Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

for

A potent fluorescent transmembrane HCl anion transporter promotes cancer cell death by perturbing cellular pH

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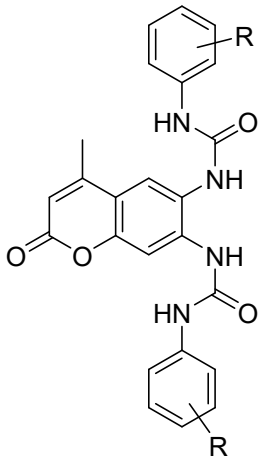
S1. General methods and material

All reagents and solvents were purified and dried by standard techniques. Reactions were monitored by TLC analysis using silica gel GF/UV 254. NMR spectra were recorded on Bruker 400 MHz FT-NMR spectrometer and Varian Gemini-300BB 300 MHz FT-NMR spectrometers (Varian Inc., Palo Alto, CA). ^{1}H spectra were run at 300 and 400 MHz and ^{13}C spectra were run at 75 and 101 MHz, in the stated solvent. Chemical shifts (δ_H) are reported relative to TMS as internal standard and coupling constant (J) values are reported in Hertz. The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Electrospray (ESI single quadrupole) mass spectra have their ion mass to charge values (m/z) stated with their relative abundances as a percentage in parentheses. Peaks assigned to the molecular ion are denoted as [M+H] or [M+Na]. Column chromatography was performed using silica gel 60 (0.063-0.200 mm). Low resolution mass spectra (LRMS) and high resolution mass spectra (HRMS) were recorded using positive/negative ion electrospray ionization (ESI) on Bruker amaZon SL mass spectrometer.

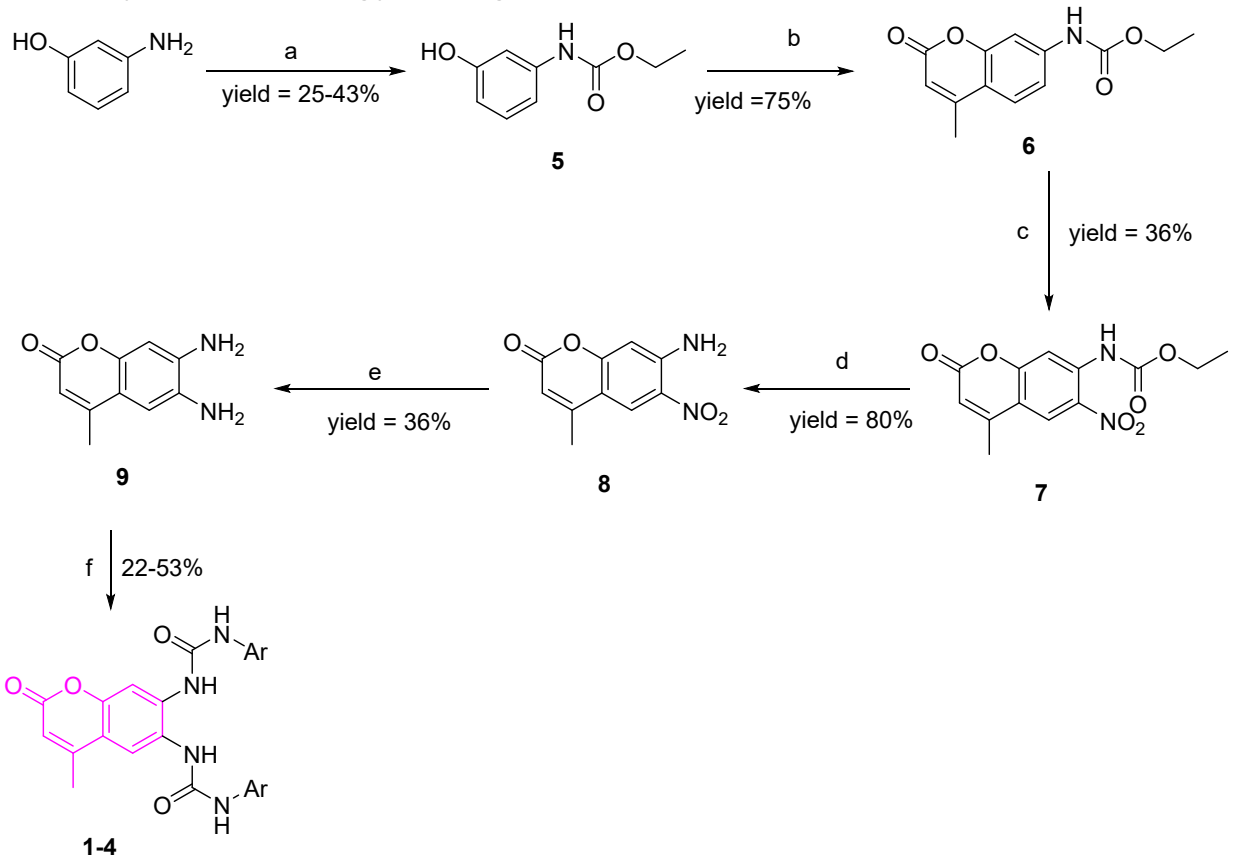
S2. Synthesis and charachterization:

S2.1. Overview of compounds:

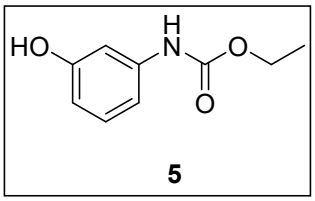


1, R = H
2, R = 4-CN
3, R = 4-CF_3
4, R = 3,5-BisCF_3

S2.2. Synthetic strategy of target anion transporters **1-4**

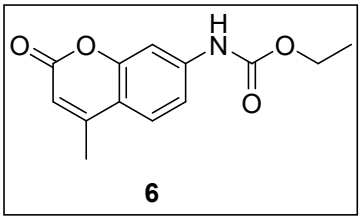


Scheme S1. a- Ethylchloroformate/DEE; b-Ethylacetoacetate, 70% H_2SO_4; c- $Al(NO_3)_3.9\ H_2O$, acetic anhydride.; d- Conc H_2SO_4, Glacial acetic acid, reflux.; e- Sn/HCl, reflux.; f- Suitable isocyanate, DCM, 45 °C, or Suitable isocyanate, 45 °C.



5

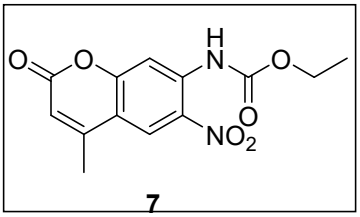
Synthesis of ethyl (3-hydroxyphenyl)carbamate **5**:[1] To a suspension of *m*-aminophenol (10.0 g, 92.0 mmol) in diethyl ether (400 mL), ethyl chloroformate (10.0 g, 92.0 mmol) was added at once and a white precipitate appeared immediately. The reaction mixture was stirred for 2 h and the resulting salt was removed by filteration, while the solvent was removed under vacuum to give 8 g of white solid which was used in the next step without any further purification.[1]



6

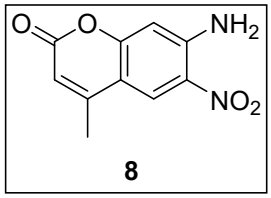
Synthesis of ethyl(4-methyl-2-oxo-2*H*-chromen-7-yl)carbamate **6:**[1] To a mixture of ethyl (3-hydroxyphenyl)carbamate **5** (8.0 g, 44.0 mmol) and ethylacetoacetate (6.8 mL, 53.0 mmol), 100 mL of 70% H_2SO_4 was added. The reaction mixture was stirred for 4 h at room temperature. The resutling solid was collected by filteration, washed with water (2 x 30 mL) and recrystallized from ethanol to give **6** (8.2 g, 75%) as a colorless needles. **^{1}H NMR (400 MHz, DMSO-*d*$_6$) *δ***: 1.40 (t, *J* = 7.0 Hz, 3H, $CH_2\underline{CH_3}$), 2.50 (s, 3H, CH_3), 4.31 (q, *J* = 7.0 Hz, 2H, $\underline{CH_2}CH_3$), 6.32 (s, 1H, ArH), 7.51 (dd, *J* = 2.0, 9.0 Hz, 1H, ArH), 7.65 (d, *J* = 2.0 Hz, 1H, ArH), 7.77 (d, *J* = 9.0 Hz, 1H, ArH), 10.24 (s, 1H, NH); **^{13}C NMR (101 MHz, DMSO-*d*$_6$) *δ***: 14.9, 18.4, 61.1, 104.8, 112.3, 114.6, 114.7, 126.3, 143.3, 153.6, 153.8, 154.3,160.5.

Synthesis of ethyl (4-methyl-6-nitro-2-oxo-2*H*-chromen-7-yl)carbamate **7**:[2] To a suspension of 7-carbethoxyamino-4-methylcoumarin **6** (4.1 g, 16.6 mmol) in acetic anhydride (100 mL), aluminium nitrate nonahydrate (5.6 g, 6.5 mmol) was added portionwise and the reaction mixture was stirred for 16 h. The mixture was slowly poured into ice cold water (200 mL) and the resulting yellow residue was purified *via* flash chromatography (hexane: ethyl acetate = 9:1) to give 7 (1.7 g, 36%). **^{1}H NMR (500 MHz, DMSO-*d*$_6$) *δ***: 1.27 (t, *J* = 7.0 Hz, 3H, $CH_2\underline{CH_3}$), 2.45 (s, 3H, CH_3), 4.18 (q, *J* = 7.0 Hz, 2H, $\underline{CH_2}CH_3$), 6.46 (s, 1H, ArH), 7.72 (s, 1H, ArH), 8.35 (s, 1H, ArH), 10.10 (s, 1H, NH); **^{13}C NMR (126 MHz, DMSO-*d*$_6$) *δ***: 14.7, 18.3, 62.2, 109.9,



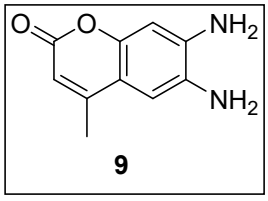
7

114.9, 115.7, 123.9, 136.1, 136.9, 152.8, 153.5, 156.3, 159.3; **LRMS ESI$^-$ (*m/z*)**: 291 (100%, M-H)$^-$.



8

Synthesis of 7-Amino-4-methyl-6-nitro-2*H*-chromen-2-one **8**:[2] 6-Nitro-7- carbethoxyamino-4-methylcoumarin **7** (1.7 g, 5.8 mmol) was heated at reflux in a mixture of concentrated sulfuric acid (6 g) and glacial acetic acid (6 g) for 4 h. After cooling the reaction mixture, was poured into ice water (30 mL) and let stand overnight. Ice was added, and neutralization of the acidic solution was accomplished using NaOH (50%). The formed yellow precipitate was filtered, washed thoroughly 3 times with ice water (3 x 10 mL) and dried to give **8** as a yellow powder (1.0 g, 80%). **^{1}H NMR (400 MHz, DMSO-*d*$_6$) *δ***: 2.38 (s, 3H, CH_3), 6.18 (s, 1H, ArH), 6.81 (s, 1H, ArH), 7.80 (br. s, 2H, NH_2), 8.33 (s, 1H, ArH); **^{13}C NMR (101 MHz, DMSO-*d*$_6$) *δ*:** 18.2, 103.3, 110.4, 111.6, 124.9, 128.8, 148.6, 153.4, 157.6, 159.7. **LRMS ESI$^-$ (*m/z*)**: 219 (100%, M-H)$^-$.



9

Synthesis of 6,7-Diamino-4-methyl-2*H*-chromen-2-one **9**:[2] Conc. hydrochloric acid (15 mL) was added in three portions over 20 minutes to a mixture of tin powder (1.1 g, 9.2 mmol) and 6-nitro-7-amino-4-methyl-coumarin (1 g, 4.5 mmol) and the reaction mixture was heated at reflux for 3 h. After cooling, the reaction mixture was poured into ice water (50 mL) and let stand overnight. Ice was added, and neutralization of the acidic solution was accomplished using NaOH (50%). The formed precipitate was extracted with ethyl acetate (3 x 200 mL) and all the organic layers washed with water (2 x 10 mL), brine (1 x 10 mL) and dried over magnesium sulfate. Ethyl acetate was removed under vacuum to give **9** as a dark yellow powder (0.31 g, 36%).**^{1}H NMR (300 MHz, DMSO-*d*$_6$) *δ***: 2.26 (s, 3H, CH_3), 4.72 (br. s, 2H, NH_2), 5.63 (br. s, 2H, NH_2), 5.87 (s, 1H, ArH), 6.44 (s, 1H, ArH), 6.76 (s, 1H, ArH); **^{13}C NMR (75 MHz, DMSO-*d*$_6$) *δ***: 18.1, 99.0, 107.1, 107.6, 109.2, 131.9, 141.3, 148.1, 153.1, 161.1. **LRMS ESI$^-$ (*m/z*)**: 191 (100%, M-H)$^-$. **HRMS (ESI$^+$)** calcd for $C_{10}H_{10}N_2NaO_2$ (M + Na^+): 213.0635, found: 213.06340 (0.22 ppm).

Synthesis of target compounds **1-4**:

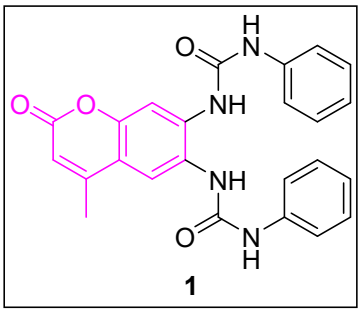
General procedure A:

6,7-Diamino-4-methylcoumarin **9** (80 mg, 0.42 mmol) was suspended in CH_2Cl_2 and added dropwise to the corresponding isocyanate derivative solution with stirring. The suspension was stirred at 45 °C overnight and after cooling the solvent removed under vacuum. The resulting residue was heated in ethanol (25 mL) and filtered while hot to give anion transporters **1**, **2** and **4.**

Procedure B:

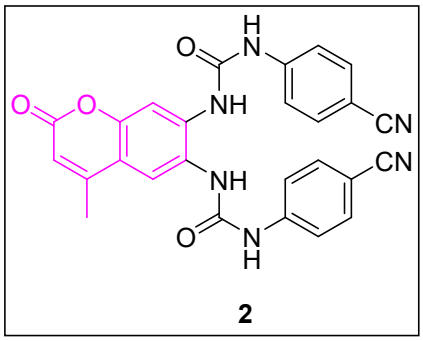
Under inert atmosphere, 4-trifluoropmethylphenyl isocyanate (0.79 g, 4.2 mmol) was added to 6,7-diamino-4-methylcoumarin **9** (80 mg, 0.42 mmol) with constant stirring. The reaction mixture was held at 45 °C overnight and after cooling the unreacted 4-trifluorpmethylphenyl isocyanate was removed under vacuum and the remaining residue was heated in ethanol (25 mL) and filtered while hot to give **3** as white solid.

1,1'-(4-Methyl-2-oxo-2*H*-chromene-6,7-diyl)bis(3-phenylurea) **1**: Using the general procedure A and phenyl isocyanate (200 mg, 1.68 mmol), compound **1** (70 mg, 39%) was isolated as a white solid. **^{1}H NMR (400 MHz, DMF-d_7) δ**: 2.40 (s, 3H, CH_3), 6.22 (s, 1H, ArH), 6.97 (q, *J* = 7.0 Hz, 2H, ArH), 7.27 (t, *J* = 7.0 Hz, 4H, ArH), 7.54 (m, 4H, ArH) 7.79 (s, 1H, ArH), 8.07 (s, 1H, ArH), 8.18 (s, 1H, NH), 8.68 (s, 1H, NH), 9.06 (s, 1H, NH), 9.46 (s, 1H, NH); **^{13}C NMR (101 MHz, DMF-d_7) δ**: 17.8, 107.3, 112.6, 114.6, 118.5, 118.7, 122.1, 122.5, 123.3, 125.2, 129.0, 129.1, 139.5, 140.1, 140.7, 152.1, 152.9, 153.2, 160.6. **LRMS ESI$^-$ (*m/z*)**: 427 (100%, M-H)$^-$. **HRMS (ESI$^-$)** calcd for $C_{24}H_{19}N_4O_4$ (M-H)$^-$: 427.1412, found: 427.1412 (-0.06 ppm).



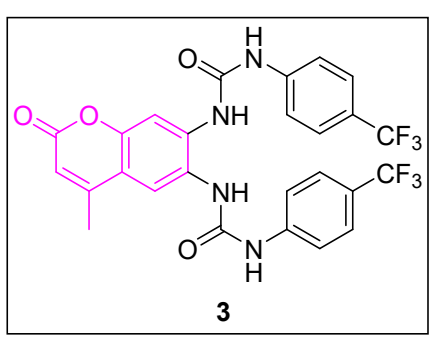
1

1,1'-(4-Methyl-2-oxo-2*H*-chromene-6,7-diyl)bis(3-(4-cyanophenyl)urea) **2**: Using the general procedure A and *p*-cyanophenyl isocyanate (242 mg, 1.68 mmol), compound **2** (106 mg, 53%) was isolated as a buff solid. **^{1}H NMR (500 MHz, DMSO-d_6) δ**: 2.39 (s, 3H, CH_3), 6.29 (s, 1H, ArH), 7.64-7.77 (m, 9H, ArH), 8.02 (s, 1H, ArH), 8.25 (s, 1H, NH), 8.65 (s, 1H, NH), 9.57 (s, 1H, NH), 9.91 (s, 1H, NH); **^{13}C NMR (126 MHz, DMSO-d_6) δ**: 18.5, 103.8, 104.2, 107.7, 113.1, 114.9, 118.6, 118.7, 119.7, 119.8, 123.7, 124.7, 133.8, 133.9, 138.5, 144.1, 144.8, 151.7, 152.4, 153.4, 154.0, 160.0. **LRMS ESI$^-$ (*m/z*)**: 513 (100%, M+Cl)$^-$. **HRMS (ESI$^+$)** calcd for $C_{26}H_{18}N_6O_4Na$ $(M+Na)^+$: 501.1282, found: 501.1287 (1.0 ppm).



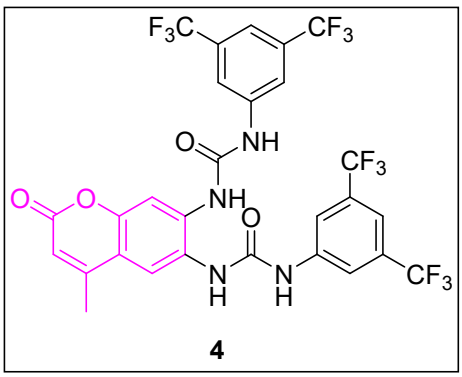
2

1,1'-(4-Methyl-2-oxo-2H-chromene-6,7-diyl)bis(3-(4-(trifluoromethyl)phenyl)urea) **3**: Using the procedure B and *p*-trifluormethylphenyl isocyanate (0.79 g, 4.2 mmol), compound **3** (53 mg, 22%) was isolated as a white solid. **^{1}H NMR (400 MHz, DMF-d_7) δ**: 2.64 (s, 3H, CH_3), 6.48 (s, 1H, ArH), 7.87 (d, *J* = 9.0 Hz, 4H, ArH), 7.95-8.05 (m, 4H, ArH), 8.21 (s, 1H, ArH), 8.40 (s, 1H, ArH), 8.51 (s, 1H, NH), 9.03 (s, 1H, NH), 9.79 (s, 1H, NH), 10.06 (s, 1H, NH); **^{13}C NMR (101 MHz, DMF-d_7) δ**: 17.8, 107.6, 112.9, 115.0, 118.4, 118.5, 122.6, 122.9, 123.0 123.3, 123.5, 123.7, 125.0, 126.4, 139.0, 143.7, 144.3, 152.1, 152.7, 153.2, 154.3, 160.5; ^{19}F (376 MHz, DMF-d_7) δ: -60.03, -60.15. **LRMS ESI$^-$ (*m/z*)**: 563 (100%, M-H)$^-$. **HRMS (ESI$^-$)** calcd for $C_{26}H_{17}F_6N_4O_4$ $(M-H)^-$: 563.1160, found: 563.1160 (-0.10 ppm).



3

1,1'-(4-Methyl-2-oxo-2H-chromene-6,7-diyl)bis(3-(3,5-bis(trifluoromethyl)phenyl)urea) **4**: Using the general procedure A and *p*-cyanophenyl isocyanate (428 mg, 1.68 mmol), compound **4** (78 mg, 27%) was isolated as a buff solid. **^{1}H NMR (400**



4

MHz, DMF-d_7) δ: 2.49 (s, 3H, CH_3), 6.31 (s, 1H, ArH),
7.68 (s, 1H, ArH), 7.71 (s, 1H, ArH), 8.05 (d, *J* = 7.0 Hz, 4H, ArH), 8.23-8.26 (m, 4H, ArH), 8.99 (s, 1H, NH), 9.41 (s, 1H, NH), 10.54 (s, 1H, NH), 10.80 (s, 1H, NH); **^{13}C NMR (101 MHz, DMF-d_7) δ:** 17.7, 107.1, 112.8, 114.7, 117.8, 119.7, 121.6, 122.4, 122.4, 124.5, 125.1, 127.8, 127.8, 131.4, 137.1, 142.1, 142.7, 151.4, 152.6, 153.1, 153.8, 160.3; **^{19}F (376 MHz, DMF-d_7) δ**: -61.36, -61.44. **LRMS ESI$^+$ (*m/z*)**: 723 (100%, M+Na)$^+$. **HRMS (ESI$^+$)** calcd for $C_{28}H_{16}F_{12}N_4O_4Na$ (M+Na)$^+$: 723.0872, found: 723.0875 (0.4 ppm).

