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Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins)

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Abstract

Ceragenins are cationic bile salt derivatives having antimicrobial activity. The interactions of several ceragenins with phospholipid bilayers were tested in different systems. The ceragenins are capable of forming specific associations with several phospholipid species that may be involved with their antimicrobial action. Their antimicrobial activity is lower in bacteria that have a high content of phosphatidylethanolamine. Gram negative bacteria with a high content of phosphatidylethanolamine exhibit sensitivity to different ceragenins that corresponds to the extent of interaction of these compounds with phospholipids, including the ability of different ceragenins to induce leakage of aqueous contents from phosphatidylethanolamine-rich liposomes. A second class of bacteria having cell membranes composed largely of anionic lipids and having a low content of phosphatidylethanolamine are very sensitive to the action of the ceragenins but they exhibit similar minimal inhibitory concentrations with most of the ceragenins and for different strains of bacteria. Although Gram negative bacteria generally have a high content of phosphatidylethanolamine, there are a few exceptions. In addition, a mutant strain of *Escherichia coli* has been made that is essentially devoid of phophatidylethanolamine, although 80% of the lipid of the wild-type strain is phosphatidylethanolamine. Furthermore, certain Gram positive bacteria are also exceptions in that they can have a high content of phosphatidylethanolamine. We find that the antimicrobial action of the ceragenins correlates better with the content of phosphatidylethanolamine in the bacterial membrane than whether or not the bacteria has an outer membrane. Thus, the bacterial lipid composition can be an important factor in determining the sensitivity of bacteria to antimicrobial agents.

Keywords: Bacterial outer membrane; Cell wall; Antimicrobial agent; Bacterial membrane; Cationic sterol derivative; Gram negative bacteria; Phosphatidylethanolamine

1. Introduction

There is growing interest in developing novel antimicrobial agents that would be effective against microbes that are resistant to traditional antibiotic therapy. Peptides with antimicrobial activity have received particular attention. Some of these peptides exist in nature as part of a natural defence system [1]. There is a large range of chemical structures among these peptides, to the extent that more generalized rules have evaded recent efforts to optimize their potency. One novel series of antimicrobial compounds is based on derivatives of bile acids with covalently attached amines. These compounds have been termed ceragenins. Many of these have a lipophilic moiety, facilitating their partitioning to membranes, thought to be important either for the mechanism of action of these agents by causing membrane damage or for facilitating the uptake of these agents into cells. In addition, ceragenins are polycationic, a feature important for selective toxicity against microbes that have exposed anionic lipids. Interestingly, there are also potent naturally occurring cationic sterol derivatives that contribute to the microbial resistance of sharks [2]. Ceragenins differ significantly in their toxicity against different species of pathogenic bacteria. In the present work we explore the relationship between the interaction of these antimicrobial agents with model membranes of different lipid

Abbreviations: DEPE, dielaidoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylene-bis-pyridinium bromide; CSA, Cationic Antimicrobial Steroid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PC, phosphatidylcholine

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composition and their toxicity to bacteria with different lipid compositions of their cell membranes. A direct correlation between phosphatidylethanolamine content and antimicrobial activity has previously been found for a pair of α/β helical peptides [3,4]. The names and structures of the ceragenins studied in the present work are given in Fig. 1.

2. Materials and methods

2.1. Materials

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). The probes 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and p-xylene-bis-pyridinium bromide (DPX) were obtained from Molecular Probes (Invitrogen). Cationic Antimicrobial Steroid (CSA) compounds were kindly provided to us by Ceragenix Pharmaceuticals, Denver, CO.

2.2. Bacteria

A mutant form of *Escherichia coli*, AD90, which has a mutated pss gene for phosphatidylserine synthase was obtained from William Dowhan of the University of Texas-Houston Medical School, as well as the pss+ strain AD90/pDD72, carrying the genes for the enzymes that catalyze the biosynthesis of phosphatidylethanolamine. *Bacillus polymyxa* and *Bacillus cereus* were obtained from Dr. Daniel R. Zeigler of the *Bacillus* Genetic Stock Center of Ohio State University and *Caulobacter crescentus* was obtained from Ellen M. Quardokus, Indiana University.

2.3. Preparation of vesicles

Lipids, with or without an added ceragenin, were dissolved in chloroform/methanol, 2/1 (v/v) at the desired lipid to ceragenin molar ratio. The solvent was then evaporated with nitrogen to deposit a film on the wall of a glass test tube. Final traces of solvent were removed for 2–3 h in an evacuated chamber. The films were suspended in the appropriate buffer by vortexing at room temperature to form multilamellar vesicles. When large unilamellar vesicles (LUVs) were prepared, the lipid films were made devoid of ceragenins. They were hydrated with buffer and suspensions were further processed with 5 cycles of freezing and thawing, followed by 10 passes through two stacked 0.1 μm polycarbonate filters (Nucleopore Filtration Products, Pleasanton, CA) in a barrel extruder (Lipex Biomembranes, Vancouver, BC) at room temperature. LUVs were stored on ice and used within a short time after preparation.

2.4. ANTS/DPX leakage studies

Aqueous content leakage from liposomes was determined using the ANTS/DPX assay [5]. Lipid films were hydrated with 12.5 mM ANTS and 45 mM DPX in 10 mM HEPES, pH 7.4. The osmolarity of this solution was adjusted with NaCl to be equal to that of the assay buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4) as measured with a cryoosmometer (Advanced Model 3MOplus Micro-Osmometer, Advanced Instruments Inc., Norwood, MA). LUVs of 0.1 μm diameter were prepared by extrusion as described above. After passage through a 2.5×20 cm column of Sephadex G-75, the void volume fractions were collected and the phospholipid concentration was determined by phosphate analysis using the method of Ames [6]. The fluorescence measurements were performed in 2 mL of buffer in a quartz cuvette equilibrated at 37 °C with stirring. Aliquots of LUVs were added to the cuvette and the fluorescence was recorded as a function of time using an excitation wavelength of 360 nm and an emission wavelength of 530 nm with 8 nm bandwidths. A 500-nm cutoff filter was placed in the emission path. Ceragenin in buffer was added to the lipid vesicles in the cuvette and the fluorescence monitored over time. At the end of each experiment, the value for 100% release was obtained by adding 20 μL of a 20% Lubrol PX solution to the cuvette and sonicated briefly to achieve complete release. Experiments were performed in duplicate; both replicates were in good agreement. Batches of LUVs prepared on different days retained the same order of potentiation.

