BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

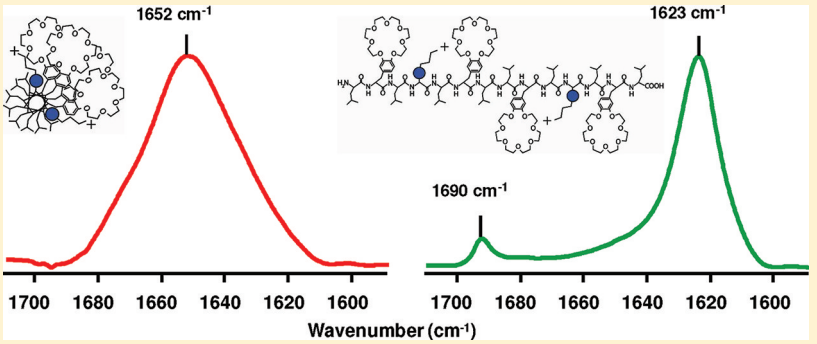
pubs.acs.org/biochemistry

Revisiting Peptide Amphiphilicity for Membrane Pore Formation

Aurélien Lorin,[†] Mathieu Noël,[†] Marie-Ève Provencher,[†] Vanessa Turcotte,[†] Carole Masson,[†] Sébastien Cardinal,[†] Patrick Lagüe,[‡] Normand Voyer,[†] and Michèle Auger*[,†]

[†]Département de chimie, PROTEO (Regroupement québécois de recherche sur la fonction, la structure et l'ingénierie des protéines), CERMA (Centre de recherche sur les matériaux avancés), and [‡]Département de biochimie, microbiologie et bioinformatique, PROTEO, Université Laval, Québec, Québec, Canada G1V 0A6

Supporting Information



ABSTRACT: It has previously been shown that an amphipathic de novo designed peptide made of 10 leucines and four phenylalanines substituted with crown ethers induces vesicle leakage without selectivity. To gain selectivity against negatively charged dimyristoylphosphatidylglycerol (DMPG) bilayers, one or two leucines of the peptide were substituted with positively charged residues at each position. All peptides induce significant calcein leakage of DMPG vesicles. However, some peptides do not induce significant leakage of zwitterionic dimyristoylphosphatidylcholine vesicles and are thus active against only bacterial model membranes. The intravesicular leakage is induced by pore formation instead of membrane micellization. Nonselective peptides are mostly helical, while selective peptides mainly adopt an intermolecular β-sheet structure. This study therefore demonstrates that the position of the lysine residues significantly influences the secondary structure and bilayer selectivity of an amphipathic 14-mer peptide, with β-sheet peptides being more selective than helical peptides.

Because of the increase in bacterial resistance against conventional antibiotics, the development of new antibiotics with novel modes of action is needed to fight against microbial infections. Ideally, these molecules should act against a wide range of pathogens and should not induce resistance.[1] Antimicrobial peptides (AMPs) meet these criteria and thus are promising candidates. Moreover, several AMPs have shown antiviral,[2−4] antifungal,[5−7] antitumor,[8−10] and immunomodulatory[2,11,12] activities in addition to important antibacterial activity.[13−15]

Natural AMPs are secreted by several living organisms and serve as the first line of defense of the organisms by killing the pathogens. Even if other mechanisms of action are proposed in the literature, it is widely accepted that cationic AMPs interact strongly with bacterial cytoplasmic membranes.[16−18] This interaction can induce membrane permeabilization responsible for cell death. Several modes of permeabilization have been suggested in the literature.[13,15,19−32] In the carpet-like model, AMPs disrupt the membrane curvature, inducing formation of micelles in a detergent-like manner.[18] The other proposed models involve pore formation, with differences in the composition and lifetime of the pores, and in the orientation of the peptides. The formation of pores in bacterial cytoplasmic membranes causes dissipation of the electrochemical gradient across the membrane, and therefore loss of cell content.[33,34]

Despite their structural differences (β-sheet, β-turn, or helical structures), most AMPs have common features.[15] They are short (12−50 residues) and possess a net positive charge (generally +2 to +9), as well as an amphipathic character. In addition, most linear AMPs adopt a helical structure. AMPs initially bind to plasma membranes before perturbing the normal phase behavior of the membrane.[35] AMPs bind preferentially to anionic bacterial membranes instead of zwitterionic eukaryotic membranes because of the overall cationic character of these peptides.[36] Other factors, such as hydrophobicity and flexibility, are also involved in membrane interaction and selectivity.[15,37] Despite this selectivity, natural AMPs are often hemolytic and are therefore not suitable as drugs. It is therefore of great interest to increase the selectivity of natural AMPs or to synthesize de novo peptides with enhanced selectivity compared to natural AMPs.

Several studies clearly show that AMP activity against zwitterionic and negatively charged model vesicles reflects

Received: August 24, 2011
Revised: September 23, 2011
Published: September 26, 2011

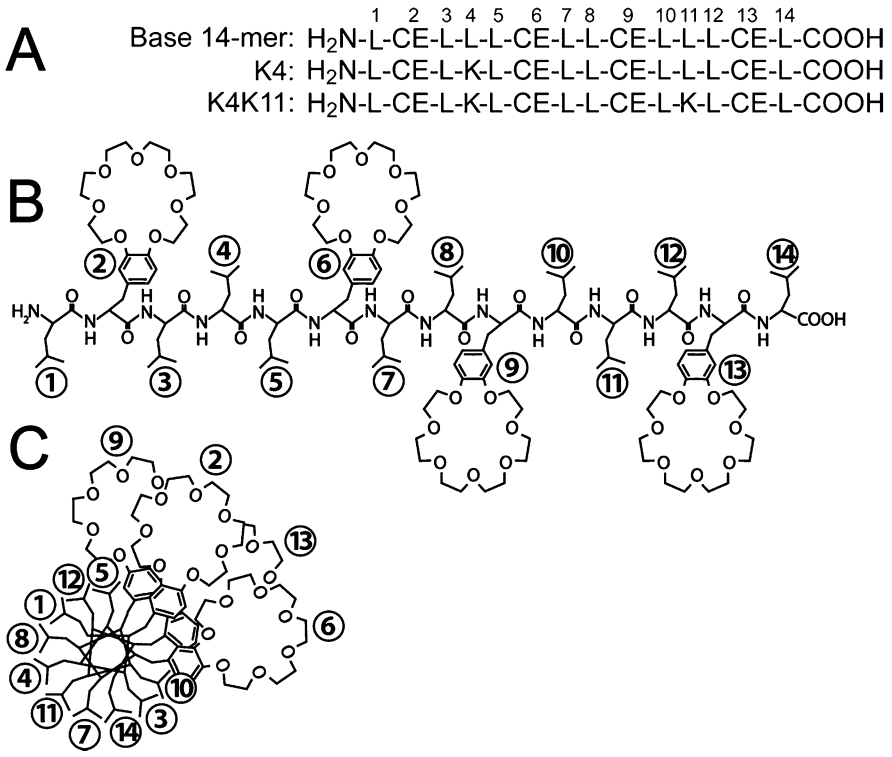


Figure 1. (A) Sequences of the neutral base 14-mer peptide and examples of positively charged analogues used in this study. The sequences of other positively charged analogues are designed using the same concept used for K4 and K4K11. (B) β-Strand diagram and (C) helical wheel projections showing the distribution of amino acid side chains of the base 14-mer peptide.

activity against humans and bacterial cells.[38−44] Vesicles composed of only phospholipids are thus frequently used to analyze the activity of AMPs and to better characterize their modes of membrane destabilization because other membrane or nonmembrane constituents present in biological cells do not interfere.[45]

The main approach used in the literature to characterize the importance of each major factor (hydrophobicity, structure, flexibility, and position of the positive charge) in the membrane activity of AMPs is to mutate or change one or several residues of natural AMP peptides.[46,47] However, when the effect of charged residues is being analyzed, this approach can be skewed by the presence of other (negatively or positively) charged residues in the peptide sequence.

In this work, an amphipathic globally neutral peptide (net charge of 0) was used as a template to study and characterize the effect of the position of one or two charged residues on its membrane interaction and selectivity. The base peptide (hereafter 14-mer) is a non-natural peptide composed of 10 leucines and four phenylalanines substituted with a 21-crown-7 macrocycle (Figure 1A).[48−50] This 14-mer peptide is shorter than the analogous 21-mer peptide, composed of 15 leucines and six 21-crown-7-phenylalanines. These two peptides adopt an amphipathic helical structure with the hydrophobic and hydrophilic sides composed of the leucine side chains and the crown ethers, respectively. The longer 21-mer peptide is long enough to span membranes and act as a synthetic ion channel where crown ethers allow ion relays.[51] However, the 14-mer peptide is too short to span lipid bilayers, and several biophysical results suggest that this peptide adopts a parallel orientation at the surface of the bilayers.[48−50] This orientation could cause the nonspecific perturbation of bilayers, inducing calcein leakage of both zwitterionic and negatively charged vesicles, in contrast to the longer 21-mer peptide.[48−50] The 14-mer peptide induces intravesicular leakage by forming pores according to the sinking raft model mechanism, like many AMPs.[12,23,52] This 14-mer peptide is thus a good template for characterizing the relationship between the position of positively charged residues and the selectivity against zwitterionic and negatively charged vesicles. Peptides with one leucine residue replaced with a lysine, arginine, or histidine residue or with two leucine residues replaced with two lysine residues were synthesized. Analogues with all possible substitutions were synthesized, giving a collection of 75 peptide analogues. The bilayer destabilizing properties of each peptide were first analyzed by measuring the calcein leakage of eukaryotic and prokaryotic mimicking vesicles, i.e., dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) unilamellar vesicles. Results show selectivity for some peptides toward DMPG vesicles. Dynamic light scattering results indicate that intravesicular leakage is not induced by micellization, suggesting pore formation. The peptide structure in the presence of vesicles was then determined by Fourier transform infrared (FTIR) spectroscopy. The results clearly indicate a relationship among the position of charged residues, the secondary structure of the peptides, and their membrane activity.

