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Developments in peptide and amide synthesis

Fernando Albericio

The solid-phase methodology is key for an effective synthesis of peptides, from a milligram scale for research to a multi-kilo scale for drug production. Indeed, small peptides containing up to 20–30 amino acids are most readily synthesized by a solid-phase strategy. Larger peptides (up to 60 amino acids) should be synthesized by a convergent approach (i.e. synthesis of protected constituent peptides in solid-phase and combination of these units in solution). Larger peptides and proteins are prepared by chemical ligation, where unprotected segments have been prepared in solid-phase.

Addresses
Department of Organic Chemistry, Martí i Franqués 1, University of Barcelona; 08028-Barcelona, Spain
e-mail: albericio@pcb.ub.es

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Abbreviations
NCL native chemical ligation
SPPS solid-phase peptide synthesis

Introduction

Recent years have witnessed a renaissance in the field of peptides. Today, more than 40 peptides are on the market, 4 more are in registration, 200 are in clinical phases and more than 400 are in advanced preclinical trials.

From a synthetic point of view, there is no doubt that the solid-phase methodology developed by R Bruce Merrifield in the 1960s has matured sufficiently to be the method of choice — either by itself or in combination with steps in solution — for the synthesis of almost all kinds of peptides. Small peptides (i.e. those containing up to 20–30 amino acids) are best synthesized by a solid-phase strategy alone [1]. Larger peptides (up to 60 amino acids) should be synthesized by a convergent approach, involving the synthesis of protected peptide segments in solid-phase and combination of these units in solution. Larger peptides and proteins are prepared by chemical ligation, where the totally unprotected segments have been prepared in solid-phase.

The purpose of this review is to provide an overview of the current state of peptide synthesis methodology, with emphasis placed on the more recent developments.

Coupling methods

The formation of the amide bond, which is a key step in the synthesis of any peptide as well as many other organic compounds of biological interest, requires activation of a carboxylic acid group [2]. Traditionally, this step is carried out with carbodiimides by conversion to the *O*-acylisourea, which is very reactive and can undergo a rearrangement to give unreactive species or cause loss of chiral integrity. Active species can be captured using a hydroxylamine derivative to give the corresponding active ester. In this regard, HOBt and HODhbt are less reactive than the *O*-acylisourea but are more stable and less prone to racemization [2] (for a glossary of reagent abbreviations, see Box 1). For more demanding couplings, Carpino *et al.* described the use of HOAt — an expensive additive compared with HOBt — and this has given excellent results [3]. The use of Cl-HOBt represents a good compromise between HOAt and HOBt in terms of reactivity and price [4]. Very recently, Carpino *et al.* described the aza derivative of HODhbt (i.e. HODhat), whose active esters are even more reactive (albeit slightly) than the OAt esters, which are considered the most reactive derivatives among these esters [5•]. Figure 1 shows the most representative coupling reagents, additives and active species used in peptide chemistry.

The same active esters can be prepared with onium (aminium/uronium and phosphonium) salts in the presence of a base. The most widely used systems are HATU, HCTU, HBTU, HDTU, HDATU and their corresponding tetrafluoroborate salts among the former class and PyAOP or PyBOP from the latter class [3,4,5•]. Couplings with aminium/uronium salts in the presence of base have proven to be more effective than those carried out with phosphonium reagents or carbodiimide in the presence of hydroxylamine derivatives [2]. On the other hand, reagents based on phosphonium salts are preferred to their aminium/uronium counterparts for cyclization, because the latter compounds can give guanilidation reactions on the amino group [2].

Other stand-alone coupling reagents are those derived from organophosphorus esters, with the main examples being the phosphate esters DepOBt, DepODhbt or DEPBT, DepOAt and the phosphinyl esters DtpOBt, DtpOAt, and DtpODhbt [6,7•]. Once again, the OAt derivative has proven superior to the ODhbt derivative and this, in turn, is better than the OBt system [7•]. These

Box 1 Glossary of reagents.

