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Properties and structure–activity studies of cyclic β-hairpin peptidomimetics based on the cationic antimicrobial peptide protegrin I

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Abstract—The properties and structure–activity relationships (SAR) of a macrocyclic analogue of porcine protegrin I (PG-I) have been investigated. The lead compound, having the sequence *cyclo*-(-Leu-Arg-Leu-Lys-Lys-Arg-Arg-Trp-Lys-Tyr-Arg-Val-D-Pro-Pro-), shows antimicrobial activity against Gram-positive and -negative bacteria, but a much lower haemolytic activity and a much reduced ability to induce dye release from phosphatidylcholine/phosphatidylglycerol liposomes, when compared to PG-I. The enantiomeric form of the lead peptide shows comparable antimicrobial activity, a property shared with other cationic antimicrobial peptides acting on cell membranes. SAR studies involving the synthesis and biological profiling of over 100 single site substituted analogues, showed that the antimicrobial activity was tolerant to a large number of the substitutions tested. Some analogues showed slightly improved antimicrobial activities (2–4-fold lowering of MICs), whereas other substitutions caused large increases in haemolytic activity on human red blood cells.

1. Introduction

The naturally occurring cationic antimicrobial peptides represent interesting targets for peptidomimetic research. These natural products have been discovered in all animal kingdoms where they are now recognized as key mediators of innate immunity to bacterial infections.[1–3] They are generally peptides of less than 50 amino acids, with a net positive charge due to multiple lysine and/or arginine residues as well as having multiple hydrophobic residues. Cationic antimicrobial peptides typically show minimal inhibitory concentrations (MICs) in the range 0.25–16 μg/mL against Gram-positive and -negative bacteria, fungi and protozoa. They have been heralded as a potential source of new antimicrobial drugs to combat the increasing threat posed by multiple drug resistant microorganisms.[4–6] Such peptides are frequently classified on the basis of their preferred structures as β-sheet, α-helical, loop or extended peptides.[7]

Most naturally occurring cationic antimicrobial peptides target and disrupt the functions of microbial cell membranes.[8–11] The excess of negative charge associated with the outer microbial cell membrane imparts some selectivity in the mode of action, although at higher concentrations animal cell membranes are frequently also disrupted. As a class of natural products, however, their mechanisms of antimicrobial action are quite complex. Some target membrane receptors such as lipopolysaccharide (e.g., polymyxin), whereas others clearly exhibit

Abbreviations: Boc, *tert*-butoxycarbonyl; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, 2-(1*H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-[1*H*-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, *N*-hydroxy-9-azabenzotriazole; HOBt, *N*-hydroxybenzotriazole; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TIPS, triisopropyl-silane; TFA, trifluoroacetic acid.

Keywords: Antibiotic; Secondary structure; Haemolysis; Protein epitope mimetic.

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other biological activities (e.g., members of the cathelicidin family[12]). It seems that cationic peptides (and peptidomimetics) may have multiple actions on cells, ranging from cell permeabilization to targeting intracellular receptors, and that these actions may vary with changes in sequence and stereochemistry.

The starting point for our peptidomimetic design was the naturally occurring antimicrobial peptide protegrin I (PG-I) (**1**) (Fig. 1), one of a family of five homologous peptides first isolated from porcine leucocytes.[13,14] PG-I is an 18-amino acid peptide containing two disulfide bridges (Cys6–15 and Cys8–13). These disulfides constrain the peptide backbone into a β-hairpin conformation, with a β-turn formed by residues 9–12, as detected by NMR in water and DMSO solution, and in DPC micelles.[15–17] Other naturally occurring disulfide bridged antimicrobial β-hairpin peptides of similar size and shape include tachyplesins[18–21] and polyphemusins[22] from horseshoe crabs, androctonin from scorpions,[23,24] thanatin from insects,[25] lactoferricin B from cows,[26] human hepcidin,[27] an antimicrobial protein from plants (Ib-AMp1),[28] and gomesin from spiders.[29,30] Related backbone cyclic antimicrobial peptides that also adopt β-hairpin structures include gramicidin S[31] and tyrocidine A,[32] both of bacterial origin, and the backbone cyclic and disulfide cross-linked peptide RTD-I (also called theta-defensin) from primate leukocytes.[33,34]

PG-I typically kills susceptible microorganisms within minutes,[35–37] consistent with a bactericidal effect caused by disruption of the cell membrane.[13,38–40] Both enantiomeric forms of PG-I show essentially the same antimicrobial activity,[37,39] a characteristic shared with other cationic peptides targeting the cell membrane.[41–43] This behaviour is consistent with the peptides interacting mainly with the fatty acyl chains in the cell membrane. However, PG-I is also able to bind the lipopolysaccharide and lipid-A in the membranes of certain Gram-negative bacteria.[44]

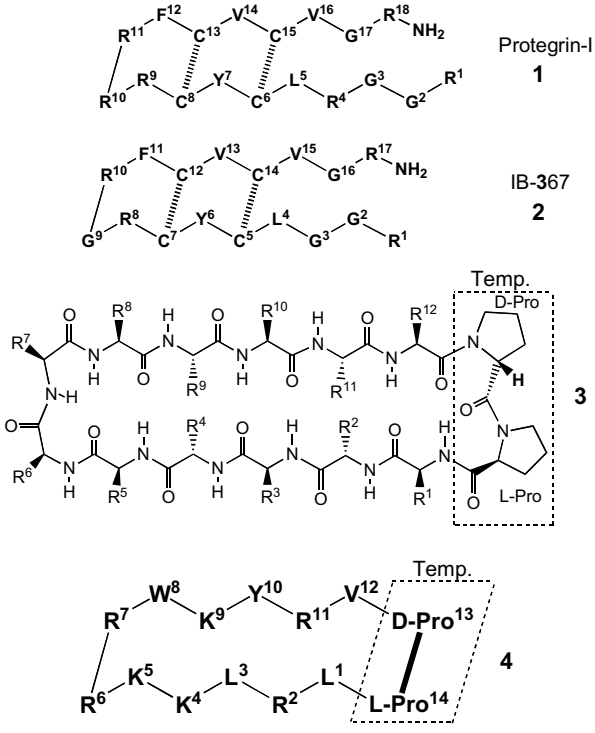


Figure 1. Structures **1**–**4**.

