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Regioselective Formation, Using Orthogonal Cysteine Protection, of an α-Conotoxin Dimer Peptide Containing Four Disulfide Bonds

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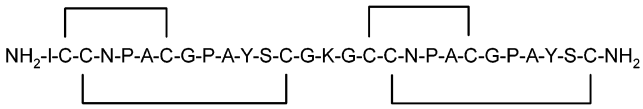
ABSTRACT



The combination of the cysteine thiol protecting groups Trt, Acm, *t*Bu, and MeBzl were used for the regioselective formation of an α-conotoxin dimer peptide containing four disulfide bridges. Additionally, a protocol is described whereby two one-pot oxidations were employed in order to improve the efficiency of the folding process. The target compound was produced in good yield.

The unambiguous formation of the disulfide bridges in complex synthetic peptides and proteins is paramount to facilitating the accurate interpretation of their biological activity. The availability of a wide variety of protecting groups for the thiol side-chain of cysteine and selective chemistries for their subsequent removal has meant that the synthesis of peptides containing one or two disulfide bonds has become relatively straightforward.[1] However, as the complexity of the target molecule increases, the demands placed on the chemistry grow with the result that highly complex peptides containing several bridges still offer a significant synthetic challenge. Consequently, new methodologies designed to simplify the folding process are still of great value. In this study, we present the synthesis and regioselective folding of an α-conotoxin dimer peptide containing four disulfide bridges using four orthogonally protected cysteine pairs.

The DMSO/TFA oxidation provides a rapid and convenient method for disulfide bond formation in peptides either possessing free thiol groups or, in some cases, directly from cysteine-protected precursors.[2] As an extension of this procedure, we have recently demonstrated the orthogonality of the cysteine derivatives *tert*-butyl (*t*Bu)[3] and 4-methylbenzyl (MeBzl)[4] in DMSO/TFA mixtures, the former being rapidly cleaved at room temperature and the latter at elevated temperatures.[5] By utilizing the large difference in the measured kinetics for the cleavage and oxidation reactions, we developed a simple one-pot procedure for the formation of two disulfide bridges in selected peptides.[6] The orthogonality was therefore introduced into the synthesis as a function of temperature rather than by cleavage under different chemical conditions. Further investigations revealed that incorporation of trityl (Trt)-protected[7] cysteine into the synthetic scheme allowed for the facile introduction of a third disulfide bridge.[8] A priori, the acetamidomethyl (Acm)[9]

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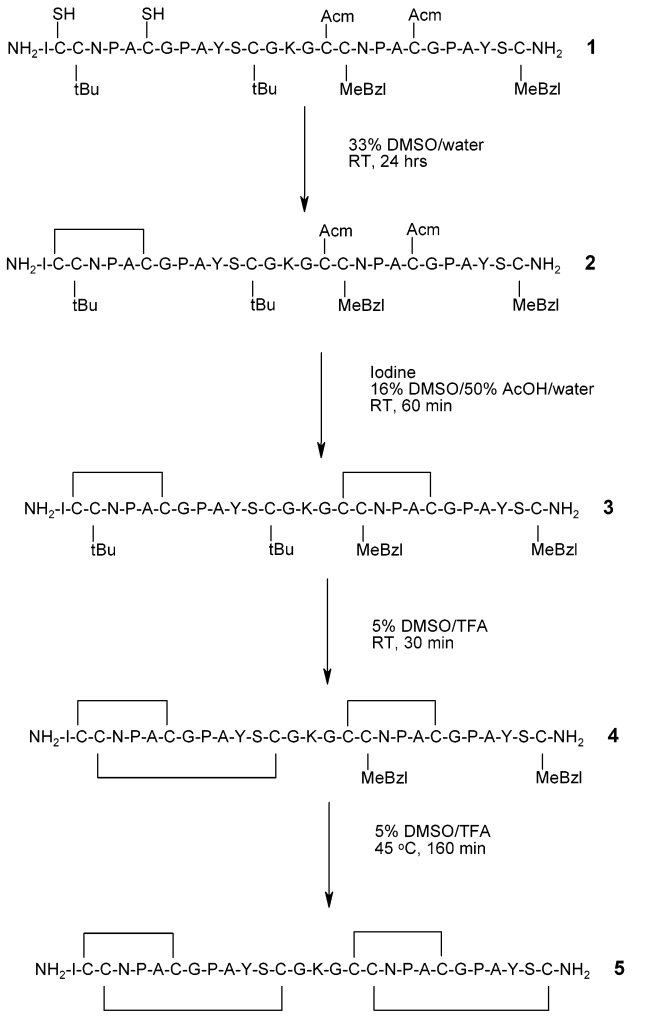
protecting group was considered to be orthogonal to trityl, *t*Bu, and MeBzl, the concomitant cleavage and oxidation with iodine giving the opportunity for selectively forming four disulfide linkages.