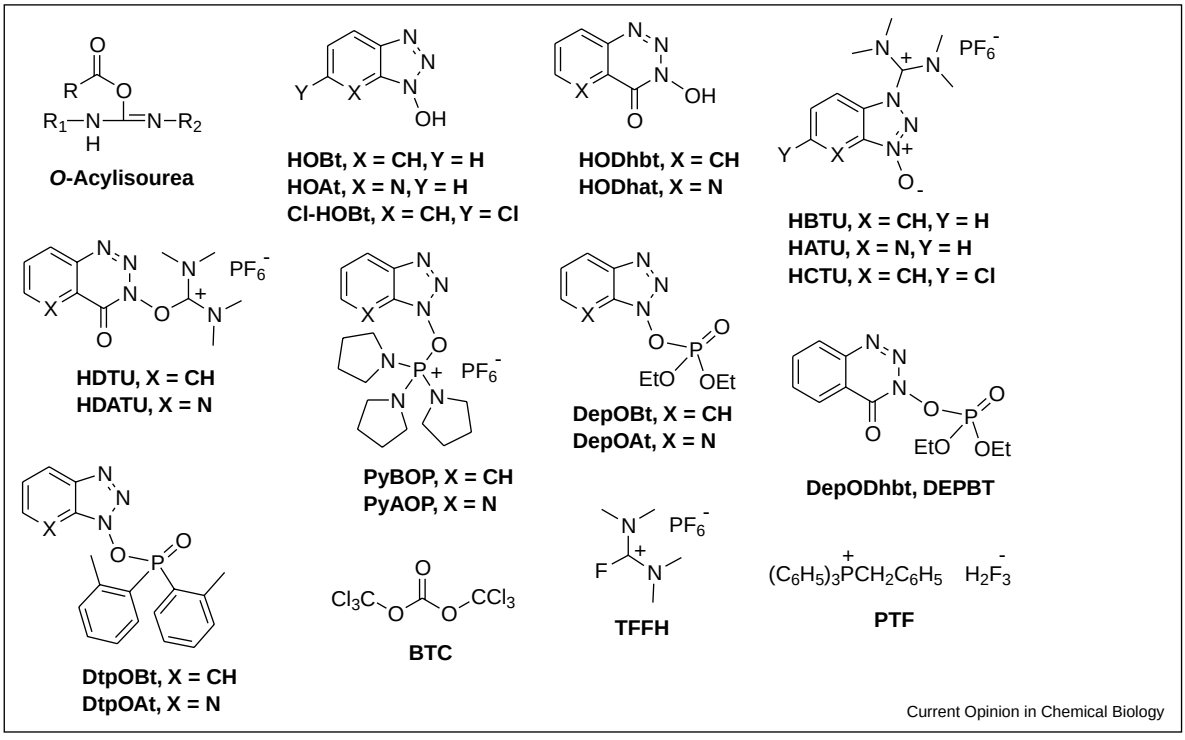
Abbr.	Reagent
Acm	Acetamidomethyl
Alloc	Allyloxycarbonyl
BAL	5-(4-Formyl-3,5-dimethoxyphenoxy)valeric acid
Boc	*t*-Butoxycarbonyl
BTC	Triphosgene, bis(trichloromethyl)carbonate
Cl-HOBt	1-Hydroxy-5-chlorobenzotriazole
CTC	2-Chlorotrityl chloride
DAST	(Diethylamino)sulfur trifluoride
DepOAt	Diethoxyphosphinyloxy-7-azabenzotriazole
DepOBt	Diethoxyphosphinyloxybenzotriazole
DepODhbt, DEPBT	3-(Diethoxyphosphinyloxy)-3,4-dihydro-4-oxo-1,2,3-benzotriazine
DIEA	*N*,*N*-Diisopropylethylamine
DIPCDI	*N*,*N*′-Diisopropylcarbodiimide
DKP	Diketopiperazine
DMF	*N*,*N*-Dimethylformamide
DPP	Didehydropeptide
DtpOAt	1-[Di(*o*-tolyl)phosphinyloxy]-7-azabenzotriazole
DtpOBt	1-[Di(*o*-tolyl)phosphinyloxy]benzotriazole
DtpODhbt	3-[Di(*o*-tolyl)phosphinyloxy]-3,4-dihydro-4-oxo-1,2,3-benzotriazine
EDC, WSC	3-(3′-Dimethylaminopropyl)-1-ethylcarbodiimide
Fmoc	Fluorenylmethoxycarbonyl
HATU	*N*-{(Dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]-pyridino-1-ylmethylene}-*N*-methylmethan-aminium hexafluorophosphate
HBTU	*N*-[(1*H*-Benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HCTU	*N*-[(1*H*-6-Chlorobenzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide
HDATU	*O*-(3,4-Dihydro-4-oxo-5-azabenzo-1,2,3-triazin-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HDTU	*O*-(3,4-Dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMFS	*N*-[(9-Hydroxymethyl)fluoren-2-yl]succinamic acid
HOAc	Acetic acid
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HODhat	3-Hydroxy-4-oxo-3,4-dihydro-5-azabenzo-1,2,3-triazene
HODhbt	3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine
NMP	*N*-Methylpyrrolidine
PTF	Benzyltriphenylphosphonium dihydrogen trifluoride
PyAOP	(7-Azabenzotriazol-1-yloxy)-tris(pyrrolidino)-phosphonium hexafluorophosphate
PyBOP	Benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
TFA	Trifluoroacetic acid
TFFH	Tetramethylfluoroformamidinium hexafluorophosphate

derivatives are more stable than aminium/phosphonium salts as they exist as neutral moieties and not as hygroscopic salts. The phosphinyl esters generally proved to be as efficient as the phosphate analogues and, in view of their extended shelf stability, are to be recommended for general use. A further advantage of these phosphorus ester derivatives is their solubility in many organic solvents, including non-polar solvents such as CH_2Cl_2 [7•]. This property can be exploited because the use of this kind of solvent usually gives better results in terms of racemization and yield in a number of cases, including segment condensation or acylation of *N*-alkyl amines.

Coupling on *N*-alkyl amines, such as N-methylamino residues or amino acids anchored to a backbone linker-based resin, can be performed using the symmetrical anhydride, which can in turn be obtained with two equivalents of protected amino acid in the presence of a single equivalent of carbodiimide in CH_2Cl_2 [8]. Another reagent that is well-suited for the acylation of *N*-alkyl amine derivatives is BTC, which is likely to function through the *in situ* generation of the corresponding acid chloride. Jung *et al.* used this method [9], which was first described by Gilon *et al.* [10], for the preparation of omphalotin A and cyclosporins, both of which are rich N-methyl amino acids containing cyclic peptides. N-methyl amino acid chlorides cannot be converted to the corresponding oxazolones in the presence of base, and this is one of the drawbacks in using acid chlorides as coupling reagents. Optimal conditions involve activation with BTC in THF in the presence of collidine and coupling in the presence of several equivalents of DIEA. This increased coupling efficiency, obtained by the combination of a weak base for carboxy activation and a stronger base for the coupling reaction, was first described by Carpino and El-Faham for the system DIPCDI/HOAt [11].

Acid fluorides are very convenient reagents for the synthesis of systems that incorporate hindered amino acids. These fluorides can be prepared and isolated by reaction of the protected amino acids with cyanuric fluoride or

Figure 1



Coupling reagents, additives and active species discussed in the text.

