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Preparation and Use of Cysteine Orthoesters for Solid-Supported Synthesis of Peptides

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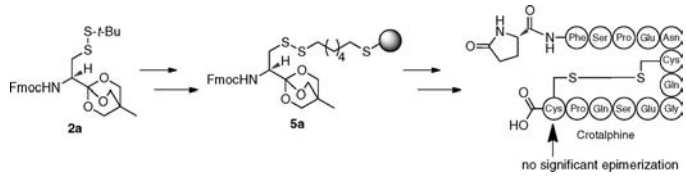
ABSTRACT



Synthesis of a chiral cysteine derivative 2 with the carboxyl protected by an acid-labile 4-methyl-2,6,7-trioxabicyclo[2.2.2]octyl (OBO) orthoester is reported. A disulfide anchoring strategy is used to link the sulfur of this OBO cysteine derivative onto modified trityl polystyrene resin for synthesis of peptides having C-terminal cysteine (Cys) residues. Fmoc-based solid phase peptide synthesis affords model tripeptides without significant epimerization. The approach is used to make the orally active analgesic crotalphine and its Cys1 diastereomer.

The acid-labile 4-methyl-2,6,7-trioxabicyclo[2.2.2] octyl (OBO) orthoester functionality was developed as a protecting group for the carboxyl of α-amino acids by Lajoie and coworkers.[1] It has proven to be very useful in recent work on construction of peptidomimetics and amino acid derivatives,[2] such as serine-derived aldehydes,[1,3] that would otherwise be likely to epimerize. Cysteine (Cys) is notably absent from the list of common α-amino acids protected with OBO because the standard approach for its direct conversion fails. However, such derivatives would be desirable as cysteine esters are especially prone to epimerization or elimination under basic conditions. These side reactions can occur to a significant extent during the esterification of a cysteine to a solid support for peptide synthesis and also during subsequent chain elongation by solid-supported peptide synthesis (SSPS) using Fmoc methodology to make peptides with a C-terminal Cys residue.[4] C-Terminal cysteines occur in many natural biologically active peptides, for example, somatostatin,[5] conotoxins,[6] neopetrosiamides,[7] and crotalphine.[8] The presence of Cys within the backbone is convenient for synthesis of larger proteins by chemical ligation.[9] In the present study

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we report the synthesis of chiral OBO-cysteine derivatives and their use in SSPS to make peptides with C-terminal Cys residues such as crotalphine, a very potent orally active analgesic peptide.[8]

The synthesis of peptides with C-terminal Cys by SSPS often relies on initial linking of the carboxy terminal cysteine to a chlorotrityl polystyrene resin.[10] The steric bulk of such linking hinders access to the α-hydrogen of the cysteine ester during Fmoc deprotection with piperidine and reduces epimerization or formation of 3-(1-piperidinyl)alanine caused by elimination of the protected thiol followed by nucleophilic attack (Figure 1). Alternatively, an elegant side chain anchoring strategy has been reported that involves connection

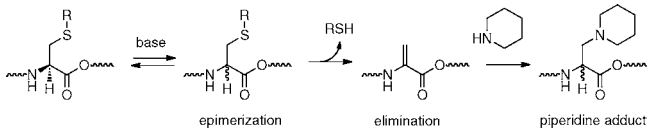
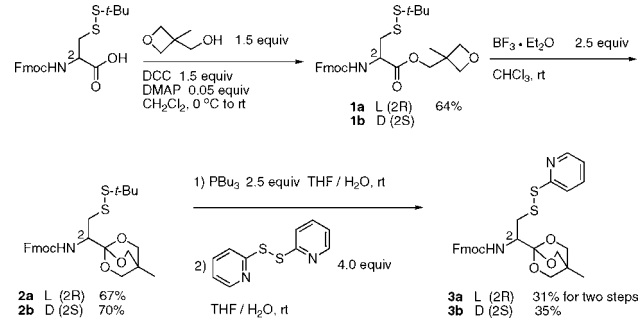


Figure 1. Common side reactions of cysteine residues linked as esters to solid support during Fmoc deprotection with piperidine.

of *N*-Fmoc cysteine *tert*-butyl ester via its sulfur to a 5-(9-xanthen-2-oxy)valeric acid moiety, which is itself linked to solid support.[11] In that approach, the bulk of the *tert*-butyl protecting group on the ester hinders epimerization and elimination reactions. A key benefit of the method is that any peptide that eliminates to form the dehydroalanine intermediate is thereby detached from the resin. However, this does potentially involve some loss of material during each Fmoc deprotection step. As an alternative, we envisaged this problem could be addressed by reducing the acidity of the cysteine α-proton by forming an OBO derivative prior to linking of the sulfur to a solid support.

