Supplementary Material (ESI) for Chemical Communications

SUPPORTING INFORMATION

Cationic Calix[4]arenes as Anion-Selective Ionophores

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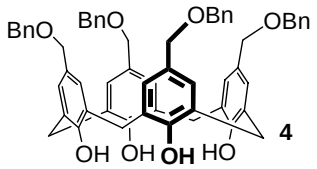
1.0 Synthesis.

1.1 General Procedures.

All reactions involving air or moisture sensitive reagents were carried out under a dry argon or nitrogen atmosphere using freshly distilled solvents. Tetrahydrofuran (THF) was distilled from $LiAlH_4$ under argon. Toluene and CH_2Cl_2 were distilled from CaH_2. Glassware was flame-dried (0.05 Torr) prior to use. When necessary, compounds were dried *in vacuo* over P_2O_5 or by azeotropic removal of water with toluene under reduced pressure. Starting materials and reagents purchased from commercial suppliers were generally used without purification unless otherwise mentioned. Reaction temperatures were measured externally; reactions were monitored by TLC on Merck silica gel plates (0.25 mm) and visualized by UV light, I_2, or by spraying with H_2SO_4-$Ce(SO_4)_2$, phosphomolybdic acid or ninhydrin solutions and drying. Flash chromatography was performed on Merck silica gel 60 (particle size: 0.040-0.063 mm) and the solvents employed were of analytical grade. Yields refer to chromatographically and spectroscopically (^{1}H- and ^{13}C-NMR) pure materials. The NMR spectra were recorded at room temperature or when indicated at 50°C, 80°C or 100°C on a Bruker DRX 400 spectrometer (^{1}H at 400.13 MHz, ^{13}C at 100.03 MHz). Chemical shifts (δ) are reported in ppm relatively to the residual solvent peak ($CHCl_3$, δ: 7.26, $^{13}CDCl_3$, δ: 77.0; CD_2HOD, δ: 3.35, $^{13}CD_3OD$, δ: 49.0; C_2DHCl_4: δ= 5.80, $^{13}CCD_2Cl_4$: δ= 72.1) and the multiplicity of each signal is designated by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; quint, quintuplet; m, multiplet; br, broad; app, apparent. Coupling costants (*J*) are quoted in Hz. Homonuclear decoupling and DEPT experiments completed the full assignment of each signal. High resolution ESI-MS spectra were performed on a Q-Star Applied Biosystem mass spectrometer.

Compound 4

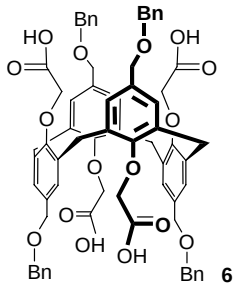
To a solution of NaOBn, generated *in situ* by addition of Na (0.52 g, 22.8 mmol) to BnOH (2.94 ml, 28.4 mmol), under nitrogen atmosphere, *p*-chloromethyl-calix[4]arene **3** (0.88 g, 1.42 mmol) in anhydrous THF (30.0 ml) was added *via cannula*. The reaction mixture was stirred overnight, acidified with HCl until pH 7 and extracted three times with $CHCl_3$ (3 × 150 ml). The combined organic phases were washed with water (2 × 70 ml), dried over Na_2SO_4, filtered and evaporated to dryness under high vacuum to remove all BnOH to give **4** (0.72 g, 56%) as a viscous oil.



4: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.62 (4H, br signal, CH*H*-bridge), 4.32 (4H, br s, C*H*H-bridge), 4.40 (8H, s, OCH_2Ar), 4.54 (8H, s, OCH_2Ph), 6.92 (8H, s, Ar*H*), 7.40 (20H, m, Ar*H*), 10.2 (4H, s, O*H*);
^{13}C NMR (100 MHz, $CDCl_3$) δ: 31.7 (× 4), 71.5 (× 4), 71.9 (× 4), 127.6 (× 4), 127.8 (× 8), 128.1 (× 4), 128.3 (× 8), 128.6 (× 8), 131.9 (× 8), 138.2 (× 4), 148.3 (× 4).
HR ESI-MS (negative ion mode): $[M-H]^-$ *m*/*z* 903.3920 (calcd for $C_{60}H_{55}O_8$ 903.3897).

Compound 6

To a solution of **4** (0.72 g, 0.79 mmol) in acetone (25.0 ml), Cs_2CO_3 (2.06 g, 6.32 mmol) and ethyl bromoacetate (0.70 ml, 6.32 mmol) were added and the resulting suspension was stirred at reflux under a nitrogen atmosphere for 24 h. The solvent was evaporated under reduced pressure. The remaining solid was taken into CH_2Cl_2 (100 ml) and washed with H_2O (2 × 100 ml). The combined organic phases were dried over Na_2SO_4, filtered and evaporated to dryness to give the crude residue **5** which was used in the next step without further purification. A solution of crude **5** in 45% aqueous KOH (3.0 ml), MeOH (10.0 ml) and THF (10.0 ml) was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the resulting solid was taken into H_2O (3 ml). The acqueous solution was acidified with 6 M HCl until pH 1, extracted with CH_2Cl_2 (2 × 10 ml). The combined organic phases were dried over Na_2SO_4, filtered and evaporated to dryness. The crude residue was flash-chromatographed (2 - 10% MeOH in CH_2Cl_2 with 0.1% CH_3COOH) to give pure **6** (0.44 g, 49%) as a viscous oil.



6

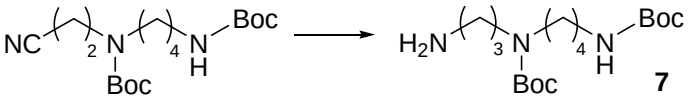
6: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.81 (8H, s, C*H*$_2$-bridge), 3.93 (8H, s, C*H*$_2$), 4.39 (8H, s, C*H*$_2$), 4.54 (8H, s, C*H*$_2$), 7.07 (8H, s, Ar*H*), 7.34 (20H, s Ar*H*);

^{13}C NMR (100 MHz, $CDCl_3$) δ: 37.3 (× 4), 66.9 (× 4), 71.2 (× 4), 72.4 (× 4), 127.6 (× 4), 127.8 (× 8), 128.3 (× 8), 129.0 (× 8), 133.1 (× 8), 135.2 (× 4), 137.9 (× 4), 153.0 (× 4), 169.3 (× 4).

HR ESI-MS (negative ion mode): $[M-H]^-$ *m*/*z* 1135.4099 (calcd for $C_{68}H_{63}O_{16}$ 1135.4116).

Compound 7

The synthesis of the protected spermidine **7** was realized following the procedure described by Nakanishi and Hu,[1] except for the last step. The final reduction of the Boc-protected nitrile, in fact, was accomplished as follows:[2] To a solution of nitrile (0.340 g, 0.99 mmol) in a mixture of 1,4-dioxane (5.0 ml) and water (0.5 ml), Raney-nickel (0.056 g, 0.48 mmol), as a 50 % suspension in water, and 10% Pd/C (0.056 g) were added together with $LiOH·H_2O$ (0.040 g, 0.96 mmol). The flask was evacuated and flushed with hydrogen three times. The reaction mixture was stirred under hydrogen atmosphere at 50°C for 20 h. The catalysts were filtered off, the solvents were removed *in vacuo*, and a mixture of water (20 ml) and CH_2Cl_2 (15 ml) was added to the crude amine. After phase separation the aqueous phase was extracted twice with CH_2Cl_2 (15 ml). The combined organic phases were dried over Na_2SO_4, filtered and evaporated to dryness. The crude residue was flash-chromatographed (0.2 - 20% MeOH in CH_2Cl_2 with 0.1% of 30% NH_4OH in water) to give **7** (0.220 g, 64%) as a viscous oil.



