Synthesis of α-Conotoxin SI, a Bicyclic Tridecapeptide Amide with Two Disulfide Bridges: Illustration of Novel Protection Schemes and Oxidation Strategies[†,1-3]

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Received June 10, 1993[®]

Abstract: Several routes are described and compared for the synthesis of α-conotoxin SI, a tridecapeptide amide of sequence H-Ile-Cys-Cys-Asn-Pro-Ala-Cys-Gly-Pro-Lys-Tyr-Ser-Cys-NH_2, with bicyclic disulfides connecting Cys^2 with Cys^7, and Cys^3 with Cys^{13}. The linear sequence was assembled smoothly on tris(alkoxy)benzylamide (PAL) supports, using stepwise Fmoc solid-phase chemistry. Side-chain protection of Cys was provided by *S*-2,4,6-trimethoxybenzyl (Tmob) at all four positions or by suitable pairwise combinations of *S*-Tmob and *S*-acetamidomethyl (Acm). Acidolytic cleavage/deprotection of these peptide-resins with trifluoroacetic acid (TFA)–CH_2Cl_2–Et_3SiH–H_2O–anisole (95:4:0.5:0.5:0.5), at 25 °C for 2 h, gave the corresponding peptide amides in high yields (90–97%); those Cys residues originally blocked by *S*-Tmob were converted to the free sulfhydryls, whereas Cys(Acm) residues remained blocked. The fully deprotected linear tetrasulfhydryl conotoxin was oxidized successfully in dilute pH 7.5 solution in the presence of 1% (v/v) DMSO, at 25 °C for 7 h, providing monomeric bicyclic peptide in an overall yield of 39%. Other simultaneous procedures gave poor yields and/or extensive oligomers and polymers, in part due to solubility problems. Further solution synthesis strategies relied on sequential disulfide pairing as dictated by the original protection scheme. A monocyclic, bis(Acm) intermediate, with the smaller loop (Cys^2 with Cys^7) already closed by the solution DMSO oxidation procedure, was cyclized further with thallium tris(trifluoroacetate) [$Tl(tfa)_3$; 1.2 equiv] in TFA–anisole (19:1) at 4 °C for 18 h. The respective oxidation yields were 62 and 65%, and the final overall yield of monomeric conotoxin reflecting cleavage from the support and the two orthogonal oxidation steps was 38%. Alternatively, the same chemical steps and solution conditions were used to test the strategy of forming the larger loop (Cys^3 with Cys^{13}) first; individual oxidation steps each proceeded in ~50% yield, and conotoxin was obtained in an overall yield of 23%. Simultaneous or orthogonal disulfide bond formation was also examined while the peptide *remained anchored* to the polymeric support. The most promising results started with the appropriate resin-bound precursor, which was treated with TFA–CH_2Cl_2–Et_3SiH–H_2O–anisole (7:92:0.5:0.5:0.5), 2 × 15 min at 25 °C, to remove selectively *S*-Tmob groups from Cys^2 and Cys^7 without significant loss of chains from the support. The resultant resin-bound dithiol was oxidized with 35 mM CCl_4–Et_3N (2 equiv each) in *N*-methylpyrrolidinone (NMP) for 4 h at 20 °C, to provide a resin-bound monocyclic bis(Acm) intermediate, which was oxidized further with $Tl(tfa)_3$ (2 equiv) in DMF–anisole (19:1) for 18 h at 4 °C. After acidolytic cleavage, overall yields of monomeric conotoxin were as high as 14%. Additional oligomeric material is apparently incorporated as a network which is retained on the support, accounting for the reduced yields (25–50%, respectively with polystyrene (PS) and polyethylene glycol–polystyrene (PEG-PS) graft supports) observed after cleavage of the peptidyl–PAL anchoring linkage. The polymer-supported routes are amenable to further optimization and may offer several advantages over solution methods.

Introduction

Conotoxins are a family of small, multiple disulfide-containing peptides isolated from the venom of marine snails and are of interest because they are competitive antagonists of nicotinic acetylcholine receptors.[4] From this family, α-conotoxin SI, a 13-residue peptide amide (Figure 1) with bicyclic disulfides connecting Cys^2 with Cys^7, and Cys^3 with Cys^{13}, is unusual insofar as it can discriminate between receptors from different vertebrates.[5] We viewed α-conotoxin SI as a challenging target for unambiguous chemical synthesis,[6] in the context of our long-term program to develop new orthogonally removable cysteine protecting groups[1,7,8a,d,e] and strategies for controlled formation of disulfide bonds.[1,7e,8] The present paper documents and contrasts a variety of solution and polymer-supported (solid-phase)[8,9] approaches for the synthesis of α-conotoxin SI, in the hope that the lessons learned may be generalized to other molecules with two or more disulfide bonds.[8b,e,10]

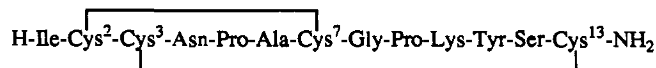

Figure 1. Structure of α-conotoxin SI.

Results and Discussion

General Plan. The linear sequence of α-conotoxin SI was assembled smoothly by solid-phase synthesis,[8a,11] using the base-labile N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) amino protecting group,[12] acidolyzable *tert*-butyl type side-chain protecting groups, and the acidolyzable tris(alkoxy)benzylamide (PAL) anchoring linkage.[13] Side-chain protection of Cys was provided by *S*-2,4,6-trimethoxybenzyl (Tmob)[7b,c] at all four positions or by suitable pairwise combinations of *S*-Tmob and *S*-acetamidomethyl

† This paper is dedicated to the memory of Dr. Mark W. Riemen, May 7, 1953–May 12, 1993, a good friend and scientist.

® Abstract published in *Advance ACS Abstracts*, October 1, 1993.

(1) Preliminary accounts of portions of this work were presented at the following: (a) Satellite meeting to 22nd European Peptide Symposium, Interlaken, Switzerland, September 14, 1992; lecture by G. Barany. (b) Second JASPEC meeting, November 9–13, 1992, Shizuoka, Japan. See: Albericio, F.; Royo, M.; Munson, M. C.; Solé, N. A.; Van Abel, R. J.; Alsina, J.; García-Echeverría, C.; Słomczyńska, U.; Eritja, R.; Pons, M.; Giralt, E.; Barany, G. In *Peptide Chemistry 1992: Proceedings of the Second Japan Symposium on Peptide Chemistry*; Yanaihara, N., Ed.; Escom: Leiden, The Netherlands, in press.

(2) Taken in part from the Ph.D. thesis of M. C. Munson, University of Minnesota, November 1993.

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(Acm).[8e,14] The starting resins were either commercially available Fmoc-PAL-Nle-MBHA-polystyrene (PS) or a novel polyethylene glycol–polystyrene graft, MPEG-Orn(Fmoc-PAL)-AM-PS.[15] Couplings of all N^{α}-Fmoc amino acid derivatives were mediated by *N,N'*-diisopropylcarbodiimide/1-hydroxybenzotriazole (DIPCDI/HOBt) in *N,N*-dimethylformamide (DMF), except Fmoc-Asn was incorporated as its pentafluorophenyl (Pfp) active ester in the presence of HOBt. Acidolytic cleavage/deprotection of the completed peptide-resins with trifluoroacetic acid (TFA)–CH_2Cl_2–Et_3SiH–H_2O–anisole (95:5:0.5:0.5:0.5), at 25 °C for 2 h, gave the corresponding peptide amides in high yields (90–97%); those Cys residues originally blocked by *S*-Tmob were converted to the free sulfhydryls, whereas Cys(Acm) residues remained blocked. Within this context, several different protection schemes were explored, and oxidation procedures were carried out both on polymer-supported peptides and on peptides after their release into solution.

