Supporting Information

Synthesis and in vitro evaluation of a peptidomimetic Inhibitor targeting the Src homology 2 (SH2) Domain of STAT6

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Contents

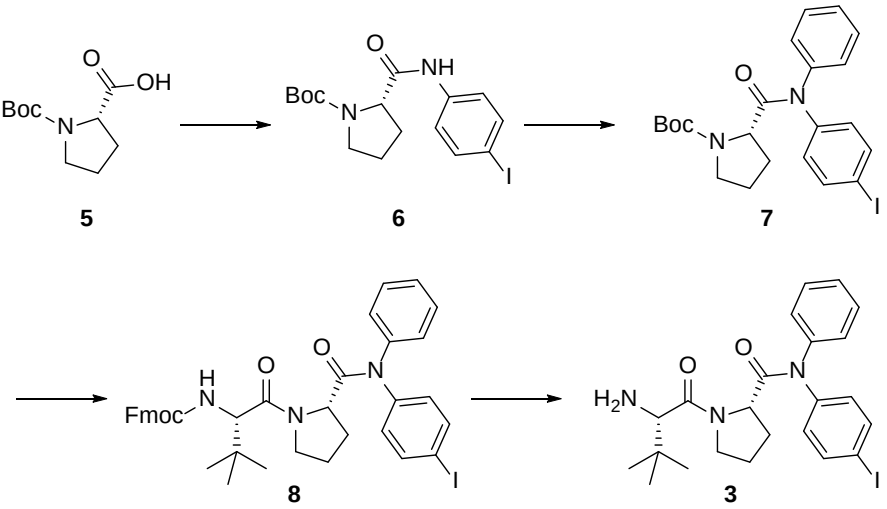
Materials

Chemicals for the syntheses were purchased from Chem-Impex International (Wood Dale, IL, USA) and Sigma Aldrich (Saint Louis, MO, USA). Normal human bronchial epithelial Beas-2B cells were a kind gift from Walter Hittelman from M. D. Anderson Cancer Center. Keratinocyte

serum-free culture medium (1X) supplemented with L-glutamine, epidermal growth factor (EGF) and bovine pituitary extract (BPE) was purchased from Gibco (Grand Island, NY, USA). Penicillin (10,000 units/mL)/streptomycin (10,000 μg/mL) solution (P/S) was from Thermo Scientific (Logan, UT, USA). Cell lysis buffer and antibodies against phospho-STAT6 (Y641) and total STAT6 were purchased from Cell Signaling Technology (Beverly, MA, USA). Lyophilized human recombinant interleukin 4 (IL-4) and 13 (IL-13) were from R&D Systems (Minneapolis, MN, USA) and PreproTech (Rocky Hill, NJ, USA) respectively. Both cytokines were reconstituted according to the manufacturer's instructions to obtain 1.0 μg/mL stock solutions. Goat anti-rabbit antibody conjugated to horseradish peroxidase, electrophoresis Criterion XT precast gels, PVDF membranes and buffers for gel-electrophoresis and western blotting were from BioRad (Hercules, CA, USA). Phenylmethanesulfonyl fluoride (PMSF) protease inhibitor was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The enhanced chemiluminescence kit (ECL) was from Amersham, (Chicago, IL, USA) and the MTS reagent kit used to assay cell viability was from Promega (Madison, WI, USA).

Synthesis and Characterization of 1

The synthesis of **3** used in our convergent methodology is summarized in Scheme S1.



Scheme S1. Synthesis of dipeptide **11**.

Synthesis of Boc-prolyl-4-iodoanilide, 6: A solution of Boc-Proline, **5** (3.0g, 13.93 mmol) 4-iodoaniline (3.0 g, 13.93 mmol) and EDC (3.5g, 18.2 mmol) in 60 mL of dry CH_2Cl_2 was stirred overnight. The reaction mixture was then transferred to a separatory funnel with an additional 20 mL of CH_2Cl_2 and washed with 5% HCl (2 × 30 mL), 10% $NaHCO_3$ (2 × 30 mL) and brine (20

mL). The organic layer was dried ($MgSO_4$) and concentrated by rotary evaporation under reduced pressure. Purification by silica gel column chromatography eluting with 15% EtOAc-hexane afforded the title product as a white solid (5.1 g, 88% yield). ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 10.1 (s, 1H), 7.65 (d, J = 8.5Hz, 2H), 7.45 (d, J = 8.5Hz, 2H), 4.24 (m, 1H, isomer), 4.17 (m, 1H), 3.41 (m, 1H), 3.33 (m, 1H), 2.2 (m, 1H), 1.74-1.93 (m, 3H), 1.4 (s, 9H, isomer), 1.26 (s, 9H, isomer). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 172.2, 171.7, 154.1, 153.6, 139.4, 137.9, 121.9, 87.2, 87.0, 79.2, 79.0, 60.9, 60.5,47.2, 47.0, 31.4, 30.6, 28.6, 28.4, 24.4, 23.9.

Synthesis of *N*-phenyl Boc-prolyl-4-iodoanilide, 7: To a stirred solution of **6** (2.0 g, 4.8 mmol) in dry CH_2Cl_2 (50 mL), was added triphenylbismuth (3.2 g, 7.2 mmol), $Cu(OAc)_2$ (1.5 g, 7.2 mmol) and dry triethylamine (1.0 mL, 7.2 mmol). The reaction was monitored by HPLC. After completion of the reaction, the solvent was evaporated and the residue was diluted with ether (150 mL) and filtered through celite. The organic layer was then washed with 5% HCl (2 × 30 mL) followed by brine and dried over $MgSO_4$. Concentration under reduced pressure and purification by silica gel chromatography using 10% EtOAc-hexane afforded **7** as a white solid (1.2 g, 48% yield). Calcd (M+H): 493.10; Found (M+H): 493.21. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 6.96-7.97 (m, 9H), 4.2 (m, 1H), 4.13 (m, 1H), 3.36 (m, 1H), 3.3 (m, 1H), 2.07 (m, 1H), 1.78-2.0 (m, 2H), 1.71 (m, 1H), 1.43 (s, 9H), 1.4 (s, 9H, isomer). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 172.5, 172.2, 153.9, 153.3, 143.0,139.4, 138.2, 131.6, 130.6, 129.6, 129.1, 128.7, 126.9, 79.3, 79.1, 57.9, 57.8, 47.4, 47.3, 31.4, 30.0, 28.8, 28.6, 24.4, 23.3.

