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Protocols for the Fmoc SPPS of cysteine containing peptides

Introduction

Peptides containing Cys can exist in either reduced (sulfhydryl) or oxidized inter/intra chain disulfide bonded forms. The synthesis of peptides containing Cys, therefore, presents special challenges to the peptide chemist. Careful planning of the synthetic strategy is essential, if the peptide possessing the correct structure is to be obtained in good yield. Choosing the most appropriate sulfhydryl protecting group is of paramount importance as a “bad” choice can lead to insurmountable difficulties during synthesis or subsequent disulfide bond formation.

It is important to remember that the procedures described here are only a starting point and cannot be guaranteed to work in every case. Problems are sequence dependent and considerable optimization of reaction conditions is often required. If difficulties do arise, our technical service will be glad to assist you whenever possible. For discussions on the management of cysteine-containing peptide, together with detailed practical protocols, see [1].

Cysteinyl protection

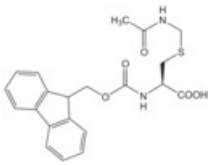
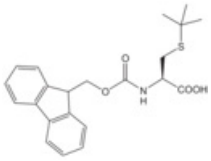
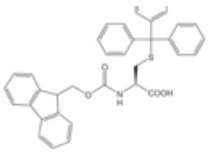
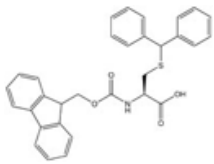
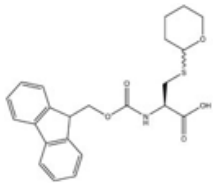
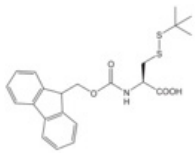
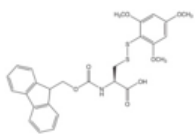
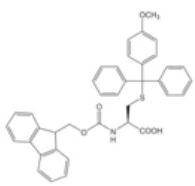
A wide variety of cysteinyl protecting groups are available for use in Fmoc SPPS. The choice depends on the nature of the desired peptide and synthetic strategic. A summary of thiol protecting groups commonly used in Fmoc SPPS is given in Table 1.

For routine synthesis of cysteinyl peptide containing free thiol groups, the trityl group is particularly recommended, as it is labile to TFA and is therefore removed during the normal cleavage procedure. The peptide is initially obtained in the reduced monomeric form but, if required, can be readily converted to a dimeric or cyclic disulfide bonded form by oxidation.

For resin selective modification of thiol groups or on-resin disulfide formation, STmp and Mmt are the most useful as they can be removed under conditions orthogonal to standard side-chain protecting groups employed in Fmoc SPPS.

The selection of protecting groups for selective disulfide is less straightforward and finding the optimal combination may take extensive experimentation. Detailed discussion on this issue is provided in section "Regioselective disulfide bond formation".

Table 1: Cysteine protecting groups

Product number	Structure	Protecting group	Cleavage reagent	Comments
852006		Acm	Hg^{2+}, Ag^{+}, I_2, Tl^{3+}, RSCl, PhSOPh-CH_3SiCl_3	Stable to TFA. Enables peptide to be purified in a protected form prior to liberation of the easily oxidizable thiol groups. Removal of Acm and simultaneous disulfide bond formation can be carried out by treatment with I_2 or Tl^3.
852007		tBu	Hg(II), HF(20 °C), TFA/DMSO, PhSOPh-CH_3SiCl_3	Stable to TFA and iodine oxidation. Treatment with $MeSiCl_3$/PhSOPh removes t-Bu and cyclizes in one step without scrambling existing disulphide bonds. Treatment with DPDS in acid leads to direct formation of Cys(Pys) in the presence of existing disulfide bridges and Cys(Acm).
852008		Trt	Hg^{2+}, Ag^{+}, I_2, Tl^{3+}, TFA, I_2, Tl^{3+}	Useful derivative for routine use in Fmoc SPPS as it generates the sulfhydryl peptide directly from the TFA cleavage reaction.
852417		Dpm	Hg^{2+}, Ag^{+}, I_2, Tl^{3+}, TFA, I_2, Tl^{3+}	Useful derivative for routine use in Fmoc SPPS as it generates the sulfhydryl peptide directly from the TFA cleavage reaction. Useable in combination with Mmt group as Dpm does not bleed during Mmt removal with 2% TFA.
852419		Thp	Hg^{2+}, Ag^{+}, I_2, Tl^{3+}, TFA, I_2, Tl^{3+}	Useful derivative for routine use in Fmoc SPPS as it generates the sulfhydryl peptide directly from the TFA cleavage reaction. Alternative to Trt group. Its uses gives rise to less racemisation and β-elimination than Trt.
852022		StBu	RSH, R_3P	Stable to TFA providing thiol scavengers are not used. Has been used in combination with Acm for selective formation of two disulfide bonds. However, removal is sluggish and STmp is preferred for on-resin deprotection.
852373		STmp	RSH, R_3P	Stable to TFA providing thiol scavengers are not used. Has been used in combination with Mmt for selective on-resin formation of two disulfide bonds.
852031		Mmt	2% TFA in DCM, Hg^{2+}, Ag^{+}, I_2, Tl^{3+}	Can be selectively removed whilst the peptide remains attached to the solid phase. Ideal for on-resin disulfide bond formation or modification of Cys side-chain.
n/a	n/a	Npys	RSH, R_3P	Stable to TFA providing thiol scavengers are not used. Activates thiol groups towards disulfide bond formation. Useful for the selective preparation of mixed disulfides.