2.5. Differential scanning calorimetry (DSC)

Measurements were made using a Nano II Differential Scanning Calorimeter (Calorimetry Sciences Corporation, Lindon, UT). The scan rate was 0.75 °C/min with a delay of 5 min between sequential scans in a series to allow for thermal equilibration. The features of the design of this instrument have been described [7]. DSC curves were analyzed by using the fitting program, DA-2, provided by Microcal Inc. (Northampton, MA) and plotted with Origin, version 5.0. MLVs were prepared as described in Preparation of vesicles. The concentration of MLV in the samples studied was maintained at 2.5 mg/mL. The cell volume is 340 μL.

2.6. ^{31}P NMR

The ^{31}P NMR spectra, from suspensions of about 25 mg of lipid, with or without added CSA-13, in PIPES buffer were obtained using a Bruker AM-500 spectrometer operating at 202.45 MHz in a 10-mm broad band probe over a 30-kHz sweep width in 16×1024 data points. A 90° pulse width of 16.6 μs was used. The sample was contained in a 5-mm diameter Shigemi NMR tube (Shigemi Co., Tokyo, Japan). Composite pulse decoupling was used to remove any proton coupling. Generally, 800 free induction decays were processed using an exponential line broadening of 100 Hz prior to Fourier transformation. Probe temperature was maintained to ±0.2 °C by a Bruker B-VT 1000 variable temperature unit. Temperatures were monitored with a calibrated thermocouple probe placed in the cavity of the NMR magnet.

2.7. Determination of minimal inhibitory concentration (MIC)

MIC values were generally determined as previously described [8]. The only exception was the determination of the MICs for the pss+ and pss− strains of

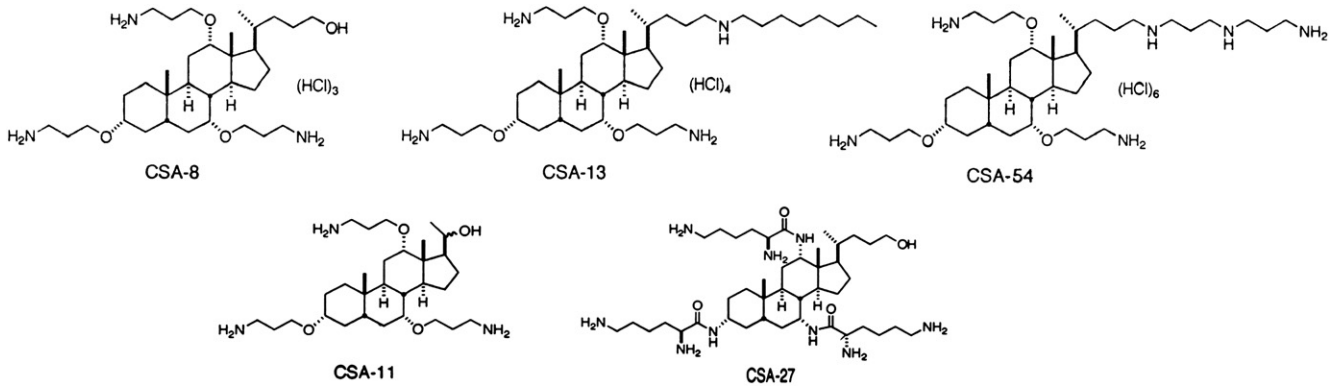

Fig. 1. Structures of the compounds studied in this work. They are bile acid derivatives, with substitutions of different chain lengths.

E. coli. These bacteria were grown in Luria–Miller (LB) broth supplemented with 50 mM $MgCl_2$ at 30 °C, conditions under which growth of the mutant strain is optimal. MIC was determined using a broth microdilution assay. Solutions of ceragenins in 0.02% acetic acid were kept in silanized glass containers at an initial concentration of 2 mg/mL. This solution was diluted 10 times with sterile LB broth containing 20 mM $MgCl_2$ and used to make serial dilutions in 96-well sterile polypropylene untreated microplates. For the assay, bacteria were regrown to midlog phase in LB broth supplemented with 20 mM $MgCl_2$ at 30 °C (exposure to less than 20 mM Mg^{2+} causes lysis of the PE minus strain) and $\sim 2\times10^5$ CFU/mL were added to each well. Incubation was done at 30 °C overnight and the plate was read at 600 nm in a microplate reader. The MIC value was the lowest concentration of ceragenin that gave no visible bacterial growth. All MIC determinations were done in duplicate.

3. Results

3.1. DSC

We probed the interaction of a group of ceragenins with representative lipids having headgroups found in the major lipid components of bacterial membranes as well as the external lipids of mammalian plasma membranes, but with acyl chains chosen so that the phospholipid would exhibit phase transitions at easily accessible temperatures. The lipids chosen for DSC were DEPE, DPPC and DPPG. DSC is a powerful tool for understanding the influence of compounds on the phase transitions of biological membrane components. PE and PG are major components of bacterial membranes while PC is abundant in mammalian membranes. These studies were done with the more active ceragenins CSA-13, CSA-54 and CSA-8. The shifts in the phase transition temperatures with increasing amounts of the ceragenins are summarized in Table 1.

3.2. Interaction of ceragenins with DEPE

DEPE undergoes a bilayer to inverted phase transition at about 65 °C that can accurately be measured by calorimetry. We have shown that the temperature of this transition (T_H) is sensitive to the presence of certain additives in the membrane and is a reliable measure of the effects of these additives on membrane curvature [9]. Membrane curvature in turn is known to play an important role in membrane fusion, including the fusion of enveloped viruses to target membranes [10,11]. We have shown that several substances that raise T_H, inhibit viral fusion. In the present series of compounds, however, we find that all three ceragenins raise T_H, i.e., promote positive curvature and would therefore be expected to inhibit viral fusion. The potency of the three compounds in raising T_H is 200±20, 18±23 and 260±16, respectively, for CSA-8, 13 and 54. The most potent ceragenin, CSA-13, is the least potent in changing curvature. Thus, positive curvature seems detrimental to anti-bacterial activity. There may be some relationship between membrane curvature and the antiviral activity of the ceragenins. For example, CSA-54 that induces the greatest positive curvature is also the most effective against Epstein–Barr Virus and HIV. CSA-54 is however not the most potent against vaccinia virus, although unlike Epstein–Barr Virus [12] or HIV [13] that infect by pH-independent fusion with the plasma membrane, vaccinia virus fusion can be promoted by acidic pH [14] which could affect its relative degree of inhibition by different ceragenins.

