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Probing and Evaluating Transmembrane Chloride Ion Transport in the Double Walled Trifluorophenyl/Phthalimide Extended Calix[4]pyrrole Based Supramolecular Receptors

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I. Materials, methods and instrumentation

All the solvents and reagents were purchased from commercial sources (Sigma Aldrich, SRL, Spectrochem Pvt. Ltd., GLR Innovations, Alfa Aesar, TCI Chemicals Pvt. Ltd., *etc.* Acetone used was of HPLC grade, and pyrrole was freshly distilled prior to its usage. Egg yolk phosphatidylcholine (EYPC) as a chloroform solution (25 mg/mL), mini extruder, polycarbonate membrane (100 nm and 200 nm) were purchased from Avanti Polar Lipid. HEPES, lucigenin, HPTS, NaOH, Triton X-100, Sephadex G-50, valinomycin and all inorganic salts were obtained from Sigma Aldrich of molecular biology grade. Analytical thin layer chromatography (TLC) was performed on readymade aluminium plates coated with silica gel, using a suitable ratio of ethyl acetate and hexane. 100-200 mesh size silica gel was used for column chromatography involving a suitable ratio of ethyl acetate and hexane. ^{1}H-NMR spectra were recorded on a 400 MHz and 500 MHz Bruker spectrometer in $CDCl_3$. The "*'' depicts the residual solvent peak. Fluoromax-4 instrument was used for fluorescence experiments. The fluorescence data was processed using OriginPro 8.5. All buffer solutions were prepared in autoclaved water. Adjustment of pH in buffer solutions was made *via* pH meter procured from Hanna instruments. Gel chromatography using Sephadex G-50 was employed to remove extravesicular dye. Chloride ion efflux was quantified through Thermo Scientific Orion Chloride ion plus ISE linked to a Fisherbrand AB 250 pH/ISE meter in order to the chloride concentration in ppm.

II. Synthetic procedures

Synthetic procedure of DPM (2): To a stirred solution of 2,4,6-trifluoroacetophenone **1** (3.0 ml, 22.40 mmol, 1 equiv.) in freshly distilled pyrrole (18.65 ml, 268.78 mmol, 12 equiv.) at 0 ºC, TFA (1.71 ml, 22.40 mmol, 1 equiv.) was added dropwise under an inert nitrogen atmosphere. The reaction was allowed to come at room temperature (rt) and stirred for 24 h. After the completion of the reaction (TLC monitoring), the reaction mixture was neutralized by 1M NaOH and extracted with CH_2Cl_2. The organic contents were dried over Na_2SO_4, filtered, and concentrated on rotary evaporator. The crude oil was subjected to column chromatography (SiO_2, 10% ethyl acetate: hexane) to get pure **2** (1.4 g, 28% yield). **^{1}H-NMR (500 MHz, $CDCl_3$):** δ 8.01 (s, 2H), 6.68 – 6.67 (m, 2H), 6.62 – 6.59 (m, 2H), 6.17 (q, J = 3.0 Hz, 2H), 5.99-5.98 (m, 2H), 2.20 (s, 3H). **^{13}C-NMR (126 MHz, $CDCl_3$):** δ 162.64, 160.64, 136.29, 117.00, 108.44, 105.52,

101.75, 101.55, 101.48, 101.28, 41.56, 28.69, 28.64, 28.60. **HRMS (ESI, Q-TOF):** *m/z* calculated for $C_{16}H_{13}F_3N_2$ $[M+H]^+$ = 291.1103, found: 291.1100.

Synthetic procedure of double walled trifluorophenyl extended C4P (3a and 3b): $BF_3.OEt_2$ (0.46 ml, 3.29 mmol, 1.1 equiv) was added dropwise under an inert nitrogen atmosphere to a stirred solution of dipyrromethane **2** (1 g, 3.44 mmol, 1 equiv.) in an excess of dry acetone (60 ml) at 0 ºC. The reaction mixture was allowed to come at rt and stirred for 3 h at the same temperature. After the reaction gets completed, the reaction mixture was neutralized by 1M NaOH, combined with water and evaporated under vacuum until most of the acetone has been removed. In the next step, the organic phase was extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4, filtered, and concentrated to give a crude mixture, which was later subjected to column chromatography (SiO_2, 5-10% ethyl acetate: hexane) to provide the *cis*-trifluorophenyl extended C4P (**3a**) in trace amount and *trans*-trifluorophenyl extended C4P (**3b**) in 11% yield.

Compound 3a: ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.26 (s, 2H), 7.19 (s, 2H), 6.56 (t, *J* = 9.5 Hz, 4H), 5.92 (s, 6H), 5.73 (s, 2H), 2.06 (s, 6H), 1.50 (s, 12H). **^{13}C-NMR (126 MHz, $CDCl_3$)**: δ 161.72, 160.10, 138.76, 138.67, 138.24, 135.00, 104.94, 103.17, 103.03, 102.74, 101.18, 41.26, 35.32, 29.70, 28.60, 28.14. **HRMS (ESI, Q-TOF):** *m/z* calculated for $C_{38}H_{34}F_6N_4$ $[M+H]^+$ =661.2760, found: 661.2781.

Compound 3b: ^{1}H-NMR (400 MHz, $CDCl_3$): *δ* 7.28 (s, 4H), 6.58 (t, *J* = 10.4 Hz, 4H), 5.94 (t, *J* = 2.8 Hz, 4H), 5.81 (t, *J* = 3.2 Hz, 4H), 2.09 (s, 6H), 1.52 (s, 12H). **^{13}C-NMR (101 MHz, $CDCl_3$)**: *δ* 162.66, 160.32, 139.05, 135.34, 118.29, 104.95, 103.69, 101.74, 101.49, 101.39, 101.15, 42.02, 35.21, 29.45, 28.19, 28.13, 28.08. **HRMS (ESI, Q-TOF):** *m/z* calculated for $C_{38}H_{34}F_6N_4$ $[M+H]^+$ = 661.2760, found: 661.2762.

Synthetic procedure for *N*-acetonyl phthalimide (5): To a stirred solution of chloroacetone (108.08 mmol, 10 g,) in DMF (100 ml), potassium phthalimide (1.1 equiv., 22 g) was added in small portions with constant stirring, and the stirring was continued at rt for 24 h. After completion of the reaction, monitored through TLC, the crude reaction mixture was dropped into surplus cold water. This, in turn, led to the development of white needle crystals, which were collected through filtration and washed with an adequate amount of water until they appeared colorless. Afterward, these crystals were vacuum dried in order to afford pure **5** (17.60 g, 80% yield).

Synthetic procedure of the DPM (6): Under an inert nitrogen atmosphere, trifluoroacetic acid (43.31 mmol, 1 equiv., 3.31 ml) was dropwise added at 0 ºC to a stirred solution of **5** (43.31mmol, 8.8g**)** in freshly distilled pyrrole (12 equiv., 36.06 ml, 519.71 mmol). The reaction was later permitted to come at rt and left undisturbed at this temperature for 14 h. Upon the completion of the reaction monitored *via* TLC, quenching of the reaction mixture was carried out by trimethylamine (5 ml). Later the organic content was extracted by means of water and CH_2Cl_2. The organic layer was dried over Na_2SO_4, followed by filtration and concentration, yielded brownish crude oil which was exposed to column chromatography (SiO_2, 3:7 ethyl acetate: hexane) to afford the DPM **6** as a pale-yellow solid (36%, 4.98 g). **^{1}H-NMR (500 MHz, $CDCl_3$):** δ 8.17 (s, 2H), 7.79-7.77 (m, 2H), 7.69-7.67 (m, 2H), 6.69-6.67 (m, 2H), 6.12 -6.10 (m, 4H), 4.24 (s, 2H), 1.72 (s, 3H). The spectral data is in accordance with the literature.[1]

Synthetic procedure of double walled phthalimide extended C4P (7): Trifluoroacetic acid (0.718 ml, 9.393 mmol, 3 equiv.) was added dropwise under inert nitrogen atmosphere to a stirred solution of DPM **6** (1g, 3.131 mmol, 1 equiv) in excess dry acetone (50 ml) at 0 ºC. The reaction mixture was allowed to reach at rt, and stirred it for 3 h. After the reaction gets completed, the reaction mixture was neutralized by triethylamine and evaporated under a vacuum until most of the acetone has been removed. Later, the organic phase was extracted with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated to give a brown-colored crude residue, which was then subjected to column chromatography (SiO_2, hexane followed by 1:9 ethyl acetate: hexane) in order to get pure receptor **7** (315 mg, 14% yield). **^{1}H-NMR (400 MHz, $CDCl_3$):** δ 7.76-7.74 (m, 4H), 7.67-7.65 (m, 4H), 7.32 (s, 4H), 5.93-5.88 (m, 8H), 4.19 (s, 2H), 4.16 (s, 2H), 1.62-1.59 (m, 18H). **^{13}C-NMR (126 MHz, $CDCl_3$):** δ 168.96, 139.24, 133.82, 131.91, 123.18, 104.52, 101.28, 46.33, 41.75, 33.84, 29.24, 26.37. **HRMS (ESI, Q-TOF):** *m/z* calculated for $C_{44}H_{42}N_6O_4$ $[M+H]^+$ = 719.3344, found: 719.3339.

