Table of contents

1. Synthesis of calixarene 4 ........................................................................................................... 2
1.1. General experimental information .................................................................................... 2
2. Binding Studies ......................................................................................................................... 6
  2.1. Experimental procedure ......................................................................................................... 6
  2.2. $^1$H NMR titration of calixarene 1 with $\text{Bu}_4\text{N}^+\text{Cl}^-$ in DMSO-$d_6$/0.5% $\text{H}_2\text{O}$ .... 7
  2.3. $^1$H NMR titration of calixarene 2 with $\text{Bu}_4\text{N}^+\text{Cl}^-$ in DMSO-$d_6$/0.5% $\text{H}_2\text{O}$ ......... 8
  2.4. $^1$H NMR titration of calixarene 3 with $\text{Bu}_4\text{N}^+\text{Cl}^-$ in DMSO-$d_6$/0.5% $\text{H}_2\text{O}$ ............ 9
  2.5. $^1$H NMR titration of calixarene 4 with $\text{Bu}_4\text{N}^+\text{Cl}^-$ in DMSO-$d_6$/0.5% $\text{H}_2\text{O}$ ........... 10
  2.6. $^1$H NMR of calixarene 4 with $\text{PrNH}_3\text{Cl}$ and $t\text{BuNH}_3\text{Cl}$ in CDCl$_3$ ......................... 11
3. Transport Studies in Vesicles by Fluorescence Assays ............................................................ 12
  3.1. Lucigenin assay – experimental procedure ....................................................................... 12
  3.2. Quantification of transport rates ......................................................................................... 13
  3.3. Transport by 2 and 4 in DPPC vesicles .............................................................................. 14
  3.4. Transport by 4 at different transporter concentrations ....................................................... 15
  3.5. Additional transport data in Na$_2$SO$_4$ ............................................................................ 15
  3.6. Experiments with anionic lipids ....................................................................................... 19
  3.7. HPTS assay – experimental procedure .............................................................................. 20
  3.8. HPTS assay with calixarene 4 ......................................................................................... 21
4. Transport Studies in Vesicles by NMR .................................................................................... 22
  4.1. General experimental information ..................................................................................... 22
  4.2. Preparation of liposomes for NMR ................................................................................... 22
  4.3. Co-transport of organic ion pairs ...................................................................................... 23
5. U-tube experiments .................................................................................................................. 24
  5.1. Experimental information .................................................................................................. 24
  5.2. Results ............................................................................................................................... 24
6. References ............................................................................................................................... 26
1. Synthesis of calixarene 4

1.1. General experimental information.

$^1$H, $^{13}$C and $^{19}$F NMR spectra were recorded on a Varian VNMRS 400 (9.4 T) equipped with an Automation Triple Resonance Broadband (ATB) probe at 298 K. 2D NMR spectra (COSY, HSQC, HMBC) were recorded for complete signal assignments. Solvent signals were used as reference for the chemical shifts. Chemical shifts are expressed in ppm and the coupling constants (J) are expressed in Hertz (Hz). High-resolution mass spectra were measured on an Agilent QTOF 6520 by electron spray ionisation. Infra-red spectra were recorded on a Bruker ALPHA FTIR spectrometer using attenuated total reflection (ATR).


Calix[6]arene-triamine 5 (150 mg, 131 μmol) was dissolved in dry DCM (1 mL) under Ar. 3,5-Bis(trifluoromethyl)phenyl isothiocyanate (79 μL, 432 μmol, 3.3 equiv) was added and the reaction mixture was stirred for 6 hours, after which it was concentrated. The crude material was purified by column chromatography over silica gel with a gradient of DCM to 1% MeOH in DCM as eluent. This afforded 4 as a white solid (170 mg, 86.7 μmol, 66%). mp 149 °C. $^1$H NMR (400 MHz, acetone-d$_6$, 298 K), δ (ppm): 0.84 (s, 27H, tBu), 1.40 (s, 27H, tBu), 2.25 (s, 9H, OMe), 3.49 (d, J = 15.1 Hz, 6H, ArCH$_2$eq), 4.16-4.20 (m, 6H, CH$_2$NH), 4.24 (t, J = 5.4 Hz, 6H, CH$_2$O), 4.60 (d, J =15.1 Hz, 6H, ArCH$_2$ax), 6.73 (s, 6H, ArHcal-arm)$^1$, 7.37 (s, 6H, ArHcal-OMe), 7.69 (s, 3H, ArH$^p$), 8.04 (t, 3H, NHCH$_2$), 8.26 (s, 6H, ArH$^t$), 9.55 (s, 3H, NHAr); $^{13}$C NMR (100 MHz, acetone-d$_6$, 298 K), δ (ppm): 29.2$^2$ (ArCH$_2$), 31.7 (CH$_3$), 31.9 (CH$_3$), 34.7 (C(CH$_3$)$_3$), 34.8 (C(CH$_3$)$_3$), 45.4 (NCH$_3$), 60.9 (OCH$_3$), 71.3 (OCH$_3$), 117.7 (CH$^{Ar}$), 123.7 (CH$^{Ax}$), 124.3 (q, J = 272 Hz, CF$_3$), 124.5 (CH$^{cal-arm}$), 129.1 (CH$^{cal-OMe}$), 131.9 (q, J = 33 Hz, CCF$_3$), 133.8 (C$^{cal}$), 134.4 (C$^{cal}$), 142.7 (CH$^{Ar}$), 146.6 (C$^{OMe}$), 146.7 (C$^{arm}$), 152.4 (C$^{cal-arm}$), 155.2 (C$^{OMe}$), 182.8 (C$^S$). $^{19}$F NMR (376.4 MHz, acetone-d$_6$, 298 K), δ (ppm): -63.5 (CF$_3$). IR, ν (cm$^{-1}$): 3251, 2973, 2935, 1522, 1471, 1382, 1377, 1175, 1131, 950, 887, 702, 682. HRMS (ESI) Calculated for C$_{102}$H$_{115}$F$_{18}$N$_6$O$_{6}$S$_3$ $^+$ [M+H]$^+$: 1958.7781, found: 1958.7766.