Table 1
Linear regression of the temperature shift with mole fraction CSA in DEPE

Compound	°C/mol fraction CSA (for T_M)	°C/mol fraction CSA (for T_H)	ΔH_M (kcal/mole)	ΔH_H (kcal/mole)
CSA-8	−43±9	200±18	39±10	3.5±0.5
CSA-13	−165±41 [a]	18±23	Not linear	0
CSA-54	−25±3	260±16	54±24	−7.3±1.3

Linear regression of the temperature shift with mole fraction CSA in DPPC

Compound	T_M, Heat (°C)	T_M, Cool (°C)	ΔH_M, Heat (kcal/mole)	ΔH_M, Cool (kcal/mole)
CSA-8	−22±2	−20±2	−7.4±2	−4.6±2.4
CSA-13	Not linear	Not linear	Not linear	Not linear
CSA-54	−9±2	−6±2	−7.9±3.3	−11.2±1.8

The temperature shift with mole fraction CSA in DPPG was not linear with any of the 3 CSAs.
[a] Not linear.

The ceragenins used in the present work also have a large effect on the gel to liquid crystalline transition of DEPE that appears at 37 °C for the pure lipid. With regard to this transition, the most potent ceragenin, CSA-13, has the strongest effect in lowering this transition temperature. This shift appears to be a result of an increase in a lower temperature component together with a decrease in the size (enthalpy) of the peak at 37 °C. Furthermore, the lower temperature peak appears to become sharper as more CSA-13 is added (Fig. 2). This behaviour suggests the formation of a complex between the CSA-13 and DEPE. This is not the case for CSA-54 that shows a gradual broadening and lowering of the peak for this transition. The behaviour of CSA-8 is intermediate. The dependence of the temperature and enthalpy of the gel to liquid crystalline transition, as well as the bilayer to hexagonal transition are shown graphically (Fig. 3).

3.3. Interaction of ceragenins with DPPC

The behaviour of ceragenins was further tested using the zwitterionic lipid dipalmitoylphosphatidylcholine (DPPC) in place of DEPE. DPPC does not exhibit a transition to the hexagonal phase and is a simpler system to test the effects on the main melting transition. The results are similar to those with DEPE but even more clear-cut (Fig. 4). Thus the broadening and resharpening of the transition peak is dramatically seen with CSA-13. It is also revealed by the biphasic plot of the dependence of ΔH on the mole fraction of CSA-13 added (Fig. 5, subscripts m and c refer to data from heating and cooling curves).

3.4. Interaction of ceragenins with DPPG

Being cationic compounds it is expected that ceragenins would interact strongly with anionic lipid. Indeed, all three ceragenins showed an abrupt shift of the phase transition temperature with increasing concentration of ceragenin, concomitant

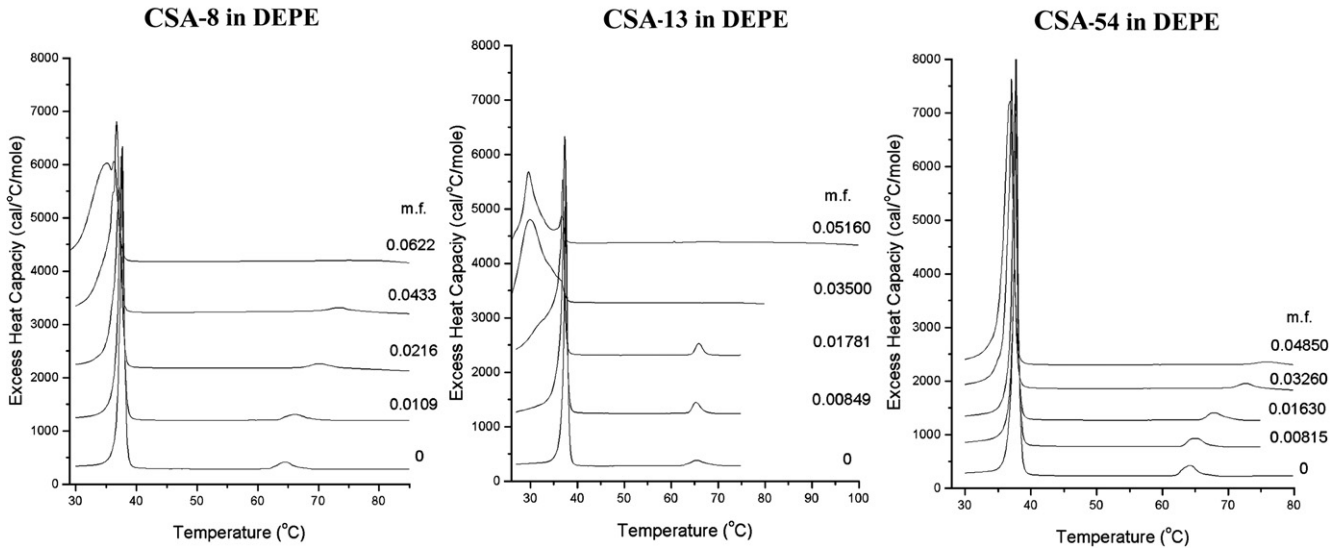

Fig. 2. DSC heating scans of DEPE with increasing mole fractions of CSA. Scan rate 0.75 o/min. Curves of excess heat capacity (cal/°C/mole) as a function of temperature are displaced along the ordinate for ease of viewing. Left curve, CSA-8; middle curve, CSA-13; and right curve, CSA-54.

with a biphasic effect on the gel to lamellar phase transition enthalpy of DPPG (Fig. 6). However, they differ in the mole fraction at which this phase separation appears, being 0.2 for CSA-8, 0.07 for CSA-13 and 0.12 for CSA-54 (Fig. 7). Again we find the biphasic effect to be strongest with CSA-13, the most active ceragenin.

3.5. ^{31}P NMR

The ^{31}P NMR powder patterns were determined for the three phospholipids used in the DSC studies, with and without the addition of CSA-13. The spectra were measured as a function of temperature. Changes with temperature were reversible, with the major change being a broadening of the powder pattern below the gel to liquid crystalline phase transition temperature. We present the results for a temperature slightly above the phase transition temperature of the lipid. Only with DPPC is there formation of a minor component corresponding to an isotropic phase, observed as a sharp peak at 0 ppm using a high mol fraction of 20 mol% CSA-13 (Fig. 8). In all three cases the CSA-13 changes the shape of the powder pattern but retains a shape corresponding to a bilayer phase.

