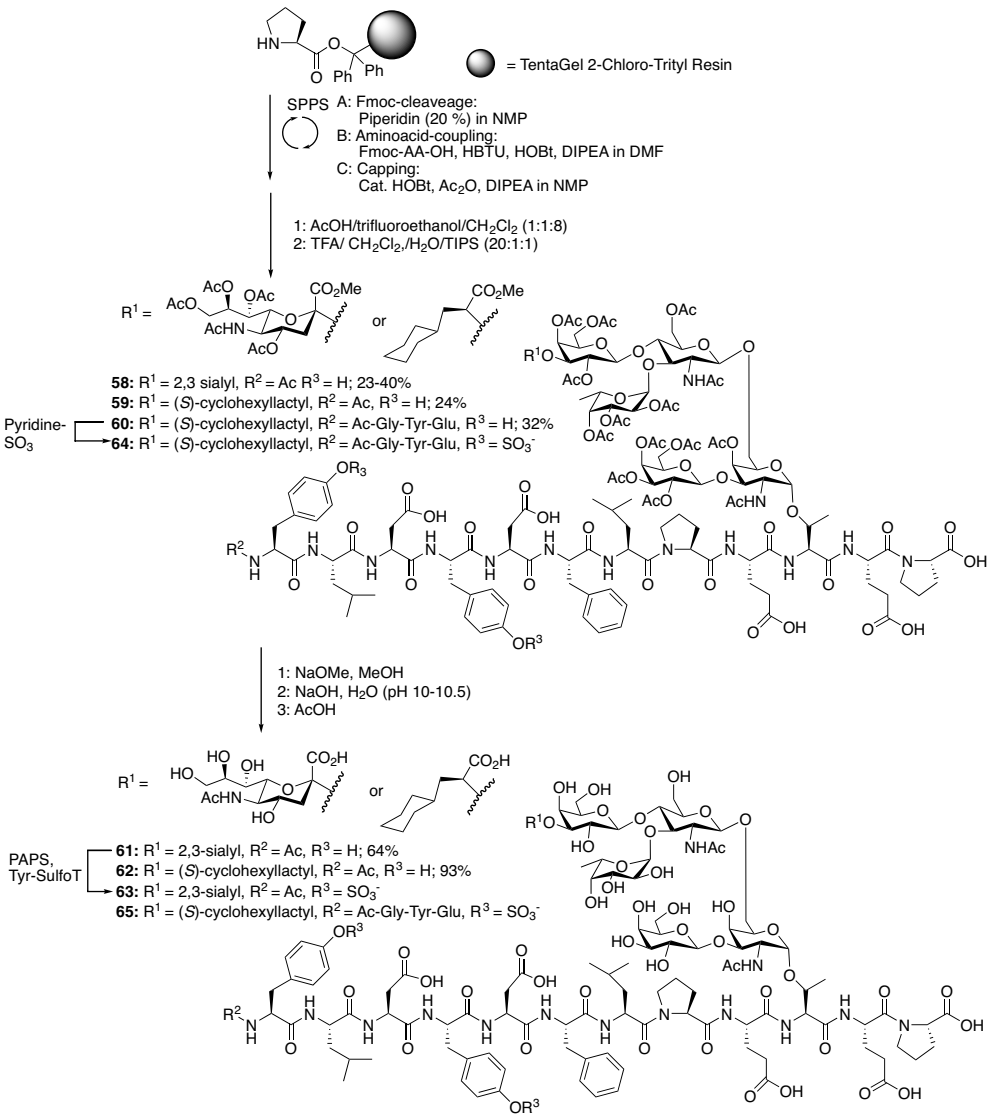


Scheme 10.9 (a) Reagents and conditions: (i) Cl_3CCN, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), CH_2Cl_2; (ii) TMSOTf, CH_2Cl_2, −40 to −30 °C. (b) Reagents and conditions: (i) Zn, AcOH; (ii) Ac_2O, pyridine, R = 2,3-sialyl 62% over two steps; (iii) H_2, $Pd(OH)_2/C$ (15–20%); (iv) Fmoc-OSu, DIPEA, CH_2Cl_2; (v) Ac_2O, pyridine; R = 2,3-sialyl 34% over three steps, R = (*S*)-cyclohexyllactyl 86% five steps; (vi) TFA, CH_2Cl_2, R = 2,3-sialyl 78%, R = (*S*)-cyclohexyllactyl 96%.

The sLe^x donor was prepared starting from peracetylated *N*-Troc glucosamine **36** [82]. By deacetylation at C-1 and treatment with *tert*-butyldiphenylsilyl (TBDPS)-Cl [83] an acid- and base-stable protecting group was introduced at the anomeric center (**37**). The *O*-acetyl protecting groups were then removed by transesterification with sodium methoxide in methanol. Introduction of a 4,6-*O*-benzylidene acetal gave the glucosamine acceptor **38**. The acceptor was reacted with a thiofucoside (**39**) [84] protected with nonparticipating *O*-benzyl groups forming a α1–3′ glycosidic bond (**40**) [85, 86]. Regioselective ring opening of the benzylidene acetal gave acceptor **41** [87], which was reacted with a partially *O*-acetyl protected galactose trichloroacetimidate donor **42** [88, 89] to give the Le^x fragment **43** in 63% yield. For incorporation of the sialyl residue, the trisaccharide was first deacetylated followed by regio- and stereoselective coupling employing ethyl xanthate donor **11** in a similar fashion as described for the sialyl-Tn and sialyl-T building blocks. The sLe^x fragment was transformed into a donor (**45**) by removal of TBDPS group using tetra-*n*-butylammonium fluoride (TBAF), and subsequently it was reacted with trichloroacetonitrile [88] to give **50** (Schemes 10.8a and 10.9a) [79, 80]. By analogy, a donor fragment containing a (*S*)-cyclohexyllactic acid [90] **51**, which is a mimic of the sialic acid was also prepared [80]. Instead of protection with a TBDPS group in the anomeric center a *O*-allyl group was employed before transformation to the final trichloroacetimidate donor (Scheme 10.8b) [80].

Both the sLe^x **50** and the sialyl mimic **51** donor fragments were then reacted with the T antigen amino acid **18** (Scheme 10.9). The couplings were performed in both cases with good β-selectivity. However, the T antigen, which had both the 4- and 6-hydroxyls free, did not react with the previously reported high regioselectivity for coupling in the 6-position [55, 91–93] when coupled to the sLe^x donor **50**. Instead, a mixture of both the major 4-coupling product **54** (59%) and the minor 6-coupling product **52** (23%) were obtained. The unexpected selectivity is obviously a result of a slow coupling reaction of such a large donor fragment. In the coupling with sLe^x mimic the desired 6-coupling product **53** was the major product (55%), but some 4-coupling product **55** was also formed (33%) (Scheme 10.9a) [79, 80]. For incorporation in peptide synthesis some additional protecting group manipulations were necessary in order to secure sufficient acid stability, which include removal of the *N*-Troc protecting group and acetylation forming the *N*-acetyl, hydrogenation of the benzyl groups, reincorporation of the Fmoc group, *O*-acetylation of free hydroxyls, and finally removal of the acid-labile *t*Bu group to give the free carboxylic acid (Scheme 10.9b) [79, 80].

10.2.1.2.2 **Synthesis of Unsulfated and Sulfated Core 2 sLe^x and Core 2 sLe^x Mimic PSGL-1 Glycopeptides** Glycopeptides of the PSGL-1 recognition domain were synthesized [79, 80], the sLe^x **58** and sLe^x mimic **59** glycopeptides containing Tyr51 and Tyr48, and the sLe^x mimic glycopeptide **60** including all three tyrosines Tyr51, Tyr48, and Tyr46. The peptides were obtained by Fmoc-SPPS described above, starting from acid-labile 2-chloro-trityl resin [94] preloaded with Fmoc-proline. The peptides were

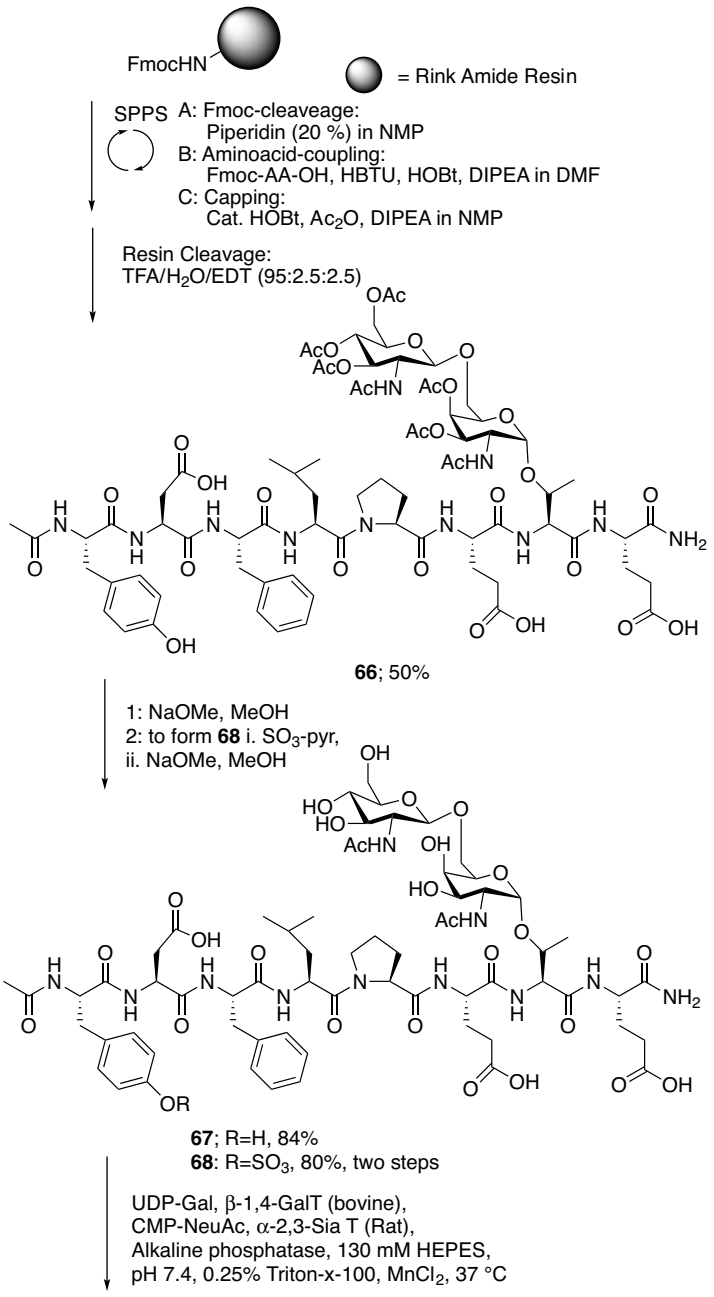


Scheme 10.10 Synthesis of PSGL-1 glycopeptides.

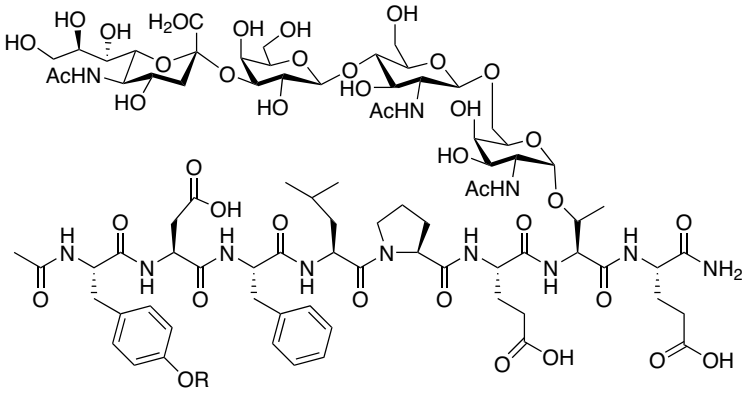
cleaved from resin with acetic acid/trifluoroethanol in dichloromethane (DCM) followed by amino acid side-chain deprotection using TFA/DCM/triisopropylsilane (TIPS)/H_2O to give the HPLC-purified glycan protected peptides **58** (23–40%), **59** (24%), and **60** (32%). The sLe^x peptide **58** and the sLe^x mimic peptide **59** were treated with sodium methoxide in methanol pH 9 for removal of the glycan *O*-acetyl protecting groups, followed by methyl ester deprotection employing sodium

hydroxide pH 10–10.5, and this gave **61** (64%) and **62** (93%). To obtain sulfatyl tyrosine PSGL-1 glycopeptides, both enzymatic and chemical approaches were investigated. The sLe^x glycopeptide **61** was sulfated using phosphoadenosyl phosphosulfate (PAPS) and recombinant tyrosine sulfotransferase [95] to give **63**. Since the enzymatic approach suffered from low reactivity and stability problems of the PAPS substrate, a chemical approach also suitable for sulfation on a preparative scale was investigated. The sLe^x mimic glycopeptide **60** protected in the glycans was reacted with pyridine–SO_3 complex in pyridine/dimethylformamide (DMF) [96]. After neutralization and workup, the *O*-acetyl and methyl ester glycan protecting groups were removed to give the PSGL-1 sLe^x mimic glycopeptide **64** with three sulfated tyrosines (Scheme 10.10) [79, 80]. The sulfated and unsulfated PSGL-1 glycopeptides were further used in binding studies to determine potential inhibitory effects to E- and P-selectin.

10.2.1.2.3 **Chemoenzymatic Synthesis of Unsulfated and Sulfated sLe^x PSGL-1 Glycopeptide** Slightly shorter sLe^x glycopeptides containing Tyr51, unsulfated and sulfated, have also been prepared by a chemoenzymatic approach [81]. In this case an *O*-acetyl and Fmoc-protected GlcNAc-β1–6-α-GalNAc threonine [96, 97] was used as a SPPS amino acid building block. The peptide synthesis performed according to Fmoc-SPPS starting with Rink amide resin [98]. The assembled peptide was cleaved from resin and side-chain deprotected using TFA to give glycopeptide **66** (50%). The *O*-acetyl glycan protecting groups were then removed by basic hydrolysis employing sodium hydroxide in methanol to give **67** (84%) or chemically sulfated on the tyrosine by the pyridine–SO_3 complex followed by treatment with the base to yield **68** (80%). The synthesized glycopeptides were used for enzymatic glycan extension. The glycosyltransferase-catalyzed reactions needed to be performed in a certain order. For example, glycan extensions by Gal and GlcNAc transferases do not occur if the glycan is already sialylated or fucosylated, and Le^x, which is fucosylated, is not a good substrate to form sLe^x. However, in this case the enzymes β1–4-galactosyl transferase (GalT) and 2,3-sialyltransferase (SiaT) could be used in a one-pot process since both have an optimal pH at 7.5. The sialylation, which is specific to the 3-position on Gal, can only proceed after the Gal residue has been coupled to the GlcNAc residue of the glycopeptides [27]. Glycosylation with GalT and SiaT in the presence of donor substrates UDP-Gal and CMP-NeuAc at pH 7.4 and 37 °C for 72 h gave the unsulfated peptide **69** in 63% yield. The glycosylation of the sulfated peptide **68** proceeded very slowly [96]. Another portion of the glycosyltransferases and additional 48 h reaction time afforded the glycopeptide **70** in 35% yield. The complete sLe^x peptides were then formed by treatment with α1–3- fucosyltransferase (FucT) and GDP-Fuc at pH 7.4 and 37 °C for 48 h to give the unsulfated glycopeptide **71** (68%) and the sulfated peptide **72** (63%) (Scheme 10.11) [81]. The value of a chemoenzymatic approach in order to produce naturally occurring complex glycopeptides has here been exemplified. However, the enzymatic approach still suffers from low availability of the glycosyltransferases, and the high substrate specificity of the enzymes is sometimes problematic, leading to low or no reactivity.

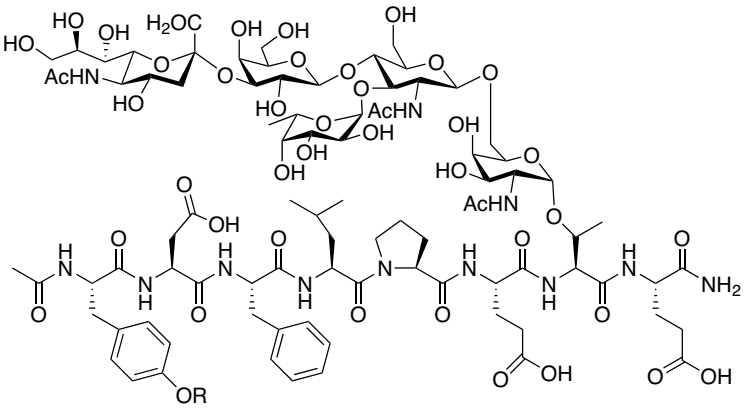


Scheme 10.11 Chemoenzymatic synthesis of PSGL-1 glycopeptides.



69; R = H, 72 h, 63%
70: R = SO_3, 96 h, 35%

GDP-Fuc, α-1,3-FucT V (human),
Alkaline phosphatase, 130 mM HEPES,
pH 7.4, 0.25% Triton-x-100, $MnCl_2$, 37 °C



71; R = H, 48 h, 68%
72: R = SO_3, 48 h, 63%

Scheme 10.11 (*Continued*)

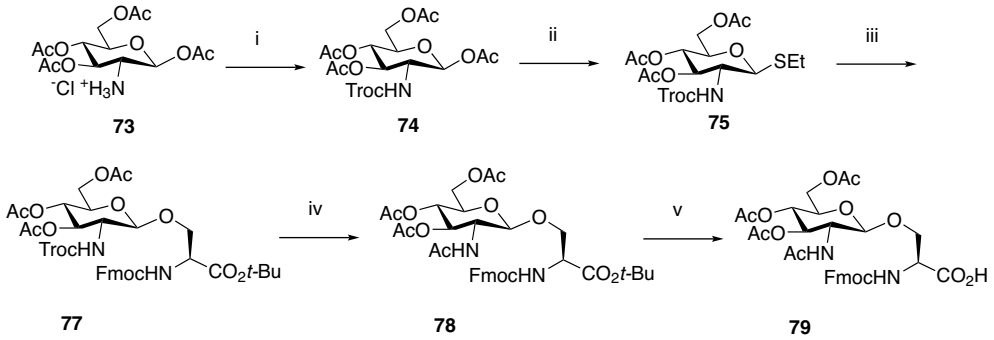
10.2.2
Synthesis of Other Types of *O*-Glycopeptides

In addition to the most common mucin-type glycosylation, serine and threonine residues can be modified with other glycans [99] like GlcNAc, glucose [100, 101], and

xylose [102, 103] via a β-glycosidic bond, and fucose [104] and mannose [105] via an α-linkage. Synthesis of a GlcNAc-modified peptide will be described here [106]. Post-translational modification with β-GlcNAc residues to the peptide backbone is clearly distinguished from the mucin-type glycosylation. The attached GlcNAc residue is not further extended with additional saccharides and, in contrast to mucin-type glycosylation, glycosylation with GlcNAc mainly occurs in the cytosol and in the nucleus. This type of *O*-glycosylation is highly dynamic and often acts in a reciprocal manner to *O*-phosphorylation of proteins [107–109]. Many different types of proteins have been found modified with GlcNAc; nuclear pore proteins, transcription factors, RNA polymerase II, proto-oncogenes, cytoskeletal proteins, kinases, and adapter proteins, to mention a few. Due to the broad function of *O*-glucosaminylation it has also been found to be involved in many diseases such as, diabetes, cancer, and Alzheimer's.

10.2.2.1 Synthesis of Fmoc-GlcNAc-Ser/Thr Amino Acids

The GlcNAc amino acid building block was prepared via coupling of a participating glucose amine donor **75**, and a Fmoc- and benzyl-protected serine or threonine [106, 110]. In this case *N*-Troc was used for protection of the amino group and for neighboring group participation forming a β-glycosidic bond. The donor glycan **75** was prepared starting from peracetylated glucosamine hydrochloride **73** through reaction with Troc-Cl followed by coupling with ethanethiol (EtSH) using BF_3 etherate. Coupling of the thioglycosidic donor **75** with Fmoc- and Bn-protected serine **75** employing dimethyl(methylthio)sulfonium triflate (DMTST) for activation gave glycosylated amino acid **77** in 98% yield. Troc group removal and *N*-acetylation by zinc in acetic acid and followed by catalytic hydrogenation with Pd(C) gave the amino acid SPPS building block **79** (Scheme 10.12) [106].

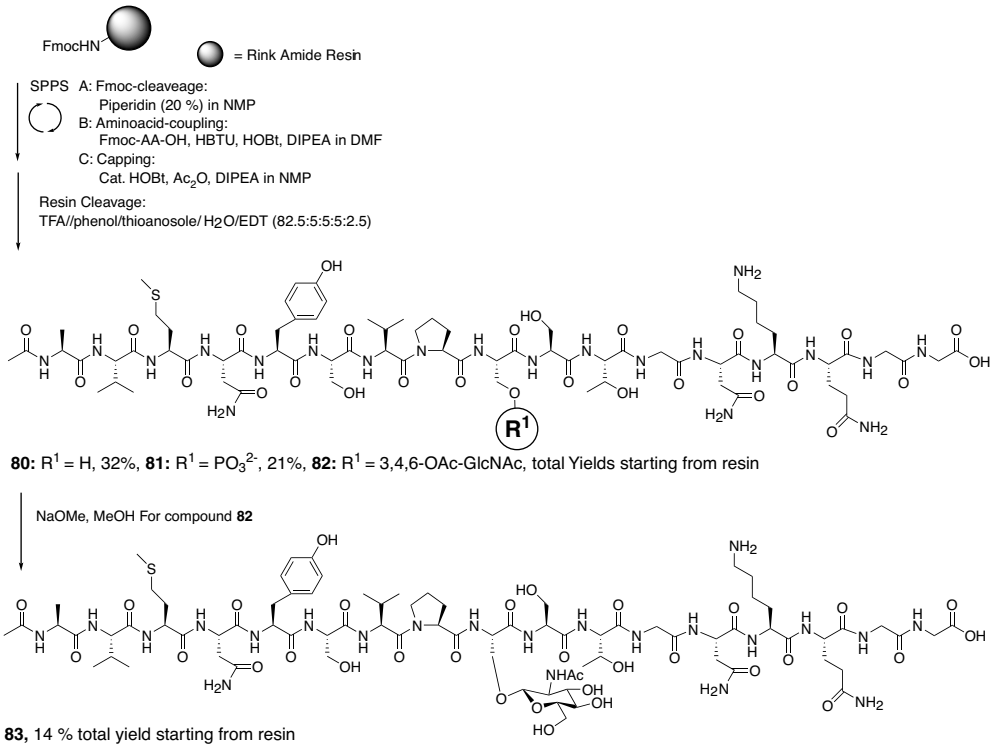


Scheme 10.12 Reagents and conditions: (i) Troc-Cl, $NaHCO_3$, H_2O, 77%; (ii) $BF_3{\cdot}Et_2O$, EtSH, 42%; (iii) Fmoc-Ser-OBzl (**76**), DMTST, DCM, 98%; (iv) Zn, Ac_2O, 66%; (v) Pd/C, H_2, EtOAc, EtOH, 83%.

10.2.2.2 Synthesis of Estrogen Receptor Peptides for Conformational Analysis

It has been found that glucosaminylation and *O*-phosphorylation of proteins often function in a reciprocal manner, and together they control the activity of cellular processes. In order to study the effect of glycosylation and phosphorylation versus no

post-translational modification, three different peptides of the N-terminal sequence Ala7–Gly23 of estrogen receptor β (ER-β) were prepared for conformational analysis via nuclear magnetic resonance, circular dichroism, and molecular dynamic simulations [106]. The peptides were synthesized according to Fmoc-SPPS employing a Rink amide resin [98]. Peptide backbone assembly was followed by cleavage from resin using a TFA cleavage cocktail. The neutral peptide **80** and the phosphopeptide **81** were used for conformational analysis, while the glycopeptide **82** was deacetylated on the glycan using sodium methoxide in methanol to give the peptide **83** (Scheme 10.13) [106]. The conformational analysis studies of the three peptides showed that peptide **80** with an unmodified Ser16 adopts a type II β-turn-like structure of the residues $S^{15}STG^{18}$ in equilibrium with random conformers, the phosphorylated peptide **81** instead adopts a more extended structure in the SSTG region, and the GlcNAc peptide **83** adopts only the type II β-turn like structure. The degree of turn formation was higher. From the studies it can be concluded that *O*-phosphorylation and *O*-glucosaminylation disturb the local structure of the N-terminal fragment of ER-β, but in different directions. In agreement with earlier proteolysis studies [111], it could be concluded that GlcNAc modification stabilized the protein structure, while the extended structure upon phosphorylation destabilized the N-terminal region and made it more accessible for proteases.



Scheme 10.13 Synthesis of ER peptides for conformational analysis.

10.3 Synthesis of *N*-Glycopeptides

The majority of *N*-glycopeptides/proteins have a pentasaccharide core, which contains two GlcNAc residues β1–4 linked, which is further β1–4 linked with mannose and branched with α-mannose in the 3- and 6-positions. Via an asparagine *N*-β1–4 linkage the reducing end of the GlcNAc residue in chitobiose is connected to the protein backbone. Glycosylation to asparagine is normally found in a typical consensus sequence of Asn–Xxx–Thr/Ser, where Xxx is any amino acid except proline. The *N*-glycans can be divided into three different classes in which the pentasaccharide core is further modified [112, 113] (Figure 10.1c and d). In the high mannose oligosaccharides two to six more mannoses are attached to the core. The complex-type saccharides are modified on the two outer core α-mannose residues of the core with *N*-acetyl lactosamine (Gal-β1–4-GlcNAc) or type 1 glycosylation (Gal-β1–3-GlcNAc) followed by addition of more *N*-acetyl lactosamine residues and/or termination with α-Gal, sialic acid, fucose, or sulfate. The complex-type *N*-glycans generate even more diversity by variation in the number of side-chains elongating from the α-mannose residues, also called antenna. Biantennary oligosaccharides linked to C-2 of α-mannose, 2,4-branched tri- and tetra-antennary *N*-glycans are the most common. Hybrid-type *N*-glycans are formed when high mannose oligosaccharides are partially processed to form complex glycans and are, therefore, a mixture of those.

The chemical synthesis of *N*-glycopeptides can be achieved by different strategies [114]. The approach in which glycosylated amino acid building blocks are incorporated during peptide backbone assembly is the most common method during synthesis of *O*-glycopeptides. This method is also common for preparation of *N*-glycopeptides, but synthesis employing large *N*-glycan amino acids often suffers from aspartimide formation during coupling and thereby low yields. The convergent approach is an alternative, although slow reaction of large *N*-glycans leads to aspartimide formation on the peptide backbone instead of formation on the amino acid. In this method the protected peptide containing an unprotected aspartate is synthesized and coupled with a glycosyl amine employing peptide coupling reagents forming an amide bond (Lansbury method) [115]. A glycosyl azide can also be used for direct coupling to the aspartyl side-chain in the presence of a tertiary phosphine via the Staudinger reaction [116]. An alternative to these methods is the chemoenzymatic glycopeptide synthesis. Glycopeptides are prepared on the monosaccharide level by the glycosylated amino acid building block method and the saccharide of the deprotected glycopeptide is then extended in solution. Saccharide extension can be performed stepwise by using different glycosyltransferases or by endoglycosidase coupling of a large intact oligosaccharide. The endoglycosidase method has problems with product hydrolysis and low transglycosylation activity. This problem has been elegantly solved by using synthetic sugar oxazolines [117]. The oxazoline saccharides are enzymatic reaction intermediates and are therefore not prone to hydrolysis. The coupling efficacy has been drastically improved in this way.

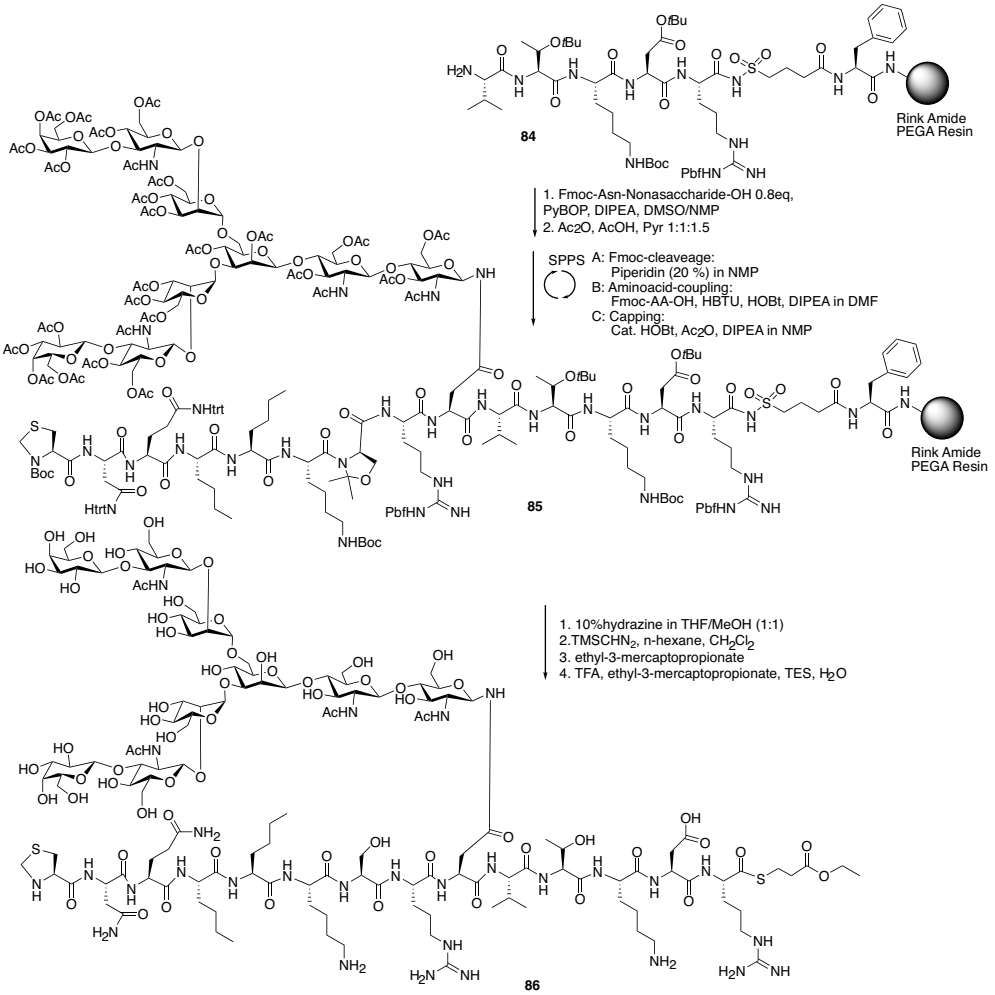
For preparation of glycan *N*-asparagine amino acid building blocks or for direct use in convergent glycopeptide synthesis, oligosaccharides with an anomeric glycosylamine are most commonly used. They can be prepared via reduction of an anomeric

azide [118–120] precursor that has already been extended with different saccharides. Alternatively the glycosyl azide can be coupled directly to the aspartate side-chain of the amino acid or peptide via Staudinger ligation [116, 121]. Unprotected saccharides can be converted to anomeric glycosyl amines by treatment with a saturated solution of ammonium hydrogen carbonate (Kochetkov method) [122, 123]. Oxazoline saccharides can be obtained by sugar peracetylation followed by oxazoline formation of the GlcNAc under treatment with TMSOTf or trimethylsilyl bromide (TMSBr)/ $BF_3{\cdot}Et_2O$ [124]. Different approaches for preparation of *N*-glycan oligosaccharides and *N*-glycopeptides will be exemplified here.

10.3.1 Synthesis of RNase C Glycoprotein

As a model to prepare homogenous *N*-glycoproteins, bovine RNase C [125] was synthesized by employing sequential native chemical ligation (NCL) [126, 127] between a *N*-glycopeptide **86** and two other peptide/protein fragments (**87** and **88**) [128]. In addition to the *N*-glycan, which was of the complex type, the glycoprotein also contained eight cysteines that needed to be correctly folded to produce the active enzyme. For preparation of the *N*-glycopeptide fragment **86**, the glycosylated amino acid approach was used. The complex-type *N*-glycan asparagine amino acid **84** was in this case prepared via isolation of a sialylglycopeptide from hen's unfertilized egg yolk [129]. The glycopeptide was treated with protease (actinase E) to give the free asparagine having a *N*-linked biantennary glycan chain with two terminal sialic acid residues. The sialic acid residues were removed by acid hydrolysis by 40 mM HCl solution [130]. The free amine on the oligosaccharide asparagine amino acid was then protected using Fmoc-OSu and $NaHCO_3$ in acetone/H_2O 1 : 1 to give the Fmoc building block **84** ready to use in the peptide synthesis. For use in sequential NCL the *N*-glycopeptide fragment RNase 26–39 **86** was prepared with a thioester in the C-terminal and a thiazolidine-protected cysteine at the N-terminus. The Fmoc-SPPS was performed starting on a double-linker PEGA resin [131]. A Rink amide linker [98] was connected to the resin in order to follow the peptide assembly via a one-step resin cleavage with TFA, in addition a Ellman sulfonamide linker [132–134] (safety catch linker) was connected to be able to cleave the synthesized peptide in a two-step procedure forming a C-terminal thioester. The peptide backbone assembly was preformed according to standard conditions. After optimization of coupling conditions the Fmoc-Asn oligosaccharide building block **84** (0.8 equiv.) was coupled by activation with benzotriazolyl-1-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and diisopropylethylamine (DIPEA) in dimethylsulfoxide (DMSO)/*N*-methylpyrrolidone (NMP) 1 : 1 with a high coupling efficacy. The unreacted amines were capped by acetic anhydride/acetic acid/pyridine (1 : 1 : 1.5), which did not result in acetylation of the sulfonamide linker, which was a problem earlier [130]. Under these conditions, the OH groups on the oligosaccharide were simultaneously acetylated and thereby protected during further peptide backbone extension. To avoid sulfoxide formation of the methionines, residues 30 and 31 were replaced by norleucine, which normally should not affect folding and enzyme activity [135, 136]. After complete peptide assembly the saccharide *O*-acetyl

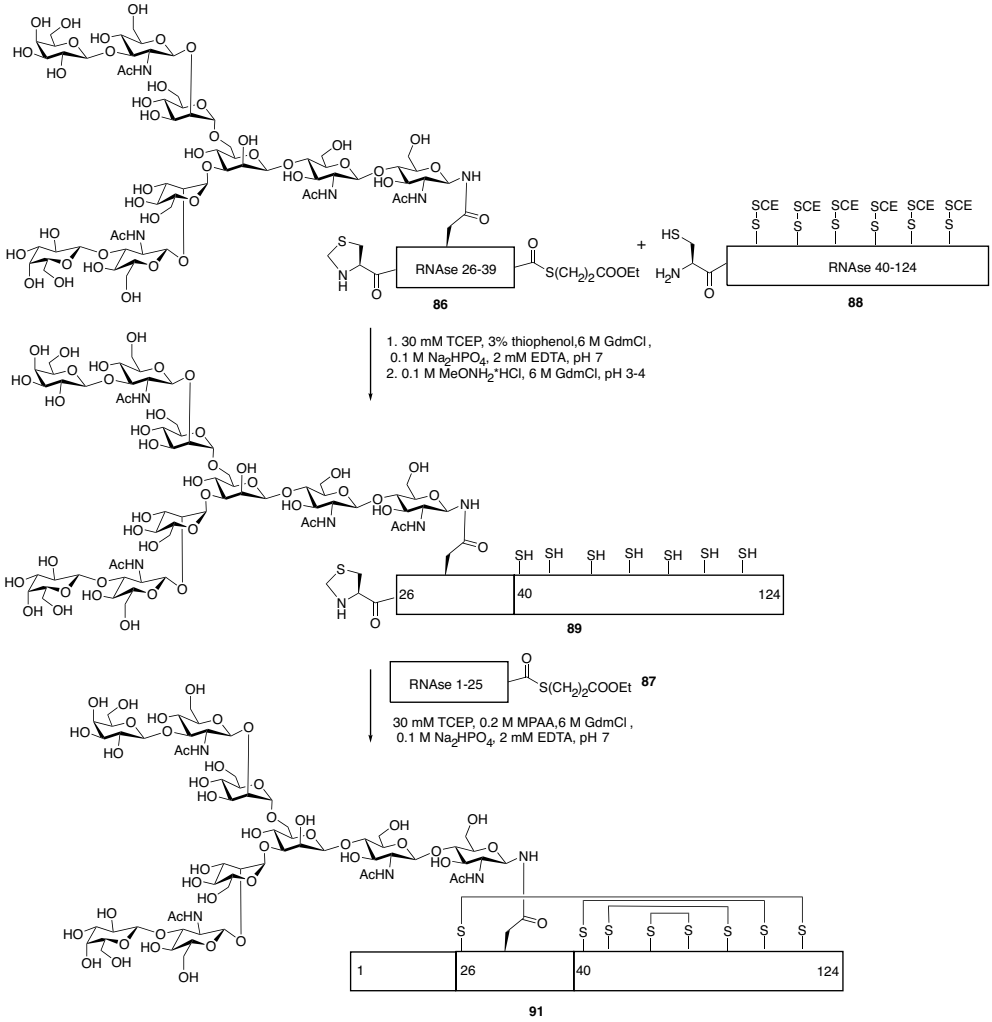
protecting groups were removed by treatment with hydrazine hydrate in MeOH/tetrahydrofuran (THF) 1 : 1 [137]. In order to cleave the peptide from resin, the sulfonamide linker was alkylated with TMS/diazomethane [138], followed by thiolysis using ethyl-3-mercaptopropionate and TFA side-chain deprotection to give the thioester peptide **86** in a total yield of 18% (Scheme 10.14) [128]. In a similar fashion the thioester peptide fragment RNase 1–25 **87** was obtained (20% total yield). For RNase C protein synthesis, a recombinant RNase 40–124 fragment **88** with a N-terminal cysteine was also prepared [139].



Scheme 10.14 Synthesis of RNase C glycopeptide fragment 26–39.

With all peptide fragments available, the chemical ligation could be performed starting with coupling between the *N*-glycopeptide RNase 26–39 fragment **86** and the

protein fragment 40–124 **88** and in the presence of thiophenol and tris(2-carboxyethyl)phosphine (TCEP) [140]. The RNase fragment 26–124 **89** was obtained and the N-terminal cysteine was deprotected using methoxyamine (0.2 M, pH 3–4, 4 h) [141], and it was then ready for use in the next ligation step. Ligation of the RNase fragment 26–124 **89** to the thioester RNase 1–25 **87** performed well in a reductive environment (TCEP) using the strongly activating thiol 0.2 M *p*-mercaptophenyl acetic acid [142] to give RNase fragment 1–124 **90**. Finally, the RNase protein was folded by dilution of the ligation coupling mixture with a 0.3 mM glutathione disulfide (GSSG) solution to give a synthetic RNase C enzyme **91** (Scheme 10.15) [128]. An enzyme assay [143] confirmed that the enzyme was active [144] with a relative activity of 56% compared with a commercial available RNase [128].



Scheme 10.15 Synthesis of RNase C glycoprotein through NCL.

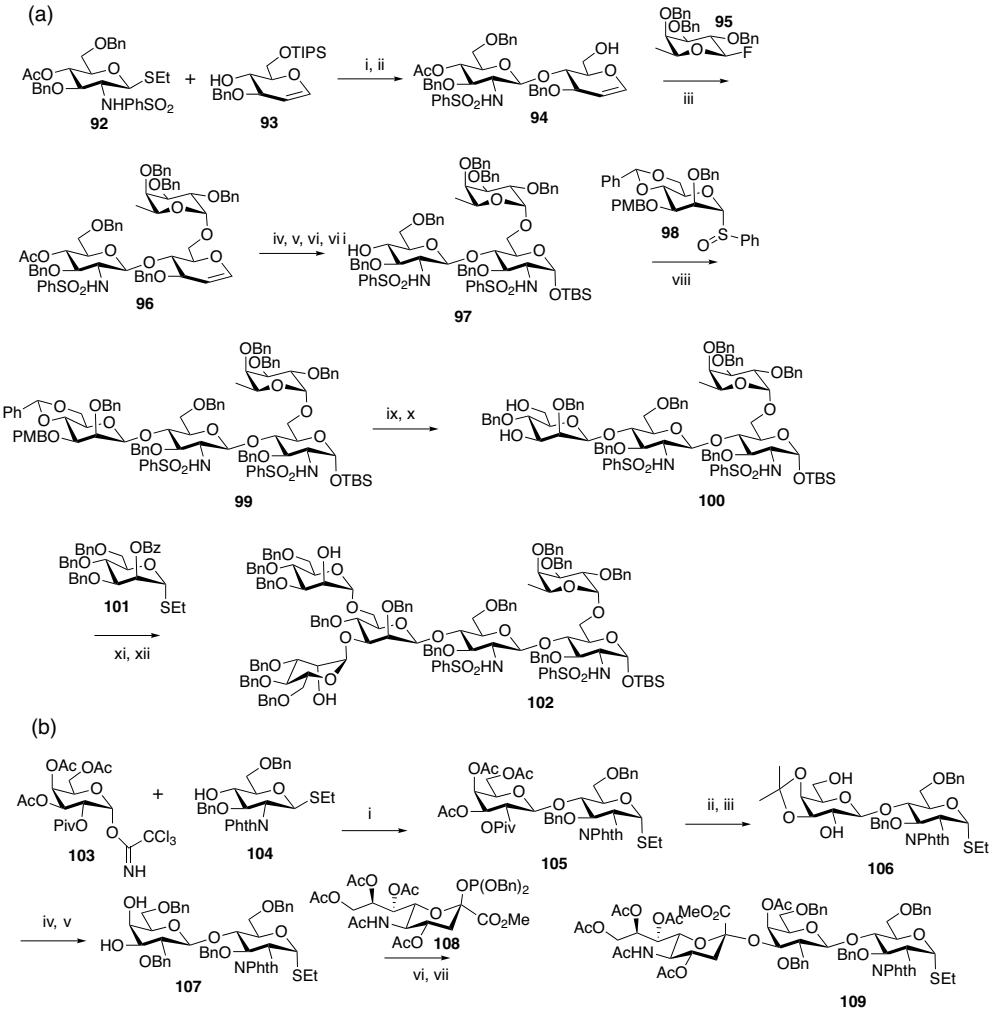
10.3.2
Synthesis of Erythropoietin *N*-Glycopeptide Fragment 1–28

The glycoprotein hormone erythropoietin (EPO) is a clinically useful red blood cell stimulant for anemia [145]. EPO has four glycosylation sites, one *O*-glycosylation site, and three *N*-glycosylation sites. EPO has been found containing many different glycoforms, and the efficacy of EPO is heavily dependent on the type and extent of glycosylation [146, 147]. It is therefore attractive to synthetically produce EPO with homogeneous glycosylation to study structure–activity relationships and for use in therapy. Three different glycopeptide fragments of the 166-amino-acid glycoprotein have been prepared for use in a EPO total synthesis containing homogenous *N*-glycans [148–150]. The synthesis of the N-terminal glycopeptide fragment 1–28 will be exemplified here [148]. A dodecasaccharide with an anomeric glucosamine **110** was prepared [150] and incorporated into the peptide via the Lansbury aspartylation [115] approach.

10.3.2.1 Synthesis of Biantennary Dodecasaccharide

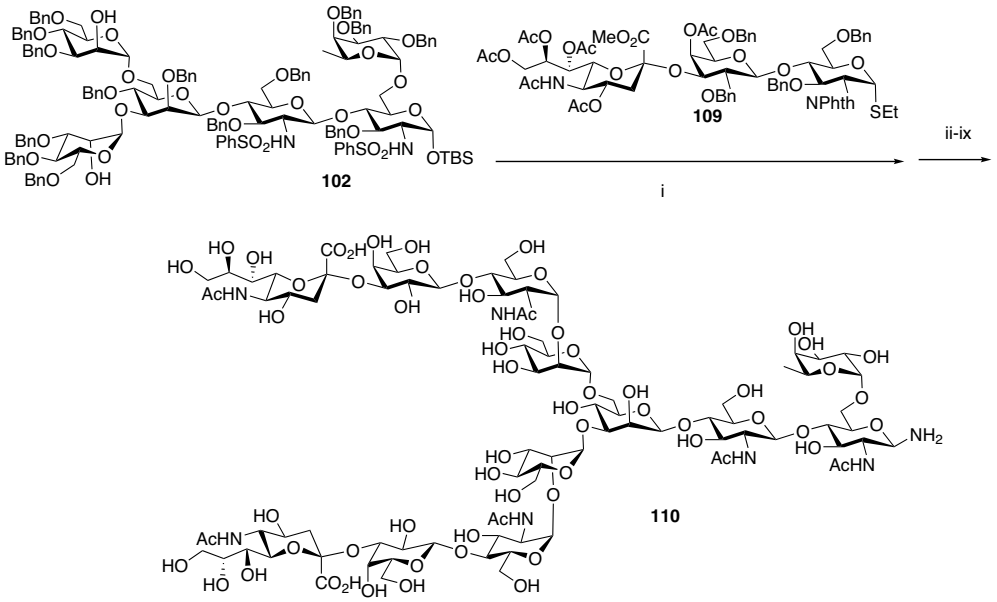
The biantennary dodecasaccharide glycan **110** could be obtained via coupling of a 2,3-sialyl lactosamine trisaccharide donor **109** to the 2-position of the terminal α-mannoses of hexasaccharide **102** containing the pentasaccharide core and a fucose in the 6-position of the anomeric glucosamine (Scheme 10.16) [151]. The hexasaccharide fragment was prepared starting with the synthesis of the disaccharide **94** available via methyl triflate mediated coupling of the monosaccharides **92** and **93** [152–154]. Further coupling with a fucose glycosyl fluoride donor **95** gave the trisaccharide glycal **96**. The glycal was functionalized with an amide in the 2-position through iodosulfonamidation [155, 156] followed by hydrolysis and *tert*-butyldimethylsilyl (TBS) protection of the anomeric center. Deacetylation in the 4-position of the nonreducing GlcN residue was followed by triflate-mediated mannosylation at −78 °C to give the β-coupling product **99** in a good anomeric ratio. Removal of the *p*-methoxy benzyl (PMB) group in the mannose 3-position using cerium ammonium nitrate (CAN) and selective ring opening of the 4,6-benzylidene acetal gave the 3,6-mannose diol **100**. The diol tetrasaccharide could then be α-mannosylated [157, 158] followed by debenzoylation to give the hexasaccharide **102**, which was ready to use for coupling with the sialyl lactosamine trisaccharide donor **109** (Scheme 10.16a) [151].

The trisaccharide thiol donor **109** was prepared via selective sialylation of the lactosamine fragment **107** (Scheme 10.17). The disaccharide was prepared through coupling of glucose imidate donor **103** with a participating pivaloyl in the 2-position and the GlcN acceptor **104** [159]. After deacetylation, isopropylidene acetal protection, benzylation, and acetal hydrolysis the disaccharide diol **107** was formed [160]. Regioselective coupling with a sialyl phosphite donor **108** [161] followed by *O*-acetyl protection of the unreacted hydroxyl gave the trisaccharide donor fragment **109** (Scheme 10.16b). Coupling with the hexasaccharide acceptor via Sinay radical cation activation [157, 158] gave the biantennary dodecasaccharide. Further protecting group manipulations of the formed dodecasaccharide were needed before it could be connected to the peptide backbone [162]. Deprotection of the sialic acid methyl



Scheme 10.16 (a) Reagents and conditions: (i) MeOTf, 2,6-di-*tert*-butylpyridine (DTBP), DCM, 64%; (ii) TBAF, AcOH, THF, 96%; (iii) $Sn(OTf)_2$, DTBP, THF, 65–72%; (iv) iodonium di-sym-collidine perchlorate (IDCP), $PhSO_2NH_2$, THF; (v) H_2O, Et_3N, THF; (vi) TBSOTf, 2,6-lutidine, DCM; (vii) NaOMe, MeOH, 44–55% (four steps); (viii) Tf_2O, (DTBP), DCM, 75–84%; (ix) CAN, MeCN, H_2O, 65–70%; (x) BH_3·THF, Bu_2BOTf, 70–74%; (xi) $(BrC_6H_4)_3NSbCl_6$, MeCN, 90%; (xii) NaOMe, MeOH, 83–85%. (b) Reagents and conditions: (i) TMSOTf, DCM, −78 °C, 79%; (ii) NaOMe, MeOH, 97%; (iii) 2,2-Dimethoxypropane (DMP), (CSA), 68%; (iv) NaH, BnBr, tetra-*n*-butylammonium iodide (TBAI), THF, 85%; (v) H_2O, aqueous HOAc, 60 °C, 92%; (vi) TMSOTf, MeCN, 55–62%; (vii) Ac_2O, pyridine, 84–87%.

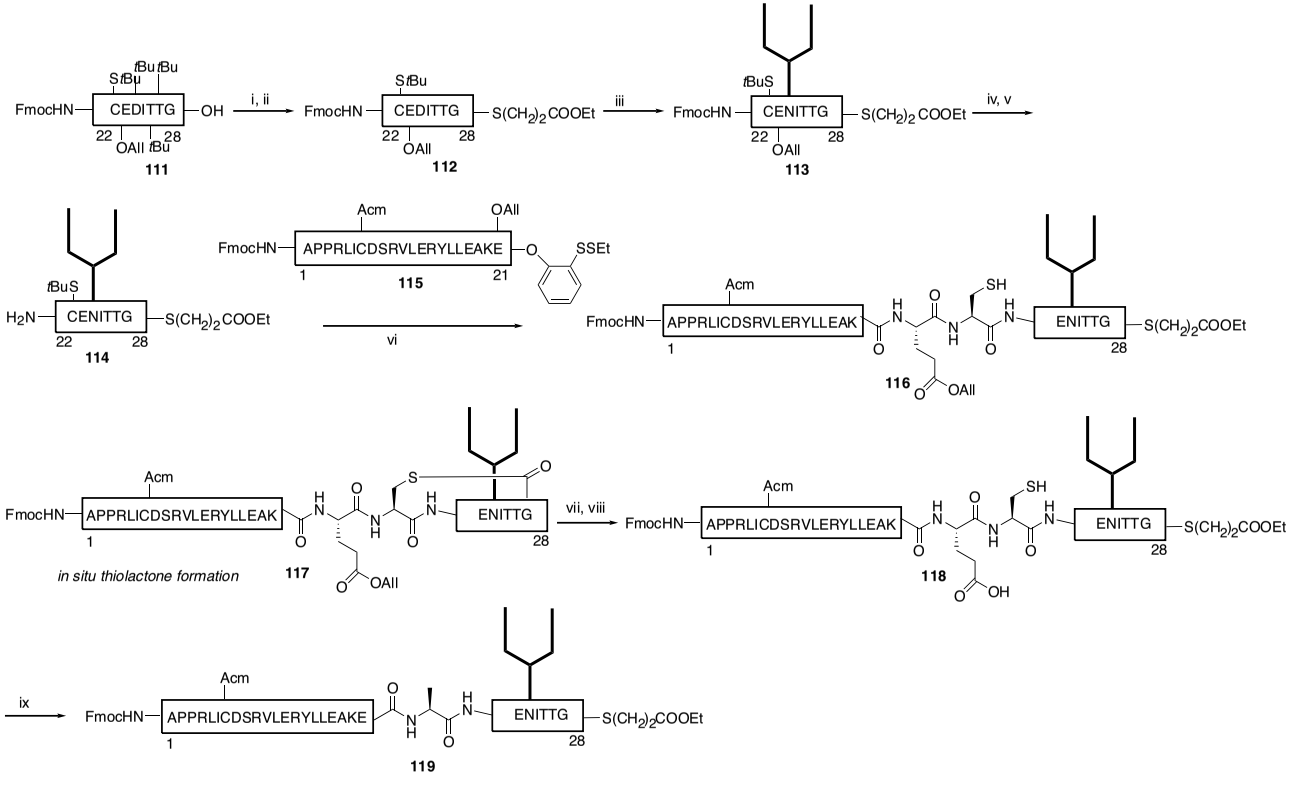
esters and removal of *O*-acetyl groups was performed using sodium methoxide in methanol. The phthalimido groups on the lactosamine chains were then converted to *N*-acetyls by treatment with ethylene diamine and subsequent acetylation with acetic anhydride in pyridine. The anomeric TBS group was then removed using TBAF followed by removal of the *O*-acetyl and *O*-benzyl protecting groups. Kochetkov anomeric amination employing a saturated solution of ammonium hydrogen carbonate [122] gave the glycosylamine **110** in good yield (Scheme 10.17) [151, 162].



