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Published on Web 06/01/2002

Mimicry of Host-Defense Peptides by Unnatural Oligomers: Antimicrobial β-Peptides

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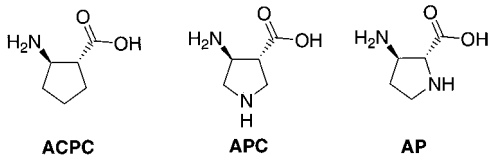
Received March 4, 2002

Abstract: We have designed β-amino acid oligomers that are helical, cationic, and amphiphilic with the intention of mimicking the biological activity of amphiphilic, cationic α-helical antimicrobial peptides found in nature (e.g., magainins). We have previously identified a 17-residue β-peptide (called β-17) with antibiotic activity similar to that of a magainin derivative against four bacterial species, including two clinical isolates that are resistant to common antibiotics. This β-peptide displays very low hemolytic activity against human red blood cells, which indicates selectivity for bacterial cells over mammalian cells. Here we examine some of the factors important for activity in this class of β-peptides. An amphiphilic helix is necessary, because a nonamphiphilic isomer proved to be inactive. The ratio of cationic to hydrophobic residues is also important. Active β-peptides induce the leakage of β-galactosidase from treated *Bacillus subtilis* cells, as do α-helical antibiotic peptides, and this similarity suggests that the β-peptide mode of action involves disruption of microbial membranes. This class of β-peptides is not degraded by proteases, which bodes well for biological applications.

Introduction

Nonnatural oligomers that are capable of adopting discrete secondary structures ("foldamers") have attracted widespread attention.[1−4] β-Amino acid oligomers ("β-peptides"), in particular, have been studied in several laboratories.[5,6] We have shown that oligomers of (*R*,*R*)-*trans*-2-aminocyclopentanecarboxylic acid (ACPC) form a 12-helix with approximately 2.5 residues per turn.[7,8] This helix is defined by 12-membered ring hydrogen bonds between each backbone carbonyl group and the amide NH of the third residue in the carboxy-terminal direction. The α-helix found in natural peptides, with approximately 3.6 residues per turn, is defined by 13-membered ring C═O- -H−N hydrogen bonds with the same directionality relative to the termini. A pyrrolidine analog of ACPC, (3*S*,4*R*)-*trans*-3-aminopyrrolidine-4-carboxylic acid (APC), is also capable of forming a 12-helical structure in aqueous solution.[9]

Chart 1. Building Blocks Used for β-Peptide Synthesis



The APC residue introduces a positive charge into the 12-helix by virtue of ring nitrogen protonation (Chart 1).

Most multicellular organisms produce antimicrobial peptides as part of their natural defense systems.[10] Many of these host-defense peptides, such as magainins[11] and cecropins,[12] are short (<30 amino acids) and form cationic, amphiphilic α-helices in the presence of target cells, micelles, vesicles, or organic solvents.[10] It is estimated that at least 150 peptides belong to this class.[13] Many studies have been performed to try to elucidate mechanisms of action, as well as parameters that are important for activity. There is still debate over the antimicrobial mechanism(s) of these peptides; possibilities include the formation of toroidal pores in the bacterial membrane[14−18] or a detergent-like disruption of the membrane ("carpet mechanism").[19,20] The

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(1) Gellman, S. H. *Acc. Chem. Res.* **1998**, *31*, 173−180.
(2) Kirshenbaum, K.; Zuckermann, R. N.; Dill, K. A. *Curr. Opin. Struct. Biol.* **1999**, *9*, 530−535.
(3) Barron, A. E.; Zuckermann, R. N. *Curr. Opin. Chem. Biol.* **1999**, *3*, 681−687.
(4) Hill, D. J.; Mio, M. J.; Prince, R. B.; Hughes, T. S.; Moore, J. S. *Chem. Rev.* **2001**, *101*, 3893−4011.
(5) Gademann, K.; Hintermann, T.; Schreiber, J. V. *Curr. Med. Chem.* **1999**, *6*, 905−925.
(6) Cheng, R. P.; Gellman, S. H.; DeGrado, W. F. *Chem. Rev.* **2001**, *101*, 3219−3232.
(7) Appella, D. H.; Christianson, L. A.; Klein, D. A.; Powell, D. R.; Huang, X. L.; Barchi, J. J.; Gellman, S. H. *Nature* **1997**, *387*, 381−384.
(8) Appella, D. H.; Christianson, L. A.; Klein, D. A.; Richards, M. R.; Powell, D. R.; Gellman, S. H. *J. Am. Chem. Soc.* **1999**, *121*, 7574−7581.
(9) Wang, X.; Espinosa, J. F.; Gellman, S. H. *J. Am. Chem. Soc.* **2000**, *122*, 4821−4822.
(10) Zasloff, M. *Nature* **2002**, *415*, 389−395.
(11) Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449−5453.
(12) Steiner, H.; Hultmark, D.; Engstrom, A.; Bennich, H.; Boman, H. G. *Nature* **1981**, *292*, 246−248.
(13) Tossi, A.; Sandri, L.; Giangaspero, A. *Biopolymers* **2000**, *55*, 4−30.
(14) Matsuzaki, K.; Murase, O.; Fujii, N.; Miyajima, K. *Biochemistry* **1995**, *34*, 6521−6526.
(15) Ludtke, S.; He, K.; Huang, H. *Biochemistry* **1995**, *34*, 16764−16769.
(16) Matsuzaki, K.; Murase, O.; Fujii, N.; Miyajima, K. *Biochemistry* **1996**, *35*, 11361−11368.
(17) Ludtke, S. J.; He, K.; Heller, W. T.; Harroun, T. A.; Yang, L.; Huang, H. W. *Biochemistry* **1996**, *35*, 13723−13728.
(18) Huang, H. W. *Biochemistry* **2000**, *39*, 8347−8352.
(19) Oren, Z.; Hong, J.; Shai, Y. *J. Biol. Chem.* **1997**, *272*, 14643−14649.