To assist examination of stereochemical integrity, both L-Cys and D-Cys OBO derivatives were targeted. Our syntheses started from commercially available and enantiomerically pure (>99%) Fmoc-L-Cys(S-*t*-Bu)-OH and Fmoc-D-Cys(S-*t*-Bu)-OH. Although the general Lajoie method[1] fails on many cysteine derivatives, the corresponding oxetane esters **1a** and **1b** were obtained in an average yield of 64% (Scheme 1). The key cyclic ortho ester rearrangement proceeded smoothly under slightly modified conditions with 2.5 equiv of $BF_3{\cdot}Et_2O$ to give L-cysteine cyclic ortho ester **2a** and D-cysteine cyclic ortho ester **2b** in 67% and 70% yield, respectively. The additional equivalents of $BF_3{\cdot}Et_2O$ may be required as coordination with the sulfur atoms may occur preferentially to the oxetane. Importantly, both compounds retained more than 99% enantiomeric purity according to chiral HPLC analyses. These Cys OBO derivatives should be useful intermediates for a variety of syntheses of natural products and peptidomimetics. In the current study,

Scheme 1. Synthesis of Cysteine Cyclic Ortho Esters



we chose to examine the utility of these cyclic ortho esters for SSPS by attachment of Cys sulfur through a disulfide bond to the solid support. Therefore, S-*t*-Bu groups were removed with tributylphosphine, and the newly formed cyclic ortho esters with free thiols were treated with 2,2′-thiopyridine disulfide to afford the desired unsymmetrical disulfide compunds **3a** and **3b** in an overall yield of 31% and 35%, respectively, over two steps. These preparations of **3a** and **3b** are readily scalable to multigram amounts.

Commercial trityl chloride resin was treated with 1,6-hexanedithiol to afford thiol resin **4** (Scheme 2). Disulfide bond exchange reactions between enantiomerically pure compounds **3** and resin **4** furnished resins **5a** and **5b**, respectively. With these key cyclic ortho ester containing resins available, two tripeptide diastereomers H-Gly-Phe-L-Cys-OH and H-Gly-Phe-D-Cys-OH were synthesized in a model study since they are known to be easily separable by RP-HPLC.[11] Using standard Fmoc-based SPPS, tripeptide-bound resins **6a** and **6b** were obtained. Fmoc deprotection, followed by acid-mediated cleavage from resin, gave tripeptide esters **7**. RP-HPLC analysis indicated that the crude peptide from resin **6a** consisted of tripeptide esters **7a** and **7b** in ∼98:2 ratio. The corresponding ratio of **7a** to **7b** in the crude mixture from resin **6b** was ∼4:96. Using lithium hydroxide mediated hydrolysis of the crude tripeptide esters, followed by reduction of the disulfide linkage using tris(2-carboxyethyl) phosphine (TCEP), afforded the corresponding crude tripeptide acids. RP-HPLC purification of these completely deprotected mixtures provided two tripeptide diastereomers **8a** and **8b**, in the ratios (HPLC) of 97:3 and 3:97, respectively. The results show that a maximum of 3% of the undesired stereoisomer may be formed and that the small amounts of epimerization occur prior to basic hydrolysis, probably during acidic cleavage. The overall isolated yield of stereochemically pure tripeptide **8a** is a respectable 25% over 10 steps based on the initial trityl chloride resin (including all couplings and deprotections). For comparison, starting from commercially available H-L-Cys(S-Trt)-2-chlorotrityl chloride resin, **8a** and **8b** were obtained in a ratio of 95:5 (Figure 2). Although this initially appears to suggest that the current methodology only gives an additional 2% stereochemical purity of the desired tripeptide isomer, it is important to note that when using chlorotrityl resin for longer peptides epimerization or elimination can occur at each stage

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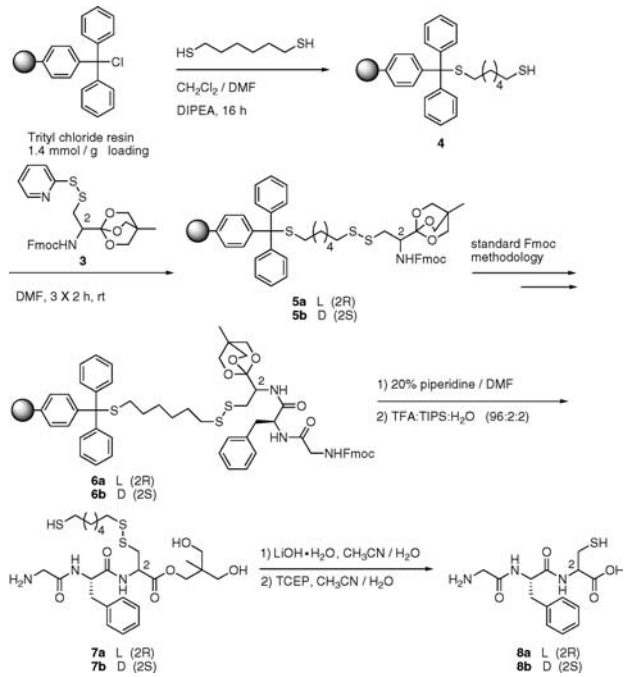
Scheme 2. Synthesis of Model Tripeptides



