Convenient Total Synthesis of a 4-Helix TASP Molecule by Chemoselective Ligation

Philip E. Dawson and Stephen B. H. Kent*

Contribution from The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037

Received April 6, 1993

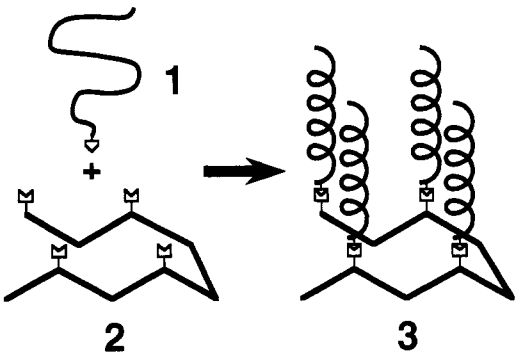
Abstract: The chemoselective ligation approach to the preparation of complex peptides has been used in a simple, convenient synthesis of a template-assembled synthetic protein (TASP) molecule. Reaction of a readily prepared synthetic *pro*-helical peptide–αCOSH, in unprotected form, with a synthetic $(BrAc)_4$ template molecule, also in unprotected form, proceeded rapidly in aqueous solution to give a uniform product in high yield. The resulting 4-helix TASP was simply purified to homogeneity, and the covalent structure was defined by ion-spray mass spectrometry [obs MW 6647.1 ± 2.8 D; calc 6645.6 (monoisotopic), 6649.9 (average isotope composition)]. The conformation of the resulting macromolecule was determined by circular dichroism (CD) and was highly helical. The chemoselective ligation of unprotected peptides represents a general approach to the synthesis of TASP molecules.

Introduction

The design and chemical synthesis of proteins has been a goal of organic chemists since the time of Emil Fischer.[1] In recent years there has been a growing interest in the *de novo* design of proteins, particularly helix-bundle proteins, and their production by chemical synthesis or recDNA-expression means.[2-4] In some cases, unexpected results have been obtained in which the experimentally observed structure of synthetic helix bundles has been different than the intended structure[5] because of the uncontrolled nature of noncovalent intermolecular association. To avoid this problem, helix-bundle proteins have been made by the preparation of covalent arrays linked through porphyrin molecules or through metal chelate complexes.[6] Preliminary evidence indicates that the expected structures have been achieved.

An alternative, earlier approach to the preparation of covalent peptide arrays of predetermined secondary and tertiary structure is the "template-assembled synthetic protein" (TASP) concept.[7] A template molecule is used to covalently anchor arrays of secondary structural elements. The distinctive feature of the TASP approach is the nonlinear topology used; the molecule is made up of an array of *branched* polypeptides,[8] rather than the folded *linear* polypeptide chain of natural proteins. This elegant concept has promised to have a profound effect on the *de novo* design of proteins. However, the reality of currently-used synthetic approaches to the preparation of TASP molecular assemblies has not lived up to the conceptual elegance. Both stepwise solid-phase (SPPS)[9] and protected-segment condensation[10] approaches have been used, with limited success. Only a minimal number of TASP molecules have been produced by arduous synthetic efforts.[6-11] Despite the exquisite care with which some of these syntheses have been performed,[9] questions still remain with respect to TASP preparations as homogeneous molecular species of defined covalent composition. A convenient, direct general preparation of these molecules in unambiguous fashion would have great utility.

Scheme I. Synthesis of a 4-Helix TASP by Chemoselective Ligation



Recently, we introduced the chemoselective ligation of unprotected peptide segments as a route to the total chemical synthesis of protein analogs of native (i.e. linear) topology.[12] This approach uses unique, mutually reactive functionalities, one type on each segment, to covalently assemble long-chain molecules from completely unprotected peptide segments. In this way, maximal advantage is taken of our ability to synthesize, handle, purify, and characterize unprotected peptides. Solubility problems are reduced, and the target molecule is produced directly in the final unprotected form.

* Corresponding author: phone, (619) 554-7048; FAX, (619) 554-6411; email, steve@kent.portal.com.