3.6. Leakage

Leakage of aqueous contents was carried out using liposomes made of lipid mixtures resembling the membrane composition of several different Gram positive and Gram

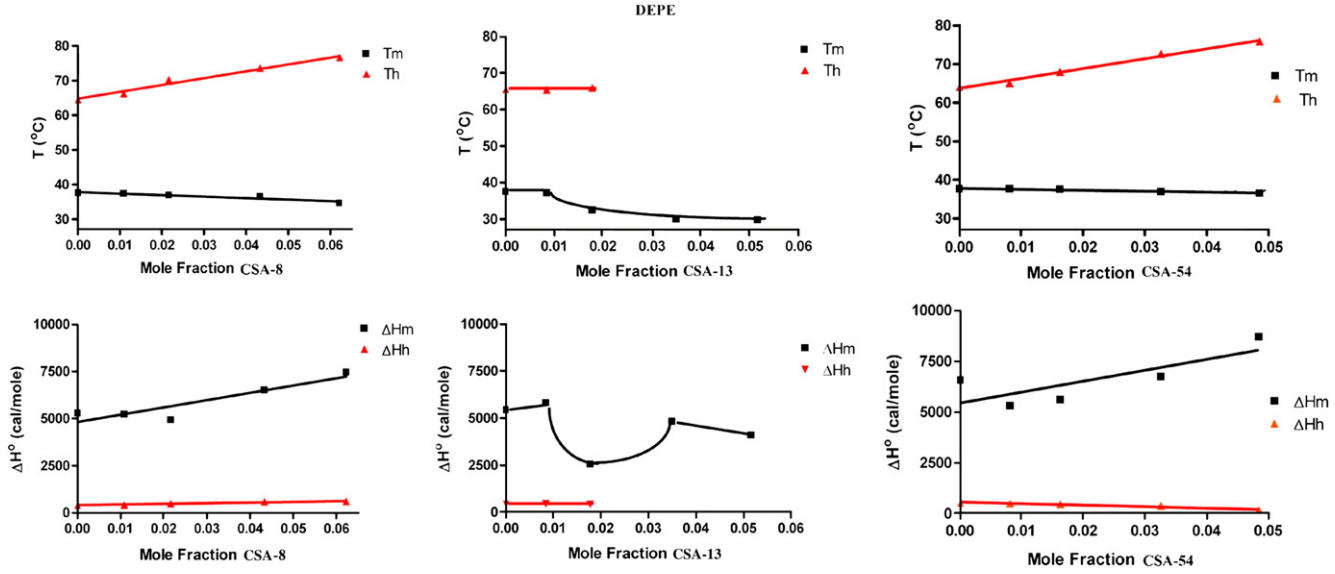

Fig. 3. Dependence of the enthalpy (.H) and transition temperature for the gel to liquid crystalline transition as well as the bilayer to hexagonal phase transition of DEPE on the presence of each one of the CSA compounds. Left curve, CSA-8; middle curve, CSA-13; and right curve, CSA-54.

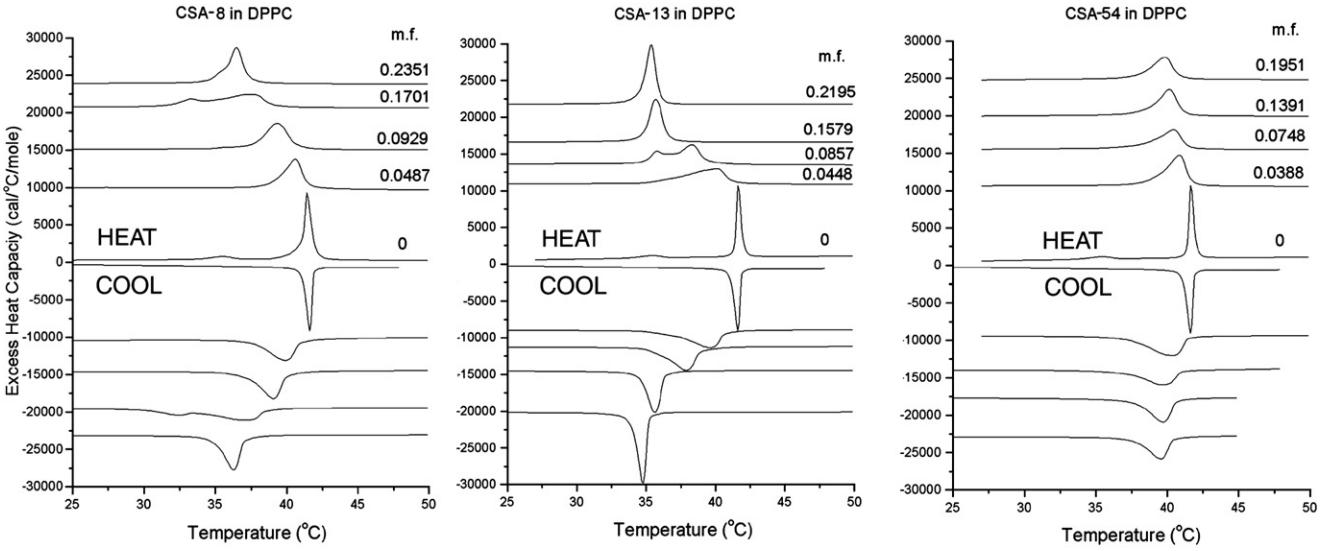

Fig. 4. DSC heating scans of DPPC with increasing mole fractions of CSA. Scan rate 0.75 o/min. Curves are displaced along the ordinate for ease of viewing. Left curve, CSA-8; middle curve, CSA-13; and right curve, CSA-54.

negative bacteria as well as those of mammalian cells. The major phospholipid components of the plasma membrane of these cells are shown in Table 2. The outer monolayer of the plasma membranes of mammalian cells is composed mostly of zwitterionic lipids, such as PC and sphingomyelin, with the anionic lipids and PE being on the inner monolayer. We have used liposomes of DOPC as a simple model for these membranes. Unsaturated acyl chains were used for the phospholipids in the leakage studies to avoid effects from phase transitions. We have also measured leakage from liposomes having a lipid composition resembling that of the Gram negative bacteria *P. mirabilis* or *E. coli* using a molar ratio of 8:2 DOPE:DOPG as well as using 8:2 DOPC:DOPG to assess the importance of the nature of the zwitterionic component. For Gram positive bacteria, we have also used a mixture of DOPE:DOPG:CL 15:80:5 corresponding to the membrane of *B. subtilis* as well as PG:CL 1:1 corresponding to the membrane of *Staphylococcus aureus* or *Streptococcus pneumoniae.* CSA-13 has the most potent membrane disrupting activity with liposomes having several different lipid compositions, followed closely by CSA-54 (Fig. 9). CSA-8 is generally less potent than either CSA-13 or CSA-54. None of the ceragenins is expected to be haemolytic, based on their lack of ability to induce leakage in liposomes of DOPC (Fig. 9). Also replacement of DOPE with DOPC in 8:2 mixtures with DOPG greatly decreases the observed leakage. In general,