10.1021/ja0260871 CCC: $22.00

net positive charge of these peptides facilitates interaction with the negatively charged membranes of bacterial cells.[21−24] Host-defense peptides are selective for bacterial versus mammalian cells, but other natural peptide toxins, such as melittin (a component of bee venom), kill both mammalian cells and bacterial cells.[25] The origin of the bacterial selectivity of peptides such as magainin is not fully understood; many factors, including hydrophobic moment, hydrophobicity, and structure, seem to influence selectivity.[13,26] Modified peptides with improved selectivity have been generated by insertion of D-amino acids[19,27,28] or by combining sequence motifs from different natural peptides.[29−33]

We thought that it would be possible to use β-peptides to mimic the structure and function of antimicrobial α-peptides because the rules governing helix formation by β-peptides are now becoming clear.[6] β-Peptides have some advantages over the naturally occurring peptides, from a biomedical perspective, such as stability to proteases.[34−36] More broadly, successful mimicry of host-defense peptides by β-peptides would raise the possibility that other functions of natural peptides and proteins could be engineered into β-peptides and other unnatural foldamers.[37−44] (Savage et al. have shown that peptide antibiotics can be mimicked by cleverly designed small molecules.[45,46])

We have reported preliminary data showing that a 17-residue β-peptide made up of APC and ACPC residues ("β-17") is indeed capable of killing bacteria.[47,48] DeGrado and co-workers have independently described a different class of β-peptides that kill *Escherichia coli*, with varying cell selectivities.[49,50] Seebach and co-workers have reported a series of β-peptides with very limited antibacterial activity.[51] Here we compare the behavior of β-17 with that of related β-peptides, in an effort to gain insight on the factors that lead to selective antibacterial activity.

Experimental Section

β-Peptide Synthesis and Purification. Fmoc-APC(Boc) and Fmoc-ACPC were synthesized as described.[52,53] Fmoc-AP(Boc) was generated by a synthesis similar to that of one previously reported for Fmoc-ACPC.[8,54] β-Peptides were synthesized on a 25 μmol scale by standard Fmoc methods with HBTU activation on Rink amide AM resin (Applied Biosystems) on a Synergy automated synthesizer (Applied Biosystems). After β-peptide synthesis was complete, the N-termini were acylated using acetic anhydride/NEt_3/CH_2Cl_2 and shaking for 1 h. The resin-bound β-peptides were cleaved from the solid support and deprotected simultaneously by using trifluoracetic acid/ethanedithiol/H_2O (38/1/1, v/v/v) and shaking for 3 h. β-Peptides were precipitated by evaporation of the deprotection solution, addition of methanol to solubilize the β-peptide, and addition of excess cold anhydrous diethyl ether. The β-peptides were purified by reversed-phase HPLC on a C_4-silica reversed-phase semipreparative column (5 μm, 10 mm × 250 mm; Vydac). The column was eluted with a linear gradient of acetonitrile in water (0.1% trifluoroacetic acid in each) at a flow rate of 3 mL/min. The purified β-peptides were shown to be >95% homogeneous by analytical HPLC with a C_4-silica reversed-phase analytical column (5 μm, 4 mm × 250 mm; Vydac), and their masses were confirmed by MALDI-TOF mass spectrometry. β-Peptide concentrations for all experiments were determined from the weight of the lyophilized β-peptide calculated as the TFA salt (assuming association of one molecule of TFA per cationic residue).

CD Spectroscopy. Circular dichroism (CD) data were obtained on an Aviv instrument at 25 °C using a quartz cell with a 1-mm path length, between 200 and 260 nm. The peptides were analyzed at 0.19−0.38 mM in methanol or Tris buffer (0.05 M, pH 7.2). The data were normalized for β-peptide concentration and number of residues (i.e., the vertical axis in CD plots is mean residue ellipticity).

Antibacterial Activity of β-Peptides. The bacteria used for these experiments were *Escherichia coli* JM109,[55] *Bacillus subtilis* BR151,[56] *Enterococcus faecium* A634 (vancomycin-resistant),[57] and *Staphylococcus aureus* 1206 (penicillin-resistant).[58] The antibacterial activity of the 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 BHI medium was added in 50 μL aliquots to 50 μL of medium containing the 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

