Supporting Information

A Transmembrane Anion Transporter Selective for Nitrate over Chloride

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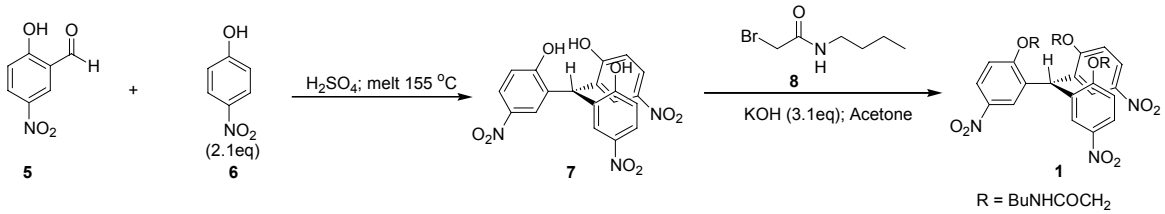
Materials and Methods. All ^{1}H NMR spectra were recorded on a Bruker DRX-400 instrument operating at 400.13 MHz and chemical shifts are reported in ppm relative to the residual solvent peak. All ^{13}C NMR spectra were recorded at 100.61 MHz and chemical shifts are reported in ppm relative to the solvent peak. ESI-MS were obtained on a JEOL AccuTOF-CS es-tof instrument with an Agilent 1100 HPLC interface. Liposome fluorimetric assays were performed using a Hitachi F-4500 spectrophotometer with temperature maintained at 25 ^{o}C by a water bath circulator (+/- 0.2 ^{o}C). Chemicals and solvents were purchased from Sigma, Aldrich, Fluka, or Acros. Lucigenin dye was purchased from Molecular Probes, and EYPC was purchased from Avanti Polar Lipids.

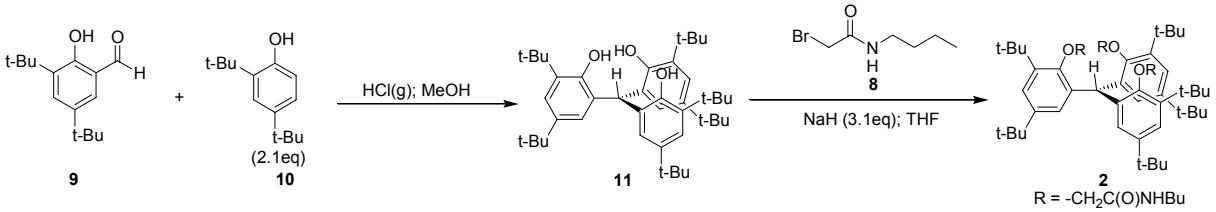
Synthetic Procedures.

Scheme 1:



Tris(5-nitro-2-butylamidomethoxyphenyl)methane (1) Nitro tripod **1** was prepared from the known intermediate **7**, which was made using a modification of literature procedures.[1-3] A mixture of 5-nitrosalicylaldehyde **5** (5.20 g, 31.1 mmol) and p-nitrophenol **6** (9.67 g, 69.5 mmol) was stirred at 110 ^{o}C under N_2 until a homogeneous yellow melt formed. Sulfuric acid (4 mL) was added to the melt and the dark-brown-orange viscous mass was stirred at 155 ^{o}C for 2 h. The reaction was cooled to rt at which point the melt solidified. To this solid, hot water was added and mechanically stirred with a glass rod. The water which was now tinted yellow was decanted to remove excess *p*-nitrophenol **6** (3 x 50 mL) leaving behind a brown solid. The brown solid was dissolved in 5% aqueous sodium hydroxide (300 mL) and the remaining insoluble black solid was filtered off. The yellow-brown solution was added dropwise with stirring to 3.5% aqueous hydrochloric acid (700 mL) causing the material to precipitate. The precipitate was filtered to give the known **7** (12.5 g, 29.2 mmol) as a brown powder.[1-3] A solution of **7** (0.100 g, 0.23 mmol) and KOH (0.041 g, 0.73 mmol) in acetone (15 mL) was stirred for 1 h at rt. Then, 2-bromo-*N*-butylacetamide (**8**; 0.14 g, 0.73 mmol) dissolved in acetone was added and the reaction mixture was allowed to reflux overnight. After cooling to rt, ethyl acetate (15 mL) was added to the reaction mixture. This solution was washed with water (15 mL) and the organic layer separated. The solvent was removed *in vacuo* and the resulting crude product was purified by column chromatography (5% MeOH- CH_2Cl_2), to yield **1** (63.5 mg, 0.083 mmol) as a yellow powder. ^{1}H NMR: 8.12 (dd, ^{3}J= 6.3 Hz, 2.7 Hz, 3 H), 7.51 (d, ^{3}J=2.7 Hz, 3 H), 6.52 (s, 1 H), 6.72 (t, ^{3}J=5.2 Hz, 3 H), 4.54 (s, 6 H), 4.20 (s, 6 H), 3.36 (q, ^{3}J = 1.5 Hz, 3 H), 3.16 (t, ^{3}J = 7.2 Hz, 6 H), 1.37 (m, 6 H), 1.25 (m, 8 H), 0.89 (t, ^{3}J = 7.3 Hz, 9 H) ^{13}C NMR: 166.8, 166.7, 159.7, 141.9, 130.2, 125.2, 124.4, 112.1, 77.2, 67.5, 38.9, 38.8, 38.3, 31.1, 19.7, 13.2. Mass calculated for $C_{37}H_{46}N_6O_{12}$: 766.3174. Mass found ESI-MS: 767.2888 m/z (M + H^+)

Scheme2:



tris(3,5-di-*tert*-buty-2-butylamidomethoxyphenyl)methane (2). Compound **11** was prepared using the literature procedure. [4] A solution of **5** (0.525 g, 0.83 mmol) and NaH (0.065 g, 2.70 mmol) in THF (15 mL) was stirred for 2 h at rt. At this point, 2-bromo-*N*-butylacetamide (**8**; 0.504 g, 2.60 mmol) dissolved in THF was added and the reaction was allowed to reflux for 8 h. The reaction mixture was cooled to rt at which point a precipitate formed. The precipitate was filtered through a fritted funnel to yield crude **2**. The resulting crude product was purified by column chromatography (MeOH:CH_2Cl_2), to yield **2** (0.29 g, 0.30 mmol) as a white powder. ^{1}H NMR: 7.22 (d, ^3J = 2.4 Hz, 3 H), 7.03 (d, ^3J = 2.4 Hz, 3 H), 6.80 (t, ^3J =5.7 Hz, 3 H), 6.25 (s, 1 H), 4.14 (s, 6 H), 3.35 (q, ^3J = 6.5 Hz, 6 H), 1.54 (m, 16 H), 1.38 (m, 6 H), 1.31 (s, 27 H), 1.17 (s, 27 H), 0.94 (t, ^3J =7.4 Hz, 3 H) ^{13}C NMR: 168.5, 151.1, 146.0, 141.8, 136.5, 126.5, 123.1, 77.2, 71.3, 38.8, 35.3, 34.5, 31.7, 31.3, 31.2, 20.0, 13.7. Mass Calculated for $C_{61}H_{97}N_3O_6$: 967.7377 Mass found (+)-ESI-MS: 968.6958 m/z (M + H^+)