Throughout this work, overall oxidation yields were determined by comparison of reversed-phase HPLC areas of synthetic conotoxin with an authentic standard of known concentration; values are reported with respect to the amount of linear peptide originally on the support. Yields in the 35–40% range were accompanied by quite clean HPLC traces (e.g., Figures 2–5), meaning that oligomers and polymers (60–65%, *assumed*) are not detected directly by this technique. Upon reduction with dithiothreitol, the mixture of monomers, oligomers, and polymers readily coalesced into a single major peak, larger in size, corresponding to tetrasulfhydryl conotoxin. Overall yields also reflect the extent of acidolytic cleavage from the resin, which becomes a significant factor for solid-phase orthogonal oxidations due to the oligomeric network problem described in the text.

Pilot experiments were carried out on peptide-resins (~25 mg, ~4 μmol of peptide) or in solution on the crude peptides released from equivalent amounts of peptide-resins. The most promising results were scaled up 4–5-fold; the reported overall yields were validated further by preparative HPLC isolation and quantitation of pure α-conotoxin SI (typical absolute recovery of monomeric bicyclic peptide from purification was 65–75%).

Solution Oxidation of Tetrasulfhydryl α-Conotoxin SI. Classically, oxidations to provide disulfides are carried out in dilute solution, in order to minimize levels of dimer and oligomer formation.[8] The fully deprotected linear tetrasulfhydryl conotoxin was oxidized successfully to α-conotoxin SI in pH 7.5 phosphate buffer containing 1% (v/v) DMSO, according to the general approach of Tam *et al.*[16] Oxidation proceeded for 7 h at 25 °C, and the overall yield of the monomeric bicyclic peptide by this *optimized* simultaneous solution oxidation approach was 39% (see Figure 2 for HPLC documentation). Oxidation rates and yields were negligible at pH ≤6 and increased markedly with higher pH and DMSO proportion; however, solubility problems were significant at pH ≥8, and too much DMSO led to difficulties in the subsequent workup or purification, e.g., melting occurring upon attempted lyophilization, or adverse effects were noted on reversed-phase HPLC columns.

(3) Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC–IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977–983. The following additional abbreviations are used: Acm, acetamidomethyl; AM, aminomethyl (resin); cocktail I, TFA–CH_2Cl_2–Et_3SiH–H_2O–anisole (7:92:0.5:0.5:0.5); cocktail II, TFA–CH_2Cl_2–Et_3SiH–H_2O–anisole (95:4:0.5:0.5:0.5); DIPCDI, *N,N'*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; HMB, hydroxymethylbenzoyl; IRAA, "internal reference" amino acid; MBHA, 4-methylbenzhydrylamine (resin); Meb, 4-methylbenzyl; Mob, 4-methoxybenzyl; MPEG, monofunctional polyethylene glycol; Nle, norleucine; NMP, *N*-methylpyrrolidinone; Orn, ornithine; PAL, tris(alkoxy)benzylamide linker of ref 13 [5-(4-(((9-fluorenylmethyloxycarbonyl)amino)methyl)-3,5-dimethoxyphenoxy)valeric acid]; PEG-PS, polyethylene glycol–polystyrene graft support; Pfp, pentafluorophenyl ester; PS, copoly(styrene-1%-divinylbenzene) polymeric support; Tmob, 2,4,6-trimethoxybenzyl; TFA, trifluoroacetic acid; $Tl(tfa)_3$, thallium(III) trifluoroacetate. Amino acid symbols denote the L-configuration. All solvent ratios and percentages are volume/volume unless stated otherwise.

(4) Reviews: (a) Olivera, B. M.; Gray, W. R.; Zeikus, R.; McIntosh, J. M.; Varga, J.; Rivier, J.; de Santos, V.; Cruz, L. J. *Science* **1985**, *230*, 1338–1343. (b) Olivera, B. M.; Rivier, J.; Scott, J. K.; Hillyard, D. R.; Cruz, L. J. *J. Biol. Chem.* **1991**, *266*, 22067–22070. (c) Olivera, B. M.; Rivier, J.; Clark, C.; Ramilo, C. A.; Corpuz, G. P.; Abagadie, F.; Mena, E. E.; Woodward, S. R.; Hillyard, D. R.; Cruz, L. J. *Science* **1991**, *249*, 257–263.

(5) Zafaralla, G. C.; Ramilo, C.; Gray, W. R.; Karlstrom, R.; Olivera, B. M.; Cruz, L. J. *Biochemistry* **1988**, *27*, 7102–7105.

(6) The synthesis of α-conotoxin SI by a nonorthogonal approach is described, without yields, in ref 5. Syntheses in the conotoxin family using orthogonal protection strategies and solution oxidations to establish two disulfides (yields generally low, i.e., 3–10%, or not reported) include the following: (a) Nishiuchi, Y.; Sakakibara, S. *FEBS Lett.* **1982**, *148*, 260–262. (b) Gray, W. R.; Rivier, J. E.; Gaylean, R.; Cruz, L. J.; Olivera, B. M. *J. Biol. Chem.* **1983**, *258*, 12247–12251. (c) Nishiuchi, Y.; Sakakibara, S. In *Peptide Chemistry: Proceedings of the 21st Symposium on Peptide Chemistry*; Munekata, Ed.; Protein Research Foundation: Osaka, Japan, 1984; pp 191–196. (d) Gray, W. R.; Luque, F. A.; Gaylean, R.; Atherton, E.; Sheppard, R. C.; Stone, B. L.; Reyes, A.; Alford, J.; McIntosh, M.; Olivera, B. M.; Cruz, L. J.; Rivier, J. *Biochemistry* **1984**, *23*, 2796–2802. (e) Atherton, E.; Sheppard, R. C.; Ward, P. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2065–2073. (f) Myers, R. A.; Zafaralla, G. C.; Gray, W. R.; Abbott, J.; Cruz, L. J.; Olivera, B. M. *Biochemistry* **1991**, *30*, 9370–9377. (g) Zhang, R.; Snyder, G. H. *Biochemistry* **1991**, *30*, 11343–11348. (h) Akaji, K.; Fujino, K.; Tatsumi, T.; Kiso, Y. *Tetrahedron Lett.* **1992**, *33*, 1073–1076.

(7) (a) Schroll A.; Barany, G. *J. Org. Chem.* **1989**, *54*, 244–247. (b) Munson, M. C.; García-Echeverría, C.; Albericio, F.; Barany, G. In *Peptides: Chemistry and Biology*; Smith, J. A., Rivier, J. E., Eds.; Escom: Leiden, The Netherlands, 1992; pp 605–606. (c) Munson, M. C.; García-Echeverría, C.; Albericio, F.; Barany, G. *J. Org. Chem.* **1992**, *57*, 3013–3018. (d) Royo, M.; García-Echeverría, C.; Munson, M. C.; Słomczyńska, U.; Eritja, R.; Giralt, E.; Barany, G.; Albericio, F. In *Innovation and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides and Oligonucleotides*; Epton, R., Ed.; Intercept: Andover, England, 1992; pp 461–465. (e) Munson, M. C.; Lebl, M.; Slaninová, J.; Barany, G. *Peptide Res.* **1993**, *6*, 155–159. These papers describe recent experimental advances from our laboratories and provide ample citations to relevant work carried out by others.

(8) Relevant principles and experimental background have been reviewed: (a) Barany, G.; Merrifield, R. B. In *The Peptides*; Gross, E.; Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 2, pp 1–284, especially pp 233–247. (b) König, W.; Geiger, R. In *Perspectives in Peptide Chemistry*; Eberle, A.; Geiger, R.; Wieland, T., Eds.; S. Karger: Basel, 1981; pp 31–44. (c) Büllesbach, E. *Kontakte (Darmstadt)* **1992**, *1*, 21–29. (d) Fields, G. B.; Tian, Z.; Barany, G. In *Synthetic Peptides: A User's Guide*; Grant, G., Ed.; W. H. Freeman and Co.: New York, 1992; pp 72–183, especially pp 100–101, 139–141. (e) Andreu, D.; Albericio, F.; Solé, N. A.; Munson, M. C.; Ferrer, M.; Barany, G. In *Peptide Synthesis and Purification Protocols*; Pennington, M. W.; Dunn, B. M., Eds.; Humana Press: Clifton, NJ, in press.

