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Modulation of Structure and Antibacterial and Hemolytic Activity by Ring Size in Cyclic Gramicidin S Analogs*

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We have evaluated the effect of ring size of gramicidin S analogs on secondary structure, lipid binding, lipid disruption, antibacterial and hemolytic activity. Cyclic analogs with ring sizes ranging from 4 to 14 residues were designed to maintain the amphipathic character as found in gramicidin S and synthesized by solid phase peptide synthesis. The secondary structure of these peptides showed a definite periodicity in β-sheet content, with rings containing 6, 10, and 14 residues exhibiting β-sheet structure, and rings containing 8 or 12 residues being largely disordered. Peptides containing 4 or 6 residues did not bind lipopolysaccharide, whereas longer peptides showed a trend of increasing binding affinity for lipopolysaccharide with increasing length. Destabilization of *Escherichia coli* outer membranes was only observed in peptides containing 10 or more residues. Peptides containing fewer than 10 residues were completely inactive and exhibited no hemolytic activity. The 10-residue peptide showed an activity profile similar to that of gramicidin S itself, with activity against Gram-positive and Gram-negative microorganisms as well as yeast, but also showed high hemolytic activity. Differential activities were obtained by increasing the size of the ring to either 12 or 14 residues. The 14-residue peptide showed no antibiotic activity but exhibited increased hemolytic activity. The 12-residue peptide lost activity against Gram-positive bacteria, retained activity against Gram-negative microorganisms and yeast, but displayed decreased hemolytic activity. Biological activities in the 12-residue peptide were optimized by a series of substitutions in residues comprising both hydrophobic and basic sites resulting in a peptide that exhibited activities comparable with gramicidin S against Gram-negative microorganisms and yeast but with substantially lower hemolytic activity. Compared with gramicidin S, the best analog showed a 10-fold improvement in antibiotic specificity for Gram-negative microorganisms and a 7-fold improvement in specificity for yeast over human erythrocytes as determined by a therapeutic index. These results indicate that it is possible to modulate structure and activities of cyclic gramicidin S analogs by varying ring sizes and further show the potential for developing clinically useful antibiotics based on gramicidin S.

The emergence of many medically relevant resistant strains of bacteria today is a major issue in human health (1). It is therefore becoming essential that new therapeutic agents be developed to combat microorganisms resistant to traditional antibiotics. We have chosen the cyclic peptide antibiotic gramicidin S (GS)[1] as our model for the basis of designing novel therapeutic agents. GS is a naturally occurring cyclic peptide (cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$) first isolated from *Bacillus brevis* by Gause and Brazhnikova (2) over 50 years ago. The use of GS analogs as antibiotics have many attractive features. These include their broad spectrum antibiotic properties (3), their small size, and hence ease of generating large amounts synthetically, as well as their simplicity in structure. Development of resistance to GS analogs is also unlikely due to the fact that the target of these analogs is the cell membrane of sensitive microorganisms, although their mechanism of action is still not well understood (4). Furthermore, GS analogs would be predicted to be stable *in vivo* as only two proteases are known to degrade GS (5, 6).

The x-ray and NMR studies of GS have confirmed that it forms a two-stranded antiparallel β-sheet structure with the strands fixed in place by two type II′ β-turns (7–9), a structure first proposed by Hodgkin and Oughton (10) in 1957 based on crystallographic data. Since its isolation in 1944, more than 200 analogs of this 10-residue peptide have been synthesized with the aim of better defining its structure-activity relationships as well as extending the activity of the compound (7, 11). From these studies, a number of structural requirements believed to be important for GS activity have been determined. These include (i) the requirement for an amphipathic structure containing basic residues on the hydrophilic face of the molecule (4, 7), (ii) a β-sheet structure, or the ability to achieve a β-sheet structure in the presence of lipid bilayers (7, 12), and (iii) a high overall hydrophobicity (3, 13, 14).

We have recently reported that the long held belief that GS is active against Gram-positive and not Gram-negative bacteria is a result of the type of assay used to measure antibacterial activity (3). The vast majority of researchers have employed agar-based assays that severely underestimate antibacterial activity against Gram-negative microorganisms. We have found that GS is active against both Gram-positive and Gram-negative microorganisms when tested using liquid-based antibacterial assays. Due to the fact that GS possesses the ability to lyse eukaryotic cells, its use as an antibiotic has been restricted to that of a topical antibiotic (15). To be useful as a broad

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[1] The abbreviations used are: GS, gramicidin S; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; MIC, minimum inhibitory concentration; NPN, 1-*N*-phenylnaphthylamine; wt, wild type; Orn, ornithine; Boc, *t*-butyloxycarbonyl.

spectrum antibiotic, it would be necessary to dissociate antieukaryotic activity from antibacterial activity. Studies with other cationic peptides have suggested this is possible (16, 17). In a previous study we found that hemolytic activity of a number of GS analogs closely paralleled antibacterial activity in liquid-based assays (3). However, one analog containing a D-His for D-Phe substitution showed increased hemolytic activity while possessing decreased antibacterial activity. It has also been reported that GS analogs containing more than 10 residues exhibit a change in activity profile (*i.e.* a change from Gram-positive specificity to Gram-negative specificity) when evaluated using agar-based assays (12, 18). Furthermore, these extended analogs were reported to possess less hemolytic activity than GS itself (12). A comparison between GS and another basic amphipathic cyclic β-sheet peptide, tachyplesin I, showed that both possessed antibacterial activity; however, tachyplesin I exhibited less hemolytic activity than GS (19). Similar results have been seen with helical peptides such as melittin and magainin 2 (20), where both peptides exhibit similar antibacterial properties but have different hemolytic activities. Together, these findings indicate that it should be possible to develop GS analogs with reduced hemolytic activity while retaining substantial antibacterial activity.

