Diastereoisomeric analogues of gramicidin S: structure, biological activity and interaction with lipid bilayers

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Analogues of a structurally equivalent version of the antimicrobial decameric cyclic peptide gramicidin S, GS10 [cyclo-(Val-Lys-Leu-D-Tyr-Pro)$_2$], were designed to study the effect of distortion in the β-sheet/β-turn structure of the cyclic peptide on its biological activity. In one approach, the hydrophobic nature of GS10 was conserved, and single amino acids in its backbone were replaced systematically with their corresponding enantiomers to give five diastereoisomeric analogues. In a related approach, a more basic and hydrophilic analogue of GS10 [cyclo-(Lys-Val-Lys-D-Tyr-Pro^5-Lys-Leu-Lys-D-Tyr-Pro^{10})], together with two of its monosubstituted diastereoisomeric analogues (featuring D-Lys^1 or D-Val^2 respectively), were synthesized. CD spectra were measured in a variety of environments, i.e. aqueous, aqueous trifluoroethanol and those containing SDS micelles or phospholipid vesicles. In comparison with GS10 spectra, CD spectra of both groups of analogues in these environments exhibited structural distortion. Moreover, compared with GS10, antimicrobial and haemolytic activities of the analogues were drastically decreased, implying the existence of a threshold minimum amphipathicity for effective biological activity. However, in both groups of analogues, there was a correlation between amphipathicity and antimicrobial and haemolytic activities. In the second group of analogues, both electrostatic and hydrophobic factors were related to their antimicrobial and haemolytic activities. In order to gain an insight into the nature of the biological activity of the two classes of cyclic peptides, the relationship of their structure to interaction with lipid membranes, and the implied mechanisms, were analysed in some detail in the present study.

Key words: antimicrobial cyclic peptides, CD spectroscopy, peptide–phospholipid vesicle interaction, β-sheet/β-turn conformation, structure–function relationships.

INTRODUCTION

Gramicidin S [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$] is a decameric cyclic antimicrobial peptide derived from *Bacillus brevis* [1]. Gramicidin S is active against a wide range of bacteria, as well as fungi [2,3]. The peptide is also very haemolytic against human erythrocytes (e.g. [3,4], and references therein). In the past 40 years, considerable effort has been directed towards studying the structure–function relationship of peptide analogues of gramicidin S [2,5–8].

Gramicidin S assumes an antiparallel double-stranded β-sheet structure with type II′ β-turns defined by Val-Pro-D-Phe-Leu residues [9–11]. A decameric analogue of gramicidin S, GS10 [cyclo-(Val-Lys-Leu-D-Tyr-Pro)$_2$], shows structural similarity with gramicidin S in a variety of environments, as revealed by CD [4,12] and NMR [13] spectroscopy. In GS10, Val-Pro-D-Tyr-Leu forms the type II′ β-turns, whereas the six alternatively placed hydrophobic and hydrophilic amino acids comprise the antiparallel β-sheet part of the molecule. Both gramicidin S and GS10 have an amphipathic nature. The plane of these cyclic molecules shows a hydrophilic face where ornithine (gramicidin S) or lysine (GS10) side chains are oriented. The other face on this plane is more hydrophobic, with valine and leucine side chains extruded. Four intermolecular hydrogen bonds between valine and leucine residues in the backbone stabilize further the overall β-sheet/β-turn structure of the molecule. The amphipathic nature of GS10 is shown in a molecular model that is based on its backbone structure ([13]; see Figure 1).

Several approaches have been undertaken in our laboratories to enhance the more desirable antimicrobial, as opposed to the less desirable haemolytic, properties of gramicidin S [3,4,14,15]. In one of these approaches, we studied the effect of the ring size on the structure, antimicrobial and haemolytic activities in a series of gramicidin S analogues, and found that haemolytic activity could be dissociated from antimicrobial activity in certain analogues lacking high amphipathicity [4]. Generally, enlargement of the ring size of GS10 (or gramicidin S) by addition of related amino acids to the backbone of the cyclic peptide (while retaining the alternative hydrophilic/hydrophobic pattern) induces more flexibility in structure, and enables the peptide to be more selective in its biological activity. An example is a 12-meric analogue of GS10 (GS12, with four lysine and four hydrophobic residues), in which there was a clear decrease in haemolytic versus antimicrobial activity, and the analogue exhibited a CD spectrum indicative of a disrupted structure in comparison with GS10 [4].

In a more recent study, by designing monosubstituted diastereoisomeric analogues of a tetradecameric β-sheet/β-turn-contain-

Abbreviations used: LPS, lipopolysaccharide; NPN, *N*-phenyl-1-naphthylamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; RP, reversed phase; SUV, small unilamellar vesicle; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

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ing analogue of gramicidin S, GS14 [cyclo-(Val-Lys-Leu-Lys-Val-D-Tyr-Pro^{7}-Leu-Lys-Val-Lys-Leu-D-Tyr-Pro^{14})] (a total of 14 diastereoisomers), antimicrobial and haemolytic activities were dissociated by systematic alterations in amphipathicity [15]. Owing to the similarity in sequence and intrinsic hydrophobicity, the amphipathicity of the diastereoisomers could be ranked on the basis of their retention time on reversed-phase (RP)-HPLC. In that series of diastereoisomeric peptides, there was a clear correlation, illustrating that high amphipathicity resulted in high haemolytic activity and low antimicrobial activity [15]. GS14 showed the highest amphipathicity, weak antimicrobial activity and high haemolytic activity, whereas the diastereoisomers with the most favourable therapeutic indices possessed some of the lowest amphipathicities, although there was a threshold value below which antimicrobial activity decreased [4,15]. Our findings in that study showed that the characteristic of high amphipathicity is not desirable in the design of conformationally constrained cyclic antimicrobial peptides, and that an optimum amphipathicity can be defined by systematic enantiomeric substitutions.

To apply a similar approach of altering amphipathicity to smaller ring sizes, we have selected the gramicidin S analogue, GS10, which is comparable with gramicidin S in its biological activity and structural features, and is more soluble in aqueous solvents. In the present study, although the overall hydrophobic nature of the molecule was conserved, the amino acids in the GS10 backbone were replaced systematically by their corresponding enantiomers to give five diastereoisomeric analogues (see peptides **1**–**5** in Table 1). In a related study, in more hydrophilic analogues of GS10, the effect of the increase in the ratio of charged-to-hydrophobic residues on structure and function of the decameric analogue of gramicidin S was examined. The hydrophobic (valine, leucine) and hydrophilic (lysine) residues in the GS10 backbone were exchanged, resulting in the two faces of GS10 being reversed, and a more basic analogue of GS10 was generated: GS10rev (cyclo-(Lys-Val-Lys-D-Tyr-Pro^{5}-Lys-Leu-Lys-D-Tyr-Pro^{10})] (see peptide **6** in Table 1). Two monosubstituted diastereoisomeric peptides related to this analogue were also synthesized (see peptides **7** and **8** in Table 1). The present paper aims to explain the structure–function relationship in the analogues, with an emphasis on the interaction of the peptides with lipid bilayers.

