Supporting Information for:

Ceramide-Mediated Transport of Chloride and Bicarbonate Across Phospholipid Membranes

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Table of Contents

General Experimental. ^{1}H NMR spectra were recorded on a Bruker DRX-400 operating at 400.13 MHz, a Bruker DRX-500 operating at 500.13 MHz, or a Bruker AVIII-600 operating at 600.13 MHz. All ^{13}C NMR spectra were recorded on a Bruker DRX-400 operating at 100.61 MHz. Chemical shifts are reported in ppm relative to the residual protonated solvent peak. ESI-MS experiments were done with a JEOL AccuTOF spectrometer. Chromatography was performed using 60-200 mesh silica gel from Baker. Thin layer chromatography was performed on Kieselgel 60 F254 silica-coated glass plates and visualized by UV and CAM stain. The pH of solutions was monitored with a Fisher Scientific AR25 dual channel pH/ion meter. Deuterated solvents were purchased from Cambridge Isotope Labs. Chemicals and solvents were purchased from Aldrich, Fisher, Acros. Phospholipids used to prepare liposomes were purchased from Avanti Polar Lipids. Liposome fluorimetric assays were recorded using a Hitachi F-4500 spectrophotometer. High-pressure extrusion was performed on the Avanti mini-extruder with a 0.1 μm polycarbonate membrane. Size-exclusion chromatography was performed using Sephadex G25 (lucigenin) or Sephacryl S200 (carboxyfluorescein).

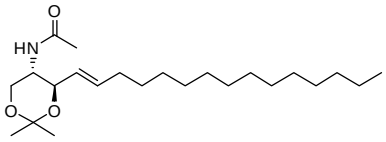
Preparation of Liposomes. A stock solution of egg-yolk phosphatidylcholine (EYPC) in $CHCl_3$ (60 mg in 3 mL) was evaporated under reduced pressure to produce a thin film that was dried *in vacuo* overnight. The lipid film was hydrated with a 1 mL solution containing 20 mM HEPES (pH 7.4), 100 mM NaCl and 2 mM lucigenin. Freeze/thaw cycles were repeated at least 9 times until no solids were visible. The frozen solution was warmed to 30-35 ^{o}C before each freeze cycle. The mixture was placed on a vortexer every 3 cycles for 30 s to facilitate hydration. The cloudy solution was extruded through a 100 nm polycarbonate membrane at least 25 times until the solution was transparent. This solution was passed down a Sephadex G25 column (11 cm x 1 cm) to remove extravesicular lucigenin. The eluant was comprised of 20 mM HEPES and 75 mM Na_2SO_4 at pH 7.4 in order to replace external Cl^- with SO_4^-. The 6-10 mL of solution isolated from gel filtration was 8-12 mM in lipid, assuming all EYPC was incorporated into the liposomes. Each stock solution of liposomes was used that day for transport assays.

Anion Transport Assays. This procedure describes a typical ion transport assay as depicted in Figure 3 of the paper. An aliquot (volume varies depending on concentration of stock solution, typically 80-120 μL) of the stock solution of EYPC liposomes was added to a cuvette and diluted to 2 mL with a solution of salt NaX and 20 mM HEPES at pH 7.4 to give a final concentration of 200 μM phospholipid. The fluorescence of intravesicular lucigenin was monitored by excitation at 372 nm, and the emission was recorded at 503 nm. At t = 30 s, a 2 μL aliquot of a 2 mM stock solution of **1** or **2** was injected to give a final concentration of compound added of 2 μM (1 mol% of compound relative to EYPC phospholipid). At the end of the experiment, 10% aqueous Triton-X was injected to lyse the liposomes. All data presented is an average of 3 runs.

Dye Release Assays. CF/DPX assays were prepared and carried out as reported by Colombini et al.[1]

Synthetic Procedures.

C2 ceramide 1,3-isopropylidene (2). C2-ceramide **1** (Avanti Polar Lipids, 10.5 mg, 30.7 μmol) was added to solution of *p*-TsOH·H_2O (2.3 mg, 12.3 μmol) in acetone (1 mL) and 2,2'-dimethoxypropane (1 mL). The reaction mixture was stirred at 25 ^oC under N_2 overnight. Solid $NaHCO_3$ was then added to the reaction mixture and the resulting mixtures was stirred for 5 min, filtered and concentrated *in vacuo*. The crude reaction product was then purified by SiO_2 column chromatography using a solvent system of 2% MeOH in CH_2Cl_2 to afford the C2 ceramide 1,3-isopropylidene **2** (11.0 mg, 94% yield). R_f= 0.22 (silica, 5% MeOH in CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$) δ= 5.75 (td, 1 H, ^3J=6.7, 15.2 Hz), 5.43 (dd, 1 H, ^3J=7.6, 15.2 Hz), 5.21 (d, 1 H, ^3J=8.0 Hz), 4.06 (dd, 1 H, ^3J=7.6, 9.2 Hz), 4.01 (dd, 1 H, ^3J=5.3, 11.3 Hz), 3.84 (m, 1H), 3.63 (dd, 1 H, ^3J=9.3, 11.3 Hz), 2.04 (m, 2 H), 1.94 (s, 3 H), 1.49 (s, 3 H), 1.42 (s, 3 H), 1.25 (m, 22 H), 0.88 (t, 3H, ^3J=6.8 Hz); ^{13}C NMR (400 MHz, $CDCl_3$) δ= 170.0, 136.8, 127.5, 99.1, 74.4, 63.0, 48.5, 32.6, 32.1, 29.9, 29.9, 29.8, 29.7, 29.6, 29.4, 29.2, 28.7, 23.6, 22.9, 20.2, 14.3; ESI-MS $[M+H]^+$ calculated for $C_{23}H_{44}NO_3^+$ 382.332, found 382.418. Crystals for x-ray structure determination were grown from DMSO.



