Supporting Information for:

Luciferin amides enable *in vivo* bioluminescence detection of endogenous fatty acid amide hydrolase activity

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Experimental Procedures

Materials and Methods

Chemicals for synthesis were obtained from Aldrich unless otherwise noted. D-luciferin was obtained from Anaspec for *in vitro* work and from Gold Bio for mouse work. Serine hydrolase inhibitors were purchased from Cayman. CycLuc1 was synthesized as previously described[3]. Protein concentrations were determined using Coomassie Plus (Thermo Scientific). Immobilized glutathione (Thermo Scientific) was used for glutathione S-transferase (GST)-tagged protein purification. Kolliphor® EL was obtained from Sigma Life Sciences (Stock # C5135) Data were plotted and analyzed with GraphPad Prism 6.0. High resolution mass spectral data were recorded on a Waters QTOF Premier spectrometer (University of Massachusetts Medical School Proteomics and Mass Spectrometry Facility). Bioluminescence assays were performed on a Xenogen IVIS-100 system in the Small Animal Imaging facility. Data acquisition and analysis were performed with Living Image® software. Data are reported as total flux (p/s) for each region of interest (ROI). For *in vitro* and cellular assays, the ROIs correspond to each well of a 96-well plate. For in vivo assays, the ROIs correspond to the indicated region of a mouse.

Plasmid Constructs

Human and rat FAAH genes (hFAAH and rFAAH) were purchased from the Mammalian Gene Collection of Thermo Fisher Scientific (clone IDs: hFAAH 5728192, rFAAH 7370226). Residues 30-579 of the rFAAH gene were PCR-amplified and cloned into the EcoRI and NotI sites of pGEX6P-1, resulting in removal of the N-terminal transmembrane domain[12]. Full length hFAAH was PCR-amplified and cloned into the KpnI and NotI sites of pcDNA3.1 to yield pcDNA3.1-hFAAH.

Protein Expression and Purification

Rat FAAH (30-579) and firefly luciferase were expressed as GST-fusion proteins using the pGEX6P-1 vector in the *E. coli* strain JM109. Cells were grown at 37°C until the OD_{600} reached 0.5-1, induced with 0.1 mM IPTG, and incubated 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. *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 incubated overnight at 4°C to cleave the GST-fusion and elute the untagged protein into storage buffer.

Purified Protein rFAAH Activity Assays

FAAH inhibitors were prepared at 4 µM in substrate buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 8 mM $MgSO_4$, and 4 mM ATP). Luciferins and luciferin amides were prepared at 40 µM in substrate buffer. Luciferase and rFAAH were prepared at 400 nM in enzyme buffer (20 mM Tris [pH 7.4], 0.1 mM EDTA, 1 mM TCEP, and 0.8 mg/mL BSA). In a black 96-well plate (Costar 3915), 25 µL FAAH inhibitor or substrate buffer was added to three wells each. rFAAH (25 µL) or enzyme buffer was added to the inhibitor and incubated at ambient temperature for 5 minutes. Luciferin or luciferin amide (25 µL) was then added to each well and incubated at ambient temperature for 20 minutes. Luminescence was then initiated by adding 25 µL of luciferase. Imaging was performed one minute after luciferase addition and integrated for 2-20s

at a final concentration of 1 μM FAAH inhibitor, 10 μM luciferin, 100 nM rFAAH, and 100 nM luciferase.

Purified rFAAH Dose-Response Assays

Purified rFAAH was prepared at concentrations ranging from 8 μM to 3.91 nM in enzyme buffer. Luciferin amides were prepared at 20 μM in substrate buffer. Luciferase was prepared at 400 nM in enzyme buffer. In a black 96-well plate (Costar 3915), 50 μL of each luciferin amide was added per well. rFAAH (25 μL) was then added to the amide and incubated at ambient temperature for 30 minutes. Luminescence was then initiated by adding 25 μL of luciferase. Imaging was performed one minute after luciferase addition for 2-20s at a final concentration of 2000 nM to 0.977 nM rFAAH, 10 μM luciferin amide, and 100 nM luciferase.

Luciferin Amide Inhibitor Assays with Purified Luciferase

Luciferin amides were prepared at 40 μM or 100 μM in substrate buffer. Luciferins were prepared at concentrations from 1000 μM to 0.488 μM in substrate buffer. Luciferase was prepared at 200 nM in enzyme buffer. In a black 96-well plate (Costar 3915), 25 μL luciferin amide or substrate buffer was added to three rows each. Luciferin (25 μL) was added to the luciferin amide. Luminescence was then initiated by adding 50 μL of luciferase. Imaging was performed one minute after luciferase addition for 2-20s at a final concentration of 0 μM, 10 μM, or 25 μM luciferin amide, 250 μM to 0.122 μM luciferin, and 100 nM luciferase.

Cell Culture

Chinese hamster ovary (CHO) cells and HeLa cells were grown in a CO_2 incubator at 37°C with 5% CO_2 and were cultured in F-12K Nutrient Mixture (GIBCO) and Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) respectively. Both media were supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin.

Transfections

CHO cells were transfected with codon-optimized firefly luciferase (luc2) as previously described[5]. HeLa cells were transfected with pcDNA3.1-luc2 plasmid[5] and either pcDNA3.1-hFAAH or empty pcDNA3.1 vector. 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 CHO cells, 0.075 µg DNA/well of pcDNA3.1-luc2 was transfected; for HeLa cells, 0.0375 µg DNA/well each of pcDNA3.1-luc2 and either pcDNA3.1-hFAAH or empty pcDNA3.1 was transfected. Assays were performed in triplicate 24 hrs after transfection.

Live Cell FAAH Activity Assays

Transfected cells were washed with HBSS. For substrate dose-response curves, the cells in 96-well plates were incubated with 50 µL of 2 µM FAAH inhibitor in HBSS or HBSS only at ambient temperature for 5 minutes. Then, 50 µL of 2x substrate was added to each well to achieve final substrate concentrations ranging from 125 µM to 0.061 µM. For inhibitor dose-response curves, the cells in 96-well plates were incubated with 50 µL of 2x FAAH inhibitor in HBSS at ambient temperature for 5 minutes (final inhibitor concentrations ranging from 10 µM to 0.21 pM). Then, 50 µL of 2x substrate was added to each well to a final luciferin concentration of 10 µM. Imaging was performed three minutes after addition of substrate.

Mice

All of the experiments involving mice were conducted in accordance with the Institutional Animal Care and Use Committee of The University of Massachusetts Medical School (docket #A-2474-14). Female FVB mice and luciferase-expressing transgenic mice (FVB-Tg(*CAG-luc,-GFP*)L2G85Chco/FathJ) were purchased from Jackson Laboratories (Bar Harbor, ME).

Striatal injection of AAV9-CMV-luc2 into FVB mice was performed as previously described[18].

Tail-vein injection of AAV

FVB mice were injected in the lateral tail vein with $1x10^{11}$ particles of AAV9-CMV-luc2 luciferase[18] suspended in sterile filtered PBS at a final volume of 200 µL. The mice were held under a heat lamp to warm the tail for better visualization of the lateral tail vein due to vasodilation and then placed into a Tailveiner (Braintree Scientific, Braintree, MA). The tails were cleaned with a 70% isopropyl alcohol wipe and injection was performed using a 27.5 gauge needle. Once the needle was withdrawn, the tail was compressed with sterile gauze to ensure complete homeostasis. The mice were monitored afterwards for recovery of normal behavior before returning to their colony. Imaging was performed weeks to months after AAV injection.

Substrate and FAAH Inhibitor Preparation for Mouse Injection

All FAAH inhibitors were prepared by direct dissolution into 18:1:1 PBS:Kolliphor:ethanol and sterile filtering through a 0.22 µm syringe filter. Each inhibitor was injected intraperitoneally (i.p.) at 5 µL/g mouse. Inhibitors were prepared at 2 mg/mL, 0.2 mg/mL, 0.02 mg/mL, and 0.002 mg/mL to achieve final concentrations of 10 mg/kg, 1 mg/kg, 0.1 mg/kg, and 0.01 mg/kg respectively. Due to limited solubility, URB597 was prepared at 0.4 mg/mL and injected at 10 µL/g to achieve a dose of 4 mg/kg.

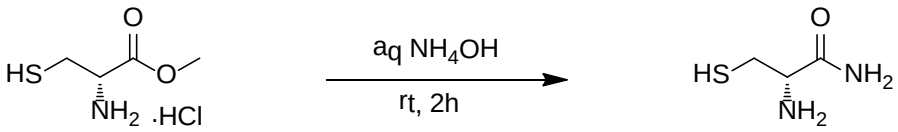
D-luciferin (100 mM) and CycLuc1 (5 mM) were prepared by direct dissolution into PBS and were sterile filtered through a 0.22 µm syringe filter. All other luciferins were prepared by diluting a 50 mM DMSO stock into PBS and sterile filtering through a 0.22 µm syringe filter. CycLuc1 ethyl ester was not soluble in PBS and was therefore prepared by diluting the 50 mM DMSO stock into 18:1:1 PBS:Kolliphor:ethanol. Each substrate was injected i.p. at 4 µL/g mouse.

Substrates were prepared at 100 mM (D-Luciferin), 5 mM (CycLuc1), 250 µM (substrates for AAV mice), and 100 µM (substrates for FVB-Tg[CAG-luc] mice) to achieve final concentrations of 400 µmol/kg, 20 µmol/kg, 1 µmol/kg, and 0.4 µmol/kg respectively.

Bioluminescence Imaging of Mice

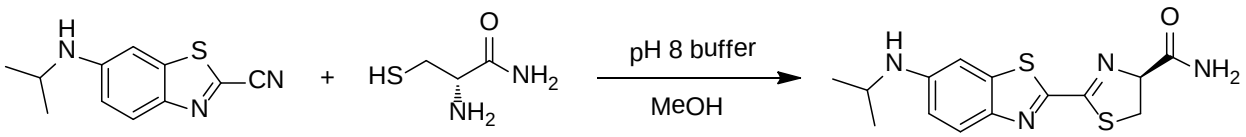
Each mouse was weighed to determine substrate and/or FAAH inhibitor dosing and anesthetized using 2.5% isoflurane in 1 L/min oxygen. FAAH inhibitors were injected i.p. at 5 µL/g mouse 30 minutes prior to luciferin injection. Luciferin substrate was injected i.p. at 4 µL/g mouse and mice were imaged dorsally at 10 minutes and ventrally at 13 minutes.

Synthetic Procedures:



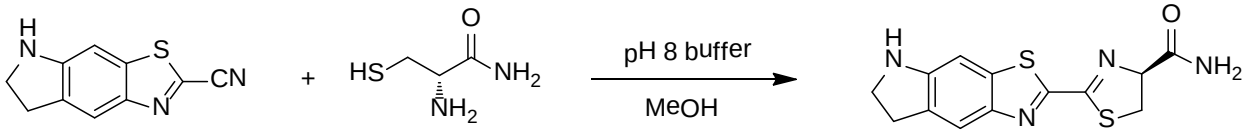
1 **2**

(*S*)-2-amino-3-mercaptopropanamide (2): D-cysteine methyl ester hydrochloride (500 mg, 2.912 mmol) was dissolved in aqueous ammonium hydroxide solution (2 mL) and the mixture was stirred at room temperature under argon for 2h. Solvents were evaporated under reduced pressure and the resulting white solid was used in the next step without further purification (300 mg, 86%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.92 (br s, 1H), 7.49 (br s, 1H), 6.54 (br s, 3H), 3.79 (t, 1H, J = 5.2 Hz), 2.90 (d, 2H, J = 5.2 Hz). ^{13}C-NMR: (100 MHz, DMSO-d_6): δ 173.0, 55.0, 26.4.



3 **2** **4**

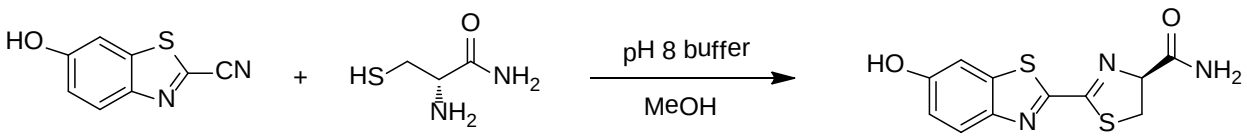
6′-isopropylaminoluciferin amide [(S)-2-(6-(isopropylamino)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxamide] (4): D-cysteine amide **2** (8 mg, 0.066 mmol) was dissolved in 50 mM aqueous sodium phosphate buffer (pH 8.0, 2 mL) and degassed using argon. This solution was added to **3** (10 mg, 0.046 mmol) in degassed methanol (2 mL). The reaction mixture was stirred at room temperature for 2h, diluted with sodium phosphate buffer and extracted with ethyl acetate (2 × 30 mL). The combined organic layers were washed with water (2 × 20 mL), dried (Na_2SO_4), filtered and evaporated to afford **4** as a yellow solid. The crude compound was purified by flash chromatography on silica gel eluting with EtOAc-hexanes (7:3) to afford the product **4** as a yellow solid (12 mg, 80%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.86 (d, 1H, *J* = 8.0 Hz), 6.92 (d, 1H, *J* = 2.4 Hz), 6.75 (dd, 1H, *J* = 9.2, 2.4 Hz), 6.71 (br s, 1H), 5.83 (br s, 1H), 5.26 (t, 1H, *J* = 9.6 Hz), 3.76 (d, 2H, *J* = 9.6 Hz), 3.69 (m, 1H), 1.26 (d, 6H, *J* = 6.4 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.4, 166.9, 154.5, 147.5, 145.7, 139.1, 125.5, 116.2, 101.4, 79.0, 44.6, 35.3, 23.0. HRMS (ESI^+) Calcd for $C_{14}H_{17}N_4OS_2$: 321.0844, Found: 321.0837.