In this report, we present the orthogonal synthesis of an α-conotoxin dimer peptide and demonstrate the compatibility of the Acm group with this folding strategy. Moreover, the synthesis was much simplified by employing two one-pot folding procedures, thus negating the need for the repetitive, cumbersome, and costly purification of the intermediate folded products at each step. In addition, to assist in the characterization of the fully folded material, we introduced the Gly−Lys−Gly linker between the two α-conotoxin units comprising a trypsin cleavage site.

Partially protected peptide **1** (Scheme 1) was assembled by Fmoc solid-phase chemistry[10] on a RINK amide resin.

Scheme 1

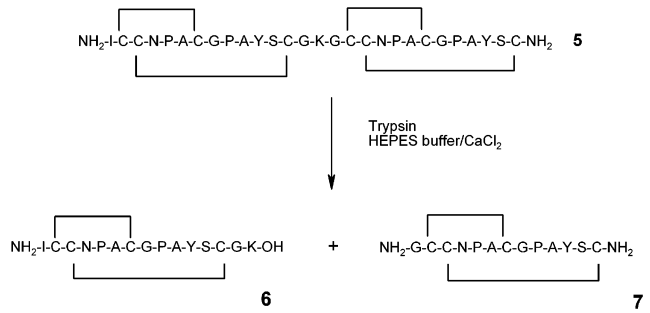


Cleavage in trifluoroacetic acid (TFA) containing the scavengers triisopropylsilane (2.5% v/v) and water (2.5% v/v) of the peptide from the solid-support gave a crude product of sufficient purity (87% by HPLC analysis) to warrant initiation of the folding protocol without prior purification. In a typical experiment, the first one-pot folding was carried out by dissolving crude peptide **1** (30 mg) in 30 mL of DMSO/water (1:2) and stirring the solution for 24 h. Complete conversion to peptide **2** was confirmed by LC-MS analysis of the reaction mixture.[11] To the solution was then added acetic acid (30 mL) followed by iodine (17 mg), and stirring was continued for an additional 60 min. Removal of the Acm groups and complete conversion to the two disulfide product **3** was confirmed by LC-MS. Ascorbic acid (20 mg) was then added, and excess solvent was evaporated in vacuo, leaving a yellow oil. The residue was diluted with water (4 mL) and charged onto a preparative HPLC column. The pure product **3** (15.3 mg) was isolated following freeze-drying in a yield of 53% from the crude starting material. The second one-pot procedure was initiated by dissolving pure product **3** in 5% DMSO/TFA (15 mL).[12] After the mixture was stirred for 30 min at room temperature, LC-MS analysis revealed the complete conversion of the *t*Bu-protected material to peptide **4**.

The solution was immediately placed in an oil bath preheated to 45 °C, and stirring was continued for an additional 160 min to effect total deprotection and folding. Analytical LC-MS revealed one major product and a lesser impurity (20%) running in front of the main peak. Following a second chromatographic purification step, 5 mg of pure product **5** was obtained in a yield of 37% calculated from purified product **3** with an MS profile consistent with the desired product.

To confirm that no reshuffling of the disulfide bonds had taken place during the folding process resulting in a bridge spanning the two α-conotoxin modules, a trypsin digest was performed. Only if the disulfide bridges were confined to the discrete α-conotoxin modules would proteolysis result in the generation of two new peptide fragments as depicted in Scheme 2. A sample of purified product **5** (0.1 mg) was

Scheme 2



dissolved in 0.5 mL each of 0.1 M HEPES buffer, pH 7.8, and 20 mM calcium chloride. To this were added 116 units of trypsin predissolved in 0.1 mL of water, and the mixture was incubated at 20 °C for 15 min. LC-MS analysis of the

