JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Subscriber access provided by ISTANBUL TEKNIK UNIV

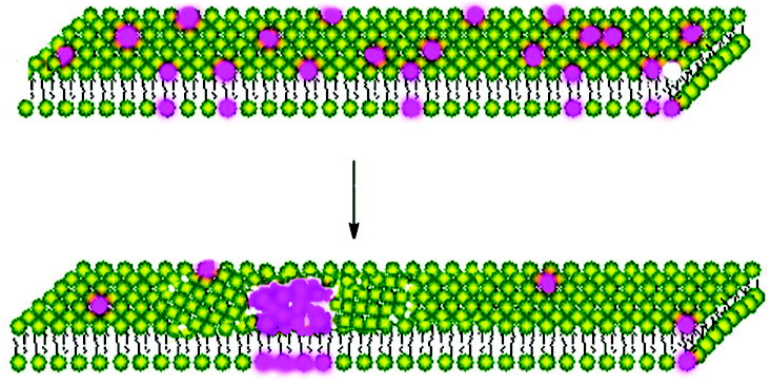
Article

Bacterial Membranes as Predictors of Antimicrobial Potency

Richard M. Epand, Shahar Rotem, Amram Mor, Bob Berno, and Raquel F. Epand

J. Am. Chem. Soc., **2008**, 130 (43), 14346-14352 • DOI: 10.1021/ja8062327 • Publication Date (Web): 01 October 2008

Downloaded from http://pubs.acs.org on February 8, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S
ARTICLES

Published on Web 10/01/2008

Bacterial Membranes as Predictors of Antimicrobial Potency

Richard M. Epand,*,† Shahar Rotem,‡ Amram Mor,‡ Bob Berno,† and Raquel F. Epand†

Departments of Biochemistry and Biomedical Sciences and of Chemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada, and Department of Biotechnology & Food Engineering, Technion—Israel Institute of Technology, Haifa 32000, Israel

Received August 7, 2008; E-mail: epand@mcmaster.ca

Abstract: A wide range of chemical structures having antimicrobial activity have been studied in an effort to treat the increasing emergence of bacteria that are resistant to traditional antibiotics. These agents have varying degrees of toxicity against different bacterial species. We demonstrate, using members of a novel class of antimicrobial agents, the oligomers of acyllysine, that one cause for the difference in species selectivity is the ability to induce the clustering of anionic lipids, resulting in their segregation into domains. This phenomenon occurs only in bacterial membranes composed of both anionic and zwitterionic lipids and not with bacteria whose membrane lipids are largely anionic. As a consequence it can be predicted which bacterial species will be most affected by antimicrobial agents that function principally by this mechanism. This finding allows for the design of new antibiotics with selective toxicity against different groups of bacteria.

Introduction

Antimicrobial and host-defense peptides are of great interest because of their potential clinical use as agents against bacterial infections.[1,2] Several novel analogues have been designed to mimic these agents in an effort to control the increasing emergence of bacteria that are resistant to traditional antibiotics. One class of such compounds are the oligomers of acyllysine (OAKs).[3] The OAKs have multiple positive charges and would be expected to preferentially bind anionic lipids.

Several mechanisms have been proposed to account for the protection by antimicrobial agents against infection including effects on the innate immune system,[4] damage to the cytoplasmic membrane of bacteria,[5] binding to DNA,[6] or inhibition of specific bacterial metabolic processes.[7] In general, these agents do not have a high specificity and are not designed to bind to a specific target. An exception is nisin, an antibiotic with a high degree of specificity of binding to lipid II. However, even nisin has recently been shown to have several modes of action.[8] Hence, in many cases there is likely more than one factor contributing to the toxicity of antimicrobial agents. The membrane plays an important role in many of the mechanisms of toxicity of these agents either as a target of the antimicrobial compound, resulting in damage to the membrane, or as a barrier that must be traversed by the toxic agent to gain access to an intracellular target. Mechanisms of membrane damage have generally focused on how the antimicrobial agents can disrupt membrane bilayer organization by forming pores lined by both lipids and peptides,[9] or by a more general "carpet mechanism".[5] Since bacteria vary widely in the lipid composition of their membranes, they would be expected to exhibit different sensitivities to antimicrobial compounds that act at the cell surface. Although much of the focus in antimicrobial therapy has been in trying to design broad-spectrum antibiotics, this can lead to a depletion of the natural bacterial flora and consequently allow the rapid proliferation of resistant pathogenic microbes. This is well recognized in the case of the intestinal microbiota and the emergence of pathogenic strains such as *Clostridium difficile*.[10] There can thus be an advantage to design antimicrobial agents of more limited specificity by taking into account the difference in the lipid composition of bacterial membranes.

A factor resulting in the selective sensitivity of different bacteria to antimicrobial agents has been suggested to be a consequence of differences in their lipid composition.[11−13] For certain antimicrobial agents, the membrane content of phosphatidylethanolamine (PE) has been found to be a major

† McMaster University.
‡ Technion—Israel Institute of Technology.

