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Interplay among Folding, Sequence, and Lipophilicity in the Antibacterial and Hemolytic Activities of α/β-Peptides

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Abstract: Host-defense peptides inhibit bacterial growth but manifest relatively little toxicity toward eukaryotic cells. Many host-defense peptides adopt α-helical conformations in which cationic side chains and lipophilic side chains are segregated to distinct regions of the molecular surface ("globally amphiphilic helices"). Several efforts have been made to develop unnatural oligomers that mimic the selective antibacterial activity of host-defense peptides; these efforts have focused on the creation of molecules that are globally amphiphilic in the preferred conformation. One such endeavor, from our laboratories, focused on helix-forming α/β-peptides, i.e., oligomers containing a 1:1 pattern of α- and β-amino acid residues in the backbone [Schmitt, M. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2004**, *126*, 6848−6849]. We found, unexpectedly, that the most favorable biological activity profile was displayed by a "scrambled" sequence, which was designed *not* to be able to form a globally amphiphilic helix. Here we report new data, involving an expanded set of α/β-peptides, from experiments designed to elucidate the origins of this surprising result. In addition, we evaluate the susceptibility of α/β-peptides to proteolytic degradation. Our results support the hypothesis that the ability to adopt a globally amphiphilic helical conformation is *not* a prerequisite for selective antibacterial activity. This conclusion represents a significant advance in our understanding of the relationship among molecular composition, conformation, and biological activity. Our results should therefore influence the design of other unnatural oligomers intended to function as antibacterial agents.

Introduction

The development of new antibiotic agents is a subject of keen interest because of the emergence of pathogenic microbes that resist current chemotherapies. Eukaryotes have evolved complex mechanisms to ward off microbial infection, and these mechanisms can provide inspiration for the development of new therapeutic strategies. The host-defense system includes peptides that are toxic to a wide array of bacteria.[1] Many scientists have looked to host-defense peptides as prototypes for the development of synthetic antibiotics because development of bacterial resistance to these peptides appears to be difficult.

Among the myriad host-defense peptides, helix-forming examples such as the magainins[2] and cecropins[3] have drawn special attention because of their architectural simplicity. These peptides are typically 20−30 residues in length. They adopt α-helical conformations in the presence of bacteria or under conditions that are thought to mimic the environment provided by a bacterial cell surface (e.g., in the presence of lipid vesicles or detergent micelles).[1] The peptides bear a net positive charge, which attracts them to the negatively charged bacterial outer surface.[4−6] Global amphiphilicity is achieved in the folded state: hydrophilic (i.e., cationic) side chains are segregated along one side of the α-helix, and lipophilic side chains are segregated along the other side (Figure 1). Designed peptides that can adopt globally amphiphilic α-helical conformations often display antimicrobial activities comparable to those of natural host-defense peptides such as magainins. Natural helical antimicrobial peptides and synthetic mimics that display selective toxicity toward bacterial cells over mammalian cells typically display a cationic residue:hydrophobic residue ratio between 1:1 and 1:2, depending on primary sequence and residue composition.[7] (The 15-residue α/β-peptides discussed below each contain five positively charged residues, giving a 1:2 cationic:hydrophobic ratio.) Shai et al. have argued convincingly for a mechanism of action that involves membrane disruption via formation of mixed lipid−peptide micelles (the "carpet model").[8,9]

The simple architecture shown in Figure 1 has inspired the development of antimicrobial oligomers that do not have an α-amino acid residue backbone. Several groups have shown that helix-forming β-amino acid oligomers ("β-peptides") can kill both Gram positive and Gram negative bacteria.[10−15] Most of

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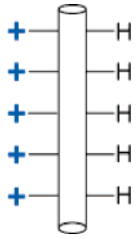

Figure 1. Graphic representation of a molecule adopting a globally amphiphilic conformation. Hydrophobic side chains (H) and cationic side chains (+) cluster on opposite sides of the structure.

these β-peptides display low lytic activity toward human red blood cells, which suggests that they share with host-defense α-peptides the ability to disrupt bacterial cell membranes in preference to eukaryotic cell membranes. Three different helical secondary structures have been endowed with antibacterial activity among β-peptides. Peptoids (*N*-alkyl-glycine oligomers) designed to adopt globally amphiphilic helical conformations have been shown to inhibit bacterial growth,[16] as have oligomers with an alternation of α- and β-amino acid residues ("α/β-peptides").[17] More dramatic structural departures from the natural prototypes have been reported as well. Savage et al. have described an imaginative extrapolation from the structure of polymyxin, a cyclic peptide, via the creation of globally amphiphilic molecules based on a steroid core.[18] These molecules were originally designed to permeabilize bacterial membranes and thereby promote the antibacterial effects of other agents; however, the amphiphilic steroids are themselves antibacterial agents. DeGrado et al. have designed small oligoamides to adopt globally amphiphilic conformations and shown that these molecules are antibacterial agents.[19,20] Other oligomers have been examined as well.[21]

Our β-peptide studies included control experiments to determine whether adoption of a globally amphiphilic helical conformation is important for antibacterial activity.[14,15] We compared sequence-isomeric β-peptides that either would or would not display a global segregation of hydrophilic (cationic) and lipophilic side chains upon folding. This strategy was implemented with two different helical β-peptide secondary structures, and in both cases sequence rearrangement ("scrambling") converted a potent antimicrobial agent into an inactive isomer.[14,15] We were therefore surprised to discover more recently a different pattern of behavior among amphiphilic α/β-peptides.[17] α/β-Peptides containing a 1:1 alteration of α- and β-amino acid residues have been shown to adopt a variety of helical conformations depending upon residue identity.[22−26] We observed significant antibacterial activity for α/β sequences designed to adopt globally amphiphilic helical conformations (**1** and **2**), but a "scrambled" sequence isomer designed *not* to be globally amphiphilic in a helical conformation (**3**) was nevertheless quite active. The α/β sequences intended to form globally amphiphilic helices were highly hemolytic, while the sequence isomer designed to form nonglobally amphiphilic helices displayed much lower hemolytic activity.

The trends observed among our initial set of α/β-peptides are significant with regard to design of antibacterial foldamers because the most favorable profile of activity (inhibition of bacterial growth at low concentrations and hemolytic activity only at high concentrations) was observed for a sequence that was designed to preclude global amphiphilicity in the helical state.[17] All efforts to date to design unnatural foldamers with antibacterial activity (including our own) have focused on achieving global segregation of lipophilic and cationic surfaces in a specific conformation. The α/β-peptide behavior we uncovered raises the possibility that alternative design approaches may be effective, and perhaps even superior. This important prospect encouraged us to conduct further investigation of sequence−activity relationships among amphiphilic helix-forming α/β-peptides, with the goal of identifying the requirements for selective inhibition of bacterial growth.

Here we provide new information on previously described[17] α/β-peptides **1**−**3** along with data acquired for new α/β-peptides **4**−**9** (Table 1). The new α/β-peptides were designed to address questions arising from our original study about the relationships among α/β-amino acid sequence, three-dimensional folding, physical properties (including susceptibility to proteolytic degradation), and biological activity. The findings reported below support the most important conclusion drawn from our initial studies: global segregation of lipophilic and hydrophilic (cationic) side chains in a helical conformation is *not* essential for selective antibacterial activity. This conclusion represents an important modification of the collective wisdom regarding the design of unnatural oligomers intended to display selective inhibition of bacterial growth.

Results

α/β-Peptide Design. Our original study focused on sequence isomeric α/β-peptides **1**−**3**.[17] Only four amino acids were used, L-lysine, L-leucine, (*S*,*S*)-ACPC, and the analogous stereoisomer of APC (Chart 1), and one lipophilic α-amino acid and one cationic and one lipophilic β-amino acid. These 15-residue α/β-peptides were designed on the basis of our structural analysis of shorter α/β-peptides (6−8 residues), which indicated formation of two different helical conformations, one containing C═O(i)→H−N(i+3) hydrogen bonds (designated the "11-helix,"