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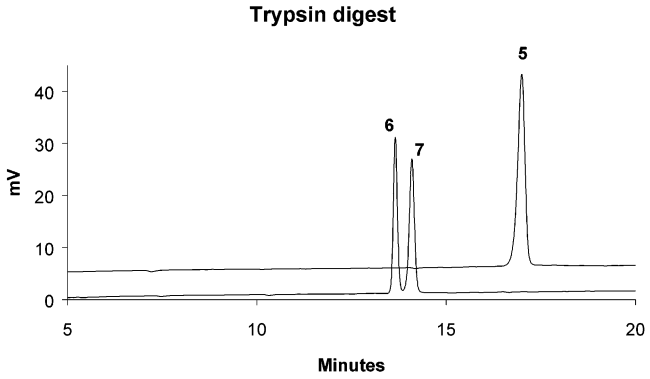

Figure 1. HPLC of peptide **5** and cleavage products **6** and **7** generated prior to and following enzymatic digestion with trypsin.

digest mixture was then carried out. Figure 1 shows an overlay of the chromatograms of product **5** prior to and following the addition of trypsin. Rapid conversion of the starting material to two new products was observed, both peaks possessing a molecular weight consistent with that of the predicted cleavage products. In addition, peptides **6** and **7** were synthesized independently by solid-phase Fmoc chemistry and found to coelute by analytical HPLC with the products from the enzymatic digestion.

The impurity from the second HPLC purification was isolated (1 mg). LC-MS of this byproduct revealed a molecular weight also consistent with peptide **5**, indicating that the folding was not completely regioselective. Trypsin hydrolysis of the byproduct was slow and resulted in the generation of only one new product, indicating the presence of a disulfide bridge spanning the cleavage site. The HPLC chromatograms revealed that the product had formed as a result of MeBzl cleavage at room temperature in DMSO/TFA. Further optimization of the conditions used for the final step with regards to temperature selection will be required to fully optimize the procedure.

In conclusion, we present herein an example of the folding of a peptide containing four disulfide bonds using full orthogonal cysteine protection. Cleavage and oxidation of the cysteine derivatives in the order Trt, Acm, *t*Bu, and MeBzl furnished the fully folded product in good yield. In addition, by employing two one-pot folding protocols, we greatly reduced the number of handling steps and the subsequent losses associated with the repetitive purification of partially folded intermediates. We have utilized this simple procedure for the efficient synthesis of peptides where the primary sequence information contributes favorably to the correct folding. It is unclear how effective this procedure will be when thermodynamically unfavorable cyclic systems are the target.

Although it is not envisaged that this methodology will be applicable to the folding of all multi-disulfide peptides, it should still provide a very useful alternative to current protocols.

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(11) All peptides were characterised by LC-MS. The analysis of peptides **1**−**4** was performed using a gradient of 5 to 50% B over 10 min on a Phenomenex Luna 5 μm, C18, 50 × 4.6 mm column at a flow rate of 2 mL/min, where A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile. The analysis of peptides **5**−**7** was performed using a gradient of 5 to 40% B over 20 min on a Vydac218TP54, 5 μm, 200 × 4.6 mm, C18 column at a flow rate of 1 mL/min, where A = 0.1% TFA/water and B = 0.1% TFA/acetonitrile. Mass analysis was performed in-line on a Thermo Finnigan AQA using positive mode ESI. A downshift of up to 1.6 amu was observed in certain mass domains, values below are not corrected. Peptide **1**: t_R, 9.50 min; expected $(MH_2)^{2+}$, 1587.7; found $(MH_2)^{2+}$, 1586.5. Peptide **2**: t_R, 8.30 min; expected $(MH_2)^{2+}$, 1586.7; found $(MH_2)^{2+}$, 1585.1. Peptide **3**: t_R, 8.48 min; expected $(MH_2)^{2+}$, 1514.6; found $(MH_2)^{2+}$, 1513.7. Peptide **4**: t_R, 7.75 min; expected $(MH_2)^{2+}$, 1457.5; found $(MH_2)^{2+}$, 1456.8. Peptide **5**: t_R, 16.99 min; expected $(MH_2)^{2+}$, 1352.5; found $(MH_2)^{2+}$, 1351.9. Peptide **6**: t_R, 13.66 min; expected MH^+, 1240.4; found MH^+; 1240.0. Peptide **7**: t_R, 14.09 min; expected MH^+, 1482.6; found MH^+, 1481.3

(12) For best results, it is important that the DMSO used in the DMSO/TFA oxidation steps is stored under Argon and over molecular sieves.