Supporting information

ION Thallos-HTL: a fluorescent thallium indicator that enables cell-selective and localizable thallium flux assays

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Materials and general methods

Reagents and solvents were used as received from commercial sources. Thin later chromatography (TLC) was carried out on silica gel F-254 plates. Preparatory TLC was performed on silica gel 60 Å extra hard layer F-254 plates. Flash column chromatography was performed using 60-20 μM (70-230 mesh) silica gel. ^{1}H and ^{13}C NMR spectra were acquired at 400/600 and 150 MHz, respectively. Chemical shifts are reported in δ (ppm) relative to the residual $CHCl_3$ (δ_H 7.26, δ_C 77.2). NMR spectra and High resolution mass spectra were obtained at the Small Molecule NMR Facility Core and the Mass Spectrometry Research Center at Vanderbilt University, respectively. The composition of the sodium gluconate buffer used in this study is 138 mM sodium gluconate, 1.26 mM $CaSO_4 \cdot 2H_2O$, 0.90 mM $MgSO_4 \cdot 7H_2O$, 5.56 mM glucose, and 20 mM HEPES.

Optical spectroscopy

Lyophilized samples of Thallos and **ION Thallos-HTL** were dissolved in DMSO (10 μL) and hydrolyzed with 0.1 M KOH (30 μL). After 15 minutes, deionized water was added to reach a final volume of 200 μL. Final concentrations of Thallos and **ION Thallos-HTL** were 213 μM and 298 μM, respectively. Complete hydrolysis was confirmed by low resolution MS. Fluorescence measurements were performed using a plate reader (Cytation 5, Biotek). Fluorescence polarization measurements were conducted on a plate reader (Flexstation 3, Molecular Devices). Fluorescence emission spectra were collected on a fluorimeter (Fluoromax Plus, Horiba Scientific). Results were processed and presented as graphics using Prism software.

Fluorescence quantum yields. Fluorescence quantum yield (Φ) values are reported relative to the standard fluorescein in 0.1 M NaOH (Φ = 0.79).[1] The unknown samples (after hydrolysis with KOH) were diluted in PBS pH 7.4 and measurements were done in the absence and presence of Tl^+ (5 mM). The refractive indexes for 0.1 M NaOH and PBS are 1.3344[2] and 1.3346,[3] respectively. The sample and the reference were excited at 450 nm. Results are presented as an average of 2 or 3 independent measurements.

Table 1. Fluorescence quantum yields

	Φ (free)	Φ (Tl^+ = 5 mM)
Thallos	0.04	0.11
ION Thallos-HTL	0.03	0.11
HaloTag-**ION Thallos-HTL**	0.09	0.21
ION Thallos-HTL*	0.04	-

*in the presence of BSA

Metal ion selectivity. To evaluate the metal ion selectivity, the fluorescence intensity of 3 μM freshly prepared hydrolyzed **ION Thallos-HTL** in 10 mM Tris HCl buffer pH 7.45 (I = 140 mM TMACl) was measured in the presence of Na^+ and K^+ at room temperature. Stock solution of 500 mM NaCl and 500 mM KCl were used to achieve solutions with final concentrations of 1 mM and 140 mM. A negative control experiment in the absence of metal (blank) and positive control in the presence of 1 mM and 4.5 mM Tl^+ were also included.

In the case of divalent ions, freshly hydrolyzed **ION Thallos-HTL** was diluted to a concentration of 3 μM in buffer containing 10 mM MOPS, 10 mM EGTA, and 100 mM KCl, pH 7.28. To achieve the desired concentrations of free Ca^{2+} and Mg^{2+} in metal buffered solutions we used MaxChelator,[4] an open-source software that calculates the concentration of free Ca^{2+} and Mg^{2+} in the presence of metal buffers.[5] We used 10 mM EGTA, I = 0.1 N, temperature = 25 °C and pH 7.28 in the calculations. The amounts of $CaCl_2$ and $MgCl_2$ calculated with MaxChelator were added to the buffered solutions to generate the desired free metal concentrations of 1 μM and 40 μM for Ca^{2+} and 1 mM and 10 mM for Mg^{2+}. Fluorescence was recorded using λ_{ex} = 490 nm, λ_{em} = 515 nm, and bandwidth = 9 nm.

Fluorescence titrations. Freshly prepared hydrolyzed stock solutions (Thallos or **ION Thallos-HTL**) were diluted to a final concentration of 3 μM in 500 μL of a high thallium (5.5 mM Tl_2S0_4 or 11 mM Tl^+) and 1500 μL of low thallium (0 mM Tl_2S0_4) sodium gluconate buffer pH 7.3. Thallium concentrations (0 mM, 0.02 mM, 0.04 mM, 0.09 mM, 0.17 mM, 0.34 mM, 0.69 mM, 1.38 mM, 2.75 mM, 5.50 mM, 11.00 mM) were obtained through a serial dilution using the high and low thallium solutions in a clear 96-well plate (VWR). Fluorescence intensities were measured using a plate reader. Spectra were recorded using λ_{ex} = 490 nm, λ_{em} = 515 nm, and

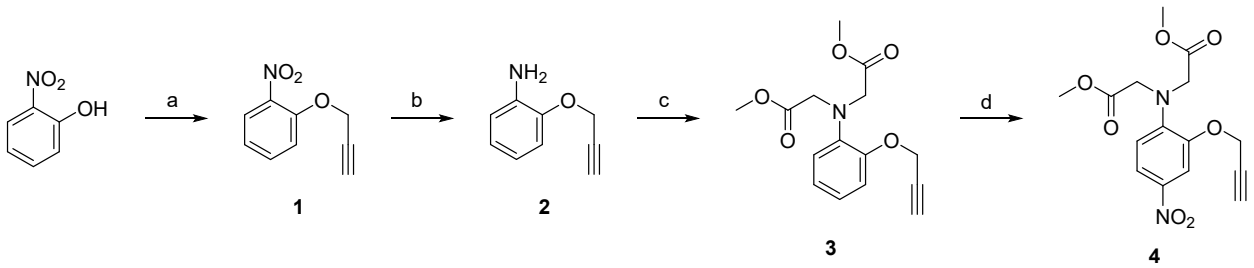
bandwidth = 9 nm. Fold change was calculated by dividing the fluorescence values by the fluorescence in the 0 mM Tl^+ group. K_d values were determined using a fit for binding-saturation with one site-total.

Fluorescence titrations in the presence and absence of HaloTag. Freshly prepared hydrolyzed **ION Thallos-HTL** was diluted in sodium gluconate buffer containing 0.1% CHAPS to a concentration of 500 nM. Then, HaloTag protein was added to this solution to give a final concentration of 500 nM for the sensor and 750 nM of protein. This solution was incubated at RT for 30 min. After the incubation, solution was split into two vials. 50 mM Tl_2SO_4 solution was added to make 400 µL of a high thallium (11 mM Tl^+) solution in one vial. An equal volume of water was added to the remaining vial to make the low thallium (0 mM Tl+) solution. In a 384-well plate (VWR) a serial dilution was done to obtain thallium concentrations of 0 mM, 0.02 mM, 0.04 mM, 0.09 mM, 0.17 mM, 0.34 mM, 0.69 mM, 1.38 mM, 2.75 mM, 5.50 mM, and 11.00 mM. Fluorescence intensities were measured using a plate reader. Spectra were recorded using λ_{ex} = 490 nm, λ_{em} = 515 nm, and bandwidth = 9 nm.

Stable cell line generation

Stable HaloTag(+) HEK-293 cells were generated from commercially available cells (HEK-293 CRL-1573™, ATTC). HEK-293 cells were seeded in a clear 6-well plate (VWR). Plasmid encoding pHTN HaloTag CMV-neo Vector was transfected into HEK-293 cells using Lipofectamine 2000 transfection reagent (Fisher Scientific) following the manufacture's protocol. Approximately 24 h-post transfection, the cells were passaged and seeded into a T-75 flask (VWR). Selection was performed using 400 µg/mL G418 disulfate (Sigma Aldrich). After a week of selection, cells were seeded at a target of 0.5 cell/well in a 96-well plate (VWR) and incubated for a week and a half. Screening for cells with high expression of HaloTag protein was done with HaloTag® TMR ligand (Promega). A clonal population with high HaloTag expression was selected for continued expansion.

Syntheses and characterization



Scheme S1. Synthesis of **compound 4**. Reagents and conditions: a) (1) K_2CO_3, DMF, RT, 15 min; (2) propargyl bromide, 65 °C, 1 h, 99% crude; b) tin, 6 M HCl, EtOH, reflux, 2.5 h; c) methyl 2-bromoacetate, proton sponge, NaI, CH_3CN, 95 °C, overnight, 77%; d) glacial acetic acid, 70% HNO_3, 0-5 °C, 30 min, 58%.

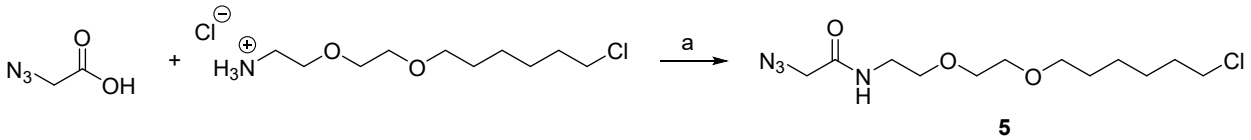
Compound 1. To a round bottom flask were added 2-nitrophenol (1.000 g, 7.19 mmol, 1.0 eq.), dry DMF (10 mL) and potassium carbonate (1.987 g, 0.01 mol, 2.0 eq.). Then, the resulting mixture was stirred at RT for 15 min. Next, propargyl bromide (1.02 mL, 9.35 mmol, 1.3 eq.) was added and the mixture was stirred at 65 °C for 1 h. Upon cooling to RT, the crude mixture was diluted with EtOAc and the heterogenous mixture was decanted. The solution was put into a separatory funnel and washed with water. The aqueous phase was back extracted with EtOAc and the combined organic layers were washed with brine. The organic phase was then dried over Na_2SO_4, filtered, and concentrated in vacuo to give a yellow solid, 1.260 g (99% crude yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.86 (dd, J = 8.0, 1.6 Hz, 1H), 7.56 (td, J = 7.6, 1.6 Hz, 1H), 7.26 (d, J = 8.4 Hz, 1H), 7.13-7.06 (m, 1H), 4.85 (d, J = 2.4 Hz, 2H), 2.58 (t, J = 2.4 Hz, 1H).

Compound 2. To a solution of **compound 1** (1.255 g, 7.08 mmol, 1.0 eq.) in EtOH (38 mL) were added 6 M HCl (12 mL, 0.07 mol, 10.0 eq.) and tin (1.892 g, 0.02 mol, 2.3 eq.). The resulting heterogenous mixture was then stirred at reflux for 2.5 h. After cooling to RT, the crude mixture was poured into icy water and the pH was adjusted to around 9 by adding aqueous 6 M KOH. The mixture was extracted three times with $CHCl_3$. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo. Analysis of the crude mixture by LCMS showed the desired compound constituted 75% of the mass. The crude mixture was used in the next step without any purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.92 (dd, J = 8.0, 1.2 Hz, 1H),

6.84 (td, J = 7.6, 1.2 Hz, 1H), 6.76-6.71 (m, 2H), 4.72 (d, J = 2.4 Hz, 2H), 3.81 (bs, 2H), 2.52 (t, J = 2.4 Hz, 1H).

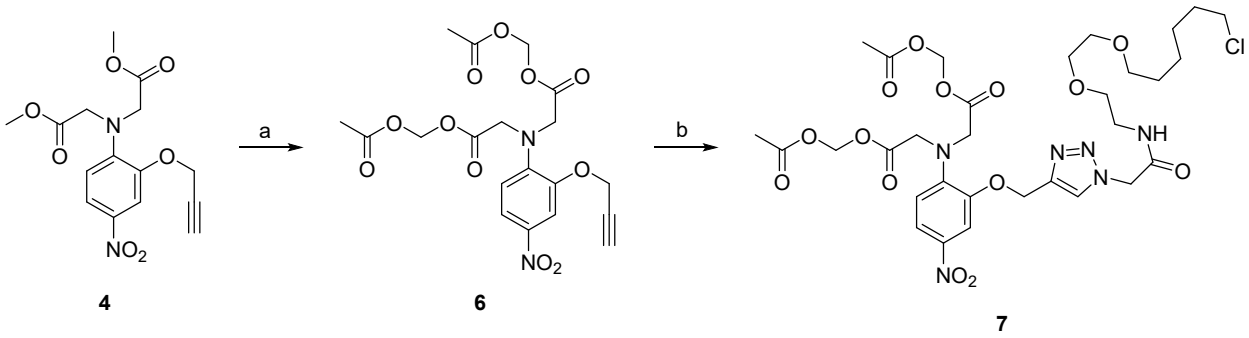
Compound 3. **Compound 2** (0.400 g, 2.72 mmol, 1.0 eq.), proton sponge (1.514 g, 7.07 mmol, 2.6 eq.) and NaI (0.306 g, 2.04 mmol, 0.8 eq.) were added to a heavy wall pressure vessel. Then, dry CH_3CN (2 mL) and methyl 2-bromoacetate (0.77 mL, 8.15 mmol, 3.0 eq.) were added and the reaction mixture was stirred overnight at 95 °C. After cooling to RT, the crude mixture was diluted with CH_3CN and filtered off. The filtrate was concentrated in vacuo. Then, the residue was dissolved with a mixture of $CHCl_3$ and MeOH, adsorbed on silica and chromatographed (SiO_2, 2:1 Hex:EtOAc). **Compound 3** was recovered as pale orange oil, 0.612 g (77% isolated yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.99-6.86 (m, 4H), 4.70 (d, J = 2.4 Hz, 2H), 4.16 (s, 4H), 3.73 (s, 6H), 2.49 (t, J = 2.4 Hz, 1H).