III. Copies of ^{1}H-NMR, ^{13}C-NMR and HRMS spectra

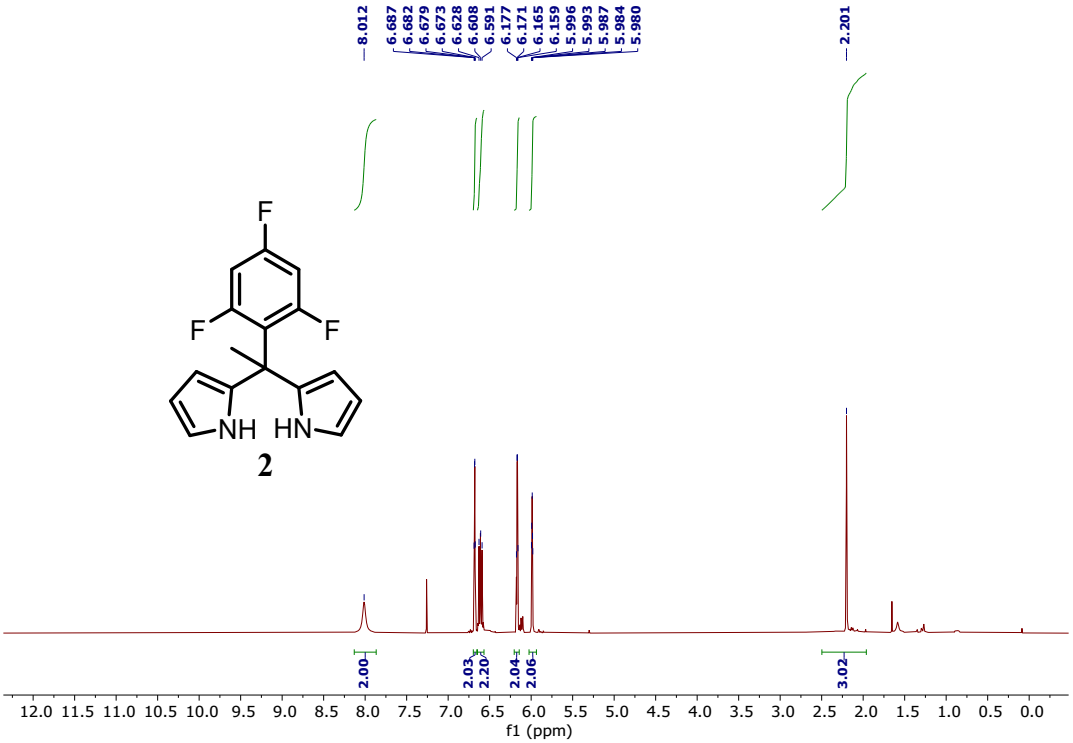

Figure S1. ^{1}H-NMR spectrum of trifluorophenyl functionalized dipyrromethane (**2**) recorded in $CDCl_3$.

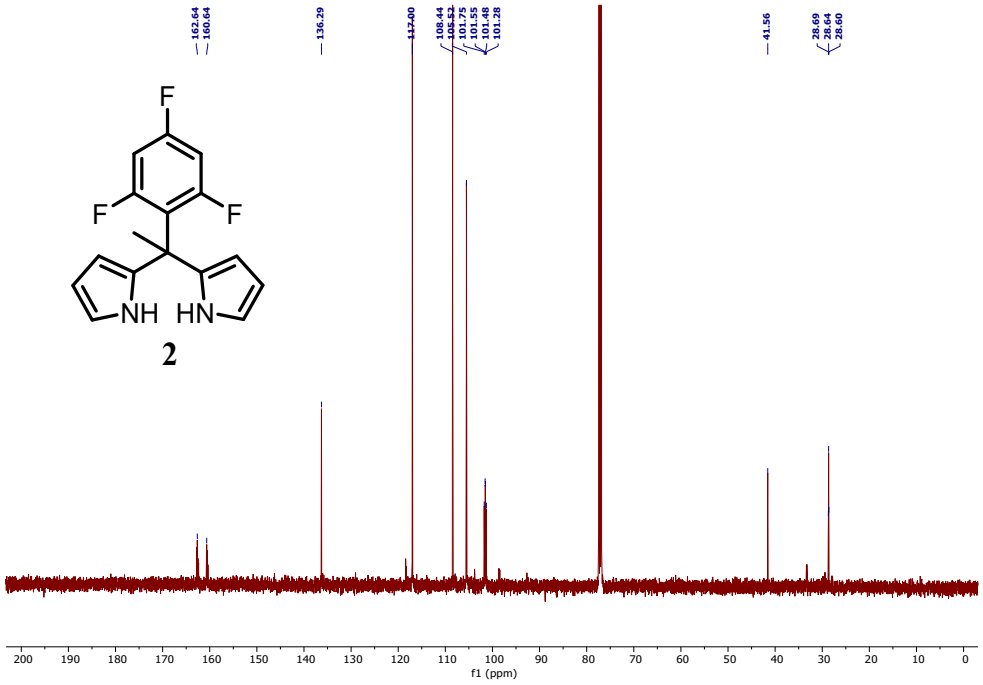

Figure S2. ^{13}C-NMR spectrum of trifluorophenyl functionalized dipyrromethane (**2**) recorded in $CDCl_3$.

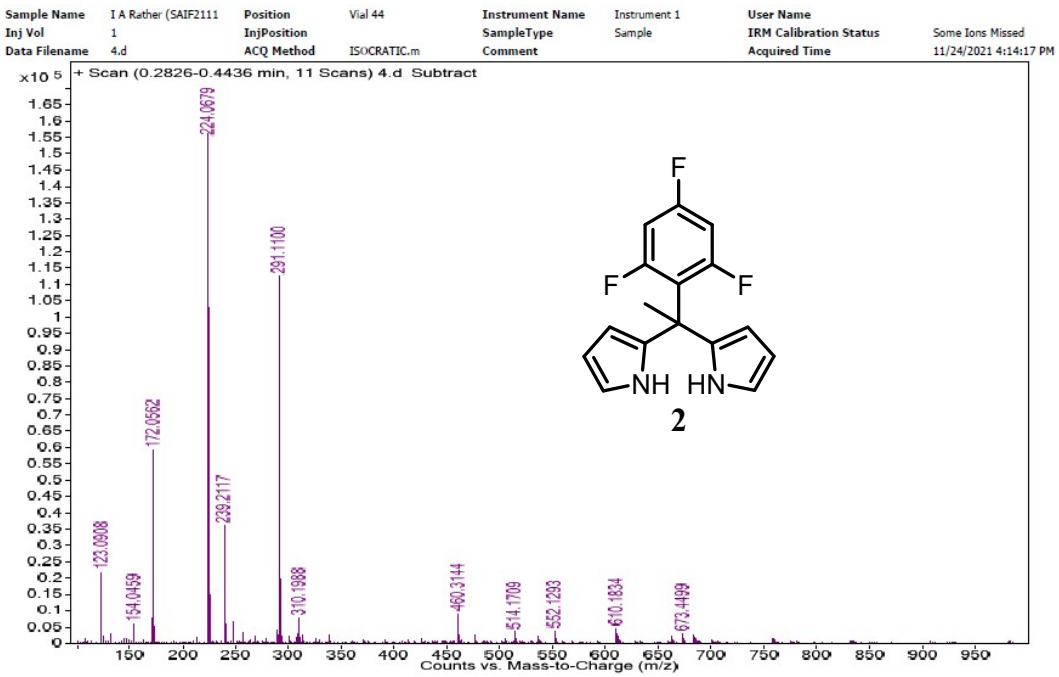

Figure S3. HRMS spectrum of trifluorophenyl functionalized dipyrromethane (**2**). (*m/z* calculated for $C_{16}H_{13}F_3N_2$ $[M+H]^+ = 291.1103$).

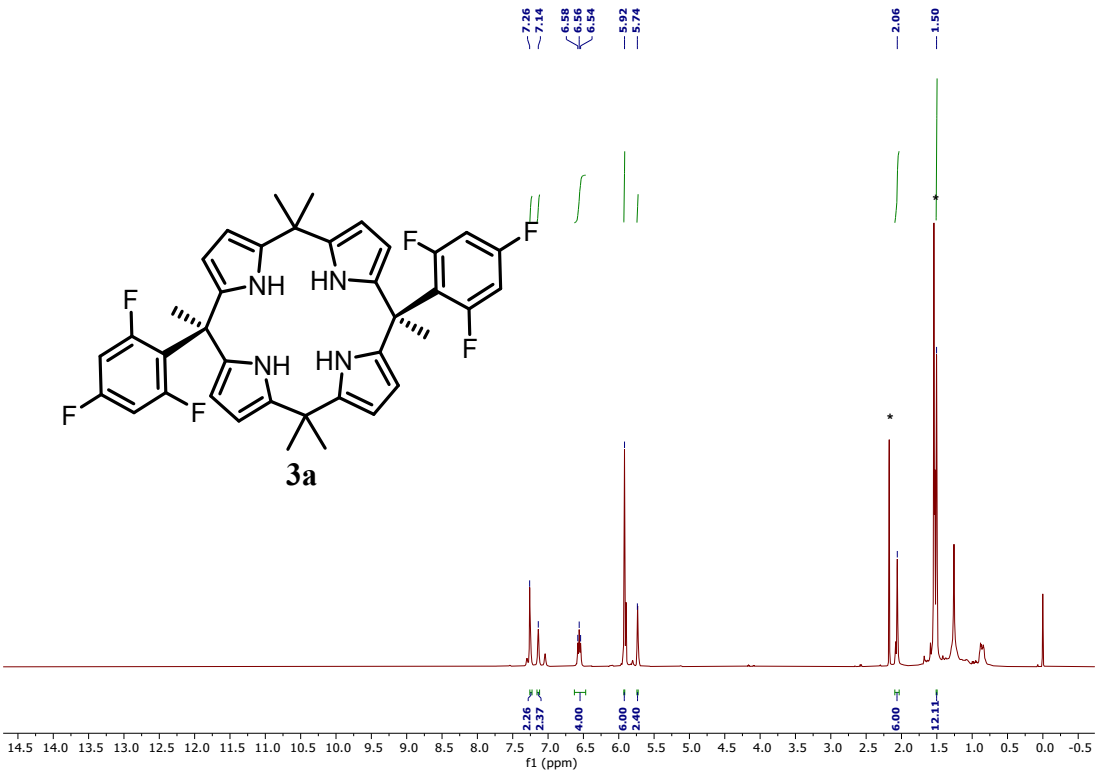

Figure S4. ^{1}H-NMR spectrum of *cis*-trifluorophenyl extended C4P (**3a**) recorded in $CDCl_3$. (* represents peaks of residual solvents water and acetone)

