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Pore formation in and enlargement of phospholipid liposomes by synthetic models of ceramides and sphingomyelin

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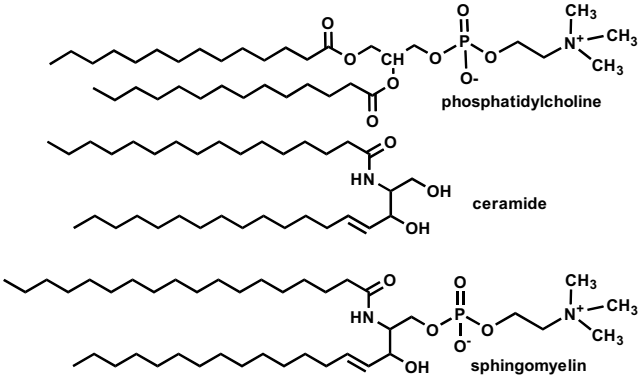
Abstract—A family of compounds having twin octadecyl anchor chains and various polar headgroups were designed to be ceramide mimics. The compounds prepared increase the apparent permeability of phospholipid vesicles to chloride and carboxyfluorescein anions. In addition, significantly larger vesicles are observed after exposure to these compounds suggesting the possibility of vesicular fusion.

1. Introduction

Sphingosines and ceramides are part of the astonishing diversity of lipid structures found in membranes.[1–3] Most major membrane lipids have hydrocarbon, midpolar, and headgroup regimes that support lipid bilayer assembly. The minor lipid components display more structural variation, which probably reflects individual functional roles.[4] Sphingolipids[5] are substituted aminoalcohols of eukaryotic plasma membranes that are not found in bacterial or plant cells. Sphingomyelin is an analog of phosphatidylcholine whose structure possesses an amide link and a secondary hydroxyl group in the midpolar regime. In eukaryotic cells, sphingomyelinase cleaves this molecule removing the phosphocholine headgroup to form the hydroxylated lipid, ceramide. This important lipid molecule coordinates cellular activities as diverse as differentiation and programmed cell death (apoptosis).[6] The structure of ceramide suggests that it does not assume the bilayer-forming role as strongly as sphingomyelin or the dominant phospholipids. However, despite their wide distribution in animal membranes[7] and their role in critical cellular functions,[8] the biological chemistry of these hydroxylated lipids is not well established.[9]



Our interest in ceramide mimics was particularly piqued by recent reports that these lipids and certain analogs form pores or channels as part of their function in apoptosis. Siskind and Colombini reported the formation of large and stable pores in phospholipid membranes by using C2- and C16-ceramide derivatives. They suggested that these pores are one mechanism responsible for the passage of cytochrome *c* from mitochondria.[10] This

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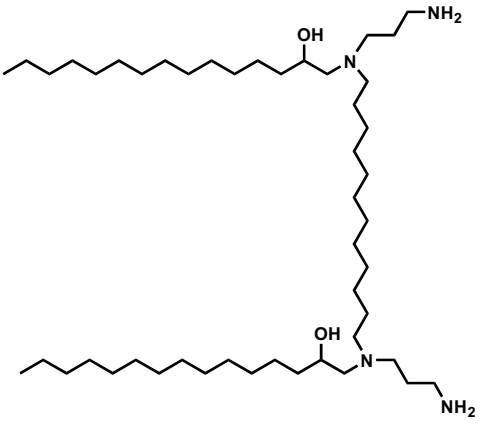
postulate assigns a pore-forming role to ceramide in the mortality decision of apoptosis. Bieberich et al. have used ceramide analogs in an effort to induce apoptosis in human cancer cells.[11] They prepared hydroxylated fatty amide derivatives of the general type CH_3-$(CH_2)_nCON(CH_2CH_2OH)_{1,2}$. Chanturiya et al. have recently reported several synthetic lipids that are structurally related to ceramide.[12] They present evidence that these compounds form channel-like pores in phospholipid bilayers. These studies all suggest that hydroxylated lipids, which lack the phosphocholine head group, form membrane pores.

In our own efforts to develop an anion-selective channel,[13,14] we prepared a family of molecules using dialkylamine derivatives of diglycolic acid as a membrane anchor.[15,16] The components of this anchor system were chosen so that assembly could be straightforward and modular and so that the final resulting amphiphile would be similar to the corresponding portion of a phospholipid. For example, the amine's alkyl groups correspond to the alkyl chains of fatty acid esters in a phospholipid.



BHHD-TADC

Our goal in this work was to determine if simple analogs of our chloride-conducting channel[15] would also show membrane activity. We recognized that the anchor systems themselves were, at least in some measure, structurally and functionally similar to ceramides and to other reported synthetic structures. We expected the structures we developed to insert in the bilayer with the long hydrocarbon tails aligned with the phospholipid acyl chains. The structural model proposed by Siskind and Colombini requires the formation of a hexameric (or oligomeric) H-bond network to form the pore and has the hydrocarbon chains radiating laterally from a hexameric pore that is perpendicular to the acyl chain long axis within the bilayer. We comment below on this proposed structure but acknowledge that pore formation was demonstrated convincingly when ceramide was present in phospholipid bilayers.