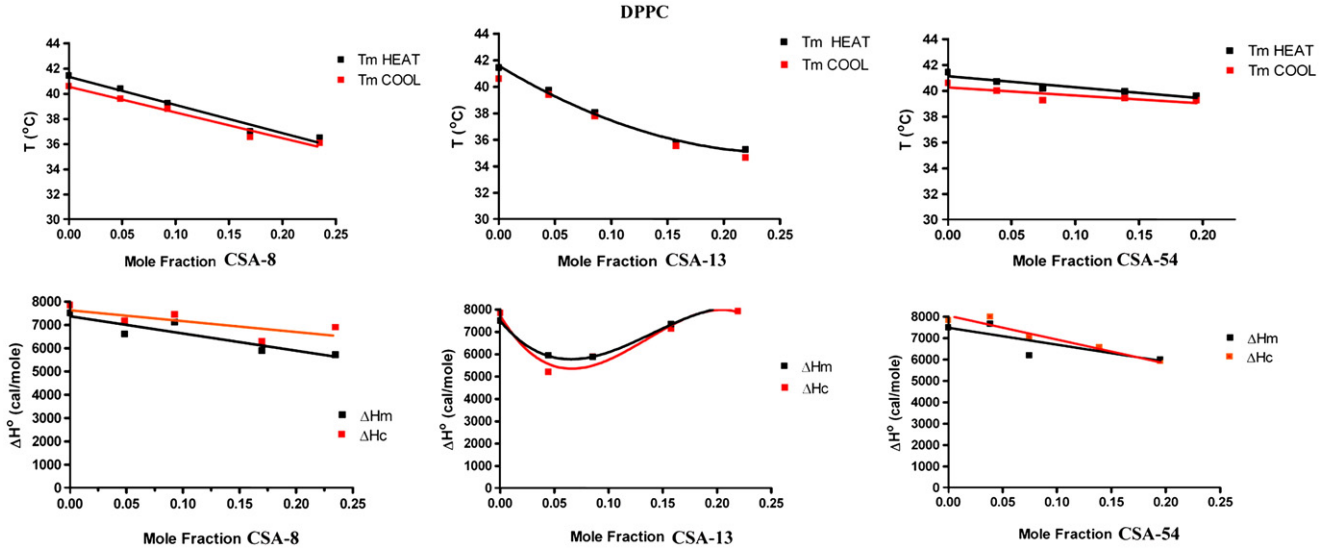

Fig. 5. Dependence of the enthalpy (.H) and transition temperature for the gel to liquid crystalline transition of DPPC on the presence of one of the CSA compounds. Red are values from cooling scans and black from heating scans. Left curve, CSA-8; middle curve, CSA-13; and right curve, CSA-54.

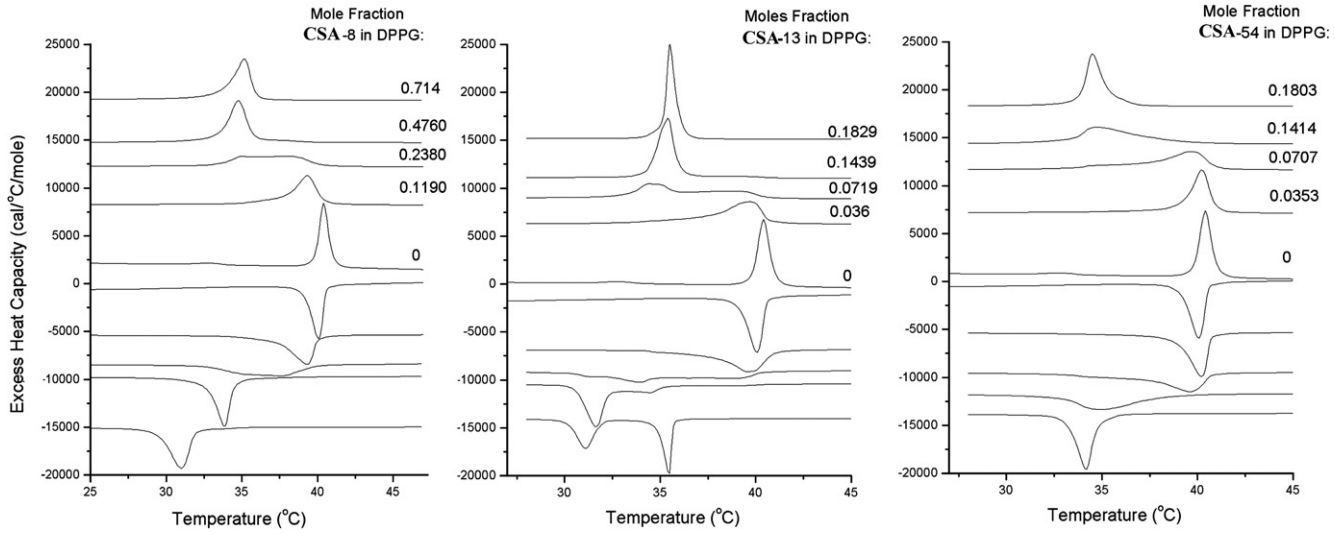

Fig. 6. DSC heating scans of DPPG with increasing mole fractions of CSA. Scan rate 0.75 o/min. Curves are displaced along the ordinate for ease of viewing. Left curve, CSA-8; middle curve, CSA-13; and right curve, CSA-54.

CSA-11 and CSA-27 are weakly active, except for CSA-27 with PG:CL 1:1.

3.7. Minimal inhibitory concentration

The MIC for the ceragenins against several bacterial species has been previously determined (Table 3). CSA-13 is the most potent of the compounds tested and CSA-11 and CSA-27 are the least active. All of the ceragenins have very weak haemolytic activity. In addition, the MICs against Gram negative bacteria are about 10-fold higher than against Gram positive bacteria. This contrast is most clearly seen with CSA-8. However, there are two exceptions to this. *P. mirabilis* is more resistant to these compounds than the other species, but even more striking is the high MIC for the Gram positive bacteria, *Bacillus anthracis*, that is closer to the MIC for the Gram negative bacteria. To our knowledge, the lipid composition of *B. anthracis* has not been determined. However, *B. anthracis* belongs to a group of closely related bacteria that also includes *B. cereus* and *Bacillus thuringiensis* [15]. The lipid composition of *B. cereus* is known (Table 2) and it is unusually rich in PE for a Gram positive bacteria. We have therefore determined the consequences of changing PE content in the bacterial membrane on their sensitivity to the ceragenin compounds.