Compound 4. To a vial containing **compound 3** (0.390 g, 1.34 mmol, 1.0 eq.) was added glacial acetic acid (58 µL, 1.00 mmol, 0.8 eq.). Then, the mixture was cooled down in an ice bath and 70% nitric acid (64 µL, 1.00 mmol, 0.8 eq.) was added dropwise. The reaction mixture was stirred at 0-5 °C for 30 min. Next, the crude mixture was diluted with EtOAc and washed with saturated $NaHCO_3$ aqueous solution. The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo. Analysis by LCMS showed 46% conversion. The residue was subjected to react again under the same reaction conditions adjusting for the remaining mass of starting material (35 µL CH_3COOH and 52 µL HNO_3). After working up the reaction as before, the residue was dissolved with $CHCl_3$, adsorbed on silica and chromatographed (SiO_2, 2:1 Hex:EtOAc) to give a yellow solid (0.262 g, 58% isolated yield). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.86 (dd, J = 9.0, 2.4 Hz, 1 H), 7.83 (dd, J = 2.4 Hz, 1 H), 6.70 (d, J = 8.4 Hz, 1H), 4.72 (d, J = 2.4 Hz, 2 H), 4.21 (s, 4 H), 3.79 (s, 6H), 2.57 (t, J = 2.4 Hz, 1H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 171.0, 147.3, 145.7, 141.0, 119.2, 116.3, 109.9, 77.2, 76.8, 57.1, 54.3, 52.4. HRMS (ESI) calcd from $C_{15}H_{17}N_2O_7$ $[M+H]^+$ 337.1030, found 337.1048.



Scheme S2. Synthesis of **compound 5**. Reagents and conditions: a) HATU, DIPEA, DMF, Ar, RT, overnight, 87%.

Compound 5. Azidoacetic acid (0.015 g, 0.15 mmol, 1.0 eq.), 2-(2-((6-chlorohexyl)oxy)ethoxy)ethanamine hydrochloride (0.039 g, 0.15 mmol, 1.0 eq.) and HATU (0.057 g, 0.15 mmol, 1.0 eq.) were added to a Schlenk flask. The flask was placed under high vacuum, followed by backfilling with Ar. Next, dry DMF (4 mL) and DIPEA (0.12 mL, 0.67 mmol, 4.5 eq.) were added. The reaction mixture was stirred at RT overnight. The crude mixture was diluted with $CHCl_3$, and it was washed with brine twice. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The desired product was purified by reverse phase preparatory HPLC (10-95% CH_3CN/H_2O, linear gradient, with constant 0.1% v/v formic acid). A colorless oil was recovered, 0.040 g (87% isolated yield). ^1H NMR ($CDCl_3$, 400 MHz) δ 6.73 (bs, 1H), 3.97 (s, 2H), 3.65-3.60 (m, 2H), 3.60-3.56 (m, 4H), 3.56-3.44 (m, 6H), 1.83-1.73 (m, 2H), 1.66-1.57 (m, 2H), 1.51-1.42 (m, 2H), 1.42-1.33 (m, 2H).

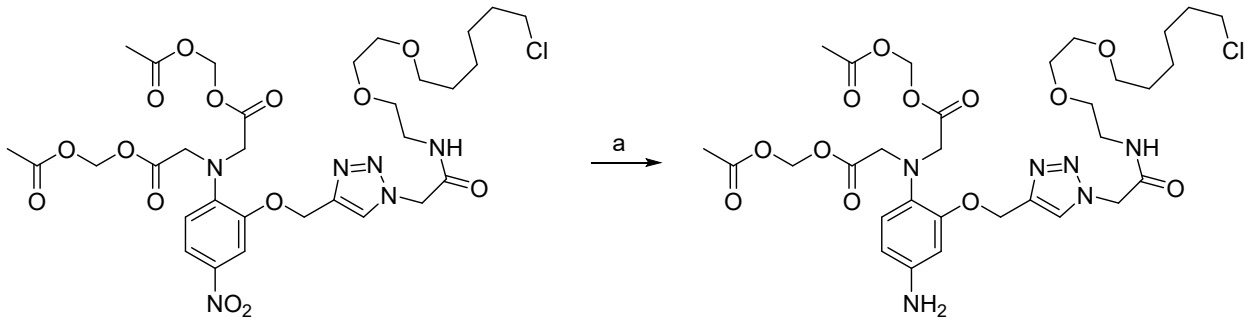


Scheme S3. Synthesis of **compound 7**. Reagents and conditions: a) (1) KOH aq., MeOH:1,4-dioxane 1:1 v/v, RT, overnight; (2) 2 M HCl; (3) bromomethyl acetate, DIPEA, DCM, 0-5 °C to RT, overnight, 65%; b) **compound 5**, CuI, DIPEA, THF, RT, overnight, 81%.

Compound 6. To a solution of **compound 4** (0.200 g, 0.60 mmol, 1.0 eq.) in a mixture of MeOH (5 mL) and 1,4-dioxane (5 mL) was added an aqueous solution (5 mL) of KOH (0.334 g, 5.95 mmol, 10.0 eq.). The reaction mixture was stirred at RT overnight. To the crude mixture were added 2 M HCl (7.4 mL) and brine, then the mixture was extracted three times with $CHCl_3$. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo to give a yellow solid, 0.158 g (86% crude yield). The recovered carboxylic acid intermediate (0.158 g, 0.51 mmol, 1.0 eq.) was dissolved in dry DCM (4 mL), then DIPEA (0.63 mL, 3.59 mmol, 7.0 eq.) was added and the solution was cooled to 0-5 °C in an ice bath. Next, bromomethyl acetate

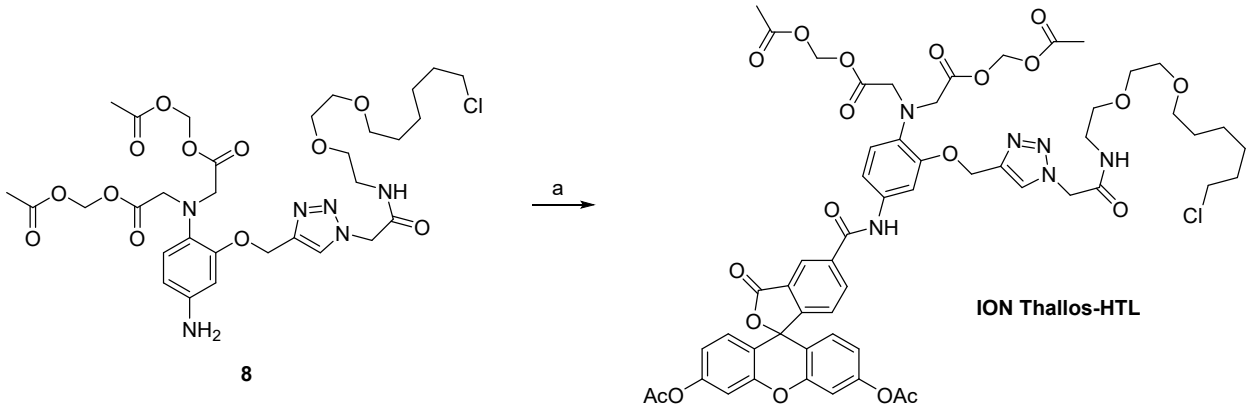
(0.2 mL, 2.05 mmol, 4.0 eq.) was added dropwise, the ice bath was removed, and the reaction mixture was stirred at RT overnight. The crude mixture was diluted with $CHCl_3$ and washed with brine. The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ and applied to 3 preparatory TLC plates, resolving with a 1:1 Hex:EtOAc mixture. **Compound 6** was obtained as a yellow solid (0.150 g, 65% isolated yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.88-7.83 (m, 2H), 6.72-6.67 (m, 1H), 5.83 (s, 4H), 4.75 (d, *J* = 2.4 Hz, 2H), 4.24 (s, 4H), 2.59 (t, *J* = 2.4 Hz, 1H), 2.14 (s, 6H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 169.6, 169.4, 147.5, 145.1, 141.5, 119.0, 116.6, 109.8, 79.8, 77.4, 77.1, 57.1, 54.1, 20.9. HRMS (ESI) calcd from $C_{19}H_{21}N_2O_{11}$ $[M+H]^+$ 453.1140, found 453.1157.

Compound 7. **Compound 6** (0.090 g, 0.20 mmol, 1.0 eq.), **compound 5** (0.122 g, 0.40 mmol, 2.0 eq.) and CuI (0.004 g, 0.02 mmol, 0.1 eq.) were added to a Schlenk flask. The flask was placed under high vacuum and backfilled with Ar using a balloon. Next, THF (1.8 mL) and DIPEA (0.06 mL, 0.34 mmol, 1.7 eq.) were added. The reaction mixture was stirred at RT overnight. The crude mixture was diluted with $CHCl_3$, and washed with brine twice. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ and applied to 3 preparatory TLC plates, resolving with EtOAc. **Compound 7** was obtained as a yellow solid (0.122 g, 81% isolated yield. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.89-7.86 (m, 2H), 7.84 (dd, *J* = 8.4, 1.8 Hz, 1H), 6.73 (t, *J* = 4.8 Hz, 1H), 6.68 (d, *J* = 8.4 Hz, 1H), 5.72 (s, 4H), 5.25 (s, 2H), 5.07 (s, 2H), 4.20 (s, 4H), 3.61-3.58 (m, 2H), 3.57-3.51 (m, 6H), 3.49-3.44 (m, 2H), 2.11 (s, 6H), 1.77 (q, *J* = 6.6 Hz, 2H), 1.61 (q, *J* = 6.6 Hz, 2H), 1.46 (q, *J* = 7.2 Hz, 2H), 1.37 (q, *J* = 7.2 Hz, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 169.7, 169.3, 164.9, 148.3, 145.0, 142.7, 141.6, 125.3, 118.7, 116.6, 109.4, 79.8, 71.4, 70.4, 70.2, 69.3, 62.8, 54.1, 53.1, 45.2, 39.7, 32.6, 29.5, 26.8, 25.5, 20.8. HRMS (ESI) calcd from $C_{31}H_{44}ClN_6O_{14}$ $[M+H]^+$ 759.2599, found 759.2605.



Scheme S4. Synthesis of **compound 8**. Reagents and conditions: a) Pd/C, H_2, DCM, RT, 2.5 h, quant.

Compound 8. To a solution of **compound 7** in dry DCM (8 mL) was added Pd/C. The mixture under stirring, was bubbled with H_2 for 5 min and stirring continued at RT for 2.5 h. The crude mixture was then filtered off through a PTFE syringe filter and the filtrate was concentrated under reduced pressure. Analysis by LCMS showed reaction completion, the product was used in the next step without any purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.90 (s, 1H), 6.83 (d, J = 8.4 Hz, 1H), 6.57 (t, J = 4.8 Hz, 1H), 6.34 (d, J = 2.4 Hz, 1H), 6.23 (dd, J = 8.4, 2.4 Hz, 1H), 5.71 (s, 4H), 5.24 (s, 2H), 5.05 (s, 2H), 4.09 (s, 4H), 3.60-3.57 (m, 2H), 3.56-3.50 (m, 6H), 3.49-3.43 (m, 4H), 2.08 (s, 6H), 1.77 (q, J = 6.8 Hz, 2H), 1.60 (q, J = 7.2 Hz, 2H), 1.50-1.41 (m, 2H), 1.41-1.24 (m, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 170.3, 169.8, 165.2, 152.1, 144.8, 143.2, 130.7, 125.1, 123.0, 108.4, 103.0, 79.3, 71.4, 70.5, 70.2, 69.4, 62.9, 54.3, 53.1, 45.2, 39.7, 32.6, 29.5, 26.8, 25.5, 20.8. HRMS (ESI) calcd from $C_{31}H_{46}ClN_6O_{12}$ $[M+H]^+$ 729.2857, found 729.2863.



Scheme S5. Synthesis of **ION Thallos-HTL**. Reagents and conditions: a) 5-carboxyfluorescein diacetate, HATU, DIPEA, DMF, Ar, 37%.