---

1 “cal” represents the aromatic units of the calix cavity bearing either the thiourea arm or the methoxy group, respectively labelled as “cal-arm” and “cal-OMe”.  
2 Determined from HSQC.
Figure S1. $^1$H NMR spectrum (400 MHz, 298 K, acetone-$d_6$) of calixarene 4. Residual solvent signals and water are respectively labelled “S” and “W”.

Figure S2. $^{13}$C NMR spectrum (100 MHz, 298 K, acetone-$d_6$) of calixarene 4. Solvent signals are labelled “S”.
Figure S3. $^{19}$F NMR spectrum (376.4 MHz, 298 K, acetone-d$_6$) of calixarene 4.

Figure S4. COSY spectrum (400 MHz, 298 K, acetone-d$_6$) of calixarene 4.
Figure S5. HSQC spectrum (\(^1\text{H} \) at 400 MHz, 298 K, acetone-d\(_6\)) of calixarene 4.

Figure S6. HMBC spectrum (\(^1\text{H} \) at 400 MHz, 298 K, acetone-d\(_6\)) of calixarene 4.
2. Binding Studies

2.1. Experimental procedure

Binding constants were measured by titrating \( \text{Bu}_4\text{N}^+\text{Cl}^- \) into solutions of the different calixarenes in DMSO-\( d_6 \)/0.5% H\(_2\)O at 298 K. Solutions of receptors 1-4 (1 mM, 1.5 mL) were prepared in DMSO-\( d_6 \)/0.5% H\(_2\)O (MilliQ) and stock solutions of 0.5 M \( \text{Bu}_4\text{N}^+\text{Cl}^- \) were prepared by dissolving the salt (dried under high vacuum) into the different receptor solutions. 500 µL of the solutions of pure receptors were transferred into NMR tubes. Initial spectra were recorded on a Varian VNMR S 600 (13.2 T) equipped with a triple probe. Aliquots of the guest solutions were then added to the NMR tube and \(^1\)H NMR spectra were recorded after each addition of guest (see Figures S7, S9, S11 and S13).

The shifts of the two NH signals were followed and the data were fitted to both 1:1 and 1:2 (host:guest) binding models using the Matlab fitting program developed in Thordarson group and the different binding stoichiometries were compared by looking at residual distribution analysis.\(^{[1,2]}\) By analyzing the residuals of the different binding models, we chose to provide the binding constants obtained with a 1:1 binding model in the main text as they provide the best fit for calixarenes 1 and 3. However, for calixarenes 2 and 4, a sinusoidal distribution of residuals is observed for the 1:1 binding model, while a more random distribution is observed for the 1:2 binding models (Figures S10 and S12). This suggests that a second binding event is likely to occur with receptors 2 and 4 and that the binding constants obtained with a 1:1 binding model might be underestimated (see Table S1).

The incorporation of PrNH\(_3^+\) in the calixarene cavity of 4 has also been observed by \(^1\)H NMR spectroscopy by adding PrNH\(_3\)Cl to a solution of 4 (1 mM in CDCl\(_3\)), see Figure S15.

**Table S1.** Binding data for calix[6]arenes 1-4

<table>
<thead>
<tr>
<th>X</th>
<th>R</th>
<th>Calix[6]arene</th>
<th>( K_a (\text{Bu}_4\text{N}^+\text{Cl}^-) [\text{M}^{-1}] )(^a)</th>
<th>( K_a (\text{Bu}_4\text{N}^+\text{Cl}^-) [\text{M}^{-1}] )(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>H</td>
<td>1</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>O</td>
<td>CF(_3)</td>
<td>2</td>
<td>28</td>
<td>58</td>
</tr>
<tr>
<td>S</td>
<td>H</td>
<td>3</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>S</td>
<td>CF(_3)</td>
<td>4</td>
<td>38</td>
<td>120</td>
</tr>
</tbody>
</table>

\(^a\) Binding constants in DMSO-\( d_6 \)/H\(_2\)O (200/1). Obtained from \(^1\)H NMR titrations of the different calixarenes with \( \text{Bu}_4\text{N}^+\text{Cl}^- \) at 25 °C and fitting of the data to a 1:1 model.