Synthesis of Fmoc-*tert*-butylglycyl-*N*-phenyl-prolyl-4-iodoanilide 8: A solution of **7** (1.00 g, 2.03 mmol) in 5 mL of neat trifluoroacetic acid (TFA) was stirred for 1 h. Excess TFA was removed under vacuum. The residue was then treated with Fmoc-Tle-OH (0.8 g, 2.23 mmol), HBTU (0.85 g, 2.23 mmol), DIPEA (1.2 mL, 6.7 mmol) in 50 mL of dry CH_2Cl_2 overnight. The organic layer was diluted with an additional 50 mL of CH_2Cl_2 and washed with 5% HCl (3 × 30 mL) followed by 10% $NaHCO_3$ (1 × 30mL) and brine. After drying ($MgSO_4$) and concentration under vacuum, the crude product was purified by silica gel chromatography using 35% EtOAc-hexane to give **8** as white foam (1.2 g, 78% yield). Calcd (M+H): 728.1985; Found (M+H): 728.2027. ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.75-7.8 (m, 3H), 7.6-7.7 (m, 4H), 7.4-7.5 (m, 2H), 7.38-7.43 (m, 4H), 7.3-7.37 (m, 3H), 7.24 (m, 1H), 1.03 (m, 1H), 5.66 (d, J = 9.8 Hz, 1H), 4.5 (m, 1H), 4.4-4.47 (m, 2H), 4.3 (m, 1H), 4.2 (m, 1H), 3.9 (m, 1H), 3.7-3.81 (m, 4H), 2.15 (m,

1H), 2.06 (m, 2H), 1.88 (m, 1H), 1.12 (s, 9H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 172.0, 170.3, 156.4, 143.9, 141.3, 138.1, 130.0, 129.0, 128.2, 127.7, 127.1, 127.05, 127.0, 125.2, 125.1, 119.9, 67.1, 59.2, 58.7, 48.7, 47.2, 36.0, 31.6, 29.7, 26.5, 25.4.

Synthesis of *tert*-butylglycyl-*N*-pheny-prolyl-4-iodoanilide 3: Compound **8** (0.5 g, 0.68 mmol) was treated with 4.0 mL of 20% piperidine in DMF for 30 min. The reaction mixture was concentrated under vacuum and subjected to RP-HPLC (2.5 × 25 cm Phenomenex Luna C18 column eluting with a linear gradient of MeCN in H_2O). Pure fractions containing the desired material were collected, recombined and lyophilized to afford 0.166 g (47% yield) of **3** as a white powder. Calcd (M+H): 506.1304; Found (M+H): 506.1296.

Synthesis of prodrug 1: To a stirred solution of **3** (0.05 g, 0.1 mmol) and the active ester **9** (synthesized as described in Mandal et al.; see ref. 1) (0.05 g, 0.1 mmol) in 3 mL of dry NMP, 40 µL of N-methylmorpholine and DMAP (0.002 g, 0.02 mmol) were added. The reaction was monitored by HPLC and once completed, the product was purified from the crude by RP-HPLC (2.5 × 25 cm Phenomenex Luna C18 column eluting with a linear gradient of MeCN in H_2O). Combined pure fractions were then lyophilized to yield 64 mg of pure **1** as a white powder (66% yield). HRMS (M + 1) calcd 994.2716; found 994.2730. ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.6-7.8 (m, 7H), 7.3-7.5 (m, 4H), 7.3-7.2 (m, 3H), 7.0 (m, 1H), 6.6 (d, 1H, *J* = 15.7 Hz), 5.76 (dd, 2H, *J* = 5.2, 12.6 Hz), 5.7 (dd, 2H, J = 5.2, 12.6 Hz), 4.9 (d, 1H, J = 10Hz), 4.5 (m, 1H), 4.03 (m, 1H), 3.8 (m, 1H), 2.2 (m, 1H), 2.1 (m, 2H), 1.9 (m, 1H), 1.24 (s, 18H), 1.14 (s, 9H). ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 176.7, 170.6, 166.0, 140.7, 138.2, 137.4, 130.1, 128.8, 128.1, 127.9, 126.9, 122.0, 82.6, 82.5, 57.5, 49.3, 38.7, 36.2, 29.7, 26.7, 26.5, 25.3.

In Vitro Evaluation of 1

Inhibition of STAT6(Tyr641) phosphorylation in intact Beas-2B cells. Beas-2B cells were cultured in Keratinocyte serum-free culture medium containing EGF and BPE and supplemented with 0.5% P/S solution. Cells were grown in a 37 °C humidified incubator with 5% CO_2 until 90% confluent and seeded on 6-well plates at a density of 3.0×10^5 cells/well the day before treatment. Powdered prodrug **1** was dissolved in DMSO to obtain a 10 µM stock solution and aliquots were added to the cultures to reach the desired final concentrations. Cells were incubated at 37 °C for 2 h and stimulated with IL-4 or IL-13 (2 ng/mL) for 1 h. Cells were then washed twice with ice-cold phosphate buffer saline (PBS) and lysed with lysis buffer containing

