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A Macrocyclic Fluorescent Probe for the Detection of Citrate

Supplementary Information

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Experimental

Synthesis

General synthesis of tetrabutylammonium carboxylates

Carboxylic acid (1eq, ~100 mg) was dissolved in water (5 mL) at room temperature. A solution of tetrabutylammonium hydroxide (2 or 3 equivalents, 1M in methanol) was added dropwise with stirring. The resulting solution was stirred at room temperature for half an hour. The methanol was removed *in vacuo*, and the remaining aqueous solution lyophilized until a dry solid was obtained. Salts were stored in a vacuum dessicator at room temperature, or under nitrogen atmosphere at -20 °C (oxalate, malonate, succinate) and were lyophilized prior to use.

Tetrabutylammonium oxalate

^{1}H NMR (300 MHz, DMSO-*d*6) δ 3.22 – 3.09 (m, 16H), 1.65 – 1.48 (m, 16H), 1.38 – 1.23 (m, 16H), 0.93 (t, *J* = 7.3 Hz, 24H).

Tetrabutylammonium malonate

^{1}H NMR (300 MHz, MeCN-*d*3) δ 3.14 – 3.06 (m, 16H), 2.70 (s, 2H), 1.67 – 1.54 (m, 16H), 1.42 – 1.29 (m, 16H), 0.97 (t, *J* = 7.3 Hz, 24H).

Tetrabutylammonium succinate

^{1}H NMR (300 MHz, DMSO-*d*6) δ 3.26 – 3.12 (m, 16H), 1.89 (s, 4H), 1.65 – 1.49 (m, 16H), 1.40 – 1.23 (m, 16H), 0.93 (t, *J* = 7.3 Hz).

Tetrabutylammonium terephthalate

^{1}H NMR (300 MHz, DMSO-*d*6) δ 7.64 (s, 4H), 3.26 – 3.12 (m, 16H), 1.65 – 1.49 (m, 16H), 1.40 – 1.23 (m, 16H), 0.93 (t, *J* = 7.3 Hz).

Tetrabutylammonium citrate

^{1}H NMR (300 MHz, DMSO-*d*6) δ 3.22 – 3.09 (m, 24H), 2.29 (d, *J* = 14 Hz, 2H), 1.98 (d, *J* = 14 Hz, 2H), 1.65 – 1.48 (m, 24H), 1.38 – 1.23 (m, 24H), 0.93 (t, *J* = 7.3 Hz, 36H).

tert-Butyl (3-(aminomethyl)benzyl)carbamate (4)

A solution of di-*tert*-butyl dicarbonate (819 mg, 3.75 mmol) in dichloromethane (10 mL) was added dropwise over a period of five minutes to a solution of freshly-distilled *m*-xyleneamine (2.5 mL, 18.9 mmol) in dichloromethane at 0 °C. The resulting mixture was allowed to warm to room temperature overnight (14h). Water was added, and the aqueous phase extracted with dichloromethane. The combined organic phases were washed repeatedly with water until TLC analysis showed no starting

diamine remaining. The organic phase was washed with brine (x1), dried (Na_2SO_4), and the solvent removed *in vacuo* to afford the title compound as a clear oil that solidified upon standing (835 mg, 94%), with all analytical data matching that previously reported in the literature.[1]

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30 (t, *J* = 7.3 Hz, 1H), 7.25 – 7.13 (m, 3H), 4.84, (s, 1H), 4.31 (d, *J* = 5.7 Hz, 2H), 3.90 (s, 2H), 1.46 (s, 9H).

tert-Butyl (3-(isothiocyanatomethyl)benzyl)carbamate (6)

Thiophosgene (180 µL, 2.35 mmol) was added dropwise to a mixture of *tert*-butyl (3-(aminomethyl)benzyl)carbamate (**5**)(371 mg, 1.57) in dichloromethane (80 mL) and saturated aqueous $NaHCO_3$ solution (16 mL) at room temperature. The resulting mixture was stirred at room temperature for 1 h. The reaction mixture was extracted with dichloromethane (x3), and the organic extracts combined, washed with brine, and dried (Na_2SO_4). Removal of the solvent *in vacuo* afforded the title compound as an off-white solid (437 mg, *quant.*) that was used in following steps without further purification. All analytical data matched that previously reported in the literature.[2]

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 (t, *J* = 7.8 Hz, 1H), 7.29 – 7.19 (m, 3H), 4.88 (s, 1H), 4.81 (s, 2H), 4.33 (d, *J* = 5.7 Hz, 2H), 1.47 (s, 9H).

Di-_tert_-butyl ((((thiocarbonylbis(azanediyl))bis(methylene))bis(3,1-phenylene))bis(methylene))dicarbamate (7)

A solution of amine **4** (370 mg, 1.57 mmol) in dichloromethane (10 mL) was added to a solution of thioisocyanate **6** (437 mg, 1.57 mmol) in dichloromethane (10 mL) at room temperature. The resulting solution was stirred at room temperature for 16 h. The solvent was removed *in vacuo* to afford the title compound as a white foam (807 mg, *quant.*), with all analytical data matching that previously reported in the literature.[3]

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.29 (t, *J* = 7.8 Hz, 2H), 7.23 – 7.10 (m, 6H), 6.13 (s, 2H), 4.96 (s, 2H), 4.63 (d, *J* = 4.5 Hz, 4H), 4.25 (d, *J* = 5.5 Hz, 4H), 1.45 (s, 18H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 182.7, 156.3, 139.5, 138.1, 129.1, 126.8, 126.7, 126.6, 79.9, 48.5, 44.4, 28.5; HRMS (ESI) Calc. for $C_{27}H_{28}N_4O_4SNa$ (MNa^+) 537.2506 found 537.2503 ; IR (ATIR, neat) 3348, 2979, 1686, 1523, 1249, 1163, 698 cm^{-1}.

3,6-Dichloro-1,8-diisothiocyanato-_9H_-carbazole (5)

Thiophosgene (250 µL, 3.26 mmol) was added dropwise to a mixture of carbazole diamine **3** (287 mg, 1.08 mmol) in ethyl acetate (22 mL) and saturated aqueous $NaHCO_3$ solution (11 mL) at room temperature. The resulting mixture was stirred at room temperature for a further 30 mins. The aqueous phase was extracted with ethyl acetate (x3) and with dichloromethane (x3). The combined organic phases were each washed with brine, dried (Na_2SO_4), and the solvent removed *in vacuo* to afford the title compound as a brown solid (378 mg, *quant.*).

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (s, 1H), 7.87 (s, 2H), 7.39 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 135.8, 133.5, 125.5, 124.5, 124.0, 120.7, 115.3; HRMS (ESI) Calc. for $C_{14}H_4Cl_2N_3S_2$ (M^-) 347.9229 found 347.9221; IR (ATIR, neat) 3330, 2010, 1253, 1227, 850, 578 cm^{-1}.

6^3,6^6-dichloro-6^9*H*-3,5,7,9,13,15-hexaaza-6(1,8)-carbazola-1,11(1,3)-dibenzenacyclohexadecaphane-4,8,14-trithione (2)

TFA (2 mL) was added dropwise to a solution of di-Boc-protected diamine **7** (179 mg, 0.348 mmol) in dichloromethane (5 mL) at room temperature. The resulting solution was stirred at room temperature for a further 2 h, at which point the solvent was removed under a flow of nitrogen. The free base of the amine was obtained by passing the compound through Amberlyst A-26(OH) resin, eluting with methanol. Due to limited stability of this compound it was carried through directly to the following step.

The intermediate diamine (0.348 mmol) was dissolved in a mixture of dichloromethane (14 mL) and acetonitrile (5 mL), and added dropwise over 20 mins to a solution of diisothiocyanate **5** (125 mg, 0.357 mmol) and DIPEA (300 µL, 1.72 mmol) in dichloromethane (140 mL) at room temperature. The resulting mixture was stirred at room temperature for 24 h. The solvent was removed *in vacuo*. Flash column chromatography, eluting with 10% THF/90% dichloromethane, afforded the title compound as an off-white solid (84 mg, 38%).

^{1}H NMR (300 MHz, DMSO-*d*6) δ 10.93 (s, 1H), 9.65 (s, 2H), 8.22 – 8.02 (m, 4H), 7.93 (s, 2H), 7.63 (s, 2H), 7.37 – 7.10 (m, 8H), 4.83 – 4.53 (m, 8H); HRMS (ESI) Calc. for $C_{31}H_{28}Cl_2N_3S_3$ $(M + H)^+$ 644.0940 found 644.0935; IR (ATIR, neat) 3257, 2923, 2854, 1532, 1294, 1225, 694 cm^{-1}. Attempts to obtain full NMR characterization of MThuA were hindered by limited solubility of the compound, and broadening of NMR peaks due to conformational flexibility. Hence, the ^{13}C NMR spectrum was obtained in the presence of an excess of $(TBA)_2HPO_4$. ^{1}H NMR (phosphate-bound)(300 MHz, DMSO-*d*6) δ 9.08 (s, 2H), 7.81 (s, 4H), 7.30 – 7.15 (m, 8H), 4.72 (s, 4H), 4.67 (s, 4H); ^{13}C NMR (phosphate-bound)(125 MHz, DMSO-*d*6) δ 183.3 (very broad, observed in HMBC spectrum), 180.7, 139.5, 138.6, 130.7, 128.4, 128.1, 127.3, 127.1, 127.0, 123.4, 122.7, 115.9, 113.7, 48.7, 48.3.

Anion Screens

Anion screens were performed as follows. A 25 µM stock solution of receptor **2** in the specified solvent mixture (40 – 50 mL) was prepared. A "blank" spectrum with no added anion was recorded using this solution. All anions used were anhydrous tetrabutylammonium salts, with the exceptions of tetraethylammonium bicarbonateand tetrabutylammonium fluoride trihydrate. Anions used were obtained from commercial sources or prepared according to literature methods. Hygroscopic salts were lyophilized prior to use. Anions were weighed into separate vials, and diluted with 1 – 3 mL of the receptor **2** stock solution, such that the solution contained 100 equivalents of anion relative to receptor **2**. Spectra of each mixture were recorded using the same cuvette, which was thoroughly cleaned and dried between each use. Spectra were recorded on a Horiba Duetta spectrophotometer with temperature control enabled (25 °C) using 10 mm quartz cuvettes.

Titrations

Anion titrations were performed as follows. A 25 µM stock solution of receptor **2** in the specified solvent mixture (40 – 50 mL) was prepared. A "blank" spectrum with no added anion was recorded using this solution. All anions used were anhydrous tetrabutylammonium salts, with the exceptions of tetraethylammonium bicarbonate and tetrabutylammonium fluoride trihydrate. Anions used were obtained from commercial sources or prepared according to literature methods. Hygroscopic salts were lyophilized prior to use. Anions were weighed into separate vials, and diluted with 0.5 – 1.5 mL of the receptor **2** stock solution, such that the solution contained 5 – 10 mM anion. Small measured aliquots were added to the receptor **2** stock solution, and the solution stirred for at least 30 seconds before the acquisition of a spectrum. Fluorescence spectra were recorded on a Horiba Duetta spectrophotometer with temperature control enabled (25 °C) using 10 mm quartz cuvettes.

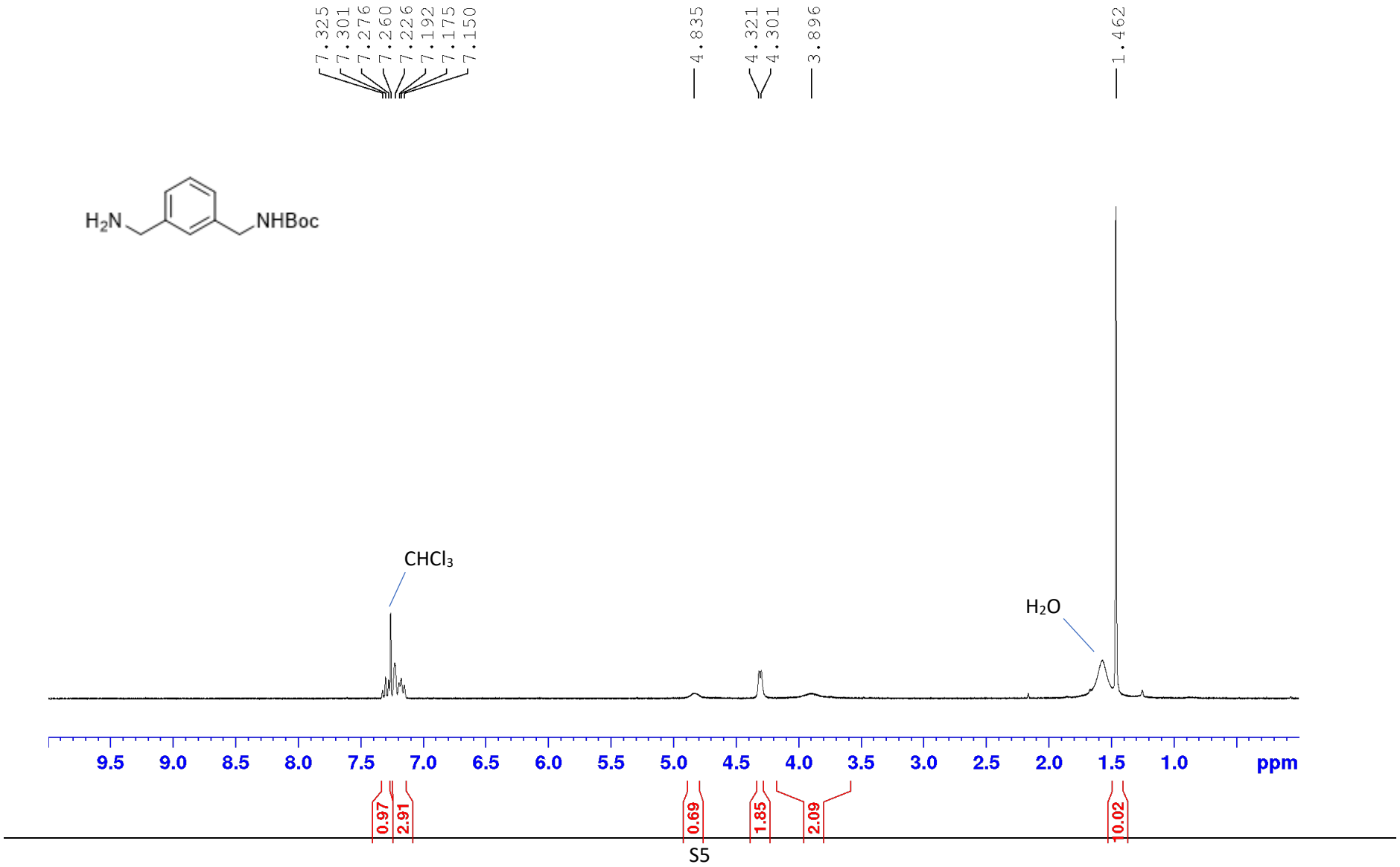
Absorption spectra were obtained in the specified solvent on a Varian Cary 400 UV-Vis spectrophotometer using 10 mm quartz cuvettes.

Cell Studies

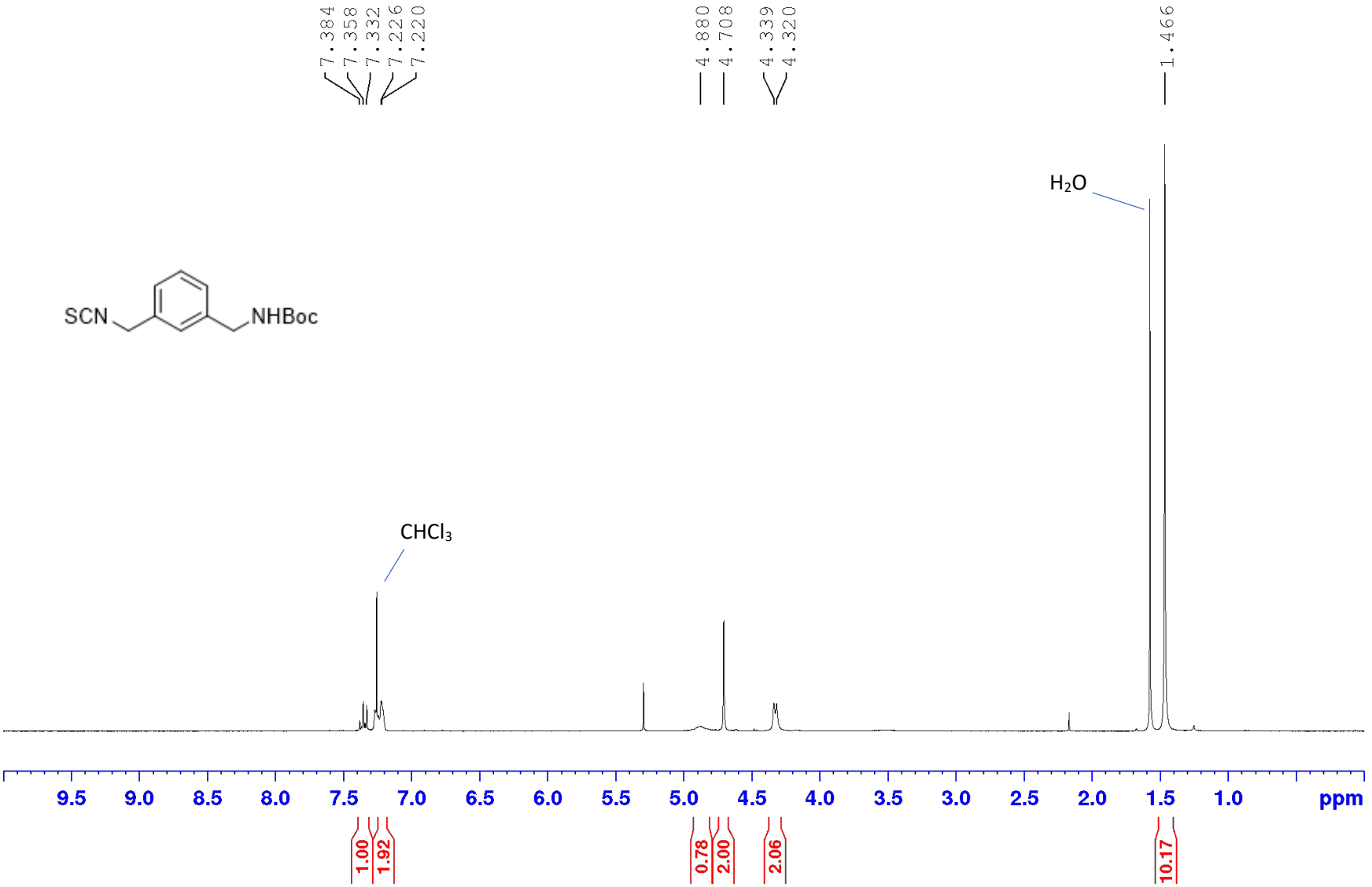
Cell staining was achieved by prior fixation and permeabilization of splenocytes using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, followed by incubation of receptor **2** for 20 minutes at room temperature. Evaluation of metabolic activity and associated changes to intracellular citrate level was determined using murine macrophages. Splenocyte-derived macrophages were first activated for 1 hour with lipopolysaccharides (LPS), LPS and oligomycin, or with PBS only. The macrophages were harvested, then permeabilized and incubated with receptor **2**. Analysis of different immune cell subsets isolated from murine spleens was achieved using receptor **2**, including plasmacytoid dendritic cell ($MHCII^+B220^+CD11c^+$), conventional dendritic cells ($MHCII^+CD11c^+$), macrophages ($CD11b^+F4/80^+$), monocytes ($Ly6C^{int/high}$), gamma delta T cell ($CD3^+gdTCR^+$), neutrophil ($CD11b^+Ly6G^+$), conventional ($CD3^+$ and $CD4^+/CD8^+$) and regulatory T cells ($CD3^+CD4^+CD25^+FoxP3^+$) as well as B cells ($B220^+$). Splenocytes were obtained by mechanical disruption followed by filtration through a 100um cell strainer to obtain a single-cell suspension. Red blood cells were then removed using RBC lysis buffer (BioLegend). Samples were resuspended in FACS buffer (2% FBS containing 1mM EDTA) for antibody staining. Briefly, cells were first stained with TruStain FcX anti-CD16/32 (93) for non-specific antibody blocking and LIVE/DEAD Fixable Blue Dead Cell Stain (Invitrogen) for dead cell exclusion. The following anti-mouse antibodies were used for the identification of various immune subsets: CD45-BV785 (30-F11), MHCII-BV510 (M5/114.15.2), B220-BUV737 (RA3-6B2), CD3-PE-CF594 (145-2C11), TCRgd-PE/Cy5 (GL3), CD4-PerCP (RM4-5), CD8-BV785 (53-6.7), CD25-BV605 (PC61), FoxP3-APC (REA788), CD11c-FITC (N418), Ly6C-BV605 (HK1.4), Ly6C-BV421 (AL-21), Ly6G-BV650 (1A8), F4/80-BV711 (BM8), CD11b-APC/Cy7 (M1/70). For detection of intracellular targets, cells were fixed and permeabilized using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set according to the manufacturer's instructions. All samples were acquired using the LSRII 5L (BD Bioscience) flow cytometer and analysed using the FlowJo v10 software.

