Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

pH switchable anion transport by an oxothiosquaramide

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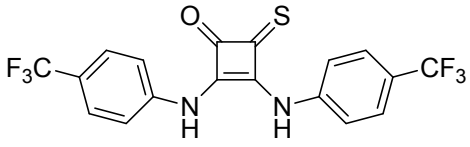
S1. Synthesis

^{1}H NMR spectra were recorded using a Bruker Avance III 500 at a frequency of 500.13 MHz, and are reported as parts per million (ppm) with DMSO-d_6 (δ_H 2.50 ppm) or CD_3CN (δ_H 1.94 ppm) as internal references. The data are reported as chemical shift (δ), multiplicity (br = broad, s = singlet, d = doublet, m = multiplet), coupling constant (*J,* Hz) and relative integral. ^{13}C NMR spectra were recorded using a Bruker Avance III 500 at a frequency of 125.76 MHz and are reported as parts per million (ppm) with DMSO-d_6 (δ_C 39.52 ppm) or CD_3CN (δ_C 118.26 ppm) as internal references. High resolution ESI spectra were recorded on a BrukerBioApex Fourier Transform Ion Cyclotron Resonance mass spectrometer (FTICR) with an Analytica ESI source, operating at 4.7 T or a BrukerDaltonics Apex Ultra FTICR with an Apollo Dual source, operating at 7 T. Infrared (IR) absorption spectra were recorded on a Bruker ALPHA Spectrometer with Attenuated Total Reflection (ATR) capability, using OPUS 6.5 software. LC-MS was performed on a Shimadzu LC-MS 2020 instrument consisting of a LC-M20A pump and a SPD-20A UV/Vis detector coupled to a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. Separations were performed on a Waters Sunfire 5 μm, 2.1 x 150 mm column (C18) operating at a flow rate of 0.2 mL min^{-1}. Separations were performed using a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a linear gradient of 0-100% B over 30 min. Analytical TLC was performed using precoated silica gel plates (Merck Kieselgel 60 F254). Squaramide **1** and thiosquaramide **2** were synthesised as previously described, [1] thionating agent P_4S_{10}.pyridine was synthesised according to the method of Bergman *et. al* [2] and all other reagents were commercially available and used as supplied. All reactions were carried out under an inert atmosphere and undertaken using anhydrous solvents dried using an Innovative Technologies PureSolve solvent purification system.

4-thioxo-2,3-bis((4-(trifluoromethyl)phenyl)amino)cyclobut-2-enone (3).



3,4-bis(4-(trifluoromethyl)phenylamino)cyclobut-3-ene -1,2-dione (0.2 g, 0.5 mmol, 1.0 equiv) and P_4S_{10}·pyridine (0.19 g, 0.5 mmol, 1.0 equiv) were suspended in anhydrous MeCN (15 mL) and heated at 90° C for 4 hrs. The solvent was removed under reduced pressure and the crude residue was stirred in 2M HCl before being subjected to flash column chromatography eluting with 5% MeCN in DCM to yield **3** as a yellow solid. (0.054 g, 26%). **^{1}H NMR** (500.13 MHz, CD_3CN): 7.35 (d, *J* = 5.0 Hz, 2 H, Ar

H),7.53 (d, *J* = 5.0 Hz, 2 H, Ar H), 7.61 (d, *J* = 5.0 Hz, 2 H, Ar H),7.65 (d, *J* = 5.0 Hz, 2 H, Ar H), 8.34 (br s, 1 H, NH); 8.82 (br s, 1 H, NH); **^{13}C NMR** (125.76 MHz, CD_3CN): 121.4, 122.7, 127.3, 127.5, 141.4, 142.0, 169.8, 180.6; **^{19}F NMR** (470 MHz, CD_3CN): -62.8, -62.7; **HRMS** (ESI) m/z calcd. for $C_{18}H_{11}F_6N_2OS$ $[M + H]^+$ 417.0491, found 417.0489; **ν_{max} (film)/cm^{-1}:** 835, 964, 1016, 1057, 1112, 1165, 1237, 1318, 1417, 1448, 1529, 1584, 1608, 1631, 1758, 3010, 3201.

S2. NMR and HPLC-MS data

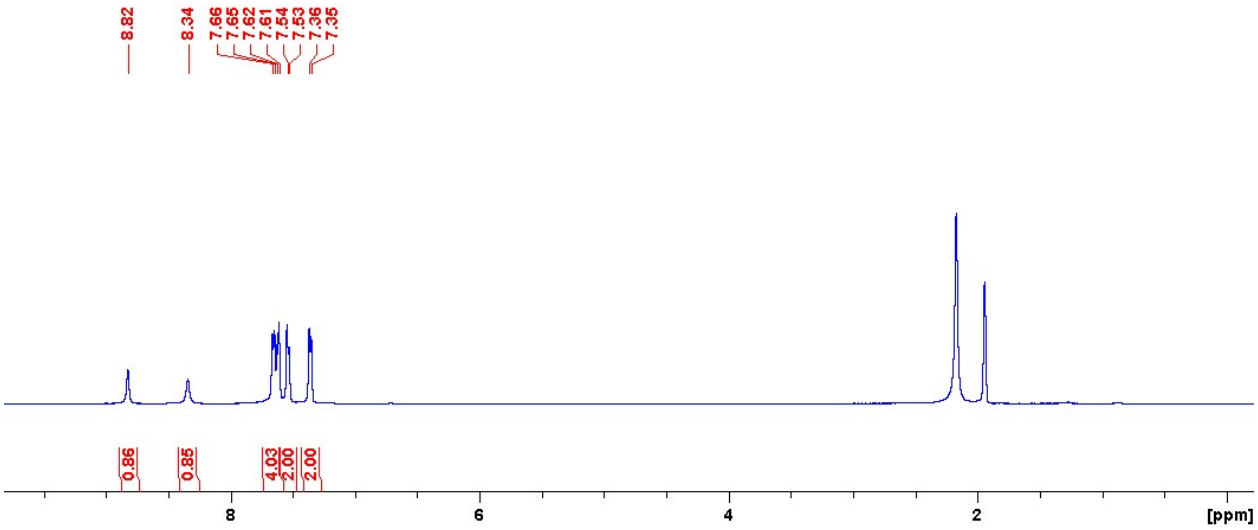


Figure S1: ^{1}H NMR (CD_3CN, 500 MHz) spectra of **3**.

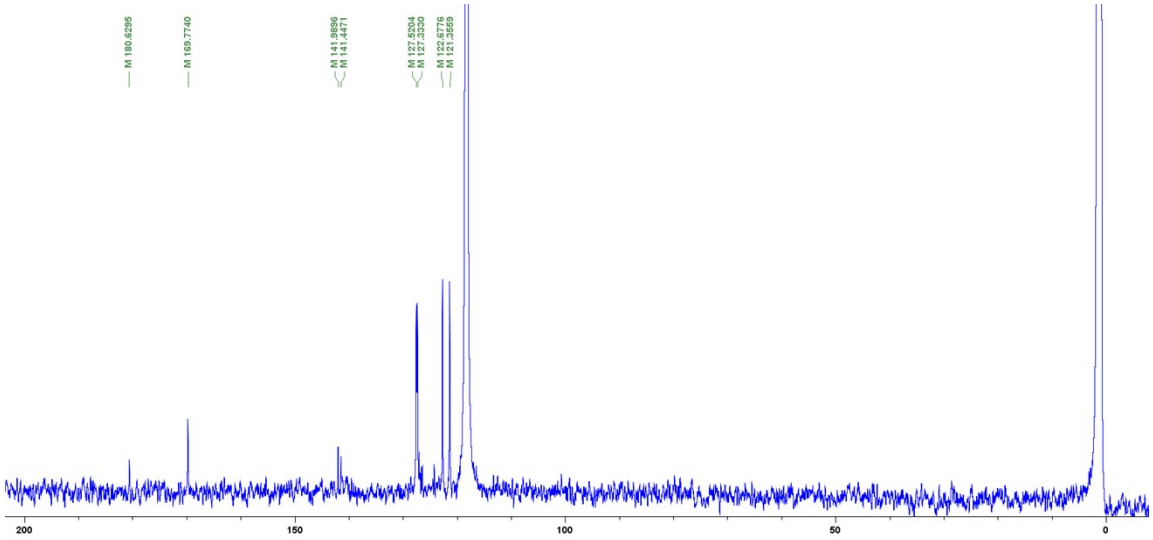


Figure S2: ^{13}C NMR (CD_3CN, 125 MHz) spectra of **3**.

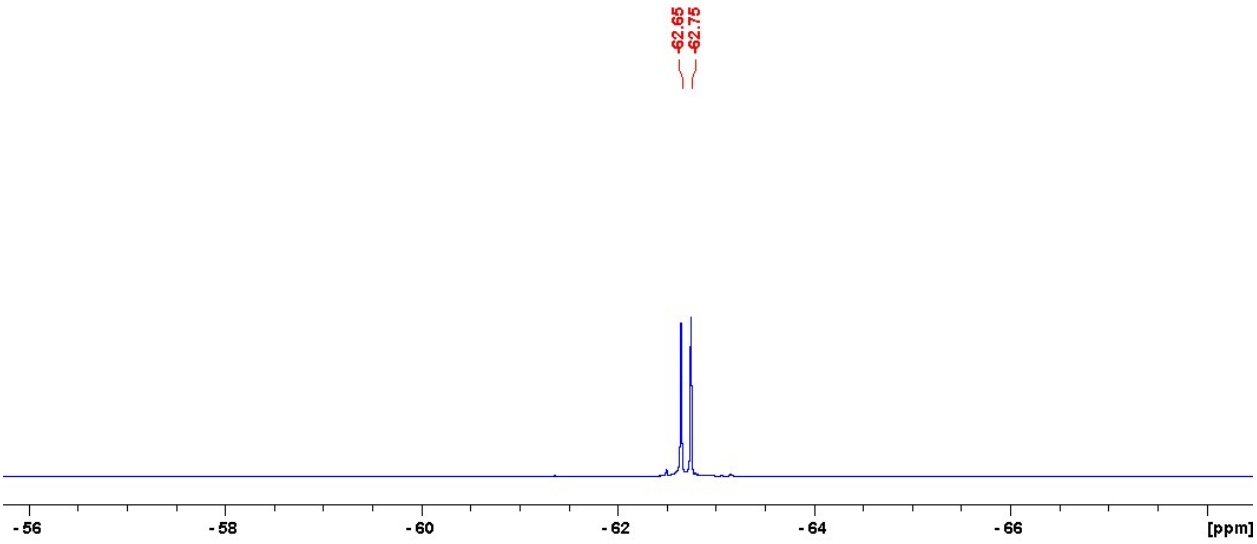
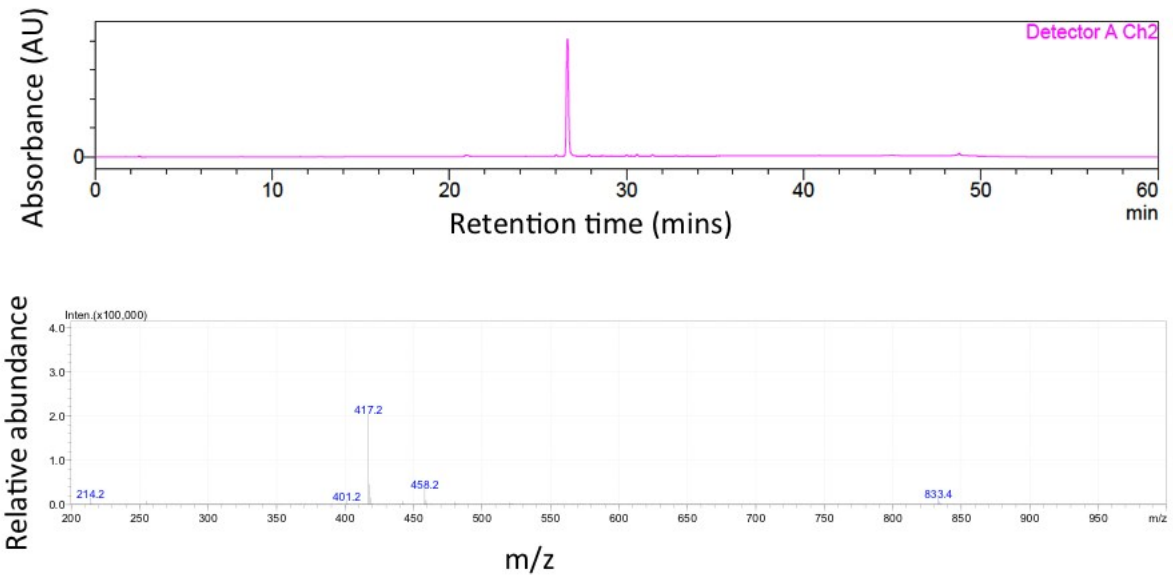


Figure S3: ^{19}F NMR (CD_3CN, 470 MHz) spectra of **3**.

