THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 1, Issue of January 4, pp. 67–74, 2002
Printed in U.S.A.

Optimization of Microbial Specificity in Cyclic Peptides by Modulation of Hydrophobicity within a Defined Structural Framework*

Received for publication, August 14, 2001
Published, JBC Papers in Press, October 26, 2001, DOI 10.1074/jbc.M107825200

Leslie H. Kondejewski‡§, Darin L. Lee¶, Masood Jelokhani-Niaraki‡‖, Susan W. Farmer, Robert E. W. Hancock**, and Robert S. Hodges‡¶‡‡§§**

*From the ‡Protein Engineering Network of Centres of Excellence and the ¶Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada, the **Department of Microbiology and the Canadian Bacterial Diseases Network, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, and the ‡‡Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262*

In the present study we have utilized the structural framework of the analog GS14K4 (cyclo(VKL*d*-KV*d*-YPL KVKL*d*-YP, where *d* denotes a D-amino acid)), to examine the role of hydrophobicity in microbial activity and specificity. The hydrophobicity of GS14K4 was systematically altered by residue replacements in the hydrophobic sites of the molecule to produce a series of analogs that were either less or more hydrophobic than the parent compound. Circular dichroism spectroscopy and reversed-phase high performance liquid chromatography analysis showed that the molecules were structurally similar and only differed in overall hydrophobicity. The hydrophobicity of GS14K4 was found to be the midpoint for hemolytic activity, with more hydrophobic analogs exhibiting increased hemolytic activity and less hydrophobic analogs showing decreased hemolytic activity. For antimicrobial activity there were differences between the hydrophobicity requirements against Gram-positive and Gram-negative microorganisms. The hydrophobicity of GS14K4 was sufficient for maximum activity against Gram-negative microorganisms and yeast, with no further increases in activity occurring with increasing hydrophobicity. With Gram-positive microorganisms significant increases in activity with increasing hydrophobicity were seen in three of the six microorganisms tested. A therapeutic index (calculated as a measure of specificity of the peptides for the microorganisms over human erythrocytes) served to define the boundaries of a therapeutic window within which lay the optimum peptide hydrophobicity for each microorganism. The therapeutic window was found to be at a lower hydrophobicity level for Gram-negative microorganisms than for Gram-positive microorganisms, although the limits were more variable for the latter. Our results show that the balance between activity and specificity in the present cyclic peptides can be optimized for each microorganism by systematic modulation of hydrophobicity.

The cationic antimicrobial peptides represent a potentially new class of antibiotics to combat those microorganisms that have developed resistance to the traditional antibiotics, and, as such, their structure-activity relationships have been actively pursued (1, 2). Although their mode of action is not fully understood, it is well established that these peptides must interact with the plasma membrane of susceptible microorganisms, where either their accumulation in the membrane causes increased permeability and loss of barrier function or they cross the membrane to access cytoplasmic targets (2). Studies with gramicidin S have indicated that this peptide is very membrane-active (3). The concept that the site of action of certain peptides is the cell membrane (as opposed to microbial enzymes) is consistent with observations of rapid (minutes to hours), concentration-dependent action, with the development of resistant microbial strains being reduced or eliminated (4). Moreover, they have also displayed toxicity to higher eukaryotic cells, an unsurprising result if the target is indeed the cell membrane. Differences in the lipid composition and the membrane potential gradient between prokaryotic and higher eukaryotic cell membranes have been used to explain the differences in specificity of these peptides (5). Because it is believed that killing with peptides like gramicidin S occurs after peptide interaction with membranes, efforts have been made to enhance specificity to provide the greatest discrimination between prokaryotic and higher eukaryotic membranes.

Two major classes of the cationic antimicrobial peptides are the α-helical and the β-sheet peptides (6–8). The α-helical class (*e.g.* cecropins (9), magainins (10, 11), and melittin (12)) consists of linear peptides that exist as disordered structures in aqueous media and become helical upon interaction with hydrophobic solvents or phospholipid vesicles. Unlike the α-helical peptides, β-sheet peptides are cyclic peptides constrained in this conformation either by disulfide bonds (tachyplesins (13, 14), protegrins (15, 16), and polyphemeusins (17, 18)) or by cyclization of the backbone (gramicidin S (19) and tyrocidines (20)). From structure-activity relationship studies of cationic antimicrobial peptides, a number of factors important for antimicrobial and anti-eukaryotic cell activity have been identified. These include the presence of both hydrophobic and basic residues, an amphipathic nature that describes the relative

* This work was supported by the Government of Canada's Network of Centers of Excellence program supported by the Canadian Institute of Health Research, the Natural Sciences and Engineering Research Council through the Protein Engineering Network of Centers of Excellence, Inc., the Canadian Bacterial Diseases Network, and National Institutes of Health Grant GM R01GM61855 (to R. S. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Caprion Pharmaceuticals Inc., 7150 Rue Alexander-Fleming, Saint-Laurent, Quebec H4S 2C8, Canada.

‖ Present address: Department of Chemistry, Wilfrid Laurier University, Waterloo, Ontario N2L 3C5, Canada.

§§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, 4200 East 9th Ave., B121, Denver, CO 80262. Tel.: 303-315-8837; Fax: 303-315-1153; E-mail: robert.hodges@uchsc.edu.

positioning of the basic and hydrophobic residues, and secondary structure, either preformed or inducible in lipid-mimicking environments (1, 2, 21, 22). The difficulty in interpreting results from such studies is that it is difficult to systematically study one variable while holding other variables constant, because sequence changes generally lead to structural changes that affect amphipathicity. Conformationally constrained peptides have the advantage that sequence changes alter conformation to a lesser degree than in their linear counterparts and thus facilitate the dissection of the relative contributions of each variable.

In structure-activity studies on cyclic peptides related to gramicidin S (GS),[1] we have previously found that amphipathicity plays a critical role in defining the scope of biological activities in these peptides (23–25). Furthermore, it was seen that antimicrobial activity could be dissociated from hemolytic activity and that the differences between these two activities could be maximized by the selection of the appropriate amphipathicity (24). It was seen that a high amphipathicity is not desirable in these peptides, because this results in high hemolytic activity coupled with low antimicrobial activity. Through systematic reduction of amphipathicity utilizing enantiomeric substitutions within the framework of the highly amphipathic β-sheet peptide, GS14 (cyclo(VKLKV*d*-YPLKVKL*d*-YP), where *d* denotes a D-amino acid), it was possible to define the optimum amphipathicity leading to the highest therapeutic index (24). In the present study, we have utilized the structural framework of the GS14 diastereomer possessing the highest therapeutic index, GS14K4 (cyclo(VKL*d*-KV*d*-YPLKVKL*d*-YP)), and have systematically altered the hydrophobicity to define the optimal hydrophobicity leading to the greatest antimicrobial activity and specificity within this structural framework.