of chain elongation during Fmoc deprotection. Hence, for longer peptides, the use of the current approach should be increasingly advantageous.

To test this concept and examine the utility of this methodology in making longer peptides with C-terminal cysteines, we applied this technique to the synthesis of the 14 residue peptide crotalphine (**12a**) and its D-Cys1 diastereomer **12b** (Scheme 3). Crotalphine was initially isolated from the venom of the Brazilian rattlesnake, *Crotalus durissus terrificus*.[8a] It is reported to be a nontoxic peptide that possesses potent analgesic activity when administered orally to rats at nanogram levels. It appears to act via a long lasting opioid antinociceptive effect in neuropathic pain that surpasses that observed with standard analgesic drugs (e.g., morphine).[8b] Interestingly, crotalphine produces no increased tolerance upon repeated administration and appears to have no addictive properties.[8a]

The syntheses of crotalphine and its Cys1 diastereomer were initiated from resins **5a** and **5b**, respectively. Using standard Fmoc-based SPPS, peptide bound resins **9a** and **9b** were obtained (Scheme 3). Cleavage from the resin as before provided the crude crotalphine derivatives **10**. RP-HPLC analysis indicated the crude material obtained from resin **9a** consisted of ester **10a** and **10b** in a ~98:2 ratio. The corresponding ratio of **10a** to **10b** upon cleavage of resin **9b** was ~1:99. Separate lithium hydroxide mediated hydrolyses of each of the esters **10** to its corresponding acid, followed by reduction of the disulfide linkage using TCEP, afforded crude reduced (free thiols) crotalphine precursor **11a** and its D-Cys1 diastereomer **11b**. These are separable by HPLC (Figure 3). Reverse-phase HPLC purification of each of these compounds provided the reduced (free thiols) crotalphine diastereomers **11a** and **11b** as stereochemically pure compunds. Intramolecular disulfide bond formations of **11a** and **11b** by aerobic oxidation gave the desired natural crotalphine **12a** and its D-Cys1 diastereomer **12b**. The overall yields for the two peptides were 5% and 2%, respectively, based on the loading of the initial commerical trityl chloride resin (a total of 33 steps including all couplings and deprotections). MALDI-MS and LC-MS/MS analysis confirmed that the sequence of the synthetic crotalphine **12a** is the same as that of the natural crotalphine. A sample of **12a** was also synthesized using commercially available H-L-Cys(S-Trt)-2-chlorotrityl chloride resin with SSPS and Fmoc methodology. The yield of this synthesis was ~3%. The amount of epimerization using the conventional chlorotrityl resin proved difficult to ascertain as a variety of side products coelute on HPLC with the D-Cys1 diastereomer. However, there is a noticeable M+51 peak in the mass spectra of crude peptide made by the chlorotrityl method that corresponds to a β-piperidinyl alanine adduct. However, as expected, no

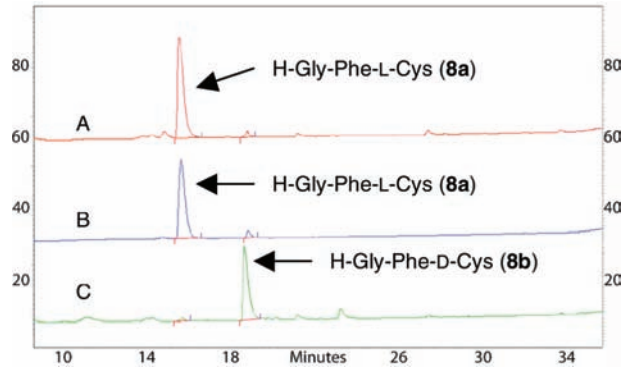


Figure 2. Reverse-phase HPLC analyses of the stereochemical purity of tripeptide H-Gly-Phe-Cys-OH. (A) Crude tripeptide from cyclic ortho ester resin **5a**. (B) Crude tripeptide from H-L-Cys(Trt)-2-chorotrityl resin. (C) Crude tripeptide from cyclic ortho ester resin **5b**. (For experimental details see Supporting Information.)