Fmoc SPPS using protected cysteine derivatives

In constrast to other Fmoc-protected amino acids, Fmoc-protected Cys derivatives can undergo significant racemization during standard coupling reactions. The problem is particularly acute when base-mediated methods like HBTU/DIPEA are used for carboxyl activation. Microwave heating and pre-activation exacerbate the problem. Fortunately, racemization is negligible if coupling is performed under acidic/neutral conditions using preformed symmetrical anhydrides [2] or OPfp esters [3] or with DIPCDI/HOBt or DIPCDI/Oxyma activation.

The synthesis of a peptide acid containing a C-terminal cysteine residue requires special consideration as extensive epimerization [4] and β-piperidinylalanine formation [5] can occur during chain extension. These side-reactions are most problematic where the cysteine residue is anchored to a Wang-type resin. Fortunately, the use of trityl-type resins [6] like 2-chlorotrityl resin, NovaSyn TGT, NovaPEG Trityl resins appear to reduce these issues to acceptable levels and are strongly recommended for the synthesis of peptide acids containing C-terminal cysteine.

Synthesis of cysteinyl peptides

Cys(Trt)/Cys(Thp)/Cys(Dpm)

For the synthesis of peptide containing free sulfhydryl groups the use of Fmoc-Cys(Trt)-OH is most cost effective approach. The trityl group is labile to TFA and is therefore removed during the normal course of the cleavage reaction.

Due to the high stability of the trityl cation and the strongly nucleophilic nature of the thiol group, this reaction is reversible and so special attention needs to be given to the cleavage conditions to ensure complete deprotection. Problems with incomplete deprotection of Cys(Trt) residues can normally be overcome by employing cleavage cocktails which contain TIS [7a]. This reagent is extremely effective at quenching the trityl cation, converting it irreversibly to triphenylmethane. It may be substituted by triethylsilane, although care must be taken with peptides containing unprotected tryptophan as silanes are known to cause reduction of indoles.

With peptides containing multiple Cys(Trt) residues, best results are obtained if the peptide is precipitated directly into diethyl ether from the TFA cleavage mixture. Addition of 2.5% ethanedithiol to the cleavage cocktail helps ensure the peptide is maintained in the reduced state and minimizes side-products due to alkylation of this cysteine thiol group. It is essential to use sufficient cleavage cocktail for the amount of peptide cleaved. Generally 30 mL per 0.5 mmole is sufficient. (approx 16 eq. of EDT/Cys residue) However, if the peptide contains multiple Cys residues and many t-butyl protecting groups, the concentration of EDT or volume of cocktail used needs to be increased to ensure effective scavenging.

Recently, Fmoc-Cys(Thp)-OH has been introduced as an alternative to Fmoc-Cys(Trt)-OH, where the sulfhydryl group is protected with the acid-cleavable tetrahydropyranyl (Thp) group [7b]. The use of Fmoc-Cys(Thp)-OH has been shown to give superior results to the corresponding S-Trt, S-Dpm, S-Acm, and S-StBu derivatives. Significantly lower racemisation and β-piperidinylalanine formation was observed for C-terminal cysteine residues attached to Wang resins during prolonged piperidine treatments. Racemisation during DIPCDI/Oxyma Pure coupling of Fmoc-Cys(Thp)-OH was only 0.74% compared with Fmoc-Cys(Trt)-OH (3.3%) and Fmoc-Cys(Dpm)-OH (6.8%). Complete removal of Thp group was effected by treatment with TFA/water/TIS 95:2.5:2.5 in 2 hours. The Thp group is, however, stable to 1% TFA in DCM, facilitating the synthesis of protected peptide fragments on hyper-acid labile resins such as 2-chlorotrityl or HMPB resins. Initial evidence suggests S-Thp peptides have enhanced solubility compared to those protected with Trt.