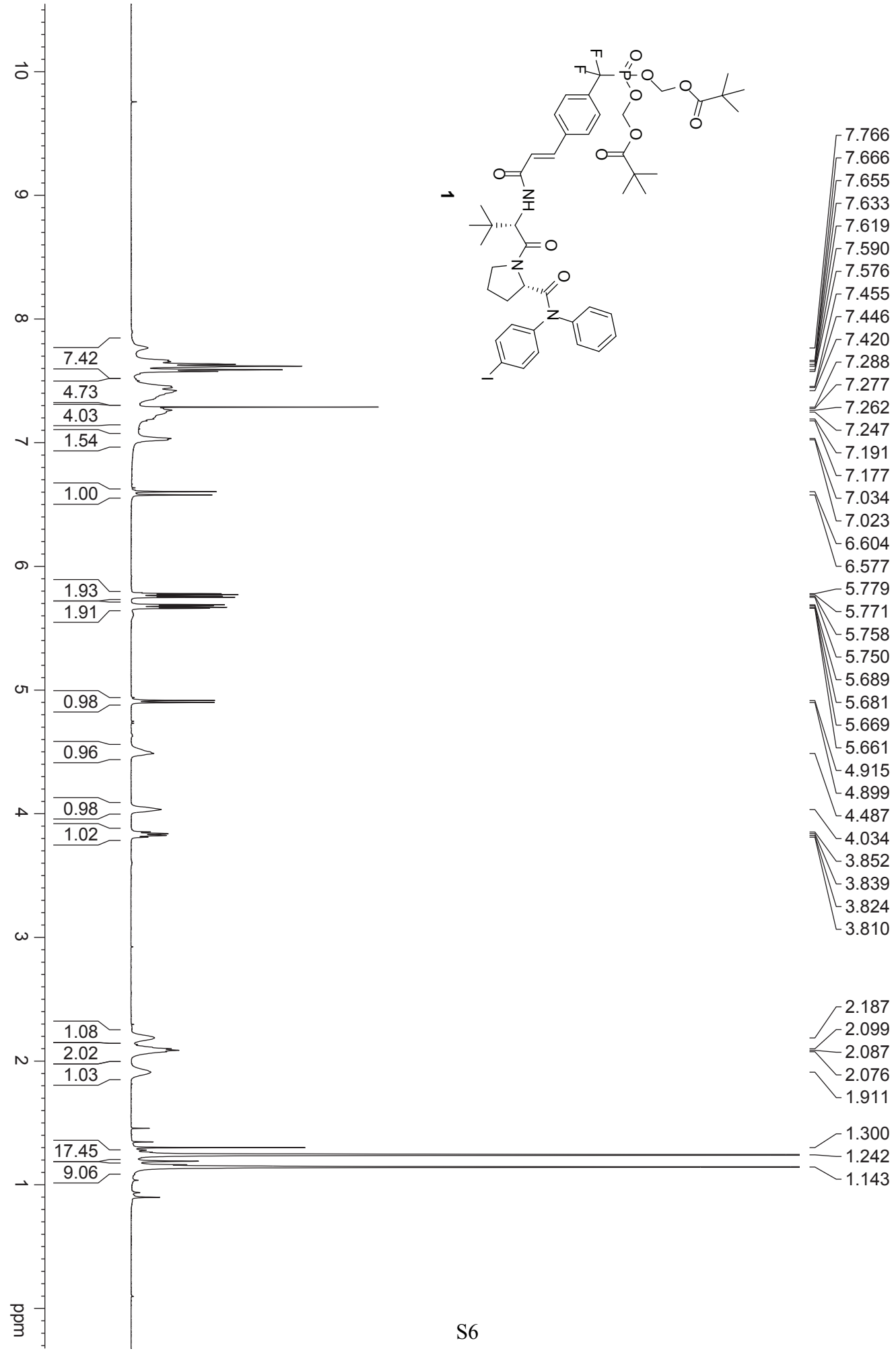
1% 100 mM PMSF solution. After centrifugation at 13200 rpm for 10 min, the supernatants were transferred to clean tubes, and aliquots (5 µL) were used to measure the concentration of proteins using the BioRad DC protein assay.

Western Blotting. Aliquots of the whole cell lysate (15 µg proteins) were separated on a 4-12% SDS-PAGE polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked with a 3% BSA solution in TBST (1X tris-buffered saline contacting 0.1 % Tween 20) and probed with phospho-STAT6(Y641) rabbit polyclonal antibody followed by incubation with secondary antibody. Levels of phospho-STAT6(Y641) protein were detected with ECL by capturing the resulting chemiluminescence signal on x-ray films. PVDF filters were then stripped with stripping buffer (25 mM glycine, pH 2, 10% SDS) at rt for 30 min, blocked with 3% BSA and probed with STAT6 antibody whose signal was detected as described above. Western blot band intensities relative to phospho-STAT6(Y641) were quantified by densitometric analysis using ImageJ.

Effect of 1 on Beas-2B cell proliferation. Effects of prodrug **1** on cell proliferation were determined by MTS assay. Beas-2B cells were seeded on a 96-well plate (2000 cell/well). Compound **1** was dissolved in DMSO to make a 10 mM stock solution. This stock was subjected to serial dilution and equal amounts of each dilution were added to the cultures. After incubation at 37 °C for 72 h, the culture medium was removed and a 1:5 MTS/PBS solution was added to each well. The dish was then incubated for an additional 1 h and viable cells were estimated by measuring the optical density of the samples at 490 nm.

