De Novo Design, Synthesis, and Characterization of Antimicrobial β-Peptides

Dahui Liu and William F. DeGrado*

Contribution from the Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6059

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Abstract: β-Peptides are a class of polyamides that have been demonstrated to adopt a variety of helical conformations. Recently, a series of amphiphilic $\mathbf{L}_{+2}$ helical β-peptides were designed, which were intended to mimic the overall physicochemical properties of a class of membrane-active antimicrobial peptides, including magainin and cecropin. Although these peptides showed potent antimicrobial activity, they also showed significant activity against human erythrocytes. Operating under the assumption that their lack of specificity arose from excessive hydrophobicity, two additional β-peptides H-(β^3-HAla-β^3-HLys-β^3-HVal)$_n$-NH_2 ($n = 4$, 5) were designed and synthesized. Both have high antimicrobial activities, but very low hemolytic potencies. The peptides bind in an $\mathbf{L}_{+2}$ conformation to phospholipid vesicles, inducing leakage of entrapped small molecules. The peptides have a low affinity for membranes consisting of neutral phosphatidylcholine lipids, but bind avidly to vesicles containing 10 mol % of acidic phosphatidylserine lipids. Differences in vesicle leakage kinetics for the two peptides suggest that chain length could affect their mechanisms of disrupting cell membranes. Thus, insights gained from the study of variants of natural α-peptides have provided a useful guide for the design of nonnatural antimicrobial β-peptides.

Introduction

Proteins fold into well-defined three-dimensional structures, which are essential to their unique biological activities. For decades, the question of how a protein's amino acid sequence dictates its conformation has captivated the attention of many chemists, biophysicists, and biochemists. As the fundamental understanding of this problem has progressed, it has become increasingly clear that proteins composed of α-amino acids are not the only molecules able to fold into well-defined three-dimensional structures.[1,2] Indeed, a variety of other oligomers have been shown to adopt secondary structures, including peptoids,[3−5] β-peptides,[1,2,6−8] γ-peptides,[9,10] δ-peptides,[11−14] and others.[15−21] These nonnatural polymers present new systems for testing the rules of protein folding and structural stabilization, while also providing an excellent medium for the design of biomimetic structures with practical applications in the areas of pharmaceuticals and materials science.

β-Peptides can adopt a variety of different helical conformations, differing in chirality as well as the radius of the structure. Bode et al.[22] suggested a nomenclature based on the handedness of the helix (left or right as **L** or **R**, respectively) as well as the directionality of hydrogen bonding interactions within the main chain. For example, an $\mathbf{L}_{-3}$ helical conformation would designate a left-handed helix consisting of main chain−main chain hydrogen bonds between an amide proton of residue *i* and the carbonyl oxygen of residue $i - 3$ preceding it in sequence.

* To whom correspondences should be addressed: (e-mail) wdegrado@mail.med.upenn.edu.

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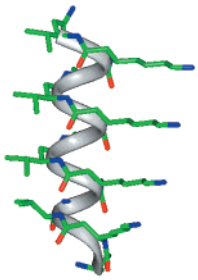


Figure 1. Molecular model of **1** in a $\mathbf{L}_{+2}$ helix conformation. Carbon atoms are shown in green, nitrogen in blue, and oxygen in red.

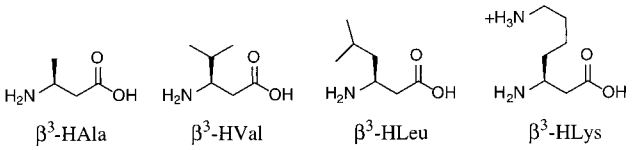
Structural investigations of poly(α-isobutyl-L-aspartate) provided the first indication that β-peptides may form helical conformations.[23−26] More recently, the synthesis of β-peptides of defined sequence has allowed high-resolution NMR and crystallographic studies of this class of compounds. Gellman's group initially focused on β-peptides containing the conformationally constrained cyclic amino acid, 2-aminocyclohexanecarboxylic acid (ACHC), while Seebach's group studied β-peptides prepared from acyclic residues with a diverse collection of side chains. Both classes of compounds adopt an $\mathbf{L}_{+2}$ helix.[27−31] The $\mathbf{L}_{+2}$ helix (Figure 1) is also referred to as the 14-helix[27] and a 3_1 helix.[28−30] The $\mathbf{L}_{+2}$ conformation is very stable relative to the α-helical conformation of α-peptides, in both organic solvents and water.[27,32−36] The features stabilizing the $\mathbf{L}_{+2}$ have been probed through synthesis of a variety of peptides from variously substituted β-amino acids. A single substituent at C1 or C2 tends to favor helix formation,[30,37−40] while the inclusion of these centers into a cyclohexane ring[27] in *trans*-2-aminocyclohexane carboxylic acid strongly stabilizes the $\mathbf{L}_{+2}$ helix.

