Supporting Information for:

Membrane Transporters for Anions That Use a Relay Mechanism

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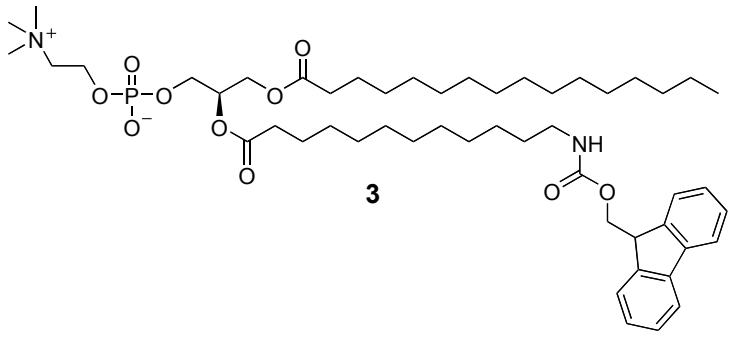
1. Synthesis and Spectra



3

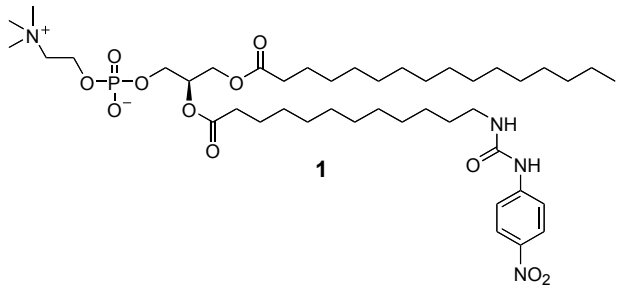
Phospholipid 3: The compound was synthesized following a modified procedure by Hadju and coworkers.[s1] To a 250 ml round bottom was added crushed glass culture tubes (6 x16 mm), lyso-phosphatidylcholine (1 g, 2.0 mmol), DCC (2.1 g, 10.1 mmol), DMAP (1.2 g, 10.1 mmol), Fmoc-12-aminododecanioc acid (4.4 g, 10.1 mmol), and anhydrous $CHCl_3$ (100 mL). The reaction was allowed to proceed with sonication for 5 hours at 20° C. The glass was then filtered from the reaction and Dowex 8X ion exchange resin was added to remove the DMAP. The solution was allowed to stir for 45 minutes. The resin was filtered and the solution was reduced in vacuo yielding a light tan solid. The crude product was purified on a silica gel column using an eluent of 65:25:4, chloroform: methanol: water. Fractions containing the product were combined and the solvent removed to yield pure **3** as a white solid product (1.57 g, 85% yield). ^{1}H NMR (300 MHz, 2:1 $CDCl_3$: MeOD) δ 7.56 (d, J = 7.5 Hz, 2H), 7.41 (d, J = 7.5 Hz, 2H), 7.19 (t, J = 7.2 Hz, 2H), 7.10 (t, J = 7.5 Hz, 2H), 5.75 (m, 1H), 5.02 (m, 1H), 4.22 (m, 1H), 4.15 (m, 1H), 4.05 (br, 2H), 3.97 (m, 1H), 3.80 (t, J = 6.0 Hz, 1H), 3.40 (m, 2H), 3.01 (s, 9H), 2.95 (m, 2H), 2.10 (m, 4H), 1.40 (m, 4H), 1.27 (m, 2H), 1.08 (br, 38H), 0.65 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, 2:1 $CDCl_3$: MeOD) δ 173.7, 173.3, 157.0, 143.7, 141.0, 127.4, 126.8, 124.8, 120.0, 70.2, 66.2, 63.4, 62.4, 58.8, 58.7, 53.8, 47.0, 40.8, 34.0, 33.8, 33.5, 31.7, 30.0, 29.4, 29.3, 29.2, 29.1, 28.9, 26.5, 24.6, 22.4, 13.7. Nominal mass (FAB+) m/z 916 [M+H].

Compounds **1** and **2** were prepared by removing the Fmoc group from **3** and reacting the liberated amine with the appropriate isocyanate.