Figure S4: Analytical LCMS trace of receptor **3**; Rt 26.66 min (0-100% B over 30 min, 0.1% Formic Acid, λ = 254 nm); Calculated Mass $[M+H]^+$: 417; Found $[M+H]^+$: 417.2.



S3. Chloride Binding Titrations

^{1}H NMR titrations were performed by additions of aliquots of chloride as the tetrabutylammonium (TBA) salt (0.15 – 0.2 M), in a solution of the receptor (2.5×10^{-3} M) in either 0.5% H_2O in DMSO-d_6 or CD_3CN to a 2.5×10^{-3} M solution of the receptor in either 0.5% H_2O in DMSO-d_6 or CD_3CN. Typically, up to 12 equivalents of the anion were added to the solution. Both salt and receptor were dried under high vacuum prior to use. ^{1}H NMR spectra were recorded on a BrukerAvance III 500 spectrometer at a frequency of 500.13 MHz and calibrated to the residual protio solvent peak in DMSO-d_6 (δ = 2.50 ppm) or CD_3CN (δ = 1.94 ppm). Stack plots were made using MestReNova Version 6.0. Where possible and when the change in chemical shift was larger than 0.2 ppm, non-linear curve fitting of the experimentally obtained titration isotherms (equivalents of anion *vs.* chemical shift of the squaramide NH protons or thiosquaramide NH protons) using the commercially available software program HypNMR® (Hyperquad® package) enabled the calculation of association constants (K_a/M^{-1}) using a 1:1 model.

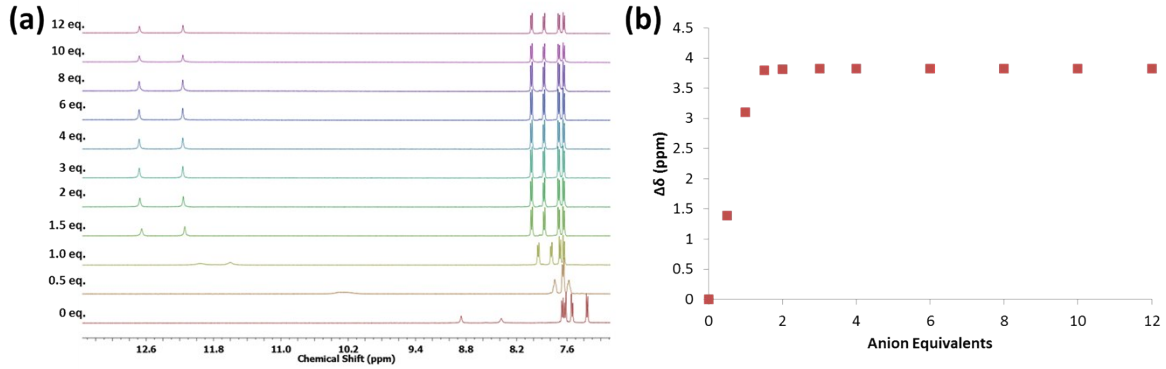


Figure S5: (a): ^{1}H NMR stackplot of **3** with TBACl in 0.5% H_2O in CD_3CN at 300 K. (b): Binding Isotherm for NH proton at δ = 8.9 ppm.

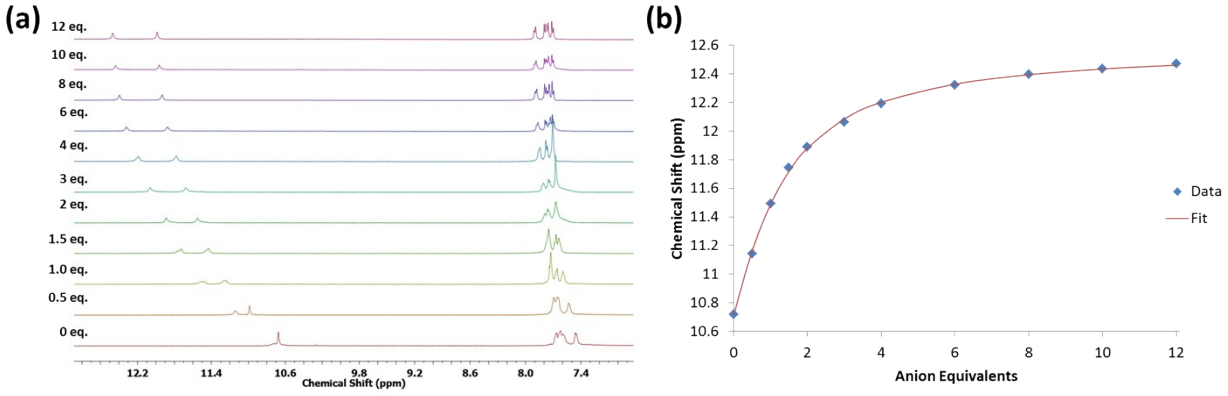


Figure S6: (a): ^{1}H NMR stackplot of **3** with TBACl in 0.5% H_2O in DMSO-d_6 at 300 K. (b): Fitplot for NH proton at δ = 10.7 ppm.

S4. Determination of pK_a values

pK_a values were experimentally determined using the wavelength of maximum difference in absorbance between the UV/Vis spectra of the anionic and the neutral species. For each compound the absorbance was measured on a solution in MeCN/H_2O (9:1) containing 0.1M $TBAPF_6$ with pH values between 2.5 and 14. The solutions were adjusted to acidic pH using an excess of HNO_3 before being slowly basified by small aliquots of an aqueous 0.1 M NaOH solution. The absorbance values at the wavelength of maximal difference were plotted against the pH values. A four parameter sigmoid curve using Sigma Plot (Systat Software Inc., Chicago, IL, USA) was fitted through the data points with the point of inflexion corresponding to the pK_a value.

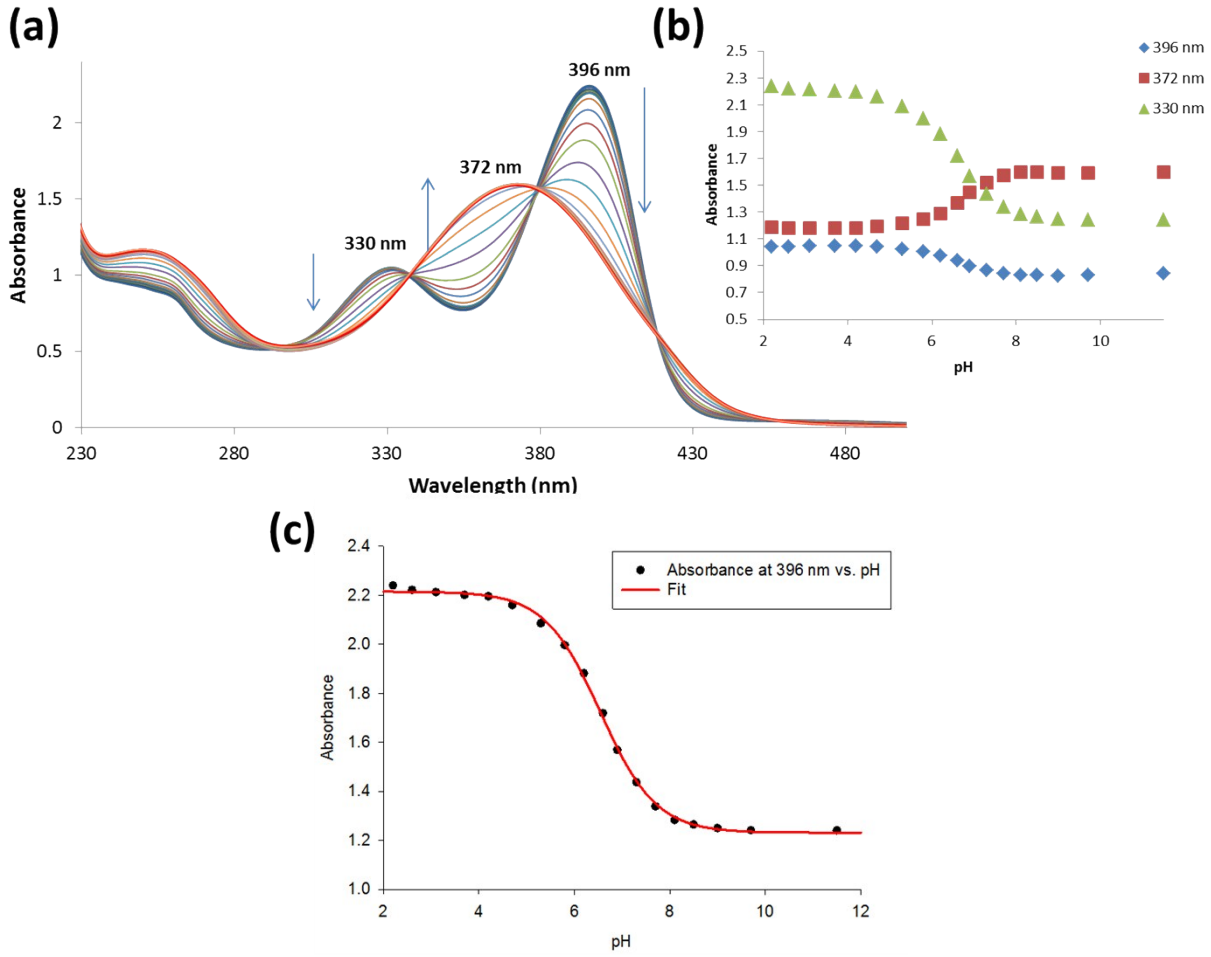


Figure S7: (a) Absorption spectra taken over the course of a pH-spectrophotometric titration of **3** (6×10^{-5} M) in an acetonitrile/water mixture (9/1 v/v; 0.1 M in $TBAPF_6$). (b) Comparison plots of absorbance at 396 nm (♦), 372 nm (■) and 330 nm (▲) vs. pH.(c) Four parameter sigmoid curve fit with the point of inflexion corresponding to the pK_a value.

S5. Anion transport studies

S5.1. General protocol

This procedure describes a typical membrane transport tests. Internal and external solutions can vary and are detailed in the caption of the figures. Chloride concentrations during transport experiments were determined using an *Accumet* chloride-selective electrode. The electrode was calibrated against sodium chloride solutions of known concentrations prior to each experiment and the calibration was fitted against a logarithmic function, $y=A\cdot log_{10}(x)+B$, where y is the electrode read-out in mV and x is the chloride concentration in M. POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) was supplied by *Genzyme* and was stored at –20°C as a solution in chloroform (1 g POPC in 35 mL chloroform). Cholesterol was supplied by *Sigma-Aldrich*. Octaethylene glycol monododecyl ether was used as detergent and was supplied by *TCI*.

A lipid film of POPC or 7:3 POPC:cholesterol was formed from a chloroform solution under reduced pressure and dried under vacuum for at least 4 hours. The lipid film was rehydrated by vortexing with a metal chloride (MCl) salt solution. The lipid suspension was then subjected to nine freeze-thaw cycles, where the suspension was alternatingly allowed to freeze in a liquid nitrogen bath, followed by thawing in a water bath. The lipid suspension was allowed to age for 30 min at room temperature and was subsequently extruded 25 times through a 200 nm polycarbonate membrane (*Nucleopore*^{TM}) using a LiposoFast-Basic extruder set (*Avestin, Inc*). The resulting unilamellar vesicles were dialyzed (*Spectra/Por® 2* Membrane MWCO 12-14 kD) against the external medium to remove unencapsulated MCl salts. Internal and external solutions vary from experiment to experiment, but in general an ionic strength of 500 mM was used.

S5.2. Chloride/nitrate transport at pH 7.2 and pH 4.0

Unilamellar POPC vesicles containing NaCl, prepared as described in Section S5.1., were suspended in the external medium consisting of a $NaNO_3$ solution buffered to pH 7.2 or pH 4.0 with phosphate or citrate salts respectively. The lipid concentration per sample was 1 mM. A DMSO solution of the carrier molecule was added to start the experiment and the chloride efflux was monitored using a chloride sensitive electrode. At 5 min, the vesicles were lysed with 50 μL of octaethylene glycol monododecyl ether (0.232 mM in 7:1 water:DMSO v/v)

and a total chloride reading was taken at 7 min. The initial value was set at 0% chloride efflux and the final chloride reading (at 7 minutes) was set as 100% chloride efflux. All other data points were calibrated to these points.