Electrospray Ionization Mass Spectroscopy: Stock solutions of TBA-NO_3 (3.6 mM), TBA-Cl (3.6 mM), nitro tripod **1** (3.6 mM), and t-butyl tripod **2** (3.6 mM) were prepared in CH_2Cl_2. The temperature of the ESI-MS was set at 272 °C. The ESI-MS spectrum was obtained in both positive mode (Orifice 1 voltage sweep: 25 V, ring lens voltage: 8 V) and negative mode (Orifice 1 voltage sweep: - 25 V, ring lens voltage: - 8V). A 10 µL aliquot of TBA salt solution was added to 10 µL of a solution of either **1** or **2** and the resulting solution was diluted to 1 mL with CH_2Cl_2 in a separate vial. From this new solution, 2 µL was injected onto the ESI-MS column. Additionally, compound **1** (10 µL) was mixed with equal amounts of TBA-Cl and TBA-NO_3 (10 µL each) and diluted to 1 mL. From this solution, 2 µL was injected onto the ESI-MS. The samples were eluted with 1:1 H_2O-MeOH containing 1% trifluoroacetic acid.

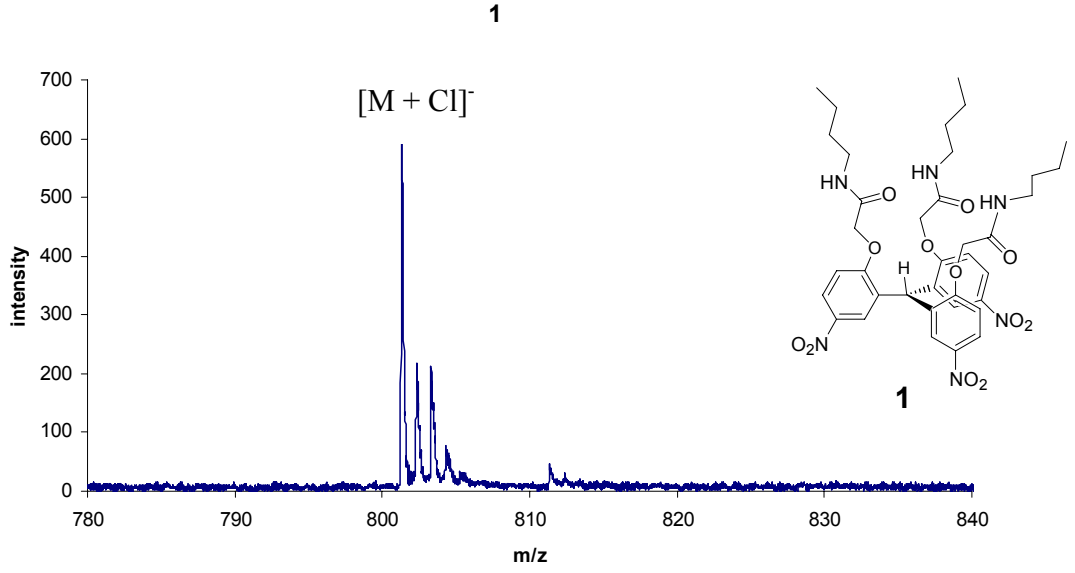


Figure 1: Negative mode ESI-MS for compound **1** in the absence of any TBA salts.