\(^b\) Binding constants in DMSO-\( d_6 \)/H\(_2\)O (200/1). Obtained from \(^1\)H NMR titrations of the different calixarenes with \( \text{Bu}_4\text{N}^+\text{Cl}^- \) at 25 °C and fitting of the data to a 1:2 model.
2.2. $^1$H NMR titration of calixarene 1 with Bu$_4$N$^+$Cl$^-$ in DMSO-$d_6$/0.5% H$_2$O.

Figure S7. Partial $^1$H NMR spectra (600 MHz, 298 K, DMSO-$d_6$/0.5% H$_2$O) from the titration of calixarene 1 (1 mM) with Bu$_4$N$^+$Cl$^-$ in DMSO-$d_6$/0.5% H$_2$O at 298 K. The number of equivalents of Bu$_4$N$^+$Cl$^-$ relative to 1 is shown.

1:1 binding model

Figure S8. Observed (*) and calculated (-) binding curves for the titration of 1 (1 mM) with Bu$_4$N$^+$Cl$^-$ at 298 K in DMSO-$d_6$/0.5% H$_2$O. Both NH ($\delta = 8.64$ and $\delta = 6.38$) were used for fitting to a 1:1 binding model and find $K_a$,$_{1:1} = 10.4 \pm 0.7$ M$^{-1}$. Plots of the residuals of the fittings are provided.
2.3. $^1$H NMR titration of calixarene 2 with Bu$_4$N$^+$Cl$^-$ in DMSO-d$_6$/0.5% H$_2$O.

**Figure S9.** Partial $^1$H NMR spectra (600 MHz, 298 K, DMSO-d$_6$/0.5% H$_2$O) from the titration of calixarene 2 (1 mM) with Bu$_4$N$^+$Cl$^-$ in DMSO-d$_6$/0.5% H$_2$O at 298 K. The number of equivalents of Bu$_4$N$^+$Cl$^-$ relative to 2 is shown.

**Figure S10.** Observed (*) and calculated (-) binding curves for the titration of 2 (1 mM) with Bu$_4$N$^+$Cl$^-$ at 298 K in DMSO-d$_6$/0.5% H$_2$O. Both NH ($\delta = 9.41$ and $\delta = 6.7$) were used for fitting to a i) 1:1 binding model and find $K_a, 1:1 = 27.9 \pm 2.8$ M$^{-1}$, and ii) 1:2 binding model and find $K_a, 1:1 = 57.8 \pm 9.5$ M$^{-1}$ and $K_a, 1:2 = 2.1 \pm 1.9$ M$^{-1}$. Plots of the residuals of the fittings are provided below their respective binding curves.
2.4. $^1$H NMR titration of calixarene 3 with Bu$_4$N$^+\text{Cl}^-$ in DMSO-d$_6$/0.5% H$_2$O.

**Figure S11.** Partial $^1$H NMR spectra (600 MHz, 298 K, DMSO-d$_6$/0.5% H$_2$O) from the titration of calixarene 3 (1 mM) with Bu$_4$N$^+\text{Cl}^-$ in DMSO-d$_6$/0.5% H$_2$O at 298 K. The number of equivalents of Bu$_4$N$^+\text{Cl}^-$ relative to 3 is shown.

1:1 binding model

**Figure S12.** Observed (*) and calculated (-) binding curves for the titration of 3 (1 mM) with Bu$_4$N$^+\text{Cl}^-$ at 298 K in DMSO-d$_6$/0.5% H$_2$O. Both NH ($\delta = 9.7$ and $\delta = 7.8$) were used for fitting to a 1:1 binding model and find $K_a, 1:1 = 10.75 \pm 0.7$ M$^{-1}$. Plots of the residuals of the fittings are provided.
2.5. H NMR titration of calixarene 4 with Bu₄N⁺Cl⁻ in DMSO-d₆/0.5% H₂O.

**Figure S13.** Partial ¹H NMR spectra (600 MHz, 298 K, DMSO-d₆/0.5% H₂O) from the titration of calixarene 4 (1 mM) with Bu₄N⁺Cl⁻ in DMSO-d₆/0.5% H₂O at 298 K. The number of equivalents of Bu₄N⁺Cl⁻ relative to 4 is shown.