General Procedure: To a round bottom flask was added **3** (0.11 mmol) and anhydrous chloroform. To the solution was added dropwise 2 molar equivalents of DBU. The solution

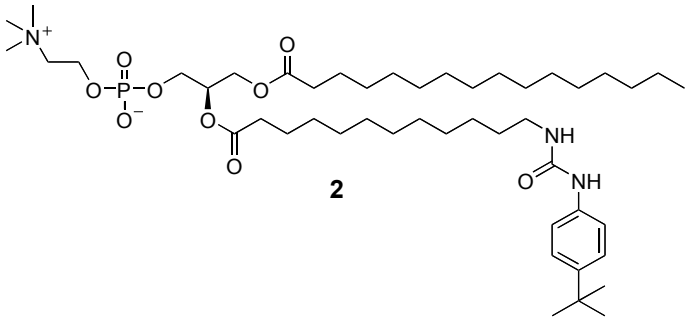
[s1] Rosseto, R.; Hajdu, J. *Tetrahedron Lett*. **2005**, *46*, 2941-2944.

was allowed to stir for 30 minutes and the reaction was monitored by TLC. The solution was then cooled to 0° C in an ice bath and 2 molar equivalents of the corresponding isocyanate was added via syringe pump over 2 hours. The solution was warmed to room temperature and stirred for 16 hours. After the reaction was complete, the solvent was removed and the crude product purified by column chromatography using a silica gel column and an eluent of 65: 25: 4, chloroform: methanol: water.