β-Peptides can also adopt a number of other conformations, analogous to the secondary structures of α-peptides. Seebach has documented a "12/10/12" conformation, featuring a central C10 turn flanked on either side by a C12 turn.[30,38] A repeating pattern of C12 hydrogen bonding also stabilizes an $\mathbf{L}_{-3}$ helical conformation in *trans*-2-aminocyclopentane carboxylic acid (ACPC)-containing peptides.[41] Finally, appropriately substituted β-amino acids are able to stabilize the formation of antiparallel hairpin and sheet-like structures.[39,40,42,43]

The favorable conformational and enzymatic properties of β-peptides suggest that they might have interesting biological applications.[44−47] Recently, we designed a series of peptides with potent cytolytic properties.[48] Previous studies had identified a basic amphiphilic α-helix as a feature that underlies the potent cell-killing behavior of a large class of peptides that includes melittin, magainins, cecropins, and many other antimicrobial peptides.[49−54] These peptides kill their target cells by disrupting the integrity of cellular membranes, either through the formation of discreet channels or by more generalized disruption of the bilayer structure. It has long been hypothesized that the overall physicochemical properties of these helices, and not their precise amino acid sequences, are responsible for their cell-killing activities.[49] To test this hypothesis, we prepared a series of β-peptides with a high potential to adopt an amphiphilic $\mathbf{L}_{+2}$ helix.[48]

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



The hydrophobic side chains in this series of peptides occur with a repeat matching the 3-residue repeat of the $\mathbf{L}_{+2}$ helix. Thus, the positively charged β^3-HLys and the hydrophobic β^3-HVal and β^3-HLeu side chains segregate to opposite faces of the helix in these peptides. Circular dichroism spectroscopy indicated that, although the peptides were unstructured in dilute aqueous solution, an $\mathbf{L}_{+2}$ helical conformation was induced upon binding to the surface of micelles. Further, the formation of this secondary structure was necessary for hemolytic and antimicrobial activity.

More recently, Gellman and co-workers described an antimicrobial β-peptide, composed of conformationally constrained,

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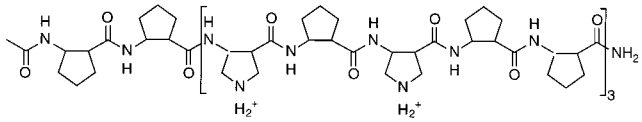
cyclic β-amino acids, that adopts an $\mathbf{L}_{-3}$ helical conformation.[55] These peptides displayed potent antimicrobial activity, and, in contrast to our earlier series of peptides, displayed minimal activity against mammalian cells.



β-17

There are a large number of differences between these two series of peptides including their helix type, hydrophobicity, and rigidity, which may account for their differential selectivities. Previous studies have shown that the selectivity of antimicrobial α-peptides reflects a delicate balance of hydrophobicity, size, helical propensity, and charge distribution.[50,54] In particular, the hydrophobicity is critical, and inclusion of too many hydrophobic amino acids will lead to compounds with very low selectivity for mammalian versus bacterial cells. Therefore, in the current study the hydrophobic content of our previous series of compounds was decreased by changing a single β^3-HVal for β^3-HAla, yielding H-(β^3-HAla-β^3-HLys-β^3-HVal)$_n$-NH$_2$ (**1**, $n = 4$; **2**, $n = 5$). Previous studies of analogues of natural antimicrobial peptides revealed that C-terminal amides are more active than the corresponding free carboxyl acid.[50] Hence **1** and **2** were designed to have C-terminal carboxamide groups. Here, we describe the antimicrobial potencies of these peptides, and additionally explore their mode of action using phospholipid bilayer vesicles.