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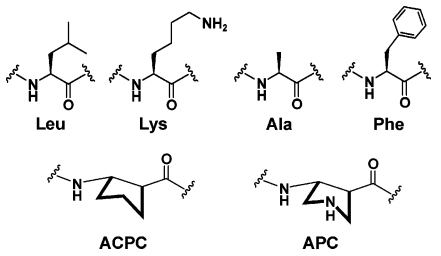
Table 1. Sequences of α/β-Peptides **1**−**9**[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	APC	Leu	ACPC	Lys	ACPC	Leu	APC	Leu	ACPC	Lys	ACPC	Leu	APC	Leu	ACPC
2	APC	Leu	ACPC	Leu	APC	Leu	ACPC	Lys	ACPC	Leu	ACPC	Lys	ACPC	Leu	APC
3	ACPC	Leu	APC	Lys	APC	Leu	ACPC	Lys	APC	Leu	ACPC	Leu	ACPC	Leu	ACPC
4	APC	Leu	APC	Leu	ACPC	Lys	ACPC	Leu	ACPC	Leu	APC	Leu	ACPC	Lys	ACPC
5	APC	Leu	ACPC	Leu	APC	Leu	ACPC	Leu	APC	Leu	ACPC	Lys	ACPC	Leu	APC
6	APC	Phe	ACPC	Leu	APC	Leu	ACPC	Phe	APC	Phe	ACPC	Lys	ACPC	Leu	APC
7	APC	Leu	ACPC	Lys	ACPC	Ala	APC	Ala	ACPC	Lys	ACPC	Ala	APC	Leu	ACPC
8	APC	Leu	ACPC	Ala	APC	Ala	ACPC	Lys	ACPC	Leu	ACPC	Lys	ACPC	Ala	APC
9	ACPC	Ala	APC	Lys	APC	Leu	ACPC	Lys	APC	Ala	ACPC	Ala	ACPC	Leu	ACPC

[a] Numbers across the top of the table indicate residue position in each α/β-peptide. Numbers in the first column of the table are the compound numbers by which each α/ β-peptide is referred in the text. All α/β-peptides have a free N-terminus and an amidated C-terminus.

Chart 1



based on hydrogen-bonded ring size) and the other containing C═O(*i*) →H−N(*i*+4) hydrogen bonds (designated the "14/15-helix").[23] For a given α/β-residue sequence, these two helices lead to different three-dimensional arrangements of side chains, as indicated by the "helix-wheel" diagrams in Figure 2. Sequence isomer **1** was designed to be globally amphiphilic in the 11-helical state; in other words, the lipophilic side chains (from leucine α-residues and ACPC β-residues) should cluster along one side of the 11-helical conformation of **1**, and the cationic side chains (from lysine α-residues and APC β-residues) should cluster along the other side. Global segregation of lipophilic and cationic side chains does *not* occur, however, in the 14/15-helical conformation of **1**. Isomer **2** was designed to be globally amphiphilic in the 14/15-helical state but not in the 11-helical state. Isomer **3** ("scrambled") was designed not to be globally amphiphilic in either helical conformation: in both the 11-helical and 14/15-helical conformations, **3** should have cationic and lipophilic side chains distributed around the helical axis. We expected that **1** and/or **2** would display the greatest antibacterial potency, and that **3** would be relatively inactive. Our results, however, indicated that the antibacterial activity of **3** was comparable to that of **1** and **2**.[17]

The unexpected pattern of antibacterial activities among **1**−**3** led us to undertake the research described here. We designed several new α/β-peptides for comparative analysis, with the aim of identifying the molecular features that determine biological activity among these helical foldamers. As was the case with **1**−**3**, each new α/β-peptide contains five cationic residues. Our first new design effort focused on the scrambled isomer, **3**, because the antibacterial efficacy of this α/β-peptide was so surprising.[17] All five cationic residues in **3** occur within the first nine residues (numbering from the N-terminus). Thus, in an extended conformation of **3** the cationic side chains would be clustered at one end of the molecule, and the other end of the α/β-peptide would be lipophilic. We wondered whether the antibacterial activity of **3** could result from this unintended "longitudinal" amphiphilicity. This question was addressed by the design of a new scrambled sequence isomer, **4**, that, like **3**, should not be globally amphiphilic in either the 11-helical or the 14/15-helical conformation (Figure 2). Sequence isomers **3** and **4** differ in that the cationic side chains are more evenly distributed within the primary structure of **4** than within the primary structure of **3**.

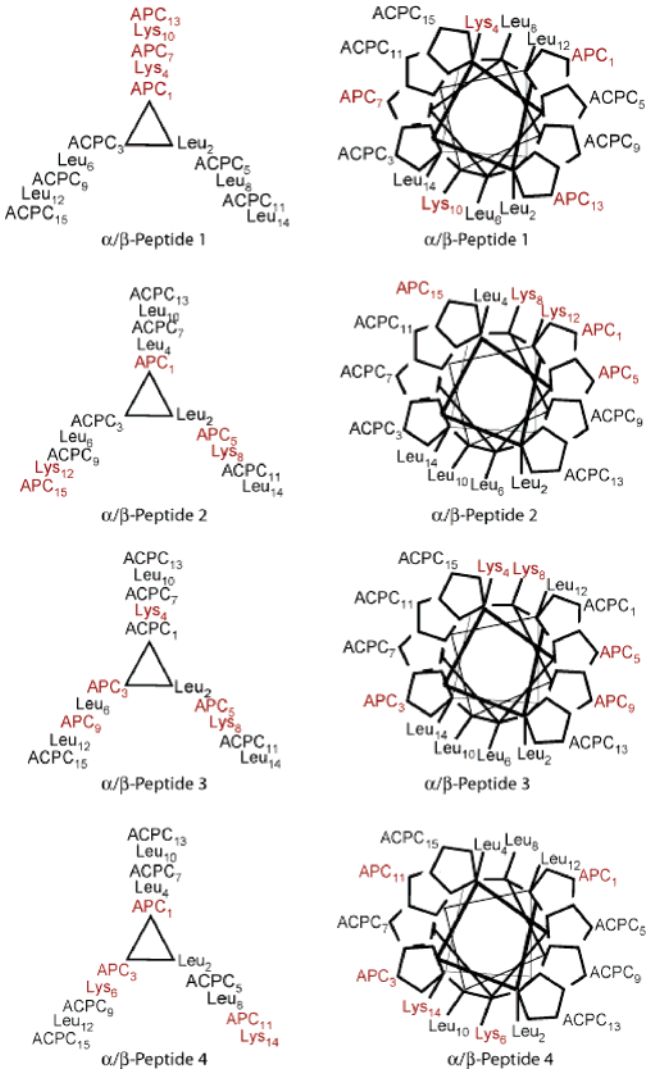


Figure 2. Helical wheel diagrams for α/β-peptides **1**−**4**. Each α/β-peptide is shown in both the 11-helical (left) and the 14/15-helical (right) conformations. Residues depicted in red bear a positive charge in aqueous solution.

A second concern regarding the original α/β-peptide sequences centered on **2**, which was designed to be globally amphiphilic in the 14/15-helical conformation but displayed weaker antibacterial activity than did **1** or **3**.[17] We wondered

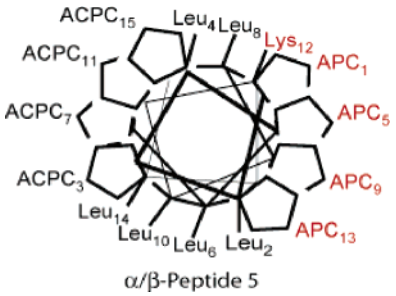


Figure 3. Helical wheel diagram for α/*β*-peptide **5** in the 14/15-helical conformation. Residues depicted in red bear a positive charge in aqueous solution.

whether the sequence of **2** failed to allow sufficient clustering of cationic side chains along one side of the 14/15-helical conformation. This question was addressed by the design of **5**, the 14/15-helical conformation of which should manifest a greater segregation of cationic and lipophilic side chains, relative to **2** (Figure 3). α/*β*-Peptide **5** is not quite a sequence isomer of **2** because **5** contains one more APC residue and one fewer Lys residue than does **2**. In addition, we prepared and evaluated **6**, an analogue of **5** in which three of the six Leu residues are replaced by Phe. α/*β*-Peptide **6** is expected to be globally amphiphilic in the 14/15-helical conformation, as is **5**, and we anticipated that the aromatic side chains in **6** would promote proton signal dispersion in the NMR spectrum and therefore facilitate structural analysis.

Finally, we wondered whether the unexpected trends among the original sequences, **1**−**3**,[17] arise because these molecules are too lipophilic. High lipophilicity can lead to high hemolytic activity among antibacterial α-peptides.[7] We addressed this concern by preparing and analyzing sequence isomeric α/*β*-peptide set **7**−**9**. The overall lipophilicity should be lower among **7**−**9** relative to **1**−**3**, because three of the five leucine residues in **1**−**3** have been replaced by alanine in **7**−**9**. α/*β*-Peptide **7** is a triple Leu→Ala mutant of **1**. Thus, **7** should be globally amphiphilic in the 11-helical conformation but not in the 14/15-helical conformation; the nonpolar face displayed by 11-helical **7** is less lipophilic than is the nonpolar face displayed by 11-helical **1**. Similarly, **8** is a triple Leu→Ala mutant of **2** (14/15-helical designs), and **9** is a triple Leu→Ala mutant of **3** (scrambled designs).

