

Supporting Material for

# **Aminoluciferins Extend Firefly Luciferase Bioluminescence into the Near-Infrared and can be Preferred Substrates over D-Luciferin**

*David M. Mofford, Gadarla Randheer Reddy, and Stephen C. Miller\**

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation St, Worcester, MA 01605

\*Correspondence should be addressed to [Stephen.miller@umassmed.edu](mailto:Stephen.miller@umassmed.edu)

## Table of Contents:

Experimental Procedures	S2-S4
Synthetic Procedures and Schemes	S5-S21
Supplementary Table 1	S22
Supplementary Figures 1-7	S23-29
Supplementary References	S30
NMR Spectra	S31-

## General

Chemicals were purchased from Aldrich, TCI, Oakwood, or Matrix Scientific unless otherwise noted. Data were plotted with GraphPad Prism 6.0. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury 400 MHz NMR spectrometer. Small molecule mass spectral data were recorded on a Waters QTOF Premier spectrometer. D-luciferin was obtained from Anaspec and 6'-aminoluciferin was obtained from Marker Gene Technologies Inc. CycLuc1, CycLuc2, 6'-MeNH-LH<sub>2</sub>, and 6'-Me<sub>2</sub>NLH<sub>2</sub> were synthesized as previously described.<sup>1</sup> Protein concentrations were determined using Coomassie Plus (Thermo Scientific). Immobilized glutathione (Thermo Scientific) was used for glutathione S-transferase (GST)-tagged protein purification. Unless otherwise stated, all protein purification steps were performed at 4°C.

## Plasmid Constructs

R218K luciferase was created as previously described.<sup>2</sup> The triple mutant R218K/L286M/S347A was generated by introducing L286M and S347A mutations identified in Harwood et al.<sup>2</sup> into the R218K luciferase, using the Quickchange site-directed mutagenesis kit (Stratagene).

## Luciferase Expression and Purification

Luciferases were expressed and purified as GST-fusion proteins from the vector pGEX6P-1 as previously described.<sup>2</sup> Briefly, JM109 cells were grown at 37°C until the OD<sub>600</sub> reached 0.5-1, induced with 0.1 mM IPTG, and incubated with shaking at 20°C overnight. Cells were pelleted at 5000 rpm in a Sorvall 2C3C Plus centrifuge (H600A rotor) at 4°C for 15 min, then flash frozen in liquid nitrogen and purified immediately or stored at -80°C. The E. coli pellets from 1 L of culture were thawed on ice, resuspended in 25 mL lysis buffer (50 mM Tris [pH 7.4], 500 mM NaCl, and 0.5% Tween 20) containing 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonification (Branson Sonifier). Dithiothreitol (DTT) was added at 10 mM, and the resulting cell lysate was clarified by centrifugation at 35,000 rpm in a Beckman 50.2 Ti rotor for 60 min at 4°C. The supernatant was batch-bound to immobilized glutathione for 1 hr at 4°C, and the beads were washed with lysis buffer containing 10 mM DTT, followed by wash buffer (50 mM Tris [pH 8.1], 250 mM NaCl, and 10 mM DTT) and storage buffer (50 mM Tris [pH 7.4], 0.1 mM EDTA, 150 mM NaCl, 1 mM TCEP). Twenty units of PreScission Protease (GE Healthcare) were added, and incubation continued for 4 h or overnight at 4°C to cleave the GST-fusion and elute the untagged luciferase protein.

## Purified Protein Luminescence Assays

Luminescence assays were initiated by adding 30 µL of purified luciferase in enzyme buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 1 mM TCEP, and 0.8 mg/mL BSA) to 30 µL 2x substrate in substrate buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 8 mM MgSO<sub>4</sub>, and 4 mM ATP) in a black 96-well plate (Costar 3915). Imaging was performed one minute after enzyme addition using a Xenogen IVIS-100 at a final enzyme concentration of 10 nM and final substrate concentrations ranging from 0.12 to 250 µM. Data acquisition and analysis was performed with Living Image® software. Data are reported as total flux (p/s) for each ROI corresponding to each well of the 96-well plate.

## Bioluminescence Emission Scans

Each purified luciferase in enzyme buffer was rapidly injected into a cuvette containing substrate in substrate buffer to a final enzyme concentration of 100 nM and a final substrate concentration of 10 µM. The emission from 400 to 800 nm was recorded in a SPEX FluoroMax-3 fluorimeter with closed excitation slits 10 s after injection.

## Burst Kinetics Assays

Using a Turner Biosystems 20/20<sup>n</sup> luminometer, 40 µL of purified luciferase in enzyme injection buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 0.625 mM TCEP, and 0.5 mg/mL BSA) was rapidly injected into a clear Eppendorf tube containing 10 µL of substrate in substrate injection buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 20 mM MgSO<sub>4</sub>, and 10 mM ATP) to a final enzyme concentration of 0.2 nM and a final luciferin

substrate concentration of 250  $\mu\text{M}$ . Measurements were taken every 0.2 s for 1 s pre-injection and 120 s post-injection. Data acquisition was performed with SIS for 2020n v1.9.0 software. Data are reported as Relative Light Units (RLU). To correct for the wavelength sensitivity of the PMT in the 20/20<sup>n</sup>, total flux was also measured using the IVIS-100 as described above with a final enzyme concentration of 10 nM and a final substrate concentration of 250  $\mu\text{M}$ . Data from the IVIS and from the 20/20<sup>n</sup> at the 60 s time point were normalized to the WT + LH<sub>2</sub> value. The correction factor of each enzyme/substrate pair was calculated by dividing the normalized IVIS data by the normalized 20/20<sup>n</sup> data. All 20/20<sup>n</sup> data were then multiplied by their respective correction factors.

### **Cell Culture**

Chinese hamster ovary-K1 (CHO-K1) cells were grown in a CO<sub>2</sub> incubator at 37°C with 5% CO<sub>2</sub> and were cultured in F-12K Nutrient Mixture (GIBCO) supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin.

### **Transfections**

The WT firefly luciferase Luc2 gene (Promega) and codon optimized mutants were cloned into the BamHI and NotI sites of pcDNA 3.1 and transfected into CHO-K1 cells for live and lysed cell experiments. Transient transfections were performed at RT using Lipofectamine 2000 on cells plated at 60–75% confluency in 96-well black tissue culture-treated plates (Costar 3916) for live cell assays, or 6-well plates for lysed cell assays. For live cells, 0.075  $\mu\text{g}$  DNA/well was transfected; for lysed cells, 2.25  $\mu\text{g}$  DNA/well was transfected. Assays were performed in triplicate 24 hr after transfection.

### **Live and Lysed CHO Cell Luminescence Assays**

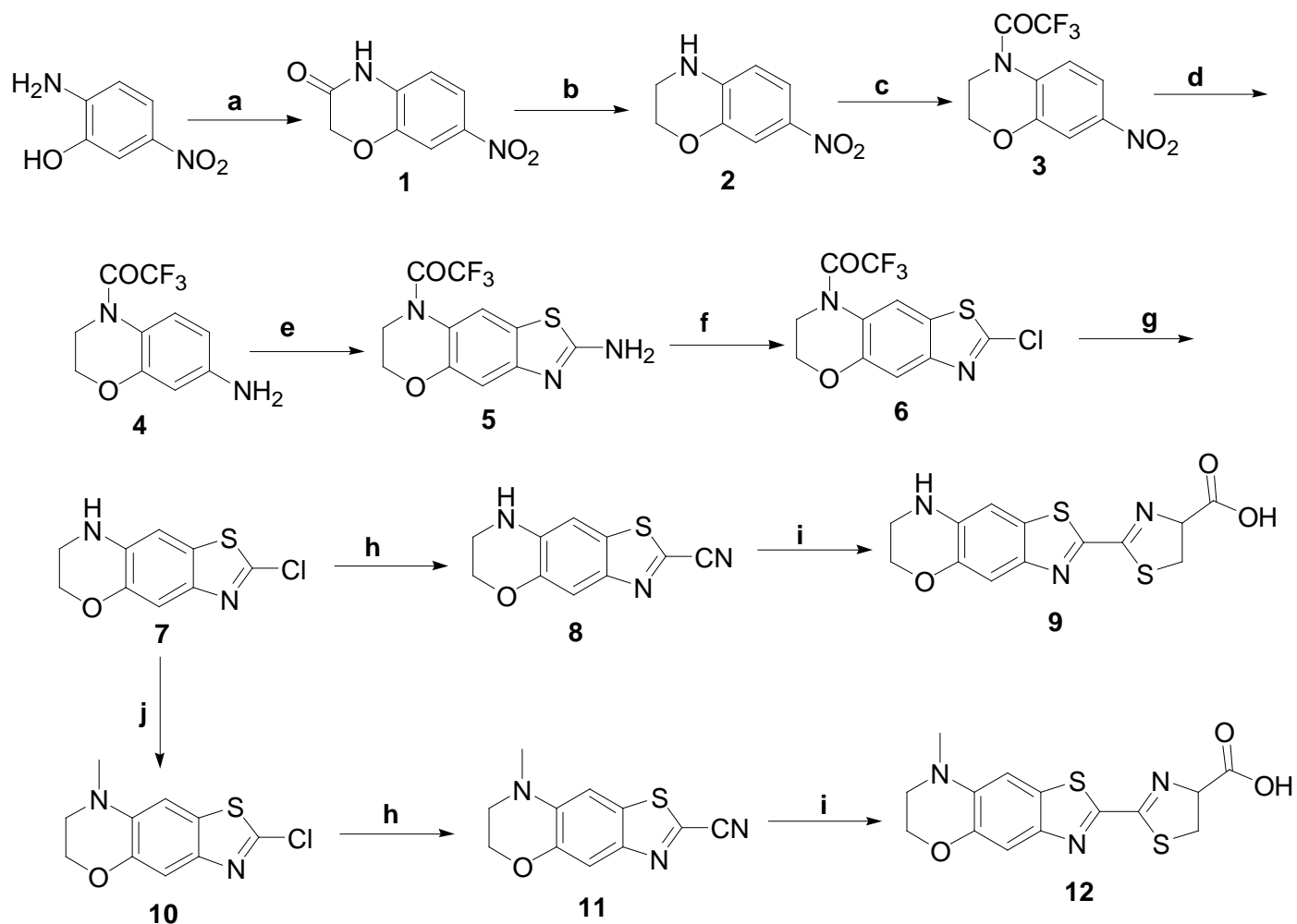
Transfected CHO cells were washed with HBSS. For live cell imaging, the cells in 96-well plates were incubated with 60  $\mu\text{L}$  of substrate in HBSS at final concentrations ranging from 0.122 to 1000  $\mu\text{M}$ . Imaging was performed using the IVIS-100 with no emission filter, three minutes after addition of substrate. Additional images were taken through a Cy5.5 bandpass filter (695-770 nm) four minutes after substrate addition. Cells grown in 6-well plates were first lysed for 20 min at RT with Passive Lysis Buffer (1 mL per well). Luminescence assays were initiated by adding 30  $\mu\text{L}$  of lysate to 30  $\mu\text{L}$  of 2x substrate in lysed cell substrate buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 8 mM MgSO<sub>4</sub>, 4 mM ATP, 6 mg/mL BSA, 250  $\mu\text{M}$  Coenzyme A, and 33 mM DTT) in a black 96-well plate (Costar 3915) with final substrate concentrations ranging from 0.122 to 250  $\mu\text{M}$ . Imaging was performed with the emission filter open one minute after addition of substrate. Additional images were taken through the Cy5.5 filter two minutes after substrate addition.

### **Western Blot**

Transfected CHO cells in 6-well plates were washed with HBSS and lysed for 20 min at RT with Passive Lysis Buffer (250  $\mu\text{L}$  per well). Cell debris was pelleted by centrifugation at 13,000 RPM at 4°C for 10 minutes. Total protein concentration of the supernatant was determined using Coomassie Plus Bradford Reagent and 18  $\mu\text{g}$  total lysate protein was used for electrophoresis. Samples were prepared by adding 167 mM DTT and 1x SDS gel loading buffer to each lysate to a final volume of 27  $\mu\text{L}$ . Samples were run on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated in blocking buffer (5% nonfat dry milk in western wash buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween-20]) for 45 minutes. Luciferase primary antibody (Promega, Cat. # G7451) was diluted 1:1000 in blocking buffer to 6 mL final volume and incubated with the membranes at room temperature for 2 hours. Membranes were washed with Western wash buffer (4 x 6 mL) and incubated at room temperature with 6 mL secondary anti-goat antibody-HRP (Santa Cruz, Cat. # SC-2033) diluted 1:4000 in blocking buffer. The membranes were again washed (4 x 6 mL) and incubated with SuperSignal West Dura Extended Duration Substrate (Pierce) for 5 minutes. The chemiluminescence was imaged using an LAS-3000 CCD camera and quantified by Multi Gauge software (FujiFilm). Antibody was then stripped by washing the membranes with Stripping Buffer (100 mM glycine, 20 mM magnesium acetate, 50 mM potassium chloride, pH 2.2 [Bio-Rad]; 2 x 10 mL), and western wash buffer (3 x 6 mL). Membranes were then re-blocked and

incubated overnight at 4°C with 6 mL primary anti- $\alpha$ -tubulin antibody (Sigma Aldrich, Cat. # T9026) diluted 1:3000 in blocking buffer. Membranes were washed with western wash buffer (4 x 6 mL) and incubated at room temperature with 6 mL secondary anti-mouse antibody-HRP (GE Healthcare, Cat. # RPN4201) diluted 1:10000 in blocking buffer for 45 minutes. The membranes were again washed (4 x 6 mL) and incubated with SuperSignal West Dura Extended Duration Substrate (Pierce) for 5 minutes. The chemiluminescence was imaged using an LAS-3000 CCD camera and quantified by Multi Gauge software (FujiFilm). Purified recombinant enzymes were tested using the same procedure by loading 50 ng for electrophoresis and incubating with primary luciferase antibody overnight at 4°C.

## Synthetic Methods



Reagents and conditions: (a) Methyl bromoacetate,  $K_2CO_3$ , DMF, 90 °C, 24h; (b)  $BH_3$ -THF (1M in THF), THF, reflux, 30 min; (c) TFAA, TEA,  $CH_2Cl_2$ , rt, 45 min; (d)  $SnCl_2 \cdot 2H_2O$ , EtOH, reflux, 4h; (e) KSCN,  $Br_2$ , AcOH, rt, 21h; (f) *t*-Butyl nitrite, CuCl,  $CH_3CN$ , rt, 2h, 65°C, 1h; (g)  $NaBH_4$ ,  $CH_3OH$ , rt, 15 min; (h) KCN, DMSO, 130°C, 3h; (i) D-cysteine, 50 mM aqueous sodium phosphate buffer, pH 8,  $CH_3OH$ ; (j) HCHO,  $NaBH(OAc)_3$ , DCE, rt, 1h.

### Supplementary Scheme 1.

**7-Nitro-4H-benzo [1, 4] oxazin-3-one (1):** Methyl bromoacetate (3.58 g, 39 mmol), 2-amino-5-nitrophenol (5 g, 32.5 mmol) and  $K_2CO_3$  (11.5 g, 81.0 mmol) in DMF (15 mL) were heated at 90°C for 24h. The reaction mixture was allowed to cool, then diluted with water. The resulting precipitate was collected by filtration and dried under vacuum to give **1** as a brown solid (4.5 g, 71%).  $^1H$ -NMR (400 MHz,  $d_6$ -DMSO):  $\delta$  11.30 (s, 1H), 7.89 (dd, 1H,  $J = 2.5, 8.7$  Hz), 7.75 (d, 1H,  $J = 2.5$  Hz), 7.04 (d, 1H,  $J = 8.7$  Hz), 4.71 (s, 2H). HRMS (ESI<sup>+</sup>) Calcd for  $C_8H_7N_2O_4Na$ : 217.0225, Found: 217.0243.

**7-Nitro-3,4-dihydro-2H-benzo[1,4]oxazine (2):** A solution of **1** (2 g, 10.3 mmol) in THF (10 mL) was treated with 1M  $BH_3/THF$  (35 mL). The solution was refluxed for 30 min then cooled to 0 °C, and quenched with 1N HCl (20 mL). The solution was stirred for 30 min then concentrated and extracted with ethyl acetate (2 x 100 mL). Evaporation of the ethyl acetate yielded **2** (1.5 g, 80 %).  $^1H$ -NMR (400 MHz,  $d_6$ -DMSO):  $\delta$  7.66 (dd, 1H,  $J = 9.2, 2.4$  Hz), 7.53 (s, 1H), 7.45 (d, 1H,  $J = 2.4$  Hz), 6.60 (d, 1H,  $J = 8.8$  Hz) 4.12 (t, 2H,  $J = 6.8$  Hz), 3.41-3.31 (m, 2H). HRMS (ESI<sup>+</sup>) Calcd for  $C_8H_9N_2O_3$ : 181.0613, Found: 181.0619.

**2,2,2-Trifluoro-1-(7-nitro-2,3-dihydro-benzo[1,4]oxazin-4-yl)-ethanone (3):** 7-Nitro-4H-benzo[1,4]oxazin-3-one (1.5 g, 8.3 mmol) and triethylamine (0.924 g, 10.0 mmol) were stirred in dichloromethane (20 mL) as trifluoroacetic anhydride (4.5 g, 12.5 mmol) was added dropwise. This mixture was stirred for 45 min; then water (50 mL) was added. After stirring for 10 min, the mixture was acidified with 5M HCl. The organic layer was washed with brine, dried and evaporated to give a yellow solid (2.1 g, 91 %).  $^1H$ -NMR (400 MHz,  $CDCl_3$ ):  $\delta$  8.17 (s, 1H), 7.82-7.79 (m, 2H), 4.47 (t, 2H,  $J = 4.8$  Hz), 4.05 (t, 2H,  $J = 4.8$  Hz).  $^{13}C$ -NMR (100 MHz,  $CDCl_3$ ):  $\delta$  155.1 (q,  $^2J_{CF} = 37$  Hz), 147.3, 146.0, 129.5, 124.4, 116.2 (q,  $^1J_{CF} = 287$  Hz), 115.9, 113.4, 65.9, 43.4.  $^{19}F$ -NMR (376 MHz,  $CDCl_3$ ): -68.9. HRMS (ESI<sup>+</sup>) Calcd for  $C_{10}H_8F_3N_2O_4$ : 277.0436, Found: 277.0438.

**1-(7-Amino-2,3-dihydro-benzo[1,4]oxazin-4-yl)-2,2,2-trifluoro-ethanone (4):**  $SnCl_2 \cdot 2H_2O$  (4.89 g, 21.73 mmol) was added to a solution of **3** (2 g, 7.24 mmol) in ethanol (20 mL) and the reaction mixture was heated to reflux for 4h. After cooling to room temperature the solvent was evaporated under vacuum. The resulting residue was dissolved in saturated  $NaHCO_3$  (50 mL) and extracted with ethyl acetate (2 x 100 mL). After washing with brine and evaporation of the organic layer under vacuum, the residue was purified by flash chromatography (25 % ethyl acetate: hexane) to give **4** (1.2 g, 67 %).  $^1H$ -NMR (400 MHz,  $CDCl_3$ ):  $\delta$  7.78 (d, 1H,  $J = 9.2$ ), 6.28 (dd, 1H,  $J = 9.2, 2.4$  Hz), 6.22 (d, 1H,  $J = 2.4$  Hz), 4.33 (t, 2H,  $J = 4.8$  Hz), 3.92 (t, 2H,  $J = 4.8$  Hz), 3.56 (s, 2H).  $^{13}C$ -NMR (100 MHz,  $CDCl_3$ ):  $\delta$  153.9 (q,  $^2J_{CF} = 36$  Hz), 148.0, 146.0, 124.9, 116.7 (q,  $^1J_{CF} = 286$  Hz), 115.5, 112.4, 108.2, 102.8, 65.9, 43.9.  $^{19}F$ -NMR (376 MHz,  $CDCl_3$ ): -68.8. HRMS (ESI<sup>+</sup>) Calcd for  $C_{10}H_{10}F_3N_2O_2$ : 247.0694, Found: 247.0707.

**1-(2-Amino-6,7-dihydro-5-oxa-1-thia-3,8-diaza-cyclopenta[b]naphthalen-8-yl)-2,2,2-trifluoro-ethanone (5):** A solution of **4** (1 g, 4.06 mmol) and potassium thiocyanate (1.57 g, 16.2 mmol) in AcOH (20 mL) was stirred at 20 °C for 10 min. Bromine (0.64 g, 4.06 mmol) was added dropwise over 20 min. The reaction mixture was stirred at room temperature for an additional 21 h. The reaction mixture was then poured onto crushed ice and the pH was adjusted to 8 using  $NH_4OH$ . The resulting precipitate was vacuum-filtered and dried to give **5**, which was used for next step without further purification (1.2 g, 97 %). HRMS (ESI<sup>+</sup>) Calcd for  $C_{11}H_9F_3N_3O_2S$ : 304.0367, Found: 304.0349.

**1-(2-Chloro-6,7-dihydro-5-oxa-1-thia-3,8-diaza-cyclopenta[b]naphthalen-8-yl)-2,2,2-trifluoro-ethanone (6):** To a mixture of t-butyl nitrite (0.56 g, 5.44 mmol), cupric chloride (0.43 mg, 4.3 mmol) and acetonitrile (10 mL) was added **5** (1.1 g, 3.63 mmol) in portions over 1h. The reaction mixture was stirred at room temperature for 2h, then heated to 65 °C for 1h. The mixture was cooled, filtered and the filtrate was poured into 6N HCl and extracted with ethyl acetate. After concentration the crude product was used for the next step without further purification (1 g, 85 %).  $^1H$ -NMR (400 MHz,  $CDCl_3$ ):  $\delta$  8.46 (s, 1H), 7.50 (s, 1H), 4.44 (t, 2H,  $J = 4.8$  Hz), 4.05 (t, 2H,  $J = 4.8$  Hz).  $^{13}C$ -NMR (100 MHz,  $CDCl_3$ ):  $\delta$  155.5, 154.9 (q,  $^1J_{CF} = 35$  Hz), 149.9, 146.9,

128.5, 123.5, 116.4 (q,  $^2J_{CF} = 287$  Hz), 116.0, 110.8, 65.8, 43.7.  $^{19}\text{F}$ -NMR (376 MHz,  $\text{CDCl}_3$ ): -68.9. HRMS (ESI<sup>+</sup>) Calcd for  $\text{C}_{11}\text{H}_7\text{ClF}_3\text{N}_2\text{O}_2\text{S}$ : 322.9869, Found: 322.9859.

**2-Chloro-7, 8-dihydro-6H-5-oxa-1-thia-3, 8-diaza-cyclopenta[b]naphthalene (7):** Compound **6** (1 g, 3.11 mmol) was stirred in methanol (20 mL) as  $\text{NaBH}_4$  (461 mg, 12.4 mmol) was added in portions over five minutes. The mixture was stirred for 15 min, diluted with water and extracted with ethyl acetate. The organic layer was washed with brine and evaporated. The crude product was purified by flash chromatography (25 % ethyl acetate: hexane) to give **7** (0.35 g, 49 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.32 (s, 1H), 6.83 (s, 1H), 4.27 (t, 2H,  $J = 4.8$  Hz), 4.04 (s, 1H), 3.46 (t, 2H,  $J = 4.0$  Hz).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  148.8, 144.5, 144.3, 134.1, 129.7, 110.2, 104.9, 65.2, 40.8. HRMS (ESI<sup>+</sup>) Calcd for  $\text{C}_9\text{H}_8\text{ClN}_2\text{OS}$ : 227.0046, Found: 227.0020.

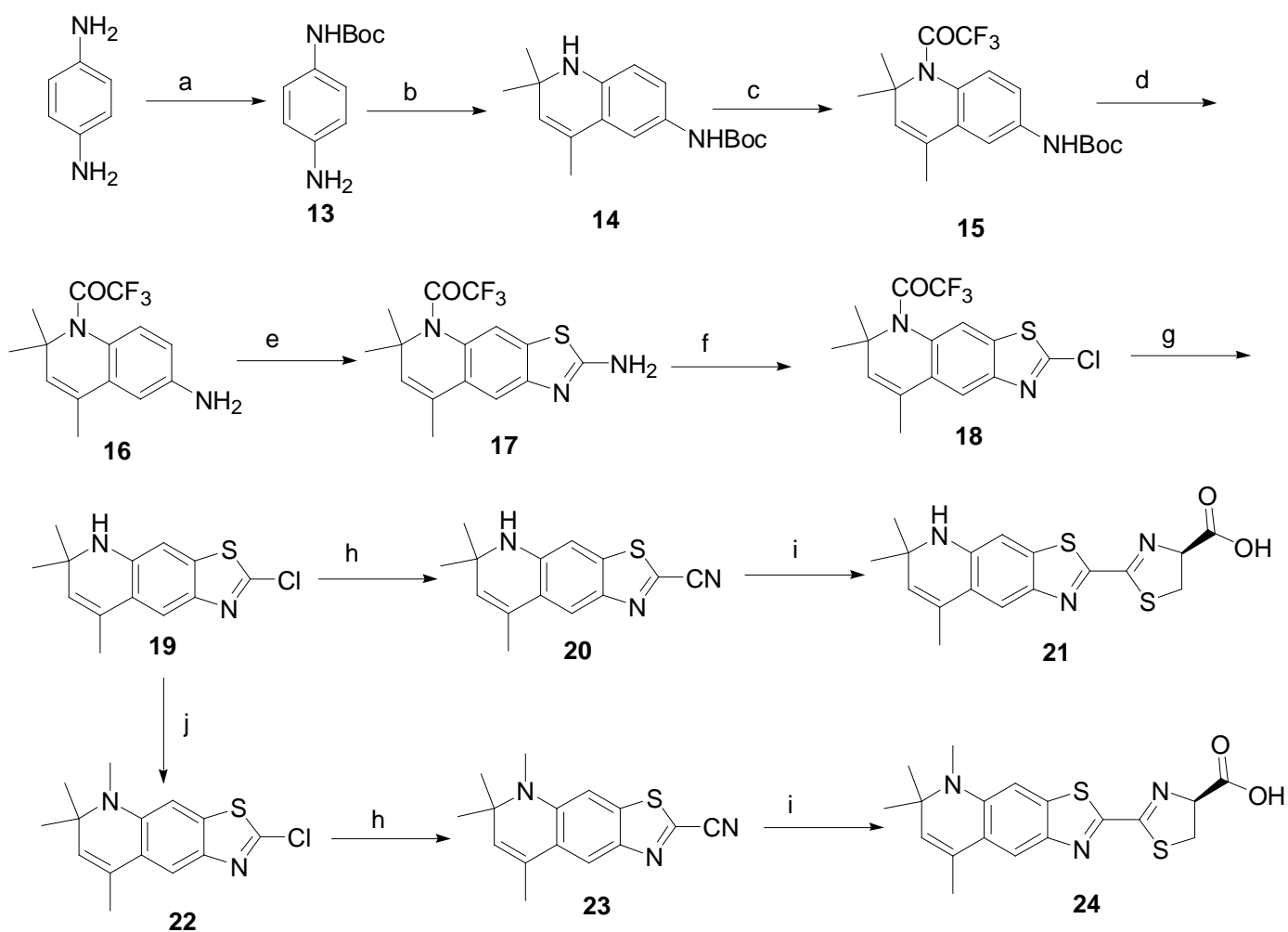
**7,8-Dihydro-6H-5-oxa-1-thia-3,8-diaza-cyclopenta[b]naphthalene-2-carbonitrile (8):** Potassium cyanide (28.7 mg, 0.44 mmol) was added to a solution of **7** (50 mg, 0.22 mmol) in DMSO (5 mL) and stirred for 3h at 130°C. The reaction was cooled to room temperature, poured into water, and extracted with ethyl acetate (2 x 20 mL). The organic phase was evaporated to dryness and purified by flash chromatography using 10% ethyl acetate: hexane as eluent (13 mg, 27%).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.54 (s, 1H), 6.95 (s, 1H), 4.41 (s, 1H), 4.31 (t, 2H,  $J = 4.8$  Hz), 3.55-3.52 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  145.8, 145.6, 137.3, 130.5, 118.6, 114.0, 111.4, 103.2, 64.9, 40.5. HRMS (ESI<sup>+</sup>) Calcd for  $\text{C}_{10}\text{H}_8\text{N}_3\text{OS}$ : 218.0388, Found: 218.0416.

**CycLuc3; 2-(7,8-Dihydro-6H-5-oxa-1-thia-3,8-diaza-cyclopenta[b]naphthalen-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (9):** D-cysteine (2.9 mg, 0.016 mmol) was dissolved in 1 mL of 50 mM aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **8** (3 mg, 0.013 mmol) in 1 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer and washed with ethyl acetate. The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 5 mL). The solvent was removed by rotary evaporation to yield **9** as an orange red solid (2 mg, 47 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3 + 2$  drops  $\text{CD}_3\text{OD}$ ):  $\delta$  7.43 (s, 1H), 6.91 (s, 1H), 5.27 (t, 1H,  $J = 4.8$  Hz), 4.23 (t, 2H,  $J = 4.4$  Hz), 3.73-3.61 (m, 2H), 3.43 (t, 2H,  $J = 4.4$  Hz).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3 + 2$  drops  $\text{CD}_3\text{OD}$ ):  $\delta$  172.3, 166.9, 156.0, 146.3, 144.8, 136.1, 130.7, 110.8, 104.2, 78.2, 64.9, 40.4, 35.2. HRMS (ESI<sup>+</sup>) Calcd for  $\text{C}_{13}\text{H}_{12}\text{N}_3\text{O}_3\text{S}_2$ : 322.0320, Found: 322.0354.

**2-Chloro-8-methyl-7,8-dihydro-6H-5-oxa-1-thia-3,8-diaza-cyclopenta[b]naphthalene (10):** Compound **7** (50 mg, 0.22 mmol) and formaldehyde (26.4 mg, 0.88 mmol) were dissolved in dichloroethane (10 mL). Sodium triacetoxyborohydride (70.3 mg, 0.33 mmol) was added and the reaction mixture was stirred for 1h at ambient temperature. The reaction was quenched with 1N NaOH (10 mL). The product was extracted with ethyl acetate (2 x 20 mL), washed with brine and dried ( $\text{MgSO}_4$ ). The solvent was removed by rotary evaporation and the resulting solid was purified by flash chromatography using 8% ethyl acetate: hexane as eluent (20 mg, 37 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.31 (s, 1H), 6.86 (s, 1H), 4.33-4.31 (m, 2H), 3.35-3.33 (m, 2H), 2.94 (s, 3H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  148.6, 144.6, 143.5, 136.7, 130.1, 109.2, 101.9, 64.9, 48.9, 39.2. HRMS (ESI<sup>+</sup>) Calcd for  $\text{C}_{10}\text{H}_{10}\text{ClN}_2\text{OS}$ : 241.0202, Found: 241.0206.

**8-Methyl-7,8-dihydro-6H-5-oxa-1-thia-3,8-diaza-cyclopenta[b]naphthalene-2-carbonitrile (11):** Potassium cyanide (11.5 mg, 0.17 mmol) was added to a solution of **10** (20 mg, 0.083 mmol) in DMSO (3 mL) and stirred for 3h at 130°C. The reaction was cooled to room temperature, poured into water, and extracted with ethyl acetate (2 x 20 mL). The organic phase was evaporated to dryness and purified by flash chromatography using 10% ethyl acetate: hexane as eluent (7 mg, 36 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.50 (s, 1H), 6.94 (s, 1H), 4.33 (t, 2H,  $J = 4.8$  Hz), 3.45 (t, 2H,  $J = 4.8$  Hz), 3.03 (s, 3H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  145.9, 145.2, 139.4, 131.2, 114.1, 110.3, 107.9, 100.1, 64.5, 48.7, 38.9. HRMS (ESI<sup>+</sup>) Calcd for  $\text{C}_{11}\text{H}_{10}\text{N}_3\text{OS}$ : 232.0545, Found: 232.0552.

**CycLuc4; 2-(8-Methyl-7,8-dihydro-6H-5-oxa-1-thia-3,8-diaza-cyclopenta[b]naphthalen-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (12):** D-cysteine (3.6 mg, 0.02 mmol) was dissolved in 1 mL of 50 mM aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **11** (4 mg, 0.017 mmol) in 1 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer and washed with ethyl acetate. The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 5 mL). The solvent was removed by rotary evaporation to yield **12** as an orange red solid (4 mg, 70%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub> + 2 drops CD<sub>3</sub>OD): δ 7.41 (s, 1H), 6.92 (s, 1H), 5.26 (t, 1H, *J* = 5.2 Hz), 4.26 (t, 2H, *J* = 4.8 Hz), 3.72-3.61 (m, 2H), 3.35 (t, 2H, *J* = 4.8 Hz), 2.95 (s, 3H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub> + 2 drops CD<sub>3</sub>OD): δ 172.2, 166.8, 155.8, 145.9, 145.1, 138.2, 131.3, 109.9, 101.2, 78.2, 64.6, 50.0, 38.9, 35.2. HRMS (ESI<sup>+</sup>) Calcd for C<sub>14</sub>H<sub>14</sub>N<sub>3</sub>O<sub>3</sub>S<sub>2</sub>: 336.0477, Found: 336.0467.



Reagents and conditions: (a)  $\text{Boc}_2\text{O}$ , 1,4 dioxane, 0 °C - rt, overnight; (b) Acetone, t-butyl catechol,  $\text{MgSO}_4$ , Iodine, 63 °C, 2 days; (c) TFAA, TEA,  $\text{CH}_2\text{Cl}_2$ , rt, 45 min; (d) 15% TFA in DCM, rt, 5h; (e) KSCN,  $\text{Br}_2$ , AcOH, rt, 21h; (f) t-Butyl nitrite, CuCl,  $\text{CH}_3\text{CN}$ , rt 2h then 65 °C 1h; (g)  $\text{NaBH}_4$ ,  $\text{CH}_3\text{OH}$ , rt, 15 min; (h) KCN, DMSO, 130 °C, 3h; (i) D-cysteine, 0.1 M aqueous sodium phosphate buffer, pH 8,  $\text{CH}_3\text{OH}$ ; (j)  $\text{CH}_3\text{I}$ ,  $\text{K}_2\text{CO}_3$ , DMF, 65 °C, overnight.

### Supplementary Scheme 2.

**(4-Amino-phenyl)-carbamic acid tert-butyl ester<sup>3</sup> (13):** To a solution of phenylenediamine (5 g, 46 mmol) in dioxane (90 mL) on ice, a solution of Boc<sub>2</sub>O (2 g, 9.2 mmol) in dioxane (10 mL) was slowly added. The mixture was stirred overnight at room temperature. The solvent was evaporated and the remaining solid was dissolved in DCM/MeOH (9/1) and washed with saturated NaHCO<sub>3</sub> (100 mL), H<sub>2</sub>O (100 mL) and brine. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated. Purification by column chromatography (20-50% EtOAc in hexanes) gave the desired product (1.8 g, 95%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.13-7.11 (m, 2H), 6.64-6.61 (m, 2H), 6.24 (br s, 1H), 3.51 (br s, 2H), 1.50 (s, 9H). HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>17</sub>N<sub>2</sub>O<sub>2</sub>: 209.1290, Found: 209.1287.

**(2,2,4-Trimethyl-1,2-dihydro-quinolin-6-yl)-carbamic acid tert-butyl ester<sup>3</sup> (14):** Following the procedure of Bonger *et al.*,<sup>3</sup> a solution of **13** (1.5 g, 7.21 mmol) in 17 mL acetone was added MgSO<sub>4</sub> (4.3 g, 36 mmol) and tert-butyl catechol (35 mg, 0.21 mmol, 3.0 mol %). Iodine (91 mg, 0.036 mmol, 5.0 mol %) was then added to the reaction mixture and the reaction was refluxed for 2 days. The mixture was diluted with ethyl acetate (50 mL), filtered and concentrated. The resulting residue was dissolved in DCM (50 mL) and washed with 3M NaOH (4 x 20 mL) and water (3 x 20 mL) to remove tert-butyl catechol. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated under vacuum. The crude product was purified by column chromatography using 8% ethyl acetate: hexane to obtain **14** as brown-yellow oil which solidified over time (950 mg, 45%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.05 (s, 1H), 6.94 (d, 1H, *J* = 7.2 Hz), 6.44 (br s, 1H), 6.35 (d, 1H, *J* = 8.0 Hz), 5.31 (s, 1H), 3.61 (br s, 1H), 1.94 (d, 3H, *J* = 4.0 Hz), 1.50 (s, 9H), 1.23 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 153.8, 140.0, 129.4, 128.7, 128.6, 122.2, 120.8, 116.2, 113.3, 79.9, 51.9, 30.8, 28.7, 18.7. HRMS (ESI<sup>+</sup>) Calcd for C<sub>17</sub>H<sub>25</sub>N<sub>2</sub>O<sub>2</sub>: 289.1916, Found: 289.1941.

**[2,2,4-Trimethyl-1-(2,2,2-trifluoro-acetyl)-1,2-dihydro-quinolin-6-yl]-carbamic acid tert-butyl ester (15):** Following the general procedure of Williamson and Ward,<sup>4</sup> trifluoroacetic anhydride (1.03 g, 4.9 mmol) was added dropwise to an ice-cooled solution of **14** (950 mg, 3.2 mmol) in dichloromethane (10 mL) and pyridine (1.06 mL). The reaction was warmed to room temperature over 1h, and then quenched by the slow addition of water (10 mL). The organic phase was separated, washed with 10% hydrochloric acid (3 x 15 mL) and water (15 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and the solvent was removed under vacuum. Flash chromatography using 5% ethyl acetate: hexane provided **15** (900 mg, 73%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 7.39 (d, 1H, *J* = 4.0 Hz), 7.12 (dd, 1H, *J* = 8.4, 4.0 Hz), 6.81 (d, 1H, *J* = 8.0 Hz), 6.51 (br s, 1H), 5.57 (d, 1H, *J* = 1.2 Hz), 2.05 (d, 3H, *J* = 4.0 Hz), 1.52 (s, 9H). <sup>19</sup>F-NMR (376 MHz, CDCl<sub>3</sub>): -67.35. <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 158.5 (q, <sup>2</sup>*J*<sub>CF</sub> = 36 Hz), 152.8, 137.3, 135.2, 129.8, 128.8, 128.6, 123.3 (q, <sup>4</sup>*J*<sub>CF</sub> = 3 Hz), 117.2, 116.4 (q, <sup>1</sup>*J*<sub>CF</sub> = 289 Hz), 113.9, 81.1, 59.9, 28.5, 25.7, 18.0. HRMS (ESI<sup>+</sup>) Calcd for C<sub>19</sub>H<sub>23</sub>F<sub>3</sub>N<sub>2</sub>O<sub>3</sub>Na: 407.1559, Found: 407.1537.

**1-(6-Amino-2,2,4-trimethyl-2H-quinolin-1-yl)-2,2,2-trifluoro-ethanone (16):** 15% TFA in DCM (13 mL) was added to a solution of **15** (900 mg, 2.34 mmol) in DCM (10 mL) The reaction was stirred for 5h at room temperature followed by addition of toluene. The mixture was concentrated, dissolved in DCM (25 mL) and washed with sat aq NaHCO<sub>3</sub> (2 x 10 mL). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated under vacuum to yield the crude product which was used in the next step without further purification (600 mg, 90%). HRMS (ESI<sup>+</sup>) Calcd for C<sub>14</sub>H<sub>16</sub>F<sub>3</sub>N<sub>2</sub>O: 285.1215, Found: 285.1197.

**1-(2-Amino-6,6,8-trimethyl-6H-thiazolo[4,5-g]quinolin-5-yl)-2,2,2-trifluoro-ethanone (17):** A solution of **16** (600 mg, 2.11 mmol) and potassium thiocyanate (819 mg, 8.45 mmol) in AcOH (20 mL) was stirred at 20 °C for 10 min. Bromine (335 mg, 2.11 mmol) was added over 20 min and the reaction mixture was stirred further at room temperature for 21 h. The reaction mixture was poured onto crushed ice and the pH was adjusted to 8 using NH<sub>4</sub>OH. The resulting precipitate was vacuum-filtered and dried to give the title compound, which was used for the next step without further purification (650 mg, 91 %). HRMS (ESI<sup>+</sup>) Calcd for C<sub>15</sub>H<sub>15</sub>F<sub>3</sub>S<sub>2</sub>O: 342.0888, Found: 342.0851.

**1-(2-Chloro-6,6,8-trimethyl-6H-thiazolo[4,5-g]quinolin-5-yl)-2,2,2-trifluoro-ethanone (18):** To a mixture of t-butyl nitrite (294 mg, 2.85 mmol), cupric chloride (225 mg, 2.28 mmol) and acetonitrile (20 mL) was added **17** (650 mg, 1.90 mmol) in portions over 1h. The reaction mixture was stirred at room temperature for 2h heated to 65 °C for 1h. The mixture was filtered and the filtrate was poured into 6N HCl and extracted with ethyl acetate (2 x 50 mL). After concentration the crude product was purified by flash chromatography (500 mg, 73 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.81 (s, 1H), 7.24 (s, 1H), 5.67 (d, 1H, *J* = 1.2 Hz), 2.12 (d, 3H, *J* = 1.6 Hz), 1.54 (s, 6H). <sup>19</sup>F-NMR (376 MHz, CDCl<sub>3</sub>): -67.08. <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 158.3 (q, <sup>2</sup>*J*<sub>CF</sub> = 36 Hz), 153.7, 150.0, 135.6, 135.0, 132.4, 128.8, 128.3, 117.6, 116.3 (q, <sup>1</sup>*J*<sub>CF</sub> = 289 Hz), 114.6 (q, <sup>4</sup>*J*<sub>CF</sub> = 3 Hz), 60.4, 25.4, 18.2. HRMS (ESI<sup>+</sup>) Calcd for C<sub>15</sub>H<sub>13</sub>ClF<sub>3</sub>N<sub>2</sub>OS: 361.0389, Found: 361.0367.

**2-Chloro-6,6,8-trimethyl-5,6-dihydro-thiazolo[4,5-g]quinoline (19):** The trifluoroacetamide (500 mg, 1.4 mmol) was stirred in methanol (5 mL) as NaBH<sub>4</sub> (217 mg, 5.8 mmol) was added. The mixture was stirred for 15 min, diluted with water (50 mL) and extracted with ethyl acetate (2 x 50 mL). The organic layer was washed with brine and evaporated to give **19** (250 mg, 67 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.57 (s, 1H), 6.68 (s, 1H), 5.44 (d, 1H, *J* = 4.0 Hz), 3.89 (br s, 1H), 2.03 (d, 3H, *J* = 1.2 Hz), 1.30 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 147.4, 143.8, 142.6, 136.9, 130.4, 128.2, 122.5, 117.6, 102.8, 52.5, 31.3, 18.9. HRMS (ESI<sup>+</sup>) Calcd for C<sub>13</sub>H<sub>14</sub>ClN<sub>2</sub>S: 265.0566, Found: 265.0557.

**6,6,8-Trimethyl-5,6-dihydro-thiazolo[4,5-g]quinoline-2-carbonitrile (20):** Potassium cyanide (49 mg, 0.75 mmol) was added to a solution of **19** (100 mg, 0.37 mmol) in DMSO (5 mL) and stirred for 3h at 130 °C. The reaction was cooled to room temperature, poured into water, and extracted with ethyl acetate (2 x 20 mL). The organic phase was evaporated to dryness and the crude material purified by flash chromatography using 10% ethyl acetate: hexane as eluent (55 mg, 58%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.77 (s, 1H), 6.76 (s, 1H), 5.51 (d, 1H, *J* = 1.2 Hz), 4.24 (br s, 1H), 2.07 (d, 3H, *J* = 1.2 Hz), 1.35 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): 145.5, 145.2, 137.5, 131.3, 129.5, 127.7, 123.9, 119.6, 114.2, 101.3, 53.1, 31.9, 18.9. HRMS (ESI<sup>+</sup>) Calcd for C<sub>14</sub>H<sub>14</sub>N<sub>3</sub>S: 256.0909, Found: 256.0907.

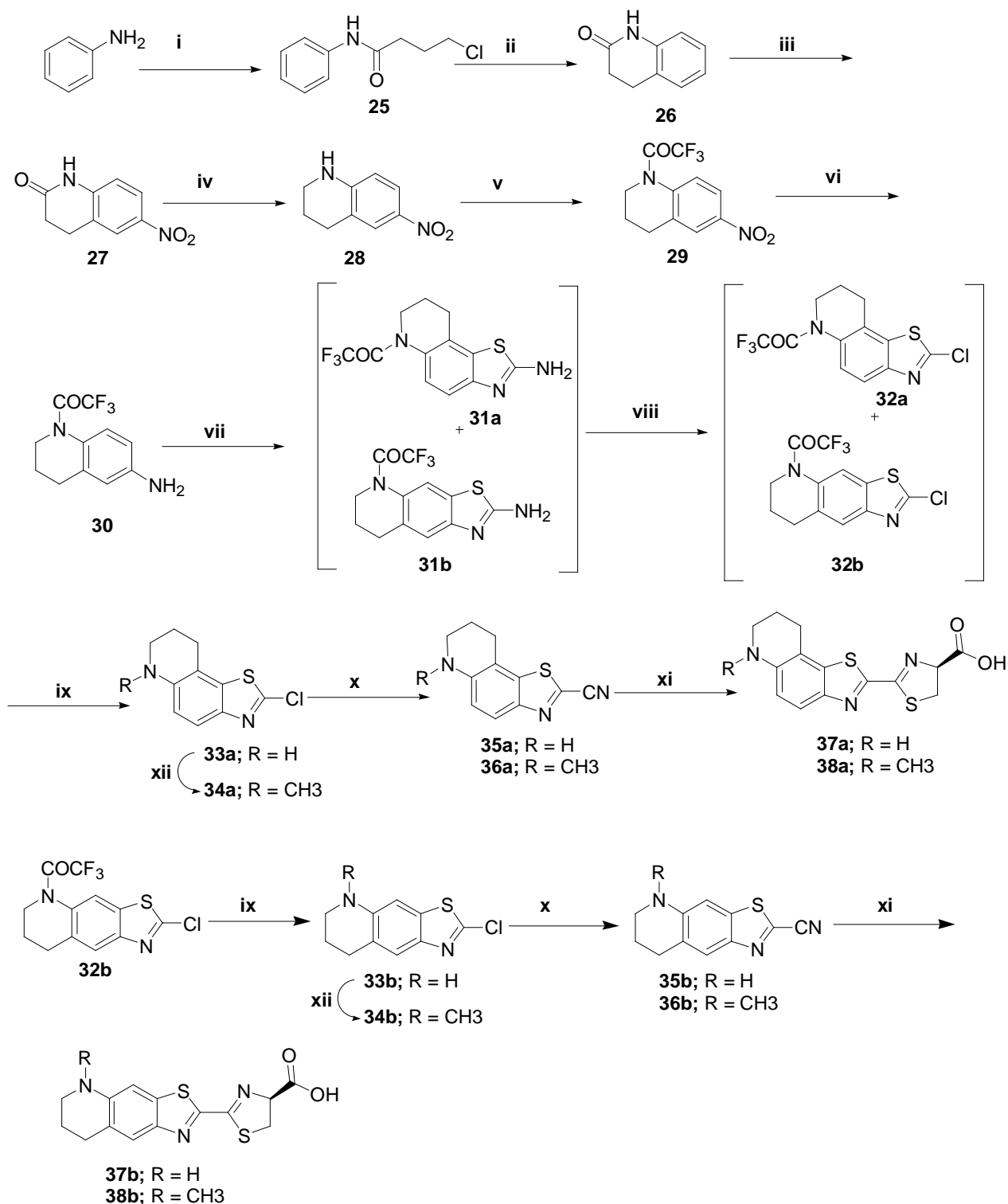
**CycLuc5; 2-(6,6,8-Trimethyl-5,6-dihydro-thiazolo[4,5-g]quinolin-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (21):** D-cysteine (5 mg, 0.028 mmol) was dissolved in 1 mL of 0.1M aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **20** (6 mg, 0.023 mmol) in 1 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer (2 mL) and washed with ethyl acetate (2 x 5 mL). The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 5 mL). The solvent was removed by rotary evaporation to yield **21** (3.2 mg, 38 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.80 (s, 1H), 6.83 (s, 1H), 5.48 (d, 1H, *J* = 1.2 Hz), 5.40 (t, 1H, *J* = 12.0 Hz), 3.76 (d, 2H, *J* = 12.0 Hz), 2.06 (d, 1H, *J* = 4.0 Hz), 1.34 (d, 1H, *J* = 1.2 Hz). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): 172.0, 167.6, 154.2, 146.9, 137.7, 130.4, 127.9, 123.2, 119.7, 102.5, 77.9, 53.1, 34.9, 31.6, 19.0. HRMS (ESI<sup>+</sup>) Calcd for C<sub>17</sub>H<sub>18</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub>: 360.0840, Found: 360.0812.

**2-Chloro-5,6,6,8-tetramethyl-5,6-dihydro-thiazolo[4,5-g]quinoline (22):** CH<sub>3</sub>I (80 mg, 0.56 mmol) was added to a mixture of **19** (100 mg, 0.37 mmol) and K<sub>2</sub>CO<sub>3</sub> (153 mg, 1.11 mmol) in DMF (5 mL). The reaction mixture was heated to 65°C and stirred overnight. The reaction was then cooled to room temperature, poured into water and extracted with ethyl acetate (2 x 50 mL). The organic phase was evaporated under vacuum to yield **22** (60 mg, 58%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.56 (s, 1H), 6.75 (s, 1H), 5.43 (d, 1H, *J* = 1.2 Hz), 2.83 (s, 1H), 2.03 (d, 3H, *J* = 1.2 Hz), 1.33 (s, 1H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): 147.4, 144.5, 142.9, 137.5, 132.0, 127.9, 124.1, 117.0, 100.8, 56.9, 31.5, 27.6, 18.9.

**5,6,6,8-Tetramethyl-5,6-dihydro-thiazolo[4,5-g]quinoline-2-carbonitrile (23):** Potassium cyanide (29 mg, 0.45 mmol) was added to a solution of **22** (60 mg, 0.22 mmol) in DMSO (5 mL) and stirred for 3h at 130 °C. The reaction was cooled to room temperature, poured into water, and extracted with ethyl acetate (2 x 20 mL). The organic phase was evaporated to dryness and the crude material purified by flash chromatography using

10% ethyl acetate: hexane as eluent (30 mg, 50 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.72 (s, 1H), 6.79 (s, 1H), 5.49 (s, 1H), 2.89 (s, 3H), 2.06 (d, 3H,  $J = 1.2$  Hz), 1.39 (s, 6H).  $^{13}\text{C-NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  146.7, 144.5, 138.4, 132.7, 129.3, 127.3, 125.5, 118.7, 114.4, 99.2, 57.7, 31.8, 28.5, 19.0. HRMS ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{15}\text{H}_{16}\text{N}_3\text{S}$ : 270.1065, Found: 270.1068.

**CycLuc6; 2-(5,6,6,8-Tetramethyl-5,6-dihydro-thiazolo[4,5-g]quinolin-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (24):** D-cysteine (3.1 mg, 0.017 mmol) was dissolved in 1 mL of 0.1 M aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **23** (4 mg, 0.014 mmol) in 1 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer (2 mL) and washed with ethyl acetate (2 x 5 mL). The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 5 mL). The solvent was removed by rotary evaporation to afford **24** (2.5 mg, 47 %).  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.75 (s, 1H), 6.83 (s, 1H), 5.45 (s, 1H), 5.40 (t, 1H,  $J = 12.0$  Hz), 3.78-3.74 (m, 2H), 2.90 (s, 3H), 2.05 (s, 3H), 1.38 (s, 6H).  $^{13}\text{C-NMR}$  (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  172.8, 167.9, 153.7, 145.9, 145.5, 138.7, 132.0, 127.7, 124.6, 118.7, 100.2, 78.3, 57.4, 35.0, 31.7, 28.34, 28.3, 19.1. HRMS ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{18}\text{H}_{20}\text{N}_3\text{O}_2\text{S}_2$ : 374.0997, Found: 374.1011.



i) 3-chloropropionyl Chloride, pyridine, acetone, 2h, 55 °C; ii) AlCl<sub>3</sub> 120 °C iii) HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>; iv) BH<sub>3</sub>.THF (1M in THF), THF; v) TFAA, TEA, CH<sub>2</sub>Cl<sub>2</sub>; vi) SnCl<sub>2</sub>-2H<sub>2</sub>O, DMF; vii) KSCN, Br<sub>2</sub>, AcOH; viii) t-Butyl nitrite, CuCl, CH<sub>3</sub>CN, rt for 2h and 65 °C for 1h; ix) NaBH<sub>4</sub>, CH<sub>3</sub>OH; x) KCN, DMSO, 130 °C; xi) D-cysteine, aq. CH<sub>3</sub>OH, pH 8; xii) NaBH(OAc)<sub>3</sub>, HCHO, DCE

Supplementary Scheme 3.

**4-Chloro-N-phenyl-butyramide (25):** 3-chloro propionyl chloride (16.24 g, 129 mmol) was added to a mixture of aniline (10 g, 107.4 mmol) and pyridine (21.20 g, 268.4 mmol) in acetone and the reaction mixture was heated for 2h at 55 °C. After cooling to room temperature, the mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 1N HCl, water and brine and the solvent was removed by rotary evaporation to give **25** (20 g, 94.5%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.84 (s, 1H), 7.51 (d, 2H, *J* = 7.2 Hz), 7.31 (t, 2H, *J* = 7.2 Hz), 7.12 (t, 1H, *J* = 7.2 Hz), 3.85 (t, 2H, *J* = 6.4 Hz), 2.80 (t, 2H, *J* = 6.4 Hz). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 168.4, 137.7, 129.3, 124.9, 120.5, 40.5, 40.2. HRMS (ESI<sup>+</sup>) Calcd for C<sub>9</sub>H<sub>11</sub>ClNO: 184.0529, Found: 184.0518.

**3,4-Dihydro-1H-quinolin-2-one (26):** 4-Chloro-N-phenyl-butyramide **25** (10 g, 54.6 mmol) and AlCl<sub>3</sub> (29 g, 218.4 mmol) was heated at 120 °C for 2h, followed by cooling to room temperature. Crushed ice was added to the reaction mixture and the solid was filtered by vacuum. The compound was purified by flash chromatography using 30 % ethyl acetate: hexane as eluent (5.8 g, 72.2%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 9.03 (s, 1H), 7.19-7.15 (m, 2H), 6.98 (t, 1H, *J* = 8.0 Hz), 6.83 (d, 1H, *J* = 8.0 Hz), 2.98 (t, 2H, *J* = 7.6 Hz), 2.65 (t, 2H, *J* = 7.6 Hz). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 172.2, 137.4, 128.2, 127.8, 123.9, 123.3, 115.7, 30.9, 25.6. HRMS (ESI<sup>+</sup>) Calcd for C<sub>9</sub>H<sub>10</sub>NO: 148.0762, Found: 148.0739.

