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α-Conotoxin ImI Incorporating Stable Cystathionine Bridges Maintains Full Potency and Identical Three-Dimensional Structure

Zoltan Dekan, Irina Vetter, Norelle L. Daly, David J. Craik, Richard J. Lewis, and Paul F. Alewood*

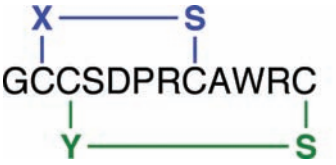
Institute for Molecular Bioscience, The University of Queensland, St Lucia 4072, Queensland, Australia

Supporting Information

ABSTRACT: The two disulfide bonds of α-conotoxin ImI, a peptide antagonist of the α7 nicotinic acetylcholine receptor (nAChR), were systematically replaced with isosteric redox-stable cystathionine thioethers. Regioselective thioether formation was accomplished on solid support through substitution of a γ-chlorohomoalanine by an intramolecular cysteine thiol to produce hybrid thioether/disulfide analogues (**2** and **3**) as well as a dual cystathionine analogue (**4**) that were found to be structurally homologous to α-conotoxin ImI by ^{1}H NMR. The antagonistic activity at the α7 nAChR of cystathionine analogue **3** ($pIC_{50} = 6.41 \pm 0.09$) was identical to that of α-conotoxin ImI (**1**, $pIC_{50} = 6.41 \pm 0.09$), whereas those of **2** ($pIC_{50} = 5.96 \pm 0.09$) and **4** ($pIC_{50} = 5.89 \pm 0.09$) showed a modest decrease. The effect of oxidation of the thioethers to sulfoxides was also investigated, with significant changes in the biological activities observed ranging from a >30-fold reduction (**2**S═O) to a 3-fold increase (**3**S═O_B) in potencies.

Disulfide bonds are a dominant structural feature of many peptides and proteins that are essential for maintaining well-defined three-dimensional structures that allow high-affinity interactions with specific molecular targets.[1] However, many disulfide bonds are susceptible to reduction and scrambling in vivo by glutathione[2] as well as redox enzymes,[3] potentially resulting in the loss of the desired biological activity of the peptide. A number of alternative linkages with increased redox stabilities including lactams,[4] diselenides,[5−10] thioethers,[11−16] and dicarba[17,18] analogues have previously been introduced into biologically active peptides with some success in maintaining structure and activity.

Lanthionine thioethers, in which one of the sulfur atoms of the disulfide bond is removed, have been assessed extensively[19] as disulfide bond replacements or as synthetic structural constraints, and are also found in some naturally occurring bioactive peptides such as the lantibiotics.[20,21] Although lanthionines make up an interesting class of compounds and have previously been used successfully as synthetic disulfide bond replacements, they are not isosteric with cystine. The cystathionine (Ctt) thioether, where one of the sulfur atoms of a disulfide bond is substituted with a methylene group, should more closely approximate the geometry of cystine than dicarba or lanthionine analogues and is therefore hypothesized to cause the minimum of structural perturbations.[16,22,23]



	X	Y
1	S	S
2	CH_2	S
3	S	CH_2
4	CH_2	CH_2

Figure 1. Cystathionine analogues of α-ImI.

Common synthetic approaches for thioether cyclization of peptides include thiol addition to an alkene, substitution of a leaving group by a thiol, desulfurization of a disulfide, and cyclization through amide bonds using preformed orthogonally protected thioether building blocks.[19] While each of these methods has its own advantage, sequence-independent stereo- and regioselectivity as well as simplicity of synthesis are desired for general application to a wider range of peptides, especially where multiple Ctt's are required.

This study focuses on the synthesis and characterization of the potent and selective α-conotoxin ImI (α-ImI)[24] incorporating either one or two cystine bond thioether replacements. It was anticipated that such replacements would generate a mimetic stable to reduction while maintaining structure and function identical to those of the native molecule. An Fmoc solid-phase Ctt synthesis strategy was undertaken via the intramolecular alkylation of a selectively deprotected cysteine thiol by γ-chlorohomoalanine (ClhAla)[25] on the growing protected peptide chain to incorporate regiospecifically either one or two Ctt moieties.

