Correlation of the Antibacterial Activities of Cationic Peptide Antibiotics and Cationic Steroid Antibiotics[1]

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The antibacterial activities of cationic steroid antibiotics and cationic peptide antibiotics have been compared. Depolarization of bacterial membranes, activation of bacterial stress-related gene promoters, and changes in bacterial morphologies caused by these antibiotics suggest that cationic steroid and peptide antibiotics share mechanistic aspects. Modified cationic steroid antibiotics display improved selectivity for prokaryotic cells over eukaryotic cells presumably due to increased charge recognition.

Introduction

Cationic peptide antibiotics (CPAs) have received increasing attention because they are found in organisms ranging from bacteria to mammals and because of their potent antimicrobial activities.[2] Reported CPAs can be classified according to their structure; the two major classes include CPAs that adopt α-helical conformations and those that form β-sheets.[2,3] In general, CPAs in both classes adopt facially amphiphilic[4] conformations, with cationic groups on one face of the molecule and hydrophobic groups on the other. Demonstrating the importance of cationic/hydrophobic segregation, amphiphilic helices comprised of β-peptides have been prepared and have shown potent antibacterial properties.[5] Models for the mode of action of CPAs include the "carpet model" and the "barrel-and-stave model".[6] In the former model, amphiphilic peptides associate with the negatively charged bacterial membrane, and once the local concentration of the peptide reaches a sufficient level, patches of the membrane are removed and the membrane structure is compromised. In the latter model, cationic peptides act as the staves of a barrel in forming stable pores in bacterial membranes.

Four general characteristics of CPAs have been used to describe their activities.[7] (i) Many CPAs display selective toxicity for prokaryotic cells over eukaryotic cells. This selectivity is likely caused by affinity of the cationic peptides for the net negative charge found on bacterial cells in contrast to eukaryotic lipid bilayers, which are typically made up of zwitterionic phospholipids. (ii) Most CPAs exhibit rapid bacterial killing times. In general, these antibiotics are believed to be membrane active, as opposed to other types of antibiotics that target a single biochemical pathway and require longer periods of time to inhibit cell growth and division. (iii) Examples of CPAs display a broad spectrum of antibacterial activity. Because they are targeted to the charged character of bacterial membranes, they effectively kill multiple types of bacteria. (iv) CPAs generally do not induce the formation of resistant organisms. To become resistant to CPAs, bacteria have to change their membrane structure, which alters the permeability barrier provided by the membrane and can make the bacteria more susceptible to other antibiotics. An additional characteristic of certain CPAs is the ability to effectively permeabilize the outer membranes of Gram-negative bacteria, sensitizing these organisms to hydrophobic antibiotics.[8]

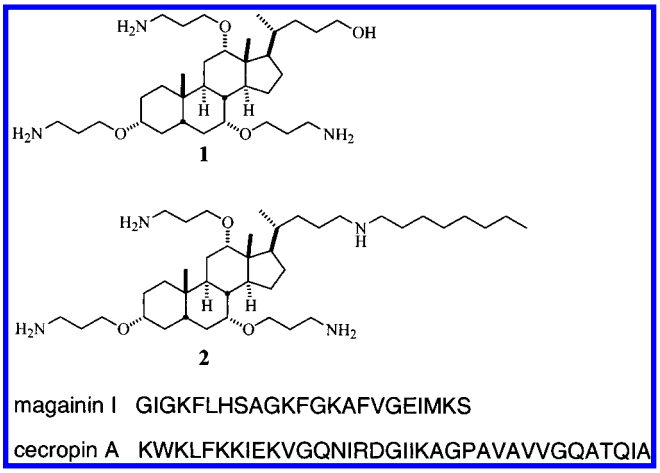

Figure 1. Structures of **1** and **2** and sequences of magainin I and cecropin A (one letter amino acid code is used).

Because of their complexity and size, many CPAs are difficult to synthesize and derivatize,[2] which complicates their biological study and application as antibiotics. Simpler compounds with similar activities would be desirable provided that they could be easily prepared and derivatized. One of the features of some CPAs that has impeded their development for clinical use is their hemolytic activity (disruption of red blood cells). Smaller molecules that display controlled hemolytic activity could provide a better understanding of the factors that govern prokaryote vs eukaryote membrane selectivity.[9]

We have developed multiple antimicrobial compounds (e.g., **1** and **2**, Figure 1), comprised of steroids,[10] that display antibacterial behaviors that are similar to those of CPAs. These cationic steroid antibiotics (CSAs) have proven to be broad-spectrum antibiotics active even

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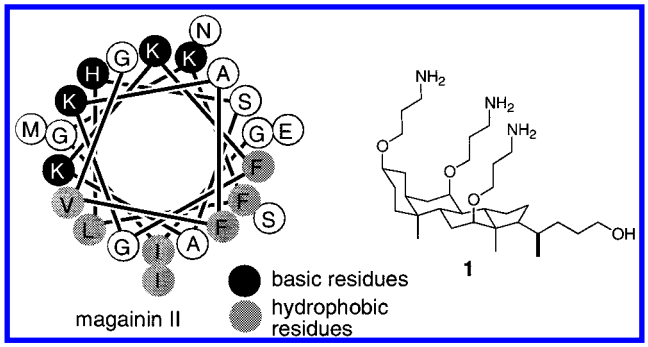


Figure 2. Helix wheel representation of magainin II demonstrating the segregation of basic and hydrophobic residues. Perspective drawing of **1**.