Fmoc-Cys(Dpm)-OH is a valuable alternative to Fmoc-Cys(Trt)-OH for introduction of Cys residues during Fmoc SPPS [7c]. The regioselective synthesis of cyclic peptides containing two disulfide bridges can be readily achieved using a combination of Dpm and Mmt sulfhydryl protecting groups. S-Dpm protection is stable to 1–3% TFA, in contrast to S-Trt which is slowly cleaved, but is removed with 95% TFA. These properties enable S-Mmt groups to be removed with dilute TFA on the solid phase without loss of S-Dpm groups. The free sulfhydryls can then be oxidized to form the first disulfide bridge. Subsequent treatment with a TFA/DMSO/anisole cocktail cleaves the peptide from the resin, removes the S-Dpm groups and effects formation of the second disulfide bridge in one step.

Cys(Acm) and Cys(tBu)

Acm and tBu protection can also be used to prepare cysteinyl peptides. However, this approach is no longer in general use.

The Acm and t-butyl groups are stable to the conditions required for the removal of all other side-chain protecting groups. Therefore, intermediate purification of the peptide is possible prior to cleavage of these cysteine protecting groups. Removal of these protecting groups can be achieved by treatment with mercury (II) acetate or silver trifluoromethanesulfonate. In the latter case, treating the silver salt of the cysteinyl peptide with aq. HCl-DMSO leads to direct disulfide bond formation [8].

CAUTION: Mercury and silver salts are toxic and corrosive; great care must be taken when using these reagents. Proper eye protection, lab. coat and gloves are mandatory. Follow local, state/provincial and federal safety regulations. Use in an efficient fume cupboard.

Method 1: Deprotection of Acm protected peptides with Hg(II) [9]

For convenience, these reactions may be carried out in a centrifuge tube.

- Dissolve the (Acm) peptide in 10% aq. AcOH (5–10 mg/mL) and adjust the pH of the solution, very carefully, to 4.0 with glacial AcOH.
- Add mercury (II) acetate (10 eq./Acm) and readjust the pH to 4.0 with AcOH or aq. NH_3. Stir the mixture gently at r.t. under a blanket of N_2.
- Add β-mercaptoethanol (20 eq./Acm) and leave the mixture to stand for 5 h.
- Remove the precipitate by centrifugation and desalt the supernatant by HPLC.

Method 2: Deprotection of t-butyl protected peptides with Hg(II) [10]

- Dissolve the (tBu) peptide in ice-cold TFA (5–10 mg/mL).
- To the resulting solution add mercury (II) acetate (10 eq./tBu) and stir the mixture gently at rt for 3 h under a blanket of N_2.
- Remove the TFA by evaporation under reduced pressure at r.t. and redissolve the residue in 10% aqueous acetic acid.
- Add β-mercaptoethanol (20 eq./tBu) and leave the mixture to stand for 5 h.
- Remove the precipitate by centrifugation and desalt the supernatant by HPLC.

Method 3: Removal of the Acm group with Ag(I) [11]

- Dissolve the (Acm) peptide in TFA/anisole (99:1)(1 mg/mL).
- To the resulting solution add silver trifluoromethanesulfonate (100 eq./Acm), and then stir at 4 °C for 2 h.
- Precipitate the peptide silver salt with ether and isolate by centrifugation.

Reduced peptide

- Treat peptide with dithiothreitol (DTT)(40 eq./Acm) in 1 M acetic acid at 25 °C for 3 h. Centrifuge and desalt the supernatant by HPLC.

Oxidized peptide

- Treat the peptide with aq. 1M HCl/DMSO (1:1) o/n at rt. Remove AgCl by filtration and isolate product by HPLC.

Cys(t-butylthio) and Cys(STmp)

Insertion of Cys(tButhio) [12] or Cys(STmp) [13] residues into a sequence allow selective deprotection of the thiol group on the solid phase, enabling either modification of Cys residues or on-resin disulfide bridge formation.

