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Structure-guided de novo design of α-helical antimicrobial peptide with enhanced specificity*

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Abstract: In the present study, the 26-residue peptide sequence Ac-KWKSFLKTFKSAKK-TVLHTALKAISS-amide (peptide P) was utilized as the framework to study the effects of introducing hydrophilic amino acid lysine on the nonpolar face of the helix on peptide biological activities. Lysine residue was systematically used to substitute original hydrophobic amino acid at the selected locations on the nonpolar face of peptide P. In order to compensate for the loss of hydrophobicity caused by lysine substitution, leucine was also used to replace original alanine to increase peptide overall hydrophobicity. Hemolytic activity is correlated with peptide hydrophobicity. By introducing lysine on the nonpolar face, we significantly weaken peptide hemolytic activity as well as antimicrobial activity. However, by utilizing leucine to compensate the hydrophobicity, we improve antimicrobial activity against both Gram-negative and -positive bacteria. Peptide self-association ability and hydrophobicity were also determined. This specific rational approach of peptide design could be a powerful method to optimize antimicrobial peptides with clinical potential as peptide antibiotics.

Keywords: antimicrobial peptides; α-helical structures; de novo design; hydrophobicity; specificity.

INTRODUCTION

Antibiotic resistance, due to the extensive clinical use of classical antibiotics [1], has become a great concern in recent years, prompting an urgent need for a new class of antibiotics. Antimicrobial peptides have been proposed as potent candidates of a new class of antibiotics, with characteristics including an ability to kill target cells rapidly, an unusually broad spectrum of activity, activity against some of the more serious antibiotic-resistant pathogens in clinics, and the relative difficulty in selecting resistant mutants in vitro [2]. It is generally accepted that the cytoplasmic membrane is the main target of antimicrobial peptides, whereby peptide accumulation in the membrane causes increased permeability and loss of barrier function resulting in leakage of cytoplasmic components and cell death [3].

Although antimicrobial peptides are extremely active to kill a broad spectrum range of Gram-negative and -positive bacteria, the specificity for prokaryotic and eukaryotic cells could be a serious challenge of peptides used in clinical practices. A large number of antimicrobial peptides are toxic to human

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cells, thus, they have no value to be developed as new drugs. In the previous work [4], we showed that by introducing hydrophilic amino acid to the center of the nonpolar face of an amphipathic α-helical antimicrobial peptide, we dramatically decrease hemolytic activity of peptides against human red blood cells (hRBCs), hence, we increase the therapeutic index. In addition, hydrophobicity of the nonpolar face of α-helical antimicrobial peptides has been demonstrated as the most crucial parameter influencing the specificity of peptides whose sole target is the cytoplasmic membrane [5].

In the previous work, we designed an α-helical antimicrobial peptide V13K with high antimicrobial activity against various Gram-negative and -positive bacteria and, more importantly, negligible hemolytic activity [6]. In the present study, in order to investigate the role of hydrophobicity and the effect of hydrophilic amino acid on the nonpolar face of the helical structure on the mechanism of action of α-helical antimicrobial peptides, we systematically introduced hydrophilic lysine residue to the nonpolar face of peptide V13K and report here that hydrophilic amino acid can be very efficient in enhancing the specificity of antimicrobial peptides, and hydrophobicity on the nonpolar face of antimicrobial peptides also plays an important role in biological activities.

MATERIALS AND METHODS

Materials and bacterial strains

Rink amide 4-methylbenzhydrylamine (MBHA) resin (0.8 mmol/g) and all of the *N*-α-Fmoc (9-fluorenyl-methoxycarbonyl) protected amino acids were purchased from GL Biochem (Shanghai, China). Coupling reagents for peptide synthesis *O*-benzotriazole-1-yl-*N*,*N*,*N*',*N*'-tetramethyl-uronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), *N*,*N*'-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were purchased from Sigma. Dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), piperidine, and 2,2,2-trifluoroethanol (TFE) were analytical grade and purchased from a local supplier. Acetonitrile (HPLC grade) was obtained from Fisher.

Bacterial strains used for antimicrobial activity study include *Escherichia coli* DH5α, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Pseudomonas aeruginosa* H188, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC6633, *Streptococcus pneumoniae* ATCC49619, and *Staphylococcus epidermidis* ATCC12228.

Peptide synthesis and purification

The peptides were synthesized by the solid-phase method using Fmoc chemistry [7]. Rink amide MBHA resin (125 mg, 0.1 mmol) was well swelled in DCM (4 ml) overnight before the synthesis. The Fmoc protecting group was removed at each cycle with 5 ml 20 % piperidine in DMF for 20 min at 25 °C, then the resin was washed with DCM, isopropanol, and DMF twice, respectively. Amino acid couplings were carried out by adding Fmoc amino acid, HBTU, HOBt, and DIEA in DMF/DCM to the resin. After acetylation, peptides were cleaved from the resin by treatment of TFA/H_2O/TIS (90:5:5) for 2 h. The peptide was precipitated by dripping into cold ether. The crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (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 (0.1 % acetonitrile/min) at a flow rate of 2 ml/min, while mobile-phase A was 0.1 % aqueous TFA in water, and B was 0.1 % TFA in acetonitrile. The purity of peptides was verified by analytical RP-HPLC as described below. The peptides were further characterized by mass spectrometry and amino acid analysis.

Analytical RP-HPLC of peptides

Peptides were analyzed on a Shimadzu LC-20AB high-performance liquid chromatograph. Runs were performed on a Zorbax 300 SB-C_8 column (150 × 4.6-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 1 ml/min, where solvent A was 0.1 % aqueous TFA, and solvent B was 0.1 % TFA in acetonitrile. Temperature profiling analyses were performed in 5 °C increments, from 5 to 80 °C.

Circular dichroism (CD) spectroscopy

CD spectra were measured with a 0.02-cm path length quartz cuvette on a Jasco J-810 spectropolarimeter (Jasco, Easton, MD) at 25 °C. Data were collected from 250 to 190 nm at a sensitivity of 100 millidegrees, response time of 1 s, bandwidth of 1.0 nm, and a scan speed of 100 nm/min. The concentration of 75 μM peptides was measured in benign buffer (50 mM KH_2PO_4/K_2HPO_4, 100 mM KCl, pH 7) or benign buffer with 50 % TFE. The mean residue molar ellipticities were calculated by the equation $[\theta] = \theta/10lc_Mn$ [8]. θ is the ellipticity in millidegrees, l is the optical path length of the cuvette in centimeters, c_M is the peptide concentration in mol/liter, and n is the number of residues in the peptide. The values of mean residue molar ellipticities of the peptide analogs at 222 nm were used to determine the relative helicity of the peptides.