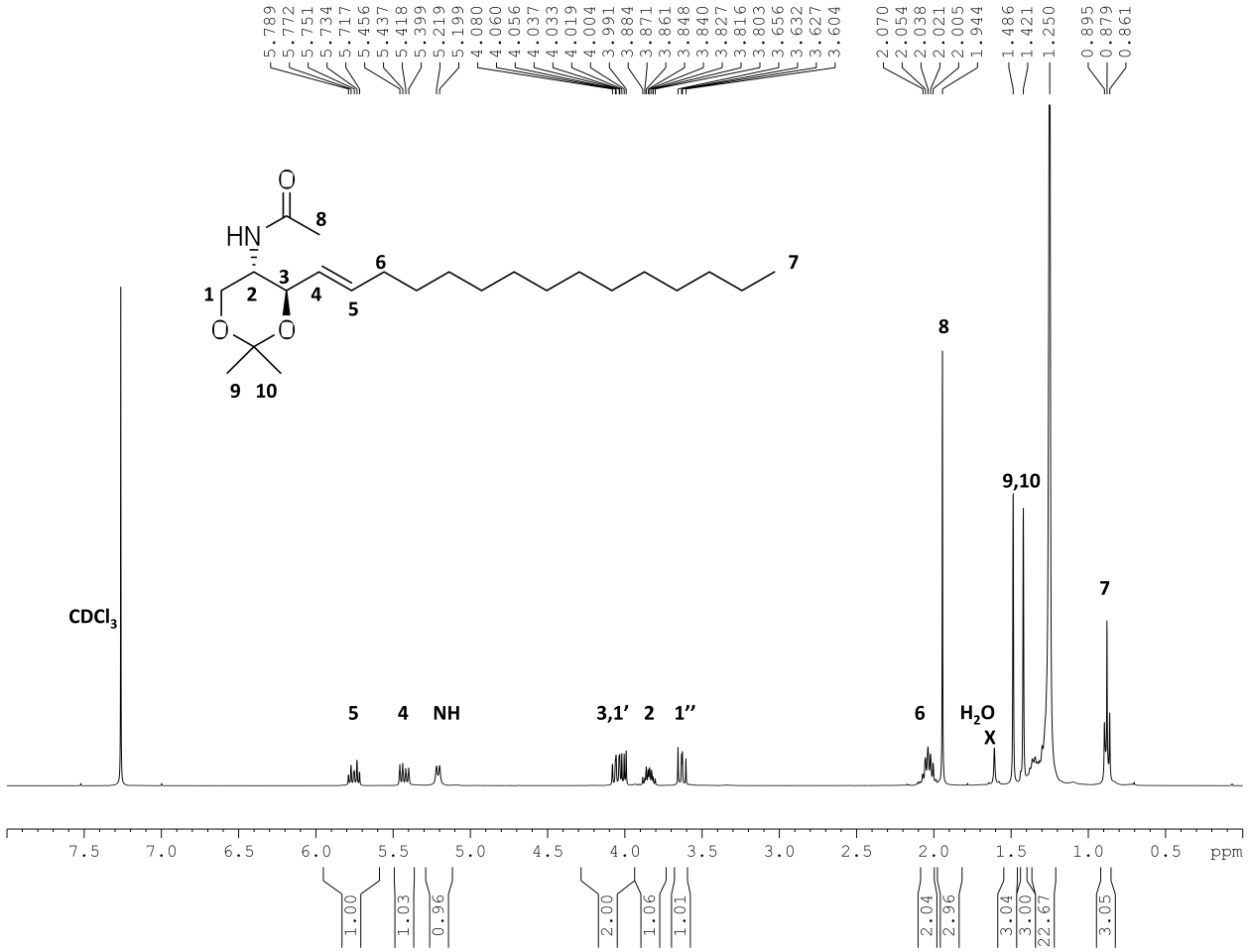


Figure S1. ^{1}H NMR spectrum (400 MHz) of C2-ceramide 1,3-isopropylidene **2** in $CDCl_3$.

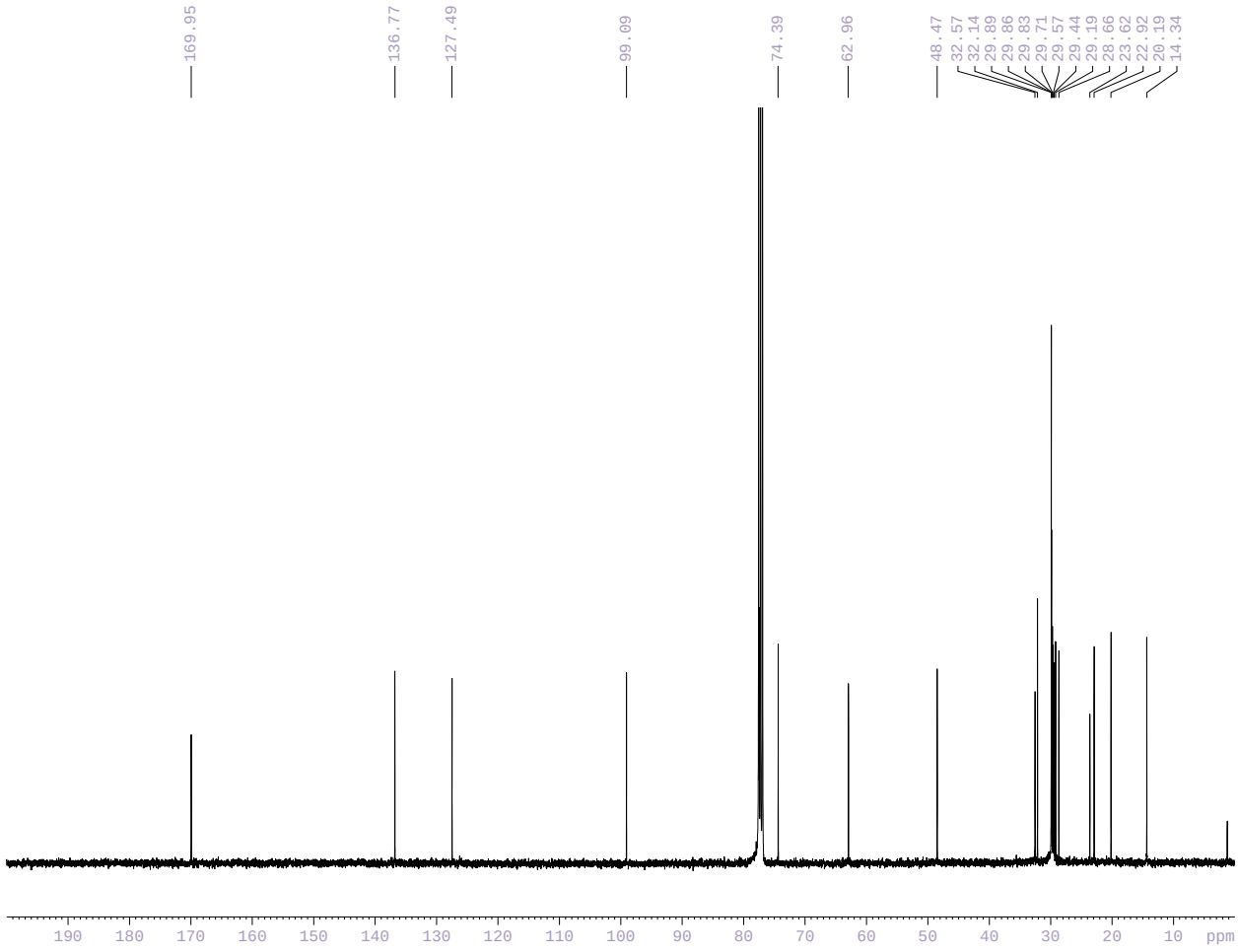


Figure S2. ^{13}C NMR spectrum (100 MHz) of C2-ceramide 1,3-isopropylidene **2** in $CDCl_3$.