The t-butylthio group is stable to TFA, providing thiols are not used as scavengers in the cleavage reaction. It is removed by reduction with either thiols [12] or trialkylphosphines [14, 15]. Recently, Góngora-Benítez, et al. [16] demonstrated the effectiveness of 20% β-mercaptoethanol, 0.1 M NMM in DMF for removing tbutylthio on solid phase, where β-mercaptoethanol alone or phosphines were unsuccessful.

However in practice, it often proves extremely difficult to remove the tButhio group on the solid support. For this reason, Albericio has recently introduced the STmp group [13]. The STmp group appears to be extremely easily removed by mild thiolysis, as Albericio has reported removing four STmp groups on the solid phase with only three 5 minute treatments of 0.1 M N-methylmorpholine (NMM) in DMF containing 5% mercaptoethanol.

Method 4: On-resin removal of STmp with thiols

- Treat peptidyl resin with 5% β-mercaptoethanol, O.1 NMM in DMF for 5 mins at rt.
- Wash resin with DMF and repeat thiol treatment twice more.

Cys(Mmt)

On-resin deprotection of Cys(Mmt) residues with 2% TFA in DCM can be effected in a batch-wise or continuous flow manner. In the latter case, the reaction can be monitored spectrophotometrically by following release of the trityl cation at 460 nm. For batch synthesis, a trityl scavenger such as TIS or TES can be added to the reaction to enhance cleavage. However, the color indication provided by the trityl cation will be lost.

Ideally, the reaction should be carried out in a sealed sintered glass funnel to prevent evaporation of the highly volatile DCM, and the filtration reaction should be carried out by applying N_2 pressure rather than by use of a vacuum.

Method 5: Removal of the Mmt with 2% TFA in DCM

Batch-wise method

- Pre-swell the dry resin (1 g) with DCM in a sintered glass funnel (of a type with a tap and stopper). Remove excess DCM.
- Add 94:1:5 DCM/TFA/TIS (10 mL), seal funnel and shake for 2 min. Remove solvent by applying N_2 pressure.
- Repeat step 2 five times.
- Wash resin with DCM and dry under vacuum.

Flow method

- Pre-swell resin (1 g) with DCM and pack into reaction column.
- Pump 1% TFA in DCM (2 mL/min) through resin. The reaction can be followed by measuring the absorbance of the column eluant using a 0.1 mm flow cell at 460 nma.
- Once reaction is finished, as indicated by the absorbance returning to baseline, flush column with DCM.

If the peptide contains other trityl-based protecting groups, the level will not return to baseline owing to slow leaching of Trt groups.

In practice, complete removal of the Mmt group can be difficult to achieve, particularly if the Mmt is situated towards to the C-terminus of the peptide. Extended 2% TFA treatment may lead to loss of protecting groups from amino acid side chains.

Handling of sulfhydryl peptides

Sulfhydryl peptides are readily oxidized by atmospheric oxygen, so they should be freeze dried immediately after cleavage and stored dry under argon. To minimize premature oxidation, cysteine-containing peptides should be handled in acidic (0.1% TFA) degassed buffers. For analysis, HPLC buffers should also be degassed by He sparging. Peptides containing multiple Cys residues may require reduction following cleavage.

Disulfide bond formation by oxidation of cysteinyl peptides

Random oxidation of sulfhydryl peptides

The simplest method for the formation one or more disulfide bridges is by random oxidation of the free sulhydryl peptide. In the case of peptides containing more than two Cys residues, the composition of the products formed may vary depending on whether the oxidation is done under thermodynamic or kinetic control.

Thermodynamic control

Of these techniques, the easiest procedure to perform is the air oxidation which gives the thermodyamincally most stable product. The procedure involves simply exposing an aqueous solution of peptide to the atmosphere in a volatile buffer and then isolating the peptide by lyophilization. Small monocyclic peptides can be readily prepared by carrying out the air oxidation immediately after TFA cleavage of Cys(Trt) without purification of the intermediate sulfhydryl peptide. The disadvantage of this procedure is that reactions can sometimes be very slow. Activated charcoal has been shown to effectively catalyze this process [17].

Method 6: Air oxidation

- Dissolve the cysteinyl peptide in 0.1 M deaerated ammonium bicarbonate (0.1–10 mg/mL).
- Leave the mixture to stand open to atmosphere until the reaction is complete. (The reaction can either be monitored by HPLC or by the Ellman test).
- Isolate the product by lyophilization.

NOTE: The linear peptide concentration may require optimization to minimize polymer formation. Polymeric material can be removed by desalting on a Sephadex G-15 column.