Biophysical studies of the interaction of PG-I with model membrane bilayers confirm that the peptide inserts into membranes, forms oligomeric aggregates that contact both the polar head groups and the hydrophobic core, and causes thinning and disruption of lipid packing. The average orientation, mobility and depth of insertion seems to depend upon the constitution (e.g., charge, lipid chain length, degree of unsaturation) of the membrane lipids used and the presence of cholesterol.[45–50] The degree of insertion into anionic containing monolayers is significantly larger than that of zwitterionic bilayers. Lipid A films are completely destabilized,[47] consistent with the preferential damage caused to bacterial over mammalian cell membranes.

Detailed structure–activity relationship (SAR) studies on PG-I revealed that linear analogues, lacking the disulfide bridges, have no regular secondary structure and exhibit reduced antimicrobial activity.[51–55] Although a large number of amino acid substitutions in the PG-I structure are tolerated, the amphiphilicity of the β-hairpin, and total number of positively charged residues impact significantly on the activity. SAR studies[52] culminated in the discovery of analogue IB367 (**2**) with Cys5–14 and Cys7–12 disulfide bridges, which has now been tested in the clinic to treat ulcerative oral mucositis, ventilator associated pneumonia, and respiratory infections associated with cystic fibrosis. However, a property limiting the wider application of PG-I as an antibiotic is its mammalian toxicity. One key indicator of this toxicity is its high haemolytic action on human red blood cells, causing ca. 37% lysis (see below) at a PG-I concentration of 100 μg/mL.

In earlier work,[56,57] we reported an approach to PG-I peptidomimetics of type **3** (Fig. 1) based on the use of β-hairpin-stabilizing organic templates. The template is chosen for its ability to promote a β-hairpin loop structure, as depicted in **3**, thereby dispensing with the need for disulfide bridges to constrain the loop into a β-hairpin geometry. We have used this design principal to prepare β-hairpin peptidomimetics of antibody hypervariable loops,[58] protease inhibitors based on the Bowman–Birk fold,[59] RNA-binders that inhibit the interaction of Tat with TAR-RNA,[60] and in the design of novel p53-HDM2 inhibitors.[61]

The PG-I mimetic **4** (Fig. 1), described earlier, adopts a β-hairpin structure in dodecylphosphocholine micelles, and exhibits good antimicrobial activity (MICs 6–12 μg/mL) against Gram-positive and Gram-negative bacteria. However, compared to PG-I, **4** shows a much reduced haemolytic activity on human erythrocytes; ca. 1% lysis at a peptide concentration of 100 μg/mL. In this work, we report new data on **4** and an extensive SAR study of this mimetic.

2. Results and discussion

2.1. Enantioselectivity

It was shown earlier[56] that **4** has a rapid bactericidal action upon both Gram-negative and Gram-positive bacteria, effectively reducing the number of viable bacterial cells by three orders of magnitude within a few minutes. The enantiomer of **4** was prepared here, and shown to have essentially similar or even slightly higher antimicrobial activity (data not shown). The enhancement in activity of *ent*-**4** is small (less than or equal to a factor of two in MIC compared to **4**), which possibly reflects a higher proteolytic stability in bacterial cultures. As with PG-I, however, the comparable activity of the enantiomer suggests that the mechanism of action does not involve binding to a chiral receptor, but rather reflects a nonenantiospecific interaction with membrane lipids.

2.2. Activity on model lipid membranes

The ability of **4** to bind and cause leakage through lipid bilayers was assessed by measuring dequenching of calcein fluorescence upon release from a concentrated solution within liposomes, which are used as model cell membranes. Large unilamellar liposomes containing calcein were prepared from various binary ratios of zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and negatively charged 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-(1-glycerol)] (POPG). As a control, the dye release assays were also performed with PG-I.

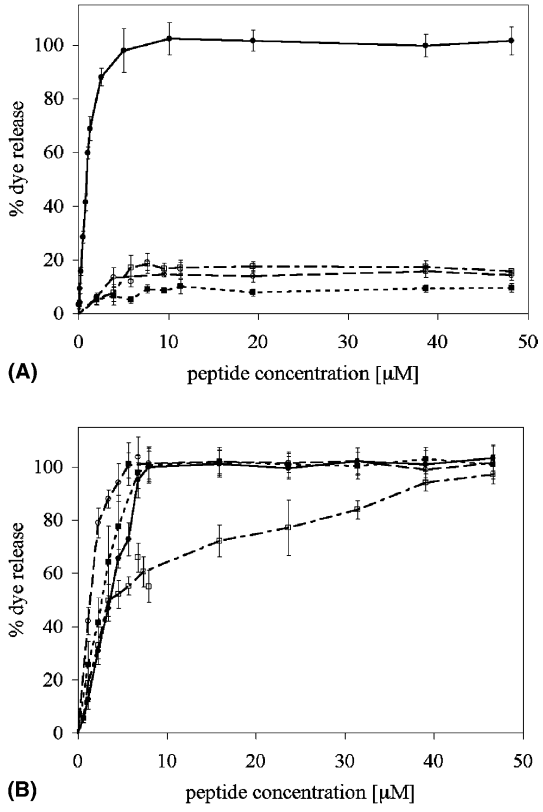


Figure 2. Concentration dependence of calcein release from liposomes induced by A, peptide mimetic **4**; and B, by PG-1. The liposomes were composed of pure POPG (●); pure POPC (□); POPC/POPG (80:20) (■) and POPC/POPG (70:30) (○). See Experimental.