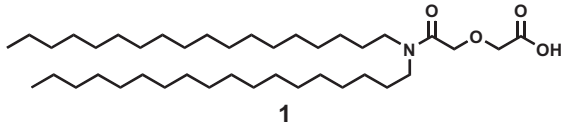
The pore-forming, ceramide-mimetic compounds reported by Chanturiya et al. are dimers connected by a dodecyl chain. Each 'twin' structure possesses two primary nitrogens, two tertiary nitrogens, and two hydroxyl groups. The six heteroatoms presumably comprise the amphiphile's headgroup and the two tetradecyl or hexadecyl chains are the tails (shown, called BHHD-TADC). There is no comment in the report about why particularly the dodecyl connector chain was used. There is also no mention of whether the individual subunits connected to form BHHD-TADC were studied. A mechanism by which pore formation may occur is presented, although no structural model for the pores is advanced. The evidence presented for anion-selective pore formation by these twin-chained structures is convincing.

We report here an effort to develop synthetic ceramide mimics and to assess their properties. We present evidence that these individual ceramide mimetic monomers are capable of forming stable aggregates. In addition, they form stable pores in phospholipid liposomes that have been assayed for release of chloride and the fluorescent dye carboxyfluorescein.

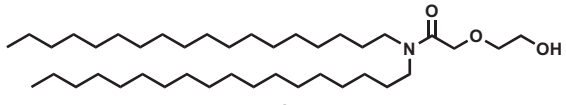
2. Results and discussion

2.1. Compounds used in this study

The amphiphiles used in this study were prepared from $(C_{18}H_{37})_2NCOCH_2OCH_2COOH$, **1**, which we have used as a membrane anchor unit in the preparation of a synthetic chloride channel.[15] Compound **1** was formed by the reaction of diglycolic anhydride in refluxing toluene (48 h) with dioctadecylamine; it was obtained in 87% yield as a white solid (mp 80–81 °C). Treatment of **1** with ethyl chloroformate and triethylamine followed by reduction with $NaBH_4$ in the presence of CH_3OH gave **2** (mp 51–52 °C) in 88% yield. Compound **3** was also obtained from **1** by coupling of the acid with diethanolamine chlorotripyrrolidinophosphonium hexafluorophosphate (PyCloP) and diisopropylethylamine (DIEA). Diol **3** was obtained as a white solid (mp 60–61 °C) in 72% yield after chromatography.



1



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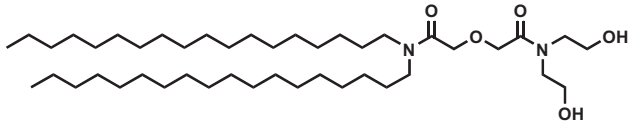

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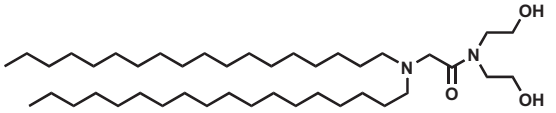

4

Diol **4** is a derivative of glycine rather than of diglycolic acid. It was prepared from ethyl bromoacetate by alkylation with dioctadecylamine (Na_2CO_3, KI, 95%) in refluxing butyronitrile. Heating with 2 N aqueous NaOH quantitatively saponified the ester. Activation of the carboxyl group (ethyl chloroformate, Et_3N, THF) followed by reaction with diethanolamine gave **4** as a white solid (81%, mp 54–55 °C). Details are presented in Section 4.

2.2. Aggregation behavior

Compounds **1**–**4** all have twin 18-carbon tails and polar headgroups. It seemed reasonable to assume that these compounds could form aggregates in aqueous suspension. An isopropyl alcohol solution of each compound (~7 mM) was prepared. An aliquot (2.5–40 μL) of each was diluted into 2 mL of doubly distilled water. Spontaneous aggregate formation (no sonication) was assayed by using dynamic light scattering (Coulter N4MD submicron particle analyzer).

Compound **3** formed aggregates, but they quickly precipitated. Measurement of aggregate size shortly after dilution revealed that only about 10% of the monomers remained as part of the suspended material. The apparent aggregate size was approximately 300 nm (3000 Å). In the case of compound **4**, a higher percentage of the monomer formed aggregates (~50%) and the average size of the particles was smaller (~150 nm). After 24 h, aggregates could no longer be detected, indicating either that they were unstable or that they precipitated, or both.

In contrast, compounds **1** and **2** spontaneously formed stable aggregates. In the case of compound **2**, no significant amount of precipitate was observed. Assemblies formed from **2** were between 150–250 nm in size as reported by the light scattering instrument's internal software. The aggregates were still present after 24 h. Aggregates formed spontaneously from **1** when the monomers were suspended in aqueous solution in the concentration range approximately 0.1–1.3 mM. Light scattering detected particles of ~100–150 nm diameter formed along with a precipitate that was typically more than 20-fold larger (~3000 nm). No clear trend was observed in aggregate size over this range. Thus, 90–100% of smaller aggregates were observed at concentrations of 0.1, 0.4, 0.5, and 0.7 mM. At concentrations of 0.2, 0.3, 0.6, 0.8, 0.9, 1.0, 1.1, 1.2, and 1.3 mM, a bimodal distribution was observed in which about half small and half large particles were detected. The aggregation behavior of these monomers is summarized in Table 1.

We conclude that aggregate formation is generally facile from **1** but either the aggregates are not stable or neither size is energetically favored. The key observation remains that aggregates form spontaneously from **1** and this often, but not always, occurs in the absence of any detectable precipitate.

The behavior of **1** was examined more closely. Aggregates were prepared by a procedure similar to the one employed for vesicle preparation described in detail in Section 4. The unimodal diameter was initially 202 ± 48 and 318 ± 48 nm after 24 h. When the aggregates were prepared in the presence of 10 equiv of NaCl, the unimodal diameters were essentially unchanged: they were initially and after 24 h, respectively, 228 ± 66 and 243 ± 86 nm. In the presence of 10 equiv of KCl, the corresponding values were 210 ± 64 and 207 ± 57 nm.

