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Disulfide Formation Strategies in Peptide Synthesis

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Disulfide bonds play an important role in both proteins and peptides. They cause conformational constraints and increase the stability of such molecules. In nature, disulfide bonds are very common in animal and plant peptide toxins. These disulfide-rich peptides typically bind very selectively with high affinities to their targets. Disulfide-rich peptides are of great importance as potential therapeutics. Robust, convenient, and efficient methods are needed in order to prepare disulfide-rich peptides to facilitate the drug discovery process. This microreview explores new cysteine protecting groups that replace obsolete protecting groups, reduce racemization, or facilitate regioselective disulfide formation, new disulfide formation strategies to assist in the synthesis of complex disulfide-rich peptides, and the use of selenocysteine to direct disulfide formation.

Introduction

Cysteine (Cys) is unique among the proteinogenic amino acids because of its capacity to form disulfide bonds in peptides and proteins.[1] A disulfide bond is a reversible covalent bond between the side chain thiol moieties of two Cys residues; it causes conformational constraints, resulting in more rigid molecules that stabilize the peptide or protein fold.[2] Disulfide-rich peptides offer prime examples of the attractive properties disulfide bonds can confer. For instance, peptides are highly prone to metabolic degradation through enzymatic digestion and typically have short circulatory half-lives in the range of a few minutes.[3] However, the disulfides in disulfide-rich peptides can strongly increase metabolic and thermal stability. This is illustrated by the cyclotide kalata B1, which contains three disulfide bonds. Kalata B1 is extracted from a plant by boiling in water, it is orally available, and it survives strongly proteolytic gastric juices.[4] Upon reduction of its three disulfide bonds, however, this peptide becomes readily susceptible to degradation by several proteolytic enzymes.

Cyclotides are disulfide-rich miniproteins derived from plants. They contain between 28 and 37 residues with a

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head-to-tail cyclized backbone.[5,6] The main structural feature of a cyclotide is the conserved Cys knot motif composed of three disulfide bonds; this, combined with the cyclized backbone, renders these molecules exceptionally stable. The therapeutic potential of cyclotides is vast because they show stability and a range of natural bioactivities, including antiviral and antimicrobial. They also serve as scaffolds on which to graft bioactive sequences.[7–10] The synthesis of cyclotides is readily performed by Boc SPPS (solid-phase peptide synthesis), followed by cyclization and oxidative folding in solution.[11] The cyclization of the backbone is typically achieved by intramolecular native chemical ligation (NCL).[12] In this approach, two peptide fragments can be joined through the transthioesterification of the Cys thiol and a thioester, followed by an S–N acyl shift to form an amide bond.[13–15] The NCL technique has revolutionized the chemical synthesis of proteins and complex peptides and is an important application of Cys.[16]

The conformational stability of disulfide-rich peptides can lead to highly potent and selective binding of a disulfide-rich peptide to its target.[17] Disulfide-rich venom peptides from snakes, scorpions, cone snails, and spiders illustrate the diverse pharmacology of this class of molecules.[18,19] Conotoxin peptides are derived from cone snail venom. These peptides contain several disulfide bonds and have evolved to produce a highly efficient and complex pharmacological cocktail, which the snail uses to immobilize prey with the aid of a harpoon-like radula.[20] Conotoxins act on neurological targets, such as ion channels and receptors. Given their wide diversity, selectivity, and potency, these molecules are of great interest from a therapeutic perspective.[21]

The ω-conotoxin ziconotide (Prialt), containing three disulfide bonds, was introduced as the first conotoxin therapeutic for the treatment of acute and chronic pain (Figure 1, a).[22] Linaclotide, another disulfide-rich peptide, also with three disulfide bonds, is used for the treatment of chronic idiopathic constipation and irritable bowel syndrome with constipation in adults (Figure 1, b).[23,24] Several disulfide-rich peptides are currently in clinical trials. This indicates the potential of disulfide-rich peptides as therapeutic agents.[20]

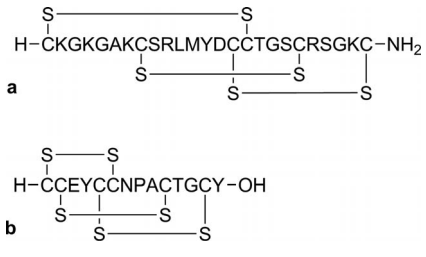


Figure 1. Disulfide connectivity of a) ziconotide, and b) linaclotide.

The importance of disulfide-containing peptides is not limited exclusively to therapeutic peptides. A wide range of applications involve the use of peptide disulfides, including bioconjugation,[25] β-sheet stabilization,[26] siRNA delivery,[27] increasing in vivo stability,[28] stabilization of peptide-based nanocarriers, and peptide backbone linkers.[29,30]

The increasing relevance of disulfide-rich peptides leads to the varied chemistry of peptide disulfide bond formation and the need for new chemistries enabling the convenient and straightforward preparation of peptides with complex disulfide connectivity.[31] Typically, three strategies can be used to synthesize disulfide-containing peptides: (I) preparation of disulfides on solid phase, (II) a mixture of disulfide formation on solid phase and subsequent disulfide formation in solution, and (III) formation of all disulfides in solution.[32] With strategy I, the disulfide is formed on a resin with medium to low loading, and the kinetic phenomenon referred to as pseudo-dilution favors intramolecular disulfide formation.[33] Disulfide-rich peptides can be prepared regioselectively by strategy I when appropriate orthogonal protection schemes are used. Strategy II can be used to form the first disulfide on solid phase, which restricts the conformational freedom, followed by formation of one or more disulfides in solution. As a result of the conformational restrictions conferred by the first disulfide, native conformations can be more easily obtained. Finally, strategy III, also called oxidative folding, involves the formation of all disulfides in solution and can work efficiently with natural peptide or protein sequences.[34] However, mixtures of peptides with distinct disulfide connectivities are frequently obtained and can thus be difficult to purify.[35]