EXPERIMENTAL

Peptides

Gramicidin S was obtained from Sigma (St Louis, MO, U.S.A.) and purified by RP-HPLC [4]. All of the other reagents were of high-purity reagent grade, and were used as purchased.

Linear sequences of the peptides were synthesized by solid-phase procedures using Boc-Chemistry. t-Butoxycarbonyl-Pro-phenylacetamidomethyl (Boc-Pro-PAM) resin (Advanced ChemTech, Louisville, KY, U.S.A.) was used to initiate the synthesis, as described previously [3,4]. Lysine side chains were protected by formyl groups throughout the syntheses. After cleavage from resin and purification by RP-HPLC, linear peptides were cyclized in a head-to-tail manner (with proline at the C-terminus) utilizing benzotriazole-1-yl-oxy-Tris-[(dimethylamino)-phosphonium hexafluorophosphate], 1-hydroxybenzotriazole and *N*,*N*-di-isopropylethylamine in *N*,*N*-dimethylformamide, and lysine residues of the cyclized peptides were then deprotected in dilute methanolic HCl at 40 °C [4]. The deprotected cyclic peptides were purified further by RP-HPLC, and their final purity was confirmed by analytical RP-HPLC (see below) and electrospray MS. Concentrations of the pure peptides in aqueous stock solutions were determined by amino acid analysis ($\pm 5\%$ error).

Analytical RP-HPLC

Gramicidin S, GS10 and peptides **1**–**8** were analysed on a Zorbax SB-C8 column [150 mm × 2.1 mm internal diam., 5 μm particle size, 30 nm (300 Å) pore size; Rockland Technologies, Wilmington, DE, U.S.A.] on a Hewlett–Packard 1100 chromatograph at 70 °C. A linear AB gradient [solvent A, 0.05% trifluoroacetic acid (TFA) in water; solvent B, 0.05% TFA in acetonitrile] of 1% solvent B/min at a flow rate of 0.25 ml/min was used.

Molecular modelling

Molecular modelling of peptides was on the basis of global energy minimization of peptide structures with appropriate parameters, using the Insight II software (Biosym Technologies, San Diego, CA, U.S.A.) on a Silicon Graphics workstation (also see [15]).

CD measurements

CD spectra were measured on Jasco 500 and Jasco 720 spectropolarimeters (Tokyo, Japan). Ellipticities are reported as the mean residue ellipticity, $[\theta]_{m.r.w}$. Spectra were measured in buffer solutions composed of final concentrations of Tris (10 mM), NaF (150 mM) and EDTA (0.1 mM), at pH 7.4. All of the measurements were in quartz cells with 0.1-cm pathlength, at 25 °C.

Preparation of micelles and phospholipid vesicles

SDS (Fisher Scientific, Fair Lawn, NJ, U.S.A.) micelles were prepared by dissolving the appropriate amount of SDS in the buffer solution.

Phosphatidylcholine (PC) (from egg yolk; 100 mg/ml in chloroform), phosphatidylethanolamine (PE) [from *Escherichia coli*; 10 mg/ml in chloroform/methanol (9:1, v/v)], phosphatidylglycerol (PG) [from egg yolk; 10 mg/ml in chloroform/methanol (49:1, v/v)], and cholesterol were purchased from Sigma.

Appropriate amounts of phospholipids in organic solvents were dried under nitrogen, and then under reduced pressure overnight. The dried lipid was redissolved in buffer, and then sonicated with a probe sonicator for between 15–30 min. Small unilamellar vesicles (SUVs) thus prepared were then centrifuged in a microcentrifuge for 20 min at 14000 ***g*** to remove titanium particles. SUVs were added to peptide solutions in buffer 30–60 min before measurement of CD spectra. PE/PG (7:3, w/w) and PC/cholesterol (9:1, w/w) SUVs were prepared as two-component lipid-bilayer systems. Lipid concentrations were determined by the assay of Bartlett [16]. Errors in the reported phospholipid concentrations are in the range of $\pm 5\%$.

Bacterial strains and haemolytic activity

Bacterial and fungal strains were prepared as described previously [3,4]. The activity of peptides against these microorganisms was measured by a liquid broth assay method [3,4]. Gram-positive bacterial strains that were utilized were *Staphylococcus aureus* (SAP0017 and K147), *Staph. epidermidis*, *Bacillus subtilis*, *Enterococcus faecalis* and the coryneform bacterial strain *Corynebacterium xerosis*. Gram-negative bacterial strains were composed of *Pseudomonas aeruginosa* (K799 and Z61), *E. coli* (UB1005 and DC2), and *Salmonella typhimurium* [C587 (14028S)

and C610 (MS4252S)]. The yeast strain employed was *Candida albicans*. Freshly collected human blood cells were prepared as described previously, and haemolytic measurements were performed in a liquid-based assay [3,4]. The reported values for both assays were determined after 24 h at 37 °C.

Bacterial outer-membrane interaction

Interaction of peptides with the outer membrane of *E. coli* UB1005 bacteria, leading to membrane permeabilization, was carried out as described previously [3,4,15]. In brief, *E. coli* cells were suspended in sodium-Hepes buffer (5 mM, pH 7.0), containing glucose (5 mM) and carbonyl cyanide *m*-chlorophenyl hydrazone (5 mM). *N*-Phenyl-1-naphthylamine (NPN) was then added to 1 ml of cells in a quartz cuvette to give a final concentration of 10 mM. At this stage, the background fluorescence was recorded. Aliquots of peptide were added to the cuvette, and the increase in fluorescence was recorded as a function of time until no further increase in emission intensity was observed. A fresh cuvette of cells with NPN was used for each peptide, and control experiments were performed to demonstrate that enhanced fluorescence was due to uptake of NPN into cells, and not a result of non-specific peptide–NPN interactions.

Interaction of cationic peptides with bacterial lipopolysaccharide (LPS) was measured as dansyl-polymyxin displacement from *Ps. aeruginosa* LPS, as described previously [4]. In brief, peptides were titrated into cuvettes containing 3 μg of LPS/ml and 2.5 μg of dansyl-polymyxin ($\approx 90\,\%$ 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 inhibition as a function of the inverse of the inhibitor concentration gave a value for IC_{50}, i.e. the concentration of inhibitor resulting in 50 % displacement of dansyl-polymyxin from LPS (the $-1/x$ intercept).

