Electronic Supplementary Information – Experimental Studies

Hydrazones in anion transporters: The detrimental effect of a second binding site

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1. General experimental information

All reagents and solvents were obtained from Sigma Aldrich, Fluorochem, Alfa Aesar and VWR, and were used without further purification unless otherwise stated. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol were purchased from Sigma Aldrich and Acros, respectively.

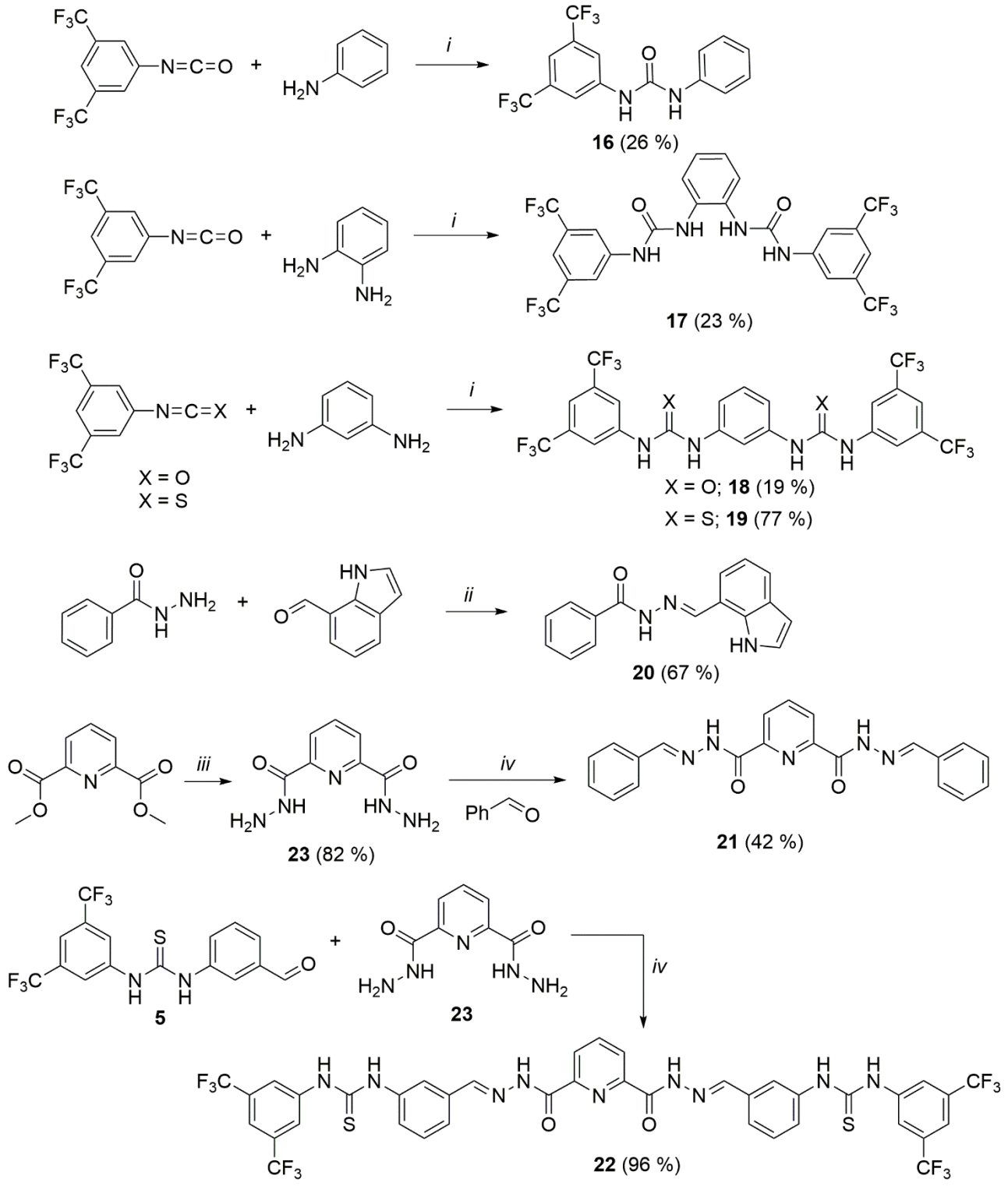
^{1}H and ^{13}C NMR spectra were recorded on a Jeol JNM-ECZ400R/S3 Spectrometer equipped with an Automation Triple Resonance Broadband (ATB) probe at 298 K. ^{1}H NMR spectra for titrations were recorded on a Varian VNMRS 400 (9.4 T) equipped with an Automation Triple Resonance Broadband (ATB) probe at 298 K. Solvent signals were used as reference for the chemical shifts. Chemical shifts are expressed in ppm and the coupling constants (J) are expressed in Hertz (Hz). High-resolution mass spectra were measured on an Agilent QTOF 6520 by electron spray ionisation. Fluorescence measurements were carried out on a FluoroMax-4 (Horiba) spectrofluorometer equipped with photomultiplier detector, double monochromator and Xenon light source. Dynamic light scattering (DLS) measurements were carried out on a Zetasizer Ultra (Malvern) at a 173° scattering angle using a 632.8 nm laser source at 25.0 °C.

Lipid solutions of POPC and cholesterol were prepared using chloroform that had been deacidified by passage through a column containing basic alumina. POPC solutions were stored at -20 °C and cholesterol solutions were freshly prepared. All aqueous solutions were prepared using deionised water that had been passed through a Millipore filtration system.

2. Synthesis and characterization of compounds 1-22

In addition to compounds **1**-**19**, described in the main text, we also prepared and studied compounds **20**-**22**. The results obtained for these additional acylhydrazones are discussed in Section 7 of this document.

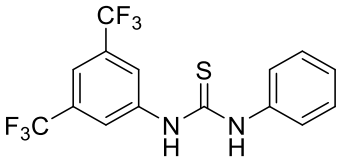
Scheme S1. Synthesis of compounds 16-22



i: DCM, r.t., overnight; *ii*: EtOH, reflux, 16 h.; *iii*: MeOH, $N_2H_4H_2O$ (16 eq.), reflux, 5h.; *iv*: EtOH, r.t., 4 - 48 h.

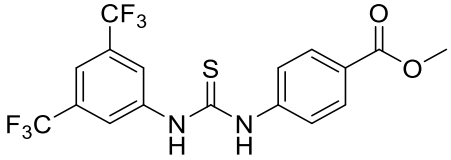
Synthetic procedures

1-(3,5-bis(trifluoromethyl)phenyl)-3-phenylthiourea (1):



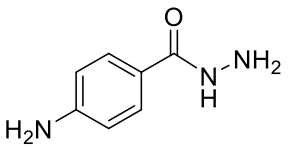
Aniline (35 µL, 0.39 mmol) was dissolved in dichloromethane (2 mL), 3,5-bis(trifluoromethyl)phenyl isothiocyanate (67 µL, 0.37 mmol) was added and the mixture was stirred at room temperature for 4 hours. The solvent was subsequently removed in vacuo and the remnant dried in high vacuum to yield the desired compound as an off-white solid (126 mg, 94%). **^{1}H NMR** (300 MHz, DMSO-d_6) δ 10.29 (s, 1H), 10.22 (s, 1H), 8.26 (s, 2H), 7.78 (s, 1H), 7.50 – 7.32 (mult., 5H), 7.19 (t, J = 7.2 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 179.84, 141.87, 138.65, 129.99 (q, $J_{C\text{-}F}$ = 32.9 Hz), 128.79, 125.22, 124.06, 123.48 (q, $J_{C\text{-}F}$ = 3.4 Hz), 123.25 (q, $J_{C\text{-}F}$ = 272.8 Hz), 116.83 (m). **HRMS** (ESI+): calcd. for $C_{15}H_{11}F_6N_2S$ $[M+H]^+$: 365.0542, found: 365.0540.

methyl 4-(3-(3,5-bis(trifluoromethyl)phenyl)thioureido)benzoate (2)



Methyl 4-aminobenzoate (200 mg, 1.32 mmol) was dissolved in DCM (2 mL), 3,5-bis(trifluoromethyl)phenyl isothiocyanate (200 µL, 1.10 mmol) was added and the mixture was stirred at room temperature overnight. A solid precipitated from the reaction mixture and it was filtered and washed with cold DCM (1 mL). Then, the solid was dissolved in DCM (4 mL), heptane (15 mL) was added on top of the DCM and after 2 hours the mixture was filtered to afford the product as a white solid (386 mg, 83%). ***R$_f$***: 0.78 (DCM-AcOEt 9:1). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 10.54 (s, 1H), 10.43 (s, 1H), 8.23 (s, 2H), 7.91 (d, J = 8.8 Hz, 2H), 7.77 (s, 1H), 7.64 (d, J = 8.8 Hz, 2H), 3.80 (s, 3H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 180.3, 166.3, 143.9, 142.1, 130.7 (q, $J_{C\text{-}F}$ = 32.9 Hz), 130.5, 125.8, 124.1, 123.7 (q, $J_{C\text{-}F}$ = 272.5 Hz), 123.0, 117.7, 52.5. **HRMS** (ESI+): calcd. for $C_{17}H_{12}F_6N_2O_2SNa$ $[M+Na]^+$: 445.0421, found: 445.0429.

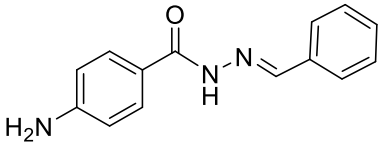
4-aminobenzohydrazide (11)



Methyl 4-aminobenzoate (500 mg, 3.31 mmol) was dissolved in MeOH (5 mL) and hydrazine monohydrate (2.5 mL) was added to the solution under stirring, as a co-solvent. The solution was stirred at 90˚C and the

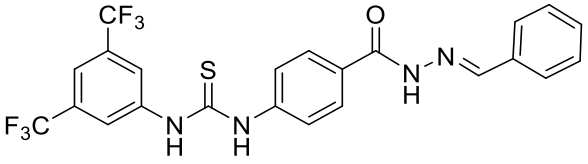
reaction was monitored by TLC (SiO_2, DCM-AcOEt 9:1). After 5 hours all the starting material had been consumed. When the mixture reached room temperature a solid precipitated. It was filtered and washed with cold MeOH (1 mL) to afford the product as a white solid (335 mg, 67%). ***R_f***: 0.06 (AcOEt). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 9.23 (s, 1H), 7.51 (d, *J* = 8.7 Hz, 2H), 6.49 (d, *J* = 8.7 Hz, 2H), 5.53 (s, 2H), 4.26 (s, 2H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 167.0, 152.1, 129.0, 120.5, 113.2.

(E)-4-amino-N'-benzylidenebenzohydrazide (12)



4-aminobenzohydrazide **11** (100 mg, 0.66 mmol) was suspended EtOH (15 mL), 67 µL benzaldehyde (1 eq) were added and the mixture stirred at room temperature. After 3.5 hours the mixture was a clear solution and TLC analysis (SiO_2, AcOEt) showed that there was no remaining benzaldehyde (Rf = 1) in the solution and a new spot different to the starting materials appeared (Rf = 0.71). More benzaldehyde (7 µL, 0.06 mmol) was added to the mixture and it was stirred for 1.5 h more to cause the complete disappearance of the starting hydrazide **12** (Rf = 0.06). The mixture was concentrated and dried under vacuum for 1.5 h to afford the product as a white solid (160 mg, 100%), which was used for the next reaction without further purification. ***R_f***: 0.71 (AcOEt). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 11.41 (s, 1H), 8.39 (s, 1H), 7.74 – 7.62 (mult., 4H), 7.50 – 7.35 (mult., 3H), 6.60 (d, *J* = 8.6 Hz, 2H), 5.75 (s, 2H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 152.8, 146.3, 136.3, 130.2, 129.8, 129.3, 127.3, 120.0, 113.1.

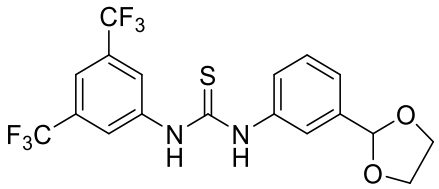
(E)-1-(4-(2-benzylidenehydrazinecarbonyl)phenyl)-3-(3,5-bis(trifluoromethyl)phenyl)thiourea (3)



The aniline derivative **12** (50 mg, 0.21 mmol) was dissolved in dry DMF (0.5 mL), 3,5-bis(trifluoromethyl)phenyl isothiocyanate (46 µL, 0.25 mmol) was added and the mixture was stirred at room temperature. The reaction was monitored by TLC (SiO_2, AcOEt) and after 20 minutes of stirring all the starting aniline **12** had been consumed and a new compound had appeared (Rf = 0.79). AcOEt (5 mL) was added to the mixture and the resulting solution was washed with HCl (0.5 M, 2x 3 mL) and brine (3 mL), dried with anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting solid was digested in Et_2O (4 mL), filtered and washed with more Et_2O (5 mL) to afford the product as a pale solid (54 mg, 61%). ***R_f***: 0.79 (AcOEt). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 11.83 (s, 1H), 10.53 (s, 1H), 10.41 (s, 1H), 8.47 (s, 1H), 8.28 (s, 2H), 7.93 (d, *J* = 8.4 Hz, 2H), 7.82 (s, 1H), 7.74 (d, *J* = 6.4 Hz, 2H), 7.65 (d, *J* = 8.4 Hz, 2H), 7.51 – 7.39 (mult., 3H). **^{13}C**

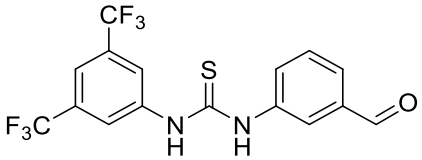
NMR (100 MHz, DMSO-d_6): δ = 180.4, 163.1, 148.1, 142.5, 142.2, 130.7 (q, $J_{C\text{-}F}$ = 32.8 Hz), 130.6, 129.9, 129.4, 128.6, 127.6, 124.1, 123.8 (q, $J_{C\text{-}F}$ = 273.2 Hz), 123.4, 117.7, 100.2. **HRMS** (ESI+): calcd. for $C_{23}H_{17}F_6N_4OS$ $[M+H]^+$: 511.1022, found: 511.1023.

1-(3-(1,3-dioxolan-2-yl)phenyl)-3-(3,5-bis(trifluoromethyl)phenyl)thiourea (6)



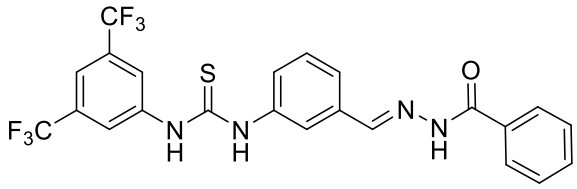
3-(1,3-dioxolan-2-yl)aniline (500 mg, 2.88 mmol) was dissolved in DCM (5 mL), 3,5-bis(trifluoromethyl)phenyl isothiocyanate (525 µL, 2.88 mmol) was added and the resulting orange solution was stirred at room temperature overnight. DCM (25 mL) was added and the organic solution washed with aqueous HCl (0.5 mM, 20 mL), brine (20 mL) and dried with anhydrous Na_2SO_4. Then the solvent was evaporated under reduced pressure, and the solid obtained was washed with heptane. This was further purified by column chromatography (SiO_2, DCM-AcOEt 96:4) to afford the product as a white solid (951 mg, 76%), with a trace of aldehyde **4**. ***R_f***: 0.36 (DCM-AcOEt 96:4). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 10.28 (s, 1H), 10.20 (s, 1H), 8.21 (s, 2H), 7.75 (s, 1H), 7.46 (s, 1H), 7.45 (d, *J* = 7.0 Hz, 1H), 7.36 (t, *J* = 7.5 Hz, 1H), 7.22 (d, *J* = 7.5 Hz, 1H), 5.70 (s, 1H), 4.05 – 3.86 (mult., 4H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 180.51, 142.3, 139.5, 139.2, 130.6 (q, $J_{C\text{-}F}$ = 32.9 Hz), 129.2, 125.3, 124.0, 123.9, 123.8 (q, $J_{C\text{-}F}$ = 272.7 Hz), 122.6, 117.4, 103.0, 65.4. **HRMS** (ESI+): calcd. for $C_{18}H_{14}F_6N_2O_2SNa$ $[M+Na]^+$: 459.0578, found: 459.0591.

1-(3,5-bis(trifluoromethyl)phenyl)-3-(3-formylphenyl)thiourea (4)



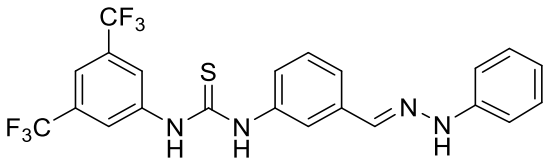
Acetal **6** (500 mg, 2.88 mmol) was dissolved in DCM (1.6 mL), TFA (14.4 mL) was added as a co-solvent and the resulting solution was stirred at room temperature. After 1 hour TLC analysis (SiO_2, DCM-AcOEt 96:4) revealed that the solution contained mainly one compound, different to the starting material. The solvents were removed under reduced pressure, the crude was redissolved in DCM (3 mL) and heptane was added to cause precipitation of the product as a yellow solid (178 mg, 99%). ***R_f***: 0.65 (DCM-AcOEt 9:1). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 10.43 (s, 1H), 10.35 (s, 1H), 9.97 (s, 1H), 8.21 (s, 2H), 7.98 (s, 1H), 7.78 (s, 1H), 7.76 (d, *J* = 8.0 Hz, 1H), 7.70 (d, *J* = 7.8 Hz, 1H), 7.57 (t, *J* = 7.8 Hz, 1H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 193.4, 180.8, 142.2, 140.2, 137.3, 130.7 (q, $J_{C\text{-}F}$ = 32.9 Hz), 130.6, 130.1, 127.0, 124.8, 124.2, 123.8 (q, $J_{C\text{-}F}$ = 272.6 Hz), 117.7. **HRMS** (ESI+): calcd. for $C_{16}H_{11}F_6N_2OS$ $[M+H]^+$: 393.0491, found: 393.0483.

(E)-1-(3-((2-benzoylhydrazono)methyl)phenyl)-3-(3,5-bis(trifluoromethyl)phenyl)thiourea (5a)



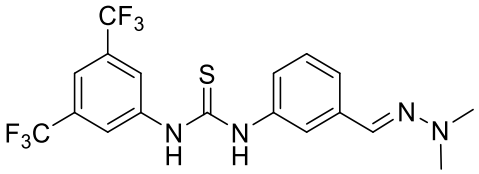
Aldehyde **4** (50 mg, 0.13 mmol) was dissolved in EtOH (8 mL), benzhydrazide (10 mg, 0.07 mmol) was added and the resulting solution was stirred at room temperature for 48 h. The solvent was removed under reduced pressure and the solid obtained was washed with Et_2O (2x 1 mL) to afford the product as a white solid (52.2 mg, 84%). ***R_f***: 0.24 (DCM-AcOEt 9:1). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 11.83 (s, 1H), 10.36 (s, 1H), 10.26 (s, 1H), 8.43 (s, 1H), 8.22 (s, 2H), 7.88 (d, *J* = 7.4 Hz, 2H), 7.79 (s, 1H), 7.77 (s, 1H), 7.63 – 7.25 (mult., 5H), 7.43 (t, *J* = 8.1 Hz, 1H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 180.6, 163.7, 147.8, 142.3, 139.8, 135.5, 133.9, 132.3, 130.6 (q, $J_{C\text{-}F}$ = 32.9 Hz), 129.8, 129.04, 128.2, 126.3, 124.6, 124.2, 123.8 (q, $J_{C\text{-}F}$ = 273.0 Hz), 122.8, 117.6. **HRMS** (ESI+): calcd. for $C_{23}H_{17}F_6N_4OS$ $[M+H]^+$: 511.1022, found: 511.1022.

(E)-1-(3,5-bis(trifluoromethyl)phenyl)-3-(3-((2-phenylhydrazono)methyl)phenyl)thiourea (5b)



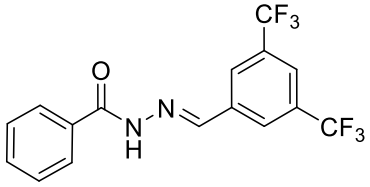
Aldehyde **4** (40 mg, 0.10 mmol) was dissolved in EtOH (5 mL), phenylhydrazine (16.5 mg, 0.15 mmol) was added and the resulting solution was stirred at room temperature for 1.5 h. The solvent was removed under reduced pressure and the crude was purified by column chromatography (SiO_2, DCM-heptane 2:1). Finally, precipitation in DCM-heptane afforded the product as a white solid (30 mg, 61%). ***R_f***: 0.46 (DCM). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 10.39 (s, 1H), 10.33 (s, 1H), 10.27 (s, 1H), 8.28 (s, 2H), 7.87 (s, 1H), 7.82 (s, 1H), 7.76 (s, 1H), 7.43 – 7.30 (mult., 3H), 7.23 (t, *J* = 7.6 Hz, 2H), 7.07 (d, *J* = 8.1 Hz, 2H), 6.77 (t, *J* = 7.3 Hz, 1H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 180.5, 145.7, 142.4, 139.6, 137.2, 136.3, 130.6, 129.6 (q, $J_{C\text{-}F}$ = 32.8 Hz), 129.7, 129.6, 124.2, 123.8 (q, $J_{C\text{-}F}$ = 272.3 Hz), 121.2, 119.4, 117.5, 112.5. **HRMS** (ESI+): calcd. for $C_{22}H_{17}F_6N_4S$ $[M+H]^+$: 483.1073, found: 483.1073.

(E)-1-(3,5-bis(trifluoromethyl)phenyl)-3-(3-((2,2-dimethylhydrazono)methyl)phenyl)thiourea (5c)



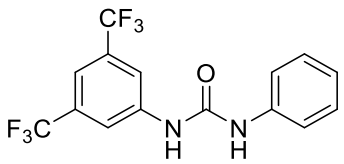
Aldehyde **4** (45 mg, 0.11 mmol) was dissolved in EtOH (5 mL), N,N-dimethylhydrazine (13 µL, 0.17 mmol) was added and the resulting solution was stirred at room temperature overnight. The solvent was removed under reduced pressure and the crude was purified by column chromatography (SiO_2, DCM). Finally, precipitation in DCM-pentane afforded the product as a white solid (25.3 mg, 51%). R_f: 0.83 (DCM-AcOEt 9:1). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 10.23 (broad, 2H), 8.24 (s, 2H), 7.78 (s, 1H), 7.56 (s, 1H), 7.45 – 7.18 (mult., 3H), 7.27 (s, 1H), 2.91 (s, 6H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 180.4, 142.4, 139.4, 138.2, 131.8, 130.5 (q, $J_{C\text{-}F}$ = 33.1 Hz), 129.4, 124.1, 123.8 (q, $J_{C\text{-}F}$ = 272.1 Hz), 123.4, 123.0, 121.1, 117.4, 43.1. **HRMS** (ESI+): calcd for $C_{18}H_{17}F_6N_4S$ $[M+H]^+$: 435.1073, found: 435.1078.

N'-(3,5-bis(trifluoromethyl)benzylidene)benzohydrazide (**7**)



Benzoylhydrazine (100 mg, 734 µmol) and 3,5-bis(trifluoromethyl)benzaldehyde (121 µL, 734 µmol) was dissolved in ethanol (10 mL) and heated to reflux. After 5 hours the reaction mixture was concentrated in vacuo. The remnant was subjected to flash column chromatography on silica gel using 1:1 ethyl acetate/heptane as the eluent to yield the desired compound as an off-white solid (200 mg, 75%). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 12.28 (s, 1H), 8.61 (s, 1H), 8.39 (s, 2H), 8.16 (s, 1H), 7.93 (d, J = 7.3 Hz, 2H), 7.61 – 7.48 (mult., 3H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 163.62, 144.33, 137.30, 133.03, 132.07, 130,86 (q, $J_{C\text{-}F}$ = 34.5 Hz), 128.54, 127.79, 127.18, 123.18 (q, $J_{C\text{-}F}$ = 272 Hz), 122.88. **HRMS** (ESI+): calcd. for $C_{16}H_{10}F_6N_2ONa$ $[M+Na]^+$: 383.0595, found: 383.0587.

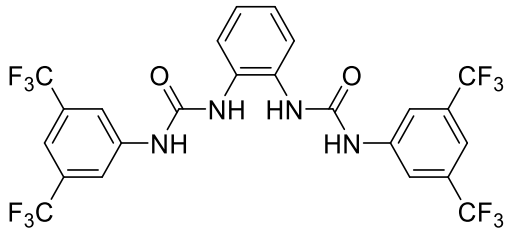
1-(3,5-bis(trifluoromethyl)phenyl)-3-phenylurea (**16**)



A flame dried 5 ml flask under an argon atmosphere was charged with dichloromethane (2 mL), aniline (36 µL, 391 µmol) and 3,5-bis(trifluoromethyl)phenyl isocyanate (68 µL, 391 µmol). The resulting mixture was stirred overnight after which the solvent was removed in vacuo. The remnant was subjected to column

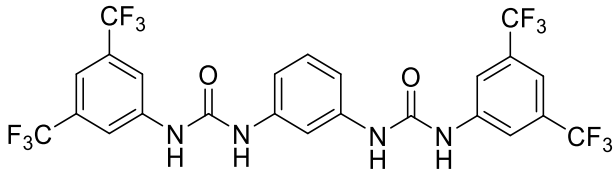
chromatography on silica gel using 50% ethyl acetate/heptane as the eluent to yield the desired compound as an off-white solid (35 mg, 26%) **^{1}H NMR** (400 MHz, DMSO-d_6): δ 9.42 (s, 1H), 8.99 (s, 1H), 8.13 (s, 2H), 7.62 (s, 1H), 7.51 – 7.45 (m, 2H), 7.33 – 7.27 (mult., 2H), 7.01 (t, J = 7.6, 1.1 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 152.98, 142.44, 139.55, 131.26 (q, $J_{C\text{-}F}$ = 32.5 Hz), 129.33, 123.88 (q, $J_{C\text{-}F}$ = 272.9 Hz), 123.07, 119.42, 118.46, 114.82. **HRMS** (ESI+): calcd. for $C_{16}H_{10}F_6N_2ONa$ $[M+Na]^+$: 371.0595, found: 371.0595.

1,1'-(1,2-phenylene)bis(3-(3,5-bis(trifluoromethyl)phenyl)urea) (**17**)



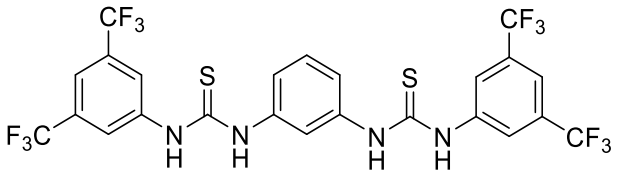
A flame dried 10 ml round bottom flask under an argon atmosphere was charged with o-phenylene diamine (50 mg, 0.46 mmol), dry dichloromethane (6 ml) and 3,5-bis(trifluoromethyl)phenyl isocyanate (183 μL, 1.06 mmol). The resulting suspension was left to stir overnight after which the solvent was removed in *vacuo*. The remnant was subjected to column chromatography on silica gel using 5% methanol/DCM as the eluent to yield the desired compound as a white solid (55 mg, 19%) **^{1}H NMR** (400 MHz, DMSO-d_6): δ 9.79 (s, 2H), 8.31 (s, 2H), 8.12 (s, 4H), 7.61 (s, 2H), 7.60 – 7.56 (mult., 2H), 7.20 – 7.15 (mult., 2H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 153.16, 141.95, 131.26, 130.71 (q, $J_{C\text{-}F}$ = 32.4 Hz), 124.99, 124.91, 123.34(q, $J_{C\text{-}F}$ = 272.5 Hz), 117.92, 114.32. The NMR characterization data are in agreement with those previously reported for this compound.[1]

1,1'-(1,3-phenylene)bis(3-(3,5-bis(trifluoromethyl)phenyl)urea) (**18**)



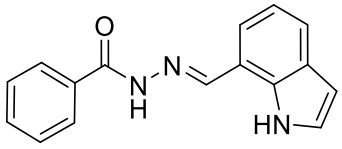
A flame dried 5 ml flask under an argon atmosphere was charged with dichloromethane (4 mL), m-phenylene diamine (51 mg, 475 μmol) and 3,5-bis(trifluoromethyl)phenyl isocyanate (164 μL, 950 μmol). The resulting mixture was stirred overnight after which the solvent was removed in vacuo. The remnant was subjected to column chromatography on silica gel using 50% ethyl acetate/heptane as the eluent to yield the desired compound as an off-white solid (40 mg, 23%) **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.27 (s, 2H), 9.04 (s, 2H), 8.10 (s, 4H), 7.75 (t, J = 2.0 Hz, 1H), 7.60 (s, 2H), 7.18 (dd, J = 8.9, 7.1 Hz, 1H), 7.12 – 7.07 (mult., 2H). **^{13}C NMR** (101 MHz, Methanol-d_4) δ 153.22, 141.67, 139.31, 131.93 (q, $J_{C\text{-}F}$ = 31.9 Hz), 129.03, 123.61(q, $J_{C\text{-}F}$ = 271.9 Hz), 118.04, 114.65, 114.03, 110.34. **HRMS** (ESI+): calcd. for $C_{24}H_{15}F_{12}N_4O_2$ $[M+H]^+$: 619.0998, found: 619.0997.

1,1'-(1,3-phenylene)bis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea) (19)



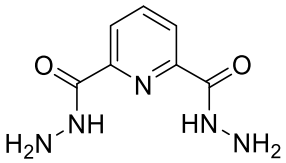
1,3-phenylenediamine (50 mg, 0.46 mmol) was dissolved in DCM (2 mL), 3,5-bis(trifluoromethyl)phenyl isothiocyanate (169 µL, 0.92 mmol) was added and the mixture was stirred at room temperature for 4 hours. The solvent was subsequently removed, and the crude product was purified by column chromatography on silica gel (going from 10% to 30% ethyl acetate in heptane as eluent) to yield the desired bisthiourea as a white solid (232 mg, 77%). **^{1}H NMR** (300 MHz, DMSO-d_6) δ 10.39 (s, 2H), 10.07 (s, 2H), 8.13 (s, 4H), 7.73 – 7.70 (mult., 3H), 7.40 (t, *J* = 8.0 Hz, 1H), 7.23 (dd, *J* = 8.0, 2.0 Hz, 2H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 179.80, 141.69, 138.93, 129.88(q, $J_{C\text{-}F}$ = 32.9 Hz), 129.43, 124.00, 123.19(q, $J_{C\text{-}F}$ = 272.0 Hz), 120.39, 119.33, 117.05. **HRMS** (ESI+): calcd. for $C_{24}H_{12}F_{12}N_4S_2$ $[M+H]^+$: 651.0541, found: 651.0536.

N'-((1H-indol-7-yl)methylene)benzohydrazide (**20**)



Benzohydrazide (100 mg, 734 µmol) and 1H-indole-7-carbaldehyde (106 mg, 734 µmol) were dissolved in ethanol (10 ml) and heated to reflux. After 16 hours the reaction mixture was concentrated in vacuo. The remnant was subjected to flash column chromatography on silica gel using 50% ethyl acetate/heptane as the eluent to yield the desired compound as an off-white solid (130 mg, 67%). **^{1}H NMR** (400 MHz, DMSO-d_6): δ 12.12 (s, 1H), 10.85 (s, 1H), 8.69 (s, 1H), 8.03 – 7.95 (mult., 2H), 7.72 (d, *J* = 7.8 Hz, 1H), 7.66 – 7.54 (mult., 4H), 7.37 (d, *J* = 7.1 Hz, 1H), 7.15 (t, *J* = 7.6 Hz, 1H), 6.63 – 6.56 (mult., 1H). **^{13}C NMR** (101 MHz, DMSO-d_6): δ 163.16, 148.38, 133.15, 131.99, 131.90, 128.58, 128.20, 127.64, 126.30, 124.13, 122.92, 119.22, 117.83, 102.04. **HRMS** (ESI+): calcd. for $C_{16}H_{13}N_3ONa$ $[M+Na]^+$: 286.0951, found: 286.0953.

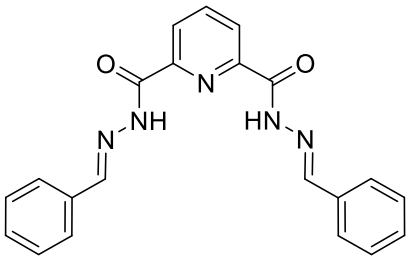
pyridine-2,6-dicarbohydrazide (23)



Dimethyl 2,6-pyridinedicarboxylate (500 mg, 2.56 mmol) was dissolved in MeOH (15 mL), hydrazine monohydrate (2 mL, 41 mmol) was added and the resulting solution was stirred at room temperature for 2

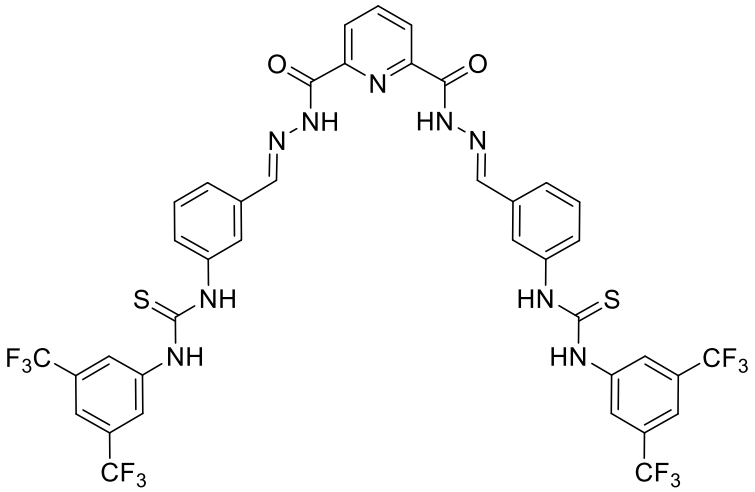
hours. A white solid precipitated from the mixture and it was filtered and washed with MeOH (5 mL) to afford the product (410 mg, 82%). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 10.59 (s, 2H), 8.11 – 8.07 (mult., 3H), 4.58 (d, *J* = 4.2 Hz, 4H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 162.5, 148.9, 139.8, 124.2.

N'2,N'6-di((E)-benzylidene)pyridine-2,6-dicarbohydrazide (21)



The bis-hydrazide **23** (40 mg, 0.20 mmol) was suspended in EtOH (10 ml), benzaldehyde (63 µL, 0.61 mmol) and a drop of acetic acid were added and the mixture was stirred at room temperature for 48 h. The mixture contained a solid in suspension, as the initial mixture did, but NMR analysis suggested that the product was the main compound in the mixture. The solid was separated by filtration to afford the product as a white solid (32 mg, 42%). ***R*$_f$**: 0.58 (DCM-EtOH 95-5). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 12.34 (s, 2H), 8.80 (s, 2H), 8.37 (d, *J* = 7.5 Hz, 2H), 8.30 (dd, *J* = 8.5, 6.8 Hz, 1H), 7.84 (d, *J* = 7.5 Hz, 4H), 7.59 – 7.42 (mult., 6H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 160.0, 150.7, 148.8, 140.6, 134.7, 131.0, 129.5, 127.9, 126.1. **HRMS** (ESI+): calcd. for $C_{21}H_{18}N_5O_2$ $[M+H]^+$: 372.1455, found: 372.1459.

1,1'-(((1*E*,1'*E*)-(2,2'-(pyridine-2,6-dicarbonyl)bis(hydrazin-2-yl-1-ylidene))bis(methanylylidene))bis(3,1-phenylene))bis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea) (22)



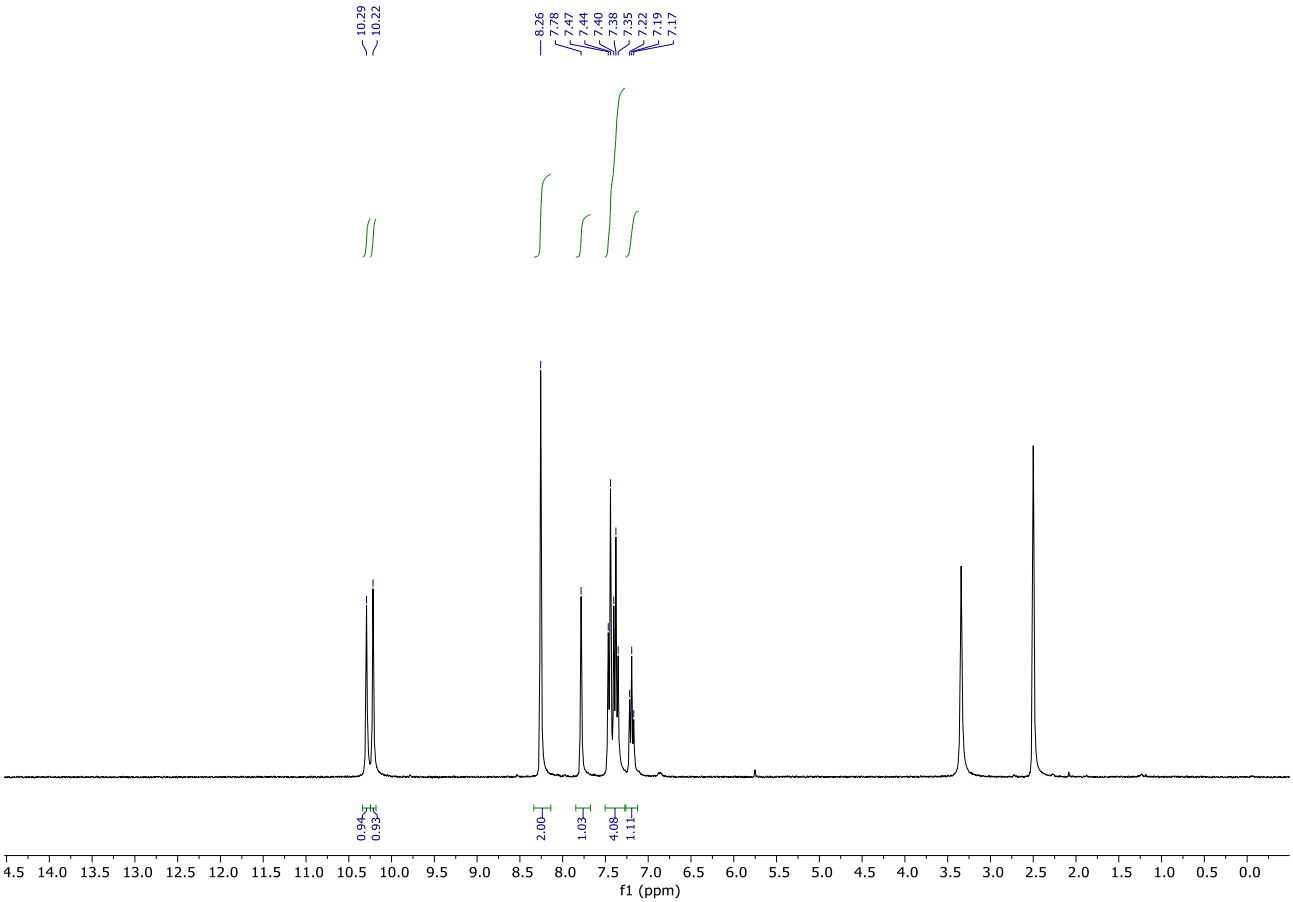
Aldehyde **4** (39 mg, 0.10 mmol) was dissolved in EtOH (2 ml), bis-hydrazide **23** (39 mg, 8.8 mmol) and a drop of acetic acid were added, and the resulting suspension was stirred at room temperature. After 4 hours the mixture had turned into a clear solution. The solvent was removed under reduced pressure and the crude was suspended in Et_2O (1 mL), filtered and washed with heptane (5 mL) to afford the product as a pale solid

(41 mg, 96%). R_f: 0.65 (AcOEt). **^{1}H NMR** (400 MHz, DMSO-d_6): δ = 12.30 (s, 2H), 10.39 (s, 2H), 10.30 (s, 2H), 8.74 (s, 2H), 8.36 – 8.33 (mult., 2H), 8.29 – 8.23 (mult., 1H), 8.23 (s, 4H), 7.90 (s, 2H), 7.77 (s, 2H), 7.60 (d, *J* = 7.71 Hz, 2H), 7.56 (d, *J* = 8.40 Hz, 2H), 7.47 (t, *J* = 7.71 Hz, 2H). **^{13}C NMR** (100 MHz, DMSO-d_6): δ = 180.2, 159.4, 149.9, 148.8, 142.3, 140.0, 135.3, 130.6 (q, J_{C-F} = 33.1 Hz), 129.9, 126.6, 125.8, 124.7, 124.1, 123.8 (q, J_{C-F} = 272.9 Hz), 123.9, 117.6. **HRMS** (ESI+): calcd. for $C_{39}H_{26}F_{12}N_9O_2S_2$ $[M+H]^+$: 944.1454, found: 944.1452.

