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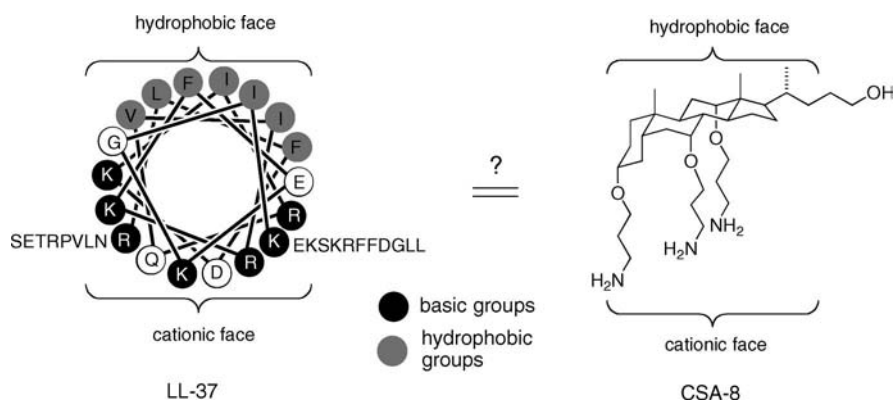
Ceragenins: Cholic Acid-Based Mimics of Antimicrobial Peptides

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RECEIVED ON DECEMBER 4, 2007

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The prevalence of drug-resistant bacteria drives the quest for new antimicrobials, including those that are not expected to readily engender resistance. One option is to mimic Nature's most ubiquitous means of controlling bacterial growth, antimicrobial peptides, which have evolved over eons. In general, bacteria remain susceptible to these peptides. Human antimicrobial peptides play a central role in innate immunity, and deficiencies in these peptides have been tied to increased rates of infection. However, clinical use of antimicrobial peptides is hampered by issues of cost and stability. The development of nonpeptide mimics of antimicrobial peptides may provide the best of both worlds: a means of using the same mechanism chosen by Nature to control bacterial growth without the problems associated with peptide therapeutics. The ceragenins were developed to mimic the cationic, facially amphiphilic structures of most antimicrobial peptides. These compounds reproduce the required morphology using a bile-acid scaffolding and appended amine groups. The resulting compounds are actively bactericidal against both Gram-positive and Gram-negative organisms, including drug-resistant bacteria. This antimicrobial activity originates from selective association of the ceragenins with negatively charged bacterial membrane components. Association has been studied with synthetic models of bacterial membrane components, with bacterial lipopolysaccharide, with vesicles derived from bacterial phospholipids, and with whole cells. Comparisons of the antimicrobial activities of ceragenins and representative antimicrobial peptides suggest that these classes of compounds share a mechanism of action. Rapid membrane depolarization is caused by both classes as well as blebbing of bacterial membranes. Bacteria express the