(20) Shai, Y. *Trends Biochem. Sci.* **1995**, *20*, 460−464.
(21) Andreu, D.; Merrifield, R. B.; Steiner, H.; Boman, H. G. *Biochemistry* **1985**, *24*, 1683−1688.
(22) Pouny, Y.; Rapaport, D.; Mor, A.; Nicolas, P.; Shai, Y. *Biochemistry* **1992**, *31*, 12416−12423.
(23) Gazit, E.; Lee, W. J.; Brey, P. T.; Shai, Y. C. *Biochemistry* **1994**, *33*, 10681−10692.
(24) Strahilevitz, J.; Mor, A.; Nicolas, P.; Shai, Y. *Biochemistry* **1994**, *33*, 10951−10960.
(25) Habermann, E. *Science* **1972**, *177*, 314−322.
(26) Giangaspero, A.; Sandri, L.; Tossi, A. *Eur. J. Biochem.* **2001**, *268*, 5589−5600.
(27) Shai, Y.; Oren, Z. *J. Biol. Chem.* **1996**, *271*, 7305−7308.
(28) Oren, Z.; Shai, Y. *Biochemistry* **1997**, *36*, 1826−1835.
(29) Boman, H. G.; Wade, D.; Boman, I. A.; Wahlin, B.; Merrifield, R. B. *FEBS Lett.* **1989**, *259*, 103−106.
(30) Wade, D.; Andreu, D.; Mitchell, S. A.; Silveira, A. M. V.; Boman, A.; Boman, H. G.; Merrifield, R. B. *Int. J. Pept. Protein Res.* **1992**, *40*, 429−436.
(31) Andreu, D.; Ubach, J.; Boman, A.; Wahlin, B.; Wade, D.; Merrifield, R. B.; Boman, H. G. *FEBS Lett.* **1992**, *296*, 190−194.
(32) Merrifield, R. B.; Juvvadi, P.; Andreu, D.; Ubach, J.; Boman, A.; Boman, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3449−3453.
(33) Kang, J. H.; Shin, S. Y.; Jang, S. Y.; Lee, M. K.; Hahm, K. S. *J. Pept. Res.* **1998**, *52*, 45−50.
(34) Hintermann, T.; Seebach, D. *Chimia* **1997**, *51*, 244−247.
(35) Seebach, D.; Abele, S.; Schreiber, J. V.; Martinoni, B.; Nussbaum, A. K.; Schild, H.; Schulz, H.; Hennecke, H.; Woessner, R.; Bitsch, F. *Chimia* **1998**, *52*, 734−739.
(36) Frackenpohl, J.; Arvidsson, P. I.; Schreiber, J. V.; Seebach, D. *ChemBioChem* **2001**, *2*, 445−455.
(37) Gademann, K.; Ernst, M.; Hoyer, D.; Seebach, D. *Angew. Chem., Intl. Ed.* **1999**, *38*, 1223−1226.
(38) Werder, M.; Hauser, H.; Abele, S.; Seebach, D. *Helv. Chim. Acta* **1999**, *82*, 1774−1783.
(39) Gademann, K.; Ernst, M.; Seebach, D.; Hoyer, D. *Helv. Chim. Acta* **2000**, *83*, 16−33.
(40) Gademann, K.; Seebach, D. *Helv. Chim. Acta* **2001**, *84*, 2924−2937.
(41) Gademann, K.; Kimmerlin, T.; Hoyer, D.; Seebach, D. *J. Med. Chem.* **2001**, *44*, 2460−2468.
(42) Xuereb, H.; Maletic, M.; Gildersleeve, J.; Pelczer, I.; Kahne, D. *J. Am. Chem. Soc.* **2000**, *122*, 1883−1890.
(43) Tanatani, A.; Hughes, T. S.; Moore, J. S. *Angew. Chem., Int. Ed.* **2001**, *41*, 325.
(44) Rouhi, A. M. *Chem. Eng. News* **2001**, *79*, 50−51.
(45) Li, C.; Peters, A. S.; Meredith, E. L.; Allman, G. W.; Savage, P. B. *J. Am. Chem. Soc.* **1998**, *120*, 2961−2962.
(46) Ding, B.; Guan, Q.; Walsh, J. P.; Boswell, J. S.; Winter, T. W.; Winter, E. S.; Boyd, S. S.; Li, C.; Savage, P. B. *J. Med. Chem.* **2002**, *45*, 663−669.
(47) Porter, E. A.; Wang, X.; Lee, H. S.; Weisblum, B.; Gellman, S. H. *Nature* **2000**, *404*, 565.
(48) Erratum: Porter, E. A.; Wang, X.; Lee, H. S.; Weisblum, B.; Gellman, S. H. *Nature* **2000**, *405*, 298.
(49) Hamuro, Y.; Schneider, J. P.; DeGrado, W. F. *J. Am. Chem. Soc.* **1999**, *121*, 12200−12201.
(50) Liu, D. H.; DeGrado, W. F. *J. Am. Chem. Soc.* **2001**, *123*, 7553−7559.
(51) Arvidsson, P. I.; Frackenpohl, J.; Ryder, N. S.; Liechty, B.; Petersen, F.; Zimmermann, H.; Camenisch, G. P.; Woessner, R.; Seebach, D. *ChemBioChem* **2001**, *2*, 771.
(52) Lee, H. S.; LePlae, P. R.; Porter, E. A.; Gellman, S. H. *J. Org. Chem.* **2001**, *66*, 3597−3599.
(53) LePlae, P. R.; Umezawa, N.; Lee, H. S.; Gellman, S. H. *J. Org. Chem.* **2001**, *66*, 5629−5632.
(54) (a) Gellman, S. H.; Appella, D. H.; Christianson, L. A.; Klein, D. A.; Krauthauser, S.; Chung, Y. J.; Wang, X. U.S. Patent 9,834,509, 2000; *Chem. Abstr.* **2000**, *132*, 322149. (b) Gomez-Vidal, J. A.; Silverman, R. B. *Org. Lett.* **2001**, *3*, 2481−2484.
(55) Yanisch-Perron, C.; Viera, J.; Messing, J. *Gene* **1985**, *33*, 103−119.
(56) Young, F. E.; Smith, C.; Reilly, B. E. *J. Bacteriol.* **1969**, *98*, 1087−1097.
(57) Nicas, T. I.; Wu, C. Y. E.; Hobbs, J. N.; Preston, D. A.; Allen, N. E. *Antimicrob. Agents Chemother.* **1989**, *33*, 11121−11124.
(58) Weisblum, B.; Demohn, V. *J. Bacteriol.* **1969**, *98*, 447−452.

absorbance at 590 nm with a microplate reader. Antibacterial activity is stated as the minimal inhibitory concentration (MIC), the concentration at which growth of the bacteria was totally inhibited. A synthetic magainin analog, Ala[8,13,18]-magainin II amide (Sigma),[59] was used for comparison.

Hemolytic Activity of β-Peptides. Freshly drawn human red blood cells (hRBC, blood type A) were washed several times with Tris buffer and centrifuged until a clear supernatant was observed. A 1% v/v suspension of hRBC in Tris was used. Two-fold serial dilutions of β-peptide in Tris 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. The 1% hRBC suspension (80 μL) was added to each well. Melittin (Sigma) at 200 μg/mL was used as the 100% hemolysis point, and Tris containing no peptide was used as the 0% hemolysis point. The plate was incubated at 37 °C for 1 h and then centrifuged at 3500 rpm for 5 min. (*Note*: Our previous hemolysis experiments were performed at 25 °C.[47]) The supernatant (80 μL) was diluted with Millipore water (80 μL), and hemoglobin was detected by measuring OD_{415}.

