Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron Letters
Tetrahedron Letters 45 (2004) 6795–6798

Synthesis of reversible cyclic peptides

Alfonso Ortiz-Acevedo and Gregg R. Dieckmann*

Department of Chemistry, The University of Texas at Dallas, 2601 North Floyd Road, Richardson, TX 75080, USA

Received 21 May 2004; revised 30 June 2004; accepted 2 July 2004
Available online 28 July 2004

Abstract—A novel approach for the synthesis of cyclic peptides that can exist in either linear or cyclized conformations is described. Synthesis of the peptides was achieved via a modified solid phase methodology. The reversible linear/cyclized (i.e., open/closed) states are controlled via the reduction/oxidation of a disulfide bond incorporated into the backbone of the peptide chain.

The use of biological macromolecules, such as DNA, polysaccharides, and polypeptides, to modify the properties of nanoscale particles has provided notable success and generated increased interest in the interface between biology and nanotechnology.[1–3] Recently we have designed/synthesized amphiphilic helical peptides that noncovalently bind single-walled carbon nanotubes (SWNTs) and effectively solubilize SWNTs in water, yielding unbundled, individual SWNTs several microns in length.[4,5] One property that we would like to improve is the affinity of peptides for the SWNTs. The utility of peptide-wrapped nanotubes should increase significantly if the peptides could be stabilized in a nanotube-bound state. We hypothesize this could be achieved by peptides designed to wrap around the circumference of a SWNT and then form head-to-tail covalent attachments, yielding closed rings on the nanotube. Toward that end, we have designed and synthesized a new class of peptides, called reversible cyclic peptides (RCPs), which have the ability to switch between a linear (open) state and a cyclic (closed) state.

The design of the RCPs is based on a specific class of cyclic peptides, which contain equal numbers of L- and D-amino acids arranged in an alternating L/D sequence.[1–3,6,7] L/D-peptides place all amino acid side chains on one face of the backbone, verses all L- or D-peptides where side chains radiate from alternating faces. In L/D-peptides, side chain sterics encourage a ring-like conformation with all side chains on the outside surface of the backbone. Although Cys side chains have been used previously to induce peptide cyclization,[1,3] the unique approach presented here involves the introduction of two thiols via modifications to the N- and C-termini of a linear L/D-peptide. The thiols are incorporated into the peptide backbone, thus facilitating reversible peptide cyclization via formation of a disulfide bond, while allowing the adoption of backbone dihedral angles appropriate for an unstrained cyclic conformation (not possible if a Cys-Cys side chain disulfide were used).

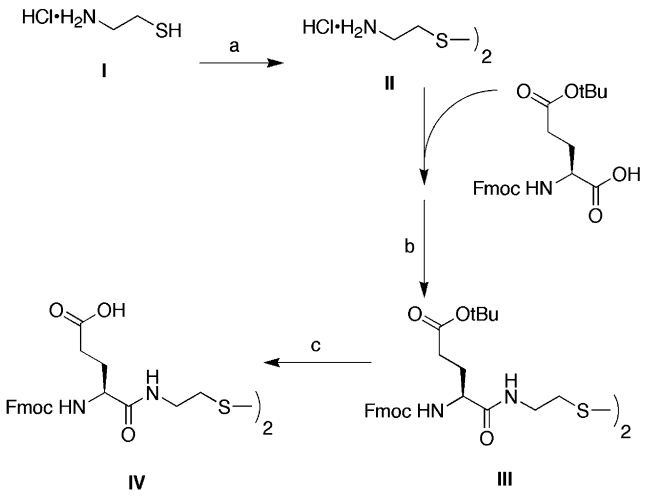
RCPs were synthesized using a modified solid phase methodology. The modified C-terminus of each RCP was generated by coupling an orthogonally protected N-α-Fmoc-L-glutamic acid γ-*tert*-butyl ester (Fmoc-Glu(*t*-Bu)-OH) to the amino end of a cysteamine. This orientation provides a thiol directed along the peptide backbone that can be utilized for disulfide bond formation. The N-terminal thiol was generated by coupling 2-mercaptoacetic acid to the free amine of the extended peptide backbone. Upon cleavage of the peptide from the resin and reduction of all disulfides, RCPs were produced containing two free thiols oriented in an

Abbreviations: Reversible cyclic peptide (RCP); 9-Fluorenylmethoxycarbonyl (Fmoc); Dimethyl sulfoxide (DMSO); Dimethylformamide (DMF); Diisopropylethylamine (DIEA); 1H-Benzotriazolium, 1-[bis-(dimethylamino) methylene]-6-chloro-, 3-oxide, hexafluorophosphate(1-) (9CI) (HCTU); Dichloromethane (DCM); Ethyl acetate (EtOAc); Thin layer chromatography (TLC); Trifluoroacetic acid (TFA); Solid phase peptide synthesis (SPPS); 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU); N-Hydroxybenzotriazole H_2O (HOBt); *N*-Methylpyrrolidone (NMP); β-Mercaptoethanol (BME); Electrospray ionization-mass spectrometry (ESI-MS); Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP); 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB).

