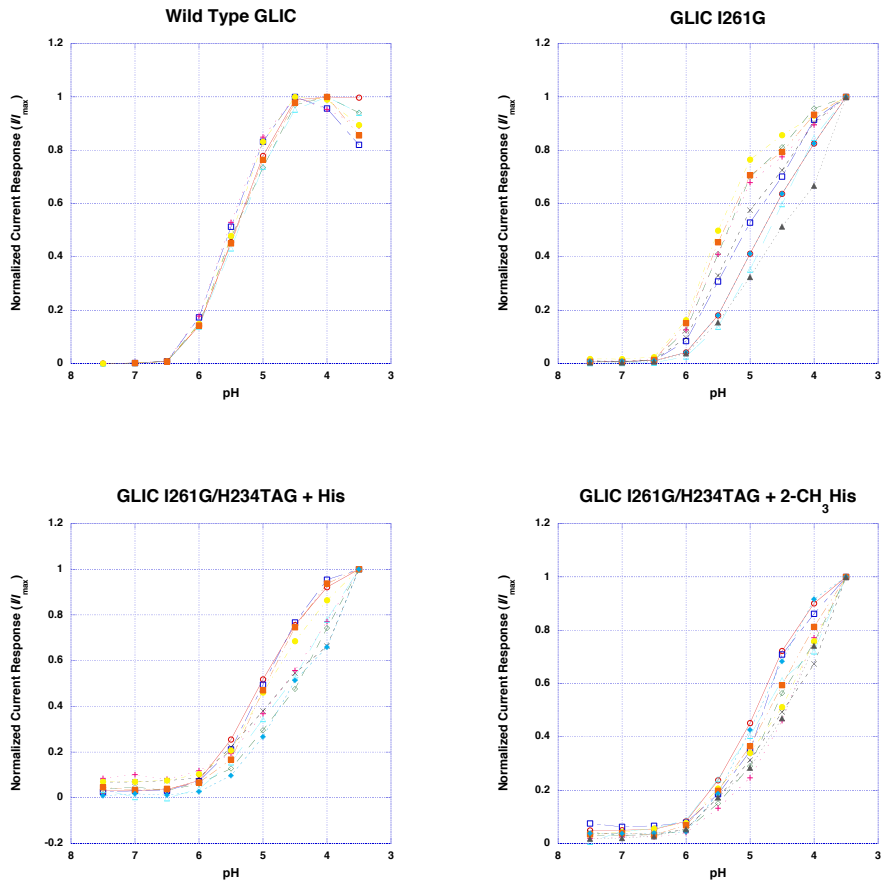


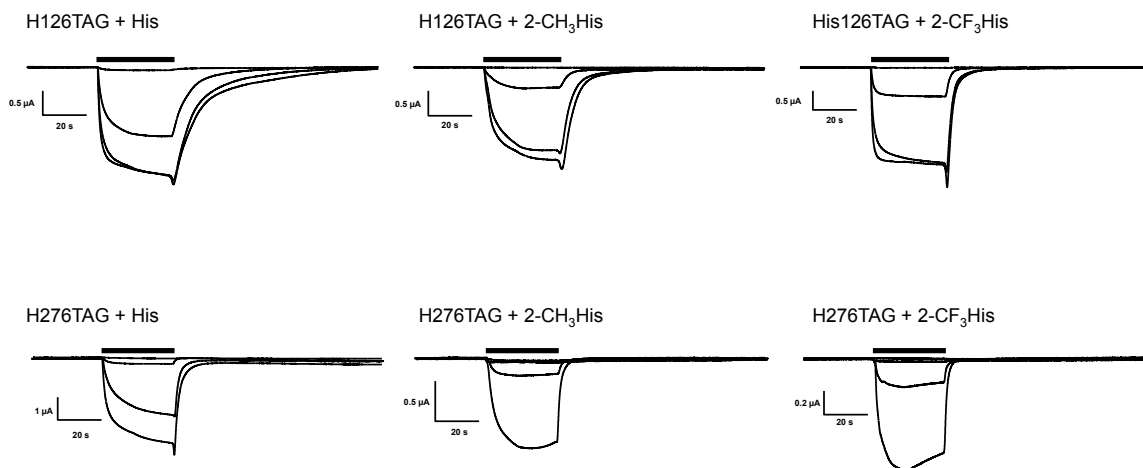
Supplemental Data

Figure S1, related to Table 1. Representative dose-response data for selected mutants. Each color represents peak current data recorded from a single oocyte.



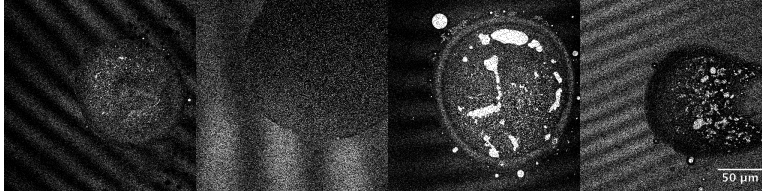
Cells expressing receptors containing the I261G mutation gave inconsistent pH-response curves with low Hill coefficients. Saturating  $H_3O^+$  concentrations were not generally tolerated by the oocytes.

**Figure S2, related to Figure 5.** Representative current traces for nonsense suppression experiments at His126 and His276. Running buffer was at pH 8; responses are shown for acidification to pH 7, 6, 5, and 4, indicated by the black bar.

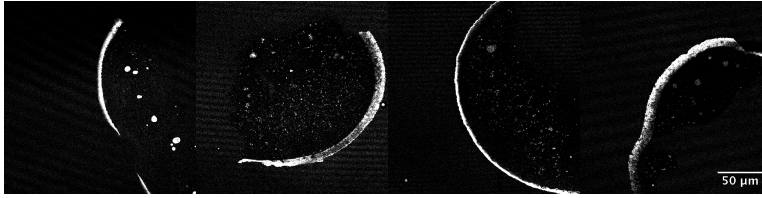


**Figure S3, related to Figure 6.** Immunofluorescence experiments. Oocytes expressing each mutant were immunolabeled and sliced, then imaged by confocal microscopy (see Supplemental Experimental Procedures). While it was possible to verify surface expression of the nonfunctional H234F mutant, expression from nonsense suppression experiments was too low to distinguish levels of fluorescence from those observed in the negative controls.

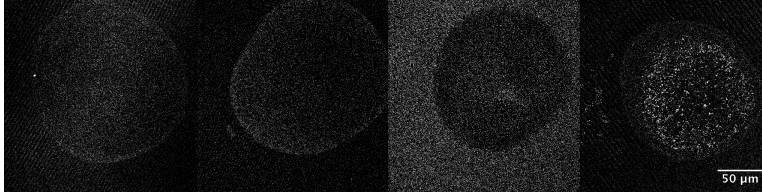
***Mock-Injected***



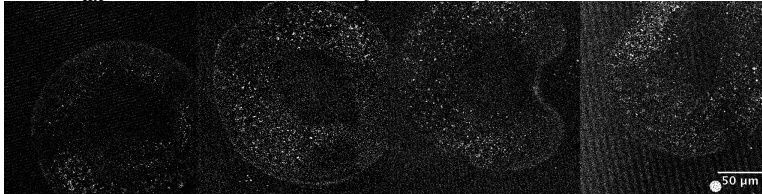
***GLIC C<sub>HA</sub>-H234F***



***GLIC C<sub>HA</sub>-I261G/H234TAG + uncharged tRNA***



***GLIC C<sub>HA</sub>-I261G/H234TAG + 2-CH<sub>3</sub>His***



***GLIC C<sub>HA</sub>-I261G/H234TAG + 2-CF<sub>3</sub>His***



## Supplemental Experimental Procedures

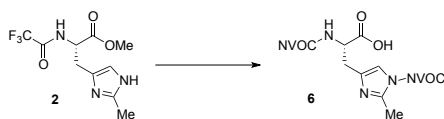
### Chemical Synthesis and Characterization of New Compounds