(1) Fischer, E. In *Molecules and Life*; Fruton, J. S., Ed.; Wiley Interscience: New York, 1972; pp 113–114.

(2) Hecht, M. H.; Richardson, J. S.; Richardson, D. C.; Ogden, R. C. *Science* **1990**, *249*, 884–891.

(3) Regan, L.; DeGrado, W. F. *Science* **1988**, *241*, 976–978.

(4) (a) DeGrado, W. F.; Wasserman, Z. R.; Lear, J. D. *Science* **1989**, *243*, 622–628. (b) Zhou, N. E.; Kay, C. M.; Hodges, R. S. *Biochemistry* **1992**, *31*, 5739–5746.

(5) Lovejoy, B.; Choe, S.; Cascio, D.; McRorie, D. K.; DeGrado, W. F.; Eisenberg, D. *Science* **1993**, *259*, 1288–1293.

(6) (a) Sasaki, T.; Kaiser, E. *J. Am. Chem. Soc.* **1989**, *111*, 380–381. (b) Ghadiri, R. M.; Soars, C.; Choi, C. *J. Am. Chem. Soc.* **1992**, *114*, 4000–4002. (c) Akerfeldt, K. S.; Kim, R. M.; Camac, D.; Groves, J. T.; Lear, J. D.; DeGrado, W. F. *J. Am. Chem. Soc.* **1992**, *114*, 9656–9657.

(7) Mutter, M. In *Peptides—Chemistry and Biology, Proceedings of the 10th American Peptide Symposium*; Marshall, G. R., Ed.; Escom: Leiden, The Netherlands, 1988; pp 349–353.

(8) Mutter, M.; Vuilleumier, S. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 535–554.

(9) Mutter, M.; Tuchscherer, G. G.; Miller, C.; Altmann, K.-H.; Carey, R. I.; Wyss, D. F.; Labhardt, A. M.; Rivier, J. E. *J. Am. Chem. Soc.* **1992**, *114*, 1463–1470.

(10) (a) Dörner, B.; Carey, R. I.; Mutter, M.; Labhardt, A. M.; Steiner, V.; Rink, H. *Innovation and Perspectives in Solid Phase Synthesis*; Epton, R., Ed.; Intercept Limited: Andover, MA, 1992; pp 163–170. (b) Ernest, I.; Vuilleumier, S.; Fritz, H.; Mutter, M. *Tetrahedron Lett.* **1990**, *31*, 4015–4018.

(11) (a) Mutter, M.; Altmann, K.-H.; Tuchscherer, G.; Vuilleumier, S. *Tetrahedron* **1988**, *44*, 771–785. (b) Mutter, M.; Hersperger, R.; Gubernator, K.; Müller, K. *Proteins* **1989**, *5*, 13–21. (c) Tuchscherer, G.; Servis, C.; Corradin, G.; Blum, U.; Rivier, J.; Mutter, M. *Protein Sci.* **1992**, *1*, 1377–1386.

(12) Schnölzer, M.; Kent, S. B. H. *Science* **1992**, *256*, 221–225.