Experimental Section

Methods and Material. Fmoc α-amino acid pentafluorophenyl esters were purchased from Nova Biochem, HBTU and HOBt from Advanced ChemTech., Fmoc PAL-PEG-PS resin (loading = 0.17 mmol/g) from PerSeptive Biosystems, SOPC and SOPS from Avanti Polar-Lipids, Inc., calcein from Lancaster Synthesis, and all other reagents from Aldrich; all were used without further purification. All Fmoc β-amino acids were synthesized from the corresponding Fmoc α-amino acid pentafluorophenyl esters via Arnst−Eistert homologation following a published procedure.[56] Peptides were manually synthesized in a standard glass peptide synthesis vessel. The purification was carried out on a Waters HPLC using a Vydac C4 column. Solvent A was composed of 0.1% TFA in water and solvent B was composed of 90% acetonitrile, 10% water, and 0.1% TFA. Mass spectra were measured on a Hewlett-Packard 1100 ESI spectrometer and a PerSpective Biosystems Voyager-DE RP MALDI-TOF mass spectrometer. NMR spectra were obtained on a Bruker AC-250 spectrometer. UV−vis spectra were measured on a Hewlett-Packard 8453 spectrometer. Fluorescence measurements were carried out using a Hitachi F-2500 fluoresence spectrophotometer. CD spectra were obtained on an AVIV 62DS spectropolarimeter.

Synthesis of H-(β^3-HAla-β^3-HLys-β^3-HVal)$_n$-NH$_2$ ($n = 4, 5$). Fmoc PAL-PEG-PS resin (588 mg, 0.1 mmol) was allowed to swell in DMF (5 mL) for 30 min before the synthesis. The Fmoc was deprotected with 20% piperidine/DMF (3 × 5 mL × 5 min) and washed with DMF (5 × 5 mL × 2 min). Amino acid couplings were carried out by adding a 2 mL solution of amino acid (0.25 mmol), HBTU (95 mg, 0.25 mmol), HOBt (34 mg, 0.25 mmol), and DIEA (139 μL, 0.8 mmol) in DMF to the resin, shaking for 4 h, and washing with DMF (5 × 5 mL × 2 min). The peptides were cleaved from the resin by treatment of TFA/TIS (95:5) for 2 h. The solution was concentrated and the peptide was precipitated by addition of cold ether. Peptides were purified by HPLC on a reverse phase C4 column, with a linear gradient from 20% to 50% solvent B in 50 min for **1**, and from 30% to 60% solvent B in 60 min for **2**. LRMS (MALDI-TOF) *m*/*e* calcd for **1** ($C_{68}H_{132}N_{17}O_{12}$) ($MH^+$) 1379.1, found 1379.6. LRMS (MALDI) *m*/*e* calcd for **2** ($C_{85}H_{164}N_{21}O_{15}$) ($MH^+$) 1719.4, found 1720.1.

Circular Dichroism Studies. CD spectra were measured on an AVIV 62DS spectropolarimeter using both 1 mm and 10 mm quartz cuvettes. Sample stock solutions were prepared in water and diluted into appropriate buffers. Peptide concentrations were determined from the dry weight of lyophilized peptide.

Hemolysis Assay. Hemolysis experiments were carried out by incubating a 0.25% suspension of human erythrocytes (RBC's) with peptides of different concentrations in 150 mM sodium chloride and 10 mM Tris buffer, pH 7.0. The sample was prepared by combining 400 μL of the RBC suspension and 100 μM of the peptide solution. After the sample was incubated at 37 °C for 1 h, it was centrifuged at 14 000 rpm for 5 min, and the OD_{414} of the supernatant was measured. Melittin (50 μM) was used to define 100% hemolysis. The hemolytic HC50 was obtained by plotting hemolysis percentage versus peptide concentration, and the concentration required for 50% hemolysis was interpolated by exponential curve fitting to the graph.

Antimicrobial Assay. Antimicrobial assay was performed by incubation of K91 *E. coli* and peptide of different concentrations in minimal media, pH 7.4. The peptide solution (50 μL) and K 91 *E. coli* culture (20 μL, grown in minimal media for 24 to 36 h) were mixed with 1 mL of minimal media. After incubation at 37 °C for 8 h, the OD_{600} was measured. The peptide doses required for 50% and 100% suppression of bacterial growth were interpolated by exponential curve fitting to the graph of suppression percentage versus peptide concentration.

Peptide Binding to Phospholipid Bilayers. The binding of peptides to phospholipid bilayers was measured using CD spectra of the peptide in the presence of varying concentrations of phospholipid vesicles. The peptides showed very small molar ellipticities at 214 nm in the absence of vesicles. Upon addition of phospholipid vesicles, the ellipiticity at 214 nm became more intense as a consequence of helix formation due to interactions between the peptide and phospholipid surface. Small unilamellar vesicles (SUV) of SOPC/SOPS were prepared by sonicating large phospholipid vesicles in 10 mM phosphate buffer, pH 7. CD spectra were taken before and after the addition of aliquots of vesicle to 2.0 mL of peptide solution in 10 mM phosphate buffer, pH 7. The dissociation constant K_d was determined by least-square fitting (KaleidaGraph) using eq 1 for single-site binding:[57]