**Figure S14.** Observed (*) and calculated (-) binding curves for the titration of 4 (1 mM) with Bu₄N⁺Cl⁻ at 298K in DMSO-d₆/0.5% H₂O. Both NH (δ = 10.25 and δ = 8.35) were used for fitting to a) 1:1 binding model and find $K_a, 1:1 = 38.5 \pm 5.0$ M⁻¹, and ii) 1:2 binding model and find $K_a, 1:1 = 119.9 \pm 21.5$ M⁻¹ and $K_a, 1:2 = 4.6 \pm 2.0$ M⁻¹. Plots of the residuals of the fittings are provided below their respective binding curves.
2.6. $^1$H NMR of calixarene 4 with PrNH$_3$Cl and tBuNH$_3$Cl in CDCl$_3$.

In agreement with results reported for calixarenes 1-3, the incorporation of PrNH$_3^+$ in the calixarene cavity is also observed for calixarene 4. Figure S15 indeed show high field signals ($\delta_{CH_3} = -1.91$ ppm and $\delta_{CH_2CH_2} = -1.19$ ppm) of the included propylammonium ion upon complexation.

Figure S15. $^1$H NMR spectra (600 MHz, 298 K, CDCl$_3$) of a) calixarene 4 (1 mM) in CDCl$_3$ and b) calixarene 4 (1 mM) in CDCl$_3$ with ca. 4 equivalent of PrNH$_3$Cl. ▼ = PrNH$_3^+$; solvents and water are respectively labelled “S” and “W”.

Figure S16. $^1$H NMR spectra (400 MHz, 298 K, CDCl$_3$) of a) calixarene 4 (1 mM) in CDCl$_3$ and b) calixarene 4 (1 mM) in CDCl$_3$ with ca. 4 equivalent of tBuNH$_3$Cl. ▼ = tBuNH$_3^+$; solvents and water are respectively labelled “S” and “W”. The variation observed between the two spectra is probably due to an interaction with Cl$^-$. However, no signal is observed at high fields (<0.5 ppm) which could correspond to complexed tBuNH$_3^+$. 
3. Transport Studies in Vesicles by Fluorescence Assays

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1‘-rac-glycerol) (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and cholesterol were purchased from Sigma Aldrich and 2-dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids. Chloroform was deacidified prior to use by passing it through a column containing basic alumina. Stock solutions (ca. 20 mM) of lipids were prepared in chloroform and stored at -20 °C. The water used for the aqueous solutions was purified with a Milli-Q purification system (Millipore).

3.1. Lucigenin assay – experimental procedure

Phospholipid and cholesterol solutions were combined with solutions of transporters in deacidified chloroform in a 5 mL round-bottomed flask. Volumes were calculated from the lipid concentrations to obtain a final concentration of 0.4 mM in lipids (phospholipid + cholesterol), with a 7:3 POPC to cholesterol ratio for POPC experiments, and a 1:1000 transporter to lipid ratio. The solvents from the mixture were evaporated under a flow of dry air and the resulting lipid film was dried in vacuum for at least 1 h. The lipid film was then hydrated with 500 µL of an aqueous solution of 10,10'-dimethyl-9,9'-biacridinium nitrate (lucigenin, 0.8 mM) and NaNO₃ (225 mM) or Na₂SO₄ (112.5 mM), sonicated for ca. 30 s and stirred for at least 1 h at room temperature for POPC experiments and 45 °C for DPPC experiments. The heterogeneous multilamellar vesicles were broken down into unilamellar vesicles by 10 freeze-thawing cycles, diluted to 1 mL with the appropriate solution (NaNO₃ or Na₂SO₄), and extruded 29 times through polycarbonate membranes (200 nm pore size) at room temperature for POPC/cholesterol vesicles or at 60 °C for DPPC vesicles to give homogeneous large unilamellar vesicles. The external lucigenin was removed by passing the vesicle solution through size exclusion columns (Sephadex G-25) eluted with its respective salt solution. The resulting vesicles were further diluted with the same salt solution to obtain a final concentration of 0.4 mM in lipids (calculated from the initial concentrations in lipids).

Fluorescence measurements were performed on 3 mL of the final vesicle solution in a quartz cell with a stir bar, and the fluorescence intensity of lucigenin (excitation at 430 nm and emission at 505 nm) was recorded over time. The temperature of the cuvette holder was controlled with a water bath at 25 °C for POPC vesicles, and 25 °C and 50 °C for DPPC vesicles using a SLM 8000 fluorescence spectrometer, or 25°C and 45 °C using a Horiba Fluoromax 4 spectrometer. The temperatures provided on the graphs are the actual temperatures of the solutions, measured with a thermocouple. 75 µL of aqueous NaCl or PrNH₃Cl (1 M, in the same salt solution as the liposomes, to give an external chloride concentration of 25 mM) was added ca. 30 s after starting the experiment and the fluorescence was recorded for an additional 11 minutes before lysing of the vesicles with 50 µL of Triton X-100 (5 wt.%) in water.

