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A method for the one-pot regioselective folding of the heat-stable bacterial enterotoxin ST peptide residues 5–18

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Abstract—A one-pot method for the regioselective formation of the three disulfide bridges of the heat-stable bacterial enterotoxin STa, residues 5–18, employing a temperature-controlled orthogonal protecting group scheme is reported. The protecting groups trityl, *t*-butyl and 4-methylbenzyl were chosen for the selective formation of the three cystine residues. Cleavage of the peptide from the solid support afforded a partially *S*-protected crude product with two free thiol groups. The first disulfide linking Cys 6 to Cys 14 was formed by oxidation of the dithiol peptide in a mixture of water/acetonitrile/DMSO. Following solvent removal, the second disulfide between Cys 5 and Cys 10 was formed directly by simultaneous cleavage and oxidation of the *t*-butyl groups in TFA/DMSO/anisole at room temperature. Subsequent heating of this solution initiated cleavage of the 4-methylbenzyl groups with concomitant oxidation of Cys 9 and Cys 17 yielding the desired three disulfide product.

The Guanalyl Cyclase C (GCC) receptor has been shown to be expressed only on the apical membrane of intestinal cells and on the membranes of primary and metastatic colorectal adenocarcinomas.[1] Consequently our interest in ligands for this receptor stems from the potential utility of GCC targeted agents for the early detection, diagnosis and staging of colorectal cancer.[2] The bacterial peptides derived from *E. Coli*, known as the heat-stable enterotoxins possess a high affinity for the GCC receptor.[3] Activation of GCC results in the overproduction of cGMP in intestinal endothelial cells with the subsequent imbalance in fluid uptake and ion secretion resulting in severe diarrhoea.[4]

The enterotoxins have a tightly packed central core comprising three disulfide bonds critical for the correct orientation of the pharmacophore.[5] During the course of our work we have developed new methods which have proven successful in the rapid and routine synthesis of these complex peptides and their analogues. In this study we present an efficient route for the assembly and one-pot regioselective folding of the STa peptide residues 5–18.

We have previously reported that multiple disulfide bonds in partially protected peptides are formed readily in a one-pot procedure using the cysteine protecting group pairings of *t*-butyl (*t*-Bu) and 4-methylbenzyl (4-MeBn).[6] In TFA:DMSO:anisole mixtures at room temperature the *t*-Bu groups are rapidly cleaved with the simultaneous formation of a disulfide bond, while the 4-MeBn protection remains intact. Subsequent heating of the solution brings about cleavage and oxidation of the 4-MeBn groups yielding the second disulfide. The *t*-Bu and 4-MeBn groups are therefore orthogonal to one another.

In this study we present an extension of this work applying the procedure to the one-pot folding of the STa peptide residues 5–18 by introducing the Trityl (Trt) protecting group for the third cysteine pairing as shown in Scheme 1. Partially protected STa 5–18, NH_2-Cys(*t*-Bu)-Cys-Glu-Leu-Cys(4-MeBn)-Cys(*t*-Bu)-Asn-Pro-Ala-Cys-Ala-Gly-Cys(4-MeBn)-Tyr-OH, **1**, was obtained following assembly by 9-fluoromethoxycarbonyl (Fmoc) solid-phase chemistry[7] on Fmoc-Tyr(*t*-Bu)-SASRIN resin and cleavage in trifluoroacetic acid (TFA) containing water and triisopropylsilane as scavengers.

In a typical experiment crude peptide **1** (100 mg) was dissolved in 600 mL of water/acetonitrile (v/v 6:4) containing DMSO (24 mL) and the pH of the solution