Table 1. Aggregation behavior of compounds **1**–**4** in aqueous solution[a]

Compd No.	Precipitate formed?	% Monomer in aggregate	Aggregate diameter (nm)	Aggregate stable 24 h?
1	—[b]	—[b]	100–150	Yes
2	No	100	150–250	Yes
3	Yes	~10	~300	No
4	Yes	~50	~150	No

[a] Result of three repetitions, initial concentration ~7 mM.
[b] Precipitate occasionally formed, see text.

2.3. Effect of compounds 1–4 on phospholipid vesicles

The major goal of the work described here was to assess the effect of compounds **1**–**4** on the properties of bilayer membranes. In particular, we wished to determine if these extremely simple structures engendered pore formation. The model for these tests was a synthetic liposome system prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate monosodium salt (DOPA). Chloroform was evaporated from the commercially available monomer solutions to afford dry lipid films of DOPC–DOPA (20 mg, 7:3 w/w). These were dissolved in diethyl ether (0.5 mL) and then exposed to 0.5 mL of an aqueous salt/buffer phase (600 mM KCl, 10 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH = 7.0). After 10–20 s sonication, the organic solvent was evaporated in vacuo from the opalescent dispersion. The resulting suspension was filtered (200 nm membrane filter) and passed through a Sephadex G25 column equilibrated with 400 mM K_2SO_4 (see Section 4 for additional details). In all cases, the vesicle preparations were 200 nm in diameter (light scattering) and stable at room temperature for 48–72 h. In the case of carboxyfluorescein (CF) release experiments, the internal buffer was 20 mM CF, 100 mM KCl and 10% HEPES (pH = 7.0) while the external buffer was 100 mM KCl and 10% HEPES (pH = 7.0).

2.4. Anion release induced by 1-4 and C_{16}-ceramide

Pore forming activity was studied in the phospholipid vesicles described above. Both chloride and carboxyfluorescein (CF) release were assessed in different experiments.

2.5. Chloride release from liposomes

Chloride release was assayed directly with a chloride-selective electrode. The electrode was allowed to equilibrate and then vesicles were added. The voltage output

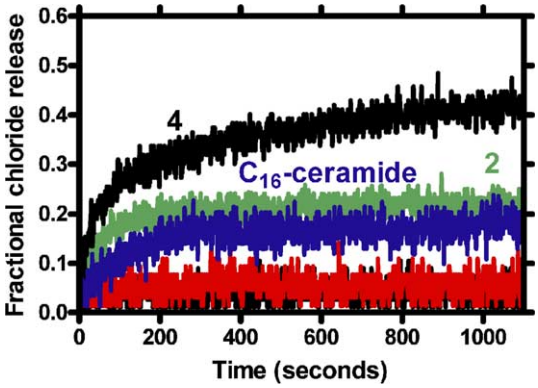

Figure 1. Graph showing fractional chloride release by C_{16}-ceramide and compounds **1**–**4** (96 μM). The traces are: **4**, black (top); **2**, green (second from top); C_{16}-ceramide, blue (middle); **3**, red and **1**, black (nearly superimposed at the bottom).

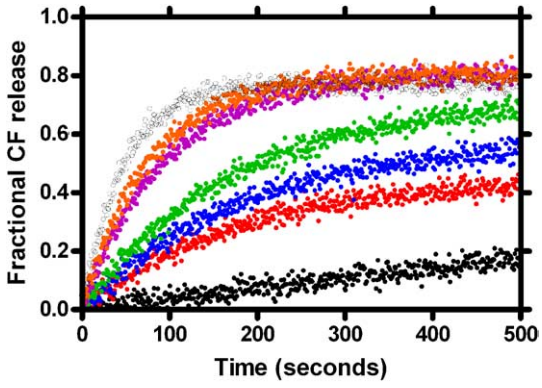

Figure 2. Concentration dependent release of CF by **4** from vesicles (~3 μM phospholipid (DOPC–DOPA, 7:3)) in 100 mM KCl, 10 mM HEPES, and pH 7.0. The concentrations are, from bottom to top, 0.5, 1.0, 2.9, 3.9, 7.7, 11.5, and 15.2 μM.

was recorded and, after 5 min, aliquots of the compound solution (5–9 mM in 2-PrOH) were added. No more than 20 μL of each compound was added to avoid any deleterious effect of isopropyl alcohol on the liposomes. Complete lysis of the vesicles was induced by addition of an aqueous solution of Triton X-100 (100 μL, 2% by weight) and data were normalized to the ultimate release value. Fractional chloride release data obtained by this method are summarized in the graph of Figure 1.

The reference compound for this comparison is natural C_{16}-ceramide. Its chloride release trace is shown in blue approximately in the middle of the graph. Compound **2** is approximately as effective at releasing chloride ion but **4** is significantly better as judged by this method.

Compounds **1** and **3** did not show chloride release activity (bottom traces in Fig. 1). We speculate that for **3**, at least, this might be due to aggregate formation and precipitation upon addition to the liposomal solution. Limited Cl^- release from vesicles was observed when either **2** or **4** was present. This reduced Cl^- transport might be caused by a number of factors including precipitation of the added compound or simply a lack of activity in this context. Another possibility is that impermeant SO_4^{2-} ion cannot transport into the vesicles and the charge imbalance thus created hinders the exit of chloride. Notwithstanding the variations exhibited by these compounds, C_{16}-ceramide, **2**, and **4** did show clear evidence of chloride release under these conditions.