7

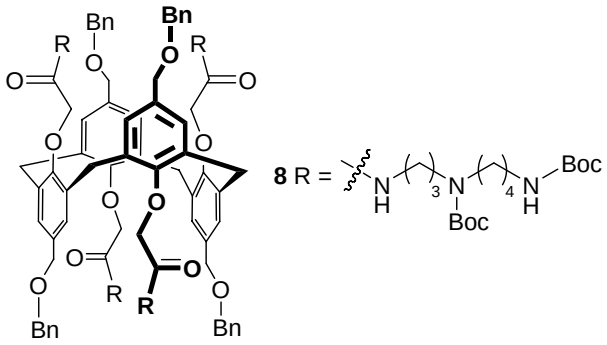
7: ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.42 (9H, m, (C*H*$_3$)$_3$CO), 1.43 (11H, m, (C*H*$_3$)$_3$CO and C*H*$_2$), 1.52 (2H, m, C*H*$_2$), 1.63 (2H, quint, *J* = 6.7 Hz, C*H*$_2$), 2.67 (2H, t, *J* = 6.7 Hz, C*H*$_2$), 3.09-3.26 (6H, m, C*H*$_2$), 4.66 (1H, m, N*H*Boc);

^{13}C NMR (100 MHz, $CDCl_3$) δ: 25.6, 27.3, 28.3 (× 6), 31.8, 39.1, 40.1, 43.8, 46.3, 79.1, 79.2, 155.5, 155.8.

HR ESI-MS: $[M+H]^+$ *m*/*z* 346.2718 (calcd for $C_{17}H_{36}N_3O_4$ 346.2706).

Compound 8

To a solution of tetraacid **6** (0.17 g, 0.15 mmol) in anhydrous toluene (6.0 ml), $SOCl_2$ (0.40 ml, 4.50 mmol) was added and the resulting mixture was stirred at reflux under a nitrogen atmosphere for 2.5 h. The solvent was evaporated under reduced pressure. To a solution of the residue in anhydrous toluene (6.0 ml), anhydrous NEt_3 (0.84 ml, 0.60 mmol) and protected spermidine **7** (0.414 g, 1.20 mmol) were added. The mixture was left at r.t. for 24 h and then it was treated with sat. aq. NH_4Cl. The aqueous layer was extracted three times with CH_2Cl_2 (3 × 20 ml) and the combined organic phases were washed with sat. aq. $NaHCO_3$, dried over $MgSO_4$, filtered and evaporated to dryness. The crude residue was flash-chromatographed (0.2 - 10% MeOH in CH_2Cl_2) to give pure **8** (0.25 g, 68%) as an amorphous solid.



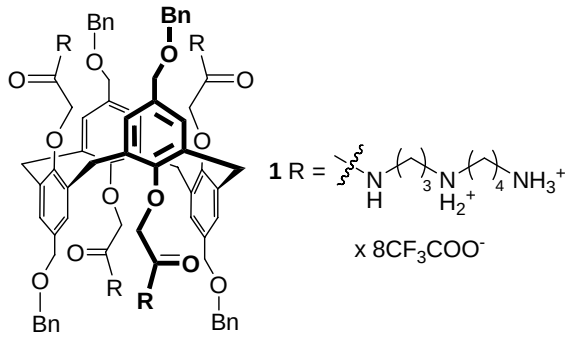
8: ^{1}H NMR (400 MHz, $C_2D_2Cl_4$, 100°C) δ: 1.30 (36H, s, $(CH_3)_3CO$), 1,32 (36H, s, $(CH_3)_3CO$), 1.35 (16H, m, CH_2), 1.54 (8H, quint, J = 6.7 Hz, CH_2), 2.92-3.05 (32H, m, CH_2N), 3.33 (8H, br s, CH_2-bridge), 3.74 (8H, br s, CH_2), 4.22 (8H, br s, CH_2), 4.34 (4H, br s, N*H*Boc), 4.36 (8H, br s, CH_2), 6.32 (4H, br s, N*H*CO), 6.91 (8H, s, Ar*H*), 7.15 (20H, s Ar*H*);

^{13}C NMR (100 MHz, $C_2D_2Cl_4$, 80°C) δ: 24.1 (× 4), 25.7 (× 4), 26.7 (× 12), 26.8 (× 12), 27.8 (× 4), 35.1 (× 4), 36.0 (× 4), 38.8 (× 4), 43.1 (× 4), 45.2 (× 4), 68.8 (× 4), 70.1 (× 4), 70.6 (× 4), 77.1 (× 4), 77.6 (× 4), 125.7 (× 4), 125.8 (× 8), 126.5 (× 8), 128.2 (× 8), 132.3 (× 4), 132.7 (× 8), 136.7 (× 4), 153.3 (× 4), 153.8 (× 4), 154.0 (× 4), 166.4 (× 4).

HR ESI-MS: $[M+Na]^+$ *m*/*z* 2468,4202 (calcd for $C_{136}H_{196}N_{12}NaO_{28}$ 2468,4180).

Compound 1

The tetraamide **8** (0.016 g, 0.006 mmol) was dissolved in a 1:1 mixture of TFA and CH_2Cl_2 (3.0 ml) and the mixture was stirred overnight. Volatiles were removed *in vacuo* and the oily residue was washed twice with diethyl ether and dried (high vacuum, r.t., overnight) to give **1** (0.015 g, quant.) as an amorphous solid.



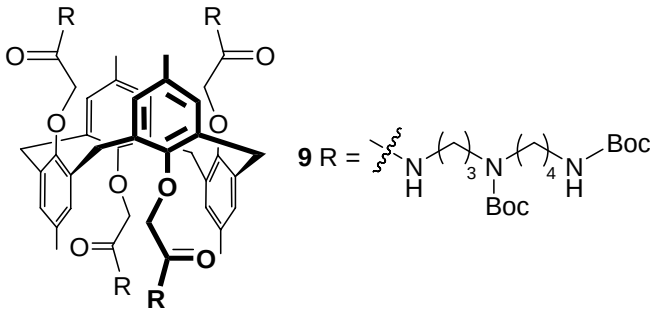
1: ^{1}H NMR (400 MHz, CD_3OD, 50°C) δ: 1.79 (16H, m, C*H*$_2$), 1.87 (8H, m, C*H*$_2$), 2.93-3.00 (32H, m, C*H*$_2$N), 3.84 (8H, br s, C*H*$_2$-bridge), 4.02 (8H, br s, C*H*$_2$), 4.45 (8H, br s, C*H*$_2$), 4.56 (8H, br s, C*H*$_2$), 7.19 (8H, s, Ar*H*), 7.39 (20H, s Ar*H*);

^{13}C NMR (100 MHz, CD_3OD, 50°C) δ: 24.2 (× 4), 25.5 (× 4), 27.5 (× 4), 37.0 (× 4), 38.2 (× 4), 40.0 (× 4), 46.5 (× 4), 49.0 (× 4, overlapped with *C*D_3OD), 71.2 (× 4), 72.9 (× 4), 73.4 (× 4), 117.8 (× 8, q, *J* = 297 Hz, *C*F_3), 128.8 (× 4), 128.9 (× 8), 129.5 (× 8), 130.6 (× 8), 135.3 (× 4), 135.3 (× 8), 139.8 (× 4), 156.4 (× 4), 163.1 (× 8, brs, $CF_3CO_2^-$), 171.8 (× 4).

HR ESI-MS: $[M+H]^+$ *m*/*z* 1646.0156 (calcd for $C_{96}H_{133}N_{12}O_{12}$ 1646.0166).

