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SYNTHESIS OF A 4-HELIX BUNDLE-LIKE TEMPLATE-ASSEMBLED SYNTHETIC PROTEIN (TASP) BY CONDENSATION OF A PROTECTED PEPTIDE ON A CONFORMATIONALLY CONSTRAINED CYCLIC CARRIER

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Abstract: We describe the condensation in solution of a fully protected helix model peptide to a cyclic peptide template including a new turn-inducing mimic to obtain a 4-helix bundle protein-like TASP. This approach allows the total synthesis of chemically and structurally well-defined protein models in the 6-10 kDa range.

The introduction of the "TASP" idea[1] has provided a novel, broadly applicable method for the construction of protein tertiary structures[2]. TASP molecules are constructed by the covalent attachment of peptide sequences with a high potential for amphiphilic secondary structure formation to a carrier molecule ("template"), resulting in a non-linear peptide chain architecture. As a key feature of this approach, the template is so designed as to direct and reinforce the folding of the secondary structure elements into the target tertiary structure in order to circumvent the well-known "folding problem"[2,3] often encountered in the *de novo* design of proteins. We have previously reported the synthesis of TASP molecules by standard solid-phase peptide synthesis methodology using specially designed linear oligopeptides[1a-c] and peptides cyclized by disulfide formation[1d] as templates. In this preliminary report, we present the total synthesis of a 4α-helix bundle TASP by condensation in solution[4] of a fully protected helix model peptide fragment to a conformationally constrained cyclic peptide template (Figure).

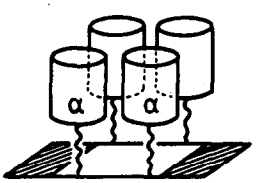
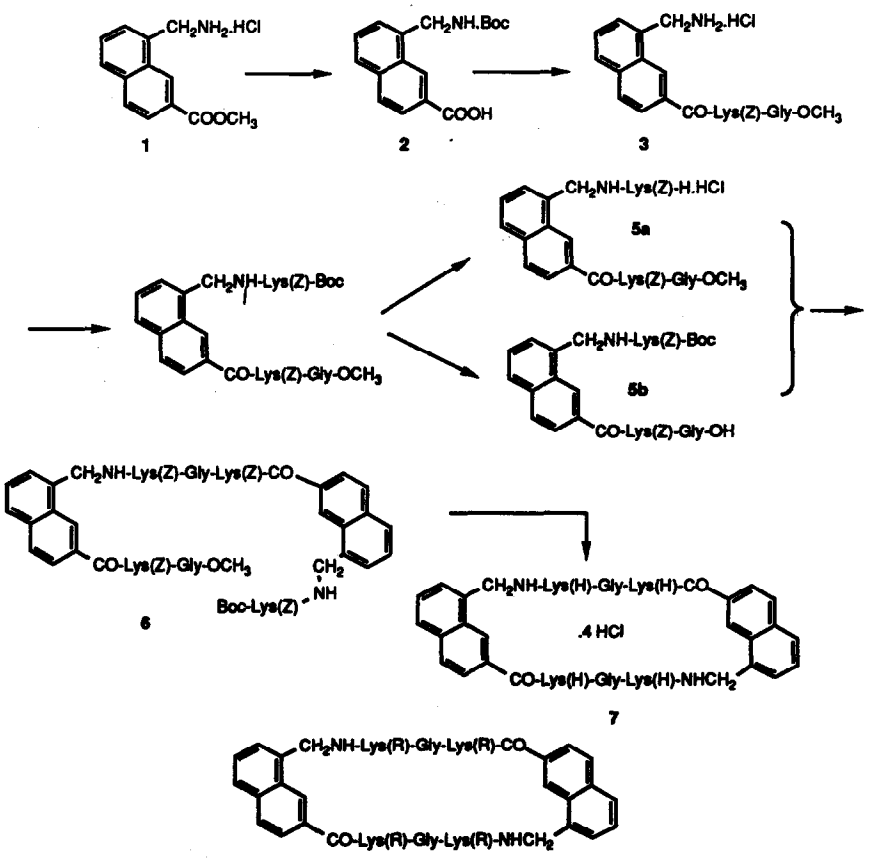


Figure Schematic representation of a 4-helix bundle-like TASP molecule; the 4 peptide blocks α are covalently attached to a conformationally constrained cyclic template designed to direct the folding of the amphiphilic helices α to the predetermined packing arrangement.