ION Thallos-HTL. **Compound 8** (0.053 g, 0.07 mmol, 1.0 eq.), 5-carboxyfluorescein diacetate (0.034 g, 0.07 mmol. 1.0 eq.) and HATU (0.028, 0.07 mmol, 1.0 eq.) were placed in a Schlenk flask. The flask was put under high vacuum and then backfilled with Ar. Next, dry DMF (4 mL) and DIPEA (50 μL, 0.26 mmol, 3.5 eq.) were added and the reaction mixture was stirred at RT overnight. The crude mixture was diluted with $CHCl_3$ and washed with brine two times. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The desired product was purified by reverse phase preparatory HPLC (10-95% CH_3CN/H_2O, linear gradient). **ION Thallos-HTL** was recovered as a white solid (0.032 g, 37% isolated yield). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.70 (s, 1H), 8.57 (s, 1H), 8.30 (d, *J* = 8.4 Hz, 1H), 7.84 (s, 1H), 7.36-7.32 (m, 1H), 7.29 (d, *J* = 7.8 Hz, 1H), 7.18 (s, 1H), 7.11 (d, *J* = 1.8 Hz, 1H), 6.88 (d, *J* = 9.0 Hz, 1H), 6.85-6.78 (m, 4H), 6.59 (t, *J* = 5.4 Hz, 1H), 5.73 (s, 4H), 5.24 (s, 2H), 5.03 (s, 2H), 4.18 (s, 4H), 3.60-3.54 (m, 4H), 3.52-3.43 (m, 6H), 3.41-3.37 (m, 2H), 2.32 (s, 6H), 2.09 (s, 6H), 1.73 (q, *J* = 6.6 Hz, 2H), 1.56 (q, *J* = 7.2 Hz, 2H), 1.41 (q, *J* = 7.2 Hz, 2H), 1.33 (q, *J* = 7.2 Hz, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 170.1, 169.7, 169.0, 168.5, 165.3, 163.8, 155.4, 152.4, 151.7, 149.9, 144.0, 137.5, 136.1, 135.4, 133.3, 128.9, 126.6, 125.6, 124.8, 123.8, 120.3, 118.1, 115.8, 114.7, 110.7, 109.2, 82.2, 79.4, 71.5, 70.4, 70.1, 69.3, 62.5, 53.9, 52.9, 45.2, 39.6, 32.6, 29.5, 26.8, 25.5, 21.3, 20.9. HRMS (ESI) calcd from $C_{56}H_{60}ClN_6O_{20}$ $[M+H]^+$ 1171.3545, found 1171.3552.

Metal ion selectivity

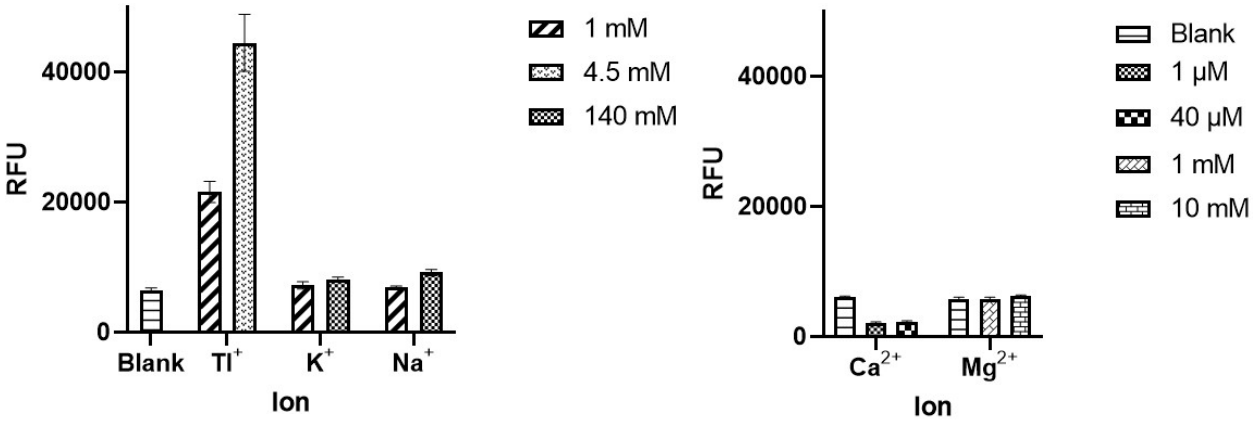


Figure S1. Fluorescence intensity responses of **ION Thallos-HTL** (3 µM, λ_{ex} = 490 nm, λ_{em} = 515 nm) to various metals. Left, monovalent cations in 10 mM Tris HCl buffer pH 7.45; right, divalent cations in 10 mM MOPS, 10 mM EGTA, 100 mM KCl pH 7.28.

Emission spectra and fluorescence traces

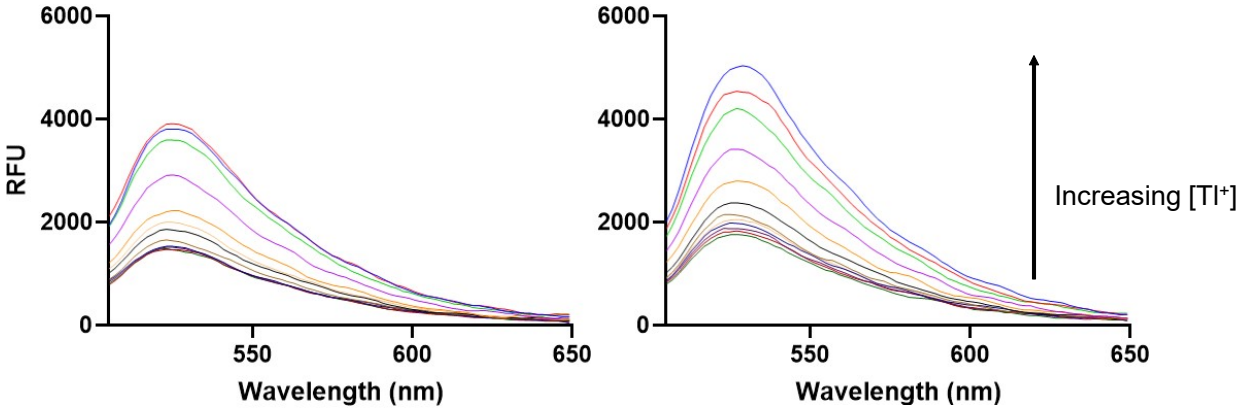


Figure S2. Emission spectra of **ION Thallos-HTL** free (500 nM) (left) and HaloTag bound (right) in the presence of thallium (0-2.25 mM Tl_2SO_4) in PBS, pH 7.4.

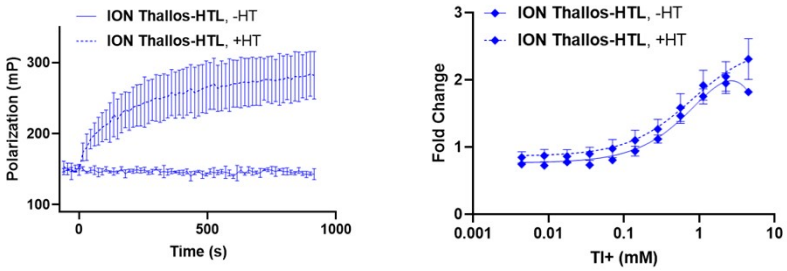


Figure S3. Fluorescence polarization traces of **ION Thallos-HTL** (500 μM) in the presence and absence of HaloTag (750 mM) in PBS, pH 7.4 containing 0.01% CHAPS (left). Fluorescence intensity fold change as a function of thallium (0-2.25 mM Tl_2SO_4) of ION Thallos-HTL free and HaloTag bound in PBS, pH 7.4.

Fluorescence images

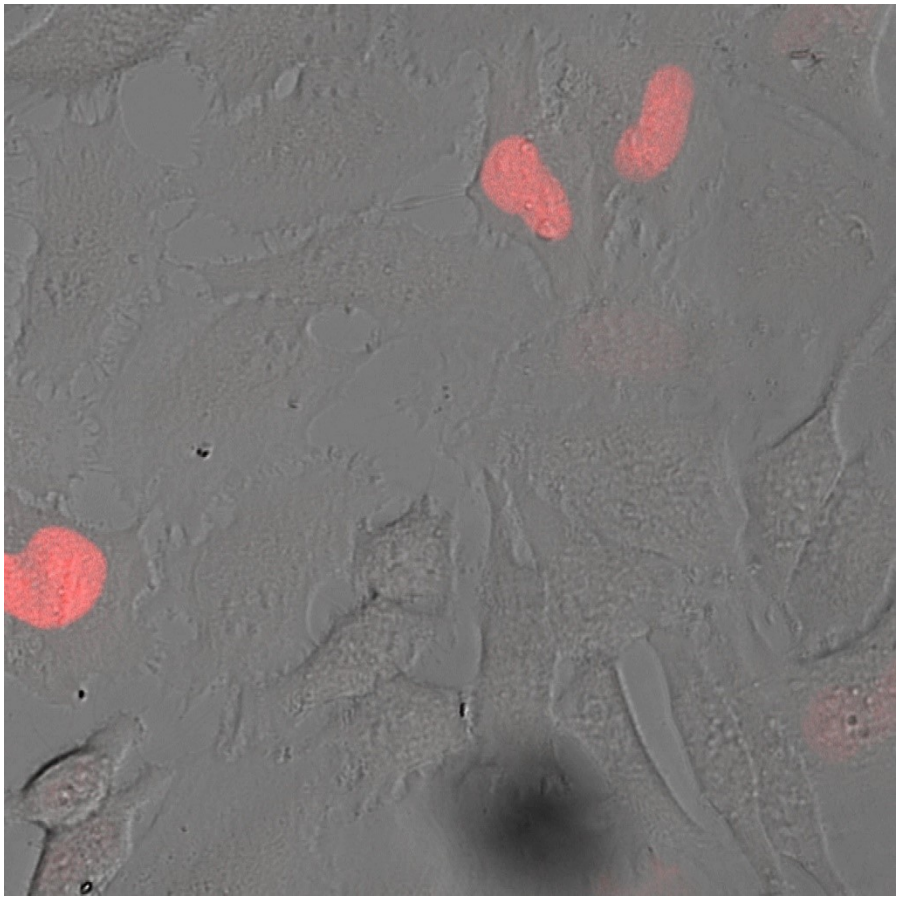


Figure S4. Image of HeLa cells transiently expressing HaloTag-NLS and labeled with Janelia Fluor 585 HaloTag ligand. Merged image of the bright field and fluorescence channel in false color.

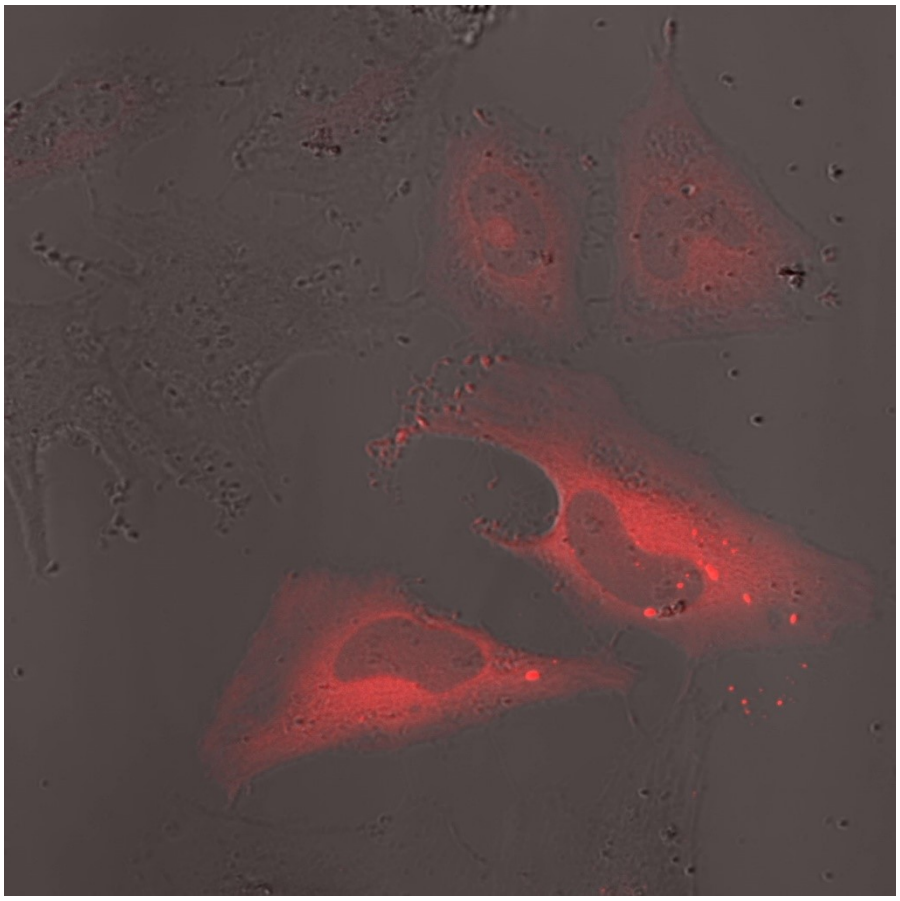


Figure S5. Image of HeLa cells transiently expressing HaloTag-NES and labeled with Janelia Fluor 585 HaloTag ligand. Merged image of the bright field and fluorescence channel in false color.