Each experiment was repeated at least 3 times and the fluorescence data were averaged after removing the initial drop (due to the quenching of remaining external fluorophore) and normalizing each fluorescence value (F) to the initial value (F₀). Traces of 500 seconds of transport are plotted.
3.2. Quantification of transport rates

As the inverse of the normalized fluorescence trace \( \frac{F_0}{F} \) is proportional to the chloride concentration inside vesicles,[4] fitting \( F_0/F \) to a single exponential (1) decay and double exponential decay (2) can provide the half-life and the initial rate, respectively.

\[
\frac{F_0}{F} = y - a e^{-bt} \quad \Leftrightarrow \quad t_\frac{1}{2} = \frac{\ln 2}{b} \quad \text{(1)}
\]

\[
\frac{F_0}{F} = y - a e^{-bt} - c e^{-dt} \quad \Leftrightarrow \quad I_0 = \frac{d}{dt} \left( \frac{F_0}{F} \right) \bigg|_{t=0} = a \cdot b + c \cdot d \quad \text{(2)}
\]

The values and errors obtained for the different receptors are provided in Table S2.
3.3. Transport by 2 and 4 in DPPC vesicles

Figure S17. Normalized fluorescence traces for chloride transport by calixarene 2 into 200 nm DPPC LUVs (carrier:lipid = 1:1000) dispersed in 225 mM NaNO₃ upon addition of and NaCl as chloride source.

Figure S18. Normalized fluorescence traces for chloride transport by calixarene 4 into 200 nm DPPC LUVs (carrier:lipid = 1:1000) dispersed in 225 mM NaNO₃ upon addition of and NaCl as chloride source.

The absence of transport observed at 25 °C points towards a carrier mechanism, because transport would be expected at both temperatures if calixarenes 2 and 4 were forming channels. Regen and co-workers suggested that the lower activity observed at 25 °C could be caused by a lower transporter concentration in the bilayer as the transporters could be expelled from the bilayer when it is in the gel phase, and would thus not be indicative of a mobile carrier mechanism.⁵ In the case of calixarenes 2 and 4, this theory is not likely as these molecules are lipophilic enough (clogP > 20) not to be expelled into the aqueous phase. Furthermore, the protocol to prepare the vesicles involve a size exclusion column at room temperature. If the receptors were expelled from the bilayer, part of them would be separated from the liposomes in the column and the transport performances would significantly decrease.
3.4. Transport by 4 at different transporter concentrations

To further support the carrier mechanism, as mentioned in the main text, chloride/nitrate exchange experiments were performed with calixarene 4 at different calixarene:lipid ratios (Figure S19a). A roughly linear trend was observed between the carrier loading and the initial rates (Figure S19b). Since the calixarenes (< 2 nm) are too small to span the lipid bilayer (ca. 4 nm), multiple calixarenes would be required to form a channel, which is in contrast with the observed linear dependence on the concentration of 4 as a non-linear relationship would be expected if the calixarene were forming channels or active aggregates.\(^6\)

![Figure S19](image1.png)

**Figure S19.** a) Normalized fluorescence traces for chloride transport by calixarene 4 into 200 nm POPC/cholesterol LUVs (carrier:lipid = 1:250, 1:1000 and 1:2500) dispersed in 225 mM NaNO\(_3\) upon addition of NaCl as chloride source. b) Initial rates as a function of carrier:lipid ratio.

3.5. Additional transport data in Na\(_2\)SO\(_4\)

![Figure S20](image2.png)

**Figure S20.** Normalized fluorescence traces for chloride transport by calixarene 2 into 200 nm POPC/cholesterol (7:3) LUVs (carrier:lipid = 1:1000) dispersed in 112.5 mM Na\(_2\)SO\(_4\) upon addition of different chloride sources.
Figure S21. Normalized fluorescence traces for chloride transport by calixarene 2 into 200 nm POPC/cholesterol (7:3) LUVs (carrier:lipid = 1:25k) dispersed in 112.5 mM Na₂SO₄ upon addition of PrNH₃Cl (blue line) or dispersed in 225 mM NaNO₃ upon addition of NaCl (orange line).

Figure S22. Normalized fluorescence traces for chloride transport by calixarene 4 into 200 nm POPC/cholesterol (7:3) LUVs (carrier:lipid = 1:25k) dispersed in 112.5 mM Na₂SO₄ upon addition of PrNH₃Cl (blue line) and tBuNH₃Cl (green line) or dispersed in 225 mM NaNO₃ upon addition of NaCl (orange line).
Figure S23. Normalized fluorescence traces for chloride transport by tren-based tripodal receptor 5 into 200 nm POPC/cholesterol (7:3) LUVs (carrier:lipid = 1:25k) dispersed in 112.5 mM Na$_2$SO$_4$ upon addition of PrNH$_3$Cl (blue line) or dispersed in 225 mM NaNO$_3$ upon addition of NaCl (orange line).