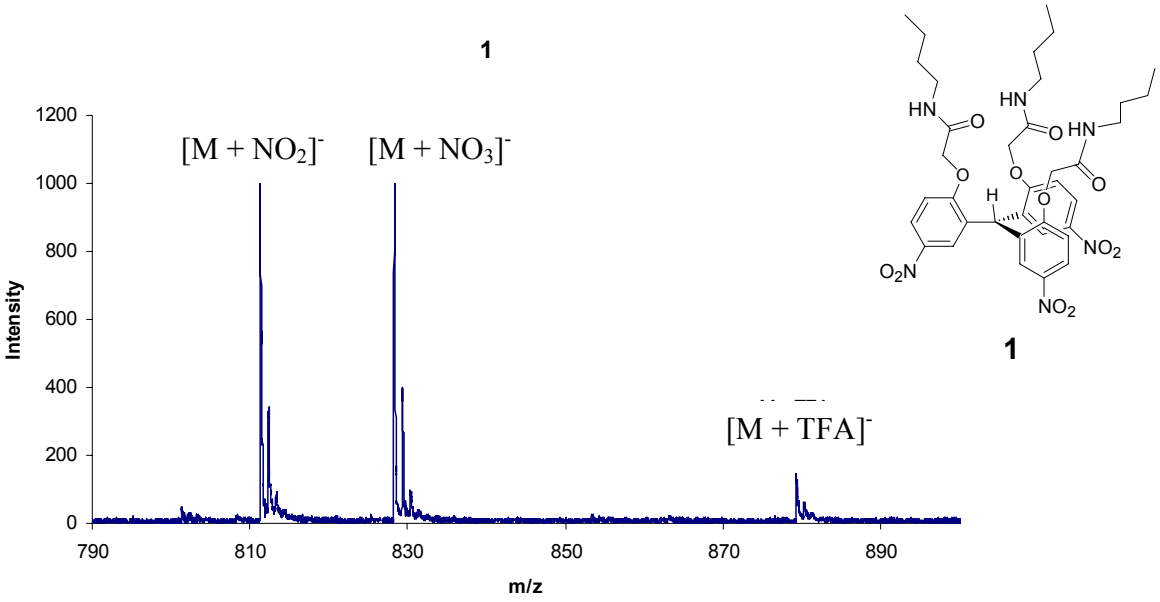


Figure 2: Negative-mode ESI-MS of **1** with $TBANO_3$ (1:1 mol%). We believe the significant $[M + NO_2]^-$ peak originates from the reduction of the $[M + NO_3]^-$ ion in the mass spectrometer in the (-)-mode ESI-MS.

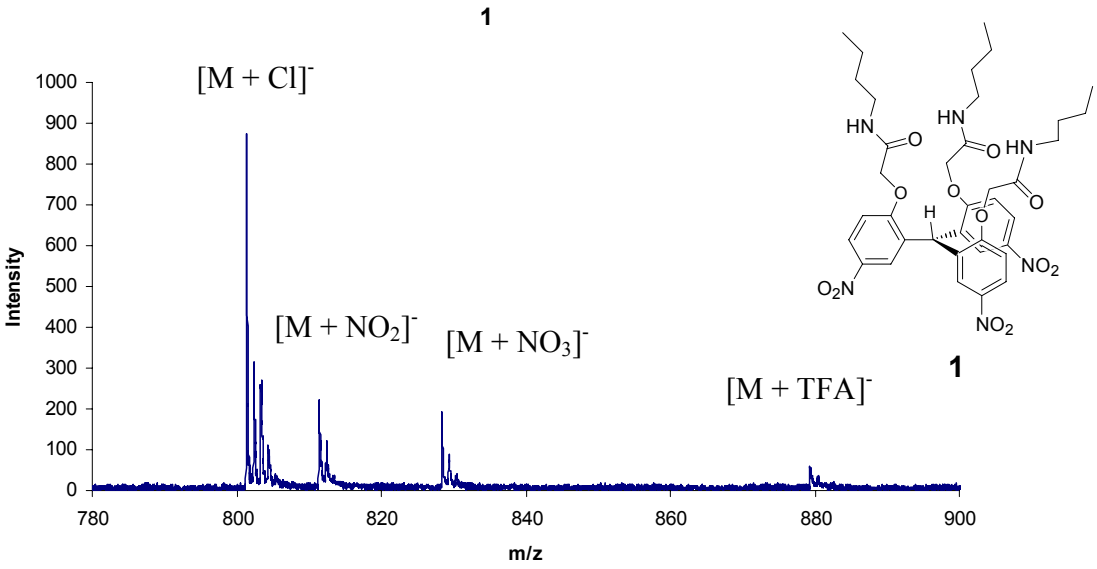


Figure 3: Negative mode ESI-MS of **1**. The mixture contained 1:1:1 TBACl: $TBANO_3$: **1**.

