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'100 years of peptide synthesis': ligation methods for peptide and protein synthesis with applications to β-peptide assemblies*

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Dates:
Received 4 October 2004
Revised 25 October 2004
Accepted 20 November 2004

*Dedicated to Murray Goodman, a pioneer in peptide chemistry.

To cite this article:
Kimmerlin, T. & Seebach, D. '100 years of peptide synthesis': ligation methods for peptide and protein synthesis with applications to β-peptide assemblies*. *J. Peptide Res.*, 2005, **65**, 229–260.
DOI 10.1111/j.1399-3011.2005.00214.x

Key words: peptide chemistry; chemical ligation; thioligation; β-amino acids; β-peptide

Abstract: A brief survey of the history of peptide chemistry from Theodore Curtius to Emil Fischer to Bruce Merrifield is first presented. The discovery and development of peptide ligation, i.e. of actual chemical synthesis of proteins are described. In the main chapter, 'Synthesis of Proteins by Chemical Ligation' a detailed discussion of the principles, reactivities and mechanisms involved in the various coupling strategies now applied (ligation, chemical ligation, native chemical ligation) is given. These include coupling sites with cysteine and methionine (as well as the seleno analogs), histidine, glycine and pseudo-prolines, 'unrestricted' amino-acid residues (using the Staudinger reaction), as well as solid-phase segment coupling by thioligation of unprotected peptides. In another section, 'Synthesis of β-peptides by Thioligation', couplings involving β^2- and β^3-peptides are described (with experimental details).

Introduction

Peptides and proteins play a central role in numerous biological and physiological processes in living organisms; they are involved as hormones and neurotransmitters in intercellular communication, act as antibodies in the immune system to protect organisms against foreign invaders, and are also involved in the transport of various substances through biological membranes. Understanding the control, at the molecular level, of the mechanisms and principles governing structural and functional properties of bioactive proteins is an important objective in biological and medical research. The first requirement for the study of proteins is to assess their ease of availability in terms of purity and quantity. There are three main routes to consider: (i) native

protein isolation, (ii) recombinant techniques for the expression of proteins in microorganisms, and (iii) chemical synthesis. Each of these methods has its advantages and disadvantages but only chemical peptide synthesis permits the incorporation of unnatural amino acids and the production of large quantities of pure peptides. Since the first synthesis of a dipeptide by Emil Fischer in 1901, peptide science has made tremendous progress and with recent innovations it is currently possible to 'routinely' synthesize proteins, well over 200 amino acids in length. The following sections focus on the development of peptide synthesis. First, a brief overview of the history of synthetic peptides will be given, followed by a presentation of the most recent methodology developed for the synthesis of large peptides: the so-called 'chemical ligation'. Applications of this method in the synthesis of β-peptides are described.

A Brief History of Peptide Chemistry

The publication in 1901, by E. Fischer (with E. Fourneau) (1), of the preparation of the dipeptide glycylglycine by hydrolysis of the diketopiperazine of glycine, is considered to be the beginning of peptide chemistry. However, T. Curtius had synthesized and characterized a related peptide 20 years earlier, during his PhD studies with H. Kolbe, preparing the first N-protected dipeptide, benzoylglycylglycine, by treatment of the silver salt of glycine with benzoylchloride (2) (Fig. 1).

The intensive work of T. Curtius with diazo compounds led to the development of the first practical method for peptide synthesis: the azide-coupling method, which was successfully employed in the synthesis of benzoylglycine peptides of a defined length (Fig. 2) (3). E. Fischer, on the contrary, developed a method of peptide coupling based on the use of acylchlorides, prepared from the corresponding free amino acid using PCl_5 and acetyl chloride as solvent (4).

The major problems, and therefore limitations, of both methods stemmed from difficulties at that time in obtaining enantiomerically pure L-amino acids and from the absence of an easily removable amino-protecting group. The introduction in 1931, by M. Bergmann (a former student of E. Fischer) and L. Zervas, of the carbobenzoxy (Cbz) group (Fig. 3 left) for the temporary protection of the amino function solved part of the problem and led to a new era in peptide synthesis. From this point, numerous small peptides such as glutathione (5), and carnosine (6) were synthesized, culminating some 20 years later in the synthesis of an active hormone, the octapeptide oxytocin, by V. du Vigneaud *et al.* (7) (see Fig. 4). V. Du Vigneaud was awarded the Nobel Prize 2 years later. It should be mentioned here that M. Bergmann and L. Zervas showed, during their synthesis of glutathione, that the use of the Cbz-protecting group prevented racemization during the formation of the acyl chloride, whilst with *N*-acyl- or *N*-benzoyl-protected amino acids, the reaction led to an almost complete racemization. It was later shown that this configurational

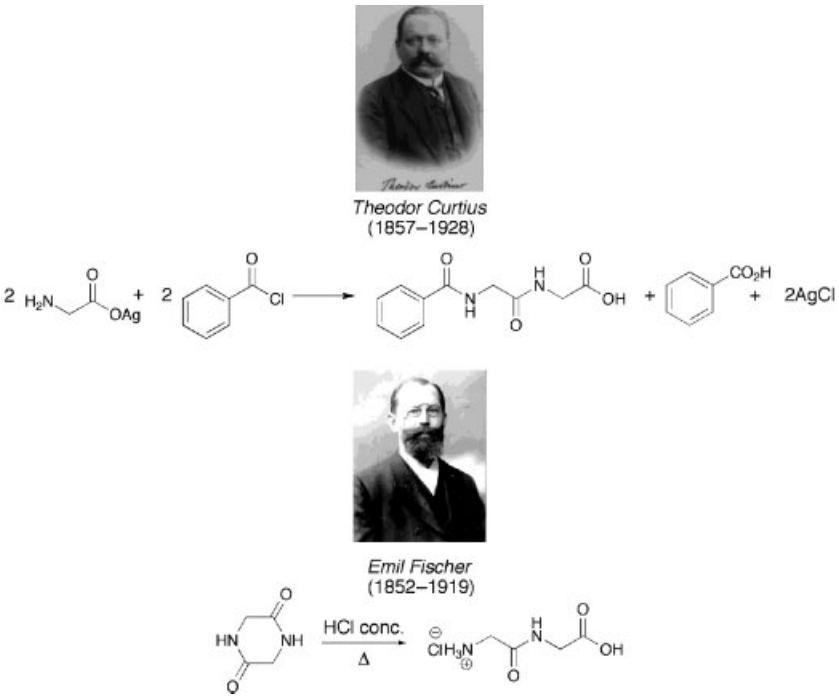


Figure 1. The first synthesis of a dipeptide by *T. Curtius* and later by E. Fischer.

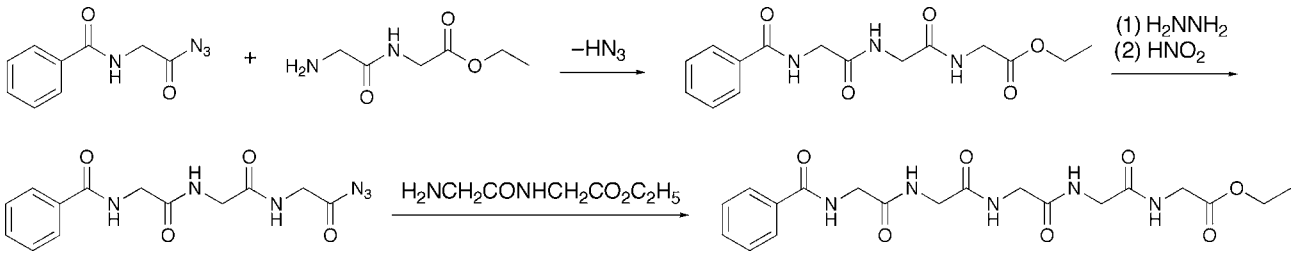


Figure 2. Synthesis of benzoylpentaglycine by the azide coupling methodology.

Figure 3. Cbz and Boc deprotection.

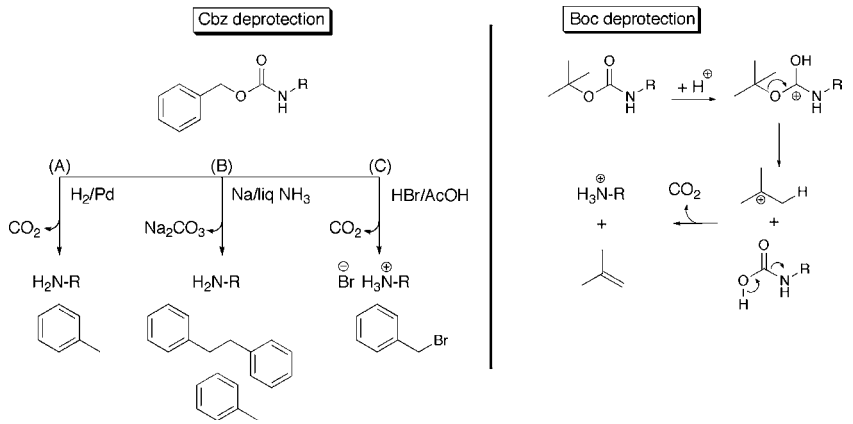
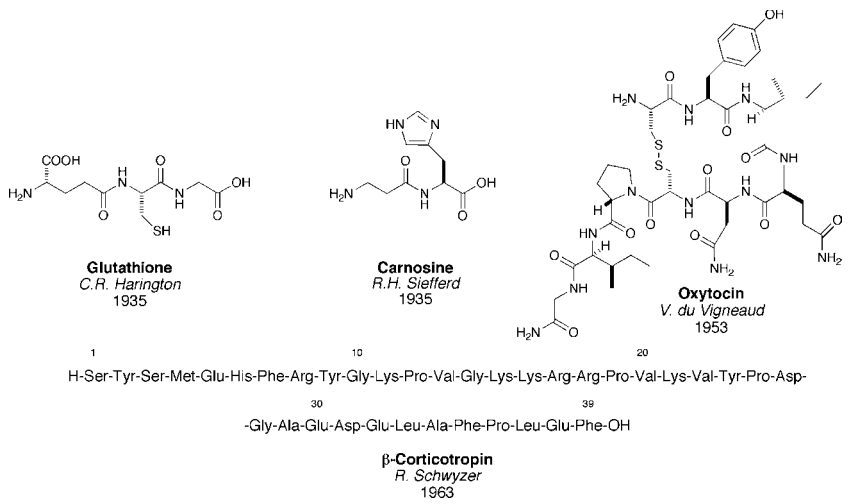


Figure 4. Molecular formulae of some of the first synthetic biologically active peptides.



stability is a general property of urethane-protecting groups.

The introduction, in 1957, by L. A. Carpino (8) and F. C. McKay and N. F. Albertson (9) of a new, acid-labile protecting group, the *tert*-butyloxycarbonyl group (Boc) (Fig. 3 right), which is stable toward hydrogenation, *Birch* reduction, strong alkali and therefore totally orthogonal to the Cbz (or modified Cbz) group and also to benzyl esters and ethers, greatly enhanced the arsenal of protecting groups available to the peptide chemist at the time. The combination of Boc- and Cbz-protecting groups was then used for the synthesis of several peptides, the most spectacular example, for this period, being the synthesis of β-corticotropin Adrenocorticotrophic Hormone (ACTH), a 39-residue porcine hormone, in 1963, by R. Schwyzer and P. Sieber (10) (Fig. 4).

The development of new protecting groups was accompanied by intensive research toward discovering new coupling methods. The most important innovation in this regard was probably the introduction of carbodiimides in 1955 by J. C. Sheehan and G. P. Hess (11) and H. G. Khorana (12) (Fig. 5). The carbodiimide activation method has, however, a high propensity for racemization because of the

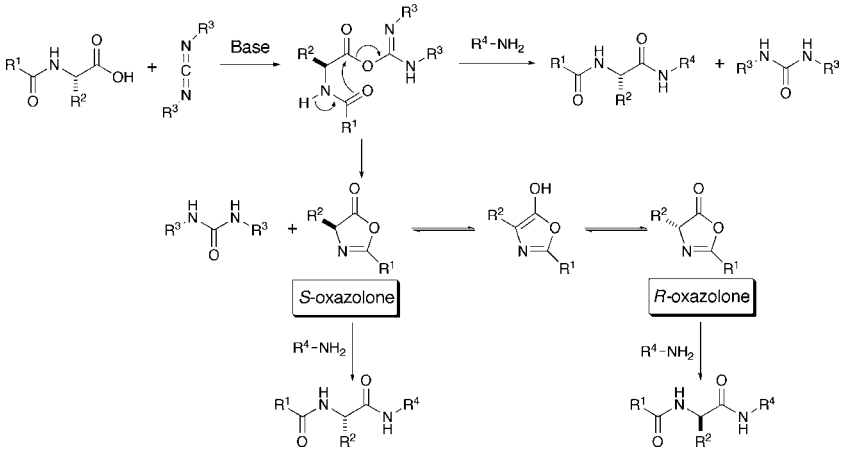


Figure 5. Coupling reaction through carbodiimide activation: mechanism of racemization via the formation of a 5(4H)-oxazolone.

high reactivity of the *O*-acyl-isourea, which can lead, through intramolecular cyclization, to the formation of an oxazolone: this cyclic intermediate can easily racemize via an aromatic intermediate as shown in Fig. 5. The racemization mechanism through the formation of the oxazolone has been extensively investigated. In their pioneering work, Goodman and McGahren (13) established all the factors affecting the optical purity of the oxazolone intermediate during the peptide bond formation, such as the nucleophilicity/basicity ratio of the incoming amino group and solvent effects.

Activation of the carboxylic group can always lead to racemization via the oxazolone intermediate; however, the introduction of 'additives' such as 1-hydroxybenzotriazole (HOBt) to the reaction mixture was then shown to minimize this by the formation of a less-reactive HOBt ester. Over the years, numerous other coupling reagents have been developed, and a selection of the most widely used ones is shown in Fig. 6.

The next major breakthrough in peptide chemistry came in 1963 when B. Merrifield published a historic paper describing the principles and the applications of his invention:

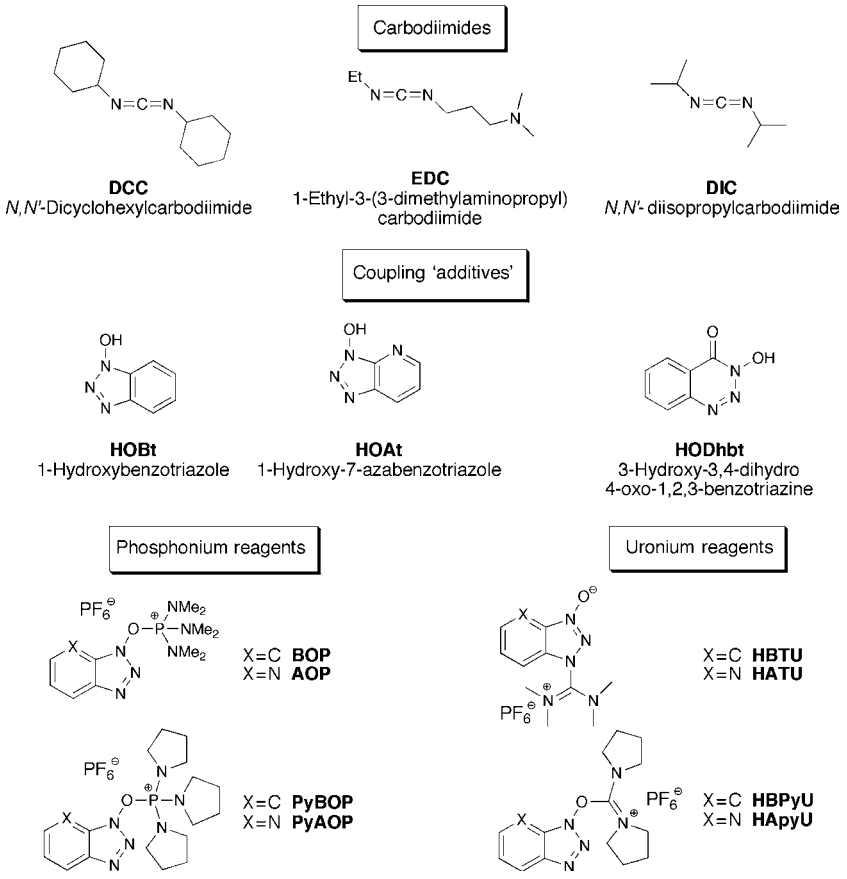


Figure 6. Molecular formulae of coupling reagents.