5 **2** **6**

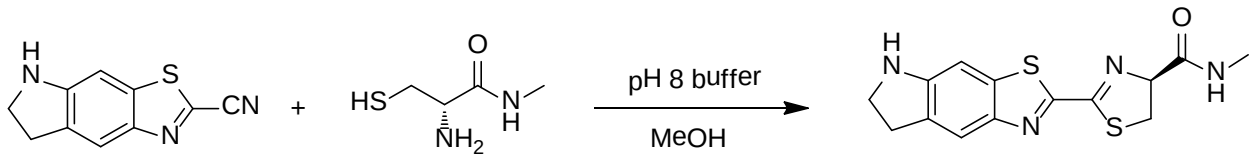
CycLuc1-amide [(S)-2-(6,7-dihydro-5H-thiazolo[4,5-f]indol-2-yl)-4,5-dihydrothiazole-4-carboxamide] (6): D-cysteine amide **2** (9 mg, 0.074 mmol) was dissolved in 50 mM aqueous sodium phosphate buffer (pH 8.0, 2 mL) and degassed using argon. This solution was added to **5** (10 mg, 0.049 mmol) in degassed methanol (2 mL). The reaction was stirred for 2 h, diluted

with sodium phosphate buffer and extracted with ethyl acetate (2 × 30 mL). The combined organic layers were washed with water (2 × 30 mL), dried (Na_2SO_4), filtered and evaporated to afford **6** as a yellow solid. The crude compound was purified by flash chromatography on silica gel eluting with EtOAc-hexanes (8:2) to afford the product **6** as a yellow solid (10 mg, 67%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.79 (s, 1H), 6.95 (s, 1H), 6.69 (br s, 1H), 5.54 (br s, 1H), 5.26 (t, 1H, *J* = 8.0 Hz), 3.75 (d, 2H, *J* = 8.0 Hz), 3.71 (t, 2H, *J* = 8.0 Hz), 3.18 (t, 2H, *J* = 8.0 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.4, 166.9, 154.6, 152.3, 147.3, 137.2, 131.9, 120.6, 98.7, 79.0, 48.0, 35.2, 29.4. HRMS (ESI$^+$) Calcd for $C_{13}H_{13}N_4OS_2$: 305.0531, Found: 305.0498.



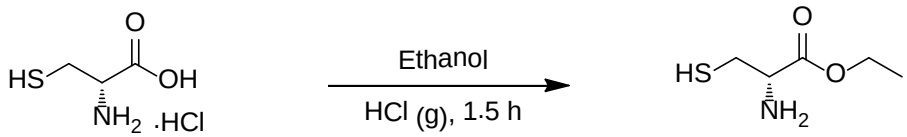
7 **2** **8**

D-luciferin-amide [(S)-2-(6-hydroxybenzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxamide] (8): D-Cysteine amide **2** (10 mg, 0.083 mmol) was dissolved in 50 mM aqueous sodium phosphate buffer (pH 8.0, 2 mL) and degassed using argon. This solution was added to **7** (10 mg, 0.056 mmol) in degassed methanol (2 mL). The reaction was stirred for 2 h, diluted with sodium phosphate buffer and extracted with ethyl acetate (2 × 30 mL). The combined organic layers were washed with water (2 × 30 mL), dried (Na_2SO_4), filtered, and evaporated. The crude compound was purified by flash chromatography on silica gel eluting with EtOAc-hexanes (8:2) to afford the product **8** as a white solid (13 mg, 83%). ^{1}H-NMR (400 MHz, DMSO-d6): δ 10.22 (br s, 1H), 7.94 (d, 1H, *J* = 8.8 Hz), 7.43 (s, 1H), 7.04 (dd, 1H, *J* = 9.2, 2.4 Hz), 5.38 (t, 1H, *J* = 10.0 Hz), 3.61-3.76 (m, 2H). ^{13}C-NMR (100 MHz, DMSO-d6): δ 171.8, 164.7, 160.0, 157.5, 146.9, 137.9, 125.5, 117.8, 107.4, 79.8, 35.1. HRMS (ESI$^+$) Calcd for $C_{11}H_{10}N_3O_2S_2$: 280.0215, Found: 280.0188.



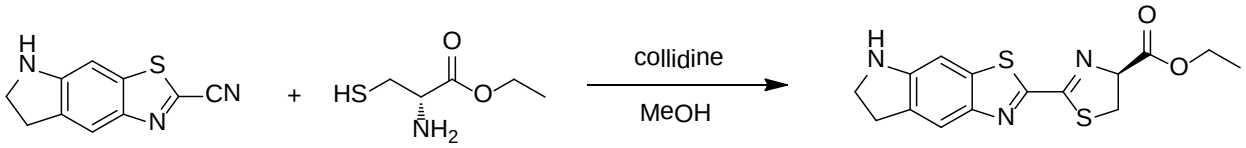
11 **10** **12**

CycLuc1-methyl amide [(S)-2-(6,7-dihydro-5H-thiazolo[4,5-f]indol-2-yl)-N-methyl-4,5-dihydrothiazole-4-carboxamide] (12): D-Cysteine methyl ester hydrochloride (500 mg, 2.912 mmol) was dissolved in 40% aqueous methylamine solution (3 mL) and the mixture was stirred at room temperature under argon for 2h. The solvents were evaporated under reduced pressure and the resulting white solid contains both product **10** and the corresponding disulphide. The crude compound was directly used in the next step. D-cysteine methylamide **10** (10 mg, 0.074 mmol) was dissolved in 50 mM aqueous sodium phosphate buffer (pH 8.0, 2 mL) and degassed using argon. This solution was added to **11** (10 mg, 0.049 mmol) in degassed methanol (2 mL). The reaction mixture was stirred at room temperature for 2 h, diluted with sodium phosphate buffer and extracted with ethyl acetate (2 × 30 mL). The combined organic layers were washed with water (2 × 30 mL), dried (Na_2SO_4), filtered, and evaporated to afford **12** as a yellow solid. The crude compound was purified by flash chromatography on silica gel eluting with EtOAc-hexanes (7:3) to afford the product **12** as a yellow solid (12 mg, 77%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.79 (s, 1H), 6.98 (s, 1H), 6.73 (br s, 1H), 5.23 (t, 1H, J = 10.0 Hz), 3.82-3.68 (m, 4H), 3.18 (t, 2H, J = 7.6 Hz), 2.89 (d, 3H, J = 5.2 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 171.5, 166.9, 154.8, 151.9, 147.4, 137.1, 131.9, 120.6, 99.0, 79.2, 48.0, 35.7, 29.4, 26.5. HRMS (ESI$^+$) Calcd for $C_{14}H_{15}N_4OS_2$: 319.0687, Found: 319.0609.



13 **14**

(S)-ethyl 2-amino-3-mercaptopropanoate (14): D-Cysteine hydrochloride **13** (2 g, 12.7 mmol) was dissolved in 50 mL ethanol solution and gentle steam of HCl gas was bubbled through the solution for 80 minutes and resulting solution was then stirred at room temperature overnight. Solvent was removed under reduced pressure. To the resulting white residue, chloroform (10 mL) was added, stirred for 10 min and resulting slurry was filtered, washed with chloroform (10 mL) and dried under high vacuum to give the product **14** as a white solid in quantitative yield. ^{1}H-NMR (400 MHz, DMSO-d6): δ 8.81 (br s, 2H), 4.32 (t, 1H, *J* = 8.0 Hz), 4.20 (m, 2H), 3.07 (dd, 1H, *J* = 16.0, 4.0 Hz), 2.95 (dd, 1H, *J* = 16.0, 4.0 Hz), 1.22 (t, 3H, *J* = 8.0 Hz). ^{13}C-NMR (100 MHz, DMSO-d6): δ 168.6, 62.8, 51.8, 37.5, 14.6.



15 **14** **16**

CycLuc1-ethyl ester [(S)-ethyl 2-(6,7-dihydro-5H-thiazolo[4,5-f]indol-2-yl)-4,5-dihydrothiazole-4-carboxylate] (16): D-cysteine ethyl ester **14** (37 mg, 0.24 mmol) was dissolved in methanol (2 mL) and collidine (29 mg, 0.24 mmol) was added. The solution was degassed using argon. This solution was added to **15** (50 mg, 0.24 mmol) in degassed methanol (2 mL). The reaction was stirred for 6 h at room temperature, and then the solvents were evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel eluting with EtOAc-hexanes (1:1) to afford the product **16** as a yellow solid (70 mg, 87%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.78 (s, 1H), 6.99 (s, 1H), 5.32 (t, 1H, *J* = 9.6 Hz), 4.29 (q, 2H, *J* = 7.2 Hz), 3.76-3.64 (m, 4H), 3.17 (t, 2H, *J* = 8.4 Hz), 1.33 (t, 1H, *J* = 7.2 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.6, 166.7, 155.3, 151.6, 147.5, 137.2, 131.8, 120.4, 99.3, 78.5, 62.2, 48.0, 35.4, 29.4, 14.4. HRMS (ESI^+) Calcd for $C_{15}H_{16}N_3O_2S_2$: 334.0684, Found: 334.0644.

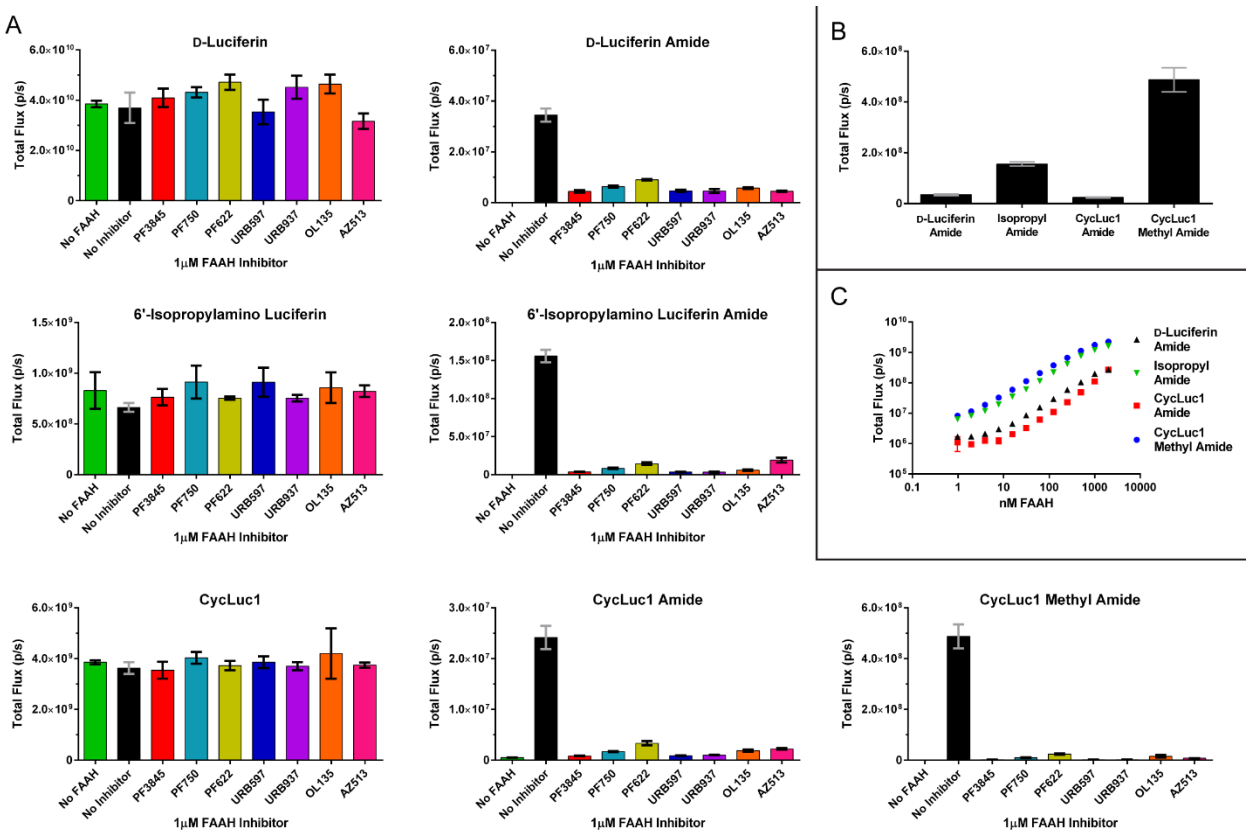


Fig S1. Luciferin amides report on rat FAAH activity *in vitro*. (*A*) Photon flux from the indicated luciferin analog (10 μM) in the absence of FAAH or presence of FAAH with and without a FAAH inhibitor. (*B*) Direct comparison of each luciferin amide after treatment with rFAAH without inhibitor from (A). (*C*) Dependence of photon flux on the concentration of rFAAH(30-579) after 30 min incubation with the indicated luciferin amide at pH 7.4, ambient temperature. All assays were performed in triplicate and are represented as the mean ± SEM.

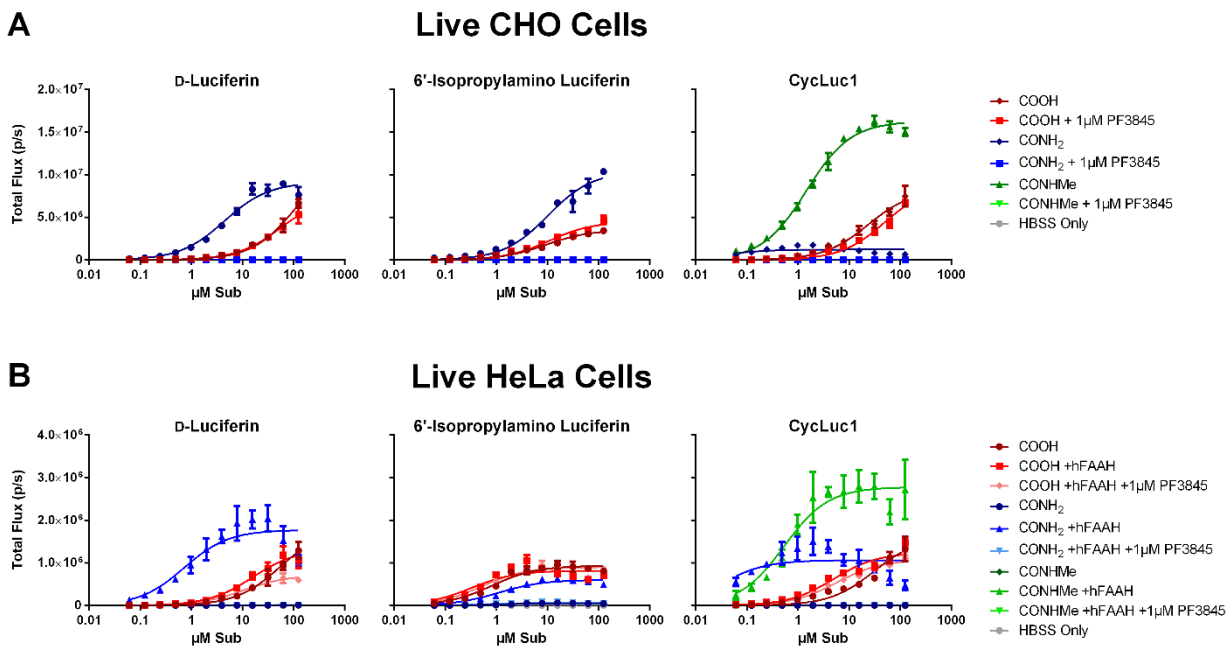


Fig S2. Luciferin amides report on FAAH activity in live cells and improve signal over parent luciferins. (*A*) Live CHO cells transfected with luciferase were incubated with 1 μM PF3845 for five minutes and then imaged with varying concentrations of the indicated luciferin analog. (*B*) Live HeLa cells co-transfected with either luciferase and human FAAH or luciferase and empty vector were incubated with 1 μM PF3845 for five minutes and then imaged with varying concentrations of the indicated luciferin analog. The assay was performed in triplicate and is represented as the mean ± SEM. Curves were fit to the Michaelis–Menten equation by nonlinear regression.

