Biochemical Pharmacology, Vol. 58, pp. 1775–1780, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00259-2

Effect of D-Amino Acid Substitution on the Stability, the Secondary Structure, and the Activity of Membrane-Active Peptide

*Sung Yu Hong, Jong Eun Oh and Keun-Hyeung Lee**

PROTEIN CHEMISTRY LABORATORY, MOGAM BIOTECHNOLOGY RESEARCH INSTITUTE, YOUNGIN CITY, KYUNGGI-DO 449-910, KOREA

ABSTRACT. Several diastereomers and an enantiomer of KKVVFKVKFKK, an antimicrobial peptide that acts on the lipid membrane of pathogens were synthesized to investigate the effect of D-amino acid substitution on stability, secondary structure, and activity. The stability of the peptide in serum was improved greatly by the D-amino acid substitutions. D-Amino acid substitutions at the N- and/or C-terminal of the peptide, which had little effect on the α-helical structure, and all D-amino acid substitutions that formed a left-handed α-helix maintained antimicrobial activity, whereas D-amino acid substitutions in the middle of the amino acid sequence disrupted the α-helical structure, resulting in the complete loss of activity. This result confirmed that the peptide did not interact with chiral receptors, enzymes, or any chiral component of the membrane. D-Amino acid substitutions at the termini reduced the inhibition of the activity by heat-inactivated serum, which indicated that local change of chirality or change of secondary structure induced by D-amino acid substitutions might affect the interactions between the peptide and certain components in the serum. The present study suggests that partial D-amino acid substitution is a useful technique to improve the *in vivo* activity of antimicrobial peptides. BIOCHEM PHARMACOL **58**;11:1775–1780, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. antimicrobial peptide; cleavage site; diastereomer; enantiomer; α-helical structure; serum protease; stability

Infectious diseases are an increasing phenomenon today, mainly as the result of an increase in antibiotic-resistant pathogens. The development of resistant strains brought about by the widespread use of current antibiotics encourages the exploration of novel antimicrobial molecules with unexploited mechanisms. Host defense peptides have been discovered from various natural sources [1–3]. More than 100 different peptides have been isolated and characterized. Some of them possess a broad range of activities against bacteria and fungi. These antimicrobial peptides have received attention because they have a low toxicity against mammalian cells and act on the lipid membrane of pathogens [4]. In this antimicrobial mechanism, a resistant strain that has a different lipid membrane composition cannot occur easily, and peptides that are not required to enter the cytoplasm have less risk of degradation by special enzymes produced by the resistant strains and removal by the export mechanism of the resistant strains. However, membrane-active peptides themselves, like other biological active peptides, may not be useful as therapeutic agents because of their low stability *in vivo*; the amide bonds in the peptides are easily cleaved by proteases [5]. Studies of magainin 2, a well-known antimicrobial peptide, showed that its biological activity decreases in the presence of protease [6, 7]. A facile method for increasing the stability of a peptide is to replace a natural amino acid with a D-amino acid. Synthesized enantiomers of naturally occurring membrane-active peptides such as cecropin A, magainin 2 amide, and melittin are resistant to enzymatic degradation and show activity similar to that of their natural form [8–10]. However, an enantiomer may not be suitable as a therapeutic agent because its extremely long half-life may increase the side-effects. An alternative method to overcome this limitation is to replace the amino acid in the most susceptible site with a D-amino acid. However, D-amino acid replacement at a specific position may decrease the activity of membrane-active peptides because it may change the secondary structure of the peptides, which plays an important role in antimicrobial activity [11]. Therefore, to improve the stability of antimicrobial peptides without affecting their activity, the effect of the replacement of D-amino acid on the secondary structure must be considered.