**6-Nitro-3, 4-dihydro-1H-quinolin-2-one (27):** HNO<sub>3</sub> (1.5 mL, 34.01 mmol) was added drop wise to a solution of sulphuric acid (10 mL) and 3, 4-Dihydro-1H-quinolin-2-one **26** (5 g, 34.01 mmol) at 0°C. The reaction mixture then stirred for 1h at 0°C. The solution poured on to crushed ice and the resulting precipitate was vacuum filtered and purified by flash chromatography using 5% methanol: dichloromethane gave a pure yellow solid **27** (2 g, 30.6%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 9.40 (s, 1H), 8.12-8.09 (m, 2H), 6.93 (d, 1H, *J* = 9.2 Hz), 3.10 (t, 2H, *J* = 7.2 Hz), 2.73 (t, 2H, *J* = 7.6 Hz). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 172.1, 143.4, 143.1, 124.3, 124.2, 124.1, 115.7, 30.2, 25.3. HRMS (ESI<sup>+</sup>) Calcd for C<sub>9</sub>H<sub>8</sub>N<sub>2</sub>O<sub>3</sub>Na: 215.0433, Found: 215.0417.

**6-Nitro-1,2,3,4-tetrahydro-quinoline (28):** A solution of **27** (1.5 g, 7.8 mmol) in THF(10 mL) was treated with 1M BH<sub>3</sub>/THF (46.8 mL). The solution was refluxed for 30 min then cooled to 0°C, and quenched with 1N HCl (20 mL). The solution was stirred for 30 min then concentrated and extracted with ethyl acetate (2 x 100 mL). The organic layer was evaporated and purified by flash chromatography using 20 % ethyl acetate : hexane as eluent to yield **28** (0.8 g, 57.6%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.90-7.87 (m, 2H), 6.36 (d, 1H, *J* = 9.2 Hz), 4.69 (s, 1H), 3.43-3.40 (m, 2H), 2.80 (t, 2H, *J* = 6.8 Hz), 1.98-1.92 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 150.6, 126.1, 124.5, 120.0, 112.4, 41.9, 27.1, 21.0. HRMS (ESI<sup>+</sup>) Calcd for C<sub>9</sub>H<sub>11</sub>N<sub>2</sub>O<sub>2</sub>: 179.0820, Found: 179.0824.

**2,2,2-Trifluoro-1-(6-nitro-3,4-dihydro-2H-quinolin-1-yl)-ethanone (29):** 6-Nitro-1,2,3,4-tetrahydro-quinoline **5** (0.5 g, 2.3 mmol) and triethylamine (0.3 mL, 2.6 mmol) were stirred in dichloromethane (10 mL) as trifluoroacetic anhydride (550 mg, 2.6 mmol) was added drop wise. This mixture was stirred for 45 min then water (50 mL) was added. After stirring for 10 min, the mixture was acidified with 5M HCl. The organic layer was washed with brine, dried and evaporated to give **29** as a yellow solid (0.63 g, 98.5%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 8.09-8.06 (m, 2H), 7.91-7.88 (m, 1H), 3.89 (t, 2H, *J* = 6.8 Hz), 3.00 (t, 2H, *J* = 6.4 Hz), 2.18-2.11 (m, 2H). <sup>19</sup>F-NMR (376 MHz, CDCl<sub>3</sub>): -68.9. <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 156.4 (q, <sup>2</sup>*J*<sub>CF</sub> = 37 Hz), 145.3, 142.5, 132.5, 125.4, 124.8, 121.7, 116.5 (q, <sup>1</sup>*J*<sub>CF</sub> = 286 Hz), 45.4 (q, <sup>4</sup>*J*<sub>CF</sub> = 4 Hz), 26.6, 23.1. HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>9</sub>F<sub>3</sub>N<sub>2</sub>O<sub>3</sub>Na: 297.0463, Found: 297.0475.

**1-(6-Amino-3,4-dihydro-2H-quinolin-1-yl)-2,2,2-trifluoro-ethanone (30):** SnCl<sub>2</sub>.2H<sub>2</sub>O (1.47 g, 6.56 mmol) was added to a solution **29** (0.6 g, 2.18 mmol) in ethanol (15 mL) and the reaction mixture was heated to reflux for 4h. After cooling to room temperature the solvent was evaporated under vacuum. The resulting residue was dissolved in NaHCO<sub>3</sub> (20 mL) and extracted with ethyl acetate (2 x 50 mL). The organic layer was evaporated

under vacuum to give desired product and used for next step without purification (450 mg, 84.5%). HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>12</sub>F<sub>3</sub>N<sub>2</sub>O: 245.0902, Found: 245.0870.

**1-(2-Amino-8,9-dihydro-7H-thiazolo[5,4-f]quinolin-6-yl)-2,2,2-trifluoro-ethanone (31a):** A solution of **30** (400 mg, 1.62 mmol) and potassium thiocyanate (636 mg, 6.55 mmol) in AcOH (10 mL) was stirred at 20 °C for 10 min. Then bromine (256 mg, 1.62 mmol) was added over 20 min dropwise. The reaction mixture was stirred further at room temperature for 21 h. On completion of reaction following a TLC examination; the reaction mixture was poured on to crushed ice the pH was adjusted to 8 using NH<sub>4</sub>OH. The resulting precipitate was vacuum-filtered and dried to give the title compound and was used for the next step without further purification (440 mg, 90.2%). HRMS (ESI<sup>+</sup>) Calcd for C<sub>12</sub>H<sub>11</sub>F<sub>3</sub>N<sub>3</sub>OS: 302.0575, Found: 302.0555.

**1-(2-Chloro-8,9-dihydro-7H-thiazolo[5,4-f]quinolin-6-yl)-2,2,2-trifluoro-ethanone (32a):** To a mixture of t-butyl nitrite (205 mg, 1.99 mmol), cupric chloride (157.6 mg, 1.59 mmol) and acetonitrile (10 mL) was added **31a** (400 mg, 1.328 mmol) in portions over 1h. The reaction mixture was stirred at room temperature for 2h heated to 65 °C for 1h. The mixture was filtered and the filtrate was poured into 6N HCl and extracted with ethyl acetate (2 x 20 mL). After concentration the crude product (310 mg, 73%) was purified by flash chromatography (6% ethyl acetate: hexane) to yield **32a** (220 mg) and **32b** (25 mg).

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) for **32a**: δ 7.85-7.78 (m, 2H), 3.91 (t, 2H, *J* = 5.6 Hz), 2.94 (t, 2H, *J* = 6.8 Hz), 2.26-2.20 (m, 2H). <sup>19</sup>F-NMR (376 MHz, CDCl<sub>3</sub>): -69.2. <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 156.1 (q, <sup>2</sup>*J*<sub>CF</sub> = 35 Hz), 153.1, 148.9, 136.6, 134.9, 123.9, 120.5, 116.8 (q, <sup>1</sup>*J*<sub>CF</sub> = 286 Hz), 45.6 (q, <sup>4</sup>*J*<sub>CF</sub> = 3 Hz), 26.1, 23.4. HRMS (ESI<sup>+</sup>) Calcd for C<sub>12</sub>H<sub>9</sub>F<sub>3</sub>N<sub>2</sub>OS: 321.0076, Found: 321.0084.

<sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) for **32b**: δ 8.24 (s, 1H), 7.74 (s, 1H), 3.90-3.87 (m, 2H), 3.01 (s, 2H), 2.16-2.11 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 154.5, 149.4, 135.1, 134.1, 130.7, 122.6, 117.3, 116.8 (q, <sup>1</sup>*J*<sub>CF</sub> = 286 Hz), 112.5, 45.4, 26.6, 23.5. <sup>19</sup>F-NMR (376 MHz, CDCl<sub>3</sub>): -69.1. HRMS (ESI<sup>+</sup>) Calcd for C<sub>12</sub>H<sub>9</sub>F<sub>3</sub>N<sub>2</sub>OS: 321.0076, Found: 321.0085.

**2-Chloro-6,7,8,9-tetrahydro-thiazolo[5,4-f]quinoline (33a):** The trifluoroacetamide (150 mg, 0.468 mmol) was stirred in methanol (5 mL) as NaBH<sub>4</sub> (69.3 mg, 1.875 mmol) was added in portions over five minutes. The mixture was stirred for 15 min, diluted with water (10ml) and extracted with ethyl acetate (2 x 20 mL). The organic layer was washed with brine and evaporated to give **33a** (90 mg, 87.3%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.47 (d, 1H, *J* = 8.8 Hz), 6.57 (d, 1H, *J* = 8.4 Hz), 4.06 (s, 1H), 3.29 (t, 2H, *J* = 5.6 Hz), 2.67 (t, 2H, *J* = 6.0 Hz), 2.01-1.95 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 147.0, 143.0, 142.7, 137.9, 121.0, 115.1, 112.6, 41.8, 26.4, 21.8. HRMS (ESI<sup>+</sup>) Calcd for C<sub>10</sub>H<sub>10</sub>ClN<sub>2</sub>S: 225.0253, Found: 225.0259.

**6,7,8,9-Tetrahydro-thiazolo[5,4-f]quinoline-2-carbonitrile (35a):** Potassium cyanide (46.42 mg, 0.714 mmol) was added to a solution of **33a** (80 mg, 0.357 mmol) in DMSO (5 mL) stirred for 3h at 130 °C. The reaction cooled to room temperature and poured into water and extracted with ethyl acetate (2 x 20 mL) the organic phase was evaporated to dryness and purified by flash chromatography using 10% ethyl acetate: hexane as eluent (30 mg, 39.1%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.77 (d, 1H, *J* = 8.4 Hz), 6.76 (d, 1H, *J* = 8.4 Hz), 4.36 (s, 1H), 3.43-3.40 (m, 2H), 2.80 (t, 2H, *J* = 6.8 Hz), 2.11- 2.05 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 145.1, 144.4, 138.5, 129.4, 123.7, 117.3, 114.3, 111.2, 41.7, 26.5, 21.3. HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>10</sub>N<sub>3</sub>S: 215.0595, Found: 215.0585.

**CycLuc9; 2-(6,7,8,9-Tetrahydro-thiazolo[5,4-f]quinolin-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (37a):** D-cysteine (4.8 mg, 0.027 mmol) was dissolved in 1 mL of 0.1 M aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **35a** (5 mg, 0.023 mmol) in 1 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer (2 mL) and washed with ethyl acetate (2 x 10 mL). The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2

x 5 mL). The solvent was removed by rotary evaporation to yield **37a** (4 mg, 54%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub> + 1 drop CD<sub>3</sub>OD): δ 7.68 (d, 1H, *J* = 8.8 Hz), 6.67 (d, 1H, *J* = 8.8 Hz), 5.30 (t, 1H, *J* = 10 Hz), 3.75-3.64 (m, 2H), 3.33 (t, 2H, *J* = 5.6 Hz), 2.76 (t, 2H, *J* = 6.4 Hz), 2.06-1.99 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub> + 1 drop CD<sub>3</sub>OD): δ 172.4, 166.8, 154.7, 145.4, 143.9, 138.5, 123.0, 116.3, 112.2, 78.2, 41.6, 35.3, 26.2, 21.5. HRMS (ESI<sup>+</sup>) Calcd for C<sub>14</sub>H<sub>14</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub>: 320.0527, Found: 320.0491.

**2-Chloro-6-methyl-6,7,8,9-tetrahydro-thiazolo[5,4-f] quinoline (34a):** 2-Chloro-6,7,8,9-tetrahydro-thiazolo[5,4-f] quinoline **33a** (50 mg, 0.22 mmol) and 37% formaldehyde (26.7 mg, 0.89 mmol) were dissolved in 1,2-dichloroethane (5 mL). Sodium triacetoxyborohydride (70 mg, 0.33 mmol) was added and the reaction mixture was stirred for 1h at ambient temperature. The reaction was quenched with 1N NaOH (5 mL). The product was extracted with ethyl acetate (2 x 20 mL), washed with brine and dried over sodium sulfate. The solvent was removed by rotary evaporation and the resulting solid was purified by flash chromatography using 5 % ethyl acetate: hexane as eluent (40 mg, 76 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.62 (d, 1H, *J* = 9.2 Hz), 6.80 (d, 1H, *J* = 8.4 Hz), 3.27 (t, 2H, *J* = 5.6 Hz), 2.96 (s, 3H), 2.75 (t, 2H, *J* = 6.4 Hz), 2.13-2.07 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 147.4, 144.9, 142.4, 137.7, 121.1, 113.9, 111.9, 51.4, 40.0, 27.4, 22.1. HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>12</sub>ClN<sub>2</sub>S: 239.0410, Found: 239.0380.

**6-Methyl-6,7,8,9-tetrahydro-thiazolo[5,4-f]quinoline-2-carbonitrile (36a):** Potassium cyanide (16 mg, 0.25 mmol) was added to a solution of **34a** (30 mg, 0.12 mmol) in DMSO (5 mL), then stirred for 3h at 130 °C. The reaction was cooled to room temperature, then poured into water and extracted with ethyl acetate (2 x 20 mL). The organic phase was evaporated to dryness and purified by flash chromatography using 10% ethyl acetate: hexane as eluent (10 mg, 36 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.86 (d, 1H, *J* = 8.8 Hz), 6.96 (d, 1H, *J* = 9.2 Hz), 3.37 (t, 2H, *J* = 6 Hz), 3.04 (s, 3H), 2.80 (t, 2H, *J* = 6.4 Hz), 2.15-2.09 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 146.7, 143.8, 138.0, 129.6, 123.8, 114.4, 113.9, 112.5, 51.2, 39.8, 27.4, 21.6. HRMS (ESI<sup>+</sup>) Calcd for C<sub>12</sub>H<sub>12</sub>N<sub>3</sub>S: 230.0752, Found: 230.0742.

**CycLuc10; 2-(6-Methyl-6,7,8,9-tetrahydro-thiazolo[5,4-f]quinolin-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (38a)** D-cysteine (2.8 mg, 0.015 mmol) was dissolved in 1 mL of 0.1M aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **36a** (3 mg, 0.013 mmol) in 1 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer and washed with ethyl acetate (2 x 5 mL). The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 5 mL). The solvent was removed by rotary evaporation to yield **38a** as an orange red solid (2 mg, 46 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.79 (d, 1H, *J* = 8.8 Hz), 6.89 (d, 1H, *J* = 9.6), 5.30 (t, 1H, *J* = 9.6 Hz), 3.76-3.63 (m, 2H), 3.31-3.28 (m, 2H), 2.98 (s, 3H), 2.78 (t, 2H, *J* = 6.8 Hz), 2.11-2.05 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub> + 1 drop CD<sub>3</sub>OD): δ 172.4, 166.9, 154.9, 153.7, 145.6, 145.0, 138.1, 123.1, 113.3, 78.2, 51.3, 40.1, 35.3, 27.1, 21.6. MS (ESI<sup>+</sup>) Calcd for C<sub>15</sub>H<sub>16</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub>: 334.0678, Found: 334.0675.

**2-Chloro-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinoline (33b):** Compound **32b** (25 mg, 0.07 mmol) was stirred in methanol (5 mL) as NaBH<sub>4</sub> (11 mg, 0.31 mmol) was added in portions over five minutes. The mixture was stirred for 15 min, diluted with water (10 mL) and extracted with ethyl acetate (2 x 20 mL). The organic layer was washed with brine and evaporated to give **33b** (15 mg, 85 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.47 (s, 1H), 6.73 (s, 1H), 4.13 (br s, 1H), 3.36 (t, 2H, *J* = 5.6 Hz), 2.89 (t, 2H, *J* = 6.0 Hz), 1.99-1.93 (m, 2H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 147.3, 143.9, 143.2, 135.6, 123.0, 122.3, 103.5, 42.2, 27.7, 21.9. HRMS (ESI<sup>+</sup>) Calcd for C<sub>10</sub>H<sub>10</sub>ClN<sub>2</sub>S: 225.0253, Found: 225.0243.

**5,6,7,8-Tetrahydro-thiazolo[4,5-g]quinoline-2-carbonitrile (35b):** Potassium cyanide (8.7 mg, 0.13 mmol) was added to a solution of **33b** (15 mg, 0.06 mmol) in DMSO (2 mL), then stirred for 3h at 130°C. The reaction was cooled to room temperature, then poured into water and extracted with ethyl acetate (2 x 20 mL). The organic phase was evaporated to dryness and purified by flash chromatography using 10% ethyl acetate: hexane as eluent (8 mg, 56 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.70 (s, 1H), 6.79 (s, 1H), 4.48 (s, 1H), 3.42 (t, 2H, *J* =

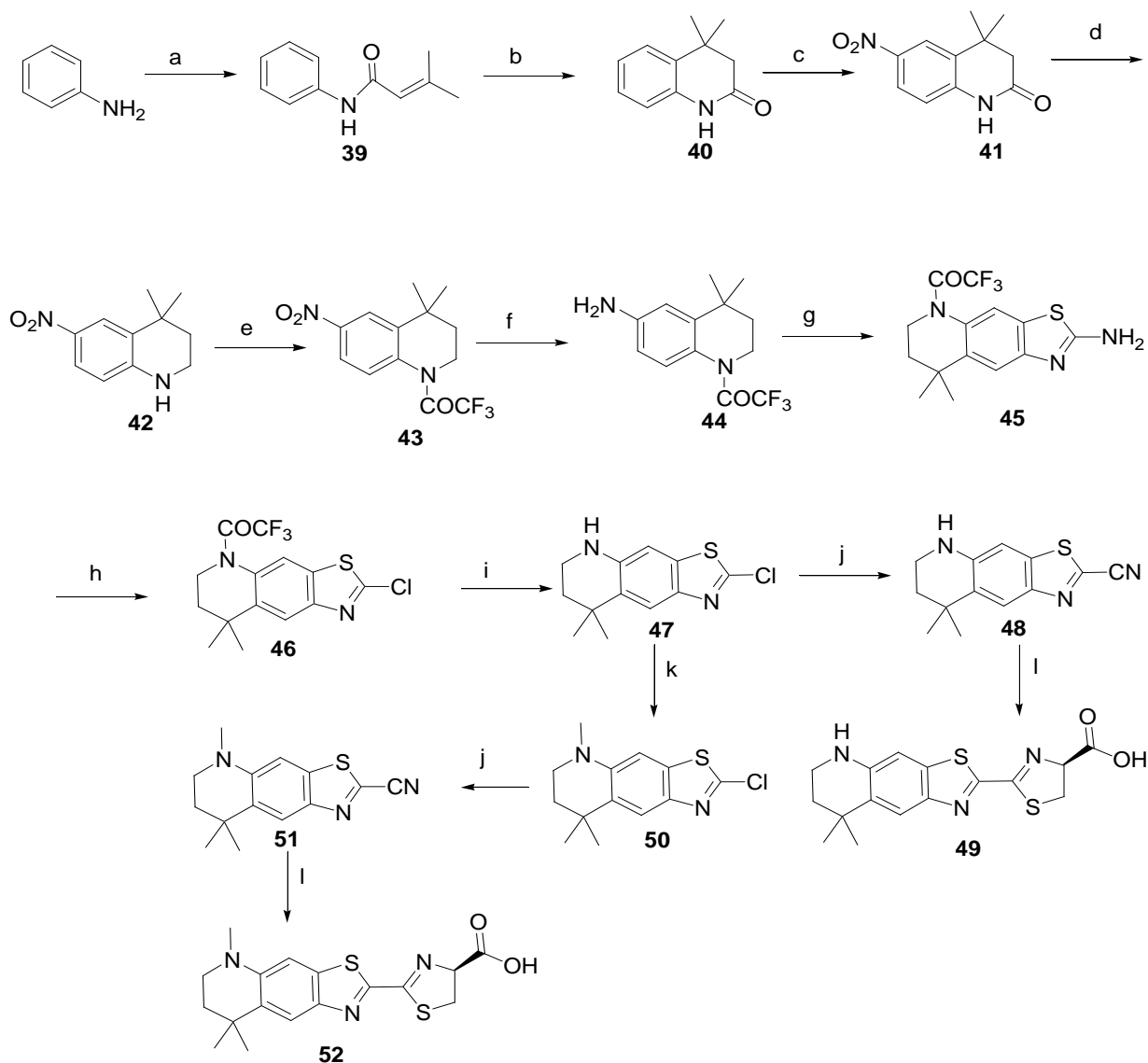
6 Hz), 2.93 (t, 2H,  $J = 6$  Hz), 2.01-1.95 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  146.6, 144.8, 129.2, 125.0, 124.5, 119.9, 114.3, 101.9, 42.1, 27.9, 21.4. HRMS ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{11}\text{H}_{10}\text{N}_3\text{S}$ : 216.0595, Found: 216.0594.

**CycLuc7; 2-(5,6,7,8-Tetrahydro-thiazolo[4,5-g]quinolin-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (37b):** D-cysteine (4.8 mg, 0.027 mmol) was dissolved in 1 mL of 0.1M aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **35b** (5 mg, 0.023 mmol) in 1 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer and washed with ethyl acetate (2 x 10 mL). The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 10 mL). The solvent was removed by rotary evaporation to yield **16a** as an orange red solid (3.5 mg, 47 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3 + 1$  drop  $\text{CD}_3\text{OD}$ ):  $\delta$  7.64 (s, 1H), 6.84 (s, 1H), 5.28 (t, 1H,  $J = 8.4$  Hz), 3.74-3.63 (m, 2H), 3.34 (t, 2H,  $J = 6.0$  Hz), 2.88 (t, 2H,  $J = 6.0$  Hz), 1.97-1.91 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3 + 1$  drop  $\text{CD}_3\text{OD}$ ):  $\delta$  172.3, 166.9, 154.6, 145.9, 144.8, 136.3, 124.5, 123.6, 103.6, 78.1, 42.0, 35.2, 27.7, 21.5. HRMS ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{14}\text{H}_{14}\text{N}_3\text{O}_2\text{S}_2$ : 320.0527, Found: 320.0537.

**2-Chloro-5-methyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinoline (34b):** 2-Chloro-5,6,7,8-tetrahydro-thiazolo[4,5-g] quinoline **33b** (30 mg, 0.13 mmol) and 37% formaldehyde (16 mg, 0.53 mmol) were dissolved in 1,2-dichloroethane (3 mL). Sodium triacetoxyborohydride (41.3 mg, 0.19 mmol) was added and the reaction mixture was stirred for 1h at ambient temperature. The reaction was quenched with 1N NaOH (5 mL). The product was extracted with ethyl acetate (2 x 20 mL), washed with brine and dried over sodium sulfate. The solvent was removed by rotary evaporation and the resulting solid was purified by flash chromatography using 5 % ethyl acetate: hexane as eluent (15 mg, 48%).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.46 (s, 1H), 6.78 (s, 1H), 3.30 (t, 2H,  $J = 5.6$  Hz), 2.93 (s, 3H), 2.86 (t, 2H,  $J = 6.4$  Hz), 2.02-1.96 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  146.9, 145.7, 142.4, 136.2, 124.1, 122.0, 100.4, 51.3, 39.5, 28.5, 22.5. HRMS ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{11}\text{H}_{12}\text{ClN}_2\text{S}$ : 239.0410, Found: 239.0390.

**5-Methyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinoline-2-carbonitrile (36b):** Potassium cyanide (7.8 mg, 0.12 mmol) was added to a solution of **34b** (15 mg, 0.06 mmol) in DMSO (3 mL), then stirred for 3h at  $130^\circ\text{C}$ . The reaction was cooled to room temperature, then poured into water and extracted with ethyl acetate (2 x 20 mL). The organic phase was evaporated to dryness and purified by flash chromatography using 10% ethyl acetate: hexane as eluent (6 mg, 43 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.67 (s, 1H), 6.83 (s, 1H), 3.40 (t, 2H,  $J = 6.0$  Hz), 3.00 (s, 3H), 2.91 (t, 2H,  $J = 6.4$  Hz), 2.03-1.99 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  147.8, 144.0, 137.1, 128.7, 126.2, 123.7, 114.5, 98.9, 51.4, 39.4, 28.7, 22.0. HRMS ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{12}\text{H}_{12}\text{N}_3\text{S}$ : 230.0752, Found: 230.0762.

**CycLuc8; 2-(5-Methyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinolin-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (38b):** D-cysteine (1.2 mg, 0.007 mmol) was dissolved in 1 mL of 0.1M aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **36b** (1.5 mg, 0.006 mmol) in 1 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer and washed with ethyl acetate (2 x 5 mL). The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 5 mL). The solvent was removed by rotary evaporation to yield **38b** as an orange red solid (1 mg, 50 %).  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.67 (s, 1H), 6.88 (s, 1H), 5.39 (t, 1H,  $J = 9.6$  Hz), 3.78-3.76 (m, 2H), 3.39 (t, 2H,  $J = 5.6$  Hz), 3.00 (s, 3H), 2.90 (t, 2H,  $J = 6.8$  Hz), 2.05-1.97 (m, 2H).  $^{13}\text{C}$ -NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  171.7, 168.4, 152.8, 147.1, 145.1, 137.4, 125.2, 123.7, 99.8, 77.9, 51.4, 39.4, 34.8, 28.7, 22.2. HRMS ( $\text{ESI}^+$ ) Calcd for  $\text{C}_{15}\text{H}_{16}\text{N}_3\text{O}_2\text{S}_2$ : 334.0684, Found: 334.0679.



**Supplementary Scheme 4.**

**3-Methyl-but-2-enoic acid phenylamide (39):** To 3,3-dimethylacryloyl chloride (9.6 mL, 86.4 mmol) in dry DCM (200 mL) was added aniline (8.04 g, 86.4 mmol) followed by diisopropylethylamine (28.56 mL, 172.8 mmol). The mixture was stirred at ambient temperature for 2 hr. Saturated sodium bicarbonate (50 mL) was added to quench the reaction. The organic layer was separated and washed with sat'd NaHCO<sub>3</sub> (100 mL) and water (100 mL). The resulting solution was dried over Na<sub>2</sub>SO<sub>4</sub> and the filtrate was evaporated to afford 3-methyl-but-2-enoic acid phenyl amide as a brown solid in quantitative yield (15.2 g). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.53 (d, 1H, *J* = 7.2 Hz), 7.34-7.29 (m, 2H), 7.09 (t, 1H, *J* = 7.2 Hz), 7.03 (s, 1H), 5.72-5.70 (m, 1H), 2.22 (d, 3H, *J* = 1.6 Hz), 1.91 (s, 3H) <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 165.9, 153.1, 138.7, 129.0, 124.1, 120.3, 119.2, 27.5, 20.2. HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>14</sub>NO: 176.1075, Found: 176.1045

**4,4-Dimethyl-3,4-dihydro-1H-quinolin-2-one (40):** To 3-Methyl-but-2-enoic acid phenylamide (15.0 g, 85 mmol) in DCM (200 mL) was added AlCl<sub>3</sub> (17.0 g, 128 mmol) and the mixture was stirred at 50 °C for 2 hr. After cooling to room temperature, the mixture was treated with 1N HCl (100 mL) and extracted with DCM (2 X 50 mL). This solution was then washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. The filtrate was evaporated to afford 4,4-Dimethyl-3,4-dihydro-1H-quinolin-2-one as a brown solid in quantitative yield (15 g). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 10.39 (s, 1H), 7.26 (d, 1H, *J* = 7.2 Hz), 7.14 (t, 1H, *J* = 7.2 Hz), 7.03- 6.94 (m, 2H), 2.49 (s, 2H), 1.31 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 172.5, 136.3, 132.6, 127.7, 124.5, 123.8, 116.5, 45.5, 34.1, 27.9. HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>14</sub>NO: 176.1075, Found: 176.1053.

**4,4-Dimethyl-6-nitro-3,4-dihydro-1H-quinolin-2-one (41):** Nitric acid (4.4 g, 68 mmol) was added dropwise to a solution of sulfuric acid (20 mL) and **40** (8.0 g, 45 mmol) at 0°C. The reaction mixture was stirred for 1h at 0°C, then poured over crushed ice and extracted with ethyl acetate (2 x 200 mL). The crude product was purified by flash chromatography using 25% ethyl acetate: hexane as eluent to give **41** (4.8 g, 48%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 8.45 (s, 1H), 8.21 (d, 1H, *J* = 2.4 Hz), 8.11 (dd, 1H, *J* = 2.4, 8.4 Hz), 6.88 (d, 1H, *J* = 9.2 Hz), 2.56 (s, 2H), 1.40 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 172.2, 143.9, 141.8, 133.4, 124.1, 121.1, 116.4, 44.7, 34.4, 27.8. HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>12</sub>N<sub>2</sub>O<sub>3</sub>Na: 243.0746, Found: 243.0715.

**4,4-Dimethyl-6-nitro-1,2,3,4-tetrahydro-quinoline (42):** A solution of **41** (4.5 g, 20 mmol) in THF (30 mL) was treated with 1M BH<sub>3</sub>/THF (120 mL). The solution was refluxed for 30 min then cooled to 0°C, and quenched with 1N HCl (20 mL). The solution was stirred for 30 min then concentrated and extracted with ethyl acetate (2 x 100 mL). Evaporation of the ethyl acetate yielded **42** (3.5 g, 84 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 8.08 (d, 1H, *J* = 2.4 Hz), 7.85 (dd, 1H, *J* = 2.4, 8.8 Hz), 6.38 (d, 1H, *J* = 9.2 Hz), 5.01 (s, 1H), 3.42 (t, 2H, *J* = 6.0 Hz), 1.69 (t, 2H, *J* = 6.4 Hz), 1.29 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 149.8, 137.4, 128.9, 124.3, 123.2, 112.8, 38.4, 35.4, 32.1, 29.9. HRMS (ESI<sup>+</sup>) Calcd for C<sub>11</sub>H<sub>15</sub>N<sub>2</sub>O<sub>2</sub>: 207.1134, Found: 207.1132.

**1-(4,4-Dimethyl-6-nitro-3,4-dihydro-2H-quinolin-1-yl)-2,2,2-trifluoro-ethanone (43):** 7-Nitro-4H-benzo[1,4]oxazin-3-one (3.5 g, 16.9 mmol) and triethylamine (1.8 g, 18.6 mmol) were stirred in dichloromethane (20 mL) as trifluoroacetic anhydride (3.9 g, 18.6 mmol) was added dropwise. This mixture was stirred for 45 min; then water (50 mL) was added. After stirring for 10 min, the mixture was acidified with 5M HCl. The organic layer was washed with brine, dried and evaporated under vacuum, the residue was purified by flash chromatography (10% ethyl acetate: hexane) to give **43** (2.7 g, 52 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 8.26 (d, 1H, *J* = 2.8 Hz), 8.04 (dd, 1H, *J* = 2.4, 9.2 Hz), 7.85 (d, 1H, *J* = 8.8 Hz), 3.90 (t, 2H, *J* = 6.0 Hz), 1.96 (t, 2H, *J* = 5.6 Hz), 1.40 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 156.2 (q, *J* = 36.6 Hz), 145.7, 141.1, 140.9, 125.5, 122.7, 121.5, 116.6, (q, *J* = 286.5 Hz), 43.1, (q, *J* = 3.7 Hz), 38.3, 33.9, 30.8. <sup>19</sup>F-NMR: -68.8. HRMS (ESI<sup>+</sup>) Calcd for C<sub>13</sub>H<sub>14</sub>F<sub>3</sub>N<sub>2</sub>O<sub>3</sub>: 303.0956, Found: 303.0943.

**1-(6-Amino-4,4-dimethyl-3,4-dihydro-2H-quinolin-1-yl)-2,2,2-trifluoro-ethanone (44):** SnCl<sub>2</sub>·2H<sub>2</sub>O (5.5 g, 24.8 mmol) was added to a solution of **43** (2.5 g, 8.2 mmol) in DMF (10 mL) and the reaction mixture was stirred at room temperature overnight. The reaction mixture was basified to pH8 using saturated NaHCO<sub>3</sub> and extracted with ethyl acetate (2 x 100 mL). After washing with brine and evaporation of the organic layer under

vacuum, the residue was purified by flash chromatography (15 % ethyl acetate: hexane) to give **44** (2.0 g, 89 %). HRMS (ESI<sup>+</sup>) Calcd for C<sub>13</sub>H<sub>16</sub>F<sub>3</sub>N<sub>2</sub>O: 273.1215, Found: 273.1195.

**1-(2-Amino-8,8-dimethyl-7,8-dihydro-6H-thiazolo[4,5-g]quinolin-5-yl)-2,2,2-trifluoro-ethanone (45):** A solution of **44** (1.5 g, 5.5 mmol) and potassium thiocyanate (2.13 g, 22 mmol) in AcOH (20 mL) was stirred at 20°C for 10 min. Bromine (0.87 g, 5.5 mmol) was added dropwise over 20 min. The reaction mixture was stirred at room temperature for an additional 21 h. The reaction mixture was then poured onto crushed ice and the pH was adjusted to 8 using NH<sub>4</sub>OH. The resulting precipitate was vacuum-filtered and dried to give **45**, which was used for next step without further purification (1.5 g, 82 %).

**1-(2-Chloro-8,8-dimethyl-7,8-dihydro-6H-thiazolo[4,5-g]quinolin-5-yl)-2,2,2-trifluoro-ethanone (46):** To a mixture of t-butyl nitrite (0.69 g, 6.7 mmol), cupric chloride (0.54 mg, 5.4 mmol) and acetonitrile (10 mL) was added **45** (1.5 g, 4.5 mmol) in portions over 1h. The reaction mixture was stirred at room temperature for 2h, and then heated to 65 °C for 1h. The mixture was cooled, filtered and the filtrate was poured into 6N HCl and extracted with ethyl acetate. After washing with brine and evaporation of the organic layer under vacuum, the residue was purified by flash chromatography (10 % ethyl acetate: hexane) to give **46** (0.8 g, 51 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 8.19 (s, 1H), 7.26 (s, 1H), 3.91 (t, 2H, *J* = 6.0 Hz), 1.94 (t, 2H, *J* = 6.0 Hz). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 146.9, 143.2, 143.1, 135.5, 131.3, 120.1, 103.6, 38.5, 36.5, 32.4, 30.9. HRMS (ESI<sup>+</sup>) Calcd for C<sub>14</sub>H<sub>13</sub>ClF<sub>3</sub>N<sub>2</sub>OS: 349.0389, Found: 349.0366.

**2-Chloro-8,8-dimethyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinoline (47):** Compound **46** (0.7 g, 2 mmol) was stirred in methanol (20 mL) as NaBH<sub>4</sub> (0.29 g, 8 mmol) was added in portions over five minutes. The mixture was stirred for 15 min, diluted with water and extracted with ethyl acetate. The organic layer was washed with brine and evaporated. The crude product was purified by flash chromatography (15 % ethyl acetate: hexane) to give **47** (0.35 g, 69 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.69 (s, 1H), 6.62 (s, 1H), 4.33 (s, 1H), 3.29 (t, 2H, *J* = 6.0 Hz), 1.67 (t, 2H, *J* = 6.0 Hz). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 146.9, 143.2, 143.1, 135.5, 131.3, 120.1, 103.6, 38.5, 36.5, 32.4, 30.9. HRMS (ESI<sup>+</sup>) Calcd for C<sub>12</sub>H<sub>14</sub>ClN<sub>2</sub>S: 253.0566, Found: 253.0540.

**8,8-Dimethyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinoline-2-carbonitrile (48):** Potassium cyanide (51 mg, 0.79 mmol) was added to a solution of **47** (100 mg, 0.39 mmol) in DMSO (5 mL) and stirred for 3h at 130°C. The reaction was cooled to room temperature, poured into water, and extracted with ethyl acetate (2 x 50 mL). The organic phase was evaporated to dryness and purified by flash chromatography using 20% ethyl acetate: hexane as eluent (45 mg, 47%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>): δ 7.95 (s, 1H), 6.81 (s, 1H), 4.52 (s, 1H), 3.44 (t, 2H, *J* = 6.4 Hz), 1.77 (t, 2H, *J* = 6.0 Hz), 1.37 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 145.7, 145.1, 136.0, 133.4, 129.1, 122.3, 114.3, 102.1, 38.5, 35.8, 32.6, 30.4. HRMS (ESI<sup>+</sup>) Calcd for C<sub>13</sub>H<sub>14</sub>N<sub>3</sub>S: 244.0909, Found: 244.0895.

**CycLuc11; 2-(8,8-Dimethyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinolin-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (49):** D-cysteine (43 mg, 0.24 mmol) was dissolved in 5 mL of 50 mM aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **48** (30 mg, 0.12 mmol) in 5 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer and washed with ethyl acetate. The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 50 mL). The solvent was removed by rotary evaporation to yield **49** as an orange red solid (20 mg, 48 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub> + 2 drops CD<sub>3</sub>OD) : δ 7.91 (s,1H), 6.82 (s,1H), 5.31 (t, 1H, *J* = 9.2 Hz), , 3.75-3.64 (m,2H), 3.37 (t, 2H, *J* = 5.6 Hz), 1.74 (t, 2H, *J* = 5.6 Hz), 1.33 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub> + 2 drops CD<sub>3</sub>OD): δ 172.4, 167.1, 154.4, 146.1, 144.1, 144.0, 136.1, 132.4, 121.9, 103.5, 78.2, 38.4, 36.1, 35.2, 32.5, 30.7. HRMS (ESI<sup>+</sup>) Calcd for C<sub>16</sub>H<sub>18</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub>: 348.0840, Found: 348.0816.

**2-Chloro-5,8,8-trimethyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinoline (50):** Compound **47** (200 mg, 0.79 mmol) and formaldehyde (95 mg, 3.17 mmol) were dissolved in dichloroethane (20 mL). Sodium

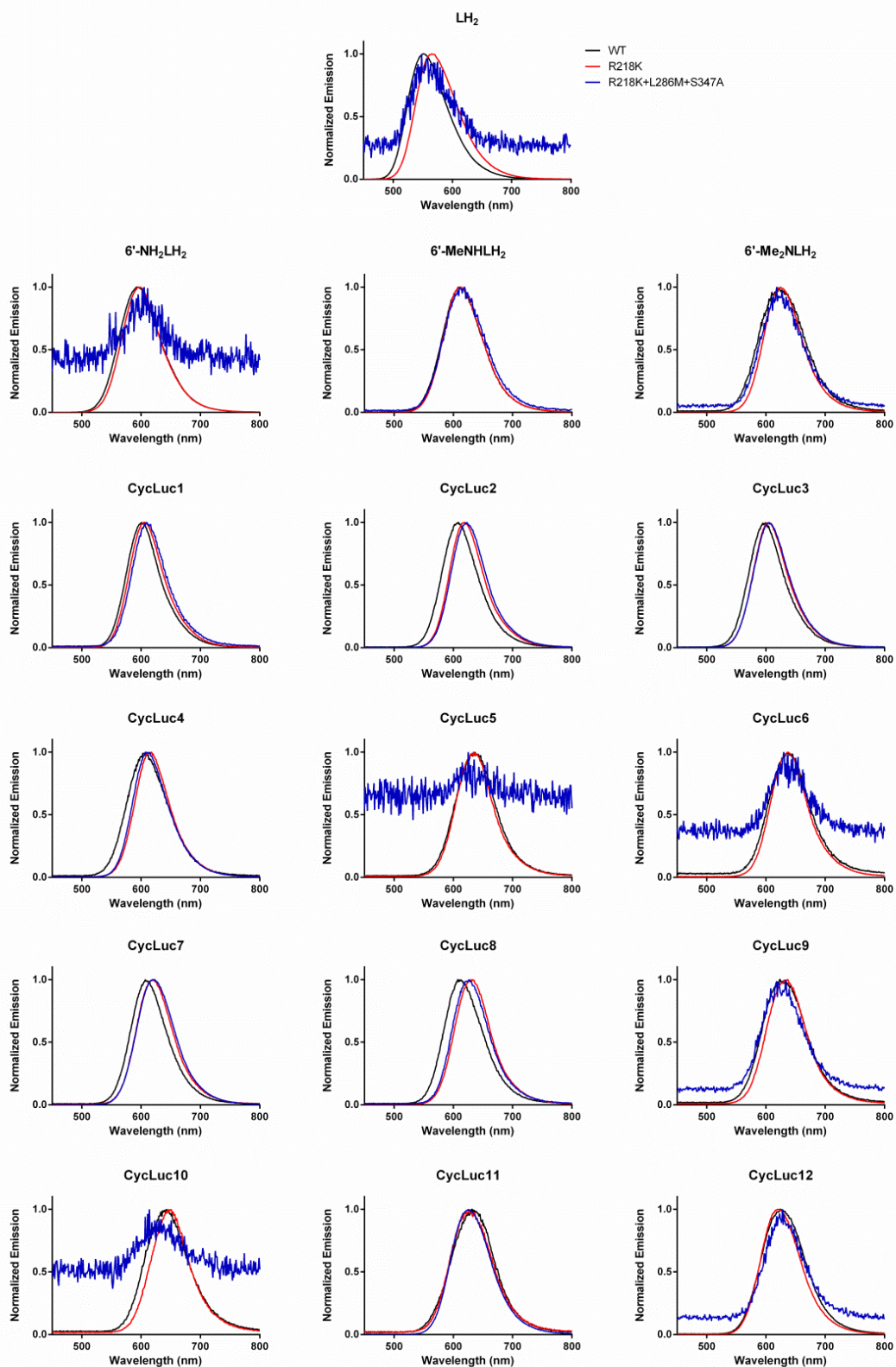
triacetoxyborohydride (250 mg, 1.18 mmol) was added and the reaction mixture was stirred for 1h at ambient temperature. The reaction was quenched with 1N NaOH (20 mL). The product was extracted with ethyl acetate (2 x 100 mL), washed with brine and dried (MgSO<sub>4</sub>). The solvent was removed by rotary evaporation and the resulting solid was purified by flash chromatography using 10% ethyl acetate: hexane as eluent (95 mg, 45 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) : δ 7.71 (s, 1H), 6.77 (s, 1H), 3.32 (t, 2H, *J* = 6.0 Hz), 2.94 (s, 3H), 1.77 (t, 2H, *J* = 5.6 Hz), 1.32 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 146.9, 144.5, 142.5, 135.9, 132.7, 119.5, 100.4, 47.8, 39.7, 36.8, 32.7, 30.7. HRMS (ESI<sup>+</sup>) Calcd for C<sub>13</sub>H<sub>16</sub>ClN<sub>2</sub>S: 267.0723, Found: 267.0709.

**5,8,8-Trimethyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinoline-2-carbonitrile (51):** Potassium cyanide (28.6 mg, 0.44 mmol) was added to a solution of **50** (60 mg, 0.22 mmol) in DMSO (5 mL) and stirred for 3h at 130°C. The reaction was cooled to room temperature, poured into water, and extracted with ethyl acetate (2 x 50 mL). The organic phase was evaporated to dryness and purified by flash chromatography using 15% ethyl acetate: hexane as eluent (25 mg, 44 %). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) : δ 7.91 (s, 1H), 6.84 (s, 1H), 3.42 (t, 2H, *J* = 6.0 Hz), 3.02 (s, 3H), 1.78 (t, 2H, *J* = 6.4 Hz), 1.34 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 146.7, 144.2, 136.7, 134.9, 128.5, 121.1, 114.5, 98.9, 47.8, 39.6, 36.0, 32.8, 30.0. HRMS (ESI<sup>+</sup>) Calcd for C<sub>14</sub>H<sub>15</sub>N<sub>3</sub>SNa: 280.0884, Found: 280.0880.

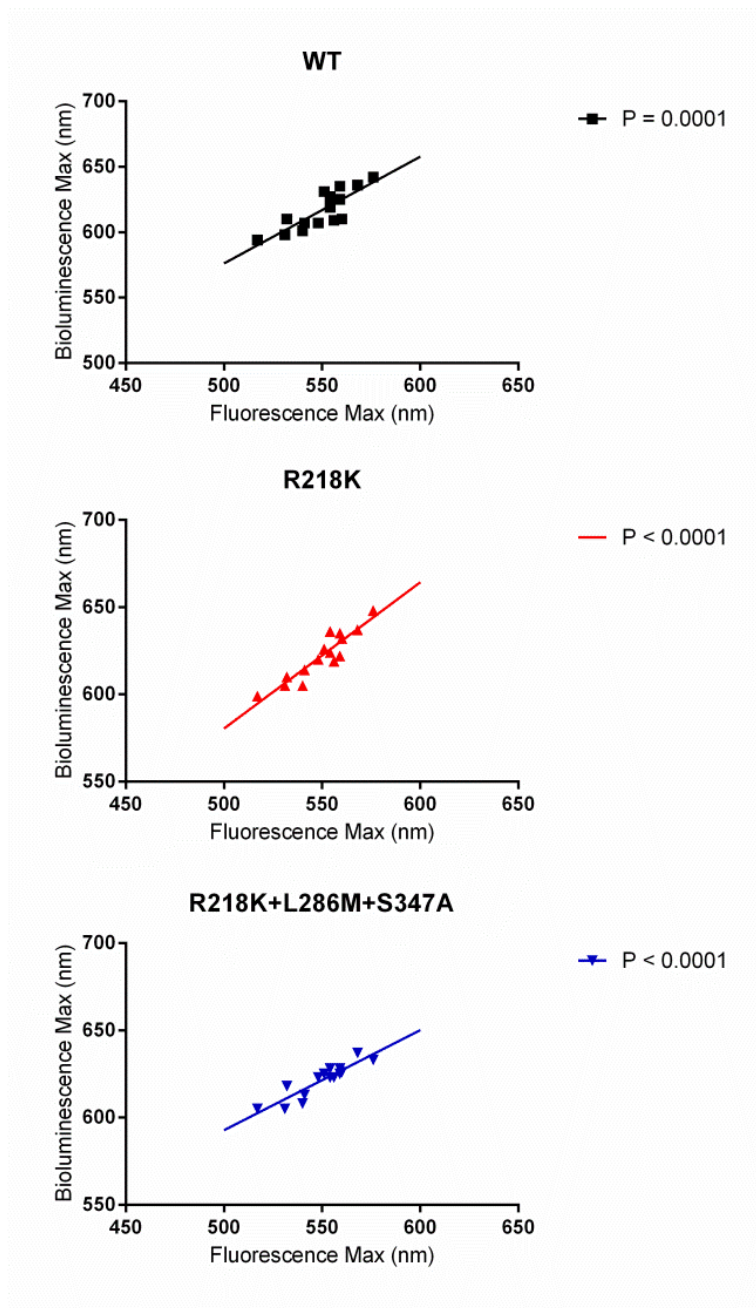
**CycLuc12; 2-(5,8,8-Trimethyl-5,6,7,8-tetrahydro-thiazolo[4,5-g]quinolin-2-yl)-4,5-dihydro-thiazole-4-carboxylic acid (52):** D-cysteine (27 mg, 0.155 mmol) was dissolved in 5 mL of 50 mM aqueous sodium phosphate buffer, pH 8 and degassed under argon. This solution was added to **51** (20 mg, 0.077 mmol) in 5 mL of degassed methanol. The reaction was stirred for 1h, and then diluted with sodium phosphate buffer and washed with ethyl acetate. The aqueous phase was acidified to pH 4 with 1M HCl and extracted with ethyl acetate (2 x 50 mL). The solvent was removed by rotary evaporation to yield **52** as an orange red solid (15 mg, 53%). <sup>1</sup>H-NMR (400 MHz, CDCl<sub>3</sub>) : δ 7.92 (s, 1H), 6.88 (s, 1H), 5.40 (t, 1H, *J* = 10 Hz), 3.75 (dd, 2H, *J* = 2, 9.6 Hz), 3.39 (t, 2H, *J* = 6.0 Hz), 3.00 (s, 3H), 1.78 (t, 2H, *J* = 6.4 Hz), 1.33 (s, 6H). <sup>13</sup>C-NMR (100 MHz, CDCl<sub>3</sub>): δ 173.3, 168.2, 153.16, 145.8, 145.2, 137.1, 133.9, 121.2, 100.1, 77.9, 47.8, 39.8, 36.3, 34.9, 32.8, 30.4. HRMS (ESI<sup>+</sup>) Calcd for C<sub>17</sub>H<sub>20</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub>: 362.0997, Found: 362.0979.

	Fluorescence (PBS)	WT	R/K	R/K+L/M+S/A
LH <sub>2</sub>	528 nm	551 nm	566 nm	558 nm
6'-NH <sub>2</sub> LH <sub>2</sub>	517	594	599	605
6'-MeNHLH <sub>2</sub>	532	610	610	618
6'-Me <sub>2</sub> NLH <sub>2</sub>	554	619	624	623
CycLuc1	540	601	605	608
CycLuc2	556	609	619	623
CycLuc3	531	598	605	605
CycLuc4	544	607	614	613
CycLuc5	559	635	635	625
CycLuc6	567	636	637	637
CycLuc7	548	607	620	623
CycLuc8	560	610	632	626
CycLuc9	554	627	636	628
CycLuc10	576	642	648	633
CycLuc11	551	631	626	625
CycLuc12	559	625	622	628

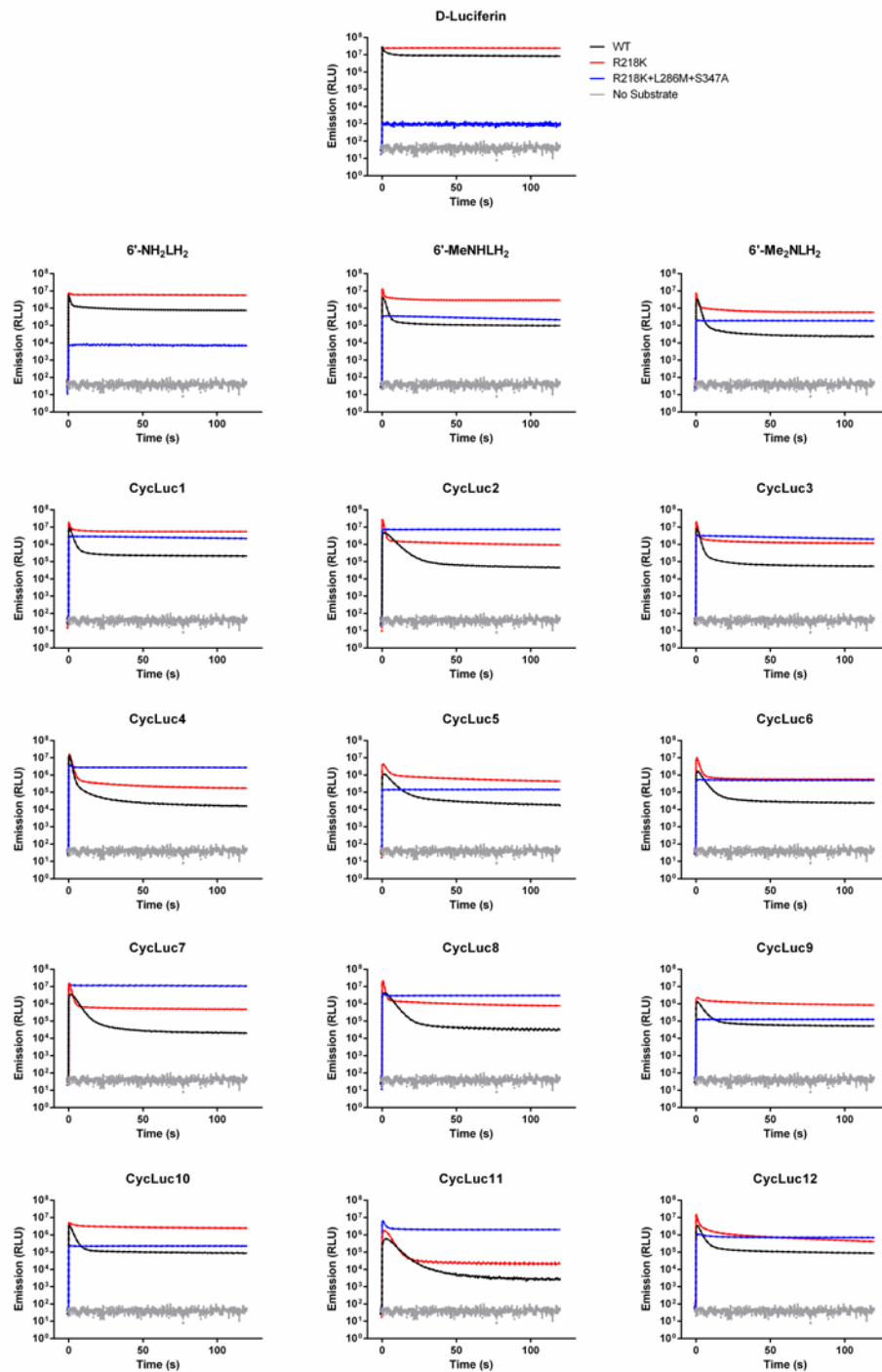
**Supplementary Table 1.** Fluorescence and bioluminescence emission wavelengths of all luciferins.



