

# **SUPPORTING MATERIAL FOR**

## **Rapid and scalable assembly of firefly luciferin substrates**

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## Materials and Methods

### General synthetic methods

All reagents purchased from commercial suppliers were of analytical grade and used without further purification. Reaction progress was monitored by thin-layer chromatography (TLC) using EMD 60 F<sub>254</sub> plates and visualization with UV light, ceric ammonium molybdate (CAM), chloranil, or KMnO<sub>4</sub> stain. Compounds were purified via flash-column chromatography using Sorbent Technologies 60 Å, 230-400 mesh silica gel, unless otherwise noted. Anhydrous solvents were dried by passage over neutral alumina with the exception of DMF, which was passed over activated molecular sieves. Reaction vessels were either flame- or oven-dried prior to use. NMR spectra were acquired with Bruker Advanced spectrometers. All spectra were acquired at 298 K, unless otherwise specified. <sup>1</sup>H-NMR spectra were acquired at either 500 or 400 MHz, and <sup>13</sup>C-NMR spectra were acquired at 125 or 100 MHz. Coupling constants (*J*) are provided in Hz and chemical shifts are reported in ppm relative to either residual non-deuterated NMR solvent or a calculated DSS reference for those <sup>13</sup>C-spectra acquired in D<sub>2</sub>O. Low and high-resolution electrospray ionization (ESI) mass spectra were collected at the University of California-Irvine Mass Spectrometry Facility.

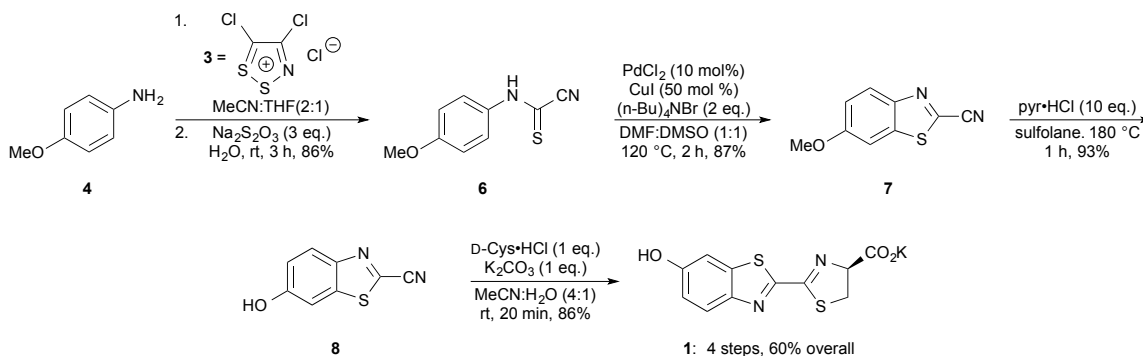
### 4,5-Dichloro-1,2,3-dithiazolium chloride (Appel's salt, **3**)<sup>1</sup>

Chloroacetonitrile (69.5 ml, 1.10 mol) was combined with S<sub>2</sub>Cl<sub>2</sub> (360 mL, 4.5 mol) and anhydrous CH<sub>2</sub>Cl<sub>2</sub> (25 mL) in a flame-dried reaction vessel equipped with a gas outlet. The mixture was thoroughly mixed then left to stand without agitation at rt under N<sub>2</sub>. Over 3 h, the yellow solution darkened and vigorously evolved gas. The reaction was allowed to stand under an inert atmosphere at rt for an additional 48 h. The precipitate was filtered and washed with anhydrous CH<sub>2</sub>Cl<sub>2</sub> (3 x 100 mL) under a N<sub>2</sub> atmosphere to provide a dark green solid (207 g, 90%). Appel's salt **3** was found to be shelf stable for >1 year when stored in desiccator at rt. This material is also commercially available from Oakwood Chemicals (CAS #: 75318-43-3).

### Synthetic procedures for D-luciferin synthesis

**Method A (Scheme 2):** This synthesis was previously reported.<sup>2</sup>

### Method B (Scheme 3):



### ***N*-(*p*-Methoxyphenyl)cyanothioformamide (6)**

*p*-Anisidine (**4**, 1.23 g, 10.0 mmol) and **3** (2.19 g, 10.5 mmol) were stirred in 90 mL of anhydrous solvent (2:1 MeCN:THF) under N<sub>2</sub> for ~1 h (until **4** was consumed). A solution of sodium thiosulfate (4.74 g, 30.0 mmol in 20 mL H<sub>2</sub>O) was then added, and the mixture was vigorously stirred for an additional 3 h. The reaction mixture was filtered to remove elemental sulfur, and the volatiles were removed *in vacuo*. The remaining aqueous mixture was again filtered to remove residual solids and the filtrate acidified with 1 M NaHSO<sub>4</sub>. Cyanothioformamide **6** precipitated from solution and was collected by vacuum filtration. The material was washed with additional H<sub>2</sub>O, then dried to provide **6** as a vivid orange solid (1.7 g, 86%). Compound **6** was characterized as a mixture of tautomers. <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>) δ 12.1 (br s, 1H), 7.92 (d, *J* = 9.1, 1.5H), 7.48 (d, *J* = 8.9, 0.4H), 7.02 (m, 2H), 3.84 (s, 3H); <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>) δ 162.4, 160.0, 132.1, 125.4, 115.2, 114.9, 56.2.

### **6-Methoxy-1,3-benzothiazole-2-carbonitrile (7)**

In Method B, compound **7** was prepared according to our previously published method.<sup>2</sup>

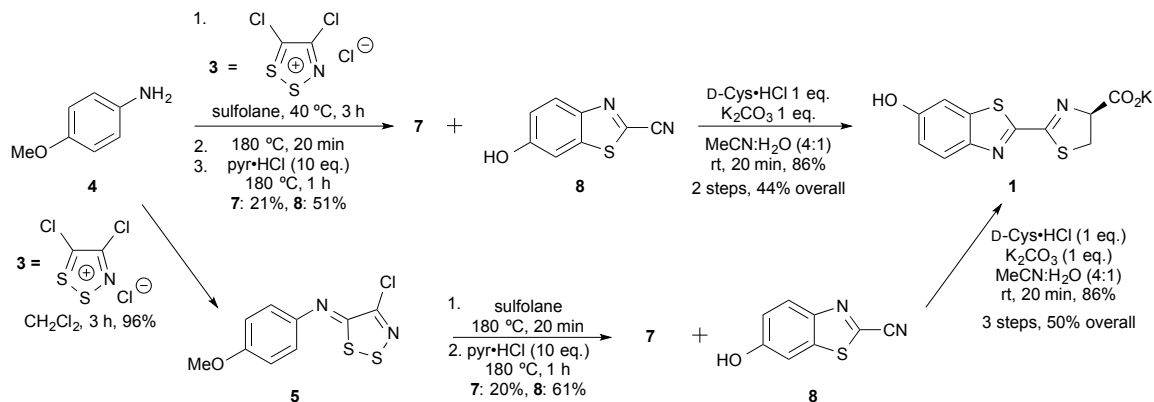
### **6-Hydroxy-1,3-benzothiazole-2-carbonitrile (8)**

Pyridine hydrochloride (6.7 g, 53 mmol) and **7** (1.0 g, 5.3 mmol) were placed in a rigorously dried sealed tube, along with dry sulfolane (2.5 mL). The reaction mixture was sealed under N<sub>2</sub> and stirred at 180 °C for 1.5 h. The mixture was allowed to cool to rt, and the remaining residue was suspended in MTBE (100 mL), then washed with H<sub>2</sub>O (2 x 20 mL) and brine (1 x 50 mL). The organics were then dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo*. The crude product was purified via column chromatography (eluting with 3:1 hexanes:ethyl acetate) to provide **8** (810 mg, 93%) as a pale yellow solid. <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>) δ 9.43 (br s, 1H) 8.05 (dd, *J* = 3.3, *J* = 9.0, 1H), 7.61 (d, *J* = 2.4, 1H), 7.27 (dd, *J* = 2.4, *J* = 9.0, 1H); <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>) δ 159.8, 147.5, 138.9, 133.9, 126.9, 119.7, 114.5, 107.5.

### **D-Luciferin (1)**

In Method B, compound **1** was synthesized as previously reported.<sup>2</sup>

### **Method C (Scheme 4):**



#### **4-Chloro-5-([4-methoxyphenyl]imino)-5H-1,2,3-dithiazole (5)**

4,5-Dichloro-1,2,3-dithiazole (**3**) (21.9 g, 105 mmol) was added to a 500-mL round-bottom flask, followed by anhydrous CH<sub>2</sub>Cl<sub>2</sub> (300 mL) and *p*-anisidine (12.3 g, 100 mmol). The green mixture was stirred at rt under N<sub>2</sub> for 3 h or until TLC (4:1 hexanes:EtOAc) showed full consumption of *p*-anisidine. The solvent was then removed *in vacuo*, and the crude material was stirred in warm hexanes (50 °C, 10 min) to solubilize residual sulfur. The suspension was quickly filtered and washed with additional warm hexanes. The precipitate (containing the hydrochloride salt) was then suspended in H<sub>2</sub>O (500 mL) and extracted with MTBE (5 x 200 mL). The combined organics were washed with H<sub>2</sub>O (200 mL) and brine (100 mL) and concentrated *in vacuo* to afford **5** (25 g, 96%) as a yellow solid. In some cases, this material was taken on directly to the next step. <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>) δ 7.31 (d, *J* = 8.9, 2H) 7.07 (d, *J* = 8.9, 2H), 3.85 (s, 3H); <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>) δ 159.8, 156.7, 149.6, 144.4, 123.2, 116.1, 56.4.