Keywords: Cyclic peptides; Disulfide bond; Solid phase synthesis.

* Corresponding author. Tel.: +1-972-883-2903; fax: +1-972-883-2925; e-mail: dieckgr@utdallas.edu

0040-4039/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2004.07.041



Scheme 1. Reaction conditions: (a) DMSO/H_2O; (b) HCTU, DIEA in DMF; (c) TFA/DCM; overall yield for steps a–c: 91.3%.

appropriate way to form a closed ring upon intramolecular disulfide formation.

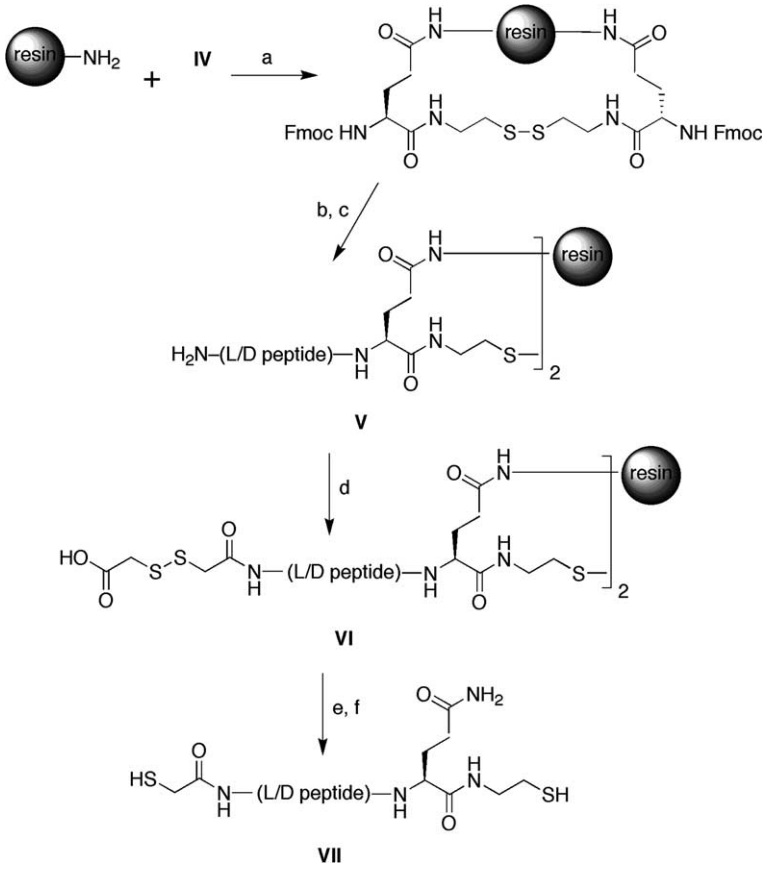
The C-terminal thiol of each RCP was introduced via a glutamic acid residue that has been coupled to a cysteamine molecule (Scheme 1). Cystamine dihydrochloride (**II**), the disulfide of cysteamine hydrochloride (**I**), was generated by oxidizing **I** with DMSO in a minimal amount of water with stirring overnight.[8–10] The solution was monitored for the presence of free thiol with the Ellman test.[11,12] Once a negative test was achieved, cold absolute ethanol was slowly added to precipitate the product (**II**), which was collected on a glass fritted funnel and dried under high vacuum. A solution of **II** in DMF was then added to a round bottom flask containing Fmoc-Glu(*t*-Bu)-OH (2.2 equiv), DIEA, and HCTU (2.2 equiv) in DMF and the reaction was allowed to stir overnight at room temperature, generating the side chain protected derivative (**III**).