S3. ^{1}HNMR and ^{13}C NMR Data

Figure S1: 7-Carbethoxyamino-4-methylcoumarin (6)

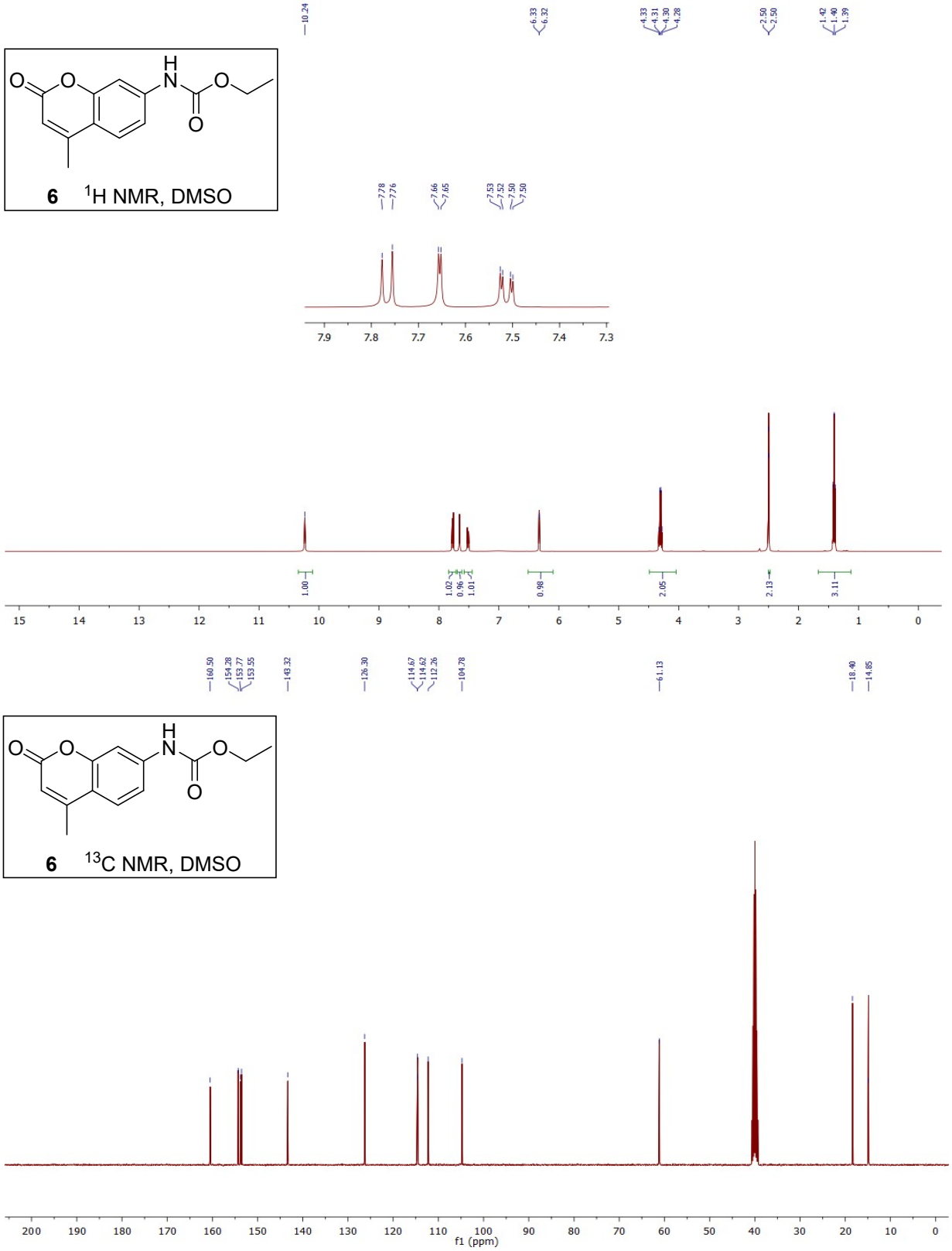
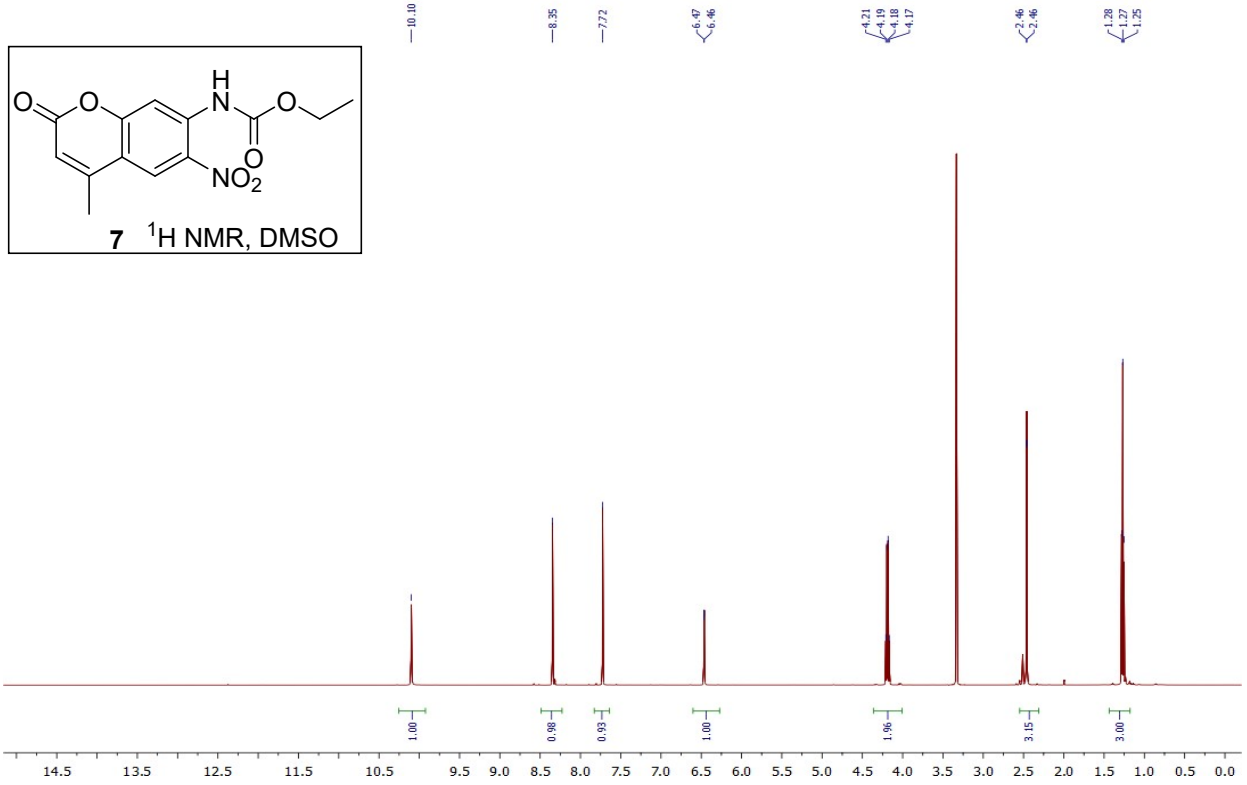


Figure S2: 6-Nitro-7- carbethoxyamino-4-methylcoumarin (7)



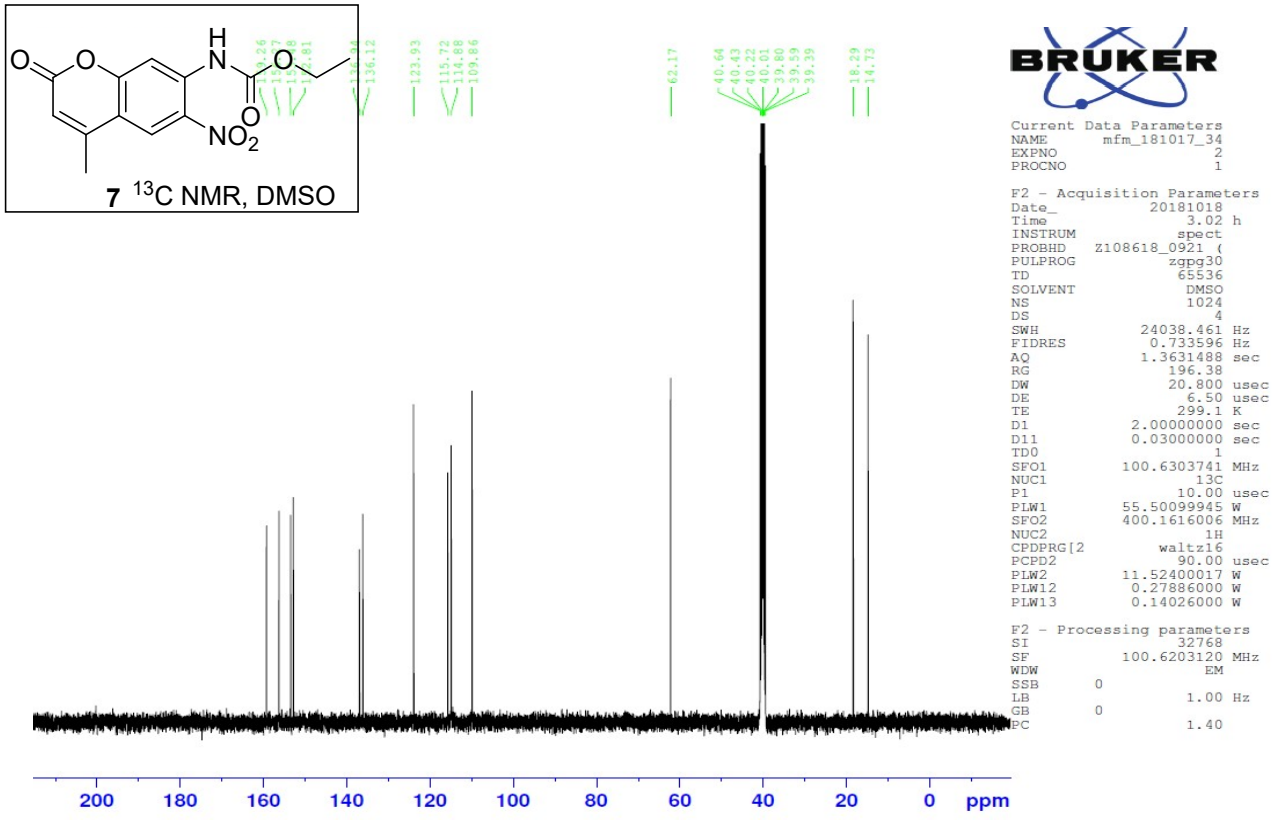
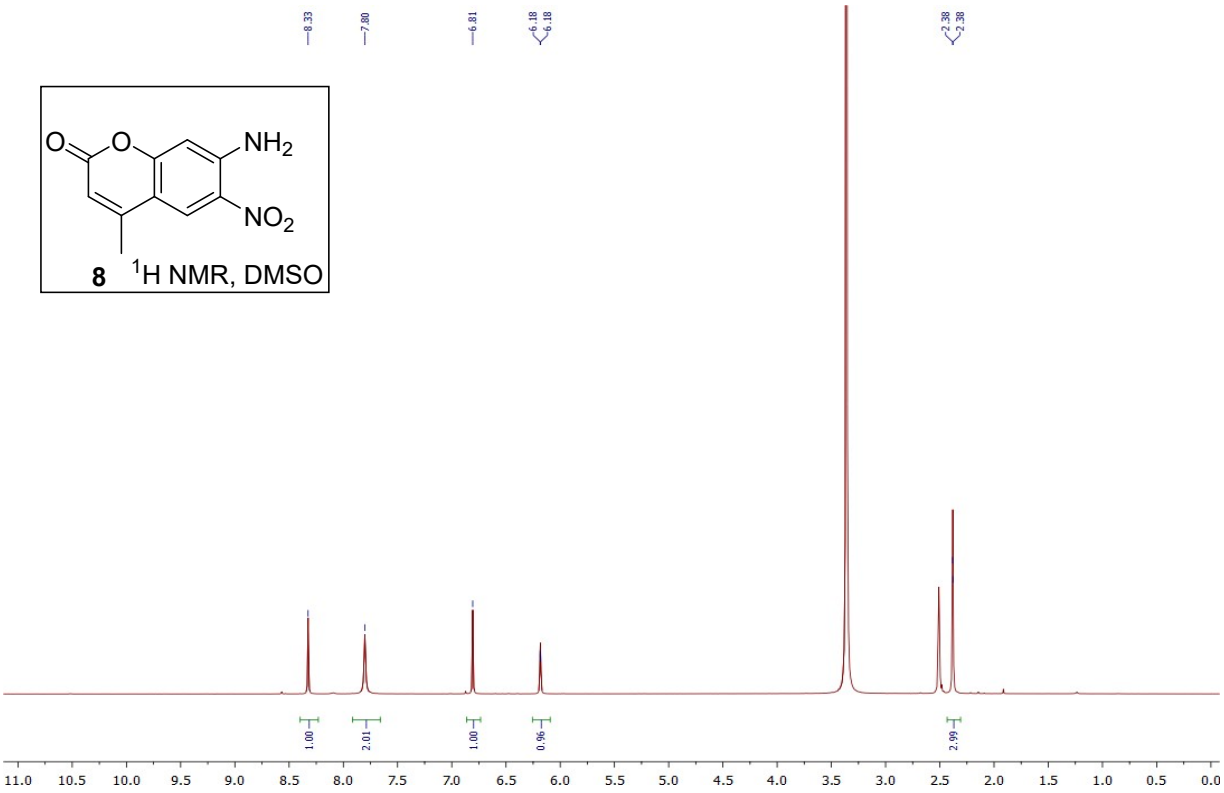


Figure S3: 6-Nitro-7-amino-4-methylcoumarin (8)



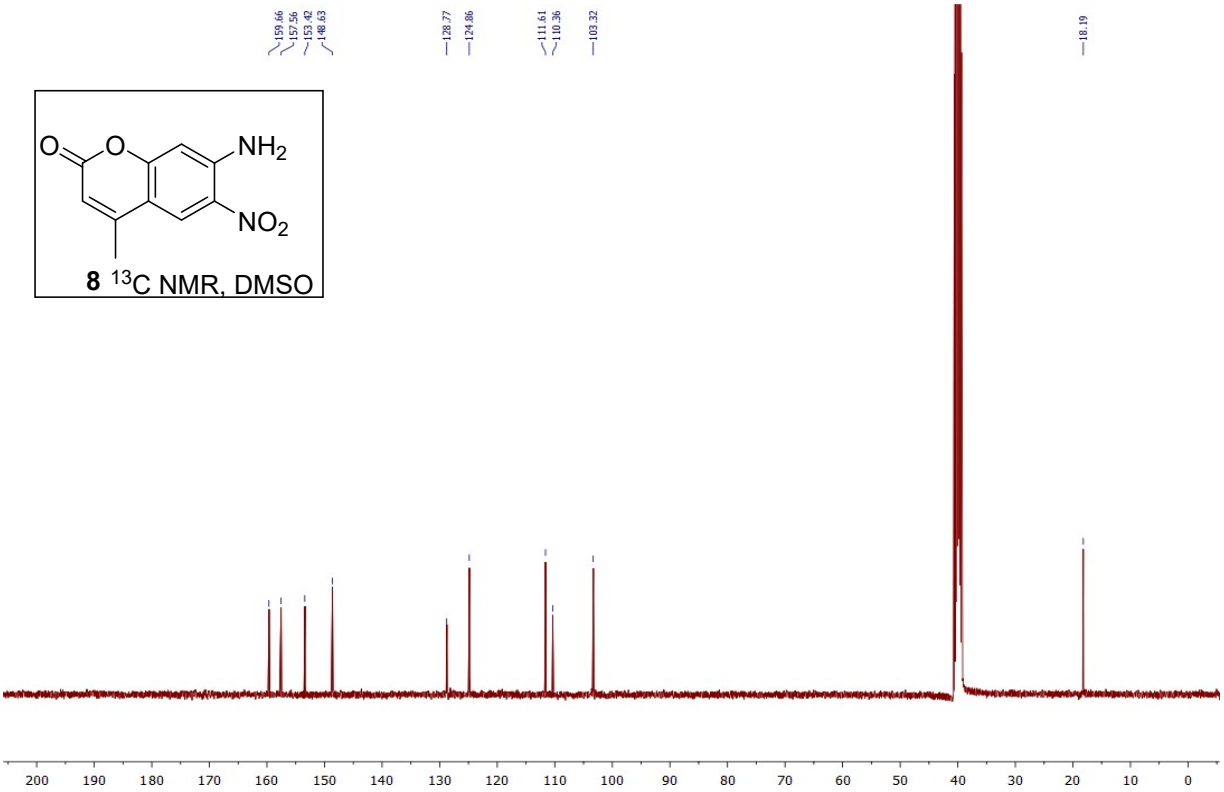
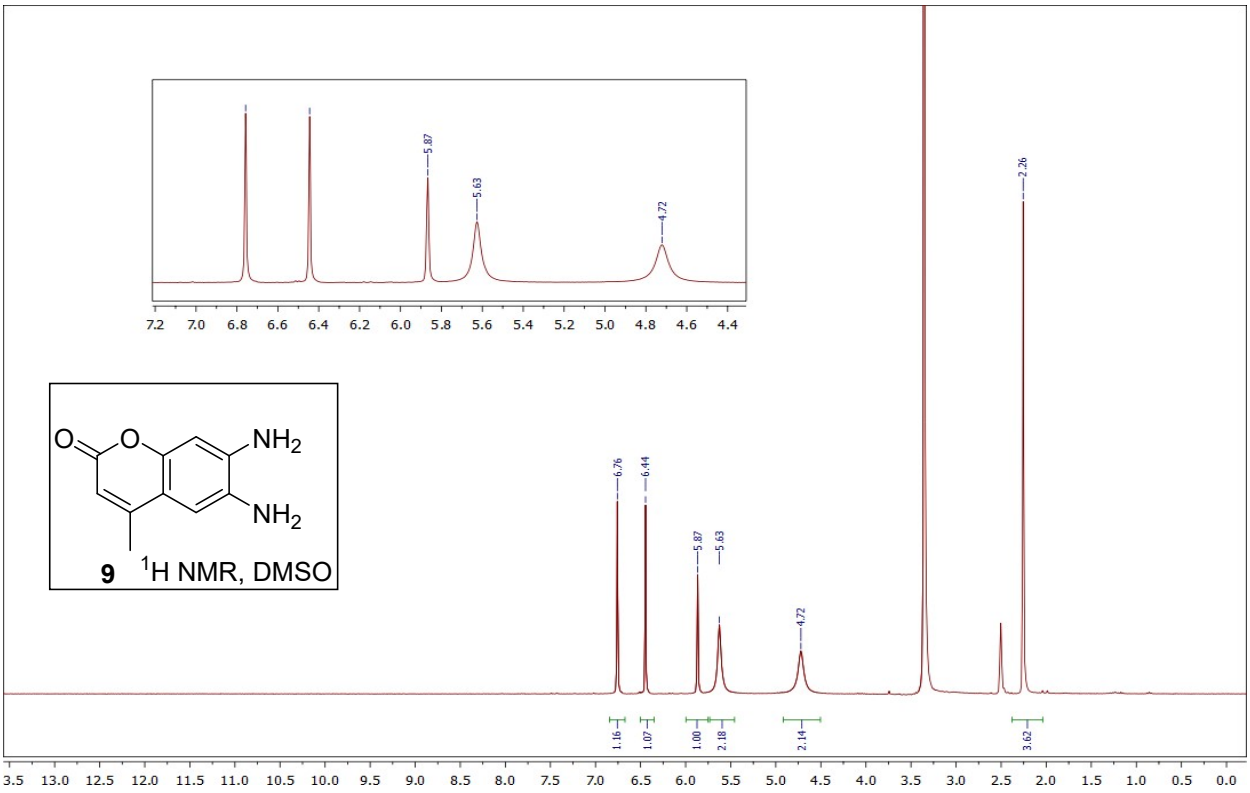


Figure S4: 6,7-Diamino-4-methylcoumarin (9)