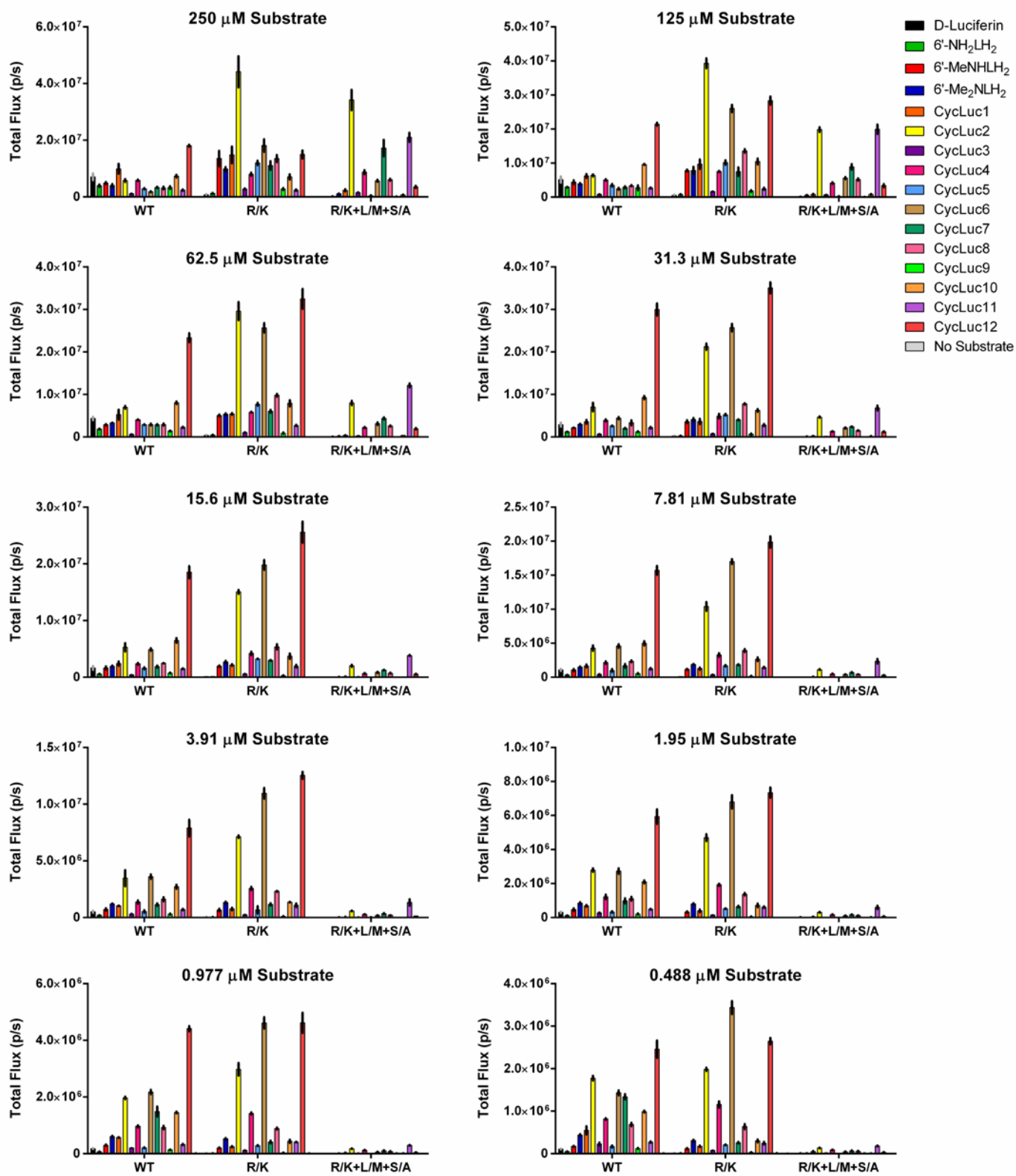
**Supplementary Figure 1.** Normalized emission spectra of all luciferins with each luciferase.



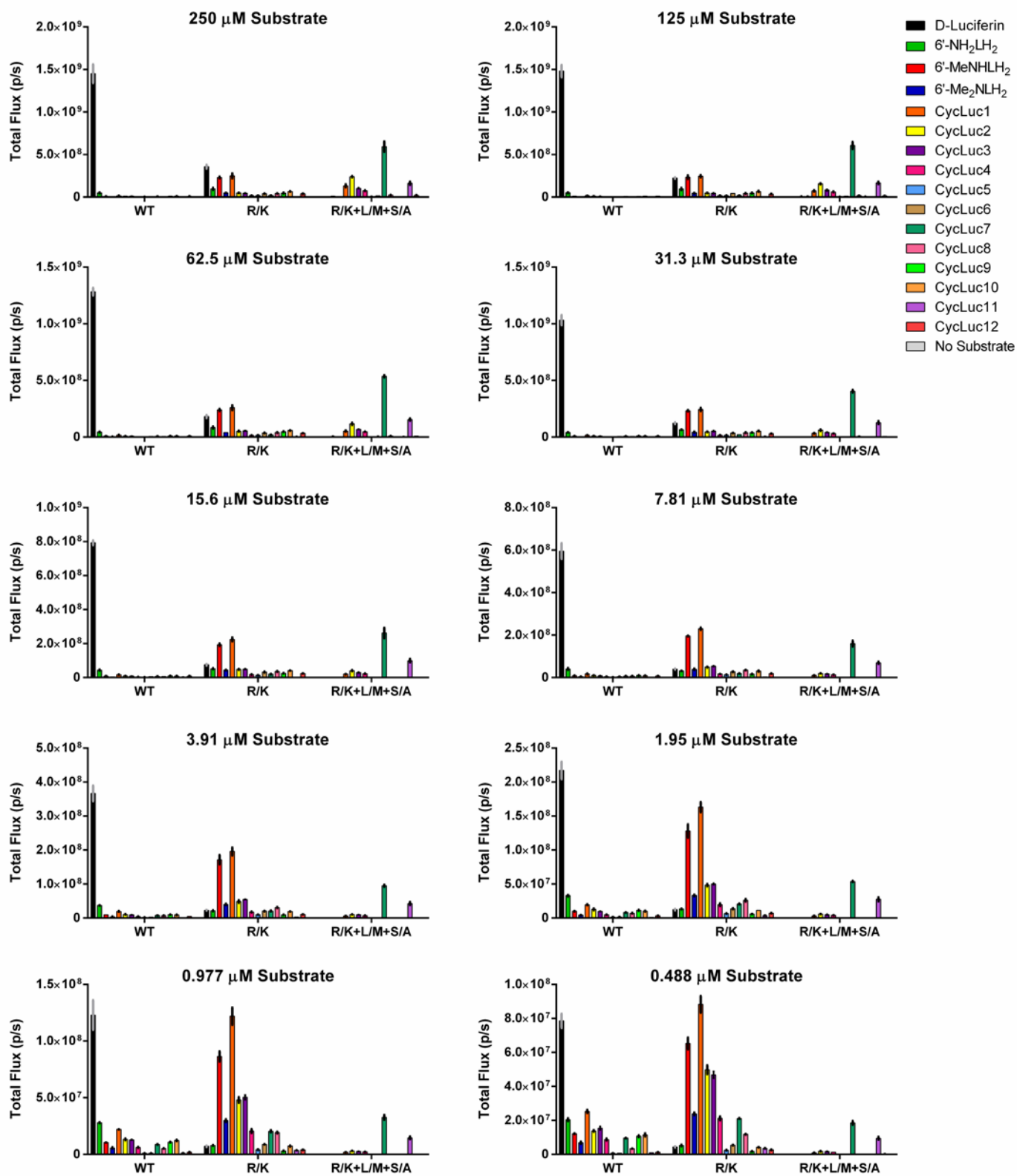
**Supplementary Figure 2.** Fluorescence peak emission wavelength for each aminoluciferin correlated with the bioluminescence peak wavelength with the indicated luciferase. Pearson correlation values are  $r = 0.83$  (WT),  $r = 0.92$  (R218K) and  $r = 0.91$  (triple mutant).



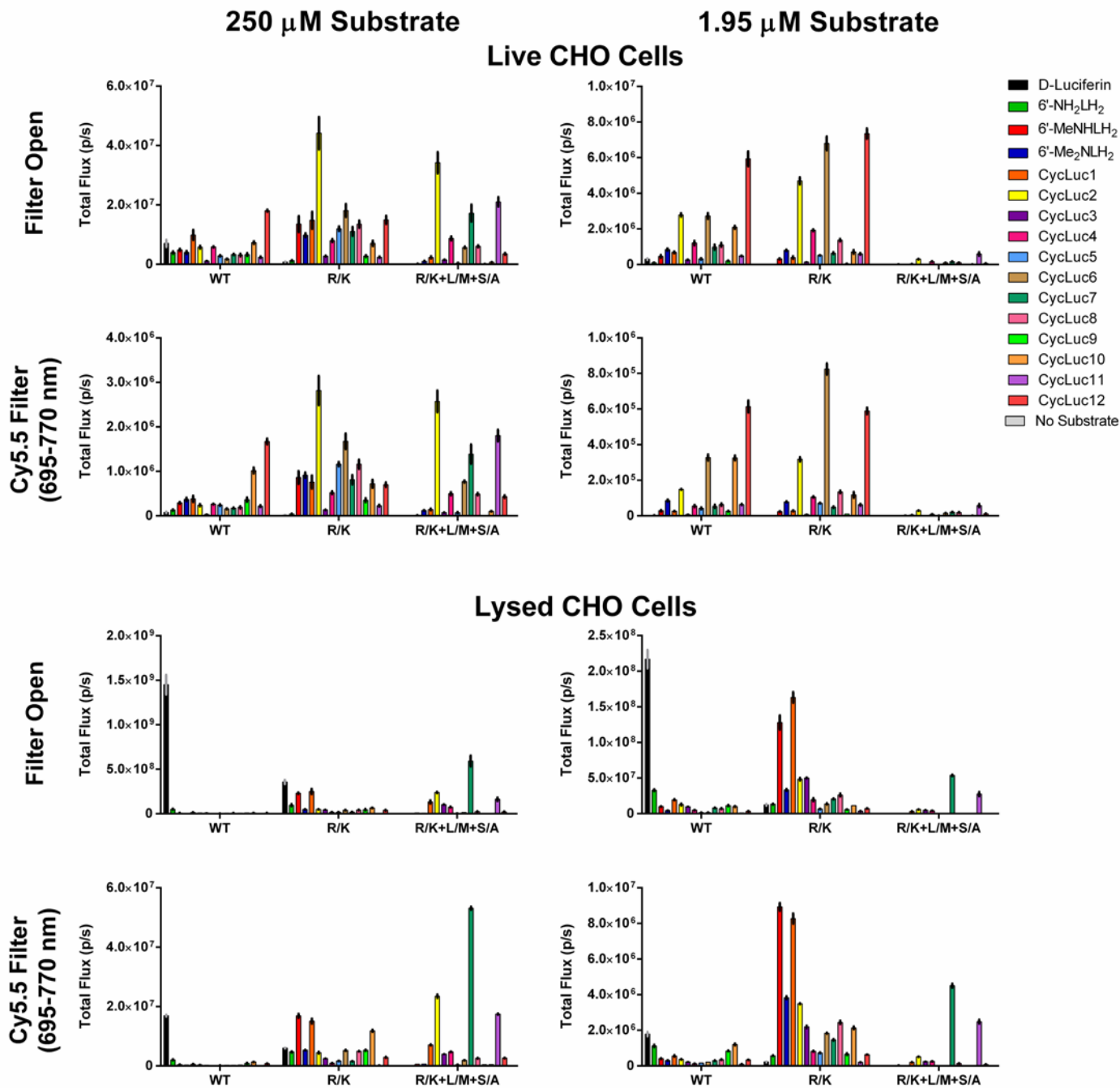
**Supplementary Figure 3.** Burst kinetics profiles of all luciferins with each luciferase, presented on the same log scale. Purified enzyme (0.2 nM final) was rapidly injected into substrate (250  $\mu$ M final). Light emission was recorded every 0.2 s for 1 s pre-injection and 120 s post-injection. Background luminescent signal in the absence of substrate is shown for reference (in gray). The assays were performed in triplicate and are represented as the mean  $\pm$  SEM.



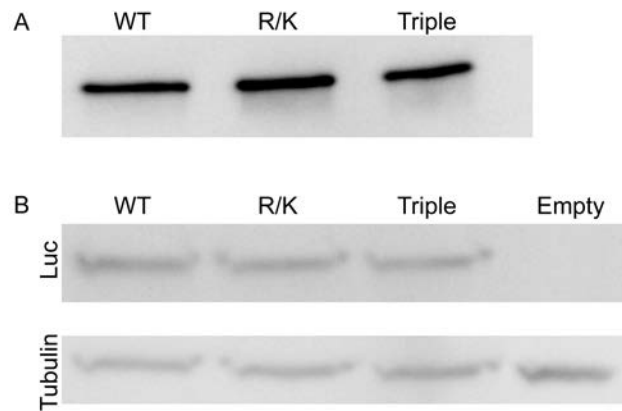
**Supplementary Figure 4.** Photon flux from live luciferase-expressing CHO-K1 cells incubated with the indicated concentration of each substrate. Note that the y-axis scale at different substrate concentrations differs. The assays were performed in triplicate and are represented as the mean  $\pm$  SEM.



**Supplementary Figure 5.** Total photon flux from lysed luciferase-expressing CHO-K1 cells treated with the indicated concentration of substrate. Note that the y-axis scale at different substrate concentrations differs. The assay was performed in triplicate and is represented as the mean  $\pm$  SEM.



**Supplementary Figure 6.** Total photon flux from live and lysed luciferase-expressing CHO-K1 cells treated with 250  $\mu$ M or 1.95  $\mu$ M luciferin, compared to flux filtered through a Cy5.5 bandpass filter. The assay was performed in triplicate and is represented as the mean  $\pm$  SEM.



**Supplementary Figure 7.** Western blot analysis of luciferase expression. A) Purified recombinant luciferases are equally recognized by an anti-luciferase antibody. B) CHO cell lysates transfected with WT or mutant luciferases yield equivalent expression.  $\alpha$ -tubulin is shown as a loading control.

Supplementary References:

1. Reddy, G.R.; Thompson, W.C.; Miller, S.C., *J. Am. Chem. Soc.* **132**, 13586-13587 (2010).
2. Harwood, K.R.; Mofford, D.M.; Reddy, G.R.; Miller, S.C., *Chemistry & Biology*, **18**, 1649-1657 (2011).
3. Bonger, K.M.; Hoogendoorn, S.; van Koppen, C.J.; Timmers, C.M.; Overkleeft, H.S.; van der Marel G.A., *ChemMedChem*, **4**, 2098-2102 (2009).
4. Williamson, N.M.; Ward, A.D., *Tetrahedron*, **61**, 155-165 (2005).



—11.30

7.90  
7.90  
7.88  
7.88

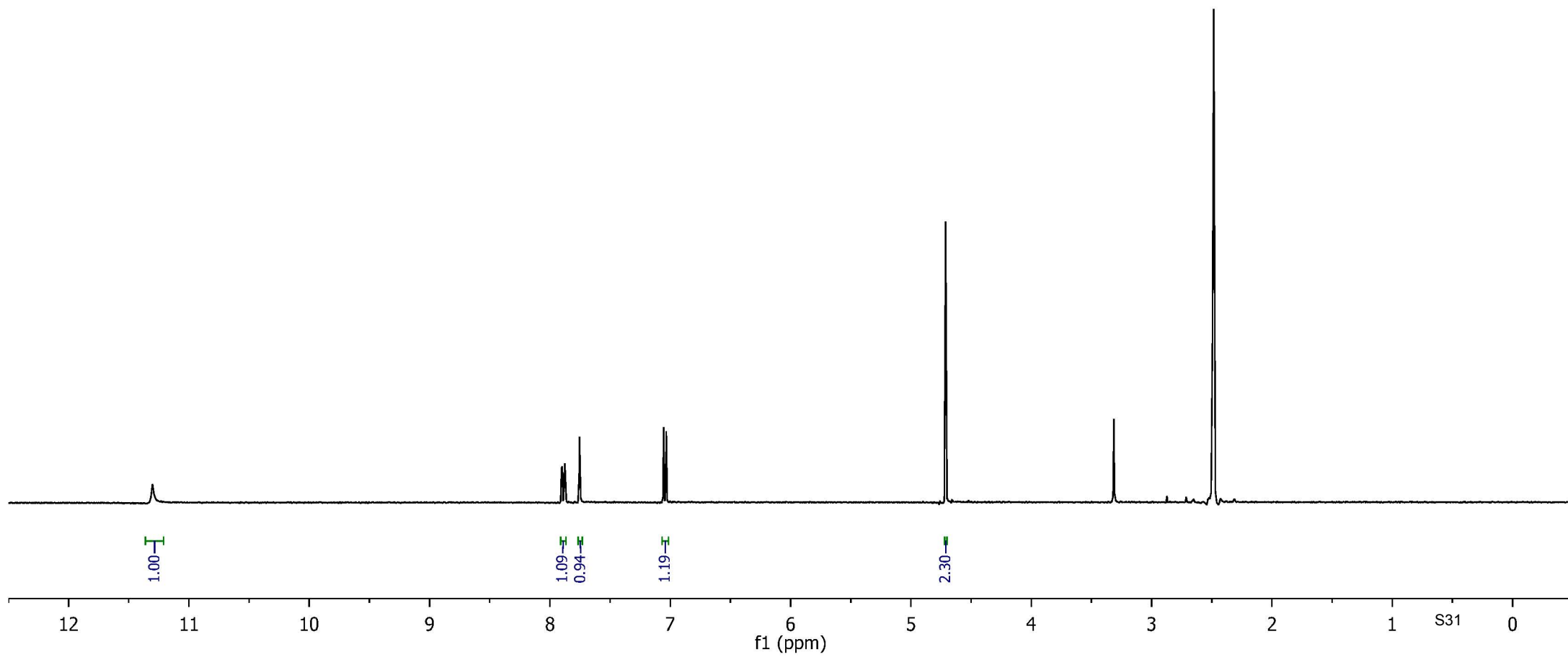
7.75  
7.75

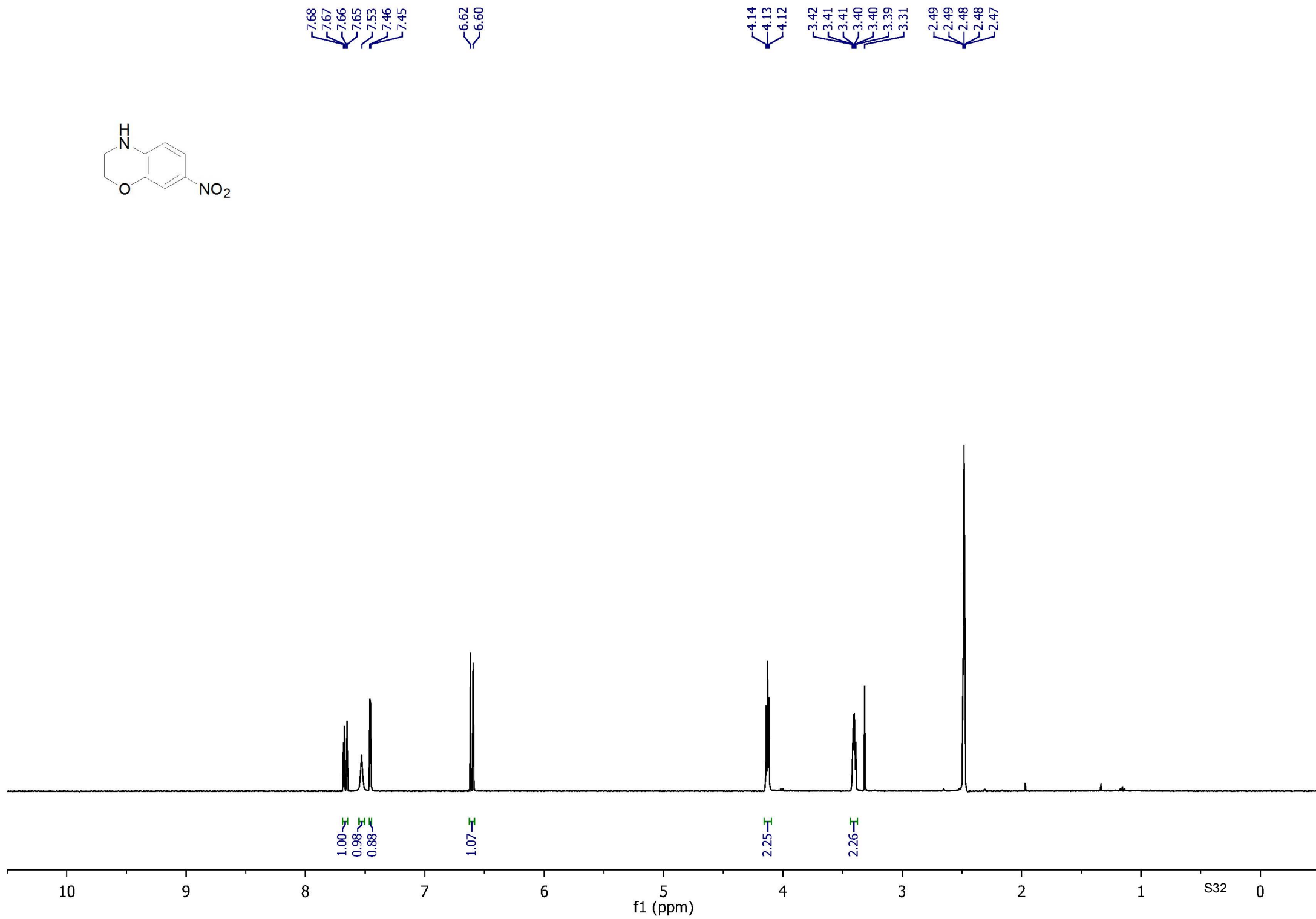
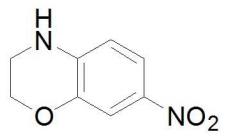
7.06  
7.03

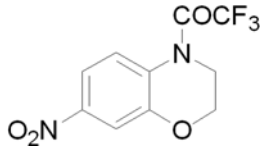
—4.71

—3.31

2.49  
2.49  
2.48  
2.48  
2.48

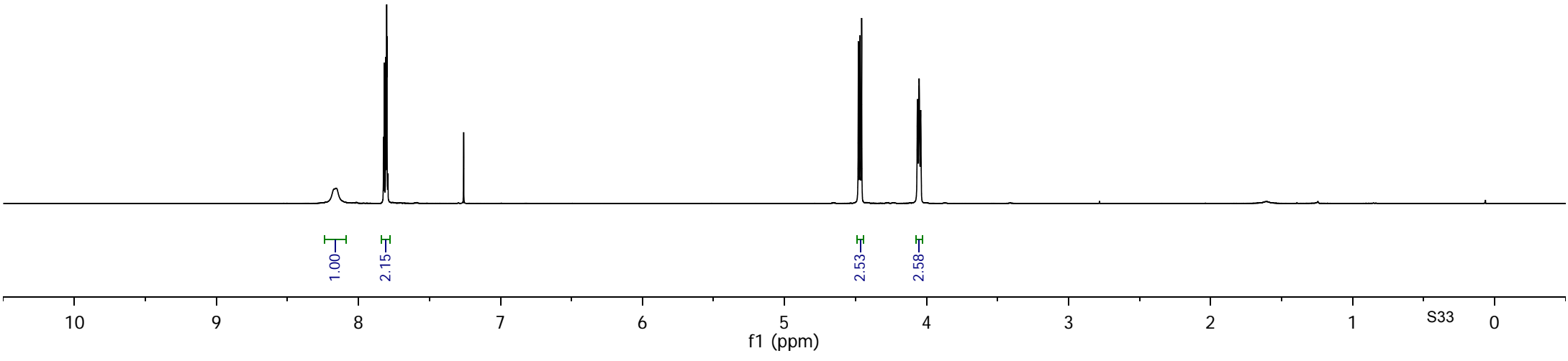


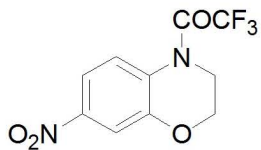




8.16  
7.82  
7.82  
7.81  
7.80  
7.79  
7.78

4.48  
4.47  
4.46  
4.06  
4.05  
4.04





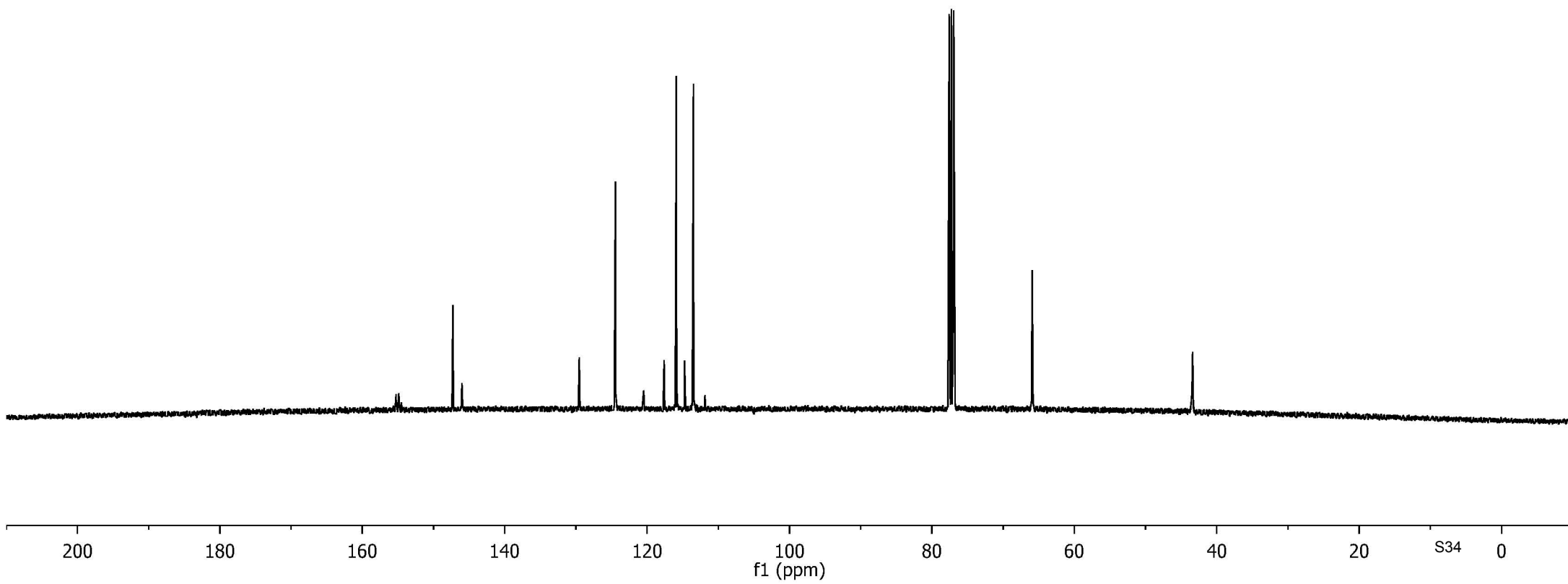
155.62  
155.25  
154.88  
154.51  
147.28  
146.00

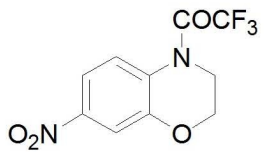
129.53  
124.45  
120.48  
117.61  
115.90  
114.74  
113.49  
111.88

77.57 cdcl3  
77.26 cdcl3  
76.94 cdcl3

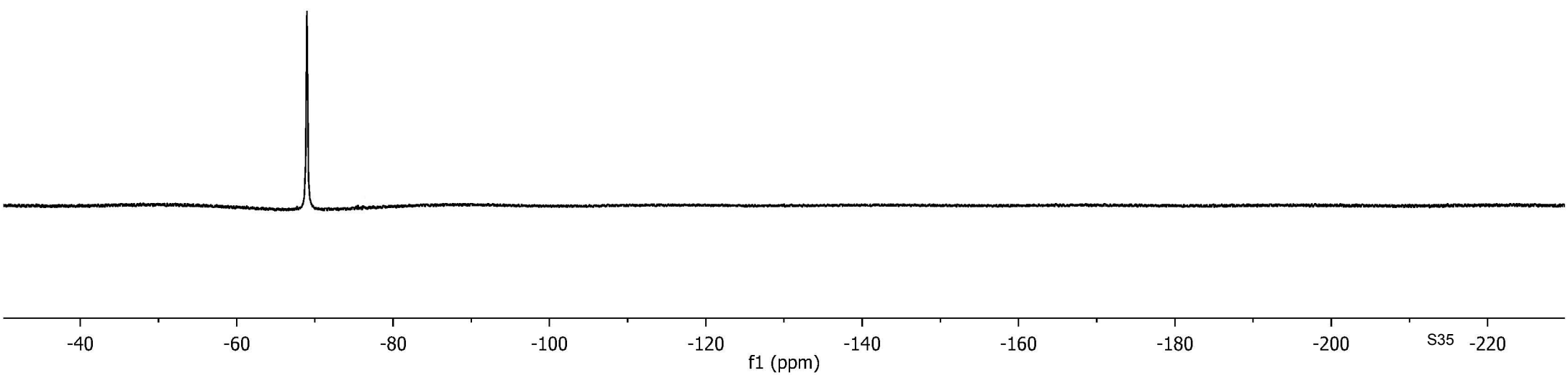
65.92

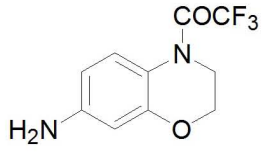
43.42





86.89



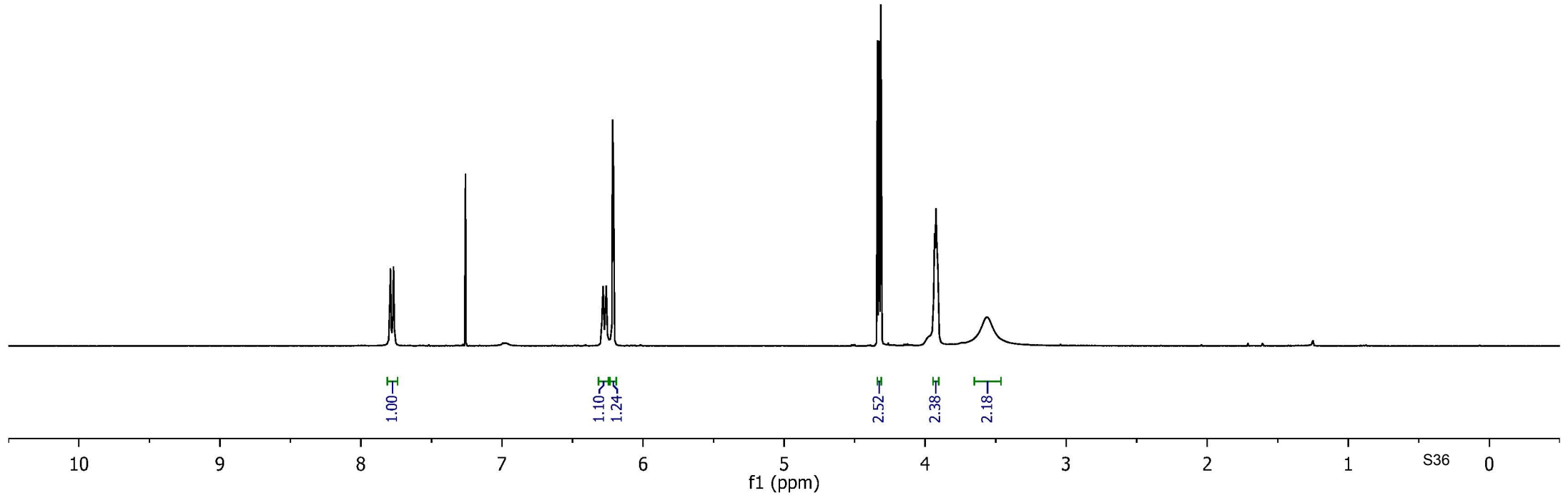


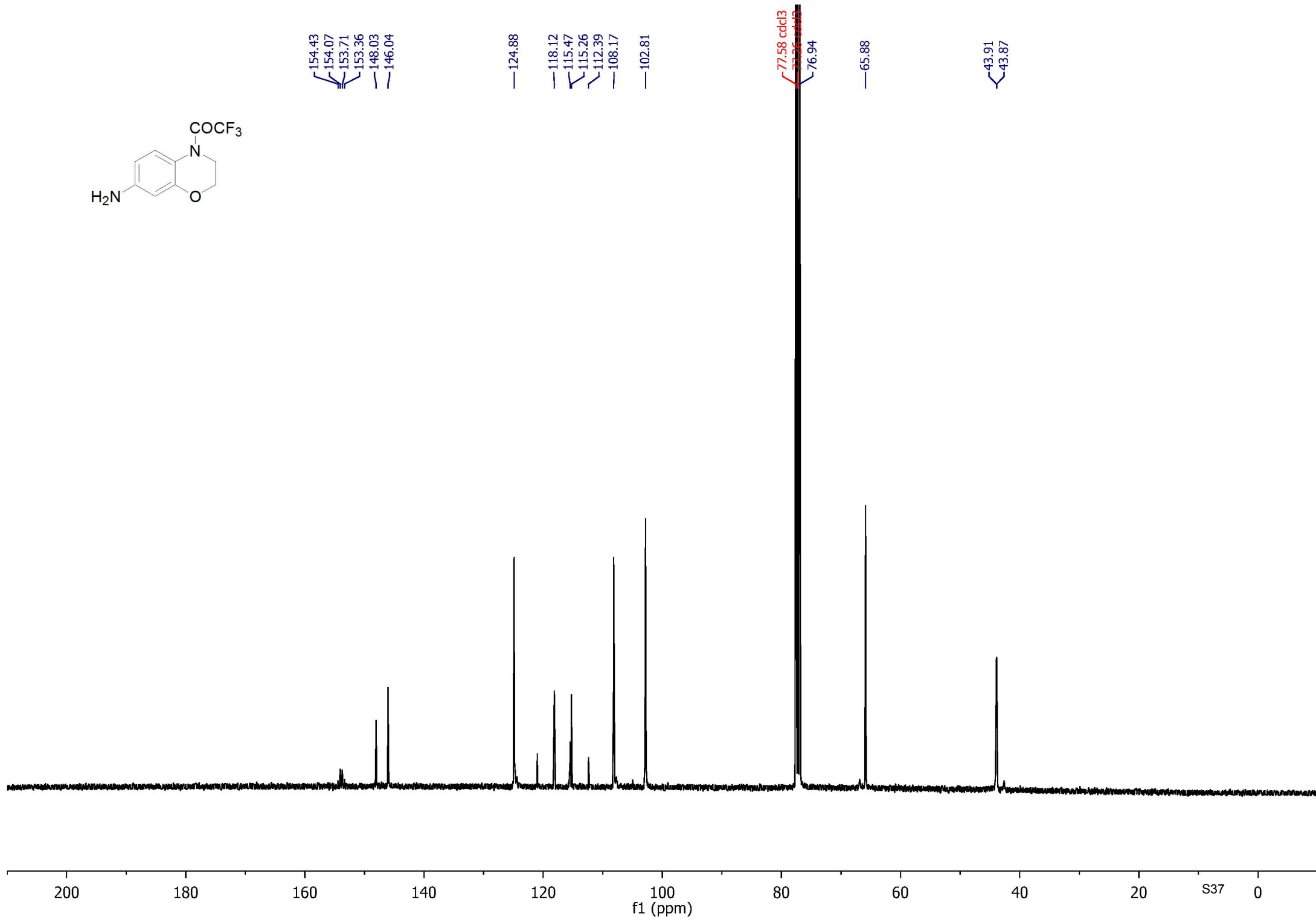
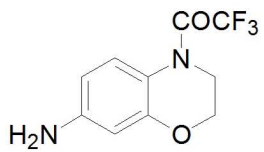
7.79  
7.77

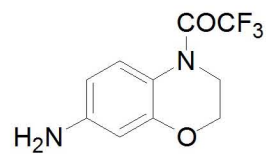
7.26

6.29  
6.28  
6.27  
6.26  
6.22  
6.21

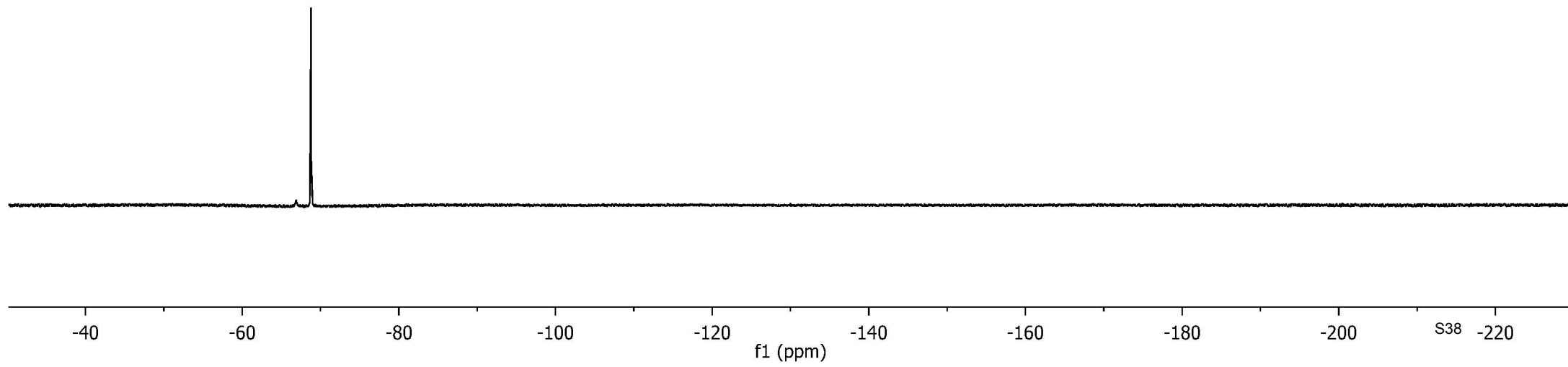
4.34  
4.33  
4.31  
3.93  
3.92  
3.91  
3.56

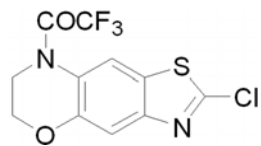






68.79





8.49

7.49

7.26

4.46

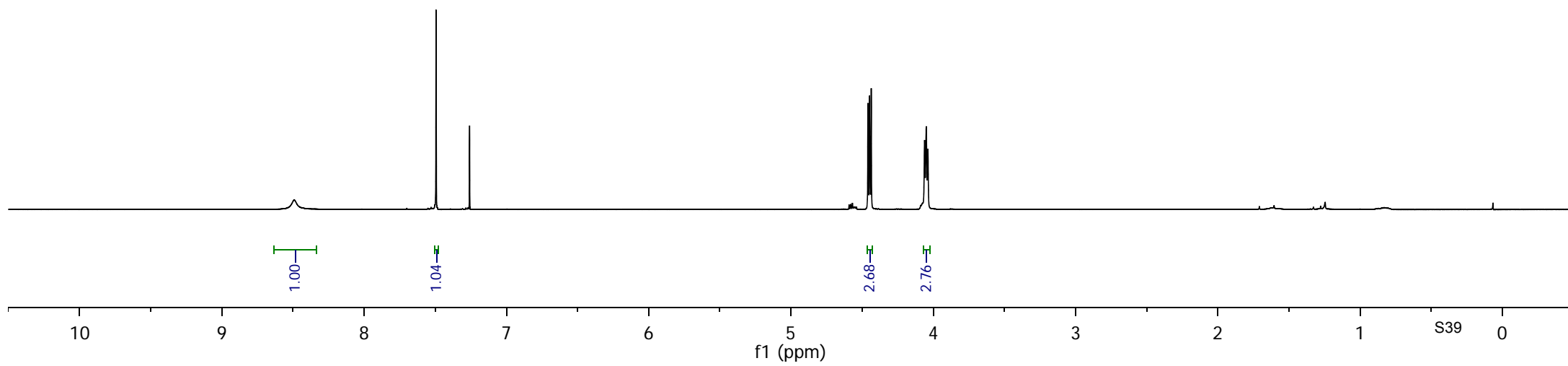
4.45

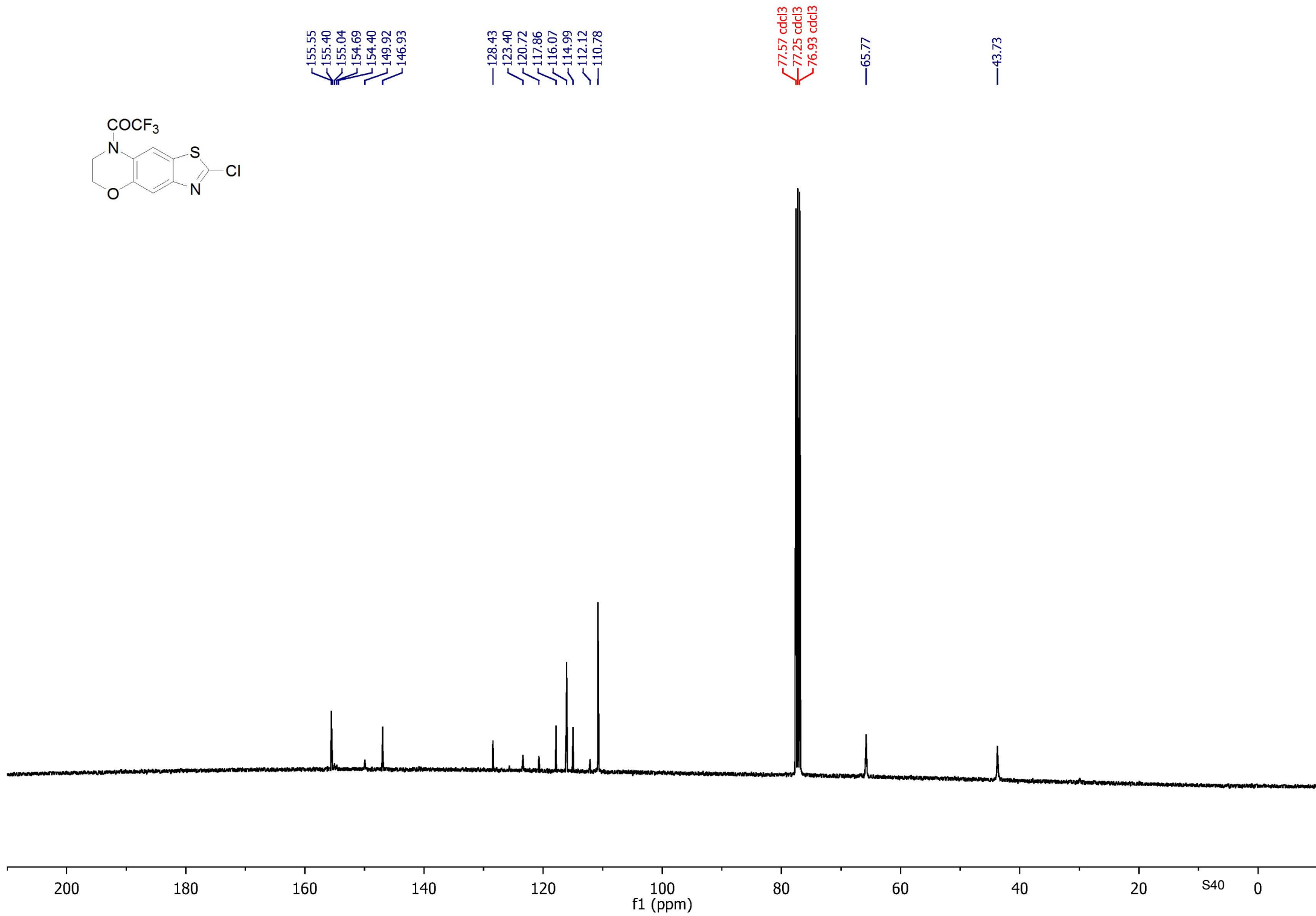
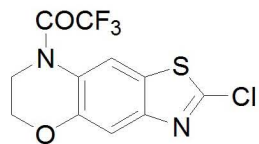
4.44

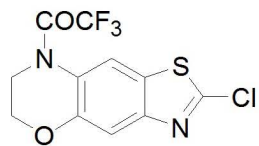
4.06

4.05

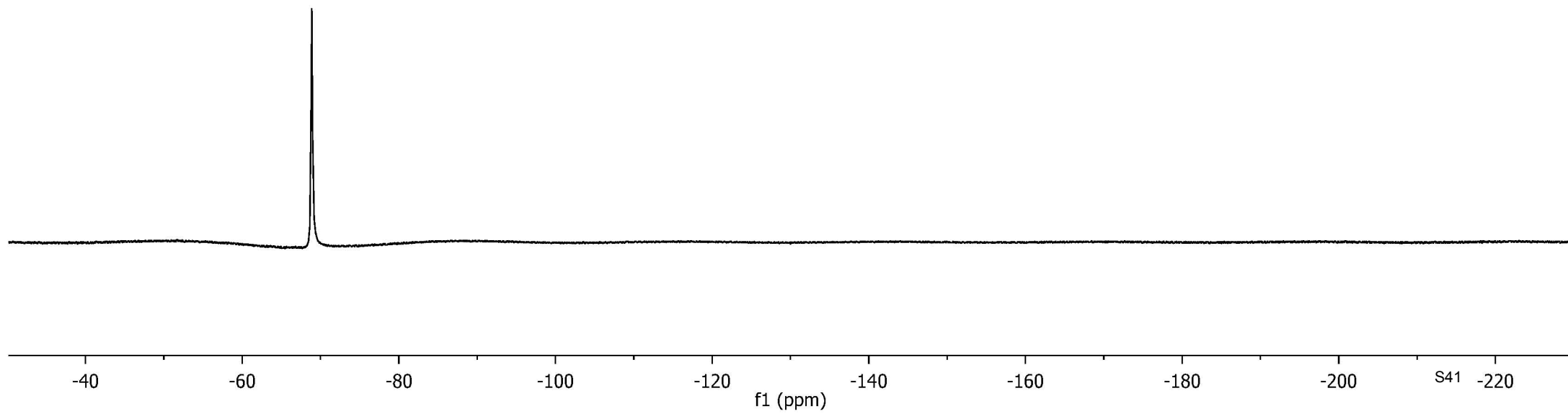
4.04

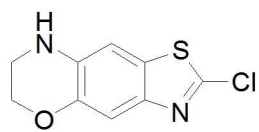






06.89



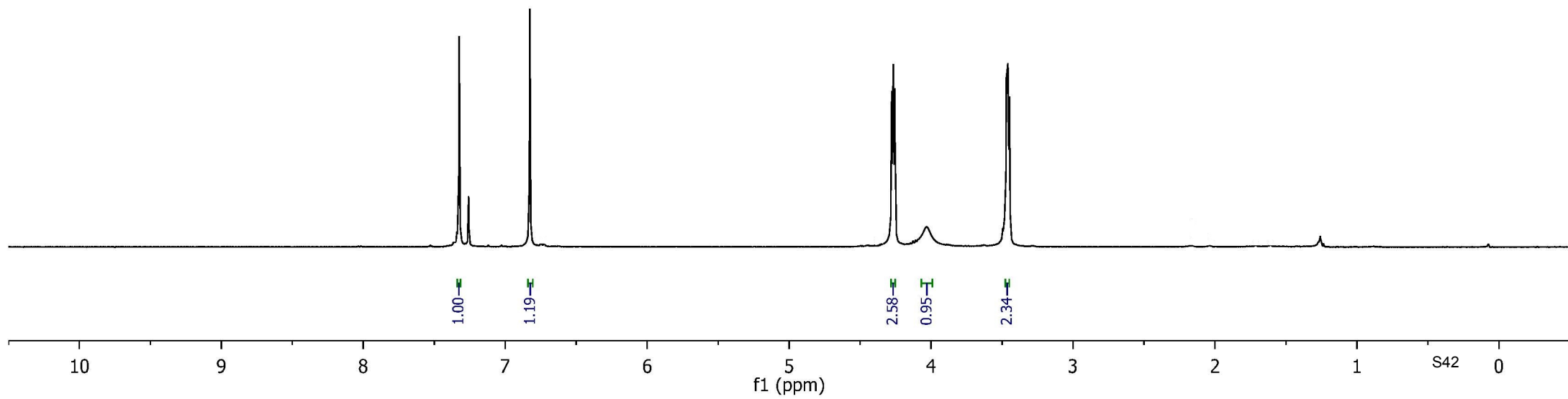


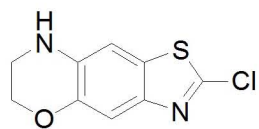
7.32  
7.26

6.83

4.28  
4.27  
4.26  
4.04

3.47  
3.46  
3.45





148.85  
144.51  
144.28

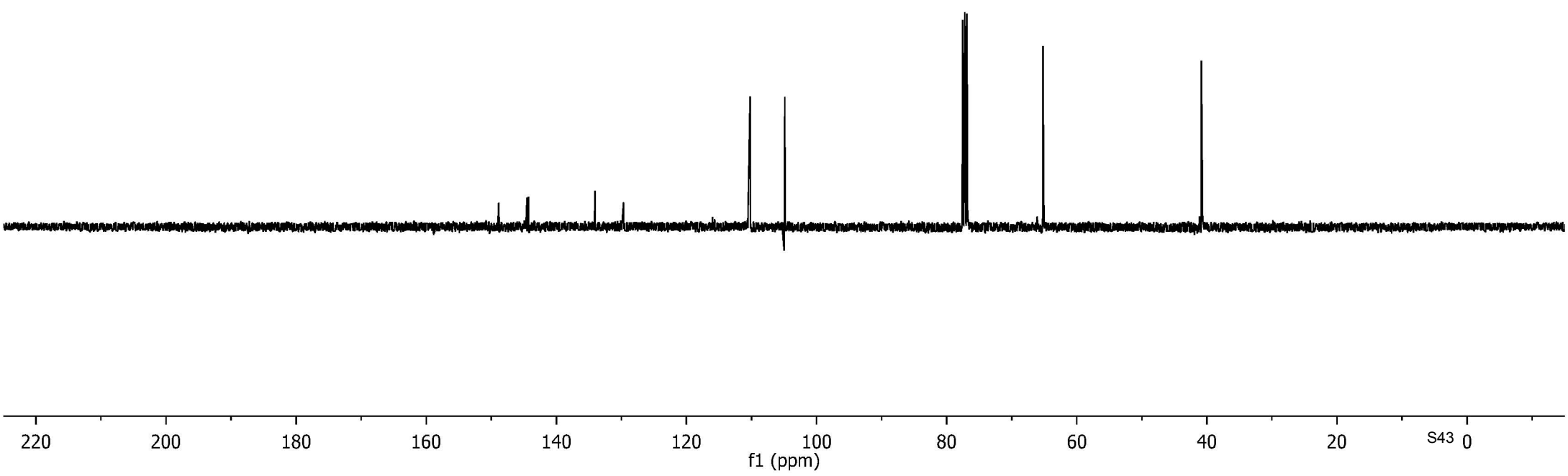
134.06  
129.66

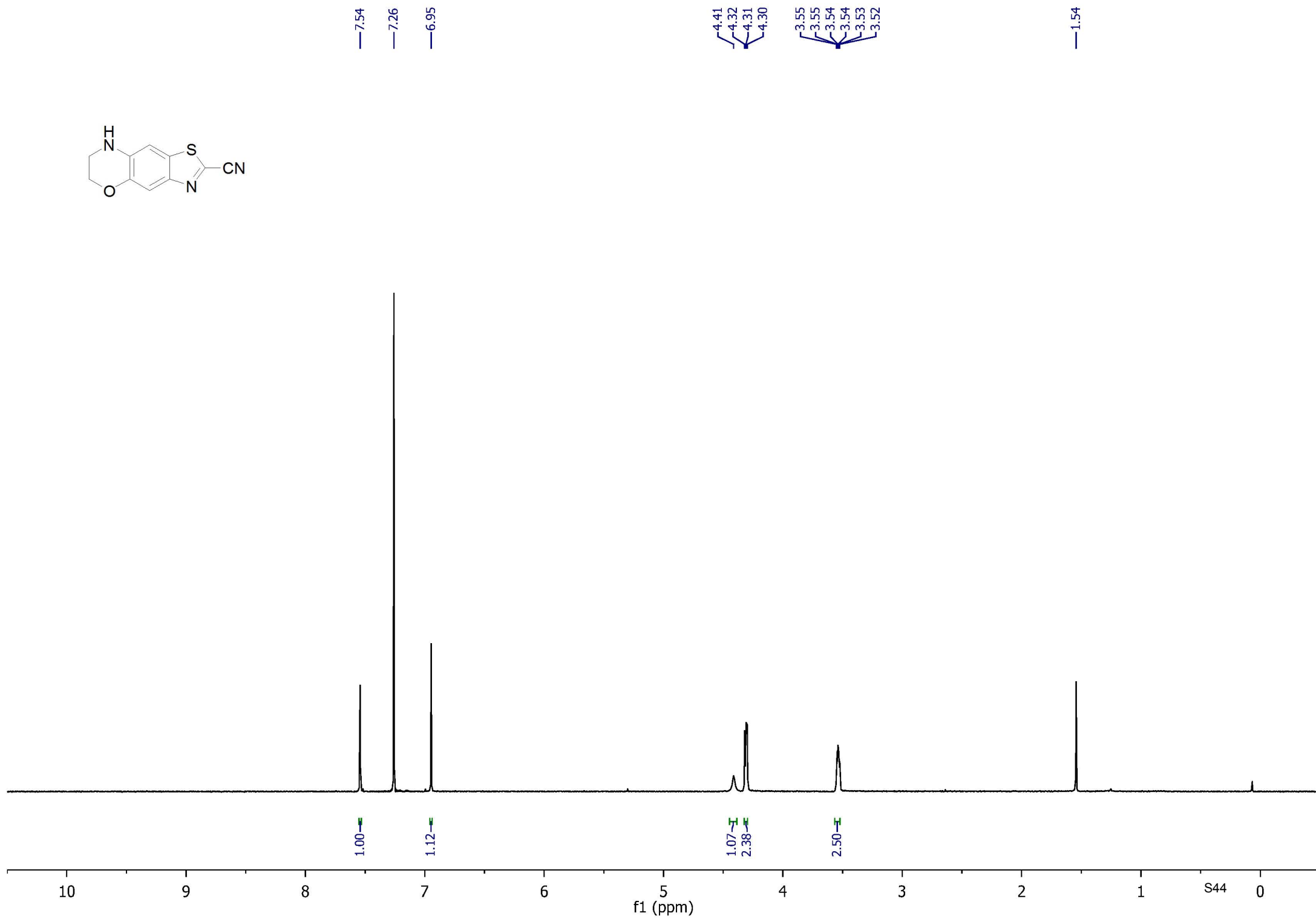
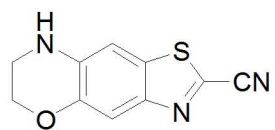
110.22  
104.89