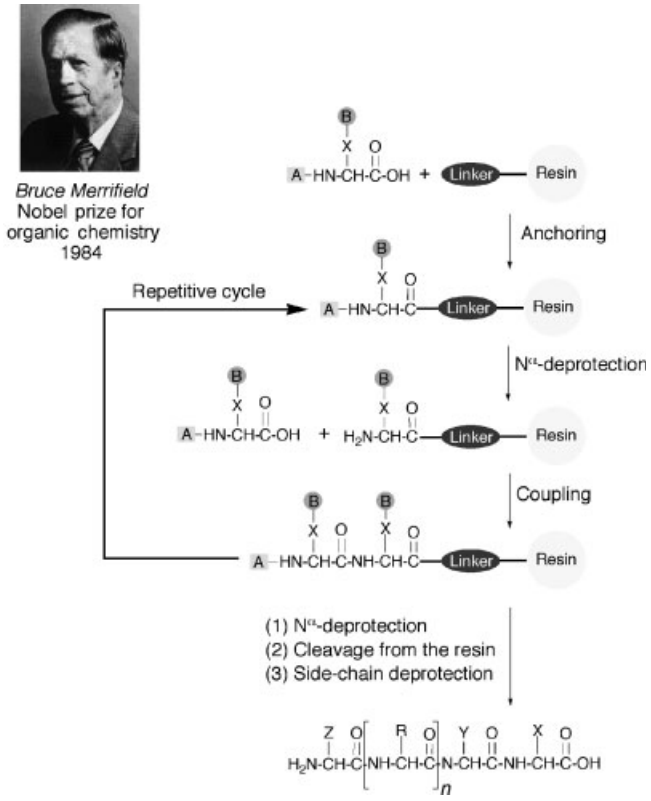


Figure 7. Schematic presentation of the principles of solid-phase peptide synthesis.

solid-phase peptide synthesis (SPPS) (14). In contrast to the solution-phase methodology, where after each reaction the product has to be isolated and purified before the next step, the growing peptide in the solid-phase approach is linked to an insoluble support and therefore, after each reaction step, the byproducts are simply removed by filtration and washing (Fig. 7). Furthermore, because of the repetitive nature of peptide synthesis (deprotection, washing, coupling, washing, deprotection, ...), the use of an insoluble support in a single reaction vessel allows for automatization of the processes.

In the beginning, the solid support used was a styrene–divinylbenzene co-polymer, functionalized by chloromethylation. The resulting benzyl chloride derivative (the Merrifield resin) was used for anchoring the first amino acid to the resin via an ester linkage. The peptide was then assembled using a carbodiimide as coupling reagent, with a combination of Boc as the protecting group for the N-terminus and benzyl for the side-chain functionalities. Upon completion of the last coupling, the peptide could be cleaved from the resin, with concurrent deprotection of the side-chain-protecting groups, using liquid hydrogen fluoride (HF). The use of liquid HF can, however, lead to numerous side reactions, including catalytic Friedel–Crafts reactions between the aromatic groups of the resin and the side

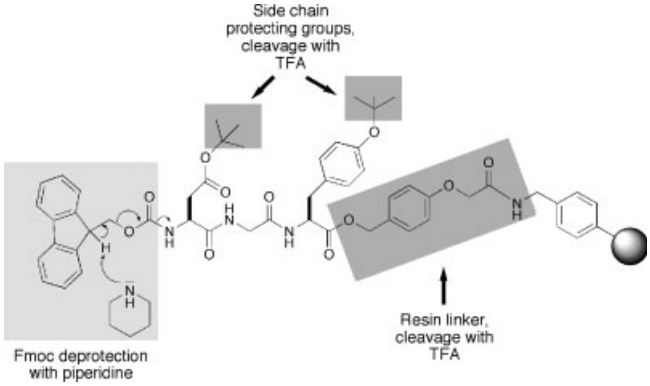


Figure 8. Fmoc strategy in solid-phase peptide synthesis. The Fmoc group is cleaved under basic conditions with piperidine, while the side-chain-protecting groups and the linker are cleaved under acidic conditions using TFA.

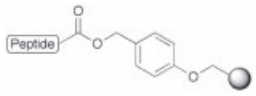
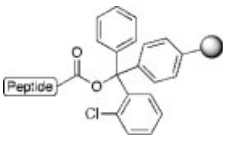
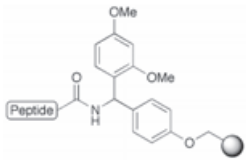
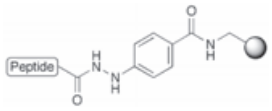
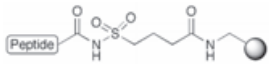
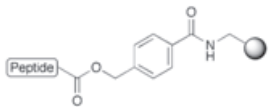
chains of the peptide, and/or promotion of an N → O acyl shift involving the side-chain groups of serine and threonine. These side reactions were minimized by the introduction by Tam *et al.* (15) of a two-stage, low–high HF concentration cleavage protocol.

In 1970, L. A. Carpino and G. Y. Han (16) introduced a totally different protecting-group strategy. This was based on the use of the base-labile 9-fluorenylmethyloxycarbonyl (Fmoc) group for the protection of the α-amino group, thereby allowing the orthogonal protection of side-chain groups through use of acid-labile protection (Fig. 8).

The mechanization of the SPPS process permits, in a fully automatic manner, the incorporation of more than 10 amino acid residues per day and since the first introduction of this methodology, has accounted for the synthesis of thousands of peptides. In 1984, B. Merrifield was awarded the Nobel Prize in chemistry for his invention. In recent years, numerous new types of resins have been developed, allowing for the preparation, through the Boc/benzyl (Bzl) or the Fmoc/*t*-Butyl (Bu) strategy, of peptides bearing different functionalities at the C-terminus such as peptide-acid, -amide, -thioester, or -alcohol (a selection of derivatized resins for Fmoc and Boc SPPS is given in Tables 1 and 2).

There is an important aspect of peptide chemistry that has not been mentioned so far, but which is an essential consideration: the purification and analysis of peptides. Parallel to the intensive development of synthetic methodology for the production of peptides, considerable progress has been made to address these factors. Today, high-pressure (high-performance) liquid chromatography (HPLC) is the most widely used method and indeed a highly effective method for purification, allowing the routine separation of complex product mixtures (17). Mass spectrometry, on the contrary, is the most powerful tool for

Table 1. **Resins for Fmoc/^{t}Bu solid-phase peptide synthesis (the solid supports are generally either cross-linked polystyrene or polyethylene glycol-based polymers)**

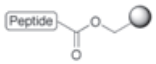
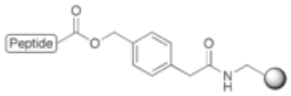
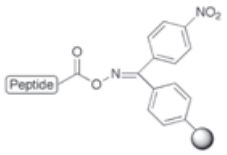
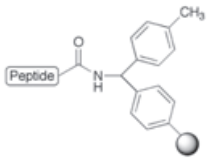
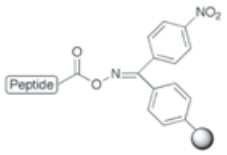
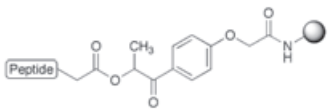
Peptide acid	Resin	Cleavage
	Wang resin	95% TFA
Protected peptide acid		
	2-Chlorotrityl resin	1% TFA in DCM
Peptide carboxamide		
	Rink amide resin	95% TFA
Peptide ester and *N*-alkylamide		
	4-Fmoc-hydrazinobenzoyl resin	$Cu(OAc)_2$, RNH_2 or $Cu(OAc)_2$, ROH, pyridine
Peptide thioester		
	4-Sulfamylbutyryl resin	(1) $TMSCH_2N_2$ (2) RSH
Peptide alcohol		
	HMBA resin (4-hydroxymethylbenzoic acid)	$NaBH_4$/EtOH

product analysis: determining the exact mass of an analytical sample is the best proof that a synthesis was, at least in part, successful or not. The development of new technologies in mass spectrometry permits the sequencing of peptides or proteins and therefore represents a direct analysis of their primary structures (18).

Synthesis of Proteins by Chemical Ligation

Since its introduction, the solid-phase methodology has advanced significantly and now allows for the routine synthesis of peptides and small proteins with sizes up to 50 amino acid residues. There are only a few examples describing the synthesis of longer chains, such as ribonuclease A (124 residues) (19) and human immunodeficiency virus (HIV)-1 TaT (86 residues) (20,21) or the green fluorescent protein, a 238-residue peptide chain synthesized by fragment coupling on solid phase (22). These examples are, however, rather exceptional as the solid-phase methodology is usually limited because of the accumulation of side products arising from incomplete deprotection or coupling reactions. Unfortunately, the average length of a protein is approximately 250 amino acids and consist of two functional domains of 15 kDa in size (23,24). In order to circumvent this limitation of solid-phase methodology for the preparation of longer proteins, new approaches have been developed. The most useful and important one of these is chemical ligation, which allows for the coupling of unprotected peptide fragments in aqueous solution.

Table 2. **Resins for Boc solid-phase peptide synthesis (the solid supports are generally either cross-linked polystyrene or polyethylene glycol-based polymers)**

Peptide acid	Resin	Cleavage
	Merrifield	HF, TFMSA
	PAM (4-hydroxymethyl-phenylacetamidomethyl)	HF, TFMA
Protected peptide acid		
	Oxime resin	NaOH/dioxane
Peptide carboxamide		
	MBHA resin (4-methylbenzhydrylamine)	HF, TFMSA
Peptide ester		
	Oxime resin	MeOH/DMF/TEA
Peptide hydrazide		
	Brominated PPOA resin [4-(2-bromopropionyl)phenoxy]-acetic acid	NH_2NH_2/DMF

Discovery and first approach: prior ligation followed by intramolecular acyl shift

In 1953, Wieland *et al.* (25) reported the possibility of generating an amide bond in aqueous solution through an intramolecular acyl shift (Fig. 9).

It is exactly this principle that was used 25 years later by Kemp *et al.* (26) and Kemp and Kerkman (27) in the development of the first ligation methodology for the coupling of two peptide fragments: the 'prior thiol capture strategy'. The principle is described in Fig. 10.

Peptide fragment **A** is derivatized at the C-terminus in the form of an ester group, using a hydroxy-group that serves as a template to bring the acyl group and the amino group in close proximity. The thiol functionality present in the hydroxy-template moiety is referred to as the 'capture site' and serves to attach the second peptide fragment to the template. In the first step, this thiol function is deprotected and allowed to react with the thiol function of the N-terminal cysteine residue present in peptide fragment **B**. A subsequent intramolecular acyl transfer leads to the formation of a new amide bond between peptides **A**

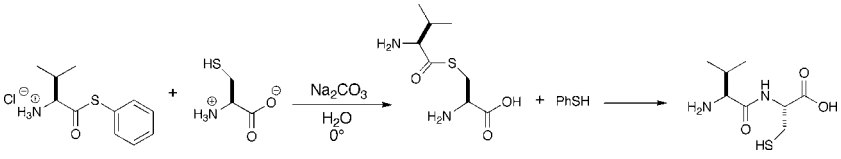


Figure 9. The first example of formation of an amide bond through an acyl shift.

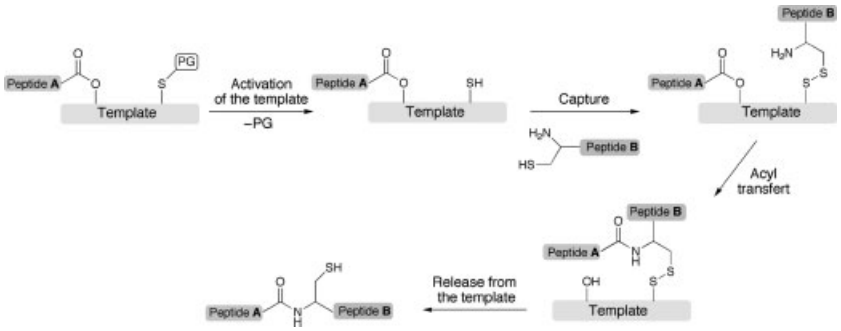


Figure 10. The principle of the 'prior thiol-capture strategy' of Kemp.

and **B**. Finally, the resulting peptide is released from the template. Extensive research into the design of the best template led to a dibenzofuran derivative shown in yellow in Fig. 11 (28). The kinetics of the acyl transfer was studied on a model reaction (Fig. 11) and it was shown that peptide bond formation is extremely sensitive to steric interactions (29). Indeed, the reaction took 51 h with R = Val, which has a branched side chain, and only 3 h with R = Ala (Table 3).

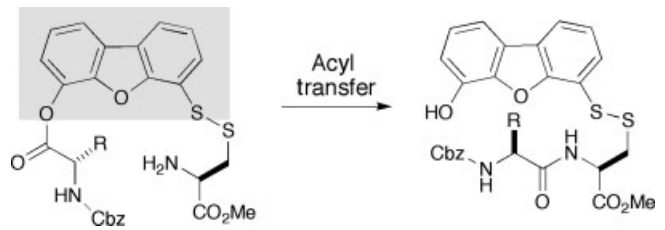


Figure 11. Model reaction for the determination of the kinetics of the acyl transfer. The part of the molecule in the yellow box is the template developed by Kemp.

This methodology was then successfully used for the synthesis of the 29-residue C-terminal segment of the protein basic pancreatic trypsin inhibitor (BPTI) (30). As shown in Fig. 12, a linear strategy was applied: four fragments were coupled sequentially through a prior thiol-capture reaction, from the C- to the N-terminus. Fragment **1** was prepared in solution while the protected fragments **2**, **3**, and **4** were prepared by solid-phase methodology on an amino-methyl polystyrene resin, derivatized with the dibenzofuran template. The reaction sequence for each fragment coupling is as follows:

- *Step* 1: The first step is the thiol-capture reaction, leading to the formation of a disulfide bond between the thiol functionality of the N-terminal cysteine present in one fragment and the thiol group of the template attached at the C-terminus of the second fragment.
- *Step* 2: The second step is the acyl transfer which leads to the formation of the amide bond between the two fragments.

Table 3. **Half-lifes for the intramolecular acyl-shift reactions shown in Fig. 11, as a function of the acyl fragment**

(Cbz)HN, R	Acyl transfer [half-time (h)]	(Cbz)HN, R	Acyl transfer [half-life (h)]
AlaGly	2.0	Asn	3.2
Ala	3.0	Asn(Mbh)	2.4
Leu	4.0	Asp(^{t}Bu)	1
Pro	34	Asp	≈0.1
Val	51	Arg(Ans)	1.8
Lys(Cbz)	3.2	Thr(^{t}Bu)	2.5

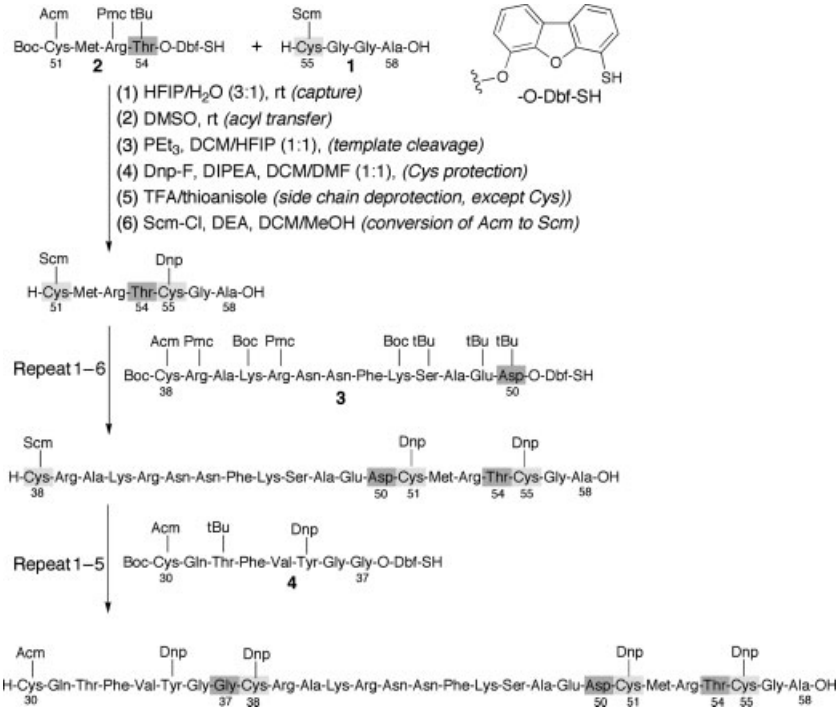


Figure 12. Synthesis of a portion of the basic pancreatic trypsin inhibitor using the prior thiol-capture strategy.

- *Step* 3: The disulfide-linked template is then cleaved using PEt_3 and the free thiol group of the cysteine residue (present at the ligation site) is protected with 2,4-dinitrophenyl (DNP) to prevent interference during the next fragment coupling.
- *Step* 4: All the side-chain-protecting groups except those of the cysteine residues are removed using TFA.
- *Step* 5: Finally, the acetamidomethyl (Acm) group of the N-terminal cysteine is replaced by a sulfenylcarbomethoxy (Scm) group (the Scm group allows for clean and rapid formation of the disulfide linkage in the capture steps: R–S–Scm + R′–SH $\rightarrow$ R–S–S–R′).