Figure S24. Normalized fluorescence traces for chloride transport by tren-based tripodal receptor 6 into 200 nm POPC/cholesterol (7:3) LUVs (carrier:lipid = 1:25k) dispersed in 112.5 mM Na$_2$SO$_4$ upon addition of PrNH$_3$Cl (blue line) or dispersed in 225 mM NaNO$_3$ upon addition of NaCl (orange line).
Figure S25. Normalized fluorescence traces for chloride transport by triethylbenzene-based tripodal receptor 7 into 200 nm POPC/cholesterol (7:3) LUVs (carrier:lipid = 1:25k) dispersed in 112.5 mM Na$_2$SO$_4$ upon addition of PrNH$_3$Cl (blue line) and tBuNH$_3$Cl (green line) or dispersed in 225 mM NaNO$_3$ upon addition of NaCl (orange line).

Table S2. Transport data for transporters 2 and 4-7.

<table>
<thead>
<tr>
<th>Receptor (carrier:lipid ratio)</th>
<th>$t_{1/2}$ (s)$^{[a]}$</th>
<th>Initial rate (s$^{-1}$) $^{[b]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cl/NO$_3^-$ antiport</td>
<td>PrNH$_3$Cl</td>
</tr>
<tr>
<td>2 (1:1000)</td>
<td>162 ± 5</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>4 (1:1000)</td>
<td>47 ± 1</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>5 (1:250)</td>
<td>159 ± 13</td>
<td>139 ± 6</td>
</tr>
<tr>
<td>6 (1:25k)</td>
<td>42 ± 5</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>7 (1:25k)</td>
<td>108 ± 2</td>
<td>86 ± 3</td>
</tr>
</tbody>
</table>

$^{[a]}$ Half-lives are calculated from fitting the inverse of normalized fluorescence curves to single exponential decay equations.

$^{[b]}$ Initial rates are calculated from fitting the inverse of normalized fluorescence curves to double exponential decay equations. The errors provided are the standard deviation obtained by fitting separately three different traces of the same experiment.
3.6. Experiments with anionic lipids

![Normalized fluorescence traces for chloride transport by calixarene 4 into 200 nm POPC/POPG/cholesterol (35:35:30) LUVs (carrier:lipid = 1:1000) dispersed in a) 225 mM NaNO\textsubscript{3} and b) 112.5 mM Na\textsubscript{2}SO\textsubscript{4}, upon addition of different chloride sources.]

Figure S26. Normalized fluorescence traces for chloride transport by calixarene 4 into 200 nm POPC/POPS/cholesterol (35:35:30) LUVs (carrier:lipid = 1:1000) dispersed in a) 225 mM NaNO\textsubscript{3} and b) 112.5 mM Na\textsubscript{2}SO\textsubscript{4}, upon addition of different chloride sources.

The lucigenin assay with calixarene 4 in NaNO\textsubscript{3} and in Na\textsubscript{2}SO\textsubscript{4} was performed with LUVs containing POPG or POPC to assess the influence of the presence of anionic lipids on the transport of chloride and propylammonium chloride. As could be expected for anion transport, the rate Cl\textsuperscript{-}/NO\textsubscript{3}\textsuperscript{-} antiport decreased in the presence of 35% anionic lipids in the lipid bilayer (see Figure S26a and S27a). These results suggest that the complexation of chloride at the lipid-water interphase, necessary for transport, is impeded by the electrostatic repulsion between chloride and the negatively charged membrane. With the same lipid composition in Na\textsubscript{2}SO\textsubscript{4}, we observe a similar decrease in rates for the cotransport of PrNH\textsubscript{3}Cl (see Figure S26b and S27b). A smaller effect of the anionic lipids on the cotransport of a neutral ion pair could eventually have been expected. The observed behavior can be explained by the complexation process of the ion pair which occurs \textit{via} the complexation of the anion prior to the complexation of the primary ammonium cation. The complexation of organic ion pairs by calix[6]arenes has been described in organic solvents as a two-step binding process, with the anion complexed first and acting as an allosteric activator for the complexation of the primary ammonium cation.\textsuperscript{[3,7]} Considering this latter mechanism of complexation, it is conceivable that the cotransport of PrNH\textsubscript{3}Cl is affected by the presence of anionic lipids in a similar way as the Cl\textsuperscript{-}/NO\textsubscript{3}\textsuperscript{-} antiport.
3.7. HPTS assay – experimental procedure

The preparation of vesicles for the HPTS assay is similar to the lucigenin assay, except that the lipid films were hydrated with 500 µL of an aqueous solution of 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium (HPTS, 1 mM) and Na₂SO₄ (112.5 mM, pH of the solution adjusted to 7.7 with aliquots of 0.5 M Na₂HPO₄). The final pH of 7.5 for the vesicles suspension was measured with a pH meter.