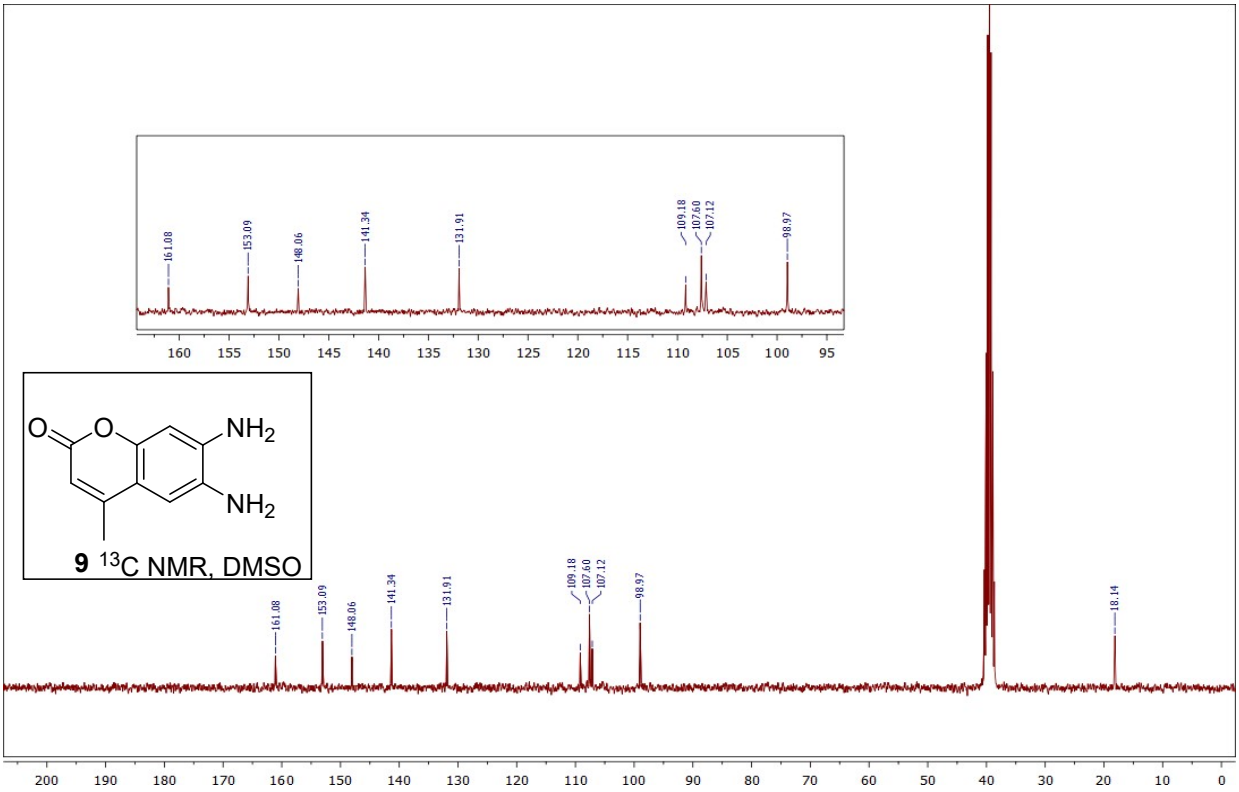
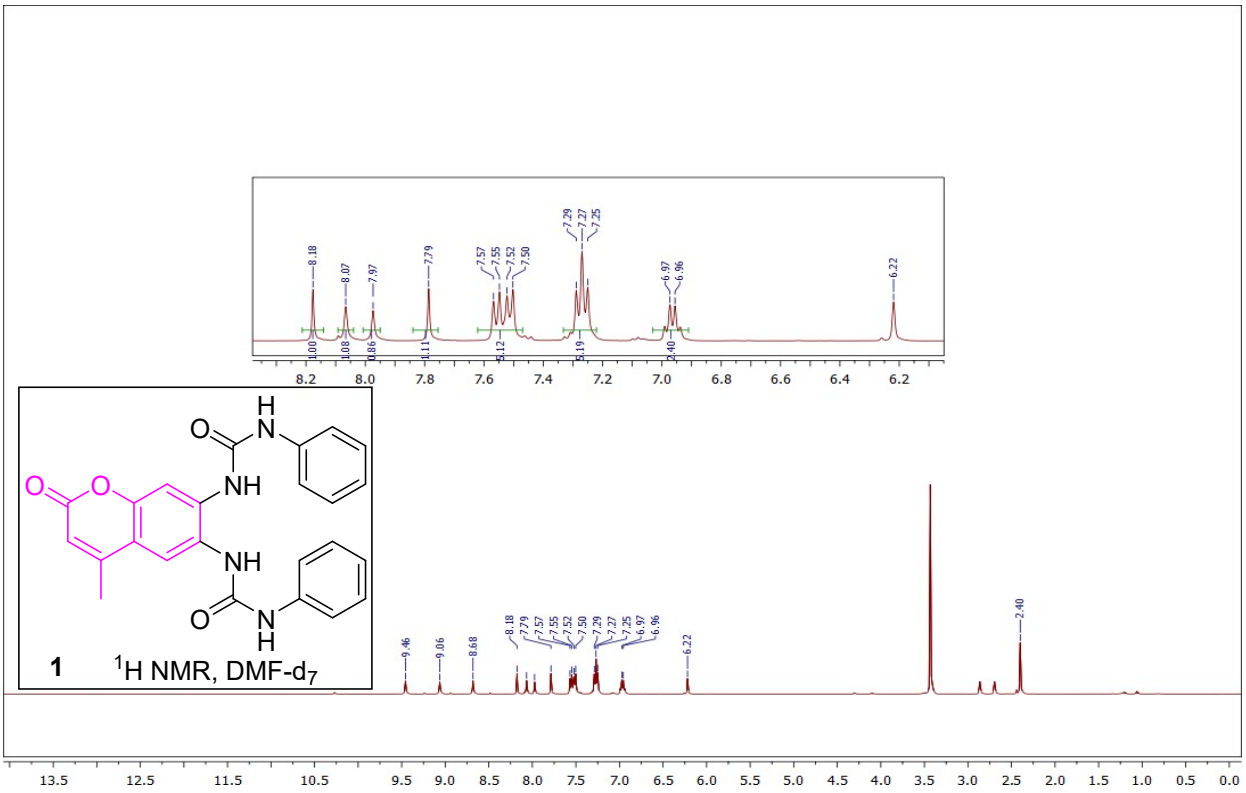
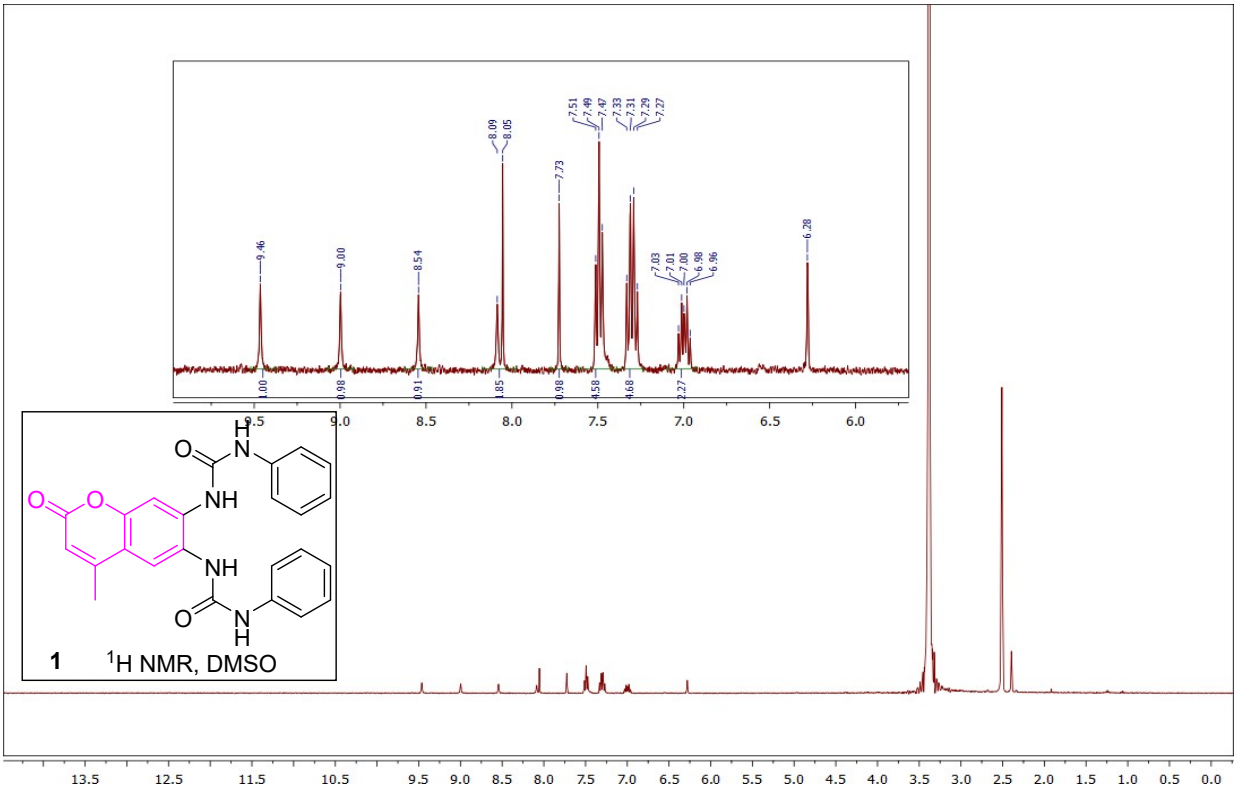
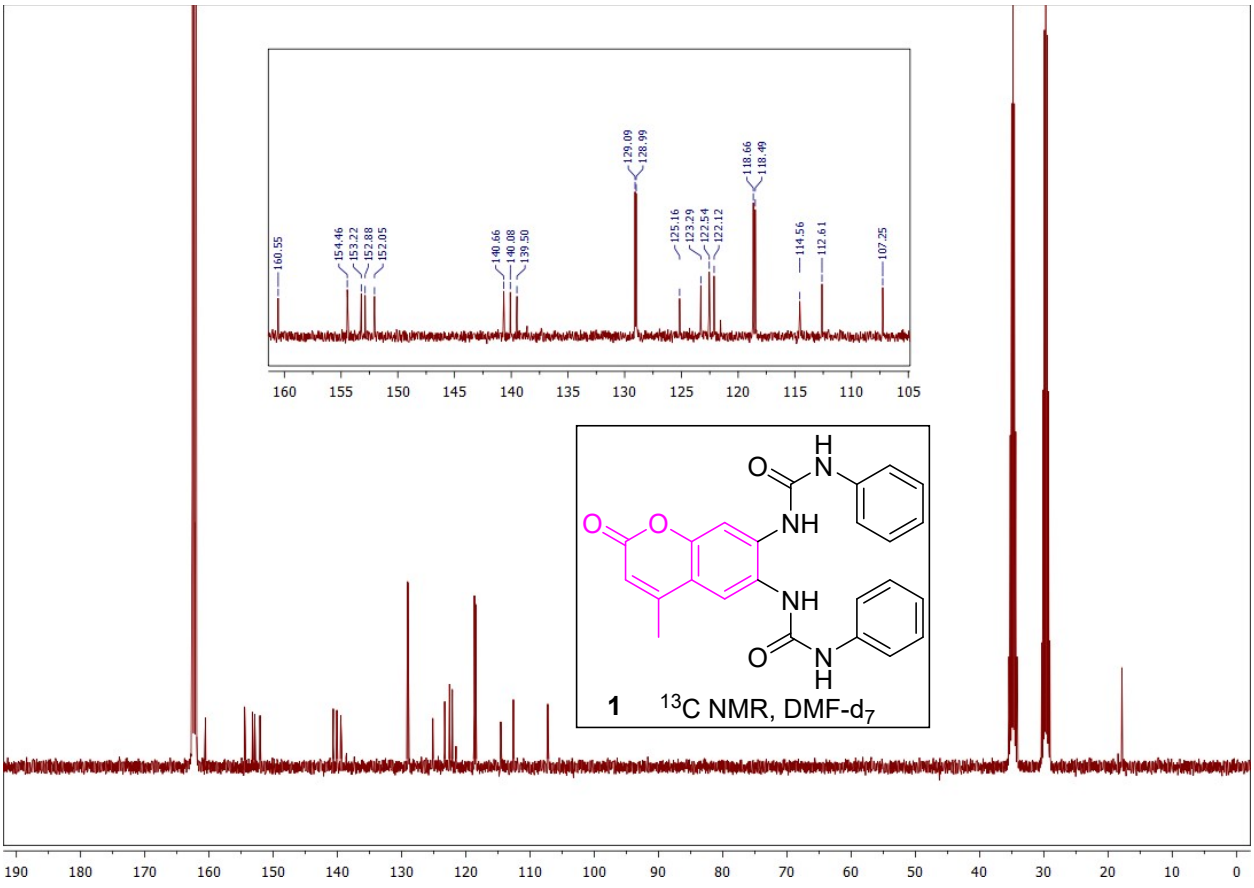


Figure S5: 1,1'-(4-methyl-2-oxo-2*H*-chromene-6,7-diyl)bis(3-phenylurea) (1)






160.55
154.46
153.22
152.88
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140.66
140.08
139.50
129.09
128.99
125.16
123.29
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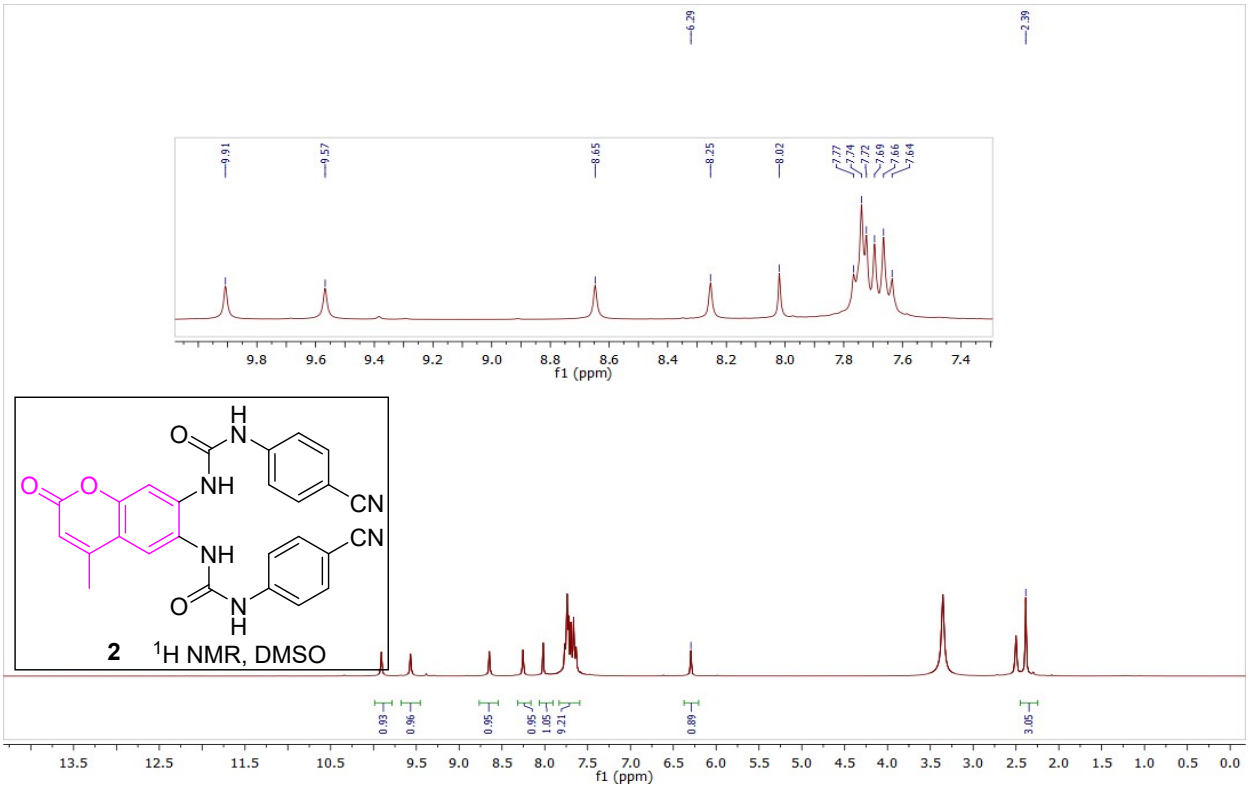
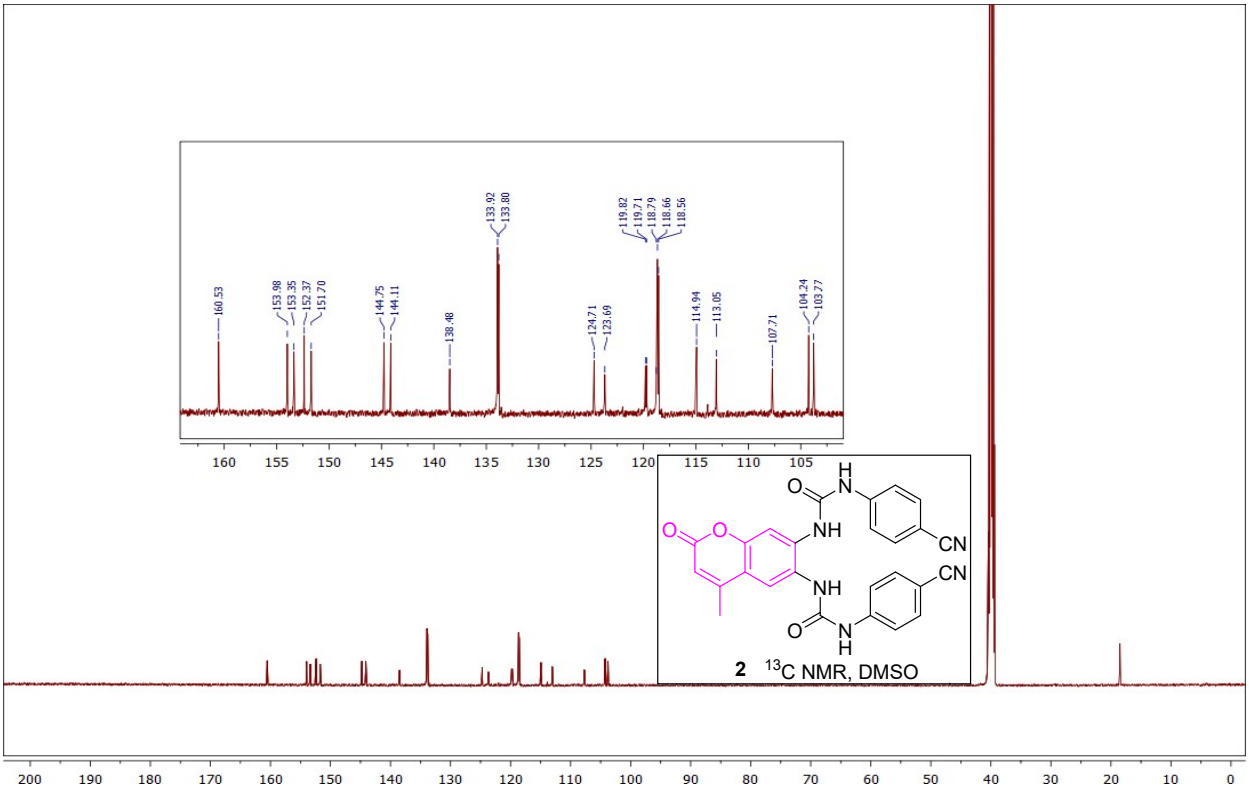
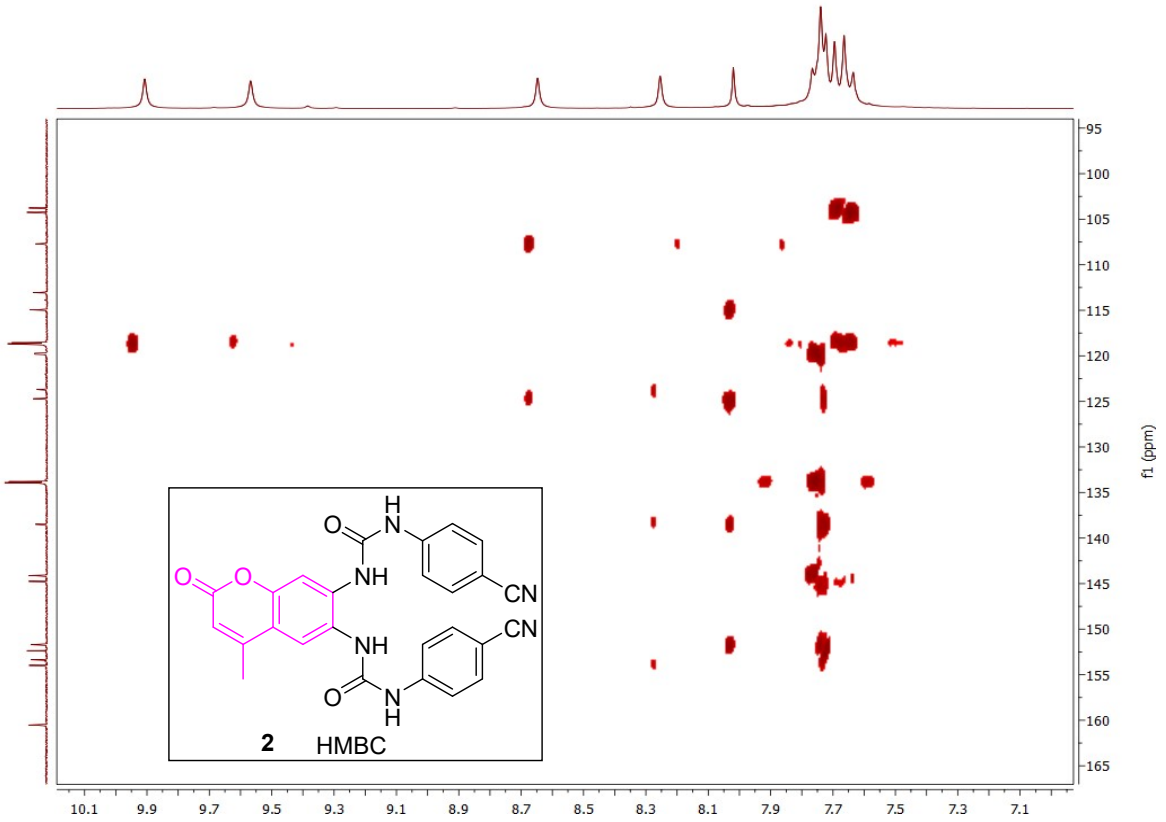
122.12
118.66
118.49
114.56
112.61
107.25
160 155 150 145 140 135 130 125 120 115 110 105
O
H
N
NH
O
O
NH
O
N
H
1 ^{13}C NMR, DMF-d_7
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0

Figure S6: 1,1'-(4-methyl-2-oxo-2*H*-chromene-6,7-diyl)bis(3-(4-cyanophenyl)urea) (2)