2.6. Carboxyfluorescein release from liposomes

In contrast with the modest Cl^- release, the effect of compounds **2**–**4** upon CF dequenching was large. The dequenching of CF has often been used to characterize liposome disruption because it provides high sensitivity and time resolution. We have analyzed the concentration dependence of dequenching for compounds **2**–**4**. The activity of **4** was greatest (as in the chloride release experiments) and achieved 80% dequenching as compared to liposome disruption by detergent. The dequenching of all three compounds was characterized as to rate (time constant) and extent. The rate of dequenching was concentration dependent as shown for **4** in Figure 2.

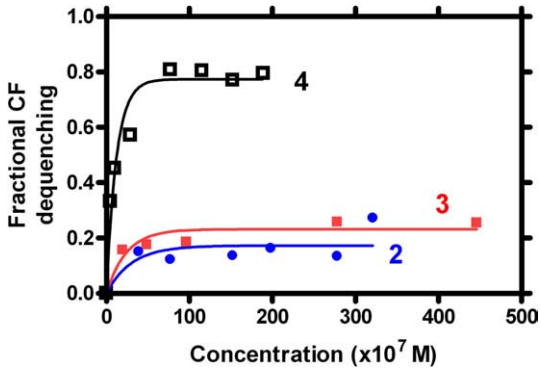

Figure 3. Fractional total fluorescence (at λ = 520) dequenching for **2**, **3**, and **4** as a function of concentration.

Fluorescence dequenching was studied for **2**, **3**, and **4** over a range of concentrations from 0.48 to 15.2 mM or about 30-fold. The extent of dequenching was greater for compound **4** than for either **2** or **3**. The behavior of these compounds with CF corresponds generally to the chloride release activity observed for these compounds as noted above. A graph is shown in Figure 3 that presents the fractional total CF dequenching as a function of concentration.

2.7. Pore formation in liposomes

Both chloride and carboxyfluorescein anions are released from phospholipid vesicles by **3** and **4**. Each of these compounds contains twin hydrocarbon chains that should align in the bilayer with the acyl chains. We speculate that formation of membrane pores involves two or more monomers as we have found for the amphiphilic heptapeptide derivatives that we previously reported. This model contrasts with that suggested by Siskind and Columbini for the compounds they studied. The authors describe the latter model as 'a column of ceramide residues held together by intermolecular hydrogen bonds between amide and carbonyl groups. This column

would span the hydrophobic portion of the membrane and, in association with other columns, would form pores of various sizes. . ..' The figure in the paper shows a pore of unspecified size presumably created by a lateral NH···O hydrogen bond network. It is unclear how the figure was created but when we examined six CPK model monomers, we were unable to assemble the structure. When the NH···O H-bonds were present, the acetyl methyl groups blocked the pore. When the six monomers were separated sufficiently to 'unblock' the pore, the H-bonds became impossibly long. If such a hexamer did form, models suggest that a vertical stack of hexamers could be stabilized by the proposed H-bond formation involving the hydroxyl groups.

The molecular models do not show unequivocally what orientation the alkyl side chains would have. As shown in the Siskind/Columbini paper, they radiate laterally into the hydrocarbon slab. While we have no evidence to contradict this arrangement, it seems odd that the long hydrocarbon chains would be perpendicular, rather than parallel, to the lipid chains. It is noted that the 'nonapoptogenic precursor, C2-D-*erythro*-dihydroceramide, lacks the 4,5-*trans* double bond present in ceramide. Rotation about the 4,5 single bond in dihydroceramide could inhibit the formation of the large stable pore structures either by making these structures unstable or preventing their formation.' It is unclear to us why rotation about the single bond would prevent pore formation, based on the proposed model. In the absence of evidence to the contrary, we assume that pores form from the compounds reported here by inserting into the phospholipid bilayer with the polar residues near the phospholipid headgroups and the hydrocarbon chains parallel to the membrane's acyl chains.

2.8. Change in diameter of existing liposomes

Carboxyfluorescein within liposomes self-quenches. When it is released into the bulk medium, fluorescence increases as quenching decreases owing to lower local concentration of the dye. Fluorescence can also increase if the dye is not released and the liposomes increase in size. A larger volume will dilute intravesicular CF and produce the increased fluorescence. The investigation of this possibility led to an interesting finding of the present work: the effect of compounds **2**–**4** on the sizes of the liposomes to which they are added. The graph of Figure 4 shows the results of the following experiment. Vesicle suspensions containing 3.26×10^{5} M lipid were sized by dynamic light scattering (see Section 4) and found to have average diameters of 197 nm. Compounds **2**–**4** were added to these vesicles in separate experiments. Each compound was added portion-wise (5 mM in 2-PrOH) to the vesicles and, after waiting 10–15 min for equilibration to occur, light scattering was used to determine the vesicle diameter.

A surprisingly large increase in liposome diameter was detected (by light scattering measurements) shortly after addition of any of the three compounds. This is shown graphically in Figure 4. Compound **1** had little effect on the vesicle diameter (not shown in the graph) but

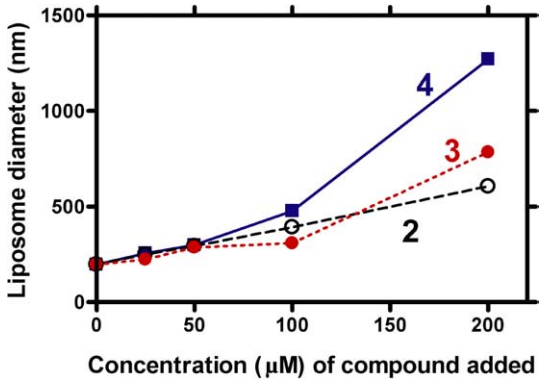

Figure 4. Plot of increase in liposome diameter from ~200 nm upon addition of **2** (open circles), **3** (filled circles), and **4** (filled squares).

compounds **2**, **3**, and especially **4** led to dramatic size increases. We note that the ultimate volume increases for **2**, **3**, and **4**, respectively, were ~60-, ~30-, and ~270-fold.