Compound 9

To a solution of tetra amide **8** (0.049 g, 0.020 mmol) in MeOH (5.0 ml), 10% Pd/C (0.049 g) was added. The flask was evacuated and flushed with hydrogen three times. The reaction mixture was stirred vigorously, under hydrogen atmosphere, overnight. It was filtered through a short pad of Celite® and concentrated. The crude residue was flash-chromatographed (0.2- 10% MeOH in CH_2Cl_2) to afford **9** (0.020, 49%) as an amorphous solid.



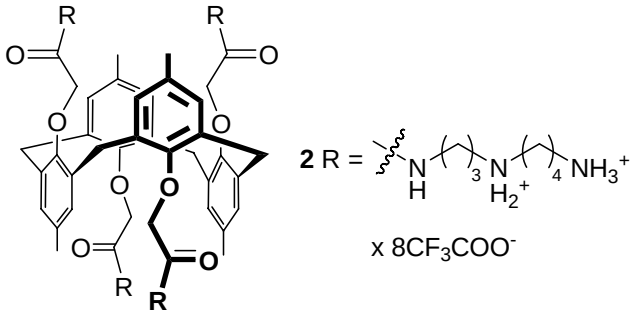
9: ^{1}H NMR (400 MHz, $C_2D_2Cl_4$, 80°C) δ: 1.28 (36H, s, (C*H*$_3$)$_3$CO), 1,32 (36H, s, (C*H*$_3$)$_3$CO), 1.32 (8H, m, C*H*$_2$, overlapped), 1.42 (8H, m, C*H*$_2$), 1.61 (8H, quint, *J* = 6.7 Hz, C*H*$_2$), 1.97 (12H, s, C*H*$_3$), 2.94-3.11 (32H, m, C*H*$_2$N), 3.30 (4H, br s, N*H*Boc), 3.59 (8H, br s, C*H*$_2$-bridge), 4.43 (4H, br s, N*H*CO), 6.68 (8H, s, Ar*H*);

^{13}C NMR (100 MHz, $C_2D_2Cl_4$, 80°C) δ: 18.9 (× 4), 24.1 (× 4), 25.7 (× 4), 26.7 (× 24), 27.8 (× 4), 35.3 (× 4), 35.9 (× 4), 38.8 (× 4), 43.2 (× 4), 45.2 (× 4), 68.7 (× 4), 77.2 (× 4), 77.6 (× 4), 129.5 (× 8), 131.2 (× 4), 132.6 (× 8), 151.6 (× 4), 153.8 (× 4), 154.1 (× 4), 166.6 (× 4).

HR ESI-MS: $[M+Na]^+$ *m*/*z* 2044.2561 (calcd for $C_{108}H_{172}N_{12}NaO_{24}$ 2044.2505).

Compound 2

Compound **9** (0.018 g, 0.009 mmol) was dissolved in a 1:1 mixture of TFA and CH_2Cl_2 (3.0 ml) and the mixture was stirred overnight. Volatiles were removed *in vacuo* and the oily residue was washed with diethyl ether. The white precipitate was washed again with diethyl ether and dried (high vacuum, r.t., overnight) to give **2** (0.019 g, quant.) as an amorphous solid.



2: ^{1}H NMR (400 MHz, CD_3OD, 50°C) δ: 1.83 (16H, m, CH_2), 1.90 (8H, m, CH_2), 2.21 (12H, s, CH_3), 2.98-3.12 (32H, m, CH_2N), 3.82 (8H, br s, CH_2-bridge), 3.90 (8H, br s, OCH_2CO), 6.96 (8H, s, Ar*H*);

^{13}C NMR (100 MHz, CD_3OD, 50°C) δ: 21.2 (× 4), 24.2 (× 4), 25.5 (× 4), 27.5 (× 4), 37.2 (× 4), 38.2 (× 4), 40.1 (× 4), 46.6 (× 4), 49.0 (× 4, overlapped with *C*D_3OD), 71.1 (× 4), 117.8 (× 8, q, *J* = 297 Hz, *C*F_3), 131.7 (× 8), 134.4 (× 4), 135.4 (× 8), 154.7 (× 4), 163.1 (× 8, bs, $CF_3CO_2^-$), 172.0 (× 4).

HR ESI-MS: $[M+H]^+$ *m*/*z* 1243.8278 (calcd for $C_{68}H_{108}N_{12}NaO_8$ 1243.8311).

2. Ionophoric activities

2.1 General Procedures.

L-α-phosphatidyl-DL-glycerol sodium salt (EYPG, 20 mg/mL chloroform solution), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) ammonium salt (PE-LRB) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) ammonium salt (PE-NBD) were purchased from Avanti Polar Lipids; egg yolk phosphatidylcholine (EYPC, 100 mg/mL chloroform solution), bis(*N*-methylacridinium) nitrate (lucigenin), calcein and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) were from Sigma; Triton® X-100 and HEPES buffer were from Fluka; Fluram® and L-Glu from Acros; all salts were of the best grade available from Aldrich and were used without further purification.

Ultra-filtration was performed on Microcon® YM-10 filters from Millipore.

Size exclusion chromatography (SEC) was performed using Sephadex™ G-75 or pre-packed columns Sephadex™ G-25 M (PD-10) from Amersham Biosciences.

Liposome were prepared by extrusion using a 10 mL Lipex™ Thermobarrel EXTRUDER (Northern Lipids Inc.) connected to a thermostatic bath (25°C). The 100 μm policarbonate membranes are Nucleopore Track-Etch Membranes from Whatman.

Fluorescence spectra were recorded on Perkin-Elmer LS-50B fluorimeter, ^{23}Na-NMR-spectra on Jeol GX-270 spectrometer (270 MHz).

All fluorimetric experiments were conducted at 25°C, ^{23}Na-NMR experiments were conducted at 27°C.

The ionophores concentration is given in percent with respect to the total concentration of lipid. Mather solutions of ionophores were prepared in methanol. Control experiments showed that the amount of methanol added to the vesicular suspension in the different experiments (maximum amount 1.6 % in volume) did not affect the permeability of the membrane

2.2 ^{23}Na-NMR Transport Assays.[3]

A mixture of 150 μL of EYPC chloroform solution (100 mg/mL, 20 μmol), 40 μL of EYPG chloroform solution (20 mg/mL, 1 μmol) and an aliquot of methanolic solution of the desired ionophore was first evaporated with Ar-flux to form a thin film and then dried under high vacuum for 3h. The lipid film was hydrated in 1 mL of LiCl solution (100 mM in H_2O/D_2O 90:10) for 30 minutes at 40°C. The lipid suspension was submitted to 5 freeze-thaw cycles (-196°C/40°C) using liquid nitrogen and a thermostatic bath, and then extruded under nitrogen pressure (15 bar) at room temperature (10 extrusions through a 0.1 μm polycarbonate membrane).

In a 5 mm NMR-tube were mixed 350 μL of the lipid dispersion and 350 μL of a *shift reagent* solution (4 mM $DyCl_3 \cdot 6H_2O$, 12 mM $Na_5P_3O_{10}$, 40 mM NaCl in H_2O/D_2O 90:10). During the experiment the spectra

were recorded every 30 minutes over 14 h (accumulation *in continuo*), the areas of the signals were calculated with Jeol Delta software. The % of Na^+ inside liposome was calculated from the known liposome entrapped volume (1.5 %). The same experiment has been repeated adding an aliquot of methanolic solution of ionophore to the preformed vesicle (single side addition) with identical results.