f1 (ppm)
2 HMBC

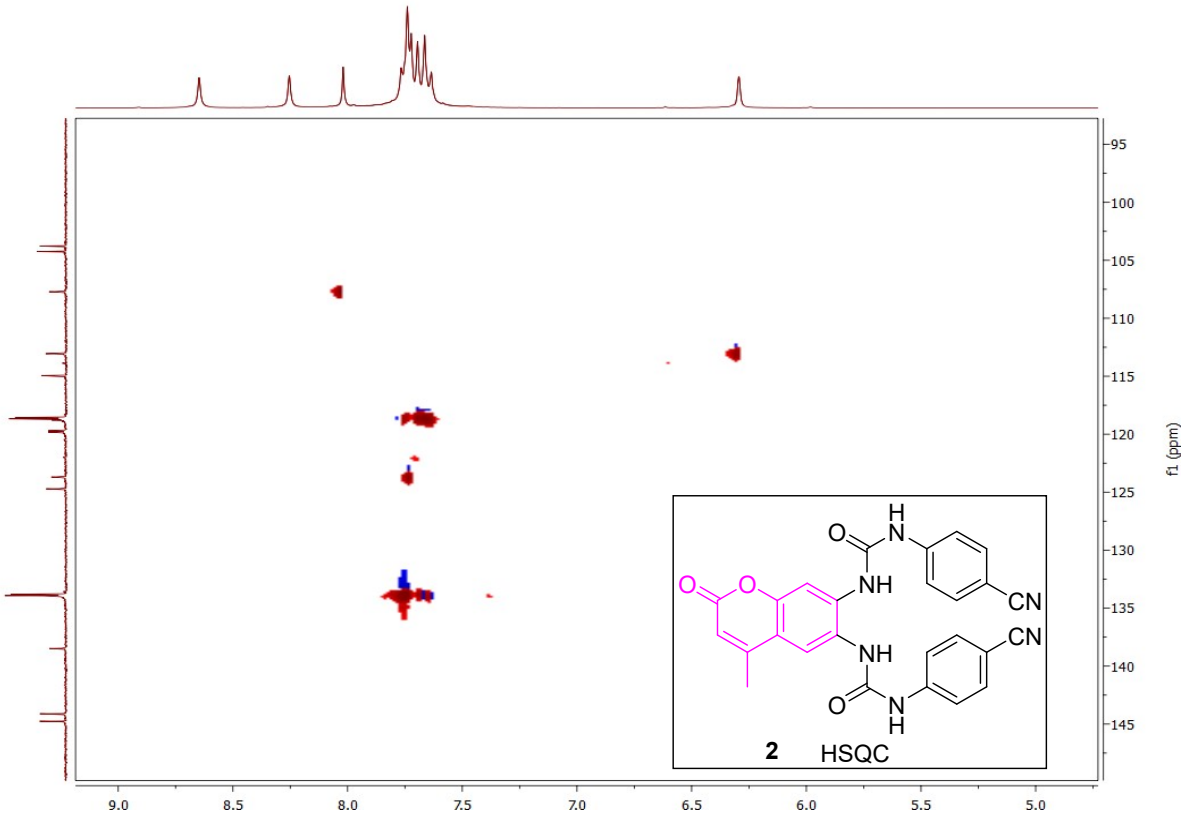
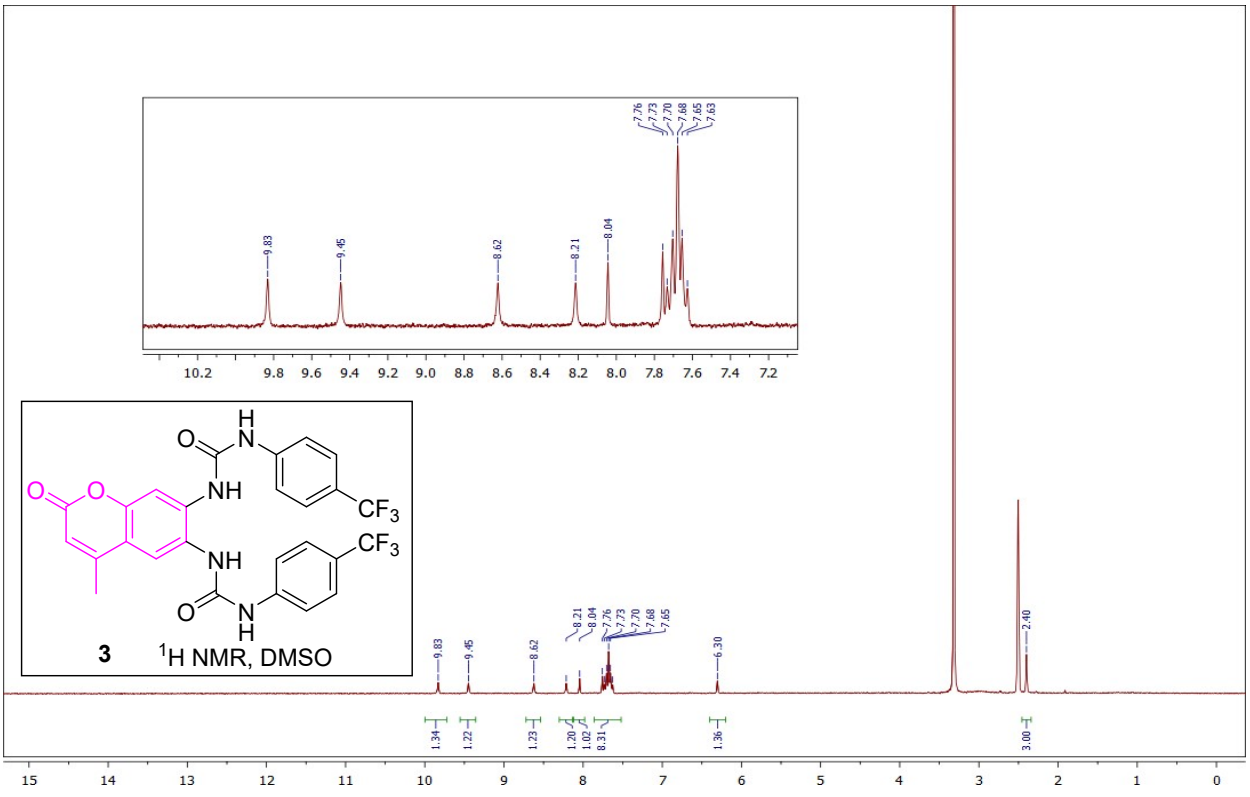
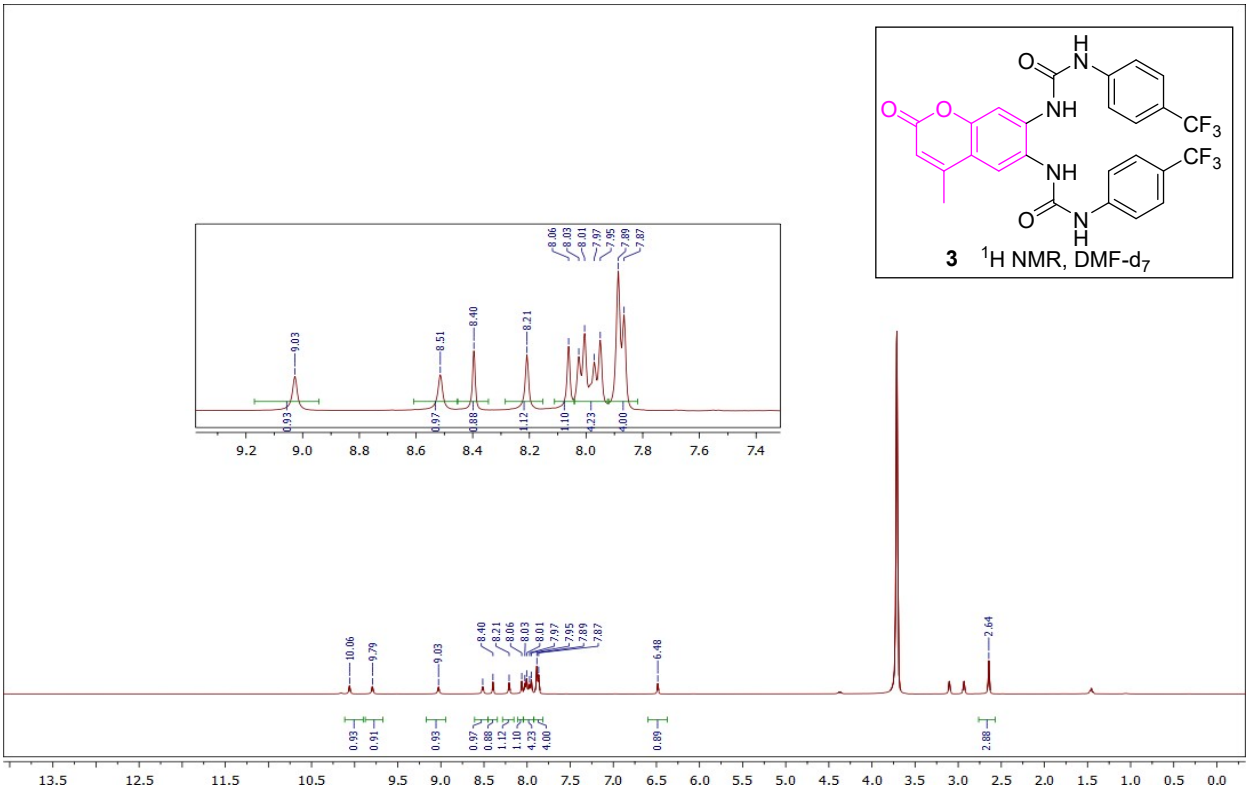
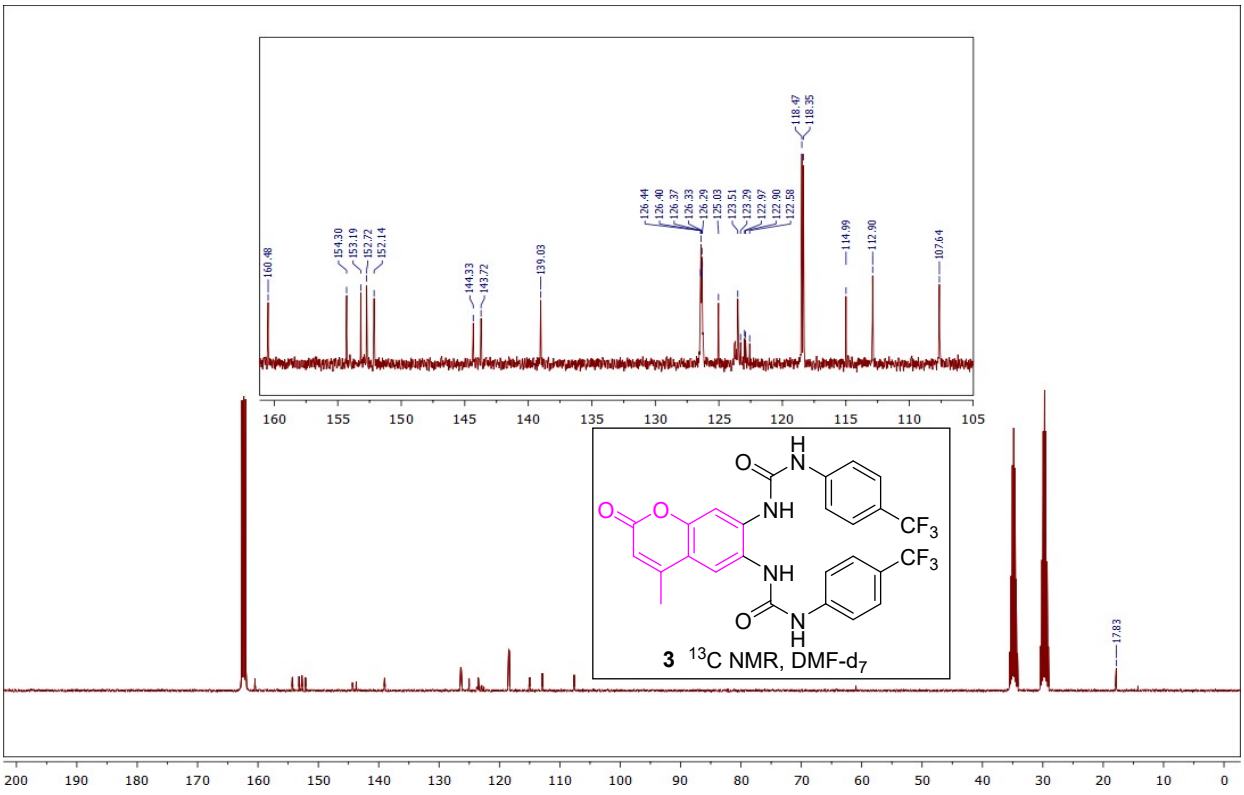

f1 (ppm)
2 HSQC

Figure S7: 1,1'-(4-methyl-2-oxo-2H-chromene-6,7-diyl)bis(3-(4-(trifluoromethyl)phenyl)urea) (3)



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154.30
153.19
152.72
152.14
144.33
143.72
139.03
126.44
126.40
126.37
126.33
126.29
125.03
123.51
123.29
122.97
122.90
122.58
118.47
118.35
114.99
112.90
107.64
17.83
3 13C NMR, DMF-d7

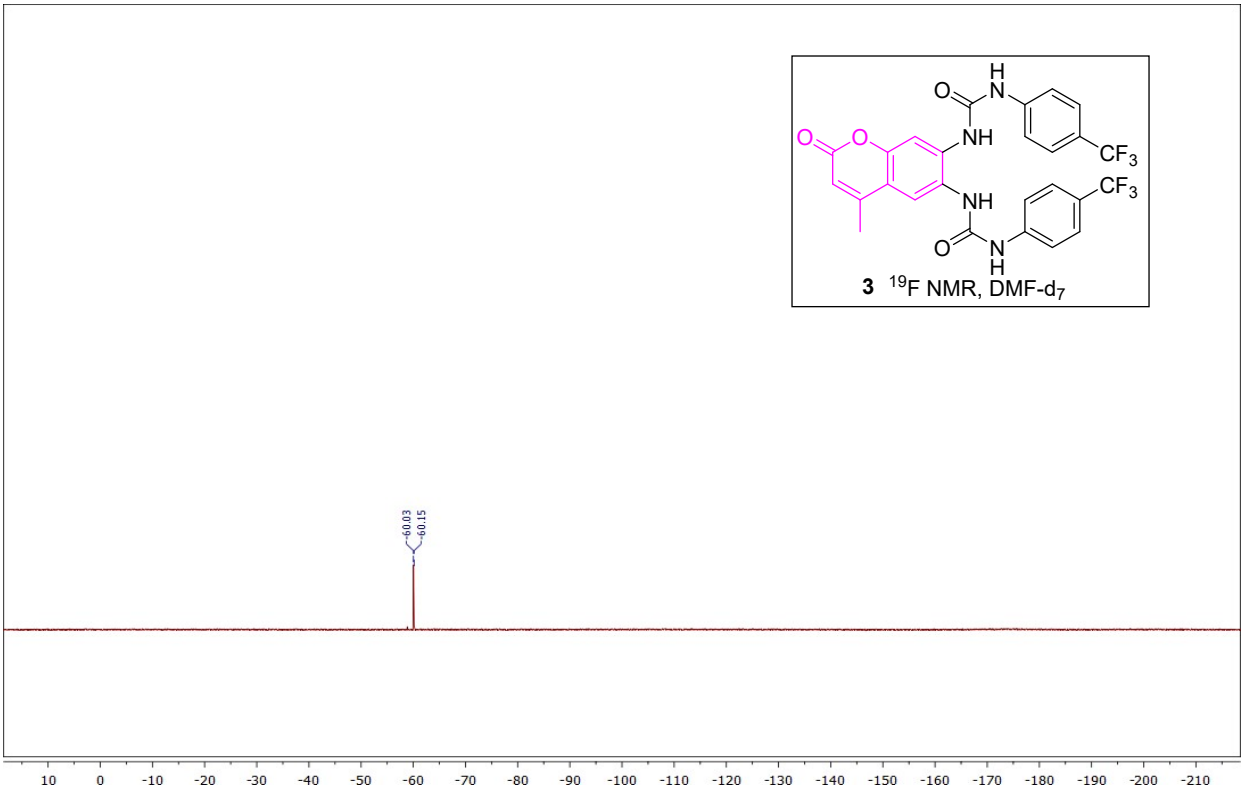
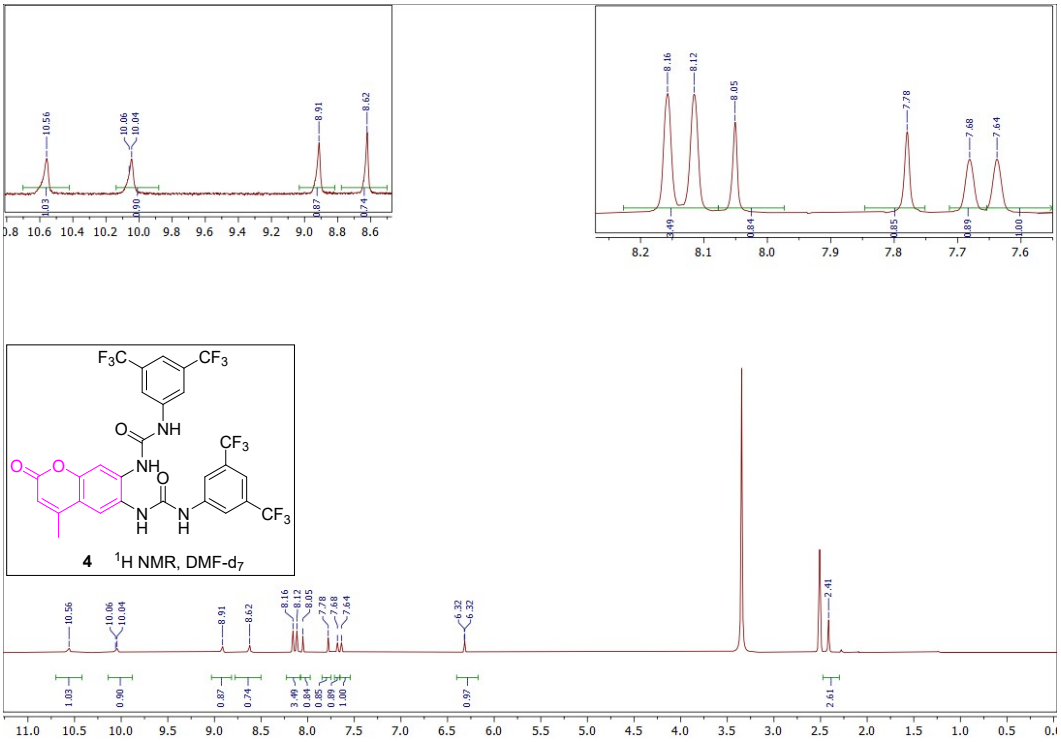
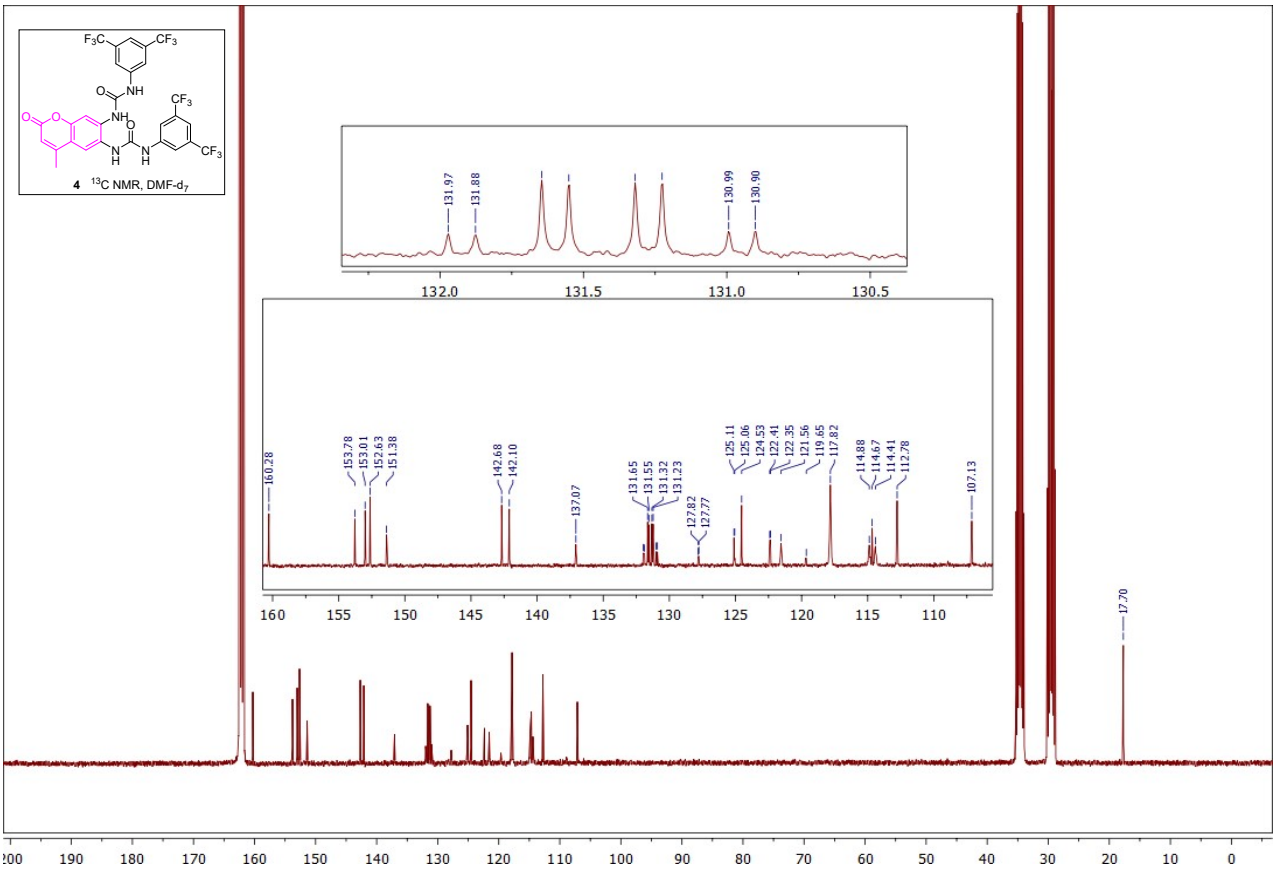
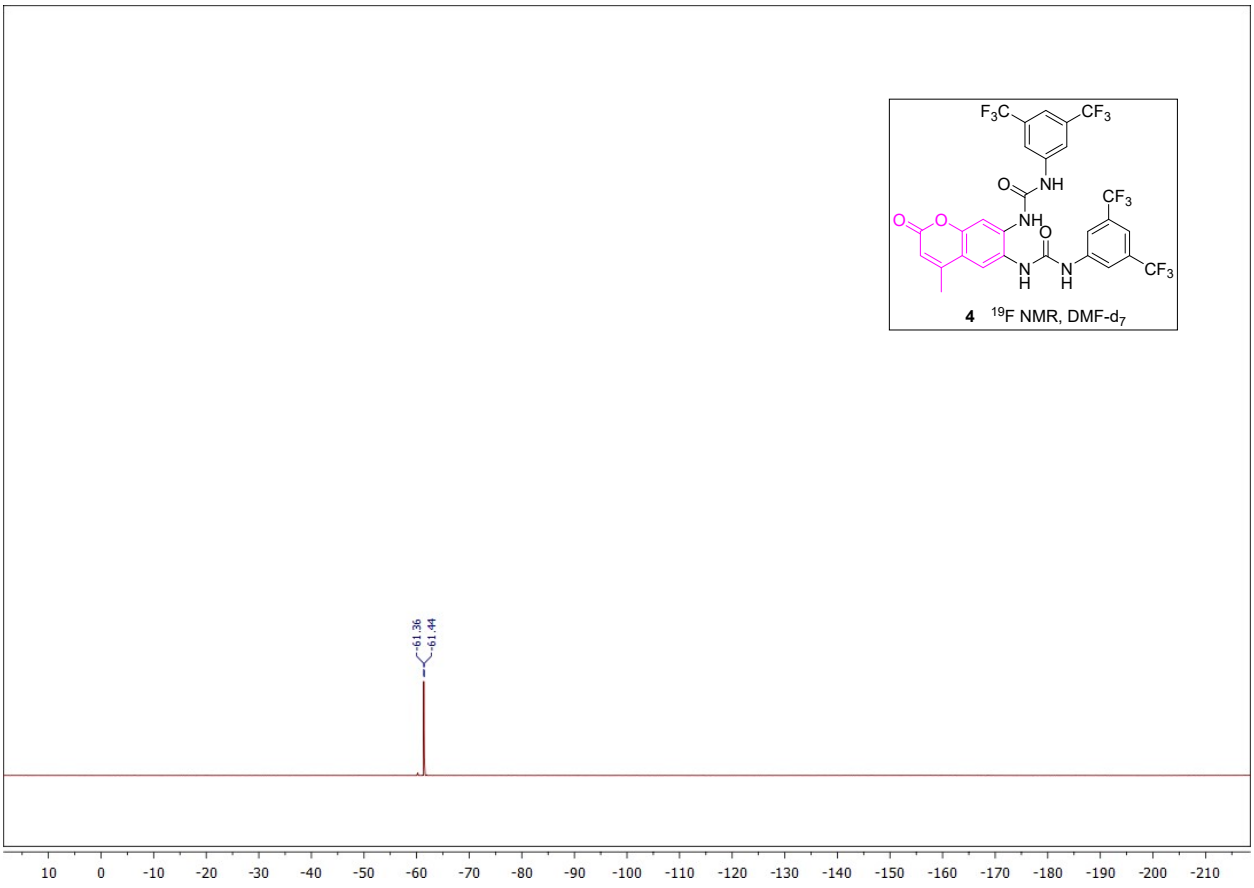

60.03
60.15
3 19F NMR, DMF-d7

Figure S8: 1,1'-(4-methyl-2-oxo-2H-chromene-6,7-diyl)bis(3-(3,5-bis(trifluoromethyl)phenyl)urea) (4)






F3C
CF3
O
NH
NH
CF3
O
O
O
N
H
N
H
CF3
4 19F NMR, DMF-d7
61.36
61.44
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
-130
-140
-150
-160
-170
-180
-190
-200
-210

S4. X-ray Crystallography:

S5.1. X-ray of complex 1

Single crystals of transporter **1** $C_{26}H_{26}N_4O_5$ were crystallised from a mixture of dimethylformamide and ethanol. A suitable crystal was selected and in Paratone on a micromount on a SuperNova, Dual, Cu at home/near, Atlas diffractometer. The crystal was kept at 100 K during data collection. Using Olex2[3], the structure was solved with the ShelXS[4] structure solution program using Direct Methods and refined with the ShelXL[5] refinement package using Least Squares minimization.

Crystal Data for $C_{26}H_{26}N_4O_5$ (*M* =474.51 g/mol): monoclinic, space group P2/c (no. 13), *a* = 28.2772(10) Å, *b* = 11.1135(9) Å, *c* = 7.9823(3) Å, *β* = 90.234(4)°, *V* = 2508.5(2) Å^3, *Z* = 4, *T* = 100(2) K, μ(Cu Kα) = 0.728 mm^{-1}, *Dcalc* = 1.256 g/cm^3, 19257 reflections measured (7.956° ≤ 2Θ ≤ 145.31°), 4894 unique (R_{int} = 0.0918, R_{sigma} = 0.0957) which were used in all calculations. The final R_1 was 0.0661 (I > 2σ(I)) and wR_2 was 0.2088 (all data). CCDC deposition number 1999498.

Figure S9: Packing diagram viewed down the *b* axis showing intermolecular hydrogen-bonding interactions (green dots). All hydrogens in the packing diagram have been omitted for clarity, except those involved in hydrogen bonding interactions.

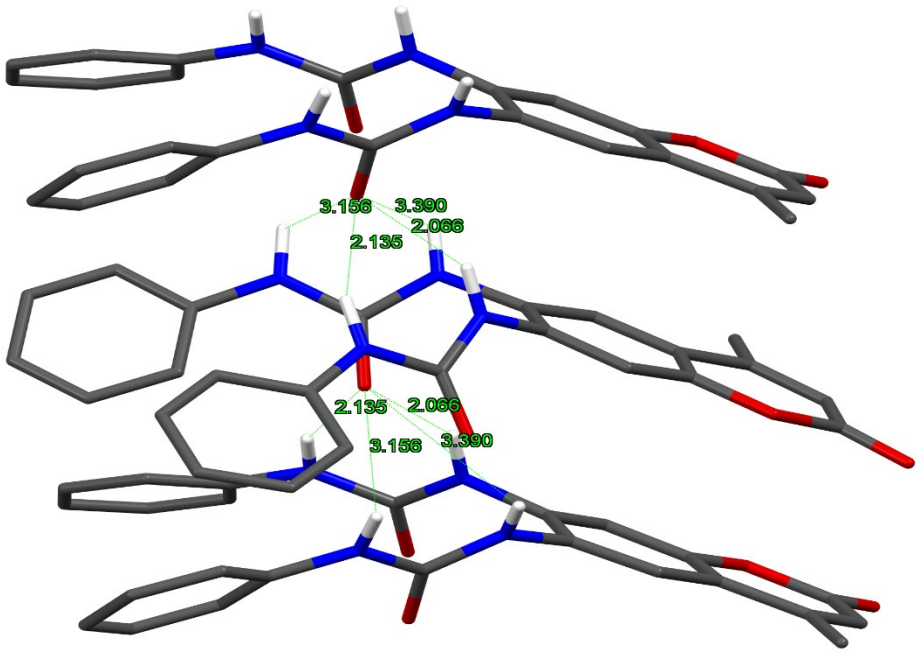
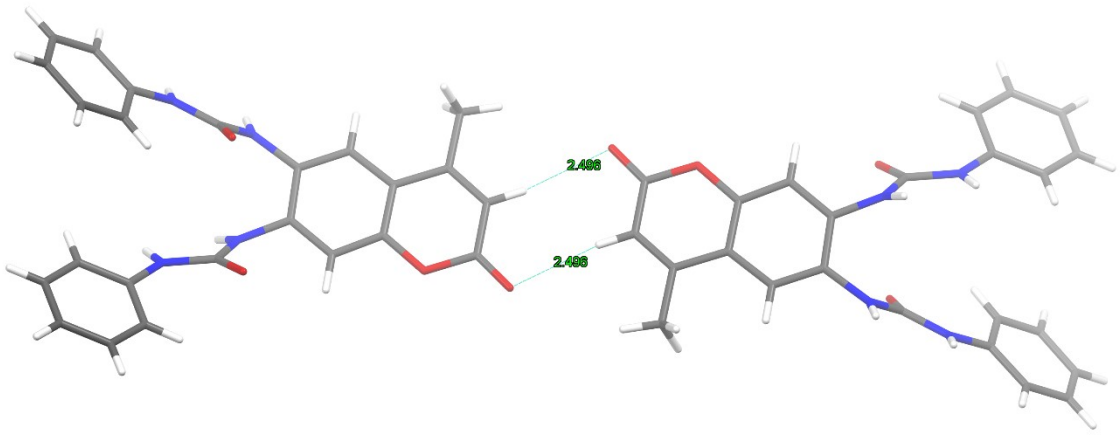


Figure S10: Packing diagram showing intermolecular electrostatic attraction (cyan dots) or receptor **1**.