(1) Hancock, R. E. W.; Sahl, H. G. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.
(2) Zasloff, M. *Nature* **2002**, *415*, 389–395.
(3) Radzishevsky, I. S.; Rotem, S.; Bourdetsky, D.; Navon-Venezia, S.; Carmeli, Y.; Mor, A. *Nat. Biotechnol.* **2007**, *25*, 657–659.
(4) Brown, K. L.; Cosseau, C.; Gardy, J. L.; Hancock, R. E. *Trends Immunol.* **2007**, *28*, 260–266.
(5) Shai, Y. *Biopolymers* **2002**, *66*, 236–248.
(6) Marchand, C.; Krajewski, K.; Lee, H. F.; Antony, S.; Johnson, A. A.; Amin, R.; Roller, P.; Kvaratskhelia, M.; Pommier, Y. *Nucleic Acids Res.* **2006**, *34*, 5157–5165.
(7) Breukink, E.; Wiedemann, I.; van Kraaij, C.; Kuipers, O. P.; Sahl, H. G.; de Kruijff, B. *Science* **1999**, *286*, 2361–2364.
(8) Hasper, H. E.; Kramer, N. E.; Smith, J. L.; Hillman, J. D.; Zachariah, C.; Kuipers, O. P.; de Kruijff, B.; Breukink, E. *Science* **2006**, *313*, 1636–1637.
(9) Matsuzaki, K. *Biochim. Biophys. Acta* **1998**, *1376*, 391–400.
(10) Silverman, N.; Paquette, N. *Science* **2008**, *319*, 734–735.
(11) Epand, R. F.; Schmitt, M. A.; Gellman, S. H.; Sen, A.; Auger, M.; Hughes, D. W.; Epand, R. M. *Mol. Membr. Biol.* **2005**, *22*, 457–469.
(12) Arnt, L.; Rennie, J. R.; Linser, S.; Willumeit, R.; Tew, G. N. *J. Phys. Chem. B* **2006**, *110*, 3527–3532.
(13) Epand, R. F.; Schmitt, M. A.; Gellman, S. H.; Epand, R. M. *Biochim. Biophys. Acta* **2006**, *1758*, 1343–1350.

 10.1021/ja8062327 CCC: $40.75 © **2008 American Chemical Society**

determining factor.[14,15] PE is a lipid with intrinsic negative curvature that has a strong tendency to form inverted phases. There is evidence the antimicrobial activity of certain synthetic oligomers is a consequence of their ability to promote the formation of nonlamellar structures.[15]

Recently, it has been suggested that cationic antimicrobial agents can affect membranes by inducing the separation of membrane lipid components, causing the formation of phase boundary defects. Phase boundary defects have been suggested to be responsible for the increased leakage of liposomes at the phase transition temperature where gel and liquid crystalline domains coexist.[16] However, in biological membranes the complexity of the composition of the membrane would not sustain such defects over a period of time. Other molecules diffusing into the defect regions would stabilize these boundary regions. Addition of a polycationic compound would be expected to induce lateral phase separation of anionic and zwitterionic lipid components as a result of preferential binding to anionic lipids. This phase separation will result, at least transiently, in the formation of phase boundary defects.

In addition to domains induced by an antimicrobial agent, bacterial membranes naturally contain domains.[17] Curvature resulting from the morphology of the bacteria may influence the formation of these domains,[18] in addition to modulation of intrinsic curvature by certain antimicrobial compounds.[15] An alternative or additional mechanism to membrane damage and leakage caused by phase boundary defects resulting from the rearrangement of lipids in the membrane is the possibility that the clustering of anionic lipids by the cationic antimicrobial agents removes anionic lipids from the natural domains in the bacterial membranes, resulting in loss of their function. It has been suggested that membrane domains have important functions in bacteria [19,20] that would be altered by sequestering anionic lipids by some cationic antimicrobial agents.

The importance of induced lateral phase separation has been specifically proposed as a mechanism contributing to the antimicrobial activity of a designed α/β-peptide,[13] a flexible sequence-random polymer,[21] and cateslytin.[22] The type of rearrangement of membrane lipids leading to phase boundary defects is illustrated in Figure 1. Such a mechanism may be important for antimicrobial compounds that have a sequence rich in cationic residues and have conformational flexibility to adapt to the distance between charges on the membrane surface.

A particularly attractive example of antimicrobial peptides that have the potential of preferentially interacting with anionic lipids and promoting their phase segregation are the OAKs.[3,23−25]

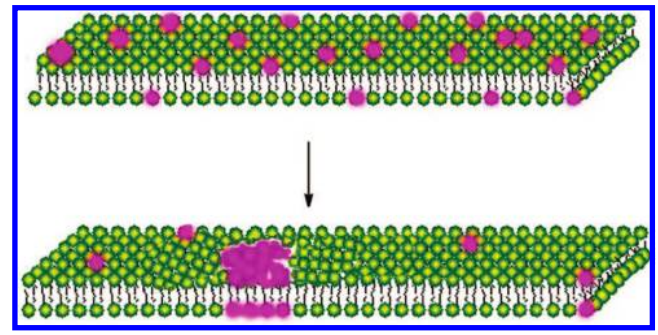


Figure 1. Schematic diagram of phase separation in a membrane bilayer. Lipids with two different kinds of headgroups, represented by red and green balls, can be induced to phase separate by addition of a substance that interacts preferentially with one of the two lipid components. This will create phase boundary defects between domains enriched in either of the two lipids. It is suggested that such a process can occur when cationic antimicrobial agents preferentially bind to an anionic lipid in the presence of a zwitterionic lipid.

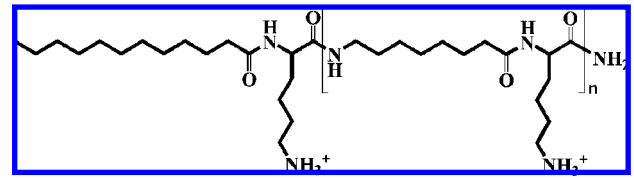


Figure 2. Chemical structure of a typical OAK. The brackets define α_8 (aminooctanoyllysyl) subunits where $n = 7$ or 5 in the OAKs used in this work, $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}5\alpha_8$, respectively.

These compounds are linear sequences of alternating acyl chains and cationic Lys residues (see Figure 2). OAKs are rich in positive charge, and because of the acyl groups, they are relatively hydrophobic and can partition well into membranes. These compounds do not form ordered secondary structures and are therefore capable of adapting their conformation to facilitate binding to multiple anionic groups on a membrane surface. We compare two OAKs, $C_{12}K\text{-}5\alpha_8$ and $C_{12}K\text{-}7\alpha_8$. There is evidence that $C_{12}K\text{-}7\alpha_8$ kills bacteria by a membrane effect while $C_{12}K\text{-}5\alpha_8$ acts through inhibition of DNA functions.[25] In the present study we demonstrate the ability of one of these OAKs, the octamer $C_{12}K\text{-}7\alpha_8$, to interact with anionic lipids and to induce lateral phase separation. Because of the importance of lateral phase separation for the antimicrobial action of $C_{12}K\text{-}7\alpha_8$, we are able to predict the bacterial species that will be sensitive to the action of this compound.