Compound Spectra

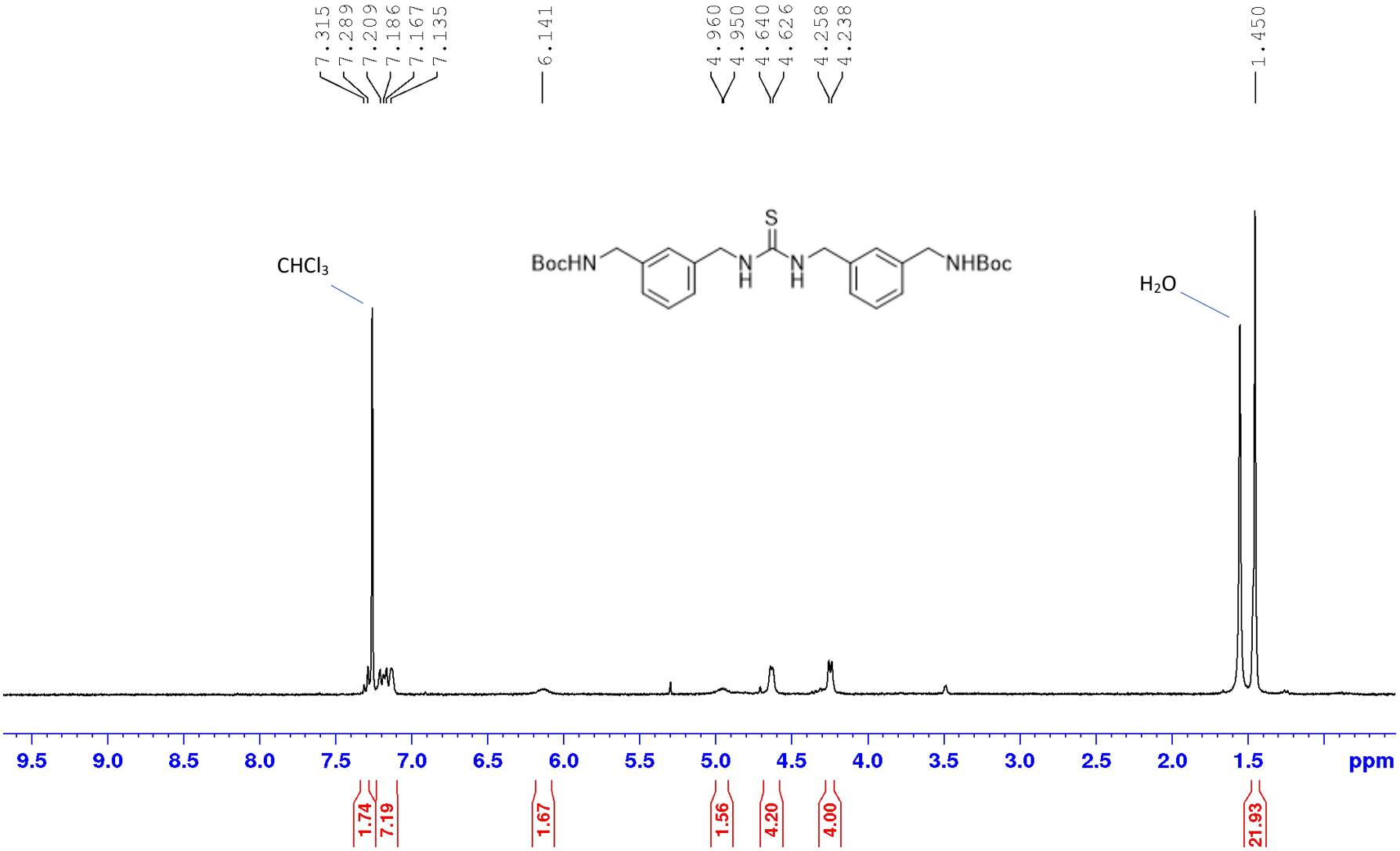
***tert*-Butyl (3-(aminomethyl)benzyl)carbamate (4) – 300 MHz, $CDCl_3$**



***tert*-Butyl (3-(isothiocyanatomethyl)benzyl)carbamate (6) – 300 MHz, $CDCl_3$**



Di-*tert*-butyl ((((thiocarbonylbis(azanediyl))bis(methylene))bis(3,1-phenylene))bis(methylene))dicarbamate (7) – 300 MHz, $CDCl_3$



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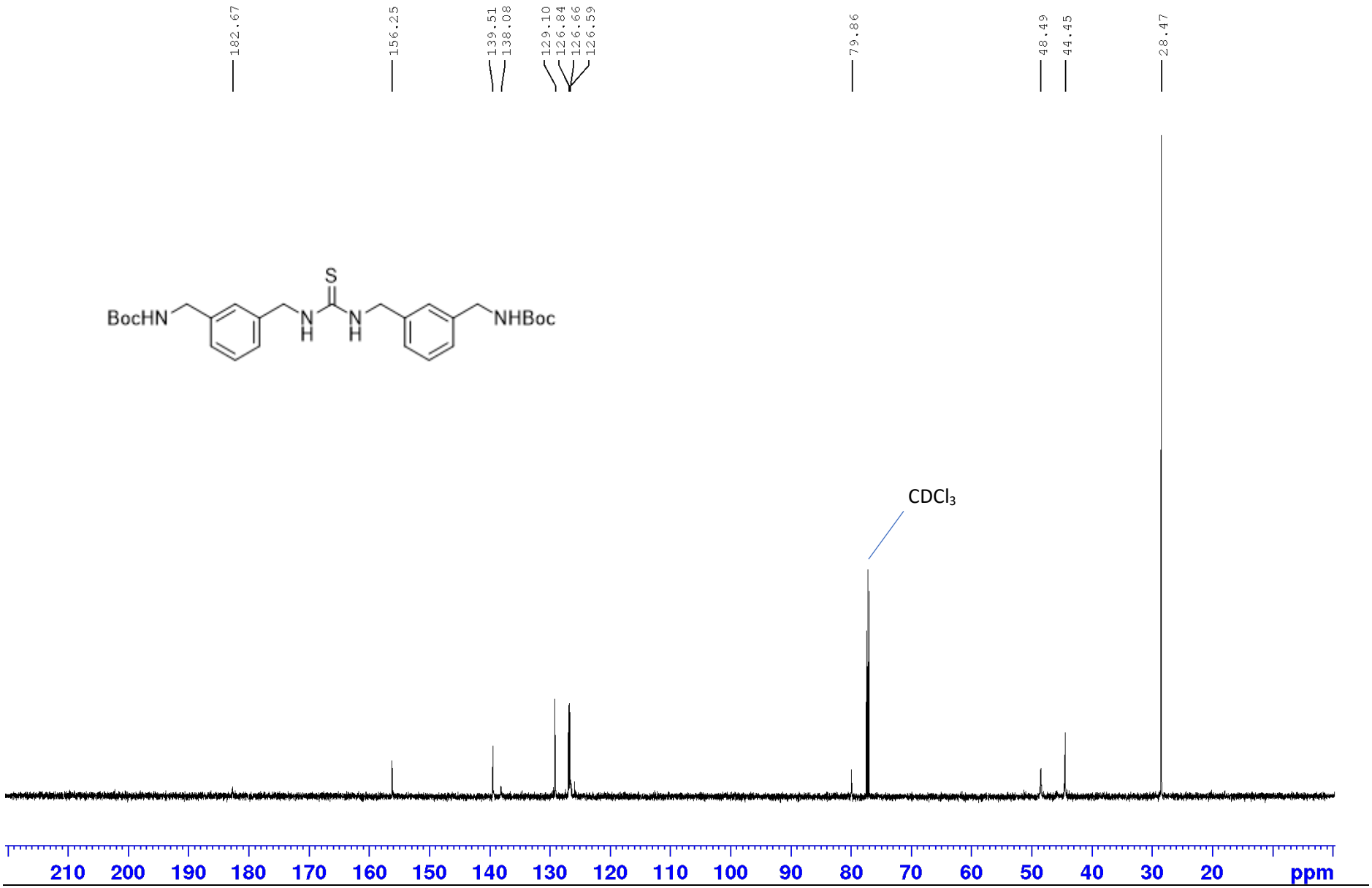

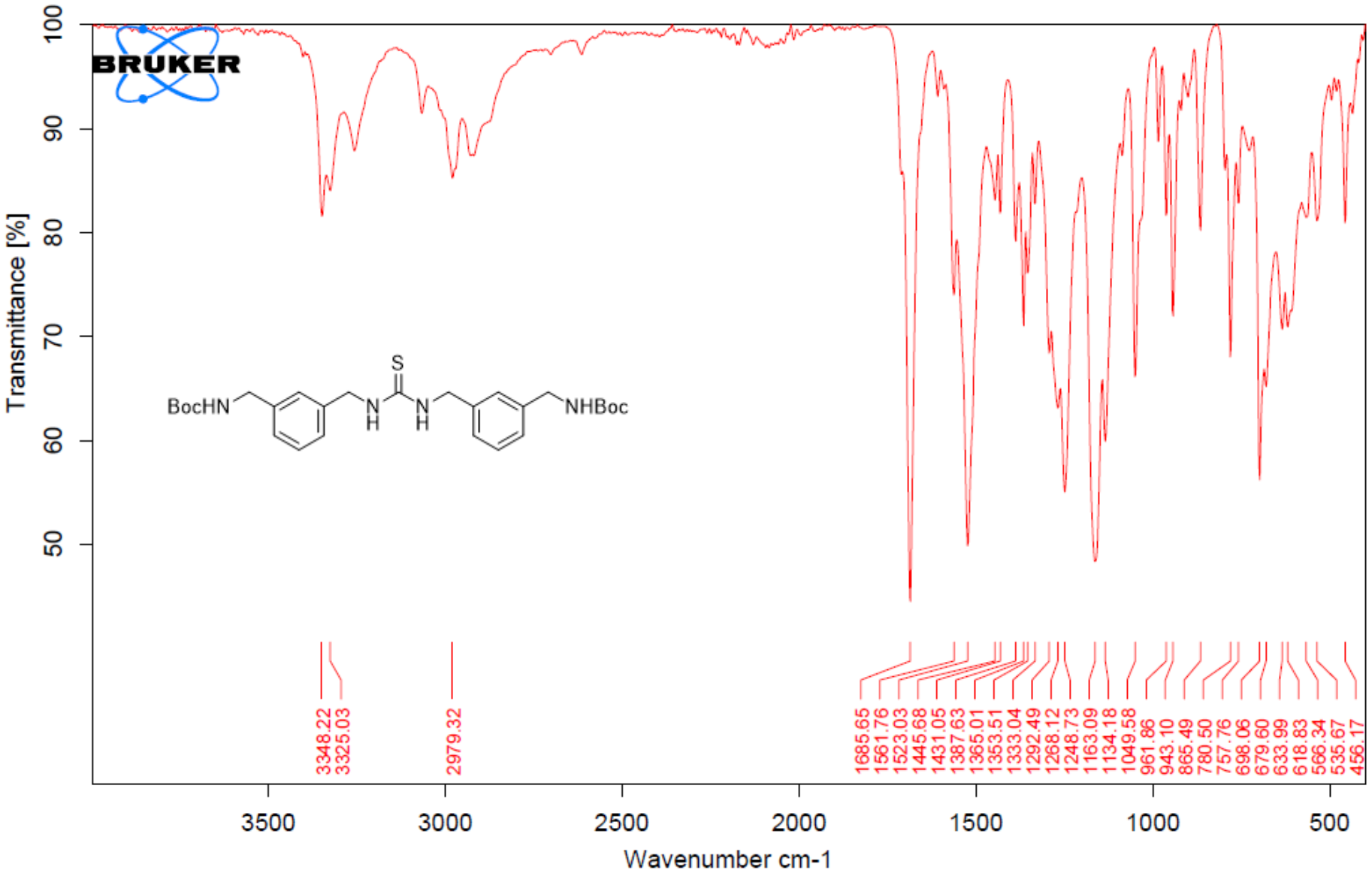
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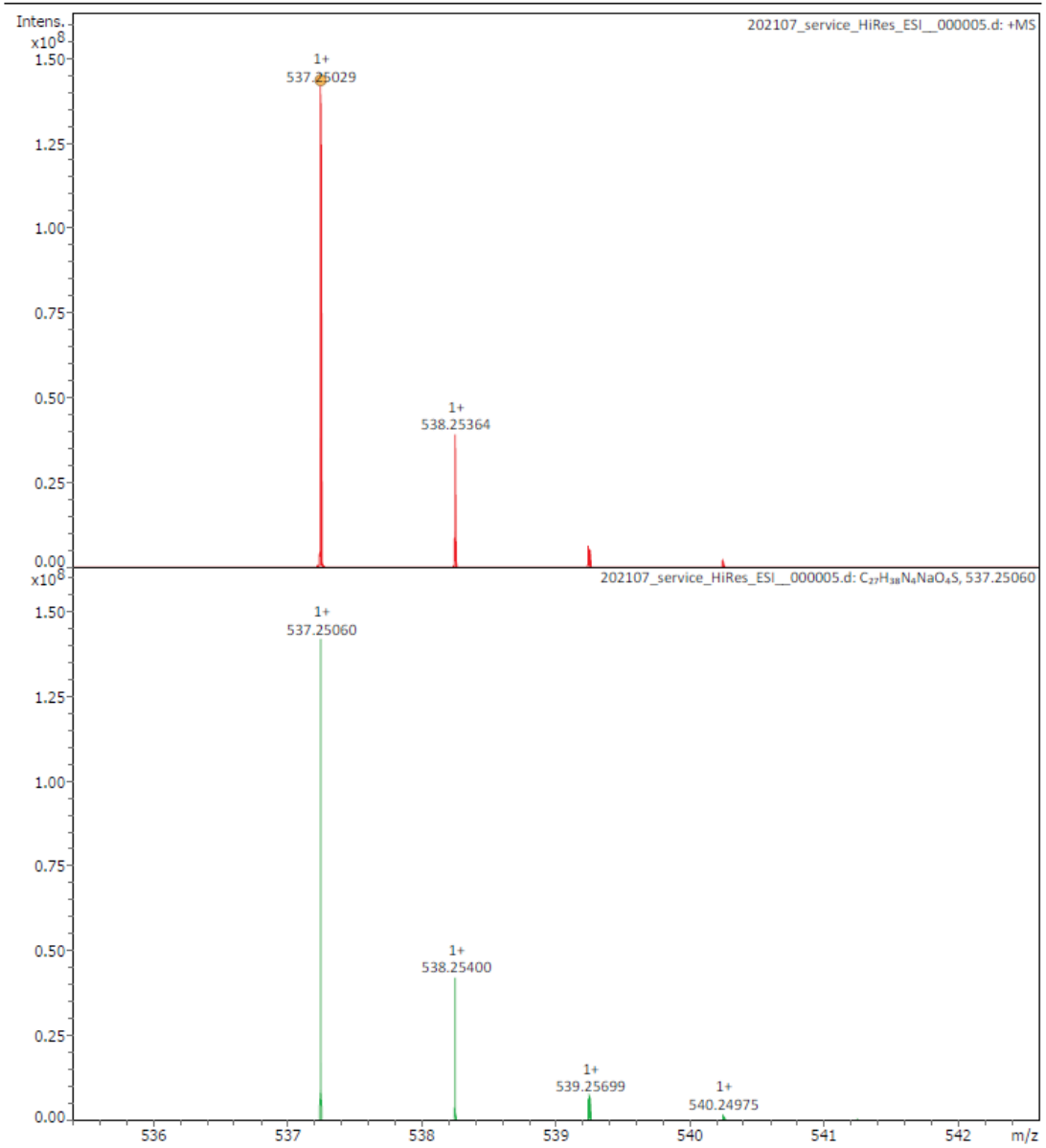
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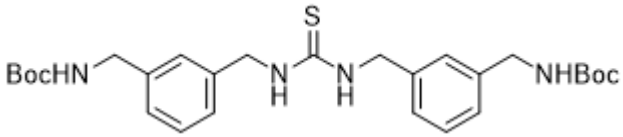
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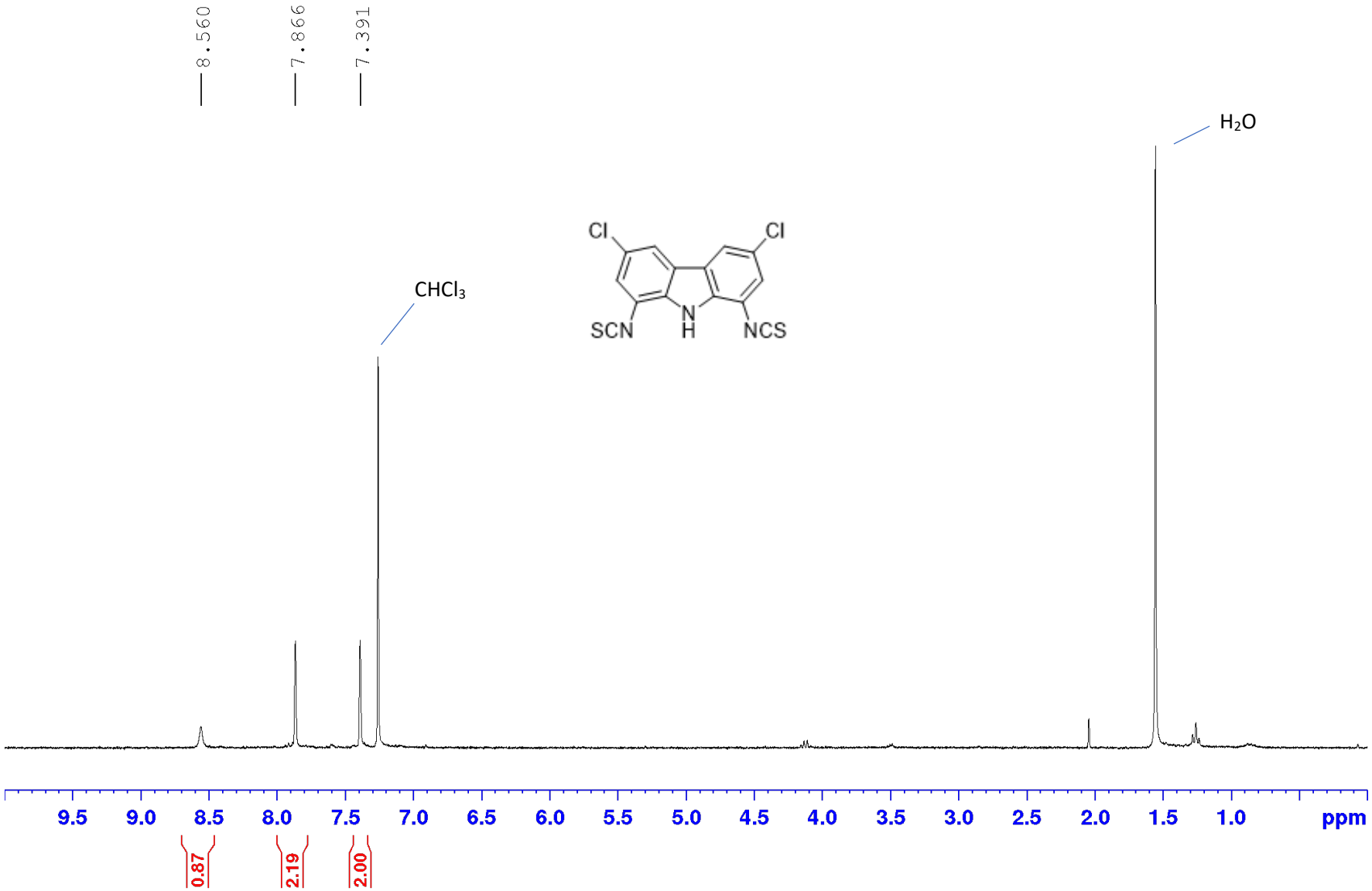
3,6-Dichloro-1,8-diisothiocyanato-*9H*-carbazole (5) – 300 MHz, $CDCl_3$