RESULTS

Peptide design and synthesis

GS10 and its analogues, peptides **1**–**8** (Table 1), were designed on the basis of the amino acid sequence of gramicidin S, preserving its alternating hydrophobic/hydrophilic amino acid (peptides **1**–**5**), and reversing the pattern of two charged residues and four hydrophobic ones to four charged residues and two hydrophobic ones in the two strands of the β-sheet (peptides **6**–**8**). Monosubstituted diastereoisomers of GS10 were designed to perturb the backbone locally, leading to a change in peptide amphipathicity, and to study the effects on biological activities. Design of peptide **6** and its diastereoisomeric analogues was aimed at studying the effect of increasing the number of charged residues and decreasing the number of hydrophobic ones on the two faces of the β-sheet, and also the effect of the corresponding enantiomeric substitutions on peptide structure and biological activity.

RP-HPLC analysis of peptide structure

GS10 and peptides **1**–**5** have identical intrinsic hydrophobicities, i.e. they have the same overall composition and hydrophobicity, regardless of single enantiomeric substitutions. However, upon RP-HPLC analysis, these analogues were resolved and demonstrated different retention times, ranging from 37.0 to 48.8 min (Table 1). The β-sheet structure of GS10 is known to impart an amphipathic nature to the molecule (Figure 1A). The hydrophobic face of GS10 makes up the preferred hydrophobic-binding domain (containing two valine and two leucine residues) that interacts with the hydrophobic RP-HPLC matrix. The finding that all diastereoisomeric analogues exhibited lower retention times than GS10 indicates that the overall amphipathicity of the peptides was reduced by the enantiomeric substitutions. Similar results have been observed with α-helical peptides [17], as well as with β-sheet peptides related to GS14 [15]. GS10rev and its diastereoisomers, peptides **6**–**8**, were intrinsically less hydrophobic than GS10, and showed much lower retention times (Table 1). In peptides **1**–**8**, structural factors clearly contribute to the decrease in the observed retention times (see below).

CD spectroscopic analysis of peptide structure

The structural features of GS10 analogues were monitored by CD spectroscopy. Figure 2(A) depicts CD spectra of monosubstituted stereoisomers of GS10 in an aqueous environment. Gramicidin S and GS10 show equivalent structures, with the minima for a combination of β-sheet and β-turn motifs [18].

Table 1 Peptide sequences, retention times, haemolytic activity and LPS-binding affinity

Amino acids are represented by the single-letter code in the sequences (O represents ornithine, and the superscripted numbers show the position of enantiomeric substitutions in peptides). The peptide concentrations shown for the haemolytic activities were after 24 h; for the LPS-binding affinity experiments, peptide concentrations shown are those required to displace 50% of dansyl-polymyxin B from LPS, as described in the Experimental section.

Peptide	Sequence	Retention time on RP-HPLC (min)	Haemolytic activity (μg/ml)	LPS-binding affinity (μg/ml)
Gramicidin S	Cyclo-(VOL-d-FPVOL-d-FP)	55.0	12.5	434
GS10	Cyclo-(VKL-d-YPVKL-d-YP)	48.8	25.0	381
Diastereoisomeric analogues of GS10				
5	Cyclo-(VKL-d-Y-d-P^{5}VKL-d-YP)	42.7	> 200	492
3	Cyclo-(VK-d-L^3-d-YPVKL-d-YP)	41.6	> 200	238
1	Cyclo-(-d-V^{1}KL-d-YPVKL-d-YP)	41.0	> 200	311
4	Cyclo-(VKLY^{4}PVKL-d-YP)	39.8	> 200	172
2	Cyclo-(V-d-K^{2}L-d-YPVKL-d-YP)	37.0	> 200	315
GS10rev series				
6 (GS10rev)	Cyclo-(KVK-d-YPKLK-d-YP)	24.3	> 200	122
7	Cyclo-(-d-K^{1}VK-d-YPKLK-d-YP)	23.5	> 200	132
8	Cyclo-(K-d-V^{2}K-d-YPKLK-d-YP)	22.7	> 200	162

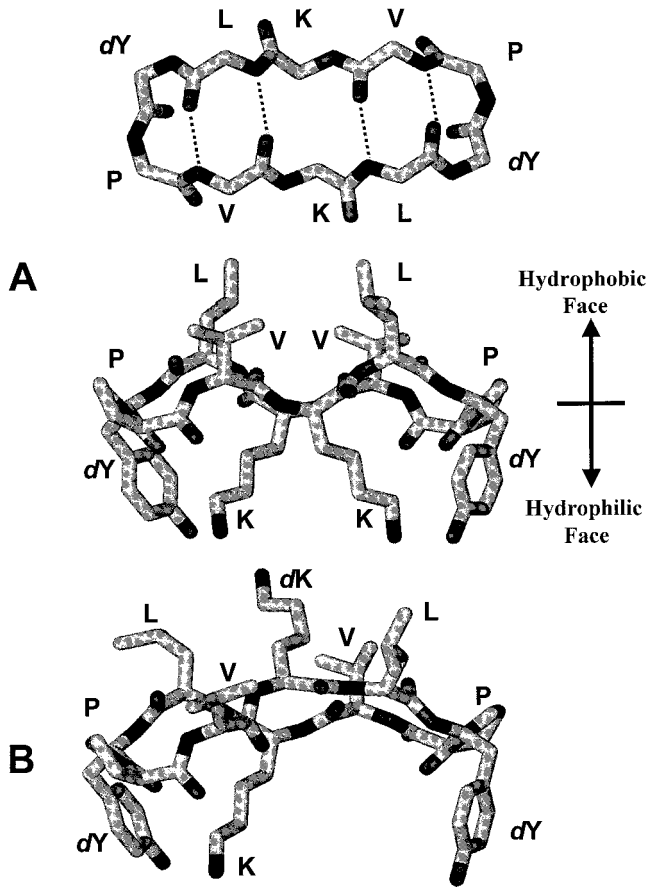

Figure 1 Top and side views of the molecular model based on the GS10 structure, with hydrogen bonds shown in dotted lines (A), and the molecular model of D-Lys^{2}GS10 (B)

Amino acids are represented by the single-letter code. *d*Y, D-tyrosine; *d*K, D-lysine.