Results and Discussion

Isothermal Titration Calorimetry (ITC). We directly demonstrate, using ITC, that $C_{12}K\text{-}7\alpha_8$ binds to 1-palmitoyl-2-oleoylphosphatidylethanolamine−tetraoleoylcardiolipin (POPE−TOCL) (3:1) with an affinity of $3.2 \times 10^5\ M^{-1}$. This is similar to the value obtained by surface plasmon resonance for binding to POPC−POPG (3:1),[25] indicating that the interaction of this OAK with lipids is not highly specific and is not sensitive to the differences in lipid structure. The reaction with POPE−TOCL (3:1) is exothermic with $\Delta H = -1.7$ kcal/mol of $C_{12}K\text{-}7\alpha_8$, consistent with an electrostatic interaction (Figure 3). $C_{12}K\text{-}5\alpha_8$ has lower affinity for POPE−TOCL (3:1), and no significant

(14) Epand, R. F.; Savage, P. B.; Epand, R. M. *Biochim. Biophys. Acta* **2007**, *1768*, 2500–2509.
(15) Yang, L.; Gordon, V. D.; Mishra, A.; Som, A.; Purdy, K. R.; Davis, M. A.; Tew, G. N.; Wong, G. C. L. *J. Am. Chem. Soc.* **2007**, *129*, 12141–12147.
(16) Hays, L. M.; Crowe, J. H.; Wolkers, W.; Rudenko, S. *Cryobiology* **2001**, *42*, 88–102.
(17) Matsumoto, K.; Kusaka, J.; Nishibori, A.; Hara, H. *Mol. Microbiol.* **2006**, *61*, 1110–1117.
(18) Mukhopadhyay, K.; Kohli, A.; Prasad, R. *Antimicrob. Agents Chemother.* **2002**, *46*, 3695–3705.
(19) Norris, V.; Fishov, I. *Biochimie* **2001**, *83*, 91–97.
(20) Romantsov, T.; Helbig, S.; Culham, D. E.; Gill, C.; Stalker, L.; Wood, J. M. *Mol. Microbiol.* **2007**, *64*, 1455–1465.
(21) Epand, R. F.; Mowery, B. P.; Lee, S. E.; Stahl, S. S.; Lehrer, R. I.; Gellman, S. H.; Epand, R. M. *J. Mol. Biol.* **2008**, *379*, 38–50.
(22) Jean-Francois, F.; Castano, S.; Desbat, B.; Odaert, B.; Roux, M.; Metz-Boutigue, M. H.; Dufourc, E. J. *Biochemistry* **2008**, *47*, 6394–6402.
(23) Radzishevsky, I.; Krugliak, M.; Ginsburg, H.; Mor, A. *Antimicrob. Agents Chemother.* **2007**, *51*, 1753–1759.
(24) Radzishevsky, I. S.; Kovachi, T.; Porat, Y.; Ziserman, L.; Zaknoon, F.; Danino, D.; Mor, A. *Chem. Biol.* **2008**, *15*, 354–362.
(25) Rotem, S.; Radzishevsky, I. S.; Bourdetsky, D.; Navon-Venezia, S.; Carmeli, Y.; Mor, A. *FASEB J.* **2008**, *22*, 2652–2661.

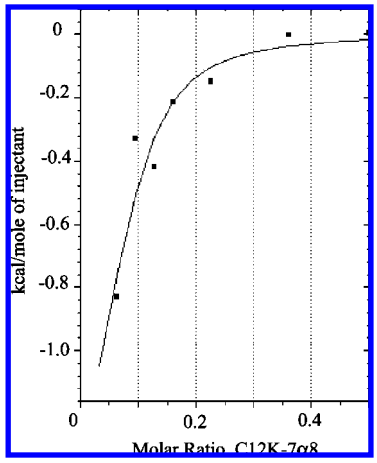


Figure 3. ITC of POPE−TOCL reacted with OAK. ITC with 100 μM small unilamellar vesicles (SUVs) of POPE−TOCL (3:1) placed in the calorimeter cell and titrated with 10 μL injections (4.5 μM each) of C12K-7α8 placed in the syringe at 30 °C. Titrations were carried out in 20 mM Pipes, pH 7.4 (0.14 M NaCl, 1 mM EDTA).

heat of reaction was observed when a comparable titration was done with this shorter (hexamer) homologue. Thus, the shorter homologue, C_{12}K-5α_8, with fewer positive charges, that does not kill bacteria by affecting their membranes also does not induce lateral phase separation in this lipid mixture.

Circular Dichroism (CD). The temperature dependence of the conformation of the OAKs was determined using the ellipticity at 222 nm. In buffer solution there is essentially little structure,[3] and no temperature dependence of the ellipticity at 222 nm for either OAK used in this work between 10 and 80 °C (not shown), indicating that in buffer the OAKs are largely devoid of structure. However, in the presence of SUVs of POPE−TOCL (3:1) and at a lipid to peptide ratio of 10, we found a difference in the folding of these two peptides (Figure 4). There is a qualitative difference in the shape of the spectra with either of the two OAKs in the presence of lipids. The spectrum with C_{12}K-7α_8 has a maximum at about 217 nm, while with C_{12}K-5α_8 the peaks are at higher wavelengths, suggesting a different folding (Figure 4, top). This large difference in conformation between the two homologues likely contributes to the difference in the mechanism of action of the two peptides. The concentration of peptide in these samples is 150 μM. It is known that OAKs self-associate at this concentration,[23] and this may contribute to an increase in structure. In the presence of lipid, C_{12}K-7α_8 denatures in the temperature range 60−70 °C (Figure 4, middle). The denaturation is irreversible, and no refolding occurs on cooling. The unfolding of C_{12}K-5α_8 is also irreversible, but in comparison to C_{12}K-7α_8, it begins at lower temperatures and is a broader denaturation curve, as is expected of a shorter homologue (Figure 4, bottom). In the temperature range used for differential scanning calorimetry (DSC) (see below) there is little change in conformation.