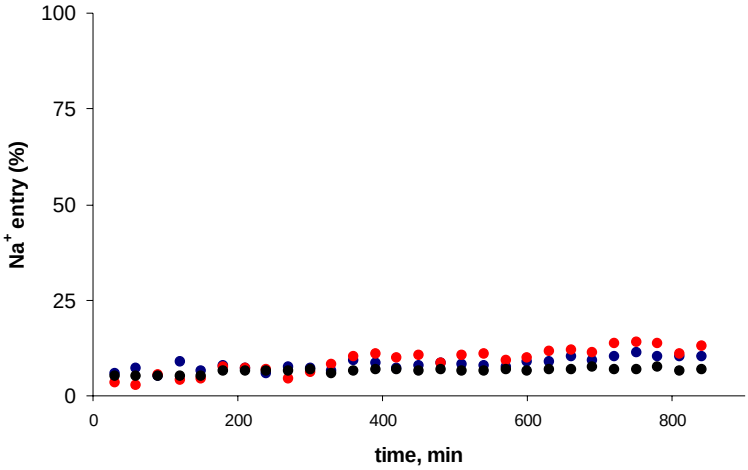


Figure S1. Kinetic profiles for the entry of Na^+ into 95:5 EYPC/EYPG vesicles containing **1** (1%, ●), **2** (1 %, ●), and without additives (●) at 27 °C. The total concentration of lipids was 10.5 mM. The results clearly show that ionophore **1** and **2** are unable to transport Na^+ across the lipid bilayer.

2.3 Ionophore Content Assays. The vesicle suspension is prepared in the same way as LUV's for ^{23}Na-NMR assays ($mol_{compound}/mol_{lipide}$ = 1%), using 1 mL of 100 mM LiCl for hydration. 550 µL of LUV solution was diluted with 1000 µL of 100 mM LiCl; 500 µL of the resulting solution were ultra-filtered on Microcon® YM-10 filters (25 minutes, 20°C, 13000 r.p.m.). In the case of compounds **1** and **2** the ionophore content in the eluate after ultra-filtration ($[ion]_{eluate}$) and in the mother solution ($[ion]_{tot}$) were measured by diluting an aliquot of 100 µL in a fluorimetric cell with 400 µL of 100 mM LiCl, 2000 µL borate buffer (pH 9), 150 µL of 5 % (v/v) Triton® X-100 and 100 µL of 10 mM Fluram® solution in acetone. The emission spectra was recorded in the 430-530 nm range (excitation 390 nm). The maximum of emission intensity was correlated to the ionophore concentration using a calibration curve obtained measuring the emission intensity of solutions of ionophore at known concentrations (prepared diluting the methanol mother solution with a 100 mM LiCl aqueous solution). In the case of compound **8** the ionophore content was measured by UV-Vis spectroscopy (λ_{max}: 210 nm) diluting an aliquot of the ultrafiltrated solution and mother solution in methanol. Absorbances were correlated with concentrations by using calibration curves obtained by UV-VIS measurements on solutions at known concentration in methanol.

The partition coefficient was calculated using the following equation:

$$PC = \frac{[ion]_{tot} - [ion]_{eluate}}{[ion]_{tot}}$$

The results were 0.53, 0.16, 0.98 for compound **1, 2,** and **8** respectively.

2.4 Calcein-Release Assay.[4] The LUV suspension was prepared as previously described except for the addition of the ionophore, using a mixture of 72 µL EYPC chloroform solution (100 mg/mL, 9.5 µmol) and 19 µL EYPG chloroform solution (20 mg/mL, 0.5 µmol). The lipid cake was hydrated in 2.0 mL of 50 mM calcein solution (1 mM HEPES, pH 7.4). The LUV suspension was separated from extravesicular dye by size exclusion chromatography (SEC) (stationary phase: column ∅1x25cm Sephadex™ G-75, mobile phase: buffer 1 mM HEPES, 150 mM NaCl, pH 7.4) and diluted with the same HEPES buffer to give a stock solution with a lipid concentration of 0.4 mM (assuming 100% of lipid was incorporated into liposomes). The vesicle suspension (550 µL stock solution) was placed in a fluorimetric cell and diluted to 2200 µL with the buffer solution used for preparation. The total lipid concentration in the fluorimetric cell was 0.1 mM. Calcein emission was monitored at 520 nm with excitation at 490 nm. During the experiment 10 µL of 0.22 mM solution of ionophore **1** were added through an injector port every 400 s up to 50 µL. Maximal changes in dye emission were obtained by lysis of the liposomes with detergent (40 µL of 5% aqueous Triton® X-100).

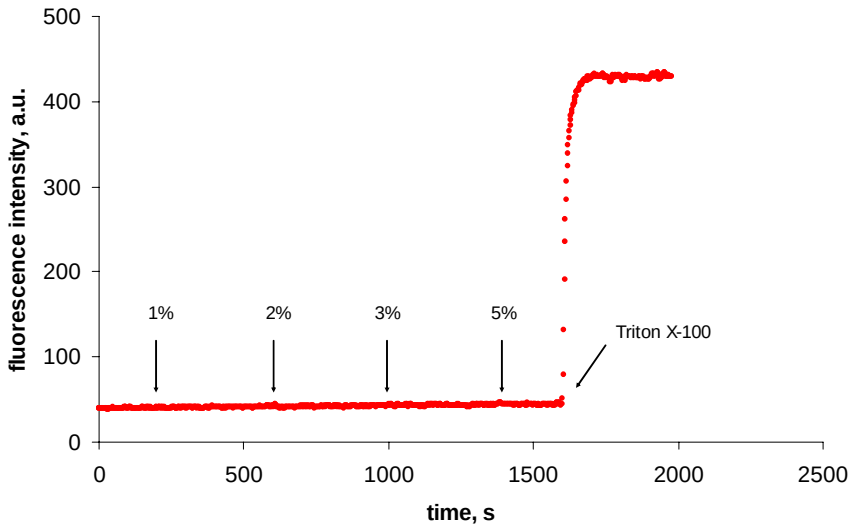


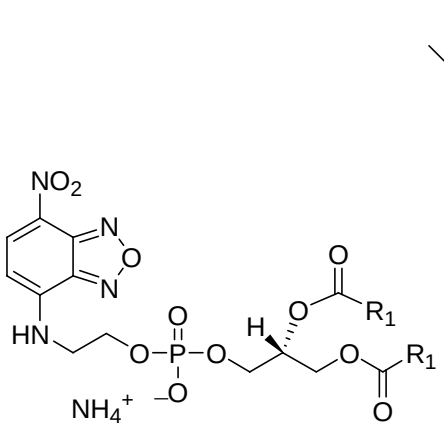
Figure S2. Fluorescence time course for calcein release from LUV (λ_{ex} = 490 nm, λ_{em} = 520 nm). The arrows indicate the time of the addition of aliquots of solution of **1** (10 µL of a 0.22 mM solution). The total concentration of ionophore added to the liposome suspension is indicated above the arrow. The final addition of detergent lysis the liposomes and the calcein is fully released. The effect of **1** on calcein release is negligible.

2.5 Vesicle Fusion Assay.[5] Ionophore-induced vesicle fusion was monitored using fluorescence resonance energy transfer (FRET) between PE-NBD and PE-LRB probes. The LUV suspension was prepared as previously described, except for the addition of the ionophore, using a mixture of 100 μL of EYPC chloroform solution (100 mg/mL, 13.16 μmol), 25 μL of EYPG chloroform solution (20 mg/mL, 0.65 μmol) and a solution of PE-NBD in CH_2Cl_2 (1.30 mL, 1.06 10^{-4} M, 1% $mol_{PE\text{-}NBD}/mol_{lipide}$). The lipid cake was hydrated in 1.0 mL HEPES buffer (25 mM, 100 mM NaCl, pH 7). A separated LUV suspension was obtained in the same way using a solution of PE-LRB in CH_2Cl_2 (2.03 mL, 6.81 10^{-5} M, 1% $mol_{PE\text{-}LRB}/mol_{lipide}$).

