Heme-Coordinating Inhibitors of Neuronal Nitric Oxide Synthase. Iron-Thioether Coordination is Stabilized by Hydrophobic Contacts Without Increased Inhibitor Potency

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CONTENTS:

Supplementary Table S1 Crystallographic data collection and refinement statistics

Data set[1]	nNOS-**1**	nNOS-**3**	nNOS-**4**	nNOS-**5**
Data collection				
PDB code	3JT3	3JT4	3JT5	3JT6
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions				
a, *b*, *c* (Å)	51.6, 111.1, 164.3	52.0, 111.2, 164.3	52.0, 111.2, 164.4	51.8, 110.8, 164.6
Resolution (Å)	2.15(2.19-2.15)	1.80(1.83-1.80)	2.10(2.14-2.10)	2.20(2.24-2.20)
R_{sym} or R_{merge}	0.054 (0.55)	0.074 (0.47)	0.078 (0.64)	0.097 (0.59)
$I / \sigma I$	10.4 (2.2)	12.9 (2.0)	7.9 (3.2)	7.2 (1.9)
No. unique reflections	50,880	87,410	56,332	47,686
Completeness (%)	97.1 (86.6)	97.9 (86.2)	99.9 (100.0)	97.0 (82.5)
Redundancy	4.4 (3.8)	5.5 (3.2)	8.1 (8.3)	3.9 (3.8)
Refinement				
Resolution (Å)	2.15	1.80	2.10	2.20
No. reflections used	48.281	82,962	53,481	45,262
R_{work} / R_{free}	0.194/0.248	0.199/0.231	0.174/0.210	0.187/0.244
No. atoms				
Protein	6,659	6,702	6,671	6,671
Ligand/ion	157	161	162	161
Water	163	432	330	200
R.m.s. deviations				
Bond lengths (Å)	0.014	0.013	0.013	0.014
Bond angles (°)	1.641	1.371	1.387	1.554

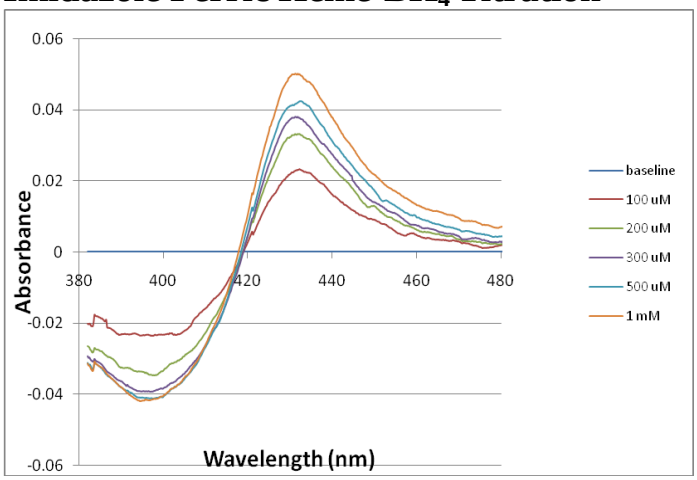
	nNOS-**6**	nNOS-**8**	Reduced nNOS -**4**	Reduced nNOS -**5**
Data collection				
PDB code	3JT7	3JT8	3JT9	3JTA
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions				
a, *b*, *c* (Å)	51.9, 110.6, 164.0	52.2, 111.4, 164.7	51.8, 110.9, 164.3	51.8, 111.5, 164.4
Resolution (Å)	2.10(2.14-2.10)	1.95(1.98-1.95)	2.10(2.14-2.10)	2.18(2.22-2.18)
R_{sym} or R_{merge}	0.081 (0.49)	0.064 (0.45)	0.074(0.51)	0.069(0.44)
$I / \sigma I$	7.8 (1.8)	9.0 (1.7)	7.7(2.0)	9.9(1.8)
No. unique reflections	56,046	70,347	55,755	50,917
Completeness (%)	99.7 (97.8)	99.1 (94.5)	99.1/99.1	99.4(95.7)
Redundancy	4.0 (3.6)	4.0 (3.7)	4.1/4.0	4.4(3.8)
Refinement				
Resolution (Å)	2.10	1.95	2.10	2.18
No. reflections used	53,221	66,790	52,945	48,311
R_{work} / R_{free} [2]	0.171/0.213	0.187/0.222	0.185/0.228	0.195/0.243
No. atoms				
Protein	6,676	6,674	6,707	6,671
Ligand/ion	169	157	149	161
Water	476	411	232	103
R.m.s. deviations				
Bond lengths (Å)	0.011	0.012	0.014	0.014
Bond angles (°)	1.302	1.334	1.467	1.517

[1] See Table 1 for chemical formula of inhibitors. Reduced nNOS refers to the data collected with the dithionite reduced crystals.

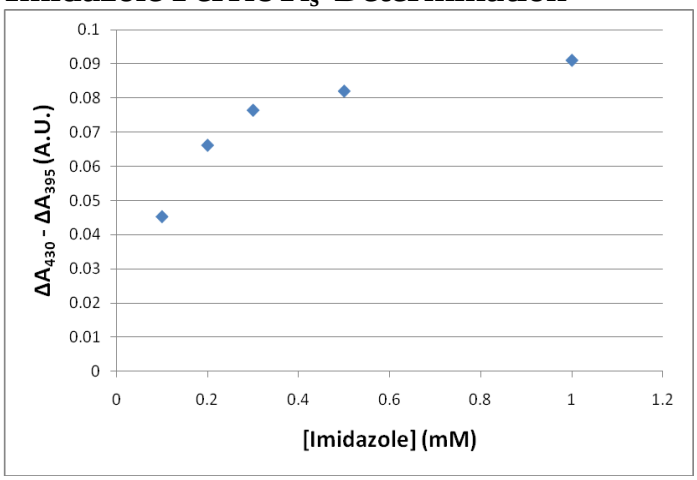
[2] R_{free} was calculated with the 5% of reflections set aside throughout the refinement. The set of reflections for the R_{free} calculation were kept the same for all data sets according to those used in the data of the starting model.

Supplementary Figure S1: Difference spectra and Lineweaver-Burk plots for imidazole, L-nitroarginine, **1**, and **5-8**

Imidazole Ferric Heme-BH_4 Titration

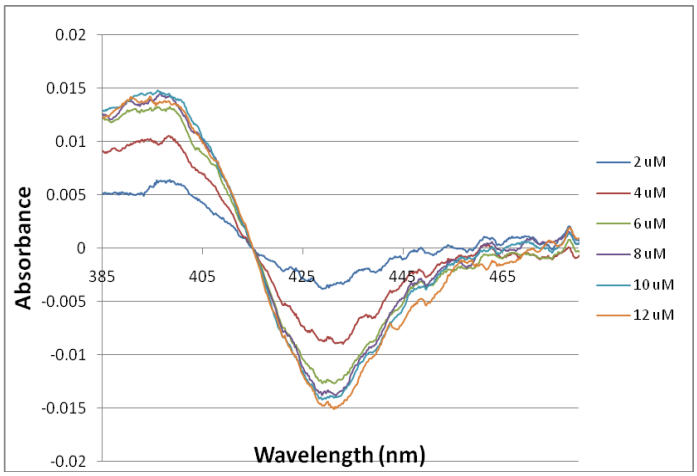


Imidazole Ferric K_s Determination

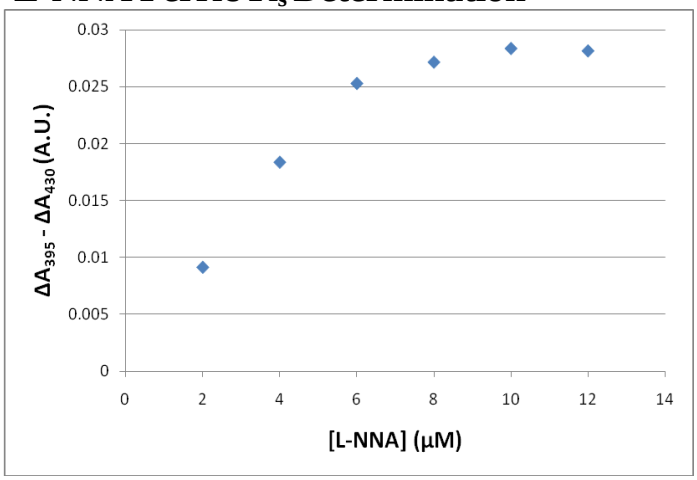


Imidazole Ferric K_s = 120 ± 20 µM
(K_s from experiment depicted above: 112 µM)

L-NNA Ferric Heme-Imidazole Titration



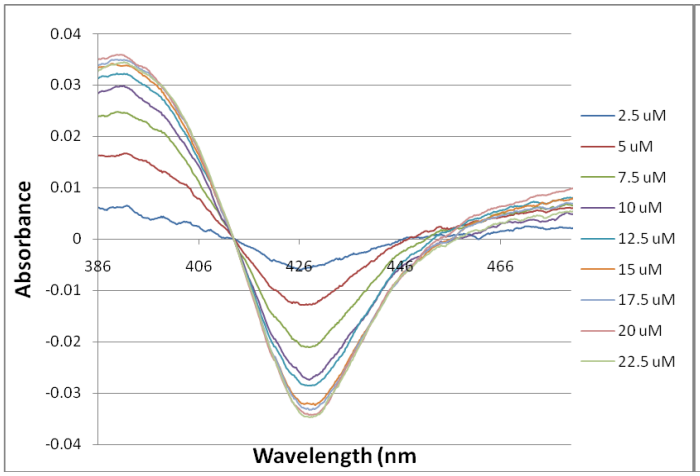
L-NNA Ferric K_s Determination



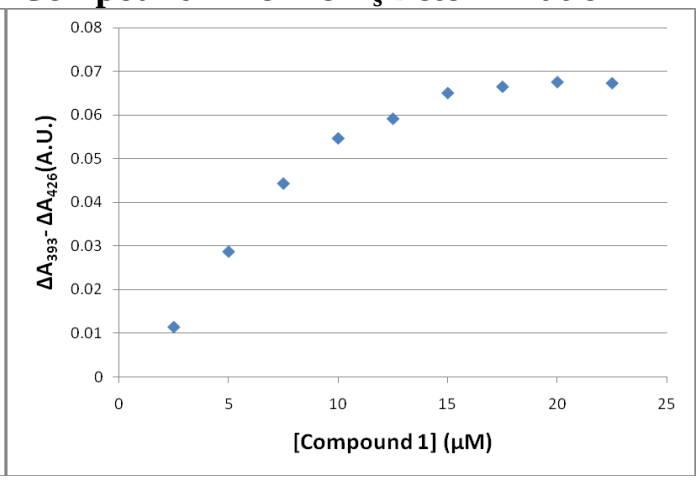
Spectra were normalized to zero absorbance at 415 nm.
The actual K_s was calculated from the apparent K_s as described.[1]

L-NNA Ferric K_s = 1.07 ± 0.01
(K_s from experiment depicted above: 1.06 µM)

Compound 1 Ferric Heme-Imidazole Titration



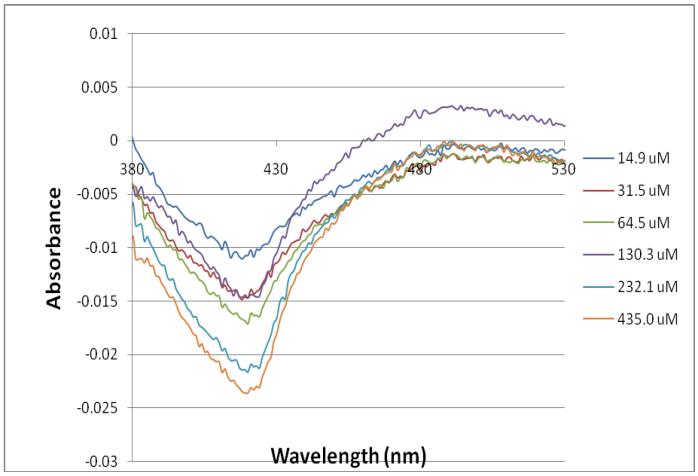
Compound 1 Ferric K_s Determination



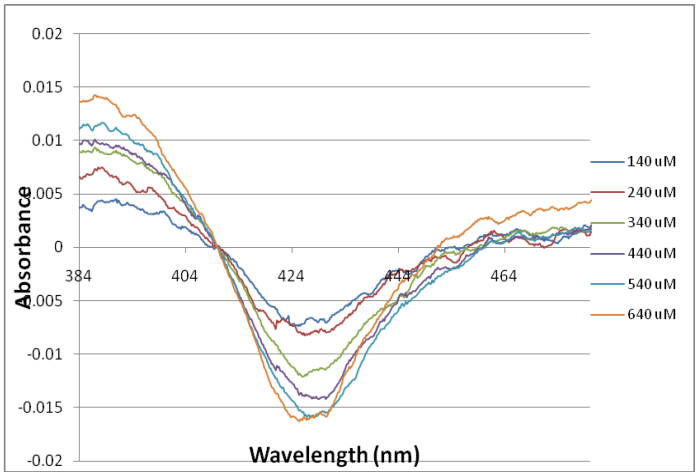
Spectra were not normalized.
Imidazole concentration was 300 μM.
The actual K_s was calculated from the apparent K_s as described.[1]

Compound 1 Ferric K_s = 2.2 ± 0.2 μM
(K_s from experiment depicted above: 2.1 μM)

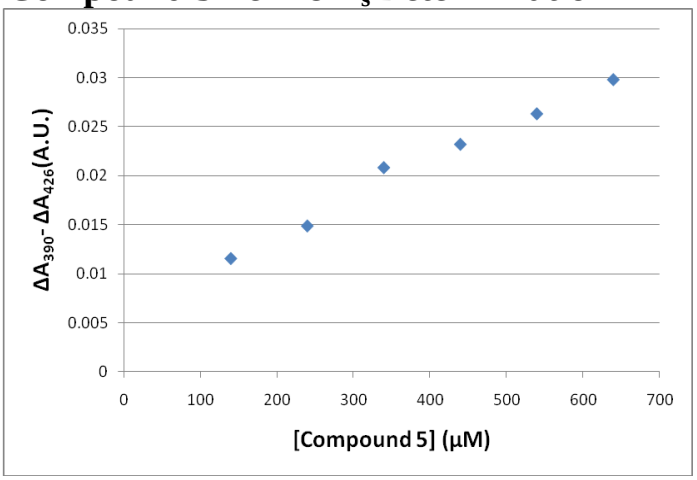
Compound 1 Ferrous Heme-BH_4 Titration



Compound 5 Ferric Heme-Imidazole Titration



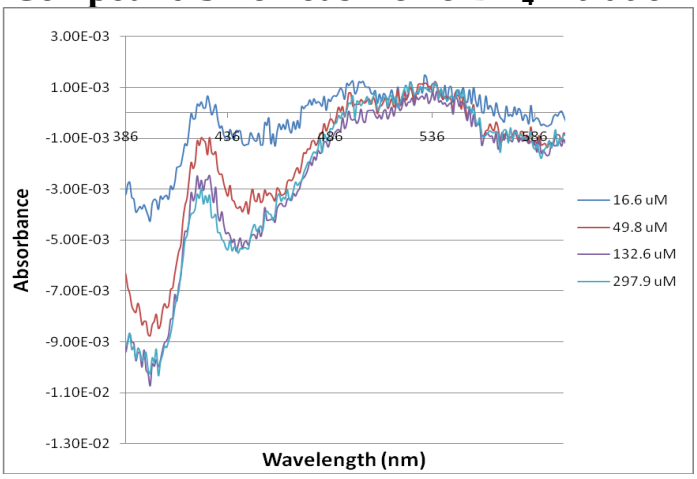
Compound 5 Ferric K_s Determination



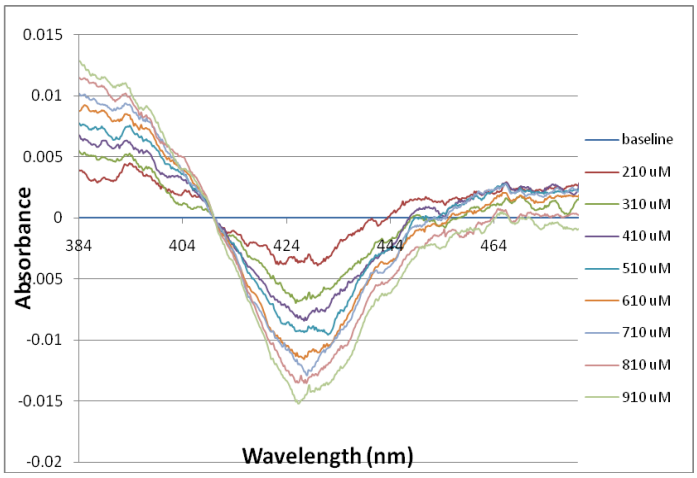
Spectra were normalized to zero absorbance at 410 nm.
Imidazole concentration was 300 uM.
The actual K_s was calculated from the apparent K_s as described.[1]

Compound 5 Ferric K_s = 170 ± 10 μM
(K_s calculated from the experiment depicted above: 180 μM)

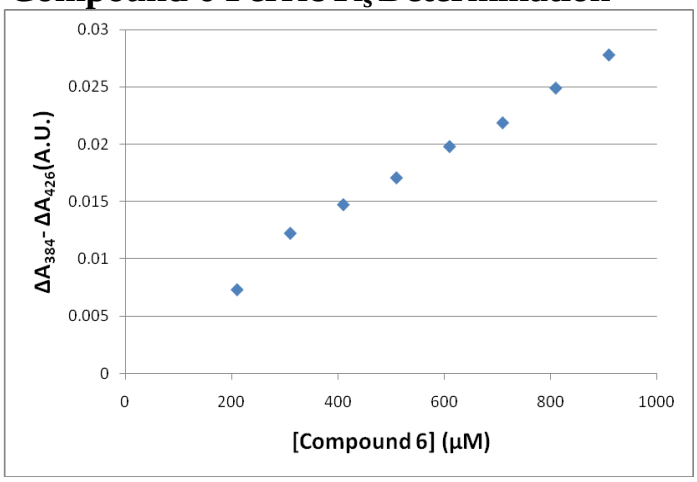
Compound 5 Ferrous Heme-BH_4 Titration



Compound 6 Ferric Heme-Imidazole Titration



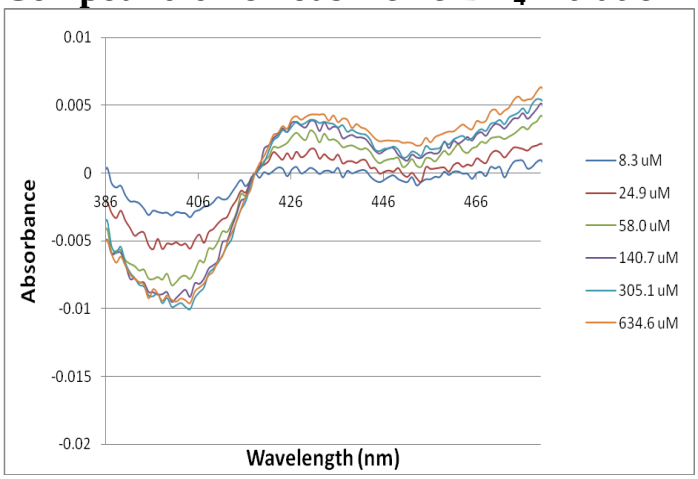
Compound 6 Ferric K_s Determination



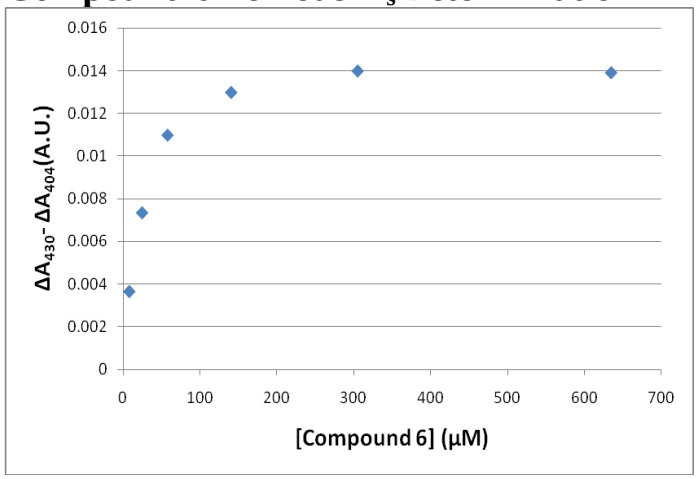
Spectra were normalized to zero absorbance at 410 nm.
Imidazole concentration was 300 uM.
The actual K_s was calculated from the apparent K_s as described.[1]

Compound 6 Ferric K_s = 550 ± 50 μM
(K_s calculated from the experiment depicted above: 570 μM)

Compound 6 Ferrous Heme-BH_4 Titration



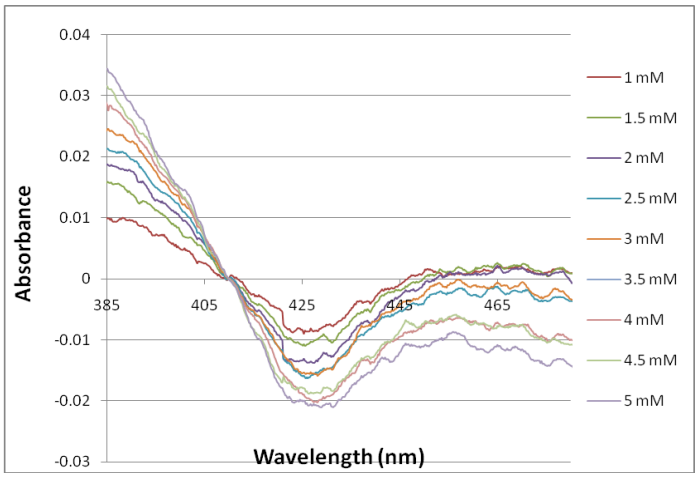
Compound 6 Ferrous K_s Determination



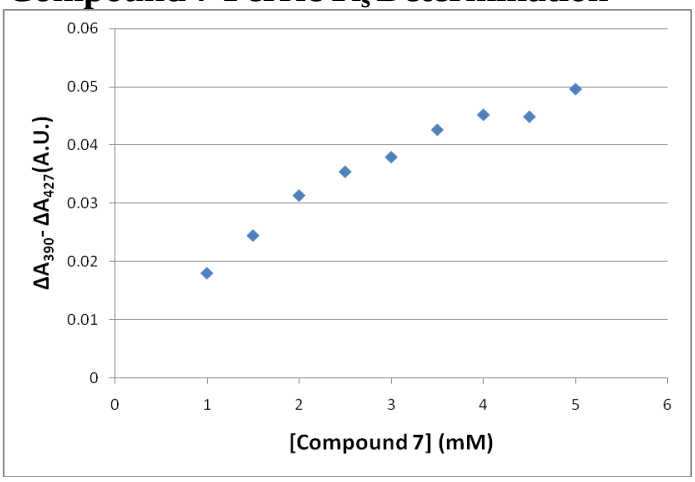
Spectra were normalized to zero absorbance at 418 nm.