The cyclic peptide carrier **7** used here (Scheme) consists of two Lys-Gly-Lys tripeptide units linked at both ends in an antiparallel way by two molecules of 8-aminomethyl-2-naphthoic acid (AMNA) recently introduced as a novel turn-inducing mimic[5]. Computer-assisted molecular modelling indicates a low-energy conformation for this cyclic peptide with distances between the lysine ε-amino groups which favour the attachment of four peptide blocks to well-packed arrangements[6].

The cyclic carrier **7** was synthesized by classical methods in solution starting from the AMNA derivative **1** (Scheme). A characteristic feature of the synthesis is the construction of the open-chain octapeptide intermediate **6** from two molecules of the tetrapeptide **4** which, for that purpose, had been deblocked at its N-end (**5a**) and at the C-end (**5b**), respectively. The synthetic strategy made use of N-α-amino Boc protection and DCC/HOBt activation throughout except in the cyclization step, where diphenylphosphoric azide (DPPA) was used successfully (88% yield) as activating agent. Hydrogenolysis (Pd/C in HCl-AcOH) of the protecting groups afforded the carrier **7** as an amorphous, fluffy powder[7,8]. A detailed NMR analysis of **7** (2D, DMSO, H_2O/D_2O) confirmed its centrosymmetrical geometry but was also indicative of some conformational flexibility in the macrocyclic carrier molecule in solution.



8: R = Ac-Asp(OtBu)-Ala-Aib-Thr(OtBu)-Ala-Ala-Aib-Asn-Ala-Aib-Lys(Boc)-Lys(Boc)-Leu-Gly

9: R = Ac-Asp-Ala-Aib-Thr-Ala-Ala-Aib-Asn-Ala-Aib-Lys-Lys-Leu-Gly

Scheme

The peptide sequence (Asp-Ala-Aib-Thr-Ala-Ala-Aib-Asn-Ala-Aib-Lys-Lys-Leu-Gly) presented here as an example for fragment condensation to the cyclic carrier is based on the 87-97 Hen Egg-white Lysozyme sequence and was modified in order to increase its amphiphilic character and helical content.[9] Full details of the synthesis of this and related peptides will be reported elsewhere[10].
The condensation reaction of the fully protected fragment to carrier **7** proceeded smoothly using standard procedures[13]. Only little partially coupled material could be detected by HPLC, indicating that the high density of reaction sites on the carrier molecule does not act adversely on the coupling kinetics. Similar findings have been made in the analogous case of solid-phase couplings using high-loading resins[15]. The target molecule in both protected (**8**) and side-chain deprotected (**9**) forms was fully characterized by tlc, HPLC, amino acid analysis and mass spectrometry[13] after purification by semipreparative HPLC.

In summary, the combination of classical and solid-phase methods of peptide synthesis and the use of fragment condensation techniques facilitates the obtension of the target molecule in good yield and purity. This strategy thus appears ideally suited for the design and synthesis of protein models using the TASP approach. The use of orthogonal protection techniques allows for a variety of packing arrangements and the attachment of different individual peptide blocks in either parallel or antiparallel fashion. Most notably, the use of cyclic conformationally constrained carrier molecules ("templates") as described here represents a new tool for protein *de novo* design. Considering the energetics of the folded and unfolded states in TASP molecules[2], a correlation between the "rigidity" of the template and the thermodynamic stability of the folded TASP molecule is predicted.