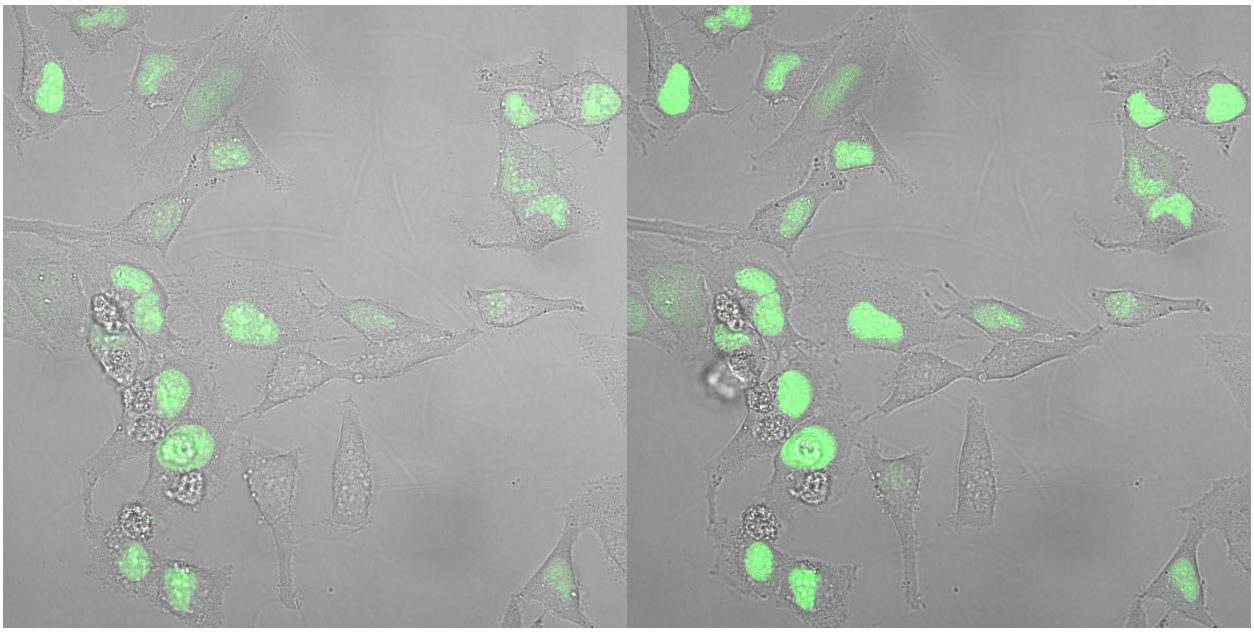


Figure S6. Images of HeLa cells transiently expressing HaloTag-NLS and labeled with **ION Thallos-HTL**. Basal fluorescence (left) and fluorescence after the addition of 0.84 mM Tl_2SO_4. Calculated 1.4-fold increment in fluorescence intensity as mean fluorescence of 16 individual cells. Merged images of the bright field and fluorescence channel in false color.

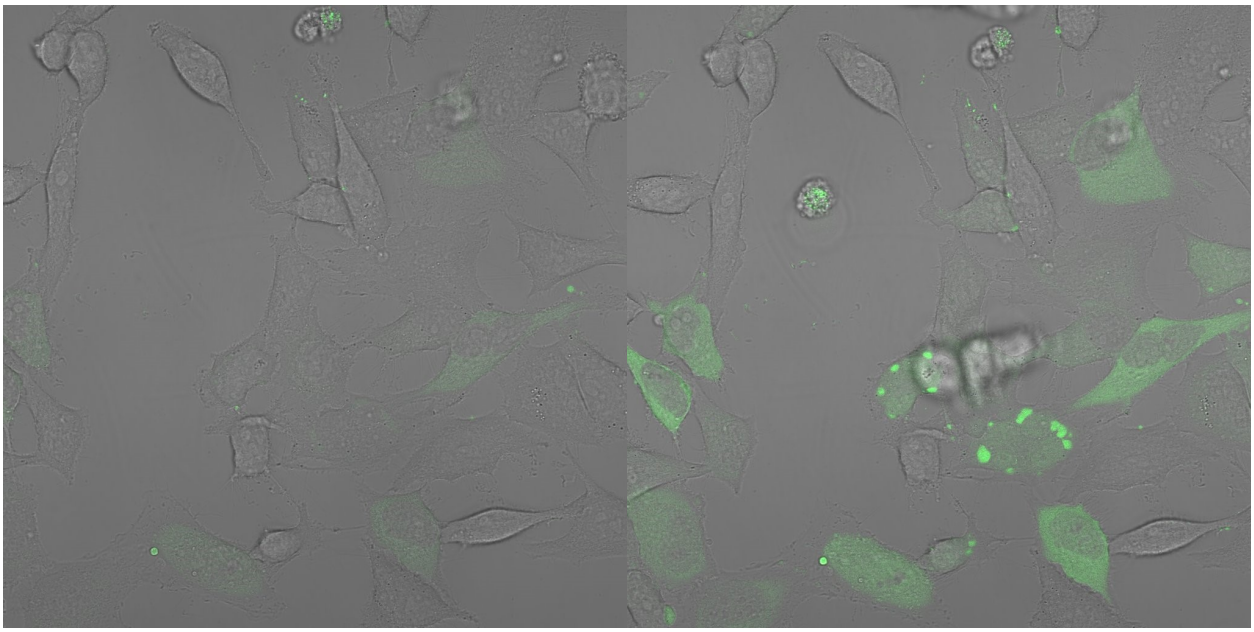


Figure S7. Images of HeLa cells transiently expressing HaloTag-NES and labeled with **ION Thallos-HTL**. Basal fluorescence (left) and fluorescence after the addition of 0.84 mM Tl_2SO_4. Calculated 1.6-fold increment in fluorescence intensity as mean fluorescence of 16 individual cells. Merged images of the bright field and fluorescence channel in false color.

Fluorescence signal quantifications

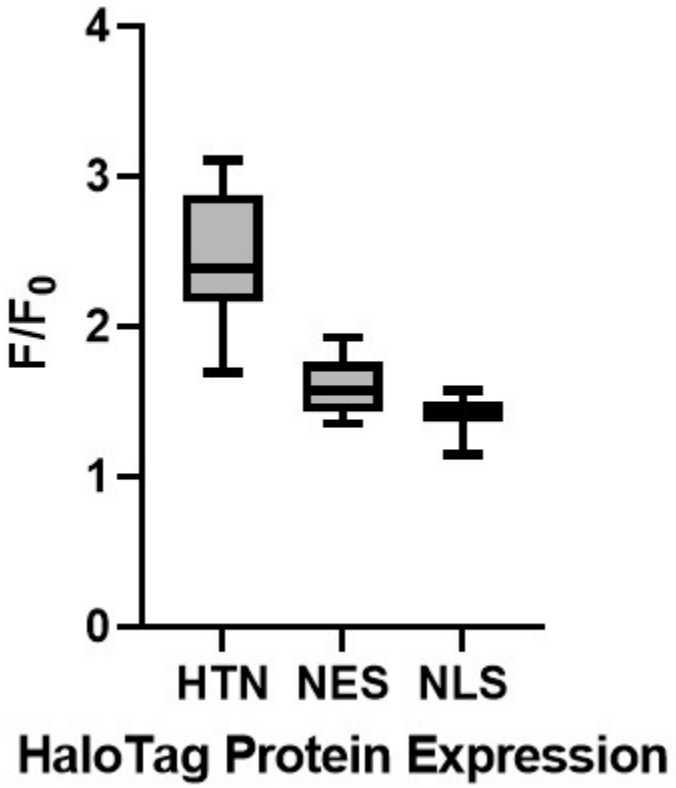


Figure S8. Ratio of the fluorescence intensities at the region of interest after addition of Tl^+ (F) over before addition (F_0). Box and whiskers plot with center lines showing the mean for 16 cells. Box delimits the 25th and 75th percentiles and the whiskers show the max and min values.

High-throughput screening (HTS) data

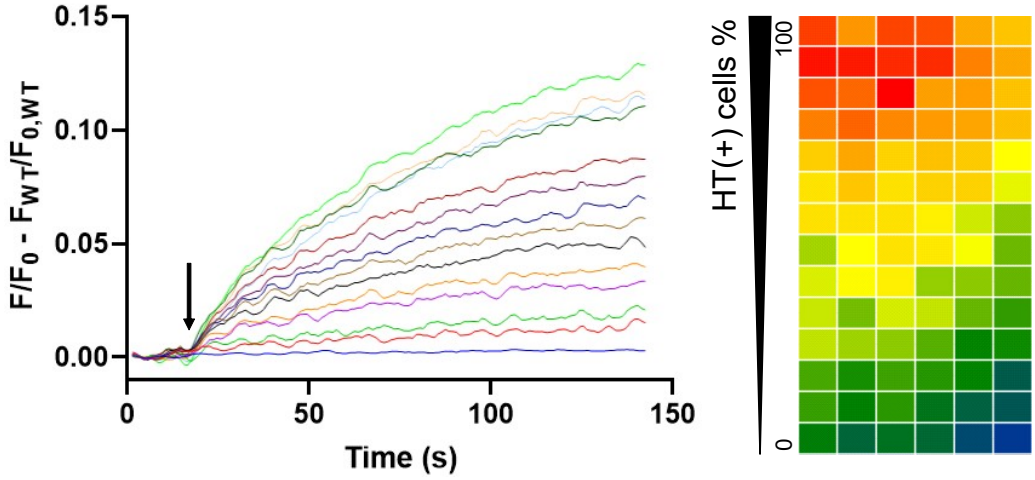


Figure S9. HaloTag (+) cell percentage-dependent fluorescence intensity. Traces from a Tl^+ flux assay in a mixed population of HT(+) HEK-293 cells and WT HEK-293 (0-100%) labeled with **ION Thallos-HTL**. The arrow indicates the time of addition of 0.84 mM Tl_2SO_4. Slope of kinetic traces after the addition of Tl^+ is displayed as a heat map [low slope (green-blue) and larger slope (red-orange)].

Copies of ^{1}H and ^{13}C NMR spectra

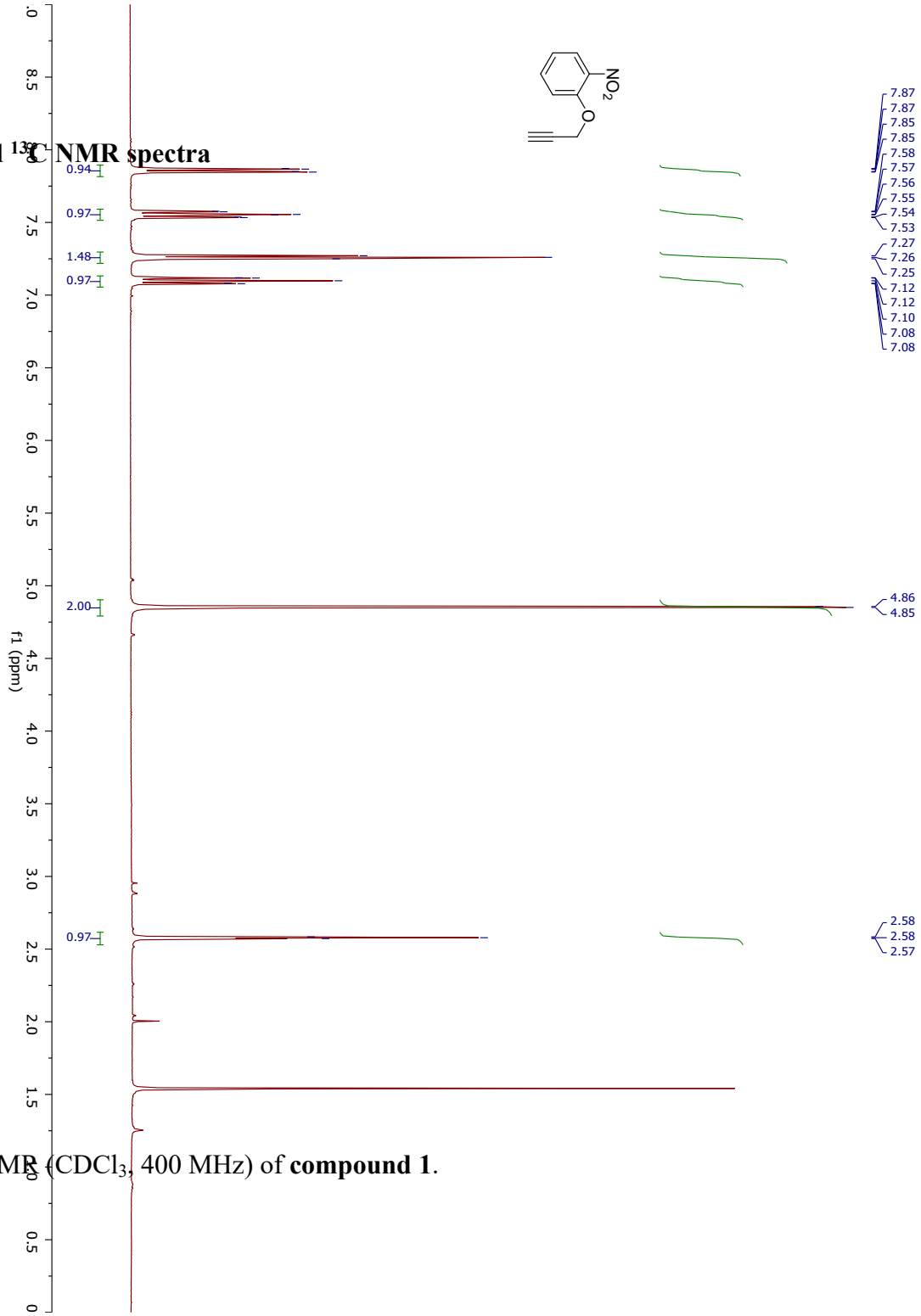


Figure S10. ^{1}H NMR ($CDCl_3$, 400 MHz) of **compound 1**.