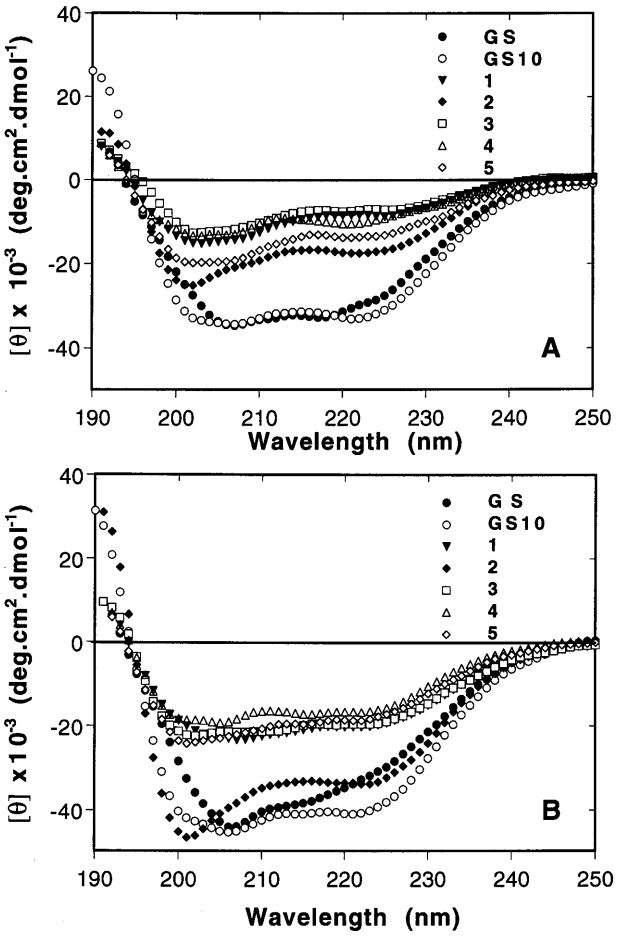

Figure 2 CD spectra of gramicidin S, GS10 and peptides 1–5 (A) in buffer, and (B) in 50% TFE/buffer (by vol.)

Peptide concentrations were at 100 μM. GS, gramicidin S.

The double minima for GS10 spectra arise at approx. 206 and 222 nm, with $[\theta]_{222}/[\theta]_{206} = 0.94$. Compared with GS10, all of the analogues (peptides **1**–**5**) reflected major structural changes, since their ellipticities were substantially decreased. We have clearly shown previously that a correlation exists between changes in the CD spectra and changes in the backbone structure of cyclic peptides, using NMR spectroscopy [15]. In the same study, the CD and NMR structural data were related with changes in the amphipathicity (measured by RP-HPLC) and biological activity [15]. Part of the typical spectrum for 'GS10-like' structures (215–225 nm) can be more sensitive to changes in the β-sheet part of the structure, as the β-sheet CD spectrum shows a minimum in this range. Compared with GS10, the minimum in the 200–210 nm range of the spectra of analogues was slightly blue-shifted to lower wavelengths. This range of the spectra is likely to be more sensitive to the changes in the turn part of the GS10 molecule, since β-sheet spectrum has little or no negative ellipticity in this area. Despite the significant change in the structure resulting from single enantiomeric substitutions (peptides **1**–**5**), CD spectra of these peptides indicate subtle conformational differences (compare peptides **2** and **5** with peptides **1**, **3** and **4**). Interestingly, peptide **2** (Figure 1B) with a substituted D-lysine in the non-hydrogen-bonded site showed the least disruption in both the β-sheet and/or turn parts of the molecule, and also the lowest retention time among the diastereoisomeric analogues (peptides **1**–**5**) (Table 1). The low retention time reflects a considerable change in amphipathicity compared with GS10. These findings (i.e. disruption of the backbone and alteration of the amphipathic pattern of the molecule) are supported by the molecular model of peptide **2** (Figure 1B), and are in agreement with an NMR study of D-Lys^{4}GS14 (an analogue of GS14 also with a D-lysine in the non-hydrogen-bonded site and the lowest retention time among the 14 diastereoisomeric analogues) in aqueous trifluoroethanol (TFE), in which the substituted D-lysine enantiomer side chain was located on the hydrophobic face of the molecule [19]. Thus, when CD spectra of the peptides show changes in structure, peptide retention times on RP-HPLC reflect the changes in amphipathicity, which, in combination with the CD spectra, can be clearly related to the relocation of the side chains, as well as changes in the peptide backbone. In addition to peptide **2**, the spectroscopic and RP-HPLC evidence for changes in the backbone conformation and amphipathic pattern of the other diastereoisomeric analogues are supported further by molecular modelling studies (M. Jelokhani-Niaraki and L. H. Kondejewski, unpublished work).

A similar trend in CD spectra of the GS10 analogues was observable in the aqueous 50% TFE solution (Figure 2B). TFE is widely believed to stabilize hydrogen bonds in secondary

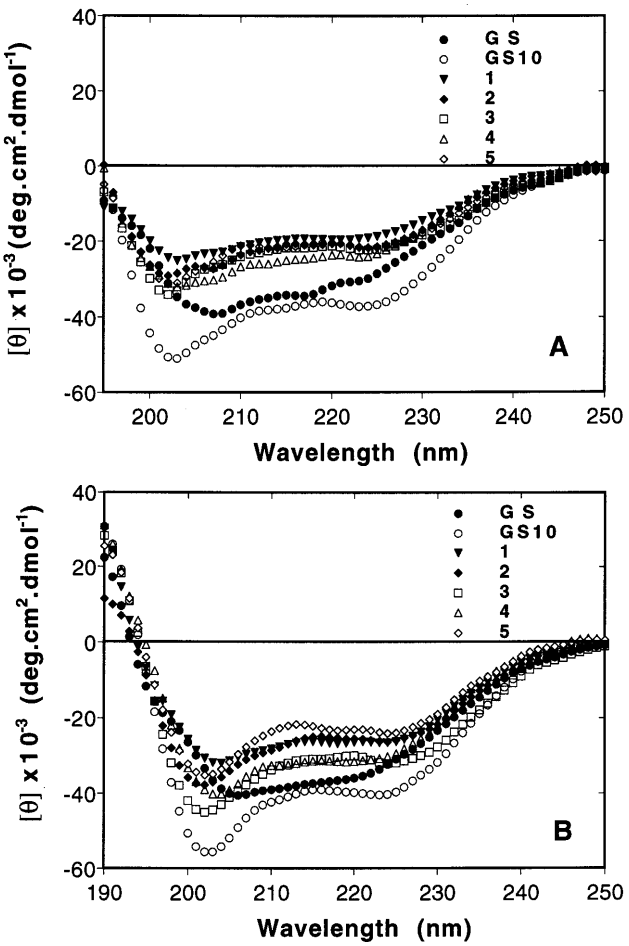

Figure 3 CD spectra of gramicidin S, GS10 and peptides 1–5 in PC (A) and PE/PG (B) vesicles

Peptide concentrations were at 10 μM, and [peptide]/[lipid] = 0.01. GS, gramicidin S.