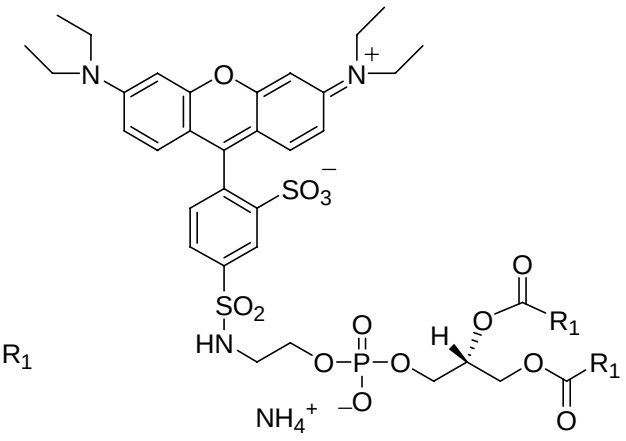
In a fluorimetric cell 20 μL of PE-NBD containing LUV were mixed with 20 μL of PE-LRB containing LUV's and diluted with 3000 μL of HEPES buffer (25 mM, 100 mM NaCl, pH 7); emission spectra were recorded in 500-650 nm range (excitation 463 nm) immediately after the addiction of an aliquot of methanolic solution of ionophore **1** (1-3 % $mol_{compound}/mol_{lipide}$) and 5 minutes after every addiction.

Excitation of PE-NBD (463 nm) leads to an emission band at 530 nm which is very close to the absorption band of PE-LRB. When the two dyes are on the membrane of the same liposome, their proximity leads, upon excitation of PE-NBD to the emission of PE-LRB (λ_{em} = 590 nm) and a concomitant decrease of the emission of PE-NBD because of energy transfer between the two systems. Starting from two separate vesicle populations containing PE-NBD and PE-LRB, respectively, the fusion process lead to one unique vesicle population containing both fluorescent labeled lipids. The vesicle fusion is, therefore, signaled by the occurrence of FRET process between the two dyes.

No FRET was observed with ionophore **1** even at 3 % concentration allowing to exclude fusogenic activity.



PE-NBD



PE-LRB

2.6 HPTS assay.[6] The LUV suspension was prepared as previously described, except for the addition of the ionophore, using a mixture of 225 μL of EYPC chloroform solution (100 mg/mL, 30 μmol) and 60 μL of EYPG chloroform solution (20 mg/mL, 1.5 μmol). The lipid cake was hydrated in 1.5 mL of 0.1 mM HPTS solution (HEPES 25 mM, 100 mM NaCl, pH 7). The LUV suspension was separated from extravesicular dye by size exclusion chromatography (SEC) (stationary phase: pre-packed column Sephadex™ G-25, mobile phase: HEPES buffer) and diluted with the same HEPES buffer to give a stock solution with a lipid concentration of 5 mM (assuming 100% of lipid was incorporated into liposomes). 104 μL of lipid suspension was placed in a fluorimetric cell, diluted to 3040 μL with the same buffer solution used for the liposome preparation and kept under gently stirring. The total lipid concentration in the fluorimetric cell was 0.17 mM. An aliquot of methanolic solution of the ionophore (10-30 μL of the appropriate mother solution in order to obtain the desired $mol_{compound}/mol_{lipide}$ ratio) was then added to the lipid suspension and the cell was incubated at 25°C for 30 minutes. After incubation the time course of fluorescence was recorded for 200 s (λ_{ex} 460 nm, λ_{em} 510 nm) and then 50 μL of 0.5 M NaOH were rapidly added through an injector port and the fluorescence emission was recorded for 1200 s. Maximal changes in dye emission were obtained by final lysis of the liposomes with detergent (40 μL of 5% aqueous Triton® X-100). Fluorescence time course were normalized using the following equation:

$$FI = \frac{(F_t - F_0)}{(F_\infty - F_0)} \cdot 100$$

were F_t is the fluorescence intensity measured at time t, F_0 is the fluorescence intensity at ionophore addition, F_∞ is the fluorescence intensity at saturation after lyses with Triton.

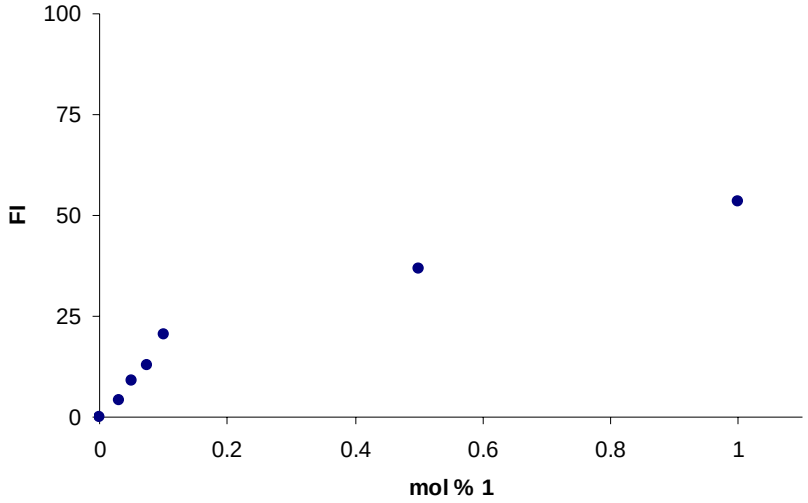


Figure S3. Normalized fluorescence intensity in the HPTS assay (FI) relative to the initial fluorescence "burst" as a function of **1** concentration (in percent with respect to the total concentration of lipids). The relative amount of the "burst" increases with the concentration of the ionophore and is almost negligible at low concentration. The "burst" is observable also in the case of ionophore **2** but is much smaller: about 4% at ionophore concentration of 3% and almost negligible at lower concentration.

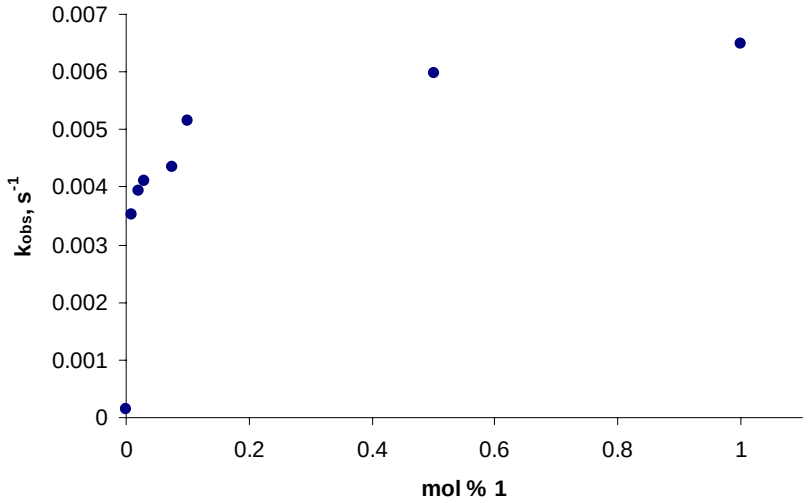


Figure S4. Dependence of the pseudo-first-order rate constant (k_{obs}, s^{-1}) from the concentration of ionophore **1**. The rate constants have been calculated for the second slower process observed in the HPTS assay. The rate of the transport process increases with the concentration of ionophore following a saturation profile. However, the saturation behaviour is at least partly explained with the presence of the fluorescence "burst" which increases with the concentration of ionophore (see Fig. S3). This implies that the second process starts at different value of pH gradient because the burst in fluorescence is due to a partial collapse of the pH gradient. Consequently, it is not possible to gain information on the mode of action of the ionophore from such kinetic profiles.