Peptide-Induced β-Galactosidase Leakage from *B. subtilis*. *B. subtilis* BAU102[60] was grown in trypticase soy broth with 10 μg/mL erythromycin and 34 μg/mL chloramphenicol to ensure retention of the plasmid containing the *lacZ* reporter gene. The cells were grown at 37 °C to a density of 0.6 absorbance units. (*Note*: It is very important to grow the bacteria to the same turbidity each time. If the bacteria grow out of mid-logarithmic phase and are then diluted for the assay, the results are irreproducible.) The cells were then spun down, and the supernatant was replaced with fresh medium to remove any residual extracellular β-galactosidase (β-gal). Aliquots (10 μL) of peptide stock solutions (200 μg/mL) were added to wells in a sterile 96-well plate (Falcon 3075 microtiter plate). Bacterial suspension (90 μL) was then added to the wells to give a total volume of 100 μL (final peptide concentration: 20 μg/mL). The final peptide concentration used in this assay is above the MIC in all cases. The plates were incubated at room temperature for 1 h to allow for the release of β-gal. After the incubation period, the plate was centrifuged for 5 min at 5000 rpm to remove all cells and cellular debris. An aliquot (80 μL) of the supernatant was removed and placed in a separate well. 4-Methylumbelliferyl β-D-galactoside (MUG, Sigma) was used as a fluorescent indicator of β-gal activity. An aliquot of MUG in DMSO (20 μL, 0.4 mg/mL) was added to the well, and fluorescence was monitored over a period of at least 10 min. Initial velocities of MUG hydrolysis, which were taken to indicate β-gal concentration, were obtained from the linear plot of fluorescence units versus time. Water containing no peptide was added as a negative control.

Protease Stability of a β-Peptide. The stability of a truncated amphiphilic β-peptide, β-7, to trypsin (from bovine pancreas, EC 3.4.21.4) and Pronase E (type XIV protease from *Streptomyces griseus*, EC 3.4.24.31) was determined by an HPLC assay. *N*-α-Benzoyl arginine ethyl ester (BAEE) was used as a standard substrate.[61] Trypsin experiments used 2.4 mL Tris buffer (50 mM, pH 8.2, $CaCl_2$), 0.5 mL peptide (2 mg/mL stock solution), and 0.1 mL trypsin (0.1 mg/mL stock solution). Pronase experiments used 2.4 mL Tris buffer (50 mM, pH 7.5), 0.5 mL peptide (2 mg/mL stock solution), and 0.1 mL Pronase (1 mg/mL stock solution). The enzyme concentrations used in the assays were high enough to cleave the standard substrate completely within 30 min. The β-peptide was incubated with enzyme at room temperature, and the peak corresponding to this β-peptide was monitored in the HPLC trace over time. A C_4-silica reversed-phase analytical column (5 μm, 4 mm × 250 mm; Vydac) with a linear gradient of 5−95% B over 45 min was used (A: 0.1% TFA/H_2O, B: CH_3CN/H_2O/TFA (80/20/0.1)).

Table 1. Antimicrobial Activities of Ala[8,13,18]-Magainin II Amide and β-Peptides (μg/mL)[a]

	E. coli	*B. subtilis*	*E. faecium*	*S. aureus*
magainin	6.3	3.2	25	25
β-17 (APC40)	6.3	0.8	12.5	3.2
AP40	25	1.6	50	12.5
APC60	100	50	>200	>200
AP60	>200	25	>200	>200
scrambled	200	200	>200	>200

[a] Minimal inhibitory concentration (MIC, in μg/mL) is defined here as the lowest concentration of peptide required for complete inhibition of growth, as determined from the absorbance at 590 nm. Bacteria in BHI medium (~10^6 CFU/ml) were incubated for 6 h at 37 °C with a 2-fold-dilution series of peptide in medium in a sterile 96-well plate. Microbial growth was determined by the increase in OD_{590} over the 6-h period. Each MIC is the result of at least two separate trials; each trial is the result of an assay run in duplicate. Results are accurate within a factor of 2. The more conservative MIC for each set of trials is reported.

Results and Discussion

β-Peptide design. The first peptide examined in this study, β-17, was designed to form a helix that would display cationic residues on 40% of the helical surface and hydrophobic residues on 60% of the surface (Figure 1). This β-peptide is very simple in that it is composed of only two different residues. The sequence has three pentad repeats preceded by two hydrophobic residues (Figure 1). Shorter β-peptides that contained only one or two pentad repeats, β-7 and β-12, were also evaluated (data not shown). β-7 did not display any activity in our assays, and β-12 was only marginally active. Magainin II is approximately 34.5 Å long when it is in an α-helical conformation (calculation based on 23 residues, 3.6 residues/turn, and a pitch of 5.4 Å). β-17 is approximately 36 Å long in a 12-helical conformation (2.6 residues/turn and a pitch of 5.5 Å).

Assays with four bacteria showed β-17 to be an antibiotic (Table 1); as a result, other β-peptides were designed to explore some of the requirements for activity (Figure 1). Analog APC60 has reversed residue proportions (60% cationic APC/40% hydrophobic ACPC) relative to those of β-17. (β-17 can be referred to as APC40 by this nomenclature.) A more hydrophobic analog, APC20, with cationic residues displayed on only 20% of the helical surface, was also prepared; however, APC20 proved too insoluble for reversed-phase HPLC purification. AP40 and AP60, isomers of β-17 and APC60, contain an alternative cationic β-amino acid, (*R*)-β-amino-D-proline (AP), in place of APC.[54] We were interested to see whether shifting the position of the cationic nitrogen in the five-membered ring would have any effect on biological activity.