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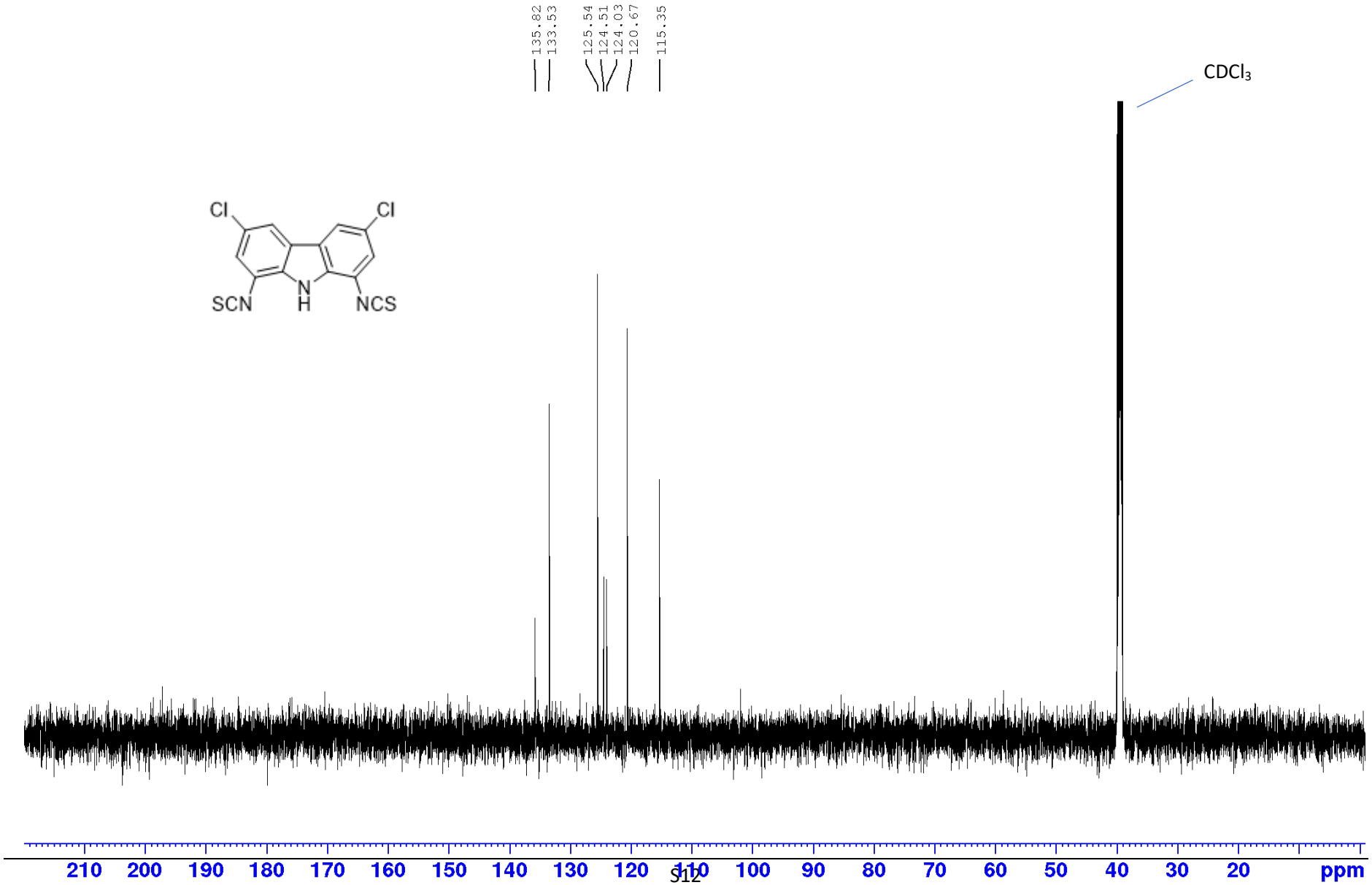

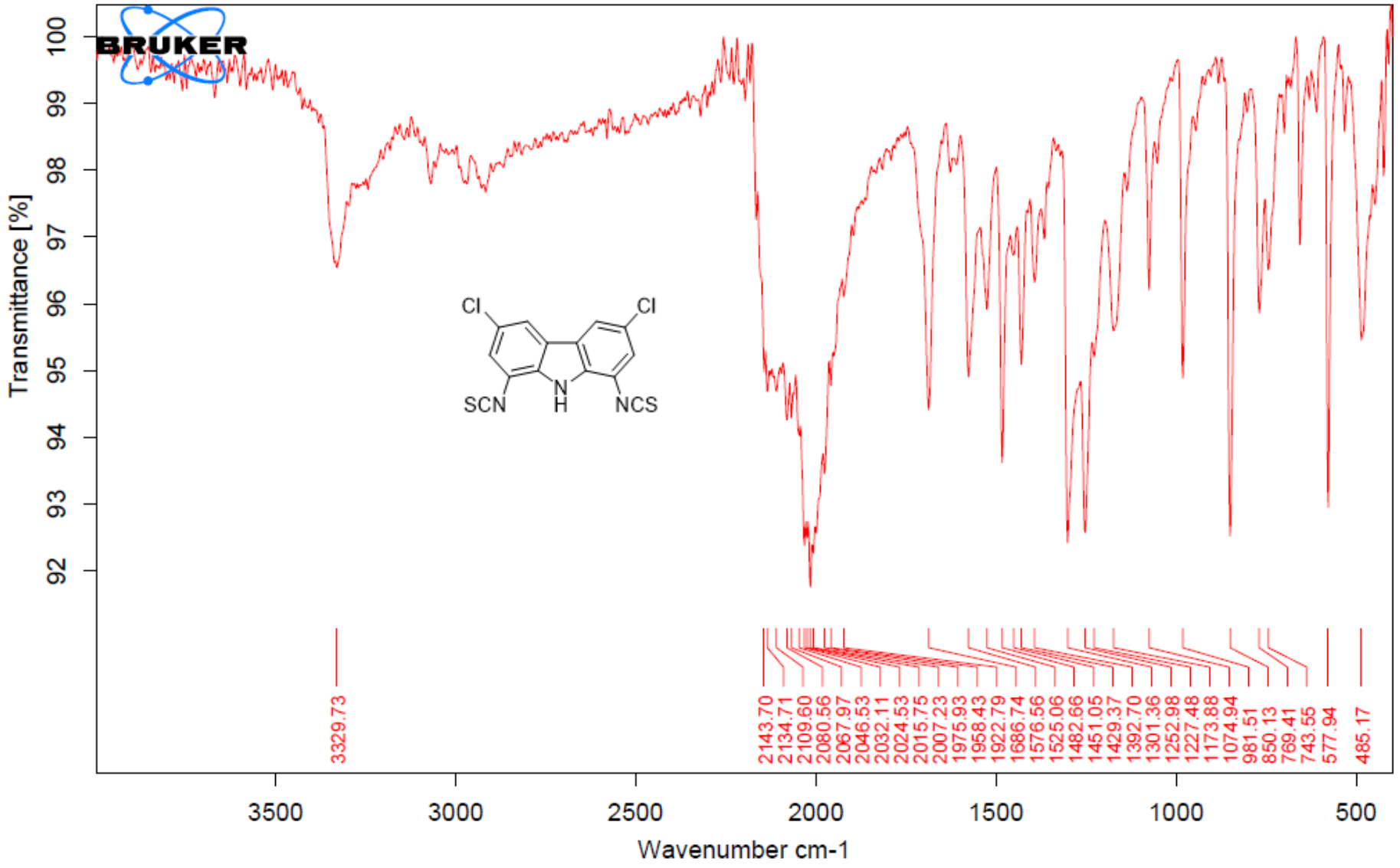
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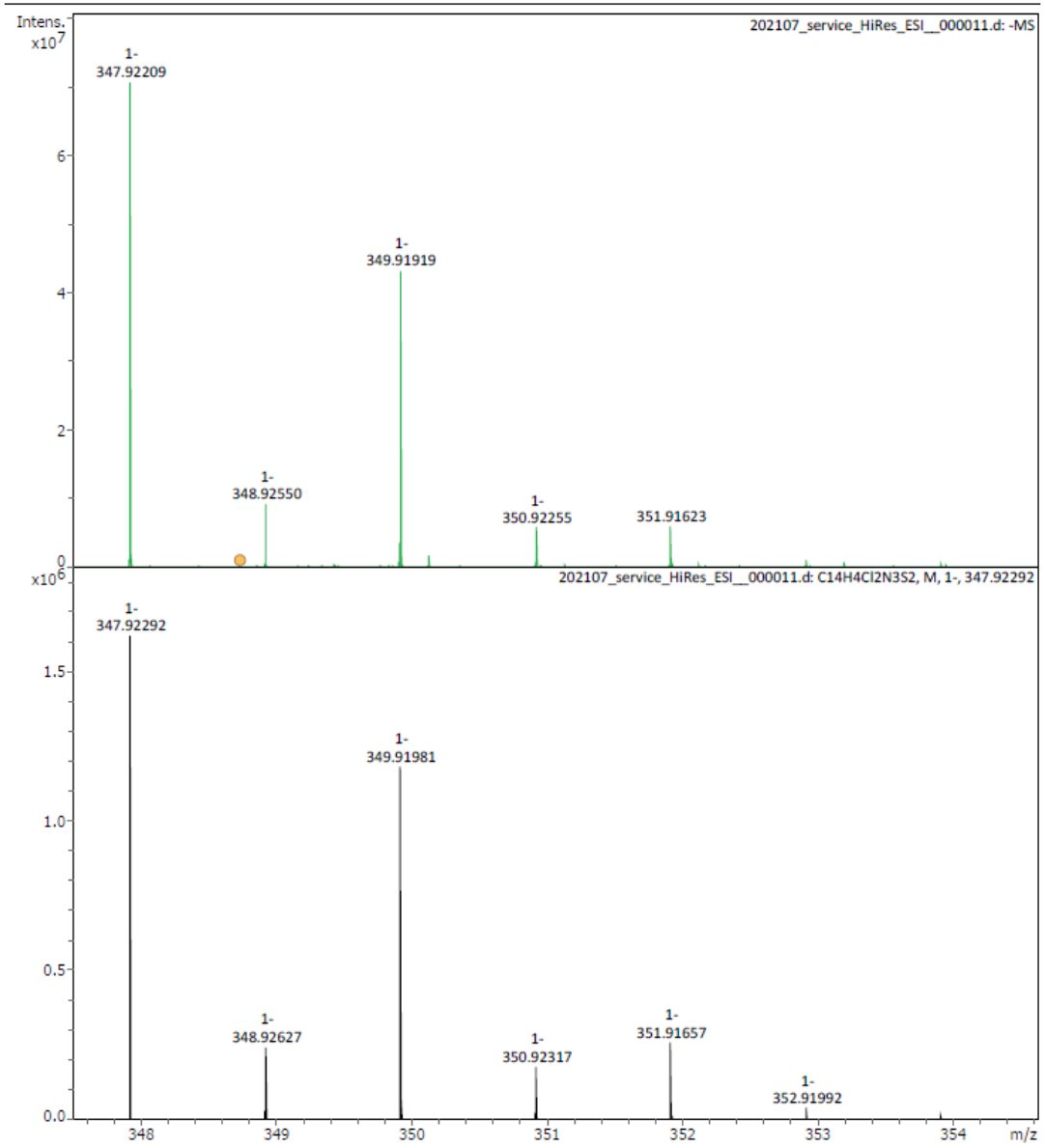
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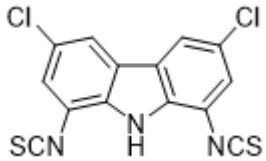
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Cl
SCN
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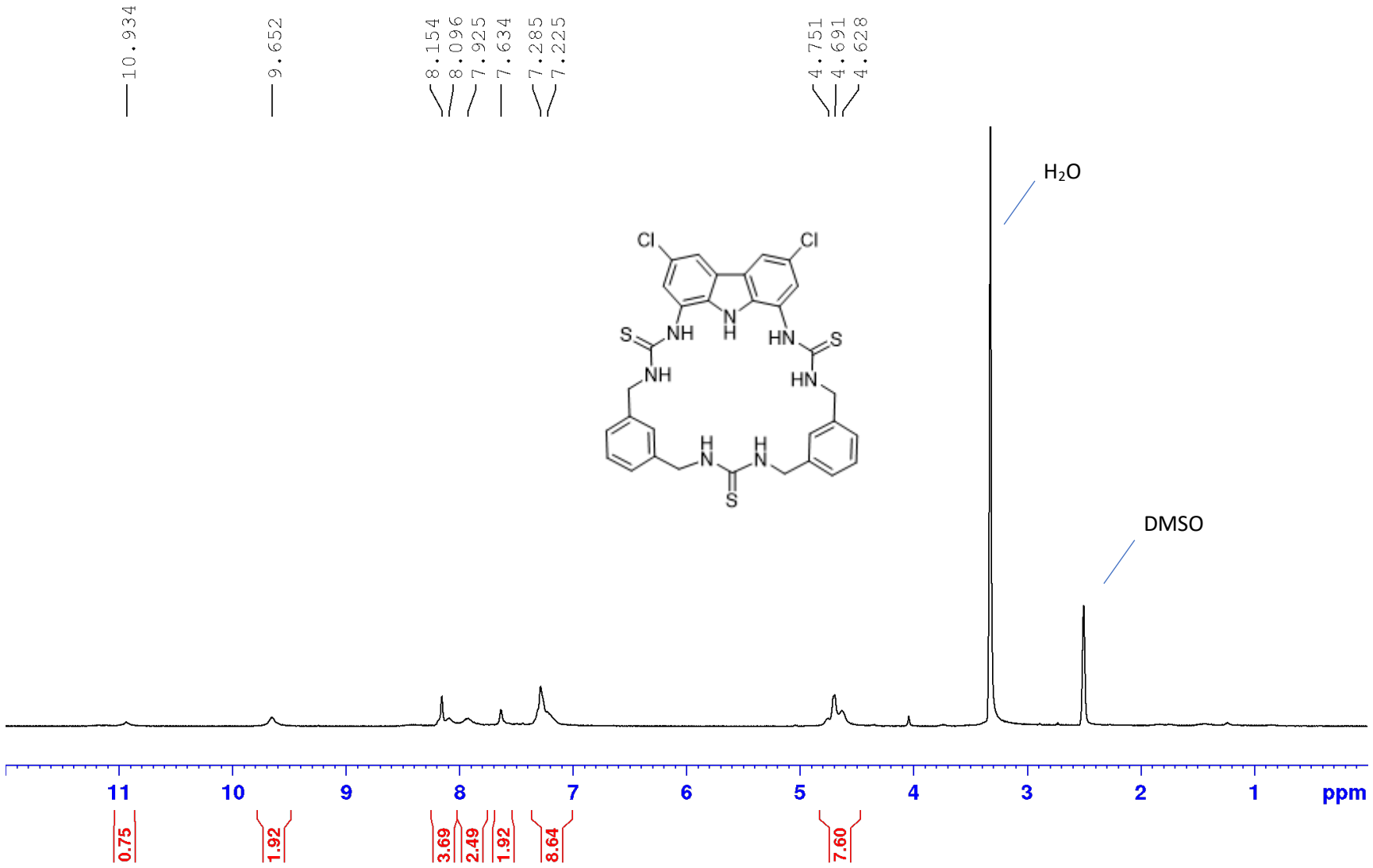
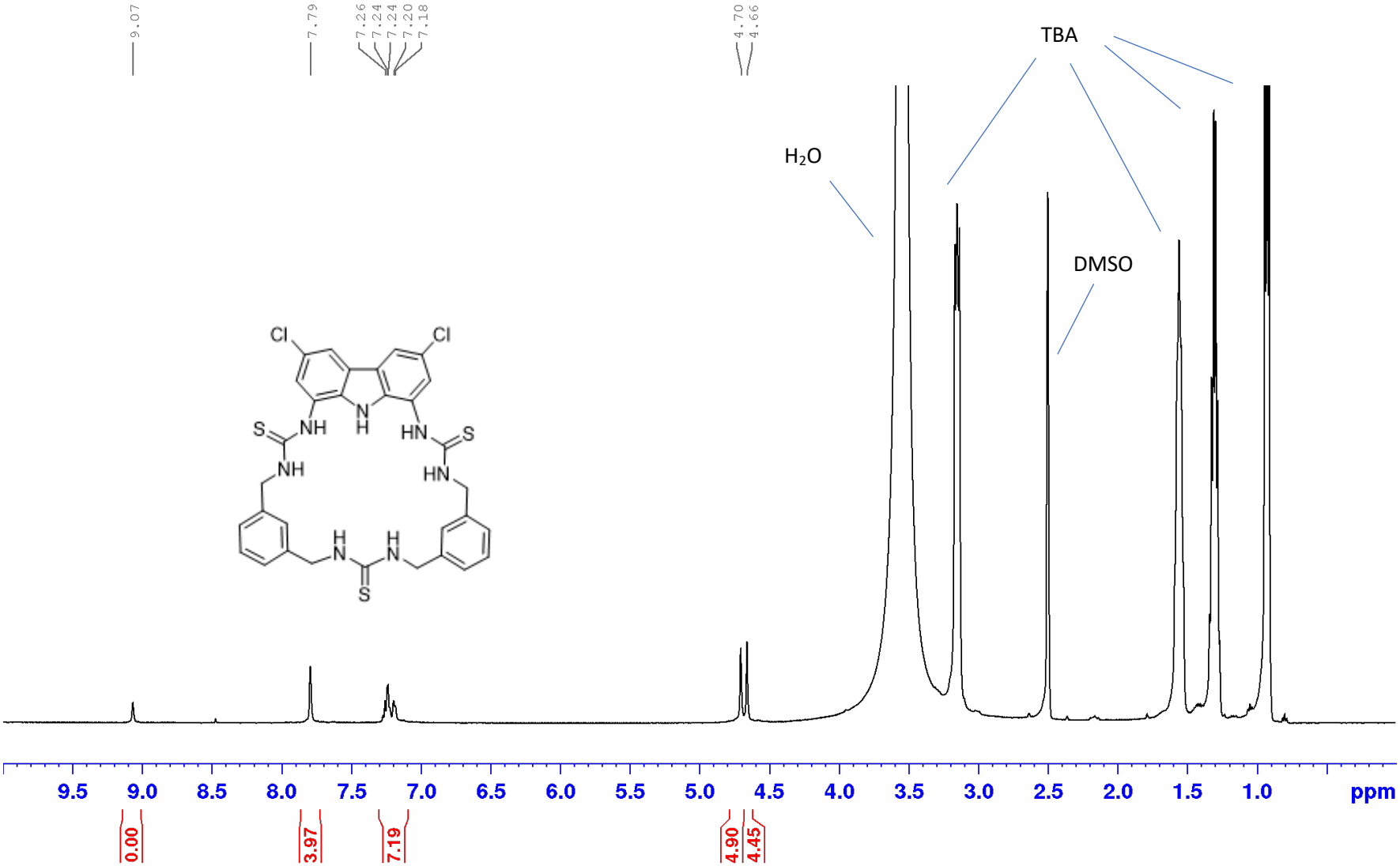
H
NCS