against multidrug-resistant strains of Gram-negative and Gram-positive bacteria.[11] In addition, examples of CSAs permeabilize the outer membranes of Gram-negative bacteria effectively sensitizing them to hydrophobic antibiotics.[8,12] Because of this activity, we have postulated that the bactericidal properties of CSAs are due to membrane disruption, and we have found that CSAs display a moderate degree of selectivity for prokaryotic over eukaryotic membranes.[12]

In an effort to better understand the mechanism of action of two representative CSAs (**1** and **2**), we have directly compared their activities to those of one of the best-studied groups[2] of CPAs, the magainin and cecropin antibiotics (Figure 1). The magainins, isolated from the skin secretions of the frog *Xenopus laevis*,[13] and cecropins, isolated from the giant silk worm moth *Hyalaphora cecropia*,[14] adopt α-helical conformations in the presence of bacterial membranes[15] and are believed to act via the carpet model.[6] Studies performed include measurements of depolarization of bacterial membranes by CSAs and CPAs, observation of bacterial gene promoters activated in response to the antibiotics, and changes in bacterial morphology as a consequence of antibacterial action. In addition, we have also prepared and characterized new CSAs that display improved selectivity for prokaryotic membranes over their eukaryotic counterparts.

Results and Discussion

The possibility that CPAs and CSAs share mechanistic aspects is better understood as the morphologies of both groups of antibiotics are considered. Both types of antibiotics display cationic facial amphiphilicity: the CPAs by virtue of their secondary (and in some cases tertiary) structure (for example, see the helix wheel of magainin II in Figure 2) and the CSAs due to the stereochemical orientation of the groups linking amines to the steroid scaffolding (Figure 2). While the facial amphiphilicity of the CSAs can be influenced by rotation about the bonds in the propyleneoxy linkers, due to the stereochemistry of the oxygen atoms on the steroid scaffolding, the amine groups are predisposed to be on one face of the molecule.[16] Considering this common feature of CPAs and CSAs, amphiphilic morphology appears essential for membrane activity. While in general the CSAs are smaller than the CPAs, the CPAs that have been identified display a wide range of molecular weights suggesting that "structure is more important than size".[2b]

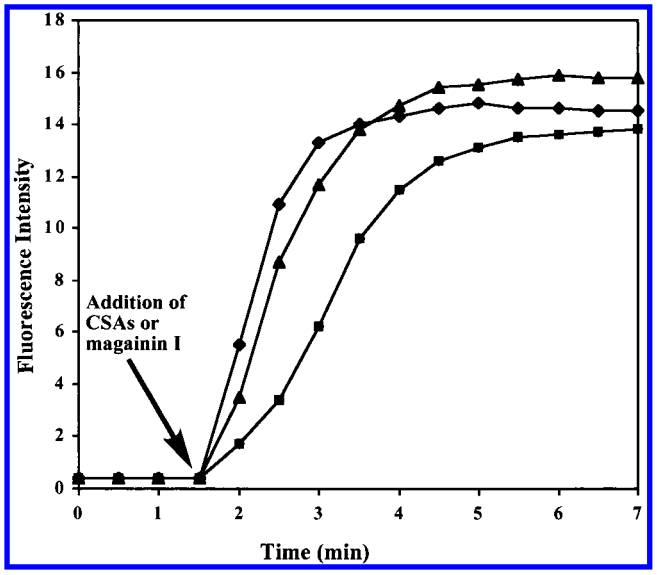


Figure 3. Depolarization of the membrane of *M. luteus* measured by an increase in fluorescence of 3,3′-diethylthiodicarbodyanine iodide: ♦, magainin (50 μg/mL or 21 μM); ■, **1** (2 μg/mL or 3.5 μM); ▲, **2** (0.5 μg/mL or 0.74 μM).

As a consequence of membrane activity of CPAs, bacterial membranes are rapidly depolarized. The activities of the magainins and nisin, both CPAs, have been characterized using the fluorescent, membrane-potential-sensitive probe 3,3′-diethylthiodicarbocyanine iodide.[17] In these experiments, the fluorescent dye is added to a bacterial culture, and the dye incorporates into the bacterial membrane. Depolarization of the membrane results in a large increase in fluorescence of the dye.[18] For our experiments, we used *Micrococcus luteus* (Presque Isle Cultures 456), a Gram-positive organism that is susceptible to the magainins. The addition of magainin I or CSAs **1** or **2** caused a rapid depolarization of the bacterial membrane (Figure 3). These results demonstrate the rapidity with which the antibiotics act and suggest that the CSAs have membrane activity comparable to magainin I, although the CSAs are active at lower concentrations.

To verify that CSAs rapidly kill bacteria, the times required for compound **2** to lower bacterial populations in culture were determined. To initial bacterial populations of 10^5 colony forming units (CFUs) per milliliter of *Escherichia coli* (ATCC 25922) or *Staphylococcus aureus* (ATCC 25923), compound **2** was added at its minimum inhibition concentrations (MICs) (0.31 and 0.59 μg/mL with *E. coli* and *S. aureus*, respectively). The numbers of CFUs were reduced by half from *E. coli* within 15 min and from *S. aureus* within 75 min.

