Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Biosynthesis of the Antibiotic Tropodithietic Acid by the Marine Bacterium *Phaeobacter inhibens*

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Identification of **1** and **2**:

Both compounds tropodithietic acid (**1**) and hydroxytropodithietic acid (**2**) were obtained from the commercial supplier Bioviotica Naturstoffe GmbH, 37127 Dransfeld, Germany. These compounds were used as standards for the identification of **1** and **2** in all culture extracts of *Phaeobacter inhibens* obtained during the course of this work.

Compounds **1** and **2** were isolated in the group of Prof. Axel Zeeck (University of Göttingen). The structure elucidation, full spectroscopic data for **1** and **2**, and the evaluation of their bioactivities are given in:

Lanfang Liang, PhD thesis, University of Göttingen, 2003.

This thesis can be downloaded from:

http://ediss.uni-goettingen.de/bitstream/handle/11858/00-1735-0000-0006-B0BB-4/liang.pdf?sequence=1

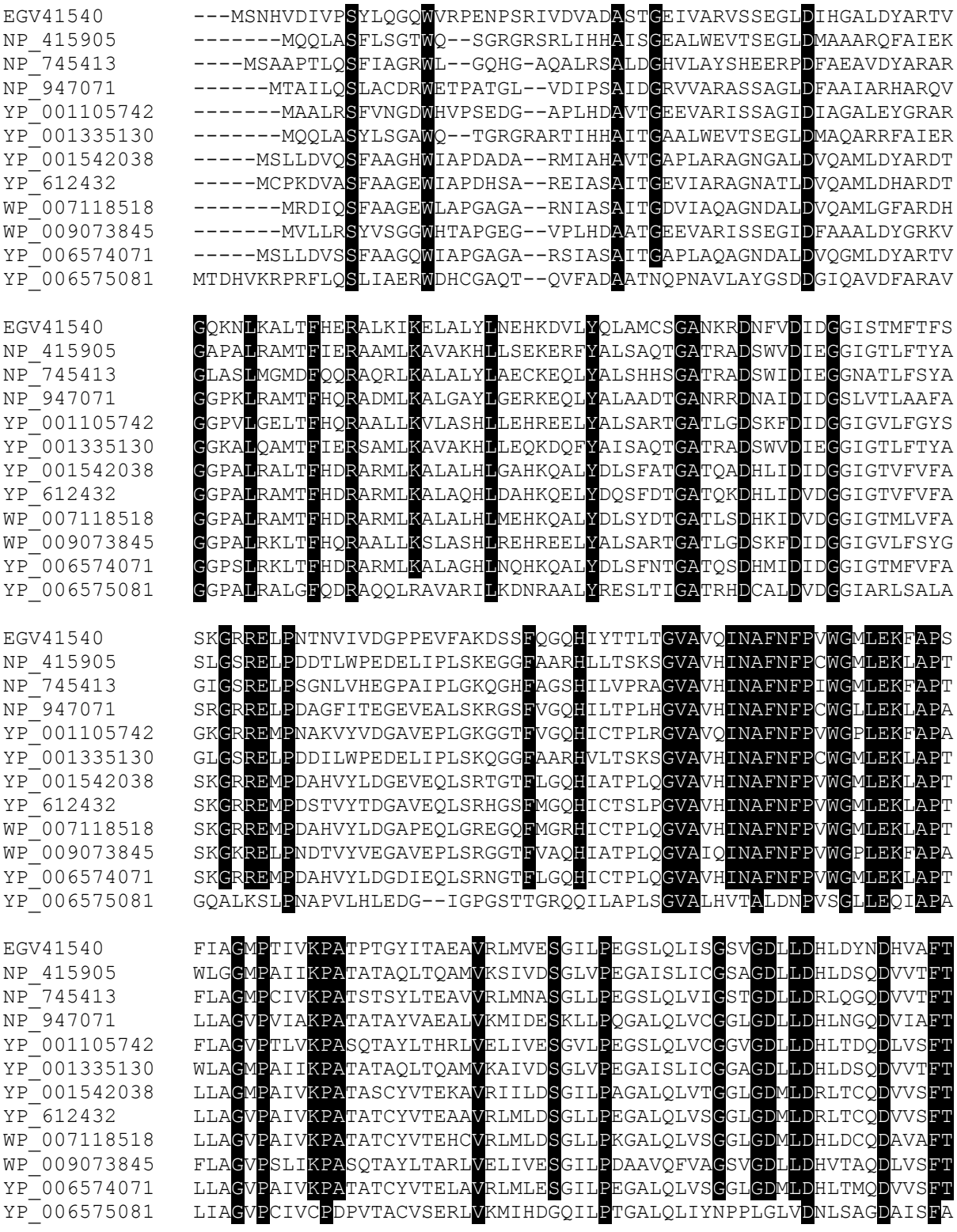
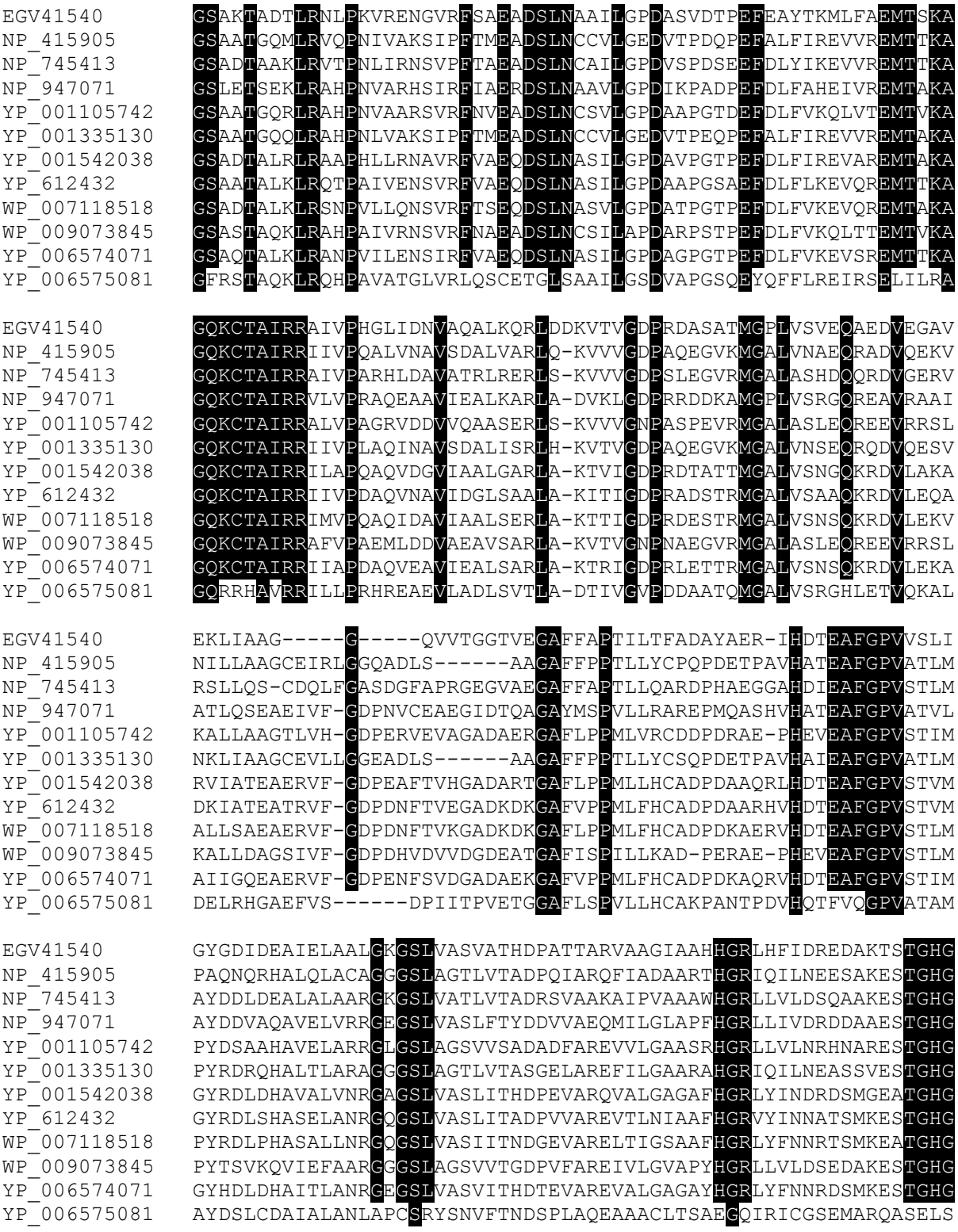


Figure 1. Alignment of amino acid sequences of PaaZ from a selection of bacteria (EGV41540: *Corynebacterium glutamicum*, NP_415905: *Escherichia coli*, NP_745413: *Pseudomonas putida*, NP_947071: *Rhodopseudomonas palustris*, YP_001105742: *Saccharopolyspora erythraea*, YP_001335130: *Klebsiella pneumoniae*, YP_001542038: *Dinoroseobacter shibae*, YP_612432: *Ruegeria* sp., WP_007118518: *Oceanibulbus indolifex*, WP_009073845: *Streptomyces* sp., YP_006574071: *Phaeobacter inhibens* PaaZ1). Consensus of *P. inhibens* PaaZ2 (YP_006575081) to the highly conserved (black) residues is in the ECH-domain 52% (75/143) and in the ALDH-domain 86% (38/44).



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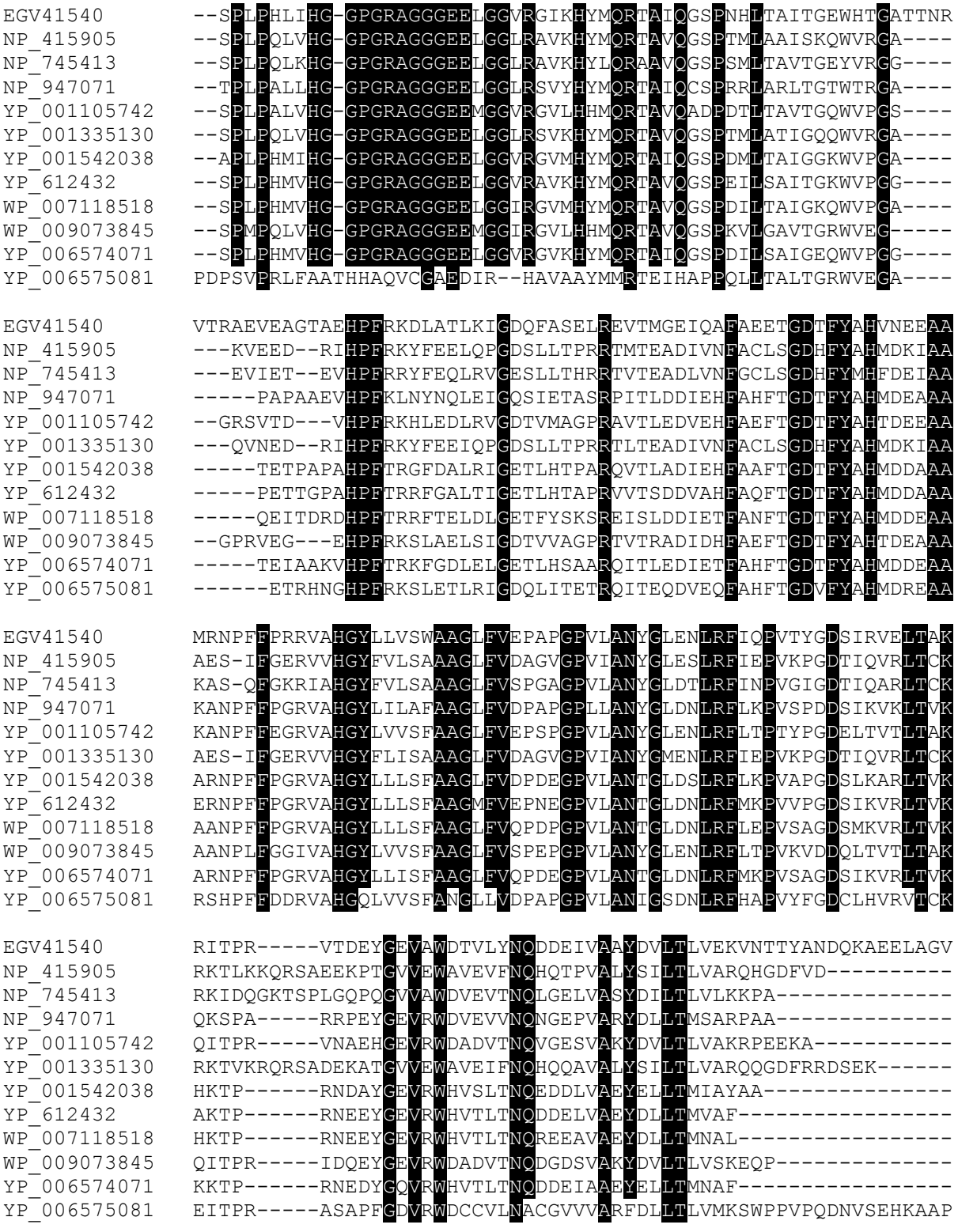

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```
EGV41540        ------------------
NP_415905       ------------------
NP_745413       ------------------
NP_947071       ------------------
YP_001105742    ------------------
YP_001335130    ------------------
YP_001542038    ------------------
YP_612432       ------------------
WP_007118518    ------------------
WP_009073845    ------------------
YP_006574071    ------------------
YP_006575081    HTAATPHRAQDPVQQPAE
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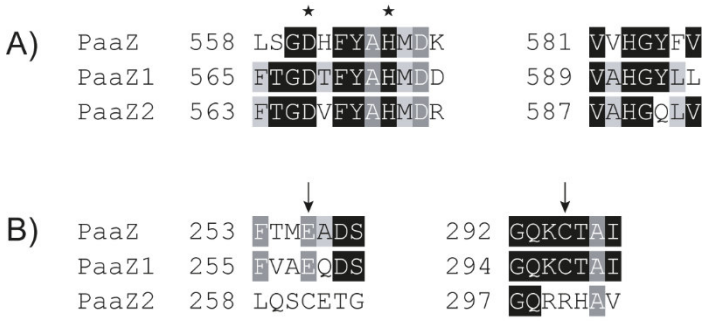

Figure 2. Amino acid sequence alignment of PaaZ from *E. coli* with PaaZ1 and PaaZ2 from *P. inhibens*. A) C-terminal ECH-domains, B) N-terminal ALDH-domains. Asterisks mark essential residues of the ECH-domain while arrows highlight Cys-295 and Glu-256 of *E. coli* PaaZ which are essential for functional ALDH-domains as discussed in the main text.