Lipophilicity Analysis via RP-HPLC. We compared net lipophilicities among α/*β*-peptides **1**−**9** via reversed-phase HPLC (RP-HPLC), an approach that is commonly employed for such comparisons among antibacterial α-peptides[27] and that we have extended to antibacterial *β*-peptides.[15] Since the stationary phase (C_8-modified silica in our case) is nonpolar and the mobile phase (water-acetonitrile) is polar, longer retention should be correlated with higher net lipophilicity. Variations in global amphiphilicity, i.e., in the segregation of hydrophilic and lipophilic residues that arises from helical folding, should be revealed by comparisons among sequence isomer sets such as **1**−**4** and **7**−**9**. Our initial comparisons among sequence isomeric α/*β*-peptides **1**−**3** revealed large variations in RP-HPLC mobility: **2**, the α/*β*-peptide designed to be globally amphiphilic in a 14/15-helical conformation, is most strongly retained, and **3**, the scrambled design, is least strongly retained.[17] This trend is consistent with NMR data indicating that α/*β*-peptides of this length prefer the 14/15-helix over the 11-helix.[17] The fact that **1** is significantly more strongly retained than is **3**, however, suggests that the α/*β*-peptide backbone can be induced to adopt an 11-helical conformation by interaction with a nonpolar surface. Other data reported below are consistent with the hypothesis that the 11-helical conformation is readily accessible to α/*β*-peptides of the type discussed here.

Figure 4 shows an overlay of RP-HPLC traces for α/*β*-peptides **1**−**9**. Several conclusions can be drawn regarding the new designs. First, the new scrambled sequence **4** is similar in net lipophilicity to isomer **3**. The comparable RP-HPLC mobilities of **3** and **4** suggest that both are valid scrambled isomers relative to **1** and **2**, and that the clustering of cationic residues in the N-terminal segment of **3** does not lead to unintended global amphiphilicity. Second, the two new 14/15-helical designs, **5** and **6**, and the original design, **2**, are all similarly retained. This similarity suggests that our goal of generating a sequence that is globally amphiphilic in the 14/15-

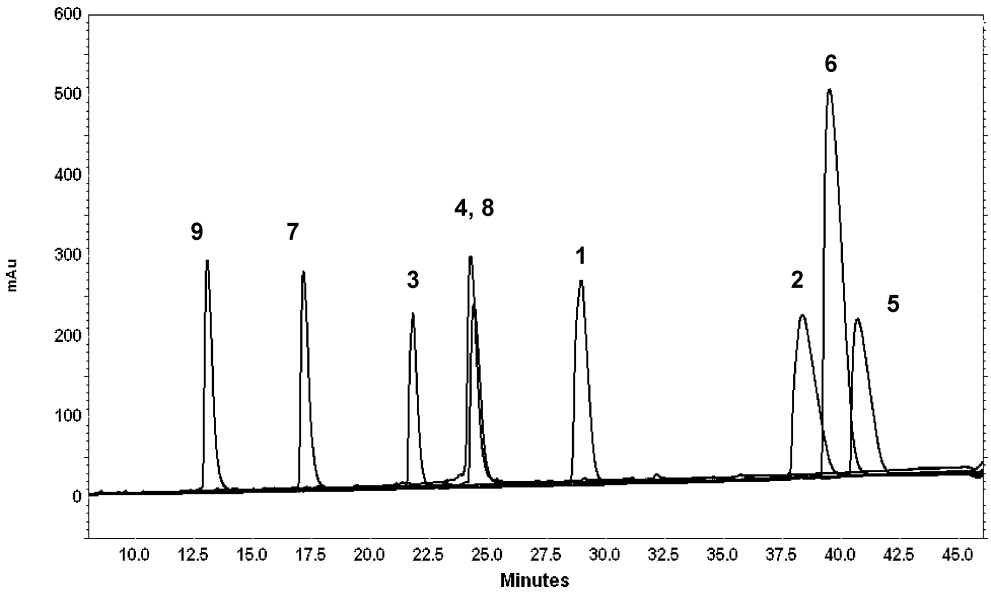
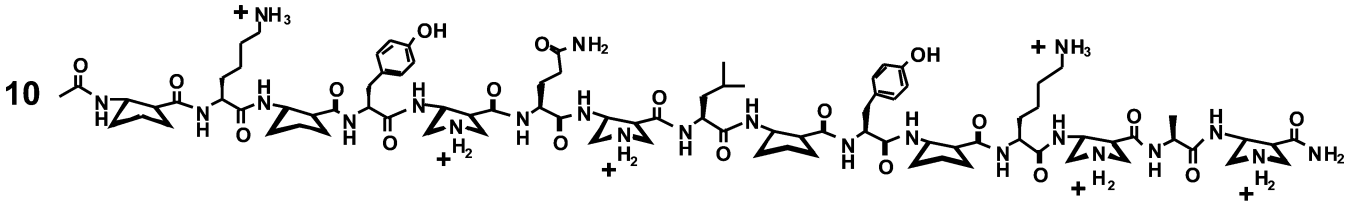


Figure 4. Overlay of RP-HPLC traces for hydrophobicity analysis of α/*β*-peptides **1**−**9**.

Chart 2



helical conformation has been achieved in each case. Third, each member of the triple Leu→Ala set, **7**−**9**, is substantially less lipophilic than its analogue among original set **1**−**3**, as expected upon replacement of three isobutyl side chains with three methyl side chains. Moreover, the retention order among **7**−**9** is analogous to the retention order among **1**−**3**: the sequence isomer designed to be globally amphiphilic in the 14/15-helical conformation is most strongly retained within each series, and the scrambled sequence isomer is least strongly retained.

Structural Analysis: Nuclear Magnetic Resonance (NMR) and Circular Dichroism (CD). Phe-containing α/β-peptide **6** provided sufficient proton resonance dispersion when dissolved in methanol to allow detection of inter-residue NOEs. In contrast, 2D NMR analysis was not possible with the other α/β-peptides discussed here (**1**−**5** or **7**−**9**) because proton resonances were heavily overlapped. High sequence redundancy within each of these α/β-peptides significantly increases the likelihood of resonance overlap.

NOEs between protons from residues that are not adjacent in sequence constitute strong evidence for α/β-peptide folding in solution. Numerous *i*,*i*+2 and *i*,*i*+3 NOEs involving backbone proton pairs were observed for **6** in CD_3OH solution. Many of these NOE patterns are consistent with either an 11-helical conformation or a 14/15-helical conformation.[23] Molecular modeling, however, suggests that certain NOE patterns should be observed for only one of these two helical secondary structures (Figure 5).[23] For example, α-residue $C_\alpha H(i)$→β-residue $C_\alpha H(i+3)$ NOEs should be characteristic for the 14/15-helix; this NOE pattern is not expected for the 11-helical conformation. Five of the seven possible NOEs of this type are observed for **6** in CD_3OH. α-Residue $C_\alpha H(i)$→α-residue NH-(i+2) NOEs, on the other hand, should be characteristic for the 11-helix; no NOEs of this type are observed for **6**. The deduced preference for the 14/15-helix in **6** is supported by observation of a few *i*,*i*+4 NOEs that are predicted for only the 14/15-helix (data in the Supporting Information). These observations suggest that the 14/15-helix is favored over the 11-helix for 15-mer **6**, as previously concluded for a different 15-residue α/β-peptide (**10**, Chart 2).[17] In that case, NOE evidence for 14/15-helical folding was obtained in aqueous solution as well as in methanol, but the NMR spectra of **6** in aqueous solution could not be assigned because of resonance overlap. In contrast to the evidence for a single type of helix with 15-residue α/β-peptides, NOE analysis of 6- to 8-residue α/β-peptides has indicated that the 11- and 14/15-helical conformations are both significantly populated.[23]

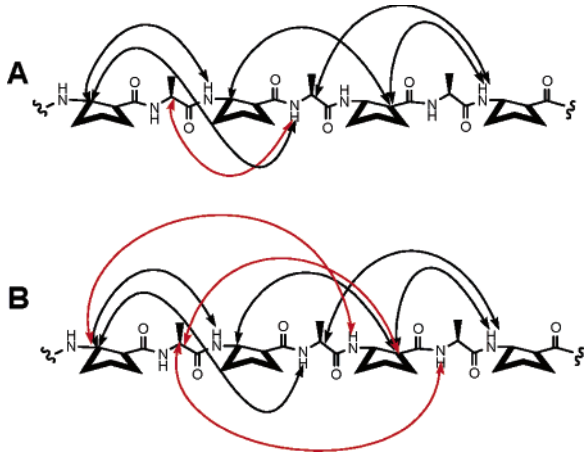


Figure 5. Characteristic NOEs for the 11-helical (A) and 14/15-helical (B) conformations. Red arrows indicate types of NOEs that can be used to distinguish between the two conformations.