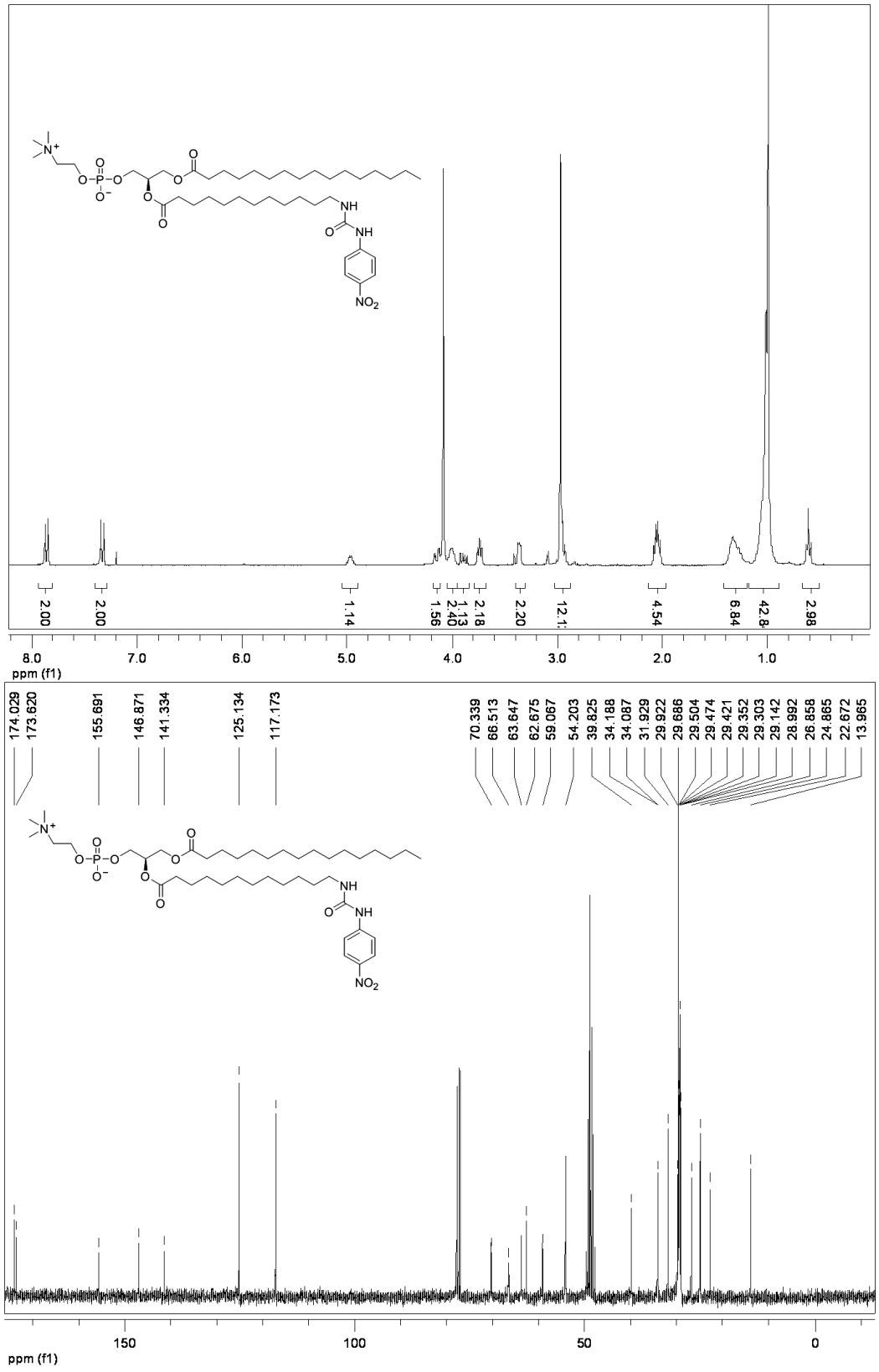
1

Phospholipid (1): Yield: 51 mg, 54%. ^{1}H NMR (300 MHz, 2:1 $CDCl_3$: MeOD) δ 7.86 (d, *J* = 8.7 Hz, 2H), 7.33 (d, *J* = 8.7Hz, 2H), 4.96 (m, 1H), 4.15 (m, 1H), 4.00 (m, 2H), 3.90 (m, 1H), 3.74 (t, *J* = 6.3 Hz, 2H), 3.36 (m, 2H), 2.97 (m, 12H), 2.05 (m, 4H), 1.34 (m, 6H), 1.02 (m, 38H), 0.61 (t, *J* = 6.3Hz, 3H). ^{13}C NMR (75 MHz, 2:1 $CDCl_3$: MeOD) δ 174.0, 173.6, 155.7, 146.9, 141.3, 125.1, 117.2, 70.3, 66.5, 63.6, 62.7, 59.0, 54.2, 39.8, 34.2, 34.1, 31.9, 29.9, 29.7, 29.5, 29.4, 29.3, 29.1, 29.0, 26.9, 24.9, 22.7, 14.0. Nominal mass (FAB+) m/z 858 [M+H].