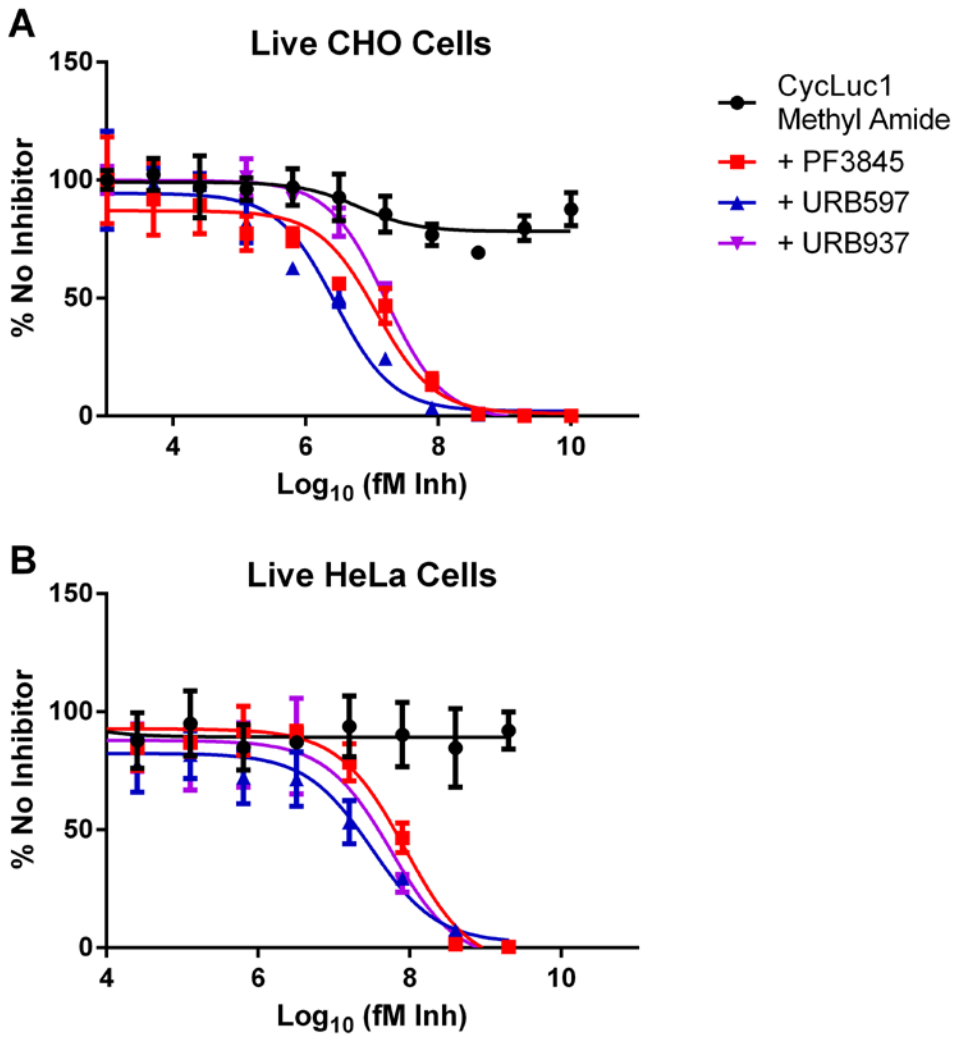


Fig S3. Luciferin amides report on inhibitor potency in live cells. (*A*) Live CHO cells transfected with luciferase were incubated with varying concentrations of FAAH inhibitor for five minutes and then imaged with CycLuc1-methyl amide (10 μM). (*B*) Live HeLa cells co-transfected with luciferase and human FAAH were incubated with varying concentrations of FAAH inhibitor for five minutes and then imaged with CycLuc1-methyl amide (10 μM). The assays were performed in triplicate and are represented as the mean ± SEM. Data was fit by nonlinear regression to log(inhibitor) vs. response (three parameters) to determine relative IC50 values. CHO cell IC50 values: PF3845, 12 nM; URB597, 2.7 nM; URB937, 16 nM. HeLa cell IC50 values: PF3845, 86 nM; URB597, 31 nM; URB937, 57 nM.

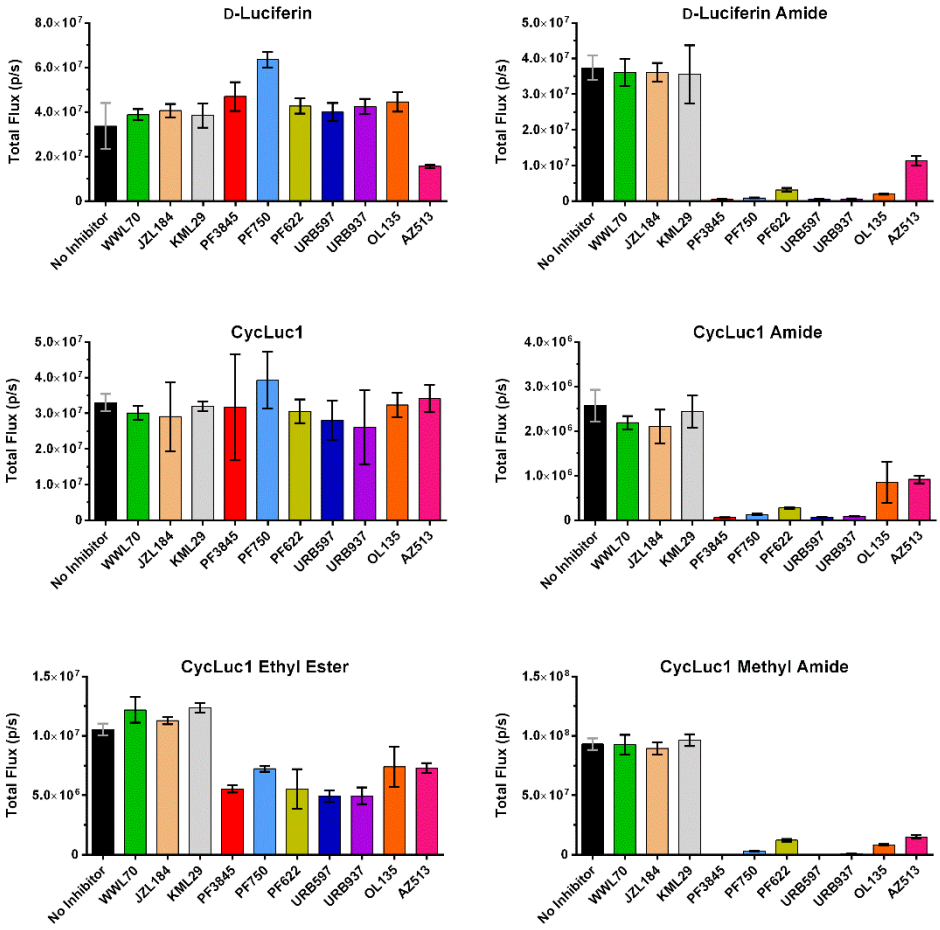


Fig S4. Luciferin amides report on FAAH activity in live CHO cells. Live CHO cells transfected with luciferase were incubated with 1 μM of the indicated serine hydrolase inhibitor for five minutes and then imaged with the indicated luciferin analog (10 μM). All assays were performed in triplicate and are represented as the mean ± SEM. FAAH inhibitors: PF3845, PF750, PF622, URB597, URB937, OL135, and AZ513; MAGL inhibitors: JZL184 and KML29; ABHD6 inhibitor: WWL70.[25]

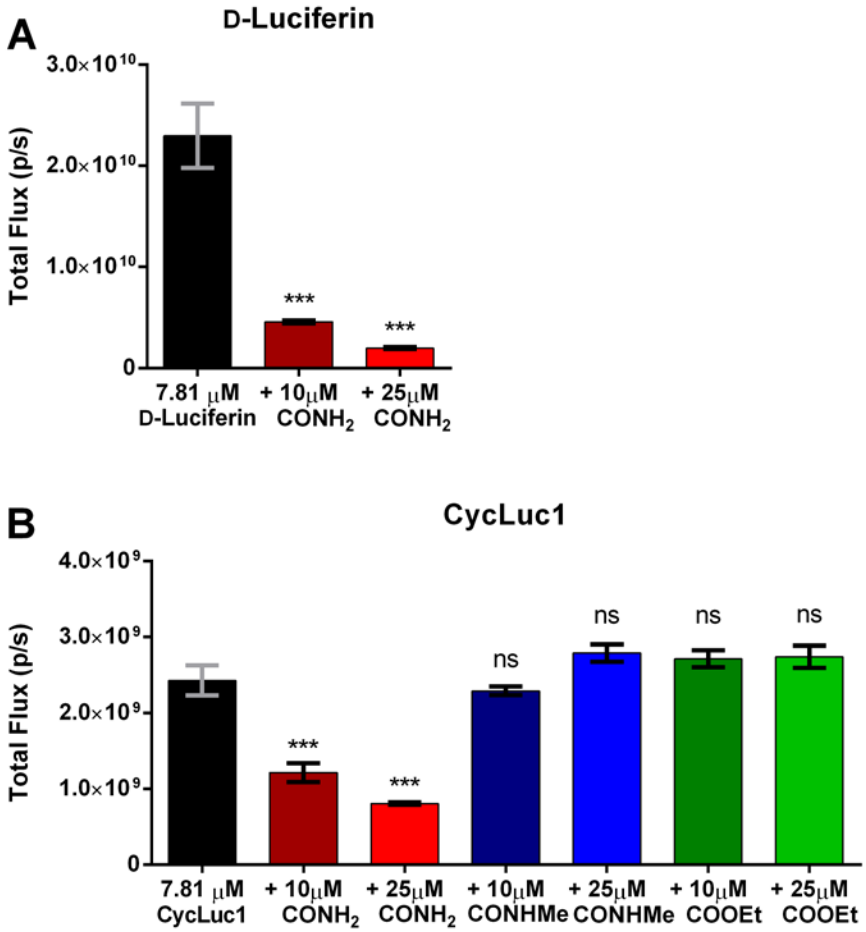


Fig S5. Luciferin primary amides can inhibit luciferase in vitro. (*A*) Purified firefly luciferase treated with 7.81 μM D-luciferin alone or in the presence of 10 μM or 25 μM D-luciferin amide. (*B*) Purified luciferase treated with 7.81 μM CycLuc1 alone or in the presence of 10 μM or 25 μM of CycLuc1-amide, CycLuc1-methyl amide, or CycLuc1–ethyl ester. The assay was performed in triplicate, is represented as the mean ± SEM. Each amide was compared to luciferin only by t-test. ns: not statistically significant; *** P <0.001.

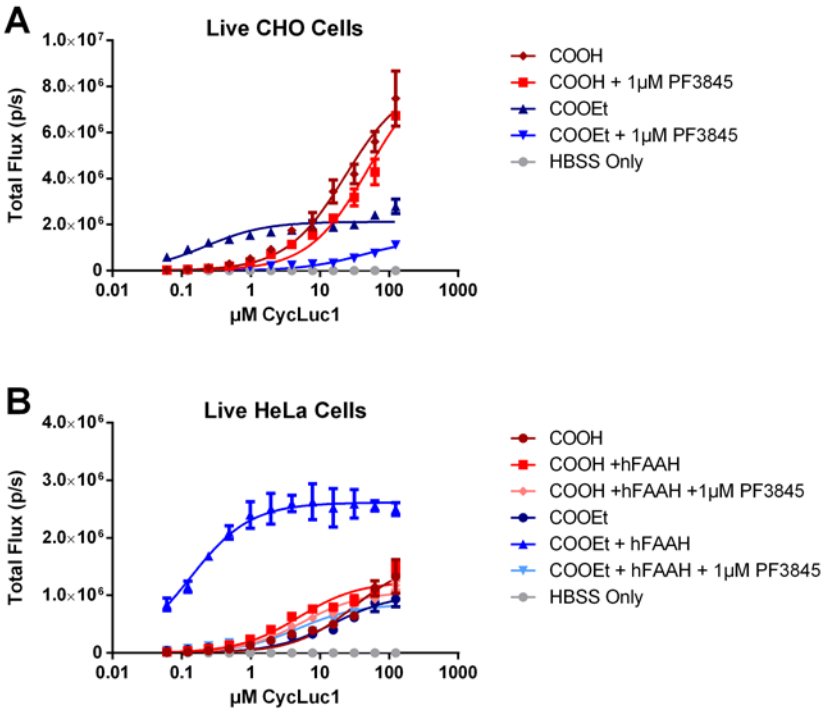


Fig S6. CycLuc1 ethyl ester supports bioluminescence from both live CHO and HeLa cells. (*A*) Live CHO cells transfected with luciferase were incubated with 1 µM PF3845 for five minutes and then imaged with varying concentrations of either CycLuc1 or CycLuc1 ethyl ester. (*B*) Live HeLa cells co-transfected with either luciferase and human FAAH or luciferase and empty vector were incubated with 1 µM PF3845 for five minutes and then imaged with varying concentrations of either CycLuc1 or CycLuc1 ethyl ester. The assay was performed in triplicate and is represented as the mean ± SEM. Curves were fit to the Michaelis–Menten equation by nonlinear regression.