Compound 6 Ferrous K_s = 22 ± 2 μM
(K_s calculated from the experiment depicted above: 20. μM)

Compound 7 Ferric Heme-Imidazole Titration



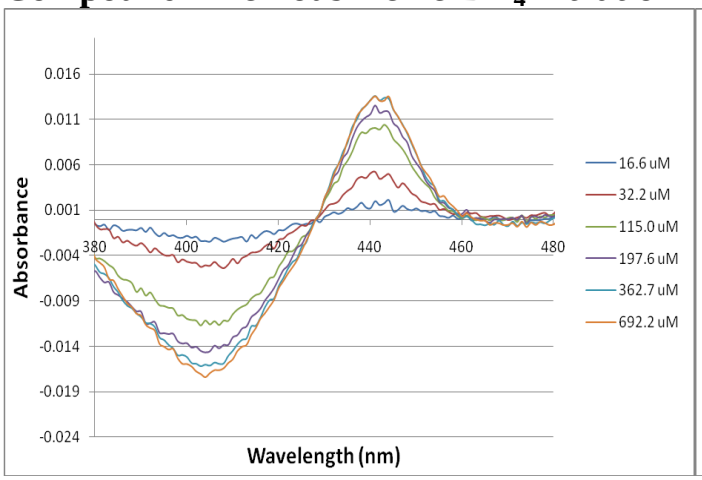
Compound 7 Ferric K_s Determination



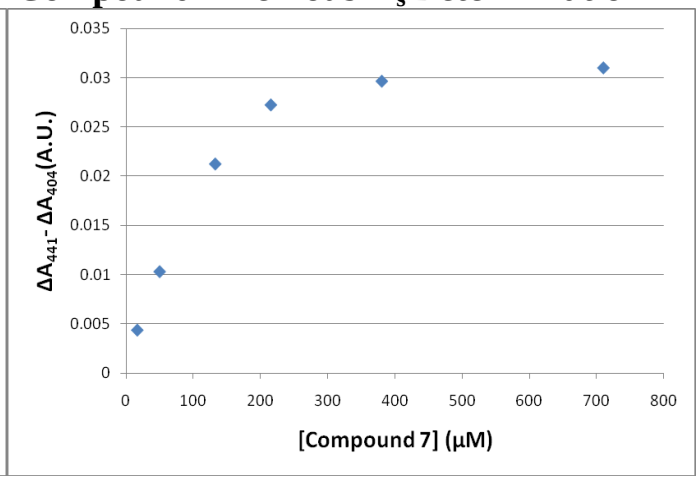
Spectra were normalized to zero absorbance at 410 nm.
Imidazole concentration was 10 uM.
Absorption due to **7** was subtracted out for each curve, and the absorptions were scaled to account for volume change.
The actual K_s was calculated from the apparent K_s as described.[1]

Compound 7 Ferric K_s = 1940 ± 90 µM
(K_s from experiment depicted above: 2010 µM)

Compound 7 Ferrous Heme-BH_4 Titration



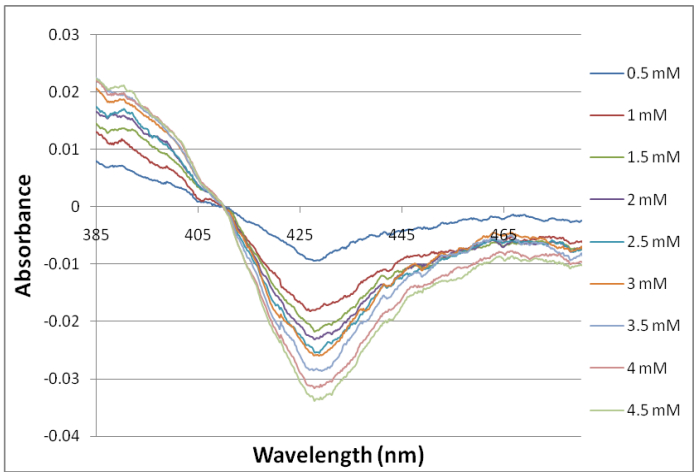
Compound 7 Ferrous K_s Determination



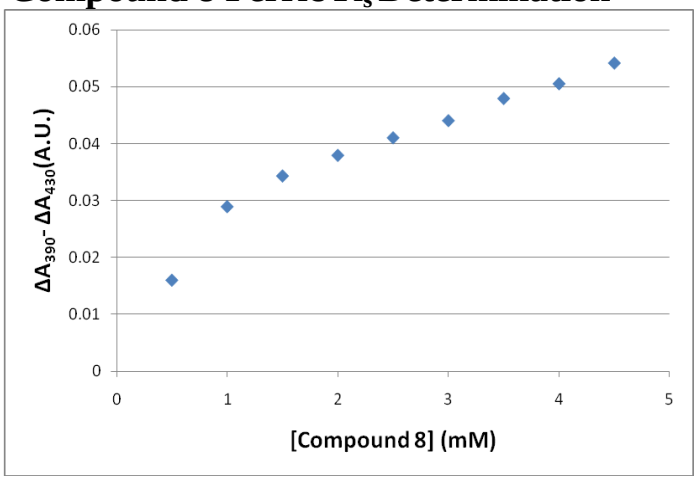
Spectra were normalized to zero absorbance at 428 nm.

Compound 7 Ferrous K_s = 110. ± 9 µM
(K_s from experiment depicted above: 104 µM)

Compound 8 Ferric Heme-Imidazole Titration



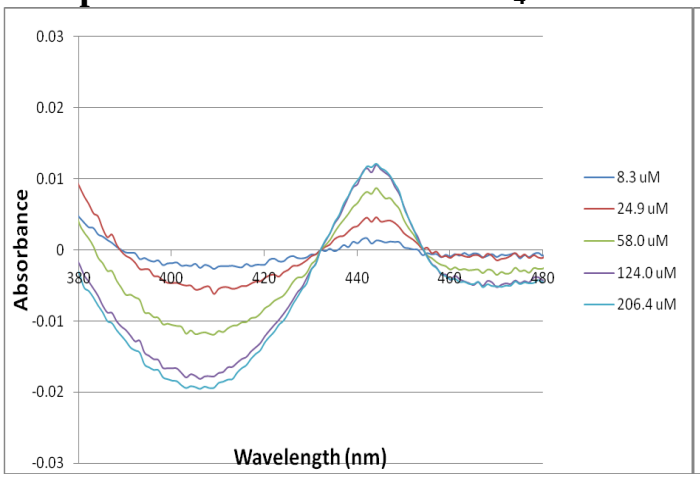
Compound 8 Ferric K_s Determination



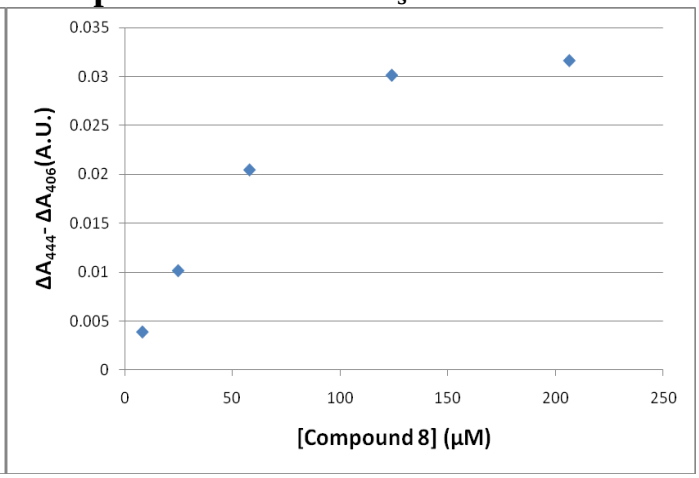
Spectra were normalized to zero absorbance at 410 nm.
Imidazole concentration was 100 uM.
Absorption due to **8** was subtracted out for each curve, and the absorptions were scaled to account for volume change.
The actual K_s was calculated from the apparent K_s as described.[1]

Apparent Compound 8 Ferric K_s = 1010 ± 40 µM
(Apparent K_s from experiment depicted above: 990 µM)

Compound 8 Ferrous Heme-BH_4 Titration



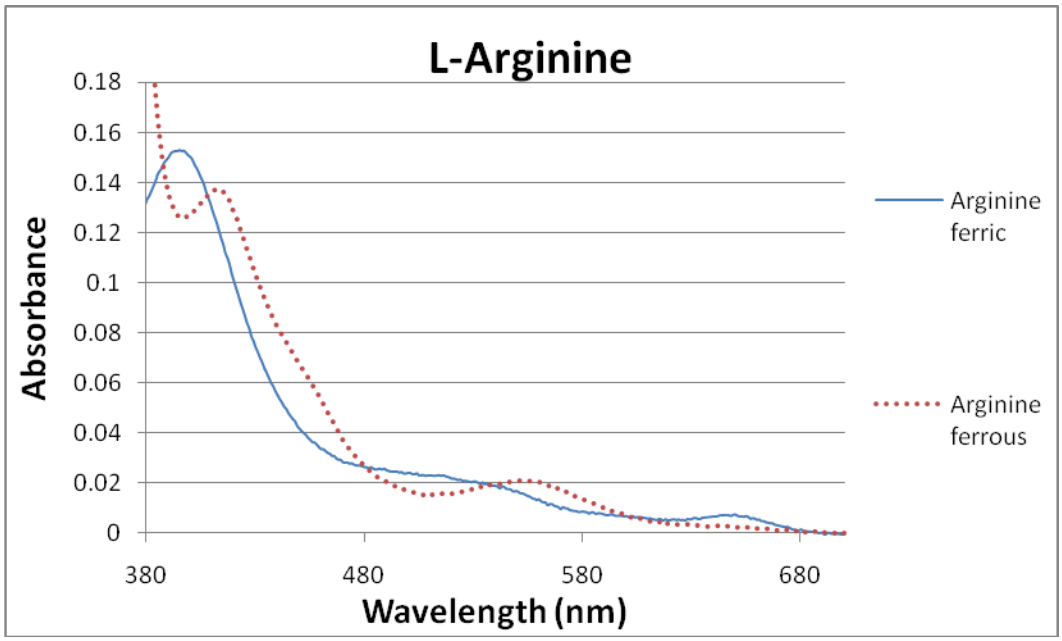
Compound 8 Ferrous K_s Determination

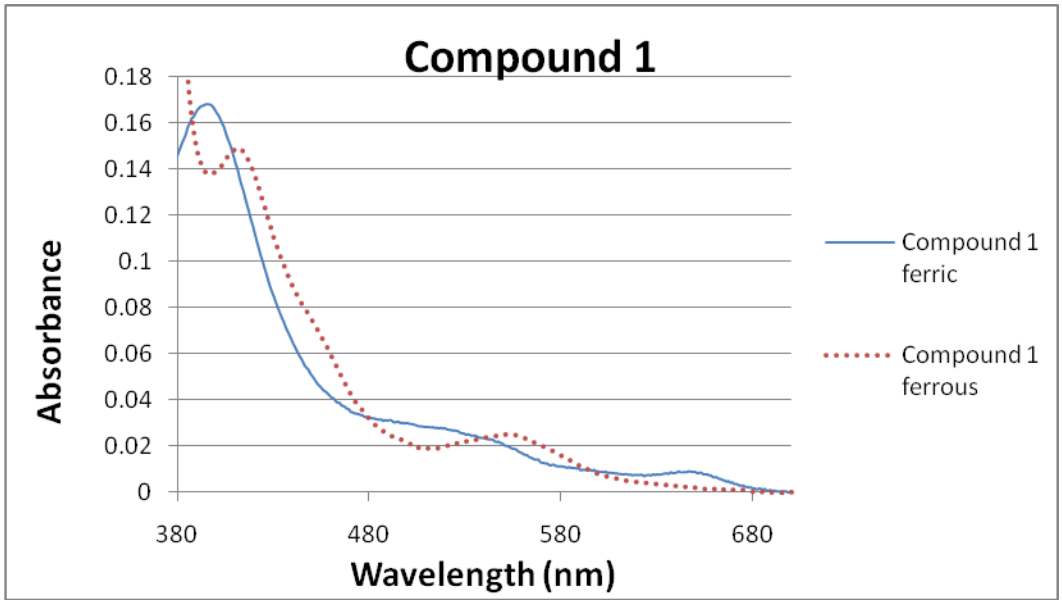


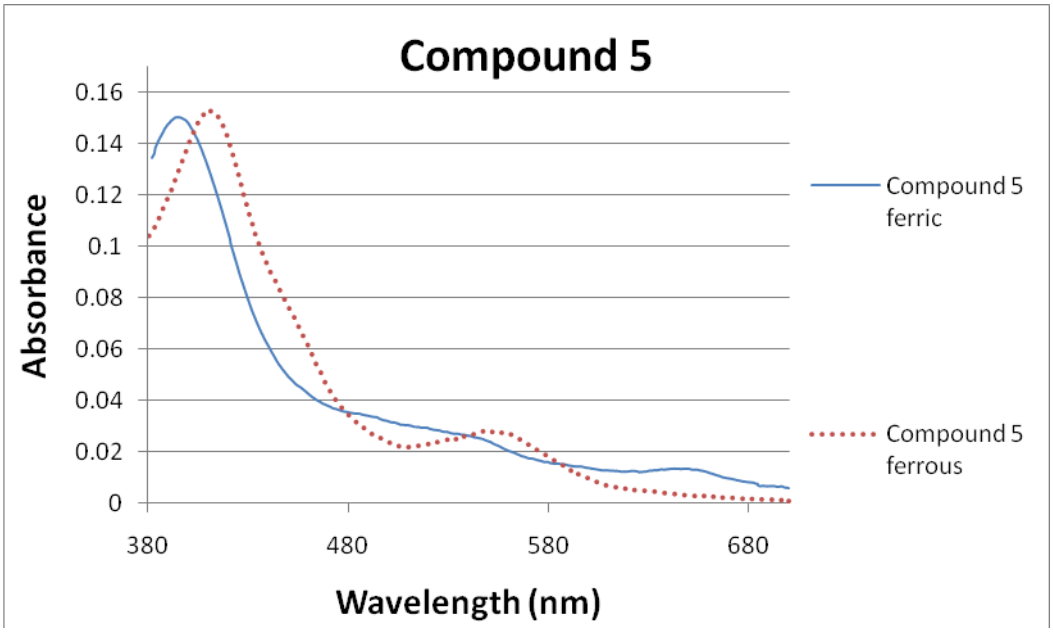
Spectra were normalized to zero absorbance at 432 nm.

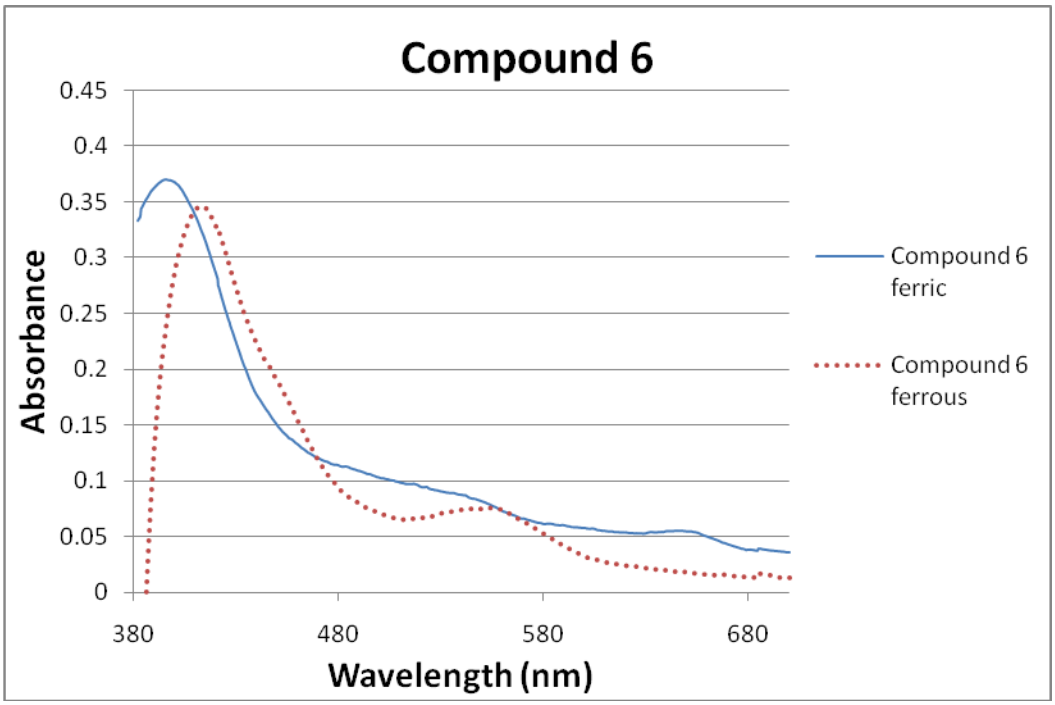
Compound 8 Ferrous K_s = 77 ± 8 µM
(K_s from experiment depicted above: 82 µM)

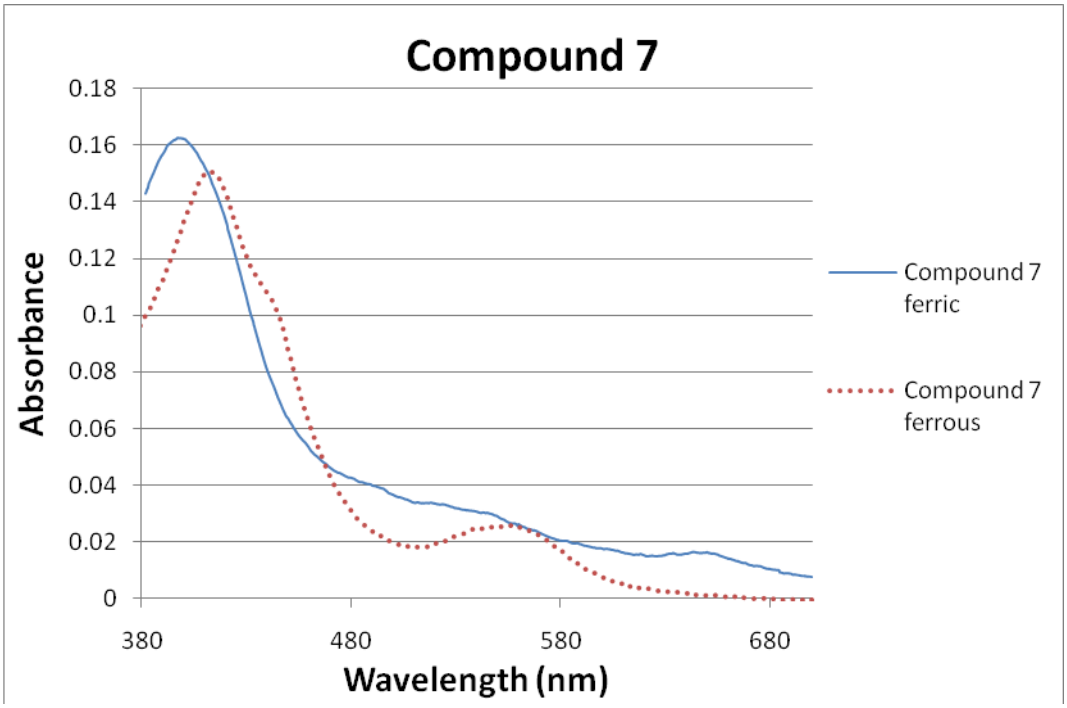
Supplementary Figure S2: Absolute absorption spectra for L-arginine (control), **1**, and **5-7** over the full visible light range.