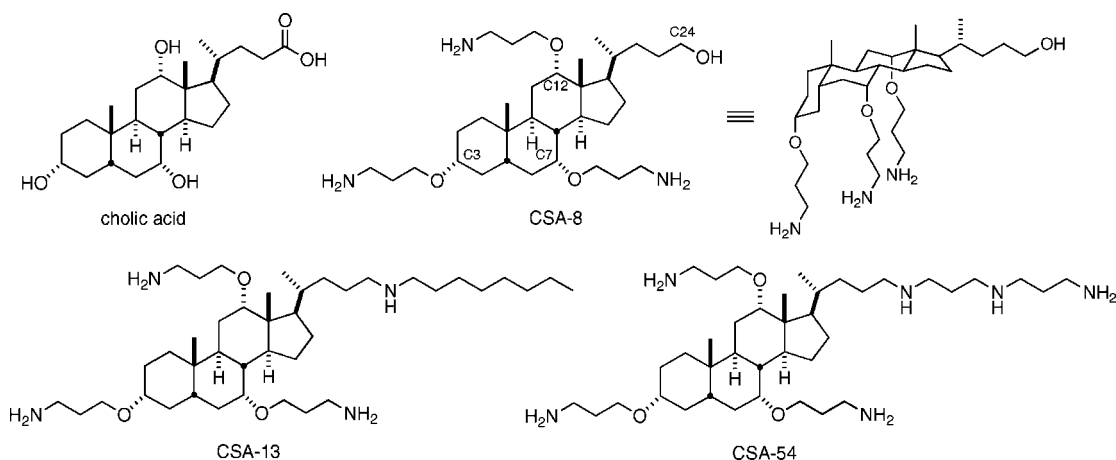


FIGURE 2. Structures of cholic acid and ceragenins CSA-8, -13, and -54, along with a perspective drawing of CSA-8 showing its facial amphiphilicity.

yield compounds effective in permeabilizing eukaryotic membranes and facilitating transfection.¹³ Davis and co-workers have made use of cholates in the development of receptors for anions that are capable of transport across lipid bilayers.^{14,15} The amphiphilic nature of the cholates is necessary for interactions with the anion and solubility in the interior of the membrane. The structural rigidity and amphiphilicity of cholates has been exploited by Kobuke and co-workers in the preparation of artificial ion channels.^{16,17} Regen and co-workers have designed cholate-based compounds, termed molecular umbrellas, capable of carrying a variety of molecules across lipid bilayers, including oligonucleotides,¹⁸ by changing their conformations based on their environment. Regen has found that these compounds have antiviral activity against lipid-enveloped viruses including HIV and herpes simplex virus.¹⁹ Furthermore, Regen and co-workers have demonstrated that oligocholates appended on an amide backbone can form pores in lipid bilayers.²⁰

We have fine-tuned the amphiphilic nature of cholic acid to better mimic the cationic charge characteristics of antimicrobial peptides and thereby generated series of nonpeptide mimics of antimicrobial peptides.^{21–23} Initially, we referred to these compounds as “cationic steroid antibiotics” (or CSAs; for examples, see Figure 2) but to avoid the suggestion that these compounds might possess steroid-like properties, we have termed the compounds “ceragenins.”

The ceragenins were designed to mimic the facially amphiphilic morphology of antimicrobial peptides, and a drawing of a representative ceragenin demonstrating its facial amphiphilicity is given in Figure 2. Our hypothesis was that reproduction of the morphology of antimicrobial peptides using a cholic acid scaffolding would yield antimicrobial compounds sharing a mechanism of action. In addition, we pro-

posed that emulsion of the spacing between amines in polymyxin B²⁴ would yield compounds with high affinity for the lipid A portion of LPS. Polymyxin B is the paradigm for small-molecule binding of lipid A, and we expected that lipid A binding would be essential for activity against Gram-negative bacteria. Modeling of polymyxin B and a cholic acid scaffolding with appended amines indicated that interamine distances were nearly identical.²⁴