Differential Scanning Calorimetry. Phosphatidylethanolamine (PE) is the principle zwitterionic lipid in most bacteria, and cardiolipin (CL) and phosphatidylglycerol (PG) are the principle anionic lipids. Bacteria vary widely in the relative amounts of these three lipids in their membranes.[26] It has been shown that

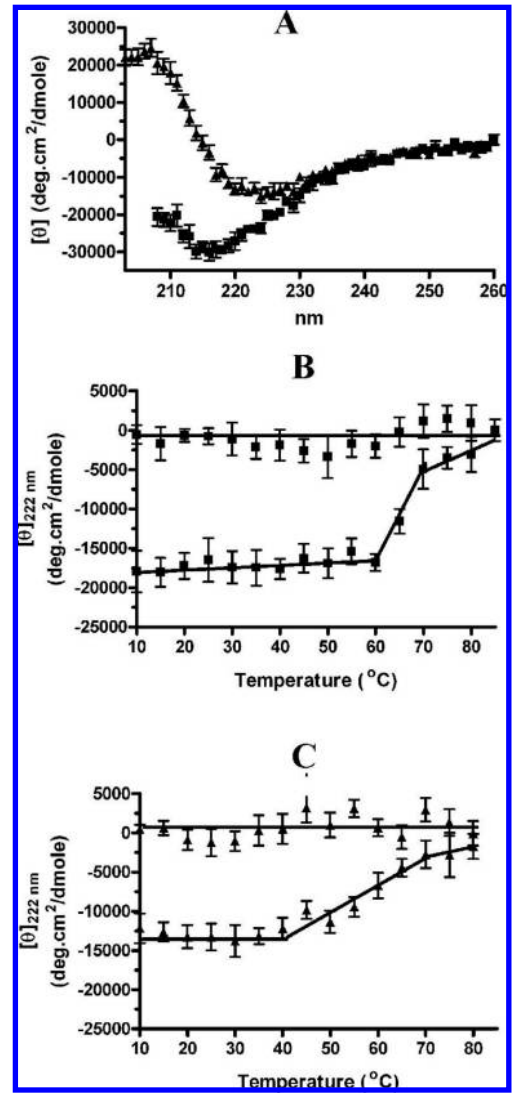


Figure 4. Circular dichroism of OAKs in SUVs of POPE−TOCL. The concentration of OAKs was 200 μg/mL with 1.5 mM POPE−TOCL (3:1) SUVs in 20 mM Pipes buffer, pH 7.4 (140 mM NaCl, 1 mM EDTA). The peptide:lipid ratio is 10. (A) Wavelength scan in mean residue ellipticity units: □, C12K-7α8; ▲, C12K-5α8. (B) Temperature scan at 222 nm of C_{12}K-7α_8 in SUVs. (C) Temperature scan of C_{12}K-5α_8 in SUVs.

CL can form segregated domains in bacterial membranes,[20,27−30] as can PG.[31] We use DSC to study phase separation between anionic and zwitterionic lipids induced by the OAKs.

The advantages of DSC for studying phase separation are that it does not require the use of bulky spectroscopic probes and it can detect the presence of domains that are too small to image with microscopy methods.[32] We have used DSC to obtain evidence for the promotion of lateral phase separation by starting with a mixture of two miscible lipids that give rise to a broad phase transition. If an added substance promotes lateral phase separation by preferentially binding to one of the components,

(26) Ratledge, C.; Wilkinson, S. G. *Microbial Lipids*; Academic Press: London, 1988; Vol. 1.
(27) Kawai, F.; Shoda, M.; Harashima, R.; Sadaie, Y.; Hara, H.; Matsumoto, K. *J. Bacteriol.* **2004**, *186*, 1475–1483.
(28) Koppelman, C. M.; Den, B. T.; Duursma, M. C.; Heeren, R. M.; Nanninga, N. *J. Bacteriol.* **2001**, *183*, 6144–6147.
(29) Mileykovskaya, E.; Dowhan, W. *J. Bacteriol.* **2000**, *182*, 1172–1175.
(30) Mileykovskaya, E. *Mol. Microbiol.* **2007**, *64*, 1419–1422.
(31) Vanounou, S.; Parola, A. H.; Fishov, I. *Mol. Microbiol.* **2003**, *49*, 1067–1079.
(32) Epand, R. M. *Biophys. Chem.* **2007**, *126*, 197–200.