The native chemical ligation

General principle of thioligation and synthesis of 'unnatural' proteins

In 1992, Schnölzer and Kent (31) introduced a novel strategy for the coupling of unprotected peptide fragments in aqueous solution. The basis for this new approach, called 'chemical ligation', is the presence in each peptide fragment of a unique, mutually reactive functionality which enables a 'chemoselective' reaction between the two components. The chemistry initially used by Schnölzer and Kent for this purpose is a nucleophilic substitution reaction between an SH group of a thioacid attached to the C-terminus of one peptide, and an alkyl bromide attached to the N-terminus of the second fragment, leading to the formation of a thioester at the ligation site. This reaction can be performed in aqueous solution: the selectivity of the reaction allows the use of unprotected peptide fragments. The characteristics of the chemical ligation methodology overcomes all the limitations of the traditional convergent approach for the synthesis of large peptides or proteins (i.e. poor solubility and difficulty in purifying the fully protected peptide fragments), and provides access to new synthetic systems. One of the first total syntheses of a protein by chemical ligation using unprotected peptides in aqueous solution was that of the human immunodeficiency virus-1 protease (HIV-1 PR) by Schnölzer and Kent (31). The HIV-1 PR protein is a homodimer of a 99-amino acid polypeptide chain with a molecular weight of 22.5 kDa. The monomer was prepared by chemical ligation of a 51-residue peptide bearing a C-terminal thioacid and a 48-residue peptide having an N-terminal alkyl bromide (Fig. 13). The reaction was performed in a phosphate buffer at pH 4.5, and was followed by analytical HPLC. After 3 h, the reaction was almost complete and the product was purified and characterized. The homodimer of this synthetic HIV-1 protease analog (Fig. 14) had the same biological activity as the native enzyme.

Native thioligation through N-terminal cysteine residues

The major disadvantage of the initial chemical ligation approach was that the reaction leads to an 'unnatural structure' at the ligation site. A second generation of ligation chemistry, referred to as 'the native chemical ligation (NCL)', was introduced in 1994 by Dawson *et al.* (33). Similar to the previous strategy, this new methodology

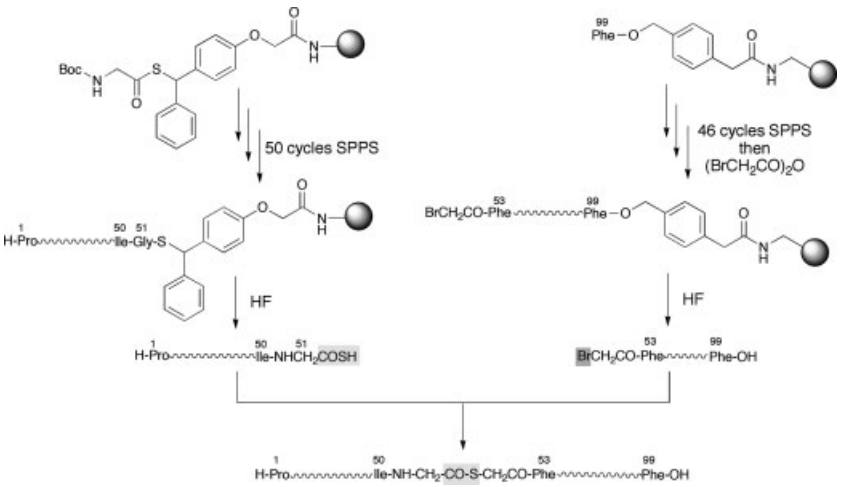


Figure 13. Total synthesis through chemical ligation of the HIV-1 PR analogue.

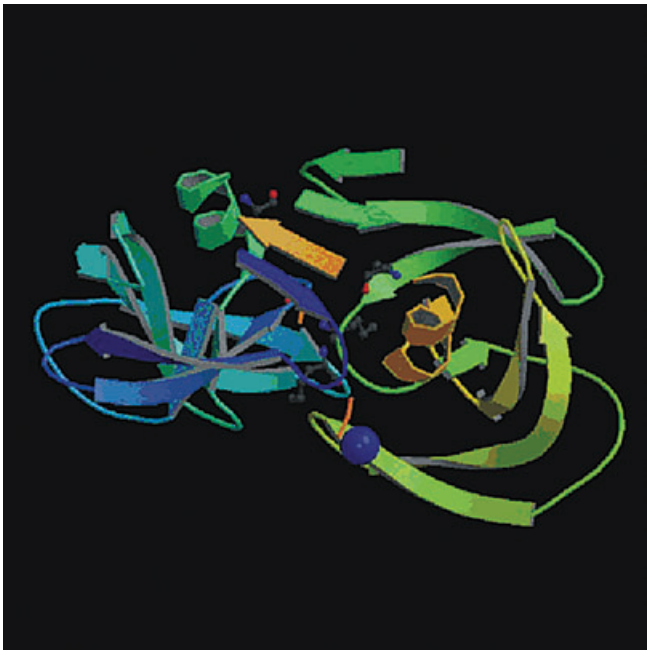


Figure 14. Structure of the synthetic HIV-1 protease complex with a substrate-based inhibitor at 2.3 Å resolution (PDB entry: 4HVP) (32).

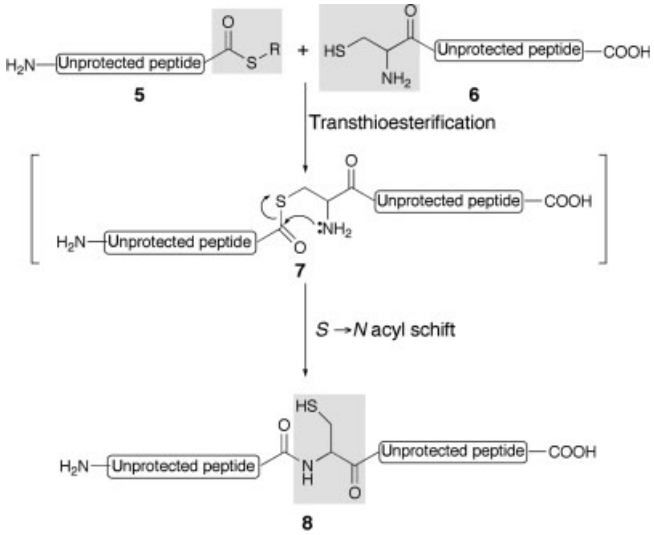


Figure 15. Synthesis of peptides by native chemical ligation of unprotected peptide fragments.

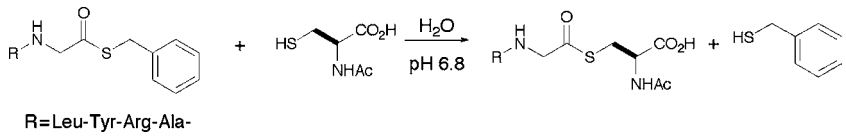
allows the coupling of two unprotected peptide fragments in aqueous solution, but now the ligation reaction leads to the formation of a 'native' amide bond at the ligation site. The principle of this strategy is outlined in Fig. 15. The first step is a chemoselective transthioesterification reaction involving the thiol group of the N-terminal cysteine present in unprotected peptide **6**, and the C-terminal thioester moiety present in peptide **5**. The thioester-linked intermediate (**7**) then undergoes a rapid intramolecular S → N acyl shift, forming the amide bond at the ligation site (**8**).

The thioester intermediate is not isolated, but evidence for its formation has been obtained by using an *N*-acetylcysteine for the ligation reaction (33). In this case, the acyl transfer cannot proceed and the thioester intermediate could be isolated (Fig. 16).

The nature of the thioester-leaving group greatly influences the rate of the reaction. Initially, benzyl thioesters were used and it has been shown that the addition of an excess of thiophenol to the reaction leads to an increase in the coupling rate (34). The thiophenol probably replaces the benzyl thiol before the ligation and therefore provides a better leaving group (Fig. 17). Furthermore, the presence of an excess of thiol suppresses the risk of oxidation of the sulfhydryl group of the N-terminal cysteine residue. This is important because a 'cystin moiety' cannot react in the ligation reaction.

An investigation of the influence of pH on the rate of the ligation reaction has shown that at pH 7 the coupling proceeds rapidly and that the reaction is almost complete after

Figure 16. Thioester formation with a cystein SH group without subsequent S → N shift.



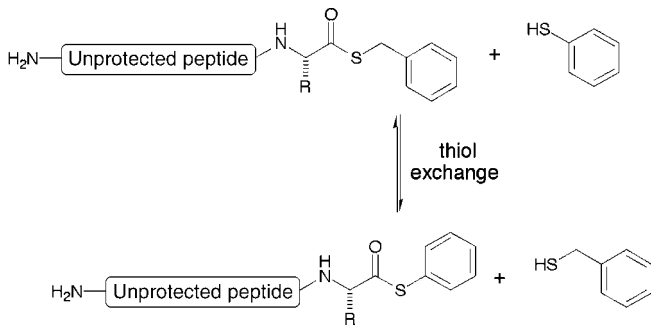


Figure 17. Transthioesterification reaction between the benzyl thioester and the phenyl thioester.

5 min, while below pH 6 the same reaction was only 50% complete after 10 min. This suggests that the ionized form of the thiol moiety of the cysteine is directly involved in the reaction (33).

The impact on the rate of the coupling reaction in the NCL of the C-terminal amino acid present in the peptide fragment bearing the C-terminal thioester has also been investigated (35). This was performed in a model reaction where each of the 20 possible thioesters was examined (Fig. 18). The results are shown in Table 4: the ligation reaction proceeding in all the cases. Similar to the results obtained by Kemp (29) (see Table 3), the coupling reaction is much slower with Pro or when β-branched amino acids like Val, Ile, Thr are used. Curiously, the rate of the reaction with Cys and His is similar to that observed with Gly and faster than that with Ala. This indicates that the side-chain groups facilitate the ligation reaction.

Finally, to complete these mechanistic studies, the extent of epimerization during the ligation process was also investigated. A model peptide was synthesized using the native chemical ligation methodology and for HPLC comparison, the peptide containing the epimer of the amino acid located next to the ligation site was prepared by standard SPPS. Analysis by HPLC revealed that no epimerization took place during the ligation reaction (36).

SPPS with a C-terminal thioester group

Peptides bearing a C-terminal thioester group are key intermediates in the synthesis of proteins by chemical ligation and consequently, several methods have been developed for their preparation on the solid phase. In the Boc strategy, the benzylic thiol **10** (Fig. 19) was used as a linker (36–38). This linker is prepared from chloride **9** by reaction with thiourea and subsequent hydrolysis of the resulting thiouronium salt using aqueous base. The first amino acid is then derivatized with this linker, and the resulting thioester **11** is finally attached to an aminomethyl polystyrene resin. Standard Boc-SPPS is then applied for preparation of the desired peptide sequence. Cleavage of the peptide from the resin with HF leads to the formation of the peptidic thioacid **12**, which is subsequently treated with benzyl bromide to yield the target peptide thioester **13**.

Figure 18. Model reaction for the determination of the effect of the amino acid residue adjacent to the ligation site (yellow box) on the kinetics of the chemical ligation reaction.

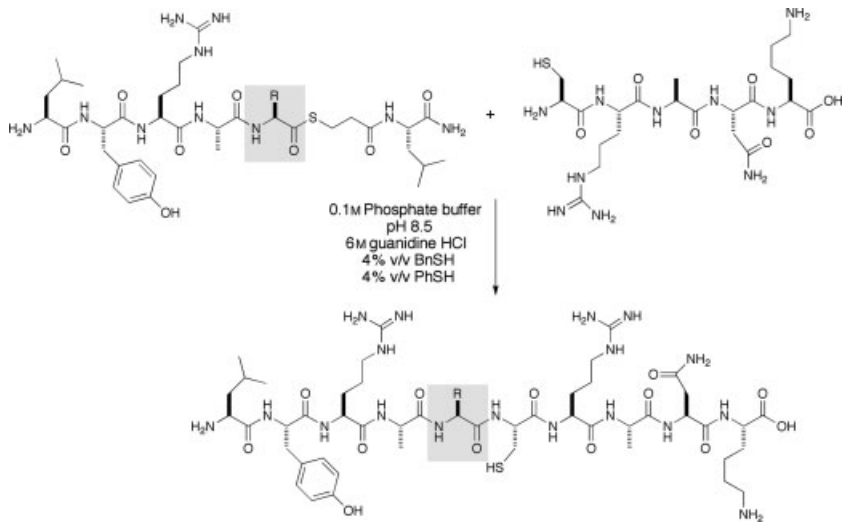


Table 4. **Rate of the coupling reaction in the chemical ligation step, depending on the C-terminal amino acid**

Coupling time (h)		Coupling time (h)		Coupling time (h)		Coupling time (h)	
Gly	≤ 4	Ala	≤ 9	Arg	≤ 24	Ile	≥48
Cys	≤ 4	Met	≤ 9	Asn	≤ 24	Leu	≥48
His	≤ 4	Phe	≤ 9	Asp	≤ 24	Pro	≥48
		Trp	≤ 9	Gln	≤ 24	Thr	≥48
		Tyr	≤ 9	Glu	≤ 24	Val	≥48
				Lys	≤ 24		
				Ser	≤ 24		

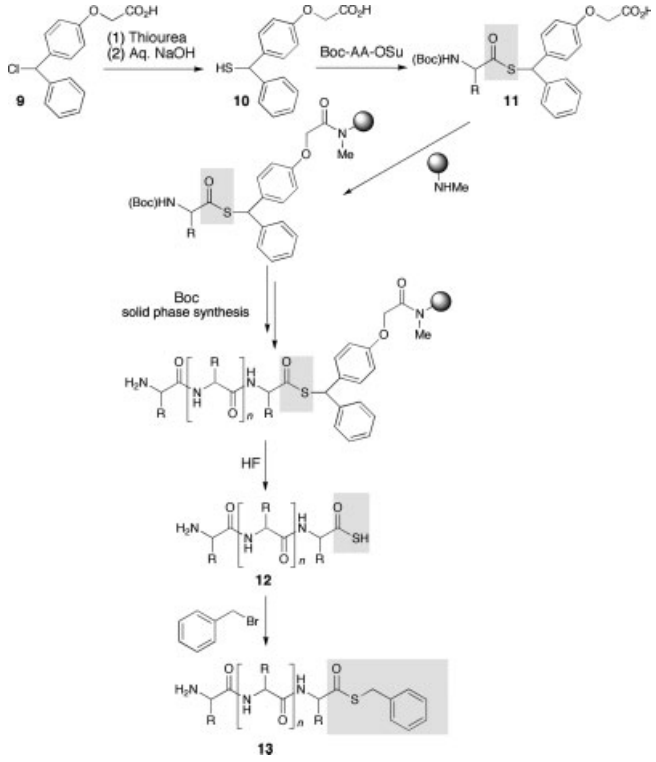


Figure 19. Kent methodology for the synthesis of a peptide thioester on solid phase.

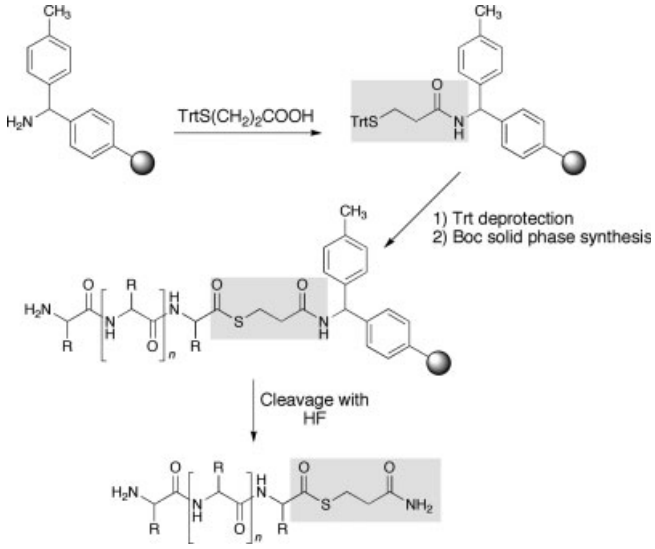


Figure 20. Boc-solid-phase synthesis of a peptide possessing a C-terminal thioester according to the method developed by Tam.

A second protocol for SPPS containing a C-terminal thioester by the Boc strategy was developed by Tam and co-workers (39–41). The method is based on the use of a 4-methylbenzhydrylamine (MBHA) resin (Fig. 20), which is first loaded with *S*-trityl mercaptopropionic acid. After removal of the trityl-protecting group, the desired polypeptide chain is assembled using standard Boc strategy. The thioester is obtained after cleavage with HF.