(9) Albericio, F.; Hammer, R. P.; García-Echeverría, C.; Molins, M. A.; Chang, J. L.; Munson, M. C.; Pons, M.; Giralt, E.; Barany, G. *Int. J. Pept. Protein Res.* **1991**, *33*, 402–413 and references cited therein. This paper defines resin-bound chemistry for disulfide-bond formation.

(10) In addition to refs 6a–h in the conotoxin family, orthogonal protection schemes directed at two disulfide bridges include the following: (a) Wünsch, E.; Moroder, L.; Göhring-Romani, S.; Musiol, H.-J.; Göhring, W.; Bovermann, G. *Int. J. Pept. Protein Res.* **1988**, *32*, 368–383. (b) Ruiz-Gayo, M.; Albericio, F.; Pons, M.; Royo, M.; Pedroso, E.; Giralt, E. *Tetrahedron Lett.* **1988**, *29*, 3845–3848. (c) Koide, T.; Otaka, A.; Arai, H.; Funakoshi, S.; Fujii, N; Yajima, H. In *Peptide Chemistry*; Yanaihara, N., Ed.; Protein Research Foundation: Osaka, Japan, 1989; pp 171–174. (d) Ponsati, B.; Giralt, E.; Andreu, D. *Tetrahedron* **1990**, *46*, 8255–8266. The general approach when three (or more) bridges need to be formed involves simultaneous oxidation of the linear polysulfhydryl precursor. In a few cases, three disulfides have been introduced by elegant versions of two-dimensional orthogonal chemistry; see: (e) Sieber, P.; Eisler, K.; Kamber, B.; Riniker, B.; Rittel, W.; Märki, F. *Hoppe-Seyler's Z. Physiol. Chem.* **1978**, *359*, 113–123. (f) Hidaka, Y.; Kubota, H.; Yoshimura, S.; Ito, H.; Takeda, Y.; Shimonishi, Y. *Bull. Chem. Soc. Jpn.* **1988**, *61*, 1265–1271. (g) Maruyama, K.; Nagata, K.; Tanaka, M.; Nagasawa, H.; Isogai, A.; Ishizaki, H.; Suzuki, A. *J. Protein Chem.* **1992**, *11*, 1–12.

(11) Reviews: (a) Merrifield, R. B. *Science* **1986**, *232*, 341–347. (b) Atherton, E.; Sheppard, R. In *Solid Phase Peptide Synthesis: A Practical Approach*; IRL Press: Oxford, 1989. (c) Full content of ref 8d.

(12) (a) Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404–3409. (b) Review: Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161–214.

(13) (a) Albericio, F.; Barany, G. *Int. J. Pept. Protein Res.* **1987**, *30*, 206–216. (b) Albericio, F.; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R. I.; Hudson D.; Barany, G. *J. Org. Chem.* **1990**, *55*, 3730–3743.

(14) (a) Veber, D.; Milkowski, J. D.; Varga, S. L.; Denkewalter, R. G.; Hirschmann, R. *J. Am. Chem. Soc.* **1972**, *94*, 5456–5461. (b) Kamber, B.; Hartmann, A.; Eisler, K.; Riniker, B.; Rink, H.; Sieber, P.; Rittel, W. *Helv. Chim. Acta* **1980**, *63*, 899–915 and references cited therein.

(15) (a) Barany, G.; Solé, N. A.; Van Abel, R. J.; Albericio, F.; Selsted, M. E. In *Innovations and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides and Oligonucleotides*; Epton, R., Ed.; Intercept: Andover, England, 1992; pp 29–38. (b) Ferrer, M.; Woodward, C.; Barany, G. *Int. J. Pept. Protein Res.* **1992**, *40*, 194–207.

(16) Tam, J.; Wu, C. W.; Liu, W.; Zhang, J. *J. Am. Chem. Soc.* **1991**, *113*, 6657–6662.

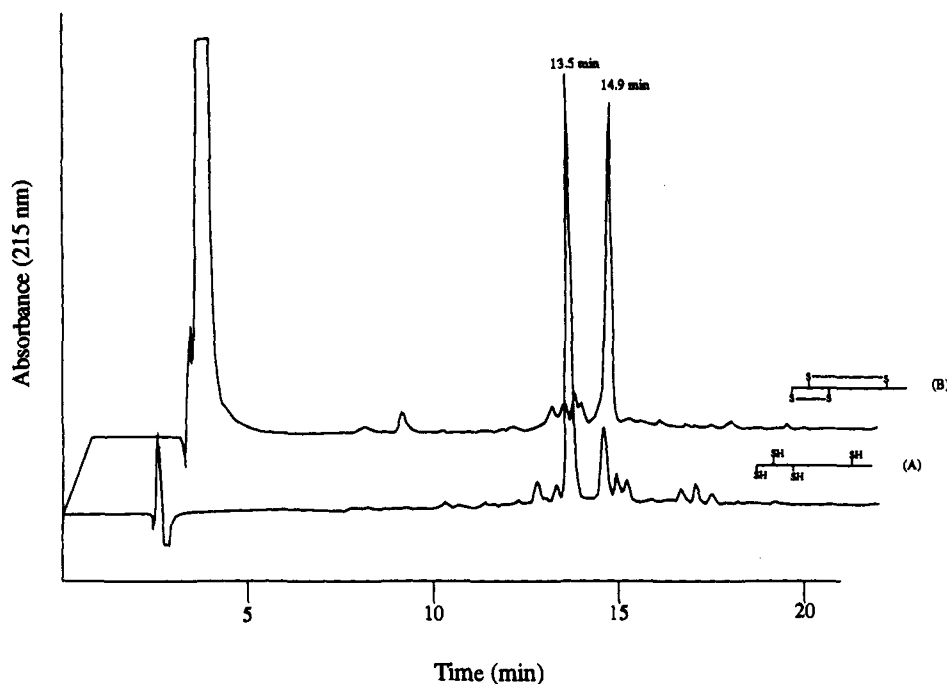


Figure 2. HPLC analysis of α-conotoxin SI before and after simultaneous DMSO oxidation in solution (see text for further details). (A) reduced conotoxin, purity 85%; (B) fully cyclized conotoxin, purity 80%. HPLC conditions are given in General.

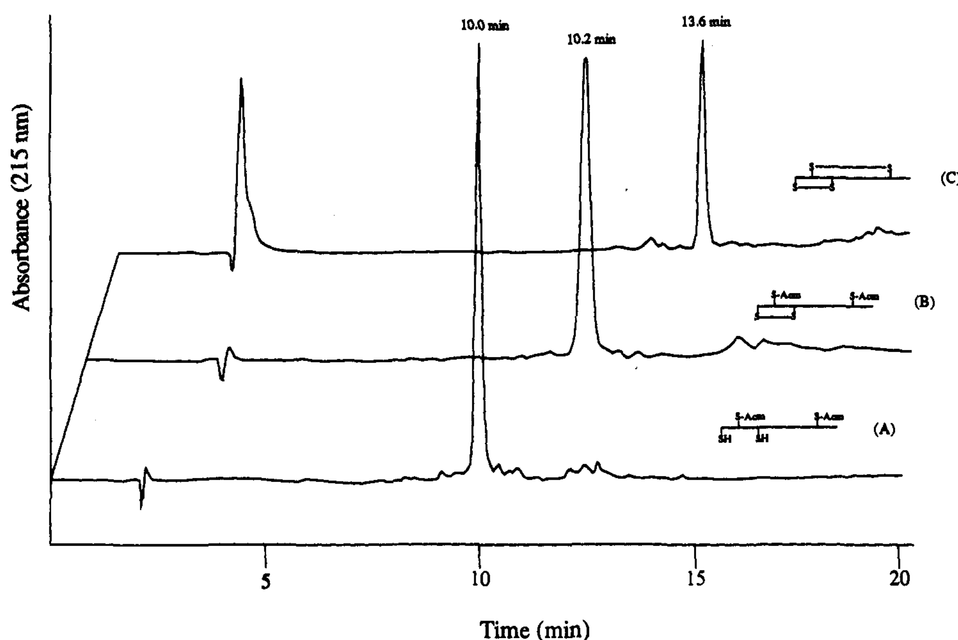


Figure 3. HPLC analysis of α-conotoxin SI at various stages during orthogonal oxidations (small loop closed first) in solution (correlate to Scheme I, right side): (A) bis(thiol), bis(Acm) intermediate, purity 95%; (B) monocyclic bis(Acm) intermediate, purity 90%; (C) fully cyclized conotoxin, purity 75%. HPLC conditions are given in General.