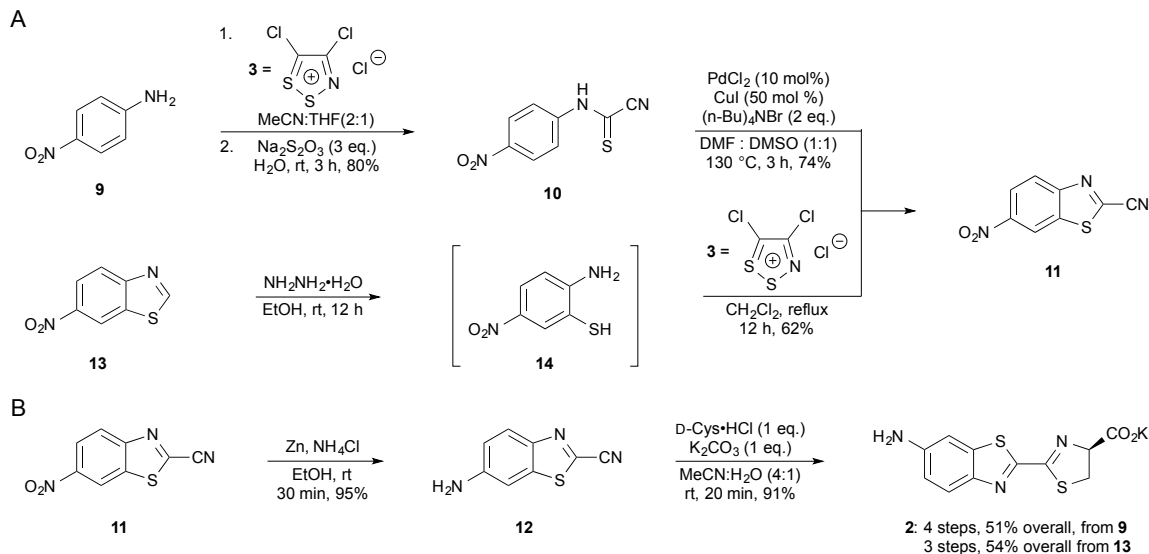
#### **6-Methoxy-1,3-benzothiazole-2-carbonitrile (7) and 6-hydroxy-1,3-benzothiazole-2-carbonitrile (8)**

*p*-Anisidine (2.46 g, 20.0 mmol) and 4,5-dichloro-1,2,3-dithiazolium chloride **3** (4.37 g, 21.0 mmol) were added to a rigorously dried sealed tube, suspended in dry sulfolane (10 mL), and placed under N<sub>2</sub>. The sealed reaction was stirred for 3 h at 40 °C, then directly transferred to a pre-heated silicon oil bath (180 °C) and stirred for an additional 20 min. Upon cooling to rt, pyridine hydrochloride (23.0 g, 0.200 mol) was added to the reaction mixture. The reaction vessel was re-sealed under N<sub>2</sub> and stirred at 180 °C for 1 h. The mixture was allowed to cool to rt, and the resulting crude residue was suspended in MTBE (400 mL), then washed with H<sub>2</sub>O (3 x 200 mL) and brine (100 mL). The organics were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated *in vacuo*. The material was then purified via column chromatography (eluting with 10:1 – 1:1 hexanes:ethyl acetate). The desired phenol **8** was isolated as a pale yellow solid (1.81 g, 51%) along with the methyl ether precursor **7** (796 mg, 21%). In some cases, the imino adduct **5** was isolated and purified prior to thermolysis and deprotection at 180 °C. In these cases, a mixture of **7** and **8** (20% and 61%, respectively) was obtained. Additional **8** was obtained by re-subjecting isolated **7** to pyr•HCl deprotection (outlined in method A above). The NMR spectra of **8** were in agreement with the values tabulated in method B above. Compound **7**: <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>) δ 8.08 (d, *J* = 9.1, 1H), 7.76 (d, *J* = 2.5, 1H), 7.30 (dd, *J* = 2.5, *J* = 9.1, 1H), 3.95 (s, 3H); <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>) δ 162.0, 148.2, 139.1, 134.8, 126.8, 120.0, 114.6, 105.1, 57.0.

#### **D-Luciferin (1)**

In Method C, compound **1** was synthesized as previously reported.<sup>2</sup>

## Synthetic procedures for 6'-aminoluciferin



### Method A (from **9**, Scheme 5):

#### (4-Nitrophenyl)cyanothioformamide (**10**)

4,5-Dichloro-1,2,3-dithiazole (**3**, 0.302 g, 1.45 mmol) and 4-nitroaniline (**9**, 0.200 g, 1.45 mmol) were added to a round-bottom flask and placed under N<sub>2</sub>. THF (6 mL) and MeCN (6 mL) were then added and the resulting solution was stirred for 5 min at rt. Pyridine (0.23 mL, 2.9 mmol) was added dropwise to the flask. The mixture was stirred for 60 min (when TLC with 7:3 hexanes:ethyl acetate indicated full consumption of **9**). A solution of sodium thiosulfate (0.686 g in 3 mL H<sub>2</sub>O) was then added, and the mixture was stirred for 50 min at rt. The reaction was then diluted with 1 M sodium bisulfate (30 mL) and extracted with EtOAc (3 x 30 mL). The organic layer was washed with 1 M sodium bisulfate (3 x 25 mL) and brine (3 x 25 mL), dried over MgSO<sub>4</sub>, filtered, and concentrated *in vacuo*. The crude material was purified via column chromatography (eluting with 9:1 to 7:3 hexanes:ethyl acetate) to provide **10** as a red-orange solid (0.24 g, 80%). <sup>1</sup>H NMR (400 MHz, acetone-*d*<sub>6</sub>, mixture of tautomers) δ 8.42 (app d, *J* = 8.3, 0.26H), 8.38 (app d, *J* = 9.2, 1.74H), 8.30 (app d, *J* = 9.2, 1.74H), 7.91 (app d, *J* = 8.4, 0.26H). <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>, mixture of tautomers) δ 167.0, 164.6, 146.6, 143.8, 126.0, 125.5, 124.1, 123.8, 114.3, 113.3; HRMS (ESI<sup>-</sup>) calcd for C<sub>8</sub>H<sub>4</sub>N<sub>3</sub>O<sub>2</sub>S [M - H]<sup>-</sup> 206.0024, found 206.0017.

#### 6-Nitro-1,3-benzothiazole-2-carbonitrile (**11**)

Palladium chloride (0.112 g, 0.634 mmol), CuI (0.458 g, 3.17 mmol), TBAB (4.08 g, 12.7 mmol) and **10** (1.30 g, 6.34 mmol) were placed in a flame-dried flask, flushed with N<sub>2</sub> and suspended in 190 mL of anhydrous DMF:DMSO (1:1). The resulting mixture was stirred at 130 °C under N<sub>2</sub> for 4 h. The reaction was then diluted with EtOAc (150 mL) and water (100 mL). The organic layer was isolated and the aqueous layer was extracted with additional ethyl acetate (4 x 100 mL). The organic layers were combined and washed with water (5 x 100 mL) and brine (2 x 100 mL), then dried over MgSO<sub>4</sub>, filtered

and concentrated *in vacuo*. The crude material was purified via column chromatography (eluting with 7:3 hexanes:ethyl acetate) to provide **11** as a fluffy, light yellow solid (0.96 g, 74%). The NMR spectra were consistent with previous reports.<sup>6</sup> <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.97 (d, *J* = 2.2, 1H), 8.53 (dd, *J* = 2.2, *J* = 9.1, 1H), 8.39 (d, *J* = 9.1, 1H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 155.4, 147.4, 141.9, 135.7, 126.2, 123.2, 118.7, 112.1.

#### **6-Amino-1,3-benzothiazole-2-carbonitrile (12)**

Ammonium chloride (6.01 g, 113 mmol) was added to a solution of **11** (2.33 g, 11.3 mmol) in reagent grade MeOH (400 mL). The mixture was stirred at rt for 5 min. Zinc powder (14.8 g, 227 mmol) was then added, and the reaction was stirred vigorously at rt for 30 min. The heterogeneous mixture was filtered through Celite using copious amounts of MeOH. The filtrate was collected and concentrated *in vacuo*. The isolated crude material was purified by column chromatography (eluting with 2:1 hexanes:ethyl acetate) to provide amine **12** (1.9 g, 94%) as a pale yellow solid. The NMR spectra were in agreement with previously published values.<sup>3</sup> <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>) δ 7.87 (d, *J* = 8.9, 1H), 7.26 (d, *J* = 2.1, 1H), 7.09 (dd, *J* = 2.1, *J* = 8.9, 1H), 5.64 (br s, 2H); <sup>13</sup>C NMR (125 MHz, acetone-*d*<sub>6</sub>) δ 151.4, 145.3, 139.5, 129.8, 126.3, 118.5, 114.8, 103.7.

#### **6'-Aminoluciferin<sup>4</sup> (2)**

D-Cysteine hydrochloride monohydrate (81.2 mg, 0.462 mmol) and **12** (71.0 mg, 0.405 mmol) were suspended in MeCN (5 mL) in a 20 mL vial. A solution of K<sub>2</sub>CO<sub>3</sub> (56.5 mg, 0.409 mmol) in 400 μL of H<sub>2</sub>O was added to the mixture, and the resulting solution stirred vigorously at rt under N<sub>2</sub>. A bright yellow precipitate formed as the reaction proceeded. After 20 min of stirring this precipitate was vacuum filtered and dried. The crude material was triturated with wetted MeCN and vacuum filtered once more to provide the analytically pure potassium salt **2** in excellent yield (117 mg, 91%) as a bright yellow powder. <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O) δ 7.53 (d, *J* = 8.8, 1H), 7.07 (s, 1H), 6.88 (d, *J* = 8.5, 1H), 5.09 (app t, *J* = 8.9, 1H), 3.72 (app t, *J* = 10.5, 1H) 3.54 (dd, *J* = 8.3, *J* = 10.5, 1H); <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O) δ 180.4, 168.2, 159.1, 149.4, 147.9, 139.9, 126.4, 120.2, 108.9, 82.7, 38.9.

#### **Method B (from 13, Scheme 5):**

#### **6-Nitro-1,3-benzothiazole-2-carbonitrile (11)**

Benzothiazole **13** (901 mg, 5.00 mmol) and hydrazine monohydrate (2.5 ml, 50 mmol) were suspended in reagent grade ethanol (50 mL), and the mixture was stirred at rt for 12 h. The deep red solution was then cooled in an ice bath. HCl (5 M solution) was added until a brilliant yellow precipitate formed. The precipitate was filtered and washed with cold H<sub>2</sub>O, affording **14** upon drying (under vacuum). Aminothiols **14** was then suspended in dry CH<sub>2</sub>Cl<sub>2</sub> (10 mL) and dithiazolium chloride **3** (619 mg, 3.00 mmol) was added. The resulting solution was stirred at reflux for 12 h. The crude mixture was adsorbed to silica gel, concentrated and purified by column chromatography (eluting with 20:1 to 5:1 hexanes:ethyl acetate) to provide nitro benzothiazole **11** (380 mg, 62%) as a pale orange solid. The NMR spectra were consistent with previous reports.<sup>6</sup> <sup>1</sup>H NMR (500 MHz, acetone-*d*<sub>6</sub>) δ 9.35 (d, *J* = 2.1, 1H), 8.57 (dd, *J* = 2.1, *J* = 9.1, 1H), 8.48 (d, *J* = 9.1, 1H);

$^{13}\text{C}$  NMR (125 MHz, acetone- $d_6$ )  $\delta$  156.8, 148.7, 144.3, 137.6, 127.0, 124.3, 121.2, 113.9.

### 6-Amino-1,3-benzothiazole-2-carbonitrile (**12**)

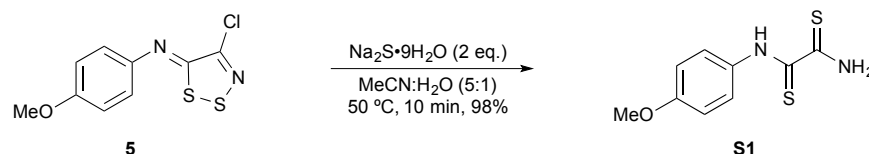
In Method B, compound **12** was prepared according to the procedure described above in Method A.

### 6'-Aminoluciferin (**2**)

In Method B, compound **2** was prepared according to the procedure described above in Method A.