PG-I induced substantial leakage of calcein from vesicles composed of POPC and POPG (80:20 and 70:30), as well as from pure POPC and POPG liposomes (Fig. 2B). Similar experiments have been described already for PG-I[62,63] and the related cationic peptide antibiotics polyphemusin and gramicidin S.[64] Mimetic **4** showed comparable leakage activity only toward the highly negatively charged, and hence more susceptible POPG liposomes (Fig. 2A). Liposomes containing 20% or 30% zwitterionic POPC, or POPC alone, experienced much reduced dye release with **4** compared to that caused by PG-I under comparable conditions. These data show that mimetic **4** possesses membranolytic activity in this model system, but at a level significantly reduced compared to that for PG-I. This parallels the reduced haemolytic behaviour on human red blood cells observed for **4**, compared to that seen for PG-I.

2.3. Structure–activity relationships

In order to probe how the antimicrobial activity of **4** is related to its sequence, libraries of analogues were synthesized, by substituting a variety of different amino acids at each of the 12 positions around the β-hairpin. For the synthesis of these analogues, a linear precursor was first assembled on 2-chlorotrityl chloride resin using Fmoc-chemistry, as described earlier.[56,57] The linear, side chain protected precursors were then cleaved from the resin with 1% TFA in CH_2Cl_2, macrocyclization was performed in dilute DMF solution with HATU/HOAt, and a final deprotection step was performed with a mixture of TFA/TIPS/H_2O (95/2.5/2.5). The desired macrocycle was typically the main component in the product. This was purified by preparative HPLC and characterized by electrospray ionization MS. The purity of the peptides used for biological assays was $\geqslant$95%.

The sequences of the analogues, their antimicrobial activities against three test organisms, and their haemolytic activity on fresh human red blood cells, are reported in Tables 1–3. We focus first on analogues arising through substitutions in the hydrophobic residues in **4**, namely Leu-1, Leu-3, Trp-8, Tyr-10 and Val-12. The results (Table 1) show that good antimicrobial activities were retained in many of the analogues, although large improvements were not observed. It has been noted often, that antimicrobial peptides acting on bacterial cell membranes typically have MICs at best in the low μM range, but not significantly below this (e.g., into the low nM range). Many of the analogues tested contained hydrophobic residues of differing size, yet showed antimicrobial activities close to those of **4**. Substituting a polar residue for a nonpolar residue, however, often had deleterious effects on MICs.

The haemolytic activity on red blood cells was also investigated (see Tables 1–3). It is apparent that substitutions at position-10 can cause large increases in haemolytic

Table 1. Minimal inhibitory concentrations (MICs) in μg/mL against the test organisms shown, and the percentage haemolysis of human red blood cells caused at a peptide concentration of 100 μg/mL

Position-1 (Leu)	Leu	Phe	Cha	Tyr	Trp	Hfe	1-Nal	2-Nal	ClF	His	Bip	Orn
E. coli ATCC 25922	12.5	18	25	12.5	25	12.5	12.5	25	6.2	25	12.5	50
P. aeruginosa ATCC 27853	6.2	9.4	25	25	50	12.5	12.5	12.5	3.1	100	6.2	100
S. aureus ATCC 25923	12.5	9.4	6.2	25	25	25	12.5	6.2	6.2	100	6.2	100
Haemolysis (%)	1.4	1.1	7.1	0.6	2.2	6.8	13.2	7.7	11.5	0.2	17.6	0.7
Position-3 (Leu)	Leu	Phe	Cha	Trp	Hfe	1-Nal	2-Nal	Val	ClF	His	Bip	Orn
E. coli ATCC 25922	12.5	25	25	12.5	12.5	12.5	6.2	6.2	50	25	12.5	50
P. aeruginosa ATCC 27853	6.2	25	12.5	12.5	6.2	12.5	25	6.2	50	100	12.5	100
S. aureus ATCC 25923	12.5	50	12.5	12.5	12.5	12.5	12.5	25	50	100	25	100
Haemolysis (%)	1.4	2.6	3.1	4.5	3.8	5.2	5.8	1.1	13.1	1.2	7.7	1.5
Position-8 (Trp)	Trp	Phe	Tyr	Y(B)	Hfe	1-Nal	2-Nal	Val	ClF	Leu	Ile	Orn
E. coli ATCC 25922	12.5	25	25	25	25	12.5	25	25	12.5	25	25	50
P. aeruginosa ATCC 27853	6.2	12.5	25	6.2	9.4	6.2	6.2	12.5	6.2	12.5	12.5	50
S. aureus ATCC 25923	12.5	50	25	6.2	25	12.5	9.4	25	12.5	50	25	25
Haemolysis (%)	1.4	2.1	1.7	4.5	3.1	2.7	3.5	2	3.3	1.3	2.3	3.1
Position-10 (Tyr)	Tyr	Phe	Cha	Trp	Hfe	1-Nal	2-Nal	Val	ClF	Leu	Bip	Orn
E. coli ATCC 25922	12.5	12.5	25	25	25	25	18	50	12.5	6.2	6.2	50
P. aeruginosa ATCC 27853	6.2	12.5	50	12.5	12.5	12.5	50	50	9.4	9.4	6.2	50
S. aureus ATCC 25923	12.5	12.5	12.5	9.4	9.4	12.5	12.5	50	12.5	12.5	6.2	50
Haemolysis (%)	1.4	30.1	57.6	3.9	31.8	29.3	39.6	4.9	34.8	26.6	33	0.1
Position-12 (Val)	Val	Phe	Cha	Trp	Hfe	1-Nal	2-Nal	Tyr	ClF	Leu	Bip	Orn
E. coli ATCC 25922	12.5	6.2	50	12.5	12.5	25	25	25	12.5	25	12.5	50
P. aeruginosa ATCC 27853	6.2	12.5	12.5	6.2	12.5	12.5	12.5	25	6.2	12.5	6.2	25
S. aureus ATCC 25923	12.5	12.5	6.2	12.5	12.5	6.2	18	50	12.5	25	6.2	100
Haemolysis (%)	1.4	10.1	35.2	10.5	21.7	3.7	23.3	6	6.4	4.5	18.4	0.3