Isolation of **III** was achieved by a series of liquid–liquid extractions followed by column chromatography. The reaction mixture was dissolved in a 3 × volume of DCM, and excess reagents were extracted by washing with a 5% sodium bicarbonate solution followed by several water washes. Excess DCM was removed by a slow air stream over the solution, and the impure product concentrate was loaded onto a silica column and eluted using 1:1 DCM–EtOAc. TLC and HPLC monitoring of column fractions confirmed the presence of **III** in early fractions. Product fractions were consolidated and the elution solvent removed under reduced pressure, generating a white solid. The solid (**III**) was redissolved in DCM and crystallized from DCM/hexanes. The desired bis-(N-α-Fmoc-L-glutamic acid α-amidoethyl) disulfide (**IV**) was generated by stirring a solution of **III** in 50% TFA in DCM for 2 h. Monitoring the side chain deprotection by HPLC indicated the reaction was complete after 1 h. The final product (**IV**) was isolated in 91.3% overall yield as a solid by removing the TFA and DCM under reduced pressure. Comparisons of the NMR spectra (^1H and ^{13}C) of **III** and **IV**, as well as the ESI-MS of **IV**, verified the identities of **III** and **IV**.[13,14]

The molecule **IV** was then used to synthesize RCPs of desired lengths utilizing established Fmoc-based SPPS protocols (Scheme 2). Two peptides of 11 and 15 amino acids were synthesized in the following way to yield RCPs RC5 and RC7 (Fig. 1). Compound **IV** was loaded on amide resin using a standard solid phase coupling procedure involving HBTU, HOBt, and DIEA in NMP, allowing the coupling reaction to proceed overnight. Peptides of the desired lengths were then synthesized by automated-SPPS utilizing amino acids of alternating chirality. Removal of the Fmoc group from the N-terminal amino acid with a solution of 20% piperidine in DMF afforded the free amine **V** that was subsequently coupled with a dithio diglycolic acid to yield **VI**. Compound **VI** was washed with BME and several DCM washes prior to being dried under high vacuum. The BME washes were incorporated to remove the N-terminal thiol protection via disulfide exchange with BME and to cleave any peptide that was not directly coupled to the resin. Allowing these peptides to continue into the next step would generate an undesired glutamic acid derivative of the target peptide. Cleavage of the peptides from the solid support was accomplished using a standard 10 mL cleavage cocktail of TFA, thioanisole, ethanedithiol, and anisole (90:5:3:2) yielding the crude product **VII**. Purification of **VII** by HPLC yielded the pure product (overall yield = 6.3% for RC5, 5.4% for RC7) displaying the appropriate molecular weight of the linear (reduced) peptide, $m/z = 1310$ [reduced RC5 + H]$^+$ and $m/z = 1710$ [reduced RC7 + H]$^+$, as indicated by ESI-MS analysis.

Characterization of the solution behavior of the RCPs provided verification of the intended design. All RCP solutions were prepared using degassed deionized water to prevent oxidation of the free thiols. The peptide concentration of RCP stock solutions was determined by utilizing the tyrosine absorbance ($\varepsilon = 1420\,M^{-1}cm^{-1}$ at 275 nm). The Ellman test was used to determine the free thiol concentration of stock solutions, as well as to monitor the oxidation of experimental solutions.[11]

Direct transitions from the linear to the cyclized states of RC5 and RC7 were observed by shifts in the HPLC retention times of 100 μM peptide solutions that were oxidized by atmospheric oxygen (Fig. 2). Aliquots of the HPLC peaks corresponding to the oxidized RCPs were submitted for mass spectral analysis. The molecular ion for each cyclized peptide appeared at 2 mass units less than that of the corresponding linear RCP monomer, $m/z = 1308$ [oxidized RC5 + H]$^+$ and $m/z = 1708$ [oxidized RC7 + H]$^+$ (Fig. 2). The reversibility of the proposed linear/cyclized states of the peptides was observed when solutions of cyclized RCP monomers were treated with the reducing agent TCEP to regenerate the linear state. HPLC traces of the solutions after TCEP addition (data not shown) clearly showed the peptides eluting at the retention times pertaining to the linear states.



Scheme 2. Reaction conditions: (a) HBTU, HOBt and DIEA in NMP; (b) standard SPPS protocols using alternating L- and D-amino acids; (c) 20% piperidine in DMF; (d) HBTU, HOBt, DIEA, and dithio diglycolic acid in NMP; (e) BME; (f) thioanisole, ethanedithiol, and anisole in TFA; overall yield for steps a–f: 6.3% (RC5), 5.4% (RC7).