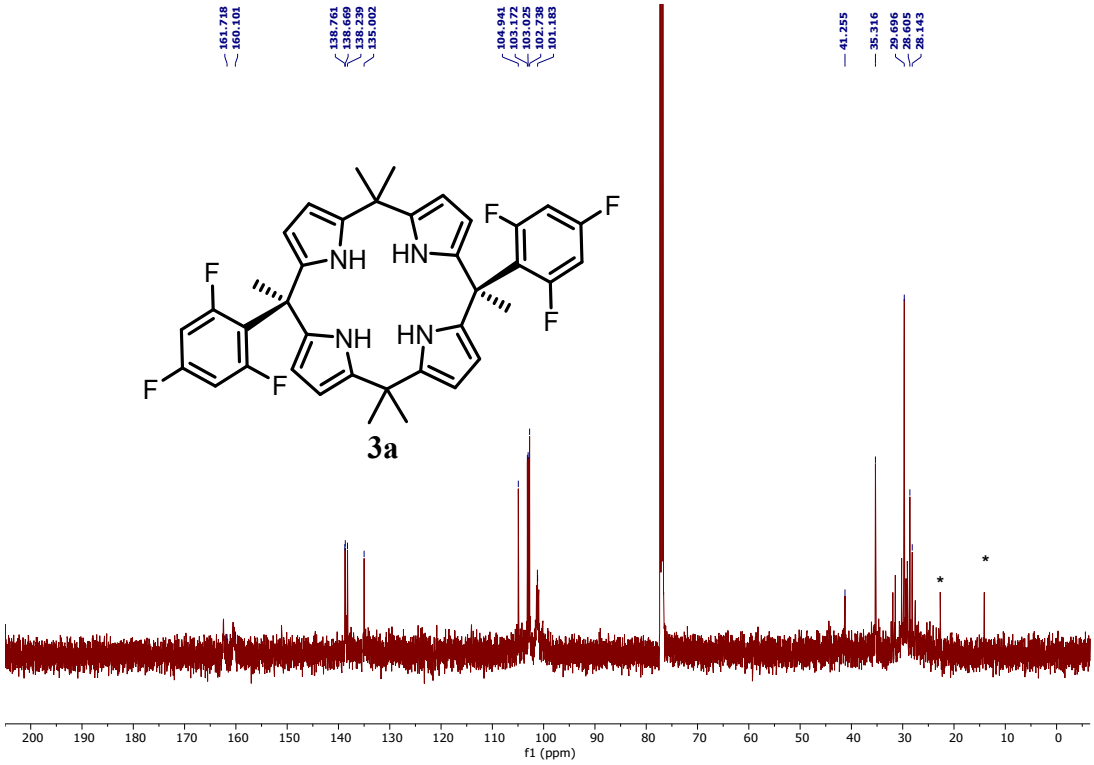

Figure S5. ^{13}C-NMR spectrum of *trans*-trifluorophenyl extended C4P (**3a**) recorded in $CDCl_3$. . (* represents peaks of residual solvent hexane)

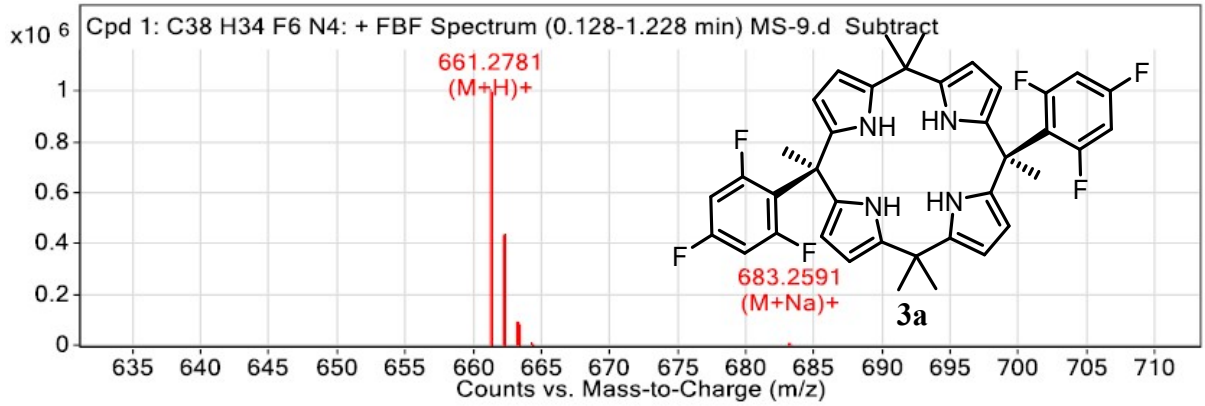

Figure S6. HRMS spectrum of *cis*-trifluorophenyl extended C4P (**3a**). (*m/z* calculated for $C_{38}H_{34}F_6N_4$ $[M+H]^+ = 661.2760$).

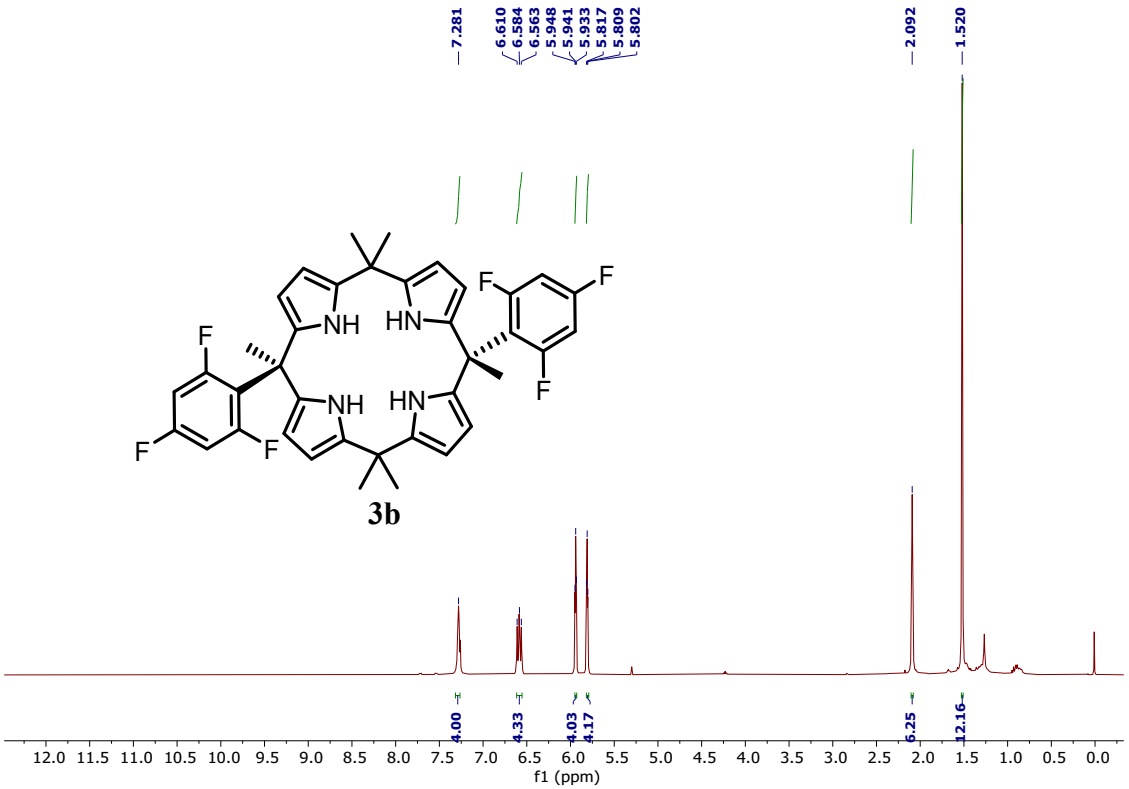

Figure S7. ^{1}H-NMR spectrum of *trans*-trifluorophenyl extended C4P (**3b**) recorded in $CDCl_3$.

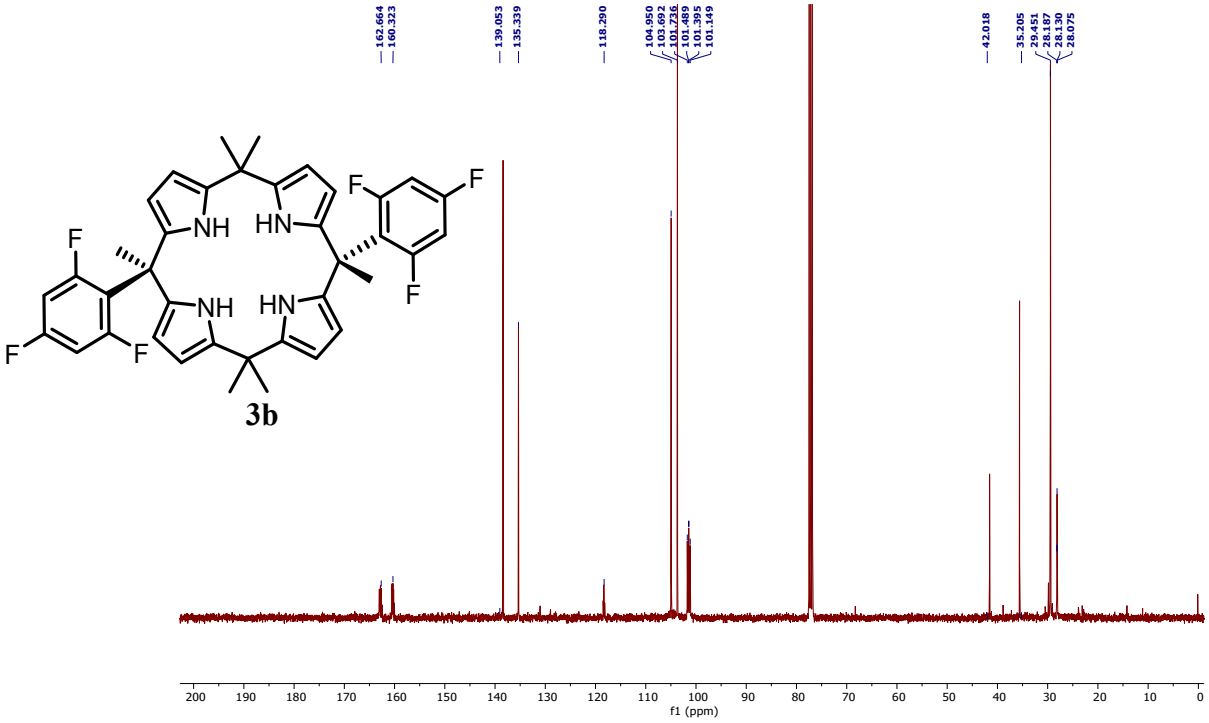

Figure S8. ^{13}C-NMR spectrum of *trans*-trifluorophenyl extended C4P (**3b**) recorded in $CDCl_3$.