**Materials.** Commercial reagents and solvents, purchased from Sigma Aldrich and VWR, were used as received. Triethylamine was freshly distilled from CaH<sub>2</sub> prior to use. Deuterated solvents for NMR experiments were purchased from Cambridge Isotope Laboratories and used without further purification. Compounds **1** and **4**,<sup>2</sup> and pdCpA<sup>3</sup> were synthesized as previously reported. Reactions involving air- or moisture-sensitive reagents were performed in anhydrous solvents under an atmosphere of Ar. Flash chromatography was performed using silica gel from Merck (230-400 mesh). Analytical TLC was performed using plates from Sigma-Aldrich (60 Å pore size, F<sub>254</sub>, 0.25 mm) and was visualized by UV irradiation (254 nm) or staining with potassium permanganate.

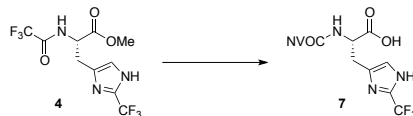
**Instrumentation.** <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained using Varian NMR instruments. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual <sup>1</sup>H resonance of the solvent. <sup>13</sup>C NMR spectra were referenced to the residual <sup>13</sup>C resonance of the solvent. Splitting patterns are designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. Mass spectroscopic data were acquired using an Agilent 1100 Series LC/MSD equipped with an ESI-APCI source. HPLC preparative purification was performed using a Waters 1525 binary pump, a Waters 2996 photodiode array detector, and an Atlantis Prep T3 OBD 5 μm 19x150mm column.



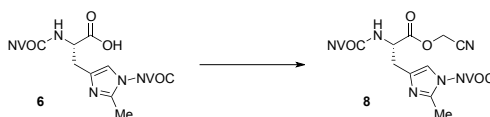
**(S)-methyl 3-(2-methyl-1H-imidazol-4-yl)-2-(2,2,2-trifluoroacetamido)propanoate (2).** To a solution of **1** (3.0 g, 11.3 mmol) in a mixture of 0.38 M aqueous sulfuric acid (30 mL, 11.3 mmol) and glacial acetic acid (30 mL) was added silver nitrate (960 mg, 5.65 mmol) at room temperature. The mixture was heated to 80 °C with stirring, and ammonium persulfate (12.9 g, 56.5 mmol) was slowly added as a solution in 60 mL of water over 10 minutes *via* cannula. After another 20 minutes, the reaction was poured over 100 g ice and allowed to come to room temperature. The mixture was basified with concentrated aqueous ammonium hydroxide to pH 10, then extracted with ethyl acetate (3 x 100 mL). The combined organic layers were dried over sodium sulfate, then filtered and concentrated. The desired product was partially purified by column chromatography (SiO<sub>2</sub>, 10% MeOH/DCM) to give **2** as a white solid (105 mg, 3%). <sup>1</sup>H-NMR (300 MHz; CD<sub>3</sub>OD) δ: 6.70 (s, 1H), 4.72 (dd, *J* = 9.5, 5.0 Hz, 1H), 3.73 (s, 3H), 3.14 (dd, *J* = 14.9, 5.0 Hz, 1H), 2.97 (dd, *J* = 14.8, 9.6 Hz, 1H), 2.32 (s, 3H); <sup>13</sup>C NMR (126 MHz; CD<sub>3</sub>OD) δ: 172.0, 158.8 (q, *J<sub>F</sub>* = 37.60), 146.0, 133.9, 117.2 (q, *J<sub>F</sub>* = 286.59), 117.1, 54.2, 53.0, 29.5, 13.3; <sup>19</sup>F NMR (282 MHz; CD<sub>3</sub>OD) δ: -78.9; LRMS (ESI) 278.0 [M-H]<sup>-</sup>.



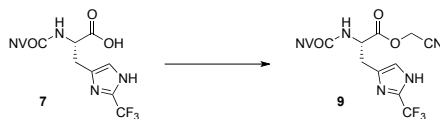
**(S)-3-(1-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)-2-methyl-1H-imidazol-4-yl)-2-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)amino)propanoic acid (6).** A solution of 6 N hydrochloric acid (10 mL) was added to a scintillation vial charged with **2** (190 mg, 0.68 mmol). A stir bar was added, and the solution was heated to 80 °C and stirred overnight. The reaction was then cooled to room temperature and concentrated to a brown solid, which was dissolved in methanol and concentrated several times to remove residual hydrochloric acid. The crude product was then directly dissolved in a mixture of water (6 mL) and dioxane (6 mL). Sodium carbonate (505 mg, 4.76 mmol) and 6-nitroveratryl chloroformate (375 mg, 1.36 mmol) were added in one portion, and the reaction was allowed to return to room temperature with stirring for 5 h. The mixture was then concentrated to a solid *in vacuo*, and the residue was suspended in water (50 mL) and acidified to pH ~2 with 1 N aqueous hydrochloric acid, then extracted with ethyl acetate (5 x 30 mL). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated. The residue was purified on a plug of silica (2% AcOH/EtOAc) to give **6** as a tan solid (138 mg, 0.21 mmol, 34% yield). <sup>1</sup>H NMR (400 MHz; d<sub>6</sub>-DMSO) δ: 7.72 (s, 1H), 7.61 (s, 1H), 7.22 (s, 1H), 7.17 (s, 1H), 7.10 (s, 1H), 7.02 (d, *J* = 6.3 Hz, 1H), 5.62 (d, *J* = 13.2 Hz, 1H), 5.57 (d, *J* = 14.0 Hz, 1H), 5.29 (d, *J* = 15.4 Hz, 1H), 5.23 (d, *J* = 15.7 Hz, 1H), 3.92 (s, 3H), 3.85-3.95 (m, 1H), 3.88 (s, 3H), 3.82 (s, 3H), 3.78 (s, 3H), 2.94 (dd, *J* = 14.8, 2.8 Hz, 1H), 2.71 (dd, *J* = 15.2, 8.2 Hz, 1H), 2.45 (s, 3H); <sup>13</sup>C NMR (126 MHz; d<sub>6</sub>-DMSO) δ: 173.3, 155.1, 153.5, 153.3, 148.6, 148.3, 147.4, 145.9, 139.7, 138.9, 138.6, 128.9, 124.7, 114.0, 111.6, 109.7, 108.2, 107.8, 65.7, 61.9, 56.4, 56.10, 56.06, 56.0, 54.9, 31.2, 16.2; LRMS (ESI) 646.2 [M-H]<sup>-</sup>.