CPAs have been effectively characterized by the bacterial promoters that they activate.[19] In response to external inducing agents, bacteria activate promoters for a number of stress response genes. These promoters have been cloned and coupled to a bacterial luminescence reporter operon (*luxCDABE*) on a plasmid introduced into *E. coli*.[19] Strains of these engineered bacteria respond to specific inducing agents by producing bacterial luciferase and its substrate, which results in bacterial luminescence. For example, strain DPD2170 responds to osmotic stress caused by high concentrations (0.5 M) of sucrose or sodium chloride by activating the

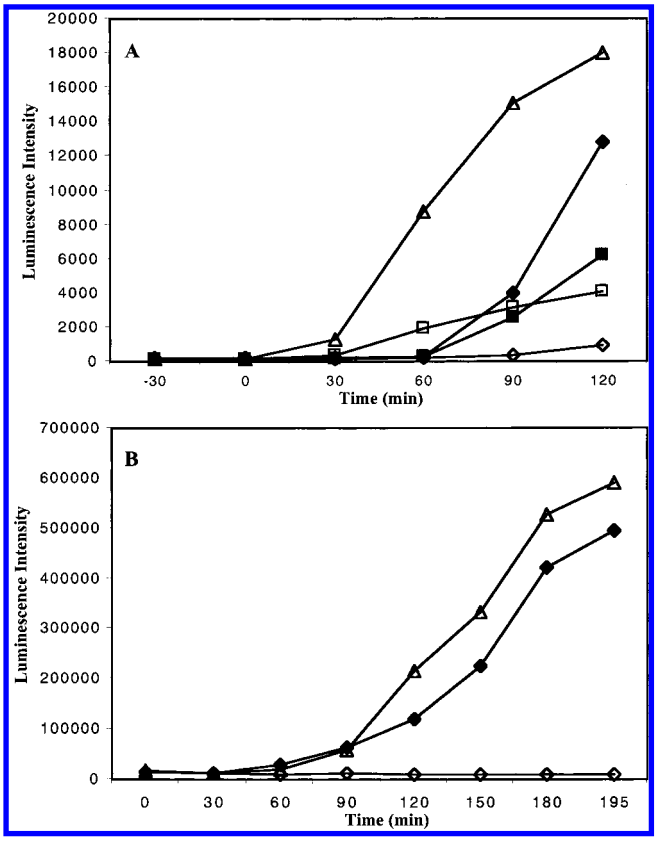


Figure 4. Luminescence of *E. coli* strains DPD2170 and DPD2194 induced by **1**. (A) *E. coli* (DPD2170) (*osmY* − *luxCDABE*): Δ, sucrose (0.5 M); □, NaCl (0.3 M); ◊, control; ♦, **1** (0.10 × MIC); ■, **1** (0.05 × MIC). (B) *E. coli* (DPD2194) (*micF* − *luxCDABE*): Δ, methyl viologen (0.1 μg/mL); ◊, control; ♦, **1** (0.10 × MIC).

promoter for the *osmY* gene. Activation of this promoter, which is coupled to the luminescence operon in this strain, causes an increase in luminescence. Similarly, strain DPD2191 responds to oxidative stress caused by methyl viologen by activation of the *micF* promoter, which causes an increase in luminescence.

At sublethal doses, CPAs magainin I and magainin II cause activation of the *osmY* promoter but not the *micF* promoter[19] where activation is defined by a doubling of background luminescence. In contrast, cecropins A and B activate both the *osmY* and the *micF* promoters.[19] Using the strain DPD2170, we found that sublethal doses (one-tenth or one-twentieth of the MIC) of **1** caused strong activation of the *osmY* (Figure 4) suggesting that the CSA caused the bacteria to experience osmotic stress in a manner similar to the magainins and cecropins. Higher concentrations caused bacterial death and a loss of luminescence. CSA **2** only caused a small increase in luminescence over the background (data not shown). The difference in the abilities of **1** and **2** to cause activation of the *osmY* promoter may be related to their differing abilities to traverse the outer membrane of Gram-negative bacteria.[10c] CSA **2** effectively traverses the outer membrane and is bactericidal at relatively low concentrations. The ability of **1** to activate the *micF* promoter was also measured; a low concentration of the CSA caused a large increase in the luminescence of DPD2191 (Figure 4). Taken together, results from experiments with strains DPD2170 and DPD2191 suggest that bacteria respond to CSA **1** in a manner similar to the response to the cecropin CPAs.

Proposed steps in the carpet model of CPA activity include (i) binding of peptide to anionic phospholipids via ionic interactions and (ii) reorientation of the CPA juxtaposing hydrophobic residues with the hydrophobic core of the membrane, followed by (iii) disintegration of the membrane.[20] Disintegration of bacterial membranes can be observed via transmission electron microscopy (TEM).

TEM was used to observe the morphological changes that bacteria undergo when treated with CSAs. It was necessary to use high concentrations of bacteria (~10^9 CFU/mL) to prepare samples for TEM. To ensure that the ratios of CSAs to bacteria were similar in MIC measurements[21] and in preparing the TEM samples, MIC values were converted from units of micrograms per milliliter to moles of CSA per bacterium. Using MIC values with these units, TEM images were captured of *E. coli* (ATCC 25922) treated with **1** and **2** at one-half of their MIC values (Figure 5). As compared to images of untreated bacteria, the TEM images of treated bacteria clearly show disintegration of the bacterial membranes. In fact, the formation of vesicles apparently lacking intracellular bacterial components can be seen. Disintegration of the bacterial membranes by the CSAs is consistent with a carpet model of action.

The desirable activities of CPAs (i.e., broad spectrum of activity, rapid killing times, and small likelihood of development of resistant bacteria) are due, in general, to their membrane activity; however, membrane activity presents potential problems.[22] The most important of which is selectivity for binding and perturbation of bacterial (prokaryotic) membranes. A measure of membrane selectivity can be obtained by comparing the MIC of an antibiotic to its minimum hemolytic concentration (MHC, minimum concentration required to lyse red blood cells). Compounds that display similar MIC and MHC values offer little or no membrane selectivity; compounds that are selective for prokaryotic membranes have low MIC values and high MHC values.