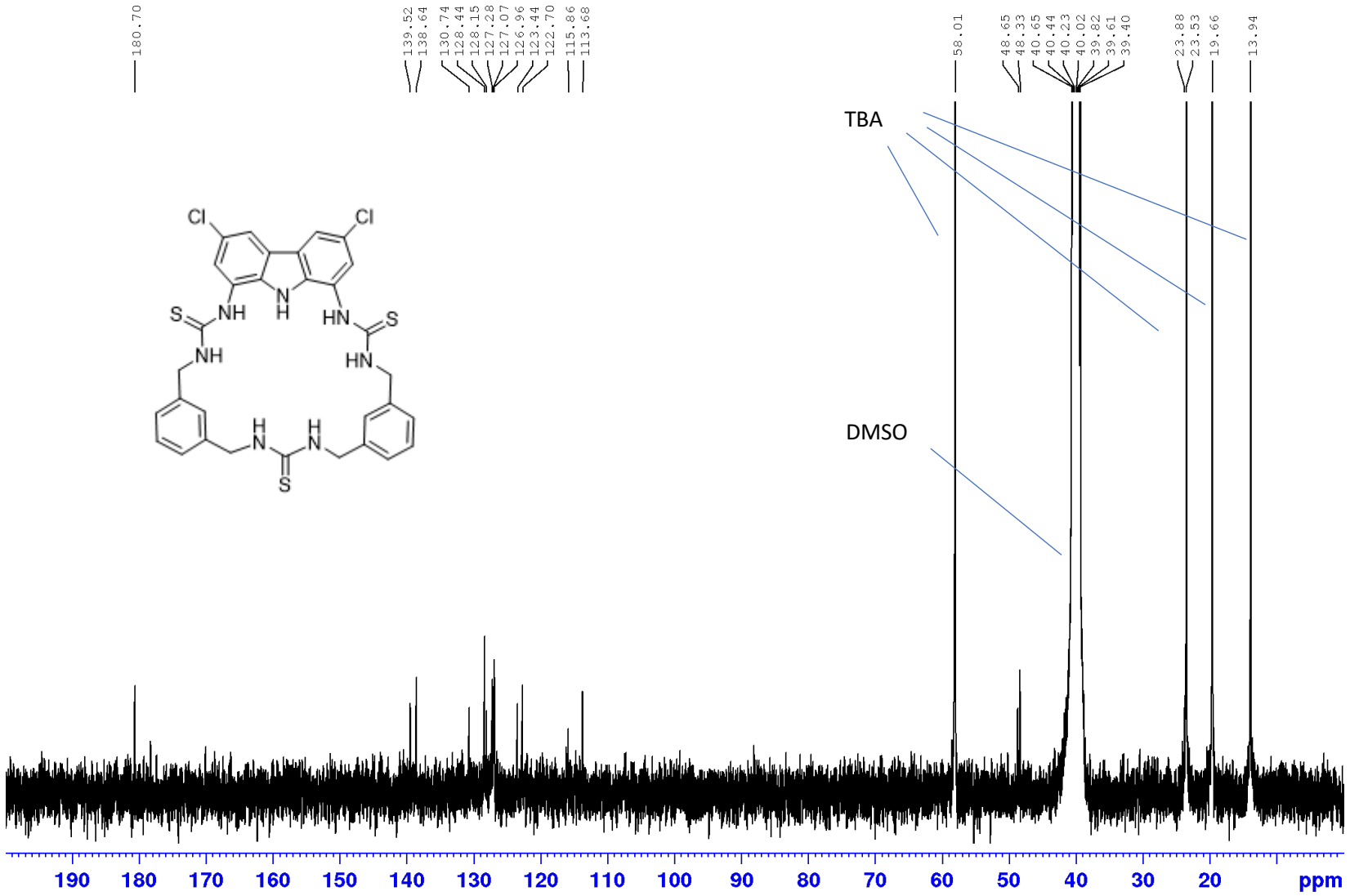
6^3,6^6-dichloro-6^9*H*-3,5,7,9,13,15-hexaaza-6(1,8)-carbazola-1,11(1,3)-dibenzenacyclohexadecaphane-4,8,14-trithione (2) –400 MHz, DMSO-*d6*

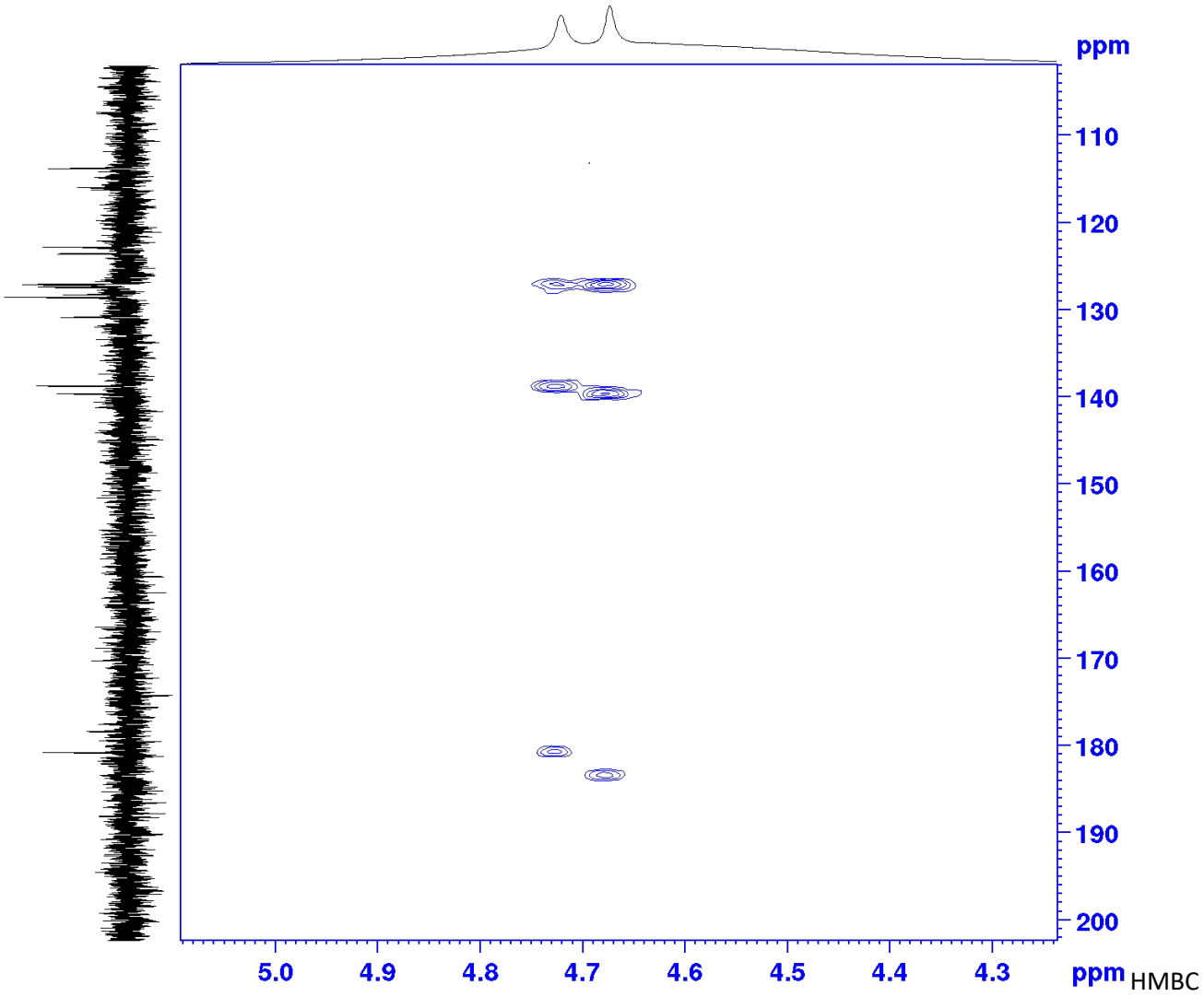


6^3,6^6-dichloro-6^9*H*-3,5,7,9,13,15-hexaaza-6(1,8)-carbazola-1,11(1,3)-dibenzenacyclohexadecaphane-4,8,14-trithione (2)(bound to TBA_2HPO_4) –400 MHz, DMSO-*d6*



6^3,6^6-dichloro-6^9*H*-3,5,7,9,13,15-hexaaza-6(1,8)-carbazola-1,11(1,3)-dibenzenacyclohexadecaphane-4,8,14-trithione (2)(bound to TBA_2HPO_4) –400 MHz, DMSO-*d6*

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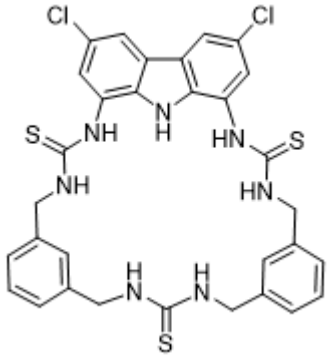
4.9
4.8
4.7
4.6
4.5
4.4
4.3
ppm

HMBC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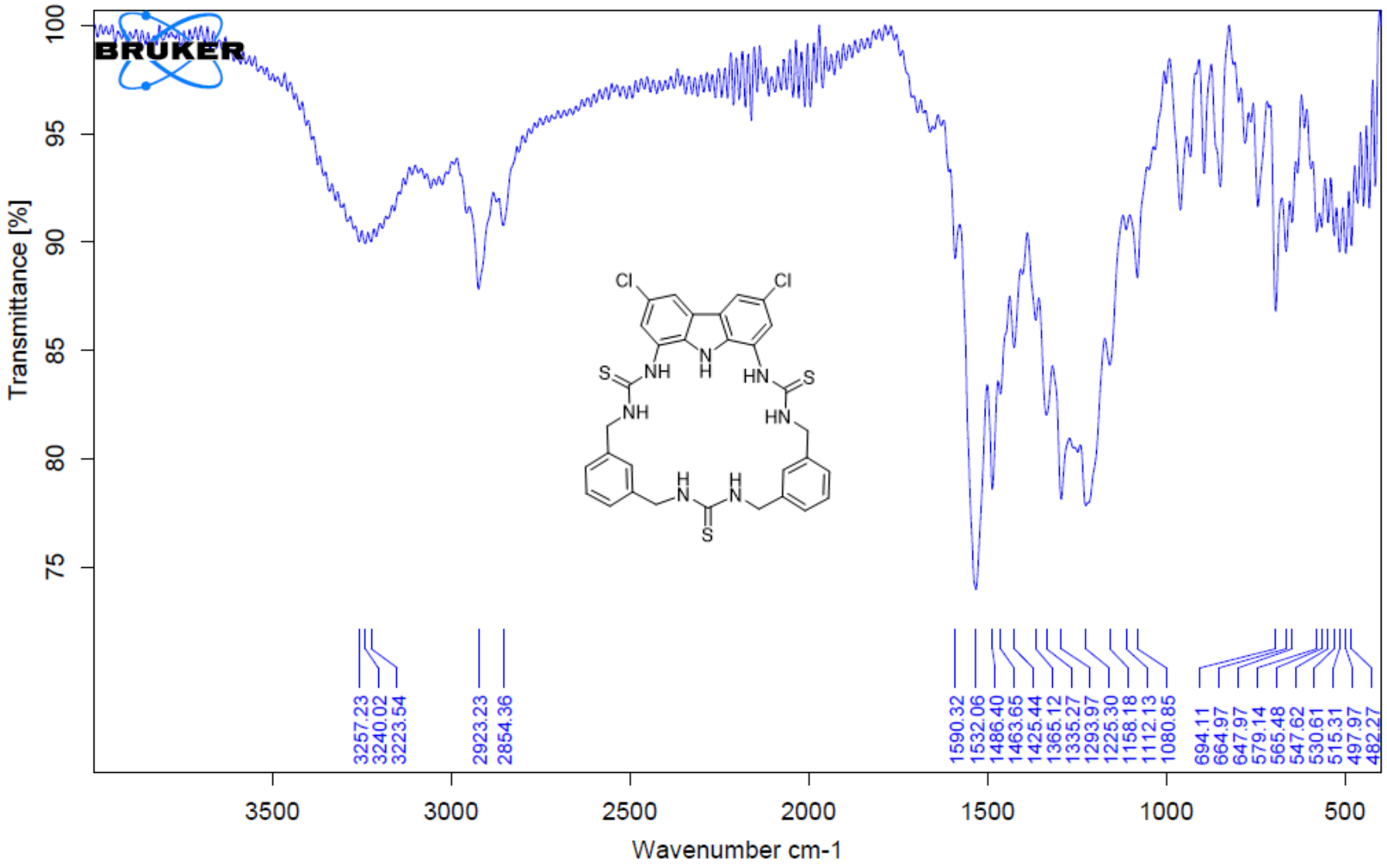
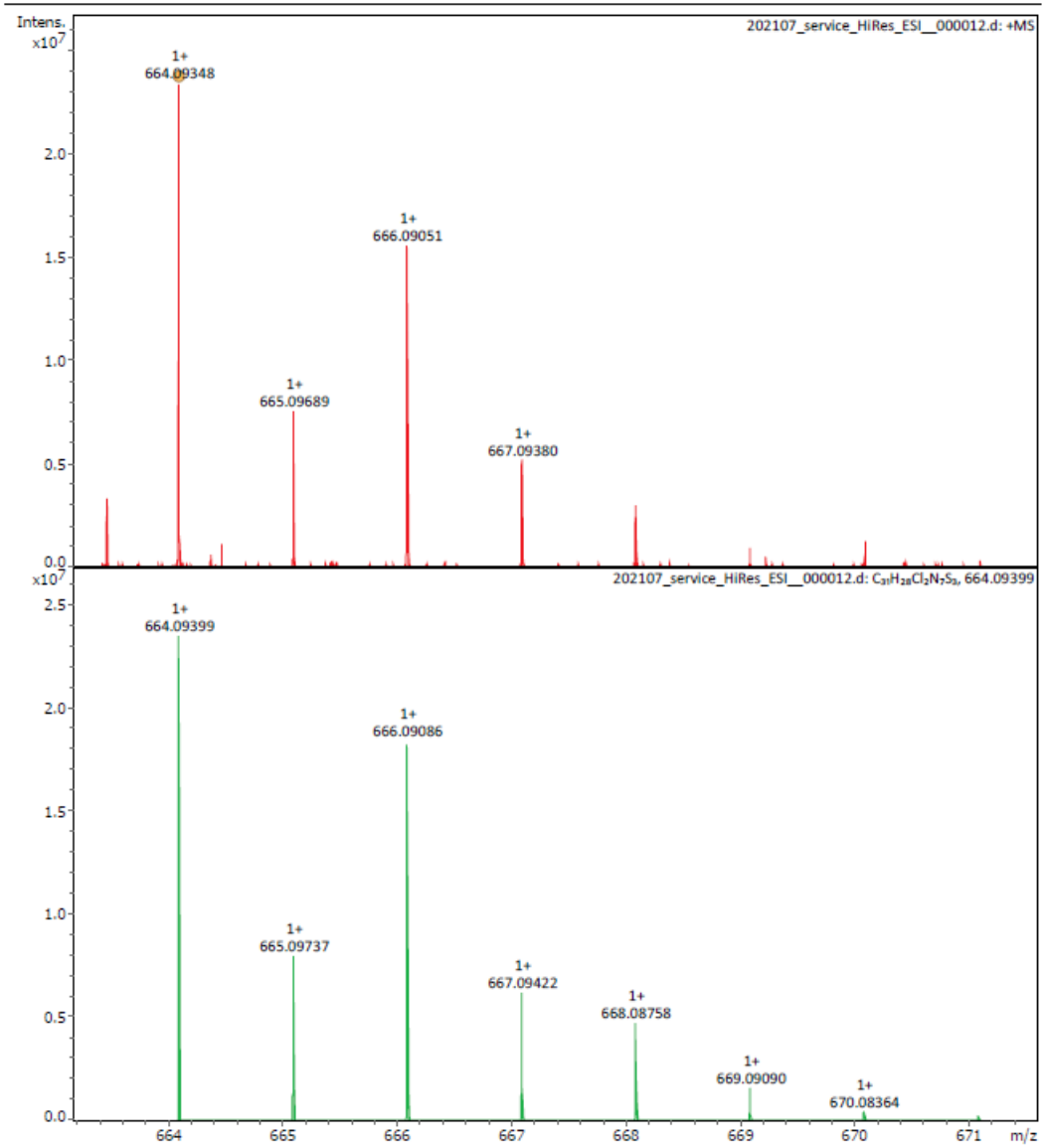
Cl
S
NH
NH
N
H
HN
S
HN
H
N
H
N
S


BRUKER
Transmittance [%]
100
95
90
85
80
75
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1
3257.23
3240.02
3223.54
2923.23
2854.36
1590.32
1532.06
1486.40
1463.65
1425.44
1365.12
1335.27
1293.97
1225.30
1158.18
1112.13
1080.85
694.11
664.97
647.97
579.14
565.48
547.62
530.61
515.31
497.97
482.27
Cl
Cl
N
H
S
NH
NH
HN
S
HN
H
N
H
N
S