$$[P](r - 1) - [PL]/n - K_d + [PL]/(nr) = 0 \quad (1)$$

where [P] and [PL] are peptide and phospholipid molar concentrations, respectively, and n is the number of phospholipids per peptide binding site. The fraction of peptide bound, r, is calculated from eq 2:[58]

$$r = (\theta_{obs} - \theta_0)/(\theta_b - \theta_0) \quad (2)$$

in which θ_{obs} is the mean residue ellipticity at 214 nm at a given concentration of phospholipid. θ_b and θ_0 are the mean residue ellipticities of the peptides in free solution and when bound to the vesicle, respectively. Since phospholipid vesicles cause light scattering, it was difficult to obtain data at very high vesicle concentrations, necessary to directly measure θ_b. Therefore θ_b was considered as a variable during the curve fitting.

Peptide-Induced Leakage of Vesicle Contents. The leakage of vesicle contents to the external media was monitored by the release of calcein encapsulated in large unilamellar vesicles. The vesicles were prepared by reverse-phase evaporation in 10 mM sodium phosphate buffer, pH 7, followed by a single extrusion through a 0.2 μm pore size polycarbonate filter. The non-trapped calcein was removed by eluting through a size exclusion Sephadex G-25 column, with 90 mM sodium chloride, 10 mM sodium phosphate, pH 7. The kinetics of leakage were monitored by following the increase of calcein fluorescence intensity at 515 nm (excitation at 490 nm, slit width 3.8 nm)

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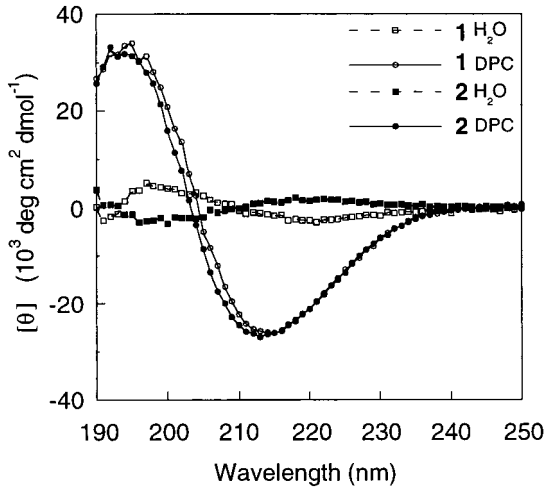

Figure 2. CD spectra of **1** and **2** in 10 mM Tris buffer (pH 7.0) in the absence and presence of DPC micelles (5 mM), plotted in mean residue ellipticity. The spectra were measured in 1 mm cuvettes with peptide concentrations between 50 and 100 μM.

due to the inhibition of self-quenching. Complete leakage was achieved by addition of 10 μL of 0.2% Triton 100 to the 2 mL solution, and the corresponding fluorescence intensity was used as 100% leakage for the calculation of the leakage fraction. Multiple exponentials were fit to the data using Igor Pro 3.12.

Results

Circular Dichoism Studies. Circular dichoism (CD) spectroscopy is a rapid method to determine secondary structures of proteins and peptides. For peptides of β-amino acids, the standard of structure determination is not as well developed as that for peptides and proteins of α-amino acids. Nevertheless, typical CD spectra of several types of helices adopted by β-peptides have been reported and provide useful references.[2,28,29,38,41] The CD spectrum of the $\mathbf{L}_{+2}$ helix has a positive cotton effect centered around 195 nm and a negative one centered around 215 nm, arising primarily from an exciton-coupled $\pi-\pi^*$ transition.[22]

The CD spectra of **1** and **2** were measured in the presence and absence of dodecylphosphocholine (DPC) micelles (Figure 2). In contrast to some of our earlier antimicrobial β-peptides that showed limited solubility in aqueous buffers,[8] peptides **1** and **2** show good solubility in water and do not form large insoluble aggregates at concentrations up to 100 μM. In homogeneous aqueous solution, the CD spectra of both peptides are relatively flat, indicating that they failed to form the $\mathbf{L}_{+2}$ helix. These data suggest that the peptides might adopt random coil conformations in the absence of stabilizing interactions with DPC micelles. The random coil conformation would maximize the peptides' conformational entropy as well as minimize the repulsion among positively charged β^3-HLys side chains. However, upon addition of DPC micelles (5 mM DPC), a strong positive peak around 195 nm and a negative peak around 215 nm were observed, indicative of an $\mathbf{L}_{+2}$ helix. The magnitudes of the mean residue ellipticities of **1** and **2** are comparable, suggesting complete helix with 12 residues ($n = 4$), similar to results obtained in a previous study of analogous β-peptides.[48] The position of the minimum shifts to slightly lower energy for the shorter peptide (213 nm for **2** vs 214 nm for **1**).