2.6.1 Determination of cation selectivity with the HPTS assay.[7] The experiment is performed as described above except for the use of the appropriate chloride salt (MCl; M = Li^+, Na^+, K^+) in the liposome preparation. The base pulse is then obtained by addition of 50 μL of 0.5 M MOH (M = Li^+, Na^+,

K^+). In this way only the alkaline cation under investigation is present inside and outside the liposome.

2.6.2 Determination of anion selectivity with the HPTS assay.[8] The experiment is performed as described above except for the use of the appropriate Na^+ salt (NaX; X= F^-, Cl^-, Br^-, I^-, NO_3^-, ClO_4^-, SO_4^{2-}, Ac^-, glutamate) in the liposome preparation. In this way only the anion under investigation is present inside and outside the liposome except for OH^-. The rates of transport of anions have been corrected for the membrane permeability of the different anions in the absence of ionophore determined in separate experiments. With these experimental conditions the rate of influx of the anion in the absence of ionophore with fluoride and acetate were too high to allow the determination of the ionophore activity.

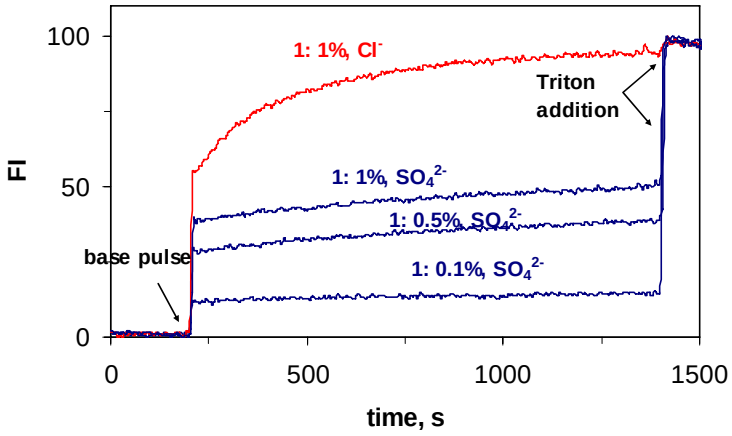


Figure S5. Normalized fluorescence change in HPTS emission (FI, λ_{ex} 460 nm, λ_{em} 510 nm) as a function of time in the presence of different concentrations of ionophore **1** and of chloride (red curve) or sulphate (blue curves) as the only transportable anion. Conditions: 25 mM HEPES, 100 mM NaCl or Na_2SO_4, pH 7.0, base pulse by addition of 50 μL of 0.5 M NaOH. The time of addition of NaOH for the base pulse and of Triton X-100 for the final liposome lysis are indicated by arrows. The experiment shows that the fluorescence "burst" is observable also in the presence of the non-transported SO_4^{2-} anion and that the relative amount of the "burst" increases with the concentration of the ionophore. This indicates that the first process is not anion selective as expected for the flux of ions through transient-pores.

2.6.3 Determination of cation and anion selectivity with the HPTS assay using the Matile's protocol.[6]

The vesicle suspension (104 μL stock solution, prepared as described above, point 2.6) was placed in a fluorimetric cell and diluted to 3040 μL with the appropriate buffer solution (25 mM HEPES, 100 mM MCl with M= Li^+, Na^+, K^+, Rb^+, Cs^+, or 100 mM NaX with X= F^-, Cl^-, Br^-, I^-, NO_3^-, ClO_4^-, SO_4^{2-}, Ac^-, pH 7). The total lipid concentration in the fluorimetric cell was 0.17 mM. An aliquot of methanolic

solution of the ionophore (10-30 µL of the appropriate mother solution in order to obtain the desired $mol_{compound}/mol_{lipide}$ ratio) was then added to the lipid suspension and the cell was incubated at 25°C for 30 minutes. After incubation the time course of fluorescence was recorded for 200 s (λ_{ex} 460 nm, λ_{em} 510 nm) and then 50 µL of 0.5 M MOH (with M= Li^+, Na^+, K^+, Rb^+, Cs^+ depending on the cation present in the extravesicular buffer solution) were rapidly added through an injector port and the fluorescence emission was recorded for 1200 s. Maximal changes in dye emission were obtained by final lysis of the liposomes with detergent (40 µL of 5% aqueous Triton® X-100). Fluorescence time course were normalized as previously described.

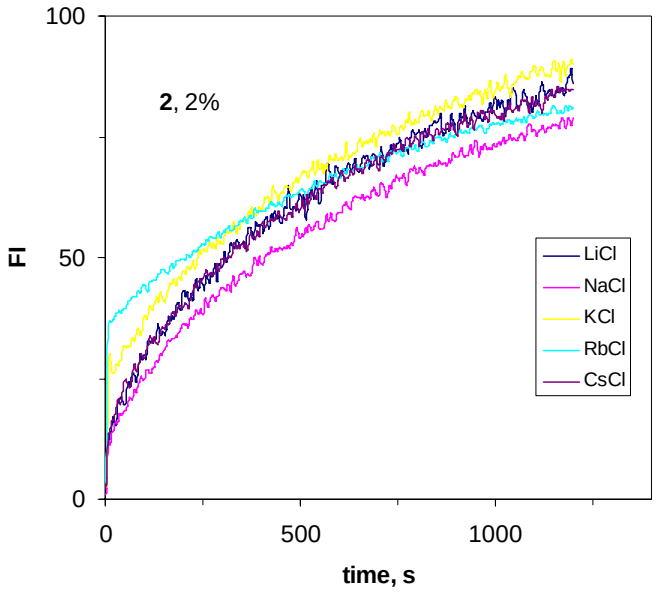


Figure S6. Determination of cation selectivities for ionophore **2** (2.0 % concentration) using the HPTS assay and following the Matile's protocol (λ_{ex} 460 nm, λ_{em} 510 nm) in 95:5 EYPC/EYPG LUVs (0.17 mM total lipid concentration, total volume 3 mL). Conditions: 25 mM HEPES, 100 mM NaCl inside vesicle, 100 mM MCl outside vesicle, pH 7.0, base pulse by addition of 50 µL of 0.5 M MOH. The experiment confirm the independence of the rate of transport from the nature of group I cations.

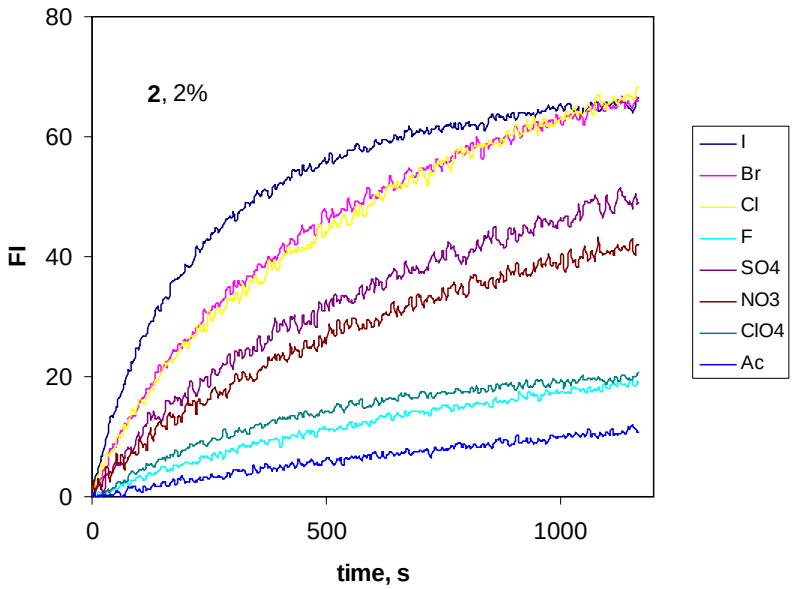


Figure S7. Determination of anion selectivities for ionophore **2** (2.0 % concentration) using the HPTS assay and following the Matile's protocol (λ_{ex} 460 nm, λ_{em} 510 nm) in 95:5 EYPC/EYPG LUVs (0.17 mM total lipid concentration, total volume 3 mL). Conditions: 25 mM HEPES, 100 mM NaCl inside vesicle, 100 mM NaX outside vesicle, pH 7.0, base pulse by addition of 50 μL of 0.5 M NaOH. The selectivity sequence is: $I^- > Br^- \approx Cl^- >> SO_4^{2-} \approx NO_3^- >> ClO_4^- \approx F^- >$ Acetate. The sequence is in accord with the one determined with the experiment in which only the anion under investigation is present (Figure 3) but with some differences probably due to the different experimental conditions. In the Matile's protocol, indeed, NaCl is always present inside the vesicle and the external added anion qualifies for OH^-/X^- antiport or H^+/X^- symport only after exchange with internal chloride. This makes the interpretation of the selectivity sequence less straightforward.