Despite the fact that thioesters are sensitive to basic conditions, Fmoc-compatible strategies for the preparation of peptide thioesters have also been developed. Ingenito *et al.*'s (42) methodology is based on the use of an acylsulfonamide 'safety-catch linker' **14** (Fig. 21). This linker, first introduced by Kenner *et al.* (43) and later modified by Backes and Ellmann (44), is stable to both strongly basic and acidic conditions. The peptide is assembled using the standard Fmoc protocol to afford solid-phase bound peptides of type **15**. After the final peptide coupling, the resin is activated for cleavage by treatment with diazomethane to give an *N*,*N*-methylacylsulfonamide **16**, or by treatment with iodoacetonitrile to give an *N*,*N*-cyanomethylacylsulfonamide **17**. The peptide is then released from the activated resin by nucleophilic displacement involving a thiol group to yield **18**, and finally the side chains are deprotected in solution to give the desired peptide thioester **19**.

Sewing and Hilvert (45) reported a second useful strategy (46). The target peptide is assembled by standard Fmoc chemistry on a 4-hydroxymethyl-phenylacetamidomethyl

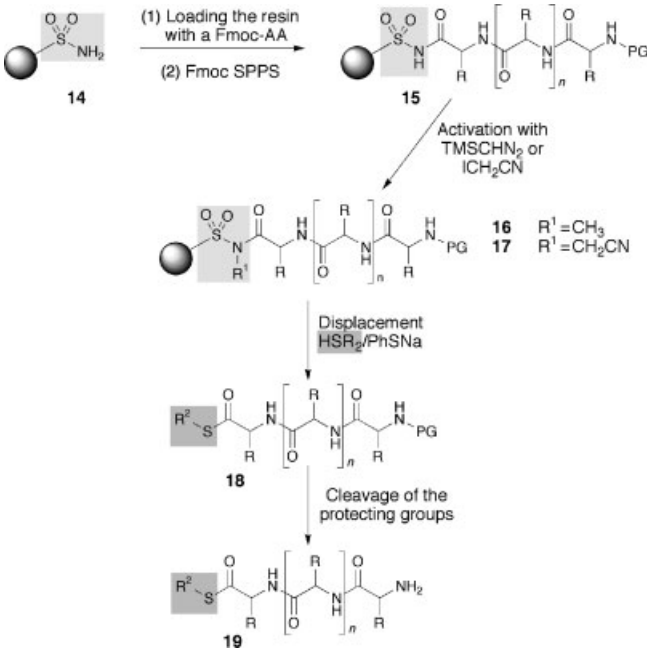


Figure 21. Fmoc-solid-phase synthesis of a peptide thioester on a sulfonamide 'safety-catch' resin.

(PAM) or 4-hydroxymethylbenzoic acid (HMBA) resin (see Tables 1 and 2), and the cleavage performed by activation of the ester linkage with $AlMe_2Cl$ in the presence of a large excess of a nucleophilic thiol (Fig. 22). The side-chain-protecting groups are subsequently removed in solution by treatment with TFA.

Besides the synthetic methodology described here, recombinant strategies based on protein splicing have also been developed for the preparation of peptides bearing a C-terminal thioester [for an extensive review see Ref. (47)].

A protein and a glycopeptide synthesis by native thioligation

The first example is the synthesis of a membrane protein: the 136-residue mechanosensitive ion channel from *Mycobacterium tuberculosis* (Tb-MscL) (Fig. 23) by Clayton *et al.* (48).

Membrane proteins are difficult to synthesize because of their large hydrophobic segment. They tend to couple inefficiently, form aggregates, and are generally difficult to purify and solubilize. In order to overcome these problems, Kochendoerfer applied the native chemical ligation methodology to the successive coupling of three fragments as shown in Fig. 24 (48). The wild-type protein does not contain any cysteine residues, and these were introduced by replacing two amino acids of the original sequence that are not critical for channel function. The two thioester fragments **20** and **21** were prepared on a thioester-generating resin similar to the procedure described above (Fig. 20), and the third fragment **22** was prepared on a PAM resin (Table 2) using standard Boc chemistry. As a result of the limited solubility of the peptide fragments, the ligation reactions were performed at 40 °C in a phosphate buffer (pH 7.5) containing a high concentration of denaturant (8 M urea). After the last ligation reaction, the 'non-native' cysteine residues were selectively masked by treatment with bromoacetamide. The synthetic Tb-MscL protein prepared in this fashion displayed almost the same ion-channel activity as the native protein.

The second example illustrates the usefulness of the native chemical ligation methodology for the synthesis of large and highly functionalized biomolecules. As shown in Fig. 25, Warren *et al.* (50) applied the native chemical ligation approach to the development of a convergent strategy for the synthesis of complex glycopeptides. The unprotected glycosylamines **23** and **24** are first attached to peptides **25** and **26**, respectively, via the side chain of the Asp residue using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) as a coupling reagent [Landsbury aspartylation (51)]. After reductive cleavage of the disulfide bond in **27**, and Fmoc deprotection of **28**, the glycopeptides are coupled together via the native chemical ligation. It appears that the cleavage of the disulfide bond in **27** with sodium 2-mercaptoethanesulfonate (MES-Na) leads almost instantaneously to the formation of the MES-Na-derived thioester which can then react with the sulfhydryl functionality of the N-terminal cysteine present in fragment **29**, giving after an intramolecular acyl shift, the desired amide bond (**30**). This methodology was successfully used for the synthesis of normal and transformed fragments of prostate-specific antigen glycopeptides (52).

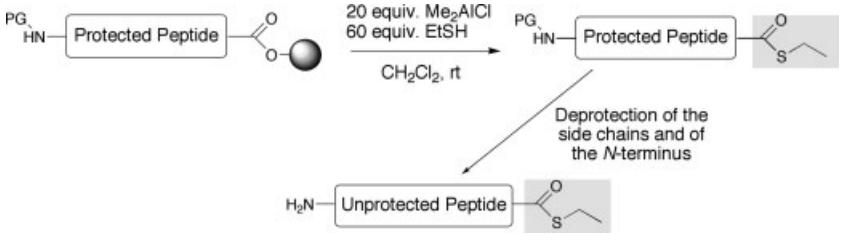


Figure 22. Formation of a C-terminal peptide thioester by dimethylaluminum thiolate-mediated cleavage.

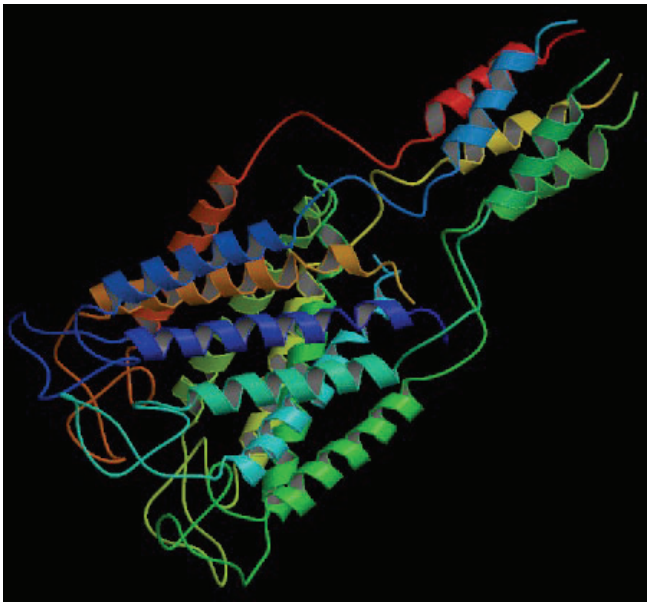


Figure 23. Structure of the homopentamer of the MscL homologue from *Mycobacterium tuberculosis* (PDB entry: 1MSL) (49).

Ligations without cysteine at the coupling site

The native chemical ligation methodology introduced by the Kent group (33,53) has proven very useful in the synthesis of large peptides and proteins. This strategy has, however, some limitations, principally because of the fact that a cysteine is required at the ligation site, and that naturally occurring proteins do not always contain a cysteine residue in the right position of their sequences. Therefore, several modifications of the initial method have been introduced to overcome this drawback.

Methionine, histidine, selenocysteine and homoselenocysteine ligation

Tam and Yu (54) have shown that the chemical ligation reaction can also be performed with a peptide bearing an N-terminal homocysteine residue instead of a cysteine. In this case, the intramolecular acyl shift proceeds through a six-membered ring (vs. a five-membered ring for cysteine). The product of this ligation contains a homocysteine at the ligation site, which is subsequently methylated with methyl *p*-nitrobenzenesulfonate to produce a methionine side chain (Fig. 26) (55).

Similar to cysteine, it has been shown by Hilvert and co-workers (56,57) and van der Donk and co-workers (58,59) that the native chemical ligation can also be mediated by selenocysteine and selenohomocysteine (60) amino acids instead of cysteine. This is of particular interest in biology as the specific physical properties of selenium (SeH is more acidic and is a better nucleophile than SH) (61) are useful for the mechanistic investigation of bioactive peptides and proteins containing a Cys in the active site. Furthermore, selenium-containing peptides can be used as a probe in nuclear magnetic resonance (NMR) spectroscopy (62). Finally, selenocysteine can also be reduced to an alanine or converted through oxidative elimination to a dehydroalanine residue (57) (Fig. 27).

Zhang and Tam have shown that the imidazole side chain of histidine, if located at the N-terminus of a peptide, can also be used as a nucleophile in the ligation reaction. In this case, the C-terminus of the second peptide fragment is activated as a disulfide, generated *in situ* by treatment of the thioacid **31** with 5,5′-dithiobis(2-nitrobenzoic acid) (Fig. 28). The reaction between the two fragments, peptide **33** having an N-terminal histidine and peptide **32** bearing an activated thioacid, first generates an amide **34** which then undergoes a $N^{im} \rightarrow N^{\alpha}$ acyl transfer, via a six-member d-ring intermediate, leading to the ligated product **35** (63).

Thioligation with a removable auxiliary – an application of the thiol-capture strategy

Another way to overcome the N-terminal cysteine residue requirement in the native chemical ligation methodology is

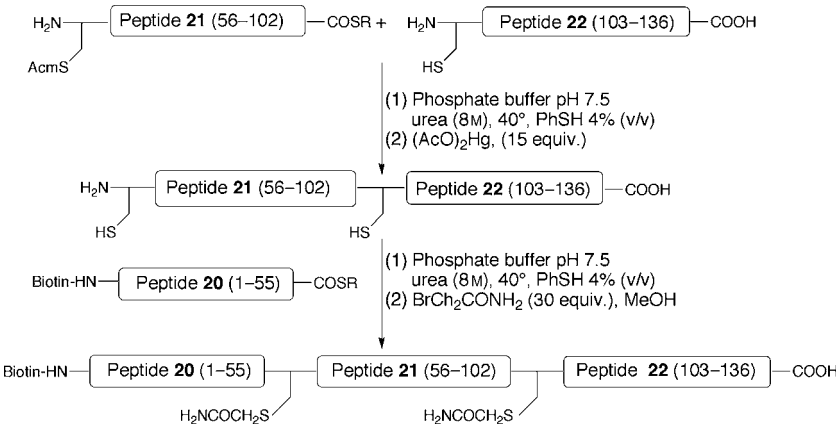
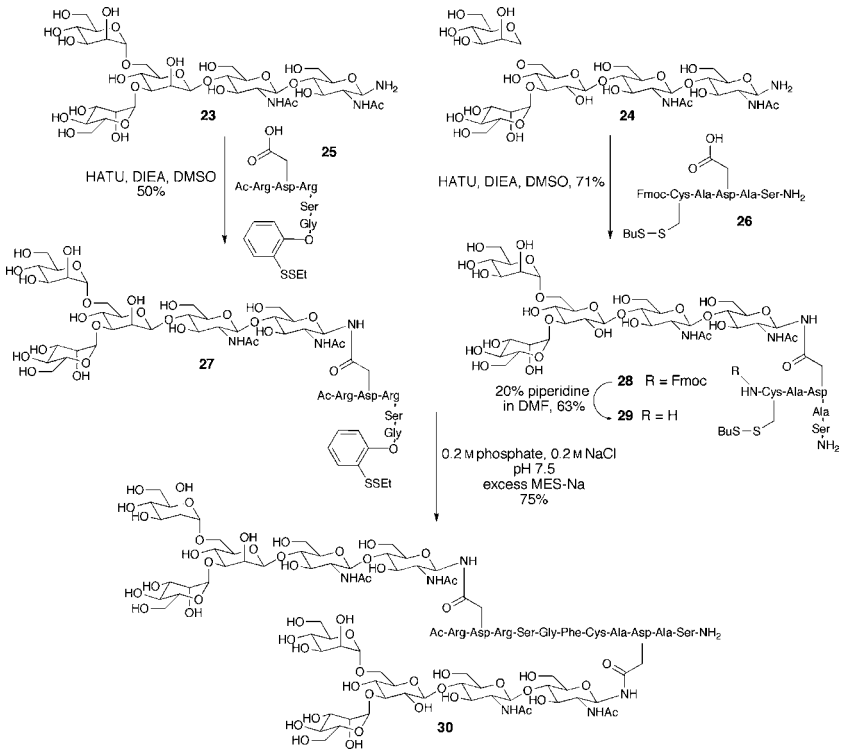


Figure 24. Synthesis by native chemical ligation of the Tb-MscL protein; a mechanosensitive ion channel protein from *Mycobacterium tuberculosis*.

Figure 25. Danishefsky's synthesis of a glycopeptide by native chemical ligation.



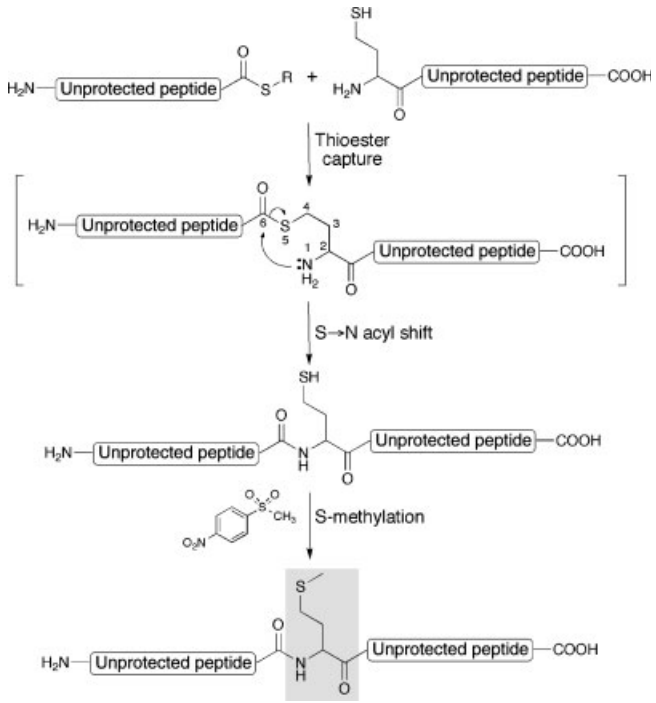


Figure 26. Native chemical ligation with homocysteine.

to mimic the characteristic of this Cys by the use of a removable auxiliary. For this purpose, the Canne *et al.* replaced the N-terminal Cys by an oxyethanethiol-substituted N-terminus (Fig. 29) (64). The first step in the ligation reaction involve a thioester exchange between peptide **36**, bearing a C-terminal thioester, and the thiol function present at the N-terminus of peptide **37**. The ligation product **38** then undergoes an S → N acyl shift with formation of an amide bond (**39**) having an *N*-(oxyalkyl) substituent on nitrogen. The N–O bond can then be cleaved under reducing conditions, e.g. with zinc dust, to give peptide **40**.

Several groups have shown that the N-terminal cysteine can also be replaced by a 2-mercaptobenzylamine linker (Fig. 30) (65–67). In this case, the intramolecular acyl shift proceeds through a six-membered ring with the linker subsequently removed by treatment with an acid such as trifluoromethanesulfonic acid (TFMSA).

A third type of auxiliary for the native chemical ligation of unprotected peptide fragments was introduced by Kawakami and Aimoto (69) in Japan and shortly after by Marinzi *et al.* (68). The auxiliary attached at the N-terminus of one peptide fragment is based on an *o*-nitrobenzyl scaffold (Fig. 31), with which the thioester present in the second peptide fragment can react. The reaction is performed under standard conditions and provides a substituted amide at the ligation site as shown in Fig. 31. The photolytic properties of the *o*-nitrobenzyl group permits the use of mild conditions for the cleavage of the auxiliary from the ligated product (68,69).

Other ligation strategies

Besides the native chemical ligation methodology described previously, and characterized by the reaction of a

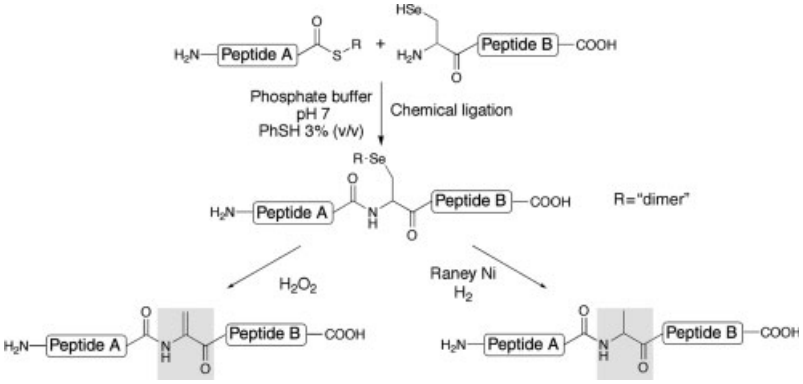


Figure 27. Synthesis of peptides by selenocysteine-mediated chemical ligation.