MATERIALS AND METHODS

Materials. DMPC and DMPG were purchased from Avanti Polar Lipids (Alabaster, AL) and used without purification. Calcein, HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], EDTA (ethylenediaminetetraacetic acid disodium), TFE (trifluoroethanol), and PMMA [poly(methyl methacrylate)] cuvettes were purchased from Sigma-Aldrich (St. Louis, MO). Water used for buffer preparation was distilled and deionized using a Barnstead (Boston, MA) NANOpurII

system (resistivity of 18.2 MΩ/cm) with four purification columns. Deuterium oxide was obtained from CDN isotopes (Pointe-Claire, QC). Sephadex G-50 was purchased from GE Healthcare Biosciences AB (Uppsala, Sweden). All solvents were of reagent grade or HPLC (high-performance liquid chromatography) grade, purchased commercially, and used without any further purification except for dimethylformamide (DMF) (degassed with N_2) and dichloromethane (distilled). Fmoc (fluorenylmethyloxycarbonyl)-protected amino acids were purchased from Matrix Innovation (Quebec, QC). All other chemicals were of reagent grade.

Peptide Synthesis. The 14-mer peptides (Figure 1) were prepared by solid-phase synthesis using the Wang resin as a solid support and N-Fmoc-protected amino acids. N-Fmoc-21-crown-7-phenylalanine was synthesized as previously described.[53] Coupling of all amino acids was conducted using HBTU/HOBt [2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/hydroxybenzotriazole] after a deprotection with piperidine (20% in DMF). Peptide purity was checked by reverse-phase HPLC using an Agilent 1050 chromatograph (Agilent Technologies, Santa Clara, CA), with a gradient of solvents A [90% H_2O/5% CH_3CN/5% 2-propanol/0.1% trifluoroacetic acid (TFA)] and B (50% CH_3CN/50% 2-propanol/0.1% TFA) over 45 min. Final characterization was conducted using a model 6210 Agilent time-of-flight mass spectrometer (Agilent Technologies) with electrospray ionization.

Peptides are N-terminally free in contrast to the previously synthesized 14-mer, which was Boc (butoxycarbonyl)-protected at the N-terminus.[48,52,54] The effect of N-terminal deprotection on the leakage properties of the 14-mer peptide was analyzed with a calcein leakage assay at a peptide/lipid molar ratio of 1/60. No significant difference was measured.

Calcein Leakage Assay. Vesicle leakage was monitored using an assay based on the dequenching of the water-soluble and membrane-impermeable dye calcein.[50,55,56] DMPC or DMPG powders were hydrated with 120 μL of buffer containing 100 mM HEPES, 5 mM EDTA, and 80 mM calcein (pH 7.4) to give a 40 mM liposomal suspension. This suspension was then submitted to five freeze−thaw cycles from liquid nitrogen temperature to 37 °C to ensure good lipid hydration. The suspension was passed 15 times through a polycarbonate membrane (0.1 μm diameter pores) using a mini-extruder (Avanti Polar Lipids). Unencapsulated calcein surrounding calcein-containing LUVs (large unilamellar vesicles) was removed by size exclusion chromatography at 37 °C using a column filled with Sephadex G-50 gel swollen in the external buffer [100 mM HEPES and 5 mM EDTA (pH 7.4)]. Lipid concentrations were quantified by the Bartlett phosphate method.[57] Vesicles were then kept at 37 °C before their use in leakage assays. The diameter of the calcein-entrapped vesicles was measured at 37 °C by dynamic light scattering at a fixed angle (173°) using a laser particle sizer (Malvern Zeta-Sizer Nano Series, Malvern, Worcestershire, U.K.).

Leakage of calcein from vesicles was followed by measuring the dequenching of calcein released into the medium. Fluorescence was recorded using a λ_{exc} of 490 nm and a λ_{em} of 515 nm on a Varian Cary Eclipse spectrometer (Varian Inc., Palo Alto, CA). The temperature was set to 37 °C. In a 1 cm PMMA cuvette, 3.4 mL of external buffer solution and 50 μL of vesicle solution were introduced. Stirring and acquisition were then started. After 1 min, an appropriate amount (10−20 μL) of TFE or peptide previously solubilized in TFE (at 1 mg/mL) was added. After 25 min, 10 μL of a 10% Triton X-100 solution were added to completely solubilize vesicles, and the fluorescence intensity after the release of all calcein, I_{max}, was measured at 30 min.

The percentage of calcein leakage was calculated according to the following equation:

$$\%\ \text{leakage} = \frac{I_{pept} - I_0 - (I_{TFE} - I_0')}{I_{max} - I_0}$$

where I_{pept} is the fluorescence intensity at time t after addition of peptide, I_0 the fluorescence intensity before addition of peptide, I_{TFE} the fluorescence intensity at time t after addition of TFE, I_0' the fluorescence intensity before addition of TFE, and I_{max} the fluorescence intensity after addition of Triton X-100 at 30 min.

Dynamic Light Scattering. DMPC and DMPG vesicles for these studies were prepared in the same way as for the calcein leakage assays. However, no calcein was used during the preparation. The hydrodynamic diameter of DMPC and DMPG vesicles at 37 °C in the presence or absence of peptides (added as a TFE solution) was measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS instrument (Malvern). The light source was a 4 mW He−Ne laser (633 nm), and the scattered light was measured at an angle of 173° to the incident beam using an avalanche photodiode detector.

Infrared Studies. Multiple lyophilizations in 10 mM HCl were first performed on the peptide powder to remove all traces of TFA.[58] Then, dry lipids (2.5 mg) and peptides at a peptide/lipid molar ratio of 1/60 were codissolved in a chloroform/methanol mixture (1/1) to ensure thorough mixing. The solvent was removed under a stream of nitrogen, followed by storage under vacuum overnight to remove all traces of organic solvent. The dry sample was hydrated with 10 μL of 100 mM HEPES, 5 mM EDTA (pH 7.4) buffer with 20% (w/w) lipids in deuterium oxide. The resulting suspension underwent at least five freeze (liquid N_2)−thaw (37 °C)−vortex shaking cycles to ensure the formation of multilamellar vesicles. For the last cycle, samples were thawed at room temperature and then placed between CaF_2 windows (BioTools Inc., Wauconda, IL).

All spectra were recorded with a Nicolet Magna 560 spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with a nitrogen-cooled MCT (mercury−cadmium−telluride) A detector. A total of 128 scans were averaged at each temperature with a resolution of 2 cm^{-1}. For recording transmission spectra, the CaF_2 windows containing the samples were inserted in a thermoelectrically regulated homemade cell. All data manipulations were performed with Grams/AI 8.0 (Galactic Industries, Salem, MA). The spectra were corrected for the water vapor and CaF_2 contribution by subtraction of a reference spectrum. The 1790−1550 cm^{-1} spectral region was baseline-corrected using a cubic function. Non-amide I contributions (ester carbonyl and 1610−1550 cm^{-1} regions) were subtracted using a peak fitting procedure.

Spectral decomposition was conducted in such a way that the number of components was minimized, with a result of four to six bands, and the observations made from the second-derivative spectra were respected. Spectra were decomposed using mixtures of Lorentzian and Gaussian line shapes. The limits for the different amide I′ components and their structural assignments were chosen according to Goormaghtigh et al.[58] and were 1612−1626 and 1682−1695 cm^{-1} for intermolecular β-sheets, 1627−1638 cm^{-1} for β-sheets, 1650−1659 cm^{-1} for α-helices, and 1660−1682 cm^{-1} for turns.

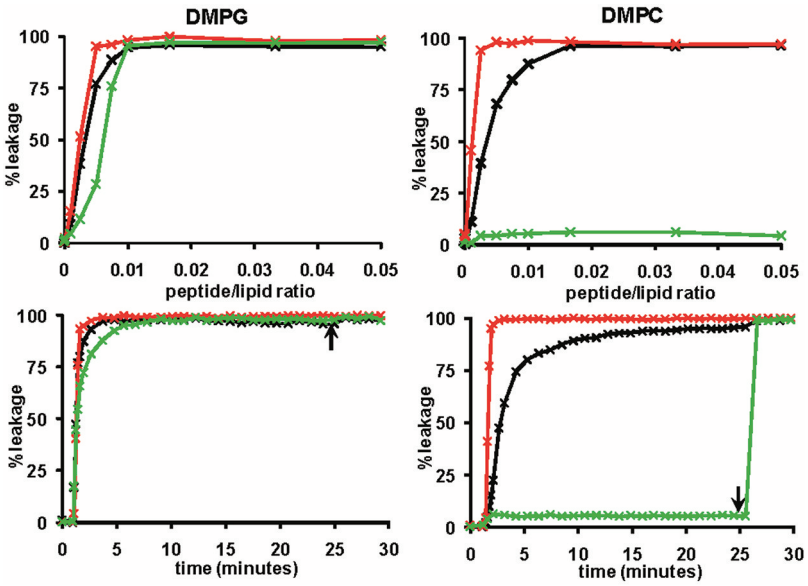


Figure 2. Percentage of calcein leakage from DMPG (left) and DMPC (right) vesicles as a function of peptide/lipid ratio (top). The percentage is calculated from the fluorescence intensity measured after 25 min (just before the addition of Triton X-100). Percentage of calcein leakage from DMPG (left) and DMPC (right) vesicles at a peptide/lipid ratio of 1/60 as a function of time (bottom). The peptide was added at 1 min. Triton X-100 was added at 25 min. Legends for the four panels: base 14-mer peptide (black), 14-mer with lysine in position 4, K4 (green), and 14-mer with lysine in position 5, K5 (red).