An increase in fluorescence upon dilution is possible since the liposomes are enlarging. In order to gain additional insight into this phenomenon, a vesicle sample prepared for a CF dequenching experiment was passed over a Sephadex G25 column (equilibrated with 100 mM KCl, 10 mM HEPES, pH = 7). If the vesicles were lysed with Triton X-100 prior to passage over the column, 90% of the CF eluted in a fairly narrow band. The same band was obtained when the vesicles were incubated with **4** (30 μM) for 300 s and then passed over the column, indicating that CF is actually released from the liposomes. As shown in Figure 2, such conditions produced an almost complete dequenching of the CF. A similar experiment was conducted with compound **3**, which is able to produce only partial dequenching (Fig. 2). In this case, the fluorescence was observed in two bands when passed over the Sephadex column. About half of the fluorescence intensity was observed in the band that eluted at the retention time observed for the lysed vesicles and with **4**. The other half of the fluorescence signal was in a fraction that eluted earlier. The faster-eluting peak corresponds to the fraction of CF that is not released, but partially dequenches owing to the enlarged liposomes. Evidently, the CF dequenching observed can be attributed to two different components because the dye that is retained within the enlarged liposomes can contribute to the observed fluorescence increase.

There are two obvious explanations for liposomal enlargement of the type observed here. Compounds **2**–**4** could simply insert into the bilayer. The increased number of monomers would increase the size of the vesicle and, consequently, its volume and diameter. Alternately, these compounds could induce vesicular fusion. In principle, the fusion of two liposomes would essentially double the number of monomers in a single vesicle, but the internal concentrations of CF would not be increased unless water enters the liposome. Increasing the number of lipid monomers would increase the surface area of the liposome relative to

the volume and result in a change of liposome shape. In our case, we assume that water enters the liposome since pores are formed upon addition of the ceramide analogs. In this case, the number and surface area of the bilayer monomers characterize the size of a spherical vesicle, and twice the number of monomers in the bilayer would produce a proportionately larger, spherical vesicle.

The initial outer sphere radius of the vesicles shown in Figure 4 is 98 nm. The outer surface is, therefore, $\sim 1.21 \times 10^5\,nm^2$. If the membrane thickness is approximately 5 nm, then the inner radius is 93 nm and the surface area is $1.09 \times 10^5\,nm^2$. The vesicles used in this study are 2:1 (mol/mol) DOPC/DOPA. Based on the composition, published headgroup area values, and our own examination of molecular models, we estimate an average lipid in this system to have a surface area of $0.5\,nm^2$. Using this value, there are 241,374 lipids in the outer leaflet and 217,373 lipids in the inner leaflet, for a total of 458,747 lipids per vesicle.

In each case above, the concentration of compound added is 0.20 mM (200 μM in Fig. 4), which translates to 2.41×10^{17} molecules in the 2 mL experimental volume. The starting concentration of phospholipids in the vesicle formation experiments was 3.26×10^{-5} M, which equals 3.93×10^{16} lipids. If there are 4.58×10^5 lipids per liposome, a maximum of 8.56×10^{10} liposomes will be formed. If the 2.41×10^{17} ceramide mimics distribute evenly among $\sim 10^{11}$ liposomes, each vesicle will add 2.8×10^6 molecules. This would give a total surface area increase between the inner and outer bilayer leaflets of $9.3 \times 10^4\,nm^2$. This is based on an estimate made from molecular models that the average surface area of the ceramide derivatives is $\sim 0.33\,nm^2$. The actual increase in surface area observed is approximately $1 \times 10^7\,nm^2$, in the case of compound **4** (this number depends on the monomer, see Fig. 4).

These estimates assume that all of the synthetic lipid monomers are absorbed into liposomal membranes. Some aggregates could form directly from the ceramide mimics themselves (see Table 1). Even so, the number of new monomers required to increase each vesicle to the observed size falls far short, suggesting that these synthetic amphiphiles may actually foster vesicular fusion.

3. Conclusion

These studies report that dramatic changes in membrane bilayer activity occur when contacted by simple, synthetic ceramide mimics. Compounds **1**–**4** are hydroxylated amphiphiles related to structures developed as part of a synthetic anion channel program. Although **1**–**4** possess twin, hydrophobic tails, they lack the peptide structure of the synthetic chloride channels.[15] They readily incorporate in phospholipid vesicles but their activity thereafter is quite different from that of the synthetic ion channels. The permeabilities of chloride and of the fluorescent dye carboxyfluorescein are both increased by the presence of these lipids, with the apparent efflux of CF being affected to a greater extent. The apparently greater CF release may be a consequence of the higher compound/lipid ratio used in the CF dequenching experiments compared to the chloride release assay. In any event, these synthetic lipids increase vesicle size to an extent too great to be accounted for by incorporation of new monomers into existing structures. We speculate, therefore, that these ceramide mimics may cause vesicular fusion.