Many CPAs, including the magainins, exhibit very good selectivity for prokaryotic over eukaryotic membranes.[2] Other examples, such as mellitin, are much less selective. The membrane selectivity of the magainins has been attributed to recognition of the bulk charges on bacterial membranes comprised of anionic phospholipids, whereas mellitin associates strongly with zwitterionic phospholipids.[23] Differences in helix dipoles have also been used to explain the difference in hemolytic activities of the magainins and mellitin.[9]

The membrane selectivity of reported CSAs varies; some have demonstrated good selectivity, while others have proven to be potent hemolytic agents.[12,24] For example, **1** exhibits low MIC values (>5 μg/mL) against Gram-positive bacteria, while its MHC is relatively high (100 μg/mL).[12] Compound **1** is also a potent sensitizer of Gram-negative bacteria to other hydrophobic antibiotics. Compound **2** is very active against both Gram-negative and Gram-positive bacteria (MICs of <4 μg/mL) and is more strongly hemolytic (MHC of 29 μg/mL).

Because bacterial membranes are generally negatively charged,[23] cationic compounds would be expected to display affinity for prokaryotic membranes. To en-

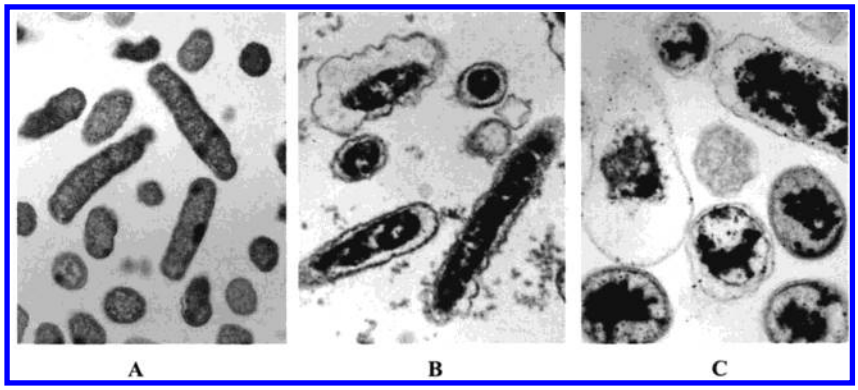


Figure 5. TEM images of *E. coli* (ATCC 25922). (A) Control (magnification 7000). (B) Treated with **1** at 0.5 × MIC (magnification 7000). (C) Treated with **2** at 0.5 × MIC (magnification 15 000).

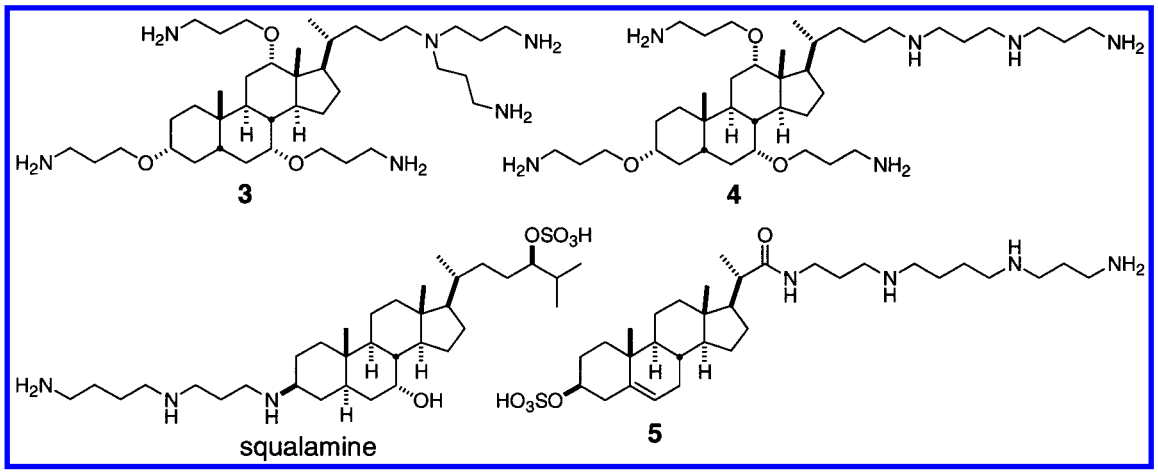
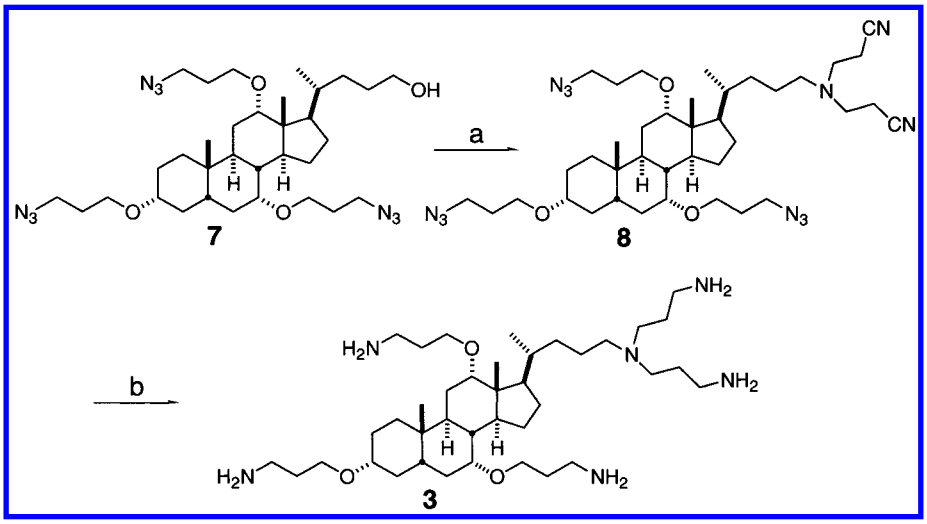


Figure 6. Structures of CSAs **3**, **4**, squalamine, and **5**.