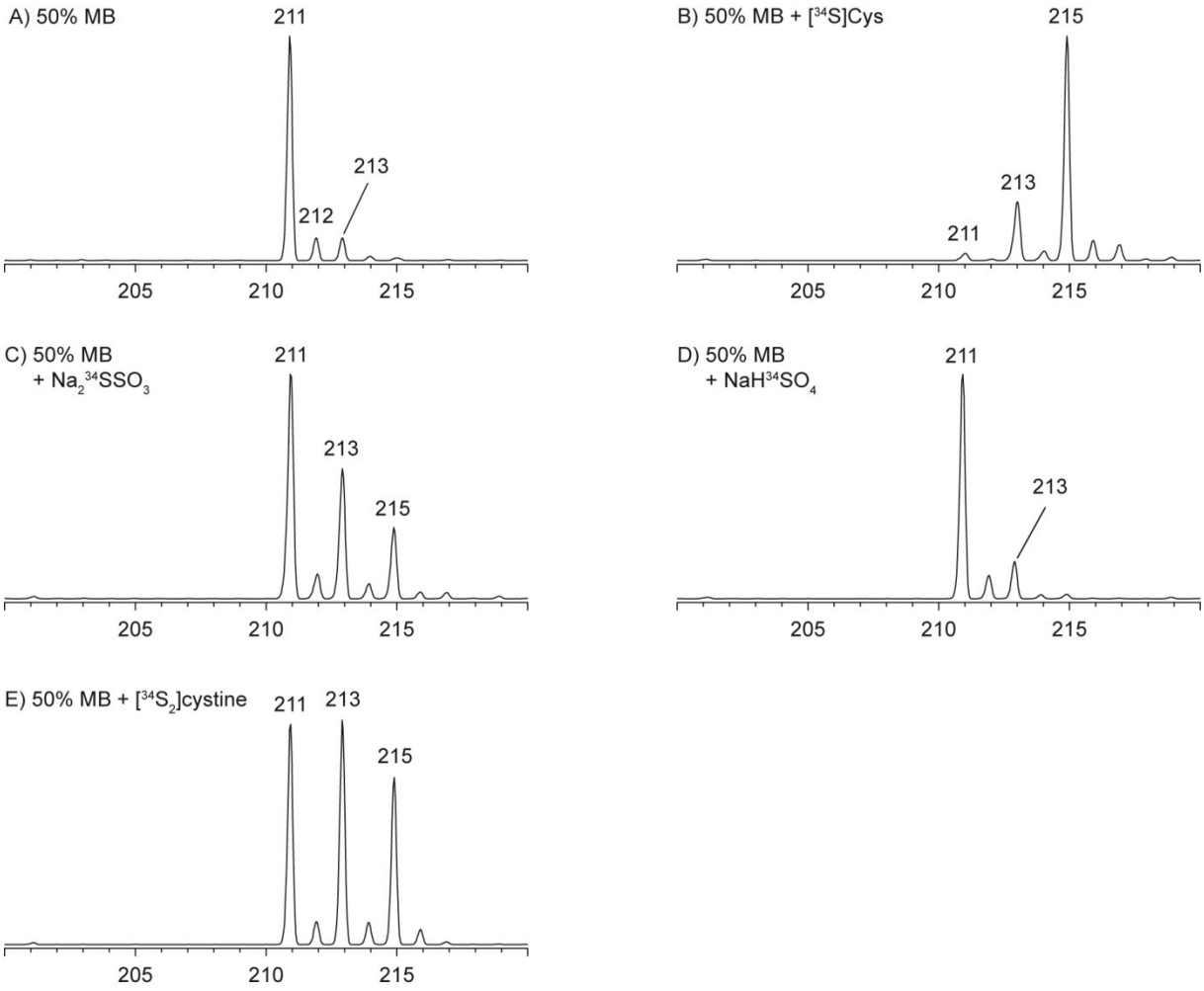

Figure 3. Incorporation of the ^{34}S-labeling into TDA (**1**). A) *P. inhibens* control; B) after feeding of 1 mM [^{34}S]Cys; C) after feeding of 1 mM $Na_2{}^{34}SSO_3$; D) after feeding of 1 mM $NaH^{34}SO_4$; E) after feeding of 0.5 mM [$^{34}S_2$]cystine.

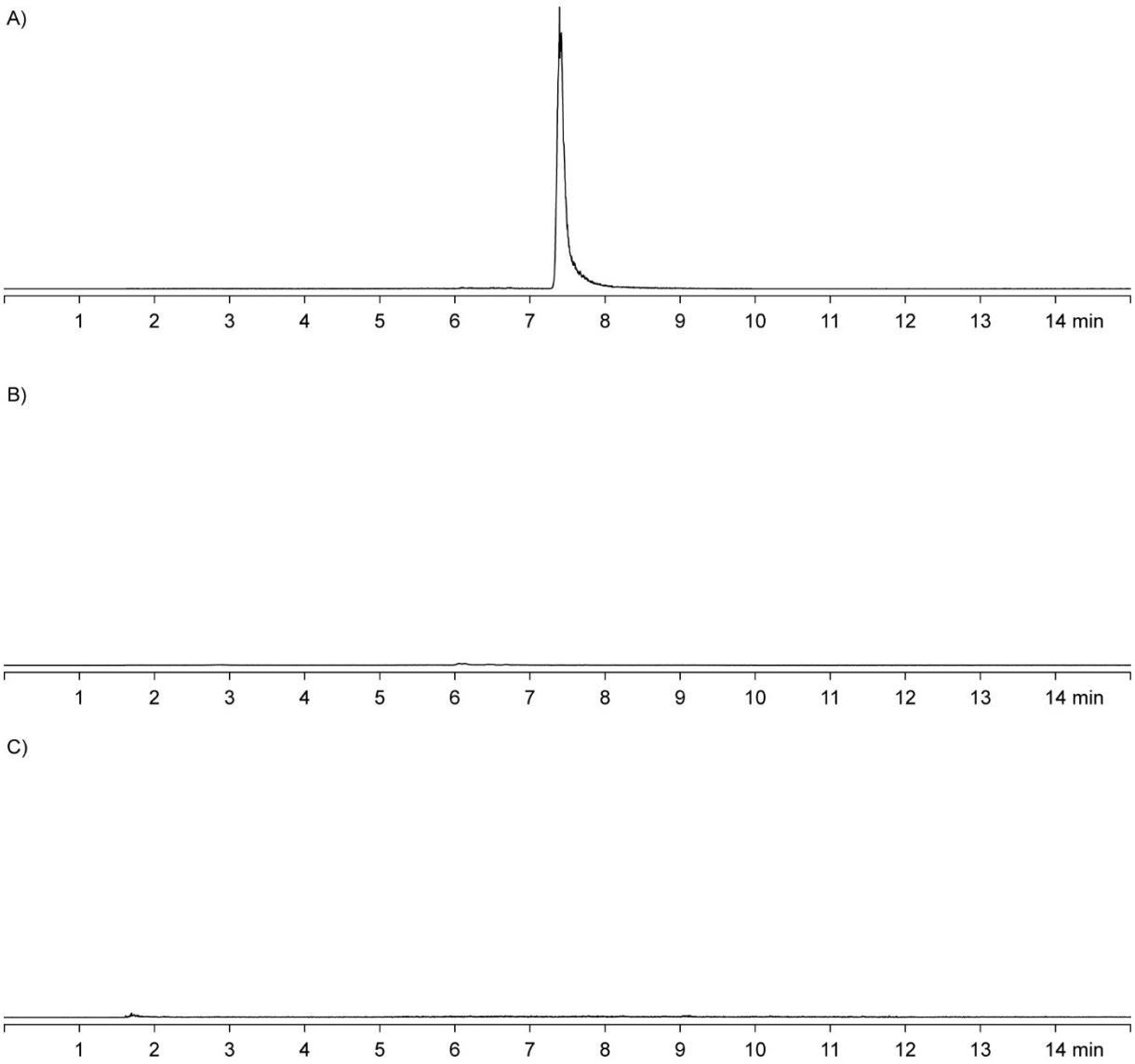

Figure 4. HPLC chromatograms (mass range m/z = 210.5–211.5) of culture extracts from *P. inhibens*. A) wild type, B) strain CP58, C) strain NB01. Tropodithietic acid (**1**) elutes at 7.4 min.

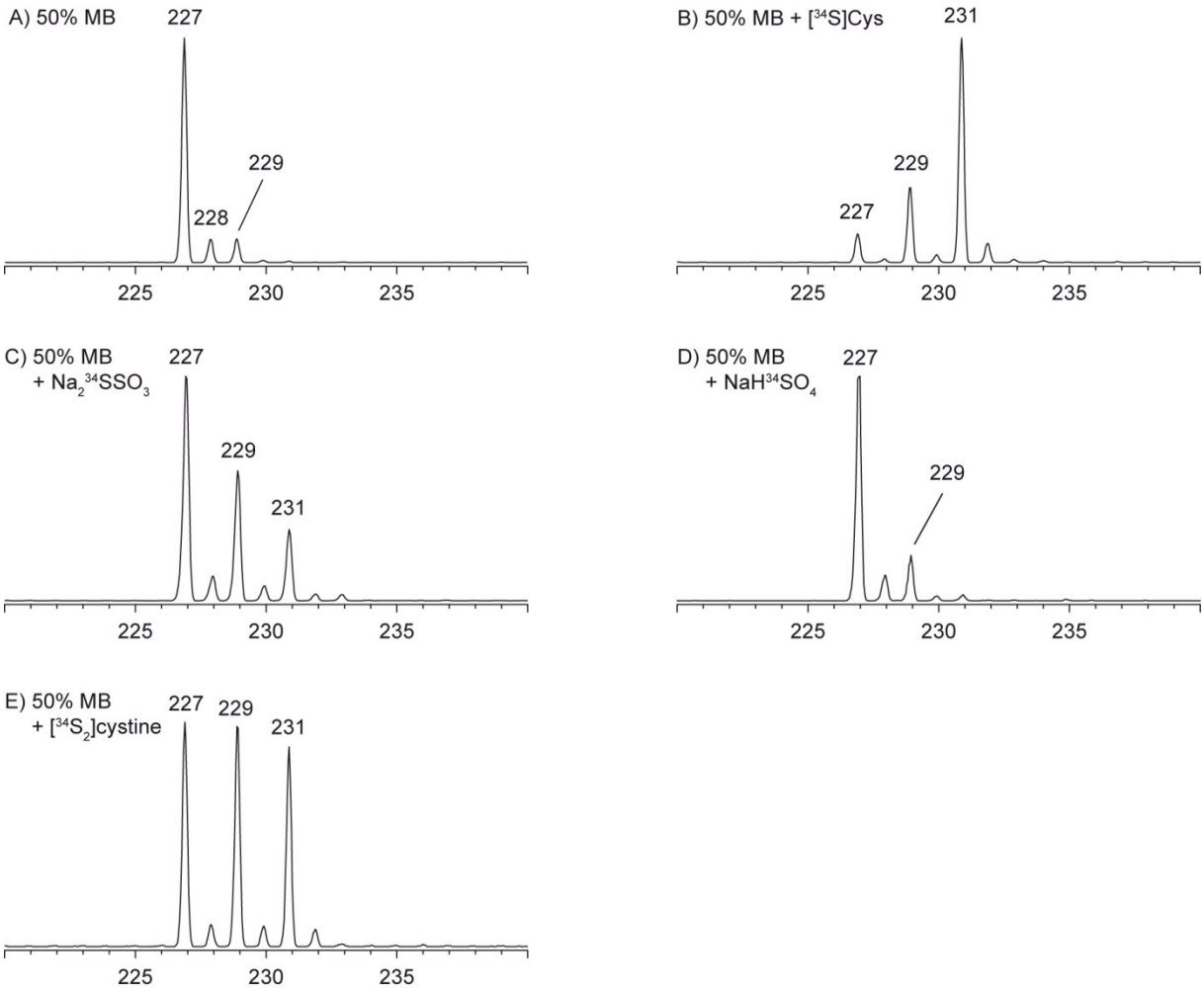

Figure 5. Incorporation of the ^{34}S-labeling into hydroxytropodithietic acid (**2**). A) *P. inhibens* control; B) after feeding of 1 mM [^{34}S]Cys; C) after feeding of 1 mM $Na_2{}^{34}SSO_3$; D) after feeding of 1 mM $NaH^{34}SO_4$; E) after feeding of 0.5 mM [$^{34}S_2$]cystine.