α-ImI was chosen as a model bioactive peptide for the synthesis of Ctt analogues **2**, **3**, and **4** (Figure 1), as the two disulfide bonds are largely buried and disulfide bond replacements must be finely tuned if full structural and functional integrity is to be maintained. α-ImI is a selective peptide antagonist of α7 nicotinic acetylcholine receptors (nAChR) with the sequence of GCCSDPRCAWRC-NH_2, containing two disulfide bonds with the connectivity Cys 1−3, Cys 2−4. α-ImI is a well-characterized molecule that provides an ideal model for the evaluation of the effects of disulfide bond mimics on structure and function, as it allows a direct comparison of Ctt replacements with its previously reported diselenide[7] and dicarba[18] counterparts.

Cystathionine-containing α-ImI analogues **2**−**4** were prepared as outlined in Scheme 1. The linear precursors **5** were synthesized on-resin using standard Fmoc solid-phase peptide synthesis (SPPS) followed by conversion of Hse(TBDMS) to ClhAla and deprotection of Cys(StBu) [where Hse is homoserine,

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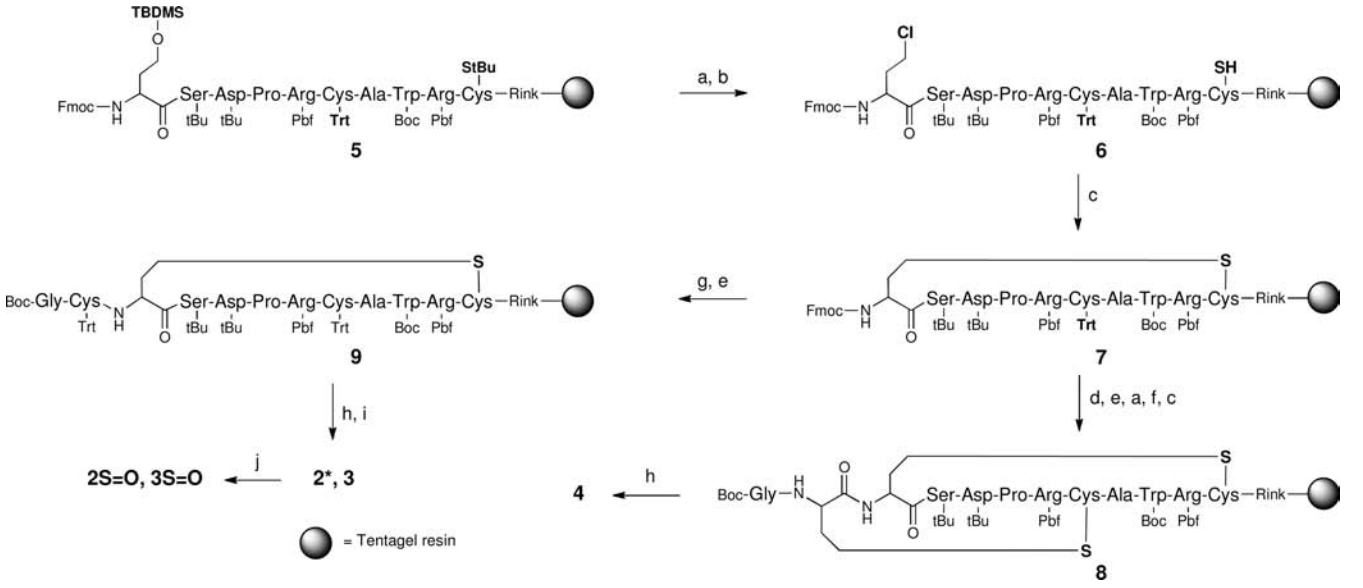
Scheme 1. Solid-Phase Synthesis of Cystathionine Analogues of α-ImI, 4 and 3[a]