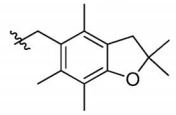
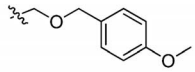
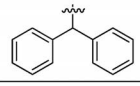
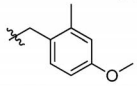
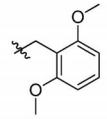
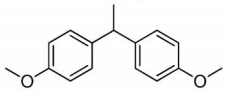
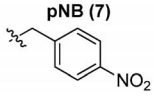
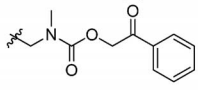
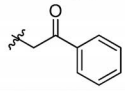
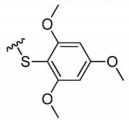
During the synthesis of Cys-containing peptides, care has to be taken to prevent or reduce side reactions at Cys.[36,37] This residue is prone to base-catalyzed racemization during coupling, so coupling under neutral conditions with use of carbodiimide reagents is recommended.[38,39] Alkylation of the Cys thiol can be a problem during cleavage of the peptide from the resin with high concentrations of TFA.[40] In order to avoid such problems, cleavage time should be minimized and appropriate scavengers should be present.

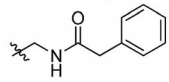
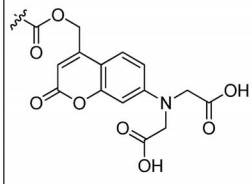
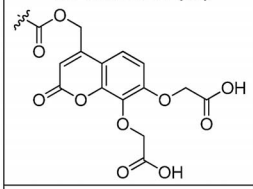
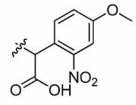
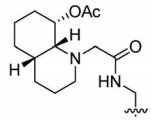
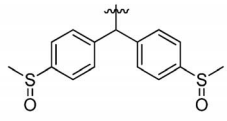
Here we examine the state of the art of peptide disulfide bond formation strategies. This review covers such strategies from 2006 until now and encompasses new methods with widely known reagents, new reagents, new approaches, and recently introduced Cys protecting groups. There are several excellent reviews of disulfide formation strategies, Cys chemistry, and protecting groups from 2006 and before.[32,41–45]

Cys Protecting Groups

This section reviews the new Cys protecting groups that have emerged as a result of the need to synthesize increasingly complex peptides (Table 1). The applicability of several known Cys protecting groups has led to increased flexibility and milder deprotection conditions. The new Cys protecting groups can introduce additional levels of orthogonality/compatibility, thus facilitating the regioselective preparation of complex peptides with rich disulfide connectivity.

Table 1. List of new and recently reevaluated Cys protecting groups.

Cys protecting group	Deprotection conditions	Stable to	Ref.
Pbfm (1) 	**acid-labile** 95% TFA/TES (or TIS) or I_2	Fmoc removal	[46]
MBom (2) 	**acid-labile** 95% TFA	Fmoc removal	[47–48]
Dpm (3) 	**acid-labile** 90–95% TFA	Fmoc removal	[48–50]
4MeO-2MeBn (4) 	**acid-labile** 90–95% TFA	Fmoc removal	[49]
2,6diMeOBn (5) 	**acid-labile** 90–95% TFA	Fmoc removal	[49]
Ddm (6) 	**acid-labile** 10% TFA	Fmoc removal	[48–49]
pNB (7) 	**reducing agent-labile** Zn/AcOH (in solution) or $SnCl_2$/HCl (on-resin)	Fmoc removal, HF, TFA	[51]
Pocam (8) 	**reducing agent-labile** Zn/AcOH	Fmoc removal	[52]
Pac (9) 	**reducing agent-labile** Zn/AcOH	Fmoc removal, TFA	[53]
***S*-Tmp (10)** 	**reducing agent-labile** thiols or trialkylphosphines	Fmoc removal	[54]

Cys protecting group	Deprotection conditions	Stable to	Ref.
Phacm (11) 	**enzyme-labile** Immobilized penicillin G acylase	Fmoc removal, TFA, HF	[55–56]
BCMACMOC (12) 	**photo-labile** light irradiation ($\lambda \geq 402$ nm)	Fmoc removal (1% DBU/DMF, piperidine causes S–N acyl shift) TFA	[57]
7,8BCMCMOC (13) 	**photo-labile** light irradiation ($\lambda \geq 325$ nm)	Fmoc removal, TFA	[57]
C4MNB (14) 	**photo-labile** light irradiation ($\lambda \geq 325$ nm)	Fmoc removal, TFA	[57]
Hqm (15) 	**hydrazine-labile** 5% hydrazine (aq), I_2 or AgOAc	Fmoc removal, TFA, TFMSA (at 0°C)	[58]
Msbh (16) 	**safety catch** NH_4I/dimethyl sulfide/TFA	Fmoc removal, TFA, HF	[59]

Acid-Labile Cys Protecting Groups

The acid-labile Cys protecting group 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-methyl (Pbfm, **1**) has been described as a replacement for Trt in Fmoc chemistry.[46] Trt-containing peptides are widely used, and the protecting group is significantly hydrophobic. To reduce issues with regard to the hydrophobicity inherent to the Trt protecting group, Pbfm was introduced. This group can be easily removed with 1% TFA in the presence of 5% triethylsilane (TES) and also with use of a high TFA concentration in the total deprotection and cleavage from resin. The successful on-resin oxidative removal of Pbfm has been performed with I_2 in various solvents with the model peptide oxytocin. Satisfactory results were obtained, thus demonstrating that this protecting group is a viable less hydrophobic alternative to Trt.