4. Experimental section

4.1. General methods

All reaction solvents were freshly distilled and reactions were conducted under N_2 unless otherwise stated. Et_3N was distilled from KOH and stored over KOH. Dichloromethane was distilled from CaH_2. Column chromatography was performed on silica gel 60 (230–400 mesh). Thin layer chromatography was performed with silica gel 60 F_{254} plates with visualization by UV light (254 nm) and/or by phosphomolybdic acid (PMA) spray. ^1H NMR spectra (300 MHz, $CDCl_3$ unless otherwise noted) are reported in ppm (δ, internal TMS) followed by integrated intensity, multiplicity (b = broad; s = singlet; d = doublet; t = triplet; m = multiplet, br s = broad singlet, etc.), and coupling constants (Hz). ^{13}C NMR spectra (75 MHz) are referenced to $CDCl_3$ (δ 77.0). Infrared spectra were recorded in KBr unless otherwise noted and were calibrated against the 1601 cm^{-1} band of polystyrene. Melting points were determined on a Thomas Hoover apparatus in open capillaries. Combustion analyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and are reported as percents.

4.2. Dioctadecylcarbamoylmethoxyacetic acid, $(C_{18}H_{37})_2NCOCH_2OCH_2COOH$, 1

Dioctadecylamine (2.0 g, 3.8 mmol) and diglycolic anhydride (0.44 g, 3.8 mmol) were heated for 48 h under reflux in toluene (50 mL). The solvent was evaporated and the crude product crystallized from $CHCl_3$ to give **1** as a white solid (2.12 g, 87%), mp 80–81 °C (lit.[17] mp 80–81 °C). ^1H NMR: 0.87 (6H, t, $J = 6.9$ Hz, $-CH_2CH_3$), 1.25 [60H, *pseudo*-s, $CH_3(CH_2)_{15}CH_2CH_2N$], 1.55 [4H, br s, $CH_3(CH_2)_{15}CH_2CH_2N$], 3.07 [2H, t, $J = 7.8$ Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 3.34 [2H, t, $J = 7.8$ Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 4.21 (2H, s, $COCH_2O$), 4.38 (2H, s, $COCH_2O$). ^{13}C NMR $CDCl_3$: 14.2, 22.8, 26.9, 27.0, 27.5, 28.7, 29.4, 29.5, 29.6, 29.7, 29.8, 32.0, 47.0, 71.4, 73.2, 171.0, 172.2. IR (KBr): 2918, 2850, 1748, 1602, 1488, 1472, 1463, 1431, 1356, 1224, 1159, 1135, 1045, 1013, 990, 920, 885, 729, 720, 689, 643 cm^{-1}.

4.3. 2-(2-Hydroxyethoxy)-*N*,*N*-dioctadecylacetamide, $(C_{18}H_{37})_2NCOCH_2OCH_2CH_2OH$, 2

Ethyl chloroformate (0.092 mL, 0.96 mmol) was added during 2 min at −10 °C to dioctadecylcarbamoylmeth-

oxyacetic acid (0.61 g, 0.96 mmol) Et_3N (0.135 mL, 0.96 mmol) in dry THF (10 mL). After stirring for 30 min, the mixture was filtered and the salt (Et_3NCl) was washed with THF (2 × 3 mL). Sodium borohydride (0.115 g, 3.04 mmol) was added to the filtrate in one portion. Methanol (0.61 mL, 15.0 mmol) was added dropwise to the reaction mixture over a period of 1 h at 10 °C. The reaction was quenched with 1 N HCl (6.5 mL) and the organic layer was separated. The aqueous phase was extracted with CH_2Cl_2 (3 × 10 mL). The organic layer was dried ($MgSO_4$), the solvent was evaporated, and the crude material was chromatographed (SiO_2, 2% MeOH in CH_2Cl_2) to give **2** as a white solid (0.52 g, 88%), mp 51–52 °C. ^{1}H NMR: 0.87 (6H, t, J = 6.9 Hz, CH_2CH_3), 1.25 [60H, *pseudo*-s, $CH_3(CH_2)_{15}CH_2CH_2N$], 1.52 [4H, br s, $CH_3(CH_2)_{15}CH_2CH_2N$], 3.10 [2H, t, J = 7.8 Hz, $CH_3(CH_2)_{16}CH_2N$], 3.30 [2H, t, J = 7.8 Hz, $CH_3(CH_2)_{16}CH_2N$], 3.65–3.72 (4H, m, OCH_2CH_2OH), 4.21 (2H, s, $COCH_2O$). ^{13}C NMR: 14.1, 22.7, 26.9, 27.0, 27.6, 28.8, 29.3, 29.7, 31.9, 46.1, 46.6, 61.7, 68.8, 74.2, 169.9. IR ($CHCl_3$): 3393, 2921, 2852, 1646, 1467, 1378, 1308, 1130, 1076, 756, 721 cm^{-1}. Anal. Calcd for $C_{40}H_{81}NO_3$: C, 76.98; H, 13.08; N, 2.24. Found: C, 76.71; H, 13.05; N, 2.14.

4.4. 2-{[Bis(2-hydroxyethyl)carbamoyl]methoxy}-*N*,*N*-dioctadecylacetamide, $(C_{18}H_{37})_2NCOCH_2OCH_2CON(CH_2CH_2OH)_2$, 3