S5. ^{1}H NMR Binding Studies with TBACl and $TBAH_2PO_4$

Proton NMR titrations binding studies were performed on Bruker 400 MHz FT-NMR spectrometer at 298 K. Solution of receptors **1**-**4** in DMSO-d_6/0.5% H_2O were prepared in 2 mM concentrations. The guest anion, tetra-*n*-butylammonium (TBA) chloride or $TBAH_2PO_4$, was prepared with the same receptor solution, to ensure the overall receptor concentration stays constant whilst the guest anion concentration changes. Using small aliquots of the guest salt, the receptor solution was titrated, and after each addition, chemical shifts were reported in ppm in refence to residual solvent peaks. The isotherm based on the four NH chemical shifts were globally fitted using the online fitting program Bindfit.[6]

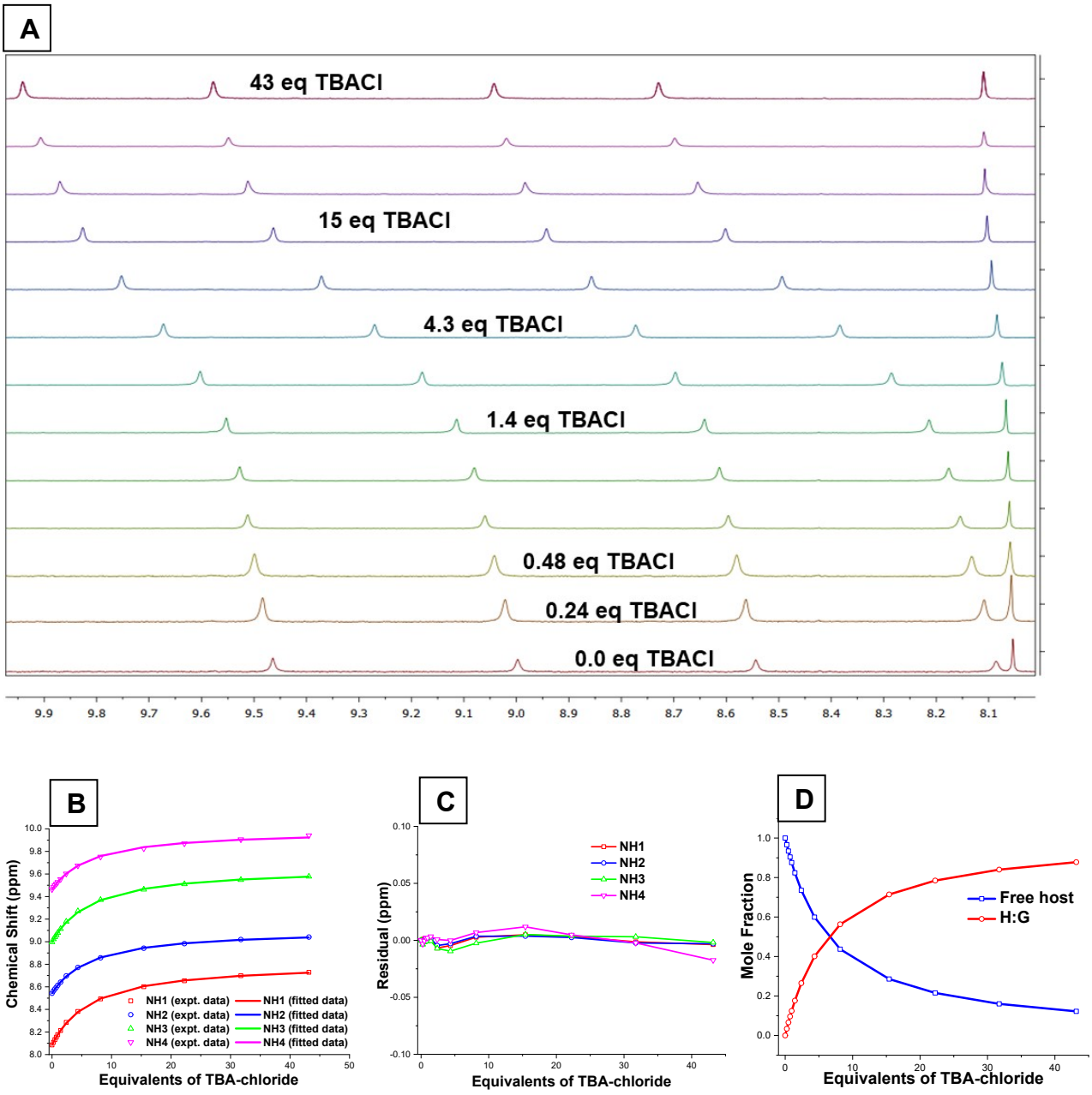


Figure S11: ^{1}H NMR spectroscopic titration of receptor **1** (2 Mm) with TBACl in DMSO-d_6 with 0.5% water at 298 K. (a) Stack plot. (b) Fitplot for 4 NH protons at δ = 8.09, 8.54, 9.00 and 9.46 ppm using global analysis with 1:1 (K_a = 81 M^{-1}, error: 1%). (c) Plot of the residuals for using global analysis. (d) Calculated mole fractions.

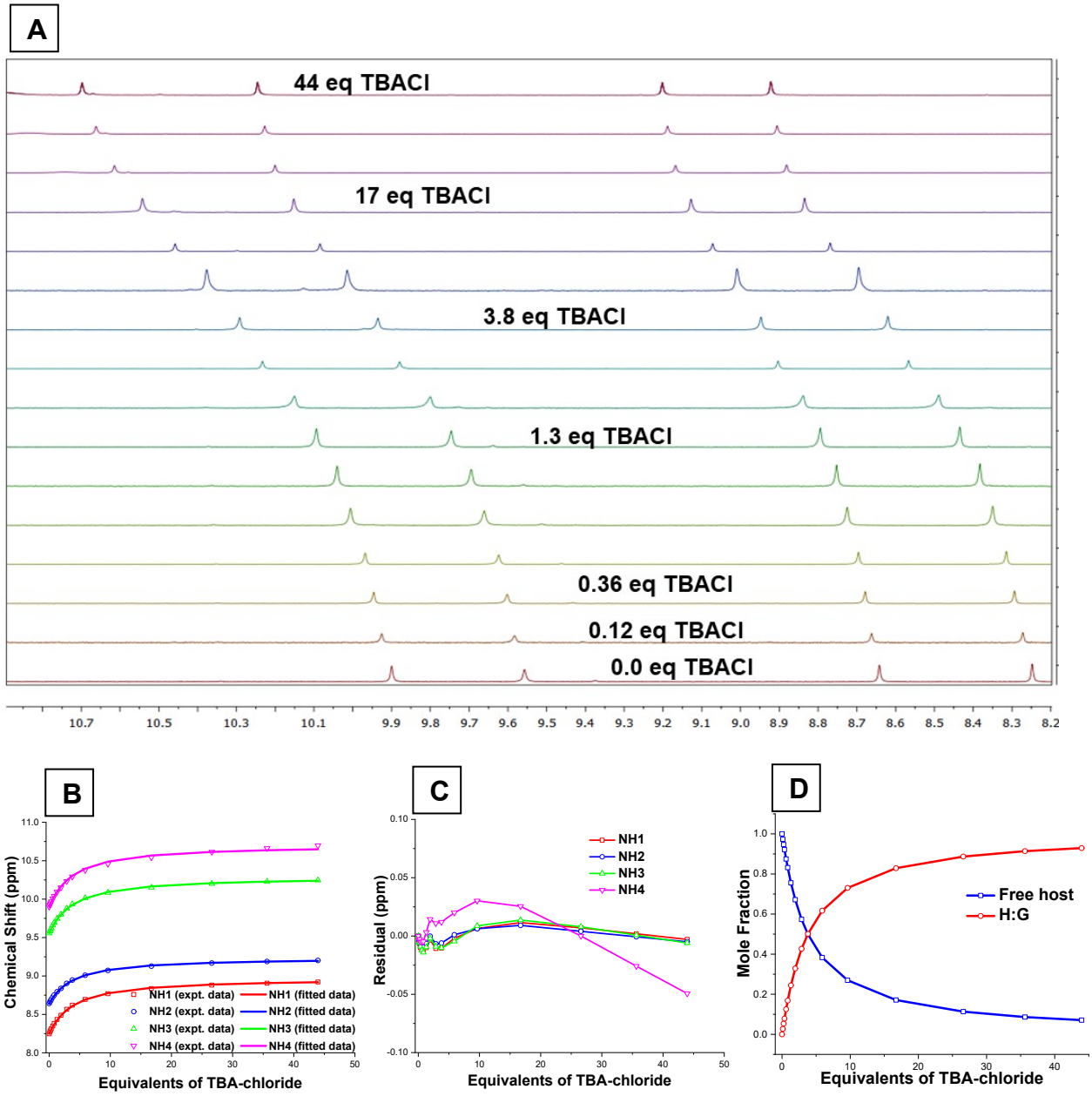


Figure S12: ^{1}H NMR spectroscopic titration of receptor **2** (2 Mm) with TBACl in DMSO-d_6 with 0.5% water at 298 K. (a) Stack plot. (b) Fitplot for 4 NH protons at δ = 8.25, 8.64, 9.56 and 9.90 ppm using global analysis with 1:2 (K_{11}: 186 M^{-1}; K_{12}: 2 M^{-1}). (c) Plot of the residuals for using global analysis. (d) Calculated mole fractions.

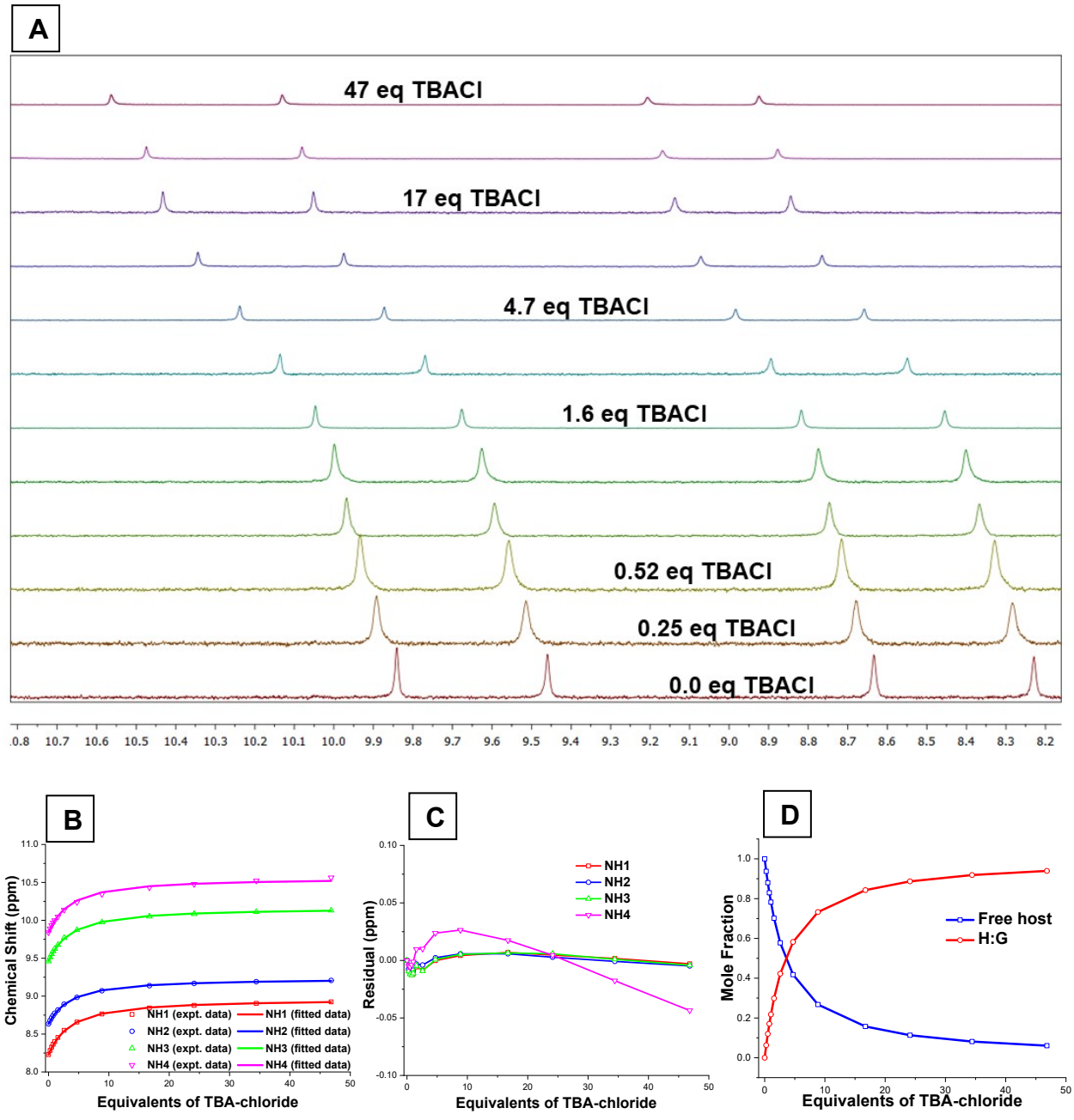


Figure S13: ^{1}H NMR spectroscopic titration of receptor **3** (2 Mm) with TBACl in DMSO-d_6 with 0.5% water at 298 K. (a) Stack plot. (b) Fitplot for 4 NH protons at δ = 8.23, 8.63, 9.46 and 9.84 ppm using global analysis with 1:2 (K_{11}: 239 M^{-1}; K_{12}: 9 M^{-1}). (c) Plot of the residuals for using global analysis. (d) Calculated mole fractions.

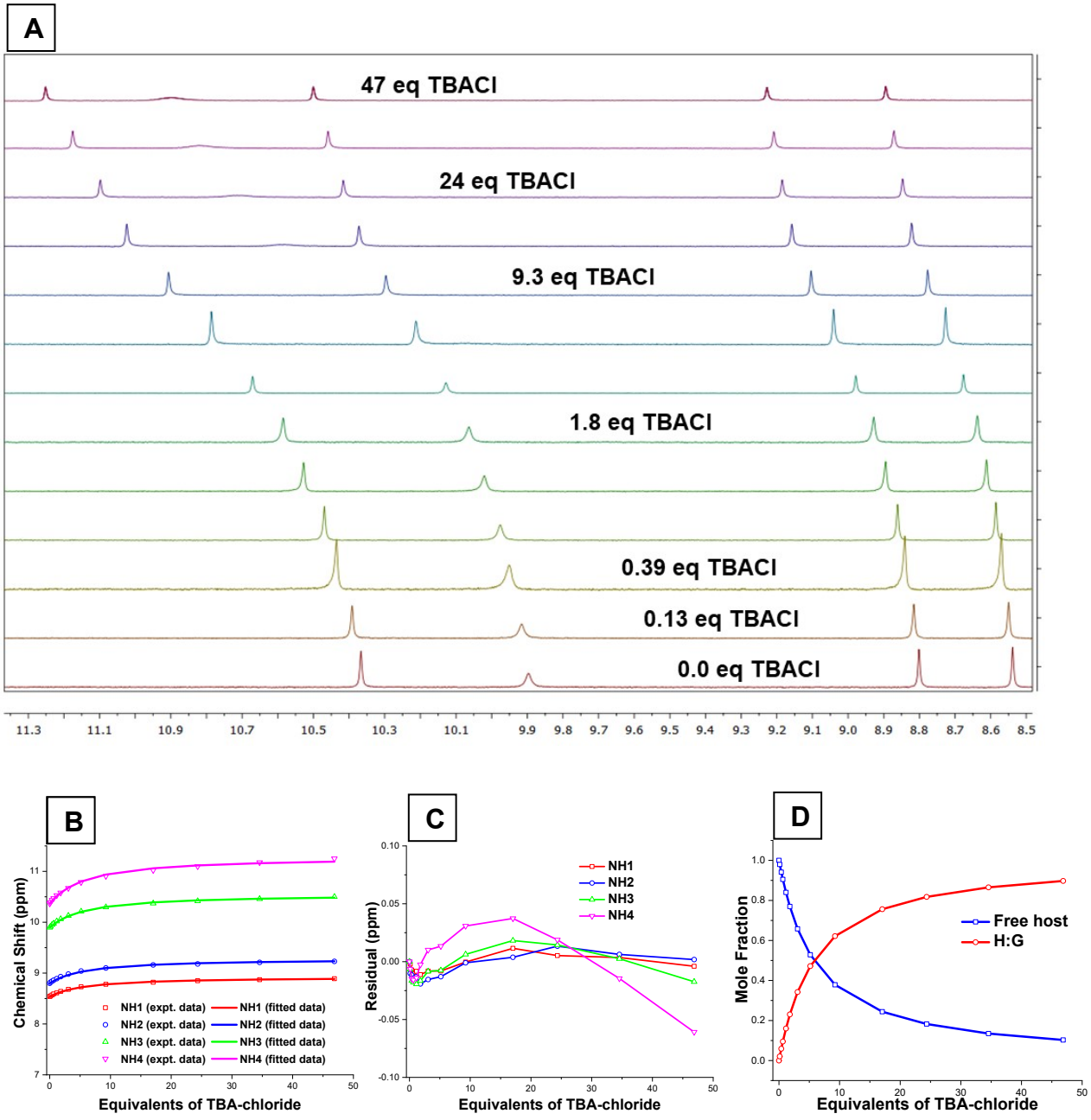


Figure S14: ^{1}H NMR spectroscopic titration of receptor **4** (2 Mm) with TBACl in DMSO-d_6 with 0.5% water at 298 K. (a) Stack plot. (b) Fitplot for 4 NH protons at δ = 8.54, 8.80, 9.90 and 10.37 ppm using global analysis with 1:2 (K_{11}: 178 M^{-1}; K_{12}: 5 M^{-1}). (c) Plot of the residuals for using global analysis. (d) Calculated mole fractions.

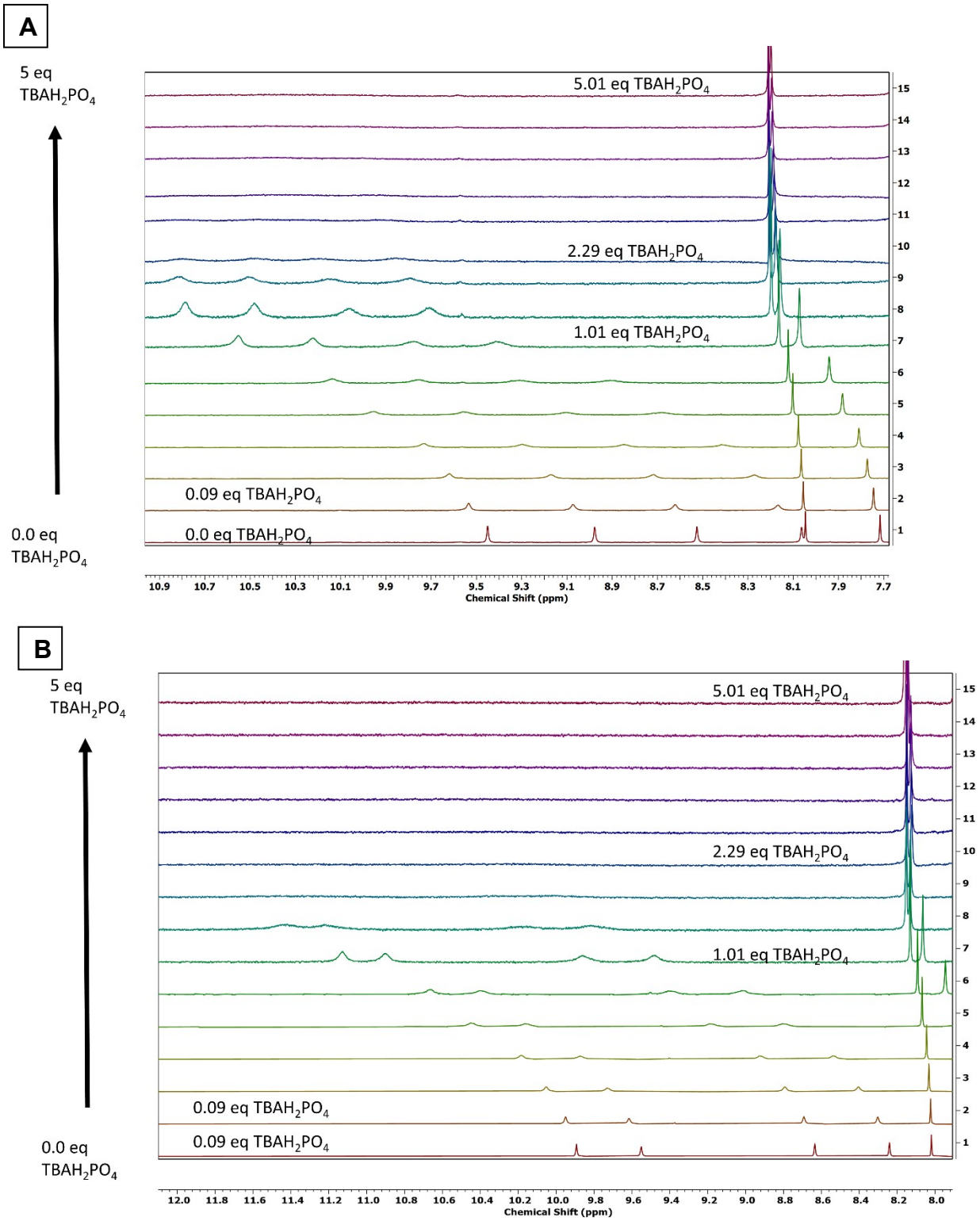


Figure S15: ^{1}H NMR spectroscopic titration of (a) receptor **1** (2 Mm) and (b) receptor **2** (2 Mm) with $TBAH_2PO_4$ in DMSO-d_6 with 0.5% water at 298 K.