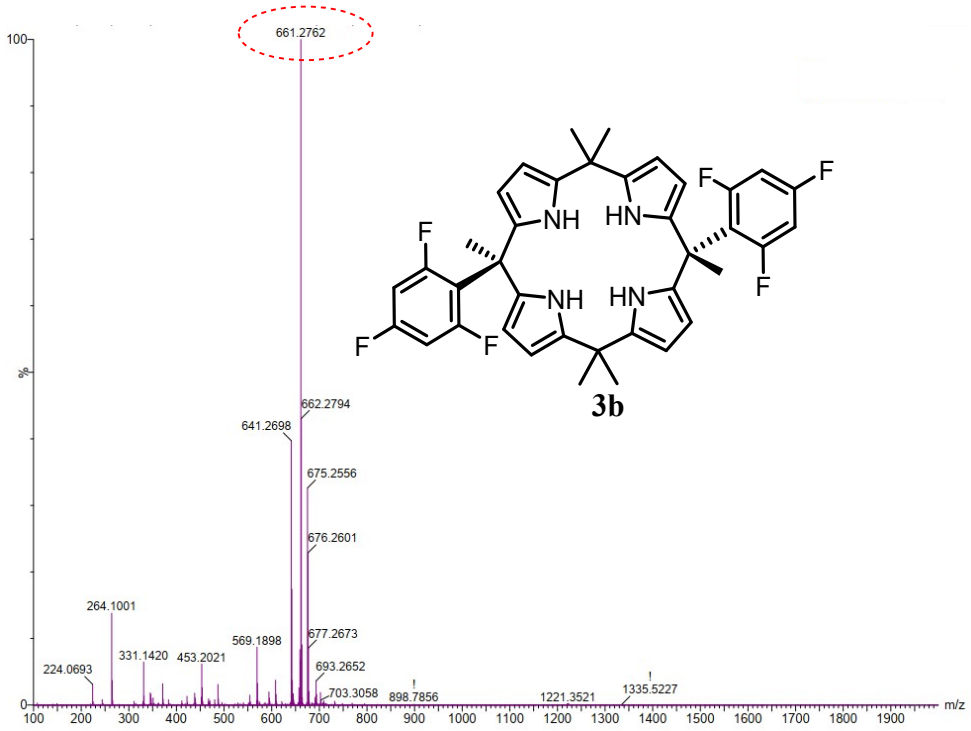

Figure S9. HRMS spectrum of *trans*-trifluorophenyl extended C4P (**3b**). (*m/z* calculated for $C_{38}H_{34}F_6N_4$ $[M+H]^+ = 661.2760$).

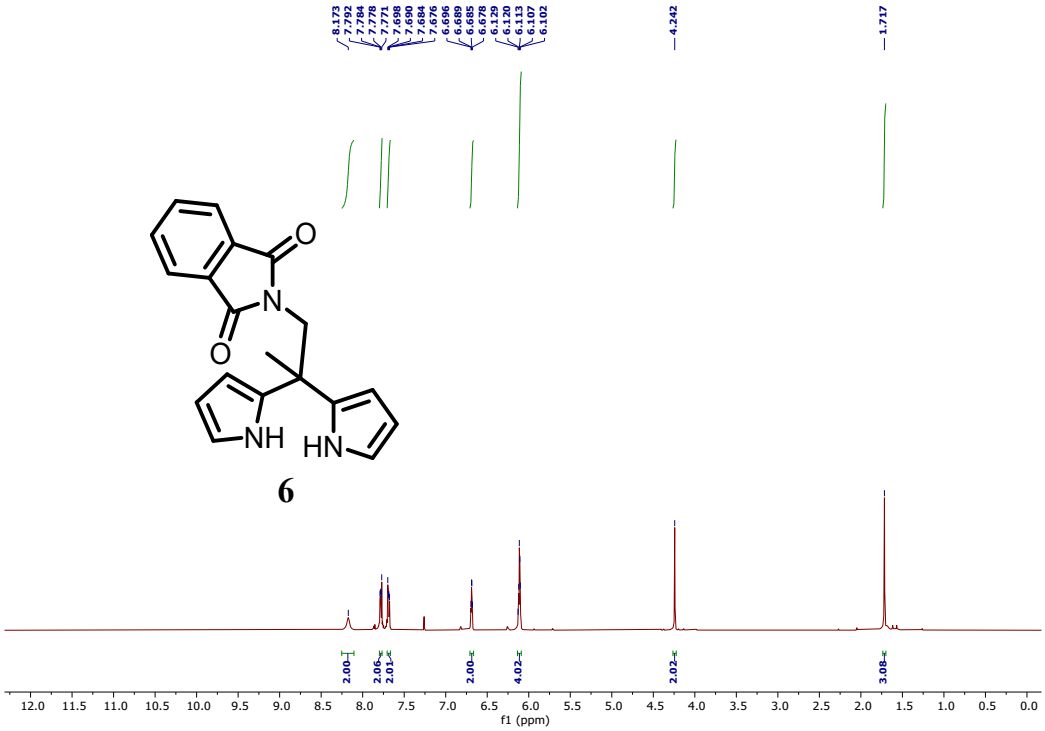

Figure S10. ^{1}H-NMR spectrum of phthalimide based dipyrromethane (**6**) recorded in $CDCl_3$.

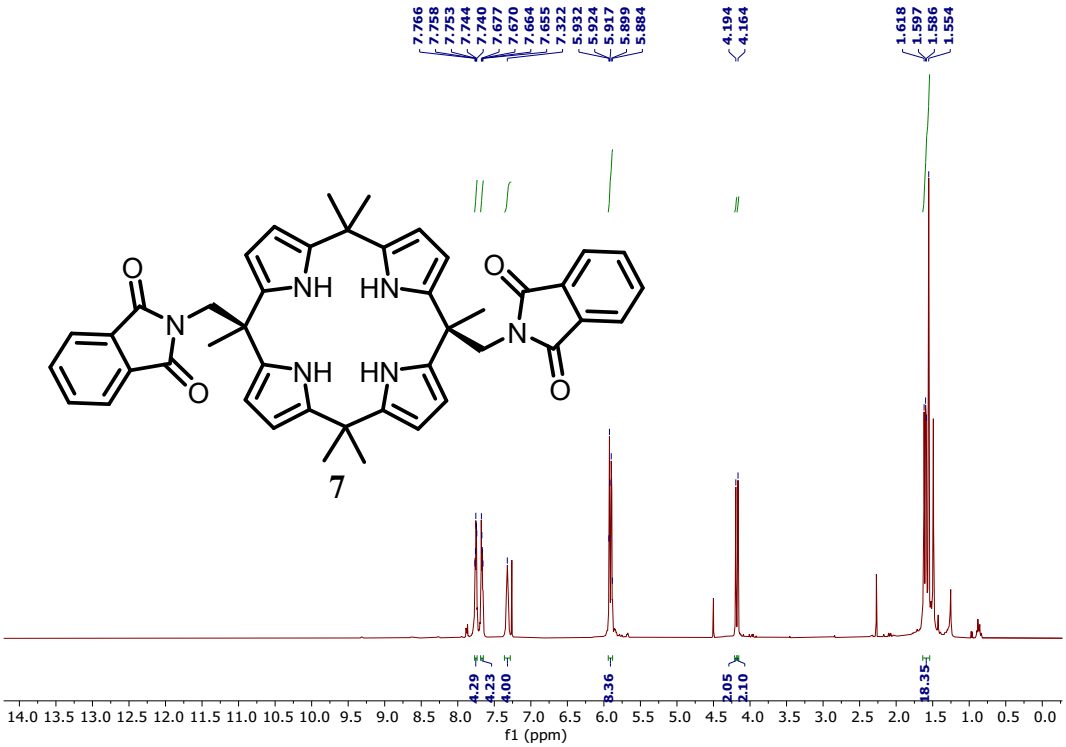

Figure S11. ^{1}H-NMR spectrum of double walled phthalimide extended C4P (**7**) recorded in $CDCl_3$. (* represents residual solvent water).

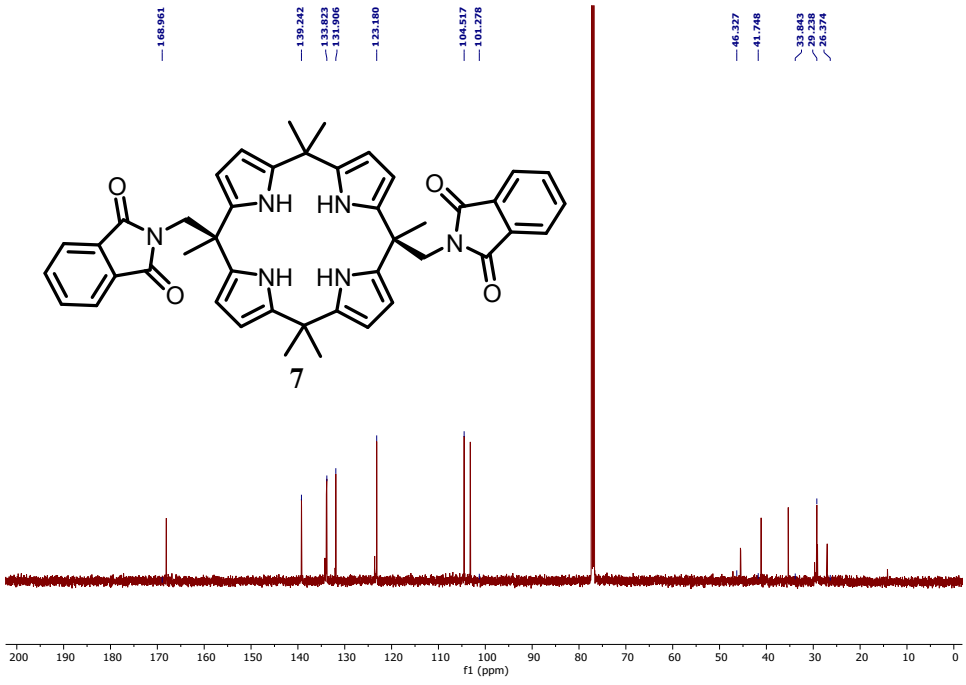

Figure S12. ^{13}C-NMR spectrum of double walled phthalimide extended C4P (**7**) recorded in $CDCl_3$.