Recently, a novel antimicrobial depsipeptide, KKVVFKVKFKK-NH_2, named KSLK, was developed by combinatorial libraries [12] and the addition of a lysine residue at the C-terminal (unpublished data). This peptide has a broad spectrum of antibacterial and antifungal activities. A liposome leakage assay suggested that this peptide acts on the membrane of pathogens. In the present study, we chose

* Corresponding author: Dr. Keun-Hyeung Lee, Protein Chemistry Laboratory, Mogam Biotechnology Research Institute, 341 Pojung-Ri, Koosung-Myun, Youngin City, Kyunggi-Do 449-910, Korea. Tel. (82) 331-262-3851; FAX (82) 331-262-6622; E-mail: lkh@kgcc.co.kr

Received 5 October 1998; accepted 5 May 1999.

this peptide as a model peptide and investigated the effect of D-amino acid substitution on the stability, secondary structure, and activity of membrane-active peptides. We measured the stability of the peptide in the presence of serum and determined the cleavage site by serum protease. On the basis of this result, we designed and synthesized several diastereomers of the model peptide. The diastereomers with D-amino acid in their N- and C-termini were more stable than the model peptide in the presence of serum, and they had antifungal and antibacterial activity similar to that of the peptide. CD* spectroscopy study indicated that the secondary structure of the peptide was not disrupted by the D-amino acid replacement at the termini. These results suggest that partial D-amino acid substitution of membrane-active peptides is an effective method to improve *in vivo* stability without affecting the secondary structure and activity.

MATERIALS AND METHODS

Peptide Synthesis

The peptides were synthesized automatically by the solid phase method using standard Fmoc chemistry [13–15] on an automatic peptide synthesizer, 431A (Applied Biosystem). The peptides were purified by a preparative reversed-phase HPLC (RP-HPLC) system to give final products of more than 95% purity by analytical HPLC analysis. The electrospray mass spectrometer on Platform II from VG (Fisons Instruments) was used to measure the mass of the purified peptides.

Determination of the Stability and Degradation Site of the Peptides in the Presence of Serum

One milliliter of 25% mouse serum/RPMI medium (v/v) in 1.5-mL Eppendorf tubes was temperature-equilibrated at 37° for 15 min before adding 10 μL of peptide stock solution in RPMI-1640 (10 mg/mL) to make the final peptide concentration 100 μg/mL. The initial time was recorded, and 100 μL of reaction solution was removed at known time intervals and added to 100 μL of 10% aqueous TCA solution. The cloudy reaction sample was cooled 4° for 15 min and spun at 13,000 *g* for 15 min to precipitate serum protein. HPLC studies indicated that no loss of KSLK by self-aggregation or binding to serum protein occurred in the process of TCA precipitation (data not shown). The analysis for the remaining peptide was carried out by RP-HPLC using an analytical C_{18} column. Kinetic analysis for the half-life was carried out by a linear least squares analysis of the logarithm of the peak area versus time. Each half-life was determined from two independent experiments performed in duplicate, and all pseudo-first order plots were linear, showing a correlation coefficient of greater than 0.95 (7 points). To determine the cleavage site of the peptide, the degraded peptide fragments were separated using a RP-HPLC C_{18} column and characterized using an amino acid sequencer on a 492 protein sequencer (Perkin-Elmer) and a mass spectrometer.

FIG. 1. Cleavage site of the model peptide in the presence of serum. The solid arrows indicated the most susceptible sites, and the dotted arrow indicated a less susceptible site.

CD Measurement

CD spectra were recorded on a J-715 spectropolarimeter (Jasco) using a quartz cell of 1-mm pathlength in the wavelengths ranging from 195 to 245 nm. The concentration of the peptides was 100 μg/mL in 10 mM sodium phosphate buffer (pH 7.4) containing 50% TFE (v/v). The peptide concentration was confirmed by amino acid analysis after CD measurements. Two scans were averaged for each peptide with a scan speed of 10 nm/min. The α-helical content of the peptide was determined from the mean residue ellipticity [θ] at 222 nm [16].