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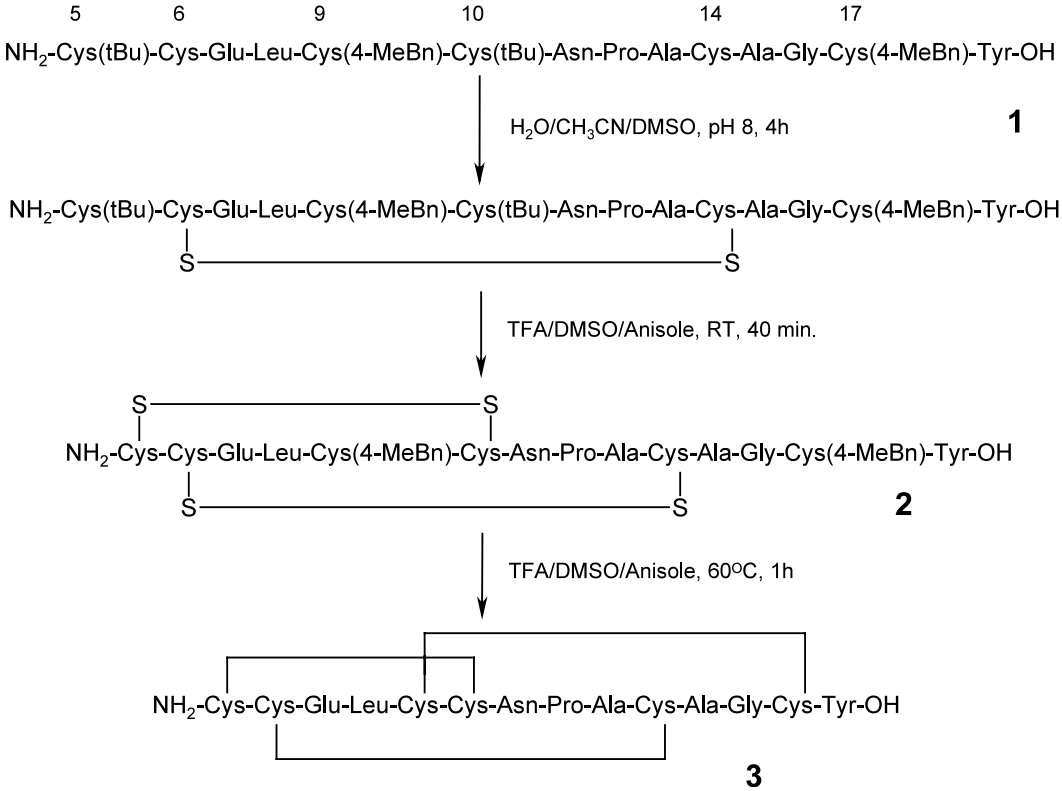

Scheme 1. One-pot method for the regioselective folding of the three disulfide bonds of STa peptide 5–18.

adjusted to 8 by addition of dilute ammonia solution. The oxidation was monitored by analytical HPLC and complete conversion to the disulfide linking Cys residues 6 and 14 observed after 4 h (Fig. 1a). The pH of the peptide solution was then adjusted to 2 by the dropwise addition of TFA and the aqueous phase removed either in vacuo or by freeze-drying. The residue was then re-dissolved in TFA (250 mL) containing DMSO (10 mL) and anisole (0.5 mL) and the solution stirred at room temperature for 40 min. Analysis of an aliquot of the reaction mixture by HPLC and MALDI-TOF revealed the complete conversion of starting material to product **2** (Fig. 1b) containing the Cys 6, 14; Cys 5, 10 disulfide pairings.

Formation of the final Cys 9 to Cys 17 bridge was brought about by heating at 60°C for a further hour with stirring. Excess TFA was then removed in vacuo and the crude fully folded product **3** (80 mg) liberated following addition and trituration with diethyl ether (Fig. 2a). Preparative HPLC was then used to isolate the pure STa peptide (21 mg) in 25% yield (Fig. 2b).

Confirmation of structure was performed by co-injection[8] with an authentic sample of ST5–18 synthesised by conventional differential protecting groups[2] as well as amino acid analysis[9] and accurate mass analysis.[10] In addition, due to the possibility of co-elution with disulfide isomers, the correct folding pattern was further confirmed by in vitro bioassay. In a radioligand binding assay product **3** bound to the GCC receptor in a dose-dependent fashion demonstrating an inhibition constant in the low nanomolar range (Ki, 10^{-9} M) in good agreement with results previously reported in the literature.[11]