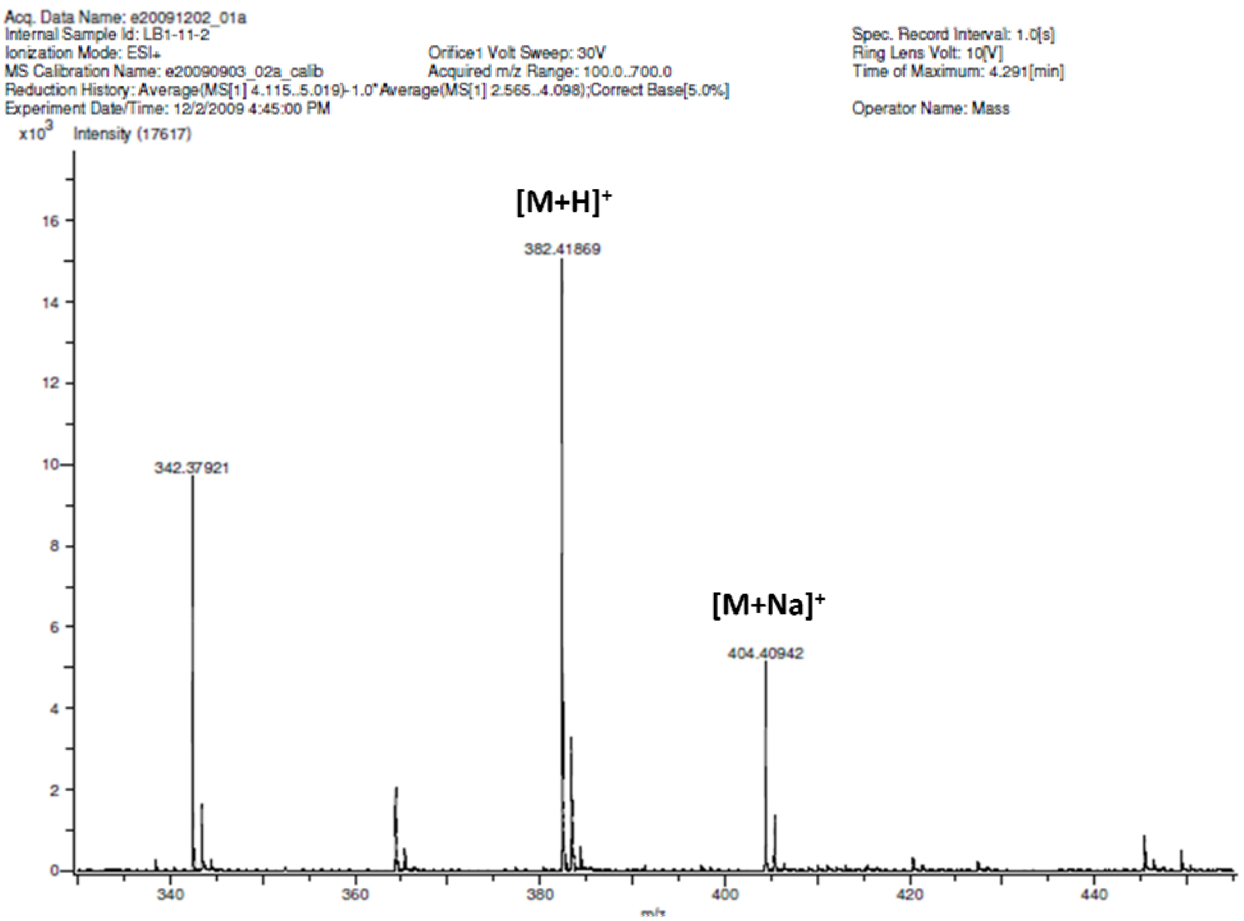


Figure S3. ESI-MS (positive mode) of C2-ceramide 1,3-isopropylidene **2**. ESI-MS $[M+H]^+$ calculated for $C_{23}H_{43}NO_3$ 382.332, found 382.418.

Crystal Structure Information for CCDC # 762206 (UM # 1793)

Compound name : C2-ceramide 1,3-isopropylidene **2**
Chemical formula : $C_{23}H_{43}NO_3$
Final R_1 [I>2σ(I)] : **5.86 %**

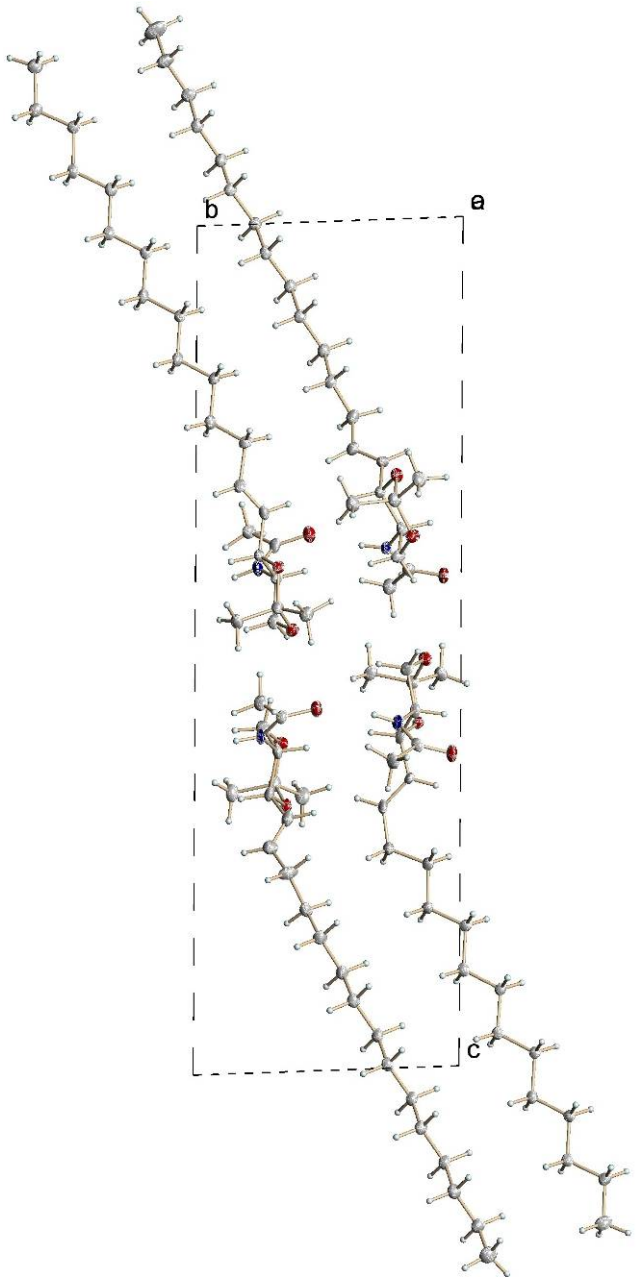


Figure S4. A view of 4 symmetrically independent molecules in the unit cell of UM#1793. Anisotropic atomic displacement ellipsoids for the non-hydrogen atoms are shown at the 30% probability level. Hydrogen atoms are displayed with an arbitrarily small radius.