To a solution of **1** (0.5 g, 0.78 mmol) and diethanolamine (0.08 g, 0.76 mmol) in CH_2Cl_2 (30 mL), containing diisopropylethylamine (0.41 mL, 2.35 mmol), chlorotripyrrolidinophosphonium hexafluorophosphate (0.33 g, 0.78 mmol) was added at room temperature and the reaction mixture was stirred for 4 days. The solvent was evaporated and the crude product was purified by chromatography (SiO_2, 5% MeOH in $CHCl_3$), and crystallized from hexanes to give a white solid (0.39 g, 72%), mp 60–61 °C. ^{1}H NMR: 0.88 [6H, t, J = 6.6 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 1.26 [60H, *pseudo*-s, $CH_3(CH_2)_{15}CH_2N$], 1.53 [4H, br s, $CH_3(CH_2)_{15}CH_2CH_2N$], 3.11 [2H, t, J = 7.8 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 3.28 [2H, t, J = 7.8 Hz, $CH_3(CH_2)_{16}CH_2N$], 3.54 (2H, t, J = 4.8 Hz, $CONCH_2CH_2OH$), 3.64 (2H, t, J = 4.8 Hz, $CONCH_2CH_2OH$), 3.80–3.90 (4H, m, $CONCH_2CH_2OH$), 4.26 (2H, s, $COCH_2O$), 4.32 (2H, s, $COCH_2O$). ^{13}C NMR: 14.1, 22.7, 26.8, 27.0, 28.8, 29.3, 29.7, 31.9,46.1, 46.9, 50.5, 51.6, 55.4, 59.9, 60.7, 68.8, 71.0, 168.4, 170.4. IR ($CHCl_3$): 3376, 2919, 2851, 1641, 1467, 1377, 1134, 1049, 909, 735 cm^{-1}. Anal. Calcd for $C_{44}H_{88}N_2O_5$: C, 72.87; H, 12.23; N, 3.86. Found: C, 72.63; H, 12.34; N, 3.89.

4.5. Preparation of 2-dioctadecylamino-*N*,*N*-bis(2-hydroxyethyl)acetamide, 4

Dioctadecylaminoacetic acid ethyl ester $(C_{18}H_{37})_2NCH_2COOCH_2CH_3$. A solution of dioctadecylamine (5.20 g, 10.0 mmol), ethyl bromoacetate (1.43 g, 8.5 mmol), Na_2CO_3 (1.6 g, 10.0 mmol), and KI (30 mg, 0.2 mmol) in *n*-PrCN (75 mL) was heated at reflux for 2 h. The mixture was cooled and filtered to remove inorganic salts. This insoluble material was washed with $CHCl_3$ (2 × 20 mL) and the combined organic layer was washed with brine (50 mL) and concentrated in vacuo. Column chromatography (SiO_2, 10% hexanes in $CHCl_3$ then 100% $CHCl_3$) gave 4.94 g (95%) of slightly yellow oil. ^{1}H NMR: 0.87 [6H, t, J = 6.6 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 1.26 [63H, overlapping signals due to $CH_3(CH_2)_{15}CH_2CH_2N$ and OCH_2CH_3], 1.43 [4H, br s, $CH_3(CH_2)_{15}CH_2CH_2N$], 2.54 [4H, t, J = 6.6 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 3.30 (2H, s, NCH_2CO), 4.16 (2H, q, J = 7.2 Hz, $-OCH_2CH_3$). ^{13}C NMR: 14.1, 14.3, 22.7, 27.4, 27.5, 29.3, 29.5, 29.6, 29.7, 31.9, 54.5, 55.3, 60.2, 171.7. IR (neat): 2923, 2853, 1744, 1467, 1369, 1178, 1113, 1033, 720 cm^{-1}.

Dioctadecylaminoacetic acid $(C_{18}H_{37})_2NCH_2COOH$. A solution of $(C_{18}H_{37})_2NCH_2COOCH_2CH_3$ (2.0 g, 3.3 mmol) in EtOH (30 mL) containing 2 N NaOH (20 mL) was refluxed overnight. After cooling down, the pH of the reaction mixture was adjusted to 6 and the precipitated solid was filtered off. The solid material was dissolved in $CHCl_3$ (50 mL), filtered, and the solvent evaporated. The product was dried on high vacuum pump to give a white solid (1.9 g, 100%), mp 102–103 °C. ^{1}H NMR: 0.88 [6H, t, J = 6.6 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 1.25 [60H, *pseudo*-s, $CH_3(CH_2)_{15}CH_2CH_2N$], 1.70 [4H, br s, $CH_3(CH_2)_{15}CH_2CH_2N$], 3.13 [4H, t, J = 7.8 Hz, $CH_3(CH_2)_{16}CH_2N$], 3.58 (2H, s, NCH_2CO), 9.95 (1H, br s, $COOH$) ^{13}C NMR: 14.1, 22.7, 24.0, 26.8, 29.2, 29.3, 29.4, 29.5, 29.7, 31.9, 54.2, 55.2, 167.6. IR ($CHCl_3$): 3325, 2955, 2918, 2850, 2359, 2253, 1735, 1607, 1471, 1348, 1326, 1296, 1259, 1242, 1188, 1169, 1148, 1060, 1042, 944, 908, 736 cm^{-1}.

4.6. 2-Dioctadecylamino-*N*,*N*-bis(2-hydroxyethyl)acetamide, $(C_{18}H_{37})_2NCH_2CON(CH_2CH_2OH)_2$, 4