2

Phospholipid (2): Yield: 66 mg, 70%. ^{1}H NMR (300 MHz, 2:1 $CDCl_3$: MeOD) δ 7.11 (m, 4H), 5.02 (m, 1H), 4.23 (m, 1H), 4.05 (m, 2H), 3.98 (m, 1H), 3.78 (t, *J* = 6.3 Hz, 2H), 3.41 (m, 2H), 3.01 (s, 9H), 2.97 (m, 2H), 2.11 (m, 4H), 1.38 (m, 4H), 1.30 (m, 2H), 1.08 (m, 38H), 0.68 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, 2:1 $CDCl_3$: MeOD) δ 173.8, 173.4, 156.3, 138.8, 131.3, 120.0, 113.8, 70.1, 66.2, 63.4, 62.4, 58.9, 53.9, 39.6, 33.9, 31.7, 29.8, 29.4, 29.2, 29.1, 29.0, 28.9, 28.8, 26.6, 24.6, 22.4, 13.7. Nominal mass (FAB+) m/z 869 [M+H].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6.0
5.0
4.0
3.0
2.0
1.0
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39.825
34.188
34.087
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29.686
29.504
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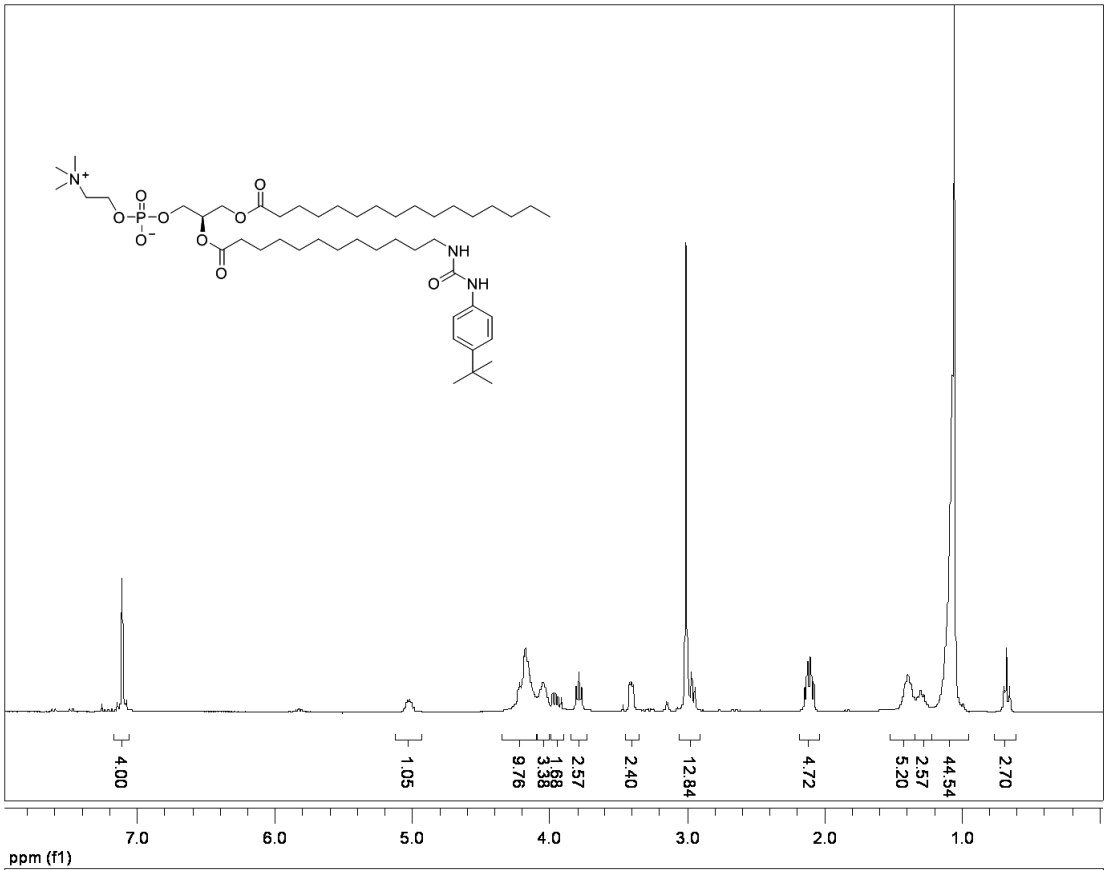