Measurement of antibacterial activity

Minimal inhibitory concentrations (MICs) were determined using a broth dilution method [9]. Briefly, bacteria were grown overnight at 37 °C in Mueller Hinton Broth, diluted in the same medium and transferred into 96-well microtiter plates (90 μl/well). Peptides were serially diluted by 0.2 % bovine serum albumin containing 0.01 % acetic acid and added to the microtiter plates in a volume of 10 μl of each well to give a final concentration of 5×10^5 colony-forming units/ml. MICs were determined as the lowest peptide concentration that inhibited bacterial growth after incubation for 24 h at 37 °C.

Measurement of hemolytic activity (MHC)

Peptide samples were serially diluted by phosphate-buffered saline (PBS) in 96-well plates (round-bottom) to give a volume of 60 μl sample solution in each well. Human erythrocytes anticoagulated by $EDTAK_2$ were collected by centrifugation (1000 × g) for 5 min, and washed twice by PBS, then diluted to a concentration of 2 % in PBS. 60 μl of 2 % erythrocytes were added to each well to give final 1 % human erythrocytes in each well and reactions were incubated at 37 °C for 4 h. The plates were then centrifuged for 10 min at 3000 rpm and supernatant (80 μl) was transferred to a 96-well plate (flat-bottom). The release of hemoglobin was determined by measuring the absorbance of the supernatant at 540 nm. Peptide samples were diluted in a 2-fold serial in order to determine the minimum concentration with no hemolysis. Erythrocytes in PBS and distilled water were used as controls of 0 and 100 % hemolysis, respectively.

Calculation of therapeutic index (MHC/MIC ratio)

Therapeutic index values were determined by the ratio of MHC/MIC, indicating the specificity of peptides against bacterial and eukaryotic cells, respectively. When there was no hemolytic activity at 1000 μg/ml, a minimal hemolytic concentration of 2000 μg/ml was used to calculate the therapeutic index; in contrast, for the antimicrobial activity, 500 μg/ml would be used if there was no activity at the upper limit of MIC 250 μg/ml.

RESULTS AND DISCUSSION

Peptide design

The parent peptide (peptide P), also known as peptide V13K in our previous work [6], is a 26-residue amphipathic peptide which adopts an α-helical conformation in a hydrophobic environment and contains a hydrophilic lysine residue on the center of the nonpolar face (position 13) (Fig. 1). In the present study, we used peptide P as a framework to systematically introduce hydrophilic amino acid lysine on the nonpolar face of the helix. Peptide sequences are shown in Table 1. Figure 1 shows the peptide analogs represented as helical nets. We selected central locations and positions further away from the center of peptide sequence on both N- and C-terminals as the lysine-substituting positions. We used single and double lysine substitution to investigate the effect of hydrophilic and charged residue on the nonpolar face on peptide biological activities. In addition to the decrease of the hydrophobicity of peptides, we also utilized leucine to replace original alanine in order to compensate the loss of overall hydrophobicity by introducing lysine on the nonpolar face of peptide P. Therefore, the criteria of introducing lysine on the nonpolar face of the helix are as follows: (a) for single lysine-substituting analogs, we selected positions 5, 9, 17, and 21 of peptide P as single lysine substitution positions. Positions 9 and 17 are closer to the center of sequence than positions 5 and 21 on N- and C-terminals, respectively (Fig. 1). Based on the model peptide studies [10], the central location substitutions had greater effect on peptide secondary structure. Moreover, at these positions, the original amino acids are leucine or phenylalanine, which is bulky hydrophobic amino acid contributing more to peptide hydrophobicity than smaller hydrophobic amino acids, thus, such substitutions will have greater effect on peptide hydrophobicity. The single lysine-substituting peptides are named as F5K, F9K, L17K, and L21K, respectively. In order to study the sole effect of charged residue on the nonpolar face of the peptide, we increased the peptide hydrophobicity on the nonpolar face by utilizing leucine to replace alanine on the same side of peptide sequence with substituting lysine, in order to compensate the decrease of hydrophobicity caused by lysine substitutions. Alanine 12 and 20 were selected due to their position on the N- and C-terminal, respectively. Hence, peptides F5K/A12L, F9K/A12L, L17K/A20L, and L21K/A20L were made, representing higher hydrophobicity compared to F5K, F9K, L17K, and L21K, respectively (Fig. 1); (b) for double lysine-substituting analogs, substitutions were made as double central substitution (F9K/L17K) or double substitution closer to the both terminals (F5K/L21K), in order to explore the location effect of lysine substitution on the peptide nonpolar face. Similarly, hydrophobicity-compensating analogs were made to dissociate the hydrophilic property and the charge effect of lysine residue, as F9K/L17K/A12L/A20L and F5K/L21K/A12L/A20L, respectively (Fig. 1).

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 self-association ability. As shown in the previous study [11], 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 ($[\theta]_{222}$ = –3950).