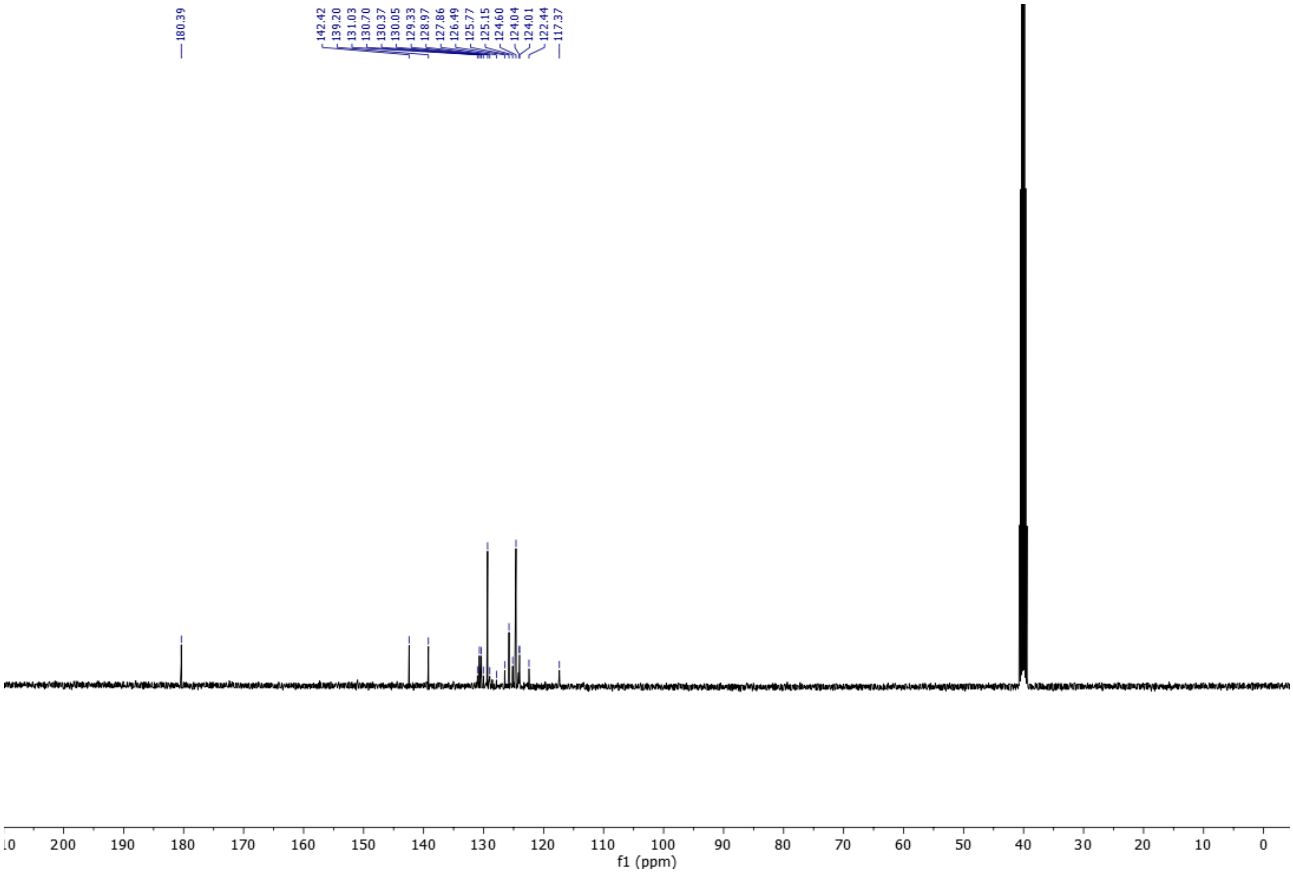
NMR spectra

1-(3,5-bis(trifluoromethyl)phenyl)-3-phenylthiourea (1)

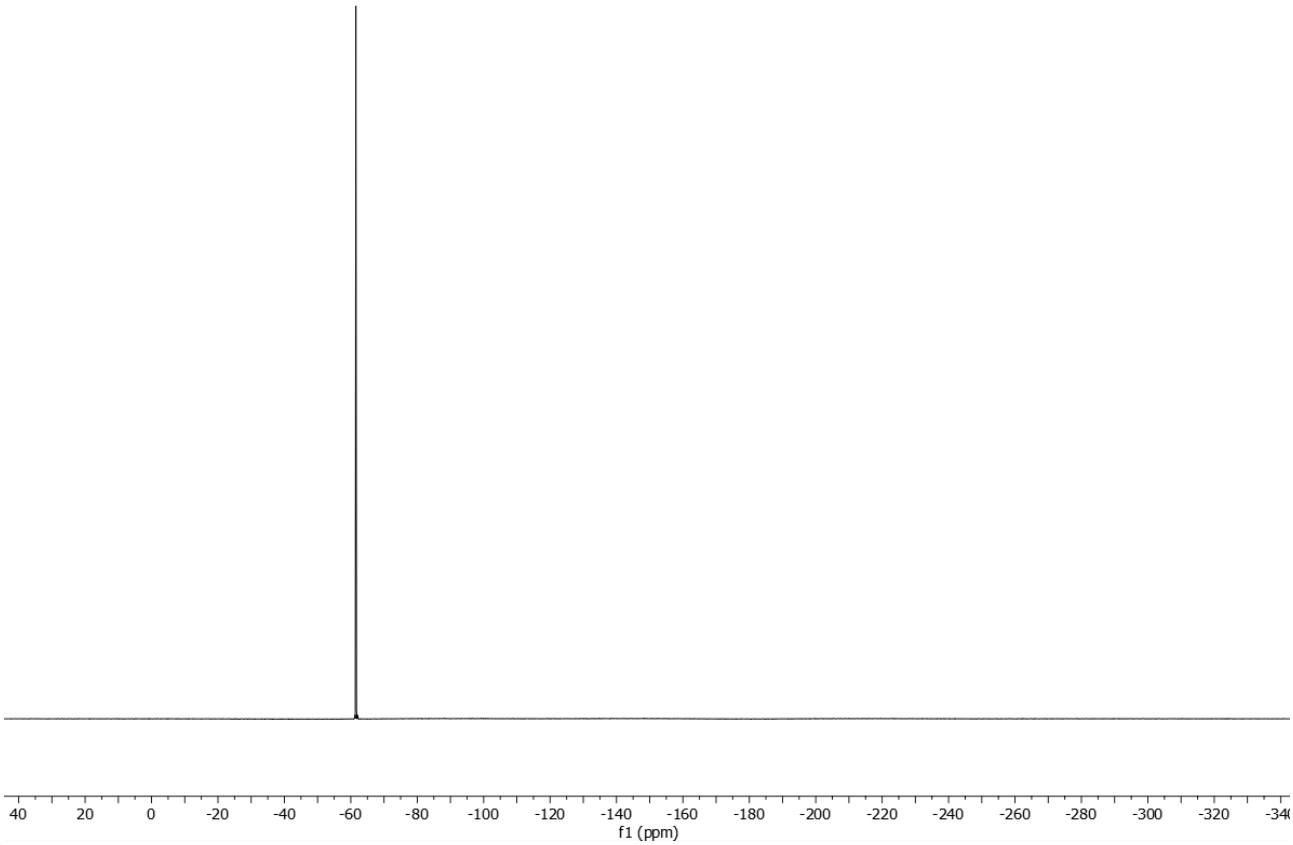
^{1}H NMR:



^{13}C NMR:

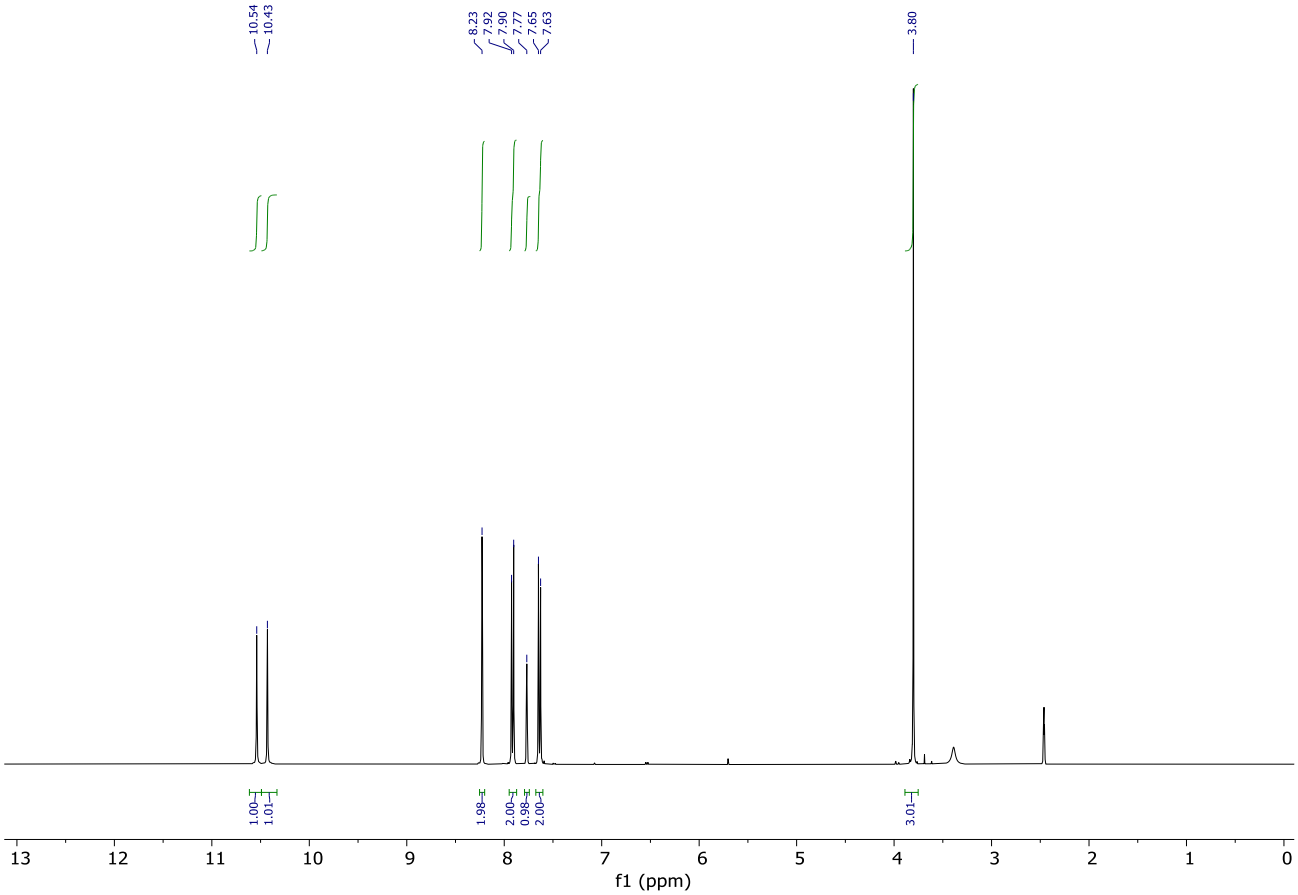


^{19}F NMR:

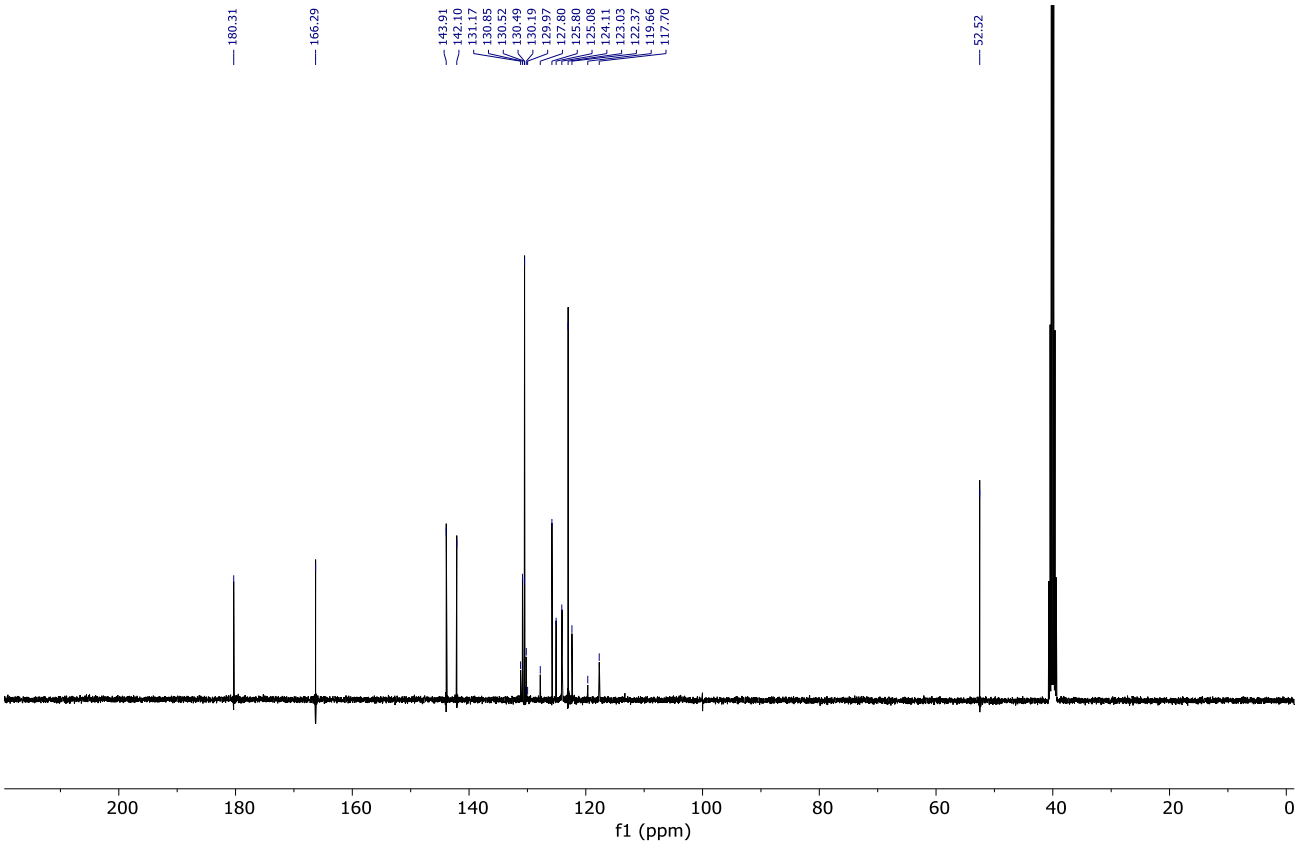

40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
-180
-200
-220
-240
-260
-280
-300
-320
f1 (ppm)

methyl 4-(3-(3,5-bis(trifluoromethyl)phenyl)thioureido)benzoate (2)

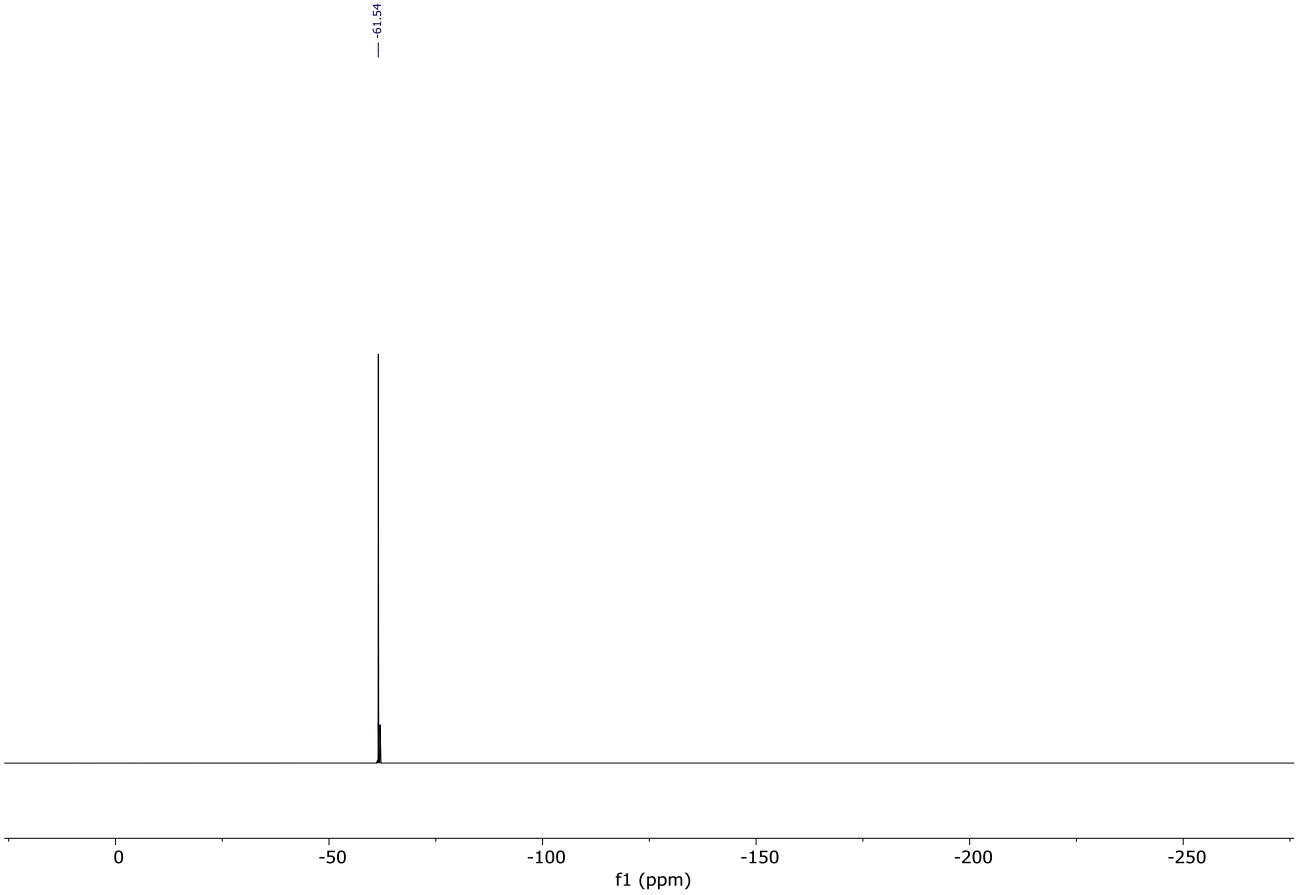
^{1}H NMR:



^{13}C NMR:

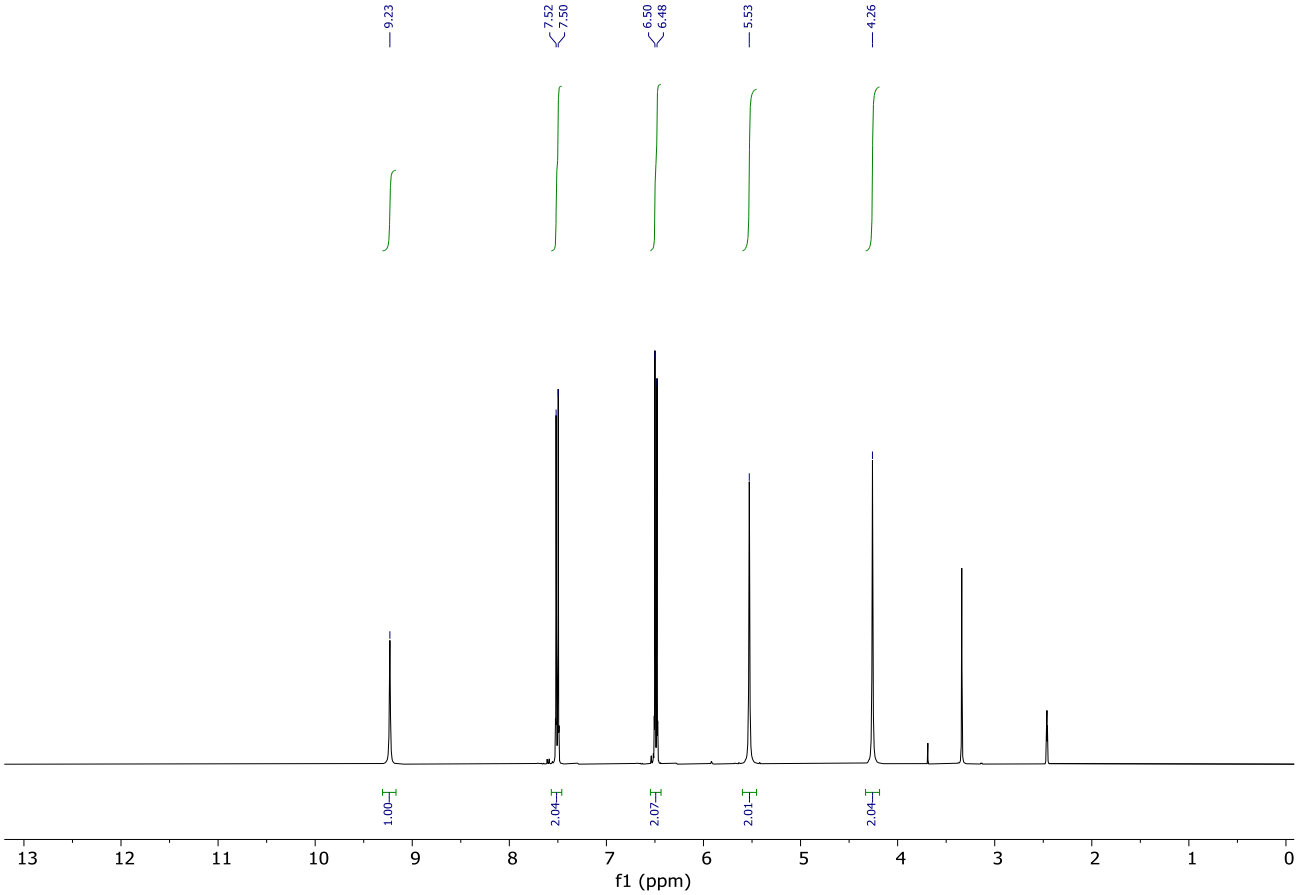


^{19}F NMR:

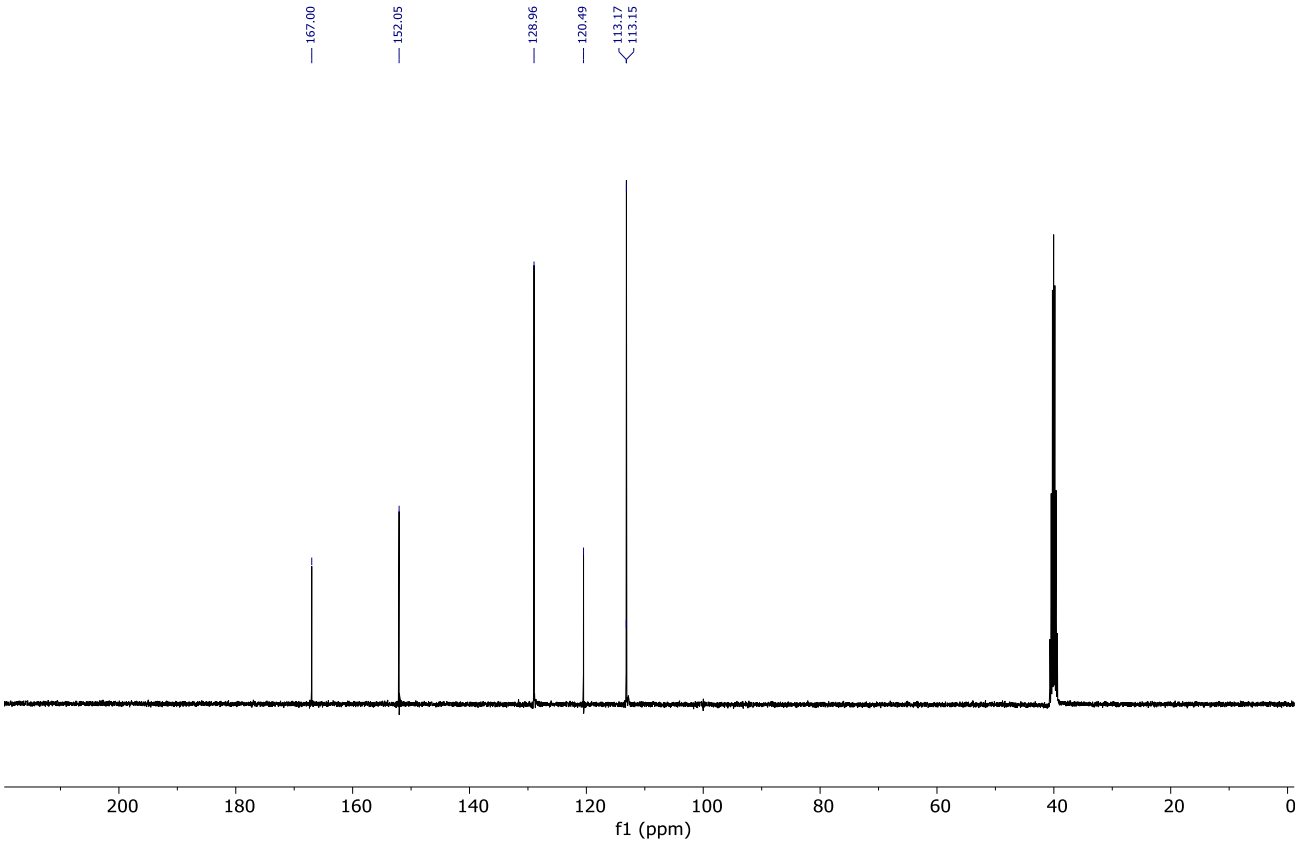


4-aminobenzohydrazide (11)

^{1}H NMR:

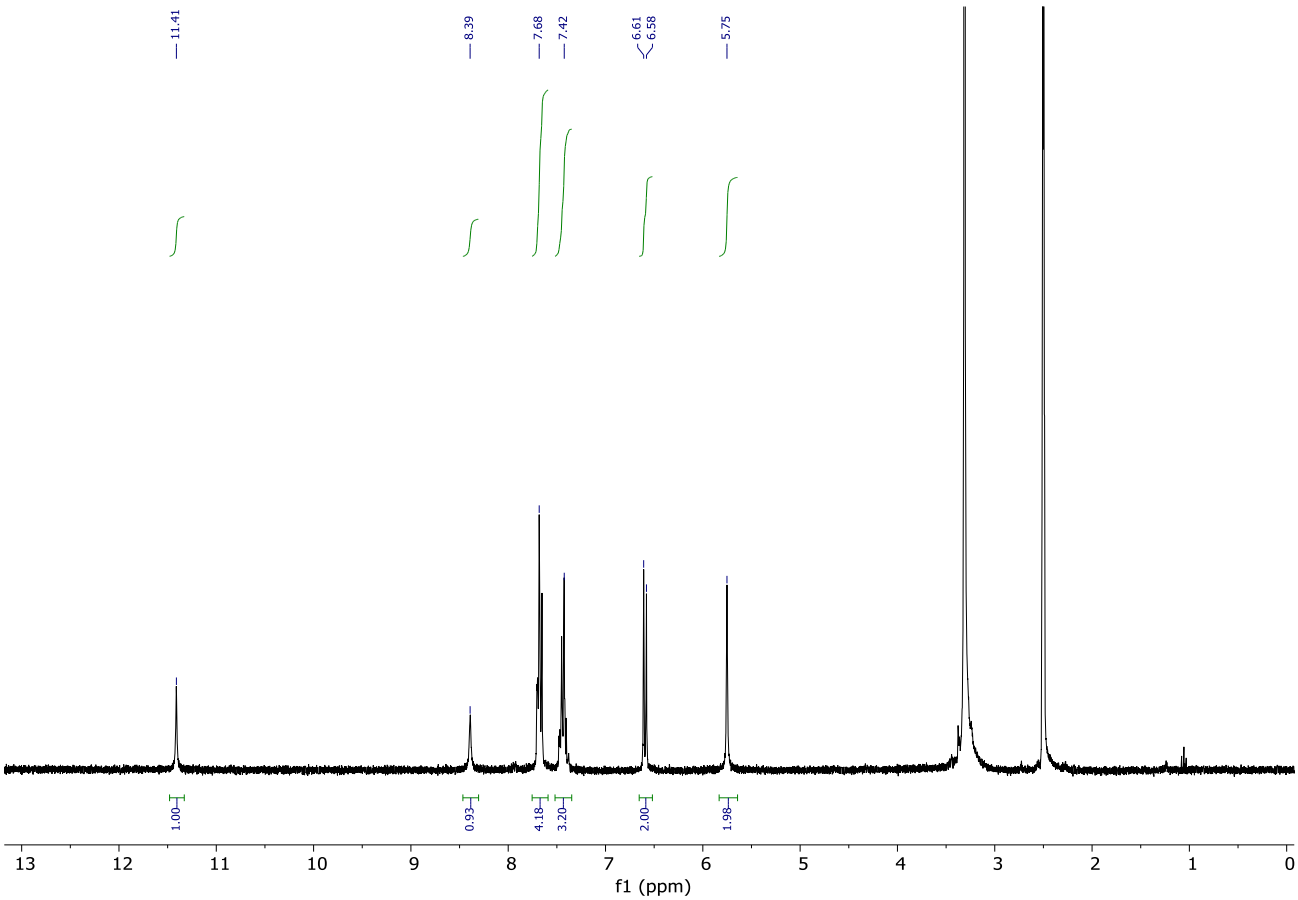


^{13}C NMR:

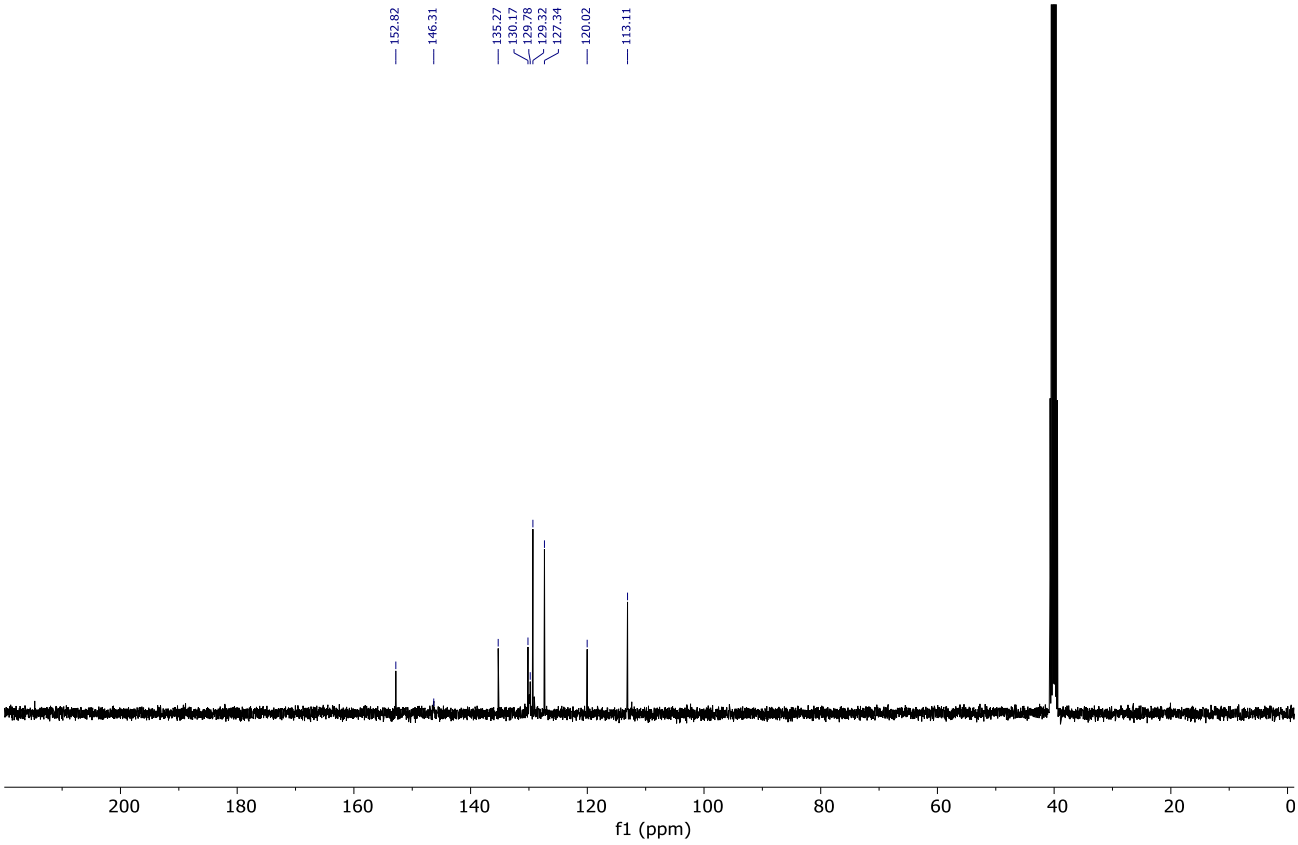


(E)-4-amino-N'-benzylidenebenzohydrazide (12)

^{1}H NMR:

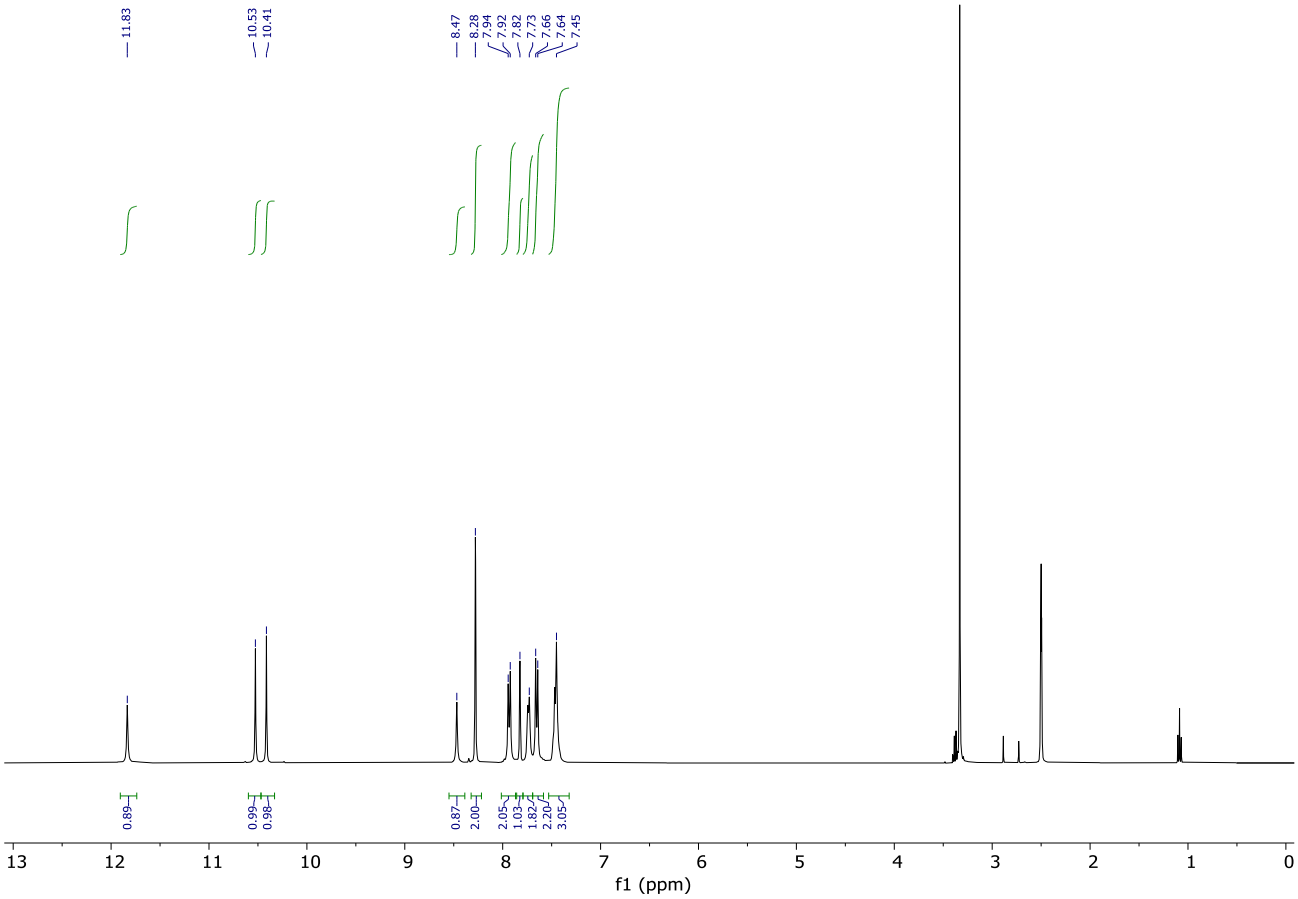


^{13}C NMR:

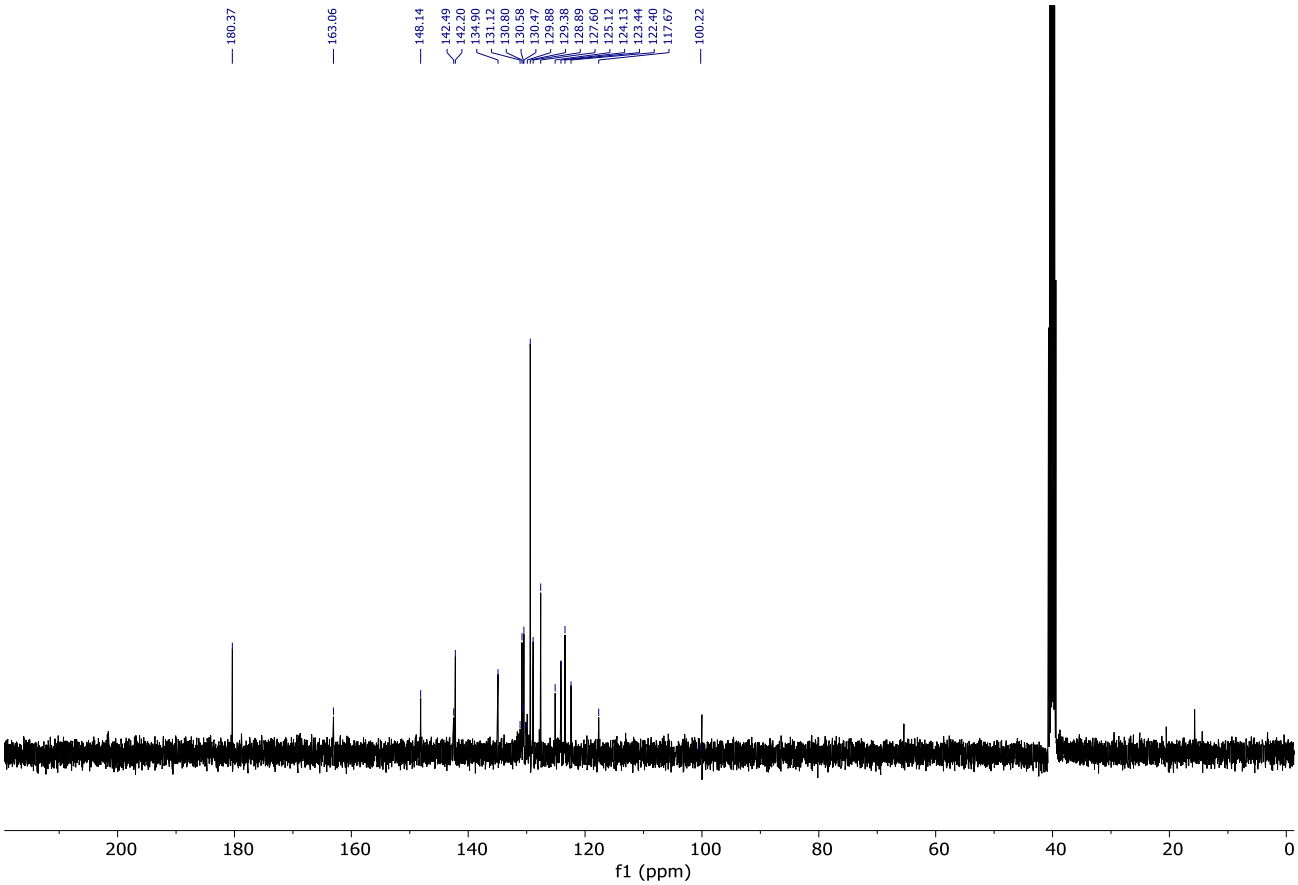


(E)-1-(4-(2-benzylidenehydrazinecarbonyl)phenyl)-3-(3,5-bis(trifluoromethyl)phenyl)thiourea (3)

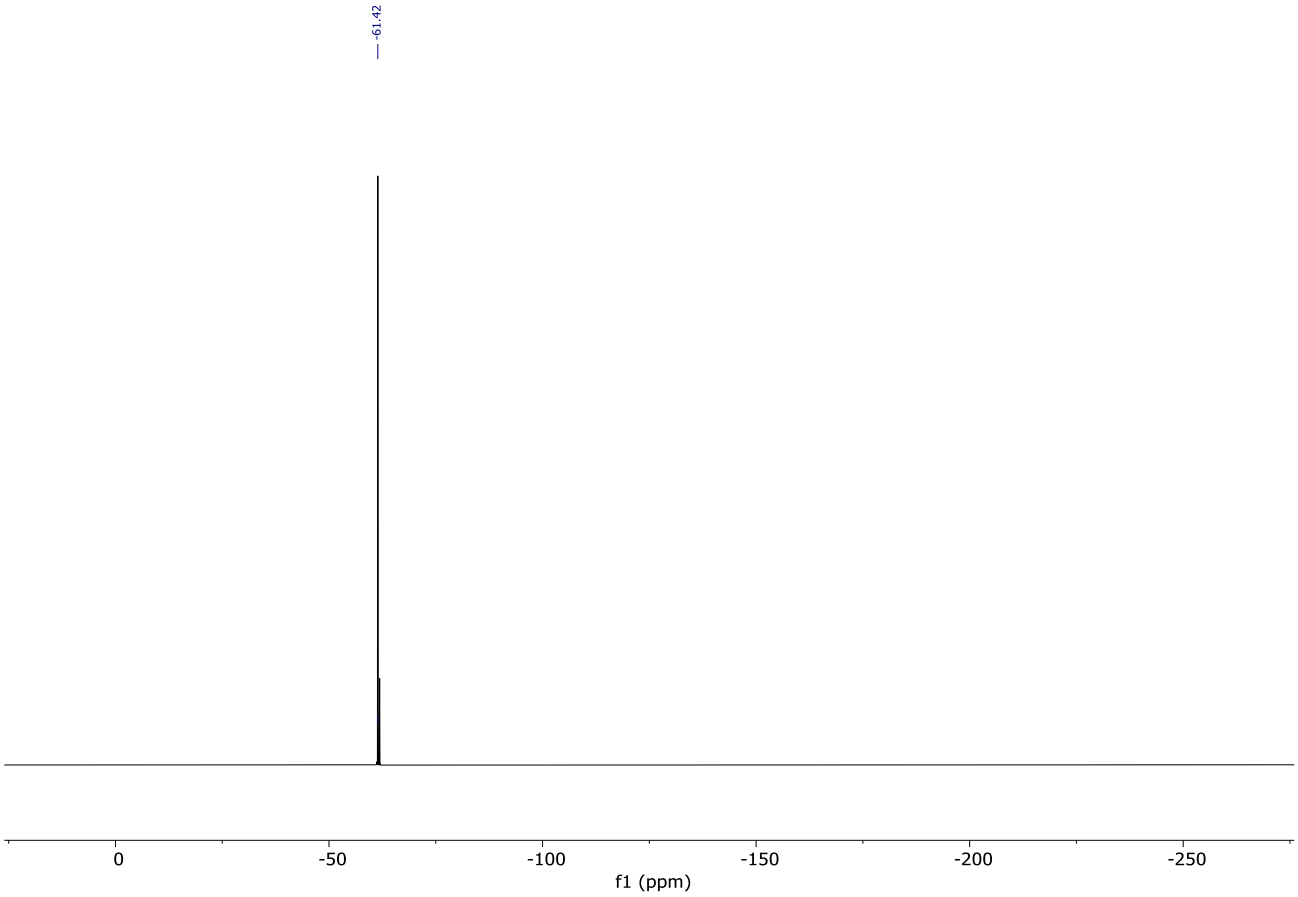
^{1}H NMR:



^{13}C NMR:

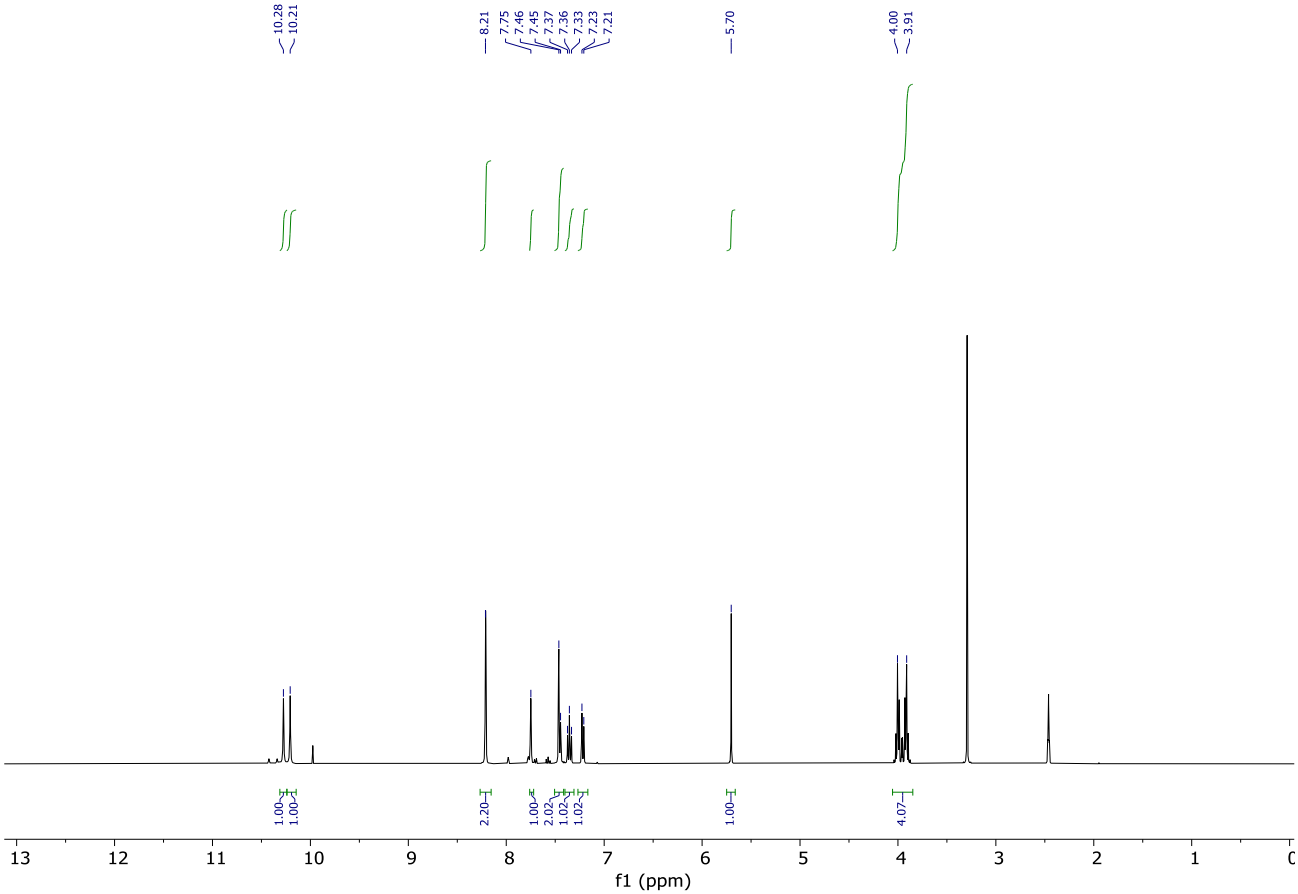


^{19}F NMR:

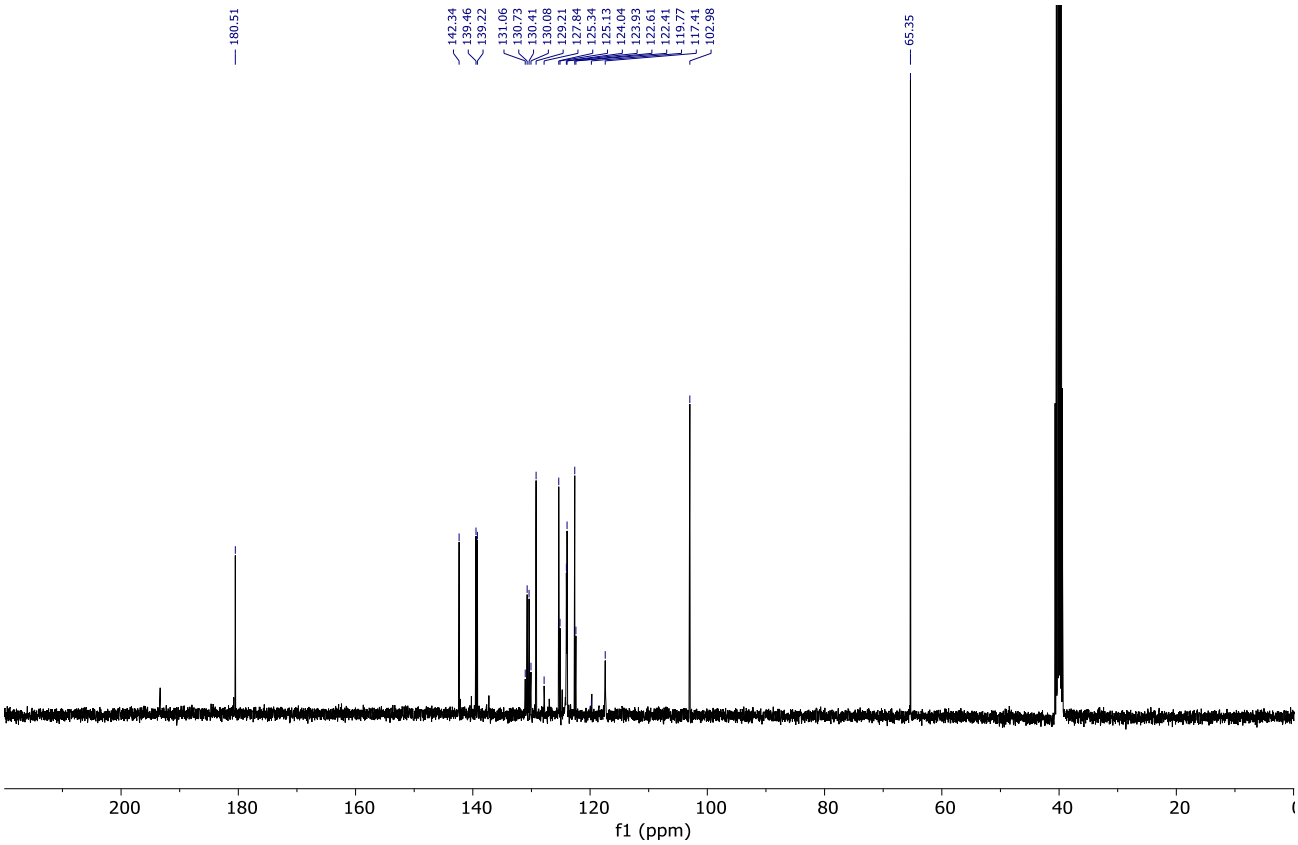


1-(3-(1,3-dioxolan-2-yl)phenyl)-3-(3,5-bis(trifluoromethyl)phenyl)thiourea (6)

^{1}H NMR:

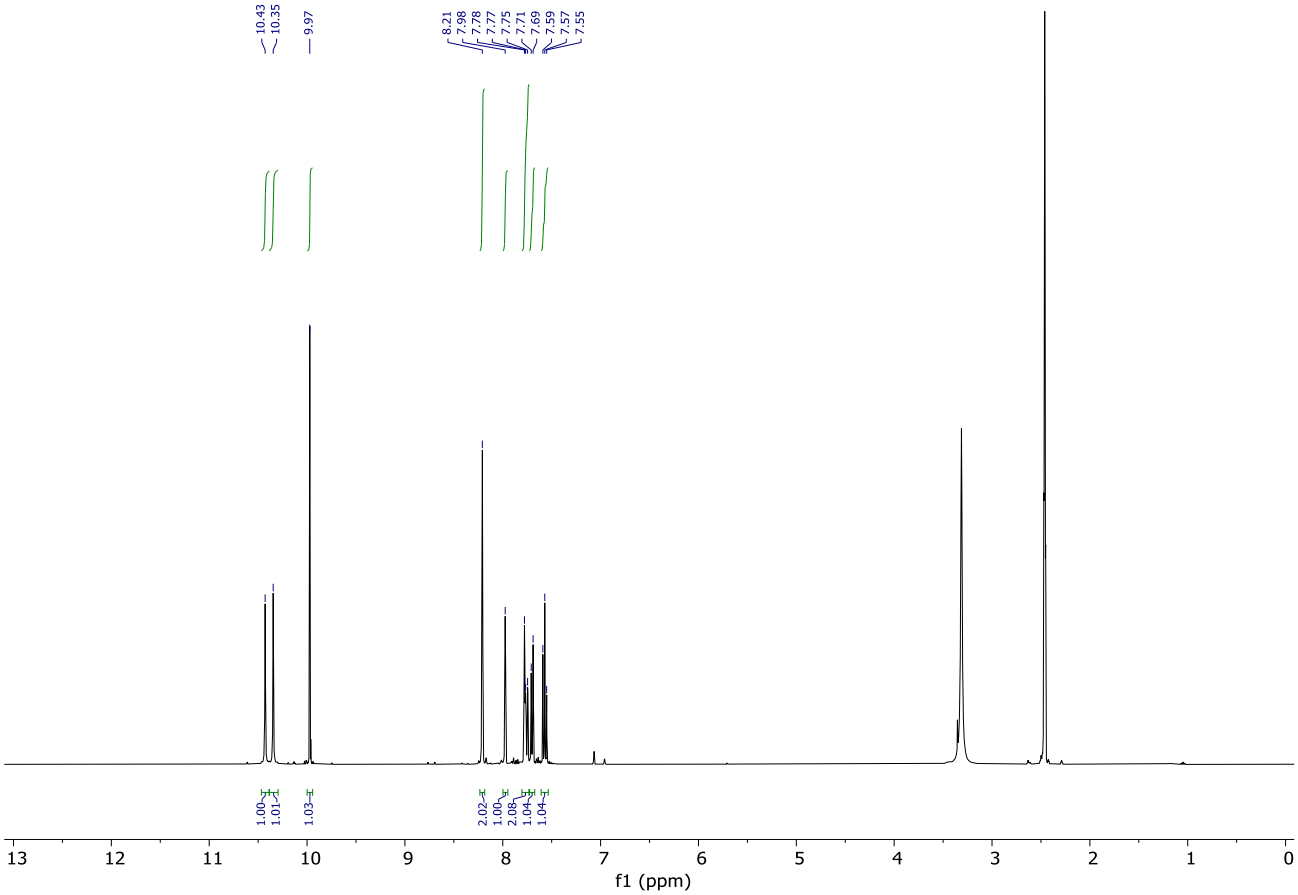


^{13}C NMR:

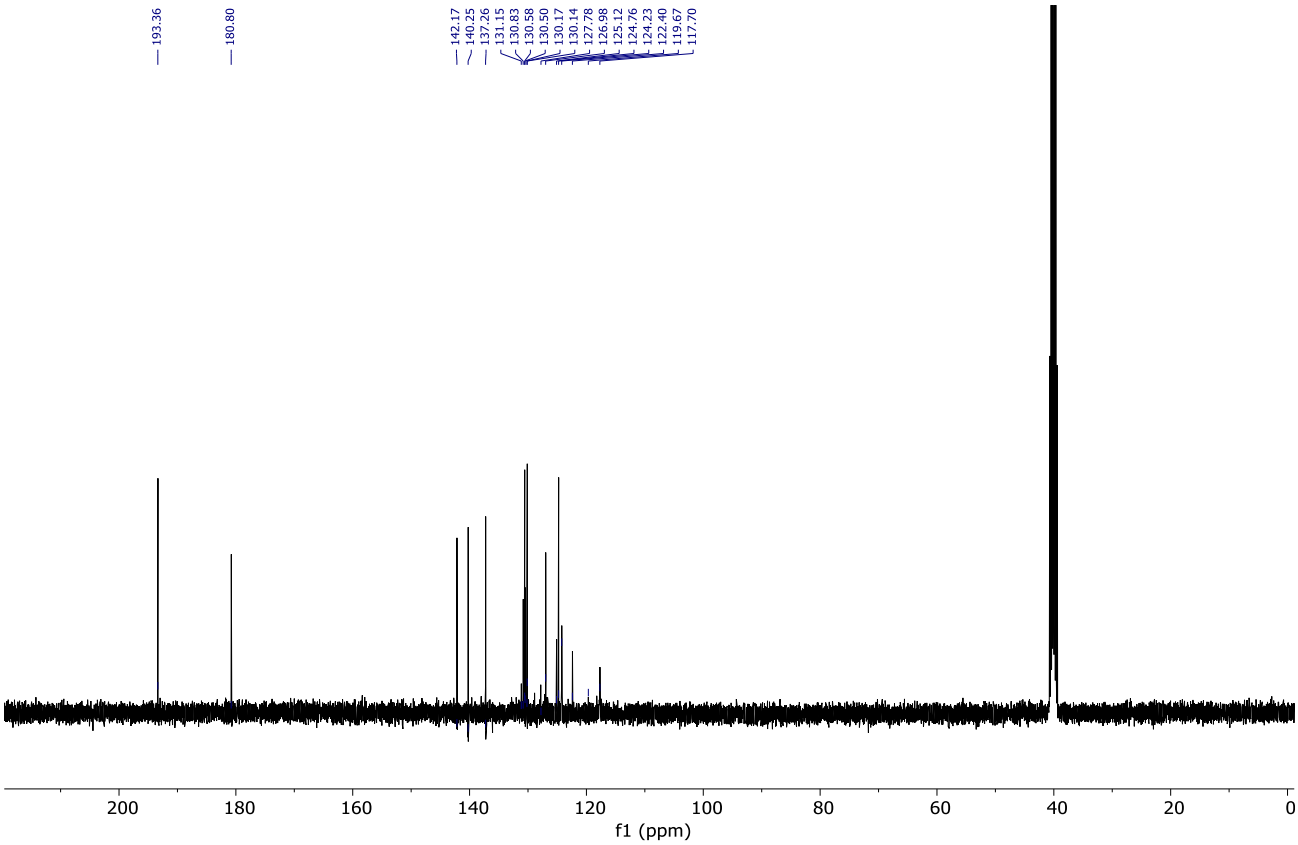


1-(3,5-bis(trifluoromethyl)phenyl)-3-(3-formylphenyl)thiourea (4)

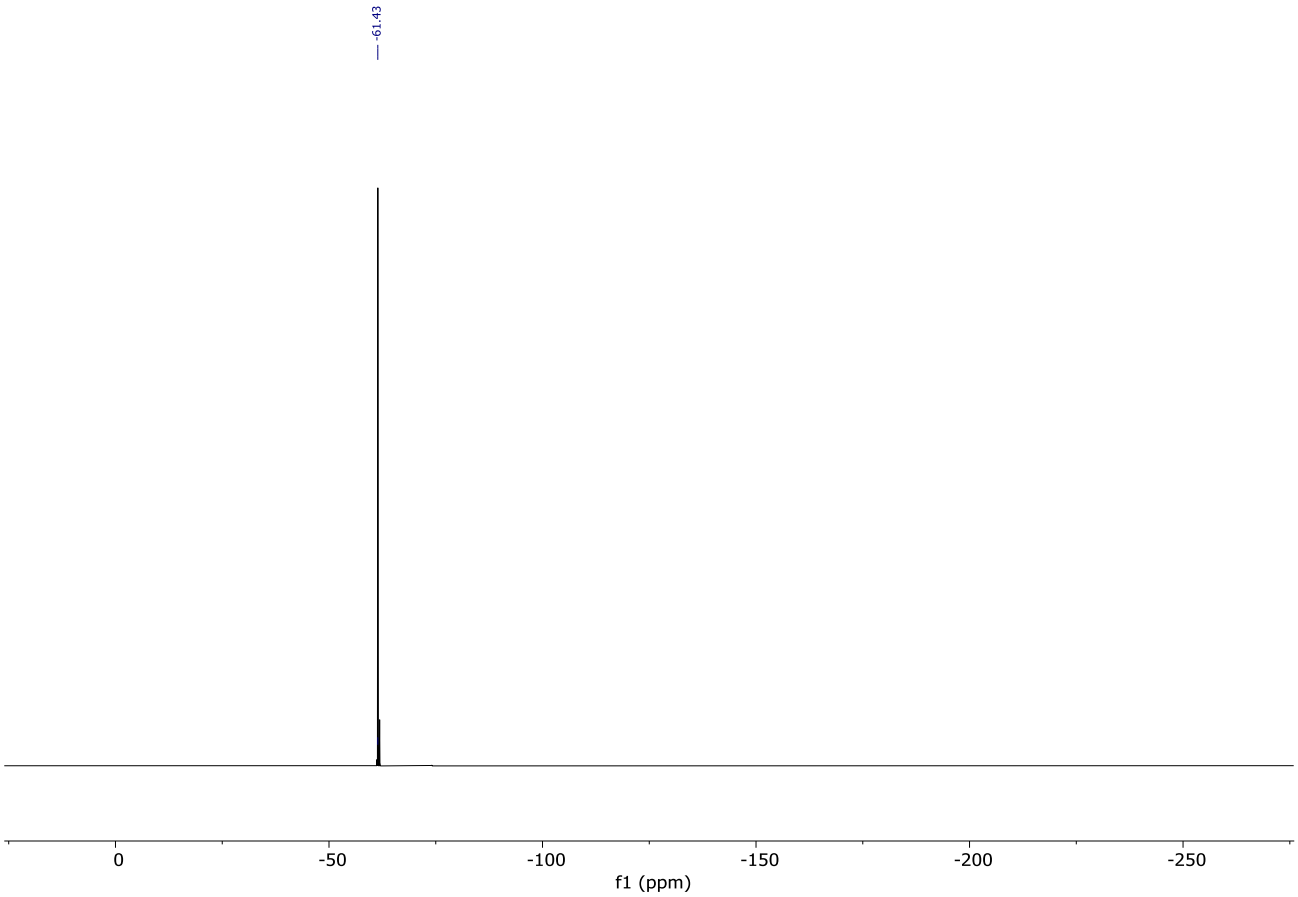
^{1}H NMR:



^{13}C NMR:

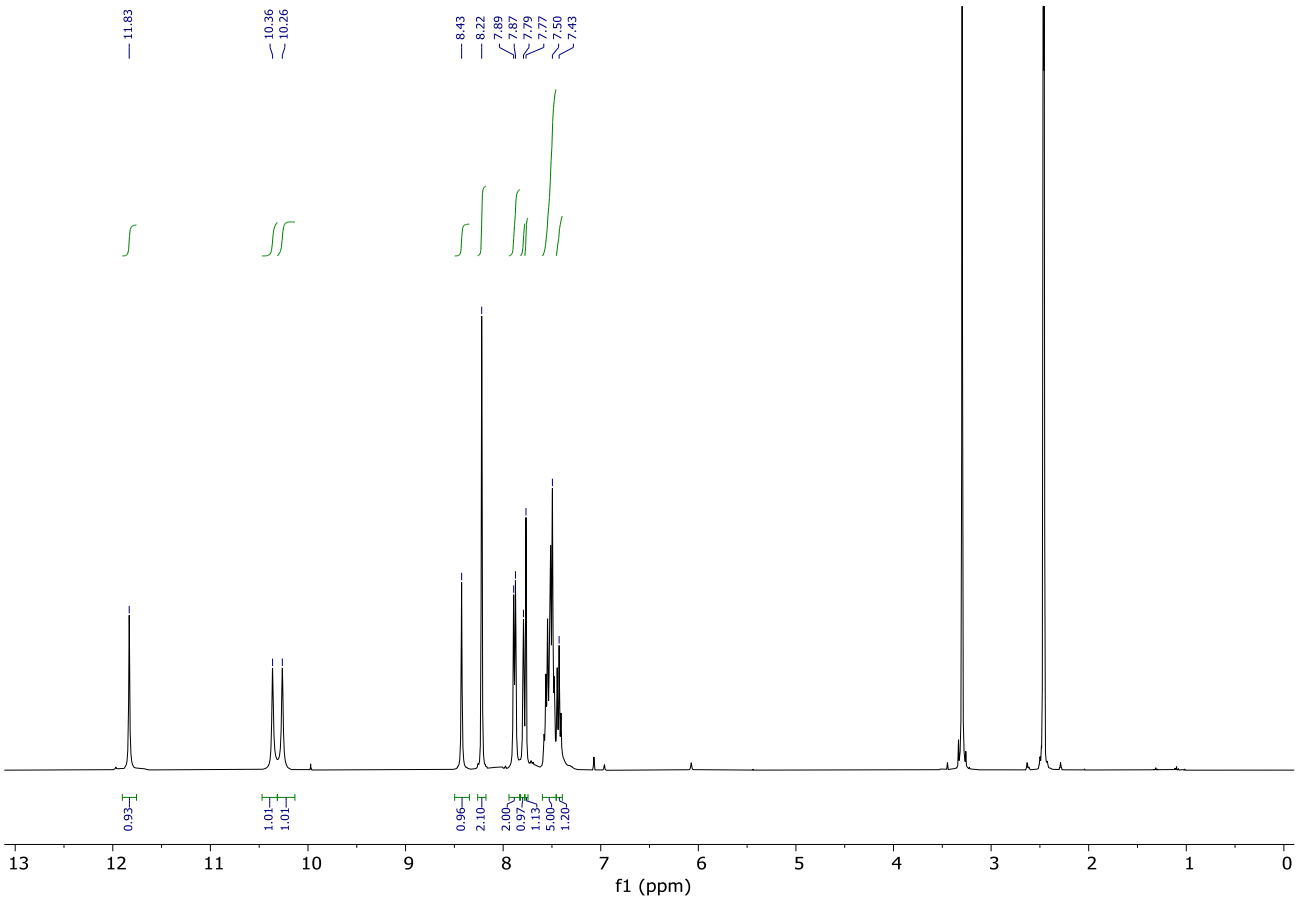


^{19}F NMR:

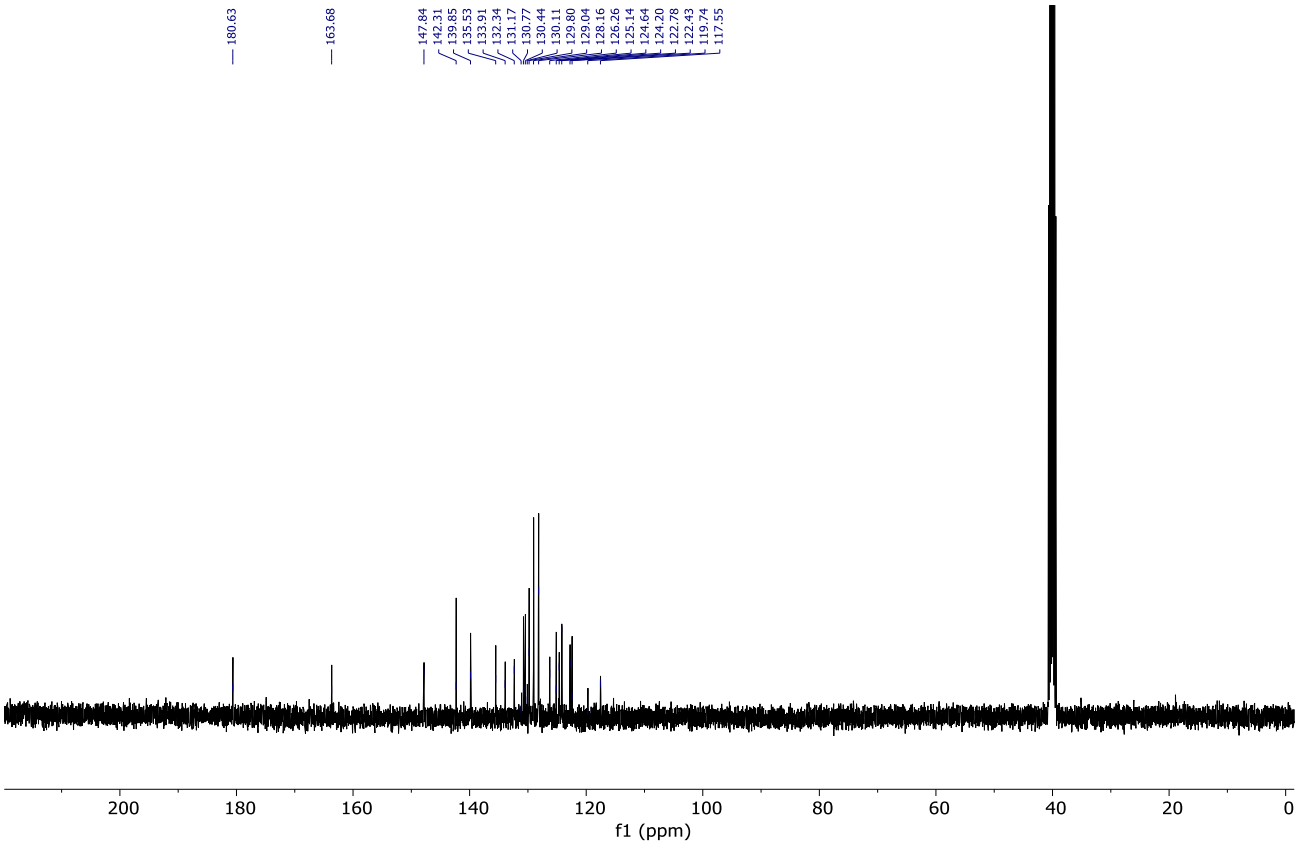


(E)-1-(3-((2-benzoylhydrazono)methyl)phenyl)-3-(3,5-bis(trifluoromethyl)phenyl)thiourea (5a)

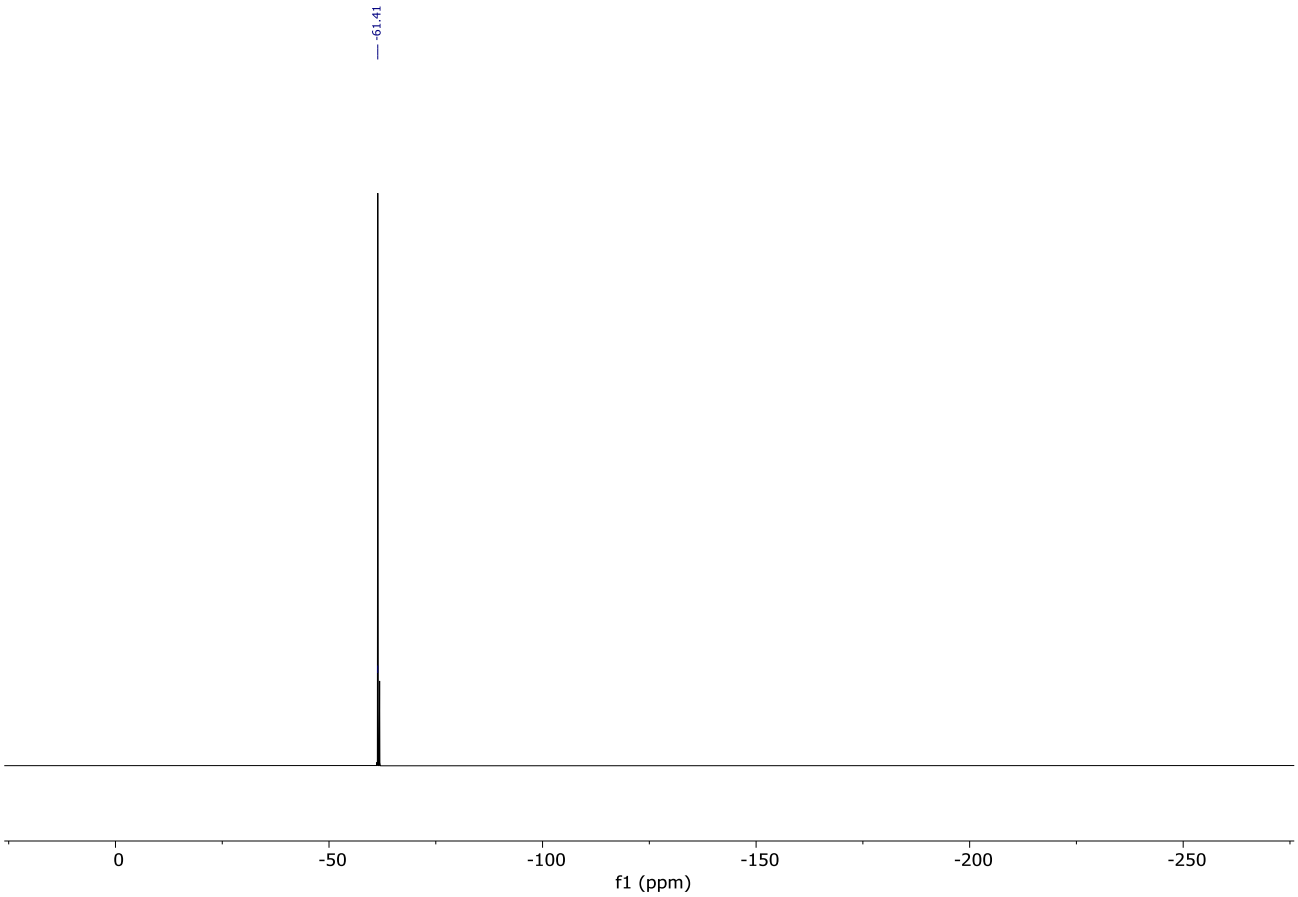
^{1}H NMR:



^{13}C NMR:

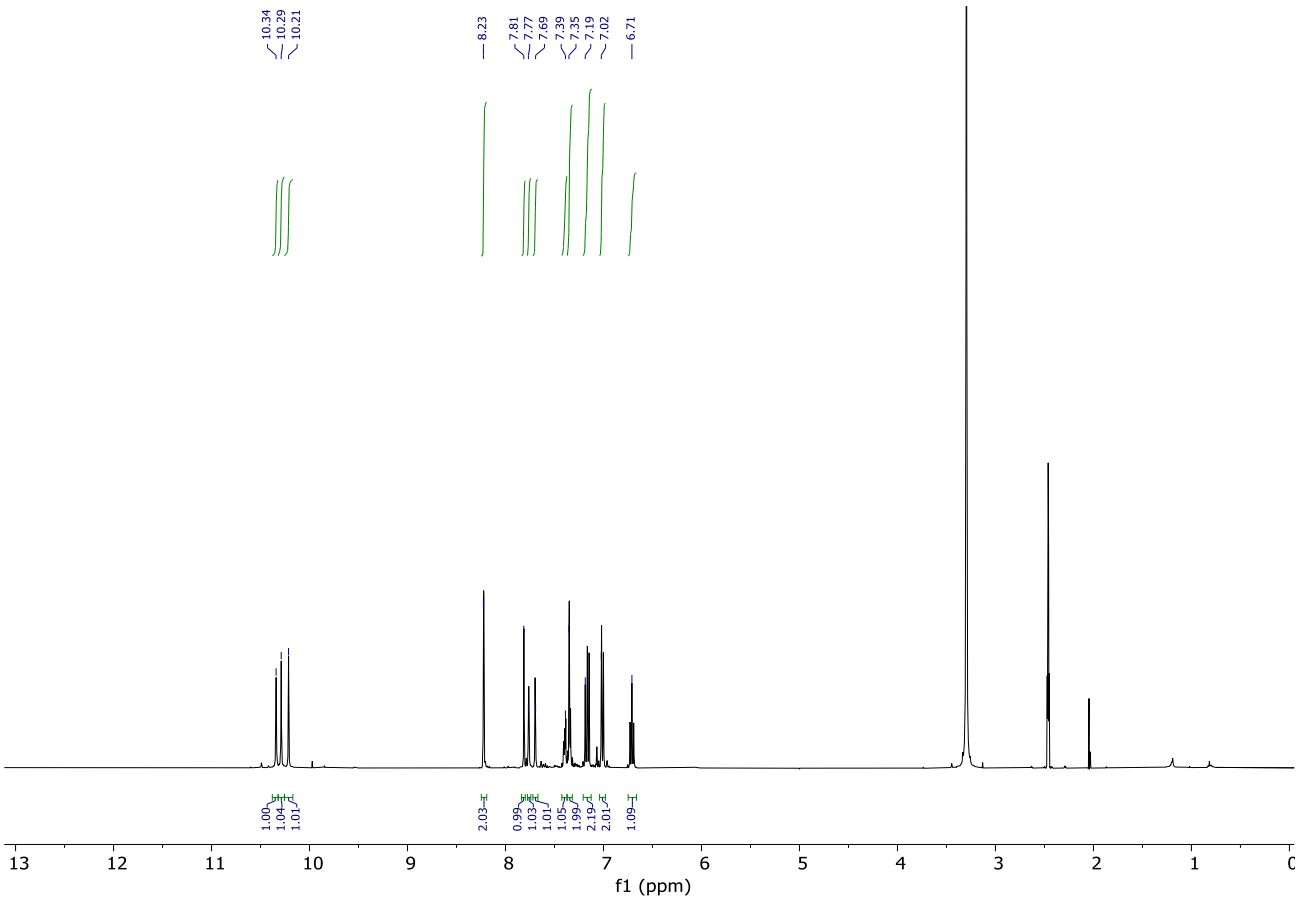


^{19}F NMR:

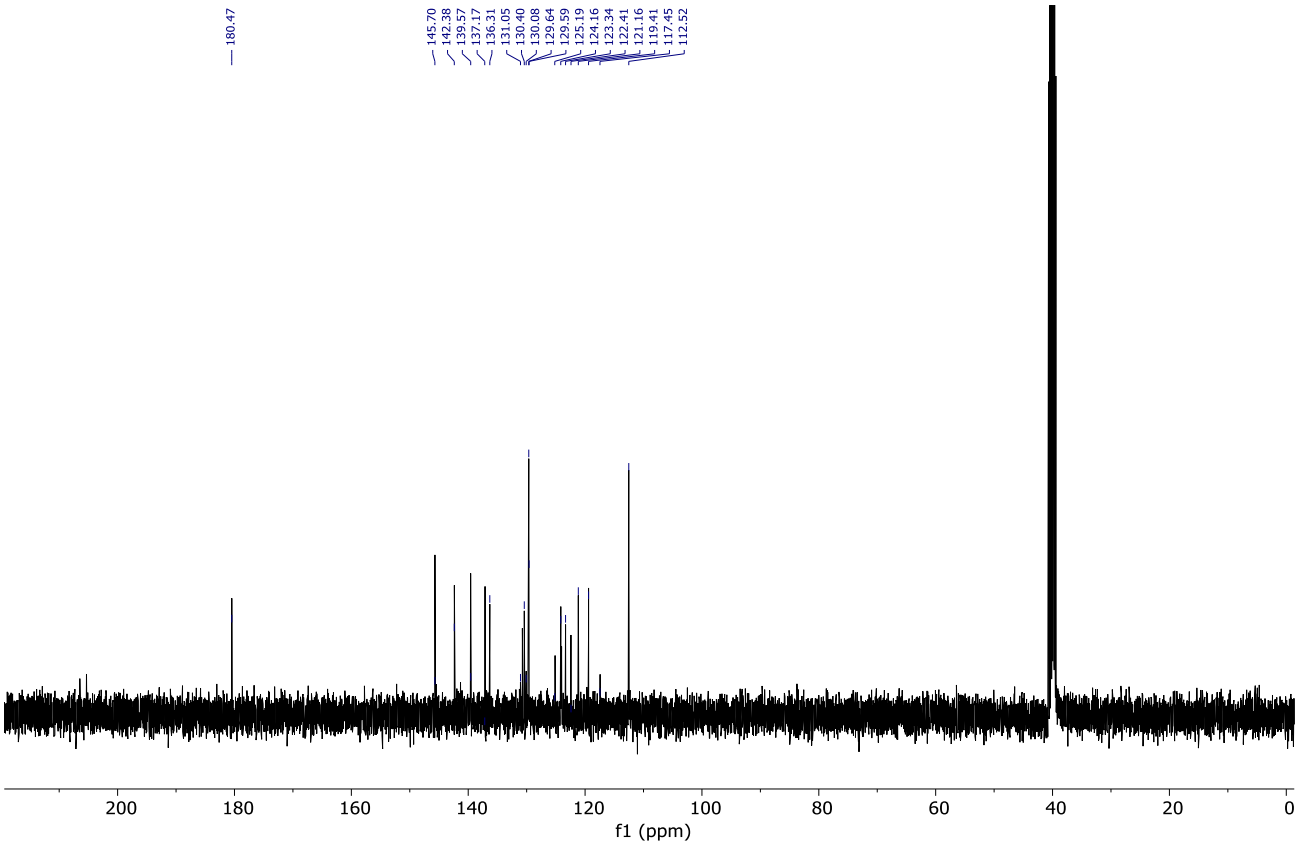


(E)-1-(3,5-bis(trifluoromethyl)phenyl)-3-(3-((2-phenylhydrazono)methyl)phenyl)thiourea (5b)

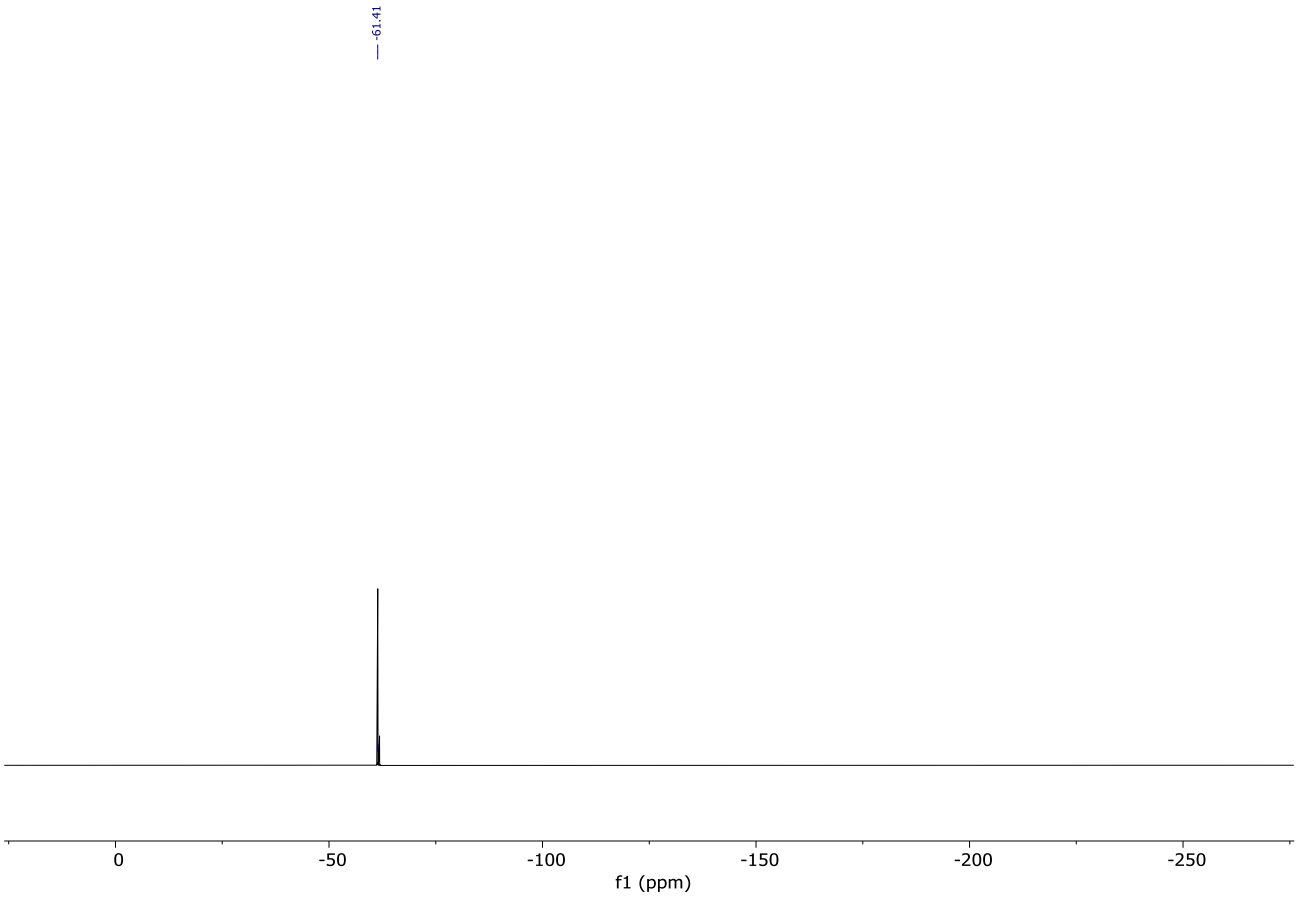
^{1}H NMR:



^{13}C NMR:

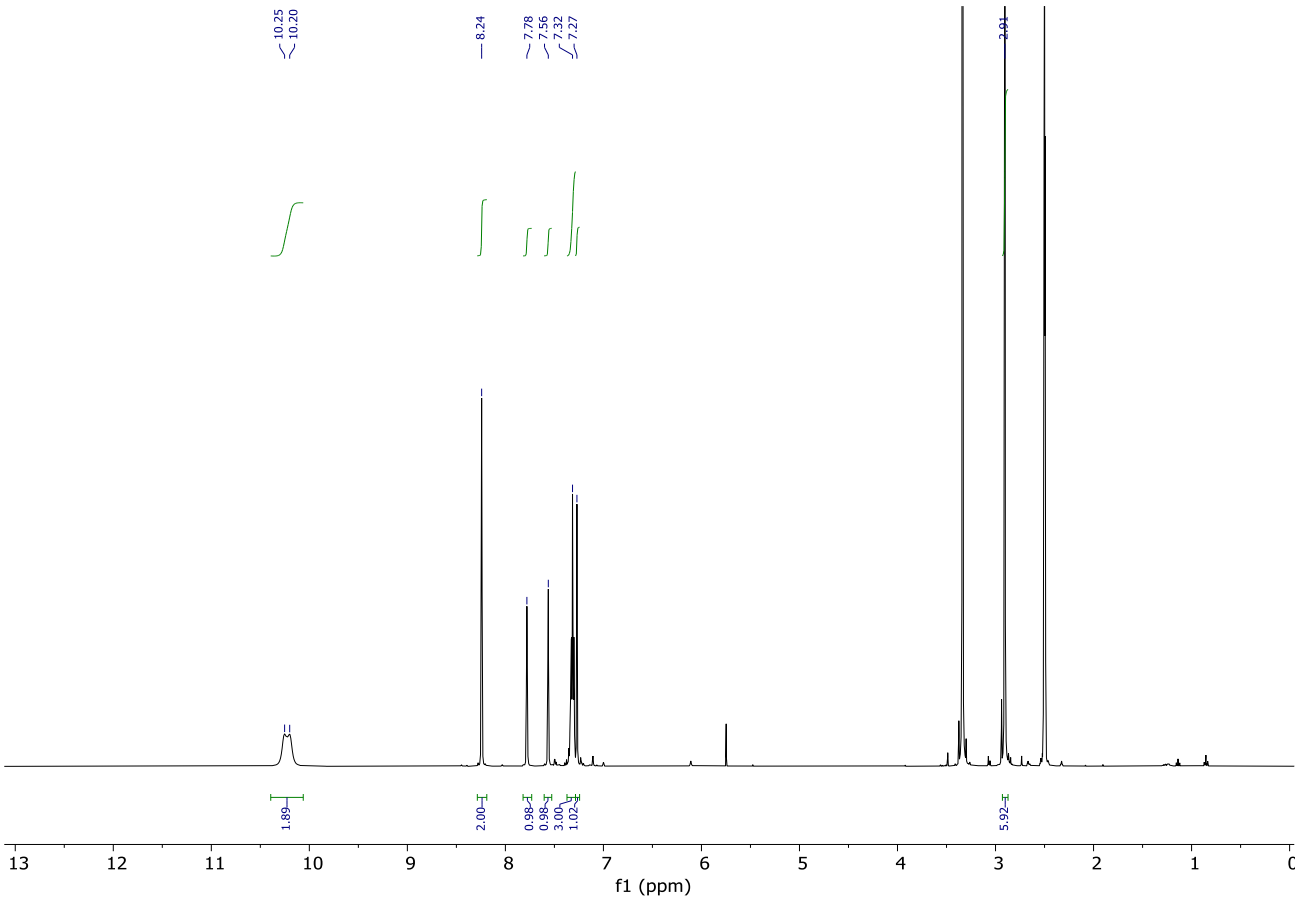


^{19}F NMR:

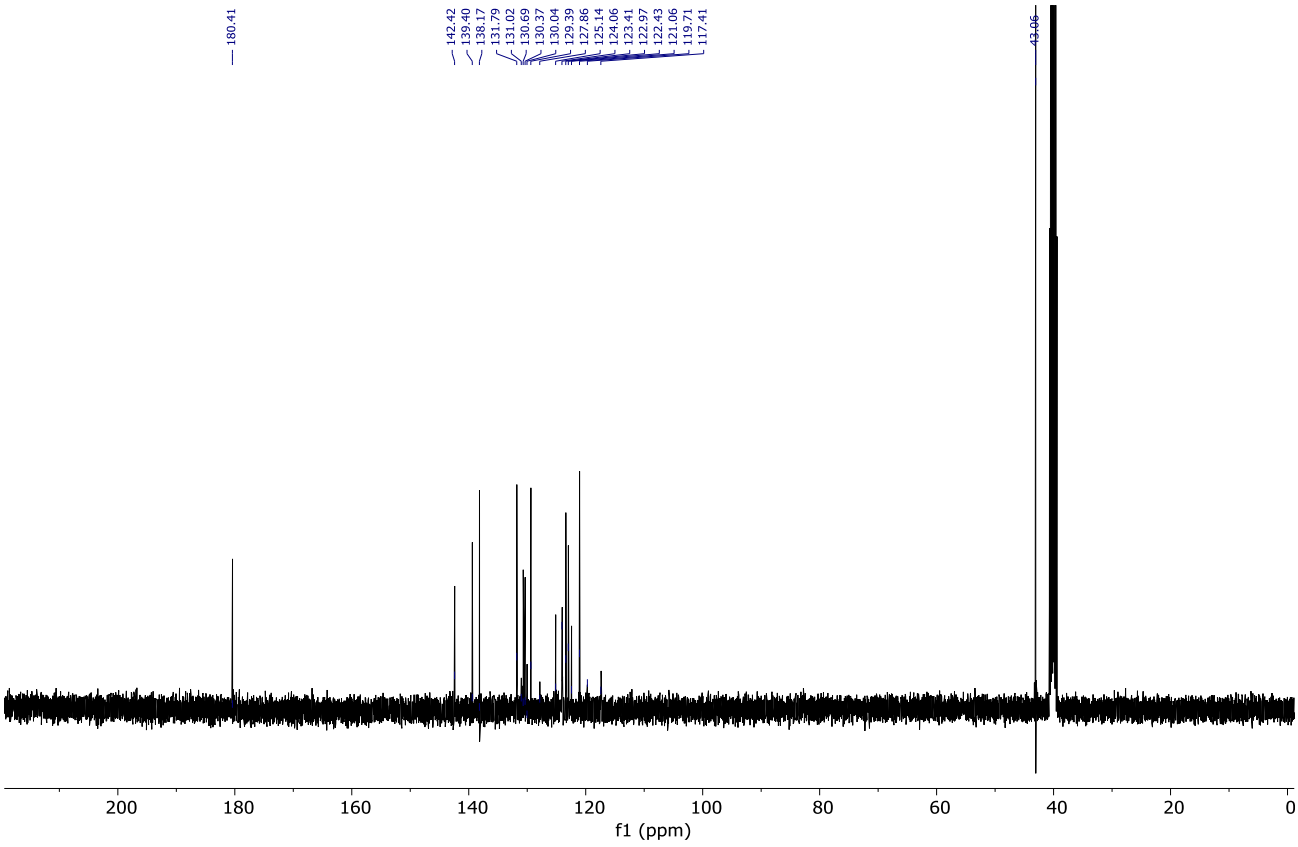


(E)-1-(3,5-bis(trifluoromethyl)phenyl)-3-(3-((2,2-dimethylhydrazono)methyl)phenyl)thiourea (5c)

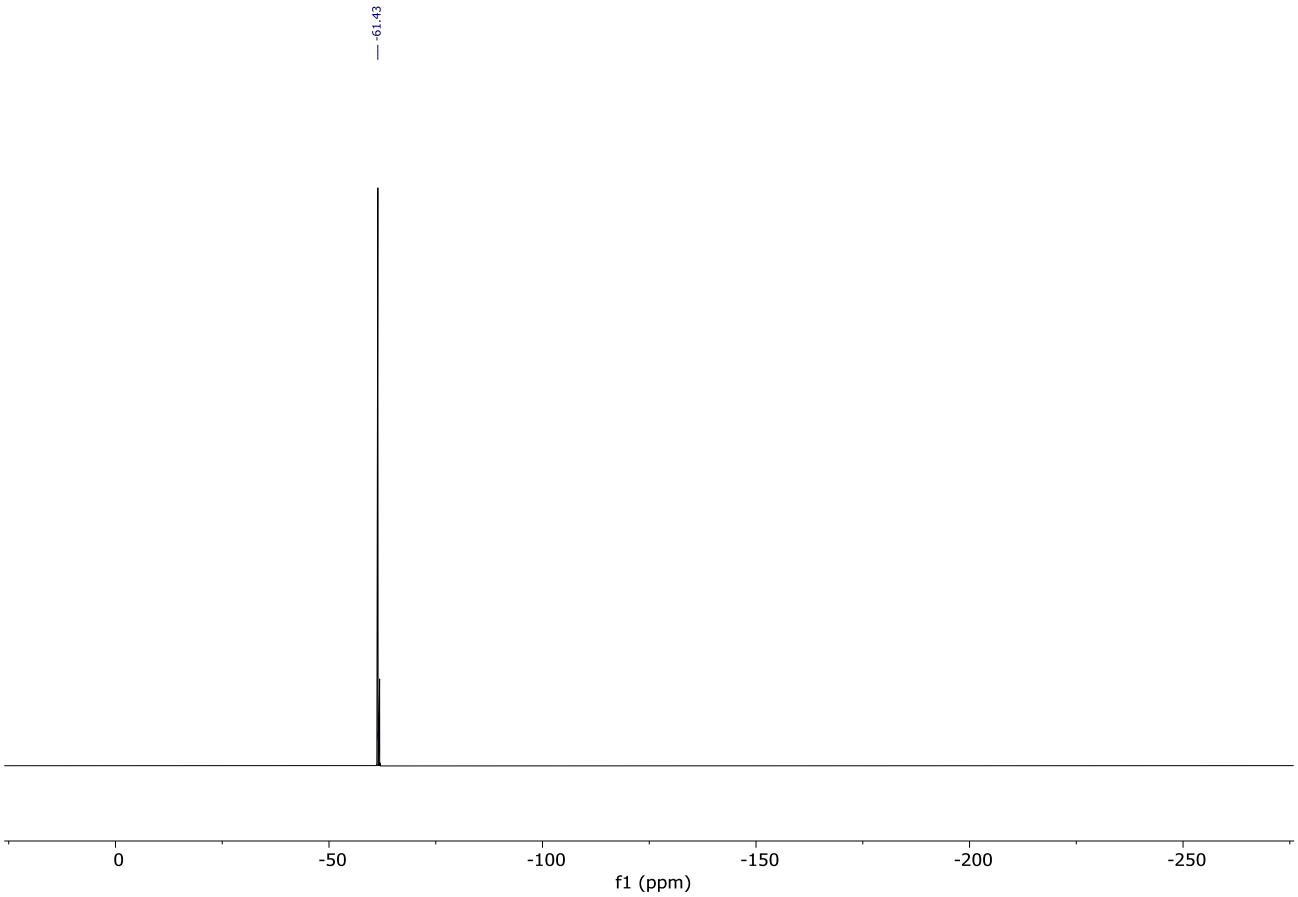
^{1}H NMR:



^{13}C NMR:

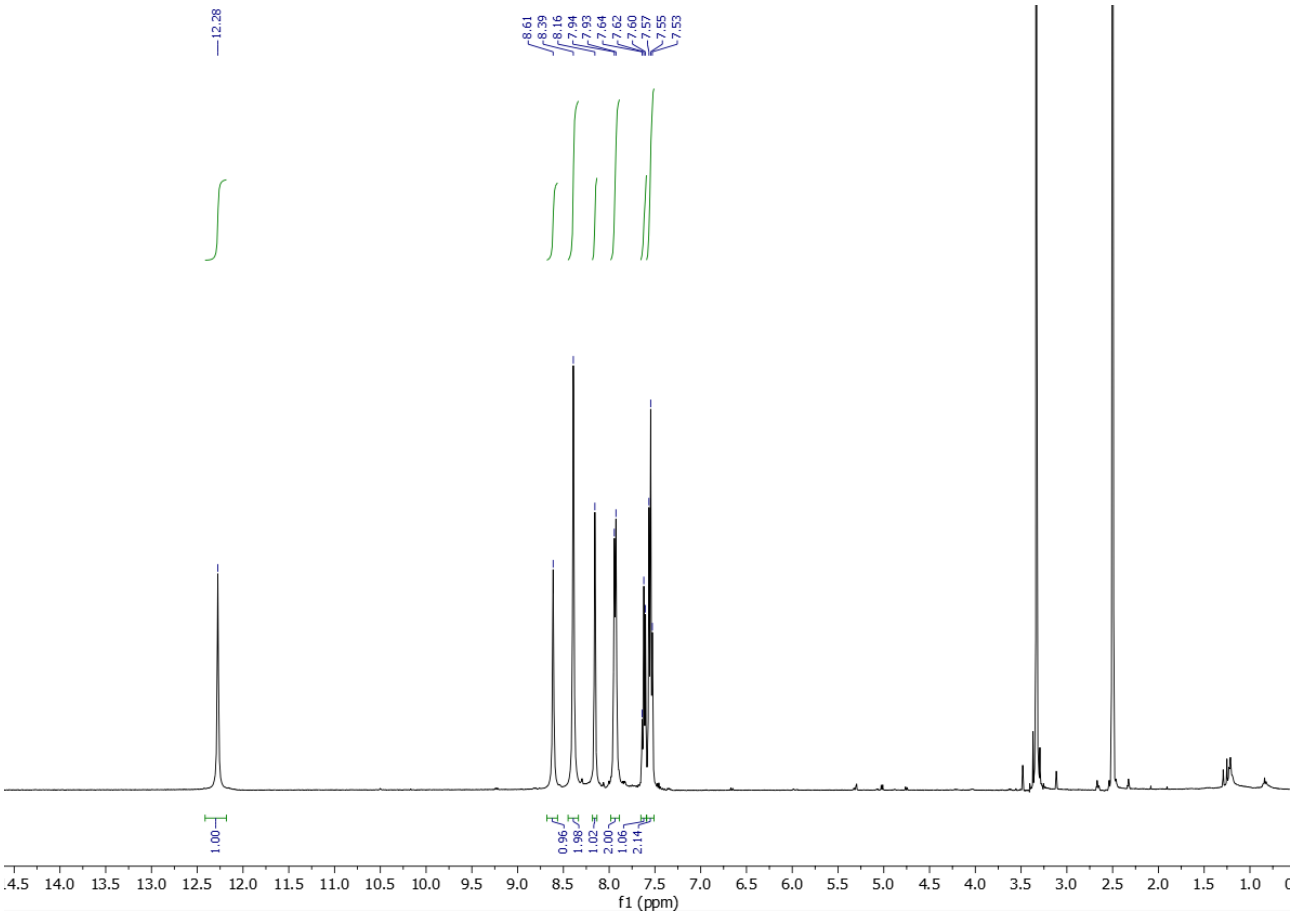


^{19}F NMR:

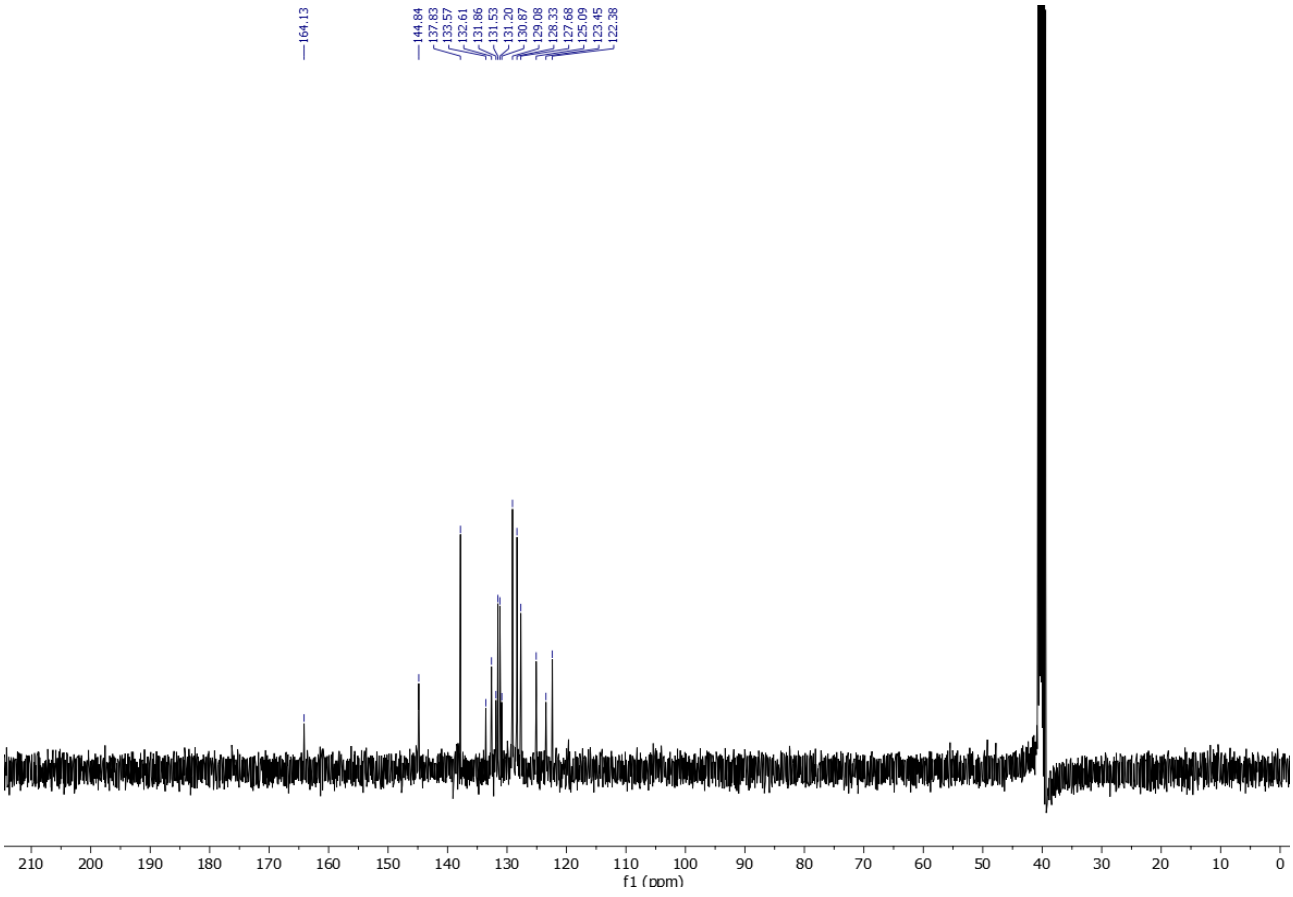


N'-(3,5-bis(trifluoromethyl)benzylidene)benzohydrazide (7)

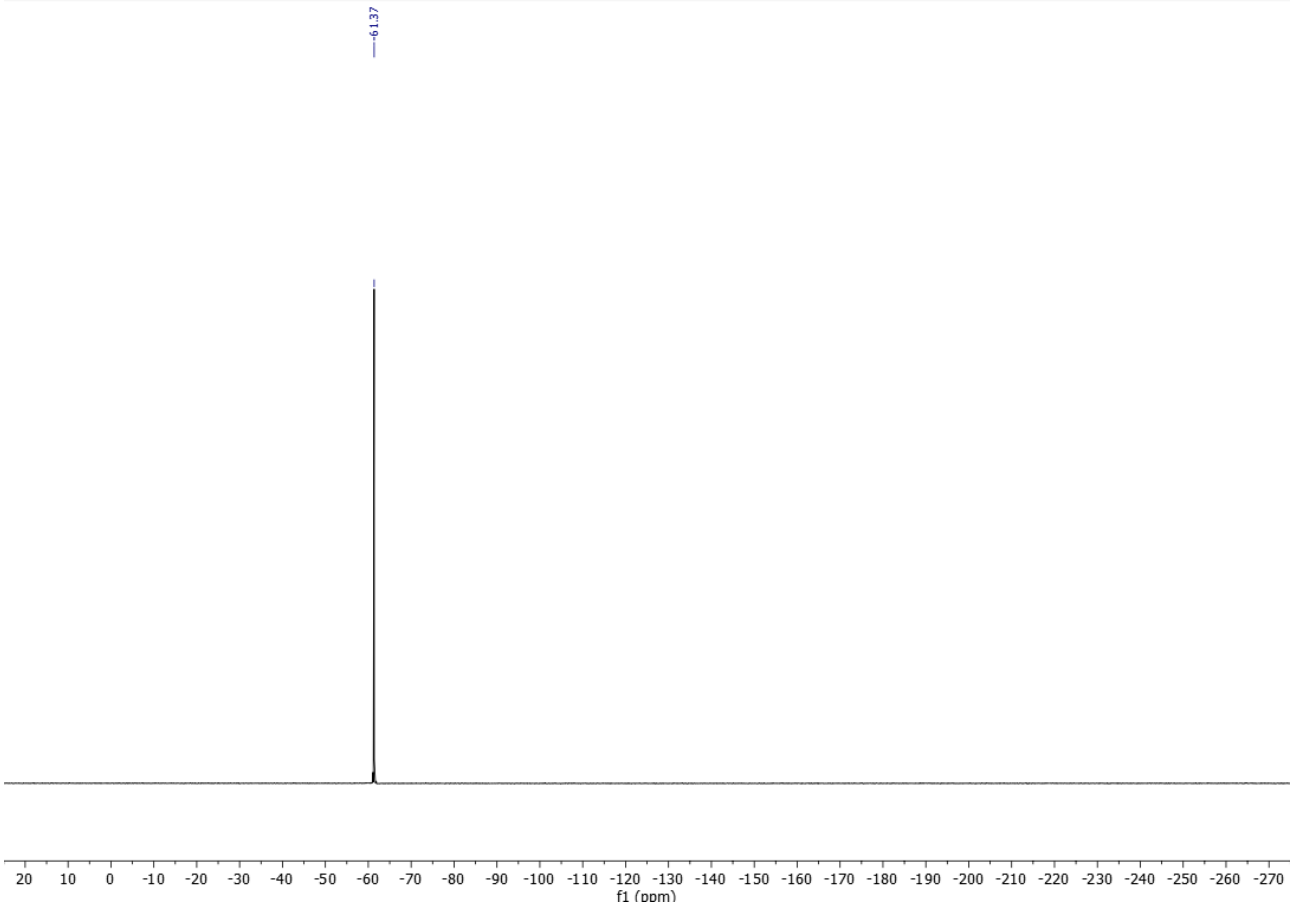
^{1}H NMR:



^{13}C NMR:

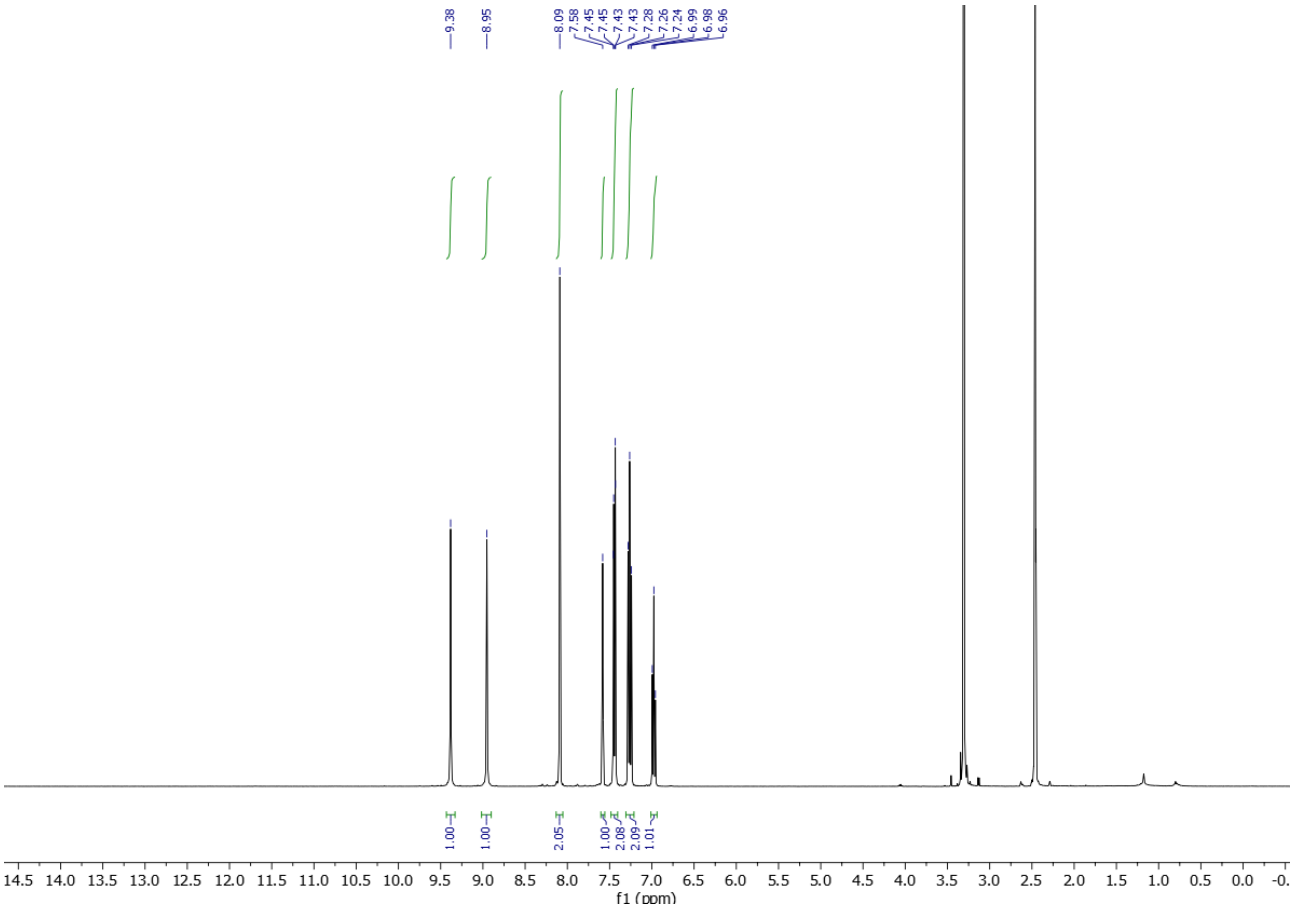


^{19}F NMR:

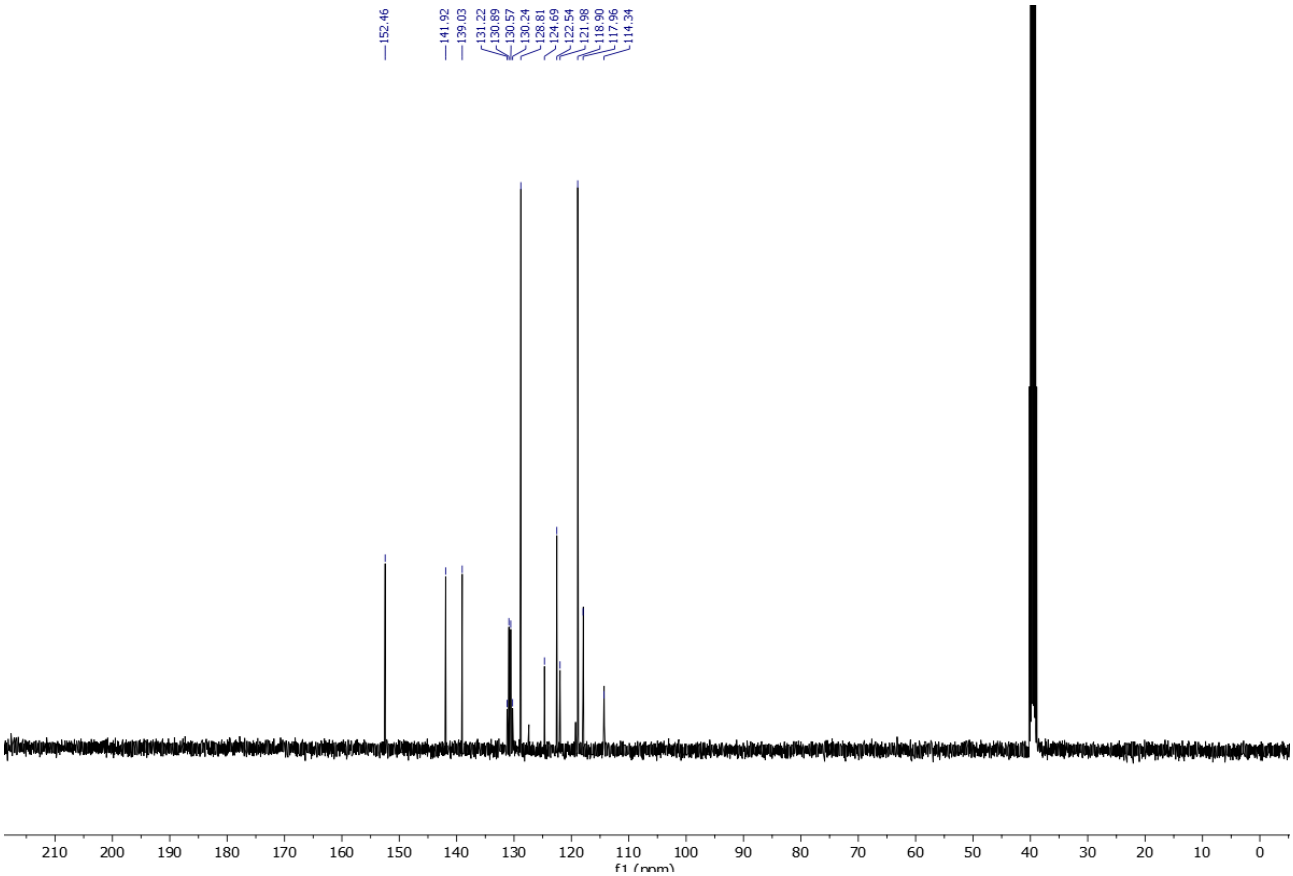

-61.37
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220 -230 -240 -250 -260 -270
f1 (ppm)

1-(3,5-bis(trifluoromethyl)phenyl)-3-phenylurea (16)

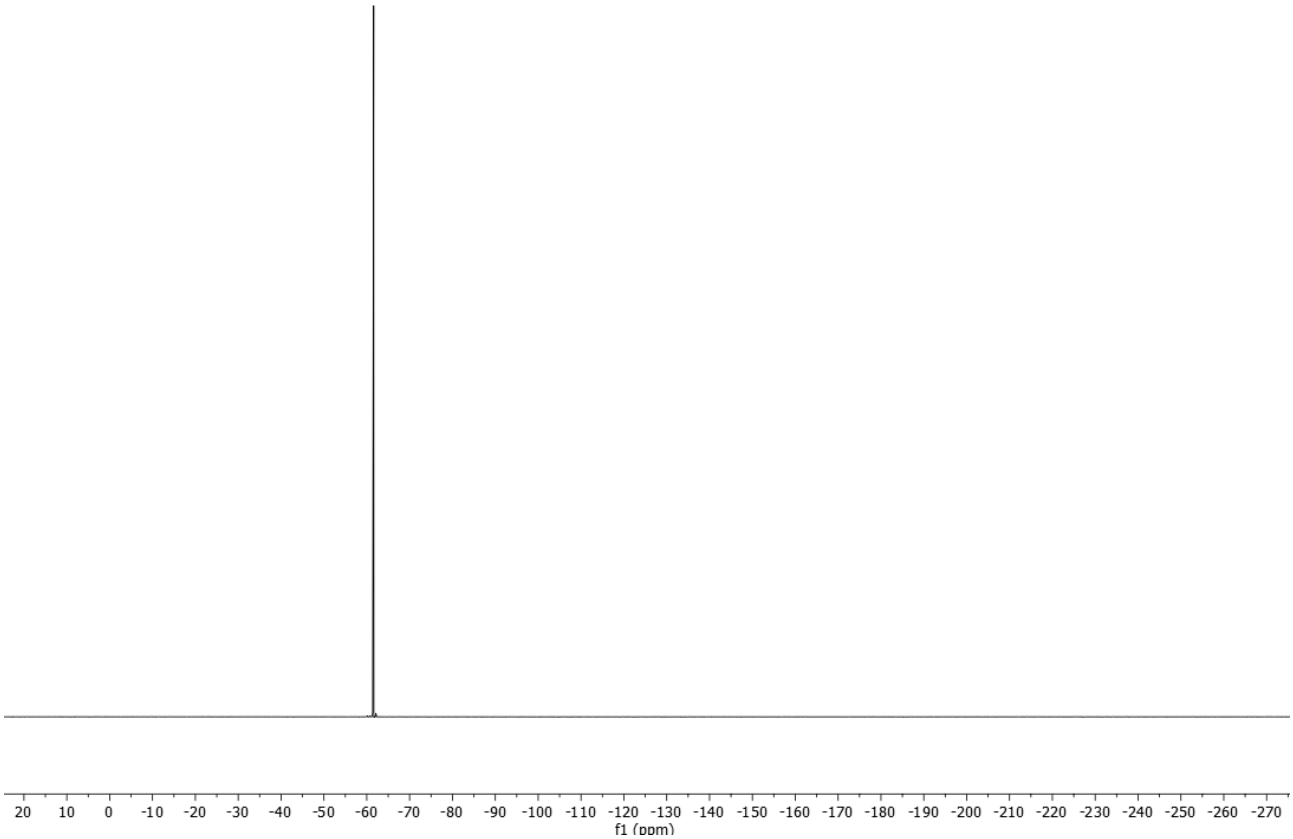
^{1}H NMR:



^{13}C NMR:

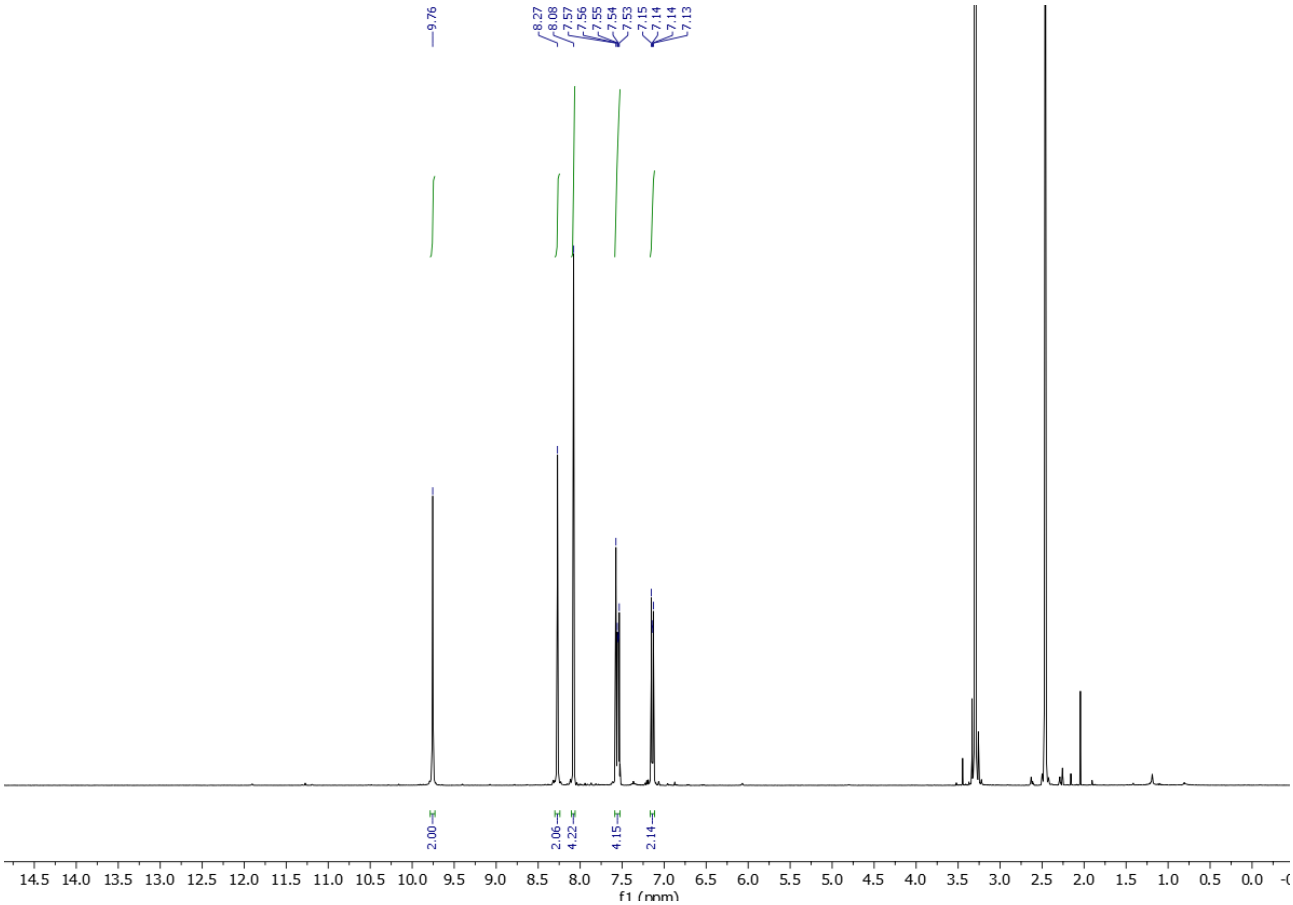


^{19}F NMR:

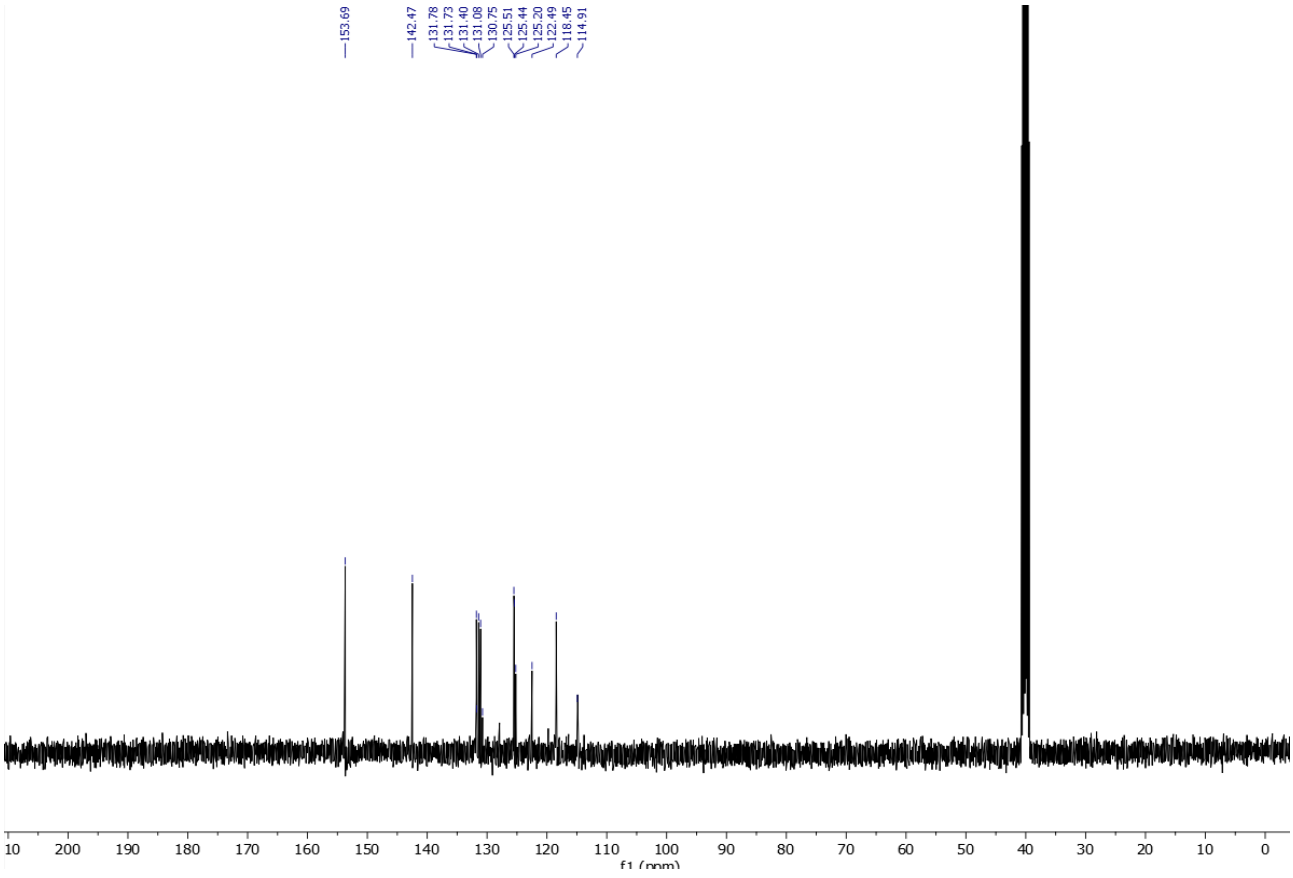

20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220 -230 -240 -250 -260 -270
f1 (ppm)

1,1'-(1,2-phenylene)bis(3-(3,5-bis(trifluoromethyl)phenyl)urea) (17)

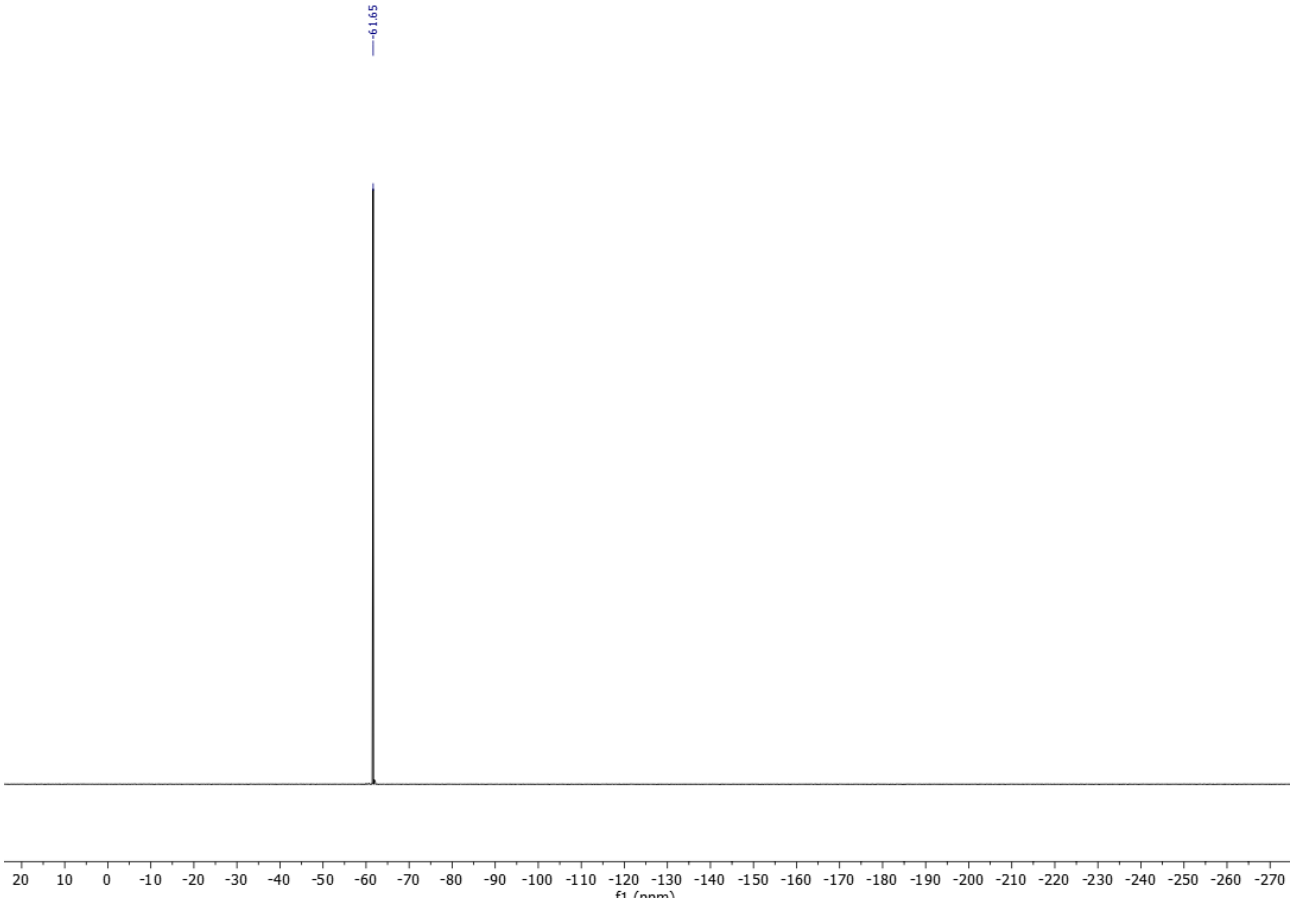
^{1}H NMR:



^{13}C NMR:

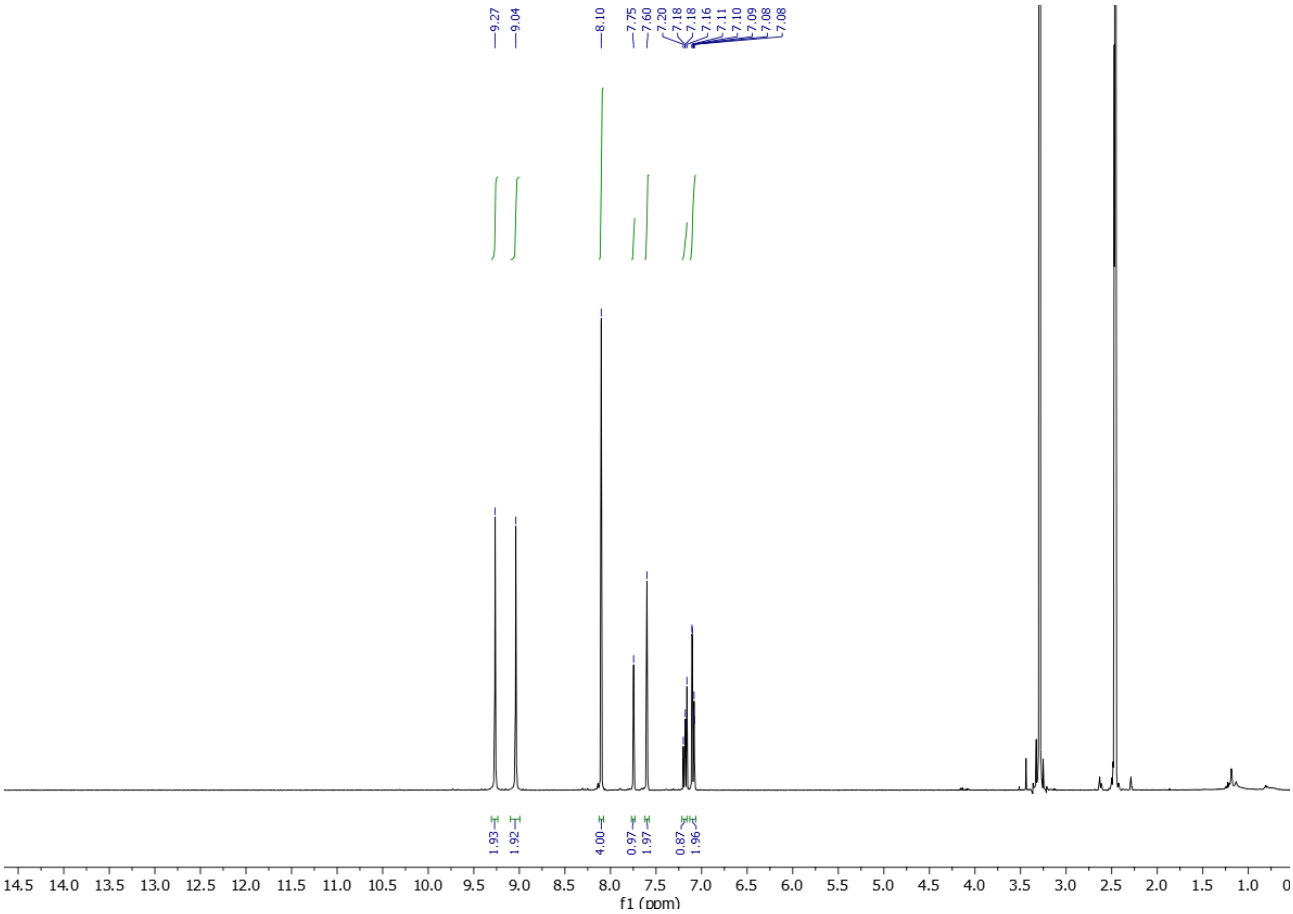


^{19}F NMR:

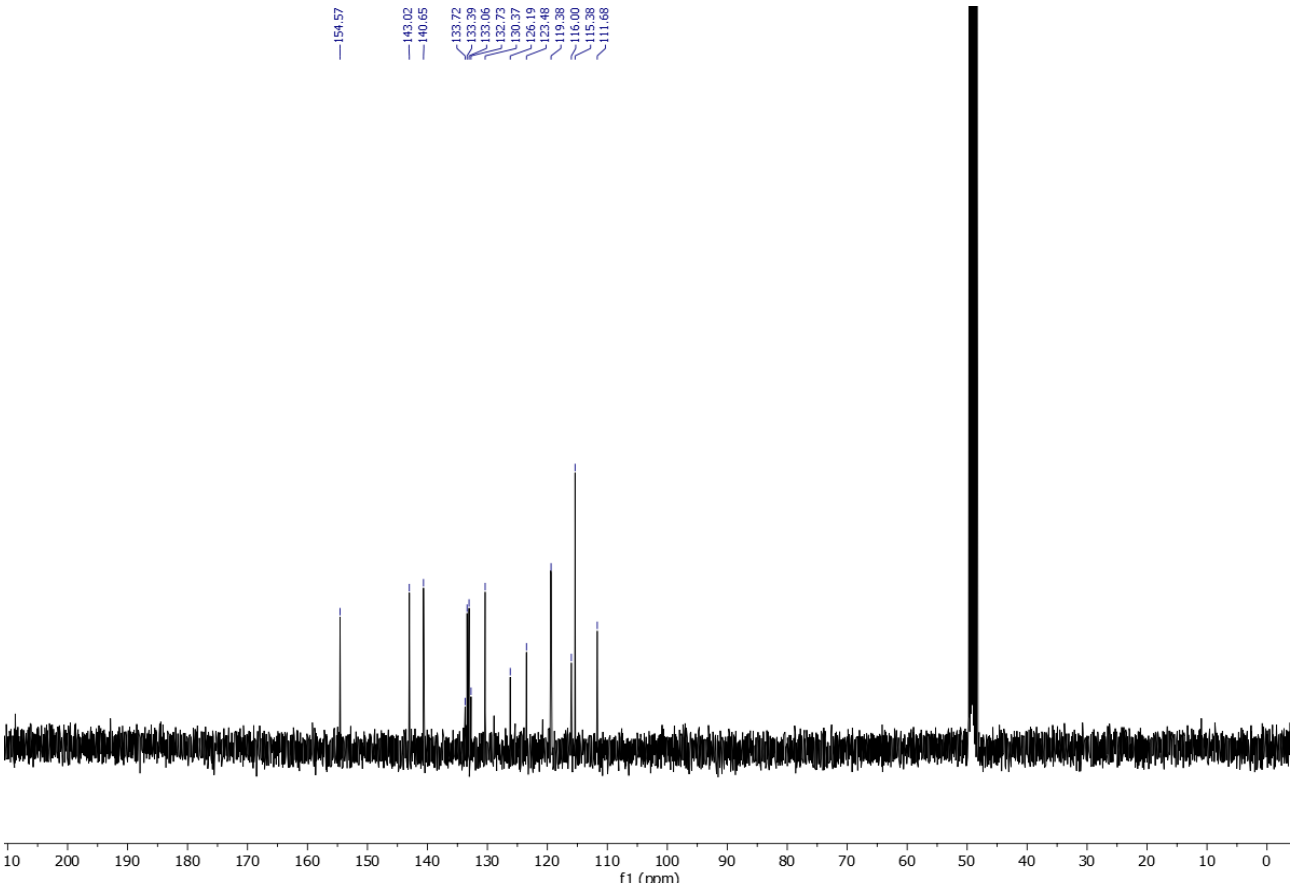

-61.65
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220 -230 -240 -250 -260 -270
f1 (ppm)

1,1'-(1,3-phenylene)bis(3-(3,5-bis(trifluoromethyl)phenyl)urea) (18)

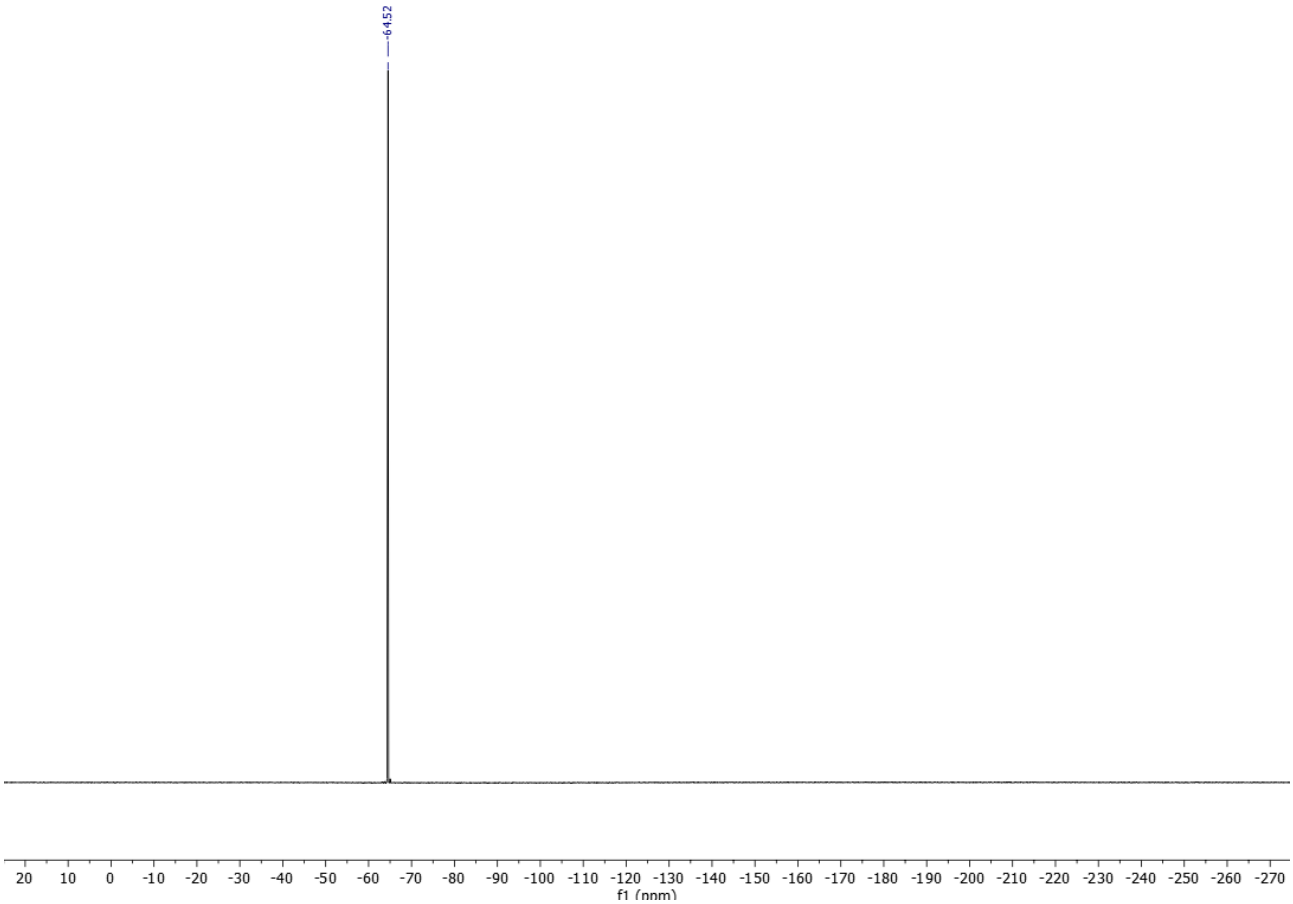
^{1}H NMR:



^{13}C NMR:

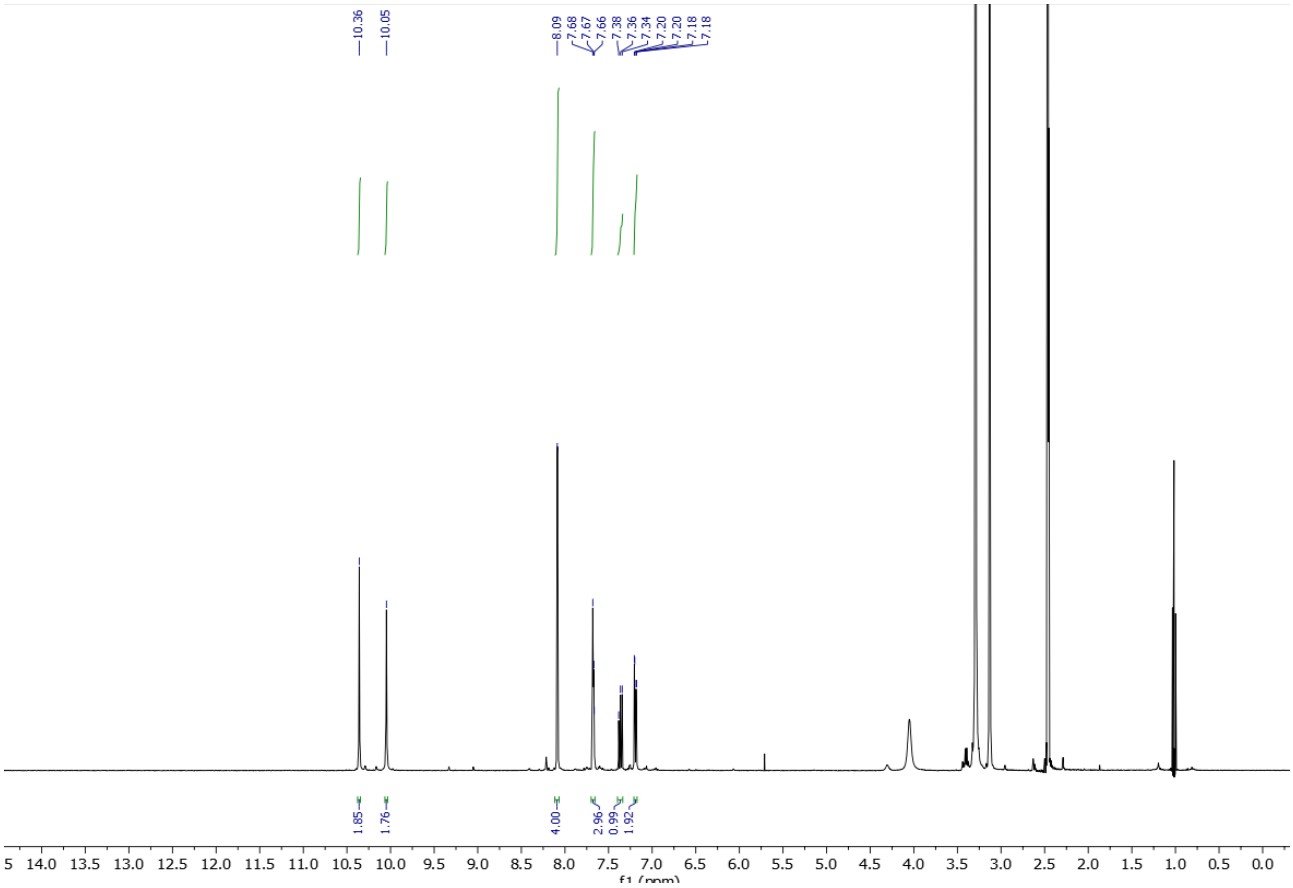


^{19}F NMR:

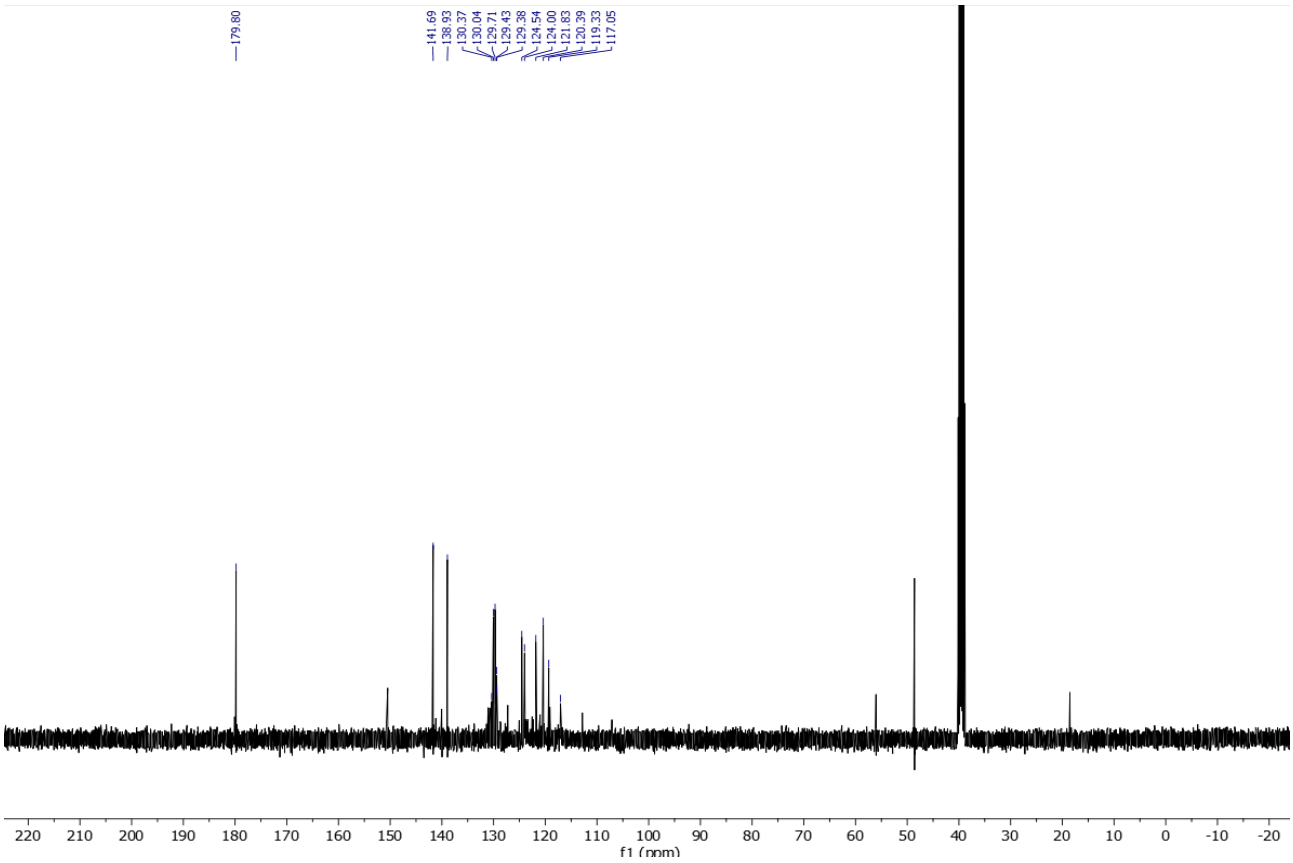

-64.52
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220 -230 -240 -250 -260 -270
f1 (ppm)

1,1'-(1,3-phenylene)bis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea) (19)

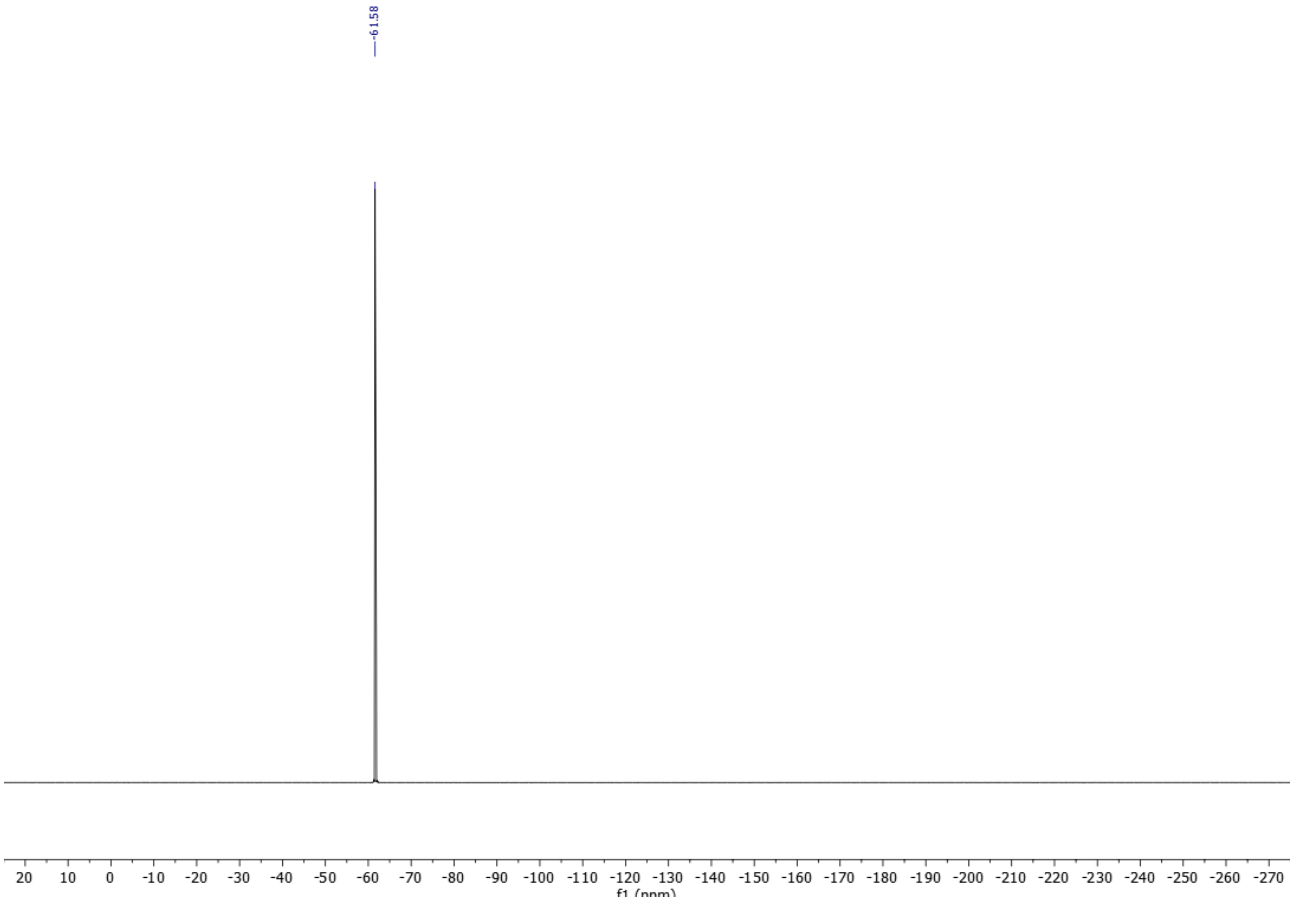
^{1}H NMR:



^{13}C NMR:

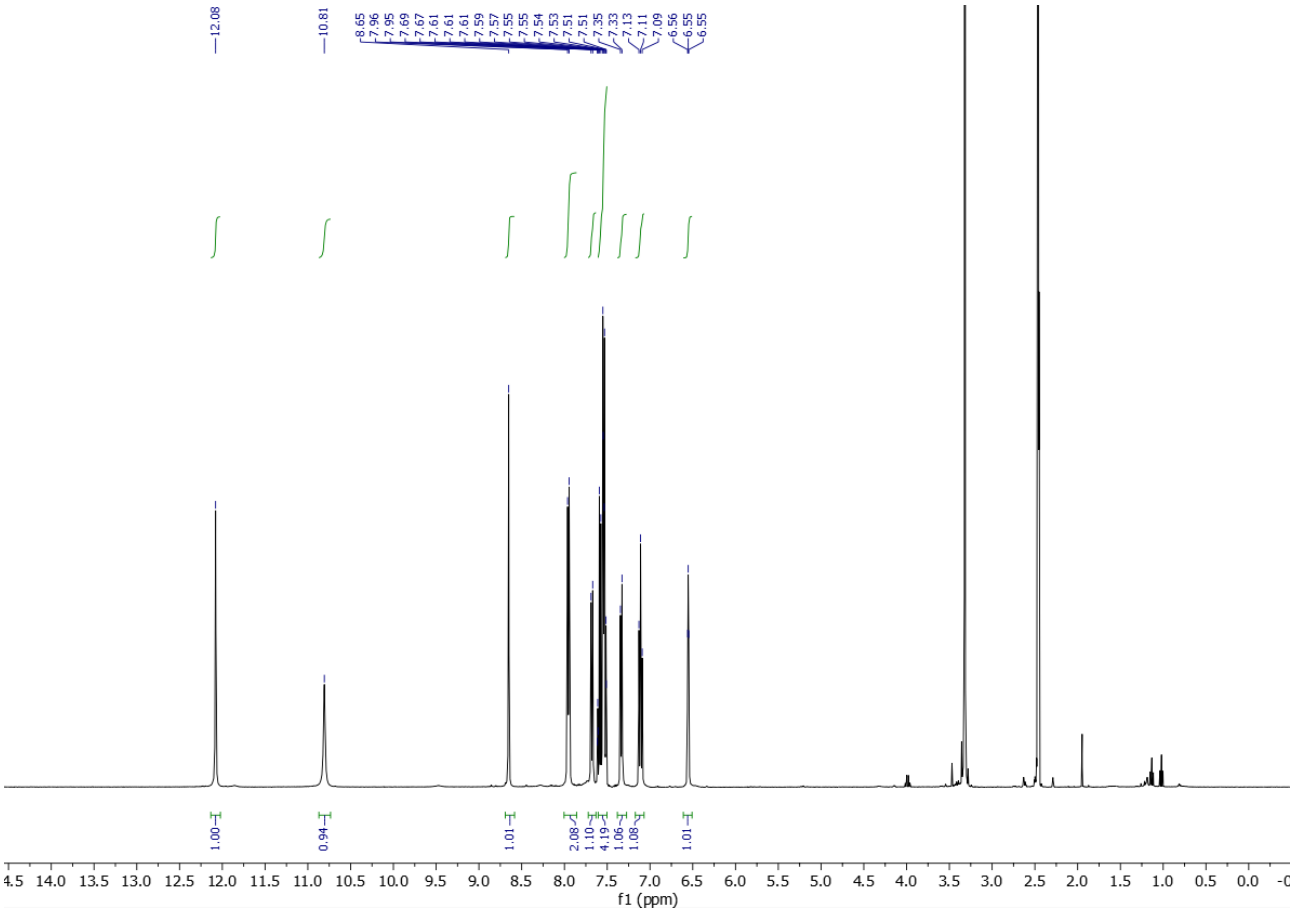


^{19}F NMR:

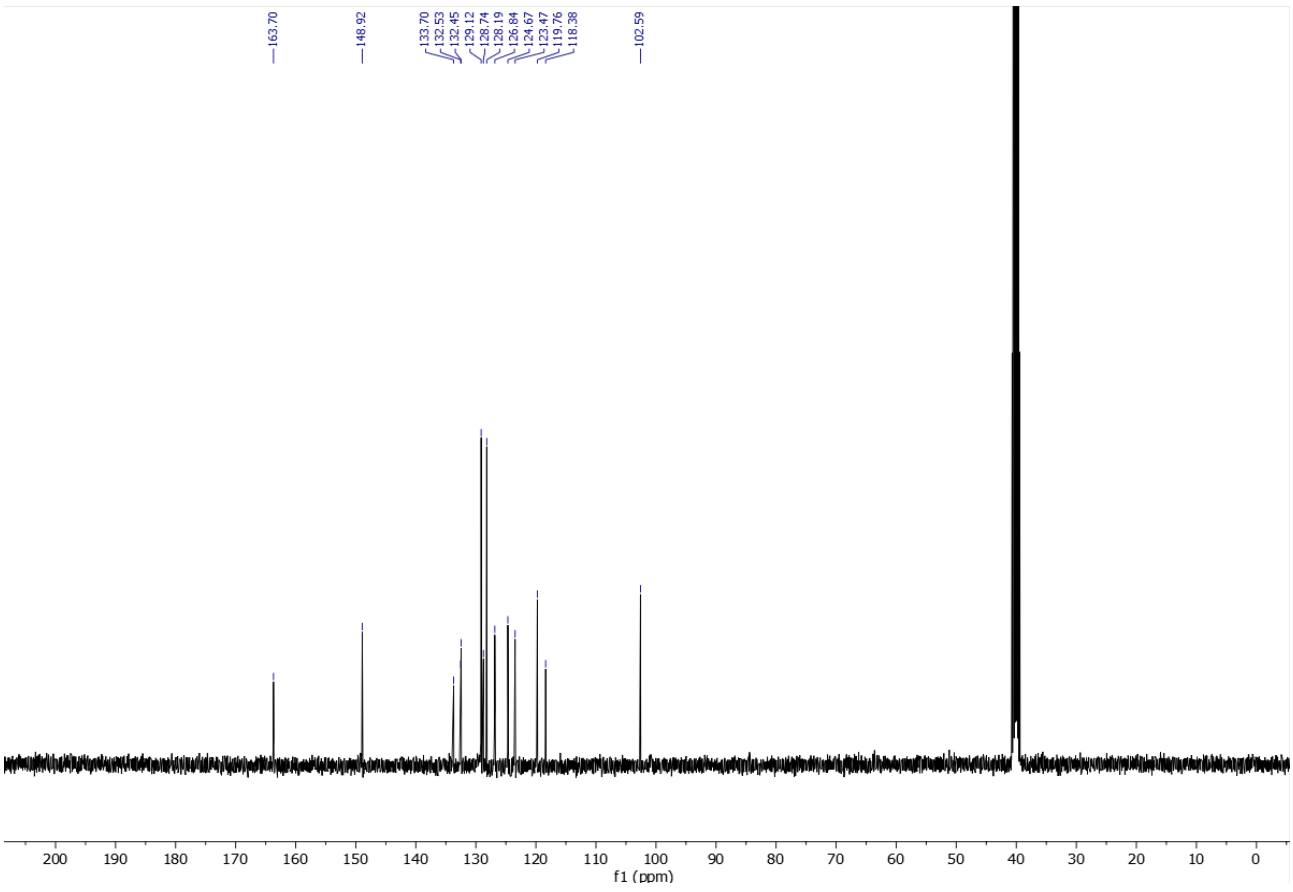

-61.58
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220 -230 -240 -250 -260 -270
f1 (ppm)

N'-((1H-indol-7-yl)methylene)benzohydrazide (20)

^{1}H NMR:

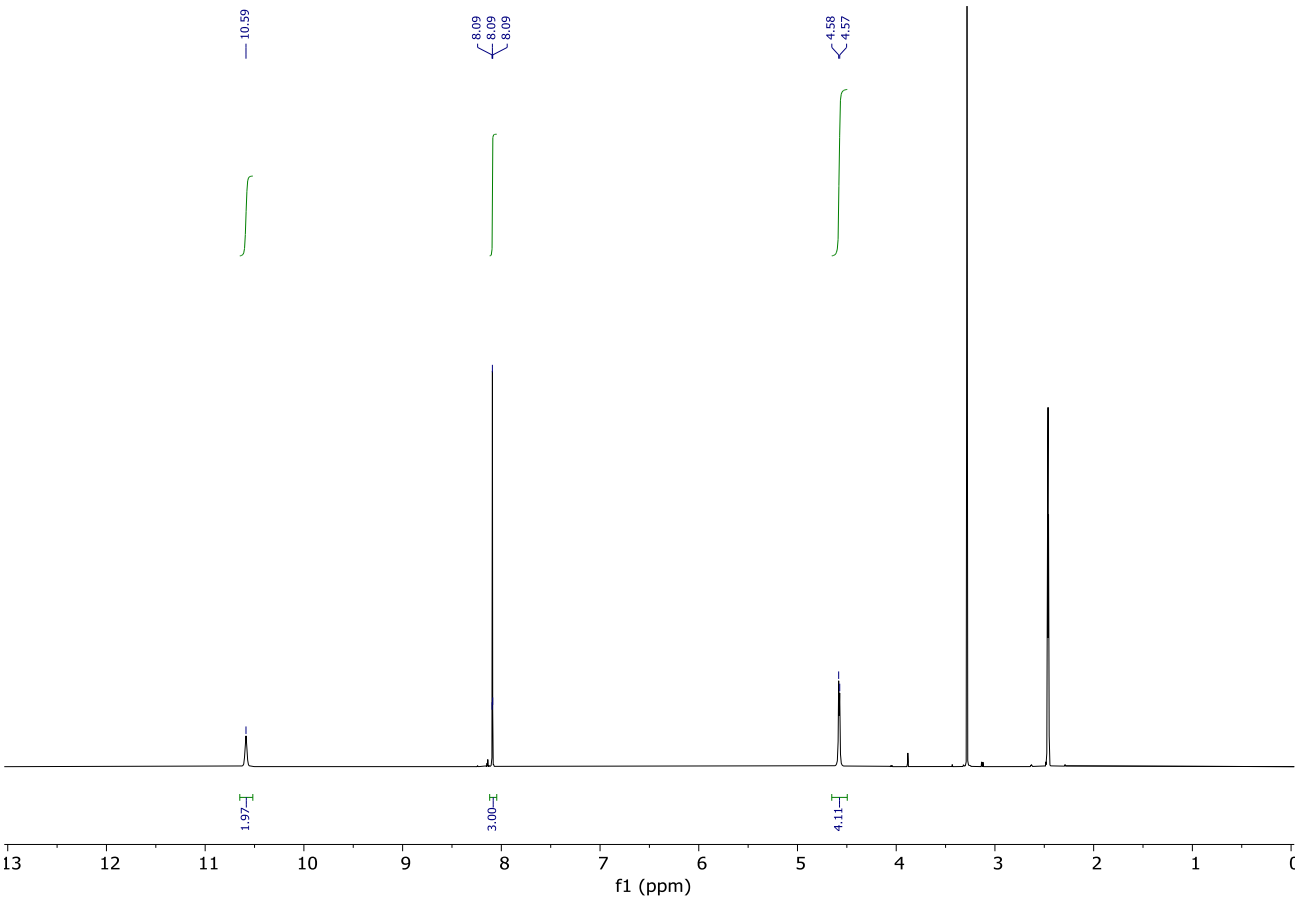


^{13}C NMR:

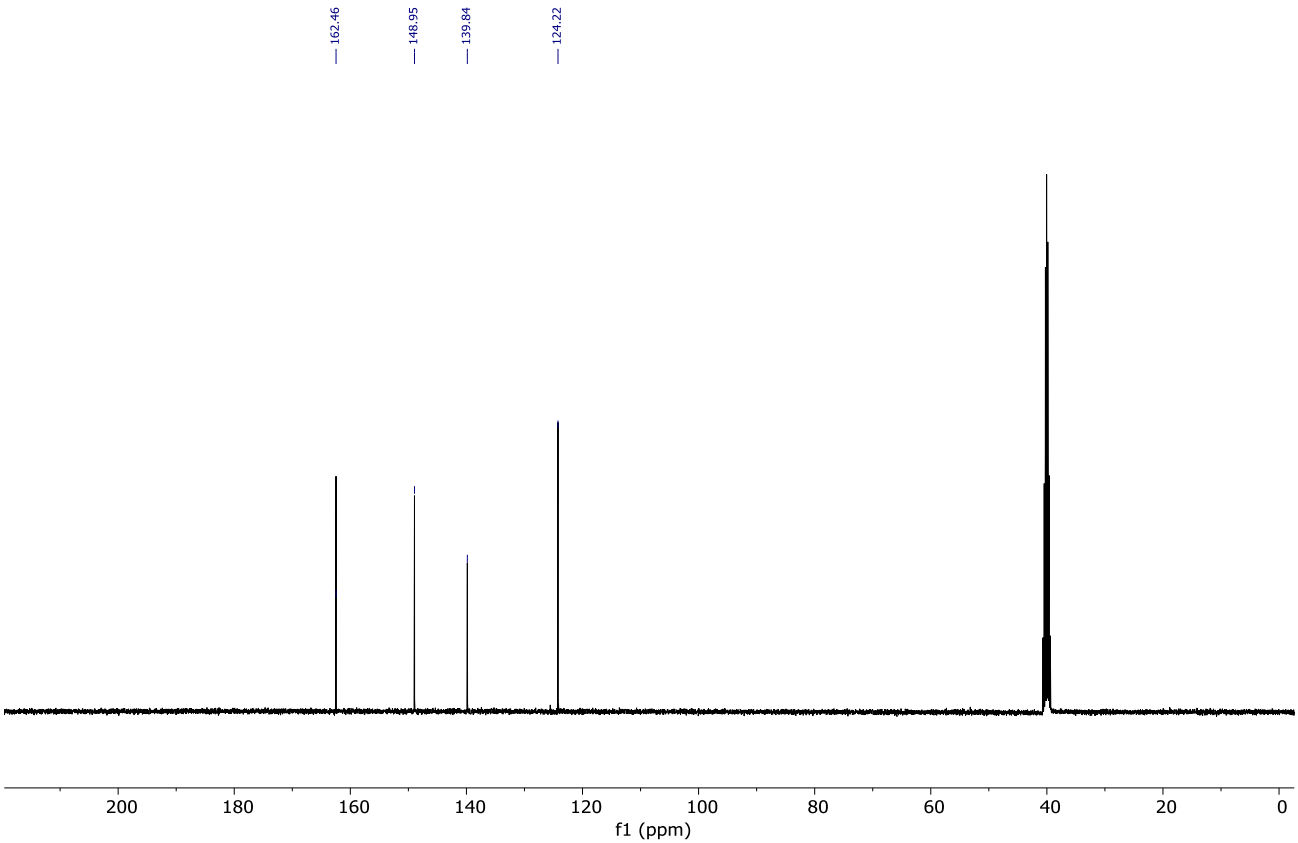


pyridine-2,6-dicarbohydrazide (23)

^{1}H NMR:

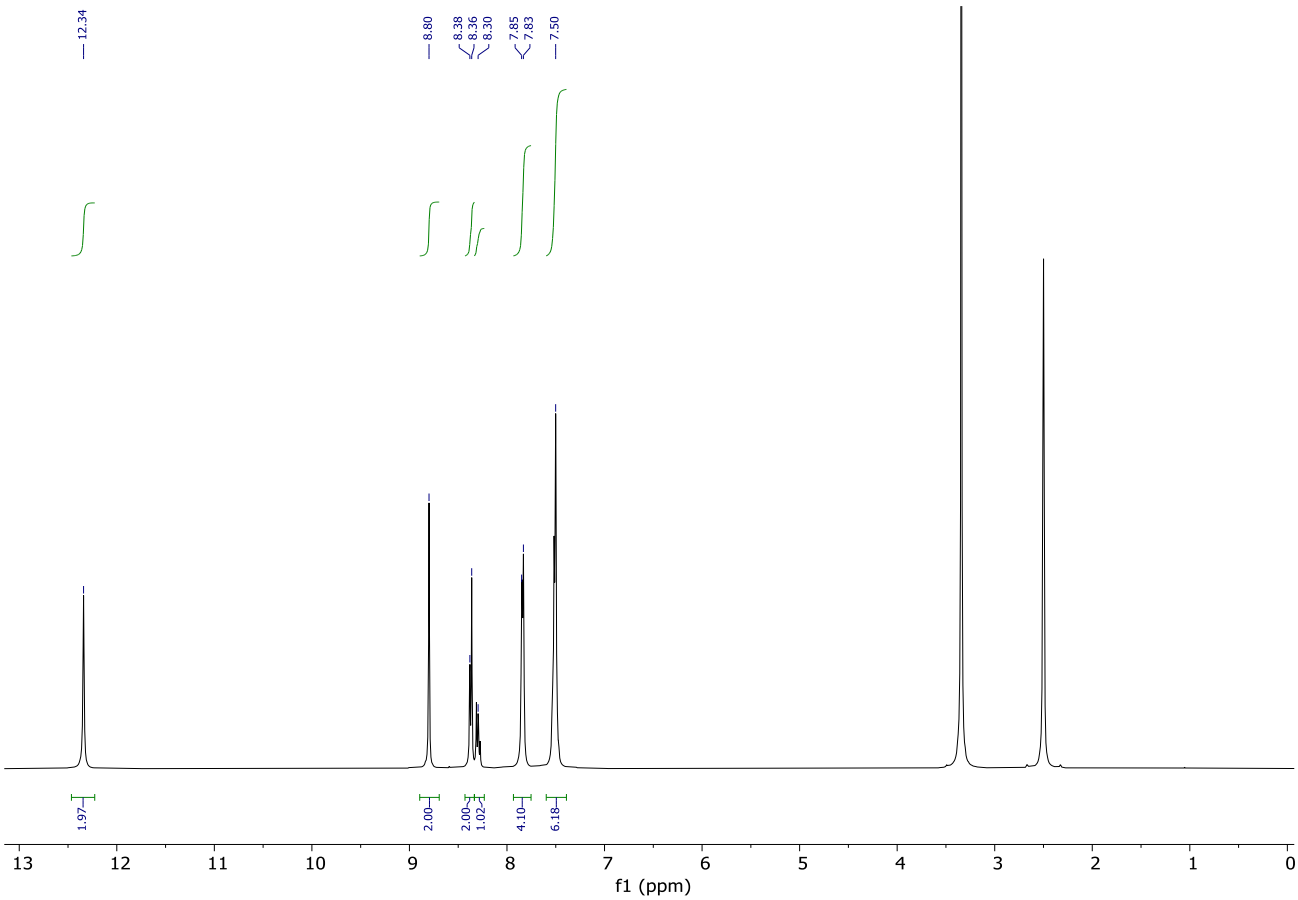


^{13}C NMR:

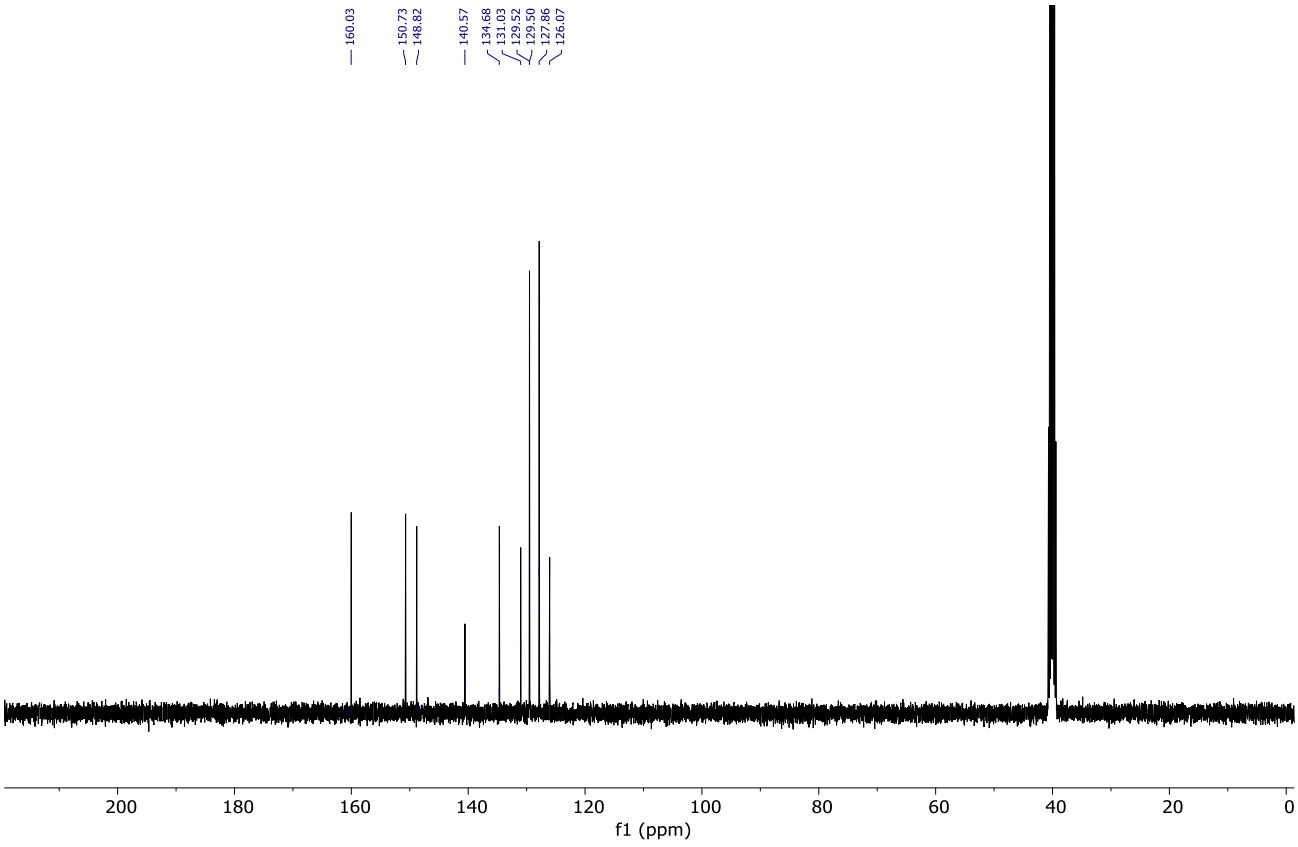


N'2,N'6-di((E)-benzylidene)pyridine-2,6-dicarbohydrazide (21)

^{1}H NMR:

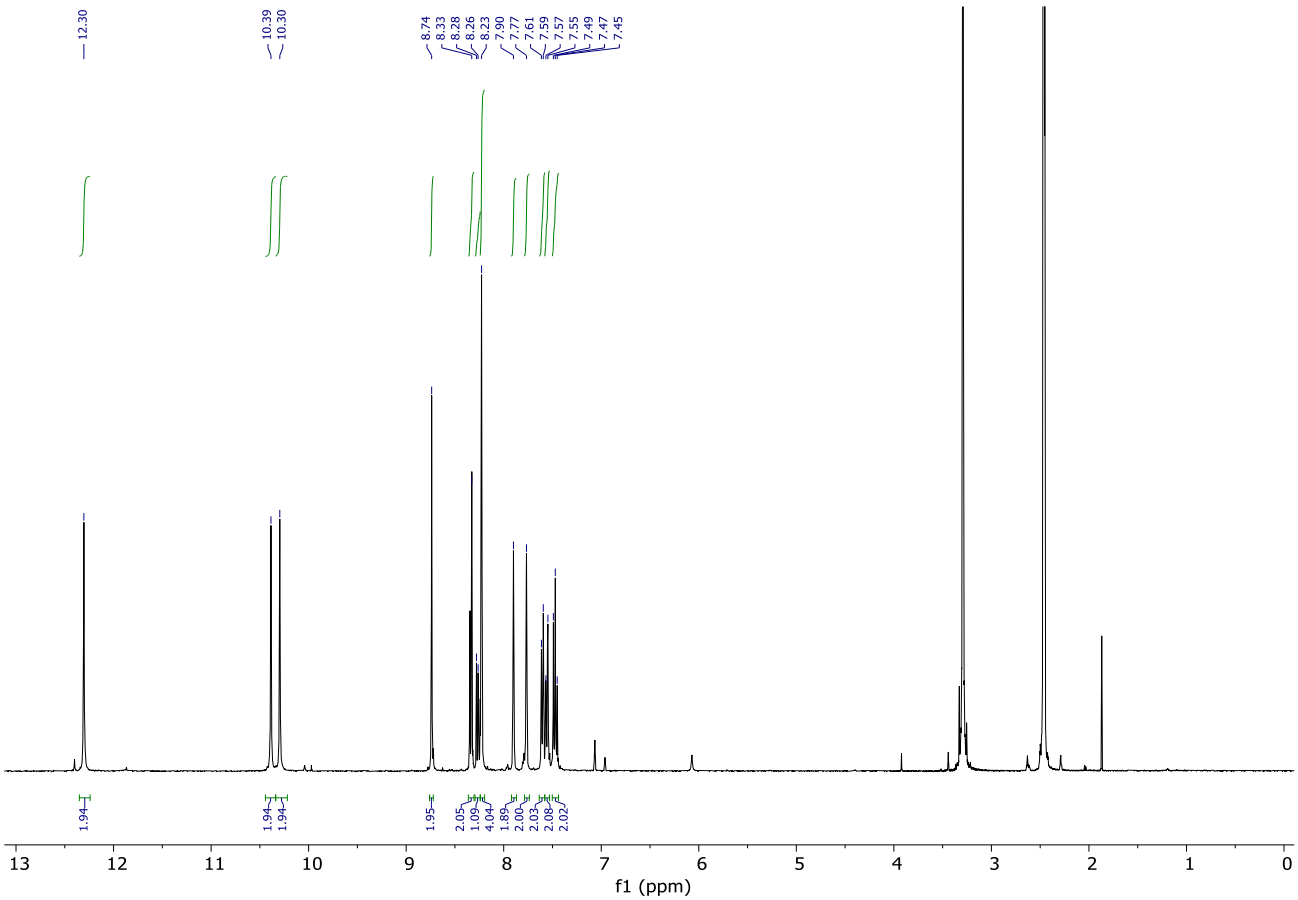


^{13}C NMR:

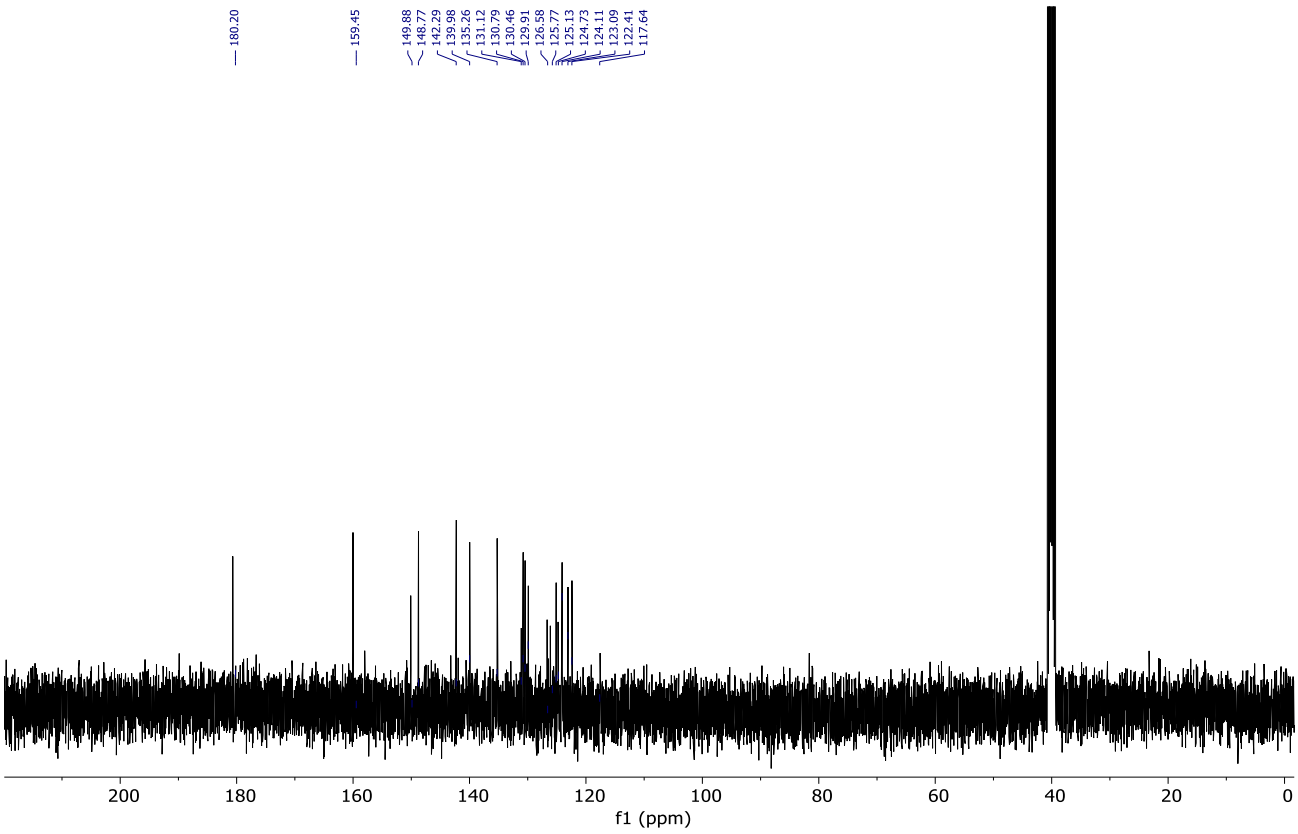


1,1'-(((1*E*,1'*E*)-(2,2'-(pyridine-2,6-dicarbonyl)bis(hydrazin-2-yl-1-ylidene))bis(methanylylidene))bis(3,1-phenylene))bis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea) (22)

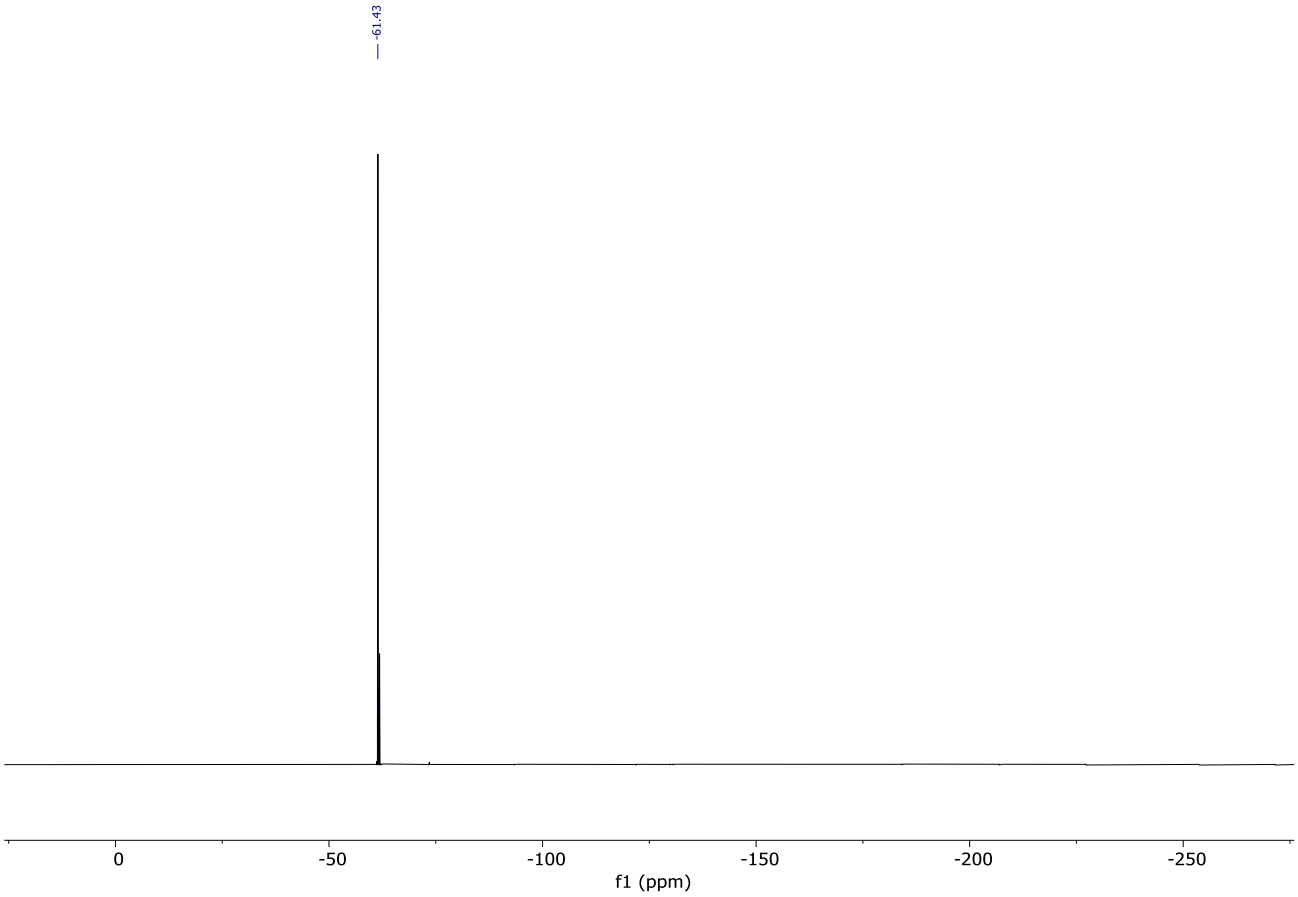
^{1}H NMR:



^{13}C NMR:



^{19}F NMR:



3. Binding Studies

3.1. Experimental procedure

Binding constants were measured by adding increasing amounts of tetrabutylammonium chloride (TBACl) into solutions of the different receptors in DMSO-d6/0.5% H_2O at 298 K. Solutions of receptors **1-7** and **19-22** (2 mM, 1.5 mL) were prepared in DMSO-d6/0.5% H_2O (MilliQ) and stock solutions of 300-400 mM TBACl were prepared by dissolving the salt (dried under high vacuum) into the different receptor solutions. 600 µL of the solutions of pure receptors were transferred into NMR tubes. Aliquots of the TBACl solutions were then added to the NMR tube and ^{1}H NMR spectra were recorded after each addition of guest (see Figures S1 - S25).

Binding affinities of select receptors for 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were measured by adding increasing amounts of POPC into solutions of the different receptors in 75% $CDCl_3$/24.5% DMSO-d6/0.5% H_2O at 298 K. Solutions of receptors **1, 3** and **7** (1 mM, 1.5 mL) were prepared in 75% $CDCl_3$/24.5% DMSO-d6/0.5% H_2O (MilliQ) and stock solutions of 100 mM POPC were prepared by dissolving the solid in the different receptor solutions. 500 µL of the solutions of pure receptors were transferred into NMR tubes. Aliquots of the POPC solutions were then added to the NMR tube and ^{1}H NMR spectra were recorded after each addition of guest (see Figures S26 – S31).

The chemical shifts of the signals most affected by the addition of the guest were followed and the data were fitted to a 1:1 (host:guest) binding models using the Bindfit v0.5 applet (available as freeware from Supramolecular.org). In the cases where the 1:1 models did not afford a good fitting for all the signals, the data were also fitted to 1:2 models. Moreover, the data obtained for compounds **19** and **22** were directly fitted to 1:2 models, because of their rigidity and relative disposition of the different binding groups in the structures. In general, the signals used for the fittings were those assigned to the two thiourea NH and the CH in *ortho* position of the 3,5-bis(trifluoromethyl)phenyl substituent, and in some cases other signals such as the acylhydrazone NH and CH were added to the fit.

3.2. ^{1}H NMR Titrations

The titration of thiourea **1** with TBACl was performed in triplicate to calculate an experimental error of the 14 % (K = 31 ± 4 M^{-1} with the error expressed as the standard deviation) (Figures S1 and S2). Since the main purpose of these titrations is to show that all the monothioureas have similar binding affinities (*i.e.,* K_{11} of the same order of magnitude), an error of 10-15 % was assumed for the titrations with compounds **2**-**6**.