DAST or, alternatively, generated *in situ* through the aminium reagent TFFH, which itself can be used as a stand-alone coupling reagent [12]. In this case, total conversion to fluoride is achieved when the reaction is carried out in the presence of an anionic polyhydrogen fluoride additive such as PTF [13•]. The use of carbodiimides or aminium salts in the presence of PTF also leads to the corresponding fluorides.

Solid-phase synthetic strategies

The vast majority of peptides have either a carboxylic acid or an amide in the side-chain of their C-terminal residue [1]. In addition to the most common resins [1], Barlos (or CTC) resin [14] (Figure 2), which was originally designed for the preparation of protected peptides using an Fmoc/*t*Bu strategy due to extremely mild conditions (1% TFA, hexafluoroisopropanol, HOAc/trifluoroethanol) used to remove the peptide from the resin, is becoming the resin of choice for the preparation also of unprotected *C*-terminal acid peptides. There are several advantages of the Barlos resin when compared with the Wang resin. First, it can be used for both free and protected peptides. Second, minimization of DKP formation due to the hindered trityl group and the presence of the Cl substituent in position 2. Third, the absence of racemization during the incorporation of the first protected amino acid by nucleophilic substitution. And fourth, the possibility of a straightforward side-chain incorporation of several protected amino acids and other functionalities such as alcohols, amines or hydrazines (see below).

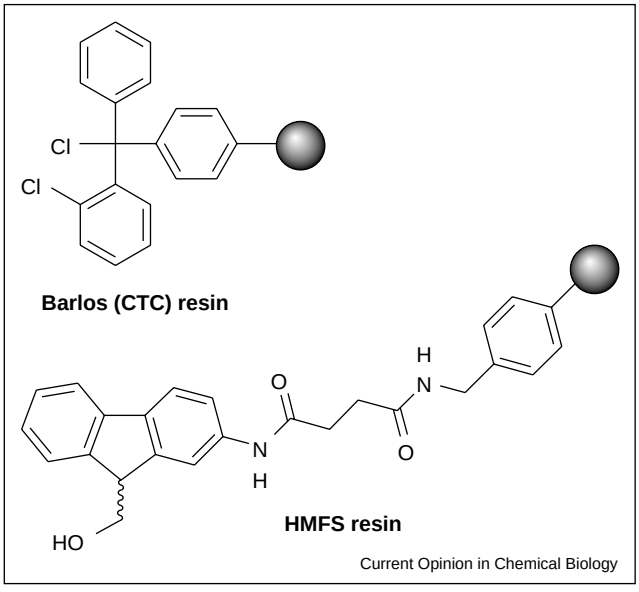
For the preparation of protected peptides using a Boc/Bzl scheme, the HMFS handle (Figure 2), which is based on a fluorenylmethyl ester, was developed by our group (see below for applications) [15].

In addition to acid and amide peptides, many C-terminally modified peptides are found in nature and are of potential interest as therapeutic agents. In recent years, a number of resins and strategies have been developed for the preparation of this kind of peptide. Representative resins or linkers used for the most important functionalities are shown in Figure 3 and an in-depth discussion of this topic can be found in the recent review published by our group [16••].

Although solid-phase peptide synthesis has been typically carried out in the C→N direction starting with the C-terminal residue. Several approaches have been developed for the side-chain anchoring of a partially protected trifunctional amino acid to an appropriate solid support, and even by attachment of the growing peptide to the support through a backbone amide nitrogen.

Wang resins can be used for side-chain anchoring of Asp, Glu, Tyr (by a Mitsunobu reaction) and, after conversion

Figure 2



Resins for acid peptides discussed into the text.

to an active carbonate, Lys. PAL and Rink resins can mask the amide function of Asn and Gln. Once again, Barlos resin has a more general applicability and can easily accommodate Asp, Glu, Cys, Tyr, Ser, Thr, Lys and His through the side-chain [17]. Furthermore, our group has recently described a resin based on a chroman system that is suitable for guanidine attachment (Figure 4) [18].

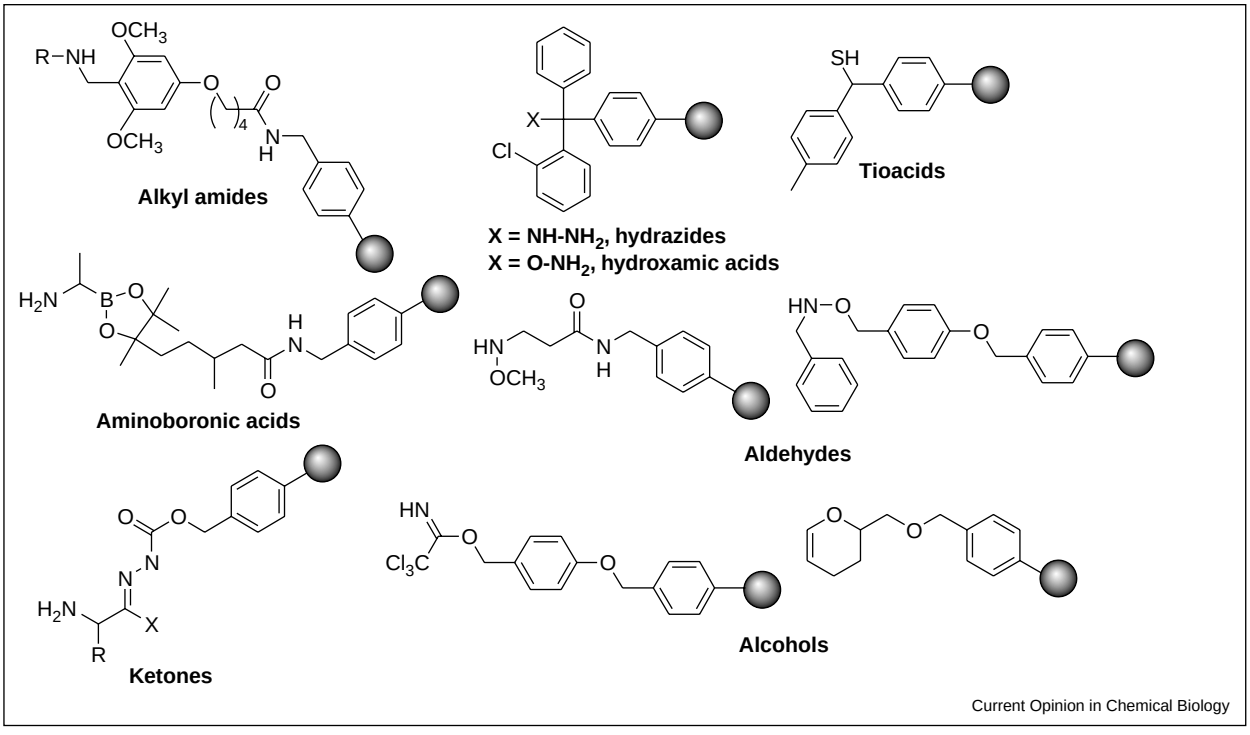
As far as backbone anchoring is concerned, our group has developed a novel and completely general strategy for this kind of attachment [8]. In this approach, the backbone amide linkage was established by a reductive amination procedure between the α-amine of the intended C-terminal residue (or an appropriately protected C-terminal modified derivative) and an aldehyde-functionalized solid support (BAL-resin) (Figure 4), followed by N-acylation using the N^{α}-protected second amino acid residue. Further work based on our initial studies has led to the development of other BAL-type resins [8].