Reference for Supporting Information

1. Mandal, P.K.; Liao, W. S.-L.; McMurray, J.S. *Organic. Lett*. **2009**, *11*, 3394-3397.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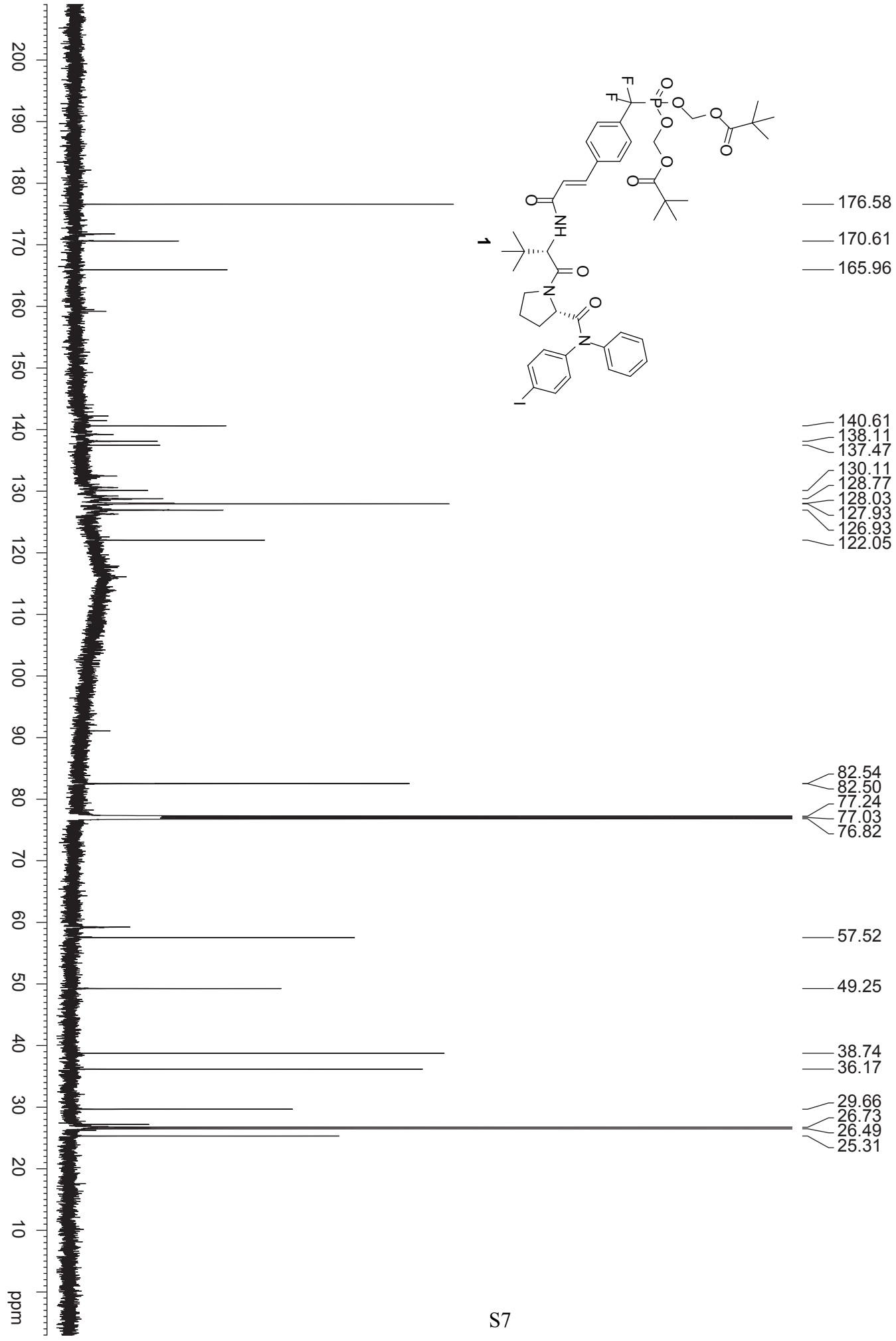