The 4-methoxybenzyloxymethyl (MBom, **2**) acid-labile Cys protecting group was introduced to suppress Cys race-

mization in Fmoc chemistry.[47] Cys is prone to racemization upon activation with phosphonium or uronium reagents, and carbodiimides are recommended as coupling reagents in order to reduce racemization.[39] However, carbodiimides are not equally efficient with respect to phosphonium and uronium reagents, and switching to another coupling system for Cys introduction is not practical, especially when using automated peptide synthesizers.[59] MBom has a similar acid-lability to Trt, and upon acidolysis it releases a molecule of highly reactive formaldehyde; this necessitates addition of the efficient formaldehyde scavenger methoxyamine hydrochloride.[60] The model peptide H-Gly-Cys-Phe-NH_2 was prepared by the uronium activation method and used to determine the levels of racemization with the protecting groups MBom, Trt, and Acm. With conventional SPPS, the observed extents of racemization were as follows: MBom (0.4%), Trt (8.0%), and Acm (4.8%). With microwave-assisted SPPS at 50 °C they were MBom (0.8%), Trt (10.9%), and Acm (8.8%), and with microwave-assisted SPPS at 80 °C they were MBom (1.3%), Trt (26.6%), and Acm (15.3%). These results demonstrate the significant level of racemization suppression achieved with MBom in comparison with standard Cys protecting groups. The MBom protecting group has applications in peptide synthesis in which Cys racemization poses a significant problem.

Diphenylmethyl (Dpm, **3**), 4-methoxy-2-methylbenzyl (4MeO-2MeBn, **4**), 2,6-dimethoxybenzyl (2,6diMeOBn, **5**), and 4,4′-dimethoxydiphenylmethyl (Ddm, **6**) have recently been introduced by our group as acid-labile Cys protecting groups.[49] We were searching for a replacement for the Mob group that is stable to low concentrations of TFA but readily removable with 95% TFA. The protecting groups Dpm, 4MeO-2MeBn, and 2,6diMeOBn were found to be appropriate for this purpose. Dpm was chosen as the most suitable, due to greatest cost-effectiveness and ease of preparation. In contrast, Ddm was readily removed with 10% TFA and could be applied as a racemization-suppressing replacement for the Trt protecting group.[48] Dpm was found to be fully compatible with the widely used acid-labile Cys protecting groups Trt and Mmt for use in Fmoc chemistry.[50] This was shown in an experiment with a peptide containing three acid-labile protecting groups: namely Dpm, Trt, and Mmt. The Dpm group was removed under the standard conditions used for total deprotection and cleavage from the resin (90–95% TFA + scavengers). Dpm was successfully used in the regioselective synthesis of the hinge fragment IgG1 and α-conotoxin ImI, with use of either Mmt or Trt as compatible protecting groups. Racemization can be an issue with Cys protecting groups, and Dpm was compared with Trt by use of the model peptide H-Gly-Cys-Phe-NH_2.[48] The peptide was prepared by uronium activation, and the degree of racemization was determined. With conventional SPPS, the extent of racemization was as follows: Dpm (1.2%) and Trt (8.0%). With microwave-assisted SPPS at 50 °C it was Dpm (3.0%) and Trt (10.9%), and with microwave-assisted SPPS at 80 °C it was Dpm (4.5%) and Trt (26.6%). These results show that Dpm can significantly reduce racemization relative to Trt and can be used as an alternative to Trt when Cys racemization is an issue. The commercially available Dpm group [Fmoc-Cys(Dpm)-OH] has applicability as an acid-labile protecting group that is fully compatible with Trt and Mmt. This is especially relevant given that Trt and Mmt are not fully compatible as acid-labile protecting groups and need diligent optimization to prevent overlap of the deprotection conditions. In addition, Dpm can be used to replace Trt because it reduces Cys racemization during coupling.

Because Cys racemization is inevitable during coupling, it is important to determine the extents of racemization produced with new Cys protecting groups. In a recent paper by Hibino et al., the acid-labile Cys protecting groups MBom (**2**), Dpm (**3**), Ddm (**6**), and Trt were evaluated for their propensity to reduce Cys racemization.[48] The highest level of racemization was observed during coupling with uronium activation in the presence of base with Trt-protected Cys (8%, as described above), whereas the protecting groups MBom (**2**), Dpm (**3**), and Ddm (**6**) caused 0.4, 1.2, and 0.8% racemization, respectively. For coupling under the recommended conditions for Cys, namely carbodiimide coupling without base, the levels of racemization are expected to be less. Taking this and the cost-effectiveness into account, we recommend the use of the commercially available Dpm or Ddm as the Cys protecting groups of choice with acceptable low levels of Cys racemization.

Reducing-Agent-Labile Cys Protecting Groups

The rarely used *p*-nitrobenzyl (pNB, **7**) Cys protecting group has recently been reevaluated for use in Boc chemistry.[51,61] In contrast with Acm, pNB is completely stable to HF, thus making it a suitable alternative Cys protecting group for Boc-based peptide synthesis. The protecting group is removed in a two-stage process, the first of which is Zn/AcOH reduction in solution or $SnCl_2$/HCl reduction on resin to give the *p*-aminobenzyl-protected (pAB-protected) Cys. After the reduction, the pAB-protected peptide is oxidized with I_2 to give the disulfide. The stability of pNB to I_2 was tested, and no reaction was observed, thus indicating orthogonality to Acm. The pNB protecting group can be used in disulfide formation in conjunction with Boc chemistry and also in regioselective synthesis. However, its compatibility with other Cys protecting groups requires further research.

N-Methyl-phenacyloxycarbamidomethyl (Pocam, **8**) was introduced as a new reducing-agent-labile Cys protecting group for Fmoc chemistry.[52] The group was developed as a general Cys protecting group for use in conjunction with the condensation reactions of the thioester method.[62] Pocam is not acid-stable, and 4 h of TFA treatment at 4 °C was required to achieve resin cleavage without significant Pocam removal. The protecting group is readily removed with Zn/AcOH in solution and is compatible with Acm. Orthogonality to Acm was demonstrated in a regioselective synthesis of SI α-conotoxin. Pocam is a Cys protecting group with limited applicability because of its instability to

acid. Also, it is obsolete because of the Pocam replacement Pac, which was subsequently introduced by the same group.