Shown are the amino acid substitutions made at the positions Leu-1, Leu-3, Trp-8, Tyr-10 and Val-12 in **4**. The first entry in each row shows the activities of **4** without substitution. The normal three letter code is used for proteinogenic amino acids, and Cha = L-cyclohexylalanine; Hfe = L-homophenylalanine; 1-Nal = L-1-naphthylalanine; 2-Nal = L-2-naphthylalanine; ClF = L-4-chlorophenylalanine; Bip = L-4-biphenylalanine; Orn = L-ornithine; Y(B) = L-(*O*-benzyl)tyrosine.

activity. Substituting Tyr-10 to Phe, for example, or introducing larger aromatic groups enhances haemolytic activity dramatically. Also, the biphenyl side chain at position-10 improves slightly the MICs, but induces much higher haemolysis than seen with **4**. A significant worsening of the haemolytic activity is seen with several other substitutions made at position-12. On the other hand, similar substitutions at positions-1 and -3, on the other side of the hairpin, or at position-8, were much less deleterious. An explanation for these selectivity effects is not yet available, since the precise molecular mechanism(s) of haemolysis is(are) unknown.

The results of substitutions of cationic residues in **4** are summarized in Table 2. Of the many substitutions tested, none gave rise to significant improvements in MICs, although in some cases a significant increase in haemolytic activity was observed.

In a final round of screening, substitutions were also made in the D-Pro-L-Pro template (Table 3), by substituting the L-Pro-14 by a different L-amino acid. Some analogues showed good antimicrobial activity (e.g., Pro-14 to L-Phe) without an increased haemolytic activity, whereas in other cases the haemolytic activity was also increased significantly. A further library of analogues was prepared by substituting L-Pro-14 for an (4-*N*-acylamino)proline analogue (**14A**–**S**), or a (4-*O*-alkoxy)proline derivative (**14T** and **14U**) (Fig. 3). The functionalized proline derivatives **7** and **9** required for the synthesis of these peptides were prepared from *N*-Boc-(2*S*,4*S*)-4-azidoprolinemethyl ester[65] (**5**) or *N*-Boc-(2*S*,4*R*)-4-hydroxyproline (**8**), as shown in Scheme 1. Most of the analogues **14A**–**U** contain additional hydrophobic groups, which should facilitate interactions with a lipid bilayer. However, any beneficial effects on the MIC of a new substituent were typically small and usually accompanied by an increase in the haemolytic activity (see Table 3). Only **14A** has an improved antimicrobial profile, without a significant increase in haemolytic activity.

In summary, the SAR studies of **4** have involved the synthesis and biological profiling of over 100 single site substituted analogues. These studies revealed that the antimicrobial activity of **4** was tolerant to a large number of the substitutions tested. Some analogues show slightly (2–4-fold) improved MIC values against the panel of test organisms. The SAR studies also revealed that the haemolytic activity of **4** on human red blood cells, was significantly increased following substitutions, in particular, at positions-10 and -12 in the putative hairpin. Finally, several new derivatives have been identified, which show an improved antimicrobial profile,

Table 2. Minimal inhibitory concentrations (MICs) in μg/mL against the test organisms shown, and the percentage haemolysis of human red blood cells at a peptide concentration of 100 μg/mL

Position-2 (Arg)	Arg	Tyr	Trp	Leu	His	Thr	Gln
E. coli ATCC 25922	12.5	6.2	6.2	12.5	25	25	12.5
P. aeruginosa ATCC 27853	6.2	12.5	18	12.5	12.5	12.5	12.5
S. aureus ATCC 25923	12.5	25	12.5	25	50	25	25
Haemolysis (%)	1.4	1.4	0.2	1.6	1.8	1.3	1.1
Position-4 (Lys)	Lys	Arg	Trp	Leu	His	Thr	Gln
E. coli ATCC 25922	12.5	12.5	50	25	12.5	25	6.2
P. aeruginosa ATCC 27853	6.2	6.2	100	25	12.5	25	25
S. aureus ATCC 25923	12.5	12.5	100	25	25	25	25
Haemolysis (%)	1.4	1.2	4	0.5	0.4	0.7	1
Position-5 (Lys)	Lys	Arg	Trp	Leu	His	Thr	Gln
E. coli ATCC 25922	12.5	12.5	25	12.5	18	25	12.5
P. aeruginosa ATCC 27853	6.2	6.2	100	25	25	12.5	12.5
S. aureus ATCC 25923	12.5	12.5	100	12.5	50	25	25
Haemolysis (%)	1.4	2.4	6	27.4	4.1	1.3	1.1
Position-6 (Arg)	Arg	Tyr	Trp	Leu	His	Thr	Gln
E. coli ATCC 25922	12.5	12.5	25	25	12.5	25	25
P. aeruginosa ATCC 27853	6.2	25	25	12.5	12.5	25	25
S. aureus ATCC 25923	12.5	50	50	25	50	100	100
Haemolysis (%)	1.4	1.8	9.4	2.6	0.3	2.5	0.6
Position-7 (Arg)	Arg	Tyr	Trp	Leu	His	Gln	Thr
E. coli ATCC 25922	12.5	12.5	12.5	25	6.2	12.5	25
P. aeruginosa ATCC 27853	6.2	12.5	6.2	25	12.5	12.5	6.2
S. aureus ATCC 25923	12.5	50	25	100	50	50	12.5
Haemolysis (%)	1.4	4.6	1.3	2.5	6.7	2.5	2.7
Position-9 (Lys)	Lys	Tyr	Trp	Leu	Thr	Gln	His
E. coli ATCC 25922	12.5	12.5	12.5	6.2	6.2	6.2	12.5
P. aeruginosa ATCC 27853	6.2	18.8	18.8	12.5	12.5	12.5	25
S. aureus ATCC 25923	12.5	25	25	12.5	25	25	12.5
Haemolysis (%)	1.4	2.3	1.6	3.5	2.7	3.7	1.4
Position-11 (Arg)	Arg	Tyr	Trp	Leu	Orn	Cit	
E. coli ATCC 25922	12.5	6.2	6.2	12.5	25	25	
P. aeruginosa ATCC 27853	6.2	12.5	12.5	25	6.2	25	
S. aureus ATCC 25923	12.5	25	12.5	50	25	50	
Haemolysis (%)	1.4	4.4	9.9	5.9	2.2	1.4	