RESULTS

Calcein Leakage. Calcein leakage is a well-established assay for studying vesicle permeabilization.[56] In this assay, a change in the fluorescence of self-quenched vesicle-entrapped calcein is related to calcein release in the external medium. We used this assay to measure the propensity of the base peptide 14-mer and its derivatives bearing one or two positively charged residues depicted in Figure 1 to induce leakage of DMPC and DMPG vesicles. The base 14-mer peptide induces significant calcein leakage of DMPC and DMPG vesicles even at a peptide/lipid ratio of 1/1000 (Figure 2, top). The leakage percentage increases up to a peptide/lipid molar ratio of 1/60, where a plateau is observed. The maximum levels of intravesicular calcein release of DMPC and DMPG vesicles are 95 and 98%, respectively. The 14-mer peptide with lysine at position 5, i.e., K5, also induces significant calcein leakage of DMPC and DMPG vesicles (more than 99% at a peptide/lipid ratio of 1/60). At a lower peptide/lipid molar ratio (between 1/1000 and 1/100), the extent of leakage induced by K5 is larger than that induced by the base 14-mer peptide with a more pronounced effect on DMPC vesicles. The larger difference observed is confirmed by the study of the rate of leakage at a peptide/lipid ratio of 1/60 (Figure 2, bottom). Indeed, the leakage of DMPC vesicles in the presence of K5 is faster than in the presence of the base 14-mer peptide, while no significant effect on the rate of leakage with DMPG vesicles is noted.

The peptide with the lysine residue at position 4, i.e., K4, also induces a large amount of leakage of anionic DMPG liposomes (97% of calcein release at a peptide/lipid molar ratio of 1/60). The leakage induced by K4 is similar to that of K5 (maximum leakage obtained within 5 min), indicating a fast perturbation of DMPG bilayers (Figure 2, bottom). However, K4 has no significant effect on zwitterionic DMPC vesicles even at a peptide/lipid molar ratio of 1/60 (5% of calcein release).

Calcein leakage of DMPC and DMPG vesicles induced by other peptides containing one positively charged residue (Lys, Arg, and His) was also studied at a peptide/lipid molar ratio of 1/60. Figure 3 clearly shows that peptides containing a positively charged residue near a crown ether phenylalanine

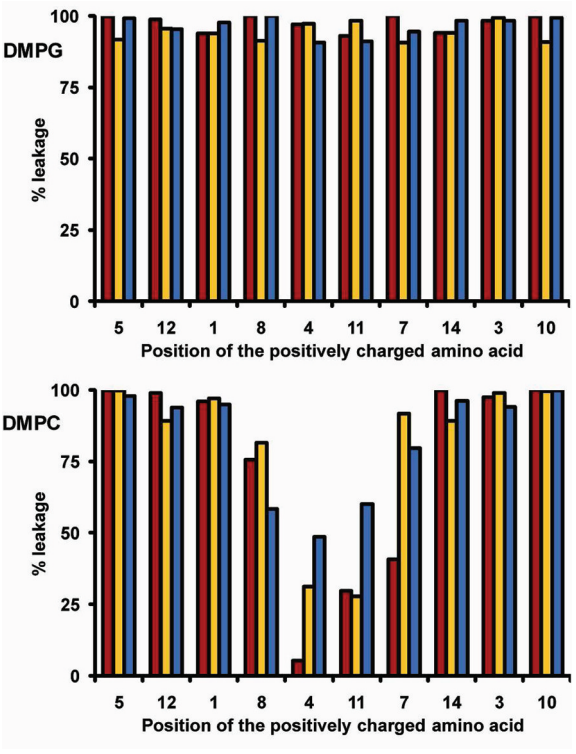


Figure 3. Percentage of calcein leakage of DMPG (top) and DMPC (bottom) vesicles induced by 14-mer with one charged residue at a peptide/lipid ratio of 1/60 as a function of the charged residue position. Leakage is shown with respect to the positively charged residue position when the peptide is in its helical form: red for the 14-mer with lysine, yellow for the 14-mer with arginine, and blue for the 14-mer with histidine.

dx.doi.org/10.1021/bi201335t | *Biochemistry* 2011, 50, 9409–9420

residue when the peptides adopt a helical conformation (positions 3, 5, 10, and 12) induce significant leakage of both DMPC and DMPG vesicles. However, the farther the charged residue is from the crown ether phenylalanine side of the helix (Figure 1C), the lower the percentage of leakage of DMPC vesicles. Peptides inducing the least leakage are those in which the charged residues are located on the opposite side of the crown ether phenylalanine residues when the peptide adopts a helical conformation (positions 4, 7, 8, and 11). This relationship between the position of the positively charged residue and the leakage percentage of DMPC vesicles is observed whatever the nature of the charged residue. Interestingly, the charged residue position has no significant effect on the leakage of DMPG vesicles. Indeed, all peptides induce a significant leakage (>90%) at a peptide/lipid molar ratio of 1/60 (Figure 3). For the rest of this study, only peptides with lysine residues as positively charged residues were used.

Tables 1 and 2 compare the calcein leakage of DMPG and DMPC vesicles induced by peptides containing one or two lysine residues at all possible positions. All peptides, regardless of the number and position of lysine in their sequence, induce strong DMPG vesicle leakage [≥90% (Table 1)]. However, leakage of DMPC vesicles is highly dependent on the number and position of lysines (Table 2). Indeed, even if the majority of the peptides induce ≥90% leakage, some peptides induce much lower levels of calcein leakage of DMPC vesicles. Peptides such as K4K7, K4K8, K4K11, and K7K11 do not induce significant leakage. Peptides with one or two lysine residues at positions 4, 7, 8, and 11, induce the least leakage of DMPC vesicles. It is important to note that peptides with two lysine residues at positions 4, 7, 8, and 11 induce less leakage than peptides with only one lysine residue at those positions, indicating a cumulative effect. Hereafter, peptides inducing little leakage of DMPC liposomes are coined selective peptides, while the others are coined nonselective peptides.

Table 1. Percentages of Calcein Leakage of DMPG Vesicles Induced by 14-mer Peptides with or without Lysine Residues[a]

position of second lysine	position of first lysine									
	1	3	4	5	7	8	10	11	12	14
no lysine	94	98	97	>99	>99	>99	>99	93	99	94
1	–	–	–	–	–	–	–	–	–	–
3	98	–	–	–	–	–	–	–	–	–
4	98	95	–	–	–	–	–	–	–	–
5	>99	90	99	–	–	–	–	–	–	–
7	92	98	98	98	–	–	–	–	–	–
8	>99	99	98	92	98	–	–	–	–	–
10	>99	99	98	96	99	93	–	–	–	–
11	94	91	>99	95	92	95	95	–	–	–
12	>99	90	98	92	>99	>99	91	95	–	–
14	>99	90	99	93	>99	96	98	>99	>99	–

[a]The measurements were performed at a peptide/lipid ratio of 1/60. The percentage of calcein leakage induced by the base 14-mer peptide without lysine is 96%.

Table 2. Percentages of Calcein Leakage of DMPC Vesicles Induced by 14-mer Peptides with or without Lysine Residues[a]

position of second lysine	position of first lysine									
	1	3	4	5	7	8	10	11	12	14
no lysine	96	97	5	>99	41	76	>99	30	99	>99
1	–	–	–	–	–	–	–	–	–	–
3	96	–	–	–	–	–	–	–	–	–
4	99	95	–	–	–	–	–	–	–	–
5	>99	>99	94	–	–	–	–	–	–	–
7	18	94	3	23	–	–	–	–	–	–
8	94	59	7	92	3	–	–	–	–	–
10	97	>99	84	99	97	55	–	–	–	–
11	14	48	7	45	3	6	77	–	–	–
12	96	98	87	96	64	96	98	98	–	–
14	97	96	68	96	97	83	97	51	97	–

[a]The measurements were performed at a peptide/lipid ratio of 1/60. The percentage of calcein leakage induced by the base 14-mer peptide without lysine is 96%.

Vesicle Integrity. In this study, DLS was used to investigate the effect of the peptides on the morphology of the lipid vesicles. The preparation of the lipid vesicles was the same as that used for the calcein experiments except that the intravesicular milieu was devoid of calcein. The results indicate that DMPG and DMPC vesicles have a diameters of ∼111 and ∼155 nm, respectively (Figure 4 and the Supporting Information). Triton X-100 is known to induce micellization of vesicles. This detergent was thus used as a control to mimic membrane disintegration induced as in the carpet-like model. Addition of Triton X-100 decreases the signal intensity at values below the detection limit, indicating formation of small vesicles and micelles that cannot be detected by DLS (data not shown). After the addition of selective or nonselective peptides, a peak corresponding to large unilamellar vesicles is still observed with only small changes in the corresponding diameter. No peak with a smaller diameter is noted. These results indicate that the peptides do not induce intravesicular leakage by micellization of the vesicles. The change in the diameter of the vesicles, more often observed as an increase in diameter, can be explained by the adsorption of peptides on vesicles. This observation can also be explained by the increase in the dispersity of diameters observed for some peptide/vesicle samples.