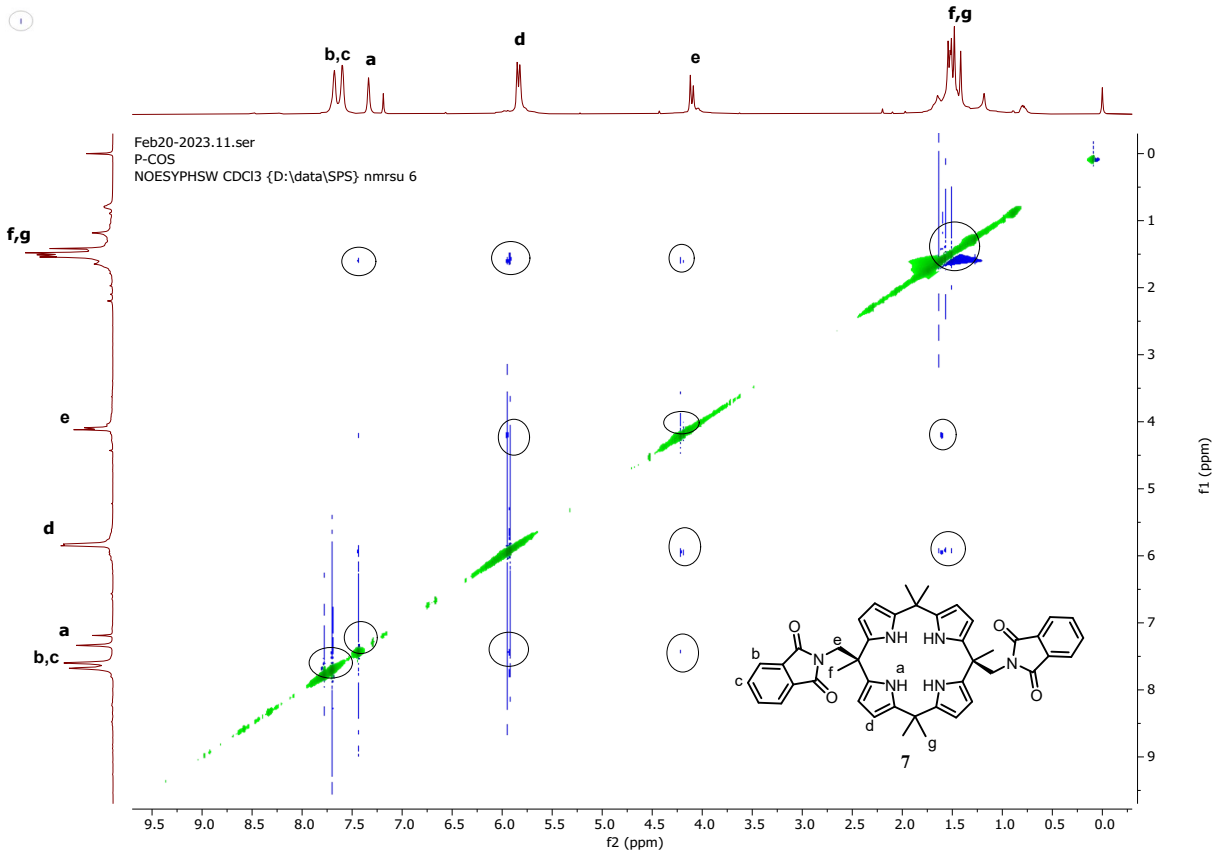

Figure S13. 2D NOESY NMR spectrum of phthalimide two-walled C4P (**7**) recorded in $CDCl_3$.

Note: The NOESY spectrum supports the following contentions:

1. The pyrrole NH-protons (a) have corresponding signals with *meso*-CH_3 protons (f,g).
2. The β-pyrrolic CH-protons (d) have corresponding signals with *meso*-CH_3 protons (f,g).
3. The methylene (-CH_2) protons (e) have corresponding signals with *meso*-CH_3 protons (f).
4. The pyrrole NH-protons (a) have corresponding signals with -CH_2 protons (e).
5. The phenyl CH-protons (b, c) have corresponding signals with -CH_2 protons (e).

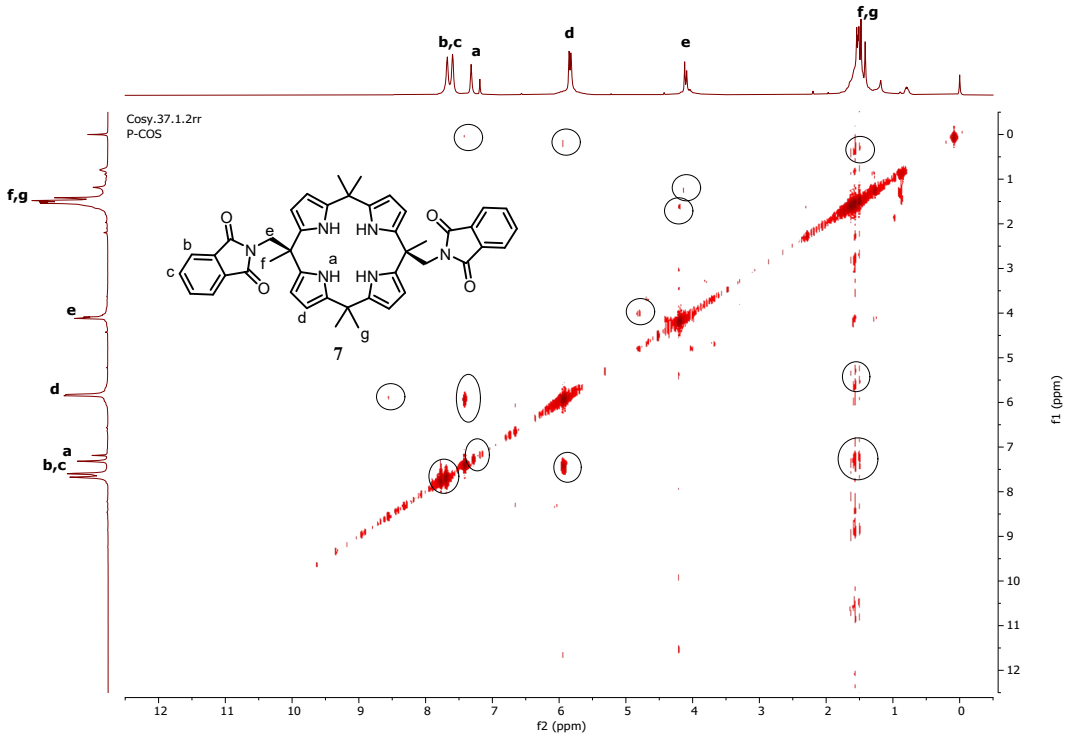

Figure S14. 2D COSY NMR spectrum of phthalimide two-walled C4P (**7**) recorded in $CDCl_3$.

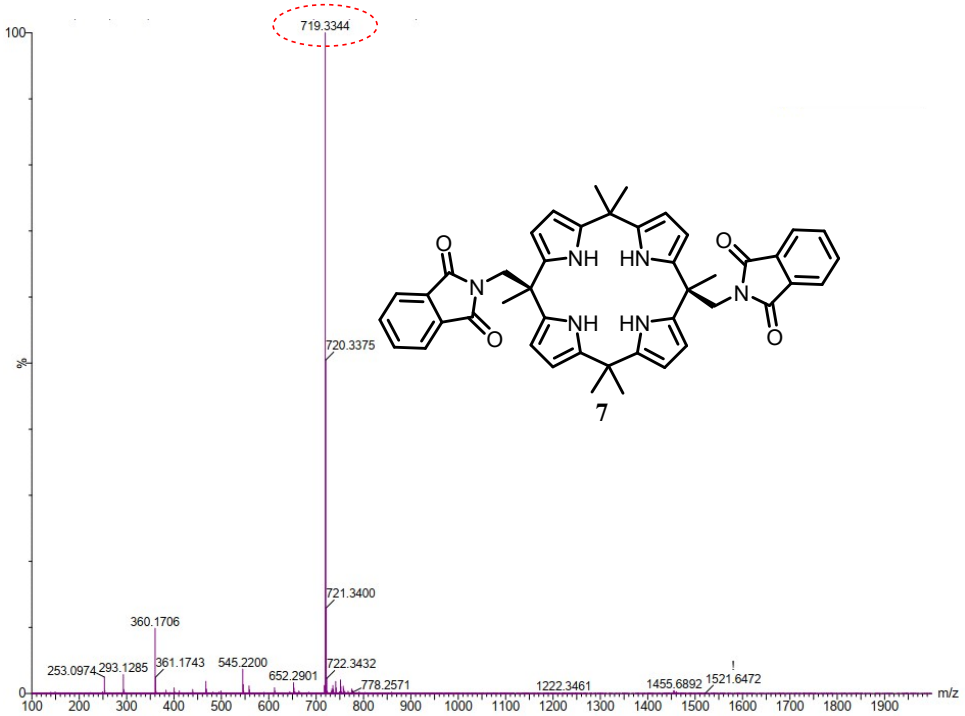

Figure S15. HRMS spectrum of double walled phthalimide extended C4P (**7**) (*m/z* calculated for $C_{44}H_{42}N_6O_4$ $[M+H]^+$ =719.3340).

IV. Crystal data

Table S1. Crystal data of *trans*-trifluorophenyl extended C4P (**3b**)

Identification code	JMI_SHD_ISD_15032022
Chemical formula	$C_{44}H_{48}F_6N_6O_2$
Formula weight	806.88 g/mol
Temperature	298(2) K
Wavelength	0.71073 Å
Crystal size	0.210 x 0.250 x 0.310 mm
Crystal system	triclinic
Space group	P -1
Unit cell dimensions	a = 10.0181(12) Å, α = 114.177(4)° b = 11.2952(14) Å, β = 115.501(4)° c = 11.6127(15) Å, γ = 94.382(4)°
Volume	1029.8(2) $Å^3$
Z	1
Density (calculated)	1.301 g/cm^3
Absorption coefficient	0.100 mm^{-1}
F(000)	424

V. Anion binding studies

^{1}H-NMR spectroscopy

^{1}H-NMR titration experiments were carried out at ambient temperature on a 400 MHz Bruker spectrometer. In order to calibrate spectra, the residual solvent signal (DMSO-d_6, δ = 2.50) was chosen as an internal reference. Both the receptor (**3b**) and TBACl salt were dried in a high vacuum before use. The titrations were executed upon incremental addition of TBACl (1.0 M in DMSO-d_6) to the solution of receptor **3b** (0.0025 M in DMSO-d_6). All ^{1}H-NMR data were processed by means of MestreNova 6.0 and the data collected was fitted in different binding modes using a supramolecular calculator known as BindFit.