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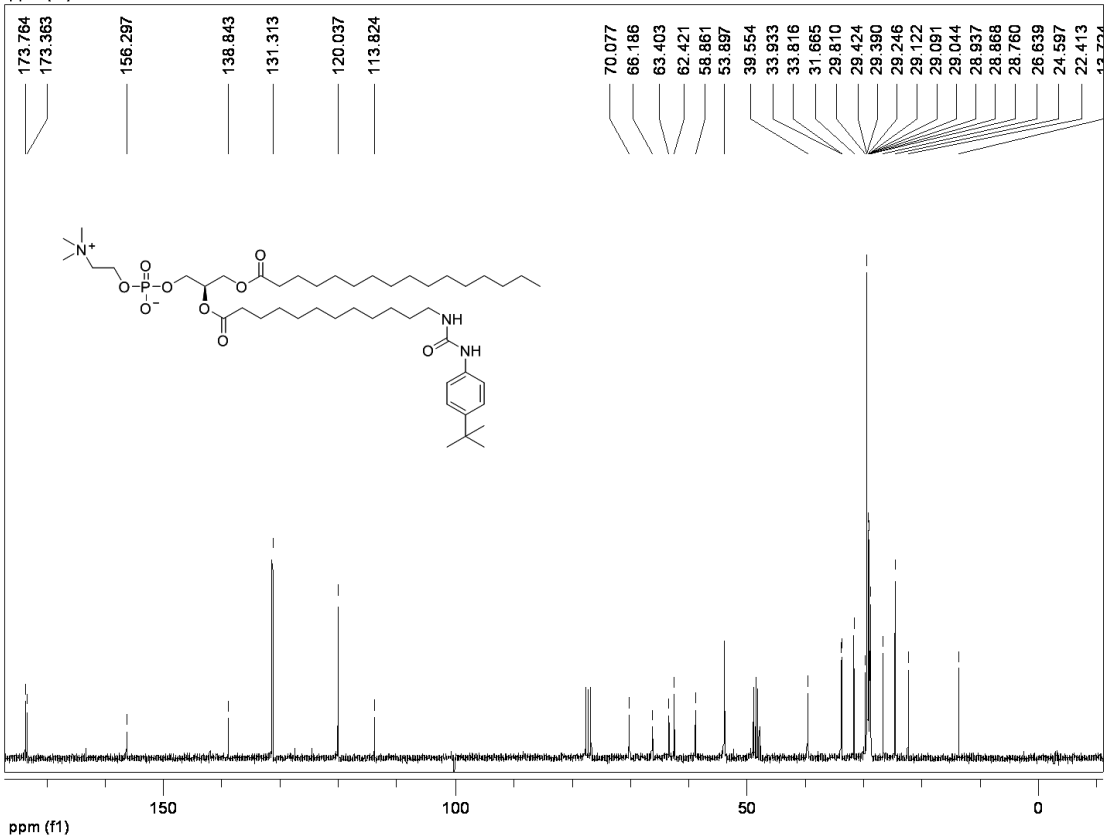
ppm (f1)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39.554
33.933
33.816
31.665
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29.424
29.390
29.246
29.122
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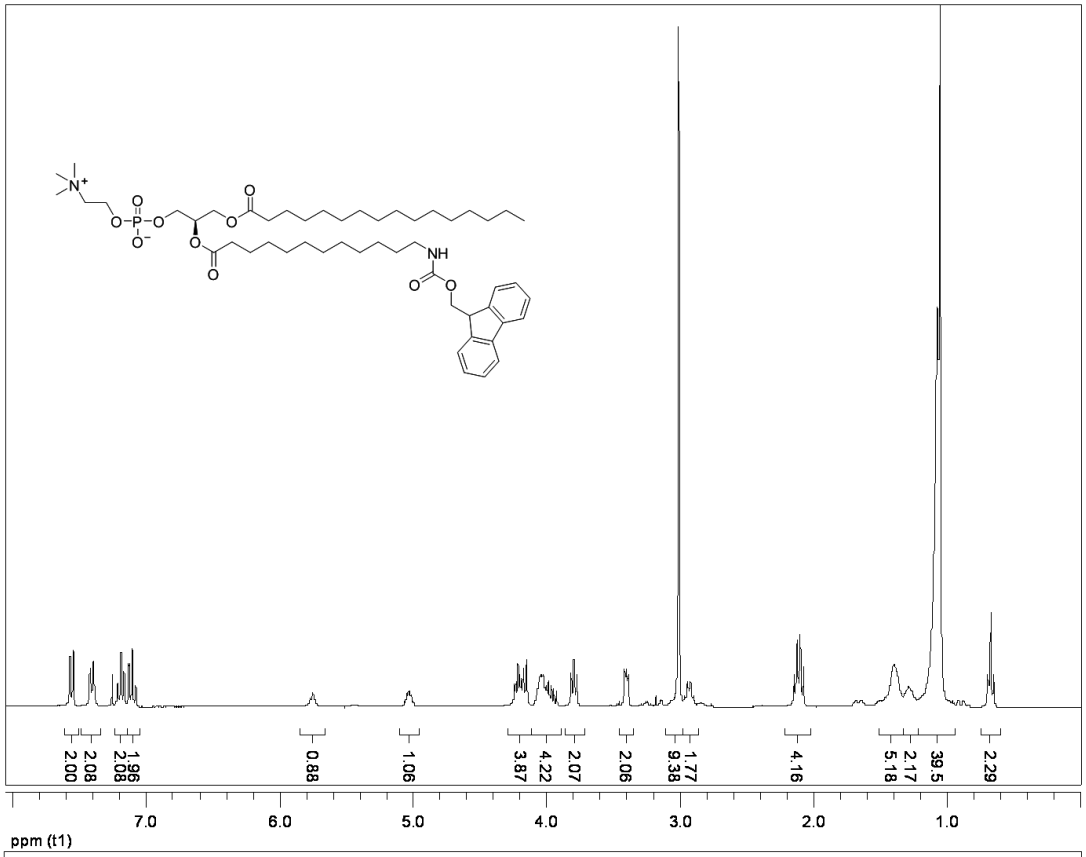
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4.22
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2.06
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4.16
5.18
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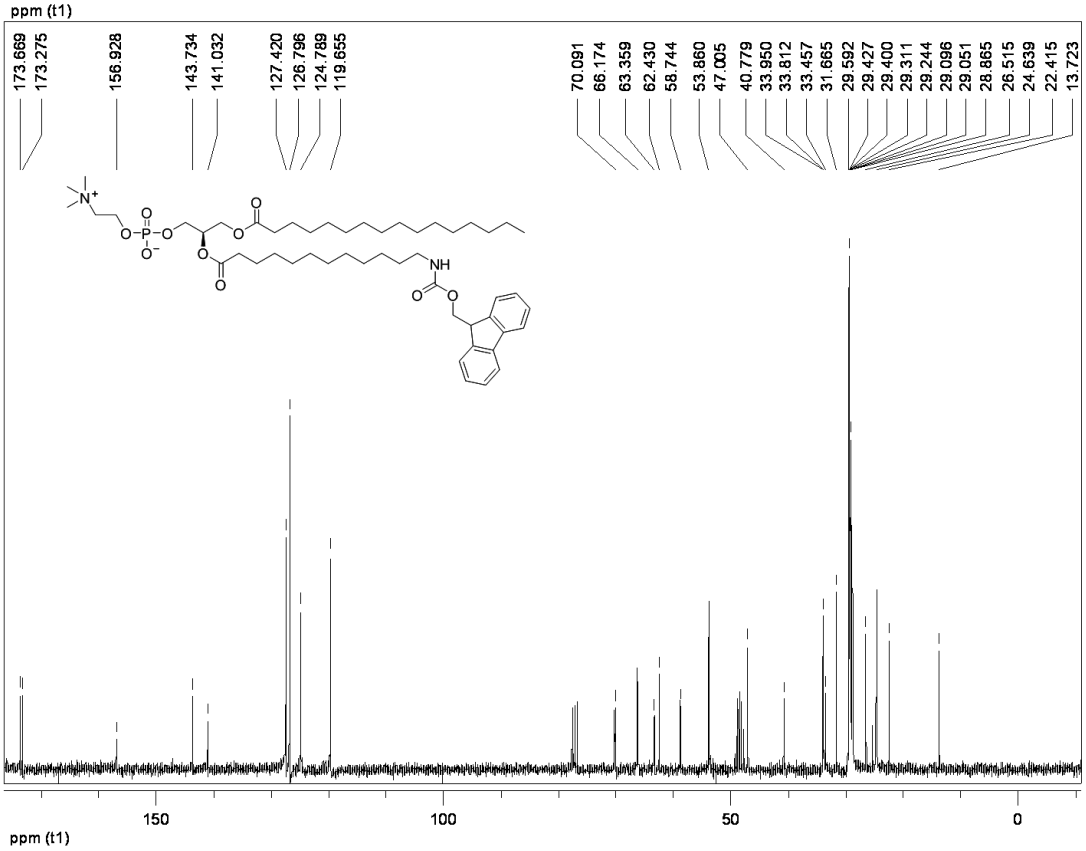
5.0
4.0
3.0
2.0
1.0
ppm (t1)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29.400
29.311
29.244
29.096
29.051
28.865
26.515
24.639
22.415
13.723
150
100
50
0
ppm (t1)