^{1}H NMR Titrations. Stock solutions of the nitro and *t*-butyl tripods, **1** and **2** (3.0 mM), in CD_2Cl_2 were prepared and divided into nine NMR tubes (300 μL). Stock solutions of the salts (TBA-NO_3 and TBA-Cl) were prepared in two different concentrations labeled solution **A** (100 mM) and solution **B** (4 mM). Aliquots of anion stock solution were added to each NMR tube in increasing volumes (in μL) as follows: solution **A** – 0, 30, 60, 120; and solution **B** – 0, 15, 30, 75, 150, and 300. This provided final anion concentrations of: 0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 mM, and anion:tripod ratios from 0 to 13 equivalents. The total solution volume was kept constant at 600 μL (solvent was added to make up this volume where necessary) to afford a concentration of 1.5 mM for the tripods **1** and **2**. Changes in the chemical shifts of the amide NH and bridgehead CH hydrogens of tripods **1** and **2** were

monitored by ^{1}H NMR (400 MHz). Titration curves were generated to fit a 1:1 binding model using the Associate v 1.6 software (F. N. Diederich, ETH Zürich) for determination of association constants by non-linear regression analysis of the spectroscopic data.

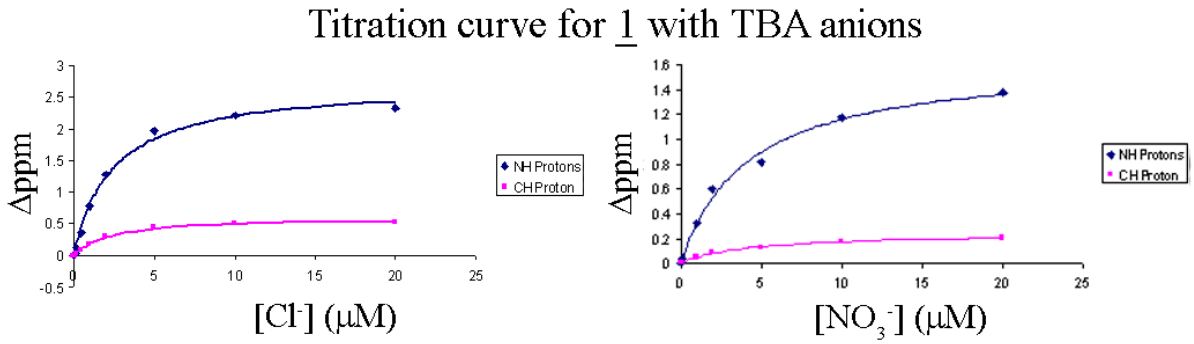
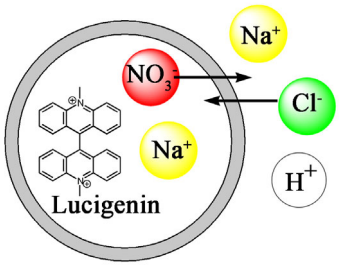


Figure 4: The ^{1}H NMR titration curves for tripod **1** binding to either Cl^- or NO_3^- in CD_2Cl_2.

Table 1: Summary of binding results for the association of nitro-tripod **1** with chloride and nitrate ions.

Anion	Δδ ppm	K_{assoc} (L/mol)
Cl^-	NH = 2.32 CH = 0.52	NH = 815.6 ± 108.1 CH = 827.9 ± 158.1
NO_3^-	NH = 1.37 CH = 0.21	NH = 325.5 ± 113.3 CH = 328.2 ± 95.3