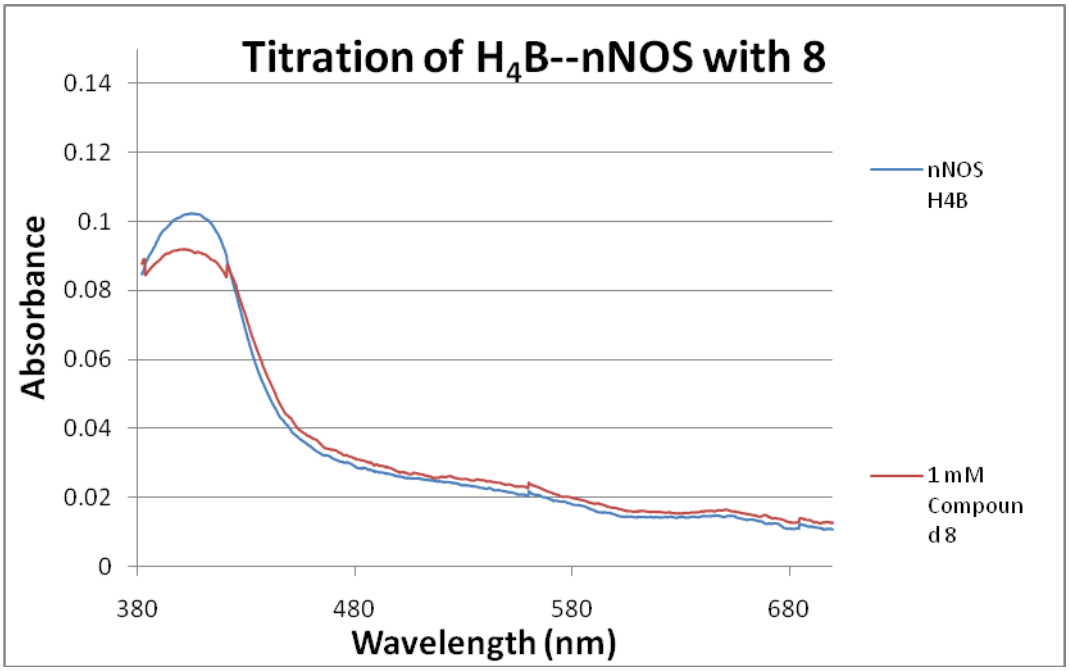



Compound 5
Absorbance
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
380
480
580
680
Wavelength (nm)
Compound 5 ferric
Compound 5 ferrous

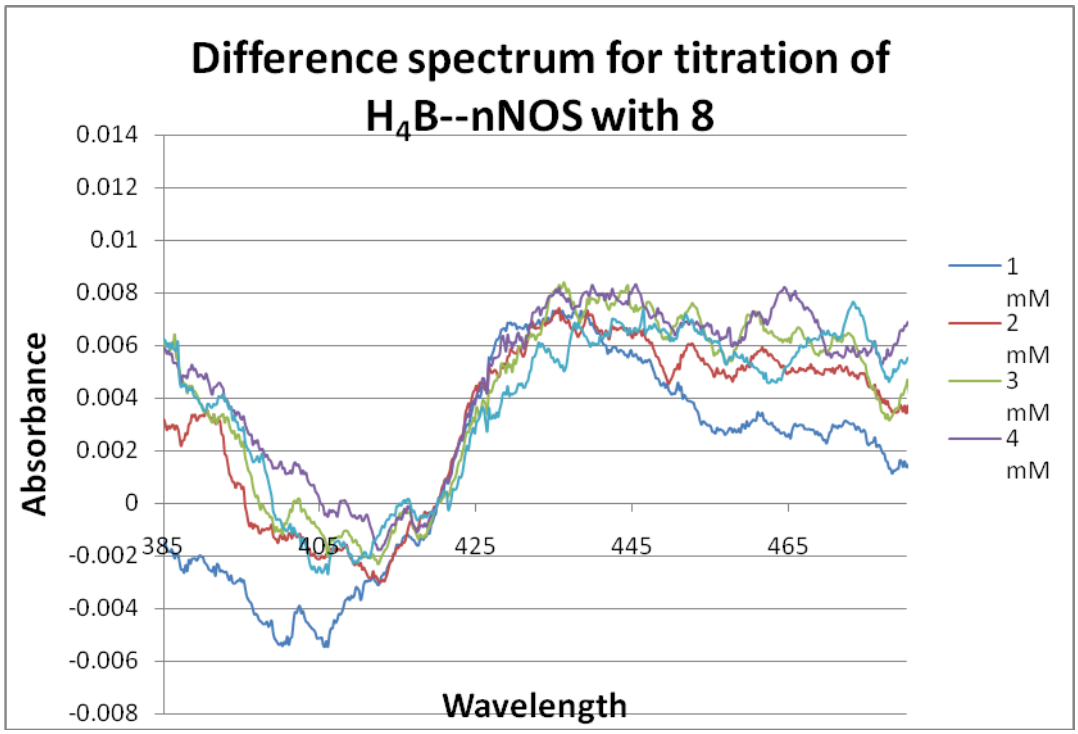

Compound 6
Absorbance
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
380
480
580
680
Wavelength (nm)
Compound 6 ferric
Compound 6 ferrous


Compound 7
Absorbance
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
380
480
580
680
Wavelength (nm)
Compound 7 ferric
Compound 7 ferrous

Supplementary Figure S3: Inverse titration for **8**

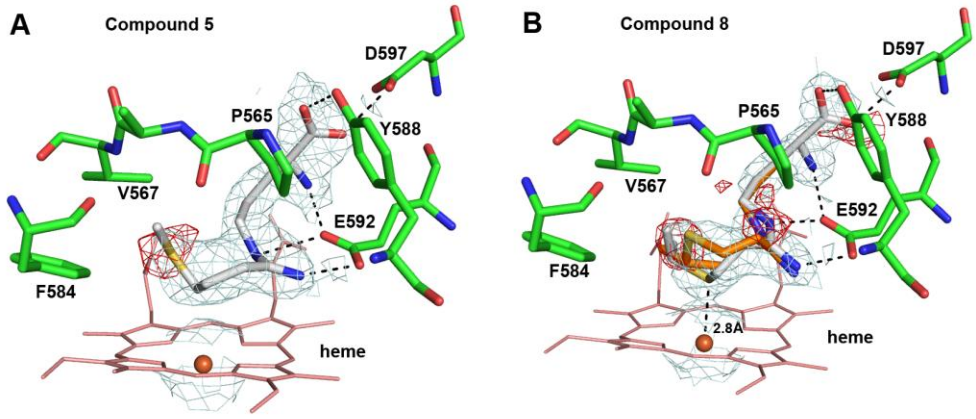


There was a substantial drop in the Soret peak height upon addition of **8**, which was quite different from what was observed with the other type I ferric ligands, for which the Soret peak increased in intensity and was blue-shifted.



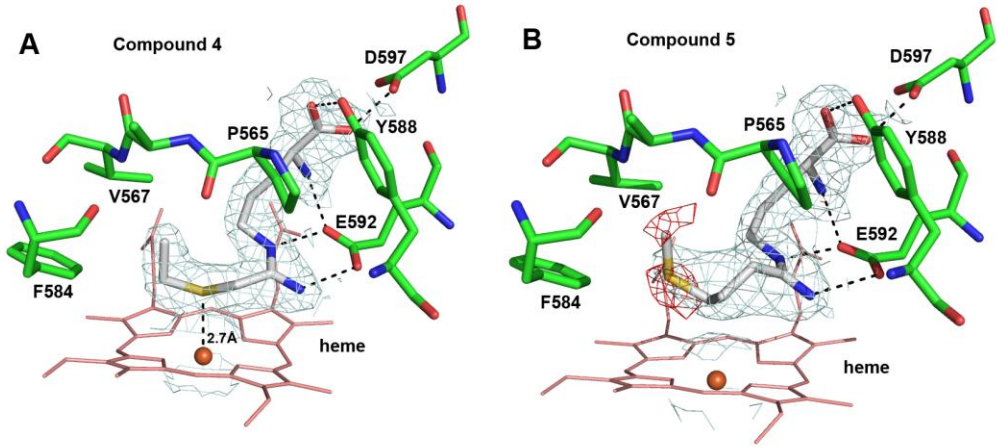
Upon titration of H_4B—nNOS with **8**, the following type II difference spectrum was obtained. Note that spectrum intensity was scaled based on the volume change for this titration, and the absorbance of **8** in the near UV region was subtracted out.

Supplementary Figure S4: Active site structures of nNOS heme domain with **5** (panel A) and **8** (B) bound.



The density maps of 2Fo-Fc (pale cyan) at 1 σ contour level and Fo-Fc (red) at -3.0 σ for the bound inhibitor and for the heme iron atom are shown. Hydrogen bonds and the Fe-S distance are depicted with dashed lines. The inhibitor models shown were built according to partially observed densities. For **5,** the density is missing for the sulfur atom and its methyl tail, thus their locations are uncertain. One conformation of **5** is shown but an alternate tail position is possible. The closest Fe-S distance in the current model is 4.5Å. For **8**, the density is visible only up to where the sulfur atom is because of a clear Fe-S interaction. However, the isopropyl tail was built in so long as it makes tolerable van der Waals contacts with protein. Two alternate conformations were modeled with one of them in a type I (sulfur swings away) mode. The shortest Fe-S distance is 2.8 Å.

Supplementary Figure S5: Active site structures of nNOS heme domain with **4** (panel A) and **5** (B) bound derived from data collected with dithionite reduced crystals.



The density maps of 2Fo-Fc (pale cyan) at 1 σ contour level and Fo-Fc (red) at -3.0 σ for the bound inhibitor and the heme iron atom are shown. Hydrogen bonds and the Fe-S distance are depicted with dashed lines. For **4** hardly any difference can be found in its binding mode compared to the nNOS – **4** structure using an untreated crystal except that the Fe-S distance is slightly shorter at 2.7 Å. The density for **5** looks almost identical in structures with or without dithionite reduction; the thioether tail is disordered beyond the sulfur atom. Data were collected to 2.4 Å resolution with a reduced nNOS crystal for compound **3** as well, but the data quality was poor; thus, the refinement was not completed. From the available density it seems that the alternate type I conformation has been eliminated. The type II binding mode gives a Fe-S distance of about 2.7 Å, essentially no different from that in the untreated structure.

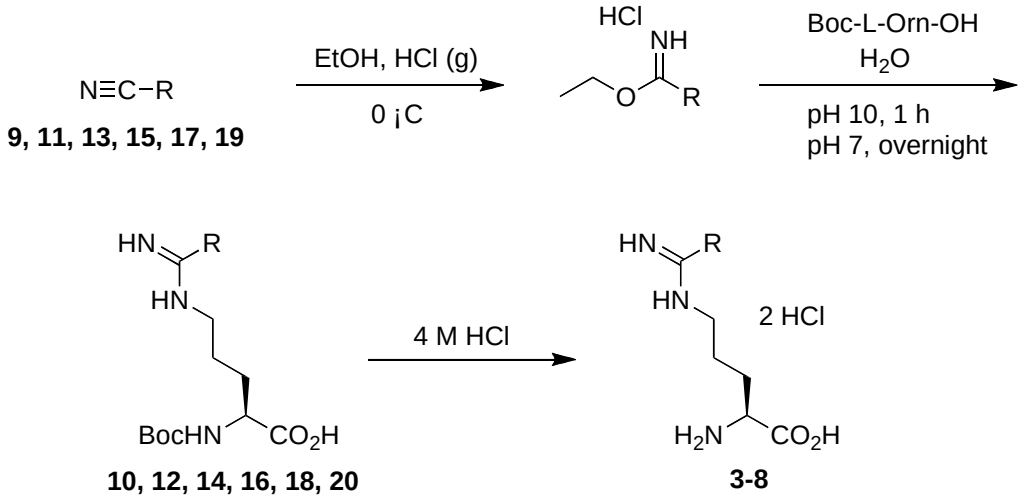
Experimental Section

CHEMICAL SYNTHESIS

Materials and analytical methods.

All reagents and solvents were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI) or Novabiochem (Gibbstown, NJ) and were used without further purification. ^{1}H NMR spectra were recorded in $CDCl_3$ or D_2O on a Varian Mercury 400 MHz, Varian Inova 500 MHz, or P Inova 500 MHz spectrometer (100.6 MHz for ^{13}C NMR on the Mercury 400, and 125.7 MHz for ^{13}C NMR on the Inova 500 and P Inova 500). All ion exchange chromatography was performed using Dowex 50WX8-200 resin. Chemical shifts are reported as δ values in parts per million with the $CDCl_3$ and D_2O peaks set at 7.26 and 4.80 ppm, respectively. An Orion research model 701H pH meter with a general combination electrode was used for pH measurements. High-resolution mass spectra were carried out using an Agilent (Wilmington, DE) 6210 ToF-LC/MS mass spectrometer; analyses were performed in the positive ion ESI mode using the reflectron configuration.

Synthetic Scheme



9: R = -$(CH_2)_2SCH_2CH_3$ **10**: R = -$(CH_2)_2SCH_2CH_3$ **3**: R = -$(CH_2)_2SCH_2CH_3$

11: R = -$CH_2SCH_2CH_3$ **12**: R = -$CH_2SCH_2CH_3$ **4**: R = -$CH_2SCH_2CH_3$

13: R = -$(CH_2)_3SCH_3$ **14**: R = -$(CH_2)_3SCH_3$ **5**: R = -$(CH_2)_3SCH_3$

15: R = -$CH_2S(CH_2)_2CH_3$ **16**: R = -$CH_2S(CH_2)_2CH_3$ **6**: R = -$CH_2S(CH_2)_2CH_3$

17: R = -$(CH_2)_2S(CH_2)_2CH_3$ **18**: R = -$(CH_2)_2S(CH_2)_2CH_3$ **7**: R = -$(CH_2)_2S(CH_2)_2CH_3$

19: R = -$(CH_2)_2SCH(CH_3)_2$ **20**: R = -$(CH_2)_2SCH(CH_3)_2$ **8**: R = -$(CH_2)_2SCH(CH_3)_2$

3-Ethylmercaptopropionitrile (9)

Ethanethiol (2.52 g, 41 mmol) and catalytic NaOMe (0.20 g, 3.7 mmol) were added to a 100-mL round-bottomed flask, and the solution was cooled to 0 °C. Acrylonitrile (4.72 g, 81 mmol) was added dropwise over a period of approximately 10 min, during which time the temperature of the reaction was not allowed to exceed 10 °C. The reaction was allowed to come to room temperature over the course of 10 h with stirring. The solution was concentrated using rotary evaporation, then placed on an oil pump overnight to yield **9** (4.58 g, 97%) as an orange liquid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 2.80 (t, *J* = 7.0 Hz, 2H), 2.66-2.63 (m, 4H), 1.29 (t, *J* = 7.5Hz, 3H).

N^1-Boc-N^5-(1-Imino-3-(ethylthio)propyl)-L-ornithine (10)

A 250-mL three-necked flask was charged with **9** (10.32 g, 90 mmol) and anhydrous, reagent grade ethanol (50.0 mL, 911 mmol). The solution was cooled to 0 °C and allowed to stir for approximately 10 min. HCl gas was then bubbled through the reaction solution for 1 h. The reaction solution was allowed to warm to room temperature overnight with stirring. Nitrogen was blown over the solution for several hours to remove excess solvent, and the remaining white solid was dried on an oil pump to yield ethyl imidic ester **10** (12.92 g, 89%), which was used in the next step without further purification. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 12.50 (br s, 1H), 11.66 (br s, 1H), 4.67 (q, *J* = 7.0 Hz, 2H), 3.07 (t, *J* = 7.0 Hz, 2H), 2.90 (t, *J* = 7.0 Hz, 2H), 2.65 (q, *J* = 7.0 Hz, 2H), 1.50 (t, *J* = 7.0 Hz, 3H), 1.27 (t, *J* = 7.5 Hz, 3H).

N^{α}-Boc-L-Orn-OH[23] (0.72 g, 3.1 mmol) and deionized water (5 mL) were added to a 20 mL vial. The mixture was stirred at room temperature until all of the N^{α}-Boc-L-Orn-OH was dissolved. NaOH (2.5 M) was added to the solution until the pH was above 10.0. A solution of the ethyl imidic ester (1.54 g, 7.8 mmol) in 2 mL of anhydrous ethanol was added dropwise to the N^{α}-Boc-L-Orn-OH solution. Additional NaOH (2.5 M) was added as needed to keep the pH of the reaction solution above 10.0. When the dropwise addition of the ethyl imidic ester solution was complete, the solution was stirred for 1 h at pH 10.5, neutralized using 1 M HCl, and left to stir overnight. The solvent was removed by rotary evaporation, and the crude material was dissolved in 30 mL of deionized water and washed with ethyl acetate (3 x 30 mL). One-eighth of the crude material was purified further using cation exchange chromatography with 5% aqueous pyridine elution to yield **10** (0.090 g, 76%) as a hygroscopic white solid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.89 (m, 1 H), 3.30 (t, *J* = 5.5 Hz, 2 H), 2.89 (t, *J* = 7.0 Hz, 2 H), 2.76 (t, *J* = 7.0 Hz, 2 H), 2.60

(q, *J* = 7.5 Hz, 2 H), 1.90-1.64 (m, 4 H), 1.40 (s, 9 H), 1.23 (t, *J* = 7.0 Hz, 3 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 179.3, 165.9, 157.5, 81.0, 55.4, 41.6, 32.7, 29.2, 27.6, 27.4, 25.2, 23.3, 13.7. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{15}H_{30}N_3O_4S$ 348.1952, found 348.1965.

N^5-(1-Imino-3-(ethylthio)propyl)-L-ornithine (3)

Compound **10** (0.052 g, 0.14 mmol) was dissolved in 4 M HCl (3 mL) and left to stir overnight. The acidic solution was concentrated using rotary evaporation, and residual water was removed using a lyophilizer to provide **3** (0.035 g, quantitative yield) as a hygroscopic yellow solid. ^{1}H-NMR (400 MHz, D_2O) δ 4.13 (t, *J* = 6.4 Hz, 1 H), 3.40 (t, *J* = 6.8 Hz, 2 H), 2.94 (t, *J* = 7.2 Hz, 2 H), 2.81 (t, *J* = 6.8 Hz, 2 H), 2.63 (q, *J* = 7.2 Hz, 2 H), 2.15-2.01 (m, 2 H), 1.93-1.77 (m, 2H), 1.25 (t, *J* = 7.2 Hz, 3 H). ^{13}C-NMR (100 MHz, D_2O): δ 171.7, 166.2, 52.5, 41.3, 32.7, 27.4, 27.0, 25.1, 22.6, 13.7. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{10}H_{22}N_3O_2S$ 248.1427, found 248.1439.

2-(Ethylthio)acetonitrile (11)

Ethanethiol (2.75 mL, 37.1 mmol), NaOMe (2.00g, 37.1 mmol), and methanol (80 mL, 2.18 mol) were added to a 500-mL round-bottomed flask. Bromoacetonitrile (2.07 mL, 29.7 mmol) was then added dropwise over a period of approximately 5 min. The reaction mixture was left to stir for 24 h. The methanol was removed by rotary evaporation, and the resulting material was dissolved in $CHCl_3$, washed with water (4 x 50 mL), and dried over sodium sulfate. The $CHCl_3$ was removed by rotary evaporation to yield **11** (1.61, 54%) as a colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.37 (s, 2 H), 2.79 (q, *J* = 7.5 Hz, 2 H), 1.36 (t, *J* = 7.5 Hz, 3 H).

N^1-Boc-N^5-(1-Imino-3-(ethylthio)ethyl)-L-ornithine (12)

Compound **11** (1.62 g, 16.0 mmol) was allowed to react with anhydrous ethanol (9.3 mL, 160 mmol) in the presence of HCl (g) according to the procedure for **10** to yield the ethyl imidic ester as a crude, light-orange solid that was used in the next reaction without purification. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 12.41 (br s, 1 H), 11.70 (br s, 1 H), 4.69 (q, *J* = 7.0 Hz, 2 H), 3.64 (s, 2 H), 2.76 (q, *J* = 7.0 Hz, 2 H), 1.52 (t, *J* = 7.0 Hz, 3 H), 1.31 (t, *J* = 7.5 Hz, 3 H).

N^{α}-Boc-L-Orn-OH (0.400 g, 1.7 mmol) was allowed to react with the ethyl imidic ester (0.73 g, 4.3 mmol) and purified according to the procedure for **10** to yield **12** (0.11 g, 55%) as a hygroscopic light orange solid. ^{1}H-NMR (500 MHz, D_2O): δ 3.89-3.79 (m, 1 H), 3.52 (m, 1 H),[2] 3.31 (t, *J* = 5.5 Hz, 2 H), 2.58 (q, *J* = 7.5 Hz, 2 H), 1.79-1.62 (m, 4 H), 1.39 (s, 9 H), 1.20 (t, *J* = 7.5 Hz, 3 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 179.1, 165.3, 157.4, 80.9, 55.3, 41.9, 31.8, 29.2, 27.6, 26.0, 23.3, 13.5. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{14}H_{28}N_3O_4S$ 334.1795, found 334.1807.