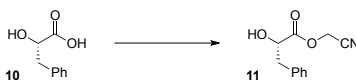
**(S)-2-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)amino-3-(2-(trifluoromethyl)-1H-imidazol-4-yl)propanoic acid (7).** Prepared according to the procedure for **6**, starting with **4** (230 mg, 0.87 mmol). The crude mixture was purified by precipitation from a hexanes/ethyl acetate mixture, giving **7** as a tan solid (202 mg, 0.29 mmol, 33% yield over 2 steps). <sup>1</sup>H NMR (500 MHz; d<sub>6</sub>-DMSO) δ: 7.89 (d, *J* = 8.1 Hz, 1H), 7.69 (s, 1H), 7.13 (s, 1H), 7.08 (s, 1H), 5.38 (d, *J* = 15.1 Hz, 1H), 5.31 (d, *J* = 15.1 Hz, 1H), 4.29 (td, *J* = 8.8, 4.5 Hz, 1H), 3.87 (s, 3H), 3.86 (s, 4H), 3.05 (dd, *J* = 14.7, 3.9 Hz, 1H), 2.93 (dd, *J* = 14.6, 9.9 Hz, 1H); <sup>13</sup>C NMR (126 MHz; d<sub>6</sub>-DMSO) δ: 172.8, 155.5, 153.5, 147.6, 139.0, 133.7 (q, *J<sub>F</sub>* = 39.5 Hz), 128.0, 118.9 (q, *J<sub>F</sub>* = 268.3), 110.0, 108.2, 62.3, 56.1, 53.7; <sup>19</sup>F NMR (282 MHz; CD<sub>3</sub>CN) δ: -64.0; LRMS (ESI) 461.1 [M - H]<sup>-</sup>.



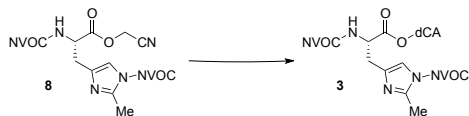
**(S)-4,5-dimethoxy-2-nitrobenzyl 4-(3-(cyanomethoxy)-2-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)amino-3-oxopropyl)-2-methyl-1H-imidazole-1-carboxylate (8).** To a solution of **6** (80 mg, 0.12 mmol) in a mixture of DMF (1.2 mL) and chloroacetonitrile (1.2 mL) was added triethylamine (54 μL, 0.39 mmol). The reaction was stirred for 24 h at room temperature, then diluted with ether (10 mL) and washed with water (3 x 10 mL). The organic layer was then concentrated to an oil, which was dissolved in ethyl acetate. The product was precipitated by addition of hexanes, giving **8** as a tan solid (25 mg, 29%). <sup>1</sup>H NMR (500 MHz; d<sub>6</sub>-DMSO) δ: 8.09 (d, *J* = 7.8, 1H), 7.72 (s, 1H), 7.66 (s, 1H), 7.28 (s, 2H), 7.11 (s, 1H), 5.64 (s, 2H), 5.35 (d, *J* = 15.0, 1H), 5.31 (d, *J* = 14.7, 1H), 5.01 (s, 2H), 4.46-4.42 (m, 1H), 3.90 (s, 3H), 3.89 (s, 3H), 3.85 (s, 6H), 2.89-2.87 (m, 2H), 2.48 (s, 3H); <sup>13</sup>C NMR (126 MHz; d<sub>6</sub>-DMSO) δ: 170.8, 155.5, 153.4, 153.2, 148.5, 148.4, 147.7, 146.8, 140.1, 139.1, 135.8, 127.6, 124.3, 115.6, 115.5, 112.4, 110.3, 108.3, 108.1, 66.0, 62.7, 56.4, 56.2, 56.11, 56.06, 53.2, 49.5, 29.1, 16.3; LRMS (ESI) 685.1 [M-H]<sup>-</sup>.



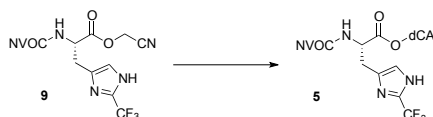
**(S)-cyanomethyl 2-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)amino-3-(2-(trifluoromethyl)-1H-imidazol-4-yl)propanoate (9).** Prepared according to the procedure for **8**, starting with **7** (150 mg, 0.32 mmol). Following precipitation, **9** was obtained as a tan solid (80 mg, 0.16 mmol, 49% yield). <sup>1</sup>H-NMR (300 MHz; CDCl<sub>3</sub>) δ: 9.71 (s, 1H), 7.73 (s, 1H), 7.07 (s, 1H), 7.03 (d, *J* = 2.0 Hz, 1H), 6.50 (d, *J* = 8.8 Hz, 1H), 5.63 (d, *J* = 15.5 Hz, 1H), 5.49 (d, *J* = 15.8 Hz, 1H), 4.80 (d, *J* = 15.7 Hz, 1H), 4.77-4.72 (m, 1H), 4.67 (d, *J* = 15.7 Hz, 1H), 3.97 (d, *J* = 10.3 Hz, 6H), 3.28 (dd, *J* = 15.1, 4.8 Hz, 1H), 3.16 (dd, *J* = 14.5, 4.8 Hz, 1H); <sup>13</sup>C-NMR (126 MHz; d<sub>6</sub>-DMSO) δ: 170.6, 155.5, 153.4, 147.8, 139.2, 137.53, 133.9 (q, *J<sub>F</sub>* = 38 Hz), 127.5, 118.8 (q, *J<sub>F</sub>* = 265 Hz), 117.3, 115.4, 110.3, 108.2, 62.7, 56.09, 56.04, 53.76, 49.5, 29.3; <sup>19</sup>F NMR (282 MHz; CDCl<sub>3</sub>) δ: -63.7; LRMS (ESI) 500.1 [M - H]<sup>-</sup>.



**(S)-cyanomethyl 2-hydroxy-3-phenylpropanoate (11).** Prepared according to the procedure for **8**, starting with **10** (100 mg, 0.60 mmol). Following precipitation, **11** was obtained as a colorless oil (85 mg, 0.41 mmol, 69% yield). <sup>1</sup>H NMR (500 MHz; CDCl<sub>3</sub>) δ: 7.33-7.30 (m, 2H), 7.28-7.25 (m, 1H), 7.22-7.20 (m, 2H), 4.75 (d, *J* = 15.7, 1H), 4.72 (d, *J* = 15.7, 1H), 4.53 (q, *J* = 5.6, 1H), 3.14 (dd, *J* = 14.0, 4.8, 1H), 3.02 (dd, *J* = 14.0, 6.7, 1H), 2.70 (d, *J* = 6.5, 1H); <sup>13</sup>C NMR (126 MHz; CDCl<sub>3</sub>) δ: 172.5, 135.4, 129.5, 128.7, 127.3, 113.7, 71.3, 48.9, 40.4; LRMS (ESI) 204.0 [M-H]<sup>-</sup>.