Scheme 1[a]

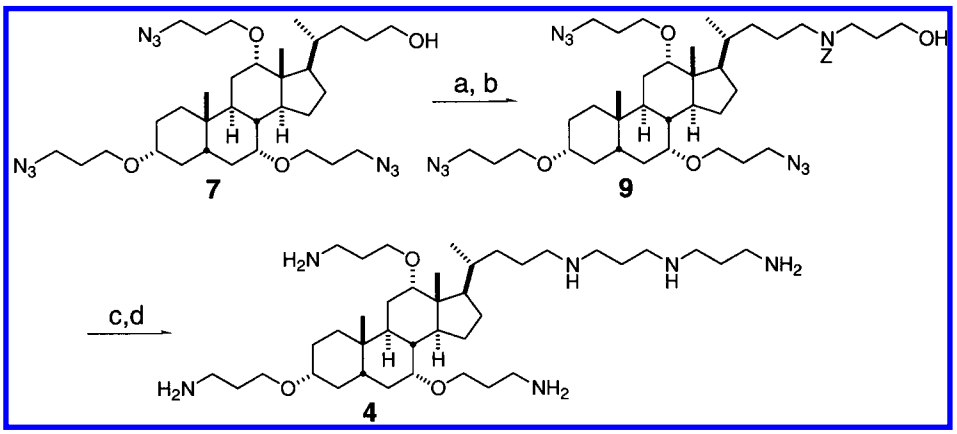


[a] Reagents (yields in parentheses): (a) MsCl, Et_3N, CH_2Cl_2; 3,3′-iminodipropionitrile, NaI, Na_2CO_3, THF (85%). (b) H_2, PtO_2, EtOH; H_2, PtO_2, HCl, EtOH (85%).

hance the ability of CSAs to bind to bacterial membranes, we prepared compounds **3** and **4** (Figure 6) in which polyamine groups, in a branched and a linear fashion, are appended at C-24. Polyamines, extending from a steroid scaffolding, are found in squalamine (Figure 6), a naturally occurring CSA obtained first from the Dogfish shark[25] and in squalamine mimics (e.g., **5**, Figure 6) developed by Regen and co-workers.[24]

The syntheses of **3** and **4** are outlined in Schemes 1 and 2, respectively. To prepare the CSA with a branched polyamine, the methanesulfonate of **7**[10c] was prepared and then reacted with 3,3′-iminodipropionitrile to give **8** in good yield. Reduction of the azides with hydrogen and platinum oxide was possible without affecting the nitrile groups. Under acidic conditions, the nitriles were reduced giving **3** in pure form. In preparing **4**, the linear polyamine was added in a stepwise manner. The methanesulfonate of **7** was reacted with 3-aminopropanol, and the resulting amine was protected as the benzyloxycarbamate giving **9**. Mesylation of the resulting

Scheme 2[a]



[a] Reagents (yields in parentheses): (a) MsCl, Et_3N, CH_2Cl_2; 3-aminopropanol, NaI, Na_2CO_3, THF (75%). (b) Benzyl chloroformate, Et_3N, CH_2Cl_2 (80%). (c) MsCl, Et_3N, CH_2Cl_2; *N*-*Z*-Propylenediamine, NaI, Na_2CO_3, DMSO (76%). (d) H_2, PtO_2, EtOH; H_2, $Pd(OH)_2$, AcOH, EtOH (95%).

Table 1. MIC Values of **1**, **3**, and **4** with Gram-Negative and Gram-Positive Bacteria and MHC Values.

	MIC and MHC values (μg/mL)		
	1	**3**	**4**
Gram-Negative Rods			
E. coli (ATCC 25922)	36	6.6	7.3
Salmonella typhimurium (ATCC 14028)	43	25	12
Gram-Positive Cocci			
S. aureus (ATCC 25923)	2.0	4.6	2.0
Streptococcus pyogenes (ATCC 19615)	4.2	3.0	1.6
minimum hemolytic concentrations	100	>200	>200

alcohol and reaction with the monobenzyloxycarbamate of propylenediamine completed incorporation of the linear polyamine. Deprotection to give **4** was accomplished by first reducing the azides with hydrogen in the presence of platinum oxide followed by removal of the carbamate groups by hydrogenation with palladium hydroxide.

The antibacterial activities of **3** and **4** were determined using both Gram-negative and Gram-positive bacteria (Table 1). As compared to **1**, both **3** and **4** were more active against Gram-negative organisms and displayed comparable activity against Gram-positive bacteria. As observed with **1**, CSAs **3** and **4** gave lower MIC values against Gram-positive bacteria. This difference in the activities of **3** and **4** against the two classes of organisms is presumably due to the permeability barrier provided by the outer membrane.[10c]

As anticipated, the hemolytic properties of **3** and **4** were better than other CSAs reported. That is, hemolysis was not observed up to concentrations of 200 μg/mL (Table 1), and due to limited solubility, it was not possible to use higher concentrations. Consideration of the relatively low MIC values of **3** and **4** and high MHC values suggests that the cationic character of these compounds causes them to be selective for prokaryotic membranes over eukaryotic membranes. This level of membrane selectivity increases the likelihood that CSAs may find therapeutic uses.