To a solution of $(C_{18}H_{37})_2NCH_2COOH$ (0.5 g, 0.81 mmol) containing triethylamine (0.23 mL, 1.62 mmol) in dry THF (10 mL), ethyl chloroformate (0.078 mL, 0.81 mmol) was added over a period of 2 min at −10 °C. After stirring for an additional 30 min, the mixture was filtered and the salt ($Et_3N^+Cl^-$) was washed with THF (2 × 3 mL). Diethanolamine (0.09 g, 0.81 mmol) was added to the filtrate in one portion and the reaction was stirred overnight at room temperature. Solvent was evaporated and the residue was dissolved in EtOAc (50 mL), washed with 5% citric acid (25 mL), $NaHCO_3$ (25 mL), and brine (25 mL), dried over $MgSO_4$ and evaporated. The crude product was purified by chromatography (SiO_2, 100% $CHCl_3$) to give a white solid (0.44 g, 81%), mp 54–55 °C. ^{1}H NMR $CDCl_3$: 0.88 [6H, t, J = 6.6 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 1.25 [60H, *pseudo*-s, $CH_3(CH_2)_{15}CH_2CH_2N$], 1.48 [4H, br s, $CH_3(CH_2)_{15}CH_2CH_2N$], 2.50 [4H, t, J = 8.0 Hz, $CH_3(CH_2)_{15}CH_2CH_2N$], 3.29 (2H, s, NCH_2CO), 3.55 (2H, t, J = 5.8 Hz, $CONCH_2CH_2OH$), 3.56 (2H, t, J = 5.8 Hz, $CONCH_2CH_2OH$), 3.75 (2H, t, J = 4.8 Hz $CONCH_2CH_2OH$), 3.81 (2H, t, J = 4.8 Hz $CONCH_2CH_2OH$). ^{13}C NMR: 14.1, 22.7, 25.3, 27.5, 29.3, 29.4, 29.6, 29.7, 31.9, 49.8, 51.8, 54.4, 57.8, 58.6, 61.2.

IR ($CHCl_3$): 3391, 2925, 2853, 2253, 1630, 1467, 1378, 1092, 908, 733, 651 cm^{-1}. Anal. Calcd for $C_{42}H_{86}N_2O_3$: C, 75.61; H, 12.99; N, 4.20. Found: C, 75.52; H, 13.20; N, 4.21.

4.7. Aggregate formation

Compounds **1**–**4** were dissolved in 2-PrOH to give solutions of concentration ~7 mM. Aliquots (2.5–40 μL) of these solutions were then added to 2 mL of doubly distilled water. Dilution with doubly distilled water resulted in the spontaneous formation of aggregates. The sizes and distributions of any aggregate(s) that formed were sized by using dynamic light scattering (Coulter N4MD submicron particle analyzer).

4.8. Vesicle preparation

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate monosodium salt (DOPA) were obtained from Avanti Polar Lipids as chloroform solutions. Dry lipid films of DOPC–DOPA (20 mg, 7:3) were dissolved in diethyl ether (0.5 mL) and 0.5 mL of an aqueous phase (600 mM KCl, 10 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH = 7.0) was added. The mixture was sonicated for 10–20 s to give an opalescent dispersion, after which the organic solvent was removed under reduced pressure. The suspension was filtered through a 200 nm filter membrane (5×) using a mini extruder and passed through a Sephadex G25 column, which had been equilibrated by an external buffer (400 mM K_2SO_4, 10 mM HEPES, pH = 7.0). The vesicles collected were subsequently characterized using laser light scattering, and their diameter was ~200 nm, as measured by a Coulter N4MD submicron particle analyzer.

4.9. Alternate aggregate preparation technique (compound 1)

A solid hydration procedure was employed to prepare the aggregates, where the amphiphile was initially dissolved in CH_2Cl_2. About 10^{-6} moles were placed in a test tube and dried to a thin film under vacuum (~2 h). Deionized water (pH 6.80) was added to gain the desired concentration (1 mM). Following bath sonication at 20–30 °C for 15 min, the milky solution obtained was filtered through a Nucleopore polycarbonate membrane. Dynamic light scattering characterized the aggregates formed. Following sonication, extrusion often resulted in loss of material, which remained associated on the polycarbonate membranes. Aggregates were prepared at a concentration of 1 mM and diluted to 25–50 nm in water prior to light-scattering experiments. The values presented in the text were obtained immediately after preparation and after 24 h had elapsed.

4.10. Assay for chloride ion release

The liposomes were prepared as previously described but they were loaded with 600 mM KCl and 10 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES, pH = 7.0) and equilibrated with an external buffer (400 mM K_2SO_4, 10 mM HEPES, pH = 7.0). The pH of the buffers prepared was adjusted using 10% NaOH. The external buffer (2.0 mL) was placed in a small disposable beaker containing a stir bar. An ion-selective electrode (Accumet Chloride Combination Electrode) was allowed to equilibrate, and the vesicle solution was added. The voltage output was recorded, and after 5 min, aliquots of the amphiphile solution (5–9 mM in 2-PrOH) were added. No more than 20 μL were added to avoid the effect of isopropyl alcohol on the liposomes. Complete lysis of the vesicles was induced by addition of an aqueous solution of the detergent Triton X100 (100 μL, 2%). The lipid concentration used in the studies presented here is ~0.67 mM. Data were collected as described in Section 4.

4.11. Assay for carboxyfluorescein release

The liposomes were prepared as described above from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphate monosodium salt (7:3 w/w, 20 mg), except that they were loaded with 20 mM 5(6)-carboxyfluorescein (CF), 100 mM KCl and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH = 7.0. In order to dissolve CF, sodium hydroxide (1 M) was added dropwise until pH 11. The solution was then acidified carefully to pH 7.0 using hydrochloric acid (1 M). The free dye was removed by gel filtration and exchanged for 100 mM KCl and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH = 7.0). These were diluted to 3 μM lipid and dequenching of intravesicular carboxyfluorescein was followed spectrofluorometrically by emission at 520 nm (excitation at 497 nm) in a thermostated cuvette. For each experiment, the initial (F_0) and total (F_{triton}, 1% Triton X100) fluorescence was determined and used to complete the dequenching for each time point: $(F-F_0)/(F_{triton}-F_0)$. In the CF release experiments, it is important to remember that the concentration of the compound being studied is in large excess compared with the concentration of vesicles as phospholipids. This ensures that the kinetics of pore activation are being studied rather that the kinetics of insertion.

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