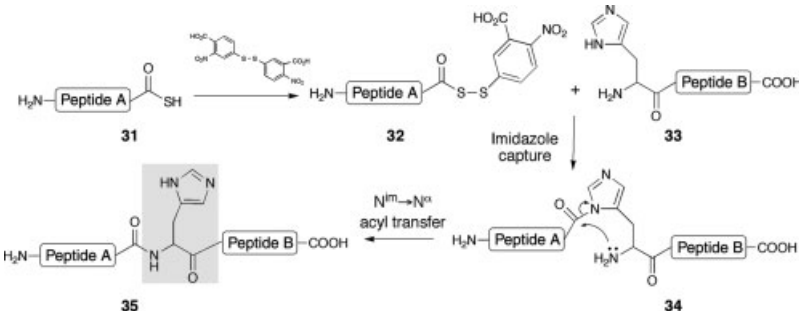


Figure 28. Histidine-mediated native chemical ligation of unprotected peptide fragments.

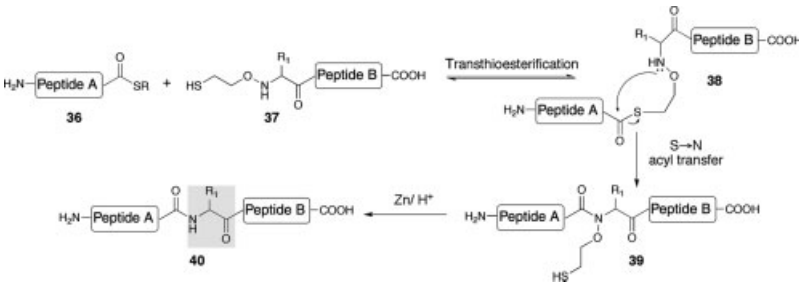


Figure 29. Chemical ligation through an N^{α}-(oxyethanethiol).

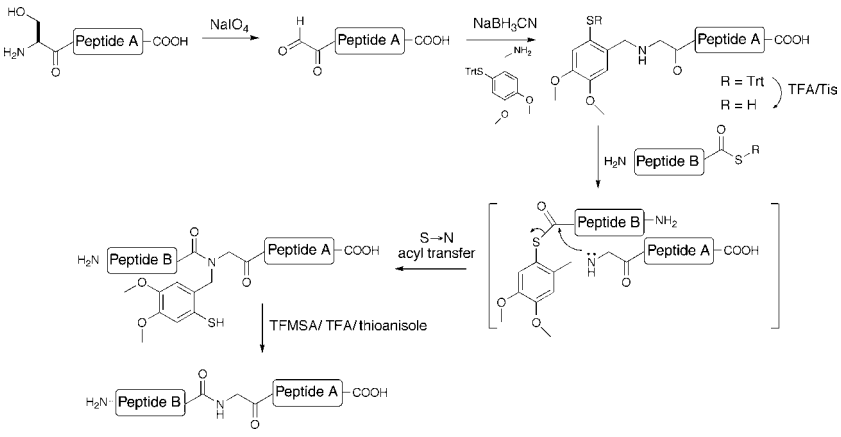
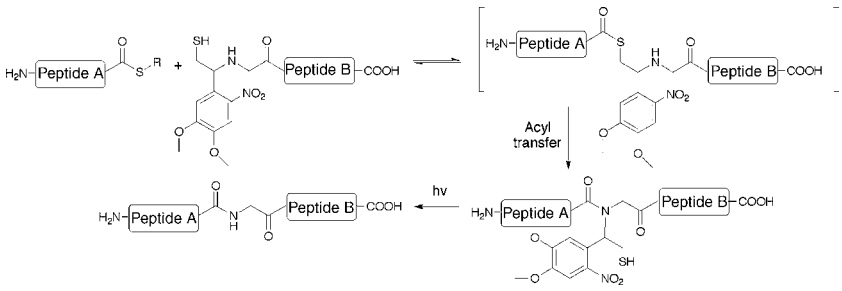


Figure 30. Native chemical ligation through a mercaptobenzylamine linker.

N-terminal Cys with a C-terminal thioester, numerous other approaches for the coupling of unprotected peptide fragments have been developed. Each of these methods can be catagorized by the types of reactions used to bring the peptide fragments together (the capture step) before the acyl transfer reaction.

Figure 31. Native chemical ligation through a photoremovable auxiliary.



Imine ligation with incorporation of pseudo-prolines

The imine ligation developed by Tam and co-workers (70–73) involves a peptide segment having a C-terminal glycoaldehyde (**41**) and a peptide bearing an N-terminal Cys (**42**), Thr (**43**) or Ser (**44**) residue (Fig. 32). The first step in this ligation process is the formation of the imines **45**, **46**, or **47** by addition of the N-terminal amino group of peptides **42**, **43**, or **44** to the C-terminal aldehyde function of peptide **41**. The presence of a nucleophilic SH or OH functionality in the side chain of the N-terminal amino acid of the segment leads, through a ring-chain tautomerism, to a thiazolidine **48** or an oxazolidine **49**, **50**, respectively. This heterocyclic intermediate then undergoes an intramolecular O → N acyl shift via a favorable bicyclic five-membered ring intermediate to form an amide bond and a hydroxymethyl-substituted pseudo-proline (*Ψpro*) moiety at the ligation site (**51**, **52**, **53**).

The peptide bearing the C-terminal aldehyde functionality was prepared on a benzaldehyde polystyrene resin (74). Treatment of this resin with glycerol in the presence of a catalytic amount of *p*-toluenesulfonic acid leads to the formation of a benzylic acetal. The peptide sequence can then be assembled using standard Fmoc solid-phase strategy. After cleavage with TFA, the diol of the glyceric ester is oxidatively cleaved, using $NaIO_4$, to give the desired peptide having an aldehyde function at its C-terminus (Fig. 33).

The formation of the thiazolidine ring during the imine ligation is 10^4 times faster than that of the oxazolidine ring (75) and the reaction can be performed in aqueous solution, whilst for the oxazolidine anhydrous conditions are required (for oxazolidine ligation the best solvent was found to be a 1 : 1 mixture of pyridine and acetic acid) (72). An important aspect in the imine ligation process is the fact that during the formation of the heterocycle, a new asymmetric carbon is created at position C(2) of the pseudo-proline ring (Fig. 34). Furthermore, the new amide bond formed in the acyl-transfer step can adopt a *cis* or *trans* conformation (Fig. 34). The configuration of the new center and the *cis–trans* conformational ratio was determined in a model reaction as shown in Fig. 34. In the case of the thiazolidine ester (**55** + **58**), both diastereoisomers were formed and are stable enough to be separated by HPLC. However, after the acyl transfer only one diastereoisomer was detected. The oxazolidine ester intermediates (**54** + **56**, **55** + **56**, **54** + **57**, and **55** + **57**) were not stable enough to be detected by HPLC; however, as with the thiazolidine a single

Figure 32. Imine ligation.

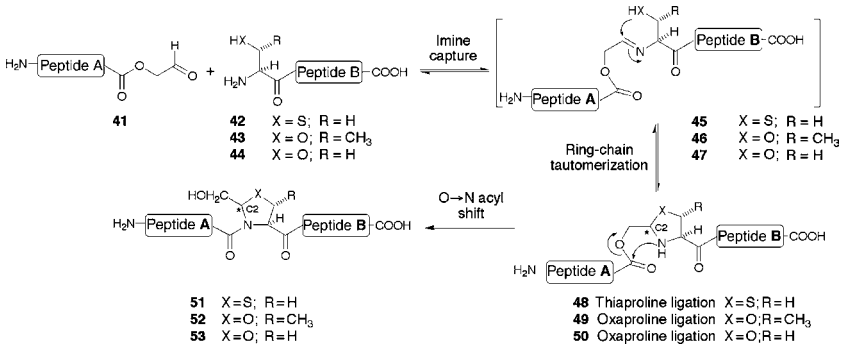
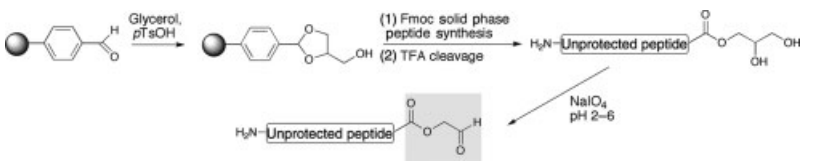
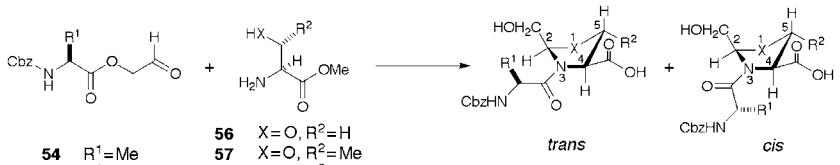


Figure 33. Solid-phase synthesis of a peptide bearing a C-terminal aldehyde function.





54 R^1=Me
55 R^1=iPr
56 X=O, R^2=H
57 X=O, R^2=Me
58 X=S, R^2=H

Figure 34. Model reactions for the determination of the stereochemical course of the pseudo-proline-ligation. The preferred formation of the 2,4-*cis*-substituted heterocycles is rationalized in papers by the Seebach group (76,77).

Table 5. **Result of the *pseudo*-proline-ligation reaction**

Segments	*X*	R^1	R^2	C(2)	*cis*/*trans* amide conformation
54 + **56**	O	Me	H	R	68 : 32
55 + **56**	O	iPr	H	R	56 : 44
54 + **57**	O	Me	Me	R	54 : 46
55 + **57**	O	iPr	Me	R	43 : 57
55 + **58**	S	iPr	H	R	40 : 60

diastereoisomer was isolated after the acyl transfer. The configuration of the new centre was assigned in both cases to be (*R*) by 2D NMR experiments (Table 5) (72). The diastereoselectivity of the ligation reaction can be explained as follows: the formation of the thiazolidine or oxazolidine ring is not stereoselective and therefore two diastereoisomers are formed. However, in the acyl transfer reaction, the C(2)-(*R*)-diastereoisomer reacts faster than the (*S*)-epimer, and gives the more stable *cis*-substituted product and because of ring-chain tautomerization the (*S*)-epimer undergoes a re-equilibration to the (*R*/*S*) mixture. The *cis*–*trans* amide rotamer ratio was determined by NMR spectroscopy and it was shown that this ratio varied, depending on the amino acids located in the vicinity of the pseudo-proline ring (72).

The imine-ligation methodology was then successfully applied by Miao and Tam (78) for the synthesis of five analogs of a proline-rich helical antimicrobial peptide, the 59-residue bactenecin 7 (Bac7) (Fig. 35). They used a three-segment ligation strategy, applying the thiaproline and oxaproline ligation methodology simultaneously. Two analogs **65** and **66**, were prepared by ligation in a C to N direction based on the fact that the ligation with thiaproline is much faster than that with oxaproline, and therefore the N-terminal Ser (resp. Thr) present in **60** (resp. **61**) does not need to be protected (Fig. 36). The C-terminal glycoaldehyde ester in **60** (resp. **61**) then reacts selectively with the N-terminal Cys present in **59**. This thiaproline ligation was performed under two-stage aqueous conditions; first at pH 5.2 for 10 h which led to formation of the two diastereomeric thiazolidine-esters (see Fig. 32), and then at pH 6.6 for 20 h to give, after O → N acyl transfer, a single product **62** (or **63**) as determined by RP-HPLC. The third fragment **64** was attached through an oxaproline ligation.

The three other analogs **73**, **74**, and **75** were prepared through an N to C three-segment sequential ligation as shown in Fig. 37. Fragments **64** and **67** were first coupled by a thiaproline ligation (**68**), followed by oxidative cleavage using $NaIO_4$ of the C-terminal glycerol ester of the resulting peptide, to give glycoaldehyde ester **69**. Subsequent oxaproline or thiaproline ligation between **69** and the fragment bearing an N-terminal Ser **70**, Thr **71** or Cys **72** yielded the three Bac analogs **73**, **74**, and **75**, respectively.

Circular dichroism (CD) investigations revealed that the five analogs formed a stable polyproline helical structure in aqueous solution. However, the replacement of the Pro residues present in Bac 7 by OPro and SPro in the analogs **65**, **66**, **73**, **74** and **75** resulted in a minor population of polyproline type I structure, while Bac 7 adopts a polyproline type II helical structure. The antimicrobial activities of the Bac 7 analogs were similar to that of the natural product.

Oxime and hydrazone ligations

Similar to the imine ligation, hydrazide- or aminoxy-derivatized N-terminal peptides can be ligated, through hydrazone or oxime linkages respectively, to peptides having a C-terminal aldehyde function. Hydrazides and oximes are good nucleophiles and highly reactive toward aldehydes under acidic condition where the basic side chains are protonated and therefore excluded from the reaction. The reaction is extremely selective and is compatible with unprotected side-chain functionality with the exception of Cys (thiazolidine formation). This type of ligation has been used to prepare cell-permeable lipopeptides (79), a dimeric transcription-factor-related protein containing 172 amino acids (38), and peptide dendrimers (80,81), for examples.

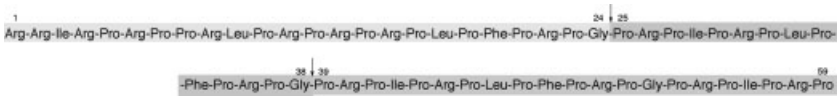


Figure 35. Primary sequence of the 59-residue antimicrobial peptide bactenecin 7 (Bac 7).

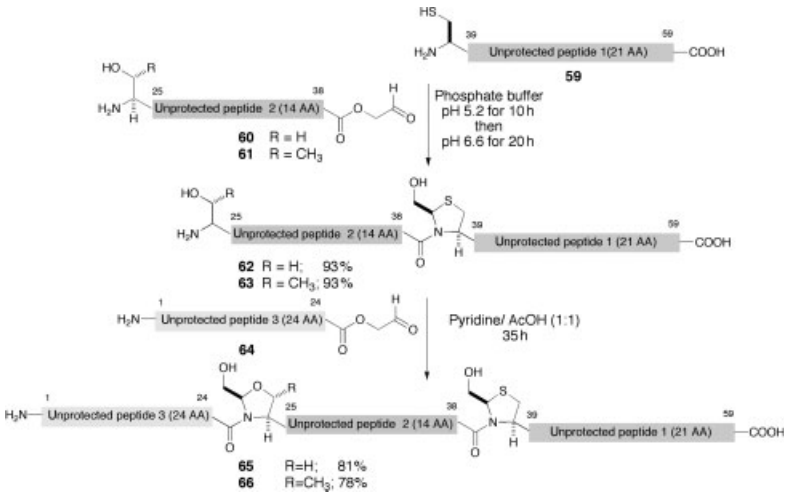


Figure 36. Three-segment tandem ligation in a C to N direction affording two analogs of Bac 7.

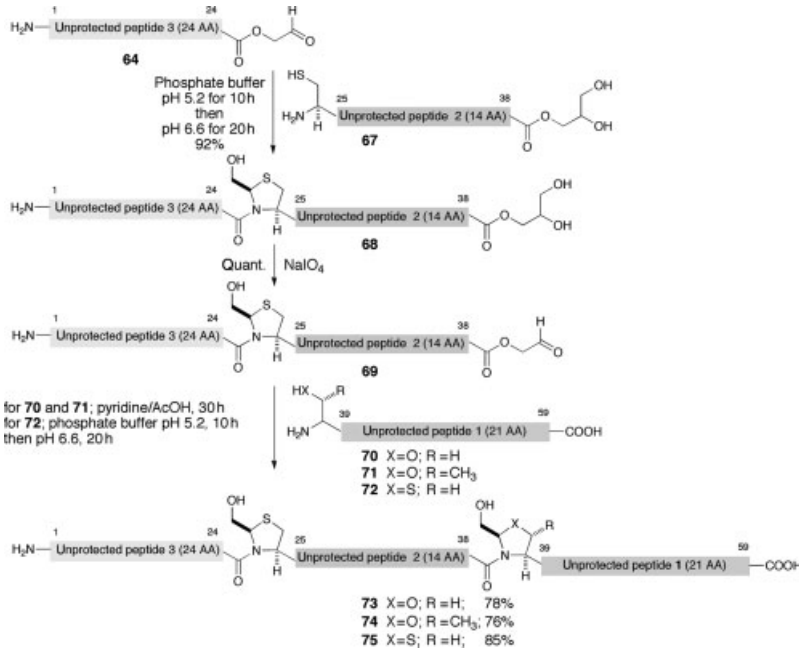


Figure 37. Synthesis of three Bac 7 analogs through an N to C three segment sequential ligation.