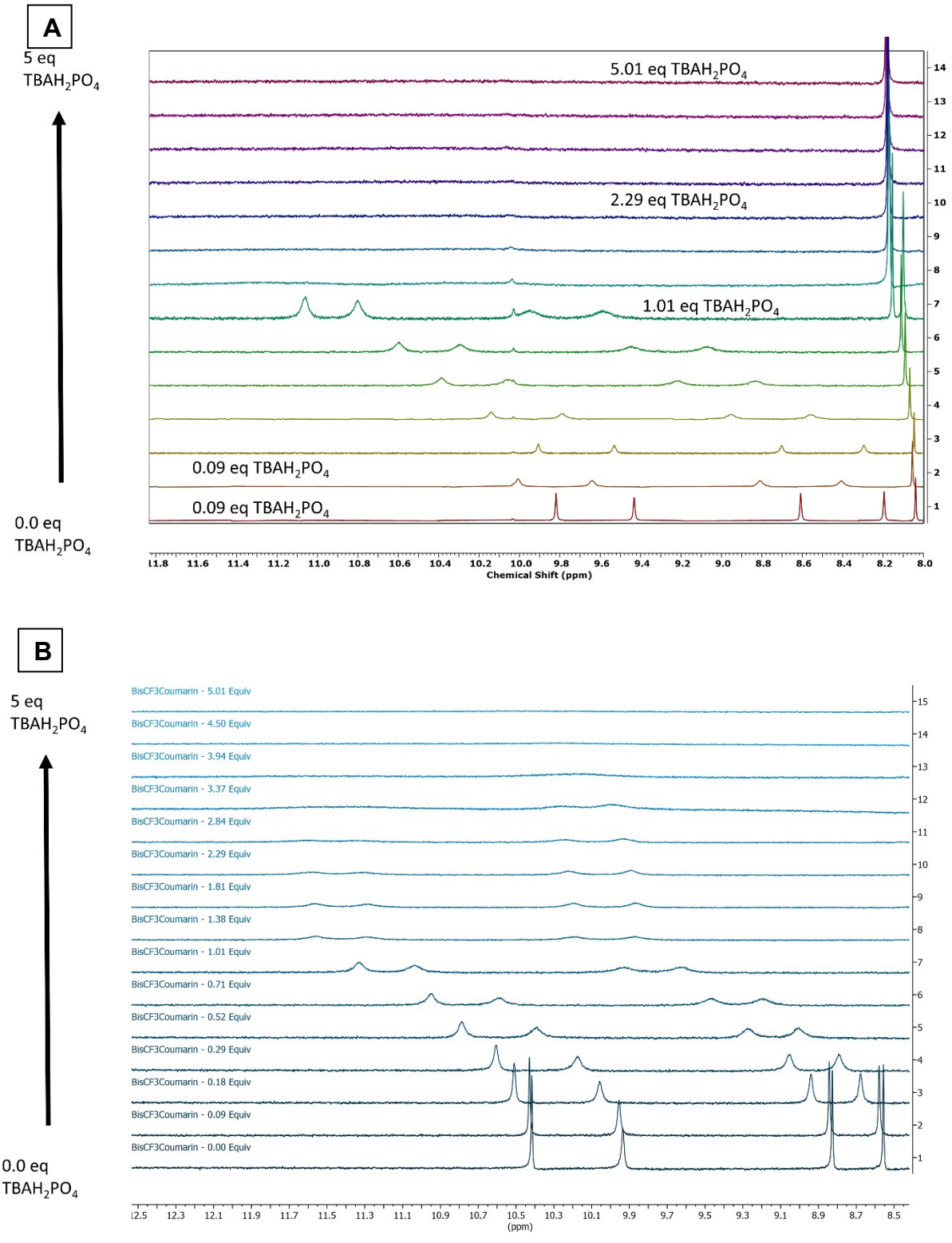


Figure S16: ^{1}H NMR spectroscopic titration of (a) receptor **1** (2 Mm) and (b) receptor **2** (2 Mm) with $TBAH_2PO_4$ in DMSO-d_6 with 0.5% water at 298 K.

S6. Anion Transport Studies

S6.1: Ion selective electrode (ISE) assays:

a- Cl/NO_3 exchange assay:

Unilamellar vesicles were prepared as reported.[7] Briefly, ~ 30 mg of POPC (1-palmitoyl-2-oleoylphosphatidylcholine) was dissolved in 1 mL chloroform in a round-bottomed flask and the solvent was removed in *vacuo* to form a thin lipid layer. The thin film was dried under high vacuum for at least 6 h and was suspended on the internal solution (4 mL) and vortexed using a lab dancer to form large multilamellar vesicles, which was subjected to nine freeze-thaw cycles alternating between water (at room temperature) and liquid nitrogen. Further, the formed lipid was left to rest for 30 minutes and then subjected to extrusion through a 200 nm polycarbonate membranes 25 times to form the unilamellar vesicles. The formed vesicles were subjected to dialysis for 4 h in the desired external solution to remove any unencapsulated internal salts. Finally, using the required external solution, the lipid was diluted to 1.0 mM. The pH of the internal and external solutions was maintained at 7.2 using phosphate buffer with a 500 mM total ionic strength. Test compound in DMSO (10 μL) was added to start the experiment and chloride selective electrode was used to monitor the chloride efflux. Detergent (50 μL) was added after 300 seconds to lyse the vesicles, while the 100% chloride efflux reading was taken at 420 seconds.

a- Hill plots for Cl/NO_3:

Hill plots were performed for Cl/NO_3 exchange assay by conducting transport assay at different concentrations of tested compounds. Receptor concentration vs chloride efflux at 270 s (the endpoint of transport assay) were plotted and fitted to the Hill equation using Origin 2019b:

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

Where y is the chloride efflux at 270 s (%) and x is the tested compound concentration (mol% relative to lipid concentration)

V_{max} is the maximum efflux possible and considered as 100% as this is experimentally the maximum chloride efflux possible.
k (EC_{50} value) is the carrier concentration needed to reach $V_{max}/2$.
Each data point on each Hill plot are an average of at least two repeated runs. Error bars represent standard deviation about the mean.

b- KCl efflux – cationohore coupling:

In this assay, 300 mM total ionic strength of both K gluconate external solution and KCl internal solution were marinated. The vesicles were made in a similar way to Cl/NO_3 exchange assay except that gel filtration, using sephadex, replaced dialysis to allow exchange of any unencapsulated KCl for KGlu. External KGlu solution (10 mL) was used to dilute the lipid solution obtained after sephadex to obtain a lipid stock of known concentration. A cationohore, monensin or valinomycin, (10 µL, 0.5 mM) was added first to the lipid solution at concentration 0.1 mol% with respect to lipid concentration. Then, receptor was added after 30 seconds of the cationohore addition to start the experiment.

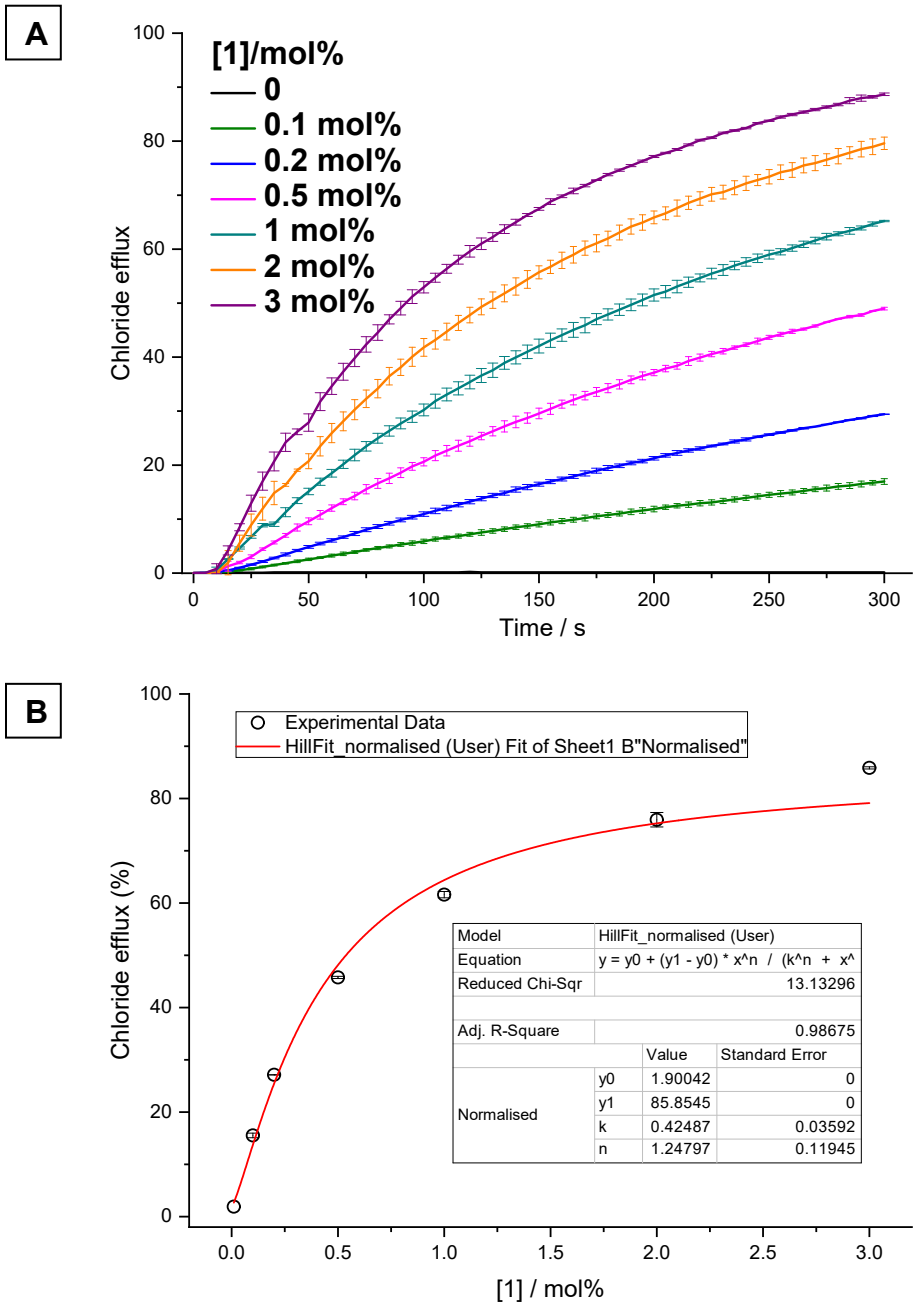


Figure S17: A-Dose response curve and B- Hill plot analysis of chloride efflux facilitated by compound **1** from unilamellar POPC vesicles that were loaded with a 489 mM KCl solution buffered to pH 7.2 with 5 mM phosphate, and were suspended in a 489 mM KNO_3 solution buffered to pH 7.2 with 5 mM phosphate salts. DMSO was used as a control. Each point is the average of at least two repeats.

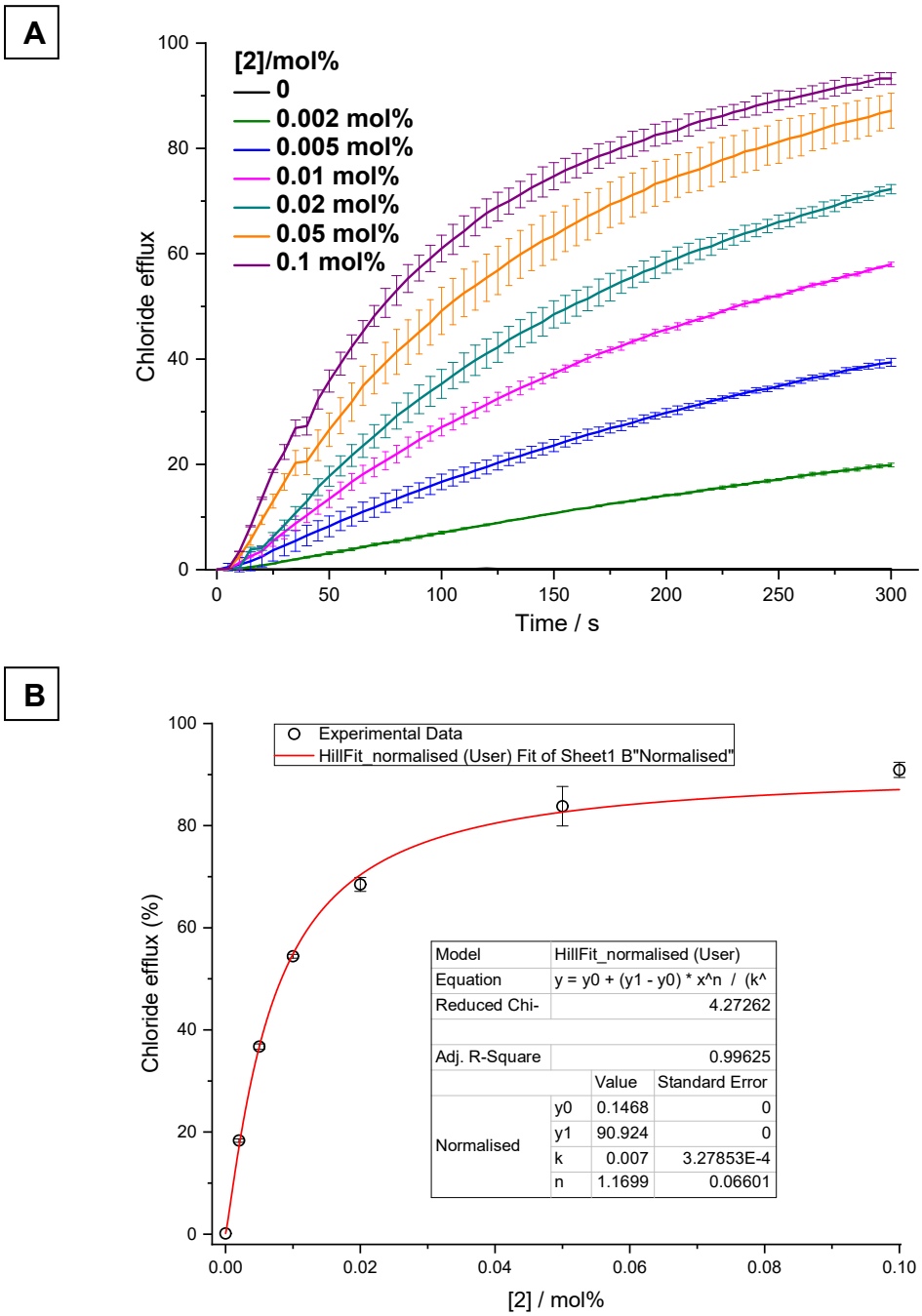


Figure S18: A-Dose response curve and B- Hill plot analysis of chloride efflux facilitated by compound **2** from unilamellar POPC vesicles that were loaded with a 489 mM KCl solution buffered to pH 7.2 with 5 mM phosphate, and were suspended in a 489 mM KNO_3 solution buffered to pH 7.2 with 5 mM phosphate salts. DMSO was used as a control. Each point is the average of at least two repeats.

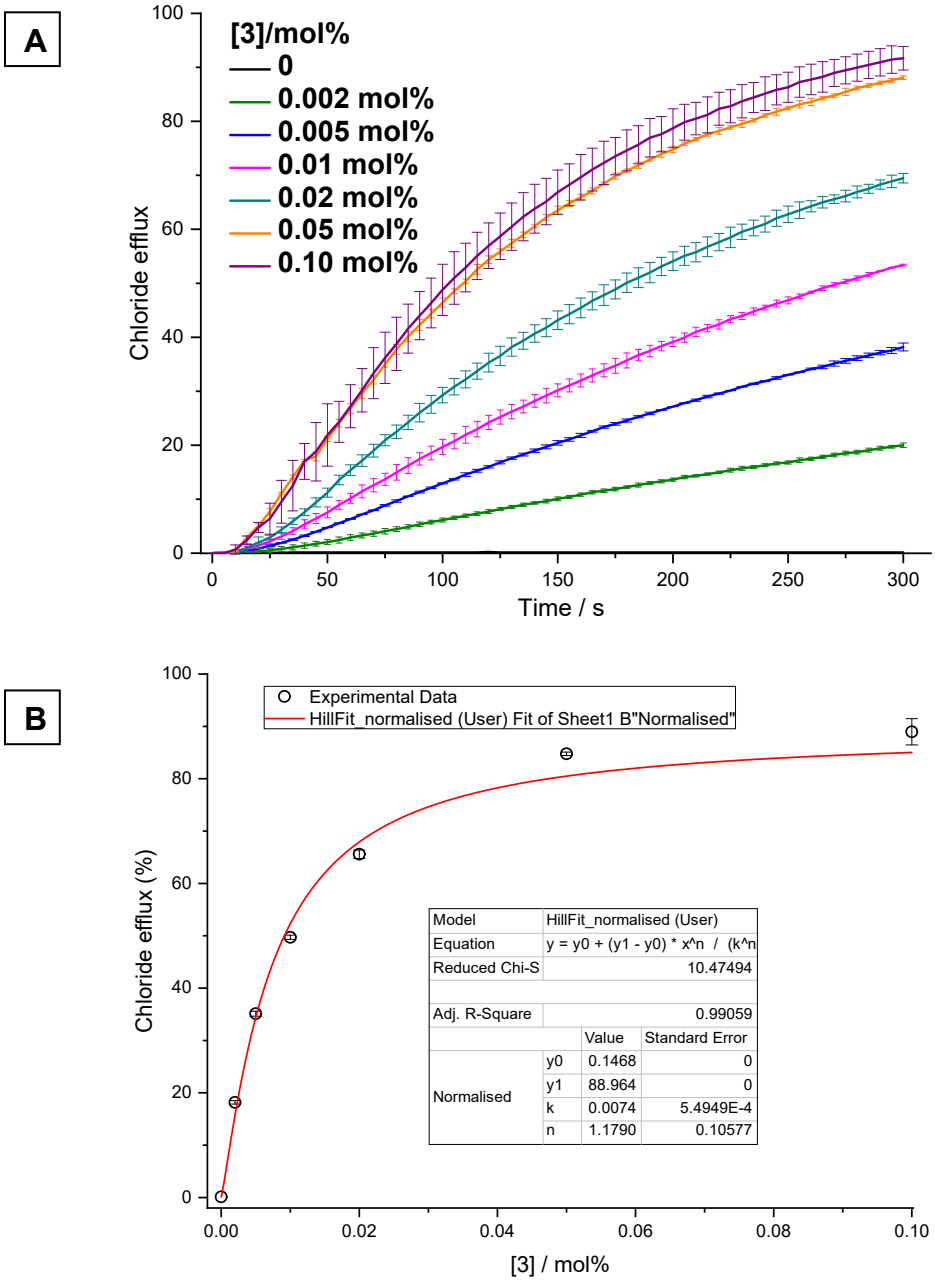


Figure S19: A-Dose response curve and B- Hill plot analysis of chloride efflux facilitated by compound **3** from unilamellar POPC vesicles that were loaded with a 489 mM KCl solution buffered to pH 7.2 with 5 mM phosphate, and were suspended in a 489 mM KNO_3 solution buffered to pH 7.2 with 5 mM phosphate salts. DMSO was used as a control. Each point is the average of at least two repeats.

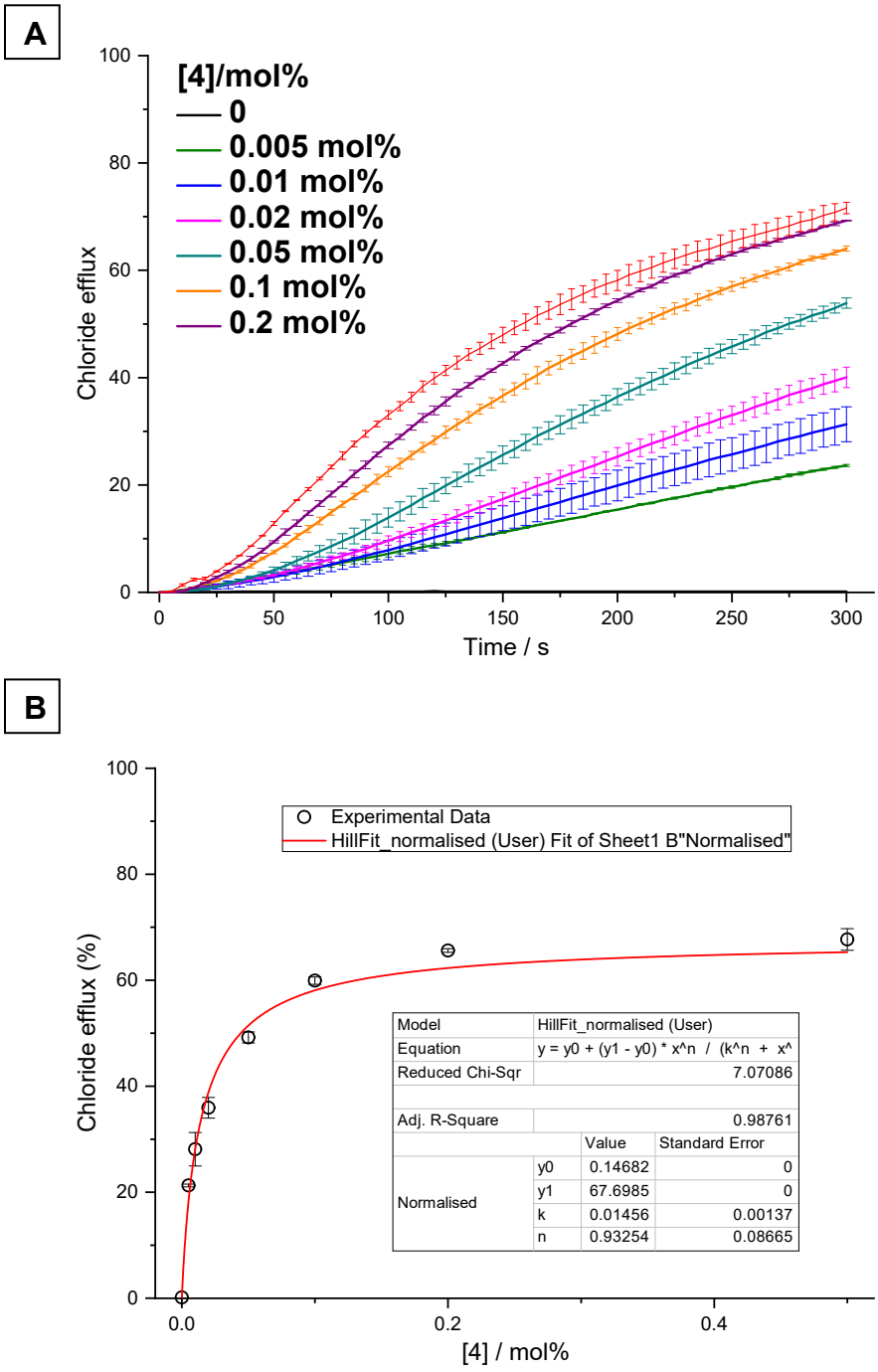


Figure S20: A-Dose response curve and B- Hill plot analysis of chloride efflux facilitated by compound **4** from unilamellar POPC vesicles that were loaded with a 489 mM KCl solution buffered to pH 7.2 with 5 mM phosphate, and were suspended in a 489 mM KNO_3 solution buffered to pH 7.2 with 5 mM phosphate salts. DMSO was used as a control. Each point is the average of at least two repeats.