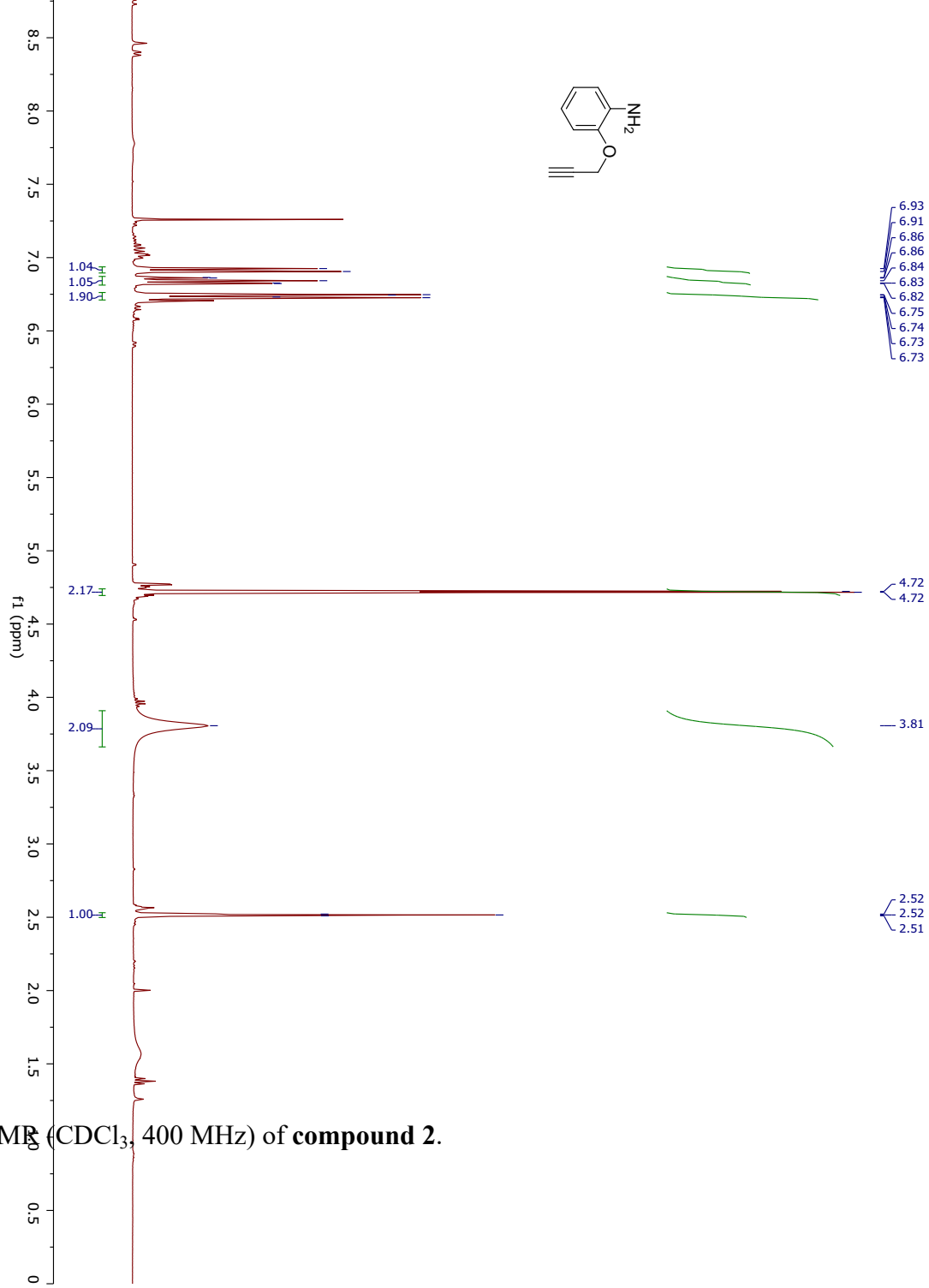


Figure S11. ^{1}H NMR ($CDCl_3$, 400 MHz) of **compound 2**.

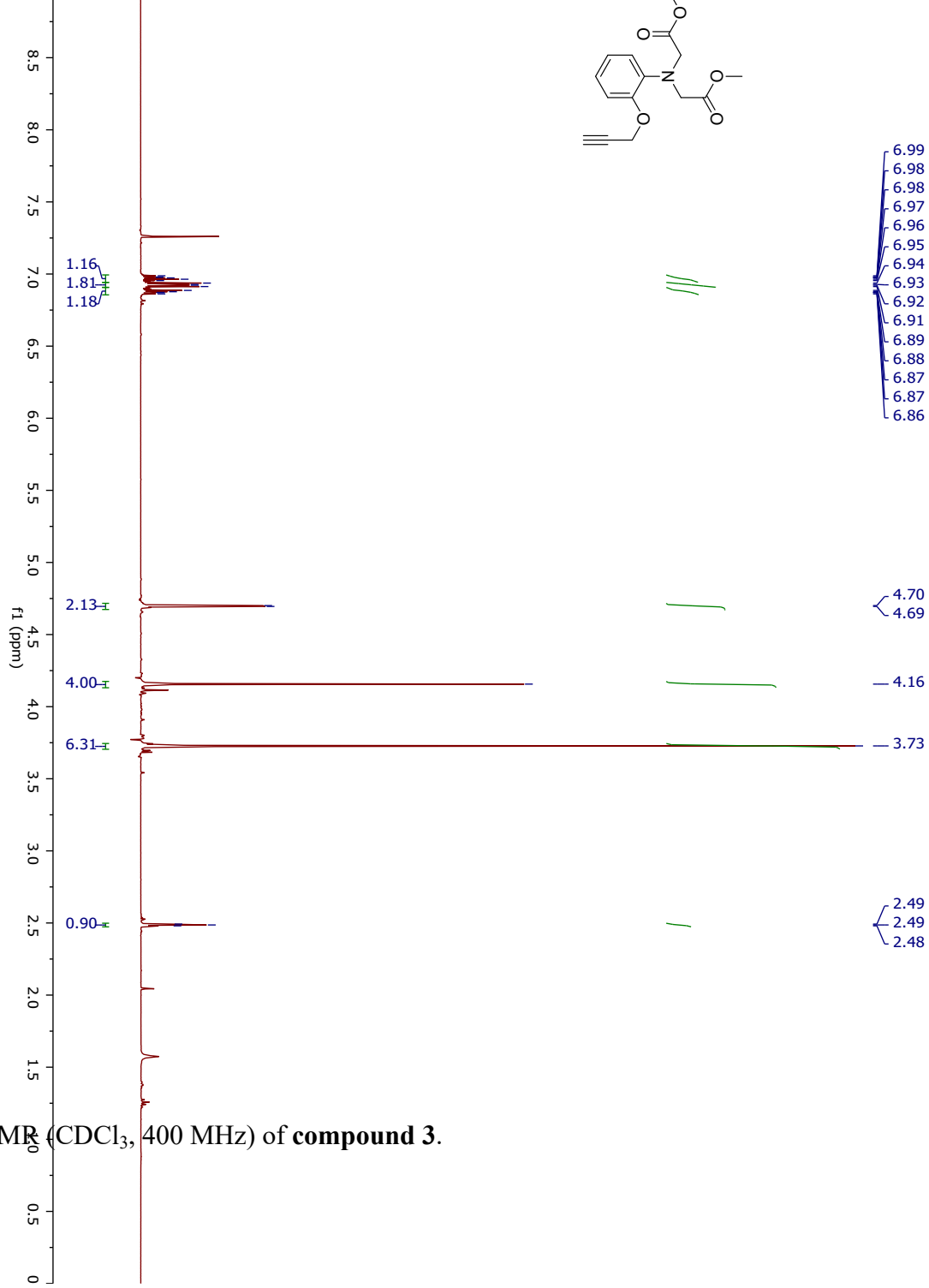


Figure S12. ^{1}H NMR ($CDCl_3$, 400 MHz) of **compound 3**.

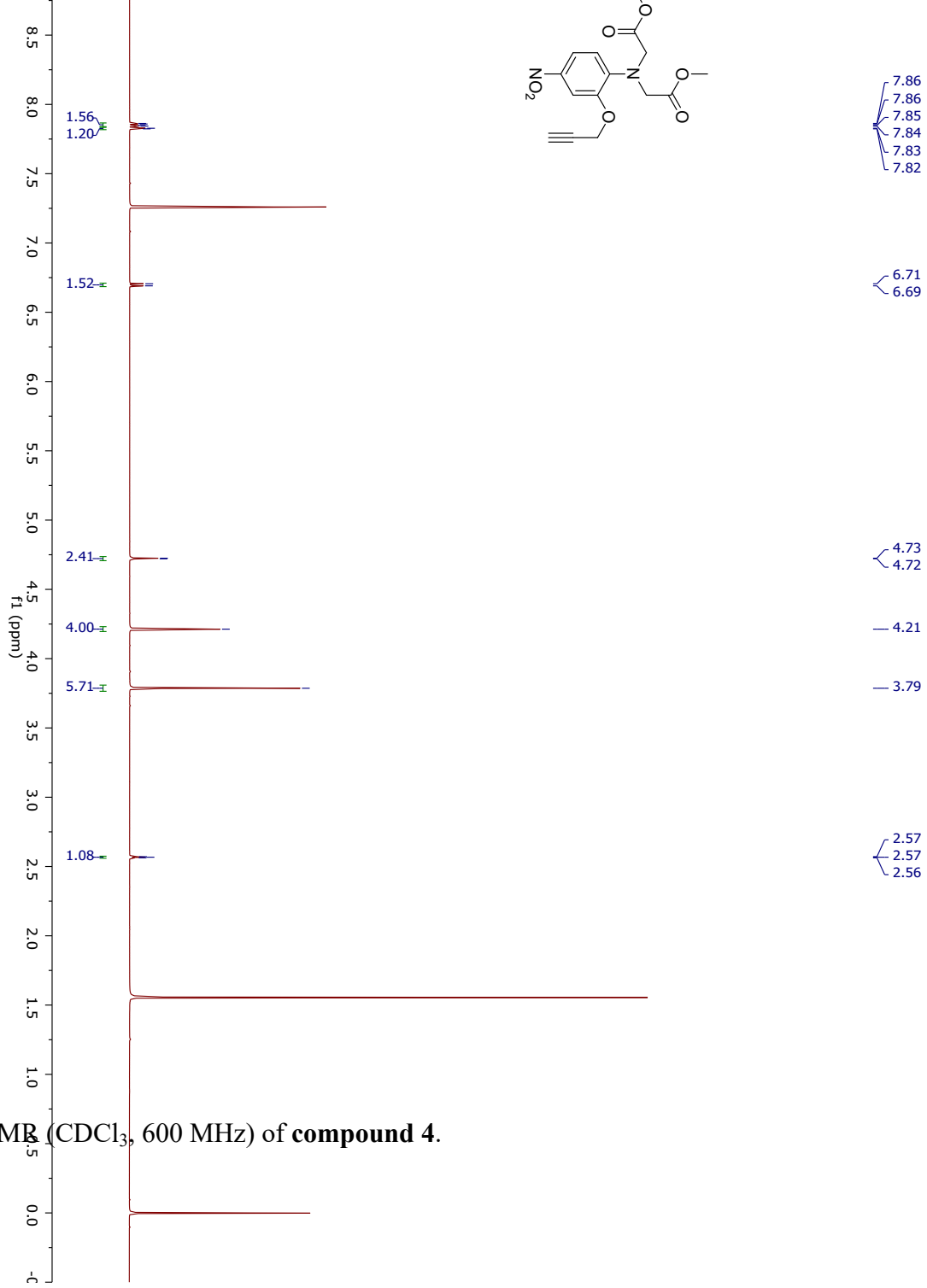


Figure S13. ^{1}H NMR ($CDCl_3$, 600 MHz) of **compound 4**.