The amino acid substitutions made at the positions Arg-2, Lys-4, Lys-5, Arg-6, Arg-7, Lys-9 and Arg-11 in **4** are shown. The first entry in each row shows the activities of **4** without substitution. The normal three letter code is used for proteinogenic amino acids, and Cit = L-citrulline; Orn = L-ornithine.

compared to **4**, whilst maintaining a much lower haemolytic activity in comparison to PG-I.

3. Experimental

3.1. Peptide synthesis

The peptidomimetic libraries were made by parallel synthesis on a Syro-II (Microsyntec, Germany) peptide synthesizer typically using 12–24 reaction vessels, and a method described earlier.[56] A typical procedure is described. In one reaction vessel, 2-chlorotrityl chloride resin (0.5 g, 0.83 mmol/g) was suspended in CH_2Cl_2 and then treated with the first amino acid (0.415 mmol) and DIEA (4 equiv) with shaking at rt for 18 h. The resin was then washed extensively (with CH_2Cl_2/MeOH/DIEA, 17/2/1; then CH_2Cl_2; then DMF; then CH_2Cl_2; and Et_2O, three times each) and dried under vacuum. Following removal of the Fmoc-group using 40% piperidine/DMF, chain elongation was performed sequentially with 5 equiv of each protected amino acid and HOBt/HBTU (each 5 equiv) for activation, diisopropylethylamine (5 equiv) as base and DMF as solvent. After completion of the synthesis, the resin was washed with CH_2Cl_2 and MeOH, and then treated with 1% TFA in CH_2Cl_2 (three times). The filtrate was neutralized with DIEA in CH_2Cl_2 and evaporated to give the linear protected peptide. The product (100 mg) was then cyclized overnight at rt in DMF (9 mL) with HOAt and HATU (each 3 equiv) and 1% v/v DIEA. After evaporation of the DMF, the product was dissolved in CH_2Cl_2,

Table 3. Amino acid substitutions made at position-14 in **4**

Position-14 (Pro)	Pro	Gly	Arg	Tyr	Phe	Trp	Leu	Ile	Val	Gln	Cha
E. coli ATCC 25922	12.5	12.5	6.2	12.5	6.2	6.2	12.5	6.2	12.5	25	12.5
P. aeruginosa ATCC 27853	6.2	25	12.5	25	6.2	12.5	12.5	25	12.5	50	6.2
S. aureus ATCC 25923	12.5	50	25	25	12.5	12.5	50	25	50	100	12.5
Haemolysis (%)	1.4	1.1	1.7	1	0.7	4.1	2.7	2	1	1.2	11.7

Position-14 (Pro)	Ybzl	Hfe	2-Nal	1-Nal	Nle	Bip	ClF	S(B)	Orn	hCha
E. coli ATCC 25922	25	12.5	12.5	25	12.5	12.5	12.5	25	25	6.2
P. aeruginosa ATCC 27853	6.2	12.5	12.5	12.5	6.2	6.2	6.2	6.2	25	6.2
S. aureus ATCC 25923	12.5	12.5	12.5	12.5	50	12.5	12.5	12.5	100	6.2
Haemolysis (%)	14.7	5.9	18.2	13.1	1.6	13.1	4.2	19.7	0.8	13.8

Position-14 (Pro)	**14A**	**14B**	**14C**	**14D**	**14E**	**14F**	**14G**	**14H**	**14I**	**14J**
E. coli ATCC 25922	3.1	6.2	6.2	6.2	12.5	50	25	12.5	9.4	12.5
P. aeruginosa ATCC 27853	3.1	6.2	6.2	6.2	25	100	50	25	12.5	6.2
S. aureus ATCC 25923	6.2	6.2	6.2	6.2	100	100	100	50	25	25
Haemolysis (%)	2.7	19.6	23.6	18	0.5	1.9	0.6	0.5	1.7	2.7

Position-14 (Pro)	**14K**	**14L**	**14M**	**14N**	**14O**	**14P**	**14Q**	**14R**	**14S**	**14T**	**14U**
E. coli ATCC 25922	50	12.5	12.5	12.5	12.5	12.5	12.5	25	25	12.5	18
P. aeruginosa ATCC 27853	25	6.2	12.5	12.5	6.2	12.5	9.4	25	6.2	6.2	12.5
S. aureus ATCC 25923	100	12.5	12.5	25	25	25	25	50	25	12.5	12.5
Haemolysis (%)	0.9	3.4	3.9	0.8	2.2	1	0.2	0.7	3	3.4	11

