De Novo Design of Antibacterial β-Peptides

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Recently, it has been demonstrated that a number of homodisperse, sequence-specific polymers fold into well-defined three-dimensional structures in solution.[1−4] Particularly significant progress has been made in elucidating the conformational properties of short polymers of β-amino acids (β-peptides).[2,3,5] This class of polyamides folds into turns, helices, and sheetlike structures, analogous to the secondary structures of proteins. β-Peptides are also chemically stable and resistant to enzymatic degradation,[6] suggesting that they might provide an attractive medium for the construction of biomimetic polymers. Here, we describe the design of β-peptides that mimic the activities of a class of natural membrane-active peptide toxins and antibiotics,[7,8] which includes magainins, bombolitin, cecropins, melittin, and mastoparans.[9−12] The biological active conformation of these peptides has been shown to consist of a positively charged, amphiphilic α-helix (Figure 1). These helices kill cells by disrupting the structural integrity of their phospholipid membranes.[10−19] The overall physicochemical properties of these helices, and not their precise sequences or chirality,[20,21] have been proposed to be the key features required for activity. Thus, it should be possible to design amphiphilic β-peptide-based toxins and antibiotics.

A particularly stable secondary structure formed by β-peptides is the L+2 helix (also known as a 14-helix, or a 3_1 helix; Figure 1), which shows a 3-residue geometric repeat.[2,3] β-Peptides in which hydrophobic side chains occur with a similar 3-residue repeat will have a high potential to form an amphiphilic L+2 helix. The thermodynamic stability of such a helix should also be dependent on its chain length and amino acid composition.[2,3,22] To experimentally address these parameters, we investigated the properties of a series of peptides of graded lengths:

Fmoc-(β^3-HVal-β^3-HLys-β^3-HLeu)$_n$-OH; $n = 2-4$
H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_n$-OH; $n = 2-4$
H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_n$-OH; $n = 2-6$

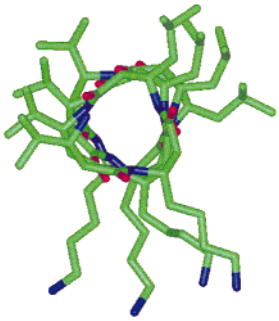
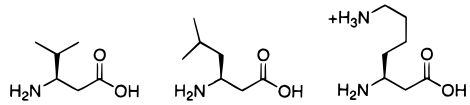


Figure 1. Molecular model of the amphiphilic β-peptide H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_4$-OH in an L+2 helical conformation. This axial view shows the segregation of hydrophobic and positively charged residues on opposite sides of the helix. Carbon atoms are shown in green, nitrogen in blue, and oxygen in red.



The above peptides were synthesized by solid-phase peptide synthesis, using tripeptide blocks prepared on a 2,4-dialkoxybenzyl ester polymer support as described in the Supporting Information. The Fmoc group in the first series of peptides provided a convenient probe for determining the concentration of the peptides. To determine the effect of this hydrophobic probe on the properties of the compounds, a parallel series of peptides was synthesized without this group.

The ability of these peptides to adopt an L+2 helix in aqueous solution in the presence and absence of micelles and phospholipid bilayers was assessed using CD spectroscopy, which provides a rapid method to assess the secondary structure formation of β-peptides. The relationship between secondary structure formation and the CD spectra of peptides composed of cyclic β-amino acids is not yet fully developed.[23] However, numerous studies with β-peptides assembled from the acyclic building blocks used in this work have demonstrated that the L+2 conformation gives rise to a strong minimum at 215 nm and a maximum at 195 nm in the π-π^* region.[2] The CD spectra of Fmoc-(β^3-HVal-β^3-HLys-β^3-HLeu)$_n$-OH ($n = 2-4$) in aqueous solution failed to exhibit these features associated with the L+2 helical conformation. This finding is consistent with previous studies showing that complete formation of the L+2 conformation by β-peptides of this length requires the addition of organic solvents or conformationally constrained amino acids.[24−26] However, the addition of dodecyl phosphocholine (DPC) micelles resulted in a length-dependent increase in the magnitude of $[\theta]_{215\ nm}$, reaching an intensity