MATERIALS AND METHODS

Peptide Synthesis and Purification—All peptides were synthesized by solid phase peptide synthesis using *t*-butyloxycarbonyl chemistry, cleaved from the resin, purified by preparative RP-HPLC, N- to C-terminally cyclized, and repurified by preparative RP-HPLC as reported previously (23, 24). The purity of linear and cyclic peptides was verified by analytical RP-HPLC as described below and correct peptide masses verified by mass spectrometry on a Fisons VG Quattro triple quadrupole mass spectrometer (Manchester, UK) (26).

Analytical RP-HPLC—Retention times of peptides were determined by RP-HPLC on a Zorbax SB-C8 column (150 × 2.1 mm inner diameter; 5-μm particle size; 300 Å pore size; Rockland Technologies, Wilmington, DE) using a Hewlett Packard 1100 chromatograph at 25 °C with a linear AB gradient of 1% B/min (where solvent A was 0.05% aqueous trifluoroacetic acid and solvent B was 0.05% trifluoroacetic acid in acetonitrile) at a flow rate of 0.25 ml/min.

Circular Dichroism Measurements—CD spectra were recorded on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) as described (23). Spectra were recorded in either 5 mM sodium acetate buffer, pH 5.5, or 5 mM sodium acetate buffer, pH 5.5, containing 50% trifluoroethanol (TFE).

Measurement of Antibacterial and Antifungal Activity—Minimal inhibitory concentrations (MICs) were determined using a standard microtiter dilution method in LB no-salt medium as described previously (23, 27). Briefly, the cells were grown overnight at 37 °C in LB and diluted in the same medium. Serial dilutions of antibiotics 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 antibiotic concentration that inhibited growth. The bacterial strains utilized were the same as reported (23).

Measurement of Hemolytic Activity—Freshly collected human blood with heparin was centrifuged to remove the buffy coat, and the erythrocytes obtained were washed three times in 0.85% saline and stored at 4 °C. Serial dilutions of the peptides in saline were prepared in round bottom microtiter plates using 100-ml volumes. Red blood cells were diluted with saline to ½₅ packed volume of cells, and 50 μl were added to each well. The plates were incubated with rocking at 37 °C, and the concentration required for complete lysis determined visually after 24 h.

Displacement of Dansyl-polymyxin from LPS—Dansyl-polymyxin displacement from *Pseudomonas aeruginosa* LPS was measured to determine the binding of the affinity of the peptides to LPS as described previously (28). Briefly, peptides were titrated into cuvettes containing 3 μg of LPS/ml and 2.5 μM dansyl-polymyxin (~90% saturation of LPS-binding sites) in 1 ml of 5 mM sodium HEPES buffer, pH 7.0, and the decrease in fluorescence was recorded. A plot of the inverse of the percentage of inhibition as a function of the inverse of inhibitor concentration gave a value for IC_{50}, the inhibitor concentration resulting in 50% displacement of dansyl-polymyxin from LPS ($-1/x$ intercept).

Permeabilization of Outer Membranes to NPN—Permeabilization studies were carried out as described previously (29, 30). Briefly, *Escherichia coli* UB1005 cells were suspended in 5 mM sodium HEPES buffer, pH 7.0, containing 5 mM glucose and 5 mM carbonyl cyanide *m*-chlorophenylhydrazone. NPN was added to 1 ml of cells in a quartz cuvette to give a final concentration of 10 mM, and the background fluorescence was recorded. Aliquots of peptide were added to the cuvette, and fluorescence was recorded as a function of time until there was no further increase in fluorescence.

RESULTS

Peptide Design

In a previous study we defined the optimum amphipathicity leading to a high specificity for microorganisms over human erythrocytes through the use of systematic enantiomeric substitutions within the framework of a 14-residue head-to-tail cyclic peptide (24). In the present investigation we have attempted to further optimize the biological properties of the diastereomeric peptide possessing the optimum amphipathicity, GS14K4 (cyclo(VKL*d*-KV*d*-YPLKVKL*d*-YP)), by modulation of hydrophobicity. Under the assumption that this "optimum structure" leading to the optimum amphipathicity (relative distribution of hydrophobic and basic residues) is retained, we have systematically replaced the hydrophobic residues in GS14K4 to obtain peptides that are either less hydrophobic or more hydrophobic than GS14K4 (Table I). GS14K4 has two D-tyrosines, three leucines, and three valine residues in its sequence that were selected to alter hydrophobicity in the analogs used in this study. Hydrophobicity was increased relative to GS14K4 by replacing two D-Tyr residues with two D-Phe residues (peptide Y2/F2), three Val with three Leu (peptide V3/L3), or a combination of both (peptide Y2/F2, V3/L3). Hydrophobicity was reduced relative to GS14K4 by replacing three Val with three Ala (V3/A3), three Leu with three Ala (L3/A3), or all six hydrophobes with Ala (V3L3/A6).

Peptide Structure

CD Spectroscopy—The secondary structure of the peptides was evaluated by CD spectroscopy. Similar to GS, GS14 exhibited a CD spectrum with large negative ellipticities at 206 and 223 nm (Fig. 1) that is indicative of β-sheet structure in these cyclic peptides (23, 24, 31). This β-sheet conformation imparts a highly amphipathic nature to GS14 (24). The incorporation of a D-Lys residue in the framework of GS14 (GS14K4) resulted in a CD spectrum more characteristic of a disordered structure under aqueous conditions, indicating disruption of both β-sheet structure and amphipathicity of GS14 (Fig. 1*A*). The NMR solution structure of GS14K4 has recently been determined in this laboratory and has verified the disruption of ordered structure and amphipathicity and showed that the structural basis for the decreased amphipathicity of GS14K4 is the placement of D-Lys[4] on the hydrophobic face of the molecule (25). All of the GS14K4 hydrophobicity analogs, whether less or more hydrophobic, exhibited CD spectra similar to GS14K4, indicating that all possessed a similar disrupted β-sheet/β-turn conforma-

[1] The abbreviations used are: GS, gramicidin S; LPS, lipopolysaccharide; MIC, minimal inhibitory concentration; NPN, *N*-phenyl-1-napthylamine; RP-HPLC, reversed-phase high performance liquid chromatography; TFE, trifluoroethanol; dansyl, 5-dimethylamino-naphthalene-1-sulfonyl.