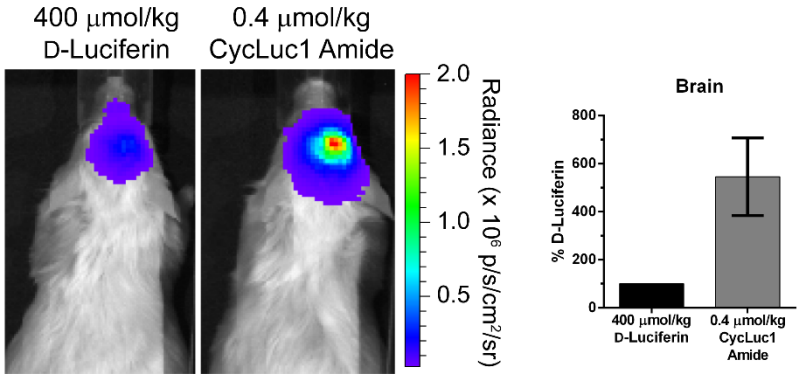


Fig S7. CycLuc1-amide increases total photon flux from the brain at 1000-fold lower dose than D-luciferin. Mice striatally injected with AAV9-CMV-luc2 were treated with 0.4 µmol/kg CycLuc1-amide or 400 µmol/kg D-luciferin. Quantification was normalized to D-luciferin signal for each mouse and is represented as the mean ± SEM for n=3 mice.

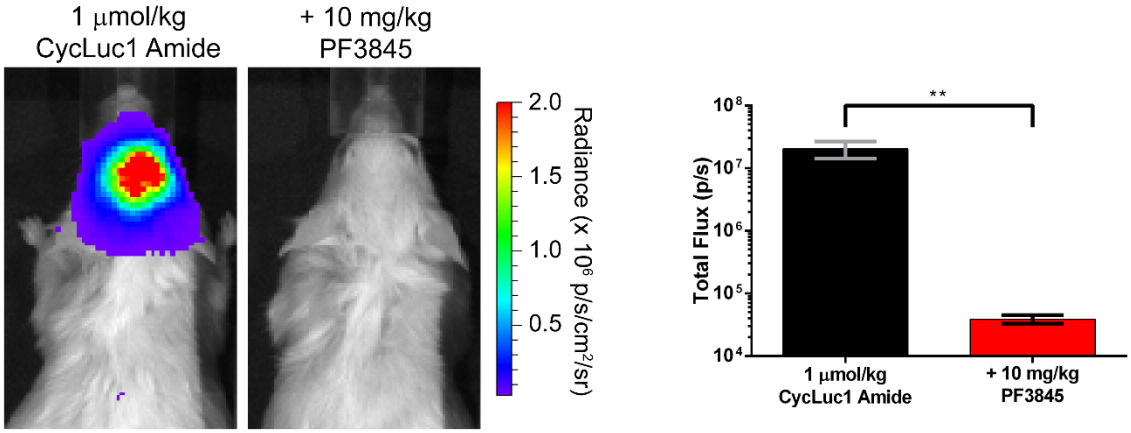


Fig S8. Inhibition of FAAH by PF3845 results in loss of signal from CycLuc1-amide in the brain. Mice striatally injected with AAV9-CMV-luc2 were imaged with CycLuc1-amide alone or after pre-treatment with 10 mg/kg PF3845. Quantification is represented as the mean ± SEM for n=3 mice and was compared by t test. ** P <0.01.

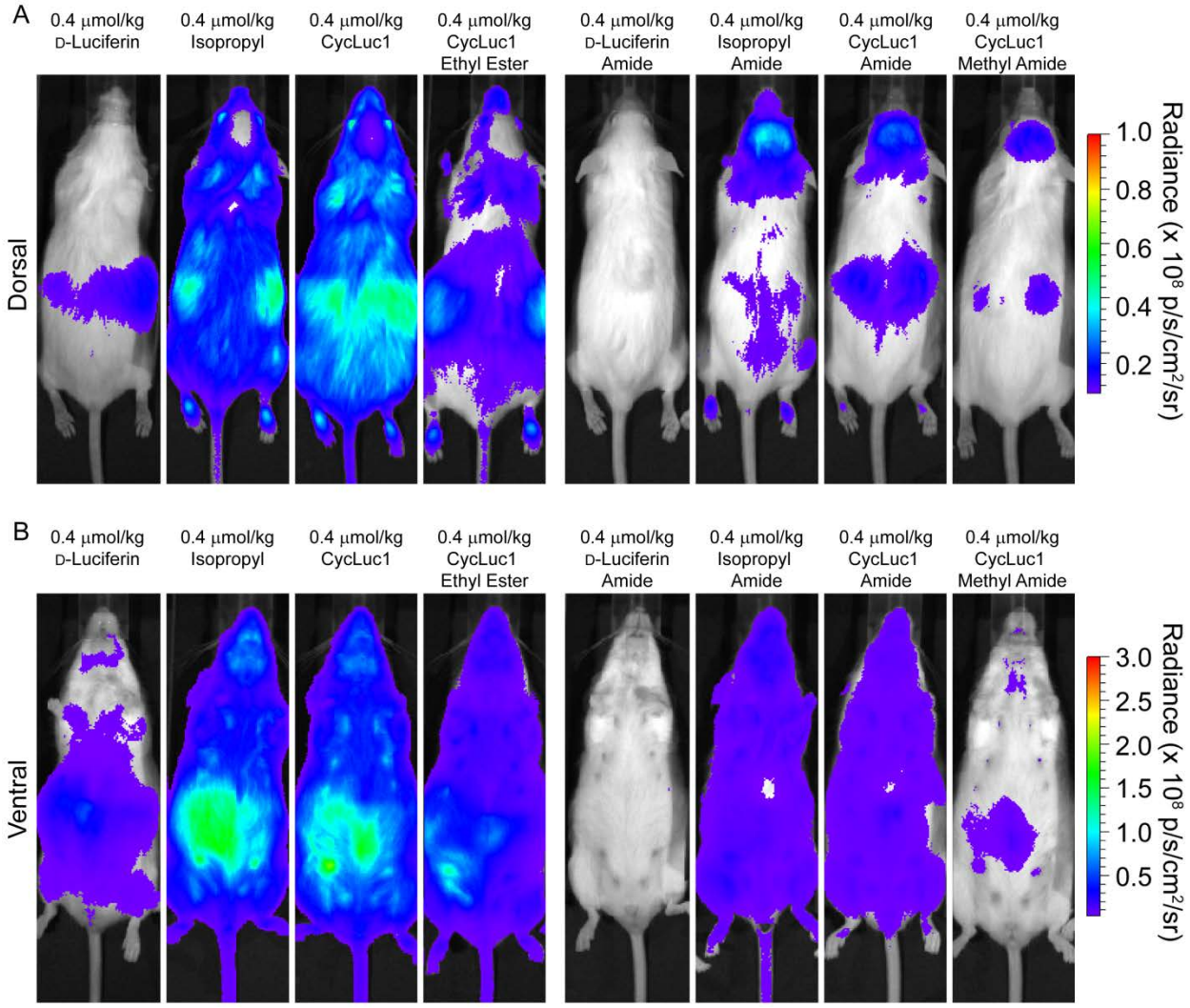


Fig S9. Bioluminescence from mice that ubiquitously express luciferase after treatment with luciferins and luciferin amides. (*A*) Dorsal view of FVB-Tg(CAG-luc) mice injected with the indicated substrate. (*B*) Ventral view of mice in (*A*).

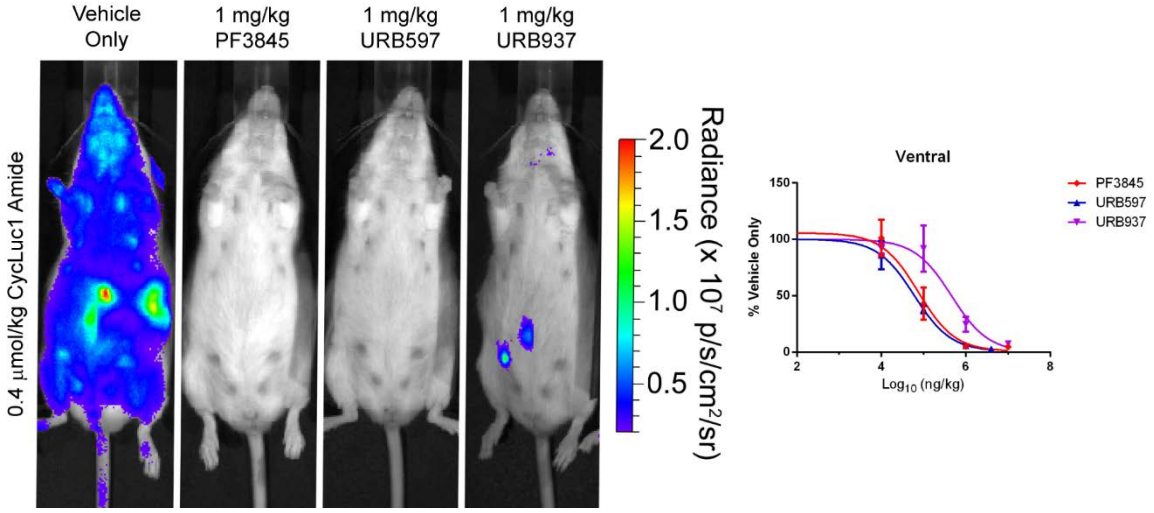


Fig S10. Ventral view of ubiquitously-expressing luciferase mice treated with CycLuc1-amide. Mice were pre-treated with vehicle only (18:1:1 PBS:Kolliphor:ethanol), or the indicated FAAH inhibitors. Quantification was normalized to the average vehicle-only signal and is represented as the mean ± SEM for n=3 mice. Data was fit by nonlinear regression to determine relative ventral IC50 values (PF3845: 0.08 mg/kg, URB597: 0.06 mg/kg, URB937: 0.46 mg/kg).

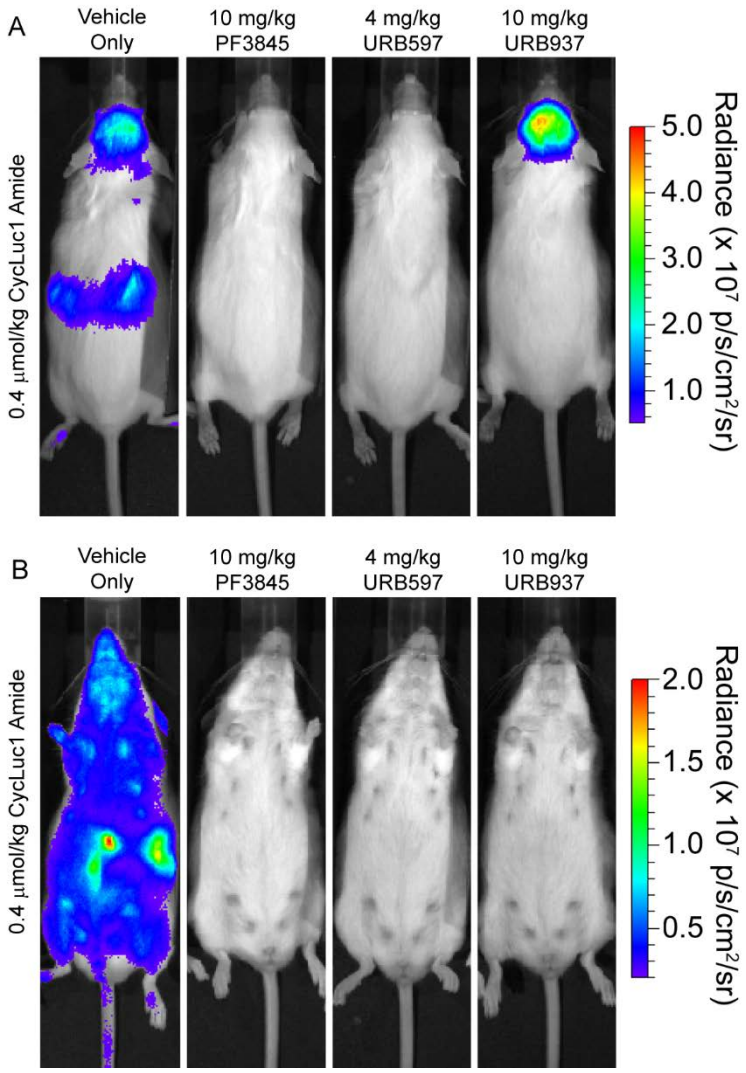


Fig S11. Mice ubiquitously-expressing luciferase treated with high inhibitor dose. Mice were pre-treated with vehicle only (18:1:1 PBS:Kolliphor:ethanol), or the indicated FAAH inhibitors and imaged with CycLuc1-amide.