### *Synthesis of dithiooxamide byproduct:*

**Scheme S1:** Mild and efficient synthesis of monoaryl-dithiooxamide **S1**



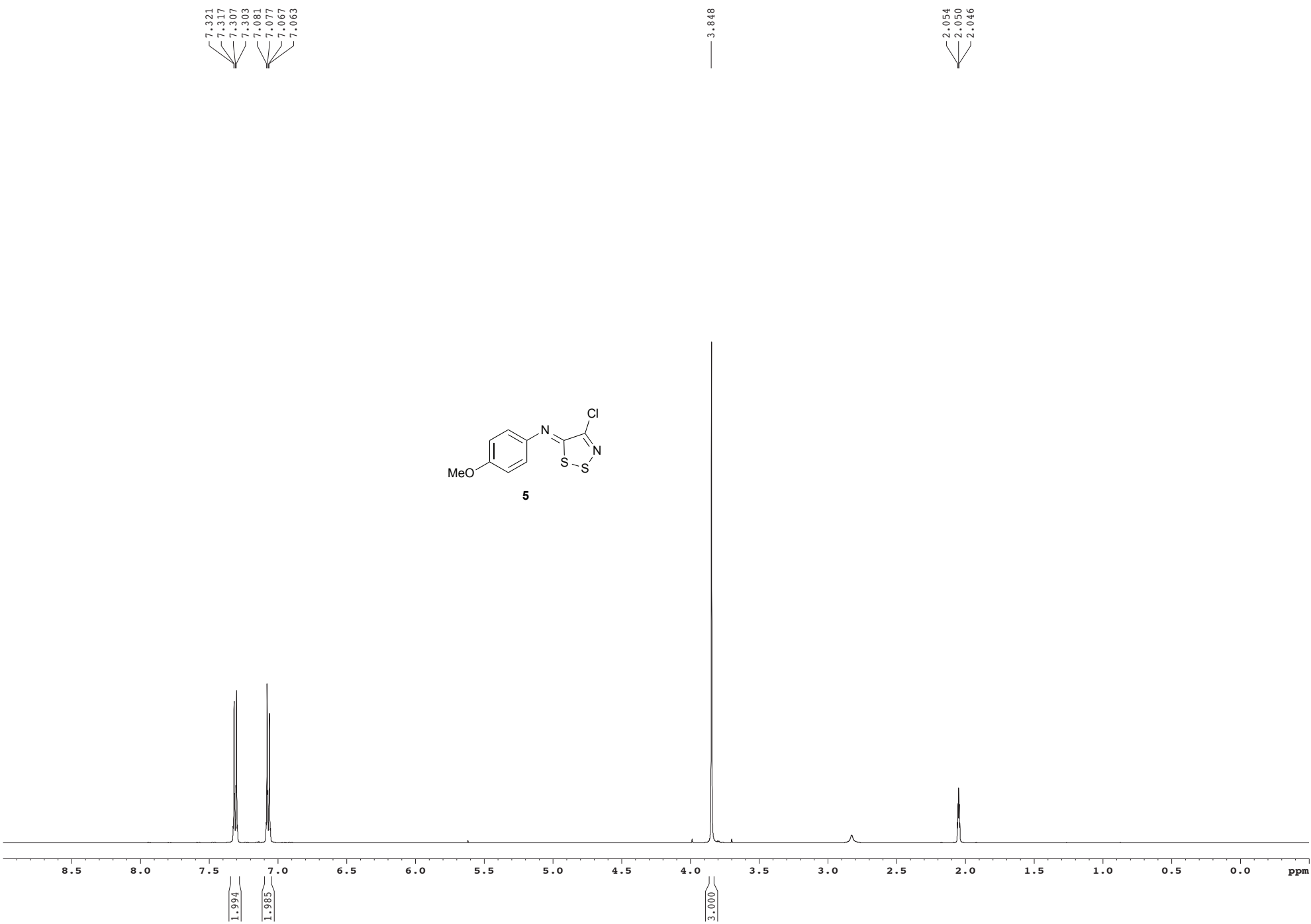
### N'-(4-Methoxyphenyl)ethanedithioamide (**S1**)

Imino adduct **5** (262 mg, 1.01 mmol) was suspended in MeCN (10 mL) and a solution of  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  (482 mg in 2 mL  $\text{H}_2\text{O}$ ) was added. The reaction was then vigorously stirred at 50 °C for 10 min. Upon cooling to rt, the reaction mixture was filtered through filter paper to remove elemental sulfur and the MeCN evaporated *in vacuo*. Upon removal of the organics, a fine red solid precipitated. This material was isolated via vacuum filtration and washed with excess  $\text{H}_2\text{O}$ . The material was further dried under vacuum to provide **S1** as a bright red solid (224 mg, 98%). The NMR spectra were in agreement with previously published values.<sup>5</sup>  $^1\text{H}$  NMR (500 MHz, acetone- $d_6$ )  $\delta$  12.1 (br s, 1H), 9.89 (br s, 2H), 8.04 (d,  $J = 9.1$ , 2H), 7.05 (d,  $J = 9.1$ , 2H), 3.87 (s, 3H);  $^{13}\text{C}$  NMR (125 MHz, acetone- $d_6$ )  $\delta$  191.8, 181.9, 159.9, 133.1, 125.1, 115.3, 56.4.