CD was used to compare folding propensities among α/β-peptides **1**−**9**. CD in the far-UV region (190−250 nm) is commonly employed to examine secondary structures of α-amino acid peptides and proteins.[28] The signal in this region arises largely from the backbone amide chromophores. Information obtained in this way is of inherently low resolution, but structural conclusions can be drawn in the case of α-peptides because CD signals have been extensively correlated with high-resolution structural data (e.g., from crystallography and NMR). Distinctive far-UV region CD signatures have been observed for β-peptides,[29] α/β-peptides,[25] and other peptidic oligomers,[30] but in these cases structural interpretations can be problematic because relatively little high-resolution structural information is available for correlation.

Figure 6A shows far-UV CD data for α/β-peptides **4**, **6**, and **8** in methanol. All three molecules show a minimum near 204 nm; the intensity of the minimum varies somewhat among the three. The other six α/β-peptides discussed here (**1**−**3**, **5**, **7**, and **9**) have very similar CD signatures in methanol (data in Supporting Information), and the intensities of the 204 nm minima vary between that of **4** and that of **8** in Figure 6A. The 204 nm minimum for **6** displays intermediate intensity within this set. Since NOE data suggest that **6** adopts only one folded conformation under these conditions, the 14/15-helix (presumably in equilibrium with the unfolded state), we tentatively assign the 204 nm minimum to this secondary structure. It may be that the variations in CD intensity at 204 nm among **1**−**9** reflect differences in the 14/15-helical population; however, further work (including identification of the CD signature for the unfolded state) will be required to address this possibility. It seems likely that that 14/15-helical and 11-helical secondary

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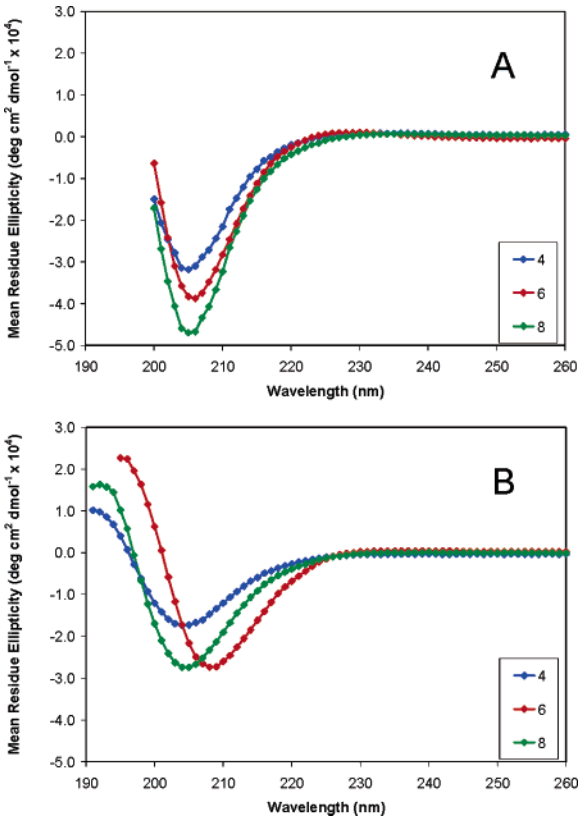

Figure 6. CD spectra of α/*β*-peptides **4**, **6**, and **8** in methanol (A) and 10 mM aqueous Tris buffer (pH 7.2) (B).

structures will have similar CD signatures, as is true of α- and 3_{10}-helical secondary structures among α-peptides.[31]

Figure 6B shows far-UV CD data for **4**, **6**, and **8** in aqueous buffer (10 mM Tris, pH 7.2). The minima for all three α/*β*-peptides are considerably less intense in water than in methanol, which suggests that the extent of folding is significantly smaller in water than in methanol. This conclusion is consistent with extensive precedent showing that α-peptides and *β*-peptides are generally less prone to folding in water than in methanol.[28,32−34] The minimum for **4** and **8** has shifted slightly to shorter wavelength in aqueous buffer relative to methanol. In contrast, the CD signature of **6** in aqueous buffer differs from those of **4** and **8**, and most of the other α/*β*-peptides discussed here, in that the minimum occurs around 209 nm. (α/*β*-Peptide **2** in aqueous buffer shows a shift to longer wavelength relative to **2** in methanol, but the shift is smaller than for **6**.) The significance of this shift, relative to **6** in methanol and relative to the other α/*β*-peptides, is unclear. Poor proton resonance dispersion observed for **6** in aqueous solution precluded NOE analysis under these conditions.

We examined the effect of adding a detergent, either sodium dodecyl sulfate (SDS; anionic headgroup) or dodecyl phosphatidyl choline (DPC; zwitterionic headgroup), on α/*β*-peptide CD spectra because detergent micelles are regarded as models for cell membranes. Micelles have been observed to promote helical folding of both α- and *β*-peptides that can form globally amphiphilic helical conformations.[7,10,15,35,36] For most of the α/*β*-peptides, detergent caused the CD signature to shift from that seen in aqueous buffer toward the signature in methanol (i.e., minimum moves toward 204 nm and absolute intensity increases; data in Supporting Information). The extent of detergent-induced shifts, however, varied considerably. Several of the α/*β*-peptides designed to be globally amphiphilic in the 14/15-helical conformation, for example, were particularly susceptible to micelle-induced promotion of folding, according to the CD data. For both **2** and **6**, the CD signatures in the presence of either SDS or DPC matched the methanol signature, while for **5** the SDS signature matched the methanol signature, but the DPC signature was not quite as intense (albeit more intense than the far-UV CD spectrum in aqueous buffer alone). For the 14/15-helical design with decreased lipophilicity, **8**, both types of micelles caused an increase in far-UV intensity relative to aqueous buffer, but the minimum near 204 nm was significantly less intense in the presence of micelles than in methanol. The diminished effects of micelles on **8** relative to **2**, **5**, and **6** are consistent with relatively lower affinity of **8** for micelles arising from the display of a less lipophilic surface in the 14/15-helical conformation. Scrambled α/*β*-peptides **3** and **4** were less strongly affected by detergent than was sequence isomer **2**. For **3**, addition of SDS caused no change in CD relative to aqueous buffer, and addition of DPC caused only a modest change. For **4**, both SDS and DPC caused modest changes. Overall, these results suggest that interaction of our cationic α/*β*-peptides with detergent micelles can promote helical folding, although the extent of this effect varies with sequence and composition. Further studies will be required to correlate trends among the CD data with high-resolution structural data.

Table 2. Antimicrobial Activities (MIC, μg/mL) of α/*β*-Peptides **1**−**9**

	E. coli	*B. subtilis*	*S. aureus*	*E. faecium*	hemolytic at MIC[a]?
1	12.5	3.1	3.1	3.1−6.3	yes (3.1)
2	≥200	6.3	50	25	yes (1.6)
3	6.3	6.3	12.5	6.3−12.5	no (100)
4	12.5	≤1.6	6.3	6.3	no (100)
5	25	3.1	12.5	12.5	yes (1.6)
6	25	3.1	6.3	12.5	yes (1.6)
7	6.3	12.5	12.5	25	no (≥400)
8	12.5	3.1	12.5	3.1−6.3	no (100)
9	50	50	≥200	≥200	no (≥400)
M[b]	12.5	3.1	50	50	no (50)[c]

[a] Numbers in parenthesis indicate the minimum concentration at which hemolysis is greater than 10%. [b] MICs for Ala^{3,8,18}-magainin II amide, the α-peptide positive control. [c] Ala^{3,8,18}-magainin II amide is active and highly selective against nonresistant strains of bacteria. This analogue shows only moderate activity and no selectivity for antibiotic-resistant strains.

Antibacterial Activity. Table 2 summarizes the effects of the six new α/*β*-peptides, **4**−**9**, on the growth of four bacterial species. These effects are presented in terms of the minimum inhibitory concentration (MIC), i.e., the lowest concentration at which bacterial growth in liquid culture is blocked. Also shown, for comparison, are the antibacterial activities previously reported for α/*β*-peptides **1**−**3**[17] and for Ala^{8,13,18}-magainin II amide, a synthetic magainin derivative that is more active against

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a variety of bacteria than are the natural magainin peptides.[37] One of the bacteria is Gram negative (*E. coli*); the rest are Gram positive. The strains of *S. aureus* and *E. faecium* we used are clinical isolates that are resistant to conventional antibiotics.[38,39]

α/β-Peptide **4**, the new scrambled sequence isomer of **1** and **2**, is quite comparable to the original scrambled design, **3**, in its activity toward all four bacteria. This similarity strengthens the conclusion we deduced from the similarity in RP-HPLC retention displayed by **3** and **4**: both **3** and **4** are valid scrambled sequences relative to **1** and **2**.