To date, no systematic study has been undertaken to reduce hemolytic activity while retaining antibacterial activity in GS analogs. Furthermore, hemolytic activity of GS analogs has only been evaluated in a few cases. Development toward a more clinically useful antibiotic requires a systematic study in order to define the features responsible for both hemolytic and antibacterial activity. Owing to the lack of understanding regarding the features responsible for specific biological properties in GS analogs, we have undertaken to better define the role of ring size in the present study. We show that hemolytic and antibacterial activities can be dissociated by selecting analogs with appropriate ring sizes and that desired activities can be optimized by specific residue replacements.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The following bacterial strains were utilized: *Escherichia coli* UB1005 (wt) and its antibiotic super-susceptible derivative DC2 (21); *E. coli* SC9251 and its polymyxin B- resistant mutant pmrA SC9252 (22); *Pseudomonas aeruginosa* H187 (wt) (23); methicillin-sensitive *Staphylococcus aureus* K147 (24); *S. aureus* SAP0017, a methicillin-resistant clinical isolate from Dr. A. Chow (University of British Columbia); *Bacillus subtilis*, an environmental wt lab strain. *Enterobacter faecalis* are ATCC 29212 cells, and *Staphylococcus epidermidis* was a clinical isolate from Dr. D. Speert (University of British Columbia). Antifungal activity was tested using a clinical lab isolate of *Candida albicans*.

Peptide Synthesis and Purification—All peptides were synthesized by solid phase peptide synthesis using precoupled Boc-Pro-phenylacetamidomethyl resin (Novabiochem, San Diego, CA) on an Applied Biosystems model 430A peptide synthesizer (Foster City, CA) using standard *t*-butyloxycarbonyl chemistry (25) as reported previously (26). Side chain protecting groups were 2-bromobenzyloxycarbonyl for tyrosine and formyl for lysine and ornithine. Side chain formylation was carried out by the procedure of Kitagawa *et al.* (27) using either N^{α}-Boc-lysine or N^{α}-Boc-ornithine. Peptides were cleaved from the resin using anhydrous hydrogen fluoride (20 ml/g resin) in the presence of 10% anisole for 1 h at −5 °C. Peptides were extracted from the resin with glacial acetic acid and lyophilized. Crude linear peptides were purified by reversed-phase HPLC on a Synchropak RP-4 preparative C8 column (250 × 21.2 mm inner diameter, 6.5-μm particle size, 300-Å pore size) (Synchrom, Lafayette, IN) using a Beckman System Gold HPLC system (San Ramon, CA). The flow rate was 5 ml/min with a linear AB gradient of 0.25% B/min where solvent A was 0.05% trifluoroacetic acid/H_2O and solvent B was 0.05% trifluoroacetic acid/acetonitrile. Purity of peptides was verified by analytical reversed-phase HPLC on a Zorbax SB-C8 column (250 × 4.6 mm inner diameter, 5-μm particle size, 300-Å pore size) (Rockland Technologies, Wilmington, DE) using a Hewlett-Packard 1090 chromatograph with a linear AB gradient of 2% B/min and a 1 ml/min flow rate. Identity of peptides was confirmed by mass spectrometry on a Fisons VG Quattro triple quadrupole mass spectrometer (Manchester, United Kingdom) fitted with an electrospray ionization source operating in positive ion mode.

Cyclization of Peptides—Pure linear formylated peptides were cyclized at a concentration of 2 mg/ml in *N*,*N*-dimethylformamide using 3 eq of each of benzotriazolyl *N*-oxytri-dimethylamino-phosphonium hexafluorophosphate, 1-hydroxybenzotriazole, and diisopropylethylamine. The progress of the cyclization reaction was monitored by analytical reversed-phase HPLC and was typically complete after 12 h. Cyclic peptides were deformylated (10% HCl in methanol, 37 °C for 24 h) and purified by preparative reversed-phase HPLC. Purified cyclic peptides were homogeneous by analytical reversed-phase HPLC and gave correct primary ion molecular weights by mass spectrometry as well as appropriate amino acid analysis ratios. Peptide concentration used for all assays was based on weight and may underestimate the actual amount of peptide.

Circular Dichroism Measurements—Circular dichroism spectra were recorded on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) equipped with a Jasco DP-500N data processor. The instrument was routinely calibrated with an aqueous solution of *d*-10-(+)-camphorsulfonic acid at 290.5 nm. Ellipticity is reported as mean residue ellipticity ($[\theta]$) in degrees·cm^2/dmol calculated from the following equation: $[\theta] = [\theta]_{obs}(mrw)/10lc$, where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue weight, c is the peptide concentration in g/ml, and l is the optical path length of the cell in cm. Spectra were recorded in 10 mM sodium acetate buffer, pH 5.5, and were the average of four scans obtained by collecting data at 0.1-nm intervals between 255 and 190 nm.

Molecular Modeling—Models of ring size analogs were built using Insight II (Biosym Technologies Inc., San Diego, CA) on a Silicon Graphics workstation. Models of the 10-, 12-, and 14-residue peptides were constructed by specifying standard antiparallel β-sheet ϕ, ψ values of −139° and 135°, respectively (28). Two type II′ β-turns were incorporated into each structure designating D-Tyr and Pro residues as residues $i + 1$ and $i + 2$ of the turns. Dihedral angles (ϕ, ψ) used for the turns were 60°, −120°, and −80°, 0° for $i + 1$ and $i + 2$ residues, respectively (28). The N termini of the analogs was arbitrarily chosen as a residue contained within the strands for the models only; chemically synthesized peptides used Pro as the C terminus in all cases.