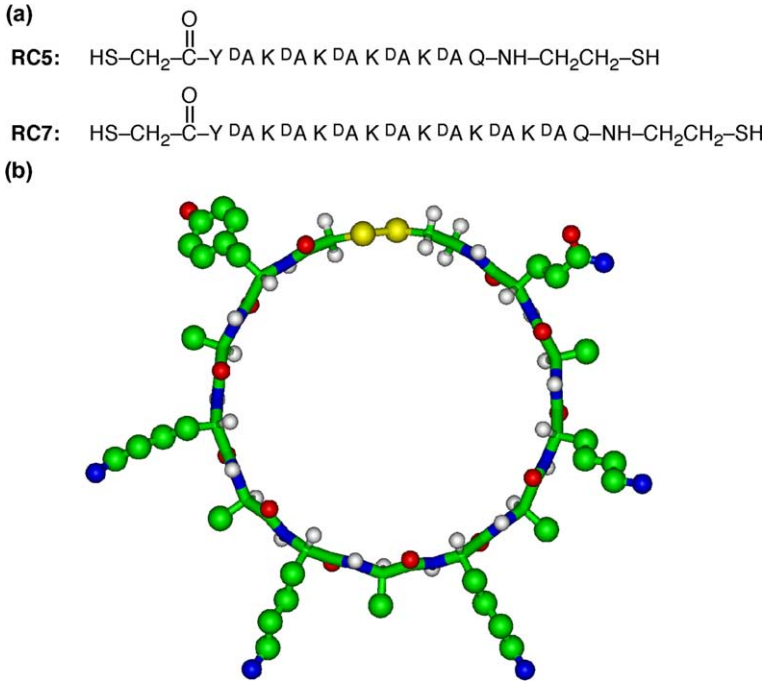


Figure 1. (a) Sequences of RC5 (11 amino acids) and RC7 (15 amino acids); (b) molecular model[15] of RC5 demonstrating the cyclized conformation of the peptide. The final model highlights the placement of amino acid side chains on the outside of the ring, as well as the orientation of the backbone carbonyl and NH groups perpendicular to the ring plane. Color scheme: green, carbon; red, oxygen; blue, nitrogen; white, hydrogen; yellow, sulfur.

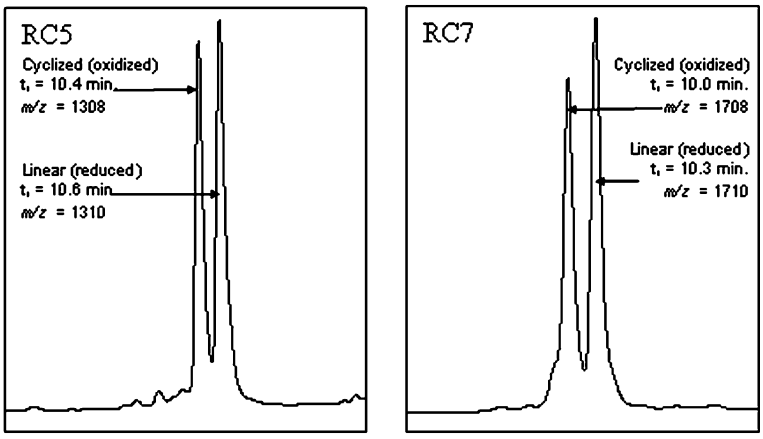


Figure 2. Chromatograms of 100 μM RCP solutions displaying the presence of both linear and cyclized conformations of the peptides in solution.

In summary, we have described the design and synthesis of a new class of cyclic peptide incorporating a backbone disulfide bond to introduce reversibility of linear/cyclized states. Synthesis was achieved by modifying the N- and C-termini of the peptide backbones, introducing thiols at both ends, which then form a disulfide bond through oxidation. Characterization of the peptides by HPLC and MS analysis verified the design of the RCPs, which can be used in their linear or cyclized conformations.

Acknowledgements

We would like to thank Dr. Zoltan Kovacs and Dr. Mark Woods for helpful discussions. Support for this research by the MIRROR Federal Initiative (A.O.-A.) and the American Chemical Society Petroleum Research Fund (PRF# 40835-AC3; G.R.D.) is gratefully acknowledged.