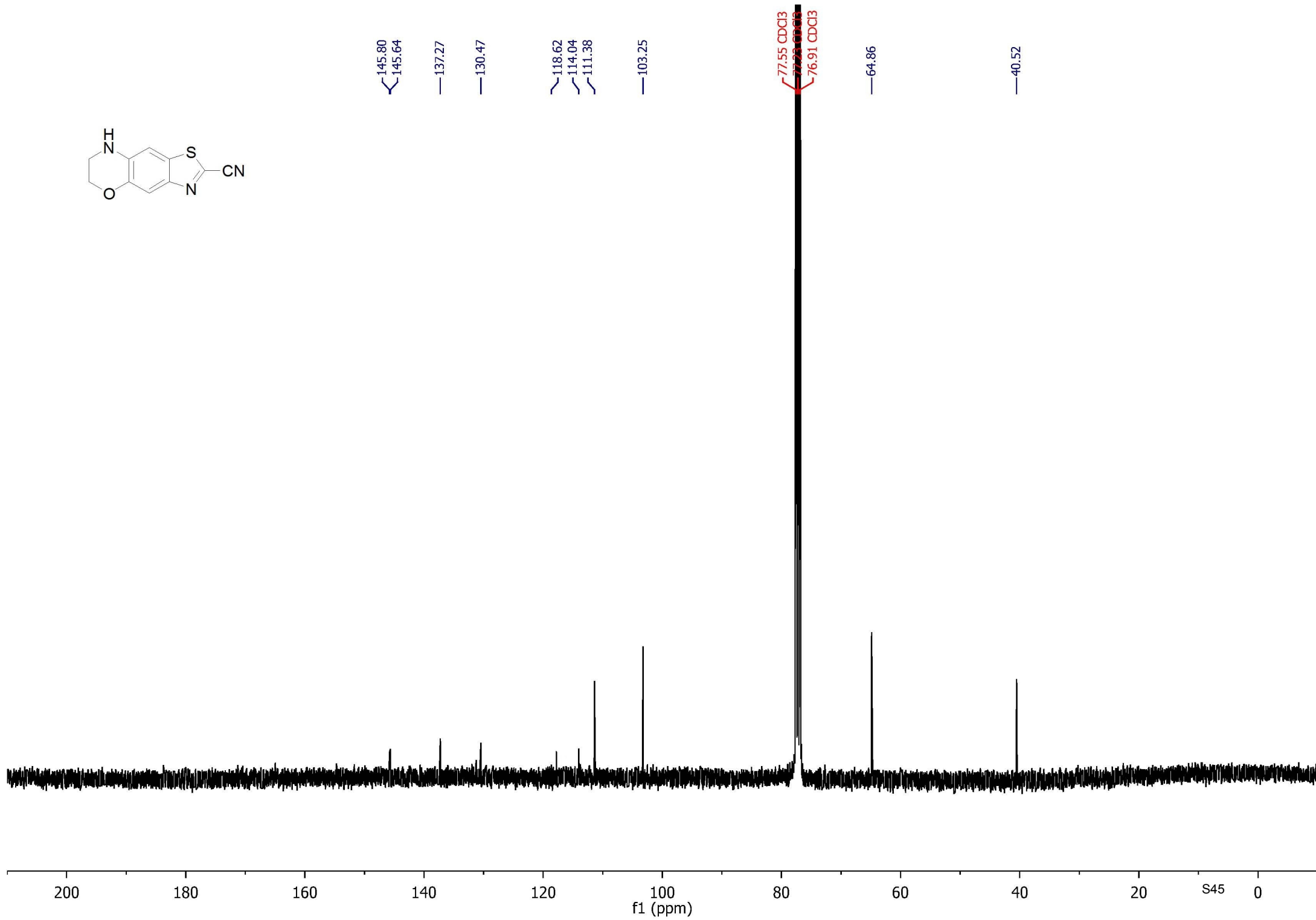
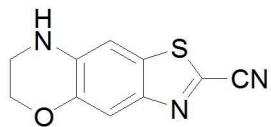
77.53 cdd13  
77.21 cdd13  
76.89 cdd13

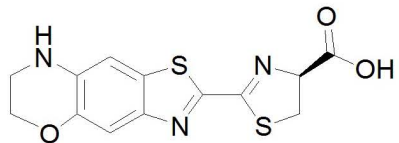
65.21

40.85





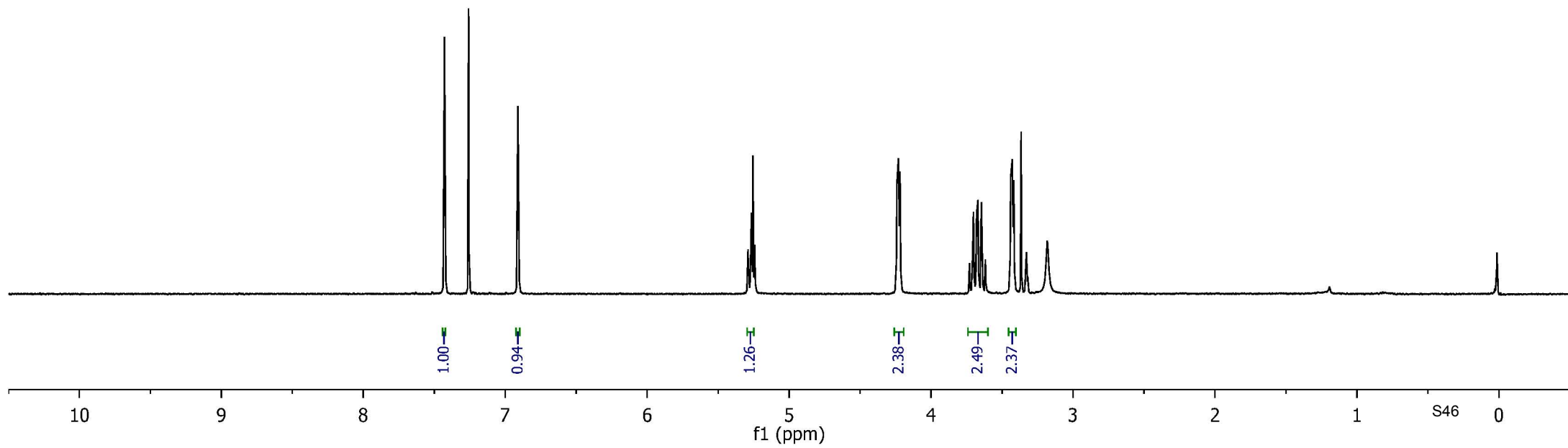


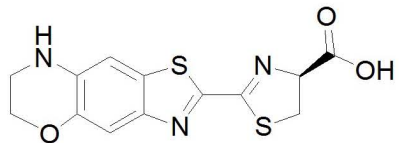


7.43  
7.26  
6.91

5.29  
5.27  
5.24

4.24  
4.23  
4.22  
3.73  
3.71  
3.70  
3.68  
3.67  
3.65  
3.62  
3.44  
3.43  
3.42  
3.34  
3.33  
3.33  
3.32  
3.32





—172.34

—166.91

—156.04

—146.32

—144.78

—136.12

—130.73

—110.81

—104.24

78.16

77.61 ccd13

77.29 ccd13

76.97 ccd13

—64.98

50.06

49.85

49.64

49.42

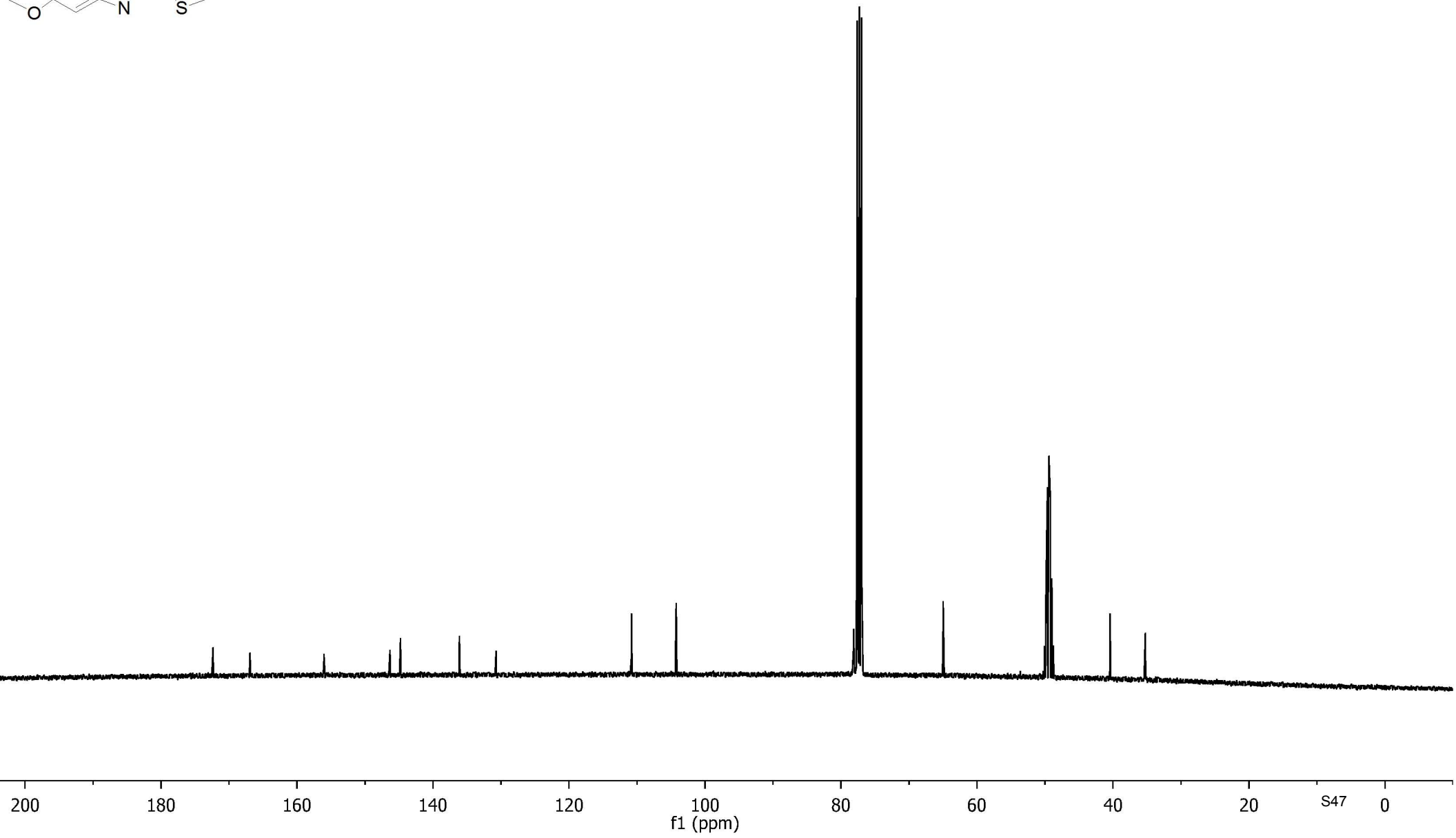
49.21

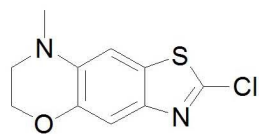
49.00

48.78

40.43

—35.24





7.31  
7.26

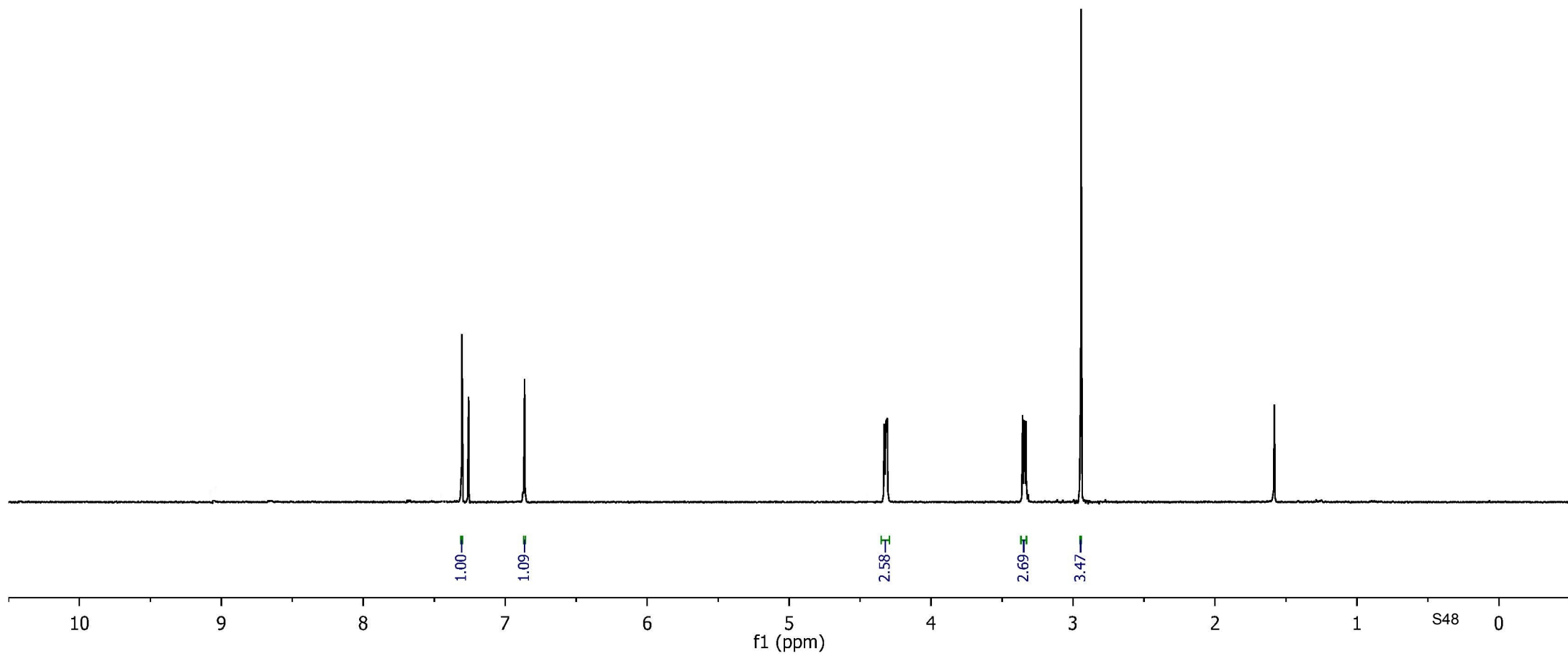
6.86

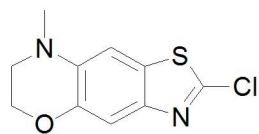
4.33  
4.32  
4.31

3.35  
3.34  
3.33

2.94

1.58





148.64  
144.63  
143.51

136.73

130.05

109.24

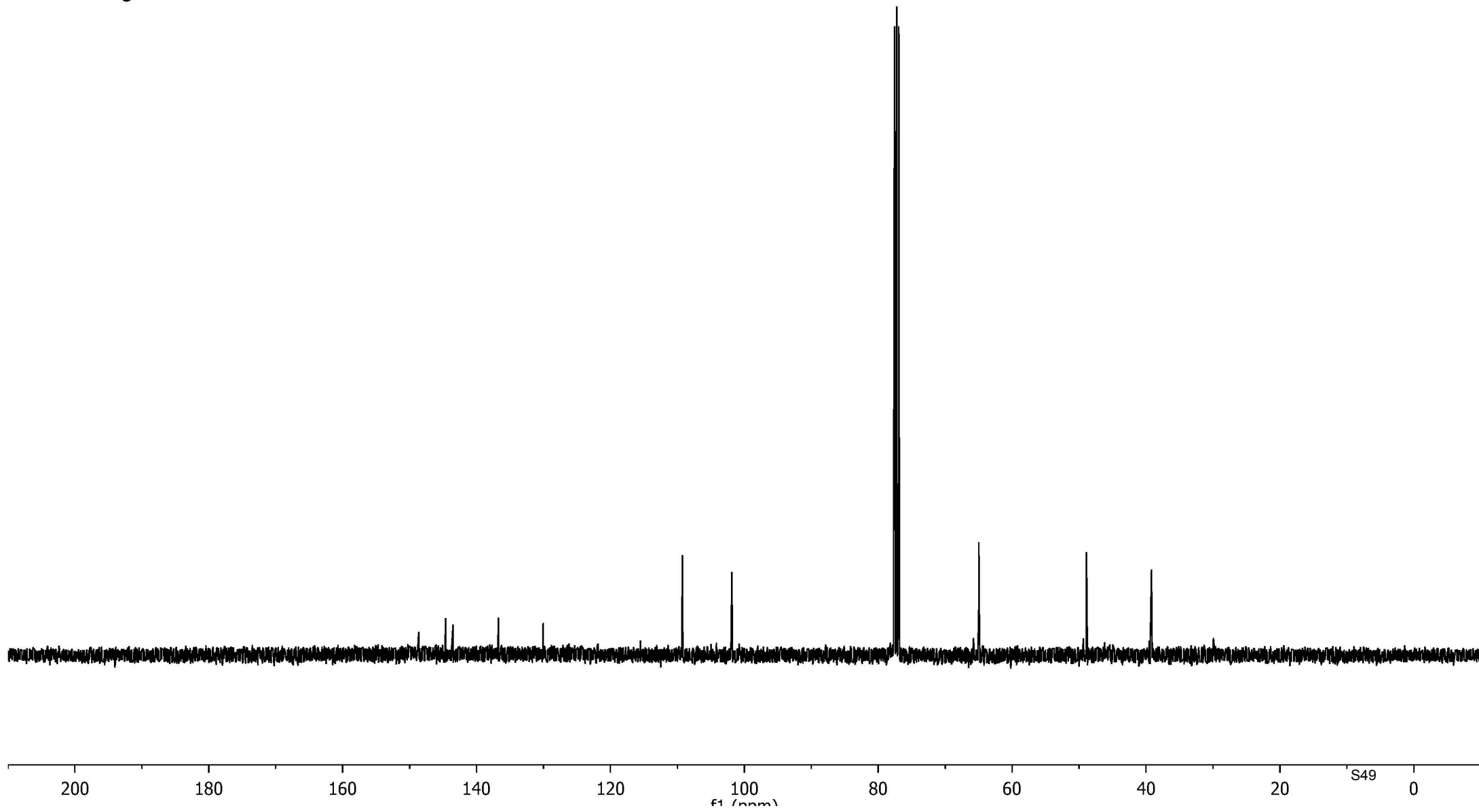
101.86

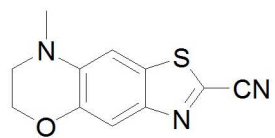
77.57 CDCl<sub>3</sub>  
77.25 CDCl<sub>3</sub>  
76.93 CDCl<sub>3</sub>

64.98

48.89

39.19





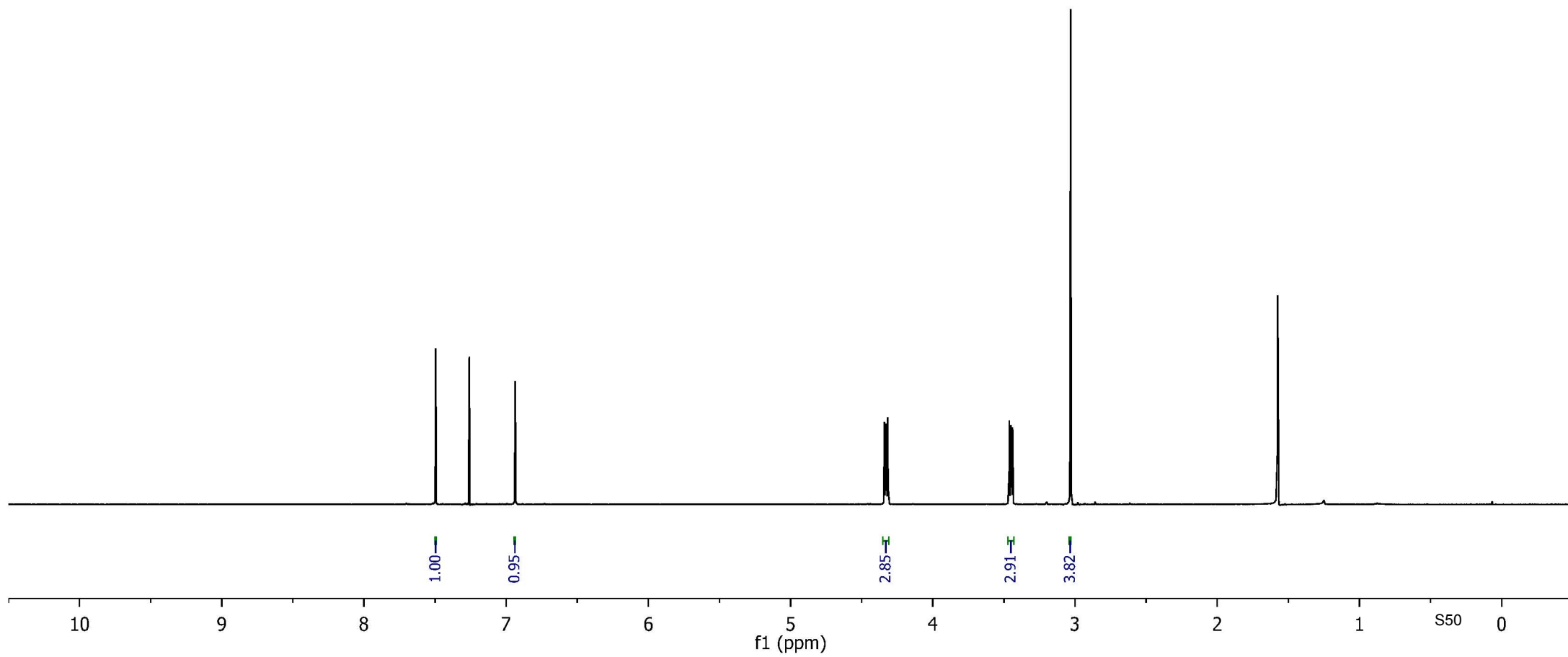
7.50  
7.26  
6.94

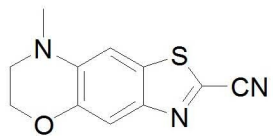
4.34  
4.33  
4.32

3.46  
3.45  
3.44

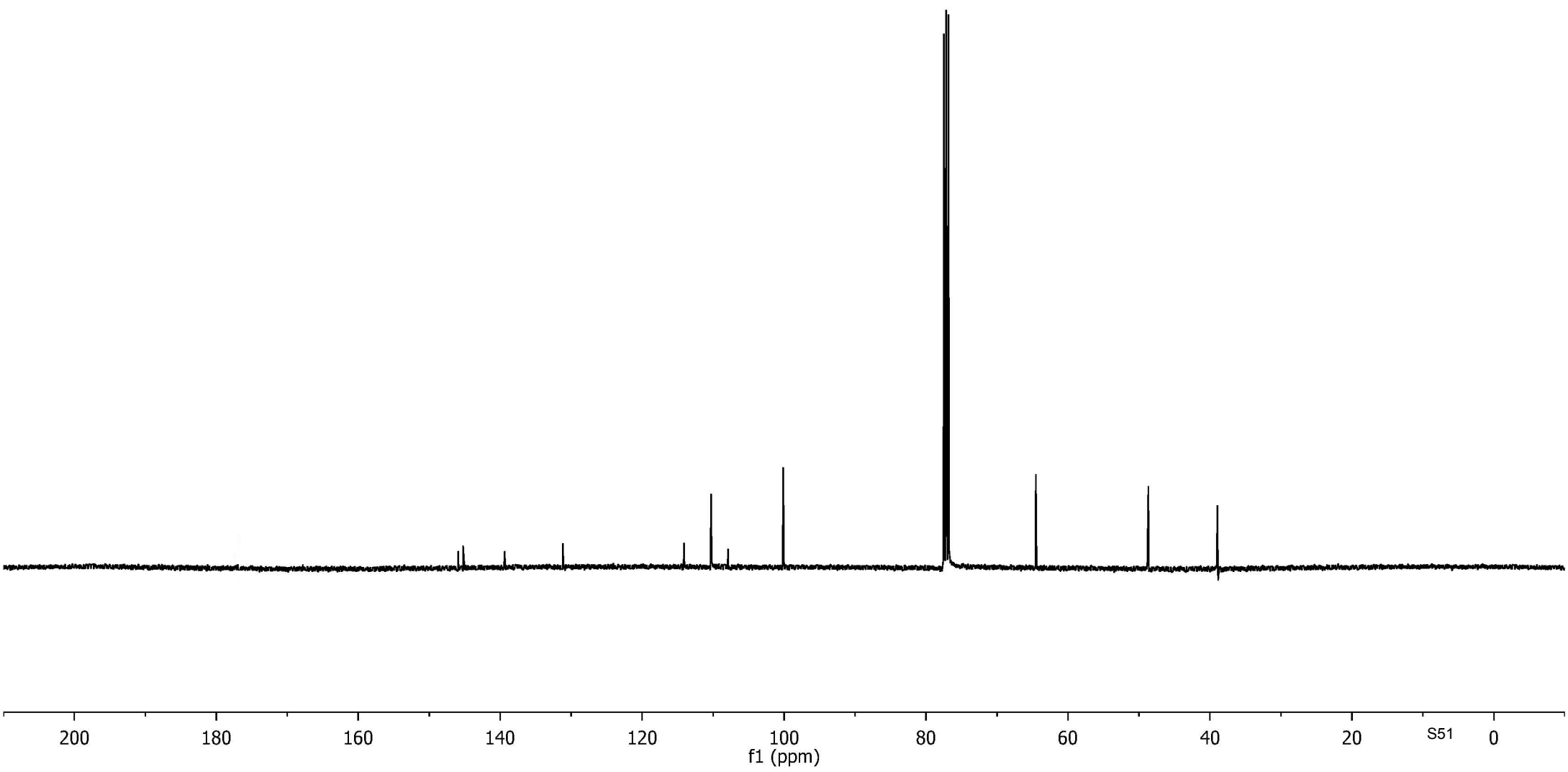
3.03

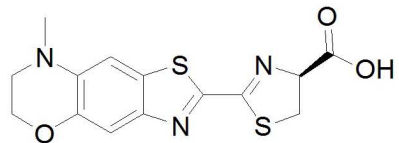
1.57





145.93  
145.24  
139.40  
131.17  
114.08  
110.27  
107.89  
100.12  
77.50 cdc13  
77.18 cdc13  
76.86 cdc13  
64.53  
48.68  
38.96





7.41  
7.26  
6.92

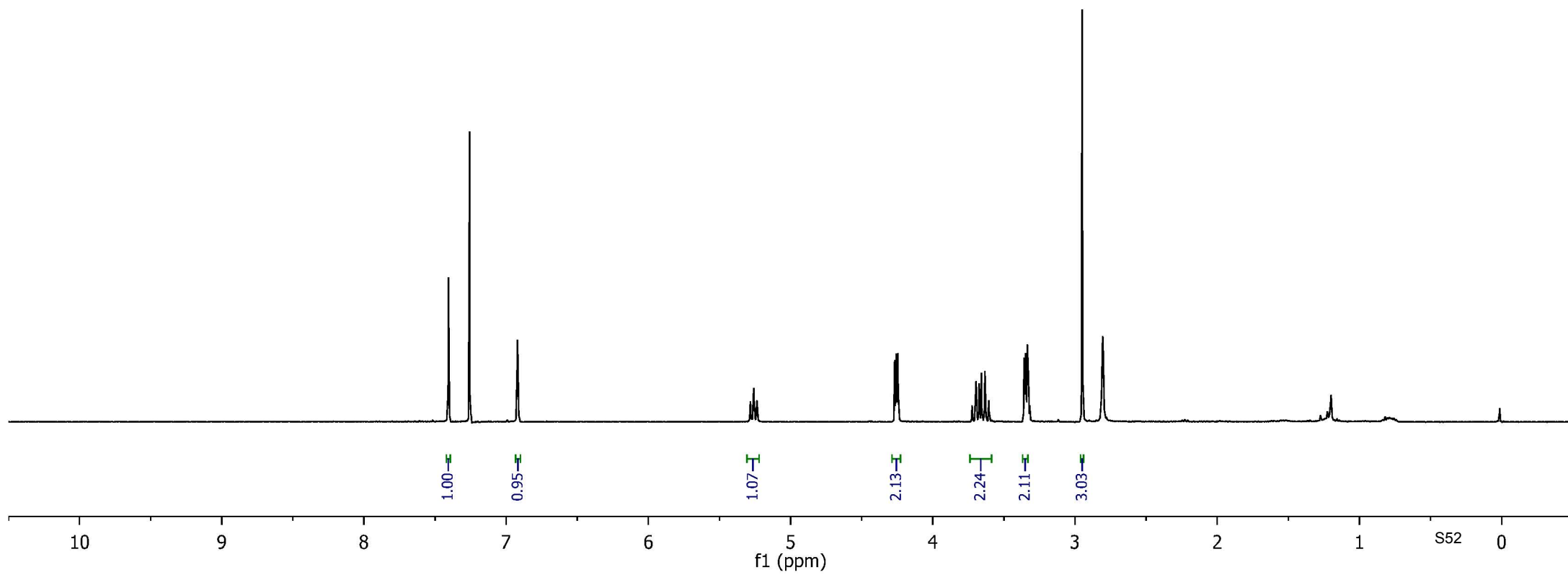
5.28  
5.26  
5.24

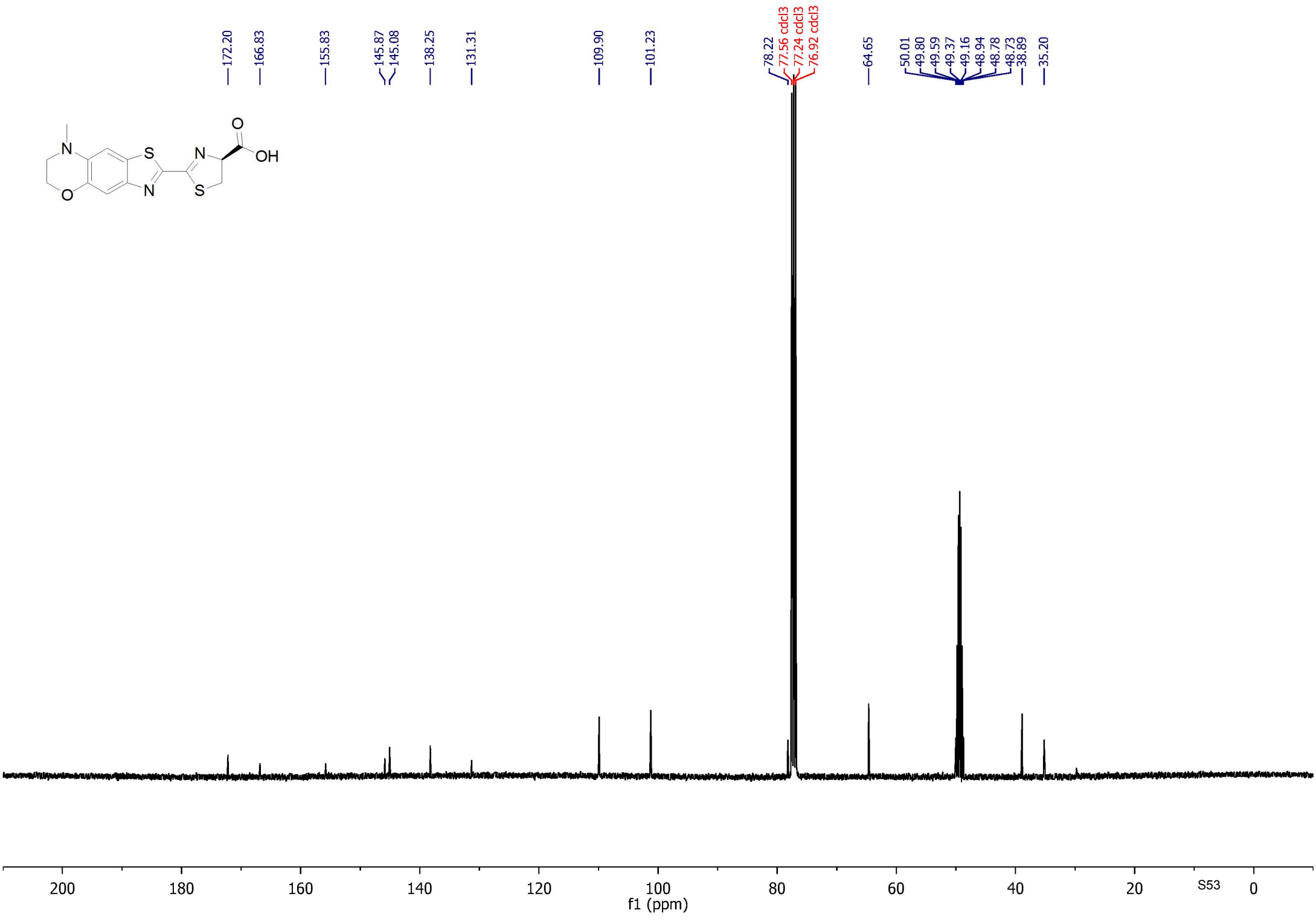
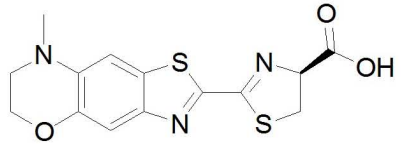
4.27  
4.26  
4.25

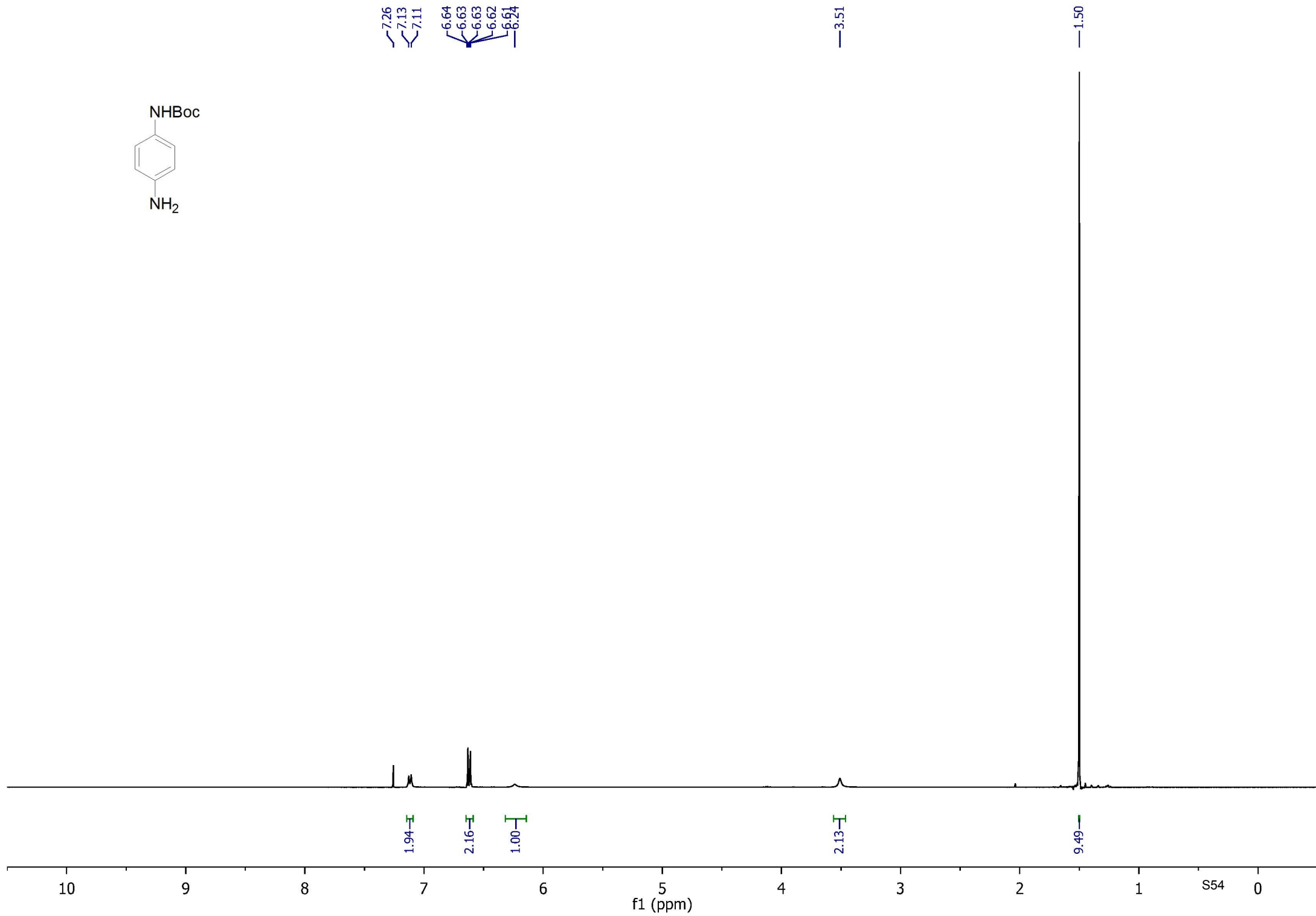
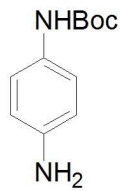
3.70  
3.67  
3.66

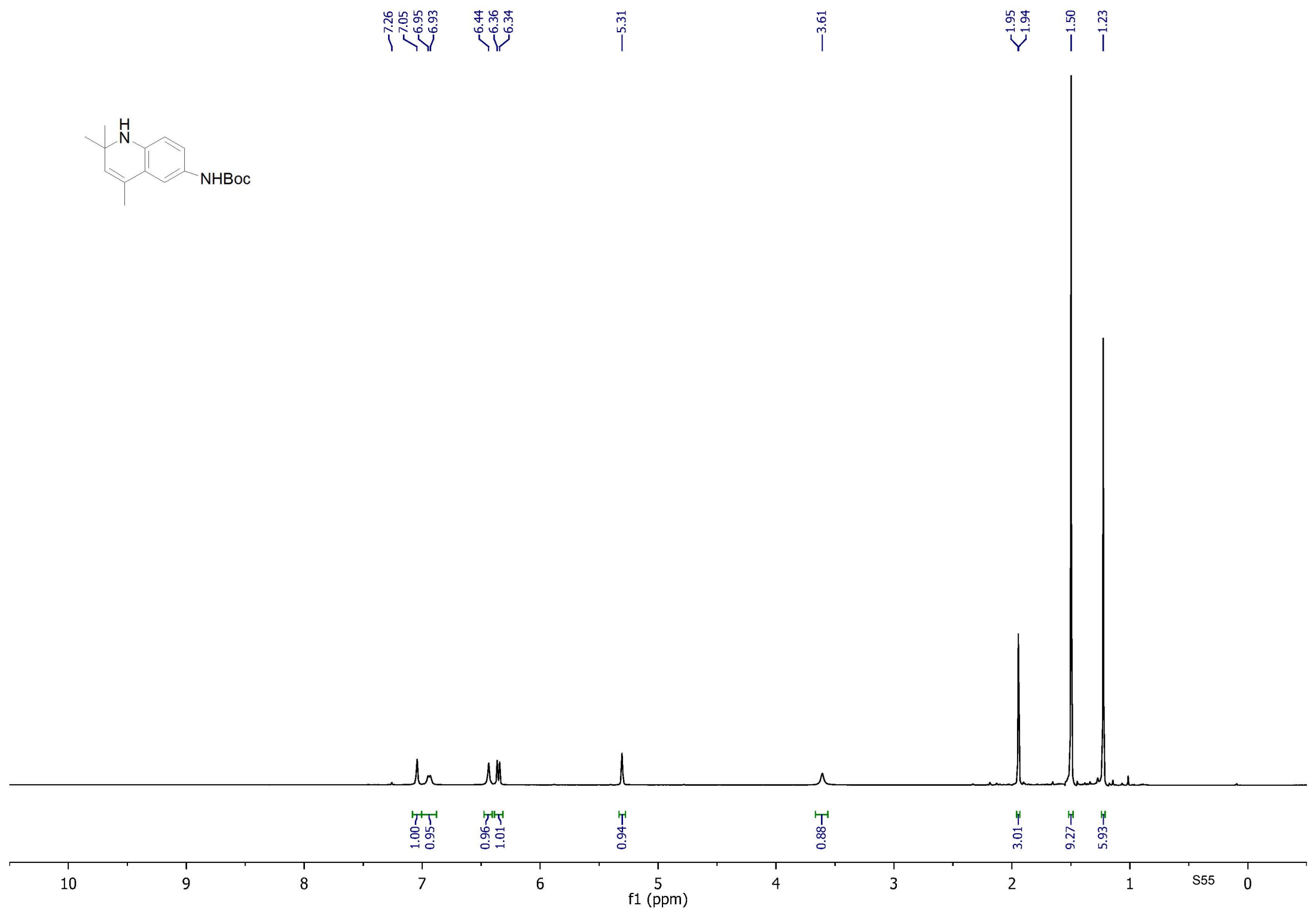
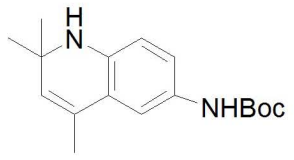
3.36  
3.35  
3.34

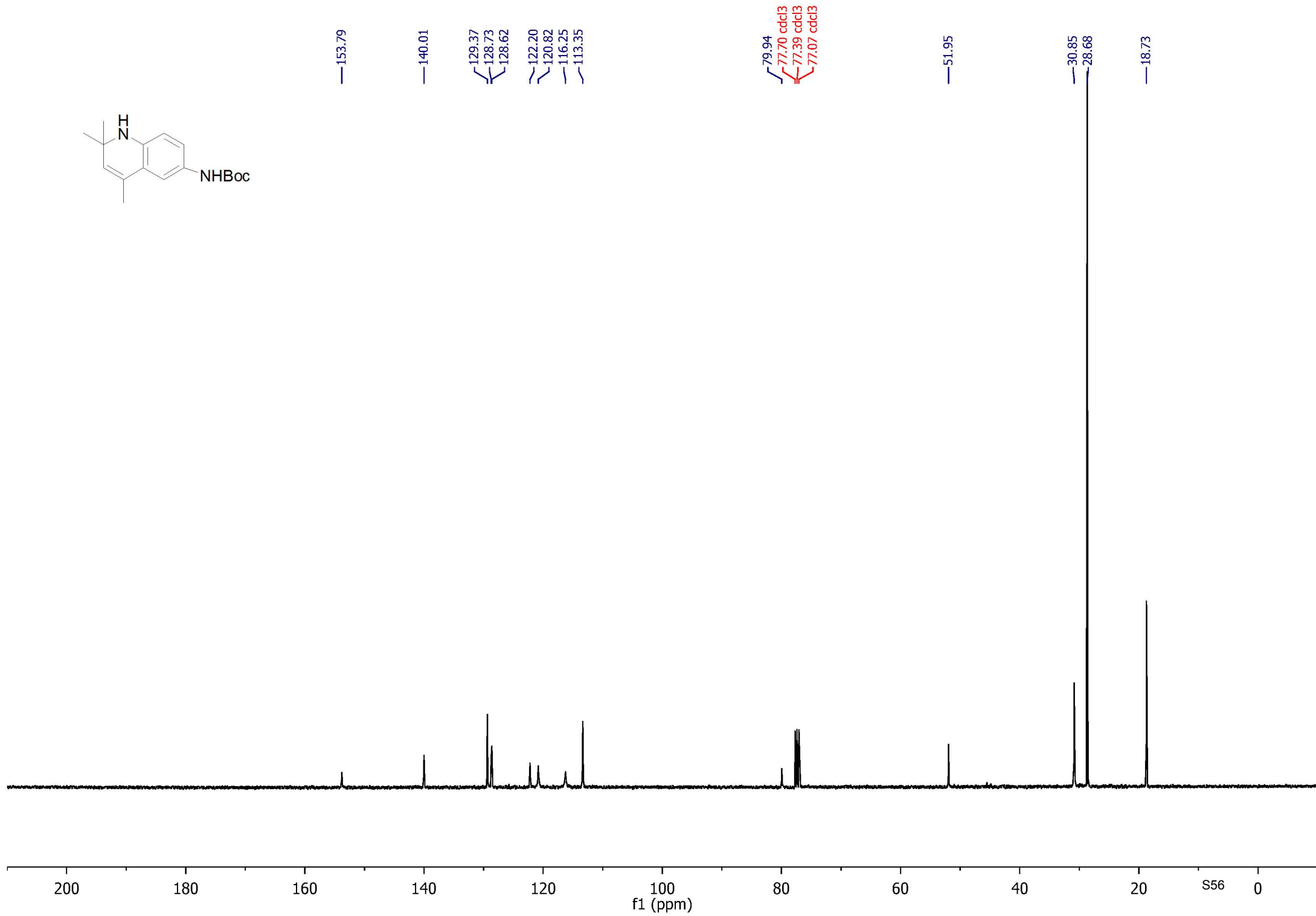
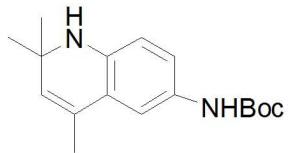
3.33  
2.93  
2.81

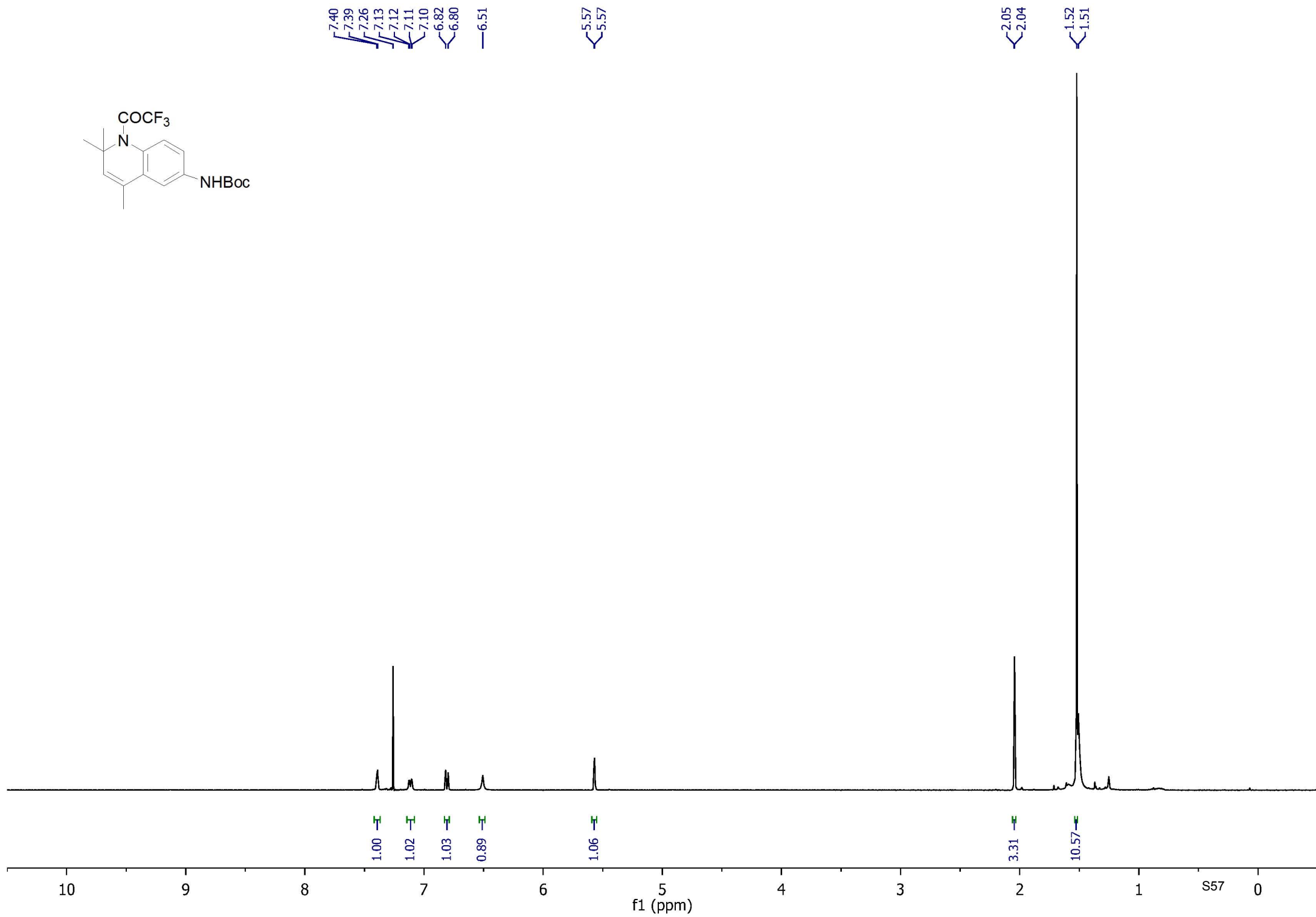
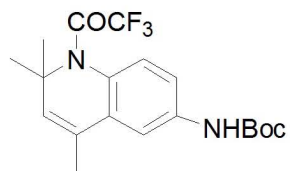


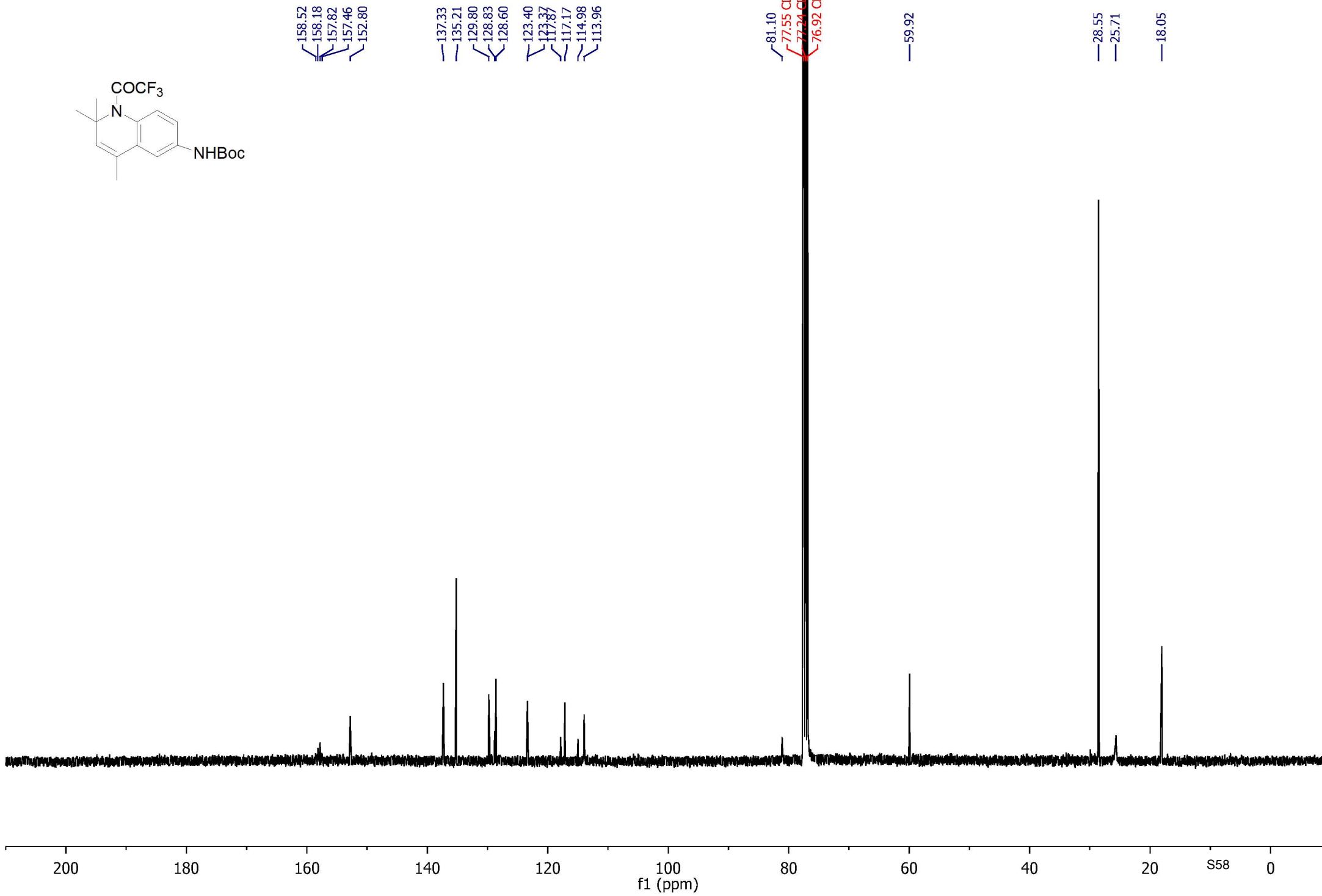
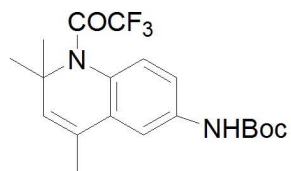


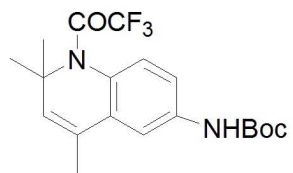












67.24



-40

-60

-80

-100

-120

-140

-160

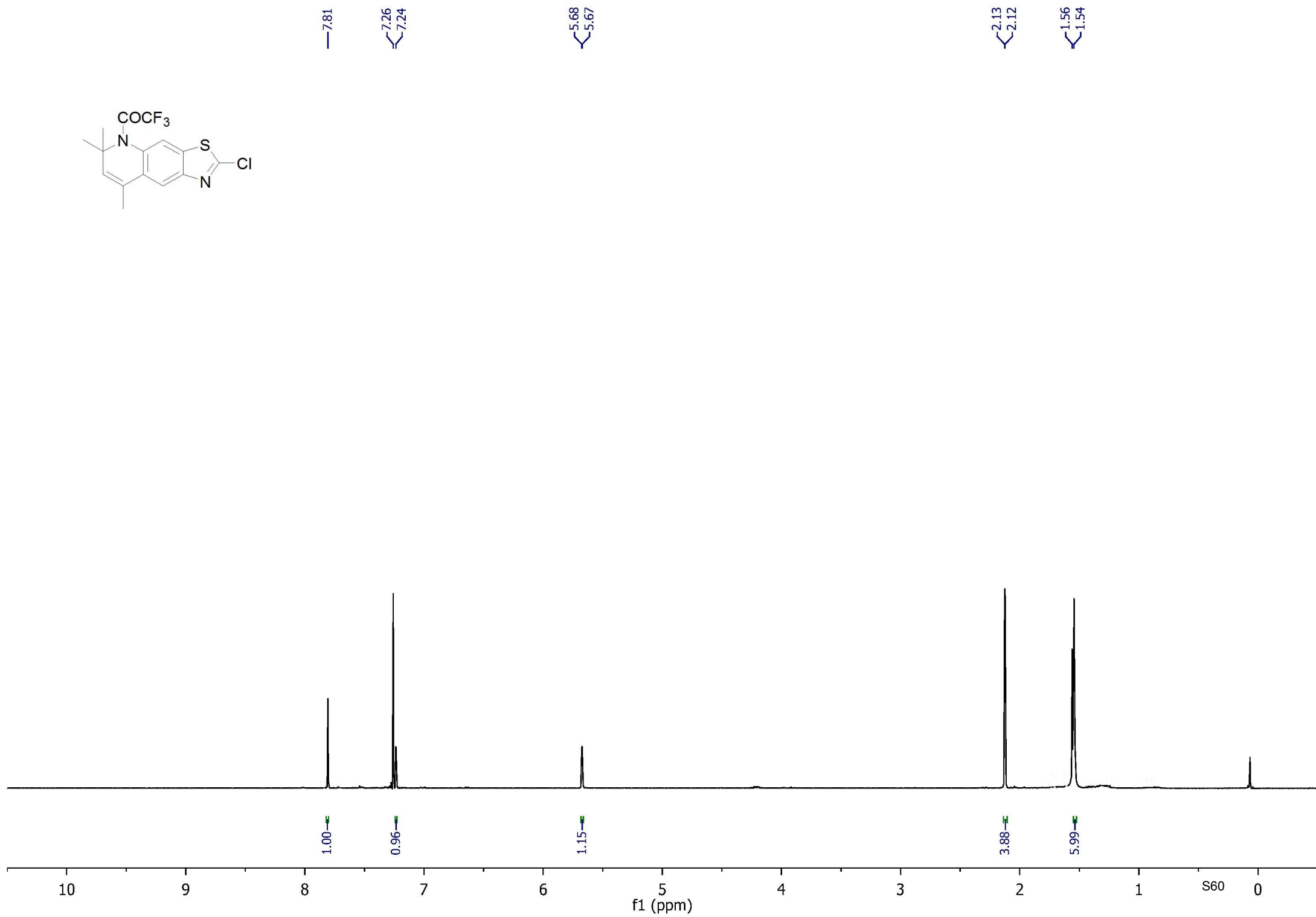
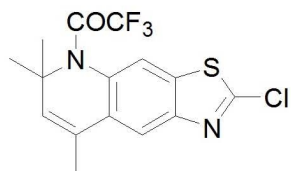
-180