Orthogonal Solution Syntheses of α-Conotoxin SI. Further strategies were based on sequential disulfide pairing as dictated by the protection scheme (Schemes I and II; Figures 3 and 4). The bis(thiol), bis(Acm) intermediate (Scheme I, right side, second line) was dissolved in pH 8.0 phosphate buffer and oxidized readily with 1% (v/v) DMSO to the monocyclic bis(Acm) precursor. A 62% yield was obtained upon 3-h reaction at 25 °C; at temperatures below 5 °C, negligible oxidation occurred. The resultant intermediate with the smaller loop (Cys^2 with Cys^7) already closed was cyclized further with the Fujii reagent,[17] thallium tris(trifluoroacetate) ($Tl(tfa)_3$; 1.2 equiv), in TFA–anisole (19:1), at 4 °C for 18 h. The yield of this second oxidation was ~65%, and the final overall yield of α-conotoxin reflecting cleavage from the support and the two orthogonal oxidation steps was 38%. Alternatively, the same chemical steps and solution conditions were used on a different dithiol intermediate (Scheme II, right side), hence testing the strategy of forming the larger loop (Cys^3 with Cys^{13}) first. The two individual oxidation steps each proceeded in ~50% yield, and conotoxin was obtained in an overall yield of 23%.

Comparing the two solution approaches (Scheme I, right side, vs Scheme II, right side), it is of interest that whereas the rates of the initial monocyclization steps were comparable, the rates of the second steps to complete the bicyclo framework differed significantly. Thus, formation of the small loop with the large

(17) Fujii, N.; Otaka, A.; Funakoshi, S.; Bessho, K.; Watanabe, T.; Akaji, K.; Yajima, H. *Chem. Pharm. Bull.* **1987**, *35*, 2339–2347 and references cited therein.

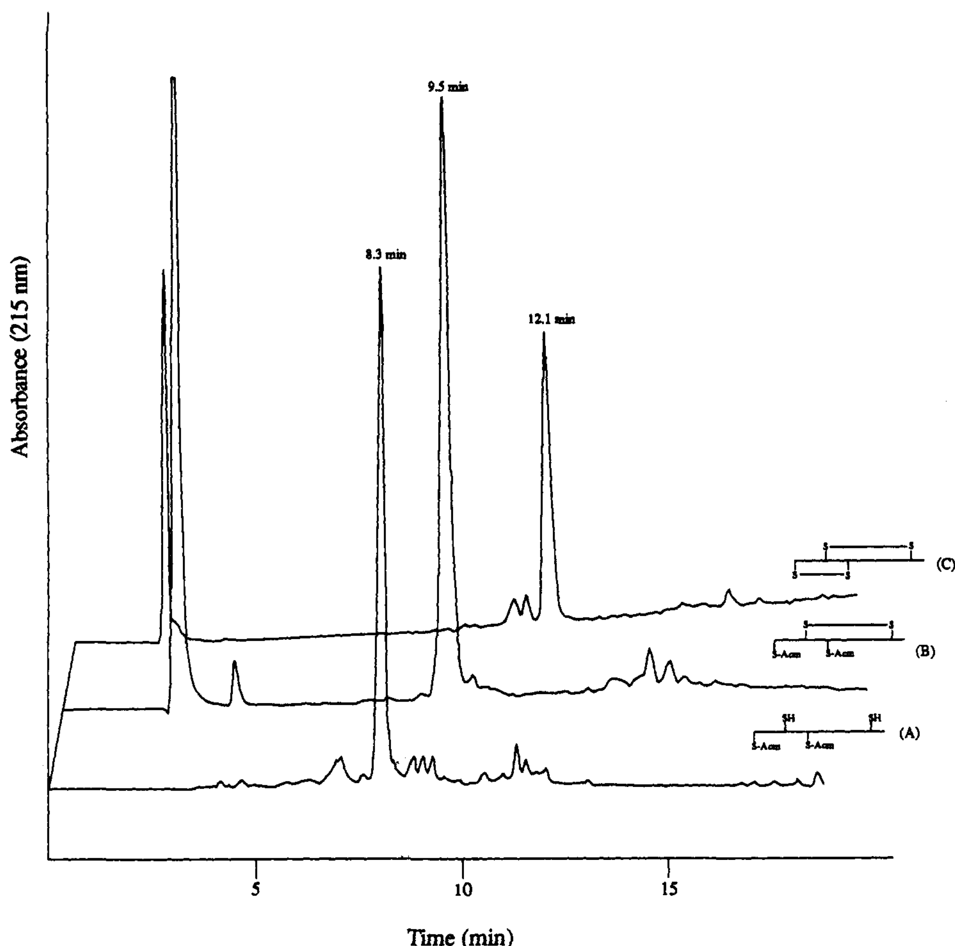


Figure 4. HPLC analysis of α-conotoxin SI at various stages during orthogonal oxidations (large loop closed first) in solution (correlate to Scheme II, right side): (A) bis(thiol), bis(Acm) intermediate, purity 84%; (B) monocyclic bis(Acm) intermediate, purity 89%; (C) fully cyclized conotoxin, purity 75%. HPLC conditions are given in General.

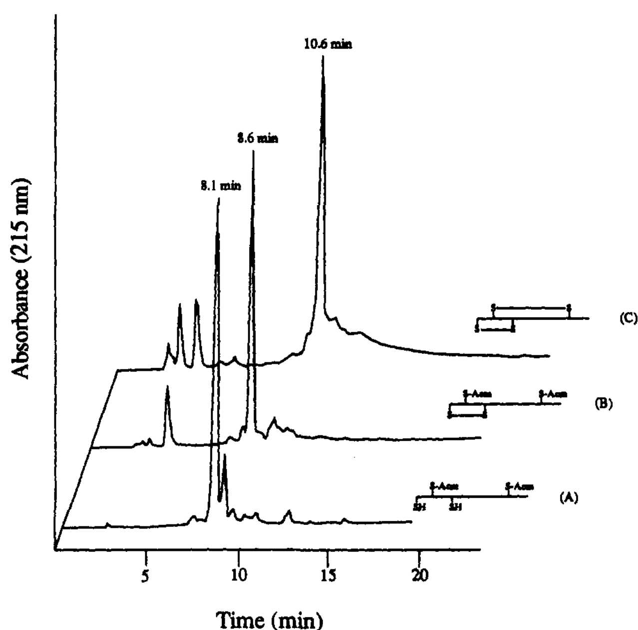


Figure 5. HPLC analysis of α-conotoxin SI at various stages during *polymer-bound* orthogonal oxidations (small loop closed first) (correlate to Scheme I, left side). (A) bis(thiol), bis(Acm) intermediate, purity 80% (with additional ~15% of the monocyclic bis(Acm) intermediate); (B) monocyclic bis(Acm) intermediate, purity 90%; (C) fully cyclized conotoxin. HPLC conditions are given in General.