Cellular Assays. The activity and selectivity of **1** and **2** were examined using *E. coli* and human erythrotytes (RBC) as models for bacterial cells and mammalian cells, respectively. The peptide concentration required for 50% bacterial growth suppression (IC_{50}), the minimal inhibitory concentration (MIC), and the peptide concentration required for 50% RBC lysis (HD_{50}) are listed in Table 1. Both peptides show high antimicrobial activity with MIC values of 26 and 20 μg/mL for **1** and **2**, respectively. The values are slightly higher than the natural antimicrobial peptide magainin (MIC 3.2 μg/mL) and the "β-17" β-peptide (MIC 6.3 μg/mL) recently reported by Gellman and co-workers.[55] The small increase in potency with chain length between **1** and **2** has been observed in our previous studies.[48] To test the selectivity of the peptides toward mammalian cells, their hemolytic activity was also measured. The results indicate that they show very strong selectivity for bacteria. The HD_{50} is 920 μM for **1** and 320 μM for **2**.

Table 1. Cellular Activities and Membrane-Binding Properties of **1** and **2**[a]

peptide	H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_4$-OH[b]	**1** (n=4)	**2** (n=5)
IC_{50} [mM]	2.5 ± 1	5 ± 1	3 ± 1
HC_{50} [μM]	37 ± 5	910 ± 260	320 ± 60
MIC [μg/mL]	9	26	20
selectivity	15	180	110
K_d(SOPS/SOPC (1:9)) [μM]		1.4 ± 0.28	0.3 ± 0.06
K_d(SOPC) [μM]		*c*	15 ± 8

[a] The assays were conducted as described in the Experimental Section. [b] These data differ somewhat from the values quoted in our earlier report.[48] The peptide described in the earlier report was contaminated with a small amount of the Fmoc-containing peptide, which resulted in increased hemolytic potency. [c] No binding was detected by CD spectra.

Membrane Binding Studies. We also examined the binding of the peptides to small unilamellar phospholipid membranes, which better approximate biological membranes than the micelles used to obtain CD spectra. Although it was not possible to obtain CD spectra below 200 nm with single unilamellar vesicles (SUV's), it was possible to monitor conformational changes associated with binding by monitoring the ellipticity at 214 nm. Absorbance spectra of the samples were carefully examined to ensure that light-scattering artifacts did not affect the quality of the results.[59,60]

Mammalian cell membranes consist primarily of neutral phosphatidylcholine, while bacterial membranes are more negatively charged due to the presence of large amounts of lipopolysaccharides or acidic polysaccharides.[61] We therefore measured the binding of **1** and **2** to bilayers composed of stearoyl-oleoyl-phosphatidylcholine (SOPC) as well as bilayers that additionally contain 10 mol % of the corresponding negatively charged phosphatidylserine derivative (SOPS). Both peptides exhibit tight binding to SOPS/SOPC (1:9) vesicles. The curves are typical of single-site binding isotherms (Figure 3), allowing the determination of the dissociation constant for binding as well as the stoichiometry of the peptide/phospholipid complex as described in the Experimental Section. Compound **1** binds to SOPS/SOPC (1:9) vesicles with a dissociation constant of 1.4 μM, and a limiting binding stoichiometry of approximately 42 phospholipid molecules per peptide. Consistent with its increased length, peptide **2** bound with greater affinity (0.2 μM) and a larger number of phospholipids (52) were required to create a binding site for this peptide.

By contrast to the high affinity displayed by these peptides for SOPC/SOPS membranes, they showed very limited binding

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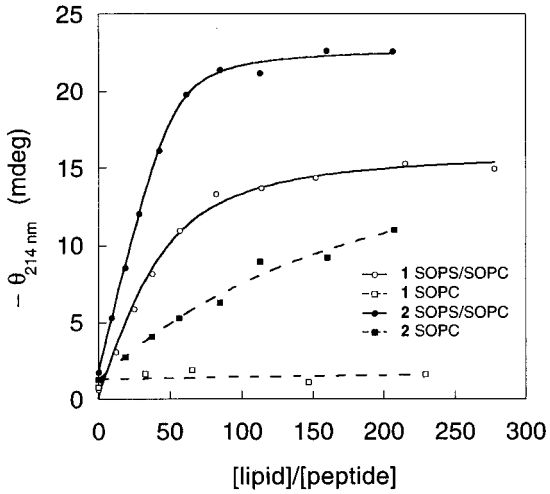