Intens.
x10^7
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1+
666.09051
1+
665.09689
1+
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1+
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1+
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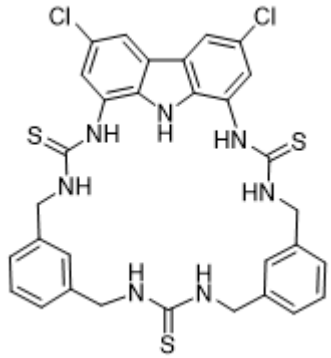
1+
668.08758
1+
669.09090
1+
670.08364
m/z


Cl
Cl
S
NH
N
H
HN
S
NH
HN
H
N
H
N
S

Additional Figures

Figure S1—DFT modelling of receptor 2 with A: adipate; B: terephthalate

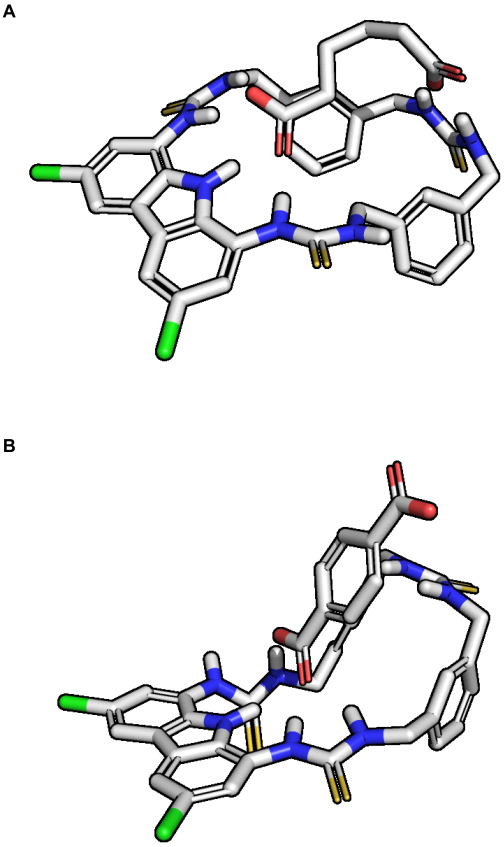


Figure S2—Variable temperature ^{1}H NMR

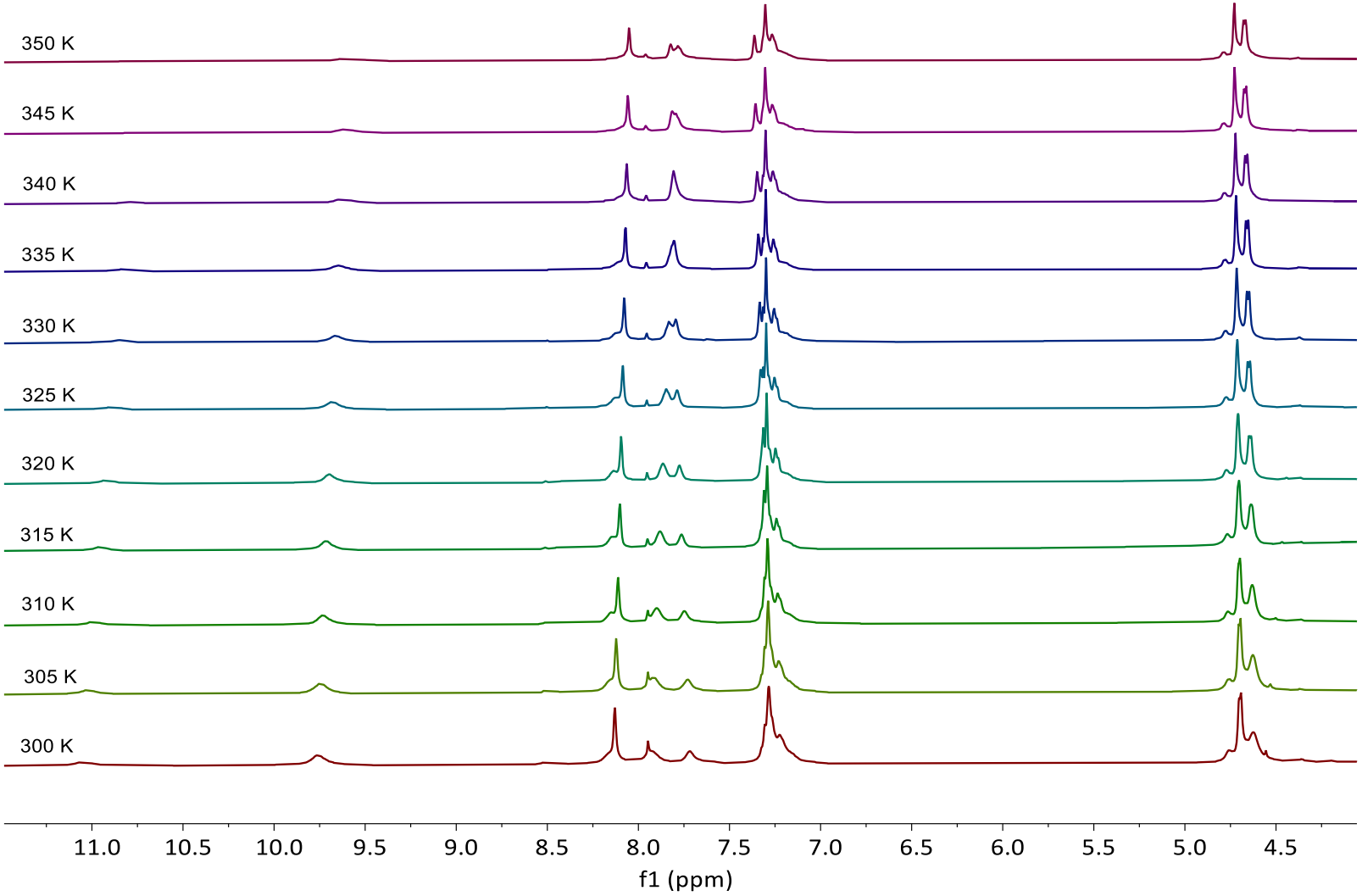


Figure S3—Terephthalate ^{1}H NMR titration

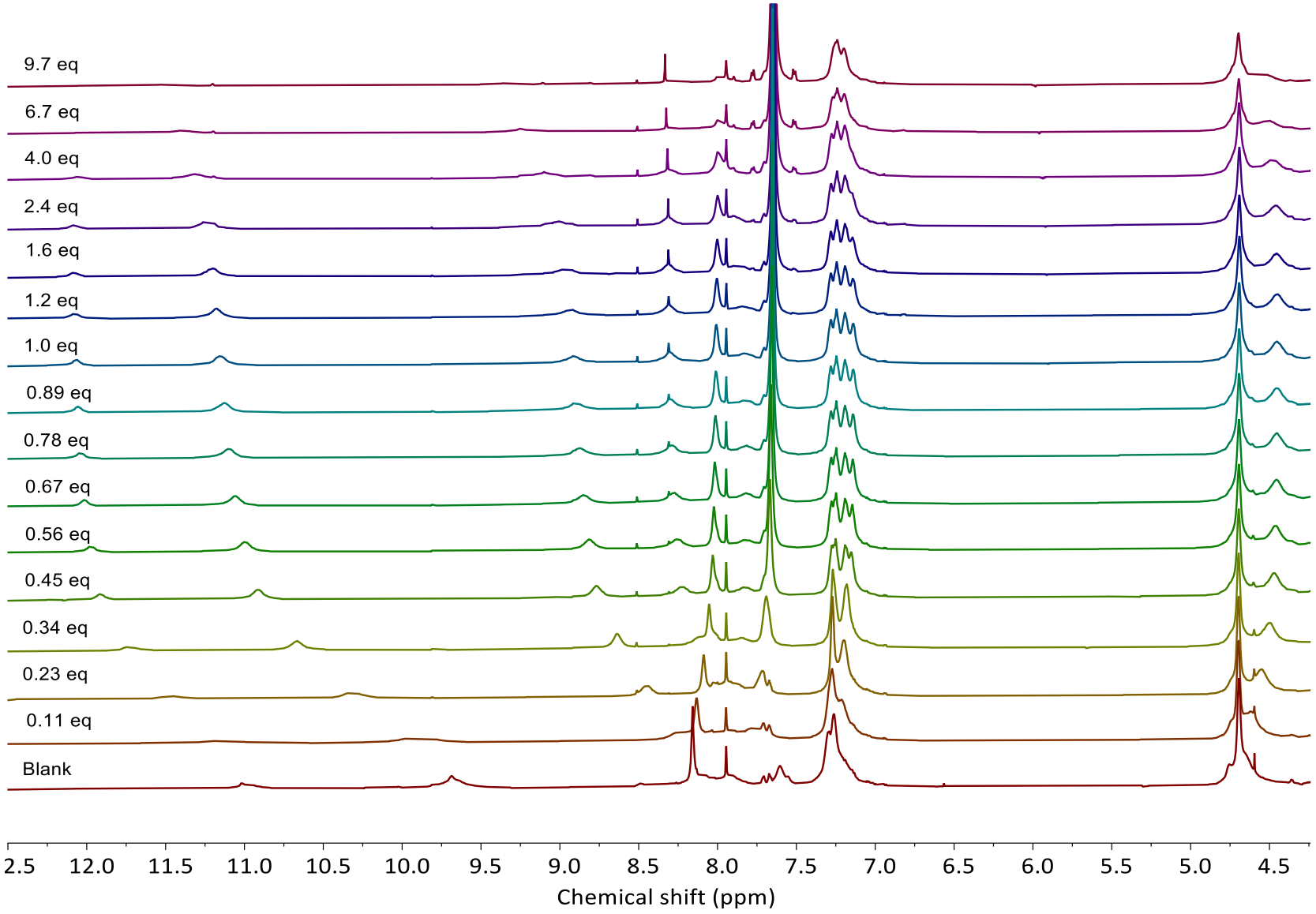


Figure S4—Propiolate ^{1}H NMR titration

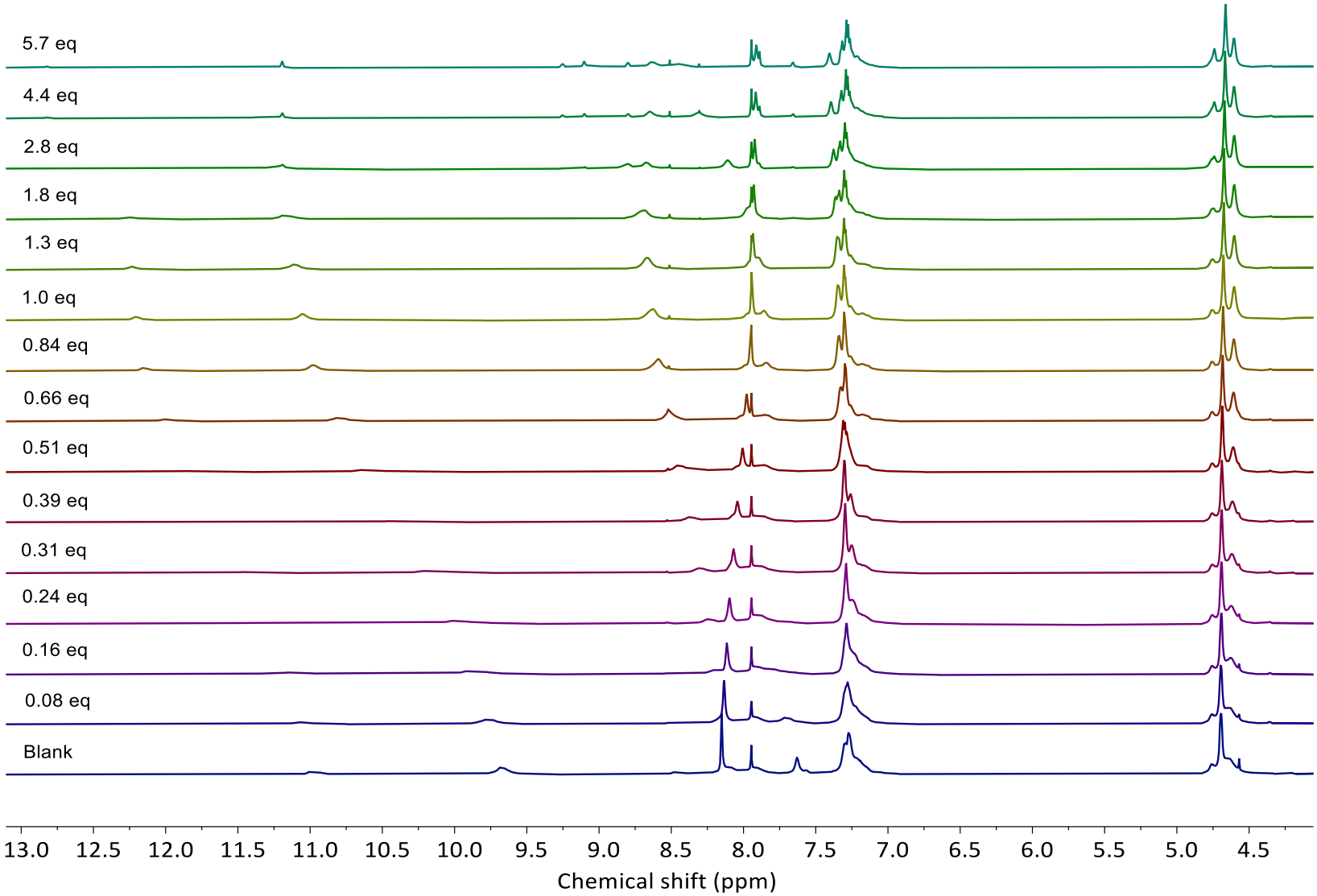


Figure S5—Malonate ^{1}H NMR titration

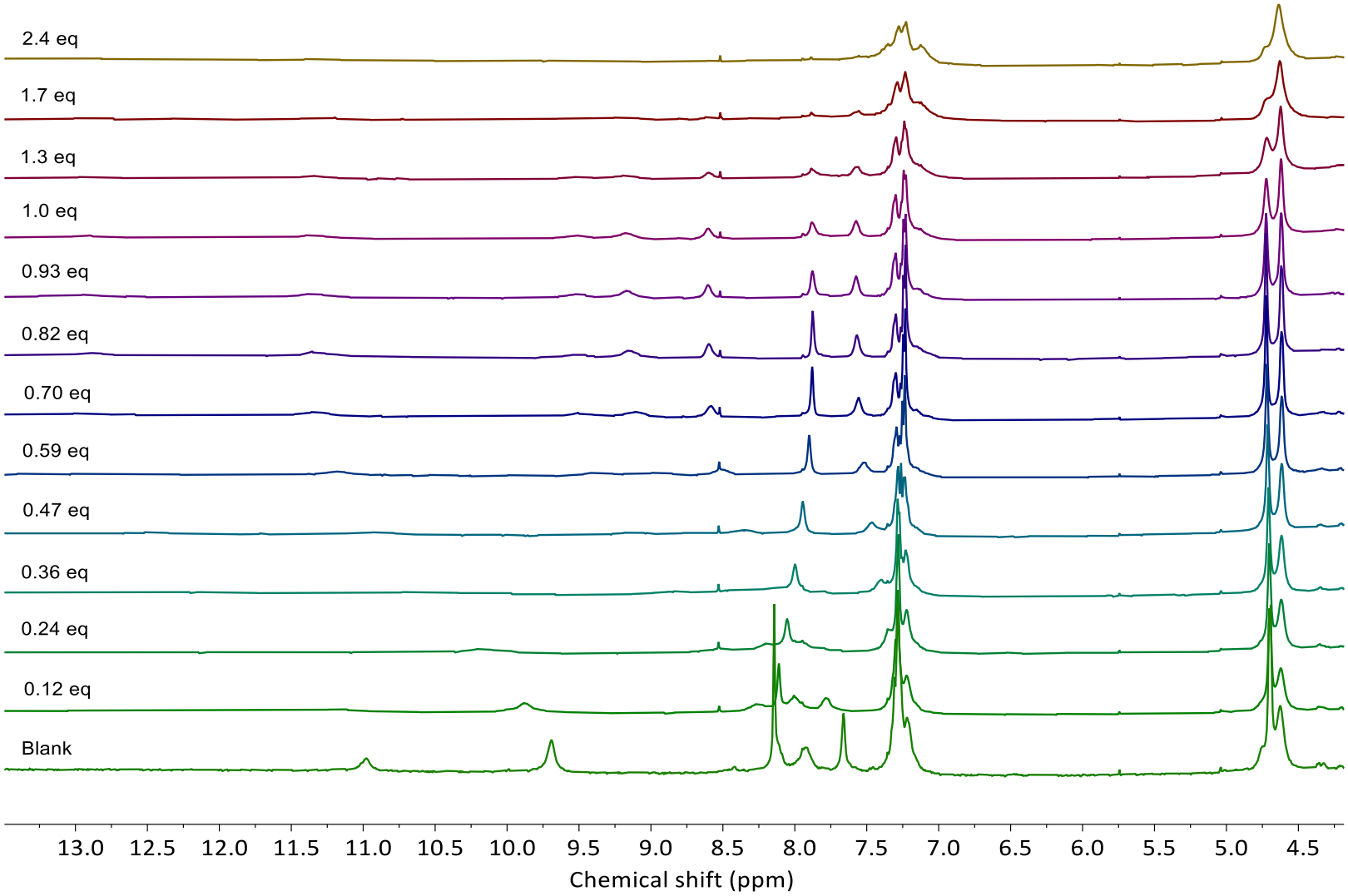
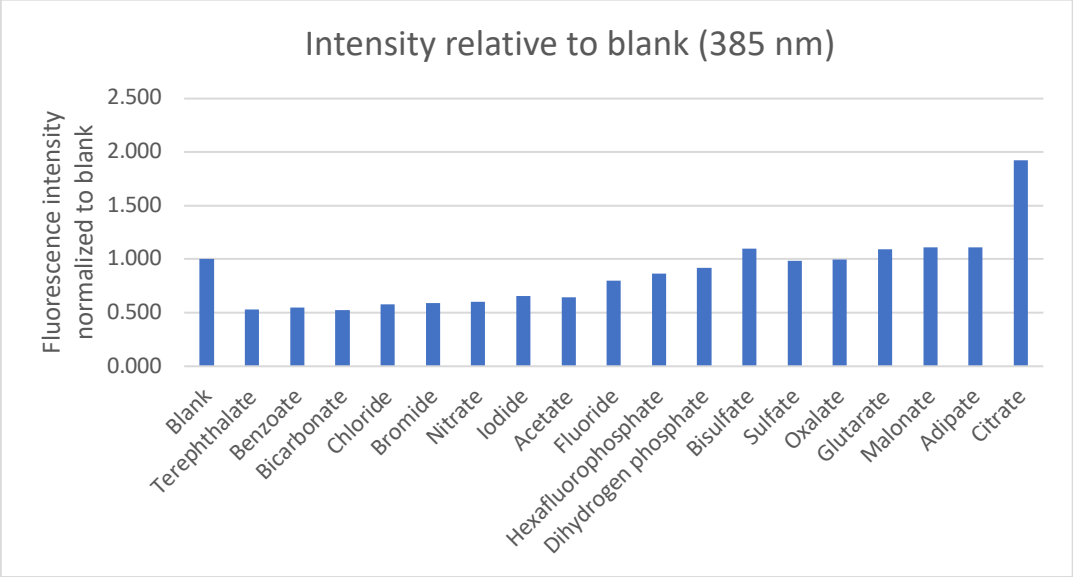
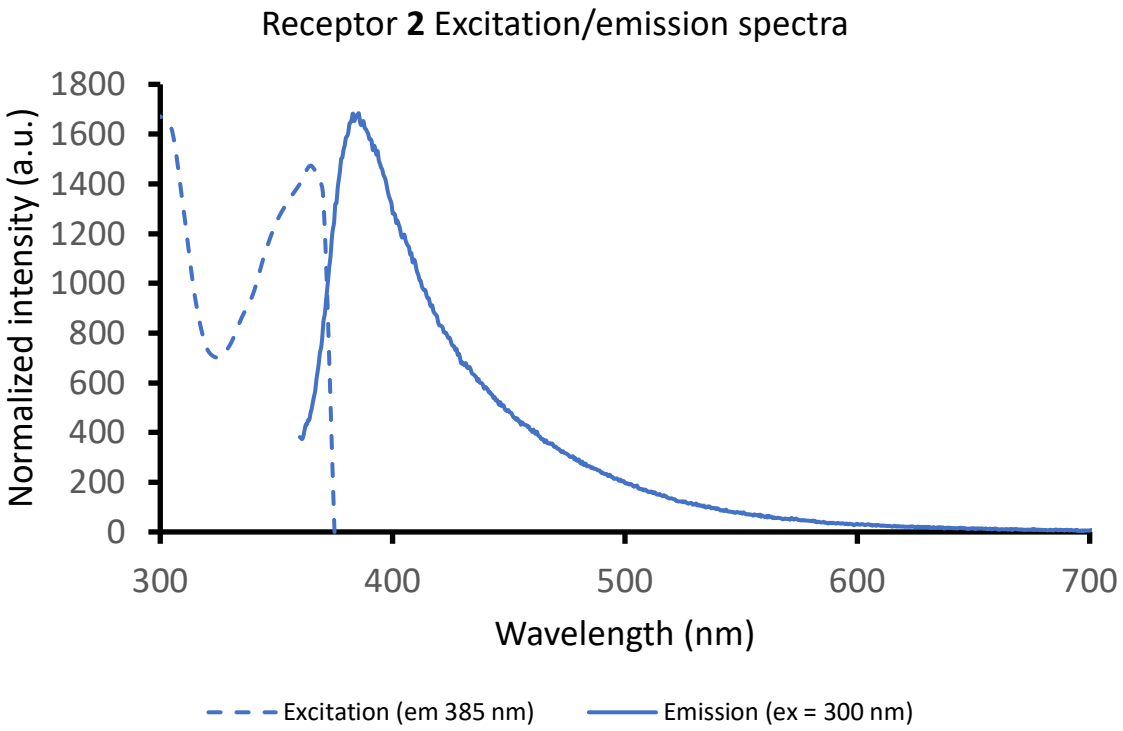