MICs in μg/mL against the test organisms are shown, and the percentage haemolysis of human red blood cells caused at a peptide concentration of 100 μg/mL. The first entry shows the activity of **4** without substitution. The normal three letter code is used for proteinogenic amino acids, and Nle = L-norleucine; S(B) = L-(*O*-benzyl)serine; hCha = L-homocyclohexylalanine; see also Tables 1 and 2 and Figure 3 for **14A**–**U**.

washed with water and dried. The cyclic protected peptide was treated with TFA/H_2O/TIPS (95/2.5/2.5) at rt for 2.5 h. After evaporation, the residue was dissolved in H_2O/acetic acid (3/1) and extracted with diisopropyl ether. The aqueous phase was evaporated and the product purified by reverse phase HPLC-MS (C18 column, gradient from 5% to 95% MeCN/H_2O + 0.1% TFA). For analysis of purified products (C18 column, flow 1 mL/min, gradient from 5–95% MeCN/H_2O + 0.1% TFA over 20 min) the HPLC instrument was interfaced with a Finnigan AQA electrospray ionization (ESI) mass spectrometer (MS). The ESI-MS of all mimetics was measured to an accuracy of 1 in 1000, and the spectra confirmed in every case the expected molecular weight (see Tables 4A and 4B).

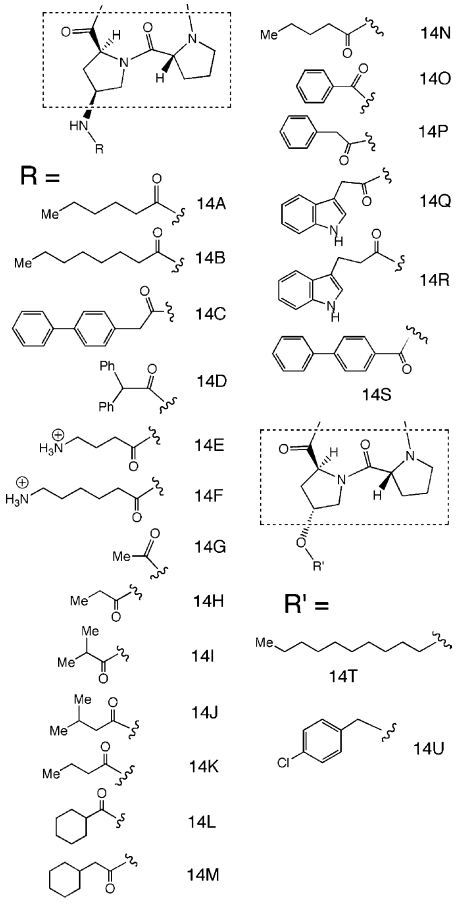


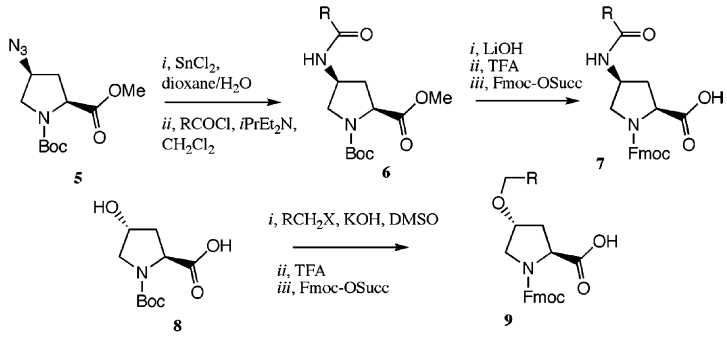
Figure 3. Structures of the analogues **14A**–**U**. Compare structure **4**.

3.2. Antimicrobial assays

The antimicrobial activities were determined by the standard NCCLS broth microdilution method. Innocula of the microorganisms were diluted into Mueller–Hinton (MH) broth to give approximately 10^6 colony forming units (CFU) per mL. Aliquots (50 μL) of the innocula were added to MH broth (50 μL) containing the peptide in serial twofold dilutions. Antimicrobial activities are expressed as the minimal inhibitory concentration (MIC) in μg/mL at which 100% inhibition of growth was observed after 18–20 h incubation at 37 °C.

3.3. Haemolytic activity

Fresh human red blood cells were washed three times with phosphate buffered saline (PBS), and then incubated with peptide at a concentration of 100 μg/mL for 1 h at 37 °C. The final erythrocyte concentration was ca. 0.9×10^9/mL. The values of 0% and 100% lysis



Scheme 1.

Table 4A. Sequences, retention times (t_R, min) on HPLC, synthesis yield (%), and the mass confirmed by ESI-MS for the mimetics described in Tables 1 and 2