TABLE I
Peptide sequences, physical properties, and biological activities of peptides used in this study

Peptide	Linear sequence[a]	Peptide hydrophobicity (observed retention time)[b]	Predicted relative hydrophobicity[c]	LPS binding affinity[d]	Hemolytic activity[e]
		min	*min*	*μM*	*μg/ml*
GS14	VKLKVYPLKVKLYP	49.5	49.6	3	1.5
Y2/F2, V3/L3	LKLKLFPLKLKLFP	46.2	55.3	25	12.5
V3/L3	LKLKLYPLKLKLYP	42.5	47.9	46	25
Y2/F2	VKLKVFPLKVKLFP	42.3	49.9	59	40
GS14K4	VKLKVYPLKVKLYP	38.9	42.5	93	200
V3/A3	AKLKAYPLKAKLYP	32.2	34.4	163	>800
L3/A3	VKAKVYPAKVKAYP	28.6	29.0	243	>800
V3L3/A6	AKAKAYPAKAKAYP	21.7	20.9	291	>800

[a] Linear sequences of cyclic peptides. One-letter amino acid code is used; underlined residues represent D-amino acids.
[b] Observed retention time on RP-HPLC at 25 °C, which is a measure of peptide hydrophobicity.
[c] Calculated using the HPLC hydrophobicity scale of Sereda *et al.* (50) with values for Leu, Phe, Val, Tyr, and Ala as 8.5, 7.9, 6.7, 4.2, and 4.0, respectively. Values for Pro and L-Lys were taken from Guo and co-workers (33) of 2.0 and −2.1, respectively. The value for D-Lys that has been shown to be on the hydrophobic face was taken as −9.2 (49).
[d] Peptide concentration to displace 50% of dansyl-polymyxin B from LPS.
[e] Peptide concentration for 100% lysis of human erythrocytes. The hemolytic activity of GS was 12.5 μg/ml.

tion under aqueous conditions. In the presence of a hydrophobic structure-inducing solvent such as trifluoroethanol, the spectrum of GS14 was greatly enhanced, indicating a stabilization of the β-sheet structure (Fig. 1*B*). Also, all of the GS14K4 hydrophobicity analogs exhibited some inducibility of structure in the presence of TFE. The measured negative ellipticities in the presence of TFE showed a correlation with predicted hydrophobicity (Table I) at both 205 nm (r = 0.83) as well as at 220 nm (r = 0.80), indicating that the structure of the more hydrophobic analogs was stabilized more efficiently by TFE (data not shown). These findings suggest that TFE-mediated structure induction in these cyclic peptides is a consequence of interactions between the hydrophobic portion of the alcohol with hydrophobic groups present in the peptides. Similar hydrophobic-hydrophobic peptide-alcohol interactions have been noted with melittin, an α-helical peptide (32).

RP-HPLC Analysis—Retention time on RP-HPLC can be used as a measure of peptide hydrophobicity (33, 34); however, it is well known that the formation of a hydrophobic preferred binding domain caused by peptide secondary structure can affect peptide interactions with reversed-phase matrices (35–38). We have shown previously with a complete set of GS14 diastereomers that the observed retention times on RP-HPLC are a measure of peptide amphipathicity in such a homologous series of peptides (24, 39). This can be seen in the comparison of the observed retention times between GS14 and GS14K4 (Table I). Both of these peptides have exactly the same composition and sequence and therefore the same intrinsic hydrophobicity but differ only in structure. The 10.6-min difference in retention times between GS14 and GS14K4 (49.5 and 38.9 min, respectively) showed the differences in the ability of the peptides to form a hydrophobic preferred binding domain and at the same time segregate basic residues to the opposite side of the molecule. Retention time differences between these two molecules were therefore a measure of peptide amphipathicity and showed that GS14K4 has a substantially lower amphipathicity compared with GS14. We have attempted to modulate the hydrophobicity of the present analogs through residue substitutions in the framework of GS14K4 under the assumption that the optimum structural features leading to the optimum amphipathicity present in GS14K4 will also be present in the analogs. As shown in Table I, three of the designed analogs were more hydrophobic than GS14K4, and three analogs were less hydrophobic. A plot of the predicted relative hydrophobicity using RP-HPLC retention time data for the hydrophobicity analogs against their observed retention times (Fig. 2) yielded a linear relationship (r = 1.0), indicating that all of the analogs

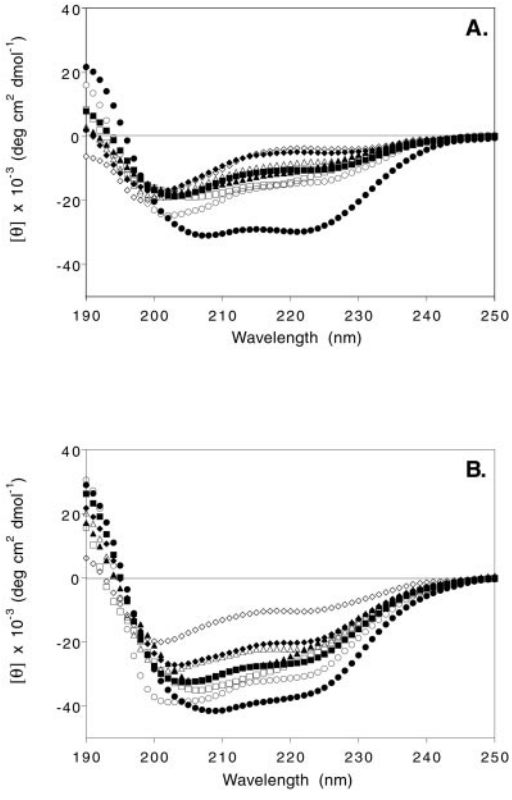

FIG. 1. **CD spectra of GS14 and GS14K4 hydrophobicity analogs.** *A*, CD spectra recorded in 5 mM sodium acetate buffer, pH 5.5, at 20 °C. The samples were GS14 (*closed circle*); GS14K4 (*open circle*); Y2/F2 (*open square*); V3/L3 (*closed square*); L3/A3 (*open triangle*); Y2/F2, V3/L3 (*closed triangle*); V3L3/A6 (*open diamond*); and V3/A3 (*closed diamond*). *B*, CD spectra recorded in 5 mM sodium acetate buffer, pH 5.5, containing 50% TFE at 20 °C. The *symbols* for the samples are identical to those for *A*.

can present their hydrophobic residues in the same orientation for interaction with the reversed-phase matrix. This indicates that the analogs possessed essentially similar structures in a hydrophobic environment and had a similar relative distribution of hydrophobic and basic residues and only differ in their intrinsic hydrophobicity.

Interaction of Peptides with Bacterial Outer Membranes

Interaction with Bacterial Lipopolysaccharide—Cationic antimicrobial peptides interact initially with negatively charged

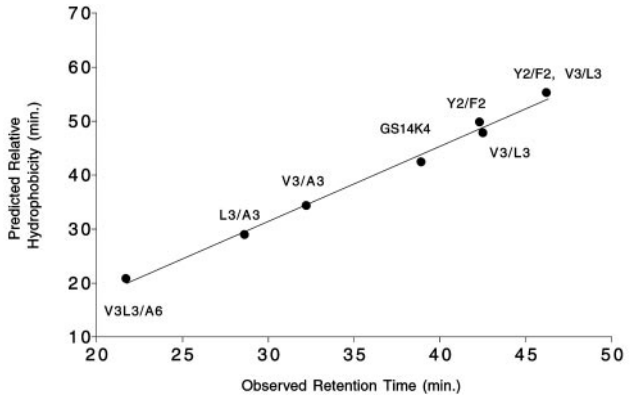

FIG. 2. **Correlation between predicted relative hydrophobicity and observed RP-HPLC retention times of GS14K4 analogs.** The predicted RP-HPLC retention times were calculated using the RP-HPLC-derived hydrophobicity scale of Guo *et al*. (34) for random coil peptides and the RP-HPLC-derived hydrophobicity scale of Sereda *et al*. (50) for α-helical peptides. The observed retention times were determined as described under "Materials and Methods."