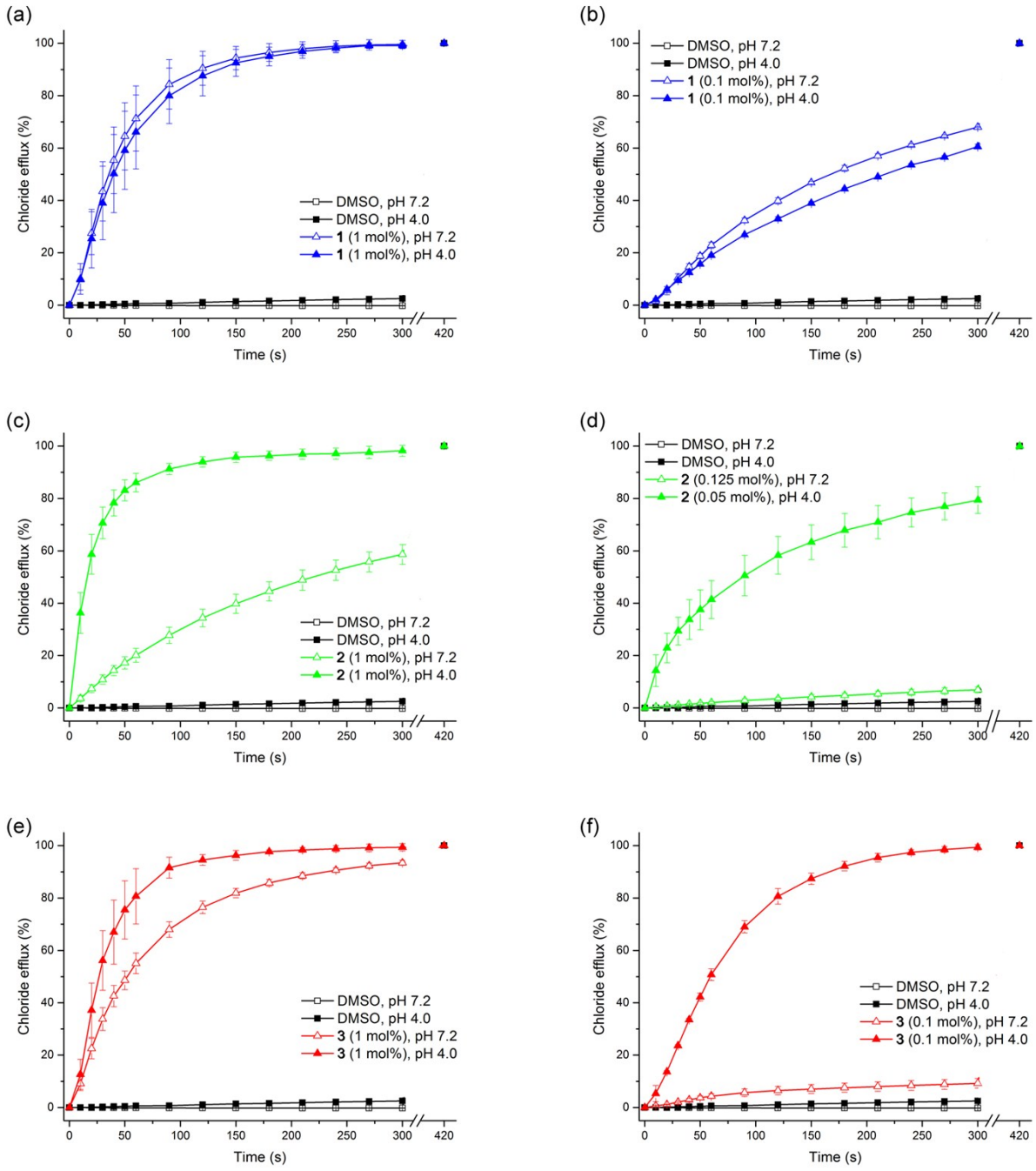


Figure S8: Chloride efflux from POPC vesicles at pH 7.2 (empty symbols) and pH 4.0 (filled symbols). POPC vesicles were loaded with a 489 mM NaCl solution buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with 5 mM citrate salts, and were suspended in a 489 mM $NaNO_3$ solution buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with 5 mM citrate salts. At the end of the experiment (300 s), detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of a minimum of 6 independent trials. DMSO was used as a control. (a) **1** (1 mol%); (b) **1** (0.1 mol%); (c) **2** (1 mol%); (d) **2** (0.05-0.125 mol%); (e) **3** (1 mol%); (f) **3** (0.1 mol%).

S5.3. Hill plots

During Hill plots the chloride/nitrate transport assays are performed as described above for various concentrations of carrier. The chloride efflux (%) 270 s after the addition of carrier is plotted as a function of the carrier concentration. Data points can then be fitted to the Hill equation using *OriginPro 9.1*:

$$y = V_{\max}\frac{x^n}{k + x^n} = 100\%\frac{x^n}{EC_{50} + x^n}$$

where y is the chloride efflux at 270 s (%) and x is the carrier concentration (mol% carrier to lipid). V_{max}, k and n are the parameters to be fitted. V_{max} is the maximum efflux possible (often fixed to 100%, as this is physically the maximum chloride efflux possible), n is the Hill coefficient and k is the carrier concentration needed to reach $V_{max}/2$ (when V_{max} is fixed to 100%, k equals EC_{50}). From the Hill plot it is therefore possible to directly obtain $EC_{50,270s}$ values, defined as the carrier concentration (mol% carrier to lipid) needed to obtain 50% chloride efflux after 270 s. The Hill plots of compounds **1**-**2** at pH 7.2 and pH 4.0 have been previously published.[1,3]

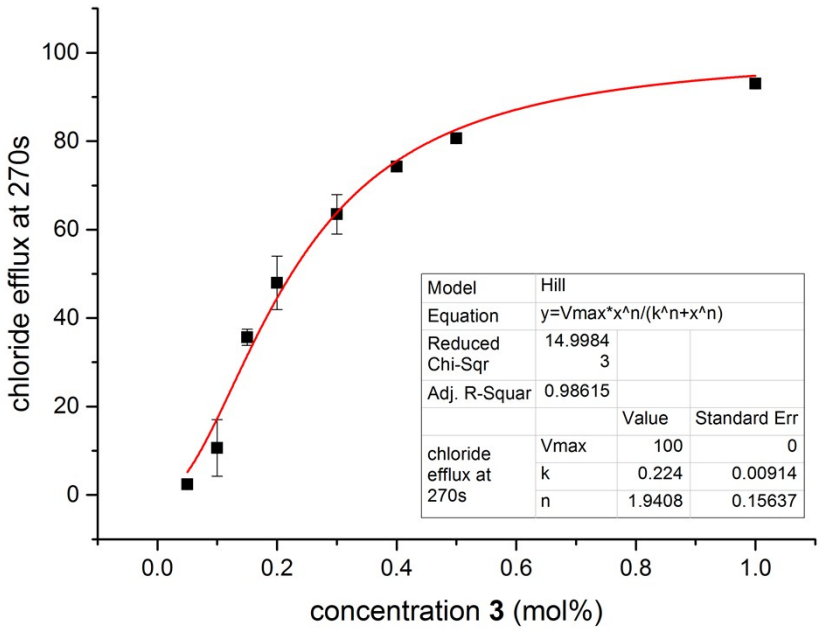


Figure S9: Hill plot for chloride release mediated by receptor **3** from unilamellar POPC vesicles loaded with 489 mM NaCl buffered to pH 7.2 with 5 mM sodium phosphate salts. The vesicles were dispersed in 489 mM $NaNO_3$ buffered to pH 7.2 with 5 mM sodium phosphate salts. Chloride efflux was measured at 270 s. Each points represent the average of a minimum of three repeats and error bars represent standard deviations. Concentrations used: 0.05 mol%, 0.1 mol%, 0.15 mol%, 0.2 mol%, 0.3 mol%, 0.4 mol%, 0.5 mol%, 1 mol% with respect to lipid.

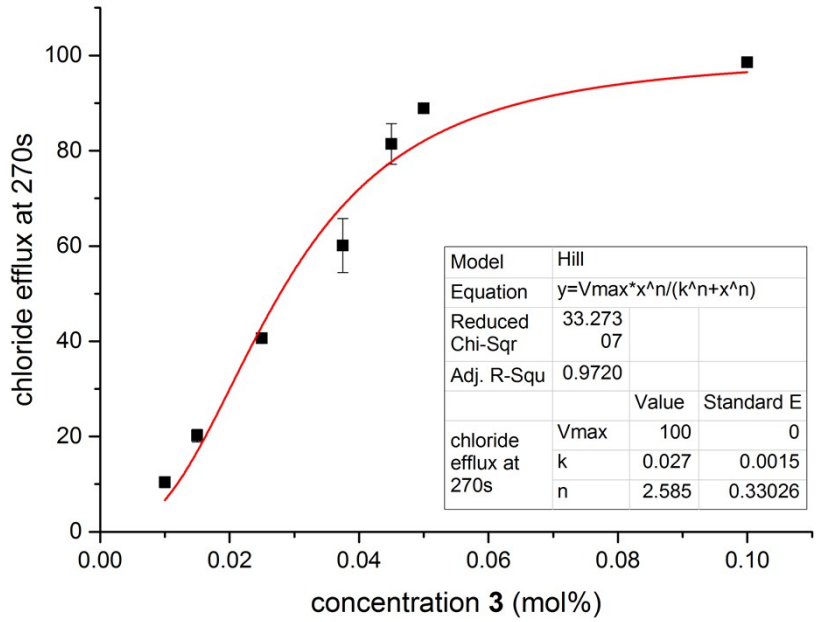


Figure S10: Hill plot for chloride release mediated by receptor **3** from unilamellar POPC vesicles loaded with 489 mM NaCl buffered to pH 4.0 with 5 mM citrate salts. The vesicles were dispersed in 489 mM $NaNO_3$ buffered to pH 4.0 with 5 mM citrate salts. Chloride efflux was measured at 270 s. Each points represent the average of a minimum of three repeats and error bars represent standard deviations. Concentrations used: 0.01 mol%, 0.015 mol%, 0.025 mol%, 0.0375 mol%, 0.045 mol%, 0.05 mol%, 0.1 mol% with respect to lipid.

S5.4. Mobile carrier tests

Cholesterol tests

For the cholesterol tests, the chloride/nitrate transport experiments (see Section S5.2) were repeated with vesicles consisting of 3:7 cholesterol:POPC. Cholesterol is believed to increase the viscosity of the membrane, hence slowing down diffusion in the lipid bilayer. This effect should be more pronounced in the case of a mobile carrier mechanism, while no effect is expected for an ion channel. However, it must be noted that cholesterol also has other effects on the membrane and the exact behaviour is hard to predict. Unlike what was previously found for **1-2,**[1,3] Figure S9 shows little difference in the transport ability of **3** in POPC and POPC:cholesterol membranes. Additional tests (calcein and U-tube assays) were therefore performed with mixed oxothiosquaramide **3** to elucidate its mode of action.

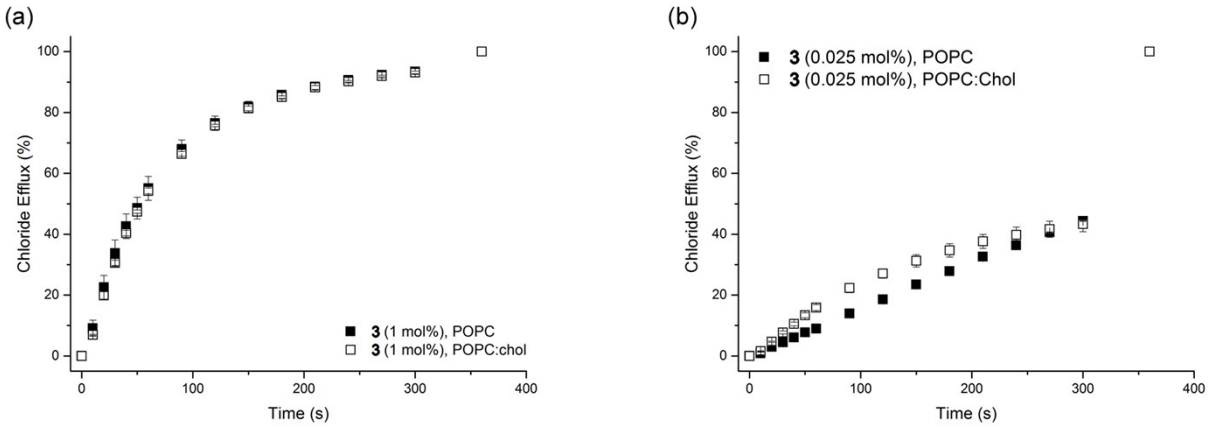


Figure S11: Chloride efflux promoted by compound **3** from unilamellar POPC vesicles (filled symbols) or unilamellar 7:3 POPC:cholesterol vesicles (empty symbols) loaded with 489 mM NaCl buffered to pH 7.2 with 5 mM sodium phosphate salts or to pH 4.0 with 5 mM citrate salts. The vesicles were dispersed in 489 mM $NaNO_3$ buffered to pH 7.2 with 5 mM sodium phosphate salts or to pH 4.0 with 5 mM citrate salts. At the end of the experiment, detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of three trials. (a) 1 mol% of compound **3** at pH 7.2. (b) 0.025 mol% of compound **3** at pH 4.0.