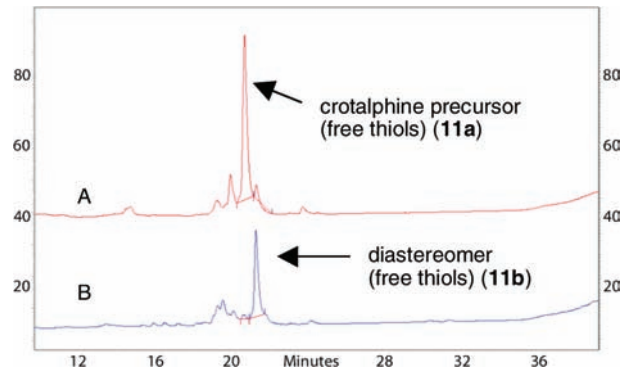
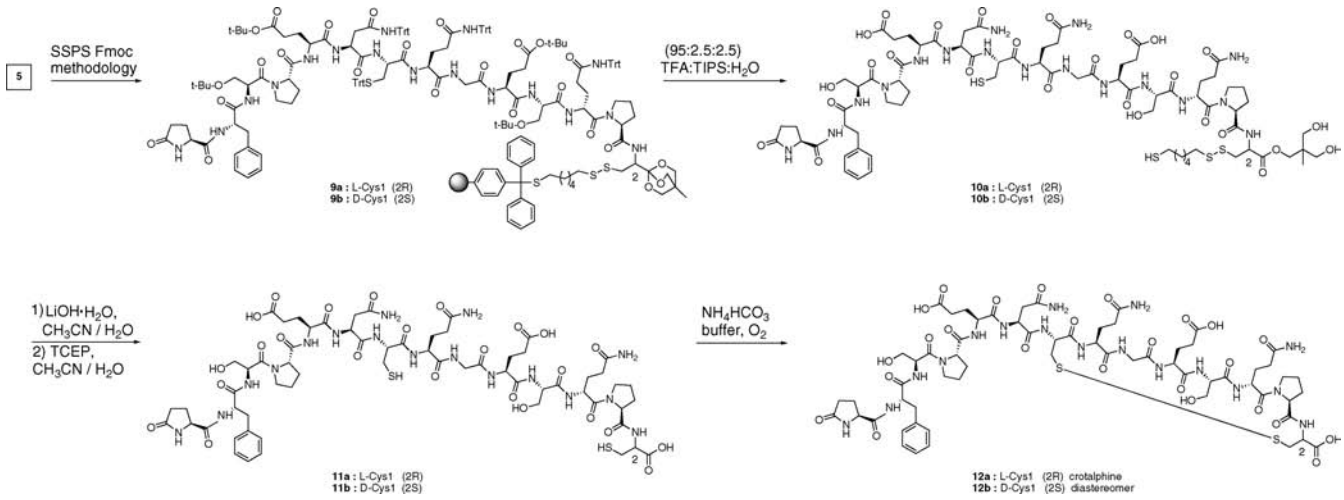


Figure 3. Reverse-phase HPLC analyses of crude crotalphine precursor **11** (free thiols). (A) Crude crotalphine precursor (free thiols) from cyclic ortho ester resin **5a**. (B) Crude diastereomer (free thiols) from cyclic ortho ester resin **5b**. (For details, see Supporting Information.)

Scheme 3. Synthesis of Crotalphine (**12a**) and Its D-Cys1 Diastereomer **12b**



M+51 peak was present in the crude material made by our current OBO cysteine method. The crude sample made by the chlorotrityl method was purified by HPLC and then compared by coinjection with **12a** obtained by the new S-linked OBO cysteine method. A single peak in HPLC was obtained.

In conclusion, we have developed a method to synthesize a novel OBO cysteine derivative **2** in which the carboxyl group of the Cys residue is replaced by a cyclic ortho ester. This should be a versatile synthon for making cysteine derivatives that would otherwise be liable to epimerize or eliminate thiolate. After incorporation of this moiety onto solid support via a disulfide linkage to give **5**, Fmoc-based SPPS generated the model tripeptides Gly-Phe-Cys **8** with excellent diastereomeric purity. A natural analgesic peptide crotalphine (**12a**) and its diastereomer **12b** were also synthesized from **5** by this method without significant cysteine epimerization. We believe this methodology will be a useful alternative for both manual synthesis and automated synthesis of bioactive peptides with C-terminal cysteines.

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Supporting Information Available: Experimental procedures and spectral data for all synthetic compounds, HPLC analyses, MALDI-TOF spectra, and MS/MS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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