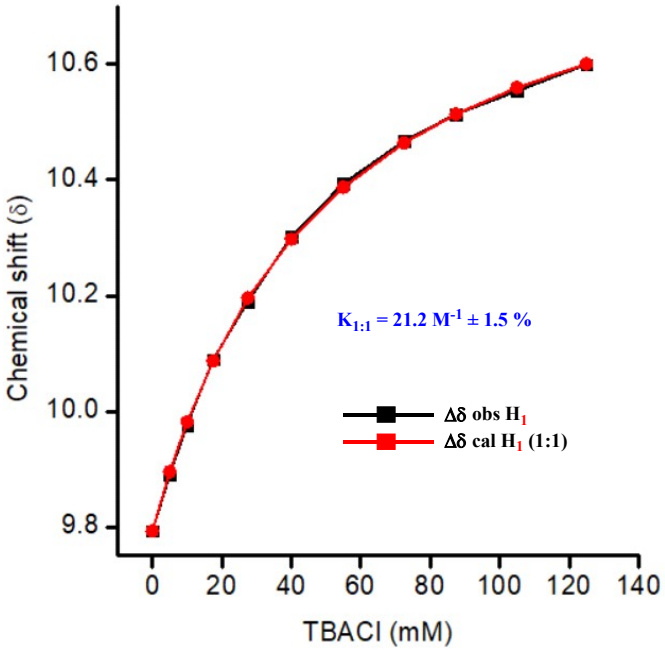

Figure S16. The plot of chemical shift (δ) of H_1 proton of receptor **3b** *vs* concentration of TBACl added, fitted to 1:1 binding model of BindFit v0.5. The binding constant obtained is the mean of the three independent experiments. *http://app.supramolecular.org/bindfit/view/25dc6274-d259-434d-bb59-15d063a865d5*.

Mass Spectrometric Studies

Electrospray ionization time-of-flight (ESI-TOF) mass spectrometer was used for recording high-resolution mass spectra (HRMS). For mass spectrometric analysis, stock solutions of receptors (**3** and **7**) and tetramethylammonium chloride (TMACl) (1 mM each) were prepared in CH_3CN of spectroscopic grade. After that, solutions were diluted in order to acquire 10 μM concentrations in CH_3CN and mixed in equal proportions. The sample was subsequently electrosprayed with a flow rate of 5.0 μL/min. A constant spray and the highest intensities were achieved with a capillary voltage of 2500 V at a source temperature of 80 °C. The parameters for the sample cone (20 V) and extractor cone voltage (5 V) were optimized for maximum intensities of the desired complexes.

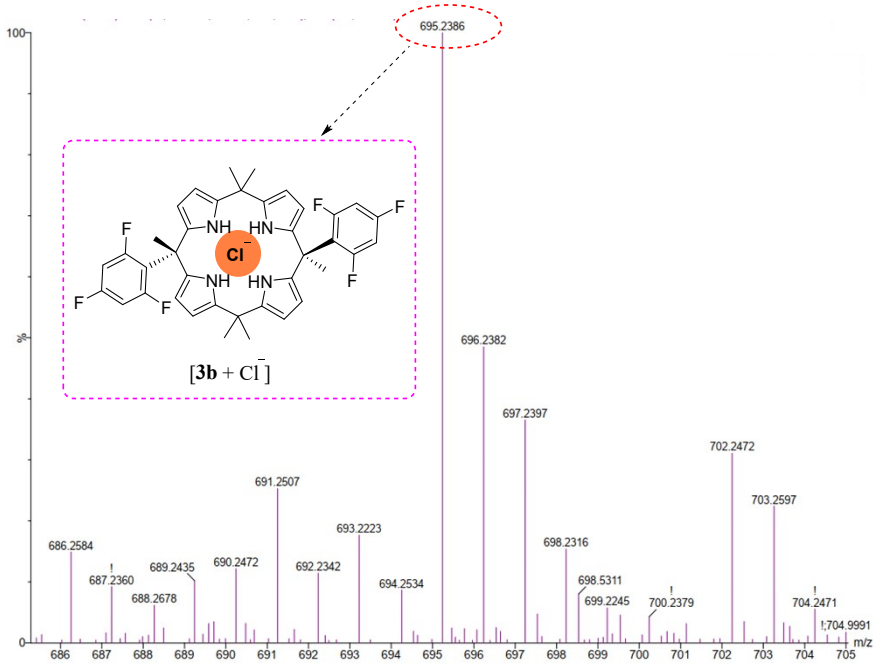

Figure S17. ESI-MS spectrum of 1:1 mixture (1 mM each) of **3b** and chloride ion (used as TMACl) recorded in CH_3CN. (*m/z* calculated for [**3b**+Cl^-] = 695.2381]).

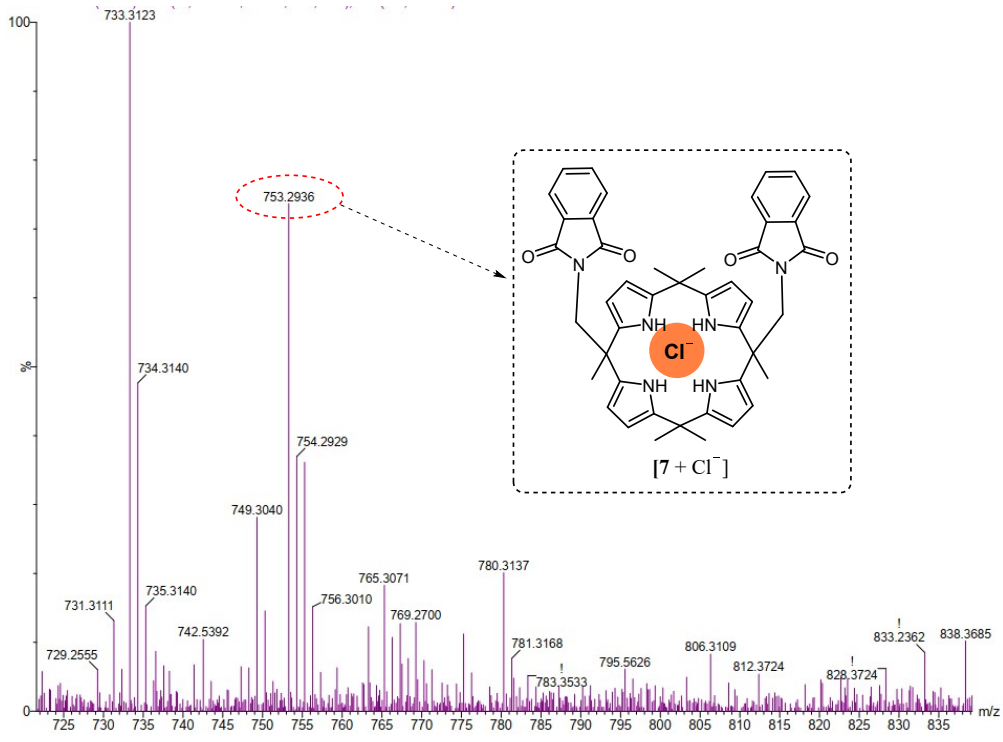

Figure S18. ESI-MS spectrum of 1:1 mixture of receptor **7** and TMACl recorded in CH_3CN. (*m/z* calculated for [**7**+Cl^-] = 753.2961).

VI. Ion transport studies

Ion transporting activity studies across EYPC-LUVs⊃HPTS

Preparation of HEPES buffer and stock solutions: The HEPES buffer of pH = 7.0 was prepared by dissolving an appropriate amount of solid HEPES (10 mM) and NaCl (100 mM) in autoclaved water. The pH was adjusted to 7.0 by the addition of aliquots from 0.5 M NaOH solution. The stock solution of all carriers was prepared using HPLC grade DMSO.

Preparation of EYPC-LUVs⊃HPTS in NaCl: In 10 mL clean and dry round bottom flask, a thin transparent film of egg yolk phosphatidylcholine (EYPC) was formed using a 1 mL EYPC lipid (25 mg/mL in $CHCl_3$) by providing continuous rotation and purging nitrogen gas. The transparent thin film was completely dried under a high vacuum for 4-5 h. After that, the transparent thin film was hydrated with 1 mL HEPES buffer (1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0), and the resulting suspension was vortexed at 10 min intervals during 1 h. This hydrated suspension was subjected to 15 cycles of freeze-thaw (liquid N_2, 55 °C) followed by extrusion through 100 nm (pore size) polycarbonate membrane 21 times to obtain the vesicles of an average 100 nm diameter. The unentrapped HPTS dyes were removed by size exclusion chromatography using Sephadex G-50 (Sigma-Aldrich) with eluting of HEPES buffer (10 mM HEPES, 100 mM NaCl, pH = 7.0). Finally, collected vesicles were diluted to 6 mL to get EYPC-LUVs⊃HPTS. *Final conditions:* ~ 5 mM EYPC, Inside: 1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0, Outside: 10 mM HEPES, 100 mM NaCl, pH = 7.0.

Ion transport activity by HPTS assay: In clean and dry fluorescence cuvette, 1975 μL of HEPES buffer (10 mM HEPES, 100 mM NaCl, pH =7.0) and 25 μL of EYPC–LUVs⊃HPTS vesicle was added. The cuvette was placed in a slowly stirring condition using a magnetic stirrer equipped in a fluorescence instrument ($t = 0$ s). The time-dependent HPTS emission intensity was monitored at λ_{em} = 510 nm (λ_{ex} = 450 nm) by creating pH gradient between intra- and extravesicular media by the addition of 0.5 M NaOH (20 μL) at $t = 20$ s. Then different concentrations of transporter molecules (**3** and **7**) in DMSO were added at t = 100 s. Finally, the vesicles were lysed by the addition of 10% Triton X-100 (25 μL) at $t = 300$ s to disturb pH gradient.

The time axis was normalized according to Equation S1:

$$t = t - 100 \qquad \text{Equation S1}$$

The time-dependent data were normalized to percent change in fluorescence intensity using Equation S2:

$$I_F = [(I_t - I_0) / (I_\infty - I_0)] \times 100 \qquad \text{Equation S2}$$

where, I_0 is the initial intensity, I_t is the intensity at time t, and I_∞ is the final intensity after the addition of Triton X-100.

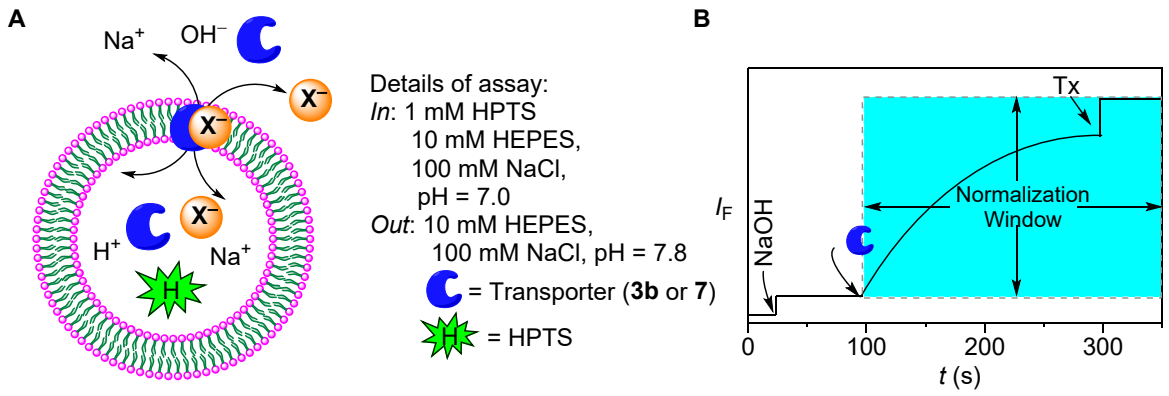

Figure S19. Representations of fluorescence-based ion transport activity assay of transporters (**3b** and **7**) using EYPC-LUVs⊃HPTS (A), and illustration of ion transport kinetics showing normalization window (B).