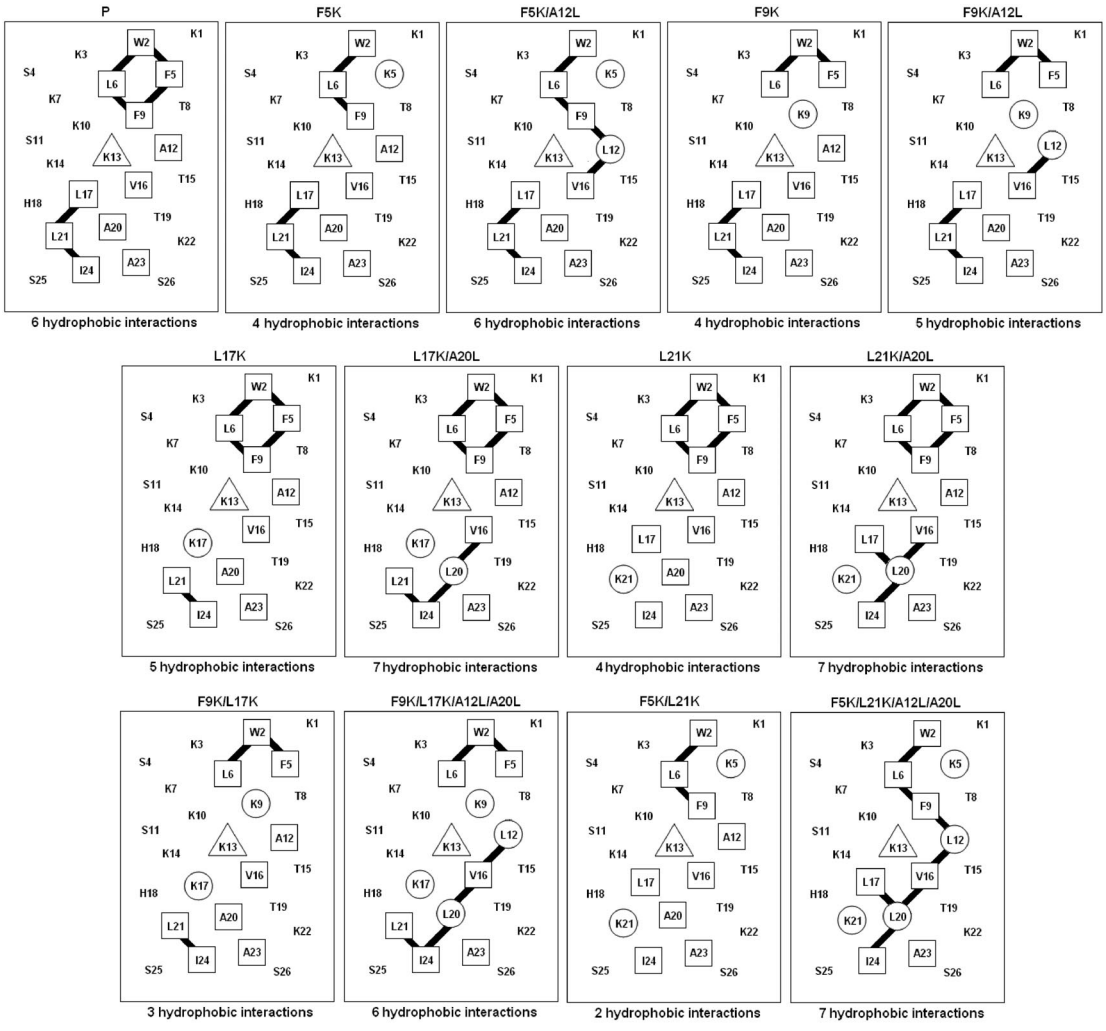

Fig. 1 Helical net representation of the designed peptides analogs. The hydrophobic amino acid residues on the nonpolar faces are boxed. The substituting hydrophilic amino acids on the nonpolar face are circled. The lysine at the position 13 on the nonpolar face is in triangle. The $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions are shown as black bars, and the number of hydrophobic interactions on the nonpolar face is indicated. The one-letter code is used for the amino acid residues.

Table 1 Sequences of peptides used in this study.

Peptide	Amino acid sequence[a]
P	Ac-K-W-K-S-F-L-K-T-F-K-S-A-K-K-T-V-L-H-T-A-L-K-A-I-S-S-amide
F5K	Ac-K-W-K-S-***K-***L-K-T-F-K-S-A-K-K-T-V-L-H-T-A-L-K-A-I-S-S-amide
F5K/A12L	Ac-K-W-K-S-***K-***L-K-T-F-K-S-***L***-K-K-T-V-L-H-T-A-L-K-A-I-S-S-amide
F9K	Ac-K-W-K-S-F-L-K-T-***K-***K-S-A-K-K-T-V-L-H-T-A-L-K-A-I-S-S-amide
F9K/A12L	Ac-K-W-K-S-F-L-K-T-***K-***K-S-***L***-K-K-T-V-L-H-T-A-L-K-A-I-S-S-amide
L17K	Ac-K-W-K-S-F-L-K-T-F-K-S-A-K-K-T-V-***K-***H-T-A-L-K-A-I-S-S-amide
L17K/A20L	Ac-K-W-K-S-F-L-K-T-F-K-S-A-K-K-T-V-***K-***H-T-***L-***L-K-A-I-S-S-amide
L21K	Ac-K-W-K-S-F-L-K-T-F-K-S-A-K-K-T-V-L-H-T-A-***K-***K-A-I-S-S-amide
L21K/A20L	Ac-K-W-K-S-F-L-K-T-F-K-S-A-K-K-T-V-L-H-T-***L-K-***K-A-I-S-S-amide
F9K/L17K	Ac-K-W-K-S-F-L-K-T-***K-***K-S-A-K-K-T-V-***K-***H-T-A-L-K-A-I-S-S-amide
F9K/L17K/A12L/A20L	Ac-K-W-K-S-F-L-K-T-***K-***K-S-***L***-K-K-T-V-***K-***H-T-***L-***L-K-A-I-S-S-amide
F5K/L21K	Ac-K-W-K-S-***K-***L-K-T-F-K-S-A-K-K-T-V-L-H-T-A-***K-***K-A-I-S-S-amide
F5K/L21K/A12L/A20L	Ac-K-W-K-S-***K-***L-K-T-F-K-S-***L***-K-K-T-V-L-H-T-***L-K-***K-A-I-S-S-amide
C[b]	Ac-E-L-E-K-G-G-L-E-G-E-K-G-G-K-E-L-E-K-amide

[a]One-letter codes are used for the amino acid residues; Ac, N^{α}-acetyl; amide, C-terminal amide; the bold and italic letters denote the substituting amino acids of the peptide P. All amino acids are L-amino acids.
[b]Peptide C is a random coil peptide used as a control in the RP-HPLC temperature profiling.

Peptide secondary structure

In order to show the effect of amino acid substitutions on peptide secondary structure, CD spectra of the peptide analogs were measured under benign (non-denaturing) pH and ionic strength (100 mM KCl, 50 mM aq. PO_4, pH 7 referred to as KP buffer) and also in 50 % TFE to mimic the hydrophobic environment of the membrane. Table 2 shows the molar ellipticity values at different environments and the helicity of peptide analogs relative to that of peptide F9K/L17K/A12L/A20L in the presence of 50 % TFE ($[\theta]_{222}$ –36 250), which is the greatest value among all analogs. In Table 2, it is clear to see that in KP buffer, similar to the peptide P, lysine-substituting peptides generally exhibited negligible α-helical structure with single/double lysine-substituted analogs or with leucine substitutions to compensate the loss of hydrophobicity. In contrast, in the presence of 50 % TFE, a mimic of the membrane's hydrophobicity and α-helix inducing ability, high helical structure could be induced, indicating that peptide analogs exist as α-helical structure when entering into the cell membrane. Figure 2 shows the CD spectra of peptide P, peptide F9K/A12L, and peptide F9K/L17K/A12L/A20L in benign conditions and in the presence of 50 % TFE.