U-tube experiment

In a U-tube experiment the lipid bilayer is substituted with a bulk organic phase. In these conditions ion channel formation is virtually impossible. The organic phase consisted of 15 mL nitrobenzene (for solubility reasons) and contained 1 mM of carrier and 2 mM of $TBA^+PF_6^-$ to provide for counterions during transport. A control experiment was executed with neat nitrobenzene (no carrier) containing 2 mM of $TBA^+PF_6^-$. The same aqueous phases were used as for the vesicle experiments. The donating phase contained 489 mM NaCl and was buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with citrate salts (15 mL). The receiving phase contained 489 mM $NaNO_3$ and was buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with citrate salts (15 mL). The change in chloride concentration of the receiving phase was monitored with a chloride-selective electrode and the electrode was calibrated to convert the potential readings (mV) to chloride concentrations (mM). The experiments were conducted at room temperature and the results are shown in Figure S10. U-tubes assays had previously shown that squaramide **1** functions as a mobile carrier,[1] while U-tubes test could not be performed for thiosquaramide **2** due to the instability of the thiosquaramide. Figure S10 shows that compound **3** is unable to transport large amounts of anions across a bulk membrane. This could potentially be due to degradation of **3** over the course of the 5-day experiment. However, compound 3 does display a steeper curve compared

to a blank experiment at pH 4.0, which could point towards a minimal amount of chloride transport.

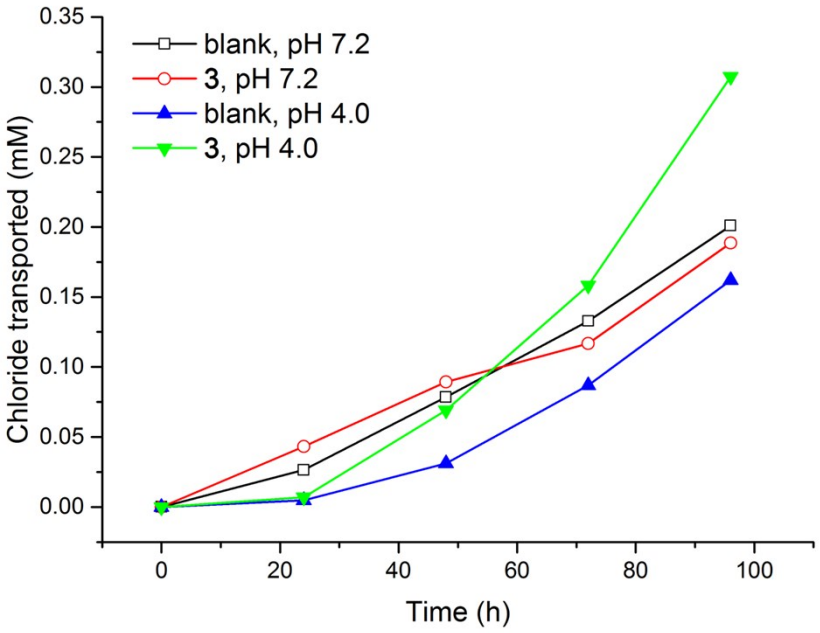


Figure S12: Graph showing the change in the chloride concentration of the receiving aqueous phase in a U-tube experiment over a period of 5 days at both pH 7.2 and pH 4.0. The donating phase contained 489 mM NaCl and was buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with citrate salts. The receiving phase contained 489 mM $NaNO_3$ and was buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with citrate salts. The bulk membrane was a nitrobenzene layer containing 2 mM $TBAPF_6$ and 1 mM carrier (or no carrier for a blank experiment).

Calcein leakage assays

A lipid film of POPC was formed from a chloroform solution under reduced pressure and dried under vacuum for at least 6 hours. The lipid film was rehydrated by vortexing with a NaCl solution (100 mM calcein, 489 mM NaCl, 5 mM phosphate buffer at pH 7.2 or 5 mM citrate buffer at pH 4.0). The lipid suspension was then subjected to nine freeze-thaw cycles and allowed to age for 30 min at room temperature before extruding 25 times through a 200 nm polycarbonate membrane. The unincorporated calcein was removed by size exclusion chromatography on a Sephadex G-50 column using a sodium nitrate solution as eluent (489 mM $NaNO_3$, 5 mM phosphate buffer at pH 7.2 or 5 mM citrate buffer at pH 4.0).

The thus obtained unilamellar POPC were suspended in a $NaNO_3$ solution buffered to pH 7.2 with sodium phosphate salts or to pH 4.0 with citrate buffer. The lipid concentration per sample was 1 mM. The fluorescence emission of the encapsulated calcein at 520 nm after

excitation at 490 nm was recorded using a *Varian Cary Eclipse Fluorescence Spectrophotometer*. At t = 10 s, a solution of the carrier molecule in DMSO (1 mol% w.r.t. lipid) was added to start ion transport. At the end of the experiment the vesicles were lysed with 30 µl of octaethylene glycol monododecyl ether (0.232 mM in 7:1 water:DMSO v/v). The fractional calcein release (FR) was calculated as follows (with I_t = fluorescence intensity at time t, I_0 = fluorescence intensity at time 0 and I_{max} = fluorescence intensity after addition of detergent):

$$FR = \frac{I_t - I_0}{I_{max} - I_0}$$

The results depicted in Figure S11 shown no sign of leakage of the calcein dye from the vesicles. This indicates that the vesicles are stable under the experimental conditions and that oxothiosquaramide **3** does not induce vesicles leakage and does not cause the formation of large channels in the POPC membranes and most likely functions as mobile carriers for chloride ions. Calcein leakage assays were not performed for compounds **1-2** have been previously reported and showed similar results.[1]

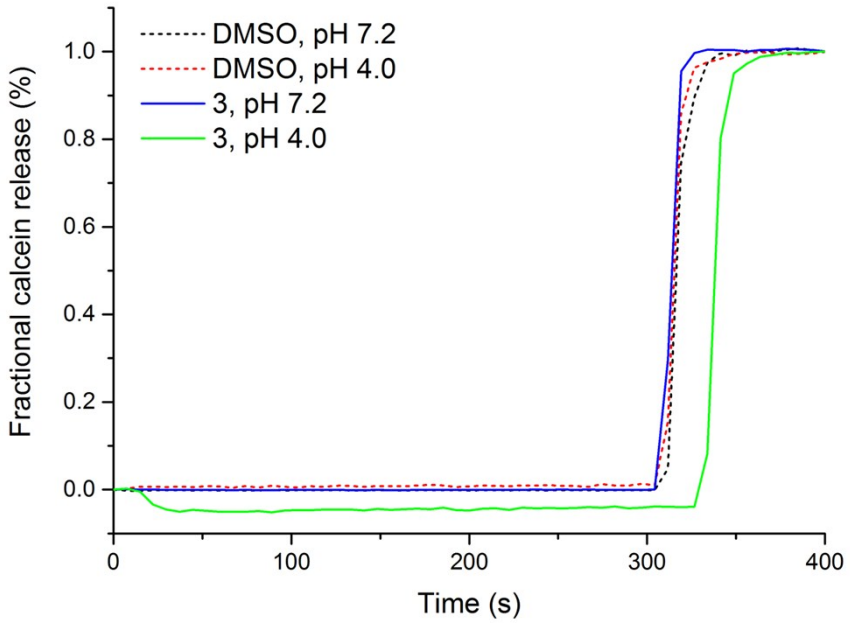


Figure S13: Calcein leakage by **3** (1 mol% carrier-to-lipid) from unilamellar POPC vesicles loaded with 100 mM calcein and 489 mM NaCl, buffered to pH 7.2 with 5 mM sodium phosphate salts or to pH 4.0 with 5 mM citrate salts. The vesicles were dispersed in 489 mM $NaNO_3$ buffered to pH 7.2 with 5 mM sodium phosphate salts or to pH 4.0 with 5 mM citrate salts. At t = 10 s, a DMSO solution of the transporter was added to start the experiment. At the end of the experiment, detergent was added to lyse the vesicles. The results are shown as the fraction of calcein leaked from the vesicles.

S5.5. Antiport tests

Unilamellar POPC vesicles containing 489 mM MCl (M = Na, Cs) solution buffered to pH 7.2 or pH 4.0 with 5 mM phosphate or citrate salts, prepared as described in Section S5.1., were suspended in the external medium consisting of a 162 mM Na_2SO_4 solution buffered to pH 7.2 or pH 4.0 with 5 mM phosphate or citrate salts. The lipid concentration per sample was 1 mM. A DMSO solution of the carrier molecule was added to start the experiment and chloride efflux was monitored using a chloride sensitive electrode. At 5 min, the vesicles were lysed with 50 μL of octaethylene glycol monododecyl ether (0.232 mM in 7:1 water:DMSO v/v) and a total chloride reading was taken at 7 min. The initial value was set at 0% chloride efflux and the final chloride reading was set as 100% chloride efflux. All other data points were calibrated to these points.

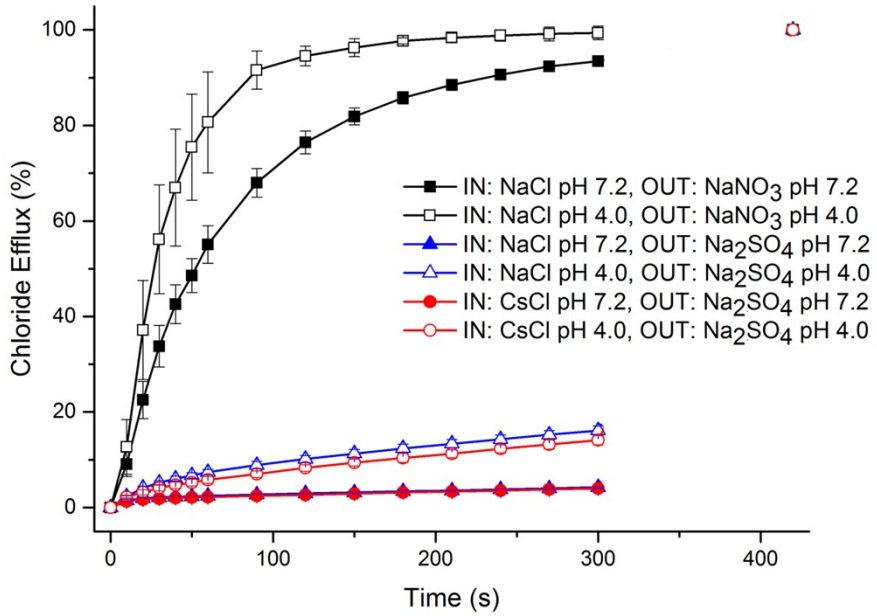


Figure S14: Chloride efflux from POPC vesicles at various conditions mediated by **3** (1 mol% with respect to lipid). POPC vesicles were loaded with a 489 mM MCl (M = Na, Cs) solution buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with 5 mM citrate salts, and were suspended in a 489 mM $NaNO_3$ or 162 mM Na_2SO_4 solution buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with 5 mM citrate salts. At the end of the experiment (300 s), detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Conditions at pH 7.2 are shown in filled symbols, while conditions at pH 4.0 are shown in empty symbols. Each point represents the average of a minimum of 3 independent trials. DMSO was used as a control (not shown).

Based on the results shown in Figure S12, similar conclusions can be made as for the previously reported oxosquaramide and thiosquaramide analogues[1,3]:

- The small difference in chloride efflux from vesicles loaded with NaCl and vesicles loaded with CsCl indicates that the counterion does not play a significant role in the transport process. Metal-chloride symport by the mixed oxothiosquaramide is therefore unlikely.
- The large difference in activity seen when nitrate or sulfate is the external anion suggests that the anion in the external solution is important for the transport process. The most likely mechanism is therefore chloride/nitrate antiport.

S5.6. HPTS assays

The fluorescence of the HPTS dye in the conditions of the transport experiments was first calibrated against pH. For this, a lipid film of POPC was formed from a chloroform solution under reduced pressure and dried under vacuum for at least 6 hours. The lipid film was rehydrated by vortexing with a NaCl solution (1 mM HPTS (8-hydroxypyrene-1,3,6-trisulphonic acid), 489 mM NaCl, 5 mM citrate buffer at pH 6.0). The lipid suspension was then subjected to nine freeze-thaw cycles and allowed to age for 30 min at room temperature before extruding 25 times through a 200 nm polycarbonate membrane. The unincorporated HPTS was removed by size exclusion chromatography on a Sephadex G-25 column using a sodium sulfate or sodium nitrate solution as eluent (162 mM Na_2SO_4 or 489 mM $NaNO_3$, 5 mM citrate buffer at pH 6.0). The thus obtained unilamellar POPC liposomes were suspended in a Na_2SO_4 or $NaNO_3$ solution buffered to pH 6.0 with citrate buffer. The lipid concentration per sample was 1 mM. The vesicles were then lysed with octaethylene glycol monododecyl ether. Small aliquots of a concentrated NaOH solution were added to increase the pH (total range pH 6 – pH 8). At each addition, the pH of the solution was measured using a pH electrode and at the same time the HPTS fluorescence was monitored by excitation at both 403 nm and 460 nm and recording the emission at 510 nm using a *Varian Cary Eclipse Fluorescence Spectrophotometer*. A calibration curve could then be obtained by fitting a plot of the pH (y) against the ratio of HPTS emission after excitation at 460 nm and 403 nm (I_{460}/I_{403}, x) to the following equation (See Figure S13): $y = a + b \cdot ln(x+c)$.