Both side-chain and backbone anchoring have broad applicability for the preparation of C-terminal modified as well as cyclic peptides.

Cyclic peptides

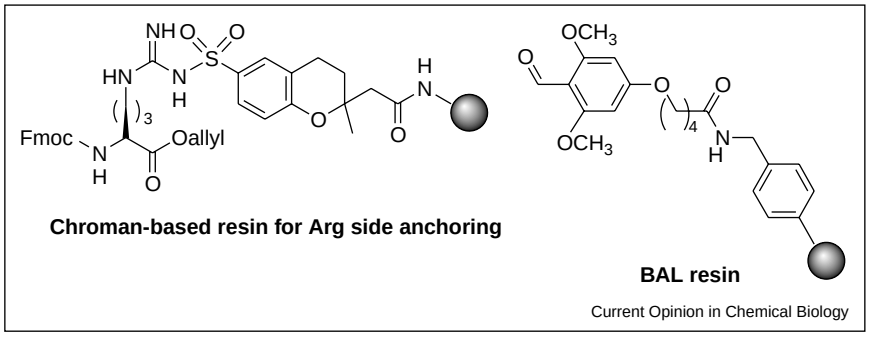
In recent years, special attention has been paid to the development of methods for the preparation of cyclic peptides. Of the numerous syntheses published in recent years, the syntheses of tentoxin [19] and scytalidamide A [20] incorporate several features that merit further discussion.

Figure 3



Representative resins for the preparation of C-terminal modified peptides.

Figure 4

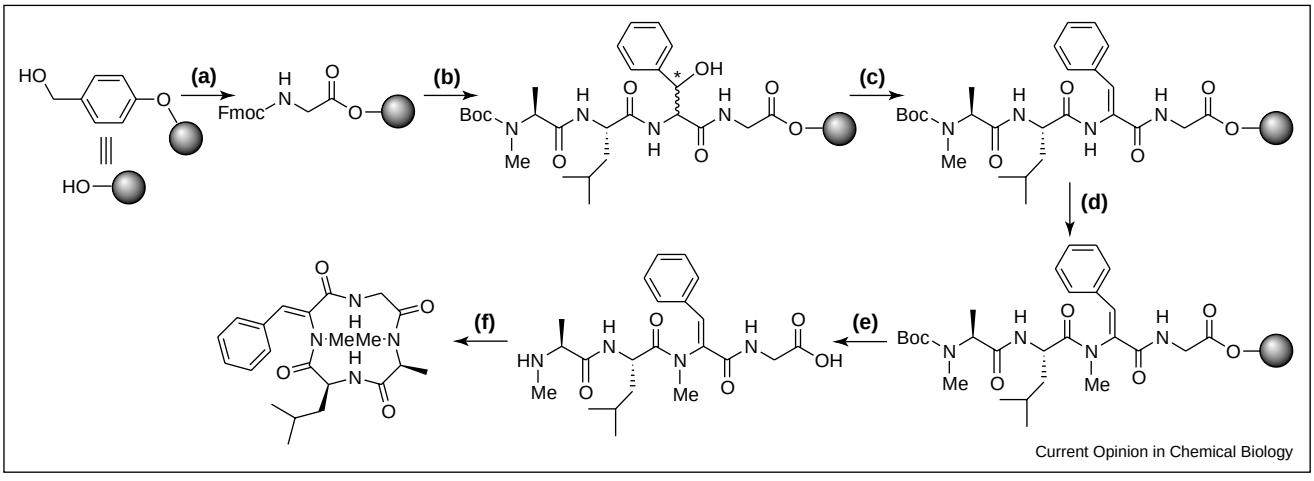


Resins for Arg side anchoring and for backbone anchoring.