An interesting application of the use of a hydroxyl amino group is the synthesis of the glycopeptide analog **78** (82) which was prepared through an oxime ligation of four disaccharide **77** (T-antigen: β-D-Gal(1 → 3)-α-D-GalNac) units to a cyclic decapeptide **76** derivatized with four-aldehyde functionalities (Fig. 38).

The Staudinger ligation

An elegant way to overcome the limitation of the original native chemical ligation was developed independently by two research groups: Raines (83–86) and Bertozzi (87,88) and is based on the Staudinger reaction. The Staudinger reaction, which was first reported in 1919 (89), is the reaction of a phosphane with an azide to produce an iminophosphorane. This iminophosphorane intermediate can then be trapped by different electrophiles (Fig. 39) [for an extensive review on the Staudinger reaction see Ref. (90)].

This reaction can be applied to the coupling of two peptide fragments, one bearing a C-terminal phosphinothioester group and the second an N-terminal azido group [for a review on the application of the Staudinger reaction for peptide ligation see Ref. (91)]. In the first step of this Staudinger ligation, the phosphinothioester reacts with the azide to give an iminophosphorane, which then undergoes an intramolecular S → N acyl shift leading to an amidophosphonium salt. The amidophosphonium salt is then

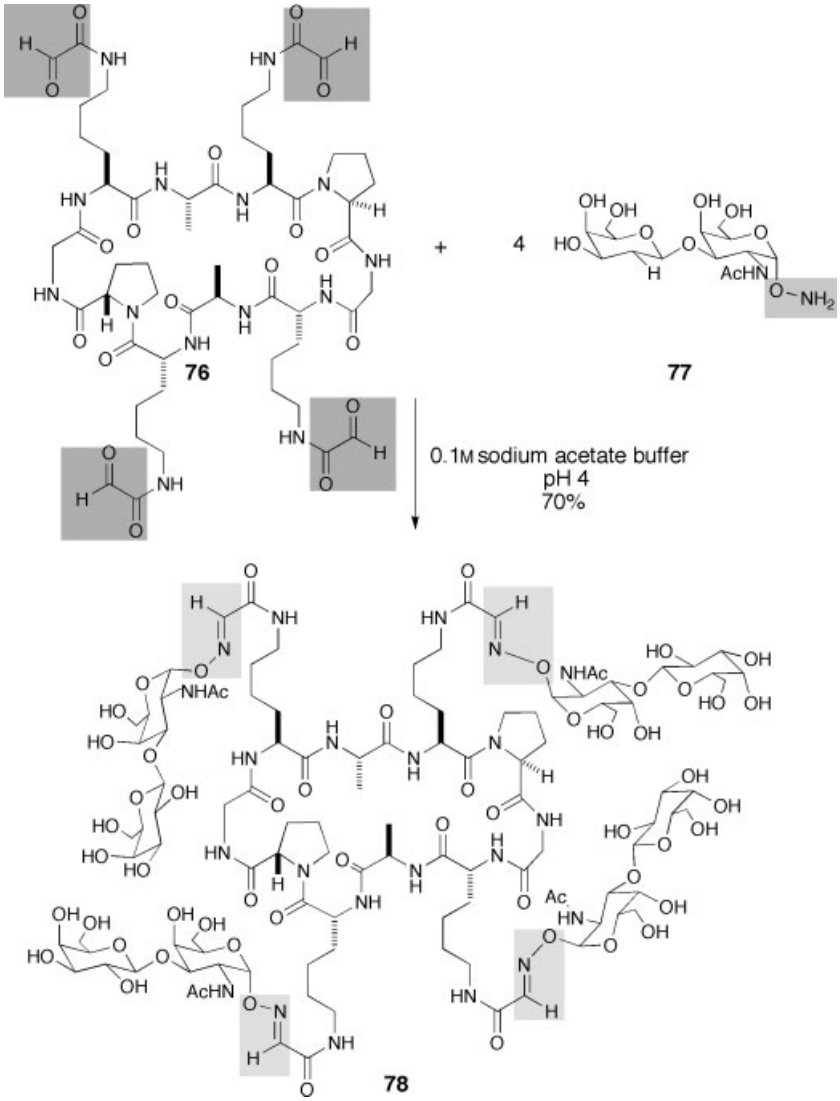


Figure 38. Synthesis of a glycopeptide using oxime ligation.

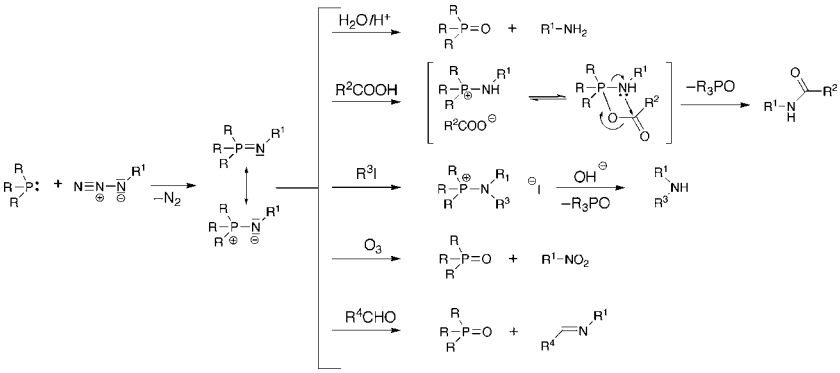


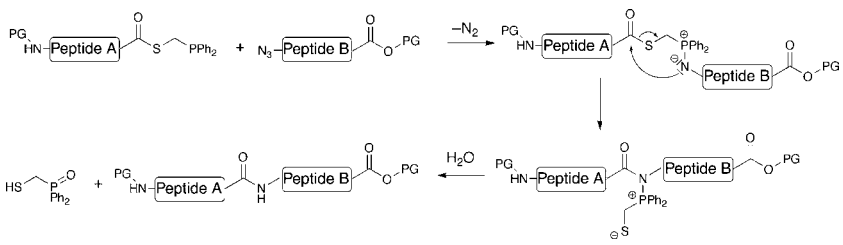
Figure 39. The Staudinger reaction.

hydrolyzed to produce the amide product and a phosphine oxide. The high reactivity of the aza-ylide does not, however, permit the presence of unprotected side-chain functionalities and therefore the Staudinger ligation is limited to the coupling of fully protected peptide fragments (Fig. 40).

Solid-phase thioligation of unprotected peptide segments

Recently, Kent (92) and Dawson (35,93) have developed a solid phase approach for the coupling of unprotected peptide fragments using the native chemical ligation methodology. There are two ways to assemble the desired polypeptide

Figure 40. Synthesis of a peptide through the Staudinger ligation, both peptide segments have to be protected at their side-chain functionalities.



chain by the solid-phase chemical ligation technique: it can be prepared either in the N → C direction or in the C → N direction.

In the first case, an unprotected peptide segment bearing a C-terminal thioester is bound to the resin through its N-terminus. The peptide thioester is prepared on a thioester-generating resin and derivatized at its N-terminus with a cleavable linker containing a keto moiety as shown in Figs 41 and 42.

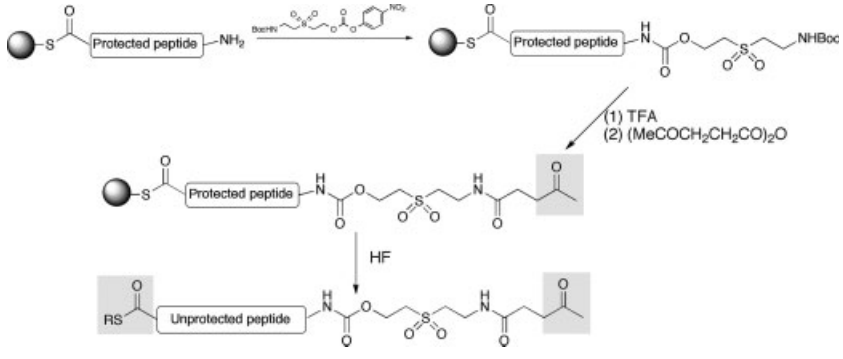
This keto function is used to attach the unprotected peptide thioester, via the formation of an oxime linkage, to a water-compatible cellulose-based resin that has been derivatized with an aminoxy group (Fig. 42). A second unprotected peptide segment containing an N-terminal Cys and a C-terminal thiocarboxylic acid group can then be coupled to the resin-bound peptide via the chemical ligation reaction. Under the conditions used for the chemical ligation reaction (phosphate buffer pH 7, 1% thiophenol) the thiocarboxylic acid is ionized and therefore unreactive toward the N-terminal Cys. This avoids unwanted intramolecular side reactions involving the incoming bifunctional (N-terminal Cys and C-terminal thiocarboxylate), unprotected peptide segment. After completion of the chemical ligation reaction, the pH is lowered to 4–5 and the thiocarboxylic function is transformed into a thioester by treatment with bromoacetic acid. At this pH, the unprotected nucleophilic side chains of Lys, Cys, Ser, and Thr are unreactive. After the reaction, the pH is returned to 7 and another chemical ligation reaction can be performed. At the end of the synthesis, the peptide is cleaved from the resin with aqueous sodium hydroxide at pH 12–14 and then purified by RP-HPLC.

For the solid-phase chemical ligation in the C to N direction (Fig. 44), the first unprotected peptide bearing an N-terminal Cys is loaded on a water-compatible cellulose-based resin via the formation of an oxime between the keto group (introduced through a linker at the C-terminus of the peptide; see Fig. 43) and the aminoxy-group of the resin (Fig. 44).

The next peptide segment bearing a C-terminal thioester and a protected N-terminal Cys is then coupled via the chemical ligation methodology. After the coupling reaction, excess reagent and soluble byproducts are washed out and the N-terminal Cys is subsequently deprotected: the acetamidomethyl (Acm) group was chosen as a temporary protecting group of the Cys. The Acm group is stable to both TFA and HF (used for the synthesis of the unprotected peptide segment) and is also stable to nucleophiles such as the thiols used during the chemical ligation reaction. On the contrary, the Acm group is selectively and easily cleaved by treatment with mercuric acetate. After the last ligation reaction, the peptide is cleaved from the resin by treatment with aqueous base.

Kent and co-workers (92) have successfully used this solid-phase ligation methodology for the synthesis of the human group V secretory phospholipase A2 (GV-PLA2). GV-PLA2 is a protein consisting of 118 amino acids with six disulfide bonds. Three native chemical ligation reactions,

Figure 41. Solid-phase synthesis of an unprotected peptide segment having a C-terminal thioester and a keto-functionalized linker at its N-terminus.



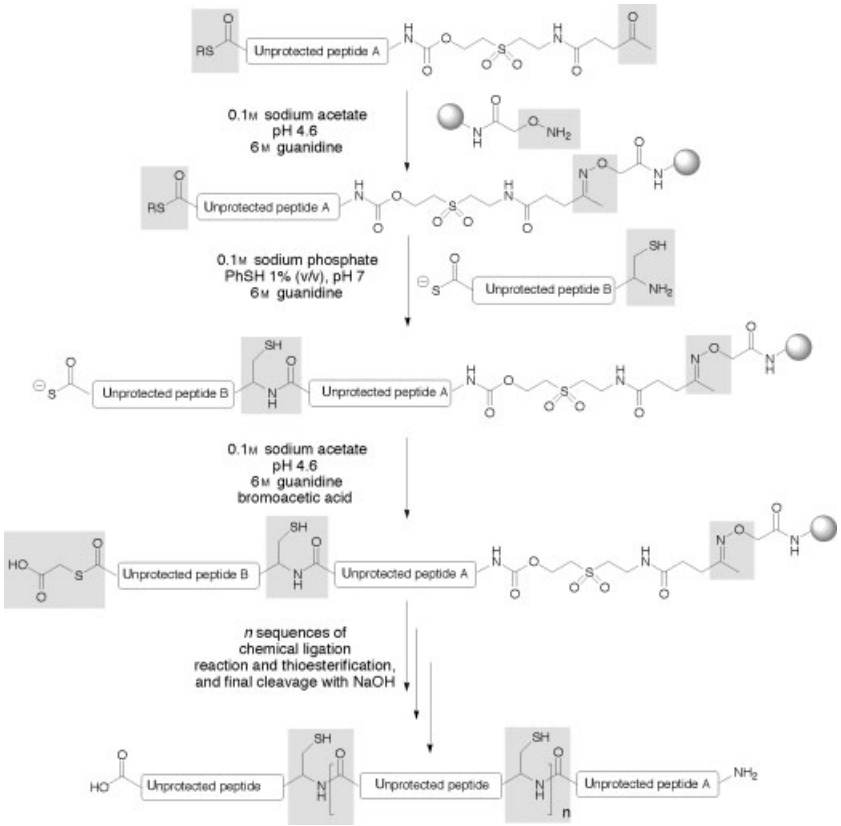


Figure 42. Solid-phase chemical ligation in the N to C direction of unprotected peptide fragments on a water-compatible cellulose-based resin.

with peptide segments ranging from 25 to 33 amino acid units, were used for the solid-phase assembly of the protein. The synthetic protein obtained in this way was characterized by electrospray mass spectrometry and shown to be identical to the native one. Furthermore, this synthetic protein displayed an enzymatic activity profile similar to that of the native protein.

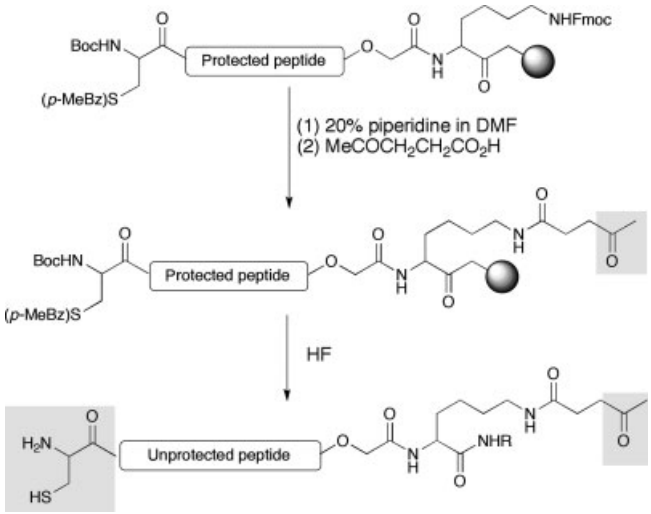


Figure 43. Solid phase synthesis of a peptide bearing an N-terminal Cys and keto functionalized linker at the C-terminus.

Synthesis of β-peptides by thioligation

The discovery in 1996 by Seebach *et al.* (94) that short chains of β-amino acids consisting of homologated proteinogenic α-amino acids generally form more stable secondary structures than their natural counterparts has opened a new era in peptide chemistry [for a review on β-peptides see Ref. (95)]. Since this pioneering work, β-peptides have been the subject of extensive investigation (96–100). Besides the numerous secondary structures such as helices (97–100) turns (96) and sheets (96) identified and characterized until now, β-peptides have also been shown to display interesting biological properties (101,102) – to be stable to metabolism in rat (103), plant and insect (104), and to be resistant to enzymatic degradation (105,106,107). Small β-peptides are synthesized using the standard Fmoc solid-phase (108,109) or Boc solution-phase methodology developed for α-peptide chemistry. For longer β-peptides however, some modification of the typical SPPS protocols had to be introduced, mainly because of the fact that β-peptides have a high folding propensity thereby leading to longer coupling times and incomplete Fmoc deprotection. Furthermore, β-amino acids are expensive building blocks [β^3-amino acids are commercial, while the β^2-amino acids have to be synthesized and their preparation can take up to 15 steps (110,111)] and cannot

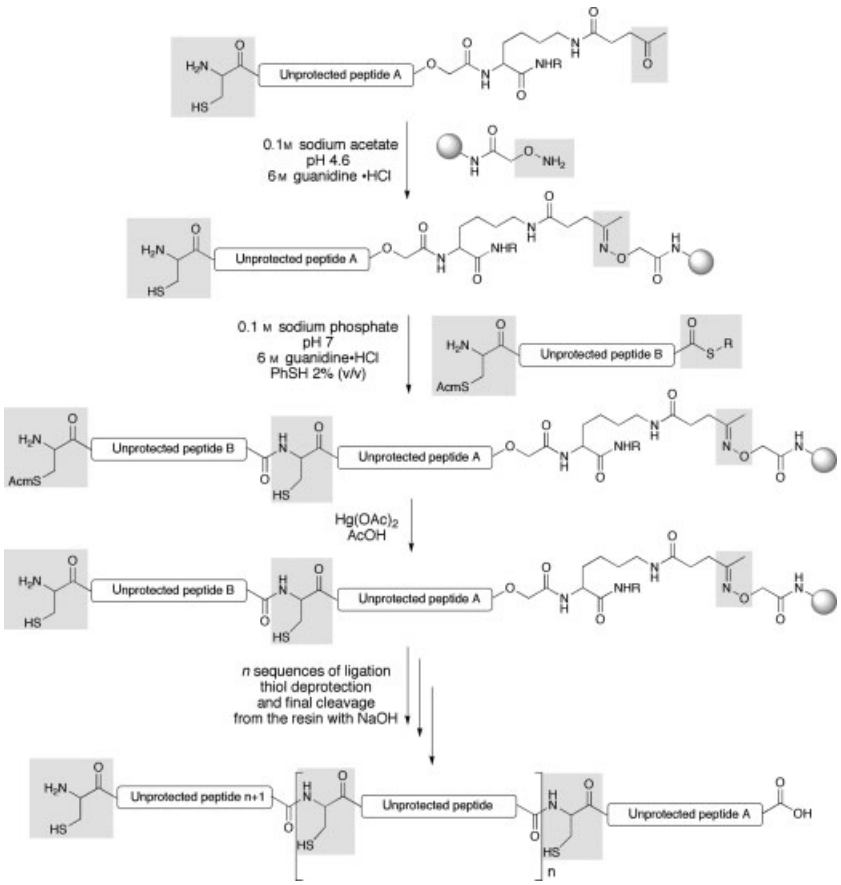


Figure 44. Solid-phase chemical ligation in the C to N direction of unprotected peptide fragments on a water-compatible cellulose-based resin.

therefore be used in excess. The problem of incomplete Fmoc deprotection was solved by the replacement of piperidine in the deprotection step by a stronger base such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (109,112) and it has been shown that the use of di- or tripeptides as building blocks for solid-phase synthesis of longer β-peptides can be advantageous (especially for the synthesis of mixed β^3/β^2 peptides) (113). In order to overcome the limitation of SPPS for the preparation of longer β-peptides, Seebach and coworkers have recently also tested the chemical ligation methodology with these unnatural derivatives and also for the coupling of α- with β-peptides (114).