**4,5-dimethoxy-2-nitrobenzyl 4-((2*S*)-3-(((5-(((5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-((phosphonoxy)methyl)tetrahydrofuran-3-yl)oxy)(hydroxy)phosphoryl)oxy)methyl)-2-(6-amino-9*H*-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl)oxy)-2-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)amino)-3-oxopropyl)-2-methyl-1*H*-imidazole-1-carboxylate (**3**). A 0.5-dram vial was charged with 10 mg pdCpA•2.4 TBAH and a stir bar, then evacuated and backfilled several times with argon. A 0.02 *M* solution of **8** in DMF (0.5 mL, 0.01 mmol) was then added, and the mixture was stirred under argon overnight. The product was then purified using reverse-phase HPLC to give **3** as a white solid. MALDI-MS (6-aza-2-thiothymine matrix) 1026.9 [M-NVOC+H]<sup>+</sup>.**

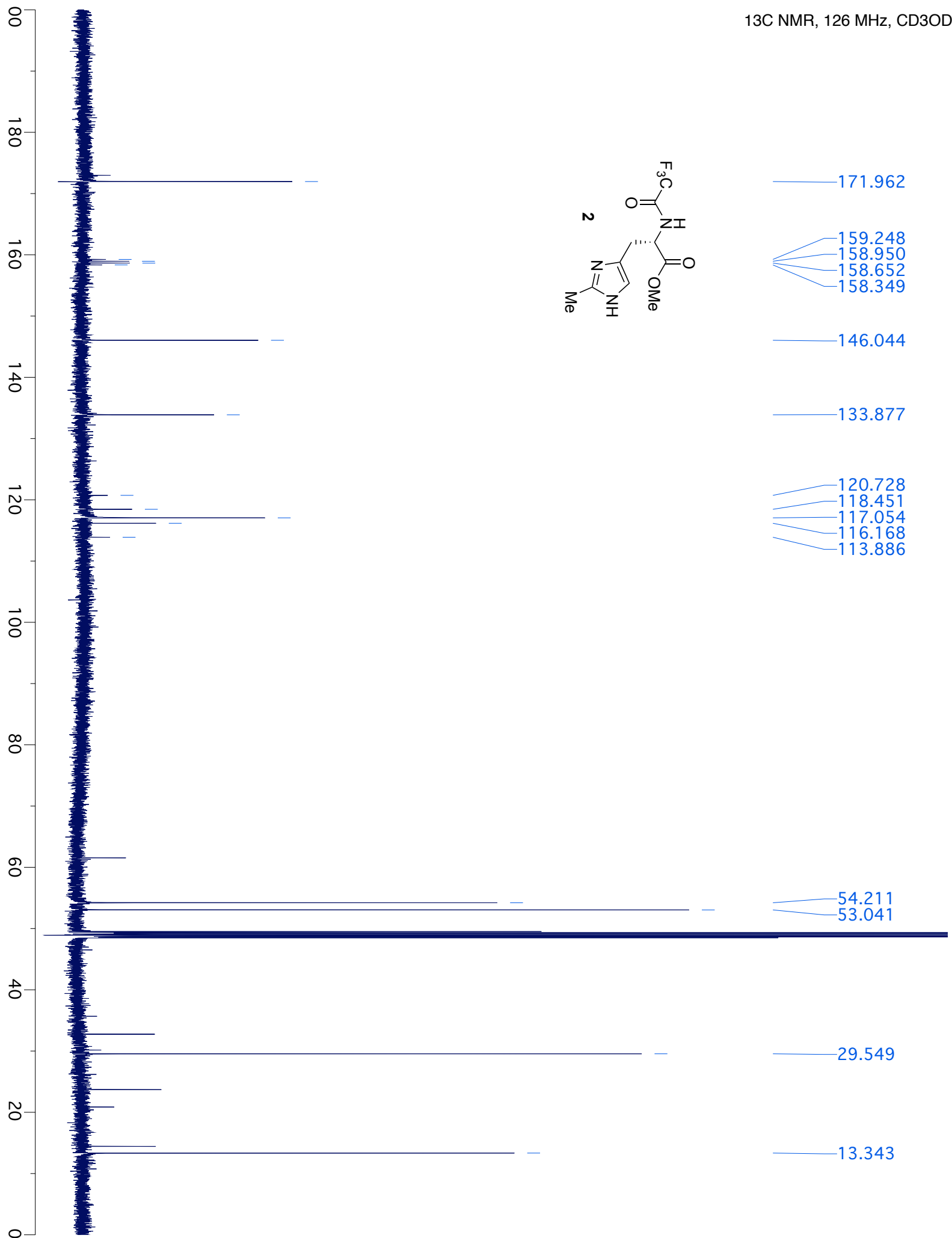


**(2*S*)-5-(((5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-((phosphonoxy)methyl)tetrahydrofuran-3-yl)oxy)(hydroxy)phosphoryl)oxy)methyl)-2-(6-amino-9*H*-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl 2-(((4,5-dimethoxy-2-nitrobenzyl)oxy)carbonyl)amino)-3-(2-(trifluoromethyl)-1*H*-imidazol-4-yl)propanoate (**5**). Prepared according to the procedure for **3**, starting with **9**. MALDI-MS (6-aza-2-thiothymine matrix) 1081.2 [M+H]<sup>+</sup>.**

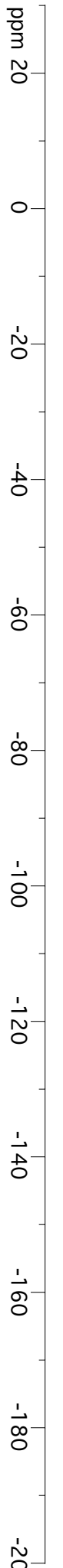
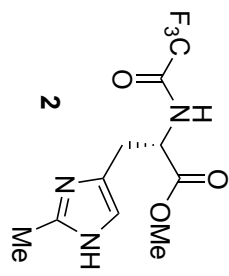


**(2*S*)-5-(((5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-2-((phosphonoxy)methyl)tetrahydrofuran-3-yl)oxy)(hydroxy)phosphoryl)oxy)methyl)-2-(6-amino-9*H*-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl 2-hydroxy-3-phenylpropanoate (**12**). Prepared according to the procedure for **3**, starting with **11**. MALDI-MS (6-aza-2-thiothymine matrix) 785.4 [M+H]<sup>+</sup>.**