Method 7: Glutathione oxidation

- Dissolve the cysteinyl peptide in 0.2 M deaerated phosphate buffer, pH 7.5 (0.1–10 mg/mL) containing 1 mM EDTA, reduced (5 mM) and oxidized (0.5 mM) glutathione.
- Stir the mixture for 1 to 2 days whilst monitoring reaction by HPLC.
- Isolate the product by lyophilization and desalt by HPLC or GPC.

Oxidation with a mixture of cysteine and cystine or reduced and oxidized glutathione is useful for oxidation of peptides containing multiple disulfide bridges. The presence of excess reduced component leads to the slow rearrangement of the intermediate bridged peptides to the most thermodyamically stable isomer.

Kinetic oxidation

Various oxidants have been used for rapid formation of disulfide bridges to produce the kinetically most favored product. These include potassium ferricyanide [18], iodine [19], DMSO [20, 21], N-chlorosuccinimide (NCS) [22], and DPDS (2,2'-dithiopyridine) [23].

Oxidation with iodine is extremely rapid and can be accomplished by dropping a solution of iodine into the rapidly stirred peptide solution until the solution is premanantly colored yellow.

DMSO oxidation is very mild and works at pHs from 3 to 8. No side-reactions have been reported involving sensitive residues (Met, Trp, Tyr).

NCS in DMF has been used for oxidation of sulfhydryls on the solid phase, with as little as 2 equivalents affecting oxidation in 15 mins [22]. This reagent can cause oxidation of methionine.

Method 8: Iodine oxidation of free sulfhydryl peptides

- Dissolve the cysteinyl peptide (0.1–10 mg/mL) in degassed AcOH/water or MeOH/water.
- Add a 0.06 M solution of iodine in MeOH dropwise with rapid stirring until the solution has a slight yellow color. Quench excess iodine with 1 M asborbic acid.
- Isolate the product by lyophilization and desalt by HPLC or GPC.

Method 9: DMSO oxidation

- Dissolve the cysteinyl peptide in AcOH. Dilute to (0.1–10 mg/mL with water.
- Add ammonium carbonate to pH 6, followed by DMSO (10% by volume).
- Stir the mixture for 4–24 h whilst monitoring reaction by HPLC.
- Isolate the product by HPLC.

Method 10: DPDS oxidation [23]

- Dissolve the cysteinyl peptide (0.1–10 mg/mL) in 0.1 M ammonium bicarbonate.
- Add 5 mM solution of DPDS in MeOH (3 eq.).
- Stir the mixture for 30–120 min whilst monitoring reaction by HPLC.
- Acidify with TFA and isolate product by HPLC.

Method 11: NCS on-resin oxidation [22]

- Remove Cys-STmp or Mtt groups on solid phase. Wash resin with DMF.
- Treat peptidyl resin with NCS (2 eq.) in DMF for 15 mins at rt.
- Wash resin with DMF and DCM. Cleave the peptide from the resin. Thiol scavengers should not be used as these reagents will cause reduction of the disulfide bond. In most cases, EDT can be substituted with TIS.

Disulfide bond formation by oxidation of protected cysteinyl peptides

Disulfide bond formation by iodine oxidation

Treatment of peptides containing Cys(Acm)/Cys(Trt) residues with iodine results in simultaneous removal of the sulfhydryl protecting groups and disulfide bond formation. Peptides containing a single Cys residue are converted to the symmetrical dimer, whilst those containing multiple residues are converted to mixtures of cyclic monomer and polymers, depending on the solvent and concentration. The rate of reaction is very solvent dependent, allowing a degree of selectivity between Cys(Trt) and Cys(Acm); in dipolar solvents, such as aq. MeOH and aq. AcOH, both Cys(Trt) and Cys(Acm) react rapidly, whereas in non-polar solvents, such as DCM and $CHCl_3$, the oxidation of Cys(Acm) is extremely sluggish [24].

The most common application of this method is the conversion of side-chain deprotected peptides containing two Cys(Acm) residues into the cyclic disulfide bridged monomer; the use of trityl protection is not appropriate as this group will be removed during TFA cleavage. The reaction is normally carried out in high dilution in mixtures of aq. MeOH or aq. AcOH, with the exact choice of solvent being dependent on the sequence. Reactions are fastest in aq. MeOH; but for peptides containing Tyr, His and Trp, aq. AcOH is to be preferred as its use limits iodination of these sensitive residues.