Circular dichroism spectra of the TASP molecule **9** are indicative of well-defined structural features. A comparative study of the effect of cyclic and linear carriers on the stabilization of helix blocks is in progress and will be reported in due course.

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References and Notes:

1. (a) M. Mutter (1988) *Peptides Chemistry and Biology*, Proc. 10th Amer. Peptide Symp. (G. R. Marshall, Ed.), Escom, Leyden, p. 349. (b) M. Mutter, E. Altmann, K.- H. Altmann, R. Hersperger, P. Koziej, K. Nebel, G. Tuchscherer, S. Vuilleumier (1988) *Helv. Chim. Acta* **71**, 835. (c) M. Mutter, R. Hersperger, K. Gubernator, K. Müller (1989) *Proteins* **5**, 13. (d) J. Rivier, C. Miller, M. Spicer, J. Andrews, J. Porter, G. Tuchscherer, M. Mutter (1990) in *Proc. 1 Int. Symp. Solid Phase Synthesis* (R. Epton, Ed.), Oxford, in press.

2. M. Mutter, S. Vuilleumier (1989) *Angew. Chem.* **101**, 551; *Angew. Chem. Int. Ed.* **28**, 535.

3. (a) J. S. Richardson, D. C. Richardson (1989) *Trends Biochem. Sci.* **14**, 304. (b) W. DeGrado (1988) *Adv. Protein Chem.* **39**, 51.

4. For some recent examples of fully protected fragments obtained by solid phase synthesis see *e. g.* (a) J.-M. Sabatier, M. Tessier-Rochat, C. Granier, J. van Rietschoten, E. Pedroso, A. Grandas, F. Albericio, E. Giralt (1987) *Tetrahedron* **43**, 5973. (b) E. T. Kaiser, H. Mihara, G. A. Laforet, J. W. Kelly, L. Walters, M. A. Findeis, T. Sasaki (1989) *Science* **243**, 187. (c) Y.-Z. Lu, S.-H. Ding, J.-Y. Chu, A. M. Felix (1990) *Int. J. Peptide Protein Res.* **35**, 95.

5. I. Ernest, J. Kalvoda, G. Rhis, M. Mutter, *Tetrahedron Letters*, accompanying paper.

6. K. Gubernator, K. Müller, I. Ernest, H. Fritz and M. Mutter, in preparation.

7. Cyclic carrier **7** (tetrahydrochloride): Tlc Rf 0.19 (butanol:acetic acid:pyridine:water); HPLC (Vydac C-18, 4-24% in 30'; A: H_2O (0.1% TFA); B: CH_3CN (0.1% TFA)) 24.2'; MS (FAB): (M 992, 7 free base): 993.6 $((M+H)^+$; $C_{52}H_{76}N_{12}O_8 \cdot Cl_4$ ($\cdot 2H_2O$): found C 52.99, H 6.71, N 14.34, Cl 12.28; req. C 53.14, H 6.86, N 14.30, Cl 12.07.

8. All intermediates of the synthesis of **7** were fully characterized by elemental analysis, IR and NMR spectroscopy and, when required, mass spectrometry. A full paper including all data is in preparation.

9. M. Mutter, K.- H. Altmann, K. Müller, S. Vuilleumier, T. Vorherr (1986) *Helv. Chim. Acta* **69**, 985. Because of concerns about the danger of racemization in fragment condensation reactions due to slower reaction kinetics and elevated temperatures, care was taken to include a C-terminal glycine in designing the peptide helix model sequence.

10. S. Vuilleumier, M. Mutter, in preparation. Briefly, the peptide sequence was assembled by solid-phase peptide synthesis, using the N-α-amino Fmoc, side-chain Boc/tBu orthogonal protecting scheme[11] and the Sasrin® resin[12], which allows the cleavage from the resin of the side-chain protected, N-terminally acetylated fragment in good yield and purity. The fragment Ac-Asp(OtBu)-Ala-Aib-Thr(OtBu)-Ala-Ala-Aib-Asn-Ala-Aib-Lys(Boc)-Lys(Boc)-Leu-Gly-OH (M. W. 1669.0) was purified to homogeneity by semipreparative RP-HPLC and fully characterized by tlc, HPLC, amino acid analysis, racemization analysis and FAB-MS.