Couplings with formation of β^2-, β^3-, β^2/β^3- and α/β-peptides

The β-peptides bearing a C-terminal thioester were prepared using the sulfonamide 'safety-catch' resin (**79**, **80**, **81**, **82**, **83**, **84**) or by applying Hilvert's methodology on an HMBA resin (**85**, **86**, **87**) (see Figs 21 and 22) (114,115). The peptides with N-terminal β^3-, β^2hCys or α-Cys (**88**, **89**, **90**, **91**) were prepared by using standard Fmoc SPPS on a Wang resin [in the case of β^2-peptide **83** and **90** Fmoc-protected β^2-dipeptides were used as building blocks (115)] (Figs 45 and 46).

The ligation reactions between β^3-, β^2-, or α-peptides bearing a C-terminal thioester, and peptides bearing an N-terminal β^3hCys, β^2hCys or Cys residue were carried out under standard conditions (34): aqueous solution, pH 7.5 phosphate buffer, 4% (v/v) PhSH. In the case of the β-peptides containing an N-terminal β^2hCys **89** and **90**, the intramolecular acyl S → N shift proceeds through a six-membered, rather than the five-membered heterocycle which is involved with peptides containing Cys or a β^3hCys (Fig. 47).

All the ligation reactions were monitored by analytical HPLC and lead to complete disappearance of the starting materials (see Fig. 48 for a typical example). After completion of the reaction, the products of ligation [**92** (**80** + **88**), **93** (**82** + **88**), **94** (**83** + **90**), **95** (**80** + **91**), **96** (**81** + **89**), **97** (**79** + **89**) and **98** (**84** + **89**)] were purified by preparative RP-HPLC and identified by high-resolution mass spectra. The yields of the purified coupling products were in the range of 40 to 70% (Fig. 49).

The preparation of compound **94** (a β^2-icosapeptide containing the 20 β^2-amino acids with proteinogenic side chains) best exemplifies the 'power' and the usefulness of the ligation methodology for the synthesis of larger β-peptides. This β^2-icosapeptide was prepared using a convergent

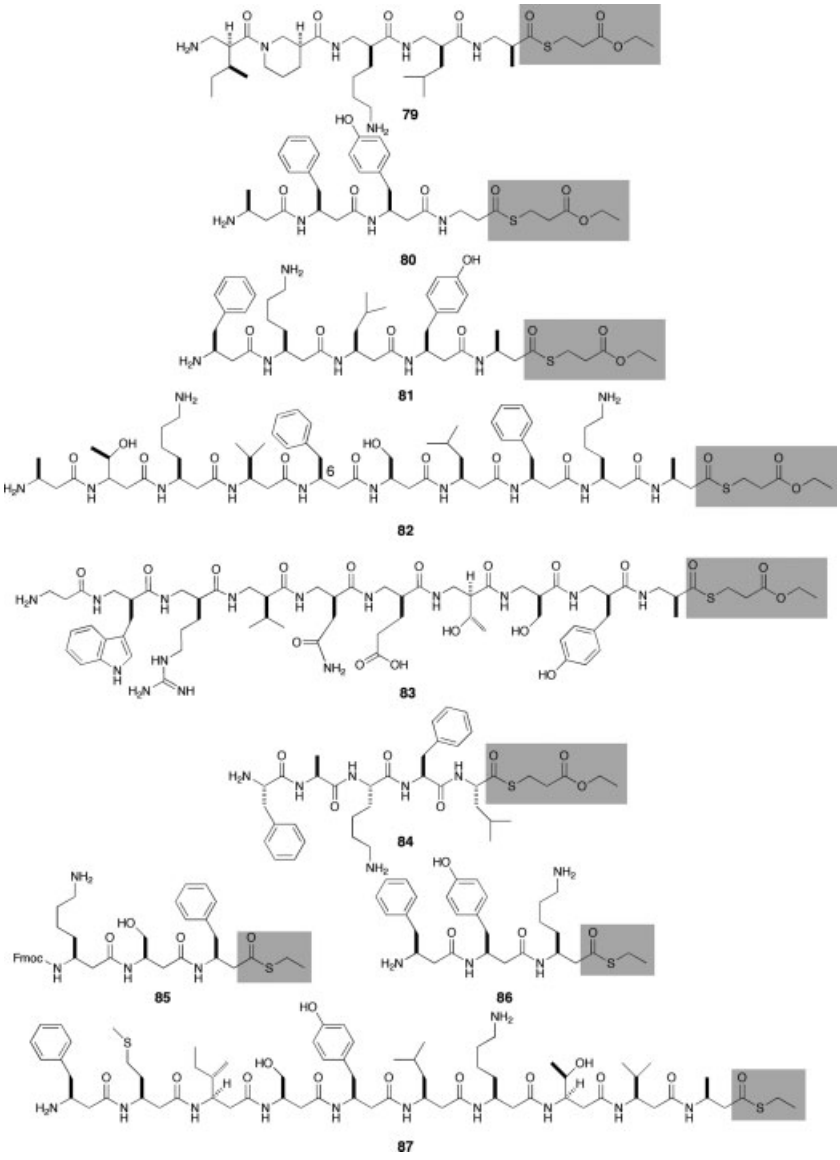


Figure 45. Formulae of β^3-, β^2-, and α-peptides with C-terminal thioester. Details on the preparation and characterization of **79, 81,** and **84,** are given in the experimental section.

strategy. First each of the 20 β^2-amino acid was synthesized in enantiomerically pure form (115–117). They were then used for the preparation of N-Fmoc-protected β^2-dipeptides for the subsequent solid-phase synthesis of two β^2-decapeptides (**90** and **83**); one containing an N-terminal β^2-homocysteine and the other a C-terminal thioester. Finally, application of the thioligation methodology yielded the expected icosapeptide **94**. This ligation reaction was the last step in a 159-step synthesis! (115).

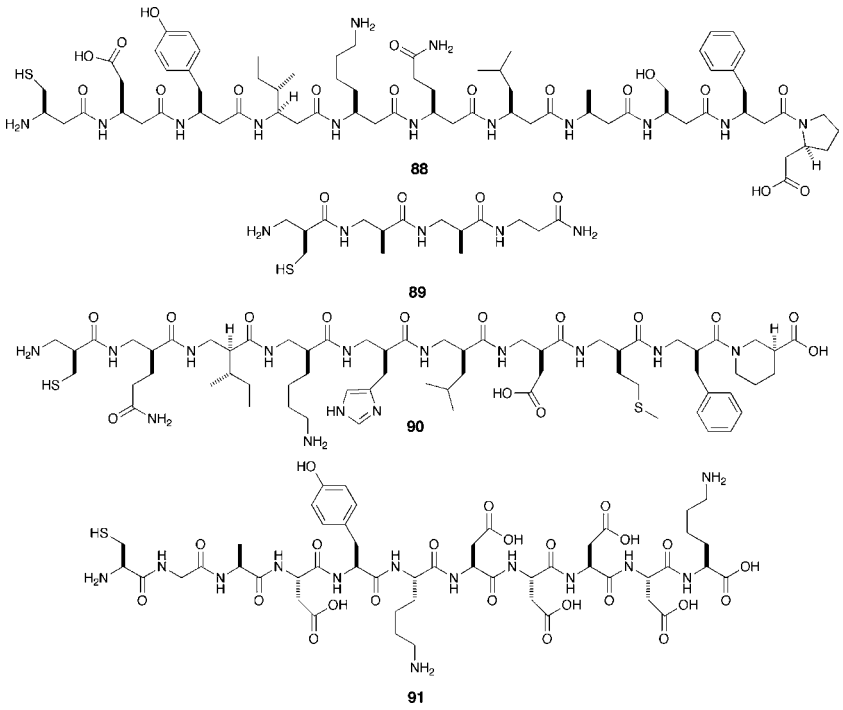
This result clearly shows that thioligation is applicable to β-peptides, and furthermore, that this strategy can also be used for the synthesis of mixed α/β and β^2/β^3 peptides. Having in mind the numerous useful properties of β-peptides, one can easily introduce these unnatural residues into natural α-proteins via the chemical ligation methodology in order to prepare hybrid proteins with enhanced chemical stability. The first experiment in this direction has been performed recently with the introduction of a β-dipeptide into an RNase without modification of the catalytic activity (118).

Experimental details for couplings involving β-peptides

General

Abbreviations: DIPCDI: diisopropylcarbodiimide, DIPEA: diisopropylethylamine, DMPA: 4-(dimethylamino)pyridine, HATU: h.v: high vacuum, Melm: 1-methyl-imidazole, MSNT: 1-(mesitylene-2-sulphonyl)-3-nitro-1H-1,2,4-triazole, TFA: trifluoroacetic acid, TIS: triisopropylsilane, TNBS: 2,4,6-trinitrobenzensulfonic acid. The β^3-amino acids were purchased from Fluka (Fluka Chemie GmbH Buchs SG, Switzerland), and the α-amino acid from Novabiochem (Novabiochem AG, Läufelfingen, Switzerland). Anal. HPLC: Merck HPLC system (LaChrom, pump type

Figure 46. Formulae of peptides containing an N-terminal β^3-, β^2-, or α-cysteine. Details on the preparation and characterization of **89** are given in the experimental section, below.



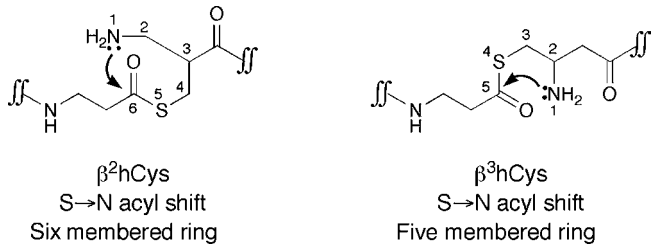


Figure 47. Formulae of the thioester-linked intermediate formed during the thioligation reaction with β^2hCys and β^3hCys (for six-membered ring intermediates in coupling between α-peptides see Fig. 26, 28, 29 and 30).

L-7150, UV detector L-7400, Interface D-7000, HPLC Manager D-7000). Macherey-Nagel C_8-column (Nucleosil 100-5 C_8 250 × 4 mm). Prep. HPLC: Merck HPLC system (LaChrom, pump type L-7150, UV detector L-7400, Interface D-7000, HPLC Manager D-7000) Macherey-Nagel C_8 column [Nucleosil 100-7 C_8 (250 × 21 mm)]. CD spectra: CD spectra were recorded on a Jasco J-710 spectropolarimeter from 190 to 250 nm at 25°C in 1-mm rectangular cells. All spectra were corrected for the corresponding solvent spectrum. Peptide concentrations were typically 0.2 mM. The molar ellipticity [θ] in deg cm²/mol (λ in nm) is calculated for the corresponding peptide or normalized. Smoothing was done by Jasco software. Solvents: MeOH (HPLC grade), aq. buffer pH 7.5: 0.1 M KH_2PO_4/0.1 M NaOH. Mass spectra: IonSpec Ultima 4.7 T FT Ion Cyclotron Resonance (ICR, HR-MALDI, in a 2.5-dihydroxybenzoic acid matrix), or Finnigan MAT TSQ 700 (ESI) mass spectrometer; in *m/z* (% of basis peak).

Reversed-phase (RP) HPLC analysis and purification

Reversed-phase HPLC analysis was perfomed on a Macherey-Nagel C_8 column [Nucleosil 100-5 C_8 (250 × 4 mm)] by using a linear gradient of A (0.1% TFA in H_2O) and B (MeCN) at a flow rate of 1.2 mL/min with UV detection at 220 nm; t_R in min. RP-HPLC purification was perfomed on a Macherey-Nagel C_8 column [Nucleosil 100-5 C_8 (250 × 21 mm)] by using a linear gradient of A and B at a flow rate of 18 mL/min (Merck HPLC system).

Anchoring of N-Fmoc-protected β- or α-amino acid on the 4-sulfamylbutyryl AM resin: General Procedure 1 (GP 1)

A solution of the Fmoc-protected β- or α-amino acid (4 eq.), 1-Melm (4 eq.), and DICPI (4 eq.) in CH_2Cl_2/DMF (4 : 1) was added to the resin that had been preswelled in CH_2Cl_2 for 1 h. The suspension was mixed by Ar bubbling for 18 h at room temperature. The resin was then filtered off, washed with DMF (4 mL, 4 × 1 min), CH_2Cl_2 (4 mL, 4 × 1 min), and dried under h.v. overnight. The loading was determined by measuring the absorbance of the benzofulvene piperidine adduct as in Ref. (112).

SPPS of the 4-sulfamylbutyryl AM resin, or the Rink amide resin: General Procedure 2 (GP 2)

The Fmoc group of the first amino acid attached to the resin (or from the Rink amide resin) was removed using 20% piperidine in DMF (4 mL, 4 × 10 min) under Ar bubbling. After filtration, the resin was washed with DMF (4 mL, 4 × 1 min). Solid-phase synthesis was continued by

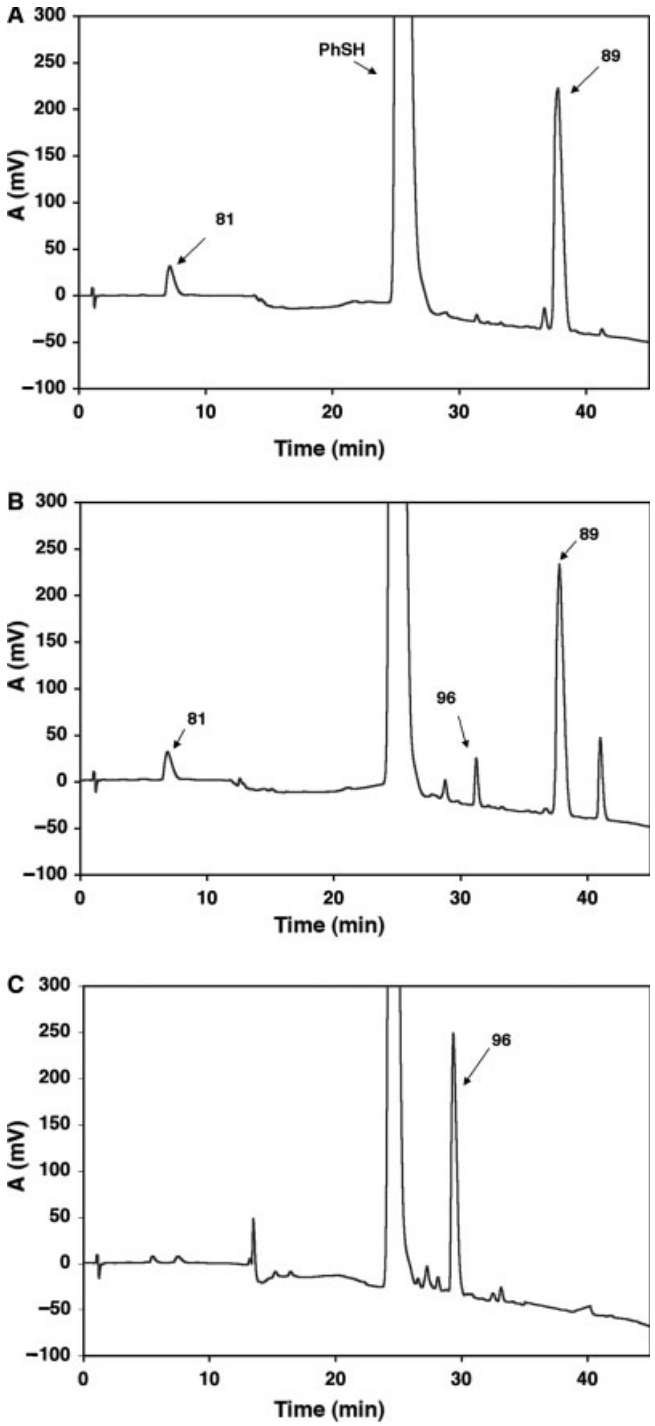


Figure 48. Analytical HPLC traces of the ligation reaction between β^3-pentapeptide (81) with C-terminal thioester and the β^2-tetrapeptide **89** with an N-terminal β^2hCys. (A) Time zero; (B) reaction after 4 h; (C) after 16 h. The peak at 30 min corresponds to the expected ligation product **96** as determined by high-resolution mass spectrometry after purification by preparative RP-HPLC (see experimental section).