0002-7863/93/1515-7263$04.00/0

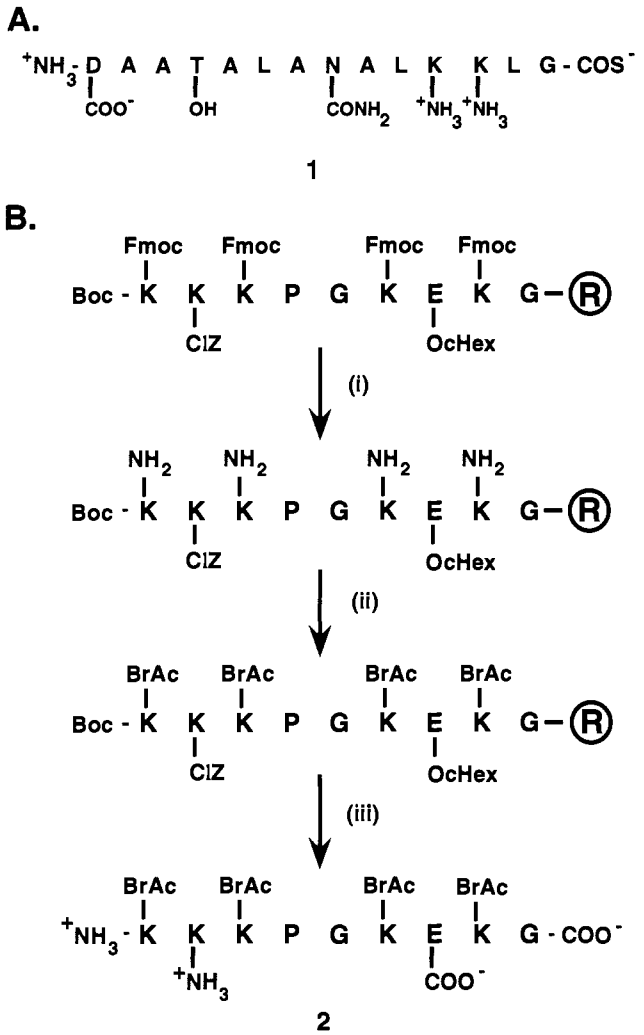


Figure 1. Unprotected synthetic peptides for use in the TASP synthesis. A. The fourteen residue *pro*-helix peptide–$^{\alpha}$thiocarboxylate **1**, prepared by SPPS on a thioester resin.[14] B. The nine-residue peptide template **2**, prepared by SPPS using N$^{\alpha}$Boc protection and both base- and acid-labile side-chain protection to allow differential modification of the side chains: (i) 50% piperidine in DMF; (ii) bromoacetic anhydride in DCM; (iii) HF/10% *p*-cresol, 1 h, 0 °C. Details in Experimental Section.

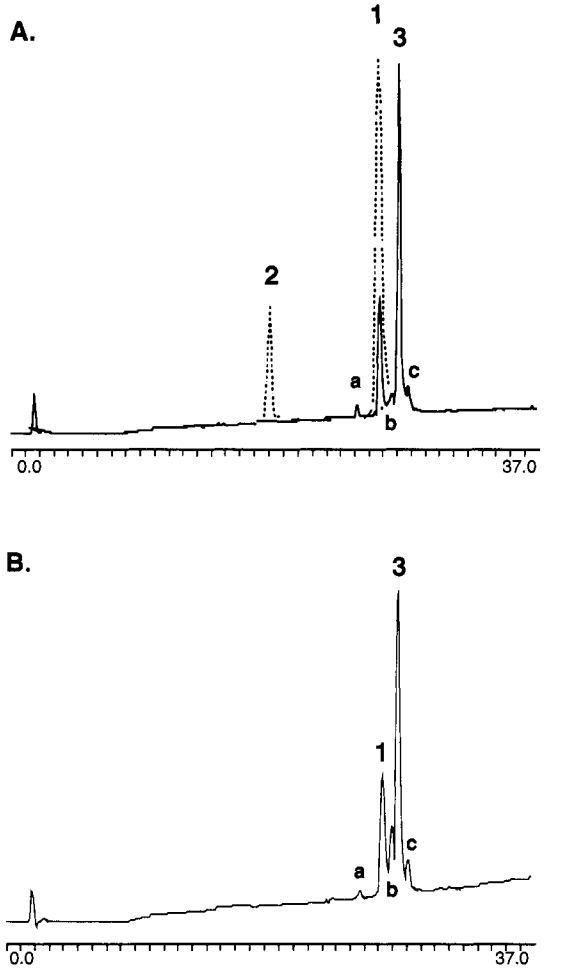


Figure 2. Reaction of the *pro*-helix peptide–$^{\alpha}$thiocarboxylate **1** with the $(BrAc)_4$ template peptide **2**, in aqueous solution at pH 5. A. Analytical HPLC of the reaction mixture after 5 h of reaction. The product 4-helix TASP **3** is indicated. Positions and original amounts of the reactants are indicated by dashed-line peaks. Minor components were identified by ion-spray MS and are **a**, *pro*-helix peptide–$^{\alpha}$carboxylate, formed by hydrolysis of the αthiocarboxylate, **b**, $(pro\text{-helix})_3(BrAc)_1$ template, and **c**, dimer $(pro\text{-helix}\text{-}^{\alpha}COS\text{-})_2$, formed by atmospheric oxidation. B. Analytical HPLC of the reaction mixture after 70 min. Products are labeled and identified as above.