We prepared a "scrambled" isomer of β-17 to determine whether amphiphilicity is required for activity, as suggested by precedents with natural antimicrobial peptides.[62] The sequence of the scrambled isomer distributes the positive charges around the 12-helix circumference, preventing formation of an amphiphilic helix (Figure 1).

Secondary Structure. Figure 2 shows CD data for five β-peptides (β-17, APC60, AP40, AP60, and scrambled) in methanol and in aqueous buffer. Each β-peptide exhibits the

(59) Chen, H. C.; Brown, J. H.; Morell, J. L.; Huang, C. M. *FEBS Lett.* **1988**, *236*, 462−466.
(60) Ulijasz, A. T.; Grenader, A.; Weisblum, B. *J. Bacteriol.* **1996**, *178*, 6305−6309.
(61) Schwert, G. W.; Takenaka, Y. *Biochim. Biophys. Acta* **1955**, *16*, 570−575.
(62) Dathe, M.; Wieprecht, T.; Nikolenko, H.; Handel, L.; Maloy, W. L.; MacDonald, D. L.; Beyermann, M.; Bienert, M. *FEBS Lett.* **1997**, *403*, 208−212.

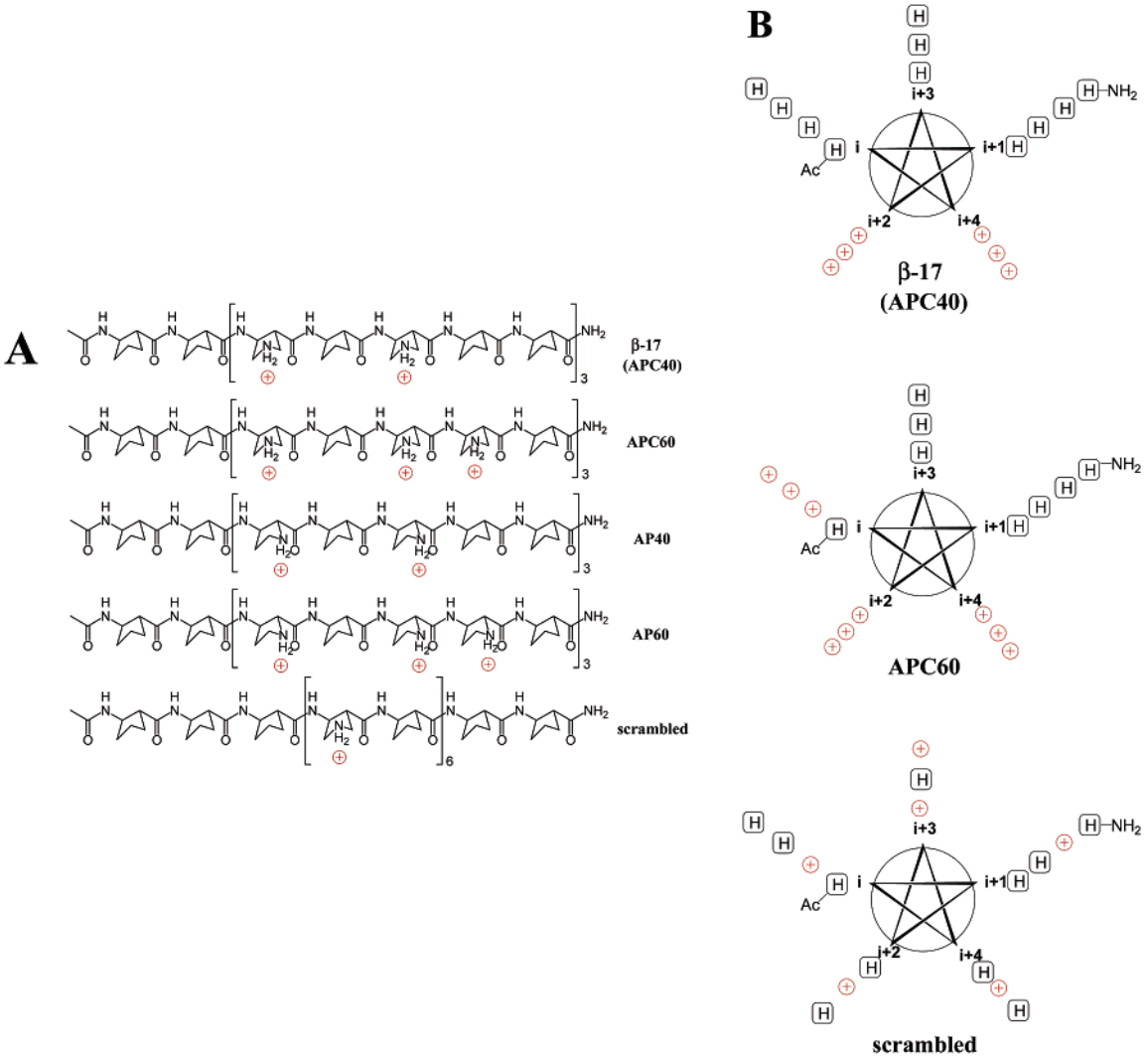


Figure 1. Design of antimicrobial β-peptides. (A) Five β-peptides investigated in this study. (B) Helical wheel diagrams for β-17, APC60, and the scrambled peptide. These diagrams represent the β-peptide in the 12-helical conformation, viewed along the helix axis. β-17 can also be designated APC40, to be consistent with the nomenclature used for the other β-peptides discussed here. "H" indicates the hydrophobic ACPC residue; "+" indicates the cationic APC residue. "Ac" indicates the acetylated N-terminus; "NH_2" indicates the amidated C-terminus.