N^5-(1-Imino-3-(ethylthio)ethyl)-L-ornithine (4)

Compound **12** (0.15 g, 0.42 mmol) was deprotected according to the procedure for **3** to yield **4** (0.098 g, quantitative yield) as a yellow hygroscopic solid. ^{1}H-NMR (500 MHz, D_2O): δ 4.11 (t, *J* = 6.5 Hz, 1 H), 3.56-3.55 (m, 1.4 H),[2] 3.37 (t, *J* = 7.0 Hz, 2 H), 2.58 (q, *J* = 7.5 Hz, 2 H), 2.03-1.98 (m, 2 H), 1.88-1.82 (m, 1 H), 1.80-1.73 (m, 1 H), 1.19 (t, *J* = 7.5 Hz, 3 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 171.4, 165.6, 52.2, 41.6, 31.9, 26.9, 26.1, 22.6, 13.6. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_9H_{20}N_3O_2S$ 234.1276, found 234.1282.

4-(Methylthio)butanenitrile (13)

To a 250 mL roundbottom flask were added MeOH (100 mL, 2.72 mol) and NaSMe (1.91 g, 27.2 mmol). The mixture was stirred until a cloudy solution formed. 4-Bromobutyronitrile (2.15 mL, 21.7 mmol) was added dropwise over a period of 10 min, during which time the solution became clear. The solution was left to stir overnight. The solvent was removed by rotary evaporation, and the resulting crude material was dissolved in $CHCl_3$ (30 mL), washed with H_2O (3 x 30 mL), and dried over sodium sulfate. The $CHCl_3$ was removed by rotary evaporation to yield **13** (2.48 g, 99%) as a colorless liquid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 2.63 (t, *J* = 7.0 Hz, 2H), 2.52 (t, *J* = 7.0 Hz, 2H), 2.11 (s, 3H), and 2.00-1.93 (m, 2H).

***N*^{1}-Boc-*N*^{5}-(1-Imino-3-(methylthio)butyl)-L-ornithine (14)**

Compound **13** (2.48 g, 21.6 mmol) was allowed to react with anhydrous ethanol (5.1 mL, 88 mmol) in the presence of HCl (g) according to the procedure for **10** to afford the ethyl imidic ester as a white solid (3.78 g, 87%), which was used without further purification. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 11.51 (br s, 1 H), 10.71 (br s, 1 H), 4.60 (q, *J* = 7.0 Hz, 2 H), 2.88 (t, *J* = 8.0 Hz, 2 H), 2.60 (t, *J* = 7.0 Hz, 2 H), 2.11 (s, 3 H), 2.10-2.04 (m, 2 H), 1.52 (t, *J* = 7.0 Hz, 3 H).

N$^{\alpha}$-Boc-L-Orn-OH (0.400 g, 1.7 mmol) was allowed to react with the ethyl imidic ester (0.85 g, 4.3 mmol) and purified according to the procedure for **10** to yield **14** (0.15 g, 74%) as a light orange solid. ^{1}H-NMR (500 MHz, D_2O): δ 3.90-3.79 (m, 1 H), 3.27 (t, *J* = 5.5 Hz, 2 H), 2.56 (m, 4 H), 2.08 (s, 3 H), 1.95 (t, *J* = 6.0 Hz, 2 H), 1.81-1.59 (m, 4 H), and 1.37 (s, 9 H). ^{13}C-NMR (100 MHz, D_2O): δ 179.0, 167.1, 157.2, 80.8, 55.3, 41.5, 31.8, 31.5, 29.2, 27.6, 25.6, 23.2, and 13.9. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{15}H_{30}N_3O_4S$ 348.1952, found 348.1966.

N^5-(1-Imino-3-(methylthio)butyl)-L-ornithine (5)

Compound **14** (0.15 g, 0.41 mmol) was deprotected according to the procedure for **3** to yield **5** (0.10 g, quantitative yield) as a yellow hygroscopic solid. ^{1}H-NMR (500 MHz, D_2O) δ 4.04 (t, *J* = 6.5 Hz, 1 H), 3.24 (t, *J* = 7.0 Hz, 2 H), 2.50-2.45 (m, 4 H), 1.98 (s, 3 H), 1.95-1.83 (m, 4 H), 1.76-1.72 (m, 1 H), 1.68-1.63 (m, 1 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 171.3, 167.5, 52.2, 41.2, 31.8, 31.6, 26.9, 25.7, 22.6, 14.0. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{10}H_{22}N_3O_2S$ 248.1427, found 248.1437.

2-(Propylthio)acetonitrile (15).

1-Propanethiol (4.52 g, 59.4 mmol) was allowed to react with bromacetonitrile (4.75 g, 39.6 mmol) in the presence of methanol (200 mL, 5.44 mol) and NaOMe (3.21 g, 59.4 mmol) and purified according to the procedure for **11** to yield **15** (3.23 g, 71%) as a colorless liquid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.30 (s, 2 H), 2.73 (t, *J* = 7.0 Hz, 2 H), 1.72-1.66 (m, 2 H), and 1.04 (t, *J* = 7.5 Hz, 3 H).

N^1-Boc-N^5-(1-Imino-3-(propylthio)ethyl)-L-ornithine (16)

Compound **15** (3.23 g, 28.1 mmol) was allowed to react with anhydrous, reagent-grade ethanol (10.0 mL, 171 mmol) in the presence of HCl gas according to the procedure for **10**, resulting in a crude mixture of the ethyl imidic ester and ethanol as a thick, white solution, which was used in the next step without purification. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 12.00 (br s, 1 H), 11.32 (br s, 1 H), 4.68 (q, *J* = 6.8 Hz, 2H), 3.66 (s, 2 H), 2.70 (t, *J* = 6.8 Hz, 2H), 1.69-1.62 (m, 2 H), 1.52 (t, *J* = 6.8 Hz, 3 H), 1.01 (t, *J* = 7.2 Hz, 3 H).

N^{α}-Boc-L-Orn-OH (0.65 g, 2.8 mmol) was allowed to react with the ethyl imidic ester (1.39 g, 7.0 mmol) and purified according to the procedure for **10** to yield **16** (0.14 g, 70%) as a colorless hygroscopic solid. ^{1}H-NMR (500 MHz, D_2O) δ 3.91-3.80 (m, 1 H), 3.52 (m, 1.2 H),[2] 3.34 (t, *J* = 5.5 Hz, 2 H), 2.53 (t, *J* = 6.5 Hz, 2 H), 1.81-1.62 (m, 4 H), 1.57 (m, 2 H), 1.41 (s, 9 H), 0.94 (t, *J* = 7.0 Hz, 3 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 179.1, 165.4, 157.4, 80.9, 55.3, 42.0, 33.9, 31.9, 29.3, 27.7, 23.3, 21.9, 12.5. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{15}H_{30}N_3O_4S$ 348.1952, found 348.1966.

N^5-(1-Imino-3-(propylthio)ethyl)-L-ornithine (6)

Compound **16** (0.14 g, 0.38 mmol) was deprotected according to the procedure for **3** to yield **6** (0.94 g, quantitative yield) as a yellow hygroscopic solid. ^{1}H-NMR (500 MHz, D_2O) δ 4.09 (t, *J* = 6.5 Hz, 1 H), 3.51 (m, 0.8 H),[2] 3.36 (t, *J* = 7.0 Hz, 2 H), 2.52 (t, *J* = 7.0 Hz, 2 H), 2.04-1.90 (m, 2 H), 1.85-1.78 (m, 1 H), 1.76-1.69 (m, 1 H), 1.54-1.47 (m, 2 H), 0.87 (t, *J* = 7.5 Hz, 2 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 171.3, 165.7, 52.2, 41.6, 34.0, 32.1, 26.9, 22.6, 21.9, 12.5. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{10}H_{22}N_3O_2S$ 248.1427, found 248.1443.

3-1-Propylmercaptopropionitrile (17)

1-Propanethiol (8.68 g, 114 mmol) was allowed to react with acrylonitrile (12.09 g, 228 mmol) in the presence of catalytic NaOMe (0.152 g, 2.8 mmol) according to the procedure for **9** to yield **17** (14.75 g, quantitative yield) as an orange liquid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 2.79 (t, 7.5, 2H), 2.64 (t, *J* = 7.5Hz, 2H), 2.58 (t, *J* = 7.5 Hz, 2H), 1.68-1.60 (m, 2H), 1.01 (t, *J* = 7.0 Hz, 3H).

N^1-Boc-N^5-(1-Imino-3-(propylthio)propyl)-L-ornithine (18)

Compound **17** (15.05 g, 116 mmol) was allowed to react with anhydrous, reagent grade ethanol (40.6 mL, 696 mmol) in the presence of HCl gas according to the procedure for **10** to yield the ethyl imidic ester as an orange oil (24.38 g, 99%), which was used in the next step without further purification. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 12.38 (br s, 1H), 11.53 (br s, 1H), 4.64 (q, *J* = 7.0 Hz, 2H), 3.06 (t, *J* = 7.0 Hz, 2H), 2.86 (t, *J* = 7.0 Hz, 2H), 2.56 (q, *J* = 7.5 Hz, 2H), 1.66-1.59 (m, 2H), 1.48 (t, *J* = 7.0 Hz, 3H), 0.97 (t, *J* = 7.5 Hz, 3H).

N^{α}-Boc-L-Orn-OH (0.34 g, 1.4 mmol) was allowed to react with the ethyl imidic ester (0.76 g, 3.6 mmol) and purified according to the procedure for **10** to yield **18** as a hygroscopic orange solid (0.11 g, 48%). ^{1}H-NMR (500 MHz, D_2O) δ 3.93-3.84 (m, 1 H), 3.30 (t, *J* = 5.5 Hz, 2 H), 2.87 (t, *J* = 7.0 Hz, 2 H), 2.75 (t, *J* = 7.0 Hz, 2 H), 2.56 (t, *J* = 7.5 Hz, 2 H), 1.84-1.61 (m, 4 H), 1.60-1.54 (m, 2 H), 1.41 (s, 9 H), 0.94 (t, *J* = 7.5 Hz, 3 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 179.2, 165.9, 157.4, 80.9, 55.3, 41.6, 33.2, 32.7, 29.2, 27.8, 27.6, 23.3, 22.1, 12.5. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{16}H_{32}N_3O_4S$ 362.2108, found 362.2117.

N^5-(1-Imino-3-(propylthio)propyl)-L-ornithine (7)

Compound **18** (0.070 g, 0.18 mmol) was deprotected according to the procedure for **3** to yield **7** (0.47 g, quantitative yield) as an orange hygroscopic solid. ^{1}H-NMR (500 MHz, D_2O) δ 4.08 (t, *J* = 6.0 Hz, 1 H), 3.30 (t, *J* = 6.0 Hz, 2 H), 2.82 (t, *J* = 6.5 Hz, 2 H), 2.72 (t, *J* = 6.5 Hz, 2 H), 2.50 (t, *J* = 7.5 Hz, 2 H), 2.06-1.90 (m, 2 H), 1.85-1.68 (m, 2H), 1.54-1.49 (m, 2H), 0.86 (t, *J* = 7.5 Hz, 3H). ^{13}C-NMR (125.7 MHz, D_2O): δ 171.3, 166.2, 52.3, 41.3, 33.2, 32.8, 27.8, 27.0, 22.6,

22.1, 12.6. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{11}H_{24}N_3O_2S$ 262.1584, found 262.1595.

3-2-Propylmercaptopropionitrile (19).

2-Propanethiol (8.68 g, 114 mmol) was allowed to react with acrylonitrile (12.09 g, 228 mmol) in the presence of catalytic NaOMe (0.152 g, 2.8 mmol) according to the procedure for **9** to yield **19** (14.42 g, 98%) as an orange liquid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 3.07-2.99 (m, 1H), 2.81 (t, *J* = 7.0 Hz, 2H), 2.63 (t, *J* = 7.0 Hz, 2H), 1.29 (d, *J* = 7.0 Hz, 6H).

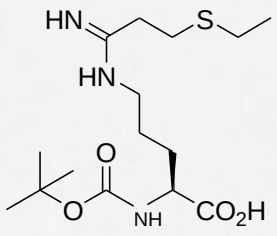
***N*^{1}-Boc-*N*^{5}-(1-Imino-3-(isopropylthio)propyl)-L-ornithine (20)**

Compound **19** (14.42 g, 112 mmol) was allowed to react with anhydrous, reagent grade ethanol (40 mL, 696 mmol) in the presence of HCl gas according to the procedure for **10** to yield the ethyl imidic ester as an orange oil (23.81 g, quantitative yield), which was used in the next step without further purification. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 12.38 (br s, 1H), 11.53 (br s, 1H), 4.67 (q, *J* = 7.5 Hz, 2H), 3.11-3.03 (m, 3H), 2.91 (t, *J* = 7.0 Hz, 2H), 1.51 (t, *J* = 7.0 Hz, 3H), 1.27 (d, *J* = 7.0 Hz, 6H).

N$^{\alpha}$-Boc-L-Orn-OH (0.16 g, 0.7 mmol) was allowed to react with the ethyl imidic ester (0.37g, 1.8 mmol) and purified according to the procedure for **10** to yield **20** (0.065 g, 77%) as a hygroscopic light orange solid. ^{1}H-NMR (500 MHz, D_2O) δ 3.91-3.80 (m, 1 H), 3.29 (t, *J* = 5.5 Hz, 2 H), 3.04-2.98 (m, 1 H), 2.90 (t, *J* = 6.5 Hz, 2 H), 2.75 (t, *J* = 6.5 Hz, 2 H), 1.85-1.62 (m, 4 H), 1.41 (s, 9 H), 1.24 (d, *J* = 6.5 Hz, 6 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 179.2, 165.9, 157.4, 80.9, 55.4, 41.7, 34.7, 32.9, 29.3, 27.7, 26.4, 23.3, 22.4. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{16}H_{32}N_3O_4S$ 362.2108, found 362.2119.

N^5-(1-Imino-3-(isopropylthio)propyl)-L-ornithine (8)