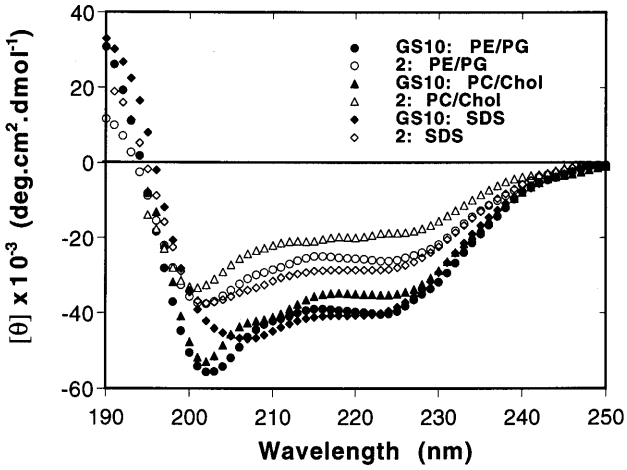

Figure 4 Comparison of GS10 and peptide 2 in PE/PG and PC/cholesterol vesicles, and SDS micelles

The following concentrations/ratios were employed: [peptide] = 10 μM; [peptide]/[lipid] = 0.01. For SDS micelles, [peptide] = 100 μM; [peptide]/[SDS] = 0.01. Chol, cholesterol.

structures such as α-helices, β-turns and β-hairpins ([20], and references therein). There was an overall increase in ellipticity of the cyclic peptides in aqueous TFE compared with the aqueous environment (more apparent in gramicidin S, GS10 and peptide **2**). However, despite minor changes in the ratio of the minima of the CD spectra of peptides, neither relative position of the minima nor the general shape of the spectra showed any basic difference between conformations in the two environments. In its CD profile, peptide **2** in aqueous TFE exhibited negative ellipticities comparable with those of GS10 in the 200–210 nm range. This result agrees with the fact that peptide **2** was the least disrupted structure in the buffer medium, and thus has the structure most susceptible to change in TFE among peptides **1**–**5**. Overall, the backbone structure of the GS10 analogues (peptides **1**–**5**) and GS10 itself is generally stable towards environmental changes in these milieus. For example, the minima for GS10 are positioned at approx. 207 and 221 nm, with $[\theta]_{221}/[\theta]_{207} = 0.91$, which is comparable with the minima ratio of 0.94 for the peptide structure in buffer.

Interaction of gramicidin S and a considerable number of other antimicrobial peptides with the plasma membrane of susceptible cells, leading to membrane destabilization and cell death, is believed to be crucial for their biological action [21]. To study the structure of GS10 and its analogues in imitations of biological membranes, spectra of the peptides were measured in phospholipid-bilayer systems and micelles. The bilayer systems employed were PC, PC/cholesterol and PE/PG vesicles. PE and PG are the major components of the bacterial membrane lipids, whereas PC, PE and cholesterol are some of the main lipids of mammalian erythrocytes. Neutral vesicles, PC and PC/cholesterol are considered to be the mimics of relatively neutral surfaces of human erythrocytes, whereas negatively charged PE/PG vesicles can approximate for bacterial cytoplasmic membranes. SDS micelles were also used both to mimic the membrane environments and to study the interaction of peptides with densely charged monolayers. Clearly, the membrane mimics utilized here are oversimplified versions of the cell membranes, which are complex and highly elaborate biological entities.

CD spectra of GS10 and peptides **1**–**5** in PC vesicles are shown in Figure 3(A). The spectrum of GS10 in PC bilayers showed a double minimum at 202 and 223 nm, with $[\theta]_{223}/[\theta]_{202} = 0.73$. Other analogues showed the same overall pattern, with a double minimum in the ranges of 200–205 nm and 220–225 nm. GS10 and peptides **1**–**5** demonstrated a similar behaviour in PE/PG vesicles (Figure 3B). The GS10 spectrum showed a double minimum at 203 and 223 nm, with $[\theta]_{223}/[\theta]_{203} = 0.74$. The ratio of the minima of spectra recorded in the PC and PE/PG systems were in the range of 0.50 to 0.85, and were approximately similar for each peptide in the two environments. It is also clear from Figure 3 that the CD spectra in the negatively charged PE/PG bilayers revealed slightly more enhanced ellipticities than the spectra in zwitterionic PC bilayers.

The spectra for GS10 (Figure 1A) and its D-Lys^{3}GS10 analogue (peptide **2**) (Figure 1B) in different environments are shown in Figure 4. In these spectra, the typical behaviour of GS10 analogues in two other systems (PC/cholesterol vesicles and SDS micelles) are compared with the PE/PG system. CD spectra of GS10 in PE/PG and PC/cholesterol vesicles were quite comparable. Moreover, it appeared that addition of cholesterol to PC vesicles (at $\approx 20\,\%$ molarity) did not substantially affect the conformation of the molecule. However, the CD profile of GS10 in SDS micelles is different from that in vesicles. Together with lipid vesicles, SDS micelles are considered to be mimics of biological membrane environments. In many instances, but not

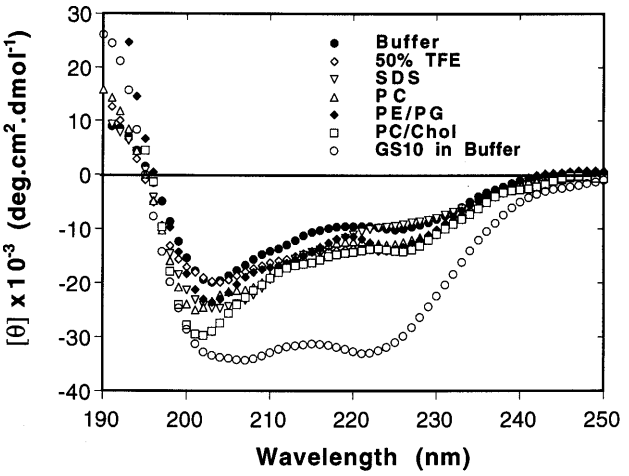

Figure 5 CD spectra of GS10rev (peptide 6) in different environments

Peptide concentrations used were 100 μM for buffer, 50% aqueous TFE and SDS (10 mM) solutions, and 10 μM for PC, PC/cholesterol and PE/PG vesicles, where [lipid] = 1 mM. Chol, cholesterol.