Phenacyl (Pac, **9**) was introduced as a reducing-agent-labile Cys protecting group for general Fmoc SPPS and use in the condensation reaction of the thioester method.[53] The protecting group is stable to general Fmoc chemistry reaction conditions but shows some instability to strong acids (1 M TFMSA/TFA). Pac can be readily removed with Zn/AcOH in solution, and these conditions are compatible with Acm and Mob. There is partial lability to Acm and Mob deprotection conditions, so Pac must be removed before Acm or Mob. During peptide elongation, low yields were observed on use of DCC/HOBt. This result was partially attributed to imine formation; consequently, mildly basic coupling conditions with use of DIPEA are recommended. The protecting group has been successfully applied to several peptides, including a regioselective synthesis of tachyplesin – a 17-residue peptide with two disulfide bonds – using Pac and Acm. Pac can be used as a compatible protecting group with Acm and Mob. However, some limitations need to be considered, such as the use of basic coupling conditions, which can promote Cys racemization.

The thiol-labile Cys protecting group trimethoxyphenylthio (*S*-Tmp, **10**) was introduced by our group as a replacement for the *tert*-butylthio (*St*Bu) group for use in Fmoc chemistry.[54] The reducing-agent-labile *St*Bu is orthogonal to all other Cys protecting groups.[63] Unfortunately, it is very difficult, and sometimes impossible, to remove *St*Bu with reducing agents.[64] *S*-Tmp can be easily removed in 5 min by use of a deprotection mixture containing dithiothreitol (DTT, 0.1 M NMM in 5% DTT/DMF). We used the *S*-Tmp protecting group to prepare several model peptides and oxytocin, and also for the regioselective synthesis of a two-disulfide-containing SI α-conotoxin and the preparation of the 18-residue T22 peptide, containing two disulfide bonds. *S*-Tmp is commercially available and, given its ease of removal, it can be used in place of *St*Bu.

An Enzyme-Labile Cys Protecting Group

Phenylacetamidomethyl (Phacm, **11**) was pioneered by our group as an enzyme-labile Cys protecting group for both Fmoc and Boc SPPS.[65] Recently, the applicability of Phacm has been broadened by exploration of the versatility of immobilized enzyme biocatalysis for the removal of this protecting group.[55] The stability of Phacm to SPPS conditions and its lability profile are similar to those of Acm. In addition, Phacm can be removed by the enzyme penicillin G acylase (PGA) from *E. coli*, which offers mild deprotection conditions. Phacm can be removed with PGA immobilized on aminoacrylic resin. Immobilization of the enzyme allows capture of the supported PGA by convenient filtration after deprotection, and the resin can be easily recycled. The biocatalytic deprotection conditions are mild, work under a broad range of conditions, prevent scrambling of disulfide bonds in disulfide-rich peptides, and are orthogonal to acid-labile protecting groups such as Trt. Disulfide formation can be initiated by addition of 10% DMSO to the deprotection mixture. Phacm removal with immobilized PGA is limited because it requires aqueous conditions, which can lead to solubility issues with certain peptide sequences. Phacm was used in the regioselective synthesis of the two-disulfide-containing peptides T22 and RGD-4C. A more recent application of this Cys protecting group was in the synthesis of the marine natural product thiocoraline.[56] The commercially available Cys protecting group Phacm is an attractive alternative to Acm that does not require harsh deprotection conditions.

Photolabile Cys Protecting Groups

The related protecting groups [7-bis(carboxymethyl)-amino-coumarin-4-yl]methoxycarbonyl (BCMACMOC, **12**), [7,8-bis(carboxymethoxy)coumarin-4-yl]methoxycarbonyl (7,8BCMCMOC, **13**), and α-carboxy-4-methoxy-2-nitrobenzyl (C4MNB, **14**) have recently been introduced as new photolabile Cys protecting groups.[57] They were used in an innovative orthogonal scheme for peptide chemistry that employs chromatic orthogonality. Chromatic orthogonality is achieved when selective deprotection is achieved by means of light irradiation with different wavelengths. Distinct wavelengths for photolysis of the different protecting groups were observed. For the photolysis of BCMACMOC, a wavelength of 402 nm was used, whereas for 7,8BCMCMOC and C4MNB irradiation at 325 nm initiated deprotection. Wavelength-selective deprotection was demonstrated with two model peptides in which either BCMACMOC and 7,8BCMCMOC or C4MNB and BCMACMOC were used as chromatic orthogonal pairs. Efficient selective deprotection was observed in both cases, thus demonstrating the potential of chromatic orthogonal schemes in peptide chemistry. However, coumarin-based protecting groups have a number of significant disadvantages, such as incompatibility with piperidine, a compound used for Fmoc removal. Piperidine causes S-to-N acyl shifts in both BCMACMOC and 7,8BCMCMOC and requires 1% DBU/DMF for Fmoc removal. In addition, the N terminus of the peptide requires acetylation prior to deprotection. These photolabile protecting groups have served to demonstrate an innovative orthogonal protection scheme that can be used in peptide chemistry. However, due to their drawbacks, they cannot be used in standard SPPS and thus require alternative protocols.