Preparation of EYPC liposomes for HPTS assays.[5] This assay describes the experiment depicted in Figure 2 of the paper. EYPC lipid (60 mg) was dissolved in 5 mL of a chloroform/methanol mixture (5 % MeOH). The resulting solution was evaporated under reduced pressure to produce a thin film that was dried *in vacuo* for 2 h. The lipid film was hydrated with 1 mL of a solution of 10 mM sodium phosphate (pH=6.4) containing 100 mM $NaNO_3$ or 100 mM NaCl and 0.1 mM HPTS. After 10 freeze/thaw cycles, the liposomes were extruded through a 100 nm polycarbonate membrane 21 times at rt. The liposome solution was passed through a Sephadex (G-25) column to remove excess dye (eluant, sodium phosphate buffer, pH 6.4, 100 mM $NaNO_3$ or 100 mM NaCl). The isolated liposomes were diluted in 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$ or 100 mM NaCl) to give a concentration of 25 mM in EYPC, assuming 100 % retention of lipid during the gel filtration process. In a typical experiment, 50 μL of the stock EYPC liposomes was diluted into 2 mL of 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a solution that is 0.5 mM in lipid. Compounds **1**, **2**, and **3** were added to give a 2:100 ligand:lipid ratio. To the cuvette containing the EYPC-transporter mixture was added 20 μL of 0.5 M NaOH solution through an injection port to give an external pH of ~7.3. The fluorescence of the intravesicular pH was monitored at excitation 403 nm and 460 nm monitoring the emission at 510 nm for 500 s. After 470 s, 0.04 mL of 10 % Triton-X detergent was added to lyse the liposomes. The internal pH of the vesicles was determined by measuring the fluorescence of HPTS dye in various solutions ranging in pH (5.0 - 9.0) and fitting the HPTS emission intensities according to the calibration equation $pH = (I_o/I_1 + 6.9201)/1.1106$, where I_o is the emission intensity with excitation at 460 nm and I_1 is emission intensity with excitation at 403 nm. The calibration was performed by measuring the HPTS emission intensities and the pH values of a 470 pM HPTS solution in 10 mM phosphate buffer containing 100 mM NaCl or 100 mM $NaNO_3$ and obtaining a calibration curve ($pH = (I_o/I_1 + 6.9201)/1.1106$). Experiments were done in triplicate.



Preparation of EYPC liposomes for lucigenin assays.[6] EYPC lipid (60 mg) was dissolved in 5 mL of a chloroform/methanol mixture (5 % MeOH). The resulting solution was evaporated under reduced pressure to produce a thin film that was dried *in vacuo* for 2 h. The lipid film was hydrated with 1 mL of a solution of 10 mM sodium phosphate (pH=6.4) containing 100 mM $NaNO_3$ and 1 mM lucigenin. After 10 freeze/thaw cycles, the liposomes were extruded through a 100 nm polycarbonate membrane 21 times at room temperature. The liposome solution was passed through a Sephadex (G-25) column to remove excess dye (eluant, sodium phosphate buffer, pH 6.4, 100 mM $NaNO_3$). The isolated liposomes were diluted in 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a concentration of 25 mM in EYPC, assuming 100 % retention of lipid during the gel filtration process. In a typical experiment, 50 uL of the stock EYPC liposomes was diluted into 2 mL of 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a solution that is 0.5 mM in lipid. Compounds **1**, **2** and **3** were added to give a 2:100 ligand:lipid ratio. To the cuvette containing the EYPC-transporter mixture was added 20 µL of 2.35 M NaCl solution through an injection port to give an external chloride concentration of 25 mM. The fluorescence of the intravesicular chloride concentration was monitored at excitation 372 nm and emission at 504 nm for 500 s. After 470 s, 0.04 mL of 10 % Triton-X detergent was added to lyse the liposomes. The internal liposome chloride concentration was determined in accordance to previous literature reports from our lab.[6] Experiments were done in triplicate.

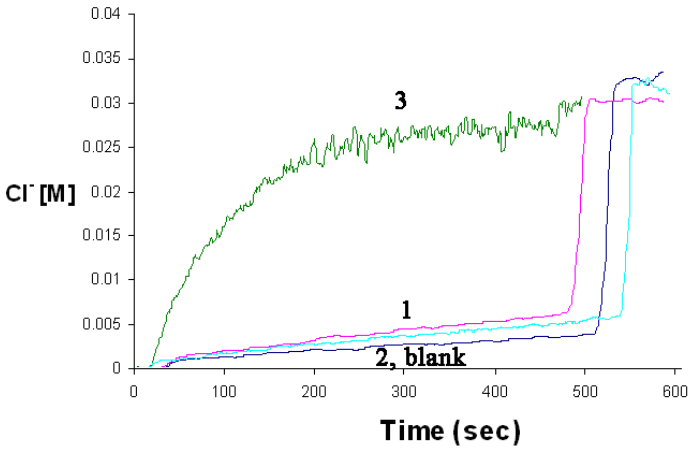


Figure 5: Cl^- transport assay utilizing encapsulated lucigenin to monitor chloride transport.