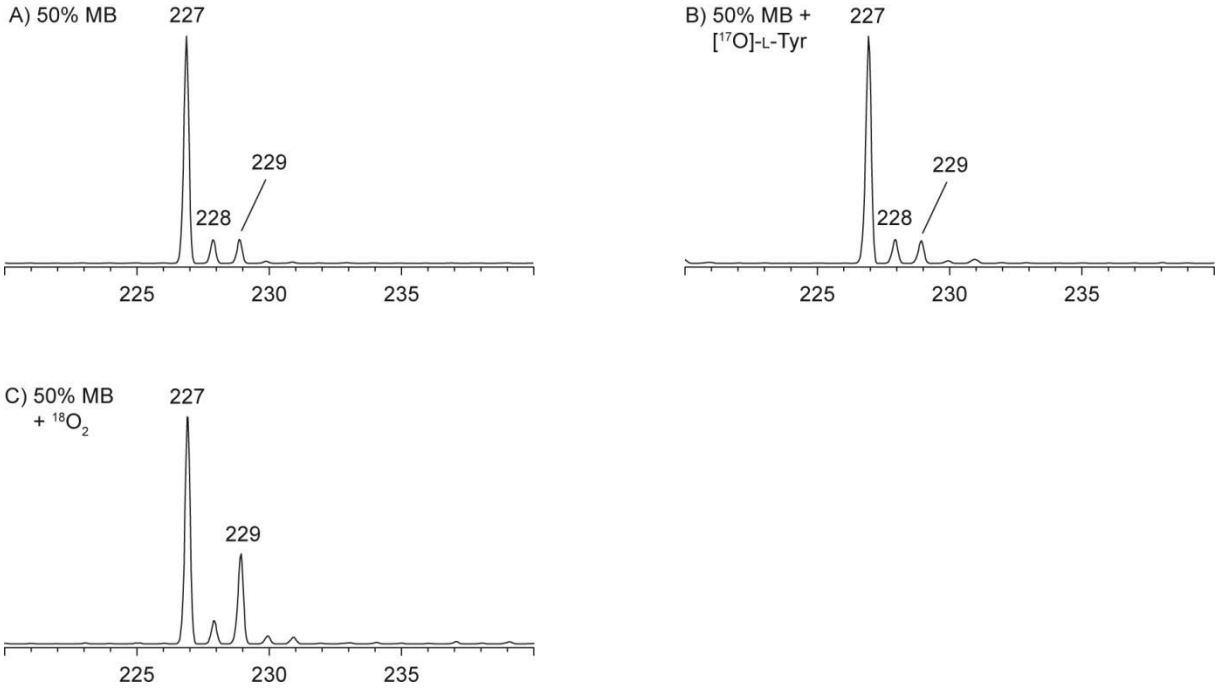

Figure 6. Feeding experiments with [*phenol*-^{17}O]-L-tyrosine and $^{18}O_2$ and incorporation of labeling into hydroxytropodithietic acid (**2**). A) *P. inhibens* control; B) after feeding of 1 mM [^{17}O]-L-Tyr; C) after incubation in an atmosphere with synthetic air containing $^{18}O_2$.

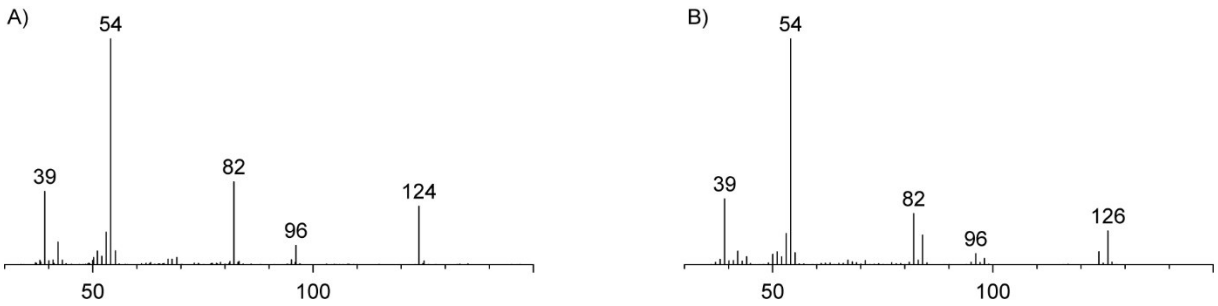

Figure 7. Mass spectra of tropone hydrate (**24**). A) *P. inhibens* control; B) *P. inhibens* after incubation in an atmosphere with synthetic air containing $^{18}O_2$.

Table 1. Bioinformatic analysis of the TDA gene cluster of *P. inhibens* DSM 17395.

Gene	Predicted function	Locus Tag[a]	Accession no.[b]
tdaA	Transcriptional regulator	PGA1_262p00980	YP_006575099
tdaB	Glutathione-S-transferase	PGA1_262p00970	YP_006575098
tdaC	Prephenate dehydratase-like protein	PGA1_262p00960	YP_006575097
tdaD	Thioesterase	PGA1_262p00950	YP_006575096
tdaE	Acyl-CoA dehydrogenase	PGA1_262p00940	YP_006575095
tdaF	Phosphopantothenoylcysteine decarboxylase	PGA1_262p00810	YP_006575082

[a] Locus tags of the targeted genes. [b] Accession number of the targeted proteins.

Table 2. Oligonucleotide primers used in this study.

Primer	Sequence
NB064f_patB-LA_KpnI	CGCCTCCATGGTACCGATCATGTCCTGCC
NB064r_patB-LA_BamHI	CTCCCGTGGATCCCGGGCGAAGCTAAC
NB065f_patB-RA_BamHI	GCGATCGGATCCGCAACGCCTTCTC
NB065r_patB-RA_HindIII	GTCCTCAAGCTTGCCGGTGATCTCG
NB033f_pBBR1MCS1-5_BamHI	GGTCATAGCTGGATCCTGTGTGAAATTG
NB033r_pBBR1MCS1-5_BamHI	GCGCACGGATCCCCGAAAAGTG

Experimental Procedures and Spectral Data

Bacterial strains, plasmids, growth conditions and feeding experiments. *Phaeobacter inhibens* DSM17395, strain CP58[1], and strain NB01 were grown in half strength Marine Broth (MB 2216, Roth) and shaking (160 rpm) or on corresponding solid agar medium (20 g/L agar) at 28°C. When required, the medium was spiked after autoclavation with gentamycin sulfate (25 μg/mL) or kanamycin sulfate (60 μg/mL). *Escherichia coli* strains were grown in 2YT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH = 7.2) at 37°C with shaking (200 rpm) or on corresponding solid agar medium (20 g/L agar). When required, the medium was spiked after autoclavation with ampicillin (100 μg/mL) or kanamycin sulfate (50 μg/mL). For the feeding experiments, half strength MB liquid medium (50 mL) was spiked after autoclavation with 1 mM $Na_2{}^{34}SSO_3 \cdot 5\ H_2O$, $NaH^{34}SO_4$ (50% ^{34}S-enchrichment), [^{34}S]-L-cysteine hydrochloride, [*phenol*-^{17}O]-L-tyrosine (35–40% ^{17}O-enchrichment), or 0.5 mM [$^{34}S_2$]-L-cystine dihydrochloride, respectively. The flasks were inoculated with 1 mL of the preculture and incubated for 16 h at 28°C. For the feeding experiment with $^{18}O_2$, synthetic air was prepared from N_2 (790 mL) and $^{18}O_2$ (97.1% enrichment, 210 mL) in a balloon and connected to an inoculated flask. The culture flask volume (300 mL) diminished the effective labeling to ca. 75%. After 16 h, each culture was acidified with aqueous HCl (2 M, 5 mL), extracted with an equal volume of ethyl acetate, dried with $MgSO_4$, and concentrated in vacuo. The residue was taken up in methanol (2 mL) and analyzed via HPLC-MS. Collection of volatiles was done with a CLSA as reported previously.[2]

Construction of *P. inhibens* mutant strains. Primers used in this study are listed in Table 2. For deletion of *patB*, primers were designed to amplify chromosomal fragments upstream and downstream of the gene. The amplified upstream region was cloned into the *Kpn*I and *Bam*HI sites of pUC-19. The amplified downstream region was cloned into the *Bam*HI and *Hin*dIII sites of the previously obtained plasmid carrying the respective upstream region. The kanamycin resistance gene (amplified from pBBR1MCS-2 and digested with *Bam*HI was cloned into the or *Bam*HI site of the respective plasmid that already carried the upstream and downstream regions. *P. inhibens* DSM 17395 was transformed by electroporation with this plasmid, leading to the strain NB01 (Δ*patB*::*kan*).

HPLC-MS analyses. HPLC-MS analyses were carried out on a Thermo LTQ XL system with a C_{18} column (Hypersil GOLD, 3 μm, 150 × 2.1 mm). MS-Conditions were as follows: source type: HESI, capillary temperature: 275 °C, source heater temperature: 40 °C, sheath gas flow: 15, aux gas flow: 10, negative polarity, source voltage: 4 kV. The LC was programmed as follows:

Time (min)	Water	Acetonitrile	Methanol/water 1:1 + 2% formic acid
0.0	0.925	0.025	0.050
1.5	0.925	0.025	0.050
8.0	0.025	0.925	0.050
15.0	0.025	0.925	0.050
17.0	0.925	0.025	0.050
22.0	0.925	0.025	0.050

Commercially available tropodithietic acid (**1**) eluted at 7.4 min and hydroxytropodithietic acid (**2**) at 6.7 min.

General Synthetic Methods. Chemicals were purchased from Acros Organics (Geel, Belgium) or Sigma-Aldrich Chemie GmbH (Steinheim, Germany), and used without further purification. $^{34}S_8$ (99.93% enriched) was purchased from Campro Scientific GmbH (Berlin, Germany). $^{18}O_2$ (97.1% enriched) and [*phenol*-^{17}O]-L-tyrosine (35–40% enriched) were purchased from Euriso-Top GmbH (Saarbrücken, Germany). Solvents were purified by distillation, and dried according to standard methods. Oxygen and/or moisture sensitive reactions were carried out under inert atmosphere (N_2) in vacuum-heated flasks with dried solvents. Thin-layer chromatography (SiO_2, TLC) was performed on 0.20 mm Macherey-Nagel silica gel plates (Polygram SIL G/UV$_{254}$). Column chromatography was performed on Merck silica gel 60 (0.040 - 0.063 mm) using standard flash chromatographic methods. The NMR spectra were recorded on Bruker AV II-300 (300 MHz), DRX-400 (400 MHz) or AV III-400 (400 MHz) spectrometers, and were referenced against TMS or TMSP (δ = 0.00 ppm) for ^{1}H-NMR and $CHCl_3$ (δ = 77.01 ppm) for ^{13}C-NMR. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br. = broad. Infrared spectra were recorded on a Bruker Tensor 27 ATR spectrometer. UV spectra were recorded on a Varian Cary 100 Bio spectrometer. Specific rotations were measured using a Dr. Kernchen Propol Digital Automatic Polarimeter. GC-MS analyses for the synthetic compounds were carried out on a HP6890 GC system connected to a HP5973 Mass Selective Detector fitted with a BPX-5 fused silica capillary column (25 m x 0.22 mm i.d., 0.25 μm film, SGE Inc., Melbourne, Australia). Conditions were as follows: inlet pressure: 77.1 kPa, He 23.3 mL min^{-1}; injection volume: 1 μL; injector: 250 °C; transfer line: 300 °C; electron energy: 70 eV. The GC was programmed as follows: 50 °C (5 min isothermic), increasing at 10 °C min^{-1} to 320 °C, and operated in split mode; carrier gas (He): 1.0 mL min^{-1}. Retention indices were determined from a homologous series of *n*-alkanes (C_8–C_{32}).

K^{34}SCN: Elemental $^{34}S_8$ (0.189 g, 5.56 mmol, 1.0 equiv.) was added to a solution of KCN (0.362 g, 5.56 mmol, 1.0 equiv.) in water (22 mL) and was stirred under reflux until the sulfur was consumed (48 h). Filtration and concentration in vacuo gave K^{34}SCN (0.546 g, 5.52 mmol, 99%) as a colorless solid. ^{13}C-NMR (75 MHz, D_2O): δ = 136.1 (C_q) ppm.