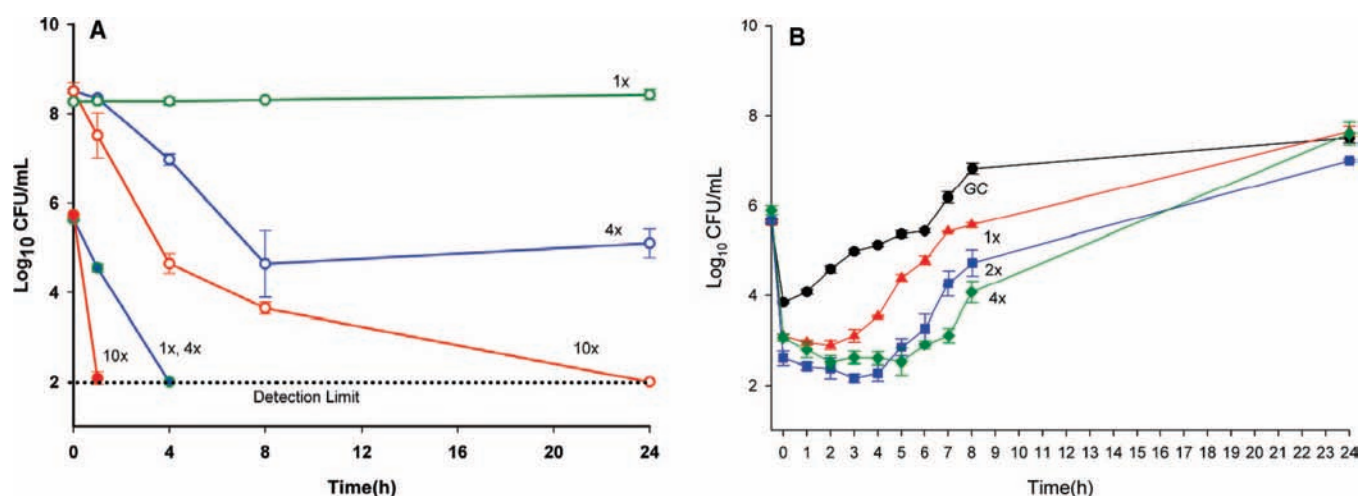
Studies with ceragenins have focused on four areas: (1) direct antibacterial activities (Gram-negative and -positive bacteria), including work with drug-resistant organisms; (2) abilities of ceragenins to sensitize Gram-negative bacteria to hydrophobic antibiotics; (3) correlation of the antimicrobial activities of ceragenins with antimicrobial peptides; and (4) methods of attaching ceragenins to polymers for prevention of bacterial colonization of surfaces.

Antibacterial Activities of Ceragenins

The most direct means of testing the hypothesis that antimicrobial peptide activity can be mimicked with a cholic acid scaffolding was to assay the antibacterial activities of ceragenins. This activity was measured with both Gram-negative and Gram-positive bacteria. Gram-negative bacteria contain two lipid bilayers: an outer membrane and a cytoplasmic membrane. As a consequence, they do not absorb “Gram’s stain” (crystal violet). Gram-positive bacteria have only one lipid bilayer but produce a larger layer of peptidoglycan. Antibacterial activities are typically compared using minimum inhibition concentrations (MICs). These are the minimum concentrations at which a compound inhibits bacterial growth in nutrient media. Minimum bactericidal concentrations (MBCs) are the concentrations at which a compound completely eradicates an inoculum of bacteria. Many antimicrobial agents are

TABLE 1. MIC Values of CSA-13 and Other Antibiotics against Clinical Isolates of *S. aureus*

isolate no.	MIC values ($\mu\text{g/mL}$)							CSA-13
	cipro-floxacin	erythro-mycin	gentamicin	oxacillin	rifampin	tetracycline	vancomycin	
SA509 ²⁶	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	>32	0.25
SA510 ²⁶	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	>32	0.125
SA512 ²⁶	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	>32	0.125
CN225 ²⁶	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	8	0.06
CN226 ²⁶	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	8	0.125
JMI967 ²⁷	>8	>8	16	>8	0.25	>8	1	0.5
JMI1182 ²⁷	>8	>8	>16	>8	0.25	>8	1	0.5
JMI1552 ²⁷	>8	>8	16	>8	>2	>8	1	0.5
JMI1718 ²⁷	>8	>8	>16	>8	0.25	>8	0.5	0.5
JMI2131 ²⁷	0.25	>8	>16	>8	2	>8	1	0.5

^a Not determined.**FIGURE 3.** (A) Time–kill curves of CSA-13 activity (1, 4, and 10 times the MIC) against a clinical isolate of vancomycin-resistant *S. aureus* (Michigan 2002). Closed circles are with an initial inoculum of 10^6 colony forming units (CFU)/mL, and open circles are with an initial inoculum of 10^8 to 10^9 CFU/mL. (B) Concentration-dependent PAE of CSA-13 with vancomycin-resistant *S. aureus* (Michigan 2002) measured at 1, 2, and 4 times the MIC. GC = growth control of the organism.

bacteriostatic, that is, they inhibit bacterial growth, but only actively kill bacteria at concentrations much higher than the MIC. Antimicrobial peptides are generally bactericidal with MBC values only slightly higher than MIC values.