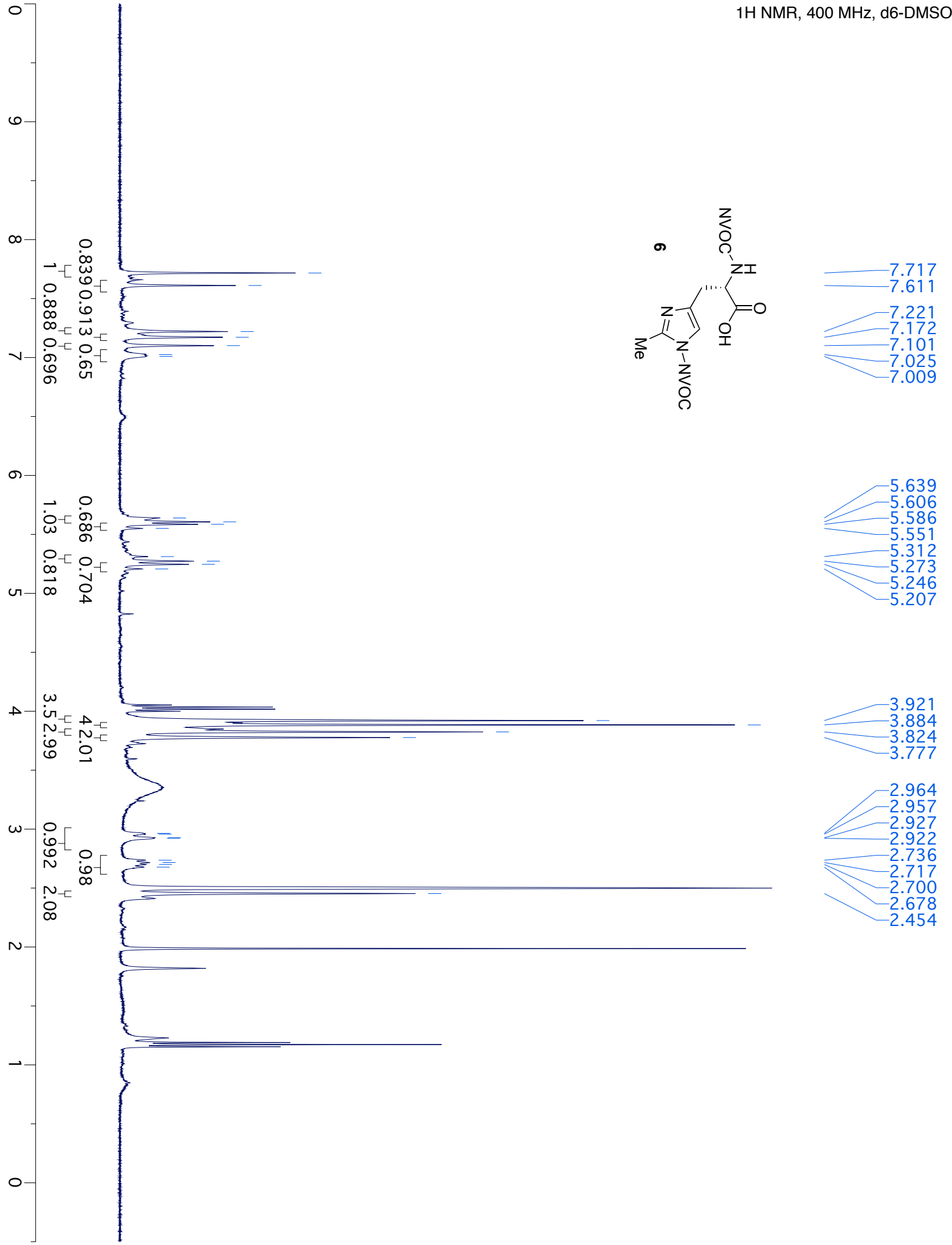
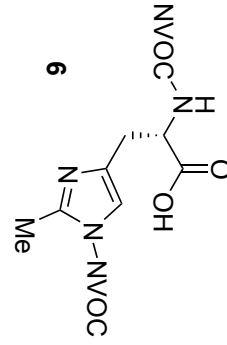