Tentoxin contains a didehydro residue, which is N-alkylated along with the alanine residue. The synthesis (Figure 5) was carried out on a Wang resin by incorporation of the protected amino acid (Fmoc, although the final residue was incorporated as the Boc-protected system) using conventional coupling methods. The DPP was prepared in solid-phase by incorporating the unprotected precursor of the didehydro residue (in this case hydroxyphenylalanine), activation of the hydroxy group with EDC in the presence of CuCl and concomitant elimination. Although the linear sequence was undertaken with the diastereomeric mixture of Phe(β-OH), this method gave exclusively the *Z* isomer, which is the thermodynamically more stable product. N-methylation of the didehydrophenylalanine residue was also carried out on solid-phase using MeI as the alkylating agent and K_2CO_3 as the base in the presence of 18-crown-6 as a phase transfer reagent in DMF. These conditions led to regioselective methylation of the didehydro residue due to the greater acidity of the corresponding amide bond. Cleavage of the resin and cyclization with DIDCDI/HOBt in the presence of DIEA — to avoid trifluoroacetylation — gave the target product with excellent purity. This point of cyclization ensures a low level of DKP formation during the synthetic elongation.

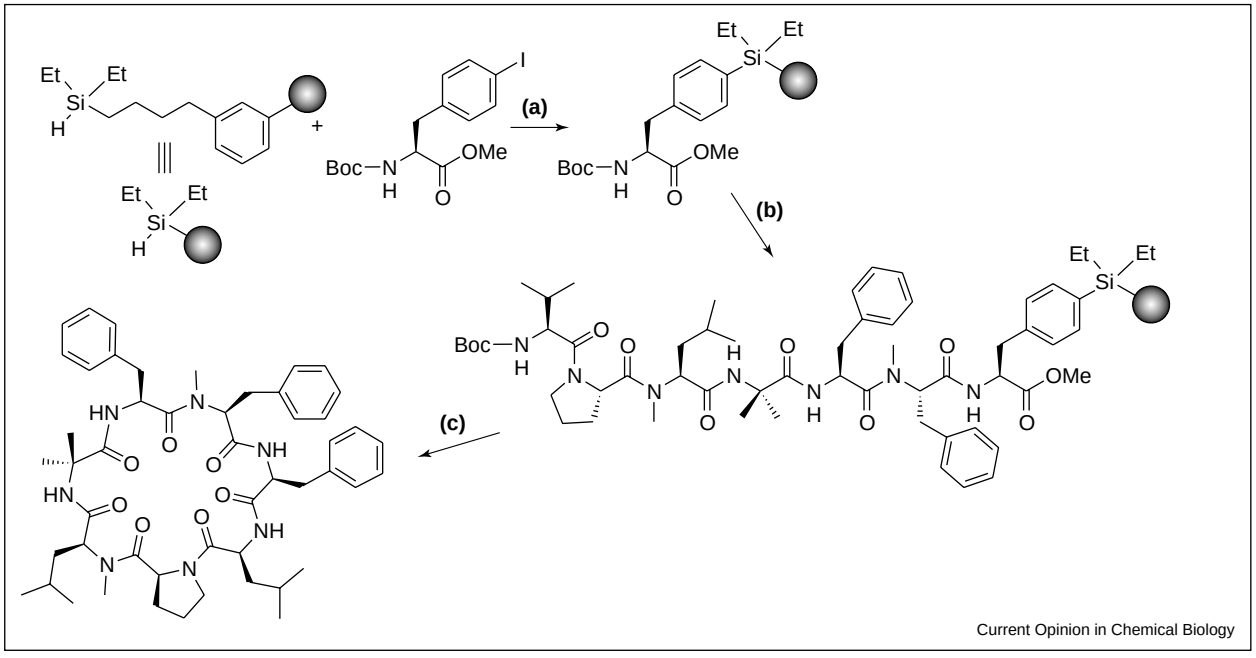
Scytalidamide A is a cyclic heptapeptide that contains several N-methyl amino acids. It can be seen from Figure 6 that a Phe residue was incorporated into the solid support through a silane linker, with the carboxylic group protected as its methyl ester. Elongation of the peptide chain was achieved with Boc-amino acids using HATU/DIEA as the coupling method. Removal of the terminal protecting groups was followed by on-resin cyclization with PyBOP/DIEA to avoid guanidine formation. Peptide cleavage was carried out with neat TFA. The same peptide was also obtained using a BAL strategy.

Figure 5



Tentoxin synthesis on a Wang resin. **(a)** Fmoc-Gly-OH, DIPCDI/DMAP, CH_2Cl_2; **(b)** SPPS: (b′) piperidine/DMF (2:8); (b″) Fmoc/Boc-aa-OH, DIPCDI/HOBt, DMF; **(c)** EDC·HCl, CuCl, CH_2Cl_2/DMF (9:1); **(d)** MeI, K_2CO_3, 18-crown-6, DMF; **(e)** TFA/H_2O (19:1); **(f)** DIPCDI/HOBt/DIEA, CH_2Cl_2/DMF (99:1).

Figure 6



Synthesis of the cyclic heptapeptide scytalidamide A. **(a)** KOAc/NMP, $Pd_2(dba)_3{\cdot}CHCl_3$, 100°C; **(b)** SPPS: (b′) TFA/H_2O (1:1); (b″) Boc-aa-OH/HATU/DIEA (5:5:15), NMP; **(c)**: (c′) LiOH, H_2O/THF; (c″) TFA/H_2O (1:1); (c‴) PyBOP/DIEA (5:15), (c⁗) neat TFA.

Medium-large size peptides synthesized by convergent strategies

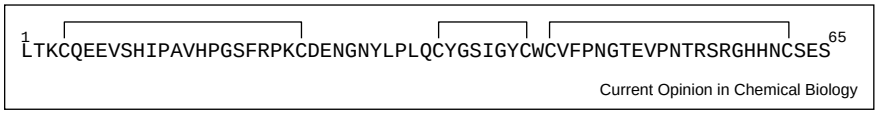
The synthetic routes to a small protein and T-20, which is the pharmaceutical peptide that is currently being synthesized on the largest scale, are discussed in this section.