A Hydrazine-Labile Cys Protecting Group

Hqm (**15**), a hydroxyquinoline-based Acm derivative, is a hydrazine-labile Cys protecting group for use in Fmoc and Boc SPPS.[58] The Hqm protecting group is compatible with Acm, because it can be removed with 5% aqueous hydrazine at pH 8.5, to which Acm is stable (although at pH 8.5 disulfide isomerization can occur and should be monitored for). This was illustrated in the regioselective synthesis of human neutrophil defensin hNP2 – a 29-resi-

due peptide with three disulfide bonds – with use of the protecting groups Trt, Hqm, and Acm. It should be noted that Acm deprotection reagents I_2 and AgOAc remove Hqm in about 30 min, so Hqm must be removed before Acm. Hqm is a versatile Cys protecting group that brings a new layer of orthogonality to disulfide formation strategies through the use of aqueous hydrazine as a mild deprotection reagent.

A Safetly-Catch Cys Protecting Group

The 4,4′-bis(dimethylsulfinyl)benzhydryl (Msbh, **16**) Cys protecting group was developed according to the safety-catch principle.[59] A safety-catch protecting group is stable under certain conditions but labile to these conditions after chemical modification of the protecting group.[66] In the case of Msbh, the protecting group is highly stabile to acids such as TFA and HF. However, once the electron-withdrawing sulfoxide groups are reduced, the resulting sulfides become electron-donating, which leads to high acid lability of the protecting group. The NH_4I/dimethylsulfide/TFA mixture was used to afford deprotection and concomitant disulfide formation. A drawback of Msbh removal is its incompatibility with the sensitive amino acid Trp. Msbh was found to be suitable for both Boc and Fmoc chemistry, which was demonstrated in the synthesis of oxytocin. Subsequently the four-disulfide-containing peptide hepcidin was prepared by an orthogonal strategy. Full compatibility of Msbh with the deprotection conditions for the Cys protecting groups Trt, Acm, Meb, and Mob was observed. The safety-catch Cys protecting group Msbh was found to be compatible to both Boc and Fmoc chemistries and therefore is broadly applicable in both strategies for the regioselective synthesis of complex disulfide-rich peptides.

New Disulfide Formation Strategies

The selenoxide reagent *trans*-3,4-dihydroxyselenolane oxide (DHS) was developed as a water-soluble disulfide formation reagent (Figure 2).[67]

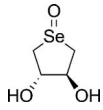
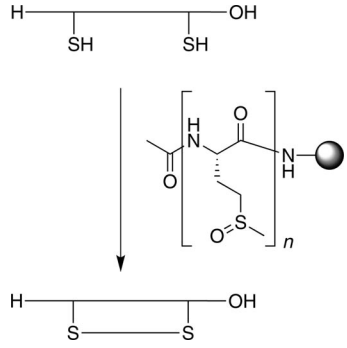


Figure 2. Structure of DHS.

DHS is a strong and selective Cys oxidant that can be used to form disulfide bonds rapidly in proteins or peptides. The proposed oxidation mechanism follows a two-step process resulting in a disulfide bond and reduced DHS. DHS was used to study the folding pathway of bovine pancreatic ribonuclease A and described as a useful reagent for the determination of oxidative folding pathways in proteins.[68] Recently, DHS was successfully used for the oxidative folding of a three-disulfide-containing recombinant hirudin analogue (CX-397) and the four-disulfide-containing hen egg white lysozyme (HEL).[69,70] The oxidative folding was highly efficient and proceeded rapidly under aqueous conditions at neutral pH at room temperature. DHS is a strong stoichiometric oxidant for the rapid formation of disulfide bonds, and it has found applications in the study of oxidative folding in disulfide-rich substrates.

Supported methionine sulfoxide has been reported as a new immobilized disulfide-forming reagent for Cys oxidation in solution or parallel peptide synthesis in solution.[71] The immobilized reagent can be readily prepared from the resin aminopoly(ethylene glycol)polyacrylamide (PEGA) either through consecutive Met couplings or by oligomerization through the use of Met-derived *N*-carboxyanhydrides and subsequent oxidation to Met sulfoxide with hydrogen peroxide (Scheme 1).

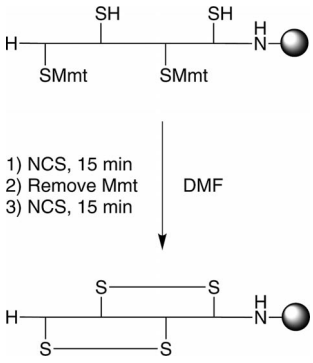


Scheme 1. Disulfide formation mediated by supported Met sulfoxide.

Disulfide formation with supported Met sulfoxide proceeds in an analogous manner to DMSO oxidation. DMSO oxidation has one major drawback: namely the difficulty of removal of excess DMSO from the oxidized peptide. Supported Met sulfoxide does not have this disadvantage, because the resin can be conveniently filtered off upon completion of disulfide formation. Several peptides were oxidized with immobilized Met sulfoxide, and efficient oxidations were reported with resins prepared both by the *N*-carboxyanhydride method and with consecutive Met couplings. The efficiency of the supported Met sulfoxide was increased by the presence of longer Met sulfoxide chains, which increased the number of Met sulfoxide groups per gram of resin. Disulfide formation with immobilized Met sulfoxide was typically complete between 4 and 48 h with use of 5 equiv. of resin in an aqueous buffer. Supported Met sulfoxide has application as a disulfide-forming reagent that can be easily removed in general peptide synthesis and also in parallel peptide synthesis.

N-Chlorosuccinimide (NCS) has recently been introduced by our group as a versatile and efficient disulfide-forming reagent for Fmoc SPPS.[72] Disulfide formation with NCS is efficient both on the solid phase and in aqueous solution.[73] The formation of disulfides was complete within 15 min, and the oxidized peptide was obtained in high purity. For on-resin disulfide formation, a solution of NCS (2 equiv.) in DMF is added to the resin and the mixture is shaken for 15 min. The excess reagent is easily removed by washing. NCS was found to be compatible with

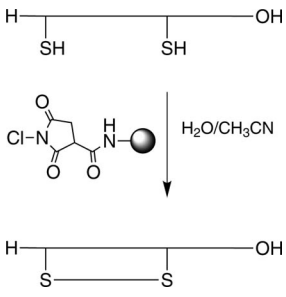
the oxidation-prone Trp, and Met could be used by lowering the excess of NCS to 1.05 equiv. Additionally, the acid-labile Cys protecting groups Trt and Mmt were also found to be compatible. Several single-disulfide-containing model peptides were efficiently oxidized with NCS. Subsequently, a regioselective synthesis of SI α-conotoxin including two consecutive on-resin NCS oxidations was performed (Scheme 2).