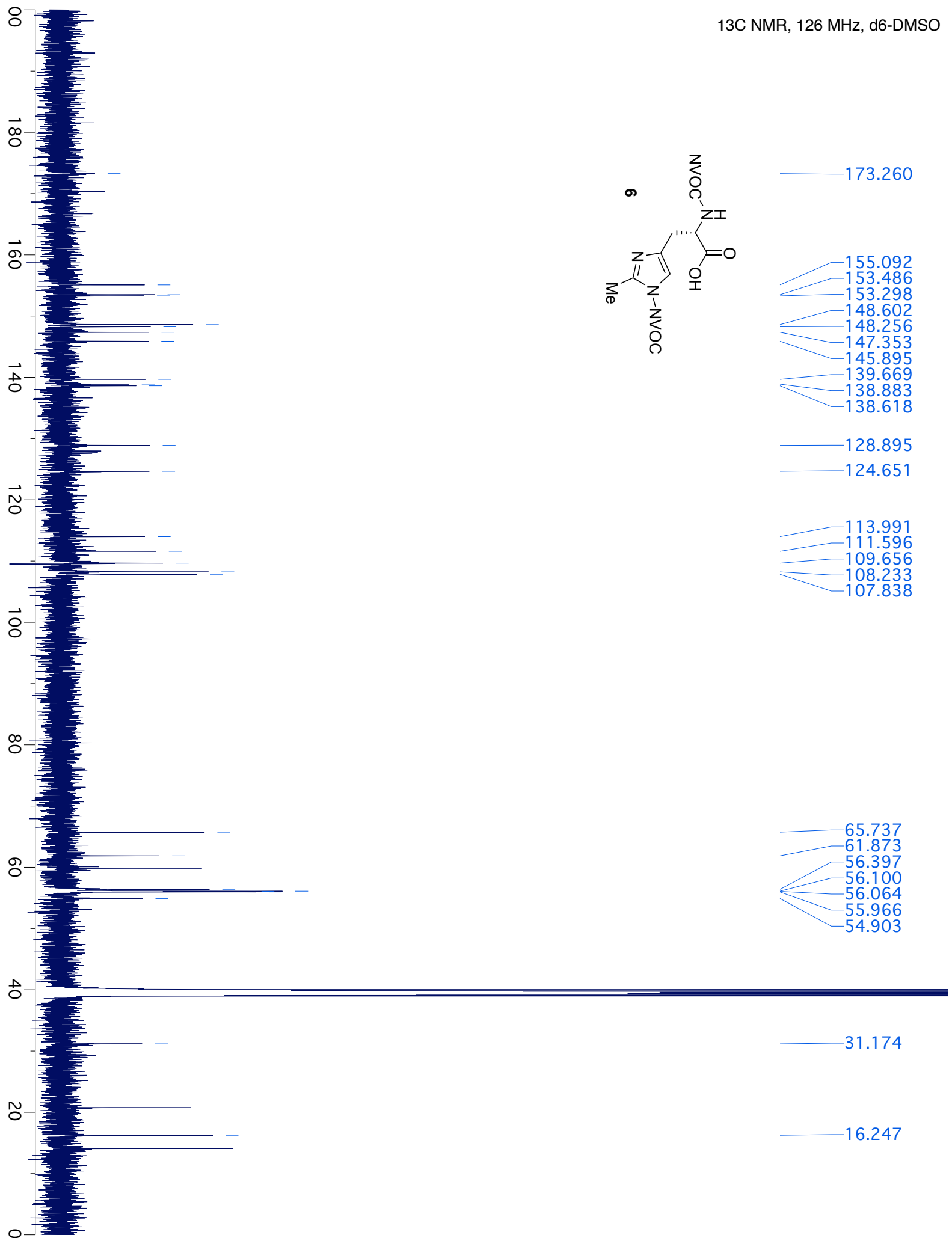


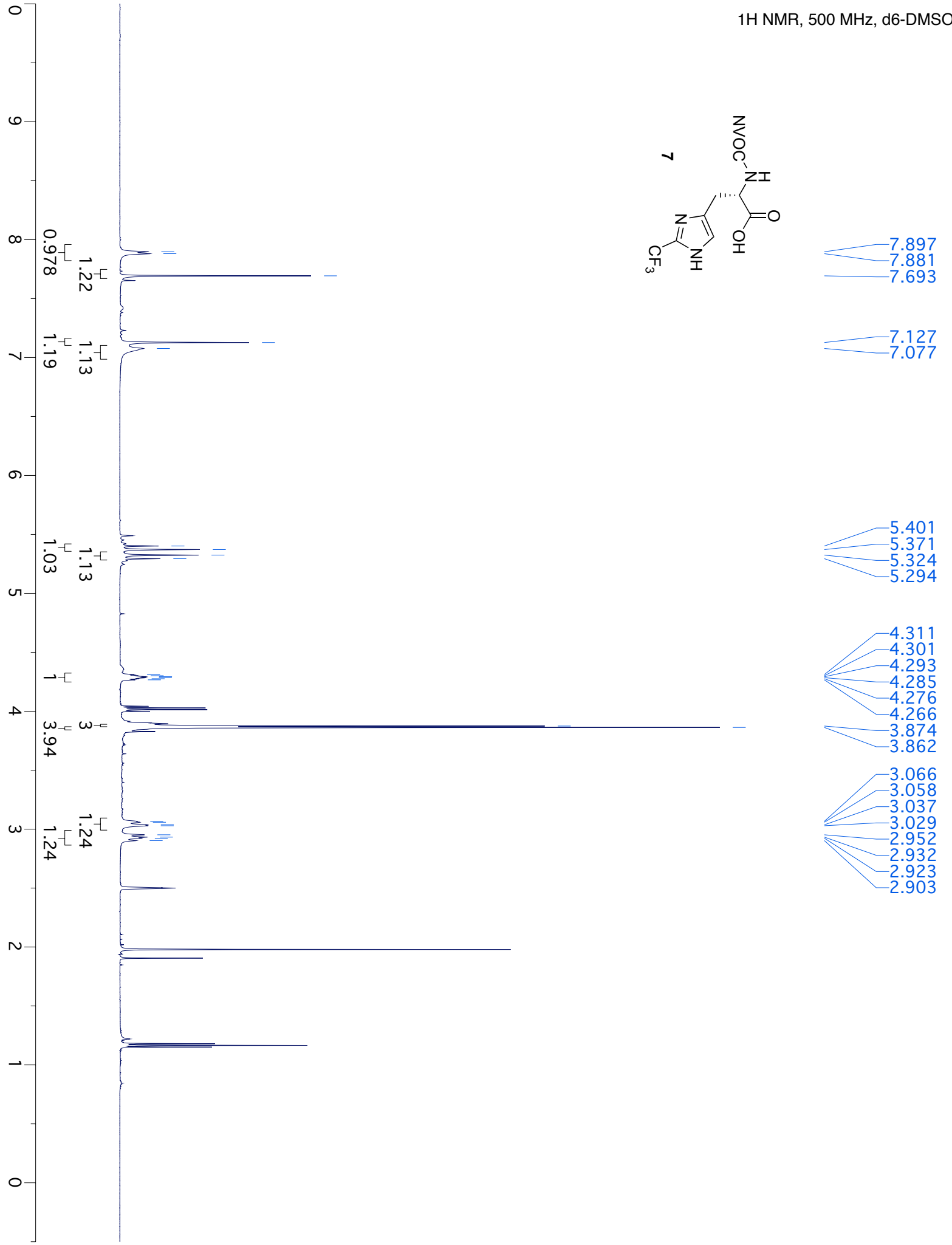
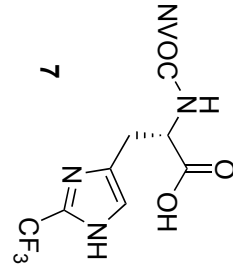


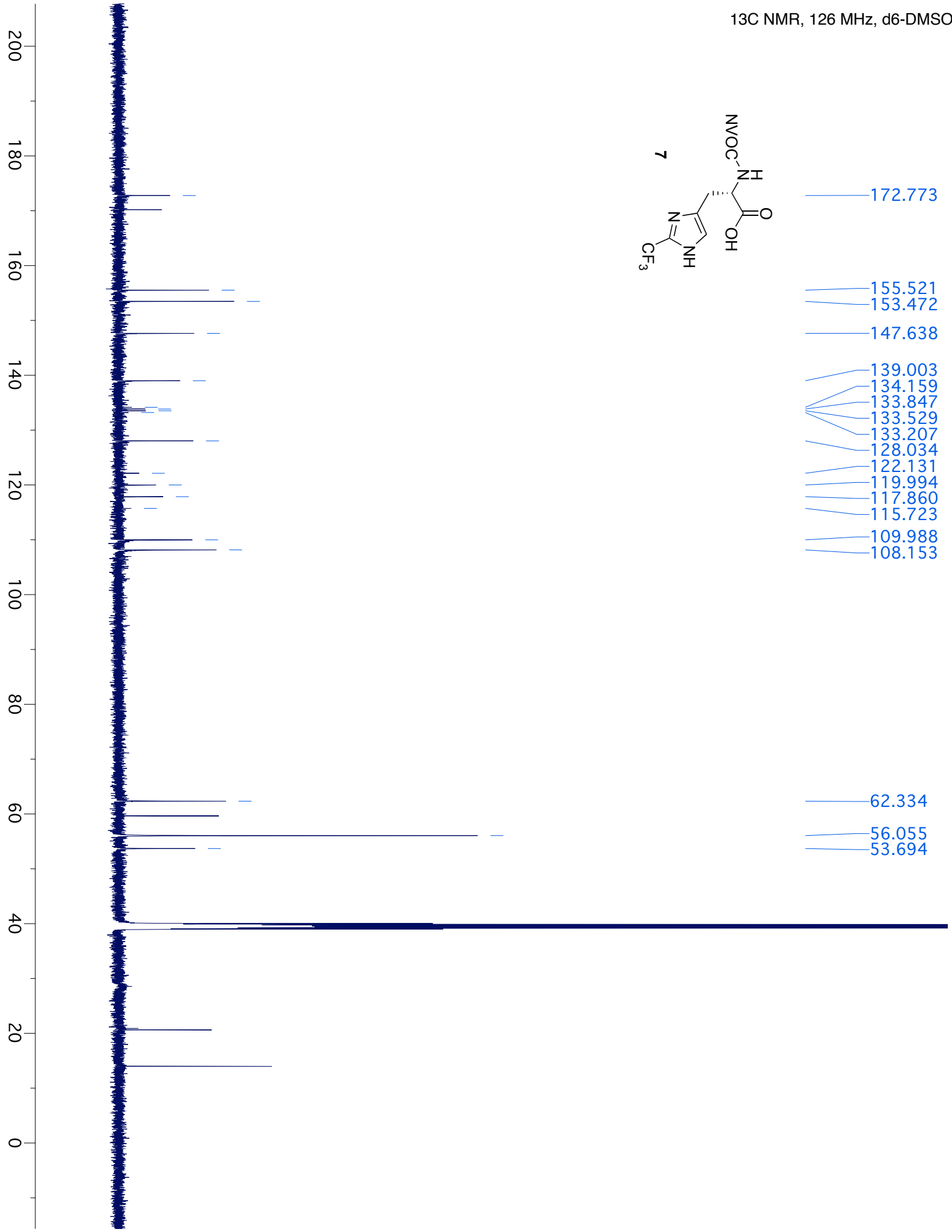
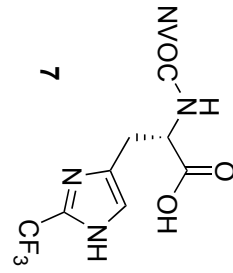