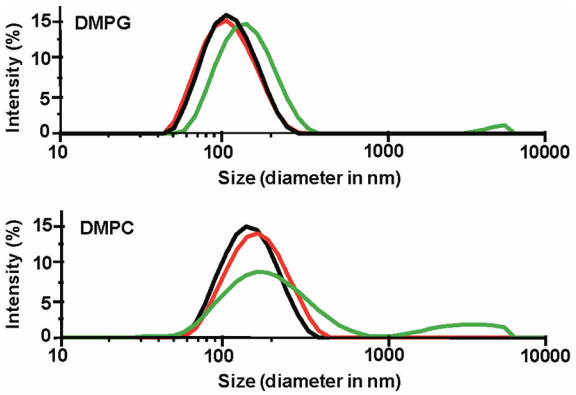


Figure 4. Size distribution of DMPG (top) and DMPC (bottom) vesicles without peptides (black) and with K4 (green) and K5 (red) peptides at a peptide/lipid ratio of 1/60.

For some samples, a second peak is observed at a higher diameter (Figure 4 and the Supporting Information). This peak represents 1−5% of the intensity for DMPG samples and can represent 20% of the intensity for DMPC samples, as in the case of K4 (Figure 4). This second peak is observed after the addition of selective peptides and never after the addition of

dx.doi.org/10.1021/bi201335t | Biochemistry 2011, 50, 9409−9420

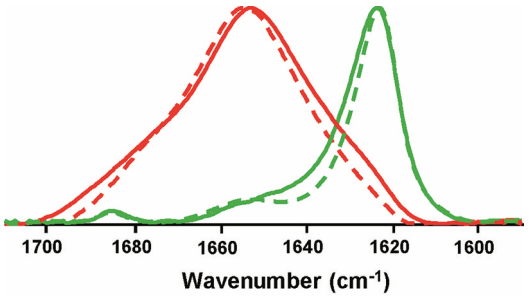


Figure 5. Infrared spectra of K4 (green) and K5 (red) peptides in the presence of DMPC (solid lines) or DMPG (dashed lines) vesicles. Only the amide I′ band (1600−1700 cm^{-1}) is shown. The measurements were performed at 37 °C.

nonselective peptides. This peak can be due to vesicle aggregation and/or fusion[59] or peptide aggregation.[60]

Peptide Secondary Structure. Calcein leakage studies clearly indicated a relationship between the location of the positively charged residue or residues and the ability of the peptide to induce leakage of zwitterionic DMPC vesicles. To determine if the location of the charged residue or residues has an effect on the peptide structure, we used FTIR spectroscopy. The amide I′ band is highly sensitive to the secondary structure of peptides and can be used as an indicator of peptide structure.[61,62]

The structures of peptides with one or two lysine residues at positions 3, 5, 10, and 12 (nonselective peptides) and at positions 4, 7, 8, and 11 (selective peptides) in the presence of DMPC and DMPG were thus analyzed through the amide I′ band. Figure 5 shows that the shape of the amide I′ band is different between selective and nonselective peptides. Indeed, the maximum of the amide I′ band is centered at ∼1652 ± 2 cm^{-1} for nonselective peptides, which is indicative of a helical structure. Curve-fitting methods were used to quantify the proportion of each secondary structure adopted by the peptides.[63] Figure 6 shows that all studied nonselective peptides are mainly structured as α-helices (50−75%). The structure of the base 14-mer peptide, which is also a nonselective peptide, is mainly helical, as well. No significant difference is observed when the peptides are in the presence of DMPC or DMPG vesicles. This indicates that the nature of the lipid does not have a significant effect on the secondary structure of the peptides.

Selective peptides have a different amide I′ band: it is centered around 1623 ± 2 cm^{-1}, which is indicative of an intermolecular β-sheet structure. The presence of a second peak, around 1690 cm^{-1}, confirms the presence of an intermolecular β-sheet structure. Measurements of each structural component confirm that selective peptides are mainly structured as intermolecular β-sheets (40−75%) whatever the nature of the lipid vesicles (Figure 6).

DISCUSSION

AMPs constitute a promising way of fighting against multiresistant bacteria. Indeed, AMPs have many advantages, including their broad-spectrum activity (antibacterial, antiviral,

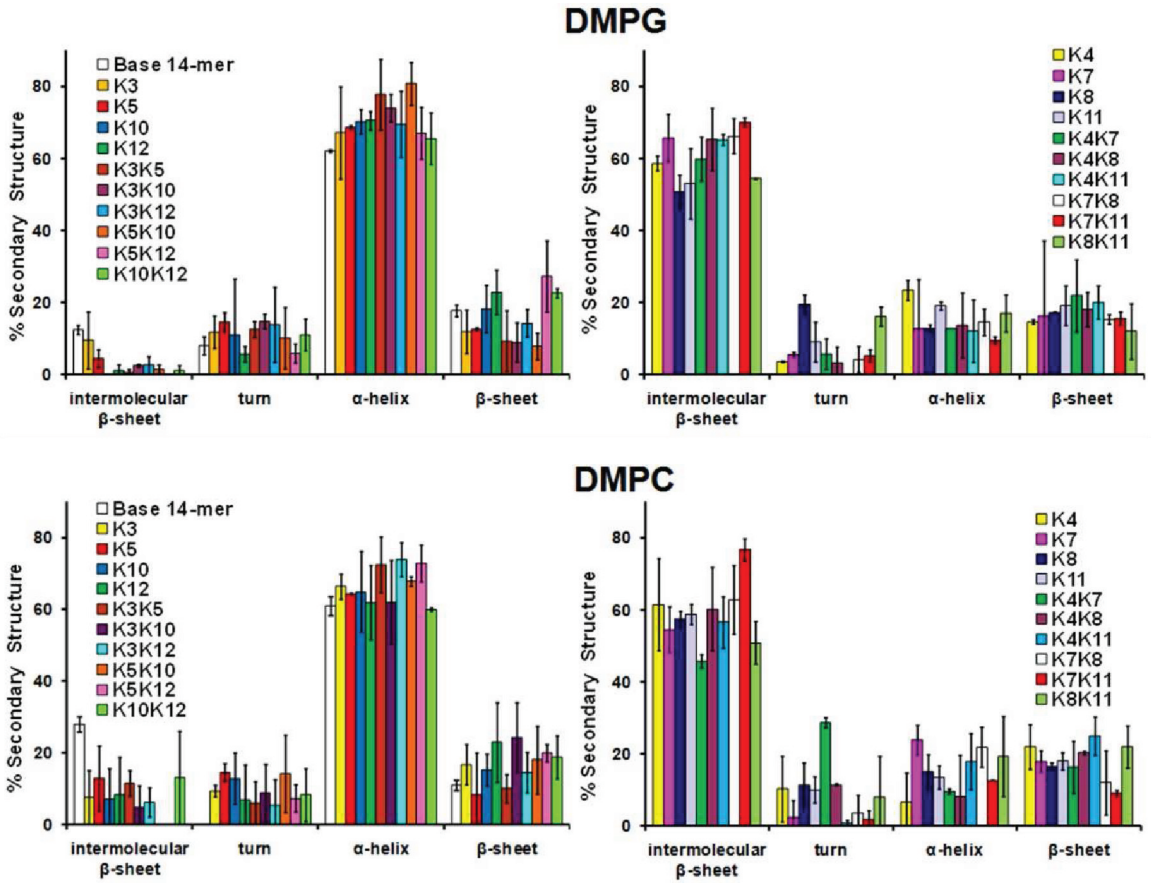


Figure 6. Percentage of secondary structure adopted by nonselective (left) or selective peptides (right) in the presence of DMPG bilayers at a peptide/lipid ratio of 1/60 (top). Percentage of secondary structure adopted by nonselective (left) or selective peptides (right) in the presence of DMPC bilayers at a peptide/lipid radio of 1/60 (bottom). The measurements were performed at 37 °C.

dx.doi.org/10.1021/bi201335t | Biochemistry 2011, 50, 9409−9420

and antifungal), their rapid and potent bactericidal activity, and their low level of induced resistance.[64] However, despite many efforts to increase bacterial activity and selectivity, non-natural or modified AMPs have received Food and Drug Administration approval for medical applications, and to date, only one cationic synthetic antimicrobial peptide has demonstrated efficacy in phase 3a clinical trials.[14,15] A better understanding of each parameter involved in the activity of antimicrobial peptides is thus crucial for the development of new AMPs or peptidomimetics with increased efficiency and selectivity.

The base 14-mer peptide investigated in this study is a good template for studying the effect of charges and their position on the activity of a mimic AMP peptide. Indeed, this short peptide is amphiphilic in its helical form and induces membrane destabilization, like AMPs. The influence of a positive charge and its position on its bilayer activity and selectivity is thus not "perturbed" by the presence of other charged residues.