Methyl *N*-(*tert*-butoxycarbonyl)-*O*-tosyl-L-serinate (13):[3] To a solution of methyl *N*-(*tert*-butoxycarbonyl)-L-serinate (5.96 g, 27.2 mmol, 1.0 equiv.) in dry pyridine (50 mL) was added 4-toluenesulfonyl chloride (7.28 g, 38.1 mol, 1.4 equiv.) at –10 °C. After stirring at –10 °C overnight, the reaction was quenched with water, the mixture extracted with ethyl acetate, dried with $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 2:1) to give **13** (7.26 g, 19.5 mmol, 72%) as a colorless oil. TLC (hexane/ethyl acetate 2:1, R_f = 0.42). ^{1}H-NMR (400 MHz, $CDCl_3$, TMS): δ = 7.76 (d, ^3J(H,H) = 8.4 Hz, 2H, 2 CH), 7.36 (d, ^3J(H,H) = 8.2 Hz, 2H, 2 CH), 5.32 (d, ^3J(H,H) = 8.1 Hz, 1H, NH), 4.51 (dt, ^3J(H,H) = 8.2 Hz, ^3J(H,H) = 3.1 Hz, 1H, CH), 4.40 (dd, ^2J(H,H) = 10.1 Hz, ^3J(H,H) = 3.1 Hz, 1H, CH_2), 4.29 (dd, ^2J(H,H) = 10.1 Hz, ^3J(H,H) = 3.1 Hz, 1H, CH_2), 3.70 (s, 3H, CH_3), 2.45 (s, ^1J(C,H) = 127.4 Hz, 3H, CH_3), 1.42 (s, 9H, 3 CH_3) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 168.9 (C_q), 154.9 (C_q), 145.1 (C_q), 132.3 (C_q), 129.9 (2 CH), 128.0 (2 CH), 80.4 (C_q), 69.5 (CH_2), 52.90 (CH), 52.85 (CH_3), 28.2 (3 CH_3), 21.6 (CH_3) ppm. IR (ATR): $\tilde{\nu}$ = 3399 (m), 2891 (w), 2927 (w), 1739 (s), 1704 (s), 1597 (w), 1510 (s), 1444w), 1370 (w), 1343 (s), 1243 (s), 1211 (m), 1155 (s), 1098 (m), 1058 (s), 1035 (w), 993 (s), 938 (s), 889 (s), 862 (m), 839 (m), 812 (s), 780 (s), 759 (m), 732 (s), 705 (w), 667 (s), 637 (m), 553 (s), 534 (s) cm^{-1}. UV/Vis (CH_2Cl_2): λ_{max} (ε, L mol^{-1} cm^{-1}) = 273 (475), 262 (608), 231 (7980) nm.

[^{34}S]Methyl *N*-(*tert*-butoxycarbonyl)-*S*-cyano-L-cysteinate (14):[4] A suspension of **13** (1.81 g, 4.85 mmol, 1.0 equiv.) and K^{34}SCN (0.506 g, 5.11 mmol, 1.05 equiv.) in dry acetonitrile (20 mL) was stirred under reflux overnight. After concentration in vacuo, the residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 3:1) to give **14** (0.399 g, 1.52

mmol, 32%) as a pale yellow oil. TLC (hexane/ethyl acetate 3:1, R_f = 0.27). GC (BPX-5): I = 1753. $[\alpha]^{22}_D$ = +95.1 (c 1.67 in $CHCl_3$). ^{1}H-NMR (400 MHz, $CDCl_3$, TMS): δ = 5.57 (d, ^3J(H,H) = 7.0 Hz, 1H, NH), 4.69 (m, 1H, CH), 3.84 (s, ^1J(C,H) = 148.5 Hz, 3H, CH_3), 3.55 (dd, ^2J(H,H) = 14.0 Hz, ^3J(H,H) = 4.6 Hz, 1H, CH_2), 3.45 (dd, ^2J(H,H) = 14.0 Hz, ^3J(H,H) = 4.5 Hz, 1H, CH_2), 1.46 (s, ^1J(C,H) = 126.8 Hz, 9H, 3 CH_3) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 169.2 (C_q), 154.8 (C_q), 111.3 (C_q), 80.9 (C_q), 53.3 (CH), 53.1 (CH_3), 36.3 (CH_2), 28.2 (3 CH_3) ppm. MS (EI, 70 eV): m/z (%) = 262 (<1) $[M]^+$, 203 (1), 188 (1), 145 (3), 128 (2), 103 (19), 88 (6), 57 (100), 41 (53). IR (ATR): $\tilde{\nu}$ = 3367 (w), 2979 (w), 2157 (w), 1745 (m), 1703 (s), 1503 (m), 1438 (w), 1393 (w), 1366 (m), 1349 (m), 1317 w), 1247 (m), 1216 (m), 1158 (s), 1054 (m), 1013 (m), 914 (w), 857 (w), 779 (w), 731 (s), 647 (w) cm^{-1}. UV/Vis (CH_2Cl_2): λ_{max} (ε, L mol^{-1} cm^{-1}) = 268 (269), 228 (289) nm.

[^{34}S]Methyl *N*-(*tert*-butoxycarbonyl)-L-cysteinate (15):[5] To a solution of **14** (1.32 g, 5.04 mmol, 1.0 equiv.) and water (2.99 g, 166 mmol, 33 equiv.) in dry THF (111 mL) was added SmI_2 (4.48 g, 11.1 mol, 2.2 equiv.) at room temperature. After stirring for 5 min, the reaction was quenched with a saturated aqueous NH_4Cl solution, the mixture extracted with diethyl ether, dried with $MgSO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 3:1) to give **15** (0.855 g, 3.61 mmol, 72%) as a colorless oil. TLC (hexane/ethyl acetate 3:1, R_f = 0.47). GC (BPX-5): I = 1539. ^{1}H-NMR (400 MHz, $CDCl_3$, TMS): δ = 5.42 (d, ^3J(H,H) = 7.3 Hz, 1H, NH), 4.61 (m, 1H, CH), 3.79 (s, ^1J(C,H) = 147.9 Hz, 3H, CH_3), 3.00 (ddd, ^2J(H,H) = 14.1 Hz, ^3J(H,H) = 9.2 Hz, ^3J(H,H) = 4.6 Hz, 1H, CH_2), 2.96 (ddd, ^2J(H,H) = 13.9 Hz, ^3J(H,H) = 9.0 Hz, ^3J(H,H) = 4.6 Hz, 1H, CH_2), 1.46 (s, 9H, 3 CH_3) 1.40 (t, ^3J(H,H) = 8.9 Hz, 1H, SH) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 170.8 (C_q), 155.1 (C_q), 80.3 (C_q), 54.8 (CH), 52.6 (CH_3), 28.3 (3 CH_3), 27.3 (CH_2) ppm. MS (EI, 70 eV): m/z (%) = 237 (<1) $[M]^+$, 181 (10), 164 (5), 136 (6), 120 (25), 104 (8), 88 (19), 78 (18), 57 (100), 41 (53). IR (ATR): $\tilde{\nu}$ = 3367 (w), 2978 (w), 2568 (w), 1744 (m), 1704 (s), 1501 (m), 1438 (w), 1392 (w), 1366 (m), 1349 (m), 1249 (m), 1212 (m), 1159 (s), 1061 (m), 1025 (m), 910 (w), 858 (w), 800 (w), 780 (w), 733 (m) cm^{-1}. UV/Vis (CH_2Cl_2): λ_{max} (ε, L mol^{-1} cm^{-1}) = 228 (191) nm.

[^{34}S]-L-Cysteine hydrochloride (16):[3] A solution of **15** (0.211 g, 0.89 mmol, 1.0 equiv.) in aqueous HCl (1 M, 10 mL) was stirred under reflux for 3 h. After concentrating in vacuo, the residue was purified by ion-exchange column chromatography (Dowex® 50-8, elution with 1 M HCl) to give **16** (0.107 g, 0.67 mmol, 75%) as a pale yellow solid. $[\alpha]^{22}_D$ = −24.5 (c 0.99 in H_2O, pH = 1.89). ^{1}H-NMR (400 MHz, D_2O, TMSP): δ = 4.29 (dd, ^3J(H,H) = 5.6 Hz, ^3J(H,H) = 4.3 Hz, 1H, CH), 3.17 (dd, ^2J(H,H) = 15.1 Hz, ^3J(H,H) = 5.6 Hz, 1H, CH_2), 3.11 (dd, ^2J(H,H) = 15.1 Hz, ^3J(H,H) = 4.3 Hz, 1H, CH_2) ppm. ^{13}C-NMR (100 MHz, D_2O): δ = 173.4 (C_q), 57.5 (CH), 26.8 (CH_2) ppm. HRMS (ESI) $[M - HCl + H^+]/z$ calcd. 124.02282, found 124.02284.

[$^{34}S_2$]-L-Cystine dihydrochloride (17):[6] [^{34}S]-L-cysteine hydrochloride (**16**, 11.9 mg, 74.4 µmol, 1 equiv.) was added to 0.15 mL of a solution of sodium iodide (11.2 mg, 74.7 µmol, 0.01 equiv.) in water (14.8 mL) and aqueous H_2O_2 (30%, 0.8 mL) at room temperature. After stirring at room temperature for 30 min, the reaction mixture was filtered, washed with water, and dried in vacuo to give **17** (9.5 mg, 30.0 µmol, 81%) as a colorless solid. $[\alpha]^{22}_D$ = −86.9 (c 1.01 in 3% NaOH-H_2O, pH = 13.30). ^{1}H-NMR (300 MHz, 3%NaOD-D_2O, TMSP): δ = 3.57 (dd, ^3J(H,H) = 7.5 Hz, ^3J(H,H) = 4.7 Hz, 1H, CH), 3.11 (dd, ^2J(H,H) = 13.6 Hz, ^3J(H,H) = 4.7 Hz, 1H, CH_2), 2.91 (dd, ^2J(H,H) = 13.6 Hz, ^3J(H,H) = 7.6 Hz, 1H, CH_2) ppm. ^{13}C-NMR (75 MHz, 3% NaOD-D_2O): δ = 183.5 (C_q), 57.5 (CH), 46.3 (CH_2) ppm.