2.6.4 Ionophoric activity and anion selectivity of compound 8.

The ionophoric activity and the anion selectivity of the fully Boc-protected compound 8 was evalulated with the HPTS assy as described above (see paragraph 2.6.2).

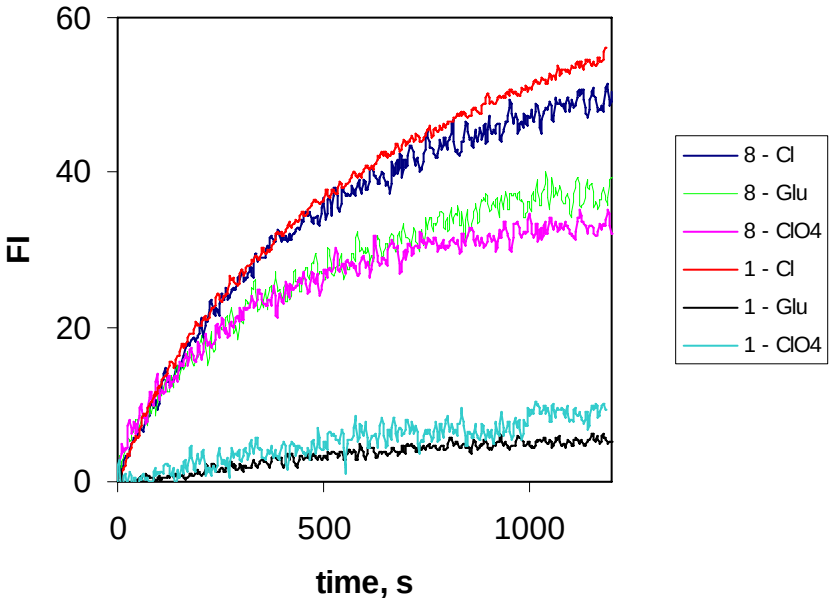


Figure S8. Normalized fluorescence change in HPTS emission (FI, λ_{ex} 460 nm, λ_{em} 510 nm) as a function of time in the presence of 0.03 % ionophore **8** and, for comparison, **1** and of chloride, glutamate and perchlorate as the only transportable anions. Conditions: 25 mM HEPES, 100 mM NaX, pH 7.0, base pulse by addition of 50 μL of 0.5 M NaOH. Ionophore **8** transports chloride (blue curve) with apparent activity similar to **1** (red curve). However, tacking in account the different partition coefficient (about twice for **8** respect to **1**) the real activity of **8** is about half than that of **1**. Moreover, **8** shows a much lower selectivity in the transport of glutamate (green curve) and perchlorate (pink curve) respect to **1** (black and light blue curves). The selectivity ratios calculated as ratio of activities at 1000 s are: **1**, Cl^-/Glu = 9.4; Cl^-/ClO_4^- = 5.4; **8**, Cl^-/Glu = 1.3; Cl^-/ClO_4^- = 1.3.

2.7 Chloride transport with the lucigenin assay.[9] The LUV suspension was prepared as previously described, except for the addition of the ionophore, using a mixture of 300 μL of EYPC chloroform solution (100 mg/mL, 40 μmol) and 80 μL of EYPG chloroform solution (20 mg/mL, 2 μmol). The lipid cake was hydrated in 2.0 mL of lucigenin solution 1 mM (25 mM HEPES, 225 mM $NaNO_3$, pH 7). The LUV suspension was separated from extravesicular dye by size exclusion chromatography (SEC) (stationary phase: column ∅1x25cm Sephadex™ G-75, mobile phase: buffer 25 mM HEPES, 225 mM $NaNO_3$, pH 7) and diluted with the same HEPES buffer to give a stock solution with a lipid concentration of 6.5 mM (assuming 100% of lipid was incorporated into liposomes). The vesicle suspension (187 μL of stock solution) was placed in a fluorimetric cell and diluted to 3040 μL with the same buffer solution used for the preparation. The total lipid concentration in the fluorimetric cell was 0.4 mM. An aliquot of methanolic solution of the ionophore (10-30 μL of the appropriate mother solution in order to obtain the desired $mol_{compound}/mol_{lipide}$ ratio) was then added to the lipid suspension and the cell was incubated at 25°C for 30 minutes. To the gently stirred mixture were added 50 μL of NaCl 1.46 M

through an injector port to establish a chloride gradient. The final external concentration of NaCl was 24 mM. Maximal changes in dye emission were obtained by lysis of the liposomes with detergent (40 μL of 5% aqueous Triton X-100). Lucigenin emission was monitored at 506 nm (excitation at 455 nm) during an entire experiment period and recorded as a function of time.

In the case of anion induced chloride transport blockage experiments five minutes before the addition of the NaCl solution 50 μL of a 1.46 M solution of $NaClO_4$ or glutamate were added to the vesicular suspension containing the ionophore. Then 50 μL of NaCl 1.46 M were added and the fluorescence emission was recorded as a function of time. The final external concentrations of NaCl and $NaClO_4$ or glutamate were 24 mM.

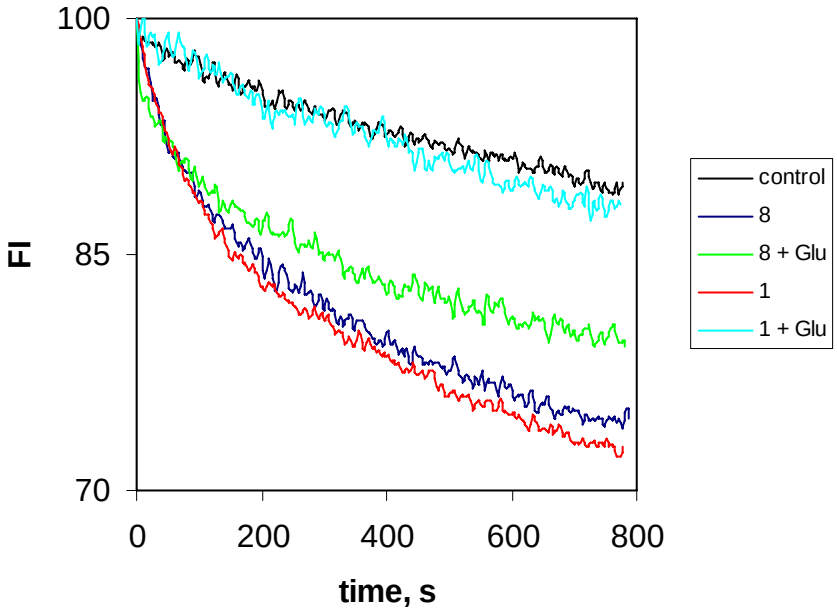


Figure S9. Normalized fluorescence change in lucigenin emission (FI, λ_{ex} 455 nm, λ_{em} 506 nm) in the presence of 3% ionophores **8** and **1** after the addition of NaCl (50 μL of 1.46 M solution, final external concentration 24 mM) to 95:5 EYPC/EYPG LUVs loaded with lucigenin (1 mM lucigenin, 0.4 mM total lipid concentration, 25 mM HEPES, 225 mM $NaNO_3$, pH 7, total volume 3 mL). In the runs in the presence of glutamate (Glu), five minutes before the addition of NaCl, an equimolar amount of sodium glutamate was added to the vesicular suspension (50 μL of 1.46 M solution, final external concentration 24 mM NaX and 24 mM NaCl). As observed above (Fig S8), ionophore **8** transports chloride (blue curve) with apparent activity similar to **1** (red curve). However, tacking in account the different partition coefficient (about twice for **8** respect to **1**) the real activity of **8** is about half than that of **1**. Moreover, the blockage effect is much more evident in the case of **1** (light blue curve) respect to **8** (green curve) confirming the lower anion selectivity of compound **8.**