Antimicrobial Assay

An *in vitro* antifungal assay was performed using a modified checkerboard microdilution technique [17, 18] with a 96-well microplate. RPMI-1640 medium (pH 7.0) (Gibco-BRL) was used as the assay medium. Freshly grown *Candida albicans* cells (ATCC 36232) on slopes of Sabouraud dextrose agar (SDA) (Merck) were suspended in physiological saline, and the concentration was adjusted to 10^4 cells/1 mL of 2× concentrated medium for use as the inoculum. The peptide solutions were added into the wells in the plate (100 μL/well), and serial 2-fold dilutions were prepared. The final concentration of the peptide solution ranged from 0.78 to 100 μg/mL. After inoculation (100 μL/well, 5×10^3 cells/mL), the plate was incubated at 30° for 48 hr, and then the absorbance at 620 nm was measured. The mouse serum used in the antifungal study was heat-inactivated at 70° for 30 min. The *in vitro* antibacterial assay was done by the same method used for the antifungal assay described above except for the assay medium and the incubation temperature. In the antibacterial assay, antibiotic medium 3 (pH 7.0 at 25°; Difco) was used, and cells were incubated at 37° for 24 hr. The MIC was defined as the lowest concentration of the peptide that completely inhibited the growth of the test organism. Each MIC was determined from three independent experiments performed in duplicate.

* *Abbreviations:* CD, circular dichroism; Fmoc, 9-fluorenylmethoxycarbonyl; TCA, trichloroacetic acid; TFE, trifluoroethanol; and MIC, minimal inhibition concentration.

TABLE 1. Sequences and characteristics of KSLK, its diastereomers, and its enantiomer

Peptides	Sequences*	Retention times (min)	% of α-Helicity	Half-life (min)
KSLK	KKVVFKVKFKK-NH_2	26.4	66	6
Diastereomer 1	**KK**VVFKVKFKK-NH_2	26.0	45	16
Diastereomer 2	KKVVFKVKF**KK**-NH_2	26.1	40	12
Diastereomer 3	**K**KVVFKVKFK**K**-NH_2	25.8	50	32
Diastereomer 4	**KK**VVFKVKF**KK**-NH_2	25.6	23	42
Diastereomer 5	KKVVF**K**VKFKK-NH_2	23.5	NA†	
Diastereomer 6	**KK**VVF**K**VKF**KK**-NH_2	24.0	NA	
Enantiomer	**KKVVFKVKFKK**-NH_2	26.2	49	>400

*Underlined bold amino acids are D-amino acids.
†α-Helicity of the peptide was not analyzed because the peptide did not have a well-defined ordered structure.

RESULTS

Design, Synthesis, and Stability of KSLK and Its Diastereomers

The half-life of KSLK and its diastereomers in the presence of serum was measured. Figure 1 showed the kinetic analysis of the degradation of the peptide in the presence of serum. The half-life was calculated by a linear least squares analysis of the logarithm of the peak area versus time. As shown in Table 1, the half-life of KSLK was about 6 min, indicating that KSLK was degraded easily by serum protease. However, the addition of a protease inhibitor did not prevent the degradation of KSLK completely when exposed to serum. Even in the presence of a protease inhibitor, 30% KSLK was lost within 90 min (data not shown). To find out the most susceptible sites in the presence of serum, the degraded fragments of KSLK in the presence of serum were isolated using a C_{18} analytical HPLC system, and then the fragments were characterized using a mass spectrometer and an amino acid sequencer. As shown in Fig. 2, analysis of the major fragment indicated that the most susceptible site must be the N-terminal amide bond and the next susceptible site was the C-terminal amide bond. Analysis of a minor fragment indicated that the amide bond in the middle region of the peptide was also cleaved. Amino acids in the susceptible sites of the model peptide were replaced by D-amino acids. As shown in Table 1, lysines at the N-terminal and/or the C-terminal were replaced by D-lysine to prevent the degradation catalyzed by the exopeptidase. Also, the diastereomers containing D-lysine in the middle of the amino acid sequence were synthesized to prevent the degradation catalyzed by the endopeptidase. The double D-amino acid replacements at the N-terminal of the peptide increased the half-life by approximately 3-fold, whereas the double D-amino acid replacements at the C-terminal increased the stability by 2-fold, which confirmed that the N-terminus was more susceptible than the C-terminus. When a lysine residue at the N- and C-terminals was replaced simultaneously with D-lysine, the half-life was increased dramatically by 5-fold. Double D-amino acid substitutions at the N- and C-terminals of the peptide