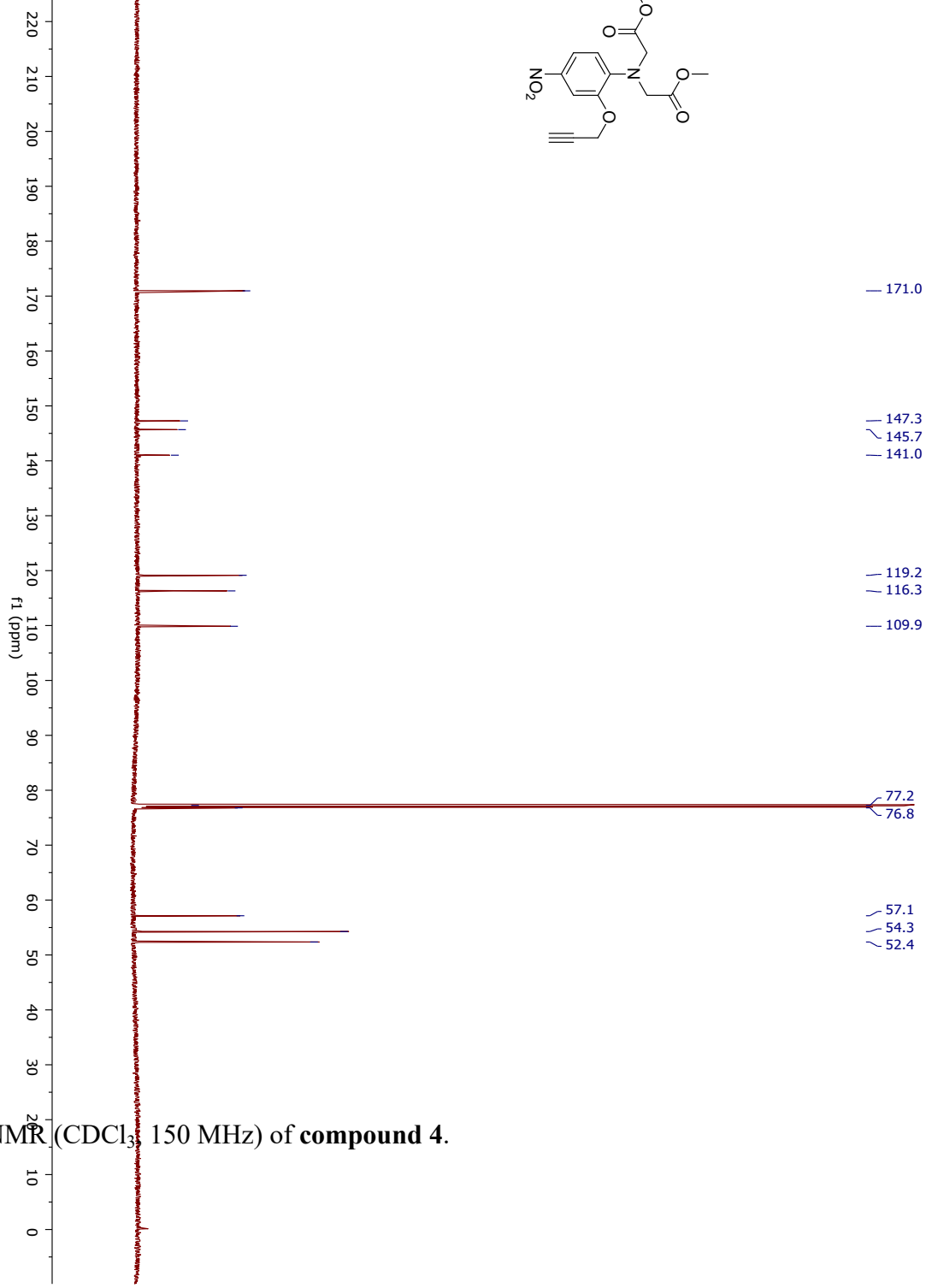


Figure S14. ^{13}C NMR ($CDCl_3$, 150 MHz) of **compound 4**.

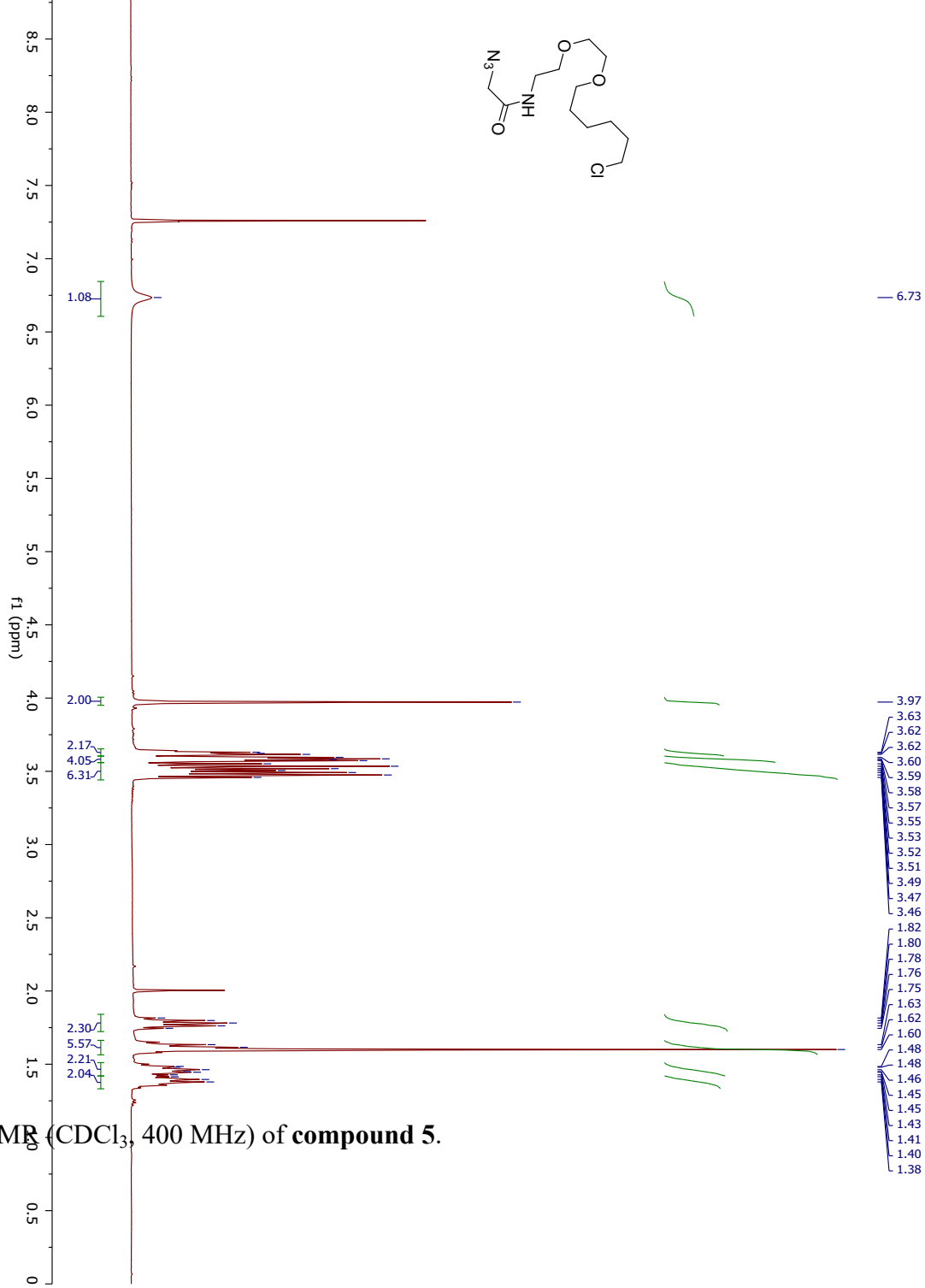


Figure S15. ^{1}H NMR ($CDCl_3$, 400 MHz) of **compound 5**.

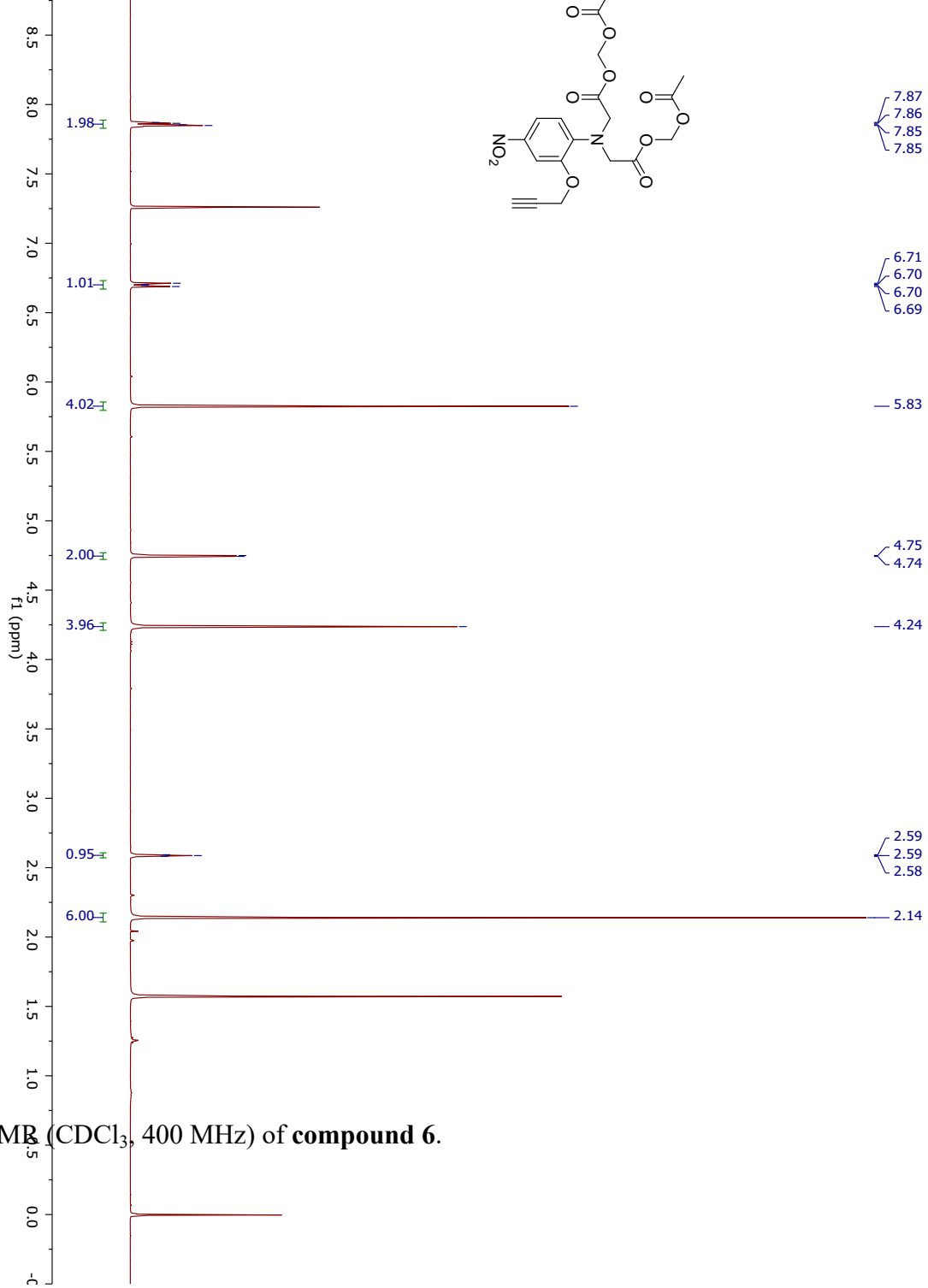


Figure S16. ^{1}H NMR ($CDCl_3$, 400 MHz) of **compound 6**.