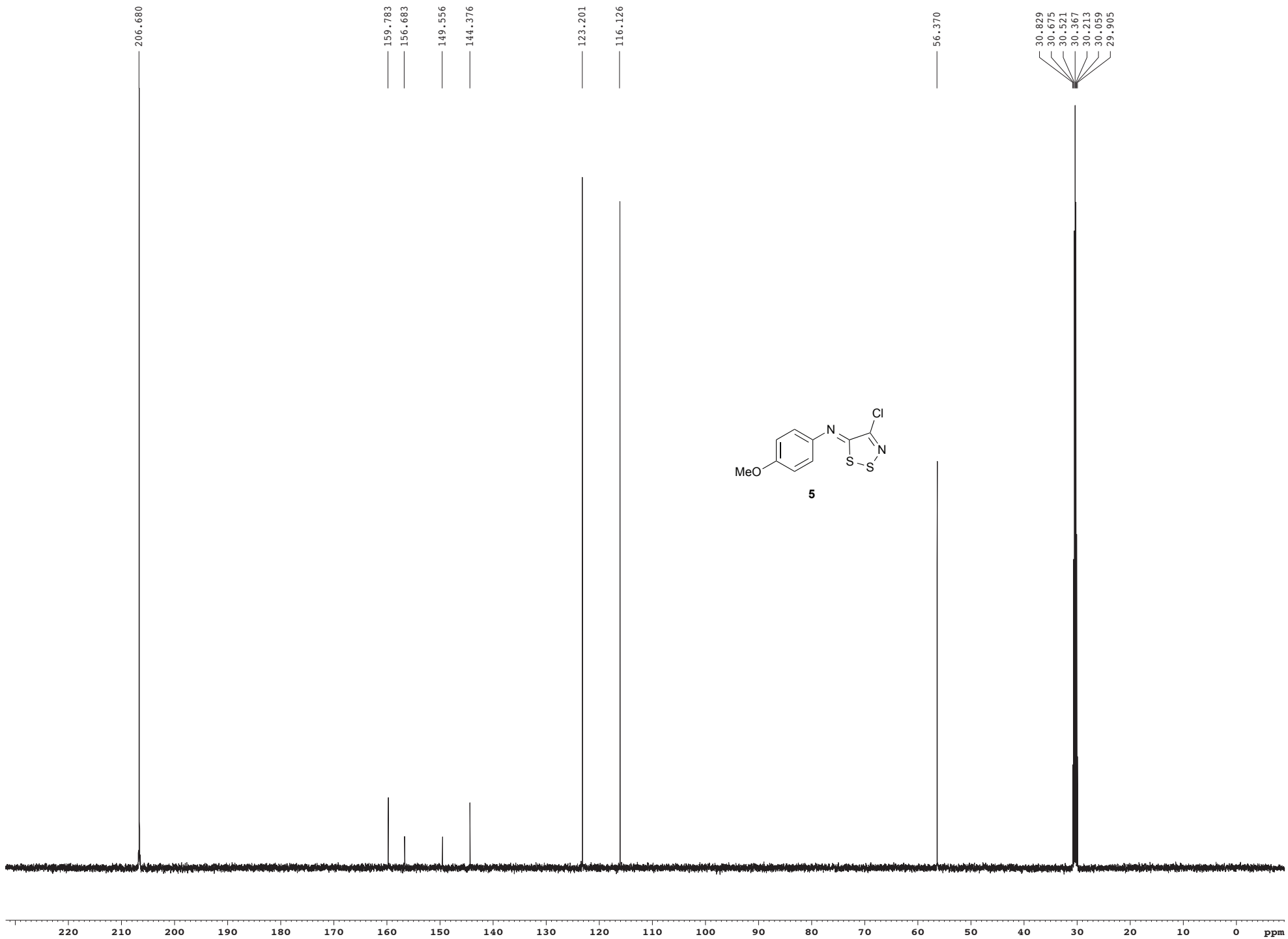
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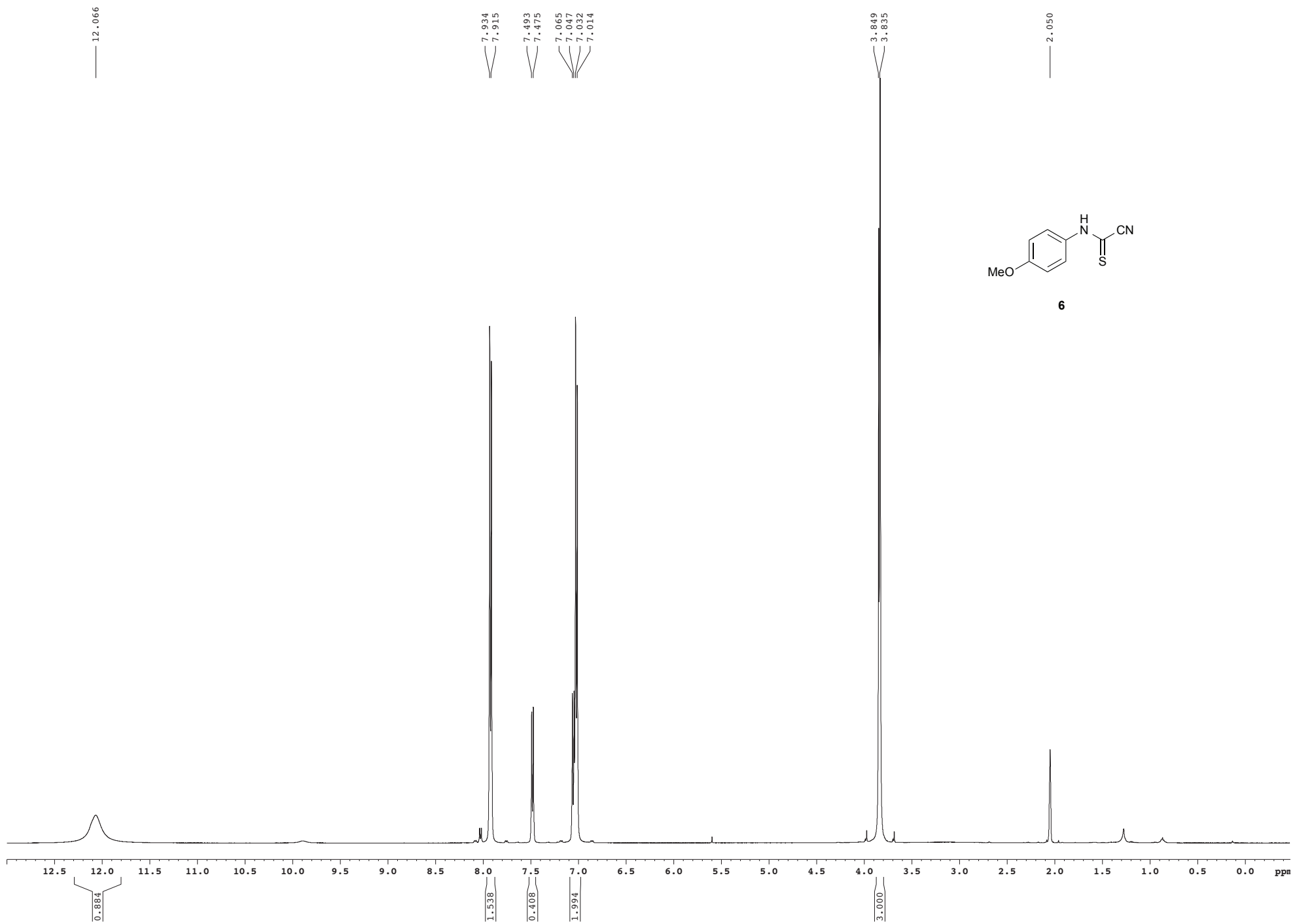
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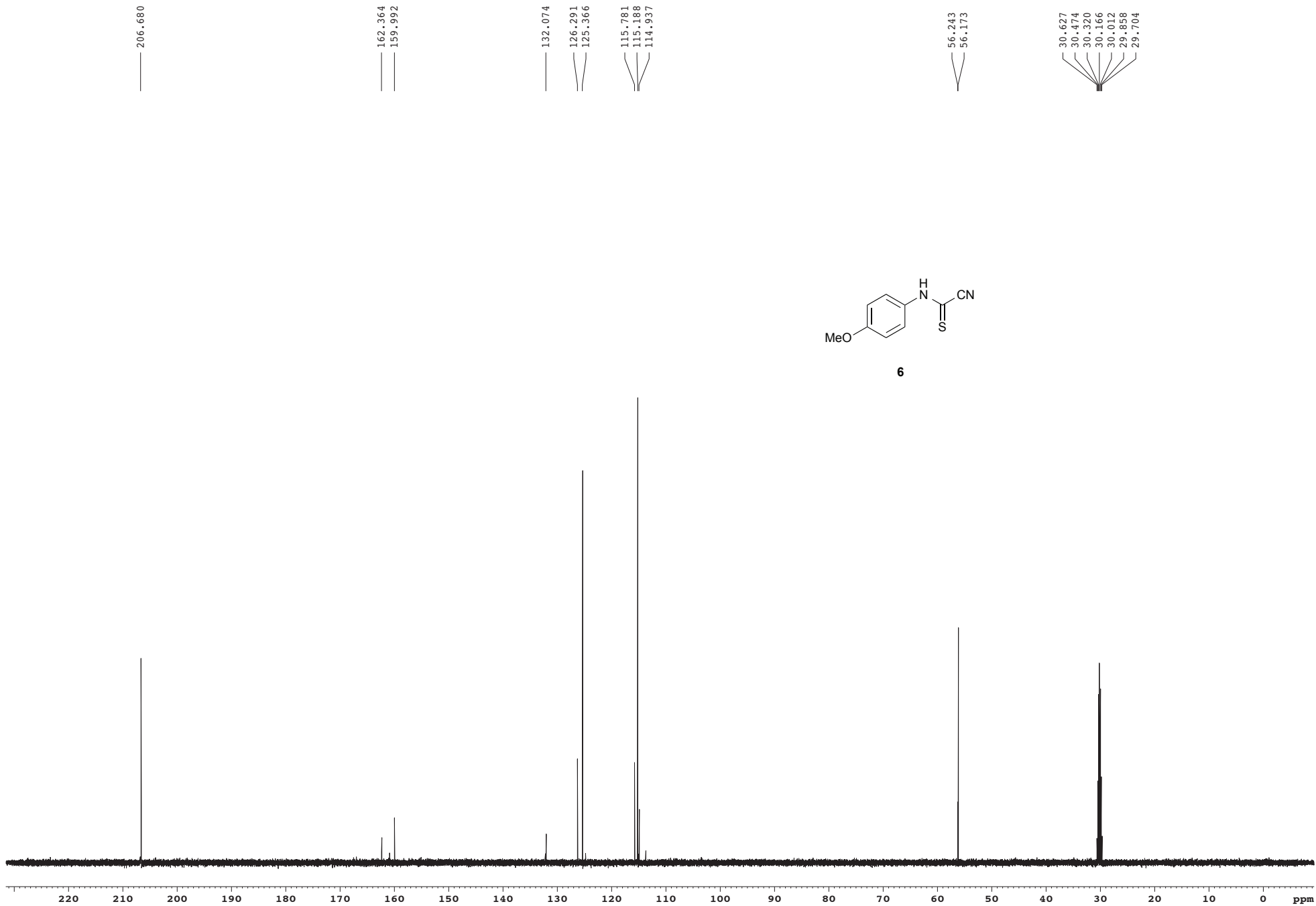
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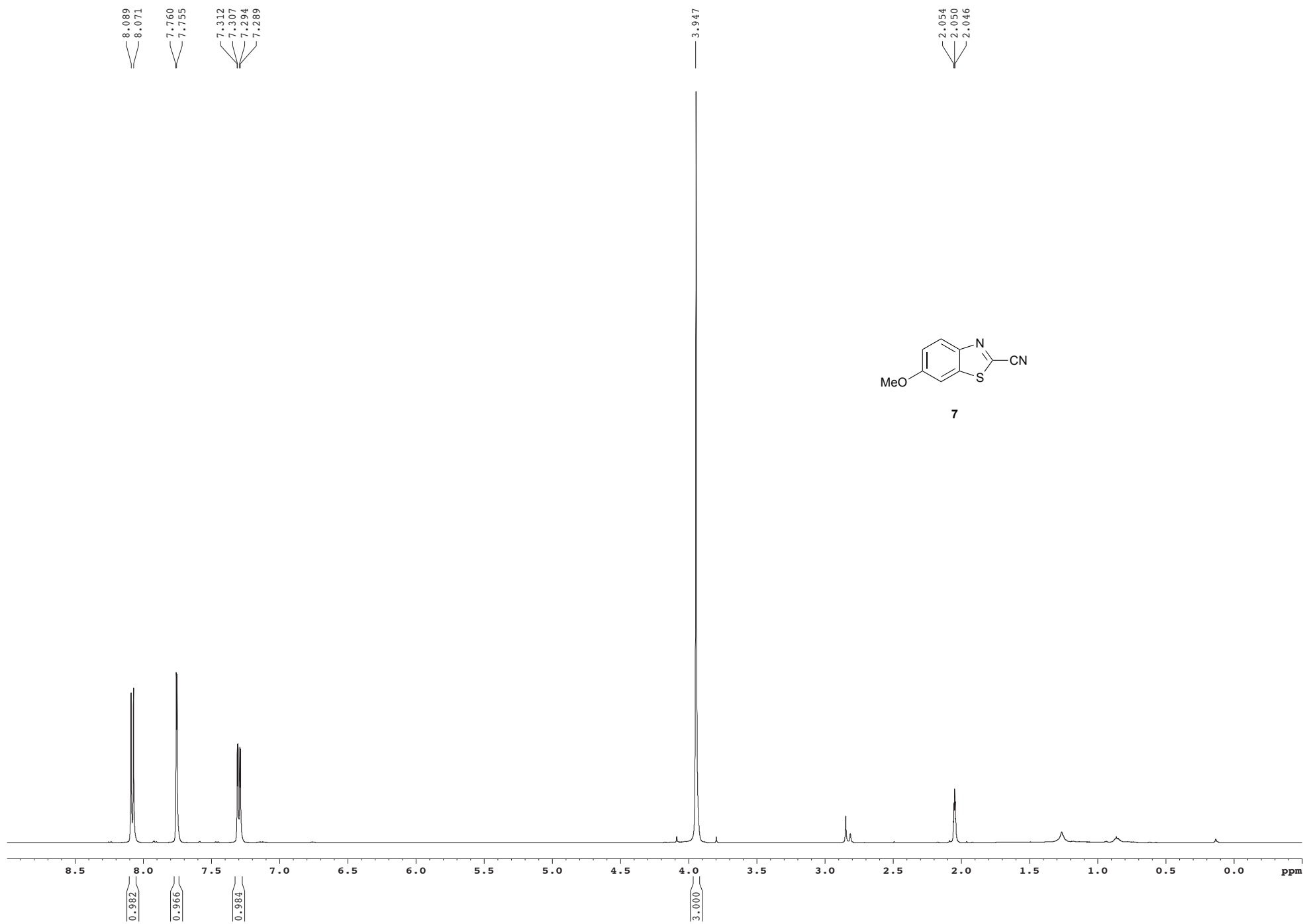