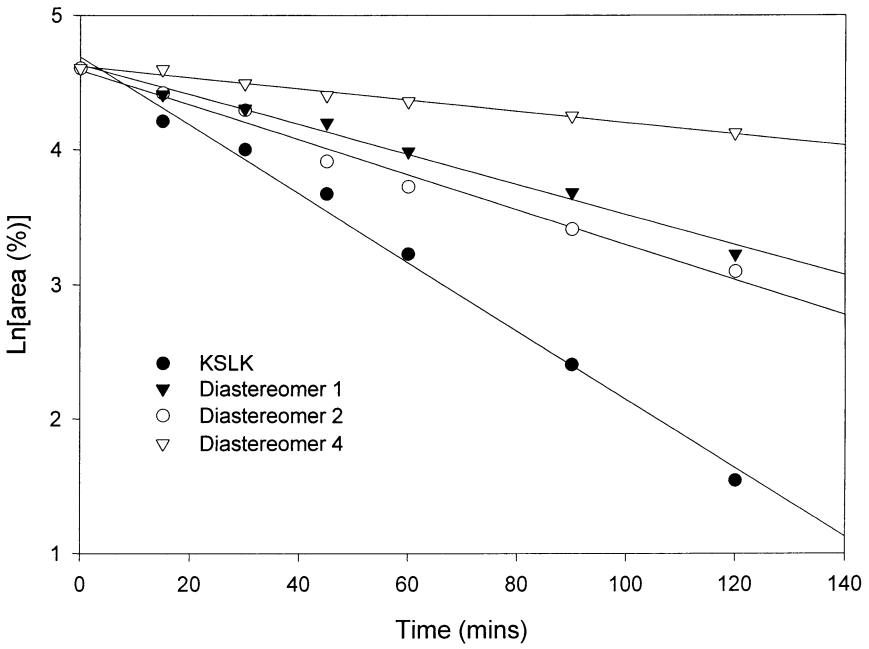

FIG. 2. Kinetic analysis of the degradation of the peptides in the presence of 25% serum (v/v), carried out by a linear least squares analysis of the logarithm of the peak area versus time. Each half-life was determined from two independent experiments performed in duplicate.

TABLE 2. Antimicrobial activity of KSLK, its diastereomers, and its enantiomer

	MIC* (μg/mL)					
Peptides	*Candida albicans* (ATCC 36232)	*Staphylococcus aureus* (ATCC 6538)	*Micrococcus luteus* (ATCC 9341)	Methicillin-resistant *S. aureus* (SR 1550)†	*Escherichia coli* (ATCC 2592)	*Pseudomonas aeruginosa* (ATCC 9027)
KSLK	3.12	3.12	6.25	25	12.5	6.25
Diastereomer 1	3.12	3.12	6.25	25	12.5	6.25
Diastereomer 2	3.12	3.12	6.25	25	12.5	6.25
Diastereomer 3	3.12	3.12	6.25	25	6.25	6.25
Diastereomer 4	3.12	6.25	6.25	50	12.5	12.5
Diastereomer 5	>100	>100	>100	>100	>100	>100
Diastereomer 6	>100	>100	>100	>100	>100	>100
Enantiomer	3.12	3.12	6.25	25	12.5	6.25

*Each MIC was determined from three independent experiments performed in duplicate.
†Characteristics of this strain are described in Ref. 19.

increased the half-life by 7-fold. This result revealed that the stability of the peptide could be synergistically increased by simultaneous blocking the two major cleavage sites and that the double D-amino acid substitution increased the stability much more than did the single D-amino acid substitution. The half-life of the enantiomer was more than 400 min, which indicated that the enantiomer was very stable in the presence of serum.