Compound **20** (0.15 g, 0.42 mmol) was deprotected according to the procedure for **3** to yield **8** (0.11 g, quantitative yield) as a hygroscopic orange solid. ^{1}H-NMR (500 MHz, D_2O) δ 3.82 (t, *J* = 6.0 Hz, 1 H), 3.04 (t, *J* = 6.0 Hz, 2 H), 2.73-2.67 (m, 1 H), 2.59 (t, *J* = 7.0 Hz, 2 H), 2.46 (t, *J* = 7.0 Hz, 2 H), 1.79-1.63 (m, 2 H), 1.57-1.42 (m, 2H), 0.89 (d, *J* = 6.5 Hz, 6 H). ^{13}C-NMR (125.7 MHz, D_2O): δ 171.1, 166.0, 52.1, 41.2, 35.6, 34.3, 32.8, 26.2, 22.3, 22.1. HRMS (ESI, H_2O) (*m/z*): $M+H^+$ calcd for $C_{11}H_{24}N_3O_2S$ 262.1584, found 262.1594.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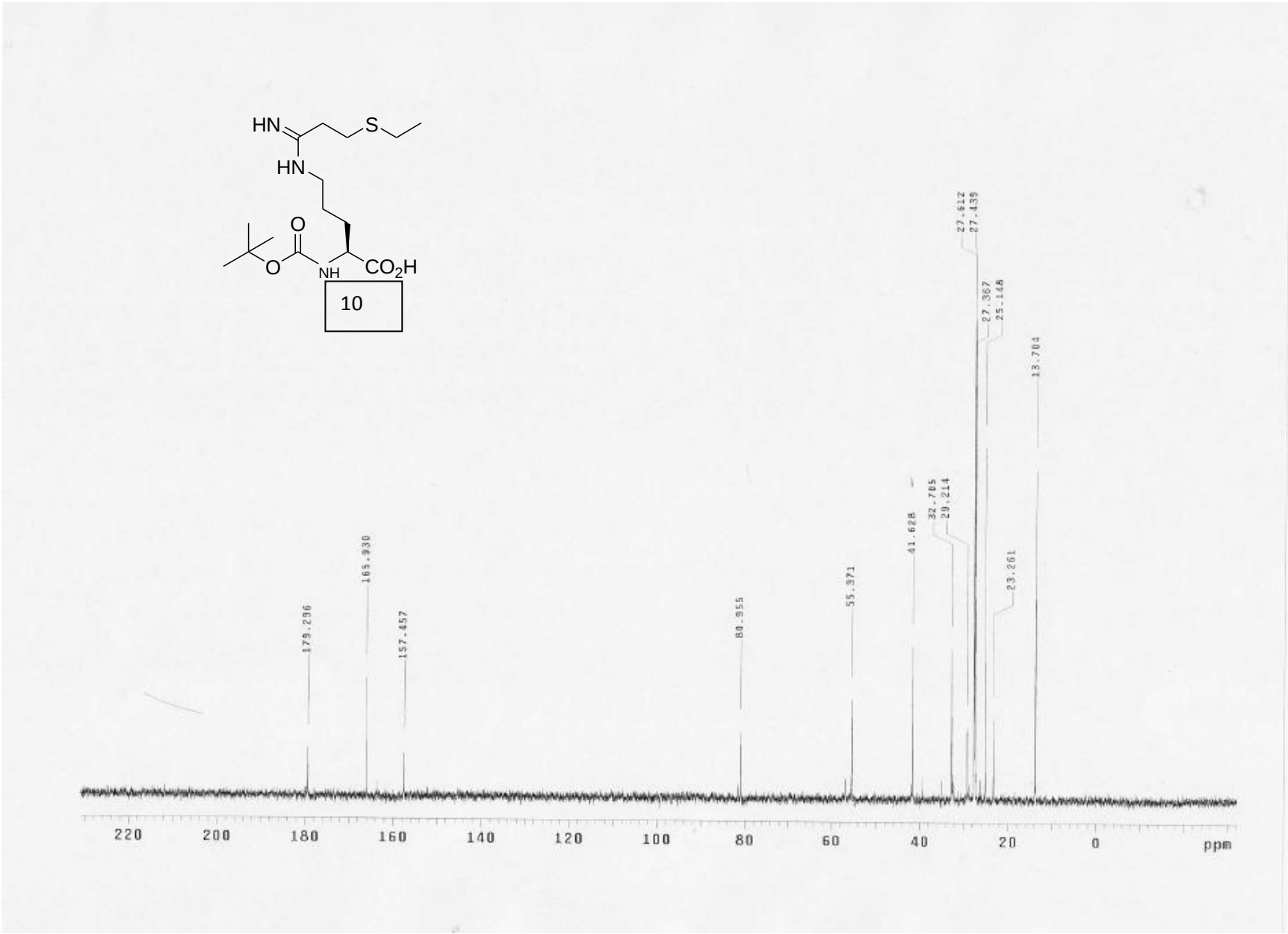
4.42
3.20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O
O
NH
CO2H
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32.705
29.214
27.612
27.439
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23.261
13.704
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200
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120
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60
40
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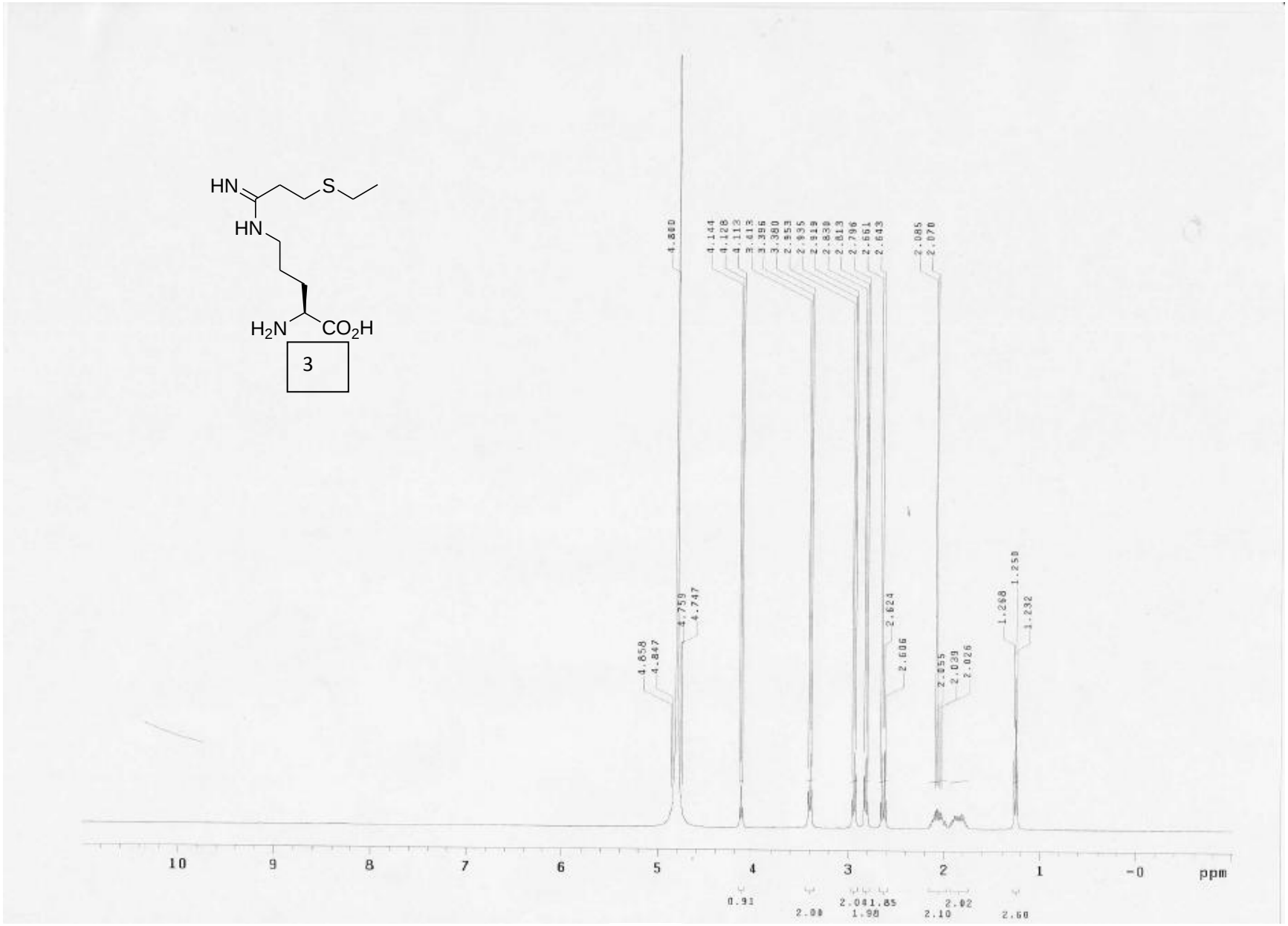
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S
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CO2H
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3.396
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2.935
2.919
2.839
2.813
2.796
2.661
2.643
2.085
2.070
4.858
4.847
4.759
4.747
2.624
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2.055
2.039
2.026
1.268
1.258
1.232
10
9
8
7
6
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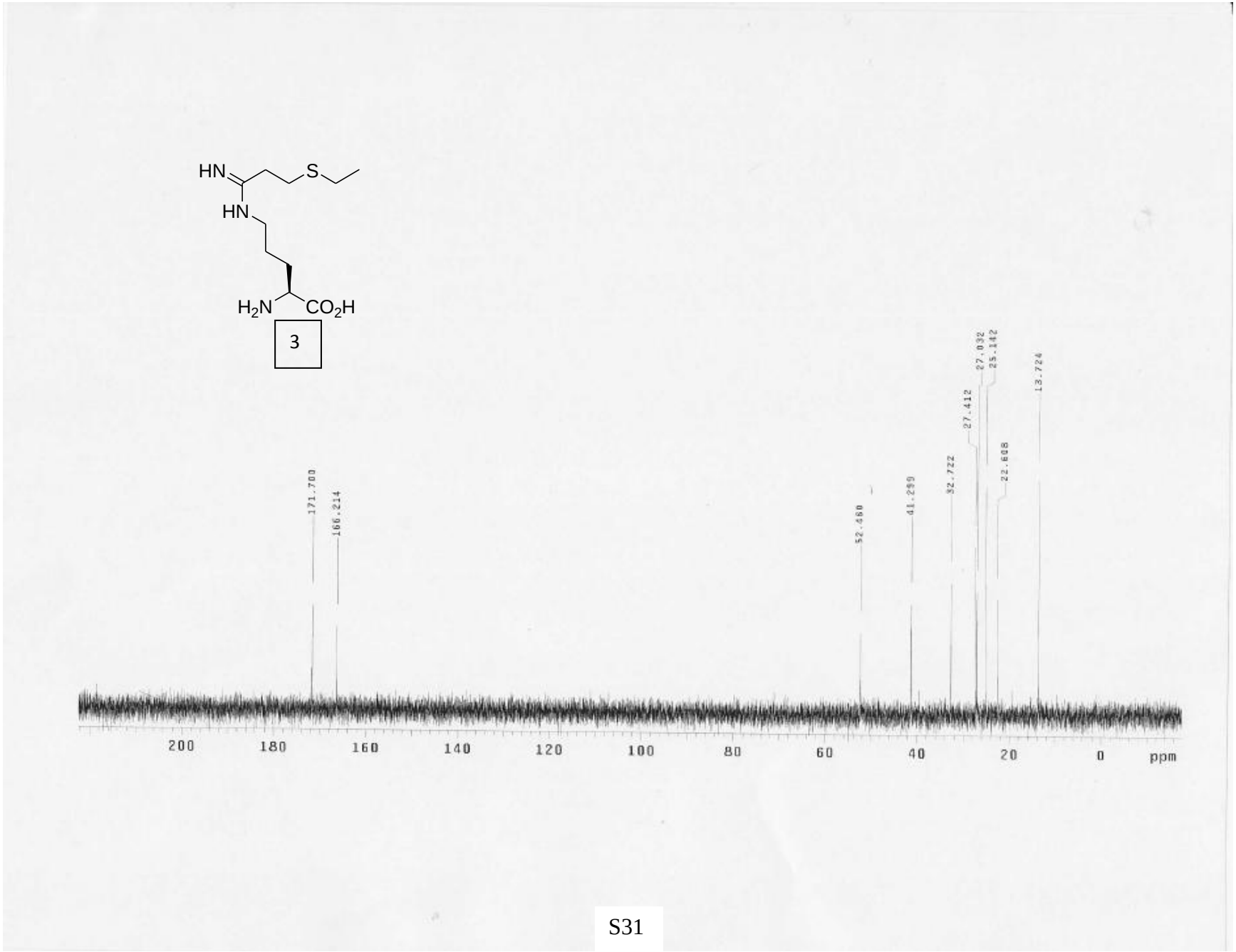
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H2N
CO2H
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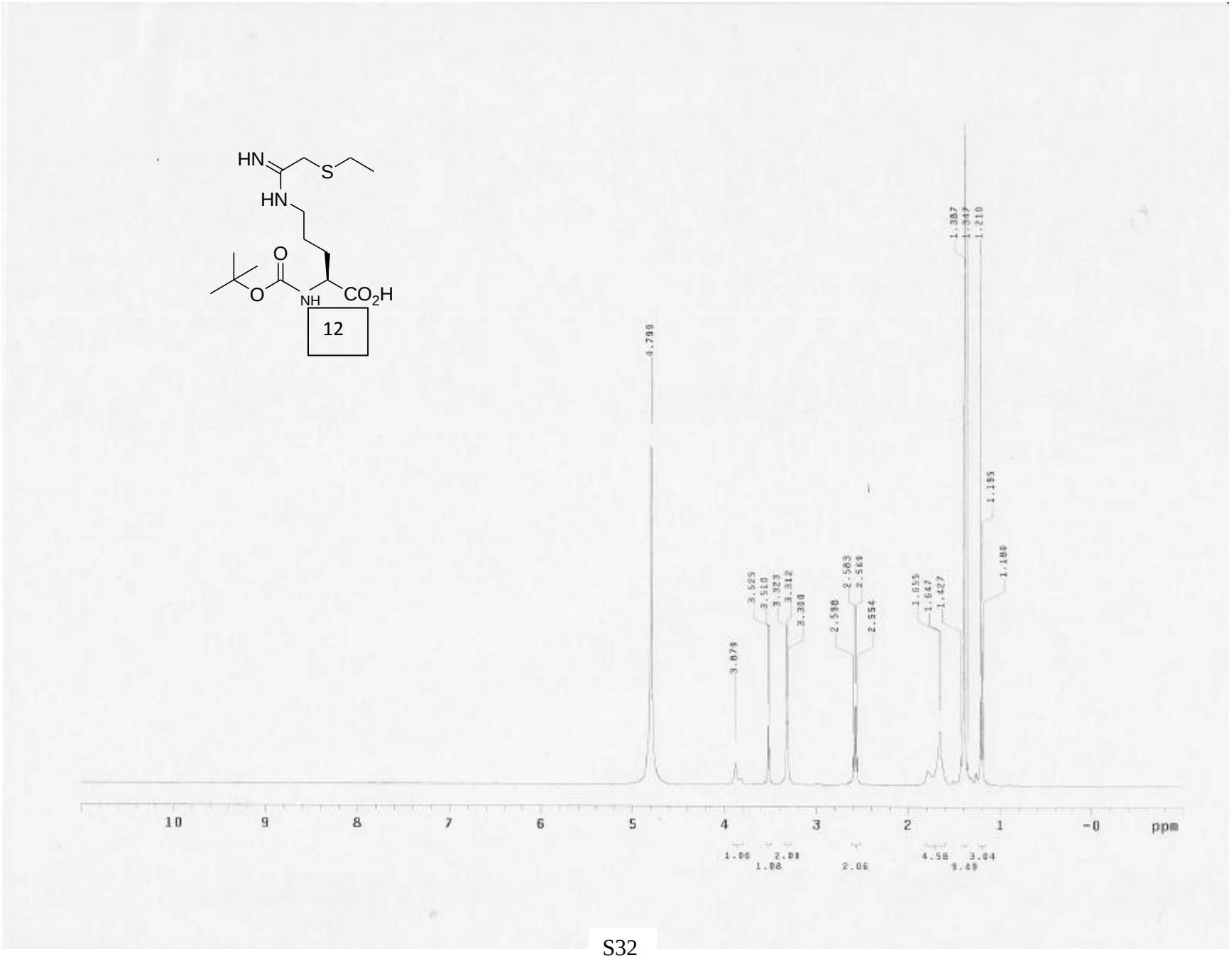
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O
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CO2H
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3.525
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3.300
2.588
2.563
2.559
2.554
1.655
1.647
1.427
1.387
1.210
1.155
1.100
10
9
8
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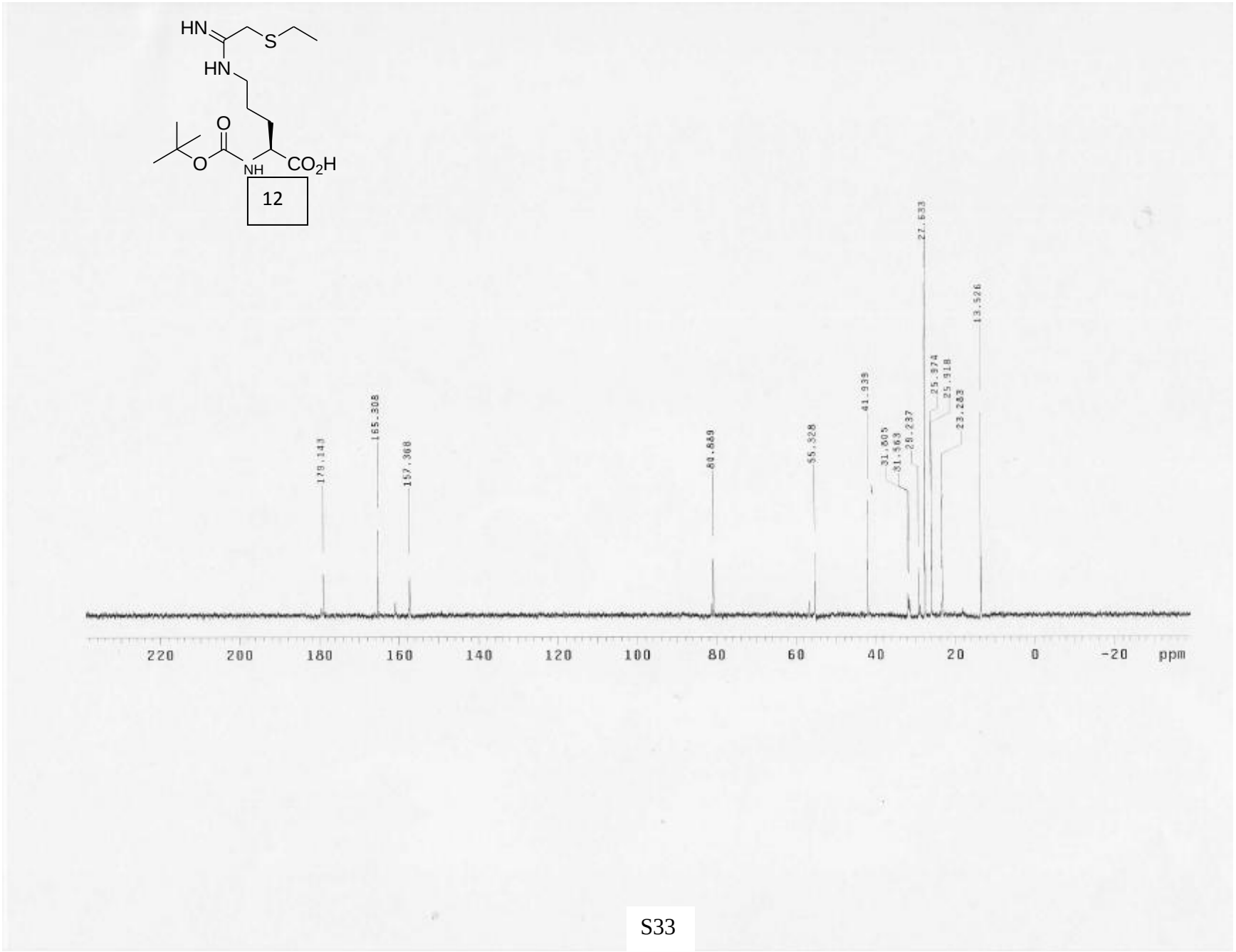
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HN
O
O
NH
CO2H
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31.563
28.237
27.633
25.974
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20
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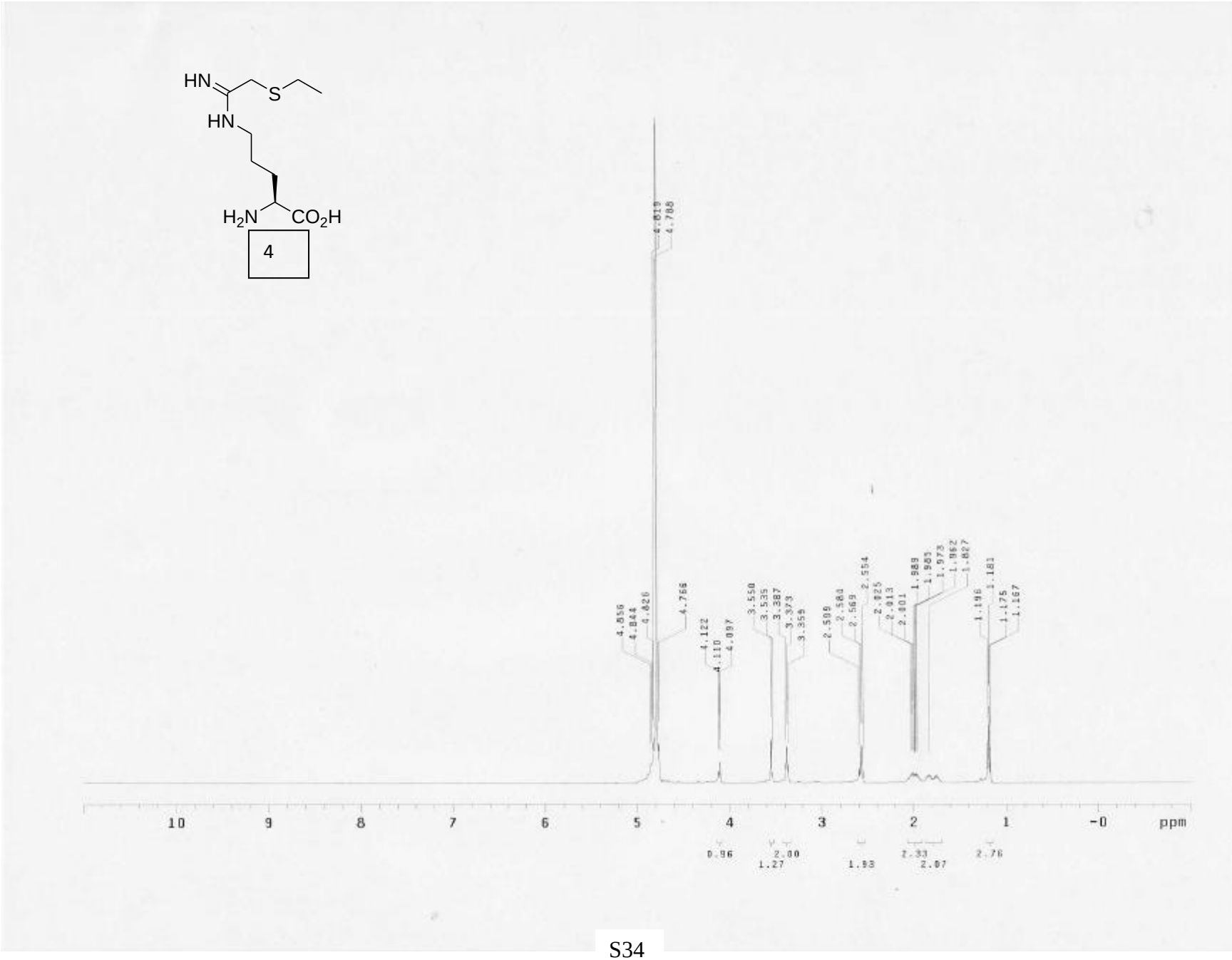
ppm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HN
H2N
CO2H
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4.820
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4.122
4.110
4.097
3.550
3.535
3.387
3.373
3.359
2.589
2.584
2.569
2.554
2.025
2.013
2.001
1.989
1.985
1.973
1.962
1.827
1.196
1.181
1.175
1.167
10
9
8
7
6
5
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2.33
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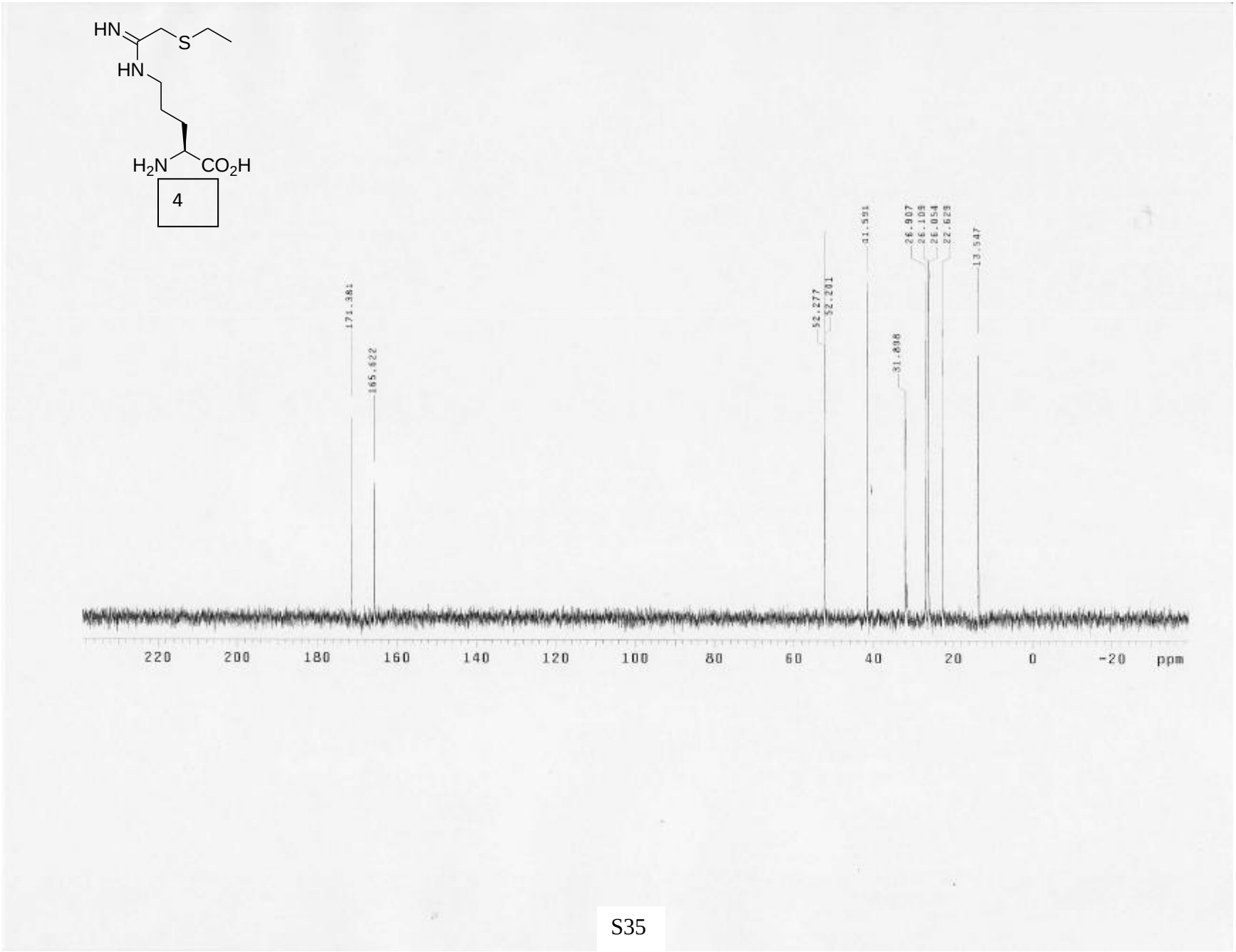
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HN
H2N
CO2H
4
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52.277
52.201
31.591
31.808
26.907
26.109
26.054
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13.547
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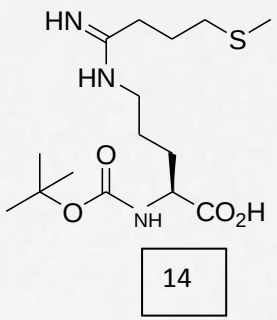
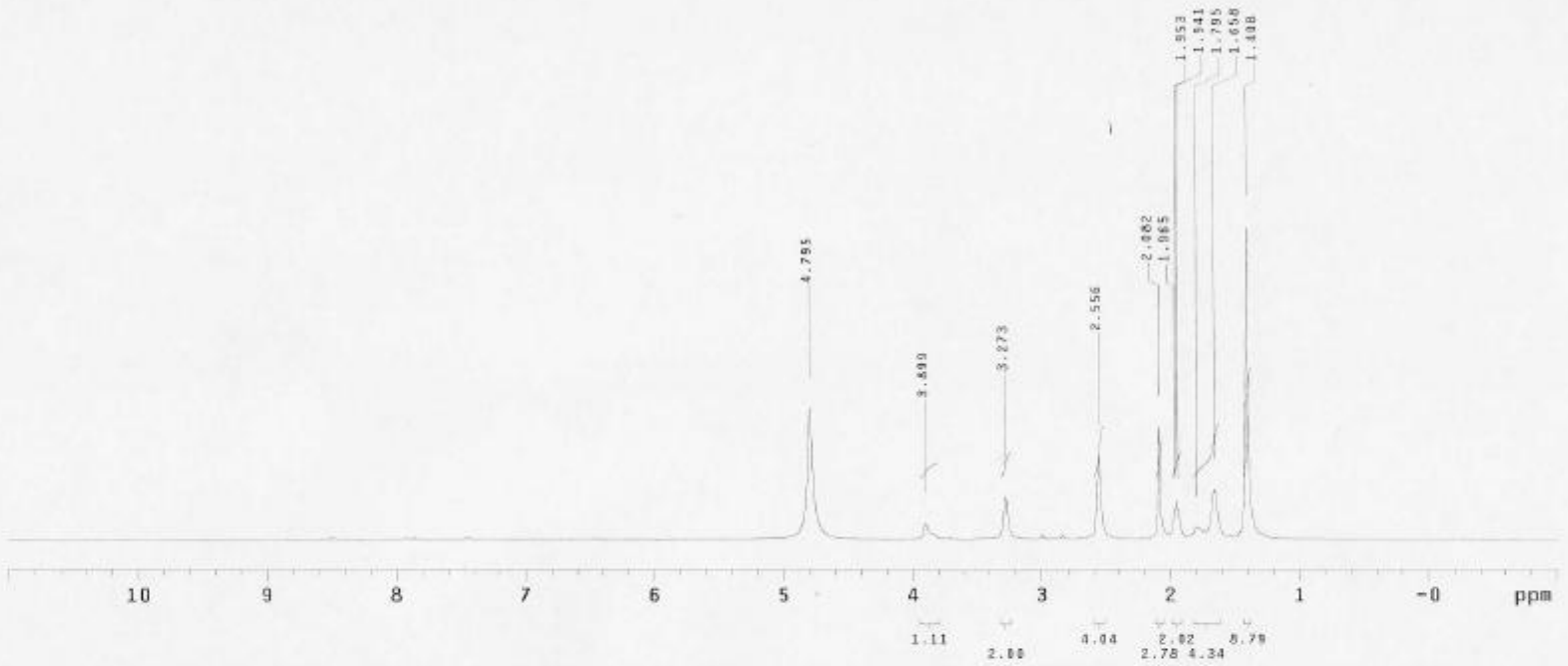
200
180
160
140
120
100
80
60
40
20
0
-20
ppm