Conclusions

CSAs display the general activities attributed to CPAs: they display selectivity for bacterial cells, they rapidly kill bacteria, they are active against a broad spectrum of bacteria, and because they are membrane active, there is little likelihood of formation of resistant organisms. In addition, CSAs **1** and **2** depolarize bacterial membranes at rates similar to that of magainin I, and **1** causes activation of the same bacterial gene promoters as the cecropins. TEM images of bacteria subjected to CSAs are consistent with membrane activity similar to that of the facially amphiphilic CPAs magainins and cecropins. Taken together, these results suggest that CSAs share at least aspects of the mechanisms of action of CPAs that function via the carpet model. Because in general CSAs are simpler to prepare than CPAs and are easier to derivatize and purify, they are well-suited for further investigations elucidating how small molecules can selectively disrupt bacterial membranes. Enhanced cell selectivity of **3** and **4**, most likely caused by charge recognition, increases the probability that CSAs can be used to fight infection and demonstrates a means by which membrane active compounds can be made more cell selective.

Experimental Section

Materials and Spectroscopic Methods. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR were recorded on Varian Unity 500 MHz or Varian Unity 300 MHz instruments. Mass spectrometric data were obtained on a JEOL SX 102 A spectrometer. Optical density measurements were made using an HP 8453 spectrophotometer. Fluorescence measurements were made using a Perkin-Elmer LS50B fluorimeter. Luminescence experiments were made using an MGM Optocomp 1 luminometer. Tetrahydrofuran (THF) and CH_2Cl_2 were dried over Na or CaH. Chemicals were obtained from Fluka, Aldrich, and ARCOS and were used as received unless otherwise noted.

Measurement of Membrane Depolarization. An *M. luteus* culture was grown up in Mueller−Hinton broth, and cells were harvested by centrifugation, washed twice in a buffer containing 250 mM sucrose and 5 mM $MgSO_4$, and suspended in the same buffer at an optical density (600 nm) of 0.085. The dye 3,3′-diethylthiodicarbocyanine iodide was added giving a 1 μM concentration. The dye was allowed to incorporate for 15 min before measurements. An excitation wavelength of 600 nm and an emission wavelength of 660 nm were used to monitor depolarization. Samples were slowly stirred during the measurement, and measurements were taken every 30 s.

Establishing Killing Times for *E. coli* and *S. aureus*. The microorganisms were grown to a concentration of 10^5 CFU/mL by incubating colonies for 120 min (*E. coli*) or 20 min (*S.*

aureus) in Mueller−Hinton broth at 37 °C. The bacterial suspensions were challenged with the CSA, and at specific time intervals, samples were plated out on Mueller−Hinton agar plates. The plates were then incubated for 24 h at 37 °C, and CFUs were counted.

Observation of Activation of *osmY* and *micF* Promoters. *E. coli* strains DPD 2170, DPD 2191, and DPD 2194 were provided by Tina K. Van Dyk (Dupont). Cultures (12 mL) were grown to optical densities (600 nm) of 0.05 in LB broth containing ampicillin (50 μg/mL). Inducers were added at the concentrations described, and the samples were incubated at 30 °C. At 30 min intervals, 1 mL samples were removed and their luminescence was measured. Samples were discarded after the measurements.

Preparation of TEM Samples. Cultures of *E. coli* (ATCC 25922) were grown to 10^9 CFU/mL in Mueller−Hinton broth, and the bacteria were pelleted by centrifugation. The supernatant was removed, and the CSA was added in Mueller−Hinton broth. The samples were incubated for 90 min at 37 °C, and the bacteria were centrifuged. The supernatant was removed, and 2% glutaraldehyde was added for fixing. After the mixture was centrifuged and the supernatant was removed, a solution of 1.5% low-melting agarose was added at 37 °C. A pellet of the cells in agarose was formed via centrifugation and was used for TEM sectioning.

Preparation of 3α,7α,12α-Tris(3-azidopropoxy)-5β-cholan-24-(*N*,*N*-bis(2-cyanoethyl))amine (8). MsCl (27.4 mg, 0.12 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a solution of 3α,7α,12α-tris(3-azidopropoxy)-5β-cholan-24-ol (**7**) (77.0 mg, 0.12 mmol) and Et_3N (48.5 mg, 0.48 mmol) in CH_2Cl_2 (20 mL) at 0 °C under N_2 during 10 min. The mixture was stirred for another 1 h and then washed with brine (3 × 10 mL), and the organic phase was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the resulting material was used in the next step. A mixture of the mesylate, 3,3′-iminodipropionitrile (2 mL, 16.6 mmol), NaI (5 mg), and Na_2CO_3 (127 mg, 1.20 mmol) in THF (2 mL) was refluxed under N_2 overnight. Water (10 mL) was added, and the product was extracted by ethyl acetate (3 × 10 mL). The combined extracts were dried over Na_2SO_4. The product **8** (76.4 mg, 85%) was obtained as an oil after column chromatography (hexane/EtOAc 2/1). ^{1}H NMR ($CDCl_3$): δ 3.68−3.59 (m, 2H), 3.55−3.37 (m, 8H), 3.28−3.09 (m, 4H), 2.86 (t, J = 6.20 Hz, 4H), 2.57−2.46 (m, 6H), 2.19−2.08 (m, 4H), 1.96−1.60 (m, 15H), 1.58−1.48 (m, 3H), 1.46−1.21 (m, 9H), 1.09−1.01 (m, 2H), 0.96 (d, J = 6.4 Hz, 3H), 0.89 (s, 3H), 0.69 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 118.55, 80.43, 79.60,75.84, 64.80, 64.27, 64.11, 60.32, 54.02, 49.68, 48.80, 48.78, 48.56, 46.36, 46.23, 42.51, 41.72, 39.62, 35.58, 35.24, 35.13, 34.89, 33.36, 31.52, 29.57, 29.50, 28.89, 27.68, 27.47, 23.79, 23.34, 22.83, 22.65, 17.90, 16.94, 12.38. High-resolution fast atom bombardment mass spectroscopy (HRFAB-MS) (thioglycerol + Na matrix): m/e ([M^+ + Na]) 771.5140 (100%), calcd 771.5122.