Figure 3. Lipid binding experiments of **1** (3.92 μM) and **2** (5.27 μM) in 10 mM sodium phosphate buffer (pH 7.0). **1** with SOPS/SOPC 1:9.

to pure SOPC bilayers, as assessed by the CD method. The addition of SOPC SUV vesicles to a solution of **1** failed to give rise to any increase in its signal at 214 nm. Thus, this peptide failed to bind SOPC vesicles in a helical conformation, although this method does not rule out the possibility that it might bind in a random coil conformation. Upon addition of SOPC vesicles to peptide **2**, one observes a small increase in helix content, consistent with a binding constant of approximately 15 μM. The much lower affinity of **2** for SOPC vesicles versus SOPS/SOPC (1:9) vesicles is consistent with its selective activity against bacterial cells.

Peptide-Induced Leakage of Liposomal Contents. Although the mechanism of action of amphiphilic α-helical antimicrobial peptides is still a matter of debate, it is generally believed their primary mode of action involves the disruption of cellular membranes.[50−52,62−70] To assess the extent to which peptides **1** and **2** disrupt phospholipid membranes, we measured their abilities to induce leakage of a dye, calcein, entrapped within the interiors of large unilamellar vesicles (LUV) of SOPS/SOPC (1:9). The time course of the leakage of encapsulated calcein was detected by its fluorescence at 515 nm (Figure 4).

Although a complete kinetic study would be beyond the scope of this article, several features are immediately apparent. First, at low peptide/phospholipid ratios, many of the kinetic traces in Figure 4 appear to level off at less than 100% leakage, indicating that at these peptide/lipid ratios the process of leakage does not go to completion, even after long times. Second, the time courses are biphasic, suggesting that at least two different processes contribute to the leakage. The kinetics of vesicle lysis are well described by the sum of two exponentials (eq 3).

$$F_t = F_f(1 - e^{-k_f t}) + F_s(1 - e^{-k_s t}) + C \qquad (3)$$

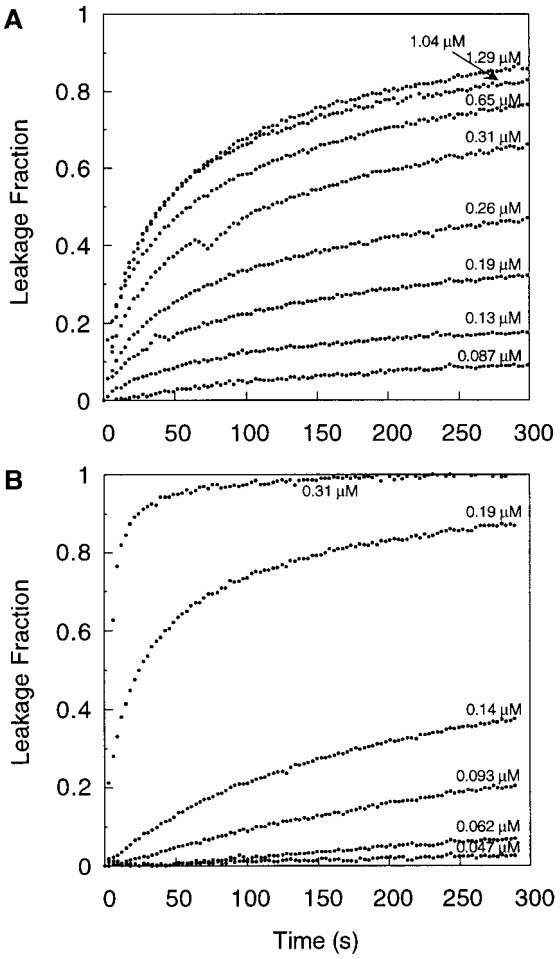

Figure 4. Time course of peptide induced leakage of liposomal contents for **1** (A) and **2** (B).

The parameters describing the fast component are k_f and F_f, the rate constant and the fractional leakage extrapolated to infinite time. The corresponding parameters for the slow phase are F_s and k_s, respectively. The constant C was found to be very close to 0 for each kinetic run, verifying that the double exponential equation is sufficient to describe the process.

Figure 5A−C shows F_f, F_s, and the sum of these parameters versus the fraction of peptide bound (relative to the theoretical maximum bound as determined in the binding studies). The sum of F_s and F_f provides the total lysis extrapolated to infinite time (Figure 5C). Interestingly, complete lysis of the vesicles occurs at subsaturating concentrations of bound peptide, reaching ∼90% lysis at ∼30−40% binding, for both peptides. For compound **1** at low concentrations, the extent of lysis associated with the slow phase dominates (Figure 5A). However, as more peptide is bound, the F_f makes an increasingly large contribution, approaching that of F_s at the highest peptide concentrations. A similar picture emerges for **2**, except that F_f actually dominates at high concentrations of peptide (Figure 5B).