The MHCII-associated p41 invariant chain fragment (P41icf) is a 66 amino acid peptide with three disulfide bridges (Figure 7) [21•].

After several unsuccessful stepwise syntheses using both Fmoc/*t*Bu and Boc/Bzl and one convergent solid-phase synthesis (synthesis of protected segments and coupling on solid-phase), the peptide was successfully synthesized by a combination of solid-phase (segment syntheses) and solution (segments assembling and disulfide formation) methods using a Boc/Bzl strategy (Figure 7). Six of the seven fragments into which P41icf was divided have a Gly residue at the C-terminal position to avoid racemization. The Pac group was chosen as a temporary carboxylic protecting group. The C-terminus was permanently protected with Bzl. The protected segments were prepared using an HMFS-based resin. The side-chain protecting groups were completely stable under the basic conditions (20% morpholine in DMF) required to detach the peptide from the resin and were removed at the end with anhydrous HF. The thiol groups of the six Cys residues were protected with the Acm group, which is stable to HF and can be removed by treatement with I_2 or Hg^{2+}. Each protected peptide was purified by recrystallization, reprecipitation or silica-gel chromatography using appropriate eluents. Fragment coupling reactions were carried out according to the scheme shown in Figure 8, using EDC as the coupling reagent in the presence of HOBt or HODhbt and DMF or NMP as solvent. The degree of purity of this peptide, around 65% by RP-HPLC, is extremely good considering its size.

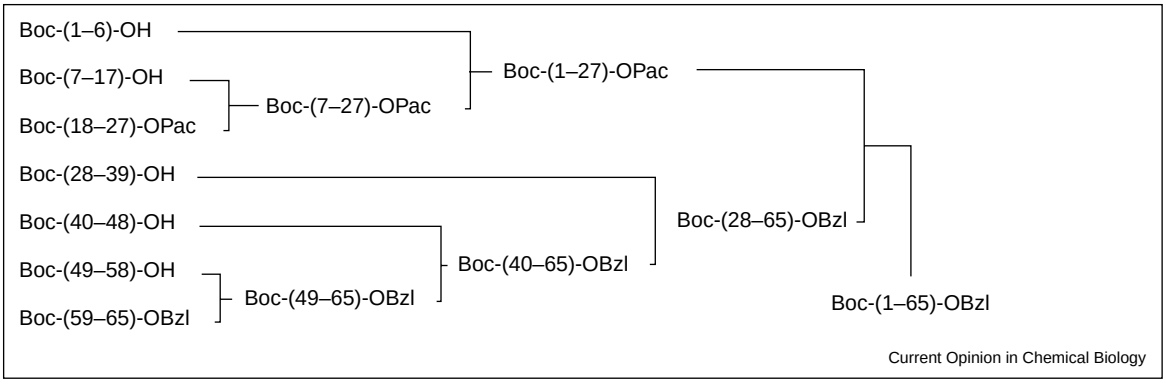
The final protected peptide was treated with HF/anisole (9:1) in the presence of HCl·H-Cys-OH·H_2O as a scavenger. The crude product was purified by RP-HPLC

Figure 7



Amino acid sequence of P41icf.

Figure 8



Coupling strategy for the synthesis of protected P41icf.

and the (6Acm)-peptide obtained in this way was treated with $Hg(OAc)_2$ in aqueous HOAc to remove the Acm groups. Hg^{2+} was removed by the addition of β-mercaptoethanol followed by gel filtration. Several different sets of conditions for the oxidative folding of the reduced peptide were investigated. The best results were obtained when the reaction was performed in 0.1 M $AcONH_4$ buffer (pH 7.8) containing 1 mM EDTA and 0.5 M Gu·HCl at a peptide concentration of 7×10^{-6} M in the presence of reduced and oxidized glutathione (GSH/GSSG) for 3 days at room temperature. Monomeric oxidized P41icf was isolated by RP-HPLC.

The 36-mer peptide T-20 [22••] (Figure 9) was divided into 3 fragments [(1–16), (17–26), (27–35)] plus the Phe that constitutes the C-terminal residue. The fragments were synthesized by an Fmoc/*t*Bu strategy on Barlos resin using HBTU-mediated couplings. The purity of the product in each case was greater than 90% by HPLC. Coupling of H-Phe-NH_2 to Fmoc-(27–35)-OH and the rest of the couplings to achieve the target sequence were carried out with HBTU/DIEA in the presence of either HOAt or 5-Cl-HOBt to minimize racemization. The purity of the crude T-20 after global side-chain deprotection with TFA in the presence of scavengers was around 75% and this was then purified by RP-HPLC. T-20 was obtained in approximately 30% yield from the loaded resin. This process is currently being used to prepare several hundred kilograms of product.

Proteins by native chemical ligation

A chemical strategy similar to that outlined above for P41icf has allowed the preparation of green fluorescent protein (GFP), a 238 residue protein that is the largest peptide/protein prepared by chemical methods [23]. However, despite this success, native chemical ligation (NCL) should be the method of choice for the preparation of large peptides and proteins [24,25•]. Chemical ligation strategies rely on the principle of chemoselective reaction. Several non-natural chemical ligations, which lead to a non-amide linkages (thioester, thioether, hydrazone, oxime and pseudoproline), have been investigated [26] but efforts have mainly been directed towards NCL processes where the native sequence is obtained.