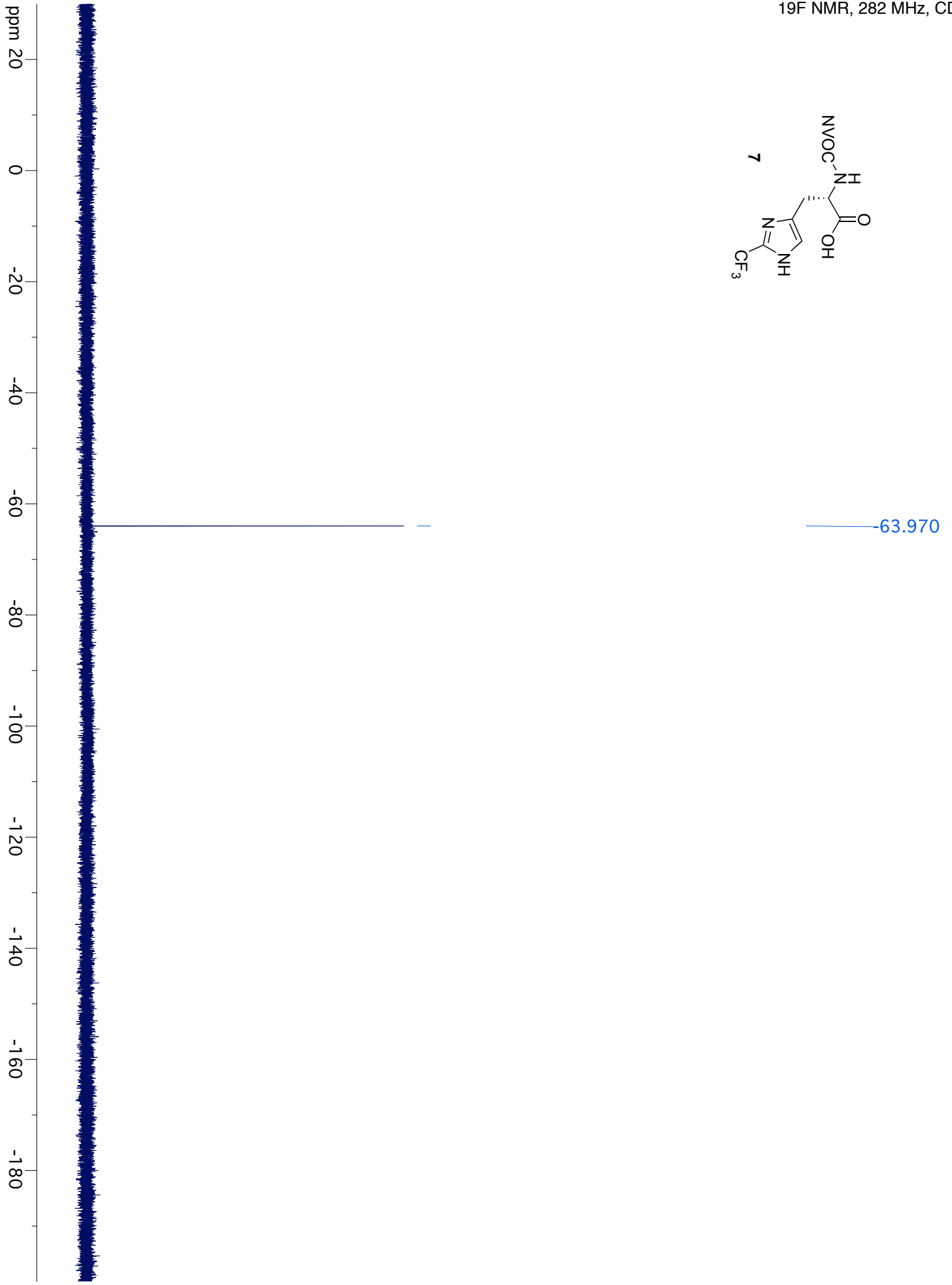
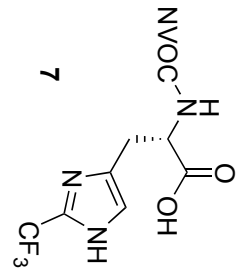
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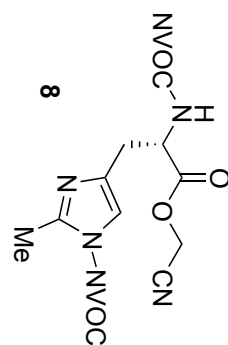












8.101  
8.085

7.721  
7.661

7.282  
7.112

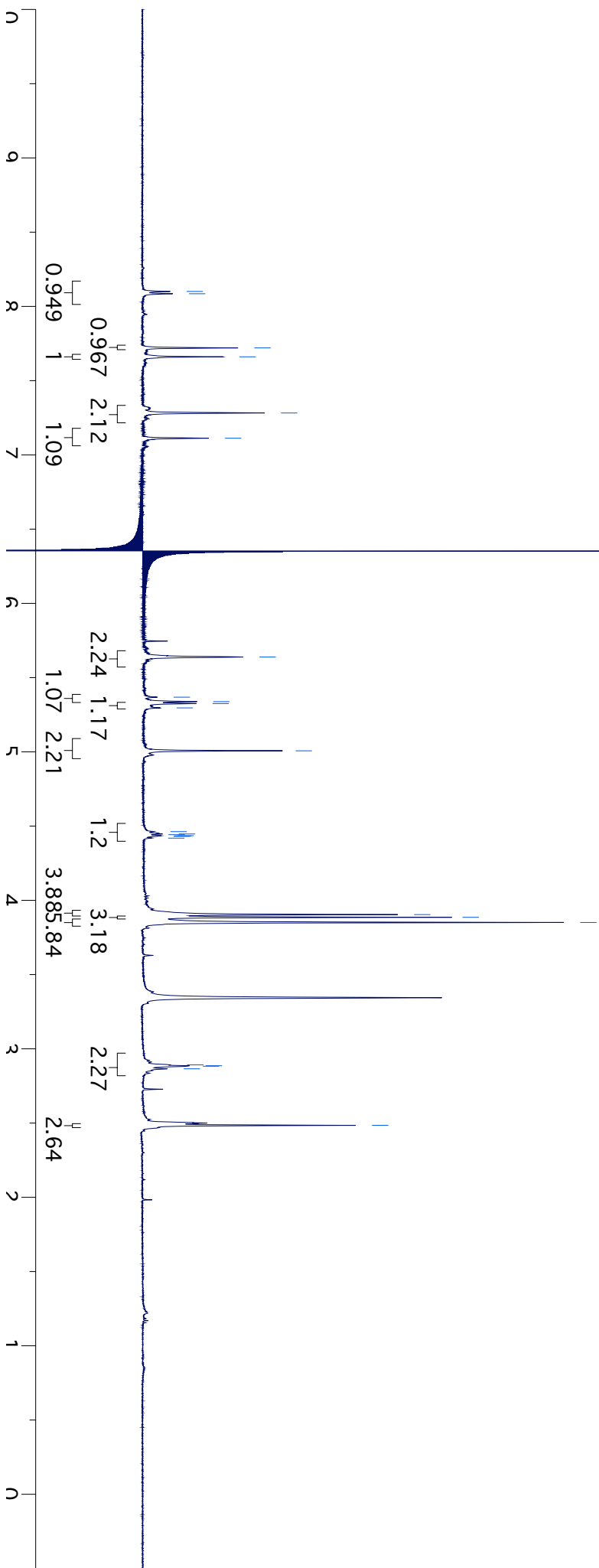
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5.338  
5.325  
5.296