Table 1. Crystal data and structure refinement for CCDC # 762206

Crystal ID	Davis/Harrell C2-ceramide 1,3-isopropylidene **2** 150K	
Empirical formula	$C_{23}H_{43}NO_3$	
Formula weight	381.58	
Temperature	150(2) K	
Wavelength	0.71073 Å	
Crystal size	0.51 × 0.12 × 0.06 mm^3	
Crystal habit	colorless needle	
Crystal system	Triclinic	
Space group	P1	
Unit cell dimensions	a = 8.9474(16) Å	α = 87.563(2)°
	b = 9.1515(16) Å	β = 82.486(2)°
	c = 29.391(5) Å	γ = 88.842(2)°
Volume	2383.5(7) $Å^3$	
Z	4	
Density, ρ_{calc}	1.063 g/cm^3	
Absorption coefficient, μ	0.069 mm^{-1}	
F(000)	848 $\bar{e}$	
Diffractometer	Bruker Smart Apex II CCD area detector	
Radiation source	fine-focus sealed tube, MoKα	
Detector distance	6.000 cm	
Detector resolution	8.333 pixels/mm	
Total frames	4230	
Frame size	512 pixels	
Frame width	-0.30°	
Exposure per frame	40 sec	
Total measurement time	54.0 hours	
Data collection method	ω and φ scans	
θ range for data collection	2.10 to 25.00°	
Index ranges	$-10 \le h \le 10$, $-10 \le k \le 10$, $-34 \le l \le 34$	
Reflections collected	30116	
Independent reflections	8310	
Observed reflection, I>2σ(I)	6931	
Coverage of independent reflections	99.1 %	
Variation in check reflections	0 %	
Absorption correction	Semi-empirical from equivalents SADABS (Sheldrick, 1996)	
Max. and min. transmission	0.996 and 0.873	
Structure solution technique	direct	
Structure solution program	SHELXS-97 (Sheldrick, 1990)	
Refinement technique	Full-matrix least-squares on F^2	
Refinement program	SHELXL-97 (Sheldrick, 1997)	
Function minimized	$\Sigma w(F_o^2 - F_c^2)^2$	
Data / restraints / parameters	8310 / 3 / 989	
Goodness-of-fit on F^2	1.000	
Δ/σ_{max}	0.000	
Final R indices: R_1, I>2σ(I)	0.0586	
wR_2, all data	0.1290	
R_{int}	0.0454	
R_{sig}	0.0436	
Weighting scheme	$w = 1/[\sigma^2(F_o^2)+(0.02P)^2+3.17P]$, $P = [\max(F_o^2,0)+2F_o^2]/3$	
Absolute structure parameter	unknown	
Largest diff. peak and hole	0.297 and -0.210 $\bar{e}/Å^3$	

$R_1 = \Sigma||F_o|-|F_c||/\Sigma|F_o|$, $wR2 = [\Sigma w(F_o^2 - F_c^2)^2/\Sigma\, w(F_o^2)^2]^{1/2}$

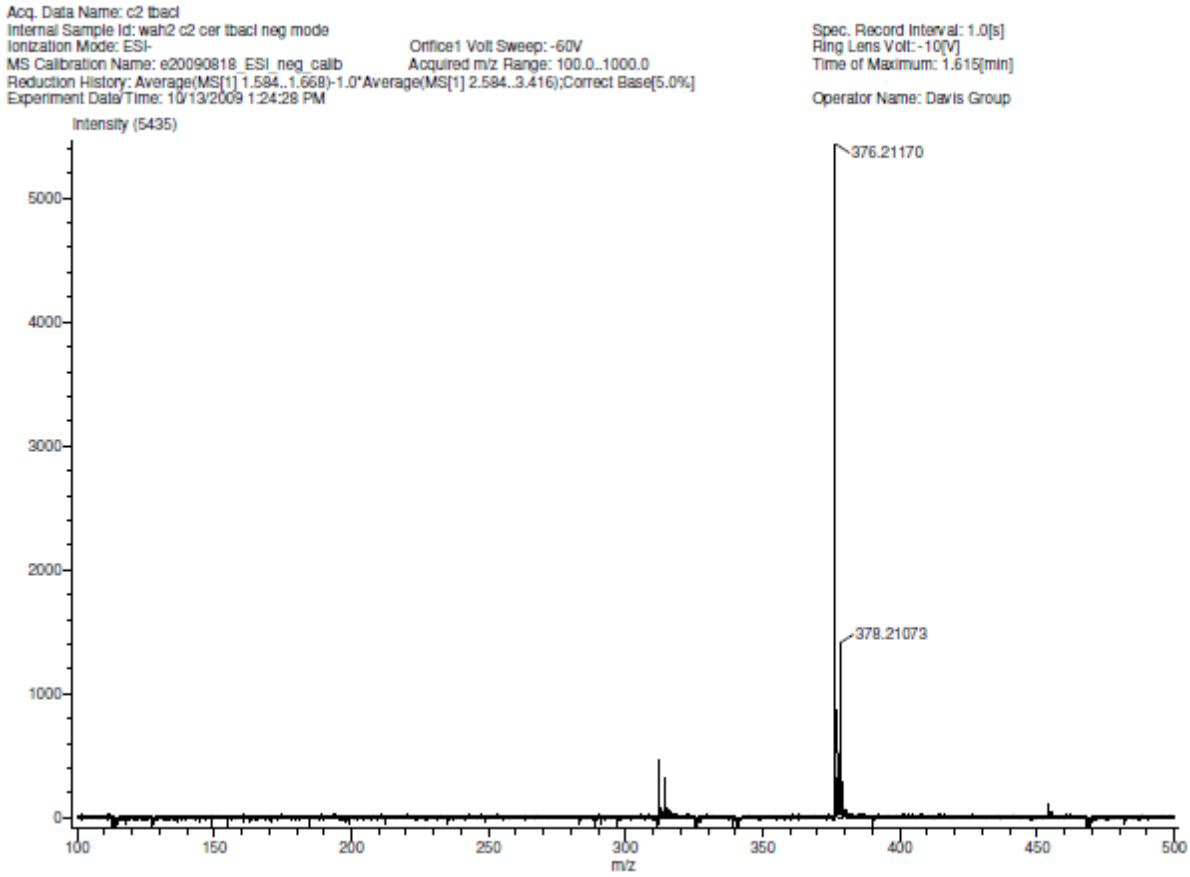


Figure S5. ESI-MS (negative mode) of ceramide **1**·Cl^- adduct. A solution of C2 ceramide **1** (2 mM) and TBACl (6 mM) in CD_2Cl_2 was injected using MeOH as the eluant. ESI-MS $[M+ Cl]^-$ calculated for $C_{20}H_{39}ClNO_3^-$ 376.26, found 376.21. Experimental variables are listed above the spectrum.