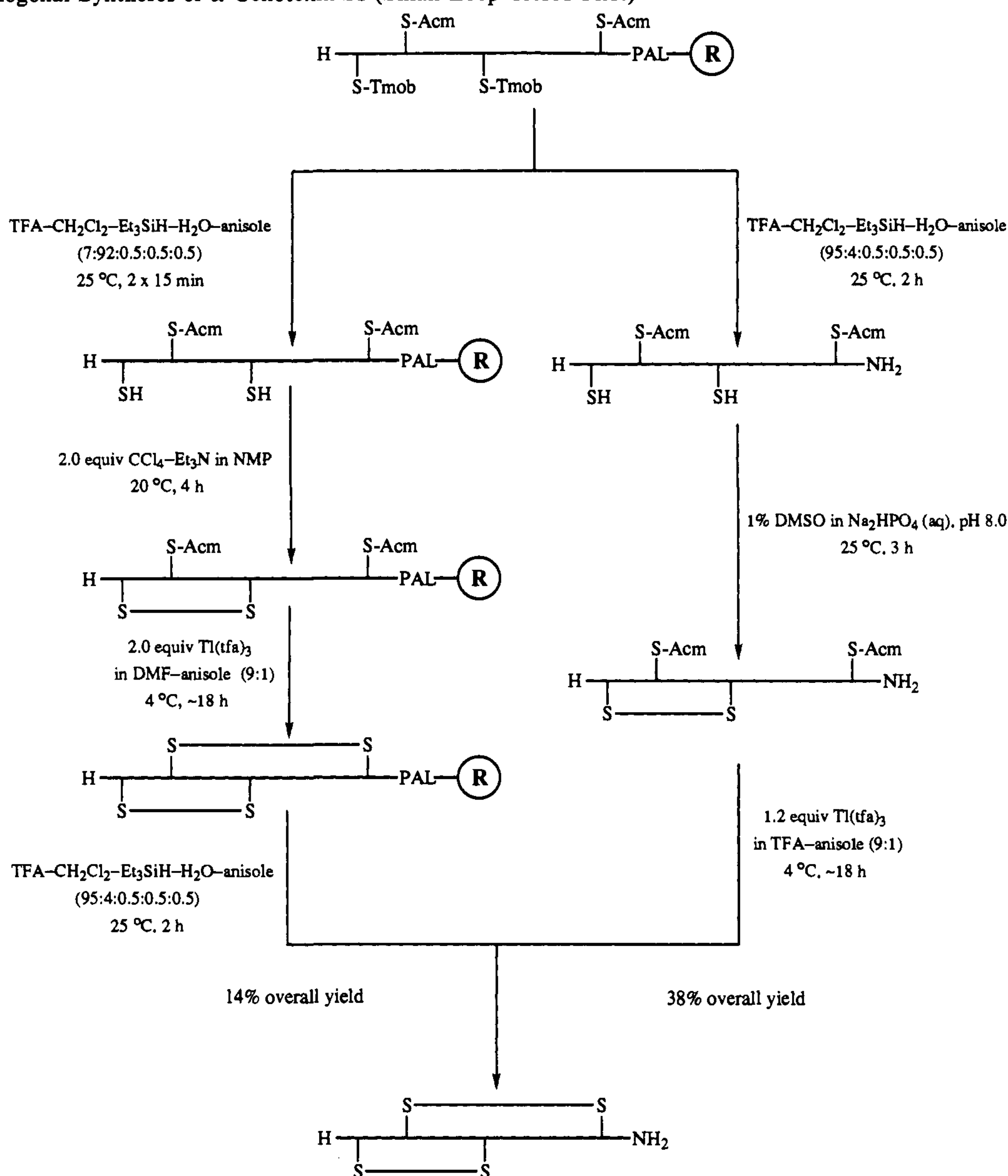
loop already in place occurred 3–4-fold more rapidly than formation of the large loop with the small loop already in place.

Regarding the initial oxidation step, Tam's DMSO procedure was very effective and compares well with lengthy aerations, or potassium ferricyanide treatments, reported in the literature.[6,8c,10]

Orthogonal Solid-Phase Synthesis of α-Conotoxin SI. Disulfide bond formation can be carried out while the peptide *remains anchored* to the polymeric support. Such approaches are predicated on the assumption that, for polymer-bound reactants, intramolecular processes are preferred due to the *pseudodilution* phenomenon;[8a,9] the solid-phase mode might also be preferred due to the relative ease of removal of excess oxidizing agents. The most promising results started with the fully protected resin-bound intermediate (Scheme I, top line), which was treated briefly with TFA–CH_2Cl_2–Et_3SiH–H_2O–anisole (7:92:0.5:0.5:0.5), for 2 × 15 min. This procedure removed selectively the Tmob groups without significant loss of chains from the support. The resultant resin-bound dithiol (Scheme I, left side, second line) was oxidized under novel conditions adapted from Wenschuh *et al.*,[18] 35 mM CCl_4–Et_3N (2 equiv each) in *N*-methylpyrrolidinone (NMP), for 4 h at 20°C, to provide the monocyclic bis(Acm) intermediate (Scheme I, left side, third line). Net yields after oxidation estimated *after* cleavage from the support (quantitation of intermediate which matched this same product from solution orthogonal synthesis) were 34–42% under the best conditions. Next, the large loop was closed by modified Fujii oxidation[9,17] (Scheme I, left side, fourth line): $Tl(tfa)_3$ (2 equiv) in DMF–anisole (19:1), for 18 h at 4 °C. Acidolytic cleavage of the resultant resin-bound protected conotoxin by the usual procedure occurred in surprisingly low yield: 27% from PS and 50% from PEG-PS (reason discussed below). In this context, the observed

(18) Wenschuh, E.; Heydenreich, M.; Runge, R.; Fischer, S. *Sulfur Lett.* **1989**, *8*, 251–260.

Scheme I. Orthogonal Syntheses of α-Conotoxin SI (Small Loop Closed First)



overall yields of fully cyclized conotoxin were a respectable 10% from PS and 14% from PEG-PS (see Figure 5 for HPLC documentation).

The resin-bound Wenschuh oxidation was carried out at 20 °C; negligible reaction occurred at < 5 °C, and reactions at >35 °C gave mainly oligomers and polymers. Relatedly, treatment of the resin-bound bis(thiol), bis(Acm) intermediate with DMSO–pH 8.0 phosphate (1:4) failed to provide any monocyclized disulfide, as primarily starting material was recovered upon acidolytic cleavage. The amount of $Tl(tfa)_3$ used in the Fujii oxidation of the bis(Acm) intermediate (Scheme I, left side) represented a compromise: reaction was incomplete with lower amounts, but reagent in excess of 1 equiv was found to promote destruction of monomeric disulfide-containing product and/or increased formation of oligomers and polymers (in accord with previous observations[9]). The oligomers apparently form a network which becomes either physically or chemically (through one or more covalent attachment points) entangled in the resin support. This interpretation is consistent with the observed reduced yields after acidolytic cleavage of the peptidyl–PAL anchoring linkage. When such resins, which retained substantial peptide after sequential synthesis, oxidations, acid cleavage, and washes, were treated *further* with dithiothreitol–Et_3N (~75 mM each) in DMF, for 3 h at 25 °C, essentially *all* of the peptide was released as the free unprotected tetrasulfhydryl conotoxin. The fact that acidolytic cleavage yields were substantially better from PEG-PS than from PS (50% vs 27%) is consistent with the idea that the former supports, by virtue of their superior solvation properties, promote higher levels of the desired intramolecular cyclization reactions.

Given the successful alternative orthogonal *solution* syntheses already mentioned, it was natural to explore a solid-phase scheme in which the large loop of α-conotoxin SI would be formed first. Unfortunately, Wenschuh oxidation at the appropriate stage (Scheme II, left side) gave negligible monocyclized disulfide. It is important to note that the larger loop includes three residues which remained blocked by *tert*-butyl ether and carbamate derivatives while oxidations were carried out on the resin, whereas those acidolyzable protecting groups were removed prior to solution oxidations. An extra level of orthogonality with allyl derivatives for side-chain protection[19] might alleviate this problem.