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(1) Kirshenbaum, K.; Barron, A. E.; Goldsmith, R. A.; Armand, P.; Bradley, E. K.; Truong, K. T. V.; Dill, K. A.; Cohen, F. E.; Zuckermann, R. N. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4303−4308.
(2) Seebach, D.; Overhand, M.; Kuhnle, F. N. M.; Martinoni, B.; Oberer, L.; Hommel, U.; Widmer, H. *Helv. Chim. Acta* **1996**, *79*, 913.
(3) Gellman, S. H. *Acc. Chem. Res.* **1998**, *31*, 173−180.
(4) Smith, A. B., III; Keenan, T. P.; Holcomb, R. C.; Sprengeler, P. A.; Guzman, M. C.; Wood, J. L.; Carroll, P. J.; Hirschmann, R. *J. Am. Chem. Soc.* **1992**, *114*, 10672−10674.
(5) DeGrado, W. F.; Schneider, J. P.; Hamuro, Y. *J. Pept. Res.* **1999**, *54*, in press.
(6) Seebach, D.; Matthews, J. L. *Chem. Commun.* **1997**, 2015−2022.
(7) Simmaco, M.; Mignogna, G.; Barra, D. *Biopolymers (Pept. Sci.)* **1999**, *47*, 435−451.
(8) Oren, Z.; Shai, Y. *Biopolymers (Pept. Sci.)* **1999**, *47*, 451−464.
(9) DeGrado, W. F. *Adv. Protein Chem.* **1988**, *39*, 51−124.
(10) Maloy, W. L.; Kari, U. P. *Biopolymers* **1995**, *37*, 105−22.
(11) Zasloff, M. *Curr. Opin. Immunol.* **1992**, *4*, 3−7.
(12) Boman, H. G.; Faye, I.; Gudmundsson, G. H.; Lee, J. Y.; Lidholm, D. A. *Eur. J. Biochem.* **1991**, *201*, 23−31.
(13) DeGrado, W. F.; Musso, G. F.; Lieber, M.; Kaiser, E. T.; Kézdy, F. J. *Biophys. J.* **1982**, *37*, 329−338.
(14) Kaiser, E. T.; Kezdy, F. J. *Science* **1984**, *223*, 249−55.
(15) Kaiser, E. T.; Kezdy, F. J. *Annu. Rev. Biophys. Biophys. Chem.* **1987**, *16*, 561−81.
(16) Fletcher, J. E.; Jiang, M. S. *Toxicon* **1993**, *31*, 669−95.
(17) Dempsey, C. E. *Biochim. Biophys. Acta* **1990**, *1031*, 143−61.
(18) Matsuzaki, K. *Biochim. Biophys. Acta* **1998**, *1376*, 391−400.
(19) Wieprecht, T.; Dathe, M.; Epand, R. M.; Beyermann, M.; Krause, E.; Maloy, W. L.; MacDonald, D. L.; Bienert, M. *Biochemistry* **1997**, *36*, 12869−80.
(20) Bisello, A.; Sala, S.; Tonello, A.; Signor, G.; Melotto, E.; Mammi, S.; Peggion, E. *Int. J. Biol. Macromol.* **1995**, *17*, 273−82.
(21) Wade, D.; Boman, A.; Wahlin, B.; Drain, C. M.; Andreu, D.; Boman, H. G.; Merrifield, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 4761−5.
(22) Zimm, B. H.; Bragg, J. K. *J. Chem. Phys.* **1959**, *31*, 526−535.
(23) Appella, D. H.; Christianson, L. A.; Karle, I. L.; Powell, D. R.; Gellman, S. H. *J. Am. Chem. Soc.* **1999**, *121*, 6206−6212.
(24) Gung, B. W.; Zhu, D.; Stalcup, A. M.; Cottrell, C. E. *J. Org. Chem.* **1999**, *64*, 2176−2177.
(25) Abele, S.; Guichard, G.; Seebach, D. *Helv. Chim. Acta* **1998**, *81*, 2141−2156.
(26) Appella, D. H.; Barchi, J. J.; Durell, S. R.; Gellman, S. H. *J. Am. Chem. Soc.* **1999**, *121*, 2309−2310.