Figure S6—Screening data: all anions



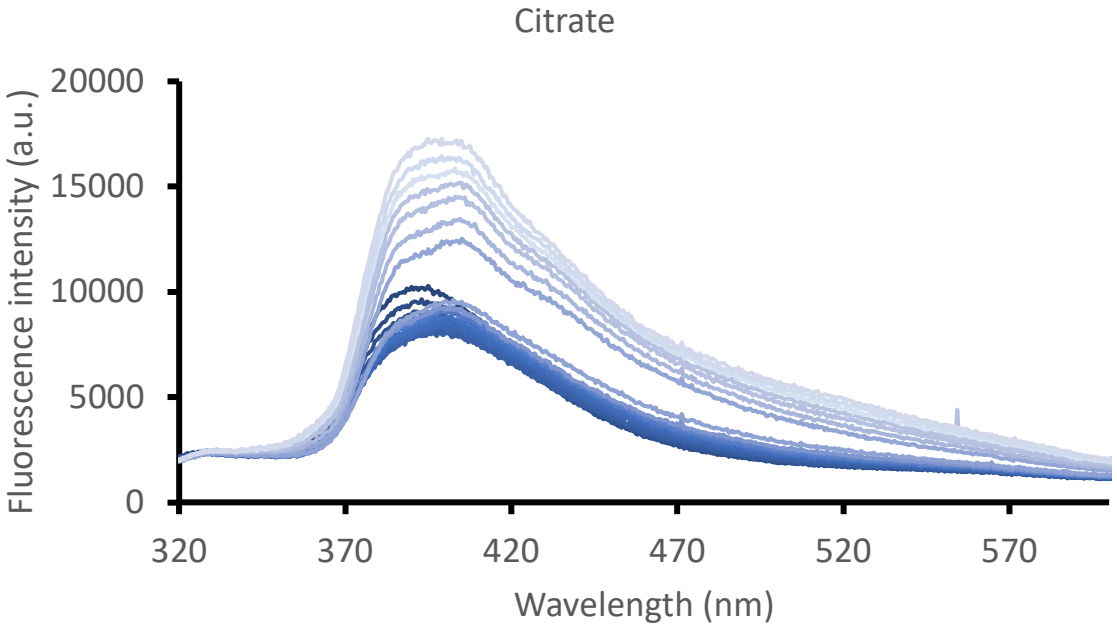
Changes in fluorescence intensity at 385 nm of receptor **2** (25 μM) in 9:1 CH_2Cl_2:methanol relative to intensity of blank with: 100 equivalents of anion (λ_{Ex} = 300 nm).

Figure S7—Excitation/emission spectrum of receptor 2

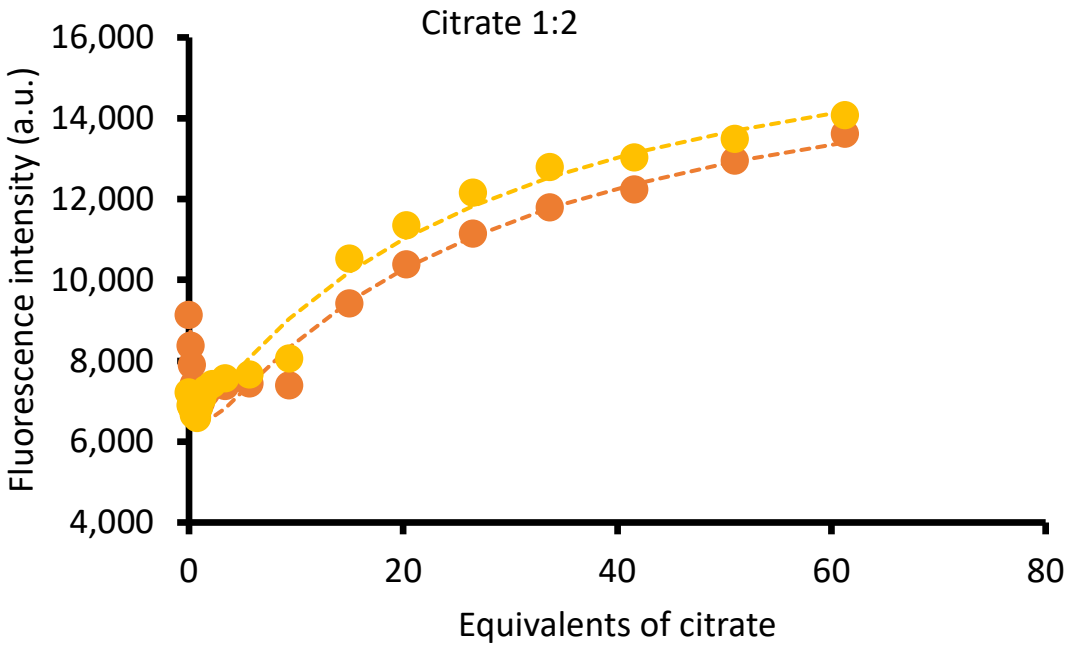


Excitation (λ_{em}= 385 nm) and emission (λ_{ex}= 300 nm) spectra of receptor **2** (25 µM in DMSO).

Figure S8—Fluorescence titration of receptor 2 (25 μM) with citrate in 9:1 CH_2Cl_2/MeOH



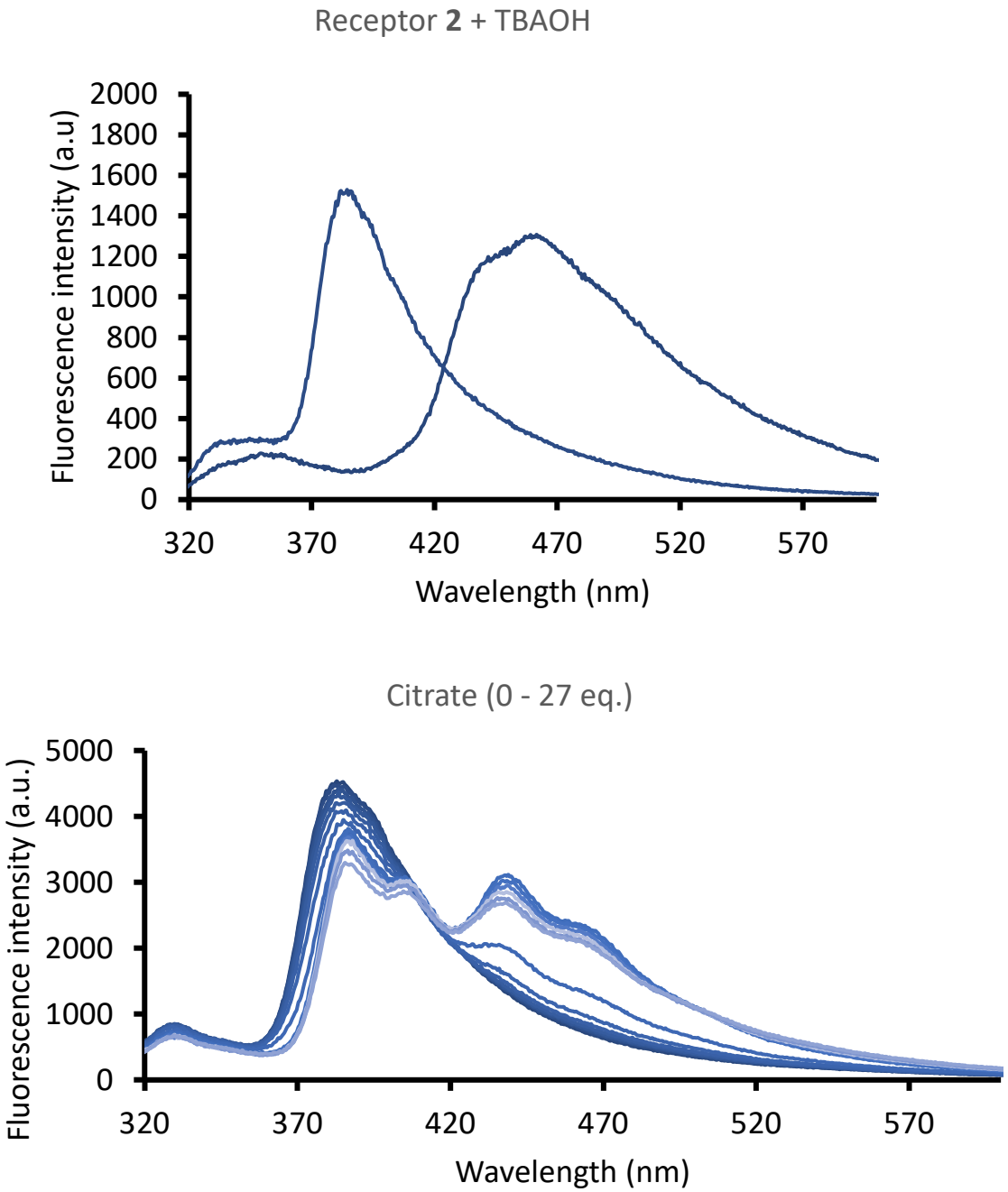
Representative data and fit of 1:2 binding model between receptor **2** and citrate. Coloured dots represent data collected at 380 nm (orange) and 420 nm (yellow), and dotted lines the expected fit.



http://app.supramolecular.org/bindfit/view/532ac009-1054-4c00-856b-1e0603d80eec

Figure S9—Deprotonation of receptor 2 (25 µM) in DMSO

Receptor **2** undergoes excited state deprotonation in the presence of carboxylates in aqueous DMSO, leading to a new emission maximum at 465 nm. This matches the spectrum of receptor **2** deprotonated with TBAOH.



Addition of dicarboxylate species as weakly basic as tetrabutylammonium terephthalate (pKa 2.95, 5.41 in H_2O) leads to excited state deprotonation of the receptor in DMSO solutions (data for 1% H_2O shown below).

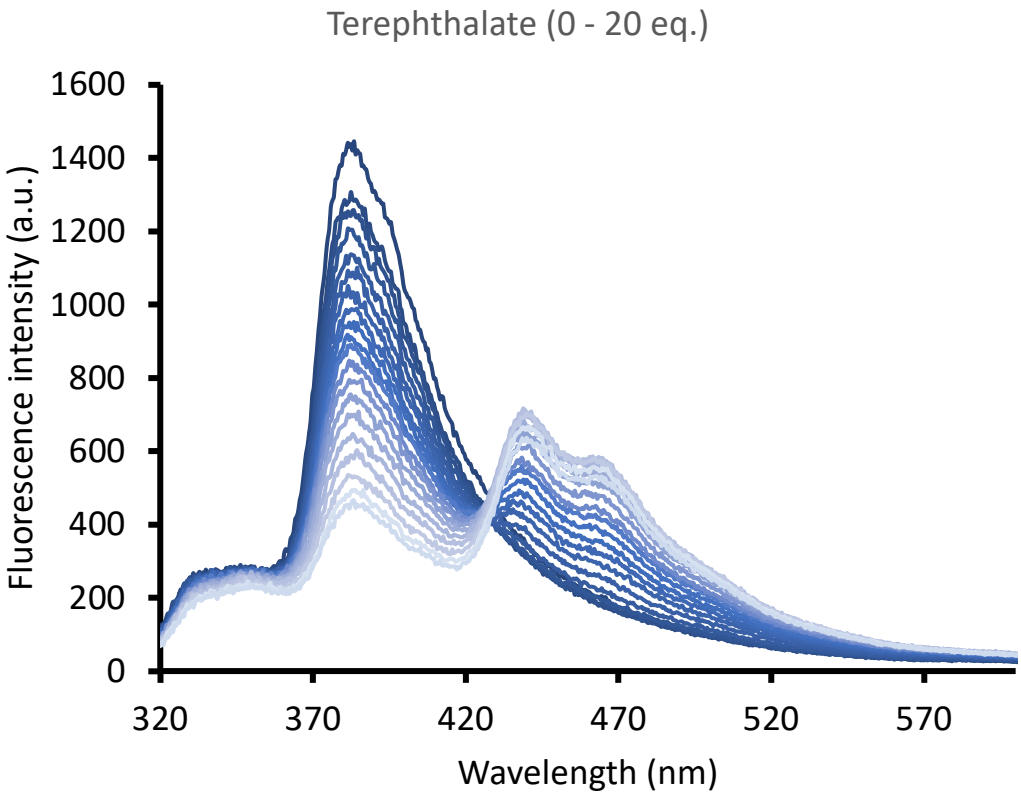
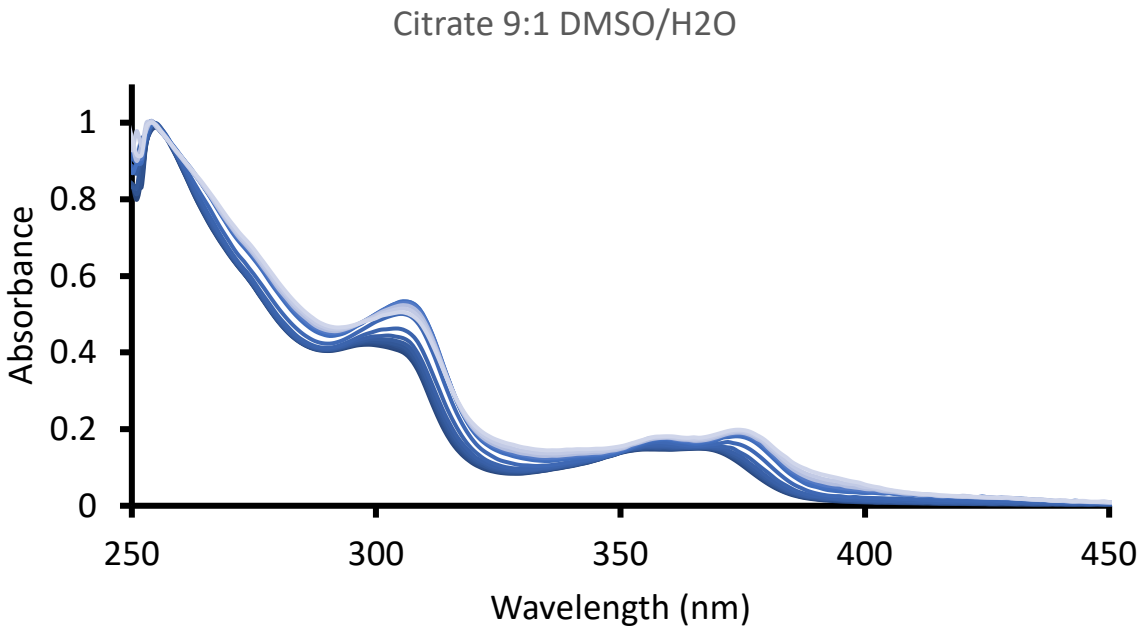
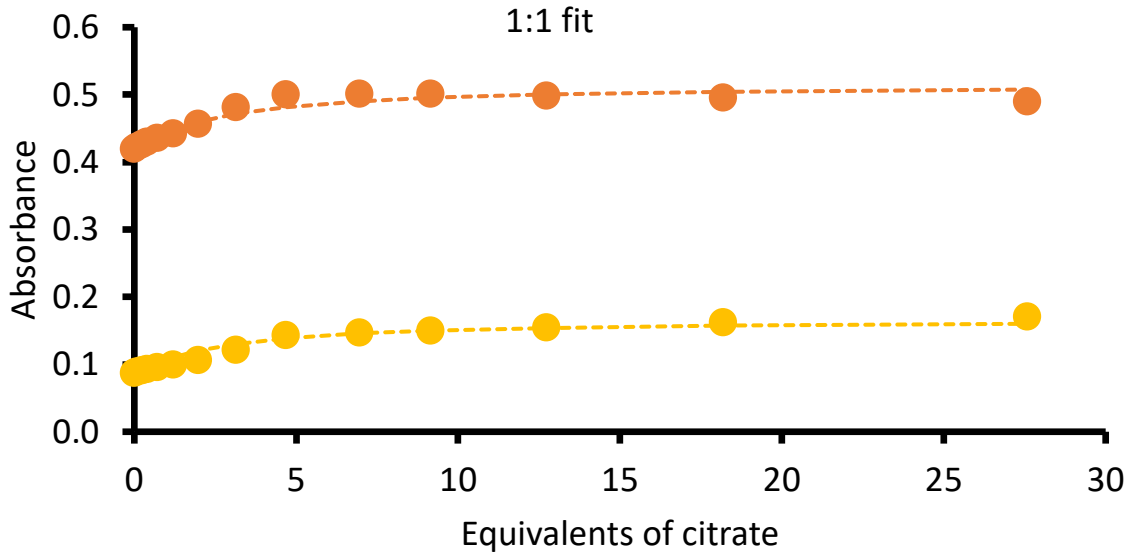


Figure S10—UV/Vis titration of receptor 2 (25 µM) with citrate in 9:1 DMSO/H_2O



Representative data and fits to binding models of receptor **2** and citrate. Coloured dots represent data collected at 300 nm (orange) and 325 nm (yellow), and dotted lines the expected fit.