Scheme 10.17 Reagents and conditions: (i) $(BrC_6H_4)_3NSbCl_6$, MeCN, 50%; (ii) NaOMe, MeOH; (iii) $H_2NCH_2CH_2NH_2$; (iv) Ac_2O, pyridine; (v) TBAF, AcOH; (vi) NaOMe, $MeOH/H_2O$; (vii) Na, NH_3; (viii) Ac_2O, $NaHCO_3$; (ix) NH_4HCO_3, H_2O (steps ii–ix 63% overall yield).

10.3.2.2 **Synthesis of *N*-Glycopeptide Fragment 1–28**

For preparation of the *N*-glycopeptide fragment 1–28 (**119**) direct coupling of the large *N*-glycan to the 28-amino-acid peptide backbone was not possible. The low reactivity was most probably an effect of the steric bulk of the large saccharide, which resulted in aspartimide formation on the peptide backbone instead of glycan coupling. The shorter peptide fragment 22–28 (**112**) was therefore chosen for coupling of the saccharide to the aspartic acid residue, which could then be connected to peptide fragment 1–21 (**115**) via NCL (Scheme 10.18). The protected peptide fragment 22–28 (**111**) was obtained in 95% yield by standard Fmoc-SPPS starting from Fmoc-Gly TentaGel™ trityl resin. Condensation of the C-terminal carboxylic acid with ethyl thiopropionate followed by removal of side-chain *t*Bu protecting groups gave thioester peptide **112** (45% yield). Coupling of the dodecasaccharide **110** to the peptide using HATU and DIPEA, gave the glycopeptide fragment **113** in 65% yield. After *N*-glycan coupling the N-terminal Fmoc protecting group was removed followed by deallylation



Scheme 10.18 Reagents and conditions: (i) ethylthiopropionate, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI), HOBt, DMF; (ii) 88% TFA, 5% H_2O, 5% phenol, 2% TIPS, 45% over two steps; (iii) HATU, DIPEA, DMSO, dodecasaccharide **110**; (iv) piperidine, 65% over two steps; (v) $Pd(PPh_3)_4$, $PhSiH_3$, DMSO, 90%; (vi) 6 M guanidine·HCl, Na_2HPO_4, TCEP·HCl, thiopropionic acid, 20%; (vii) $Pd(PPh_3)_4$, $PhSiH_3$, DMSO, 90%; (viii) 6 M guanidine·HCl, Na_2HPO_4, TCEP·HCl, thiopropionic acid, 65%; (ix) 6 M guanidine·HCl, Na_2HPO_4, TCEP·HCl, thiopropionic acid, VA-044 (radical initiator), 37 °C, 67%.

of the protected glutamate side-chain. The glycopeptide **114**, which had a S-*t*Bu disulfide-protected cysteine at the N-terminus could then be used for coupling with the peptide fragment 1–21 (**115**) containing an *O*-disulfide phenolic ester [163] at the C-terminus. The peptide fragment 1–21 was prepared by Fmoc-SPPS starting from Fmoc-Lys(Boc) TentaGel trityl resin to give the protected peptide. The C-terminus reacted with a glutamic acid functionalized with a disulfide phenolic ester followed by TFA-mediated peptide side-chain deprotection. NCL of the two peptide fragments **114** and **115** started by reduction of the phenolic ester and cysteine disulfides employing TCEP [140]. The coupling product **116** formed *in situ* a thiolactone (**117**) between the cysteine thiol and the C-terminal glycine carboxylic acid, which was opened again by reaction with thiopropionic acid. Finally, the desired EPO *N*-glycopeptide fragment 1–28 was formed by desulfurization of the cysteine forming an alanine residue [164–166] by treatment with a water soluble radical initiator VA-044, TCEP, and thiopropionic acid at 37 °C to give **119** in a yield of 67% (Scheme 10.18) [148].

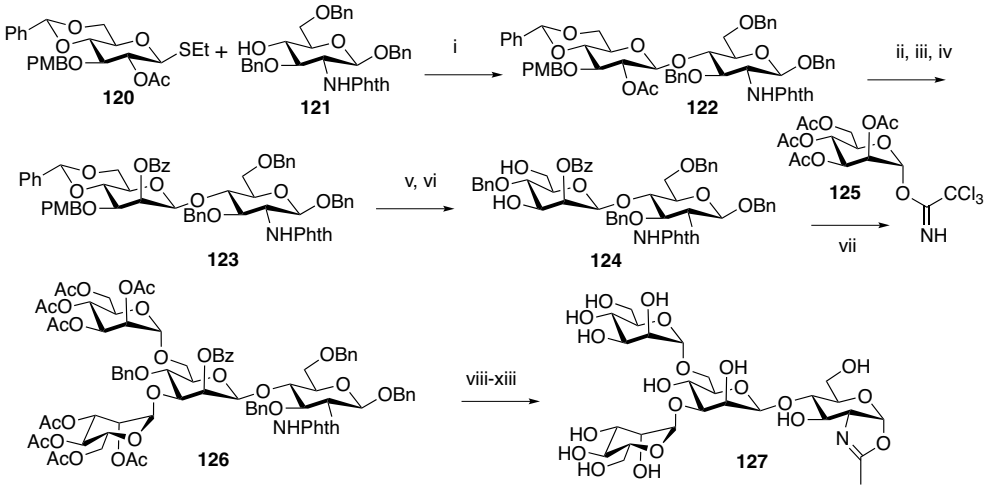
10.3.3
Chemoenzymatic Synthesis of a HIV GP120 V3 Domain *N*-Glycopeptide

The third variable (V3) domain of HIV-1 glycoprotein GP120 is an important target for development of HIV-1 vaccines [167]. This domain has three conserved *N*-glycosylation sites. In order to study how *N*-glycosylation affects conformation, immunogenicity, and proteolytic stability, a cyclic *N*-glycopeptide consisting of the V3 domain and the *N*-glycan pentasaccharide core was synthesized [168]. A chemoenzymatic approach was used employing endoglycosidase (Endo) A. The endoglycosidases normally have hydrolytic activity cleaving the β1–4 glycosidic bond in the chitobiose fragment of the pentasaccharide core, but it has also been found that they have transglycosylation activity. Via the endoglycosidase-catalyzed transglycosylation reaction a large oligosaccharide can be attached to a GlcNAc-containing peptide or protein in one step, but the coupling efficacy is normally low and thereby problems with product hydrolysis also occur. By using synthetic oxazoline oligosaccharide donor substrates, which mimic the 1,2-oxazolinium ion intermediate, coupling efficacy could be drastically improved [117]. The synthesis of the HIV GP120 V3 domain containing 47 amino acids and two *N*-linked core pentasaccharides will exemplify the potential of this approach.

10.3.3.1 Synthesis of the Oxazoline Tetrasaccharide Donor

The oxazoline tetrasaccharide donor **127** was prepared via activation of the peracetylated core tetrasaccharide **126**, which in turn could be obtained via α-mannose coupling to the Man-β1–4-GlcN disaccharide **124** (Scheme 10.19) [169]. Synthesis of the disaccharide **122** was achieved through β-glycosylation of Glc donor **120** with the GlcN monosaccharide **121**. The glucose residue was then inverted to a mannose by forming the triflate and nucleophilic substitution with tetrabutylammonium benzoate to give **123**. By removal of the PMB protecting group in the 3′-position and selective benzylidene acetal ring opening gave the 3′,6′-diol **124**, which was ready for coupling with mannose imidate **125** activated with TMSOTf to form the α-mannose product **126**. Conversion of the phthalimido group to NHAc with ethylendiamine and subsequent

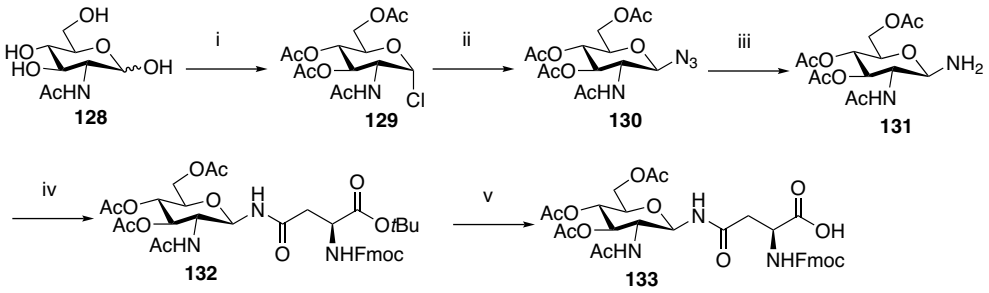
acetic anhydride treatment was followed by palladium-catalyzed removal of the benzyl groups and an additional acetylation to give the peracetylated tetrasaccharide. The oxazoline product was formed by treatment with $TMSBr/BF_3{\cdot}Et_2O$ [124]. Deacetylation with sodium methoxide in methanol gave donor **127** ready to use in the endoglycosidase-catalyzed glycopeptide coupling (Scheme 10.19) [169].



Scheme 10.19 Reagents and conditions: (i) NIS, AgOTf, CH_2Cl_2, 74%; (ii) NaOMe, MeOH; (iii) Tf_2O, pyridine, CH_2Cl_2; (iv) Bu_4NOBz, toluene, 82% over three steps; (v) 2,3-dichloro-5,6-dicyano-benzoquinone (DDQ), CH_2Cl_2, H_2O, 75%; (vi) $BH_3{\cdot}THF$, Bu_2BOTf, 78%; (vii) TMSOTf, CH_2Cl_2, 80%; (viii) $H_2NCH_2CH_2NH_2$; (ix) Ac_2O, pyridine; (x) H_2/Pd-C; (xi) Ac_2O, pyridine, 73% over four steps; (xii) TMSBr, $BF_3{\cdot}Et_2O$, collidine; (xiii) NaOMe, MeOH, 80% over two steps.

10.3.3.2 Synthesis of Fmoc-GlcNAc-Asn Amino Acid Building Block

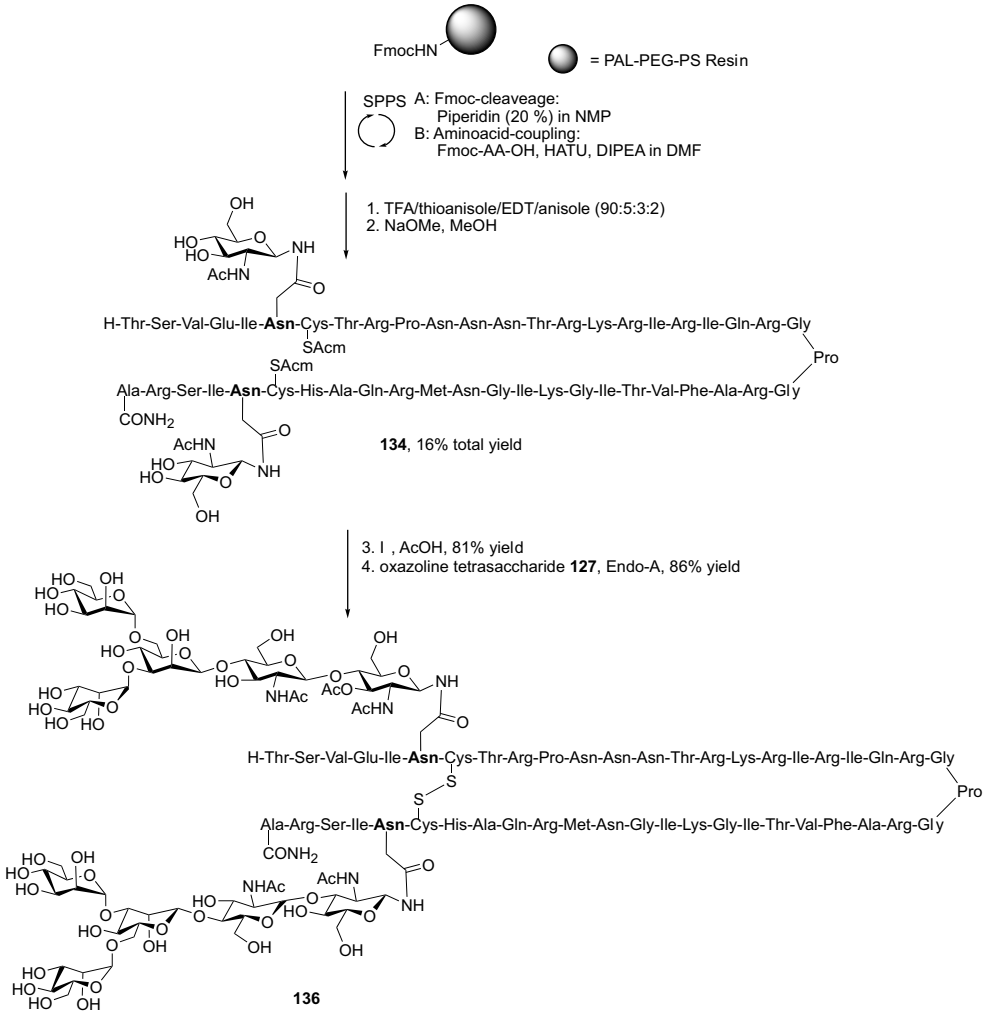
The GlcNAc-Asp amino acid building block **133** was obtained starting with GlcNAc **128**. Treatment with acetyl chloride gave the peracetylated α-chloride **129**, and substitution employing NaN_3 and phase-transfer catalysis gave the β-azide **130** [119, 170, 171]. The azide was reduced by catalytic hydrogenation to give the amine **131** [119, 172]. After coupling [172, 173] with Fmoc-Asp(OH)-O*t*Bu and acidic removal of the carboxylic acid *t*Bu protective group gave the glycosylated amino acid building block **133** (Scheme 10.20).



Scheme 10.20 Reagents and conditions: (i) AcCl; (ii) NaN_3, $(nBu_4N)HSO_4$; (iii) H_2, Pd/C; (iv) Fmoc-Asp(OH)-O*t*Bu, 1,3-dicyclohexylcarbodiimide (DCC), HOBt; (v) TFA.

10.3.3.3 Synthesis of V3 Cyclic GlcNAc Peptide and Endo A Coupling with $Man_3GlcNAc$ Oxazoline Donor

A cyclic 47-mer glycopeptide was prepared corresponding to the amino acid residues 290–336 of HIV GP120, with two GlcNAc residues in the conserved *N*-glycosylation sites N295 and N332 [168]. The glycopeptide was obtained employing Fmoc-SPPS using PAL–poly(ethylene glycol)–polystyrene resin. The glycosylated amino acid Fmoc-(Ac_3GlcNAc)-Asn-OH **133** activated with HATU and DIPEA was used to incorporate the GlcNAc residues on the peptide backbone (Scheme 10.21). The two cysteines, later used for cyclization of the peptide, were protected with Acm protecting groups, which are stable under both acidic and basic conditions. After full backbone assembly, the peptide was cleaved from the resin using a TFA cleavage



Scheme 10.21 Chemoenzymatic synthesis of HIV GP 120 V3 domain *N*-glycopeptide.

cocktail. The crude peptide was treated with 5% aqueous hydrazine to remove the glycan *O*-acetyl protecting groups and purified with reversed-phase HPLC to give the linear glycopeptide **134** in a total yield of 16%. The glycopeptide was treated with iodine in 10% acetic acid to give the free cysteines, which directly cyclized. Endo A [174] catalyzed transglycosylation of the cyclic GlcNAc peptide **135** with the $Man_3GlcNAc$ oxazoline donor **127** (3 : 1 donor/acceptor ratio, phosphate buffer pH 6.5) was completed after 2 h to give the diglycosylated pentasaccharide core peptide **136** in 86% yield (Scheme 10.21) [167]. Due to the high transglycosylation efficiency, no enzymatic hydrolysis was observed. Although the substrate specificity of the endoglycosidases needs to be further studied, this approach seems to be promising for the synthesis of larger peptides or proteins with complex *N*-glycosylation. By combining Endo H, which hydrolyzes the *N*-glycan, with Endo A or Endo M and sugar oxazolines, it was recently shown that it is possible to change the *N*-glycosylation forming a homogenous glycoprotein [175].

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11
Recent Developments in Neoglycopeptide Synthesis

Margaret A. Brimble, Nicole Miller, and Geoffrey M. Williams

11.1
Introduction

With an estimated 50% of naturally occurring peptides being glycosylated, glycoproteins are clearly an important class of compounds. Since structures for the carbohydrate chains are not genetically encoded nor is biosynthesis controlled by direct transcription, glycosylation takes place via complex co- and post-translational modifications. Depending on substrate availability and competition between carbohydrate processing enzymes, variation in the nature and extent of glycosylation often occurs, resulting in a heterogeneous mixture of different glycoforms of the same protein backbone. Most frequently carbohydrates are linked to proteins by an *N*-glycosidic bond to the side-chain of an asparagine within the consensus sequence Asn–Xxx–Ser/Thr (Figure 11.1). The majority of motifs for *N*-linked glycans are β-*N*-acetyl glucosamine (GlcNAcβ)-Asn as these are derived from a common biosynthetic oligosaccharide precursor, although less usual motifs such as glucose, GlcNAcα, or *N*-acetyl galactosamine (GalNAc) can be also found. Alternatively, residues may be linked via an *O*-glycosidic bond to serine or threonine (Figure 11.1). For *O*-linked glycans, GalNAcα-Ser/Thr (so-called Tn antigen) or GlcNAcβ-Ser/Thr (Figure 11.1) predominate, although less-common linkages to tyrosine and hydroxylysine also occur. Finally, even *C*-linked glycosides, specifically that of Manα-Trp, have also now been observed in nature [1, 2].

The structural diversity of glycopeptides and glycoproteins is essential for the broad scope and subtlety of their biological function [3, 4]. They are involved in cell signaling, neuronal development, and inflammatory responses, and the epitopes provided by glycans play a key role in immune surveillance. Moreover, glycans can also change the structural and physicochemical properties of the peptides themselves (e.g., by influencing folding or enhancing proteolytic stability). Consequently, there is a constant imperative to access pure, homogeneous glycoproteins in order to gain a better understanding of the role of glycosylation in molecular biology and to investigate the potential of glycoproteins as therapeutic agents. Unfortunately, the isolation of glycoproteins from natural sources is complicated and although new

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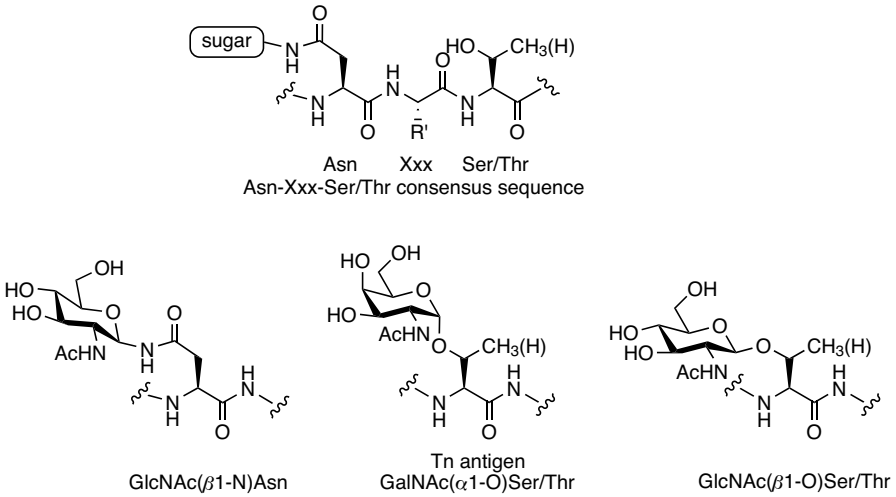


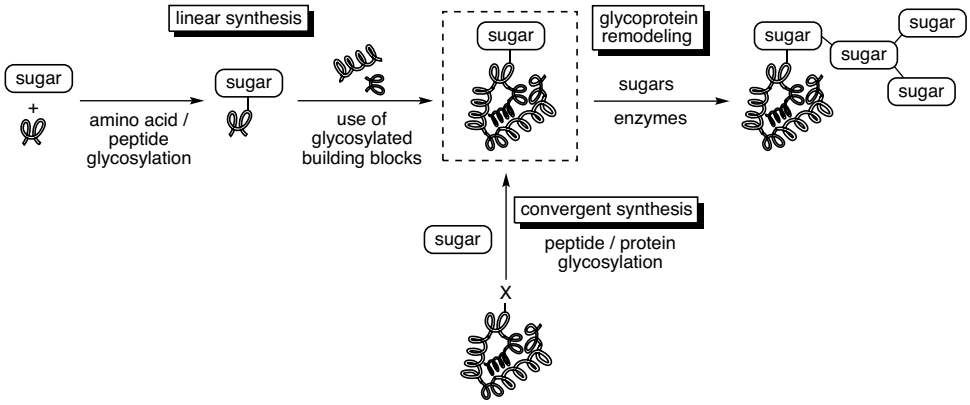
Figure 11.1 Representative structures of *N*- and *O*-linked glycopeptides.

synthetic methods allow access to larger targets, the synthesis of complex glycopeptides remains restricted to the expertise of a few specialized laboratories worldwide. Recent developments in the field of glycopeptide and glycoside synthesis are the subject of several excellent reviews [4–9].

Glycopeptide and glycoprotein mimetics with non-native linkages, so-called neoglycopeptides and neoglycoproteins, respectively, are often readily accessible and usually available as a single neoglycoform. By exchanging natural features with groups that are more easily obtained through chemical synthesis, these neoglycoconjugates provide useful alternatives for the study of structure–activity relationships. Furthermore, enhanced stability towards chemical and enzymatic degradation can be achieved by introducing the more stable *S*- and *C*-glycosidic bonds, thereby providing superior properties for potential therapeutic applications.

Glycopeptides and glycopeptide mimetics up to 50 amino acid residues in length are generally synthesized by stepwise solid-phase peptide synthesis (SPPS). SPPS can be performed by incorporating protected glycosylated amino acid building blocks in a linear fashion (Scheme 11.1), but the drawbacks to this approach are complex protecting group manipulations and the formation of unstable glycosidic bonds at an early stage in the synthesis. Furthermore, for longer peptides, yields and purity decrease because of aggregation effects and the accumulation of byproducts. In a more convergent approach, the glycosidic linkage is formed on a preassembled peptide backbone in a site-selective fashion late in the synthesis (Scheme 11.1), although this requires not only the carbohydrate, but also the amino acid residues to be protected in an orthogonal fashion. The carbohydrate components of assembled glycopeptides may then be extended by chemical or enzymatic methods.

Despite the challenges, syntheses of complex glycopeptide and neoglycopeptide structures have been accomplished [10–14]. The size limitation associated with SPPS has been overcome by assembling manageable glycopeptide fragments into



Scheme 11.1 Strategies for glycopeptide synthesis.

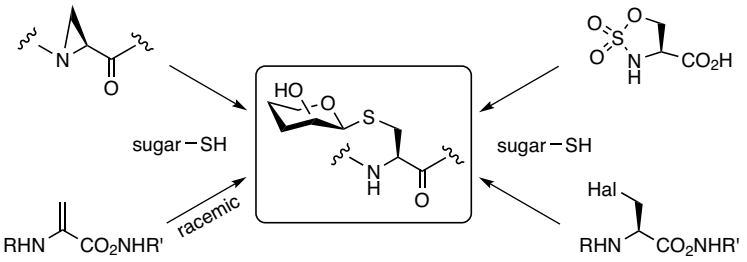
full-length proteins using chemical ligation techniques [4, 15–18]. Global modifications of protein side-chains with similar reactivity have been made in order to introduce new groups, but this process tends to be rather indiscriminate. Refinements through the use of modern enzymatic, molecular, and cell biological techniques have enabled highly specific, site-selective introduction of natural and functionalized amino acids. Thus, in combination with chemoselective glycosylation methods, site-selective glycoprotein synthesis is becoming increasingly feasible [5, 17]. This chapter summarizes the recent developments for the synthesis of unnatural glycosylated amino acids, neoglycopeptides, and neoglycoproteins, with an emphasis on the chemical synthesis of these constructs. The reader is also referred to several other comprehensive reviews on the synthesis of glycopeptide mimetics [2, 5, 9, 19, 20].

11.2
Neoglycoside and Neoglycopeptide Synthesis

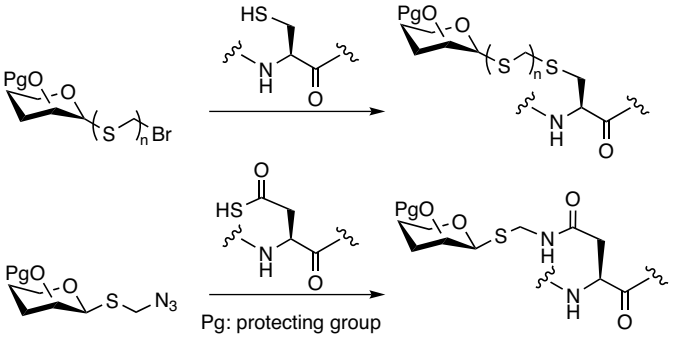
11.2.1
S-Glycosides

The *S*-glycosidic linkage is a commonly used synthetic method to attach sugars, owing to its ease of introduction, and relative stability to acidic, basic, and enzymatic hydrolysis when compared to *O*-glycosides [19]. *S*-Glycosides may be readily generated by S_N2 reactions between an anomerically stable sugar thiolate (nucleophile) and a suitable peptidic substrate acceptor, examples of which are given in Scheme 11.2.

Despite Michael addition to dehydroalanine (Dha) yielding a racemic mixture, it nevertheless provides a commonly used technique due to an increasing number of facile methods to generate dehydroalanine in peptides and proteins, such as oxidative elimination of phenylselenocysteine. Aziridine-containing peptides have been converted to *S*-glycosides, although it is necessary to incorporate the moiety as a



Scheme 11.2 Reaction of sugar thiols with glycosyl acceptors.



Scheme 11.3 Preparation of *S*-glycosides.

dipeptide to avoid intramolecular aziridine *N*-deacetylation on Fmoc deprotection. Serine-derived sulfamidates enable *S*-glycosidation in aqueous media and displacement of iodide or bromide in halo-amino acids provides an alternative route to access *S*-glycosides [5].

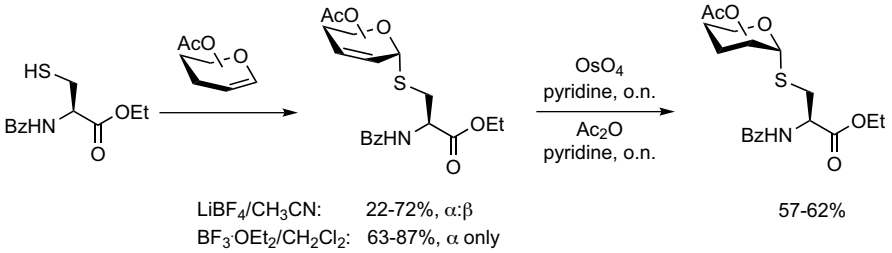
The Staudinger ligation between glycosylthiomethyl azides and thioacid-containing peptides has also been successfully employed [5].

Alternatively, *S*-glycosides may be synthesized by reaction of glycosyl halides with a corresponding peptide nucleophile (Scheme 11.3). Examples include glycosyl bromides and glycosylthiomethyl bromides as well as mesylates.

S-Glycosides with an α-configuration can also be obtained via acid-catalyzed S_N1 reactions. Ferrier reaction between a glycal and protected L-cysteine or DL-homocysteine using $BF_3{\cdot}OEt_2$ as a Lewis acid (Scheme 11.4) afforded a product with excellent α-selectivity [21]. Subsequent dihydroxylation of glucal conjugates afforded mannose derivatives, and, similarly, galactal conjugates gave a mixture of talose and glucose.

11.2.2 *N*-Glycosides

Neoglycopeptides with nitrogen linkers in the form of carbamate, urea, and thiourea have been reported [2, 5]. Carbamate-tethered glycosides have been obtained by



Scheme 11.4 Synthesis of *S*-glycosides via Ferrier reaction [21].

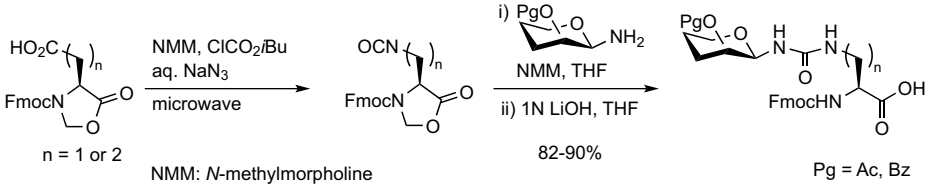
reaction of a sugar isocyanate [22, 23] with a peptide hydroxyl group or, more recently, in reactions between either oxycarbonyl chlorides or pentafluorophenyl carbonates with sugar amines [24].

Sureshbabu *et al*. described the production of urea-linked glycopeptides in which C-terminal peptidyl isocyanates [25] and amino acid-derived oxazolidinone isocyanates [26] were obtained via Curtius rearrangement of the corresponding acid azides and subsequently trapped by glycosylamines (Scheme 11.5). This approach was further refined to a one-pot procedure, using Deoxo-Fluor and trimethylsilyl azide to generate the acid azide [27].

The isocyanates were also transformed to more stable yet equally reactive pentafluorophenyl carbamates before conversion to the ureas [25, 26]. In subsequent reports, preactivation of the carboxy group was avoided by using diphenyl phosphoryl azide as an azido-transfer reagent [28].

Novel *C*-glycosyl amino acids with a thiourea linker have been reported by Dondoni *et al*. [29]. Direct cyanation of an anomeric glycosyl acetate with trimethylsilyl cyanide followed by reduction with lithium aluminum hydride furnished the crude glycosylmethyl amines, which were then transformed into corresponding isothiocyanates and converted to thiourea-tethered glycosides by reaction with β-amino-L-alanine. When using perbenzylated glycosyl donors, Fmoc protection of the amino acid partner afforded better yields than Boc protection owing to diminished steric hindrance [29].

Danishefsky *et al*. [30] reported the synthesis of *N*-thioformyl glycosides and glycopeptides from glycosyl isonitriles and the thioacid-functionalized side-chain of aspartic acid using microwave irradiation. Hydrogenation over Raney-nickel afforded the *N*-linked glycopeptides [30]. New mono- and diglycosylated amino acids



Scheme 11.5 Synthesis of urea-linked glycosides [26].

have been synthesized from acetylated azidoethyl mannoside via Staudinger ligation, thereby avoiding the $O \rightarrow N$-acetyl group migration that occurs under standard peptide coupling conditions [31].

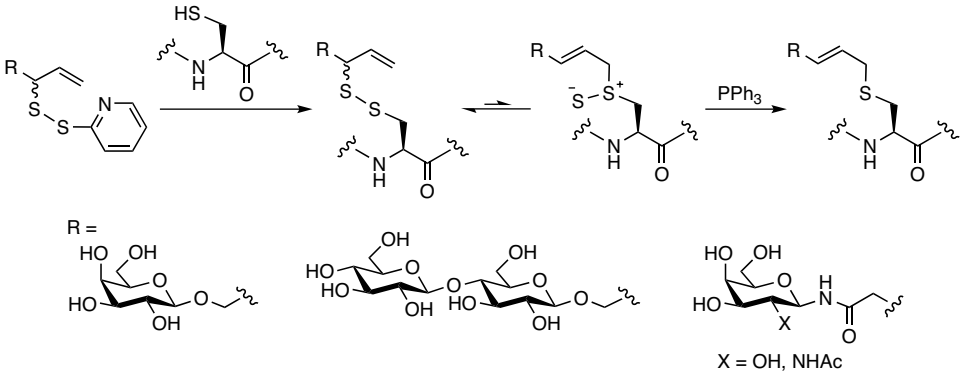
In a study on glycosylation of tyrocidine A, sugars were connected by amide coupling, either directly or using a short alkyl amine spacer, to aspartic acid to provide glycosylated analogs at the asparagine residue in the macrocyclic peptide. Those with no spacer showed diminished antibacterial activity, whereas a short spacer gave activity consistent with that of the parent molecule [32].

Reductive amination is a flexible tool to incorporate carbohydrates, both at the N-terminus and at internal nitrogens in a peptide chain [33]. Ozonolysis of *O*-allyl mannoside and glycosylation of the N^{ε}-amino group of lysine with the resulting mannosidic aldehyde afforded mono- and diglycosylated building blocks for SPPS [34].

11.2.3
O-Glycosides

O-Glycoside-linked neoglycopeptides and glycopeptide mimics are often synthesized in a similar fashion to *O*-linked glycopeptides, but using unnatural building blocks. 1,5-Anhydro derivatives of galactose were coupled with amino acid-derived aziridines in the presence of $BF_3{\cdot}OEt_2$. Aziridine ring opening and protecting group manipulation afforded the glycosylated building block for further incorporation into a 13-mer peptide derived from mucin 1 (MUC1) [35]. A hydroxylated cyclobutane amino acid was incorporated into a diamide trimer and glycosylated using modified conditions of the Koenigs–Knorr reaction [36].

Disulfide ligation followed by subsequent phosphine-mediated desulfurative allylic rearrangement enabled the synthesis of thioether bridged *N*- and *O*-glycosides under mild reaction conditions, proceeding with retention of configuration of the peptide backbone stereochemistry and high *trans* selectivity with respect to the spacer (Scheme 11.6) [37].



Scheme 11.6 Glycoconjugation with Crich's third-generation glycosyl donors [37].

11.2.4
C-Glycosides

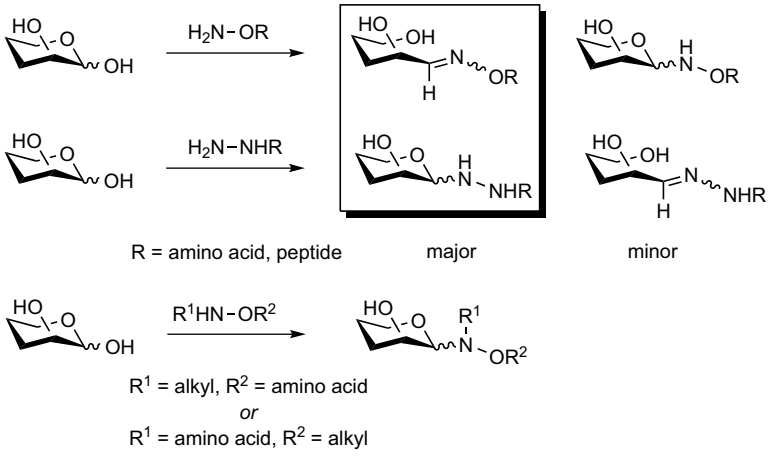
Although *C*-glycosyl amino acids were reported in the 1940s, their incorporation into neoglycopeptides was only first reported in 1992. Since the discovery of naturally occurring *C*-glycosides in 1994 the development of stable *C*-glycopeptide mimics has advanced significantly [2, 5].

A lysine analog with the side-chain constrained by incorporating a glucose scaffold was synthesized via Lewis acid-promoted reductive ring opening of a glucose-derived C1-epoxide in the presence of tributyltin hydride. The resulting α-hydroxyl ester was converted to an azide, then subsequently transformed to a protected amino acid derivative that was then coupled to a tryptophan building block [38]. Other examples of amino acid-derived *C*-glycosides [39] have recently been published by Sridhar *et al.* [40].

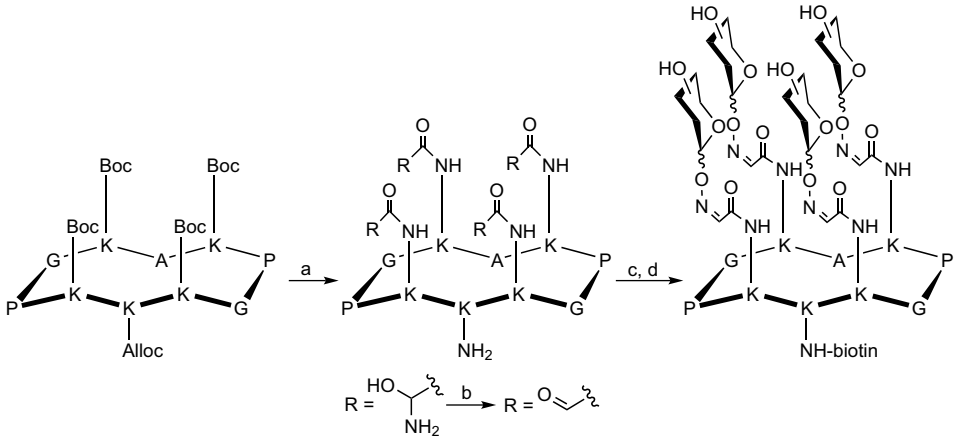
11.2.5
C=N Linkage

Formation of oxime and related C=N bonds between carbonyl and amino-oxy- or hydrazide-functionalized compounds is widely used in neoglycopeptide synthesis, due to the high chemoselectivity of the reaction, the ability to perform reactions in water, and little or no requirement for protecting groups [2, 5]. When carried out with reducing sugars generally a mixture of cyclic and acyclic forms result, the latter usually dominating with alkoxyamines and the former with hydrazides or *N*, *O*-disubstituted hydroxylamines (Scheme 11.7) [2].

Dumy *et al.* [41] used oxime formation to generate regioselectively addressable functionalized templates (RAFTs). A suitably derived template was reacted with



Scheme 11.7 Oxime and hydrazone-linked glycosides.



a) 1. TFA/CH_2Cl_2; 2. BocSer(*t*Bu)/PyBOP, DIEA/DMF; 3. $Pd(Ph_3)_4$/$PhSiH_3$, CH_2Cl_2; 4. TFA/TIS/H_2O;
b) $NaIO_4$/H_2O; c) sugar-ONH_2, AcOH; d) biotin-OSu, DIEA/DMF.

Scheme 11.8 Tetravalent RAFT glycoclusters [41].

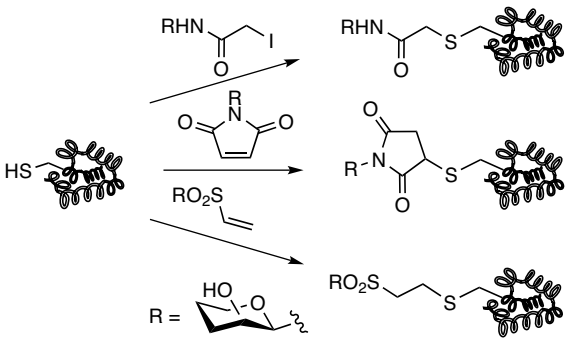
amino-oxylated lactose to produce tetravalent glycoclusters in an effort to enhance lectin binding (Scheme 11.8).

This chemistry was also used for the chemoselective modification of acetal-protected dextran particles using alkoxyamine-functionalized fluorophores or an alkoxyamine-terminating poly(arginine) sequence derived from cell-penetrating peptides. The cellular uptake and the use of the particles as drug delivery vehicles were monitored using an encapsulated luciferase reporter [42].

11.3
Protein Side-Chain Modifications

11.3.1
Modifications of Cysteine Side-Chains

The use of chemoselective methods to modify natural and unnatural residues of unprotected peptides and proteins has found widespread application in chemical biology and in the development of therapeutics [5, 43]. However, controlling chemical modification directly is often difficult, owing to the diversity of functionalities within a protein and the different reactivities of these groups. Development of methods for site-specific modification is therefore important. The thiol group of a cysteine side-chain is an ideal target for chemoselective modification, owing to the ease with which it can be derivatized. Since the natural abundance of cysteine is low, the use of site-directed mutagenesis conveniently allows construction of proteins with a single cysteine on a predetermined site for site-selective modification. This section highlights the recent developments in cysteine side-chain modifications; more details can be found in other reports [5, 44].

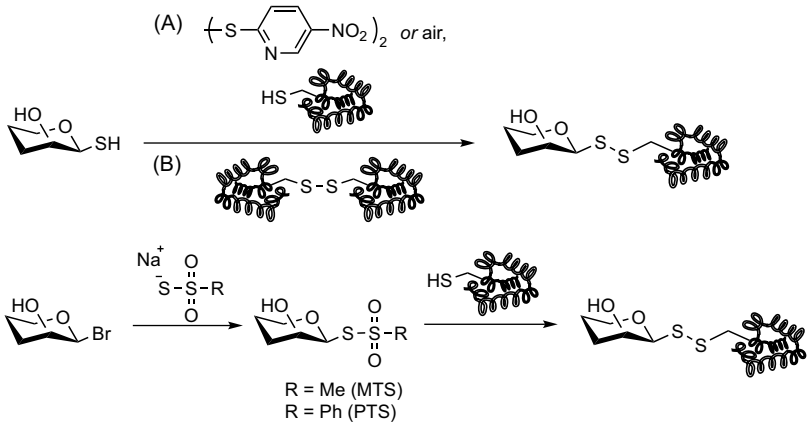


Scheme 11.9 Alkylation of cysteine side-chain.

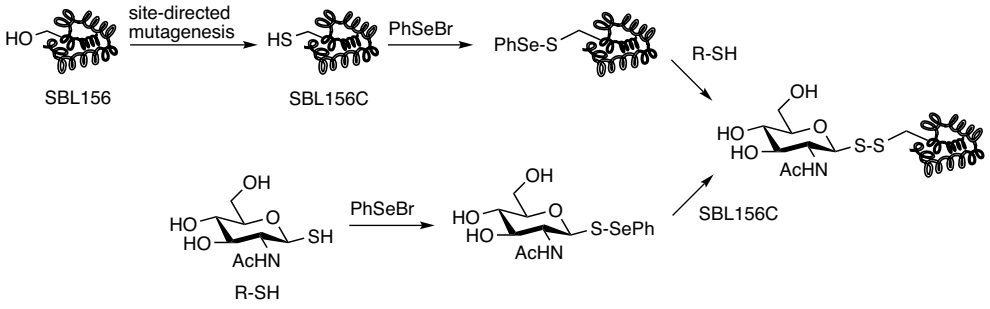
The thiol residue can be readily alkylated by electrophiles, such as α-halocarbonyls. Iodoacetamides were used as early as 1935 [45] and continue to find application in cysteine glycosylation (Scheme 11.9) [46, 47]. Use of chloroacetamide has suppressed the alkylation of lysine, which was observed as a side-reaction in some cases. Glycosyl maleimides, maleimide-activated spacers, and related Michael acceptors have also been used for cysteine glycosylation (Scheme 11.9) [5].

Formation of disulfides is an obvious means of derivatization and has been achieved by Boons *et al.* using dithiopyridyl [48] activation, aerial oxidation (Scheme 11.10A) and disulfide exchange (Scheme 11.10B) [49]. Use of activated thiols such as methanethiosulfonate (MTS) [50, 51] and phenylthiosulfonate (PTS) [52] also allows selective disulfide formation with a high reaction rate, thereby avoiding the need for a large excess of reagents (Scheme 11.10).

A novel sulfotyrosine mimic was installed site-selectively into a galactosidase reporter enzyme via MTS-mediated disulfide formation. Subsequent modification of azidohomoalanine Aha43 with sialyl-Lewis X (sLe^x) via azide alkyne cycloaddition (see Section 11.4) afforded double modified P-selectin glycoprotein ligand-1



Scheme 11.10 Glycosylation via disulfide formation.



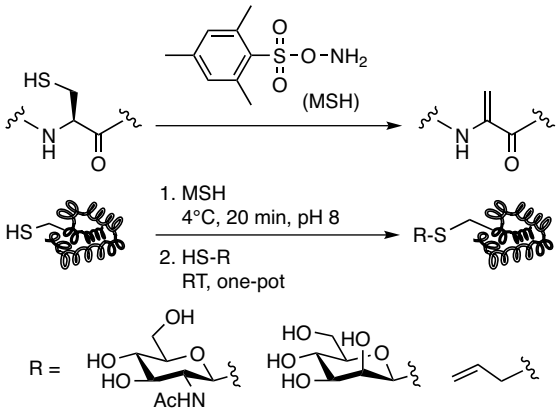
Scheme 11.11 *S*-Glycosides via phenylselenylsulfide intermediates [55].

(PSGL-1), which was used in biological studies as a reporter for mammalian brain inflammation [53].

Phenylselenylsulfide intermediates, generated by preactivation of glycosyl thiol or cysteine protein with PhSeBr, have been used to conjugate glycoproteins [54]. This technique was exemplified by the introduction of three different mono- and trisaccharides at position 156 in one of the two loop regions of the single cysteine mutant of subtilisin (SBL) derived from *Bacillus lentis* (Scheme 11.11) [55]. Studies on the influence of glycosylation revealed that proteolytic stability was higher in the glycosylated form and that degradation rates diminished with the larger glycosides.

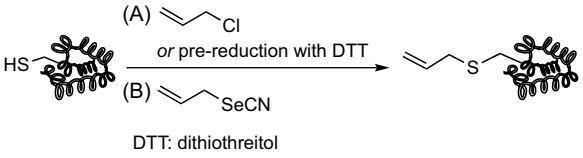
The cysteine disulfide of modified proteins has been converted to more stable thioethers by treatment firstly with hexamethylphosphorus triamide, to induce elimination to dehydroalanine, and then addition of a thiol to afford an epimeric mixture of thioethers [56, 57].

Dehydroalanine, which is also accessible by oxidative elimination of alkylcysteines [58] and selenocysteines [59] with hydrogen peroxide or by treatment of cysteine with excess *O*-mesitylenesulfonylhydroxylamine (MSH), is a useful Michael acceptor and versatile means for further modification of proteins such as glycosylation or generation of allylcysteine in one pot (Scheme 11.12) [60, 61].



Scheme 11.12 Oxidative elimination of cysteine with MSH [60, 61].

Allylcysteine has been glycosylated using a Krimse–Doyle reaction [62], but more generally it can be considered a privileged substrate for cross-metathesis (see Section 11.5). While addition of thiols to dehydroalanine usually results in racemization and possible reduction of natural disulfide bonds, Davis *et al.* [63] usefully reported that the direct alkylation of cysteine, with either allyl chloride or a reductive allyl selenenylsulfide rearrangement after treatment with allyl selenocyanate, avoided the problem and provided single diastereomers of the required allylated product (Scheme 11.13). It was noted, however, that allylation on strongly basic lysine or histidine residues is a possible side-reaction when using allyl chloride.

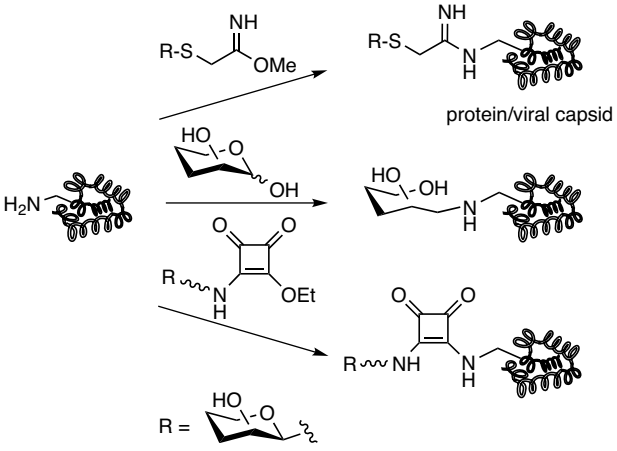


Scheme 11.13 Direct allylation of cysteine [63].

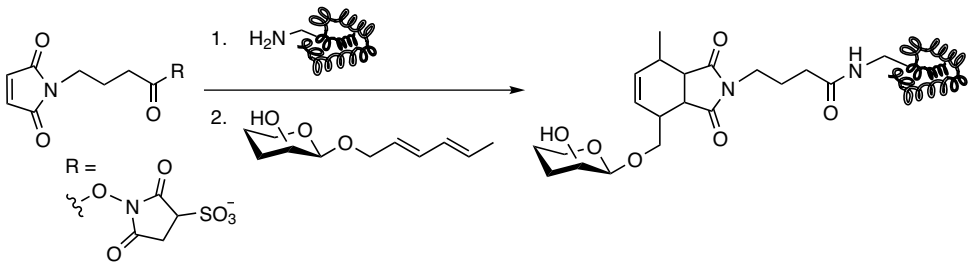
11.3.2
Modifications of Lysine Side-Chains

In contrast to cysteine, lysine is present in high abundance on protein surfaces; hence, it becomes difficult to target these in a site-selective manner. The two major routes to indiscriminately modify lysine residues are generation of 2-iminomethoxymethyl thioglycosides (IME methodology) and reductive amination (Scheme 11.14) [2, 5].

Cyanomethyl thioglycosides were converted to the corresponding methyl imidates, which were subsequently conjugated to lysine residues on the cell surface of



Scheme 11.14 Lysine side-chain modifications.



Scheme 11.15 Glycosylation using Diels–Alder chemistry [68].

adenovirus [64]. The extent of glycosylation could be controlled by varying the reaction conditions and ranged from 1500 to 20 000 glycans.

Aldehyde-functionalized glycosides or linkers have been conjugated to lysine amino groups via reductive amination [65]. In order to reduce steric hindrance and increase the loading with glycosides spacer arms have been introduced [66].