We have explored the utility of the chemoselective ligation approach in the synthesis of TASP molecules. The approach used is shown in Scheme I. The TASP is assembled from short unprotected peptide segments, which are synthesized in straightforward fashion by standard methods and which are readily purified to high levels of homogeneity. The target 4-helix TASP molecule was designed on the basis of the work of Mutter.[11a] The final molecule contains a total of four copies of the helix-forming peptide **1**, one copy attached to the side chain of each of four lysine residues in a template molecule. The template molecule **2** contains a central Gly-Pro sequence to facilitate the formation of a reverse-turn structure and to thus promote the association of the helix-forming peptides.[11a]

Results and Discussion

The synthetic peptide **1**, intended to form a 13-residue amphipathic helix under suitable helix-promoting conditions, was prepared with an additional C-terminal -Gly$^{\alpha}$COSH residue (Figure 1A). This *pro*-helix peptide was reacted with the template peptide **2** containing four lysine side chains modified to contain bromoacetyl moieties (Figure 1B). Peptides were chemically synthesized by manual stepwise solid-phase methods according to published procedures.[13] Acidolytic cleavage of the *pro*-helix peptide from a thioester resin[14] generated the peptide–$^{\alpha}$COSH **1**. A combination of base-labile and acid-labile protecting groups was used to generate the template molecule **2** with four of the five lysine side chains modified with bromoacetyl groups[15] (Figure 1B). The range of functionalities present in the two peptides is shown in Figure 1.

The unprotected peptides were simply ligated in the desired fashion by nucleophilic reaction between the $^{\alpha}$COSH moiety of the *pro*-helix peptide **1** and the bromoacetyl functionalities of the template molecule **2**. The reaction proceeded cleanly over several hours at ambient temperature in aqueous buffer at pH 5.0 to give a near-quantitative yield of the 4-helix TASP molecule **3** (Figure 2A). The reaction was monitored by direct ionspray MS[16] of the reaction mixture and by analytical HPLC. An excess of the

(13) (a) Kent, S. B. H. *Annu. Rev. Biochem.* **1988**, *57*, 957–989. (b) Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180–193.

(14) (a) Blake, J. *Int. J. Pept. Protein Res.* **1981**, *17*, 273–274. (b) Yamashiro, D.; Li, C. H. *J. Int. J. Pept. Protein Res.* **1988**, *31*, 322.

(15) Robey, F. A.; Fields, R. L. *Anal. Biochem.* **1989**, *177*, 373–377.

(16) (a) Although salts generally interfere with the evaporative ionization process, the dilute NH_4OAc buffer used was compatible with direct ion-spray MS of synthetic peptides. (b) Schnölzer, M.; Jones, A.; Alewood, P.; Kent, S. B. H. *Anal. Biochem.* **1992**, *204*, 335–343.

