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Rational Design of α-Helical Antimicrobial Peptides with Enhanced Activities and Specificity/Therapeutic Index*

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In the present study, the 26-residue peptide sequence Ac-KWKSFLKTFKSAVKTVLHTALKAISS-amide (V_{681}) was utilized as the framework to study the effects of peptide hydrophobicity/hydrophilicity, amphipathicity, and helicity (induced by single amino acid substitutions in the center of the polar and nonpolar faces of the amphipathic helix) on biological activities. The peptide analogs were also studied by temperature profiling in reversed-phase high performance liquid chromatography, from 5 to 80 °C, to evaluate the self-associating ability of the molecules in solution, another important parameter in understanding peptide antimicrobial and hemolytic activities. A higher ability to self-associate in solution was correlated with weaker antimicrobial activity and stronger hemolytic activity of the peptides. Biological studies showed that strong hemolytic activity of the peptides generally correlated with high hydrophobicity, high amphipathicity, and high helicity. In most cases, the D-amino acid substituted peptides possessed an enhanced average antimicrobial activity compared with L-diastereomers. The therapeutic index of V_{681} was improved 90- and 23-fold against Gram-negative and Gram-positive bacteria, respectively. By simply replacing the central hydrophobic or hydrophilic amino acid residue on the nonpolar or the polar face of these amphipathic derivatives of V_{681} with a series of selected D-/L-amino acids, we demonstrated that this method has excellent potential for the rational design of antimicrobial peptides with enhanced activities.

The extensive clinical use of classical antibiotics has led to the growing emergence of many medically relevant resistant strains of bacteria (1, 2). Moreover, only three new structural classes of antibiotics (the oxazolidinone, linezolid, the streptogramins, and the lipopeptide, daptomycin (3–5)) have been introduced into medical practice in the past 40 years. Therefore, the development of a new class of antibiotics has become critical. The cationic antimicrobial peptides could represent such a new class of antibiotics (6–8). Although the exact mode of action of antimicrobial peptides has not been established, all cationic amphipathic peptides interact with membranes, and it has been proposed that the cytoplasmic membrane is the main target of some peptides, whereby peptide accumulation in the membrane causes increased permeability and loss of barrier function (9, 10). The development of resistance to membrane active peptides whose sole target is the cytoplasmic membrane is not expected because this would require substantial changes in the lipid composition of cell membranes of microorganisms.

Two major classes of the cationic antimicrobial peptides are the α-helical and the β-sheet peptides (6, 7, 11, 12). The β-sheet class consists of cyclic peptides constrained in this conformation either by intramolecular disulfide bonds, *e.g.* defensins (13) and protegrins (14), or by an N-terminal to C-terminal covalent bond, *e.g.* gramicidin S (15) and tyrocidines (16). Unlike the β-sheet peptides, α-helical peptides are linear molecules that mainly exist as disordered structures in aqueous media and become amphipathic helices upon interaction with the hydrophobic membranes, *e.g.* cecropins (17), magainins (18), and melittins (19). From numerous structure/activity studies on both natural and synthetic antimicrobial peptides, a number of factors believed to be important for antimicrobial activity have been identified, including the presence of both hydrophobic and basic residues, an amphipathic nature that segregates basic and hydrophobic residues, and an inducible or preformed secondary structure (α-helical or β-sheet).

The major barrier to the use of antimicrobial peptides as antibiotics is their toxicity or ability to lyse eukaryotic cells. This is perhaps not a surprising result if the target is indeed the cell membrane (6–9). To be useful as a broad spectrum antibiotic, it would be necessary to dissociate anti-eukaryotic activity from antimicrobial activity, *i.e.* increasing the antimicrobial activity and reducing toxicity to normal cells. Recent studies on a number of α-helical and β-sheet peptides have attempted to delineate features responsible for these activities and found that high amphipathicity (20–23), high hydrophobicity (20, 23–25), as well as high helicity or β-sheet structure (23, 26, 27) were correlated with increased toxicity as measured by hemolytic activity. In contrast, antimicrobial activity was found to be less dependent on these factors, compared with hemolytic activity (20–24, 26–28). Therefore, specificity (or therapeutic index, which is defined as the ratio of MHC[1] (he-

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[1] The abbreviations used are: MHC, minimal hemolytic concentration; RP-HPLC, reversed-phase high performance liquid chromatography; MIC, minimal inhibitory concentration; TFE, trifluoroethanol; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

molytic activity) and MIC (antimicrobial activity), MHC/MIC for bacteria over erythrocytes) could be increased in one of the following three ways: increasing antimicrobial activity, decreasing hemolytic activity while maintaining antimicrobial activity, or a combination of both increasing antimicrobial activity and decreasing hemolytic activity.

We believe that a synthetic peptide approach to examining the effect of small incremental changes in hydrophobicity/hydrophilicity, amphipathicity, and helicity of cationic antimicrobial peptides will enable rapid progress in the rational design of peptide antibiotics. Our previous studies (22, 23, 28) have successfully utilized such an approach to dissociate antimicrobial and hemolytic activities of *de novo* designed cyclic β-sheet gramicidin S analogs, by systematic alterations in amphipathicity/hydrophobicity through D-amino acid substitutions. In recent work, we demonstrated that the helix-destabilizing properties of D-amino acids offer a systematic approach to the controlled alteration of the hydrophobicity, amphipathicity, and helicity of amphipathic α-helical model peptides (29). By single substitutions of different D-amino acids into the center of the hydrophobic face of an amphipathic α-helical model peptide, we demonstrated that different D-amino acids disrupted α-helical structure to different degrees, whereas the destabilized structure could still be induced to fold into an α-helix in hydrophobic medium. The advantage of this method of single D- or L-amino acid substitutions at a specific site is that it enables a greater understanding of the mechanism of action of these peptides. In this study, we have utilized the structural framework of an amphipathic α-helical antimicrobial peptide V_{681} (30, 31) to systematically change peptide amphipathicity, hydrophobicity, and helicity by single D- or L-amino acid substitutions in the center of either the polar or nonpolar faces of the amphipathic helix. Peptide V_{681}, with excellent antimicrobial activity and strong hemolytic activity (30, 31), was selected as an ideal candidate for our study. By introducing different D- or L-amino acid substitutions, we report here that hydrophobicity/amphipathicity and helicity have dramatic effects on the biophysical and biological activities, and by utilizing this method, a significant improvement in antimicrobial activity and specificity can be achieved. In addition, it is plausible that high peptide hydrophobicity and amphipathicity also result in greater peptide self-association in solution. Because we have developed a novel method to measure self-association of small amphipathic molecules, namely temperature profiling in reversed-phase chromatography (32, 33), we have applied this technique for the first time to investigate the influence of peptide dimerization ability on biological activities of α-helical antimicrobial peptides.

Thus, our objectives in this study were 3-fold. First was to demonstrate the importance of the peptide self-association parameter in the *de novo* design of amphipathic α-helical antimicrobial peptides. Second was to test the hypothesis that disruption of α-helical structure in benign conditions by D-amino acid substitutions or substitutions of hydrophilic/charged L-amino acids on the nonpolar face can dramatically alter specificity in a similar manner to our previous work on cyclic β-sheet antimicrobial peptides (23, 28). Third was to observe whether these substitutions will enhance antimicrobial activity, decrease toxicity, and improve antimicrobial specificity while maintaining broad spectrum activity for Gram-negative and Gram-positive bacteria.

MATERIALS AND METHODS

Peptide Synthesis and Purification—Syntheses of the peptides Ac-KWKSFLKTFK$X_{D/L}$AVKTVLHTALKAISS-amide and Ac-KWKSFLK-TFKSA$X_{D/L}$KTVLHTALKAISS-amide, with substitution sites at positions 11 and 13, respectively, were carried out by solid phase peptide synthesis using *t*-butyloxycarbonyl chemistry and 4-methylbenzhydrylamine resin (0.97 mmol/g), as described previously (29). The crude peptides were purified by preparative RP-HPLC using a Zorbax 300 SB-C_8 column (250 × 9.4-mm inner diameter; 6.5-μm particle size, 300-Å pore size; Agilent Technologies) with a linear AB gradient (0.2% acetonitrile/min) at a flow rate of 2 ml/min, where mobile phase A was 0.1% aqueous trifluoroacetic acid in water, and B was 0.1% trifluoroacetic acid in acetonitrile. The purity of peptides was verified by analytical RP-HPLC as described below. The peptides were further characterized by electrospray mass spectrometry and amino acid analysis.

Analytical RP-HPLC of Peptides—Peptides were analyzed on an Agilent 1100 series liquid chromatograph (Little Falls, DE). Runs were performed on a Zorbax 300 SB-C_8 column (150 × 2.1-mm inner diameter; 5-μm particle size, 300-Å pore size) from Agilent Technologies using a linear AB gradient (1% acetonitrile/min) and a flow rate of 0.25 ml/min, where solvent A was 0.05% aqueous trifluoroacetic acid, pH 2, and solvent B was 0.05% trifluoroacetic acid in acetonitrile. Temperature profiling analyses were performed in 3 °C increments, from 5 to 80 °C.

Characterization of Helical Structure—The mean residue molar ellipticities of peptides were determined by CD spectroscopy, using a Jasco J-720 spectropolarimeter (Jasco, Easton, MD), at 25 °C under benign (nondenaturing) conditions (50 mM KH_2PO_4/K_2HPO_4, 100 mM KCl, pH 7), hereafter referred to as KP buffer, as well as in the presence of an α-helix inducing solvent, 2,2,2-trifluoroethanol (TFE) (50 mM KH_2PO_4/K_2HPO_4, 100 mM KCl, pH 7 buffer, 50% TFE). A 10-fold dilution of an ~500 μM stock solution of the peptide analogs was loaded into a 0.02-cm fused silica cell and its ellipticity scanned from 190 to 250 nm. The values of molar ellipticities of the peptide analogs at a wavelength of 222 nm were used to estimate the relative α-helicity of the peptides.

CD Temperature Denaturation Study of Peptide V_{681}—The native peptide V_{681} was dissolved in 0.05% aqueous trifluoroacetic acid containing 50% TFE, pH 2, loaded into a 0.02-cm fused silica cell, and peptide ellipticity scanned from 190 to 250 nm at temperatures of 5, 15, 25, 35, 45, 55, 65, and 80 °C. The spectra at different temperatures were used to mimic the alteration of peptide conformation during temperature profiling analysis in RP-HPLC. The ratio of the molar ellipticity (222 nm) at a particular temperature (t) relative to that at 5 °C $([\theta]_t - [\theta]_u)/([\theta]_5 - [\theta]_u)$ was calculated and plotted against temperature in order to obtain the thermal melting profiles, where $[\theta]_5$ and $[\theta]_u$ represent the molar ellipticity values for the fully folded and fully unfolded species, respectively. $[\theta]_u$ was determined in the presence of 8 M urea with a value of 1500 degree·cm^2·dmol^{-1} to represent a totally random coil state (34). The melting temperature (T_m) was calculated as the temperature at which the α-helix was 50% denatured $(([\theta]_t - [\theta]_u)/([\theta]_5 - [\theta]_u) = 0.5)$, and the values were taken as a measure of α-helix stability.