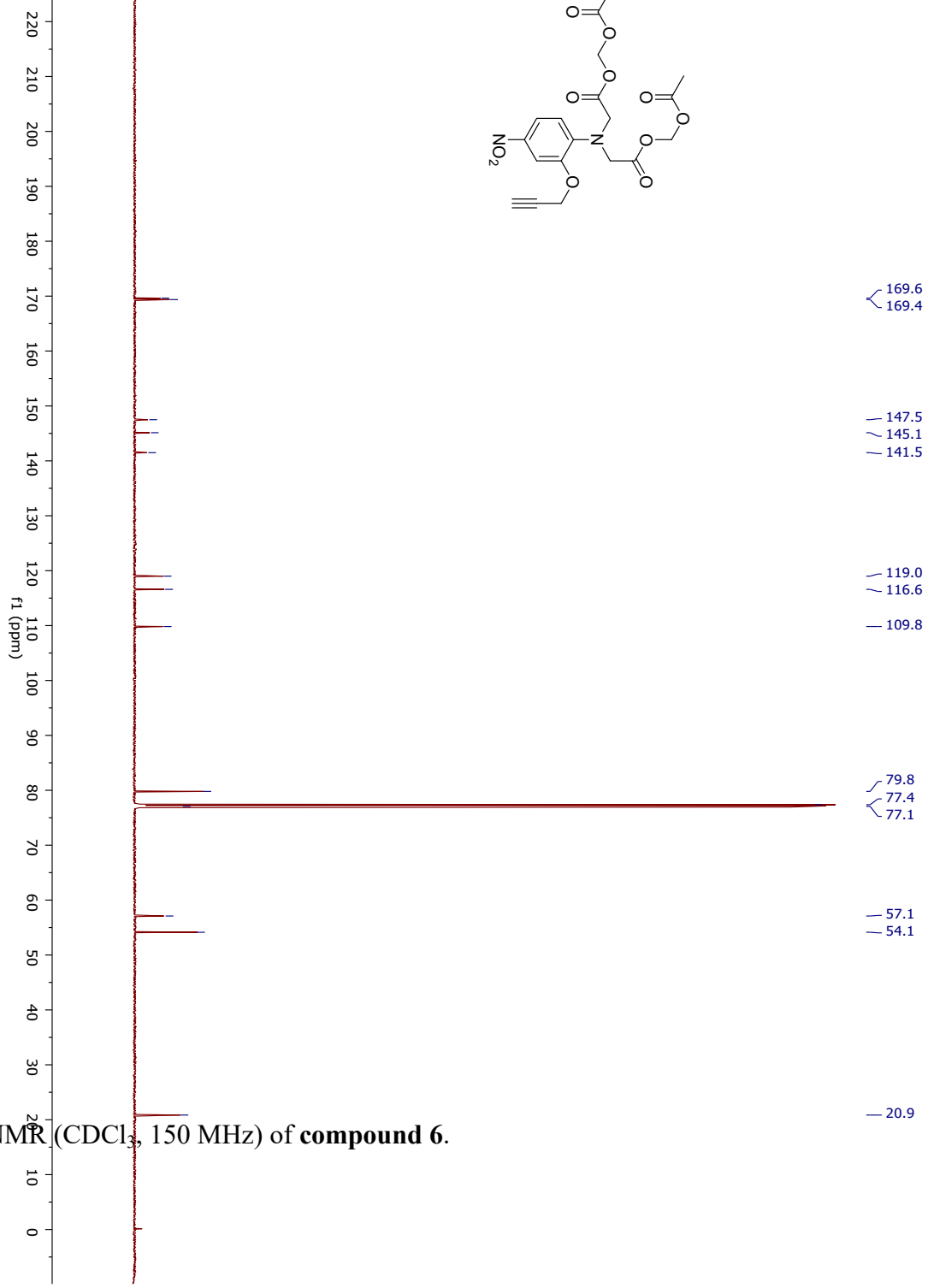


Figure S17. ^{13}C NMR ($CDCl_3$, 150 MHz) of **compound 6**.

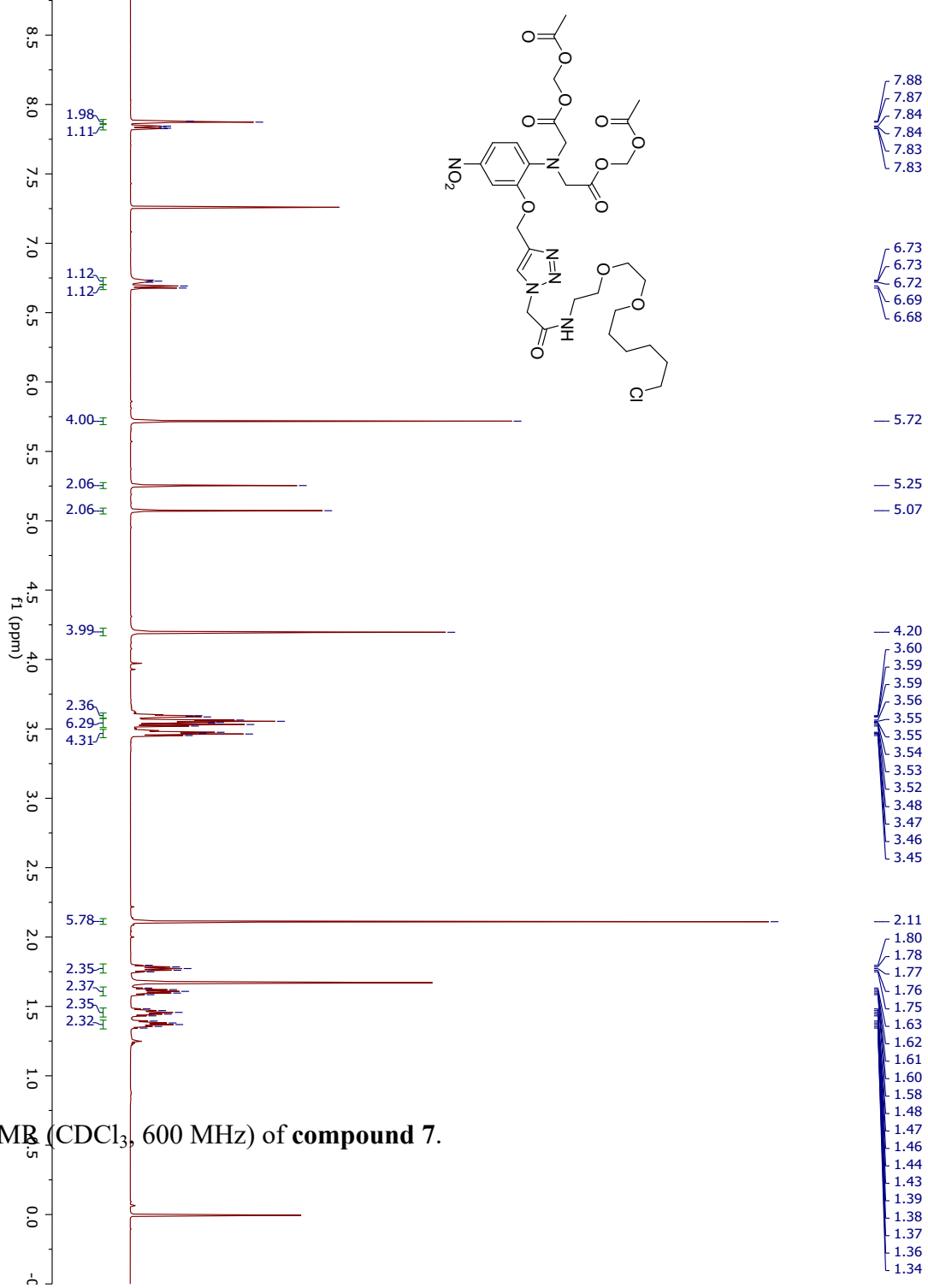


Figure S18. ^{1}H NMR ($CDCl_3$, 600 MHz) of **compound 7**.

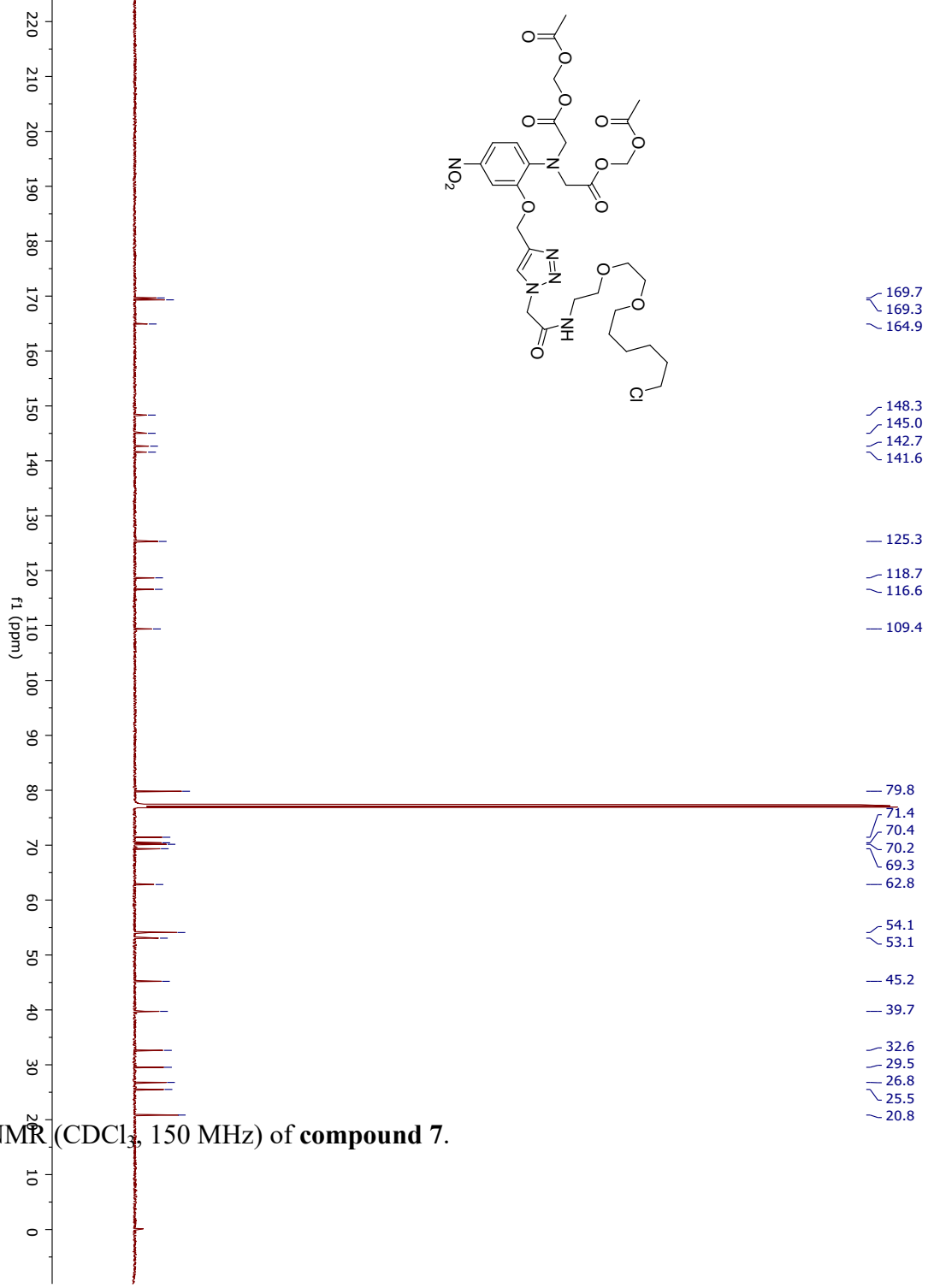


Figure S19. ^{13}C NMR ($CDCl_3$, 150 MHz) of **compound 7**.

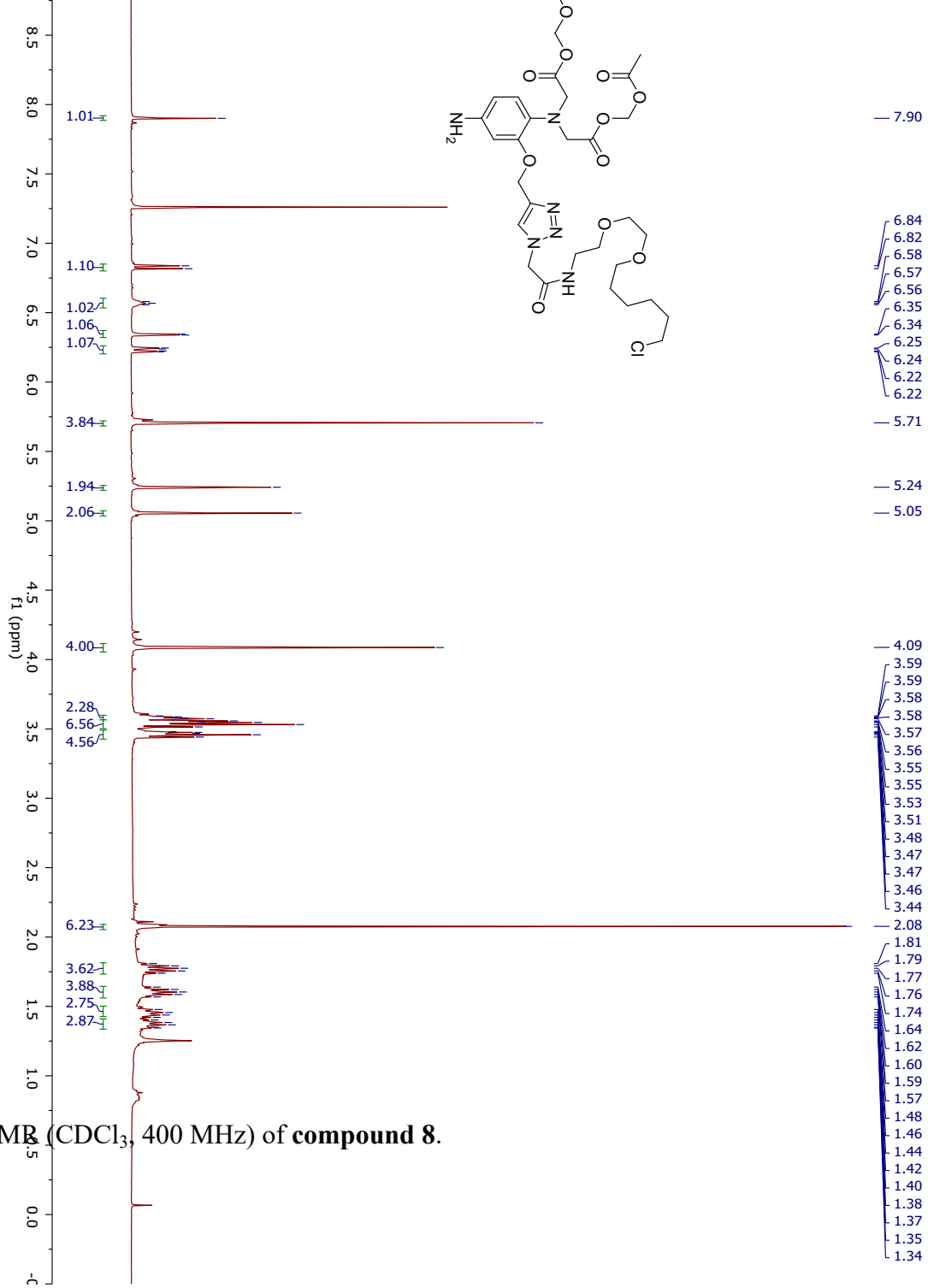


Figure S20. ^{1}H NMR ($CDCl_3$, 400 MHz) of **compound 8**.