Preparation of 3α,7α,12α-Tris(3-aminopropoxy)-5β-cholan-24-(*N*,*N*-bis(3-aminopropyl))amine (3). Compound **8** (76.4 mg, 0.10 mmol) in dry ethanol (10 mL) was hydrogenated (800 psi) in the presence of PtO_2 (5 mg, 0.02 mmol) at room temperature for 48 h. The catalyst was filtered out, and the filtrate was concentrated under reduced pressure. The product was separated by column chromatography (CH_2Cl_2/MeOH/NH_4OH 3/3/1) to give the corresponding triamine (60.4 mg, 89%). ^{1}H NMR ($CDCl_3$): δ 3.68−3.41 (m, 8H), 3.38−3.05 (m, 6H), 2.88−2.71 (m, 12H), 2.53−2.41 (m, 8H), 2.18−2.04 (m, 3H), 1.81−1.13 (m, 24H), 0.96 (d, J = 6.4 Hz, 3H), 0.89 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (80% $CDCl_3$,and 20% CD_3OD): δ 118.63, 80.30, 79.20, 75.80, 66.36, 66.25, 66.13, 53.77, 49.35, 46.69, 45.88, 42.43, 41.43, 39.64, 39.64, 39.27, 39.09, 35.36, 35.02, 34.82, 34.57, 33.12, 31.96, 31.86, 31.57, 28.36, 27.55, 27.32, 27.21, 24.01, 23.07, 22.46, 22.23, 17.77, 16.60, 12.11. HRFAB-MS (thioglycerol + H^+ matrix): m/e ([M^+ + H^+]) 671.5598, calcd 671.5588. The triamine (60.0 mg, 0.09 mmol) in dry ethanol (10 mL) and HCl (aqueous, 37%, 1 mL) was hydrogenated (800 psi) in the presence of PtO_2 (5 mg, 0.02 mmol) at room temperature for 48 h. The catalyst was filtered out, and the filtrate was concentrated under reduced pressure. Compound **3** was obtained (58.1 mg, 95%) as a yellow solid. ^{1}H NMR (dimethylsulfoxide (DMSO)-d_6): δ 4.01 (broad, 10H), 3.72−2.81 (m, 20H), 2.18−165 (m, 20H), 1.51−1.12 (m, 19H), 0.96 (d, J = 6.4 Hz, 3H), 0.89 (s, 3H), 0.69 (s, 3H). ^{13} C NMR (CD_3OD): δ 82.53, 81.89, 77.96, 67.89, 67.78, 67.23, 58.12, 56.89, 54.21, 47.56, 44.41, 43.85, 40.92, 36.89, 36.77, 36.59, 35.96, 31.99, 30.09, 29.60, 29.28, 24.94, 24.72, 24.10, 23.71, 23.12, 22.68, 22.19, 20.06, 17.55, 11.80. HRFAB-MS (thioglycerol + H^+ matrix): m/e ([M^+ + H^+]) 679.6229, calcd 679.6214.

Preparation of 3α,7α,12α-Tris(3-azidopropoxy)-5β-cholan-24-(*N*-(benzyloxycarbonyl)-*N*-(3-hydroxypropyl))-amine (9). MsCl (105 mg, 0.92 mmol) in CH_2 Cl_2 (10 mL) was added dropwise to a solution of **7** (294 mg, 0.46 mmol) and Et_3N (186 mg, 1.84 mmol) in CH_2Cl_2 (30 mL) at 0 °C under N_2 during 10 min. The mixture was stirred for another 1 h, then it was washed with brine (3 × 10 mL), and the organic phase was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the resulting material was used for the next step reaction. The mesylate, 2-aminopropanol (3 mL), NaI (10 mg), and Na_2CO_3 (488 mg, 4.6 mmol) in THF (4 mL) were refluxed under N_2 overnight. Water (20 mL) was added, and the product was extracted by ethyl acetate (3 × 20 mL) and dried over Na_2SO_4. After column chromatography (CH_2Cl_2/MeOH/NH_4OH 10/1.5/0.2), the desired alcohol was obtained as a brown oil (243 mg, 75%). ^{1}H NMR ($CDCl_3$): δ 3.78 (t, J = 5.37 Hz, 2H), 3.68−3.37 (m, 14H), 3.23−3.05 (m, 6H), 2.07 (t, J = 5.37 Hz, 2H), 2.60−2.51 (m, 2H), 2.17−2.11 (m, 3H), 1.93−1.51 (m, 17H), 1.48−1.15 (m, 6H), 1.07−0.98 (m, 2H), 0.88 (d, J = 6.84 Hz, 3H), 0.86 (s, 3H), 0.63 (s, 3H). ^{13} C NMR ($CDCl_3$): δ 80.41, 79.59, 75.87, 64.82, 64.25, 64.21, 64.10, 50.25, 49.88, 48.82, 48.76, 48.56, 46.29, 46.20, 42.48, 41.79, 40.16, 39.62, 35.57, 35.24, 35.10, 34.89, 33.42, 30.43, 29.57, 29.52, 28.89, 27.67, 27.62, 27.45, 26.18, 23.35, 22.84, 22.65, 17.86, 12.38. HRFAB-MS (thioglycerol + H^+ matrix): m/e ([M^+ + H^+]) 701.5186, calcd 701.5190. Benzyl chloroformate (65.0 mg, 0.38 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a solution of the alcohol from the previous step (242 mg, 0.35 mmol) and Et_3N (105 mg, 1.04 mmol) in CH_2Cl_2 (30 mL) at 0 °C under N_2 during 10 min. The mixture was stirred for another 2 h and then washed with brine (3 × 10 mL), and the organic phase was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (hexane/EtOAc 2/1) to give **9** (230 mg, 80%) as an oil. ^{1}H NMR ($CDCl_3$): δ 7.39−7.21 (m, 5H), 5.62 (s, 1H), 5.11 (s, 2H), 3.79−3.22 (m, 14H), 3.19−3.02 (m, 6H), 2.17−2.13 (m, 3H), 1.93−1.61 (m, 14H), 1.48−1.15 (m, 16H), 0.88 (d, J = 6.84 Hz, 3H), 0.86 (s, 3H), 0.63 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 157.51, 136.68, 128.45, 127.93, 127.70, 80.41, 79.59, 75.87, 67.37, 64.82, 64.25, 64.21, 58.30, 48.76, 48.56, 47.51, 46.46, 46.20, 45.75, 43.25, 42.48, 41.79, 39.62, 35.57, 35.24, 35.10, 34.89, 32.96, 30.58, 29.65, 29.57, 29.48, 28.89, 27.67, 27.62, 27.45, 25.57, 23.35, 22.84, 22.65, 17.86, 12.38. HRFAB-MS (thioglycerol + H^+ matrix): m/e ([M^+]) 835.5467, calcd 835.5480.