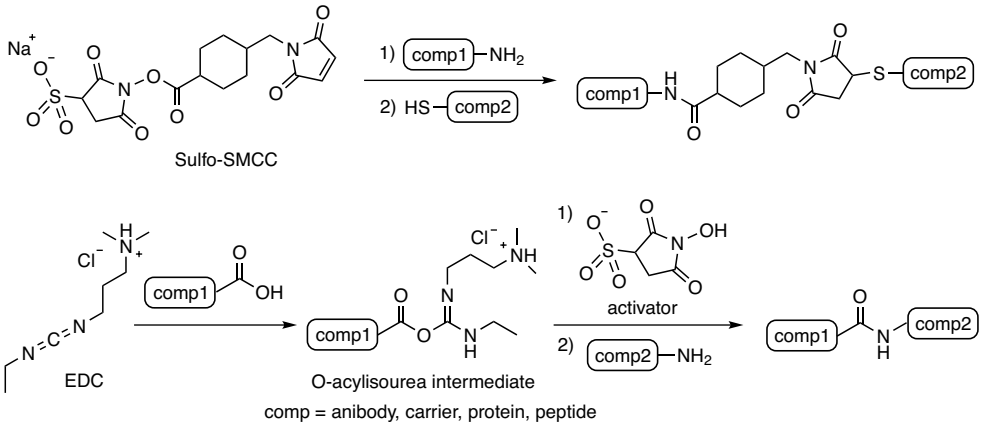
Squaric acid diesters have also been used as linkers for neoglycoconjugation (Scheme 11.14). In a recent study bovine serum albumin (BSA) was conjugated using this method with defined amounts of GalNAcβ1–4(Fucα1–3)GlcNAcβ (LDNF antigen) to test the influence of glycan density and the nature of the carrier protein on the immune response [67]. The oligosaccharide–diaminopyridine conjugate was synthesized enzymatically then reacted with diethyl squarate which enabled coupling to the BSA carrier protein.

After conjugation of human serum albumin to a sulfosuccinimidyl-derived bifunctional linker, coupling of several different glycosides to the maleimide part of the linker was performed using a Diels–Alder reaction (Scheme 11.15) [68]. In a similar vein a bifunctional linker with a terminal amino-oxy group was used to tether keto-functionalized carbohydrates [69].

11.3.3
Other Side-Chain Modifications

A range of different linkers and labels for modifying proteins using simple reaction conditions that proceed in reproducible yield are now commercially available. Maleimide activation is one of the most common methods to functionalize carrier proteins on lysine side-chains. Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) is used as a bifunctional cross-linker (Scheme 11.16). The *N*-hydroxysuccinimide (NHS) ester is reactive towards amines and the maleimide group towards sulfhydryls, with each functional derivative being stable under the conditions used for conjugation to the other.

Carboxyl groups can be linked to primary amines using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) via formation of an unstable *O*-acylisourea intermediate (Scheme 11.16). Addition of an NHS ester affords a more stable intermediate that is subsequently reacted with an amino-functionalized compound.



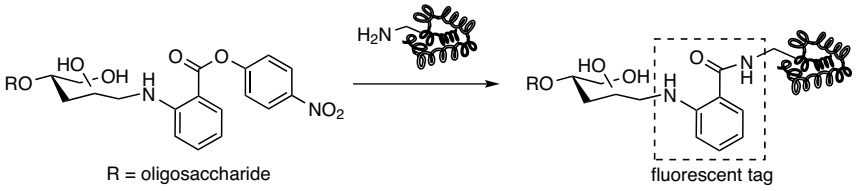
Scheme 11.16 Hetero-bifunctional linkers.

As previously discussed (see Section 11.2.5), oxime formation is a versatile route for forming glycoconjugates [2, 5]. An aldehyde tag on a GalNAc residue in an *O*-glycopeptide containing a native *O*-glycosyl-peptide bond was installed using galactose oxidase and subsequently derivatized with a variety of amino-oxy-oligosaccharides [70].

Nishimura *et al.* [71] introduced amino-oxy- and methylamino-oxy-substituted homoalanines into streptavidin, and were able to couple several unmodified glycosides in a combinatorial fashion.

Reactions of aldehydes with amines have been widely used to generate neoglycoproteins via reductive amination (see Scheme 11.14). Due to the low concentration of the aldehyde form of an unmodified sugar, up to 300 equiv. may be required for direct functionalization of protein side-chain amino groups. However, sodium sulfate has been reported to be an effective additive to enhance glycosylation of BSA using only 15 equiv. of sugar. Using slightly higher temperatures and increased reaction times, 5.1 glycosides were incorporated onto BSA compared to 9.2 using 300 equiv. of sugar [72].

Commercially available *p*-nitrophenyl anthranilate (PNPA) has recently been used as a fluorescent hetero-bifunctional linker (Scheme 11.17) [73]. Coupling of PNPA to an unmodified glycan and BSA afforded a fluorescent anthranilamide-linked glycoconjugate.



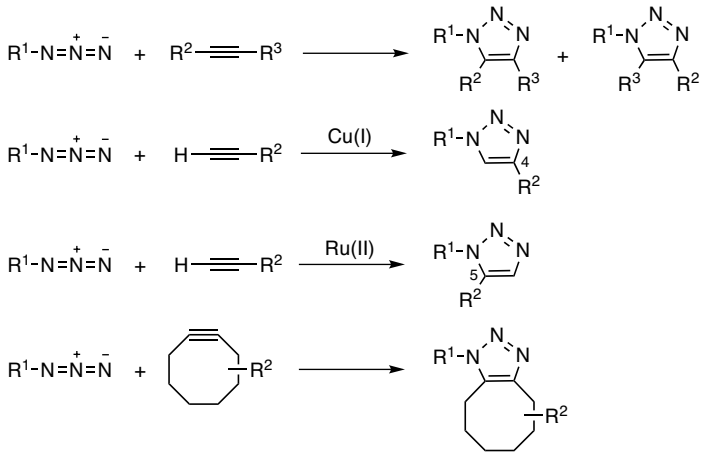
Scheme 11.17 Fluorescent anthranilamide-linked glycoconjugate [73].

11.4
Cu(I)-Catalyzed Azide–Alkyne "Click" Cycloaddition

11.4.1
General Aspects of Cu(I)-Catalyzed Azide–Alkyne cycloaddition

The concept of click chemistry was introduced by Sharpless *et al.* in 2001 [74]. Several reactions fall within the remit of click chemistry in that they are simple, modular, and proceed under fairly benign conditions. Examples include the hetero-Diels–Alder reaction, ring-opening reactions of strained heterocyclic electrophiles (e.g., epoxides, aziridines), carbonyl reactions of the nonaldol type (e.g., formation of ureas, thioureas, oximes), various additions to carbon–carbon multiple bonds, and Michael additions (e.g., thiol–ene coupling). However, the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) has assumed a particularly important role in organic synthesis under this heading.

In most cases the thermal 1,3-dipolar Huisgen cycloaddition reaction [75] between an azide and an alkyne is slow and not regioselective (Scheme 11.18). Sharpless *et al.* [76] and Meldal *et al.* [77] discovered a dramatic acceleration of reaction rate between terminal alkynes and azides by the addition of catalytic amounts of Cu(I) (Scheme 11.18). The CuAAC does not seem to be significantly affected by steric and electronic properties, hence a broad range of substrates can be used. The high selectivity of the CuAAC renders the reaction compatible with complex and diverse functionalities present in biomacromolecules, obviating the need for complex protecting group strategies, and it proceeds in both aqueous and organic media. The resulting 1,4-disubstituted triazoles form peptide bond isosteres, with similar geometry and distance between substituents, a strong dipole moment, and excellent stability towards hydrolytic cleavage, oxidation, and reduction (Figure 11.2) [78, 79].



Scheme 11.18 Azide–alkyne cycloaddition.

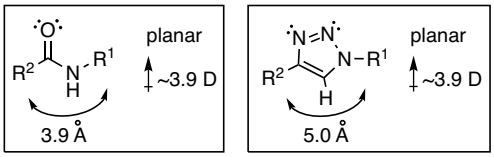


Figure 11.2 1,4-Disubstituted triazoles as peptide isosteres.

With such a range of convenient features, the CuAAC has been widely adopted in all fields of chemistry [80–86]. In recent years a range of modifications have been developed to improve reaction yield and product selectivity. Exclusive access to 1,5-substituted triazoles can also be obtained using ruthenium as catalyst in place of copper under more stringent reaction conditions (Scheme 11.18) [87]. Metal-free reaction conditions have also been developed to avoid the use of cytotoxic copper in order to carry out bioconjugation reactions in living systems (Scheme 11.18) [88–91].

Catalytically active Cu(I) species can be used directly as salts or as coordination complexes in organic solvents. Use of excess base prevents the oxidation and disproportionation of Cu(I), and accelerates the formation of Cu(I) acetylides. Cheap, commonly available, and easy to handle Cu(II) catalyst systems include $CuSO_4 \cdot 5H_2O$ or $Cu(OAc)_2$, which are used in combination with an excess of a reducing agent (e.g., sodium ascorbate). Additionally, Cu(0) and copper nanoclusters have also been used [78, 80].

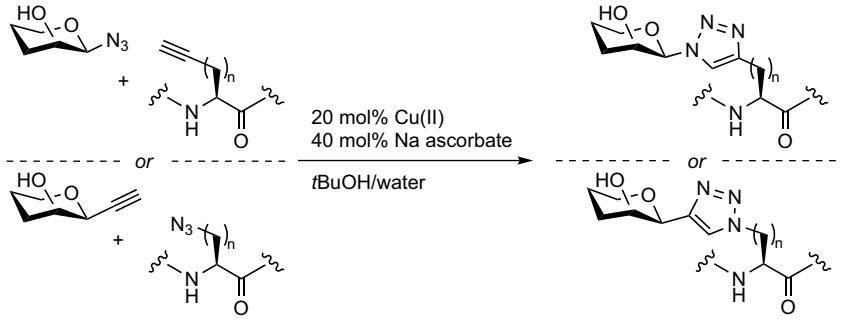
Auxiliary ligands such as tris(triazoyl) amines or bathophenanthroline are used in CuAACs requiring a low concentration of catalyst, necessary for reactions using sensitive biomacromolecules and in bioconjugation reactions. These agents stabilize Cu(I) and lower the cytotoxicity, thus preventing degradation of biomaterial [53, 78, 80].

The following sections provide an overview of the recent developments and applications of CuAACs in the synthesis of neoglycosides, neoglycopeptides, and neoglycoproteins.

11.4.2
Neoglycoside and Neoglycopeptide Synthesis via CuAAC

The first account of CuAAC in the solid-phase synthesis of peptidotriazoles and neoglycopeptides using a CuI/*N,N*-diisopropylethylamine (DIPEA) catalyst system in tetrahydrofuran (THF) was published by Meldal *et al.* [77]. The high reported efficiency proved to be equally effective for reactions of azidoglycosides with acetylene-functionalized peptides and the reverse protocol (Scheme 11.19) [92].

New additives and catalyst systems have since been developed to accelerate the CuAAC and simplify the purification process. Histidine derivatives showed a similar effect as NEt_3 or DIPEA on the acceleration of CuAAC in the reaction of the azide derived from Boc-ornithine with 4-ethynylaniline in dimethylformamide using CuI [93]. This method was applied to coupling of azido-functionalized unprotected lactose to Boc-Pra–Tyr-OMe (Pra = propargylglycine) in 78% yield. Incorporation of



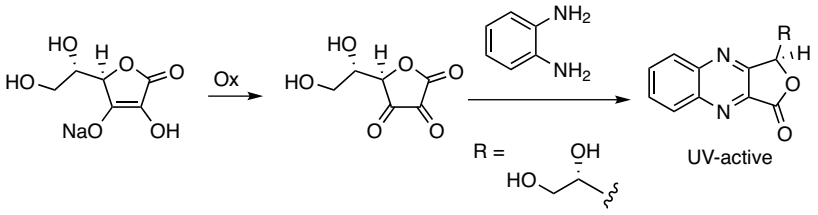
Scheme 11.19 Synthesis of neoglycopeptides via CuAAC [92].

benzyl histidine into the peptide backbone enabled additive-free CuAAC between peptide fragments on solid phase and in solution; however, no yields were reported for CuAAC in solution because of problems with product isolation [93].

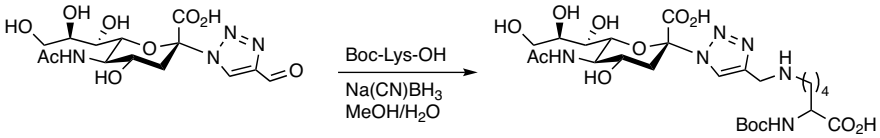
Coordination of copper to the product is sometimes an issue and a way of circumventing this is to use heterogeneous catalysts such as Cu(I)-modified zeolites in toluene [94]. Alkyne homocoupling is avoided with these catalysts and product recovery is facile, requiring a simple filtration and solvent evaporation sequence. The structural parameters of the zeolites strongly influence the outcome of CuAAC and heating is often required for large molecules. Good yields have been obtained using oligosaccharides; however, the lack of conversion when using Fmoc-Pra-OMe and a mere 47% yield using Cbz-Pra-OMe in the CuAAC with protected azido glucose suggests a limited use for Cu(I) zeolites in the synthesis of neoglycosides [94].

In another approach, *o*-phenylenediamine was introduced as an additive for the copper sulfate/sodium ascorbate system to accelerate the CuAAC of unprotected 1-glucosyl azide in water and water/*t*BuOH [95]. More importantly, the initially formed dehydroascorbate was trapped by *o*-phenylenediamine to give fluorescent quinoxaline, which in turn could be easily removed by adsorption onto activated charcoal together with Cu(II) (Scheme 11.20). This method allowed the synthesis of a phenylalanine-derived neoglycoside in 84% yield without the need for column chromatography [95].

CuAAC has found application in the synthesis of an aldehyde-containing sialic acid derivative (Scheme 11.21) [96]. Subsequent reductive amination with Boc-Lys-OH afforded the sialic acid-containing neoglycoside in 71% yield.



Scheme 11.20 Dehydroascorbate–phenylenediamine addition [95].



Scheme 11.21 Lysine functionalized with a sialic acid-containing neoglycoside [96].

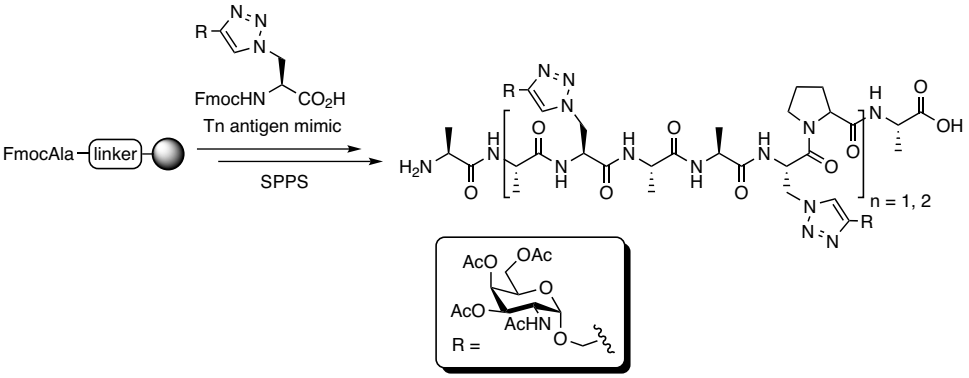
^{18}F-labeled glycosides have also been synthesized from ^{18}F-labeled mannosyl azide precursors using the copper sulfate/ascorbate catalyst system [97].

Neoglycopeptides containing the triazole moiety have found application in structure-activity–relationship studies and immunotherapy. After establishing syntheses of neoglycosides by CuAAC, Rutjes *et al.* [98] reported the synthesis of glycosylated cyclic arginine–glycine–aspartate (RGD) derivatives with either a natural amide bond or a triazole linker for selective targeting of $\alpha_v\beta_3$ integrin. Solid-phase binding assays revealed no significant difference in binding affinity and biodistribution studies suggested improved pharmacological properties of the triazole-linked compounds [98]. In a subsequent account, Rutjes *et al.* [99] synthesized several new triazole-linked glycosides from protected and unprotected glycosyl donors with propargylglycine and azidoalanine derivatives. Additionally, a dipeptide containing both free and $SiMe_3$-protected propargylglycine was glycosylated in a stepwise fashion with benzyl and acetyl-protected azidoglucose (77% overall yield). The C-termini of the neoglycosides were elongated with glycine and phenylalanine enzymatically by alcalase. Both the length of the neoglycoside side-chain and the amino acid had a strong influence on the efficiency of reaction, with yields ranging from 30 to 98% and reactions proceeding slowly over 6 days [99].

The propargylamide group of a β-amino acid has been coupled via CuAAC to different glycosyl azides and the resulting β-peptide building blocks activated as pentafluorophenyl esters. These were then used in spot syntheses to construct a glycotetrapeptide library of 256 compounds, some of which exhibited binding affinities comparable to natural ligands [100].

CuAAC was used for structure–activity relationship studies on antifreeze glycopeptides (AFGPs) [101] and neoglycopeptoids [102]. *N*-Propargylglycine was conjugated to peracetylated α- and β-azidogalactose and β-azido-*N*-acetyl-galactose using 1 equiv. CuI/sodium ascorbate, 1 equiv. azide, and 2 equiv. DIPEA in 75–86% yield. The resulting triazole linked glycosides were incorporated into short (*N*-Pro–Ala–Ala)$_n$ peptoids ($n = 1$–4) by SPPS. In a second approach, conjugation of the neoglycopeptoids was performed between sugar-azides and *N*-propargyl-functionalized peptoid backbones on Rink amide resin with 6 equiv. CuI, 3 equiv. sodium ascorbate, and 2 equiv. of the azides. Both reactions in solution and on resin required stirring overnight at room temperature.

An efficient synthesis of valuable triazole-linked α-GalNAc-containing Tn antigen mimic has been developed by Brimble *et al.* [101] (Scheme 11.22). With a catalyst loading of just 2 mol% $CuSO_4 \cdot 5H_2O$/5 mol% sodium ascorbate the CuAAC between azidoalanine and α-propargylated GalNAc gave an excellent 88% yield of the glycosylated building block in less than 30 min at 80 °C with microwave irradiation.



Scheme 11.22 Synthesis of triazole-linked AFGP analogs [101].

This was then used for microwave-enhanced automated Fmoc SPPS of analogs of AFGPs. In an alternative approach, glycoconjugation of a multi-azido-functionalized peptide backbone was performed in solution in less than 10 min, using only 10 mol% $CuSO_4 \cdot 5H_2O$ and 25 mol% sodium ascorbate [101].

The GM_2 ganglioside antigen is overexpressed in various types of cancer and hence has become an important target for specific anticancer immunotherapy. The conjugation of GM_2 oligosaccharide, accessible in gram quantities from high-density recombinant *Escherichia coli* culture, with an alkyne-functionalized *N*-terminal lysine of a peptide epitope from poliovirus afforded a synthetic vaccine that induced production of human tumor cell-specific antibodies in immunized mice [103].

The glycoside cluster effect has also been studied between the mannose-specific lectin concanavalin A and triazole-linked mannose units on RAFTs revealing a 3-fold increase in binding affinity of the tetravalent glycocluster relative to the divalent glycocluster [104].

Overkleeft *et al.* [105] designed a mannosylated fluorescent epoxysuccinate to target cysteine proteases in the endosomal/lysosomal compartments after selective mannose receptor-mediated uptake into the antigen-presenting cells. CuAAC was applied on solid phase to conjugate six mannose units to the side-chains of $(Lys)_6$ and then in solution to attach the mannose cluster to the fluorescent epoxysuccinate (Figure 11.3) [105].

11.4.3
CuAAC and Neoglycoproteins

Reactions on proteins using classical bioconjugation reactions often suffer from a lack of selectivity, with reactions occurring at unwanted positions in the macromolecule. The high chemoselectivity of the CuAAC renders it an excellent tool in biochemistry for postsynthetic modifications such as labeling, tagging, imaging, and enrichment [83, 106, 107].

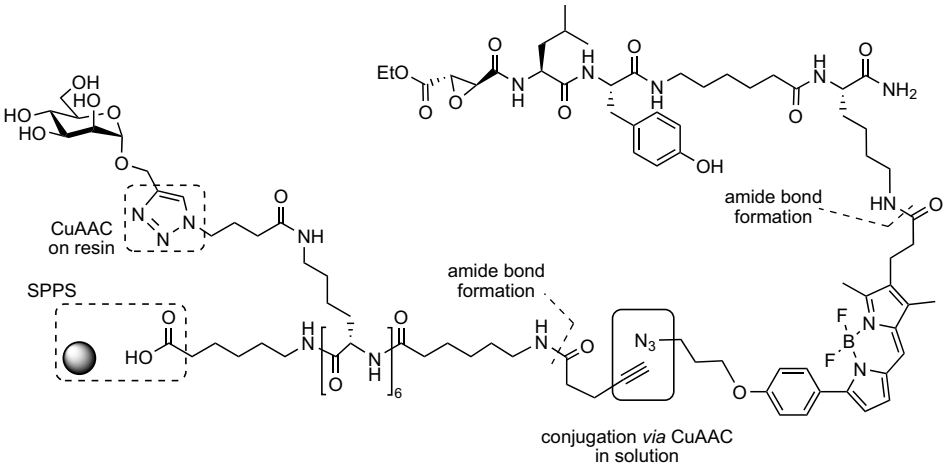
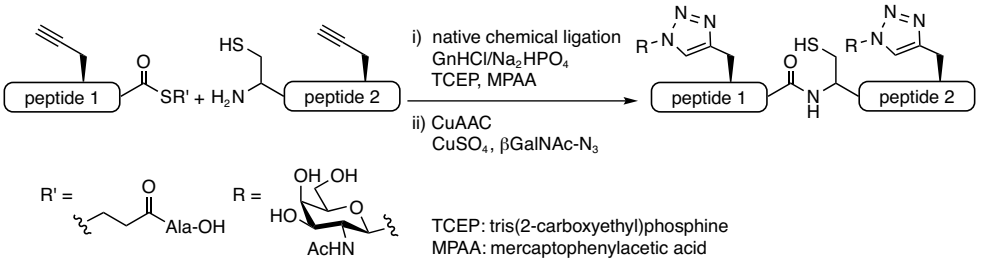


Figure 11.3 Mannosylated fluorescent epoxysuccinate construct building blocks [105].

Azido- and propargyl-functionalized amino acids or glycosides can be incorporated into proteins via biochemical and enzymatic strategies, and chemical modification. The synthesis of a pyrrolysine analog with terminal alkyne functionality and its incorporation into a calcium/calmodulin protein through the UAG codon in recombinant *E. coli* was described by Chan *et al.* [108]. CuAAC and cysteine tagging allowed introduction of a fluorophoric donor–acceptor pair to monitor conformational changes of the protein by fluorescence resonance energy transfer spectroscopy [108]. Photostable and easily accessible aliphatic azides and alkynes have been introduced site-specifically into proteins for labeling using an orthogonal synthetase/$tRNA_{CUA}$ pair that does not recognize amino acids naturally present in *E. coli* [109]. Azide and alkyne-containing amino acids have been introduced into virus-like particles composed of hepatitis B virus (HBV) and bacteriophage Qβ capsid proteins in a methionine auxotrophic strain of *E. coli* [110]. Subsequent CuAAC with the self-assembled icosahedral capsids was possible with Qβ, whereas HBV decomposed in attempts to form more than 120 triazole linkages per capsid [110].

A facile, but unselective surface modification of BSA and tobacco mosaic virus by alkylation of nucleophilic residues with alkylsulfonium salts and subsequently performing CuAAC with a nonfluorescent azidocoumarin derivative allowed monitoring of the CuAAC reaction by time-dependent emission spectroscopy [111]. In a search for biologically relevant GlcNAc proteins, MCF-7 cells were cultured in the presence of an azido analog of GlcNAc, then lysed and the cell contents allowed to undergo CuAAC with an alkylated biotin probe. Streptavidin-affinity chromatography enabled the isolation of a number of new *O*-GlcNAc glycosylated proteins [112]. In a modified approach, α-crystallin, an *O*-GlcNAc glycosylated protein, was post-translationally elongated with a UDP-azidogalactose substrate by an engineered β-1,4-galactosyltransferase enzyme [113]. CuAAC with fluorescent tetramethyl-6-carboxyrhodamine alkyne and enrichment of the peptides allowed direct in-gel fluorescence detection after one- and two-dimensional electrophoresis. Using a



Scheme 11.23 One-pot consecutive NCL plus CuAAC [114].

similar technique, cellular imaging of *O*-GlcNAc proteins in HeLa cells or cortical neurons was made possible after CuAAC with a biotin–alkyne probe and incubation with a streptavidin–AlexaFluor 488 conjugate. This nonmetabolic strategy can be applied to all cell types and tissues, and provides near quantitative labeling without apparent perturbation of signaling pathways [113].

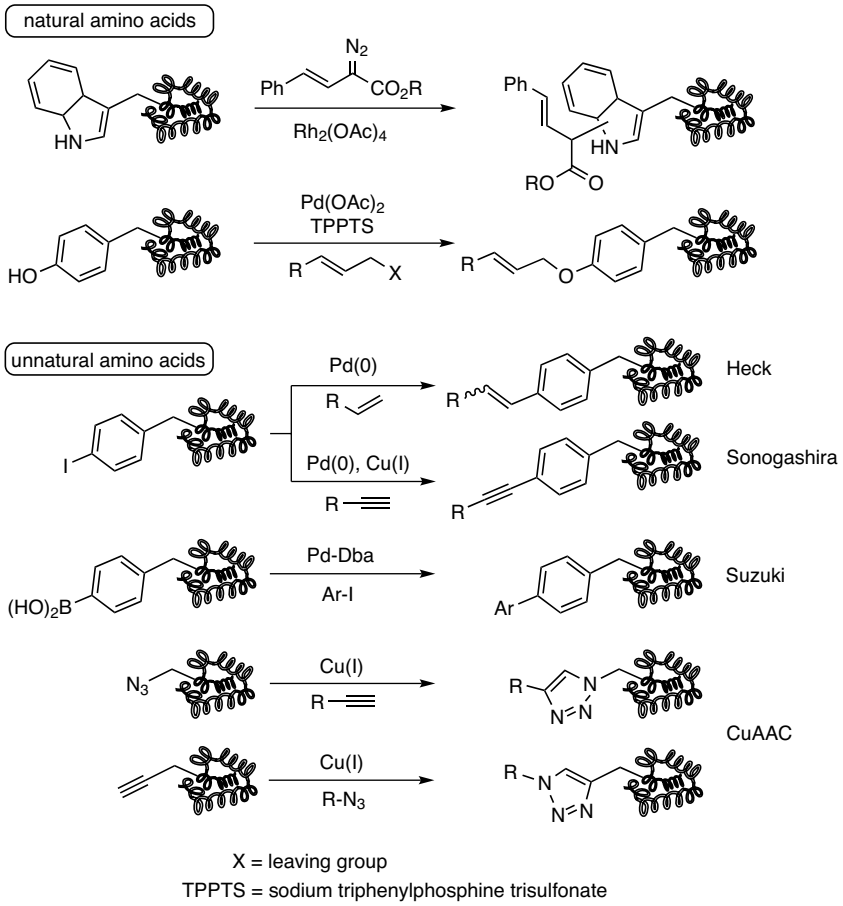
CuAAC and native chemical ligation (NCL) have been used in an efficient one-pot procedure using unprotected peptide fragments (Scheme 11.23) [114]. Ligation of peptide-thioester and *N*-terminal cysteine peptides in a buffer solution in the presence of tris(2-carboxyethyl)phosphine (TCEP) and 4-mercaptophenylacetic acid overnight afforded a 23-amino-acid residue peptide containing propargylglycine in positions 9 and 19. Subsequent addition of $CuSO_4$ (reduced to Cu(I) by TCEP) and unprotected GalNAc-azide to the solution enabled the click reaction, which was completed after 6 h. All 20 genetically encoded amino acids and the terminal cysteine, the peptide thioester, and acetamidomethyl protection of cysteine were compatible with the reaction conditions used [114].

11.5 Cross-Metathesis

Reactions catalyzed by transition metals are powerful tools for creating carbon–carbon and carbon–heteroatom bonds in the synthesis of small molecules. In recent years an increasing number of accounts have been published describing the use of transition metals to modify peptides and proteins (Scheme 11.24).

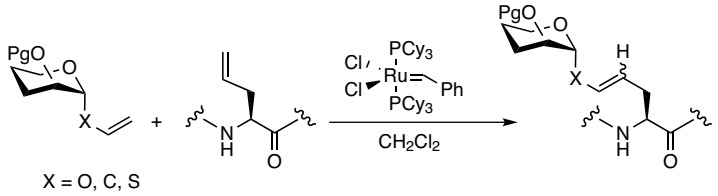
The most prominent examples are the CuAAC (see Section 11.4) and olefin metathesis. Like the CuAAC, olefin metathesis is highly chemoselective and efficient, taking place in aqueous media at ambient temperature, near-neutral pH, and at low concentration – properties extremely suitable for reactions with proteins. This section summarizes the application of olefin metathesis to the synthesis of neoglycopeptides and neoglycoproteins. An excellent review by Davis *et al.* [115] provides a more detailed discussion of the modification of peptides by cross-metathesis.

O-Allyl glycosides have been used as building blocks in olefin metathesis with even higher efficiency than *C*-allyl glycosides (Scheme 11.25). The corresponding saturated *O*- and *C*-glycosides are then accessible by hydrogenation.

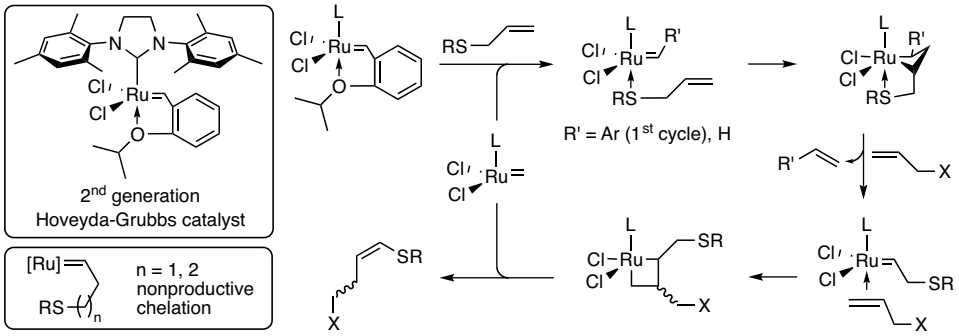


Scheme 11.24 Protein modification by transition metals [115].

Danishefsky used *O*-glycoside-containing carbohydrate epitopes synthesized by a cross-metathesis/hydrogenation sequence in the production of synthetic vaccines (see Section 11.6 and Scheme 11.25). In the quest for conditions that facilitated sustainable chemistry, water-soluble (pre)catalysts were developed by incorporation of poly(ethylene glycol) or quaternary ammonium groups into the catalyst ligands [116]. Water-insoluble catalysts, on the other hand, can be used in combination with cosolvents [117]. The incorporation of homoallylglycine (Hag) into proteins is well established, but no product formation could be observed when attempting cross-metathesis. Allyl sulfides, on the other hand, were found to be very reactive substrates and even enhanced the reactivity of the catalyst. Mechanistically, this was explained by the rapid formation of a reactive alkylidine species from the second-generation Hoveyda–Grubbs catalyst, owing to the preference for ruthenium to coordinate to sulfur rather than oxygen (Scheme 11.26) [61].



Scheme 11.25 Synthesis of neoglycosides via cross-metathesis.



Scheme 11.26 Sulfur-assisted cross-metathesis [61].

Introduction of allyl sulfides into proteins is possible using several approaches. One involves the conjugate addition of allyl thiol to dehydroalanine, which in turn is accessible by oxidative elimination of alkylcysteine [58] or phenylselenocysteine [59] or by use of MSH [60] (Scheme 11.12). To avoid epimerization, direct alkylation of cysteine [63] (Scheme 11.13) or desulfurization of disulfides is preferable (see Section 11.3.2). Initial attempts to perform cross-metathesis on *S*-allylcysteine-containing proteins resulted in the formation of a possible metalloprotein species. However, addition of $MgCl_2$ as a mild Lewis acid successfully prevented nonproductive chelation (Scheme 11.26) and allowed efficient cross-metathesis with allyl alcohols and allyl glycosides [61].

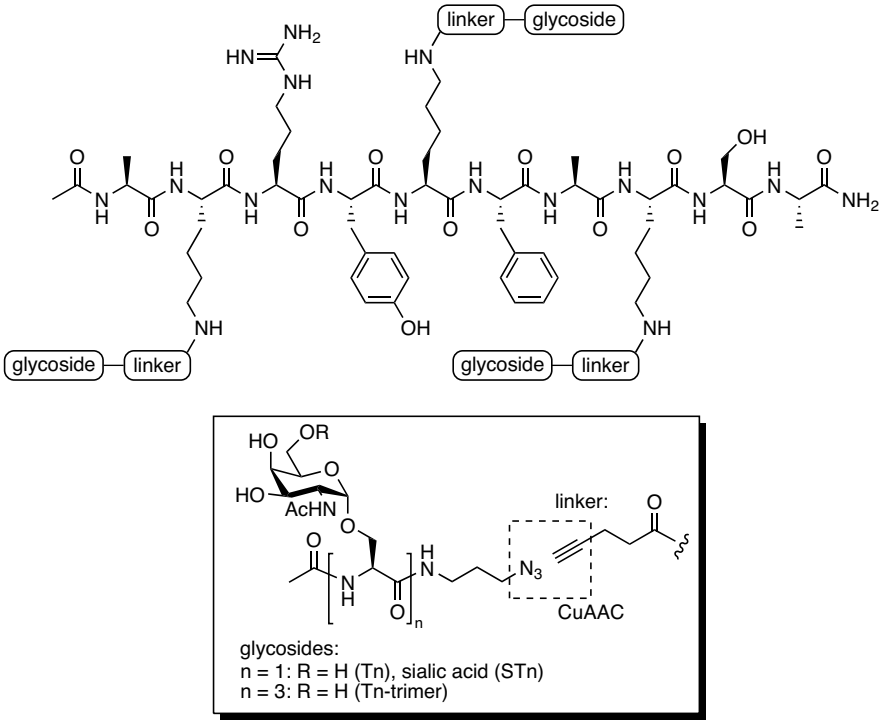
As incorporation of *S*-allylcysteine (Sac) by recombinant techniques results in only a low level of modification [61], reassignment of a UAG amber nonsense codon could provide an alternative, efficient method.

11.6
Application of Neoglycopeptides as Synthetic Vaccines

In recent years impressive progress has been made in the design of synthetic immunostimulating vaccines [118] to combat suppression by tumor cells of the immune response towards their cell surface antigens [4]. The groups of Boons [119], Danishefsky [11, 120], Kunz [121, 122] and Schmidt [123] have made notable

advances in this area. Mucin glycopeptides have become an important target for the development of immunotherapy against cancer [124], especially transmembrane mucin MUC1 [125]. The core structure of these high-molecular-weight and heavily *O*-glycosylated proteins consists of a variable number of tandem repeats rich in serine, threonine, and proline. In epithelial tumor cells MUC1 is overexpressed in a less glycosylated form, bearing short saccharide side-chains because of a downregulation of certain glycosyltransferases. Incomplete glycosylation renders the peptide backbone accessible to the immune system; thus, it is both saccharide and peptide components that contribute to immunogenic tumor-associated epitopes [125].

Neoglycosides have found wide application in the synthesis of vaccine constructs due to a greater hydrolytic stability than their naturally occurring counterparts [120]. Novel linkers are used for the installation of carbohydrates on the peptide backbone and to link the vaccine constructs to carrier proteins. Danishefsky's first-generation vaccines comprised a single carbohydrate antigen attached to an immunogenic carrier protein such as keyhole limpet hemocyanin (KLH) via reductive amination. Publications reporting more elaborate constructs with multiple repeats of carbohydrate epitopes followed using click chemistry and nanosized copper in phosphate-buffered saline buffer for the conjugation step (Scheme 11.27) [126].

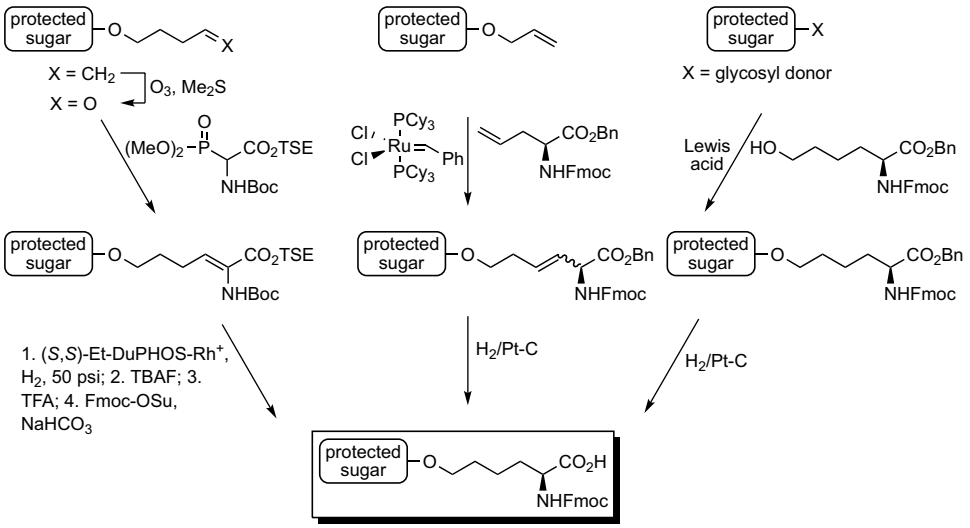


Scheme 11.27 CuAAC applied to the synthesis of vaccine constructs [126].

For example, a decapeptide backbone was prepared bearing three acetylenes, made by amidation of lysine side-chains with activated pentynoic acid. This was then reacted with azido-functionalized glycosyl amino acid displaying Tn or sialyl-Tn antigen to give the necessary multi-carbohydrate construct [126].

Novel vaccine constructs consist of different carbohydrate antigens such as Globo-H, Le^y, GM_2, sialyl-Tn, TF, and Tn with the multiantigenicity reflecting the developing heterogeneity of the tumor cell surface over its lifetime [11, 127, 128]. Administration of such multiantigenic vaccines is advantageous as a lower level of carrier protein is required and the need to validate each component of the vaccine is avoided [11]. Danishefsky *et al*. exemplified three distinct synthetic routes for preparing neoglycosides (Scheme 11.28). First, ozonolysis of an olefinated sugar followed by Horner–Emmons reaction yielded the dehydroamino acid, which was subsequently reduced enantioselectively. The second entailed cross-metathesis between an alkenyl-functionalized saccharide and Fmoc-L-allylglycine benzyl ester using the first-generation Hoveyda–Grubbs catalyst with subsequent hydrogenation. The third method involved introduction of the amino acid component by coupling hydroxynorleucine directly using glycal epoxide or trichloroacetimidate donors in the presence of a Lewis acid [11].

With the neoglycosides in hand, homogeneous penta- and hexavalent vaccine constructs with varying antigen composition and spacer length were assembled via peptide bond formation and covalent linkage to KLH. Enzyme-linked immunosorbent assays and fluorescence-activated cell sorter assays were conducted in mice, and these preclinical evaluations revealed that the individual immunological properties of the antigens were preserved in the assembled vaccine constructs [11]. Le^y was the least immunogenic of the carbohydrate antigens associated with prostate and breast



Scheme 11.28 Danishefsky's glycosyl amino acid syntheses [11].

cancer, hence it was replaced by GM_2 [127]. Optimization of conjugation conditions raised the efficiency from 228 to 505 incorporated copies of glycopeptide per KLH [127]. In further studies a tandem repeat unit from the MUC1 peptide was coupled to the multiantigenic domain via a diaminopropyl spacer to initiate production of antigens through the B cell epitope and to activate T cells through the immunogenic peptide fragment. In this way both epitopes were exposed to a maximum extent on the surface of the carrier protein (Figure 11.4) [128].

MUC5AC-derived peptide fragments and the carbohydrate antigen globotriaosyl ceramide Gb_3, both found on ovarian tumor cell surfaces, were used by Danishefsky *et al.* to synthesize a vaccine construct with a cluster of Gb_3–MUC5AC units [120].

The concept of introducing both the fucosyl GM_1 carbohydrate as well as major histocompatibility complex type II binding peptide to the carrier protein was used by Danishefsky for the synthesis of a vaccine construct for the treatment of small-cell lung cancer (Figure 11.5) [129]. Based on previous work [130], cross-metathesis between pentenyl-functionalized hexasaccharide and an allylglycine derivative, followed by hydrogenation afforded the glycoside that was added to the N-terminus of the requisite peptide fragment during SPPS. Deprotection of the C-terminus followed by introduction of the 2-sulfhydrylacetate linker enabled conjugation to maleimide-activated KLH, providing the vaccine construct [129].

Bundel *et al.* [131] synthesized a nonimmunogenic hetero-bifunctional linker based on a water-soluble triethylene glycol core with one end activated as NHS-carboxylate, and the other consisting of an acrylate moiety. The target antigens were

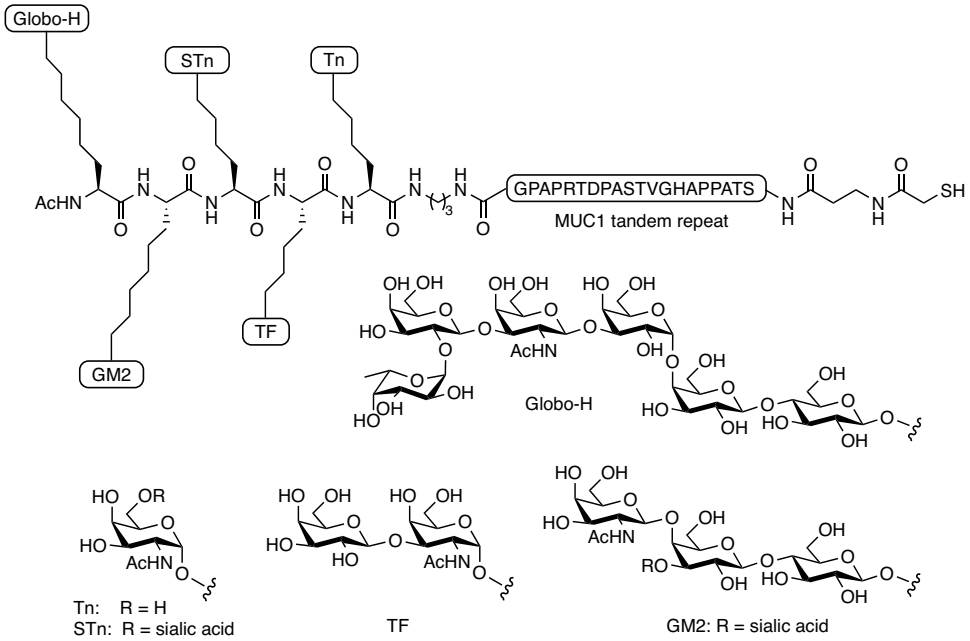


Figure 11.4 Pentavalent vaccine construct [128].

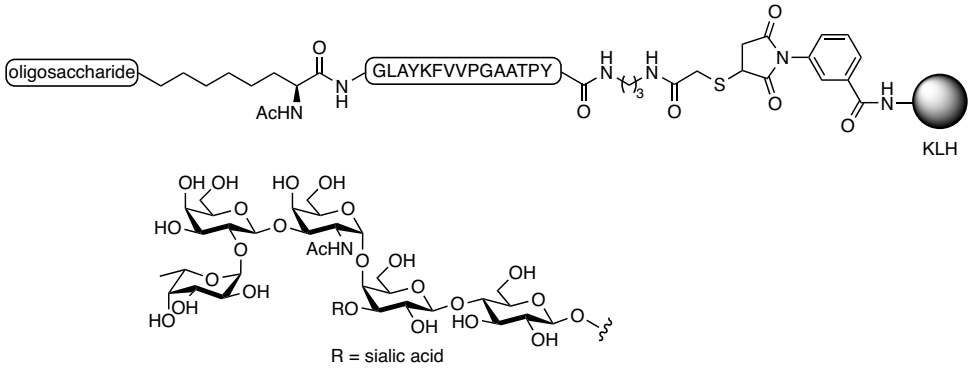


Figure 11.5 Synthetic vaccine against small-cell lung cancer [129].

equipped with an amino-functionalized tether and subsequently conjugated to the NHS-carboxylate of the linker in aqueous borate buffer at pH 8.1 to afford a stable building block for further vaccine construction. Michael addition of the appropriate thiol-modified peptide epitope or the carrier protein to the oligosaccharide acrylate was performed in Tris·HCl buffer at pH 8.9.

The group of Dumy and BenMohamed [132, 133] used the synthetic versatility of RAFT cores to develop a glycolipopeptide cancer vaccine composed of an oxime-ligated Tn antigen cluster, a T helper and cytotoxic T lymphocyte epitope peptide, and an adjuvant lipid moiety. After introduction of a cysteine activated with *S*-3-nitro-2-pyridinesulfenyl (Npys) at the free lysine side-chain, the Tn cluster, biomolecules bearing a free thiol were conjugated by disulfide bond formation.

Studies on modified glucagon-like peptide-1 (GLP-1), a potential type II diabetes treatment, have been performed by Ueda *et al.* [134] to assess binding affinities, proteolytic stability, and blood glucose-lowering ability. GLP-1 analogs were prepared by chemoenzymatic carbohydrate elongation using glycosyltransferases and by alkylation of a cysteine residue with *N*-iodoacetyl glycosylamines. The di- and triglycosylated sialyl-LacNAc analogs demonstrated significantly improved enzymatic stability and prolonged blood glucose-lowering activity.

11.7
Enzymatic, Molecular, and Cell Biological Techniques

As outlined in previous sections, the chemical synthesis of neoglycopeptides and neoglycoproteins often goes hand in hand with molecular biological and biotechnological techniques that are also used for the synthesis of glycopeptides and glycoproteins. This section provides a brief description of some enzymatic, molecular, and cell biological techniques that have been used for glycoprotein synthesis together with some recent publications. The reader is referred to textbooks and comprehensive reviews that cover this area in more detail [2, 4, 5, 106, 135, 136].

11.7.1.1 Enzymatic Glycoprotein Synthesis

Enzymes can be used to extend and remodel glycans on proteins or to perform glycosylation and ligation in an efficient and specific manner [5]. Although enzymatic methods provide ready access to complex glycosylation patterns, stringent substrate specificity and enzyme availability are limiting factors for general applications. Furthermore, in the case of glycan extension and glycoprotein remodeling the initial protein–sugar bond has to be generated beforehand. Conversion of heterogeneous glycans to a single residue by enzymatic degradation and subsequent transglycosylation allows glycoprotein remodeling, although the transglycosylation step often suffers from low yield.

Usui *et al.* [137, 138] designed artificial sialoglycopolypeptides as inhibitors of avian and human influenza virus infection. Their syntheses entailed consecutive enzymatic additions of GlcNAc and galactose residues with a differing number of sugar units built on an alkyl chain spacer linking them to a γ-polyglutamic acid backbone.

Purified chitinase fractions Chi 100 have been used to transfer oxazoline-functionalized di- and trisaccharide donors on to GlcNAc displayed on bovine ribonuclease B, with yields of 40 and 20%, respectively [139]. However, separation of products from starting materials by reversed-phase high-performance liquid chromatography proved difficult and the search continues for more efficient enzymes.

6-Deoxy-6-aminohexoses have been ligated directly to peptides using transpeptidase sortase [140]. The LPXTG pentapeptide near the C-terminus of the target protein is recognized and cleaved by sortase at the threonine–glycine peptide bond, and a new amide bond then formed between threonine and a suitable glycine-functionalized substrate. This method was used to specifically conjugate a 6-aminoglycoside antibiotic to biologically relevant peptide sequences [140]. This technique was also used for ligation of a short peptide containing LPETG and a hexahistidine tag to glycine-modified glycosylphosphatidylinositol (GPI) analogs to show proof of concept for the synthesis of anchored GPI glycoproteins [141].

11.7.2 Molecular and Cell Biological Techniques

Complex glycoproteins that contain unnatural structural elements have been generated using *in vivo* techniques. A large number of enzymatic pathways play a role in the expression of a defined glycoprotein in a host, which opens up multiple possibilities to influence glycosylation patterns: introduction or removal of genes that express glycosyltransferases will result in different glycosylations, as would expression in another cell type or species.

To increase the proportion of a particular sugar within a glycoprotein, additional glycosyltransferase genes may be introduced into the organism to augment incorporation of that sugar. Indeed, the entire glycosylation pathway in yeast has now been re-engineered by knocking out four yeast-specific glycosylation genes and introducing 14 other genes in order to generate customized glycoproteins [142, 143].

Using the cells own biosynthetic machinery, unnatural compounds displaying useful chemical handles (such as ketones and azide-derived sugars) have been

incorporated into cell surface glycoproteins. Subsequent labeling using these handles enabled visualization of glycoprotein trafficking processes on the cell surface [91, 144]. Similarly, incorporation of an azido tag into the flagella of pathogenic bacteria has been performed on *Campylobacter jejuni* cells through cultivation in a motility agar containing an azido-functionalized precursor [145]. Peptides attached at the C-terminus of a leader peptide for recognition by lacticin synthetase mutants have been generated by expression in *E. coli* [146]. After incubation with lacticin synthetases, serine and threonine residues up to 30 amino acids distant from the leader peptide have been dehydrated. The resulting dehydroalanines were subsequently glycosylated by addition of thio-sugars [146]. Expressed protein ligation has been used to introduce cysteine-containing molecules at the terminus of bacterially expressed proteins.

Site-directed mutagenesis is now considered a classical tool in genetic engineering for the introduction of a natural amino acid at a certain position in the protein, which is then available for subsequent site-selective modification. Nonproteinogenic amino acids, too, can be incorporated at a preselected site using codon suppression technology. An altered tRNA synthetase can be made to read a nonsense codon and incorporate the amino acid of choice. Cropp *et al.* [147] developed a protocol for the design of aminoacyl-tRNA synthetases in *E. coli* that selectively directs incorporation of unnatural amino acids in response to the amber codon in yeast cells. Site-specific incorporation of unnatural amino acids into mammalian cell proteins has been made possible after transfer of the aminoacyl-tRNA synthetases from yeast into mammalian cells [148, 149]. The genetically introduced natural and non-natural amino acid residues can be post-translationally modified using the methods discussed in Sections 11.1–11.6.

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Part III
Amino Acids in Combinatorial Synthesis

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12
Combinatorial/Library Peptide Synthesis

Michal Lebl

12.1
Introduction

Combinatorial chemistry is a phenomenon that appeared swiftly in the 1990s and in retrospective it is surprising that it took so long to bring it to the arsenal of modern chemists [1] as it seems to be self-evident that compounds can be synthesized in a parallel fashion so much faster and cheaper than at one-at-a-time pace. First came the parallel synthesis of peptides, pioneered by Geysen *et al*. [2] and Houghten [3], enabled by the concept of solid-phase chemistry invented by Merrifield [4–6] and Letsinger and Kornet [7]. Peptides were an obvious choice for the application of parallel synthesis, since their chemistry was well developed, and did not need long reaction times, inert atmosphere, or increased temperature and pressure. Even though solid-phase chemistry was also proposed to be applicable for the synthesis of other types of organic molecules, these early attempts were not taken seriously and were considered as more of a curiosity than mainstream technological progress [8–10]. Leznoff was one of the first to recognize the potential of solid-phase organic reactions and predicted that such reactions would be easy to automate [11]. Today, there are many examples of syntheses that could be achieved only by use of a solid support or in which a solid support acts as convenient "pseudo-diluent" allowing reactions like cyclizations being performed in manageable volumes. The fact that most chemical transformations can be performed in solid phase, sometimes actually more effectively than in solution [12], is now generally accepted and thus it seems quite strange that solid-phase synthesis met such resistance at the very beginning [13–16]. Even in the peptide arena, a number of established laboratories resisted the transition to solid-phase technology for decades. Only the successes of numerous applications in both research laboratories and industrial processes, at scales ranging from milligrams to kilograms and more (tons of the pharmaceutically relevant peptide Fuzeon® were produced using solid-phase synthesis by Roche [17]), convinced the skeptics that solid phase is the technology of the future.