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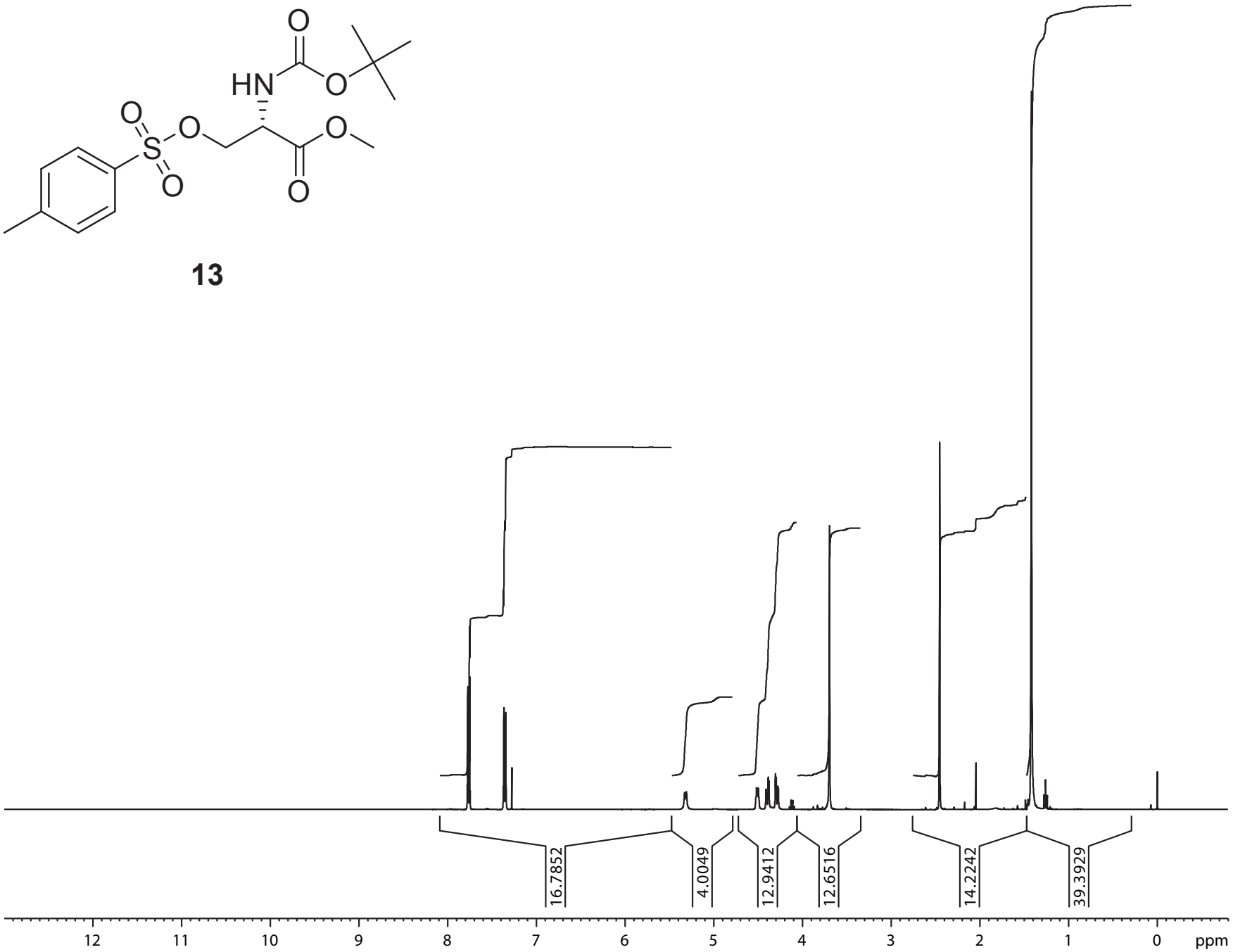
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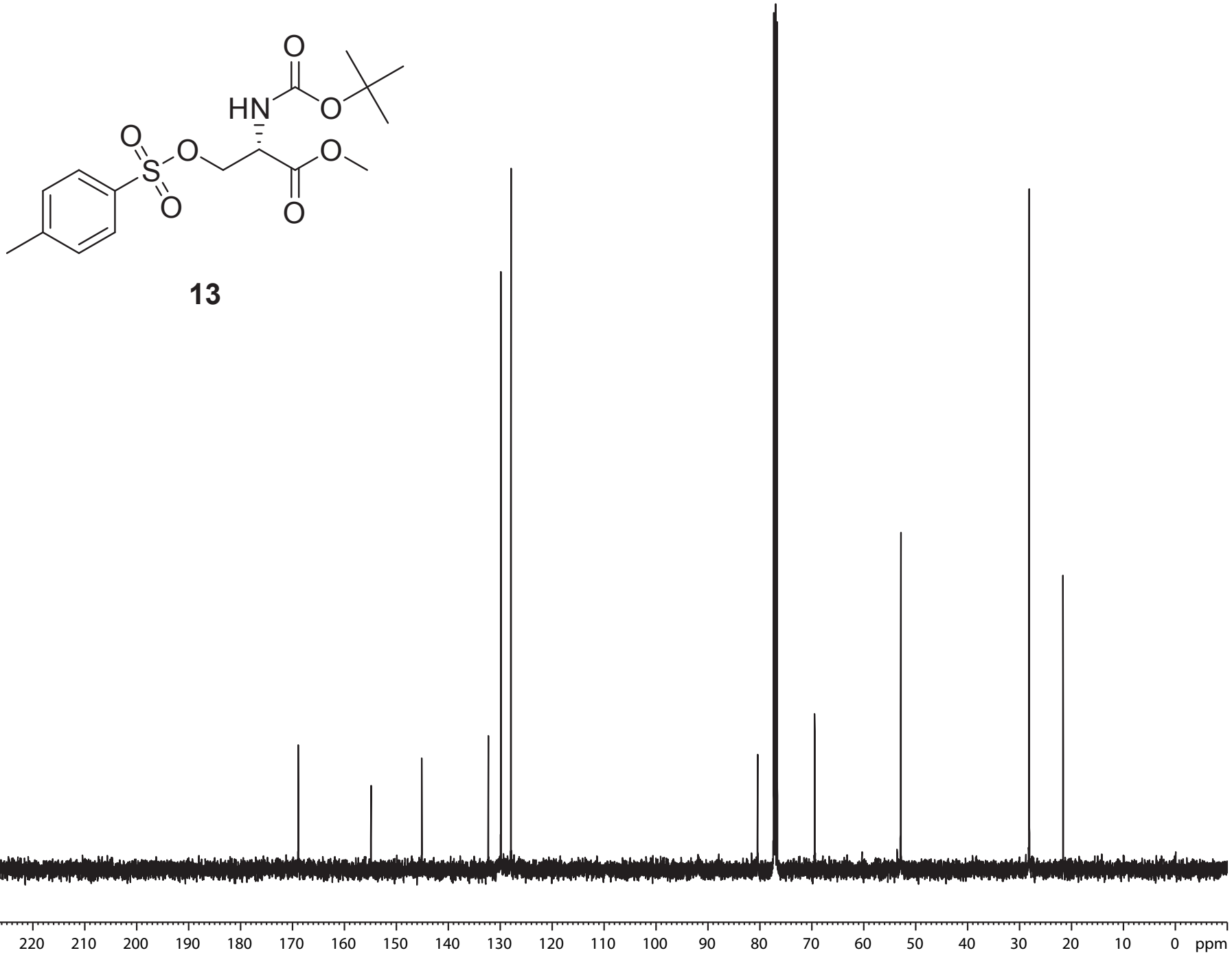
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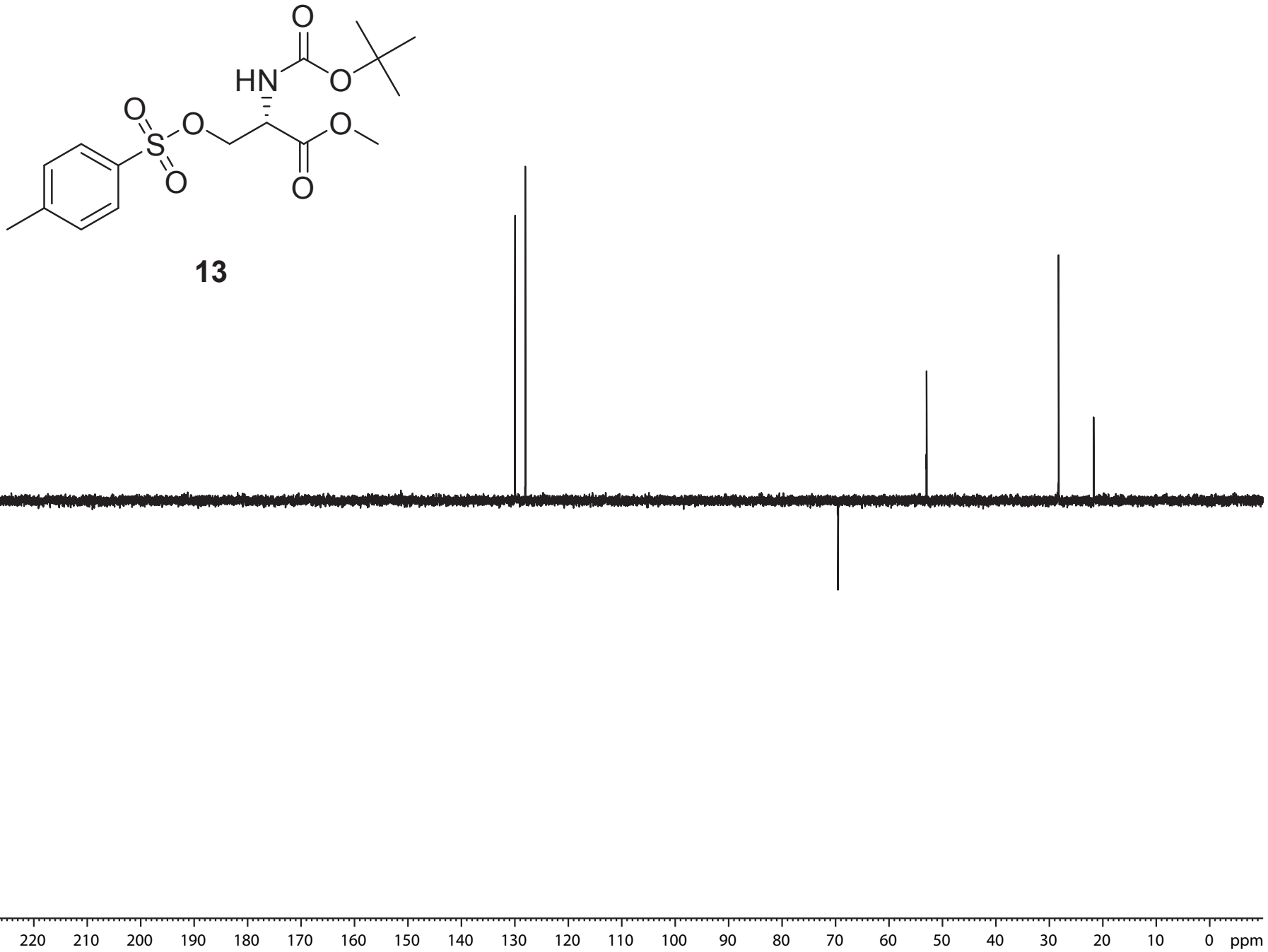
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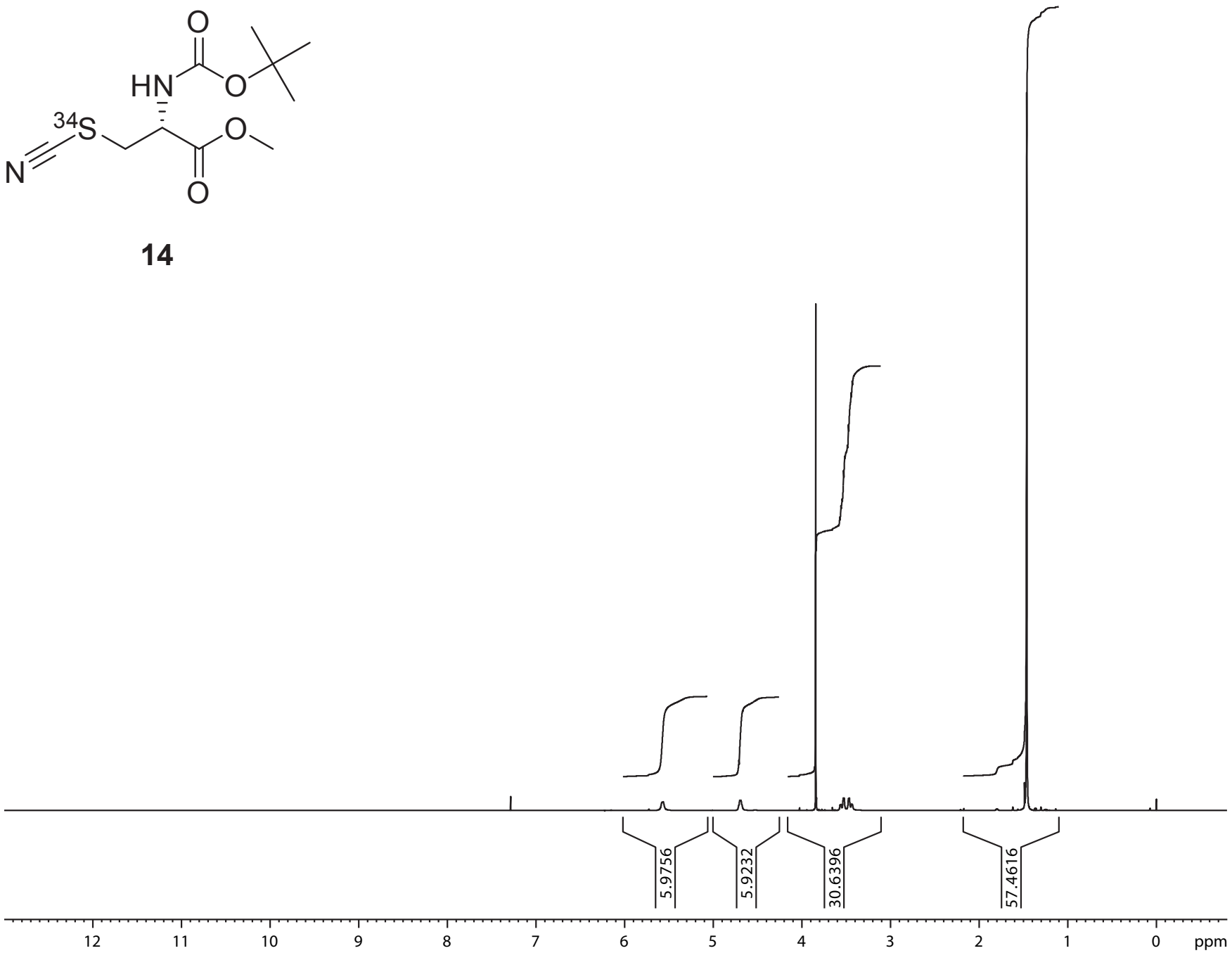
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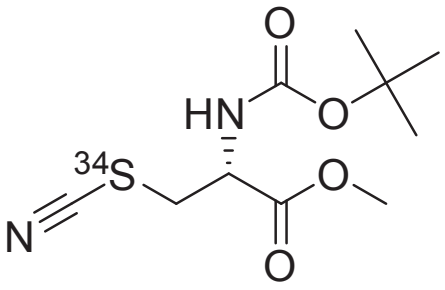
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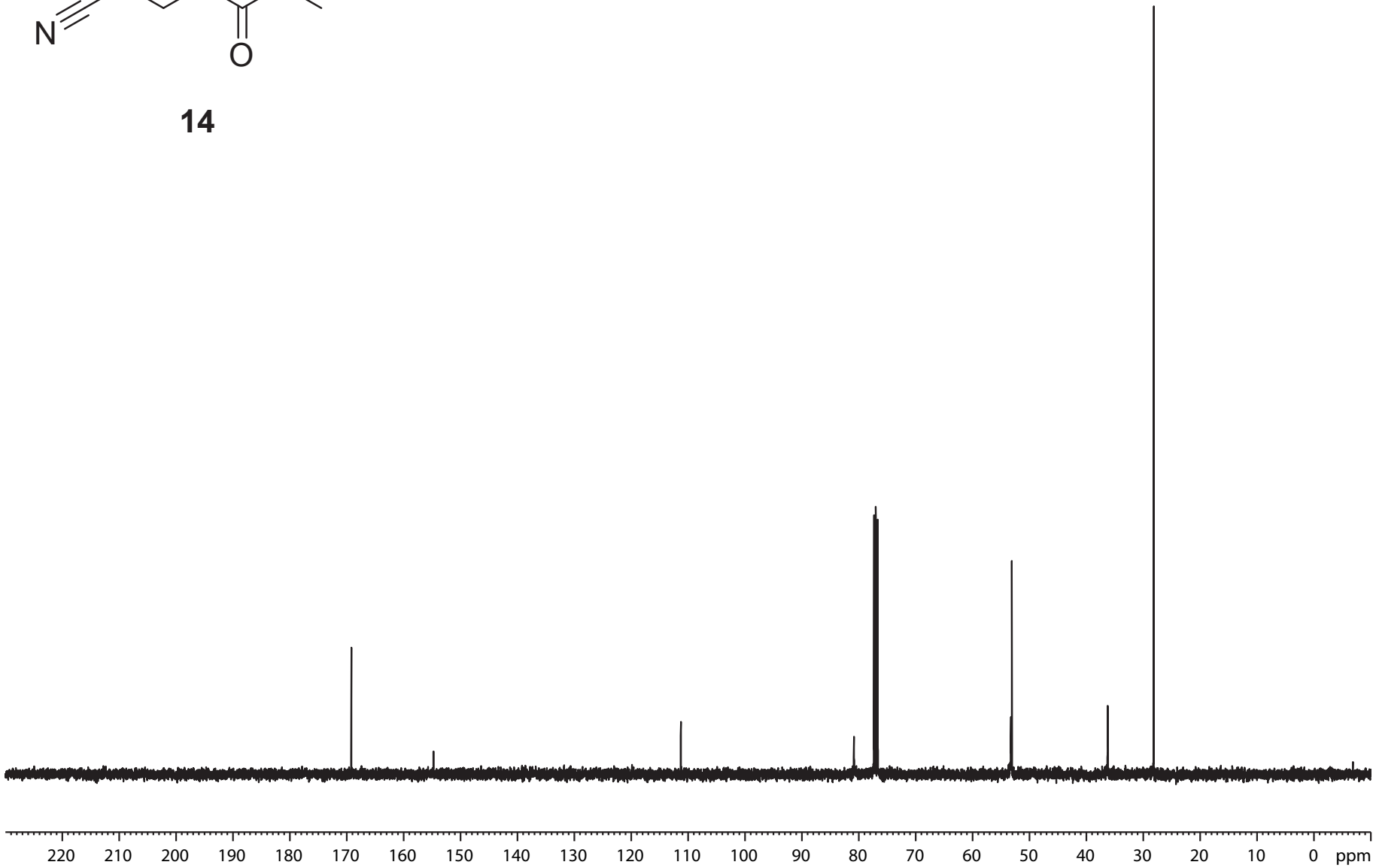