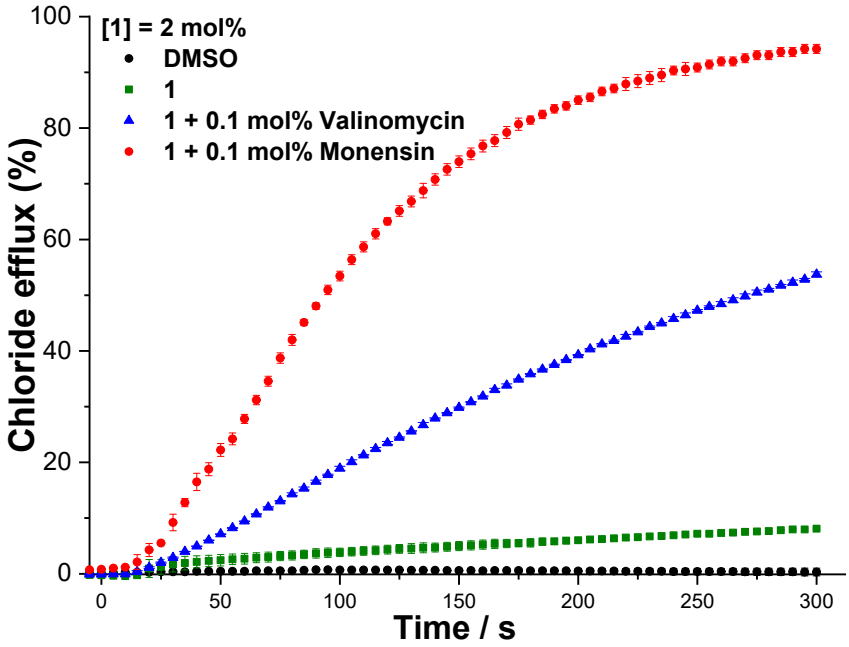


Figure S21: Electrogenic or electroneutral transport mediated by compound **1** (2 mol% with respect to lipid) in the presence of monensin or valinomycin (0.1 mol% with respect to lipid). Unilamellar POPC vesicles were loaded with a 300 mM KCl solution buffered to pH 7.2 with 5 mM phosphate and were suspended in a 300 mM KGlu solution buffered to pH 7.2 with 5 mM phosphate salts. DMSO was used as a control. Each point is the average of at least two repeats.

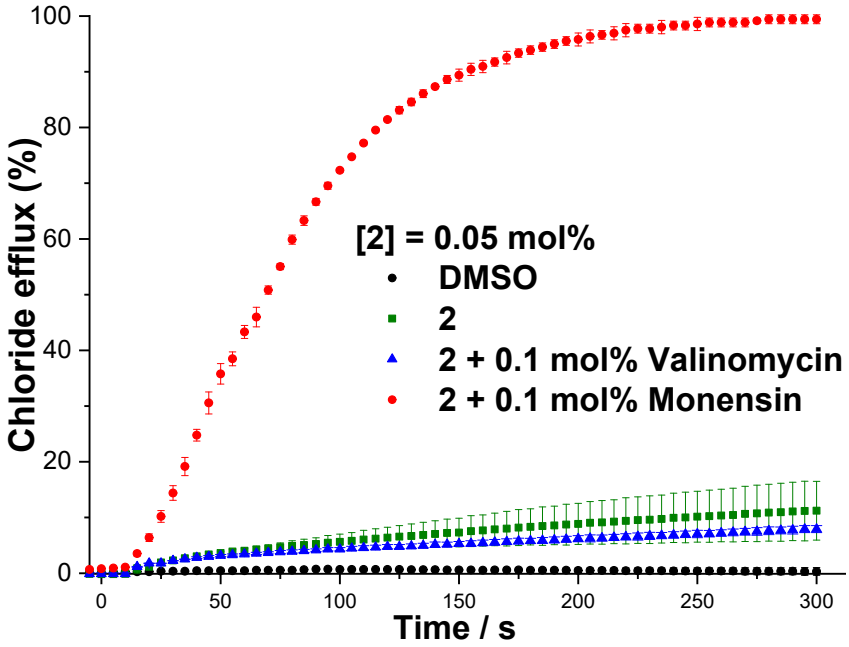


Figure S22: Electrogenic or electroneutral transport mediated by compound **2** (0.05 mol% with respect to lipid) in the presence of monensin or valinomycin (0.1 mol% with respect to lipid). Unilamellar POPC vesicles were loaded with a 300 mM KCl solution buffered to pH 7.2 with 5 mM phosphate and were

suspended in a 300 mM KGlu solution buffered to pH 7.2 with 5 mM phosphate salts. DMSO was used as a control. Each point is the average of at least two repeats.

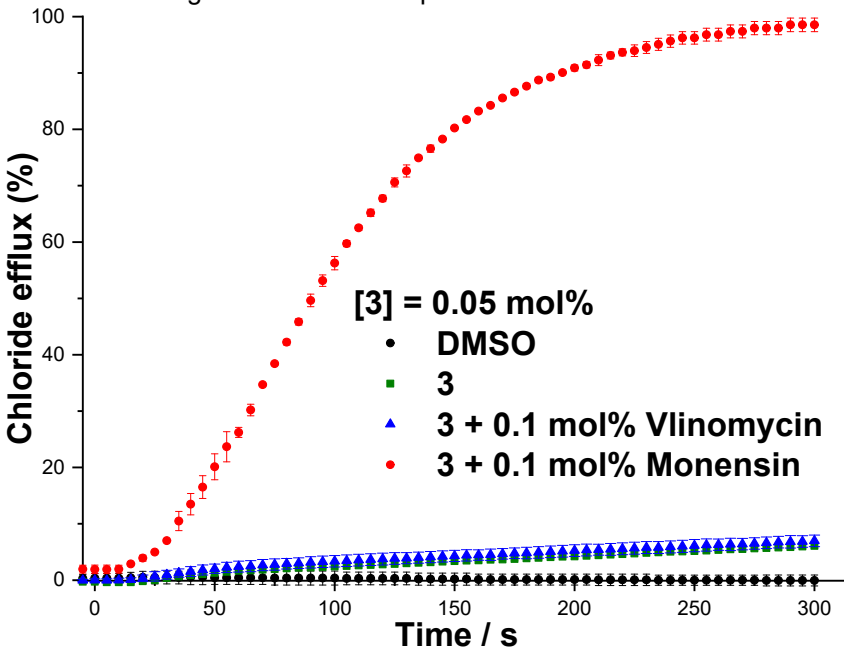


Figure S23: Electrogenic or electroneutral transport mediated by compound **3** (0.05 mol% with respect to lipid) in the presence of monensin or valinomycin (0.1 mol% with respect to lipid). Unilamellar POPC vesicles were loaded with a 300 mM KCl solution buffered to pH 7.2 with 5 mM phosphate and were suspended in a 300 mM KGlu solution buffered to pH 7.2 with 5 mM phosphate salts. DMSO was used as a control. Each point is the average of at least two repeats.

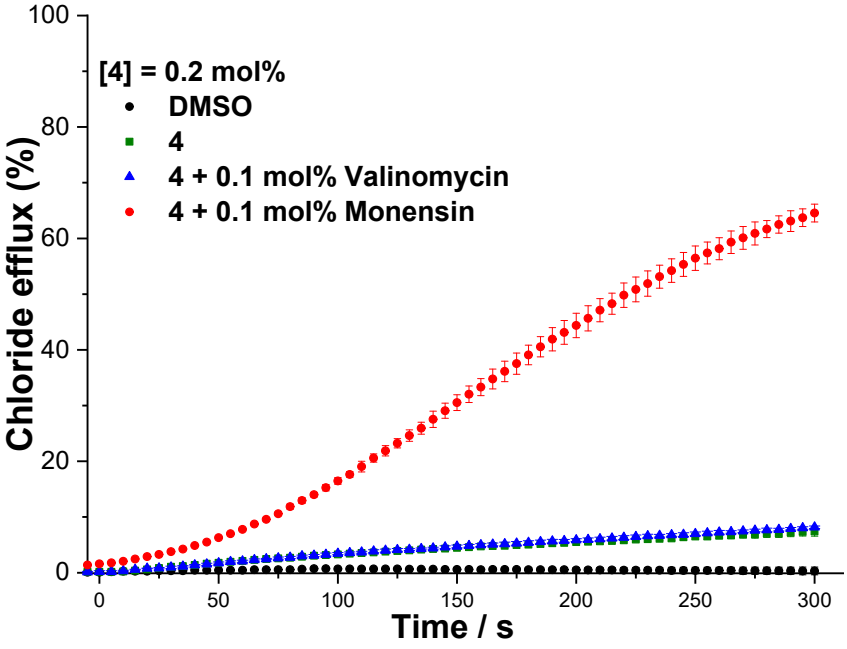


Figure S24: Electrogenic or electroneutral transport mediated by compound **4** (0.2 mol% with respect to lipid) in the presence of monensin or valinomycin (0.1 mol% with respect to lipid). Unilamellar POPC vesicles were loaded with a 300 mM KCl solution buffered to pH 7.2 with 5 mM phosphate and were

suspended in a 300 mM KGlu solution buffered to pH 7.2 with 5 mM phosphate salts. DMSO was used as a control. Each point is the average of at least two repeats.

S6.2: General preparation for HPTS assays:

Base-pulse 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) assays were conducted using unilamellar 1-palmitoyl-2-oleoylphosphatidylcholine vesicles (POPC) with a mean diameter of 200 nm loaded with the pH sensitive fluorescence dye HPTS (1 mM).[7] A chloroform solution of POPC (~ 30 mg/mL) was evaoprated under vacuum and dried for at least 6 h. The thin film was hydrated by the internal solution containing HPTS (1 mM) and was was subjected to nine freeze-thaw cycles followed by extrusion 25 times through a 200 nm polycarbonate membrane. Size exclusion chromatography using sephadex G-25 column and HPTS-free external solution as an eluent was conducted to remove unentrapped HPTS from the vesicles` solution. The internal and external solutions used were identical salt solution potassium chloride KCl (100 mM) buffered with 10 mM HEPES at pH 7.0. Finally, for each measurement, external solution (KCl) was used to dilute the lipid tock to obtain 2.5 mL lipid suspension containing 0.1 mM lipid. A base pulse of KOH (25 µL, 0.5 M) at final concentration 5 mM was added to generate a transmembrane pH gradient. After the tested receptors were added, HPTS fluorescence ratio (λ_{ex} = 460 nm, λ_{em} = 510 nm divided by λ_{ex} = 403 nm, λ_{em} = 510 nm) was recorded. Assisting ionophore (carbonyl cyanide phenylhydrazone (CCCP) or valinomycin) was used as a 5 µL DMSO. Bovine serum albumins (BSA) was added to vesicles at 1 mol% (with respect to lipid) and allowed to stir for 30 minutes to test if the transport is fatty acid independent, while, oleic acid (1 mol%) was used as a source of fatty acid to test if the transport is fatty acid dependent. Detergent (25 µL) was added at 200 seconds to destroy the pH gradient to calibrate the assay.

Results are the average of at least three repeats and the fractional fluorescence intensity (I_f) was determined using the following formula:

$$I_f = \frac{R_t - R_0}{R_d - R_0}$$

Where

- R_t is the fluorescence ratio at time t.
- R_0 is the fluorescence ratio at time 0
- R_d is the fluorescence ratio after detergent addition.

Hill plots were determined for KCl transport assay by conducting transport assays at different tested receptors concentrations. Receptor concentration vs fractional fluorescence intensity I_f at 200 s (the endpoint of transport assay) were plotted and fitted to the Hill equation using Origin 2019b:

The following formula was used to calculate hill coefficients (n) and EC_{50} (200 s) values by fitting the curves to the following equation:

$$y = y_0 + (y_{max} - y_0)\frac{x^n}{k^n + x^n}$$

Where:

- y is I_f (200 s) value of the ionophore at concentration x (receptors concentration is expressed as ionophore to lipid molar ratio).
- y_0 is I_f value at 200 s, without addition of the ionophore.
- y_{max} is the maximum *If* value.
- n is the Hill coefficient, and K is the EC_{50} (200 s) value.

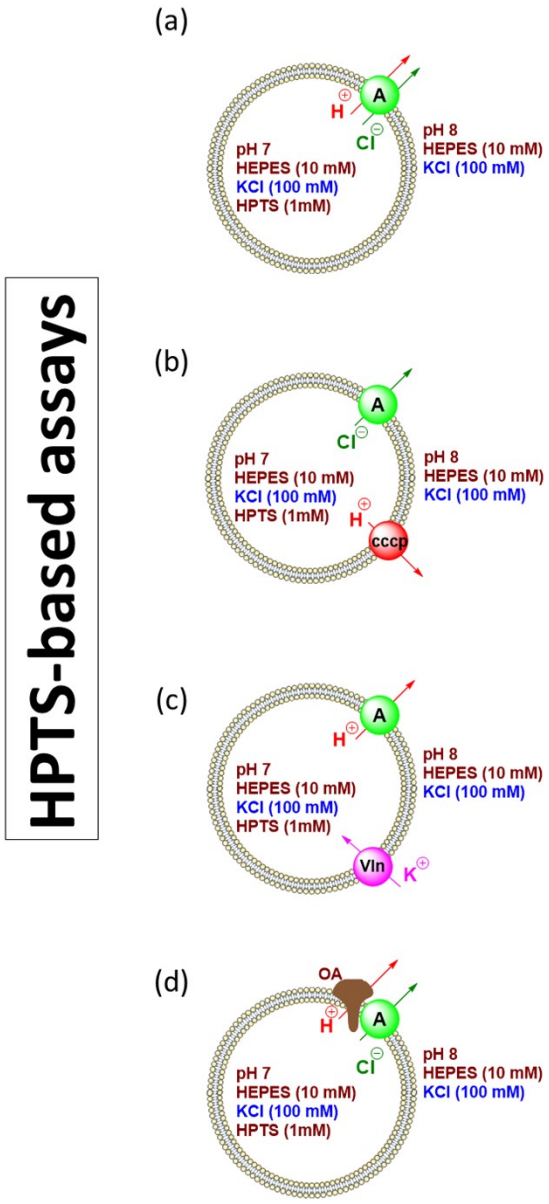


Figure S25: Schematic representation of the HPTS-based assays used in the current study (a) H^+/Cl^- symport or OH^-/Cl^- antiport (b) the presence of cccp (protonophore) to asses Cl^- uniport (c) the presence of valinomycin to measure the proton flux (d) the effect of fatty acid presence as a fuel on the transport.

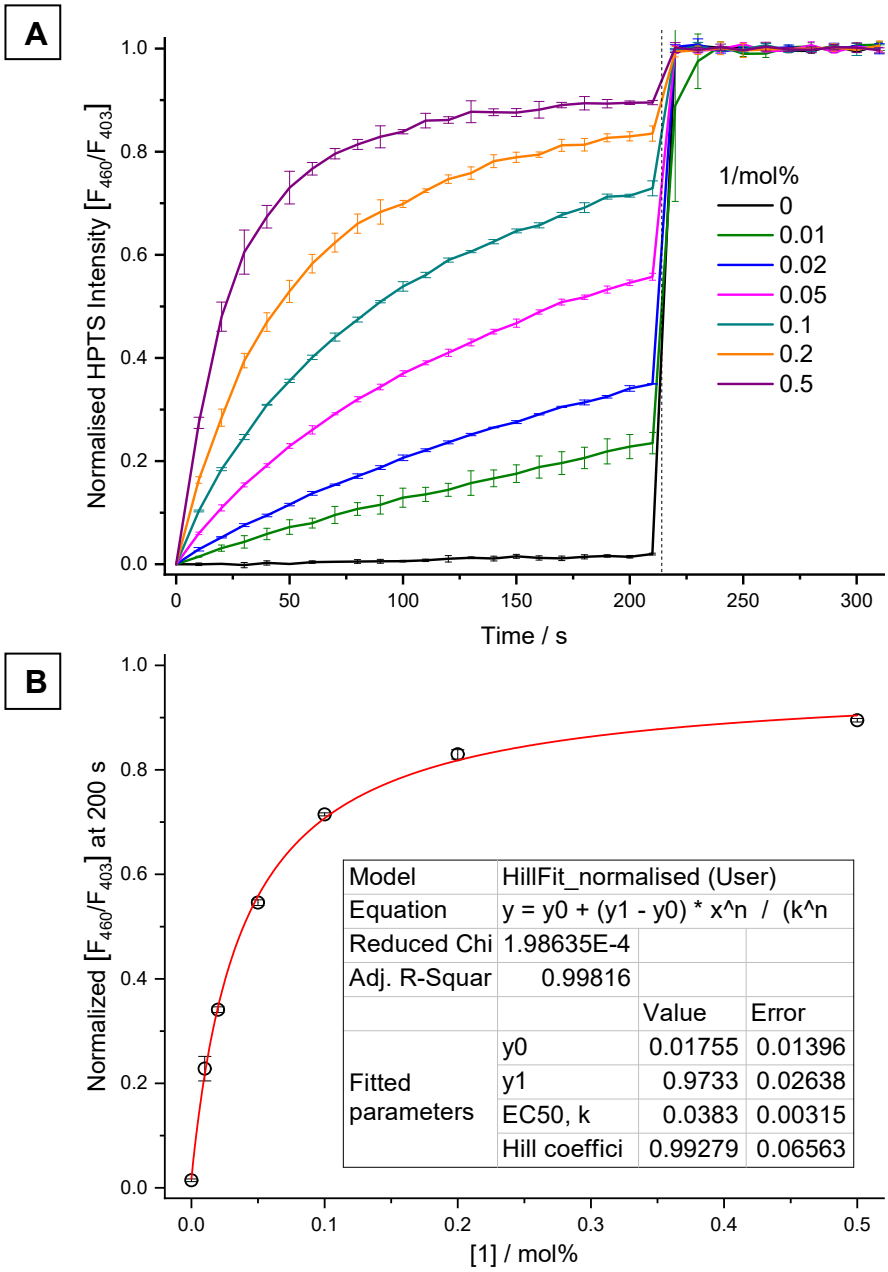


Figure S26: A-Dose response curve, B-Hill plot analysis of H^+/Cl^- symport or Cl^-/OH^- antiport facilitated by compound **1** using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM). The test compound was added at 0 s and detergent was added at 200 s. Ionophore concentrations are shown as ionophore to lipid molar ratios. Error bars represent SD from at least three repeats.

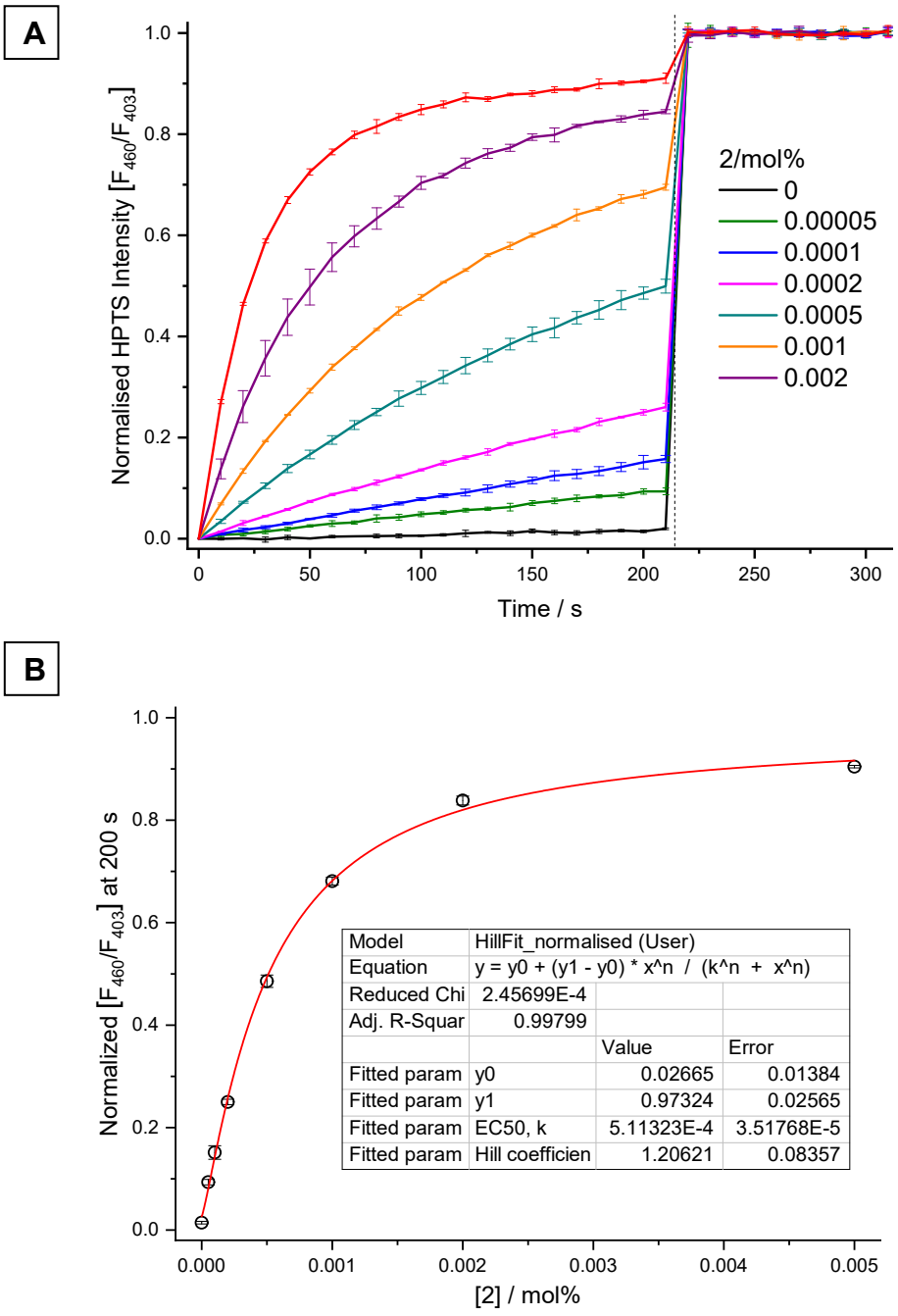


Figure S27: A-Dose response curve, B-Hill plot analysis of H^+/Cl^- symport or Cl^-/OH^- antiport facilitated by compound **2** using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM). The test compound was added at 0 s and detergent was added at 200 s. Ionophore concentrations are shown as ionophore to lipid molar ratios. Error bars represent SD from at least three repeats.