Determination of Peptide Amphipathicity—Amphipathicity of peptide analogs was determined by the calculation of hydrophobic moment (35) using the software package Jemboss version 1.2.1 (36), modified to include a hydrophobicity scale determined in our laboratory. The hydrophobicity scale used in this study is listed as follows: Trp, 33.1; Phe, 30.1; Leu, 24.7; Ile, 22.8; Met, 17.3; Tyr, 16.0; Val, 15.1; Pro, 10.4; Cys, 9.2; His, 4.7; Ala, 4.1; Arg, 4.1; Thr, 4.1; Gln, 1.7; Ser, 1.3; Asn, 1.0; Gly, 0.0; Glu, −0.3; Asp, −0.8; and Lys, −2.0.[2] These hydrophobicity coefficients were determined from reversed-phase chromatography at pH 7 (10 mM Na_2HPO_4 buffer containing 50 mM NaCl) of a model random coil peptide with single substitution of all 20 naturally occurring amino acids. We propose that this HPLC-derived scale reflects the relative differences in hydrophilicity/hydrophobicity of the 20 amino acid side chains more accurately than previously determined scales.

Measurement of Antibacterial Activity—Minimal inhibitory concentrations (MICs) were determined using a standard microtiter dilution method in a modified Luria-Bertani medium with no added salt (LB, composed exclusively 10 g of tryptone and 5 g of yeast extract/liter). Briefly, cells were grown overnight at 37 °C in LB and diluted in the same medium. Serial dilutions of the peptides were added to the microtiter plates in a volume of 100 μl followed by 10 μl of bacteria to give a final inoculum of 5×10^5 colony-forming units/ml. Plates were incubated at 37 °C for 24 h, and MICs were determined as the lowest peptide concentration that inhibited growth.

Measurement of Hemolytic Activity (MHC)—Peptide samples were added to 1% human erythrocytes in phosphate-buffered saline (0.08 M NaCl, 0.043 M Na_2HPO_4, 0.011 M KH_2PO_4), and reactions were incubated at 37 °C for 12 h in microtiter plates. Peptide samples were diluted 2-fold in order to determine the concentration that produced no

[2] J. Kovacs, C. T. Mant, and R. S. Hodges, unpublished data.

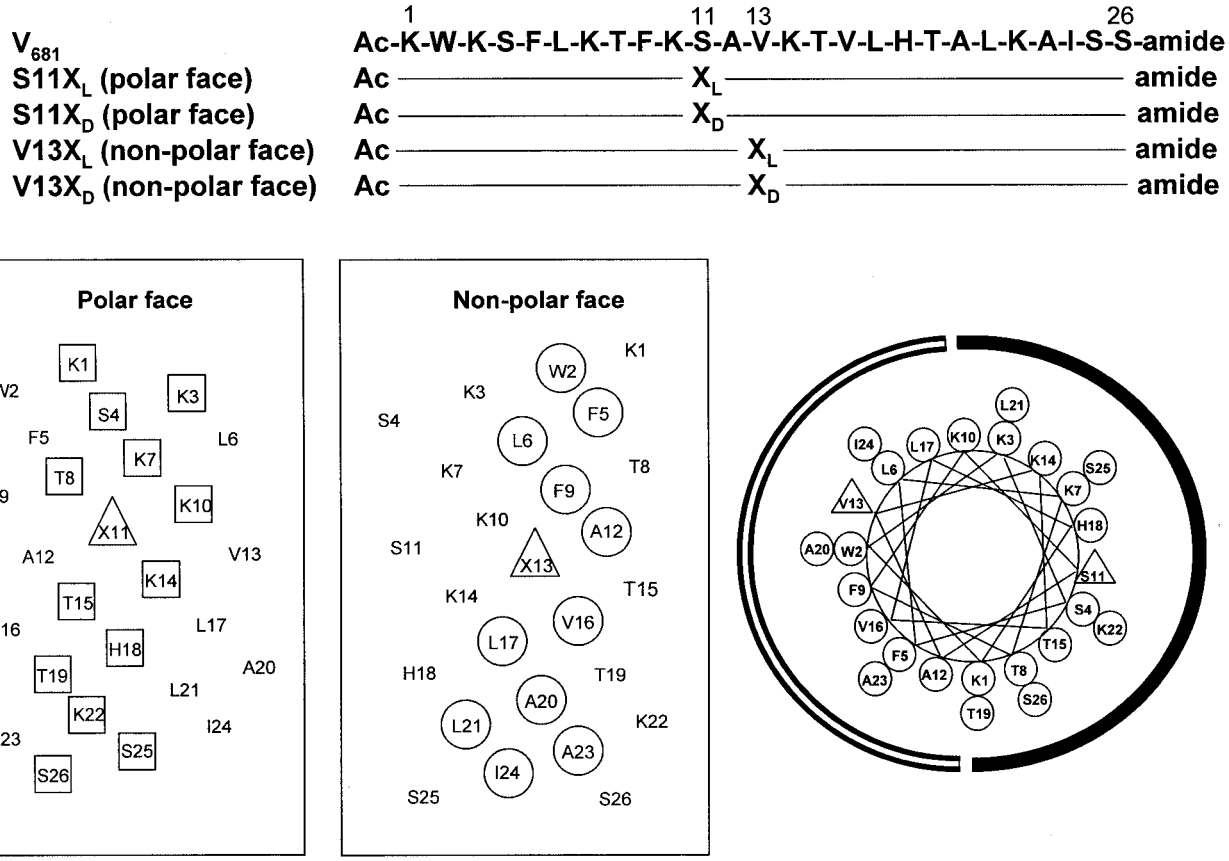

FIG. 1. **Representation of the "host" peptide V_{681} as helical nets showing the polar/hydrophilic face (*boxed residues*) and nonpolar/hydrophobic face (*circled residues*) and helical wheel and the sequences of the synthetic peptide analogs used in this work.** The hydrophobic face is indicated as an *open arc*, and the hydrophilic face is shown as a *solid arc* in the helical wheel. The substitution sites at positions 11 and 13 are in *triangles* on the polar face and the nonpolar face. S11X_L and S11X_D denote polar face substitutions of Ser-11. V13X_L and V13X_D denote nonpolar face substitutions of Val-13. *Ac* denotes N^{α}-acetyl, and amide denotes C^{α}-amide. One-letter codes are used for the amino acid residues.

hemolysis. This determination was made by withdrawing aliquots from the hemolysis assays, removing unlysed erythrocytes by centrifugation (800 × *g*), and determining which concentration of peptide failed to cause the release of hemoglobin. Hemoglobin release was determined spectrophotometrically at 562 nm. The hemolytic titer was the highest 2-fold dilution of the peptide that still caused release of hemoglobin from erythrocytes. The control for no release of hemoglobin was a sample of 1% erythrocytes without any peptide added. Because erythrocytes were in an isotonic buffer, no detectable release (<1% of that released upon complete hemolysis) of hemoglobin was observed from this control during the course of the assay.

Calculation of Therapeutic Index (MHC/MIC Ratio)—It should be noted that both the MHC and MIC values are carried out by serial 2-fold dilutions; thus, for individual bacteria and individual peptides, the therapeutic index (MHC/MIC) could vary as much as 4-fold if the peptide is very active in both hemolytic and antimicrobial activities. However, if there is no detectable hemolytic activity, then the maximum possible error in the therapeutic index would be only 2-fold from variations in the antimicrobial activity. When there was no detectable hemolytic activity at 250 μg/ml, a minimal hemolytic concentration of 500 μg/ml was used to calculate the therapeutic index.

RESULTS AND DISCUSSION

Design and Characterization of Peptide Analogs

Peptide Design—Peptide V_{681}, a 26-residue amphipathic antimicrobial peptide with a polar and nonpolar face (30), was selected as the parent peptide in this study (Fig. 1). Its polar face consisted of 14 residues: six lysine residues, one histidine, four serines, and three threonines. In contrast, the nonpolar face consisted of 12 residues: three alanines, two valines, three leucines, two phenylalanines, one isoleucine, and one tryptophan residue. In this study, we chose D-/L-amino acid substitution sites at the center of the hydrophobic face (position 13) and at the center of the hydrophilic face (position 11) of the helix, such that these substitution sites were also located in the center of the overall peptide sequence. This was based on our previous model peptide studies (29, 34, 37) that demonstrated that these central location substitutions had the greatest effect on peptide secondary structure. To study the effects of varying hydrophobicity/hydrophilicity on peptide biological activities, in the design of V_{681} analogs, five L-amino acids (Leu, Val, Ala, Ser, and Lys) and Gly were selected out of the 20 natural amino acids as the substituting residues, representing a wide range of hydrophobicity. The hydrophobicity of these six amino acid residues decreased in the order Leu > Val > Ala > Gly > Ser > Lys (29). Based on the relative hydrophobicity of amino acid side chains (29), leucine was used to replace the native valine on the nonpolar face to increase peptide hydrophobicity and amphipathicity. Alanine was selected to reduce peptide hydrophobicity/amphipathicity while maintaining high helicity. A hydrophilic amino acid, serine, was selected to decrease the hydrophobicity/amphipathicity of V_{681} in the nonpolar face. Positively charged lysine was used to decrease further peptide hydrophobicity and amphipathicity. In contrast, the same amino acid substitutions on the polar face would have different effects on the alteration of hydrophobicity/hydrophilicity and amphipathicity, because the native amino acid residue is serine on the polar face of V_{681}. As a result, on the polar face leucine, valine, and alanine were used to increase peptide hydrophobicity as well as to decrease the amphipathicity of V_{681}, whereas lysine was selected to increase peptide hydrophilicity and am-

TABLE I
Circular dichroism data of V_{681} peptide analogs

Peptides[a]	Benign[b]				50% TFE[c]			
	X_L[d]		X_D[d]		X_L[d]		X_D[d]	
	$[\theta]_{222}$	% helix[e]	$[\theta]_{222}$	% helix[e]	$[\theta]_{222}$	% helix[e]	$[\theta]_{222}$	% helix[e]
V13L	−20,600	71	−7,350	25	−28,250	98	−25,750	89
V13V[f]	−12,900	45	−2,800	10	−27,300	94	−26,000	90
V13A	−3,450	12	−2,850	10	−28,950	100	−24,650	85
V13S	−1,300	4	−1,700	6	−27,550	95	−22,200	77
V13K	−1,450	5	−2,000	7	−26,250	91	−23,600	82
V13G	−2,250	8			−24,350	84		
S11L	−10,850	37	−2,950	10	−28,550	99	−26,100	90
S11A	−13,600	47	−3,050	11	−27,600	95	−27,300	94
S11S[f]	−12,900	45	−2,800	10	−27,300	94	−26,000	90
S11V	−7,550	26	−2,400	8	−23,050	80	−20,800	72
S11K	−5,950	21	−2,500	9	−27,350	94	−27,800	96
S11G	−4,550	16			−25,950	90		

[a] Peptides are ordered by relative hydrophobicity to the parent peptide V_{681} at 5 °C (Fig. 1).
[b] The mean residue molar ellipticities, $[\theta]_{222}$ (degree·cm^2·dmol^{-1}) at wavelength 222 nm were measured at 25 °C in KP buffer (100 mM KCl, 50 mM PO_4, pH 7.0).
[c] The mean residue molar ellipticities (degree·cm^2·dmol^{-1}) at wavelength 222 nm were measured at 25 °C in the KP buffer diluted 1:1 (v/v) with TFE.
[d] X_L and X_D denote the L- and D-substitutions, respectively.
[e] The helical content (in percentage) of a peptide relative to the molar ellipticity value of peptide $V13A_L$ in 50% TFE.
[f] $V13V_L$ and $S11S_L$ are the same peptide, which is the parent peptide V_{681}.

phipathicity. In previous studies, Kondejewski and co-workers (23, 38) and Lee *et al.* (28) successfully utilized D-amino acid substitutions to dissociate the antimicrobial activity and hemolytic activity of the cyclic β-sheet gramicidin S analogs. In the present study, D-enantiomers of the five L-amino acid residues were also incorporated at the same positions on the nonpolar/polar face of V_{681} to change not only peptide hydrophobicity/hydrophilicity and amphipathicity but, more importantly, to disrupt peptide helical structure. Because glycine does not exhibit optical activity and has no side chain, the Gly-substituted analog was used as a reference for diastereomeric peptide pairs.