Scheme 2. Regioselective on-resin NCS oxidation.

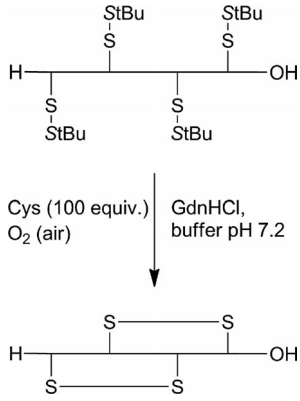
After on-resin NCS oxidation was established, the method was broadened in scope to include disulfide formation in aqueous solution. Excellent results were obtained with NCS under aqueous conditions, with flexibility in the H_2O/CH_3CN ratio. Typical conditions for disulfide formation were the addition of an NCS solution (2 equiv.) to the peptide under aqueous conditions, and disulfide formation was complete within 15 min. Thus, in view of these results, NCS is currently one of the most versatile peptide disulfide-forming reagents available.

We continued with the development of NCS as a disulfide-forming reagent to broaden its applicability in combinatorial libraries.[74] NCS was immobilized on the versatile poly(ethylene glycol)-based ChemMatrix resin. The immobilized reagent can be used in either organic or aqueous media and was found to form peptide disulfide bonds efficiently (Scheme 3). The immobilized reagent was readily removed by filtration on completion of the reaction, thus making it applicable for the preparation of combinatorial libraries.



Scheme 3. Disulfide formation with immobilized NCS.

A useful new method for the oxidative folding of synthetic polypeptides was reported.[75] The method involves the use of *St*Bu-protected peptides in the presence of a large excess of Cys and chaotropic salts under buffered conditions (Scheme 4).

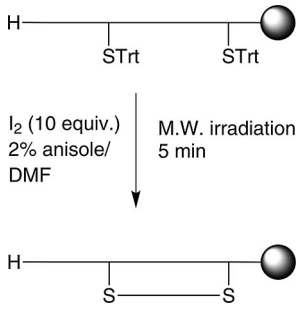


Scheme 4. Oxidative folding with *St*Bu-protected peptide.

The formation of the folded disulfide-containing peptide is governed by a thiolate/disulfide exchange and leads to the thermodynamically favored product. To test the conditions, hu-TARC, a well-studied model peptide containing 71 residues and two disulfide bonds, was chosen. The peptide was oxidized by three methods: standard oxidative folding and oxidative folding either of the fully *S*-protected peptide (4× *St*Bu) or of the partially *S*-protected peptide (2× *St*Bu). Within 24 h, 77% conversion to the native disulfide was achieved with standard oxidative folding, with fully and partially *S*-*t*Bu-protected peptides showing equal or better performance. These strategies were successfully applied to the synthesis of a chemokine (hu-CCL1 24–96/I-309) and of large fragments of *Plasmodium falciparum*, and *P. berghei* circumsporozoite malaria protein. Oxidative folding with *S*-*t*Bu-protected synthetic peptides offers several advantages, such as the reduction of dimerization, as observed with the synthesis of the 102-residue fragment *Pf* CSP-(282–383), no premature oxidation of fully S-deprotected peptides, and fewer difficulties in the isolation of Cys-rich peptides. However, the *S*-*t*Bu protecting group is relatively stable and can be difficult to remove when sterically hindered by the sequence or fold.[64]

Microwave-assisted I_2 oxidation was reported as an efficient method for the formation of peptide disulfides.[76] In this simple procedure, a supported peptide with Trt-protected Cys is subjected to microwave-assisted oxidative disulfide formation. This method was successfully applied to the on-resin disulfide formation in the vasoactive cyclic peptide urotensin-II (Scheme 5).

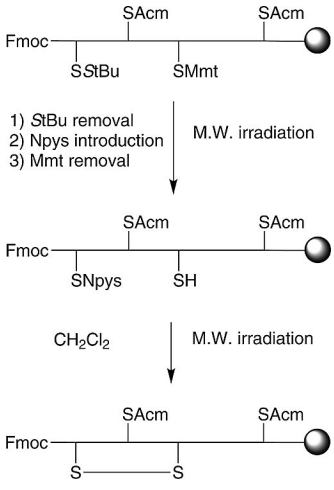
In the microwave-assisted disulfide formation of urotensin-II, both the purity and the yield were superior to those obtained in standard on-resin I_2 oxidation without microwave irradiation. However, microwave-assisted peptide synthesis with Cys-containing peptides can be hindered by increased Cys racemization. In this regard, alternative protocols are recommended if this becomes an issue.[48,77] Quan-



Scheme 5. Microwave-assisted I_2 oxidation.

titative disulfide formation was achieved in 5 min by this method. This observation demonstrates the applicability of this approach for the formation of disulfide-containing peptides. It seems to be limited to the formation of single disulfide.

Highly efficient microwave-assisted on-resin disulfide formation was recently reported. In this case it used a displacement method to form the first intramolecular disulfide in the regioselective preparation of a two-disulfide-containing peptide.[78] The displacement method requires the reaction between a free thiol and 2,2′-dithiobis(5-nitropyridine) (DTNP) to form an activated 5-nitropyridinesulfenyl (Npys) Cys. Subsequently, a highly acid-labile protecting group (Mmt) was removed, and the resin was heated by microwave irradiation to form an intramolecular disulfide (Scheme 6).