2. Transport Experiments

Materials and Equipment: The following commercial suppliers were used: all lipids (Avanti Polar Lipids); lucigenin (Molecular Probes); Lipofast extruder and porous polycarbonate membranes (200 nm) (Avestin); quartz cuvettes (Fischer-Scientific). Fluorescence spectra were obtained using a Jobin-Yvon Fluoromax-3 spectrometer with FT WinLab software and external water bath cooling unit.

Preparation of Unilamellar Vesicles: All lipids and transporter compounds were stored as solids or as chloroform solutions (10 or 20 mg/mL) in a -20º C freezer. The chloroform solutions were mixed in a 10 mL round bottom flask to give the desired molar ratios. The chloroform was removed using a rotary evaporator followed by evacuation on a high vacuum pump line for 1-3 hours. The aqueous solution to be placed inside the vesicles (1 mL) was added to the lipid film, as well as a small glass ring. Vesicle dispersion was achieved by vortexing the flask at room temperature, followed by nine freeze-thaw cycles. The unilamellar dispersion was extruded twenty-nine times through a 200 nm polycarbonate membrane to give unilamellar vesicles.

Lucigenin Assay (Typical Procedure): A suspension of unilamellar vesicles (200 nm mean diameter, 20 mM lipid) composed of POPC/Cholesterol (7:3 molar ratio) and an aqueous phase containing $NaNO_3$ (225 mM) and lucigenin (1 mM) was loaded onto a Sephadex G-50 column and eluted with aqueous $NaNO_3$ (225 mM) to remove the un-encapsulated lucigenin. The elution process was monitored with a hand-held UV/vis lamp, which allowed the fluorescent lucigenin to be visualized. The eluted vesicles were diluted with aqueous $NaNO_3$ (225 mM) to a final total volume of 25 mL (0.4 mM lipid). An aliquot of this suspension (3 mL) was placed in a cuvette, and the transport experiment was initiated by addition of a very small aliquot of aqueous NaCl (25 mM final concentration) at 25 ºC. The lucigenin fluorescence was monitored at 455/505 ex/em with a 3 nm slit width. The traces shown in each of the quenching figures are representative of three separate experiments. Each curve was fitted by non-linear computer methods to the normalized first order decay equation $(I_0-I_t)/(I_0-I_f)=1-e^{-k_{obs}t}$ where I_0 is initial fluorescence intensity, I_t is intensity at time t, I_f is intensity at time final.