Because all peptide analogs were made based on a single amino acid substitution in either the polar or nonpolar faces of V_{681}, peptides were divided into two categories, V13*X* peptides (nonpolar face substitutions) and S11*X* peptides (polar face substitutions). Each peptide was named after the substituting amino acid residue, *e.g.* the peptide analog with L-leucine substitution on the nonpolar face of V_{681} is called $V13L_L$. It is important to note that because the L-valine of the nonpolar face and L-serine of the polar face are the original amino acid residues in the V_{681} sequence (Fig. 1), peptide analogs $V13V_L$ and $S11S_L$ are the same peptide as V_{681}.

A control peptide (peptide C) designed to exhibit negligible secondary structure, *i.e.* a random coil, was employed as a standard peptide for temperature profiling during RP-HPLC to monitor peptide dimerization. As shown in the previous study (32), this 18-residue peptide, with the sequence of Ac-ELEKGGLEGEKGGKELEK-amide, clearly exhibited negligible secondary structure, even in the presence of the strong α-helix inducing properties of 50% TFE and at the low temperature of 5 °C ($[\theta]_{222}$ = −3,950).

Structure of Peptide Diastereomers—To determine the secondary structure of peptides in different environments, CD spectra of the peptide analogs were measured under benign (nondenaturing) pH and ionic strength (100 mM KCl, 50 mM aqueous KH_2PO_4/K_2HPO_4, pH 7, referred to as KP buffer) and also in 50% TFE to mimic the hydrophobic environment of the membrane. The parent peptide, V_{681}, exhibited low α-helical content in KP buffer, *i.e.* $[\theta]_{222}$ of −12,900 compared with −27,300 in 50% TFE, an increase in α-helical content from 45 to 94%, respectively (Table I). From Table I, in KP buffer, D-amino acid substituted peptides generally exhibited considerably less α-helical structure compared with their L-diastereomers. The negligible secondary structure characteristics of the D-peptides underlines the helix-disrupting properties of a single D-amino acid substitution, as demonstrated in our previous model α-helical peptide study (29). On the nonpolar face, the native L-Val residue was critical in maintaining α-helical structure. Substitution of L-Val with less hydrophobic amino acids (L-Ala, Gly, L-Ser, and L-Lys) dramatically decreased the α-helical structure ($V13V_L$, $[\theta]_{222}$ of −12,900 to values ranging from −1,300 to −3,450 for $V13S_L$, $V13K_L$, V13G, and $V13A_L$) (Table I). Even the substitution with L-Ala, which is known to have the highest α-helical propensity of all 20 amino acids (37), could not stabilize the α-helical structure. This shows the importance of hydrophobicity on the nonpolar face in maintaining the α-helical structure. In contrast, substitution with a more hydrophobic amino acid (L-Leu for L-Val) on the nonpolar face significantly increased α-helical structure ($[\theta]_{222}$ for peptide $V13L_L$ of −20,600 compared with peptide $V13V_L$ of −12,900). It is noteworthy that on the nonpolar face, the magnitude of the helical content of L-peptides in KP buffer was related to the hydrophobicity of the substituting amino acids, *i.e.* $V13L_L > V13V_L > V13A_L > V13S_L = V13K_L$, again showing the importance of hydrophobicity on the nonpolar face in maintaining the α-helical structure. Because of their helix-disruptive ability on the nonpolar face, the D-amino acid substitutions D-Val and D-Leu dramatically decreased α-helical structure in KP medium compared with their L-counterparts. Regardless of the helix-disrupting properties of L- or D-substitutions made on the nonpolar face, high helical structure could be induced by the nonpolar environment of 50% TFE, a mimic of the hydrophobicity and α-helix inducing ability of the membrane (Table I). From Table I, it is clear that although D-amino acid substituted peptides were strongly induced into helical structure in 50% TFE, they were still generally less helical than the L-diastereomers, indicating that D-substitutions were still destabilizing of α-helical structure compared with their L-diastereomers in a hydrophobic environment.

In this study, the L-substitutions on the polar face in benign medium had dramatically different effects on α-helical structure than the same substitutions on the nonpolar face. For example, $V13L_L$ ($[\theta]_{222}$ of −20,600) differed from $S11L_L$ ($[\theta]_{222}$

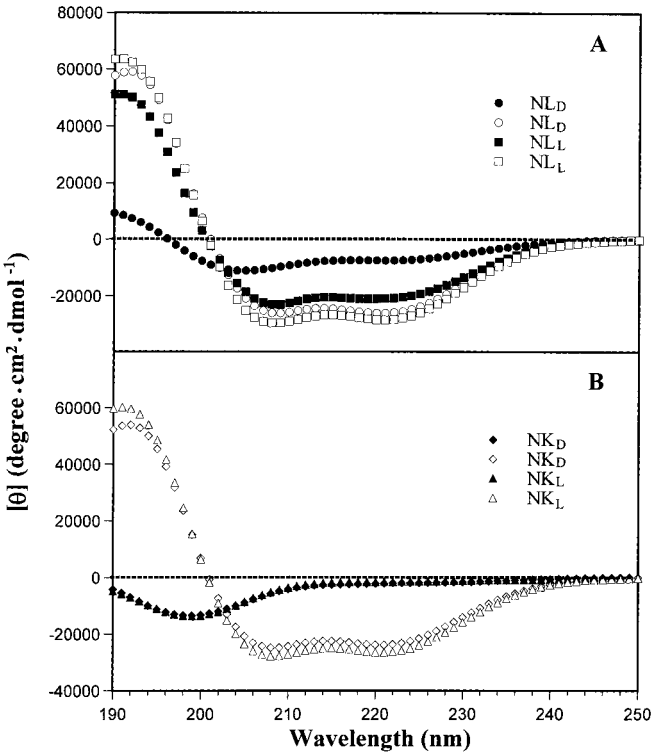

FIG. 2. **CD spectra of peptides $V13L_D$ and $V13L_L$ (*panel A*) and peptides $V13K_D$ and $V13K_L$ (*panel B*) at pH 7 and 25 °C in 50 mM aqueous PO_4 containing 100 mM KCl.** *Panels A* and *B*, *solid symbols* represent the CD spectra of peptide analogs in KP buffer without TFE, and *open symbols* represent CD spectra obtained in the presence of 50% TFE. The symbols used are as follows: *circle* for $V13L_D$ and *square* for $V13L_L$ in *panel A*; *diamond* for $V13K_D$ and *triangle* for $V13K_L$ in *panel B*.

−10,850), indicating that Leu stabilized α-helical structure on the nonpolar face and destabilized α-helical structure on the polar face. Similarly, Val destabilized α-helical structure on the polar face; on the other hand, Ala and Ser destabilized helical structure on the nonpolar face, whereas Ala and Ser stabilized the α-helical structure when substituted in the polar face, compared with the other amino acid substitutions. Taken together, even though Ala had the highest α-helical propensity of all amino acids (37), its α-helical propensity could not overcome the need for hydrophobicity on the nonpolar face ($[\theta]_{222}$ for peptides $V13A_L$, −3,450, and $V13L_L$, −20,600), whereas on the polar face, peptide $S11A_L$ exhibited high helical structure in KP buffer ($[\theta]_{222}$ −13,600) in contrast to peptide $S11L_L$ ($[\theta]_{222}$ −10,850) (Table I). It is noteworthy that Val and Leu substitutions on the polar face decreased the amphipathicity of the helix as well as increased the hydrophobicity; however, the lower helical content compared with the native $S11S_L$ indicated that there should be a balance of amphipathicity and hydrophobicity to enhance the helical content. Similar to the substitutions on the nonpolar face, all D-amino acid substitutions on the polar face were destabilizing to α-helical structure in KP medium; however, high helical structure could be induced by adding 50% TFE. As shown in Table I, nonpolar face substitutions exhibited a greater range of molar ellipticity values in KP buffer than polar face analogs, demonstrating that the amino acid residues on the nonpolar face of the helix played a more important role in peptide secondary structure than those on the polar face. As expected, Gly was destabilizing to α-helical structure whether on the nonpolar or polar face because of its low α-helical propensity (37).

Fig. 2 shows the CD spectra of the most and the least hydrophobic substitutions on the nonpolar face. In KP buffer, peptide $V13L_D$ showed much less helical structure than $V13L_L$ because of the helix-destabilizing ability of the D-amino acid, whereas in 50% TFE, both peptides could be induced to a fully helical structure (Fig. 2, *panel A*). In contrast, in KP buffer peptides $V13K_L$ and $V13K_D$ were random coils, because of the combined effects of decreasing hydrophobicity and amphipathicity by replacing the native L-Val with D-Lys or L-Lys on the nonpolar face; again, in 50% TFE, both of them were induced into highly helical structures, even though peptide $V13K_L$ demonstrated slightly more helical content than peptide $V13K_D$ (Fig. 2, *panel B*).

Peptide Self-association

Helix Stability of Peptide V_{681} in a Hydrophobic Environment—We wanted to use temperature profiling during RP-HPLC to determine the self-association ability of the various analogs of V_{681}, which would occur through interaction of the nonpolar faces of these amphipathic α-helices. Thus, it was initially important to determine the helicity and stability of the native peptide V_{681} in a hydrophobic environment such as that characteristic of RP-HPLC. By using model amphipathic α-helical peptides with all 20 amino acid substitutions in the center of the nonpolar face, we showed previously that the model amphipathic peptides were maximally induced into an α-helical structure in 40% TFE and that the stability of the α-helix during temperature denaturation was dependent on the substitution (29). In order to investigate the stability of V_{681} in a hydrophobic environment, we carried out a temperature denaturation study in solution, as monitored by circular dichroism spectroscopy. We used 50% aqueous TFE in 0.05% trifluoroacetic acid to mimic the hydrophobic conditions in the reversed phase column because the hydrophobic environment of a reversed phase column (hydrophobic stationary phase and the nonpolar organic solvent in the mobile phase) could induce α-helical structure in a similar manner to TFE. Fig. 3, *panel A,* shows the change of V_{681} helical conformation over the temperature range from 5 to 80 °C in the nonpolar medium. At 5 °C, 50% TFE induced full α-helical structure of V_{681}. During the temperature denaturation, the helical content of V_{681} decreased with increasing temperature, and even at 80 °C V_{681} remained significantly α-helical. Fig. 3, *panel B,* shows the stability profile of V_{681} with a transition temperature T_m of 79.3 °C, where T_m is defined as the temperature when 50% of α-helical structure is denatured compared with the fully folded conformation of the peptide in 50% TFE at 5 °C. These data support the view that, during temperature profiling in RP-HPLC, the peptides are fully helical at low temperatures such as 5 °C and can remain at least partially α-helical at 80 °C in solution during partitioning in RP-HPLC. In addition, because of their hydrophobic preferred binding domains, the peptides will remain α-helical when bound to the hydrophobic matrix. Overall, these results suggest that V_{681} is a very stable α-helical peptide in a nonpolar environment, whether it is in solution (such as 50% TFE), under the conditions of RP-HPLC, or from a physiological point of view, in the hydrophobic environment of the biomembrane.