It is noteworthy that for peptides (F9K/A12L and F9K/L17K/A12L/A20L) with lysine substitution at the center of the nonpolar face of helix, although exhibiting similar random coil structure in KP buffer, they show the highest helicity 94.8 and 100 %, respectively, in the presence of 50 % TFE compared to other analogs with the substitutions at different locations, indicating that central substitutions have greater effect on the change of peptide secondary structure (Table 2 and Fig. 2). In addition, peptides with leucine substituted by lysine exhibit less helical structure than peptides with phenylalanine replaced by lysine in 50 % TFE, i.e., peptides F9K and F5K have helicity values of 89.5 and 85.0 %, respectively, which are much greater values than those of peptide L17K (76.4 %) and L21K (66.2 %), respectively, which is consistent with the amino acid α-helical propensity report [12] that leucine has stronger helical propensity than phenylalanine.

Table 2 CD data of the peptide analogs.

Peptides[a]	Benign[b]		50 % TFE[c]	
	$[\theta]_{222}$	% helix[d]	$[\theta]_{222}$	% helix[d]
F9K/L17K	–2600	7.2	–31 200	86.1
F5K/L21K	–2900	8.0	–33 650	92.8
F9K	–2250	6.3	–32 400	89.5
F9K/L17K/A12L/A20L	–1800	5.0	–36 250	100.0
F5K	–1000	2.8	–30 800	85.0
L17K	–4050	11.1	–27 650	76.4
F9K/A12L	–4700	13.0	–34 350	94.8
L21K	–3750	10.5	–23 950	66.2
F5K/A12L	–3600	10.0	–31 200	86.2
F5K/L21K/A12L/A20L	–3950	11.0	–33 850	93.4
L17K/A20L	–3200	8.9	–23 950	66.1
P	–3600	10.0	–29 050	80.1
L21K/A20L	–3100	8.6	–25 250	69.6

[a]Peptides are ordered by relative hydrophobicity during RP-HPLC at 5 °C (Table 3).
[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, $[\theta]_{222}$ (degree·cm^2 dmol^{-1}) at wavelength 222 nm were measured at 25 °C in KP buffer diluted 1:1 (v/v) with TFE.
[d]The helical content (in percentage) of a peptide relative to the molar ellipticity value of peptide F9K/L17K/A12L/A20L in 50 % TFE.

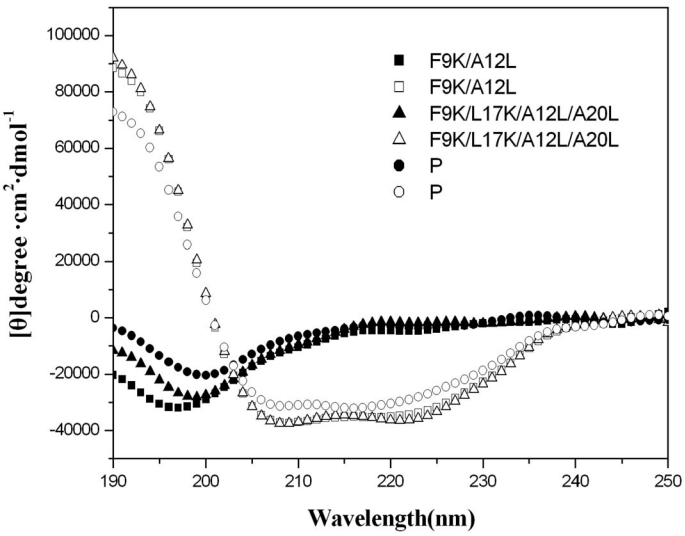

Fig. 2 CD spectra of peptide F95K/A12L, F9K/L17K/A12L/A20L, and P at pH 7 and 25 °C in 50 mM PO_4 containing 100 mM KCl. Solid symbols denote the CD spectra of peptide analogs in KP buffer without TFE, and open symbols denotes CD spectra obtained in the presence of 50 % TFE.

Peptide hydrophobicity and self-association ability

RP-HPLC retention behavior is a well-used method to represent apparent peptide hydrophobicity, and retention times of peptides are highly sensitive to the conformational status of peptides upon interaction with the hydrophobic environment of the column matrix [13]. The nonpolar face of an amphipathic α-helical peptide represents a preferred binding domain to the hydrophobic matrix of a reversed-phase column [14]. In this study, the relative hydrophobicity of peptide analogs at 5 and 80 °C is shown in Table 3. It is clear to see that most of peptide analogs with lysine substitutions resulting in the decrease of peptide hydrophobicity, except peptide L21K/A20L (L21K/A20L 38.47 min vs. peptide P 38.02 min at 5 °C, Table 3). A trend of relative hydrophobicity is clear that peptide analogs with relative central lysine substitutions exhibit greater decrease on hydrophobicity compared to those with substitutions further away from the center of the nonpolar face. For example, in Table 3, peptides F9K vs. F5K, L17K vs. L21K, F9K/L17K vs. F5K/L21K, F9K/A12L vs. F5K/A12L, L17K/A20L vs. L21K/A20L, F9K/L17K/A12L/A20L vs. F5K/L21K/A12L/A20L show the same results of the central lysine substitution, causing the greater reduction of hydrophobicity. This result is also consistent with the CD results in Table 2, that due to the stronger effect of central substitution on the peptide α-helical structure, peptides with central lysine substitution on the nonpolar face also show greater decrease on hydrophobicity during RP-HPLC. It is surprised that for peptide F9K/L17K/A12L/A20L, although the hydrophobicity of the peptide is not high, it can be induced into the strongest α-helical structure in the presence of 50 % TFE (Tables 2 and 3), which may attribute to the increase of molecule flexibility. At 80 °C, peptides exhibit the same hydrophobicity order compared to that at 5 °C, except that the values of retention time are less at high temperature due to the general temperature effect such as lower mobile-phase viscosity and a significant increase in mass transfer between the stationary and mobile phases. These effects decrease retention time with increasing temperature in a linear fashion, as shown for the random coil control peptide C (Table 3 and Fig. 3A). In Fig. 1, the number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions of each peptide analog is also shown. $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions play important role in peptide self-association and peptide apparent hydrophobicity when peptide shows as α-helical structure.