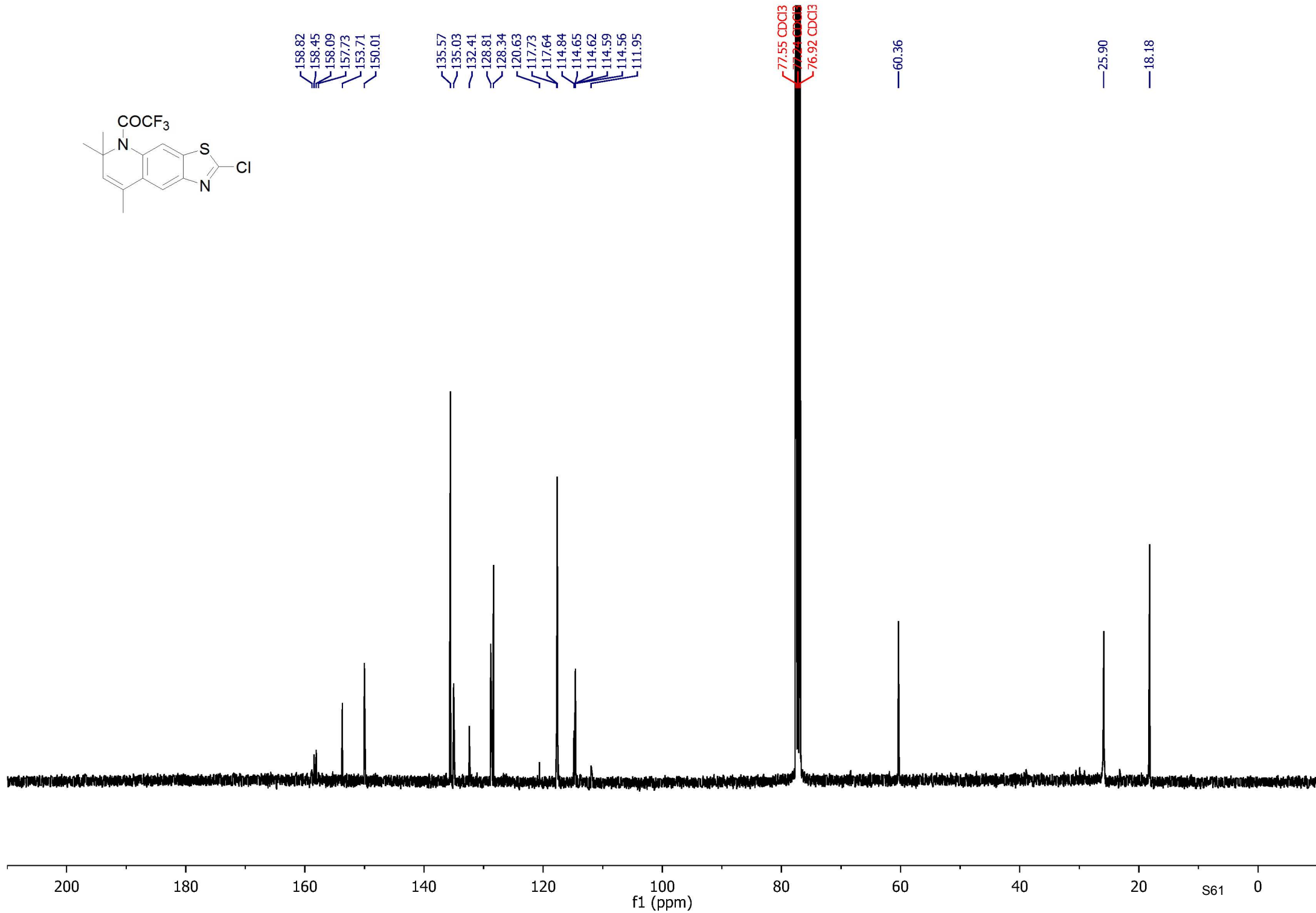
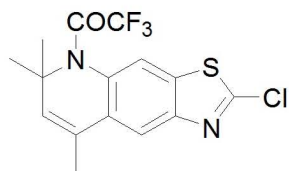
-200

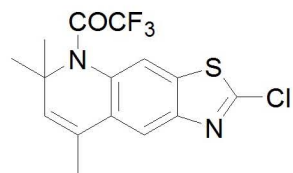
S59

-220

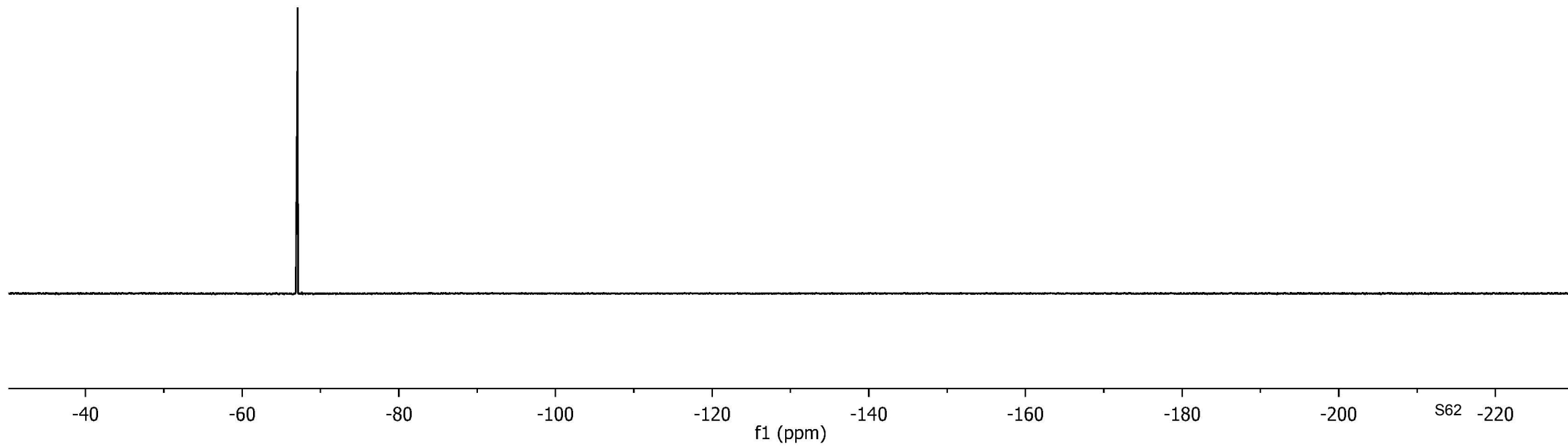
f1 (ppm)

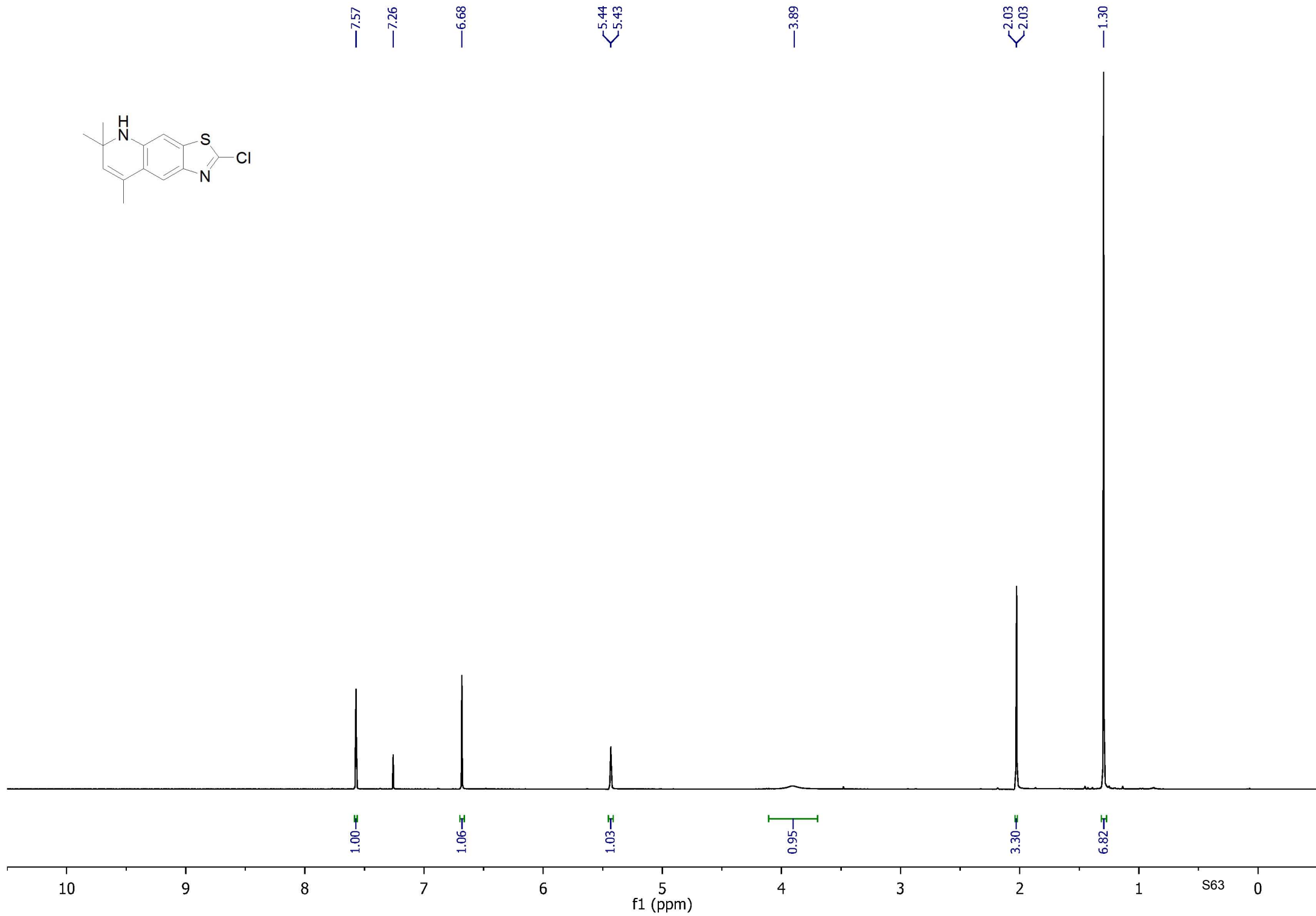
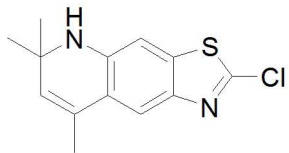


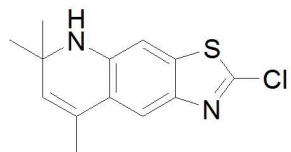




-67.10







— 147.40  
— 143.80  
— 142.60  
— 136.86  
— 130.39  
— 128.25  
— 122.46  
— 117.62

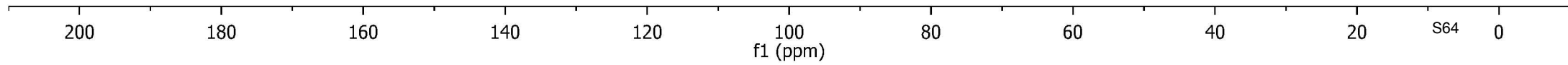
— 102.76

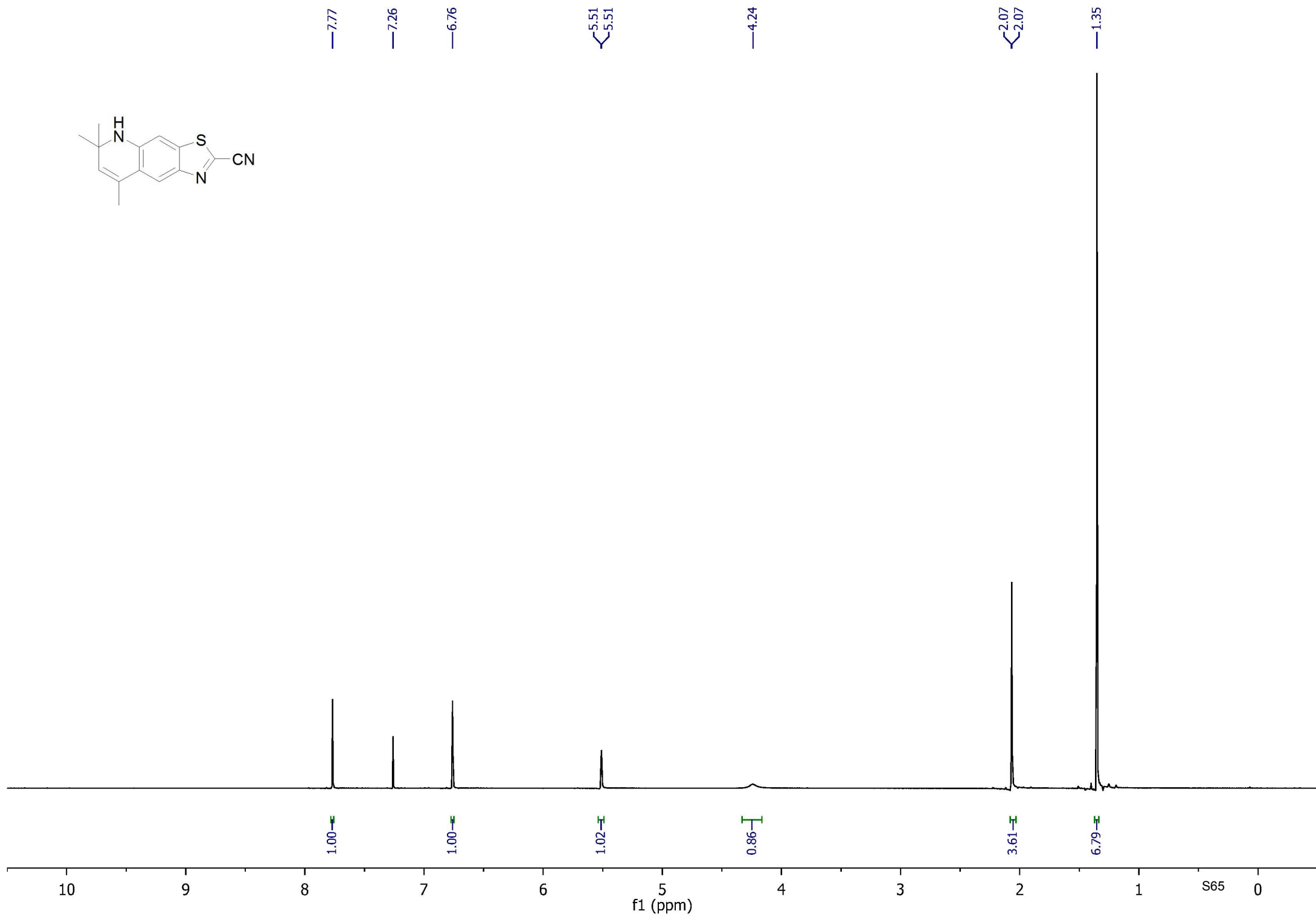
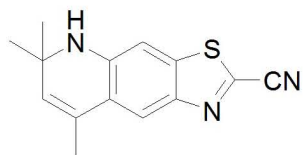
77.62 CDCl<sub>3</sub>  
77.30 CDCl<sub>3</sub>  
76.98 CDCl<sub>3</sub>

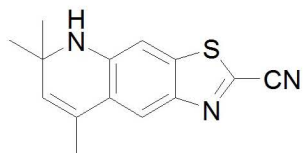
— 52.52

— 31.33

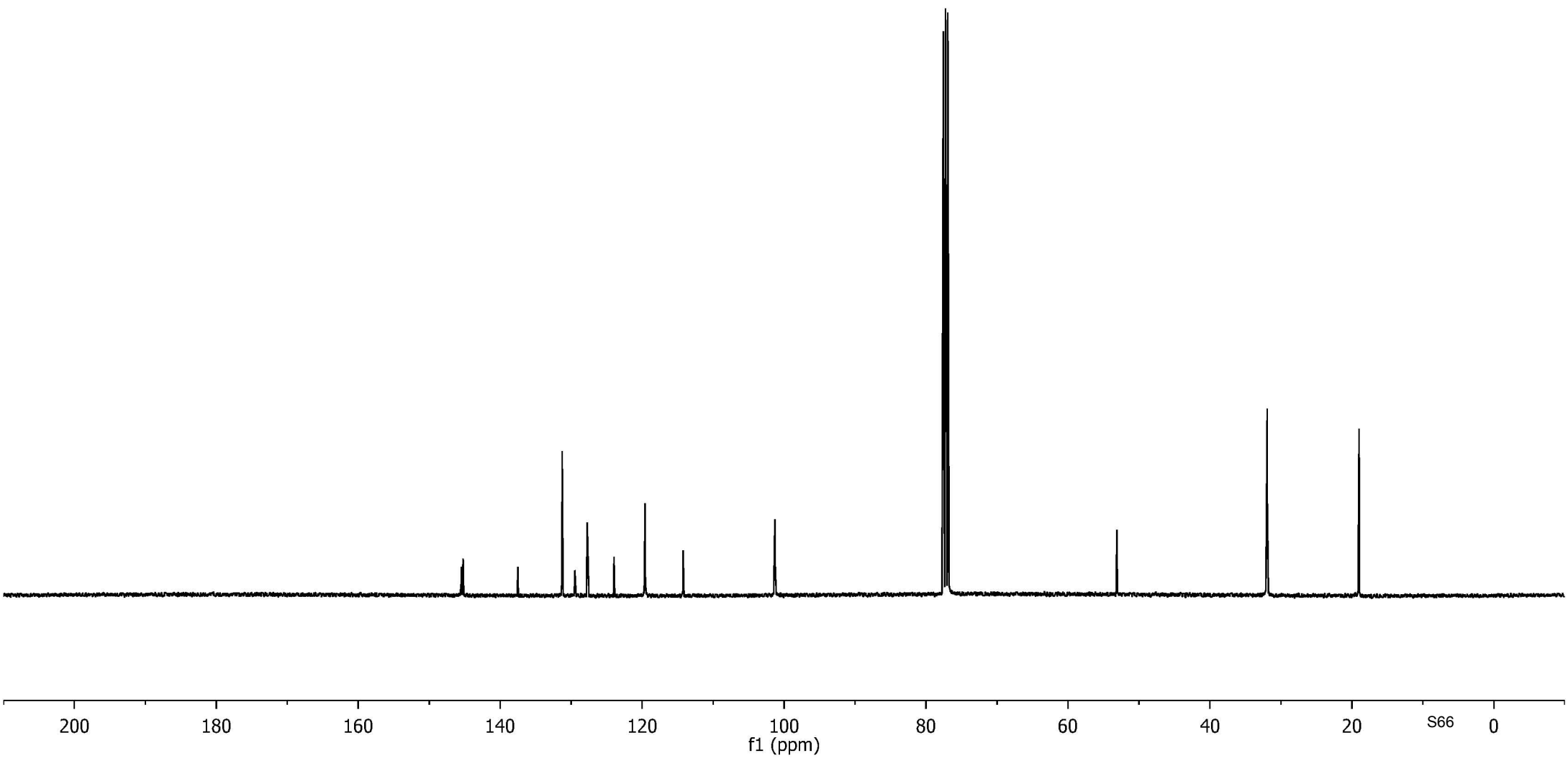
— 18.98

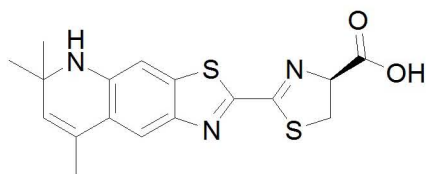






- 145.49
- 145.22
- 137.51
- 131.27
- 129.48
- 127.74
- 123.96
- 119.58
- 114.23
- 101.30
- 77.57 cdc13
- 77.25 cdc13
- 76.94 cdc13
- 53.11
- 31.94
- 18.99





7.80

7.26

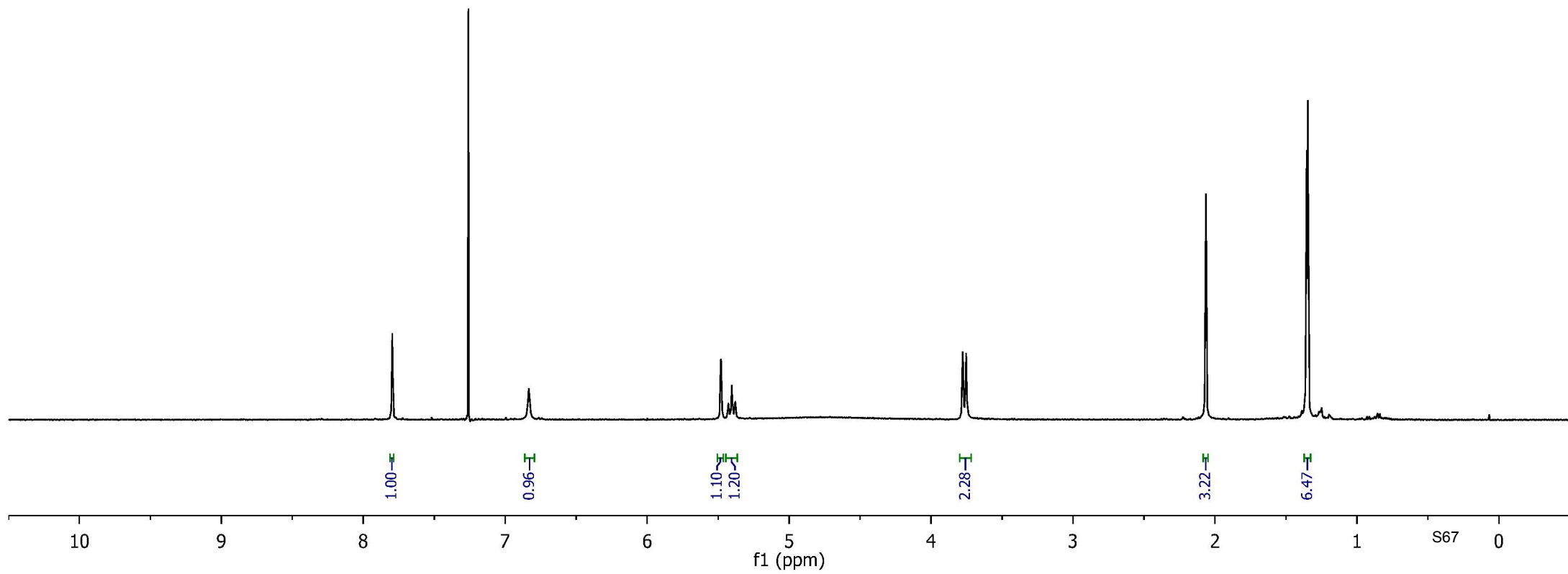
6.83

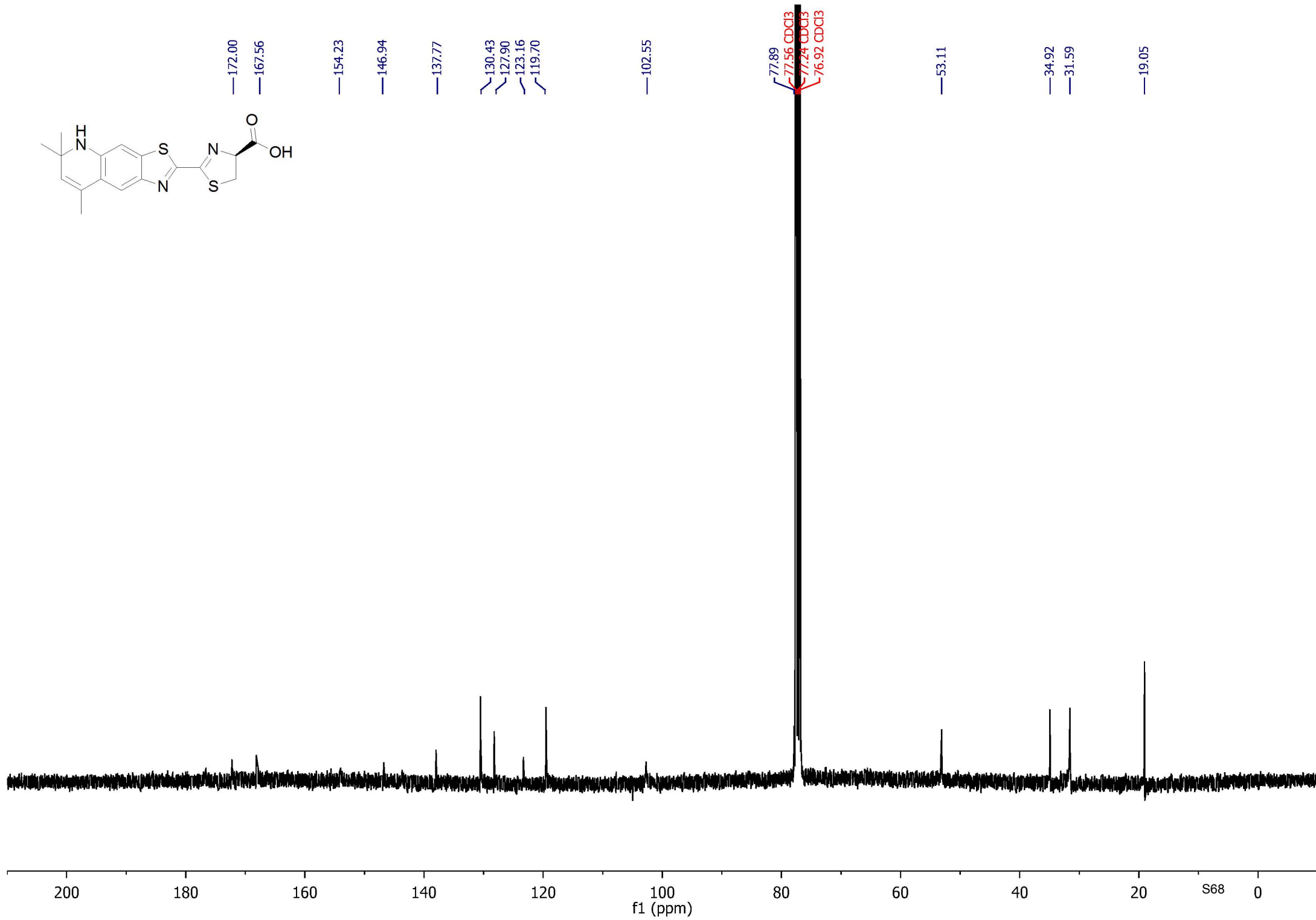
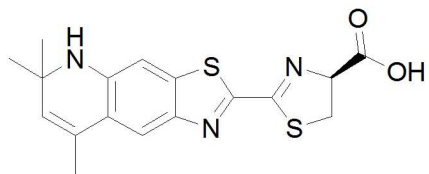
5.48  
5.48  
5.43  
5.40  
5.38

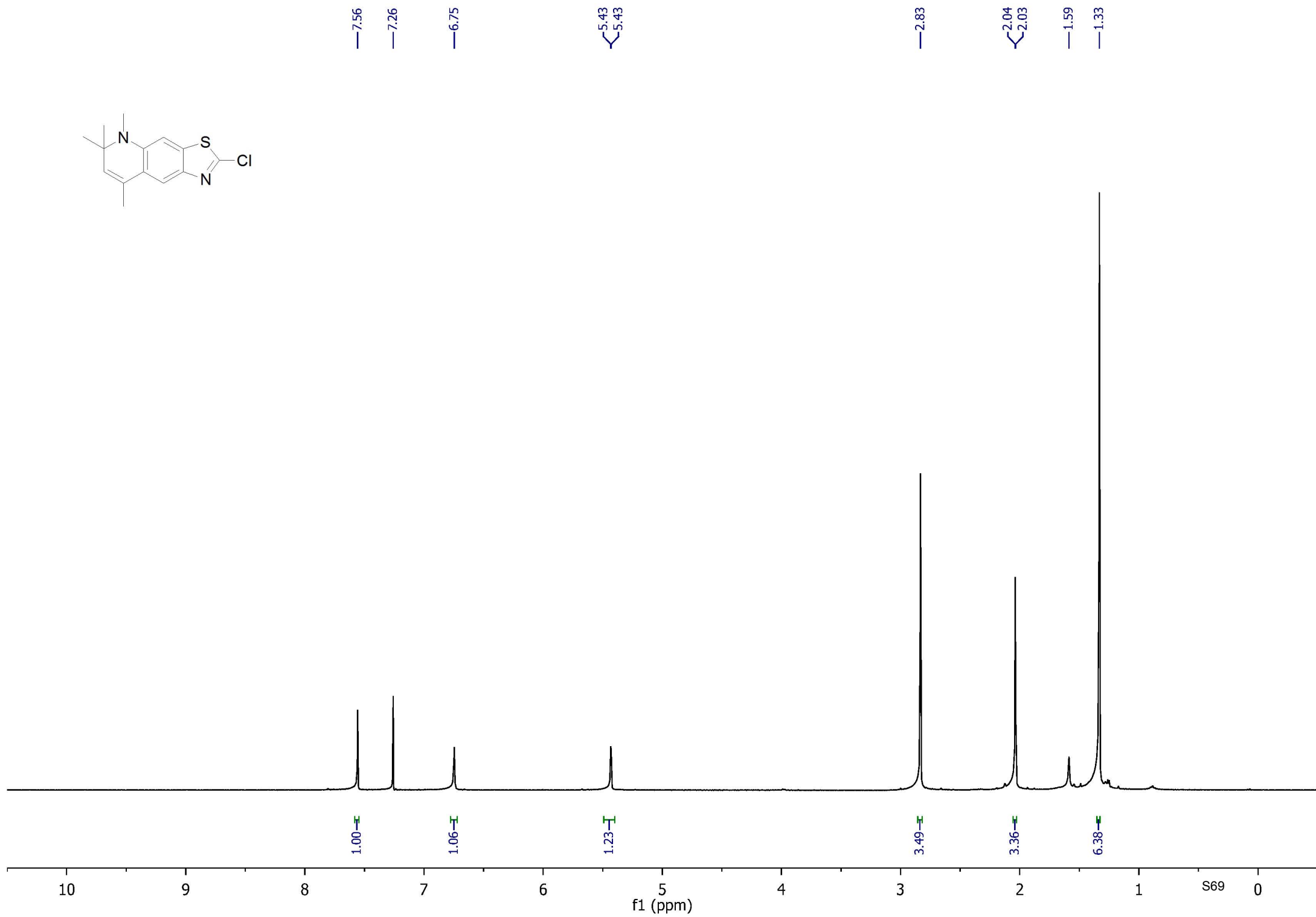
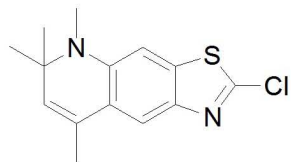
3.78  
3.75

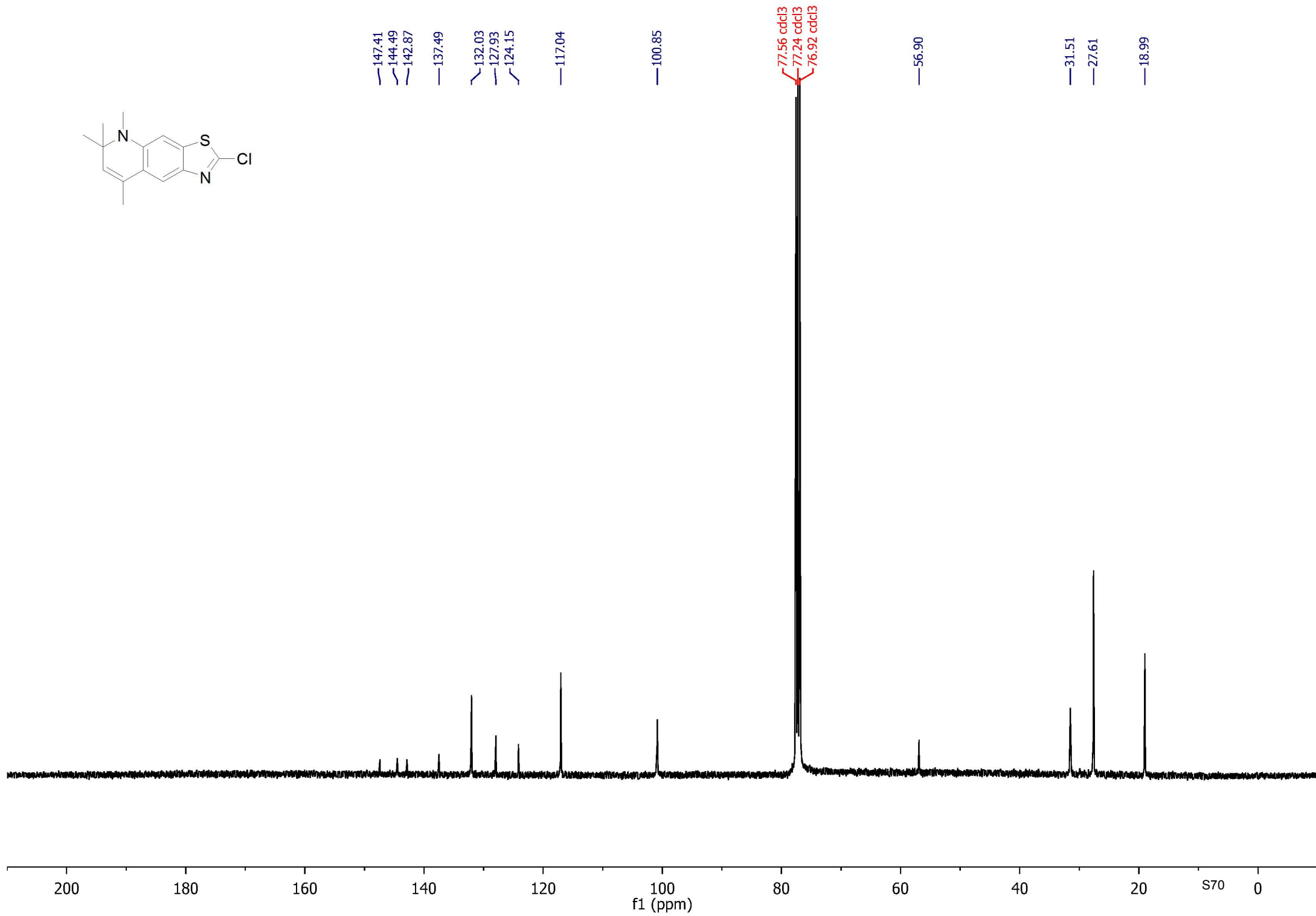
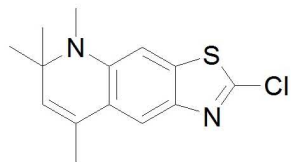
2.07  
2.06

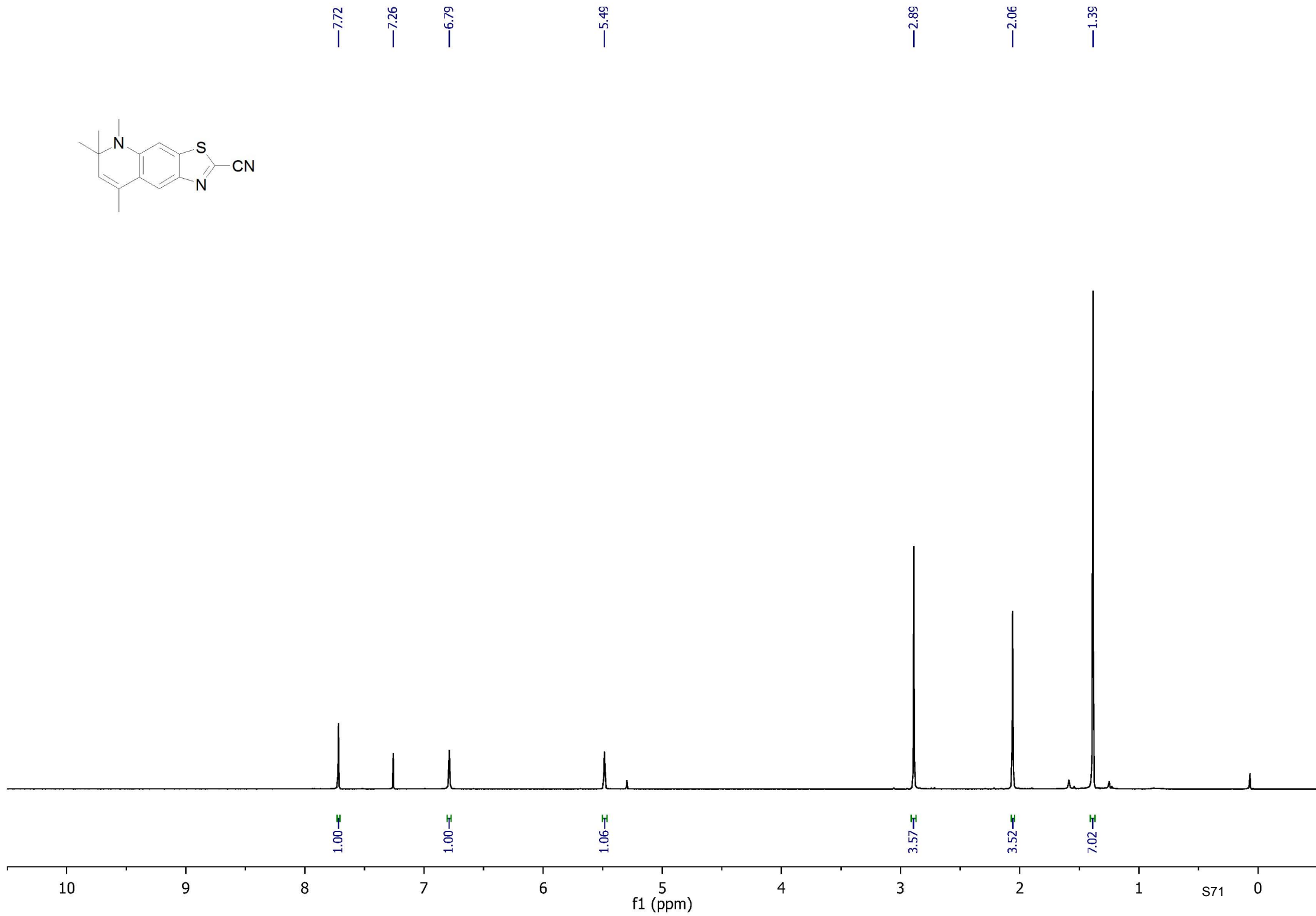
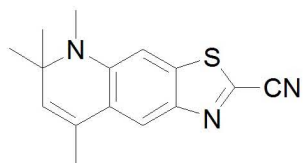
1.35  
1.35

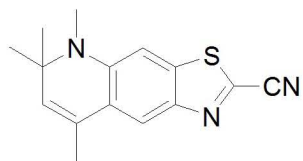












146.72

144.55

138.37

132.69

129.26

127.26

125.46

118.68

114.35

99.15

77.57 CDCl<sub>3</sub>

77.25 CDCl<sub>3</sub>

76.93 CDCl<sub>3</sub>

57.69

31.84

28.54

19.03

200

180

160

140

120

100

80

60

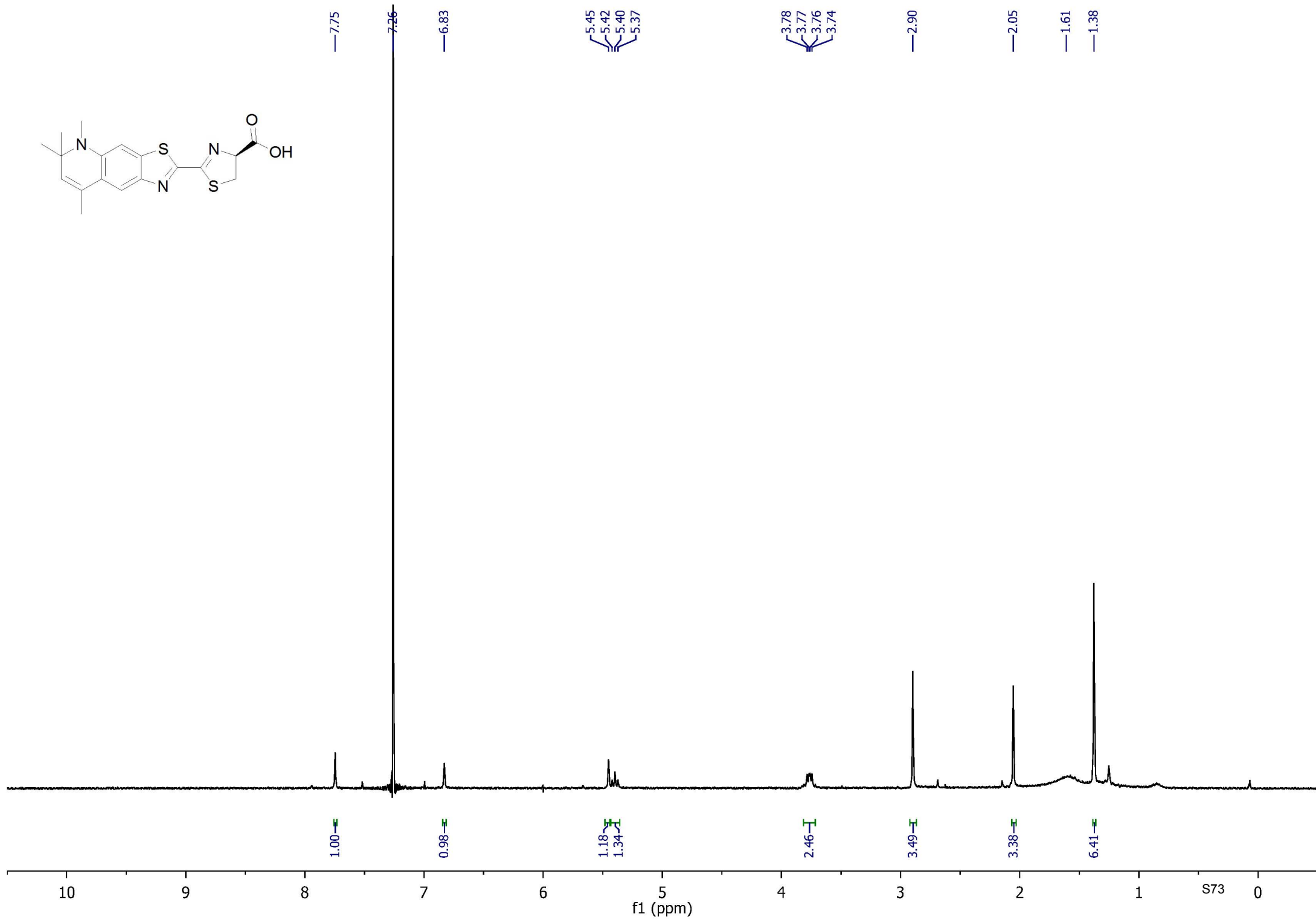
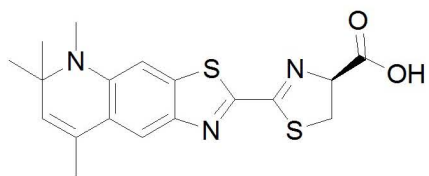
40

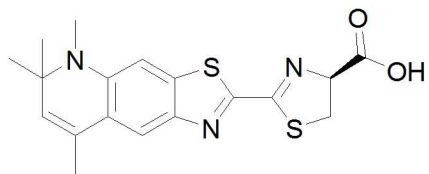
20

S72

0

f1 (ppm)





—172.84

—167.88

—153.65

—145.88

—145.45

—138.73

—132.00

—127.68

—124.62

—118.66

—100.19

—78.28

—77.56 cdcl3

—77.24 cdcl3

—76.92 cdcl3

—57.44

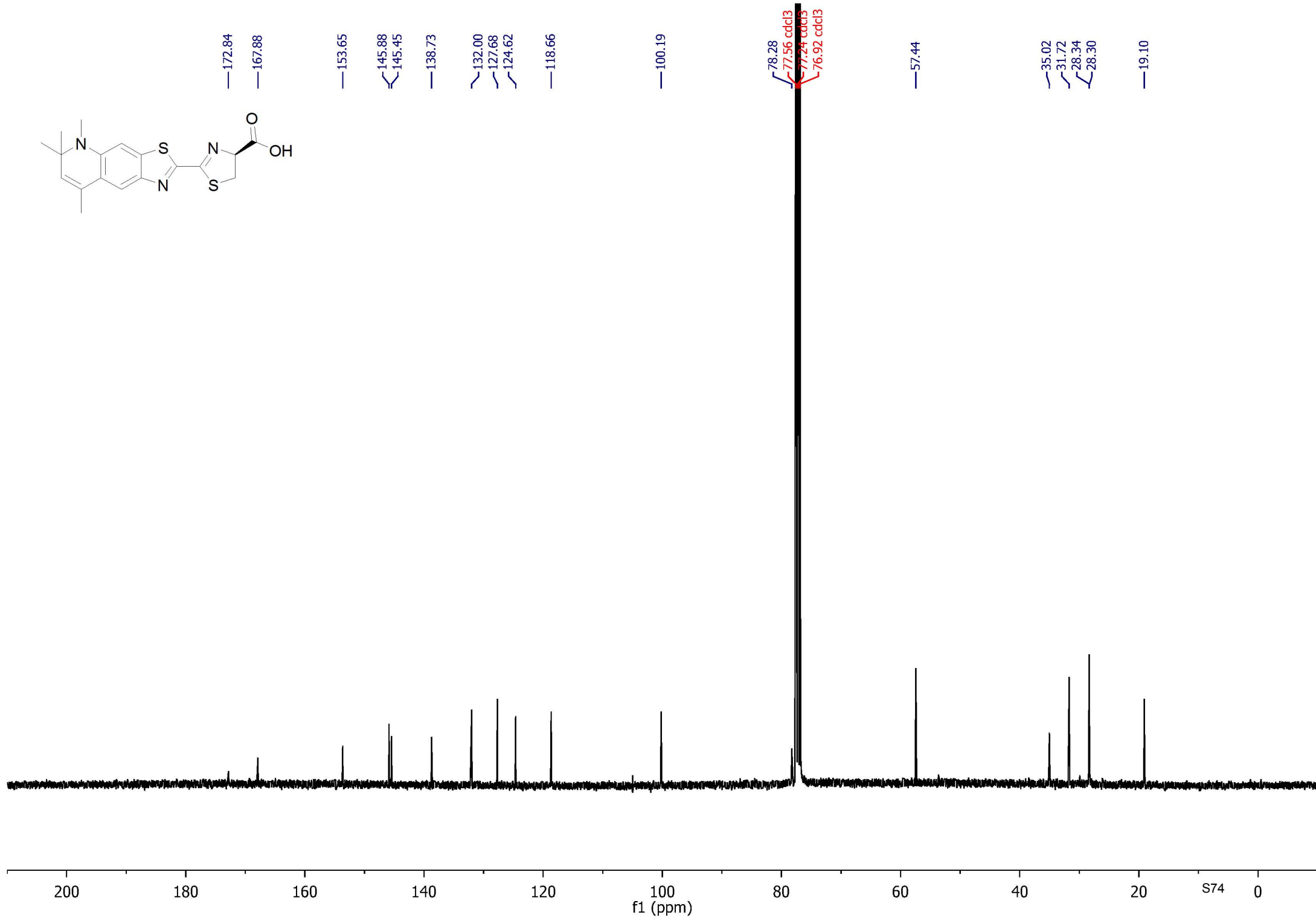
—35.02

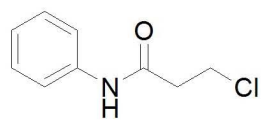
—31.72

—28.34

—28.30

—19.10

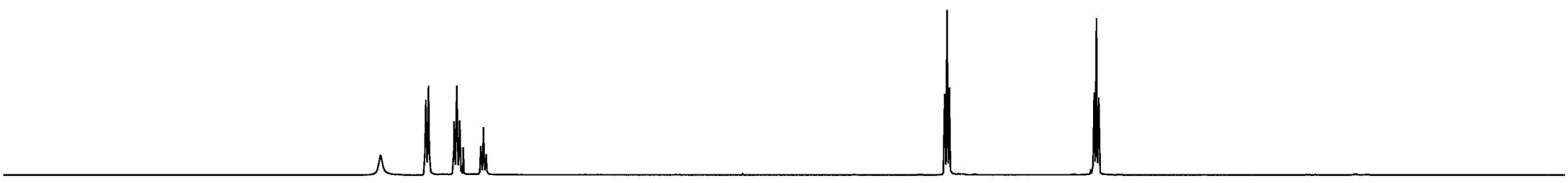




7.84  
7.52  
7.50  
7.32  
7.31  
7.29  
7.26  
7.14  
7.12  
7.10

3.87  
3.85  
3.84

2.82  
2.80  
2.78



1.00  
2.15  
2.18  
1.12

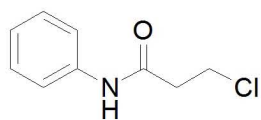
2.32

2.42

10 9 8 7 6 5 4 3 2 1 0

f1 (ppm)

S75



168.36

137.67

129.26

124.97

120.53

77.62 CDCl<sub>3</sub>

77.30 CDCl<sub>3</sub>

76.98 CDCl<sub>3</sub>

40.58

40.19

200

180

160

140

120

100  
f1 (ppm)

80

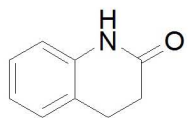
60

40

20

S76

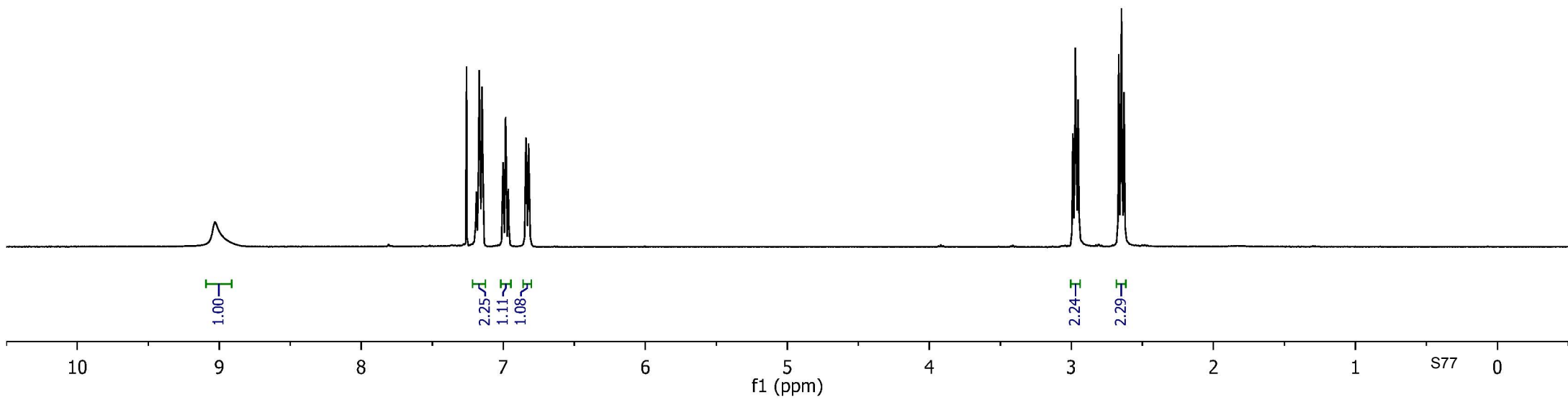
0

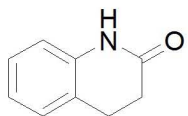


9.03

7.26  
7.19  
7.17  
7.15  
7.00  
6.98  
6.96  
6.84  
6.82

2.99  
2.97  
2.95  
2.67  
2.65  
2.63





—172.24

—137.45

128.17

127.76

123.87

123.32

—115.68

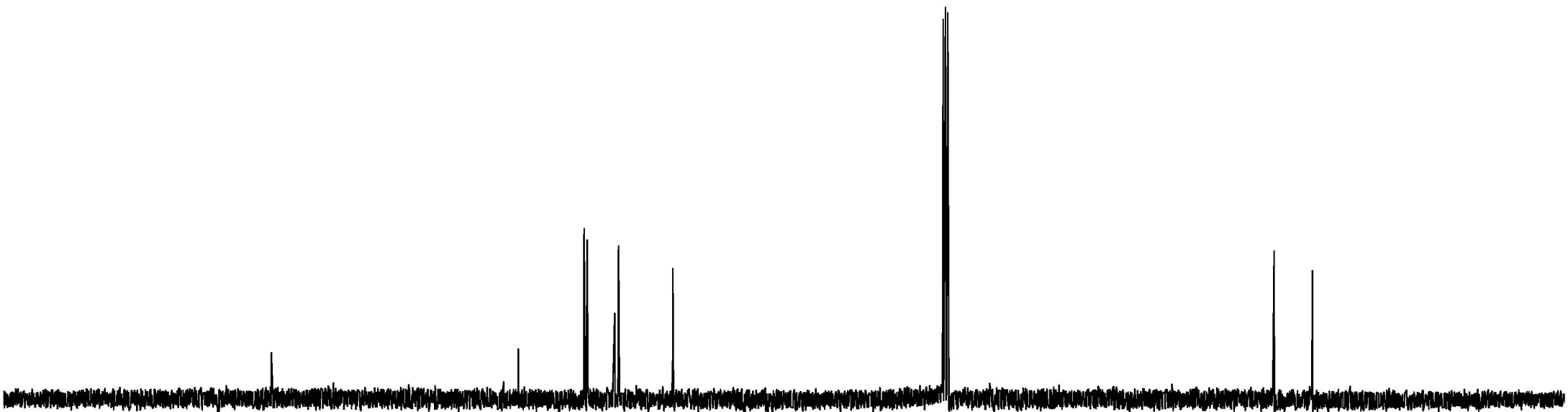
77.58 CDCl<sub>3</sub>

77.26 CDCl<sub>3</sub>

76.94 CDCl<sub>3</sub>

—30.96

—25.56



200

180

160

140

120

100

80

60

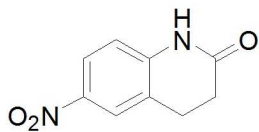
40

20

S78

0

f1 (ppm)