Solid-Phase Oxidations of Linear Tetrasulfhydryl or Tetra-(Tmob) α-Conotoxin SI. Since simultaneous oxidation can be optimized in solution, corresponding polymer-supported approaches were considered. A tetra(Tmob)-peptide-PAL-PEG-PS intermediate was treated *directly* with $Tl(tfa)_3$ (4 equiv) in

Scheme II. Orthogonal Syntheses of α-Conotoxin SI (Large Loop Closed First)

S-Tmob S-Tmob
H— —PAL—(R)
S-Acm S-Acm

$TFA-CH_2Cl_2-Et_3SiH-H_2O$–anisole
(7:95:0.5:0.5:0.5)
25 °C, 2 x 13 min

$TFA-CH_2Cl_2-Et_3SiH-H_2O$–anisole
(95:4:0.5:0.5:0.5)
25 °C, 2 h

SH SH
H— —PAL—(R)
S-Acm S-Acm

SH SH
H— —NH_2
S-Acm S-Acm

2.0 equiv CCl_4-Et_3N in NMP
15 to 40 °C, 4 to 24 h

1% DMSO in Na_2HPO_4 (aq), pH 8.0
25 °C, ~5 h

S——S
H— —PAL—(R)
S-Acm S-Acm

S——S
H— —NH_2
S-Acm S-Acm

1.2 equiv $Tl(tfa)_3$
in TFA–anisole (9:1)
0 °C, 5 h

23 % overall yield

S——S
H— —NH_2
S——S

DMF–anisole (19:1), for 3 h at 0 °C. Following acid cleavage (35% yield), three peaks were observed, upon HPLC, in a 36:8:55 ratio. The major of these peaks comigrated with monomeric bicyclic conotoxin, corresponding to a maximum overall yield of ~7% in the crude cleaved product. The two remaining peaks are presumably monomers with *mismatched* alignment of disulfides; this point needs to be proven by unambiguous orthogonal synthesis. The additional peaks did *not* occur during any of the orthogonal syntheses, either in solution or polymer-supported, that were directed at the *correct* conotoxin structure.

The resin-bound tetra(Tmob) intermediate was deblocked selectively under dilute TFA conditions already described. Wenschuh oxidation under previously optimized conditions failed, as evidenced by the absence of monomeric conotoxin after acidolytic cleavage. Only 30% of the chains were released at this point, suggesting again an extensive oligomeric network retained on the support. Additionally, polymer-supported variations of Tam's DMSO method[16] also seemed to fail, based on recovery of fully reduced conotoxin after acidolytic release of chains.

Conclusions

The studies described herein demonstrate the feasibility of several approaches for the synthesis of multiple disulfide-containing peptides. For solution procedures, experimental details are quite critical, especially with regard to solubility issues, which are often difficult to predict *a priori*. A specific illustration from this work relates to the enhanced solubility of a bis(Acm), bis(thiol) intermediate (orthogonal route) with respect to the tetrathiol (simultaneous oxidation) at the pH values most favorable for oxidation. Polymer-supported procedures are operationally more convenient and can furnish products in good initial purities, but again, success depends on strategic details. Thus, for the conotoxin model, the resin-bound approach required initial closing of the smaller loop before the larger loop, while in solution either oxidation order gave the desired product. The conditions for polymer-supported disulfide formation can undoubtedly be optimized further to result in improved yields and purities. For example, PEG-PS supports seem to provide advantageous results with respect to standard PS supports for both linear chain assembly and oxidation reactions, and the extended range of solvents that are compatible with PEG-PS offers additional variables for optimization studies.

While addressing the subtleties of oxidation conditions both in solution and on the solid phase, we recognized a need to increase the repertoire of methods for accomplishing the transformation of bis(thiol)s to disulfides. A procedure from the organosulfur literature due to Wenschuh,[18] involving as the oxidant carbon tetrachloride plus a tertiary amine in a polar aprotic solvent, has been adapted to the peptide field and is particularly suitable for the solid-phase mode (this work and ref 7e). Even so, for some

(19) (a) Belshaw, P. J.; Mzengeza, S.; Lajoie, G. A. *Synth. Commun.* **1990**, *20*, 3157–3160. (b) Lyttle, M. H.; Hudson, D. In *Peptides: Chemistry and Biology*; Smith, J. A., Rivier, J., Eds.; Escom Science Publishers: Leiden, The Netherlands, 1992, pp 583–584. (c) Loffet, A.; Zhang, H. X. In *Innovations and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides and Oligonucleotides*; Epton, R. Ed.; Intercept: Andover, England, 1992; pp 77–82.

sequences, the conditions used led either to no reaction or, conversely, to intermolecular oligomerization side reactions.

The *best* results from this work on α-conotoxin SI compare favorably to literature reports on related molecules.[6] Our Fmoc/Tmob/Acm/PAL orthogonal approaches involve milder overall conditions than the Boc/Meb, Mob/Acm/MBHA, or Fmoc/StBu/Acm/HMB strategies used by others. Ours are among the first examples where one or both disulfide-forming steps for complex targets have been carried out at the peptide-resin level.[1,7e,8e,10b] Ongoing work seeks to generalize the strategies and tactics reported herein to additional multiple disulfide-containing targets such as apamin, endothelin, and enterotoxin analogues.

Experimental Section

General. Most of the materials, solvents, instrumentation, and general methods have been described and summarized in previous publications from these laboratories.[7c,9,13,15] Analytical HPLC (Figures 2–5) was performed using a Vydac analytical C-18 reversed-phase column (10-μm particle size; 0.46 × 25.0 cm) on a Beckman system configured with two Model 112 pumps and a Model 165 variable wavelength detector controlled from an IBM computer with Beckman System Gold software. Peptide samples were chromatographed at 1.2 mL/min using 0.01 N aqueous HCl–CH_3CN (9:1 to 4:6 over 20 min), detection at 215 nm. Differences in absolute retention times of α-conotoxin SI (Figures 2–5) reflect changes in the properties of the C-18 column during the time that these various experiments were conducted; the identities of peaks were always verified by co-injection with standards. Preparative HPLC was performed using a Vydac semi-preparative C-18 reversed-phase column (10-μm particle size; 2.2 × 25.0 cm) on a Waters Deltaprep system using manual injection (1-mL loop size) and elution at 14 mL/min using 0.01 N aqueous HCl–CH_3CN (20:1 to 6:4 over 25 min), detection at 280 nm. Fast atom bombardment mass spectrometry (FABMS) to characterize synthesized peptides and peptide intermediates was carried out in glycerol or thioglycerol matrices on a VG Analytical, Ltd., 707E-HF high-resolution double-focusing mass spectrometer equipped with a VG 11/250 data system, operated at a resolution of 3000.

Suitably protected N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) amino acids were from Advanced Chemtech (Louisville, KY), except for Fmoc-Cys(Tmob)-OH, which was prepared according to ref 7c. A standard sample of α-conotoxin SI was from Bachem Bioscience (Philadelphia, PA). The 5-(4-(((9-fluorenylmethyloxycarbonyl)amino)methyl)-3,5-dimethoxyphenoxy)valeric acid (PAL handle)[13] was from Millipore (Bedford, MA). Starting resins for this work were either Fmoc-PAL-Nle-MBHA-PS (~0.27 mmol/g, from Millipore) or MPEG-Orn(Fmoc-PAL)-AM-PS graft (~0.25 mmol/g; prepared in our laboratory as outlined in ref 15). Automated chain assemblies were carried out on ~300-mg scales of starting resin, using a MilliGen/Biosearch Model 9600 (batchwise) peptide synthesizer. For stepwise chain assembly, Fmoc-amino acids (5–7 equiv) were incorporated by 1-h couplings (except Ile, 90 min) mediated by DIPCDI/HOBt (DIPCDI and HOBt, 5–7 equiv each, final concentration of 0.23 M in 5.0 mL of DMF). Asparagine, with its ω-carboxamide side chain unprotected, was incorporated as its pentafluorophenyl (Pfp) active ester (5–7 equiv, for 1 h in the presence of HOBt, *no* DIPCDI). Fmoc groups were removed during stepwise synthesis by piperidine–DMF (1:4, 3 + 7 min, 5 mL), followed by washes with DMF (5 × 2 min, 5 mL).