always, peptide structures in these systems are similar [22]. This similarity or difference essentially depends on the peptide conformation, its mode of interaction with membranes, and its environmental sensitivity. SDS micelles are densely charged, and their average sizes are much smaller than SUVs used for CD measurement [23]. Compared with the CD spectrum in SDS micelles, the minima of the GS10 spectrum in PE/PG (and the rest of the vesicle systems used in the present study) was blue-shifted and more intense in the area around 202 nm. The same conformational change was observable for peptides **1**, **3**, **4** and **5** (results not shown). Peptide **2** was different from the other analogues, since its structure in PE/PG and SDS systems was found to be very similar. It has already been shown that peptide **2** has a structure that is distinct from the rest of analogues in aqueous and 50% TFE solutions (Figure 2).

General conformational features reflected in the CD spectra of another group of GS10 analogues, GS10rev and its diastereoisomeric peptides (peptides **6**–**8**), were similar. Only GS10rev (peptide **6**) spectra are shown in Figure 5. Peptides **6**–**8** contain four lysine residues, and are considerably less hydrophobic than GS10 and peptides **1**–**5** (see the retention times in Table 1). The stable CD pattern of peptide **6** in all environments implies that it possesses a relatively rigid conformation.

An interesting feature of the CD spectra of peptide **6** in Figure 5 is that there is a clear similarity, both in shape and ellipticity, among the spectra in SDS, PC and PE/PG vesicles. In fact, the peptide in PC/cholesterol shows the most negative ellipticity among the studied systems. One would expect an intense electrostatic interaction to occur between the four lysine residues in the molecule and the negatively charged vesicles or micelles. From the data shown, it would appear that the total number of the positively charged lysine side chains, as well as their intramolecular distance and orientation, affect the lipid–peptide interaction.

It is important to mention that the cyclic peptides of the present study did not show signs of aggregation or fundamental conformational changes in the examined concentration range (10–100 μM), as confirmed by CD and UV concentration-dependent measurements (results not shown).

Biological activity

Table 1 shows the haemolytic activity of GS10 and its analogues, peptides **1**–**8**. In comparison with gramicidin S, GS10 is less haemolytic. Moreover, the less amphipathic (**1**–**5**) and less hydrophobic (**6**–**8**) peptide analogues were considerably less haemolytic. It has been already shown that there is a direct correlation between haemolytic activity and either hydrophobicity [3,4] or amphipathicity [15] in gramicidin S analogues. An increase in overall hydrophobicity and/or amphipathicity normally increases the haemolytic activity of the peptide.

Table 2 shows the biological activity of peptides against different Gram-positive bacteria and yeast. Generally, less hydrophobic GS10 is slightly less active than gramicidin S [3]. As with the haemolytic activity, all of the analogues showed a drastic decrease in biological activity, compared with gramicidin S and GS10. In the case of monosubstituted diastereoisomeric peptides **1**–**5**, antibacterial activity against *Staph. aureus* and *B. subtilis* and antifungal activity against *Ca. albicans* were suppressed. All of these analogues showed some activity against *Co. xerosis*. In all other cases, antibacterial activities were much weaker than those for either gramicidin S or GS10. Peptide **2**, with the lowest retention time on RP-HPLC and unique CD spectra, was the least active among the analogues. Analogues **6**–**8** were virtually inactive against the studied micro-organisms.

Table 3 depicts the activity of peptides against Gram-negative bacteria. As with Gram-positive bacteria and yeast, GS10 was again less active than gramicidin S. All the other analogues

Table 2 Biological activity of GS10 analogues against Gram-positive bacteria and yeast

		Minimal inhibitory concentration (μg/ml)						
Peptide	Micro-organism ...	*S. aureus* SAP0017	*S. aureus* K147	*S. epidermidis*	*B. subtilis*	*E. faecalis*	*Co. xerosis*	*Ca. albicans*
GS		1.5	1.5	1.5	3.1	1.5	1.5	4.0
GS10		6.2	6.2	4.5	6.2	3.1	1.5	6.2
5		> 200	150	100	> 200	> 100	62	> 200
3		> 200	> 200	200	> 200	> 125	50	> 200
1		> 200	> 200	150	> 200	> 125	50	> 200
4		> 200	> 200	200	> 200	> 150	100	> 200
2		> 200	> 200	> 200	> 200	> 200	125	> 200
6		> 200	> 200	> 200	> 200	> 200	200	> 200
7		> 200	> 200	> 200	> 200	> 200	150	> 200
8		> 200	> 200	> 200	> 200	> 200	> 200	> 200

Table 3 Biological activity of GS10 analogues against Gram-negative bacteria

Peptide	Gram-negative bacterium ...	Minimal inhibitory concentration (μg/ml)						
		Ps. aeruginosa K799(H187)	*Ps. aeruginosa** Z61(H188)	*E. coli* UB1005	*E. coli** DC2	*S. typhimurium* C587	*S. typhimurium** *C610*	Average† Gram-negative
GS		25	6.2	9.0	3.1	18	9.0	6.1
GS10		50	6.2	25	6.2	200	25	12.5
5		> 200	25	100	50	200	100	58
3		> 200	25	200	50	> 200	100	58
1		> 200	25	200	50	> 200	200	92
4		> 200	50	200	100	> 200	200	117
2		> 200	50	> 200	200	> 200	> 200	217
6		> 200	50	200	200	> 200	> 200	> 200
7		> 200	50	> 200	200	> 200	> 200	> 200
8		> 200	50	> 200	> 200	> 200	> 200	> 200

* Supersusceptible derivatives of Gram-negative bacteria with more permeable outer membranes. †Average minimal inhibitory concentration values (after 24 h) against supersusceptible Gram-negative bacteria, *Ps. aeruginosa* Z61, *E. coli* DC2 and *S. typhimurium* C610.