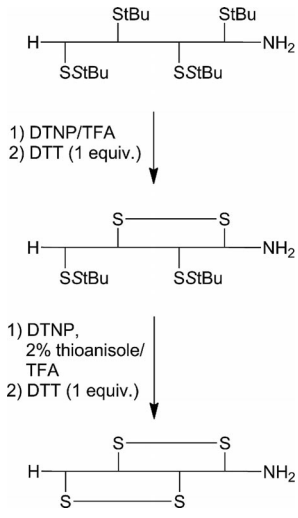


Scheme 6. Microwave-assisted displacement method for disulfide formation.

This method was successfully used in the synthesis of the two-disulfide-containing α-conotoxin MII. The best results were obtained with the preparation of the first disulfide on resin and of the second disulfide in solution. The main advantages of this method are the high purity of the crude peptide and a significant reduction in the reaction time in relation to its displacement on resin at room temperature. The time required for completion of disulfide formation was dependent on whether the short or long disulfide loop was prepared first. The formation of the short loop was straightforward, taking 20 min at room temperature or 2× 5 min with microwave heating. Conversely, the long loop required 3 days at room temperature or 6× 5 min with microwave heating. The significant reduction in time required for disulfide formation illustrates the power of microwave heating with use of a displacement method. This method has application for the regioselective preparation of disulfide-rich peptides.

The formation of complex disulfide connectivities in the regioselective preparation of disulfide-rich peptides hinges on the compatibility of Cys protecting group pairs.[32] New methods for Cys protecting group removal can increase the utility of the protecting groups. A useful new method for the removal of several commercially available Cys protecting groups by use of DTNP was recently described.[79] The deprotections were performed in TFA with or without the presence of thioanisole on the bis-protected model peptide oxytocin. The ease of deprotection in this system for the tested groups was Mob > *t*Bu > S*t*Bu > Acm. The *St*Bu protecting group was notable in this case because thioanisole was needed to afford deprotection. Without thioanisole, this group was stable to DTNP. The bis-Npys-protected peptide was observed upon removal of the Cys protecting groups with DTNP. A useful finding was that the addition of DTT (1 equiv.) led to nearly instantaneous quantitative disulfide formation.

The applicability of the DTNP deprotection method was demonstrated in the regioselective synthesis of apamin. The choice of protecting groups was *t*Bu and *St*Bu, and the first disulfide formation was initiated with DTNP/TFA to remove *t*Bu in the absence of thioanisole. After cyclization, S*t*Bu was removed by introducing thioanisole to initiate *St*Bu removal (Scheme 7).

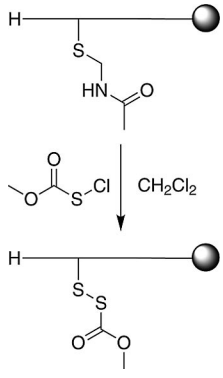


Scheme 7. Regioselective DTNP-mediated deprotection and oxidation of apamin.

DTNP-mediated deprotection of several Cys protecting groups offers a mild alternative to the commonly used harsh or toxic reagents and has applicability in the synthesis of disulfide-containing peptides. A limitation of this ap-

proach is that in some cases the removal of both protecting groups in a bis-protected peptide can be slow, thus requiring an elevated temperature and an increase in DTNP concentration.

A new on-resin Cys protecting group transformation was recently reported for the Acm protecting group.[80] Acm is widely used for direct I_2 oxidative disulfide formation. In addition, Acm can be removed by use of toxic heavy metal mercuric(II) salts. Acm can be converted into *S*-methoxycarbonylsulfenyl (Scm) in solution and can be reduced with DTT to the free thiol; this mild method circumvents the need for toxic heavy metals.[81] The Scm group can also be used to form mixed disulfides by the addition of a free-thiol-containing peptide or small molecule. Solution-based Acm to Scm conversions are often difficult, due to the differences in solubility of Acm- and Scm-containing peptides.[80] Such conditions require a large excess of Scm-Cl, which can sulfenylate both Trp and Tyr. To avoid these complications, an on-resin transformation of Acm to Scm was developed. In this, only a small excess of Scm-Cl was needed, so there was no reaction with side-chain-protected Trp and Tyr (Scheme 8).



Scheme 8. On-resin Acm to Scm conversion.

This transformation has some limitations, such as the possible reaction of N termini with Scm-Cl to form acid-labile sulfenamides. It was found that reactions with immobilized Cys(Scm)-containing peptides can be slow due to steric hindrance, and cleavage from the resin prior to Cys(Scm) reactions is recommended.

The widely used Acm protecting group is most often used for treatment with I_2 to afford oxidative deprotection and concomitant peptide disulfide formation.[82] During the removal of Acm, an excess of I_2 is used, and this needs to be rapidly quenched or absorbed in order to reduce side reactions after disulfide formation. A convenient one-step ether precipitation was recently introduced for simultaneous Acm removal, I_2 quenching, and peptide isolation. The peptide is oxidized in aqueous acetic acid (90–95%), and ice cold ether (9 volume equiv.) is added upon completion of the reaction. The mixture is cooled on dry ice for several minutes, centrifuged, and decanted to afford the crude peptide pellet. This method was successfully used on the nonapeptide vasopressin and human insulin-like peptide 3. Recovery rates of peptides obtained by this method were comparable to those achieved with the conventional method, in which ascorbic acid is used as the quenching reagent. However, ether precipitation has the advantage that it requires fewer steps, is easy to apply, and has minimal side reactions.