Measurement of Antibacterial and Antifungal Activity—MICs were determined using a standard microtiter dilution method in LB no-salt medium (10 g of tryptone and 5 g of yeast extract per liter). Briefly, 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 overnight and MICs determined as the lowest antibiotic concentration that inhibited growth.

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-μl volumes. Red blood cells were diluted with saline to 1/25 packed volume of cells and 50 μl added to each well. Plates were incubated with rocking at 37 °C, and the concentration required for complete lysis was determined visually after 4 h.

Permeabilization of Outer Membranes to NPN—Permeabilization studies were carried out as described previously (3). Briefly, *E. coli* SC9251 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 recorded. Aliquots of peptide were added to the cuvette and fluorescence recorded as a function of time until there was no further increase in fluorescence. A fresh cuvette of cells with NPN was used for each concentration for each peptide, and control experiments were performed to demonstrate that enhanced fluorescence was due to uptake of NPN into cells, as described previously (29–31).

Displacement of Dansyl-polymyxin from LPS—Dansyl-polymyxin displacement from *P. aeruginosa* LPS was measured as described previously (32). Briefly, peptides were titrated into cuvettes containing 3 μg of LPS/ml and 2.5 μM dansyl-polymyxin (approximately 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 percent inhibition as a function of the inverse of inhibitor concentration gave a value for I_{50}, the inhibitor concentration resulting in 50% displacement of dansyl-polymyxin from LPS ($-1/x$ intercept).

TABLE I
Sequences and biological and physical properties of cyclic GS ring analogs

Peptide	Linear sequence[a]	Retention time[b]	Antibiotic activity (μg/ml)[c] and therapeutic index[d]						Hemolytic activity[e]	LPS binding activity[f]	β-Sheet content[g]
			Gram-positive		Gram-negative		Yeast				
			Activity	Index	Activity	Index	Activity	Index			
		min							*μg/ml*	*μM*	
GS	FPVOLFPVOL	33.2	5	7.8	15	2.6	9	4.3	39	295	+
GS4	YPYP	16.5	>200		>200		>200		>800		−
GS6	YPKYPK	15.1	>200		>200		>200		>1600		+
GS8	YPVKYPKL	21.2	>200		>200		>200		>1600	820	−
GS10	YPVKLYPVKL	29.5	8	8.4	17	3.9	13	5.2	67	259	+
GS12	YPVKLKYPKVKL	22.3	240	1.0	32	7.8	42	6.0	250	46	−
GS14	YPVKLKVYPLKVKL	29.1	270	0.02	330	0.02	230	0.03	6	3	+
GS12LV	YPLKVKYPKLKV	22.2	240	0.4	25	3.7	50	1.8	92	33	−
GS12F	FPVKLKFPKVKL	24.3	110	3.6	16	25	13	30	400	127	−
GS12FO	FPVOLOFPOVOL	24.8	120	1.3	16	10	16	10	160	105	−
GS12FO/LL	FPLOLOFPOLOL	26.1	42	4.0	18	9.4	16	11	170	44	−

[a] Linear sequences of cyclic peptides. Underlined residues represent D-amino acids. O is ornithine.
[b] Retention time on reversed-phase HPLC.
[c] Average activity calculated from Table II.
[d] Therapeutic index = hemolytic activity/antibiotic activity.
[e] Hemolytic activity determined using human erythrocytes.
[f] Peptide concentration to displace 50% of dansyl-polymyxin from LPS as calculated from Fig. 3.
[g] Determined by CD spectroscopy as shown in Fig. 1.

RESULTS

Design of Cyclic GS Analogs—We have synthesized cyclic GS analogs ranging in ring size from 4 to 14 residues as shown in Table I. The peptides were designed to incorporate the alternating hydrophobic-hydrophilic residue pattern found in GS, where Val and Leu residues make up the hydrophobic face of the molecule and Orn residues constitute the hydrophilic face. This was accomplished by increasing the length of the ring by successively incorporating either hydrophobic (Val and Leu) or hydrophilic (Lys or Orn) residues. Peptides used for preliminary screening also incorporated additional substitutions, namely the replacement of D-Tyr for D-Phe for increased solubility and Lys for Orn. Previous studies have shown that these homologous substitutions lead to minimal changes in both structure and activity of GS analogs (3, 7). Because of the unusual activity exhibited by the 12-residue peptide, native residues were systematically incorporated into it in order to determine the importance of each in antibiotic and hemolytic activity.

CD Spectroscopy—As shown previously (33), the CD spectra of GS and various GS-related 10-residue cyclic peptides do not resemble that of a typical anti-parallel β-sheet spectrum, normally characterized by a negative CD band near 217 nm and a positive band near 195 nm (34), likely due to the large contribution of two β-turns and aromatic residues present in a small molecule. However, as discussed by Ovchinnikov and Ivanov (4), CD spectra can provide evidence as to the perturbation of a conformational state in a series of related peptides. Hence, a GS-like CD spectrum is likely a good indicator for β-structure in these cyclic peptides. As shown in Fig. 1, cyclic peptides containing 6, 10, or 14 residues exhibit GS-like CD spectra under aqueous conditions and therefore contain β-sheet structure. On the other hand, cyclic peptides containing either 8 or 12 residues exhibit completely different spectra than GS, the most noticeable difference being a substantially reduced ellipticity in the range 210–225 nm, indicating a largely disordered structure. All 12-residue peptides exhibited similar disordered CD spectra (data not shown). This periodicity of β-structure as a function of ring size has been confirmed in our laboratory by ^{1}H NMR spectroscopy using the chemical shift index (35) and detailed analysis of coupling constants. Complete NMR assignments and final structure calculations are in progress.[2]