For the iodine oxidation of protected peptide fragments in solution, it is recommended that of the two Cys residues to be joined together in the disulfide bridge, one should be protected with Acm and the other with Trt. This measure limits polymer formation and allows the use of non-polar solvent mixtures such as TFE/$CHCl_3$ which favor dissolution of the protected fragment. Iodine oxidation of peptides containing a Cys residue with a free N α-amino function has been noted to be extremely sluggish, possibly a result of the positive charge on the amino group inhibiting formation of the sulfonium intermediate.

Migration of Acm from cysteine to the side chains of Gln [25a] and Ser/Thr [25b] residues has been reported during iodine and thallium (III) oxidation reactions.

Method 12: General method for iodine oxidation

- Dissolve the Cys-Acm peptide (15 µmol) in AcOH (30 mL) Blanket under N_2.
- Add 160 mM aq. HCl (5 mL). Add 20 mM I_2 in AcOH (45 mL).
- After 30–120 min of vigorous stirring (after disappearance of starting material), quench the iodine by adding 1 M aq. sodium thiosulfate or ascorbic acid drop-wise until the mixture is colorless, and concentrate by evaporation under reduced pressure to approximately one third of the original volume. Isolate the product by HPLC.

Alternatively [26]:

- After 30–120 min of vigorous stirring, add 480 mL of cold diethyl ether. Cool on dry ice for 10 min. Isolate peptide by centrifugation.

Excess iodine can also be removed by extraction of the solution with CCl_4, or treatment with activated charcoal or Zn powder.

One-pot formation of two disulfides (based on [27])

- Dissolve the 2xCys, 2xCys-Acm peptide in AcOH (2 mg/mL) under a blanket of N_2.
- Add approx 1 eq. of I_2 as 0.5 M I_2 in MeOH dropwise until there is a persistant brown color. Add a further 9 eq. of iodine. Add water, 20% of the volume of AcOH used and stir mixture.
- Remove excess iodine as described above.

Thallium trifluoroacetate oxidation

Like iodine, $Tl(CF_3CO_2)_3$ converts peptides containing multiple Cys(Acm) residues to the corresponding disulfide bridged cystine peptides [28]. In solution, this reaction has generally been carried out in TFA, as this is an excellent solvent for both free and protected peptides. For direct disulfide bond formation on the solid phase, DMF has been employed as the solvent, allowing good resin swelling without premature cleavage of peptide chains. It is important to note that Met and Trp must be protected during $Tl(CF_3CO_2)_3$ treatment to avoid oxidation of these sensitive residues; Met(O) and Trp(Mts), and Trp(Boc) have been employed in conjunction with t-Boc [28] and Fmoc [29, 30] strategies, respectively.

CAUTION: Thallium salts are highly toxic and corrosive; great care must be taken when using these reagents. Proper eye protection, lab. coat and gloves are mandatory. Follow local, state/provincial and federal safety regulations. Use in an efficient fume cupboard.

Method 13: Solution phase oxidation with Tl(III) [28]

- Dissolve the peptide in TFA (1–10 mg/mL) with anisole (10 µL/mg), and cool in an ice bath.
- Add $Tl(CF_3CO_2)_3$ (1.2 eq.) and allow to react for 5–18 h.
- Remove TFA by evaporation in vacuo and precipitate peptide with ether. Centrifuge and decant ether, removing excess thallium reagent.
- Add fresh ether, shake tube to disperse peptide and centrifuge. Decant ether and repeat washing process three times.

Method 14: Solid phase oxidation with Tl(III) [29, 30]

- Suspend peptidyl resin in DMF/anisole (19:1) and add $Tl(CF_3CO_2)_3$ (1.2 eq. relative to peptide).
- Leave to stand at 0 °C for 80 min, and wash resin with DMF.
- Cleave the peptide from the resin with TFA. Thiol scavengers should not be used as these reagents will cause reduction of the disulfide bond. In most cases, EDT can be substituted with TIS.

Regioselective disulfide bond formation

Synthesis of multiple disulfide containing peptides by selective bridge formation is not a task to be undertaken lightly. In the synthesis of peptides containing multiple disulfide bonds the best results are often obtained by random oxidation, as the desired biologically active isomer is generally the most thermodynamically stable. Methods that work for one peptide do not necessarily work for another. Sometimes two bridges can be formed without issues, but the formation of the third can then prove intractable. Furthermore, the order in which the bridges are formed is often crucial. Selective formation of the first bridge can provide a template for the correct formation of other bridges by random oxidation. A considerable time investment is required with no guarantee of success.