Effect of L-/D-Amino Acid Substitutions on RP-HPLC Retention Behavior of Peptides—L- or D-amino acid substitutions in the nonpolar or polar face of the native V_{681} molecule may have a profound influence on the effective hydrophobicity of this peptide as monitored by RP-HPLC. It is well documented that the formation of a hydrophobic binding domain due to peptide secondary structure can affect peptide interactions with reversed phase matrices; this effect was observed particularly for amphipathic α-helical peptides (29, 39–42). Indeed, Zhou *et al.* (42) clearly demonstrated that, because of this preferred bind-

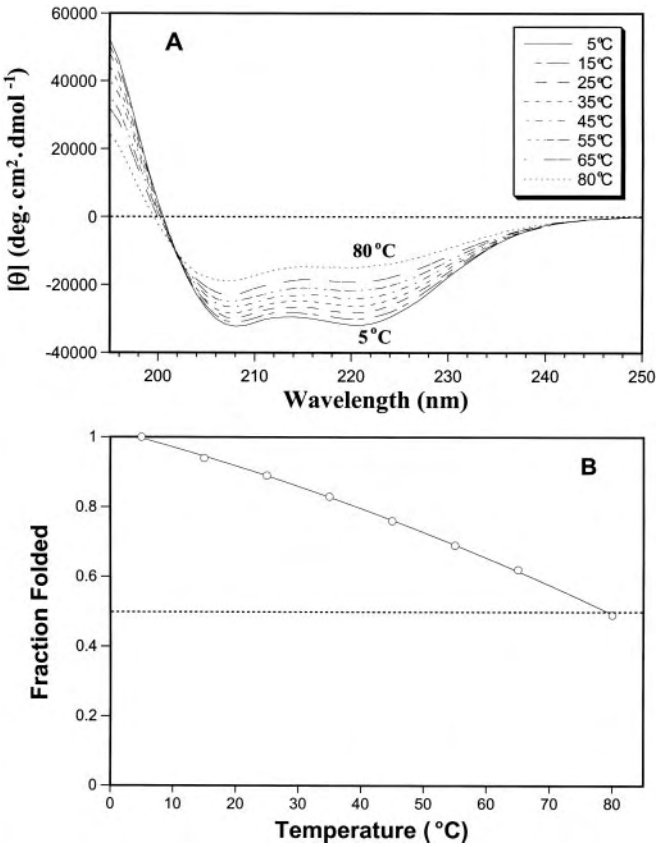

FIG. 3. **CD temperature denaturation of peptide V_{681}.** *Panel A*, the experiment was carried out in 0.05% aqueous trifluoroacetic acid, pH 2, in the presence of 50% TFE. CD spectra at different temperatures are shown as different lines in the figure. *Panel B*, stability plot of peptide V_{681} during CD temperature denaturation.

ing domain, amphipathic α-helical peptides are considerably more retentive than nonamphipathic peptides of the same amino acid composition. In addition, as noted above, the chromatography conditions characteristic of RP-HPLC (hydrophobic stationary phase and nonpolar eluting solvent) are able to induce and stabilize helical structure in potentially helical polypeptides (42–44). From Fig. 1, it can be seen that the substitution site at position 13, in the center of the nonpolar face of the helix, ensures a maximal effect on the intimate interaction of the substituting side chain with the reversed-phase stationary phase; thus, any differences in effective hydrophobicity via amino acid substitutions in the preferred binding domain can be readily monitored through consequent differences in RP-HPLC retention time. The retention time data for the peptides is shown in Table II, which records retention times at 5 °C, the maximal retention times and retention times at 80 °C during the temperature profiling. Temperatures of 5 and 80 °C were the lower and upper temperature limits of temperature profiling in RP-HPLC, representing dimerization of the peptides at 5 °C and the monomerization of peptides at 80 °C because of dissociation of the dimers. The maximal retention times represent the threshold points where peptides transform from dimeric to monomeric form. The retention profiles from 5 to 80 °C are shown in Fig. 5. Among the nonpolar face substituted peptides, peptides with more hydrophobic substitutions (whether L- or D-amino acid substitutions) were more retained during RP-HPLC, *i.e.* peptides were eluted in the order of Lys, Gly, Ser, Ala, Val, and Leu (Table II). In addition, on the nonpolar face, the L-analogs were always more retained than the D-diastereomers (Table II and Fig. 5). Because the aforementioned preferred binding domain of amphipathic helices is actually the nonpolar face of the helix, D-peptides had a smaller preferred binding domain compared with L-diastereomers, because of the helix-disruptive ability of D-amino acids, resulting in lower retention times during RP-HPLC. In contrast, on the polar face the elution order of peptides was not correlated with the order of amino acid side chain hydrophobicity, *e.g.* $S11A_L$ and $S11S_L$ were more retained than $S11V_L$ (Table II); $S11S_D$ was the most retained peptide among the D-amino acid substituted analogs on the polar face (Table II). Indeed, on the polar face peptides $S11L_L$ and $S11A_L$, with the replacement of L-Ser by L-Leu or L-Ala, had increased overall hydrophobicity as revealed by higher retention times compared with V_{681}. Although amino acid L-Val is much more hydrophobic than L-Ser, the observation that peptide $S11V_L$ was less retained than the native peptide V_{681} (with L-Ser at position 11 of the polar face) could be attributed to the helix-disrupting characteristics of the β-branched Val residue (also see Table I). In contrast, at 80 °C, $S11V_L$ became better retained than $S11S_L$. Because of the unfolding of the helical structure at high temperature, the side chain hydrophobicity of the substituting amino acid in the peptide plays a more important role in the overall hydrophobicity. In a similar manner to the nonpolar face substituted peptides, peptides with D-amino acids substituted into the polar face were dramatically less retained than their L-diastereomers. Because of the effect of the preferred binding domain, peptides with substitutions on the nonpolar face had a greater retention time range than those with polar face substitutions, *e.g.* 11.31 min for the L-peptides with nonpolar face substitutions *versus* 2.40 min for the L-peptides with polar face substitutions at 5 °C, and 11.05 *versus* 3.27 min for the D-peptides with nonpolar or polar face substitutions, respectively, at 5 °C (Table II).

Determination of Relative Hydrophobicity by RP-HPLC—Elution times during RP-HPLC have frequently been utilized as a measure of relative hydrophobicity of peptide analogs (29, 34). In the current study, peptide analogs differed only by a single amino acid substitution on either the nonpolar face or the polar face of V_{681}; thus, the retention time data in Table II can be considered to reflect the hydrophobicity difference between peptide analogs. In order to more easily visualize the variation in hydrophobicity of the peptide analogs, the retention time data in Table II were normalized relative to that of the native peptide V_{681} at 5 and 80 °C, respectively. Hydrophobicity relative to the native peptide V_{681} indicates an increase or decrease of the apparent peptide hydrophobicity with the different amino acid substitutions on the polar or nonpolar face. Again, from Table II and Fig. 5, for nonpolar face substituted peptides, there was a wide range of peptide hydrophobicity in the order L-Leu > L-Val > L-Ala > L-Ser > Gly > L-Lys at both 5 and 80 °C. On both the nonpolar and polar faces, the relative hydrophobicities of the D-peptides were always less than their L-diastereomers, indicating that the helix-disrupting characteristic of D-amino acids also leads to disruption of the preferred binding domain of the helices. On both nonpolar and polar faces, peptides exhibited a greater retention time range at 80 °C than at 5 °C, also indicating that due to the unfolding of the helical structures at 80 °C, the side chain hydrophobicity of the substituted amino acids played a more essential role in determining the overall hydrophobicity of the peptide analogs.

The hydrophobicity/hydrophilicity effects of substitutions on the nonpolar face relative to the native peptide V_{681} were large. For example, $V13V_L$ to $V13A_L$, to $V13S_L$, and to $V13K_L$ resulted in decreases in hydrophobicity of −4.45, −8.21, and −12.61 min at 80 °C, respectively (Table II). In fact, the same substitutions, *i.e.* $S11V_L$ to $S11A_L$, to $S11S_L$, and to $S11K_L$, resulted in overall hydrophobicity changes of the peptide by +0.45,

TABLE II
Relative hydrophobicity and association ability of peptide analogs during RP-HPLC temperature profiling

Peptides[a] non-polar[e]	t_R (min)[b]			Δt_R (X-V13V$_L$) (min)[c]		P_A (min)[d]
	5 °C	Max	80 °C	5 °C	80 °C	
V13L$_L$	48.16	50.45	47.02	0.06	1.19	4.22
V13V$_L$[f]	48.10	49.99	45.83	0	0	3.63
V13A$_L$	43.94	44.88	41.38	−4.16	−4.45	2.59
V13S$_L$	40.72	41.08	37.62	−7.38	−8.21	1.82
V13K$_L$	36.85	36.91	33.22	−11.25	−12.61	1.10
V13G	39.96	40.22	36.74	−8.14	−9.09	1.64
V13L$_D$	45.10	46.37	43.03	−3.00	−2.80	3.02
V13V$_D$	42.55	43.43	40.15	−5.55	−5.68	2.63
V13A$_D$	40.49	41.01	38.00	−7.61	−7.83	2.19
V13S$_D$	37.02	37.12	34.08	−11.08	−11.75	1.46
V13K$_D$	34.05	34.05	30.96	−14.05	−14.87	1.10
Polar[e]	t_R (min)[b]			Δt_R (X-S11S$_L$) (min)[c]		P_A (min)[d]
	5 °C	Max	80 °C	5 °C	80 °C	
S11L$_L$	48.78	51.23	47.51	0.68	1.68	4.33
S11A$_L$	48.25	50.57	46.63	0.15	0.80	4.15
S11S$_L$[f]	48.10	49.99	45.83	0	0	3.63
S11V$_L$	47.83	49.93	46.18	−0.27	0.35	3.91
S11K$_L$	46.38	47.90	43.89	−1.72	−1.94	3.17
S11G	45.86	47.09	43.07	−2.24	−2.76	2.82
S11S$_D$	45.47	46.60	42.59	−2.63	−3.24	2.73
S11A$_D$	45.19	46.36	42.57	−2.91	−3.26	2.82
S11L$_D$	44.73	45.85	42.14	−3.37	−3.69	2.82
S11V$_D$	42.96	43.83	40.51	−5.14	−5.32	2.54
S11K$_D$	42.20	42.87	39.29	−5.90	−6.54	2.23
C[g]	22.74		18.64			

[a] Peptides are ordered by relative hydrophobicity to the native L-Val substituted analog on the non-polar face and L-Ser substituted analog on the polar face.
[b] t_R (min) denotes the retention times at 5 °C, the maximal retention times, and the retention times at 80 °C during the temperature profiling.
[c] Values denote the difference of retention time relative to that of the parent peptide V$_{681}$ (V13V$_L$ for the non-polar face substitutions and S11S$_L$ for the polar face substitutions), representing the relative hydrophobicity of the peptide analogs.
[d] P_A denotes the association parameter of each peptide during the RP-HPLC temperature profiling, which is the maximal retention time difference of (($t_R{}^t - t_R{}^5$ for peptide analogs) − ($t_R{}^t - t_R{}^5$ for control peptide C)) within the temperature range, and ($t_R{}^t - t_R{}^5$) is the retention time difference of a peptide at a specific temperature (t) compared with that at 5 °C.
[e] Non-polar and polar denote the amino acid substituted on either the non-polar face or the polar face of the amphipathic parent peptide V$_{681}$ (see Fig. 1).
[f] V13V$_L$ and S11S$_L$ are the same peptide, which is the parent peptide V$_{681}$.
[g] Peptide C is a random coil control used to calculate P_A values; see footnote *d*.