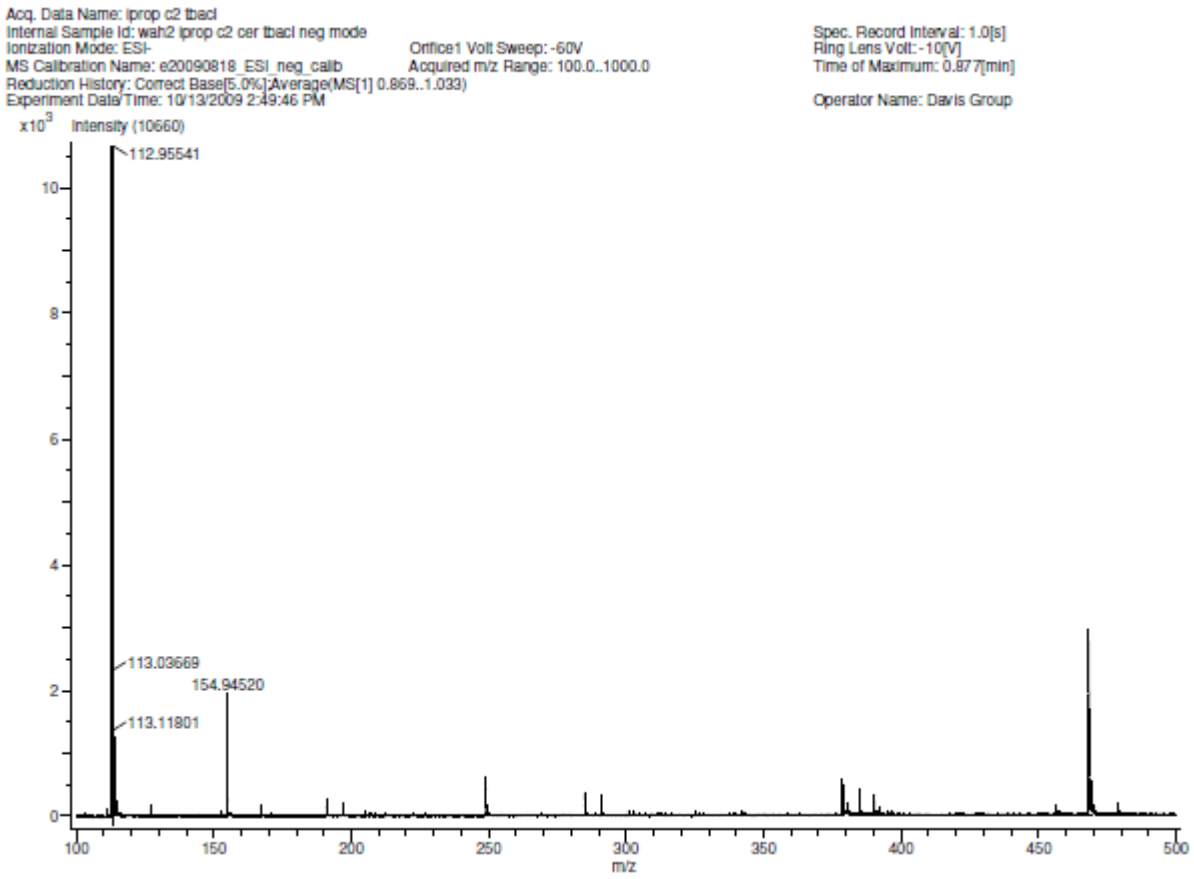


Figure S6. ESI-MS (negative mode) of a solution of C2-ceramide 1,3-isopropylidene **2** (2 mM) and TBACl (6 mM) in CD_2Cl_2 with MeOH as the eluant. ESI-MS $[M+Cl]^-$ calculated for $C_{23}H_{43}ClNO_3^-$ 416.29. Note the absence of a signal corresponding to a **2**·Cl^- adduct. Signals for ceramide derivatives are typically difficult to observe in the negative mode without the presence a bound anion. Experimental variables are listed above the spectrum.

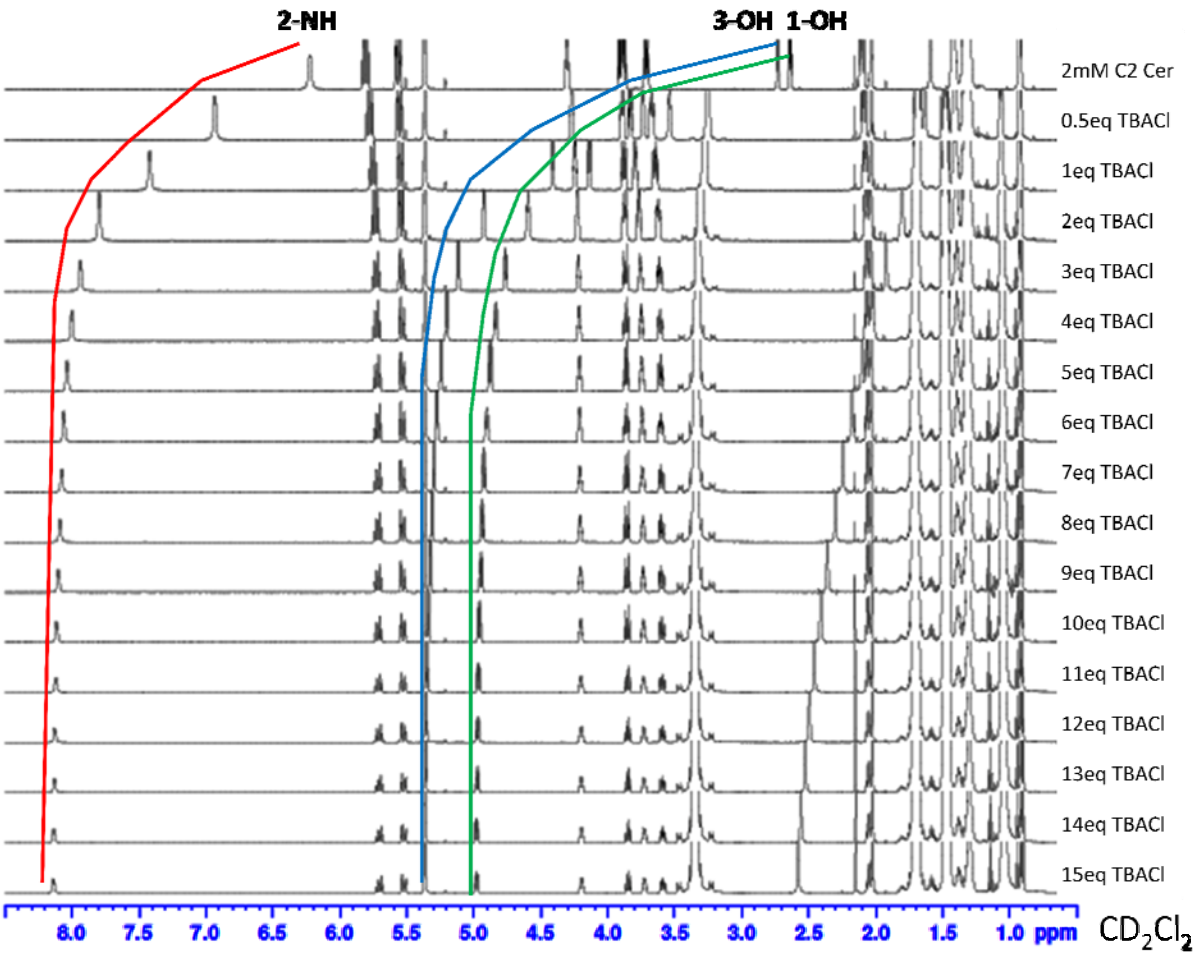


Figure S7. Stack plot from ^{1}H NMR titration of C2 ceramide **1** (2 mM) with TBACl in CD_2Cl_2.