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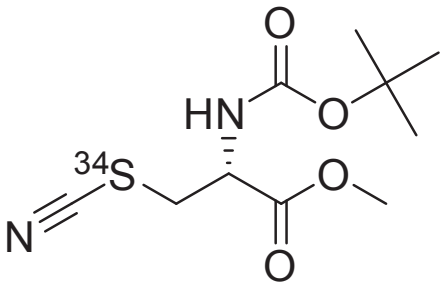
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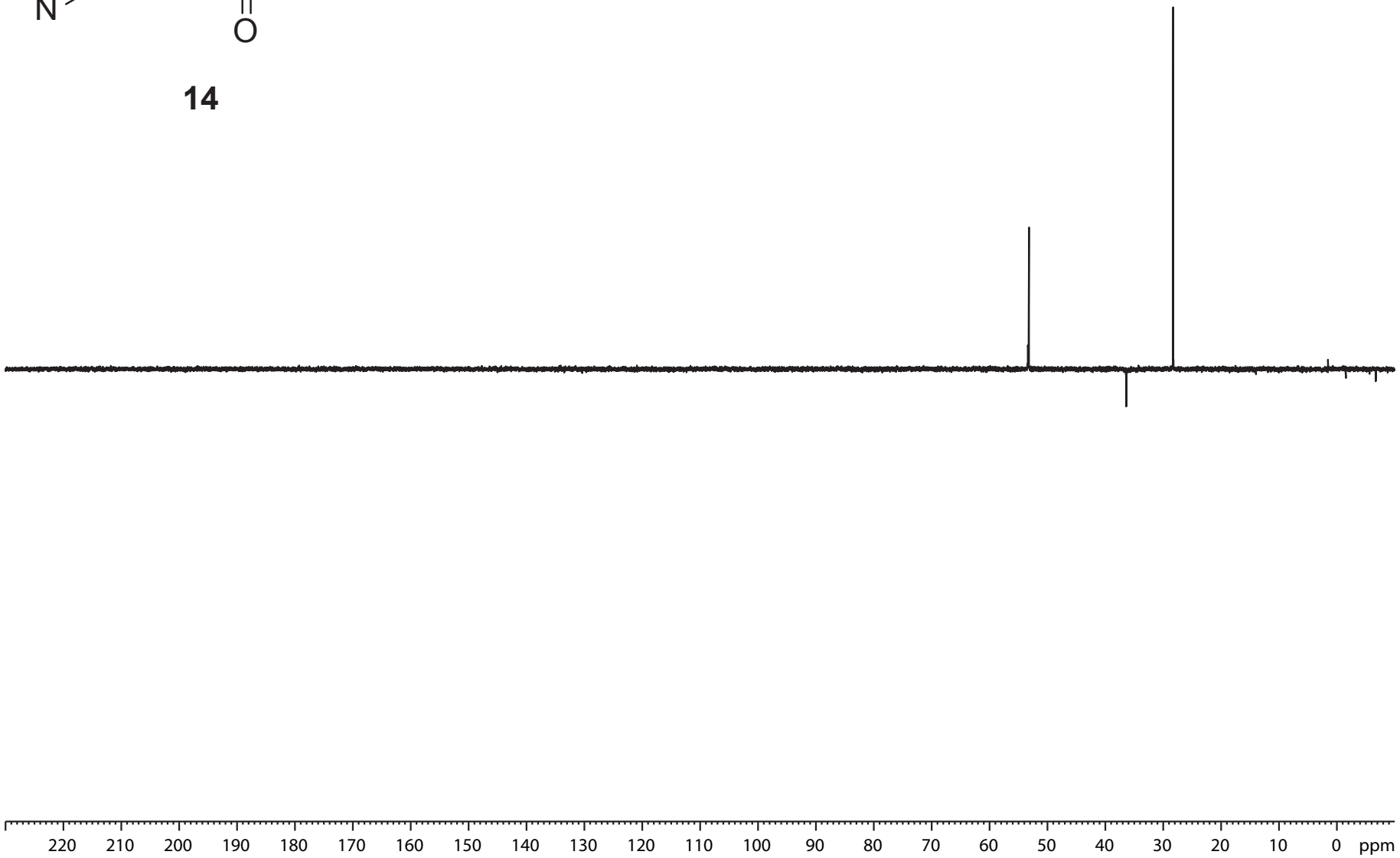
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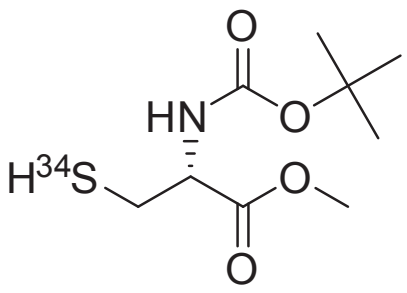
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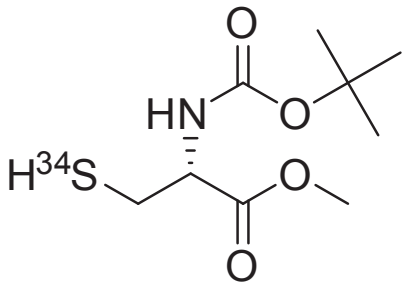
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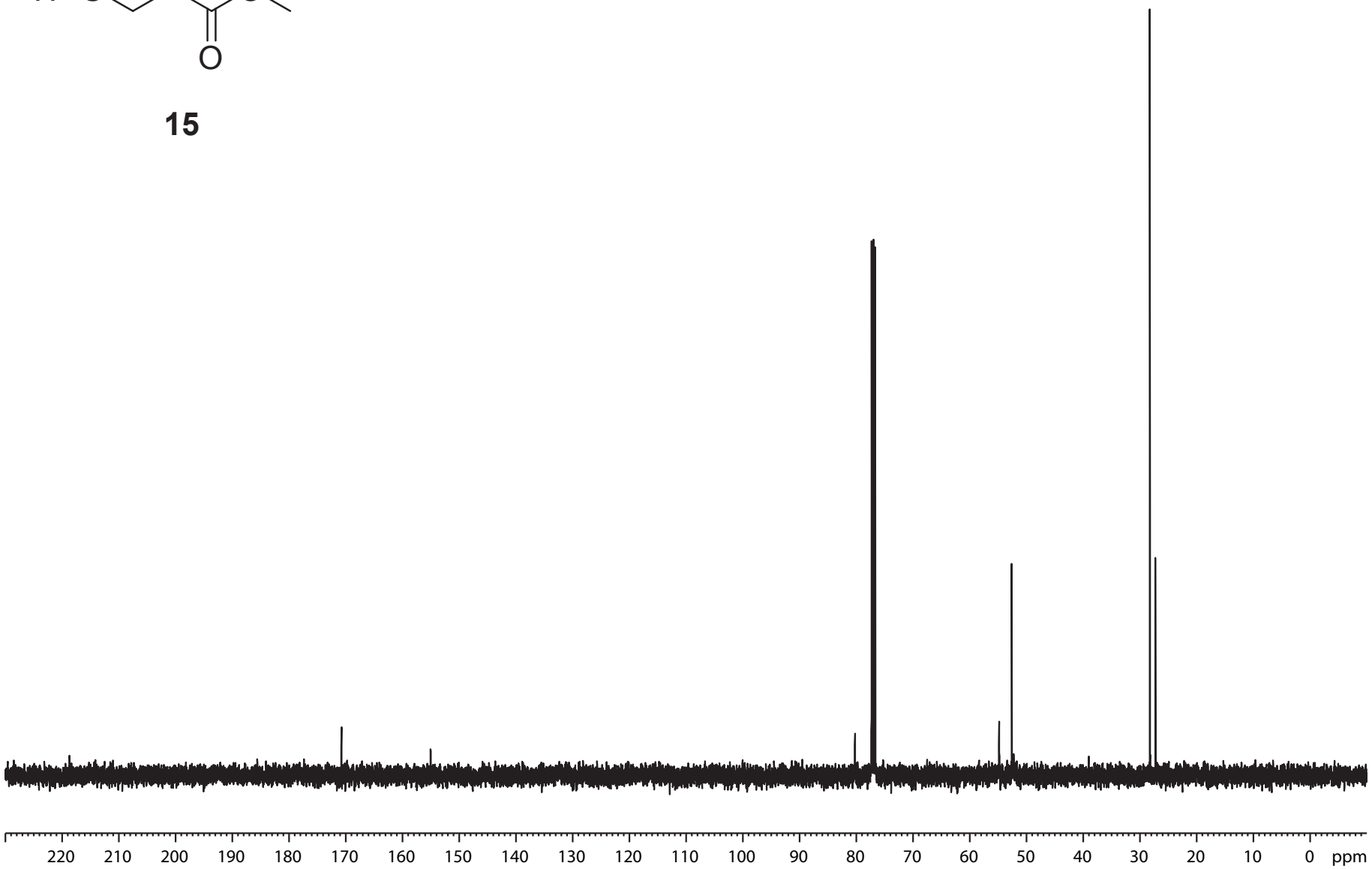
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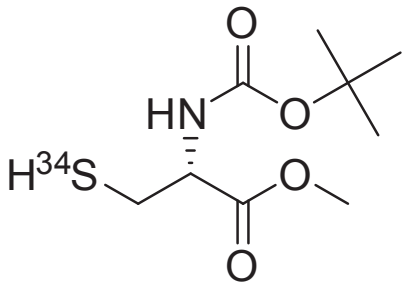