Preparation of 3α,7α,12α-Tris(3-aminopropoxy)-5β-cholan-24-(*N*-(*N*-(3-aminopropyl))-3-aminopropyl)-amine (4). MsCl (31.9 mg, 0.28 mmol) in CH_2Cl_2 (5 mL) was added dropwise to a solution of **9** (114 mg, 0.14 mmol) and Et_3N (56.6 mg, 0.56 mmol) in CH_2Cl_2 (20 mL) at 0 °C under N_2 during 10 min. The mixture was stirred for another 1 h, then it was washed with brine (3 × 10 mL), and the organic phase was dried over Na_2SO_4. The solvent was removed under reduced pressure, and the resulting material was used in the next step of the reaction. The resulting mesylate (285 mg, 1.40 mmol), *N*-benzyloxycarbamoylpropylenediamine (148 mg, 1.40 mmol), NaI (10 mg), and Na_2CO_3 (100 mg) in DMSO (2 mL) were heated to 90 °C under N_2 overnight. Water (10 mL) was added, and the mixture was extracted by ethyl acetate (3 × 20 mL). The extracts were dried over Na_2SO_4. After column chromatography (CH_2Cl_2/MeOH/NH_4OH 10/0.5/0.1), the desired amine was obtained as a brown oil (109 mg, 76%). ^{1}H NMR ($CDCl_3$): δ 7.39−7.22 (m, 10H), 5.85 (s, 1H), 5.11 (s, 2H),

5.08 (s, 2H), 3.75 (s, 1H), 3.67−3.03 (m, 22H), 2.74−2.53 (m, 4H), 2.11−2.06 (m, 4H), 1.95−1.15 (m, 29H), 0.89 (d, $J = 6.84$ Hz, 3H), 0.85 (s, 3H), 0.66 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 157.28, 156.69, 136.66, 128.61, 128.37, 128.14, 127.90, 127.74, 127.49, 80.41, 79.58, 75.82, 67.37, 66.49, 64.78, 64.27, 64.11, 48.74, 48.50, 47.53, 46.48, 46.22, 45.67, 43.98, 42.49, 41.76, 39.91, 39.60, 38.25, 35.50, 35.22, 35.10, 34.88, 32.92, 29.64, 29.56, 29.48, 28.86, 27.67, 27.62, 27.45, 25.25, 23.32, 22.84, 22.65, 17.80, 12.39. HRFAB-MS (thioglycerol + H^+ matrix): *m/e* ([$M^+ + H^+$]) 1025.6683, calcd 1025.6664. The amine from the previous step (40.0 mg, 0.04 mmol) in dry ethanol (10 mL) was hydrogenated (800 psi) in the presence of PtO_2 (5 mg, 0.02 mmol) at room temperature for 48 h. The catalyst was filtered out, and the filtrate was concentrated under reduced pressure. The residue was further hydrogenated (800 psi) in the presence of $Pd(OH)_2$ (40 mg, 20% on carbon) for 48 h. The catalyst was filtrated out, and the filtrate was concentrated under reduced pressure to give compound **4** (25.5 mg, 95%) as a yellow solid. ^{1}H NMR (CD_3OD): δ 3.92−261 (m, 26H), 2.13−1.11 (m, 33H), 0.88 (d, $J = 6.84$ Hz, 3H), 0.86 (s, 3H), 0.63 (s, 3H). ^{13}C NMR (CD_3OD): δ 80.95, 79.66, 76.49, 65.55, 65.25, 64.95, 48.21, 48.06, 47.91, 46.20, 46.09, 45.45, 44.97, 44.82, 43.06, 41.77, 39.56, 38.28, 36.85, 35.12, 34.93, 34.83, 34.61, 32.44, 29.62, 29.46, 28.32, 28.26, 28.14, 27.43, 27.25, 26.25, 25.59, 24.20, 23.10, 22.39, 21.99, 17.33, 11.52. HRFAB-MS (thioglycerol + H^+ matrix): *m/e* ([$M^+ + H^+$]) 679.6224, calcd 679.6214.

Measurement of MIC and MHC Values. These values were measured as reported previously.[11]

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