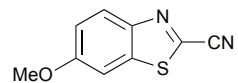
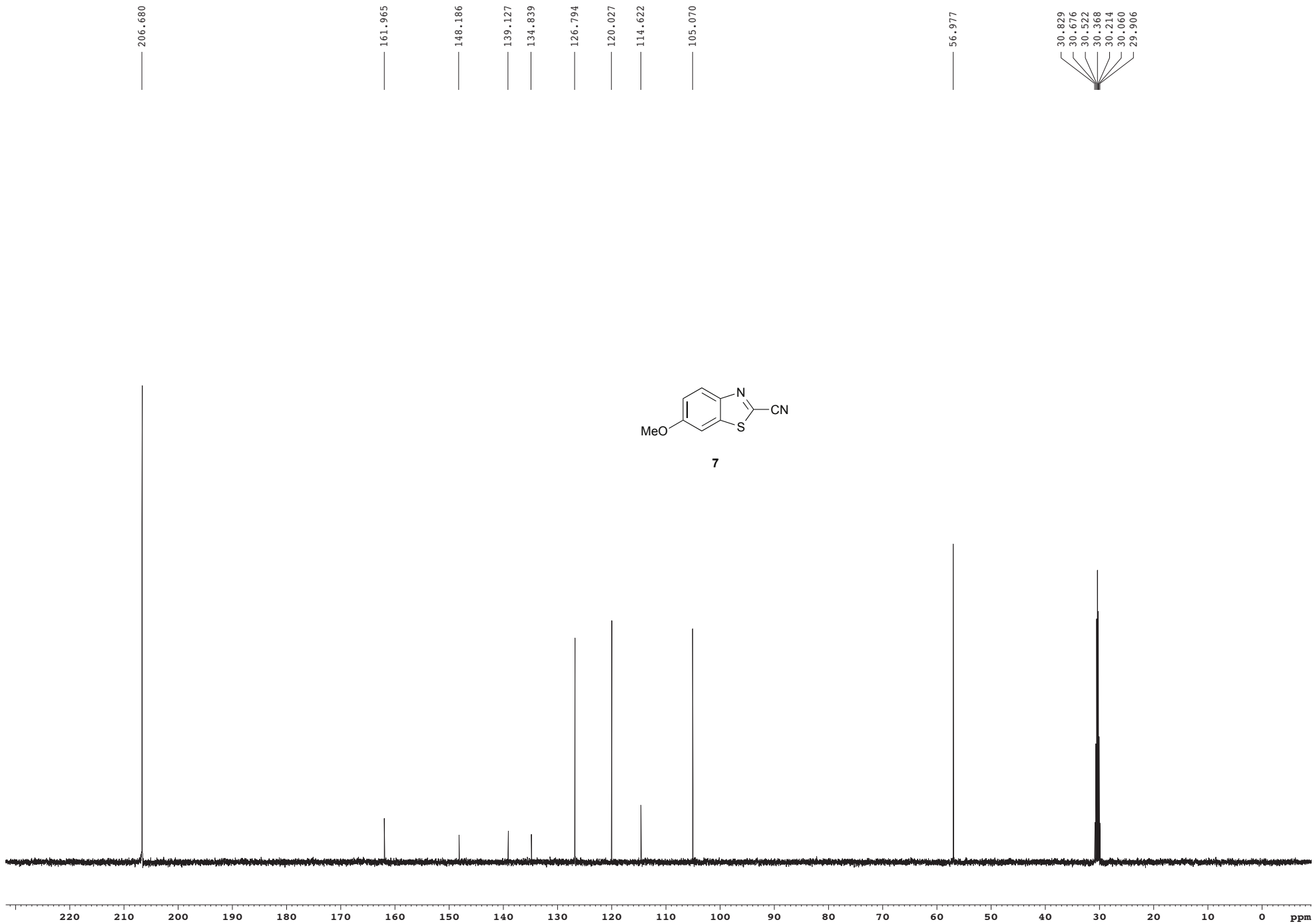