E. coli is a Gram negative bacteria that is rich in PE (Table 2). The primary route for the synthesis of PE in these bacteria is via the decarboxylation of phosphatidylserine. Both the pss gene, coding for phosphatidylserine synthetase, as well as the psd

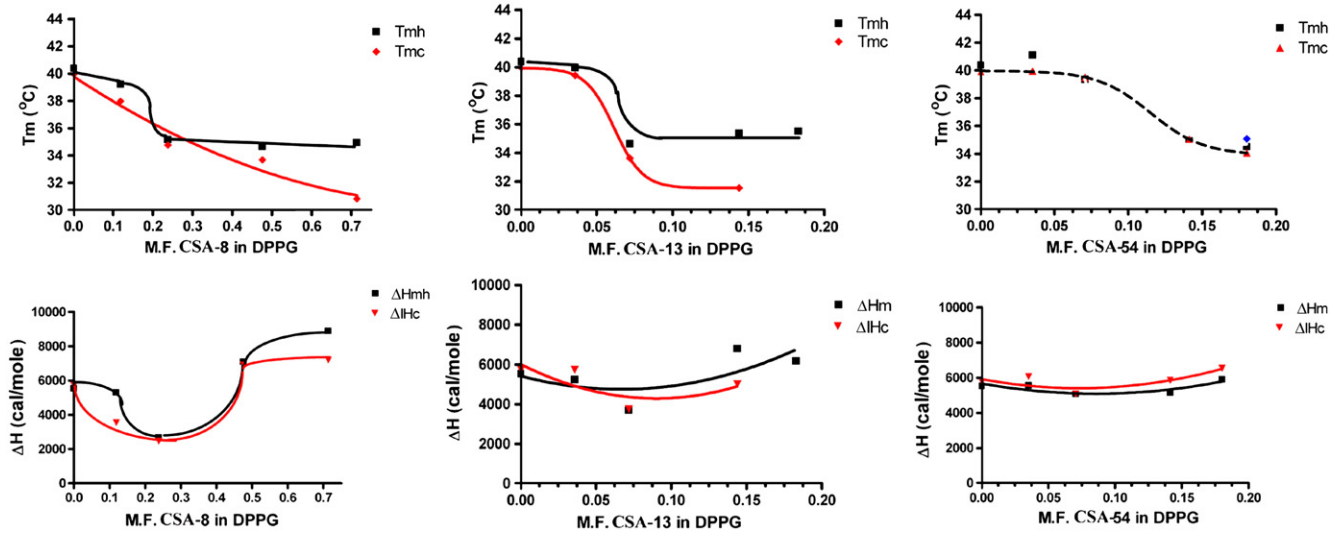

Fig. 7. Dependence of the enthalpy (.H) and transition temperature for the gel to liquid crystalline transition of DPPG on the presence of one of the CSA compounds. Red are values from cooling scans and black from heating scans. Left curve, CSA-8; middle curve, CSA-13; and right curve, CSA-54.

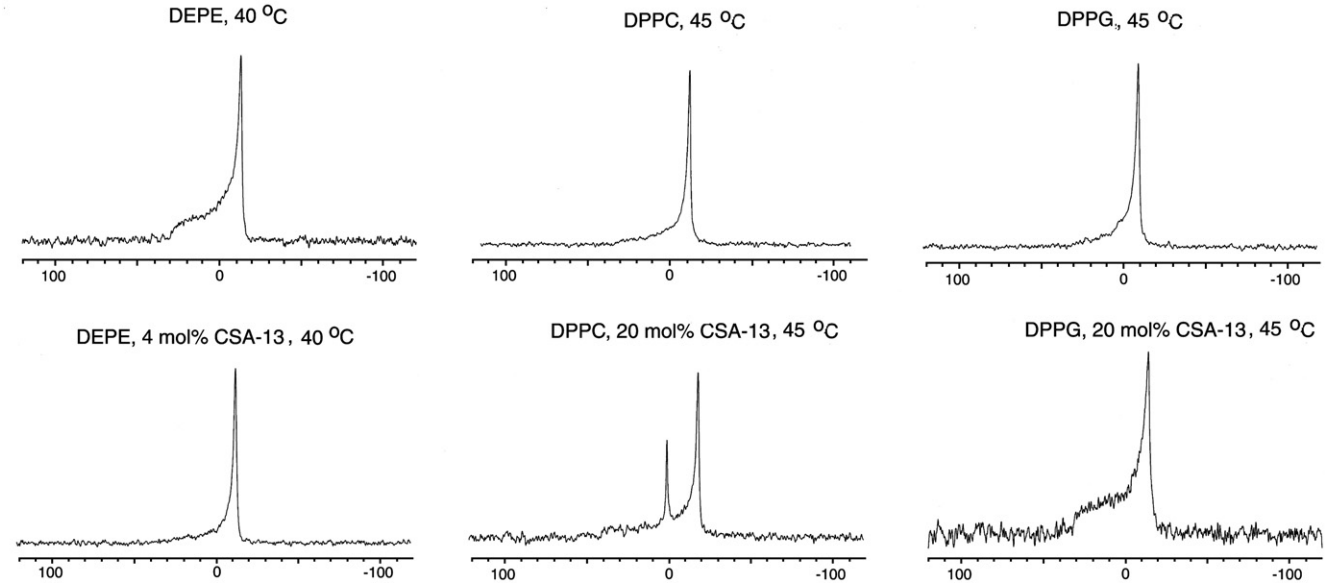

Fig. 8. ^{31}P NMR spectra of DEPE, DPPC or DPPG with or without the addition of CSA-13. In all samples the lipid concentration was 50 mg in 400 μL.

gene for phosphatidylserine decarboxylase are required for PE synthesis [16,17]. A pss− mutant of *Escherichia coli* has been constructed that is viable in the absence of PE in the membrane [18]. We have tested the antimicrobial activity of CSA-8 against this mutant compared with that against the wild-type *Escherichia coli*. We find no toxicity, up to 100 μg/mL, of CSA-8 against the +pss strain that contains the normal compliment of 80% PE. However, there was complete killing of the −pss strain at a CSA-8 concentration of 25 μg/mL. Thus, although both the +pss and −pss strains have outer membranes, the susceptibility of the strain with a low content of PE is significantly higher than the wild-type strain.