2.8 Chloride transport with the HPTS assay.[7] The vesicle stock solution was prepared as described for the HPTS assay (point 2.6) but using only HEPES buffer (25 mM, pH 7) without added salt for the swelling and for the size exclusion chromatography (SEC). 104 μL of the stock solution were placed in a

fluorimetric cell and diluted to 3040 µL with the same buffer solution used for preparation and kept under gently stirring. The total lipid concentration in the fluorimetric cell was 0.17 mM. An aliquot of methanolic solution of the ionophore (10-30 µL of the appropriate mother solution in order to obtain the desired $mol_{compound}/mol_{lipide}$ ratio) was then added to the lipid suspension and the cell was incubated at 25°C for 30 minutes. After incubation the time course of fluorescence was recorded for 200 s (λ_{ex} 460 nm, λ_{em} 510 nm) and then 50 µL of 2 M NaCl or Na_2SO_4 were rapidly added through an injector port to establish the anion gradient. The final external concentrations of NaCl or Na_2SO_4 were 33 mM. The fluorescence emission was recorded for 1200 s and the maximal changes in dye emission were obtained by final lysis of the liposomes with detergent (40 µL of 5% aqueous Triton® X-100). Fluorescence time course were normalized as previously described.

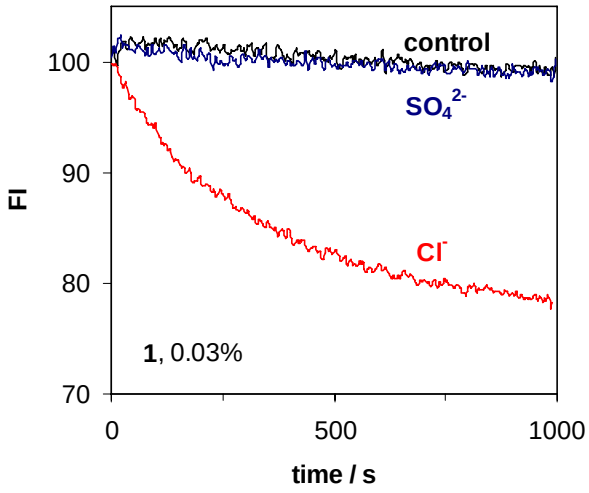


Figure S10. Normalized fluorescence change in HPTS emission (FI, λ_{ex} 460 nm, λ_{em} 510 nm) as a function of time in the presence of ionophore **1** (0.03 % concentration) after the external addition of chloride (red curve) or sulphate (blue curves) or nothing (control, black curve). Conditions: 25 mM HEPES, pH 7.0, anion pulse by addition of 50 µL of 2 M NaCl or Na_2SO_4. The decrease in the fluorescence emission of HPTS after chloride pulse indicated acidification of the inner vesicular compartment consistent with OH^-/Cl^- antiport or H^+/Cl^- symport. No effect is observed upon addition of sulphate ion confirming that this ion is not transported by ionophore **1**.

2.9 Anomalous mole fraction effect (AMFE).[10]

AMFE refers to a lower than expected activity found with mixtures of ions compared to pure ions. Its occurrence indicates that the non transported or less efficiently transported ion competes with the better transported ion for "binding sites" along the channel inhibiting its transport. AMFE was assayed using HPTS and the Matile's protocol for anion selectivity (paragraph 2.6.3) with the only difference that the

buffer used to dilute the vesicular preparation contained mixture of chloride and glutamate with total anion concentration of 100 mM.

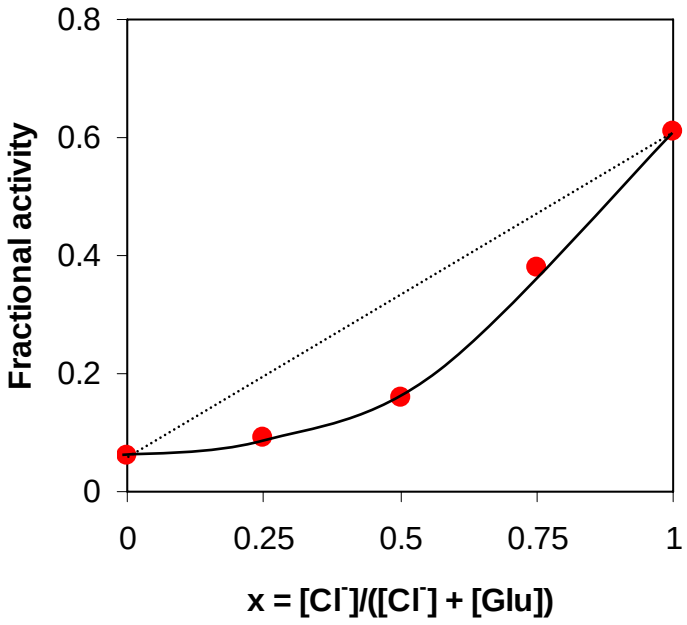


Figure S11. Mole fraction behavior of compound **1** (0.03 %) for Cl^-/Glutamate mixtures determined in the condition of Figure S7. The dotted line represents the expected behavior in case of simple additivity. The observation of a lower activity confirms the inhibition effect of the glutamate anion on the transport of chloride.

Antiproliferative Activity. J774.A1 (murine monocyte/macrophage) cells were grown as previously reported.[11] All reagents for cell culture were from Hy-Clone (Euroclone, Paignton, Devon, UK); MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide] and 6-mercaptopurine (6-MP) were from Sigma Chemicals. J774.Al (3.4×10^4 cells) were plated on 96-well microtiter plates and allowed to adhere at 37°C in 5% CO_2 and 95% air for 2 h. Thereafter, the medium was replaced with 50 μL of fresh medium and a 75 μL aliquot of 1:10 serial dilutions of ionophore **1** (or **8**) were added, and then the cells were incubated firstly for 72 hours and then for 3, 6,12, 24 and 48 hours in time course experiments (vide infra). The cell viability was assessed through an MTT conversion assay. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340, DASIT) equipped with a 620 nm filter. The viability of each cell line in response to treatment with ionophore 1

was calculated as follows: % dead cells = 100 - (OD treated/OD control) × 100. The observed IC_{50} values report the concentration that inhibited cell growth by 50% as compared to the control.

"Time course" experiments clarified that the antiproliferative activity was significant 12 hours after incubation with ionophore **1** (Table 1), excluding membrane rupture due to non-specific tensioactive effect.

Hours	3	6	12	24	48	72
IC_{50} (μM)	>100	>100	48.6±0.2	48.1±0.3	83.1±0.5	49.5±0.5

[a]Data are expressed as mean±s.e.m. of three different experiments, each in triplicate.

Table S1. Time course of anti-proliferative activity of **1** on J774.A1[a]

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