Samples for fluorescence measurements consisted of 3 mL of the final vesicle solution in a quartz cell with a stir bar, and the excitation spectra of HPTS (λ_em = 510 nm) were recorded every 30 seconds at 25 °C using a SLM 8000 fluorescence spectrometer. 75 µL of aqueous PrNH₃Cl (1 M in 112.5 mM Na₂SO₄, to give an external chloride concentration of 25 mM) was added ca. 30 s after starting the experiment and the fluorescence spectra were recorded for an additional 11 minutes. Each experiment was repeated 3 times and ratio of the fluorescence intensities between λ_exc = 460 nm and λ_exc = 403 nm was averaged and plotted over time. Traces up to 500 seconds after addition of chloride are plotted. Due to technical limitations, excitation spectra can be recorded only every 30 seconds.
3.8. HPTS assay with calixarene 4

**Figure S28.** Ratio of HPTS fluorescence intensities of excitation ($\lambda_{em} = 510$ nm) for chloride transport by calixarene 4 into 200 nm POPC/cholesterol (7:3) LUVs (carrier:lipid = 1:1000) dispersed in 112.5 mM Na$_2$SO$_4$ upon addition of PrNH$_3$Cl.

Transport of PrNH$_3$Cl in the presence Na$_2$SO$_4$ and with HPTS inside the liposome cavity was studied by addition of 25 mM PrNH$_3$Cl to the vesicles. The small drop in ratio of intensities upon chloride transport using PrNH$_3$Cl indicates a drop in pH inside the vesicles, which is due to transport of PrNH$_3^+$ or H$^+$ inside the vesicles, or OH$^-$ out of the vesicles. By comparing the observed drop (<1, **Figure S28**) to what would be expected with net transport of 25 mM H$^+$ into or OH$^-$ out of the liposomes (>2) (**Figure S29**), we can conclude that the transport of chloride observed with the lucigenin assay in sulfate with PrNH$_3$Cl as chloride salt does not correspond to either H$^+$/Cl$^-$ co-transport nor Cl$^-$/OH$^-$ antiport as main mechanisms. Furthermore, a large pH gradient across the membrane would be generated if H$^+$/Cl$^-$ co-transport or Cl$^-$/OH$^-$ antiport was the main mechanism, which would not be favorable.

**Figure S29.** Titration of HPTS (1 µM in 112.5 mM Na$_2$SO$_4$) with HCl up to 25 mM. pH is measured with a pH-meter and the ratio of excitation intensities is calculated from each excitation spectrum and plotted.
4. Transport Studies in Vesicles by NMR

$^1$H and $^{35}$Cl NMR have been used to characterize the different species that are transported in presence of preincorporated receptor.

4.1. General experimental information

$^1$H NMR spectra were recorded at 400 MHz on a Varian VNMRS 400 (9.4 T) equipped with an ATB probe. $^1$H NMR chemical shifts were referenced using solvent signals. The spectra were recorded using a 90° pulse sequence. The sodium salt of thulium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonate) (TmDOTP$^5$-) was used as shift reagent for $^1$H NMR experiments. The synthesis of the ligand, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra(methylene phosphonic acid) (H$_8$DOTP), was adapted from Lázár et al.,[8] using 1,4,7,10-tetraazacyclododecane (commercially obtained from Fluorochem) as starting material. To obtain the paramagnetic complex, H$_8$DOTP was dissolved in 0.5 M NaHCO$_3$ in D$_2$O and further mixed with an equimolar solution of Tm$_2$SO$_4$ in D$_2$O, yielding a 40 mM TmDOTP$^{5-}$ solution in D$_2$O with 0.25 M NaHCO$_3$.

$^{35}$Cl spectra were acquired at 39.2 MHz on a Varian VNMRS 400 (9.4 T). $^{35}$Cl NMR chemical shifts were referenced with 1 M NaCl ($\delta$ 0.0 ppm). The spectra were recorded using a 90° pulse sequence with an acquisition time of 100 ms and a relaxation delay of 100 ms. 5000 scans were generally acquired to obtain reliable data and a sufficient S/N ratio. Co$^{3+}$ was used as shift and relaxation agent for $^{35}$Cl NMR experiments using a 0.5 M CoSO$_4$ solution in D$_2$O.

4.2. Preparation of liposomes for NMR

Phospholipid and cholesterol solutions were combined with solutions of transporters in methanol or deacidified chloroform in a 5 mL round-bottomed flask. Volumes were calculated from the concentrations to obtain a final concentration of 10 mM in lipids (POPC + cholesterol), with a 7:3 POPC to cholesterol ratio and a 1:100 transporter to lipid ratio. The solvents from the mixture were evaporated under a flow of dry air and the resulting lipid film was dried under vacuum for at least 1 h. The lipid film was then hydrated with 500 µL of 50 mM Na$_2$SO$_4$ in D$_2$O, vortexed until a homogeneous solution was obtained, and stirred for at least 1 h. The resulting multilamellar vesicles were frozen and thawed 10 times, diluted to 1 mL with the appropriate solution (50 mM Na$_2$SO$_4$), and extruded 41 times through polycarbonate membranes (400 nm) at room temperature. A final dilution was performed to reach the desired concentration (10 mM in lipids) and volume for each experiment. Each NMR sample consisted of 500 µL of vesicle dispersion.
4.3. Co-transport of organic ion pairs

$^1$H and $^{35}$Cl NMR have been used to confirm the presence of propylammonium chloride inside the vesicles after transport in presence of sulfate. $^1$H NMR spectra of the full signals are provided in Figure S30 and a “zoom” of the relevant area is provided in Figure 3 in the main text. $^{35}$Cl NMR spectra are shown in Figure S24.