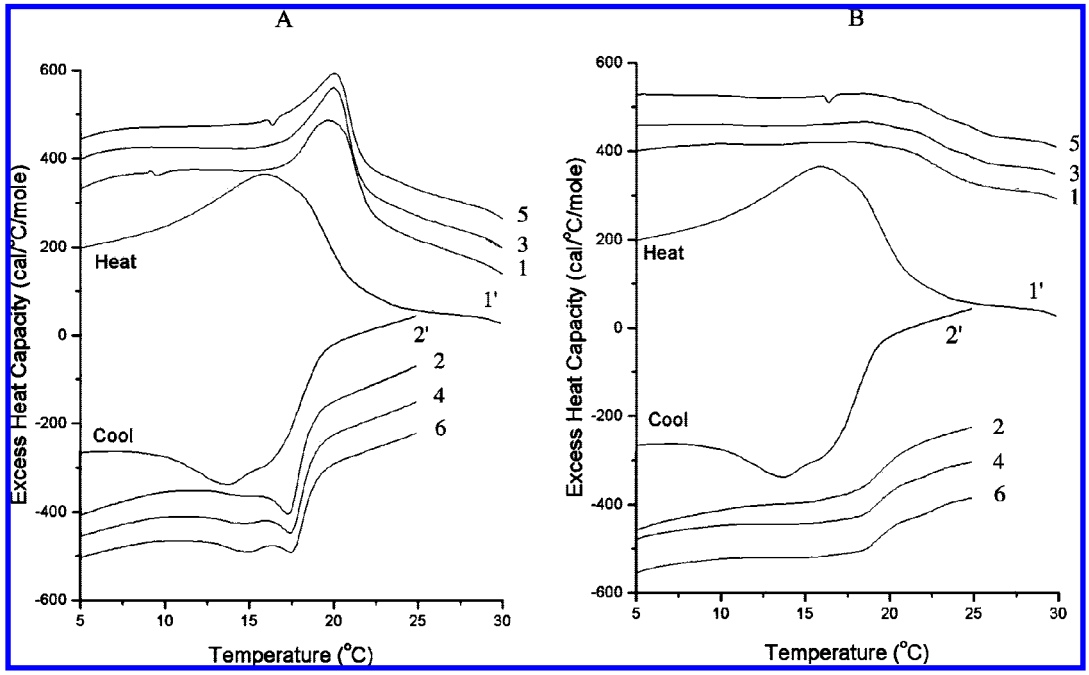


Figure 5. Phase separation of POPE−TOCL mixtures determined by DSC. DSC carried out with 2.5 mg/mL POPE−TOCL (3:1) at a scan rate of 1.0 deg/min in the absence and presence of $C_{12}K\text{-}7\alpha_8$ (A) or $C_{12}K\text{-}5\alpha_8$ (B) in 20 mM Pipes, pH 7.4 (0.14 M NaCl, 1 mM EDTA). The lipid:OAK molar ratio was 10. Odd numbers are heating scans, and even numbers are cooling scans. Labels 1′ and 2′ refer to heating and cooling scans, respectively, of the lipid in the absence of OAK.

it will segregate that component into a domain, leaving the rest of the membrane enriched in the second lipid component. As a result the second component will exhibit a more cooperative phase transition at a temperature closer to that of the pure lipid. We have previously used mixtures of POPE and TOCL to demonstrate protein-induced lateral phase separation.[33] The lipid mixture POPE−TOCL (75:25) has a broad gel to liquid crystalline phase transition centered at about 15 °C (Figure 5). The gel to liquid crystalline phase transition temperatures of the individual lipids that comprise this mixture are 25 °C for POPE and below 0 °C for TOCL. Addition of $C_{12}K\text{-}7\alpha_8$ produces a shift in the transition temperature of the mixture to higher temperatures and sharpens the transition. This behavior is consistent with the $C_{12}K\text{-}7\alpha_8$ binding to TOCL and clustering this anionic lipid, thus leaving the remaining lipid enriched in POPE and giving rise to a more cooperative transition at a temperature closer to that of pure POPE (Figure 5). By comparison, the less active analogue, $C_{12}K\text{-}5\alpha_8$, is weaker in promoting this phase separation (Figure 5). We have also compared the DSC curves of POPE−TOCL (3:1) containing 10 mol % $C_{12}K\text{-}7\alpha_8$ with those of POPE containing variable amounts of TOCL (see Figure 1 in the Supporting Information). The sample of POPE−TOCL (3:1) containing 10 mol % $C_{12}K\text{-}7\alpha_8$ has a phase transition similar to that of POPE containing about 10 mol % TOCL. We conclude that the OAK clusters some TOCL away from the mixture and that the only phase transition that is observed is the remaining lipid, i.e., the POPE-rich domain, with a decrease in TOCL from 25% to ∼12%. Although $C_{12}K\text{-}5\alpha_8$ also raises the transition temperature of this lipid mixture, it broadens the transition, suggesting that it binds to both lipids of this mixture.

A similar observation was made with POPE−dioleoylphosphatidylglycerol (DOPG) (3:1). This lipid composition mimics the cytoplasmic membrane composition of *Escherichia coli*, which has been reported to be PE−PG (80:15). DOPG was chosen because the oleoyl chains match those of TOCL. The mixture has a broad phase transition temperature centered at 15 °C (Figure 6). The phase transition temperature of DOPG is also below 0 °C. Again $C_{12}K\text{-}7\alpha_8$ shows a larger shift of one component toward higher temperatures than its counterpart $C_{12}K\text{-}5\alpha_8$ (Figure 6) presumably caused by stronger clustering of negatively charged lipid by $C_{12}K\text{-}7\alpha_8$.

Our results with both lipid mixtures indicate that $C_{12}K\text{-}7\alpha_8$ would segregate charge in a membrane containing both PE and an anionic lipid. In comparison, $C_{12}K\text{-}5\alpha_8$ does so only to a small extent. This is consistent with the observation that $C_{12}K\text{-}7\alpha_8$ reaches and permeabilizes the cytoplasmic membrane of the *E. coli* mutant ML-35p, but $C_{12}K\text{-}5\alpha_8$ does not, and also that $C_{12}K\text{-}7\alpha_8$ is more potent in dissipating a membrane potential.[25] In addition, the fact that similar behavior was observed when the anionic lipid was TOCL or when it was DOPG illustrates that $C_{12}K\text{-}7\alpha_8$ segregates anionic lipids from zwiterionic ones and that the structure of the anionic lipid is not of prime importance.

^{31}P MAS/NMR. We further investigated the induction of phase segregation by $C_{12}K\text{-}7\alpha_8$ using ^{31}P MAS/NMR. Since TOCL is a symmetrical molecule, the chemical shifts of each of the two phosphate groups are identical and are different from that of POPE. When these two lipids are mixed, their resonance lines approach, indicating at least partial miscibility of the two lipids (Figure 7). Similar measurements were done over a range of temperatures, both with and without addition of $C_{12}K\text{-}7\alpha_8$. The chemical shift values are summarized in Table 1. The only observed abrupt change of chemical shift with temperature is with pure POPE, which changes from about −0.14 ppm below 25 °C to −0.019 ppm at 30 °C. This is the temperature range in which this lipid converts from the gel state to the liquid crystalline state. The fact that such an abrupt change is not

(33) Epand, R. F.; Tokarska-Schlattner, M.; Schlattner, U.; Wallimann, T.; Epand, R. M. *J. Mol. Biol.* **2007**, *365*, 968–980.