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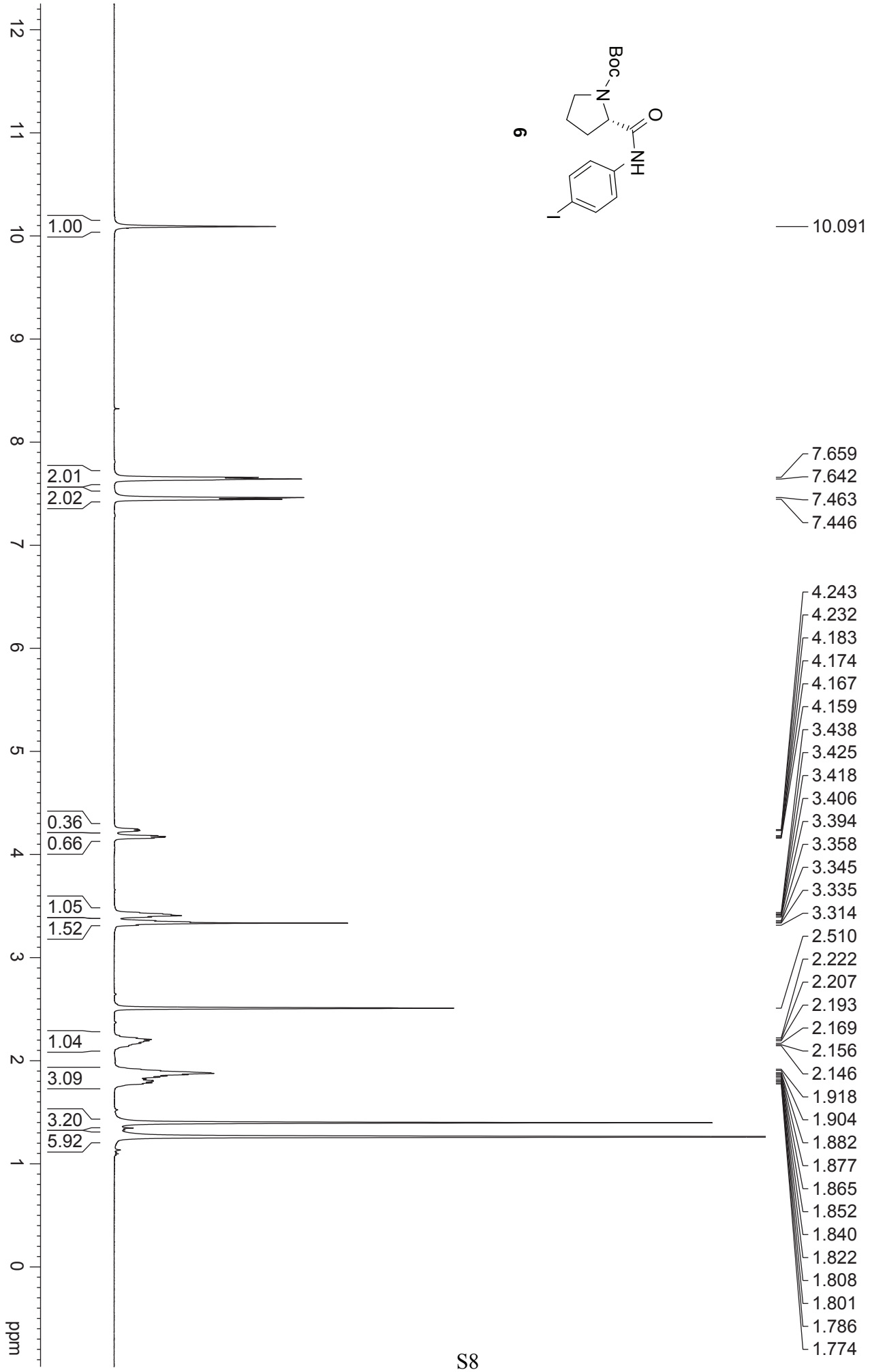
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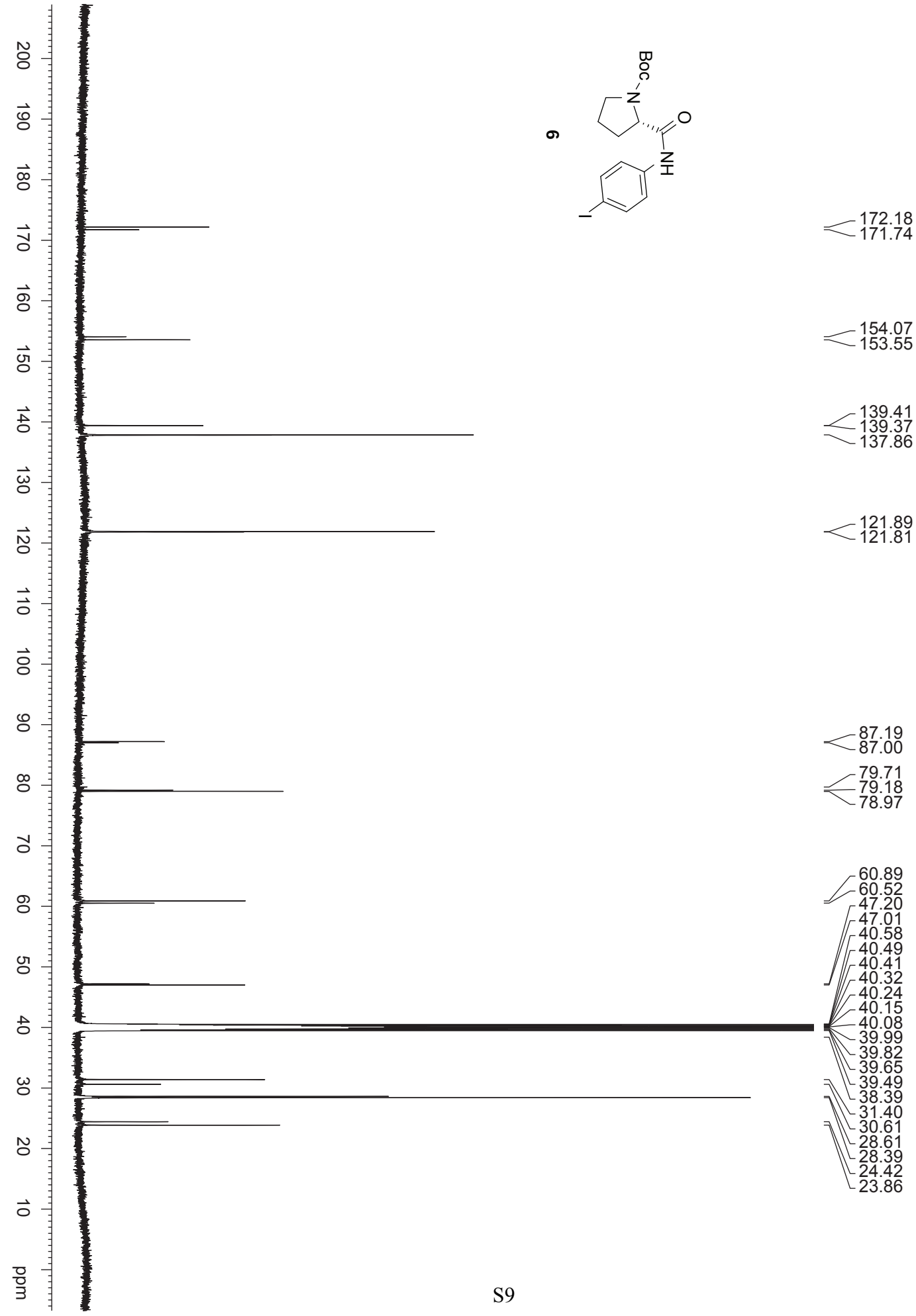