12-helical CD signature, with a maximum at 206 nm and a minimum at 223 nm in methanol.[63] The maxima are blue-shifted to 203 nm in aqueous buffer. All β-peptides show stronger signals in methanol than in aqueous solution, which agrees with data that have previously been reported for these types of β-peptides.[9,64] This solvent-dependent difference in CD intensity indicates that the 12-helix is more highly populated in methanol than in water. Helix stabilization by alcohols relative to water is well established for more flexible β-peptides[6] and for conventional peptides.[65] The fact that these β-peptides display partial 12-helix formation in water sets them apart from many conventional α-peptides. Magainins and other antimicrobial α-peptides do not display α-helicity in the absence of membranes or membrane mimics.[10,66] (We have previously shown that β-peptides containing ACPC, APC, and related residues show significant 12-helix formation in aqueous solution with as few as six residues.[9,64])

The scrambled β-peptide shows the most intense signal in both solvents, suggesting that this molecule has the highest 12-helix population. β-17, AP40, and APC60 show similar intensities in both solvents. AP60 displayed the weakest CD signal of all of the β-peptides.

Antibacterial Activity. The activities of the β-peptides against four species of bacteria (one Gram-negative, three Gram-positive) are shown in Table 1. Ala[8,13,18]-magainin II amide (GIGKFLHAAKKFAKAFVAEIMNS-NH_2), a synthetic magainin derivative that is more active than natural magainins,[59] was used as a positive control. β-17 (APC40) and AP40 had activities similar to those of the synthetic magainin (AP40 was slightly less active in some cases). Both of these peptides have a 40% cationic face.

APC60 and AP60, the two peptides with a 60% cationic face, showed very limited antibacterial activity. This result was not expected, since each of these peptides has a ca. 144° hydrophobic sector, viewed along the helix axis, and the majority of natural antimicrobial peptides have a hydrophobic sector of

(63) Applequist, J.; Bode, K. A.; Appella, D. H.; Christianson, L. A.; Gellman, S. H. *J. Am. Chem. Soc.* **1998**, *120*, 4891−4892.
(64) Lee, H. S.; Syud, F. A.; Wang, X. F.; Gellman, S. H. *J. Am. Chem. Soc.* **2001**, *123*, 7721−7722.
(65) Creighton, T. E. *Proteins: Structures and Molecular Properties*, 2nd ed.; W. H. Freeman and Company: New York, 1993.
(66) Bechinger, B.; Zasloff, M.; Opella, S. J. *Protein Sci.* **1993**, *2*, 2077−2084.

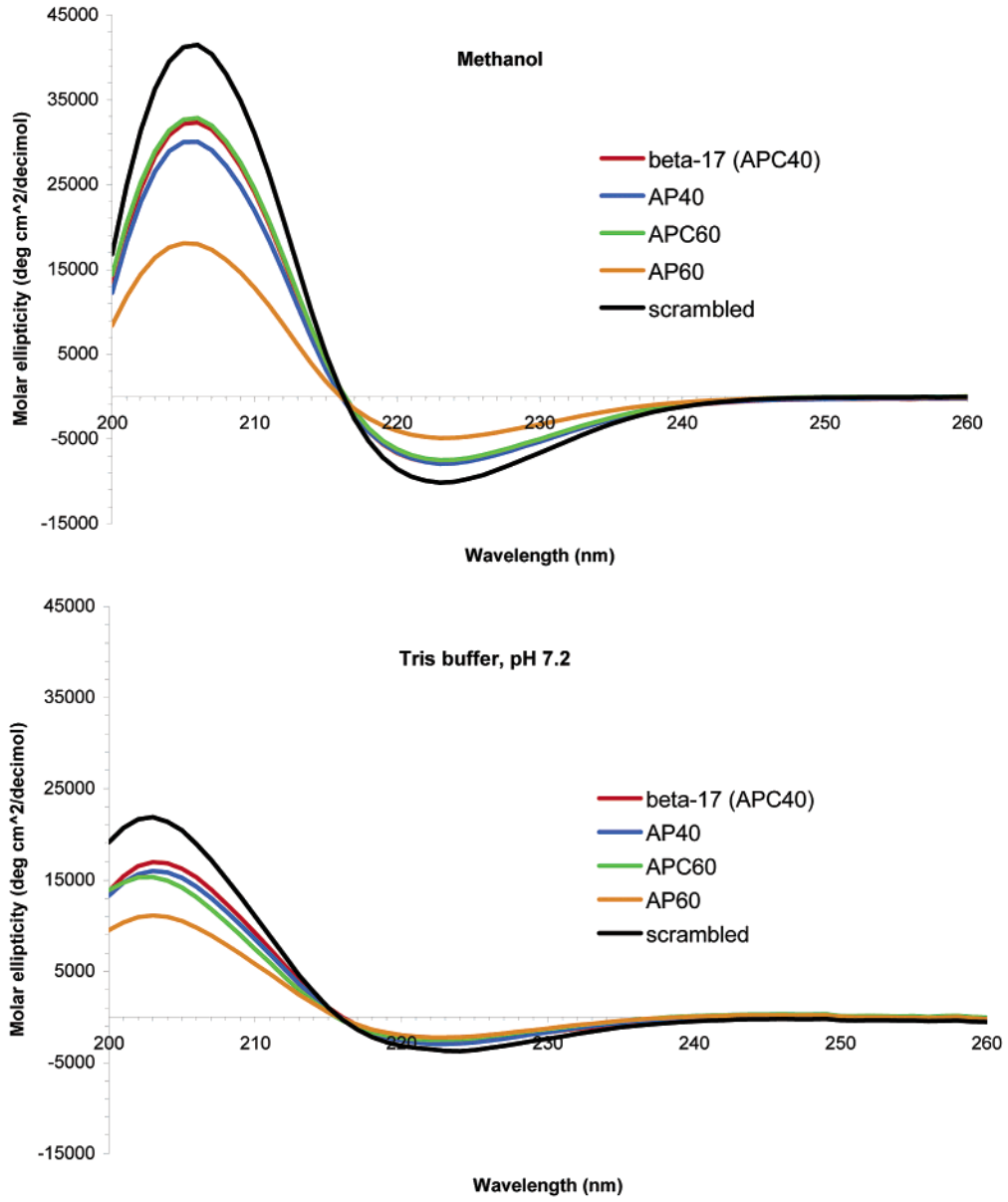


Figure 2. CD spectra of β-peptides. (Top) CD spectra recorded in methanol. (Bottom) CD spectra recorded in Tris buffer, 50 mM, pH 7.2. Concentrations of β-peptides were 0.19−0.38 mM in both sets of spectra.