Studies have shown that peptide self-association (i.e., the ability to dimerize) in aqueous solutions is a very important parameter to understand antimicrobial activity [15]. If the self-association ability of a peptide is too strong in aqueous media, it could decrease the ability of the peptide to dissociate, pass through the capsule and cell wall of microorganisms, and penetrate into the cytoplasmic membrane to kill target cells. The ability of peptides to self-associate was determined by the technique of RP-HPLC temperature profiling [16]. Figure 3A shows the retention behavior of peptide analogs during RP-HPLC from 5 to 80 °C. The control peptide C is a monomeric random coil peptide in both aqueous and hydrophobic media; thus, its retention behavior within the temperature range of 5–80 °C represents only general temperature effects on peptide retention behavior, i.e., a linear decrease in peptide retention time with increasing temperature due to greater solute diffusivity and enhanced mass transfer between the stationary and mobile phases at higher temperatures. In this study, most of the peptide analogs show the similar retention behavior compared with peptide C, indicating that their self-associating ability is not strong in solution in general. However, even within a narrow range of self-association, peptide analogs varied dramatically on the self-associating ability from Fig. 3B. 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. 3B were defined as the peptide association parameter (P_A) to quantify the association ability of peptide analogs in solution (Table 3). As seen from the data in Table 3, P_A is not consistent with peptide relative hydrophobicity during RP-HPLC. The hydrophobicity of peptide P was altered in two ways: first, by introducing lysine or leucine, the intrinsic hydrophobicity of the side-chain was change [17]; and second, peptide hydrophobicity is influenced by the number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions, which affects the continuity of the hydrophobic surface on the nonpolar face of the peptide [18]. In this study, P_A values are influenced dramatically by the number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions, e.g., peptide

F9K/L17K shows greater self-association ability than peptide F5K/L21K with P_A values of 0.57 and 0.30, respectively; in contrast, their number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions is 3 and 2, respectively, which is consistent with the difference of P_A values. Same consistency trend can also be found between peptides L17K and L21K, F9K/A12L and F5K/A12L, F9K/L17K/A12L/A20L and F5K/L21K/A12L/A20L, etc. Although consistency between P_A values and the number of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ hydrophobic interactions existing widely among peptide analogs, it is obvious that peptide hydrophobicity and self-association ability are two different properties which can be affected from different angles.

Table 3 Relative hydrophobicity and self-association ability of peptide analogs during RP-HPLC temperature profiling.

Peptides[a]	t_R(min)[b]		P_A(min)[c]
	5 °C	80 °C	
F9K/L17K	27.29	25.20	0.55
F5K/L21K	28.40	25.91	0.28
F9K	30.67	28.63	0.66
F9K/L17K/A12L/A20L	32.05	29.76	0.39
F5K	32.13	29.63	0.41
L17K	32.68	30.44	0.69
F9K/A12L	32.92	30.81	0.69
L21K	34.06	31.93	0.81
F5K/A12L	35.15	32.97	0.69
F5K/L21K/A12L/A20L	35.48	33.37	0.88
L17K/A20L	36.41	34.14	0.81
P	38.02	36.10	0.93
L21K/A20L	38.47	36.48	1.08
C[d]	22.56	19.96	

[a]Peptides are ordered by relative hydrophobicity during RP-HPLC at 5 °C.

[b]t_R(min) denotes the retention times at 5 and at 80 °C during the temperature profiling.

[c]P_A denotes the self-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.

[d]Peptide C is a random coil control used to calculate P_A values.

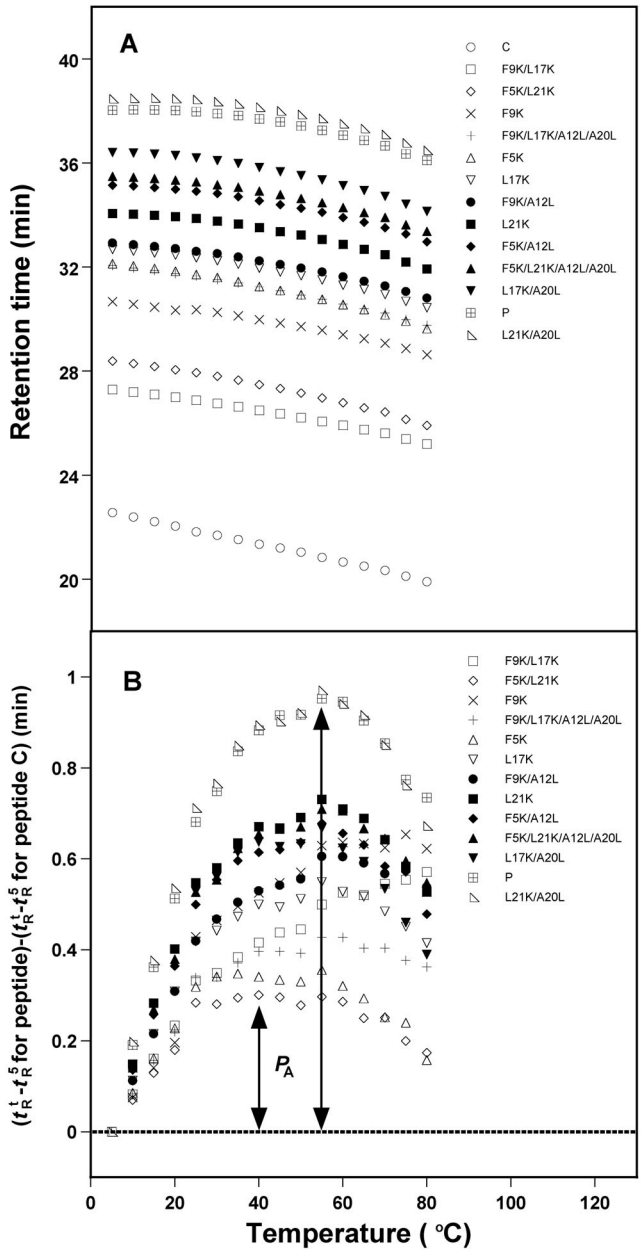

Fig. 3 RP-HPLC temperature profiles of the peptide analogs (panel A). The data are obtained as follows: RP-HPLC, Agilent Zorbax 300 SB-C_8 column (150 × 4.6-mm inner diameter, 5-µm particle size, 300-Å pore size), linear A-B gradient (1 % acetonitrile/min) at a flow rate of 1 ml/min, while eluent A is 0.1 % aqueous TFA and eluent B is 0.1 % TFA in acetonitrile. Within the temperature range of 5–80 °C, an increment of 5 °C was performed. Normalized RP-HPLC temperature profiles of peptide analogs (panel B). Column and conditions are as above A. 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 peptide analogs) minus ($t_R{}^t - t_R{}^5$ for control peptide C), where $t_R{}^t$ is the retention time at a specific temperature of an antimicrobial peptides or the random coil peptide and $t_R{}^5$ is the retention time at 5 °C.