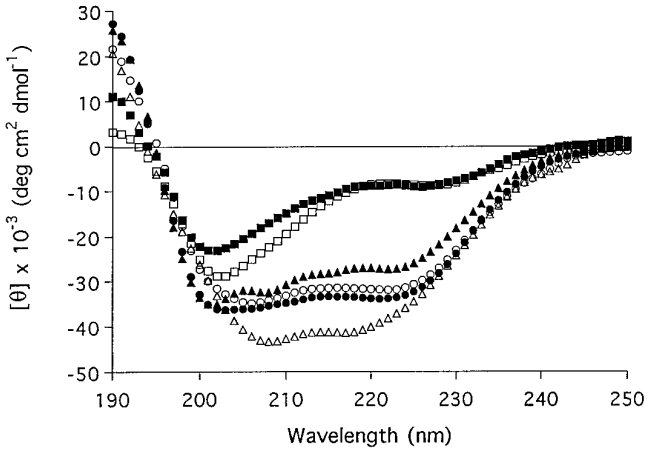

FIG. 1. **Circular dichroism spectroscopy of GS ring analogs.** Spectra were recorded in 10 mM sodium acetate buffer, pH 5.5 at 20 °C. GS6, ▲; GS8, □; GS10, ●; GS12, ■; GS14, ○; GS, △.

Modeling of Ring Structures—Models of 10-, 12-, and 14-residue peptides constructed to contain an antiparallel β-sheet structure and two type II′ β-turns as found in GS are shown in Fig. 2. From these models it is apparent that the N and C termini of the 10- and 14-residue peptides are in close proximity and will likely maintain the specified β-structure upon cyclization of the termini. The N and C termini of the 12-residue peptide on the other hand are far apart. Although the termini can be chemically linked during cyclization of the peptides, this will lead to severe distortion of the β-sheet structure in the 12-residue peptides. Furthermore, no combination of β-turn types could bring the termini together in the 12-residue peptide. These models show that the basis of the periodicity in β-structure seen by CD spectroscopy lies in the number of residues contained within the strands of these cyclic peptides. There is an absolute requirement for an odd number of residues between residues $i + 2$ and $i + 1$ of the two turns to maintain β-strand character in cyclic peptides.

[2] D. S. Wishart, L. H. Kondejewski, R. S. Hodges, and B. D. Sykes, manuscript in preparation.

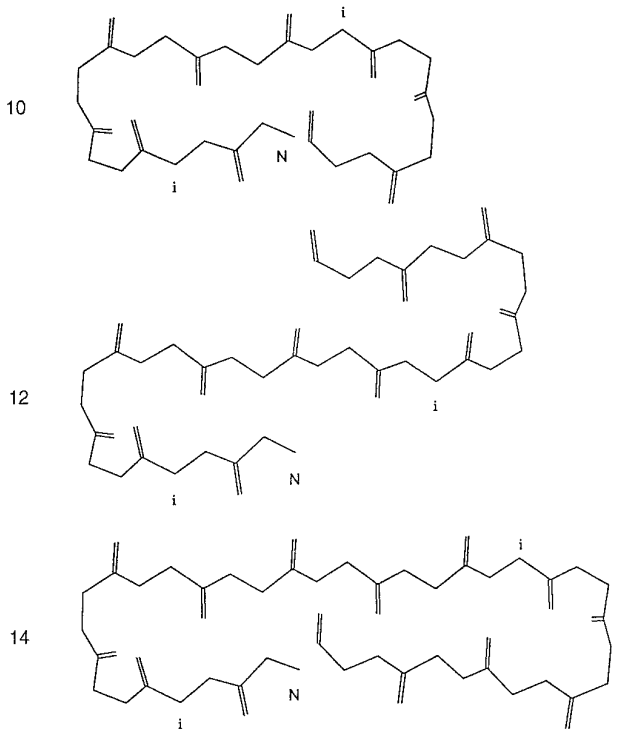

FIG. 2. **Modelling of GS ring analogs.** Backbone structures of 10-, 12-, and 14-residue peptide models constructed to contain β-sheet dihedral angles within the strands and two type II′ β-turns defined by the Xaa-D-Tyr-Pro-Xaa sequence as described under "Materials and Methods." *N* denotes the amino termini and *i* denotes Cα of the first residue in the turn.

Antibiotic Activity—Earlier work has shown that agar-based assays severely underestimate antifungal activity as well as activity against Gram-negative microorganisms (3). Consequently, a liquid-broth method was used to measure the antibiotic properties of the peptides reported in this study as shown in Table II and summarized in Table I. GS analogs composed of either 4, 6, or 8 residues (GS4, GS6, and GS8) were found to be completely inactive against all microorganisms tested. The 10-residue peptide containing the D-Phe to D-Tyr and Orn to Lys substitutions (GS10) exhibited slightly weaker antibacterial and antifungal activity compared with GS. Extension of the GS ring to contain two more Lys residues (GS12) resulted in an activity profile slightly weaker than GS10 against Gram-negative microorganisms and yeast and substantially reduced activity against Gram-positive microorganisms. Further extension of the ring to 14 residues (GS14) resulted in a product that was inactive against most microorganisms.