Against Gram-positive bacteria, nearly the entire series of ceragenins tested are very active with MIC values at or below microgram per milliliter concentrations. Table 1 lists MIC values of one of the more active ceragenins, CSA-13, against clinical isolates of multidrug-resistant *Staphylococcus aureus*, including highly vancomycin resistant strains. The MBC values of CSA-13 are similar to the MIC values against both Gram-negative and Gram-positive bacteria including *S. aureus*.²⁵

Chin, et al.²⁸ showed the MIC and MBC values of CSA-13 against vancomycin-resistant *S. aureus* to be $1 \mu\text{g/mL}$, a result that is consistent with active bactericidal activity. Concentration-dependent bactericidal activity was demonstrated with varying concentrations of 1, 4, and 10 times the MIC against different inoculum densities over a 24 h period as shown in

Figure 3A. In addition, CSA-13 displays a postantibiotic effect (PAE). PAE refers to the suppression of bacterial growth that persists after short exposure of organisms to antimicrobials. It is the antimicrobial effect due to prior exposure rather than to persisting sub-MIC concentrations of the compound. That is, PAE measures the antibacterial activity (in hours or minutes) of a compound after it has been removed from the environment indicating affinity of an antimicrobial for its target. In experiments with resistant *S. aureus*, CSA-13 displayed significant PAE, suggesting that CSA-13's affinity for the negatively charged bacterial membrane increased as the concentration increased. The PAE nearly doubled from 2 to 4 times the MIC (Figure 3B).

Controlling the growth of Gram-negative bacteria can pose a challenge due to the permeability barrier of the outer membrane. Many hydrophobic antibiotics are inactive or only weakly active against these organisms because they do not effectively traverse the outer membrane. For example, erythromycin gives MIC values of less than $1 \mu\text{g/mL}$ with most