[a] Reagents and conditions: (a) 10 equiv of Ph_3PCl_2, DCM, 6 h; (b) 100 equiv of Bu_3P in 5% H_2O/THF, 2 h; (c) 0.1 M $NaHCO_3$ in DMF/H_2O (3:2), 72 h; (d) SPPS: Fmoc-Hse(TBDMS)-OH; (e) SPPS: Boc-Gly-OH; (f) 3% TFA/5% TIPS in DCM, 10 × 3 min; (g) SPPS: Fmoc-Cys(Trt)-OH; (h) 95% TFA/2.5% TIPS/2.5% H_2O, 2 h; (i) 0.1 M NH_4HCO_3 (aq), pH 8.2, 6 h; (j) 0.1% H_2O_2/0.1% TFA (aq), 35°C, 16 h. * **2** was prepared analogously to **3** by exchanging the positions of Cys 2(Trt) and Hse 3(TBDMS), and those of Cys 8(Trt) and Cys 12(StBu)).

TBDMS is *tert*-butyldimethylsilyl, and StBu is *tert*-butylthio], which proceeded smoothly irrespective of the type of solid support used (polystyrene or Tentagel-S). Following 6 and 2 h treatments with Ph_3PCl_2 and Bu_3P, respectively, the desired product containing the Cl-hAla and the S-deprotected cysteine **6** was obtained. To optimize the on-resin intramolecular thiol alkylation, conditions were evaluated by varying the solvent (DMF or DMF/H_2O), the base (DIEA, NMM, Na_2CO_3, or $NaHCO_3$) and the type of solid support (polystyrene or Tentagel-S). Initial attempts at intramolecular alkylation of Cys 12 with ClhAla 3 (to form the 2−4 Ctt, **7**) on polystyrene solid support using previously reported conditions[15,26,27] with DMF as the solvent and either DIEA or NMM as the base did not result in the formation of the desired Ctt product. Attempts at using the same conditions on Tentagel-S resin also failed to produce a thioether bond, as only the linear precursors were recovered in each case. The use of Na_2CO_3[25] in DMF/H_2O gave no **7**, though thioether cyclization was eventually accomplished by treatment of **6** with 0.1 M $NaHCO_3$ in DMF/H_2O (3:2) on Tentagel-S resin. The same conditions were used successfully with subsequent substitution reactions to produce the dual Ctt analogue **4**, following the coupling of the second Hse(TBDMS) and final Gly residues, O(TBDMS) to -Cl conversion, and Cys(Trt) deprotection. Tryptic digest of the purified final product showed a single HPLC peak containing the mass of the cyclized Ctt peptide + 36 Da (Supporting Information Figure S2), corresponding to cleavage at both Arg residues with the fragments being held together by the two thioether bonds, confirming that both cyclizations had indeed taken place. The hybrid cystine/Ctt analogues **2** and **3** were prepared by replacing Hse 3 and Hse 2 with a Cys(Trt) residue respectively, and following the formation of one thioether bridge, completion of chain assembly **9**, cleavage from the resin, and formation of the disulfide bond by air oxidation of the crude peptides in aqueous buffer.

Although not observed during the synthesis and handling of these particular peptides, thioethers can be susceptible to the formation of sulfoxides, which may cause further structural changes to the molecule. To investigate these effects on biological activity, sulfoxide analogues of **2** and **3** were prepared by oxidation of the thioether bridge using H_2O_2.[16] Interestingly, the oxidation of **2** produced only a single product (**2**S=O, as determined by RP-HPLC and MS) rather than the expected isomeric mixture of sulfoxides as observed following the oxidation of **3**, which were isolated and designated as **3**S=O_A (earlier eluting) and **3**S=O_B (later eluting).