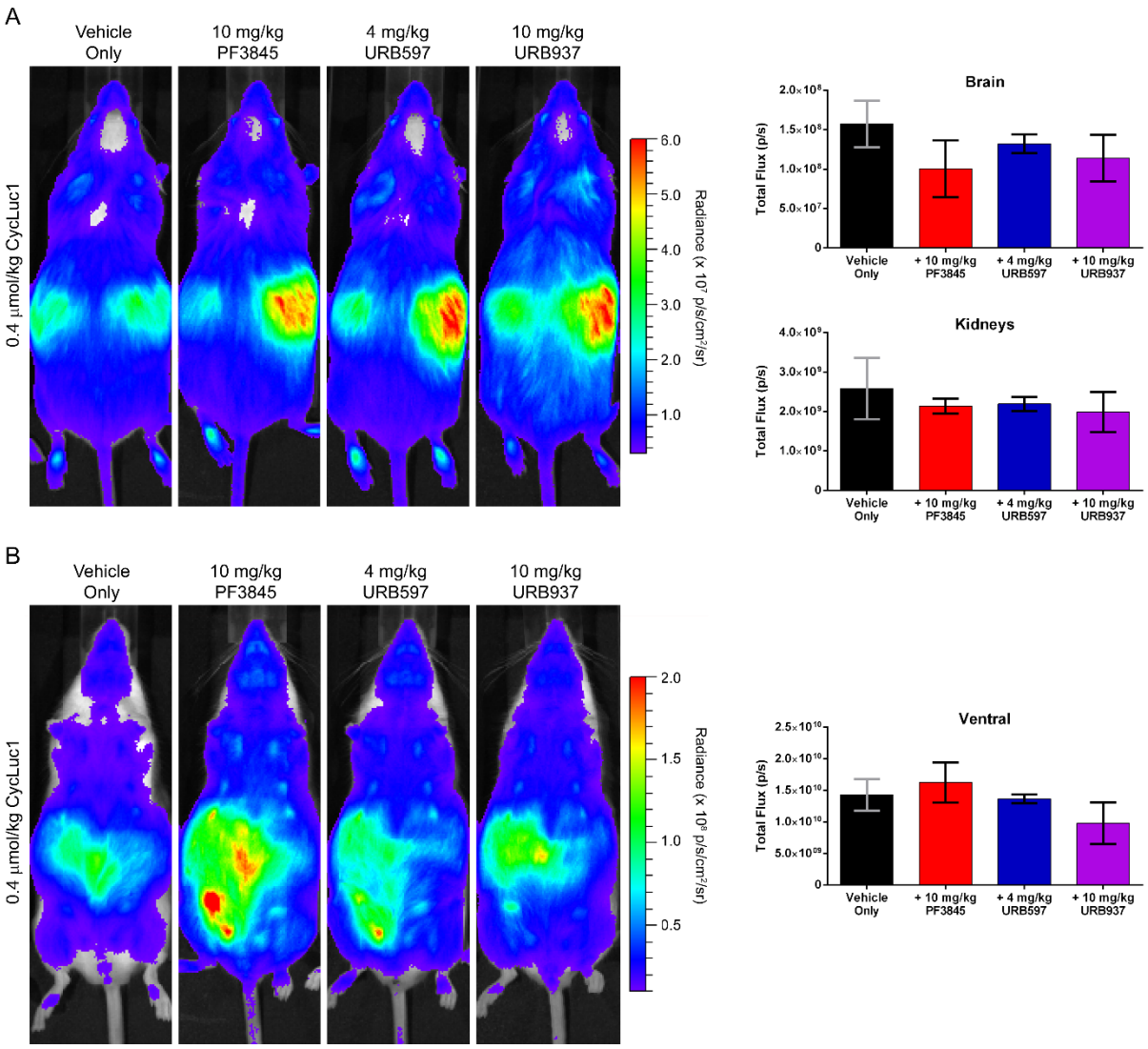


Fig S12. FAAH inhibitors do not affect parent luciferins. (*A*) Dorsal view of live FVB-Tg(CAG-luc) mice, injected with the indicated inhibitor and treated with CycLuc1. (*B*) Ventral view of mice in (*A*). Quantification is represented as the mean ± SEM for n=3 mice. Each inhibitor was compared to vehicle only by t-test. No statistically significant difference was found for any inhibitor.

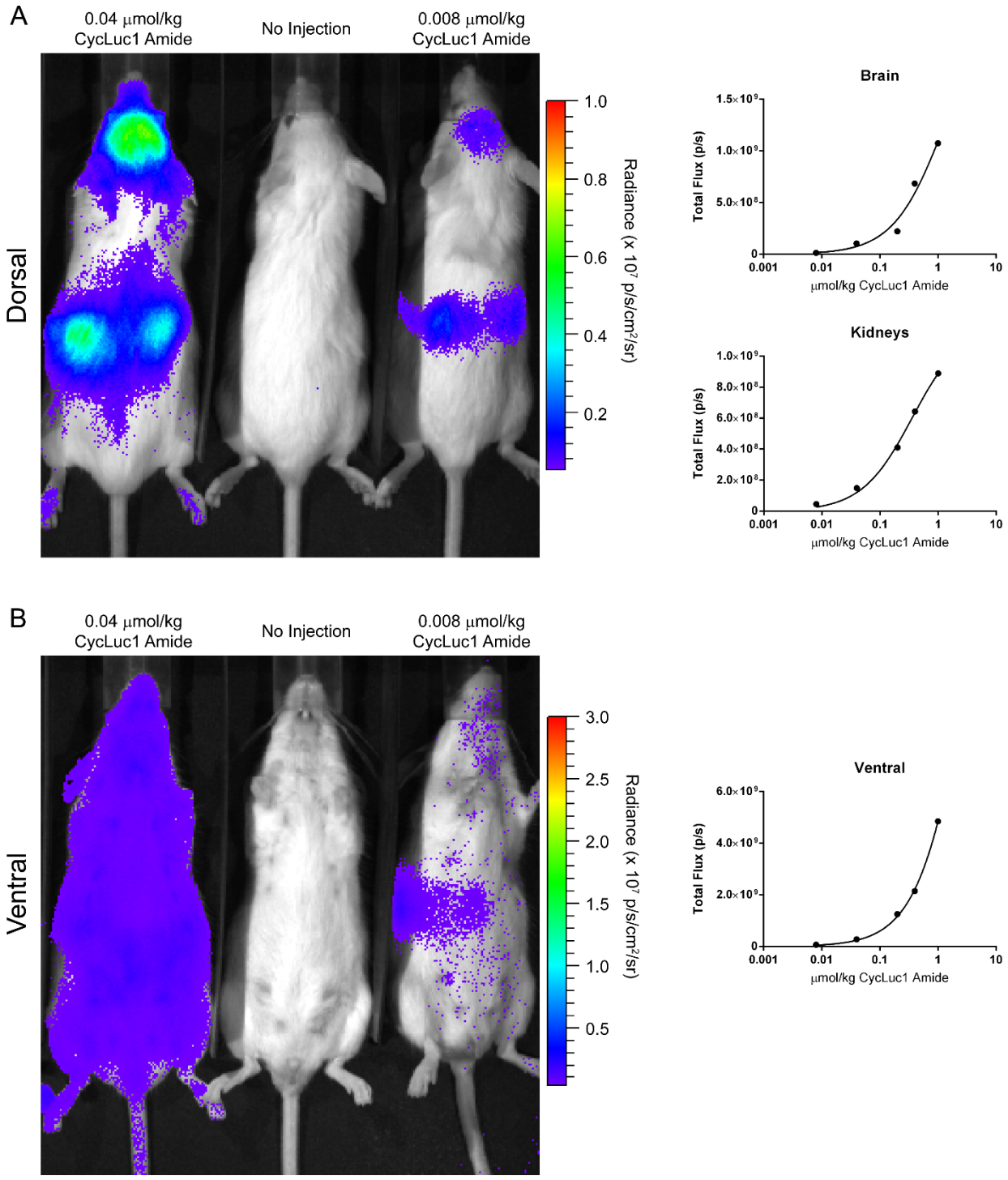
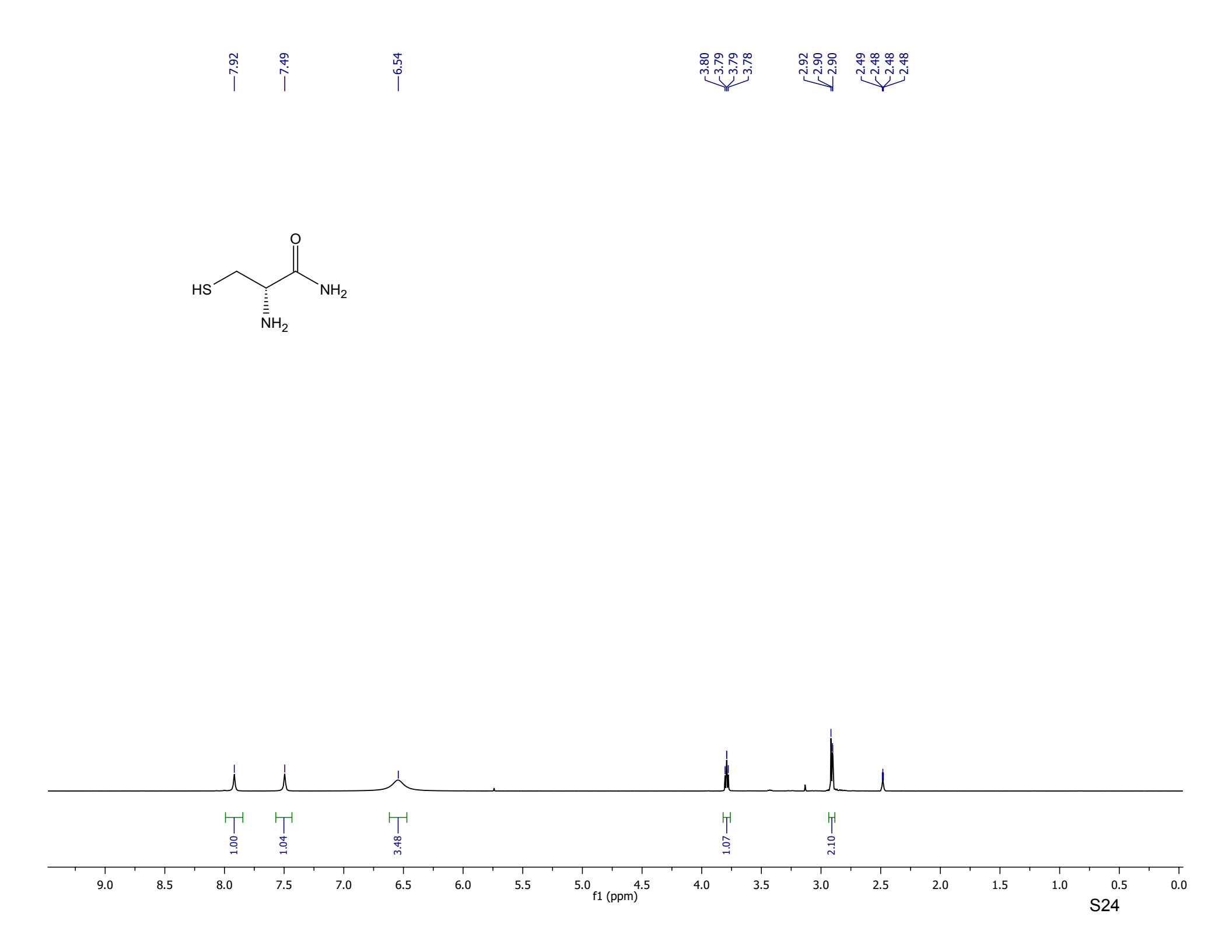


Fig S13. CycLuc1-amide can be imaged at doses as low as 8 nmol/kg and signal is not saturated at 1 µmol/kg. (*A*) Dorsal view of FVB-Tg(CAG-luc) mice injected with the indicated concentration of CycLuc1 amide. (*B*) Ventral view of mice in (*s*). Quantification is represented as n=1 mouse per substrate dose.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2.90
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2.48
2.48
O
HS
NH2
NH2
1.00
1.04
3.48
1.07
2.10
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
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3.0
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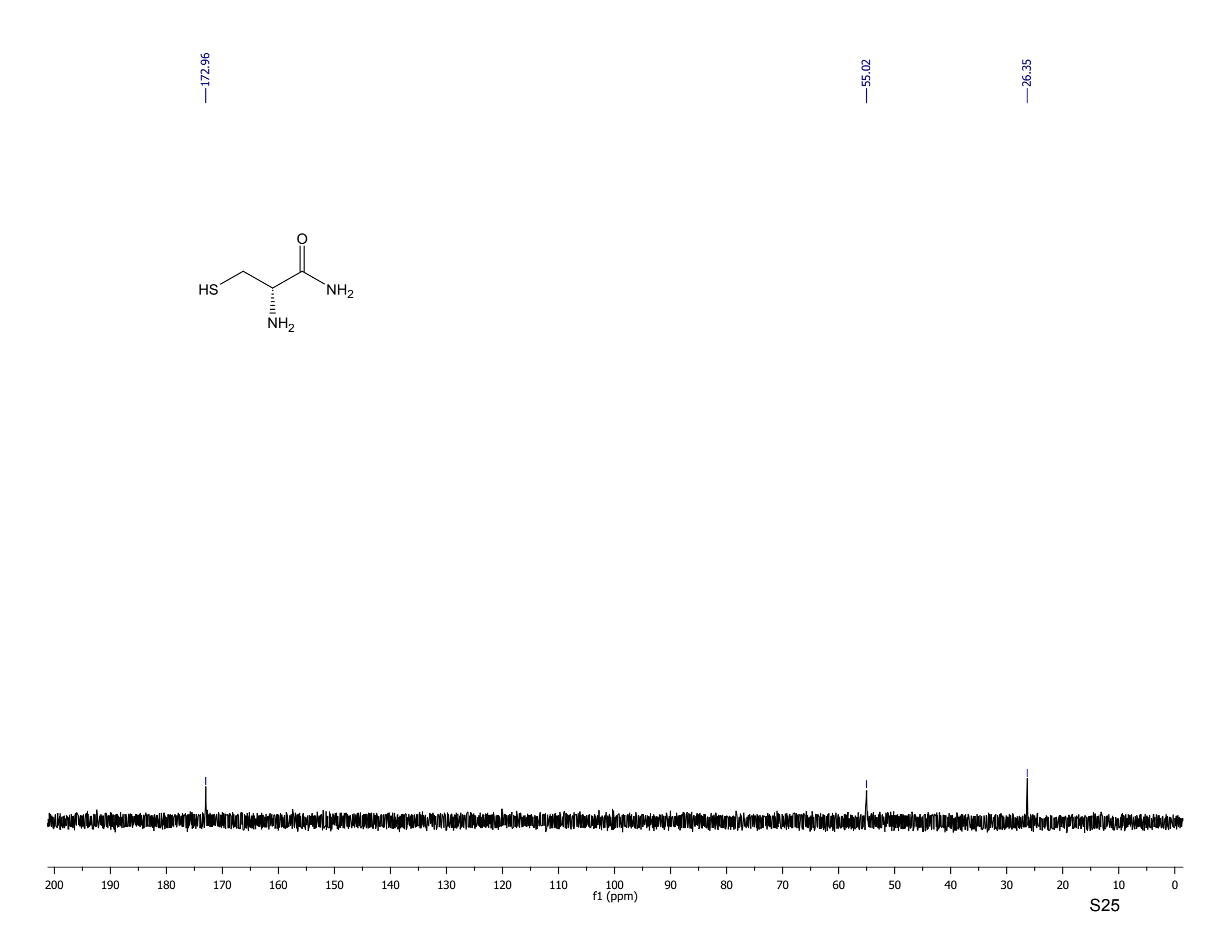
0.0
f1 (ppm)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55.02
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NH2
NH2
200
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180
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150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