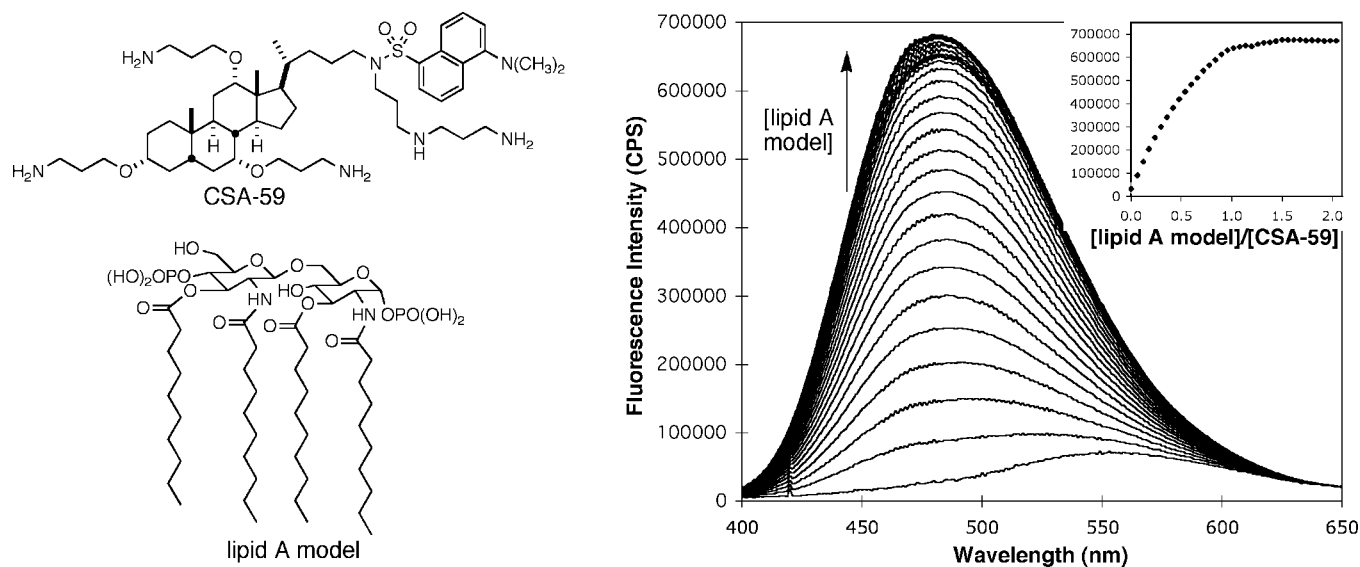


FIGURE 4. Titration of CSA-59 with the lipid A model shown. Inset shows fluorescence increase as a function of lipid A/CSA-59 ratio.

Gram-positive bacteria, but against Gram-negative organisms, MIC values are often above $50 \mu\text{g}/\text{mL}$. Association of small molecules with lipid A, which comprises a majority of the outer membrane, causes an increase in permeabilization of the outer membrane. In early investigations with ceragenins, it was discovered that the nature of the group extending from C24 (see Figure 2 for steroid numbering) dramatically influenced the bactericidal activity of the ceragenins against Gram-negative bacteria, such as *Escherichia coli*.²⁹ A lipid chain extended from C24 (as in CSA-13 in Figure 2) gives a compound that effectively kills Gram-negative bacteria at relatively low concentrations. However, if this chain is absent (as in CSA-8), the ceragenins lose activity against *E. coli* and other Gram-negative bacteria.²⁴ We have proposed that the lipid chain facilitates traversal of the ceragenin through the outer membranes of Gram-negative bacteria. Nevertheless, ceragenin CSA-8 retains the ability to associate with the outer membranes and sensitize Gram-negative bacteria to hydrophobic antibiotics.^{25,30} For example, the MIC of erythromycin with a drug-resistant strain of *Klebsiella pneumoniae* is ca. $70 \mu\text{g}/\text{mL}$, and the MIC of CSA-8 is $>30 \mu\text{g}/\text{mL}$ with the same strain. However, in combination it requires erythromycin and CSA-8 at concentrations of 1 and $0.7 \mu\text{g}/\text{mL}$, respectively, to effectively inhibit bacterial growth.³⁰

Association of Ceragenins with Bacterial Membrane Components

A key aspect to the proposed mechanism of action of the ceragenins is association with bacterial membranes. The target of ceragenins with Gram-negative bacteria is the lipid A portion of LPS. Studies of the association of a fluorophore-labeled cer-

agenin, CSA-59, with the disaccharide headgroup of lipid A (Figure 4) indicate an association constant of $1.7 \times 10^6 \text{ M}^{-1}$ and a one to one binding stoichiometry.³¹ Displacement studies with polymyxin B indicated that CSA-59 had comparable affinity for the lipid A model compound. Pioneering work by de Kruijff and co-workers has shown that the antibacterial activity of the antimicrobial peptide nisin is dependent on its affinity for lipid II, a precursor of bacterial peptidoglycan.³² Similarly, selective affinity of ceragenins for lipid A likely improves its activity against Gram-negative bacteria.

The affinity of ceragenins for LPS was also demonstrated in studies using measurement of the translocation of the transcription factor NF- κ B measured by Bucki et al.³³ Cells that participate in innate immunity respond to LPS, via TLR4, by release of a series of proinflammatory cytokines. This response can result in sepsis or septic shock. A key step in this response is the translocation of NF- κ B to the cell nucleus, and effective sequestration of LPS limits NF- κ B translocation. Addition of LPS to endothelial cells results in NF- κ B translocation (Figure 5). However, preincubation of LPS with either LL-37 or CSA-13 resulted in a loss of the response, suggesting that LL-37 and CSA-13 are comparably able to sequester LPS.