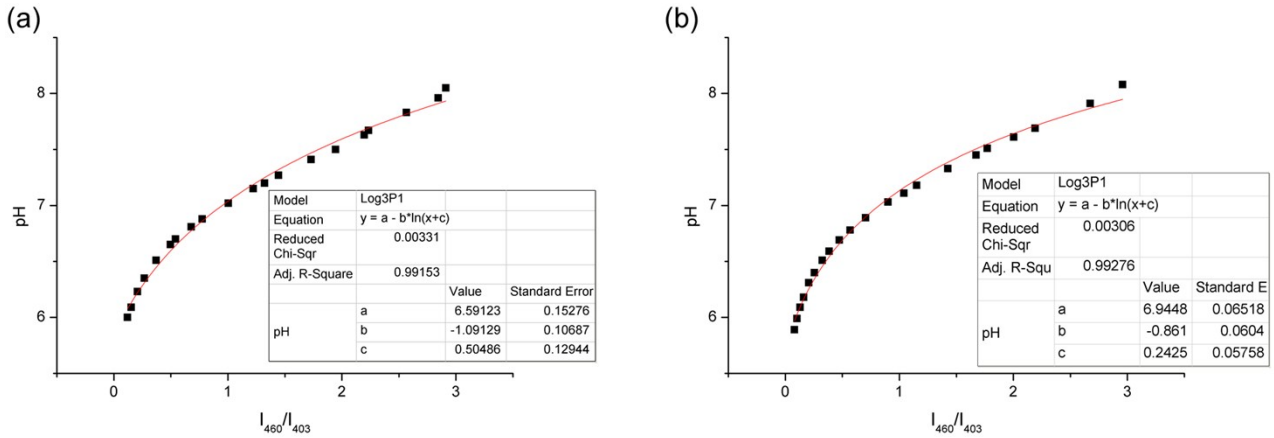


Figure S15: Calibration curve for pH (y) from the ratio of HPTS emission after excitation at 460 nm and 403 nm (I_{460}/I_{403}, x), (a) in the presence of $NaNO_3$; (b) in presence of $NaSO_4$.

For the actual HCl symport assays, a lipid film of POPC was formed from a chloroform solution under reduced pressure and dried under vacuum for at least 6 hours. The lipid film was rehydrated by vortexing with a NaCl solution (1 mM HPTS (8-hydroxypyrene-1,3,6-trisulphonic acid), 489 mM NaCl, 5 mM phosphate buffer at pH 7.2 or 5 mM citrate buffer at pH 6.0). The lipid suspension was then subjected to nine freeze-thaw cycles and allowed to age for 30 min at room temperature before extruding 25 times through a 200 nm polycarbonate membrane. The unincorporated HPTS was removed by size exclusion chromatography on a Sephadex G-25 column using a sodium sulfate or sodium nitrate solution as eluent (162 mM Na_2SO_4 or 489 mM $NaNO_3$, 5 mM phosphate buffer at pH 7.2 or 5 mM citrate buffer at pH 6.0). The thus obtained unilamellar POPC vesicles were suspended in a Na_2SO_4 or $NaNO_3$ solution buffered to pH 7.2 with sodium phosphate salts or to pH 6.0 with citrate buffer. The lipid concentration per sample was 1 mM. A DMSO solution of the carrier molecule was added to start the experiment (when a pH gradient was applied a small amount of the appropriate acid or base was added to the external solution just before the start of the experiment in order to achieve a pH gradient of one pH unit). The fluorescence of intravesicular HPTS was monitored by excitation at both 403 nm and 460 nm and recording the emission at 510 nm using a *Varian Cary Eclipse Fluorescence Spectrophotometer*. At the end of the experiment, the vesicles were lysed with 30 µL of octaethylene glycol monododecyl ether (0.232 mM in 7:1 water:DMSO v/v).

$$pH = 6.59 + 1.09 \cdot \ln\left(\frac{I_{460}}{I_{403}} + 0.50\right) \quad \textit{for external nitrate}$$

$$pH = 6.94 + 0.86 \cdot \ln\left(\frac{I_{460}}{I_{403}} + 0.24\right) \quad \textit{for external sulfate}$$

To allow a better comparison with the previously published data obtained for **1** and **2** on different days and batches of vesicles, the results are converted to change in pH (ΔpH) versus time. When nitrate is the external anion (Figure S14-S16), the results reveal that in the absence of a pH gradient the intravesicular pH does not change significantly upon the addition of (thio)squaramid-based transporters (Figure S14-S15). However, when a pH gradient is applied across the phospholipid bilayer, a fast dissipation of the gradient is observed upon the addition of oxosquaramide **1**, thiosquaramides **2** and mixed oxothiosquaramide **3**, suggesting that H^+/Cl^- symport (or OH^-/Cl^- antiport) can occur under these conditions (Figure S16). However, when sulfate is the external anion, the results are slightly different (Figure S17-S19). In this case only a small increase in internal pH was observed when both the intravesicular and extravesicular solution were buffered to pH 7.2 (Figure S17), while a large increase in internal pH was seen when a pH gradient was applied (Figure S19). However, unlike the nitrate tests (Figure S15), a large increase in internal pH was also observed when both intra- and extravesicular solution were buffered to pH 6.0 (Figure S18), hinting that H^+/Cl^- symport (or OH^-/Cl^- antiport) can occur without a pH gradient providing that sulfate is the external anion (because the chloride gradient cannot be dissipated by Cl^-/SO_4^{2-} transport).

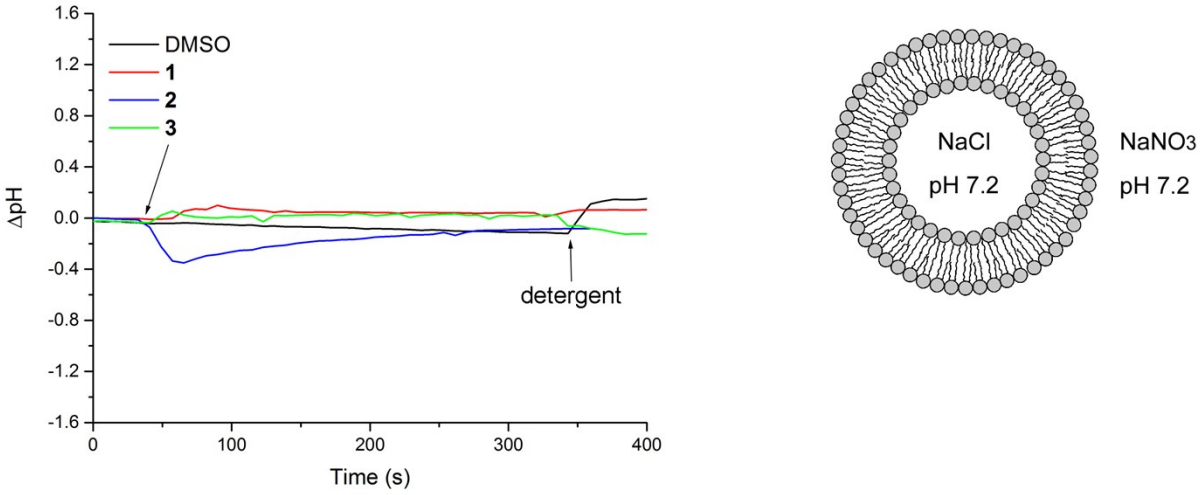


Figure S16: Investigation of HCl transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 489 mM NaCl, 1 mM HPTS, buffered to pH 7.2 with phosphate buffer and suspended in a solution of 489 mM $NaNO_3$ buffered to pH 7.2 with phosphate buffer. At time t = 50 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 350 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

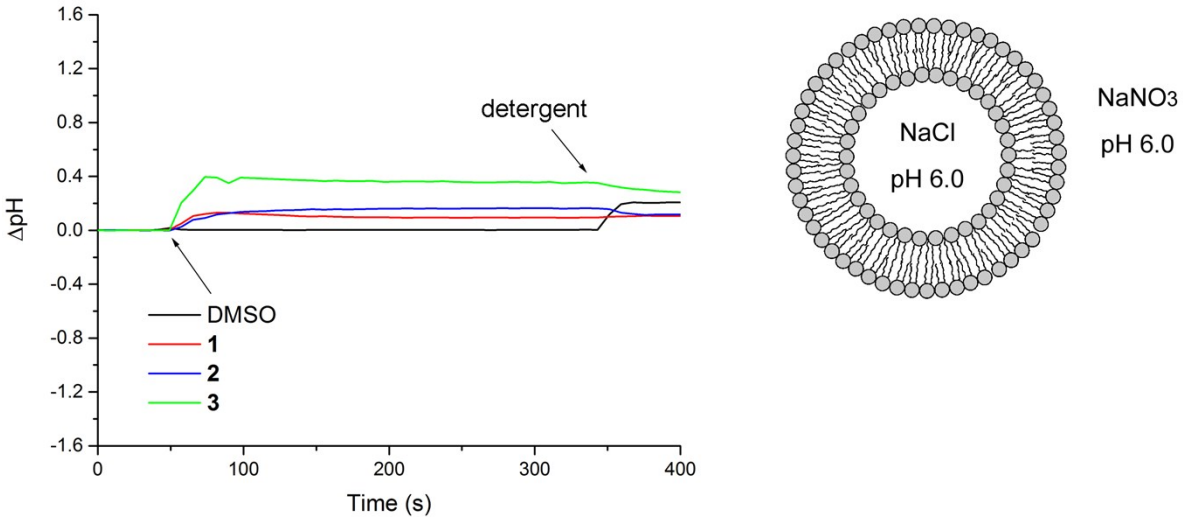


Figure S17: Investigation of HCl transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 489 mM NaCl, 1 mM HPTS, buffered to pH 6.0 with citrate buffer and suspended in a solution of 489 mM $NaNO_3$ buffered to pH 6.0 with citrate buffer. At time t = 50 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 350 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

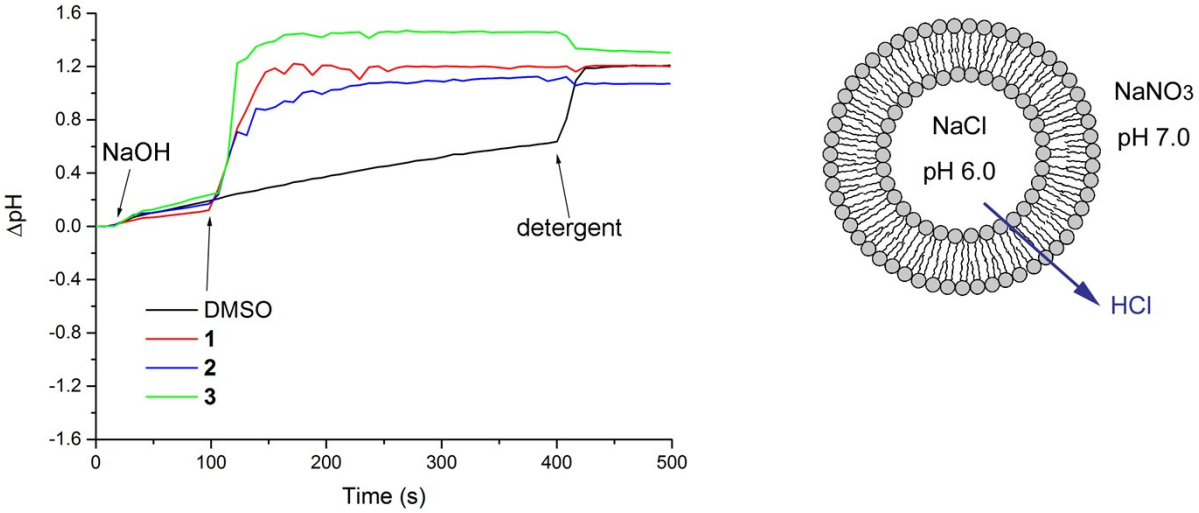


Figure S18: Investigation of HCl transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 489 mM NaCl, 1 mM HPTS, buffered to pH 6.0 with citrate buffer, and suspended in a solution of 489 mM $NaNO_3$ buffered to pH 6.0 with citrate buffer. At time t = 20 s a NaOH solution was added to achieve an external pH of 7.0. At time t = 100 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 400 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