Mimetic	1	2	3	4	5	6	7	8	9	10	11	12	t_R	%	Mass
1	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.6	51	1878.4
2	Phe	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.5	52	1912.4
3	Cha	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.6	36	1918.4
4	Tyr	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.4	38	1928.4
5	Trp	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.5	40	1951.4
6	Hfe	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	9.6	95	1926.4
7	1Nal	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	11.8	47	1962.4
8	2Nal	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	9.6	69	1962.4
9	ClF	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	11.1	53	1946.8
10	His	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.3	63	1902.3
11	Bip	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	11.4	39	1988.5
12	Orn	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	8.4	84	1879.3
13	Leu	Arg	Phe	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.6	44	1912.4
14	Leu	Arg	Cha	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.8	41	1918.4
15	Leu	Arg	Trp	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.5	41	1951.4
16	Leu	Arg	Hfe	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.6	32	1926.4
17	Leu	Arg	1Nal	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.9	43	1962.4
18	Leu	Arg	2Nal	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	11.0	42	1962.4
19	Leu	Arg	Val	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.0	47	1864.3
20	Leu	Arg	ClF	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.9	50	1946.8
21	Leu	Arg	His	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.3	40	1902.4
22	Leu	Arg	Bip	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	11.3	39	1988.5
23	Leu	Arg	Orn	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	9.2	48	1879.3
24	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Phe	Lys	Tyr	Arg	Val	10.0	54	1839.3
25	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Tyr	Lys	Tyr	Arg	Val	9.9	48	1855.3
26	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Y(B)	Lys	Tyr	Arg	Val	11.0	33	1945.4
27	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Hfe	Lys	Tyr	Arg	Val	10.3	52	1853.3
28	Leu	Arg	Leu	Lys	Lys	Arg	Arg	1Nal	Lys	Tyr	Arg	Val	10.5	34	1889.4
29	Leu	Arg	Leu	Lys	Lys	Arg	Arg	2Nal	Lys	Tyr	Arg	Val	10.5	53	1889.4
30	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Val	Lys	Tyr	Arg	Val	9.9	49	1791.2
31	Leu	Arg	Leu	Lys	Lys	Arg	Arg	ClF	Lys	Tyr	Arg	Val	10.4	49	1873.8
32	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Leu	Lys	Tyr	Arg	Val	10.1	46	1805.3
33	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Ile	Lys	Tyr	Arg	Val	10.1	57	1805.3
34	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Orn	Lys	Tyr	Arg	Val	9.7	31	1806.3
35	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Phe	Arg	Val	10.9	45	1862.3
36	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Cha	Arg	Val	11.1	42	1868.4
37	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Trp	Arg	Val	10.8	56	1901.4
38	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Hfe	Arg	Val	11.3	32	1876.4
39	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	1Nal	Arg	Val	11.6	24	1912.4
40	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	2Nal	Arg	Val	11.7	30	1912.4
41	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Val	Arg	Val	10.6	48	1814.3
42	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	ClF	Arg	Val	11.2	48	1896.8
43	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Leu	Arg	Val	10.7	18	1828.3
44	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Bip	Arg	Val	11.6	46	1938.5
45	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Orn	Arg	Val	9.4	49	1829.3

(*continued on next page*)

Table 4A (*continued*)

Mimetic	1	2	3	4	5	6	7	8	9	10	11	12	t_R	%	Mass
46	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Phe	10.6	35	1926.4
47	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Cha	11.2	60	1932.5
48	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Trp	10.4	69	1965.4
49	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Hfe	10.8	95	1940.4
50	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	1Nal	11.3	89	1976.5
51	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	2Nal	11.2	30	1976.5
52	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Tyr	10.2	42	1942.2
53	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	ClF	11.0	32	1960.8
54	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Leu	10.5	56	1892.4
55	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Bip	11.7	37	2002.5
56	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Orn	8.3	75	1893.4
57	Leu	Tyr	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.3	42	1885.4
58	Leu	Trp	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.4	55	1908.4
59	Leu	Leu	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.6	88	1835.3
60	Leu	His	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.3	40	1859.3
61	Leu	Thr	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.4	94	1823.3
62	Leu	Gln	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.4	55	1850.3
63	Leu	Arg	Leu	Arg	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.3	45	1906.4
64	Leu	Arg	Leu	Trp	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.5	35	1936.4
65	Leu	Arg	Leu	Leu	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.9	62	1863.3
66	Leu	Arg	Leu	His	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.2	30	1887.3
67	Leu	Arg	Leu	Thr	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.4	94	1851.3
68	Leu	Arg	Leu	Gln	Lys	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.3	76	1878.3
69	Leu	Arg	Leu	Lys	Arg	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.4	37	1906.4
70	Leu	Arg	Leu	Lys	Trp	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.3	45	1936.4
71	Leu	Arg	Leu	Lys	Leu	Arg	Arg	Trp	Lys	Tyr	Arg	Val	11.9	43	1863.3
72	Leu	Arg	Leu	Lys	His	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.5	53	1887.3
73	Leu	Arg	Leu	Lys	Thr	Arg	Arg	Trp	Lys	Tyr	Arg	Val	11.4	45	1851.3
74	Leu	Arg	Leu	Lys	Gln	Arg	Arg	Trp	Lys	Tyr	Arg	Val	10.4	50	1878.3
75	Leu	Arg	Leu	Lys	Lys	Tyr	Arg	Trp	Lys	Tyr	Arg	Val	10.5	65	1885.4
76	Leu	Arg	Leu	Lys	Lys	Trp	Arg	Trp	Lys	Tyr	Arg	Val	10.8	35	1908.4
77	Leu	Arg	Leu	Lys	Lys	Leu	Arg	Trp	Lys	Tyr	Arg	Val	10.8	58	1835.3
78	Leu	Arg	Leu	Lys	Lys	His	Arg	Trp	Lys	Tyr	Arg	Val	10.2	46	1859.3
79	Leu	Arg	Leu	Lys	Lys	Thr	Arg	Trp	Lys	Tyr	Arg	Val	10.4	82	1823.3
80	Leu	Arg	Leu	Lys	Lys	Gln	Arg	Trp	Lys	Tyr	Arg	Val	10.4	36	1850.3
81	Leu	Arg	Leu	Lys	Lys	Arg	Tyr	Trp	Lys	Tyr	Arg	Val	10.6	60	1885.4
82	Leu	Arg	Leu	Lys	Lys	Arg	Trp	Trp	Lys	Tyr	Arg	Val	10.8	70	1908.4
83	Leu	Arg	Leu	Lys	Lys	Arg	Leu	Trp	Lys	Tyr	Arg	Val	10.7	50	1835.3
84	Leu	Arg	Leu	Lys	Lys	Arg	His	Trp	Lys	Tyr	Arg	Val	10.3	74	1859.3
85	Leu	Arg	Leu	Lys	Lys	Arg	Gln	Trp	Lys	Tyr	Arg	Val	10.4	40	1850.3
86	Leu	Arg	Leu	Lys	Lys	Arg	Thr	Trp	Lys	Tyr	Arg	Val	10.3	45	1823.3
87	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Tyr	Tyr	Arg	Val	10.5	88	1913.4
88	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Trp	Tyr	Arg	Val	10.7	65	1936.4
89	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Leu	Tyr	Arg	Val	10.6	44	1863.3
90	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Thr	Tyr	Arg	Val	10.5	84	1851.3
91	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Gln	Tyr	Arg	Val	10.5	52	1878.3
92	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	His	Tyr	Arg	Val	10.4	49	1887.3
93	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Tyr	Val	10.6	54	1885.3
94	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Trp	Val	10.9	47	1908.4
95	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Leu	Val	10.7	65	1835.3
96	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Orn	Val	10.2	92	1836.3
97	Leu	Arg	Leu	Lys	Lys	Arg	Arg	Trp	Lys	Tyr	Cit	Val	10.5	54	1880.7