References and notes

1. Lambert, J. N.; Mitchell, J. P.; Roberts, K. D. *J. Chem. Soc., Perkin Trans. 1* **2001**, 471–484.
2. Hartgerink, J. D.; Clark, T. D.; Ghadiri, M. R. *Chem. Eur. J.* **1998**, *4*(8), 1367–1372.
3. Bong, D. T.; Clark, T. D.; Granja, J. R.; Ghadiri, M. R. *Angew. Chem., Int. Ed.* **2001**, *40*, 988–1011.
4. Dieckmann, G. R.; Dalton, A. B.; Johnson, P. A.; Razal, J.; Chen, J.; Giordano, G. M.; Muñoz, E.; Musselman, I. H.; Baughman, R.; Draper, R. K. *J. Am. Chem. Soc.* **2003**, *125*, 1770–1777.
5. Zorbas, V.; Ortiz-Acevedo, A.; Dalton, A. B.; Dieckmann, G. R.; Draper, R. K.; Baughman, R. H.; Jose-Yacaman, M.; Musselman, I. H. *J. Am. Chem. Soc.* **2004**, *126*, 7222–7227.
6. Buriak, J. M.; Ghadiri, M. R. *Mater. Sci. Eng. C* **1997**, *4*, 207–212.
7. Khazanovich, N.; Granja, J. R.; McRee, D. E.; Milligan, R. A.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1994**, *116*, 6011–6012.
8. Oiry, J.; Pue, J. Y.; Imbach, J. L.; Fatome, M.; Sentenac-Roumanou, H.; Lion, C. *J. Med. Chem.* **1986**, *29*, 2217–2225.
9. Mills, E. J.; Bogert, M. T. *J. Am. Chem. Soc.* **1940**, *62*, 1173–1180.
10. Yiannios, C. N.; Karabinos, J. V. *J. Org. Chem.* **1963**, *28*(11), 3246–3248.
11. Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. F. *Biochem. Pharm.* **1961**, *7*, 88–90.
12. Free thiol concentration was determined using the Ellman test. A stock solution of DTNB was prepared by dissolving 20 mg of DTNB in 5 mL of 100 mM potassium phosphate buffer at pH 8. Analytical solutions were prepared by mixing 100 μL of sample with 100 μL of DTNB stock solution and 2.8 mL of 100 mM potassium phosphate buffer at pH 8. A reference solution was also prepared incorporating 100 μL of water in place of the sample solution. After 15 min the absorbance ($\lambda = 410$ nm) of the analytical solutions was measured using a 1 cm cuvette. Free thiol concentration (mol/L) was determined by the equation; $[SH] = (A_{sample} - A_{reference})/136{,}50$.
13. A sample of compound **III** was analyzed by NMR and ESI prior to being converted to **IV**. ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.09 (2H, t, $J = 5$ Hz), 7.88 (4H, d, $J = 8$ Hz), 7.72 (4H, m), 7.50 (2H, $J = 7$ Hz), 7.41 (4H, t, $J = 7$ Hz), 7.32 (4H, t, $J = 7$ Hz), 4.25 (6H, m), 3.96 (2H, m), 2.77 (4H, t, $J = 6$ Hz), 2.22 (4H, t, $J = 7$ Hz), 1.80 (4H, m), 1.37 (18H, s); ^{13}C NMR (270 MHz, DMSO-d_6): δ 172.2, 172.0, 156.5, 144.4, 144.3, 141.3, 128.2, 127.6, 125.9, 120.7, 80.3, 66.2, 54.5, 47.2, 38.4, 37.5, 31.9, 28.3, 27.9; ESI-MS (*m*/*z*) $[M + H]^+ = 967$ ($MW_{calcd} = 966\,g\,mol^{-1}$).
14. Compound **IV** was dried under high vacuum prior to coupling to the amide resin. The pure compound was analyzed by NMR and ESI-MS to verify the identity. ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.11 (2H, t, $J = 5$ Hz), 7.85 (4H, d, $J = 8$ Hz), 7.70 (4H, m), 7.50 (2H, $J = 8$ Hz), 7.40 (4H, t, $J = 7$ Hz), 7.30 (4H, t, $J = 7$ Hz), 3.95 (4H, m), 3.37 (4H, m), 2.76 (4H, t, $J = 6$ Hz), 2.27 (4H, t, $J = 7$ Hz), 1.80 (4H, m); ^{13}C NMR (270 MHz, DMSO-d_6): δ 174.5, 172.3, 156.6, 144.4, 144.2, 141.2, 128.2, 127.6, 125.8, 120.6, 66.3, 54.6, 47.2, 38.4, 37.5, 30.8, 27.7; ESI-MS (*m*/*z*) $[M + H]^+ = 855$ ($MW_{calcd} = 854\,g\,mol^{-1}$).
15. Model building and energy refinement calculations were performed using InsightII and Discover (Accelrys Inc., San Diego, CA) and the Consistent Valence Force Field (CVFF).[16] Final average backbone dihedral angles: L-amino acids: $\phi = -152°$, $\psi = 156°$; D-amino acids: $\phi = 154°$, $\psi = -156°$.
16. Dauber-Osguthorpe, P.; Roberts, V. A.; Osguthorpe, D. J.; Wolff, J.; Genest, M.; Hagler, A. T. *Proteins* **1988**, *4*, 31–47.