Additional substitutions were made in the 12-residue peptides in an attempt to increase antibiotic activity because of the promising activities displayed by GS12, *i.e.* reasonable antibiotic activity coupled with low hemolytic activity (see below). The placement of hydrophobic residues Val and Leu (GS12LV) had no effect on antibiotic activity. Replacement of D-Tyr by D-Phe (GS12F) resulted in an increase in activity against Gram-negative microorganisms and yeast comparable with that of GS itself, as well as an increase in activity against Gram-positive microorganisms, although the latter activity was still low. The increased activity of GS12F compared with GS12 is likely related to increased hydrophobicity as we have previously found a direct correlation between antibiotic activity and hydrophobicity in 10-residue GS analogs (3). Further substitutions in the D-Phe containing peptide, namely Orn for Lys (GS12FO) and Leu for Val (GS12FO/LL), had no further effect on activity against Gram-negative microorganisms and yeast with these activities reaching a plateau at the level of GS itself. An increase in Gram-positive activity was seen in GS12FO/LL however, indicating that hydrophobicity is related to activity against Gram-positive microorganisms in the 12-residue peptides. This is supported by the finding that activity of the 12-residue peptide series against Gram-positive microorganisms is correlated with overall hydrophobicity as measured by retention time on HPLC (Table I). From these results it can be concluded that in a 12-residue GS analog (i) the presence of D-Phe enhances both Gram-positive and Gram-negative activity, (ii) replacement of Orn by Lys has no effect on either activity, (iii) increased hydrophobicity results in greater Gram-positive activity, and (iv) Gram-positive activity is much more sensitive to hydrophobicity than either antifungal or Gram-negative activity.

Hemolytic Activity—Hemolytic activity of the cyclic peptides was measured using a liquid-based assay comparable with that used to measure antibiotic activity. Cyclic peptides containing 4, 6, or 8 residues were found to be completely non-hemolytic as shown in Table I. Consistent with slightly reduced antibacterial activity, the hemolytic activity of GS10 was also slightly less than that of GS itself. GS12 on the other hand exhibited substantially reduced hemolytic activity compared with either GS or GS10, indicating that an increase in length to 12 residues resulted in reduced hemolytic activity. Interestingly, the 14-residue peptide that showed almost no antibacterial or antifungal properties was the most hemolytic peptide in the series. Also shown in Table I is the therapeutic index of the analogs, defined as the ratio of hemolytic activity to antibiotic activity. The index is a measure of the specificity of the peptide for microorganisms compared with normal eukaryotic cells, with a larger number indicating greater specificity toward microorganisms. For GS10 the therapeutic index was similar to that of GS, with all activities decreasing in parallel. However, for GS12 it can be seen that while specificity for Gram-positive microorganisms decreased, specificity for Gram-negative microorganisms increased almost 3-fold due to the substantially reduced hemolytic activity exhibited by this peptide. This indicated that antibiotic activity and hemolytic activity have been dissociated in this analog. These activities were also dissociated in GS14 but in the opposite direction, *i.e.* greater specificity for eukaryotic cells.

In an attempt to further optimize the therapeutic index of GS12, *i.e.* to further increase antibiotic activity while decreasing hemolytic activity, a number of substitutions were made in GS12. It can be seen that the placement of Val and Leu residues in the D-Tyr-containing peptide (GS12LV) increased hemolytic activity, resulting in a decrease in the therapeutic index compared with GS12. Replacement of D-Tyr by D-Phe in GS12F resulted in decreased hemolytic activity coupled with increased antibiotic activity giving a very high therapeutic index for this analog, an improvement of approximately 10-fold for Gram-negative microorganisms and a 7-fold improvement for yeast compared with GS (Table I). Substitution of Orn for Lys in GS12F caused an increase in hemolytic activity in GS12FO and GS12FO/LL, resulting in a poorer therapeutic index for these analogs compared with GS12F. However, the therapeutic indices of these analogs were still better than that of GS, with a 4- and 2-fold improvement in therapeutic index against Gram-negative bacteria and yeast, respectively. These results indicate that apart from the number of residues in the ring, it is the nature of the basic residues and D-amino acids, and the combination of D-amino acid, and the placement of hydrophobic residues that are responsible for modulating hemolytic activity. Our findings show that it is possible to substantially increase the specificity of GS analogs for microorga-

TABLE II
Antibiotic activity of cyclic GS ring analogs

Peptide[b]	Antibiotic activity[a]											
	Gram-negative						Gram-positive					*C. albicans*
	E. coli UB1005	*E. coli* DC2	*E. coli* SC9251	*E. coli* SC9252	*P. aeruginosa* H187	*P. aeruginosa* H188	*S. aureus* SAP0017	*S. aureus* K147	*S. epidermidis*	*E. faecalis*	*B. subtilis*	
GS	19	9	13	5	25	16	5	5	3	6	6	9
GS10	25	14	21	8	19	13	9	6	6	14	6	13
GS12	25	11	42	30	75	10	230	300	50	300	300	42
GS14	400	190	400	190	400	400	400	400	110	29	400	230
GS12F	17	9	11	14	38	8	130	150	38	100	150	13
GS12LV	21	14	22	25	58	10	300	330	25	150	400	50
GS12FO	22	5	6	9	50	5	88	130	34	260	75	16
GS12FO/LL	16	5	8	9	63	8	44	66	22	41	38	16

[a] Minimum inhibitory concentration (μg/ml).
[b] GS4, GS6, and GS8 showed no antibiotic activity.

nisms over normal eukaryotic cells, primarily by choice of the appropriate ring size, and secondly by incorporation of appropriate substitutions in the chosen ring size.

Displacement of Dansyl-polymyxin from LPS—In order to determine whether ring size affects the interaction between the peptide and bacterial membranes, we studied the interaction between the GS ring analogs and bacterial lipopolysaccharide (LPS). It has been shown previously that dansyl-polymyxin is a good probe for cationic binding sites on both purified LPS as well as whole bacterial cells (32, 36, 37). This probe fluoresces strongly when bound to LPS and only weakly in solution, and hence, any compound that displaces dansyl-polymyxin from LPS results in a decrease in observed fluorescence. The ability of various GS analogs to displace dansyl-polymyxin is shown in Fig. 3 and summarized in Table I. GS4 and GS6 exhibited essentially no probe displacement, whereas peptides containing eight or more residues showed a progressively increased ability to displace dansyl-polymyxin. GS10 was approximately as efficient as GS itself, indicating that the D-Phe to D-Tyr substitution had little effect on binding to LPS. GS14 exhibited the highest displacement ability, approaching the efficiency of polymyxin B itself.