Completed peptide-resins were stored at 4 °C, with the N-terminal Fmoc group still attached. Where called for in the synthetic plan, resin-bound deprotections and oxidations were performed in 6-mL Teflon-capped polypropylene plastic syringes fitted with porous frits, under constant slow agitation with a mechanical rotator through a 360° arc. In particular, prior to all oxidative transformations and/or acid cleavages or deprotections, N-terminal Fmoc was removed manually by treatment of the peptide-resin (~25 mg) with piperidine–DMF (1:3; 3 + 7 min, 2 mL), followed by washes with DMF (5 × 2 min, 2 mL). In addition, *S*-Tmob protecting groups were removed selectively from resin-bound chains with TFA–CH_2Cl_2–Et_3SiH–H_2O–anisole, 7:92:0.5:0.5:0.5 (dilute TFA, cocktail I, 2 mL), for 2 × 15 min at 25 °C. Peptide chains were released from supports, with concomitant removal of acid-labile side-chain protecting groups, by use of TFA–CH_2Cl_2–Et_3SiH–H_2O–anisole, 95:4:0.5:0.5:0.5 (concentrated TFA, cocktail II, 2 mL), for 2 h at 25 °C. The filtrates from the cleavage reactions were collected, combined with washes of TFA (3 × 2 min, 1 mL), and concentrated *in vacuo*. Cold diethyl ether (~5 mL) was added at ~0 °C to precipitate the crude cleaved peptides; after trituration for 2 min, the peptides were collected upon centrifugation and decantation of the ether. Solution oxidations were carried out in 10-mL round-bottom flasks under continuous and vigorous magnetic stirring, and similar ether precipitation procedures were followed to isolate the peptide intermediates and products for further analysis, purification, and/or chemical transformations.

Amino acid analyses were carried out on a Beckman 6300 analyzer. Samples were hydrolyzed with propionic acid–12 N HCl (1:1), 1 drop of phenol at 110 °C, 24 h for peptide-resins, or with 6 N aqueous HCl at 110 °C, 24 h for released peptides. Reported cleavage yields are based on amino acid ratios, with respect to norleucine (Nle) or ornithine (Orn) "internal reference" amino acid (IRAA) of recovered peptide-resins after cocktail II treatment; the values reported were consistent with direct quantitation by hydrolysis and amino acid analysis of the material released into solution. Isolated amounts of peptides reported for final yields are based on amino acid analysis, rather than weight.

Optimized Non-Orthogonal Solution Synthesis of α-Conotoxin SI from MPEG-Orn[H-Ile-Cys²(Tmob)-Cys³(Tmob)-Asn-Pro-Ala-Cys⁷(Tmob)-Gly-Pro-Lys(Boc)-Tyr(*t*Bu)-Ser(*t*Bu)-Cys¹³(Tmob)-NH-PAL]-AM-PS. The peptide-resin (25 mg) was swollen in CH_2Cl_2 (2 mL) for 5 min, filtered, and cleaved with cocktail II (90–95% cleavage yield) to provide crude, tetrasulfhydryl conotoxin in >85% purity (Figure 2A). A portion of the solid material (after ether precipitation) was characterized by FABMS: *m/z* calcd for $C_{55}H_{88}N_{16}O_{16}S_4$ 1356.5; found 1357.4 [MH^+]. The remainder of the crude cleaved peptide (~5.8 mg) was dissolved in 0.01 M phosphate, pH 7.5 (4.0 mL), and DMSO (40 μL) was added to 1% (v/v). After 7 h of constant stirring at 25 °C, the oxidation mixture was lyophilized to provide the bicyclic peptide (~80% purity; Figure 2B), with salts.

On a preparative scale starting with the peptide-resin (75 mg), the reduced peptide (16.6 mg, 11.5 μmol) was dissolved in 0.01 M phosphate, pH 7.5 (12.0 mL), and DMSO (0.12 mL) was then added. After 7-h reaction at 25 °C, oxidation was quenched by lyophilization, and the crude peptide was purified by preparative HPLC. Yield: 4.3 mg (3.2 μmol; 24% absolute yield, ~65% recovery for chromatography) of α-conotoxin SI. The identity of the product was verified by co-injection with a standard and by FABMS: *m/z* calcd for $C_{55}H_{84}N_{16}O_{16}S_4$ 1352.51; found 1353.5 [MH^+]. Amino acid analysis: Ala 0.96, Asp 0.98, Gly 0.96, Ile 0.89, Lys 1.04, Pro 2.15, Tyr 0.95, Cys and Ser not determined.

Solid-Phase Non-Orthogonal Oxidation of MPEG-Orn[H-Ile-Cys²-(Tmob)-Cys³(Tmob)-Asn-Pro-Ala-Cys⁷(Tmob)-Gly-Pro-Lys(Boc)-Tyr-(tBu)-Ser(tBu)-Cys¹³(Tmob)-NH-PAL]-AM-PS. The peptide-resin (25 mg) was swollen in DMF (2 mL) for 5 min, filtered, and then treated with 25 mM $Tl(tfa)_3$ (7.0 mg; 2.3 equiv) in DMF–anisole (19:1, 0.3 mL). After 3 h at 4 °C, the Tl reagent was removed by washing with DMF (5 × 2 min, 2 mL) and CH_2Cl_2 (5 × 2 min, 2 mL). Treatment with cocktail II provided crude peptide (3.0 mg, 35% overall cleavage yield), which constituted three HPLC peaks in the ratio 36:8:55 (t_R 12.6, 12.9, and 13.7 min, respectively). The largest and latest eluting of these peaks corresponded to the desired α-conotoxin SI, as verified by co-injection with a standard. Comparison of HPLC areas revealed that the maximum overall yield of monomeric conotoxin in the crude cleaved peptide was ~7%.

Orthogonal Solution Synthesis of α-Conotoxin SI from MPEG-Orn-[H-Ile-Cys²(Tmob)-Cys³(Acm)-Asn-Pro-Ala-Cys⁷(Tmob)-Gly-Pro-Lys-(Boc)-Tyr(tBu)-Ser(tBu)-Cys¹³(Acm)-NH-PAL]-AM-PS (Scheme I, Right Side). The peptide-resin (25 mg) was swollen in CH_2Cl_2 (2 mL) for 5 min, filtered, and treated with cocktail II (~90% cleavage yield) to provide the crude bis(thiol), bis(Acm) intermediate (>95% purity; Figure 3A). A portion of the solid (after ether precipitation) was characterized by FABMS: *m/z* calcd for $C_{61}H_{98}N_{18}O_{18}S_4$ 1498.62; found 1499.6 [MH^+] and 1521.6 [$(M + Na)^+$]. The remainder of the crude cleaved peptide (6.1 mg) was dissolved in 0.01 M phosphate, pH 8.0 (4.0 mL), and DMSO (40 μL) was added to 1% (v/v). After 3 h at 25 °C, the oxidation was quenched by lyophilization, to provide the monocyclic (bis)Acm intermediate (Cys² → Cys⁷ loop closed) (~90% purity; Figure 3B). Yield: 4.2 mg (2.8 μmol; ~62% oxidation yield); FABMS: *m/z* calcd for $C_{61}H_{96}N_{18}O_{18}S_4$ 1496.62; found 1497.8 [MH^+]. Next, the solid, crude monocyclic (bis)Acm intermediate (~2.8 μmol) was dissolved in TFA–anisole (19:1, 3.0 mL), chilled, and further cyclized upon addition of $Tl(tfa)_3$ (1.8 mg; 1.2 equiv). After ~18 h at 4 °C, the reaction mixture was concentrated; the peptide was then precipitated with diethyl ether (10 mL), triturated for 2 min, centrifuged, and decanted. The trituration/centrifugation cycle was repeated two more times to ensure that the Tl

salt was removed. This procedure provided crude, bicyclic α-conotoxin SI (~75% purity; Figure 3C). Yield: 2.1 mg (1.5 μmol; ~35% overall yield).

On a preparative scale starting with the peptide-resin (80 mg), the bis(thiol), bis(Acm) peptide (19.5 mg, 13.0 μmol) was subjected to the same series of transformations described (scaled up proportionally in all aspects), and the crude product was purified by preparative HPLC. Yield: 5.2 mg (3.8 μmol; 27% absolute yield, 70% recovery for chromatography) of α-conotoxin SI. The identity of the product was verified by co-injection with a standard and by FABMS: *m/z* calcd for $C_{55}H_{84}N_{16}O_{16}S_4$ 1352.51; found 1353.6 [MH$^+$] and 1375.5 [(M + Na)$^+$]. Amino acid analysis: Ala 0.91, Asp 1.04, Gly 1.04, Ile 0.90, Lys 1.06, Pro 2.18, Tyr 0.98, Cys and Ser not determined.