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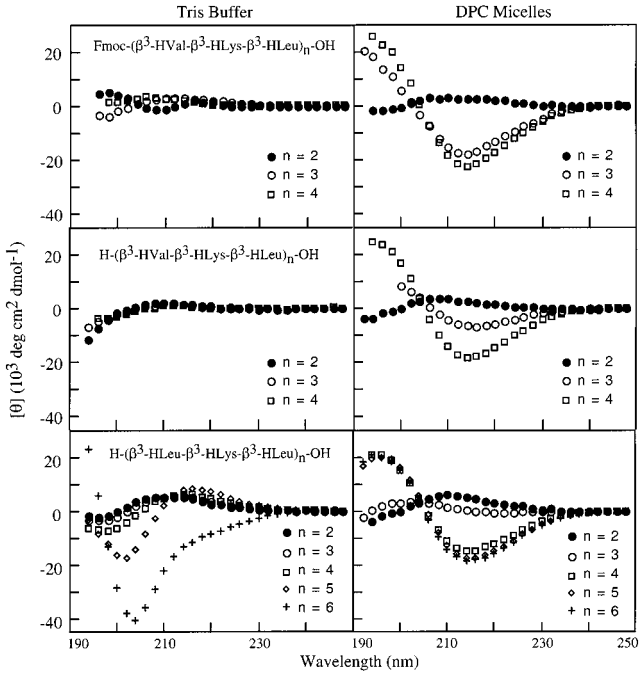

Figure 2. CD spectra of β-peptides in 10 mM Tris buffer (pH = 7.0), in the absence (left) and presence (right) of DPC micelles (monomer concentration = 5 mM). The data are expressed in mean residue ellipticity to allow comparison of peptides of differing lengths. Peptide concentrations were approximated from the dry weight of the peptides, which were isolated as TFA salts.

consistent with essentially complete helix formation at $n = 4$. Similar data were observed with small, unilamellar vesicles composed of POPC (not shown). Hydrophobic/water interfaces are similarly able to induce α-helix formation in a variety of amphiphilic α-peptides.[27]

The presence of the hydrophobic Fmoc group appears to favor binding to DPC micelles (and concomitant formation of an L+2 helical conformation), based on the slightly greater negative ellipticity at 215 nm observed for Fmoc-(β^3-HVal- β^3-HLys- β^3-HLeu)$_3$-OH versus H-(β^3-HVal- β^3-HLys- β^3-HLeu)$_3$-OH. The dependence of amino acid composition on L+2 helix formation was probed by comparing the CD spectra of H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_3$-OH versus H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_3$-OH (Figure 2). These spectra indicate that the β^3-HVal -containing β-peptide has a slightly greater propensity to form the L+2 helix than the β^3-HLeu -containing peptide. However, at chain lengths of 12 residues ($n = 4$) or longer, helix formation appeared to be complete for all three series of peptides, because further chain elongation failed to increase the intensity of $[\theta]_{215\ nm}$.

The biological activities of these peptides were measured using human erythrocytes (RBCs) and *Escherichia coli* as models for mammalian and bacterial cells, respectively. Hemolysis was monitored in 10 mM Tris, 150 mM NaCl, pH 7.0, while the bacterial assay was conducted in minimal media M9 (Table 1); both of these solutions are sufficiently transparent to allow CD and ultracentrifugation measurements (Supporting Information). Under the assay conditions, the peptides gave CD spectra similar to those in Tris buffer, with the exception of H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_n$-OH, $n = 5$ and 6. Analytical ultracentrifugation indicated that these two peptides formed large aggregates in the presence of phosphate (an essential component of minimal media). Therefore, the antibacterial activities of these two peptides were not considered.