http://app.supramolecular.org/bindfit/view/2965461d-7a8d-4bcd-8092-8d82d4e2d154



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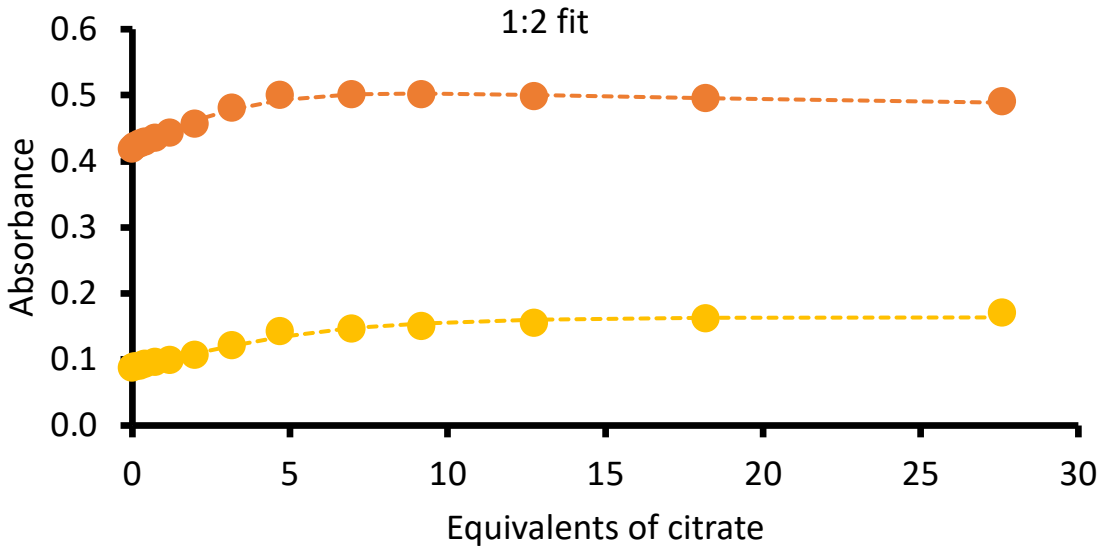

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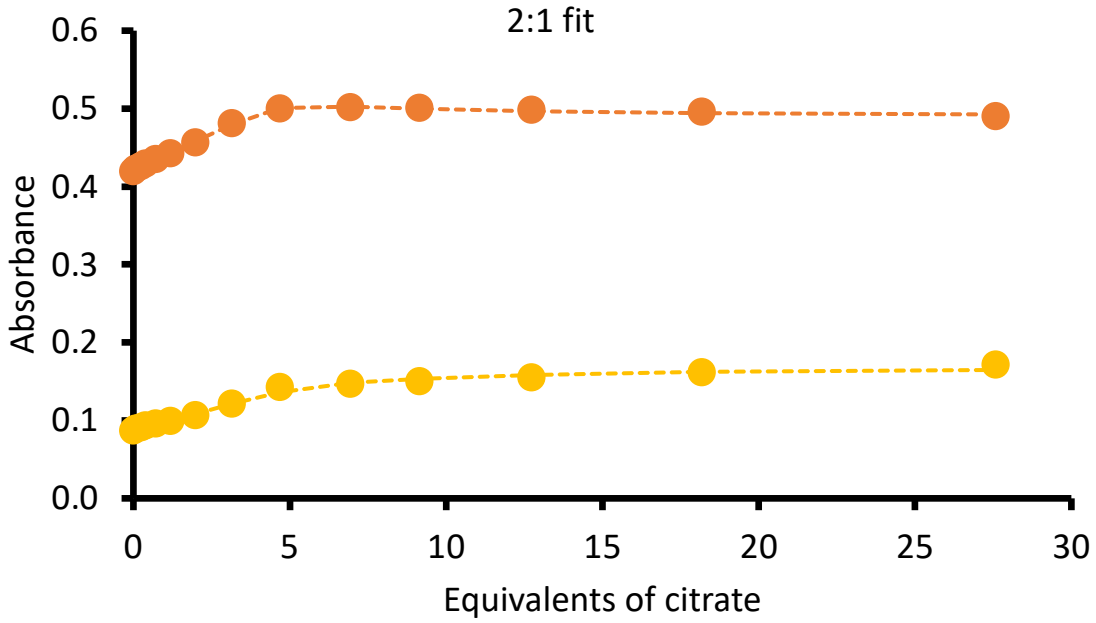
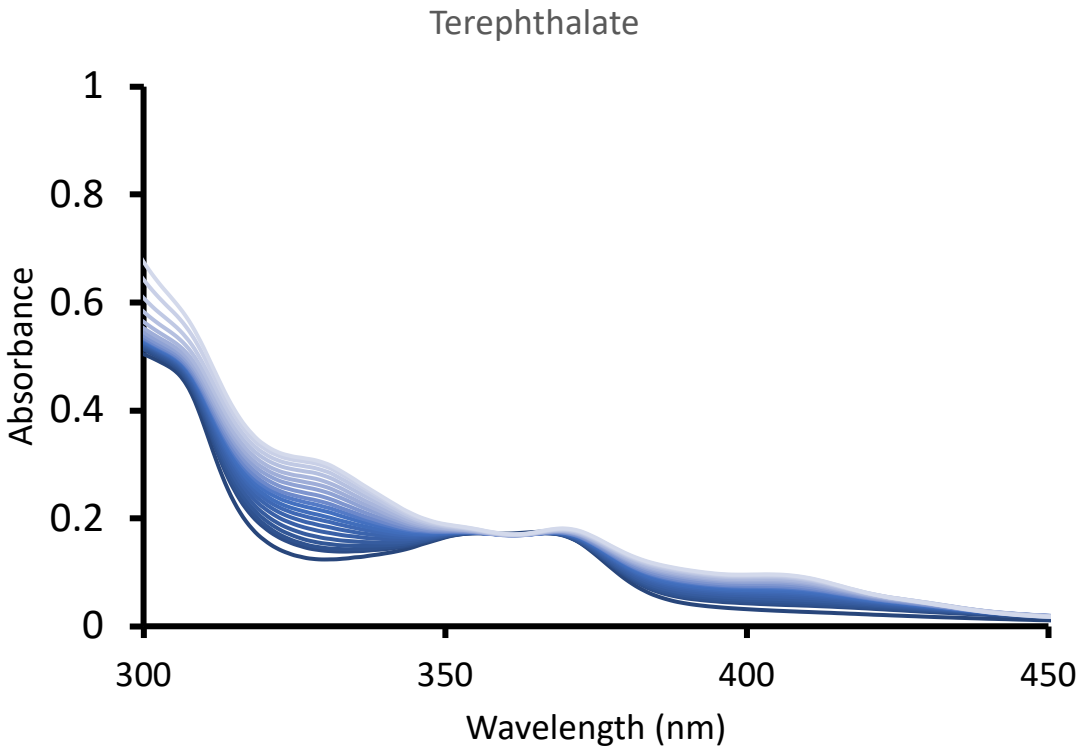
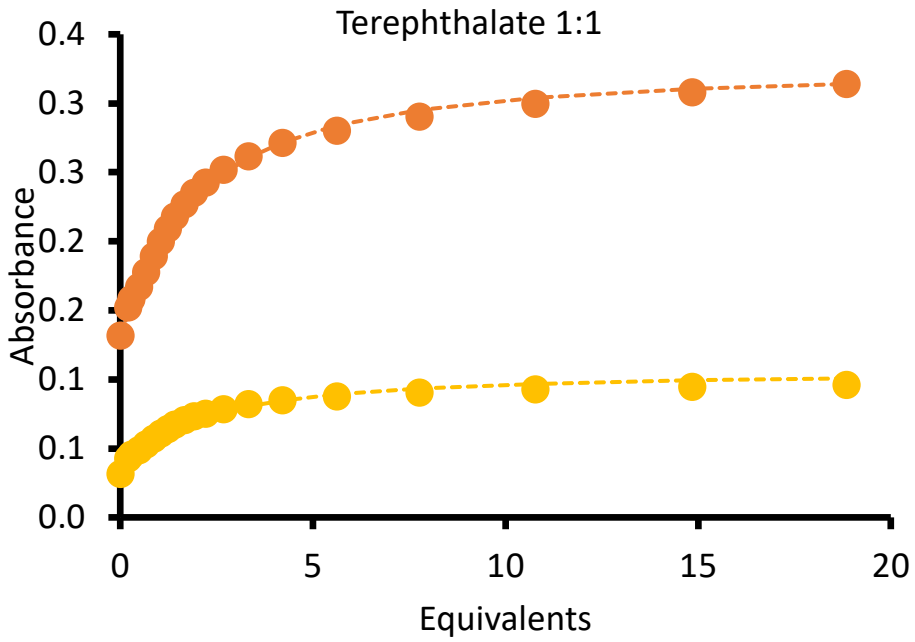


Figure S11 – Representative UV-vis titrations of receptor 2 (25 µM) with carboxylates
Terephthalate (DMSO + 1% H2O): $K_{1:1}$ = 2.83 x 10^4 M^{-1}

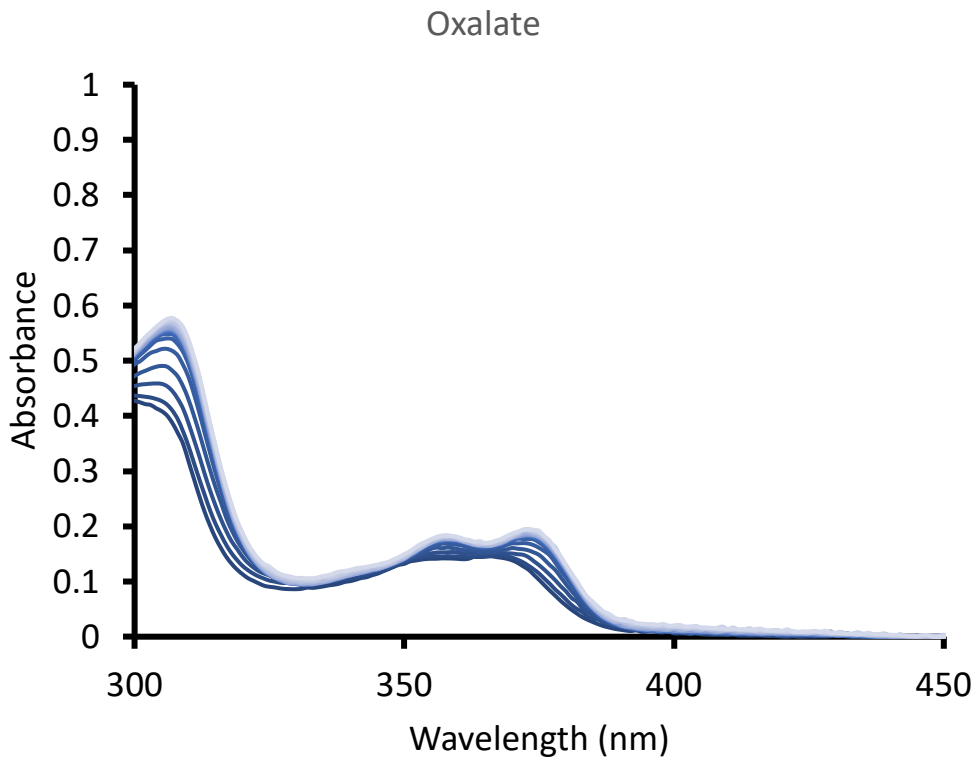


Representative data and fits to binding models of receptor **2** and terephthalate. Coloured dots represent data collected at 325 nm (orange) and 400 nm (yellow), and dotted lines the expected fit.

http://app.supramolecular.org/bindfit/view/ffc1a891-c456-48cf-98e0-0c79875fb74a



Oxalate (DMSO + 1% H2O): $K_{1:1}$ = > 10^5 M^{-1}



Representative data and fits to binding models of receptor **2** and oxalate. Coloured dots represent data collected at 300 nm (orange), 325 nm (yellow), and 380 nm (green) and dotted lines the expected fit.

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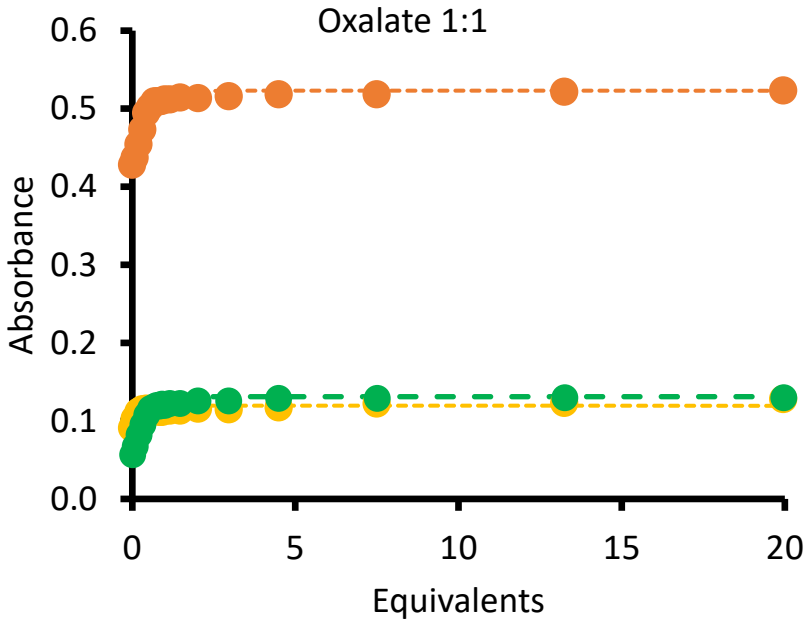
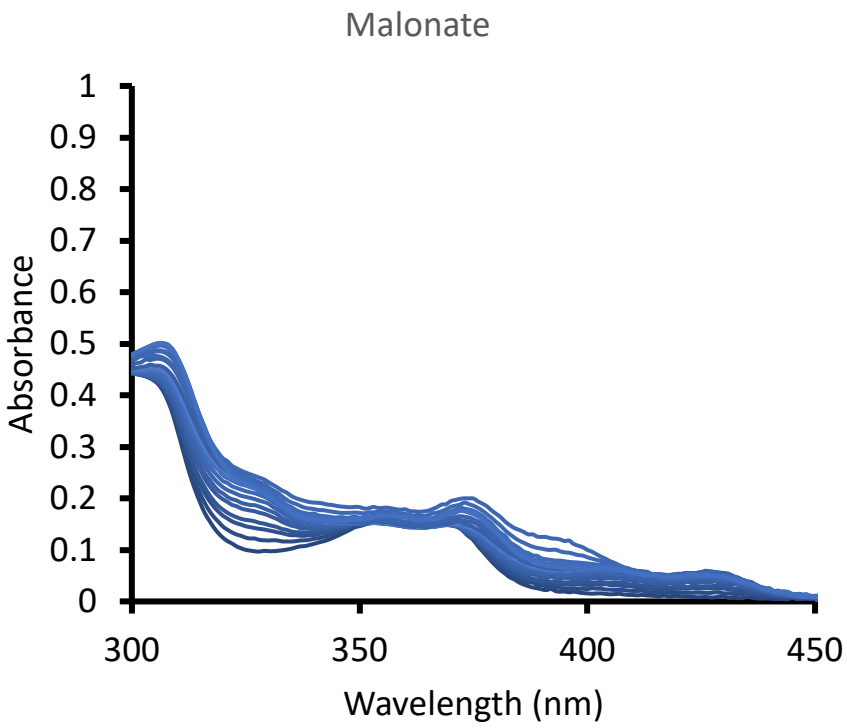


Malonate (DMSO + 1% H2O): Receptor displayed slow equilibration, with possible deprotonation of receptor. Plausible fits to 1:2 and 2:1 binding models ($K_{1:1}$ = 4.5 x 10^5 M^{-1}; $K_{1:2}$ = 2.4 x 10^4 M^{-1} or $K_{1:1}$ = 4.3 x 10^4; $K_{2:1}$ = 3.4 x 10^3).



Representative data and fits to binding models of receptor **2** and malonate. Coloured dots represent data collected at 300 nm (orange), 325 nm (yellow), and 375 nm (green) and dotted lines the expected fit.

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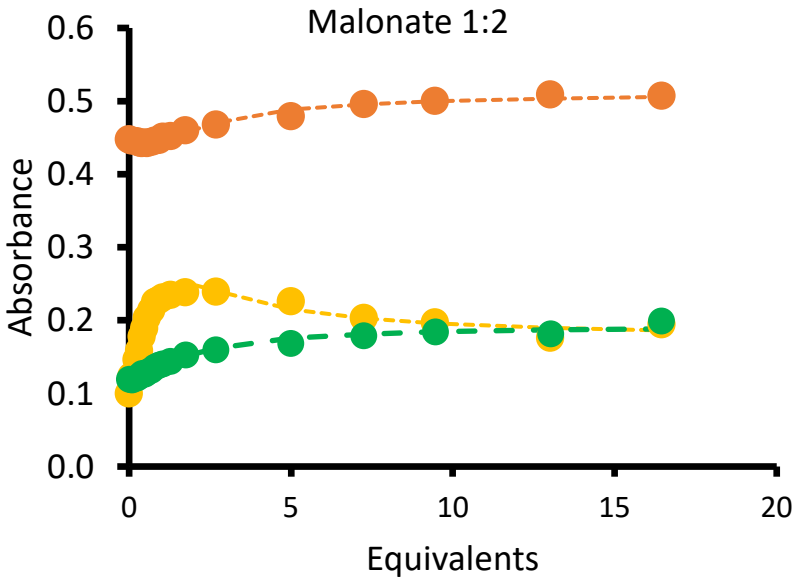
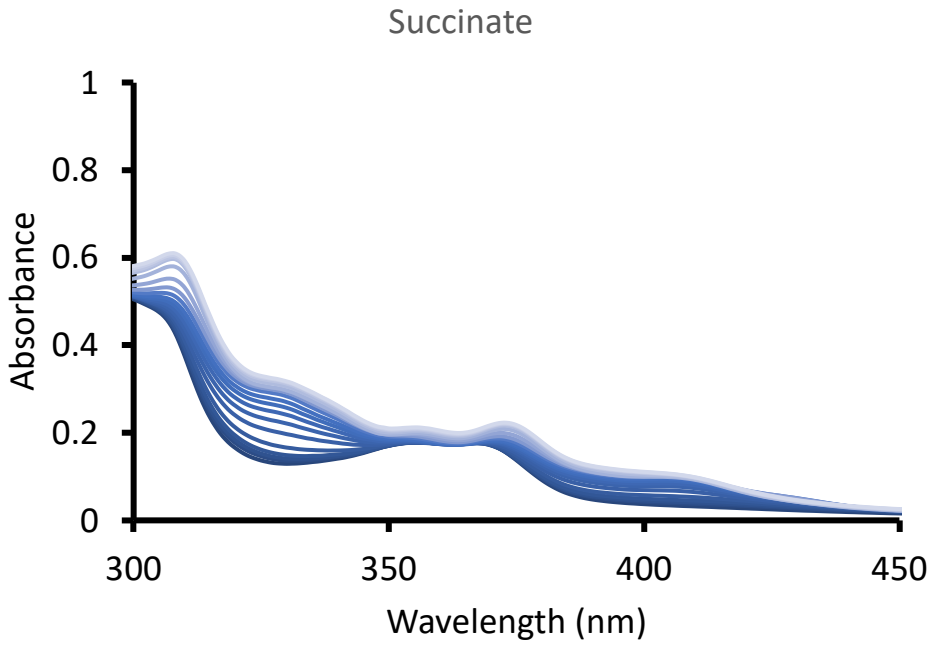


Succinate (DMSO + 1% H2O): $K_{1:1}$ = 4.38 x 10^4 M^{-1}; $K_{1:2}$ = 2.92 x 10^4 M^{-1}`



Representative data and fits to binding models of receptor **2** and succinate. Coloured dots represent data collected at 325 nm (orange) and 400 nm (yellow) and dotted lines the expected fit.