−0.35, and −2.29 min at 80 °C, respectively. This indicates that the polar face substitutions affected overall hydrophobicity of the peptide in a minor way relative to substitutions on the nonpolar face. In fact, the effect was 10 times less for Ala, >20 times less for Ser, and >5 times less for Lys.

Determination of Peptide Self-association by RP-HPLC Temperature Profiling—We believe that peptide self-association (*i.e.* the ability to dimerize) in aqueous solution is a very important parameter to understand antimicrobial activity. Since its introduction, the technique of RP-HPLC temperature profiling to monitor molecule self-association has been applied to several types of molecules, including cyclic β-sheet peptides (33), monomeric α-helices, and α-helices that dimerize (32), as well as α-helices that dimerize to form coiled-coils (45).

It is well accepted that the amphipathicity of antimicrobial peptides is necessary for their mechanism of action, because the positively charged polar face will help the molecules reach the biomembrane through electrostatic interaction with the negatively charged head groups of phospholipids, and then the nonpolar face of the peptides will allow insertion into the membrane through hydrophobic interactions, causing increased permeability and loss of barrier function of target cells (9, 10). If the self-association ability of a peptide in aqueous media is too strong (forming dimers and burying the nonpolar face), it could decrease the ability of the peptide to dissociate and penetrate into the biomembrane and to kill target cells.

Fig. 4 shows the RP-HPLC elution profiles of the random coil control peptide C, peptide V13K$_L$, and peptide V13L$_L$ at different temperatures. These examples were chosen to demonstrate the retention behavior with increasing temperature of a peptide with high association ability (V13L$_L$), low association ability (V13K$_L$), and no association ability (peptide C). Fig. 5 shows the temperature profiling of all L-/D-amino acid substituted peptide analogs during RP-HPLC from 5 and 80 °C.

Although peptides are eluted from a reversed-phase column mainly by an adsorption/desorption mechanism (46), even a peptide strongly bound to a hydrophobic stationary phase will partition between the matrix and the mobile phase when the acetonitrile content becomes high enough during gradient elution. At low temperatures, peptides exist in a dimer-monomer equilibrium during RP-HPLC partitioning, with the dimeric unbound state favored and dissociation required for rebinding; thus, the retention times are relatively low. With the increase of temperature, equilibrium is shifted toward the monomeric form in solution because of the disruption of the dimer. The higher solution concentration of monomer during partitioning increases the on-rate for the bound state, and the retention time therefore increases. It should be noted that the increased temperature also introduces other general effects on retention time because of lower mobile phase viscosity and a significant increase in mass transfer between the stationary phase and mobile phase. These effects decrease retention time with increasing temperature in a linear fashion, as shown for the random coil control peptide C (Fig. 5). Conversely, for the

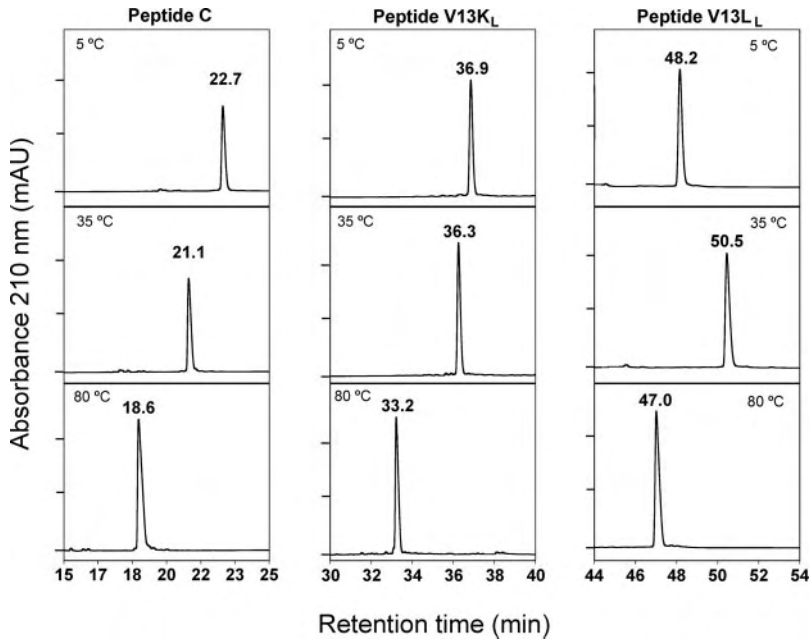

FIG. 4. **Effect of temperature on RP-HPLC profiles of peptide C, peptide V13K$_L$, and V13L$_L$.** Conditions are as follows: RP-HPLC, narrow-bore SB-C$_8$ column (150 × 2.1-mm inner diameter; 5-μm particle size, 300-Å pore size), linear A-B gradient (1% acetonitrile/min) at a flow rate of 0.25 ml/min, where eluent A is 0.05% aqueous trifluoroacetic acid and eluent B is 0.05% trifluoroacetic acid in acetonitrile. Only RP-HPLC profiles of peptide C, peptide V13K$_L$, and peptide V13L$_L$ at 5, 35, and 80 °C were selected as examples to show the temperature effect.

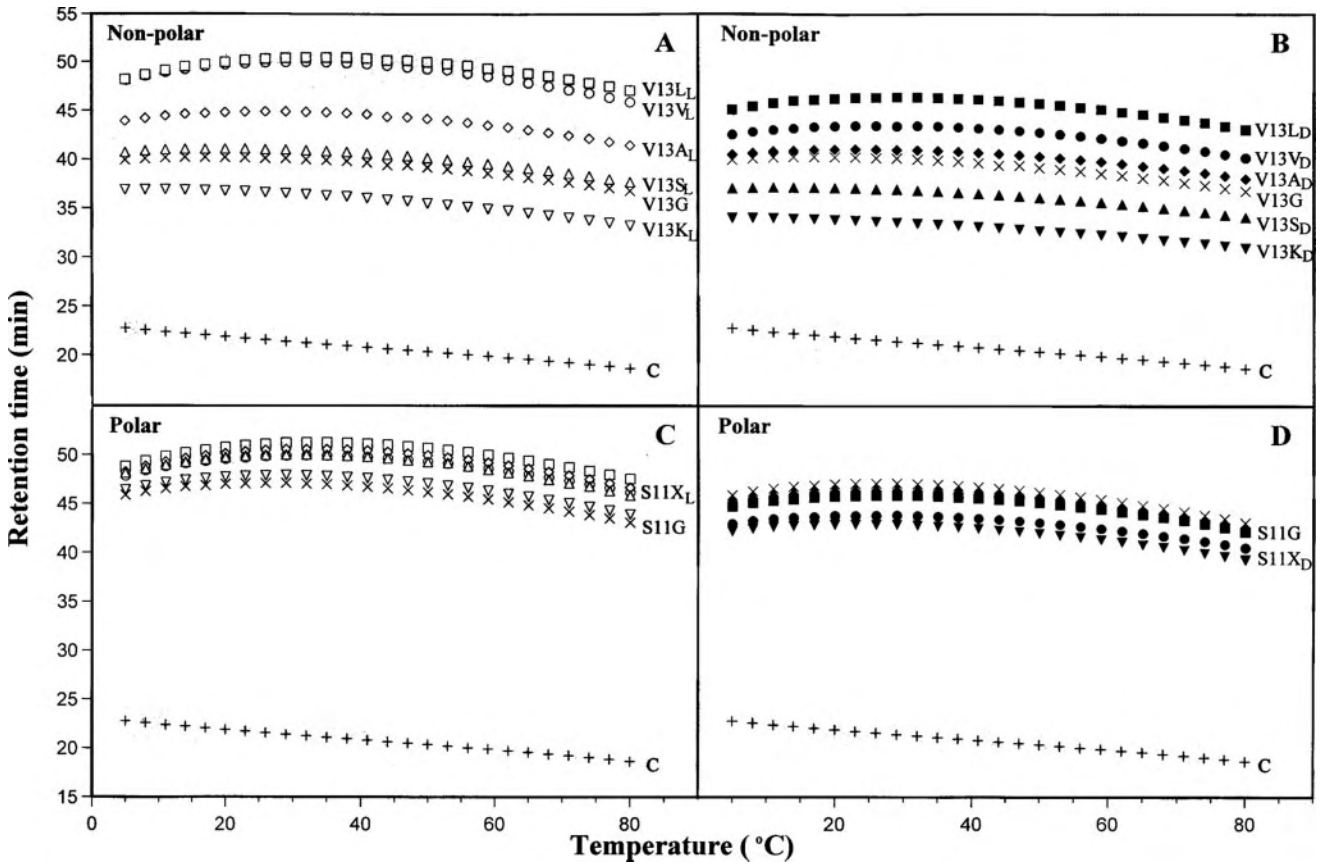

FIG. 5. **RP-HPLC temperature profiles of peptide V$_{681}$ and its analogs.** Column and conditions are as in Fig. 4. Retention data have been collected in 3 °C increments within the temperature range of 5–80 °C. *Open symbols* represent the temperature profiles of L-amino acid substituted peptides on either the nonpolar or polar face of V$_{681}$ (*panels A* and *C*); *solid symbols* represent the temperature profiles of D-amino acid substituted peptides on either the nonpolar or polar face of V$_{681}$ (*panels B* and *D*). In all panels, the substituting amino acids used in either the nonpolar or polar face of V$_{681}$ are Val (*circle*), Leu (*square*), Ala (*diamond*), Ser (*triangle*), Lys (*inverted triangle*), and Gly (×). The temperature profile of the random coil control peptide (*C*) is also shown in the figure (+).

dimerized peptides, at a given temperature dimers are disrupted and converted to monomers, and the retention time reaches the maximal value. Above this critical temperature, one will observe a decrease in retention time with increasing temperature because of the low mobile phase viscosity and increase in mass transfer. In addition, the above described temperature-induced conformational changes, as monitored by CD, may also have an impact by decreasing the retention time with increasing temperature, largely because of the destabilization of peptide α-helical structure and loss of preferred binding domain at high temperatures. To eliminate these general effects during RP-HPLC, the data from Fig. 5 were normalized