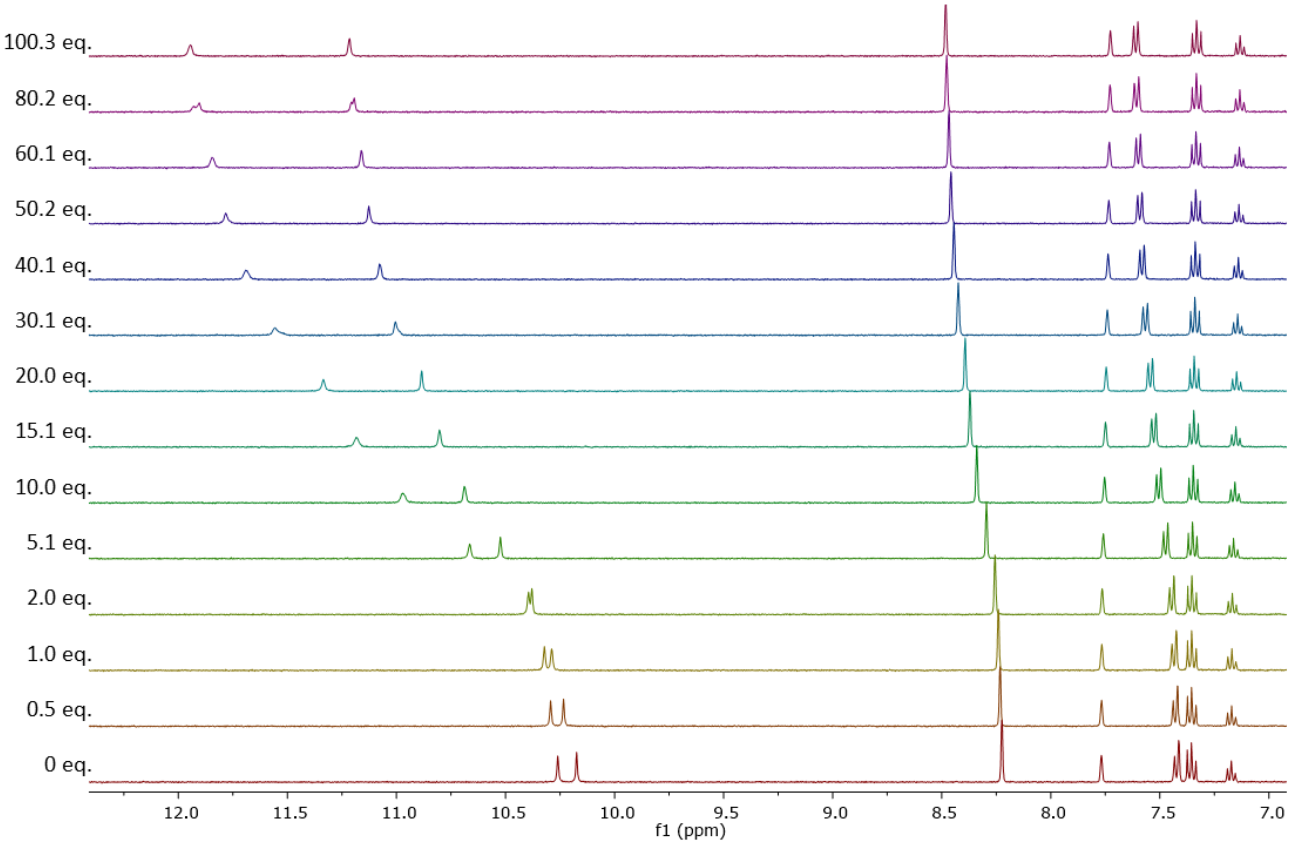
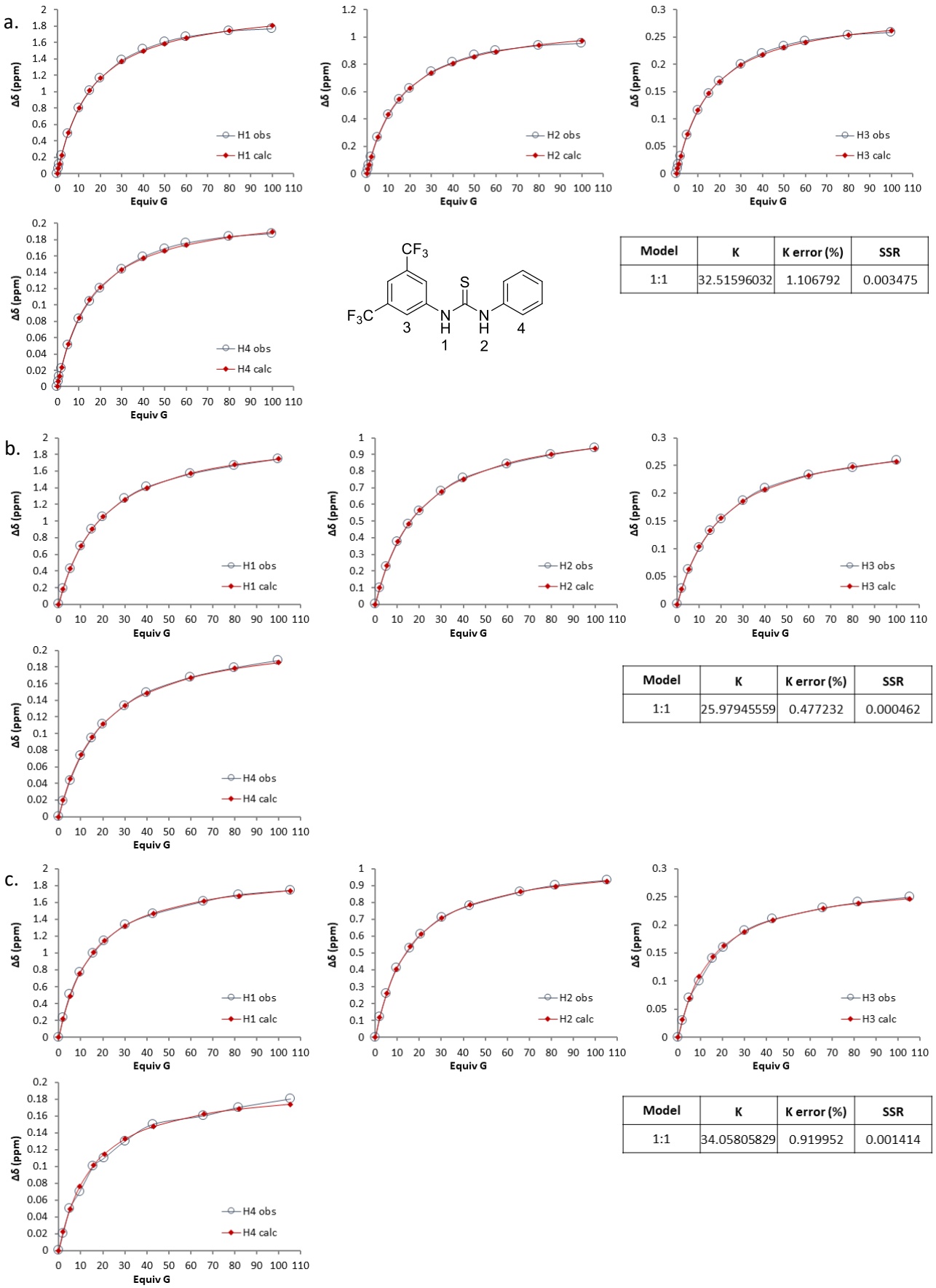


Figure S1. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **1** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **1** is shown.



Model	K	K error (%)	SSR
1:1	32.51596032	1.106792	0.003475

Model	K	K error (%)	SSR
1:1	25.97945559	0.477232	0.000462

Model	K	K error (%)	SSR
1:1	34.05805829	0.919952	0.001414

Figure S2. Observed (obs) and calculated (calc) binding curves for the titrations of **1** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3 and H4 correspond to signals starting at 10.2, 10.3, 8.2 and 7.4 ppm, respectively. Fittings from three independent titrations are shown (a-c).

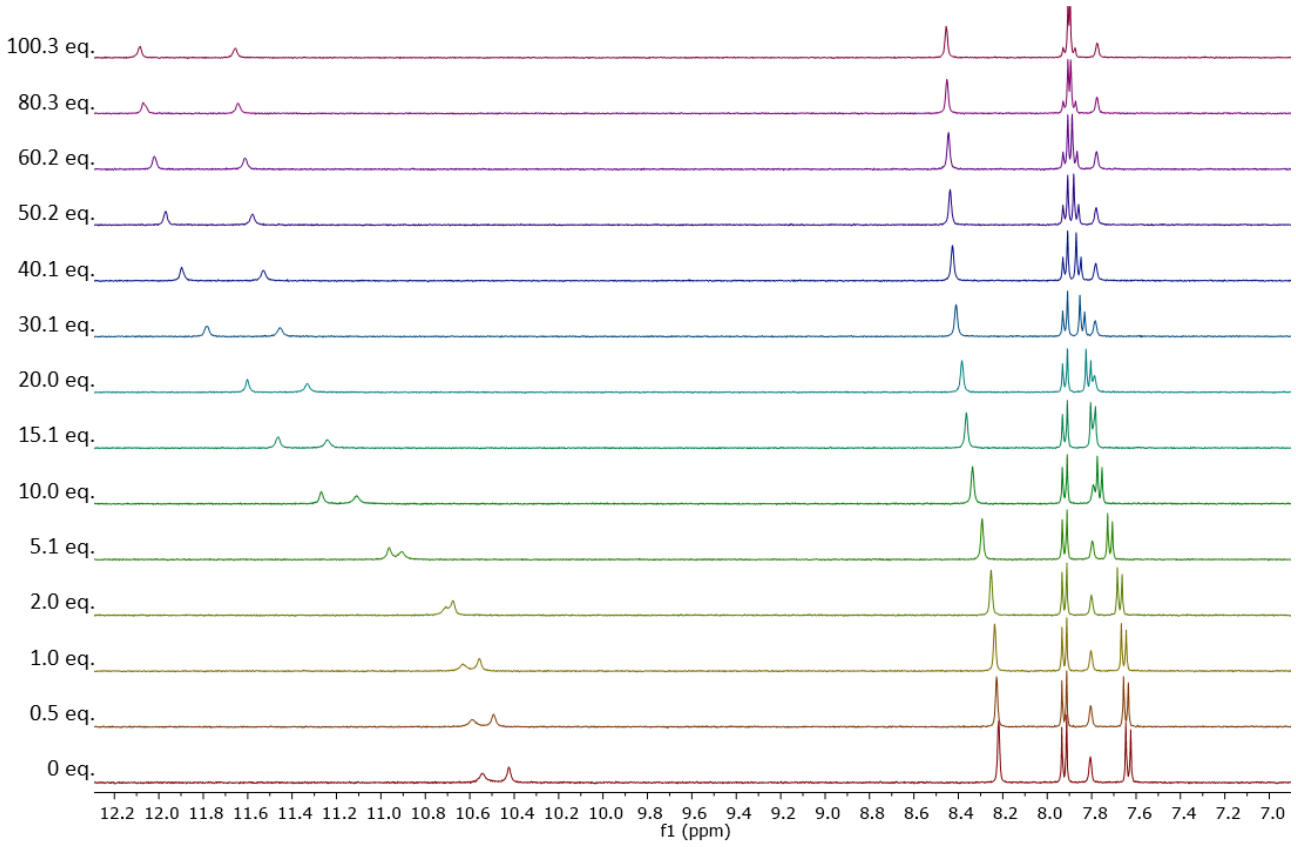
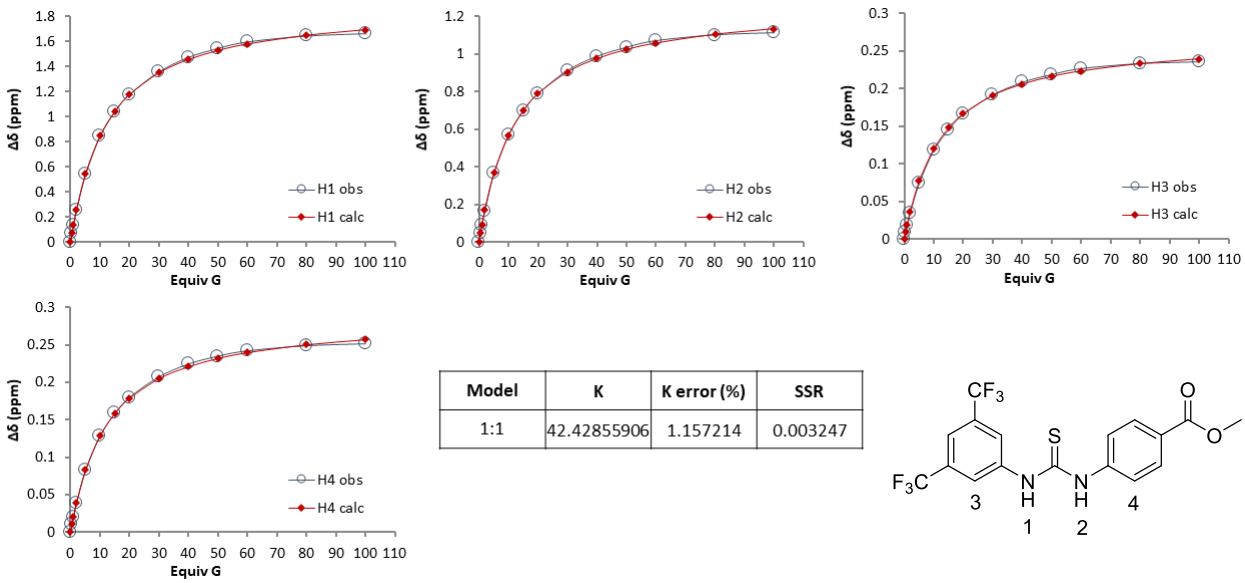


Figure S3. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **2** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **2** is shown.



Model	K	K error (%)	SSR
1:1	42.42855906	1.157214	0.003247

Figure S4. Observed (obs) and calculated (calc) binding curves for the titration of **2** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3 and H4 correspond to signals starting at 10.4, 10.5, 8.2 and 7.6 ppm, respectively.

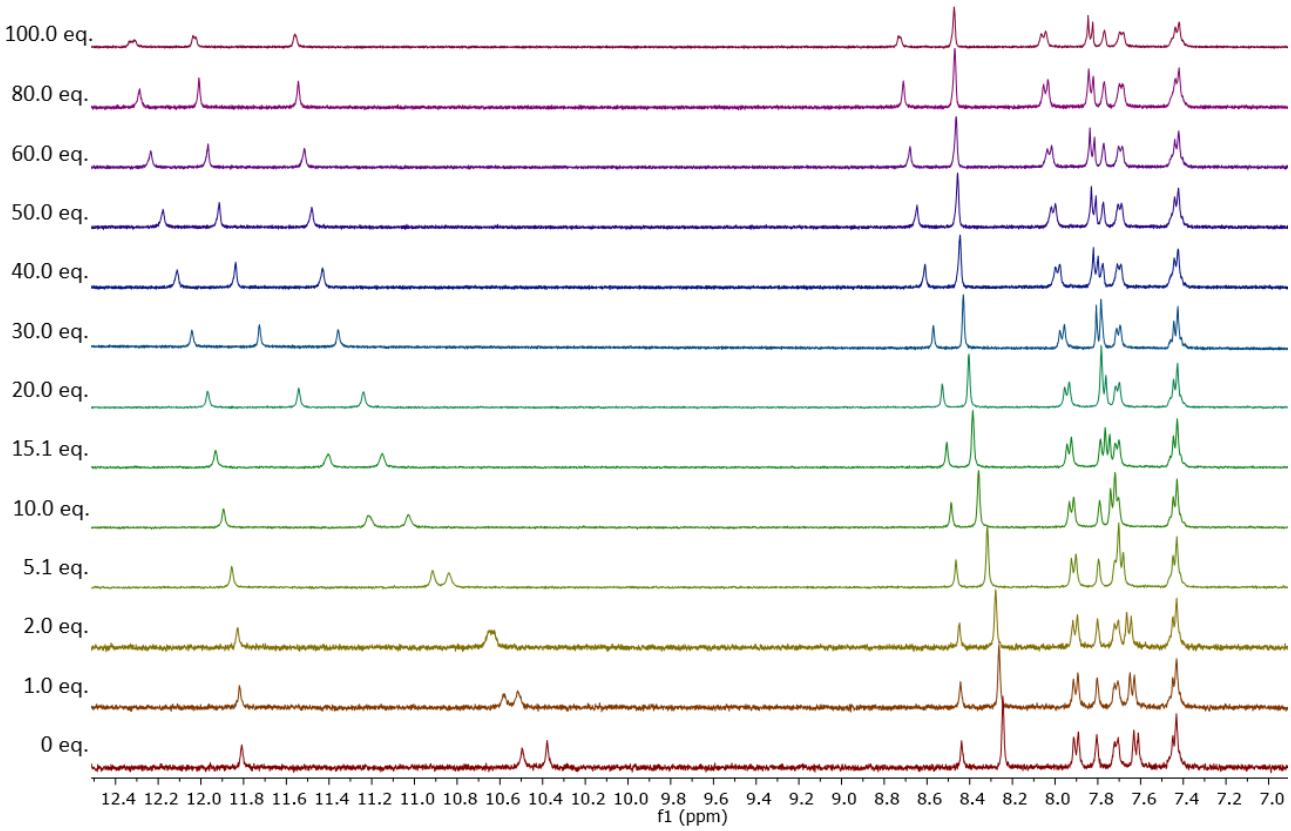
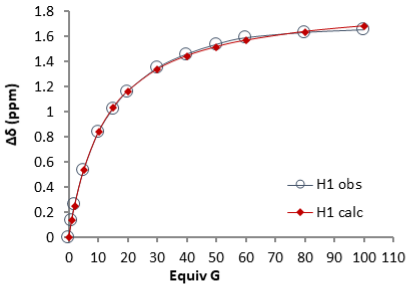
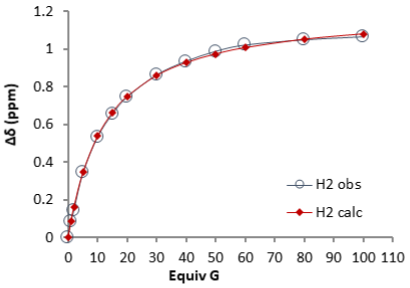
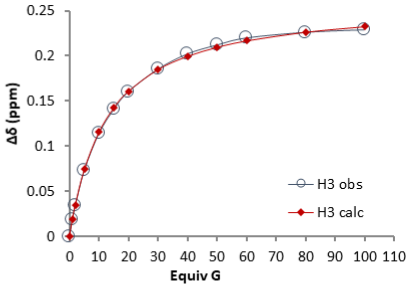


Figure S5. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **3** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **3** is shown.

a.

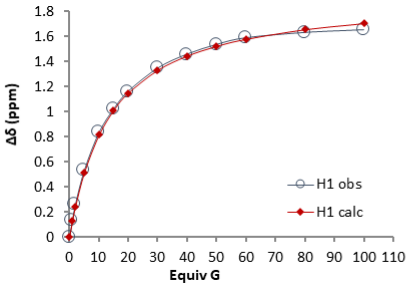


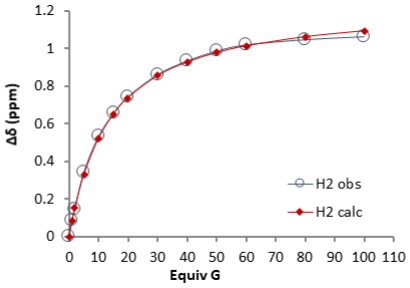


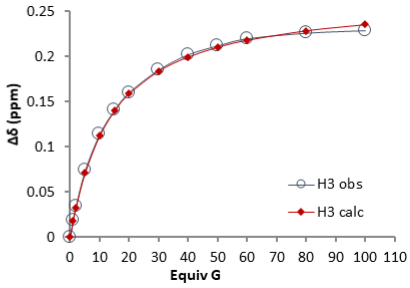


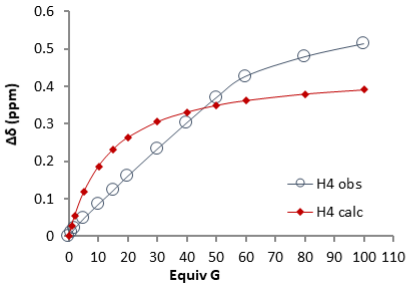
Model	K	K error (%)	SSR
1:1	41.74395272	1.481316	0.003373

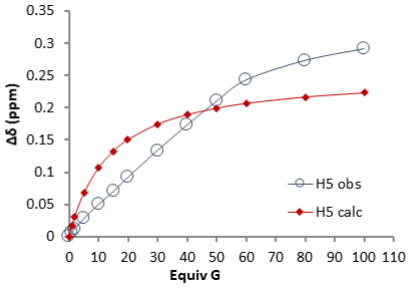
b.



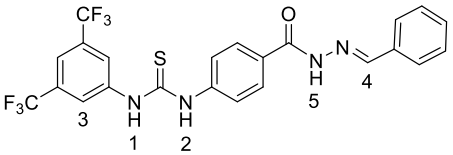




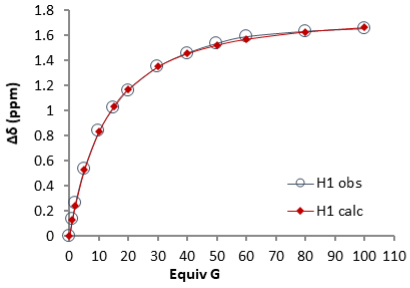


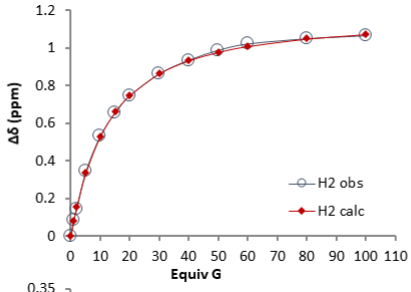


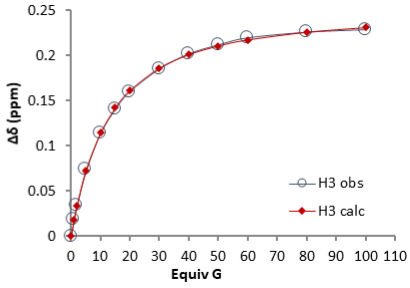
Model	K	K error (%)	SSR
1:1	37.9165476	5.909666	0.107036

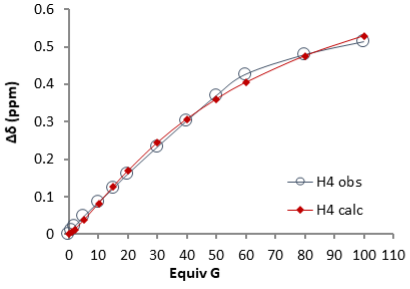


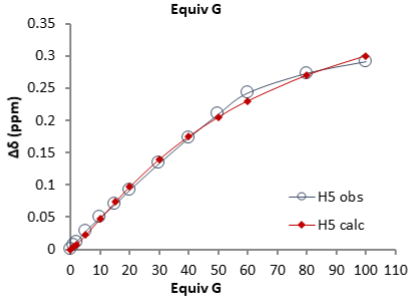
c.











Model	K_{11}	K_{12}
1:2	43.68788512	7.099549

K_{11} error (%)	K_{12} error (%)	SSR
1.691262	3.018188	0.003584

Figure S6. Observed (obs) and calculated (calc) binding curves for the titration of **3** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3, H4 and H5 correspond to signals starting at 10.4, 10.5, 8.2, 11.8 and 8.4 ppm, respectively. Fittings of 3 signals to 1:1 model (a), 5 signals to 1:1 model (b) and 5 signals to 1:2 model (c).

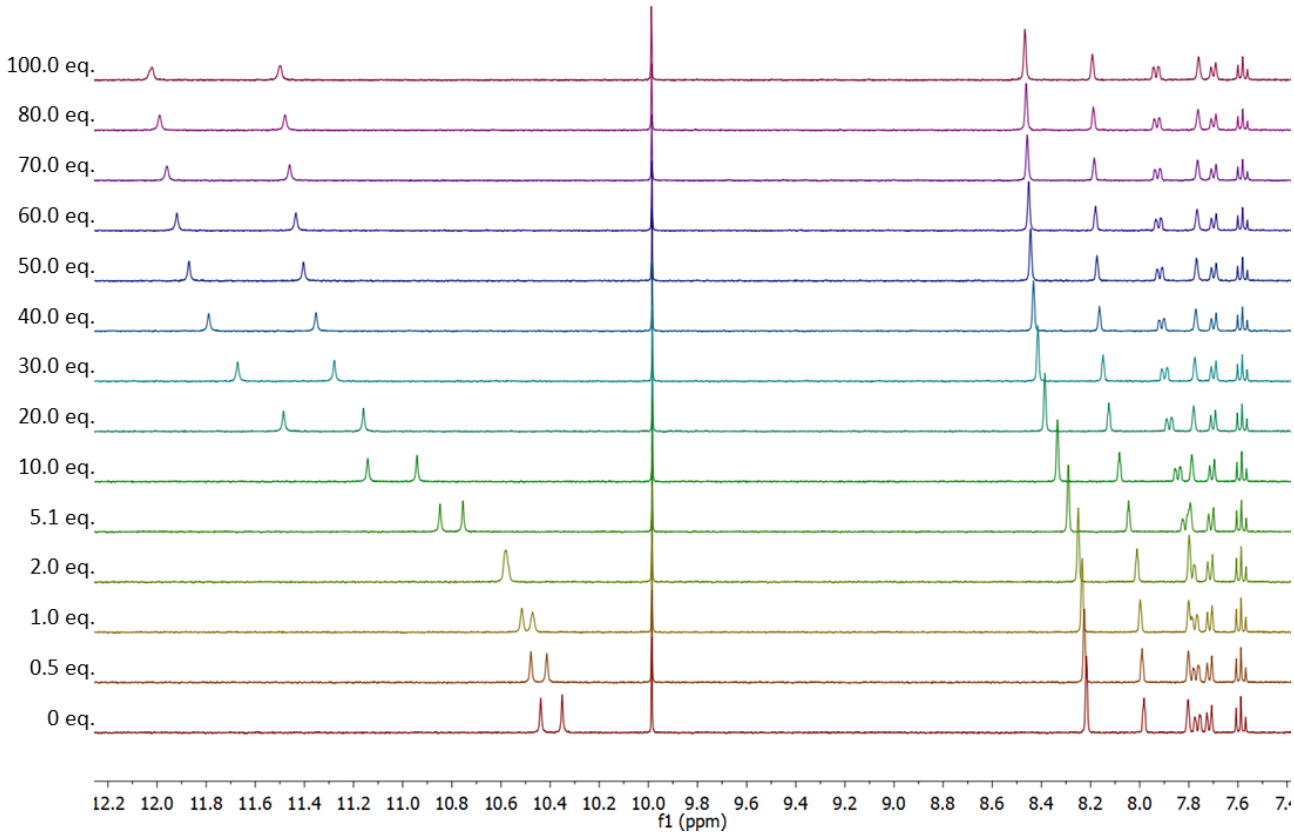


Figure S7. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **4** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **4** is shown.

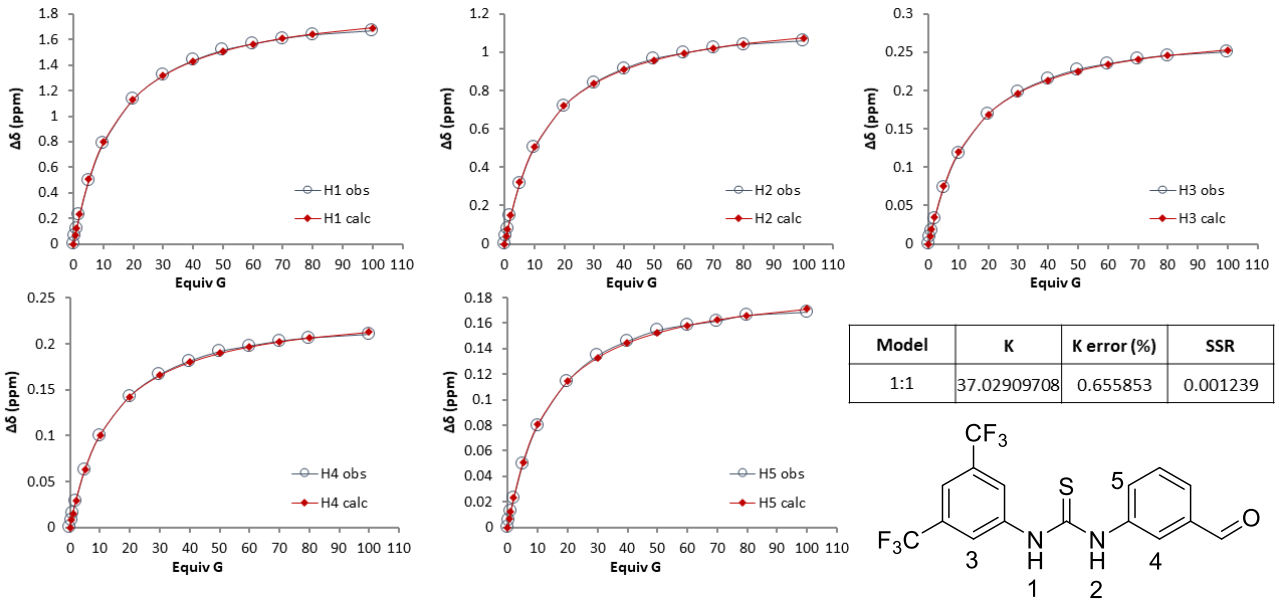
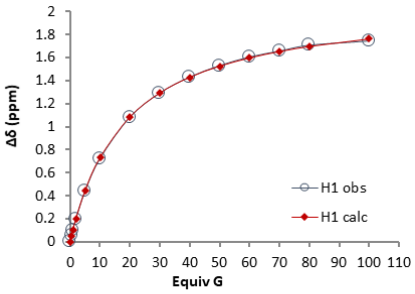
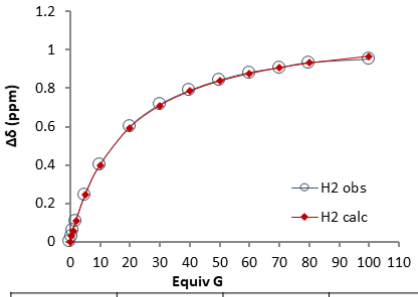
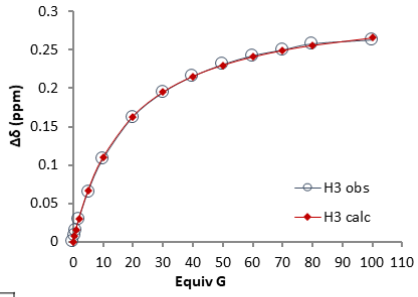


Figure S8. Observed (obs) and calculated (calc) binding curves for the titration of **4** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3, H4 and H5 correspond to signals starting at 10.35, 10.44, 8.22, 7.98 and 7.76, respectively.

a.

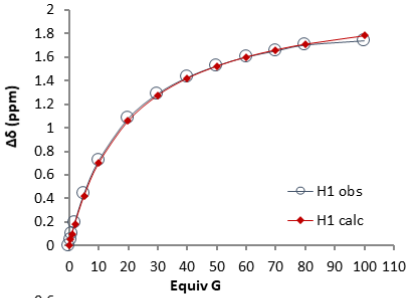


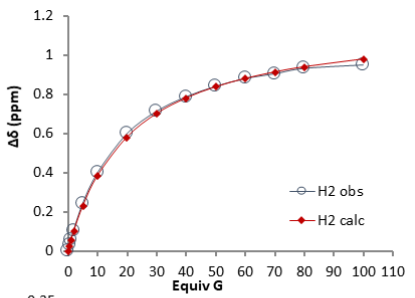


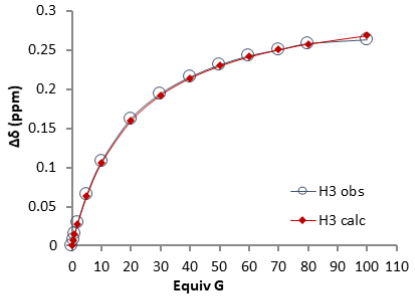


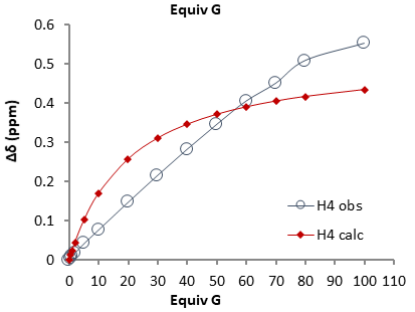
Model	K	K error (%)	SSR
1:1	27.91384399	0.720432	0.001024

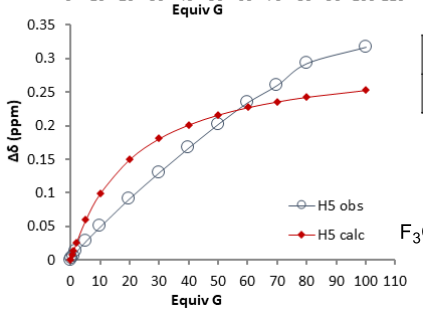
b.



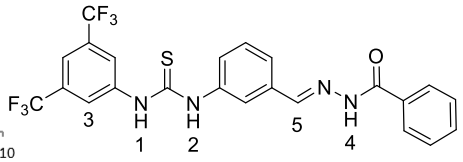




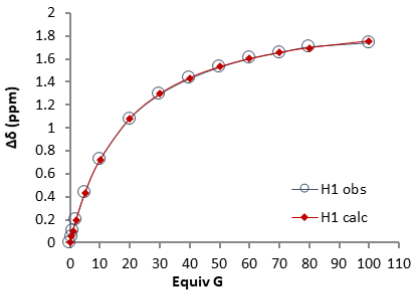


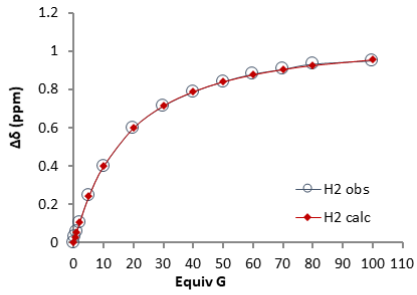


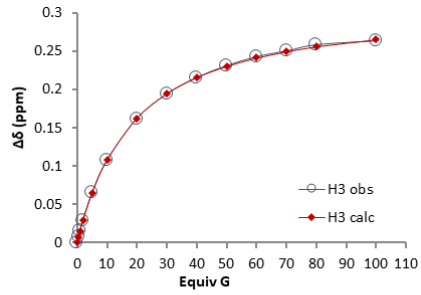
Model	K	K error (%)	SSR
1:1	25.06881382	4.665115	0.087862

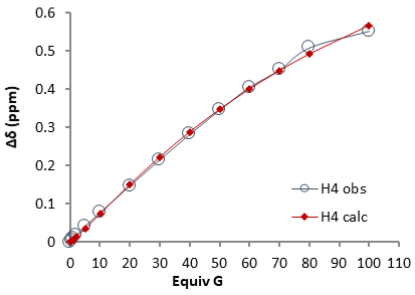


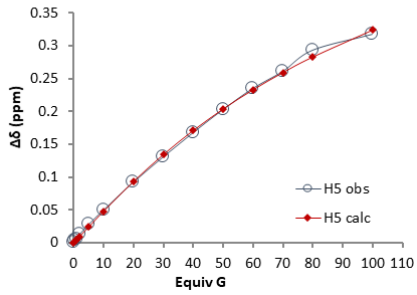
c.











Model	K_{11}	K_{12}
1:2	29.79484845	3.72182

K_{11} error (%)	K_{12} error (%)	SSR
0.906475	1.515109	0.001433

Figure S9. Observed (obs) and calculated (calc) binding curves for the titration of **5a** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3, H4 and H5 correspond to signals starting at 10.27, 10.7, 8.23, 11.85 and 8.43, respectively. Fittings of 3 signals to 1:1 model (a), 5 signals to 1:1 model (b) and 5 signals to 1:2 model (c).

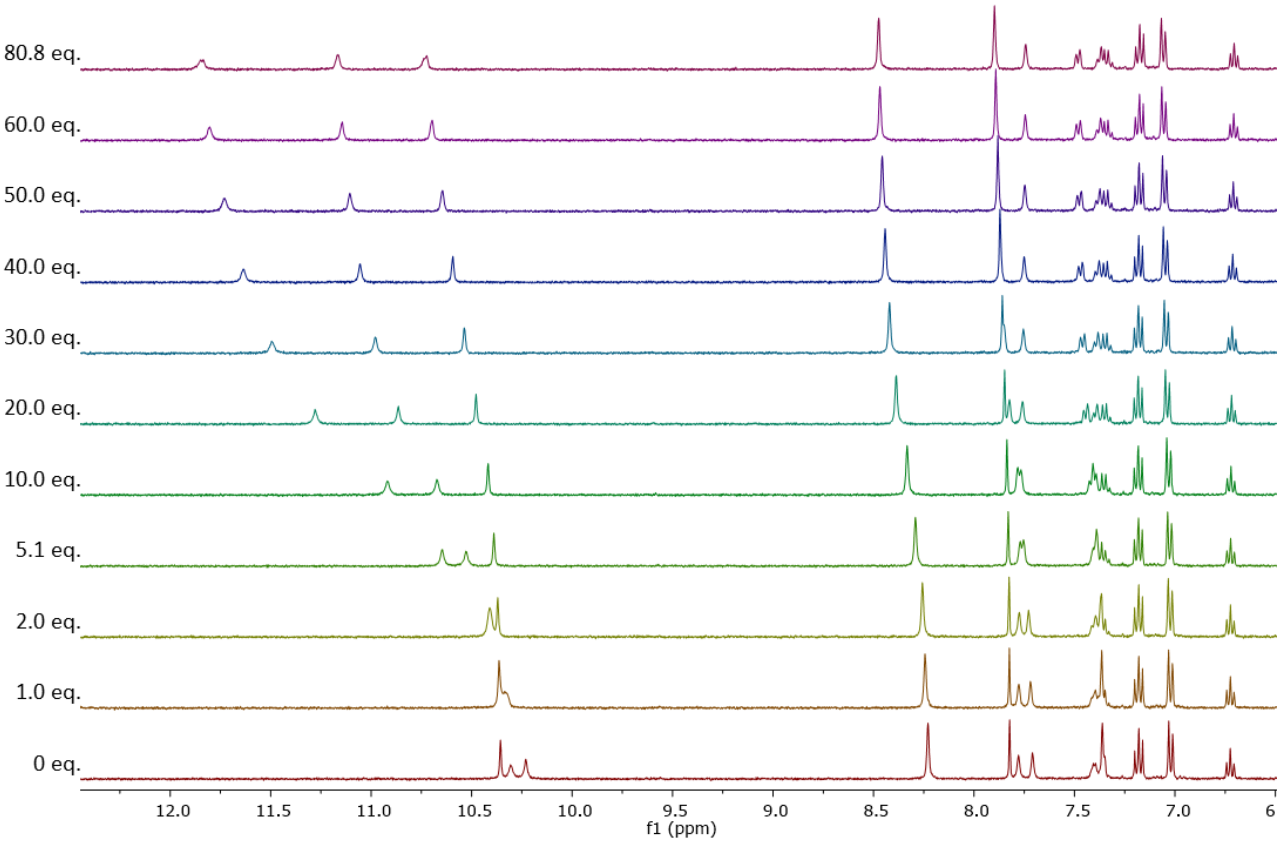
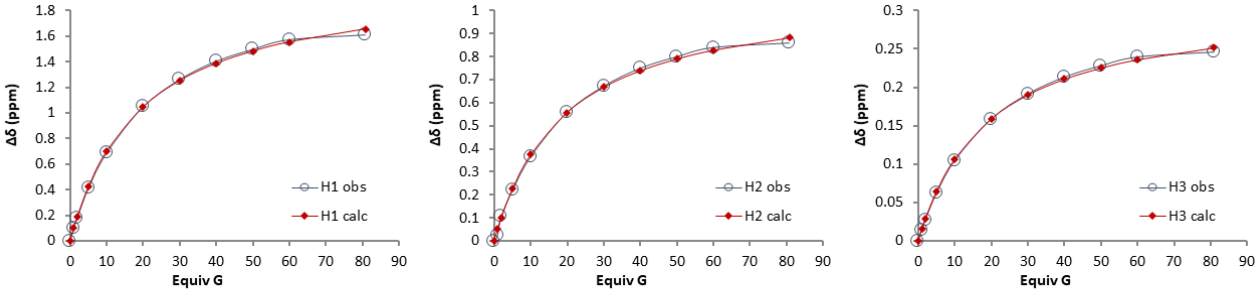


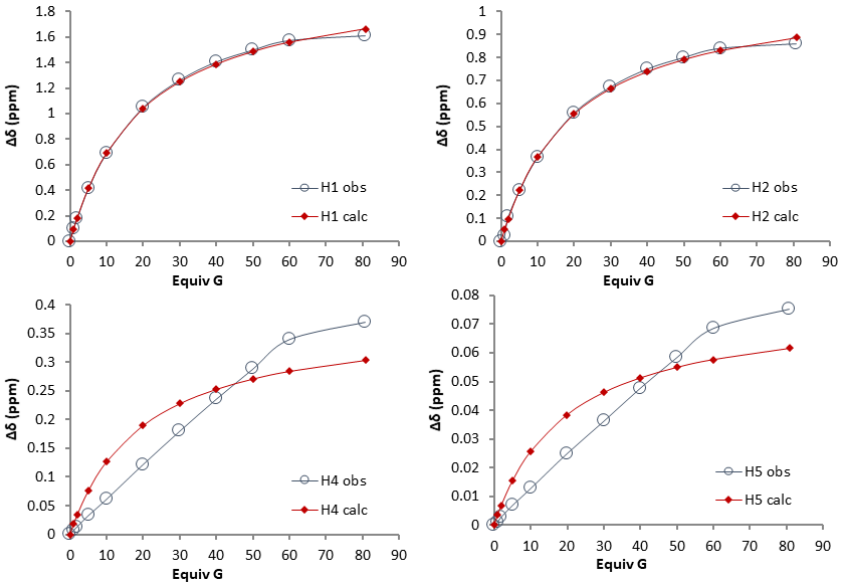
Figure S10. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **5b** (2 mM) with TBACl (0 - 160 mM). The number of equivalents of TBACl relative to **5b** is shown.

a.

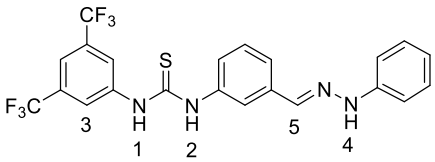


Model	K	K error (%)	SSR
1:1	27.26066941	2.07167	0.005281

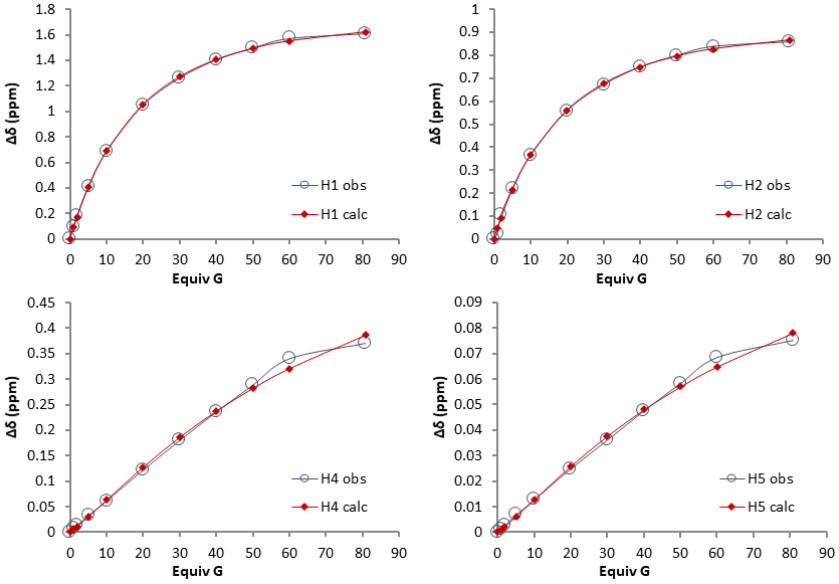
b.



Model	K	K error (%)	SSR
1:1	26.08275148	3.493021	0.028453



c.



Model	K_{11}	K_{12}
1:2	24.11673448	5.41338

K_{11} error (%)	K_{12} error (%)	SSR
1.426752	5.067326	0.002702

Figure S11. Observed (obs) and calculated (calc) binding curves for the titration of **5b** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3, H4 and H5 correspond to signals starting at 10.23, 10.31, 8.23, 10.36, 7.82, respectively. Fittings of 3 signals to 1:1 model (a), 5 signals to 1:1 model (b) and 5 signals to 1:2 model (c).

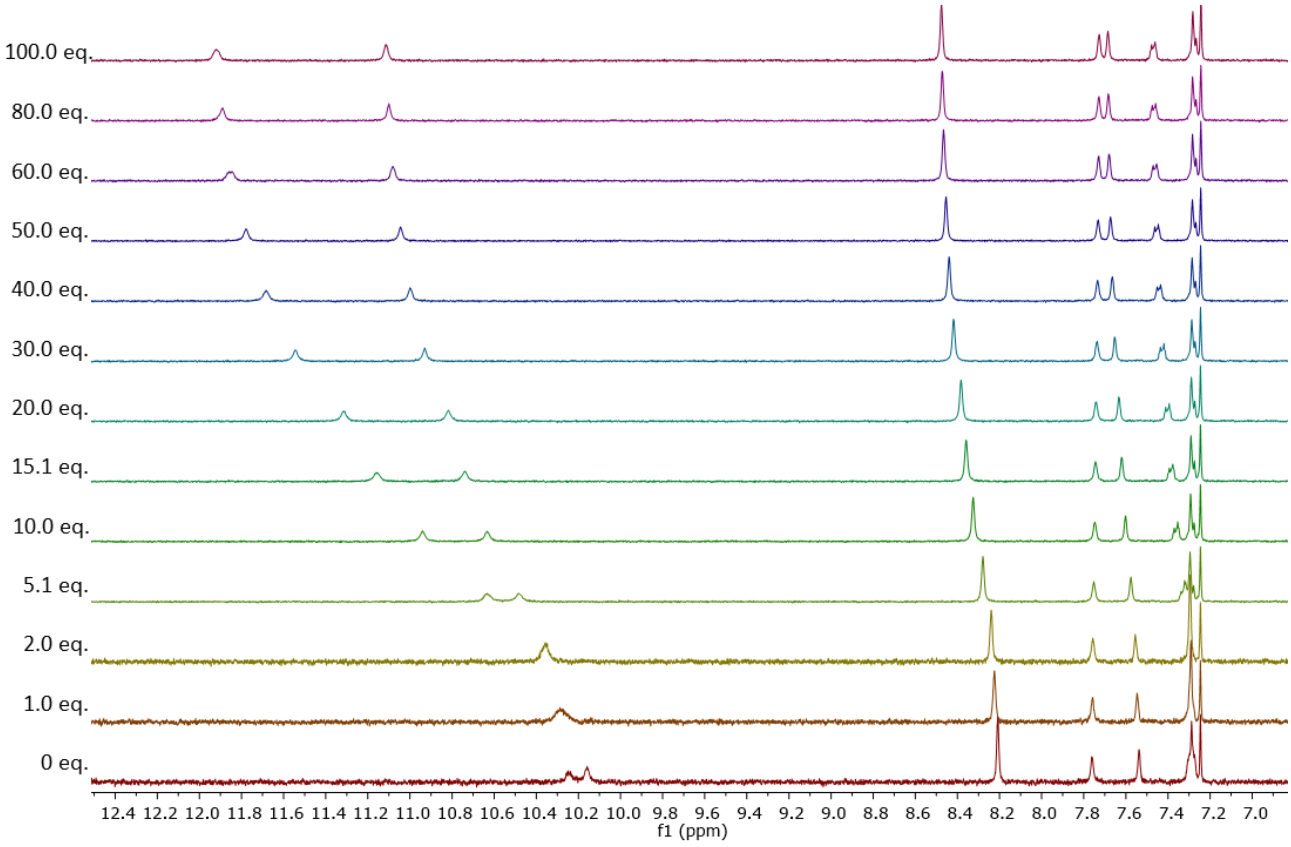
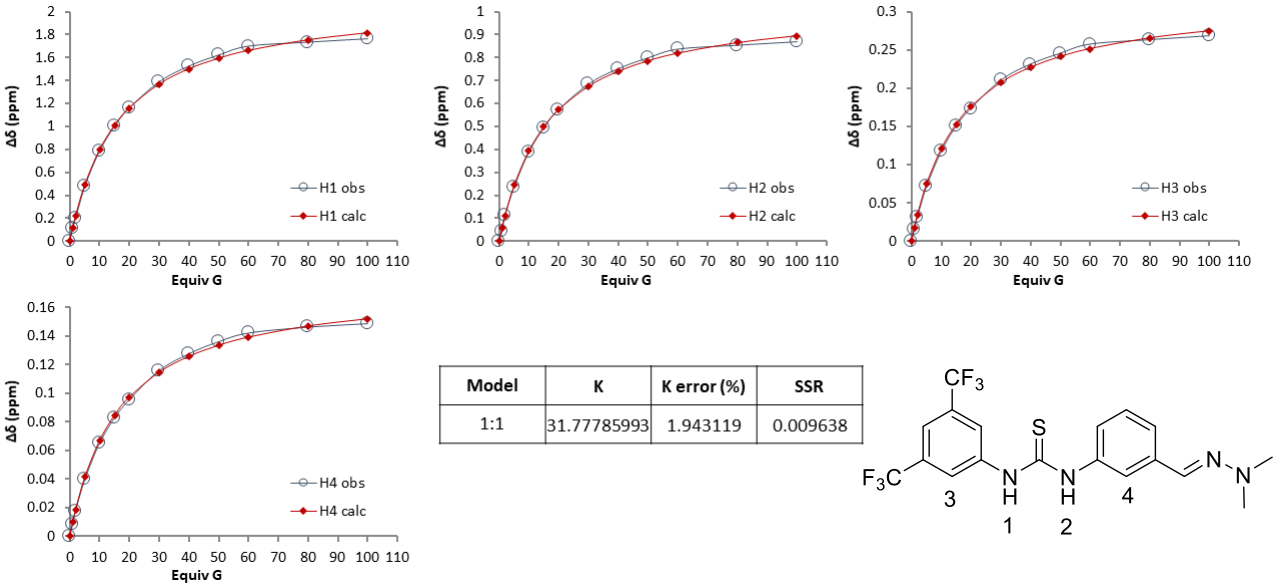


Figure S12. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **5c** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **5c** is shown.