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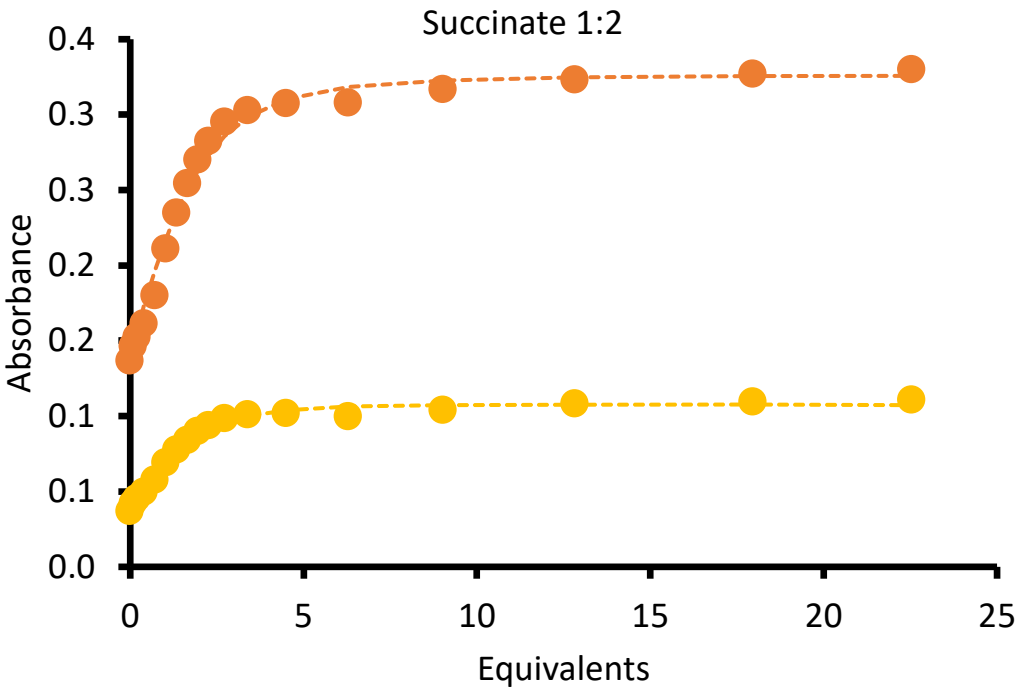
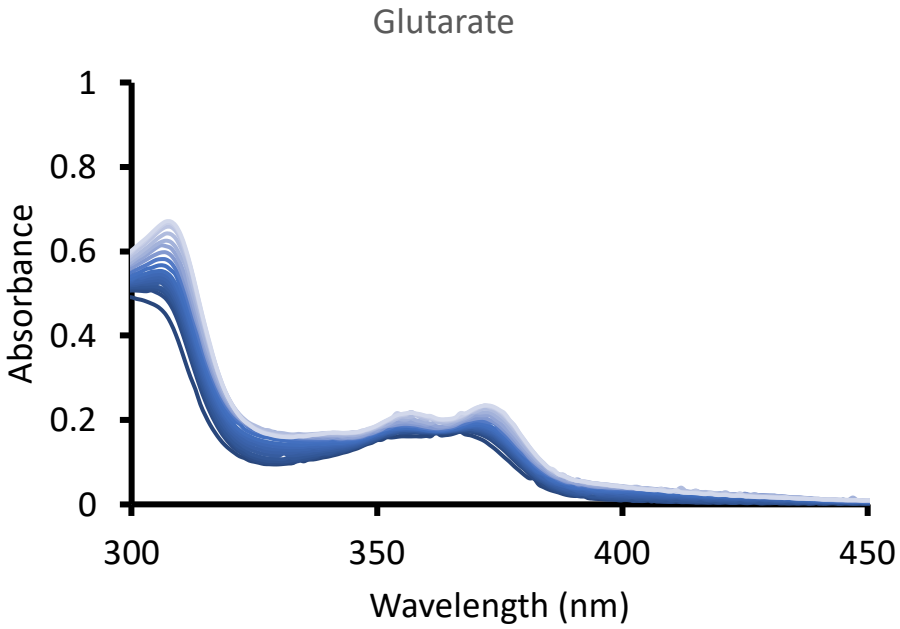


Glutarate (DMSO + 1% H_2O): $K_{1:1}$ = 1.4 x 10^5 M^{-1}; $K_{1:2}$ = 3.3 x 10^3 M^{-1}



Representative data and fits to binding models of receptor **2** and glutarate. Coloured dots represent data collected at 325 nm (orange) and 400 nm (yellow) and dotted lines the expected fit.

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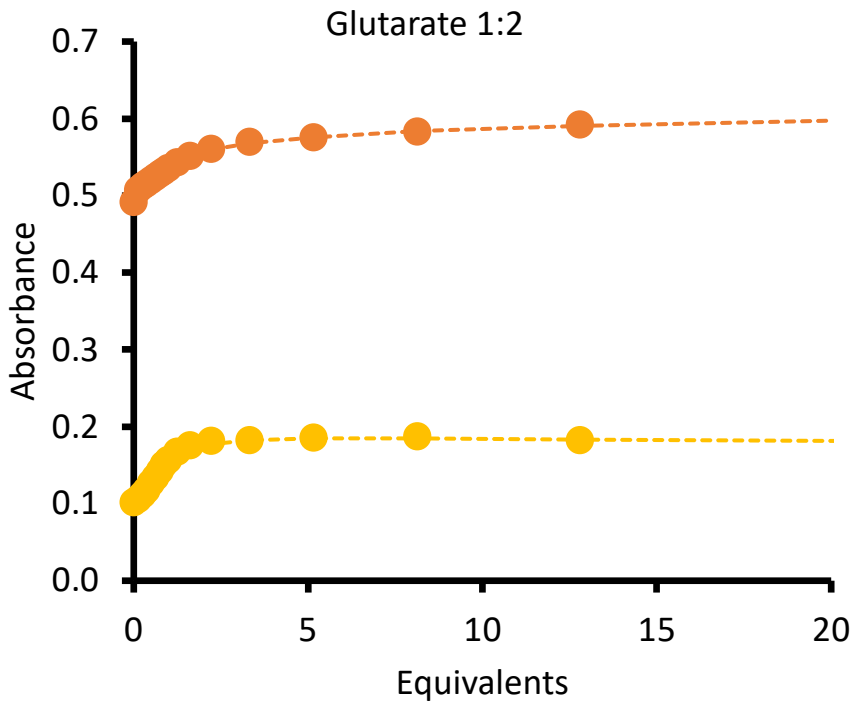
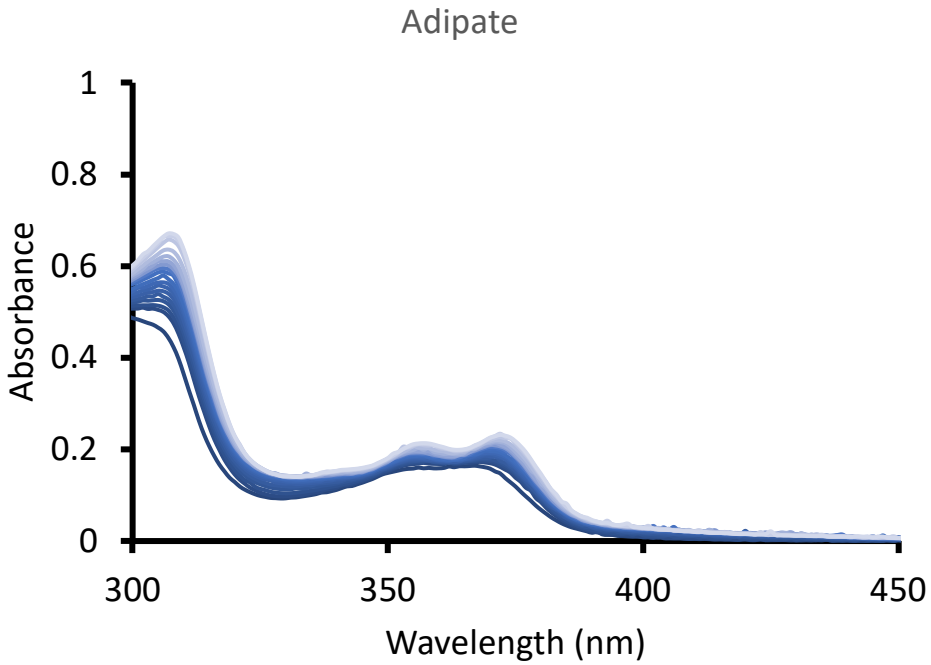


Adipate (DMSO + 1% H_2O): $K_{1:1} > 10^5\ M^{-1}$



Representative data and fits to binding models of receptor **2** and adipate. Coloured dots represent data collected at 300 nm (orange) and 325 nm (yellow) and dotted lines the expected fit.

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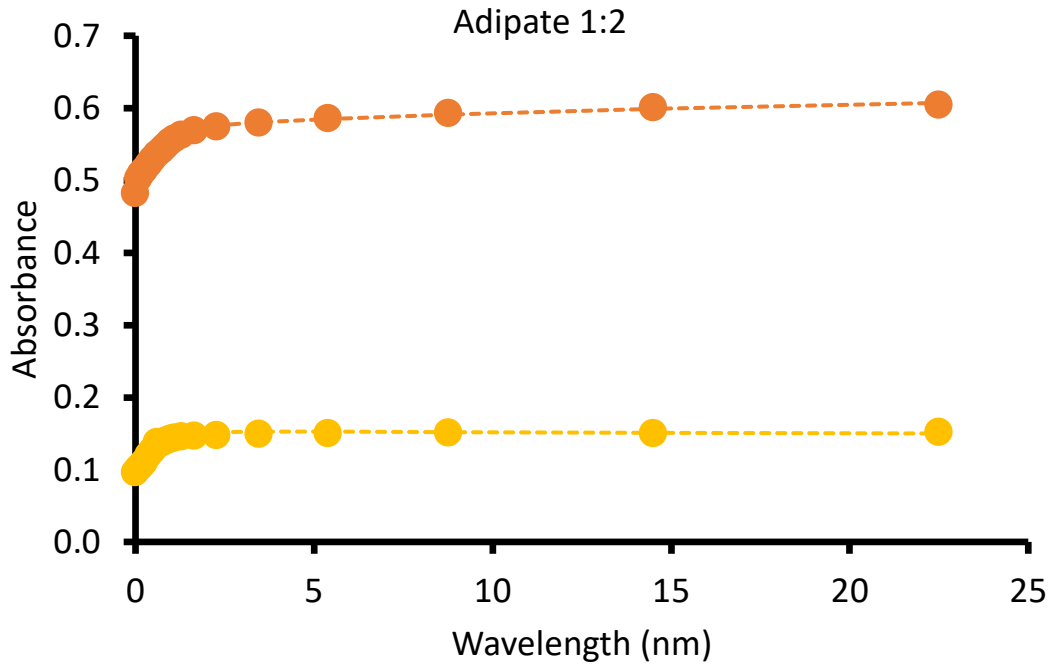


Benzoate (DMSO + 1% H_2O):

Negligible changes in absorbance—data could not be fit to any binding model.

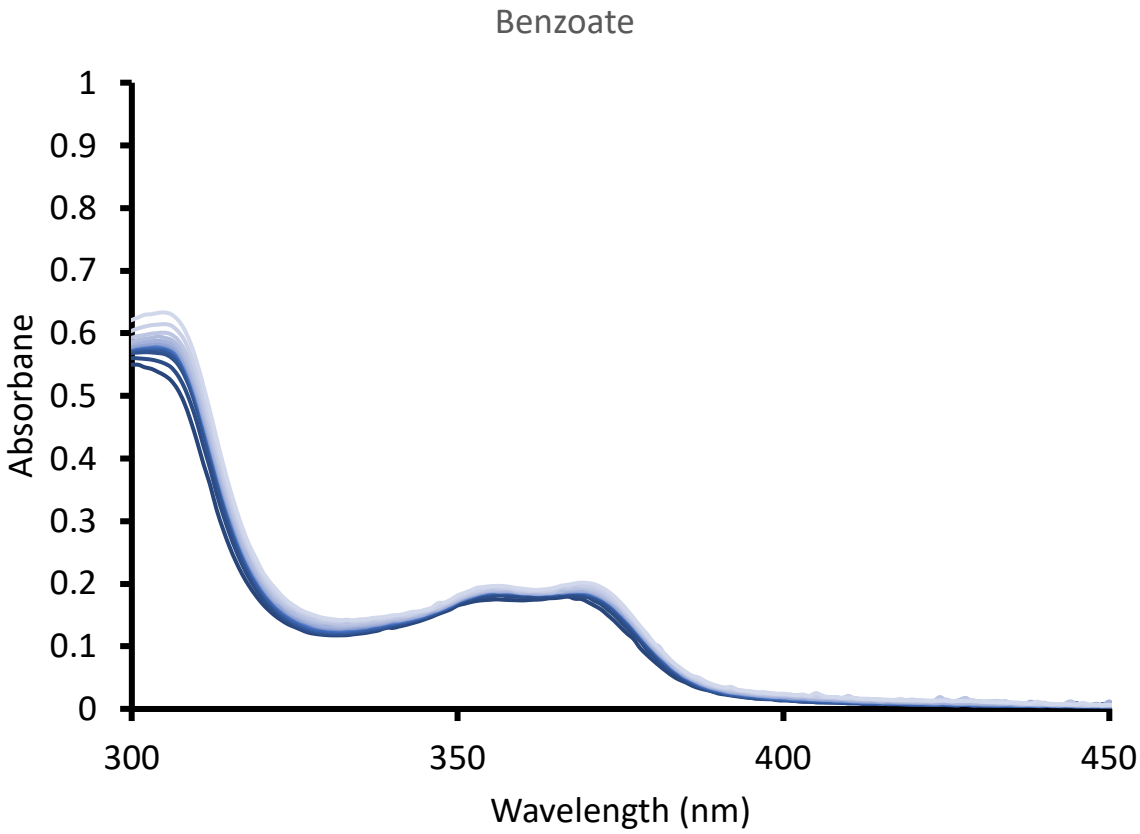
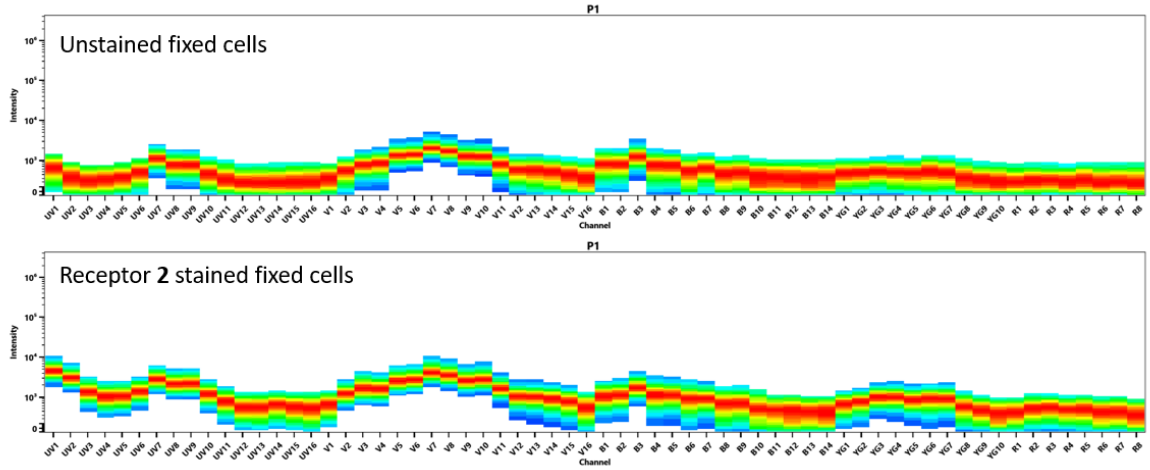


Figure S12—Spectral Signature of Receptor 2



Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)
Ultraviolet	UV1	372	15	365	380
	UV2	387	15	380	395
	UV3	427	15	420	435
	UV4	443	15	435	450
	UV5	458	15	450	480
	UV6	473	15	465	480
	UV7	514	28	500	528
	UV8	542	28	528	556
	UV9	581	31	566	597
	UV10	612	31	597	628
	UV11	664	27	650	677
	UV12	691	28	677	705
	UV13	720	29	705	734
	UV14	750	30	735	765
	UV15	780	30	765	795
	UV16	812	34	795	829
Violet	V1	428	15	420	435
	V2	443	15	436	451
	V3	458	15	451	466
	V4	473	15	466	481
	V5	508	20	498	518
	V6	525	17	516	533
	V7	542	17	533	550
	V8	581	19	571	590
	V9	598	20	588	608
	V10	615	20	605	625
	V11	664	27	651	678
	V12	692	28	678	706
	V13	720	29	706	735
	V14	750	30	735	765
	V15	780	30	765	795
	V16	812	34	795	829

Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)
Blue	B1	508	20	498	518
	B2	525	17	516	533
	B3	542	17	533	550
	B4	581	19	571	590
	B5	598	20	588	608
	B6	615	20	605	625
	B7	660	17	652	669
	B8	678	18	669	687
	B9	697	19	688	707
	B10	717	20	707	727
	B11	738	21	728	749
	B12	760	23	749	772
	B13	783	23	772	795
	B14	812	34	795	829
Yellow Green	YG1	577	20	567	587
	YG2	598	20	588	608
	YG3	615	20	605	625
	YG4	660	17	652	669
	YG5	678	18	669	687
	YG6	697	19	688	707
	YG7	720	29	706	735
	YG8	750	30	735	765
	YG9	780	30	765	795
	YG10	812	34	795	829
Red	R1	660	17	652	669
	R2	678	18	669	687
	R3	697	19	688	707
	R4	717	20	707	727
	R5	738	21	728	749
	R6	760	23	749	772
	R7	783	23	772	795
	R8	812	34	795	829

Spectral signature of fixed cells in the absence and presence of receptor **2** (50 µM). An increase in fluorescence intensity was observed for the UV1-UV3 channels (372 – 435 nm) when receptor **2** was present.

1. Berry, S. N.; Qin, L.; Lewis, W.; Jolliffe, K. A., Conformationally adaptable macrocyclic receptors for ditopic anions: analysis of chelate cooperativity in aqueous containing media. *Chemical Science* **2020,** *11*, 7015 - 7022.
2. Smith, J.; Liras, J. L.; Schneider, S. E.; Anslyn, E. V., Solid and Solution Phase Organic Syntheses of Oligomeric Thioureas. *The Journal of Organic Chemistry* **1996,** *61* (25), 8811-8818.
3. Gross, R.; Dürner, G.; Göbel, M. W., Beschleunigung von Substitutionsreaktionen eines Phosphorsäurediesters durch Bis(guanidinium)-Verbindungen. *Liebigs Ann. Chem.* **1994,** *1994* (1), 49-58.