Solid-phase chemistry has been extensively reviewed and only some of most relevant articles can be mentioned here [18]. Solid-phase peptide synthesis was

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covered in detail in the most authoritative book – *Houben–Weyl Methods of Organic Chemistry* [19]. More personal stories of the development of various types of solid-phase materials are available in the comprehensive assembly of recollections of scientists developing these supports [20, 21].

The term combinatorial, or library, synthesis was coined only after two seminal papers were published in 1991. The fact that the time was right for the arrival of a new technique was evidenced by the appearance of these two papers in the same issue of *Nature*. Houghten *et al.*'s paper [22] described the generation of peptide mixtures, which after biological testing could be deconvoluted to identify the active component of the mixture. Lam *et al.*'s paper [23] presented the generation of millions of beads each containing only one type of peptide. This mixture of beads could be tested for binding to the biomolecule (receptor, antibody, enzyme), and beads with ligands showing binding could be isolated and the structure of the peptide determined by sequencing. Both of these papers used the technique called "split and mix," or "divide, couple, and recombine," which was actually published earlier by Furka *et al.* [24, 25]. Pioneers of combinatorial chemistry were asked later to write their recollections of the history of discovery of their techniques, and their memories were collected [1] in the inaugural article of the new journal dedicated to combinatorial chemistry, appropriately named the *Journal of Combinatorial Chemistry*. Combinatorial synthesis developed far beyond peptides and it is now very difficult to find a class of molecules that is not subjected to a certain degree of combinatorial technology. This chapter is dedicated to chemically synthesized peptide libraries (biological libraries are covered in another chapter), but most of the principles discussed are applicable to any chemical entities. Earlier reviews and discussions of synthetic concepts can be found elsewhere [13–16, 26–56].

12.2 High-Throughput Synthesis of Peptides

12.2.1 Parallel Peptide Synthesis

The idea that the effectiveness of peptide synthesis could be enhanced by automation [57, 58] or by synthesizing more than one peptide at a time was around since the introduction of solid-phase synthesis, but it required prepared minds to make a quantum leap from just several parallel reactions to several hundred reactions. Three papers can be traced to the beginning of massively parallel synthetic approaches – Frank's synthesis on paper circles [59], Houghten's technique of tea-bag synthesis [3], and Geysen's pin synthesis [60].

Ronald Frank used marked pieces of cellulose paper as a substrate for DNA synthesis [59] (later extended into synthesis of peptides [61]), and showed that separation of segments requiring the coupling of the same building block into the same pool (reagent vessel) and resorting the segments before the next step of the synthesis increases the throughput of the synthesis substantially.

Houghten's approach [3] is based on the same concept of compartmentalizing the synthetic substrate (polystyrene resin beads). Beads are contained in polypropylene mesh bags, which could be individually labeled and would survive the synthetic sequence without losing the encapsulated solid carrier. This so-called tea-bag synthetic technology [62, 63] was the basis of formation of a company Multiple Peptide Synthesis and was used for the synthesis of peptides in scales reaching from milligrams to multigrams. Automation of the tea-bag technology proved difficult in the version requiring multiple resorting of hundreds of polypropylene containers. In the case that only a limited number of tea-bags are used, an automated synthesizer utilizing centrifugation for liquid removal was built [64, 65].

Geysen's pin synthesis utilizes plastic pins covered with a functionalized polystyrene layer arrayed in a grid following the pattern of 96-well microtiter plates. Simply dipping these pins into the wells with appropriate activated building blocks (amino acids) allows for simultaneous parallel synthesis. The material, shape, and functionalization of the pins went through multiple generations [66–81]. The optimized version of the multipin technology uses SynPhase™ "lanterns" or "crowns" – polymeric barrels in a shape of miniature Chinese lanterns or crowns [82, 83] (Figure 12.1). The lanterns are constructed from inert polymer core material in a barrel shape, onto which the functionalized "synthetic" layer is grafted. This geometry allows for the attachment of individual lanterns (one or more per pin) to the pin holders for individualized synthetic steps or combining all lanterns for common synthetic steps. Lanterns can be color-coded or labeled by attachment of radiofrequency transmitters. Supplies for multipin synthesis are available from the company Mimotopes (www.mimotopes.com). For a compilation of papers using SynPhase products, see http://www.mimotopes.com/files/editor_upload/File/CombinatorialChemistry/SynPhase_Publications.pdf.

A convenient and general method of peptide cleavage from the solid support is the use of gaseous ammonia. Since the ammonia treatment leaves the polymer support dry upon cleavage, further extraction of the peptide is necessary. This allows for the cleavage to be performed in parallel/batch fashion and to sort the polymer (lanterns,

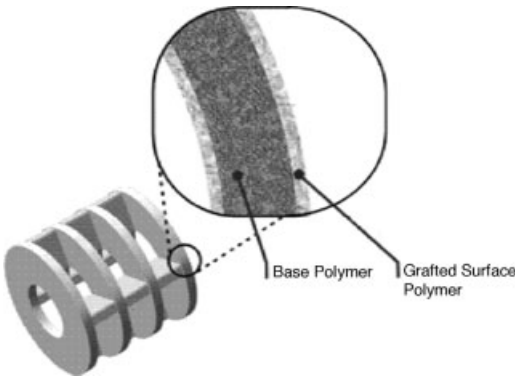


Figure 12.1 Structure of the SynPhase lantern.

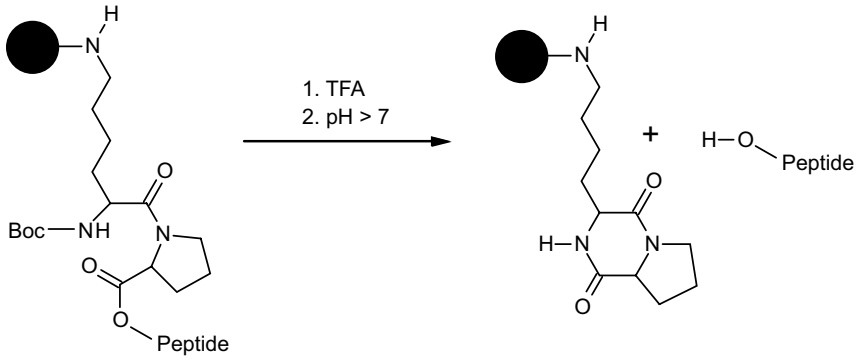


Figure 12.2 Scheme of the diketopiperazine linker release [84].

sheets, beads) by identity of the peptide later in the dry state followed by the peptide's release into a plate/vial by addition of a solvent. This ammonia release and dry-sorting technique was originally utilized in pin synthesis using the diketopiperazine linker [84] (Figure 12.2), but works well even with a simple benzyl ester linkage.

A number of papers were dedicated to the parallel synthesis in specialized synthetic blocks, where activated amino acids are delivered into individual locations by pipetting (manual or automated), and common reagents are delivered by multichannel liquid distributors and removed by filtration. Reactors in these blocks are equipped with a frit at the bottom, and utilize various techniques for simultaneous closing and opening of the exit ports (overpressure, individual plugs, membranes, squeezed tubing, etc.). These techniques and available instruments were extensively reviewed by us in 2005 [51], and therefore only new instruments and some nonmainstream techniques will be discussed here. The list of available instrumentation is kept updated at http://www.peptideresource.com/synthesizer.html.

Thuramed (a division of Creosalus; thuramed.com) introduced an elegant solution to the multiple peptide synthesizer. Their machine, Tetras™, is capable of 106 parallel syntheses, where each reactor can be operating at a different scale and using a different synthetic protocol, thus making the claim of being the only asynchronous multiple synthesizer true. Reactor cartridges are placed on the perimeter of the rotor allowing positioning of each reactor under a dedicated nozzle delivering reagents or washing solvents. This design eliminates the danger of cross-contamination and wastage of reagents needed for flushing the common lines. Reagent nozzles can be supplied from a local vessel of very small volume, minimizing the need for using larger than necessary volumes of expensive components, or from the tanks of solvents placed in a dedicated off-machine location.

The availability of solvent-resistant 96-well or even 384-well microtiter filter plates allowed for techniques in which the reactor (filter plate) was disposable. However, for institutions and companies interested in the synthesis of tens of thousands of individual peptides, even the cost of these filter plates was carefully evaluated and more economical techniques using standard deep-well microtiter plates were conceived. One of these techniques uses "surface suction" for removal of the reagent

solutions [85, 86]. To be able to use this technique, the solid support suspended in the solution must sediment when the stirring (shaking, magnetic or mechanical stirring, bubbling) is stopped. When the array of wide-mouth suction tubes is slowly lowered towards the surface of the liquid, only the surface layer is taken away by suction and the tube can be lowered close to the level of sedimented solid support without disturbing it. A simple method for the uniform distribution of solid support into the wells of microtiter plates was described [87].

Tilted centrifugation is another technique for liquid removal using classical microtiter plates and sedimenting solid support [88, 89]. Again, the solid support must sediment at the end of the reaction prior to the solution removal. Synthetic plates are placed on the perimeter of the centrifuge and tilted slightly towards the center of rotation. Rotation creates a "pocket" of the given volume, depending on the distance from the center of rotation, speed of rotation, and the plate tilt, from which the solid support cannot escape. This technique can process multitudes of plates at the same time extremely fast and was a basis of several automated solid-phase synthesizers [51, 90, 91]. This technology was tested in the synthesis of tens of thousands peptides for finding the optimal coupling reagent in multiple synthesis (surprisingly, good old *N*,*N*′-diisopropylcarbodiimide (DIC) was found to be the best due to its stability in solution and performance *in situ*ations where the speed of condensation is not the most desired feature [90]) and replacement for regulated piperidine by 4-methylpiperidine [92]. A simple synthesizer based on the same principle was built to process 24 peptides in a plastic disposable rotor [93] (Figure 12.3).

Figure 12.3 Simple centrifugal DNA and peptide synthesizer [93].

Any technique that removes liquids in parallel, but delivers solutions in a series is bound to be less efficient than a technique with balanced solution addition and removal. An ideal process of synthesis performs all steps of the synthesis at the same time at the different sections of the synthetic substrate (machine), as shown, for example, in the synthesis utilizing a cotton strip as the solid support [94]. A high-throughput synthesizer was realized for parallel synthesis of 12 000 DNA sequences by a San Diego company (Illumina, Inc.), but can be easily imagined to be built for peptide synthesis. This synthesizer has 36 384-well filter plates arranged in a circular fashion, moving in 10-s increments under stations dedicated to perform one step of the synthesis (addition of building blocks, activation, washing, deprotection) so that each plate is in a different stage of the individual cycle of the synthesis at the same time. After one round of passage around the circle, one building block is attached to the growing polymer in all wells of the plate and the process continues with another cycle. Reagents are delivered from pressurized containers by actuation of an array of solenoid valves as they pass above the wells of the microtiter plate [95].

The parallel deprotection and cleavage of peptides from the solid support is as important as the parallel synthesis. Gaseous HF was found to be a convenient reagent for processing multiple reactors [96] or even microtiter plates [97, 98] simultaneously. The ultimate convenience of processing the solid supported synthetic product is treatment of the silica gel support with HF. Silica gel dissolves into SiF_4 and evaporates, leaving behind only the deprotected peptide [99, 100].

12.2.2 Directed Sorting

The previously mentioned tea-bag technology or paper disk synthesis are examples of synthetic methodologies utilizing directed sorting – reorganization of the synthetic compartments in such a way that in each step of the synthesis the compartments requiring the attachment of the same building block (amino acid) can be combined and the reaction can be performed in the same reaction vessel. This requires either some means of labeling the individual container, or keeping the containers in an order, so that at any moment the identity of the individual container can be established.

Labeling was achieved by simply writing the code on the tea-bag [3], including a radiofrequency tag [101, 102], or a one- or two-dimensional barcode. Radiofrequency tags or barcodes allow for automating the sorting process [103, 104]. Exciting technologies can be designed using barcodes miniaturized to a level that each particle of the solid support has its own "barcode" [105–109]; unfortunately, at this level of miniaturization, the cost of the individual particles and the need for specialized equipment used for their reading may be prohibitive for widespread application.

Two-dimensional barcode labels (MiniKan™, MicroKan™, or NanoKan™) became commercially available from the company IRORI (later Discovery Partners International) and, together with the sorter system capable of sorting tens of thousands of cans in a reasonable timeframe, can make synthesis of multimilligram quantities of

tens of thousands of peptides quite feasible [110, 111]. However, alternative (and less-expensive) technologies are available for peptide synthesis and NanoKans are used in a majority of cases for parallel synthesis of small molecules. A very impressive automation was built around these solid-phase carriers [112].

Krchňák *et al.* used plastic syringes equipped with a frit for resin compartmentalization [113–116]. This manual technique was later automated and a robot handling plastic syringes was built [117]. Manual handling of multiple syringes can be also simplified by using so called Domino Blocks [118], available commercially (www.torviq.com).

A promising way of compartmentalization of the solid support is the sintering of its particles in the mixture with an inert polymer into so called "plugs" [119]. These plugs were commercially available from Polymer Laboratories (now part of Agilent Technologies).

Compartmentalization similar to tea-bags was also achieved in porous wafers made from a Teflon® ring covered on both sides by a porous Teflon membrane to form a cylinder-shaped permeable container [120]. Wafers became the basis for building a solid-phase DNA synthesizer and due to their proprietary character never made it to mainstream peptide synthesis, even though they seem to be an ideal tool for multiple synthesis. Krchňák *et al.* later designed "resin capsules" constructed from a polypropylene ring sealed with a polyetheretherketone membrane [121]. These capsules can be easily made using common tools in the chemical laboratory and sized appropriately for the synthetic vessels (plastic syringes), thus optimizing the use of reagents (one disadvantage of tea-bags is the excessive use of reagent solutions).

Convenient containers/particles for directed sorting are SynPhase lanterns mentioned earlier. They can be equipped with radiofrequency tags, or they can be arranged on pins or strings, putting them in ordered arrays. If lanterns are transferred according to a defined algorithm from one array to another without any errors, they thus make assemblies ("necklaces") that could then be used for coupling the next amino acid. The identity of each particle (lantern) can be established at the end of the synthesis by the position on a particular necklace (pin). Techniques utilizing these concepts were pursued by Krchňák *et al.* [122, 123] and later also by Furka *et al.* [124–126]. The ENCORE system [122, 127], originally developed for organic combinatorial synthesis, but easily applicable for multiple peptide synthesis, utilizes lanterns attached to pins and dedicated tools to arrange them efficiently into particular reactors.

Solid carriers capable of convenient compartmentalization and therefore parallel multiple synthesis include functionalized polymeric sheets and disks [128, 129], membranes [130], paper, cellulose [61, 131], cotton [132, 133], or functionalized acrylate-grafted polypropylene fabric [134]. Some of them will be mentioned later since they act as the support of choice for array syntheses. The case close to the author's heart is the case of multiple synthesis on cotton. Cotton is the most inexpensive solid support that, due to its polysaccharide structure, allows efficient synthesis of difficult sequences [132, 135], can be easily compartmentalized, and automated multiple [64, 65] or continuous [94] synthesizers can be designed for its use. Solid-phase synthesis on planar supports was reviewed earlier [136].

On the borderline between random library synthesis and synthesis using directed sorting is the strategy using dividable material (fabric, paper, sheets), where the material is divided after each coupling step utilizing different building blocks. This technique does not end up with a coded individual piece of the solid support and positively responding pieces must still be decoded, but it guarantees that all components of the library are synthesized and none of them is synthesized in more than one copy [137, 138].

12.3 Synthesis of Peptide Arrays

For a multitude of applications the availability of individual peptides for testing in solutions is not necessary. For example, the evaluation of binding to a biologically relevant target can be performed with the peptide still immobilized on the support. Realization of high-throughput screening can be then easily implemented on "chips" containing hundreds to hundreds of thousands individual peptides on their surface [139–143].

The first arrays of peptides were prepared on planar cellulose paper sheets by Ronald Frank [144]. The activated paper was spotted (the reason for calling this technique SPOT synthesis) with solutions of Fmoc-protected activated amino acids. After attachment of the first building block, the remaining free functional groups on the surface were acetylated (capped), thus creating an array of available synthetic locations. The Fmoc group was removed by immersing the paper sheets in a piperidine solution and after washing the free amino group-containing spots were revealed by application of bromophenol blue solution [145, 146]. The synthesis was continued by repeated cycles of delivering activated amino acids to appropriate locations, incubation, and deprotection by the immersion into the deprotecting solution. The building block delivery process, originally manual pipetting, was automated to eliminate the potential for operator error and became the flagship technology for the company Jerini Peptide Technologies (www.jpt.com). SPOT synthesis and its applications were reviewed in detail elsewhere [147–159].

The pioneering approach to the synthesis of peptides on chips was published by Fodor [160–167] and became the basis for the formation of Affymax (www.affymax.com). Later the company (in its Affymetrix incarnation; www.affymetrix.com) concentrated on production of DNA chips for genotyping and gene profiling [168, 169].

Fodor's technology [160], based on photolithography, is illustrated in Figure 12.4. The surface of the synthetic substrate protected by a photocleavable protecting group is deprotected in defined regions by light irradiation through the mask. The chip is then flooded with the activated amino acid, which couples on the deprotected regions of the surface. After the completed coupling, the activated amino acid is washed away, the next set of regions is deprotected by light, and the next amino acid is coupled to the deprotected areas. This process is repeated until all amino acids needed for the first coupling are applied. Then the amino acids for the second cycle of peptide synthesis

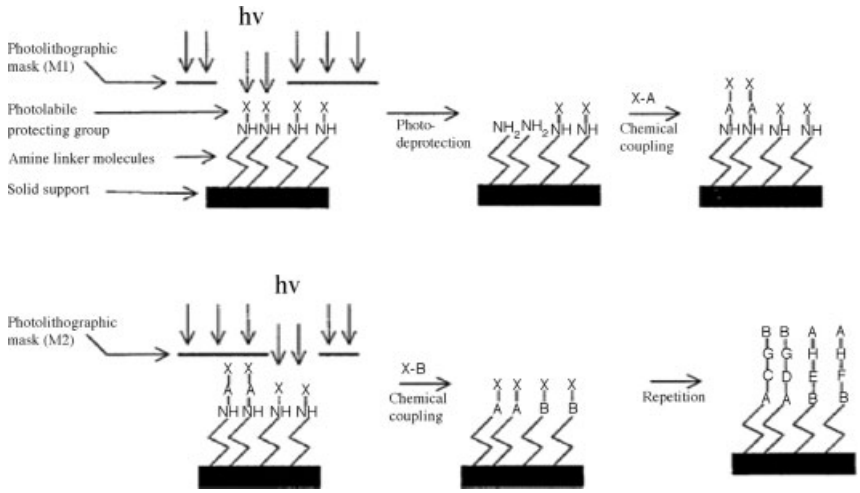


Figure 12.4 Scheme of photolithographic peptide synthesis. Irradiation of the surface covered with the growing peptides protected on the N-terminus by photocleavable protecting groups through the mask creates a patterned surface with deprotected locations onto which the next amino acid can be coupled. A mask is then repositioned to allow deprotection of the next set of locations.

are attached in the same way and the process is repeated until the peptides of desired sequence are built on all chip locations. The obvious disadvantage of this process is the multitude of repetitions necessary for each step of the synthesis and the necessity to apply activated solutions across the whole surface, even though only a fraction of it is actually engaged in the reaction extending the peptide chain. Each step of the process also requires a dedicated photo mask. The mask issue was later removed by the application of the actuated micromirror chip for selective irradiation of the synthetic chip surface [170]. This technology led to the formation of yet another company, Nimblegen (www.nimblegen.com), which is, however, totally dedicated to the synthesis of DNA arrays.

Their Maskless Array Synthesizer (MAS) technology instrument is comprised of a maskless light projector, a reaction chamber, and a fluidic unit. The Digital Micromirror Device (DMD), a solid-state array of miniature aluminum mirrors, is able to pattern 786 000 to 4.2 million individual pixels of light. These "virtual masks" reflect the desired pattern of UV light onto the microscope slide in the reaction chamber, which is coupled to the synthesizer. The UV light selectively cleaves a UV-labile protecting group at the precise location where the next building block will be coupled. A maskless photolithography peptide synthesizer was also reported recently by Korean scientists [171].

An interesting alternative to the approach described above is based on photolytic generation of an acid at a specific location of the chip. This *in situ* generated acid then deprotects the Boc-protected peptide fragment to allow the next coupling step [172–175]. Therefore, the site of the reaction is also addressed by light, but in

contrast to photosensitive protecting groups, conventional Boc chemistry building blocks can be used, making this approach cheaper. Since the Boc synthetic strategy is used, generation of a relatively strong acid is required. It is critical to ensure that the generated acid will not diffuse into the neighboring location and deprotect unwanted loci. This was achieved by using the polymeric coating which slowed the diffusion rate of the acid on a silicon-based microfluidics chip manufactured by Applied MEMS that contains either 4000 or 8000 chambers on an area of little more than 1 cm^2. Each chamber serves as an independent reaction vial to synthesize a specific type of oligonucleotide or peptide. In addition to linear peptides, photolithography was used, for example, for the synthesis of arrays of peptoids and cyclic peptides [176, 177].

A similar in concept is strategy employed by Maurer *et al.* [178], who generated deprotecting acid (for removal of the Boc group in peptide synthesis) by application of an electric current on an array of electrodes analogously to Egeland and Southern [179], who developed this technique for DNA array synthesis. An acid was generated by applying $+3$ V onto an array of platinum electrodes in a solution of diphenylhydrazine. Diffusion of the acid was eliminated by the fact that diphenylhydrazine is a weak base, which neutralized the acid everywhere besides the closest proximity to the active electrodes. Even though this technology was developed for peptide synthesis, it became the basis for DNA chip synthesis in the company Combimatrix [180].

All these technologies require repetition of each coupling step n times, where n is the number of amino acids needed for coupling in that particular step, and result in a very slow process.

A revolutionary approach to peptide arrays is being developed by the company PepPerPrint in Heidelberg, Germany [181]. They have developed glass slides modified with a multifunctional poly(ethylene glycol) (PEG)-based polymer. PEG films starting from self-assembled alkyl silane monolayers via monolayer peroxidation and subsequent graft polymerization of PEG methacrylate (PEGMA) were optimized for substitution and film thickness. The novel support material allows a versatile modification of the amino group surface density up to 40 nmol/cm^2. The polymer coating is stable to a wide range of chemical and thermal conditions, and prevents the glass surface from unspecific protein adsorption [182, 183].

Their first method of synthesis on microelectronic chips utilizes solid amino acid microparticles charged by friction and transferred to defined pixel electrodes onto the chip's surface, where they couple to a functional polymer coating simply upon melting.

The building blocks are distributed to a location on the glass substrate in the form of a specially formulated toner by electrostatic deposition analogously to printing by a "Xerox®" machine. This "printer," however, has 30 or more ink cartridges. Toner is created by encapsulating Fmoc-protected pentafluorophenylesters in diphenylformamide. The diphenylformamide is solid at room temperature and the micron-size beads of activated amino acids can be stored for months without losing activity. After distribution of amino acids to the locations on the slide, the "dust" particles

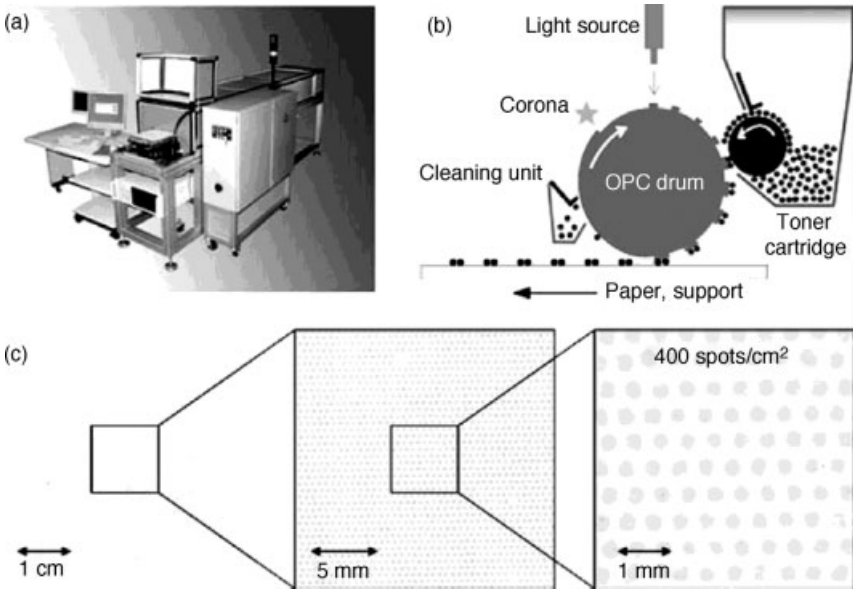


Figure 12.5 Laser printing-based peptide synthesizer. (a) Peptide laser printer with 20 different printing units aligned; the mounting for the support is visible at the front of the printer. (b) A light source (LED row) illuminates and thereby neutralizes selected areas of an organic photoconductor drum (OPC), which is first uniformly charged by a corona. Triboelectrically charged toner particles are transferred to these neutralized areas and from there by a strong electric field to a solid support. (c) Amino acid toner was printed by the peptide laser printer onto a glass slide derivatized with free amino groups. The active pentafluorophenyl esters embedded in the particles were released by heat, the residual material was washed away with DMF, and the remaining free amino groups were blocked with 10% acetic anhydride in DMF. Finally, the Fmoc protecting groups were removed with 20% piperidine in DMF and the newly introduced free amino groups were stained with 0.1% bromophenol blue in methanol. (Modified from [184].)

are melted at elevated temperature and the coupling of pentafluorophenyl ester proceeds. After coupling completion, the glass slide substrate is transferred to the compartment in which washing and deprotection is performed. By applying standard Fmoc chemistry, peptide array densities of up to 40 000 spots/cm^2 were prepared [185]. The scheme of the synthesizer is illustrated in Figure 12.5.

This method solves the problem of technologies using photo- or electro-deprotection (lithography) due to the monomer-by-monomer repeated consecutive coupling of 20 different amino acids, which adds up to an excessive number of coupling cycles. Synthesis based on electrically charged solid amino acid particles is more efficient. A laser printer or a chip addresses the different charged particles consecutively to a solid support, where, when completed, the whole layer of solid amino acid particles is melted at once. This allows coupling of all 20 different amino acids to the support in one single coupling reaction [181, 186–188].

12.4
Peptide Libraries

12.4.1
Synthesis of Peptide Mixtures

Furka realized that by the repetitive process of coupling, mixing, and recombination of the solid support resin one can create almost equimolar mixtures of peptides [24, 25, 189–191]. The same conclusion was reached independently by Houghten, and his group has shown that keeping portions of the resin support separated after the last two couplings generates mixtures differing by only two amino acids in their sequence, and allows one to test these mixtures in any biological test and define whether the defined positions play a significant part in the biological activity of those peptides [22, 27, 192–205]. In the next step, the second generation of peptide mixtures is generated, this time defining the next position of the sequence. Repetition of this process defines, by using simpler and simpler mixtures, the most active sequence from original mixtures. This technique was used for identification of antibody epitopes and epitope mapping, enzyme substrate and inhibitor discovery, receptor ligand elucidation, or identification of substances responsible for physiological effects in *in vivo* tests [206–208].

Instead of the split-and-mix technique for the generation of equimolar mixtures of peptides by coupling in "mixed positions," equikinetic mixtures of amino acids can be introduced [209] making the synthesis faster and more effective. In this method, relative reactivities of building blocks are determined first and then amino acids are mixed in such a ratio that speed of all possible extension reactions is the same (normalized). Example of ratios of amino acids used in coupling with DIC [210] and pentafluorophenyl esters [211] can be found in Table 12.1. This ratio has to be established empirically not just for different amino acids, but for different side-chain protecting groups as well. However, it was shown in a detailed study that since the equikinetic mixture cannot take into account the character of the amino acid to be extended, a significant portion of the sequence space can be under-represented in the synthesized library [212].

An alternative procedure to incorporate all amino acids in an equimolar ratio is based on coupling of a slightly subequivalent amount of a mixture containing the same amount of all amino acids, followed by the second coupling with an excess of the same mixture [213, 214]. The first coupling step guarantees that no amino acid is incorporated preferentially and the second step ensures completeness of the coupling.

Positional scanning libraries were later invented by Houghten's laboratory as an alternative synthetic and screening technique [215–222]. In this version of mixture screening, the libraries in which all positions are defined are synthesized and screened, and ligands deduced from the most active mixtures are synthesized and their activities tested. An illustrative (imaginary) example of deduction of the active sequence by this technique is given in Figure 12.6. Eighty tetrapeptide mixtures of 8000 peptides (composed of all 20 natural amino acids) were prepared with all 20

Table 12.1 Composition of amino acids mixture for coupling in equimolar quantities (isokinetic ratio).

Amino acid	Molar ratio	Amino acid	Molar ratio
Boc-Ala	1.18	Fmoc-Ala-OPfp	1.20
Boc-Arg(Tos)	2.26		
Boc-Asn	1.86	Fmoc-Asn-OPfp	5.05
Boc-Asp(OBzl)	1.22	Fmoc-Asp(OBut)-OPfp	1.00
Boc-Gln	1.85	Fmoc-Gln(Trt)-OPfp	1.66
Boc-Glu(OBzl)	1.26	Fmoc-Glu(OBut)-OPfp	1.35
Boc-Gly	1.00	Fmoc-Gly-OPfp	1.11
Boc-His(Dnp)	1.24	Fmoc-His(Trt)-OPfp	2.49
Boc-Ile	6.02	Fmoc-Ile-OPfp	13.01
Boc-Lys(2-CI-Z)	2.16	Fmoc-Lys(Boc)-OPfp	1.84
Boc-Leu	1.72	Fmoc-Leu-OPfp	1.39
Boc-Met(O)	0.80		
Boc-Phe	0.88	Fmoc-Phe-OPfp	1.15
Boc-Pro	1.50	Fmoc-Pro-OPfp	2.00
Boc-Ser(Bzl)	0.97		
Boc-Thr(Bzl)	1.66		
Boc-Trp(For)	1.32	Fmoc-Trp(Boc)-OPfp	1.74
Boc-Tyr(2-Br-Z)	1.44	Fmoc-Tyr(But)-OPfp	1.22
Boc-Val	3.91	Fmoc-Val-OPfp	9.62

The ratio depends strongly on the protecting groups and the activation procedure used (left column, DIC activation [210]; right column, coupling of Pfp esters [211]).

amino acids defined in each position of the peptide chain. In this case these 80 mixtures represented 32 000 individual peptides. The most active mixture for the defined position 1 was the one containing methionine as the defined amino acid. The most active mixtures with the defined position 2 were those ones with isoleucine and alanine, position 3 has shown significantly enhanced binding activity for asparagine and arginine, and position 4 has shown a strong preference for aspartic acid and serine. To retest these sequences in solution and identify the most active one, it would be necessary to resynthesize only those eight peptides representing the combination of all selected possibilities. If the same exercise would be performed with an iterative technique, it would be necessary to synthesize new libraries after each consecutive screening step (seven libraries; 140 mixtures in total) and these iterative libraries would be probably useless in screening other targets. Positional screening libraries are generic and can be used against a plethora of targets. The disadvantage of iterative scanning is that the amino acid residues defined at the beginning do not have to be the optimal and all subsequently synthesized libraries are biased towards these earlier defined parts of the sequence. The positional scanning library technique was successfully used to define the consensus peptide structures in a multitude of studies with libraries representing up to 10^{12} peptides, the screening of which would be impossible by the application of other techniques.

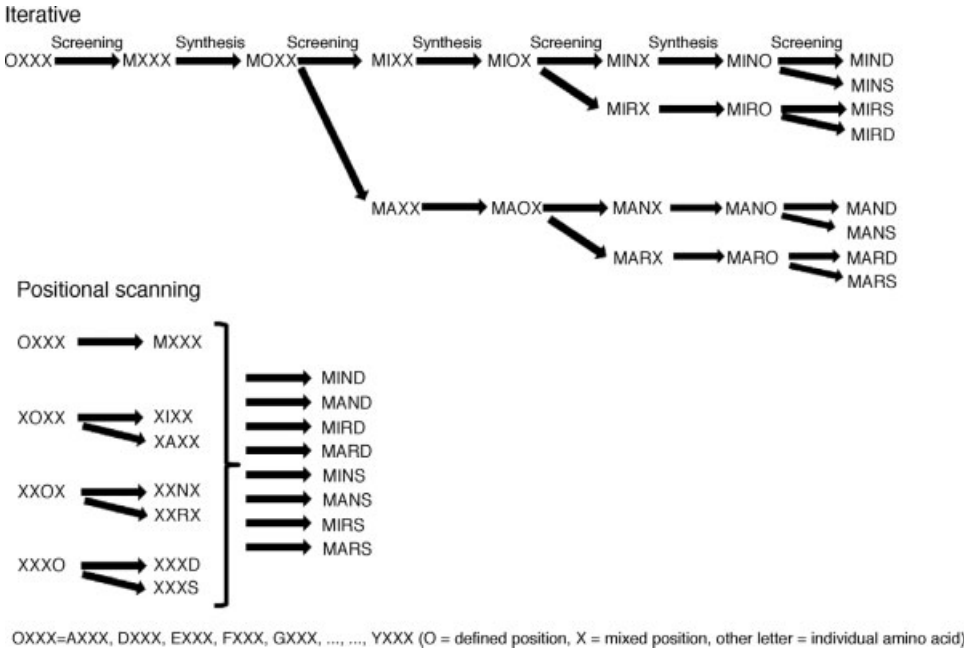


Figure 12.6 Comparison of iterative and positional scanning library techniques. (Modified from [49].)

The structure of positional scanning libraries is not limited to linear peptide sequences. Cyclic as well as scaffolded libraries with randomized "arms" attached to a semirigid scaffold based on a bicyclic peptide template were successfully synthesized and screened [197, 223, 224].

Deconvolution of so-called "omission libraries" (libraries with defined amino acid missing instead of fixed in a given position) was much less successful and did not find wide application [225].

In so-called "orthogonal" libraries used by Deprez *et al.* [226] each library member is present in two (or three) different mixtures and any two (or three) mixtures of the library have one, and only one, peptide in common. Consequently, the testing of these libraries should show activity of the mixtures containing the active compound in each sublibrary. If only one compound in the library is active, the identification of this compound by "mental deconvolution" is simple. However, if activities are observed in a number of sublibraries, the identification of the active components may be excessively complicated.

The concept of creating the orthogonal mixture can be explained on mixing the compounds synthesized in 100, 96-well microtiter plates. If aliquots are taken from all wells in a plate, 100 "plate mixes" of 96 compounds each can be created. Then aliquots are taken from all A1 wells of all plates and this create 96 "well mixes" of 100 compounds each. If the activity is found in only one plate and one well mix, then the active compound can be determined by testing 196 mixtures instead of testing 9600

individual peptides. "Column, row, diagonal, ..." mixes can also be created to confirm the finding. "Orthogonal" libraries by Deprez *et al*. are, of course, synthesized as mixtures and not created by mixing the individual compounds [226].

Mixture-based peptide libraries were also used as a basis for transformation into nonpeptidic structures by global modifications – the most notorious example being the creation of "libraries from libraries" in which the peptides were reduced into polyamines or polyalkylated [227–230].

Results achieved in Houghten's laboratories utilizing libraries of peptide mixtures can be found at http://www.tpims.org/scientists_richard-houghten.asp. An application of mixture libraries was comprehensively reviewed by Eichler [49].

Even though most of the libraries were synthesized on the solid phase, the technique using a soluble carrier can be used as well [231].

Other types of soluble peptide libraries were prepared by individual coupling of peptides with DNA [232, 233] or peptide nucleic acid [234] tags. After biotransformation of the peptide library in the solution assay (cleavage by protease, phosphorylation by kinase), the modified substrate was captured on a complementary DNA array and the structure of the substrate peptide was defined.

12.4.2
Synthesis of Peptides on a Mixture of Particles

Furka *et al*. [24] synthesized libraries in 1988, but he did not recognize the fact that each individual bead resulting from the split-and-mix synthesis contains just one peptide sequence. A fraction of the resin in every step is exposed only to one amino acid and therefore only one peptide sequence is being built on each bead (Figure 12.7). After mixing all beads together the identity of the beads is lost, but after another separation into individual reactors, again, only one amino acid can be attached to the growing chain. This fact did not escape the prepared mind of Kit Lam [23], who came up with the split-and-mix synthetic scheme to create mixtures of beads containing multiple copies of the individual sequences (one-bead–one-compound (OBOC) technology). The synthesized peptides, still attached to the beads, can be then submitted to binding studies and after isolation of beads expressing binding (observed by color, fluorescence, radioactivity, or detailed observation of cells sticking to the bead surface), the structure of active peptides can be elucidated by Edman degradation, mass spectrometry (MS), or reading the code built in during the library construction. Even though the OBOC dedicated solid-phase library synthesizer was constructed [235, 236], owing to the fact that the library design varies significantly in a number of randomized positions and used building blocks, these libraries are usually prepared by manual synthesis.

Since the application of these OBOC libraries was reviewed [23, 236–251] on a number of occasions and in a multitude of journals and monographs, this chapter will not go into the individual results achieved with OBOC libraries (as listed, for example, in [252]), but rather concentrate on the various techniques applicable for their production and testing. Results achieved by Lam's laboratory, which remains

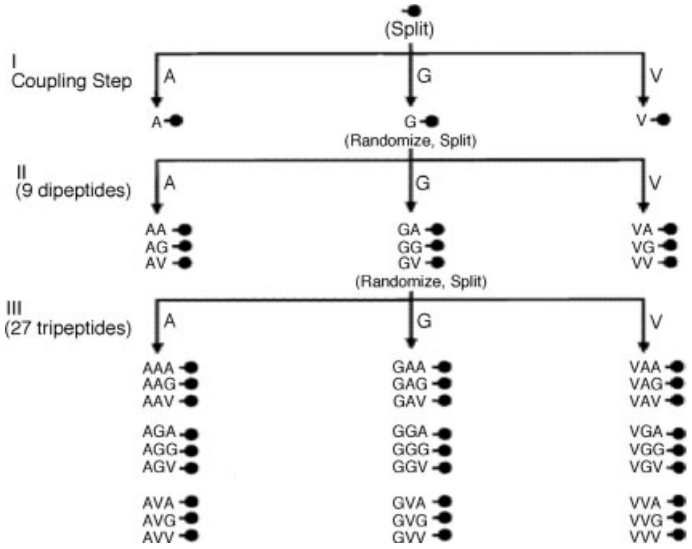


Figure 12.7 Principle of split-and-mix combinatorial synthesis [23]. A batch of beads (e.g., 1 000 000 beads) is separated into three aliquots and each aliquot is coupled with a different amino acid. Beads are mixed together and the process is repeated twice. At the end of this tripeptide synthesis, there are 27 populations of approximately 37 000 beads each with a unique sequence. Obviously, if we would try to synthesize a hexapeptide using all 20 amino acid in each coupling, we would prepare only 1.5% of possible structures with just 1 000 000 beads (64 000 000 possible sequences).

very active in developing OBOC technology and applying it especially in the area of cancer research, are available at http://oboc.ucdavis.edu/html/publications.htm.

In almost 20 years since the invention of one-bead–one-peptide technology [23], a great effort by numerous laboratories has been dedicated to eliminating its drawbacks, and making it more efficient and reliable. The greatest problem is the fact that screening is performed on the surface of the polymeric bead and not in solution (this issue will be discussed later), and that the generated signal representing the binding of the target to the bead does not correlate with the affinity of the ligand on the bead surface. First, the unspecific binding due to interaction with components of the detection system, or any unspecific protein, must be excluded, such as by using the dual-color screening system [253], or by scanning immobilized beads treated by two different labeled proteins or protein mixtures consecutively. By subtracting the two optical images it is possible to define beads binding uniquely to only one protein mixture and not to proteins common to both mixtures or to chemical and protein components of the assay itself [254].

Indeed, a ligand on a surface may have different binding properties. The resins typically used for library synthesis have a high loading capacity (e.g., 90-μm TentaGel™ resin with a loading capacity of 0.3 mmol/g has a ligand density of around 100 mM). This high loading is, however, only necessary for subsequent hit identification and not for actual biomolecule binding. High ligand density may

contribute to undesired binding of a target molecule to low-affinity ligands (due to high local ligand concentrations) and may also result in unintended multidentate interactions with the target leading to false-positives. As a result of this known fact, it is common to resynthesize and test the initial hits individually in solution. Since screening of a large library can produce hundreds of hits or more, it is not uncommon (due to the expense of sequencing and resynthesis) to define the structure and resynthesize only a fraction of "hits," potentially missing the best compound.

An alternative to the sole bead binding evaluation is the technique of "bead blot." The library of beads is incubated with the protein or protein mixture and protein-loaded beads are immobilized on a porous surface. Proteins bound to beads are then directionally eluted onto the surface and captured on a membrane superimposed on the beads. The location of the target protein is then defined by specific immunostaining and the bead responsible for its binding can be recovered for structure evaluation. This method allows evaluation of binding to a multitude of proteins contained in the original incubating mixture for which a selective labeled antibody is available [255, 256].

Meldal *et al.* [257–263] prepared a library of internally quenched fluorescent substrates on a support susceptible to penetration of the proteolytic enzymes into its structure (PEG acrylate). Appearance of fluorescence on the beads after incubation then pointed to the bead carrying the good substrate for the particular enzyme. This technique was modified into a "one-bead–two-compounds" assay [264, 265], in which the resin bead in addition to a combinatorial library member contains a reporter compound that can act as a beacon to monitor the activity of the library member. In the screen for enzyme inhibitors, the bead contains a quenched substrate and a library of potentially inhibiting peptides. Upon incubation of the beads with enzyme, the beads lacking fluorescent signal are likely to carry inhibitor, and can be selected and sequenced. This concept can be generally applied in all fields of combinatorial chemistry, including drug, catalyst, and material development.

Lam *et al.* speculated that lowering the substitution on the surface of the bead allowed an increase in the stringency of the assay [266]. They achieved it by segregating the internal volume of the beads from the surface layer. The internal content of the bead contains enough material for the sequencing and can actually be different from the bead surface. This allows for encoding nonsequencable parts of the peptide or nonpeptide structure on the bead surface, or for increasing the efficiency of Edman degradation by not including conserved parts of the peptide structure (if any) in the bead interior. Debenham *et al.* [267] used decreased substitution of the surface for the screening of high-affinity lectin ligands in an attempt to mimic the solution assay and avoid bivalent interaction. They found that actually 99.9% of the ligands had to be eliminated from the surface to prevent bidentate binding. It was later shown and explained by Chen *et al.* [268] that the decrease of the surface substitution is the best way to reduce the nonspecific binding of the macromolecular targets. High surface loading with the test compound was probably the reason for the earlier reported unsuccessful results from screening OBOC libraries.

The first attempts to segregate the surface and internal volume of the bead used enzymes to cleave the substrate-linker only on the bead surface, leaving the enzyme inaccessible core intact – to "shave the bead" [269]. The "shaved" bead can then be

functionalized with a different protecting group to segregate the two compartments – the surface and interior. This technique was later replaced by simple acylation of the amino groups of the bead matrix in a biphasic solvent environment. The water-soaked TentaGel bead surface is exposed to an organic solvent that contains the derivatizing reagent (e.g., Fmoc-OSu or Alloc-OSu), while the interior of the bead remains in water without any derivatizing reagent. In this way only the outer layer of the bead is derivatized. By varying the ratio of diethyl ether and dichloromethane (DCM) and adjusting the amount of Fmoc-OSu used, the thickness of the outer layer can be controlled [251, 270].

Astle *et al.* [271] simplified the process of hit isolation through magnetic bead sorting. The target protein was labeled with a magnetic nanoparticle. The binding of the target protein to a positive bead made the bead magnetic and allowed it to be separated from the rest of the library by placing a magnet on the side of the tube (Figure 12.8). The positive (magnetic) beads were separated into individual wells of a microtiter plate and the compounds were released by treatment with CNBr (methionine was used as part of the linker). The resulting samples (about 50%) were spotted onto a glass slide to generate a peptide microarray, which was then incubated with varying concentrations of the fluorescently labeled target protein to confirm and

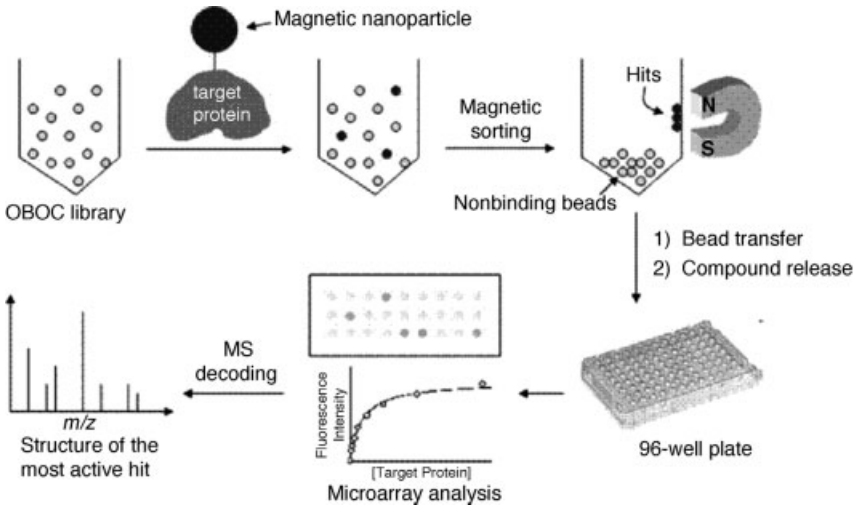


Figure 12.8 Scheme of the integrated magnetic screening and testing of hits on microarrays. TentaGel beads (75 μm) from a OBOC library are incubated with target protein, washed, and then incubated with antitarget protein antibodies linked covalently to iron oxide-containing particles (Dynabeads). Beads that bind the target protein are now magnetically labeled and retained on the side of the tube using a magnet, while nonbinding beads are removed. Each of the selected magnetic beads is separated into the well of a microtiter plate and the compounds are removed from the beads by cleavage of a linker. The compounds are then spotted onto a glass slide and formed microarrays are probed with different concentrations of the target protein to determine the intrinsic affinity of each compound for the target. The structure of selected hit is then deduced by tandem MS. In this way, no resynthesis of the hits is necessary until the best binders are confirmed [271]. (Illustration adapted from [272].)

reinvestigate its binding to these hits. A plot of the fluorescence intensity against the protein concentration gave the dissociation constant for each protein–ligand pair. This technique was tested on a 64-million mixed peptide/peptoid library and screen for binding to an anti-FLAG antibody. An initial on-bead screening produced 63 hits. A microarray analysis of the 63 hits revealed 27 low-nanomolar ligands against the antibody. The identity of the most active hits was then defined by matrix-assisted laser desorption ionization (MALDI)-MS.

The original paper [23] teaches the isolation of "positive" beads under a microscope. This technique is obviously a bottleneck in the application utilizing large libraries as it is negatively influenced by the subjective interpretation of bead color intensities. Affymetrix scientists successfully applied an automated fluorescence-activated cell sorter for selecting active beads from the peptide library coded by DNA tags tested against labeled antibodies [273]. Selectide Corporation (now part of Aventis), a company based on Kit Lam's technology, tested early on automated separation of fluorescently tagged beads using cell sorter instruments with surprisingly very limited success [236]. Later, a dedicated particle sorter COPAS™ (Union Biometrica) became a commercially available bead sorting instrument for the OBOC technology. It was successfully used, for example, for the screening of fluorescent internally quenched substrate libraries [264]. The success of this technique can be attributed to the use of highly porous solid support with minimal autofluorescence. Alternatively, the library can be first presorted to eliminate beads with high autofluorescence [274–276] or a different fluorescent labeling agent (such as quantum dots) with a different fluorescence emission wavelength can be used [277]. Validation of the selected (resynthesized) structures has to be performed in any case (as discussed, for example, by Kodadek and Bachhawat-Sikder [275]) to eliminate hits resulting from artifactual interactions.

The collaboration of scientists from the University of Edinburgh, Novartis, and Evotec Technologies (now PerkinElmer) resulted in the development of an automated system for selection and bead picking of fluorescently tagged positive beads utilizing confocal microscopy [278, 279] (Figure 12.9). Fluorescence-based detection of positive beads is complicated by the autofluorescence of the bead material or the

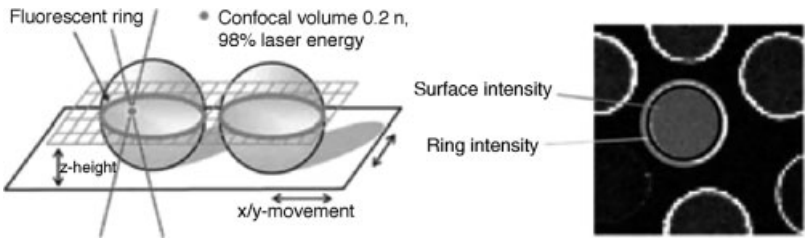


Figure 12.9 Principle of automated confocal microscopy screening of OBOC libraries. Beads in the monolayer are moved in the *xy* plane where the confocal plane is offset by about half of the bead size from the bead support. Observation of increased "ring" intensity represents the binding to macromolecular fluorescently labeled target. Fluorescence in the middle of the bead represents autofluorescence of the bead material or synthesized compound and is irrelevant to the biological interaction with the target [280].

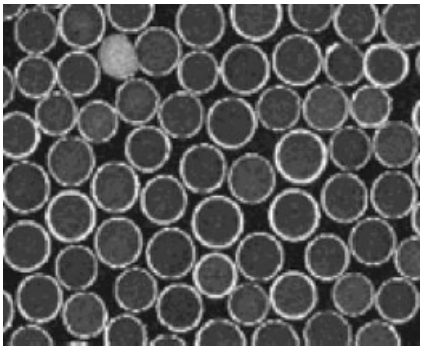


Figure 12.10 Resynthesized and retested (by confocal microscopy) beads with identical ligand show clearly the typical ring signifying the real binding effect [281]. At the same time large differences in ring intensity exemplifies the nonhomogeneity of the bead surface.

synthesized compounds, which can be higher than the fluorescence of the bound target macromolecule [276, 277]. However, the fact that the macromolecular fluorescently labeled target does not penetrate into the interior of the polymeric bead can be used for distinguishing between autofluorescence and fluorescence from the molecules bound to the surface of the bead. Confocal microscopy focuses only at a couple of microns "slice" of the bead and specific surface binding of the fluorophor-labeled target is clearly visible as the ring around the perimeter of the bead – unlike autofluorescence, which is distributed throughout the bead material. The bead library after incubation with the fluorescently labeled target is distributed as a monolayer of beads in the wells of a 96-well glass-bottom microtiter plate. Each well contains approximately 2000 beads. The plate is placed in an *xy* positioner above an inverted confocal microscope capable of autofocusing slightly under the center of the polymeric bead. The individual wells are then scanned in approximately 4 min each (for the 96-well plate, 00 000 beads can be scanned in 7 h), and a table of locations and internal and ring fluorescence intensities is built. After completion of the scan, the hits are ranked by their intensities, and the best are picked by an actuated capillary and placed into individual containers for structural evaluation. The bead picking process is slower and can retrieve about 1 bead/min [280–282]. Resynthesis and retesting of the active compounds has shown that surface inhomogeneity prevents precise ranking of the ligand quality based on the solid support fluorescence reading (Figure 12.10).