7.88
7.85
7.26
6.92
6.91
6.77
6.76
6.74
6.74
6.71
5.83
5.29
5.26
5.24
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3.75
3.70
3.69
3.67
1.27
1.25
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S
N
N
S
O
NH2
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1.10
1.25
0.74
0.93
0.94
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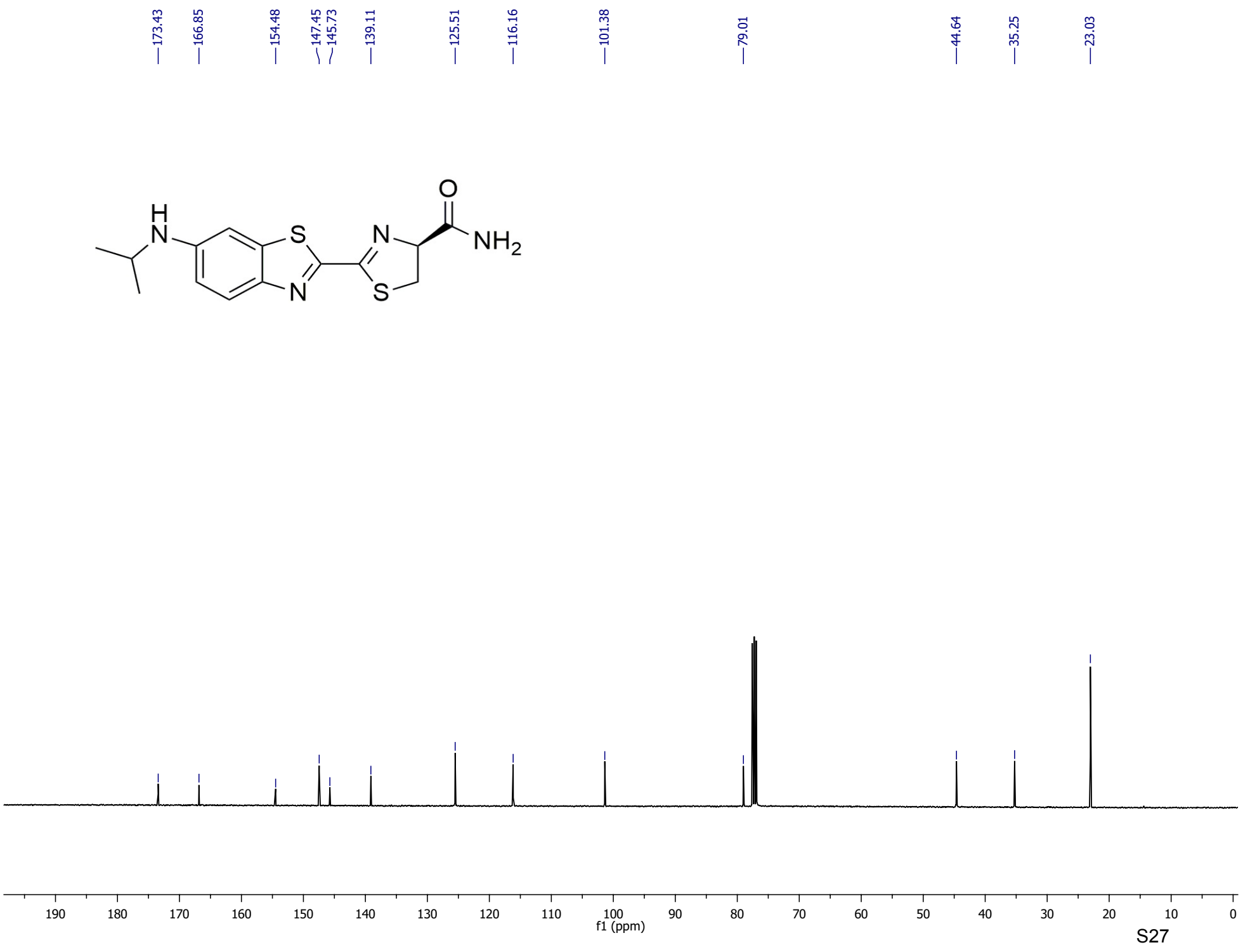
f1 (ppm)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H
N
S
N
O
NH2
N
S
190
180
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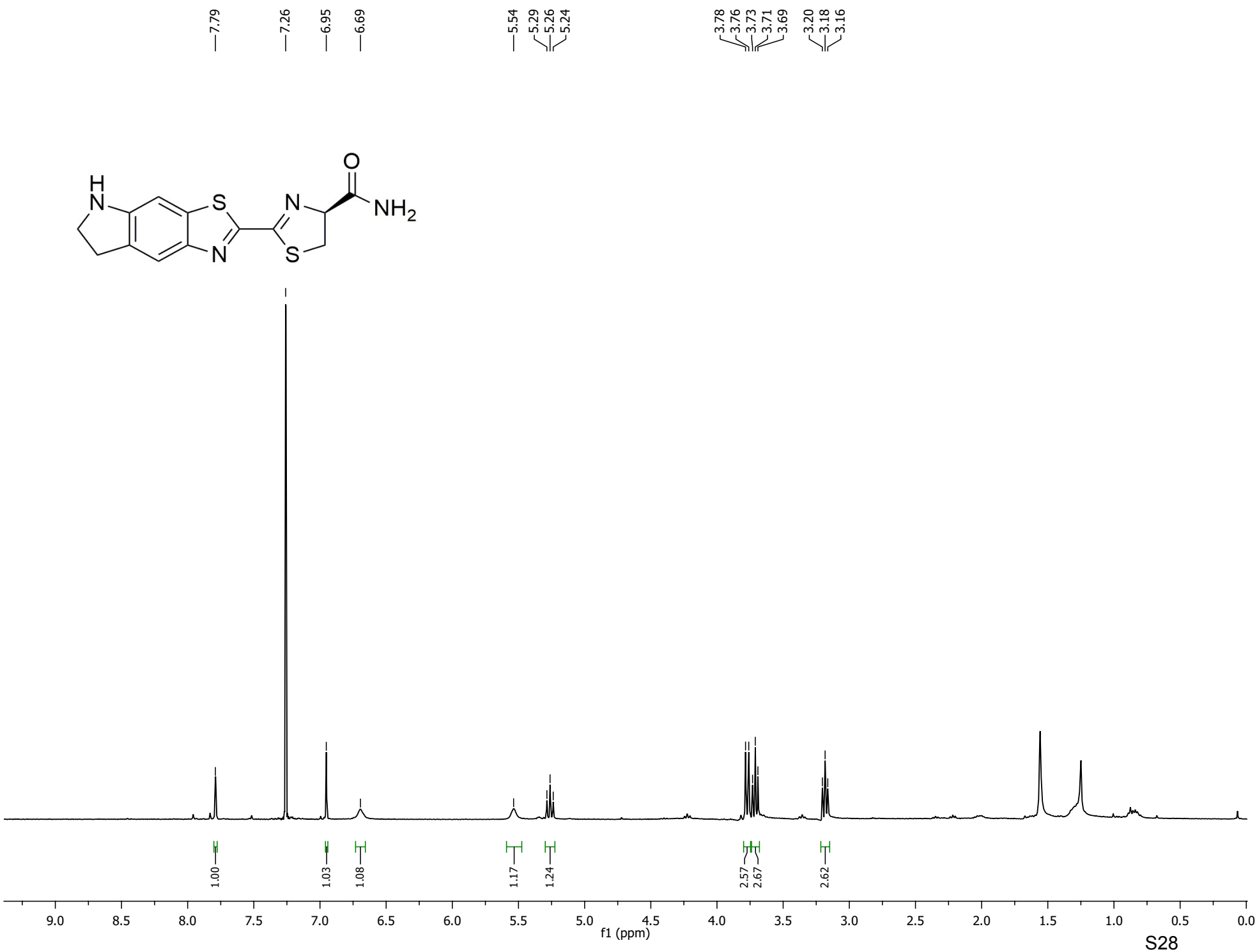
f1 (ppm)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3.20
3.18
3.16
O
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N
S
N
NH2
N
S
1.00
1.03
1.08
1.17
1.24
2.57
2.67
2.62
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8.5
8.0
7.5
7.0
6.5
6.0
5.5