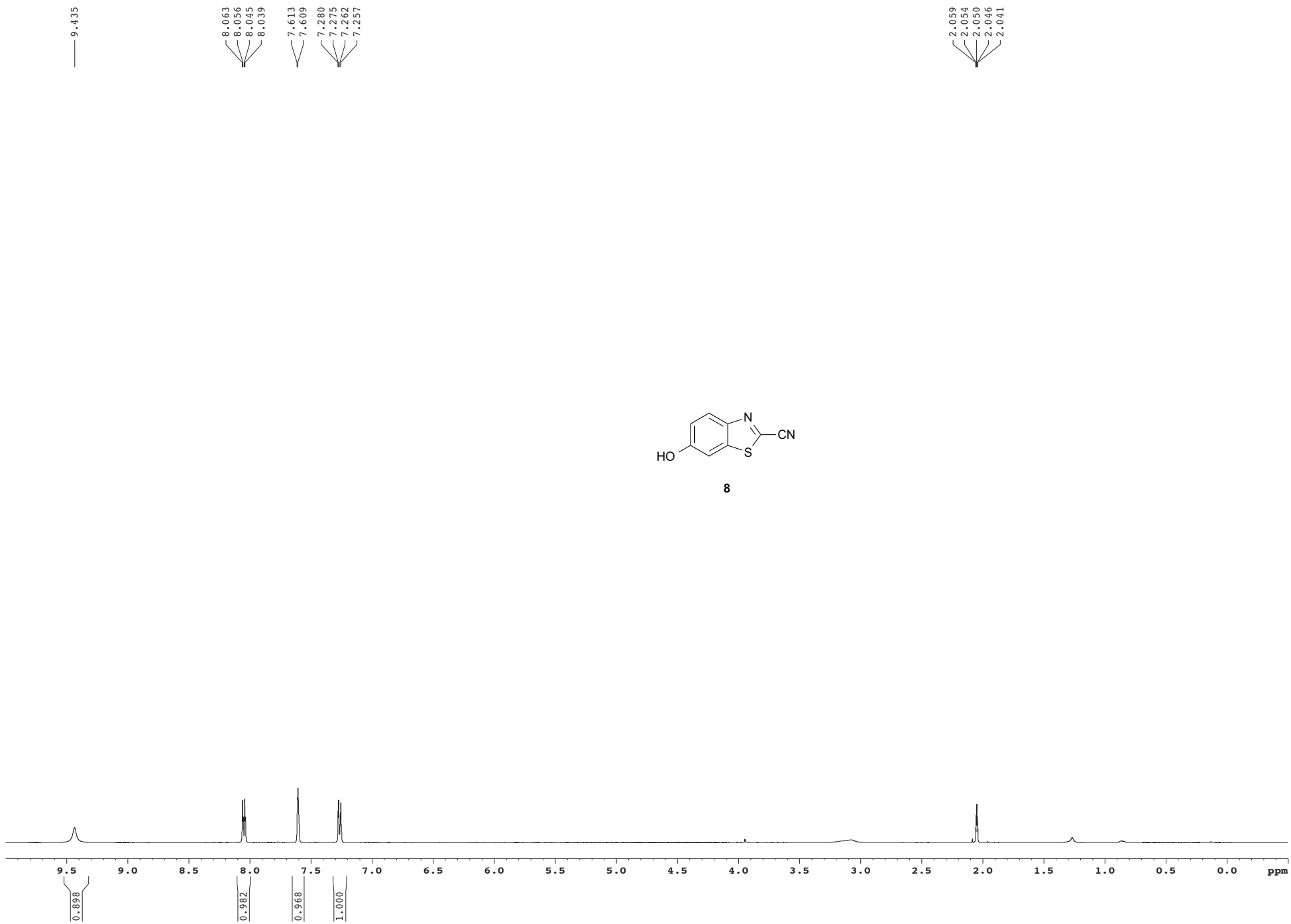


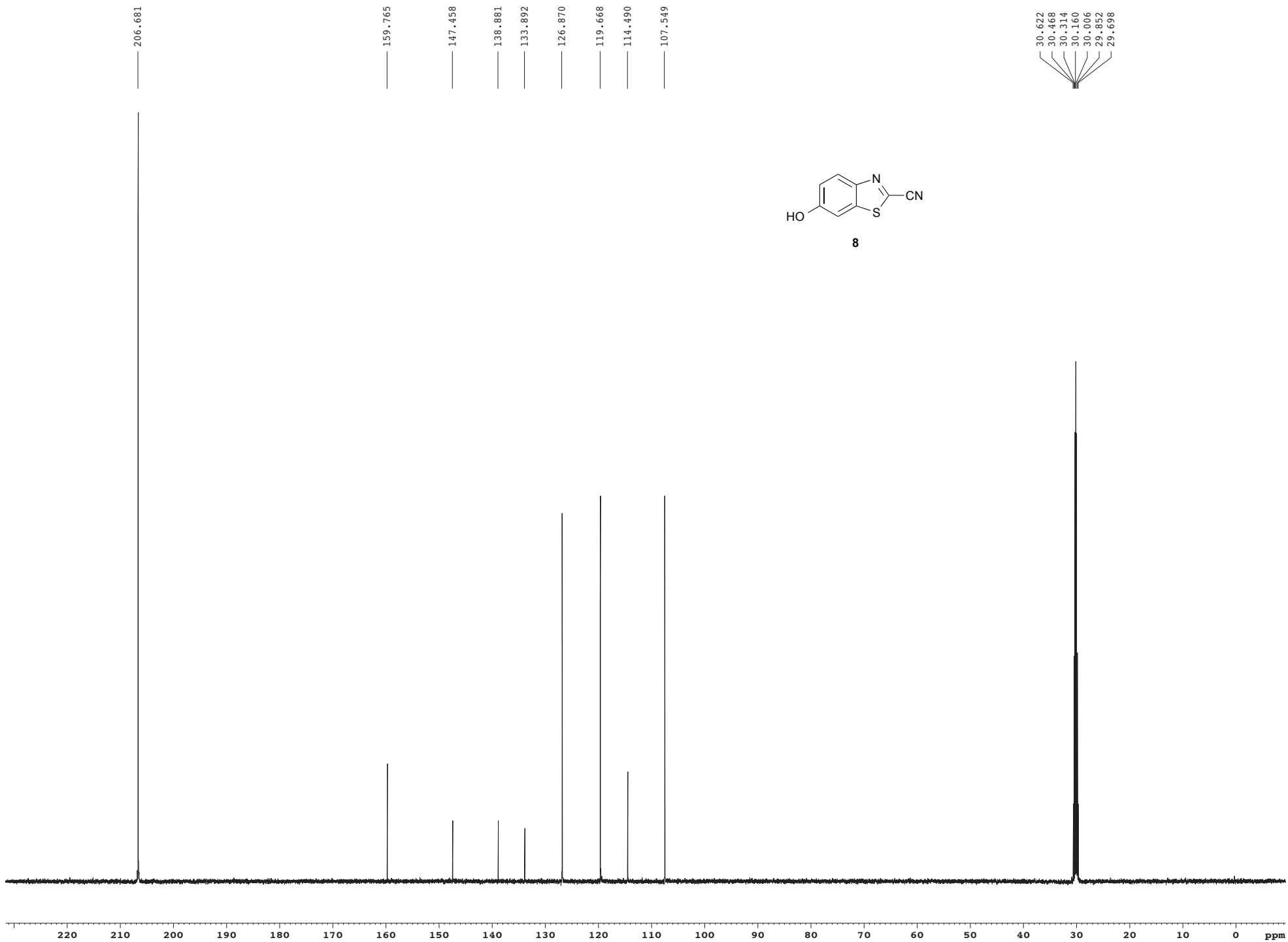


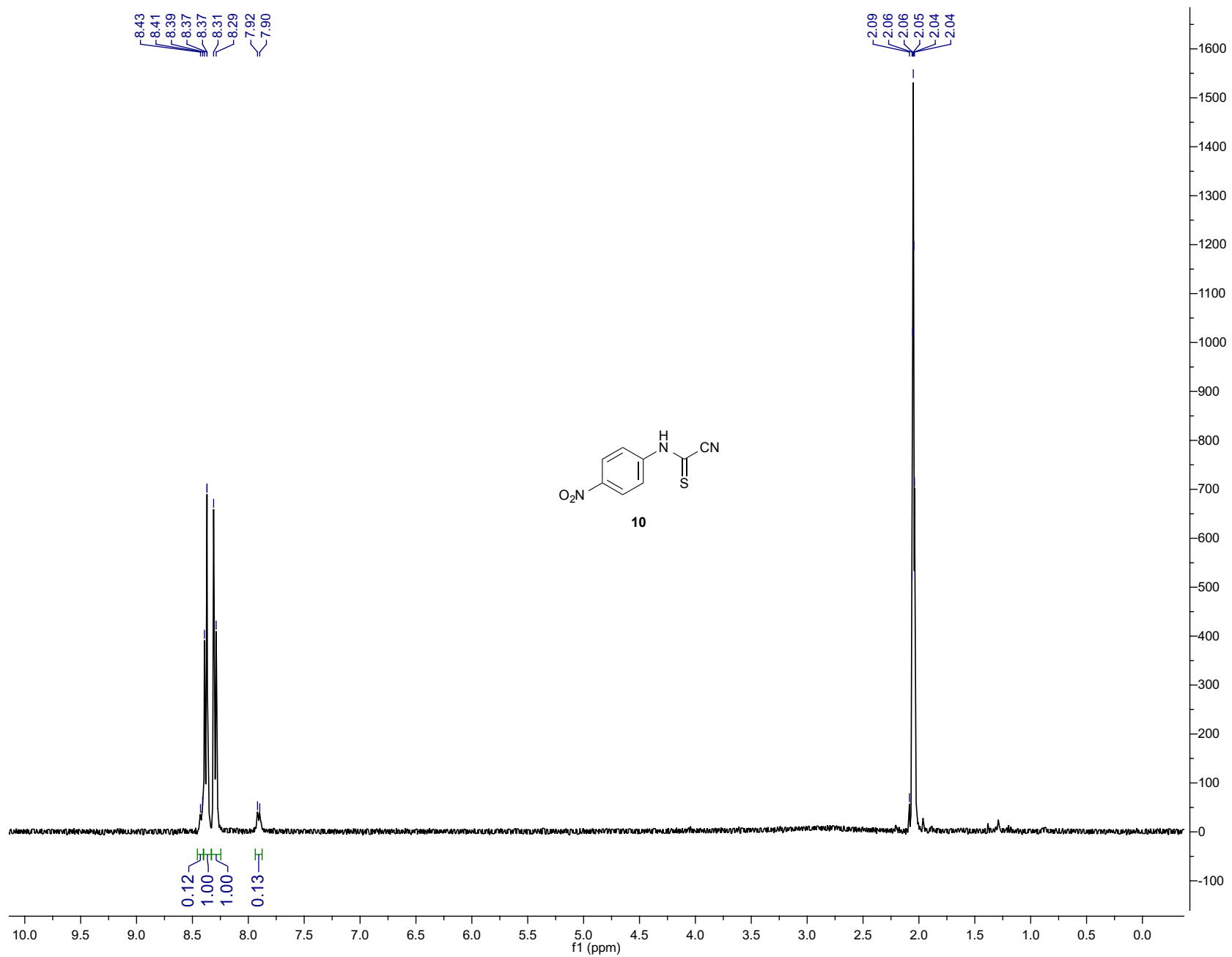




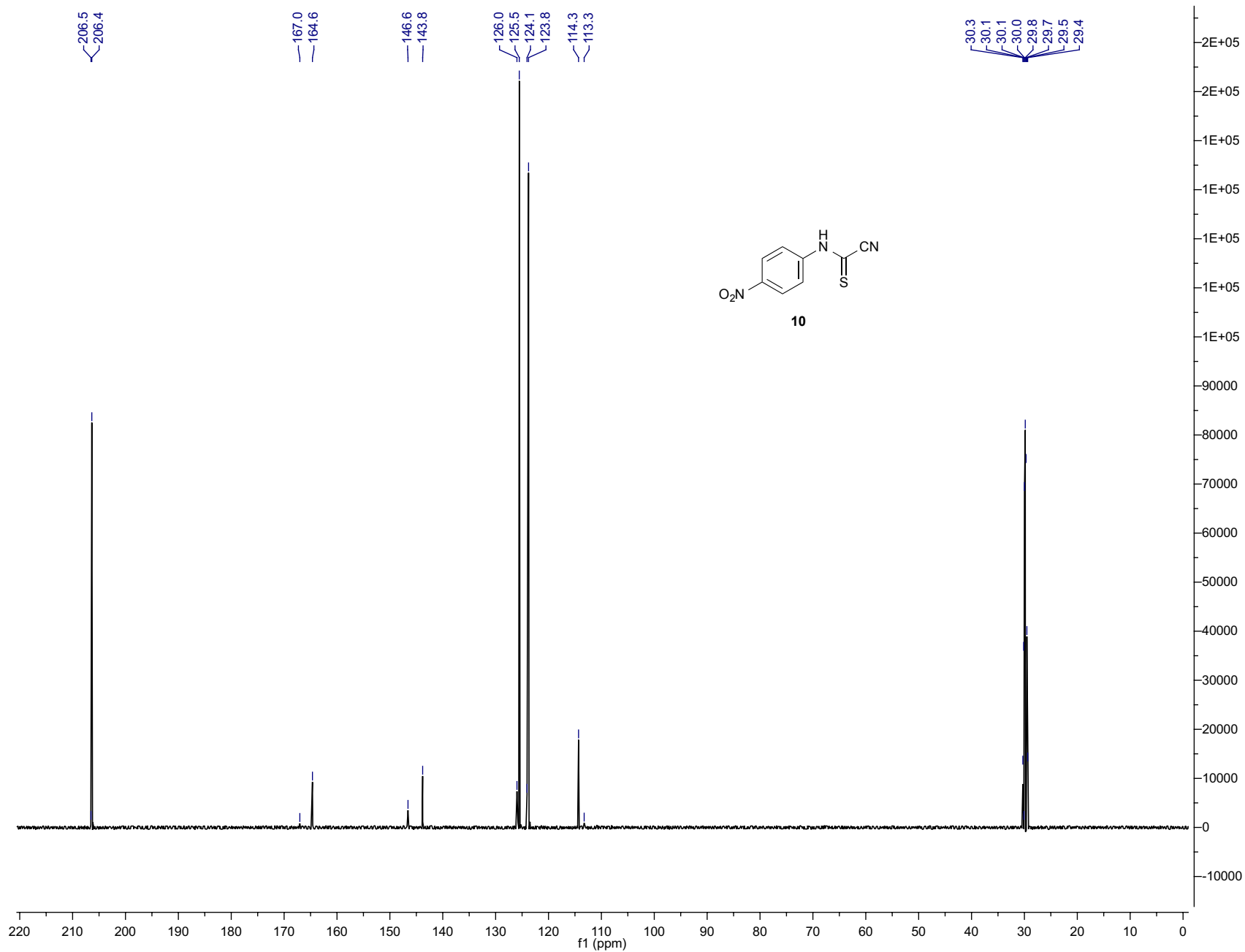
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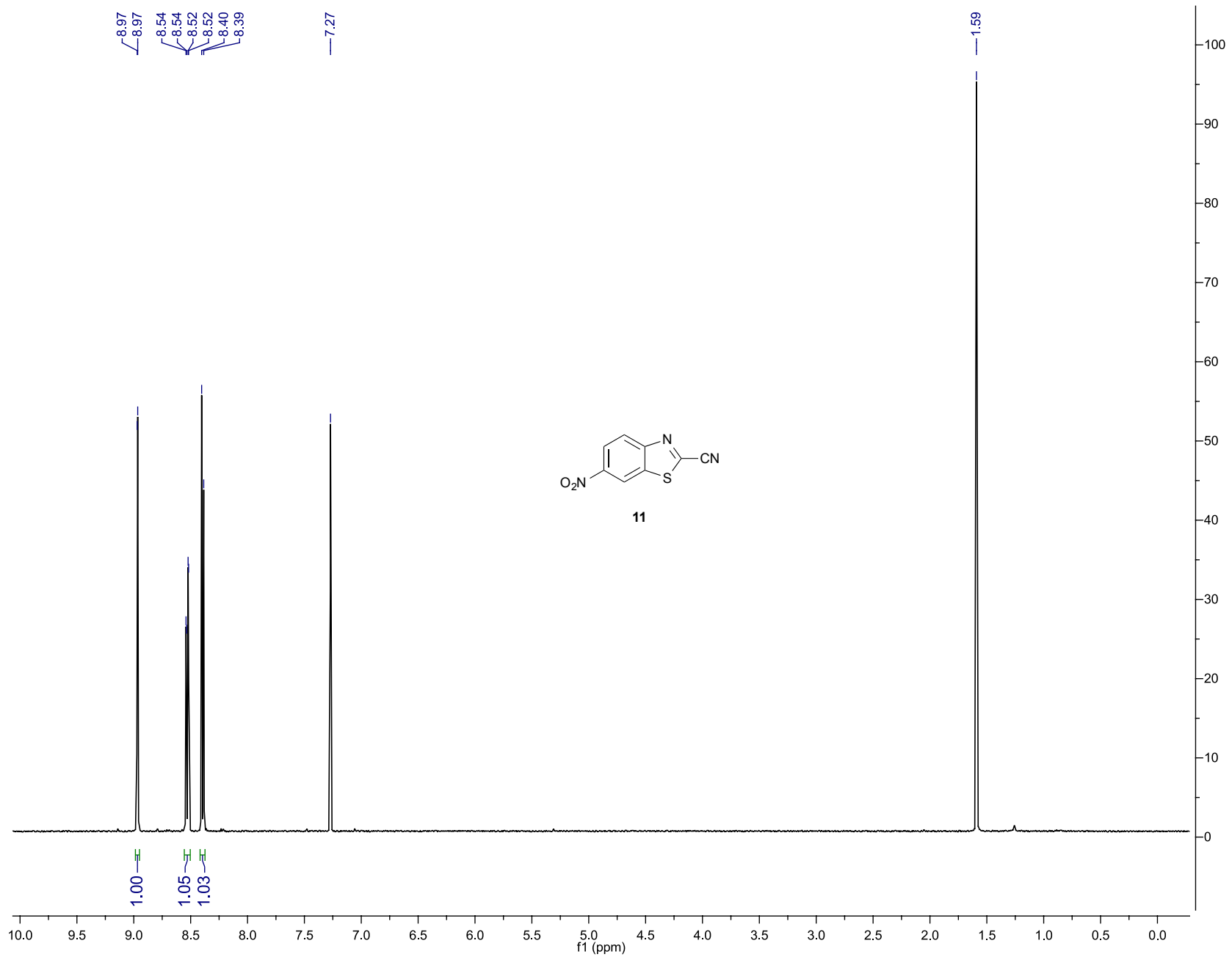