To validate the results obtained with the automated bead picking system, Hintersteiner *et al.* [281] designed a system utilizing postsynthesis/postscreening labeling for verification of the binding of ligand on the positive bead in solution assay. For the screening, the tested compound (peptide) is attached to the bead via a complex linker (Figure 12.11) composed of the segment allowing eventual detachment from the bead, followed by an amino acid with a terminal alkyne in the side-chain (labeling segment), a hydrophilic spacer to separate the library segment from the labeling segment, and the library (combinatorial) part. The identified and picked beads are then labeled via "click" reaction [283, 284] of the alkyne moiety with an

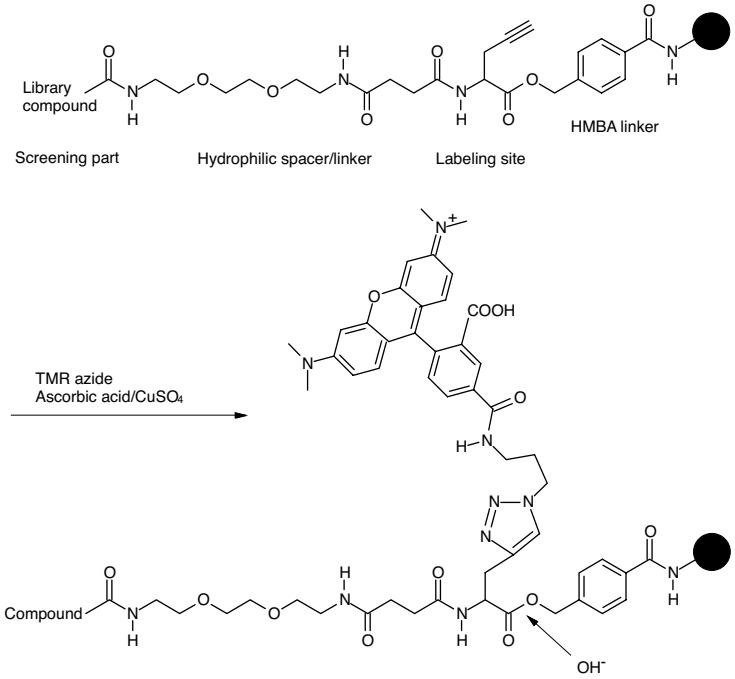


Figure 12.11 Structure of the attachment to the bead used in postsynthesis/postscreening labeling method. After on-bead screening with confocal microscopy and bead picking, individual hit beads are treated with an azide-modified dye (e.g., TMR-azide) to convert all compounds into fluorescent ligands for further affinity testing via fluorescence spectrometry. Compound is released to solution from the HMBA linker via hydrolysis [281].

azide-containing fluorophore. The ligands, now fluorescently labeled, are then cleaved from the beads and their concentration is quantified by fluorescence reading. Subsequently, binding to the unlabeled target is evaluated in a solution by two-dimensional fluorescence intensity distribution analysis (2D-FIDA). Only the structures of compounds with high and validated binding are then analyzed by MALDI-MS. The process was illustrated on an identification of ligands to the S_H2 domain of the adaptor protein Grb2. This automated technology is obviously not available for average laboratories due to the required dedicated instrumentation, but it is probably the most important addition to OBOC technology.

The fusion of OBOC technology and libraries utilizing a deconvolution of mixtures is the so-called "library of libraries" [285] (often confused with libraries from libraries, where one type of library is globally transformed into a different type of library by chemical reaction). A library of libraries is an assembly of individual beads, where each bead contains a sublibrary characterized by several defined positions in a sequence. For example, the hexapeptide library of libraries with three defined positions would be composed of 160 000 different types of beads, each of which would contain 8000 different peptides with three defined positions in their sequence.

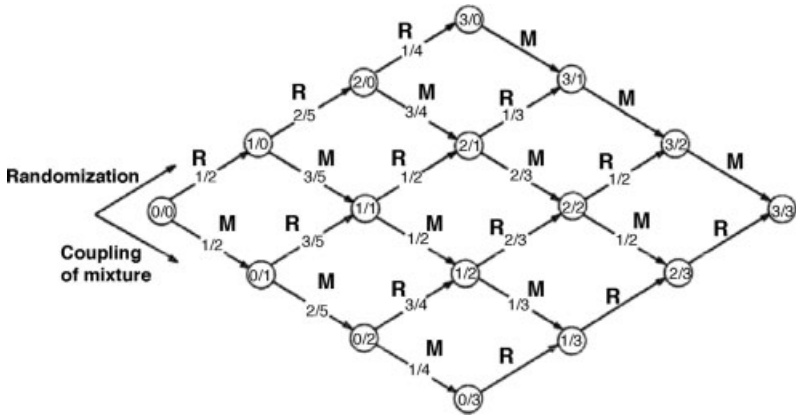


Figure 12.12 Scheme of the synthesis of a hexapeptide library of libraries with a three-amino-acid motif. Arrows carrying the symbol R represent performed randomization and arrows carrying the symbol M mean that the mixture of amino acids was coupled. Numbers inside of a circle show the number of randomized positions versus fixed position in the particular portion of the library at that particular point – the completed library (3/3) has three fixed and three mixed positions. The numbers in the arrows specify the ratio in which the portion of the library from the previous step was split for performing randomization or coupling of mixed amino acids. (Adapted from [285].)

This represents an enormous saving in comparison with an OBOC library, where we would need 64 000 000 beads to cover the same diversity. However, due to the slightly complicated synthetic scheme (see Figure 12.12), the potential of this type of library for quick definition of a peptide pharmacophore has, up to now, been underutilized. The concept of a library of libraries is clearly applicable to libraries built on planar substrates or labeled macroscopic substrates.

12.4.2.1 Determination of the Structure of a Peptide on an Individual Bead

For natural peptides the Edman degradation is still used for the sequence determination of "active" beads retrieved from a library. However, this technique is slow, cannot distinguish between L and D configurations of the building blocks, and unsequencable blocks (anything but α-amino acids) cannot be incorporated in the library structure. Therefore, alternative techniques were evaluated for structure determination.

Youngquist *et al.* [286, 287] developed a method in which during the generation of the library a small portion of the growing peptide chain is capped, so that at the end each bead contains "the history" of its synthesis. This history is then easily deconvoluted into the peptide sequence by MS.

An increase in the throughput of decoding the peptidic structures was achieved by the application of a partial Edman degradation (PED) followed by MALDI-MS [288–290]. In this method, beads carrying unique peptoid (or peptide–peptoid) sequences were subjected to multiple cycles of treatment with a 1 : 3 (mol/mol) mixture of phenyl isothiocyanate (PITC) and Fmoc-Cl to generate a series of

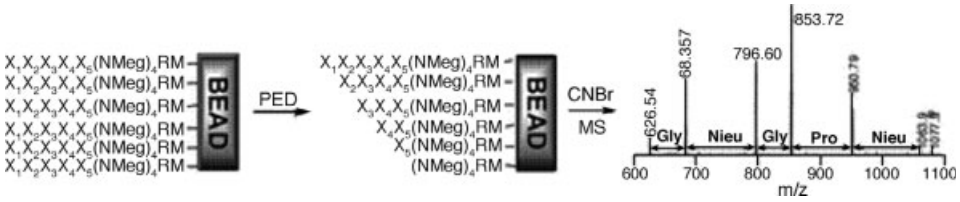


Figure 12.13 Example of rapid sequence determination by PED followed by MALDI-MS [288].

N-terminal truncation products for each resin-bound peptoid. At the end the Fmoc group was removed from the N-terminus and any reacted side-chains via piperidine treatment. The resulting mixture of the full-length sequence and its truncation products was analyzed by MALDI-MS. From the typical spectrum shown in Figure 12.13, the sequence could be easily deduced [288].

The peptide can also be tagged by sulfobenzoic acid on the N-terminus, which promotes an efficient charge site-initiated fragmentation of the backbone amide bonds to selectively enhance the detection of a single fragment ion series that contains the C-terminus of the molecule (y-ions) [291]. Another method uses a cleavable linker that is hydrophilic to help reduce nonspecific binding to biological samples and allows for the attachment of a halogen tag, which greatly facilitates postscreening sequencing by tandem MS (MS/MS). The linker is based on a tartaric acid unit, which, upon cleavage from resin, generates a C-terminal aldehyde. This aldehyde can then be derivatized with a bromine-containing amino-oxy compound that serves as an isotope tag for subsequent MS/MS analysis of y-ion fragments [292].

As it became obvious that library techniques are applicable not only to peptides, but also to small organic molecules, various techniques for coding/decoding the structure of a binding compound (complicated and available in limited quantity) on the bead surface by some entity hidden in the core of the bead (simply detectable and relatively abundant) were sought. The coding became especially relevant with the possibility to effectively differentiate the two compartments [266, 270, 293–295].

Coding for structure elucidation of nonpeptide molecules was covered in many reviews [37, 244, 296–299], often using very interesting and creative concepts, but in the context of peptide libraries is of only limited relevance.

As the MS techniques [300, 301] are becoming more sensitive and more readily available, more reports based on direct structure determination by MS/MS experiments are being published [302]. A single bead can even be physically cut into several pieces and still provide adequate quantities of peptides for this type of analysis [302, 303]. The hydrogen–deuterium exchange can simplify interpretation of spectra obtained from electrospray MS [304]. Hits from cysteine-rich libraries were alkylated to transform cysteine residues into acetamidomethyl derivatives, cleaved from the beads, and the structure determined by an MS/MS experiment [305]. Another example of the direct application of MS/MS for structure determination of peptides from noncoded library of peptides was shown by Brown *et al.* [306]. The library was synthesized on a hydroxymethyl benzoic acid (HMBA) linker. The peptides were cleaved from the beads by an application of gaseous ammonia. It was shown that even a

short (5 min) exposure to high-pressure (117 psi) ammonia gas detaches the majority of the peptide from the linker. Individual beads were then placed on a glass surface. A nanomanipulator then placed a 1-μm nanoelectrospray tip next to the bead and delivered the extraction solvent to the bead. After 30 s, the solvent was backfilled into the nanoelectrospray tip and the tip was introduced into the mass spectrometer. MS/MS analysis delivered high-quality data in 100% of tested cases.

12.4.3 Solution-Based Screening of OBOC Libraries

The disadvantage of OBOC libraries with their limitation to binding assays was quickly recognized by Lam *et al*. and this technique was modified by the introduction of selectively cleavable linkers to allow assaying of libraries in solution. Since the identity of the tested peptide is not known until the sequence is read from the "active" bead, the test must guarantee that the observed activity of the solution would be traceable back to the bead from which the active compound was cleaved. Moreover, there must be enough of peptide still available on that source bead to allow the structure determination. There are two general approaches to screen a OBOC library with solution-phase assays: (i) the 96-well two-stage release assays and (ii) the *in situ*-releasable solution-phase assay with immobilized beads. In both approaches, ligands are attached to the solid support via cleavable linker(s). The ligands are then released from each bead into solution phase where the biological assays take place. The bead of origin of the positive releasate can subsequently be identified and isolated for structure determination. The spatial relationship between the active bead and the solution activity can be realized by embedding the bead in a semisolid medium (e.g., gel), to which the peptide is slowly diffusing from the entrapped bead. Beads can be also immobilized into microwells, in which the activity will be tested.

In the 96-well two-stage releasable assay [307], the double orthogonally cleavable linker [308–310] (e.g., the linker of the structure in Figure 12.14) is incorporated into the preparation of the library. Approximately 100–500 beads are added into each well of a 96-well filtration plate (e.g., Millipore). Upon neutralization, the first aliquot of the library is released with the formation of a diketopiperazine structure on the bead. After incubation overnight, suction is applied so that the filtrates (each with 100–500 compounds) are collected in a 96-well plate placed beneath the filtration plate. The filtrates are then assayed for biological activity in solution. Beads from the positive wells are then redistributed into filtration plates, now with one bead per well. With alkali treatment (e.g., gaseous ammonia), the second aliquot of the library is released and the filtrates from each well are then tested for biological activity. The beads that correspond to the wells with active compounds are then identified and isolated for structure determination. This two-stage release assay is needed if a high number of beads (e.g., more than 5000) are assayed. If the number of beads to be assayed is limited, a single release assay with one bead per well may be sufficient, particularly if the transfer of individual beads into each well (one bead per well) can be automated. For a 100-μm bead, approximately 100 pmol of compound can, in principle, be recovered giving a final concentration of 1 μM (if the final assay volume is 100 μl). In

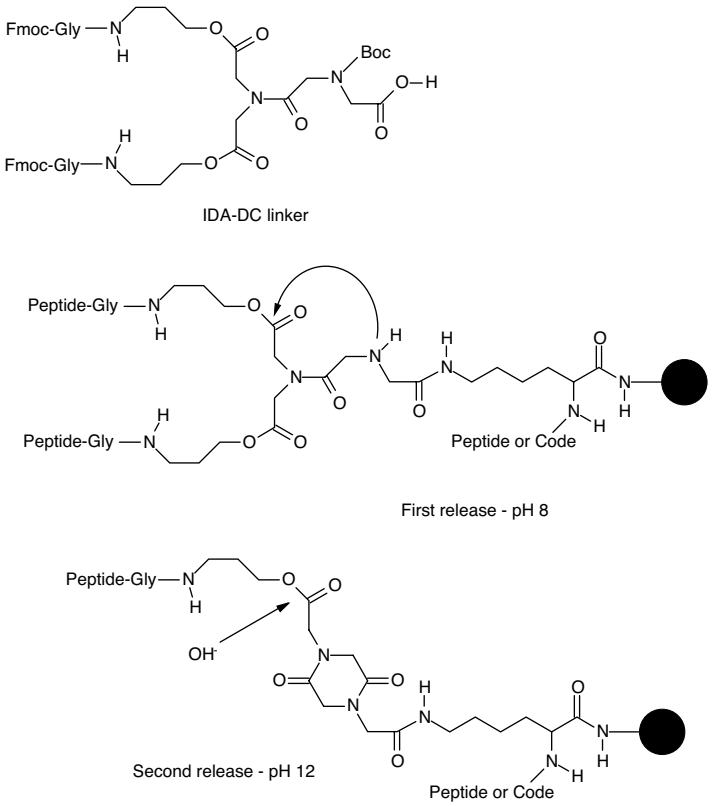


Figure 12.14 Scheme of the sequential releases from doubly cleavable linker [308]. If the linker is attached through trifunctional amino acid (Lys), a copy of nonreleasable peptide or a code is still attached to the bead after both releases.

order to increase the concentration of the recovered compound, one may (i) miniaturize the assay volume (e.g., using the 384-well plate), (ii) use bigger beads, or (iii) use beads with a higher substitution level.

For *in situ* solution-phase releasable assay, Salmon *et al.* proposed the use of soft agar to immobilize beads [311]. After the linker has been cleaved the compounds were released and diffused into the surrounding agar, where the solution-phase assay takes place. Jayawickreme *et al.* [312–314] reported a related method to identify ligands that interact with the α-melanocyte-stimulating hormone (MSH) receptor. They first immobilized the bead library on a thin film of polyethylene and exposed the beads to gaseous trifluoroacetic acid (TFA) for 10 h at room temperature. After neutralization with gaseous ammonia, the beads were layered on the surface of a dish of melanocytes growing in soft agar. As a result of pigment dispersion, the cells located underneath and around the positive beads with MSH agonist activity turned dark within 15 min. This elegant assay system has also been adapted to other G-protein-coupled receptors by transfecting those receptors into a cell line with melanocyte background. Salmon *et al.* [311] have applied a related *in situ* solution-phase releasable assay to anticancer

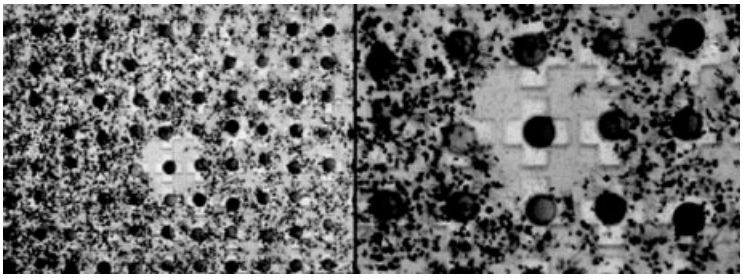


Figure 12.15 Cell viability assay on a dedicated microfabricated microtiter plate. Plate wells can accept only one bead (120 μm size). Clearing in the cell lawn clearly defines location of the source bead of the cytotoxic peptide [252].

agent discovery. The *in situ*-releasable assay is highly efficient and, in principle, only a single cleavable linker is needed since the beads are already spatially separated; the two-stage release assay as described in the 96-well releasable method is not needed. The *in situ* assay has an added advantage in that the concentration of the released compound could be rather high (e.g., above 10 μM) in close proximity to the bead and the potency of the compound could be estimated on the basis of the size of the activity ring surrounding each positive bead (Figure 12.15).

A recently published technique [252] uses a dedicated photolithographically microfabricated plate in which each well is sized to accommodate only one 120-μm TentaGel library bead. This plate is loaded with 10 000 beads and beads are covered with a suspension of cancer cells in Matrigel™ to create the three-dimensional cell culture. After the cell colonies reach a desired confluency, the library compound connected by a disulfide-containing linker is released by application of a buffer containing dithiothreitol. After an additional incubation the regions in which cytotoxic peptide was released are clearly observable by a decreased cell viability associated with particular beads. Those beads were retrieved and the structure was determined by Edman degradation.

In some instances, it may be advantageous to combine solution-phase assays with on-bead assays to screen a specific target. Positive beads isolated by this approach are more likely to be true-positives. For example, the compound-beads are partitioned into 1000 beads per well and a portion of the compound on each bead is released into the solution for biological testing. The 1000 beads from a positive well can then be recycled and an on-bead binding assay performed to identify the single positive bead. Using this approach, Salmon *et al.* successfully isolated ligands that bind to an anti-β-endorphin monoclonal antibody. This strategy may have higher throughput than the earlier discussed system for automated picking of beads from binding assays followed by solution evaluation of binding constants; however, the future is clearly favoring automated systems. It remains to be seen whether large pharmaceutical companies will be able to revitalize their development pipelines by the application of the new (highly automated and expensive) technologies, or whether simple semiautomated or completely manual techniques applied in small biotech companies or academic laboratories will still provide a major resource in supplying new drug candidates.

12.5
Future of Peptide Libraries

As can be seen in this chapter, there is a multitude of concepts for generating diversity of peptide or peptide-like structures. All of them have a clear goal of discovering a new functional molecule that can be applied in a practical application (as a pharmaceutical, diagnostic, catalyst, or anything that can be imagined). It is still to be determined which technology will be dominant in the future, but at this moment it can be predicted that the winning technique will be highly automatable, simple, with a low demand on the amount of necessary target and reagents, and, most importantly, provide the most reliable and validated results. The author's personal prediction is that it would be the automated OBOC technology applying the library of libraries strategy.

12.6
Synthetic Protocols

12.6.1
Pin Synthesis [2, 60, 76, 315]

The polyethylene rods (diameter, 4 mm; length, 40 mm) immersed in a 6% (v/v) aqueous solution of acrylic acid were irradiated at a dose of 1 000 000 rads (1 rad = 0.01 Gy). The rods so prepared were assembled into a polyethylene holder with the format and spacing of a microtiter plate. Subsequent reactions at the tips of the rods were carried out in a Teflon tray with a matrix of wells to match the rod spacing. Conventional methods of solid-phase peptide chemistry were used to couple Boc-L-lysine methyl ester to the polyethylene/polyacrylic acid via the amino group of the side-chain. Carboxy substitution of the support was determined by treating NH_2-lysine(OMe)-polyethylene/polyacrylic acid with ^{14}C-labeled butyric acid and was found to be 0.15–0.2 nmol/mm^2. Removal of the Boc group was followed by the coupling of Boc-L-alanine to complete a peptide-like spacer. Successive amino acids were added as dictated by the sequence to be synthesized. At the completion of the final coupling reaction, and after removal of the Boc protecting group, the terminal amino group was acetylated with acetic anhydride in dimethylformamide (DMF)/triethylamine. All *N,N*-dicyclohexylcarbodiimide (DCC)-mediated coupling reactions were carried out in DMF in the presence of 1-hydroxybenzotriazole (HOBt). The following side-chain protecting groups were used: *O*-benzyl for threonine, serine, aspartic acid, glutamic acid, and tyrosine; carbobenzoxy for lysine; tosyl for arginine; 4-methylbenzyl for cysteine; and 1-benzyloxycarbonylamido-2,2,2-trifluoroethyl for histidine. Side-chain-protecting groups were removed by treatment with borontris(trifluoroacetate) in TFA for 90 min at room temperature. After hydrolysis with HCl/propionic acid, sequences included in the synthesis as controls were analyzed to confirm that, although coupling at each stage had occurred, it was incomplete for several of the amino acids, notably arginine. Before testing by

enzyme-linked immunosorbent assay (ELISA), support-coupled peptides were washed several times with phosphate-buffered saline (P_i/NaCl).

12.6.2 SPOT Synthesis [144, 153]

A sheet of chromatographic paper Whatman Chrl (Maidstone, UK) was marked with a pencil (spot positions) and the sheet was dried under vacuum overnight. A solution of 0.2 M Fmoc-amino acid (Pro or β-Ala), 0.24 M DIC, and *N*-methylimidazole in DMF was soaked into the paper sheet, and the reaction was run for 3 h in a closed container. The paper sheet was washed 3 times with DMF and treated with piperidine/DMF (1 : 4, v/v) for 20 min. After three washes with DMF and two washes with ethanol, the sheet was dried in a desiccator. Solutions of Fmoc-amino acid HOBt esters (0.5 ml, 0.3 M) were then spotted onto pencil-marked spots and reactions proceed for 20 min on a plastic tray covered with a glass plate. The paper sheet was then washed twice with acetic anhydride/DMF (1 : 49, v/v) and treated with the same solution in the presence of 1% diisopropylethylamine (DIPEA) for 30 min. After washing with DMF (4 times), deprotection by piperidine/DMF (1 : 4, v/v, 5 min), and washing with DMF (4 times), the sheet was washed in bromophenol blue solution (0.01%) to reveal blue spots with available amino groups. The sheet was washed with ethanol (2 times) and dried by cold air from a hair dryer between two layers of Whatman 3MM paper. Blue spots were used as a target for spotting activated solutions of amino acids (0.3 M, 0.5–1 ml). The paper sheet was optionally respotted after 15 min (if disappearance of blue color was slow). Final deprotection was performed by immersing the dry sheet into a solution of TFA/DCM/diisobutylsilane/water (50 : 45 : 3 : 2, v/v/v/v) for 2 h. After washes with DCM (4 times), DMF (3 times), and ethanol (2 times), the sheet was dried and ready for binding assay or storage (−20 °C in a sealed plastic bag). In the case of peptide synthesis on a linker cleavable by intramolecular diketopiperazine formation, the sheet has to be washed exclusively by acidic solutions so as to not lose the synthesized peptides prematurely. The spots can be cut or punched out of the dried sheet into polypropylene tubes or into wells of a microtiter plate and after addition of neutral buffer the peptides are released into solution.

12.6.3 Synthesis in Tea-Bags [3]

Standard Boc-amino acid resin (50–100 mg; 0.2–0.8 meq/g) was contained in polypropylene 74-μm mesh packets having approximate dimensions of 15 × 20 mm. After a number was placed at the top of the unsealed bag with a black marker pen, the packet was closed and the number was permanently sealed into the polypropylene to give an easily readable label for each bag. These resin packets can be used for simultaneous multiple peptide syntheses (i.e., syntheses in which many different peptides are produced concurrently) or for multiple analog peptide syntheses (i.e., syntheses in which many analogs of a particular peptide are produced concurrently). The method is not limited to the Boc strategy, which is described in this example. The

standard deprotecting, neutralization, coupling, and wash protocols, and basically any type of the resin support, can be used for the synthesis.

Synthesis of analogs having single-amino-acid variations can be achieved by using various peptide synthesizers or completely manual methods. Between 40 and 80 individual packets containing the desired starting resin were carried through their common Boc removal, washing, and neutralization steps. After their methylene chloride washes to remove excess base, the packets containing the neutralized peptide resins were removed from the reaction vessel and added to solutions containing preformed symmetrical anhydrides of the next protected amino acid. The individual coupling steps were carried out for 60 min with stirring or shaking at room temperature. After completion of the coupling steps, the resin packets were returned to the reaction vessel, and the synthesis process was continued through additional cycles of common wash, deprotection, neutralization, and coupling steps until the syntheses were completed. Specific variations of analogous peptides, such as single-residue replacement or omission analogs or chain-lengthened or -shortened analogs, were easily accomplished by removing the individual coded packets at the point of variation during the synthesis, carrying out the desired variation separately, and, if appropriate, returning the packet to the common reaction vessel for completion. After the preparation of a series of protected peptide resins, the resin-filled packets were washed thoroughly, dried, and weighed to give an initial indication of coupling completion. The protected peptide resins, still contained within their packets, were then cleaved by using conventional HF/anisole procedures in a vessel modified to allow cleavage of 20 peptide resins at once. After extraction of the residual anisole with ether or ethyl acetate, the peptides were extracted from the resin packets by 5% acetic acid and either lyophilized directly or put through a Sephadex G-10 desalting column prior to lyophilization. The crude peptides were characterized by high-performance liquid chromatography and found to have an average purity of 84% (70–94%).

12.6.4
Synthesis on Cotton [132]

12.6.4.1 Modification of the Cotton Carrier

DMAP-Catalyzed Carbodiimide/HOBt Acylation

i) The cotton sheet (10 cm^2) was shaken successively in 25% TFA/DCM (3 ml) for 20 min, DCM (2 × 3 ml) for 3 min each, 10% DIPEA/DCM (2 × 3 ml) for 3 min each and DCM (2 × 3 ml) for 3 min each and dried between filter papers. Subsequently, the carrier was shaken overnight in 0.1 M Fmoc-amino acid/DCC/HOBt/0.03 M 4-dimethylaminopyridine (DMAP) in DMF (1 ml). The carrier was washed successively with DMF (2 × 3 ml) for 3 min each, ethanol (2 × 3 ml) for 3 min each, and DCM (2 × 3 ml) for 3 min each, and dried between filter papers. A sample of the carrier was used for the determination of substitution. After shaking in 20% piperidine in DMF (5 ml) and washing by the same solution, the absorption of the solution was read at 301 nm. The

substitution was calculated according to the formula:
Substitution (mmol/cm^2) = ($A \times V$ (ml) $\times$ 1000)/(8100 $\times$ F (cm^2))
where A is the absorption, V is the volume, and F is the area of the carrier. The absorption coefficient (8100) was determined by a calibration curve using solutions of Fmoc-Ala in 20% piperidine/DMF.

ii) The reaction was performed in the same way, with the exception that the solution was placed 4 times for 15 min in an ultrasonic bath. In this way, larger quantities of cotton were modified (3 $\times$ 300 cm).

NMI-Catalyzed Carbodiimide/HOBt Acylation The cotton or paper carrier (10 cm^2) was pretreated with TFA/DCM, as described above, and shaken overnight in 0.1 M Fmoc-amino acid/DIC/HOBt/0.2 M *N*-methylimidazole (NMI) in DMF (1 ml) or soaked with 0.5 M Fmoc-amino acid/DIC/HOBt/1 M NMI (0.2 ml) and left overnight. After that the carrier was shaken successively in DMF (2 $\times$ 3 ml) for 3 min each and DCM (2 $\times$ 3 ml) for 3 min each, and dried between filter papers.

Attachment of the Acid-Labile Handle and the Starting Amino Acid The handle HO-CH_2-C_6H_4-O-$(CH_2)_2$-COOTcp (HPP-OTcp) was synthesized according to the original procedure described by Albericio and Barany [316]. The amino acid carrier (10 cm^2) was shaken in 0.1 M HPP-OTcp/HOBt in DMF (1 ml) after the addition of a 0.01 M bromophenol blue/DMA solution (20 μl) until the disappearance of the blue color from the carrier (30–60 min) or the carrier was soaked with 0.3 M HPP-OTcp/HOBt in DMF (0.2 ml) and left after the addition of bromophenol blue until decolorization. After that, the carrier was shaken successively in DMF (2 $\times$ 3 ml) for 3 min each and DCM (2 $\times$ 3 ml) for 3 min each, and dried between filter papers. The HPP-amino acid-carrier formed by this reaction was then acylated with the starting amino acid as described above.

Peptide synthesis using Fmoc-protected amino acids using standard protocol (DIC/HOBt activation, monitored with bromophenol blue, 20% piperidine in DMF for deprotection) is recommended for assembly of peptides. Boc-based peptide synthesis gives much inferior products.

12.6.5
Split-and-Mix Synthesis of OBOC Noncleavable Libraries [237]

The library is synthesized on 130-μm TentaGel (Rapp Polymere, Tubingen, Germany) resin beads. Alternatively, ArgoGel™ (Argonaut Technologies, Foster City, CA) polydimethylacrylamide beads or Pepsyn Gel Resin (Cambridge Research Biochemicals, Northwitch, UK) can be used. In general, any resin that is compatible with organic solvents, as well as aqueous media, is adequate. Spacers, such as aminocaproic acid, aminobutyric acid, and/or β-Ala, may be attached to the resin prior to assembling the library. The resin beads are divided into 19 aliquots contained in 19 polypropylene vials or plastic syringes equipped with a plastic frit at the bottom (CSPS, San Diego, CA). (The number of amino acids used in randomization, equal to the number of reaction vessels, is limited only by the patience of the chemist.) Nineteen Fmoc-amino acids (all proteinogenic amino acids except Cys) are added

separately into each of the resin aliquots using a minimal amount of DMF. The amino acids are added in 3-fold excess, and coupling is initiated by adding a 3-fold excess of benzotriazol-l-yl-oxytris-(dimethylamino) phosphonium hexafluorophosphate (BOP) and DIPEA (or DIC) and HOBt. A trace amount of bromophenol blue is added to the reaction mixture. The vials are tightly sealed (syringes are capped) and rocked gently for approximately 30 min at room temperature or until all beads turn from blue to colorless. Completion of the coupling is confirmed by the ninhydrin test. When the coupling is incomplete, the beads are allowed to settle and the supernatant is gently removed; alternatively, in the case of synthesis in a syringe, it is expelled from the syringe. A fresh activated Fmoc-amino acid is added and the reaction proceeds for an additional 1 h. The resin is washed by DMF and the resin pools are mixed in a siliconized cylindrical glass vessel fitted at the bottom with a frit. Dry nitrogen is bubbled through to mix the resin. After washing 8 times with DMF, piperidine/DMF (1 : 4, v/v) is added. After 10 min of bubbling with nitrogen, the piperidine is removed and the resin is washed 10 times with DMF. The amount of released dibenzofulvene–piperidine adduct is determined by measuring the absorbance at 302 nm. A stable level of substitution determined in this manner throughout the library synthesis serves as one of the quality control criteria. The resin is again divided into 19 aliquots for the coupling of the next 19 amino acids. After the coupling steps are completed, the Fmoc group is removed with piperidine/DMF (1 : 4, v/v), and the resin is washed with DMF and DCM. The side-chain protecting groups are removed by treatment with reagent K (TFA/phenol/water/phenolethanedithiol, 82.5 : 5 : 5 : 5 : 2.5; v/w/v/w/v) for 5 + 120 min. This treatment is performed in the common container (glass bubbler) or, in cases that separate pools of resin are required, in individual syringes. The resin is washed thoroughly with TFA, DCM, DMF, DMF/water (1 : 1, v/v), and 0.01% HCl in water, and stored in DMF at 4 °C. Again, small individual pools can be conveniently stored in the plastic syringes in which the whole synthesis is performed. Larger library batches (up to 80 g) are stored with protected side-chains in 0.2% HOBt/DMF at 4°C. To verify the quality of the library, several randomly chosen beads are sequenced, and the average amount of peptide per bead is determined. This value is confirmed by quantitative amino acid analysis of a random sample from the library (1 mg). Whereas amino acid analysis is used to determine the overall amino acid composition of the library, sequence analysis confirms the random distribution of amino acids at each position

12.6.6
Preparation of Dual-Layer Beads [251, 270]

Preparation of topologically segregated bifunctional TentaGel resin beads with 60% Boc outside and 40% Fmoc-linker inside (outside-Boc/inside-Fmoc-linker bifunctional resin). TentaGel S NH_2 resin beads (1.0 g, 0.26 mmol) were swollen in water for 48 h. The water was drained and a solution of Alloc-OSu (31.1 mg, 0.156 mmol) in a DCM/diethyl ether mixture (50 ml, v/v, 55 : 45) was added to the resin, followed by addition of DIPEA (55 μl, 0.312 mmol). The resulting mixture was shaken vigorously

for 1 h. The resin was washed 3 times with DCM and 6 times with DMF. Fmoc-linker was then built in the inner region of the resin. The resin was washed 3 times with DCM. In the presence of argon, a solution of $PhSiH_3$ (770 µl, 6.24 mmol) in DCM (4 ml) was added to the resin followed by a solution of $Pd(PPh_3)_4$ (75.1 mg, 0.065 mmol) in DCM (12 ml). The mixture was shaken in an argon atmosphere for 30 min. This process was repeated. The resin was washed with DCM, DMF, and DCM 3 times each. A solution of di-*tert*-butyl dicarbonate (1.19 ml, 5.2 mmol) in DCM (10 ml) was added to the resin, followed by the addition of DIPEA (226.4 µl, 1.3 mmol). The mixture was shaken until the ninhydrin test was negative. The obtained outside-Boc/inside-Fmoc-linker bifunctional resin was washed with DCM, DMF, DCM, and MeOH 3 times each, and then dried *in vacuo*. The percentage of inner region was determined to be 39% using quantitative UV absorption analysis of the dibenzofulvene–piperidine adduct released by treatment with piperidine.

12.6.7
Preparation of Library of Libraries [285]

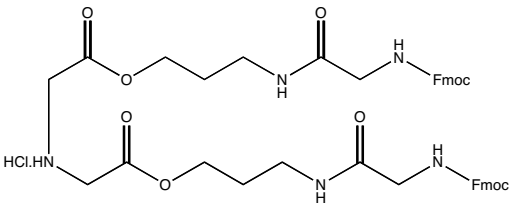
Libraries of peptides were constructed on TentaGel Resin S Amino-NH_2 (Rapp Polymere, Tubingen, Germany). Standard solid-phase peptide synthesis chemistry (Fmoc chemistry) was used. In every step the resin was divided into a number of pools in ratios according to the scheme in Figure 12.12. The mixture of protected amino acids with molar ratios adjusted according to the results from the pilot experiment was used in steps in which the amino acids in the mixture were coupled. The randomization steps were performed according to split-synthesis methodology. Finally, the Fmoc groups were removed with 20% (v/v) piperidine in DMF and the side-chain protecting groups were removed with a mixture of TFA/phenol/anisole/ethanedithiol, 94 : 2: 2: 2; v/w/v/v or with reagent K (TFA/phenol/water/thiophenol/ethanedithiol, 82.5 : 5: 5: 5: 2.5 (v/w/v/w/v)). The resin was then washed thoroughly with DMF, neutralized with 10% (v/v) DIPEA in DMF, thoroughly washed again, hydrated, and stored in 0.01% HCl at 4 °C.

12.6.8
Preparation of OBOC Libraries for Testing in Solution [308, 317]

12.6.8.1 Synthesis of Multicleavable Linker

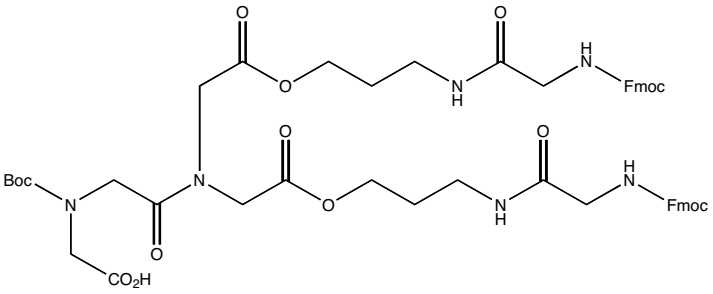
Boc-iminodiacetic acid (Boc-Ida) A solution of iminodiacetic acid (30.0 g, 225 mmol) in 1 M NaOH (225 ml) and dioxane (200 ml) was stirred and cooled in an ice-water bath. Di-*tert*-butyl pyrocarbonate (53.9 g, 247 mmol) was added in several portions and stirring was continued at room temperature for 1 h. Dioxane was evaporated *in vacuo*, and the residue was covered with a layer of ethyl acetate (100 ml) and acidified with a saturated solution of $KHSO_4$ to pH 2–3. The aqueous phase was extracted with ethyl acetate (3 × 150 ml). Combined ethyl acetate extracts were washed with water (100 ml), dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The product was crystallized from a solvent mixture of ethyl acetate and petroleum ether. Yield 47.0 g (90%).

HN(CH_2-CO-O-(CH_2)$_3$-NH<-Gly<-Fmoc)$_2$·HCl



A solution of Fmoc-Gly-NH-(CH_2)$_3$-OH (7.08 g, 20 mmol) in DMF (30 ml) was added to the solution of Boc-Ida (2.33 g, 10 mmol), HOBt (2.7 g, 20 mmol), and DIC (3.14 ml, 20 mmol) in DMF (30 ml). DMAP (0.48 g, 4 mmol) was added and the reaction mixture was stirred at room temperature for 5 h. DMF was evaporated under reduced pressure, and the oily residue was dissolved in AcOEt (100 ml), filtered, and extracted 3 times with water, 5% aqueous HCl, water, a saturated solution of $NaHCO_3$, water, and a saturated solution of NaCl in water. The organic layer was dried by anhydrous $MgSO_4$, and the AcOEt was evaporated under reduced pressure. Yield 7.2 g (80%) of a crispy foam. The product was dissolved in DCM (30 ml), TFA (30 ml) was added, and the reaction mixture was stirred for 30 min. DCM and TFA were evaporated under reduced pressure, and the oily residue was dissolved in AcOEt and the solution was washed 3 times with water and a saturated solution of $NaHCO_3$. After addition of a solution of Na_2SO_4, three layers were formed. The bottom layer containing the product was separated, acidified by shaking with 5% HCl, dissolved in chloroform, dried by anhydrous $MgSO_4$, concentrated to a small volume, and poured into a large excess of ether. The resulting precipitate was collected, washed with ether, and dried. Yield 5.4 g (64%) of a crispy foam, single spot on preparative layer chromatography, R_f 0.28 in $CHCl_3$/MeOH/AcOH (90 : 9 : 1).

Boc-N(CH_2-COOH)-CH_2-CON(CH_2-CO-O-(CH_2)$_3$-NH<- Gly<-Fmoc)$_2$ (IDA-DC)



Boc-Ida (2.33 g, 10 mmol) was dissolved in DCM/DMF (10 : 1, 50 ml), DIC (1.57 ml, 10 mmol) was added, and the reaction mixture was stirred for 30 min. Then the solution of HN(CH_2-CO-O-(CH_2)$_3$-NH<-Gly<-Fmoc)$_2$·HCl (8.4 g, 10 mmol) in DMF (50 ml) was added, the pH was brought to about 8 by addition of DIPEA, and the reaction mixture was stirred for 1 h. DMF and DCM were evaporated under reduced pressure, and the oily residue was dissolved in AcOEt

(100 ml) and washed 3 times with water, 5% aqueous HCl, water, and finally it was extracted with a saturated solution of $NaHCO_3$. The $NaHCO_3$ extracts were combined and acidified with aqueous HCl, and the solution was extracted 3 times with AcOEt and the organic phase was dried with anhydrous $MgSO_4$, and then the AcOEt was evaporated under reduced pressure. The oily residue was triturated with ether and the product crystallized. Yield 6.8 g (67%). Linker (300 mg) was dissolved in acetonitrite (ACN) (6 ml) and diluted with water (6 ml). A solution of linker (3 ml) was applied to a RP column equilibrated with 40% ACN in water (100 ml). The column was washed with 40% cerium ammonium nitrate (CAN) (100 ml), 45% CAN (100 ml), 50% CAN (200 ml), and 70% CAN (200 ml). Fractions (10 ml) were collected and their purity was checked by thin-layer chromatography in the system chloroform/toluene/MeOH: water (10 : 10 : 10 : 1). Fractions containing homogeneous linker were combined, the ACN was evaporated under reduced pressure, and the residual solution was lyophilized. Yield 188 mg, single spot on thin-layer chromatography, R_f 0.28 in $CHCl_3$/MeOH/AcOH (90 : 9 : 1). ^1H nuclear magnetic resonance (300 MHz, dimethylsulfoxide, 27 °C) δ : 1.36 (9H, Boc), 1.75 (2H, $C\beta H_2$), 3.16 (2H, $C\alpha H_2$), 3.60 (2H, Gly CH_2), 3.76–4.23 (8H, Ida CH_2), 4.11 (2H, $C\gamma H_2$), 4.19–4.34 (3H, Fmoc CH_2 and CH), 7.49 (1H, Gly NH), 7.34, 7.42, 7.72, and 7.89 (8H, Fmoc aromatic H).

12.6.8.2 **Synthesis of the Library**

TentaGel (5 g, 0.23 mmol/g, 130 μm average particle size) was swollen in DMF (swollen resin volume 25 ml) and Fmoc-Lys(Boc)/DIC/HOBt (3 equiv. each) in DMF was coupled. After 2 h the resin was washed 5 times with DMF and once with DCM and the Boc group was removed with TFA/DCM (1 : 1, v/v, 1 + 20 min). After washing with DCM (5 times) and DMF (4 times), the resin was neutralized with DIPEA/DMF (1 : 49, v/v), washed with DMF (3 times), and the linker IDA-DC (Figure 12.14, 3 equiv.) was activated by DIC and HOBt (3 equiv. each) in DMF and coupled overnight. The resin was washed with DMF (5 times) and the Fmoc group was removed with piperidine/DMF (1 : 4, v : v, 20 min). After washing with DMF (3 times) and distribution of the resin into m reaction vessels (plastic vials or fritted syringes), individual Fmoc-protected amino acids were coupled to each part of the resin using DIC and HOBt (3 equiv. each). The reaction was monitored using bromophenol blue. When complete coupling was observed in all reaction vessels (all resin particles were decolorized), completeness of the coupling was verified using the ninhydrin test. All resin portions were combined, washed with DMF (5 times), and the Fmoc group was removed as described above. This procedure (separate couplings and deprotection after combining the resin) was repeated $n - 1$ times ($n =$ number of library positions). The side-chain protecting groups were cleaved by reagent K for 2 h, and washed with TFA (3 times), DCM (5 times), DMF containing 0.1% HC1 (4 times), and 0.1% HC1 in water (5 times). The library has to be stored in an acidic solution in order to prevent premature loss of peptides.

12.6.8.3 **Quality Control of the Doubly Releasable Library**

Dried resin (5–10 mg) was shaken overnight in 2–5 ml of 0.1 M HEPES buffer (pH 8.5) in a polypropylene syringe equipped at the bottom with a polypropylene or Teflon

frit and a polypropylene plunger. The absorbance of the solution (diluted, if necessary) at 280 nm was measured and the amount of released peptide was calculated according to the following formula:

$$\text{Release (mmol/g)} = (\text{absorbance} \times \text{volume} \times \text{dilution})/(1197 \times n/x + 5559 \times m/y) \times \text{mass},$$

where mass is the quantity of library beads in grams, x is the number of amino acids in positions where Tyr is used, y is the number of amino acids in positions where Trp is used, n is the number of positions in the library where Tyr is used, and m is the number of positions in the library where Trp is used. If other amino acids with absorbance at 280 nm were used in library construction, the above formula must be modified. A solution of 0.2% NaOH was drawn into the syringe containing the library sample and the syringe was shaken for 4 h. The solution was expelled from the syringe and the absorbance measured at 280 nm. The same calculation was performed using the formula shown above using coefficients 1507 and 5377 instead of 1197 and 5559, respectively. The amount of released peptide in each step should not differ by more than 10% from the theoretical value, which was calculated according to

$$\text{Theoretical release (mmol/g)} = \text{Subst.}/(1 + \text{Subst.}(3\text{MW} + 686)/1000),$$

where Subst. is the original substitution of the resin (in mmol/g), MW is the average molecular weight of the library peptides, and 686 is the molecular weight of the linker (without Fmoc groups), plus one Lys residue, minus one molecule of water. The average molecular weight of a natural amino acid is 119.7 (19 amino acids, Cys excluded). Therefore, the average molecular weight of a pentapeptide library made from these 19 amino acids is 598.5. Starting with a resin substitution of 0.2 mmol/g, 0.134 mmol of pentapeptide should be released at each step using 1 g of dried library resin.

12.6.8.4 **Two-Stage Release Assay in 96-Well Microassay Plates**

Library beads were transferred into pH 4.5 buffer containing 1.0% carboxymethylcellulose (to slow sedimentation), shaken, and rapidly pipetted into the upper chambers of a vacuum-controlled 96-well filtration manifold (Model 09 601; Millipore, South San Francisco, CA). Approximately 500 beads were placed in each filtration well, so that each plate contains approximately 48 500 unique peptides. The filtration plates serve as "master" plates for retaining subsets of peptides in unique locations. The transfer buffer was removed by vacuum filtration, and the first stage release of peptides was accomplished by dispensing the appropriate buffer or tissue culture medium (neutral pH) to each well and incubating overnight. The released peptides were vacuum filtered into 96-well microassay test plates where the biological activity was determined. In some experiments the released peptides were distributed into several replicate plates for multiple simultaneous assays against different molecular targets. Wells identified as "positive" were marked and the beads of origin were recovered from the corresponding well(s) of the filtration master plate with the

aid of a low-power stereomicroscope. The recovered beads were transferred one by one (one bead per well) into individual microwells of 96-well filtration plates. Cleavage of the ester (second) linker was then accomplished by overnight incubation in ammonia vapors in a desiccator or dedicated pressurized chamber. After drying, the appropriate buffer was added and the plates were gently shaken for several hours. Thereafter, the peptide-containing buffer was filtered into the test plates for bioassay. The individual peptide beads corresponding to each positive well in the second-stage assay were recovered and submitted for microsequencing.

12.6.9
Synthesis of the Positional Scanning Library [210]

The positional scanning hexapeptide library was composed of six sublibraries (O1XXXXX-NH_2, XO2XXXX-NH_2, XXO3XXX-NH_2, XXXO4XX-NH_2, XXXXO5X-NH_2, and XXXXXO6-NH_2), where one position (O) is individually defined with one of 19 amino acids and the remaining five positions (X) are mixtures of 19 amino acids. Thus, the entire library is made up of 114 (19×6) distinct peptide mixtures. Amino acids are mixed for coupling in a molar ratio that ensures equimolar incorporation [210] of amino acids into peptides (Table 12.1). One hundred fourteen polypropylene mesh bags were labeled and loaded with 400 mg 4-methylbenzhydrylamine (MBHA) resin each. Nineteen Boc-protected amino acids were activated by DIC and coupled to bags 96–114, whereas the mixture of 19 amino acids was coupled to bags 1–95. Resins bags 96–114 have a defined amino acid at position 6. The other bags have a mixture of amino acids at that site. After Boc removal, 19 individual amino acids were coupled to bags 77–95 and the amino acid mixtures to the remaining bags. Resin bags 77–95 have a defined amino acid at position 5. This procedure was repeated through to the sixth coupling. The peptides were cleaved, extracted, and lyophilized. Peptide mixtures were dissolved in water at 10–20 mg/ml and stored for 1–2 weeks at 4 °C or were frozen for prolonged storage. The higher final concentration of peptide mixtures in this library compared to the dual defined peptide library compensates for the presence of 19 times more peptides when compared to the latter peptide mixtures (five versus four mixture positions).

12.6.10
Synthesis of the Dual Defined Iterative Hexapeptide Library [210]

Nineteen (or any number corresponding to the number of used building blocks, depending on the capability of the chemist) individually labeled porous polypropylene mesh packets were charged with 20 g of MBHA resin each. Each of 19 of the 20 genetically coded Boc-amino acids (Cys excluded) was activated by DIC and coupled to one of the 19 resin packets. The coupling reaction was monitored for completion by using bromophenol blue or the ninhydrin test. The resin packets were washed with DCM and dried; the resins of all packets were recombined and mixed thoroughly. This one-position resin was referred to as X-resin. The X-resin was divided into 19 equal

portions and placed into new polypropylene mesh packets. The Boc group was removed with TFA/DCM (11 : 9, v/v), and the resin was washed with DCM and 2-propanol, neutralized with DIPEA/DCM (1 : 19, v/v), and washed with DCM. The 19 amino acids were activated by DIC and coupled to the resin packets to generate 361 (19^2) dipeptides. These dipeptides were termed OX-resins. Mixing of all OX-resins affords XX-resin. The coupling steps were repeated twice more to generate a 130 321 tetrapeptide mixture resin (XXXX-resin). The XXXX-resin was divided into 400 equal aliquots and placed in labeled polypropylene mesh packets. The Boc group was removed and the resin neutralized. Two amino acids were coupled to each of the packets using standard coupling procedures. The result was a hexapeptide mixture resin (O1O2XXX-resin) with two defined (O) and four mixture (X) positions. The 400 separate peptide mixtures were deprotected and cleaved using the high/low HF method in a multivessel apparatus. The peptides were extracted from the resins with water or a mixture of acetic acid and water, the solution was lyophilized twice, and peptide mixtures were dissolved in water at 1–5 mg/ml. The peptide library was stored for 1–2 weeks at 4 °C or frozen for prolonged storage. Sonication facilitates the solubilization of peptide mixtures with hydrophobic amino acids at the defined positions.

12.6.11
Acylation Monitoring by Bromophenol Blue [145, 146]

Couplings performed in neutral solution (DIC/HOBt, preformed anhydrides, active esters) can be monitored by addition of trace amounts of bromophenol blue. The sensitivity of the method can be significantly diminished by application of a large amount of bromophenol blue and therefore no more indicator than the amount equal to 1% of available amino groups should be applied. Usually several drops of 0.1% solution of bromophenol blue in DMF or *N*-methylpyrrolidone (if the dissolution provides blue solution, it can be decolorized by the addition of HOBt) are added into the last wash before coupling or directly to the solution of activated acid. Blue-colored beads turn green, greenish yellow, and eventually yellow. The speed of some couplings can be puzzling. Most are complete within 2–5 min; in library synthesis, however, care should be taken about the slowest couplings, which may require much longer exposure. Bromophenol blue monitoring allows the evaluation of coupling at the level of individual beads. In this case a sample of the reaction slurry is placed on the Petri dish and inspected under a microscope. It is easy to detect one incompletely coupled bead in the middle of tens of thousands of beads. Successful application of bromophenol blue monitoring requires the absence of quaternary ammonium salts on the resin, as resin containing these residual functionalities never becomes bromophenol blue-negative. Before using a new batch of solid support, the resin should be peracetylated (if it is not fully protected) and treated with bromophenol blue solution. If blue coloration is observable, bromophenol blue cannot be used for monitoring. In the presence of sulfonium salts (modified side-chains of Met), the resin would be greenish even without the presence of a free amino group.