To verify that affinity for LPS translated into cell selectivity, a fluorophore-labeled ceragenin similar to CSA-59 was titrated into suspensions containing either Gram-negative bacteria (*E. coli*), Gram-positive bacteria (*S. aureus*), eukaryotic (Chinese hamster ovary) cells, or mixtures of these cell types.³¹ The fluorophore appended to the ceragenin responded via increases in its fluorescence intensity and emission wavelength upon moving from an aqueous to a hydrophobic environment. The labeled ceragenin displayed a high degree of

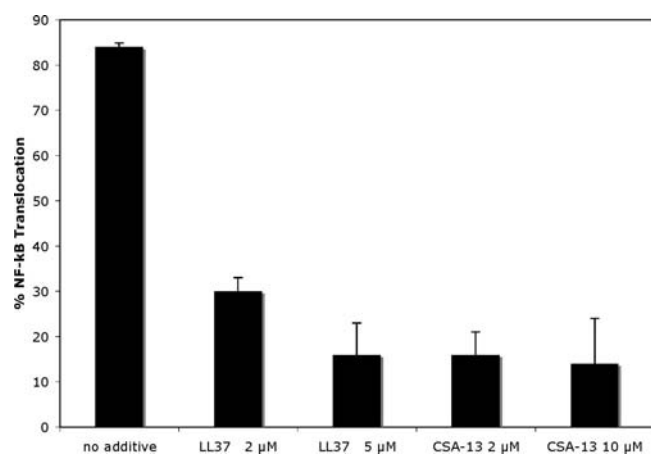


FIGURE 5. Quantification of NF-κB translocation in human aorta endothelial cells stimulated with LPS (0.1 μg/mL) with and without LL-37 or CSA-13. Error bars represent standard deviations from three experiments.

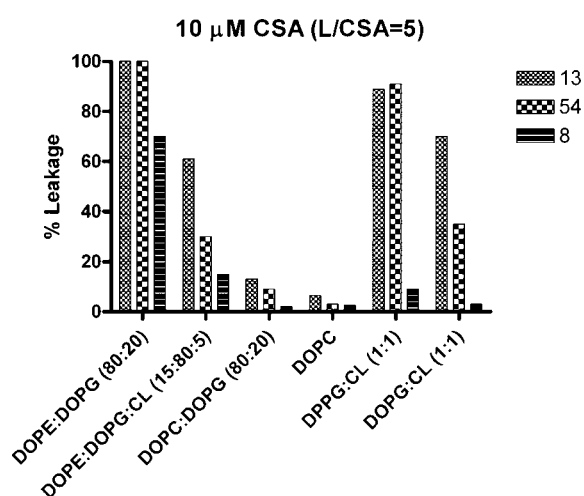


FIGURE 6. Leakage of vesicles derived from the indicated phospholipids with ceragenins CSA-8, CSA-13, and CSA-54. DOPE = dioleoylphosphatidylethanolamine; DOPG = dioleoylphosphatidylglycerol; CL = cardiolipin; DOPC = dioleoylphosphatidylcholine; DPPG = dipalmitoylphosphatidylglycerol.

selectivity ($>10^5$) for Gram-negative bacteria over the other cell types and was approximately 10^3 more selective for the Gram-positive bacteria over the eukaryotic cells.