Model	K	K error (%)	SSR
1:1	31.77785993	1.943119	0.009638

Figure S13. Observed (obs) and calculated (calc) binding curves for the titration of **5c** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3 and H4 correspond to signals starting at 10.1, 10.2, 8.2 and 7.5 ppm, respectively.

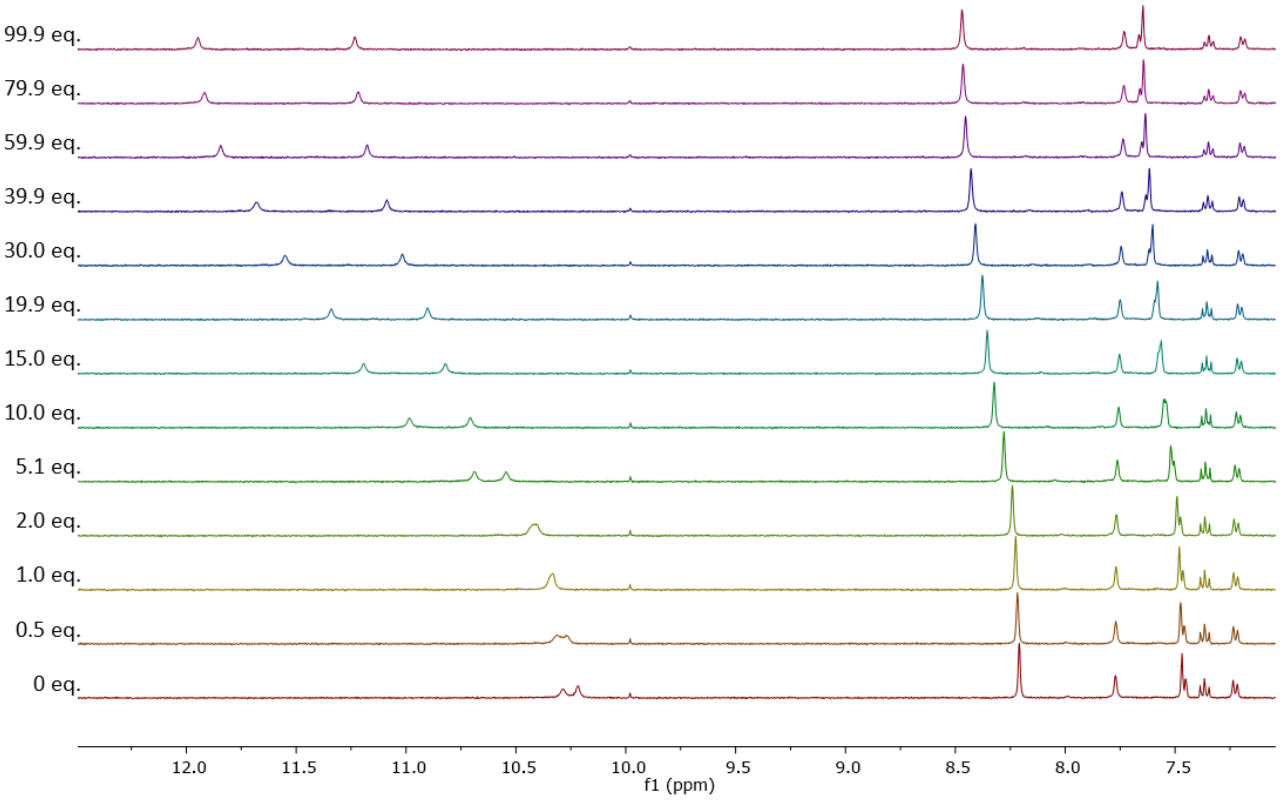
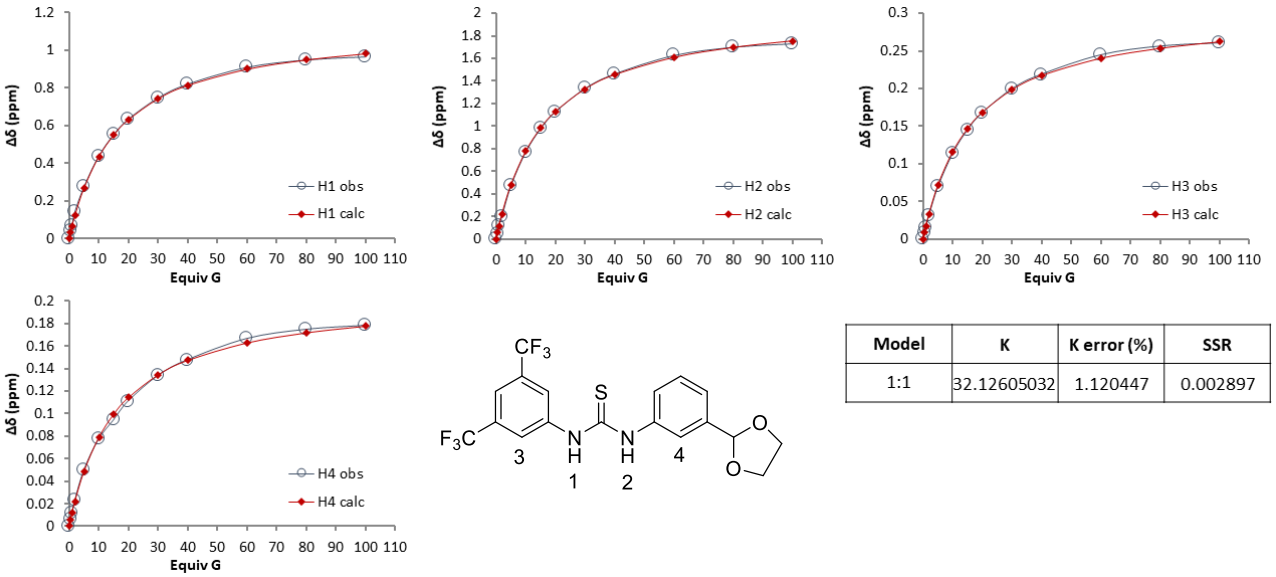


Figure S14. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **6** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **6** is shown.



Model	K	K error (%)	SSR
1:1	32.12605032	1.120447	0.002897

Figure S15. Observed (obs) and calculated (calc) binding curves for the titration of **6** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3 and H4 correspond to signals starting at 10.27, 10.22, 8.221 and 7.47, respectively.

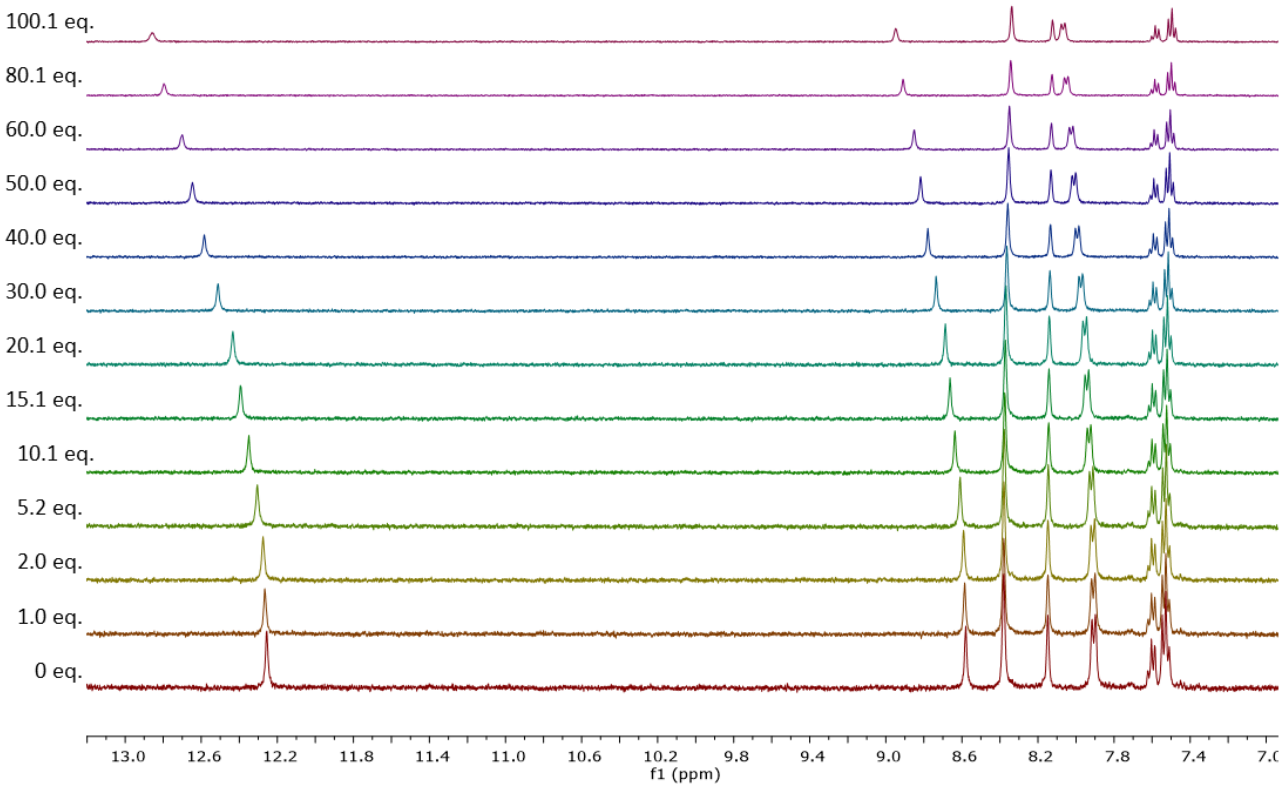
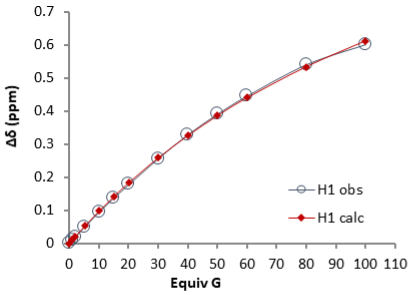
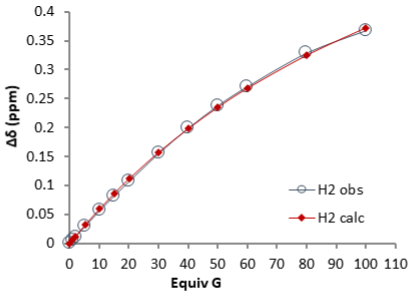
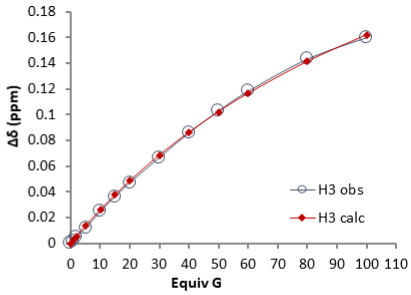


Figure S16. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **7** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **7** is shown.







Model	K	K error (%)	SSR
1:1	3.568864345	0.813921	0.000456

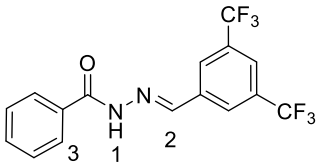


Figure S17. Observed (obs) and calculated (calc) binding curves for the titration of **7** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2 and H3 correspond to signals starting at 12.3, 8.6 and 7.9 ppm, respectively.

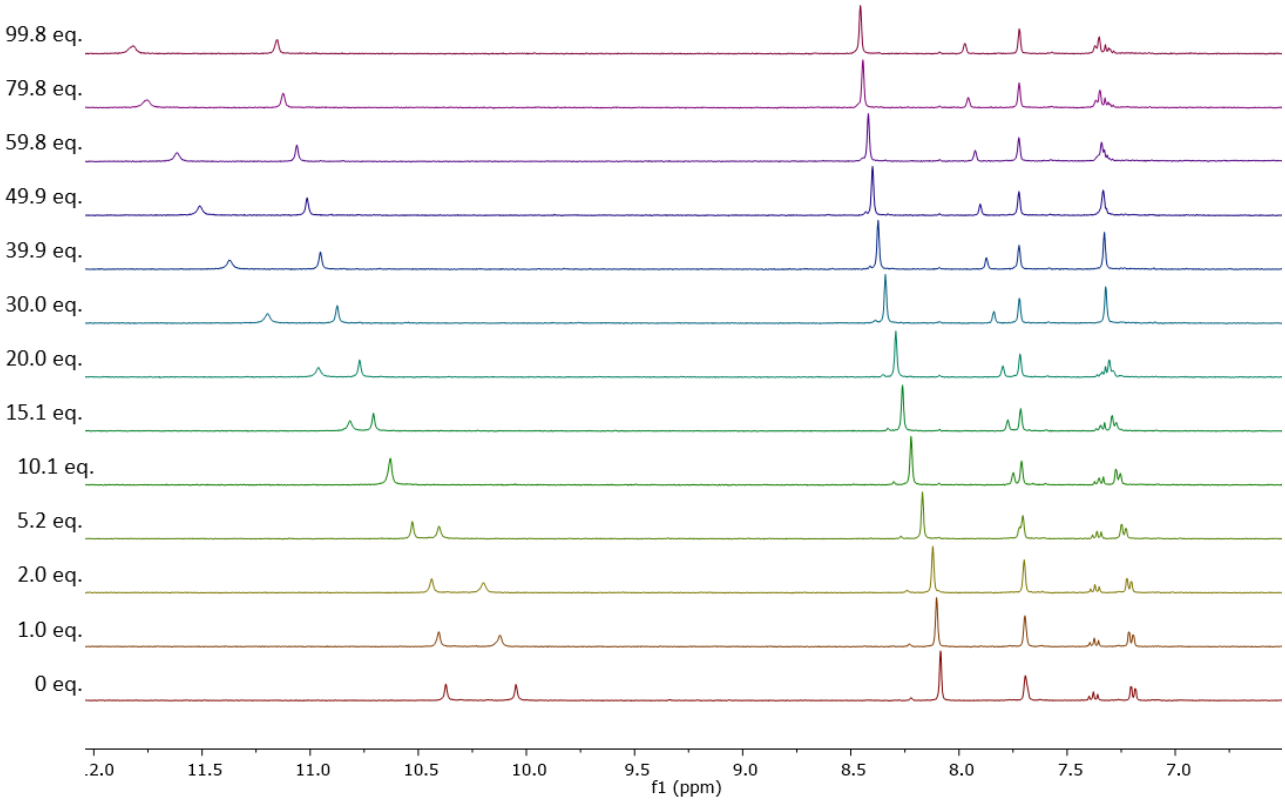
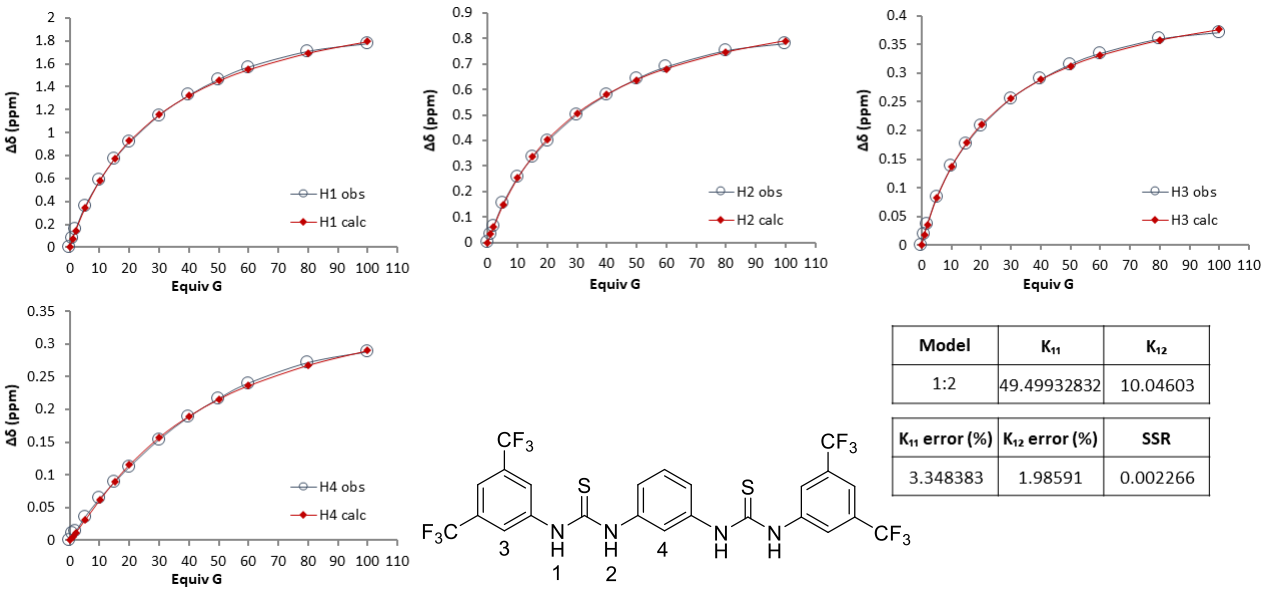


Figure S18. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of bis-thiourea **19** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **19** is shown.



Model	K_{11}	K_{12}
1:2	49.49932832	10.04603

K_{11} error (%)	K_{12} error (%)	SSR
3.348383	1.98591	0.002266

Figure S19. Observed (obs) and calculated (calc) binding curves for the titration of **19** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3 and H4 correspond to signals starting at 10.0, 10.3, 8.0 and 7.7 ppm, respectively.

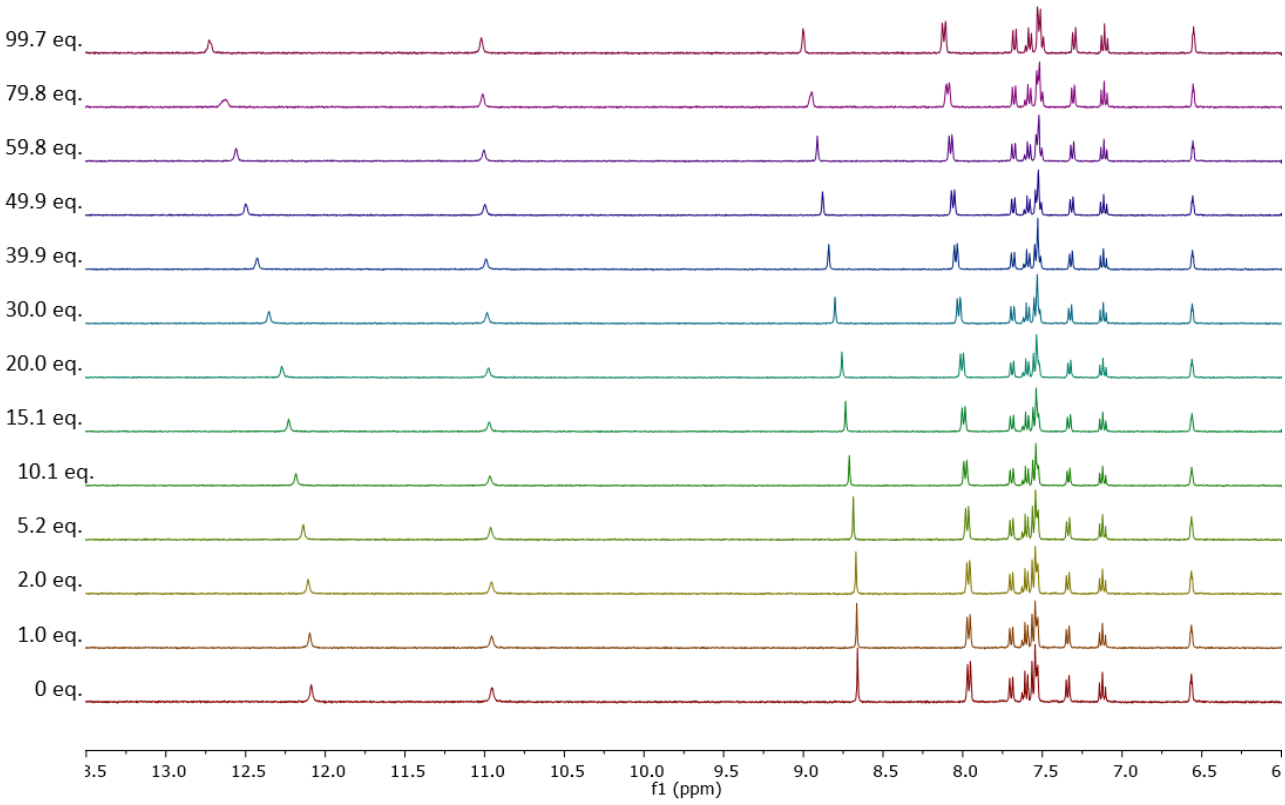
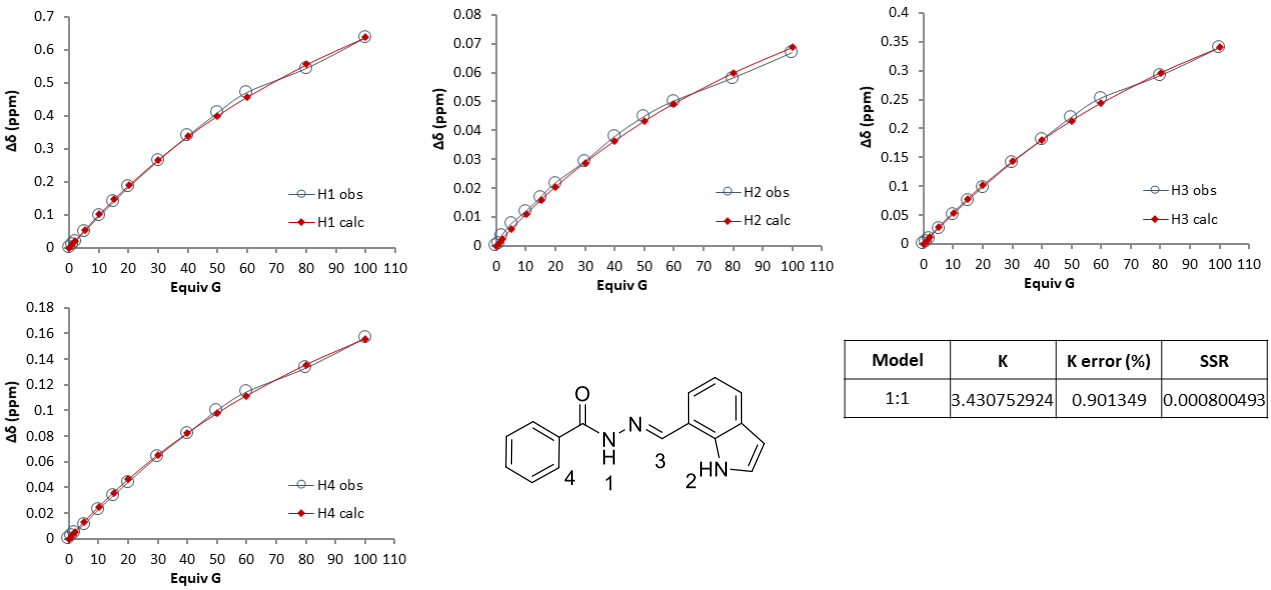


Figure S20. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of thiourea **20** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **20** is shown.



Model	K	K error (%)	SSR
1:1	3.430752924	0.901349	0.000800493

Figure S21. Observed (obs) and calculated (calc) binding curves for the titration of **20** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3 and H4 correspond to signals starting at 12.1, 11.0, 8.7, and 8.0 ppm, respectively.

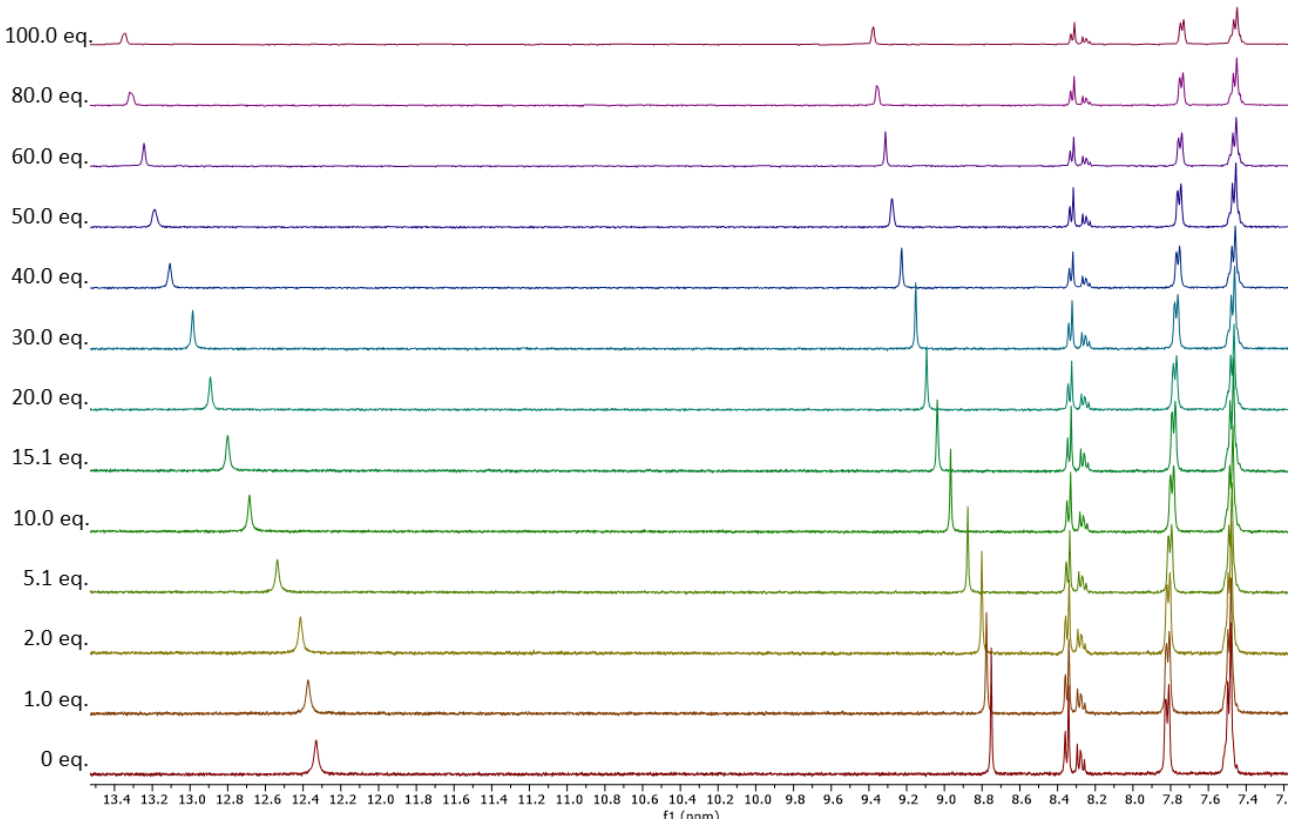
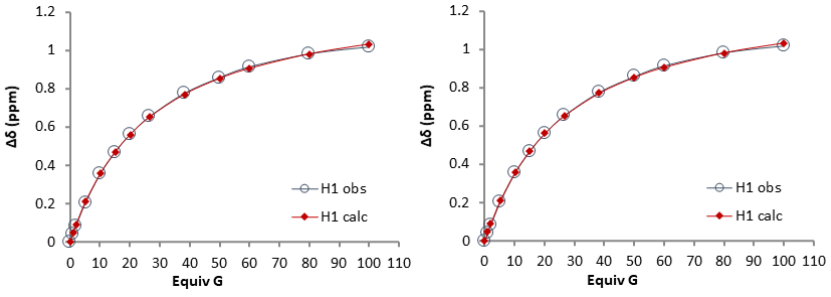


Figure S22. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of bis-acyl hydrazone **21** (2 mM) with TBACl (0 - 160 mM). The number of equivalents of TBACl relative to **21** is shown.



Model	K	K error (%)	SSR
1:1	19.20611346	1.067266	0.00061

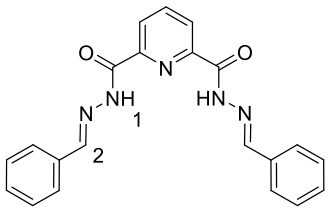


Figure S23. Observed (obs) and calculated (calc) binding curves for the titration of **21** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1 and H2 correspond to signals starting at 12.3 and 8.7, respectively.

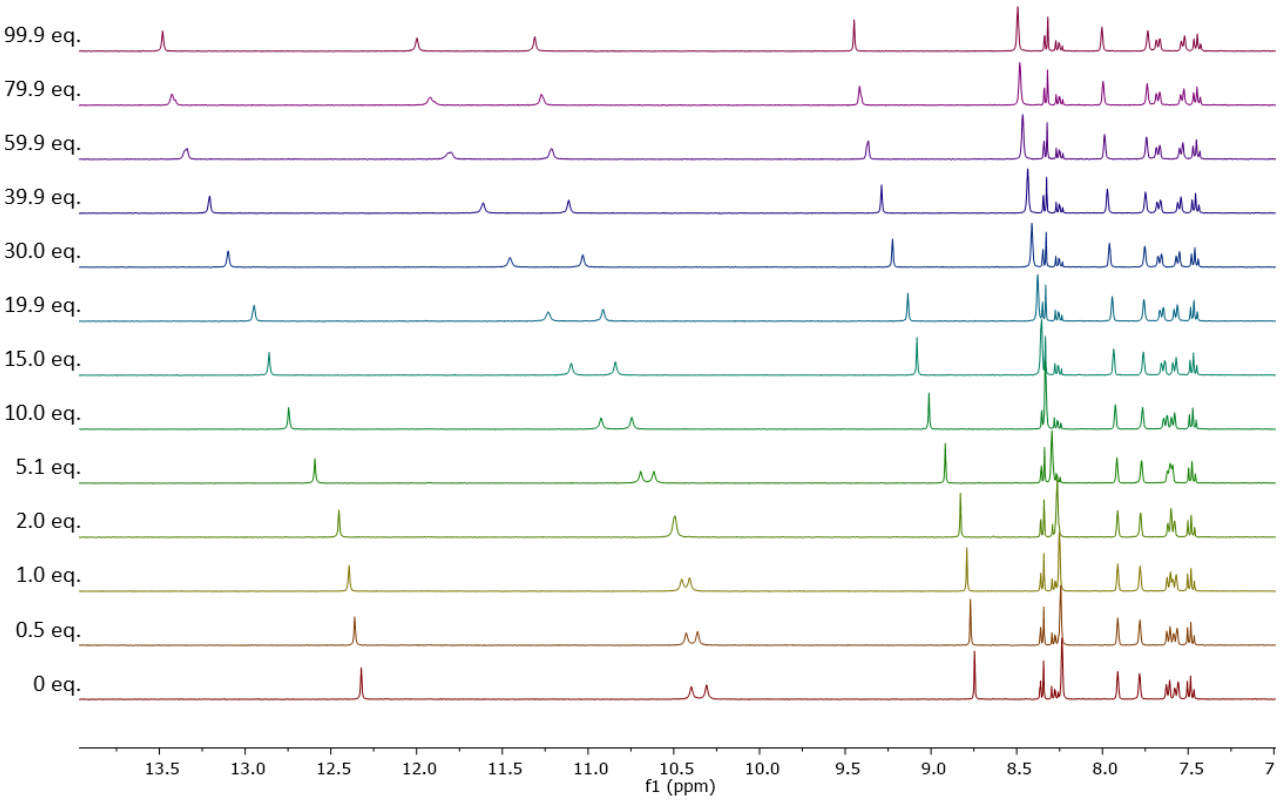


Figure S24. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, DMSO-d6/0.5% H_2O) from the titration of bis-thiourea **22** (2 mM) with TBACl (0 - 200 mM). The number of equivalents of TBACl relative to **22** is shown.

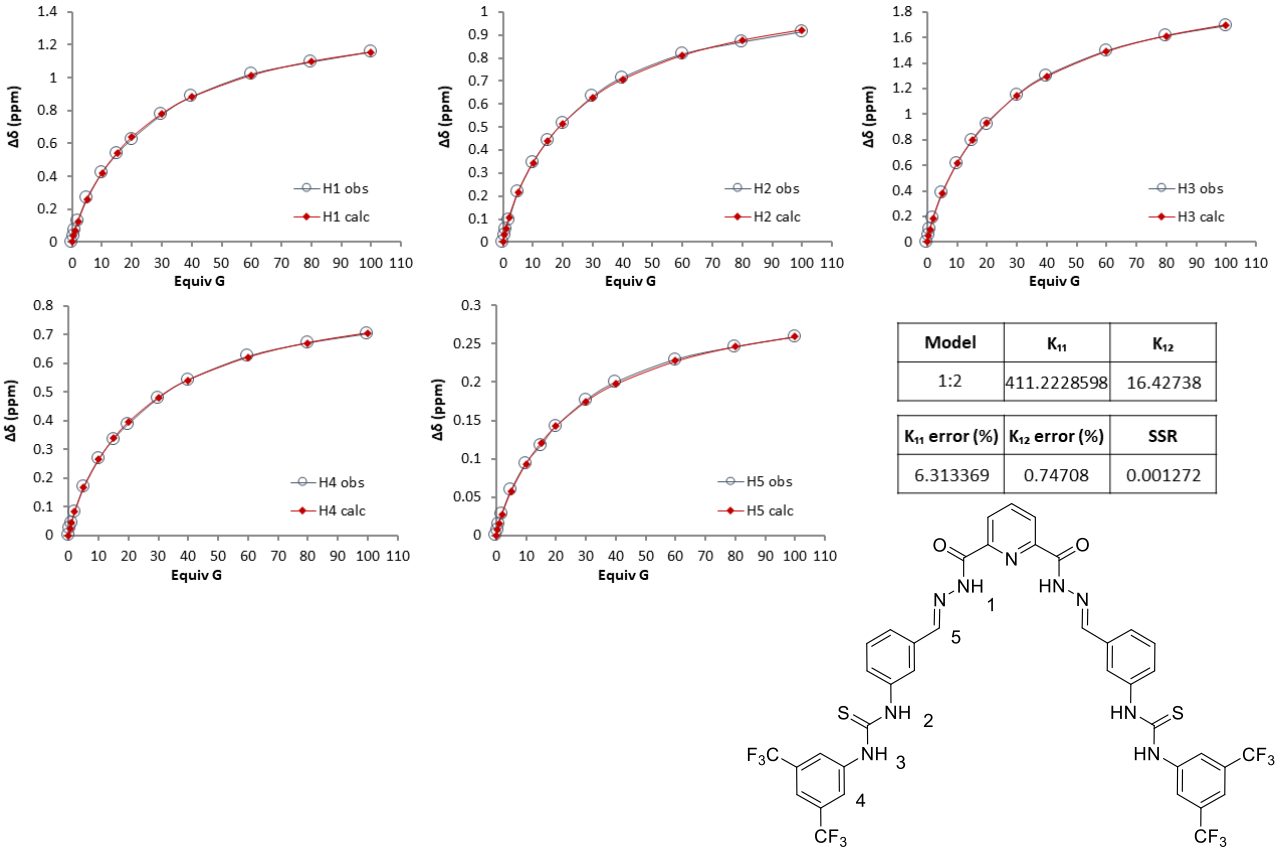


Figure S25. Observed (obs) and calculated (calc) binding curves for the titration of **22** (2 mM) with TBACl at 298K in DMSO-d6/0.5% H_2O. H1, H2, H3, H4 and H5 correspond to signals starting at 12.32, 10.40, 10.31, 8.74 and 8.24, respectively.

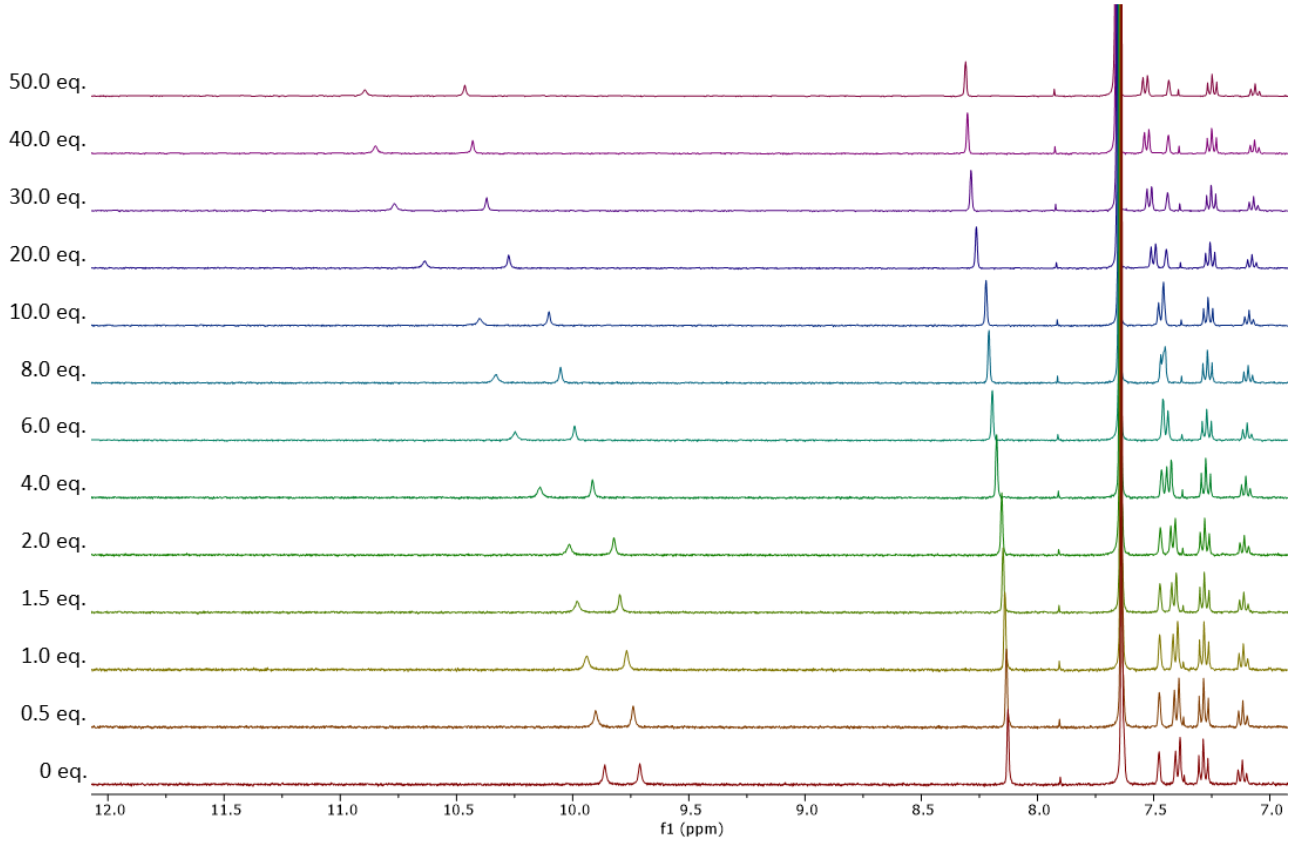
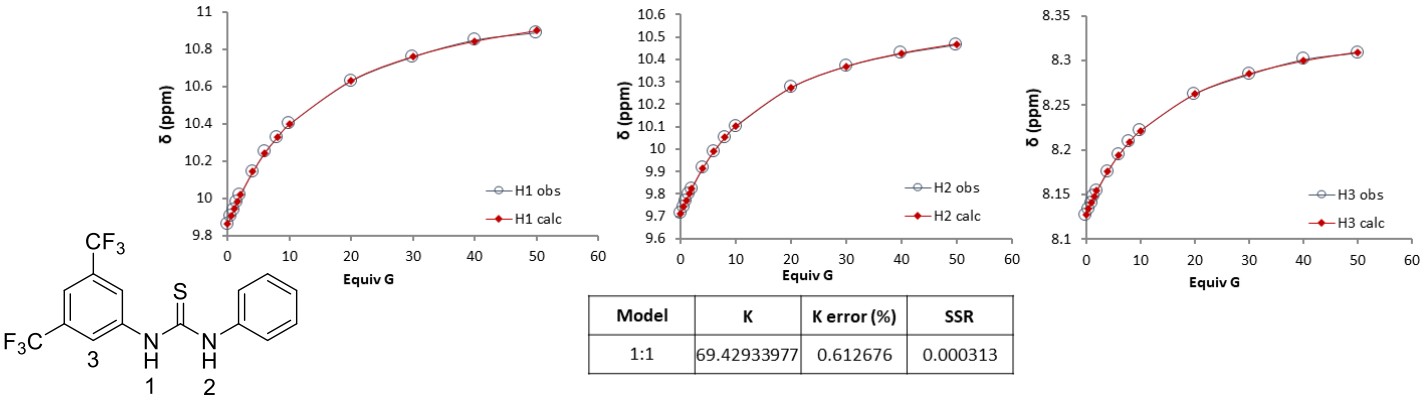


Figure S 26. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, 75% $CDCl_3$/24.5% DMSO-d6/0.5% H_2O) from the titration of **1** (1 mM) with POPC (0 - 50 mM). The number of equivalents of POPC relative to **1** is shown.



Model	K	K error (%)	SSR
1:1	69.42933977	0.612676	0.000313

Figure S 27. Observed (obs) and calculated (calc) binding curves for the titration of **1** (1 mM) with POPC at 298K in 75% $CDCl_3$/24.5% DMSO-d6/0.5% H_2O. H1, H2 and H3 correspond to signals starting at 9.86, 9.71 and 8.13 ppm, respectively.