Functional activities of the Ctt analogues are summarized in Figure 2. Replacement of the Cys 2−4 disulfide with a Ctt bridge (**3**) did not show any difference in activity (pIC_{50} = 6.41 ± 0.09) compared to α-ImI (**1**). The two isomers produced by the oxidation of the thioether of **3** each displayed different activities, with **3**S=O_A showing a 2-fold decrease (pIC_{50} = 6.10 ± 0.06) and **3**S=O_B showing a 3-fold increase (pIC_{50} = 6.92 ± 0.05) in potency compared to **1**, suggesting that significant structural changes may be occurring due to the altered geometry of the bridge.

The substitution of the Cys 1−3 disulfide (**2**) resulted in a 3-fold reduction in potency (pIC_{50} = 5.96 ± 0.09), and similarly, a 3-fold decrease in activity was observed when both the Cys 1−3 and the Cys 2−4 disulfides were replaced (**4**, pIC_{50} = 5.89 ± 0.09). The Cys 1−3 disulfide bond of α-conotoxins is predicted to be involved in binding to nAChRs through hydrophobic interactions with an internal vicinal disulfide bond of the receptor.[28] Thus, the drop in activity of **2** and **4** may be attributed to the decreased hydrophobicity of the thioether compared to the native disulfide (based on retention time, see Supporting Information Figure S1) rather than a result of structural changes. Further reduction in the hydrophobicity of this bridge by oxidation of Ctt 1−3 to its sulfoxide (**2**S=O) resulted in an even

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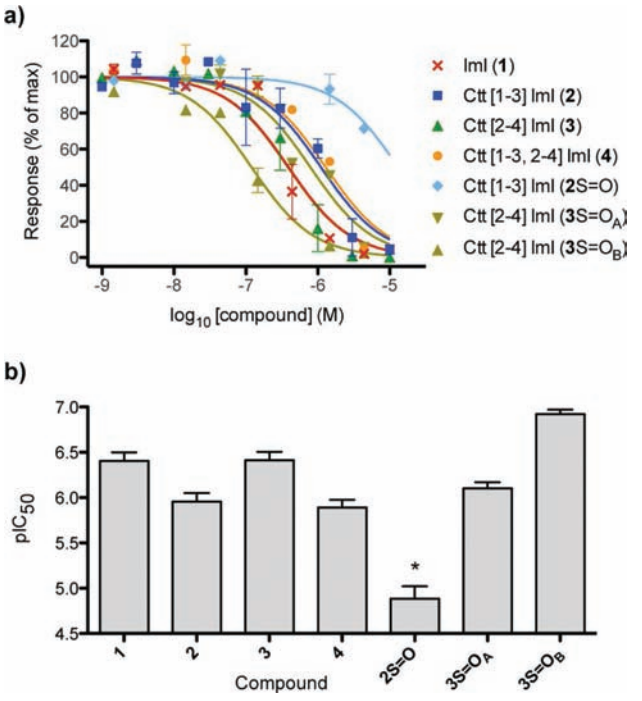

Figure 2. (a) Representative concentration−response curves showing inhibition of choline-induced Ca^{2+} responses from SH-SY5Y cells expressing the human α7 nAChR by the cystathionine analogues of α-ImI. (b) Comparison of pIC_{50} values (*IC_{50} of **2**S=O estimated from extrapolation of the concentration−response curve). Data are presented as mean ± SEM of n = 3 replicates and are representative of 3−5 independent experiments.