In order to further probe the relationship between the PE content of the bacteria and microbial resistance, we sought species of bacteria that were exceptions to the generalization that Gram negative bacteria are rich in PE and Gram positive are poor in PE. We already discussed one such example, *B. anthracis*. *B. cereus* and *B. polymyxa* are two other Gram positive bacteria with high PE. In addition, *C. crescentus* is an example of a Gram negative bacteria with low PE. The MICs for these bacteria were determined as part of the present study, under the same uniform conditions. The MIC values obtained for these bacteria species were compared with values obtained previously reported for CSA-8 against other species and good agreement was found with previous results. The new MIC

Table 2
Major phospholipid components

Bacterial species	% Total membrane phospholipid			
	PE	PG	CL	PC
Gram negative bacteria				
Proteus mirabilis [23]	80	10	5	–
E. coli [24]	80	15	–	–
Pseudomonas aeruginosa [25]	60	21	11	–
Caulobacter crescentus [a] [26–28]	–	40	–	–
Gram positive bacteria				
Bacillus polymyxa [29,30] [b]	60	3	8	–
Bacillus cereus [31]	43 [c]	40	17	–
B. anthracis [d]	43	40	17	–
B. subtilis [32]	12	70	4	–
S. aureus [33]	–	58	42	–
S. pneumoniae [34]	–	50	50	–
S. pyogenes [35]	Traces	–	Major component	–
Mammalian liver plasma membrane	23 [e]	18 [f]	1	39

[a] Neither PE or CL were detected. The major lipid component of this bacteria is monoglycosyldiglyceride, corresponding to about 45% of the lipid composition.
[b] Contains 26% lysolipids; 16% lysophosphatidylcholine and 10% lysophosphatidylserine [30].
[c] This value corresponds to bacteria grown at 37 °C. A higher value of 50–60% PE is generally found for this species when grown at 15 °C [31].
[d] Assumed to be the same as *Bacillus cereus* to which it is closely related genetically [15].
[e] PE is on the inner monolayer of mammalian plasma membranes and not exposed to the environment.
[f] Total anionic lipid = PS + PI + PA.

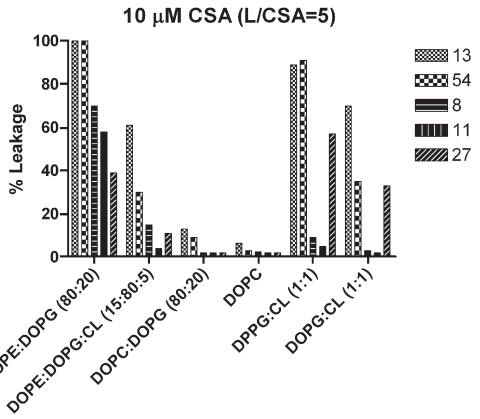

Fig. 9. CSA-induced aqueous contents leakage from liposomes of different lipid compositions. Leakage measured at 200 s using 10 μM CSA at a phospholipid to CSA ratio of 5.

Table 3

Organism	MIC Values (μg/mL) of Ceragenins[a]				
	CSA-13	CSA-54	CSA-8	CSA-11	CSA-27
Gram negative bacteria					
Proteus mirabilis	16	500	500	ND	ND
E. coli	3.0	7.0	36	80	85
P. aeruginosa	2.0	ND	20	85	ND
Gram positive bacteria					
B. anthracis	2.5	ND	20	ND	ND
S. aureus	0.4	2.0	2.0	9.0	ND
S. pyogenes	0.5	0.5	0.5	ND	ND
B. subtilis	0.5	1	0.5	ND	ND
Hemolysis					
Minimum hemolytic concentration	30	>100	100	>100	ND

ND=Not Determined.

[a] Data taken from [8,36–39] and provided by C. Genberg of Ceragenix Pharmaceuticals, Inc.

values in μg/mL for *B. polymyxa, B. cereus,* and *C. crescentus* are 35, 50 and 5, respectively.

4. Discussion

There are a wide range of cationic antimicrobial agents believed to produce toxic effects by several different mechanisms [19]. A common element among these agents is their positive charge that is believed to contribute to the specificity toward prokaryotes that have exposed anionic lipids. The mechanism of CSA-8 has been compared with that of certain cationic peptides [20]. CSA-8 causes depolarization of bacterial membranes at rates similar to that of magainin I, although at much lower concentration. CSA-8 also stimulates the activation of the same bacterial promoters as does cecropin. Also similar to many cationic antimicrobial peptides, CSA-8 also shows, by electron microscopy, the disruption of bacterial membranes. It thus seems likely that there is a relationship between the mechanism of action of the ceragenins and that of certain cationic antimicrobial peptides.

Cationic antimicrobial agents differ greatly in their selectivity for different bacteria. There are some agents that exhibit a broad range of antimicrobial action including both Gram positive as well as Gram negative bacteria. However, there are also agents, such as polymyxin, that are selective for Gram negative bacteria because they have an affinity for a component of the outer membrane [21]. There are also several cationic peptides, as well as the ceragenins, that have higher toxicity against Gram positive bacteria than against Gram negative bacteria. It is often assumed that the outer membrane of Gram negative bacteria affords them greater protection by preventing access of antimicrobial agents to the inner membrane. However, this does not appear to be the case with CSA-8, since removal of PE in the pss− mutant of E. coli leads to increased sensitivity to this antimicrobial agent. The −pss strain has an outer membrane as well as the same protein content and composition and acyl chain composition as the +pss form [17]. The −pss mutant has an increase in cardiolipin content that could cause an increase in susceptibility to toxic cationic agents. However, these cells can only be grown at very high concentrations of Mg^{2+}, above 20 mM! It is therefore likely that the divalent cations will screen the electrostatic interactions between CSA-8 and the cell membrane. This also explains why the +pss mutant has resistance to CSA-8 up to 100 μg/mL when the MIC is determined in the presence of 20 mM Mg^{2+}. What can be concluded is that even though the outer membrane is still present, the pss− mutants have greater susceptibility to CSA-8. We suggest that this must be a consequence of the loss of PE from the plasma membrane.

Although generally Gram positive bacteria have a low PE content in their membranes and Gram negative bacteria have a high PE content, exceptions can be found.

B. polymyxa, *B. cereus* and consequently also *B. anthracis* have a relatively high content of PE, although they are Gram positive bacteria (Table 2). *C. crescentus* is an example of a Gram negative bacteria with a low content of PE (Table 2). The MIC values for CSA-8 appear to be more closely related to the PE content of the bacteria than to the presence or absence of an outer membrane (Table 4). Thus the three Bacilli with high PE have MICs comparable to most of the Gram negative bacteria, while *C. crescentus* with a low PE has a low MIC comparable to that of the majority of Gram positive bacteria. *P. mirabilis* has a higher MIC than other Gram negative bacteria, possibly because the outer membrane contributes more to resistance for this bacterium than for others. It should also be noted that bacteria with high PE will also have less anionic lipid and this could contribute to the higher resistance of these organisms. The fact that the pss− mutants have lower resistance to CSA-8 does not negate this possibility since these assays had to be performed at very high concentrations of divalent cations where the role of charge interactions is likely reduced.