Antimicrobial Activity of KSLK, Its Diastereomers, and Its Enantiomer

The activities of KSLK and its isomers were tested against bacteria and fungi. As shown in Table 2, the diastereomers that had D-lysine at the N- and/or C-terminal showed the same activity as KSLK against all test microorganisms, which meant that the D-amino acid replacement of lysine residues at the N- and/or C-terminal did not affect antimicrobial activity. The enantiomer also showed the same activity as KSLK, indicating that the chirality of the peptide did not affect antimicrobial activity. However, the diastereomers with D-lysine in the middle of the amino acid sequence did not show activity up to 100 μg/mL.

The activities of KSLK and its isomers against *C. albicans* were measured in the presence of heat-inactivated serum. As shown in Table 3, the MIC of KSLK and its enantiomer was increased by more than 16-fold when heat-inactivated serum (12.5%, v/v) was added to the assay media. Even though the addition of the heat-inactivated serum also decreased the activity of the diastereomers, interestingly the activity was much less decreased than those of KSLK and its enantiomer; the activity of the diastereomers was decreased by only 2- or 4-fold.

TABLE 3. Antifungal activity of KSLK and its isomers in the presence of heat-inactivated mouse serum

	MIC (μg/mL) against *C. albicans**	
Peptides	0% Serum	12.5% Serum
KSLK	3.12	50
Diastereomer 1	3.12	12.5
Diastereomer 2	3.12	6.25
Diastereomer 3	3.12	12.5
Diastereomer 4	3.12	12.5
Enantiomer	3.12	50

*Each MIC was determined from three independent experiments performed in duplicate.

CD Spectra of KSLK, Its Diastereomers, and Its Enantiomer

To investigate the effect of D-amino acid substitution on the secondary structure of the peptide, CD spectra of KSLK and its isomers were measured. Generally, it has been reported that even two adjacent D-amino acid substitutions could not form the left-handed helix and caused a local change in the structure [20–22]. In aqueous solutions, the peptides had a random coil structure (data not shown). As shown in Fig. 3 and Table 1, CD spectra with a negative ellipticity at the double minima of 208 and 222 nm revealed that KSLK, diastereomer 1, diastereomer 2, diastereomer 3, and diastereomer 4 had a predominantly α-helical structure in the presence of 50% TFE (pH 7.4). However, the α-helical content of KSLK was decreased by the increase in the number of D-amino acids in the termini. CD spectra indicated that diastereomer 5 and diastereomer 6 did not have the α-helical structure, indicating that the incorporation of D-amino acid in the middle of the peptide destroyed the α-helical structure. The CD spectrum with a positive ellipticity of the enantiomer revealed that the enantiomer had a left-handed α-helical structure with a major conformer.

DISCUSSION

The prediction of *in vivo* peptide pharmacokinetics is quite difficult because there are many factors that determine peptide fate, including absorption rate, first-pass metabolism, hepatic and renal clearance, cellular binding and