Polymyxin nonapeptide, a delipidated version of polymyxin B, has been shown to be a much weaker inhibitor of dansyl-polymyxin binding than polymyxin B itself, suggesting that the presence of both positive charges, as well as a hydrophobic portion, are important for membrane interactions (32). As shown in Fig. 3, an increase in two positive charges from GS4 to GS6 was not sufficient to increase binding to LPS. Extension of the ring to 8 residues by incorporating two hydrophobes in GS8 did not substantially increase binding activity of the GS analogs. However, a further increase of two hydrophobes in GS10 markedly increased LPS binding. A further increase of two positive charges in GS12 increased LPS binding activity by approximately 6-fold (Table I); however, the incorporation of two more hydrophobes in GS14 resulted in a 100-fold increase in binding activity. The differences in LPS binding by the analogs appear to be due to the number of basic residues as well as the presence or absence of β-sheet structure. The β-sheet structure is likely important as this will either place the basic residues on the same face of the molecule with a specific distance between the positive charges in the case of a peptide in a β-sheet conformation or in some undefined position in the case where the peptide assumes a disordered conformation. Therefore, for two peptides with the same number of basic residues, the one in the β-sheet conformation binds stronger than a peptide in a disordered conformation. The positioning of basic residues in GS analogs and the relevance to antibacterial activity has been discussed previously by Ovchinnikov and Ivanov (4) who found a correlation between spacing of basic groups and antibacterial activity. They proposed that the optimum spacing between positive charges is one that optimizes binding of each charge to adjacent phospholipid molecules.

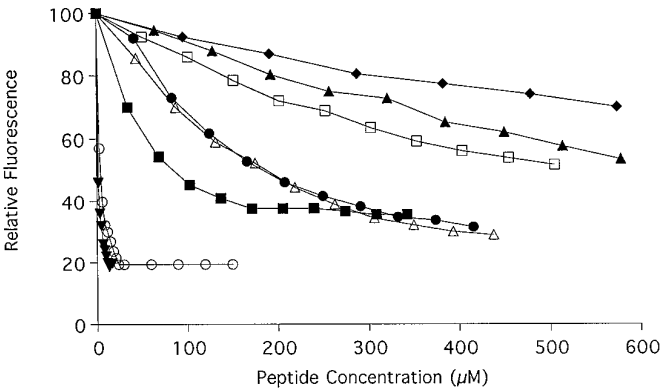

FIG. 3. **LPS-bound dansyl-polymyxin B displacement by GS ring analogs.** Solutions containing LPS-bound dansyl-polymyxin were titrated with peptides and the decrease in fluorescence monitored as described under "Materials and Methods." Samples were GS4, ◆; GS6, ▲; GS8, □; GS10, ●; GS12, ■; GS14, ○; GS, △; polymyxin B, ▼.

Permeabilization of Outer Membranes to NPN—Permeabilization of *E. coli* outer membranes by GS analogs was monitored using the hydrophobic fluorescent probe *N*-phenyl-1-naphthylamine (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 (30). Fig. 4 shows that both GS4 and GS6 caused no outer membrane destabilization at the concentrations tested. GS10, GS12, and GS14 all showed a similar capacity to disrupt the *E. coli* membrane as GS itself, whereas GS8 showed a diminished capacity to disrupt membranes.

DISCUSSION

There have been a number of reports describing the properties of GS analogs with various ring sizes (12, 18, 38–41); however, agar-based assays have been used to determine antibacterial activity in all cases. Since we have recently shown that agar-based assays severely underestimate antifungal and Gram-negative antibacterial activity compared with solution-based assays (3), we felt it necessary to reevaluate GS analogs with increased length using solution-based assays. Furthermore, hemolytic activity of these analogs has only been determined in a few cases, and no clear correlation between structure and activity has been found. In the present study we investigated the effect of ring size on a number of parameters in order to better define those features responsible for antibacterial and hemolytic activity. Apart from determining these activities, we have also evaluated the content of β-sheet struc-

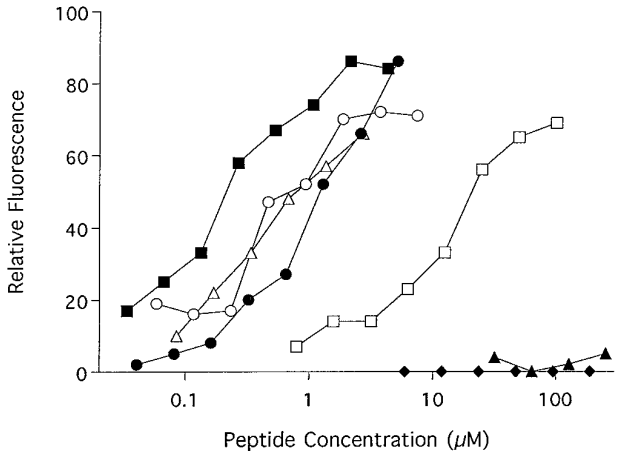

FIG. 4. **Peptide-mediated NPN uptake in *E. coli* UB1005.** *E. coli* UB1005 cells were incubated with NPN in the presence of various concentrations of peptides. Enhanced uptake of NPN was measured by an increase in fluorescence due to NPN partitioning into the hydrophobic membrane. Samples were GS4, ♦; GS6, ▲; GS8, □; GS10, ●; GS12, ■; GS14, ○; GS, △.

ture of these analogs and have investigated the binding of peptides to Gram-negative LPS and their ability to disrupt Gram-negative cell membranes.