Dose-response activity: The fluorescence kinetics of each transporter (**3b** and **7**) at different concentrations was studied over the course of time. The concentration profile data were evaluated at t = 290 s to get effective concentration, EC_{50} (i.e. the concentration of transporter needed to achieve 50% chloride efflux)[2] using Hill equation (Equation S3):

$$Y = Y_\infty + (Y_0 - Y_\infty) / [1 + (c/EC_{50})^n] \qquad \text{Equation S3}$$

where, Y_0 = Fluorescence intensity just before the transporter addition (at t = 0 s), Y_∞ = Fluorescence intensity with excess transporter concentration (**3b** or **7**), c = Concentration of transporter compound (**3b** or **7**), and n = Hill coefficient (i.e., indicative for the number of monomers needed to form an active supramolecule)[3].

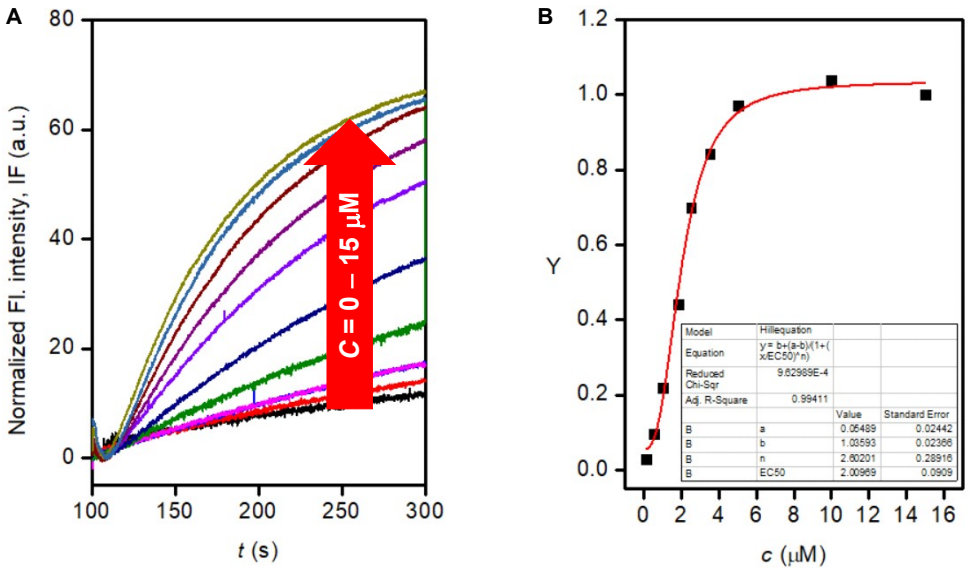

Figure S20. Concentration-dependent activity of transporter (**7**) across EYPC-LUVs⊃HPTS (A). Dose-response plot of **7** at 180 s after addition of compound (B).

Anion selectivity studies

Preparation of EYPC-LUVs⊃HPTS for anion selectivity: EYPC-LUVs⊃HPTS (~ 5.0 mM EYPC, inside: 1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0 and outside: 10 mM HEPES, 100 mM NaX, pH = 7.0; where, X^- = Cl^-, Br^-, I^-, ClO_4^-, and NO_3^-) were prepared following reported protocol.[4]

Anion Selectivity Assay: In a clean fluorescence cuvette, 1975 μL of HEPES buffer (10 mM HEPES, 100 mM NaX, at pH = 7.0; where, X^- = Cl^-, Br^-, I^-, ClO_4^-, and NO_3^- was added followed by addition of 25 μL of EYPC-LUVs⊃HPTS vesicle in slowly stirring condition by a magnetic stirrer equipped with the fluorescence instrument (at t = 0 s). HPTS fluorescence emission intensity (F_t) was monitored with time at λ_{em} = 510 nm (λ_{ex} = 450 nm). 20 μL of 0.5 M NaOH was added to the cuvette at t = 20 s to make the pH gradient between the intra- and extravesicular system. The compound **3b** was added at t = 100 s and at t = 300 s, 25 μL of 10% Triton X-100 was added to lyze all vesicles for the complete destruction of the pH gradient. For data analysis and comparison, time (X-axis) was normalized using Equation S1. Fluorescence intensities (F_t) were normalized to fractional emission intensity I_F using Equation S2.

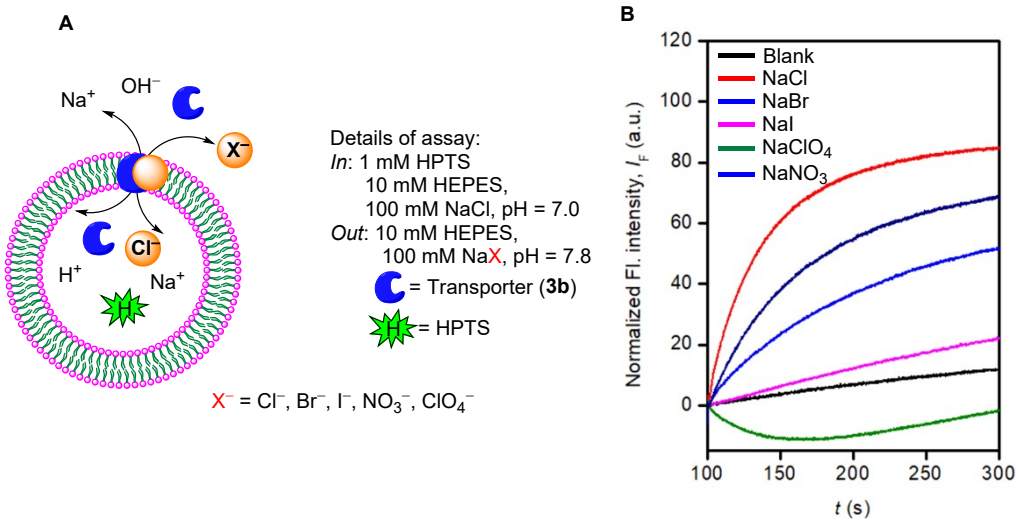

Figure S21. Schematic representations of fluorescence-based Anion selectivity assay (A) Anion selectivity of **3b** (0.5 μM) measured by varying external Anions (X^- = Cl^-, Br^-, ClO_4^-, NO_3^- and I^-) across EYPC-LUVs⊃HPTS (B).

Cation selectivity assay: Similarly, cation selectivity of transporter **3b** was explored by changing extravesicular HEPES buffer solution (10 mM HEPES, 100 mM MCl, pH = 7.0) of chloride salts (MCl) of different cations (M = Li^+, Na^+, K^+, Rb^+, and Cs^+). The time axis was normalized according to Equation S1. The fluorescence data were normalized to percent change in intensity as a course of time using Equation S2.

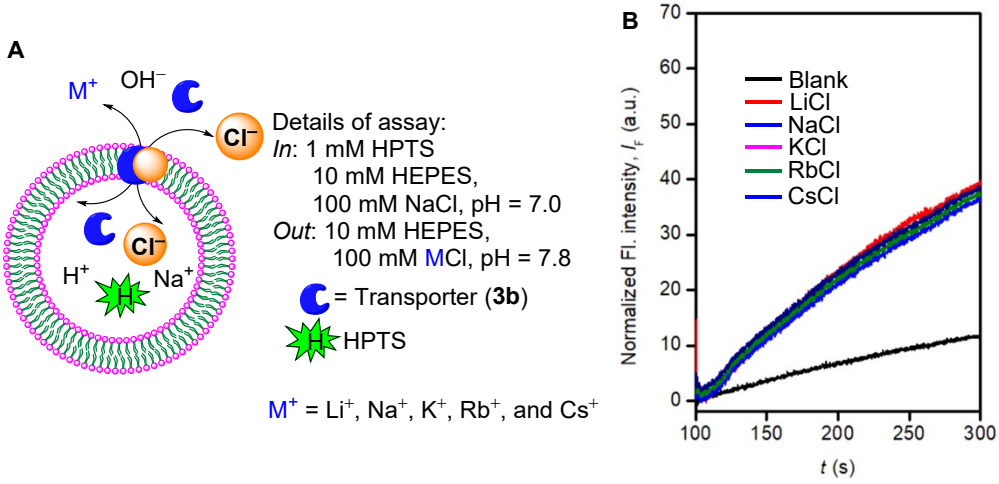

Figure S22. Schematic illustration of fluorescence-based cation selectivity assay (A). Cation selectivity of **3b** (0.3 μM) measured by varying external cations (M^+ = Li^+, Na^+, K^+, Rb^+, and Cs^+) across EYPC-LUVs⊃HPTS (B).

Chloride transport activity across EYPC-LUVs⊃lucigenin vesicles

Preparation of EYPC-LUVs⊃lucigenin vesicles: In 10 mL clean and dry round bottom flask, a thin transparent film of egg yolk phosphatidylcholine (EYPC) was formed by drying 1.0 mL egg yolk phosphatidylcholine (EYPC, 25 mg/mL in $CHCl_3$) with providing continuous rotation and purging nitrogen. The transparent thin film was kept under the high vacuum for 4 hours to remove all traces of $CHCl_3$. Then the transparent thin film was hydrated with 1.0 mL aqueous $NaNO_3$ (200 mM, 1.0 mM lucigenin) with occasional vortexing at 10 min intervals for 1 h. The resulting suspension was subjected to freeze and thaw cycles (≥ 15, liquid nitrogen, 55 ºC water bath) and 21 times extrusion through 200 nm pore size polycarbonate membrane. The size exclusion chromatography (using Sephadex G-50) was performed to remove extravesicular dye using 200 mM $NaNO_3$ solution as eluent. The collected vesicle suspension was diluted to 4 mL. Inside: 200 mM $NaNO_3$, 1 mM lucigenin, pH = 7.0; outside: 200 mM $NaNO_3$, pH = 7.0.