Since Gram-positive bacteria do not produce LPS, the question of the target of ceragenins in these molecules was raised. Epand et al. studied the interactions of ceragenins with vesicles derived from a variety of phospholipids found in bacterial membranes (Figure 6).³⁴ In this study, it was found that all three ceragenins tested caused leakage with negatively charged vesicles containing phosphatidylethanolamine (PE) but that replacement of PE with phosphatidylcholine resulted in a substantial loss of interaction and leakage. Notably, all three ceragenins studied engaged in this behavior even though they contain different number of amines. In highly

charged vesicles containing cardiolipin and phosphatidylglycerol, the role of charge became more apparent with CSA-8, which contains fewer amine groups than CSA-13 and CSA-54, becoming less active. These studies, along with DSC and ³¹P NMR studies led to the conclusion that the ceragenins exhibit low-potency activity against bacteria containing high amounts of PE and higher bactericidal activity against bacteria containing high amounts of anionic lipids.

Correlation of the Antibacterial Activities of Ceragenins and Antimicrobial Peptides

Considering the similar facially amphiphilic morphology and charge characteristics of the ceragenins and antimicrobial peptides such as LL-37 and the magainins, it is expected that these antimicrobials would display similar mechanisms of action. To correlate these mechanisms, we investigated the kinetics of bacterial membrane depolarization, changes in bacterial membrane morphology in response to nonlethal doses of the antimicrobials, and the genes that bacteria express in response to the compounds. As described above, we also compared the ability of CSA-13 and LL-37 to sequester LPS and found them to be very similar.

Membrane depolarization is a key step in the antibacterial activity of antimicrobial peptides and ceragenins. This may come from leakage, similar to that seen in the vesicle studies. To study membrane depolarization, we used a fluorescent cyanine dye that incorporates into polarized membranes and displays a large increase in fluorescence intensity upon membrane depolarization. For these studies, we used the Gram-positive bacterium *Micrococcus luteus* because it was susceptible to the amphibian antimicrobial peptide magainin I. Treatment of *M. luteus* with magainin, CSA-8, or CSA-13 resulted in rapid membrane depolarization (Figure 7).³⁵ Notably, the ceragenins caused depolarization at concentrations far below those of magainin I required for comparable depolarization, with CSA-13 more than an order of magnitude more active.

With Gram-negative bacteria, interactions with antimicrobial peptides lead to membrane blebbing, and this has been observed via electron microscopy and atomic force microscopy.^{10,11,33} Electron microscopic and AFM images show similar changes in the morphology of the membranes of Gram-negative bacteria exposed to ceragenins.^{33,35} A transmission electron microscopy image of *E. coli* treated with a sublethal dose of CSA-13 is shown in Figure 8 with substantial membrane blebbing.

Antimicrobial peptides have been characterized by the bacterial promoters they activate, and these promoters have been

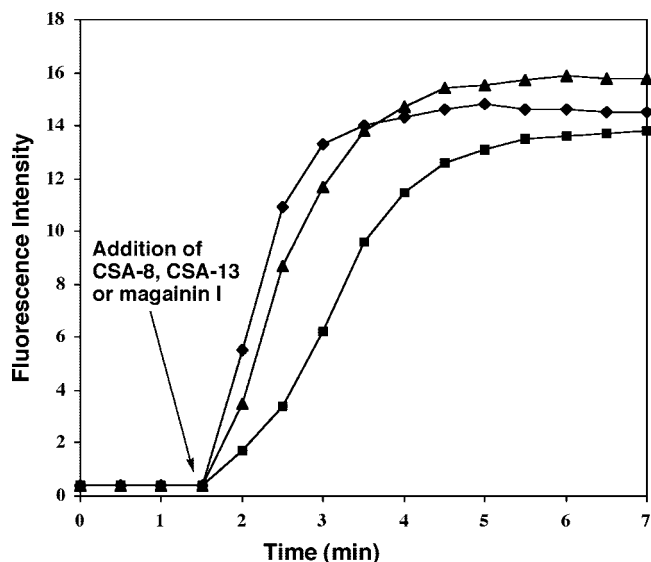


FIGURE 7. Depolarization of the membrane of *M. luteus* measured by an increase in fluorescence of 3,3'-diethylthiadicarbocyanine iodide: (■) magainin (50 $\mu\text{g}/\text{mL}$ or 21 μM); (▲) CSA-8 (2 $\mu\text{g}/\text{mL}$ or 3.5 μM); (◆) CSA-13 (0.5 $\mu\text{g}/\text{mL}$ or 0.74 μM).