All three series of peptides show length-dependent antibacterial activities, which correlate with their helical contents in DPC micelles. In DPC micelles the helical contents of the three

Table 1

peptide	hemolysis[a] HD50 (μM)	anti-bacterial assay[b] IC50 (μM)	select-ivity HD50/ IC50
Fmoc-(β^3-HVal-β^3-HLys-β^3-HLeu)$_2$-OH	>100	>100	
Fmoc-(β^3-HVal−β^3-HLys-β^3-HLeu)$_3$-OH	6.3	15	0.42
Fmoc-(β^3-HVal−β^3-HLys−β^3-HLeu)$_4$-OH	0.31	1.5	0.21
H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_2$-OH	>100	>100	
H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_3$-OH	86	41	2.1
H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_4$-OH	4.2	2.1	2.0
H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_2$-OH	>100	>100	
H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_3$-OH	>100	35	>2.9
H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_4$-OH	2.6	1.7	1.5
H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_5$-OH	0.23		
H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_6$-OH	0.081		

[a] Hemolysis experiments were performed by incubating a 0.25% suspension of human RBCs in 10 mM Tris buffer containing 150 mM NaCl at pH 7.0 with varying amounts of peptide. The hemolytic dose required to lyse 50% of the RBCs was obtained as described in the Supporting Information. [b] Antibacterial assays were performed by incubating varying amounts of peptide with cultures of K91 *E. coli* in minimal media at pH 7.4. The peptide dose required to supress 50% bacterial growth was obtained as described in the Supporting Information.

9-residue β-peptides follow the progression Fmoc-(β^3-HVal-β^3-HLys-β^3-HLeu)$_3$-OH > H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_3$-OH > H-(β^3-HLeu-β^3-HLys-β^3-HLeu)$_3$-OH. Approximately the same trend is observed in the biological data. However, the biological activities of the peptides continue to increase as their chain lengths are increased beyond the threshold required for complete helix formation in DPC micelles. This result may reflect the fact that longer helices have a higher surface area available for binding to membrane surfaces, providing enhanced affinity and greater efficacy.

These data show the utility of linear β-peptides for the design of biologically active peptides. Also, Pavone, Lombardi and co-workers have prepared a series of cyclic mixed α/β peptides that are potent antagonists of the neurokinin A receptor,[28] and Seebach and co-workers have designed cyclic peptides composed exclusively of β-amino acids that bind to the somatostatin receptor with IC_{50} values ranging from 36 to 190 μM.[29] The basic, amphiphilic β-peptides described herein are highly active against *E. coli* as well as RBCs. The potency of the longest peptide (80 nM) is considerably greater than that of melittin, whose LD_{50} is approximately 0.5 μM under these conditions.[13] Although the selectivity of the current β-peptides for bacterial versus mammalian cells is currently low, it should be possible to improve their selectivity. The effects of hydrophobic/hydrophilic balance, chain length, and helix-forming potential on selectivity and affinity is understood for α-helical antibacterial peptides of this class.[10] In a similar manner it would be of interest to design β-peptides with graded amphiphilicity and helix stability to allow optimization of affinity and selectivity. The activities of such analogues would also provide a further test for the hypothesis that the L+2 helix is essential for activity.

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Supporting Information Available: Synthetic procedures, assay conditions, and CD spectra of peptides under conditions of the biological assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA992728P

(27) DeGrado, W. F.; Lear, J. D. *J. Am. Chem. Soc.* **1985**, *107*, 7684.

(28) Pavone, V.; Lombardi, A.; Nastri, F.; Saviano, M.; Maglio, O.; D'Auria, G.; Quartara, L.; Maggi, C. A.; Pedone, C. *J. Chem. Soc., Perkin Trans. 2* **1995**, 987−993.

(29) Gademann, K.; Ernst, M.; Hoyer, D.; Seebach, D. *Angew. Chem., Int. Ed.* **1999**, *38*, 8.

(30) Peggion, E.; Mammi, S.; Schievano, E. *Biopolymers (Pept. Sci.)* **1998**, *43*, 419−431.