HN
S
HN
O
O
NH
CO2H

14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1.795
1.658
1.408
10
9
8
7
6
5
4
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2
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-0
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2.00
4.04
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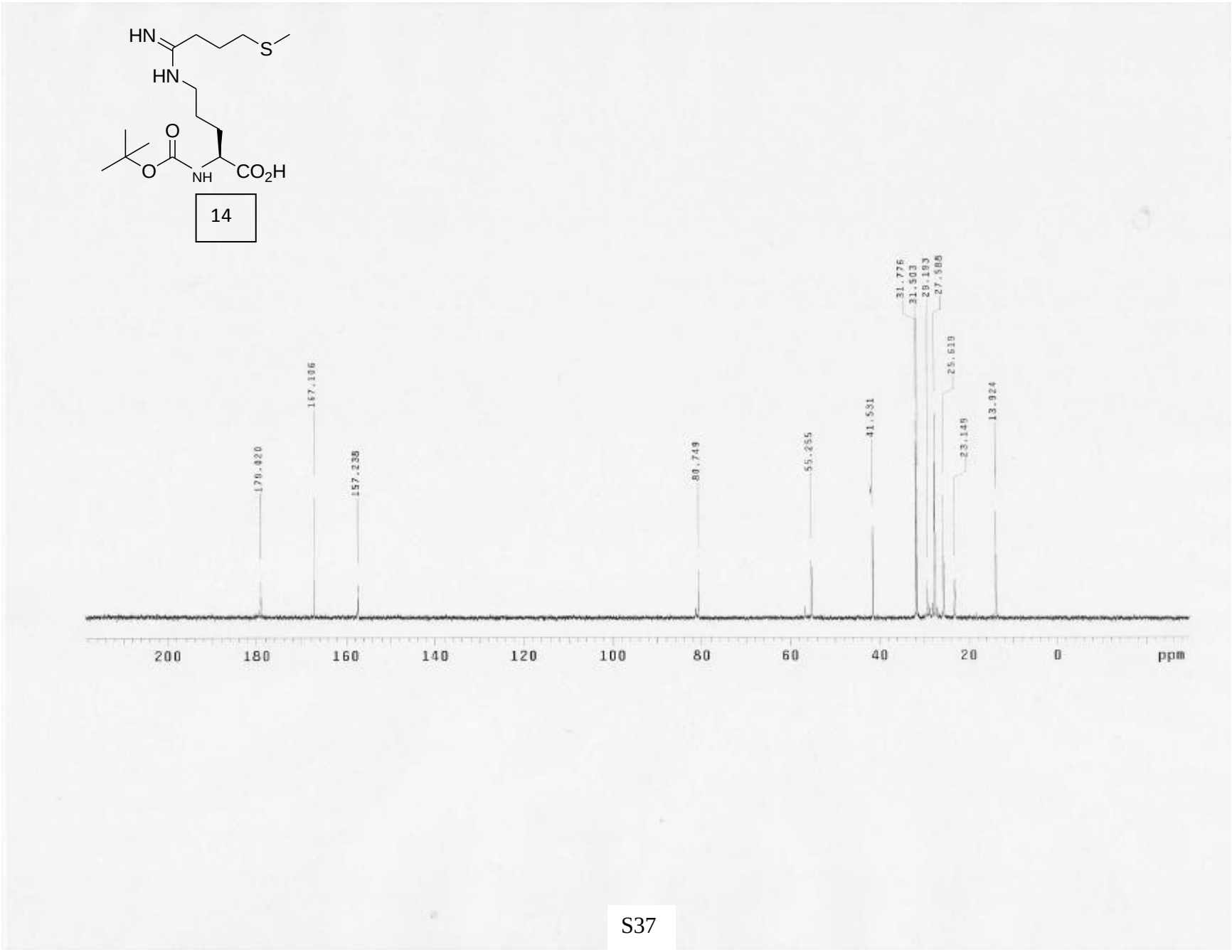
2.78
4.34
8.79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S
HN
O
O
NH
CO_2H
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157.238
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55.255
41.531
31.776
31.503
29.193
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23.149
13.924
200
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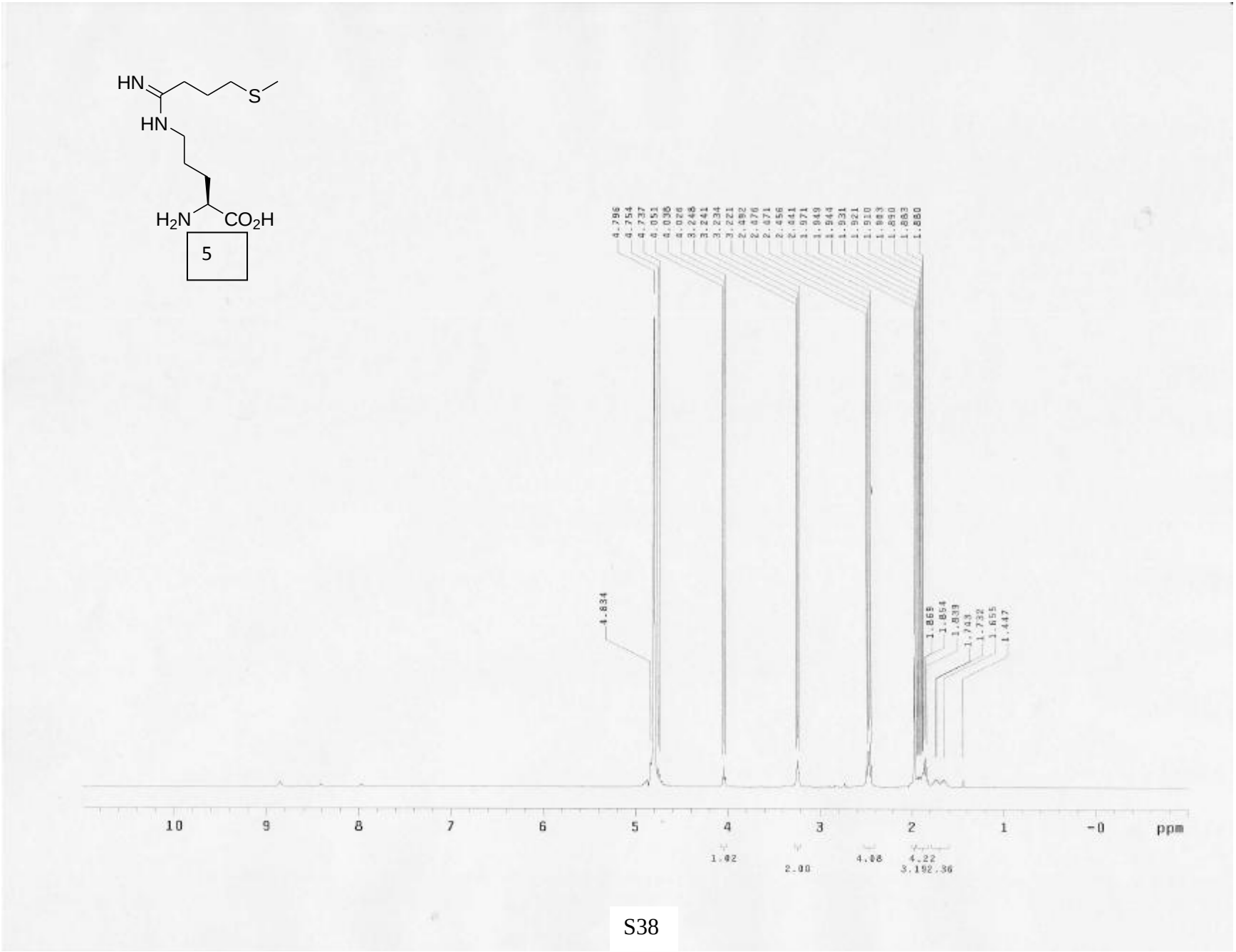
160
140
120
100
80
60
40
20
0
ppm


HN
S
HN
H2N
CO2H
5
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4.754
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4.051
4.035
4.026
3.248
3.241
3.234
3.221
2.492
2.476
2.471
2.456
2.441
1.971
1.949
1.944
1.931
1.921
1.910
1.883
1.850
1.883
1.880
4.834
1.869
1.854
1.833
1.743
1.732
1.655
1.447
10
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8
7
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5
4
3
2
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2.00
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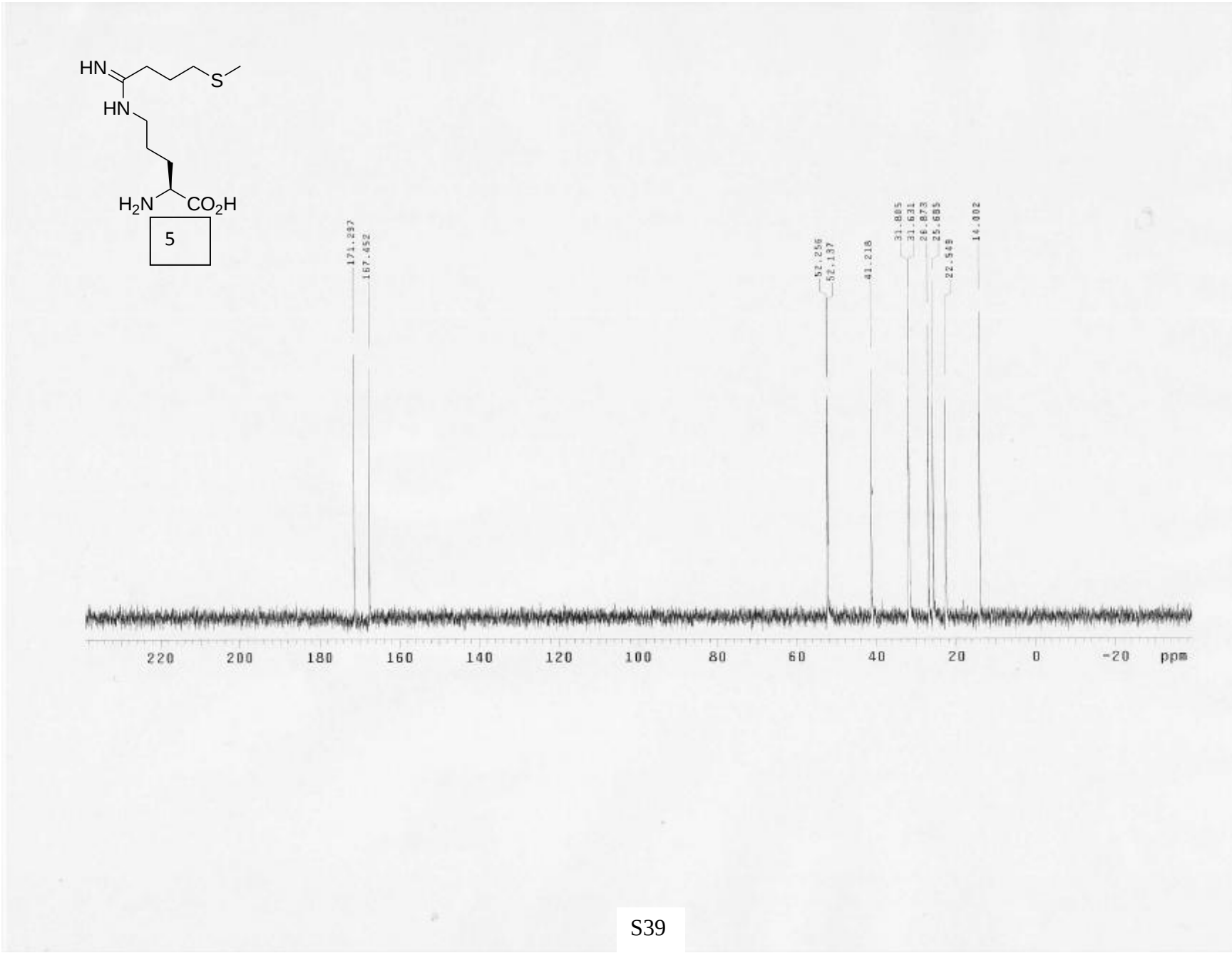
4.22
3.192.36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S
HN
H2N
CO2H
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31.805
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14.002
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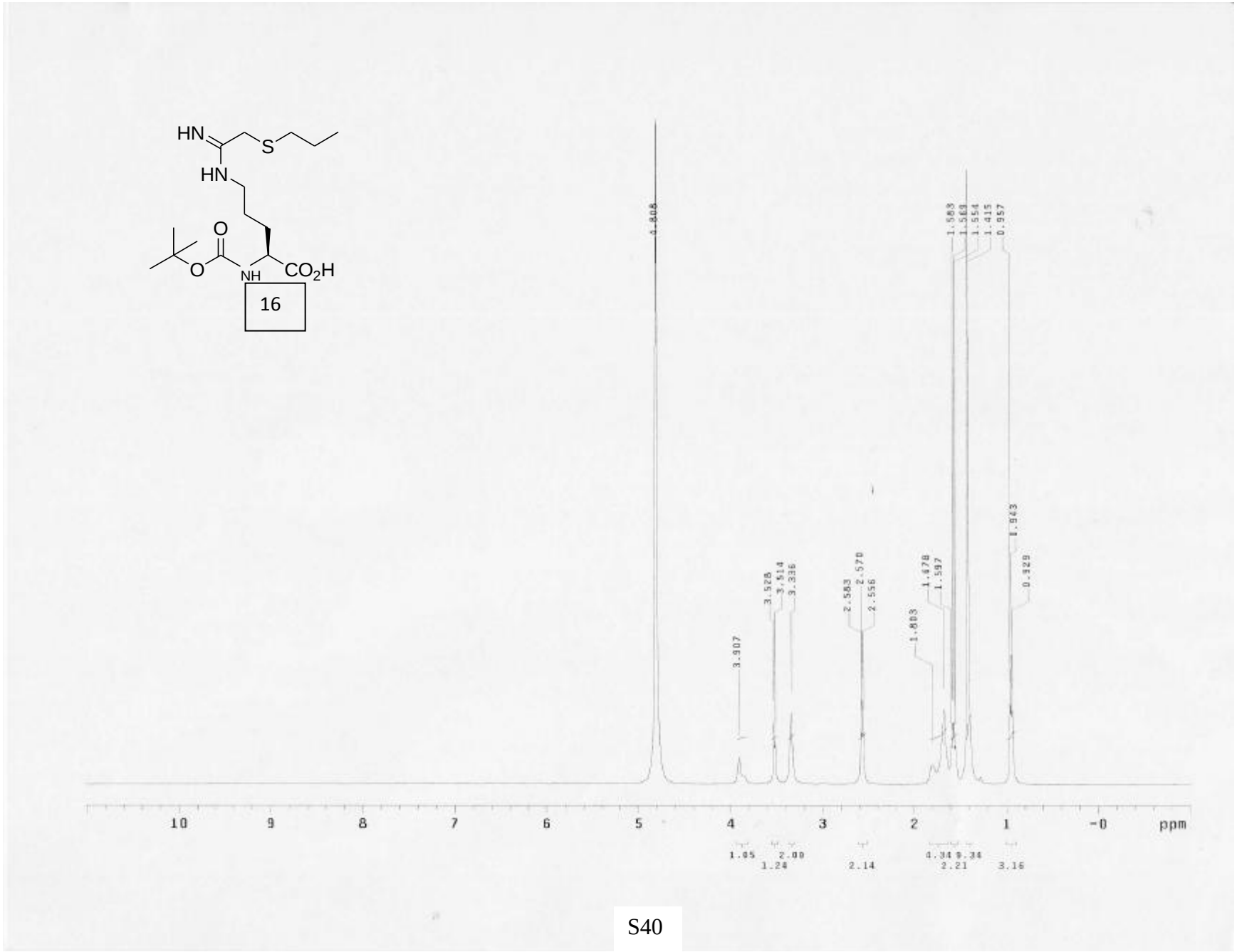
200
180
160
140
120
100
80
60
40
20
0
-20
ppm