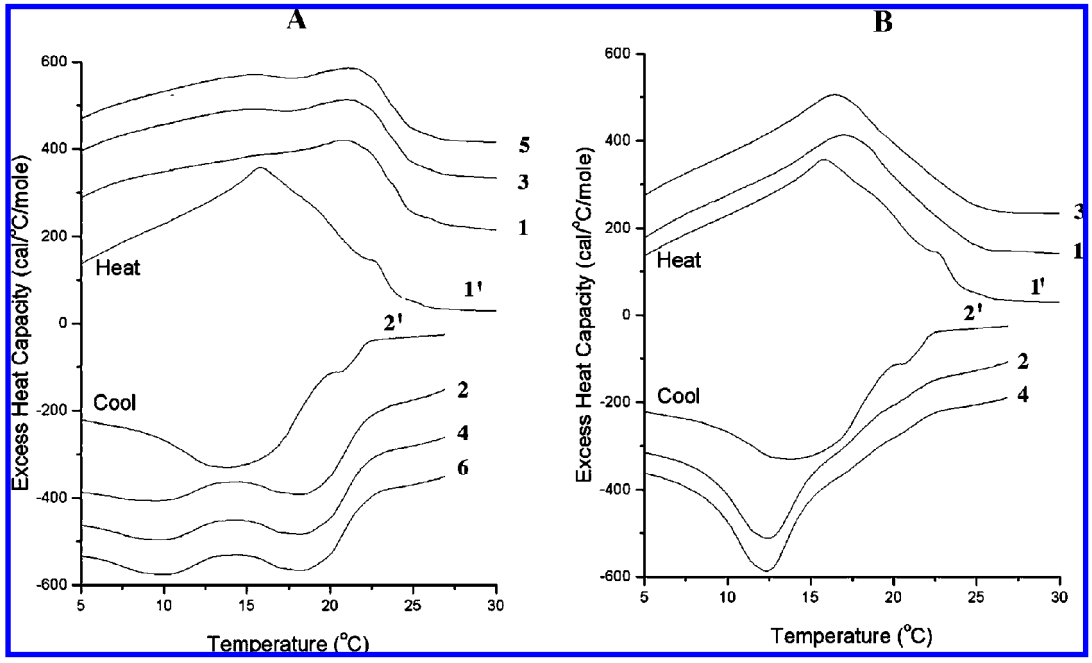


Figure 6. Phase separation of POPE−DOPG mixtures determined by DSC. DSC carried out with 2.5 mg/mL POPE−DOPG (3:1) at a scan rate of 1.0 deg/min in the absence and presence of C12K-7α_8 (A) or C12K-5α_8 (B) in 20 mM Pipes, pH 7.4 (0.14 M NaCl, 1 mM EDTA). The lipid:OAK molar ratio was 15. Odd numbers are heating scans, and even numbers are cooling scans. Labels 1′ and 2′ refer to heating and cooling scans, respectively, of the lipid in the absence of OAK.

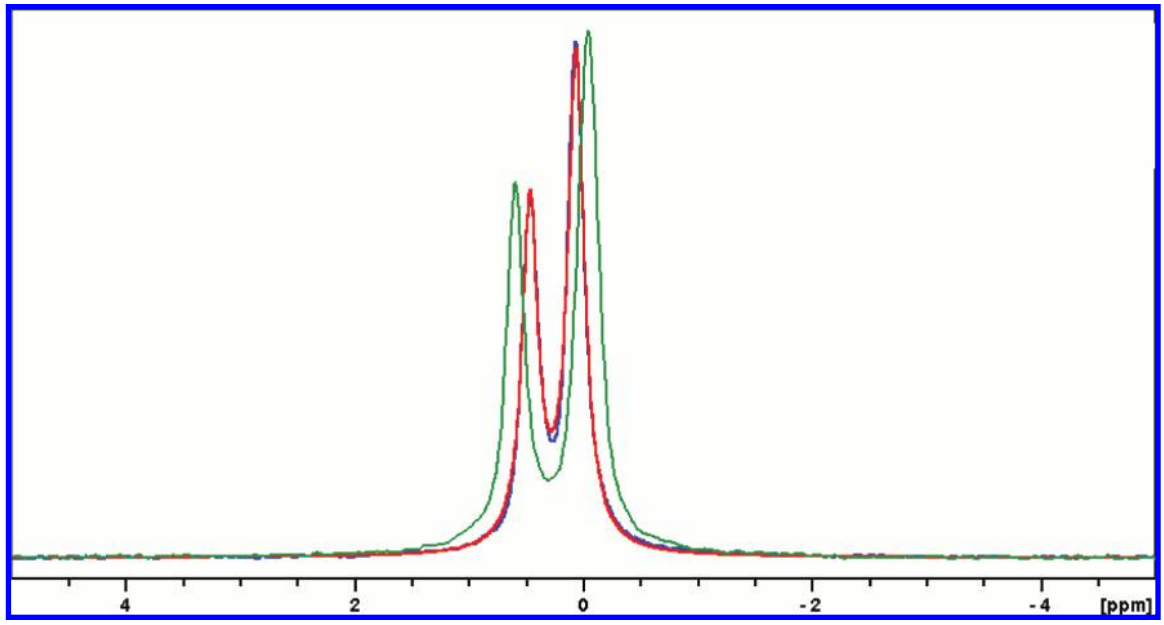


Figure 7. ^{31}P MAS/NMR spectra of the isotropic peaks of TOCL and POPE as a mixture and as separate lipids. Spectra measured at 30 °C of a mixture of POPE−TOCL (3:1) (blue spectrum). Calculated curve fit for this spectrum using the shape of the spectra and the spinning side bands (red spectrum). The excellence of the fit curve for the lipid mixture from the line shape analysis is apparent. Spectrum calculated as the arithmetical sum of the ^{31}P MAS/NMR spectra of POPE and of TOCL as pure lipids (green spectrum). Spectra measured in 20 mM Pipes buffer, pH 7.4, 140 mM NaCl, 1 mM EDTA, at a spinning frequency of 5 kHz.