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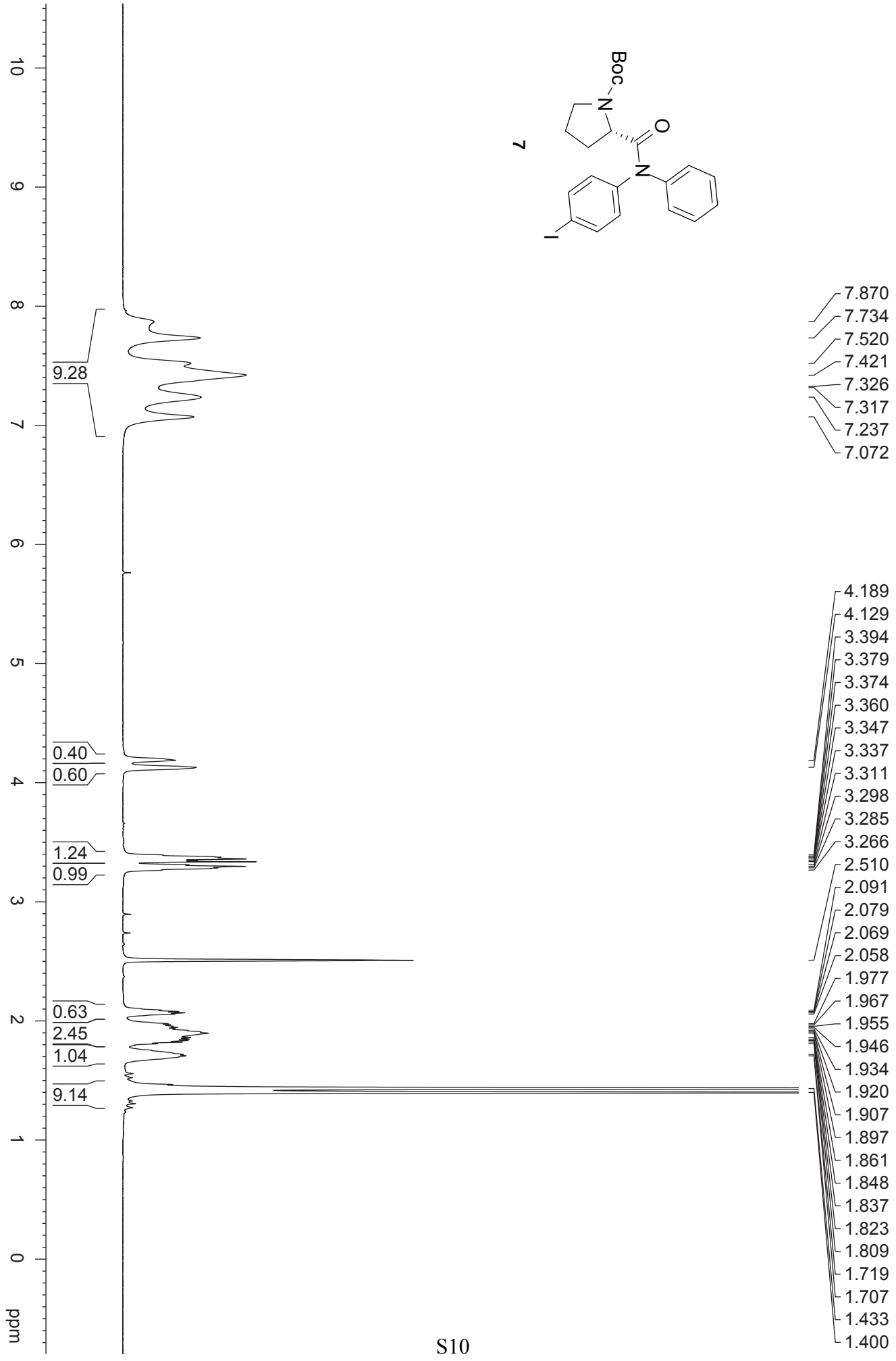
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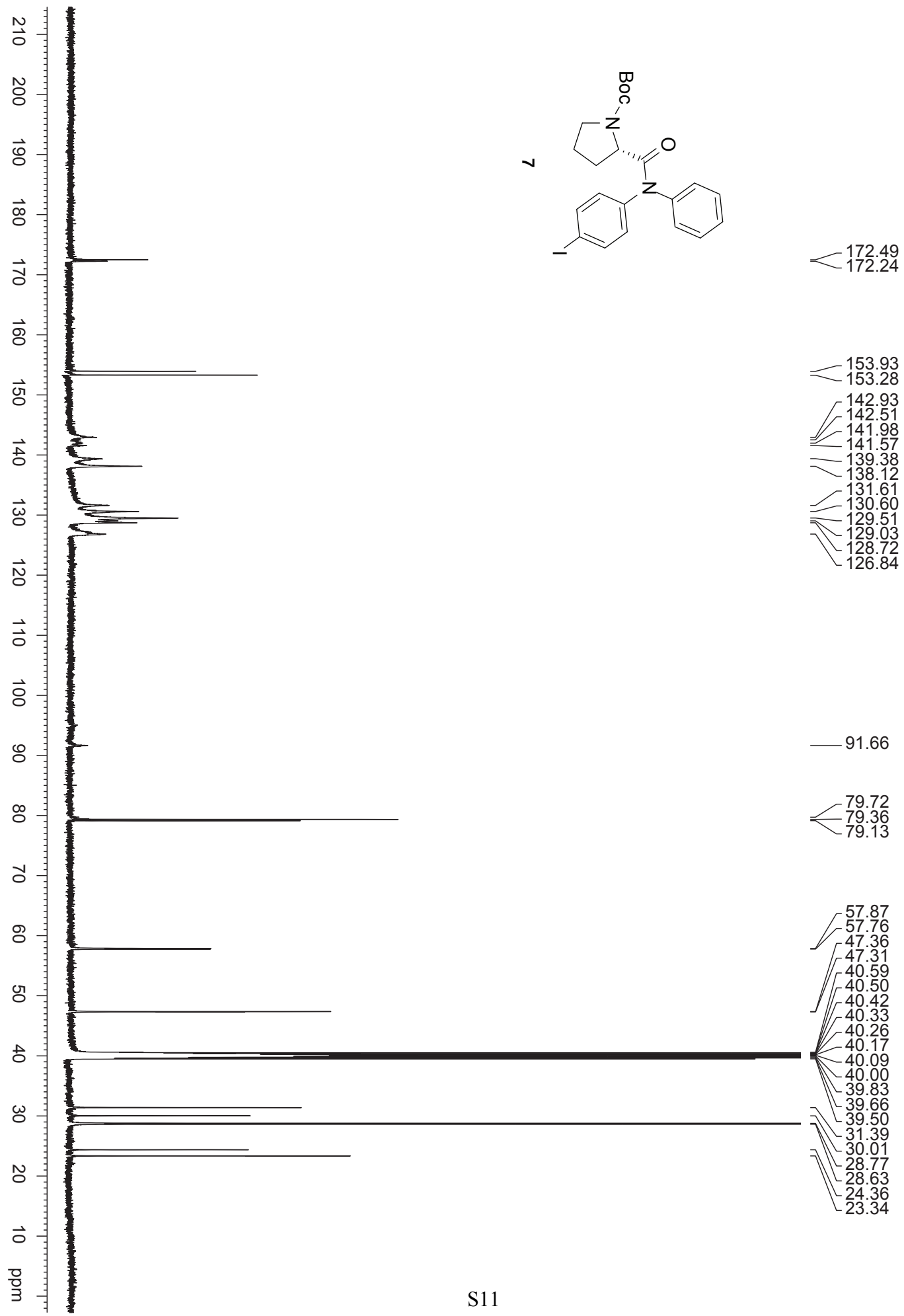