HN
S
HN
O
O
NH
CO_2H
16
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3.528
3.514
3.336
2.583
2.570
2.556
1.803
1.678
1.597
1.583
1.563
1.554
1.415
0.957
1.943
0.929
10
9
8
7
6
5
4
3
2
1
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ppm
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2.14
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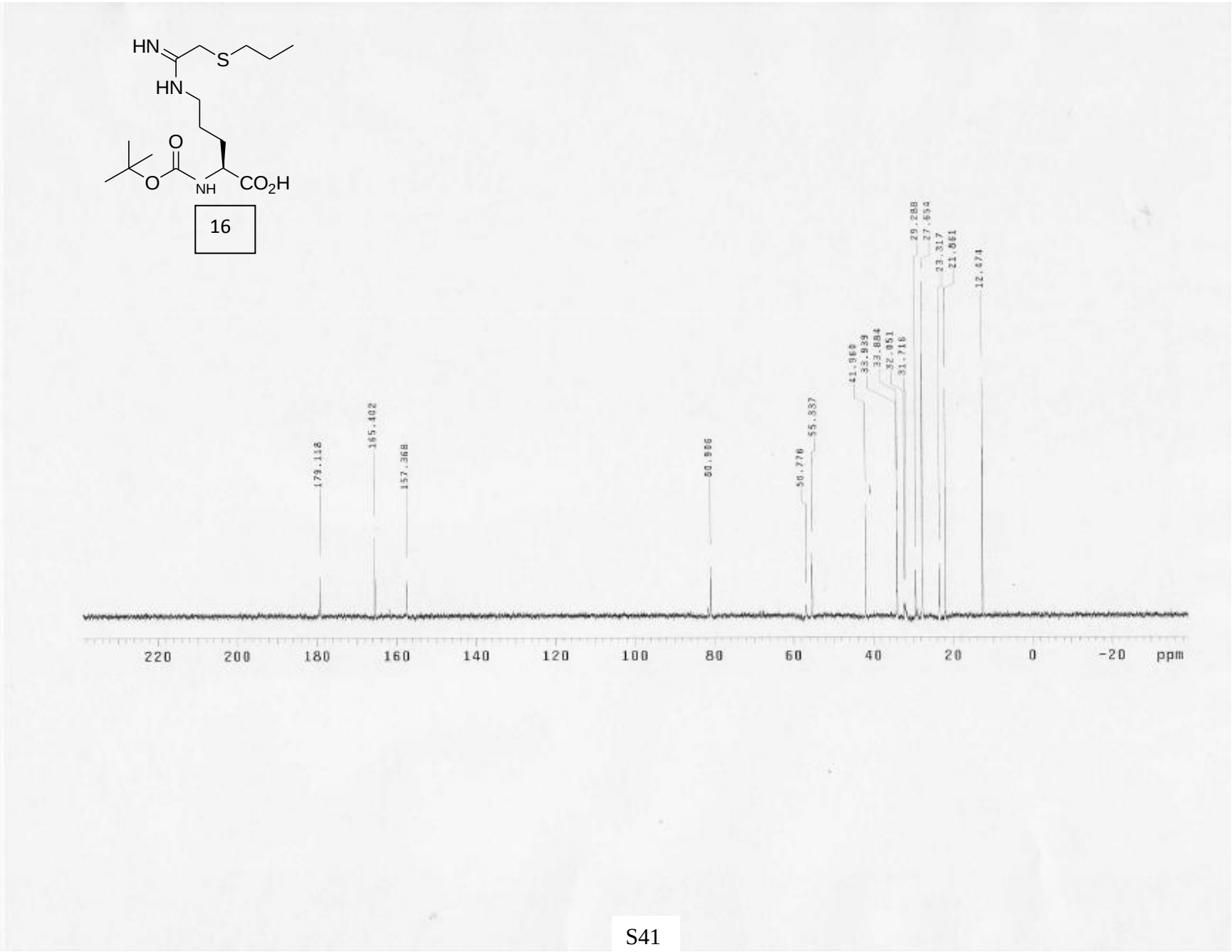
2.21
3.16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S
HN
O
O
NH
CO2H
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179.118
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157.368
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56.776
55.337
41.969
33.939
33.884
32.051
31.716
29.288
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23.317
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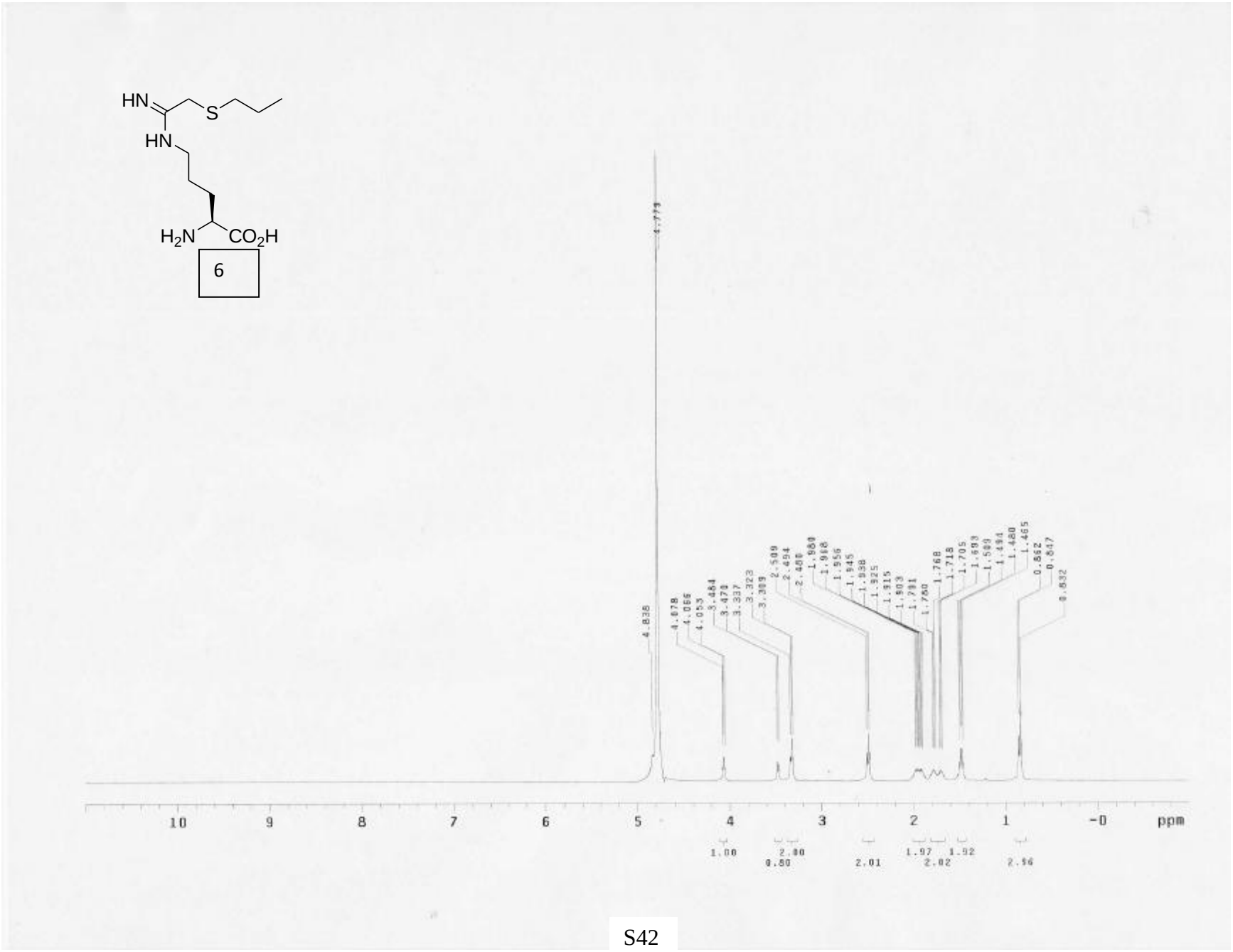
12.474
220
200
180
160
140
120
100
80
60
40
20
0
-20
ppm


HN
S
HN
H2N
CO2H
6
4.838
4.078
4.066
4.053
3.484
3.470
3.337
3.323
3.309
2.509
2.494
2.480
1.980
1.968
1.956
1.945
1.938
1.925
1.915
1.903
1.791
1.780
1.768
1.718
1.705
1.693
1.589
1.494
1.480
1.465
0.882
0.847
0.832
10
9
8
7
6
5
4
3
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ppm
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2.01
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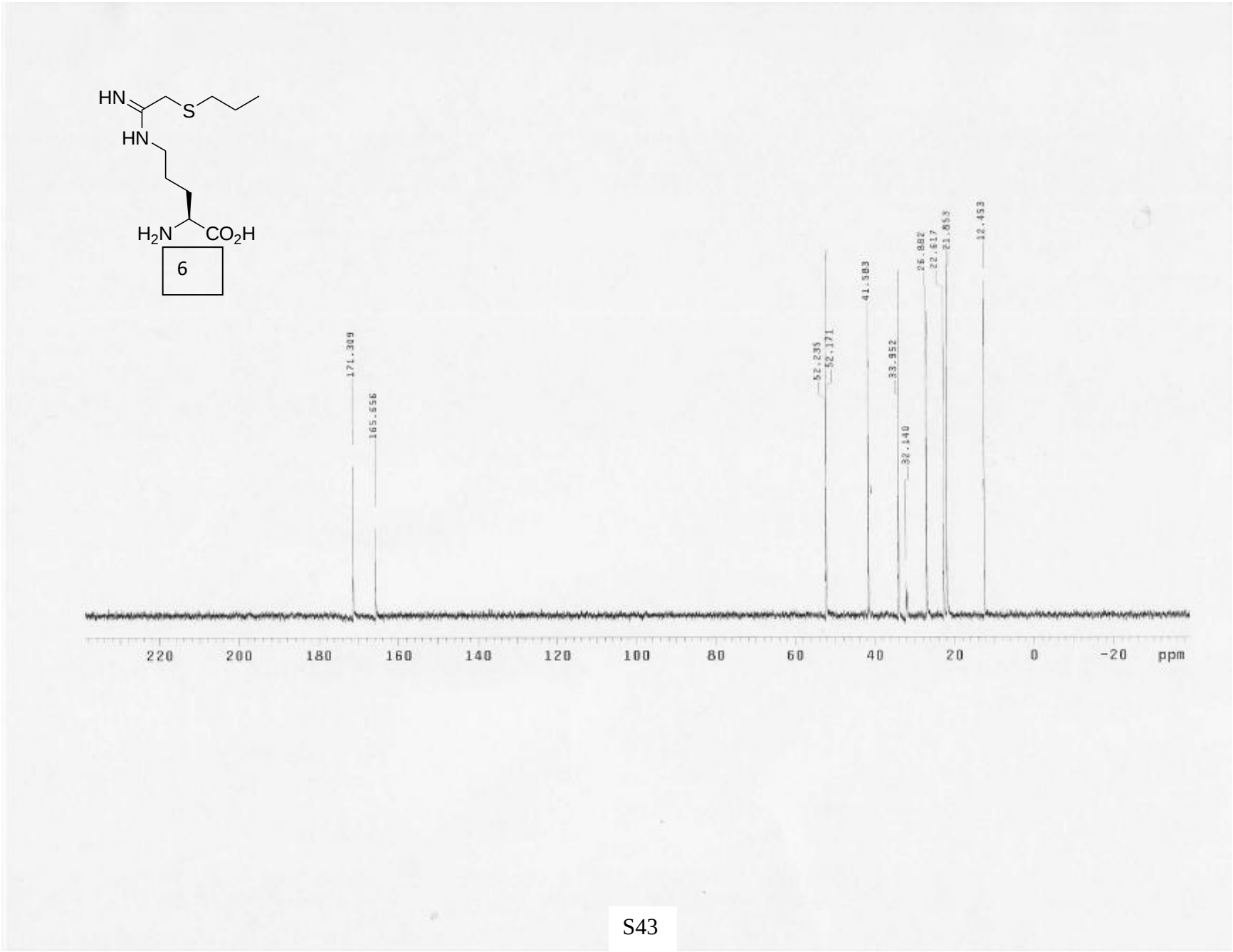
2.96


HN
S
HN
H_2N
CO_2H
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52.171
41.583
33.952
32.140
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12.453
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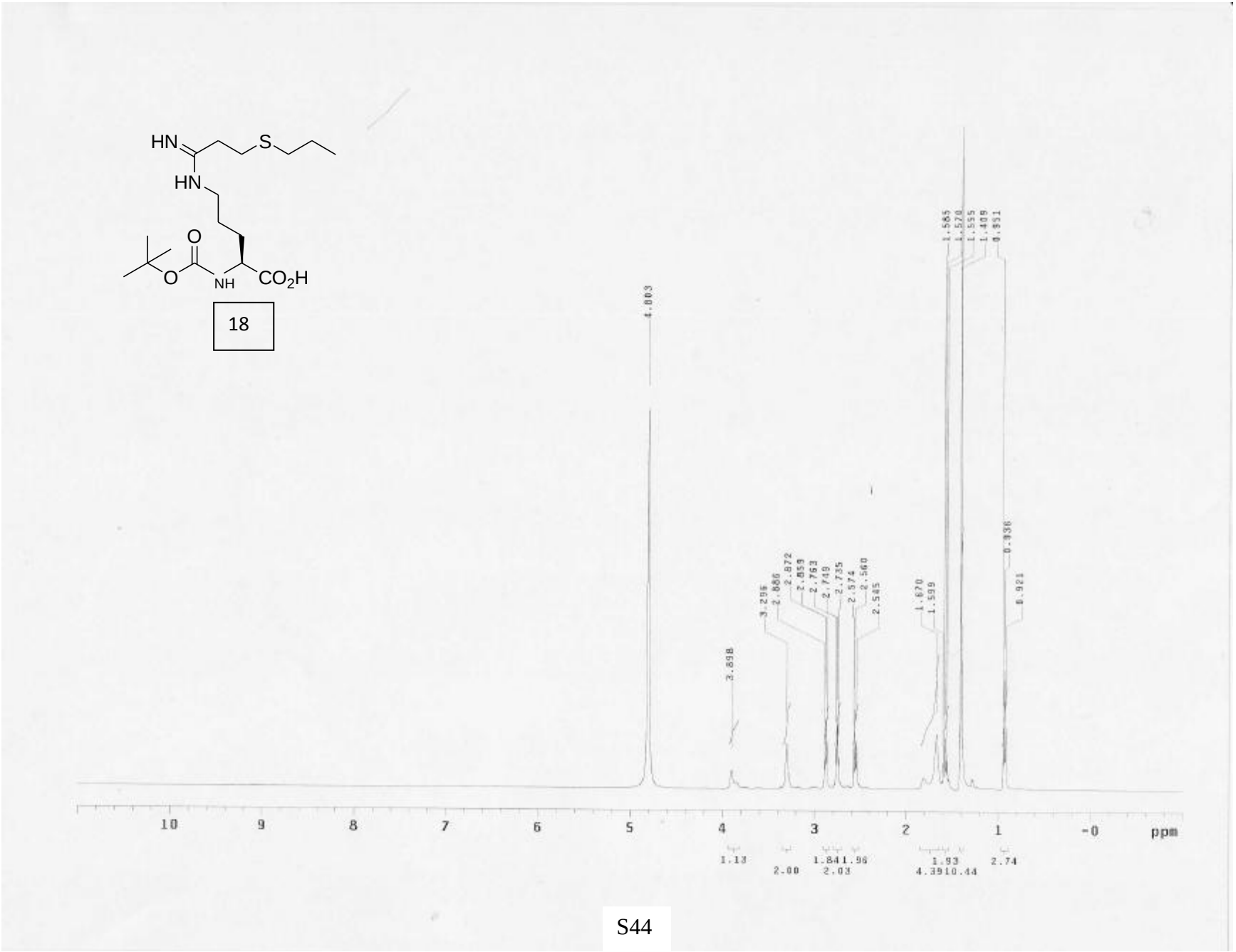
120
100
80
60
40
20
0
-20
ppm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S
HN
O
O
NH
CO2H
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2.749
2.735
2.574
2.560
2.545
1.670
1.599
1.585
1.570
1.555
1.409
0.851
0.336
0.921
10
9
8
7
6
5
4
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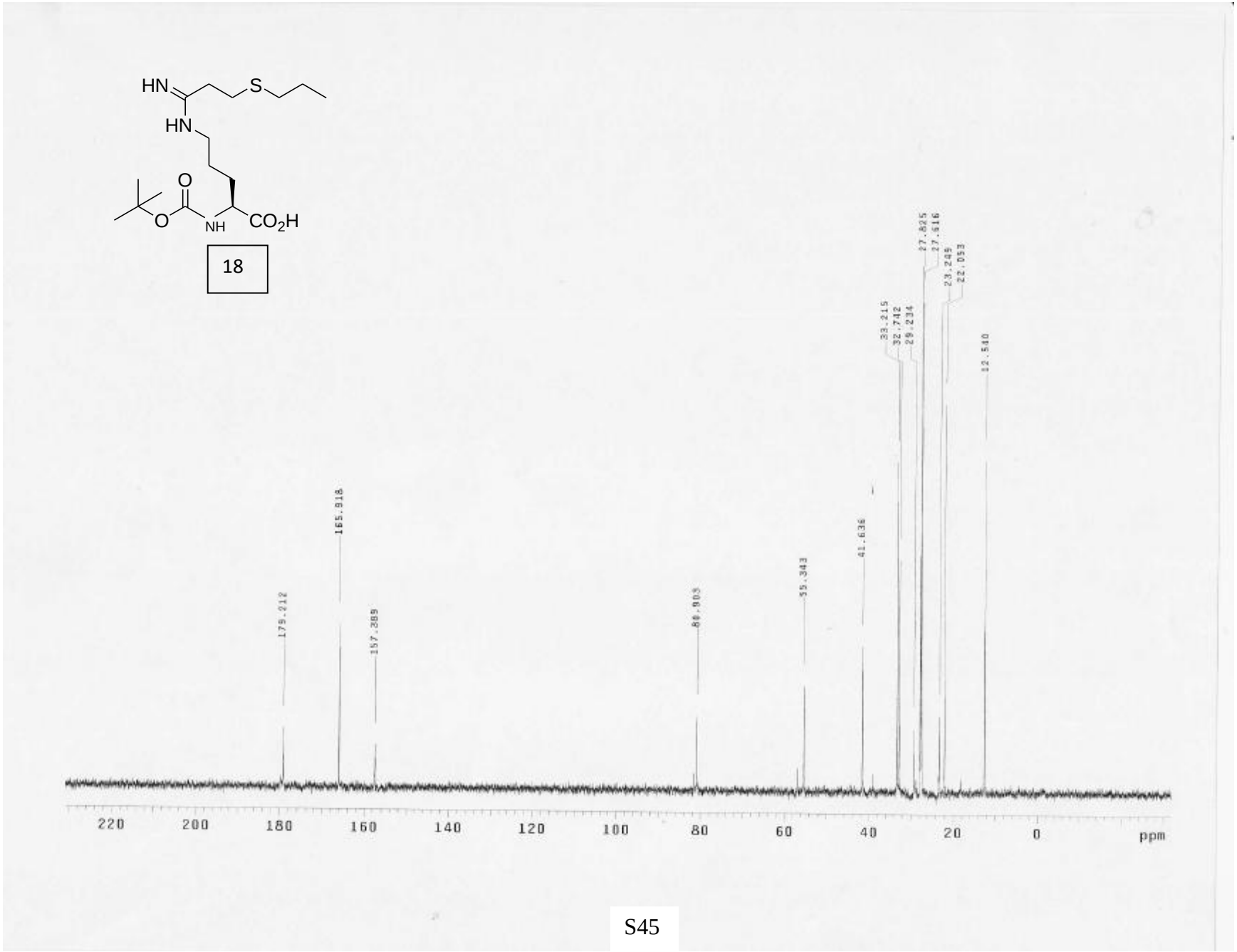
HN
S
HN
O
O
NH
CO2H
18
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165.918
157.389
80.903
55.343
41.636
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32.742
29.234
27.825
27.616
23.249
22.053
12.540
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200
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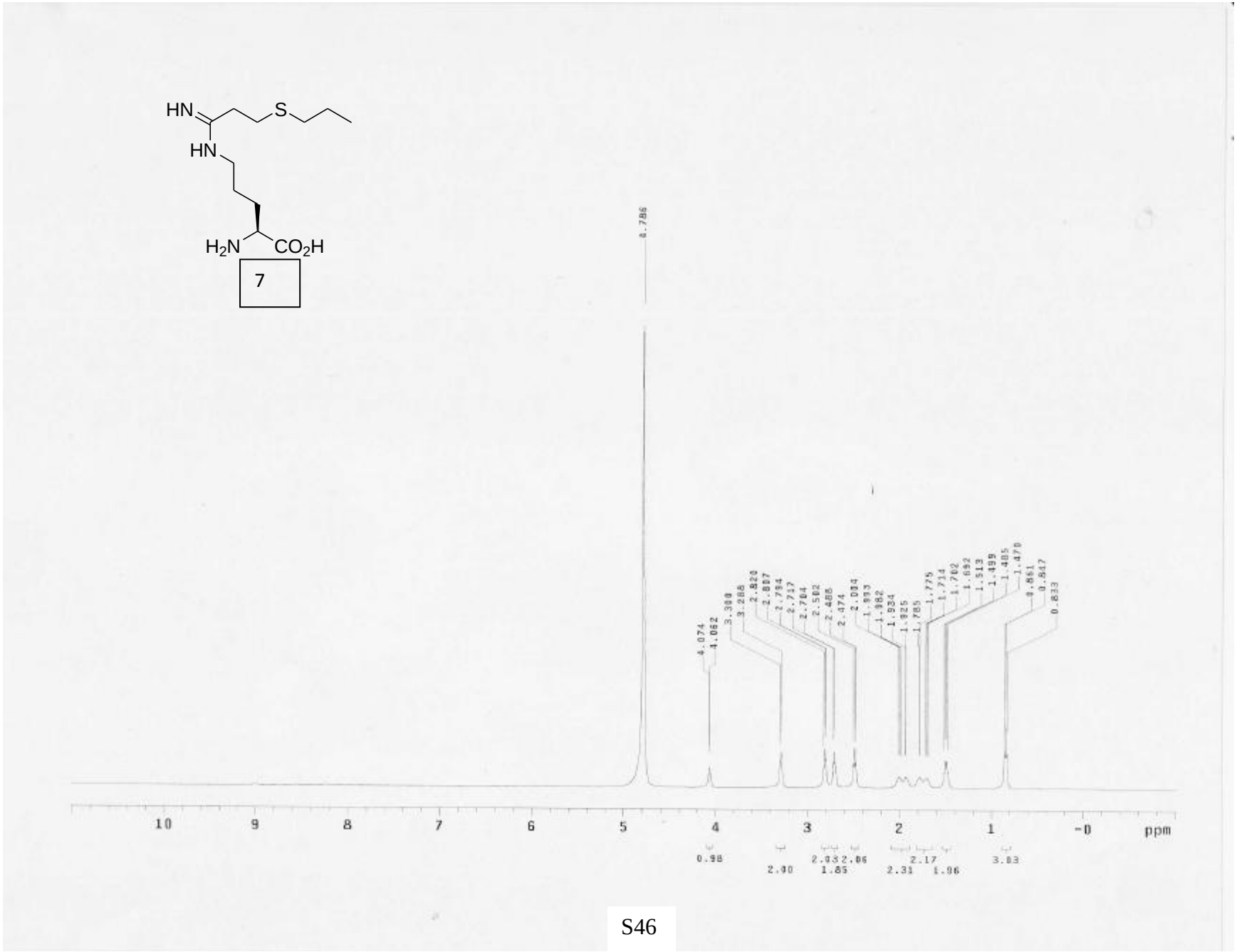
160
140
120
100
80
60
40
20
0
ppm