α/β-Peptide **5**, an analogue of **2** that is intended to achieve greater global amphiphilicity in the 14/15-helical conformation, displays somewhat enhanced antibacterial activity relative to **2**. This improvement is most significant for *E. coli*, toward which **2** is inactive.[17] The MIC for **5** against *E. coli*, in contrast, approaches that of the magainin derivative. The antibacterial activity of **6**, a triple Leu→Phe mutant of **5**, is very similar to the activity of **5** itself for all four species.

Comparison of the triple Leu→Ala mutants **7**−**9** with the original designs **1**−**3** reveals that the decrease in net lipophilicity exerts substantial effects on antibacterial activity. Within each pair of analogues (**1**/**7**, **2**/**8**, and **3**/**9**), the Leu→Ala mutations cause a different pattern of changes. For the pair designed to be globally amphiphilic in the 11-helical conformation, **1** and **7**, the Leu→Ala mutations lead to a modest weakening of activity against the three Gram positive bacteria, and perhaps a slight improvement against *E. coli*. For the 14/15-helical designs, **2** and **8**, the Leu→Ala mutations lead to improvements in activity toward all four bacteria, with the most dramatic effect displayed against *E. coli* (≥200 μg/mL for **2** vs 12.5 μg/mL for **8**). For the scrambled pair, **3** and **9**, the Leu→Ala mutations lead to a dramatic loss in activity. Within sequence isomer set **1**−**3**, the 11-helical design (**1**) and the scrambled design (**3**) display comparable antibacterial activity against the panel of bacteria, and the 14/15-helical design (**2**) is significantly less effective. In contrast, the 14/15-helical design (**8**) is the most active among sequence isomer set **7**−**9**, followed closely by the 11-helical design (**7**), and the scrambled design (**9**) shows only weak activity.

Hemolytic Activity. Host-defense peptides such as the magainins are selective for disruption of bacterial cells relative to eukaryotic cells. For in vitro studies of the type reported here, it is typical to assess eukaryotic cell susceptibility by measuring a peptide's ability to induce human red blood cell lysis ("hemolysis"). Hemolysis as a function of the logarithm of α/β-peptide concentration is shown in Figure 7 for **1**−**9**. Also shown are data for two α-peptides, the magainin derivative, representative of a host-defense peptide, and melittin, a peptide toxin that strongly disrupts both prokaryotic and eukaryotic cell membranes.[40] Melittin is highly hemolytic at relatively low concentrations (≥10 μg/mL), while the magainin derivative requires at least 10-fold higher concentration to cause significant hemolysis.

The data in Figure 7 show that the new scrambled α/β-peptide, **4**, and the original scrambled design, **3**, display comparable and relatively low hemolytic activity. This parallel provides further support for our conclusion that both of these sequence isomers are valid scrambled control compounds for comparison to **1** and **2** (which are both highly hemolytic). The new 14/15-helical designs, **5** and **6**, are very similar to the original design, **2**, in terms of hemolytic activity. Each of the triple Leu→Ala mutants, **7**−**9**, displays much reduced hemolytic activity relative to the analogue among **1**−**3**; indeed, no hemolysis at all could be detected for **7** or **9**. α/β-Peptide **8**, designed to be globally amphiphilic in the 14/15-helical conformation, is comparable to the magainin derivative in terms of hemolytic activity.

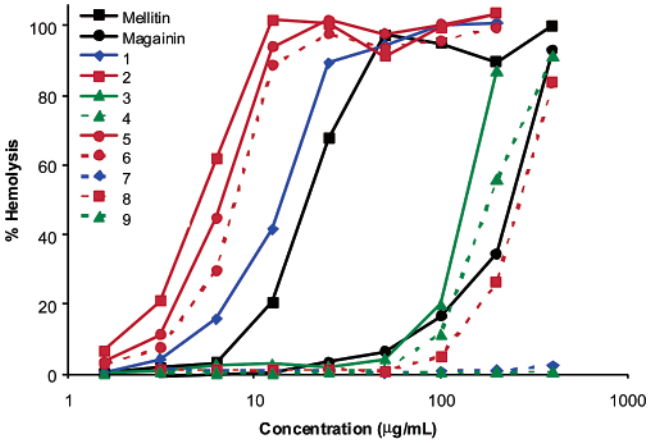

Figure 7. Hemolytic profiles for α/β-peptides **1**−**9** against human RBC, type A. Also shown are curves for the α-peptides melittin and magainin. The value for melittin at 400 μg/mL is taken to represent 100% hemolysis.

The rightmost column in Table 2 indicates whether each α/β-peptide displays significant hemolysis (≥10%) over the range of MICs for each of the four bacterial strains. These data allow one to identify **3**, **4**, **7**, and **8** as the α/β-peptides that display the most desirable profile, strongly antibacterial but weakly hemolytic.

Protease Susceptibility. Conventional peptides, composed of L-α-amino acid residues, are rapidly degraded by proteases, a feature that can limit biomedical utility. Oligo-amides with entirely unnatural backbones, such as β-peptides, are highly resistant to proteolytic cleavage;[14,41−43] this feature could be favorable with regard to long-term prospects for biological applications. We wondered whether the α/β-peptides discussed here can be cleaved by proteases. It has long been known that insertion of an acyclic β-amino acid residue among L-α-residues can protect nearby amide bonds from proteolysis,[44] and α/β-peptides containing acyclic β-residues have recently been shown to resist proteolytic cleavage.[45] Our studies are the first to explore α/β-peptides containing cyclic β-residues.

Three α/β-peptides were examined, **1**, **7**, and **10**. α/β-Peptide **7** is the triple Leu→Ala mutant of **1**; **10** has a very different α-residue content and was previously used for NMR analysis.[17] We examined three different proteases, trypsin, chymotrypsin,

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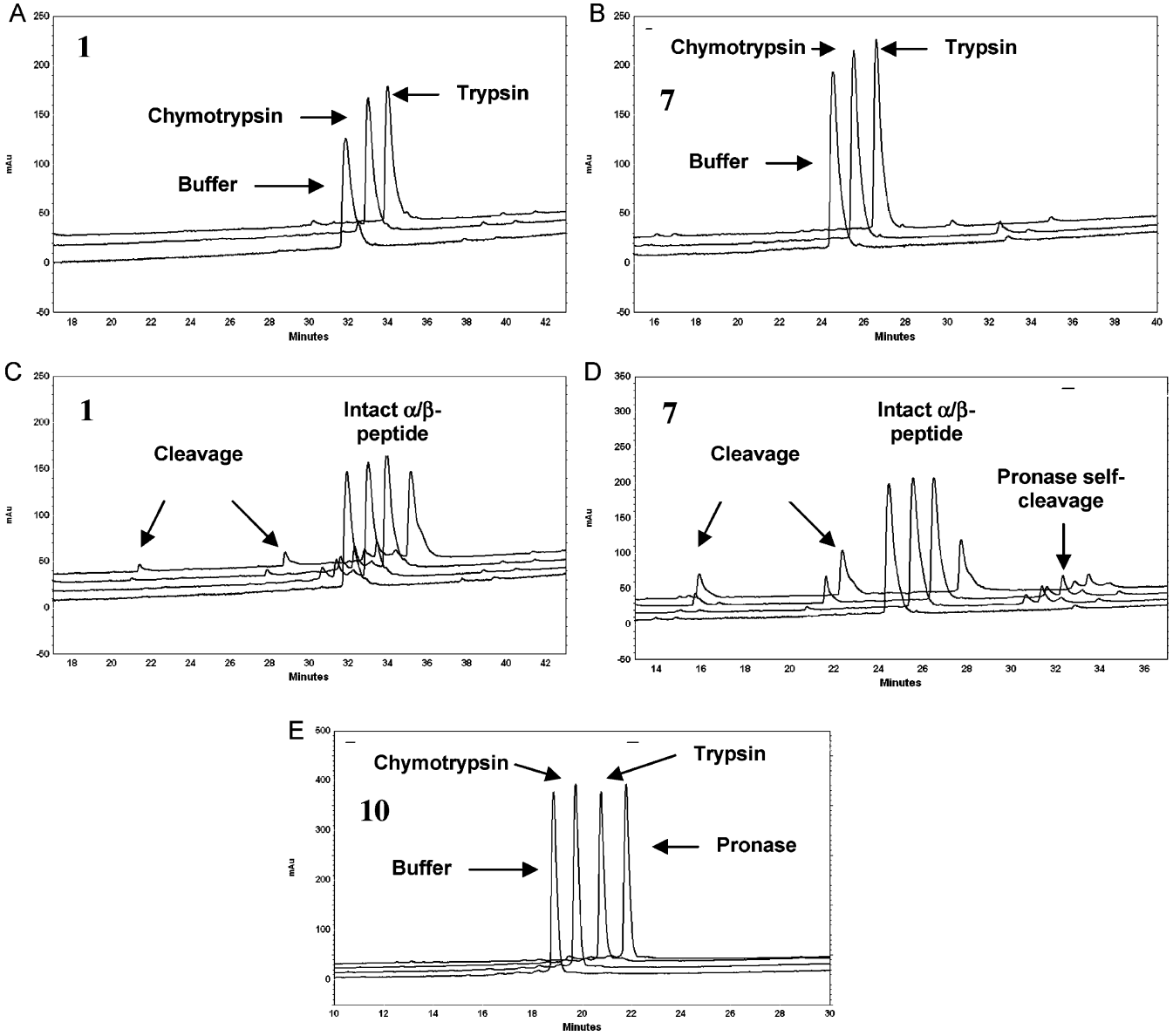