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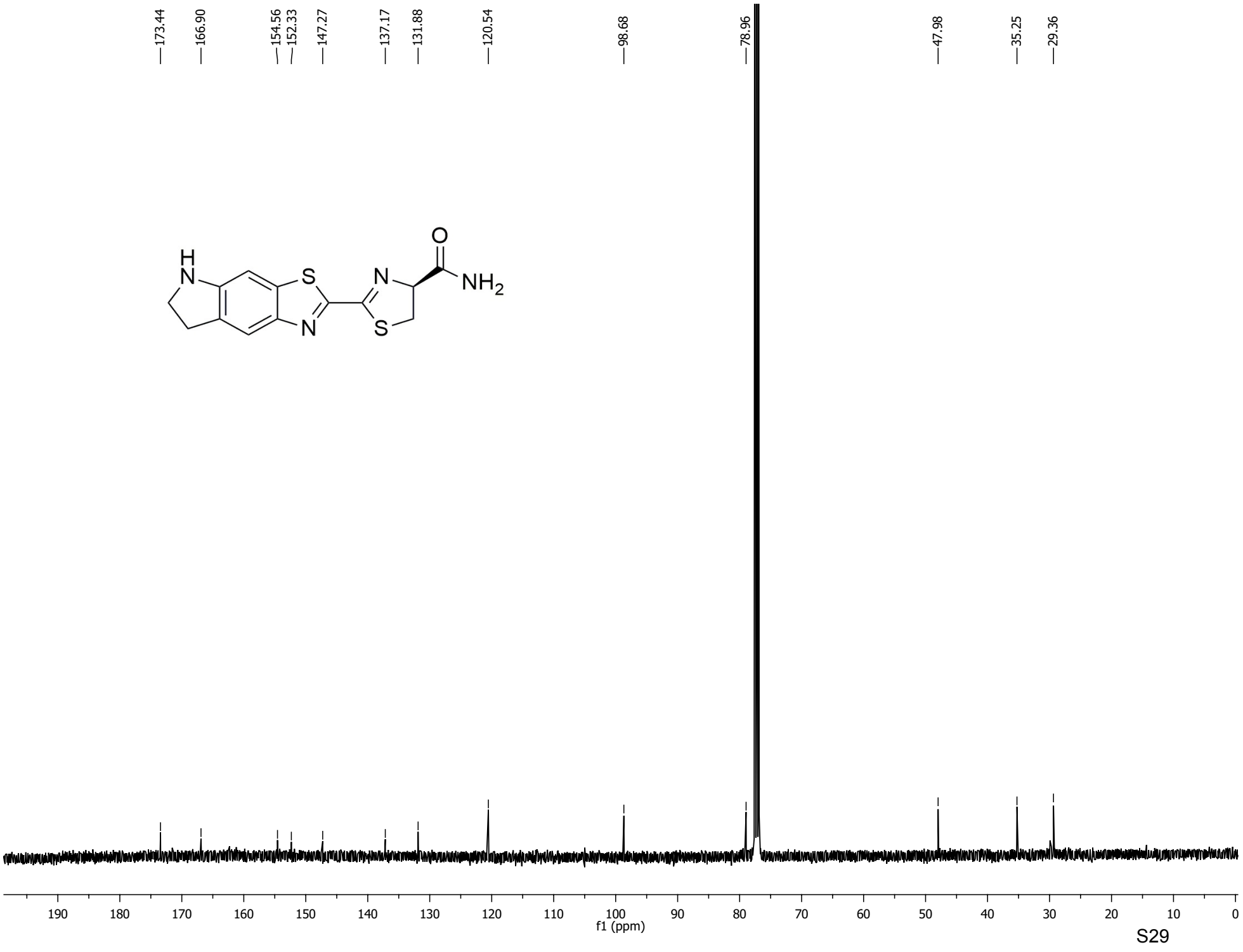
f1 (ppm)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29.36
H
N
S
N
N
S
O
NH2
190
180
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110
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90
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50
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10
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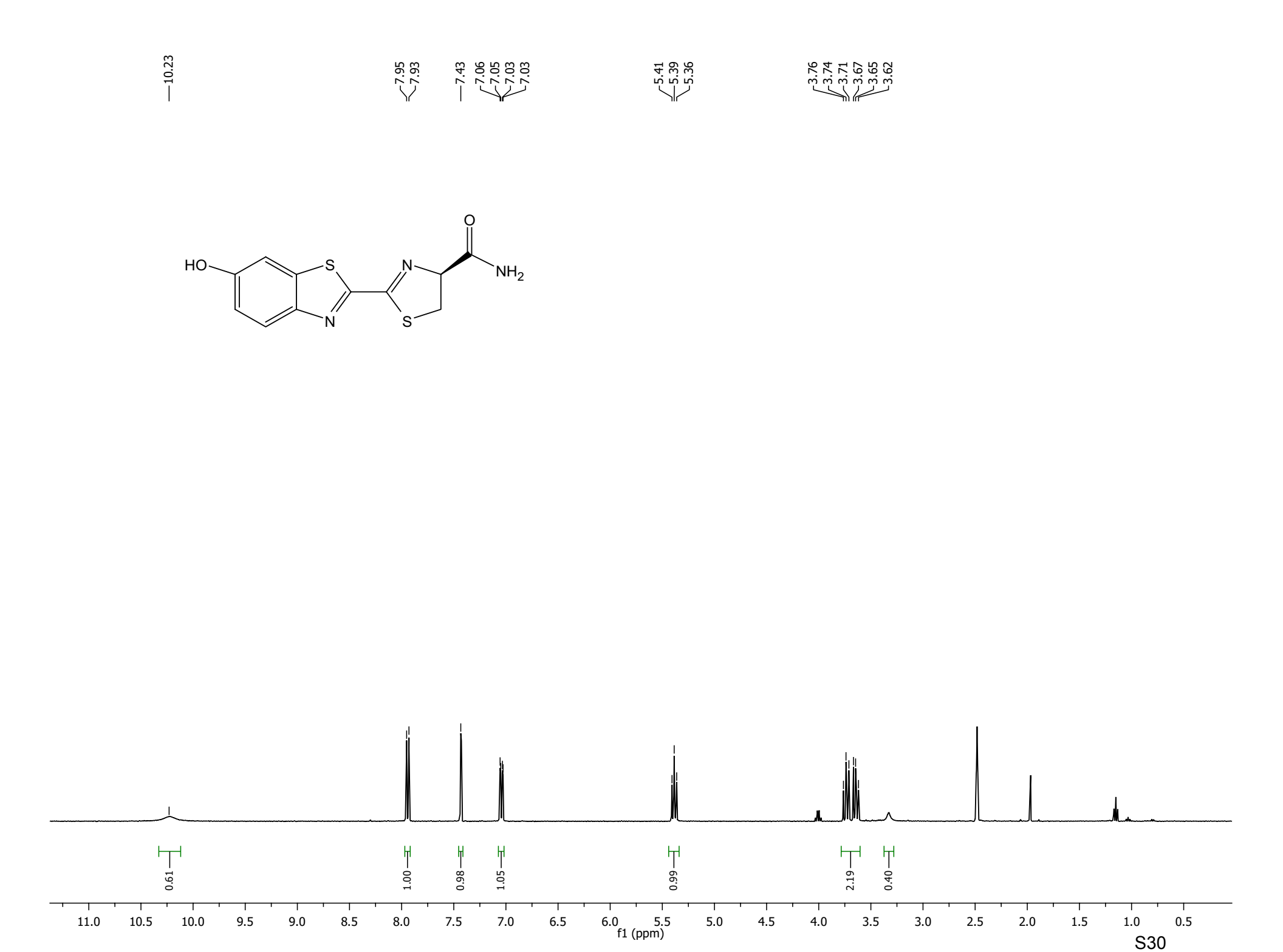
f1 (ppm)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3.65
3.62
HO
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N
S
O
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1.05
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0.40
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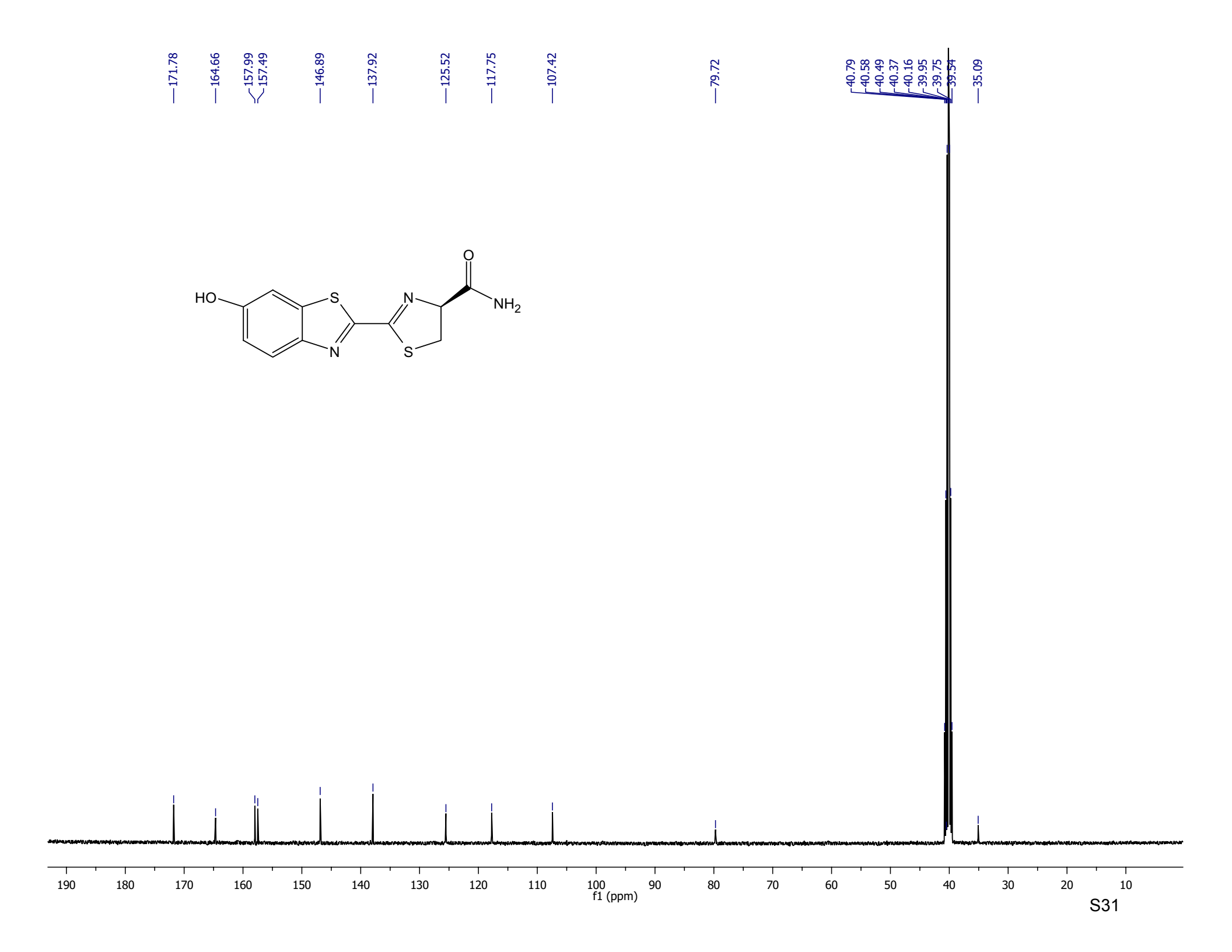
f1 (ppm)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39.95