HN
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HN
H2N
CO2H
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3.308
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2.734
2.717
2.704
2.502
2.488
2.474
2.034
1.993
1.982
1.934
1.925
1.785
1.775
1.714
1.702
1.692
1.513
1.499
1.485
1.470
0.861
0.847
0.833
10
9
8
7
6
5
4
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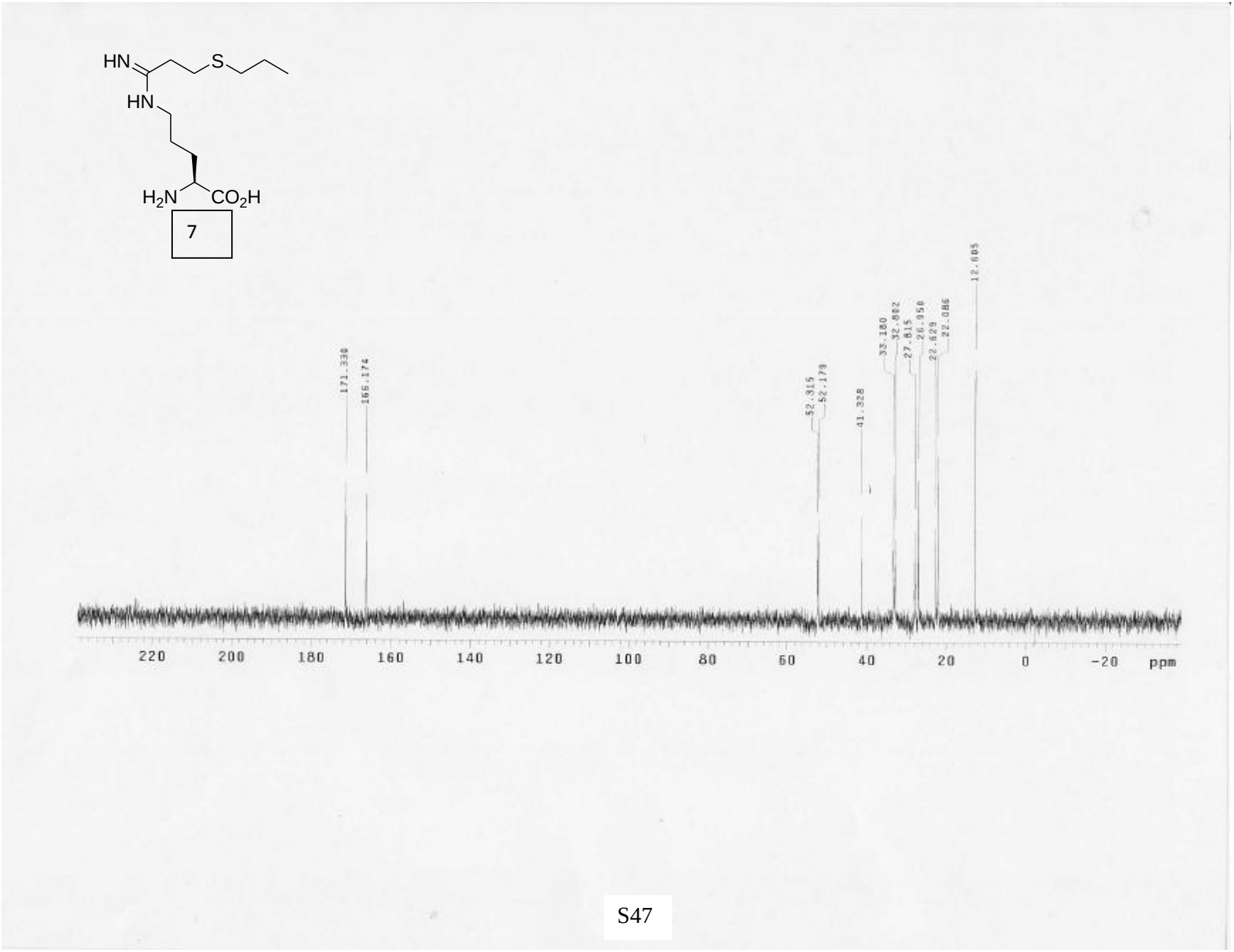
3.03


HN
S
HN
H2N
CO2H
7
171.338
166.174
52.315
52.179
41.328
33.180
32.882
27.815
26.958
22.629
22.086
12.605
220
200
180
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140
120
100
80
60
40
20
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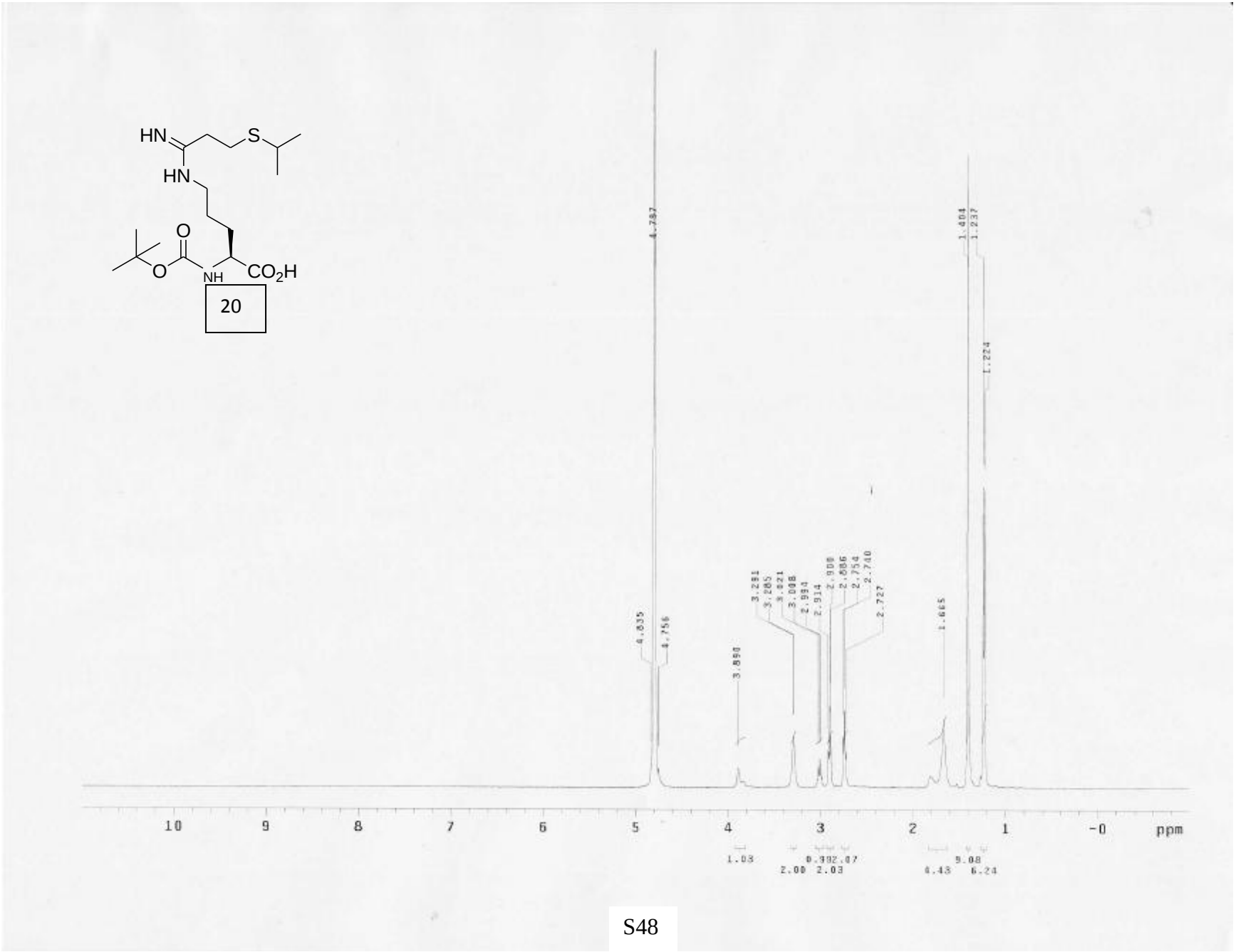
-20
ppm


HN
S
HN
O
O
NH
CO2H
20
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4.756
3.850
3.291
3.285
3.021
3.008
2.934
2.914
2.908
2.686
2.754
2.740
2.727
1.665
1.464
1.237
1.224
10
9
8
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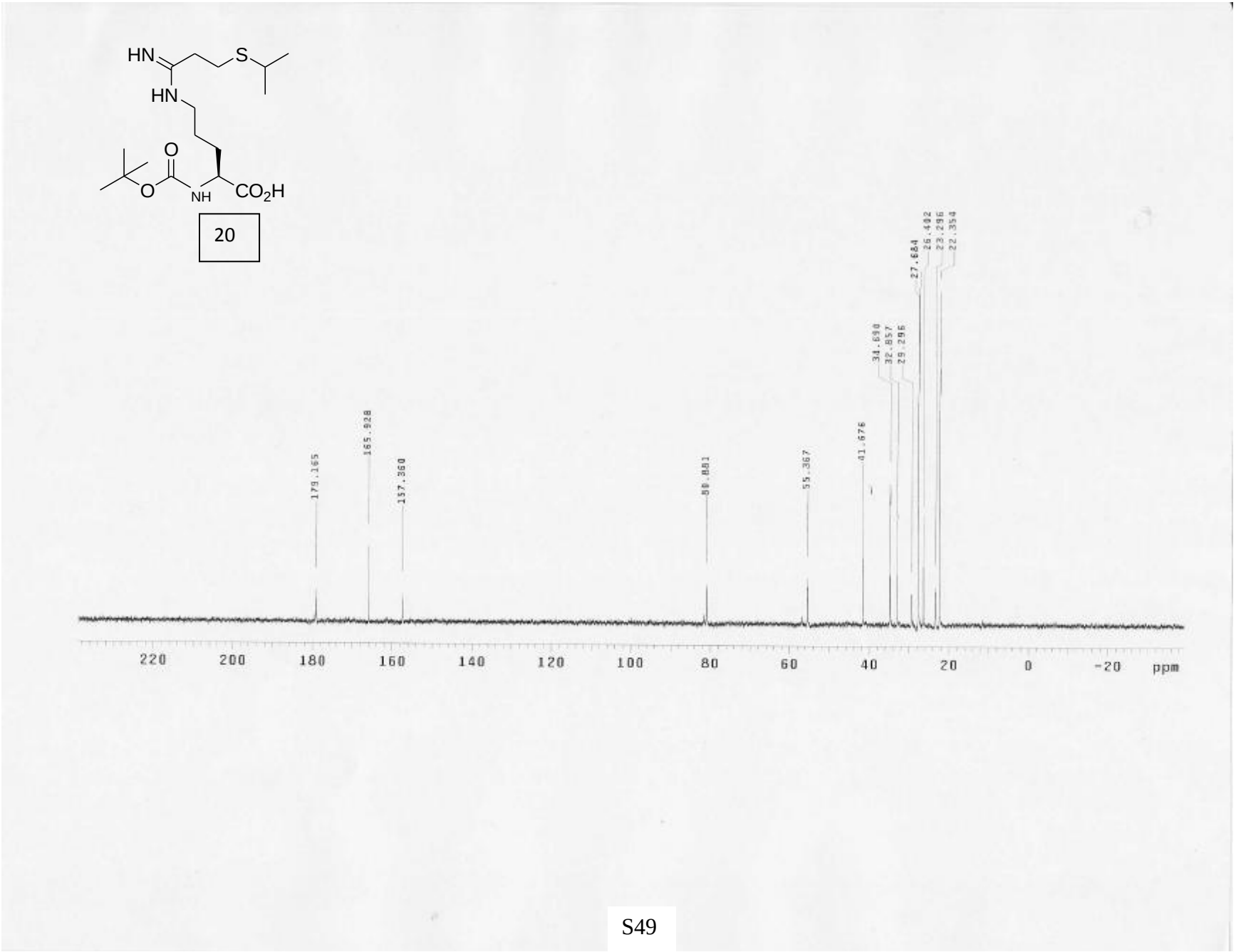
9.08
6.43 6.24


HN
S
HN
O
O
NH
CO2H
20
179.165
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137.360
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55.367
41.676
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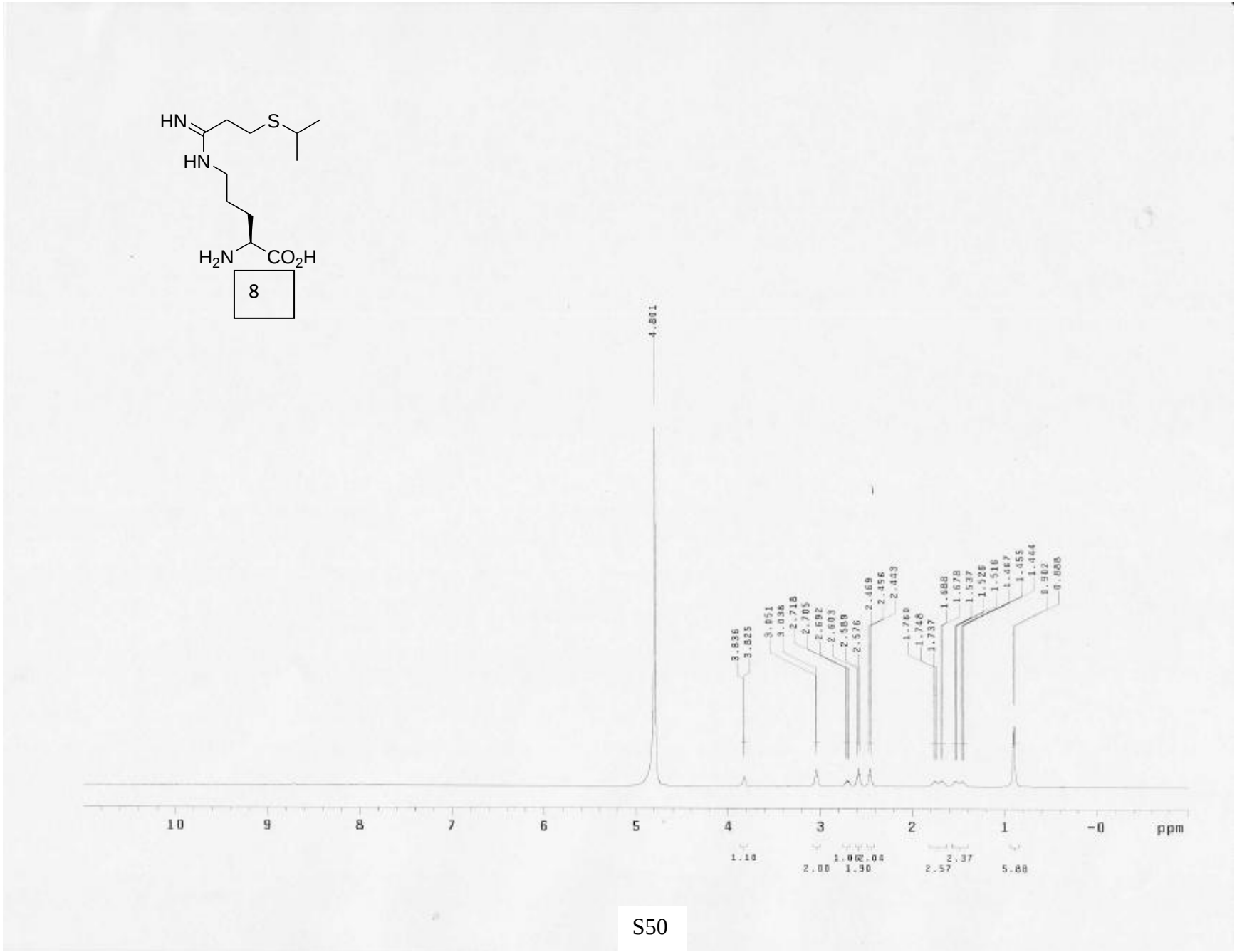
32.857
29.296
27.684
26.492
23.296
22.354
220
200
180
160
140
120
100
80
60
40
20
0
-20
ppm


HN
S
HN
H2N
CO2H
8
4.881
3.836
3.825
3.051
3.038
2.718
2.705
2.692
2.603
2.589
2.576
2.469
2.456
2.443
1.760
1.748
1.737
1.688
1.678
1.537
1.526
1.516
1.467
1.455
1.444
0.902
0.888
10
9
8
7
6
5
4
3
2
1
-0
ppm
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1.00
1.90
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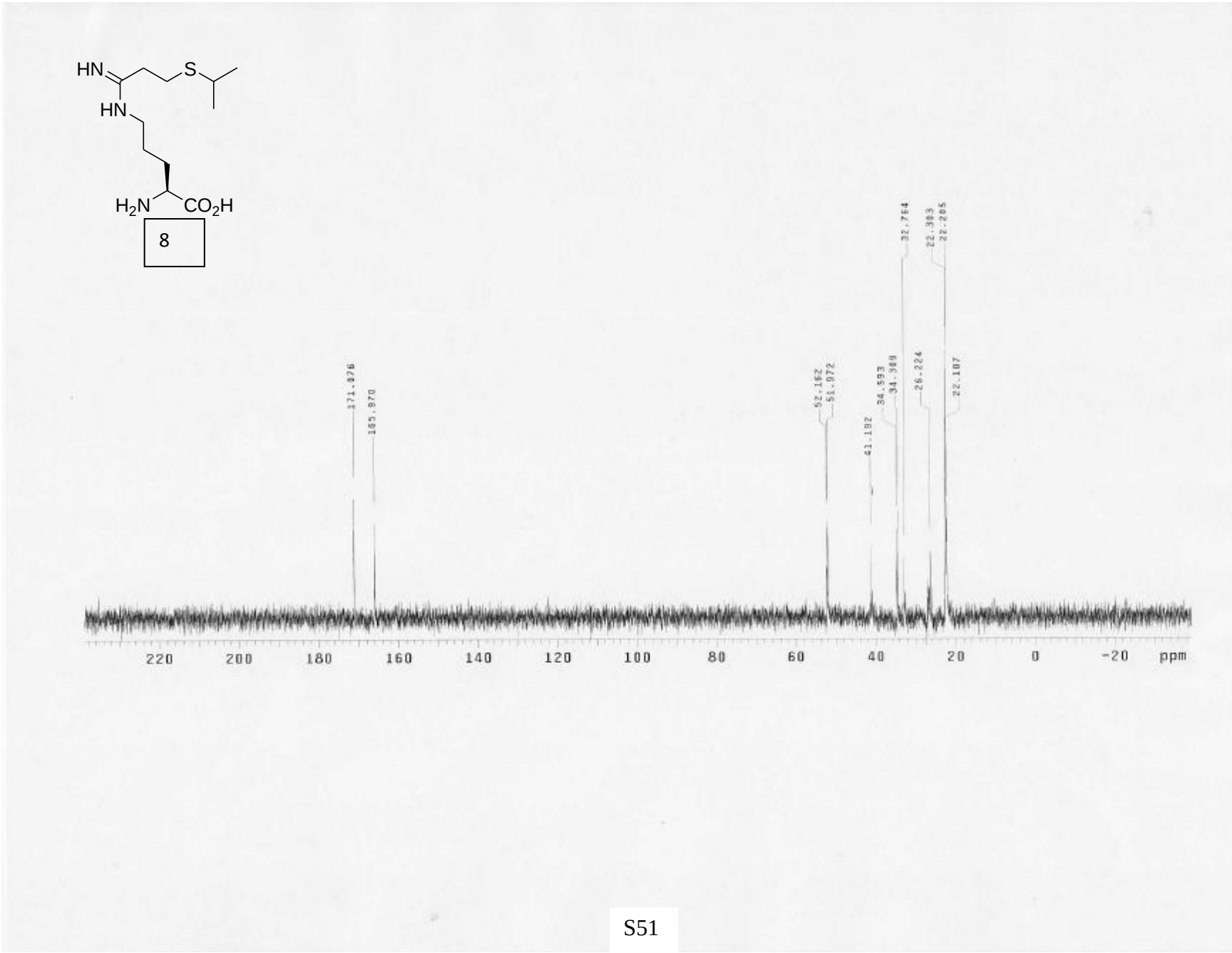
2.57
2.37
5.88


HN
S
HN
H_2N
CO_2H
8
171.876
165.870
52.162
51.072
41.192
34.593
34.369
32.764
26.224
22.383
22.265
22.107
220
200
180
160
140
120
100
80
60
40
20
0
-20
ppm

References

1. Roman, L. J.; Sheta, E. A.; Martásek, P.; Gross, S. S.; Liu, Q.; Masters, B. S. S. High-level expression of functional rat neuronal nitric oxide synthase in escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8428–8432.

2. For **4**, **6**, **12**, and **16**, the protons on the methylene group adjacent to the amidine moiety were found to exchange slowly with D_2O, causing the integration to be too low. The fact that these protons did not give rise to singlets can be explained by amidine rotamers.