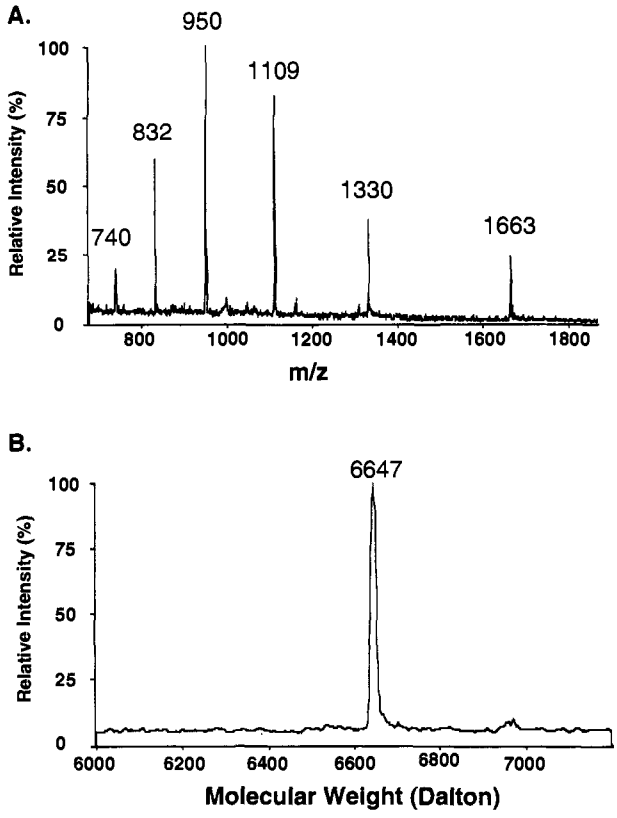


Figure 3. A . Ion-spray mass spectrometry of HPLC-purified peak **3**. Raw data is shown. The multiple charge states all arise from protonation of a single molecular species of MW 6647 D. No significant other species were detected in the HPLC fraction. B. Reconstruction of the total raw MS data to a single charge state.

peptide–$^{\alpha}$COSH component **1** was used, and the final reaction mixture was fully depleted of the $(BrAc)_4$ template molecule **2**. The ligation proceeded very quickly as monitored by HPLC. After just 70 min, the reaction had progressed almost to completion (Figure 2B). In addition to the target 4-helix TASP **3**, the only other detectable components were residual excess *pro*-helix–$^{\alpha}$COSH **1**, residual amounts of $(pro\text{-helix})_3(BrAc)_1$ template, and trace amounts of *pro*-helix–$^{\alpha}$COOH and the dimer (*pro*-helix–$^{\alpha}$COS-)$_2$. It is of some interest that the only intermediate reaction product detected (Figure 2B) was the $(pro\text{-helix})_3(BrAc)_1$ template.

The desired product was readily purified by HPLC and was lyophilized to yield a white solid. This was characterized by ionspray mass spectrometry (Figure 3) and was found to be the target 4-helix TASP **3** in high purity and with the expected mass [obs MW 6647.1 ± 2.8 D; calc for $C_{288}H_{503}N_{82}O_{88}S_4$ 6645.6 (monoisotopic), 6649.9 (average isotope composition)]. The ligated 4-helix TASP **3** was stable for days at ambient temperatures in pH 5.0 10 mM NH_4OAc and was indefinitely stable at 4 °C at pH 6.0. Circular dichroism spectroscopy was used to determine the secondary structure of the ligated TASP molecule **3** in water. The molecule was highly helical (Figure 4). Control studies of peptide **1** under identical conditions showed no helical content whatever. These conformational properties were similar to those observed for the closely related 4-helix TASP prepared by conventional means.[9,10a] Ion-spray MS under native conditions[17] showed that the 4-helix TASP occurred as a monomeric species.

The overwhelming feature of this synthetic approach is its simplicity. The ligation occurred rapidly in aqueous solution, and essentially no other reaction products were detected. The product was readily purified to give exceptionally homogeneous

(17) Baca, M.; Kent, S. B. H. *J. Am. Chem. Soc.* **1992**, *114*, 3992–3993.

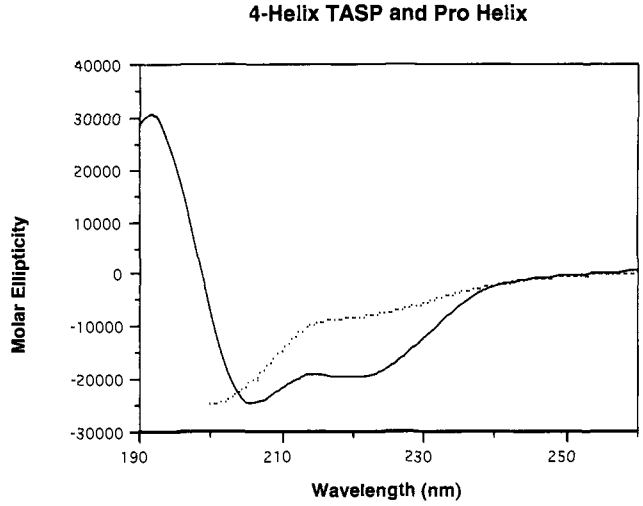


Figure 4. Circular dichroism spectrum in water of the HPLC-purified 4-helix TASP product **3** (solid line). Characteristic minima at 220 and 208 nm and a maximum at 193 nm indicate high helical content for the ligated TASP. Under identical conditions the *pro*-helix peptide **1** (dotted line) gave a weak featureless spectrum with no indication of helical structure.