It is interesting from a structural point of view that there is a periodicity in β-sheet content as a function of ring size, where 6-, 10-, and 14-residue peptides exhibit β-sheet structure as found in GS, whereas 8- and 12-residue peptides display a disordered structure. Due to the ambiguous nature of the CD spectra of small cyclic peptides, we have also confirmed this trend using NMR spectroscopy. These findings indicate an absolute requirement for an odd number of residues between the two turns (made up by the D-Xaa-Pro sequence). This is likely a reflection of the constrained nature of these peptides. Furthermore, only those peptides with an odd number of residues between the two corner residues can form an alternating hydrogen bonding pattern as found in antiparallel β-sheets (*i.e.* a hydrogen-bonded pair of residues followed by a non-hydrogen bonded pair, etc.). Molecular modelling studies have also confirmed that it is not possible for the 8- and 12-residue peptides to form β-sheet structures. Preliminary data suggest that the 12-residue peptides may take on a somewhat circular structure similar to that of valinomycin in aqueous medium.[3] Our findings confirm the relationship between β-structure and the number of residues in cyclic peptides predicted by Schwyzer (42, 43) almost 40 years ago. Based on cyclodimerization studies with 3- and 5-residue peptides, Schwyzer proposed that cyclic peptides containing 2 (2 n + 1) residues, where n = 1, 2, 3 . . . , could form β-sheet structures and later presented structural evidence that this rule held true for cyclic hexapeptides (44, 45). These findings not only have implications for design of cyclic β-sheet antibiotics but are also relevant in the design of appropriate scaffolds for use in template assembled synthetic proteins (46) since the β-sheet backbone structure (or lack of) will determine the relative positioning of the side chains.

The structural characterizations of the 14-residue peptide by CD spectroscopy presented in this study agree with those of Tamaki *et al.* (39) who obtained similar results for a homologous 14-residue peptide. In contrast, other groups have presented CD spectra more characteristic of disordered structures for homologous 14-residue peptides (12, 18, 41). Such differences are difficult to reconcile based on the similarity in sequences. We have previously observed that racemization in our 14-residue peptide resulted in CD spectra characteristic of disordered or random coil structure. Furthermore, Tamaki *et al.* (39) demonstrated that the incorporation of D-amino acids between the two turns (assumed to be made up by the D-Phe-Pro sequence as in GS itself) of similar 14-residue peptides, led to random coil-like CD spectra similar to those observed by others (12, 18, 41). It is therefore possible that the analogs reported by these groups unintentionally contain D-amino acids. Such racemization may be due to the coupling chemistry used (solution phase) during synthesis.

A previous study has shown that there are a number of determinants for antibacterial activity in membrane-active helical peptides (17, 47). These include overall hydrophobicity, amphipathicity (hydrophobic moment), and helicity. With GS, there is also evidence suggesting that high overall hydrophobicity, the presence of basic side chains, an amphipathic nature, and high β-sheet content are important for antibacterial activity (4, 7, 12). Our findings show that the 12-residue cyclic peptides are disordered in solution and hence the hydrophobic moment will likely be lower in such a structure compared with a β-sheet structure containing alternating hydrophobic and hydrophilic residues. From a thermodynamic point of view, the free energy of transfer from solution into the membrane will be lower (more favorable) for the case of an ordered β-sheet structure, compared with an unordered structure. This is because of the presence of interstrand hydrogen bonds in the β-sheet structure. In the disordered structure, amide NH and CO are likely solvated, and hence the free energy of transfer of this solvated structure into the membrane would be predicted to be greater (less favorable) than when all hydrogen bonds are formed. Our modelling studies show that the 12-residue peptide cannot achieve a perfect β-sheet structure with all potential hydrogen bonds formed (where residues i and i + 3 of a turn are H-bonded) due to the number of residues per strand.

It is generally accepted that the main site of action of GS is the cell membrane of susceptible cells. Studies by Ovchinnikov and Ivanov (4) have indicated that GS acts mainly, if not solely, on lipid membranes since enantiomeric preparations of GS exhibited identical activities as GS itself. Furthermore, the finding that divalent cations can impair antibiotic activity also indicates that GS interacts with charged lipids on the surfaces of cell membranes (29, 48). However, in the present study we found that strong membrane interactions are not sufficient, in themselves, to lead to an increase in activity against Gram-negative microorganisms. The 12- and 14-residue peptides bound Gram-negative LPS much stronger that GS itself; however, neither exhibited greater Gram-negative activity than GS. It would also appear that LPS (cell) binding and membrane destabilization are not related since the 14-residue peptide, which bound LPS with 100-fold greater affinity than GS, showed essentially the same ability to destabilize *E. coli* outer membranes to the fluorescent probe NPN. Furthermore, even though the 14-residue peptide was able to destabilize Gram-negative outer membranes to a similar extent as GS, it exhibited no activity against these microorganisms. This likely indicates that our assay to measure outer membrane destabilization does not reflect the ability of the peptide to accumulate in the membrane of Gram-positive microorganisms or to accumulate in the inner (cytoplasmic) membrane of Gram-negative microorganisms. Our findings indicate that there must be other steps required for cell death other than binding and outer membrane destabilization. These other steps are probably related to the accumulation of the peptide in the membrane. Evidence for such accumulation of GS has been reported by Zidovetzki *et al.* (49). Prokaryotic membranes con-

[3] L. H. Kondejewski, S. W. Farmer, D. S. Wishart, C. M. Kay, R. E. W. Hancock, and R. S. Hodges, unpublished results.