Figure 8. RP-HPLC traces for protease stability studies. Traces are offset by 1 min relative to the trace for each α/*β*-peptide in buffer (front trace): (A) **1** with trypsin and chymotrypsin (36 h), (B) **7** with trypsin and chymotrypsin (36 h), (C) **1** with pronase (front trace: buffer, then after 1 h, 25 h, and 88 h incubation), (D) **7** with pronase (front trace: buffer, then after 3 h, 27 h, and 90 h incubation), and (E) **10** with all three proteases (60 h).

and pronase. Trypsin cleaves peptide bonds adjacent to positively charged side chains (such as that of Lys), while chymotrypsin cuts adjacent to aromatic side chains (such as that of Tyr). Pronase displays a wide range of protease activities.[46]

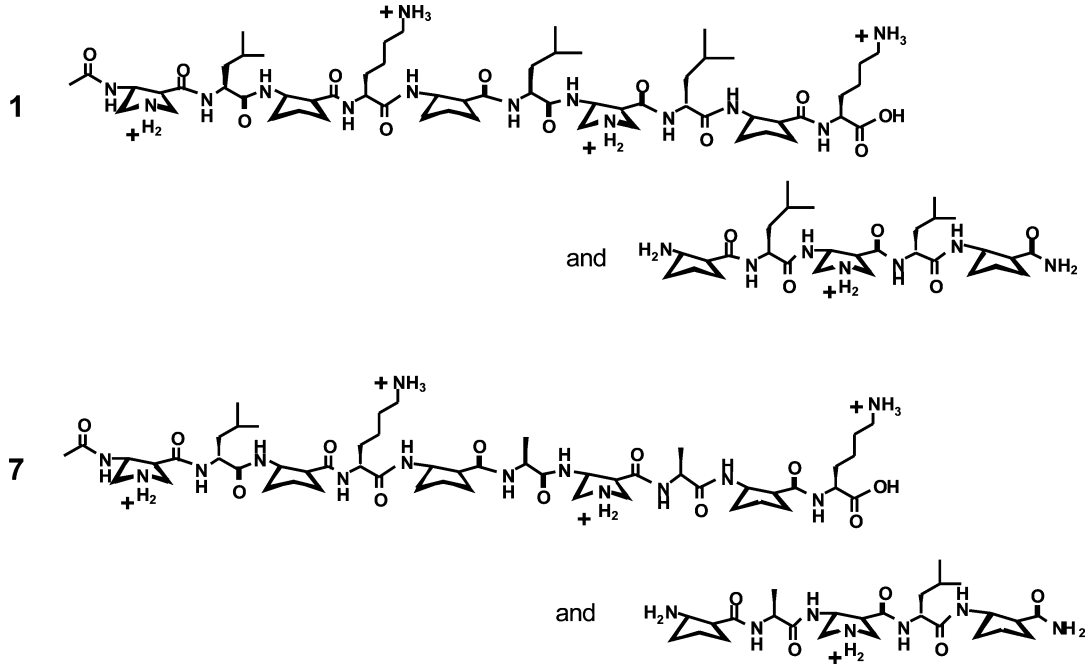
α/*β*-Peptides **1**, **7**, and **10** were all highly resistant to cleavage by trypsin or chymotrypsin (Figure 8). After incubation with these two enzymes (36 h for **1** and **7**; 60 h for **10**), no proteolysis could be detected for any of these α/*β*-peptides (Figure 8A (**1**), Figure 8C (**7**), Figure 8E (**10**)), even though all three contain Lys and **10** contains Tyr. The most aggressive among the proteases we examined, pronase, caused no degradation of **10** after 60 h (Figure 8E); however, both **1** and **7** experienced partial cleavage, at one site, after prolonged treatment with pronase (Chart 3, Figure 8B,C).

Figure 8C shows the effect of treating **1** with pronase, as monitored by HPLC. α/*β*-Peptide **1** elutes at approximately 32 min under these conditions. Additional small peaks that appear between 30 and 33 min in pronase-containing samples arise from enzymatic self-cleavage, as indicated by control studies. After one day, trace amounts of two α/*β*-peptide cleavage products can be detected, but even after nearly four days **1** is largely intact. MALDI-TOF mass spectrometry identifies a single cleavage site within **1**, between ACPC-9 and Lys-10. An analogous ACPC-Lys bond occurs between residues 3 and 4 of **1**, but no cleavage at this site is detected. Figure 8D shows the effect of pronase on **7**. This α/*β*-peptide is cleaved more rapidly than is analogue **1**, although even after nearly four days a substantial amount of intact **7** remains. Cleavage occurs between residues 9 and 10, i.e., at the position analogous to the cleavage site in **1**.

Overall, our data confirm the expectation that oligomers containing a 1:1 alternation of α- and *β*-residues are highly resistant to proteolytic degradation. Both **1** and **7** (but not **10**) contain a single amide bond that is slowly cleaved by the most

(46) *Sigma Biochemicals and Reagents Catalog*, 2002−2003.

Chart 3. Fragments of **1** (top) and **7** (bottom) after Incubation with Pronase



aggressive protease, pronase. It is unclear why this particular ACPC-Lys bond is susceptible, while other ACPC-Lys bonds in **1**, **7**, and **10** are not.

Discussion

The work described here has arisen from our general interest in developing unnatural oligomers that mimic the selective toxicity displayed by helix-forming host-defense peptides toward prokaryotic cells relative to eukaryotic cells. These particular studies were motivated by an apparent contradiction between sequence/folding/activity correlations observed among antibacterial β-peptides and those observed among α/β-peptides.[14,15,17] For β-peptides, sequences designed to be globally amphiphilic (Figure 1) in a helical conformation block bacterial growth at relatively low concentrations, while scrambled sequence isomers, which should form helical conformations that are *not* globally amphiphilic, do not block bacterial growth.[14,15] In contrast, initial studies with α/β-peptide sequence isomers **1**−**3** revealed that the scrambled version, **3**, manifests considerable antibacterial activity.[17] The sequence designed to be globally amphiphilic in the most favorable helical conformation, **2**, shows weaker antibacterial activity than does **1** or **3**. The trend in hemolytic activities among **1**−**3** is quite different from the trend in antibacterial activity: **1** and **2** are strongly hemolytic, but **3** is only weakly hemolytic.

Some of the new α/β-peptides reported here (**4**−**6**) represent tests of the extent to which our design goals were achieved with **1**−**3**. Thus, for example, **4** is a sequence isomer of **3** and constitutes an alternative scrambled design that is intended to avoid unintended global amphiphilicity that might be displayed by **3** in an extended conformation. Since the behavior of **4** is quite similar to that of **3** by every measure reported here (RP-HPLC retention, antibacterial activity, hemolytic activity, CD signature), we conclude that both are valid scrambled-sequence controls for isomers **1** and **2**. Thus, the unexpected antibacterial activity observed for **3** does not arise from a design flaw.

α/β-Peptides **5** and **6** are new designs intended to achieve global amphiphilicity in the 14/15-helical conformation. Although these α/β-peptides are not sequence isomers of the original 14/15-helical design, **2**, their comparison with **2** is reasonable because the compositions are similar and net charge is the same. These three α/β-peptides display very similar RP-HPLC retention and very similar hemolytic activities; however, they vary somewhat in antibacterial effects. The most dramatic difference is seen for *E. coli*, against which **2** is inactive but both **5** and **6** are quite active, and significant differences are seen as well for *S. aureus*. Overall, these comparisons suggest that **2** is a good 14/15-helical design, although perhaps not ideal. The modest improvements in antibacterial activity displayed by **5** and **6** relative to **2** cannot explain the quandary associated with scrambled designs **3** and **4**, however, because neither **5** nor **6** is markedly superior to **3** or **4** as an inhibitor of bacterial growth.