11. (a) E. Atherton, R. C. Sheppard (1987) in *Peptides: Analysis, Synthesis, Biology* (J. Meienhofer, S. Udenfriend, Eds.), Vol. **9**, Academic Press, Orlando, p. 1. (b) G. B. Fields, R. C. Noble (1990) *Int. J. Peptide Protein Res.* **35**, 161.

12. M. Mergler, R. Tanner, J. Gosteli, P. Grogg (1988) *Tetrahedron Lett.* **29**, 4005.

13. Carrier **7** (hydrochloride form, 0.54 mg, 0.47 µmol) in NMP (5 µl) and DIPEA (19 µl, 1.9 µmol, 4 eq., 0.1N in DMA) and the fully protected acetylated Lysozyme 87-97 model fragment carboxylic acid (6.3 mg, 3.8 µmol, 8 eq.), BOP[14] and HOBt (each 4 µl 1N in DMA, 8.4 eq) and DIPEA (38 µl, 1N in DMA, 3.8 µmol, 1 eq.) were added and the condensation reaction mixture (total volume: 70 µl (carrier concentration 6.6 mM, fragment concentration 54 mM) stirred at 45 °C for 16 h. The crude product **8** was precipitated with ether, the supernatant discarded and the obtained gum triturated with water. The resulting white solid was either partially purified by gel filtration (Sephadex LH-20 in methanol, 1x45 cm, 4 ml/h) or by semipreparative RP-HPLC C-18 (Lichrosorb, 8x250 mm, 5µ, 100Å). Tlc R_f 0.3 (Ethyl acetate:acetone:acetic acid:water 6:2:1:1); HPLC (Vydac C-4, 4.6x250 mm, 5µ, 300 Å; A: H_2O:CH_3CN 9:1 (0.05% TFA); B: H_2O:CH_3CN 1:19 (0.05% TFA), 35-80%B in 45') 33.5'; Amino acid analysis: Asp 6.72 (8), Thr 3.52 (4), Gly 6.0 (6), Ala 14.81 (16), Leu 4.35 (4), Lys 11.38 (12), (Aib (8), AMNA (2) n. d); MS (Cf252, PD) ($C_{356}H_{596}N_{80}O_{100}$, M 7588, 7597.1): 7498.5 (M - Boc)$^+$.

The side-chain protecting groups were removed by standard procedures (TFA:DCM:thioanisole 50:45:5 (2 ml), 4 h) and the crude free peptide **9** purified by semipreparative HPLC. Overall yield 1.8 mg (0.24 µmol).

Tlc R_f 0.38 (butanol:pyridine:acetic acid:water 4:1:1:2); HPLC (Vydac C-4, 10-55% in 60') 33'; Amino acid analysis: Asp 7.73 (8), Thr 3.70 (4), Gly 6.0 (6), Ala 15.76 (16), Leu 4.59 (4), Lys 12.05 (12), (Aib (8), AMNA (2) n. d.); MS (Cf252, PD) $C_{284}H_{468}N_{80}O_{84}$ (M 6340, 6347.8, 8TFA 7259.5): 6348.1 $(M+H)^+$.

14. CAUTION: The use of the BOP reagent involves the formation of hexamethylphosphoric triamide (HMPA), a possible carcinogenic hazard. A peptide coupling reagent related to BOP devoid of this toxic by-product has recently been described : J. Coste, D. Le-Nguyen, B. Castro (1990) *Tetrahedron Letters* **31**, 205.

15. R. Epton (1990) in *Proc. 1. Int. Symp. Solid Phase Synthesis* (R. Epton, Ed.), Oxford, in press.

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