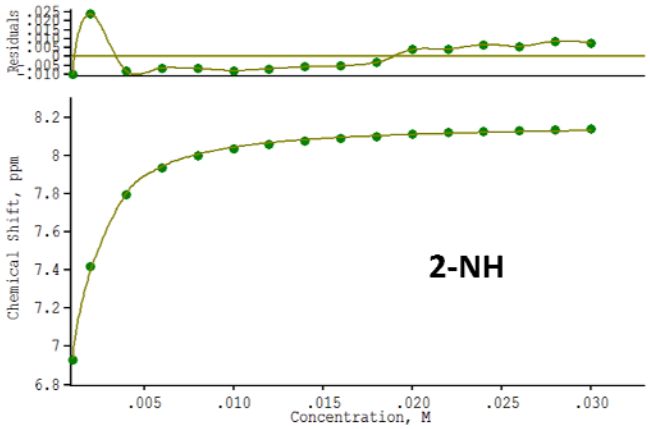


Figure S8. ^{1}H NMR data for the 2-NH proton of C2 ceramide **1** (2 mM) during titration with TBACl in CD_2Cl_2. A binding constant of $K_a = 1734 \pm 82$ M^{-1} was determined using WinEQNMR2.[2]

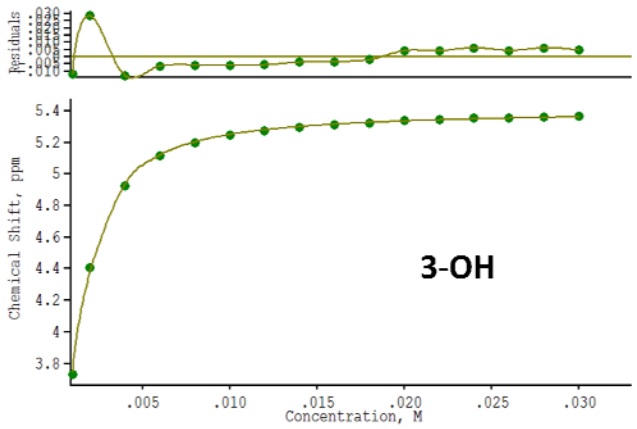


Figure S9. ^{1}H NMR data for the 3-OH proton of C2 ceramide **1** (2 mM) during titration with TBACl in CD_2Cl_2. A binding constant of $K_a = 1877 \pm 54$ M^{-1} was determined using WinEQNMR2.[2]

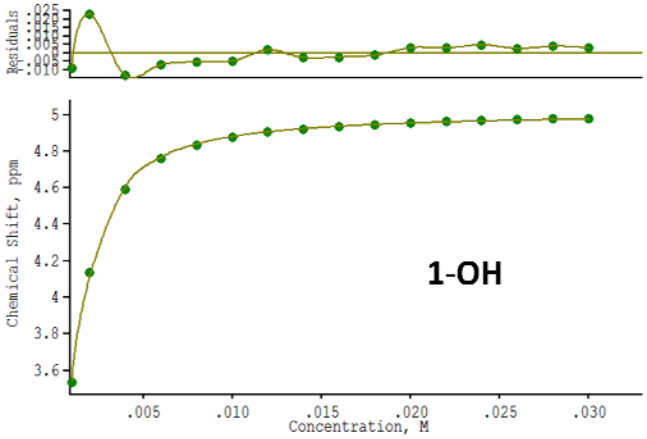


Figure S10. ^{1}H NMR data for the 1-OH proton of C2 ceramide **1** (2 mM) during titration with TBACl in CD_2Cl_2. A binding constant of $K_a = 1935 \pm 65$ M^{-1} was determined using WinEQNMR2.[2]

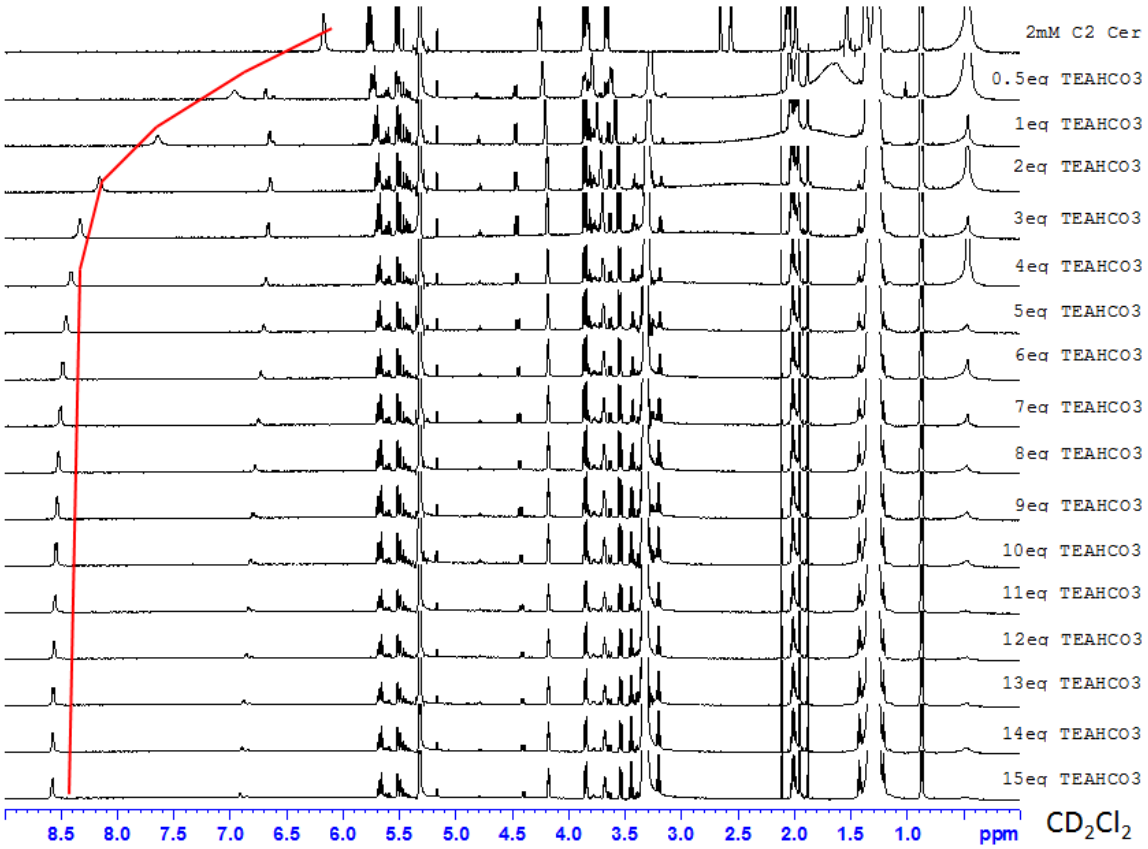


Figure S11. Stack plot from ^{1}H NMR titration of C2 ceramide **1** (2 mM) with tetraethylammonium bicarbonate ($TEAHCO_3$) in CD_2Cl_2. Note that addition of $TEAHCO_3$ results in loss of the signals for both 1-OH and 3-OH.