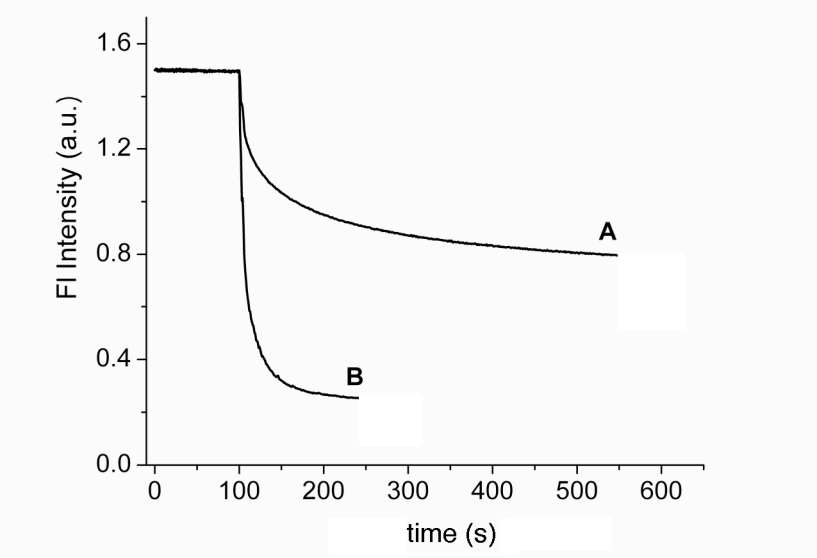

Figure S1. Effect of encapsulated counter anion on Cl^- influx. At 100 s, an aliquot of NaCl (25 mM final conc.) was added to vesicles encapsulating lucigenin (1 mM) and either (**A**) Na_2SO_4 (167 mM) or (**B**) $NaNO_3$ (225 mM). The vesicle membranes contained **1** (4 mol %) in POPC:cholesterol (7:3) at 25 ºC. The Cl^- influx induces quenching of the lucigenin fluorescence (ex: 455, em: 505 nm).

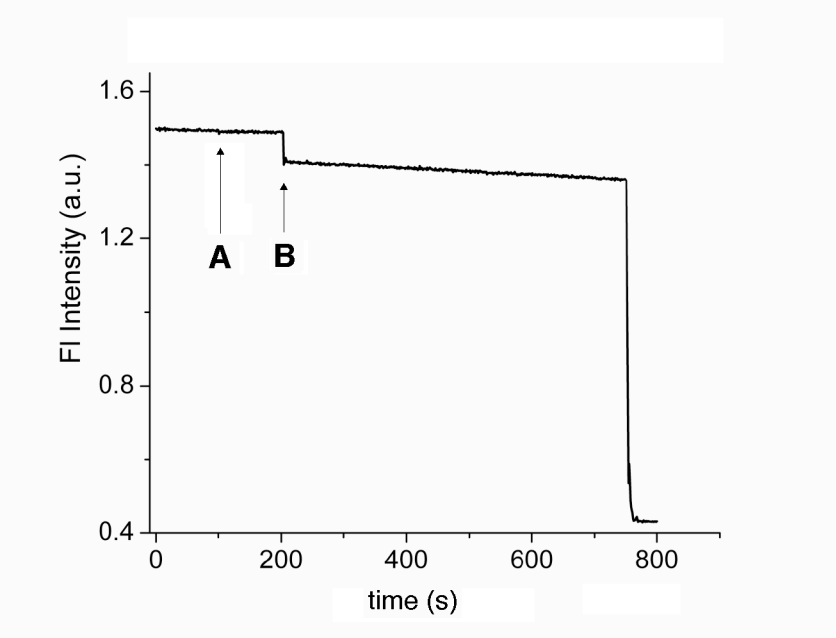

Figure S2. No Cl^- influx occurred when **1** was added externally to preformed vesicles. (**A**) At 100 s, a small aliquot of **1** (25 µM of a 2 mM DMSO stock solution) was added to 3 mL of vesicles composed of POPC/Cholesterol (7:3, 0.4 mM total lipid) and encapsulating lucigenin (1 mM) and $NaNO_3$ (225 mM). (**B**) At 200 s, an aliquot of NaCl (25 mM final conc.) was added and the change in lucigenin fluorescence (ex: 455, em: 505 nm) was monitored with time. The vesicles were lysed at 750 s. T = 25 ºC.