9.40

8.12  
8.11  
8.11  
8.10  
8.10  
8.10  
8.09

7.26

6.95  
6.92

3.12  
3.10  
3.08  
2.75  
2.73  
2.71

1.00

2.09

1.11

2.36

2.35

10

9

8

6

5

4

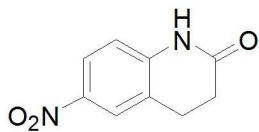
2

1

S79

0

f1 (ppm)



172.10

143.39  
143.13

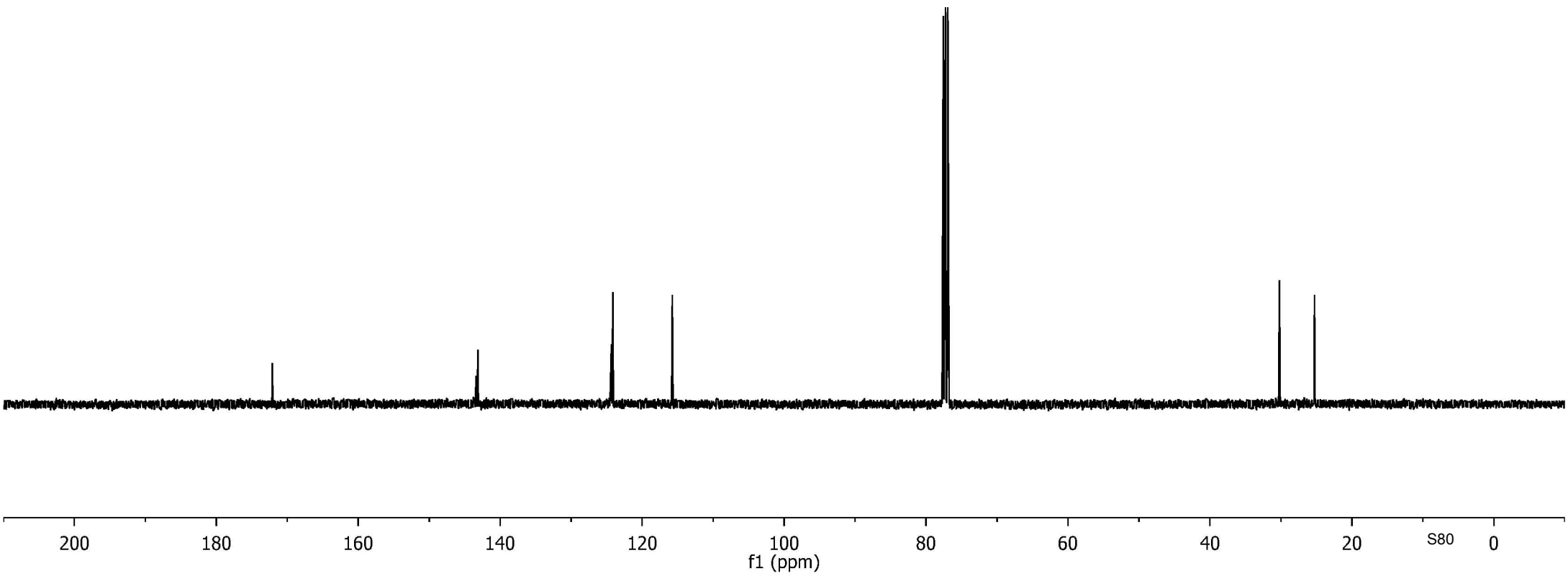
124.33  
124.19  
124.11

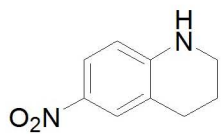
115.75

77.57 cdc13  
77.25 cdc13  
76.93 cdc13

30.21

25.27





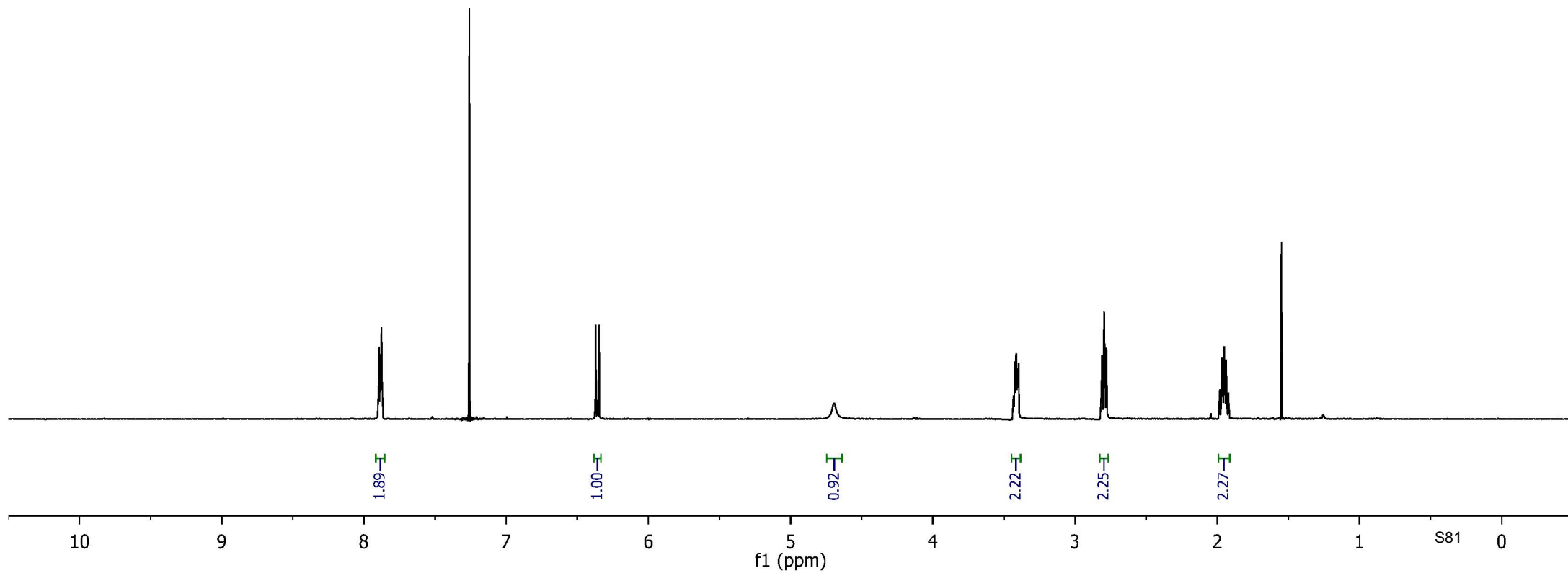
7.90  
7.89  
7.89  
7.89  
7.88  
7.88  
7.88  
7.88  
7.87  
7.26

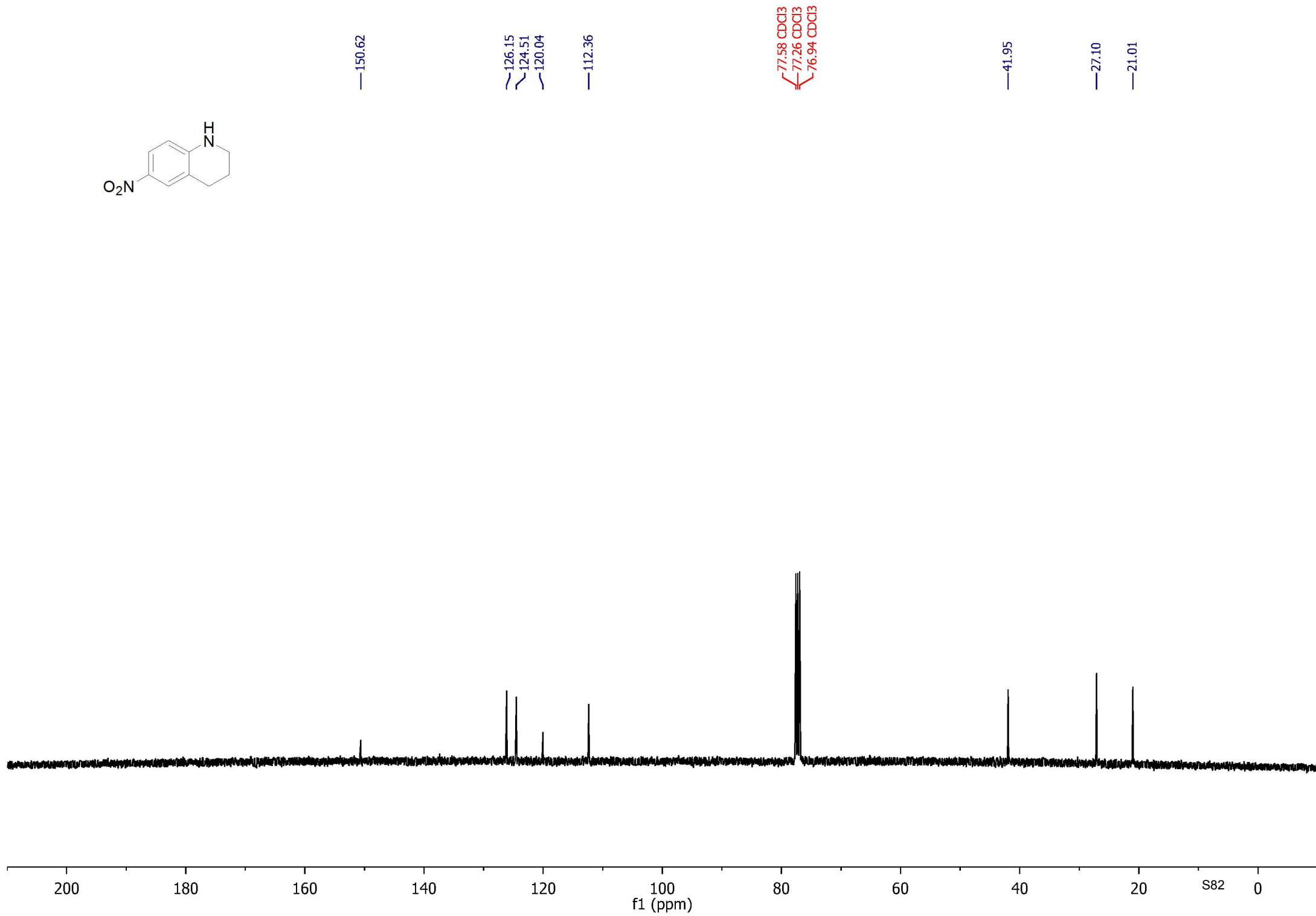
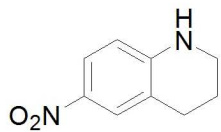
6.37  
6.35

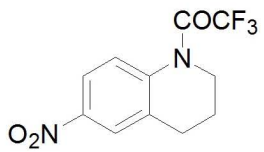
4.69

3.43  
3.42  
3.42  
3.42  
3.42  
3.41  
3.41  
3.41  
3.40  
2.81  
2.80  
2.78

1.98  
1.97  
1.97  
1.95  
1.95  
1.94  
1.94  
1.94  
1.92







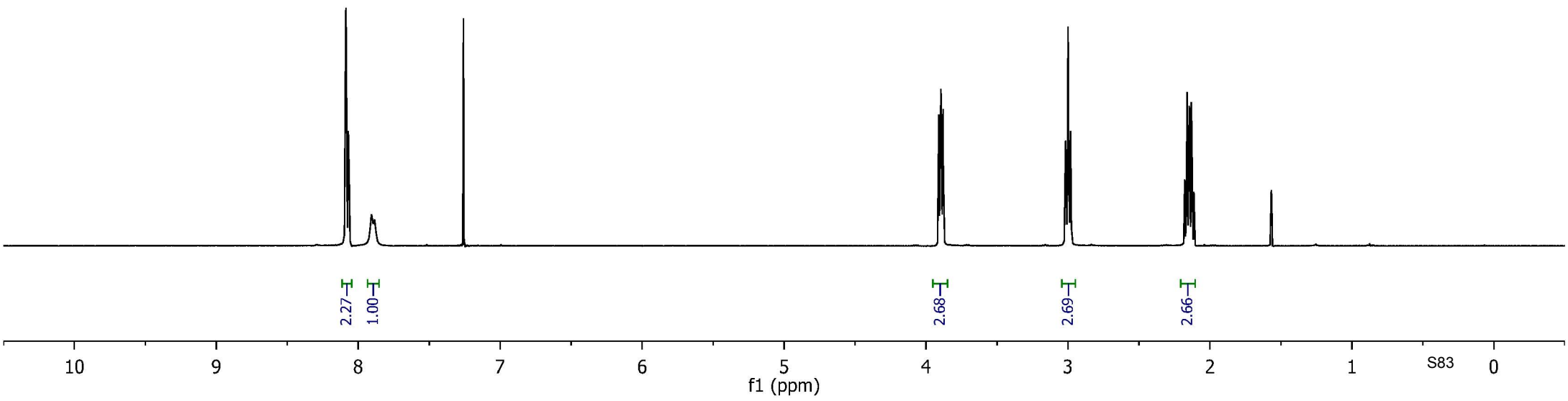
8.09  
8.09  
8.08  
8.08  
8.07  
8.07  
8.07  
8.06  
8.06  
7.91  
7.88  
7.26

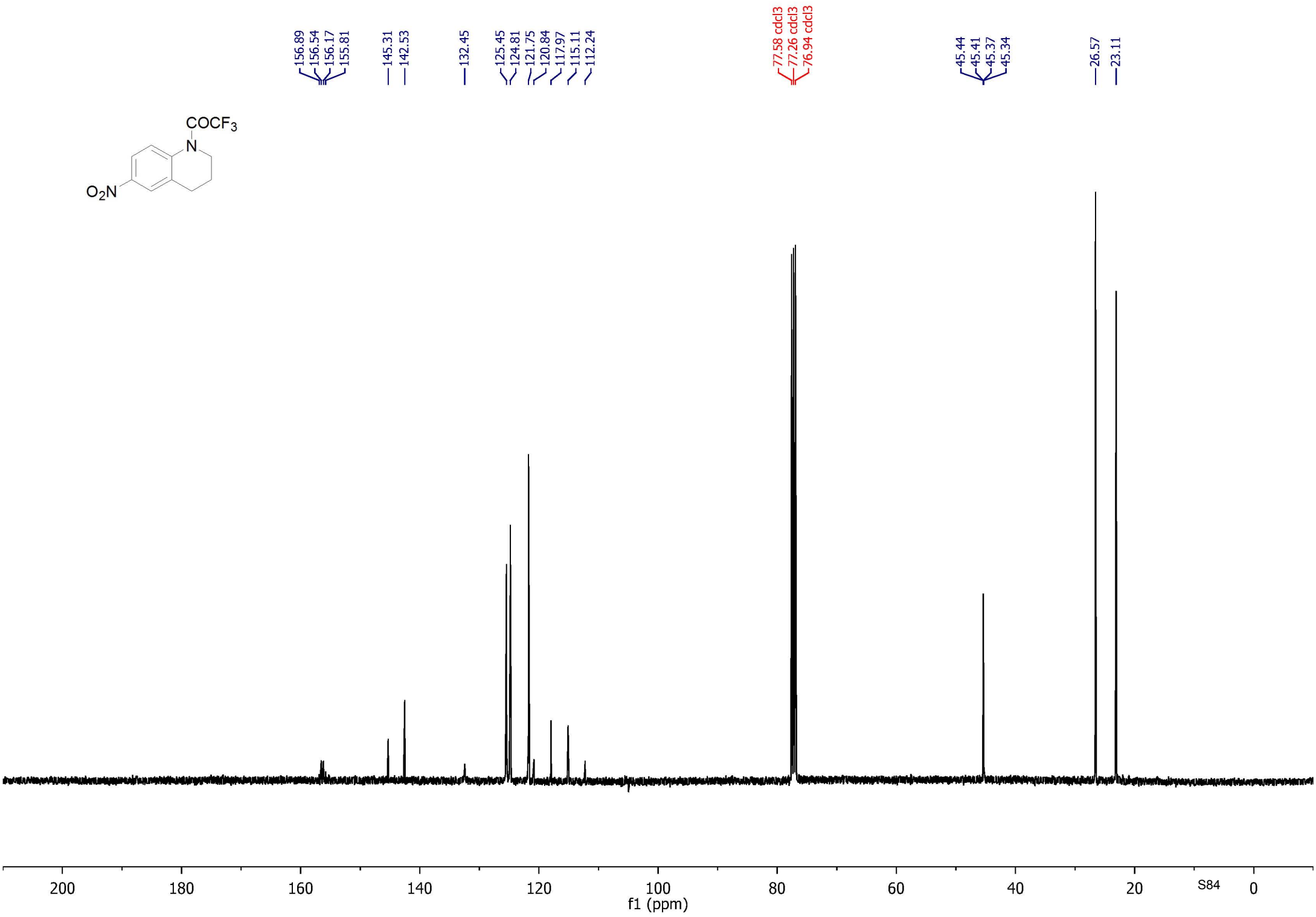
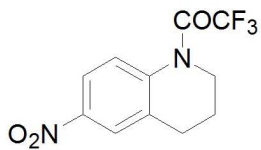
3.91  
3.89  
3.88

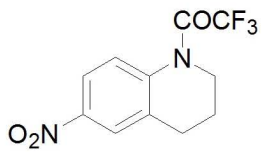
3.02  
3.00  
2.98

2.18  
2.16  
2.15  
2.13  
2.11

1.56







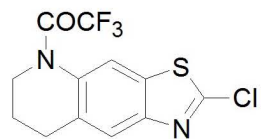
68.92



-40 -60 -80 -100 -120 -140 -160 -180 -200 -220

f1 (ppm)

S85

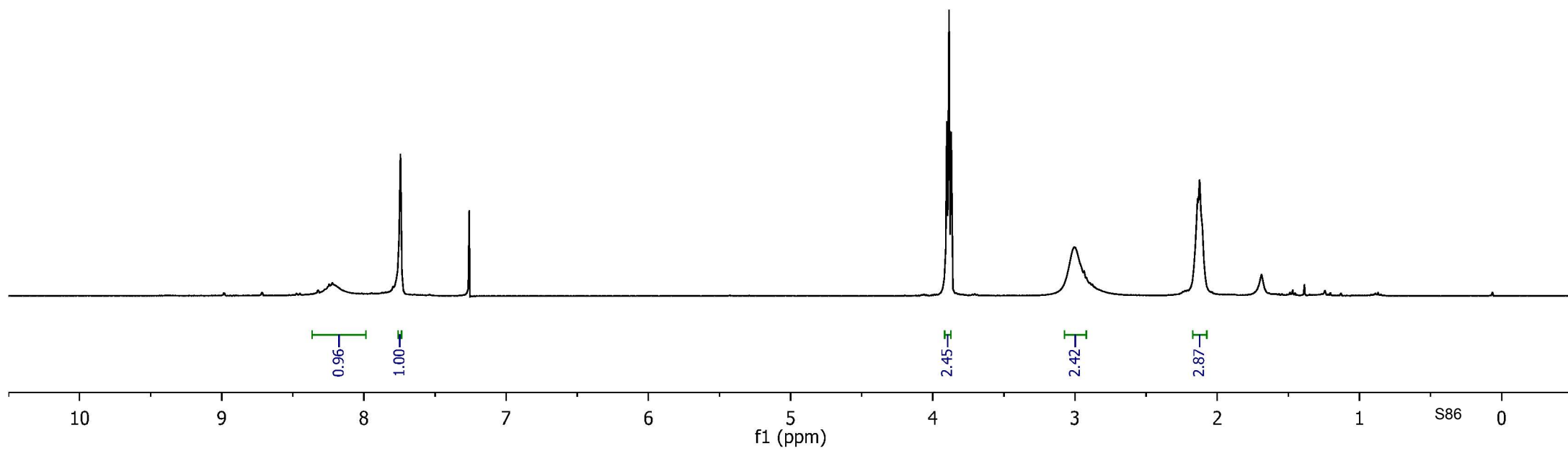


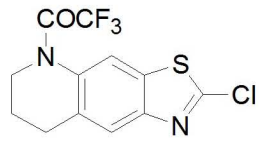
8.24  
7.74  
7.26

3.90  
3.89  
3.87

3.01

2.16  
2.16  
2.14  
2.12  
2.11





156.14  
154.53  
149.41

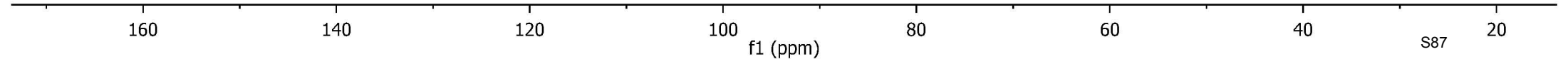
135.10  
134.12  
130.68

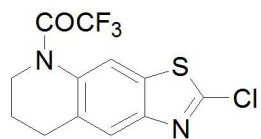
122.61  
121.08  
118.22  
117.35  
115.36  
112.49

77.58 cdcl  
76.95 cdc

45.38

26.58  
23.52



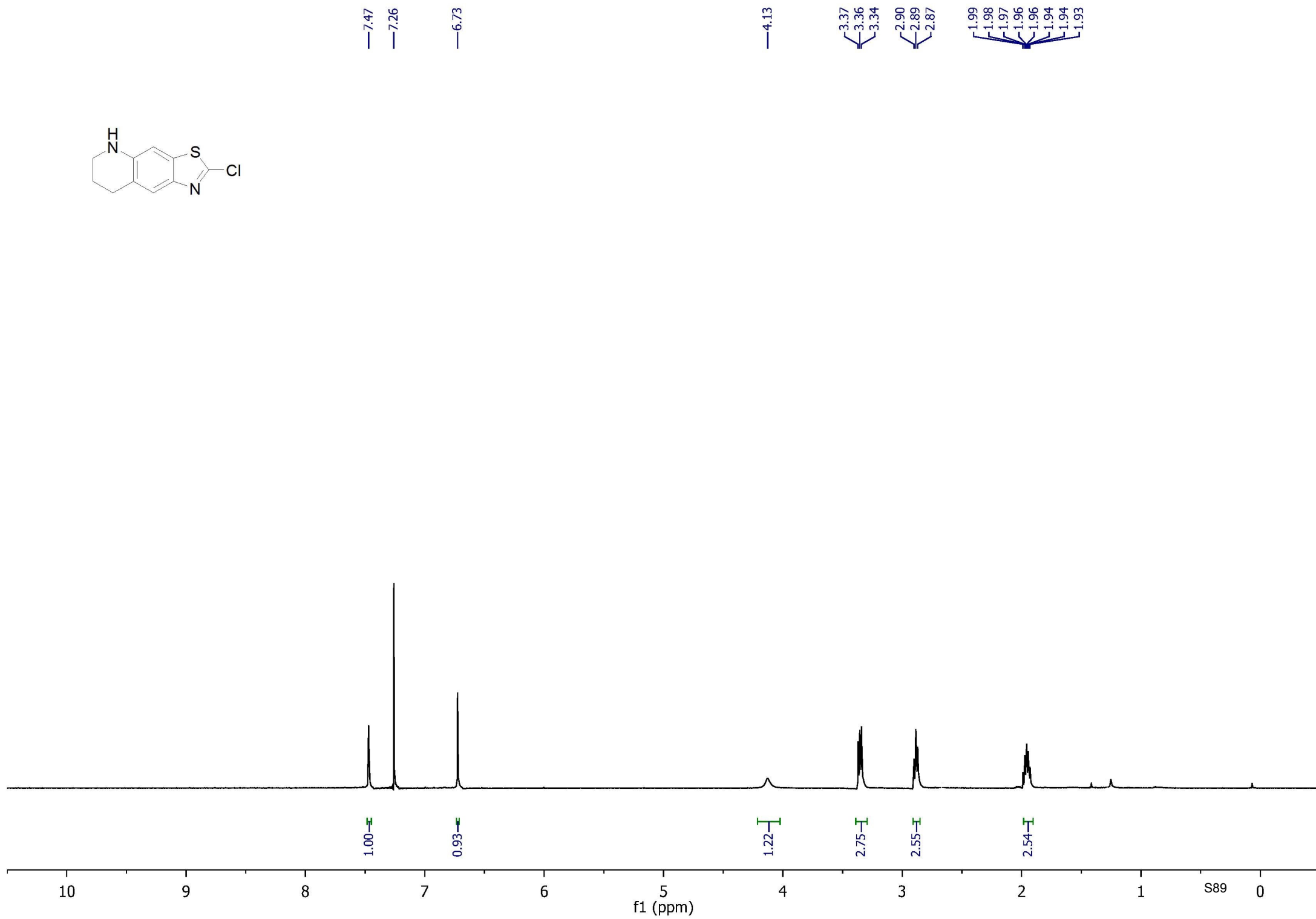
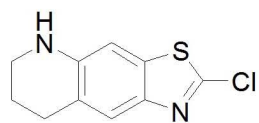


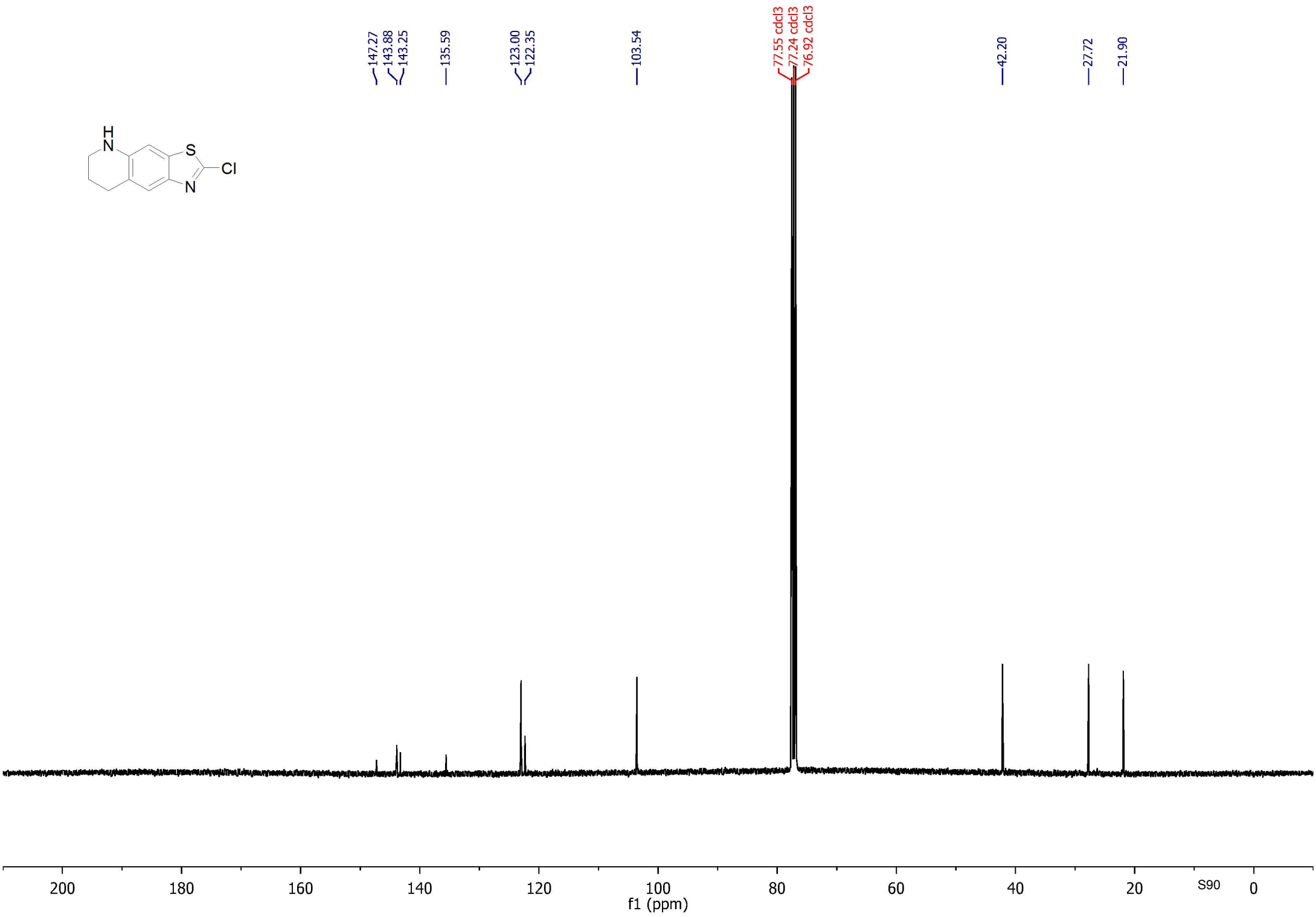
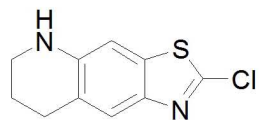
—69.16

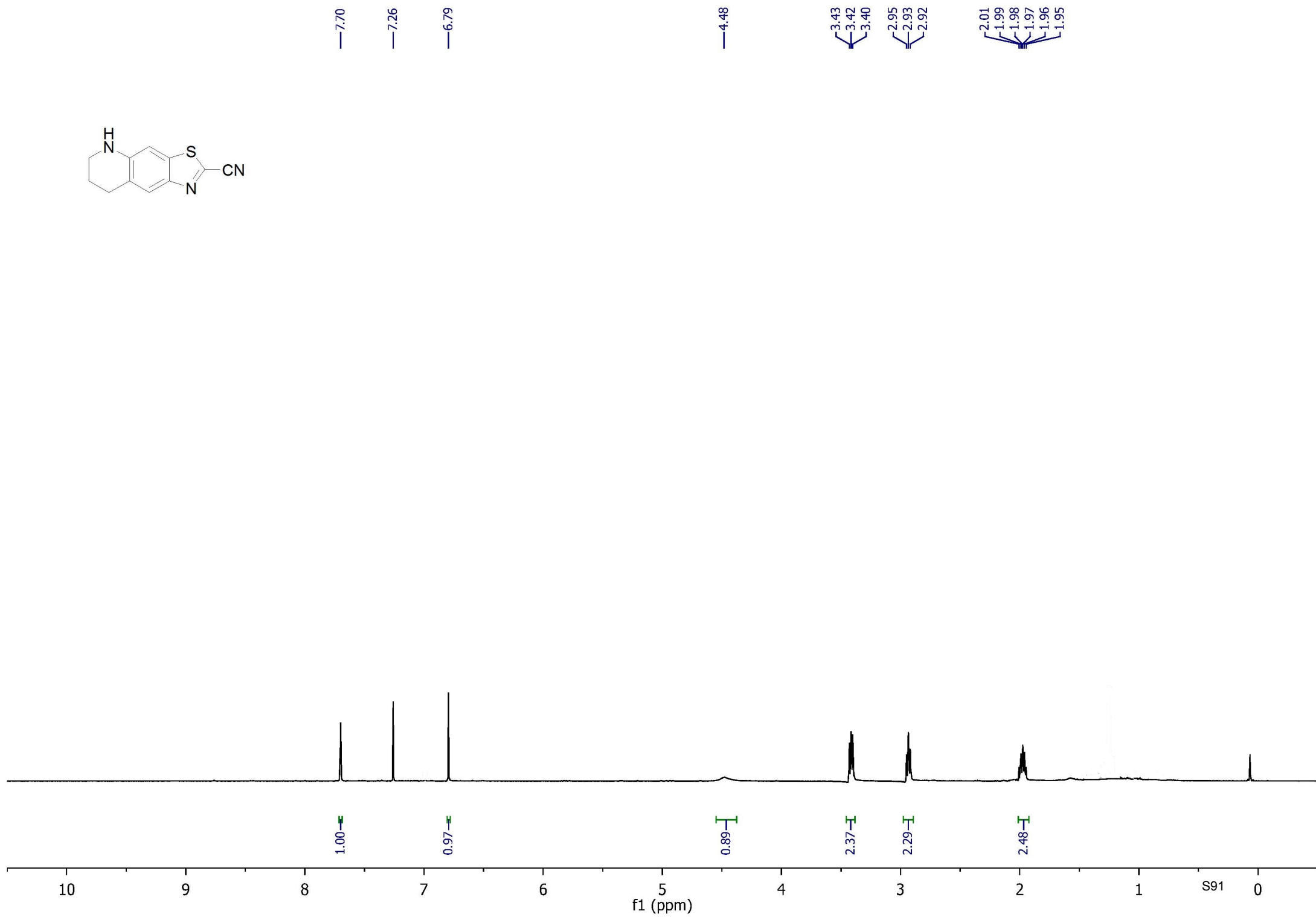
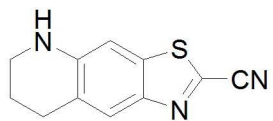


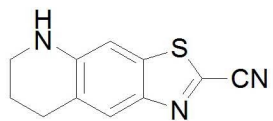
-40 -60 -80 -100 -120 -140 -160 -180 -200 -220 S88

f1 (ppm)









146.59  
144.73

136.23

129.19  
124.98  
124.50  
119.92

114.32

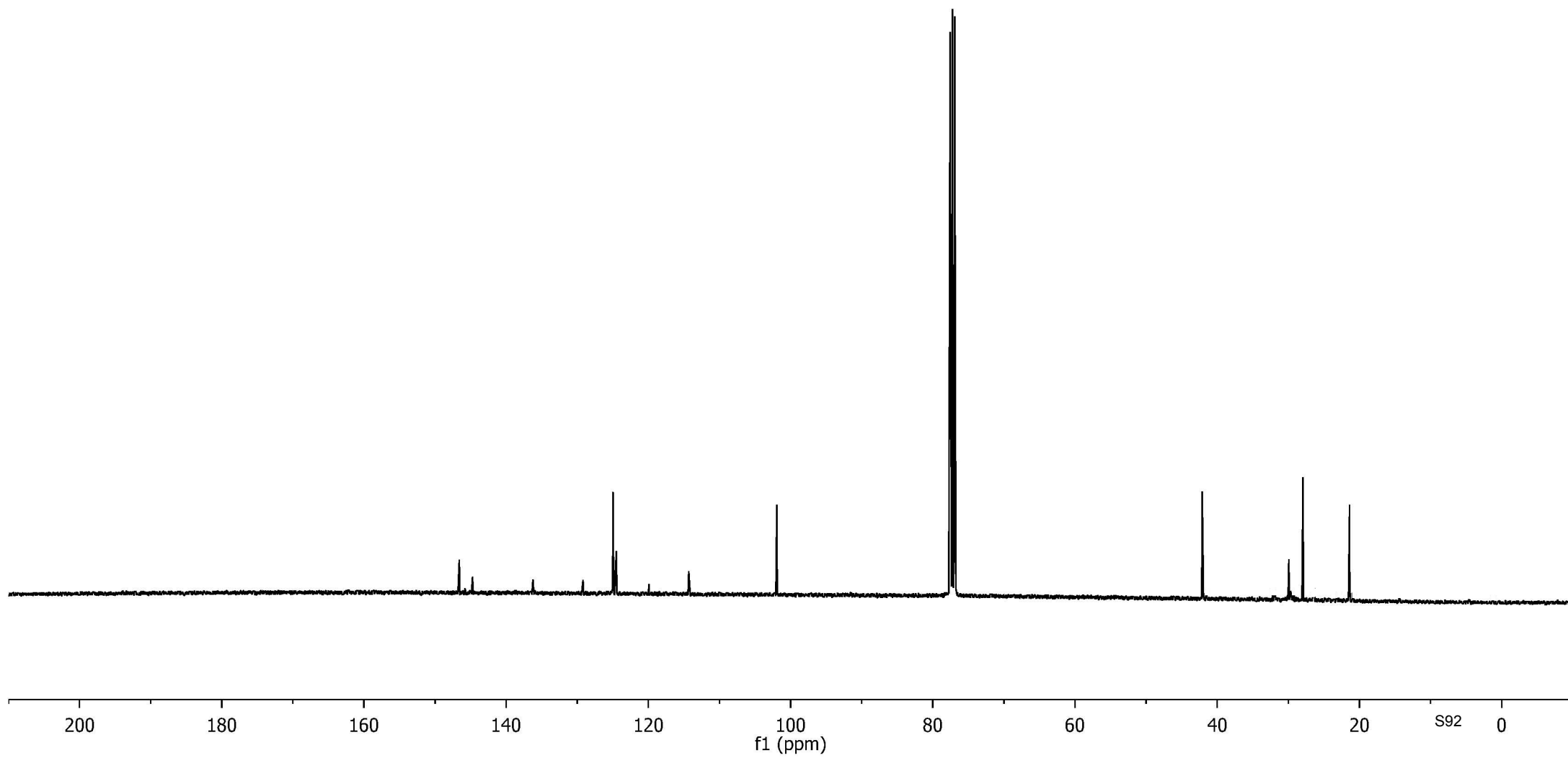
101.94

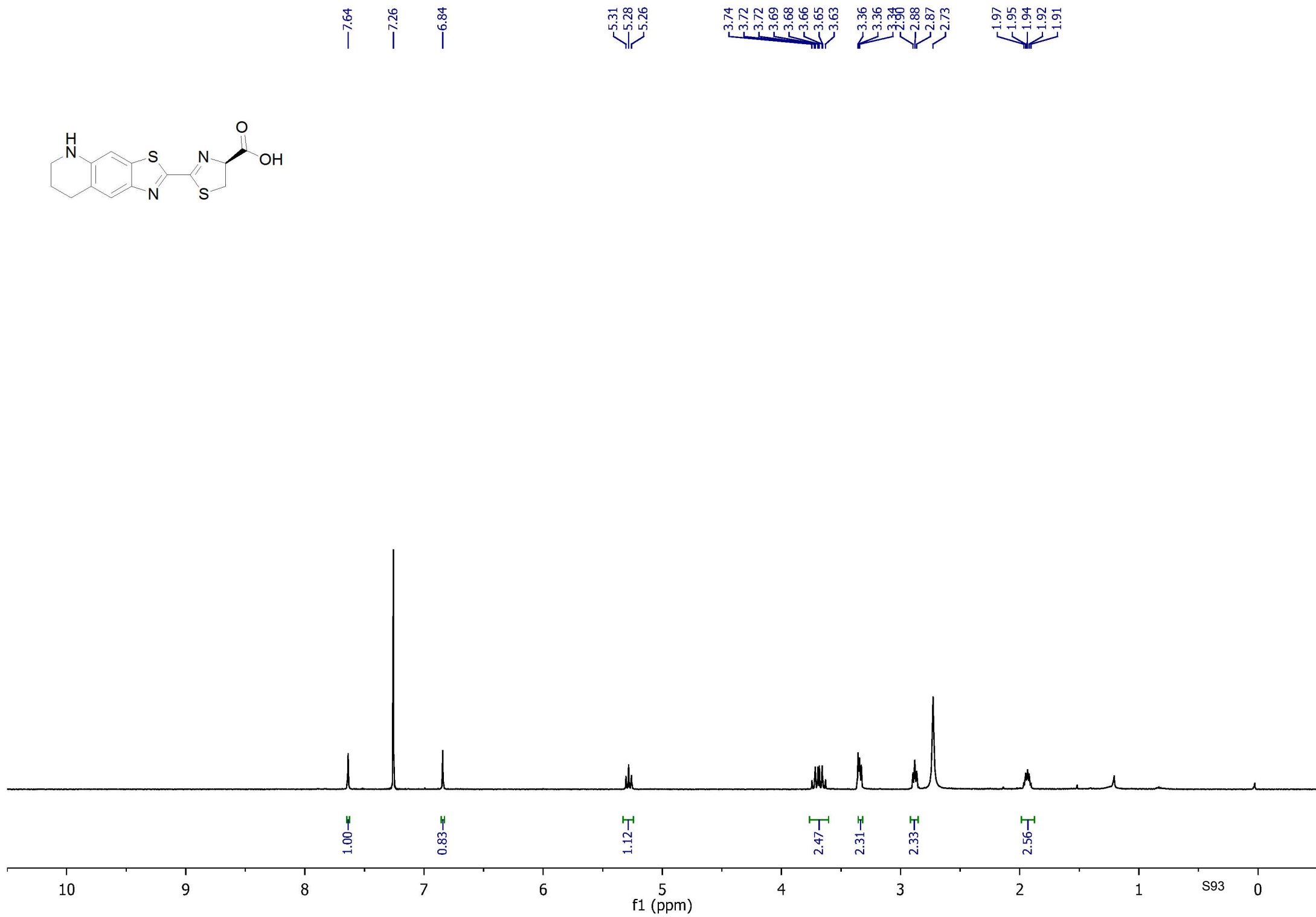
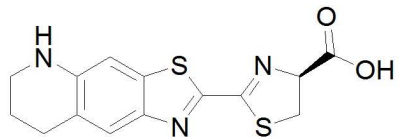
77.56 cdc13  
77.24 cdc13  
76.92 cdc13

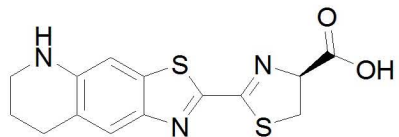
42.11

29.93  
27.94

21.39







— 172.34

— 166.96

— 154.56

~ 145.87

~ 144.85

— 136.34

~ 124.51

~ 123.61

— 103.58

~ 78.14

~ 77.60 cddcl3

~ 77.28 cddcl3

~ 76.96 cddcl3

~ 50.23

~ 50.01

~ 49.80

~ 49.58

~ 49.37

~ 49.16

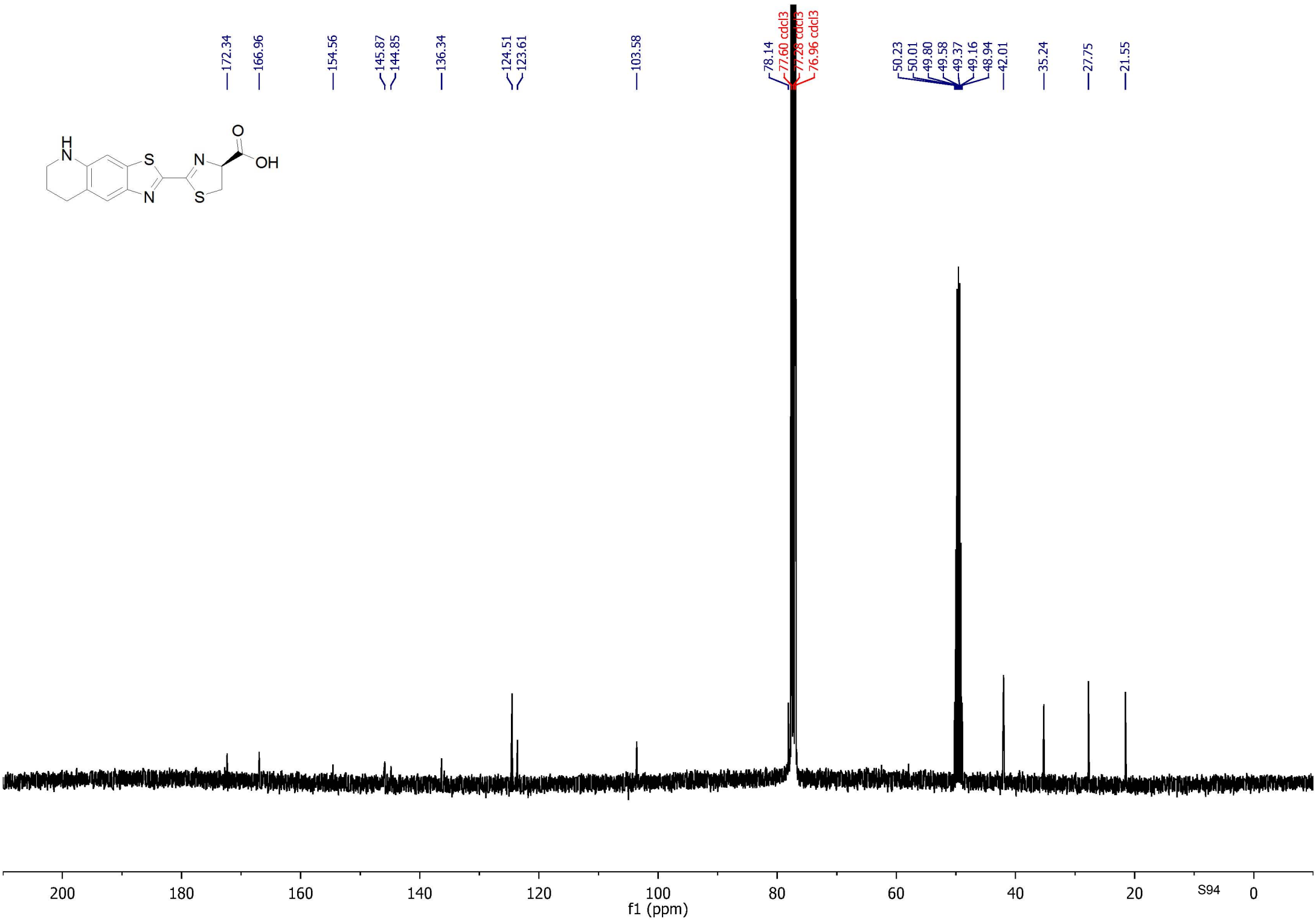
~ 48.94

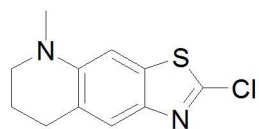
— 42.01

— 35.24

— 27.75

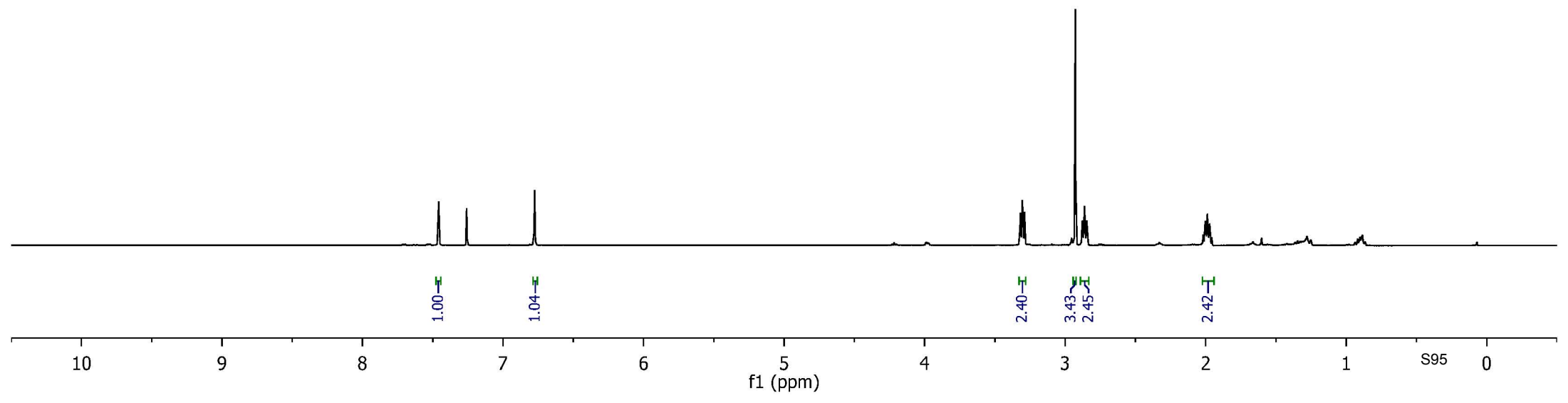
— 21.55

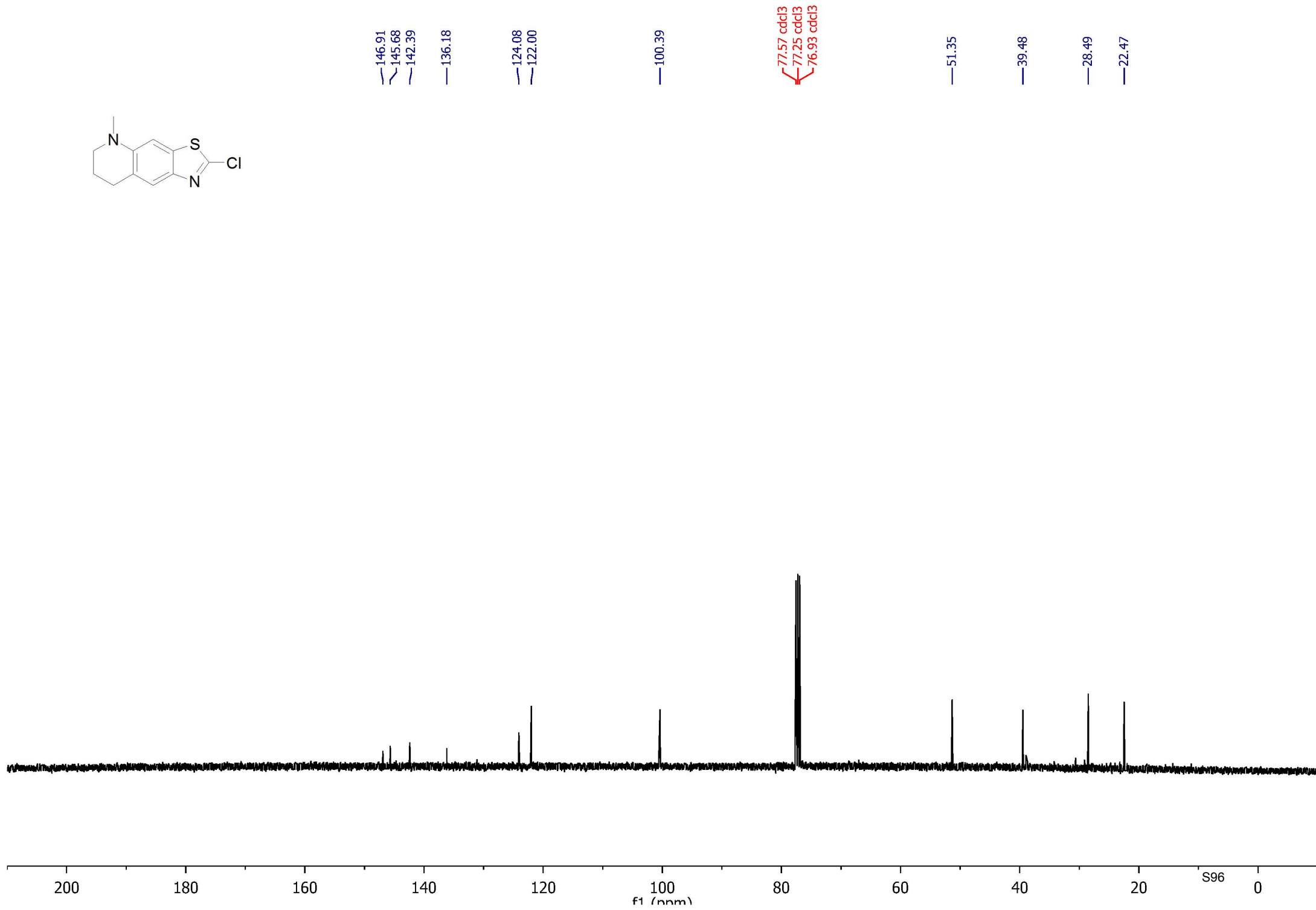
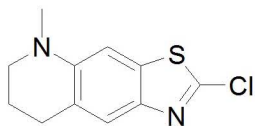


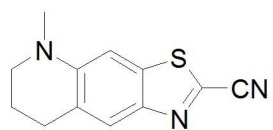


7.46  
7.26  
6.78

3.32  
3.30  
3.29  
2.93  
2.88  
2.86  
2.85  
2.02  
2.00  
1.99  
1.97  
1.96