observed with mixtures of POPE and TOCL indicates that any domain, either in the presence or in the absence of C_{12}K-7α_8, that is enriched in POPE also contains TOCL to broaden the transition, as is also observed by DSC (Figure 5). Addition of C_{12}K-7α_8 causes the POPE peak in the mixture to have a resonance position at lower frequency, closer to that of pure POPE. We suggest this is a consequence of the environment of POPE becoming more depleted of TOCL and therefore the peak moving closer to that of pure POPE. In contrast, the TOCL peak moves further from the chemical shift of pure TOCL upon addition of C_{12}K-7α_8. This occurs because C_{12}K-7α_8 neutralizes some of the negative charge on TOCL by binding to the headgroup, thus moving the chemical shift toward that for a zwitterionic lipid. This is consistent with changes in the line width as will be described below.

The line width for gel-phase POPE below 25 °C is much larger than the values at high temperatures (Table 2). The line widths of TOCL also increase with decreasing temperature, but

Table 1. Isotropic Chemical Shifts (ppm)

	TOCL peak			POPE Peak		
temp (°C)	TOCL	POPE−TOCL	POPE−TOCL + $C_{12}K\text{-}7\alpha_8$	POPE	POPE−TOCL	POPE−TOCL + $C_{12}K\text{-}7\alpha_8$
−10	0.666					
−5		0.590	0.496		0.081	0.018
0	0.640	0.553	0.491	−0.143	0.057[a]	0.061
5		0.521	0.478		0.085	0.051
10	0.636	0.486	0.463	−0.143	0.068	0.057
15		0.482	0.447		0.072	0.055
20	0.618	0.479	0.442	−0.136	0.073	0.050
25		0.466	0.443	−0.033	0.063	0.053
30	0.598	0.470	0.431	−0.019	0.065	0.041

[a] This value may be less reliable because of asymmetrical broadening of the peaks. The peak position of this resonance was 0.11 ppm.

Table 2. ^{31}P MAS/NMR Line Widths (Hz)

	TOCL peak			POPE peak		
temp (°C)	TOCL	POPE−TOCL	POPE−TOCL + $C_{12}K\text{-}7\alpha_8$	POPE	POPE−TOCL	POPE−TOCL + $C_{12}K\text{-}7\alpha_8$
−10	70.93					
−5		69.48	118.72		125.50	78.61
0	54.90	59.97	92.27	124.29	80.67	61.95
5		49.80	73.13		50.46	50.41
10	47.21	41.75	54.72	99.72	36.36	50.64
15		35.41	56.09		34.22	39.02
20	46.57	35.87	46.97	138.05	33.56	38.48
25		34.64	53.48	42.24	33.89	40.66
30	41.36	33.73	52.16	42.08	35.17	43.71

more gradually than for POPE, and the linewidths at low temperatures are narrower for TOCL. The line widths of POPE and TOCL are the same when they are in the liquid crystalline state at 30 °C. Between 15 and 35 °C the line widths of both TOCL and POPE in the lipid mixture remain at about 34 Hz and are narrower than those of either of the pure lipid components alone. Addition of $C_{12}K\text{-}7\alpha_8$ in this temperature range causes broadening of both lipid resonances of the mixture. The POPE line width in the mixture with $C_{12}K\text{-}7\alpha_8$ at these temperatures is the same as that of the pure POPE, suggesting an enrichment of a domain with POPE in the presence of $C_{12}K\text{-}7\alpha_8$. The TOCL line width in the mixture with $C_{12}K\text{-}7\alpha_8$ at these temperatures is increased even more than observed with POPE. This increase is evidence of $C_{12}K\text{-}7\alpha_8$ selectively slowing the motion of the TOCL component. This kind of selective broadening has been previously used to demonstrate preferential binding of peptides to particular lipids in a mixture.[34] Thus, changes in both the isotropic chemical shift and the line widths of the peaks are consistent with $C_{12}K\text{-}7\alpha_8$ binding preferentially to TOCL and inducing the formation of clusters of this lipid.

The chemical shift anisotropy of the lipid mixture at different temperatures is not greatly altered by the presence of $C_{12}K\text{-}7\alpha_8$ (see Table 1 in the Supporting Information). This indicates that the binding of $C_{12}K\text{-}7\alpha_8$ does not destroy the bilayer morphology of the membrane.

Antimicrobial Activity. The exposure of anionic groups at the surface of bacteria is well recognized as a factor contributing to the selective toxicity of cationic antimicrobial agents. However, not all bacteria with exposed anionic lipids are equally susceptible to antimicrobial agents. Furthermore, in the case of OAKs, Gram-negative bacteria that have less anionic lipid in their membranes are generally more sensitive to the action of

(34) Clayton, J. C.; Hughes, E.; Middleton, D. A. *Biochemistry* **2005**, *44*, 17016–17026.

Table 3. Lipid Composition and MICs of $C_{12}K\text{-}7\alpha_8$ against Various Bacteria

	total lipid concn (%)				
bacterial species	CL	PG	PE	MIC (μM)	ref
Gram-Negative Bacteria					
E. coli	5	15	80	1.6−3.1	25
Enterobacter cloacae	3	21	74	1.6	25
Yersinia kristensenii	20	20	60	1.6	25
Proteus mirabilis	5	10	80	6.2	25
Klebsiella pneumoniae	6	5	82	3.1	25
Pseudomonas aeruginosa	11	21	60	6.2	25
Gram-Positive Bacteria					
Staphylococcus aureus	42	58	0	50	25
Streptococcus pneumonia	50	50	0	50	current study
Bacillus cereus	17	40	43	12	3, 25
Bacillus polymyxa	8	3	60	6.2	current study