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13
Phage-Displayed Combinatorial Peptides

Renhua Huang, Kritika Pershad, Malgorzata Kokoszka, and Brian K. Kay

13.1
Introduction

Phage-display is a convenient vehicle for the generation and screening of combinatorial peptide libraries for a variety of purposes. With standard molecular biology techniques, it is now possible to generate phage libraries displaying 10^{10} different peptides, and screen them for peptide ligands to cell surfaces, metals, and proteins, or substrates of proteases, on the time frame of weeks.

The first reported display of an exogenous protein fragment on the surface of bacteriophage occurred in 1985 [1]. In pioneering work by Professor George Smith, a fragment of the β-galactosidase protein of *Escherichia coli* was inserted into a gene encoding a minor capsid protein of M13 bacteriophage and the resulting phage were still infectious. Not only did the resulting chimeric phage particles display epitopes of the β-galactosidase protein, but also the fusion phage could be enriched more than 1000-fold over nonrecombinant phage with antibodies to the β-galactosidase protein. Protocols were subsequently developed that permitted a single fusion phage to be isolated, through affinity selection with antibodies, from an excess of 10^8 nonrecombinant phage particles [2].

Combinatorial peptide libraries that were phage-displayed appeared in three simultaneous publications in 1990. In two of the publications, the linear epitopes of two different monoclonal antibodies were mapped by affinity selecting families of related peptides from libraries of 10^8 different peptides [3, 4]. In the third publication, peptide ligands to streptavidin were selected from a library of 10^8 different peptides [5]. These three publications were unique in that libraries of such a large size were available for the first time and were sufficiently large enough to represent nearly all 6-mer peptide permutations. In addition, these combinatorial peptide libraries were proposed to be the source of peptide ligands to receptors, and thus serve as agonists and antagonists. Since then, phage-displayed combinatorial peptides have been the source of peptides that selectively bind cell and tissue surfaces [6–11], cytosolic proteins [12–14], receptors [15–21], inert materials [22, 23], metals [24], and toxins [25–27].

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There are several advantages of working with phage-displayed combinatorial peptide libraries:

- Once the libraries have been generated, they are very stable and can be stored at −80 °C indefinitely.
- The libraries are renewable, through infection of bacteria and harvesting of the particles from the cultures.
- It is possible to identify peptide ligands of interest from a library with a modest effort, in just a few weeks.
- The identity of selected peptides can be simply deduced by DNA sequencing, which is routine, and at a low cost, at commercial sequencing facilities.

On the other hand, there are also several disadvantages:

- While the concentration of phage particles sounds impressive and approaches 10^{12}/ml, the molarity of the displayed peptides is quite low (i.e., picomolar) and, therefore, biochemical experiments necessitate the use of synthetic forms of the peptides.
- The sequence complexity of the library is below that of the number of recombinants in a library and a negative result may be due to sparse sampling.
- There is biological selection against odd numbers of cysteines [28], runs of positive charges [29], and certain residues at fixed positions within the displayed peptide [30]; consequently, selection results may be inherently biased.
- Phage-display typically only yields binding or bioactive peptides that "work" and as a result one can only infer, from the absence of data, what does "not work."

Thus, based on the last three limitations, phage-display is more of a survey tool, rather than an analytical one: whenever one isolates peptides of interest through phage-display experiments, it is generally beneficial to synthesize the isolated peptides, and sets of related sequences, for careful, qualitative and quantitative analysis.

13.1.1 Types of Phage Vectors

The most popular phage-display systems are based on bacteriophage that infects *E. coli*, and, of these, M13 bacteriophage is the most popular. (Other bacteriophage systems, such as λ [31, 32] and T7 [30, 33–35] have also been used for display purposes, but they will not be discussed here.) The M13 bacteriophage particle, referred to hereon as simply "phage," consists of a single DNA molecule and five different coat proteins. These coat proteins, termed the protein products of genes III, VI, VII, VIII, and IX (i.e., pIII, pVI, pVII, pVIII, and pIX), package the DNA into a stable macromolecule that is infectious for *E. coli*. Figure 13.1 presents a cartoon of the M13 bacteriophage particle, highlighting the relative locations of the various coat proteins and DNA. While over the years all five capsid proteins have served to display peptides or proteins [36], the most commonly used vectors display at the N-terminus of pIII or pVIII, although it has been possible to display peptides at the C-terminus of the pIII [37]. It should be noted that pVIII has been engineered to improve display

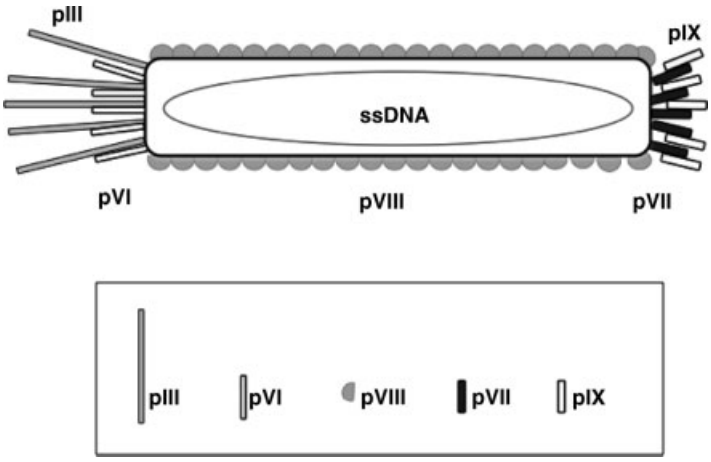
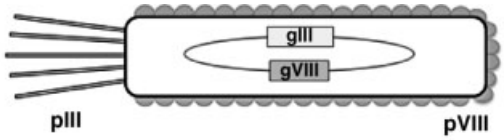


Figure 13.1 M13 bacteriophage particle. A single-stranded DNA (ssDNA) genome is encapsulated by 2700 copies of major coat protein pVIII and capped with five copies of each minor coat protein (i.e., pIII, pVI, pVII, and pIX).

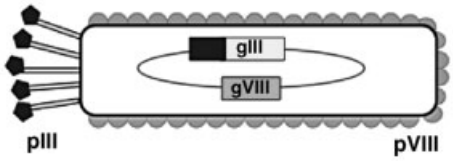
efficiency [38], along with a reduction in its number of positive charges [39], which decreases nonspecific interactions of phage particles with protein targets that have high isoelectric points.

There are two basic types of phage-display particles – those packaged in *E. coli* with proteins encoded by the viral genome and those packaged with proteins supplied by a helper virus [40]. Using a nomenclature suggested by Smith [41], in type 3 or 8 display, combinatorial peptides are displayed on all five and 2700 copies of pIII or pVIII, respectively (Figure 13.2). In type 3 + 3 or 8 + 8 display, there is a mixture of both wild-type and fusion capsid proteins incorporated into phage particles, with the recombinant pIII or pVIII proteins (i.e., fusions between the displayed peptide or protein with the capsid protein) encoded by phagemid genomes and the wild-type proteins encoded by helper phage (i.e., M13K07, VCS-M13). In type 3 + 3 and 8 + 8 display, the combinatorial peptides would be displayed once to hundreds of times, respectively, on phage particles. Finally, in type 33 and 88 display, there are two copies of gene III or VIII, respectively, in the viral genome – one wild-type and one recombinant. Consequently, phage particles bear both wild-type and recombinant capsid proteins, similar in display frequency as the 3 + 3 and 8 + 8 systems.

One major consequence of the different vectors is that the number of copies of the combinatorial peptide, which are displayed on a phage particle, will vary. In some cases, the number of copies displayed on pIII will be pentavalent (i.e., type 3 display), and in other cases it will be monovalent (i.e., type 3 + 3, type 33). Conversely, the number of copies displayed on pVIII will be around 2700 (i.e., type 8 display) and in other cases it will be 50–200 (i.e., type 8 + 8, type 88). Effectively, these differences in valency lead to differences in the apparent affinity of the peptide ligands for their targets, through the phenomenon of avidity [42] (i.e., when more than one peptide ligand is displayed on a phage particle, it has the opportunity to bind to two or more



Wild-type M13 phage



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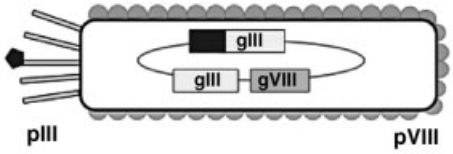
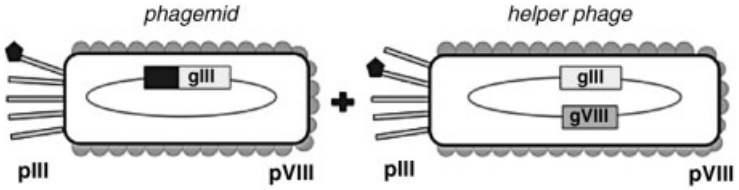
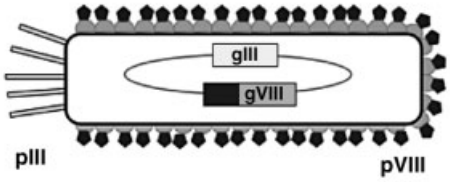


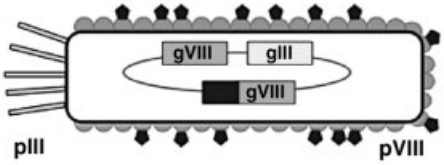
Type 33



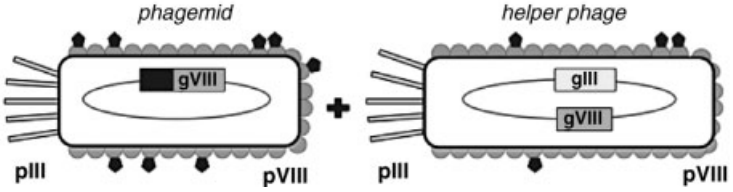
Type 3+3



Type 8



Type 88



Type 8+8

targets simultaneously). Generally, one screens pentavalent or multivalent display libraries for peptides with weak dissociation constants (i.e., 3 μM to 300 nM) and monovalent display libraries for peptides with stronger dissociation constants (i.e., below 300 nM).

13.1.2 Generation of Combinatorial Peptide Libraries

The earliest combinatorial peptide libraries were constructed in one of two ways. In one method, an oligonucleotide is annealed to sticky ends of a vector DNA molecule and converted to double-stranded DNA *in vitro*, before introducing the DNA into *E. coli* via electroporation [43]. In another method, one oligonucleotide is annealed to another, generating double-stranded DNA (after fill-in with DNA polymerase), which is then digested with restriction endonuclease enzymes, resulting in insert fragments that are then ligated with double-stranded vector DNA and electroporated into *E. coli* [28]. Electroporation is the method of choice for generating large phage-display libraries in *E. coli*, as it creates libraries of 10^9–10^{11} members.

Currently, the procedure of choice for constructing combinatorial peptide libraries is to use the method of Kunkel mutagenesis [44–46]. In this technique, single-stranded DNA is prepared from bacteriophage M13 particles, which are secreted by *E. coli* that carry the *dut*$^-$ and *ung*$^-$ mutations. (DNA molecules propagated in such bacteria errantly contain uridine at sites that would normally be thymidine.) The single-stranded DNA is annealed to an oligonucleotide, which contains around 21 complementary bases at each end and which has a degenerate region in the center. As seen in Figure 13.3, this partial double-stranded DNA then serves as a template for DNA synthesis by DNA polymerase, which is converted to double-stranded DNA *in vitro*, as is evident by an apparent change in mobility of the product DNA during agarose gel electrophoresis. This method is very efficient in generating large

Figure 13.2 Phage-display vectors. Type 3 and type 8 represent two different phage-display systems. The phage genome bears one copy of gene III (gIII, light gray box) or gene VIII (gVIII, dark gray box) fused to a segment encoding the foreign peptide (black box). As a result, the respective coat proteins (pIII or pVIII) display the same fusion protein (black pentagon). Type 33 and type 88 represent hybrid phage-display systems, where the phage genome bears, respectively, two copies of gene III or VIII, one of which is wild-type and the other recombinant. Since the wild-type pIII (or pVIII) is generally expressed at higher level than the fused form in a bacterial host, only a small number of fused pIII (or pVIII) molecules generally incorporate into the assembled phage particle. Type 3 + 3 and type 8 + 8 represent two phagemid systems. In these systems, the phage particle contains phagemid genome, which can propagate as circular plasmids in *E. coli*, but can also be packaged and secreted as phage particles, with the aid of a helper virus. In this last phage-display system, two separate genomes bear copies of gene III (or VIII): wild-type on the helper phage, recombinant on the phagemid. Two types of phage particles are synthesized and secreted by the infected bacteria (i.e., one with helper phage genome and one with phagemid genome). Both types of virions are mosaic, displaying only a small number of recombinant pIII (or pVIII).

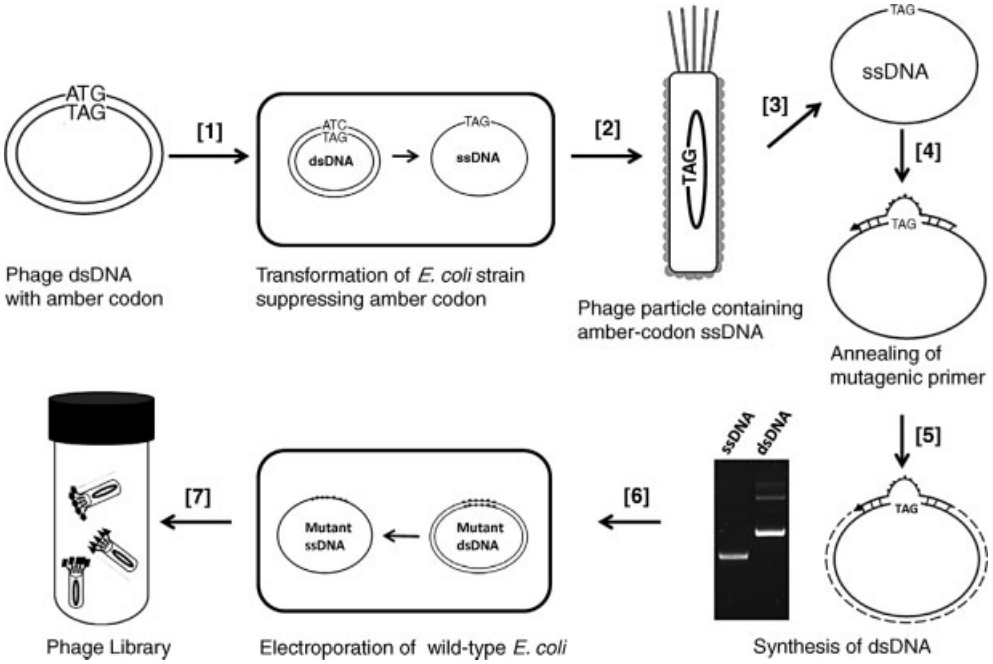


Figure 13.3 Construction of combinatorial peptide libraries. In a modified protocol of Scholle *et al.* [53], an amber codon (TAG) is used for negative selection of nonrecombinant genomes, with the goal of achieving up to a 100% mutation rate. (1) The amber codon is inserted into the N-terminal of gene III of replicative form of double-stranded M13 phage DNA (dsDNA), which is then transformed into an *E. coli* strain (i.e., TG1), which carries a mutant tRNA that recognizes the amber code and inserts a glutamine during translation. (2) Transformed *E. coli* cells produce phage particles containing single-stranded DNA (ssDNA), (3) which is used as the template of the double-stranded DNA synthesis. (4) Mutagenic primers containing randomized stretches of DNA are annealed to the single-stranded DNA. (5) The double-stranded DNA is synthesized from annealed single-stranded DNA in the presence of DNA polymerase and ligase. (6) The synthesized double-stranded DNA is transformed into wild-type *E. coli*, in which the parental strand (with the inserted amber codon) cannot be translated and amplified over generations, while the mutant strand can be translated and further amplified. (7) A phage-display peptide library is generated by the production of mutant phage particles displaying the peptides of different sequences fused to the protein III of phage surface. The library is then used for affinity selections.

(i.e., 10^9–10^{10}) libraries in less than 1 week [47]. A modified Kunkel protocol for generating combinatorial peptide libraries relies on the insertion of an amber stop codon (TAG) in the gene III of the M13 bacteriophage genome [53] (Figure 13.3). Like described above, the amber codon-containing single-stranded DNA serves as the template for the generation of double-stranded DNA after annealing to the mutagenic oligonucleotides, and when the synthesized double-stranded DNA is electroporated into wild-type strain of *E. coli*, nonrecombinant DNA molecules are lost during

amplification step due to the retained amber codon. This modified Kunkel mutagenesis approach allows efficient generation of phage-display peptide libraries consisting of as much as 100% recombinants.

Generally, the region that is randomized is degenerate and can encode any amino acid sequence of fixed length. Typically, one chooses NNK or NNS coding schemes, rather than NNN, where N is an equimolar fraction of A, C, G, or T nucleotides, K is G or T, and S is C or G. The value of using NNK or NNS codons is that the number of oligonucleotide permutations more closely represents the number of different amino acid permutations than the NNN scheme (i.e., 32^x is closer in value to 20^x than 64^x, where x is the number of amino acids being randomized). In the NNK scheme, 32 codons encode one stop codon (TAG), which is suppressed in certain strains of *E. coli*, and all 20 amino acids, which are represented once (C, D, E, F, H, I, K, M, N, Q, W, Y), twice (A, G, P, V, T), or three times (L, R, S). While it is possible to encode a subset of amino acids with other codon schemes, there has been significant interest in using 20 trinucleotide phosphoramidites [48–50], thereby permitting an exact correspondence between the complexity of the DNA library and its coding potential [51]. The commercial availability (Glenn Research, Sterling, VA, USA; www.glenresearch.com) of the trinucleotide phosphoramidites should accelerate their use in synthesizing oligonucleotides for constructing combinatorial peptide libraries, which are based on triplet codons.

While it is anticipated that the phage libraries will display most peptide permutations, it is often not the case for several reasons:

- As the phage are assembled and secreted in an oxidizing environment, there is a negative selection of peptides with an odd number of cysteines [28].
- Peptides with runs of positively charged amino acids tend to interfere with the proper insertion of pIII into the inner membrane of *E. coli* [29].
- Proteases endogenous to *E. coli* may destroy or clip the displayed peptide off the surface of the phage particles [48, 52].
- Certain amino acids adjacent to the signal sequence of the coat protein may interfere with cleavage of the precursor form by signal peptidase [53].
- As the number of sequence permutations can exceed the number of clones, such libraries have sparse representation. For example, a library of 7-mer and 8-mer peptides would need 1.2×10^9 and 2.4×10^{10} clones to represent all 7-mer (i.e., 20^7) and 8-mer (i.e., 20^8) peptides. Since the limits of electroporation are typically 10^9–10^{10}, generally not all permutations will be present in such libraries.

Recently, it has been possible to expand the chemical diversity of phage-displayed peptides through the mechanism of tRNA suppression. An amber codon, TAG, can be engineered into a peptide-coding region and when the resulting mRNA is translated *in vivo*, a mutant tRNA, which has been charged by a synthetase engineered to utilize unnatural amino acids, will complete protein synthesis. Professor Peter Schultz and colleagues at the Scripps Research Institute (La Jolla, CA, USA) have used this general approach to incorporate over 70 unnatural amino acids into proteins [54]. This technique of expanding the genetic code has been applied to phage-display, as demonstrated with the incorporation of five distinct unnatural

amino acids [55] into peptides or sulfotyrosine into the complementary determining region of a phage-displayed antibody fragment [56]. In the future, this approach is likely to be used more widely, as a way of increasing the chemical diversity of phage-displayed peptides and proteins.

13.1.3 Identifying Peptide Ligands to Protein Targets

One of the most powerful applications of phage-displayed combinatorial peptide libraries is to screen them for peptide ligands to target proteins (Figure 13.4). To accomplish this task, one immobilizes a target protein either directly or indirectly on

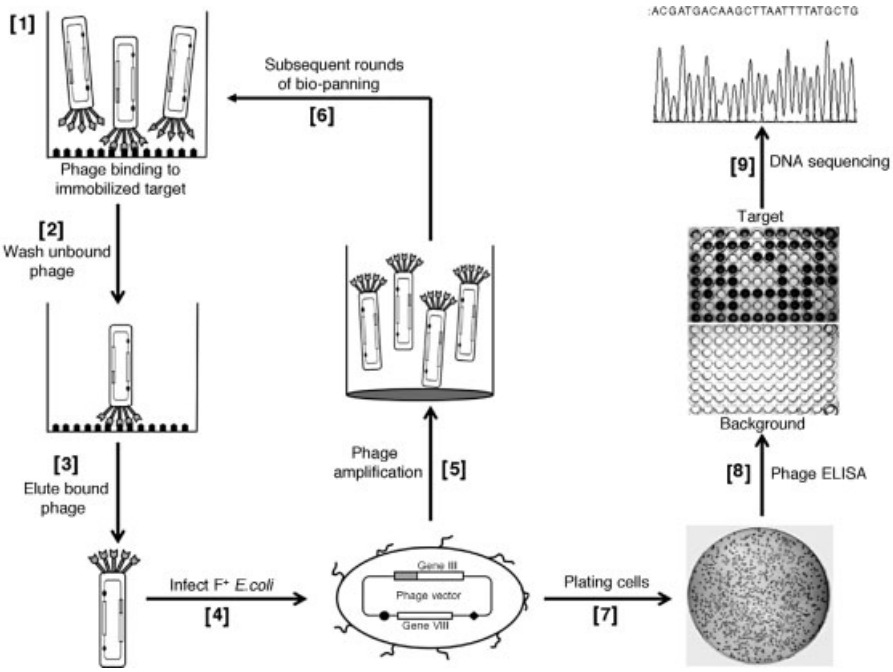


Figure 13.4 Screening for peptide ligands. (1) Phage particles, which are displaying combinatorial peptides, are first incubated with the immobilized target protein. (2) The unbound phage particles are removed by washing in the presence of detergent, followed by (3) recovery of the bound phage with exposure to low pH conditions. (4) Bacterial cells with F-pilus (F^+) are infected en masse with the eluted phage and (5) amplified overnight. (6) The resultant phage particles are subsequently used for the second round of affinity selection. (7) After two or three rounds of affinity selection, the phage-infected cells are plated, after diluting them, and phage are amplified from individual plaques. (8) The individual phage clones are analyzed, by phage ELISA, for binding to the target protein and the negative control (background). (9) DNA is isolated from the binding phage and sequenced to identify the binding peptide sequences.

a surface, incubates it with an aliquot of phage particles that are displaying combinatorial peptides, removes excess phage particles, and recovers phage that potentially binds to the target. Generally, after three rounds of affinity selection, one tests individual clones for binding. When an experiment goes well, 10–30% of the tested phage clones will bind to the target, but not to the negative control. Growth of binding phage clones, preparation of DNA, and sequencing the insert of verified binding clones would then reveal the amino acid sequence of the peptide ligands for the target. Many of these steps are discussed below.

To discover peptide ligands to a target, it is essential that the target protein be properly folded. When proteins adopt their correct three-dimensional structure, they often create pockets into which short peptides can bind, and such concave surfaces are often binding sites for small molecules or "hotspots" for protein–protein interactions [57]. For example, it is possible to isolate peptide ligands through phage-display that bind to the active sites of enzymes, such as β-glucosidase [58], β-lactamase [59], dihydrofolate reductase [60], HIV reverse transcriptase [61], and tRNA synthetases [58], to name a few. It is also possible to identify peptide ligands to a variety of protein targets, such as calmodulin [62, 63], oxidized thioredoxin [64], and protein interaction modules [65–71]. While the publication records of isolating peptide ligands to denatured proteins [72] or linear peptides are sparse [73, 74], most scientists believe that protein targets need to be well-folded and with pockets for them to yield peptide ligands when screened with phage-displayed combinatorial peptide libraries.

Another important design element of a successful affinity selection experiment is to immobilize the protein target on a surface, directly or indirectly. In direct immobilization, a polystyrene tube or microtiter plate well is coated with the target. Thus, phage that bind the target will be retained in the tube or well after washing and can be recovered for the next round of screening. It is also possible to incubate the phage-display library with a soluble protein target, and then capture the target and any bound phage particles, prior to washing away nonbound phages. For example, the target can be biotinylated [75], incubated in solution with the library of phage, and then captured on streptavidin-coated magnetic beads, prior to washing. The advantage of this type of indirect capture method is that the concentration of soluble target can be adjusted for the anticipated affinity of the interaction and the dynamics of interaction should be more complete in solution than on a surface. Regardless of the route of immobilization, a few things should be kept in mind:

- Care should be taken to minimize the selection of phage particles that display peptides that bind directly to polystyrene [76] or streptavidin [5].
- If possible, the target should be immobilized or captured in a way that retains the three-dimensional structure, or binding ability, of an interacting moiety [77].

Once phage have been recovered and confirmed to bind selectively to the target of interest, the primary structure of the peptide ligands is determined by DNA sequencing. From the predicted primary structure of the binding peptide, one looks for two kinds of patterns. (i) Was more than one copy of a peptide ligand isolated? Very

often, sibling clones are isolated two to 20 times, which strengthens the confidence of the investigator in the significance of the peptide as a ligand. (ii) Is there a motif among the selected peptide ligands? Typically, targets have a single or dominant peptide binding site, and the amino acid residues of the peptide ligands can be aligned, with the goal of identifying a motif, which represents the minimal binding sequence. While the alignment can be accomplished by eye, it is possible to use the LOGO method [78] to display consensus sequences. Tools for generating such plots are available on the web [79, 80].

To explore the significance of a motif shared between peptide ligands for a particular target, one can perform alanine scanning on the phage. In alanine scanning [50–52] (Figure 13.5), individual amino acid residues are replaced one at a time in the displayed peptide, and the impact on binding assessed in a phage enzyme-linked immunosorbent assay (ELISA, Figure 13.6). Using the same techniques for generating combinatorial peptide libraries, one can generate a set of phage particles that alanine-scan a particular peptide ligand sequence and thereby determine which residues contribute the greatest to binding the target.

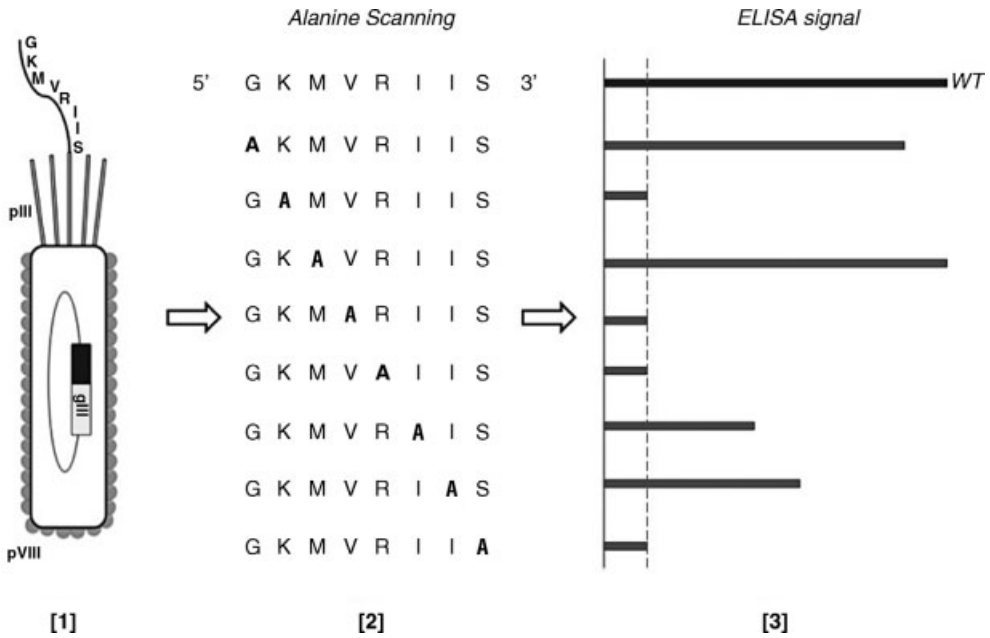


Figure 13.5 Alanine scanning. (1) The peptide sequence, NH_2-GKMVRIIS-COOH, is fused to the N-terminus of the minor coat protein pIII in a phagemid genome. (2) Alanine substitutions are created to analyze the contribution of individual amino acid residues in the peptide ligand to binding. (3) All mutated sequences displayed on recombinant phagemid particles are analyzed via ELISA. The dashed line represents background signal. The results indicate which residues from the peptide sequence are important for binding and which ones are likely not to be important.

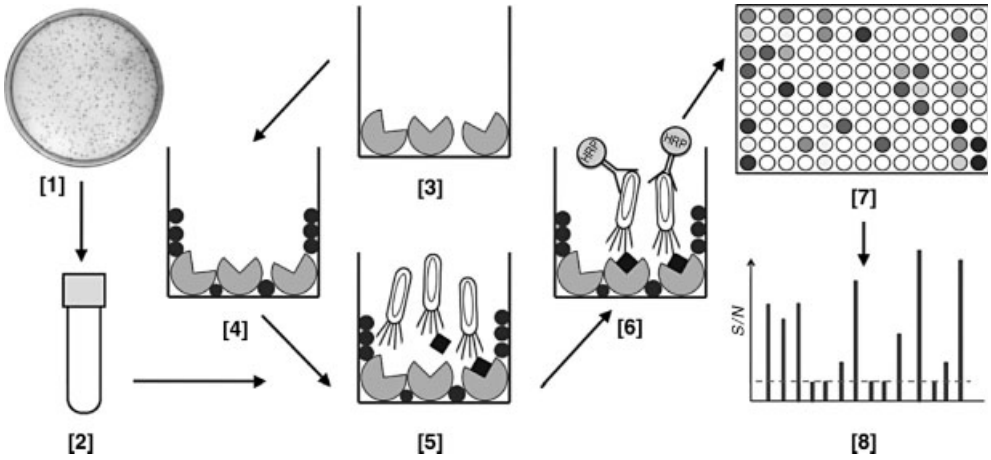


Figure 13.6 Phage ELISA. (1) Single recombinant phage clones on a Luria–Bertani Petri plate. (2) Phage particles amplified in a liquid medium. (3) A 96-well assay plate coated with target protein (light gray). (4) Remaining binding sites on a surface of the plastic well saturated with blocking agent (dark gray). (5) Peptide displaying phage particles incubated in the presence of target protein. (6) Binding of the peptide to the target protein detected via phage-specific horseradish peroxidase conjugated antibody (anti-M13-HRP). (7) Quantitative analysis performed through absorbance measurements. (8) Data plotted on the graph as the function of absorbance (signal). The dashed line represents background signal (noise). S/N = signal-to-noise ratio.

13.1.4
Mapping Protein–Protein Interactions

Protein–protein interactions are involved in virtually all known biological processes. Proteins interact with one another via some regions of the protein surface known as epitopes, which are conventionally classified as *continuous* (or *linear*) epitopes and *discontinuous* (or *conformational*) epitopes depending on their continuity in the primary structure. The structure of epitopes can be defined by X-ray crystallography, which is not readily applicable due to the unpredictable nature of crystal formation by protein complexes. For proteins with known sequences, generation of a set of synthetic peptides and scanning the entire sequence of the protein, can be used to define linear epitopes [81]. As this approach is relatively expensive and requires knowledge of the primary structure of the interacting protein, phage-displayed combinatorial peptides libraries are an attractive alternative experimental option.

Screening of phage-display libraries of random peptides can define residues or segments of peptides that contribute to a particular protein–protein interaction. Among different types of protein–protein interactions, antibody–antigen interactions are among one of the best-studied model systems [82–85]. To map the epitope on the antigen, the isolated antibody is used for biopanning with a phage-display library of random peptides. After three rounds of affinity selections, enriched phage clones are tested for their binding to the antibody. All the confirmed binders are

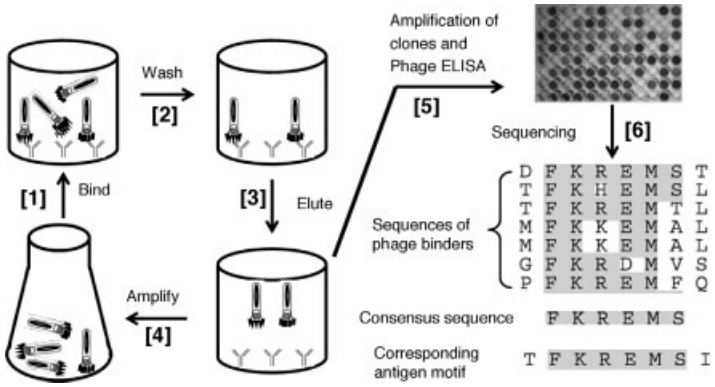


Figure 13.7 Mapping of antigenic epitope by phage-display. A monoclonal antibody is raised against a particular antigen (i.e., nucleocapsid protein of Hendra virus). To map the antigenic epitope, a phage-displayed combinatorial peptide library is screened for potential binders to the monoclonal antibody. (1) A phage-display peptide library is incubated with the immobilized monoclonal antibody. (2) Nonbinding phage particles are removed by extensive washing. (3) Bound phages are eluted and used to infect cells for further amplification. (4) Eluted phages are amplified for next round of screening. (5) After three rounds of screening, enriched clones are plated as individual colonies, which are picked for amplification and for testing their binding to the monoclonal antibody by phage ELISA. (6) Phage clones binding to the monoclonal antibody are sequenced. The consensus sequence of the binding peptides is revealed by alignment and is compared to the entire antigen sequence. The conserved sequence between the consensus sequence of binders and the antigen is the antigenic sequence. Here, the mapping experiment revealed that the isolated monoclonal antibody binds to the linear epitope, FKREMS, of the nucleocapsid protein of the Hendra virus [58].

sequenced and their sequences are aligned to reveal a consensus, which can be compared to the primary structure of the antigen to identify the epitope (Figure 13.7). Defining the epitopes of antibody–antigen interactions leads to numerous, broad applications, notably the illustration of the fundamental mechanisms of molecular recognition [86], and the development of diagnostic [87–91], therapeutic reagents [20, 92–97], and vaccines [98–103].

Very often, one can identify a peptide ligand to a protein target in just a few week's time. Such peptides tend to bind to just a small number (i.e., one or two) of sites on a target, as revealed by binning of ligand sequences into one or two consensuses and by competition experiments (Figure 13.8). When this happens, there are several potential experimental outcomes:

- The primary structure of the peptide consensus often matches that of cellular proteins, which is very useful in mapping protein–protein interactions in cells [13, 104, 105]. This method of predicting protein–protein interactions in a cell has been applied at the genomic level for protein interaction modules such as the PDZ domains of nematodes [70], the SH3 domains of yeast [71], and numerous WW domains of mammals [69].

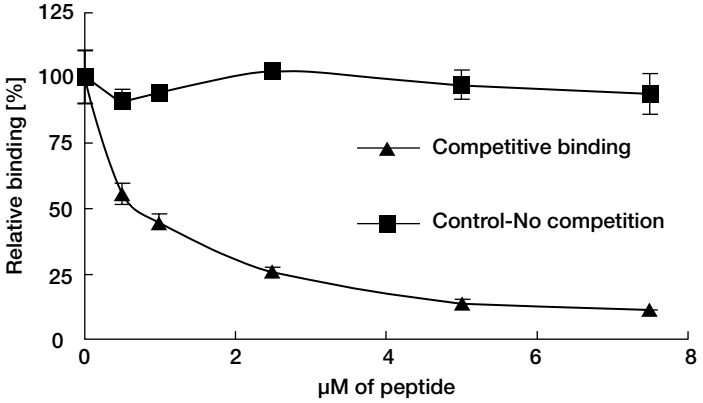


Figure 13.8 Competitive binding of peptides. In an enzyme-linked binding assay, an alkaline phosphatase-linked peptide ligand is incubated with its target in the presence of two different peptides – an experimental one that binds to the same target as the alkaline phosphatase-linked ligand (triangle) and a negative control (square). As the concentration of experimental peptide increases, the amount of alkaline phosphatase-linked ligand binding to its target decreases. Conversely, increasing the amount of control peptide did not reduce the binding of alkaline phosphatase peptide ligand to the target (unpublished data).

- Such peptide ligands can be used to follow allosteric changes in the target protein, such as when the estrogen receptor binds estrogenic compounds [106, 107] or when small G-proteins bind GDP or GTP [108, 109].
- Peptide ligands can be used to inhibit protein–protein interactions for proof-of-principle experiments regarding target validation or drug design [110–114].

13.1.5
Identifying Peptide Ligands Binding to Cell Surfaces

Very often investigators are interested in surveying what membrane proteins may be uniquely present on the surface of cancerous cells and thus may serve as biomarkers of disease. One approach is to affinity select peptide ligands to the cell surface by phage-display and then focus on those ligands that show preferential binding to cancerous instead of wild-type cells. This approach has been used to identify peptide ligands to targets present on cells grown in tissue culture [115], endothelial cells [6, 116], and various organs [6, 117–123], as well on parasites (or parasite infected cells) [101, 124, 125]. Such peptides have been attached to small molecules for efficient delivery of drugs or imaging reagents [11]. Synthetic forms of the peptides have also been used to inhibit metastasis in animal studies [126, 127], block parasite infection [128], or potentially stop viral infection [111].

While there are many notable successes with this approach, there are several technical challenges of note:

- The cell surface is very complex and thus one has no control of what the phage-displayed peptides will bind; for example, they can bind to an abundant

receptor (i.e., plasma membrane protein, extracellular matrix component) or they can bind to a rare one for which there just happens to be a good ligand in the library. Thus, there is no experimental preference for isolating peptide ligands to cell surface targets that are unique to a particular cell type, although there is some recent evidence that subtraction of ligands to common targets (i.e., shared between wild-type and cancerous cell surfaces) is possible [129].

- Binding of phage particles bearing peptide ligands to a cell surface component can lead to internalization of the phage particle [130], which means precautions need to be taken to minimize uptake (i.e., 4 °C) or phage particles need to be rescued from the interior of cells by detergent [131].
- The format for affinity selections is more complicated than typical phage selections with pure protein captured on microtiter plate wells or magnetic beads. Special care must be taken not to lose cultured cells off the substrate, as well as additional rounds of selection are necessary to yield a population of binding phage particles [132].
- Even if a peptide ligand for a cell or organ type is isolated from a library, the identity of the target is unknown, which can limit experimental verification of the usefulness of the peptide ligand and its corresponding binding partner.

13.1.6
Mapping Protease Specificity

Phage-displayed combinatorial peptide libraries have also been very useful in mapping the specificity of proteases [133, 134]. This technique, pioneered by Matthews and Wells [135], takes advantage of the fact that the M13 bacteriophage particle is resistant to proteases and remains infectious after several hours of incubation. Thus, to map the specificity of a protease, one captures a library of phage particles, displaying different peptide sequences, to a solid support, adds protease, and the only phage particles that will released will be those that contain a cleavage site in the linker region encoding the combinatorial peptide (Figure 13.9). Capture can be accomplished with a short epitope-tag [136] or a biotinylated AviTag [137]. This approach is remarkably efficient and has been used to map the specificity of a variety of proteases, such as anthrax lethal factor [138], caspase [139], furin [140], metalloproteases [141], prostate-specific antigen [142], serine proteases [143], tissue-type plasminogen activator [144], and trypsin variants [145].

13.1.7
Identifying Peptide Ligands to the Surfaces of Inert Materials

A recent application of phage-displayed combinatorial peptides has been to discover peptides that bind to inert material. While it has been known for a long time that certain peptide sequences can bind polystyrene [76], metal oxides have proven to be fruitful targets; for instance, peptides have been isolated that bind to gold [146], semiconductor materials [23], silver [147], and titanium oxide [148]. These peptides have been used to fabricate novel nanoparticles [149–154].

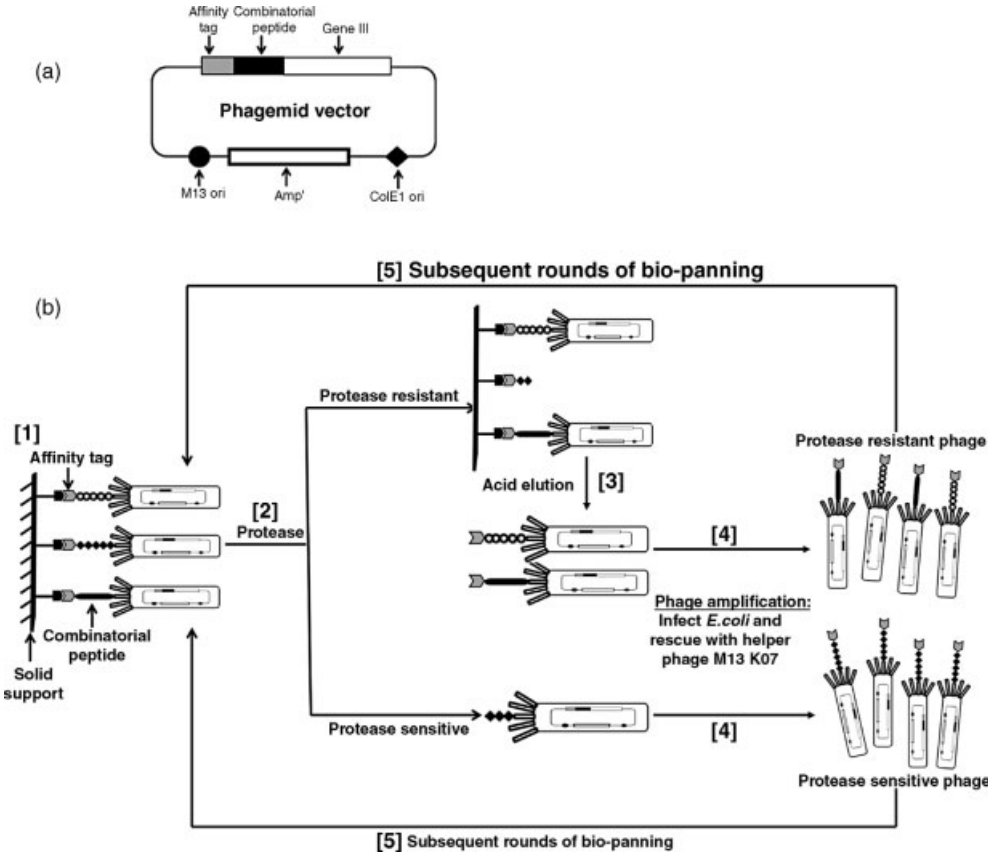


Figure 13.9 Screening for protease specificity. (a) Phagemid vector map. The combinatorial peptides are fused to the N-terminus of the gene III coding sequence. An affinity tag precedes the peptide gene III fusions. The phagemid DNA has β-lactamase gene for ampicillin resistance, and, bacterial and phage origin of replication. (b) Bio-panning using phage-displayed combinatorial peptide libraries. (1) Phage particles, which are displaying combinatorial peptides, are first immobilized to a solid support via the N-terminal affinity tag. (2) This is followed by incubation with the protease. (3) Phage particles displaying a protease cleavable sequence get eluted, whereas the protease resistant phages are eluted under acidic pH. (4) Both phage pools are amplified separately and (5) used for subsequent rounds of affinity selection. DNA samples isolated from the individual phage clones, after two or three rounds of biopanning, are sequenced to identify the protease-sensitive as well as the protease-resistant peptide sequences.

13.2 Conclusions

Thus, as described herein, phage-displayed combinatorial peptide libraries are a rich source of peptide ligands for a variety of biological and nonbiological materials. This

technique is relatively simple and inexpensive to implement in the laboratory, and can provide results in a matter of weeks. Coupled with chemical synthesis of peptides, it is a very powerful discovery tool that can lead to many basic science, industrial, and therapeutic applications.

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14
Designing New Proteins

Michael I. Sadowski and James T. MacDonald

14.1
Introduction

14.1.1
Why Design New Proteins?

Natural proteins exist that perform almost every useful function: they act as motors (both linear and rotary), chemical catalysts, inhibitors, promoters, switches, levers, generators, sensors, and structural components [1]. With such an impressive array of diverse functions already known, the substantial number of natural proteins have the potential to provide a complete tool kit for nanoscale engineering.

However, nature is a tinkerer, not a designer, and although we know of proteins that can perform all of these functions, the redeployment of natural proteins in other contexts is not always straightforward. Since there is no selection pressure for the protein to be active and stable outside the usually quite narrow range of environmental conditions in which it is used by its host organism it can be difficult to produce useful quantities of functional protein outside its native context.

For engineering purposes it is therefore necessary to understand the principles of protein structure, function, and stability in enough detail to be able to create our own. There are two principal means by which this can be achieved: computational design and directed evolution. Computational design is potentially the most cost-effective method, but there are significant limitations on our ability to simulate systems on a molecular scale and therefore at present it is necessary to optimize the details of designs either manually or by a process of directed evolution in the laboratory. Directed evolution has been used as a method of creating novel proteins, as well as for re-engineering known proteins for new applications and new functions. Combining these methods provides the advantages of both, and several recent successful designs have used this strategy.

The properties of most natural proteins depend on their chemical and physical environment, and may require several other interacting partners and very specific environmental conditions to be functional. Engineering, on the other hand, requires

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modular components – components that do not function differently depending on the environment. The proteins found in nature demonstrate that this is at least to some extent possible, with functional domains being reused in a variety of contexts as interaction partners in a new quaternary arrangement, as members of multidomain proteins, or as components of new pathways ([2–4]). Designing modular proteins is essential for the development of synthetic biology applications for novel protein components, since without modularity of this kind the use of proteins as off-the-shelf components (as we are used to with electronic components such as resistors) will not be possible [5].

The most significant goal of protein design is to create catalysts for reactions not found in living organisms. Although nature has provided us with a huge number of examples to follow there are many potential applications of proteins for which no natural equivalent is likely to exist, such as designing new materials or catalysis of reactions that involve compounds not found in nature. Although nature has already begun to react to some man-made compounds [6], we cannot expect to find everything we need in the enormous protein library provided by living organisms. Medical and biotechnological applications are especially interesting since understanding how proteins can be made to interact arbitrarily with small molecules, DNA, and other proteins is the first step towards low-level control of existing biochemical systems. In addition to its benefits as engineering there is significant interest in protein design as a means to understand natural systems and the rules governing protein behavior in general. Progress in design is likely to be significant for further understanding of evolution on the molecular level.

14.1.2 How New is "New?"

Although the idea of a new protein is simple there are several levels on which a protein can be considered new. In the most literal sense a new protein is one with an amino acid sequence not previously seen in nature. However, on a larger scale we could ask whether the protein has a structure or function that is very similar to or very different to those that have already been used by living organisms. In order to make proteins to perform arbitrary tasks it may be necessary to consider proteins that are novel both in the sense of new amino acid sequences and new structures.

Although the number of possible sequences dwarfs even those that are likely to have existed, it is not clear how much scope there is for novel structures. Protein structures have been found to cluster in groups with similar overall arrangements of α-helices and β-sheets known as folds. A key observation found relatively early in the growth of protein structure databases was that some of these fold groups are extremely highly represented in the genomes of living organisms and perform a wide range of different functions. Clearly the age of the fold group is important since older proteins will have had more opportunity for re-use, but the concept of designability (i.e., the size of the sequence space compatible with a given structure) has also been offered as an explanation for this.

This idea is potentially of great importance to the protein designer: studies on lattice proteins have shown that not all structures have an equal size of sequence space that folds into them [7], but that a relatively small number of possible structures may have a sequence space at least 1000 times greater than the majority. Such highly designable structures will be considerably more fit from the point of view of adopting multiple functions and occupying different niches within the organism, since more sequence diversity will inevitably create more functional diversity.

This has implications for the design of new proteins: more designable folds will have greater stability and can therefore tolerate more functional constraints, providing more scope for design of new functions or multifunctionality; additionally, they provide a much bigger target for design methods since the likelihood of getting the right fold despite inaccuracies in the energy function is much higher.

On this basis it may be possible to achieve almost any desired function using one of the highly populated "superfolds" [8] such as the TIM-barrel, immunoglobulin, or Rossmann fold. This is clearly a strategy that has been successful in nature, and the immune system provided some of the first templates used in protein design since immunoglobulins are by nature intended to be able to recognize arbitrary new chemical surfaces and are also known to be able to support catalysis. However, it has been shown using a simple topological description of protein folds that the space of natural protein folds is surprisingly empty [9] and with the peak of observation of new folds having occurred some years back it seems likely that this is true in general [10]. It is not yet clear to what extent this is indicative of the physical designability of protein structures or the consequences of evolutionary sampling.

Since our needs and those of nature are not in general identical we might need to consider the design of proteins using hitherto unseen protein folds. This may offer solutions to the problems of multiple constraints, for example, or may simply be more practical. Additionally, once the question of designing proteins with specific dynamical properties can be addressed it is possible that arbitrary backbone conformations will be required for many useful applications.

14.2
Protein Design Methods

Successful protein design is critically dependent on producing a sequence that folds into a unique, well-packed hydrophobic core and thus a stable structure that can be used as the basis for engineering of a catalytic active site or binding site for interactions with other molecules. Initial strategies for protein design included careful manual design using disulfide bonds to achieve stability [11] or specification of the patterning of hydrophobic and hydrophilic residues [12–14]. However, it proved difficult to achieve a fully native-like stable structure by these methods, with deficiencies in hydrophobic packing leading to only partially folded, molten-globule states. The introduction of computational modeling to optimize packing (as originally conceived by [15]) was found to solve the problem and allow the specification

of fully native-like structures [16]. Computational modeling is now the mainstay of protein design, increasingly combined with a directed evolution strategy for the optimization of stability and function. We will describe both of these methods in the next two sections. We also consider how functional constraints can be introduced in computational design methods and discuss how both methods have been used in the design of novel protein–protein interfaces.

14.2.1 Computational Design

Computational protein design can be split into two problems: (i) finding a backbone conformation that can be designed and (ii) optimizing the sequence for a given backbone (sometimes called the "inverse folding" problem). The aim of inverse folding is to minimize the $\Delta G_{\text{folding}}$ for a particular folded backbone conformation (or ensemble of conformations) such that the protein spends almost all of its time in the folded ensemble.

In theory, if one had a potential energy function that accurately represented the physical forces a protein is subject to, $\Delta G_{\text{folding}}$ could be directly computed and an optimal protein sequence for a given backbone would be simple to find. In practice, such a measurement would be computationally intractable even if a perfect potential energy function were available, owing to the astronomical number of possible conformations a protein backbone can adopt. It is therefore necessary to employ various heuristic methods to indirectly minimize $\Delta G_{\text{folding}}$.

The simplest way to find a backbone structure that is capable of being designed is to reuse experimentally solved backbone structures and/or local loop remodeling; however, there has been work on methods to design *de novo* backbone structures [17–20].