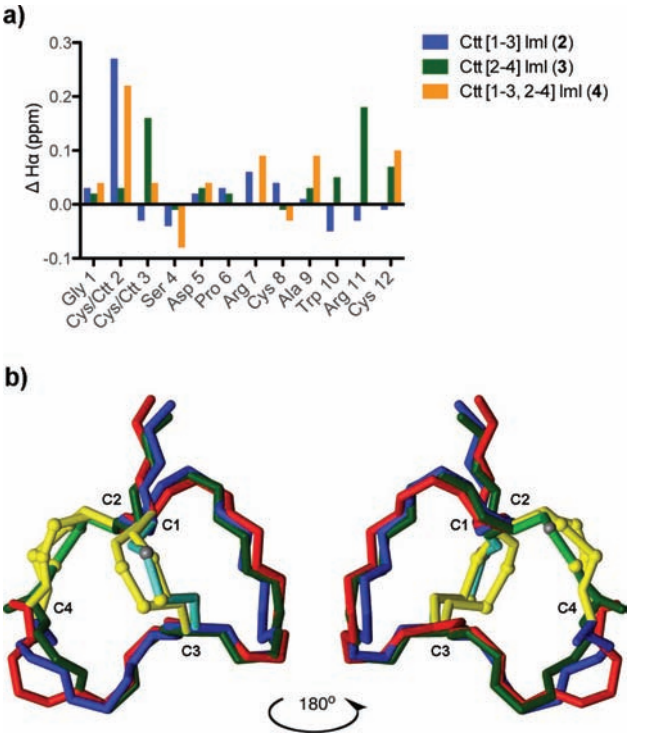

Figure 3. (a) Differences in ^{1}H NMR Hα shifts of **2**−**4** relative to **1**. (b) Overlay of the average of the 20 lowest energy structures of **1** (PDB 1IM1) (red), **2** (blue), and **3** (green). Cystine bridges are shown in yellow; Ctt 1−3 of **2** and Ctt 2−4 of **3** are shown in light blue and light green, respectively. Yellow balls represent sulfur atoms, and the Cγ of cystathionines is highlighted in gray.

greater loss of activity (>30 fold), although structural similarity to α-ImI was not confirmed in this case. This trend is consistent with the increase in activities of other α-conotoxins at nAChRs observed following the replacement of Cys 1−3 with the more hydrophobic selenocystine.[29]

Analysis of the thioethers **2**−**4** by two-dimensional ^{1}H NMR confirmed the presence of a single conformation for each analogue. The concordance of the backbone Hα chemical shifts (Δ Hα < 0.1) for most residues with those of **1**[30] indicates that both Cys 1−3 and Cys 2−4 cystine-to-cystathionine substitutions were structurally conservative (Figure 3A). The slight upfield shifts of Ctt 2 Hα of **2** and **4** and Ctt 3 Hα of **3** compared to those of Cys 2 and Cys 3 of **1**, respectively, are consistent with the increased shielding effect of the introduced methylene group compared to that of the native sulfur atom. The three-dimensional structures of **2** (BMRB 21007) and **3** (BMRB 21006) were calculated from NOE and dihedral restraints (see Supporting Information Table S1). Superposition of the backbone atoms of **2** and **3** with those of **1** (Figure 3B) resulted in low rmsd values of 0.35 and 0.37 for residues 2−10 (0.90 and 0.79 for residues 1−12) between the average structures of **1** and **2** and between those of **1** and **3**, respectively, which confirms a high degree of structural homology. The differences in the rmsd values for residues 1−12 compared to those of 2−10 are due to increased structural disorder at residues 1, 11, and 12 observed in all three sets of structures, including that of the native molecule.

In summary, each of the disulfide bonds of α-ImI was successfully replaced with isosteric cystathionines on solid support using a simple intramolecular Cys alkylation strategy. Structural analysis showed that the Ctt analogues caused minimal perturbations to the native peptide, and the functional assays revealed no or very modest losses in biological activities, confirming the conservative nature of these substitutions and further supporting their usefulness as stable disulfide bond mimetics. Oxidation of the thioether bridges to sulfoxides provided fast and convenient access to a new set of analogues with significantly altered biological activities and provided further insight into the structure−activity relationships of α-ImI.

■ ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures and additional figures (S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
p.alewood@imb.uq.edu.au

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