7.67

7.26

6.83

3.42

3.40

3.39

3.00

2.92

2.91

2.89

2.03

2.02

2.01

2.00

1.99

1.88

1.00

1.01

2.44

3.52

2.59

2.57

10

9

8

6

5

4

3

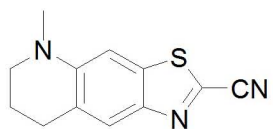
2

1

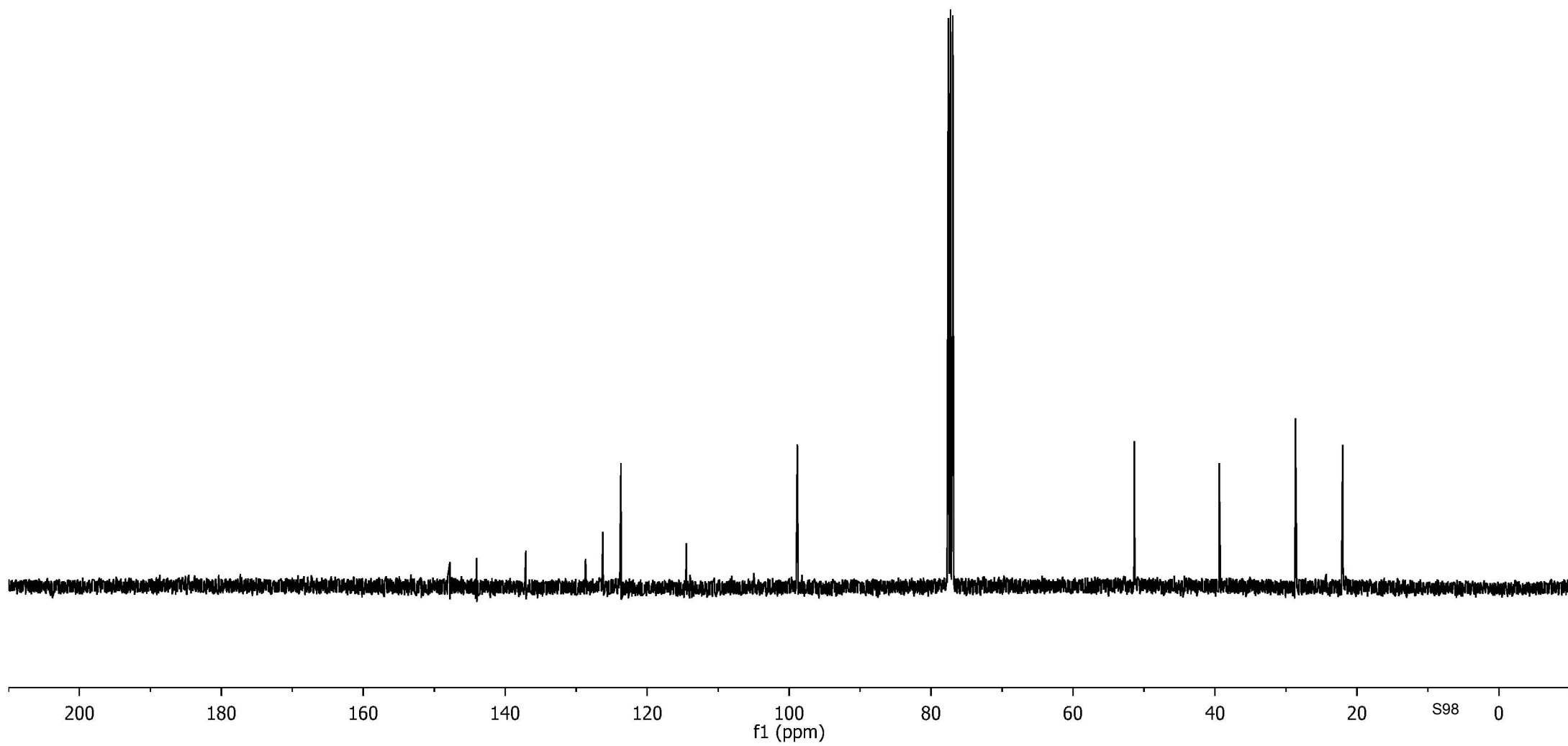
S97

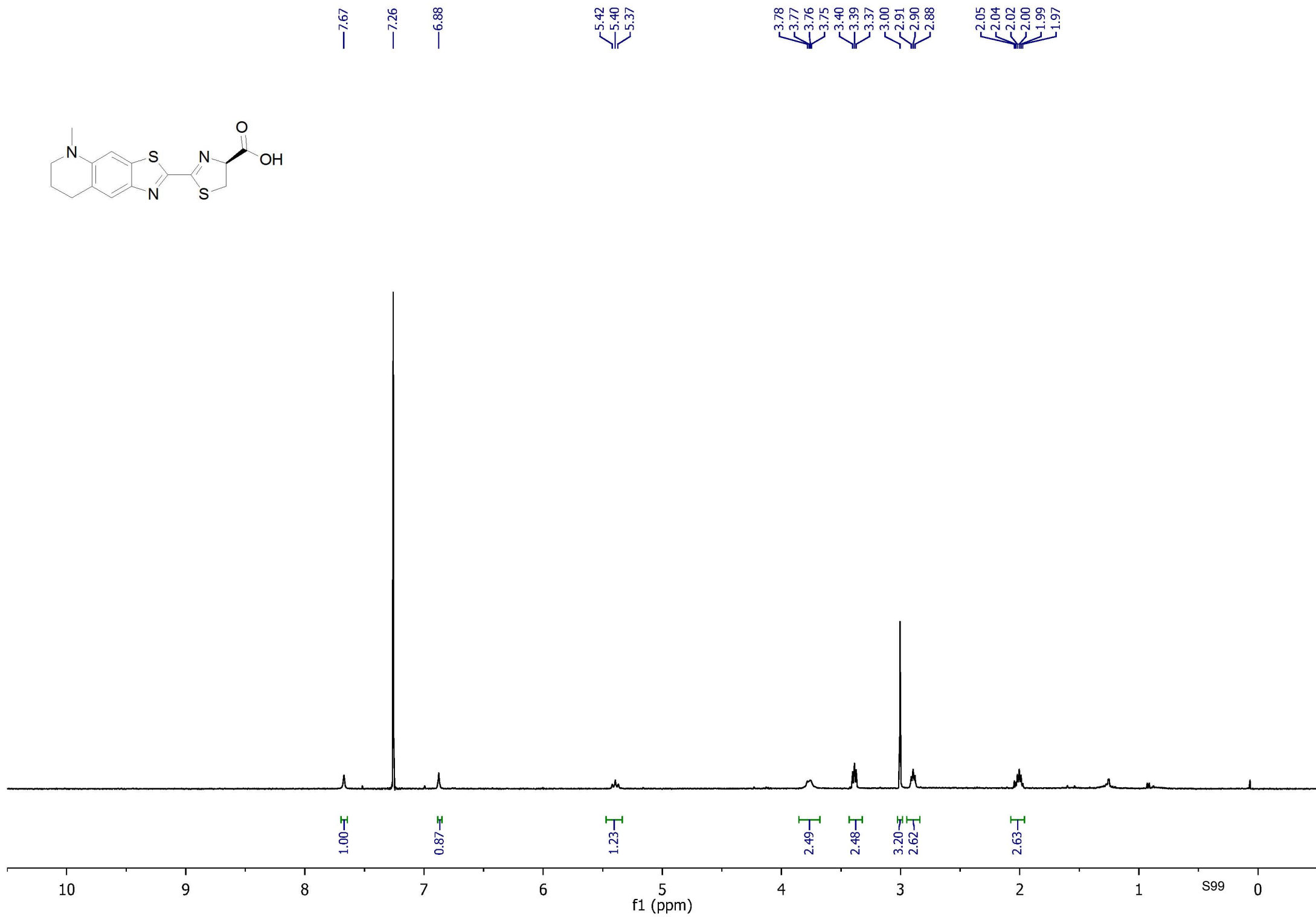
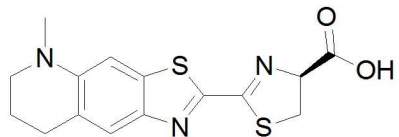
0

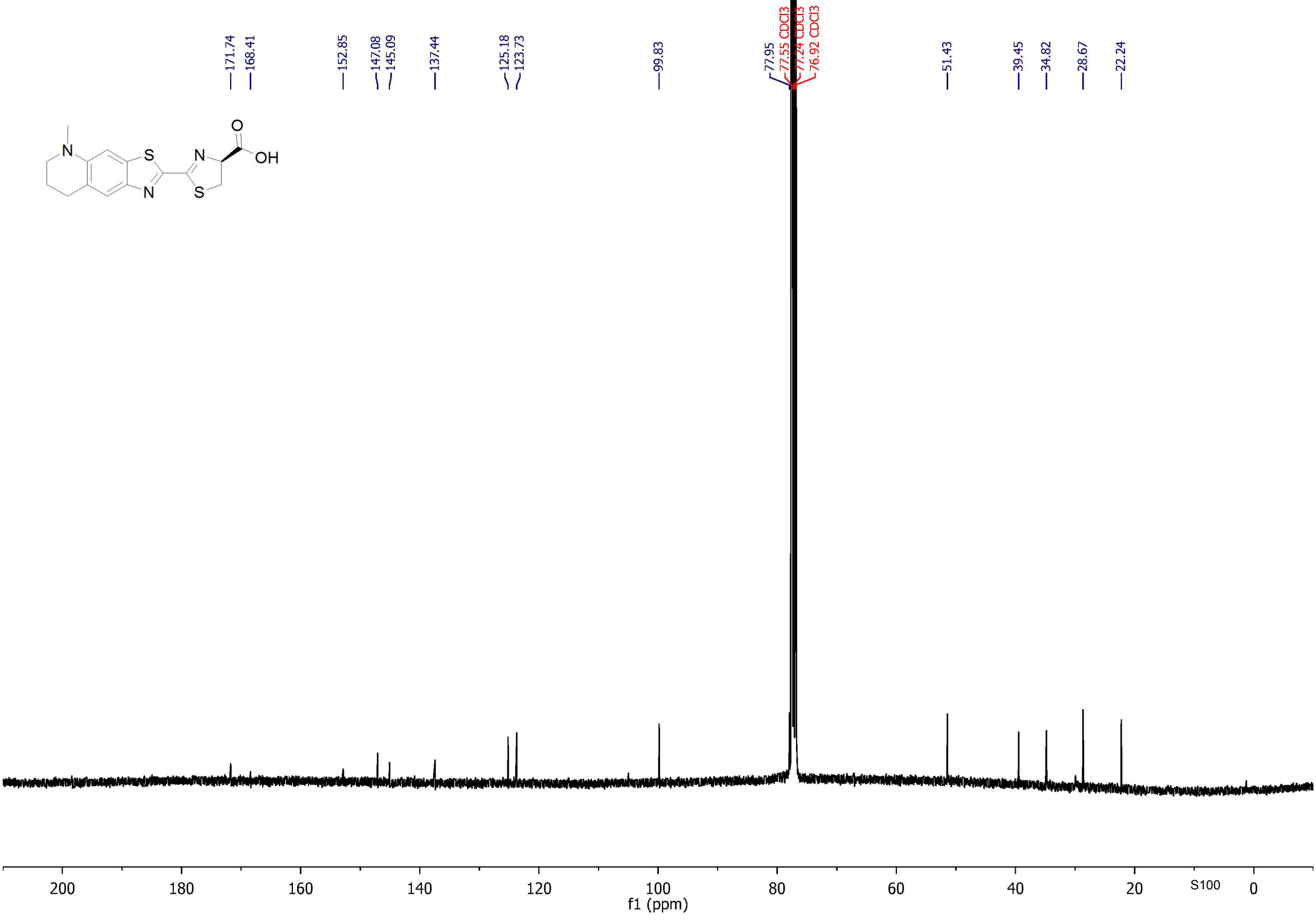
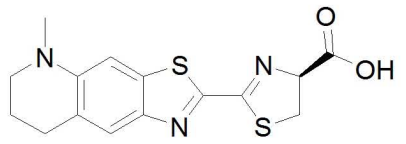
f1 (ppm)

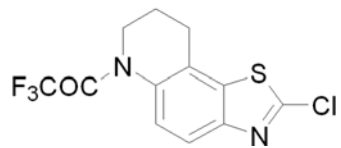


147.81  
144.03  
137.09  
128.69  
126.24  
123.70  
114.49  
98.85  
77.57 CDCl3  
77.25 CDCl3  
76.94 CDCl3  
51.38  
39.41  
28.67  
22.01









7.85  
7.80  
7.78

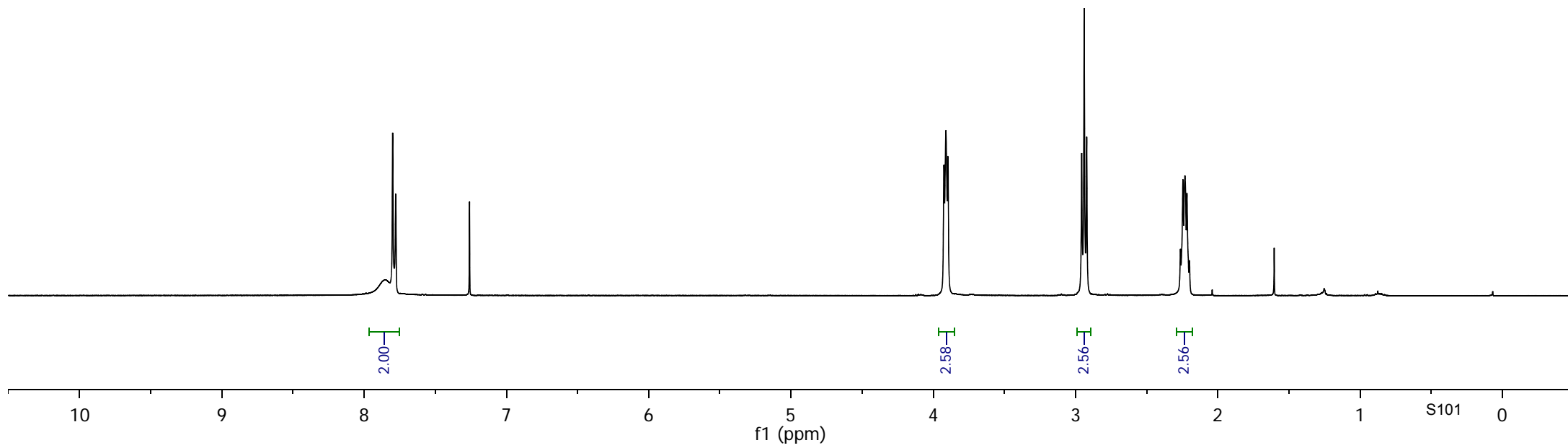
7.26

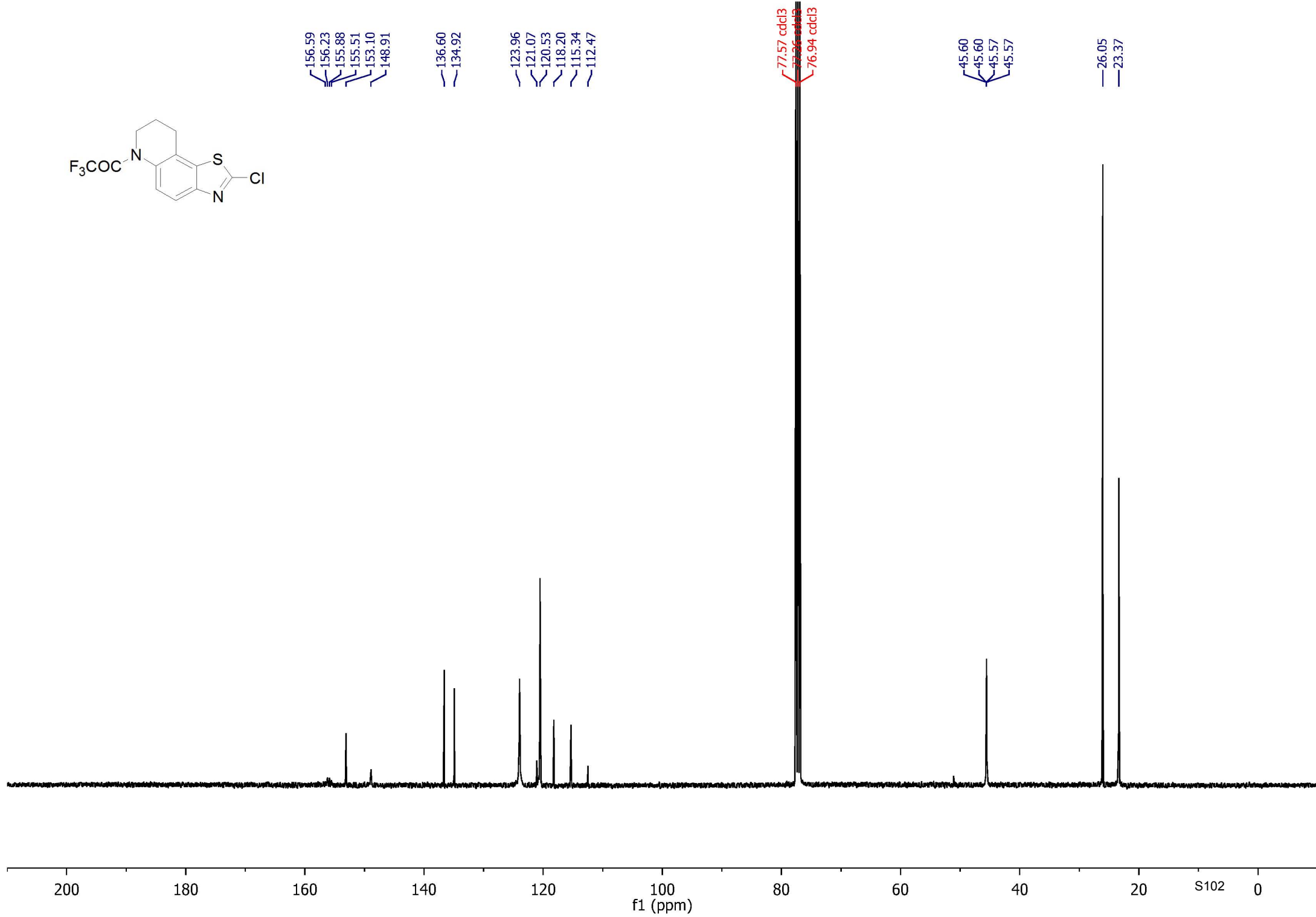
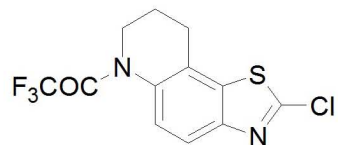
3.92  
3.91  
3.90

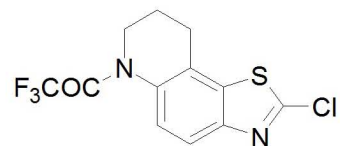
2.96  
2.94  
2.92

2.26  
2.25  
2.23  
2.22  
2.20

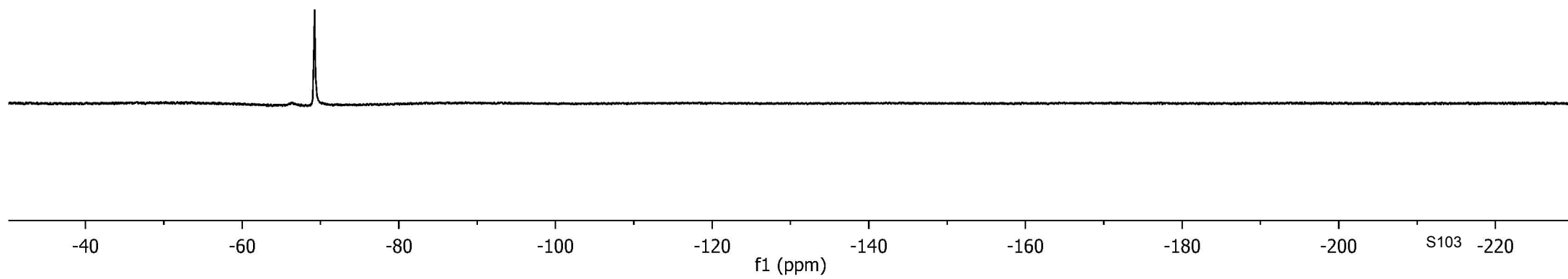
1.60

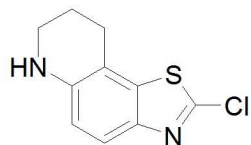






—69.25





7.50  
7.48  
7.26

6.58  
6.56

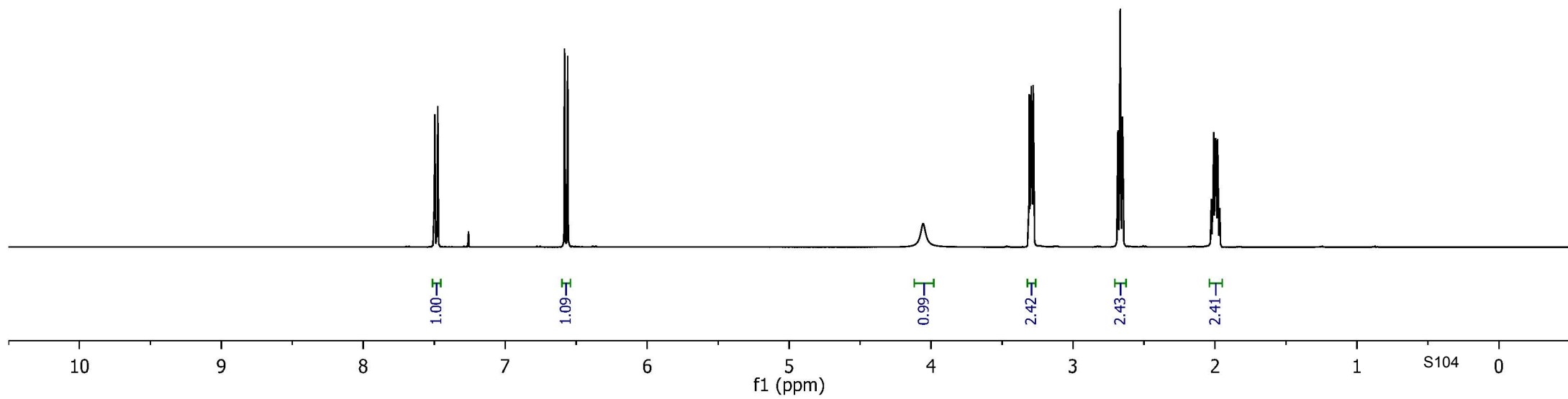
4.06

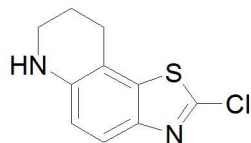
3.31  
3.29  
3.28

2.68  
2.67  
2.65

2.01  
2.01  
2.00

2.00  
1.99  
1.99  
1.98  
1.97





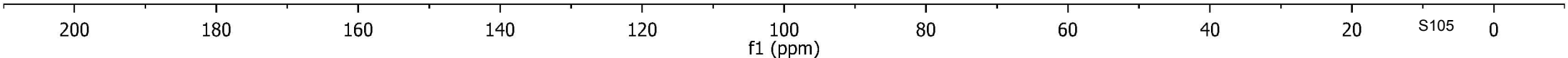
147.00  
143.00  
142.71  
137.98

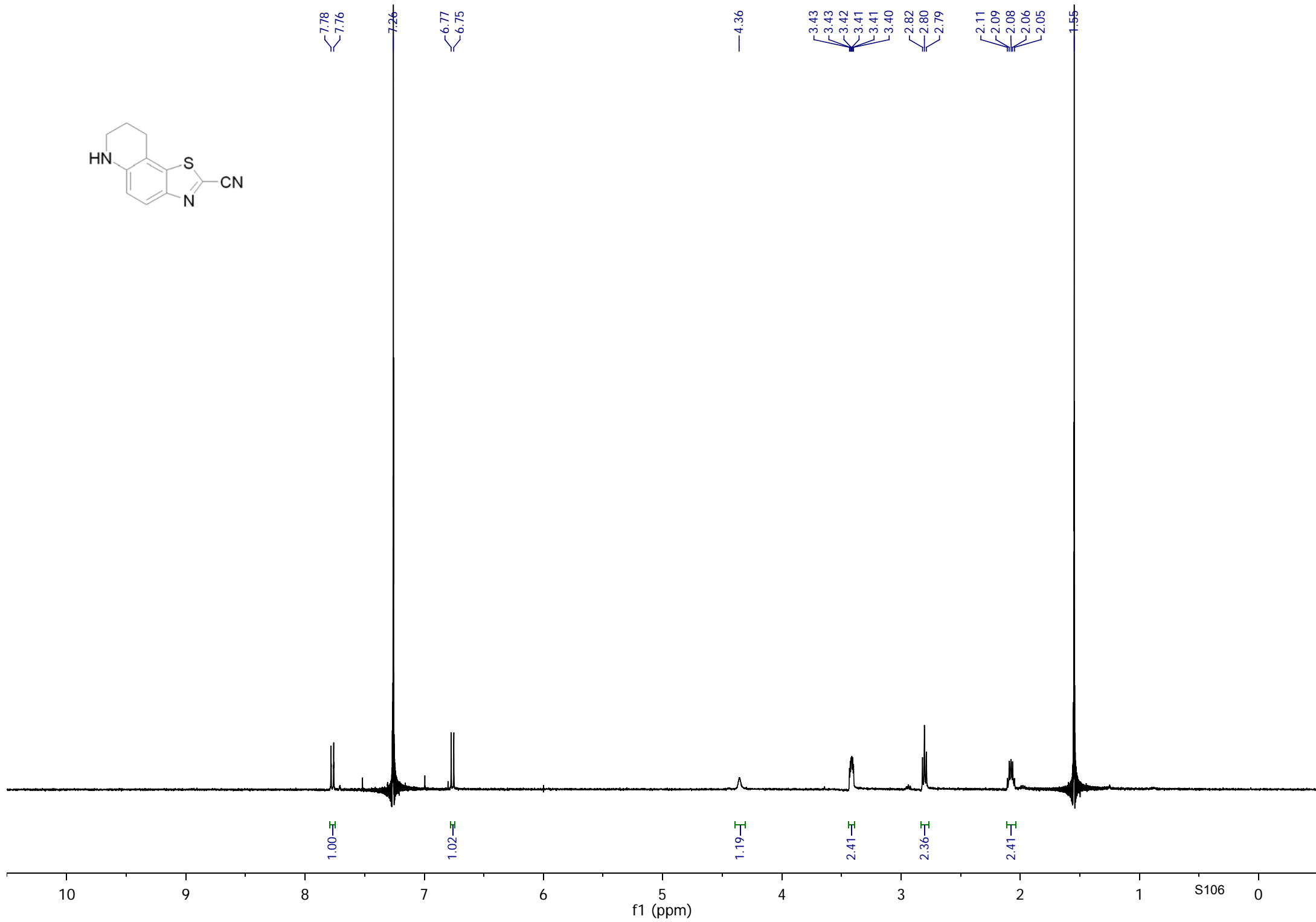
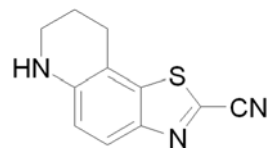
121.03  
115.11  
112.56

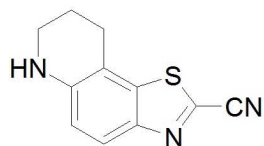
77.72 cdd13  
77.40 cdd13  
77.08 cdd13

41.82

26.45  
21.76







145.12  
144.45

138.54

129.45

123.74

117.33

114.28

111.21

77.56 cdcl3  
77.24 cdcl3  
76.92 cdcl3

41.66

26.49

21.29

200

180

160

140

120

100

80

60

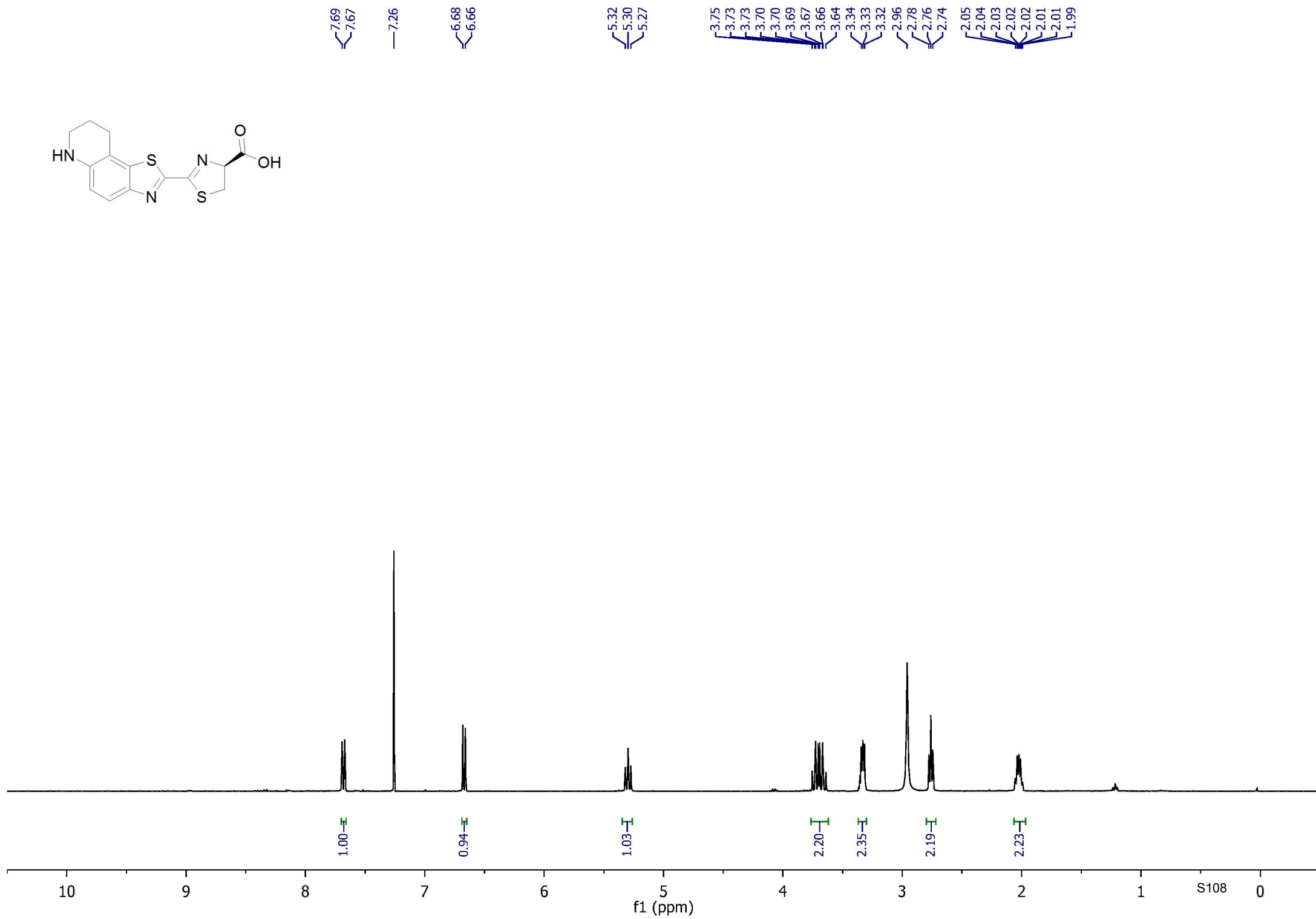
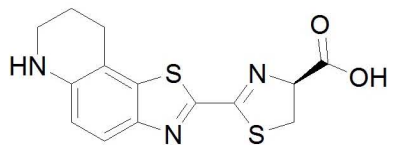
40

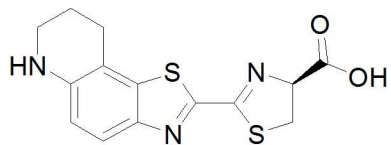
20

S107

0

f1 (ppm)





— 172.42

— 166.83

— 154.66

— 145.43

— 143.95

— 138.51

— 123.01

— 116.31

— 112.23

78.22

77.60 CDCl3

77.28 CDCl3

76.97 CDCl3

50.21

50.00

49.78

49.57

49.36

49.14

48.93

41.64

35.30

26.23

21.51

200

180

160

140

120

100  
f1 (ppm)

80

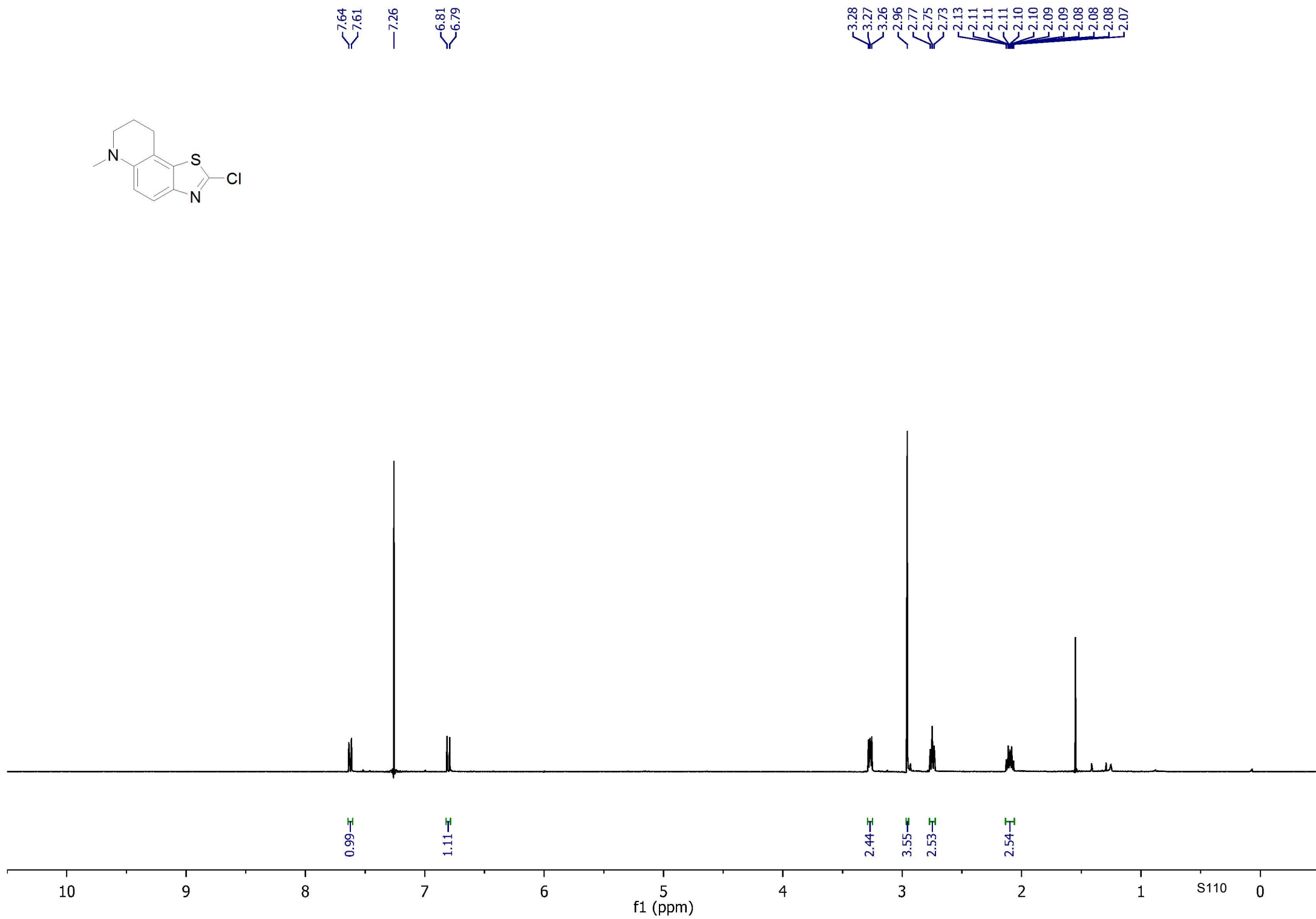
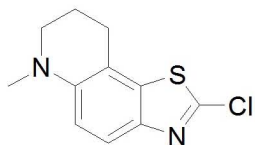
60

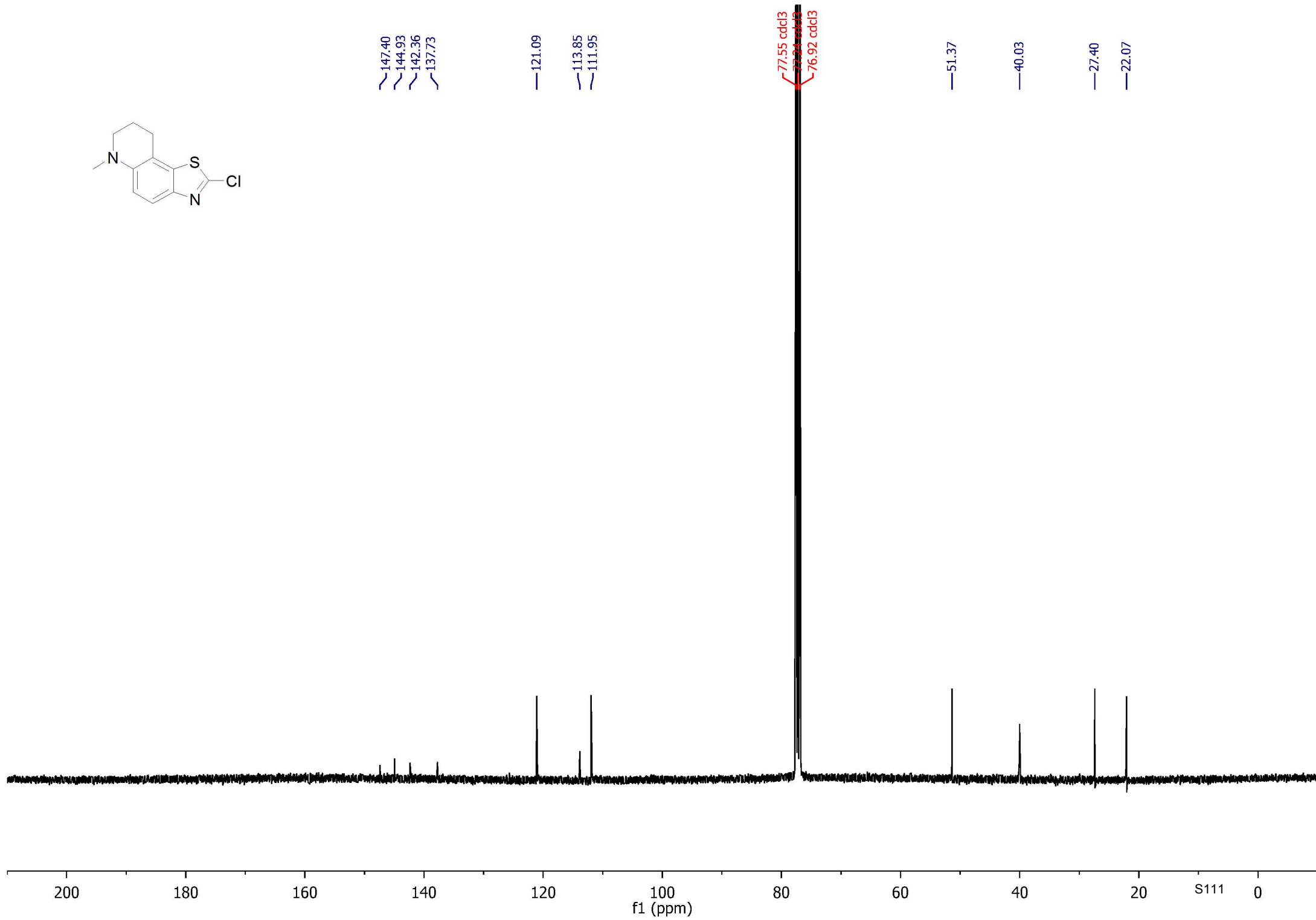
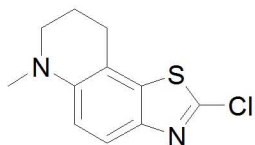
40

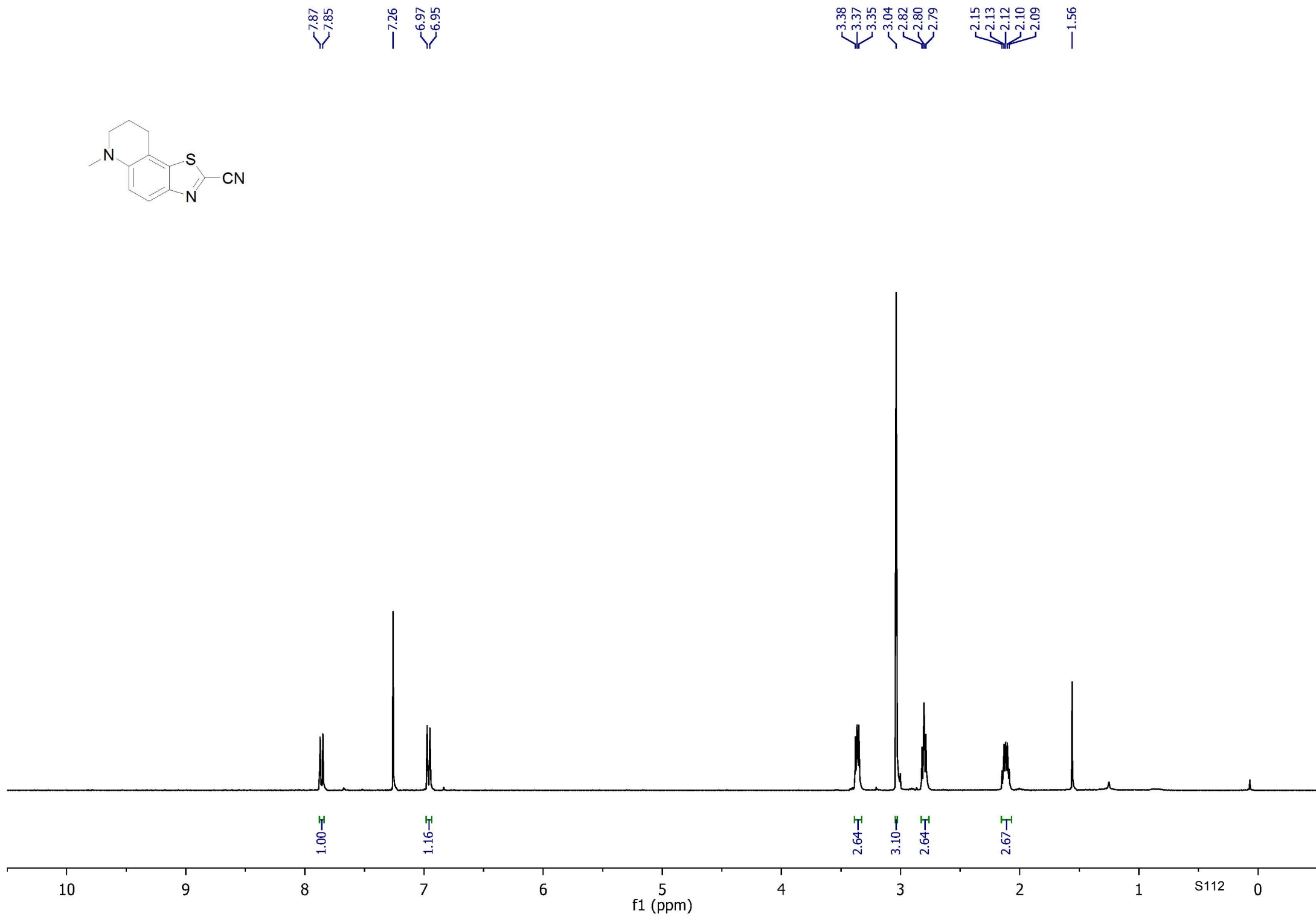
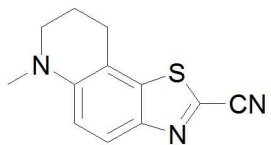
20

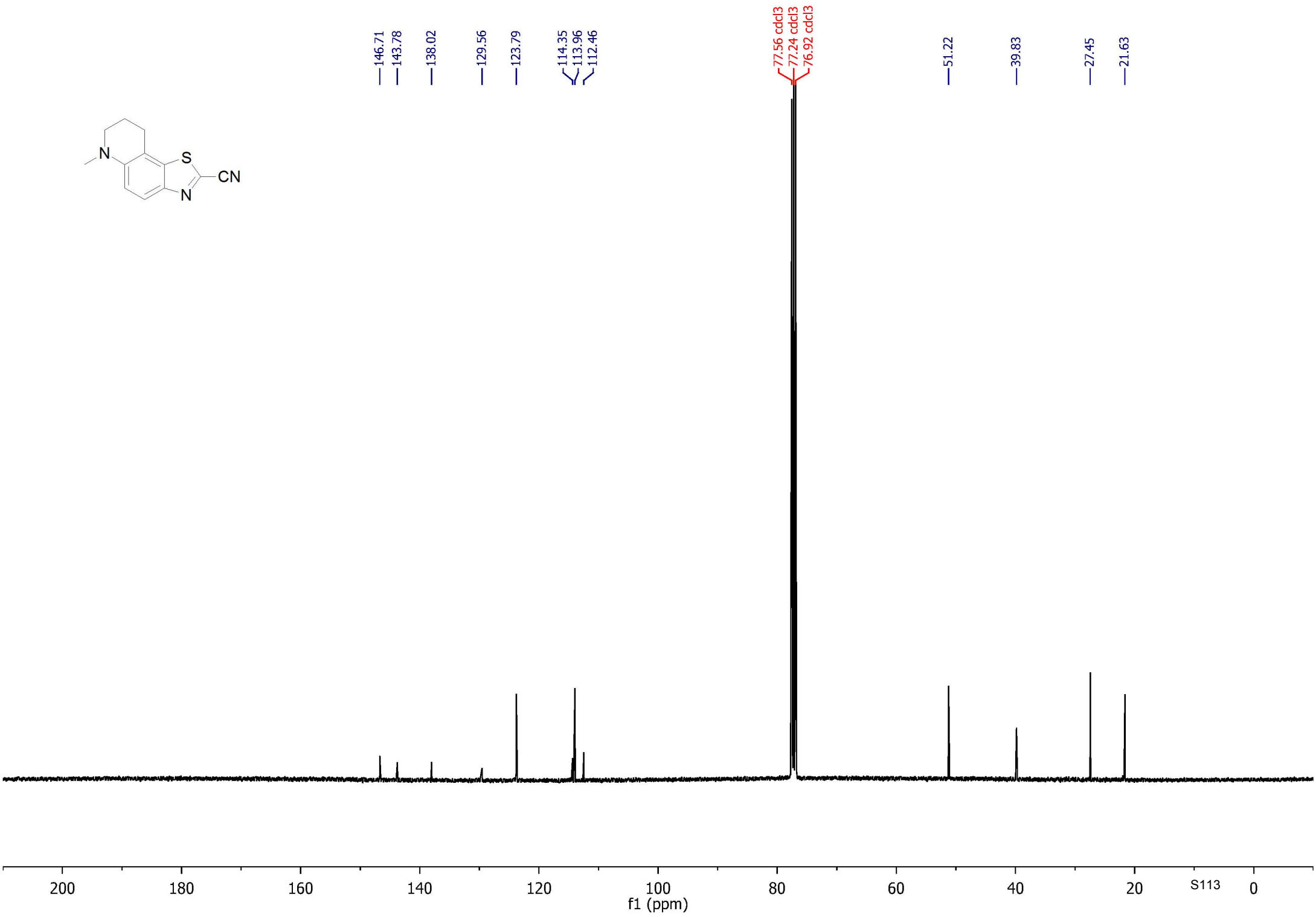
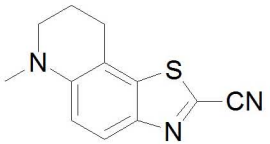
S109

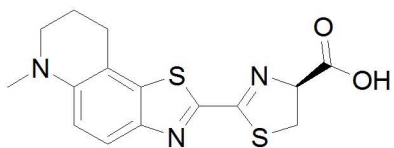
0











7.80  
7.78

7.26

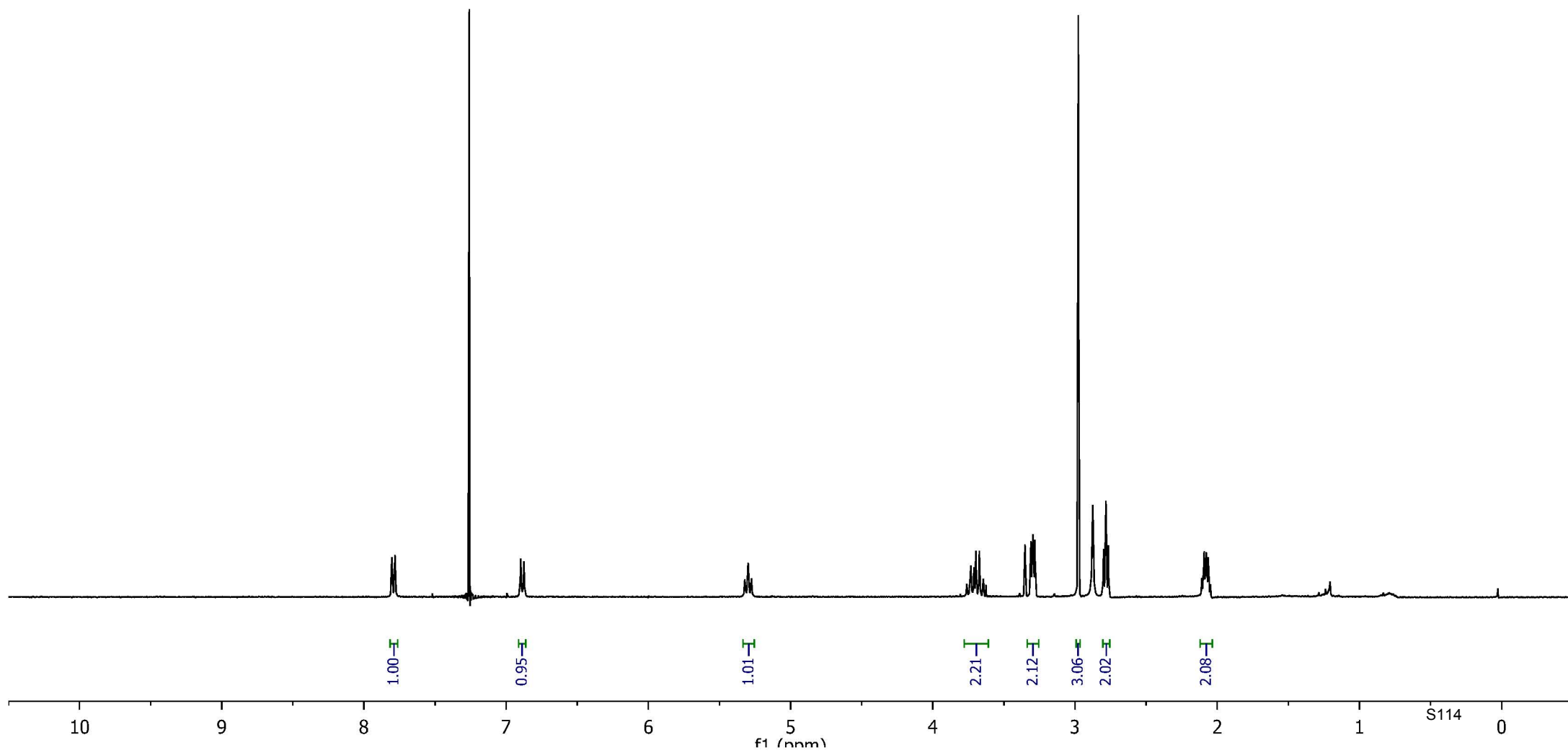
6.90  
6.88

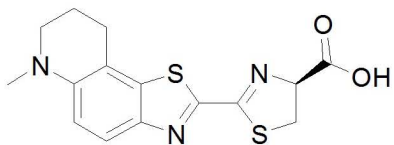
5.32  
5.30  
5.28

3.76  
3.74  
3.73  
3.71  
3.70  
3.67  
3.67  
3.65  
3.63

3.30  
3.28  
3.28  
2.88  
2.80  
2.78  
2.77

2.11  
2.09  
2.08  
2.06  
2.05





— 172.36

— 166.90

— 154.98

— 153.73

— 145.64

— 145.00

— 138.09

— 123.09

— 113.29

— 78.15

— 77.60 cdcl3

— 77.28 cdcl3

— 76.96 cdcl3

— 51.34

— 50.19

— 49.98

— 49.76

— 49.55

— 49.34

— 49.12

— 48.91

— 48.68

— 35.32

— 27.10

— 21.64

200

180

160

140

120

100

80

60

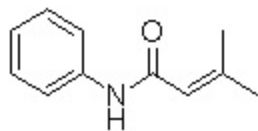
40

20

S115

0

f1 (ppm)



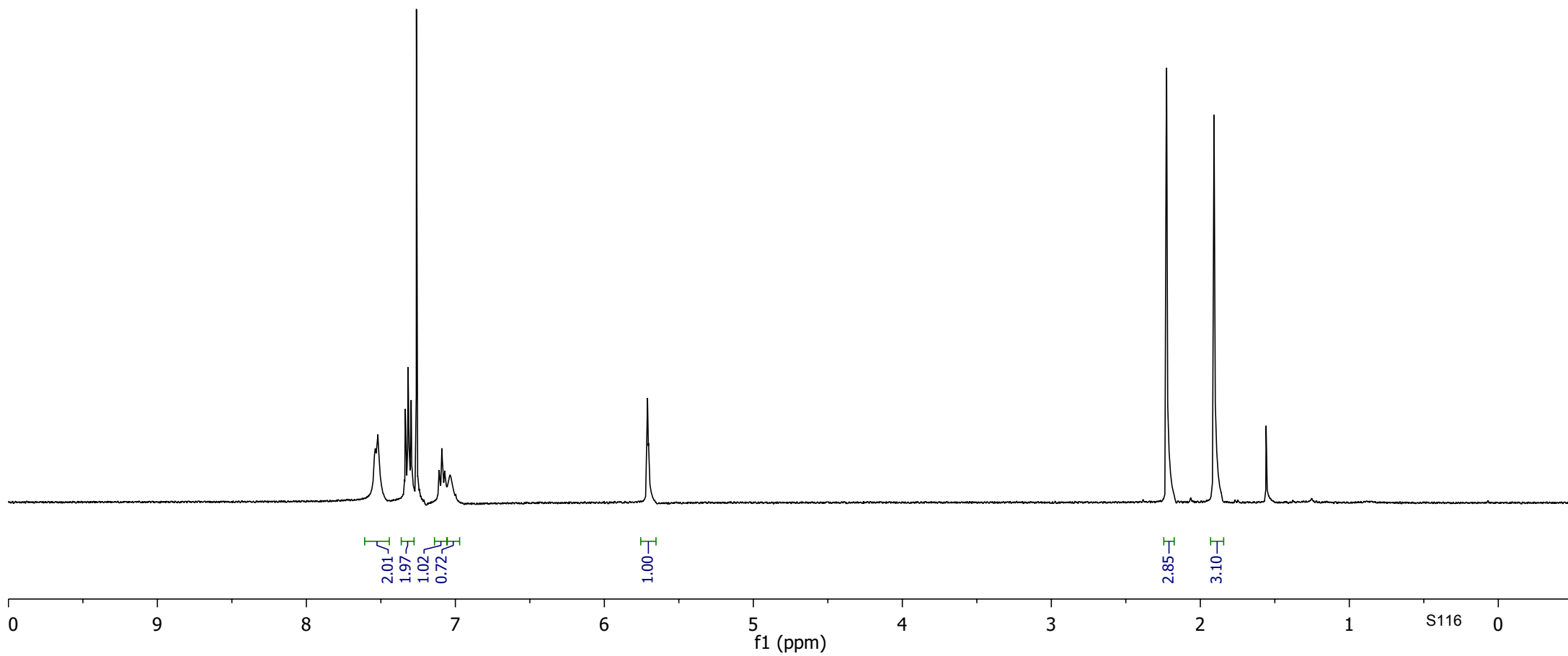
7.54  
7.52  
7.34  
7.33  
7.32  
7.32  
7.30  
7.30  
7.26  
7.11  
7.09  
7.07  
7.03