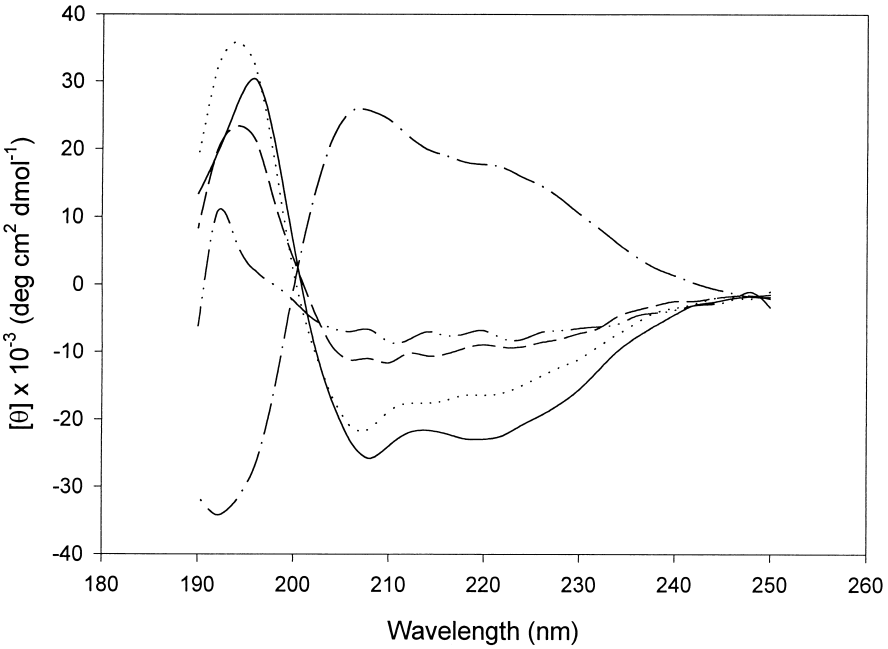

FIG. 3. CD spectra of KSLK and its isomers in 10 mM phosphate buffer (pH 7.4) including 50% TFE (v/v). Key: (—) KSLK; (· · ·) diasteromer 1; (—-) diasteromer 4; (– · · –) diasteromer 5; and (– · –) enantiomer.

uptake, and circulating peptidase [23]. However, *in vivo* peptide stability in blood can be gauged by determining the *in vitro* stability in serum [24]. Therefore, we studied the effect of D-amino acid replacement on the stability of membrane-active peptide by measuring the half-life in the presence of serum.

A facile method for increasing the stability of peptide is to replace natural amino acids in the cleavage sites by D-amino acids. However, in this case, the effect of the D-amino acid substitution on the secondary structure should be considered because change of the secondary structure induced by D-amino acid substitutions could affect the activity [11]. The present study showed that the terminal amide bonds of KSLK were the most susceptible site in the presence of serum, which indicated that exopeptidase in the serum predominantly degraded the peptide. We expected that if D-amino acid substitution did not greatly disturb the secondary structure, the D-amino acid replacement in the cleavage site could increase the stability without affecting the activity because the chirality was not a requirement for antimicrobial activity of membrane-active peptides [12]. As expected, the D-amino acid substitutions at the terminus increased the half-life of the peptide without affecting the activity under serum-free conditions. The N- or C-terminal D-amino acid substitution increased the *ex vivo* serum half-life by 2- and 3-fold, respectively. However, the simultaneous protection of both the N- and the C-terminal increased the half-life by 7-fold. This result indicated that simultaneous protection of cleavage sites by D-amino acid substitution could increase the stability synergistically.

We studied the effect of D-amino acid substitution on the secondary structure of the peptide. Even though the D-amino acid substitutions at the terminal of KSLK decreased the α-helical contents, these substitutions retained the α-helical structure, whereas the incorporation of D-amino acid in the middle of the peptide disrupted the α-helical structure. The decrease of the α-helical contents within 43% did not affect the antimicrobial activity, whereas disruption of the α-helical structure resulted in the complete loss of the activity. This result suggests that the α-helical structure of peptides is the fundamental requirement for their activity; however, it seems that there is some threshold of α-helical structure for activity. If α-helicity of the peptide is over this threshold, the structure is no longer a major factor for the activity. The enantiomer, which had a left-handed α-helical structure, showed the same activity as KSLK. The retained activity of the enantiomer confirmed that the peptide did not form tight interactions with chiral receptors or enzymes or with the chiral components of the lipid membrane.