Only One Positively Charged Residue Can Induce Selectivity. Our fluorescence results indicate that substitution at specific locations of only one leucine with a positively charged residue, namely, lysine, arginine, or histidine, is sufficient to make the nonselective base 14-mer peptide become selective, i.e., active against negatively charged DMPG vesicles but not against zwitterionic DMPC vesicles. These results confirm that cationic character is an important parameter for selective targeting of AMPs.[65] Leakage results also show that the 14-mer peptides with two lysine residues located at positions 4, 7, 8, and 11 have greater bilayer selectivity against bacterial mimic membranes than analogous peptides with only one lysine at those positions (Tables 1 and 2). However, previous studies showed that there is no linear relationship between the number of cationic charges in peptides and their antibacterial activity.[20,66,67] Indeed, magainin 2 analogues having a net charge of +6 to +7 possess stronger hemolytic activity but lower antibacterial activity than those with a net charge of +3 to +5.[67] Therefore, for several antibacterial peptides, an optimum number of cationic charges was found for selectivity. For the 14-mer peptides, further studies at lower peptide/lipid molar ratios and with more leucine to lysine substitutions will have to be performed to determine the optimum number of positively charged residues for bilayer selectivity.

Importance of the Lysine Position with Respect to the Secondary Structure of Peptides. Our FTIR results indicate that the base 14-mer peptide is mainly structured as an α-helix in the presence of lipid vesicles. This result is in agreement with previous CD (circular dichroism) experiments.[49] However, substitution of one or two leucine residues with lysines can induce important conformational changes in the peptide. Indeed, the peptide can adopt an intermolecular β-sheet structure instead of a helical structure, a dramatic change taking into account modifications made in the amino acid content. These data indicate that the structure of the 14-mer peptides is highly sensitive to their sequence. This is mainly due to their short size and the simplicity of their sequence.

Other peptides with antimicrobial properties also show structural changes after only one amino acid substitution.[68−70] For example, a glycine to arginine substitution in a synthetic ESF1 peptide induces a large increase in the level of β-sheet structure in the presence of SDS (sodium dodecyl sulfate).[68] In the case of the lysine-modified 14-mer peptides, the fact that only one substitution can switch the secondary structure completely suggests that the stabilization energy for helical or intermolecular β-sheet structures is similar. However, whatever the secondary structure adopted, the stability of peptides with this structure is high enough to sustain a noticeable temperature variation [infrared results indicate no structural change for helical and intermolecular β-sheets peptides between 12 and 70 °C (data not shown)].

The formation of intermolecular β-sheet structures is observed for specific substitutions at positions 4, 7, 8, and 11, while the peptide remains helical for substitutions at positions 3, 5, 10, and 12. The β-strand diagram and the helical wheel projection of 14-mer peptides (Figure 1B,C) allow a better understanding of the role of lysine positions in the peptide structure. Indeed, leucine to lysine substitutions supporting a helical structure are close to the hydrophilic side composed of crown ether phenylalanine residues, therefore increasing the amphipathic character of the peptides. On the other hand, substitutions disfavoring a helical structure are located on the opposite side of crown ether phenylalanine residues when considering the peptide as helical. However, with these peptides, lysine residues are close to crown ether phenylalanine residues when the peptide adopts a β-strand structure. Crown ethers are known to bind primary ammonium ions quite significantly by hydrogen bonds and electrostatic interactions.[51,71] Therefore, interactions between the side chain of lysines and the crown ether phenylalanines residues can stabilize structures when in their proximity. This is the case with the helical form when lysine residues are at positions 3, 5, 10, and 12 and with the β-strand structure when lysine residues are at positions 4, 7, 8, and 11.

Importance of the Peptide Structure with Respect to Membrane Activity. The combination of our fluorescence and infrared results indicates that peptides adopting a β-sheet structure are selective, i.e., inducing leakage of DMPG vesicles but not of DMPC vesicles. Several natural β-sheet peptides are registered as AMPs. These peptides contain a β-hairpin and are stabilized by one or several disulfide bonds between cysteine residues. Few β-sheet peptides having antimicrobial activities without cysteine (and, so, without disulfide bonds) have been reported. To the best of our knowledge, only some derivatives of the natural AMPs lactoferricin and tachyplesin I and the synthetic 18-residue peptide $(KIGAKI)_3$-NH_2 are linear β-sheet peptides with antimicrobial activities.[72−75] Thus, with these peptides, the β-sheet 14-mer peptides constitute a new class of linear peptides lacking disulfide bonds selective against negatively charged membranes. On the other hand, our fluorescence and infrared results indicate that the helical 14-mer peptides are nonselective. Several other studies show evidence that helicity of AMPs is more important for activity against zwitterionic membranes than against negatively charged membranes.[24,76−78] For example, derivatives of cytolysin, pardaxin, and melittin with a lower helical content show a decreased hemolytic activity without a significant change in antibacterial activity.[77,79] This relationship between the helical content and the lytic activity of these derivatives on zwitterionic membranes was confirmed by lipid vesicle leakage assays.[77,79] Similar results were also observed for analogues of magainin 2, crabolin, and model peptides mimicking AMPs.[68,70,80,81] In a recent paper, Blazyk and colleagues clearly show that positively charged amphipathic β-sheet peptides display enhanced selectivity in targeting bacterial cells compared to amphipathic α-helical peptides, despite the fact that both types of peptides contain the same number of charged residues.[74]

When the peptides adopt an intermolecular β-sheet structure, the hydrophobicity of the AMP accessible surface is smaller

than when they are helical. Indeed, the oligomerization limits exposure of hydrophobic areas, while in the helical amphipathic structure, all hydrophobic residues are concentrated on one side. Several studies clearly show that an increase in the hydrophobicity of AMPs induces an increase in the hemolytic activity and/or of the level of intravesicular leakage of zwitterionic vesicles.[16,47,66,81−85] For example, Wieprecht and colleagues have shown that most hydrophobic peptides derived from magainin are more active against zwitterionic vesicles than less hydrophobic peptides, but that both types of peptides have no significant activity difference on negatively charged vesicles.[84] Other studies have also shown that the influence of hydrophobicity is stronger for the hemolytic activity than for the antibacterial activity.[47,81,82,86] Therefore, the relationship between the helical content of AMPs and their hemolytic activity can be explained by the hydrophobicity of the peptide accessible surface. This relationship can also be explained by a change in the mode of membrane destabilization induced by β-sheet and helical peptides because of their hydrophobic accessible surface and/or their length. Whatever the cause, our results are consistent with those of Shai and Oren,[87] suggesting that peptides inducing membrane leakage via contact with the acidic phospholipid headgroups do not require a preferable structure, as long as a certain level of hydrophobicity and a number of positive charges are preserved.

Is Amphipathicity Necessary? Many studies suggest that an amphipathic arrangement is important for the activity of AMPs. Indeed, changing the amino acid distribution to disrupt the hydrophilic−hydrophobic segregation decreases the activity against bacteria or bacterial-mimic vesicles of many α-helical[26,82,88−90] and β-sheet[72,74] structured AMPs. The base 14-mer peptide is amphipathic in its active form, i.e., the helical form. This amphipathicity is reinforced for α-helical 14-mer peptides containing one or two positively charged residues when these residues are located in the hydrophilic side composed of the synthetic crown ether phenylalanines (Figure 1C). However, 14-mer peptides in their β-sheet conformation are not amphipathic because each β-sheet side contains two crown ether phenylalanine residues (hydrophilic residue) (Figure 1B). Because β-sheet peptides are active against only DMPG vesicles while α-helical peptides are active against both DMPG and DMPC bilayers, the amphipathicity does not seem to be a prerequisite for the activity of 14-mer peptides on bacterium-mimicking membranes. Our results are thus in contradiction with results obtained in previously cited studies. Interestingly, other results in the literature are in agreement with ours. More specifically, it was shown that scrambling the sequence of natural melittin, model amphipathic α-peptides, or unnatural oligomers to form nonamphipathic structures produced no significant change or led to a stronger antibacterial activity, while decreasing the hemolytic activity.[91−94]

More recently, a new type of AMP, called temporin-SHf, was demonstrated to have activity against bacteria and yeast but no hemolytic activity by adopting a nonamphipathic α-helix.[95] Therefore, as already suggested by Blondelle and Houghten,[96] the amphiphatic character is more important for hemolytic potency than for the inhibition of bacterial growth. This could be explained by the fact that the interaction between amphipathic peptides or polymers and membranes is favored for peptides in a surface orientation in which hydrophilic groups are exposed to the aqueous buffer or lipid headgroups, while hydrophobic groups are buried in the membrane core. Electrostatic interactions between the positively charged residues of the peptide and the negatively charged headgroups of negatively charged bilayers could then be less important than hydrophilic and hydrophobic interactions. However, these electrostatic interactions are more important in the interaction of nonamphipathic peptides with membranes. Therefore, as suggested by Schmitt and colleagues,[97] it does not seem necessary to design an amphiphilic secondary structure to generate oligomers with favorable antibacterial−hemolytic activity profiles. Our results with 14-mer analogues support this hypothesis.

Mode of Action of Cationic 14-mer Peptides. Previous fluorescence and solid-state nuclear magnetic resonance (NMR) studies on the neutral base 14-mer peptide suggested that this peptide destabilizes the target bilayers not by barrel-stave or detergent-like mechanisms, but by a pore formation mechanism similar to the "sinking raft" model.[13,23,50,52] The peptide inserts into the bilayer interface to destabilize the lipid headgroups and induces the formation of torus-shaped pores.