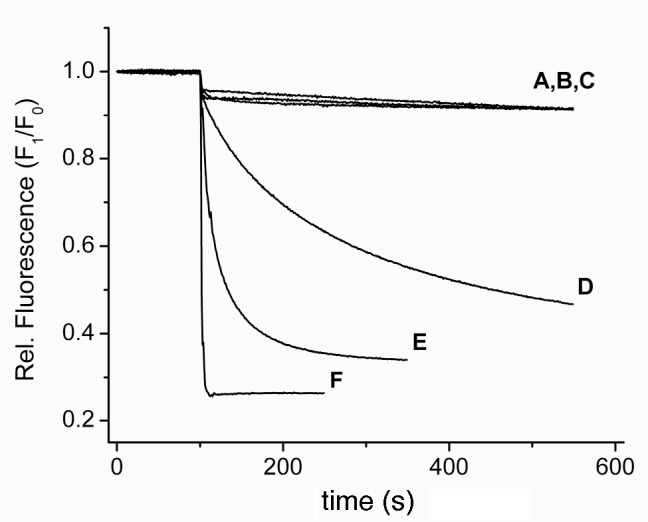

Figure S3. Cl^- influx induced by **1** (3 mol %) in vesicle membranes of different thickness. At 100 s, an aliquot of NaCl (25 mM final conc.) was added to vesicles encapsulating lucigenin (1 mM) and $NaNO_3$ (225 mM). The vesicle membranes were composed of phosphatidylcholine:cholesterol (7:3) where the two phosphatidylcholine acyl chains were: **A** nervonoyl (24:1)), **B** erucoyl (22:1), **C** eicosenoyl (20:1), **D** oleoyl (18:1), **E** palmitoleoyl (16:1), and **F** myristoleoyl (14:1). T = 25 ºC, which means that all vesicle membranes were in the fluid lamellar phase.[s2]

Transporter Concentration Studies: Shown in Figure S4 is a typical set of lucigenin quenching curves induced by Cl^- influx into vesicles containing different concentrations of transporter **1**.

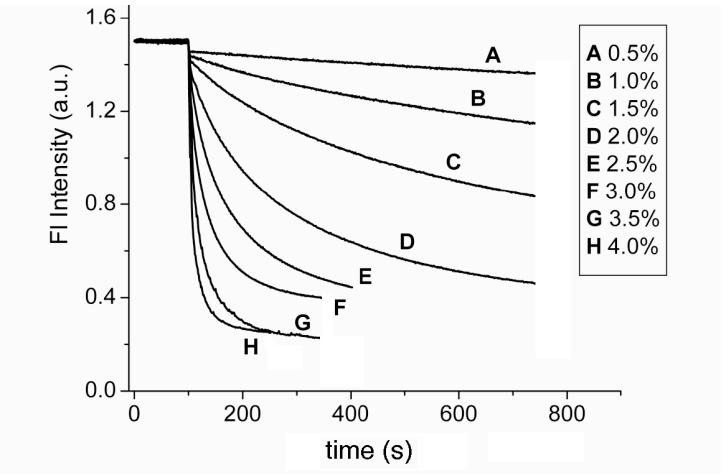

Figure S4. Concentration effect. Transporter **1** was preincorporated into POPC/Cholesterol (7:3 molar ratio) vesicles at increasing molar percentages, 0.5 (**A**), 1.0 (**B**), 1.5 (**C**), 2.0 (**D**), 2.5 (**E**),

[s2] Epand, R. M. et al. *Chem. Phys. Lipids*, **2005**, 135, 39-53.

3.0 (**F**), 3.5 (**G**), and 4.0 (**H**). The vesicles contained lucigenin (1 mM) and $NaNO_3$ (225 mM) and were pulsed with NaCl (25 mM final conc.) at 100 seconds. T = 25 ºC.

The raw data in Figure S4 was analyzed in the following way. Computer fitting of each quenching curve to the first order decay equation, described in the lucigenin assay above, provided an observed rate constant for chloride influx (k_{obs}). The points in Figure 2 of the paper are the average of three individual experiments with S.D. of ±5.0%. Figure 2 shows that increased concentration of transporter **1** produces an exponential increase in k_{obs} which is indicative of a kinetically active aggregate ($\mathbf{1_n}$). The data was modeled quantitatively using a membrane transporter association scheme with the following assumptions and definitions: [s3] all molecules of **1** are located in the vesicle membrane, monomeric **1** is inactive, K is the equilibrium constant for dissociation of $\mathbf{1_n}$ into monomeric **1**, k_2 is the intrinsic transport rate constant for $\mathbf{1_n}$, background influx of Cl^- in the absence of **1** is so low (k_{obs} = 0.0007 s^{-1}) that it can be ignored. Equations 1 – 3 are used to derive equation 4 which states that a plot of k_{obs} versus $[\mathbf{1}]^n$ gives a straight line with slope k_2/K, as shown in the insert of Figure 2 (the values for k_2/K are 0.04 for DMPC:cholesterol and 0.0003 for POPC:cholesterol).

Eq 1: $\text{Rate} = k_{obs}[Cl^-]$

Eq 2: $k_{obs} = k_2[\mathbf{1_n}]$

Eq 3: $K = [\mathbf{1}]^n/[\mathbf{1_n}]$

Eq 4: $k_{obs} = k_2[\mathbf{1}]^n/K$

[s3] Otto, S.; Osifchin, M.; Regen, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 7276-7277.