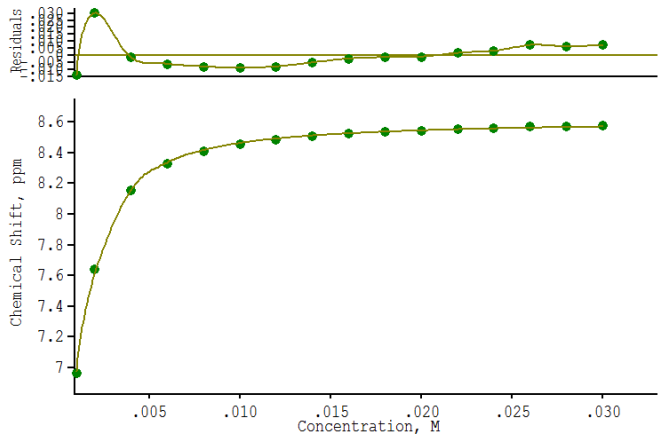


Figure S12. ^{1}H NMR data for the 2-NH proton of C2 ceramide **1** (2 mM) during titration with $TEAHCO_3$ in CD_2Cl_2. A binding constant of $K_a = 1933 \pm 58\ M^{-1}$ was determined using WinEQNMR2.[2]

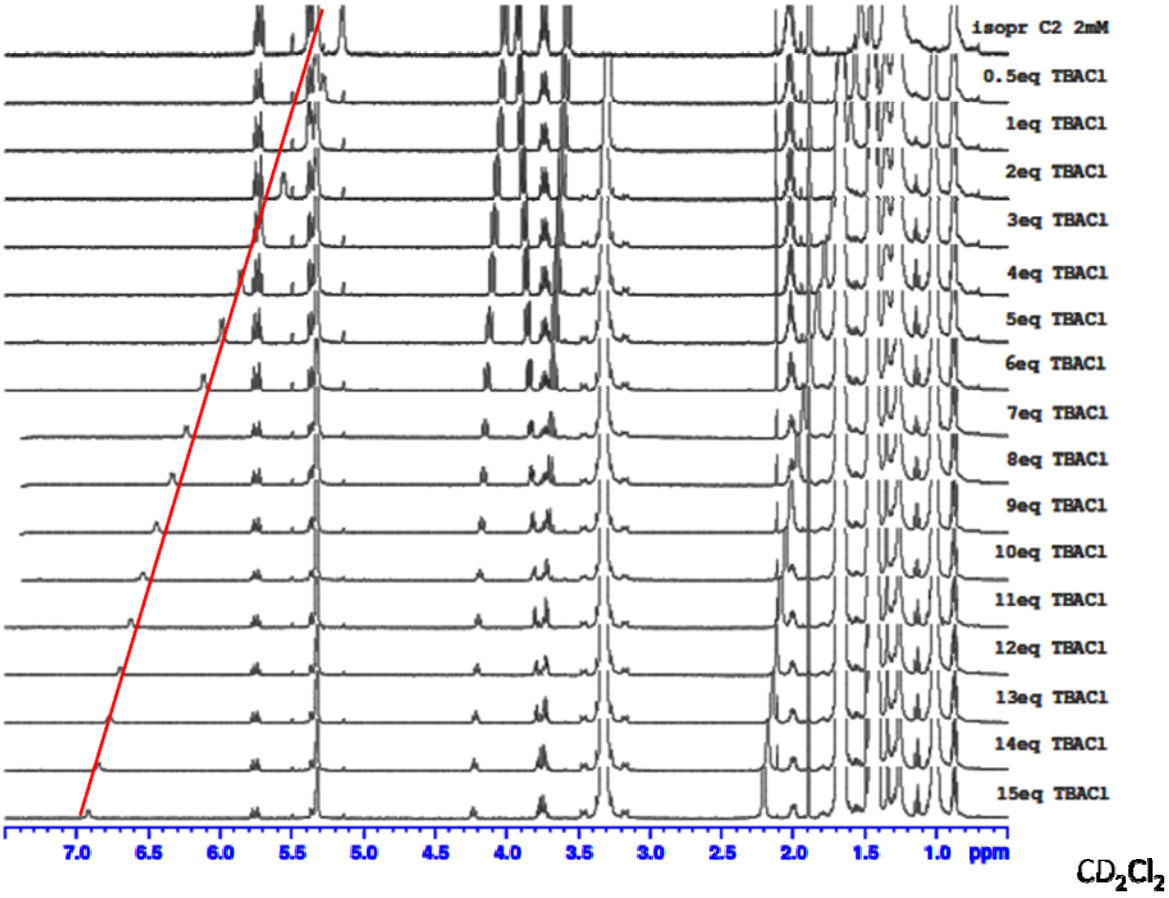


Figure S13. Stack plot from ^{1}H NMR titration of C2 ceramide 1,3-isopropylidene **2** (2 mM) with TBACl in CD_2Cl_2.

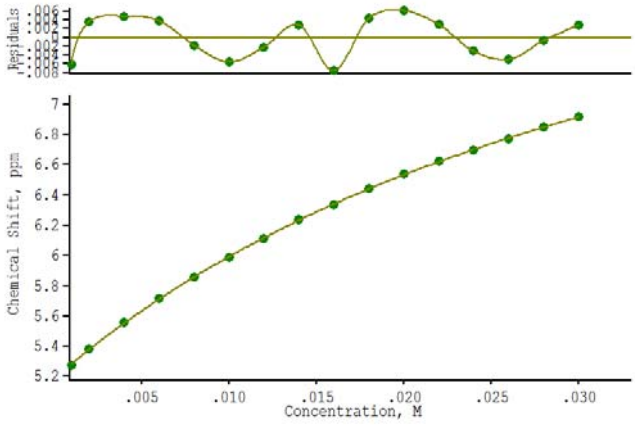


Figure S14. ^{1}H NMR data for the 2-NH proton of C2 ceramide 1,3-isopropylidene **2** (2 mM) during titration with TBACl in CD_2Cl_2. A binding constant of $K_a = 27 \pm 0.6$ M^{-1} was determined using WinEQNMR2.[2]

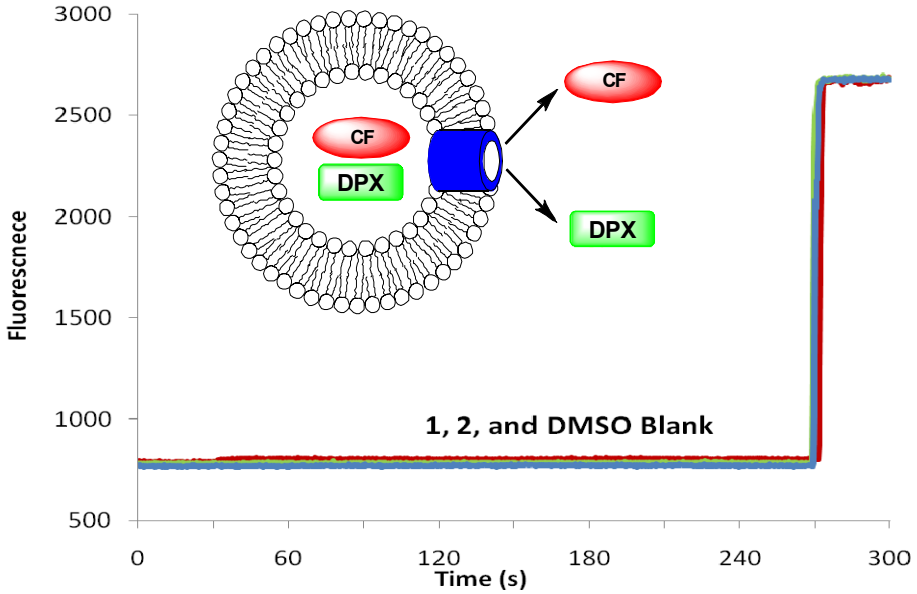


Figure S15. Dye release assay with EYPC liposomes (200 μM) containing CF/DPX and comparing compounds **1** and **2** (2 μM, 1 mol%). DPX quenches the fluorescence CF. If the compound added forms pores large enough to release small organic dyes, then an increase in fluorescence is observed as CF and DPX are released and diffuse away from one another. At concentrations of 1 mol%, neither C2 ceramide **1** nor the 1,3-isoproylidene **2** form large pores in these EYPC liposomes. The data is an average of 3 separate runs.