tain predominantly acidic phospholipids, whereas the eukaryotic membranes are composed mainly of zwitterionic phospholipids. The strong membrane interaction of GS14 with bacterial membrane phospholipids (as evidenced by its high LPS binding activity) is likely ionic in nature, and this strong interaction with the polar headgroups may prevent GS14 from entering the membranes, resulting in the lack of activity against these microorganisms. Due to the zwitterionic nature of the phospholipids of eukaryotic membranes, binding of GS14 to the polar headgroups is likely weaker to these membranes that may allow for the accumulation of peptide within the erythrocyte membrane. The differences in membrane composition may therefore explain the lack of activity of GS14 against microorganisms. The high hemolytic activity of GS14 compared with GS may be due to the increased number of basic side chains on the same face of the molecule (due to the β-sheet conformation exibited by GS14) that may be responsible for an increase in binding of GS14 to erythrocyte membranes.

Consistent with previous reports (38, 40), we found that cyclic GS analogs containing 8 or fewer residues were completely inactive against both bacteria as well as eukaryotic cells. This likely reflects a minimum requirement for the proportion of hydrophobic to hydrophilic residues as well as a minimum requirement for overall hydrophobicity of the cyclic analogs. Both binding to LPS and destabilization of *E. coli* membranes were greatly reduced in analogs containing 8 or fewer residues.

In our previous study on 10-residue cyclic peptides, we found that hydrophobicity was strongly correlated with activity against yeast as well as both Gram-positive and Gram-negative microorganisms (3), although Gram-positive activity was more sensitive to changes in hydrophobicity. In the present study, our results clearly show that within the 12-residue peptide series there is also a direct correlation between hydrophobicity and activity against Gram-positive microorganisms. There was also a correlation seen for yeast and Gram-negative bacteria, but activity against these microorganisms reached a plateau at the level of GS itself and was not increased further. Activity against Gram-positive microorganisms was also much more sensitive to changes in ring size; we found a 50-fold difference in activity between GS and the least active 12-residue analog against Gram-positive bacteria. In contrast, we found only a 2-fold difference for Gram-negative bacteria. Our results indicate that Gram-positive antibacterial activity and hemolytic activity are much more sensitive to ring size, and hence structure, than Gram-negative antibacterial activity.

Our results concerning antibacterial activity in the 12-residue peptides are comparable with those of Ando *et al.* (12, 41) who studied similar peptides using an agar-based assay. However, Ando *et al.* report that their homologous series of 14-residue peptides exhibit substantial antibacterial activity and low hemolytic activity. In contrast, our 14-residue peptide exhibited just the opposite, no antibacterial activity but extremely high hemolytic activity. Ando *et al.* (41) also reported that homologous 12- and 14-residue peptides containing Orn to Lys substitutions were essentially inactive against both Gram-positive and Gram-negative microorganisms and that neither exhibited hemolytic activity. These results also differ from our findings that show that the Orn to Lys substitution in a 12-residue peptide had no effect on antibacterial activity and that the Orn-containing analog was in fact more hemolytic than a Lys-containing analog. These discrepancies are difficult to reconcile and may be a consequence of undetected racemization occurring in the peptides prepared by Ando *et al.* As discussed above, racemization in one of our 14-residue peptides led to a loss of β-structure, similar to that reported by Ando *et al.* (12, 41). In the 12-residue peptides reported here, the lack of β-structure appears to be important in defining the activity of the peptide, *i.e.* antifungal and Gram-negative antibacterial activity coupled with low hemolytic activity.

Magainin 2 shows a similar specificity as the 12-residue peptides reported in the present study in that it exhibits antibacterial activity with low hemolytic activity. In studies aimed at determining the molecular basis for membrane specificity of magainin 2, Matsuzaki *et al.* (20) found that the absence of acidic phospholipids as well as an abundance of cholesterol in erythrocyte membranes contributed to protection from magainin attack. Bacterial cells contain predominantly zwitterionic and acidic phospholipids, and this difference likely contributes to their susceptibility to basic peptides. This, however, does not explain the change in specificity between the present 10- and 12-residue peptides. This lack of hemolytic activity displayed by the 12-residue peptides may be a result of either a lack of binding to erythrocytes or the inability to carry out GS-like membrane disruption once bound to erythrocytes. Both these possibilities are likely due to the lack of β-structure in these peptides and hence the different relative positioning of basic and hydrophobic residues compared with peptides adopting a β-sheet structure. A recent report showed similar findings in model basic helical peptides (50). Peptides that were amphipathic and helical exhibited both hemolytic and antibacterial properties, whereas a peptide that had little helical structure (due to a Pro substitution mid-chain), and therefore was not amphipathic, exhibited only antibacterial activity with low hemolytic activity.

What are the factors responsible for specific activities in GS and GS analogs? Our findings indicate that there is an interplay of a number of factors including (i) backbone conformation, which determines relative positioning of critical side chains and the ability of the peptide to partition into membranes; (ii) the nature of the basic residues, since the side chain length would likely affect their ability to interact with negatively charged phospholipids of membranes; and (iii) overall hydrophobicity that again affects the ability of the peptide to partition into membranes. We have shown that the ring size of GS analogs plays a large role in antibacterial as well as hemolytic activity. Our results indicate that it is possible to modulate these activities by selecting appropriate ring sizes. Furthermore, these activities can be further optimized by appropriate substitutions in positions comprising the basic residues and the hydrophobic residues. These findings show the potential for the development of clinically useful antibiotics possessing antibacterial activity with low hemolytic activity (a high therapeutic index) based on the cyclic nature of GS. We are currently further investigating membrane interactions with these cyclic peptides in order to better define the role of both structure and sequence in interactions with bacterial and eukaryotic membranes.

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