Orthogonal Solution Synthesis of α-Conotoxin SI from MPEG-Orn-[H-Ile-Cys^2(Acm)-Cys^3(Tmob)-Asn-Pro-Ala-Cys^7(Acm)-Gly-Pro-Lys-(Boc)-Tyr(tBu)-Ser(tBu)-Cys^{13}(Tmob)-NH-PAL]-AM-PS (Scheme II, Right Side). The peptide-resin (25 mg) was swollen in CH_2Cl_2 (2 mL) for 5 min, filtered, and treated with cocktail II (93% cleavage yield) to provide the crude bis(Acm), bis(thiol) intermediate (~84% purity; Figure 4A). A portion of the solid (after ether precipitation) was characterized by FABMS: *m/z* calcd for $C_{61}H_{98}N_{18}O_{18}S_4$ 1498.62; found 1520.0 [(M + Na)$^+$]. The remainder of the crude peptide, which included 4.2 μmol of the bis(Acm), bis(thiol) intermediate, was dissolved in 0.01 M phosphate, pH 8.0 (4.0 mL), and DMSO (40 μL) was added to 1% (v/v). After 5 h at 25 °C, the oxidation was quenched by lyophilization to provide the (bis)Acm monocyclic intermediate (Cys^3→Cys^{13} loop closed) in ~89% purity (Figure 4B). Yield: 3.5 mg (2.3 μmol; ~53% oxidation yield). FABMS: *m/z* calcd for $C_{61}H_{96}N_{18}O_{18}S_4$ 1496.60; found 1519.5 [(M + Na)$^+$]. Next, the solid, crude (bis)Acm, monocyclic intermediate (3.5 mg, 2.3 μmol) was dissolved in TFA–anisole (19:1, 2.5 mL), chilled, and further cyclized upon addition of $Tl(tfa)_3$ (1.5 mg; 1.2 equiv). After ~5 h at 4 °C, the reaction mixture was concentrated and worked up as described with the preceding procedure, to provide crude, bicyclic α-conotoxin SI in ~75% purity (Figure 4C). Yield: 1.5 mg (1.1 μmol; ~53% for first cyclization, ~50% for second cyclization; ~23% overall yield). FABMS: *m/z* calcd for $C_{55}H_{87}N_{16}O_{16}S_4$ 1352.51; found 1353.6 [MH$^+$] and 1375.5 [(M + Na)$^+$].

Orthogonal Solid-Phase Synthesis of α-Conotoxin SI from MPEG-Orn[H-Ile-Cys^2(Tmob)-Cys^3(Acm)-Asn-Pro-Ala-Cys^7(Tmob)-Gly-Pro-Lys(Boc)-Tyr(tBu)-Ser(tBu)-Cys^{13}(Acm)-NH-PAL]-AM-PS (Scheme I, Left Side). The peptide-resin (25 mg) was swollen in CH_2Cl_2 (2 mL) for 5 min, filtered, and treated with cocktail I to deprotect selectively *S*-Tmob protecting groups from Cys^2 and Cys^7. A portion of this resin-bound intermediate was released from the support with cocktail II (92% cleavage yield) to provide the crude bis(thiol), bis(Acm) intermediate in ~84% purity (Figure 5A). This solid was characterized by FABMS: *m/z* calcd for $C_{61}H_{98}N_{18}O_{18}S_4$ 1498.62; found 1499.65 [MH$^+$].

Continuing on toward the monocyclized product (closure of Cys^2→Cys^7 loop), the resin-bound bis(thiol), bis(Acm) intermediate was washed with CH_2Cl_2, DMF, and NMP (each for 5 × 2 min, 2 mL), swollen further in NMP (2 mL) for 5 min, and then treated with 35 mM CCl_4 (0.9 μL) and Et_3N (1.0 μL) (2 equiv each) in NMP (0.4 mL). After 4 h at 20 °C, the excess oxidizing agent was removed by filtration and washing with DMF (5 × 2 min, 2 mL). The peptide-resin was washed further with CH_2Cl_2 (5 × 2 min, 2 mL) and finally treated with cocktail II (~85% cleavage yield). The standard workup (see General) provided the monocyclic bis(Acm) intermediate in ~90% purity (Figure 5B). Yield: 3.1 mg (2.1 μmol; ~47% oxidation yield). FABMS: *m/z* calcd for $C_{61}H_{96}N_{18}O_{18}S_4$ 1496.62; found 1497.6 [MH$^+$] and 1521.6 [(M + Na)$^+$].

Following resin-bound closure of the Cys^2→Cys^7 loop, the peptide-resin (25 mg) was swollen in DMF (2 mL) for 5 min, filtered, and then treated with 25 mM $Tl(tfa)_3$ (4.9 mg; 2 equiv) in DMF–anisole (19:1, 0.35 mL) to effect closure of the Cys^3→Cys^{13} loop. After ~18 h at 4 °C, the Tl reagent was removed with DMF (5 × 2 min, 2 mL) and the peptide-resin washed further with CH_2Cl_2 (5 × 2 min, 2 mL). Cleavage with cocktail II (50% overall cleavage yield) followed by standard workup (see General) provided crude α-conotoxin SI (Figure 5C), which included 0.7 μmol of monomer (~14% overall yield).

On a preparative scale, the peptide-resin (90 mg) was subjected to the same series of transformations described above (scaled up proportionally in all aspects), and the crude product was purified by preparative HPLC. Yield: 2.2 mg (1.6 μmol; ~10% absolute yield based on loading of the completed peptide-resin) of α-conotoxin SI. The identity of this product was verified by co-injection with a standard and characterized by FABMS: *m/z* calcd for $C_{55}H_{87}N_{16}O_{16}S_4$ 1352.51; found 1353.6 [MH$^+$] and 1375.5 [(M + Na)$^+$]. Amino acid analysis: Ala 1.01, Asp 1.05, Gly 1.10, Ile 0.88, Lys 1.02, Pro 2.12, Tyr 1.00, Cys and Ser not determined.

Additional experiments probed the reason for the lower than usual yield upon acidolytic cleavage of the peptidyl–PAL anchoring linkage *subsequent* to resin-bound oxidations. Following removal of the Tl reagent with DMF (5 × 2 min, 2 mL), the peptide-resin (25 mg) was swollen in DMF (2 mL) for 5 min and then treated with a solution of dithiothreitol (12 mg) and triethylamine (15 μL) (75 mM each) in DMF (2 mL) for 3 h at 25 °C. The peptide-resin was then washed with DMF (5 × 2 min, 2 mL) and CH_2Cl_2 (5 × 2 min, 2 mL) and treated with cocktail II. The cleaved material matched the product isolated from a direct cleavage of the tetra(Tmob) protected peptide-resin (see Figure 2A for fully reduced α-conotoxin SI). A portion of the solid material (after ether precipitation) was characterized by FABMS: *m/z* calcd for $C_{55}H_{88}N_{16}O_{16}S_4$ 1356.5; found 1357.2 [MH$^+$].

The title experiment was also repeated starting with an Fmoc-PAL-Nle-MBHA polystyrene (PS) support (~300 mg). The protected conotoxin-resin (final loading ~ 0.23 mmol/g) was transformed by the same procedures; the first (closure of Cys^2→Cys^7) and second (closure of Cys^3→Cys^{13}) oxidation steps proceeded in ~40% and ~23% yields, respectively (optimized protocols). The yields of acidolytic cleavage (cocktail II) were 25–30%, for reasons discussed in the text. Other analytical data corresponded to the text orthogonal solid-phase synthesis on PEG-PS.

Acknowledgment. We thank Dr. Nuria Solé for the PEG-PS (ref 15a) used in this work, Dr. Ed Larka for mass spectra, and Ms. Kris Kishel for enthusiastic assistance, and we express gratitude to the National Institutes of Health (GM 28934, 42722, and 43552) for financial support.