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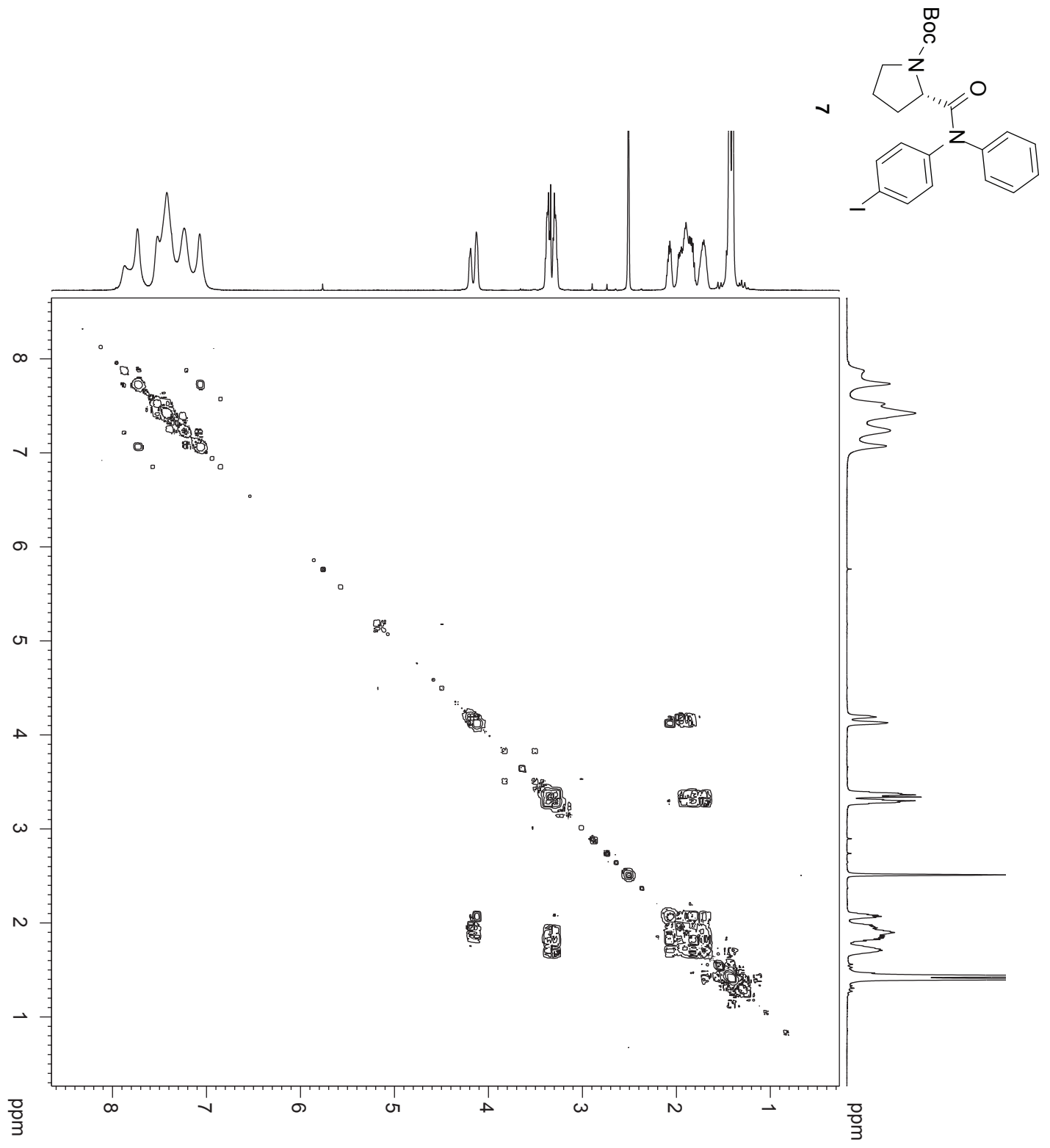
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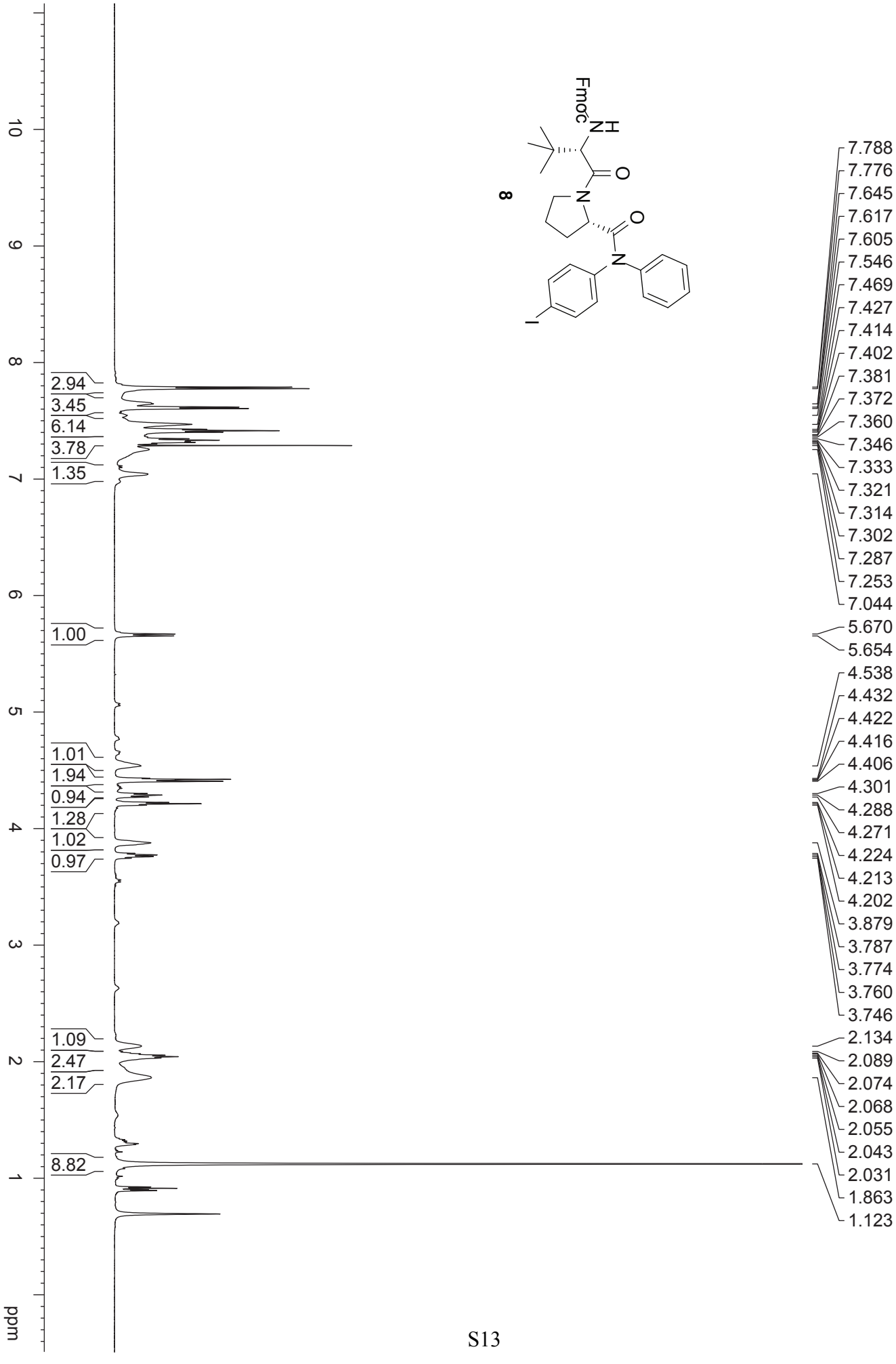
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