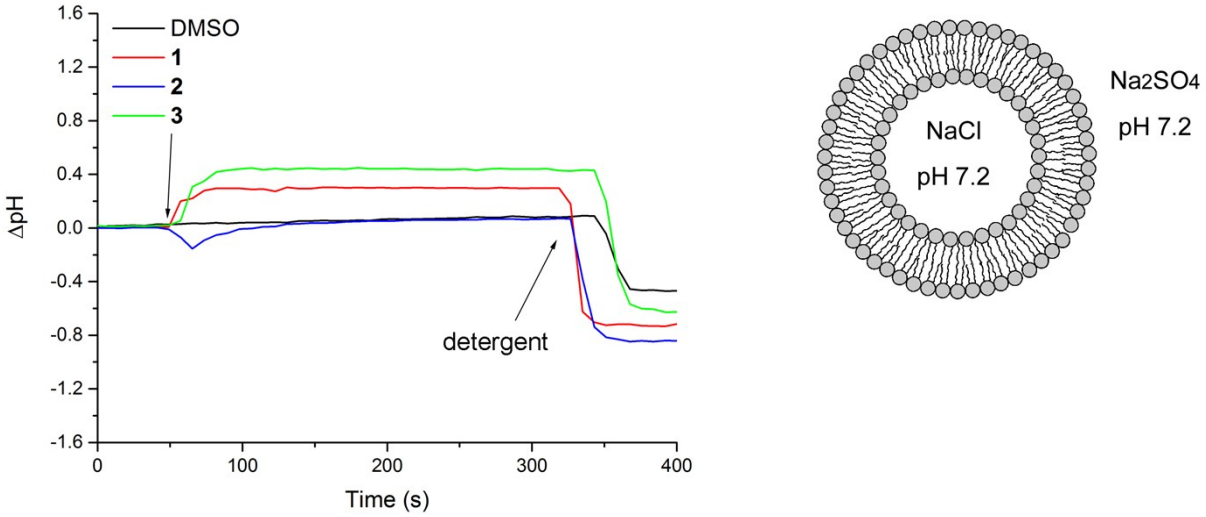


Figure S19: Investigation of HCl transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 489 mM NaCl, 1 mM HPTS, buffered to pH 7.2 with phosphate buffer and suspended in a solution of 162 mM Na_2SO_4 buffered to pH 7.2 with phosphate buffer. At time t = 50 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 350 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

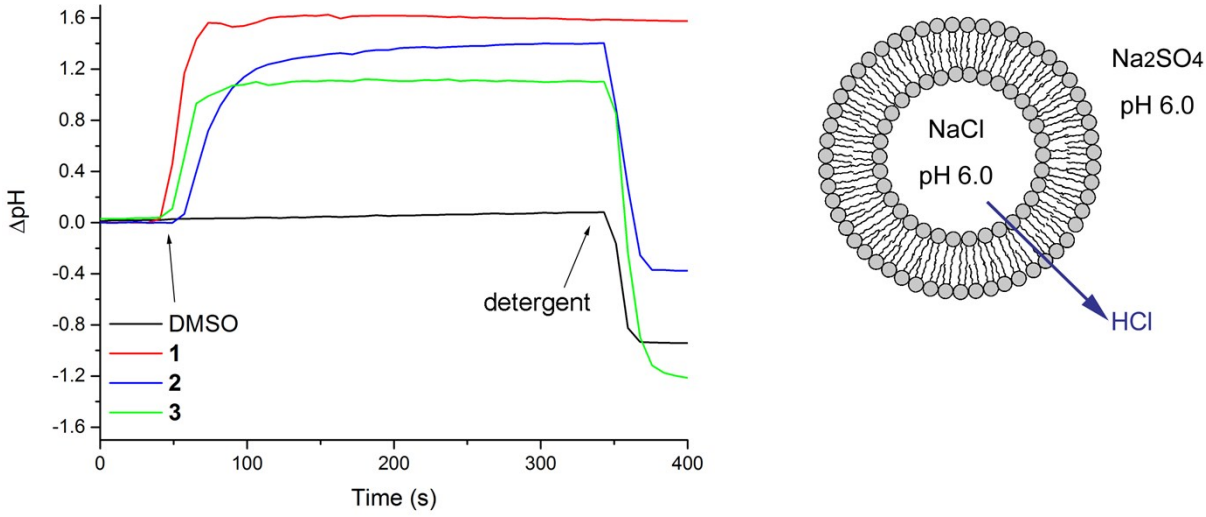


Figure S20: Investigation of HCl transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 489 mM NaCl, 1 mM HPTS, buffered to pH 6.0 with citrate buffer and suspended in a solution of 162 mM Na_2SO_4 buffered to pH 6.0 with citrate buffer. At time t = 50 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 350 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

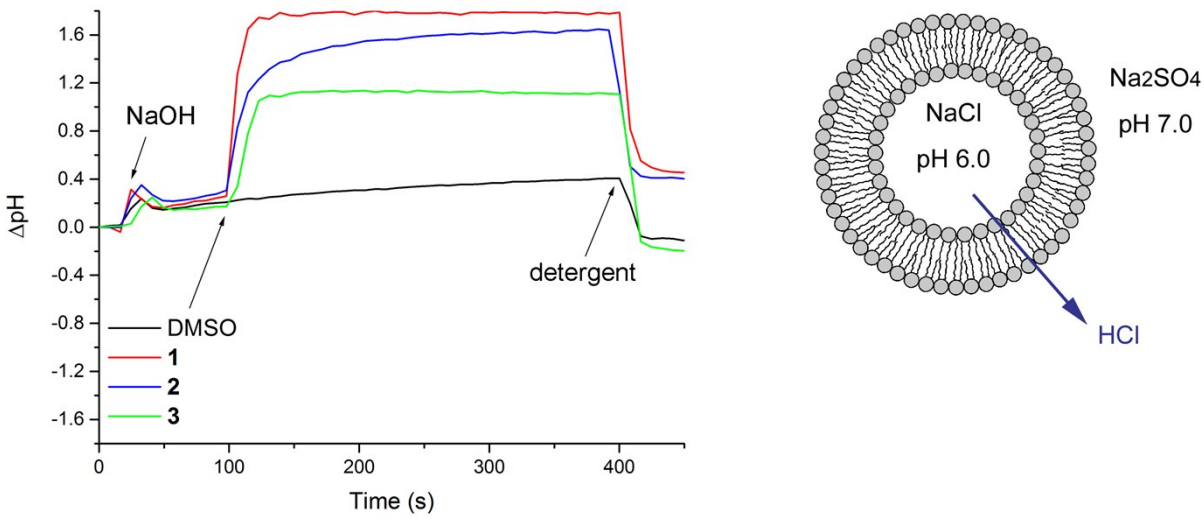


Figure S21: Investigation of HCl transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the intravesicular pH. POPC vesicles were loaded with 489 mM NaCl, 1 mM HPTS, buffered to pH 6.0 with citrate buffer, and suspended in a solution of 162 mM Na_2SO_4 buffered to pH 6.0 with citrate buffer. At time t = 20 s a NaOH solution was added to achieve an external pH of 7.0. At time t = 100 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 400 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

In order to see if any other acids can be transported across POPC bilayers, the HPTS tests were repeated with the internal and external solutions exchanged or with the pH gradient in opposite directions. The results shown in Figures S20-S23 indicated that HCl and HNO_3 can be transported by **1-3**, but HSO_4^- (or H_2SO_4) cannot be transported by these compounds.

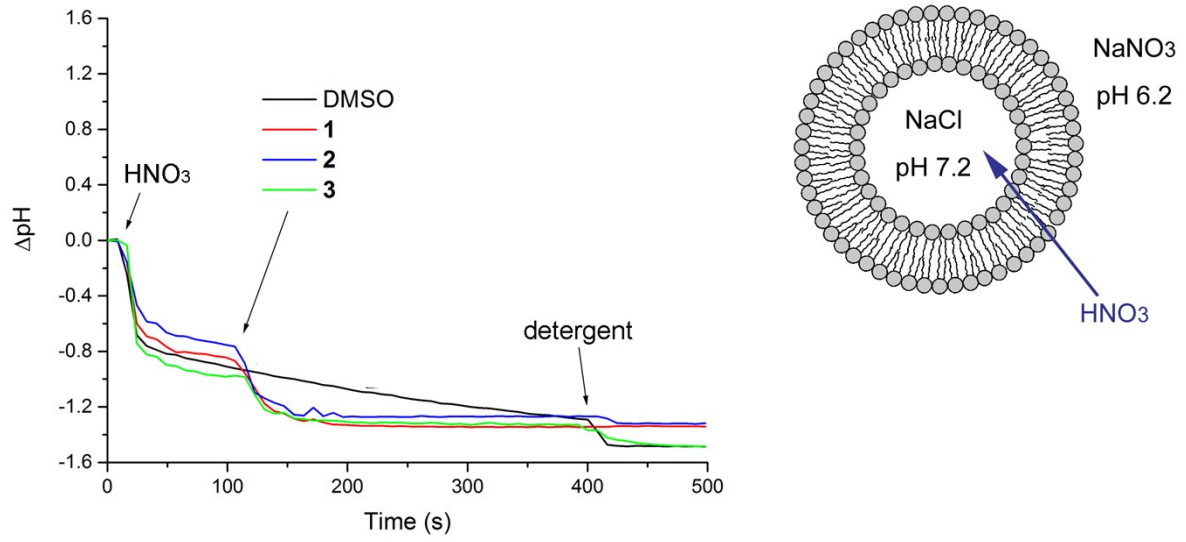


Figure S22: Investigation of HNO_3 transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 489 mM NaCl, 1 mM HPTS, buffered to pH 7.2

with phosphate buffer, and suspended in a solution of 489 mM $NaNO_3$ buffered to pH 7.2 with phosphate buffer. At time t = 10 s a HNO_3 solution was added to achieve an external pH of 6.2. At time t = 100 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 400 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

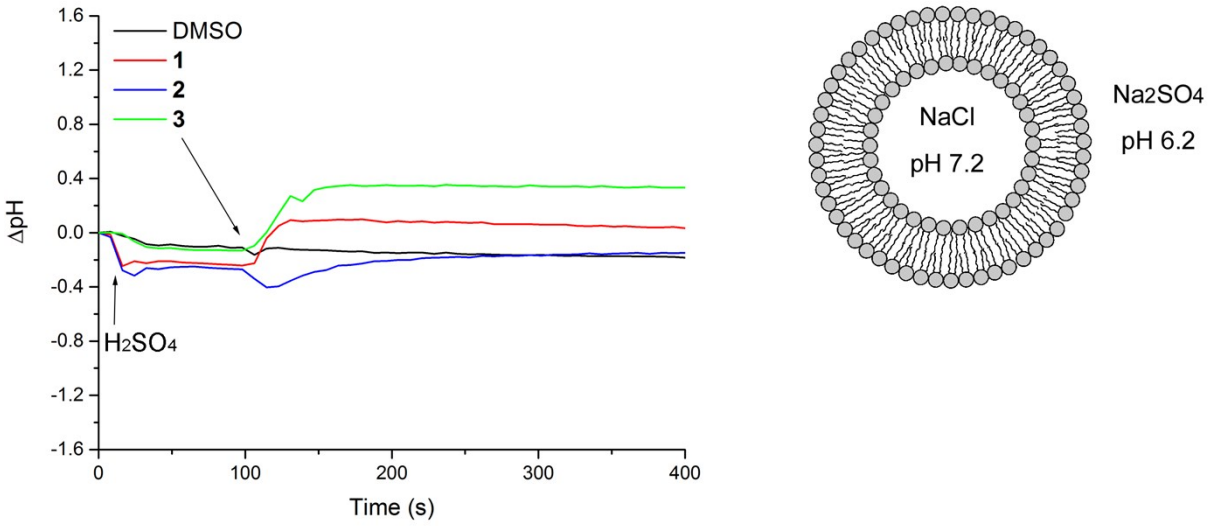


Figure S23: Investigation of HSO_4^- transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 489 mM NaCl, 1 mM HPTS, buffered to pH 7.2 with phosphate buffer, and suspended in a solution of 162 mM Na_2SO_4 buffered to pH 7.2 with phosphate buffer. At time t = 10 s a H_2SO_4 solution was added to achieve an external pH of 6.2. At time t = 100 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 400 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