material with a mass consistent with the proposed covalent structure. The observed helical secondary structure was consistent with the formation of the target 4-helix TASP molecule **3**.

The major advantages of the chemoselective ligation approach include the elimination of microheterogeneity in the final product and the generality of the approach. Peptides simultaneously built up on the template by stepwise solid-phase synthesis yield very heterogeneous crude products. Mutter and co-workers have demonstrated the use of stringent chromatographic techniques to increase the purity of the final product,[9] but these protocols have not been applied to the larger and more hydrophobic ion-channel assemblies prepared by Montal and co-workers.[18] Segment condensation of protected peptide fragments has been used to decrease heterogeneity, but problems with solubility lead to extremely slow ligation reactions, resulting in low yields.[6a] Recent work by DeGrado and co-workers used unprotected peptide fragments but is of limited applicability due to the incompatibility of the ligation reaction with all functional groups, especially the ϵ-amino group of lysine.[6c]

In contrast to these approaches, the chemoselective ligation approach allows ready purification of the unprotected peptide components, which can be ligated at high concentration in a fast, clean reaction. The target compound is obtained directly in the final unprotected form and is readily purified. The method is of general applicability because the chemistry is compatible with all functional groups found in peptides and proteins.[12] In previous work we have shown that purified unprotected peptides can be correctly ligated in solvents such as 6 M guanidine·HCl[12] or in organic/aqueous mixtures.[19] This allows great flexibility in the selecton of *pro*-helix sequences and of solvent conditions to maintain the necessary solubility for rapid reaction.

Conclusion

These results indicate the potential of the chemoselective ligation approach as a general route to the preparation of TASP-like macromolecules. Combinations of existing chemical tactics provide great versatility for the selective introduction of reactive moieties into unprotected peptide building blocks which can be used in the design and synthesis of a variety of TASP-related compounds. In particular, the thioester nucleophilic ligation chemistry described here can be combined with other ligation

(18) Grove, A.; Tomich, J. M.; Montal, M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6418–6422.

(19) Baca, M. Unpublished results from this laboratory.

chemistries originally developed for rejoining fragments of proteins[20] to generate a great diversity of nonlinear covalent molecular topologies. In conjunction with modern analytical protein chemistry, the chemoselective synthetic approach is a powerful adjunct to the design and study of conformations and activities of protein-related macromolecules.

Experimental Section

Materials and Methods. Analytical and semipreparative gradient HPLC was performed on a Rainin dual-pump high-pressure mixing system with 214-nm UV detection using Vydac C-18 analytical (5 μm, 0.46 × 15 cm) and semipreparative (10 μm, 1.0 × 25 cm) columns. Analytical runs used a 0%–67% B gradient over 30 min at 1 mL/min where buffer A is 0.1% TFA in H_2O and buffer B is 90% CH_3CN + 10% buffer A. Mass spectra were obtained using a Sciex API-III quadrupole ion-spray mass spectrometer. CD measurements were obtained using an Aviv 62 DS instrument. Peptide concentrations were determined by amino acid analysis after hydrolysis in 6 N HCl for 24 h at 110 °C.