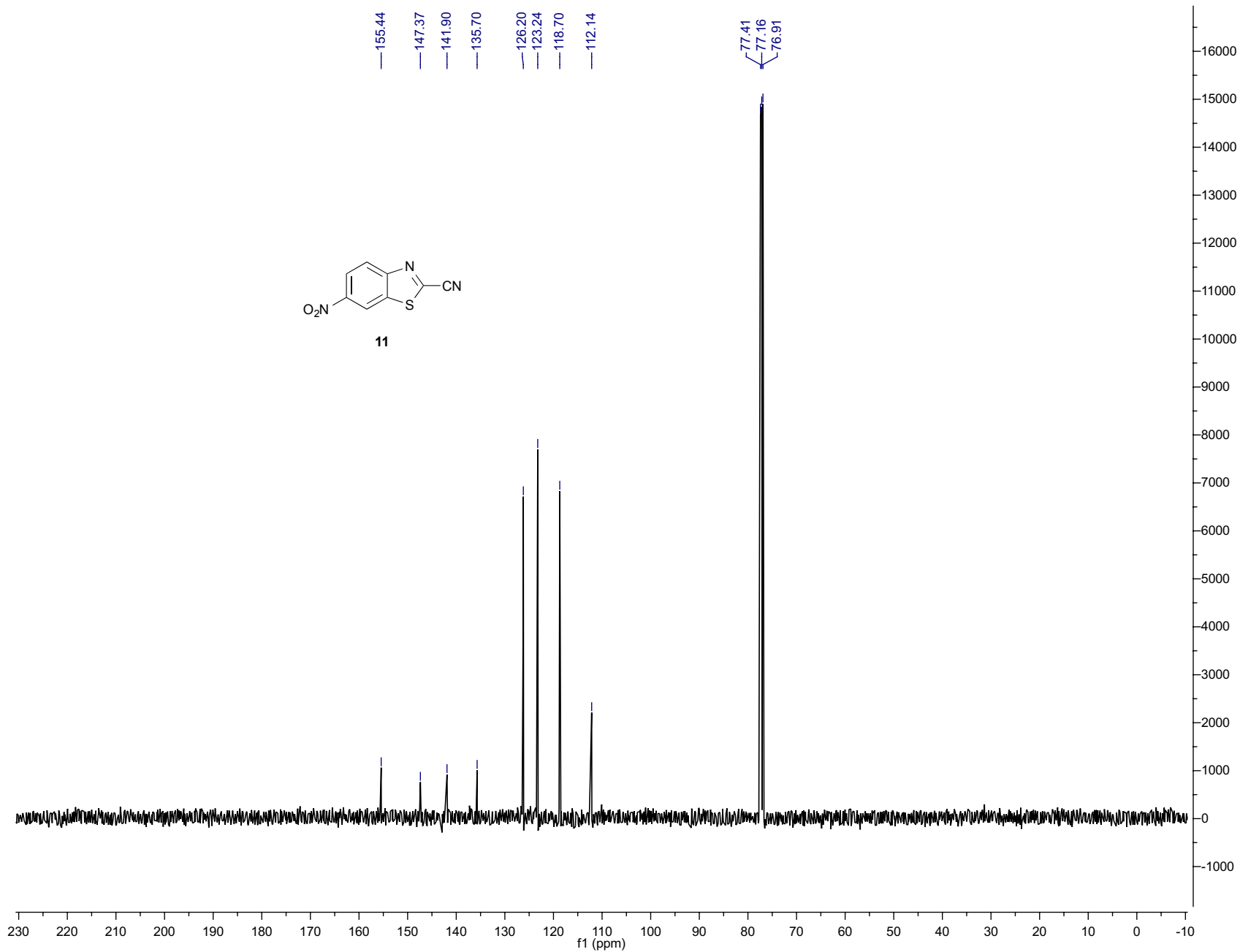
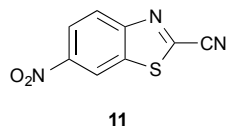