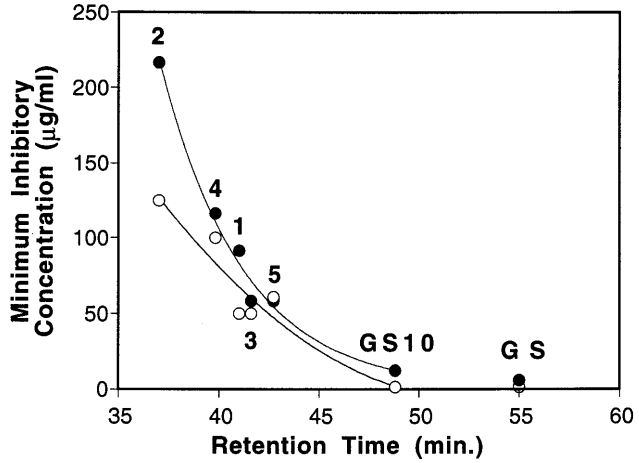

Figure 6 Relationship between amphipathicity and antimicrobial activity in GS10 diastereomeric analogues, peptides 1–5: average activity of peptides against three supersusceptible Gram-negative bacteria (*Ps. aeruginosa* Z61, *E. coli* DC2 and *S. typhimurium* C610), and activity against the coryneform Gram-positive bacteria *Co. xerosis*.

The average activity of peptides against three supersusceptible Gram-negative bacteria (*Ps. aeruginosa* Z61, *E. coli* DC2 and *S. typhimurium* C610; also see Table 3) (●) and activity against the coryneform Gram-positive bacteria *Co. xerosis*; also see Table 2) (○) are shown. Average activities of gramicidin S (GS) against similar strains are also shown for comparison.

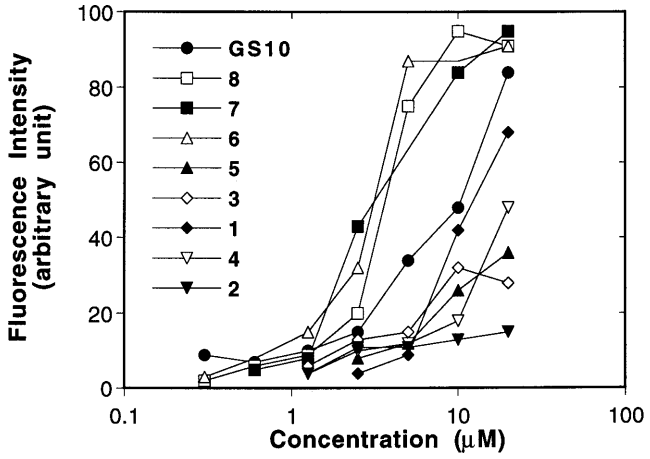

Figure 7 Permeabilization of the outer membrane of *E. coli* UB1005 induced by GS10 and its analogues

Changes in the NPN uptake by membrane are reflected as changes in fluorescence intensity of the probe (see the Experimental section for details).

showed a drastic decrease in their antibacterial activities against wild-type bacteria, but showed some activity against supersusceptible derivatives (*Ps. aeruginosa* Z61, *E. coli* DC2 and *S. typhimurium* C610) with defective outer-membrane permeability barriers. The most active peptides (although much less active than gramicidin S or GS10) were **1**, **3** and **5**, with the highest retention times among the GS10 analogues (Tables 1 and 3).

In Figure 6, the average activity of peptides **1**–**5** against *C. xerosis*, and a group of supersusceptible Gram-negative bacteria (*Ps. aeruginosa* Z61, *E. coli* DC2 and *S. typhimurium* C610) is correlated with the amphipathicity of the molecules. The more amphipathic peptides possess stronger antimicrobial activity. A similar relationship of amphipathicity and/or hydrophobicity with antimicrobial activity was observed in our previous studies with other biologically active gramicidin S analogues [3,4].

Permeabilization of the outer membrane of bacteria

Another method for assessment of peptide–membrane interaction is monitoring the changes in the NPN fluorescence [3,4,15]. The fluorescence of NPN (a non-polar fluorescent probe) is sensitive to environmental changes, and increases when the probe enters into more hydrophobic environments. Interaction of GS10 and its analogues (peptides **1**–**8**) with the outer membrane of the Gram-negative *E. coli* bacterium resulted in a general increase in NPN fluorescence (Figure 7). The results in Figure 7 can be interpreted to show that peptides possess different abilities to interact with, and permeabilize the outer membrane of, *E. coli* containing PE, PG and LPSs [24]. At lower concentrations, all peptides induced low permeabilization, whereas at higher concentrations peptides **6**–**8** caused high-probe permeation and were significantly better in this regard than GS10. In accordance with their mode of interaction with the bacterial outer membrane, peptides **1**–**5**, GS10 and peptides **6**–**8** can be divided into three separate groups, with peptides **1**–**5**

considerably less effective, and peptides **6**–**8** considerably more effective, than GS10 (Figure 7).

Displacement of dansyl-polymyxin from LPS

Cationic peptides interact with LPS, which is located solely on the outer half of the outer membrane of Gram-negative bacteria. LPS act as a permeation barrier, rendering bacteria resistant to certain antibiotics. Displacement of polymyxin B (a cationic cyclolinear nonapeptide with a high affinity for LPS) by other cationic peptides can be used as a measure of their affinity for LPS [4,15]. The LPS-binding abilities of the cyclic peptides examined in this study were evaluated in terms of their IC_{50} values (see the Experimental section). Gramicidin S, GS10 and the diastereoisomeric analogues of GS10 (peptides **1**–**5**) possessed high IC_{50} values, showing a low affinity for LPS (Table 1). Among these peptides, peptide **4** had the highest affinity for LPS. Interestingly, compared with the diastereoisomeric analogues of GS10, peptides **6**–**8** had an approximately 3-fold higher affinity for LPS (Table 1).

DISCUSSION

From the CD studies, GS10 and all of the analogues **1**–**8** showed certain similarities in structure. The double minimum pattern in all of the studied environments was universal in these peptides, despite differences in ellipticities, relative minima ratios and location of the minima. The wavelengths for minima were variable, but limited to certain ranges of the spectra, i.e. 200–210 nm and 220–225 nm. In addition, the cyclic nature and small size of the decameric molecules would restrict flexible conformations. Therefore it is plausible to assume relatively rigid conformations for these peptides. In the diastereoisomeric analogues (**1**–**5**), the overall β-sheet/β-turn structure of GS10 is distorted, and no typical structure can be assigned on the basis of CD spectra alone. Distortions reflected in the CD spectra imply that any change in the turn or sheet dihedral angles and/or hydrogen-bonding lattice may lead to local flexibility and partial structural distortion. Conformational distortion was also observable in analogues **6**–**8**.