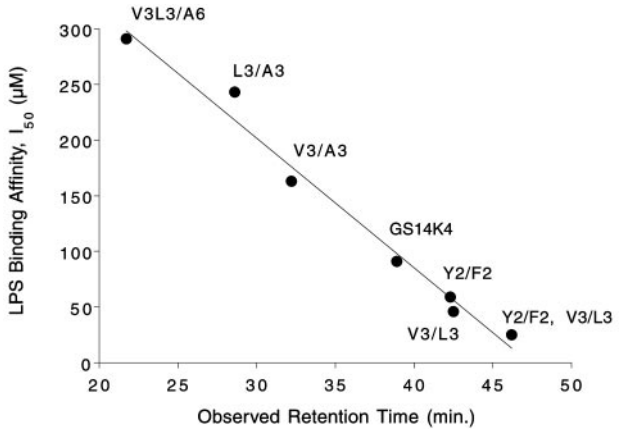

FIG. 3. **Correlation between peptide hydrophobicity and LPS binding affinity in GS14K4 analogs.** The RP-HPLC-derived retention times and LPS binding affinity listed in Table I were measured as described under "Materials and Methods."

outer membrane LPS on the surface of Gram-negative bacteria (40, 41). The interaction of the GS14K4 analogs with bacterial LPS was investigated by monitoring the displacement of LPS-bound dansyl-polymyxin B by the peptides (23, 28); the measured binding affinities are listed in Table I. There was a direct correlation ($r = 0.99$) between peptide hydrophobicity and LPS binding affinity (Fig. 3), indicating that increased peptide hydrophobicity resulted in higher binding affinity to LPS. Comparison of the LPS binding affinity of GS14 with GS14K4, two peptides that only differed in amphipathicity, shows that amphipathicity also played a large role in modulating binding affinity (Table I).

Permeabilization of Outer Membranes to NPN—Permeabilization of *E. coli* outer membranes by the analogs was monitored using the hydrophobic fluorescent probe NPN. NPN fluorescence is substantially increased when it is incorporated into the hydrophobic bacterial cell membrane (after permeabilization) compared with its fluorescence in the presence of bacterial cells under nonpermeabilizing conditions (42). Outer membrane destabilization by the GS14K4 hydrophobicity analogs is shown in Fig. 4. All of the analogs with the exception of the least hydrophobic analog (V3L3/A6) were found to exhibit similar capacities to permeabilize the outer membrane to the hydrophobic probe. Peptide V3L3/A6 was ~1/10th as effective at destabilizing the outer membrane as compared with the other analogs.

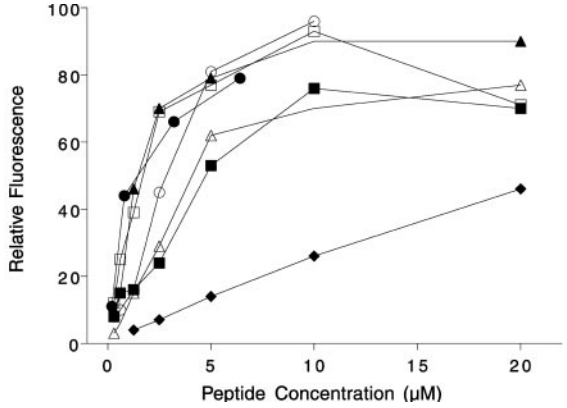

FIG. 4. **Permeabilization of *E. coli* UB1005 cells to NPN.** Peptide-mediated outer membrane destabilization was monitored by fluorescence increase because of NPN partitioning into the hydrophobic membrane interior. The samples were GS14K4 (*closed circle*); V3L3 (*open circle*); Y2/F2 (*open square*); Y2/F2, V3/L3 (*closed square*); L3/A3, (*open triangle*); V3/A3 (*closed triangle*); and V3L3/A6 (*closed diamond*).

Biological Activities of Peptides

Hemolytic Activity—The hemolytic activities of the peptides against human erythrocytes were determined as a measure of peptide toxicity toward higher eukaryotic cells (Table I). The hemolytic activity of GS14K4 is considerably lower than that of the parent compound, GS14, because of the decreased amphipathicity of GS14K4 (24). The GS14K4 hydrophobicity analogs exhibited a range of hemolytic activities, from that approaching GS14, to greatly decreased hemolytic activity (Table I). There was a correlation between the hemolytic activity and RP-HPLC-determined hydrophobicities of the analogs (Fig. 5), whereby strong hemolytic activity was seen only in those analogs that had retention times greater than 39 min (*i.e.* greater than that of GS14K4). This suggests that the hemolytic activity of the peptides is strongly influenced by the hydrophobicity of the nonpolar face of the molecule. Analogs with hydrophobicities less than GS14K4 exhibited negligible hemolytic activity.

Gram-negative Activity—The antimicrobial activities of the peptides against a number of Gram-negative microorganisms is shown in Table II, along with a therapeutic index that was calculated as a measure of specificity of the peptides for the microorganism over human erythrocytes. GS14K4 exhibited both high activity against all of the microorganisms as well as high specificity, as is evident from the high therapeutic indices. GS14, on the other hand, had essentially no activity against these microorganisms because of its high amphipathicity (24, 25). Compared with GS14K4, some of the hydrophobicity analogs exhibited similar antimicrobial activities, whereas others exhibited greatly reduced activity. We found a correlation between peptide hydrophobicity and antimicrobial activity as shown in Fig. 6 for representative Gram-negative microorganisms. It was evident that for all of the microorganisms there was a dramatic loss of antimicrobial activity below a certain hydrophobicity threshold (~32-min retention time; analog V3/A3). For most of the microorganisms, this analog represented the hydrophobicity cut-off for the peptides studied. For the majority of microorganisms, there was a limit to the maximum antimicrobial activity, with little further increase in activity obtained with increasing hydrophobicity past ~39 min (analog GS14K4). Interestingly, increasing the peptide hydrophobicity (analog V3/L3) substantially decreased antimicrobial activity against two different Gram-negative microorganisms (*P. aeruginosa* H187 and *Salmonella typhimurium* C587; Table II). Also shown in Table II and Fig. 6 are the therapeutic indices of the analogs for the Gram-negative microorganisms. It is clear from this data that there was

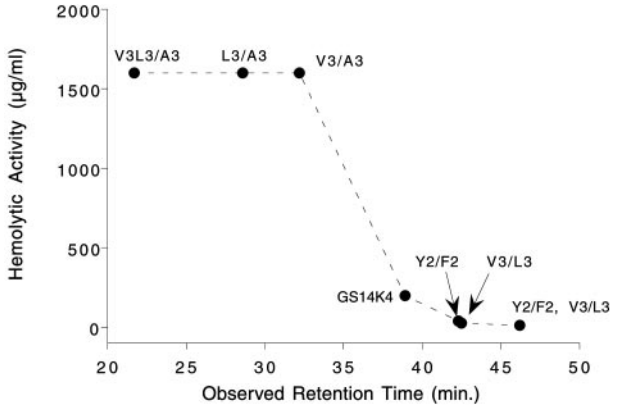

FIG. 5. **Relationship between hydrophobicity and hemolytic activity of GS14K4 analogs.** Hemolytic activity and RP-HPLC-derived retention times listed in Table I were determined as described under "Materials and Methods." Hemolytic activities reported as >800 μg/ml in Table I are plotted as 1600 μg/ml.