Nitrate Reductase assay.[7] This assay describes the experiment depicted in Figure 3 of the paper. EYPC lipid (60 mg, Avanti) was dissolved in 5 mL of a chloroform/methanol mixture (5 % MeOH). The resulting solution was evaporated under reduced pressure to produce a thin film that was dried *in vacuo* for 2 h. The lipid film was hydrated with 1 mL of a solution of 10 mM sodium phosphate (pH=7.2) containing 100 mM $NaNO_3$. After 10 freeze/thaw cycles, the liposomes were extruded through a 100 nm polycarbonate membrane 21 times at room temperature. The liposome solution was passed through a Sephadex (G-25) column eluted with 10 mM sodium phosphate (pH 7.2, 100 mM NaCl) to remove the external $NaNO_3$ ions and to give a concentration of 25 mM in EYPC, assuming 100 % retention of lipid during the gel filtration process. In a typical experiment, 50 µL of the stock EYPC liposomes was diluted into 2 mL of 10 mM sodium phosphate (pH 7.2, 100 mM NaCl, 1.1 mM NADPH, 0.3 units of nitrate reductase) to give a solution that is 0.5 mM in lipid. Tripods **1** and **2** were added to separate cuvettes and the UV spectrum was monitored over time. After 3 h. of monitoring the NADPH UV spectrum, 60 µL of sulfanilamide (0.29 M in 2 N HCl) and 216 µL of N-(1-naphthyl)ethylenediamine (0.29 M in 2 N HCl) were added to the cuvette. The solution turned a red color in the presence of nitrite indicating formation of the diazo **4** (Griess reaction) and remained colorless in the absence of nitrite (Griess reaction).

Preparation of EYPC liposomes for HPTS assays of Nitrate and Chloride with no pH gradient. This assay describes the experiment depicted in Figure 4 of the paper. EYPC lipid (60 mg, Avanti) was dissolved in 5 mL of a chloroform/methanol mixture (5 % MeOH). The resulting solution was evaporated under reduced pressure to produce a thin film that was dried *in vacuo* for 2 h. The lipid film was hydrated with 1 mL of a solution of 10 mM sodium phosphate (pH 6.4) containing 100 mM $NaNO_3$ and 0.1 mM HPTS. After 10 freeze/thaw cycles, the liposomes were extruded through a 100 nm polycarbonate membrane 21 times at room temperature. The liposome solution was passed through a Sephadex (G-25) column to remove excess dye (eluant, sodium phosphate buffer, pH 6.4, 100 mM $NaNO_3$). The isolated liposomes were diluted in 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a concentration of 25 mM in EYPC, assuming 100 % retention of lipid during the gel filtration process. In a typical experiment, 50 μL of the stock EYPC liposomes was diluted into 2 mL of 10 mM sodium phosphate (pH 6.4, 100 mM NaCl) to give a solution that is 0.5 mM in lipid. To the cuvette containing the EYPC-transporter mixture was added compounds **1**, **2** and **3** through an injection port to give a 2:100 ligand:lipid ratio. The fluorescence of the intravesicular pH was monitored at excitation 403 nm and 460 nm monitoring the emission at 510 nm for 500 s. After 470 s, 0.04 mL of 10 % Triton-X detergent was added to lyse the liposomes. The pH was calculated by fitting the HPTS emission intensities according to the calibration equation $pH = (I_o/I_1 + 6.9201)/1.1106$, where I_o is the emission intensity with excitation at 460 nm and I_1 is emission intensity with excitation at 403 nm. Experiments were done in triplicate.

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