Comparison of the CD spectra of GS10 and peptides **1**–**5** in aqueous solution and lipid vesicle systems shows a strong affinity of all peptides for lipid-bilayer surfaces, representing mammalian erythrocytes and cytoplasmic membranes of bacteria. The ellipticities were clearly enhanced in lipid vesicles, which implies that peptides were readily partitioning on to the bilayer surface. Inducing NPN probe permeation in the outer membrane of *E. coli* is in support of peptide–membrane interaction. However, NPN permeation was weak for peptides **1**–**5** containing two lysine residues (Figure 7). The strong permeabilization of the outer membrane at medium to high concentrations of peptides **6**–**8** (Figure 7) reflects the role of electrostatic forces in peptide–membrane interaction. The outer-membrane surface had a greater affinity for peptides **6**–**8** containing four positively charged lysine residues. Similarly, peptides **6**–**8** had a high affinity for LPSs of the outer membrane of *Ps. aeruginosa* (Table 1). However, this greater affinity does not cause a major conformational change in these peptides (Figure 5). It is worth noting that the enhanced ability to permeabilize the outer membrane of bacteria and the LPS-binding affinity of peptides **6**–**8** do not correlate with their biological activity (Tables 1–3). Interestingly, from the concentration-dependent behaviour shown in Figure 7, peptides **1**–**8** are divided into two structurally related groups, i.e. peptides **1**–**5** and peptides **6**–**8**, with two different membrane-interacting behaviours. GS10 is structurally different from both groups, and its permeation ability is intermediary between these groups.

There have been reports on the interaction of gramicidin S with membrane systems [25–27]. Enhancement of peptide β-sheet/turn structures upon interaction with membrane surfaces is in agreement with the occurrence of β-structures on membrane surfaces [28–30]. Compared with α-helical structures, β-structures are energetically less favoured for penetration of the membrane interior, since the amide bonds in the structure have limitations for satisfying intermolecular hydrogen bonds (Figure 1A).

Overall, on the basis of CD studies in lipid vesicles and SDS micelles, the mode of interaction of gramicidin S and GS10 with the cytoplasmic membrane surfaces would seem to entail adsorption followed by further stabilization of the secondary structure. Stabilization might be also caused by partial penetration below the membrane surface (from an increase in tyrosine fluorescence intensity in comparison with aqueous solution; results not shown). The next step is the induction of certain changes in the lipid-bilayer morphology via peptide–lipid and/or peptide–peptide interactions. This secondary step destabilizes the membrane structure. Disruption of the gramicidin S or GS10 backbone by systematic enantiomeric substitutions in peptides **1**–**5** is likely to affect the secondary step of interaction with membranes. Analogues are all adsorbed on the surface of bilayers, but their mode of intermolecular interactions can differ from that of either the gramicidin S or GS10 molecules. This change in the mode of interaction seems to be related to the changed topology of the molecules (and their general amphipathic pattern), which can directly affect the biological activity of the analogues. Changes in the interaction of peptides with membrane, and the effect on biological activity, have also been observed for amphipathic helical peptides with double enantiomeric substitutions [22,31], as well as for gramicidin S and gratisin cyclic analogues [32].

Peptides **6**–**8** are much less hydrophobic than GS10 and peptides **1**–**5** (Table 1). They also exhibit distorted structures and reduced biological activity (Figure 5 and Tables 2 and 3). Peptides **6**–**8** show an affinity for cytoplasmic membranes, but no special preference for charged vesicles to zwitterionic ones (Figure 5; data are shown only for peptide **6**). In the case of these peptides, as holds true for peptides **1**–**5**, the secondary interaction after adsorption on to lipid surfaces can be different from that of both gramicidin S and GS10. Indeed, the mode of interaction of these peptides with the outer membrane of *E. coli* is different from that of both GS10 and peptides **1**–**5** (Figure 7).

In comparison with GS10, haemolytic activity is strongly suppressed in all of the diastereoisomeric analogues. Moreover, there is a direct correlation between antimicrobial activity and amphipathicity in the analogues: a decrease in antimicrobial activity corresponded to the decrease in amphipathicity (decrease in RP-HPLC retention times) (Figure 6). In addition, in the case of gramicidin S and GS10 (where the amphipathic topology is conserved), hydrophobicity becomes the dominant factor and gramicidin S proves to be more active biologically (Figure 6). The case is not as simple for the diastereoisomeric analogues of GS14 [15]. In these analogues, unlike GS10 analogues, haemolytic activity was not strongly suppressed, but showed a decrease with a decrease in amphipathicity. GS14 was the most haemolytic peptide, with the highest retention time. In contrast, antimicrobial activity in GS14 diastereoisomeric analogues followed a reversed order: for a majority of the tested micro-organisms (Gram-positive and -negative bacteria and yeast), an increase in antimicrobial activity corresponded to a decrease in amphipathicity [15].

Reversing the amphipathic pattern of the primary structure of the cyclic molecule by alternative substitution of lysine and hydrophobic residues [GS10rev (peptide **6**) and its mono-substituted diastereoisomers (peptides **7** and **8**)] also leads to distorted structures. These conformational changes result in substantial reduction of antimicrobial and haemolytic activities. However, in the case of peptides **6**–**8**, the electrostatic factor may prevail due to the presence of four charged lysine residues. Electrostatic force cannot only induce strong binding to (and perturbation in) negatively charged membrane surfaces (Figure 7), but also acts as an impeding element for further penetration of peptides towards the cytoplasmic membrane of bacteria and into the deeper levels of membrane, which may be critical for antimicrobial activity.

In conclusion, in agreement with previous studies [2], there is a definite relationship between structure and function in the decameric gramicidin S-like structures, i.e. changes in structure and the cyclic topology of the molecule can drastically suppress its functional mode. In the diastereoisomeric analogues (peptides **1**–**5**), haemolytic activity was suppressed and dissociated from antimicrobial activity. However, their antimicrobial activity was weak. In addition, amphipathicity in these cyclic molecules plays a definite role in their interaction with lipid membranes. In the diastereoisomeric analogues of GS10 (peptides **1**–**5**) the amphipathic pattern of the GS10 molecule was changed, while its inherent hydrophobic nature was conserved. The consequent change in the haemolytic and antimicrobial activity of the peptides clearly implies the existence of a threshold minimum amphipathicity for effective biological activity. In peptides **6**–**8**, amphipathicity and overall hydrophobicity of GS10 were altered and the total positive charge of the cyclic peptide increased. Moreover, despite their strong affinity for biological membranes and ability to perturb the membrane surface, peptides **6**–**8** were not active. It is clear that, in these peptides, the balance between electrostatic, amphipathic and hydrophobic factors was disturbed. Therefore modes of interaction of peptides **6**–**8** with both the cytoplasmic membranes of erythrocytes and bacteria, and the outer membrane of Gram-negative bacteria, were fundamentally changed, resulting in suppression of their biological activity.

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