an optimal hydrophobicity for all of the microorganisms. The highest therapeutic indices for each organism are in bold type in Table II to highlight these differences. Of the analogs studied, V3/A3 possessed the best therapeutic indices and demonstrated an improvement over the therapeutic index of GS14K4. Although the antimicrobial activity of this peptide was reduced compared with GS14K4, the greatly reduced hemolytic activity was the dominating influence on the therapeutic index, resulting in indices that were in the range of 2–5-fold greater than those for GS14K4. The analogs on either side of this optimal peptide (peptides L3/A3 and GS14K4, with retention times of 28.6 and 38.9 min, respectively) therefore mark the boundaries of a "therapeutic window," inside of which would be predicted to lie the optimal peptide hydrophobicity for this series of peptides against Gram-negative microorganisms. The therapeutic windows are shaded in Fig. 6. Comparison of the therapeutic indices of the best analog, V3/A3, with those of GS itself shows that V3/A3 possesses therapeutic indices in the range of 20–200-fold greater than GS itself and thus represents a significant improvement in specificity. Overall, GS14K4 showed an activity that is near maximum antimicrobial activity (less than a 2-fold difference between GS14K4 and the most active compound in this series, L3/A3) and a reasonable therapeutic index among the analogs listed in this study (Table II).

Gram-positive and Antifungal Activity—The activity of the hydrophobicity analogs against a number of Gram-positive microorganisms and yeast is shown in Table III. GS14K4 exhibited strong activity against most of the tested microorganisms, coupled with a high therapeutic index. Unlike the case for Gram-negative microorganisms where maximum antimicrobial activity reached a plateau at the hydrophobicity level of GS14K4, a number of the more hydrophobic analogs exhibited substantially greater activity against some of the Gram-positive microorganisms as well as yeast. In most cases, however, GS14K4 exhibited the highest therapeutic indices because of the increased hemolytic activity of the more hydrophobic analogs. The highest therapeutic indices for each microorganism are in bold type. The Y2/F2 peptide (Table III), which increased the hydrophobicity of the two turns, had much better antimicrobial activity than GS14K4 and even with the greater hemolytic activity (Table I) still had the best therapeutic index. Peptide V3/A3, a slightly less hydrophobic peptide than GS14K4, exhibited a 4-fold increase in therapeutic index for *C. xeroses* over GS14K4. As with Gram-negative microorganisms above, a similar trend of antimicrobial activity and therapeutic index changes with hydrophobicity changes was seen against Gram-positive microorganisms and yeast (Fig. 7). In this case, however, the hydrophobicity threshold was more variable and dependent on the microorganism, but overall the threshold was shifted to slightly higher hydrophobicity values, indicating that a higher hydrophobicity was required for strong activity against Gram-positive microorganisms than for Gram-negative microorganisms. As with the Gram-negative microorganisms, a therapeutic window can be defined but centered at somewhat higher hydrophobicity values. For the majority of Gram-positive microorganisms and yeast, this optimal hydrophobicity was centered at 39 min (GS14K4), and the analogs on either side (peptides V3/A3 and Y2/F2, with retention times of 32.2 and 42.3 min, respectively) marked the boundaries of the optimum hydrophobicity for Gram-positive microorganisms and yeast in this series of peptides.

DISCUSSION

The backbone framework of the head-to-tail cyclic peptide, GS14K4, was used as a structural scaffold in the present study to optimize hydrophobicity within the amphipathic framework of GS14K4. All hydrophobicity analogs were found to possess similar backbone conformations under aqueous conditions as measured by CD spectroscopy, as well as similar side chain location in a hydrophobic environment as determined by RP-HPLC. Together, these results indicate that the amphipathicity (relative distribution of hydrophobic and basic residues) remained substantially similar in the analogs and that the analogs differed only in hydrophobicity.

In our previous study, we reported that we could modulate hemolytic activity through structural changes leading to changes in amphipathicity within the framework of GS14 (24); this can be seen in the large difference in hemolytic activities between GS14 and GS14K4 (Table I). Here we find that while maintaining the structural changes responsible for decreased amphipathicity, we could further modulate the hemolytic activity of the present peptides by specific residue replacements that either increased or decreased the hydrophobicity of the peptides. Together, these findings indicate that peptide hydrophobicity drives hemolytic activity. Within the context of a highly amphipathic molecule there was a high "directed hydrophobicity." Conversely, the present GS14K4 hydrophobicity analogs possessed similar relative distributions of hydrophobic and basic residues but differed in overall peptide hydrophobicity. Again, there appears to be a threshold hydrophobicity for the "hydrophobic patch" that was responsible for the observed hemolytic activity. Peptides that were less hydrophobic than GS14K4 possessed no hemolytic activity, whereas peptides that were more hydrophobic were considerably more hemolytic than GS14K4. High hydrophobicity would be expected to increase activity of membrane-active peptides, because increased hydrophobicity would be predicted to facilitate partitioning of a greater proportion of the peptide into the membrane. Similar findings relating hydrophobicity and hemolytic activity have been reported in linear α-helical peptides (43–45) as well as other cyclic peptides based on GS (46).

As with hemolytic activity, antimicrobial activity was also sensitive to changes in hydrophobicity. Increasing hydrophobicity also tended to result in peptides with a maximal amount of antimicrobial activity depending on the microbial strain tested, usually at the hydrophobicity of GS14K4. A systematic study of hydrophobicity effects on the activity of antimicrobial peptides has been reported previously (21), but based on amino acid sequence analysis, the majority of antimicrobial peptides identified from natural sources are amphipathic, containing hydrophobic regions that may facilitate partitioning into the cell membrane. Antimicrobial peptides with hydrophilic sequences, such as androctonin (47), may act via a different mechanism than amphipathic peptides, perhaps by a method

TABLE II
Activity of GS14K4 hydrophobicity analogs against Gram-negative microorganisms

Peptide	Minimal inhibitory concentration and therapeutic index[a]											
	P. aeruginosa H187		*P. aeruginosa* H188		*E. coli* UB1005		*E. coli* DC2		*S. typhinurium* C587		*S. typhinurium* C610	
	Activity	Index	Activity	Index	Activity	Index	Activity	Index	Activity	Index	Activity	Index
	μg/ml											
GS	20	0.6	6.2	2	6.2	2	3.1	4	20	0.6	10	1.3
GS14	>200	<0.01	>200	<0.01	>200	<0.01	200	0.01	>200	<0.01	>200	<0.01
Y2/F2, V3/L3	16	0.8	3.1	4	8.1	1.5	2	6.3	33	0.4	13	1
V3/L3	100	0.3	3.1	8	6.2	4	2.8	8.9	100	0.3	18	1.4
Y2/F2	19	2.7	3.1	16	5.6	8.9	1.5	33	8.1	6.2	3.1	16
GS14K4	25	8	3.1	65	6.2	32	3.1	65	11	18	4.1	49
V3/A3	150	**11**	18	**89**	13	**128**	9	**178**	38	**42**	6.2	**258**
L3/A3	>200	4	100	16	200	8	200	8	>200	4	100	16
V3L3/A6	>200	4	150	11	>200	4	>200	4	>200	4	>200	4