39.75
39.54
35.09
HO
S
N
N
S
O
NH2
190
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60
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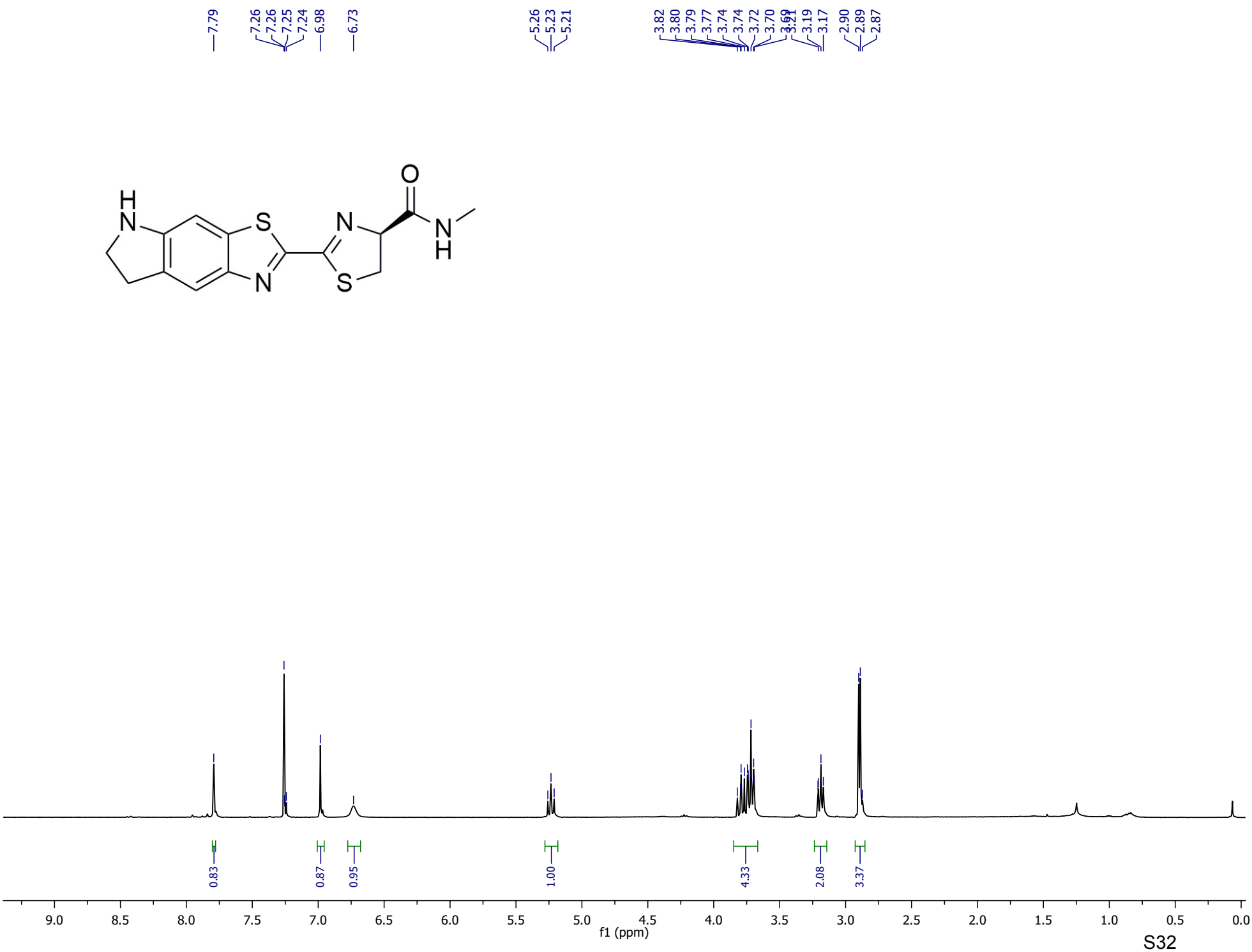
f1 (ppm)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H
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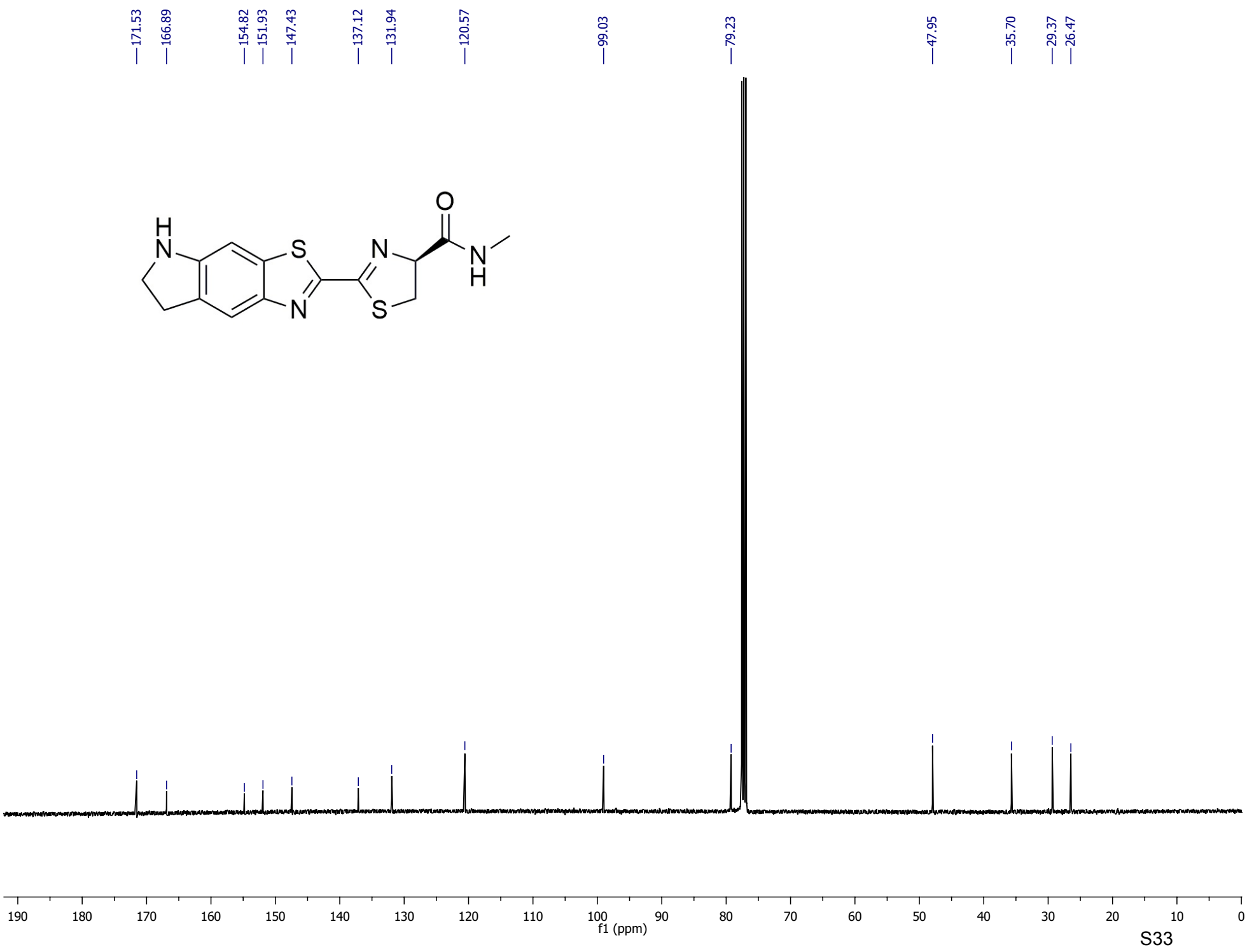
0.0
f1 (ppm)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S
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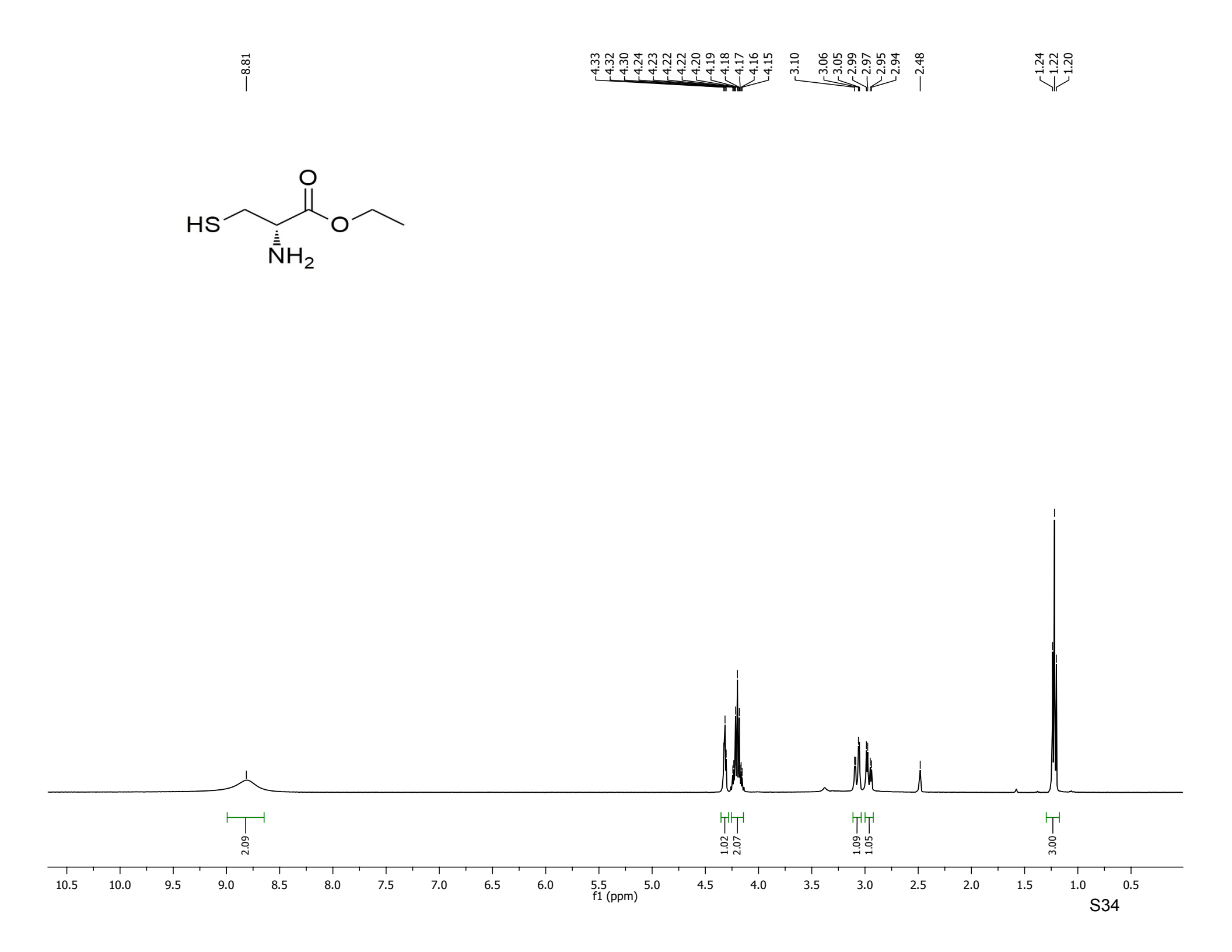
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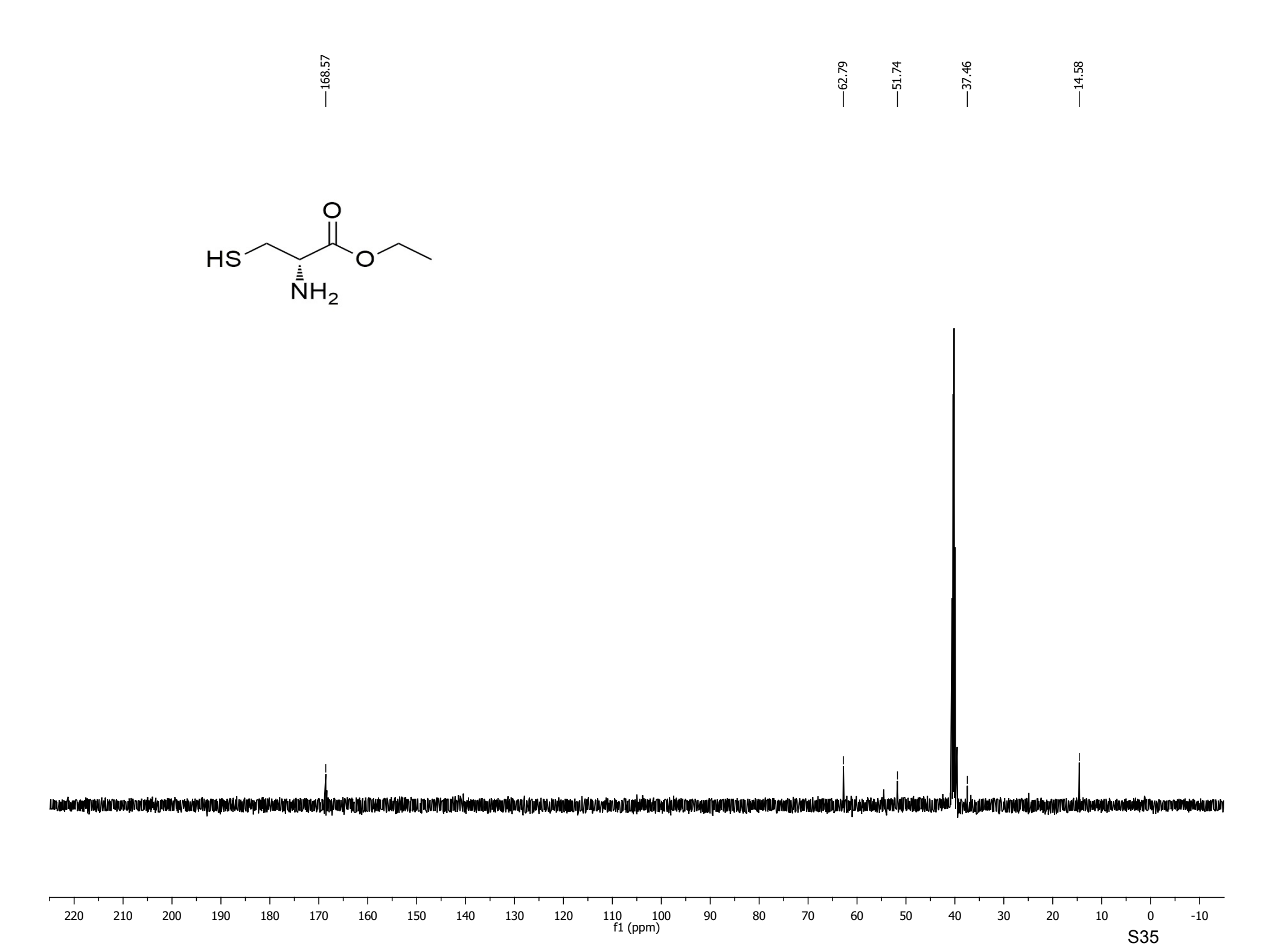
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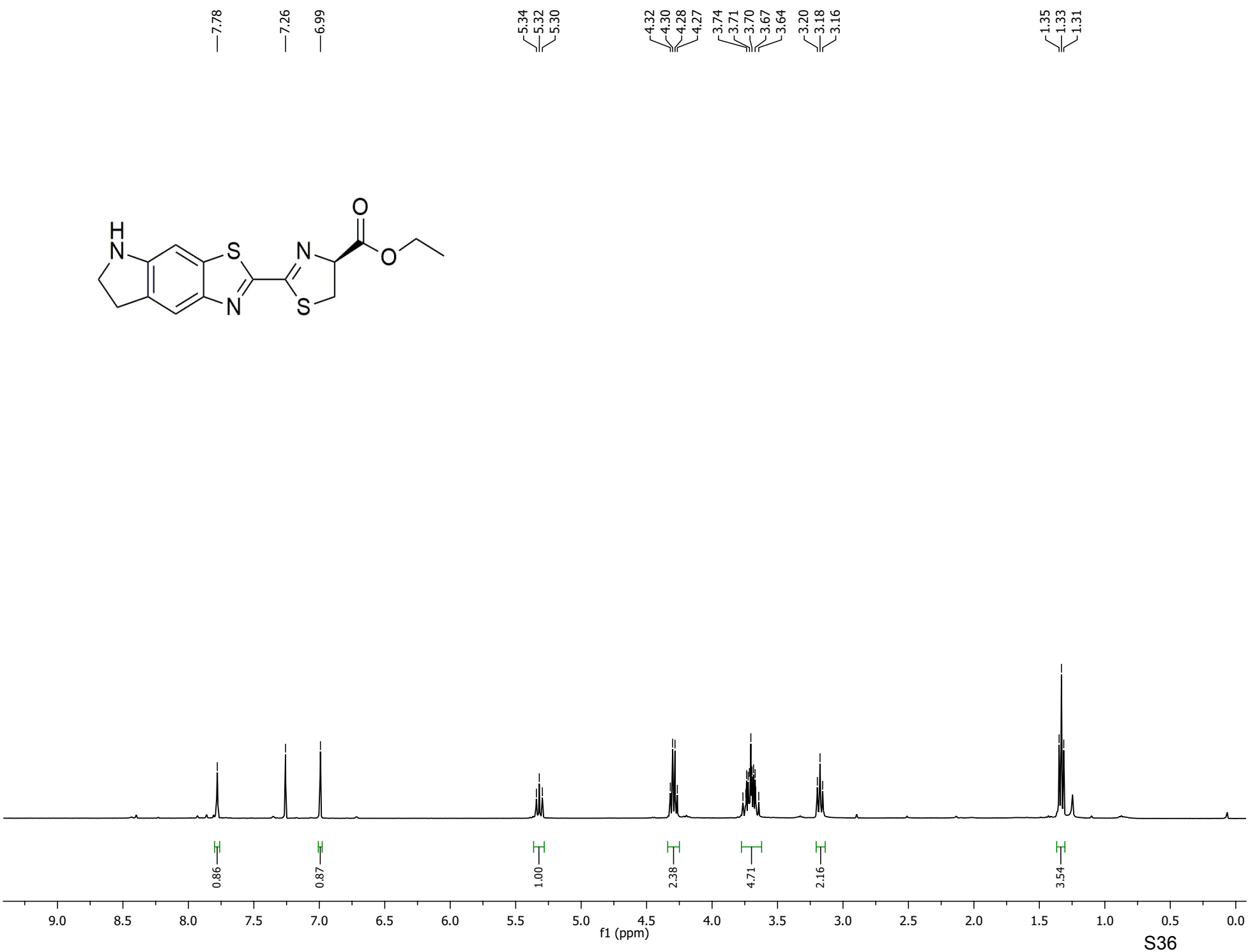
f1 (ppm)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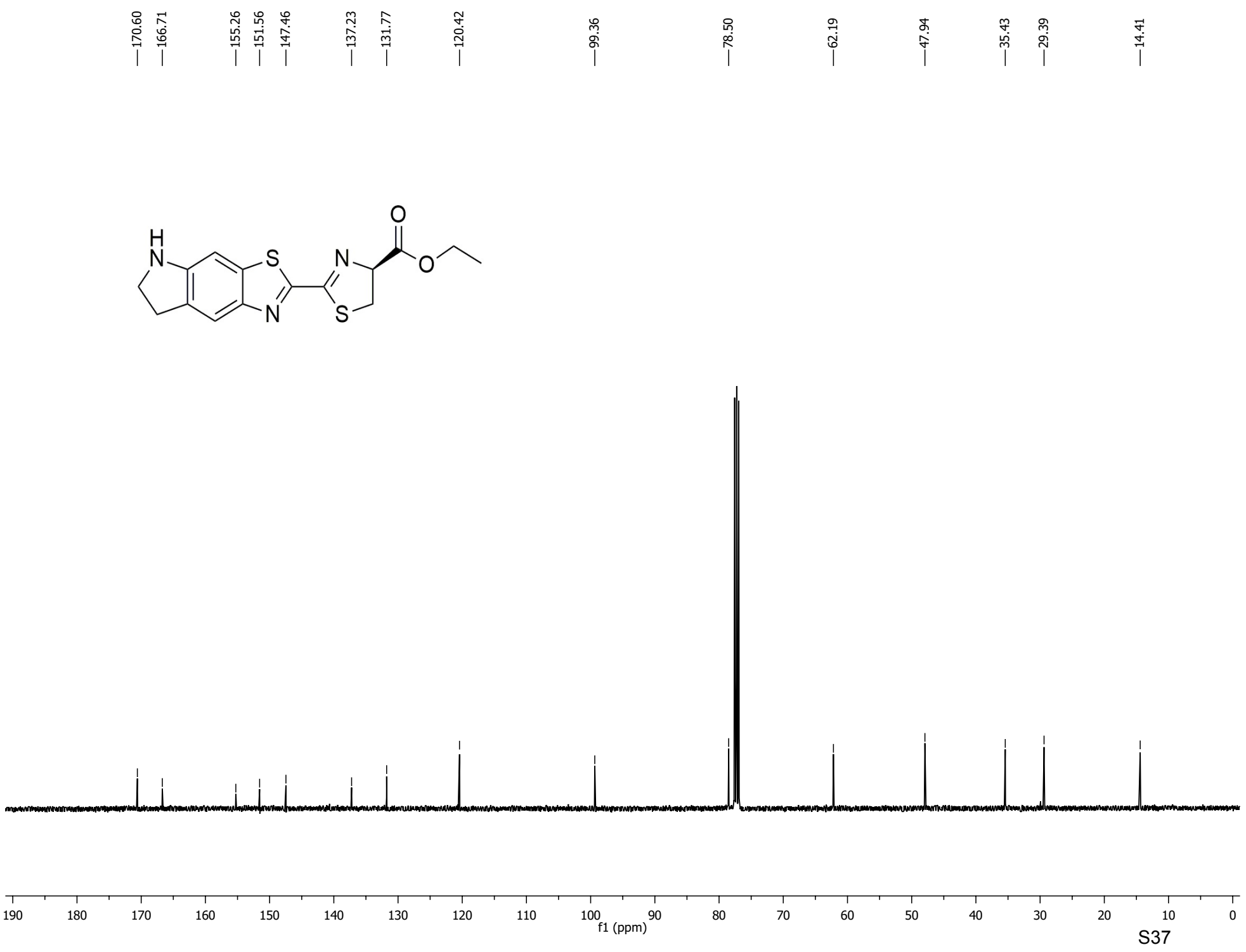
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f1 (ppm)