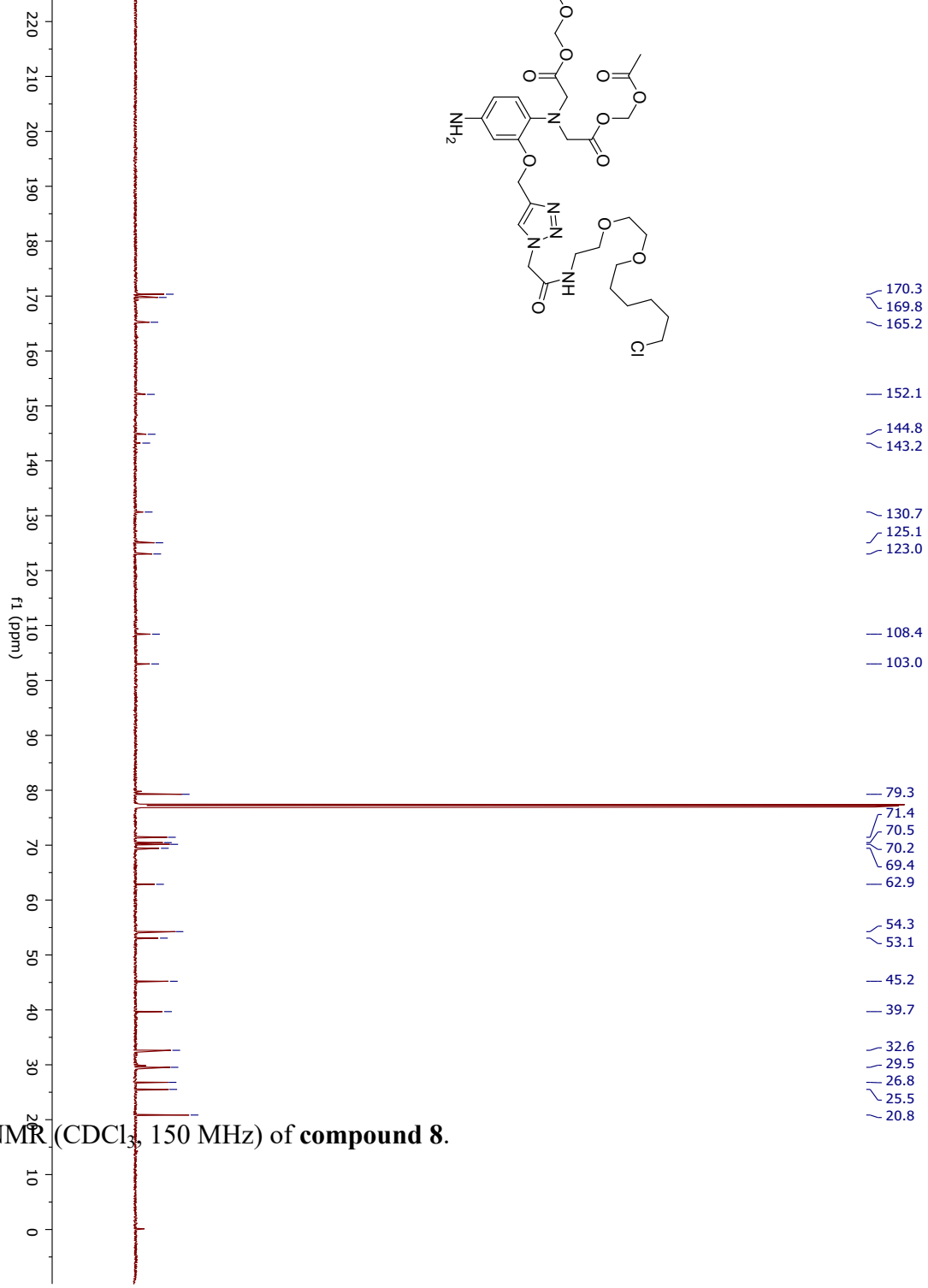


Figure S21. ^{13}C NMR ($CDCl_3$, 150 MHz) of **compound 8**.

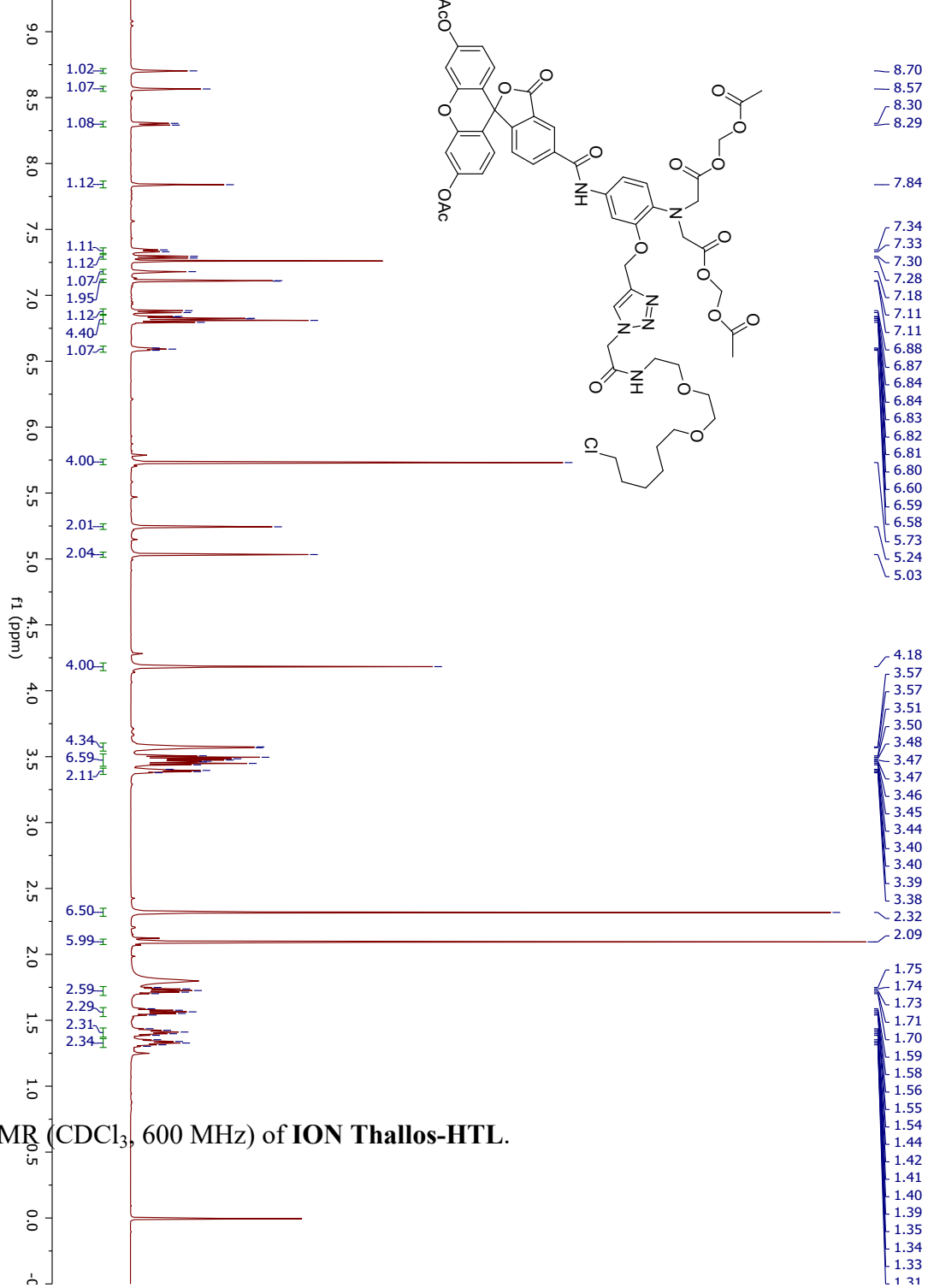


Figure S22. ^{1}H NMR ($CDCl_3$, 600 MHz) of **ION Thallos-HTL**.

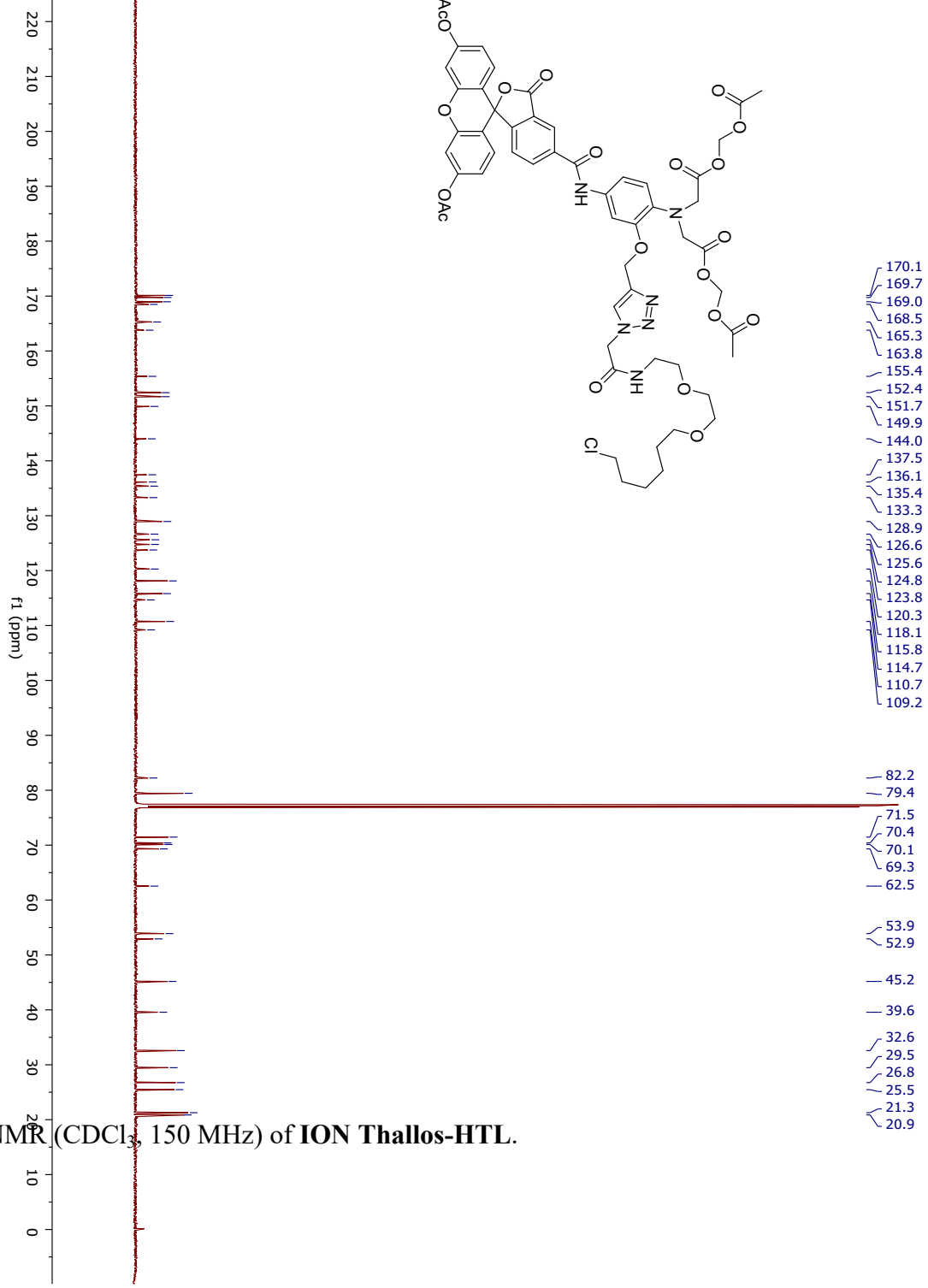


Figure S23. ^{13}C NMR ($CDCl_3$, 150 MHz) of **ION Thallos-HTL**.

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