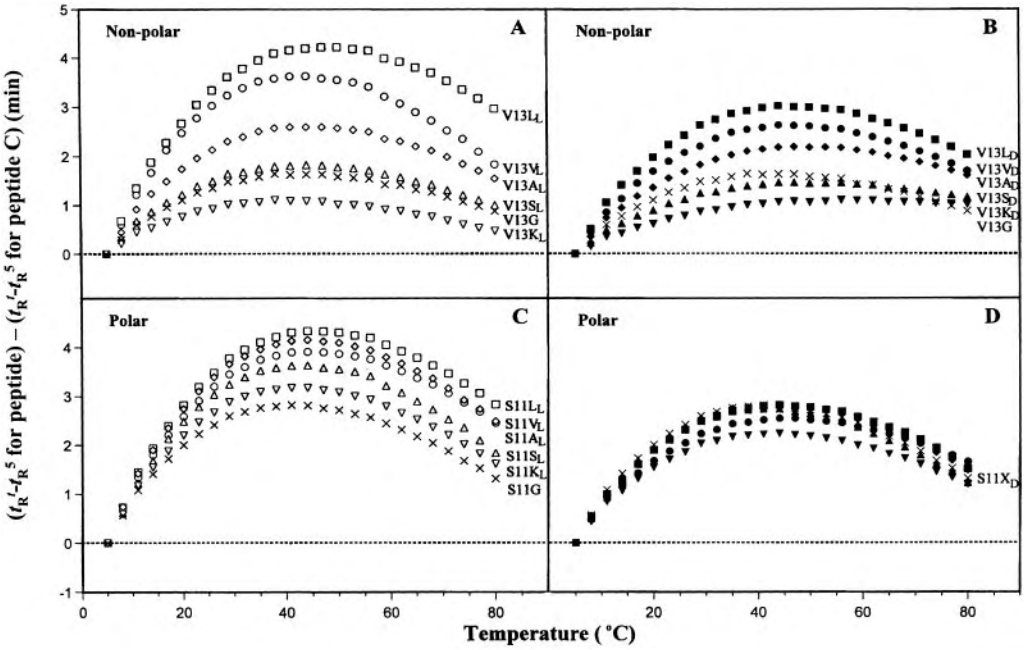

FIG. 6. **Normalized RP-HPLC temperature profiles of peptide V_{681} and its analogs.** Temperature profiles normalized to retention behavior of random coil peptide C. Column and conditions are as in Fig. 4. The retention behavior of the peptides was normalized to that of the random coil peptide C through the expression ($t_R^t - t_R^5$ for peptides) minus ($t_R^t - t_R^5$ for C), where t_R^t is the retention time at a specific temperature of an antimicrobial peptide or the random coil peptide, and t_R^5 is the retention time at 5 °C. *Open symbols* represent the temperature profiling of L-amino acid substituted peptides on either the nonpolar or polar face of V_{681} (*panels A* and *C,* respectively); *solid symbols* represent D-amino acid substituted peptides on either the nonpolar or polar face of V_{681} (*panels B* and *D*, respectively). In all panels, amino acids used for substitution in either the nonpolar or polar face of V_{681} are Val (*circle*), Leu (*square*), Ala (*diamond*), Ser (*triangle*), Lys (*inverted triangle*), and Gly (×).

relative to the temperature profile of the random coil peptide standard C and normalized to the retention time at 5 °C, the latter of which is presented as a *dotted line* in Fig. 6.

It was observed that the peptide analogs in this study showed dramatic varying dimerization ability in solution (Fig. 6). The maximal values of the change of retention times (($t_R^t - t_R^5$ for peptide) – ($t_R^t - t_R^5$ for *C*)) in Fig. 6 were defined as the peptide association parameter (P_A) to quantify the association ability of peptide analogs in solution (Table II). As seen from the data in Table II, peptides with higher relative hydrophobicity generally showed stronger self-association ability in solution. The P_A values of the peptide with nonpolar face substitutions were of the same order as their relative hydrophobicity, indicating that the hydrophobicity on the hydrophobic face of the amphipathic helix was essential during dimerization, because the dimers are formed by the binding together of the nonpolar faces of two amphipathic molecules. In contrast, the different relationship between P_A and the relative hydrophobicity of the peptides with polar face substitutions demonstrated that the hydrophobicity on the polar face of the helices plays a less important role in peptide association. Generally speaking, the P_A values of L-peptides were significantly greater than those of their D-diastereomers, indicating the importance of helical structure during dimerization (Table II). In Table II, in most cases, the peptides with polar face substitutions had greater P_A values than the corresponding peptide analogs with the same amino acid substitutions on the nonpolar face. This is exactly what one would expect because polar face substitutions have little effect on the preferred dimerization domain, whereas nonpolar face substitutions would dramatically affect the hydrophobicity and dimerization ability of the peptide.

Relationships between Peptide Self-association and Hydrophobicity, Amphipathicity, and Helicity—Amphipathicity of the L-amino acid substituted peptides was determined (Table III) by the calculation of hydrophobic moment (35) using the software package Jemboss version 1.2.1 (36), modified to include the hydrophobicity scale determined in our laboratory (see "Materials and Methods" for details). Peptide amphipathicity, for the nonpolar face substitutions, was directly correlated with side chain hydrophobicity of the substituted amino acid residue, *i.e.* the more hydrophobic the residue the higher the amphipathicity (values of 5.92 and 4.92 for $V13L_L$ and $V13K_L$, respectively); in contrast, on the polar face, peptide amphipathicity was inversely correlated with side chain hydrophobicity of the substituted amino acid residue, *i.e.* the more hydrophobic the residue, the lower the amphipathicity (compare $S11K_L$ and $S11L_L$ with amphipathicity values of 5.68 and 4.66, respectively, Table III).

TABLE III
Amphipathicity of peptide analogs

Peptide	Amphipathicity[a]	Peptide	Amphipathicity[a]
$V13L_L$	5.92	$S11L_L$	4.66
$V13V_L$[b]	5.56	$S11V_L$	5.03
$V13A_L$	5.15	$S11A_L$	5.45
V13G	5.00	S11G	5.61
$V13S_L$	5.04	$S11S_L$[b]	5.56
$V13K_L$	4.92	$S11K_L$	5.68

[a] Amphipathicity was determined by the calculation of hydrophobic moment (35) using hydrophobicity coefficients determined by reversed-phase chromatography (see "Materials and Methods" for details).
[b] Peptides $V13V_L$ and $S11S_L$ are the same peptide as V_{681}.

The native sequence, V_{681}, was very amphipathic with a value of 5.56. To place this value in perspective, the sequence of V_{681} can be shuffled to obtain an amphipathic value of 0.57 (KHAVIKWSIKSSVKFKISTAFKATTI) or a maximum value of 7.31 for the sequence of HWSKLLKSFTKALKKFAKAITSVVST.

The range of amphipathicity values achieved by single substitutions on the polar and nonpolar faces varied from a low of 4.66 for $S11L_L$ to a high of 5.92 for $V13L_L$ (Table III). Even though single substitutions changed the amphipathicity, all the analogs remained very amphipathic, *e.g.* even with a lysine

TABLE IV
Antimicrobial (MIC) and hemolytic (MHC) activities of peptide analogs against Gram-negative bacteria and human red blood cells

Peptides	MIC[a]							MHC[b] hRBC	Therapeutic index[c]
	Escherichia coli UB1005 wt[d]	*E. coli* DC2 abs[d]	*Salmonella typhimurium* C587 wt[d]	*S. typhimurium* C610 abs[d]	*Pseudomonas aeruginosa* H187 wt[d]	*P. aeruginosa* H188 abs[d]	*GM*[e]		
				μg/ml				*μg/ml*	
$V13L_L$	6.4	5.0	32.0	10.1	12.7	32.0	12.7	7.8	0.6
$V13V_L$[f]	7.1	4.5	20.2	5.7	6.4	20.2	8.8	15.6	1.8
$V13A_L$	2.5	2.5	6.4	2.5	5.0	6.4	3.8	31.2	8.1
V13G	2.5	2.5	5.0	2.5	6.4	10.1	4.1	125.0	30.2
$V13S_L$	2.5	2.5	6.4	2.0	6.4	10.1	4.2	125.0	30.1
$V13K_L$[g]	**2.5**	**1.6**	**4.0**	**1.3**	**8.0**	**5.0**	**3.1**	**>250.0**	**163.0**
$V13L_D$	3.2	2.5	16.0	3.2	6.4	10.1	5.5	7.8	1.4
$V13V_D$	2.5	1.6	5.0	2.0	4.0	8.0	3.3	62.5	19.0
$V13A_D$[g]	**1.6**	**2.0**	**5.0**	**2.0**	**4.0**	**10.1**	**3.3**	**250.0**	**75.7**
$V13S_D$	3.2	2.0	12.7	2.0	18.3	20.2	6.3	>250.0	79.9
$V13K_D$	3.2	2.5	32.0	1.0	32.0	25.4	7.7	>250.0	65.0
$S11L_L$	16.0	5.0	32.0	12.7	20.2	32.0	16.6	4.0	0.2
$S11V_L$	6.4	4.0	32.0	5.0	10.1	20.2	9.7	7.8	0.8
$S11A_L$	6.4	4.0	20.2	4.0	10.1	16.0	8.3	15.6	1.9
S11G	5.0	2.5	12.7	3.2	4.0	10.1	5.2	7.8	1.5
$S11S_L$[f]	7.1	4.5	20.2	5.7	6.4	20.2	8.8	15.6	1.8
$S11K_L$	10.1	4.0	25.4	8.0	25.4	32.0	13.7	4.0	0.3
$S11L_D$	5.0	2.5	10.1	3.2	4.0	10.1	5.0	31.2	6.2
$S11V_D$	5.0	2.5	10.1	4.0	6.4	16.0	6.1	125.0	20.5
$S11A_D$	4.0	2.5	8.0	2.0	5.0	8.0	4.3	15.6	3.6
$S11S_D$	2.5	1.6	5.0	1.6	2.0	10.1	2.9	15.6	5.3
$S11K_D$	3.2	1.6	3.2	1.6	2.0	6.4	2.6	31.2	11.8

[a] Antimicrobial activity (minimal inhibitory concentration) is given as the geometric mean of three sets of determinations.
[b] Hemolytic activity (minimal hemolytic concentration) was determined on human red blood cells (hRBC). When no detectable hemolytic activity was observed at 250 μg/ml, a value of 500 μg/ml was used for calculation of the therapeutic index.
[c] Therapeutic index = MHC (μg/ml)/geometric mean of MIC (μg/ml). Larger values indicate greater antimicrobial specificity.
[d] wt denotes the wild type strain, and abs denotes the antibiotics-sensitive strain.
[e] *GM* denotes the geometric mean of MIC values from all six microbial strains in this table.
[f] $V13V_L$ and $S11S_L$ are the same peptide, which is the parent peptide V_{681}.
[g] The boldface entries show the two best peptides with broad spectrum activity in terms of the therapeutic index against both Gram-negative and Gram-positive bacteria.

substitution on the nonpolar face $V13K_L$ has a value of 4.92.