When heat-inactivated serum was added to the assay medium, the activity of KSLK and its enantiomer against *C. albicans* was decreased greatly. The activity of the diastereomers also was decreased by the addition of the heat-inactivated serum; however, the decrease in activity was much less than those of KSLK and its enantiomer. There are two possible explanations. First, although the serum was inactivated by heating, the heat-stable serum protease can still be active. In this case, diastereomers were degraded less; therefore, they showed a potent activity. However, this suggestion is not enough to explain the fact that the enantiomer, which had the longest half-life in the presence of serum, showed the same activity trend as KSLK in the presence of heat-inactivated serum. Another explanation is that certain components in the serum interact with the peptides and inhibit the binding of the peptides on membranes of pathogen. Peck-Miller *et al.* [25] studied interactions between membrane-active peptides and com-

ponents in the heat-inactivated serum. They reported that the low-density lipoprotein in the serum interacted with the membrane-active peptides and that the α-helical structure of the peptide plays an important role in these interactions, resulting in a decrease of activity. Our results indicated that the local change of chirality or change of secondary structure induced by D-amino acid substitutions may change the decrease of activity by the interactions between some components in the serum and the peptides. Our results also suggested that partial D-amino acid replacement can change the *in vivo* stability of peptides because the cellular absorption, hepatic and renal clearance, and binding of protease to peptides may be affected by the interactions between peptides and some components in the serum. Therefore, diastereomers are a useful tool for the pharmacokinetic study of membrane-active peptides.

In conclusion, D-amino acid substitution(s) at both termini of the membrane-active peptide, which maintained the α-helical structure and the antimicrobial activity, improved the stability of peptides in the presence of serum. Interestingly, the diastereomers with D-amino acid in their termini had more improved activity in the presence of heat-inactivated serum than KSLK and its enantiomer. These results suggested that partial D-amino acid substitutions of membrane-active peptide can improve the *in vivo* activity. We expect that this technique can be utilized for the development of therapeutic agents of membrane-active peptides.