In conclusion we have further extended the utility of the methodology employing temperature-controlled orthogonality to a more complex peptide system containing three disulfide bridges. This procedure has the distinct advantage of allowing rapid regioselective formation of disulfide bonds in peptides without the need for isolation of intermediates. Although it is anticipated that limitations are inevitable due to the sensitivity of

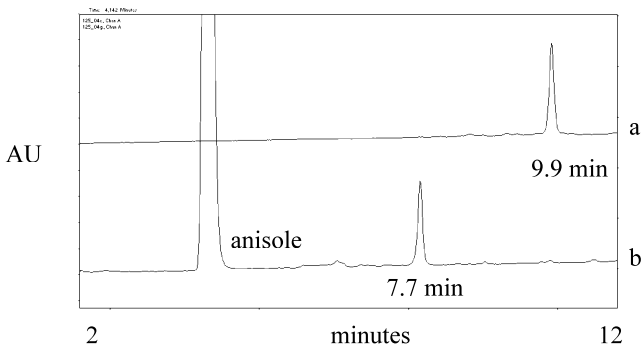

Figure 1. (a) An analytical HPLC of the crude product with one disulfide. (b) An analytical HPLC of the reaction mixture after 40 min in TFA/DMSO/anisole. The 7.7 min peak corresponds to the intermediate with two disulfides. Both samples were run on a Phenomenex, Luna C18 column (60×4.6 mm, 3 μm) at a flow rate of 2 mL/min where solvent A = 0.1%TFA/water and B = 0.1% TFA/acetonitrile employing a gradient of 20 to 50%B over 10 min.

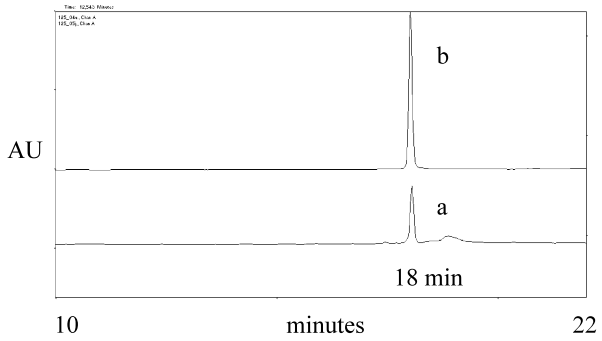

Figure 2. (a) The analytical HPLC of the crude fully folded STa peptide product. (b) The pure peptide fraction following purification. Both samples were run on a Phenomenex, Luna C18 column (250×4.6 mm, 5 μm) at a flow rate of 1 mL/min where solvent A = 0.1%TFA/water and B = 0.1% TFA/acetonitrile employing a gradient of 5 to 40%B over 20 min. Estimated purity of final purified product >98% by analytical HPLC.

some peptides to the reaction conditions, we believe that this procedure still provides a valuable addition to current methodologies. Work continues to investigate its potential utility in the field of combinatorial chemistry.

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8. HPLC co-elution: pure product **3** and an authentic sample of STa 5–18 were co-injected on a Phenomenex, Luna C18 column (250×4.6 mm, 5 μm) at a flow rate of 1 mL/min where solvent A = 0.1%TFA/water and B = 0.1% TFA/acetonitrile. The peptide was eluted using a gradient of 5 to 40%B over 20 min the product eluting as a single peak with retention time 18 min.
9. Amino acid analysis: Asn 1 (1.00); Glu 1 (1.00); Cys 6 (5.78); Pro 1 (0.98); Ala 2 (1.98); Gly 1 (1.00); Leu 1 (0.99); Tyr 1 (0.93).
10. Mass spectrometry: accurate mass measurement was performed on a laser-desorption mass spectrometer coupled with delayed extraction. In the MALDI analysis, standard peptide signals (Angiotensin I, 1296.6853 Da. and ACTH 1-16, 1936.9855 Da.) were used to accurately measure the sodiated $[M+Na]^+$ monoisotopic sample ion. The ion was analysed six times in this way and the average value 1468.3906 was consistent with the given formula $C_{55}H_{79}N_{15}O_{19}S_6$ which would have an expected sodiated quasimolecular ion mass of 1468.3899.
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