NCL requires a C-terminal thioester residue on one peptide and an N-terminal cysteine on a second peptide. Two limitations are associated with NCL. The first is related to the preparation of the thioester peptide, a stage that has mainly been performed using Boc/Bzl chemistry due to the lability of thioesters to secondary amines used to remove the Fmoc group. This problem has been circumvented through three main approaches (Figure 10). The groups of Kent [27] and Pessi [28] independently adapted Kenner's acylsulfonamide safety-catch linker (Figure 10a). Although this approach is promising, acylation of Kenner's resin is not a straightforward reaction and alkylation of the sulfonamide prior to nucleophilic cleavage can damage some of the peptide residues. Our

Figure 9

Ac-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln^{16}-Glu-Lys-Asn-Glu-Gln-Gln-Leu-Leu-Glu-Leu^{26}-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp^{35}-Phe-NH_2

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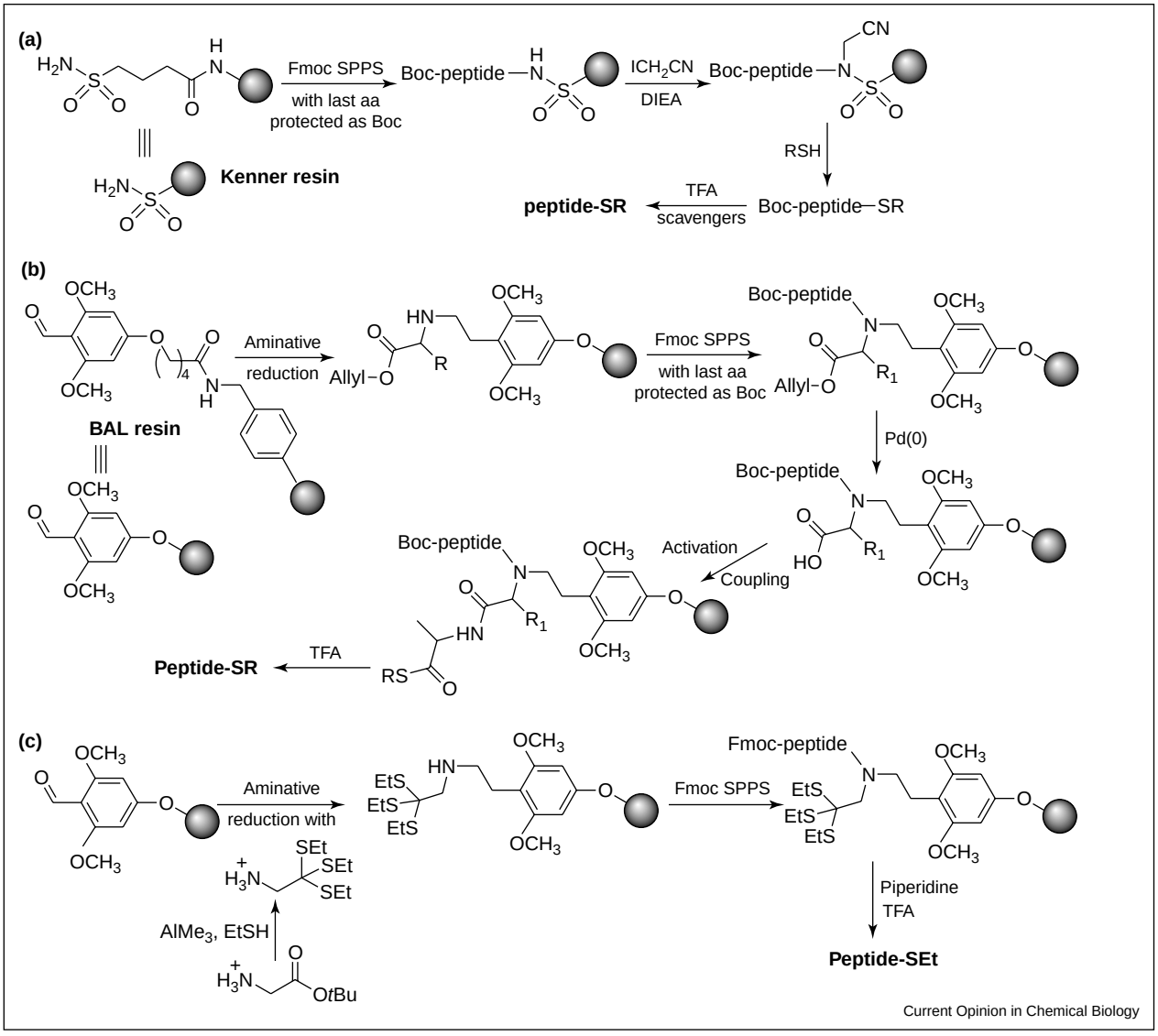
Amino acid sequence of T-20.