Despite the considerable computational challenges, there has been significant progress in computational protein design in the past decade with three groups in particular having the most high profile successes: David Baker, Steve Mayo, and Homme Hellinga. The Baker group has developed a comprehensive protein modeling package called Rosetta that is publicly available for download [21]. The Mayo group has developed a software package called ORBIT (Optimization of Rotamers by Iterative Techniques [22]), and the Hellinga group has developed a package called Dezymer [23].

All three groups use a "positive design" methodology. That is to say, their approach to the inverse folding problem is to optimize the potential energy of different amino acids and discrete side-chain conformations (called rotamers and chosen to represent those commonly observed in protein crystal structures) at each position in the backbone structure being designed while disregarding any explicit consideration of possible competing backbone conformations. Methods that take into account alternative conformations are termed "negative design," but it is often not clear which alternative states are the most important to consider and this may only be necessary in a minority of cases.

The potential energy functions used in computational protein design are variants of standard molecular mechanics force fields in some cases overlaid with additional statistical potential terms (e.g., hydrogen-bonding potential, Ramachandaran plot

amino acid propensity potentials, or pairwise potentials) and implicit solvation terms. In general, the emphasis is on modeling short-range side-chain packing interactions as these have been shown to be most important for protein stability [24, 25]. For example, in Rosetta force-field the more long-range electrostatic terms are modeled with pairwise statistical potentials.

The Dezymer and ORBIT design methods differ from the Rosetta design method by their use of different optimization techniques. Rosetta's design method uses a simulated annealing-based technique whereby different amino acid side-chains and rotamers are trialed. The trial moves are accepted if the potential energy is reduced, but rejected with a certain probability if the potential energy is increased depending on the Metropolis criterion as the temperature is gradually reduced. This is a stochastic method and is not guaranteed to find the global minimum. The methods of the Mayo and Hellinga groups use variations of the dead-end elimination (DEE) algorithm, which is a deterministic algorithm that is guaranteed to find the global minimum for a defined set of discrete variables (in this case rotamers). The basic philosophy of DEE is to eliminate side-chain rotamers (and pairs of rotamers) that cannot be part of the global minimum ("dead-ends") thereby reducing the search space [26].

The default choices for the methods above all assume a fixed-backbone conformation. Although success in design may depend mostly on the choice of a designable backbone, the use of fixed-backbone designs may create artificial restrictions on the sequence space accessible to the process. It is preferable to incorporate an element of flexibility into the design process, increasing the likelihood of finding a suitable sequence. A number of strategies for simultaneous optimization of sequence and structure have been developed and are now standard in current design methods [27].

14.2.1.1 **Computational Enzyme Design**

Based on the computational protein design methods described in the previous section, a number of laboratories have had some spectacular successes. The tools of computational protein design allow the placement of amino acid residues with atomic-level precision [18] and this is a prerequisite of any successful computational enzyme design.

Transition State Theory states that the activated complexes (i.e., the transition states) are in quasiequilibrium with their reactants and that the rate of the reaction is proportional to the concentration of this activated complex. Enzymes therefore catalyze reactions by specific binding of the substrates and stabilizing the transition state of the reaction. In order to design enzymes one must determine interactions likely to be important in stabilizing the transition state by studying existing enzymes or by *ab initio* quantum chemistry calculations.

Once a set of catalytic functional group geometries and interactions have been determined it is necessary to search a backbone scaffold library (typically a set of high resolution crystal structures) to find compatible candidate sites. One method to achieve this is to work backwards from the desired functional group geometry by using an "inverse rotamer tree" where, instead of building side-chain rotamers forwards from a known backbone structure, the side-chains are built backwards from

the functional groups to enumerate all possible compatible backbone conformations. A second approach (as used in RosettaMatch) is to independently build all possible rotamers and the associated transition state model in an ideal geometry forward from all backbone positions independently. The positions and orientations of each possible transition state model is given a hash key and the catalytic residue backbone positions are recorded for each hash key in a table. If, for a given hash key, each catalytic residue has at least one recorded backbone position in the table then a possible active site position has been found [28].

14.2.1.2 **Results of Computational Design Experiments**

The complete computational redesign of globular proteins has been shown to perform relatively well experimentally for all-α and α/β proteins [25], but less well for all-β proteins [29], and residues at core positions tend to be quite similar to the wild-type residues, showing a high degree of packing specificity [24].

The first full computational redesign of a naturally occurring protein, the second zinc-finger module of Zif268, was conducted in the Mayo laboratory [25] and its structure experimentally was verified by nuclear magnetic resonance (NMR). This encouraging result was augmented by a large-scale test of computational protein design in the laboratory of David Baker [29] involving completely redesigning nine different globular proteins without including any knowledge of the wild-type sequences and then biophysically characterizing them with circular dichroism, chemical denaturation, temperature melts, and one-dimensional NMR spectroscopy. Six of these redesigned proteins were found to have equal or higher stabilities, one protein (the λ-repressor) was destabilized, one protein (tenascin) aggregated and one protein (*src* SH3) was unfolded. These results appeared to show that proteins composed of mainly β-sheets are harder to design, perhaps due to a higher propensity for aggregation.

There have also been encouraging results in *de novo* backbone construction techniques. It is clearly the case that any backbone structure generated by some arbitrary method would not be designable. That is to say that it would not be possible to find sequences that would fold into that backbone structure. There are a number of properties that are characteristic of naturally occurring proteins and these can be used as rules to guide the construction of *de novo* backbones. For example, backbone dihedral angles are restricted to quite limited regions of the Ramachandaran plot, buried unsatisfied backbone polar groups are very unfavorable, and secondary structure elements cannot be too closely packed or spaced too far apart.

The DeGrado laboratory showed that it was possible to reconstruct the backbones of a number of small metalloproteins using idealized secondary structure elements related by a small number of symmetry operations and sequence redesign with a simple side-chain repacking algorithm [17]. The structure of one of the designed proteins (a four-helix bundle called DF1) was solved crystallographically and the backbone was found to be 1.65 Å from the model.

A related approach was taken by the Martial and Mayo laboratories to construct an ideal TIM-barrel of 216 amino acid residues from idealized secondary structure elements [30]. The β-barrel was created by setting a radius and tilt and then

optimized for hydrogen bonding. The helices were then placed around the barrel, controlled by a small number of parameters that were set to match the values found in existing TIM barrels. After designing the sequence with ORBIT's DEE algorithm, the artificial proteins were expressed in bacteria and were found to be folded in solution although the full three-dimensional structure was not determined experimentally.

The concept of building up backbone structures from idealized secondary structure elements has been further generalized by Taylor *et al.*, who have developed a scheme to generalize the structure of protein domains into a "Periodic Table" which can then be used to construct *de novo* backbone scaffolds [20, 31, 32]. The "Periodic Table" classifies compact globular protein domains into layers of secondary structure imposed by β-sheets. Ideal forms, in which each secondary structure element is represented as a line segment, are generated with simple packing rules. The axes of two packing α-helices or an α-helix packing on a β-sheet are placed 10 Å apart while adjacent strands in a β-sheet are placed 5 Å apart. The β-sheets have a predefined "twist," "curl," and "stagger" that the packing α-helices follow. Connections between the secondary structure elements in the layers then define a topology resulting in a "stick" model. It is then possible to construct rough α-carbon structures using the sticks as axes for placing idealized α-carbon secondary structure elements [33], which can then be refined into full backbone structures that are practically indistinguishable from real proteins [20].

The most dramatic success in designing *de novo* backbone structures is the construction of a novel topology, Top7, by Baker *et al.* [18]. The backbone scaffold was constructed by predetermining the secondary structure and using the predetermined hydrogen-bonding partners in the β-sheet as distance restraints. The overall fold was then assembled from fragments of known protein structures using the Rosetta *de novo* structure prediction methodology [34]. A disadvantage of the fragment replacement approach is that assembling larger and more complex topologies such as β-barrels becomes difficult as the energy landscape becomes more frustrated and the large nonlocal moves (i.e., fragment replacement) become too disruptive to the overall structure to converge to the global minimum quickly.

There have been a number of notable successes in computational enzyme design although reaction rates still fall well below naturally occurring enzymes. Notable early computational enzyme design efforts consisted of transplanting metalloenzyme active sites on to different scaffolds [35] and grafting *p*-nitrophenol acetate hydrolysis activity onto a catalytically inert thioredoxin scaffold [36]. There has also been progress in the design of receptor and sensor proteins [37], and modifying the specificity of naturally occurring enzymes [38–40].

In the past few years even more extraordinary progress has been made including the computational design of enzymes to catalyze the Kemp elimination reaction for which no naturally occurring enzyme exists [41]. In this case the activity was further increased more than 200-fold by *in vitro* directed evolution. The same algorithms were also used to design *de novo* retro aldolases in range of different scaffolds with the most successful designs incorporating an explicit water molecule in the active site [42]. X-ray crystal structures of two of the designed enzymes showed that atom

level accuracy was achieved with active site heavy atom root mean squared deviations of 1.1 and 0.8 Å.

14.2.2 Directed Evolution Methods

Directed evolution is a process of artificial selection with the aim of producing a protein with the required properties by generating randomized protein sequences, usually by molecular biology techniques, assaying the proteins for the required activity and selecting the most promising sequences to take through to the next round. The starting point can in general be either a random library, a set of designed sequences, or mutants generated from a native protein. Although conceptually simple, there are many technical complexities that can significantly affect the efficiency of the process. The most important considerations are the sequence space that can be made accessible by the underlying randomization process and the accurate selection of active variants, which we will discuss in the following two sections.

14.2.2.1 Randomization Strategies

Ideally, the sequences generated for selection should be generated by an unbiased random process. However, for real sequences it is not generally possible to do this: direct protein synthesis is expensive and limited in the lengths that it can generate, and so proteins are made as translated gene products. Typical approaches to generating randomized gene constructs are error-prone polymerase chain reaction, mutator polymerases (typically from *E. coli*), chemical mutagenesis, and random synthesis methods, which aim to introduce random genetic changes.

Each of these methods exhibits distinct preferences for generating mutations on the genetic level. A comparison of 19 methods on four genes in an *E. coli* expression system [43] demonstrated that significant biases in the location and type of substitutions exist for all of these methods. Particular problems are the introduction of premature termination codons and a tendency to introduce too few mutations compared to an unbiased method. As an additional complicating factor the degree and kind of errors varied substantially between the four genes, making a general strategy difficult to anticipate.

A more general problem for these methods is how to access the full spectrum of possible mutations on an amino acid level: the structure of the genetic code means that even a perfectly unbiased set of genetic mutations will only allow 37% of possible substitutions, a limit which is almost reached by error-prone polymerase chain reaction methods. However, the nucleotide biases reported above additionally create substantial biases in the diversity that can be accessed at particular positions in the protein. Volles and Lansbury [44] developed software to simulate several of the approaches above in order to allow the creation of unbiased random libraries.

Although the methods above aim for a theoretically ideal random distribution some bias is unavoidable given the use of molecular biology to generate proteins and,

in general, it may not always be undesirable. A bias against stop codons is preferable, for example, and for experiments starting from natural gene sequences it is possible that codon usage has evolved to limit the available diversity at some positions while increasing it at others, which may be important for preventing disruption of sensitive sites of the protein. In general, no unbiased optimization procedure is superior to any other and it is important to use problem-specific information when generating an optimization strategy for a particular task. Thus, although it may be useful to encourage unbiased mutations at some sites it seems preferable to have some basis for controlling mutational diversity to encourage exploration of potentially interesting regions of the fitness space, a point of view which has been argued by several. Unfortunately, we are limited in this respect by our poor understanding of the detailed sequence–structure–function relationship and so it seems that it is likely to be profitable not to entirely restrict the generation of diversity simply to, for example, positions proximal to the active site. When moving from a computational design to directed evolution there is the question of how the designed protein sequence is to be back-translated into a nucleotide sequence. It seems likely that a proper choice of both the initial constructs and the mutagenesis strategy in a complementary fashion is required.

14.2.2.2 **Expression Systems and Assays**

Assaying constructs for activity against the required reaction is a difficult step, which has seen several technical innovations. The general requirement is that the novel protein, substrate, and product are definitely associated within a compartment, and that the product can be easily identified.

This can be done *in vitro* (using artificial compartments) or *in vivo* (using cells). Both methods have advantages and disadvantages: *in vitro* methods permit the use of larger libraries than *in vivo* systems (possibly up to seven orders of magnitude), but have so far been found to be more successful for evolution of binding activities rather than catalytic activities; an ATP-binding protein of novel fold (Protein Data Bank (PDB) code 1uw1) was produced using these methods from an unbiased random library of 10^{12} sequences initially, followed by eight rounds of evolutionary selection. *In vivo* methods are purported to be better for catalytic purposes, but there are substantial difficulties in ensuring that the protein product directly catalyzes the reaction and does not produce cellular toxicity.

The standard technique for assay and selection is to generate fluorescence with the product and use fluorescence-activated cell sorting methods to isolate variants with activity [45]. Currently this can process up to about 10 000 at a time, and can be adapted both to cell and cell-free expression systems. The difficulty is to ensure that the product does not escape its compartment and activate fluorescence in others, which would diminish the ability to select efficiently. Bershtein and Tawfik [46] review progress in this area.

Much of the success of directed evolution has been in optimization of an existing enzymatic activity; however, there has been one reported success in the evolution of an entirely novel catalytic activity from a previously noncatalytic zinc-finger domain [47].

In general, there is a clear reason for designers of novel proteins to consider directed evolution methods in that we may never achieve a sufficiently detailed picture of the chemical environment of most proteins to be capable of designing proteins with high specificity and activity. Directed evolution can provide a means to optimize activity and specificity towards a particular product (we might imagine finding a very general specificity towards a particular substrate and then generating a series of more specific enzymes from this) or to probe the mutational landscape of a designed protein in order to improve designs. An example of this is the study of Yoshikuni *et al.* [48].

Future strategies in which the results of a directed evolution process are provided to a computational design method can be anticipated and seem likely to provide still more efficient methods.

A particularly interesting trend in directed evolution, owing to Tawfik, is the use of so-called neutral drifts. In this method selection pressure is applied towards the native function of a natural protein prior to selection for a novel function. Several published studies have shown that the results of exploring the neutral network surrounding a natural protein are that library sizes can be greatly decreased [49]. Although at present only applicable to evolving novel functions for natural proteins of known function it is conceivable that these methods might be of use in building on earlier design work.

14.2.3
Design of Protein Interfaces

An important consideration when designing novel proteins is the set of interactions with other proteins which may already exist within the context in which the new protein is to be used, which may be introduced by the addition of other novel proteins or which occur as a result of self-interactions. Interfaces with other proteins are also essential functions for biology and engineered binding (mostly from directed evolution) is increasingly of importance as an alternative to the use of monoclonal antibodies for biotechnological purposes [50].

For computational design the first question is whether a specific energy function is required for this task. Cohen *et al.* [51] studied interfaces found in the PDB, and saw that there were few principal differences between the energetic and structural properties at protein–protein interfaces and those within the protein that contribute to stability of the folded state of a monomer, with the important implication that existing energy functions for design are sufficient for this task also. The authors did identify one important difference: within interface regions the contribution of polar side-chain–side-chain interactions was substantially greater (62% of contacts compared to 36%), with backbone–backbone and backbone–side-chain interactions being more important for monomer stability. This result compares favorably with the study of Ofran and Rost [52] in which robust statistical differences in amino acid composition between intra- and interprotein interfaces were found, with the latter being dominated by salt-bridges. Despite this relatively minor difference these findings broadly justify the use of existing energy functions for single-protein design in this context.

In their authoritative review, Karanicolas and Kuhlman [53] identified two strategies that have been successfully applied to interface redesign. The first involves the engineering of favorable charge–charge pair interactions between the two interaction partners at the edge of the interface region. Since the energetics of hydrogen bonding and the intricacies of well-packed hydrophobic interfaces are as yet not sufficiently understood to engineer with any precision this has the very desirable property that the interface region is itself left undisturbed. The disadvantage is that it requires an existing imbalance of charge in the region of interest, which may be relatively unusual. Introduction of a charged surface residue may be possible where they do not exist, however as yet no such studies have been published to demonstrate that this is an effective strategy. The interaction between the integrin LFA-1 and its ligand ICAM-1 is one which has been successfully re-engineered by this approach [54]. Overall, groups have reported substantial success with this method (60%); however, as noted above, it may be of limited use in general.

The second approach is to redesign the interface directly. This uses the same design tools as for individual protein design; however, additional considerations of negative design and the lack of accurate modeling of protein dynamics add constraints. Sammond *et al*. [55] demonstrated a comparatively high success rate with this approach. From a meta-analysis of previous literature they found that the most successful published mutations were those that increased surface hydrophobicity, either by replacing a hydrophilic amino acid with a hydrophobic one or replacing a small hydrophobe by a larger one. On this basis they developed a constrained prediction pipeline in which they restricted mutations using these constraints, with the additional constraint that the mutations must not significantly (above 0.5 kcal/mol) destabilize the monomer of either partner.

They tested this approach experimentally with two complexes: the $G_{\alpha i1}$–RGS_{14} GoLoco motif (protein–peptide interaction) and the UbcH7–E6AP interaction (protein–protein). The overall success rate of this approach (percent residues predicted as stabilizing found to be stabilizing) was roughly 50%, substantially higher than the 15–30% previously reported for comparable approaches [56]. Another important success in computational interface redesign has recently been reported for calmodulin [57].

The results above show promise, but results are limited to stabilization of existing interfaces. Another important consideration is negative design; the authors of the studies above did not assess the accuracy of their methods in predicting destabilization, which may be more important for novel interface design than positive constraints, although specificity for one interaction typically destabilizes others, as has been found with calmodulin [57]. Several recent studies on this more challenging goal have been published. The simplest possible method is of course to "graft" key residues for the interaction from one protein to another. Naturally this has the limitation that we must have already seen an interaction of the type we need, however it is an approach, which has been shown to work. Liu *et al*. [58] demonstrated this with the human erythropoietin receptor and the pleckstrin homology domain of rat phospholipase D (PLCδ1-PH). By scanning the PDB with the key residues from the human erythropoietin (EPO)-EPO receptor (erythropoietin receptor EPO) bind-

ing site, the binding site of the PLCδ1-PH was identified as a suitable target for grafting. By mutagenesis of this binding site to match the EPO binding residues the authors were able to demonstrate significant affinity of PLCδ1-PH for EPOR.

Huang *et al.* [59] presented the first example of the more general case of designing a completely novel interface that produced a homodimeric conformation for a protein known to exist as a monomer in its native state: the β1 domain of streptococcal protein G. This method involved identification of potential interfaces by a restricted docking protocol requiring only helix–helix interactions using a reduced side-chain representation with an increased interaction radius to account for the missing side-chain atoms. Experimental characterization by ultracentrifugation and ^{1}H-NMR spectroscopy was consistent with a modest stability in the dimeric form on the high micromolar range. Although this is a positive result it is clear that much more work is required to generate high-affinity complexes.

Clearly there have been several successes in redesigning interface specificity for known interactions and modest successes in producing *de novo* interfaces with single specificity. However, the more challenging case of interfaces for multiple interactions has not yet been tackled experimentally. In a groundbreaking study Humphris and Kortemme [60] used computational design with Rosetta and the Rosetta interface to examine the constraints placed on a sequence by multiple partner interactions.

The authors reasoned that the additional constraints placed on interactions with multiple partners might be identified by designing interfaces from known promiscuous proteins to interact with each partner individually and comparing results with those found for designing towards all partners simultaneously. They found that in many cases the multiple interaction partners shared a large commonality of the interface and that the results of optimizing towards one partner were similar to the results of optimizing towards the entire set. However, for a subset of highly promiscuous proteins (e.g., ubiquitin) the number of constraints on the hub was very high and the sequences generated for individual optimizations were very different from those found for the entire set.

14.3
Protocol for Protein Design

Although protein design has seen an exciting series of high-profile successes it remains a scientific rather than an engineering task. While it has been shown that current methods are capable of finding sequences that fold into the desired structure, it is not guaranteed that these methods will be successful. It may prove, as theoretical studies on ideal lattices predict, that some folds are much harder to design than others. The successes that have been reported so far are very encouraging, but it is not yet clear how to decide when a backbone will be designable, whether a particular sequence is more likely to be a successful design, or indeed how often to expect success.

It is therefore necessary to choose a method based on the degree of novelty required and the depth of available resources. If the objective is to increase stability of a known protein or change the specificity of binding or catalysis then it should be possible to achieve this with a few rounds of directed evolution, provided that the

protein is experimentally tractable and a suitable assay can be found. Greater novelty is likely to require a more rational approach to be cost-effective. While computational methods cannot guarantee success, they can offer a significant enrichment of the initial population of leads over a purely random choice. Directed evolution is then likely to be required for the optimization of function and other important properties of the protein.

For such a rational approach the first question is for which backbone structure to design a sequence. Although some successes have been reported with all-β proteins the design of all-α and mixed α/β proteins is more likely to be successful. The specific choice of protein may depend on the function that is required – if the introduction of a known enzymatic or binding activity is the target of the design then screening for structures with sites that have the necessary arrangement of atoms is an obvious first step. Since proteins within a particular class may vary in their available sequence space it is best to choose a backbone structure from a fold group with a large natural population – the TIM α/β-8 barrel, ferredoxin, and immunoglobulin folds are good examples. This jointly maximizes the chance of the required protein existing and of being able to find it. With sufficient time and resources it is probably sensible to choose several scaffolds if they are compatible with the design constraints. The quality of the structure is also crucial – high-resolution crystal structures will conform better to the parameters of the energy function and result in more natural sequences, and it is obvious that no design can be successful if the structural target is not physically possible.

Having decided on one or more scaffolds it is necessary to choose which of the available design packages is to be used. At present the technical differences between the three software implementations are not of significance to the end user; however, the RosettaDesign package is probably the easiest to use and most readily available, and benefits from a large and active user community. We will therefore add some comments specific to this method, although all of the recommendations will be generally applicable.

The overall strategy is one of attempting to maximize the chance of finding good initial sequences, which requires many simulations to be run, preferably in parallel on a moderately sized cluster. The number required depends primarily on the difficulty of the design and, in general, the best recommendation is to sample as widely as resources and time permit.

Before proceeding to sequence design the structures must be relaxed in the energy function to be used for sequence optimization. This is a critical step since computational methods are very sensitive to the details of the structure, and small deviations from expected distances and angles can result in severe restrictions on the amino acids that are available at certain positions. This can be as a result of poor refinement or simply differences between the energy function used to refine the structure and that used by the design package, since a structure with low energy in one energy function might have a high energy when assessed with another.

Sequence design is best achieved using a flexible protocol, with alternating cycles of design and relaxation of the structure to the new sequence. In RosettaDesign this can be achieved by alternating runs of the *relax* and *fixbb* protocols included as default. More advanced protocols can also be created by the user, although some knowledge of

C++ programming is required. Flexible design considerably increases the likelihood of success at the expense of a much longer process for a single sequence.

In our experience, 10 alternating design/relax cycles are sufficient to balance these concerns.

Problems with correctly modeling the aqueous environment and the effects of solvent exposure lead to significant difficulties in design of the accessible surface residues. Since good surface is crucial for stability and solubility of the final product this requires careful attention and is usually achieved by position-specific restraints on the design. Design packages provide the option to restrain residues at particular sites in order to allow this. In RosettaDesign this is done using a restraint file – a simple text file that describes overall parameters for a run and lists the choice of residues available at each position in the protein. Designing stable, soluble proteins is most likely if this is used to constrain exposed positions to be hydrophilic and buried positions to be hydrophobic. Since the backbone is moving with each relaxation this has to be done between the relaxation and design steps. The choice of accessible versus buried residues can be made in many ways such as simple counts of neighboring atoms [20] or a more sophisticated modification of standard solvent accessibility calculation methods. The choice of hydrophilic and hydrophobic sets can be made on the basis of known scales of hydrophobicity, one example might be AGILMFWYV for core positions, GARNQHKMFWYDE for partially exposed positions, and RNDQEGKPST for surface positions.

Assessment of the final designed structures involves looking for low-energy structures as assessed by the design package itself and independent methods of model quality assessment such as full-atom potential functions (e.g., DFIRE [61]). The RosettaHoles method provides a good independent test of core design quality, producing a score for the number, size, and distribution of cavities within the structure with reference to the same values for high quality PDB structures. The sequence compositions can also be compared to those expected from known structures and identify structures that are likely to have problematic features: an excess of glycine and alanine is indicative of a structure with bad clashes, for example, whereas an excess of hydrophilic residues might indicate that the structure is not sufficiently compact. Surface design composition is more difficult to assess and it is likely that the lowest-energy designs will need to be checked manually for unrealistic features. Some manual intervention to alleviate these may also be required.

Following this it will be necessary to test the selected designs experimentally for solubility, structure, and function. Active variants may then require further optimization, which might proceed on manual grounds or by the use of directed evolution approaches as outlined above.

14.4 Conclusions

In this chapter, we have presented a snapshot of current methods and results in the exciting and fast-moving field of protein design. The significant successes that have

been reported thus far have served as important proof-of-concept results; however, there remain many scientific questions that must be answered and technical barriers that must be overcome before the field can mature into an engineering discipline. Larger-scale benchmarks of design methods and energy functions are a priority, and particular emphasis needs to be placed on methods for surface design to improve stability and solubility. The interface between directed evolution and computational design seems another important area for the development of new approaches and new understanding, although in the longer term it is to be hoped that computational design will be sufficient for the basic purpose of creating novel monomeric enzymes and binding proteins, with directed evolution being used for refinement and more complicated multiobjective problems in which the number of constraints becomes intractable for computational approaches.

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15
Amino Acid-Based Dendrimers

Zhengshuang Shi, Chunhui Zhou, Zhigang Liu, Filbert Totsingan, and Neville R. Kallenbach

15.1
Introduction

Dendrimers are highly branched molecules consisting of a central core that is generationally grafted with layers of building blocks (Figure 15.1) [1, 2]. The dendrimer generation is defined as the number of branching points from the core to the surface of the dendrimer. Here, amino acid-based dendrimers, or peptide dendrimers, are loosely defined as molecules formed from amino acids emanating from a branched template or scaffold, in which branched templates can be branched amino acids such as lysine [3–5], glutamic and aspartic acid [6, 7], *cis*-4-amino-L-proline [8], or other building blocks, including cyclic peptides, poly(amido amines) (PAMAM) [9], polypropylene imines (PPI) [1], poly(aryl ethers) [10, 11], and a variety of other organic branched motifs such as carbohydrates [12, 13]. In this chapter we focus mainly on peptide dendrimers that are branched with amino acids (natural or unnatural) and exclusively composed of amide bonds. As a result, dendrimers that are obtained by conjugating peptides/amino acids onto other scaffolds are discussed only briefly.

15.2
Peptide Dendrimer Synthesis: Divergent and Convergent Approaches

Dendrimers afford a precise and concentrated display of functionality on a scaffold. There are two general conceptually different synthetic routes for the construction of dendrimers: divergent and convergent approaches (Figure 15.2) [15]. In the divergent method, the dendrimer is built up from its core outward – the most obvious scheme (Figure 15.2A); in the convergent method the dendrimer is assembled through coupling of preformed branching segments onto a central core unit (Figure 15.2B). The convergent synthetic strategy was devised by Frechet *et al.* [10, 11] to overcome a serious problem with the divergent approach. Due to the progressive accumulation

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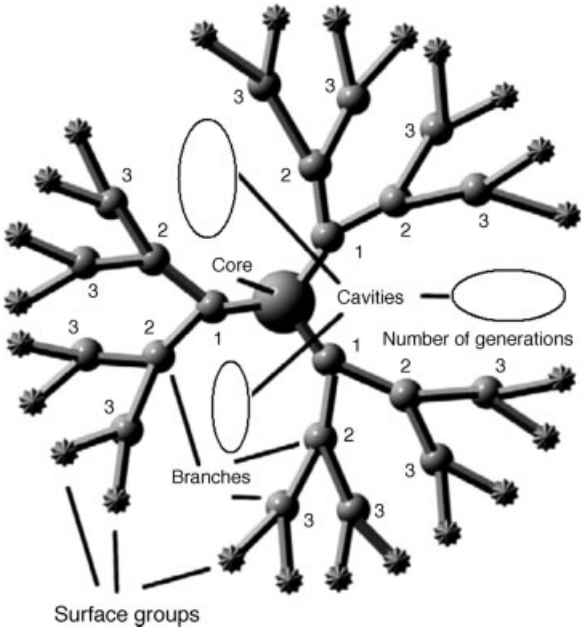


Figure 15.1 Dendrimer structure. (Adapted with permission from Fig. 1 of [14], copyright 2009, American Chemical Society.)

of defects during the process of performing the exponentially growing number of reactions as the dendrimer expands from one generation to the next, it becomes practically impossible to synthesize defect-free dendrimers beyond the fifth or sixth generations [16]. For example, given a yield of 99.5% per reaction site, only $0.995^{\exp 504} = 8.0\%$ of defect-free products would be obtained in the case of synthesis of a sixth generation PPI dendrimer constructed using the divergent

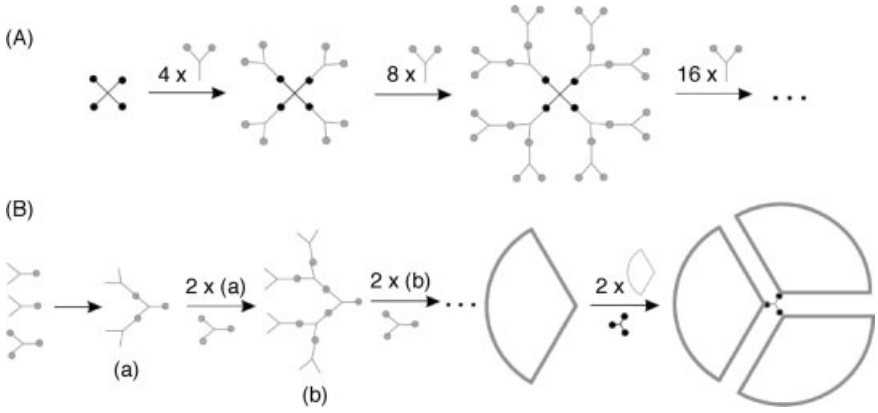
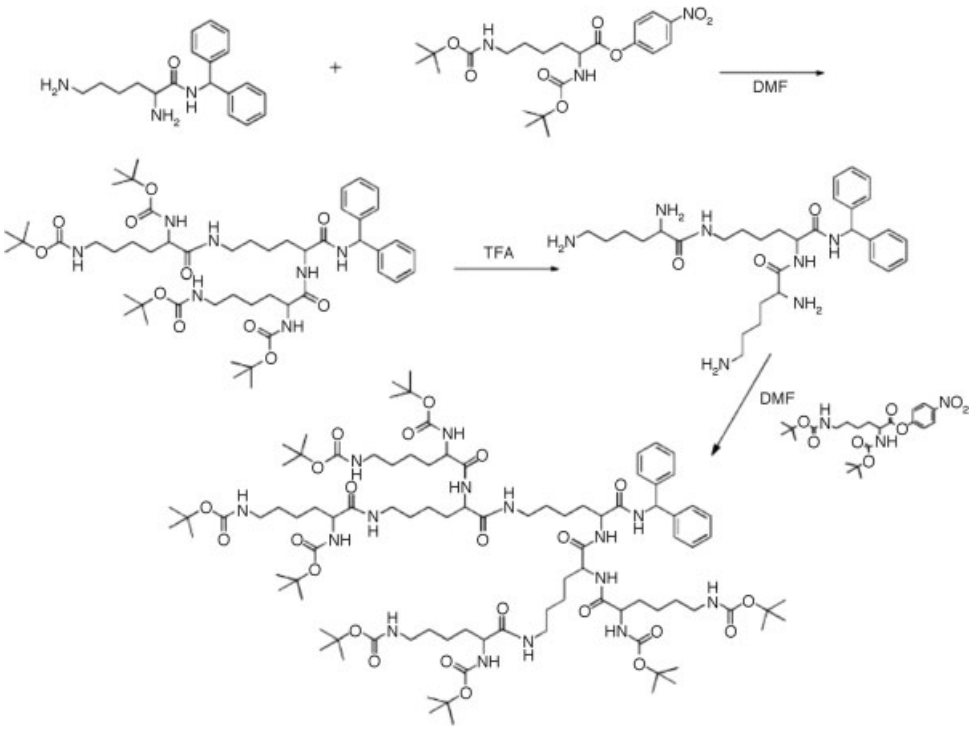


Figure 15.2 (A) Divergent approach. (B) Convergent approach. (Reproduced with permission from Fig. 1 of [17], copyright 2001, P. Pomovski and Polish Biochemical Society.)

strategy [16]. In practice, a two-stage strategy that combines the divergent and convergent approaches has been developed [8].

15.2.1
Synthesis of the First Peptide Dendrimers: Polylysine Dendrimers

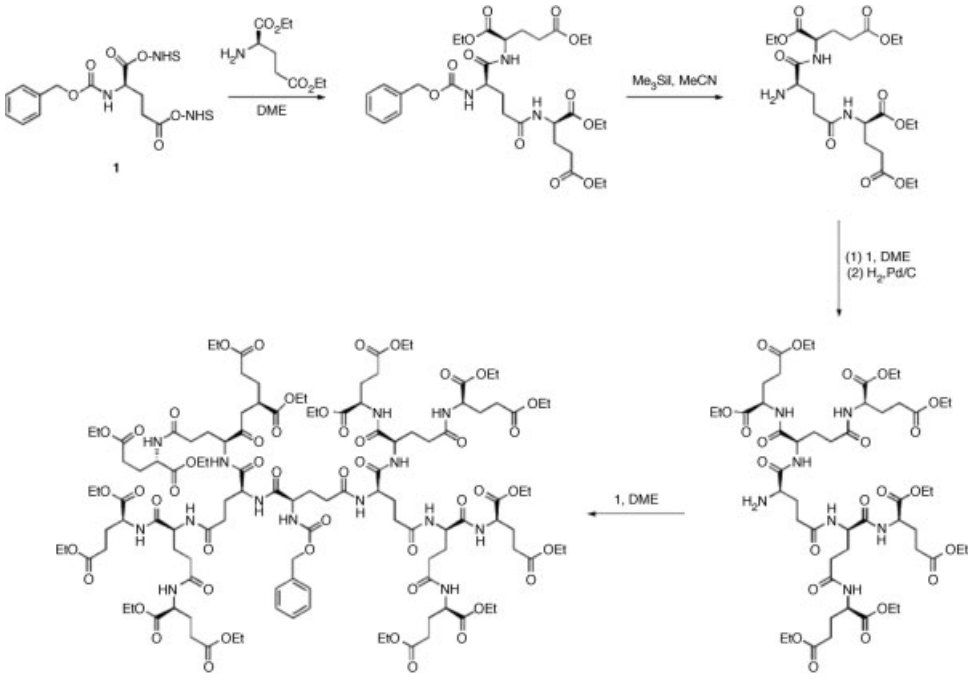
The first peptide dendrimers appear to have been synthesized by Denkewalter *et al.* and Aharoni *et al.* at the Allied Corporation in the early 1980s [3–5]. They reported the synthesis of polylysine dendrimers from $N^{\alpha},N^{\varepsilon}$-di-(*tert*-butoxycarbonyl)-protected lysine in a stepwise fashion (Scheme 15.1). This original synthesis was accomplished by a repetitive sequence of $N^{\alpha},N^{\varepsilon}$-di-(*tert*-butoxycarbonyl)-L-lysine-*p*-nitrophenyl ester couplings and trifluoroacetic acid-mediated deprotection reactions. The basic idea of this design is to make use of the bifunctionality of the amino acid lysine to form a core as well as to serve as branching units, which is possible as both N^{α}- and N^{ε}-amino groups are available reactive ends. Subsequent coupling of lysine residues propagates additional generations of the dendrimer. Different peptide sequences can be assembled on this scaffold to create a large number of desired peptide dendrimer products.



Scheme 15.1 Synthesis of polylysine dendrimers. (Reproduced with permission from Scheme 1 of [18], copyright 2009, Elsevier B.V.)

15.2.2
Glutamic/Aspartic Acid, Proline, and Arginine Dendrimers

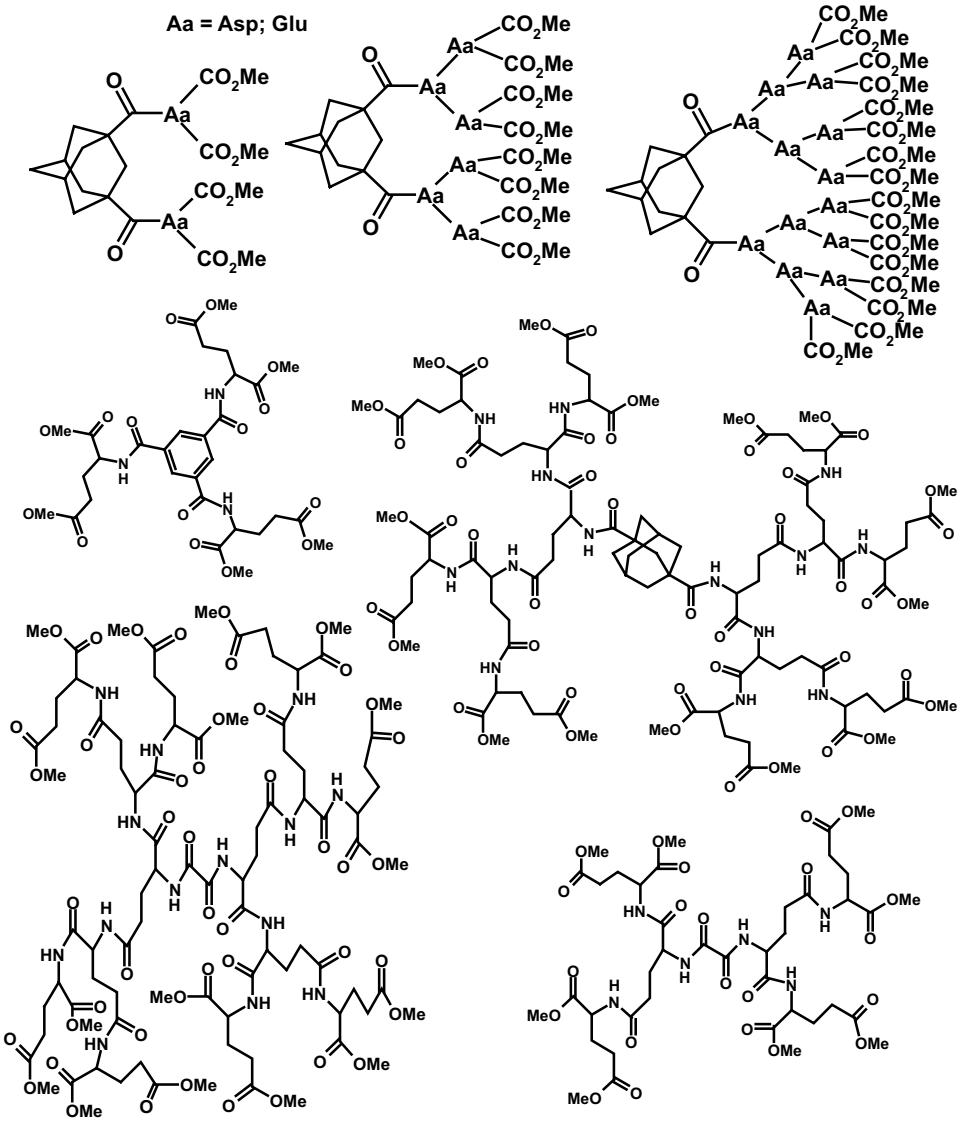
Mitchell *et al*. reported the synthesis of L-glutamic acid-based dendrimers in 1994 (Scheme 15.2) [6]. L-Glutamic acid was protected and activated as the *N*-(benzyloxycarbonyl)-L-glutamic acid bis(*N*-hydroxysuccinimide) ester, which was reacted with L-glutamic acid diethyl ester in dimethoxyethane. Selective deprotection of the benzyloxycarbonyl protective group in the product with iodotrimethylsilane resulted in the first-generation dendrimers. The first-generation dendrimers were subsequently converted to the second-generation dendrimers, during which the benzyloxycarbonyl protective group was removed by hydrogenation as iodotrimethylsilane was not effective. The final dendrimers were obtained by applying repetitive reactions to the second-generation dendrimers. The same group also prepared L-aspartic acid-based dendrimers in which L-aspartic acid served as the branching unit and 1,3,5-benzene tricarbonyl core as the core unit [7].



Scheme 15.2 Synthesis of glutamic acid dendrimers. (Reproduced with permission from Scheme 3 of [18], copyright 2009, Elsevier B.V.)

A number of other peptide dendrimers composed of glutamic/aspartic acid [19–23], proline [8, 24–26], or arginine [27–29] have been reported. Ranganathan *et al*. synthesized several series of polyglutamic/aspartic acid dendrimers including those with adamantane, an oxalyl group, or a benzenetricarbonyl unit as dendrimer

core (Scheme 15.3) [19–21]. Kraatz *et al.* synthesized two series of glutamic acid dendrimers using a ferrocene core (Figure 15.3) [22, 23]. Arginine-rich peptide dendrimers in which arginines are grafted onto polylysine dendrimeric cores have also been designed and synthesized (Scheme 15.4) [27–29]. Polyproline dendrimers based on *cis*-4-amino-L-proline as branching units and either spermidine or a cyclic Lys–Lys as the core have been constructed by Crespo *et al.* [8]. Interested readers may



Scheme 15.3 Glutamic/aspartic acid peptide dendrimers prepared by Ranganathan *et al.* (Adapted with permission from [19], copyright 1997, Elsevier B.V., [20] copyright 1998, Ovid, [21], copyright 2000, Jhon Wiley and sons, Inc.)

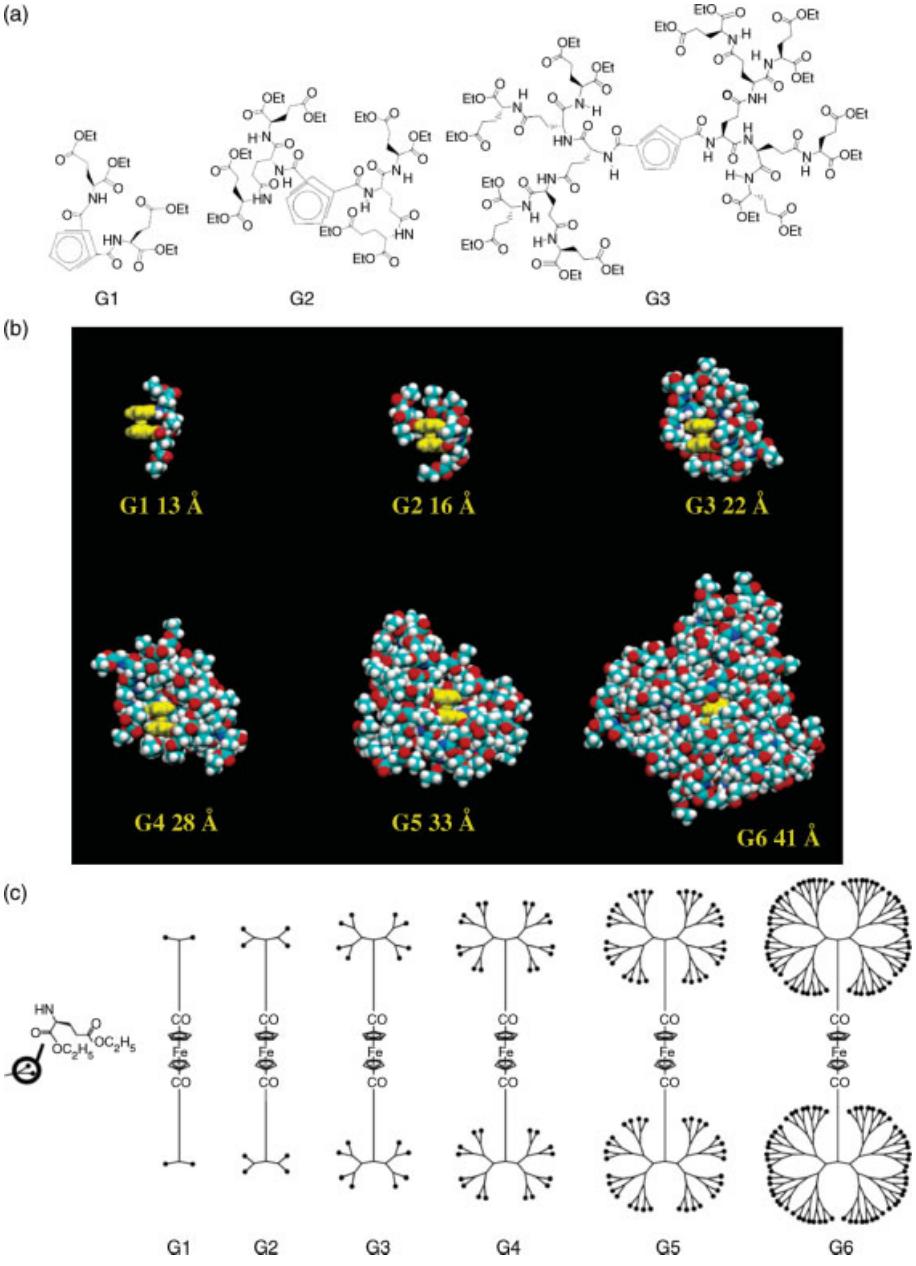
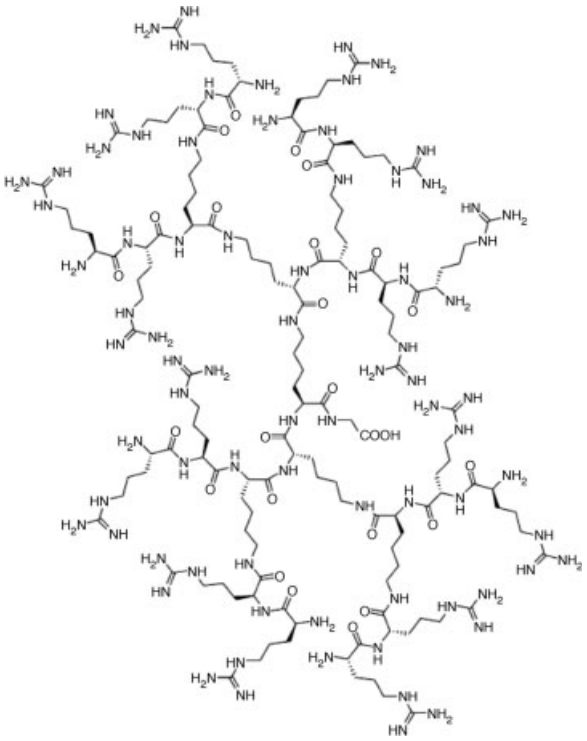


Figure 15.3 (a) Structures of disubstituted Fc–peptide dendrimers G1, G2 and G3. (b) Molecular models of disubstituted Fc–glutamic acid dendrimers G1–G6 (Fc: yellow; carbon: green; hydrogen: white; oxygen: red; nitrogen: blue). (c) Schematic representation of disubstituted Fc-glutamic acid dendrimers G1–G6. (Adapted with permission from [22, 23], copyright 2005/06, American Chemical Society.)

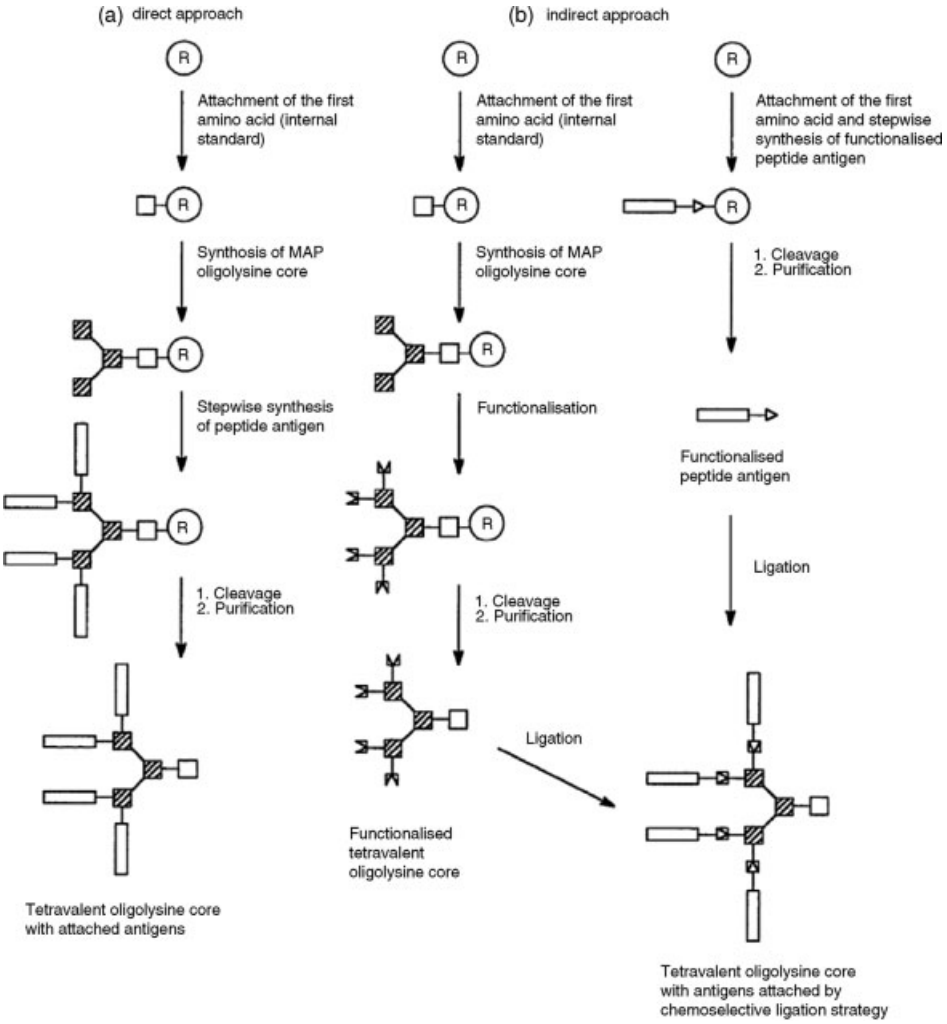


Scheme 15.4 Structure of the arginine dendrimer. (Reproduced with permission from Fig. 8 of [8], copyright 2005, American Chemical Society.)

refer to the corresponding literature for the synthetic details of these interesting products.

15.2.3
Synthesis of MAPs

In the late 1980s, Tam extended applications of polylysine peptide dendrimers in an attempt to develop potential multiple antigenic peptides (MAPs) [30, 31]. As indicated by the name, the goal was to provide a rational system for polyvalent linkage of different types of peptide antigens and attachment of immunomodulating molecules for use as vaccines. There are two general approaches to the synthesis of MAP dendrimers: the direct divergent approach and an alternative indirect approach (Scheme 15.5) [32]. The indirect synthesis route combines the divergent approach with various chemoselective ligation methods to couple different presynthesized peptides onto the polylysine dendritic matrix. The most commonly used ligation methods are based on thiol chemistry and carboxyl chemistry [32]. Some chemoselective ligation reactions tailored for polylysine or other modified polylysine scaffolds are summarized in Scheme 15.6, taken from Tam [32].