The largest changes in physical and biological properties, relative to the original α/β-peptide designs, were observed in the triple Leu→Ala series, **7**−**9**. In contrast to the trends observed in original set **1**−**3**, the sequences designed to be globally amphiphilic in helical conformations among the Leu→Ala mutants, **7** and **8**, are quite active against all four bacteria, and the scrambled isomer, **9**, shows only weak antibacterial effects. The high antibacterial activity of **7** supports our hypothesis that the 11-helical conformation can be accessed without a large energetic penalty, even if α/β-peptides of this length intrinsically prefer to form the 14/15-helix relative to the 11-helix. The pattern of behavior among **7**−**9**, globally amphiphilic design = active, and scrambled design = inactive, matches previous observations among β-peptides in two different conformational series (designed to adopt either 12- or 14-helical secondary structure[14,15]). All three of the Ala-containing α/β-peptides **7**−**9** display very weak or undetectable hemolytic activity. Therefore, **7** and **8** match the profile of activity that is characteristic of host-defense peptides, which manifest toxicity

toward bacteria at much lower concentrations than are required for lysis of red blood cells. However, the activity profile of 14/15-helical design **8** is no better than that displayed by scrambled designs **3** and **4**.

Numerous efforts have been made to correlate RP-HPLC retention trends with the biological activities of amphiphilic α-peptides.[27] This type of analysis has been motivated by the widely accepted hypothesis that such peptides act by presenting large and discrete lipophilic surfaces that interact with the nonpolar portions of lipids and thereby disrupt biological membrane barrier function. RP-HPLC retention is generally greater for molecules that display larger lipophilic surfaces, which promote interaction with the alkyl chains of the stationary phase. Among α/β-peptides **1**−**9** we find a reasonable correlation between increasing retention and increasing hemolytic activity. Thus, **2**, **5**, and **6** are most strongly retained, and these compounds are extremely hemolytic, significantly more so than melittin. α/β-Peptide **1** is next most strongly retained, and **1** is comparable to melittin in hemolytic activity. α/β-Peptides **3**, **4**, and **8** cluster at intermediate retention, and they are comparable to the magainin derivative in their relatively low hemolytic activity. The least strongly retained α/β-peptides, **7** and **9**, display no hemolytic activity under the conditions we examined. In contrast to hemolytic activity, inhibition of bacterial growth does not show obvious correlations to RP-HPLC retention trends. For example, of the two α/β-peptides with the weakest antibacterial activity (averaged over the four species), **9** is least strongly retained and **2** is among the most strongly retained. α/β-Peptide **7** is second least strongly retained, but this compound is quite active, particularly against *E. coli*. α/β-Peptides **5** and **6** display very similar retention to that of **2**, but they manifest stronger antibacterial activity, especially against *E. coli* and *S. aureus*. An interesting correlation emerges from considering both antibacterial and hemolytic activities simultaneously. The retention window between approximately 17.5 and 25 min (corresponding to 32.5−40% acetonitrile in water) contains the optimal α/β-peptide designs. More strongly retained α/β-peptides are too hemolytic, and the less strongly retained compound is too weak an inhibitor of bacterial growth.

The trends in physicochemical and biological behavior among α/β-peptides **1**−**9** suggest that the most desirable activity profile, growth inhibition toward both Gram positive and Gram negative bacteria but low propensity to induce hemolysis, can be achieved within this foldamer family in two different ways. Both strategies require a combination of lipophilic and cationic side chains. One successful path is to design α/β sequences that display global amphiphilicity (Figure 1) in a favorable conformation; this design strategy has been widely employed for α-peptides and for foldamers. The second path, which initially seems paradoxical, is to design α/β sequences that *cannot* be globally amphiphilic in the most favorable conformation. Whether the first path or the second path will be successful depends on the absolute lipophilicity of the residues that comprise the α/β-peptide, according to our results. We use the phrase "absolute lipophilicity" to describe the sum of the lipophilicities of each residue in an α/β-peptide, regardless of conformation. In contrast, we use the phrase "net lipophilicity" to encompass not only the lipophilicities of the constituent residues of an α/β-peptide, but also the contribution of primary sequence and conformation to the behavior of the molecule. For example, **1**−**3** are sequence isomers and therefore possess the same absolute lipophilicity, but these three α/β-peptides have very different net lipophilicities as revealed by the large variations in RP-HPLC retention. If the nonpolar residues bear sufficiently lipophilic side chains, as is true for **1**−**6**, then scrambled sequences are preferable to globally amphiphilic designs in terms of achieving selective antibacterial activity. In this high absolute lipophilicity regime, both globally amphiphilic and scrambled designs can generate high antibacterial activity, but the globally amphiphilic designs unavoidably display high hemolytic activity. On the other hand, if the nonpolar side chain set has a sufficiently low absolute lipophilicity, as in **7**−**9**, then a globally amphiphilic design is preferable to a scrambled sequence. In this low absolute lipophilicity regime none of the designs displays high hemolytic activity, but only the sequences intended to be globally amphiphilic in the folded state can significantly inhibit bacterial growth. The fact that **7** and **8** are roughly comparable in their antibacterial activities indicates that the 11-helical conformation is sufficiently stable to serve as a basis for globally amphiphilic designs, even though the 14/15-helical conformation appears to be intrinsically preferred relative to the 11-helical conformation for α/β-peptides of this length and composition.

The hypothesis outlined in the preceding paragraph is interesting in relation to previous reports on α- and β-peptides designed to manifest antibacterial activity. Pioneering work from two groups showed that natural toxin peptides such as melittin can be modified in ways that significantly diminish hemolytic activity but retain antibacterial activity. Blondelle and Houghten achieved this result by examining a comprehensive set of single-residue omission analogues of melittin.[47] Omission of most lipophilic residues led to a substantial loss in hemolytic activity, and a corresponding decrease in RP-HPLC retention, but usually only modest effects on antibacterial activity. These workers speculated that the ability of an α-peptide to form a globally amphiphilic α-helix is more important for hemolysis than for inhibition of bacterial growth. Oren and Shai subsequently reported a hypothesis-driven approach to "detoxified" melittin analogues: these workers prepared a diastereomeric peptide in which 4 of the 26 residues, scattered along the sequence, had the D-configuration rather than the natural L-configuration.[48] This melittin diastereomer was intended to have little or no α-helix-forming propensity, and the success of this design feature was demonstrated by CD comparisons in a helix-promoting solvent. The heterochiral melittin diastereomer displayed a dramatic decrease in hemolytic activity relative to the natural homochiral version, but the heterochiral isomer retained considerable antibacterial activity. Shai and co-workers have subsequently shown that designed heterochiral α-peptides display a variety of attractive features, including antibacterial, antiviral, and anticancer activities as well as low toxicity toward healthy eukaryotic cells.[49−51] This work has inspired other heterochiral peptide design efforts.[52] On the basis of parallels between our findings with α/β-peptides and those of Shai et al. with

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α-peptides, we suspect that the creation of heterochiral diastereomers or scrambled sequence isomers can be viewed as complementary design strategies for diminishing hemolysis while retaining bacterial growth inhibition, if one starts from an oligomer for which both activities are strong and that adopts a globally amphiphilic conformation.

How can scrambled α/β sequences and heterochiral α sequences display significant antimicrobial activity? One possibility is that such oligomers could, under appropriate circumstances, adopt irregular, nonhelical conformations that result in a global segregation of lipophilic and cationic side chains. Adoption of irregular but globally amphiphilic conformations might be induced by interaction with a membrane, as adoption of globally amphiphilic α-helical conformations is often induced by this type of interaction. This possibility would require a high level of backbone flexibility, a feature that is intrinsic to α-amino acid residues. This hypothesis predicts that more rigid backbones would make it difficult for oligomers that had been designed not to form globally amphiphilic secondary structures to undergo conformational excursions necessary for clustering of lipophilic and cationic side chains. Previous findings with antibacterial helix-forming β-peptides are consistent with this prediction: when these β-peptides are constructed largely or entirely from conformationally restricted cyclic residues, scrambled sequences are completely inactive.[14,15] In contrast, for α-peptides that form globally amphiphilic α-helices, scrambled sequence isomers retain substantial antibacterial activity.[27] Our hypothesis regarding nonhelical but globally amphiphilic conformations has recently received strong support from work of Wang et al., who carried out two-dimensional analysis of a heterochiral α-peptide in the presence of micellar SDS.[53] The results reveal a fascinating and irregular peptide conformation that segregates lipophilic side chains on one side and cationic side chains on the other. It is interesting that earlier structural analysis of a heterochiral melittin diastereomer under micellar conditions revealed α-helical folding comparable to that seen for melittin itself (homochiral).[54]