140−200°.[13] We did not actively investigate the β-peptides APC20 or APC80. APC80 would have likely been inactive, given the marginal activity of APC60, and APC20 proved too insoluble to assay (vide supra). β-17 and AP40 have a hydrophobic sector of ca. 216° of the 12-helical circumference. It is our hypothesis that a relatively large hydrophobic sector is required for activity in this β-peptide series because the ACPC residue has limited hydrophobicity. Perhaps this limitation could be overcome by appending hydrophobic side chains, which has recently been achieved with APC.[64]

The scrambled β-peptide showed little or no activity against any of the organisms. This result supports our hypothesis that the 12-helix adopted by this class of β-peptides must be amphiphilic for antibacterial activity.

Hemolytic Activity. The activities of the peptides against hRBC are shown in Figure 3. Melittin, a cytolytic peptide from bee venom,[25] was used as a positive control. β-17 (APC40) and AP40 have hemolytic activities somewhat lower than that of the magainin analog, with AP40 being the less hemolytic of the two β-peptides. Significant hemolysis occurs only at concentrations much higher than the antibacterial MIC values (Table 1), indicating selectivity for bacterial cells over mam-

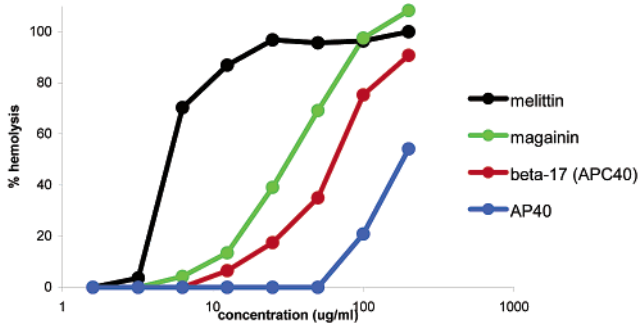


Figure 3. Hemolytic activities of β-peptides. Melittin was used as a hemolytic standard. Melittin at 200 μg/mL was used as the 100% hemolysis value. APC60, AP60, and the scrambled peptide showed no hemolytic activity under these assay conditions.

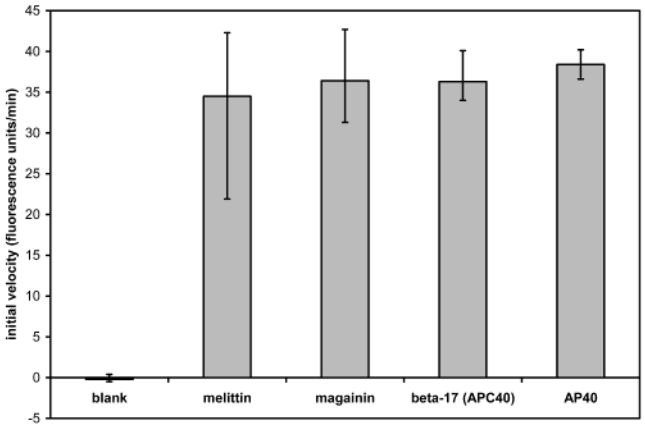


Figure 4. Peptide-induced leakage of β-galactosidase from BAU-102. The vertical axis represents relative amounts of β-galactosidase present in the medium after incubation with the peptides for 60 min, based on initial velocities of the enzyme-catalyzed reaction. Error bars are based on four separate trials with each peptide (except AP40, for which only two trials were run).

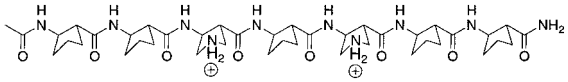
malian cells. Thus, both β-17 and isomer AP40 show selectivity toward bacteria that mimics the selectivity of natural host-defense peptides. APC60, AP60, and the scrambled peptide did not show any hemolytic activity (data not shown).

***β*-Peptide-Induced Enzyme Leakage from BAU-102.** The leakage of the enzyme β-galactosidase (MW ≈ 116 kDa) from *B. subtilis* BAU-102[60] was used as an indicator for membrane permeabilization by the β-peptides. BAU-102 has a plasmid containing *lacZ* under control of *vanRS*. Disruption of the inner cell membrane, either by the direct action of an effector molecule, or as the indirect effect resulting from inhibition of cell wall peptidoglycan synthesis, should allow β-gal (and other cell contents) to diffuse out of the bacteria. We detected this leakage by measuring the initial velocity of a reaction catalyzed by β-gal; initial velocity was taken to indicate the amount of enzyme that had leaked out of the bacterial cells.

The protocol that we developed to detect bacterial membrane permeabilization is somewhat different from protocols reported by others.[26,67,68] A typical assay monitors the cleavage of a chromo- or fluorogenic substrate in the medium by β-gal after a membrane permeabilizing peptide is added to a β-gal constitutive strain of bacteria. However, in such an assay it is difficult to deconvolute the kinetics of membrane permeabilization by the peptide from the kinetics of the enzyme itself acting on the indicator substrate. Therefore, we revised the assay to test only whether leakage occurs and to what extent it occurs over a period of 60 min. At the end of the 60 min incubation with peptide, the samples are centrifuged to remove cells and cellular debris. The fluorogenic substrate is then added to the supernatant, which should contain only β-gal released during the incubation period. The relative amount of β-gal can then be estimated by the initial velocity of the catalyzed reaction.