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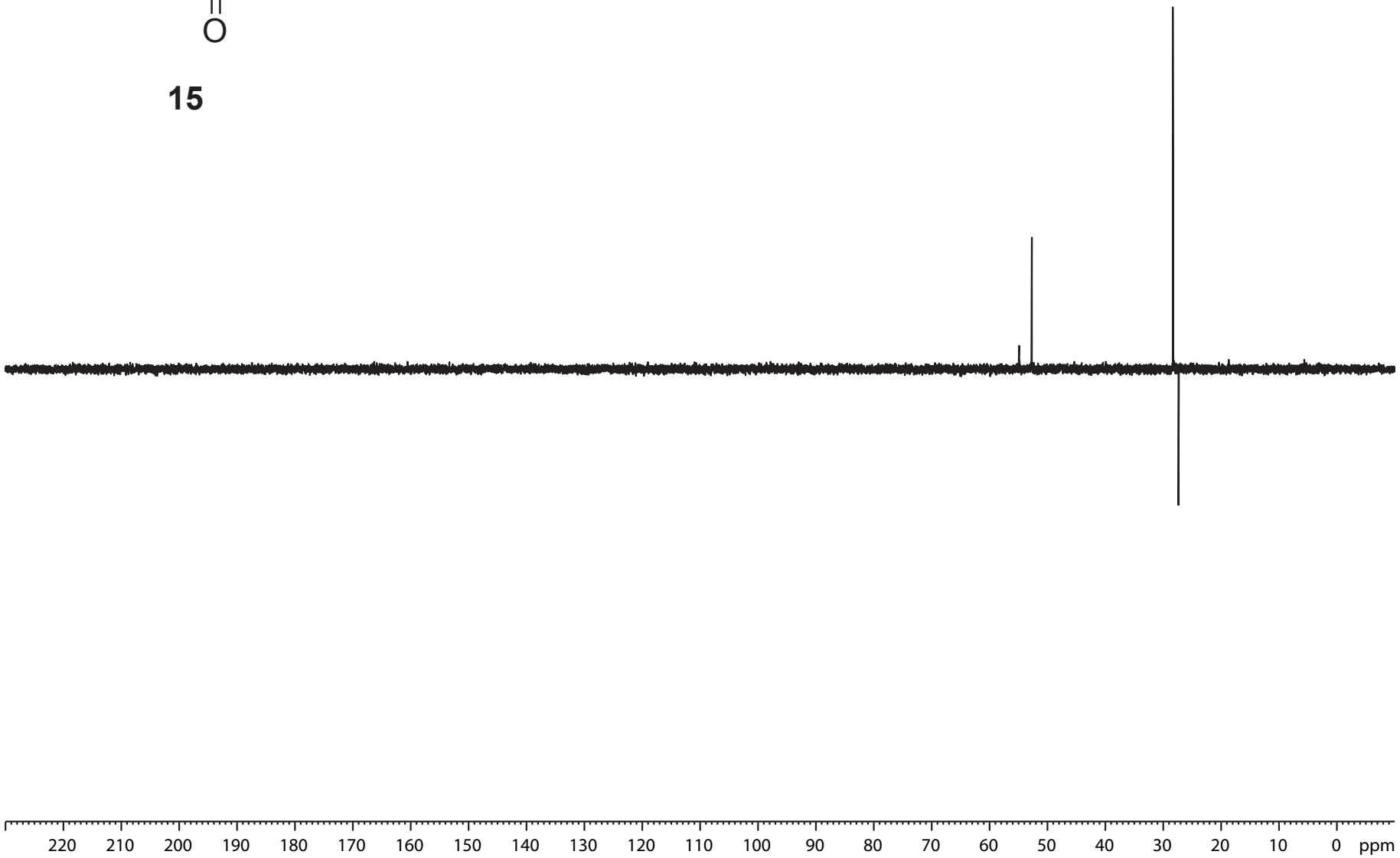
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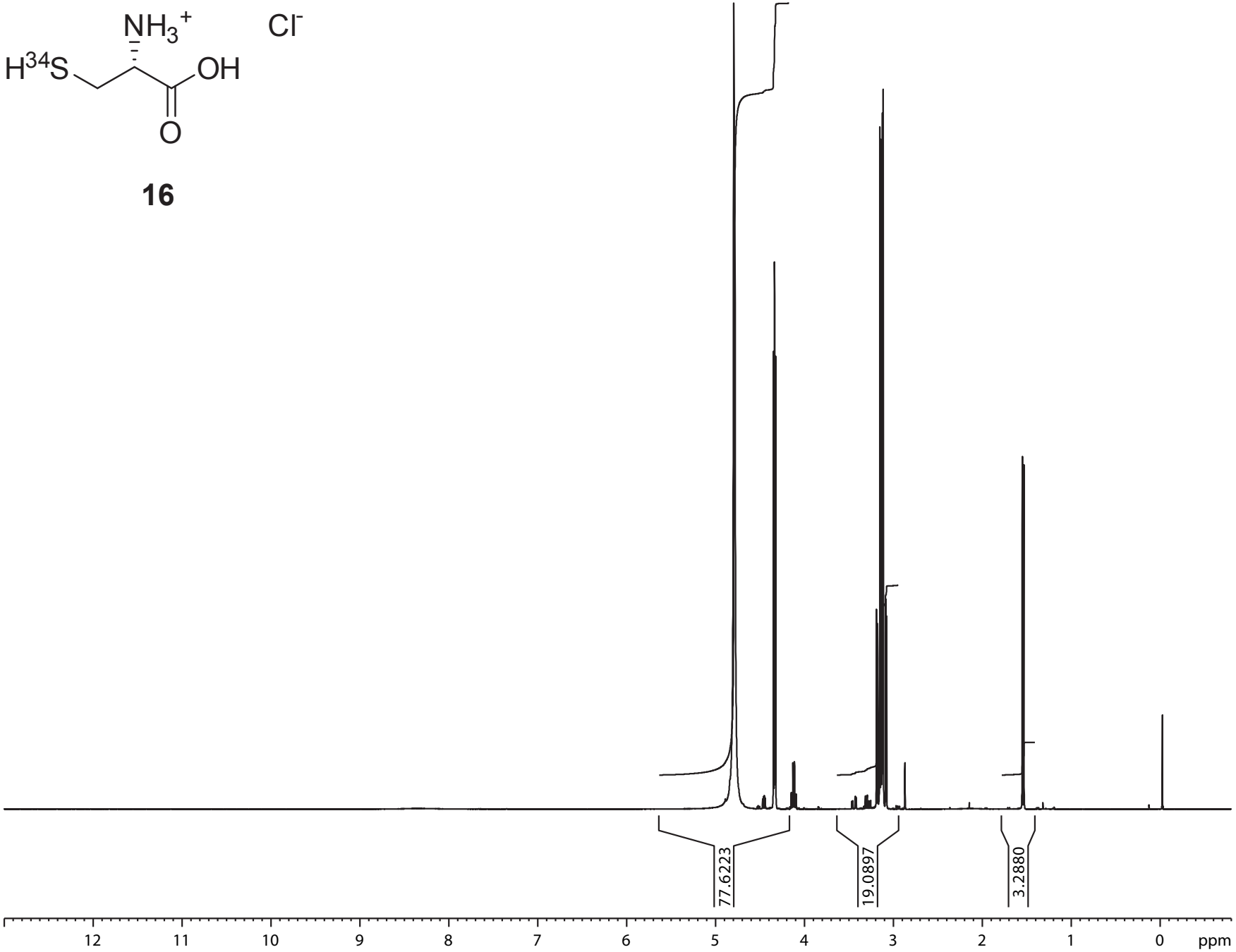
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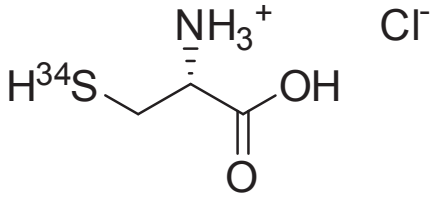
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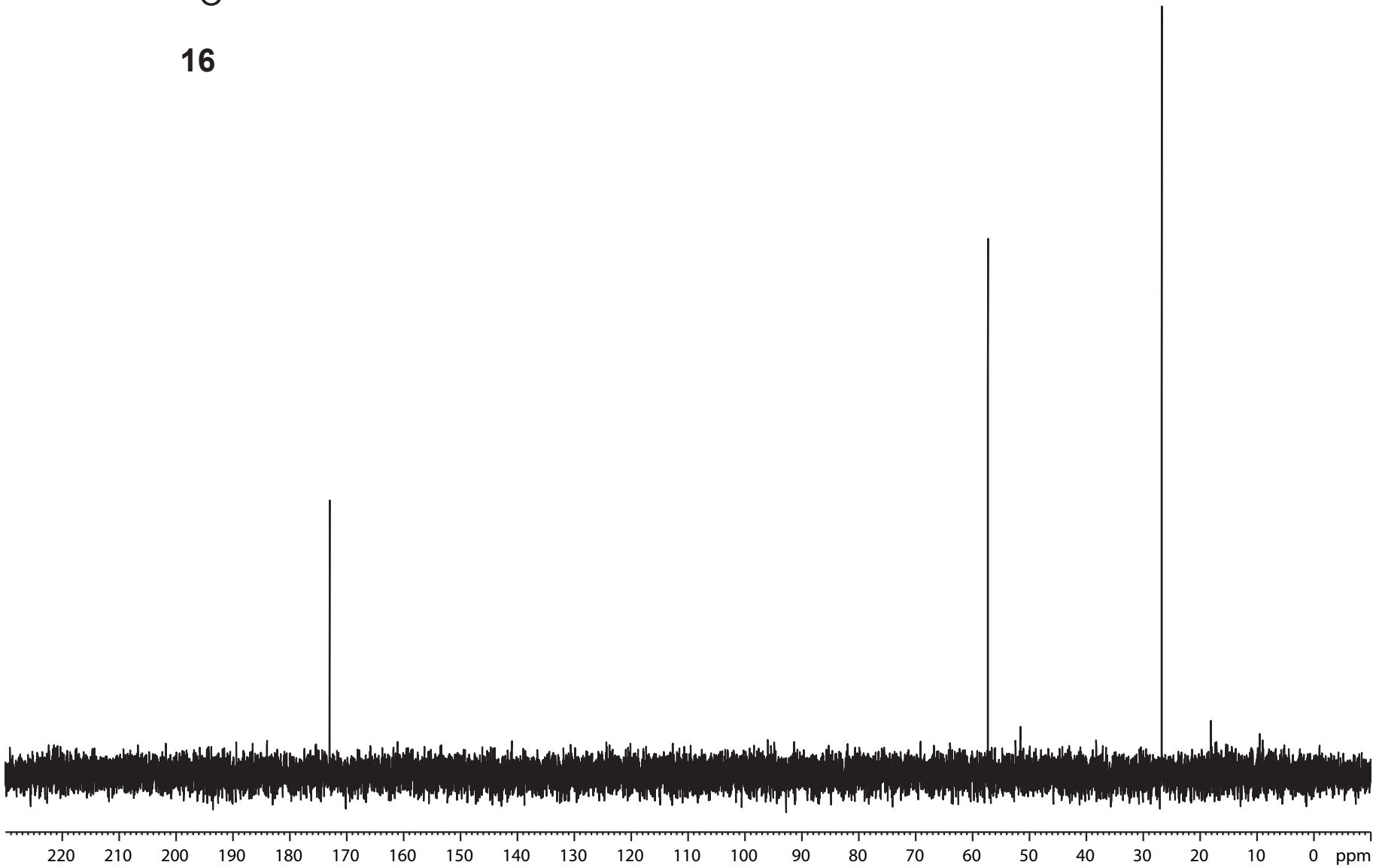
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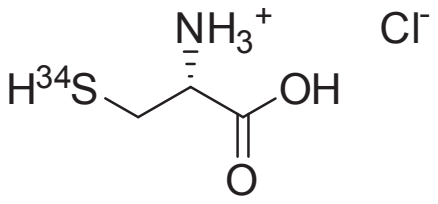
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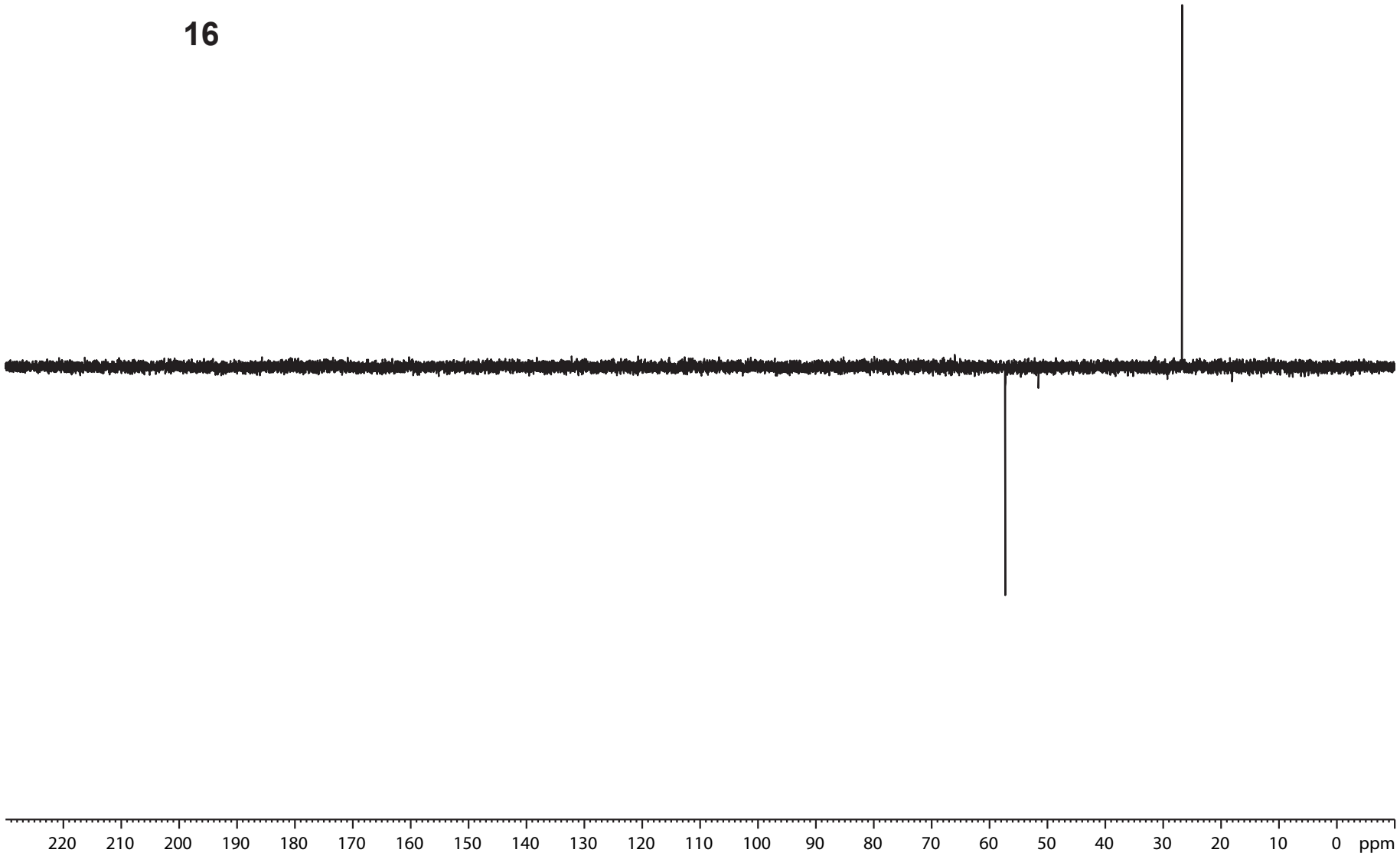