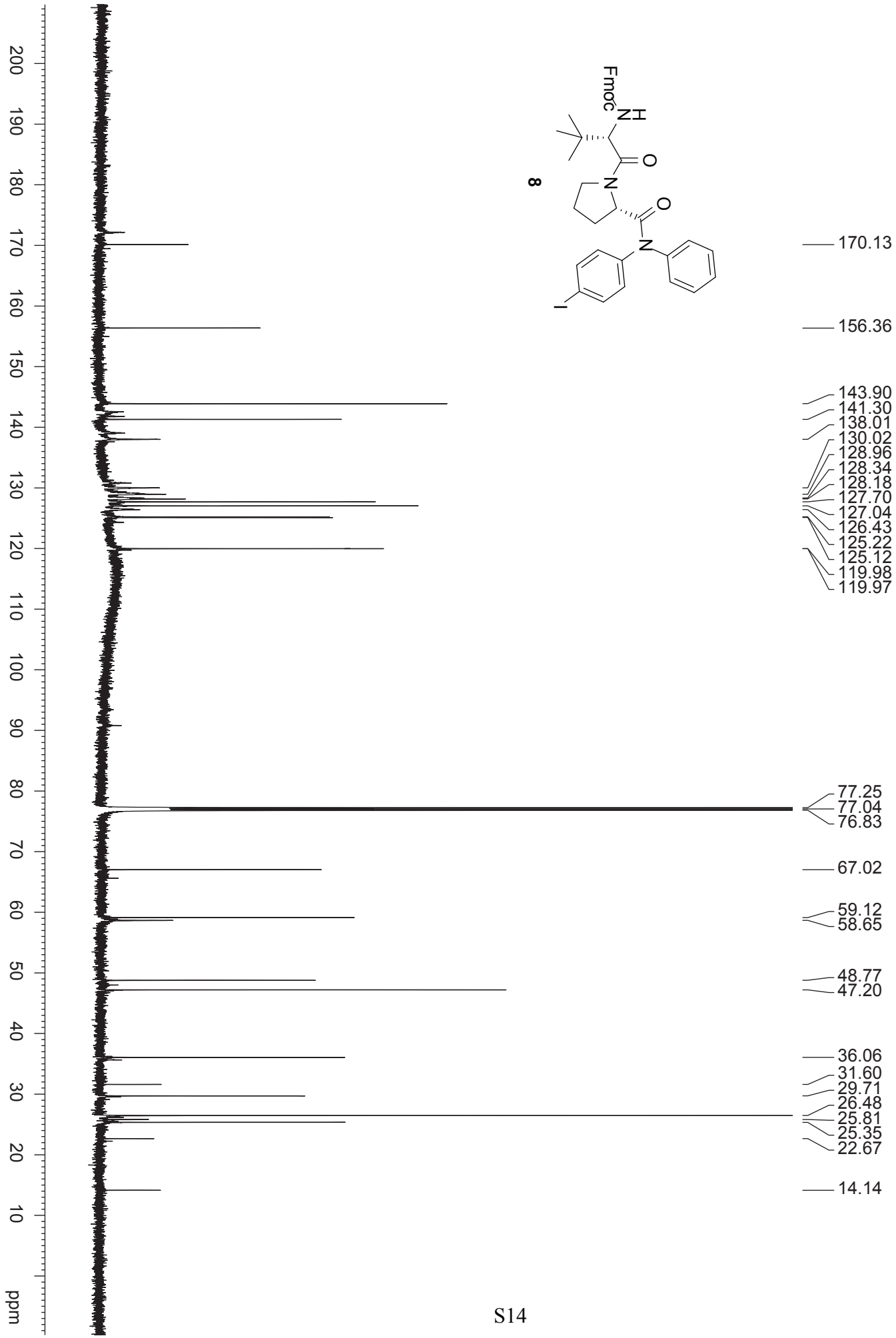
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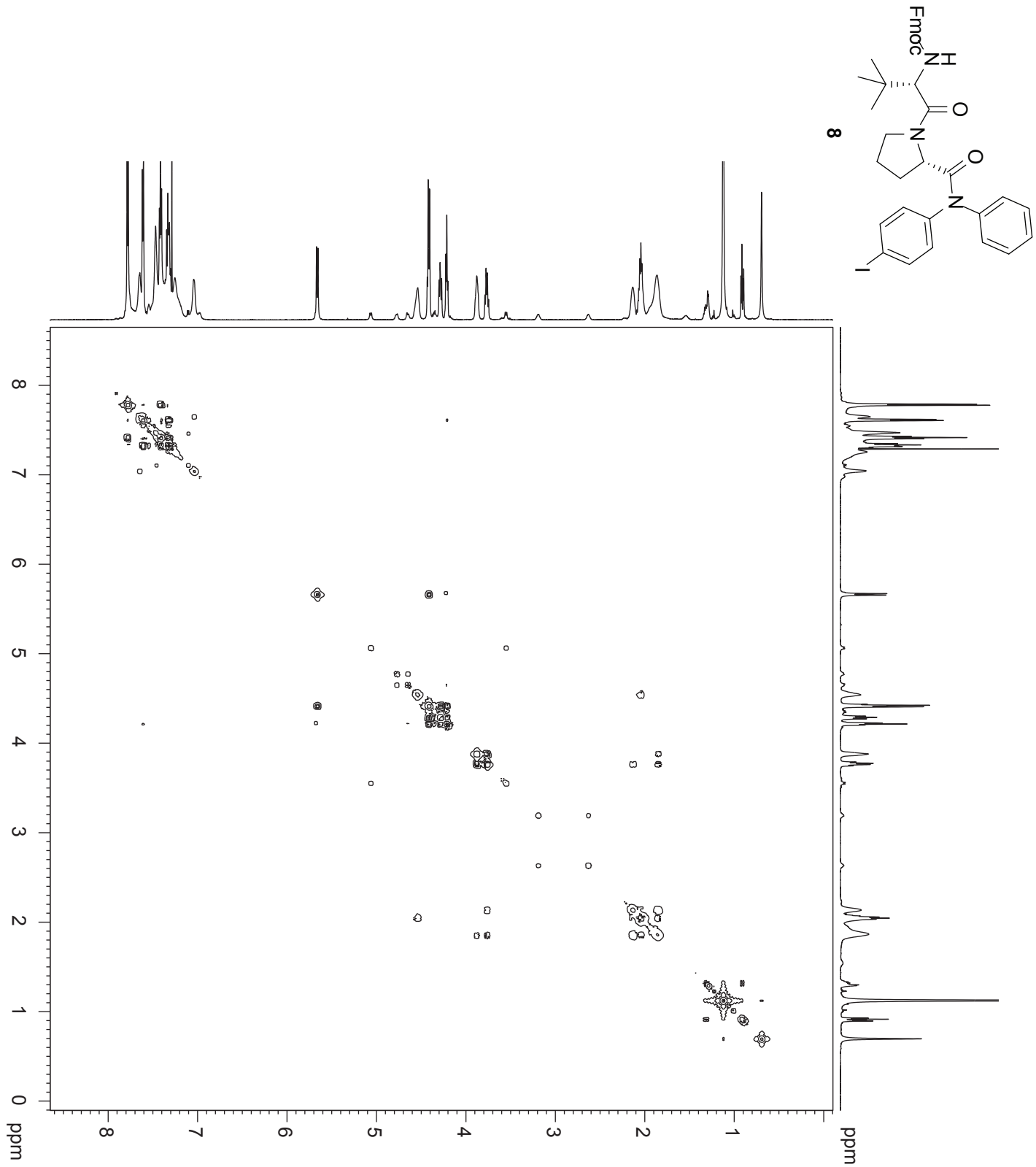
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