Hemolytic activity

The hemolytic activity of the peptides against human erythrocytes was determined as a major measure of peptide toxicity toward eukaryotic cells (Table 4). In this study, by introducing lysine on the nonpolar face of the helix, we improved the peptide hemolytic activity significantly from 500 μg/ml up to no hemolysis detected at the concentration of 1000 μg/ml (determined as the MHC). As shown in the previous studies, hemolytic activity is correlated with peptide hydrophobicity [19], that is, the more hydrophobic the peptide the more hemolytic the peptide. In Table 4, peptides with either double or single lysine substitutions at different locations generally show the improvement on hemolysis against hRBCs compared to peptide P. However, for the lysine-substituting peptides with enhanced hydrophobicity by leucine substitutions, hemolytic activity varies due to the hydrophobicity change. From Table 4, it is interesting to see that peptides with alanine replacing to leucine at the position of 20 exhibit greater hemolytic activity compared to the peptide P, only three peptides are more hemolytic than peptide P as L21K/A20L, L17K/A20L, and F5K/L21K/A12L/A20L with MHC values 125, 125, and 250 μg/ml, respectively.

Table 4 Antimicrobial (MIC) and hemolytic (MHC) activities of peptide analogs against Gram-negative bacteria and hRBCs.

Peptides[a]	MIC[b]					MHC[c]	Therapeutic
	E. coli DH5α	*E. coli* ATCC25922	*P. aeruginosa* ATCC27853	*P. aeruginosa* H188	*GM*[e]	hRBC	index[d]
			μg/ml			μg/ml	
F9K/L17K	250	125	>250	16	125.7	>1000	15.9
F5K/L21K	8	8	64	4	11.3	>1000	176.8
F9K	8	8	64	4	11.3	>1000	176.8
F9K/L17K/A12L/A20L	1	0.5	8	2	1.7	500	297.3
F5K	4	8	32	4	8.0	>1000	250.0
L17K	2	2	8	4	3.4	500	148.7
F9K/A12L	4	2	8	2	3.4	1000	297.3
L21K	4	4	16	2	4.8	500	105.1
F5K/A12L	2	1	8	2	2.4	500	210.2
F5K/L21K/A12L/A20L	1	1	4	1	1.4	250	176.8
L17K/A20L	1	1	4	4	2.0	125	62.5
P	2	1	8	4	2.8	500	176.8
L21K/A20L	2	1	8	2	2.4	125	52.6

[a]Peptides are ordered by relative hydrophobicity during RP-HPLC at 5 °C.
[b]Antimicrobial activity (MIC) was determined as the minimal concentration of peptide to inhibit microbial growth. When no antimicrobial activity was observed at 250 μg/ml, a value of 500 μg/ml was used for calculation of the therapeutic index.
[c]Hemolytic activity (MHC) was determined on hRBCs. When no detectable hemolytic activity was observed at 1000 μg/ml, a value of 2000 μg/ml was used for calculation of the therapeutic index.
[d]Therapeutic index = MHC (μg/ml)/geometric mean of MIC (μg/ml). Larger values indicate greater antimicrobial specificity.
[e]GM denotes the geometric mean of MIC values from all four microbial strains in this table.

Antimicrobial activity

Antimicrobial activity of peptides, as shown in Tables 4 and 5, was determined on a diverse group of Gram-negative and -positive bacteria. In Table 4, the geometric mean MIC values from 4 microbial strains in this table were calculated to provide an overall evaluation of antimicrobial activity against Gram-negative bacteria. In this study, peptides generally exhibit high activity against Gram-negative bacteria with MIC values from 1–10 μg/ml. It is apparent that by introducing one or two lysines in the nonpolar face of peptide P, we could change the antimicrobial activity in a wide range from extremely

high activity against Gram-negative bacteria to very low activity, as geometric mean values altering from 1.4 μg/ml for peptide F5K/L21K/A12L/A20L to 125.7 μg/ml for peptide F9K/L17K (Table 4). Compared to peptide P, five peptide analogs exhibit stronger antimicrobial activity, among which peptide analogs with double lysine and leucine substitutions show the greatest improvement on antimicrobial activity against Gram-negative bacteria, indicating the importance of peptide hydrophobicity on the mechanism of action of antimicrobial peptide [20].

Table 5 Antimicrobial (MIC) and hemolytic (MHC) activities of peptide analogs against Gram-positive bacteria and hRBCs.

Peptides[a]	MIC[b]					MHC[c]	Therapeutic
	S. aureus ATCC25923	*B. subtilis* ATCC6633	*S. pneumoniae* ATCC49619	*S. epidermidis* ATCC12228	*GM*[e]	hRBC	index[d]
			μg/ml			*μg/ml*	
F9K/L17K	>250	16	32	8	37.8	>1000	52.9
F5K/L21K	>250	32	32	8	45.0	>1000	44.5
F9K	250	8	16	8	22.5	>1000	88.9
F9K/L17K/A12L/A20L	125	8	4	2	9.5	500	52.9
F5K	250	8	8	8	18.9	>1000	105.7
L17K	250	8	8	8	18.9	500	26.4
F9K/A12L	125	8	4	4	11.2	1000	88.9
L21K	250	16	16	4	22.5	500	22.2
F5K/A12L	64	8	2	2	6.7	500	74.3
F5K/L21K/A12L/A20L	32	4	4	1	4.8	250	52.6
L17K/A20L	125	16	4	2	11.2	125	11.1
P	64	4	8	4	9.5	500	52.6
L21K/A20L	125	4	4	1	6.7	125	18.7

[a]Peptides are ordered by relative hydrophobicity during RP-HPLC at 5 °C.
[b]Antimicrobial activity (MIC) was determined as the minimal concentration of peptide to inhibit microbial growth. When no antimicrobial activity was observed at 250 μg/ml, a value of 500 μg/ml was used for calculation of the therapeutic index.
[c]Hemolytic activity (MHC) was determined on hRBCs. When no detectable hemolytic activity was observed at 1000 μg/ml, a value of 2000 μg/ml was used for calculation of the therapeutic index.
[d]Therapeutic index = MHC (μg/ml)/geometric mean of MIC (μg/ml). Larger values indicate greater antimicrobial specificity.
[e]GM denotes the geometric mean of MIC values from all four microbial strains in this table.