We have further probed the mechanism of action of CSA-8 and its relationship with other ceragenins. There is a relationship between the potency of the ceragenin against Gram negative bacteria having a high PE content and its ability to induce leakage in liposomes having a similar lipid composition of DOPE:DOPG (80:20) (Fig. 9). Thus CSA-8 is always less potent than CSA-13 for several Gram negative bacteria (Table 3) and its

Table 4
CSA-8 MIC and PE content

Bacteria	MIC (μg/mL)	% PE
Gram negative bacteria		
Proteus mirabilis	500	80
Escherichia coli	36	80
Pseudomonas aeruginosa	20	60
Caulobacter crescentus	5	~0
Gram positive bacteria		
Bacillus polymyxa	35	60
B. anthracis	20	43
Bacillus cereus	50	43
Bacillus subtilis	0.5	12
S. aureus	2	~0
S. pyogenes	0.5	~0

ability to induce leakage in liposomes having a lipid composition with 80% PE (Fig. 9). The situation is less clear for CSA-54 that has a MIC close to CSA-8 with *P. mirabilis*, where other factors may come into play, but has a MIC close to that of CSA-13 and its leakage potency with these liposomes is like that of CSA-13. In addition, all of the ceragenins are weakly haemolytic (high minimal concentration required to induce hemolysis), with CSA-13 being the most haemolytic among these compounds (Table 3). This is also in accord with the observation that none of the ceragenins is very effective in causing lysis to zwitterionic liposomes of phosphatidylcholine alone or with DOPC as the major component, although among the ceragenins tested, CSA-13 is again the most effective (Fig. 9).

Except for *B. anthracis*, the MICs for the other three Gram positive bacteria are all low and insensitive to the structure of the ceragenins, and are also similar for all three bacterial species (Table 3). Despite this, leakage studies with liposomes having the lipid composition of *B. subtilis*, *i.e.* PE:PG:CL (15:80:5) show large differences in leakage potency among the ceragenins (Fig. 9). This is also true for leakage induced in liposomes resembling the lipid composition of *S. aureus*, i.e. PG:CL (1:1) that shows a much wider variation among the ceragenins (Fig. 9) that does not correspond to the narrow range of MIC values for these bacteria (Table 3).

There thus appears to be two different mechanisms of actions of the ceragenins. One being of lower potency against bacteria with a high PE content that correlates with liposomal leakage and the other being of higher potency against bacteria that have membranes composed predominantly of anionic lipids. The bacteria with a low PE content are killed by the ceragenins by a mechanism that does not correlate with membrane leakage, suggesting that it is not through the formation of pores in the bacteria. This mechanism is insensitive to the structure of the ceragenins or even of the overall charge on the ceragenin. Electrostatic interactions of the ceragenins with anionic lipids or with LPS would be expected. However, the MICs of CSA-54 and CSA-8 are the same against *S. aureus* (Table 3), even though CSA-54 has a charge of +6 compared with +3 for CSA-8. Unlike the antimicrobial activity, charge on the ceragenin may contribute to the extent of liposomal leakage. For example, the leakage induced in the liposomes of PG:CL (1:1) by CSA-54 is much greater with CSA-8. This may also explain why CSA-27, having a charge of +6, although generally found to have weak antimicrobial activity is potent in inducing leakage in the PG:CL lipid mixture (Fig. 9). Thus charge is not the only factor determining the antimicrobial activity, even against *S. aureus*, a bacterium having a membrane composed almost entirely of anionic lipids.

It is striking that the change from DOPE:DOPG (8:2) to DOPC:DOPG (8:2) results in a dramatic loss in the membrane rupturing capacity of the ceragenins. The structural difference between PE and PC is that an ammonium group ($-NH_3^+$) is changed to a trimethylammonium group ($-N(CH_3)_3^+$). Hydrogen bonding with the N–H bonds in PE may occur, while a similar interaction is not possible with PC. Membrane curvature effects may also play a role. If the ceragenins act by forming a pore lined with both lipid and ceragenin, it would be anticipated that pore formation would be facilitated by drugs that promote positive membrane curvature as has been found with magainin [22]. However, all three of the ceragenins tested, CSA-8, CSA-13 and CSA-54 raise the bilayer to hexagonal phase transition temperature of DEPE by 200±20, 18±23 and 257±16, respectively. The observation that the curvature effects are smallest for the most potent CSA-13 suggests that promotion of positive curvature may inhibit the action of these compounds against bacteria with a high PE content.

One physical property that is particular to the ceragenins is the effect they have on the gel to liquid crystalline phase transition of DPPC and DPPG. Usually when an additive is introduced to a pure lipid component, the melting behaviour of the pure component is broadened, demonstrating that the transition is becoming less cooperative. However, in the case of the ceragenins, their interactions with both DPPC and DPPG show an initial broadening followed by the formation of a component with a new transition temperature and high cooperativity. Although not identical, the three ceragenins tested behaved in a similar manner with both DPPC and with DPPG. This property may contribute in some way to the antimicrobial activity of these compounds since the most potent analog, CSA-13, is also the ceragenin that affects the phase transitions at the lowest concentration. CSA-13 is also the only ceragenin that promotes formation of a new cooperative transition with DEPE at lower temperature (Fig. 2), indicating that it also forms complexes with PE. We also measured the ^{31}P NMR powder patterns with these mixtures and observed that CSA-13 did not induce the formation of non-lamellar phases, although they did modify the shape of the powder patterns without affecting the chemical shift anisotropy (Fig. 8). There is also another unique feature of the interaction of CSA-13 with the zwitterionic lipids, DEPE and DPPC. The ΔH of the main transition of these lipids changes in a biphasic fashion for CSA-13, while for CSA-8 and CSA-54 there is a monotonic increase of ΔH with increasing mol fraction of ceragenin with both DEPE (Fig. 3) and with DPPC (Fig. 5). This unique feature indicates that CSA-13 has specific interactions with phospholipids, a property that may contribute to its antimicrobial potency.

Thus, there is a relationship between the antimicrobial activity of these compounds and the lipid composition of a bacterial membrane. Bacteria with high PE content are more resistant to the action of these agents. Among the different ceragenins, those that are more effective in rupturing liposomes with a high PE content also have lower MICs for these bacteria with high PE. Since the ceragenins have a conformationally restricted fused ring structure and chemically modified forms are relatively easy to synthesize, the increased understanding of their specificity and mode of action provided by the present work will contribute to the design of new analogs with increased potency and broader range of activity.

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