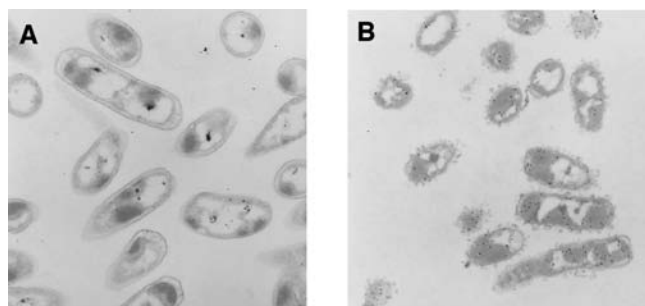


FIGURE 8. TEM images of *E. coli*: (A) control (magnification 7000); (B) treated with CSA-13 at 0.5 \times MIC (magnification 7000).

cloned and coupled to a bacterial luminescence reporter operon on a plasmid introduced into *E. coli*. At sublethal doses, the magainins cause activation of the *osmY* promoter, but not the *micF* promoter while cecropins activate both the *osmY* and *micF* promoters.³⁶ Using the same bacterial luminescence reporter, we observed that sublethal doses of ceragenins caused activation of both the *osmY* and *micF* promoters.³⁵

Conclusions

The ubiquity of antimicrobial peptides that have apparently evolved independently argues that their mechanism of action is effective in controlling bacterial growth without readily engendering resistance. Considering the prevalence of antibiotic-resistant bacteria, there is a pressing need for development of new antimicrobials, and clinical use of antimicrobial peptides has been presented as an attractive option. However, given the understanding of the active conformations of antimicrobial peptides and the ability to design and synthesize

smaller molecules that approximate these conformations, mimics of antimicrobial peptides may be more suitable for clinical development. Mimics can avoid protease degradation and salt sensitivity and may be simpler and less expensive to prepare and purify on a large scale. The ceragenins were designed to mimic antibacterial peptide activities, and direct comparisons suggest that they duplicate the antibacterial activities of antimicrobial peptides well. The ceragenins are well tolerated by cells that are routinely exposed to antimicrobial peptides, and preliminary in vivo testing of the ceragenins confirms that they are well suited to replace or augment the antibacterial activities of endogenous antimicrobial peptides. With pressing need for antimicrobials for use in topical, inhaled, or systemic applications, it is possible that ceragenins will find use in controlling bacterial growth and preventing infection.

Development of the ceragenins and studies with this class of compounds has been funded by the National Institutes of Health, Ceragenix Pharmaceuticals, and the Canadian Institutes of Health Research. P.B.S. is a paid consultant for Ceragenix Pharmaceuticals.

BIOGRAPHICAL INFORMATION

Xin-Zhong Lai received his Ph.D. in chemistry from University of Connecticut and conducted postdoctoral research in the Department of Chemistry and Biochemistry at Brigham Young University. His research interests include drug discovery and the exploration of the chemistry and biology of bioactive nature products.

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Robert Bucki is a Senior Research Investigator at the Institute for Medicine and Engineering at the University of Pennsylvania where he studies aspects of lipid biology and the effects of poly-electrolytes on antimicrobial agents. He received his M.D. and Ph.D. in physiology from the Medical University of Bialystok in Poland and did postdoctoral work at the Université Paris-Sud in Orsay.

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Paul B. Savage is the Reed M. Izatt Professor of Chemistry and Biochemistry at Brigham Young University. His laboratory studies membrane-targeting antimicrobials with an emphasis on development of mimics of antimicrobial peptides. His group also isolates, designs and prepares glycolipid antigens for natural killer T cells.

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