Surprisingly, there are major differences between the rate constants for vesicle leakage, induced by the two different peptides. It was difficult to obtain good estimates for the rates over portions of the experimental range for the two peptides (Figure 6). Nevertheless, it is apparent that the rate constant associated with the slow phase, k_s, is independent of peptide concentration for peptide **1**, but approximately third order with respect to bound peptide for **2**. Even more striking, the k_f is largely independent of peptide concentration for peptide **1**, but very high order in bound peptide concentration for peptide **2**.

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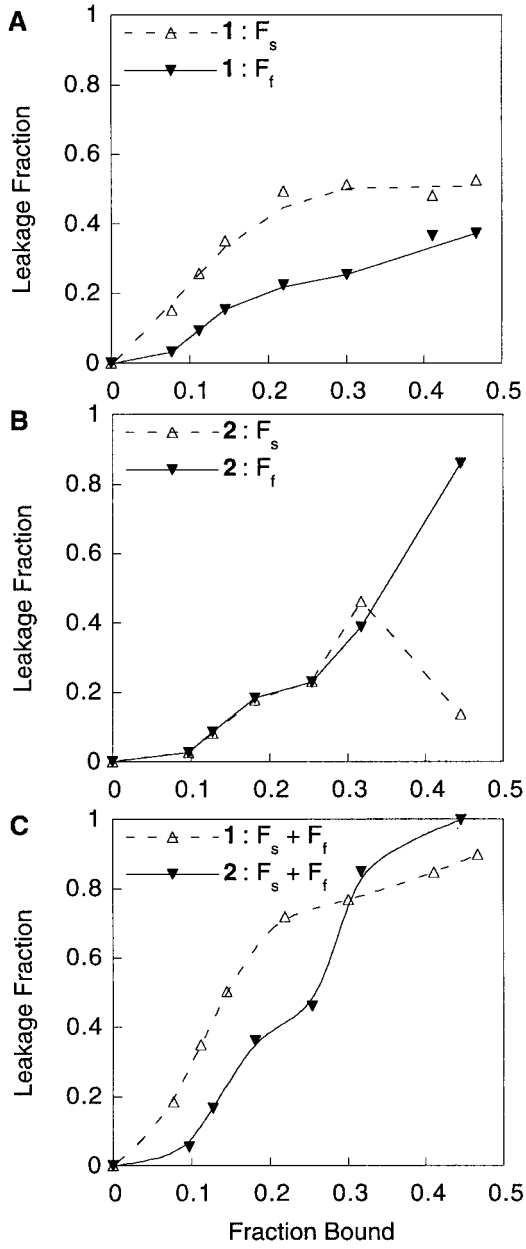

Figure 5. Fractional lysis extrapolated to infinite time by fitting the liposome leakage kinetic data to double exponential (eq 3). F_s and F_f for **1** (A) and **2** (B) and $F_s + F_f$ for both peptides (C).

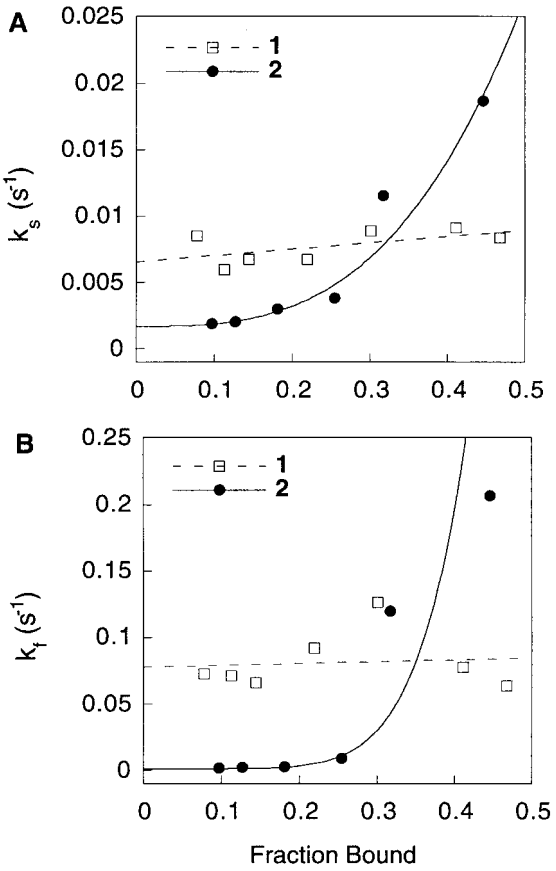

Figure 6. Rate constants from fitting the liposome leakage kinetic data to double exponential (eq 3) for **1** (A) and **2** (B).