In this study, DLS results indicate that the bilayer destabilization induced by cationic peptides designed from this base 14-mer is not accompanied by the formation of small micelles. Fluorescence results also show that the critical threshold peptide/lipid molar ratio for significant leakage is very low (around 1/10000) and is consistent with pore formation, where the amount of peptide molecules required to disrupt membranes is smaller than that for peptides acting by a carpet-like mechanism.[98] Thus, as for the base nonselective peptide, selective and non-selective cationic peptides appear to induce calcein leakage by formation of pores in the target bilayers, instead of micellization.

In a helical conformation, the 14-mer peptides exhibit a cone shape because of the presence of the large crown ethers on one side of the helix. The insertion of these cone-shaped molecules in the lipid headgroup region can induce a positive curvature of the bilayer and then increase the formation propensity of sinking raft pores in DMPC and DMPG bilayers. Even if the spatial arrangement of aggregated β-sheet 14-mer peptides is not known, the cone shape of 14-mer peptides is reduced in this conformation. These differences in the molecule shape suggest that the mechanism of action of β-sheet 14-mer selective peptides for inducing torus-shaped pores in DMPG bilayers could be different from those of the α-helical nonselective peptides. However, further experiments are required to confirm this hypothesis.

Are β-Sheet 14-mer Peptides Good Candidates for Antibiotics? Previous scattering, calorimetric, and fluorescence experiments on natural and non-natural AMPs have shown that the usual critical threshold peptide/lipid molar ratios for significant leakage vary from 1/260 to 1/5 for different natural and non-natural AMPs.[19,43,99−103] This threshold is 1/1000 for monosubstituted active 14-mer peptides in interaction with DMPG or DMPC vesicles (Figure 2). This result indicates that, compared to previously studied peptides, fewer positively charged 14-mer peptides are needed to induce leakage of lipid bilayers. Moreover, the mode of action of 14-mer peptides is fast. Indeed, the complete leakage of calcein from DMPC vesicles occurred rapidly [<5 min after peptides are injected (Figure 2)].

The low critical threshold and the fast kinetics suggest that cationic selective 14-mer peptides may be candidates for development of therapeutically useful antimicrobial agents. Moreover, cationic 14-mer peptides contain non-natural residues, and it was reported that non-natural molecules mimicking AMPs offer significant advantages over classic AMPs as therapeutical agents.[104]

■ ASSOCIATED CONTENT

Supporting Information

Effects of nonselective and selective peptides on the size of DMPC and DMPG lipid vesicles. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Chemistry, PROTEO, CERMA, Université Laval, Québec, Québec, Canada G1V 0A6. Telephone: (418) 656-3393. Fax: (418) 656-7916. E-mail: michele.auger@chm.ulaval.ca.

Funding

This work was supported by the Natural Sciences and Engineering Research Council of Canada, the Fonds québécois de la recherche sur la nature et les technologies (FQRNT), the Regroupement québécois de recherche sur la structure, la fonction et l'ingénierie des protéines (PROTEO), the Centre de recherche sur les matériaux avancés (CERMA), and the Centre québécois sur les matériaux fonctionnels (CQMF). A.L. also thanks PROTEO for the award of a postdoctoral scholarship.

■ ACKNOWLEDGMENTS

We thank Jean-François Rioux and Rodica Plesu for their technical assistance in FTIR and DLS measurements, respectively.

■ ABBREVIATIONS

AMP, antimicrobial peptide; Boc, butoxycarbonyl; CaF_2, calcium fluoride; CD, circular dichroism; CH_3CN, acetonitrile; DLS, dynamic light scattering; DMF, dimethylformamide; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid disodium; Fmoc, fluorenylmethyloxycarbonyl; FTIR, Fourier transform infrared; He−Ne, helium−neon; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, hydroxybenzotriazole; HPLC, high-performance liquid chromatography; LUVs, large unilamellar vesicles; MCT, mercury−cadmium−telluride; NMR, nuclear magnetic resonance; PMMA, poly(methyl methacrylate); SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

■ REFERENCES

(1) Marshall, S. G., and Arenas, G. (2003) Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology. *Electron. J. Biotechnol. 6*, 271−284.

(2) Agerberth, B., and Gudmundsson, G. H. (2006) Host antimicrobial defence peptides in human disease. *Curr. Top. Microbiol. Immunol. 306*, 67−90.

(3) Andersen, J. H., Jenssen, H., Sandvik, K., and Gutteberg, T. J. (2004) Anti-HSV activity of lactoferrin and lactoferricin is dependent on the presence of heparan sulphate at the cell surface. *J. Med. Virol. 74*, 262−271.

(4) Falco, A., Mas, V., Tafalla, C., Perez, L., Coll, J. M., and Estepa, A. (2007) Dual antiviral activity of human α-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): Inactivation of virus particles and induction of a type I interferon-related response. *Antiviral Res. 76*, 111−123.

(5) Rubinchik, E., Dugourd, D., Algara, T., Pasetka, C., and Friedland, H. D. (2009) Antimicrobial and antifungal activities of a novel cationic antimicrobial peptide, omiganan, in experimental skin colonisation models. *Int. J. Antimicrob. Agents 34*, 457−461.

(6) Shai, Y., Makovitzky, A., and Avrahami, D. (2006) Host defense peptides and lipopeptides: Modes of action and potential candidates for the treatment of bacterial and fungal infections. *Curr. Protein Pept. Sci. 7*, 479−486.

(7) Vogel, H. J., Schibli, D. J., Jing, W., Lohmeier-Vogel, E. M., Epand, R. F., and Epand, R. M. (2002) Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides. *Biochem. Cell Biol. 80*, 49−63.

(8) Lehmann, J., Retz, M., Sidhu, S. S., Suttmann, H., Sell, M., Paulsen, F., Harder, J., Unteregger, G., and Stockle, M. (2006) Antitumor activity of the antimicrobial peptide magainin II against bladder cancer cell lines. *Eur. Urol. 50*, 141−147.

(9) Schweizer, F. (2009) Cationic amphiphilic peptides with cancer-selective toxicity. *Eur. J. Pharmacol. 625*, 190−194.

(10) Wang, K. R., Zhang, B. Z., Zhang, W., Yan, J. X., Li, J., and Wang, R. (2008) Antitumor effects, cell selectivity and structure-activity relationship of a novel antimicrobial peptide polybia-MPI. *Peptides 29*, 963−968.

(11) Molhoek, E. M., den Hertog, A. L., de Vries, A. M., Nazmi, K., Veerman, E. C., Hartgers, F. C., Yazdanbakhsh, M., Bikker, F. J., and van der Kleij, D. (2009) Structure-function relationship of the human antimicrobial peptide LL-37 and LL-37 fragments in the modulation of TLR responses. *Biol. Chem. 390*, 295−303.

(12) Mookherjee, N., Rehaume, L. M., and Hancock, R. E. (2007) Cathelicidins and functional analogues as antisepsis molecules. *Expert Opin. Ther. Targets 11*, 993−1004.

(13) Chan, D. I., Prenner, E. J., and Vogel, H. J. (2006) Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochim. Biophys. Acta 1758*, 1184−1202.

(14) Hancock, R. E., and Sahl, H. G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol. 24*, 1551−1557.

(15) Jenssen, H., Hamill, P., and Hancock, R. E. (2006) Peptide antimicrobial agents. *Clin. Microbiol. Rev. 19*, 491−511.

(16) Dathe, M., and Wieprecht, T. (1999) Structural features of helical antimicrobial peptides: Their potential to modulate activity on model membranes and biological cells. *Biochim. Biophys. Acta 1462*, 71−87.

(17) Giuliani, A., Pirri, G., Bozzi, A., Di Giulio, A., Aschi, M., and Rinaldi, A. C. (2008) Antimicrobial peptides: Natural templates for synthetic membrane-active compounds. *Cell. Mol. Life Sci. 65*, 2450−2460.

(18) Shai, Y. (2002) Mode of action of membrane active antimicrobial peptides. *Biopolymers 66*, 236−248.

(19) Huang, H. W. (2006) Molecular mechanism of antimicrobial peptides: The origin of cooperativity. *Biochim. Biophys. Acta 1758*, 1292−1302.

(20) Kiyota, T., Lee, S., and Sugihara, G. (1996) Design and synthesis of amphiphilic α-helical model peptides with systematically varied hydrophobic-hydrophilic balance and their interaction with lipid- and bio-membranes. *Biochemistry 35*, 13196−13204.

(21) Oren, Z., and Shai, Y. (1998) Mode of action of linear amphipathic α-helical antimicrobial peptides. *Biopolymers 47*, 451−463.

(22) Papo, N., and Shai, Y. (2003) Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes? *Peptides 24*, 1693−1703.

(23) Pokorny, A., and Almeida, P. F. (2005) Permeabilization of raft-containing lipid vesicles by δ-lysin: A mechanism for cell sensitivity to cytotoxic peptides. *Biochemistry 44*, 9538−9544.

(24) Rausch, J. M., Marks, J. R., Rathinakumar, R., and Wimley, W. C. (2007) β-Sheet pore-forming peptides selected from a rational combinatorial library: Mechanism of pore formation in lipid vesicles and activity in biological membranes. *Biochemistry 46*, 12124−12139.

(25) Salditt, T., Li, C., and Spaar, A. (2006) Structure of antimicrobial peptides and lipid membranes probed by interface-sensitive X-ray scattering. *Biochim. Biophys. Acta 1758*, 1483−1498.