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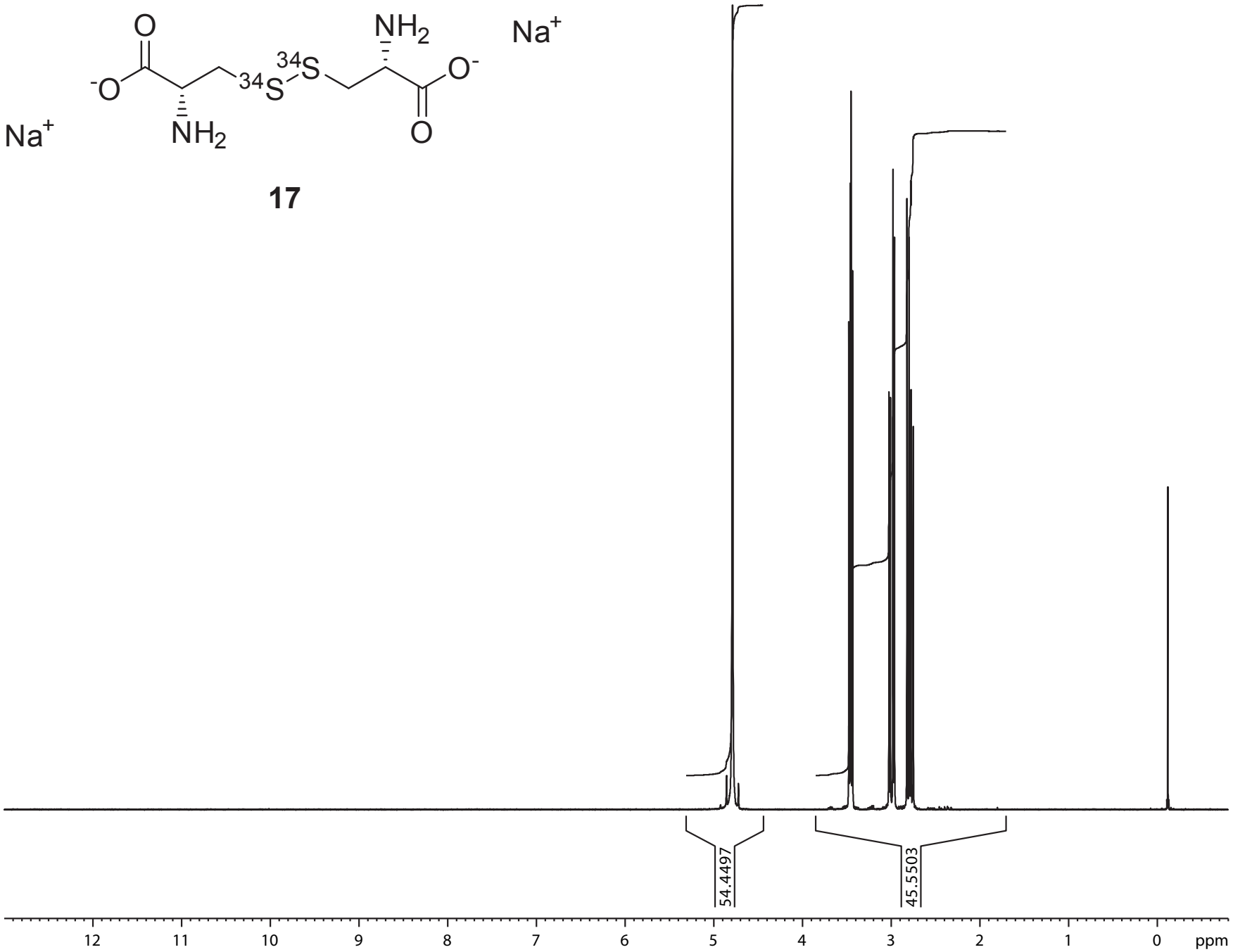
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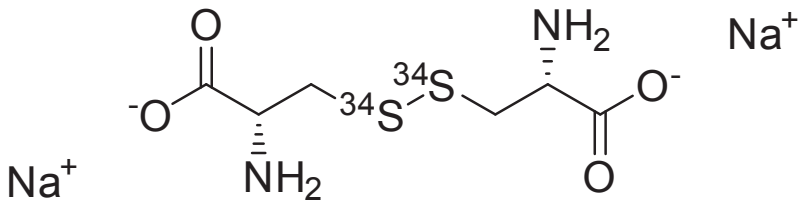
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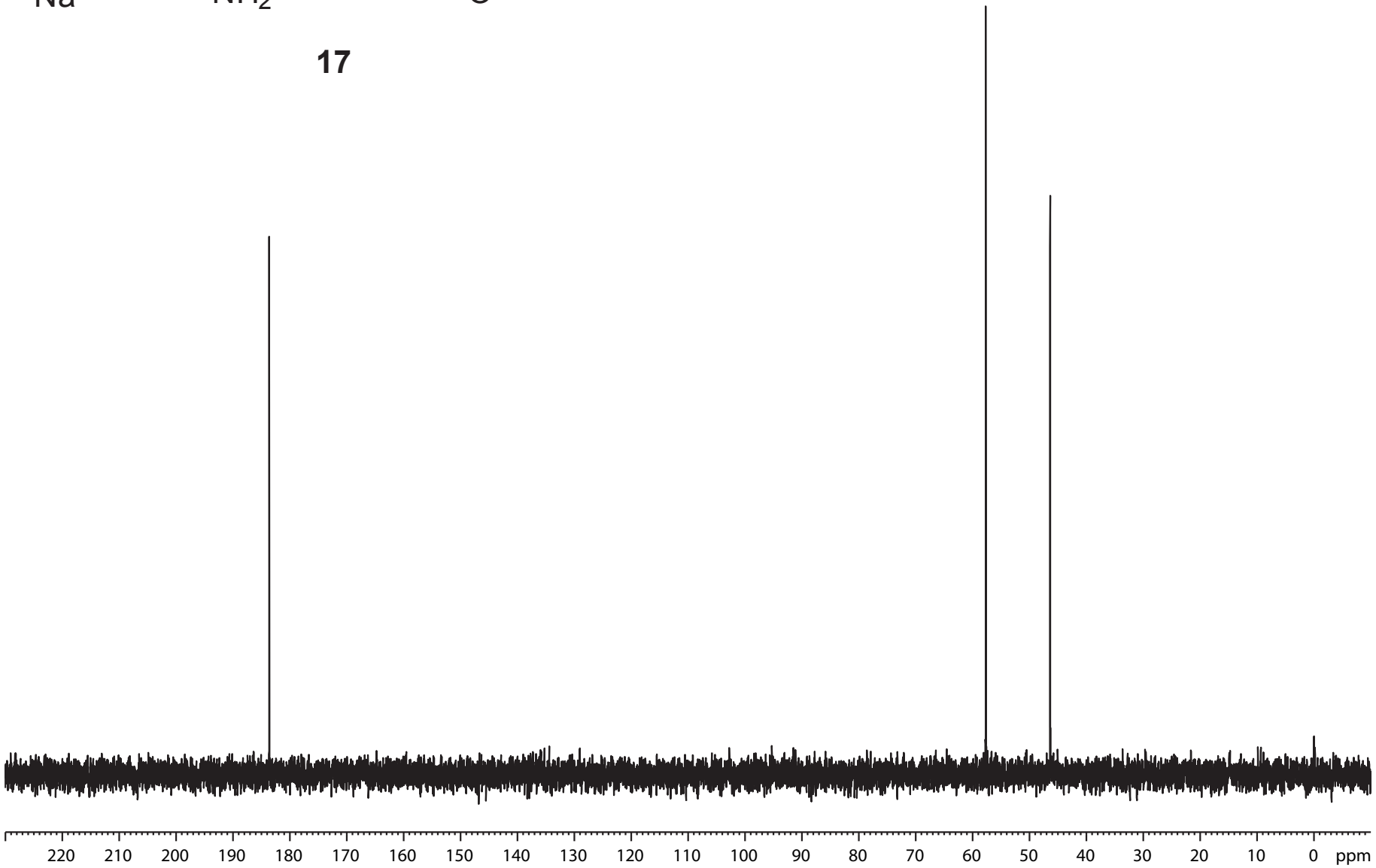
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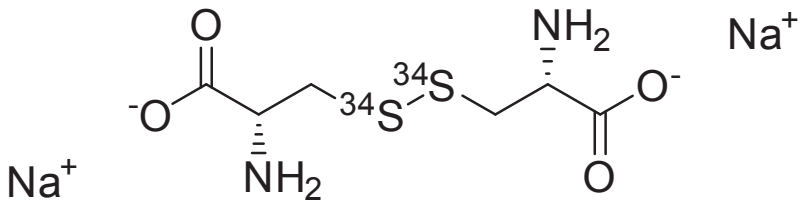
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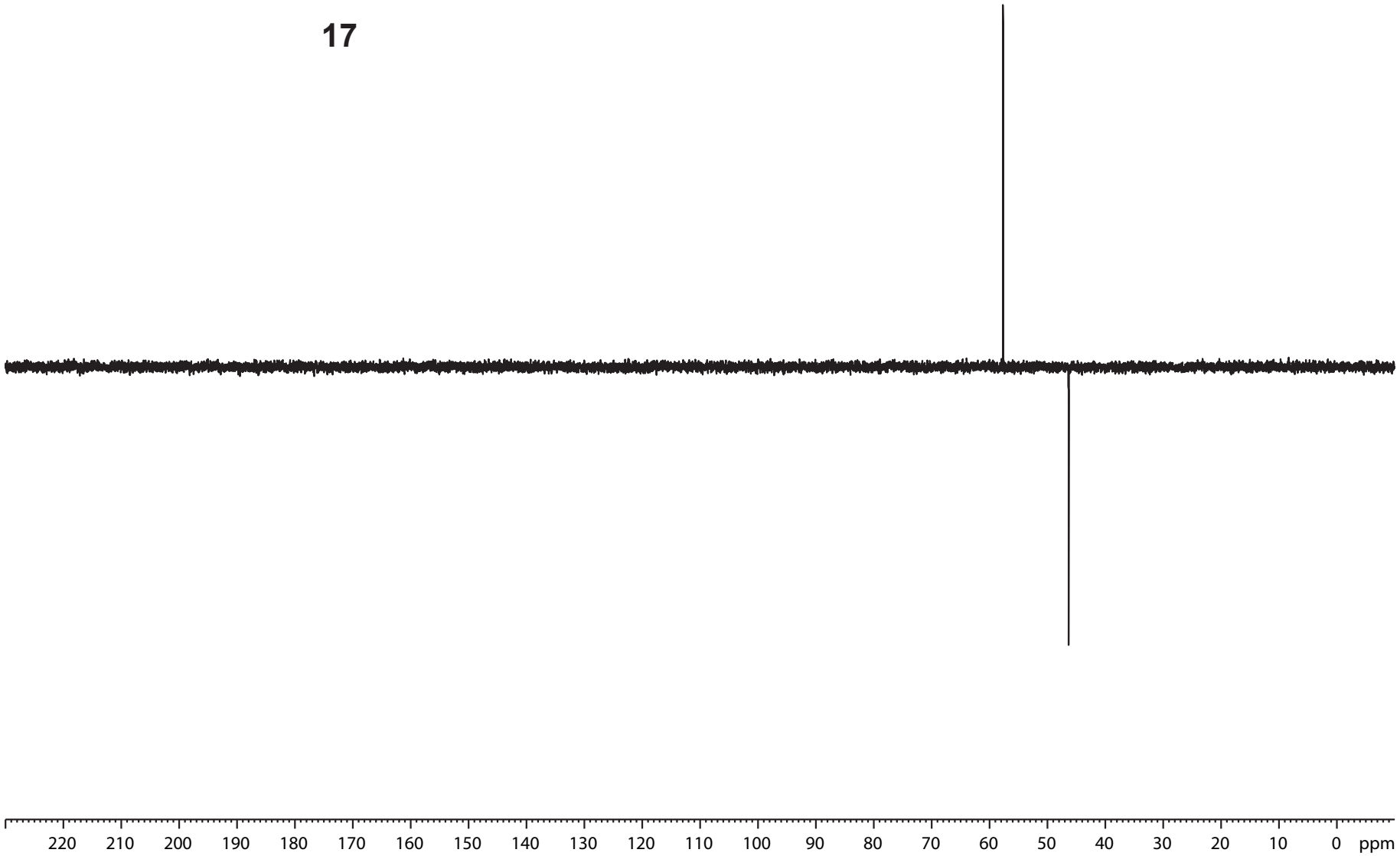
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