Ion transport activity by lucigenin assay

In a clean and dry fluorescence cuvette, 200 mM $NaNO_3$ (1975 μL) and EYPC-LUVs⊃lucigenin (25 μL) were taken. This suspension was placed in a slowly stirring condition in a fluorescence instrument equipped with a magnetic stirrer (at t = 0 s). The fluorescence intensity of lucigenin was monitored at λ_{em} = 535 nm (λ_{ex} = 455 nm) as a course of time. The chloride gradient was created by the addition of 2.0 M NaCl (33.3 μL) at t = 20 s between intra- and extravesicular system, followed by the addition of the transporter (**3b**) at t = 100 s. Finally, vesicles were lysed by the addition of 10% Triton X-100 (25 μL) at t = 300 s for the complete destruction of the chloride gradient.

The time axis was normalized according to Equation S1 and the time-dependent data were normalized to percent change in fluorescence intensity using Equation S4:

$$I_F = [(I_t - I_0) / (I_\infty - I_0)] \times (-100) \qquad \text{Equation S4}$$

where, I_0 is the initial intensity, I_t is the intensity at time t, and I_∞ is the final intensity after the addition of Triton X-100.

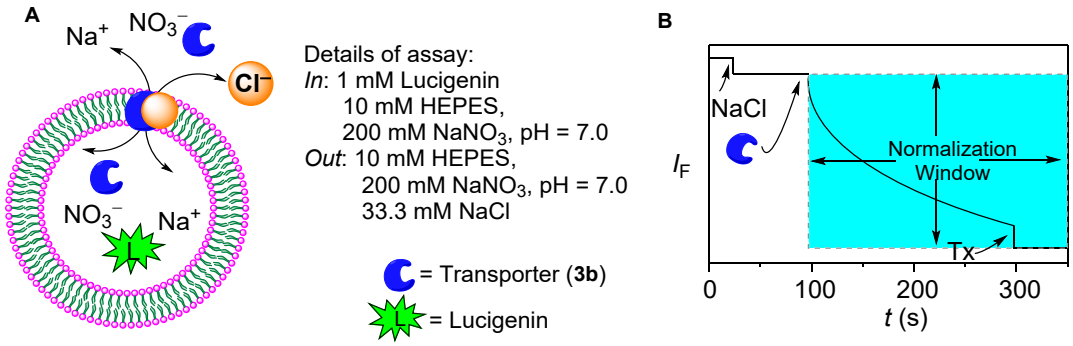

Figure S23. Representations of fluorescence-based ion transport activity assay of transporter (**3b**) using EYPC-LUVs⊃lucigenin (A), and illustration of ion transport kinetics showing normalization window (B).

Proof of antiport mechanism by lucigenin assay in the presence of external SO_4^{2-} and NO_3^- anions

Preparation of EYPC-LUVs⊃lucigenin vesicles: In a 10 mL clean and dry round bottom flask, the thin transparent film of egg yolk phosphatidylcholine (EYPC) was formed by drying 1.0 mL egg yolk phosphatidylcholine (EYPC, 25 mg/mL in $CHCl_3$) with providing continuous rotation and purging nitrogen. The transparent thin film was kept under a high vacuum for 4 h to remove all traces of $CHCl_3$. Then the transparent thin film was hydrated with 1.0 mL aqueous NaCl (200 mM, 1.0 mM Lucigenin) with occasional vortexing at 10 min intervals for 1 h. The resulting suspension was subjected to freeze and thaw cycles (≥ 15, liquid nitrogen, 55 °C water bath) and 21 times extrusion through 200 nm pore size polycarbonate membrane. The size exclusion chromatography (using Sephadex G-50) was performed to remove extravesicular dye using 200 mM NaCl solution as eluent. The collected vesicles suspension was diluted to 4 mL. Final conditions: ~ 5 mM EYPC; inside: 200 mM NaCl, 1 mM lucigenin, pH 7.0; outside: either 200 mM $NaNO_3$ or 200 mM Na_2SO_4.

Ion transport assay

In a clean and dry fluorescence cuvette, either 200 mM of $NaNO_3$ or 200 mM of Na_2SO_4 (1975 μL) and EYPC-LUVs⊃lucigenin (25 μL) were taken. This suspension was placed in a slowly stirring condition in a fluorescence instrument equipped with a magnetic stirrer (at t = 0 s). The fluorescence intensity of lucigenin was monitored at λ_{em} = 535 nm (λ_{ex} = 455 nm) as a course of

time. The transporter molecule **3b** was added at t = 100 s. Finally, vesicles were lysed by adding 10% Triton X-100 (25 μL) at t = 300 s for the complete destruction of chloride gradient. NO_3^- transport occurred with the concomitant efflux of Cl^- ions, and on the other hand, SO_4^{2-} being more hydrophilic is not transported easily, suggesting the operation of antiport mechanism.

The time axis was normalized using Eq. S5,

$$t = t - 50 \qquad \text{Equation S5}$$

and the time-dependent data were normalized to percent change in fluorescence intensity using Eq. S2.

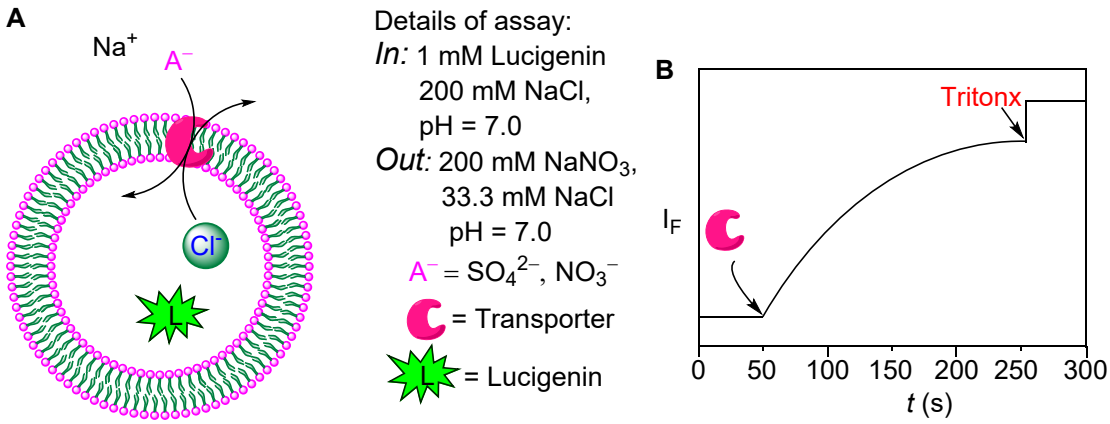

Figure S24 Representation of fluorescence-based antiport assay using EYPC-LUVs⊃lucigenin (A). Representation of ion transport kinetics showing normalization window (B).

U-tube experiments for checking Cl^- transport

These experiments were carried out to check whether the ion transport occurs via a mobile carrier mechanism or through an ion channel formation by the transporter **3b**. In a U-shaped glass tube, 15 mL solution of **3b** (1 mM) and tetrabutylammonium hexafluorophosphate (1 mM) in $CHCl_3$ was placed. Then the left arm (source arm) of the U-tube was filled with a 7.5 mL buffer solution consisting of 500 mM NaCl and 5 mM phosphate buffer set to pH 7.0. On the other hand, the right arm (receiver arm) was filled with a 7.5 mL buffer solution consisting of 500 mM $NaNO_3$ and 5 mM phosphate buffer set to pH 7.0. It is assumed that no ion channel can be formed by **3b** in the organic layer connecting to aqueous layers due to the long path length of the organic layer. Hence, the exchange of Cl^- and NO_3^- ions between two arms would be

possible if **3b** functions as a mobile carrier. Therefore, the transport of Cl^- ions from the source to the receiver arm was monitored by a chloride ion selective electrode (ISE) after fixed time intervals for an overall period of 12 days. A control experiment was also done without placing **3b** in the $CHCl_3$ layer. A gradual increase of chloride concentration, only in the presence of **3b**, confirmed the mobile carrier mechanism as the primary transport mechanism (**Figure S25**).

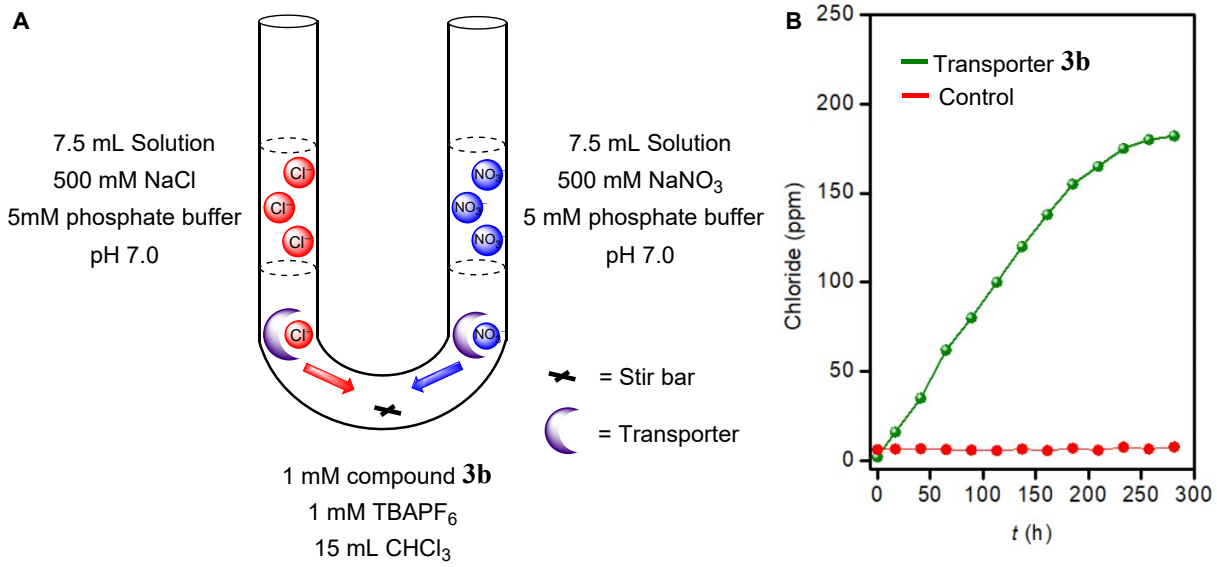

Figure S25. Schematic demonstration of U-tube experiment setup (A). Change of chloride ion concentration in a U-tube experiment with and without transporter **3b** (1.0 mM) (B).

VII. References

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