(26) Zelezetsky, I., and Tossi, A. (2006) α-Helical antimicrobial peptides: Using a sequence template to guide structure-activity relationship studies. *Biochim. Biophys. Acta 1758*, 1436−1449.
(27) Zhao, H., Sood, R., Jutila, A., Bose, S., Fimland, G., Nissen-Meyer, J., and Kinnunen, P. K. (2006) Interaction of the antimicrobial peptide pheromone Plantaricin A with model membranes: Implications for a novel mechanism of action. *Biochim. Biophys. Acta 1758*, 1461−1474.
(28) Thennarasu, S., Huang, R., Lee, D. K., Yang, P., Maloy, L., Chen, Z., and Ramamoorthy, A. (2010) Limiting an antimicrobial peptide to the lipid-water interface enhances its bacterial membrane selectivity: A case study of MSI-367. *Biochemistry 49*, 10595−10605.
(29) Bhunia, A., Domadia, P. N., Torres, J., Hallock, K. J., Ramamoorthy, A., and Bhattacharjya, S. (2010) NMR structure of pardaxin, a pore-forming antimicrobial peptide, in lipopolysaccharide micelles: Mechanism of outer membrane permeabilization. *J. Biol. Chem. 285*, 3883−3895.
(30) Thennarasu, S., Tan, A. M., Penumatchu, R., Shelburne, C. E., Heyl, D. L., and Ramamoorthy, A. (2010) Antimicrobial and membrane disrupting activities of a peptide derived from the human cathelicidin antimicrobial peptide LL37. *Biophys. J. 98*, 248−257.
(31) Gottler, L. M., and Ramamoorthy, A. (2009) Structure, membrane orientation, mechanism, and function of pexiganan: A highly potent antimicrobial peptide designed from magainin. *Biochim. Biophys. Acta 1788*, 1680−1686.
(32) Ramamoorthy, A., Lee, D. K., Narasimhaswamy, T., and Nanga, R. P. R. (2010) Cholesterol reduces pardaxin's dynamics: A barrel-stave mechanism of membrane disruption investigated by solid-state NMR. *Biochim. Biophys. Acta 1798*, 223−227.
(33) Lohner, K., and Blondelle, S. E. (2005) Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics. *Comb. Chem. High Throughput Screening 8*, 241−256.
(34) Toke, O. (2005) Antimicrobial peptides: New candidates in the fight against bacterial infections. *Biopolymers 80*, 717−735.
(35) Lohner, K. (2001) The role of membrane lipid composition in cell targeting of antimicrobial peptides. *Development of novel antimicrobial agents: Emerging strategies*, pp 149−165, Horizon Scientific Press, Wymondham, U.K.
(36) Epand, R. F., Schmitt, M. A., Gellman, S. H., and Epand, R. M. (2006) Role of membrane lipids in the mechanism of bacterial species selective toxicity by two α/β-antimicrobial peptides. *Biochim. Biophys. Acta 1758*, 1343−1350.
(37) Tachi, T., Epand, R. F., Epand, R. M., and Matsuzaki, K. (2002) Position-dependent hydrophobicity of the antimicrobial magainin peptide affects the mode of peptide-lipid interactions and selective toxicity. *Biochemistry 41*, 10723−10731.
(38) Blondelle, S. E., Lohner, K., and Aguilar, M. (1999) Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: Determination and biological specificity. *Biochim. Biophys. Acta 1462*, 89−108.
(39) Dhople, V. M., and Nagaraj, R. (1995) Generation of analogs having potent antimicrobial and hemolytic activities with minimal changes from an inactive 16-residue peptide corresponding to the helical region of *Staphylococcus aureus* δ-toxin. *Protein Eng. 8*, 315−318.
(40) Gopal, R., Park, S. C., Ha, K. J., Cho, S. J., Kim, S. W., Song, P. I., Nah, J. W., Park, Y., and Hahm, K. S. (2009) Effect of leucine and lysine substitution on the antimicrobial activity and evaluation of the mechanism of the HPA3NT3 analog peptide. *J. Pept. Sci. 15*, 589−594.
(41) Matsuyama, K., and Natori, S. (1990) Mode of action of sapecin, a novel antibacterial protein of *Sarcophaga peregrina* (flesh fly). *J. Biochem. 108*, 128−132.
(42) Rathinakumar, R., Walkenhorst, W. F., and Wimley, W. C. (2009) Broad-spectrum antimicrobial peptides by rational combinatorial design and high-throughput screening: The importance of interfacial activity. *J. Am. Chem. Soc. 131*, 7609−7617.
(43) Som, A., Vemparala, S., Ivanov, I., and Tew, G. N. (2008) Synthetic mimics of antimicrobial peptides. *Biopolymers 90*, 83−93.
(44) Zhu, W. L., and Shin, S. Y. (2009) Effects of dimerization of the cell-penetrating peptide Tat analog on antimicrobial activity and mechanism of bactericidal action. *J. Pept. Sci. 15*, 345−352.
(45) Epand, R. M., and Epand, R. F. (2003) Liposomes as models for antimicrobial peptides. *Methods Enzymol. 372*, 124−133.
(46) Jiang, Z., Vasil, A. I., Hale, J. D., Hancock, R. E., Vasil, M. L., and Hodges, R. S. (2008) Effects of net charge and the number of positively charged residues on the biological activity of amphipathic α-helical cationic antimicrobial peptides. *Biopolymers 90*, 369−383.
(47) Maloy, W. L., and Kari, U. P. (1995) Structure-activity studies on magainins and other host defense peptides. *Biopolymers 37*, 105−122.
(48) Ouellet, M., Bernard, G., Voyer, N., and Auger, M. (2006) Insights on the interactions of synthetic amphipathic peptides with model membranes as revealed by ^{31}P and ^{2}H solid-state NMR and infrared spectroscopies. *Biophys. J. 90*, 4071−4084.
(49) Ouellet, M., Otis, F., Voyer, N., and Auger, M. (2006) Biophysical studies of the interactions between 14-mer and 21-mer model amphipathic peptides and membranes: Insights on their modes of action. *Biochim. Biophys. Acta 1758*, 1235−1244.
(50) Vandenburg, Y. R., Smith, B. D., Biron, E., and Voyer, N. (2002) Membrane disruption ability of facially amphiphilic helical peptides. *Chem. Commun.*, 1694−1695.
(51) Otis, F., Racine-Berthiaume, C., and Voyer, N. (2011) How far can a sodium ion travel within a lipid bilayer? *J. Am. Chem. Soc. 133*, 6481−6483.
(52) Ouellet, M., Doucet, J.-D., Voyer, N., and Auger, M. (2007) Membrane topology of a 14-mer model amphipathic peptide: A solid-state NMR spectroscopy study. *Biochemistry 46*, 6597−6606.
(53) Boutin, J. M., Richer, J., Tremblay, M., and Bissonette, V. (2007) Synthesis and characterization of peptide nanostructures chemisorbed on gold. *New J. Chem. 31*, 741−747.
(54) Biron, E., Otis, F., Meillon, J. C., Robitaille, M., Lamothe, J., Van Hove, P., Cormier, M. E., and Voyer, N. (2004) Design, synthesis, and characterization of peptide nanostructures having ion channel activity. *Bioorg. Med. Chem. 12*, 1279−1290.
(55) Allen, T. M., and Cleland, L. G. (1980) Serum-induced leakage of liposome contents. *Biochim. Biophys. Acta 597*, 418−426.
(56) Kendall, D. A., and MacDonald, R. C. (1982) A fluorescence assay to monitor vesicle fusion and lysis. *J. Biol. Chem. 257*, 13892−13895.
(57) Bartlett, G. R. (1959) Colorimetric assay methods for free and phosphorylated glyceric acids. *J. Biol. Chem. 234*, 469−471.
(58) Goormaghtigh, E., Raussens, V., and Ruysschaert, J. M. (1999) Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes. *Biochim. Biophys. Acta 1422*, 105−185.
(59) Trikash, I., Gumenyuk, V., and Lishko, V. (2010) The fusion of synaptic vesicle membranes studied by lipid mixing: The R18 fluorescence assay validity. *Chem. Phys. Lipids 163*, 778−786.
(60) Pascal, C., Poncet-Legrand, C., Cabane, B., and Vernhet, A. (2008) Aggregation of a proline-rich protein induced by epigallocatechin gallate and condensed tannins: Effect of protein glycosylation. *J. Agric. Food Chem. 56*, 6724−6732.
(61) Rahmelow, K., and Hubner, W. (1996) Secondary structure determination of proteins in aqueous solution by infrared spectroscopy: A comparison of multivariate data analysis methods. *Anal. Biochem. 241*, 5−13.
(62) Wi, S., Pancoska, P., and Keiderling, T. A. (1998) Predictions of protein secondary structures using factor analysis on Fourier transform infrared spectra: Effect of Fourier self-deconvolution of the amide I and amide II bands. *Biospectroscopy 4*, 93−106.
(63) Cooper, E. A., and Knutson, K. (1995) Fourier transform infrared spectroscopy investigations of protein structure. *Pharm. Biotechnol. 7*, 101−143.
(64) Gordon, Y. J., Romanowski, E. G., and McDermott, A. M. (2005) A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr. Eye Res. 30*, 505−515.
(65) Yeaman, M. R., and Yount, N. Y. (2003) Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev. 55*, 27−55.