In contrast, peptides have weak antimicrobial activity against Gram-positive bacterium *S. aureus* in this study (Table 5). The Gram-positive strain, peptides showing the highest antimicrobial activity, is *S. epidermidis.* In Table 5, the geometric mean MIC values from 4 microbial strains in this table were calculated to provide an overall evaluation of antimicrobial activity against Gram-positive bacteria. Unlike against Gram-negative bacteria, only peptides F5K/A12L, F5K/L21K/A12L/A20L, and L21K/A20L show the improvement of antimicrobial activity against Gram-positive strains compared to peptide P (Table 5). Overall, it is clear that in this study peptides show better antimicrobial activity against Gram-negative bacteria than against Gram-positive bacteria.

Peptide specificity (therapeutic Index)

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 before, peptide P is a peptide with good antimicrobial activity and low hemolytic activity; hence, its therapeutic index is high, 176.8 against Gram-negative bacteria and 52.6 against Gram-positive bacteria. In this study, by

introducing hydrophilic amino acid lysine into the nonpolar face of peptide P, we further increased the therapeutic index of peptide P to 297.3 for Gram-negative bacteria (peptides F9K/L17K/A12L/A20L and F9K/A12L) and 105.7 for Gram-positive bacteria (peptide F5K), respectively.

Table 6 summarizes the fold improvement on hemolytic activity, antimicrobial activity, and therapeutic index by introducing lysine on the nonpolar face of peptide P and utilizing leucine to compensate the loss of hydrophobicity. In Table 6, albeit the peptides F9K/L17K/A12L/A20L and F5K/L21K/A12L/A20L show the strongest antimicrobial activity against Gram-negative bacterial strains, their therapeutic indices are close to that of peptide P due to the low values on hemolytic activity. Same conclusion can be drawn for therapeutic index against Gram-positive bacteria, that peptide F5K/L21K/A12L/A20L exhibits the strongest antimicrobial activity but shows the same therapeutic index value with peptide P due to the lower value on hemolytic activity. Overall, compared to peptide P, there are six peptides showing the improved or comparable therapeutic index against both Gram-negative and -positive bacteria, F9K, F5K, F9K/A12L, F5K/A12L, F9K/L17K/A12L/A20L, and F5K/L21K/A12L/A20L (Table 6). Based on these data, it is obvious that phenylalanine is not as important as leucine on the nonpolar face of peptide P, which may attribute to leucine's stronger helical propensity than phenylalanine, hence leucine plays more important role in maintaining α-helical structure of peptides. Substituting phenylalanine with lysine can cause the improvement on hemolytic activity but also the decrease of antimicrobial activity, which can be compensated by increasing the peptide hydrophobicity of using leucine to replace alanine on the nonpolar face.

Table 6 Biological activities of peptide analogs.

Peptides[a]	Hemolytic activity		Antimicrobial activity				Therapeutic index			
			MIC							
	MHC (μg/ml)	Fold[b]	*GM*[c] (μg/ml) G^-	Fold[d]	*GM*[c] (μg/ml) G^+	Fold[d]	G^-	Fold[e]	G^+	Fold[e]
F9K/L17K	>1000	0.25	125.7	0.02	37.8	0.25	15.9	11.12	52.9	0.99
F5K/L21K	>1000	0.25	11.3	0.25	45.0	0.21	176.8	1.00	44.5	1.18
F9K	>1000	0.25	11.3	0.25	22.5	0.42	176.8	1.00	88.9	0.59
F9K/L17K/A12L/A20L	500	1	1.7	1.65	9.5	1.00	297.3	0.59	52.9	0.99
F5K	>1000	0.25	8.0	0.35	18.9	0.50	250.0	0.71	105.7	0.50
L17K	500	1	3.4	0.82	18.9	0.50	148.7	1.19	26.4	1.99
F9K/A12L	1000	0.5	3.4	0.82	11.2	0.85	297.3	0.59	88.9	0.59
L21K	500	1	4.8	0.58	22.5	0.42	105.1	1.68	22.2	2.37
F5K/A12L	500	1	2.4	1.17	6.7	1.42	210.2	0.84	74.3	0.71
F5K/L21K/A12L/A20L	250	2	1.4	2.00	4.8	1.98	176.8	1.00	52.6	1.00
L17K/A20L	125	4	2.0	1.40	11.2	0.85	62.5	2.83	11.1	4.74
P	500	1	2.8	1.00	9.5	1.00	176.8	1.00	52.6	1.00
L21K/A20L	125	4	2.4	1.17	6.7	1.42	52.6	3.36	18.7	2.81

[a]Peptides are ordered by relative hydrophobicity during RP-HPLC at 5 °C (Table 3).
[b]The fold increase in hemolytic activity was determined as relative to that of the parent peptide P. Hemolytic activity (MHC, which is the maximal peptide concentration showing no hemolysis after 4 h) was determined with hRBCs. When no detectable hemolytic activity was observed at 1000 μg/ml, a value of 2000 μg/ml was used for calculation of the therapeutic index.
[c]GM, geometric mean of the MICs of four bacterial strains.
[d]The fold improvement in antimicrobial activity (geometric mean data) was determined as relative to that of parent peptide P.
[e]The fold improvement in the therapeutic index was determined as relative to that of parent peptide P. The value was determined by the ratio of MHC (μg/ml) and geometric mean of MIC (μg/ml)

These results are also consistent with the mechanism of action "membrane discrimination mechanism" [21], in which for antimicrobial activity, peptide locates in a parallel position at the interface of lipid headgroups and hydrophobic core of membrane and takes a carpet-like mechanism to cause the lysis of bacterial membrane, in contrast, for hemolytic activity of eukaryotic cells, peptides have to penetrate vertically into the hydrophobic core to form pores or channels (like barrel-stave mechanism), thus causes the lysis of eukaryotic cells. The reason to take different mechanisms for different types of cells is due to the different lipid compositions of cytoplasmic membrane as described in the previous studies [22]. In this study, introducing lysine to the nonpolar face of peptide P results in the decrease of hydrophobicity, thus improves the peptide activity on hemolysis. However, the decrease of hydrophobicity also raises the problem that peptides are not hydrophobic enough to enter into the bacterial membrane to kill the cells. Therefore, using leucine to substitute alanine to bring peptide hydrophobicity back, we obtained peptides F9K/A12L, F5K/A12L, F9K/L17K/A12L/A20L, and F5K/L21K/A12L/A20L with improved or comparable therapeutic index with peptide P. Charge of peptides may also play an important role on biological activities. By evaluating the overall biological activities of all peptide analogs in this study, F9K/L17K/A12L/A20L and F5K/L21K/A12L/A20L show as the leading compounds with the strongest antimicrobial activity and good improvement on therapeutic index (Table 6).