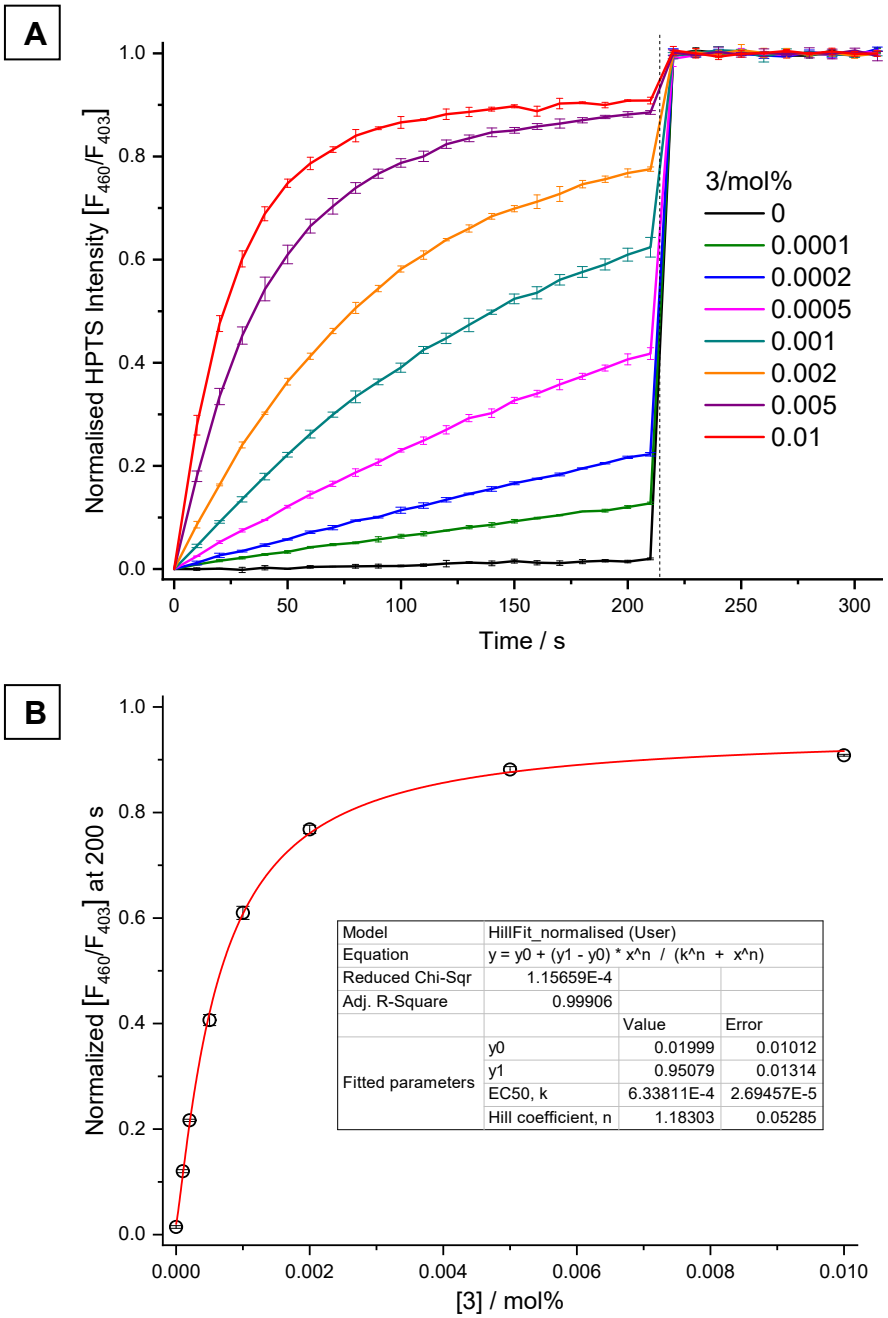


Figure S28: A-Dose response curve, B-Hill plot analysis of H^+/Cl^- symport or Cl^-/OH^- antiport facilitated by compound **3** using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM). The test compound was added at 0 s and detergent was added at 200 s. Ionophore concentrations are shown as ionophore to lipid molar ratios. Error bars represent SD from at least three repeats.

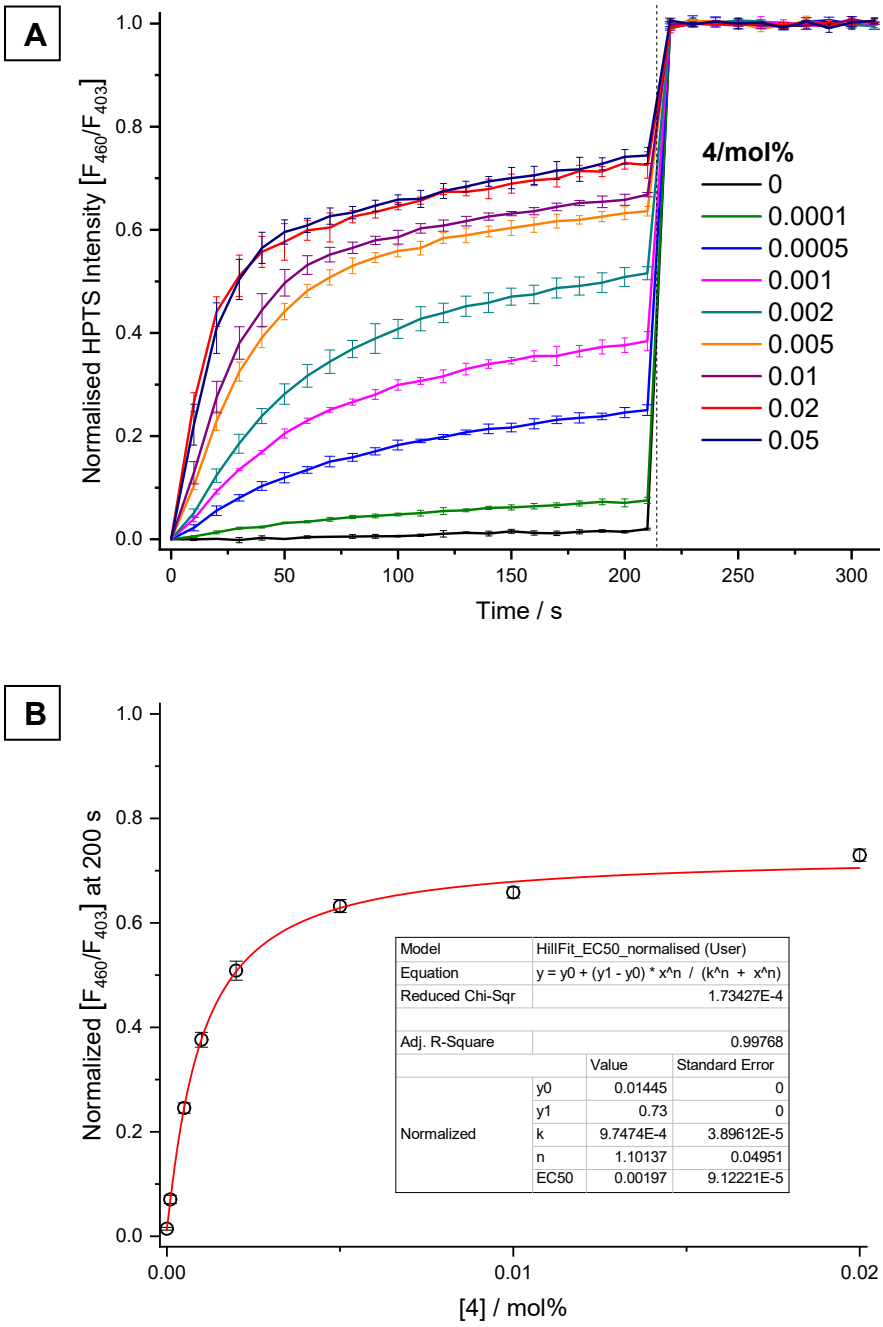


Figure S29: A-Dose response curve, B-Hill plot analysis of H^+/Cl^- symport or Cl^-/OH^- antiport facilitated by compound **4** using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM). The test compound was added at 0 s and detergent was added at 200 s. Ionophore concentrations are shown as ionophore to lipid molar ratios. Error bars represent SD from at least three repeats.

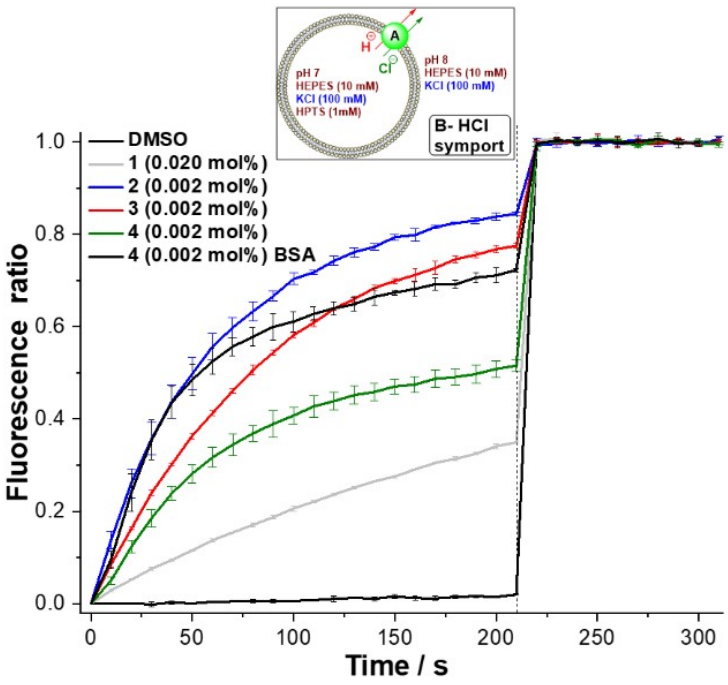


Figure S30; H^+/Cl^- symport or OH^-/Cl^- antiport facilitated by compounds **1**–**4** (0.02 mol% (rtl) for transporter **1** and 0.002 mol% (rtl) for transporters **2**-**4**) from unilamellar POPC vesicles loaded with 100 mM KCl buffered to pH 7.0 with 10 mM HEPES buffer and 1 mM HPTS internal sensor. and suspended in an external solution of 100 mM KCl buffered to pH 7.0 with 10 mM HEPES buffer. At 200 s, the detergent was added to lyse the vesicles and collapse the pH gradient for calibration of HPTS fluorescence. Transporter **4** was tested in the presence of BSA-treated lipids. DMSO was used as a control experiment.

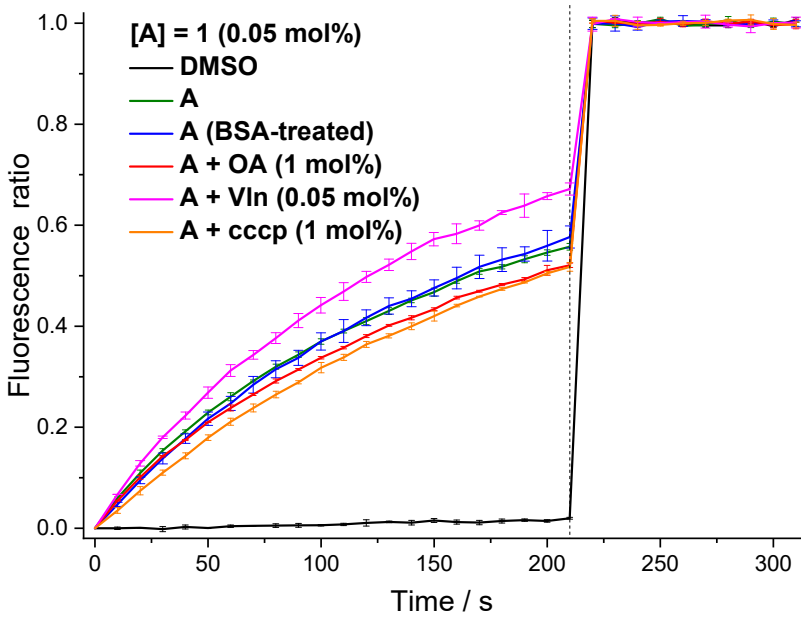


Figure S31: Using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM), different conditions were applied including using BSA-treated lipid (to test if the transport is fatty acid dependent) addition of oleic acid at 1 mol% (as a source of fatty acid), addition of the protonphore cccp at 1 mol% (to measure of chloride uniport solely), or addition of valinomycin at 0.05 mol% (as a measure of H^+ flux), on the rate of chloride transport of receptor **1** (0.05 mol%).

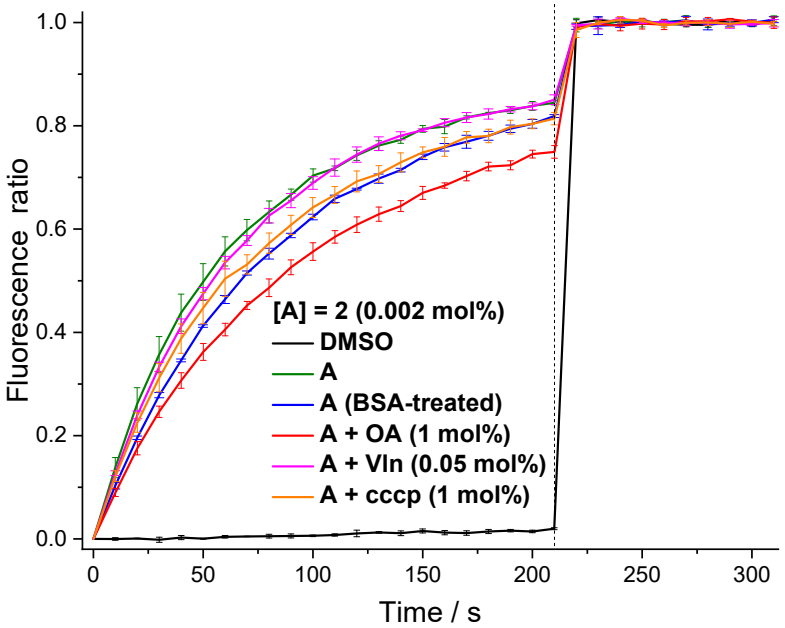


Figure S32: Using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM), different conditions were applied including using BSA-treated lipid (to test if the transport is fatty acid dependent) addition of oleic acid at 1 mol% (as a source of fatty acid), addition of the protonphore cccp at 1 mol% (to measure of chloride uniport solely), or addition of valinomycin at 0.05 mol% (as a measure of H^+ flux), on the rate of chloride transport of receptor **2** (0.002 mol%).

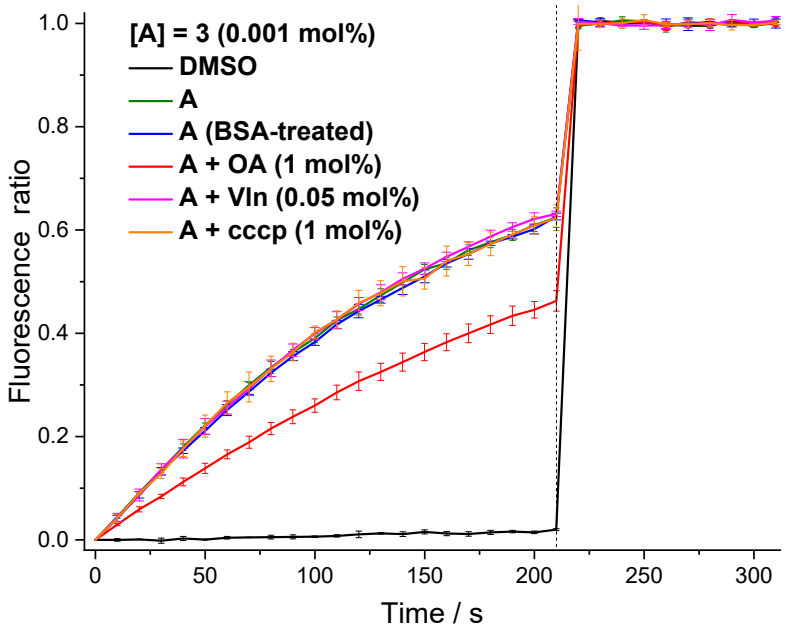


Figure S33: Using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM), different conditions were applied including using BSA-treated lipid (to test if the transport is fatty acid dependent) addition of oleic acid at 1 mol% (as a source of fatty acid), addition of the protonphore cccp at 1 mol% (to measure of chloride uniport solely), or addition of valinomycin at 0.05 mol% (as a measure of H^+ flux), on the rate of chloride transport of receptor **3** (0.001 mol%).

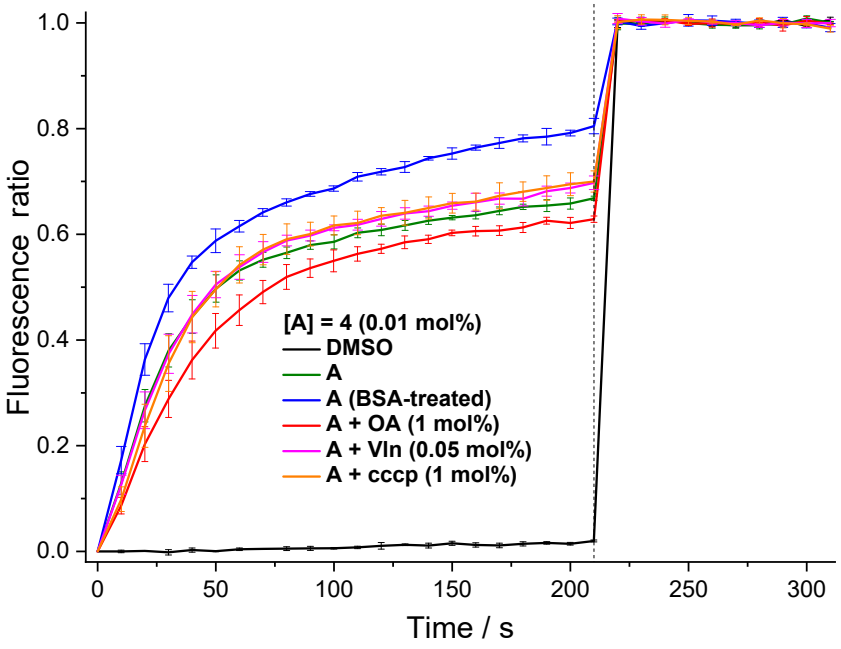


Figure S34: Using KCl-KOH assay from POPC vesicles loaded with KCl (100 mM), buffered to pH 7.0 with HEPES (10 mM), different conditions were applied including using BSA-treated lipid (to test if the transport is fatty acid dependent) addition of oleic acid at 1 mol% (as a source of fatty acid), addition of the protonphore cccp at 1 mol% (to measure of chloride uniport solely), or addition of valinomycin at 0.05 mol% (as a measure of H^+ flux), on the rate of chloride transport of receptor **4** (0.01 mol%).

S7. Biological results:

General cell culture information:

Human cell lines A549 (lung carcinoma), human colon adenocarcinoma cells (SW620) and MCF-7 (breast adenocarcinoma) were obtained from the American Type Culture Collection (ATCC, Manassas VA) and maintained in DMEM media (Biological Industries, Beit Haemek, Israel) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine, all from Biological Industries and 10% fetal bovine serum (FBS; Invitrogen- Life Technologies, Carlsbad, CA). Cells were grown at 37 °C under a 5% CO_2 atmosphere.

S7.1 Cell viability assay (MTT)

Cell viability was determined by the MTT assay. Cells (1×10^5 cells/mL) were seeded in 96-well microtiter plates and incubated for 24 h to allow cells to attach. Afterwards, they were treated for 24 h with different compound concentrations to obtain the dose-response curves (range 0.78 to 100 µM) and inhibitory concentration (IC) of 25, 50 and 75% of cell population were calculated. Compound diluent (maximum 1% DMSO) was added to control cells. Then, 10 µM of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide diluted in PBS (MTT, Sigma-Aldrich, St Louis, MO) was added to each well for an additional 2 h. The medium was removed and the MTT formazan precipitate was dissolved in 100 µl of DMSO. Absorbance was read on a Multiskan multiwell plate reader (Thermo Scientific Inc., Waltham, MA) at 570 nm. For each condition, at least three independent experiments were performed in triplicates. Cell viability was expressed as a percentage of control cells, and data are shown as the mean value ± S.D. Dose-response curves and IC values were calculated with GraphPad Prism 8 software.

Table S1: $IC_{25,}$ IC_{50} and IC_{75} values (in µM; Mean ± SD) of compounds **1**–**4** on human lung carcinoma (A549), human colon adenocarcinoma (SW620) and human breast adenocarcinoma (MCF-7) cell lines. Compounds 1-3 have ICs values above the

maximum concentration tested (>100 uM) for all cell lines. Data represents at least three independent replicates.

		A549	SW620	MCF7
1	**IC_{75}**	>100	>100	>100
	IC_{50}	>100	>100	>100
	IC_{25}	>100	>100	>100
2	**IC_{75}**	>100	>100	>100
	IC_{50}	>100	>100	>100
	IC_{25}	>100	>100	>100
3	**IC_{75}**	>100	>100	>100
	IC_{50}	>100	>100	>100
	IC_{25}	>100	>100	>100
4	**IC_{75}**	6.06 ± 1.44	0.83 ± 0.09	35.71 ± 11.93
	IC_{50}	1.86 ± 0.39	0.51 ± 0.07	17.92 ± 4.25
	IC_{25}	0.61 ± 0.23	0.32 ± 0.08	9.29 ± 2.41

S7.2 Lambda Scan and Compound Localization (Confocal Microscopy)

A549 cells were harvested (2 x 10^4 cells/well) in an 8-well sterile µ-Slide (Ibidi, Gräfelfing, Germany) for 24 hours. Next day, cells were treated with IC_{75} concentration of compound **4** for different time-points depending on the experiment. Images were captured using a Carl Zeiss LSM 880 spectral confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and managed by ZEN 2 Blue edition software (Zeiss). Representative images from three independent experiments are shown.

Lambda-Scan assay was carried out along 405 nm to 720 nm by exciting compound **4** inside the cells with four different lasers: 405 nm, 488 nm, 561 nm and 633 nm after 1 h of incubation (Figure S33).

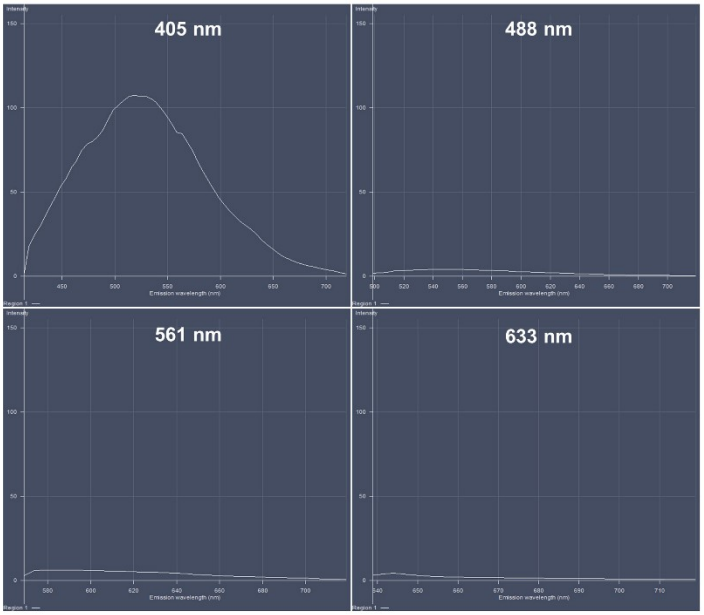


Figure S35: Compound **4** lambda scan. A549 cells were treated for 1 h with IC_{75} µM of compound **4**. Cells were excited using four different lasers (405, 488, 561 and 633 nm lasers) and emission wavelength scan (EM slit: 10 nm) was recorded for each laser. Images were captured from Carl Zeiss LSM 880 spectral confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and data was managed with ZEN 2 Blue edition software (Zeiss).

For localization analysis, compound **4** was incubated for different times up to 8 h. Along this interval, images were acquired every 30 minutes inside of the well. Representative images at 2 h, 4 h and 8 h are showed in Figure 4. Our results exposed a predominant cytoplasmatic distribution of the compound with slight accumulations in dots resembling lysosomes after 4 h. To evaluate if these accumulations were localized in lysosomes, cells were pre-incubated during 30 min with Lysotracker Red (Molecular Probes, OR, USA). This tracker has a selective localization with lysosomes based on their pH.

S7.3 Intracellular pH

Since there is a clear location of compound 4 in lysosomes we wonder if this anion transporter could affect the cytosolic pH. In order to quantify intracellular pH, A549 cells were seeded in a black well plate with clear bottom (1 x 10^4 cells/well). Next day, cells were treated for 1 h and 8 h with IC_{75} of compound **4**. Intracellular pH quantification was

done by using pH Rodo Red AM Staining kit from Molecular Probes following manufacturer protocol. The emission at 590 nm after excitation with 550 nm was detected by FLUOstar OPTIMA (BMG LabTech, Ortenberg, Germany). pH quantification was performed by extrapolating test values to an Intracellular pH Calibration Curve kit (Molecular Probes). Moreover, the intracellular pH change was also evaluated with confocal microscopy using the same kit, incubating A549 with IC_{75} of compound 4 for 1 h and 8 h (Figure 5).

S8. References:

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