Conclusions

The behavior of the α/β-peptides described here indicates that it is not necessary to design toward a regular, globally amphiphilic secondary structure in order to generate oligomers with favorable antibacterial/hemolytic activity profiles. To our knowledge, all previous efforts to create unnatural oligomers intended to display antibacterial activity (including our own) have focused on specific and generally regular globally amphiphilic conformations.[10−17,19,20]

Our findings raise the prospect of developing cell type-selective antibacterial materials that should be relatively inexpensive to produce on large scale (in contrast to discrete oligomers such as α-, β-, or α/β-peptides, which require labor-intensive stepwise synthesis). Specifically, as we have previously proposed,[55] it seems likely that random copolymers containing both lipophilic and cationic appendages could display the desired properties if two conditions are met. First, the backbone would have to be sufficiently flexible to allow the adoption of globally amphiphilic conformations regardless of the specific sequence or configurational pattern of the subunits along the backbone. Second, the backbone would have to be sufficiently "neutral" in terms of hydrophilic/lipophilic balance that the biological activity would be determined largely by side chains. Efforts to test this hypothesis are underway in several laboratories.[55−57]

Experimental Section

α/β-Peptide Synthesis and Purification. β-Amino acids (Fmoc-ACPC and Fmoc-APC(Boc)) were synthesized from β-keto esters via reductive amination as described.[58,59] Fmoc-α-amino acids were purchased from EMD Biosciences. α/β-Peptides were synthesized manually in parallel using HBTU activation on NovaSyn TGR resin (EMD Biosciences) in Alltech solid-phase extraction tubes. Coupling reactions were allowed to proceed at room temperature for 2 h; deprotection reactions were allowed to proceed at room temperature for 30 min. After synthesis was complete, N-termini were acetylated with acetic anhydride/ DIEA/CH_2Cl_2 for 2 h with rocking. α/β-Peptides were cleaved from resin with 95% TFA/5% water (v/v) for 2−4 h with rocking. The α/β-peptide solution was filtered away from the resin, and the TFA was removed under a stream of N_2(g). α/β-Peptides were precipitated by the addition of ice cold anhydrous diethyl ether. The precipitated α/β-peptide was pelleted by centrifugation, and the ether was decanted. The precipitation/centrifugation process was repeated a total of three times. The crude α/β-peptide pellet was dried under N_2(g) and dissolved in 5 mL of 50% H_2O/50% acetonitrile (v/v) and lyophilized. Crude α/β-peptides were purified by RP-HPLC on a C_4-silica column using a linear gradient of acetonitrile in water. Solvents were prepared as follows: solvent A, H_2O with 0.1% TFA; solvent B, 80% acetonitrile and 20% water (v/v) with 0.1% TFA. Masses of purified peptides were confirmed by MALDI-TOF mass spectrometry. α/β-Peptide concentrations for all experiments were determined from the weight of the lyophilized α/β-peptide powder calculated as the TFA salt (assuming association of 1 TFA molecule per cationic residue).

Nuclear Magnetic Resonance (NMR). Samples were prepared by dissolving lyophilized peptides in methanol-d_3 or 9:1 H_2O:D_2O, 100 mM acetic acid-d_4, pH 3.8. Peptide concentrations were usually 2−3 mM with trace amounts of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) added as an internal reference. Fully dissolved peptides were syringe-filtered into a 3 mm NMR tube and sonicated to ensure homogeneity. Total sample volume was approximately 250 μL.

NMR experiments were acquired on a Varian Inova-600 spectrometer at 4, 14, or 24 °C as required for best spectral resolution. COSY,[60] TOCSY,[61] and rotating frame Overhauser spectroscopy (ROESY)[62] experiments were performed for chemical shift and structure assignment. Mixing times for TOCSY experiments were 80 and 200 ms for ROESY experiments. Standard Varian pulse sequences were used, and data were processed using Varian VNMR 5.3 software and Sparky (a PC-based NMR spectra viewing program; T. D. Goddard and D. G. Kneller, SPARKY 3 University of California, San Francisco). Chemical shift assignments were made on the basis of COSY and TOCSY cross-peaks as well as sequential α-amide NOEs in the ROESY spectrum.

Circular Dichroism (CD). Measurements were performed on an AVIV model 202SF spectrometer with 5 s averaging times and a 1 nm step size. Spectra were acquired in methanol, aqueous 10 mM Tris,

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pH 7.2, 5 mM DPC, and 25 mM SDS at 25 °C. Peptide concentrations were 0.1 mM. Samples were analyzed in a quartz cuvette with a 1 mm path length. Data were corrected for baseline absorbance and normalized for path length, number of amide chromophores, and concentration.

RP-HPLC Analysis of α/β-Peptides. Net hydrophobicity of α/β-peptides was analyzed by RP-HPLC on a C_8-silica analytical column using a linear gradient of acetonitrile in water (A, H_2O with 0.1% TFA; B, acetonitrile with 0.1% TFA) from 20% to 60% over 40 min. α/β-Peptide samples were made at a concentration of 2 mg/mL in water. The gradient begins after elution of the solvent front, which occurs about 5 min after injection. Reported retention profiles represent the average of at least two separate experiments and were highly reproducible.

Antibacterial Activity of α/β-Peptides. The bacteria strains used in these assays were *Escherichia coli* JM109,[63] *Bacillus subtilis* BR151,[64] *Staphylococcus aureus* 1206 (methicillin-resistant),[39] and *Enterococcus faecium* A634 (vancomycin-resistant).[38] The antibacterial activity for the α/β-peptides was determined in sterile 96-well plates (Falcon 3075 microtiter plate) by a microdilution method. A bacterial suspension of approximately 10^6 CFU/mL in BHI medium was added in 50 μL aliquots to 50 μL of medium containing the α/β-peptide in 2-fold serial dilutions for a total volume of 100 μL in each well. The plates were incubated at 37 °C for 6 h. Growth inhibition was determined by measuring the OD at wavelengths ranging from 595 to 650 nm. Each MIC is the result of at least two separate trials; each trial is the result of an assay run in duplicate. MIC determinations were reproducible to within a factor of 2 and are reported as the highest (most conservative) of the determined values.

Hemolytic Activity of α/β-Peptides. Freshly drawn human red blood cells (hRBC, blood type A) were washed several times with Tris buffer (pH 7.2, 150 mM NaCl) and centrifuged at 3500× rpm until the supernatant was clear. Two-fold serial dilutions of α/β-peptide in Millipore water were added to each well in a sterile 96-well plate (Falcon 3075 microtiter plate), for a total volume of 20 μL in each well. A 1% v/v hRBC suspension (80 μL in Tris buffer) was added to each well. The plate was incubated at 37 °C for 1 h, and then the cells were pelleted by centrifugation at 3500 rpm for 5 min The supernatant (80 μL) was diluted with Millipore water (80 μL), and hemoglobin was detected by measuring the OD at 405 nm. The OD of cells incubated with melittin at 400 μg/mL defines 100%; the OD of cells incubated in Tris buffer defines 0%.

Protease Susceptibility. The effect of α-chymotrypsin (Sigma, EC 3.4.21.1), trypsin (Sigma, EC 3.4.21.4), or pronase E (Sigma, EC 3.4.24.31) on three different 15-mer α/β-peptides was determined by using RP-HPLC to monitor solutions containing the α/β-peptide and one enzyme. Enzyme activity was confirmed by the use of standard substrates *N*-α-benzoyl arginine (for trypsin and pronase) and *N*-α-benzoyl tyrosine (for α-chymotrypsin). Each assay contained 2.4 mL of appropriate buffer (10 mM Tris, pH 7.5 for trypsin and pronase; 10 mM Tris, pH 8.0 for chymotrypsin), 0.1 mL of protease solution (0.1 mg/mL for trypsin and chymotrypsin and 1.0 mg/mL for pronase), and 0.5 mL of a 2 mg/mL solution of α/ β-peptide. A solution containing only α/β-peptide in buffer without any protease was used as a negative control. The enzyme concentrations used in these assays were sufficient to cleave the standard substrate completely within 30 min. The α/β-peptide was incubated with protease at room temperature, and the assay was monitored by RP-HPLC using a C_4-silica analytical column using a linear gradient of 5−95% organic solvent in water (v/v) over 45 min (solvent A, H_2O with 0.1% TFA; solvent B, 80% acetonitrile and 20% water (v/v) with 0.1% TFA).

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Supporting Information Available: NMR data and circular dichroism (CD) data. This material is available free of charge via the Internet at http://pubs.acs.org.

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