(66) Blondelle, S. E., and Houghten, R. A. (1992) Design of model amphipathic peptides having potent antimicrobial activities. *Biochemistry 31*, 12688−12694.
(67) Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M., and Bienert, M. (2001) Optimization of the antimicrobial activity of magainin peptides by modification of charge. *FEBS Lett. 501*, 146−150.
(68) Dykes, G. A., Aimoto, S., and Hastings, J. W. (1998) Modification of a synthetic antimicrobial peptide (ESF1) for improved inhibitory activity. *Biochem. Biophys. Res. Commun. 248*, 268−272.
(69) Yang, S. T., Yub Shin, S. Y., Kim, Y. C., Kim, Y., Hahm, K. S., and Kim, J. I. (2002) Conformation-dependent antibiotic activity of tritrpticin, a cathelicidin-derived antimicrobial peptide. *Biochem. Biophys. Res. Commun. 296*, 1044−1050.
(70) Zhu, W. L., Park, Y., Park, I. S., Park, Y. S., Kim, Y., Hahm, K. S., and Shin, S. Y. (2006) Improvement of bacterial cell selectivity of melittin by a single Trp mutation with a peptoid residue. *Protein Pept. Lett. 13*, 719−725.
(71) Maleknia, S., and Brodbelt, J. (1993) Cavity-size-dependent dissociation of crown ether/ammonium ion complexes in the gas phase. *J. Am. Chem. Soc. 115*, 2837−2843.
(72) Blazyk, J., Wiegand, R., Klein, J., Hammer, J., Epand, R. M., Epand, R. F., Maloy, W. L., and Kari, U. P. (2001) A novel linear amphipathic β-sheet cationic antimicrobial peptide with enhanced selectivity for bacterial lipids. *J. Biol. Chem. 276*, 27899−27906.
(73) Hoek, K. S., Milne, J. M., Grieve, P. A., Dionysius, D. A., and Smith, R. (1997) Antibacterial activity in bovine lactoferrin-derived peptides. *Antimicrob. Agents Chemother. 41*, 54−59.
(74) Jin, Y., Hammer, J., Pate, M., Zhang, Y., Zhu, F., Zmuda, E., and Blazyk, J. (2005) Antimicrobial activities and structures of two linear cationic peptide families with various amphipathic β-sheet and α-helical potentials. *Antimicrob. Agents Chemother. 49*, 4957−4964.
(75) Ramamoorthy, A., Thennarasu, S., Tan, A., Gottipati, K., Sreekumar, S., Heyl, D. L., An, F. Y., and Shelburne, C. E. (2006) Deletion of all cysteines in tachyplesin I abolishes hemolytic activity and retains antimicrobial activity and lipopolysaccharide selective binding. *Biochemistry 45*, 6529−6540.
(76) Conlon, J. M., Al-Ghaferi, N., Abraham, B., and Leprince, J. (2007) Strategies for transformation of naturally-occurring amphibian antimicrobial peptides into therapeutically valuable anti-infective agents. *Methods 42*, 349−357.
(77) Shai, Y., and Oren, Z. (1996) Diastereoisomers of cytolysins, a novel class of potent antibacterial peptides. *J. Biol. Chem. 271*, 7305−7308.
(78) Subasinghage, A. P., Conlon, J. M., and Hewage, C. M. (2010) Development of potent anti-infective agents from *Silurana tropicalis*: Conformational analysis of the amphipathic, α-helical antimicrobial peptide XT-7 and its non-haemolytic analogue [G4K]XT-7. *Biochim. Biophys. Acta 1804*, 1020−1028.
(79) Oren, Z., and Shai, Y. (1997) Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: Structure-function study. *Biochemistry 36*, 1826−1835.
(80) Krishnakumari, V., and Nagaraj, R. (1997) Antimicrobial and hemolytic activities of crabrolin, a 13-residue peptide from the venom of the European hornet, *Vespa crabro*, and its analogs. *J. Pept. Res. 50*, 88−93.
(81) Wieprecht, T., Dathe, M., Schumann, M., Krause, E., Beyermann, M., and Bienert, M. (1996) Conformational and functional study of magainin 2 in model membrane environments using the new approach of systematic double-D-amino acid replacement. *Biochemistry 35*, 10844−10853.
(82) Dathe, M., Wieprecht, T., Nikolenko, H., Handel, L., Maloy, W. L., MacDonald, D. L., Beyermann, M., and Bienert, M. (1997) Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and haemolytic activity of amphipathic helical peptides. *FEBS Lett. 403*, 208−212.
(83) Giangaspero, A., Sandri, L., and Tossi, A. (2001) Amphipathic α helical antimicrobial peptides. *Eur. J. Biochem. 268*, 5589−5600.
(84) Wieprecht, T., Dathe, M., Beyermann, M., Krause, E., Maloy, W. L., MacDonald, D. L., and Bienert, M. (1997) Peptide hydrophobicity controls the activity and selectivity of magainin 2 amide in interaction with membranes. *Biochemistry 36*, 6124−6132.
(85) Wieprecht, T., Dathe, M., Krause, E., Beyermann, M., Maloy, W. L., MacDonald, D. L., and Bienert, M. (1997) Modulation of membrane activity of amphipathic, antibacterial peptides by slight modifications of the hydrophobic moment. *FEBS Lett. 417*, 135−140.
(86) Kustanovich, I., Shalev, D. E., Mikhlin, M., Gaidukov, L., and Mor, A. (2002) Structural requirements for potent versus selective cytotoxicity for antimicrobial dermaseptin S4 derivatives. *J. Biol. Chem. 277*, 16941−16951.
(87) Shai, Y., and Oren, Z. (2001) From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides 22*, 1629−1641.
(88) Chen, Y., Mant, C. T., Farmer, S. W., Hancock, R. E., Vasil, M. L., and Hodges, R. S. (2005) Rational design of α-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. *J. Biol. Chem. 280*, 12316−12329.
(89) Porter, E. A., Weisblum, B., and Gellman, S. H. (2002) Mimicry of host-defense peptides by unnatural oligomers: Antimicrobial β-peptides. *J. Am. Chem. Soc. 124*, 7324−7330.
(90) Raguse, T. L., Porter, E. A., Weisblum, B., and Gellman, S. H. (2002) Structure-activity studies of 14-helical antimicrobial β-peptides: Probing the relationship between conformational stability and antimicrobial potency. *J. Am. Chem. Soc. 124*, 12774−12785.
(91) Blondelle, S. E., and Houghten, R. A. (1991) Probing the relationships between the structure and hemolytic activity of melittin with a complete set of leucine substitution analogs. *Pept. Res. 4*, 12−18.
(92) Papo, N., Oren, Z., Pag, U., Sahl, H. G., and Shai, Y. (2002) The consequence of sequence alteration of an amphipathic α-helical antimicrobial peptide and its diastereomers. *J. Biol. Chem. 277*, 33913−33921.
(93) Schmitt, M. A., Choi, S. H., Guzei, I. A., and Gellman, S. H. (2006) New helical foldamers: Heterogeneous backbones with 1:2 and 2:1 α:β-amino acid residue patterns. *J. Am. Chem. Soc. 128*, 4538−4539.
(94) Schmitt, M. A., Weisblum, B., and Gellman, S. H. (2004) Unexpected relationships between structure and function in α,β-peptides: Antimicrobial foldamers with heterogeneous backbones. *J. Am. Chem. Soc. 126*, 6848−6849.
(95) Abbassi, F., Lequin, O., Piesse, C., Goasdoue, N., Foulon, T., Nicolas, P., and Ladram, A. (2010) Temporin-SHf, a new type of Phe-rich and hydrophobic ultrashort antimicrobial peptide. *J. Biol. Chem. 285*, 16880−16892.
(96) Blondelle, S. E., and Houghten, R. A. (1991) Hemolytic and antimicrobial activities of the twenty-four individual omission analogues of melittin. *Biochemistry 30*, 4671−4678.
(97) Schmitt, M. A., Weisblum, B., and Gellman, S. H. (2007) Interplay among folding, sequence, and lipophilicity in the antibacterial and hemolytic activities of α/β-peptides. *J. Am. Chem. Soc. 129*, 417−428.
(98) Ambroggio, E. E., Separovic, F., Bowie, J. H., Fidelio, G. D., and Bagatolli, L. A. (2005) Direct visualization of membrane leakage induced by the antibiotic peptides: Maculatin, citropin, and aurein. *Biophys. J. 89*, 1874−1881.
(99) Lamaziere, A., Burlina, F., Wolf, C., Chassaing, G., Trugnan, G., and Ayala-Sanmartin, J. (2007) Non-metabolic membrane tubulation and permeability induced by bioactive peptides. *PLoS One 2*, e201.
(100) Lee, M. T., Chen, F. Y., and Huang, H. W. (2004) Energetics of pore formation induced by membrane active peptides. *Biochemistry 43*, 3590−3599.
(101) Prenner, E. J., Lewis, R. N., and McElhaney, R. N. (1999) The interaction of the antimicrobial peptide gramicidin S with lipid bilayer model and biological membranes. *Biochim. Biophys. Acta 1462*, 201−221.
(102) Tamba, Y., and Yamazaki, M. (2005) Single giant unilamellar vesicle method reveals effect of antimicrobial peptide magainin 2 on membrane permeability. *Biochemistry 44*, 15823−15833.

(103) Zhang, L., Rozek, A., and Hancock, R. E. (2001) Interaction of cationic antimicrobial peptides with model membranes. *J. Biol. Chem. 276*, 35714−35722.
(104) Rotem, S., and Mor, A. (2009) Antimicrobial peptide mimics for improved therapeutic properties. *Biochim. Biophys. Acta 1788*, 1582−1592.