From Table II and Table III, peptides with higher relative hydrophobicity on their nonpolar face created higher amphipathicity and generally showed stronger self-associating ability in solution; in contrast, for peptides with polar face substitutions, increasing hydrophobicity lowers amphipathicity, yet the peptides still strongly self-associate, which indicates that peptide amphipathicity plays a less important role in peptide self-association when changes in amphipathicity are created on the polar face. In addition, self-associating ability is correlated with the secondary structure of peptides, *i.e.* in this study, disrupting the peptide helical structure by replacing the L-amino acid with its D-amino acid counterpart decreases the P_A values (Table I and Table II).

Biological Activity of Peptides

Hemolytic Activity—The hemolytic activity of the peptides against human erythrocytes was determined as a major measure of peptide toxicity toward higher eukaryotic cells (Table IV). As mentioned before, the native peptide V_{681} (also named as $V13V_L$ or $S11S_L$) had strong hemolytic activity, with a minimal hemolytic concentration (MHC value) of 15.6 μg/ml (Table IV). In this study, because of the alteration of hydrophobicity, amphipathicity, and stability, the hemolytic activity of the best variants of peptide V_{681} was significantly decreased to no detectable activity, a >32-fold decrease for $V13K_L$ (Table VI). From Table IV, it is clear that for the nonpolar face substituted peptides, hemolytic activity was correlated with the side chain hydrophobicity of the substituting amino acid residue, *i.e.* the more hydrophobic the substituting amino acid, the more hemolytic the peptide, consistent with our previous study on the β-sheet antimicrobial peptide gramicidin S (25). For example, the MHC of peptide $V13L_L$ was 7.8 μg/ml; in contrast, the MHC was decreased, parallel with the reduction of hydrophobicity, to an undetectable level for peptide $V13K_L$. Peptide hydrophobicity and amphipathicity on the nonpolar face were also correlated with peptide self-associating ability, thus peptides with less self-association in benign conditions also exhibited less hemolytic activity against eukaryotic cells. In contrast, for polar face substituted peptides, the relationships between self-association, hydrophobicity/amphipathicity, and hemolytic activity were less clear. Of course, the hydrophobic nonpolar face remained very similar when L-substitutions were made on the polar face; thus, dimerization and hydrophobicity of the nonpolar face would be less affected, and hemolytic activity would remain relatively strong.

In addition to hydrophobicity/amphipathicity, peptide helicity seemed to have an additional effect on hemolytic activity. In general, on both the nonpolar and polar faces, D-amino acid substituted peptides were less hemolytic than their L-diastereomers. For example, $V13A_L$ had an MHC value of 31.2 μg/ml compared with $V13A_D$ with a value of 250 μg/ml, an 8-fold decrease in hemolytic activity (Table IV). This phenomenon generally correlated with peptide self-associating ability, because D-diastereomeric analogs exhibited weaker self-associating ability than L-analogs (Table II). Additionally, D-substitutions disrupt helicity, which in turn disrupts hydrophobicity of the nonpolar face. This result was also consistent with the data of Shai and co-workers (26, 27), who demonstrated that, through multiple D-amino acid substitutions, the helicity of peptides is substantially reduced leading to decreased hemolytic activity. Thus, peptide structure is important in the cytotoxicity toward mammalian cells, although these disturbed helices can still maintain antibacterial activity.

TABLE V
Antimicrobial (MIC) and hemolytic (MHC) activities of peptide analogs against Gram-positive bacteria and human red blood cells

Peptides	MIC[a]							MHC[b] hRBC	Therapeutic index[c]
	Staphylococcus aureus 25923 wt[d]	*S. aureus* SAP0017 methR[d]	*Staphylococcus epidermidis* C621 wt[d]	*Bacillus subtilis* C971 wt[d]	*Enterococcus faecalis* C625 wt[d]	*Corynebacterium xerosis* C875 wt[d]	*GM*[e]		
	μg/ml							*μg/ml*	
$V13L_L$	32.0	25.4	8.0	3.2	32.0	2.5	10.9	7.8	0.7
$V13V_L$[f]	16.0	9.0	5.0	2.2	16.0	2.5	6.3	15.6	2.5
$V13A_L$	8.0	5.0	3.2	2.0	16.0	2.0	4.5	31.2	6.9
V13G	25.4	10.1	3.2	2.0	50.8	2.0	7.4	125.0	16.9
$V13S_L$	16.0	12.7	4.0	2.5	50.8	1.6	7.4	125.0	16.9
$V13K_L$[g]	**64.0**	**64.0**	**5.0**	**1.6**	**64.0**	**1.3**	**11.8**	**>250.0**	**42.3**
$V13L_D$	5.0	4.0	2.5	2.5	6.4	1.6	3.3	7.8	2.4
$V13V_D$	4.0	3.2	1.6	1.3	12.7	1.3	2.8	62.5	22.7
$V13A_D$[g]	**8.0**	**5.0**	**2.0**	**1.6**	**32.0**	**1.6**	**4.3**	**250.0**	**57.8**
$V13S_D$	64.0	64.0	12.7	2.5	64.0	2.0	16.0	>250.0	31.3
$V13K_D$	64.0	64.0	25.4	3.2	64.0	2.0	18.7	>250.0	26.8
$S11L_L$	32.0	32.0	16.0	5.0	50.8	2.5	14.8	4.0	0.3
$S11V_L$	16.0	12.7	8.0	2.5	20.2	1.3	6.9	7.8	1.1
$S11A_L$	16.0	12.7	4.0	2.5	20.2	2.0	6.6	15.6	2.4
S11G	8.0	5.0	4.0	2.0	12.7	2.0	4.5	7.8	1.7
$S11S_L$[f]	16.0	9.0	5.0	2.2	16.0	2.5	6.3	15.6	2.5
$S11K_L$	32.0	16.0	6.4	3.2	32.0	4.0	10.5	4.0	0.4
$S11L_D$	8.0	5.0	4.0	2.0	16.0	2.0	4.7	31.2	6.7
$S11V_D$	16.0	8.0	4.0	2.5	32.0	2.0	6.6	125.0	19.0
$S11A_D$	6.4	5.0	2.5	2.0	12.7	1.6	3.8	15.6	4.1
$S11S_D$	4.0	2.5	2.0	1.3	6.4	1.0	2.3	15.6	6.7
$S11K_D$	4.0	2.5	2.0	2.0	12.7	1.0	2.8	31.2	11.0

[a] Antimicrobial activity (minimal inhibitory concentration) is given as the geometric mean of three sets of determinations.
[b] Hemolytic activity (minimal hemolytic concentration) was determined on human red blood cells (hRBC). When no detectable hemolytic activity was observed at 250 μg/ml, a value of 500 μg/ml was used for calculation of the therapeutic index.
[c] Therapeutic index = MHC (μg/ml)/geometric mean MIC (μg/ml). Larger values indicate greater antimicrobial specificity.
[d] wt denotes the wild type strain, and methR denotes the methicillin-resistant strain.
[e] *GM* denotes the geometric mean of MIC values from all six microbial strains in this table.
[f] $V13V_L$ and $S11S_L$ are the same peptide, which is the parent peptide V_{681}.
[g] The boldface entries show the two best peptides with broad spectrum activity in terms of the therapeutic index against both Gram-negative and Gram-positive bacteria.

In the present study, peptide analogs with nonpolar face substitutions exhibited a greater range of hemolytic activity (7.8 μg/ml to not detectable) than the polar face substitutions (4–125 μg/ml), again indicating that the nonpolar face of the helix may play a more essential role during the interaction with the biomembrane of normal cells (Table IV). As expected, the peptides with the polar face substitutions showed stronger hemolytic activity than the peptides with the same amino acid substitutions on the nonpolar face, which may be attributed to the different magnitude of the hydrophobicity change by the same amino acid substitutions on different sides of the amphipathic helix. Most interestingly, in this study, all polar face substituted peptides except $S11L_D$, $S11V_D$, and $S11K_D$ showed stronger hemolysis of erythrocytes than V_{681}; in contrast, on the nonpolar face, only peptides $V13L_D$ and $V13L_L$ were more hemolytic than V_{681} (Table IV).

Antimicrobial Activity against Gram-negative Microorganisms—The antimicrobial activity of the peptides with either nonpolar face or polar face amino acid substitutions against a range of Gram-negative microorganisms is shown in Table IV. The geometric mean MIC values from six microbial strains in Table IV were calculated to provide an overall evaluation of antimicrobial activity against Gram-negative bacteria. From Table VI, it is apparent that many peptide analogs showed considerable improvement in antimicrobial activity against Gram-negative bacteria over the native peptide V_{681}, as much as 3.4-fold.

For Gram-negative bacteria, disruption of peptide helicity seemed to outweigh other factors in the improvement of antimicrobial activity, *i.e.* in most cases the peptides with D-amino acid substitutions showed better antimicrobial activity than L-diastereomers. The exceptions were peptides $V13S_D$ and $V13K_D$. The reason for the weaker activity of peptides $V13S_D$ and $V13K_D$ compared with $V13S_L$ and $V13K_L$, respectively, was possibly the combined effects of the destabilization of the helix, the decrease of hydrophobicity on the nonpolar face, and the disruption of amphipathicity, highlighting the importance of maintaining a certain magnitude of hydrophobicity and amphipathicity on the nonpolar face of the helix for biological activity, *i.e.* perhaps there is a combined threshold of helicity and hydrophobicity/amphipathicity required for biological activity of α-helical antimicrobial peptides. In this study, peptide self-associating ability (relative hydrophobicity) seemed to have no general relationship to MIC; however, most interestingly, for peptides with L-hydrophobic amino acid substitutions (Leu, Val, and Ala) in the polar and nonpolar faces, the less hydrophobic the substituting amino acid, the more active the peptide against Gram-negative bacteria (Table IV).

Antimicrobial Activity against Gram-positive Microorganisms—Table V shows the antimicrobial activity of the peptides against Gram-positive microorganisms. By introducing D-/L-amino acid substitutions, we improved the antimicrobial activity of peptide V_{681} against Gram-positive bacteria by as much as 2.7-fold (mean MIC values for V_{681} were 6.3 μg/ml compared with 2.3 μg/ml for $S11S_D$, see Table VI). Compared with peptide V_{681}, most of the peptide analogs with increased antimicrobial activity against Gram-positive microorganisms were D-amino acid substituted peptides (7 D-peptides *versus* 1 L-peptide, see Table V). It was surprising to observe that peptides with polar face substitutions showed an overall greater improvement in MIC than those with nonpolar face substitutions. Generally speaking, increasing the hydrophobicity of the native peptide V_{681} by amino acid substitutions at either the polar or the nonpolar face weakened the antimicrobial activity

TABLE VI
Effect of amino acid substitutions on the biological activity of V_{681}
Only the peptide analogs with a therapeutic index greater than V_{681} are included in this table.