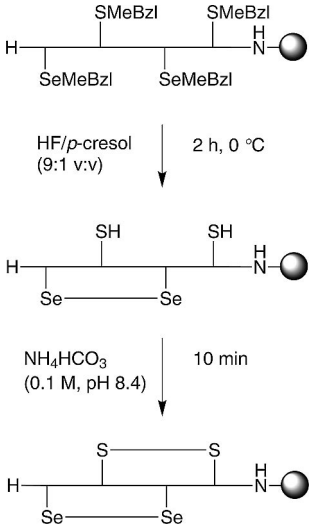
For large-scale I_2 oxidation of peptides, a new method that uses a commercial anion exchange resin to quench the reaction was developed.[83] Quenching of I_2 after the completion of disulfide formation is required in order to reduce side reactions associated with I_2 oxidation.[84] The commercially available quaternary-ammonium-based anion-exchange resin Indion 830-S was reported to be an efficient I_2 quencher for use in large-scale disulfide formation. Standard I_2 quenching with ascorbic acid gave poorer results than quenching with the anion-exchange resin. The quenching method is simple. After disulfide formation, the resin is added and stirred for 30 min, followed by filtration of the resin. This method has applications mainly in the large-scale preparation of disulfide-containing peptides.

Selenocysteine and Disulfide Formation

The natural amino acid Sec, also referred to as the 21st proteinogenic amino acid, has gained popularity in peptide chemistry in recent years because of its close relation to Cys and its capacity to form diselenide bonds in an analogous fashion to disulfide bonds.[85] A comprehensive description of selenium chemistry and its application in peptide chemistry is outside the scope of this review. However, due to the use of Sec in facilitating the formation of disulfide bonds, we highlight a few notable recent examples.

The chemistry of selenium is similar to that of sulfur but with some clear distinctions such as the stronger acidity of the selenol group and a significantly lower redox potential of the diselenide bond relative to the disulfide bond.[86] The lower redox potential leads to the strongly favored formation of a diselenide instead of a mixed selenosulfide bond. This preferential diselenide formation has been exploited to direct disulfide formation in disulfide-rich peptides.[44]

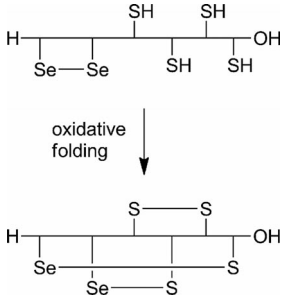
An example of preferential diselenide formation was illustrated by the on-resin selenium-directed synthesis of conotoxin derivatives.[87] The linear sequences were synthesized by BOC SPPS with use of an HF-resistant SCAL linker with methylbenzyl-protected (MeBzl-protected) Cys and Sec. Upon HF removal of the MeBzl protecting groups, the diselenide was spontaneously and quantitatively formed (Scheme 9). The subsequent oxidation of the Cys pair was rapid (<10 min at pH 8), thus demonstrating the utility of diselenide formation and direction of disulfide formation. An additional advantage of diselenide-directed disulfide formation is the preference for formation of the highly favored diselenide, thus making the determination of disulfide connectivity significantly less challenging. A useful property of Sec is that ^{77}Se can be used in NMR experiments to determine disulfide connectivity.[88] This is a clear advantage because determining disulfide connectivity can be difficult in certain peptides.



Scheme 9. On-resin Sec-directed disulfide formation.

Intramolecular redox catalysis has recently emerged as an attractive and powerful method for greatly improving the times needed to reach the native folds in disulfide-rich peptides and proteins. This method requires Sec as an intramolecular redox catalyst, through the replacement of a pair of Cys residues with Sec, to achieve the native-like fold by guiding the folding pathway to avoid kinetic traps.[89] Two very recent papers have demonstrated Sec redox catalysis in disulfide-rich peptides. The potential of this method in peptide chemistry has been briefly reviewed and highlighted by Craik.[90]

Selenium-based redox catalysis in peptide chemistry was demonstrated in the higher efficiency of selenoglutathione (GSeSeG) relative to glutathione (GSSG) as a redox auxiliary in the oxidative folding of bovine pancreatic trypsin inhibitor (BPTI).[91] This work led to the use of Sec as an intramolecular redox catalyst, which was applied to BPTI by the same group.[92] The most remarkable example used a non-native diselenide connection to modulate the folding while avoiding kinetic traps; finally a native connectivity was obtained with two less-favored Sec bonds (Scheme 10).



Scheme 10. Oxidative folding of BPTI with the assistance of an intramolecular diselenide.

A second example of this type of method was demonstrated by replacing a pair of Cys residues with Sec to guide disulfide formation in a conotoxin.[93] Native diselenide connectivity was used to enhance the folding speed over 130-fold in relation to all-Cys-containing conotoxins, without the need for additional reagents.

The increasing use of Sec in peptide chemistry and especially in combination with disulfide-rich peptides holds promise. The greater stability and reactivity of selenium makes it a useful tool in the peptide chemist's repertoire. Diselenide-containing peptides might offer therapeutic applications; however, it is yet to be determined whether diselenide bonds are biocompatible in vivo.

Conclusions

Disulfide-rich peptides are becoming increasingly relevant as potential therapeutics and molecular probes. Constant development and innovation in Cys chemistry are crucial to speed up the synthesis and to increase the yields of complex disulfide-containing peptides. Currently, the synthesis of disulfide-rich peptides is time-consuming, and new methods and reagents to accelerate this process should greatly benefit the preparation of peptides for drug research.

Natural combinatorial libraries show a remarkable hypervariability, illustrated by the great number of animal toxins. Such toxins provide us with millions of disulfide-rich toxin peptides. Unfortunately, the typical volume of venom is minute, and performing biological assays with the purified natural peptides is impractical and is highly time-consuming. Therefore, it is necessary to synthesize such compounds. Easy access to the synthetic preparation of such hypervariable sequences with control over disulfide connectivity would allow large-scale combinatorial library generation with which to tap into the enormous therapeutic potential of disulfide-rich peptides.

Acknowledgments

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