**Figure S30.** $^1$H NMR spectra (400 MHz, 298 K, D$_2$O) of POPC/cholesterol liposomes with preincorporated calixarene 4. a) 10 mM POPC/cholesterol vesicles extruded through 400 nm membrane pores in 50 mM Na$_2$SO$_4$ in D$_2$O. b) after addition of 100 mM propylammonium chloride in D$_2$O. c) spectrum recorded 1h later and after addition of 2 mM TmDOTP$^5$-

**Figure S31.** $^{35}$Cl NMR spectra (39.2 MHz, 298 K, D$_2$O) 10 mM POPC/cholesterol vesicles extruded through 400 nm membrane pores with preincorporated calixarene 4 (carrier:lipid = 1:100, blue lines) or no carrier (orange lines) in 50 mM Na$_2$SO$_4$ in D$_2$O and after addition of 100 mM PrNH$_3$Cl (a), and 1 h later after addition of 20 mM Co$^{2+}$ (b).
5. U-tube experiments

5.1. Experimental information

U-tube experiments were performed using the setup illustrated in Figure S32. The donor phase consisted of a 5 mL aqueous solution of 1 M NaCl or PrNH₃Cl and the receiving phase of 5 mL pure MilliQ water. The organic phase consisted of 4 mL of calixarene 4 (1 mM) in deacidified CHCl₃ (no carrier in blank experiments). Transport was started by stirring the organic phase (400 rpm) at room temperature and the receiving phase was analyzed after 72 h.

The concentration of chloride in the receiving phase was determined by fluorescence spectroscopy. Fluorescence samples consisted of 500 µL of the receiving phase diluted in 1 mL of an aqueous solution of lucigenin (1.5 µM). The fluorescence intensity of lucigenin was then measured ($\lambda_{\text{exc}} = 430 \text{ nm}, \lambda_{\text{em,max}} = 505 \text{ nm}$) and compared to control samples (500 µL H₂O + 1 mL lucigenin 1.5 µM) for which the intensity of the maximum is normalized to 1.

For the transport of PrNH₃Cl, 1 mL of the receiving phase was evaporated and re-dissolved in 600 µL D₂O for NMR characterization.

![Figure S32. U-tube experimental setup](image)

5.2. Results

![Figure S33. Fluorescence spectra of lucigenin combined with samples from the U-tube experiments with a) NaCl and b) PrNH₃Cl. The spectra are normalized to control samples with H₂O.](image)
Grauwels et al. Repositioning Chloride Transmembrane Transporters: Transport of Organic Ion Pairs

**Figure S34.** Stern-Volmer plot for the quenching of lucigenin in H$_2$O. The data were obtained from a titration of 1 µM of lucigenin in water with both NaCl or PrNH$_3$Cl, which gave the same relationship. The data plotted are an average of two titrations with NaCl.

**Table S3.** Concentration of chloride in the receiving phase calculated from the Stern-Volmer equation obtained from the data in Figure S34. Data are rounded to the nearest unit.

<table>
<thead>
<tr>
<th>Time</th>
<th>No transporter</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl (mM)</td>
<td>PrNH$_3$Cl (mM)</td>
</tr>
<tr>
<td>0 h</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>72 h</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure S35.** $^1$H NMR spectra (400 MHz, 298 K, D$_2$O) of 1 mL of the receiving phase from U-tube experiments with PrNH$_3$Cl evaporated and re-dissolved in 600 µL D$_2$O. a) Experiment without carrier in the bulk liquid membrane and b) with calixarene 4. 1 mM TMSP-d$_4$ (3-(trimethylsilyl)-2,2,3,3-tetadeuteropropionic acid) was added as internal reference. Integration of the reference showed that the concentration of PrNH$_3^+$ in the U-tube experiment after 72 h is equal to 15 mM (25 mM in the NMR tube).

Results shown in Figures S33-34 and summarized in Table S3 show that calixarene 4 is indeed able to carry propylammonium chloride through a bulk liquid membrane. No transport of chloride was observed when using NaCl as chloride source as (i) no anions are present in the receiving phase to allow an antiport mechanism and (ii) sodium cannot be cotransported with chloride by calixarene 4. The transport of neither chloride nor propylammonium was observed in all control experiments without carrier.
6. References