For the selective formation of two disulfide bonds, combinations of either Trt and Acm, or STmp and Acm protection can be used: the first disulfide bond is formed after selective removal of Trt or STmp protection; generation of the second disulfide bond is then carried out in a single step by treatment of the Acm protected peptide with iodine or thallium trifluoroacetate. Alternatively, with Trt and Acm, a one-pot reaction can be performed in solution, where the first disulfide is formed by the fast oxidiation of disulfhydryl peptide with a stoichiometric amount of iodine, followed by the addition of excess iodine and water to effect oxidation of the Acm-protected thiols (Method 12).

The combination of STmp and Mmt facilitate selective formation of two bridges on the solid phase [22]. STmp groups are removed by treatment with mercaptoenthanol (Method 4) and the first bridge formed by oxidation with NCS (Methd 11). Removal of Mmt with 2% TFA in DCM (Method 5), followed by oxidation again with NCS furnishes the second bridge.

Table 2: Protecting group combinations for selective synthesis of multiple disulfide bonds.

First	Second	Third	Comments
Trt	Acm		1: bridge by air oxidation or stoichiometric I_2 oxidation. 2: by excess I_2 oxidation.
tBu	MeBzl		Temperature controlled oxidation with DMSO/TFA.

First	Second	Third	Comments
STmp	Mmt		On-resin formation of both bridges with NCS oxidation.
Mmt	Dpm		On-resin formation of one bridge. Second bridge formed in solution by air oxidation.
STmp	Acm		1: On-resin with NCS oxidation. 2: with solid or solution phase I_2 oxidation.
STmp	Mmt	Acm	1 & 2: On-resin formation of both bridges with NCS oxidation. 3: I_2 oxidation in solution.
STmp	Mmt	tBu	1 & 2: On-resin formation of both bridges with NCS oxidation. 3: DMSO/TFA or $MeSiCl_3/Ph_2SO$ in solution.
Trt	Acm	tBu	1: bridge by air oxidation or stoichiometric I_2 oxidation. 2: by excess I_2 oxidation. 3: TFA/DMSO or $MeSiCl_3/Ph_2SO$.
Trt	tBu	MeBzl	1: bridge by air oxidation or stoichiometric I_2 oxidation. 2 & 3: by temperature controlled oxidation with TFA/DMSO.

t-Butyl protection, in conjunction with one step cleavage and cyclization with $MeSiCl_3/Ph_2SO$, has been used to introduce a third disulfide bridge, leading to the selective synthesis of α-conotoxin and insulin [31]. In a similar manner, a combination of tBu and MeBzl cysteine protection has been employed in a regioselective one-pot formation of the two disulfide bonds of α-conotoxin SI [32]. The first disulfide bond was formed by cleavage of the tBu groups and simultaneous oxidation with TFA/DMSO/anisole at room temperature. Subsequent heating to 70 °C resulted in cleavage of the MeBzl groups and formation of the second disulfide bridge. For other examples of the application of this approach in the synthesis of multi-bridged peptides see references [33–36]. These methods may cause oxidation of Met and Trp residues.

Method 15: $MeSiCl_3/Ph_2SO$ oxidation [31]

- Dissolve Cys-tBu peptide in TFA (1–10 mg/mL).
- Add Ph_2SO (10 eq.), CH_3SiCl_3 (100–250 eq.) and anisole (100 eq.).
- Allow reaction to proceed for 10–30 min at 25 °C.
- Quench reaction with NH_4F (300 eq.) and precipitate peptide by addtion of a large volume of cold diethyl ether and isolate by centrifugation.

Method 16: TFA/DMSO oxidation [32]

- Dissolve peptide in TFA/DMSO/anisole (97.9/2/0.1) (2 mg/mL).
- Stir at rt for 40 mins. Add an additional aliquot of TFA/DMSO/anisole (40 µL/mg peptide).
- Heat at 70 °C for 3 h. Precipitate peptide with diethyl ether and isolate by centrifugation.

Asymmetric disulfides

S-Npys protected peptides react rapidly with thiols over a wide range of pH to form mixed disulfides [37], making this protecting group particularly useful for preparation of peptide-protein conjugates or peptides with two different chains bonded by a disulfide bridge [38–41]. However, owing to the instability of the Npys group to piperidine when using Fmoc chemistry, the Cys(Npys) residue is usually introduced at the N-terminus of the peptide using Boc-Cys(Npys)-OH. Alternatively, it has been found that the Npys group can be added post-synthetically to any Trt protected Cys residue by adding 5 equivalents of 2,2′-dithio-bis-(5-nitropyridine) to the standard TFA/TIS/water (95:2.5:2.5) cleavage mixture [42].