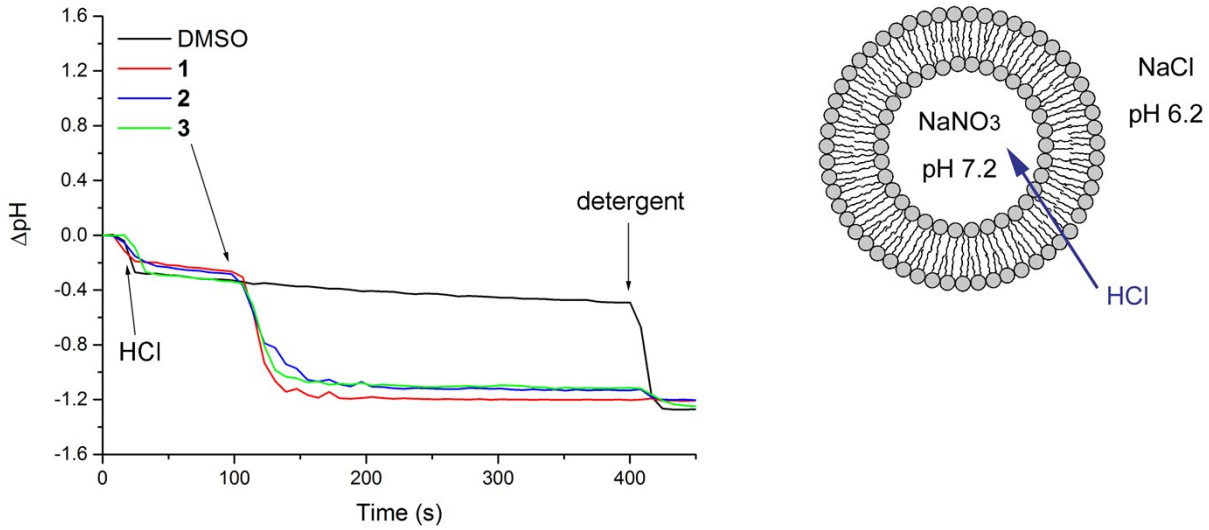


Figure S24: Investigation of HCl transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 489 mM $NaNO_3$, 1 mM HPTS, buffered to pH 7.2 with phosphate buffer, and suspended in a solution of 489 mM NaCl buffered to pH 7.2 with phosphate buffer. At time t = 10 s a HCl solution was added to achieve an external pH of 6.2. At time t = 100 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 400 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

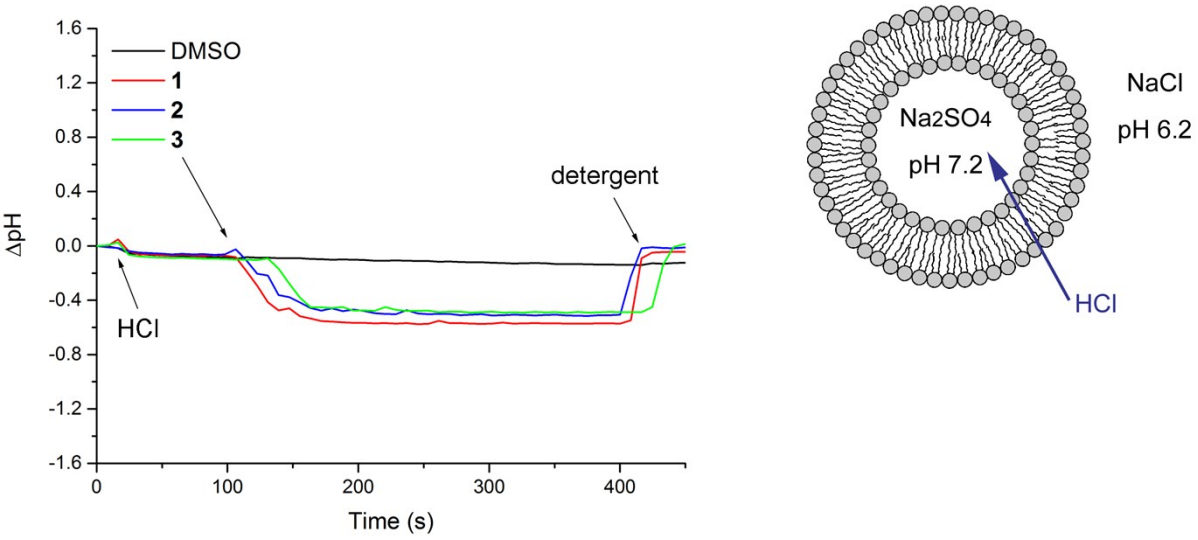


Figure S25: Investigation of HCl transport facilitated by **1**–**3** using the fluorescent dye HPTS to estimate the change in intravesicular pH. POPC vesicles were loaded with 162 mM Na_2SO_4, 1 mM HPTS, buffered to pH 7.2 with phosphate buffer, and suspended in a solution of 489 mM NaCl buffered to pH 7.2 with phosphate buffer. At time t = 10 s a HCl solution was added to achieve an external pH of 6.2. At time t = 100 s a DMSO solution of the transporter was added (1 mol% with respect to lipid) and at time t = 400 s detergent was added. Each line represents the average of 3 independent trials and DMSO was used as a control.

There is a spectral overlap between the absorbance region of the (thio)squaramides and HPTS (400-500 nm), which might cause interference. To test whether interference is an issue, aqueous HPTS solutions were prepared (1 mM HPTS, 489 mM NaCl, buffered with 5 mM phosphate salts to pH 7.2 or with 5 mM citrate salts to pH 6.0) and diluted with a NaCl solution (489 mM NaCl, buffered with 5 mM phosphate salts to pH 7.2 or with 5 mM citrate salts to pH 6.0) so that the fluorescence intensity of HPTS was similar to those obtained during the transport experiments and the emission spectra after excitation at 403 nm and 460 nm were then collected. A small amount of transporter was subsequently added (aliquots of 6 μL of a 5 mM DMSO solution were added to a 3 mL cuvette, to mimic the conditions of the transport experiments) and the emission spectra were collected. The results shown in Figure S24 reveals that there is some interference in the HPTS fluorescence by compound **3**, resulting in a reduction in fluorescence intensity. However, when the ratios of the HPTS fluorescence intensity at 510 nm after excitation at 403 nm and 460 nm are calculated, as well as the pH values based on these ratios, it is clear that the interference from the mixed oxothiosquaramide does not significantly affect the ratio of the fluorescence intensity at 510 nm, especially at the amounts used during the transport studies (6 μL of a 5 mM DMSO stock solution), although some interference is seen at pH 7.2. This implies that the results shown in Figures S14-S23 are due to real changes in intravesicular pH and not due to interference.

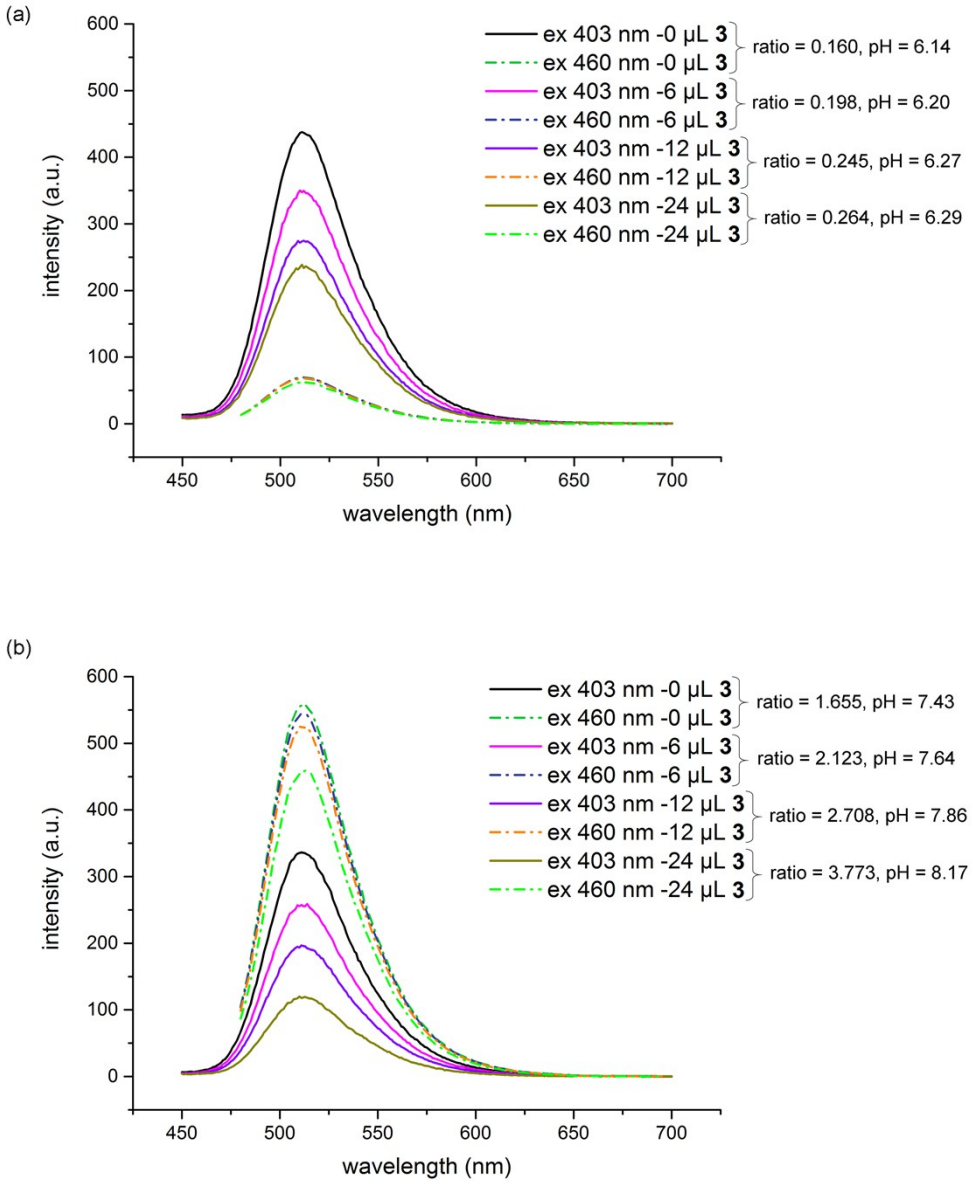


Figure S26: Investigation of possible interference in HPTS fluorescence by compound **3**. Some interference is present, but this does not affect the pH determination. (a) HPTS solution at pH 6.0 using 5 mM citrate buffer; (b) HPTS solution at pH 7.2 using 5 mM phosphate buffer.

S5.7. Apparent pK_a determination

Unilamellar POPC vesicles containing NaCl, prepared as described in Section S5.1., were suspended in the external medium consisting of $NaNO_3$ solution buffered to various pH. For the preparation of the buffer of both the internal and external solutions, an online application was used (http://www.biomol.net/en/tools/buffercalculator.htm), whereby the concentration of buffer was set to 5 mM and the ionic strength to 500 mM (the following buffers were used:

pH 4.0 – pH 6.0 = citrate, pH 7.2 = phosphate, pH 8.0 – pH 9.0 = Tris, pH 10.0 = ethanolamine, pH 11.0 = methylamine). The lipid concentration per sample was 1 mM. A DMSO solution of the carrier molecule was added (to achieve 1 mol% carrier-to-lipid concentration) to start the experiment and the chloride efflux was monitored using a chloride sensitive electrode. At 5 min, the vesicles were lysed with 50 μL of octaethylene glycol monododecyl ether (0.232 mM in 7:1 water:DMSO v/v) and a total chloride reading was taken at 7 min. The initial value was set at 0% chloride efflux and the final chloride reading (at 7 minutes) was set as 100% chloride efflux. All other data points were calibrated to these points.

In order to calculate apparent pK_a values from this data, plots were made of the transport activity versus pH and subsequently fitted to the following equation (where *y* is a measure of transport activity, *x* is the pH, *MIN* is the minimum transport activity and *MAX* is the maximum transport activity):

$$y = MAX + \frac{(MIN - MAX)}{(1 + 10^{pKa - x})}$$

In theory there are various measures of transport activity that can be use, *e.g.* percentage of chloride efflux at a given time or initial rate of chloride efflux (k_{ini}). However, when using the single-point data of percentage of chloride efflux at a given time, artificially high pK_a values can be obtained for highly active chloride transporters (see Figures S25). This is because the maximum efflux that can be achieved is 100%. A highly active transporter can achieve this 100% chloride efflux in 60 s, 90s, 300 s, etc. This means that when you plot the chloride efflux at 300 s versus pH for a highly active transporter, a platform at 100% chloride efflux is maintained for a wider range of pH than when a plot was made of the chloride efflux at 60 s versus pH. This is because in the first case 100% chloride efflux could be achieved in 60 s or in 300 s and no distinction is made between these values while they correspond to an actual difference in transport activity. In order to avoid this error, it is better to use the initial rate of chloride efflux (k_{ini}) as a measure of transport activity. The k_{ini} values were obtained by fitting the chloride efflux versus time plots with the following asymptotic function using *OriginPro 9.1*: $y = a - b \cdot c^x$, where *y* is the chloride efflux (%) and *x* is time (s). The initial rate of chloride release (k_{ini}) is then given by $k_{ini} = -b \cdot ln(c)$ and is obtained in % s^{-1}. In order to give more comparable values between the different compounds with different activities, the k_{ini} values were normalised to the highest k_{ini} value (at pH = 4.0).