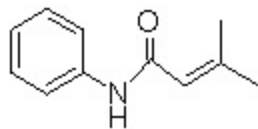
5.71

2.23  
2.23

1.91  
1.91

1.56





—165.86

—153.10

—138.68

—129.11  
—129.04

—124.11

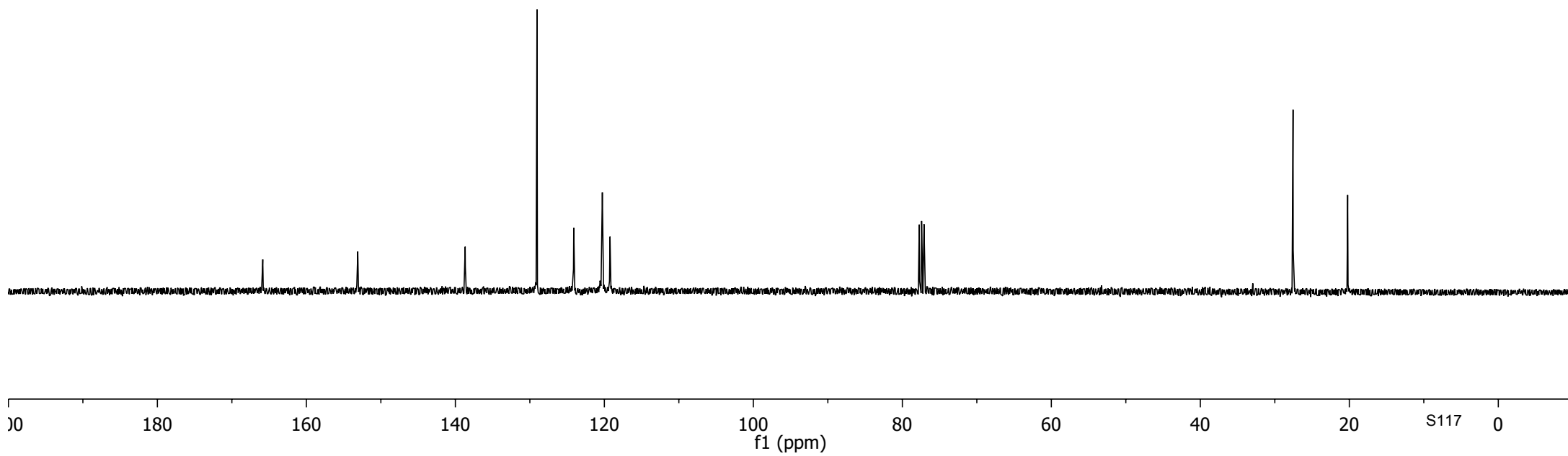
—120.27

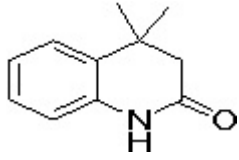
—119.24

77.73 cdd3  
77.41 cdd3  
77.09 cdd3

—27.55

—20.22



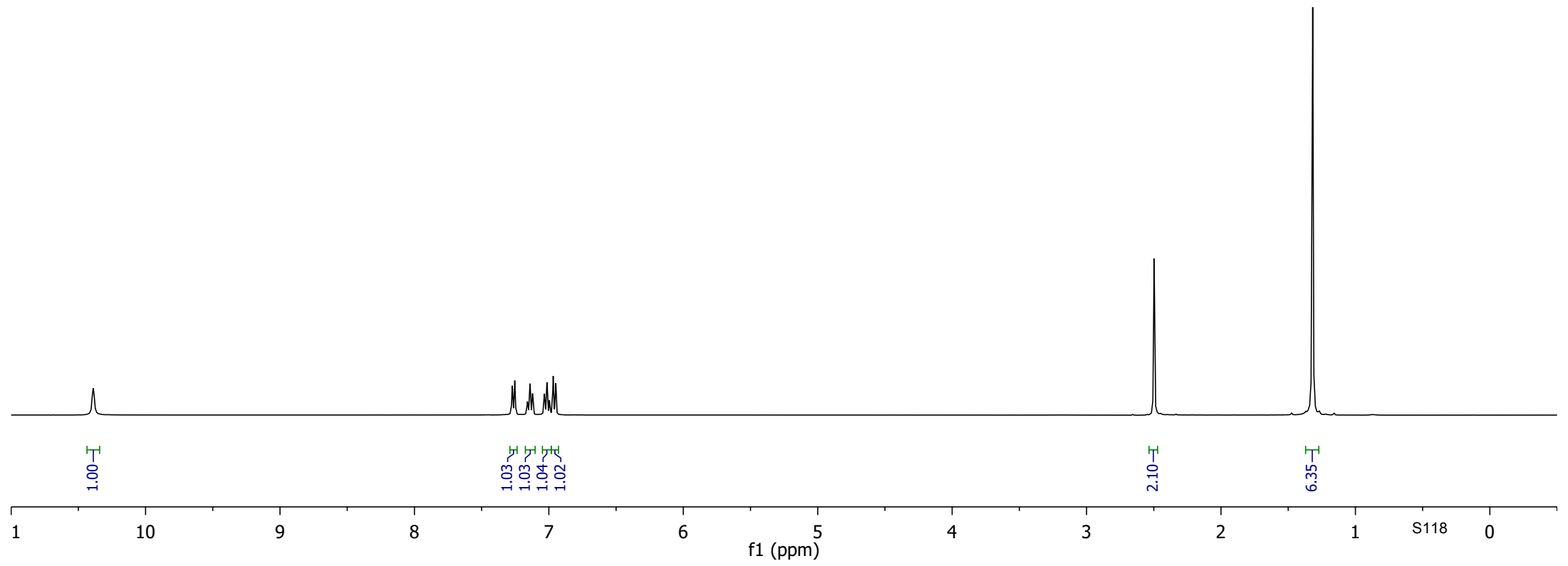


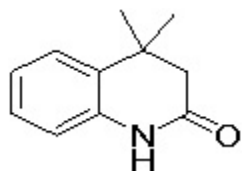
7.27  
7.25  
7.16  
7.14  
7.12  
7.03  
7.01  
7.00  
6.97  
6.95

10.39

2.50

1.32





—172.44

—136.34

—132.57

—127.73

—124.50

—123.77

—116.49

77.81 cddc3

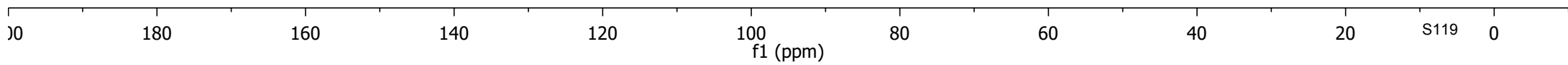
77.49 cddc3

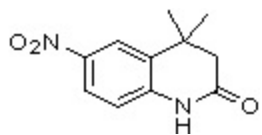
77.17 cddc3

—45.51

—34.08

—27.94





8.46  
8.22  
8.21  
8.13  
8.12  
8.11  
8.10

7.26

6.89  
6.87

2.56

1.56  
1.40



0.94  
1.00  
0.96

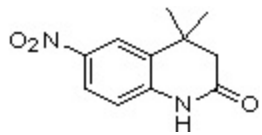
0.99

2.21

6.46

0 9 8 7 6 5 4 3 2 1 S120 0

f1 (ppm)



—172.17

—143.93

—141.87

—133.44

—124.14

—121.10

—116.41

—77.58 cddc13

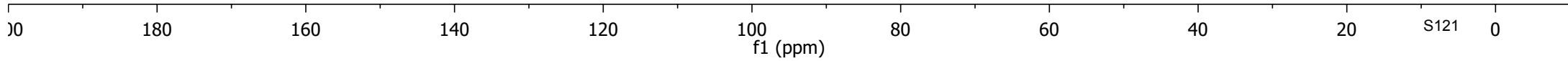
—77.26 cddc13

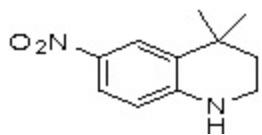
—76.94 cddc13

—44.74

—34.45

—27.76





8.08  
8.08  
7.86  
7.85  
7.84  
7.83

7.26

6.39  
6.37

5.05

3.44  
3.42  
3.41

1.71  
1.70  
1.68

1.29

1.00

0.97

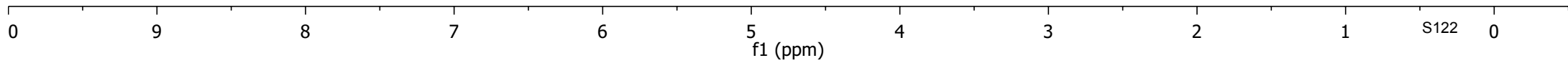
1.10

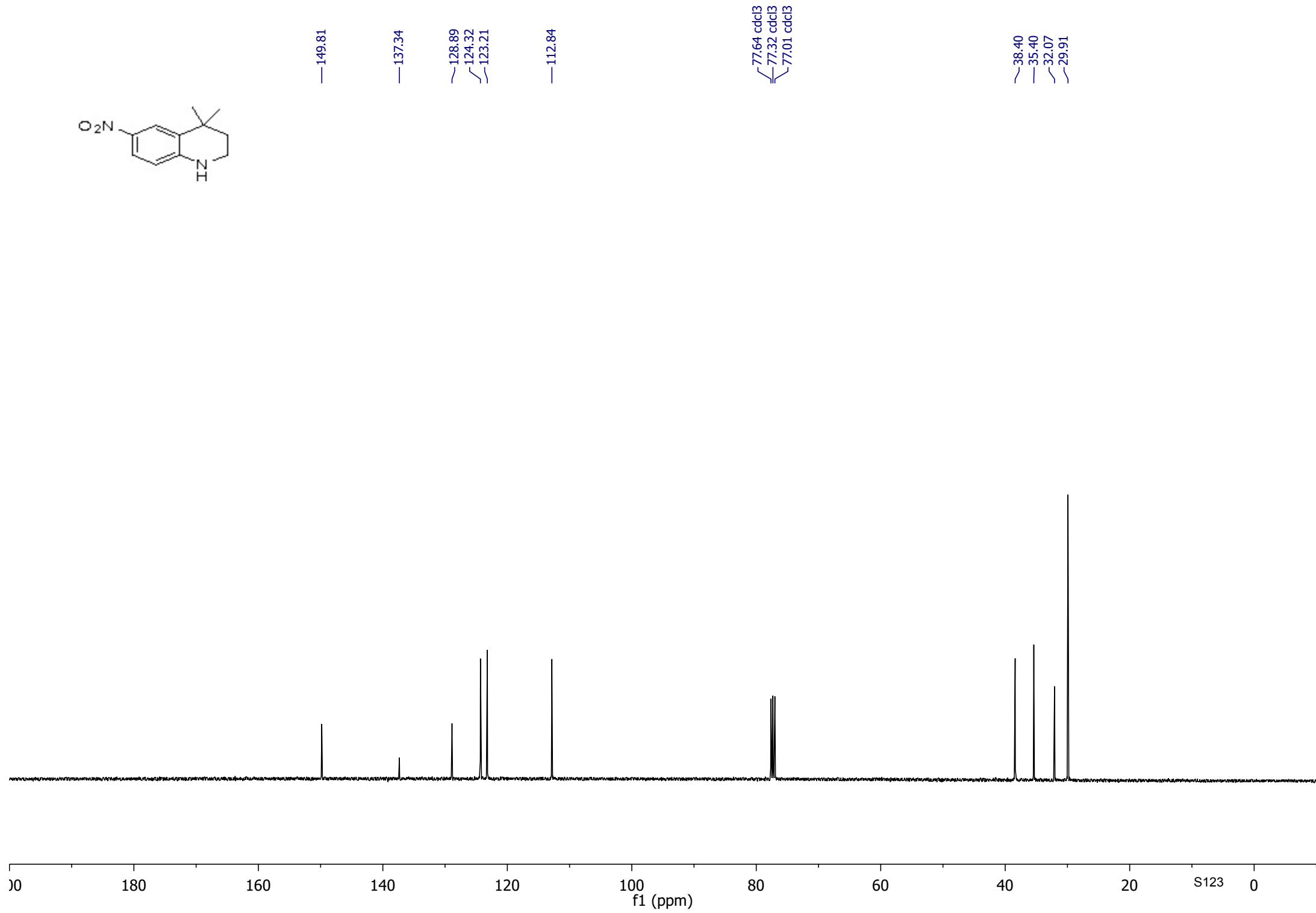
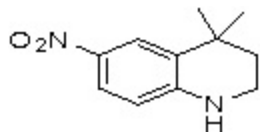
0.93

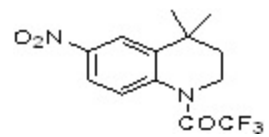
2.40

2.40

7.01





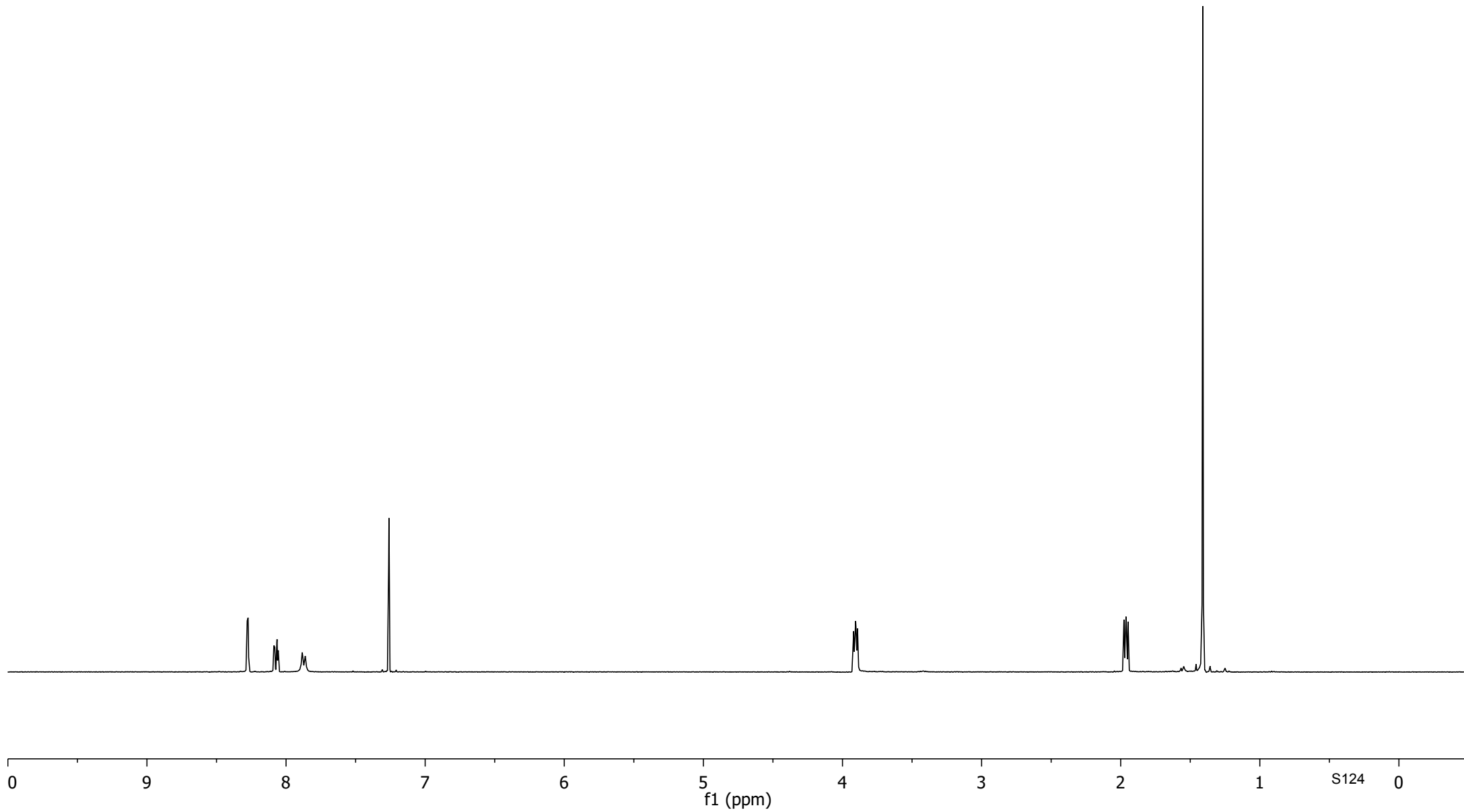


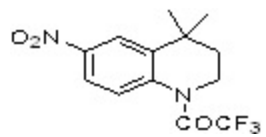
8.28  
8.27  
8.09  
8.08  
8.06  
8.06  
7.88  
7.86  
7.26

3.92  
3.91  
3.89

1.98  
1.96  
1.95

1.41





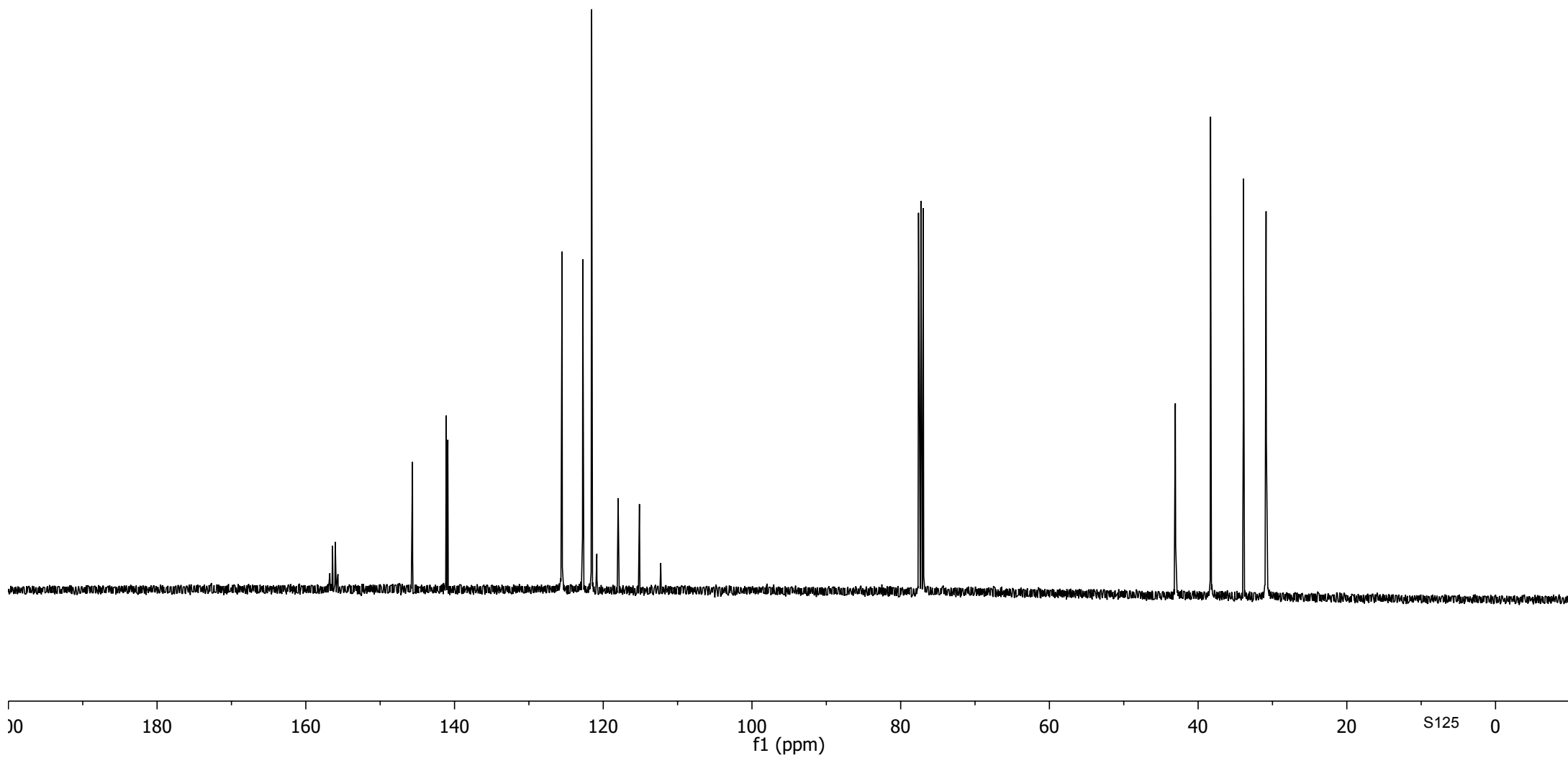
156.77  
156.40  
156.04  
155.68

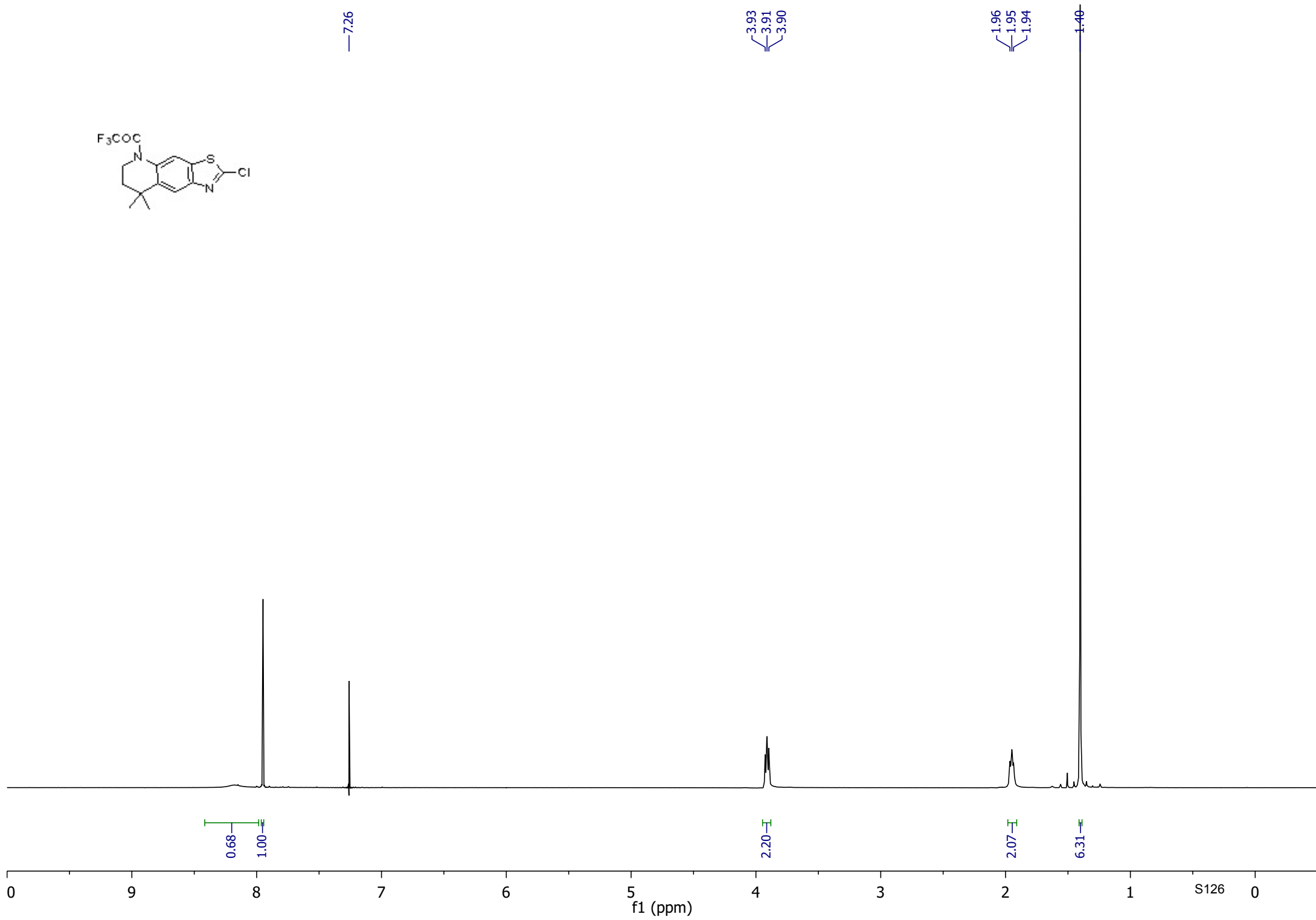
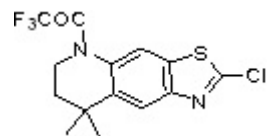
145.65  
141.12  
140.90

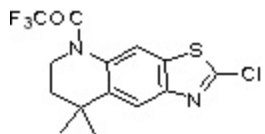
125.54  
122.75  
121.56  
120.86  
118.00  
115.13  
112.27

77.60 cddc13  
77.28 cddc13  
76.96 cddc13

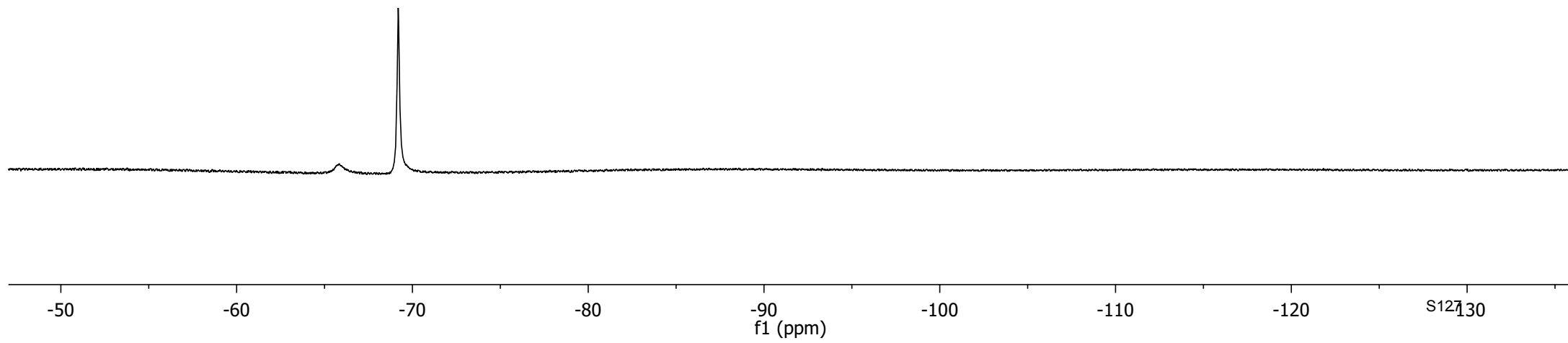
43.14  
43.10  
43.07  
43.03  
38.33  
33.92  
30.86

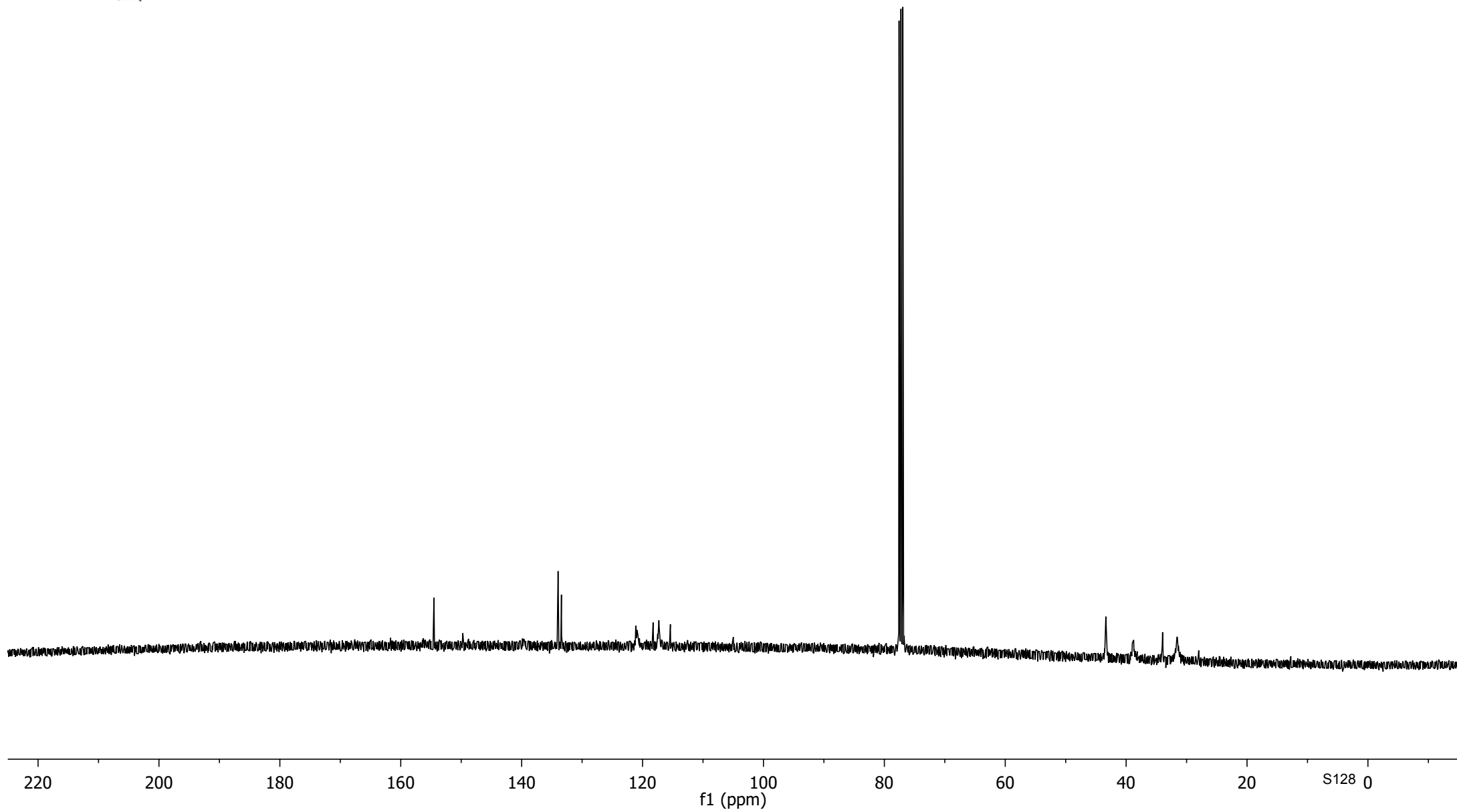
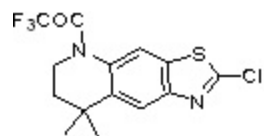


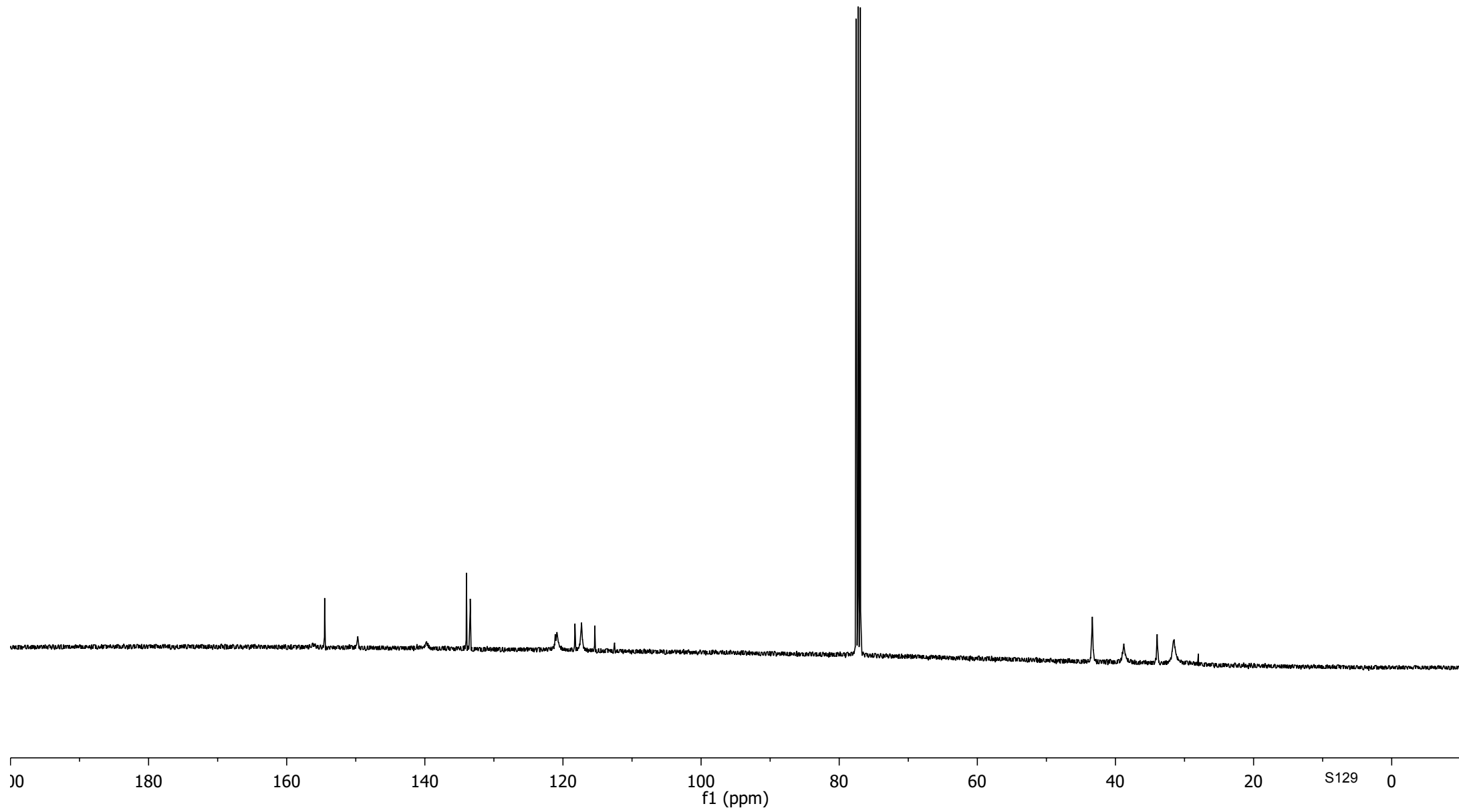
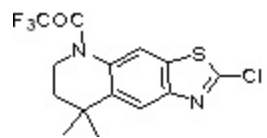


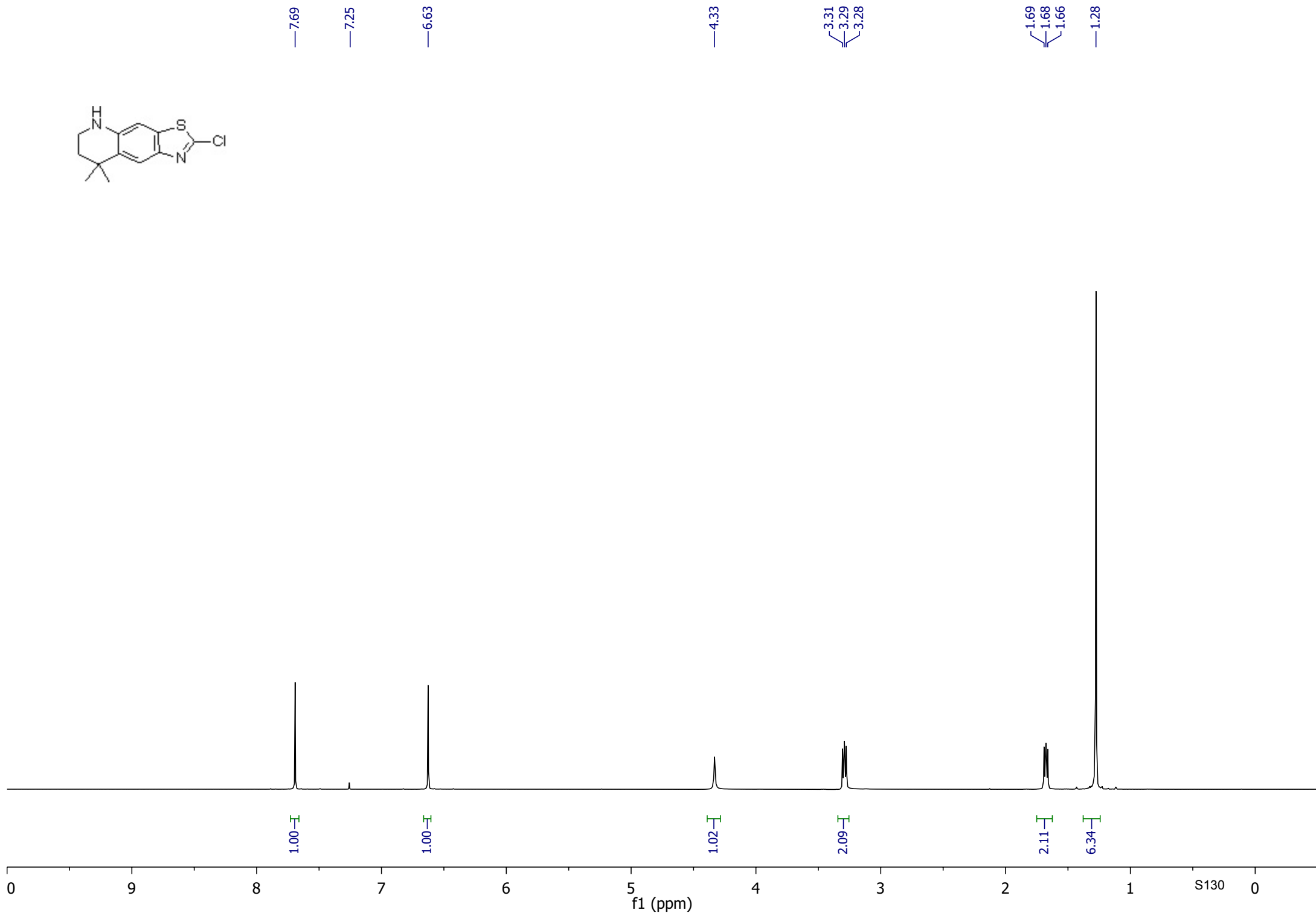
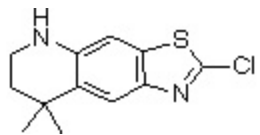


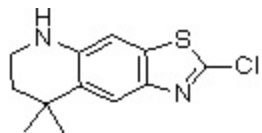
—69.19











146.89  
143.20  
143.14

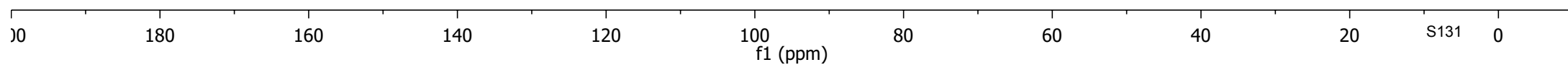
135.46  
131.28

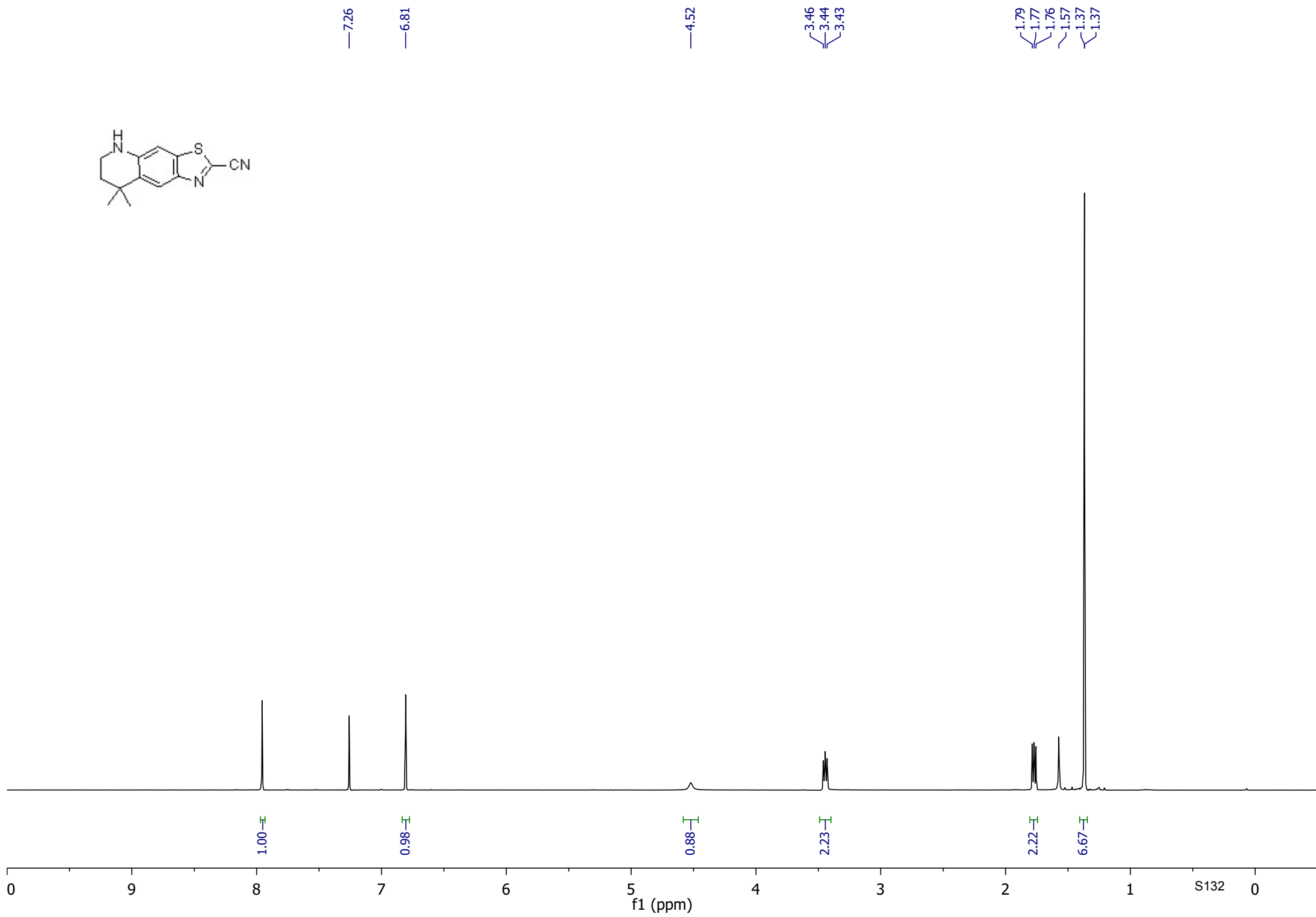
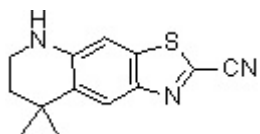
120.05

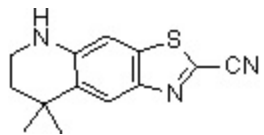
103.59

77.88 cdd13  
77.56 cdd13  
77.24 cdd13

38.53  
36.49  
32.39  
30.96







145.66  
145.03

135.99  
133.45  
129.07

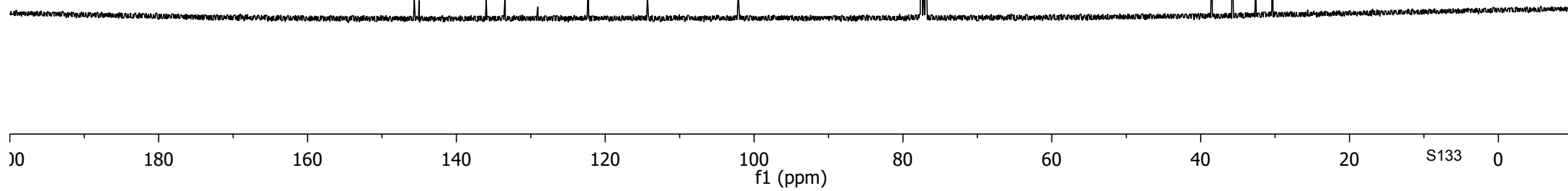
122.26

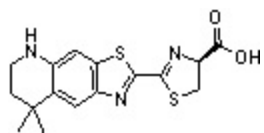
114.33

102.08

77.56 cdd13  
77.24 cdd13  
76.92 cdd13

38.52  
35.78  
32.60  
30.40





—7.91

—7.26

—6.83

5.33  
5.31  
5.28

3.75  
3.73  
3.70  
3.69  
3.67  
3.64  
3.38  
3.37  
3.35  
3.18

1.75  
1.74  
1.72

—1.33

1.00

0.85

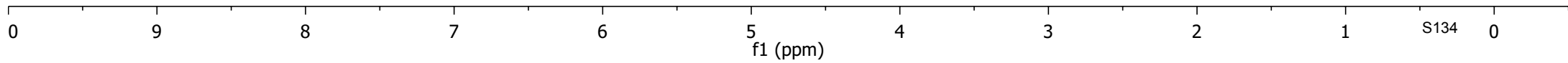
1.02

2.09

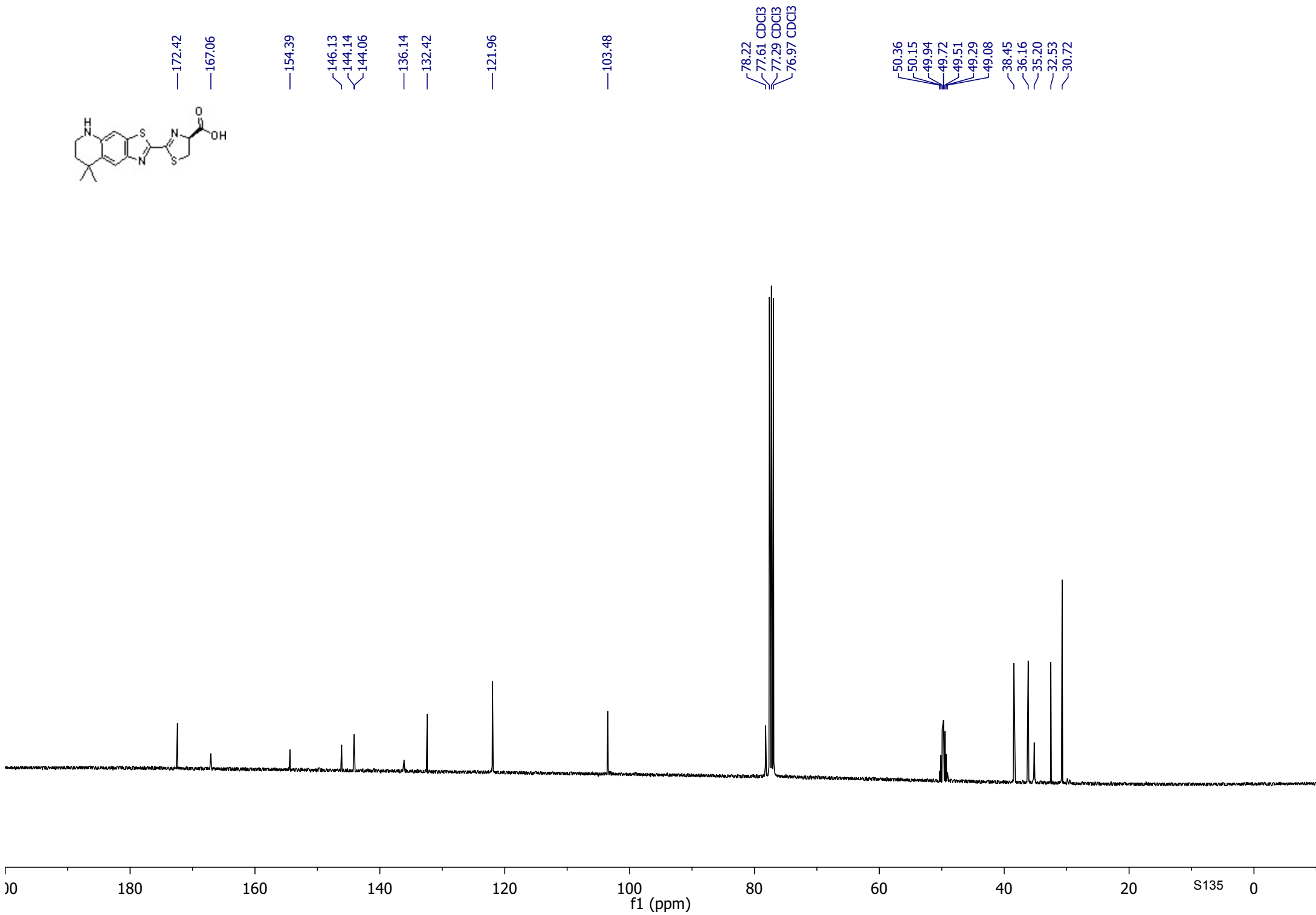
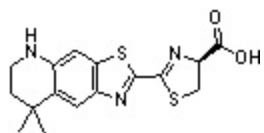
2.11

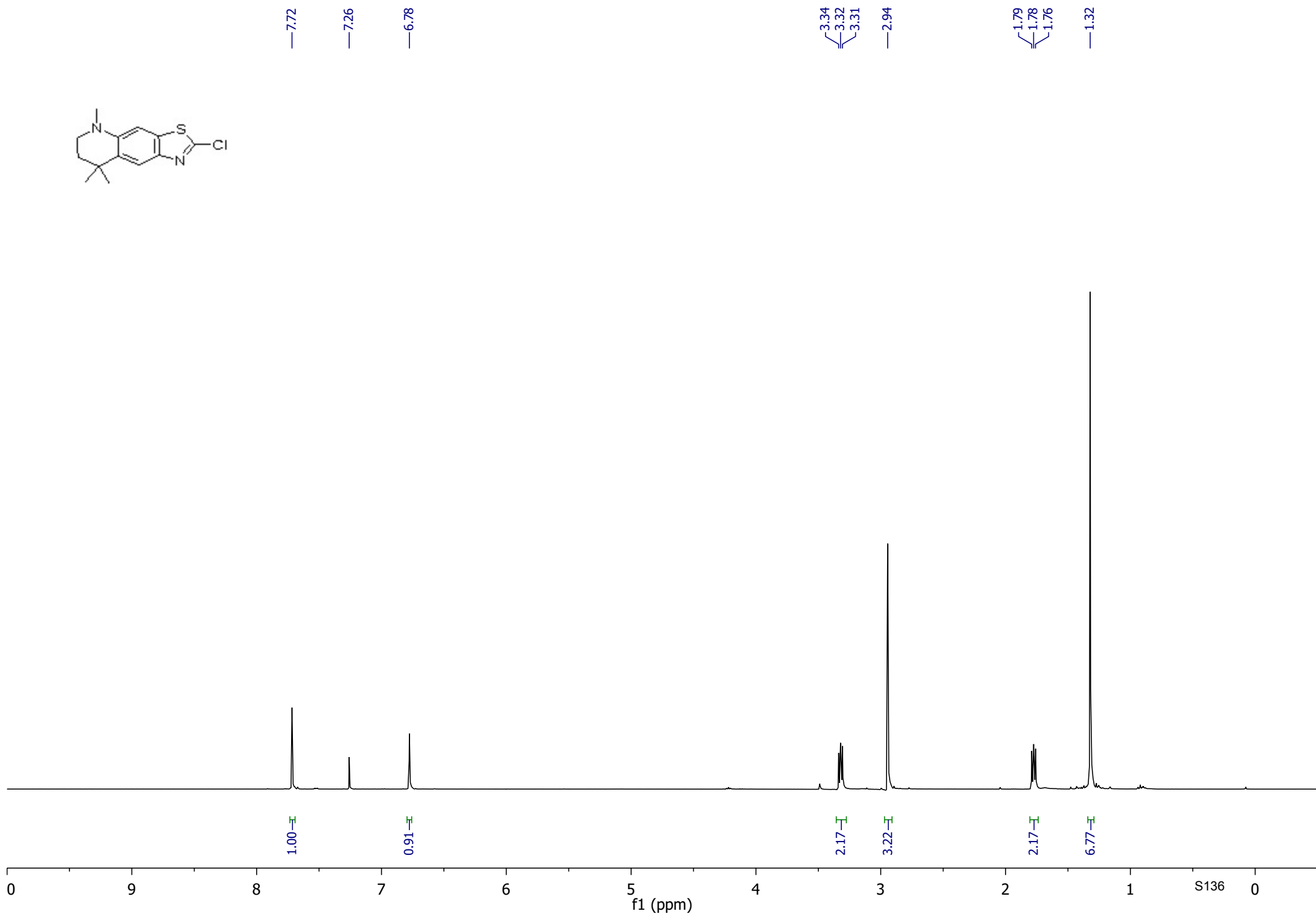
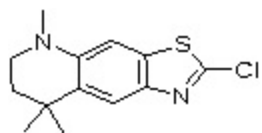
2.11

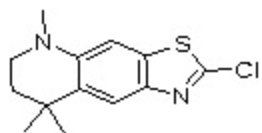
6.14



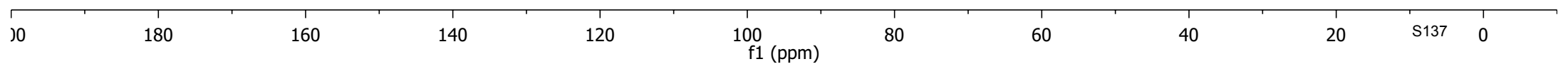
S134

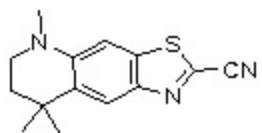






146.86  
144.48  
142.51  
135.95  
132.75  
119.46  
100.42  
77.59 cdd  
77.27 cdd  
76.95 cdd  
47.80  
39.73  
36.80  
32.75  
30.69





7.91

7.26

6.84

3.44  
3.43  
3.41

3.02

1.80  
1.78  
1.77

1.34

1.00

1.00

2.26

3.30

2.28

6.79

0

9

8

7

6

5

4

3

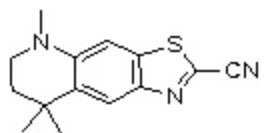
2

1

S138

0

f1 (ppm)



— 146.67  
— 144.16

— 136.75  
— 134.91

— 128.56

— 121.06

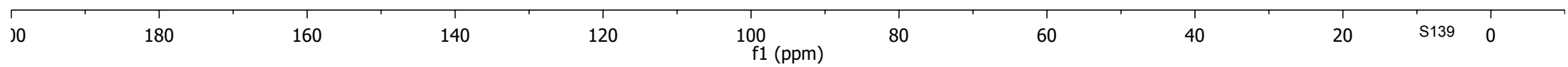
— 114.52

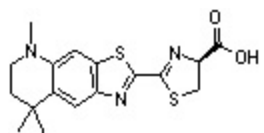
— 98.97

— 77.59 cdd13  
— 77.27 cdd13  
— 76.95 cdd13

— 47.75

— 39.64  
— 36.03  
— 32.82  
— 30.03





—7.93

—7.26

—6.88

5.43  
5.41  
5.38

3.77  
3.74

3.41  
3.39  
3.38

—3.01

1.80  
1.78  
1.77

1.34  
1.33

1.00

0.99

1.23

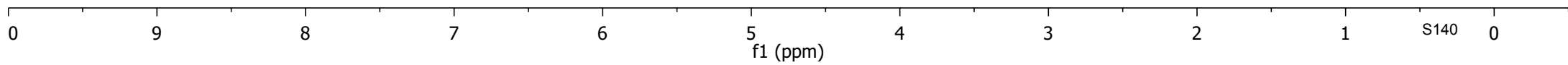
2.18

2.44

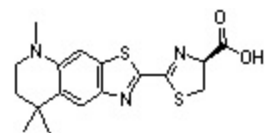
3.43

2.57

6.64



S140



173.27

168.20

153.15

145.81

145.23

137.06

133.86

121.16

100.10

77.89

77.57

77.26

76.94

47.84

39.76

36.34

34.95

32.83

30.37

30.35