Figure 10

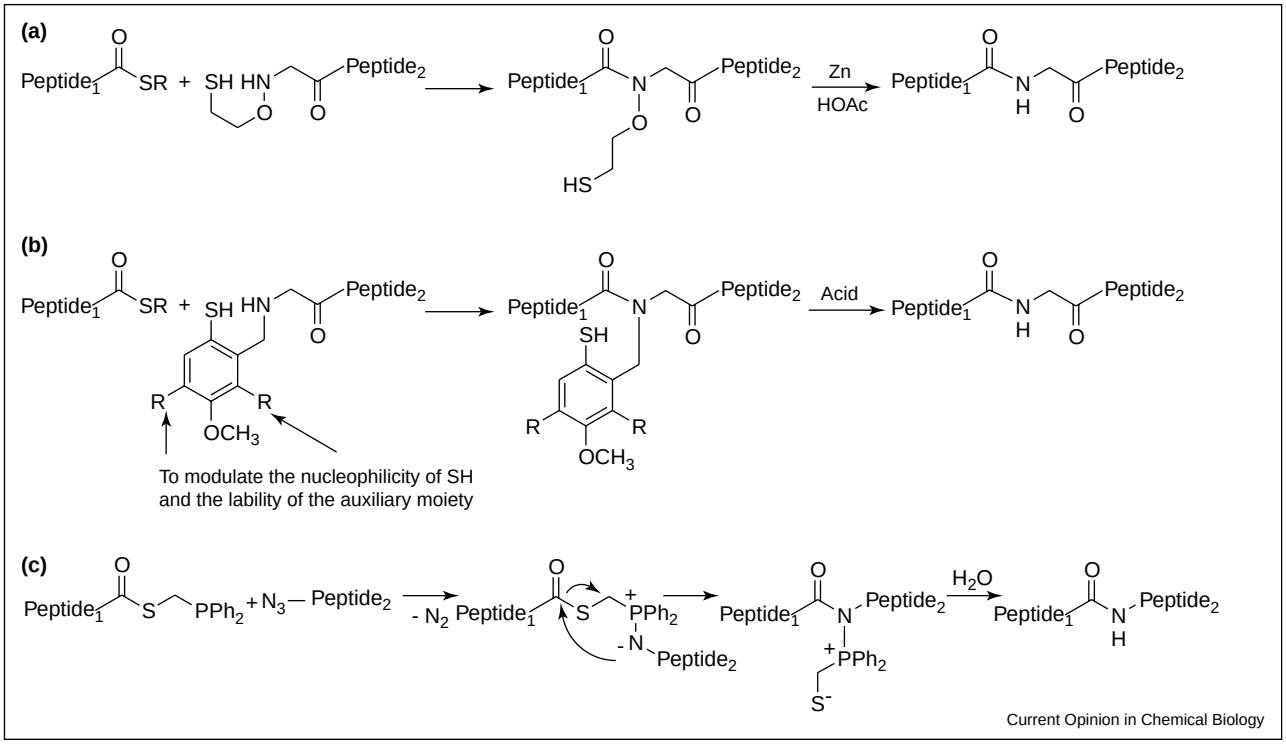


Strategies to prepare peptide thioesters using an Fmoc/*t*Bu strategy. **(a)** By Kenner's acylsulfonamide safety-catch resin. **(b)** By anchoring the penultimate residue to the BAL resin. **(c)** By anchoring an aminotrithiorthoester to BAL resin.

group has investigated two different approaches based on the BAL strategy. The first approach involves the stages shown in Figure 10b [29]. First, peptide synthesis is initiated by anchoring the penultimate residue, with its carboxyl group protected as the allyl ester, through the backbone nitrogen. This is followed by continuation with standard Fmoc/tBu protocols for peptide chain elongation in the C→N direction. Next, the allyl protecting group is selectively removed followed by solid-phase activation of the pendant carboxyl with HATU/DIEA in CH_2Cl_2 and coupling with the amino acid thioester. Finally, cleavage/deprotection releases the free peptide product into solution. Formation of DKP during the removal of the N-protecting group of the second residue and the risk of racemization prior to the introduction of the amino acid thioester are possible drawbacks of this approach. The second strategy involves anchoring an amino trithioortho ester to the BAL resin and elongation of the peptide chain [30•]. Final cleavage/deprotection cleavage renders the unprotected thioester peptide (Figure 10c). The trithioortho ester is totally stable to piperidine and avoids DKP formation.

The second limitation of NCL is related to the prerequisite of having an N-terminal cysteine residue. Several approaches have been developed to overcome this limitation. For example, homocysteine can be used and this can be methylated after ligation to render the Met-containing

Figure 11



Strategies for NCL with **(a,b)** removable acyl transfer auxiliaries and **(c)** by Staudinger chemistry.

protein [31]. Furthermore, Cys itself can be desulfurized to give an Ala residue [32]. This strategy opens the possibility of using peptides containing thio derivatives of the common amino acids, which after chemical ligation and desulfurization can give peptides with other proteinogenic amino acids [33]. Furthermore, *N*-ethanethiol (Figure 11a) [34] and *N*-2-sulfanylbenzyl auxiliaries (Figure 11b) [35–37] have been investigated. These moieties, which contain the thiol required for the ligation, are removed after the protein is formed by treatment with Zn/HOAc and under acid conditions, respectively.

A more general strategy involves Staudinger ligation between a C-terminal phosphinothioester and an N-terminal azide, which gives an amide that does not have residual atoms (Figure 11c). The initial intermediate is an iminophosphorane and this rearranges to an amidophosphonium salt, hydrolysis of which gives the amide. The use of a combination of native chemical and solid-phase Staudinger ligation enabled Raines *et al.* to prepare ribonuclease A, a protein containing 124 amino acids [38••].

A combination of NCL and SPOT synthesis has allowed the creation of large arrays of WW protein domains, an approach that constitutes a promising new tool for the identification of tailor-made specific binders [39••].

Kochendoerfer, Kent *et al.* used NCL to synthesize a homogeneous polymer-modified erythropoiesis protein. This system is a 51 kilodalton polymer consisting of a peptide chain containing 166 amino acids and two covalently attached, branched monodisperse polyethyleneglycol moieties that are negatively charged [40••].

Conclusions

The 100 year old dream of Emil Fischer about the possibility of synthesizing proteins is today becoming reality. It is now feasible that almost any peptide can be prepared by chemical methods in a laboratory. A key development in this process was the establishment by Merrifield of the solid-phase methodology. Efforts to develop new resins, coupling reagents and protecting groups continue apace and the adaptation and incorporation of biochemical processes into the chemical pathways is ongoing. Such developments will encourage us to consider peptides as natural pharmaceuticals.

Update

Recently, the first report regarding the use of room temperature ionic liquids for solution peptide synthesis have been published [41•]. The solution synthesis of hindered amino acids using 1-butyl-3-methyl imidazolium hexafluorophosphate in combination with onium salts (HATU, PyBOP), all of which are structurally similar,

has been described. Crude peptides were obtained with higher purities than those from coupling in classical solvents. This method may present advantages in the context of green chemistry.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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