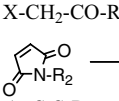
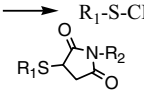
Scheme 15.5 Schematic diagram for the preparation of MAPs: (a) direct approach and (b) indirect approach. (Reproduced with permission from Fig. 1 of [33], copyright 1999, John Wiley and Sons, Inc.)

The principle of multivalency has been widely discussed as a general strategy to enhance the avidity of weak binding ligands for a given target [34]. Increasing the number of copies of a ligand on a covalent scaffold can elicit dramatic enhancements in the affinity of a single weak-binding ligand. The effect is similar to that involved in chelation, in which divalent metal ions are bound extremely tightly relative to any monovalent ion.

The effect depends on the topology and affinity of each ligand site, as outlined in Figure 15.4. In the simplest case, corresponding to a dendrimer, sites are distributed roughly uniformly over the approximate surface of a sphere, rather than a circle as

1. Thiol chemistry

a. thioalkylation $R_1\text{-SH} + \text{X-CH}_2\text{-CO-}R_2 \longrightarrow R_1\text{-S-CH}_2\text{-CO-}R_2$

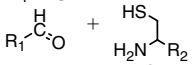
b. thiol addition $R_1\text{-SH}$ +  

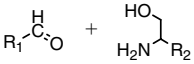
c. thiol-disulfide exchange $R_1\text{-SH} + \text{Ar-S-S-}R_2 \longrightarrow R_1\text{-S-S-}R_2$

2. Carboxyl chemistry

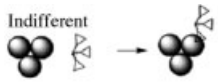
a. hydrazone  $+ \text{NH}_2\text{-NH-}R_2 \longrightarrow R_1\text{-CH=N-NH-}R_2$

b. oxime  $+ \text{NH}_2\text{-O-}R_2 \longrightarrow R_1\text{-CH=N-O-}R_2$

c. thiazolidine 

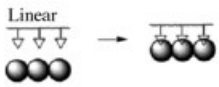
d. oxazolidine 

Scheme 15.6 Chemoselective methods for ligating unprotected peptides to form MAPs. (Adapted with permission from Table 3 of [32], copyright 1996, Elsevier B.V.)



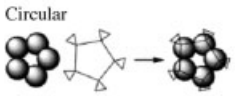
$$\Omega(\text{indifferent}) = nm$$

$$\Omega_i(\text{linear}) = (n - i{+}1)(m - i{+}1)$$



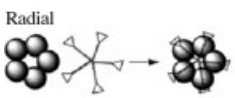
$$\Omega_i(\text{circular}) = nm$$

$$\Omega_i(\text{radial}) = \frac{n!m!}{(n-i)!(m-i)!i!}$$



m branches of the multimeric ligand

n binding sites of the multimeric receptor



$$W_i = \frac{e^{-\Delta G_i^{\circ}/RT}}{\sum_{i=1}^{i_{\max}} e^{-\Delta G_i^{\circ}/RT}}$$

$$\Delta G^{\circ}_{\text{avidity}} = \Delta G^{\circ}_{\text{inter}} + \Delta G^{\circ}_{\text{intra}} \sum_{i=1}^{i_{\max}} W_i(i-1) + RT \sum_{i=1}^{i_{\max}} W_i \ln (W_i / \Omega_i)$$

$$\Delta S^{\circ}_{\text{avidity}} = -R \sum_{i=1}^{i_{\max}} W_i \ln W_i + R \sum_{i=1}^{i_{\max}} \ln \Omega_i$$

W_i weight coefficient Ω_i the degeneracy of bound states

Figure 15.4 Thermodynamic model of the multivalency effect. (Adapted with permission from [34], copyright 2003, American Chemical Society.)

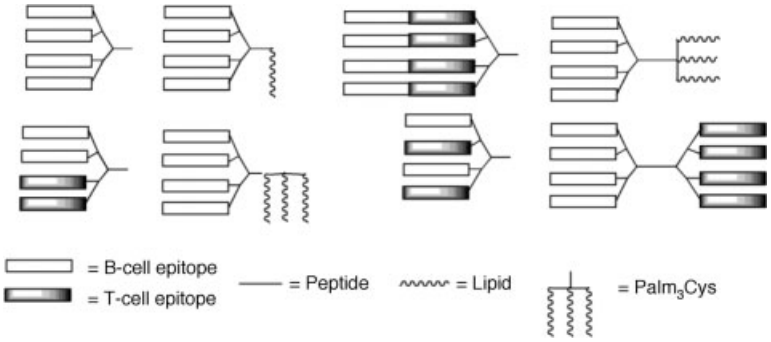


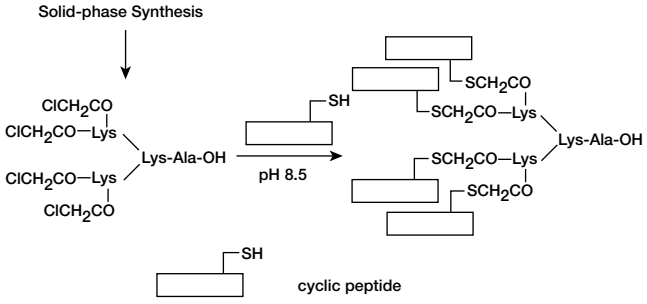
Figure 15.5 Schematic of different MAP designs comprising different peptides representing T and B cell epitopes, respectively, showing different ways to fuse these elements into MAP structures. Also shown is the possibility of including fatty acids into such structures either as single, straight chain alkyls or as the specific tripalmitatecysteinyl structure. (Reproduced with permission from Fig. 21 of [36], copyright 2004, Royal Society of Chemistry.)

shown. However, the amplification factor can easily lead to nanomolar dissociation constants for the assembly as a whole when single affinities are in the millimolar to micromolar range.

The MAP strategy affords construction of multivalent assemblies with an exceptional degree of flexibility as illustrated in Figure 15.5 [32, 33, 35, 36]. MAPs containing monoepitopes, chimeric epitopes, and diepitopes can be generated at will. Lipidated MAPs and MAPs containing cyclic peptide epitopes, which mimic protein surface loops better (Scheme 15.7) [37], can be synthesized as well. Through chemoselective ligations, the design and synthesis of MAPs that display heterogeneous epitopes are also possible.

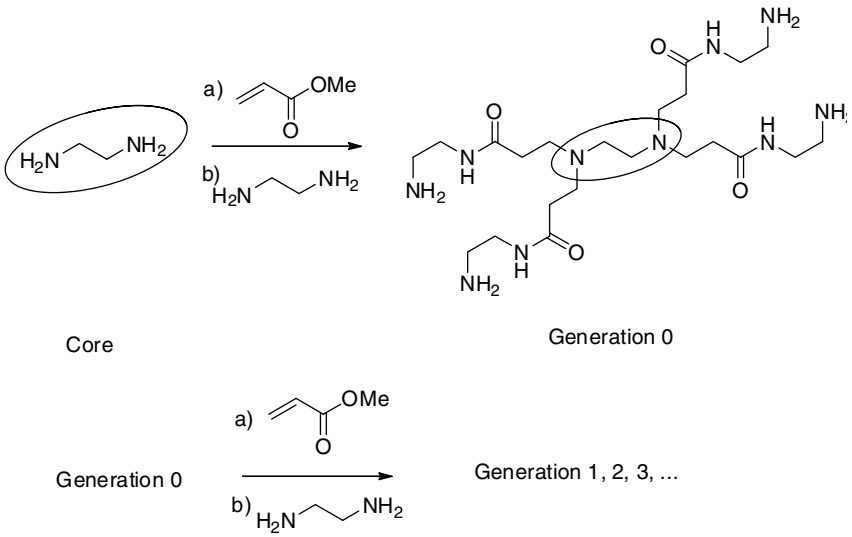
15.2.4
Synthesis of Peptide Dendrimers Grafted on PAMAM and other Peptide Dendrimers

While its core matrix is not directly synthesized from amino acids, Tomalia's PAMAM dendrimers represent an important class of macromolecular architecture



Scheme 15.7 Synthesis of cyclic peptide dendrimers through thioether linkage. (Reproduced with permission from Fig. 8 of [37], copyright 1997, American Chemical Society.)

with a high degree of molecular uniformity, specific size and shape characteristics, and a highly functionalized terminal surface constructed from stable amide bonds [2]. PAMAM dendrimers can be synthesized by the divergent method, which involves a two-step iterative reaction sequence that yields successive generations of dendrite β-alanine units around a central core [38] (Scheme 15.8). While the PAMAM core–shell increases with each generation, the surface groups amplify exponentially as in the previous cases discussed.



Scheme 15.8 Synthesis of PAMAM dendrimers by exhaustive Michael addition of amino groups with methyl acrylate, followed by amidation of the resulting esters with ethylenediamine [38]. (Copyright 2001, Elsevier B.V.)

Efforts have been made to attach surface peptide groups to the PAMAM dendrimer core, forming peptide dendrimer conjugates [39]. An example of such a conjugation is the coupling of cysteine-containing peptides with a maleimide-derivatized PAMAM dendrimer using solution-phase reaction [40, 41]. Alternatively, solid-phase synthesis of peptide–dendrimer conjugates may be achieved starting from the initial core [42, 43].

Other scaffolds have been designed with different core compositions. One such design involves assembling peptide dendrimers on a poly(ethylene glycol) resin from simple amino acid-based monomers using a cyanoethylation/hydrogenation strategy [44]. Peptide dendrimers constructed by coupling peptide segments onto polyphenylene scaffolds have been synthesized, affording shape-persistent, MAP candidates [45]. Examples of the use of carbohydrates as potential templates for multivalent peptide assembly have also been reported [12, 13]. A novel amino acid-based dendrimer was prepared from 3,5-bis(2-*tert*-butyloxycarbonyl aminoethoxy) benzoic acid methyl ester [46, 47]. Sasaki and Kaiser's design of an artificial hemeprotein via attachment of four identical peptides onto a coproporphyrin core

can be regarded as another example of peptide dendrimers [48]. Additional examples of the application of amino-, hydroxylic-, and carboxylic-based organic templates as dendrimer scaffolds using either the divergent or convergent approach are summarized in two previous reviews [49, 50].

15.3 Applications of Peptide Dendrimers

15.3.1 Initial Efforts on MAPs

In efforts to apply polylysine dendrimers as potential MAPs, increasing the functionality of small peptides was hoped to enhance their immunogenicity and therefore their applicability as vaccines [51]. As compared to conventional peptide–protein conjugates in which antigens account for only a small percentage of the total mass, MAPs potentially afford more concentrated epitopes attached to an immunologically silent lysine core. Despite many articles reporting initially encouraging results using MAPs, no vaccine based on these appears to be in use today as far as we can discover.

15.3.2 Peptide Dendrimers as Antimicrobial Agents

Naturally occurring antimicrobial peptides are found in all orders of life [52]. While over 1000 antimicrobial peptides have been identified, the problem is that they tend to be highly cytotoxic, hemolyzing red blood cells. Efforts to dissociate the hemolytic activity from the antimicrobial activity of these peptides are an important part of the search for new antibiotics capable of overcoming multidrug-resistant bacterial strains that are prevalent in hospitals and elsewhere.

Tam *et al.* have designed and synthesized several sets of dendrimeric peptides with two to eight copies of tetrapeptides or octapeptides derived from a putative microbial surface recognition motif that occurs naturally in protegrins and tachyplesins [53]. The resulting dendrimeric peptides are broadly active against four Gram-negative bacteria, three Gram-positive bacteria and three fungi with minimal inhibition concentration in the low- or submicromolar range. Hemolytic assays showed that they are not toxic to human erythrocytes with an observable hemolytic index (HI) in the range of above 2000. These results suggest that dendrimer peptides are promising antibacterial drug candidates, maintaining the antimicrobial potency while reducing cytotoxicity.

Recently, Kallenbach *et al.* have observed that a series of very short peptides with simple repeat sequences $(RW)_n$ ($n = 3$–5) are potent antimicrobial agents with IC_{50} values in the micromolar range [54]. Based on this observation, a dendrimeric peptide $(RW)_{4D}$ (Figure 15.6) was designed and synthesized [55]. Compared to the antimicrobial dendrimeric peptides designed by Tam [53], $(RW)_{4D}$ is simpler and more economical to produce, since it contains four copies of a dipeptide RW only. The

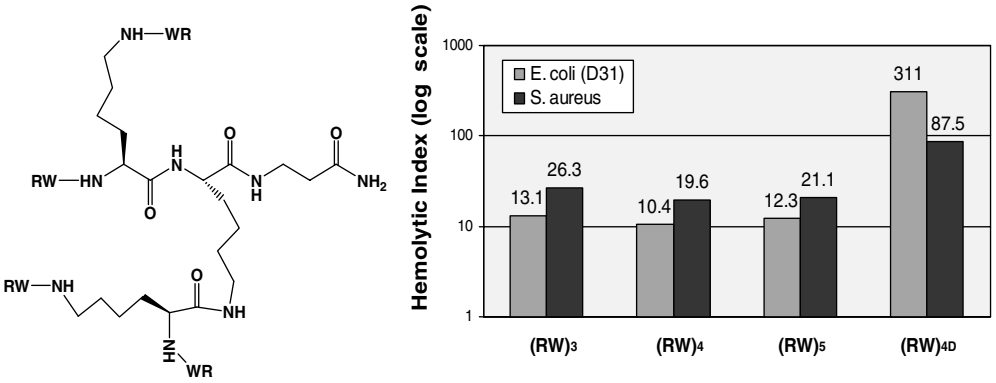


Figure 15.6 HI values. The HI, defined as the ratio of HD_{50} to IC_{50}, indicates the selectivity. Note the HI is in log scale and the values are labeled at the top of each bar. *E. coli* (D31), having a second outer membrane and containing negatively charged lipopolysaccharide, are Gram-negative; *S. aureus* have a thicker outer cell wall made of peptidoglycan and are Gram-positive.

activity of $(RW)_{4D}$ against *Escherichia coli* (D31) or *Staphylococcus aureus* is in the same range as that of the linear chains from $(RW)_n$ ($n = 3$–5). Significantly, however, as compared to $(RW)_n$ ($n = 3$–5), $(RW)_{4D}$ shows much lower hemolytic activity when evaluated by the HI. Surprisingly, $(RW)_{4D}$ is more effective against Gram-negative than Gram-positive bacteria (Figure 15.6), reversing the trend seen in natural antimicrobial peptides. Furthermore, $(RW)_{4D}$ is active against *E. coli* biofilms [56].

Biofilms are a major threat to human health, mediating infection via in-dwelling catheters or implanted devices. Recently, Reymond *et al.* [57, 58] identified two fucosyl-peptide dendrimers, FD2 (*C*-Fuc-LysProLeu)$_4$(LysPheLysIle)$_2$LysHisIleNH$_2$ and PA8 (*O*-Fuc-LysAlaAsp)$_4$(LysSerGlyAla)$_2$LysHisIleNH$_2$, through affinity screening toward fucose-specific lectin, LecB. As a human pathogen, the bacterium *Pseudomonas aeruginosa* can synthesize a fucose-specific lectin, LecB, that is suspected of playing a role on the formation of biofilms. They examined the effect of dendrimers on inhibiting *P. aeruginosa* biofilm formation and found that FD2 can completely inhibit *P. aeruginosa* biofilm formation at 50 μM ($IC_{50} \sim 10$ μM) and it is also effective at dispersing preformed biofilms. Interestingly, FD2 is not toxic to the bacterium other than biofilm inhibition and dispersion. Another recent study by Zhu *et al.* [59] has tested the inhibitory effects of a series of branched His–Lys dendrimers on a number of *Candida* species and some other fungi. They found that the four-branched dendrimer H2K4b is the most effective among all tested on fungal growth inhibition. It is important to note that these dendrimers are both active as antifungal agents and nucleic acid delivery agents. The results are consistent with the hypothesis that both activities result from some common properties of the histidine-rich dendrimer peptide – their endosomal-disrupting properties. A number of other dendrimer peptides have been reported to be effective against a variety of bacterial strains including multidrug-resistant strains [60–64]. It is generally observed that bacterial resistance to peptides or the dendrimers develops much less than to

standard antibiotics such as vancomycin. This is especially true for bacterial cells in biofilms, which enhance antibiotic resistance very significantly.

15.3.3
Peptide Dendrimers as Protein/Enzyme Mimics

The intrinsically multivalent nature of dendrimeric architecture is reflected in the presence of multiple copies of a functional group attached to a compact surface. Given multiple copies of a catalytic group positioned at such a surface, peptide dendrimers might serve as potentially effective enzyme mimics [65, 66]. Reymond *et al.* have recently identified a series of peptide dendrimers built from His–Ser repeats using diaminopropionic acid (Dap) as branching units (Figure 15.7) [67]. These dendrimers display enzyme-like Michaelis–Menten kinetics upon catalyzing the hydrolysis of pyrene trissulfonate esters. Furthermore, the catalytic activity of these molecules shows a strong positive dendritic effect, with the catalytic efficiency of these molecules increasing with the generation number (Figure 15.7). Upon increasing the generation from G1 $(Ac\text{-}His\text{-}Ser)_2Dap\text{-}His\text{-}Ser\text{-}NH_2$ to G4 $(Ac\text{-}His\text{-}Ser)_{16}(Dap\text{-}His\text{-}Ser)_8(Dap\text{-}His\text{-}Ser)_4(Dap\text{-}His\text{-}Ser)_2Dap\text{-}His\text{-}Ser\text{-}NH_2$, k_{cat}/K_M increases quadratically with contributions from both k_{cat} and K_M. Mutational

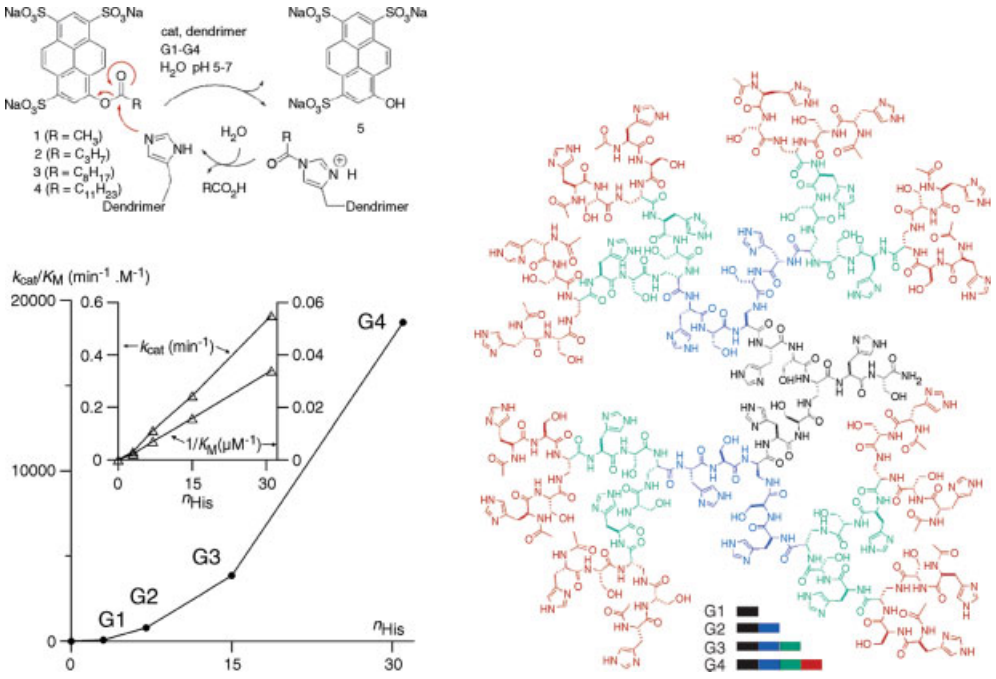


Figure 15.7 Catalysis of ester hydrolysis of peptide dendrimers G1–G4 and the dendritic effect on the hydrolysis reaction. (Reproduced with permission from Scheme 1 and Fig. 2 of [67], copyright 2004, American Chemical Society.)

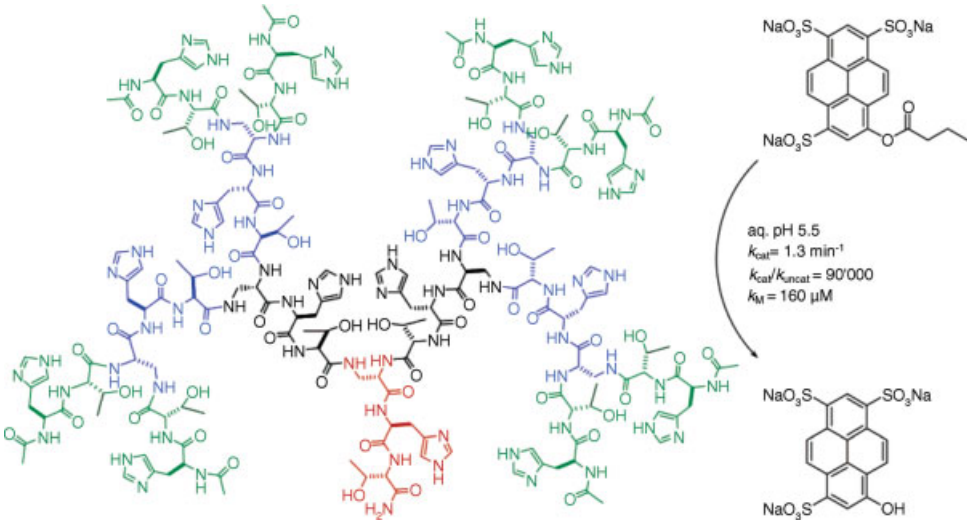


Figure 15.8 Threonine mutant dendrimer and its improved catalytic activity on ester hydrolysis reactions. (Reproduced with permission from [68], copyright 2006, American Chemical Society.)

studies on the third-generation dendrimer (Ac-His-Ser)$_8$(Dap-His-Ser)$_4$(Dap-His-Ser)$_2$Dap-His-Ser-NH$_2$ revealed that the catalytic proficiency is related to the location of histidines within the dendrimers as well as to the identities of other amino acids. The most effective catalytic dendrimer found [68] is the threonine mutant (Ac-His-Thr)$_8$(Dap-His-Thr)$_4$(Dap-His-Thr)$_2$Dap-His-Thr-NH$_2$ with a $k_{cat}/k_{uncat} = 90\,000$, corresponding to a 5-fold improvement over the original third-generation dendrimer (Figure 15.8).

Investigating the potential of peptide dendrimers to serve as protein mimics [69], the same group has recently identified a variety of vitamin B_{12} dendrimer ligands that could potentially serve as a vitamin B_{12} transporter [70]. Reymond *et al.* prepared a split-and-mix combinatorial peptide dendrimer library and screened their affinity for cobalamin. The dendritic ligands found through screening have metal-binding residues at the core and a polyanionic shell of glutamates in the outer layers.

15.3.4
Peptide Dendrimers as Ion Sensors and MRI Contrast Agents

Functionalized peptide dendrimers can target specific analytes and therefore find applications as sensors [71]. Vogtle *et al.* modified the surface of a polylysine dendrimer with 24 chromophoric dansyl units and employed it as ion sensors (Figure 15.9) [72, 73]. Dansyl units show an intense fluorescence band in the visible region, and 21 amide groups contained inside the interior of each dendrimer can interact with Co^{2+}, Ni^{2+}, Zn^{2+}, and lanthanide ions, including Nd^{3+}, Eu^{3+}, Gd^{3+}, Tb^{3+}, Er^{3+}, and Yb^{3+}. Upon titration of each ion into a dendrimer solution, the fluorescence of the dansyl units is quenched to various degrees (Figure 15.9).

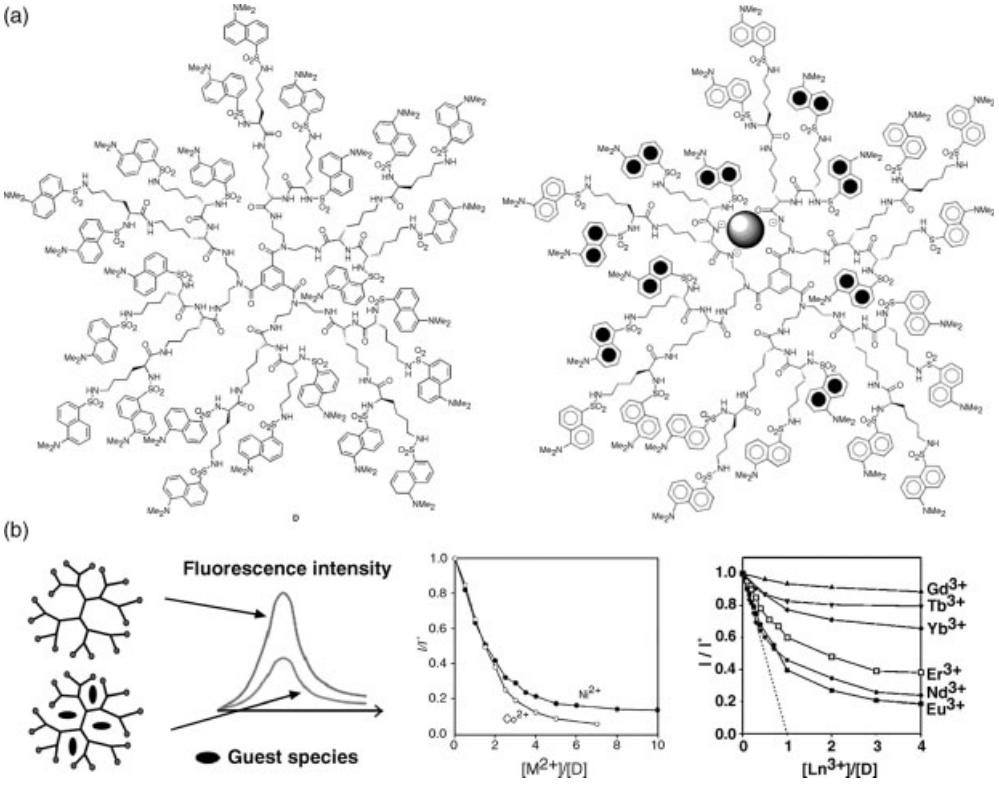


Figure 15.9 (Top) Structure of the dendrimer and the schematic representation of the dendrimer as ion sensor. (Bottom) Titration results of the dendrimers with different ions as indicated by fluorescence quench. (Adapted with permission from [72] copyright 2000, Royal Society of Chemistry, [73] copyright 2002, American Chemical Society.)

As lanthanide ions are paramagnetic, their presence can enhance the relaxation rate of water protons and therefore improve the signal-to-noise ratio of the image obtained [74]. Lanthanide ions in general, in particular Gd^{3+}, are excellent magnetic resonance imaging(MRI) contrast agents. Gadomer 17 was designed as a dendritic MRI contrast agent (Figure 15.10) [75]. It has a trimesoyl triamide core to which 18 lysine residues are attached. On the surface of the dendrimer, 24 Gd^{3+}-DOTA (tetraazacyclododecanetetraacetic acid) can bind.

In another example, a symmetric polylysine-based dendrimer was prepared by native chemical ligation (Figure 15.11) [76]. This dendrimer has two polylysine dendritic wedges. On one wedge, diethylenetriaminepentaacetic acid (DTPA), was attached to the polylysine scaffold and could be used as an efficient chelating agent for lanthanide ions, in particular Gd^{3+}; on the other wedge, an oligopeptide RGDS that binds to $\alpha_v\beta_3$ integrins was attached to allow specific targeting. The system reported has potential to be developed as a MRI contrast agent specific for cardiovascular disease.

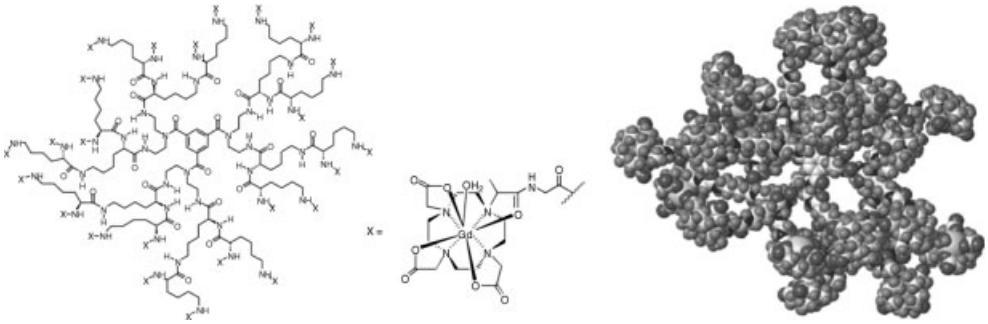


Figure 15.10 Structure and the computer generated model of Gadomer 17. (Reproduced with permission from [75], copyright 2002, Wiley-VCH.)

15.3.5
Peptide Dendrimers as DNA/RNA Delivery Vectors

Treatment of inherited disorders through gene therapy relies on the development of safe and efficient gene delivery vectors [14, 77–81]. Many investigations of viral and nonviral gene delivery systems have been carried out in recent years. Compared to the inherent danger and limitations of viral delivery systems, nonviral systems are safer and have gained a lot of interest recently despite the fact that nonviral systems are considered to be less effective. In the search for synthetic nonviral vectors, efforts

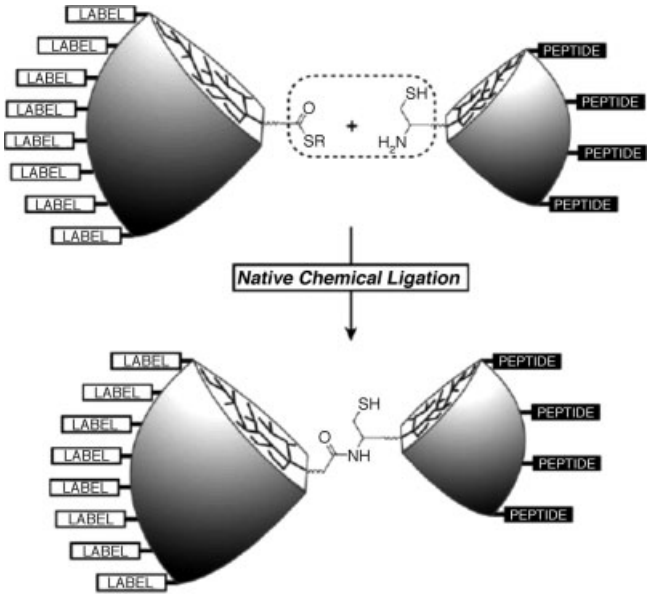


Figure 15.11 Nonsymmetric dendrimers prepared from native chemical ligation. (Reproduced with permission from Scheme 1 of [76], copyright 2006, Royal Society of Chemistry.)

have been devoted to several different types of molecular systems. These include cationic polymers, cationic dendrimers (including peptide and nonpeptide dendrimers), cationic liposomes, and cell-penetrating peptides, including those equipped with nuclear localization sequences. Here, we discuss the development of synthetic vectors based on peptide dendrimers. For the purpose of vector development, vectors need to bind and carry DNA/RNA, and protect them from enzymatic degradation; they should interact with target cell surfaces and transport DNA/RNA across membranes; they should allow their cargo DNA/RNA to escape from the endosome following internalization; and they should release therapeutic DNA/RNA at the appropriate location in the target cell and hopefully produce the desired perturbation of gene expression.

Peptide dendrimers in principle can satisfy many of the above requirements. Cationic peptide dendrimers bind negatively charged DNA/RNA with high affinity and show strong protection of DNA/RNA against enzymatic digestion. Different modifications of peptide dendrimers such as the incorporation of amino acids with long lipid chains (Figure 15.12) [82] and attachment of different penetrating peptides onto dendrimer surfaces [83–85] have been explored in an effort to take advantage of different endocytotic pathways and overcome the mammalian cell membrane barrier. There are four major internalization pathways used by mammalian cells [14, 36, 80]: macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, as well as clathrin- and caveolin-independent endocytotic pathways (Figure 15.13).

Tung *et al*. designed a series of peptides derived from a polylysine dendritic scaffold (Figure 15.14) [83]. Their idea was based on the observation that a peptide fragment of the HIV *tat* protein, RKKRRQRRR, is able to ferry various molecules across cellular and nuclear membranes. They reasoned that the peptide might help to improve plasmid DNA delivery efficiency if multiple copies of the peptides can be incorporated onto a branched scaffold. The results showed that all designed peptides interact

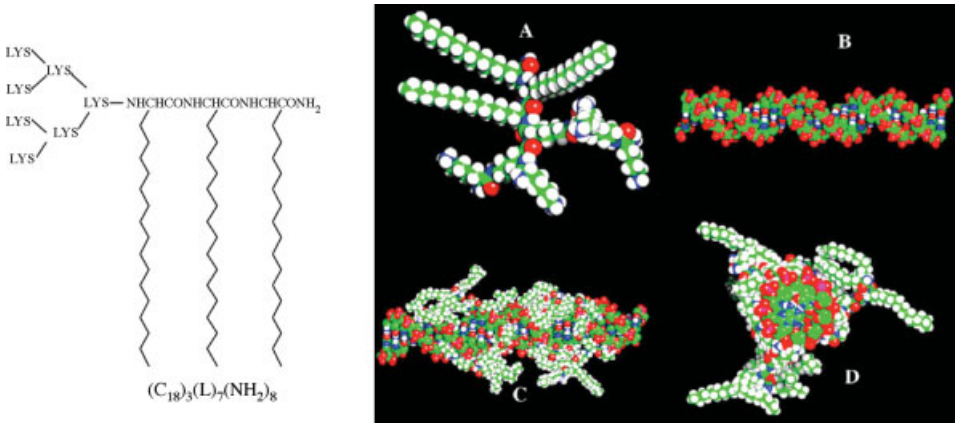


Figure 15.12 Structure of a lipid dendrimer, and computer-generated models of the dendrimer, a segment of DNA with 32 base pairs, and the dendrimer/DNA complexes (two different views). (Adapted with permission from [82, 86], copyright 2003, 2005, Elsevier B.V.)

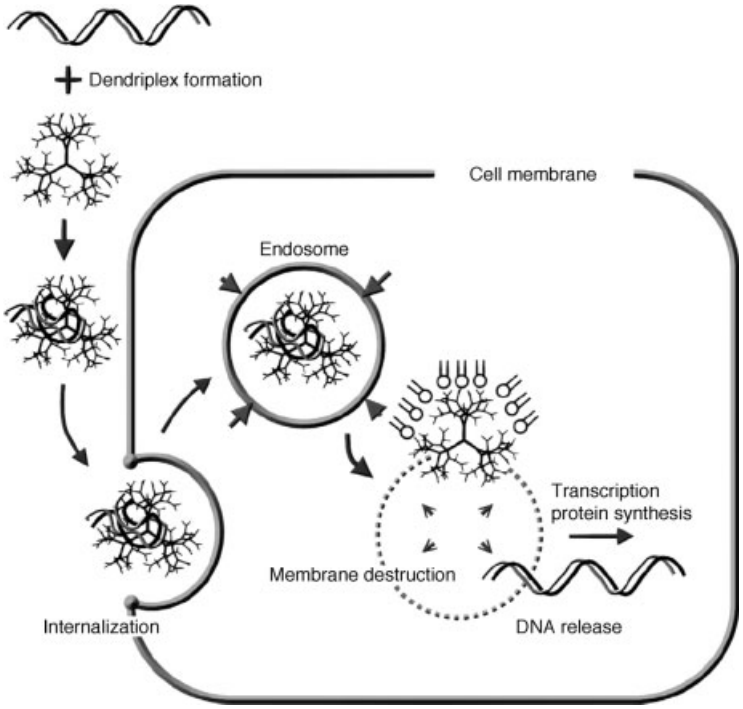


Figure 15.13 Internalization pathway of gene/dendrimer cargo. (Reproduced with permission from Fig. 2 of [14], copyright 2009, Maik Naukc/Interperiodica.)

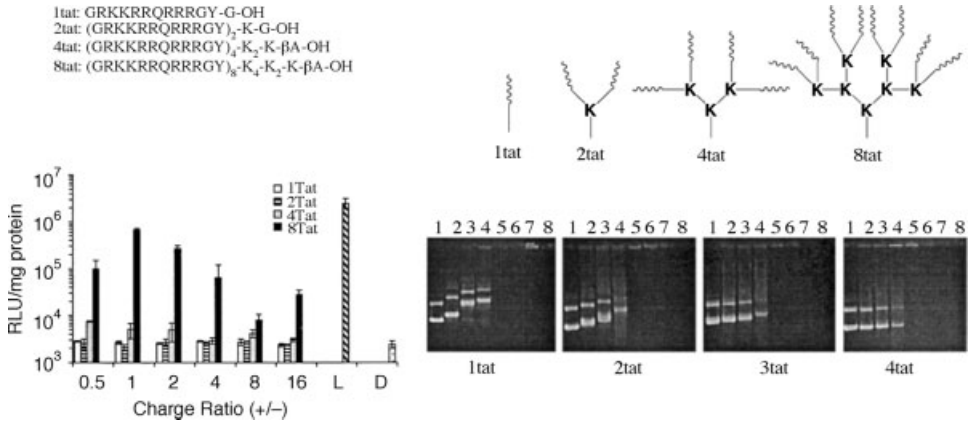


Figure 15.14 Sequences and structures of synthesized peptides (βA is β-alanine and the undulating line represents the TAT peptide) and the transfection of COS-1 cell with different peptides at various charge ratios (L: lipofectamine; D: naked DNA control) as well as gel-shift assays indicating the binding of synthesized peptides with luciferase DNA at various charge ratios. (Adapted with permission from Figs 1–3 of [83], copyright 2002, Elsevier B.V.)

with plasmid DNA as revealed by gel-shift assays (Figure 15.14). The dendrimer peptide with eight *tat* peptide chains attached showed the optimal transfection activity.

Bayele *et al.* [87] have recently designed two polylysine dendrimers that contain lipidated amino acids, S-72 and S-73 (Figure 15.15a). The uniqueness of this design is that they incorporated two functional moieties into the dendrimers: (i) a positively charged lysine module for nucleic acid interactions and (ii) a neutral lipid moiety for membrane transit. The results showed that the dendrimers can indeed serve as nucleic acid carriers and deliver the desired oligonucleotides into the nucleus (Figure 15.15).

Toth and Minchin's group have recently constructed a small combinatory library of synthetic DNA vectors (Figure 15.16) [84, 85]. The vectors consist of various combinations of the cell penetrating peptide TAT, the SV40 large T protein nuclear localization signal (NLS), and a cationic dendrimer of seven lysine residues (DEN). The vectors were assessed for their capability to form complexes with plasmid DNA

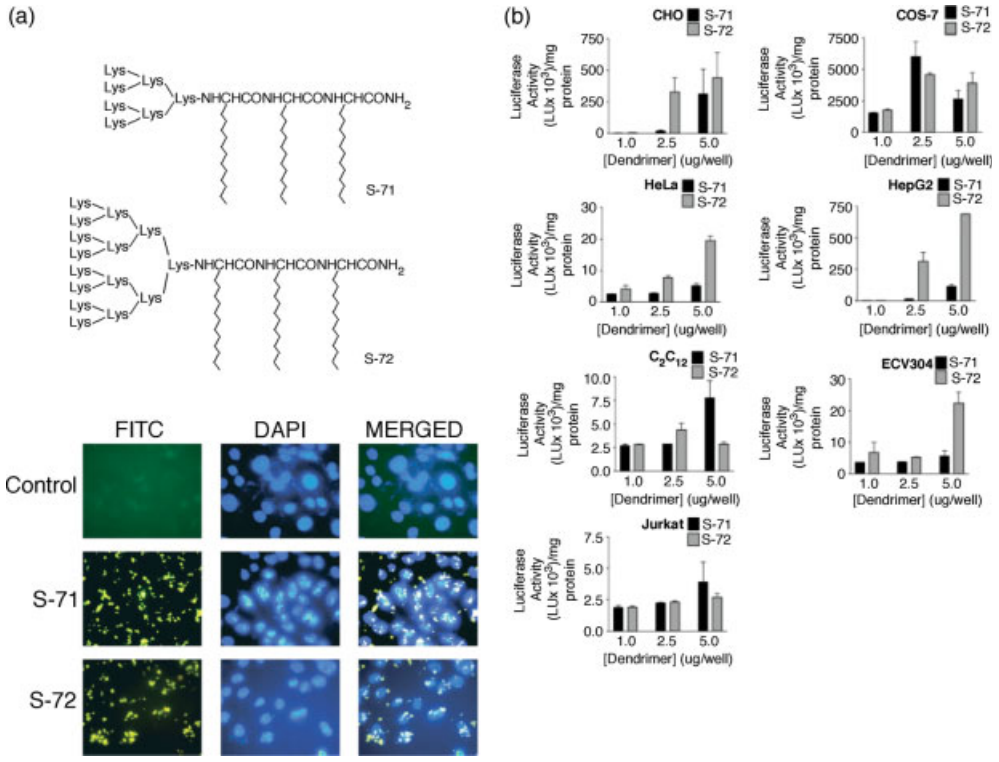


Figure 15.15 (a) Structures of lipidic peptide dendrimers and nuclear delivery of oligonucleotide by dendrimers. (b) Transfection assays on different cell types. (Adapted with permission from Figs 1, 4, and 5 of [87], copyright 2005, Wiley Liss, Inc. and The American Pharmacist Association.)

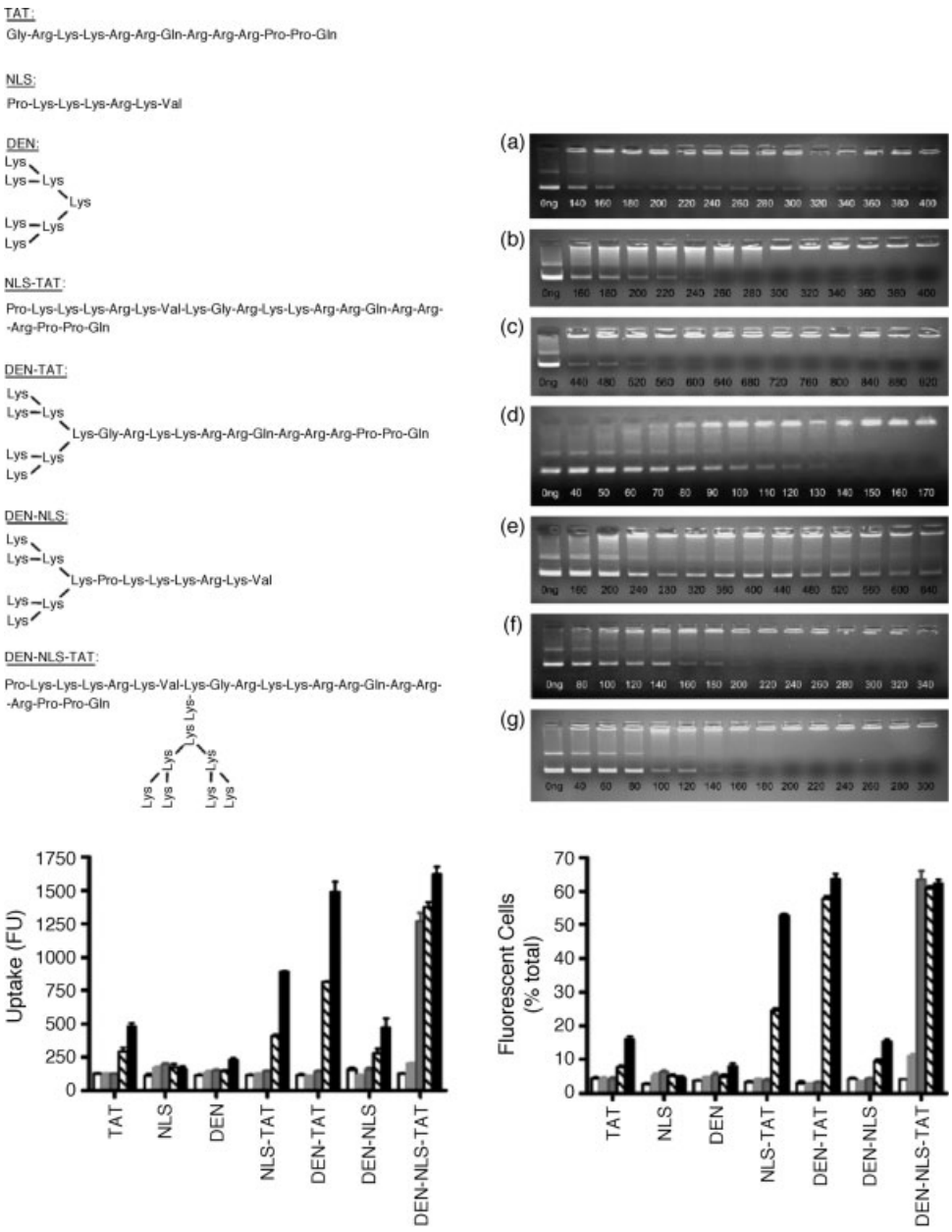


Figure 15.16 Sequences and structures of the dendrimers and gel-shift assays of the dendrimer/DNA complexes as well as the DNA uptake of different dendrimer/DNA complexes at various charge ratios. (Adapted with permission from [84, 85], copyright 2007, 2009, Elsevier B.V.)

using several biophysical assays all of which indicate positive results (Figure 15.16). A test of each member's capability to deliver exogenous DNA into human HeLa dells revealed that the best system among them is the one containing all three functionalities (i.e., TAT, NLS, and DEN). Fluorescence microscopy indicated that with the help of the DEN–NLS–TAT dendrimer the exogenous DNA was delivered but confined to intracellular compartments. Incubation of the dendrimer with a fusogenic agent such as chloroquine enhanced transgene expression by more than 500-fold. The results point to the potential importance of multifactorial assembly in design of nonviral gene delivery carriers.

Mixson *et al*. have recently investigated a series of branched peptides composed mainly of histidine and lysine residues as DNA/RNA vectors [88–90]. They found that H2K4bT with a histidine-rich tail is an optimal DNA vector [89] as the dendrimer by itself can effectively transport plasmids into a variety of cells without any covector. They interpret the successful effect of adding a histidine-rich tail to the dendrimer as due to the histidine-rich sequence's capability to buffer the acidic endosome and thereby induce its lysis. This helps exogenous DNA escape the endosomal pathway and thus DNA degradation by lysosomes is avoided. The optimal plasmid DNA carrier H2K4bT has four branches and a tail. In contrast, the optimal small interfering RNA carriers H3K8b, H3K8b(+RGD), and similar structural analogs consist of eight terminal branches [90]. They have further tested both types of vectors as DNA/RNA carriers in several *in vivo* studies. In tumor-bearing mouse models, they observed successful delivery of corresponding genes or small interfering RNAs and demonstrated inhibitory effects on tumor xenografts [91–94]. Taken together, these data suggest that development of nonviral DNA/RNA vectors through amino acid sequence modification and peptide branching with lysine residues may lead to an effective strategy for cancer treatments.

15.3.6
Other Application of Peptide Dendrimers

Fassina has developed a procedure for the direct immobilization on preactivated affinity solid supports of a peptide dendrimer with a multimeric tripeptide ligand (Met–Tyr–Phe) [95]. The peptide ligand was assembled on an eight-branched heptalysine dendritic scaffold and the resulting dendrimers were coupled to preactivated resin supports. The modified solid supports were then packed onto a glass column and applied to the affinity purification of bovine neurophysin from a crude mixture. Another important application of peptide dendrimers was reported by Grinstaff *et al*. [96–98]. They used cross-linkable precursor dendritic molecules to form a hydrogel and applied the cross-linked biomolecules to seal ocular wounds created in surgeries, such as corneal incision during a typical cataract procedure (Figure 15.17). The results suggest potential applications of peptide dendrimers as soft materials for specific biomedical purposes. Thus, since the initial design of peptide dendrimers as MAPs [30], the potential of these molecules for vaccine development has proven limited in practice. However, several fruitful biochemical and medical applications of various designed peptide dendrimers have been pur-

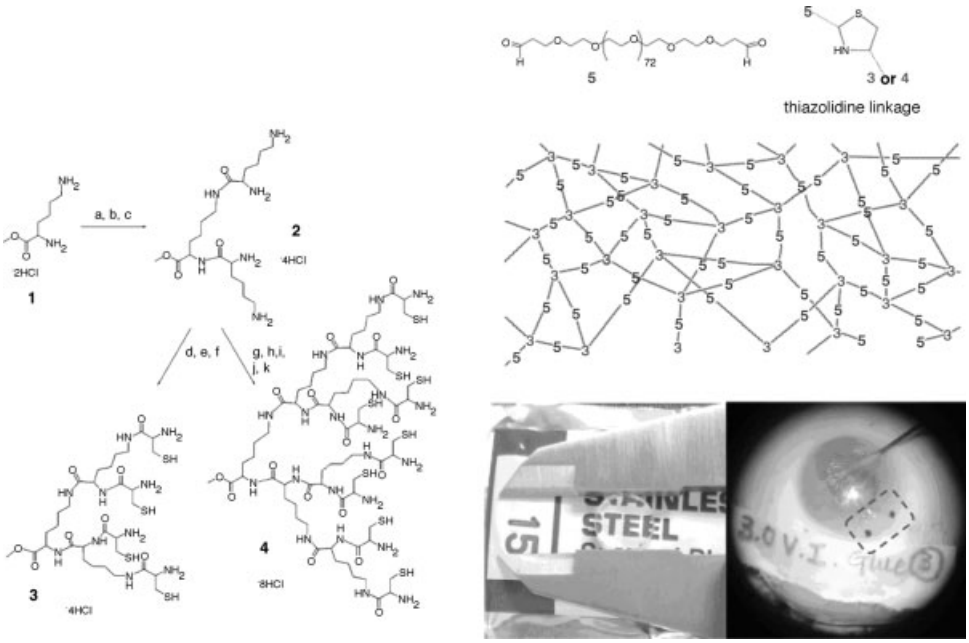


Figure 15.17 Synthesis of cross-linkable precursor dendritic molecules and their reaction with poly(ethylene glycol dialdehyde) to form an idealized cross-linked hydrogel. The left photograph shows the synthesized hydrogel and the right the result of a surgery wound repaired by the hydrogel sealant (incision is between the two purple dots and the gel is within the blue border). (Reproduced with permission from Figs 1 and 2 and Scheme 1 of [96], copyright 2004, American Chemical Society.)

sued [18, 99–103]. Peptide dendrimers have been reported to serve a variety of purposes in applications ranging from protein/enzyme mimics [48, 65, 66], to metal ion sensors [72, 73], MRI contrast agents [75], drug transport vehicles, DNA/RNA delivery vectors [91–94], and antimicrobial agents [53, 55, 62, 63].

15.4 Conclusions

Peptide dendrimers represent a unique class of molecules constructed from amino acids with branched structures. Their properties differ from linear polypeptides due to their intrinsically multivalent construction. Display of functional groups or structures on dendrimeric scaffolds has opened up new vistas for synthesis, study, and application. While numerous peptide dendrimers have been developed in recent decades, there is still additional potential to explore the structural diversity and wealth of applications for this unique class of structures [17].

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