It is important to note that there is a balance of peptide hydrophobicity for antimicrobial activity and hemolytic activity, that is, reducing hydrophobicity improves peptide hemolytic activity; in contrast, if one reduces peptide hydrophobicity too much, the peptide will lose its antimicrobial activity, e.g., peptides F9K/L17K and F5K/L21K. The de novo design approach of introducing hydrophilic amino acid into the nonpolar face of the peptide, then increasing peptide hydrophobicity by other amino acid substitutions can be a practical way to obtain antimicrobial peptides with great specificity.

CONCLUSIONS

Using a structure guided de novo approach to antimicrobial peptide design by introducing lysine on the nonpolar face of peptide P, we were able to develop peptide antimicrobial activity, hemolytic activity and therapeutic index. We have proved that using hydrophilic amino acid on the hydrophobic face of the helical structure can reduce peptide hemolytic activity against hRBCs; in contrast, increase peptide hydrophobicity while the remaining hydrophilic amino acid residues on the nonpolar face can improve antimicrobial activity and keep the cytotoxicity against eukaryotic cells low, thus, improve specificity. This specific rational approach of peptide design could be a powerful method to optimize antimicrobial peptides with clinical potential as peptide antibiotics.

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REFERENCES

1. (a) H. C. Neu. *Science* **257**, 1064 (1992); (b) N. Papo, Y. Shai. *Biochemistry* **42**, 458 (2003).
2. (a) M. Zasloff. *Nature* **415**, 389 (2002); (b) R. E. Hancock, R. Lehrer. *Trends Biotechnol.* **16**, 82 (1998).
3. (a) R. E. Hancock, R. Lehrer. *Trends Biotechnol.* **16**, 82 (1998); (b) Y. Shai. *Biochim. Biophys. Acta* **1462**, 55 (1999); (c) Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, R. S. Hodges. *Antimicrob. Agents Chemother.* **51**, 1398 (2007).

4. Y. Chen, C. T. Mant, S. W. Farmer, R. E. Hancock, M. L. Vasil, R. S. Hodges. *J. Biol. Chem.* **280**, 12316 (2005).
5. (a) R. M. Epand, H. J. Vogel. *Biochim. Biophys. Acta* **1462**, 11 (1999); (b) Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, R. S. Hodges. *Antimicrob. Agents Chemother.* **51**, 1398 (2007).
6. (a) Y. Chen, C. T. Mant, S. W. Farmer, R. E. Hancock, M. L. Vasil, R. S. Hodges. *J. Biol. Chem.* **280**, 12316 (2005); (b) Y. Chen, A. I. Vasil, L. Rehaume, C. T. Mant, J. L. Burns, M. L. Vasil, R. E. Hancock, R. S. Hodges. *Chem. Biol. Drug Des.* **67**, 162 (2006).
7. W. C. Chan, P. D. White. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Oxford University Press, Oxford (2000).
8. D. L. Lee, C. T. Mant, R. S. Hodges. *J. Biol. Chem.* **278**, 22918 (2003).
9. M. Stark, L. P. Liu, C. M. Deber. *Antimicrob. Agents Chemother.* **46**, 3585 (2002).
10. (a) Y. Chen, C. T. Mant, R. S. Hodges. *J. Pept. Res.* **59**, 18 (2002); (b) O. D. Monera, T. J. Sereda, N. E. Zhou, C. M. Kay, R. S. Hodges. *J. Pept. Sci.* **1**, 319 (1995); (c) N. E. Zhou, O. D. Monera, C. M. Kay, R. S. Hodges. *Protein Pept. Lett.* **1**, 114 (1994).
11. C. T. Mant, Y. Chen, R. S. Hodges. *J. Chromatogr., A* **1009**, 29 (2003).
12. O. D. Monera, T. J. Sereda, N. E. Zhou, C. M. Kay, R. S. Hodges. *J. Pept. Sci.* **1**, 319 (1995).
13. (a) Y. Chen, C. T. Mant, R. S. Hodges. *J. Pept. Res.* **59**, 18 (2002); (b) Y. Chen, C. T. Mant, S. W. Farmer, R. E. Hancock, M. L. Vasil, R. S. Hodges. *J. Biol. Chem.* **280**, 12316 (2005).
14. N. E. Zhou, C. T. Mant, R. S. Hodges. *Pept. Res.* **3**, 8 (1990).
15. (a) D. Avrahami, Y. Shai. *Biochemistry* **41**, 2254 (2002); (b) Y. Chen, C. T. Mant, S. W. Farmer, R. E. Hancock, M. L. Vasil, R. S. Hodges. *J. Biol. Chem.* **280**, 12316 (2005); (c) D. L. Lee, C. T. Mant, R. S. Hodges. *J. Biol. Chem.* **278**, 22918 (2003).
16. (a) C. T. Mant, Y. Chen, R. S. Hodges. *J. Chromatogr., A* **1009**, 29 (2003); (b) Y. Chen, C. T. Mant, S. W. Farmer, R. E. Hancock, M. L. Vasil, R. S. Hodges. *J. Biol. Chem.* **280**, 12316 (2005).
17. J. M. Kovacs, C. T. Mant, R. S. Hodges. *Biopolymers (Peptide Sci.)* **84**, 283 (2006).
18. Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, R. S. Hodges. *Antimicrob. Agents Chemother.* **51**, 1398 (2007).
19. (a) L. H. Kondejewski, D. L. Lee, M. Jelokhani-Niaraki, S. W. Farmer, R. E. Hancock, R. S. Hodges. *J. Biol. Chem.* **277**, 67 (2002); (b) Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, R. S. Hodges. *Antimicrob. Agents Chemother.* **51**, 1398 (2007).
20. (a) R. M. Epand, H. J. Vogel. *Biochim. Biophys. Acta* **1462**, 11 (1999); (b) Y. Shai. *Biochim. Biophys. Acta* **1462**, 55 (1999); (c) Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, R. S. Hodges. *Antimicrob. Agents Chemother.* **51**, 1398 (2007).
21. (a) Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, R. S. Hodges. *Antimicrob. Agents Chemother.* **51**, 1398 (2007); (b) Y. Chen, A. I. Vasil, L. Rehaume, C. T. Mant, J. L. Burns, M. L. Vasil, R. E. Hancock, R. S. Hodges. *Chem. Biol. Drug Des.* **67**, 162 (2006).
22. (a) Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, R. S. Hodges. *Antimicrob. Agents Chemother.* **51**, 1398 (2007); (b) Y. Chen, A. I. Vasil, L. Rehaume, C. T. Mant, J. L. Burns, M. L. Vasil, R. E. Hancock, R. S. Hodges. *Chem. Biol. Drug Des.* **67**, 162 (2006).