sequential incorporation of *N*-Fmoc-protected β^3-, β^2-, or α-amino acids. For each coupling step, the resin was treated with a solution of Fmoc-protected amino acid (4 eq.), HATU (3.8 eq.) and DIPEA (7.8 eq.) in DMF (4 mL). The suspension was then mixed by Ar bubbling for 45–60 min. Monitoring of the coupling reaction was performed with 2,4,6-trinitrobenzensulfonic acid (TNBS) (119). In the case of a positive TNBS test (indicating incomplete coupling), the suspension was allowed to react further for 1–2 h or, after filtration, the peptide resin was treated again with the same Fmoc amino acid (2 eq.) and coupling reagents. The resin was then filtered off and washed with DMF (4 mL, 4 × 1 min) prior to the subsequent Fmoc-deprotection step using 20% piperidine in DMF (4 mL, 4 × 10 min). After filtration, the resin was washed with DMF (4 mL, 3 × 1 min) and solid-phase synthesis was continued by sequential incorporation of Fmoc-protected amino acids. For each coupling step, the resin was treated as described above. After the last coupling the resin was filtered off, washed with DMF (4 mL, 4 × 1 min), CH_2Cl_2 (4 mL, 4 × 1 min), and MeOH (4 mL, 4 × 1 min), dried under h.v. for 24 h, and used for the cleavage step.

Alkylation of the peptide–acylsulfonamide resin: General Procedure 3 (GP 3)

The 4-sulfamylbutyryl AM resin was activated according to (42). After swelling the resin in THF, 4 mL of a solution of TMS-CHN_2 (2 M in hexane) was added, and the suspension was mixed by Ar bubbling for 2 h. Subsequently, the resin was filtered off, washed with THF (4 mL, 4 × 1 min) and DMF (4 mL, 4 × 1 min), and used in the displacement reaction (see below).

Thioesterificatin, deprotection and purification of the alkylated peptide-acylsulfonamide resin: General Procedure 4 (GP 4)

The activated *N*-acylsulfonamide resin was swollen in DMF and filtered off. A solution of ethyl-3-mercaptopropionate (50 eq.) and sodium thiophenate (0.5 eq.) in DMF (1 M) was added. The suspension was mixed by Ar bubbling for 24 h, then the resin was filtered and washed with DMF (4 × 4 mL). The combined filtrate and washes were collected, rotary evaporated and dried under h.v. Removal of side-chain-protecting groups was accomplished in solution by treatment of the protected peptide thioester with a solution of TFA/H_2O/TIS (95/2.5/2.5). Solvents were concentrated under reduced pressure. The precipitate, which formed upon addition of cold Et_2O to the oily residue, was collected by centrifugation. After drying, the crude thioester was purified by preparative RP-HPLC and lyophilized.

Chemical ligation reaction of unprotected β- or α-peptides: General Procedure 5 (GP 5)

Typically, the C-terminal β- or α-peptide thioester (1 eq.) and the peptide containing an N-terminal β^2hCys (1 eq.)

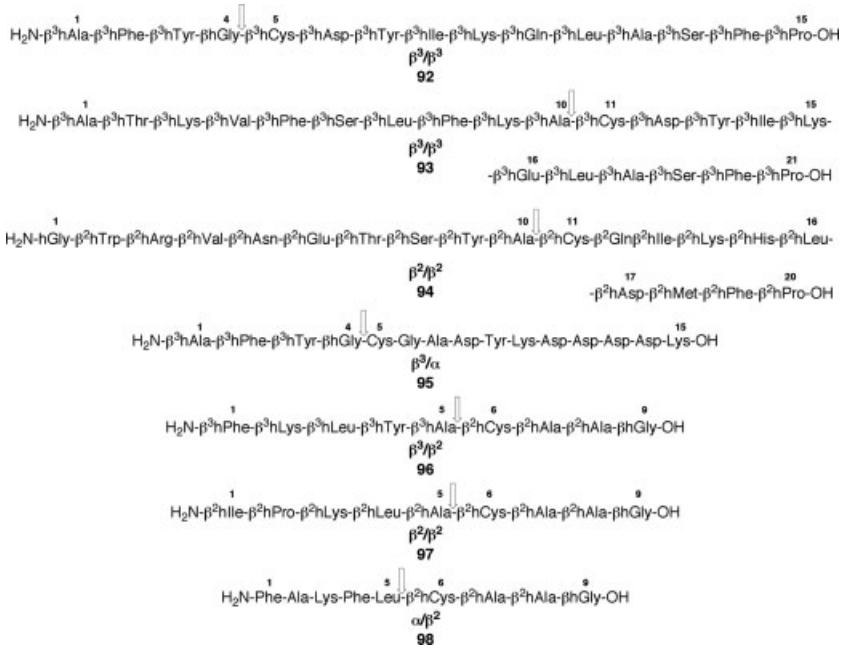


Figure 49. Formulae of the β^3-, β^2- and mixed β^3/β^2-, β^3/α, and β^2/α-peptides **92**–**98** synthesized through the thioligation methodology. The arrows indicates the site of ligation. Details on the synthesis and characterization of **96** and **98** are given in the experimental section, below.

were dissolved in 100 mM sodium phosphate buffer (pH 7.5) to give a final concentration of 2 mM. Thiophenol (4 % v/v) was added, and the mixture stirred under Ar at room temperature. The reaction was monitored by analytical C_8 RP-HPLC. Following the ligation, the product was purified by preparative C_8 RP-HPLC and lyophilized. For the synthesis of compounds **80**, **82**, **85**, **86**, **87**, **88**, **91**, **92**, **93**, **95** see Ref. (114) and for that of **83**, **90**, and **94** see Ref. (115).

H-β ^{2}hIle-β ^{2}hPro-β ^{2}hLys-β ^{2}hLeu-β ^{2}hAla-S-ethylpropionate (**79**)

The 4-sulfamylbutyryl AM resin (250 mg, 0.275 mmol) was derivatized with Fmoc-β^{2}hAla-OH (357 mg, 1.10 mmol) according to GP 1 to give a loading of 0.77 mmol/g (70%), corresponding to 0.192 mmol of anchored Fmoc-β^{2}hAla-OH. Solid-phase synthesis was performed according to GP 2 by sequential incorporation of Fmoc-β^{2}hLeu-OH; Fmoc-β^{2}hLys(Boc)-OH; Fmoc-β^{2}hPro-OH; Boc-β^{2}hIle-OH. The resin was activated for the cleavage by treatment with 4 mL of a solution of 2 M TMS-CHN_2 in THF according to GP 3. The activated *N*-acylsulfonamide resin was then treated with a solution of ethyl-3-mercaptopropionate (1.2 mL, 9.60 mmol), and sodium thiophenate (12 mg, 0.09 mmol) in DMF (5 mL) for 24 h according to GP 4. Removal of side-chain-protecting groups was accomplished in solution by treatment of the protected β^2-peptide thioester with a solution of TFA/H_2O/TIS (95/2.5/2.5) according to GP 4. Purification by RP-HPLC (5–40% B in 40 min, C_8) yielded **79** (95 mg, 68%) as a colorless fluffy solid. Anal. RP-HPLC: t_R 30.52 (5–40% B in 40 min, C_8). CD (0.2 mM in MeOH): $-5.579{\cdot}10^3$ (219.5 nm); 0 (205.6 nm); $+31.904{\cdot}10^3$ (196.8 nm). HR-MALDI-MS: 749.4659 ($[C_{36}H_{66}N_6NaO_7S]^+$; calc. 749.4606).

H-β ^{3}hPhe-β ^{3}hLys-β ^{3}hLeu-β ^{3}hTyr-β ^{3}hAla-S-ethylpropionate (**81**)

The 4-sulfamylbutyryl AM resin (205 mg, 0.225 mmol) was derivatized with Fmoc-β^{3}hAla-OH (293 mg, 0.902 mmol) according to GP 1 to give a loading of 0.75 mmol/g (68%), corresponding to 0.153 mmol of anchored Fmoc-β^{3}hAla-OH. Solid-phase synthesis was performed according to GP 2 by sequential incorporation of Fmoc-β^{3}hTyr(*t*Bu)-OH; Fmoc-β^{3}hLeu-OH; Fmoc-β^{3}hLys(Boc)-OH; Boc-β^{3}hPhe-OH. The resin was then activated for the cleavage by treatment with 4 mL of a solution of 2 M TMS-CHN_2 in THF according to GP 3. The activated *N*-acylsulfonamide resin was then treated with a solution of ethyl-3-mercaptopropionate (1.0 mL, 7.65 mmol), and sodium thiophenate (9 mg, 0.068 mmol) in DMF (5 mL) for 24 h according to GP 4. Removal of side-chain-protecting groups was accomplished in solution by treatment of the protected β^3-peptide thioester with a solution of TFA/H_2O/TIS (95/2.5/2.5) according to GP 4. Purification by RP-HPLC (5–40% B in 40 min, C_8) yielded **81** (85 mg, 67%) as a colorless fluffy solid. Anal. RP-HPLC: t_R 31.28 (5–40% B in 40 min, C_8). CD (0.2 mM in MeOH): -1.620×10^3 (218.5 nm); 0 (212.5 nm); $+15.147{\times}10^3$ (201 nm). HR-MALDI-MS: 827.4727 ($[C_{43}H_{67}N_6O_8S]^+$; calc. 827.4736).

H-Phe-Ala-Lys-Phe-Leu-S-ethylpropionate (**84**)

The 4-sulfamylbutyryl AM resin (205 mg, 0.225 mmol) was derivatized with Fmoc-Leu-OH (318 mg, 0.90 mmol)

according to GP 1 to give a loading of 0.81 mmol/g (73%), corresponding to 0.166 mmol of anchored Fmoc-Leu-OH. Solid-phase synthesis was performed according to GP 2 by sequential incorporation of Fmoc-Phe-OH; Fmoc-Lys(Boc)-OH; Fmoc-Ala-OH; Boc-Phe-OH. The resin was then activated for the cleavage by treatment with 3 mL of a solution of 2 M TMS-CHN_2 in THF according to GP 3. The activated *N*-acylsulfonamide resin was then treated with a solution of ethyl-3-mercaptopropionate (1.44 mL, 11.25 mmol), and sodium thiophenate (10 mg, 0.083 mmol) in DMF (5 mL) for 24 h according to GP 4. Removal of side-chain-protecting groups was accomplished in solution by treatment of the protected peptide thioester with a solution of TFA/H_2O/TIS (95/2.5/2.5) according to GP 4. Purification by RP-HPLC (5–40% B in 40 min, 40–98% B in 20 min, C_8) yielded **84** (92 mg, 75%) as a colorless fluffy solid. Anal. RP-HPLC: t_R 41.7 (5–40% B in 40 min, 40–98% B in 20 min, C_8). HR-MALDI-MS: 763.3820 ($[C_{38}H_{56}N_6NaO_7S]^+$; calc. 763.3823).

H-β^2hCys-β^2hAla-β^2hAla-βhGly-NH_2 **(89)**

The Rink amide resin (300 mg, 0.171 mmol) was Fmoc deprotected and solid-phase synthesis was performed according to GP 2 by sequential incorporation of Fmoc-βGly-OH; Fmoc-β^2hAla-OH; Fmoc-β^2hAla-OH; Boc-β^2Cys(Trt)-OH. After the last coupling, the resin was washed with DMF (4 mL, 5 × 3 min), CH_2Cl_2 (4 mL, 5 × 3 min), Et_2O (4 mL, 5 × 1 min) and dried under h.v. for 12 h. The dried Rink amide resin was first treated with a mixture of CH_2Cl_2/TFA/TIS 90/9/1 (3 × 3 mL), then with a mixture CH_2Cl_2/TFA/TIS 95/4/1 (3 × 3 mL) allowing the solvent to pass through the resin bed slowly. Excess TFA/CH_2Cl_2 was evaporated and deprotection was completed by stirring the oily residue in TFA/H_2O/EDT/TIS 94/2.5/2.5/1 for 1 h. The solvent was evaporated and the precipitate, which formed upon addition of cold Et_2O to the oily residue, was collected by centrifugation. After drying, the crude peptide was purified by preparative RP-HPLC and lyophilized. Purification by RP-HPLC (5–40% B in 40 min, C_8) yielded **89** (54 mg, 85%) as a colorless fluffy solid. Anal. RP-HPLC: t_R 6.71 (5–40% B in 40 min, C_8). HR-MALDI-MS: 376.2018 ($[C_{15}H_{30}N_5O_4S]^+$; calc. 376.2013).

H-β^3hPhe-β^3hLys-β^3hLeu-β^3hTyr-β^3hAla-β^2hCys-β^2hAla-β^2hAla-βhGly-NH_2 **(96)**

The β^2-peptide fragment **89** (3.6 mg, 7.36 μmol) and the β^3-peptide **81** with C-terminal thioester (7.7 mg, 7.36 μmol) were ligated in 4 mL phosphate buffer containing 4% PhSH according to GP 5. The ligation was performed at r.t. and monitored by anal. RP-HPLC. Following completion of the ligation, the reaction mixture was diluted with H_2O (4 mL) containing 0.1% TFA and purified by prep. RP-HPLC (2–40% B in 40 min, C_8) to yield the TFA salt of **96** (5.6 mg, 59 %). CD (0.2 mM in MeOH): $-10.915{\cdot}10^3$ (217.2 nm); 0 (207.1 nm); $+22.779{\cdot}10^3$ (197 nm) Anal. RP-HPLC: t_R 37.68 (2–40% B in 40 min, C_8). HR-MALDI-MS: 1068.628 ($[C_{53}H_{86}N_{11}O_{10}S]^+$; calc. 1068.6274).

H-β^2hIle-β^2hPro-β^2hLys-β^2hLeu-β^2hAla-β^2hCys-β^2hAla-β^2hAla-βhGly-NH_2 **(97)**.

The β^2-peptide fragment **89** (3.2 mg, 6.6 μmol) and the β^2-peptide **79** with C-terminal thioester (6.3 mg, 6.6 μmol) were ligated in 4 mL phosphate buffer containing 4% PhSH according to GP 5. The ligation was performed at room temperature and monitored by anal. RP-HPLC. Following completion of the ligation, the reaction mixture was diluted with H_2O (4 mL) containing 0.1% TFA and purified by prep. RP-HPLC (2–40% B in 40 min, C_8) to yield the TFA salt of **97** (4.3 mg, 55 %). CD (0.2 mM in MeOH): $-1.417{\cdot}10^3$ (215.7 nm); 0 (209.5 nm); $+13.924{\cdot}10^3$ (190.6 nm) Anal. RP-HPLC: t_R 33.12 (2–40% B in 40 min, C_8). HR-MALDI-MS: 990.6144 ($[C_{46}H_{85}N_{11}NaO_9S]^+$; calc. 990.6145).

H-Phe-Ala-Lys-Phe-Leu-β^2hCys-β^2hAla-β^2hAla-βhGly-NH_2 **(98)**

The β^2-peptide fragment **89** (1.9 mg, 3.9 μmol) and the α-peptide **84** with C-terminal thioester (3.8 mg, 3.9 μmol) were ligated in 3 mL phosphate buffer containing 3% PhSH according to GP 5. The ligation was performed at r.t. and monitored by anal. RP-HPLC. Following completion of the ligation, the reaction mixture was diluted with H_2O (4 mL) containing 0.1% TFA and purified by prep. RP-HPLC (2–40% B in 40 min, C_8) to yield the TFA salt of **98** (3.6 mg, 75 %). Anal. RP-HPLC: t_R 24.89 (2–40% B in 40 min, C_8). HR-MALDI-MS: 1004.537 ($[C_{48}H_{75}N_{11}NaO_9S]^+$; calc. 1004.5362).

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