Peptide	Gram-negative				MHC[d]	Fold[b]	Gram-positive			
	MIC[a]	Fold[b]	Therapeutic index[c]	Fold[b]			MIC[a]	Fold[b]	Therapeutic index[c]	Fold[b]
	μg/ml				*μg/ml*		*μg/ml*			
V_{681}	8.8	1.0	1.8	1.0	15.6	1.0	6.3	1.0	2.5	1.0
$V13A_L$	3.8	2.3	8.1	4.5	31.2	2.0	4.5	1.4	6.9	2.8
V13G	4.1	2.1	30.2	16.8	125.0	8.0	7.4	0.9	16.8	6.7
$V13S_L$	4.2	2.1	30.1	16.7	125.0	8.0	7.4	0.9	16.9	6.8
$V13K_L$[e]	**3.1**	**2.8**	**163.0**	**90.6**	**>250.0**	**32.1**	**11.8**	**0.5**	**42.3**	**16.9**
$V13V_D$	3.3	2.7	19.0	10.6	62.5	4.0	2.8	2.3	22.7	9.1
$V13A_D$[e]	**3.3**	**2.7**	**75.7**	**42.1**	**250.0**	**16.0**	**4.3**	**1.5**	**57.8**	**23.1**
$V13S_D$	6.3	1.4	79.9	44.4	>250.0	32.1	16.0	0.4	31.3	12.5
$V13K_D$	7.7	1.1	65.0	36.1	>250.0	32.1	18.7	0.3	26.8	10.7
$S11L_D$	5.0	1.8	6.2	3.4	31.2	2.0	4.7	1.3	6.7	2.7
$S11V_D$	6.1	1.4	20.5	11.4	125.0	8.0	6.6	1.0	19.0	7.6
$S11A_D$	4.3	2.0	3.6	2.0	15.6	1.0	3.8	1.7	4.1	1.6
$S11S_D$	2.9	3.0	5.3	2.9	15.6	1.0	2.3	2.7	6.7	2.7
$S11K_D$	2.6	3.4	11.8	6.6	31.2	2.0	2.8	2.3	11.0	4.4

[a] Antimicrobial activity (minimal inhibitory concentration) was given as the geometric mean data of Tables IV and V.
[b] Fold denotes the improvement in activity compared with the corresponding data of the parent peptide V_{681}.
[c] Therapeutic index = MHC (in μg/ml)/MIC (in μg/ml). Larger values indicate greater antimicrobial specificity.
[d] Hemolytic activity (minimal hemolytic concentration) was determined on human red blood cells (hRBC). When no detectable hemolytic activity was observed at 250 μg/ml, a value of 500 μg/ml was used for calculation of the therapeutic index and fold increased.
[e] The boldface entries show the two best peptides with broad spectrum activity in terms of the therapeutic index against both Gram-negative and Gram-positive bacteria.

against Gram-positive bacteria, *e.g.* peptides $V13L_L$ and $S11L_L$ (Table V). Amino acid substitutions of D-Ser and D-Lys on the nonpolar face significantly weakened the activity, in a similar manner to the Gram-negative activity, indicating again the importance of maintaining a certain magnitude of helicity and hydrophobicity/amphipathicity on the nonpolar face of the helix for peptide Gram-positive antimicrobial activity.

Therapeutic Index—The therapeutic index is a widely employed parameter to represent the specificity of antimicrobial reagents. It is calculated by the ratio of MHC (hemolytic activity) and MIC (antimicrobial activity); thus, larger values in therapeutic index indicate greater antimicrobial specificity. As mentioned above, the native peptide V_{681} is a peptide with good antimicrobial activity coupled with strong hemolytic activity; hence, its therapeutic index is low (1.8 and 2.5 for Gram-negative and Gram-positive bacteria, respectively; Table VI) and comparable with general toxins like melittin. In this study, by altering peptide hydrophobicity/hydrophilicity, amphipathicity, and helicity, we significantly increased the therapeutic index of peptide V_{681} against Gram-negative bacteria by 90-fold (Table VI) and Gram-positive bacteria by 23-fold (Table VI). As indicated in Tables IV and V, there was a greater range of therapeutic indices for peptides with the nonpolar face substitutions compared with the polar face substitutions, which was consistent with peptide self-association studies, indicating that the nonpolar face of the helix may play a more important role in the mechanism of action.

Table VI summarizes the data for these peptide analogs with improved therapeutic index (MHC/MIC) values relative to the native peptide V_{681}. From Table I and Table VI, it is clear that all peptides with improved therapeutic indices are those showing less stable helical structure in KP medium (either the D-amino acid substituted peptides or the hydrophilic amino acid substituted peptides on the nonpolar face). The two peptides with the best therapeutic indices among all the analogs were $V13K_L$ with a 90- and 17-fold improvement and $V13A_D$ with a 42- and 23-fold improvement compared with V_{681} against all the tested Gram-negative and Gram-positive microorganisms, respectively. It is noteworthy that the hemolytic activity of these two peptides was extremely weak; in addition, peptide $V13K_L$ exhibited improved antimicrobial activity compared with peptide V_{681} against Gram-negative bacteria, and $V13A_D$ exhibited improved antimicrobial activity against Gram-negative and Gram-positive bacteria (Table VI).

Proposed Mechanisms of Action of Antimicrobial Peptides in Biomembranes—The exact mechanism of action of cationic amphipathic antimicrobial peptides is not well understood (47–52), because their lethal action could be from membrane disruption solely or from translocation through the membrane to target receptors inside the cell. Many models have been proposed on how peptides interact with the cytoplasmic membrane. For example, the peptide may form transmembrane channels/pores by bundles of amphipathic α-helices, as their hydrophobic surfaces interact with the lipid core of the membrane and the hydrophilic surfaces point inward, producing an aqueous pore ("barrel-stave" mechanism) (53); or the peptides lie at the interface parallel with the membrane allowing their hydrophobic surface to interact with the hydrophobic component of the lipid, and the positive charge residues can still interact with the negatively charged head groups of the phospholipid ("carpet" mechanism) (54). In support of the interface model is the NMR study of the amphipathic cyclic β-sheet antimicrobial peptide of gramicidin S (55). In the latter model, the peptides are not in the hydrophobic core of the membrane, and neither do they assemble the aqueous pore with their hydrophilic faces. Neither of these mechanisms alone can fully explain the data in the present study. For example, the hemolytic activity is correlated to the peptide hydrophobicity and amphipathicity on the nonpolar face, which may be consistent with the barrel-stave mechanism, *i.e.* peptides interact with the hydrophobic core of the membrane by their nonpolar face to form pores/channels. In contrast, the antimicrobial activity is not correlated with peptide hydrophobicity/amphipathicity, showing that the barrel-stave mechanism may not be suitable to explain the mechanism of antimicrobial action. Indeed, the carpet mechanism may best explain the interaction between the peptides and the bacterial membrane.

Impact of Present Results on Proposed Mechanisms of Action—We believe that the main target for the peptides with the desired biological activities in the present study is the cytoplasmic membrane. Based on the above observations, we propose that both mechanisms are in operation for the peptides used in

this study, *i.e.* the mechanism depends upon the difference in membrane composition between prokaryotic and eukaryotic cells. If the peptides form pores/channels in the hydrophobic core of the eukaryotic bilayer, they would cause the hemolysis of human red blood cells; in contrast, for prokaryotic cells the peptides lyse cells in a detergent-like mechanism as described in the carpet mechanism.

Indeed, it is known that the extent of interaction between peptide and biomembrane is dependent on the composition of the lipid bilayer. For example, Liu *et al.* (56–58) utilized a polyleucine-based α-helical transmembrane peptide to demonstrate that the peptide reduced the phase transition temperature to a greater extent in phosphatidylethanolamine (PE) bilayers than in phosphatidylcholine (PC) or phosphatidylglycerol bilayers, indicating a greater disruption of PE organization. The zwitterionic PE is the major lipid component in prokaryotic cell membranes, and PC is the major lipid component in eukaryotic cell membranes (59, 60). In addition, although PE also exists in eukaryotic membranes, because of the asymmetry in lipid distribution, PE is mainly found in the inner leaflet of the bilayer, whereas PC is mainly found in the outer leaflet of the eukaryotic bilayer. We draw the conclusion that, in a similar fashion to the results of transmembrane α-helical peptides, the antimicrobial specificity of the antimicrobial α-helical peptides in the present study is a result of the composition differences of the lipid bilayer between eukaryotic and bacterial cells.

In support of this proposal, we have selected two examples from our study. The results for peptide $V13K_L$, the peptide with the highest therapeutic index (MHC/MIC) against Gram-negative bacteria, can be explained by using our combined model. For example, if hemolysis of eukaryotic cells requires insertion of the peptide into the hydrophobic core of the membrane, which depends on the composition of the bilayer, and interaction of the nonpolar face of the amphipathic α-helix with the hydrophobic lipid core of the bilayers, it seems reasonable to assume that disruption of the hydrophobic surface with the Lys substitution ($V13K_L$) would both disrupt dimerization of the peptide in aqueous solution, allowing the peptide to more easily enter the interface region and prevent penetration into the hydrophobic core of the membrane. Thus, the peptide is unable to cause hemolysis. On the other hand, if the mechanism for prokaryotic cells allows the interaction of monomeric peptides with the phospholipid head groups in the interface region, then no insertion into the hydrophobic core of the membrane is required for antimicrobial activity.

In contrast, the observation that the antimicrobial activity of peptide $V13L_L$ (with Leu at the substitution site) was worse than that of $V13K_L$, and its hemolytic activity was stronger (MIC values of 12.7 μg/ml for $V13L_L$ *versus* 3.1 μg/ml for $V13K_L$ against Gram-negative bacteria; hemolytic activity of 7.8 μg/ml for $V13L_L$ *versus* no detectable hemolytic activity for $V13K_L$), which can also be explained by our combined model. Thus, peptide $V13L_L$ has a fully accessible nonpolar face required for insertion into the bilayer and for interaction with the hydrophobic core of the membrane to form pores/channels (barrel-stave mechanism), whereas the hemolytic activity of peptide $V13L_L$ is dramatically stronger than peptide $V13K_L$. On the other hand, because of the stronger tendency of peptide $V13L_L$ to be inserted into the hydrophobic core of the membrane than peptide $V13K_L$, peptide $V13L_L$ actually interacts less with the water/lipid interface of the bacterial membrane; hence, the antimicrobial activity is 4-fold weaker than the peptide $V13K_L$ against Gram-negative bacteria. This supports the view that the carpet mechanism is essential for strong antimicrobial activity, and if there is a preference by the peptide for penetration into the hydrophobic core of the bilayer, the antimicrobial activity will actually decrease.

Conclusions

By utilizing a structure-based rational approach to antimicrobial peptide design based on single D-/L-amino acid substitutions in the center of the peptide nonpolar/polar face of the amphipathic α-helical antimicrobial peptide V_{681}, we were able to develop antimicrobial peptides with improved activity and specificity and clinical potential as broad spectrum antibiotics. Systematically altering peptide hydrophobicity/hydrophilicity, amphipathicity, and helicity, we were able to optimize the specificity of the parent peptide V_{681} with significantly increased therapeutic indices of 90-fold against Gram-negative bacteria and 23-fold against Gram-positive microorganisms, respectively. The hemolytic activity of these peptides has been demonstrated to have close relationships with peptide hydrophobicity, amphipathicity, and helicity. High peptide hydrophobicity, amphipathicity, and helicity usually result in strong hemolytic activity. The controlled disruption of the α-helical structure (disruption under benign conditions and inducible in hydrophobic conditions) also seems to be related to strong antimicrobial activity over a variety of Gram-negative and Gram-positive bacterial strains. Furthermore, the technique of temperature profiling in RP-HPLC appears to be a valuable tool to determine the self-association ability of molecules in solution, which we believe is an important property influencing peptide biological activity.

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