References

1. Saberwal G and Nagaraj R, Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: Facets of their conformational features, structure-function correlations and membrane-perturbing abilities. *Biochim Biophys Acta* **1197:** 109–131, 1994.
2. Boman HG, Peptide antibiotics and their role in innate immunity. *Annu Rev Immunol* **13:** 61–92, 1995.
3. Hancock RE, Falla T and Brown M, Cationic bactericidal peptides. *Adv Microb Physiol* **37:** 135–175, 1995.
4. Westerhoff HV, Juretic D, Hendler RW and Zasloff M, Magainins and the disruption of membrane-linked free-energy transduction. *Proc Natl Acad Sci USA* **86:** 6597–6601, 1989.
5. Walter R, Neidle A and Marks N, Significant differences in the degradation of Pro-Leu-Gly-NH_2 by human serum and that of other species. *Proc Soc Exp Biol Med* **148:** 98–103, 1975.
6. Merrifield RB, Juvvadi P, Andreu D, Ubach J, Boman A and Boman HG. Retro and retroenantio analogs of cecropin-melittin hybrids. *Proc Natl Acad Sci USA* **92:** 3449–3453, 1995.
7. Juretic D, Chen HC, Brown JH, Morell JL, Hendler RW and Westerhoff HV, Magainin 2 amide and analogues. Antimicrobial activity, membrane depolarization and susceptibility to proteolysis. *FEBS Lett* **249:** 219–223, 1989.
8. Westerhoff HV, Hendler RW, Zasloff M and Juretic D, Interactions between a new class of eukaryotic antimicrobial agents and isolated rat liver mitochondria. *Biochim Biophys Acta* **975:** 361–369, 1989.
9. Bessalle R, Kapitkovsky A, Gorea A, Shalit I and Fridkin M, All-D-magainin: Chirality, antimicrobial activity and proteolytic resistance. *FEBS Lett* **274:** 151–155, 1990.
10. Juvvadi P, Vunnam S and Merrifield RB, Synthetic melittin, its enantio, retro, and retroenantio isomers, and selected chimeric analogs: Their antibacterial, hemolytic, and lipid bilayer action. *J Am Chem Soc* **118:** 8989–8997, 1996.
11. Wieprecht T, Dathe M, Schumann M, Krause E, Beyermann M and Bienert M, Conformational and functional study of magainin 2 in model membrane environments using the new approach of systematic double-D-amino acid replacement. *Biochemistry* **35:** 10844–10853, 1996.
12. Hong SY, Oh JE, Kwon MY, Choi MJ, Lee JH, Lee BL, Moon HM and Lee KH, Identification and characterization of novel antimicrobial decapeptides generated by combinatorial chemistry. *Antimicrob Agents Chemother* **42:** 2534–2541, 1998.
13. Fields GB, Tian Z and Barany G, *Synthetic Peptide: A User's Guide*. W. H. Freeman & Co., New York, 1992.
14. Furka A, Sebestyen F, Asgedom M and Dibo G, General method for rapid synthesis of multicomponent peptide mixtures. *Int J Pept Protein Res* **37:** 487–493, 1991.
15. Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM and Knapp RJ, A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* **354:** 82–84, 1991.
16. Chen YH, Yang JT and Martinez HM, Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* **11:** 4120–4131, 1972.
17. Rodriguez-Tudela JL, Berenguer J, Martinez-Sugarez JV and Sanchez R, Comparison of a spectrophotometric microdilution method with RPMI-2% glucose with the National Committee for Clinical Laboratory Standards reference macrodilution method M27-P for *in vitro* susceptibility testing of amphotericin B, flucytosine, and fluconazole against *Candida albicans*. *Antimicrob Agents Chemother* **40:** 1998–2003, 1996.
18. Nakajima R, Kitamura A, Someya K, Tanaka M and Sato K, *In vitro* and *in vivo* antifungal activities of DU-6859a, a fluoroquinolone, in combination with amphotericin B and fluconazole against pathogenic fungi. *Antimicrob Agents Chemother* **39:** 1517–1521, 1995.
19. Murakami K, Nomura K, Doi M and Yoshida T, Production of low-affinity penicillin-binding protein by low- and high-resistance groups of methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* **31:** 1307–1311, 1987.
20. Dathe M, Schumann M, Wieprecht T, Winkler A, Beyermann M, Krause E, Matsuzaki K, Murase O and Bienert M, Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry* **35:** 12612–12622, 1996.
21. Rothemund S, Beyermann M, Krause E, Krause G, Bienert M, Hodges RS, Sykes BD and Sonnichsen FD, Structure effects of double D-amino acid replacements: A nuclear magnetic resonance and circular dichroism study using amphipathic model helices. *Biochemistry* **34:** 12954–12962, 1995.
22. Krause E, Beyermann M, Dathe M, Rothemund S and Bienert M, Location of an amphipathic α-helix in peptides using reversed-phase HPLC retention behavior of D-amino acid analogs. *Anal Chem* **67:** 252–258, 1995.
23. Powell MF, Grey H, Gaeta FCA, Sette A and Colon S, Peptide stability in drug development: A comparison of peptide reactivity in different biological media. *J Pharm Sci* **81:** 731–735, 1992.
24. Powell MF, Stewart T Jr, Otvos L, Urge L, Gaeta FCA, Sette A, Arrhenius T, Thomson D, Soda K and Colon S, Peptide stability in drug development. II. Effect of single amino acid substitution and glycosylation on peptide reactivity in human serum. *Pharm Res* **10:** 1268–1273, 1993.
25. Peck-Miller KA, Darveau RP and Fell HP, Identification of serum components that inhibit the tumoricidal activity of amphiphilic alpha helical peptides. *Cancer Chemother Pharmacol* **32:** 109–115, 1993.