[a] Therapeutic index = hemolytic activity/minimal inhibitory concentration. For the calculation of the therapeutic index, values of 400 μg/ml were used for MIC values reported as >200 μg/ml, and values of 1600 μg/ml were used for hemolytic activity values reported as >800 μg/ml. The therapeutic index of the analog with the highest value for each microorganism is in bold type. The underlining highlights the peptide (GS14K4) with antimicrobial activity within 2-fold of the most active peptide against each organism in this study, as well as possessing the second highest therapeutic index. The hemolytic activity of GS was 12.5 μg/ml.

that does not directly involve destabilization of the integrity of the membrane lipid bilayer but alternatively interacts with DNA in the cell nucleus to interfere with transcription processes.

The outer membrane of Gram-negative microorganisms is the first cell component that antimicrobial peptides encounter. This barrier must be breached to allow access of the peptides to the inner membrane, in a process sometimes described as "self-promoted uptake" (1). A major constituent of the outer membrane is LPS, a negatively charged molecule containing numerous carbohydrate moieties anchored to the membrane by a lipid A molecule. The interaction of the highly amphipathic GS14 peptide with bacterial membranes is extremely strong, approaching the affinity of polymyxin B itself (23). Utilizing a complete set of GS14 diastereomers, we previously found a correlation between high amphipathicity, increased LPS binding affinity, and decreased antimicrobial activity (24). This observation suggests that high affinity interactions with outer membrane components may sequester peptides to the outer membrane, hinder the movement of the peptides to their presumed site of action on the inner membrane, and inhibit peptide accumulation there, thus decreasing antimicrobial activity. In the present study, we found a direct correlation between peptide hydrophobicity and outer membrane binding affinity (the positive charge distribution is constant in these analogs). Surprisingly, however, these high affinity interactions by high hydrophobicity analogs did not appear to impede the activity of the peptides in most cases, although activity against three of the six Gram-negative microorganisms tested did decrease with increasing peptide hydrophobicity (peptide V3/L3 is significantly less active than GS14K4 against *P. aeruginosa* H187, *S. typhimurium* C587, and *S. typhimurium* C610). It should be noted that GS14 has LPS binding affinity approximately 1 order of magnitude higher than the best GS14K4 hydrophobicity analogs (Table I). The GS14K4 analogs have lower amphipathicity because of the disruption of the hydrophobic patch by D-Lys at position 4. It is possible that the amphipathicity of GS14 is too high because of the four lysine residues on the hydrophilic face, which allow high affinity LPS binding and prevent subsequent membrane translocation and destabilization; the peptide merely "sits" in the interfacial region parallel to the plane of the bilayer akin to gramicidin S (48, 49) without a change in orientation. In summary, the overall amphipathicity of the GS14K4 high hydrophobicity analogs is low enough so as *not* to prevent the peptide from partitioning to the inner membrane, whereas high LPS binding affinity (in the case of

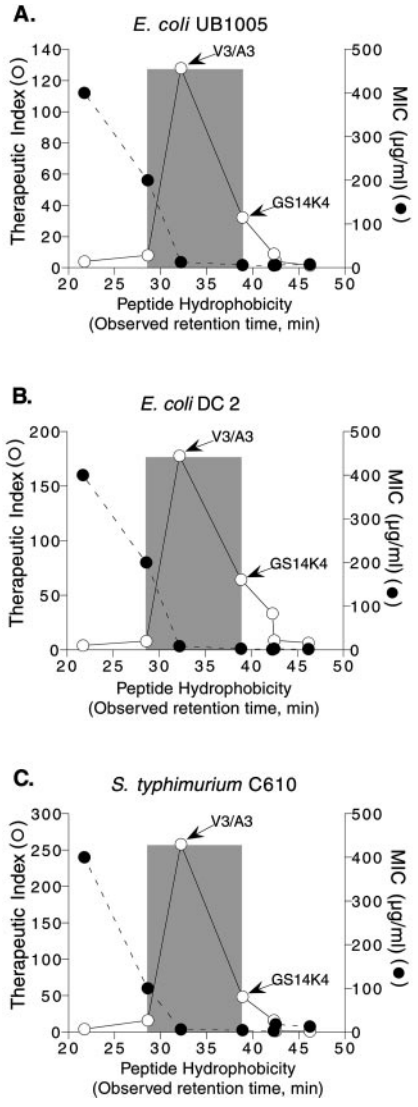

FIG. 6. **Antimicrobial activity and microbial specificity of GS14K4 hydrophobicity analogs against Gram-negative microorganisms.** The MIC (*closed circle*) and the therapeutic index (*open circle*) of the GS14K4 analogs shown in Table II are plotted as a function of peptide hydrophobicity as determined by RP-HPLC (observed retention time, Table I). *A*, *E. coli* UB1005; *B*, *E. coli* DC2; *C*, *S. typhimurium* C610. A value of 400 μg/ml was used for MIC values reported as >200 μg/ml, and a value of 1600 μg/ml was used for hemolytic activities reported as >800 μg/ml to allow calculation of the therapeutic index.

TABLE III
Antifungal and Gram-positive activity of GS14K4 hydrophobicity analogs

Peptide	Minimal inhibitory concentration and therapeutic index[a]													
	Staphylococcus aureus SAP0017		*S. aureus* K147		*Staphylococcus epidermis*		*Bacillus subtilis*		*Enterococcous faecalis*		*Corynebacterium xerosis*		*Candida albicans*	
	Activity	Index	Activity	Index	Activity	Index	Activity	Index	Activity	Index	Activity	Index	Activity	Index
	μg/ml													
GS	1.5	8.3	1.5	8.3	1.5	8.3	3.1	4	2.8	4.5	0.7	18	3.1	4
GS14	>200	<0.01	>200	<0.01	113	0.01	>200	<0.01	2.3	0.7	6.2	0.2	175	0.01
Y2/F2, V3/L3	9.4	1.3	9.4	1.3	2	6.3	6.2	2	1.5	8.3	1.2	10	3.1	4
V3/L3	13	2	23	1.1	2	13	9.4	2.7	1.5	17	1	25	4	6.3
Y2/F2	8.1	**6.2**	11	**4.4**	2.8	18	13	4.4	6.2	8.1	1.4	36	3.1	16
GS14K4	50	4	100	2	2	**100**	25	**8**	1.5	**133**	1.7	118	6.2	**32**
V3/A3	>200	4	>200	4	50	32	>200	4	>200	4	3.1	**520**	50	**32**
L3/A3	>200	4	>200	4	>200	4	>200	4	>200	4	100	16	>200	4
V3L3/A6	>200	4	>200	4	>200	4	>200	4	>200	4	150	10	>200	4

[a] Therapeutic index = hemolytic activity/antibacterial activity. For the calculation of the therapeutic index, values of 400 μg/ml were used for MIC values reported as >200 μg/ml, and values of 1600 μg/ml were used for hemolytic activity values reported as >800 μg/ml. The therapeutic index of the analog with the highest value for each microorganism is in bold type. The underlined values highlight the antimicrobial activity of peptides Y2/F2 and GS14K4, which have the highest therapeutic index or second highest therapeutic index for comparison. The hemolytic activity of GS was 12.5 μg/ml.