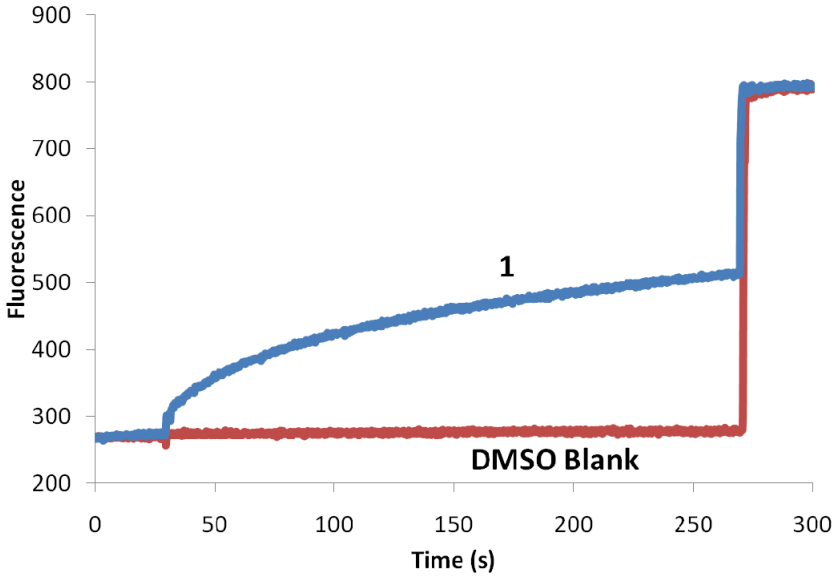


Figure S16. Dye release assay with EYPC liposomes (200 μM) containing CF/DPX with 100 μM C2 ceramide **1** (50 mol%). An increase in fluorescence is observed when using higher concentrations of C2 ceramide **1**, corresponding to the formation of large pores in the liposomal bilayer allowing for the release of intravesicular CF and/or DPX. The data is an average of 3 separate runs.

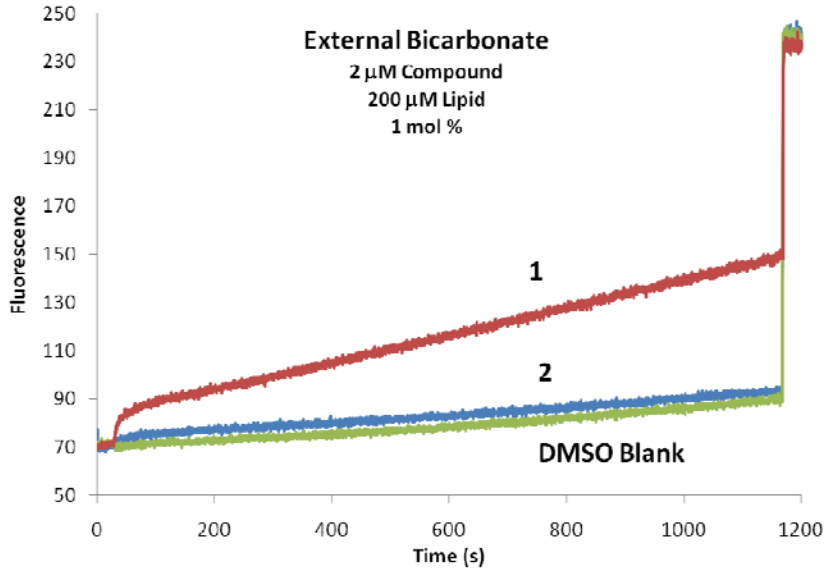


Figure S17. Anion release assay using lucigenin and NaCl containing EYPC liposomes (200 μM) with $NaHCO_3$ (100 mM) as the external anion. Lucigenin fluorescence is quenched by the internal Cl^-. Upon addition of C2 ceramide **1** (2 μM, 1 mol%) an increase in the lucigenin fluorescence is observed, corresponding to the exchange of Cl^- for HCO_3^-. Isopropylidene C2 ceramide **2** does not facilitate release of intravascular Cl^- in the presence of external HCO_3^-. The data is an average of 3 separate runs.

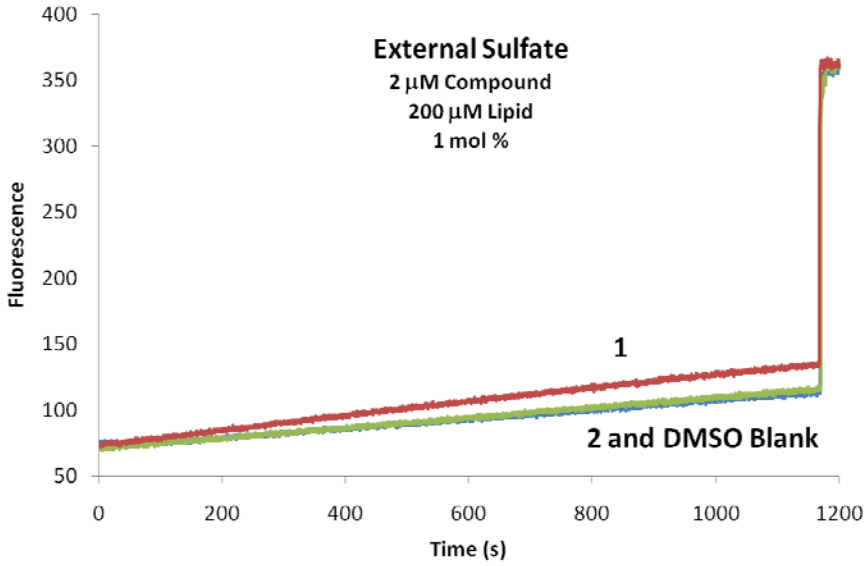


Figure S18. Anion release assay using lucigenin and NaCl containing EYPC liposomes (200 μM) with Na_2SO_4 (75mM) as the external anion. Little increase in lucigenin fluorescence was observed for C2 ceramide **1** (red line), and no increase in fluorescence is observed for isopropylidene C2 ceramide **2** (green line). The Cl^- transport activity of C2 ceramide **2** is significantly decreased in the presence of external SO_4^{2-} when compared to external NO_3^- or HCO_3^-. The data is an average of 3 separate runs.

Supporting Information References.

1. Stiban, J.; Fistere, D.; Colombini, M. *Apoptosis* **2006**, *11*, 773.
2. Hynes, M. J. *J. Chem. Soc., Dalton Trans.* **1993**, 311.