Direct conversion of Cys(tBu) to Cys(Pys) in the presence of Cys(Acm) and a disulfide bridge has been achieved by treatment with DPDS/thioanisole/TFA/TFMSA [43]. This method was used to provide activated relaxin A chain, a key intermediate for the synthesis of the insulin like hormone relaxin [43].

Method 17: tBu to Pys conversion [43]

- Dissolve Cys-tBu peptide and DPDS (4 eq.) in TFA and thioanisole (9:1 v/v) (50 mg/mL).
- The mixture was chilled on ice after which a similar volume of TFMSA in TFA (1:4 v/v) was added and the reaction continued for 45 min at 0 °C.
- Precipitate the Cys(Pys) peptide with cold diethyl ether and isolate by centrifugation. Wash pellet 4 times with fresh ether to remove excess DPDS.

3.10.8 Reduction of disulfide bonds

Reduction with DTT

Since its introduction by W.W. Cleland [44], DTT (Calbiochem cat. no. 233153) has become the standard reagent for the reduction of cystinyl peptides. It has little odor, is highly water soluble, and reduces disulfides quantitatively in aqueous media at pH 8.

Method 18: Reduction with DTT

- Dissolve peptide in 0.1 M ammonium bicarbonate (1–3 mg/mL).
- Add DTT (5-fold excess relative to thiol groups).
- Blanket mixture with N_2 and leave to stand for 6 h.
- Acidify mixture with AcOH and lyophilize.
- Remove DTT by gel-filtration.

Reduction with TCEP

TCEP is a water-soluble phosphine that reduces disulfides over a wide range of pH (1.5–9.0) [45]. Unlike, thiol-based reducing agents, TCEP is not sensitive to oxygen, so no special precautions are necessary to exclude air. The reaction is irreversible and kinetically controlled, unlike the reaction with DTT which is thermodynamically controlled [42]. The use of TCEP is ideal for the reduction of peptides that are not soluble at pH 8 required for reduction with DTT. It should be noted that prolonged contact of peptide with excess TCEP can cause desulfurization of cysteine residues.

Method 19: Reduction with TCEP

- Dissolve peptide in any suitable aqueous/organic solvent mixture (1–3 mg/mL, pH).
- Add TCEP (5-fold excess relative to disulfide).
- Gently agitate solution for 1 h.
- Purify reduced peptide by HPLC or gel-filtration.

3.10.9 Ellman test [46, 47]

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) [Calbiochem cat. no. 322123], reacts quantitatively with aliphatic sulfhydryl groups to generate a yellow anion. This reaction can be used to follow the progress of air oxidation reactions. Samples from the reaction mixture are taken at suitable intervals and the thiol content tested.

Method 20: Ellman test

- Dissolve DTNB (40 mg) in 0.1 M sodium phosphate buffer, pH 8 (10 mL).
- Take a sample from the reaction mixture and dilute with buffer to give 3 mL solution containing 0.1–0.2 µmole of peptide. Add DTNB reagent (0.1 mL) to the peptide solution and leave the mixture to stand for 15 min. Measure the absorbance at 410 nm using a 1 cm cell.
- Prepare a reference solution by adding DTNB reagent (0.1 mL) to buffer (3 mL) and measure the absorbance.
- Calculate the concentration of sulfhydryl groups using the following equation; [SH] = [A410(sample)-A410(reference)]/13650.

Cat.No.	Product	Contents
8520060005	Fmoc-Cys(Acm)-OH	5 g
8520060025		25 g
8520060100		100 g
8520070005	Fmoc-Cys(tBu)-OH	5 g
8520070025		25 g
8520070100		100 g
8520220005	Fmoc-Cys(tButhio)-OH	5 g
8520220025		25 g
8524170005	Fmoc-Cys(Dpm)-OH	5 g
8524170025		25 g
8520310001	Fmoc-Cys(Mmt)-OH	1 g
8520310005		5 g
8520310025		25g
8523730001	Fmoc-Cys(STmp)-OH	1 g
8523730005		5 g
8524190005	Fmoc-Cys(Thp)-OH	5 g
8524190025		25 g
8520080025	Fmoc-Cys(Trt)-OH	5 g
8520080100		25 g
8520080250		100 g

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