GS14), as a result of high amphipathicity, may preclude this partitioning.

Although only Gram-negative bacteria possess outer membranes, the NPN permeabilization studies reveal important characteristics of the GS14K4 hydrophobicity analogs. Because all peptides except the least hydrophobic analog, V3L3/A6, permeabilized the outer membrane, this result supports the idea that this series of peptides must possess a minimal hydrophobicity to penetrate the bacterial outer membrane, move through the periplasmic space, and interact with the inner membrane to kill bacteria. Peptide V3L3/A6 could not permeabilize the outer membrane as effectively and thus was not antimicrobial. This model could apply to both Gram-positive and Gram-negative bacteria because hydrophobic peptide-lipid interactions would certainly be required in both membrane systems to facilitate bacteriocidal effects. Similarly, V3L3/A6 was also not hemolytic, probably because it lacked the minimal hydrophobicity required for partitioning into lipid membranes and for membrane-destabilizing interactions necessary to promote cell lysis.

To maximize therapeutic index, the optimum hydrophobicity will likely differ slightly between target microorganisms and for other antimicrobial peptides with different structures. Based on therapeutic index values, the peptides L3/A3 and GS14K4, with retention times of 28.6 and 38.9 min, respectively, mark the lower and upper hydrophobicity limits of a therapeutic window, inside of which would be predicted to lie the optimal peptide hydrophobicity for this series of GS14K4 analogs against Gram-negative organisms. But against Gram-positive organisms, this therapeutic window shifts to the higher hydrophobicities between peptides V3/A3 and Y2/F2 (retention times 32.2 and 42.3 min, respectively) because of the higher hydrophobicity required for activity against these microorganisms. Designing peptides with smaller incremental changes in hydrophobicity within these therapeutic windows would allow one to arrive at the optimal peptide hydrophobicity for any given microorganism.

CONCLUSIONS

Utilizing a rational approach to antimicrobial peptide design, we first identified an appropriate amphipathicity leading to the optimum antimicrobial properties coupled with weak hemolytic activity (GS14K4). Working within this structural framework, we have now further optimized the activities and specificities of these peptides through incremental changes in the intrinsic hydrophobicity of the peptide. These changes in intrinsic hydrophobicity were accomplished through manipula-

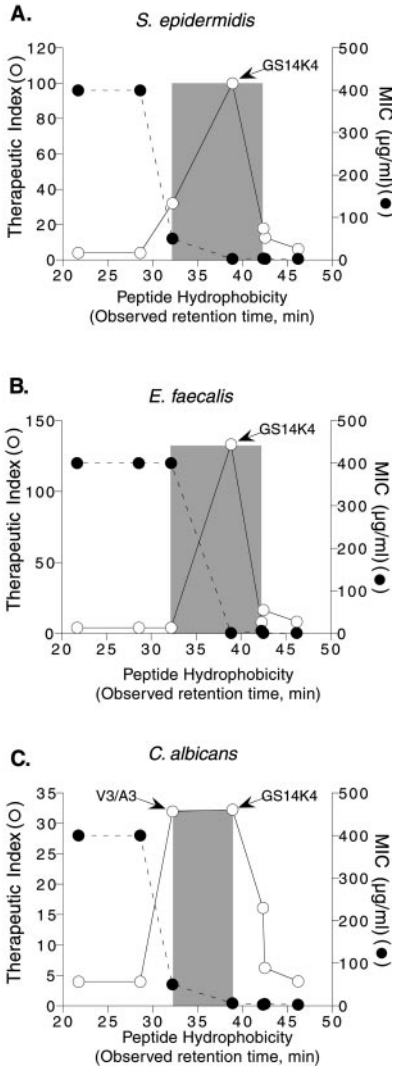

FIG. 7. **Antimicrobial activity and microbial specificity of GS14K4 hydrophobicity analogs against Gram-positive microorganisms and yeast.** The minimal inhibitory concentration (*closed circle*) and the therapeutic index (*open circle*) of the GS14K4 analogs shown in Table III are plotted as a function of peptide hydrophobicity as determined by RP-HPLC (observed retention time, Table I). *A*, *S. epidermidis*; *B*, *E. faecalis*; *C*, *C. albicans*. A value of 400 μg/ml was used for minimal inhibitory concentration values reported as >200 μg/ml, and a value of 1600 μg/ml was used for hemolytic activities reported as >800 μg/ml to allow calculation of the therapeutic index.

tion of the nature of the hydrophobic residues and result in changes in the effective hydrophobicity of the peptides existing in a defined structure. We find that both hemolytic activity and affinity for bacterial outer membranes decreased with decreasing effective hydrophobicity. Through such incremental changes in hydrophobicity we were able to define the limits of an optimal therapeutic window for each microorganism, where both hemolytic and antimicrobial activities are optimized resulting in the greatest specificity. This therapeutic window was found to be at a slightly higher effective hydrophobicity for Gram-positive microorganisms compared with Gram-negative microorganisms and yeast.

Acknowledgments—We thank Marc Genest and Jennifer Labrecque for assistance with peptide synthesis and purification and Bob Luty for CD measurements.