were determined by incubation of cells with PBS or 0.1% Triton X-100 in water, respectively. The samples were centrifuged, the supernatant diluted 20-fold in PBS, and the optical density was measured at 540 nm. The reproducibility in determinations of haemolytic activity was typically ±1%.

4. Dye release from liposomes

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Genzyme (Liestal, Switzerland). Large unilamellar vesicles (LUVs) of approxi-

Table 4B. Retention times (t_R) on HPLC, synthesis yield (%), and the mass confirmed by ESI-MS for the mimetics described in Table 3; also shown is the residue used to replace L-Pro-14 (see also Fig. 3)

Mimetic		t_R (min)	%	Av. mass	Mimic	t_R (min)	%	Av. mass
1	Pro	10.6	51	1878.4	**14A**	11.1	51	1991.5
2	Gly	9.5	58	1838.3	**14B**	11.8	43	2019.6
3	Arg	9.3	57	1937.4	**14C**	11.5	37	2087.6
4	Tyr	9.9	29	1944.4	**14D**	11.8	23	2087.6
5	Trp	10.7	25	1967.5	**14E**	9.4	27	1978.5
6	Leu	10.5	21	1894.4	**14F**	9.4	30	2006.5
7	Ile	10.4	42	1894.4	**14G**	10.0	48	1935.4
8	Val	10.5	50	1880.4	**14H**	10.0	42	1949.4
9	Gln	9.8	33	1909.4	**14I**	10.1	55	1963.4
10	Cha	11.2	36	1934.5	**14J**	10.3	44	1977.5
11	Y(B)	11.6	51	2034.6	**14K**	10.4	23	1963.4
12	Hfe	10.8	76	1942.5	**14L**	10.3	20	2003.5
13	2Nal	11.4	27	1978.5	**14M**	10.9	18	2017.5
14	1Nal	11.5	46	1978.5	**14N**	10.5	21	1977.5
15	Nle	10.4	53	1894.4	**14O**	10.5	30	1997.4
16	Bip	11.1	43	2004.5	**14P**	10.6	32	2011.5
17	ClF	10.6	67	1962.9	**14Q**	10.9	28	2050.5
18	S(B)	10.9	51	1958.5	**14R**	9.9	24	2064.5
19	Orn	9.7	76	1895.4	**14S**	11.6	22	2073.5
20	hCha	11.0	64	1948.5	**14T**	11.5	20	2034.6
					14U	11.7	21	2018.3

mately 100 nm diameter were prepared by the freeze/thaw and extrusion method.[66,67] Pure phospholipids or mixtures thereof (20 mg) were dissolved in chloroform and evaporated to dryness. The resulting lipid film was further dried overnight under vacuum. The lipid films were hydrated in 1 mL of 10 mM Tris-buffer containing 50 mM calcein, 10 mM NaCl and 0.1 mM EDTA, pH 7.4, subjected to six freeze–thaw cycles, and then extruded 13 times through a 100 nm polycarbonate membrane (Nucleopore, Pleasanton, CA) using an extruder (Lipex Biomembranes, Canada). The size distribution of the obtained LUVs was measured by dynamic light-scattering with a Nicomp model 370 particle sizing system (Santa Barbara, CA). The calcein-entrapped vesicles were separated from the free dye by gel filtration through a Sephadex G-25 column (1 × 25 cm) with standard buffer (10 mM Tris, 100 mM NaCl, 0.1 mM EDTA, pH 7.4) as eluent. The final phospholipid concentration was determined by phosphorus analysis.[68]

The peptide-induced leakage of calcein from the LUVs was monitored by measuring the fluorescence intensity of calcein released into the standard buffer at 20 °C. Dye-entrapped vesicles were dissolved in the standard buffer (2 mL) to a final phospholipid concentration of 30 μM. The release of dye was then initiated by addition of an aliquot of peptide and the increase of the calcein fluorescence intensity was recorded 5 min after addition of the peptides with a Perkin–Elmer LS55 luminescence spectrophotometer set to an emission wavelength of 519 nm. The excitation wavelength was set in the range 430–490 nm to obtain a fluorescence signal within the optimal working range of the photo detector and slit widths of 4 nm were used throughout. To measure the maximum fluorescence intensity F_{max} corresponding to 100% calcein release, 10 μL of 10% Triton X-100 were added to the samples at the end of each experiment. The apparent percent calcein leakage was calculated according to:

$$\%\ \text{Dye leakage} = \frac{(F - F_0)}{(F_{max} - F_0)} \cdot 100$$

where F is the fluorescence intensity induced by the peptide, and F_0 and F_{max} denote the fluorescence intensities without peptide or detergent and with Triton X-100, respectively.

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