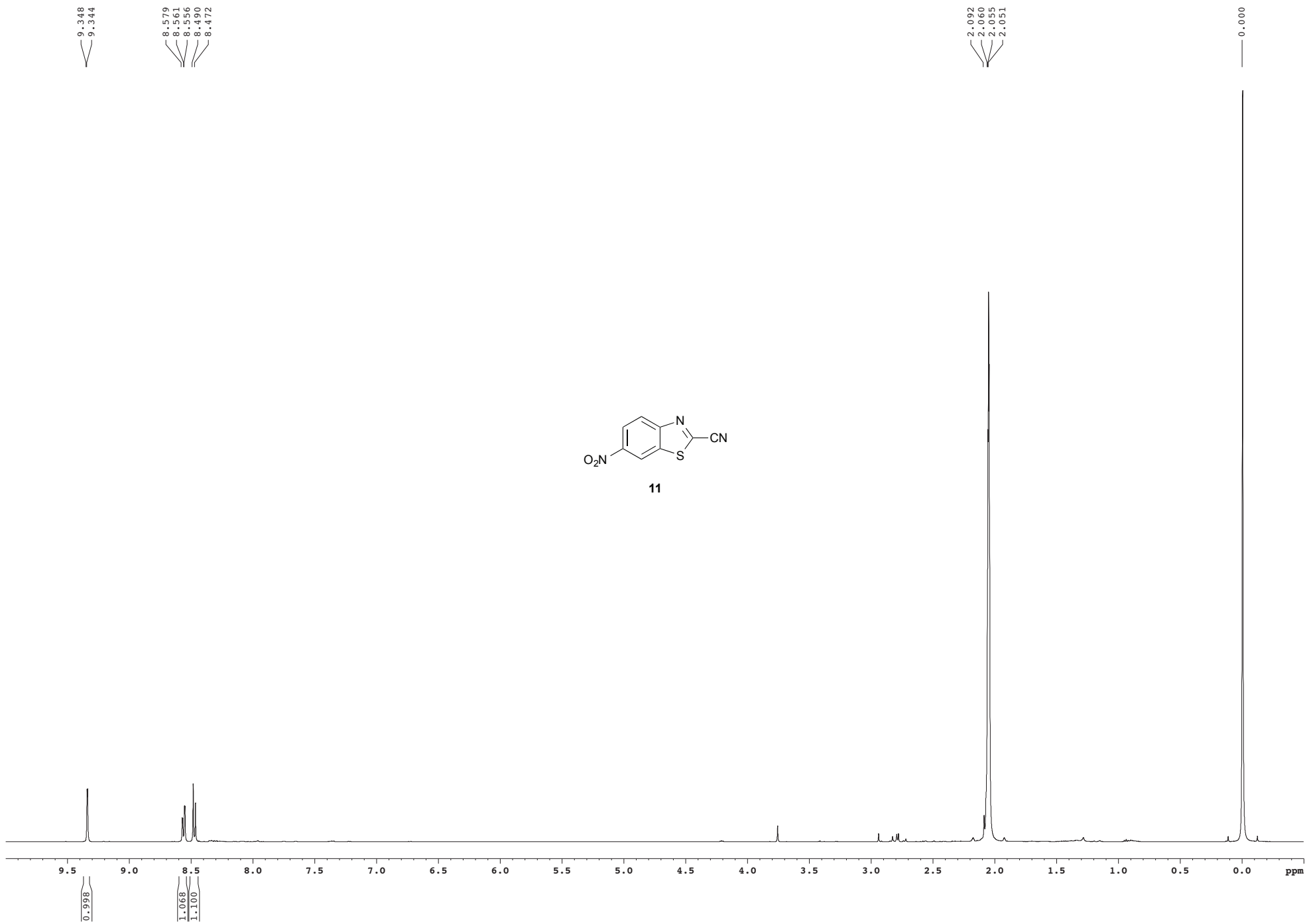


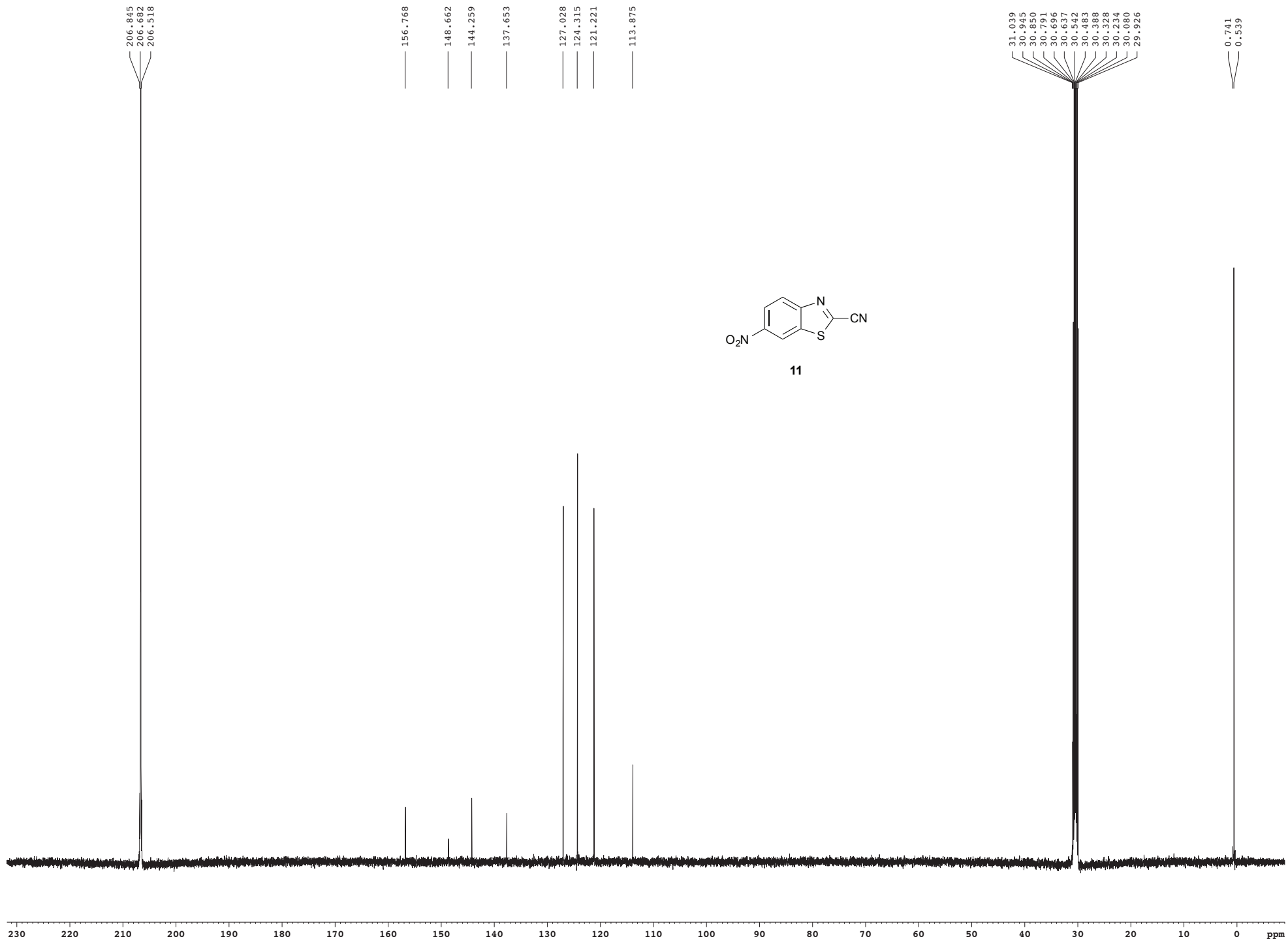


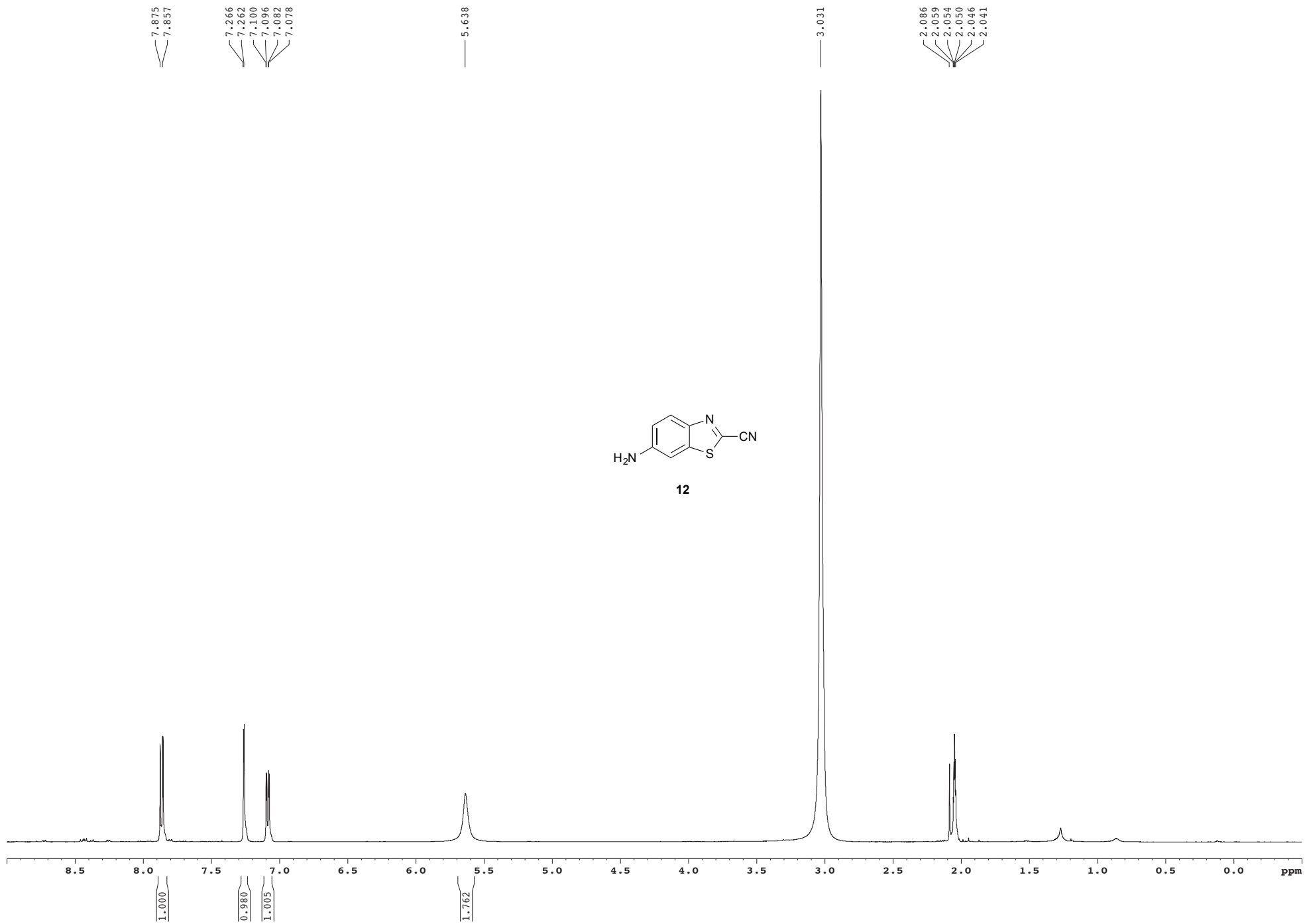


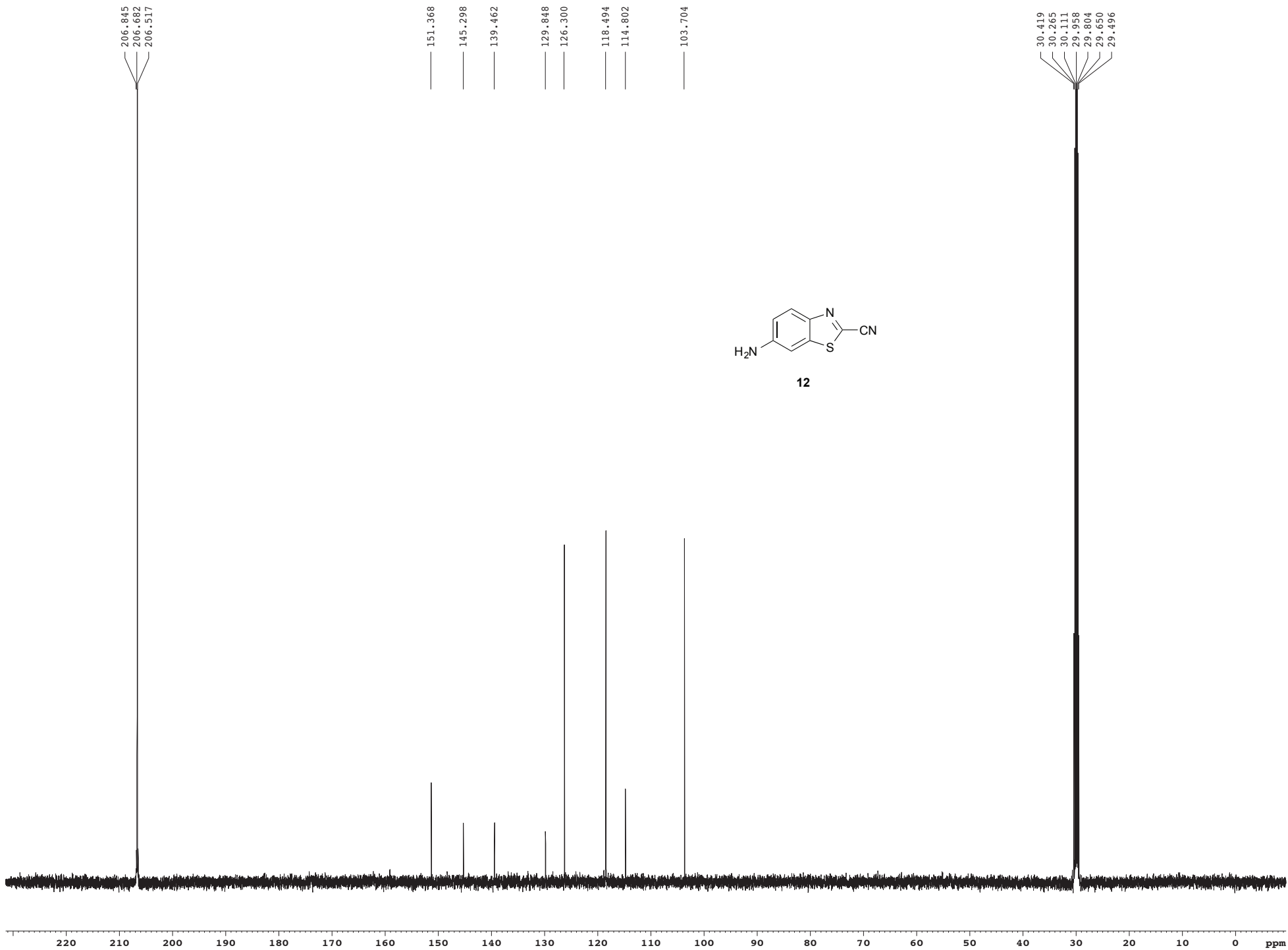


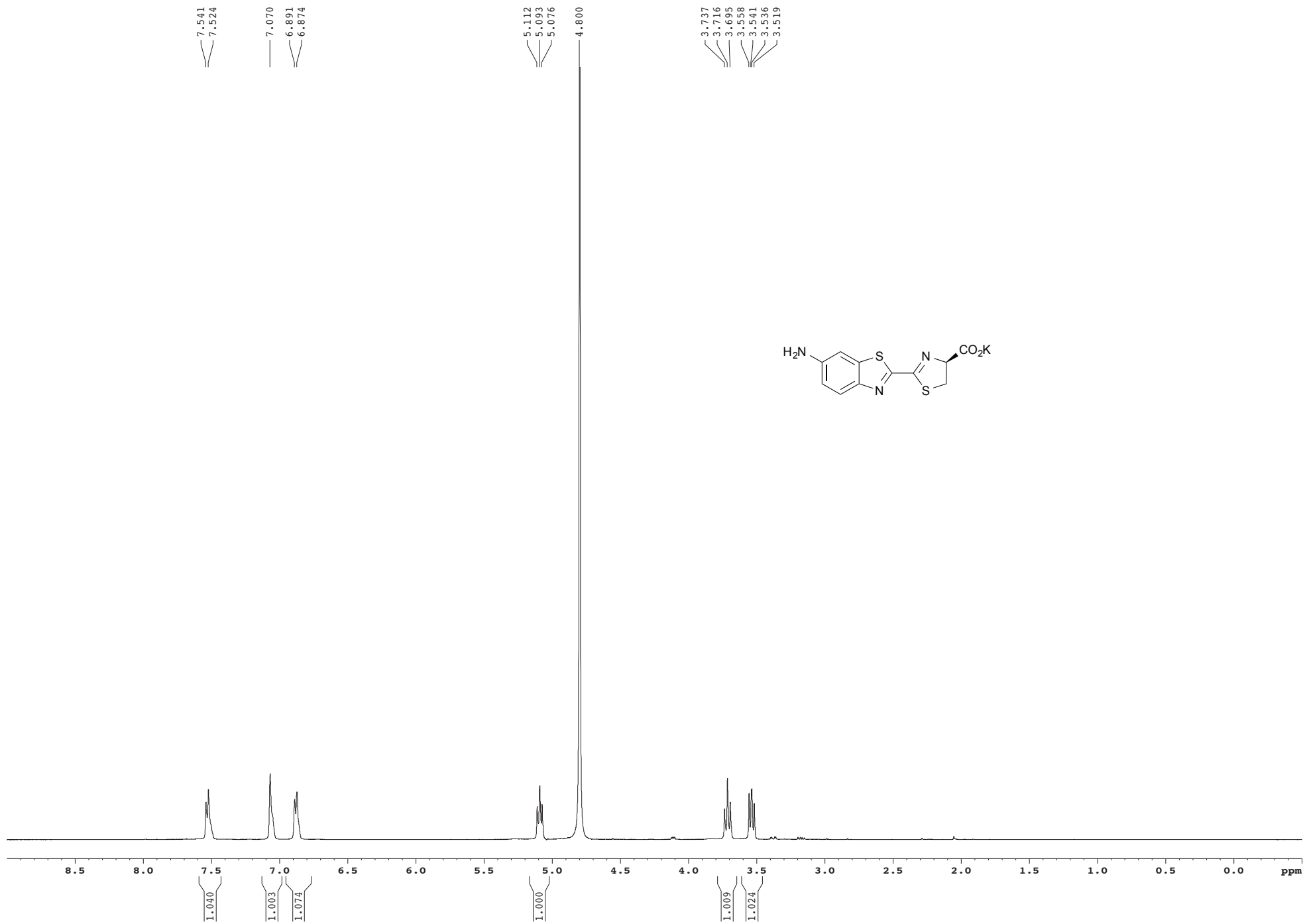














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120.231  
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38.931