REFERENCES

1. Hancock, R. E. (1997) *Lancet* **349,** 418–422
2. Hancock, R. E. W., and Lehrer, R. (1998) *Trends Biotechnol.* **16,** 82–88
3. Zhang, L., Dhillon, P., Yan, H., Farmer, S., and Hancock, R. E. (2000) *Antimicrob. Agents Chemother.* **44,** 3317–3321
4. Oren, Z., and Shai, Y. (2001) in *Development of Novel Antimicrobial Agents: Emerging Strategies* (Lohner, K., ed) pp. 183–204., Horizon Scientific Press, Wymondham, UK
5. Matsuzaki, K., Sugishita, K., Fujii, N., and Miyajima, K. (1995) *Biochemistry* **34,** 3423–3429
6. van't Hof, W., Veerman, E. C., Helmerhorst, E. J., and Amerongen, A. V. (2001) *Biol. Chem.* **382,** 597–619
7. Andreau, D., and Rivas, L. (1998) *Biopolymers* **47,** 415–433
8. Matsuzaki, K. (1999) *Biochim. Biophys. Acta* **1462,** 1–10
9. Holak, T. A., Engstrom, A., Kraulis, P. J., Lindeberg, G., Bennich, H., Jones, T. A., Gronenborn, A. M., and Clore, G. M. (1988) *Biochemistry* **27,** 7620–7629
10. Zasloff, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5449–5453
11. Bechinger, B., Zasloff, M., and Opella, S. J. (1992) *Biophys. J.* **62,** 12–14
12. Bazzo, R., Tappin, M. J., Pastore, A., Harvey, T. S., Carver, J. A., and Campbell, I. D. (1988) *Eur. J. Biochem.* **173,** 139–146
13. Nakamura, T., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T., Iwanaga, S., Niwa, M., Takao, T., and Shimonishi, Y. (1988) *J. Biol. Chem.* **263,** 16709–16713
14. Kawano, K., Yoneya, T., Miyata, T., Yoshikawa, K., Tokunaga, F., Terada, Y., and Iwanaga, S. (1990) *J. Biol. Chem.* **265,** 15365–15367
15. Harwig, S. S., Swiderek, K. M., Lee, T. D., and Lehrer, R. I. (1995) *J. Pept. Sci.* **1,** 207–215
16. Storici, P., and Zanetti, M. (1993) *Biochem. Biophys. Res. Commun.* **196,** 1363–1368
17. Miyata, T., Tokunaga, F., Yoneya, T., Yoshikawa, K., Iwanaga, S., Niwa, M., Takao, T., and Shimonishi, Y. (1989) *J. Biochem.* (*Tokyo*) **106,** 663–668
18. Zhang, L., Scott, M. G., Yan, H., Mayer, L. D., and Hancock, R. E. (2000) *Biochemistry* **39,** 14504–14514
19. Khaled, M. A., Urry, D. W., Sugano, H., Miyoshi, M., and Izumiya, N. (1978) *Biochemistry* **17,** 2490–2494
20. Kuo, M. C., and Gibbons, W. A. (1980) *Biophys. J.* **32,** 807–836
21. Pathak, N., Salas-Auvert, R., Ruche, G., Janna, M. H., McCarthy, D., and Harrison, R. G. (1995) *Proteins* **22,** 182–186
22. Izumiya, N., Kato, T., Aoyagi, H., Waki, M., and Kondo, M. (1979) *Synthetic Aspects of Biologically Active Cyclic Peptides: Gramicidin S and Tyrocidines*, Halsted Press, New York, pp. 49–89
23. Kondejewski, L. H., Farmer, S. W., Wishart, D. S., Kay, C. M., Hancock, R. E., and Hodges, R. S. (1996) *J. Biol. Chem.* **271,** 25261–25268
24. Kondejewski, L. H., Jelokhani-Niaraki, M., Farmer, S. W., Lix, B., Kay, C. M., Sykes, B. D., Hancock, R. E., and Hodges, R. S. (1999) *J. Biol. Chem.* **274,** 13181–13192
25. McInnes, C., Kondejewski, L. H., Hodges, R. S., and Sykes, B. D. (2000) *J. Biol. Chem.* **275,** 14287–14294
26. Lavigne, P., Crump, M. P., Gagne, S. M., Hodges, R. S., Kay, C. M., and Sykes, B. D. (1998) *J. Mol. Biol.* **281,** 165–181
27. Kondejewski, L. H., Farmer, S. W., Wishart, D. S., Hancock, R. E., and Hodges, R. S. (1996) *Int. J. Pept. Protein Res.5* **47,** 460–466
28. Moore, R. A., Bates, N. C., and Hancock, R. E. (1986) *Antimicrob. Agents Chemother.* **29,** 496–500
29. Ono, S., Lee, S., Kodera, Y., Aoyagi, H., Waki, M., Kato, T., and Izumiya, N. (1987) *FEBS Lett.* **220,** 332–336
30. Hancock, R. E., and Farmer, S. W. (1993) *Antimicrob. Agents Chemother.* **37,** 453–456
31. Gibbs, A. C., Kondejewski, L. H., Gronwald, W., Nip, A. M., Hodges, R. S., and Wishart, D. S. (1999) *Nat. Struct. Biol.* **5,** 284–288
32. Hirota, N., Mizuno, K., and Goto, Y. (1998) *J. Mol. Biol.* **275,** 365–378
33. Parker, J. M., Guo, D., and Hodges, R. S. (1986) *Biochemistry* **25,** 5425–5432
34. Guo, D., Mant, C. T., Taneja, A. K., Parker, J. M. R., and Hodges, R. S. (1986) *J. Chromatogr.* **359,** 499–517
35. Zhou, N. E., Mant, C. T., and Hodges, R. S. (1990) *Pept. Res.* **3,** 8–20
36. Rothemund, S., Krause, E., Beyermann, M., Dathe, M., Bienert, M., Hodges, R. S., Sykes, B. D., and Sonnichsen, F. D. (1996) *Pept. Res.* **9,** 79–87
37. Krause, E., Beyermann, M., Fabian, H., Dathe, M., Rothemund, S., and Bienert, M. (1996) *Int. J. Pept. Protein Res.* **48,** 559–568
38. Wishart, D. S., Kondejewski, L. H., Semchuk, P. D., Sykes, B. D., and Hodges, R. S. (1996) *Lett. Peptide Sci.* **3,** 53–60
39. Mant, C. T., Kondejewski, L. H., and Hodges, R. S. (1998) *J. Chromatogr.* **816,** 79–88
40. Hancock, R. E. (1981) *J. Antimicrob. Chemother.* **8,** 429–445
41. Nicas, T. I., and Hancock, R. E. (1983) *J. Gen. Microbiol.* **129,** 509–517
42. Loh, B., Grant, C., and Hancock, R. E. (1984) *Antimicrob. Agents Chemother.* **26,** 546–551
43. Wieprecht, T., Dathe, M., Beyermann, M., Krause, E., Maloy, W. L., MacDonald, D. L., and Bienert, M. (1997) *Biochemistry* **36,** 6124–6132
44. Dathe, M., Wieprecht, T., Nikolenko, H., Handel, L., Maloy, W. L., MacDonald, D. L., Beyermann, M., and Bienert, M. (1997) *FEBS Lett.* **403,** 208–212
45. Juvvadi, P., Vunnam, S., Merrifield, E. L., Boman, H. G., and Merrifield, R. B. (1996) *J. Pept. Sci.* **2,** 223–232
46. Jelokhani-Niaraki, M., Kondejewski, L. H., Farmer, S. W., Hancock, R. E., Kay, C. M., and Hodges, R. S. (2000) *Biochem. J.* **349,** 747–755
47. Hetru, C., Letellier, L., Oren, Z., Hoffmann, J. A., and Shai, Y. (2000) *Biochem. J.* **345,** 653–664
48. Prenner, E. J., Lewis, R. N., Kondejewski, L. H., Hodges, R. S., and McElhaney, R. N. (1999) *Biochim. Biophys. Acta* **1417,** 211–223
49. Prenner, E. J., Lewis, R. N., Jelokhani-Niaraki, M., Hodges, R. S., and McElhaney, R. N. (2001) *Biochim. Biophys. Acta* **1510,** 83–92
50. Sereda, T. J., Mant, C. T., Sonnichsen, F. D., and Hodges, R. S. (1994) *J. Chromatogr.* **676,** 139–153