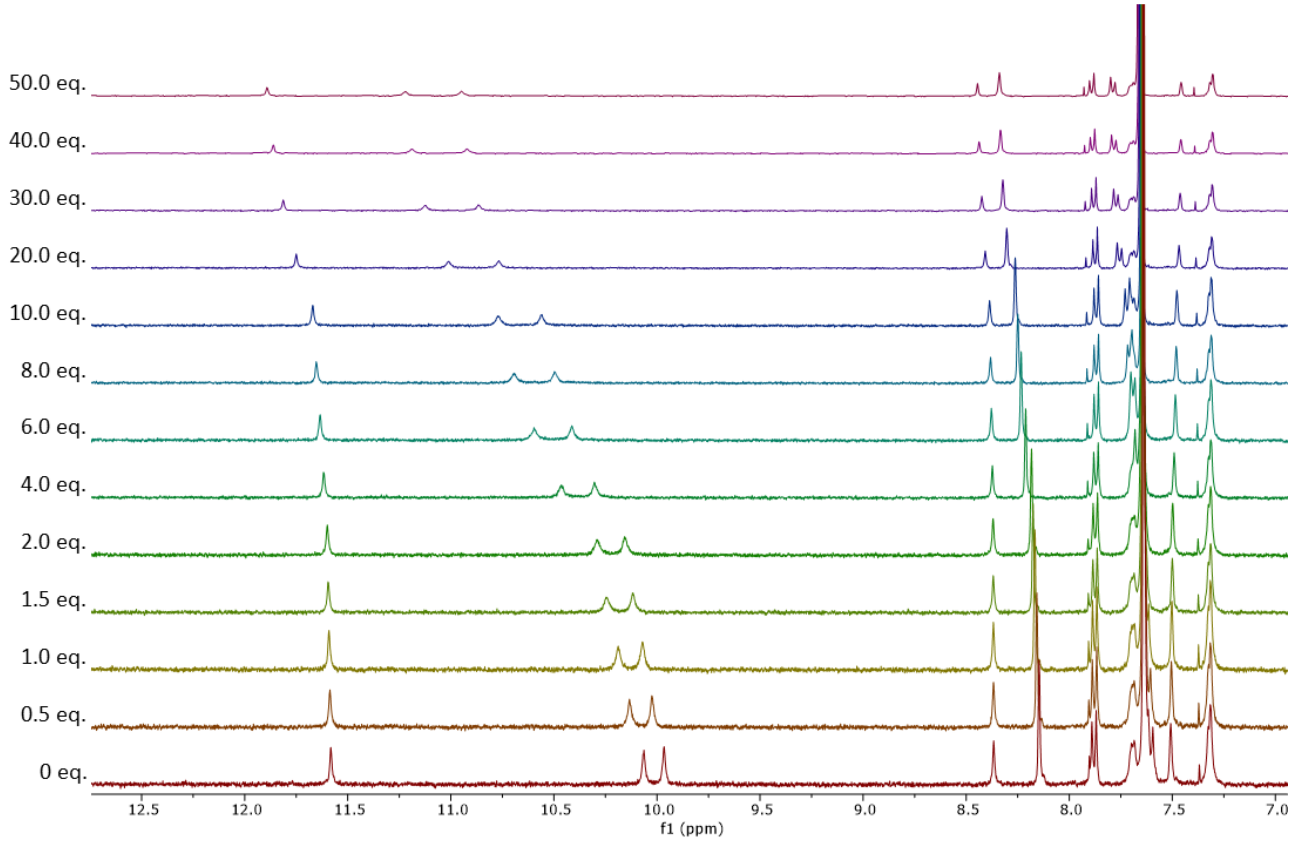
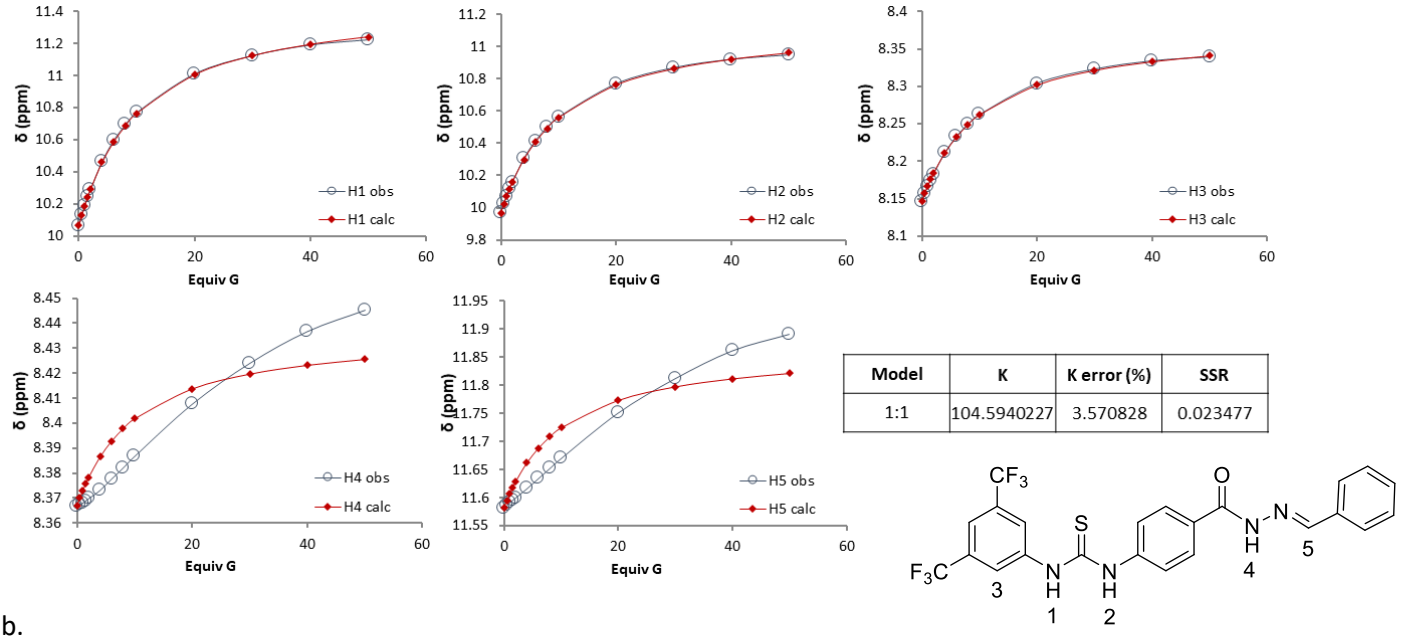


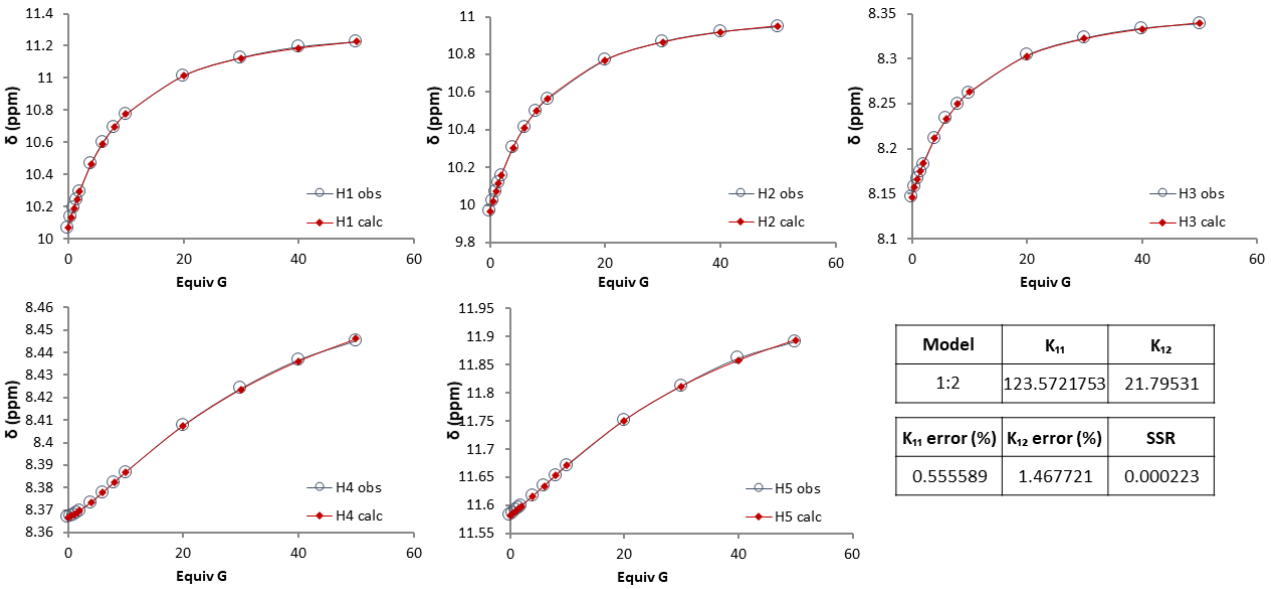
Figure S28. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, 75% $CDCl_3$/24.5% DMSO-d6/0.5% H_2O) from the titration of **3** (1 mM) with POPC (0 - 50 mM). The number of equivalents of POPC relative to **3** is shown.

a.



Model	K	K error (%)	SSR
1:1	104.5940227	3.570828	0.023477

b.



Model	K_{11}	K_{12}
1:2	123.5721753	21.79531

K_{11} error (%)	K_{12} error (%)	SSR
0.555589	1.467721	0.000223

Figure S29. Observed (obs) and calculated (calc) binding curves for the titration of **3** (1 mM) with POPC at 298K in 75% $CDCl_3$/24.5% DMSO-d6/0.5% H_2O. H1, H2, H3, H4 and H5 correspond to signals starting at 10.06, 9.97, 8.15, 8.37 and 11.58 ppm, respectively. Fittings of 5 signals to 1:1 model (a) and 1:2 model (b).

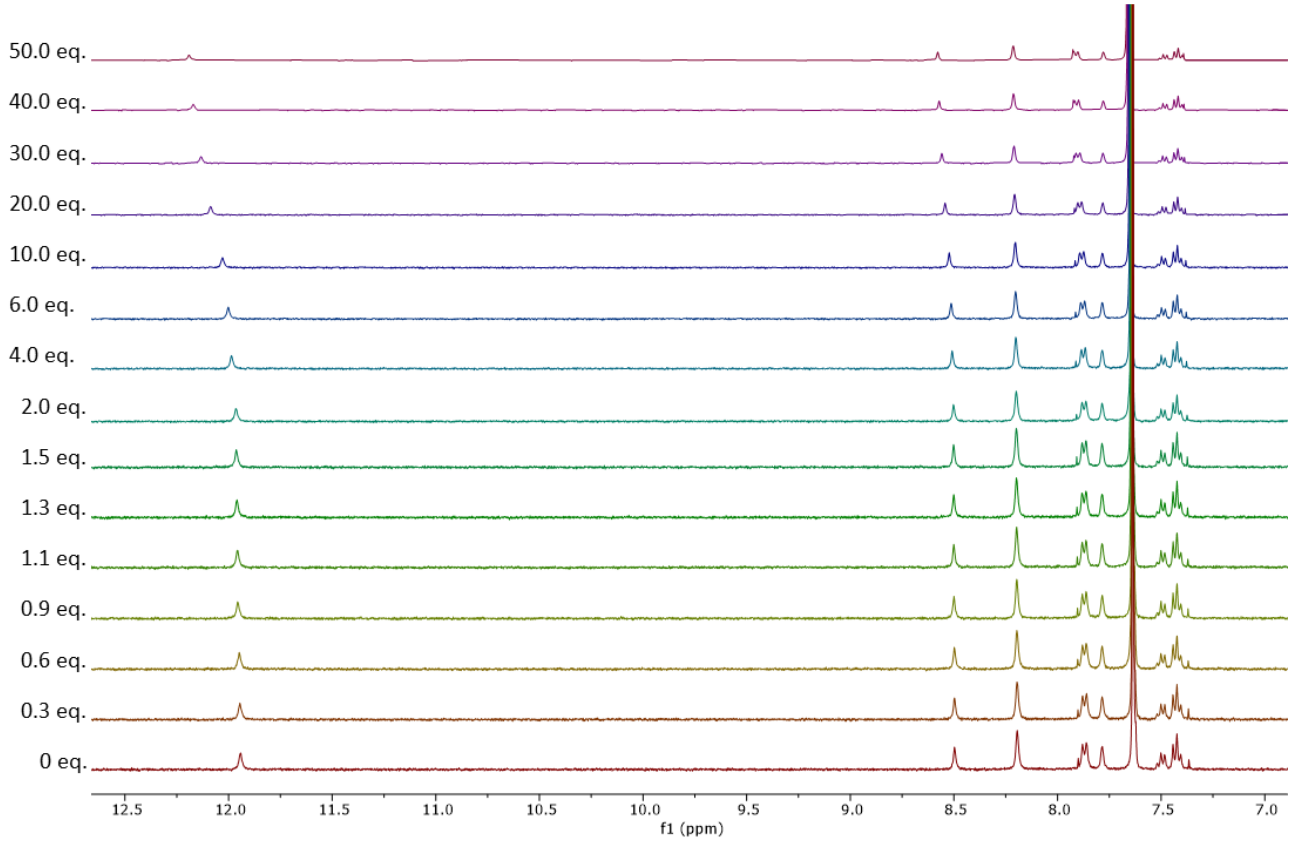
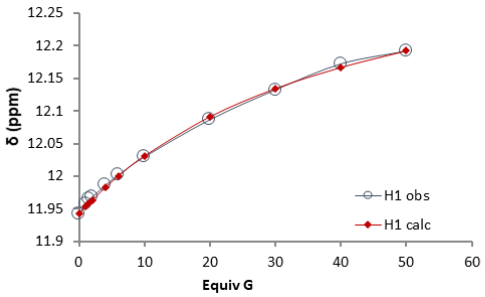


Figure S30. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, 75% $CDCl_3$/24.5% DMSO-d6/0.5% H_2O) from the titration of **7** (1 mM) with POPC (0 - 50 mM). The number of equivalents of POPC relative to **7** is shown.



Model	K	K error (%)	SSR
1:1	25.06786705	4.056964	0.000143

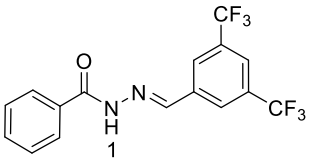


Figure S31. Observed (obs) and calculated (calc) binding curves for the titration of **7** (1 mM) with POPC at 298K in 75% $CDCl_3$/24.5% DMSO-d6/0.5% H_2O. H1 corresponds to the signals starting at 11.94 ppm.

4. Stability studies by ^{1}H NMR in CD_3OD

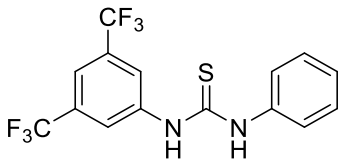
Previous studies with diaryl thioureas have evidenced that degradation of this kind of compounds can be a reason for their low activity.[2] To prove that this is not the case of our most inactive compounds, we monitored their stability in methanol using ^{1}H NMR spectroscopy.

The ^{1}H NMR spectra of different solutions of compounds **1**, **3**, **5a**, **18** and **19** in CD_3OD were monitored over time to study their relative stability. During the experiments, the solutions were stored at room temperature inside NMR tubes.

Thiourea **1** was used in these studies as a reference compound (good transport activity) and was compared to the most relevant compounds that showed poor transport activity. Acylhydrazone derivatives **3** and **5a** showed a similar stability to that observed for **1**. These three compounds showed a very low degree of degradation after one day (31 h) and the compound could still be observed after one month (752 h), together with the products that resulted from degradation.

The *meta*-phenylene bis-thiourea **19** showed to be less stable than the previous thioureas, since it was completely degraded after one month. On the other hand, the *ortho*-phenylene bis-urea **18** was clearly more stable than any of the thioureas studied, showing no sign of degradation after 24 days (578 h).

In any case, the solutions of the transporters were used for the transport experiments within eight hours after preparation, and none of the compounds studied was significantly degraded after eight hours.



1

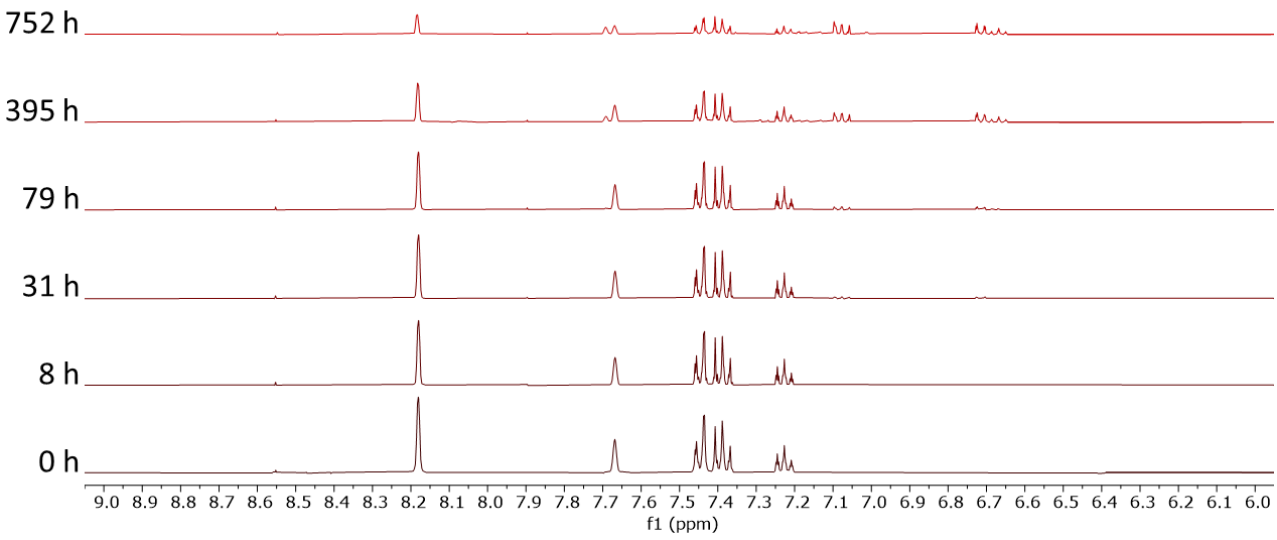
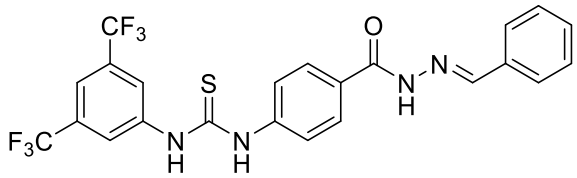


Figure S32. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, CD_3OD) of the thiourea **1** (2.3 mM) at different times after the preparation of the sample.



3

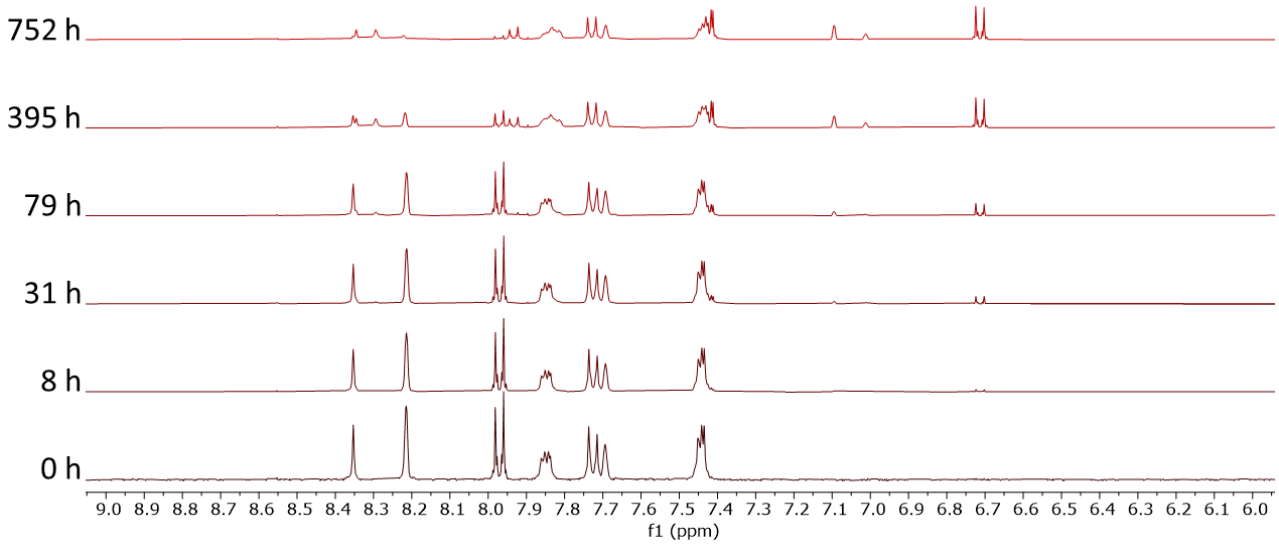
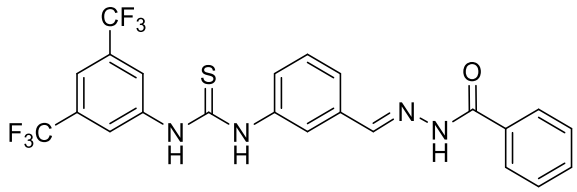


Figure S33. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, CD_3OD) of the thiourea **3** (2.3 mM) at different times after the preparation of the sample.



5a

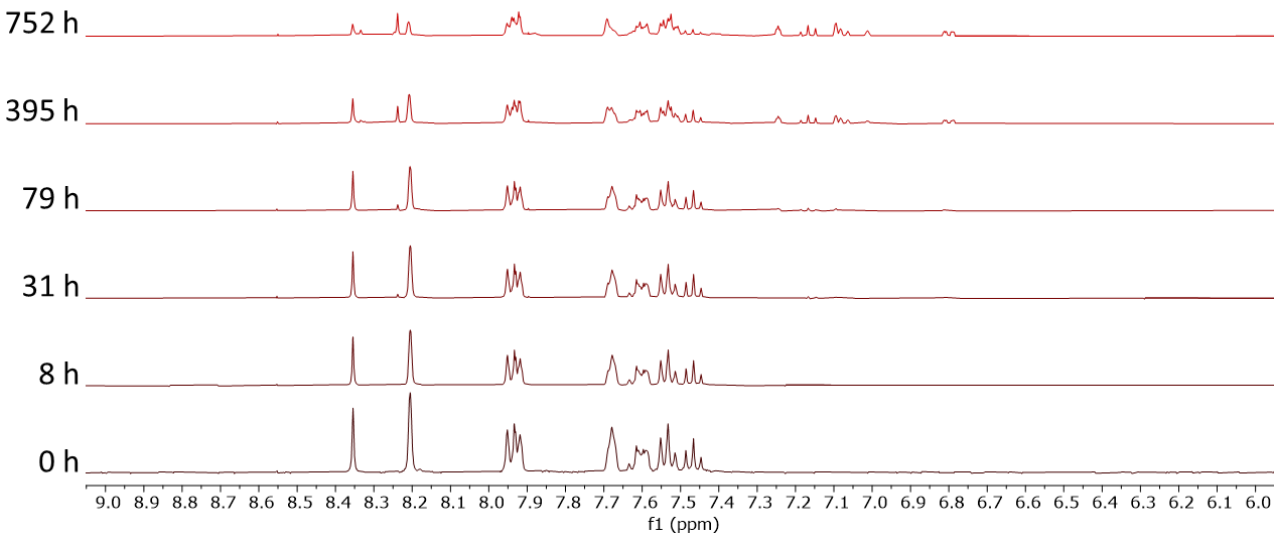
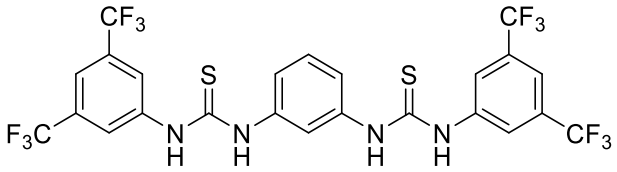


Figure S34. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, CD_3OD) of the thiourea **5a** (2.3 mM) at different times after the preparation of the sample.



19

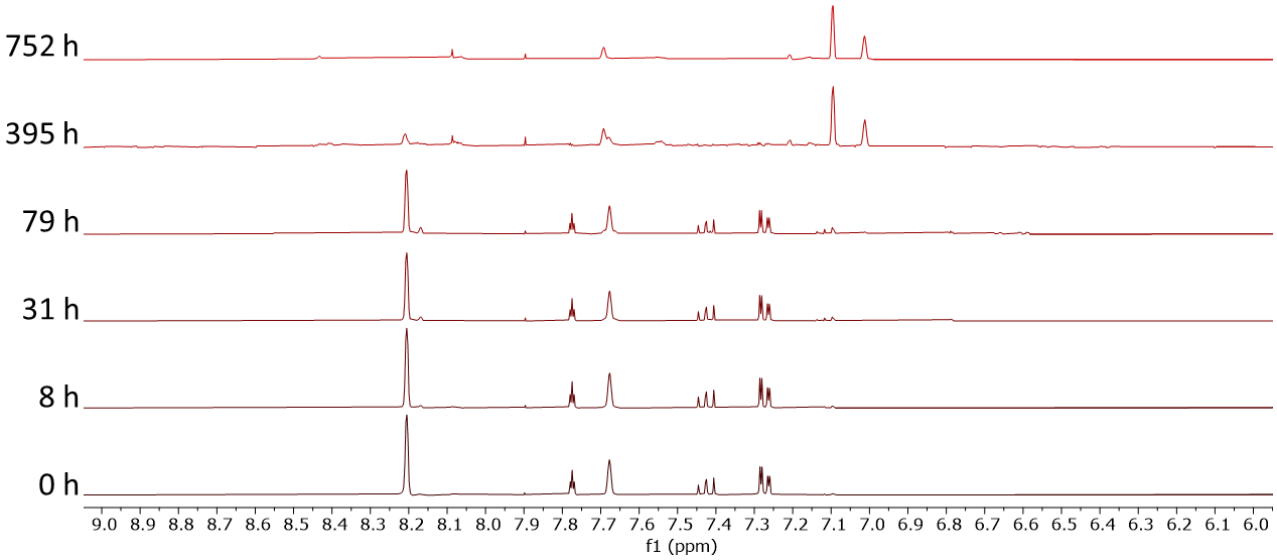
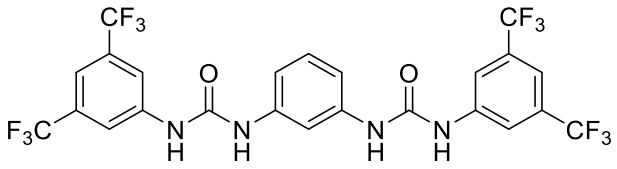


Figure S35. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, CD_3OD) of the thiourea **19** (2.3 mM) at different times after the preparation of the sample.



18

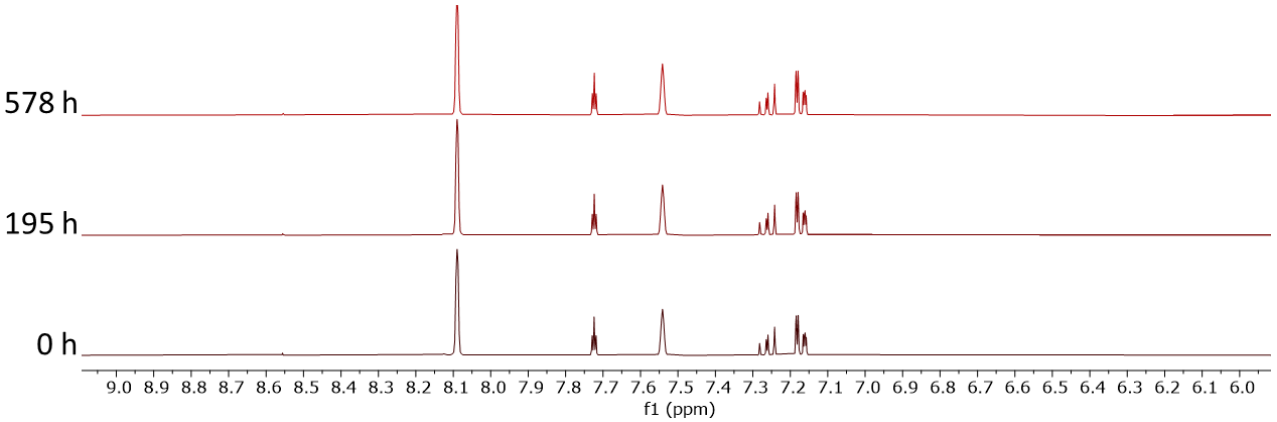


Figure S36. Selected region of the ^{1}H NMR spectra (400 MHz, 298 K, CD_3OD) of the thiourea **18** (2.3 mM) at different times after the preparation of the sample.

5. Transport studies in vesicles charged with lucigenin

5.1. Preparation of the vesicles

POPC and cholesterol solutions (15-30 mM) in deacidified chloroform were combined in a 5 mL round bottom flask. The volumes of the aliquots were calculated from the concentrations of the lipid solutions to obtain a POPC to cholesterol ratio of 7:3 (for instance by combining 7 µmol POPC and 3 µmol cholesterol). To preincorporate a compound in the membrane of the liposomes, a stock solution of compound in organic solvent (CH_3CN for compound **3** and CH_3OH for compounds **5a**, **18**, **19** and **22**) was added to the mixture. The volume of the stock solution was calculated to reach the desired compound:lipid ratio (1:100). The solvents were evaporated under a flow of nitrogen and the resulting lipid film was dried under high vacuum for at least 1 h.

The lipid film was then hydrated with 500 µL of an aqueous solution of N,N'-Dimethyl-9,9'-biacridinium dinitrate (Lucigenin, 0.8 mM) in a solution of the desired buffer (225 mM $NaNO_3$, 1 mM HEPES, pH 7.5; or 225 mM $NaNO_3$, 1 mM sodium citrate, pH 5.0). The resulting mixture was sonicated for 30 s and stirred for 1 h to give heterogeneous vesicles. Multilamellar vesicles were disrupted by 10 freeze-thaw cycles. The mixture was diluted to 1 mL (by adding 0.5 mL of buffer solution) and extruded 29 times through a polycarbonate membrane with 200 nm pores in a mini-extruder (Avestin LiposoFast-Basic). The external dye was removed by passing the liposomes through a pre-packed size exclusion column (containing 8.3 mL Sephadex G-25 medium), eluted with buffer solution. The collected large unilamellar vesicles were further diluted with buffer solution to obtain total lipid concentration of 0.4 mM (15-50 mL) and used for transport measurements the same day.

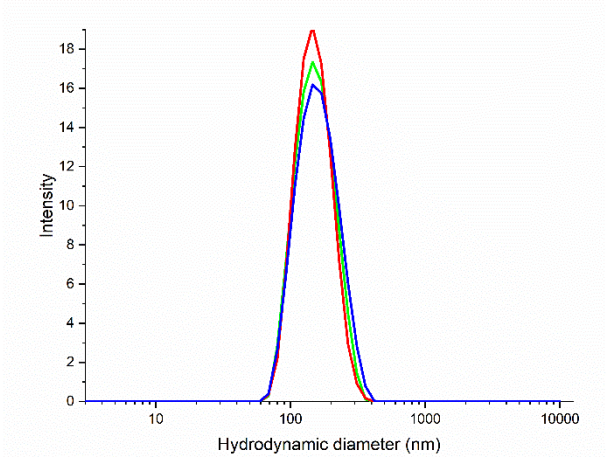


Figure S37. DLS data of LUVs with lucigenin encapsulated and suspended in 225 mM $NaNO_3$ with 1 mM HEPES at pH7.5 for 3 different batches of LUVs (each curve corresponds to the average of three consecutive measurements on the same batch of vesicles, using a refractive index of 1.45 for the liposomes and a refractive index of 1.33 and viscosity of 0.91 mPa·s for the buffer). These curves are indicative of a monodisperse suspension of vesicles with a mean diameter (z-average) of 145 nm.

5.2. Chloride transport measurements

3.00 mL of the freshly prepared liposomes solution were placed in a quartz cuvette with a small stir bar, and the cuvette was placed inside the sample compartment of a Fluoromax-4 spectrometer. The transporter was added to the liposomes as 6 µL of a 2 mM stock solution in an organic solvent (CH_3CN for compounds **1**-**4**, **5b**, **5c** and **6**-**8**, and CH_3OH for compounds **5a**, **16**-**18**, **20** and **21**; transporter:lipid molar ratio 1:100). These additions were performed using a Gilson pipette for organic solvents and placing the end of the tip close to the magnetic bar on the bottom of the cuvette, while stirring. For blank curves, 6 µL of CH_3CN were added, and for curves with the compounds preincorporated in the vesicles no organic solution was added. The

temperature was allowed to stabilize by stirring at 25 ˚C for 3-5 minutes (these conditions were maintained during the whole transport experiment), and after equilibration the transport measurement was started. During transport measurements the fluorescence intensity at 505 nm (3 nm slits) was monitored over time (15 minutes, 0.2 s interval) with excitation at 430 nm (3 nm slits).

For transport measurements 75 µL of NaCl (1 M, in 225 mM $NaNO_3$) were added to the liposomes 30 s after the start of the fluorescence recording, to create a Cl^- concentration gradient of 25 mM. The fluorescence intensity was measured for another 10 minutes, followed by lysing of the liposomes by addition of 50 µL of Triton X-100 (5% $^w/_w$ in water).

Each experiment was performed at least twice, and the data of the different runs were averaged and normalized. To normalize the curves, the initial 30-40 seconds were deleted to get rid of the fluorescence intensity in absence of NaCl (first 30 s) and the initial drop in fluorescence intensity after addition of NaCl (provoked by extravesicular lucigenin), and all the values were divided by the initial value of the resulting curve (F/F_0).

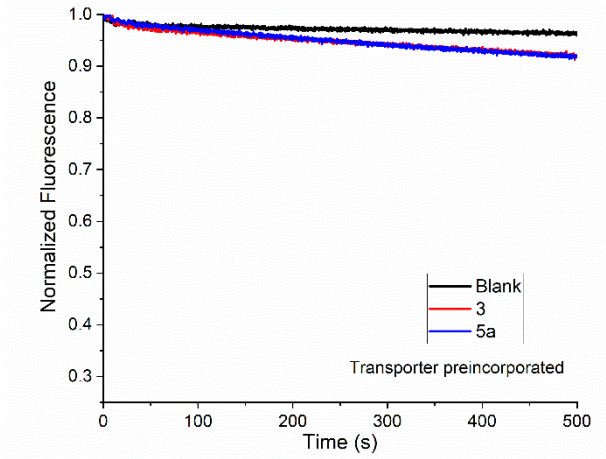


Figure S38. Transport of Cl^- by compounds **3**, **5a** (preincorporated in the vesicles at 1:100 transporter to lipid ratio) as monitored by the lucigenin assay in 225 mM $NaNO_3$ with 1 mM HEPES at pH 7.5, upon addition of 25 mM NaCl.

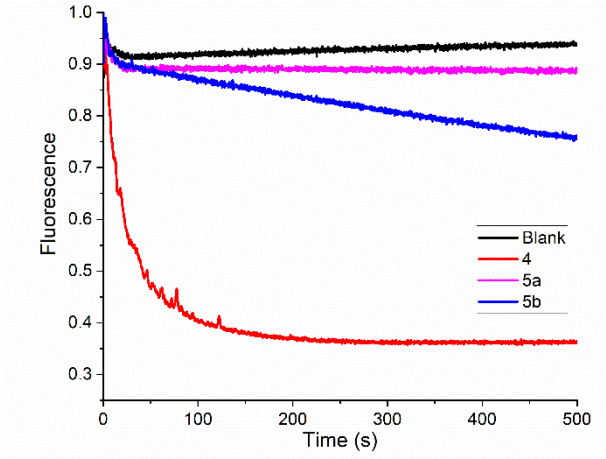


Figure S39. Transport of Cl^- by compounds **4**, **5a** and **5b** (postincorporated at 1:100 transporter to lipid ratio) as monitored by the lucigenin assay in 225 mM $NaNO_3$ with 1 mM citrate at pH 5.0, upon addition of 25 mM NaCl.

Compounds **19**, **22** are more lipophilic than any of the other compounds studied. Therefore, to ensure the presence of these compounds in the membranes of the liposomes their transport properties were studied with vesicles containing the compounds preincorporated.

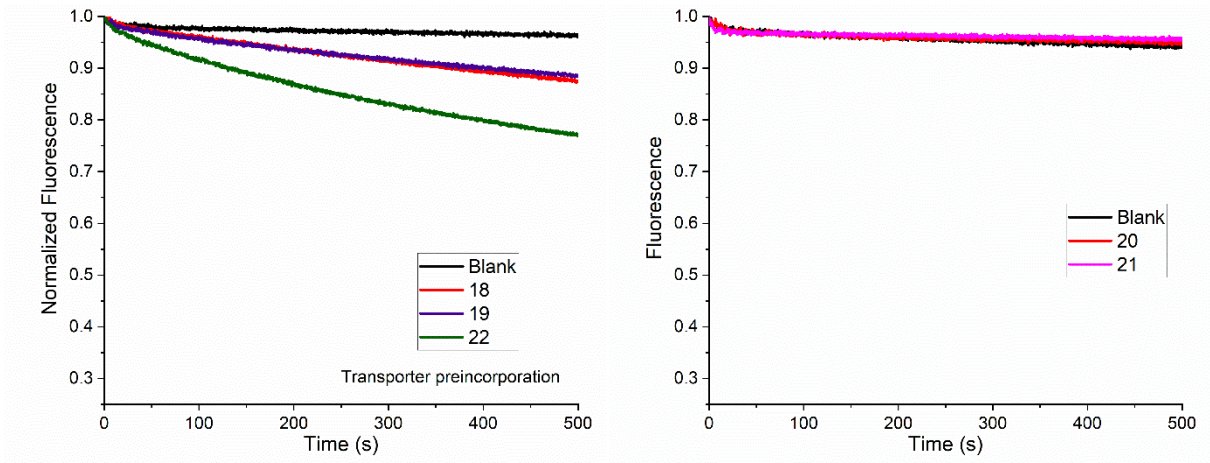


Figure S40. Transport of Cl^- by compounds **18**, **19**, **22** (preincorporated in the vesicles at 1:100 transporter to lipid ratio) and compounds **20, 21** (postincorporated in the vesicles at 1:100 transporter to lipid ratio) as monitored by the lucigenin assay in 225 mM $NaNO_3$ with 1 mM HEPES at pH 7.5, upon addition of 25 mM NaCl.

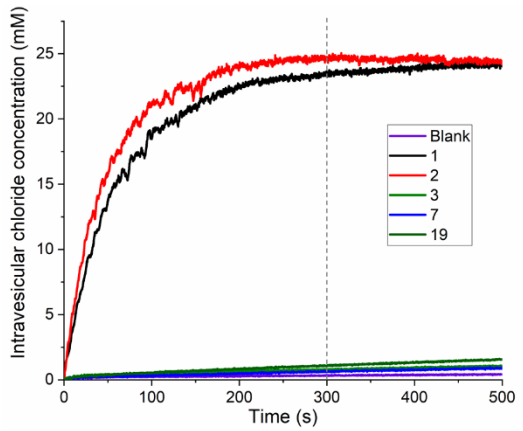
5.3. Quantitative chloride transport activity

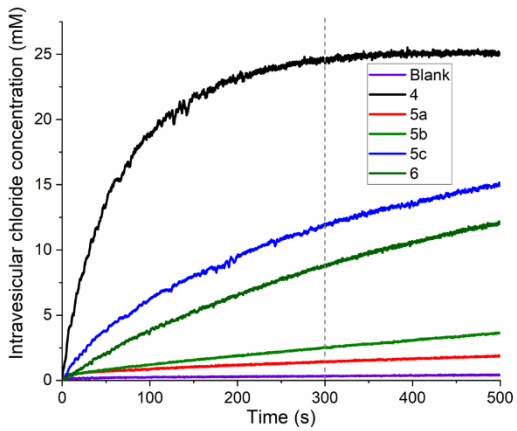
The transport activity of the compounds can be expressed as the concentration of chloride internalized inside the vesicles after 300 s. To determine this value we first obtained the inversed transport curves as F_0/F and we normalized them between 0 and 25 mM intravesicular chloride ($[Cl^-]_{in}$) based on equation (1):

$$[Cl^-]_{in} = \left(\frac{x_t - x_0}{x_{max} - x_0}\right) \times 25 \qquad (1)$$

where x_t is F_0/F at time t, x_0 is the initial F_0/F and x_{max} is F_0/F at 500 s for the curve of transporter **4**.

The resulting curves and the values of intravesicular chloride concentration at 300 s ($[Cl^-]_{in\ at\ 300s}$) are showed in Figure S35.





Compound	$[Cl^-]_{in\ at\ 300s}$ (mM)
1	23.5
2	24.8
3	0.8
4	24.7
5a	1.4
5b	2.5
5c	11.9
6	8.9
7	0.6
19	1.0

Figure S41. Transport of Cl^- by compounds **1**, **2**, **3**, **4**, **5a**, **5b**, **5c**, **6**, **7** and **19** represented as the concentration of chloride internalized inside the vesicles over time and quantitative transport activity expressed as the intravesicular chloride at 300 s for each curve.

6. Transport studies in vesicles charged with HPTS

6.1. Preparation of the vesicles

POPC and cholesterol solutions (15-30 mM) in deacidified chloroform were combined in a 5 mL round bottom flask. The volumes of the aliquots were calculated from the concentrations of the lipid solutions to obtain a POPC to cholesterol ratio of 7:3 (for instance by combining 7 µmol POPC and 3 µmol cholesterol). The solvent was evaporated under a flow of nitrogen and the resulting lipid film was dried under high vacuum for at least 1 h. The lipid film was then hydrated with 500 µL of an aqueous solution of 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS, 0.1 mM) in a solution of the desired buffer (100 mM sodium gluconate, 10 mM HEPES, pH 7.0). The resulting mixture was sonicated for 30 s and stirred for 1 h to give heterogeneous vesicles. Multilamellar vesicles were disrupted by 10 freeze-thaw cycles. The mixture was diluted to 1 mL (by adding 0.5 mL of buffer solution) and extruded 29 times through a polycarbonate membrane with 200 nm pores in a mini-extruder (Avestin LiposoFast-Basic). The external dye was removed by passing the liposomes through a pre-packed size exclusion column (containing 8.3 mL Sephadex G-25 medium), eluted with buffer solution. The collected large unilamellar vesicles were further diluted with buffer solution to obtain total lipid concentration of 0.1 mM (20 mL).

6.2. Proton transport measurements

3.00 mL of the freshly prepared liposomes solution were placed in a quartz cuvette with a small stir bar. The temperature was allowed to stabilize by stirring at 25 ˚C for 3-5 minutes inside the sample compartment of a Fluoromax-4 spectrometer (these conditions were maintained during the whole transport experiment), and after equilibration the transport measurement was started. During transport measurements the fluorescence intensity at 511 nm (2 nm slits) was monitored over time (10 minutes, 2 s interval) with excitation at two different wavelengths; 455 nm and 403 nm (3 nm slits).

For transport measurements 30 µL of TBAOH (0.5 M in H_2O) were added to the liposomes 30 s after the start of the fluorescence recording, to increase the extravesicular pH to 8.0 and create a transmembrane pH gradient. The transporter was added 60 s later as a DMSO solution (12 µL, 0.25 mM; transporter:lipid molar ratio 1:100) and the fluorescence intensity was measured for another 200 s, followed by lysing of the liposomes by addition of 50 µL of Triton X-100 (5% $^{w}/_{w}$ in water).

The transport curves were obtained as the ratio of the fluorescence intensities obtained from the two different excitation wavelengths (I_{455}/I_{403}). Each experiment was repeated 3 times and the different runs were averaged and normalized. To normalize the curves, the initial 90 seconds were deleted to get rid of the fluorescence intensity in absence of the transporter, and all the values were normalized between 0 (for the lowest value; first point of the curve) and 1 (higher value; after lysis).

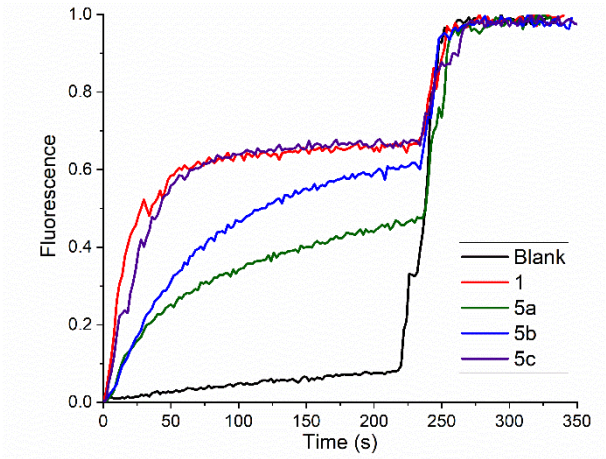


Figure S42. Transport of H^+ (or OH^-) by compounds **1**, **5a**, **5b** and **5c** (postincorporated at 1:100 transporter to lipid ratio) as monitored by the HPTS assay in 100 mM NaGluc with 10 mM HEPES at pH 7.0, upon addition of 0.5 mM TBAOH (final pH 8.0).

7. Discussion of the results obtained for acylhydrazones 20-22

To further explore the possibility of obtaining hydrazone-based anion transporters, we prepared and studied compounds **20**-**22**, which contain in their structures acylhydrazone groups with acidic NHs (Figure S37).

Acylhydrazone **20** bears an indole ring that can contribute to the binding of a chloride anion and the symmetric dipicoline-acylhydrazone **21** could coordinate a single chloride anion with the two hydrazone groups. While the former showed the same affinity for chloride as the reference acylhydrazone **7** (< 5 M^{-1}), the later gave a binding constant noticeably higher (19 M^{-1}) (Table S1). Nevertheless, none of these compounds showed significant activity as chloride transporters (Figure S34). Compound **22** also contains the dipicoline-acylhydrazone unit, and in this case it works as a linker between two thiourea motifs. This compound could form a complex with chloride with the four thiourea NHs coordinating the anion and it is an example of a more complex receptor obtained from the simpler dipicolinehydrazide and aldehyde **4** by dynamic covalent chemistry. The binding constant obtained with this compound for the first binding event was significantly higher than that of any of the thioureas described in the main text (411 M^{-1}, Table S1), and similar to that of a previously reported tris-thiourea with excellent chloride transport properties (450 M^{-1}).[3] Despite its higher affinity for chloride, compound **22** transported the anion far less efficiently than the simpler precursor **4** (Figure S34).

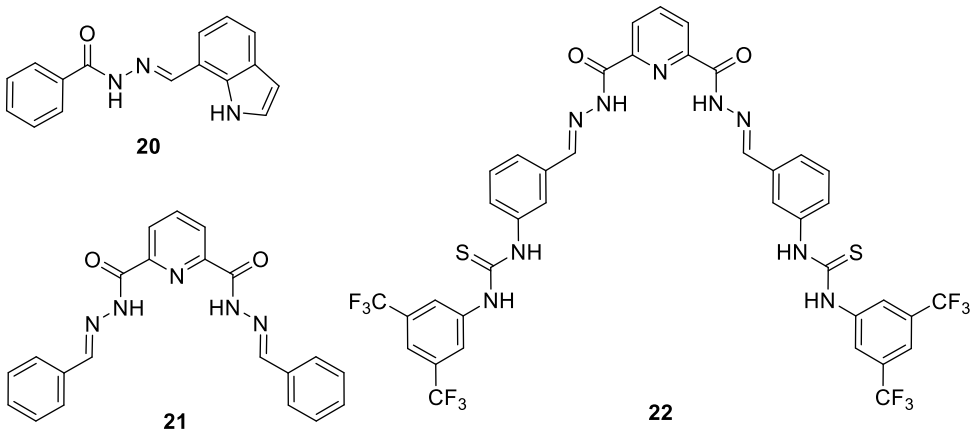


Figure S43. Structures of acylhydrazones **20**-**22**.

Table S1. logP values, chloride binding constants and relative transport activity of compounds **20**-**22.**

Compound	logP[a]	K_a (M^{-1})[b]			Relative Cl^- transport activity[c]
		1:1 model	1:2 model		
		K_{11} (M^{-1})	K_{11} (M^{-1})	K_{12} (M^{-1})	
20	2.9	3	-	-	Poor
21	4.2	19	-	-	Poor
22	11.1	-	411	16	Poor

[a] Calculated values using ChemDraw 19.0.

[b] Determined by ^{1}H NMR titration in DMSO:H_2O 99.5:0.5.

[b] Stablished from the comparison of the transport curves at 1:100 transporter-lipid molar ratio.

The poor transport properties of acylhydrazones **20**-**22** revealed that avoiding molecules with separate binding sites or increasing the binding affinity of the molecules is not enough to obtain efficient acylhydrazone-based anion transporters. As pointed out in the main text, the preorganization of the binding motifs in the structure of the transporters is a key feature to obtain highly active compounds. It is likely that due to the intrinsic conformational flexibility of the acylhydrazone groups,[4,5] these molecules exist in the membranes as conformers with a poor preorganization of the binding groups to coordinate to a single anion, probably interacting with the phospholipid head groups.

[1] U. Manna, S. Kayal, B. Nayak and G. Das, *Dalton Transactions*, 2017, **46**, 11956-11969.
[2] C. M. Dias, H. Valkenier and A. P. Davis, *Chem. Eur. J.*, 2018, **24**, 6262-6268.
[3] H. Valkenier, C. M. Dias, K. L. Porter Goff, O. Jurcek, R. Puttreddy, K. Rissanen and A. P. Davis, *Chem. Commun.*, 2015, **51**, 14235-14238.
[4] I. Hamzi, T. M. Barhoumi-Slimi and R. Abidi, *Heteroatom Chemistry*, 2016, **27**, 139-148.
[5] A. Blanco-Gómez, Á. Fernández-Blanco, V. Blanco, J. Rodríguez, C. Peinador and M. D. García, *J. Am. Chem. Soc.,* 2019, **141**, 3959-3964.