The leakage-inducing activities of melittin, Ala[8,13,18]-magainin II amide, β-17, and AP40 were investigated. All of the oligomers studied led to similar amounts of β-gal in the supernatant after the incubation period (Figure 4). In contrast, supernatant from cells with no peptide added showed no cleavage of the fluorogenic substrate. These results suggest that the β-peptides we examined act by a membrane-disrupting mechanism similar to that of the magainins.

Protease Stability of *β*-7. A truncated analog of β-17, β-7, was tested for susceptibility to proteolytic cleavage. Proteolysis



β-7

of this class of β-peptides has never been studied, but β-peptides composed of acyclic residues have been shown to be very stable to many endogenous proteases.[34−36] We examined trypsin because of the ability of this enzyme to cleave α-peptides adjacent to a positive charge (Lys or Arg). Pronase, which displays many proteolytic activities,[69] was also evaluated because of its broad activity toward many different α-peptide substrates.

β-7 was incubated with the proteases in appropriate buffers and monitored by RP-HPLC. The area of the peak representing

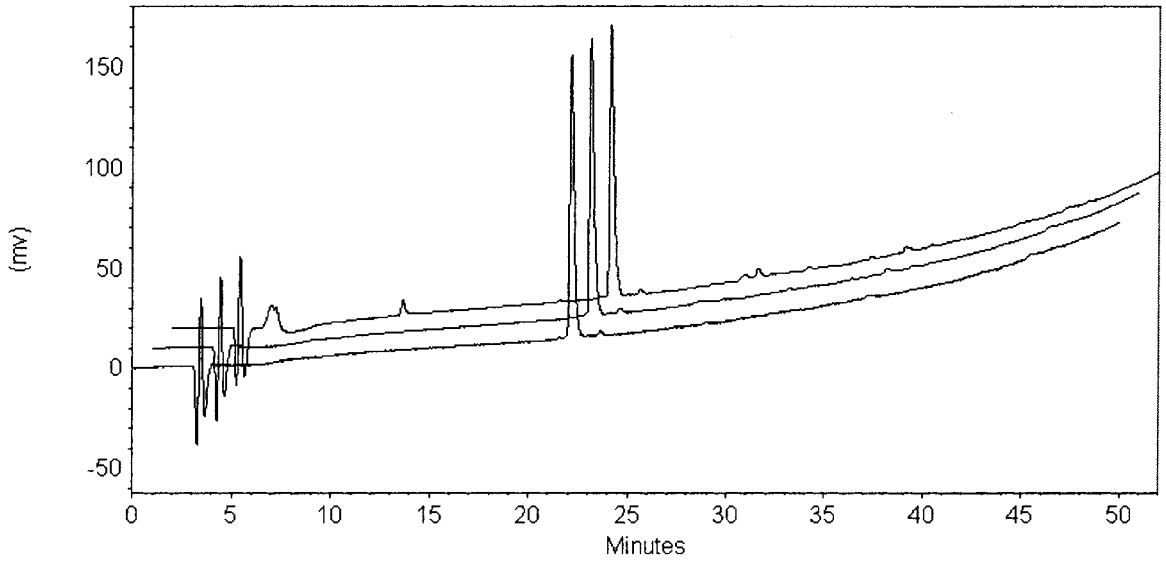


Figure 5. HPLC assay for protease stability of β-7. β-7 incubated in buffer (bottom); β-7 incubated for 50 h with trypsin (middle); β-7 incubated for 49 h with Pronase (top). The traces have been offset by one minute relative to one another along the horizontal axis so that peaks can be observed; all three major peaks corresponding to β-7 occurred at 22.1 min. Small peaks in the top trace that are not present in the other traces are not likely due to degradation of β-7; these peaks were also present in other traces containing the Pronase solution (data not shown).

β-7 did not change significantly over a period of 2 days (Figure 5). At the conclusion of the experiment, the standard substrate, *N*-α-benzoyl arginine ethyl ester (BAEE), was added to a portion of the solution and incubated for 30 min. Cleavage of the standard substrate was complete in ≤30 min, indicating retention of enzyme activity over the 2-day period at room temperature. BAEE cleavage in the presence of β-7 also indicates that the β-peptide does not inhibit enzyme activity. These results are consistent with the results of Seebach and co-workers for other β-peptides[34−36] as well as with the general knowledge that proteases are usually specific for peptides containing at least some α-amino acids. β-Amino acids have previously been incorporated into peptidomimetics to confer proteolytic stability.[70] The high stability of β-7 to proteases is significant in terms of potential biomedical applications of 12-helical β-peptides.

(67) Falla, T. J.; Karunaratne, D. N.; Hancock, R. E. W. *J. Biol. Chem.* **1996**, *271*, 19298−19303.
(68) Skerlavaj, B.; Benincasa, M.; Risso, A.; Zanetti, M.; Gennaro, R. *FEBS Lett.* **1999**, *463*, 58−62.
(69) *Sigma Biochemicals and Reagents Catalog*, 2002−2003.
(70) Steer, D. L.; Lew, R. A.; Perlmutter, P.; Smith, A. I.; Aguilar, M. I. *J. Pept. Sci.* **2000**, *6*, 470−477 and references therein.

Conclusions

We have shown that 12-helical β-peptides are capable of selectively killing a variety of bacterial species, including two clinical isolates that are resistant to common antibiotics. For this class of β-peptides, it seems that a 40% cationic face is best for activity; β-peptides containing more cationic residues are not as active. Helical amphiphilicity is also important for activity. These β-peptides appear to act by a mechanism similar to that of antimicrobial α-peptides, based on their ability to cause rapid release of a cytoplasmic enzyme from *B. subtilis* cells. The 12-helical β-peptides are impervious to proteases, which is encouraging with regard to potential biological applications.

Acknowledgment. This research was supported by the NIH (GM56414). E.A.P. was supported in part by a Biotechnology Training Grant (NIH 5 T32 GM08349). CD data were obtained in the Biophysics Instrumentation Facility at UW-Madison, supported by NSF.

JA0260871