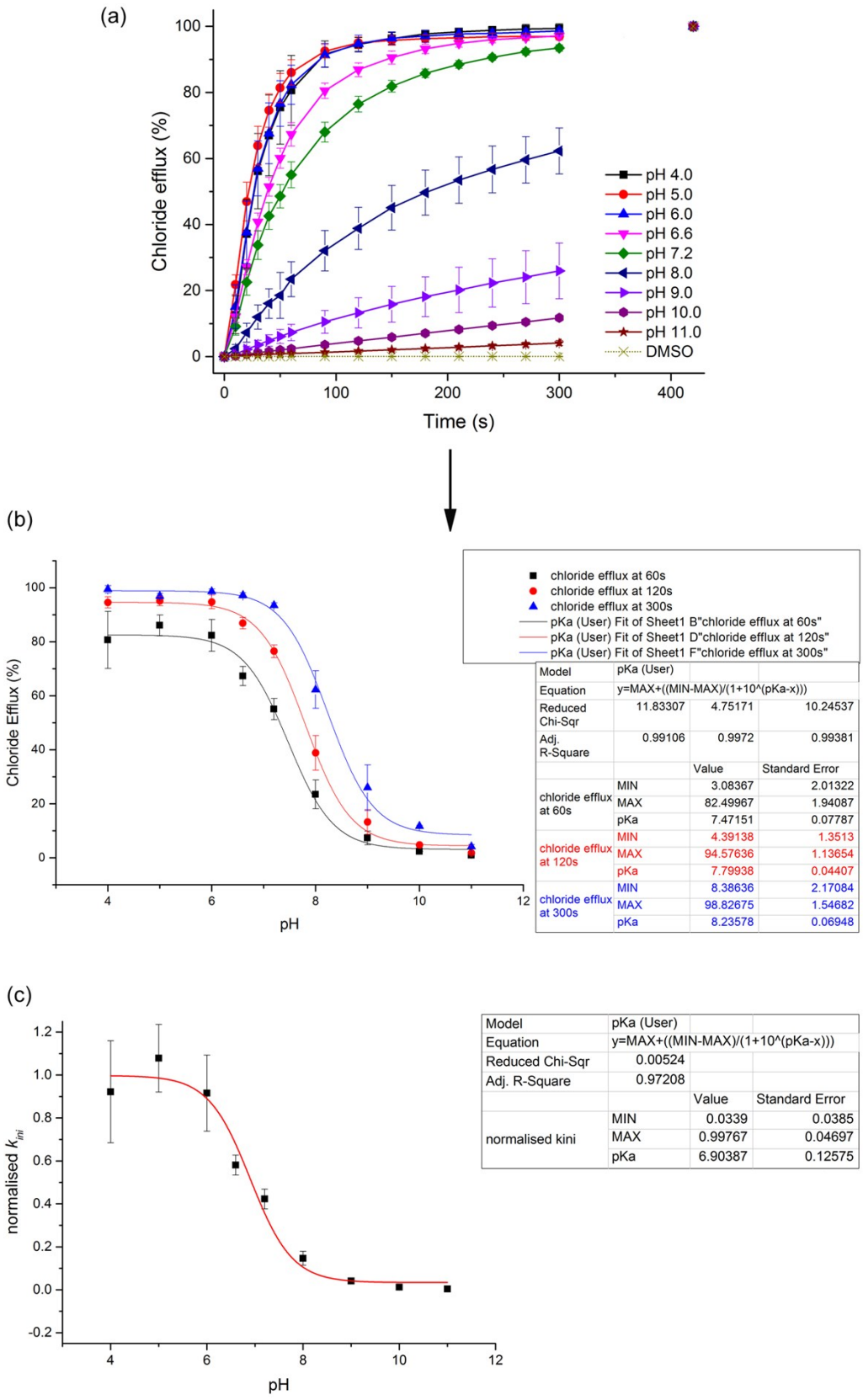


Figure S27: Apparent pK_a determination of receptor **3**. (a) Chloride efflux mediated by 1 mol% **1** from unilamellar POPC vesicles loaded with 489 mM NaCl buffered to various pH. The vesicles were dispersed in 489 mM $NaNO_3$ buffered to the same pH as the internal solution. Each points represent the average of a minimum of three repeats and error bars represent standard deviations. (b) pK_a determination based on the percentage of chloride efflux at 60 s, 120 s and 300 s. (c) pK_a determination based on normalised k_{ini} values.

S6. Oxothiosquaramide stability

As observed for thiosquaramide **2** in our previous work[1] oxothiosquaramide **3** was also found to display instability in solution over time. It was found that the ^{1}H and ^{19}F NMR spectra of **3** change over time due to degradation of the oxothiosquaramide moiety (Figure S28). To overcome the issue of instability, all of the experiments were performed on freshly made DMSO solutions that were used immediately. The ^{1}H NMR spectra obtained during the anion binding studies, indicated that the oxothiosquaramide remained stable over the course of the experiment. To check if the instability had an effect on the anion transport studies, some of the anion transport experiments were repeated with old DMSO stock solutions were used (>7 days old). A significant change in anion transport ability was seen, suggesting that the oxothiosquaramide had partially degraded. All of the transport experiments described above and in the manuscript were therefore performed with freshly made DMSO solutions and should thus be valid.

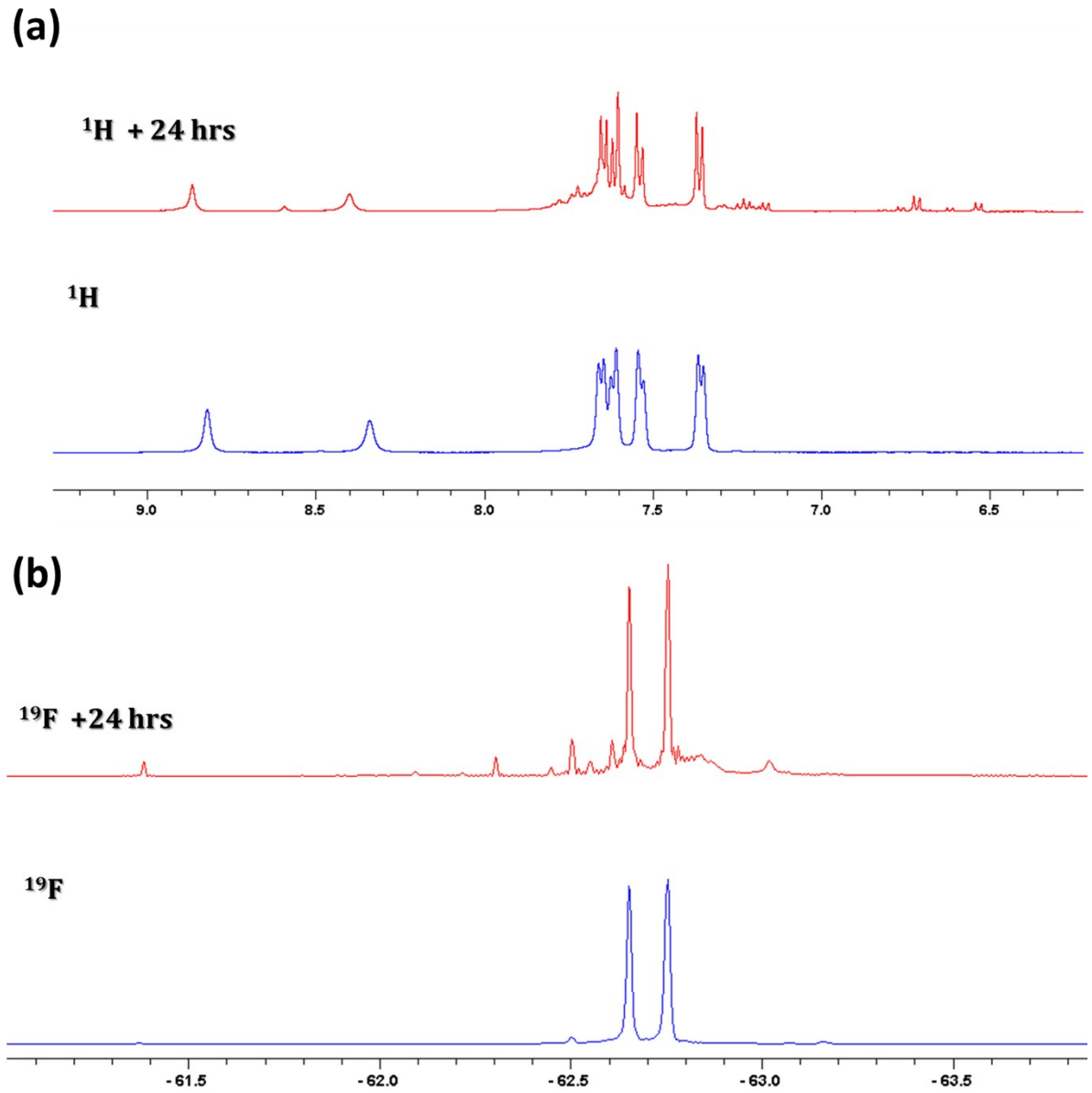


Figure S28: Changes in the (a) ^{1}H NMR and (b) ^{19}F NMR spectra of a MeCN solution of compound **3** over time.

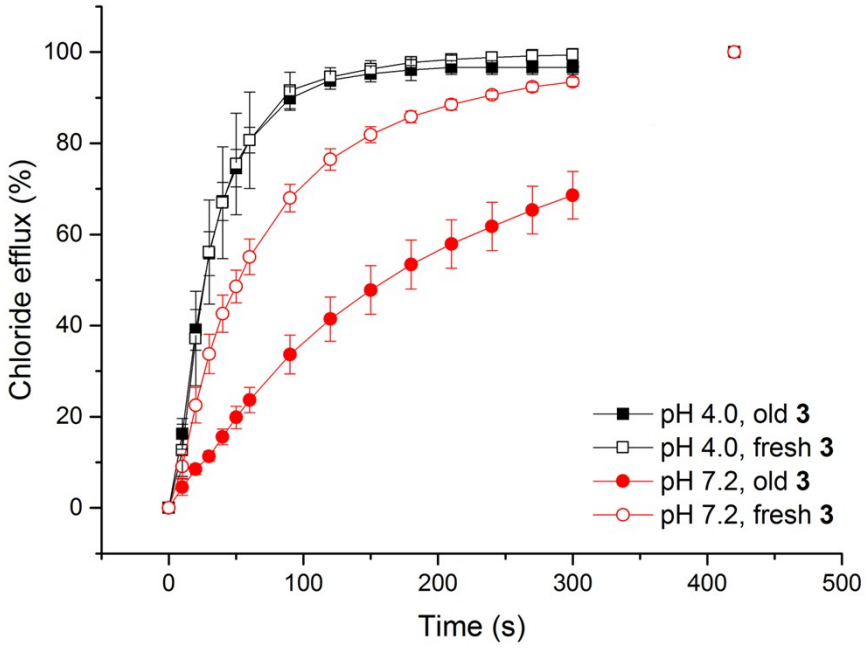


Figure S29: Chloride efflux from POPC vesicles at pH 4.0 and pH 7.2 mediated by compound **3** (1 mol% with respect to lipid) from stock solution that were either freshly prepared or were more than one week old. POPC vesicles were loaded with a 489 mM NaCl solution buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with 5 mM citrate salts, and were suspended in a 489 mM $NaNO_3$ solution buffered to pH 7.2 with 5 mM phosphate salts or to pH 4.0 with 5 mM citrate salts. The experiment was started by the addition of a DMSO solution of compound **3** (10 µL of a 5 mM stock solution - fresh or old). At the end of the experiment (300 s), detergent was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. Each point represents the average of a minimum of 3 independent trials.

S7. References

(1) Busschaert, N.; Elmes, R. B. P.; Czech, D. D.; Wu, X.; Kirby, I. L.; Peck, E. M.; Hendzel, K. D.; Shaw, S. K.; Chan, B.; Smith, B. D.; Jolliffe, K. A.; Gale, P. A. *Chem. Sci.;* **2014**, *5*, 3617-3626.
(2) Bergman, J.; Pettersson, B.; Hasimbegovic, V.; Svensson, P. H. *J. Org. Chem.* **2011**, *76*, 1546-1553.
(3) Busschaert, N.; Kirby, I. L.; Young, S.; Coles, S. J.; Horton, P. N.; Light, M. E.; Gale, P. A. *Angew. Chem. Int. Ed.* **2012**, *51*, 4426-4430.