OAKs (lower minimal inhibitory concentration, MIC). We can predict the relative sensitivity of different bacterial species to the action of $C_{12}K\text{-}7\alpha_8$ on the basis of the ability of this agent to induce lateral phase separation in the membrane. Because there are large differences in membrane lipid composition among different bacteria, the ability of $C_{12}K\text{-}7\alpha_8$ to induce lateral phase segregation occurs more readily with some bacteria than with others. Bacteria that contain both anionic and zwitterionic lipids should be more susceptible to OAKs, relative to bacteria that contain mainly anionic lipids. Separation of anionic lipids from zwitterionic lipids by OAKs will induce the formation of phase boundary defects through which leakage can occur. Gram-negative bacteria generally have a high content of the zwitterionic lipid PE as well as contain anionic lipids.[26] They should therefore in general be sensitive to OAKs. $C_{12}K\text{-}7\alpha_8$ can access and permeate the cytoplasmic membrane of the Gram-negative bacteria *E. coli*[25] and also depolarize the bacterial membrane.[3] It is found that nine different strains of Gram-negative bacteria have MICs between 1.6 and 6.2 μM with another one having a MIC of 12.5, only slightly higher[23] (also see Supplementary Table 2 of ref 3). *Serratia odorifera* is an exception with a MIC > 50 μM, but the lipid composition of *S. odorifera* membranes has not been reported. We present examples of the lipid composition of several common Gram-negative bacteria and their corresponding MICs for $C_{12}K\text{-}7\alpha_8$ (Table 3). In all of the examples the PE content is high, in the range 60−80%, and the MIC is low, in the range 1.6−6.2 μM.

We have further tested this relationship by determining the MICs for two Gram-positive bacteria, *Strep. pneumonia* and *B. polymyxa*, that had not been previously studied with OAKs. We predicted that, since *Staphylococcus pneumonia* has a low content of zwitterionic phospholipids, like *Staph. aureus*, boundary defects between zwitterionic lipid-rich membrane domains and anionic lipid-rich domains would not form in the presence of $C_{12}K\text{-}7\alpha_8$. A predicted consequence of this is that these bacteria will exhibit a relatively higher MIC, as we have found (Table 3). Interestingly, the MICs for these bacteria are higher than those for most Gram-negative bacteria that are more commonly resistant to antimicrobial agents because of the presence of an outer membrane. Gram-positive bacteria generally have a low content of zwitterionic lipid. *B. polymyxa* represents an exception to the general rule that Gram-positive bacteria have a low content of PE compared with Gram-negative bacteria. Indeed, *B. polymyxa*, like *B. cereus*, has a high PE content. We predicted that these species would be more sensitive to $C_{12}K\text{-}7\alpha_8$ since they have both anionic and

zwitterionic lipids that can segregate to form phase boundaries. We have found that these two bacterial species have a low MIC as predicted (Table 3).

In addition, there is a group of three bacterial species that have substantial amounts of the cationic lipid lysyl-PG or the zwitterionic lysyl-CL (not shown). The role of lysyl-PG in the potency of OAKs, based on our suggestion of lateral phase separation, would be expected to be biphasic. At lower concentrations it would facilitate phase separation by $C_{12}K$-$7\alpha_8$ even more than zwitterionic lipids, since there would be a greater tendency of the cationic OAKs to cluster anionic lipids and be repelled by lysyl-PG-rich positively charged domains. However, high concentrations of lysyl-PG will lower the negative charge on the bacterial surface and inhibit the sequestration of OAKs to the bacterial membrane. Thus, *Enterococcus faecalis*, which has a higher content of lysyl-PG (a large fraction of the lipids are still undetermined), has a high value for the MIC, possibly because the OAKs are not strongly sequestered to the membrane surface. In comparison, *Listeria seeligeri* and *Streptococcus agalactiae* have lower contents of lysyl-PG and also have a low MIC of 6.2 μM.[25] Two predominant lipids in this organism are CL and lysyl-CL.[35] Since lysyl-CL has equal numbers of positive and negative charges, *L. seeligeri* is another example of Gram-positive bacteria that can form domains with anionic and uncharged lipids. Hence, the MIC of *L. seeligeri* is much lower than that for bacteria whose membranes are composed largely of anionic lipids.

Induction of lateral phase separation causing defects in membranes is just one of many mechanisms of action of antimicrobial agents. It is likely to be more important for some agents than for others and for certain species of bacteria more than others. The fact that the lipid composition of the bacteria will provide a predictable feature contributing to the sensitivity of the bacteria to certain antimicrobial agents can be used to design new drugs with a more limited range of bacterial toxicities. The mechanism of inducing lateral phase separation will in general be more important for Gram-negative bacteria than for Gram-positive species since most Gram-negative bacteria have significant amounts of both anionic and zwitterionic lipids. This may be an important feature in designing antimicrobial therapies, given that Gram-negative bacteria are generally more resistant to antimicrobial agents.

In summary, we demonstrate that $C_{12}K$-$7\alpha_8$, an OAK with potent antimicrobial activity, promotes the segregation of anionic and zwitterionic lipids. We used this finding to predict which species of bacteria will be more susceptible to this agent. These species will contain both anionic and zwitterionic lipids in their cytoplasmic membranes so that the promotion of clustering of anionic lipids by $C_{12}K$-$7\alpha_8$ will produce phase boundary defects that could transiently breach the permeability barrier of the cell membrane. This phenomenon provides a rationale to predict which bacteria will be most susceptible to a particular antimicrobial agent acting by this novel mechanism and will aid in the design of new agents.

Acknowledgment. This work was funded by Grant MOP 86608 from the Canadian Institutes of Health Research.

Supporting Information Available: "Materials and Methods" section, a figure containing the DSC curves for mixtures of POPE and TOCL in different proportions, and a table of the values of the chemical shift anisotropy in the presence and absence of $C_{12}K$-$7\alpha_8$. This material is available free of charge via the Internet at http://pubs.acs.org.

JA8062327

(35) Fischer, W.; Leopold, K. *Int. J. Syst. Bacteriol.* **1999**, *49*, 653–662.