Discussion

Numerous studies on natural antimicrobial peptides revealed that an appropriate balance in the distribution of hydrophobic and polar residues is required to obtain a high selectivity for killing bacteria versus mammalian cells. If the peptides are overly hydrophobic they bind indiscriminately to cellular membranes, resulting in very low selectivity for bacterial versus mammalian membranes.[50,54] Peptides **1** and **2** are significantly less hydrophobic than the previously reported series (as assessed by their retention times on reverse-phase HPLC), as a result of the replacement of β^3-HAla for β^3-HLeu and β^3-HVal at several positions of the sequence.[71] The results from the antimicrobial assay and hemolysis experiments indicate that by reducing the hydrophobicity of the peptide, the selectivity is enhanced as predicted.

This paper also addresses a second question: Is it possible to obtain high cell selectivity within this series of β-peptides, which are prepared from relatively flexible monosubstituted β-amino acids? Earlier studies suggested that the helical potential must be appropriately tuned to provide binding specificity: if the helical potential was too low the peptides failed to be effective, whereas if the helical potential was too high they were antimicrobial, but lost their cell selectivity.[54,72−75] Previously, Gellman showed that a series of conformationally constrained β-peptides, which formed very stable helices in water, were able to form highly selective antimicrobial agents.[55] By contrast, we demonstrate that highly flexible compounds are also able to show highly selective antimicrobial activity. We conclude that there is no a priori requirement concerning the helix-forming potential (in homogeneous aqueous solution) of the compound. Clearly, a peptide with a highly stable helix will bind more tightly to membranes than a peptide with a marginally stable helix, assuming that the hydrophobicity and charge distribution were otherwise identical. Thus, by increasing the helical potential of a highly selective peptide, it might be possible to obtain a peptide that binds too avidly to mammalian membranes, thereby losing its selectivity. However, the results of Gellman and co-workers indicate that highly stable helices can show appropriate selectivity if their charge and hydrophobicity distributions are appropriate.

Several mechanisms have been proposed for the process of cell killing by antimicrobial peptides. In the carpet mechanism,

(71) HPLC retention time is listed in Table S1 in the Supporting Information.

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peptides aggregate parallel to the membrane surface.[76,77] The accumulated peptide molecules wrap the membrane surface in a carpet-like manner, leading to thinning and ultimately rupture of the membrane after the concentration of the surface-bound peptide reaches a threshold value. The so-called barrel-stave mechanism suggests that the bound peptides on the cell surface self-associate into transmembrane helical bundles that form stable aqueous pores in the membrane.[78,79] A third explanation [62] for the activity of this class of peptides is that the peptides initially bind only to the outer leaflet of the bilayer. Binding is overall favorable because it leads to a decrease in the exposure of the hydrophobic side chains to water, but also leads to an increase in the lateral surface pressure of the outer leaflet relative to the inner leaflet of the bilayer. This pressure imbalance leads to translocation of the peptides into the interior of the bilayer thereby equilibrating the surface pressure on both sides of the bilayer. However, because the peptides are very hydrophilic, the translocation is likely to occur with concomitant formation of transient openings in the membrane, which allow hydration of the polar side chains of the peptide. Leakage of the cellular contents occurs during the formation of these transient pores. Once the equilibration of the bilayer is complete, the integrity of the membrane may be largely restored. Most antimicrobial peptides probably act by more than one of these mechanisms. Interestingly, the difference in the kinetics of vesicle leakage for peptides **1** versus **2** suggests that chain length might also affect the mechanisms by which the peptides disrupt bilayers.

In conclusion, this paper describes the design and synthesis of a series of membrane-active β-peptides with potencies and specificities similar to those of naturally occurring peptides such as magainin. These results indicate that insights gained from the study of variants of natural α-peptides have provided a useful guide to the design of nonnatural antimicrobial β-peptides. They also raise the possibility of designing molecules that are even simpler in structure than these β-peptides, but nevertheless reproduce the activities of this class of antimicrobial compounds.

Supporting Information Available: The HPLC retention time and corresponding percent CH_3CN for **1**, **2**, and H-(β^3-HVal-β^3-HLys-β^3-HLeu)$_4$-OH (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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