Synthesis of *pro*-Helix Peptide 1. Except where noted, peptides were synthesized by manual stepwise solid-phase methods according to published procedures.[13] Coupling yields were monitored by quantitative ninhydrin assay. The *pro*-helix was synthesized on a Gly thioester resin[14b] using standard N^{α}Boc chemistry SPPS. The peptide was deprotected and simultaneously cleaved from the resin by treatment with HF plus 2% anisole for 1 h at 0 °C. The crude peptide was taken up in neat TFA, diluted with doubly distilled H_2O to 0.5% TFA, and lyophilized to remove residual anisole. The *pro*-helix was purified by semipreparative reversed-phase HPLC (0%–67% buffer B over 60 min at 3 mL/min) and characterized by ion-spray mass spectrometry [obs MW 1372.2 ± 0.4 D; calc 1371.8 (monoisotopic), 1372.6 (average isotope composition)].

Synthesis of Template Peptide 2. The template peptide was synthesized using a combination of N^{α}Boc chemistry SPPS and N^{ϵ}Fmoc lysine side chain protection on a Gly-OCH_2-Pam-resin (Figure 1B). To minimize the possibility of premature Fmoc removal, a separate brief neutralization with 5% diisopropylethylamine/DMF was used, rather than *in situ* neutralization in the coupling step.[13b] Following nine synthetic cycles, the Fmoc protecting groups on the lysine side chains were removed by two 5-min treatments with 50% piperidine/DMF. The free ε-amino groups were bromoacetylated using bromoacetic acid/DIC coupling.[15] The peptide was deprotected and cleaved by HF plus 10% *p*-cresol over 1 h 0 °C using standard protocols.[13b] The template was then purified to homogeneity by semipreparative reversed-phase HPLC (25%–41% buffer B over 30 min at 3 mL/min) and characterized by ion-spray mass spectrometry. [obsd MW 1482.7 ± 0.4 D; calc 1478.3 (monoisotopic), 1483.0 (average isotope compositon)].

Synthesis of 4-Helix TASP 3. Ligation was performed by combining 0.50 mg of the $(BrAc)_4$ template (FW 1484 D, 3.36×10^{-7} mol, 1.12 mM) and 2.75 mg of *pro*-helix-$^{\alpha}$COSH (FW 1372 D, 2.0×10^{-6} mol, 6.68 mM) in 300 μL of 10 mM NH_4OAc aqueous buffer, pH 5.0, at 23 °C. Reaction was monitored by analytical reversed-phase HPLC (4-μL aliquots). Peaks were collected on the basis of UV absorbance and examined by ion-spray MS.[16] After 6.5 h at 23 °C, the reaction mixture was stored at 4 °C. Product (170 μL of reaction mixture) was purified by reversed-phase HPLC (38%–54% buffer B over 30 min at 3 mL/min) and lyophilized, giving 0.26 mg of pure product, theoretical yield 1.27 mg, 20.5%. The mass was determined by ion-spray mass spectrometry [obs MW 6647.1 ± 2.8 D; calc 6645.6 (monoisotopic), 6649.9 (average isotope composition)].

Stability. Stability was monitored by analytical reversed-phase HPLC and mass spectrometry of collected peaks. 1 mM 4-helix TASP from the ligation reaction was stored at 23 °C for several days without degradation. 5 μM 4-helix TASP stored in 100 mM phosphate buffer, pH 6.0, showed no decomposition after 7 days at 4 °C.

Circular Dichroism. Both the *pro*-helix (7.5 μM) and the 4-helix TASP (1.78 μM) were dissolved in doubly distilled H_2O. Measurements were taken in a 2-mL cuvette with a path length of 1 cm at 20.0 °C, scanning from 260 to 190 nm every 0.50 nm.

Acknowledgment. This work was supported by funds from the Markey Foundation. We gratefully acknowledge the assistance of Dr. Reza Ghadiri, The Scripps Research Institute, in running the CD spectra, and Dr. Keith Rose, University of Geneva, for stimulating discussions.

(20) (a) Rose, K.; Vilaseca, L. A.; Werlen, R.; Meunier, A.; Fisch, I.; Jones, R. M. L.; Offord, R. E. *Bioconjugate Chem.* **1991**, *2*, 154–159. (b) Gaertner, H. F.; Rose, K.; Cotton, R.; Timms, D.; Camble, R.; Offord, R. E. *Bioconjugate Chem.* **1992**, *3*, 262–268.