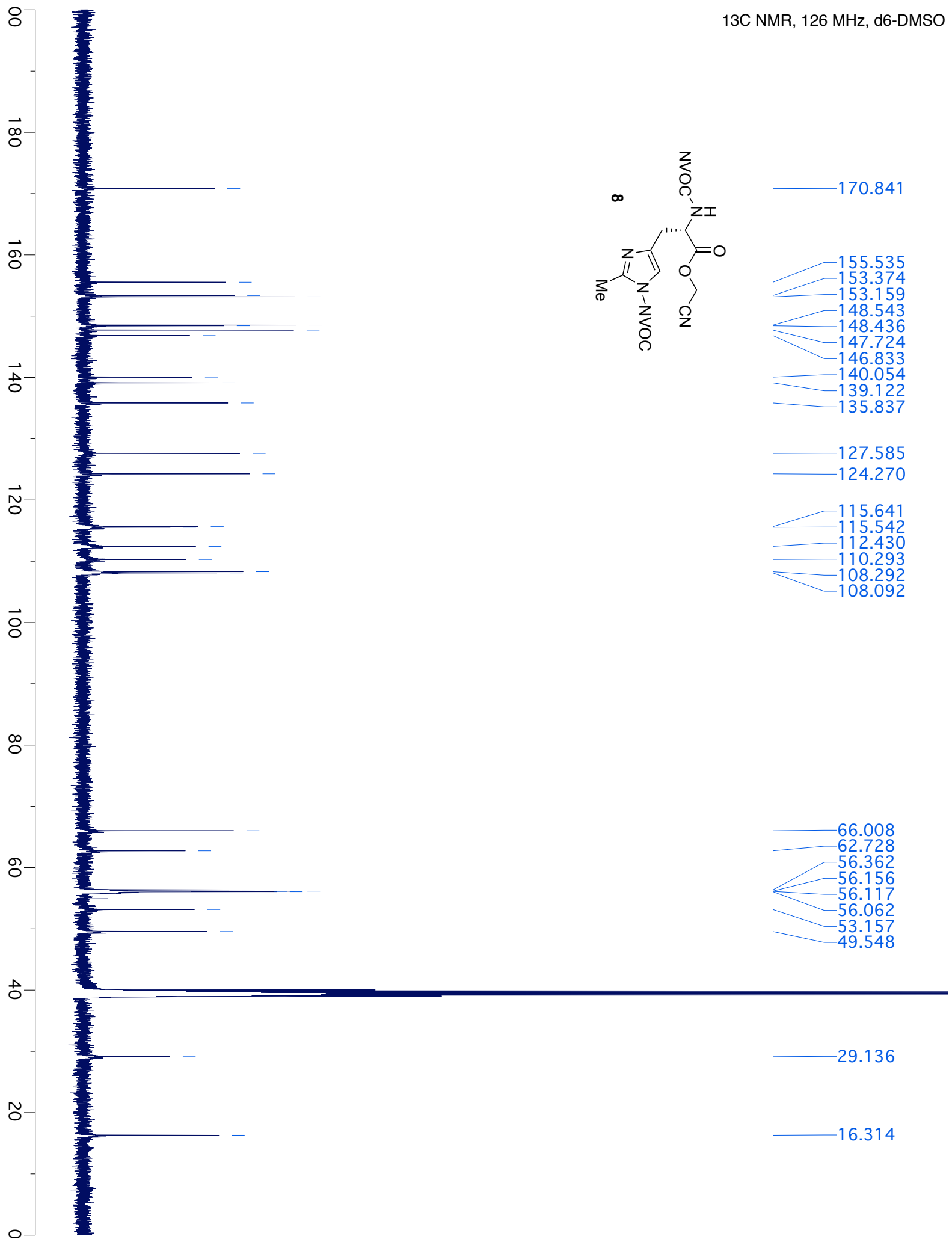
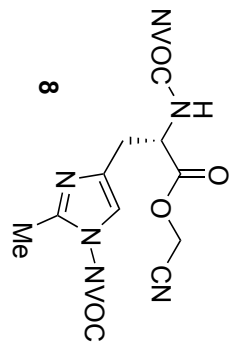
5.007  
4.463  
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4.435  
4.434  
4.433  
4.431  
4.419

3.904  
3.886  
3.851

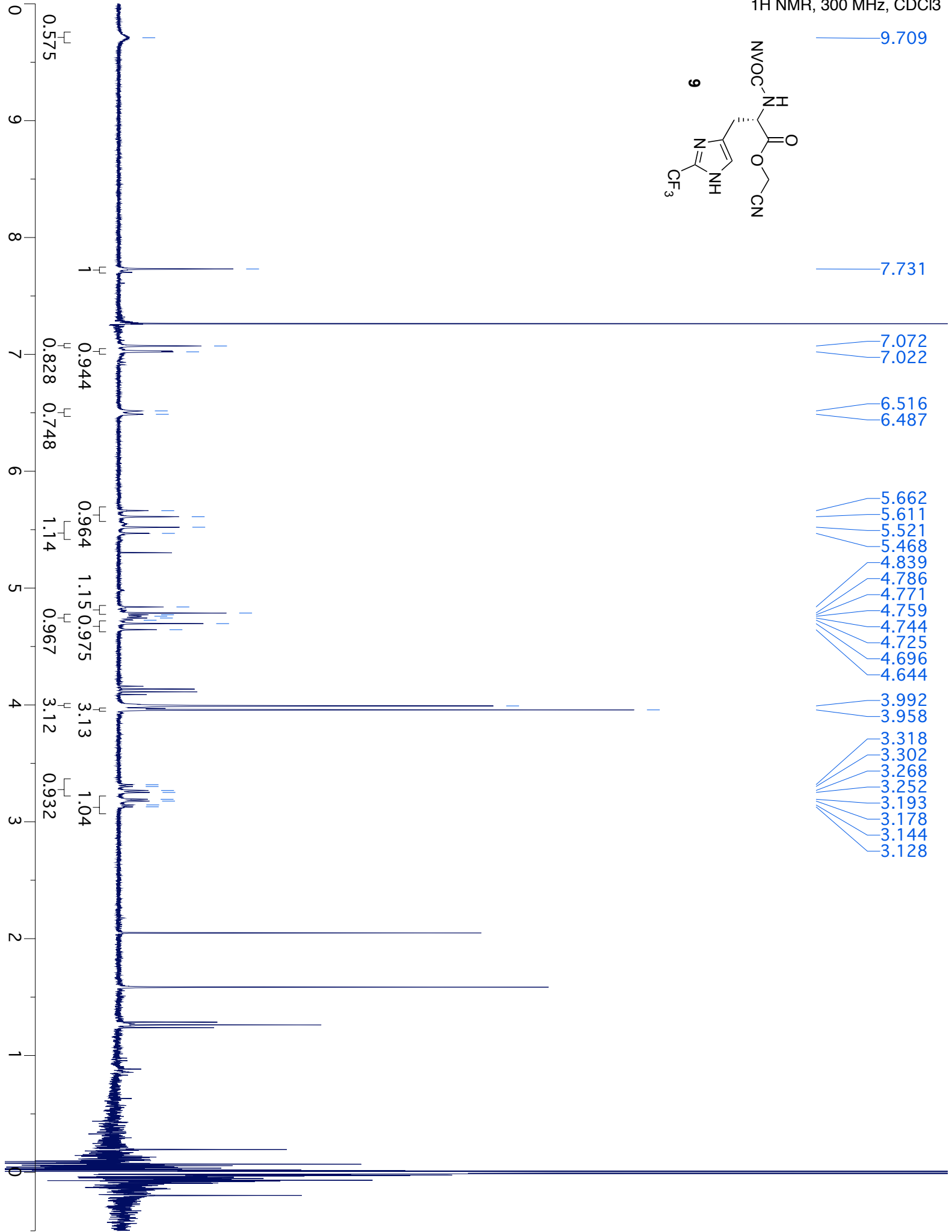
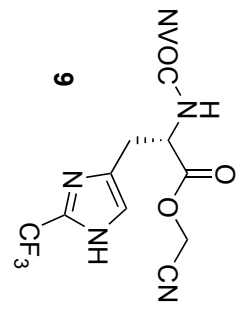
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2.883  
2.865

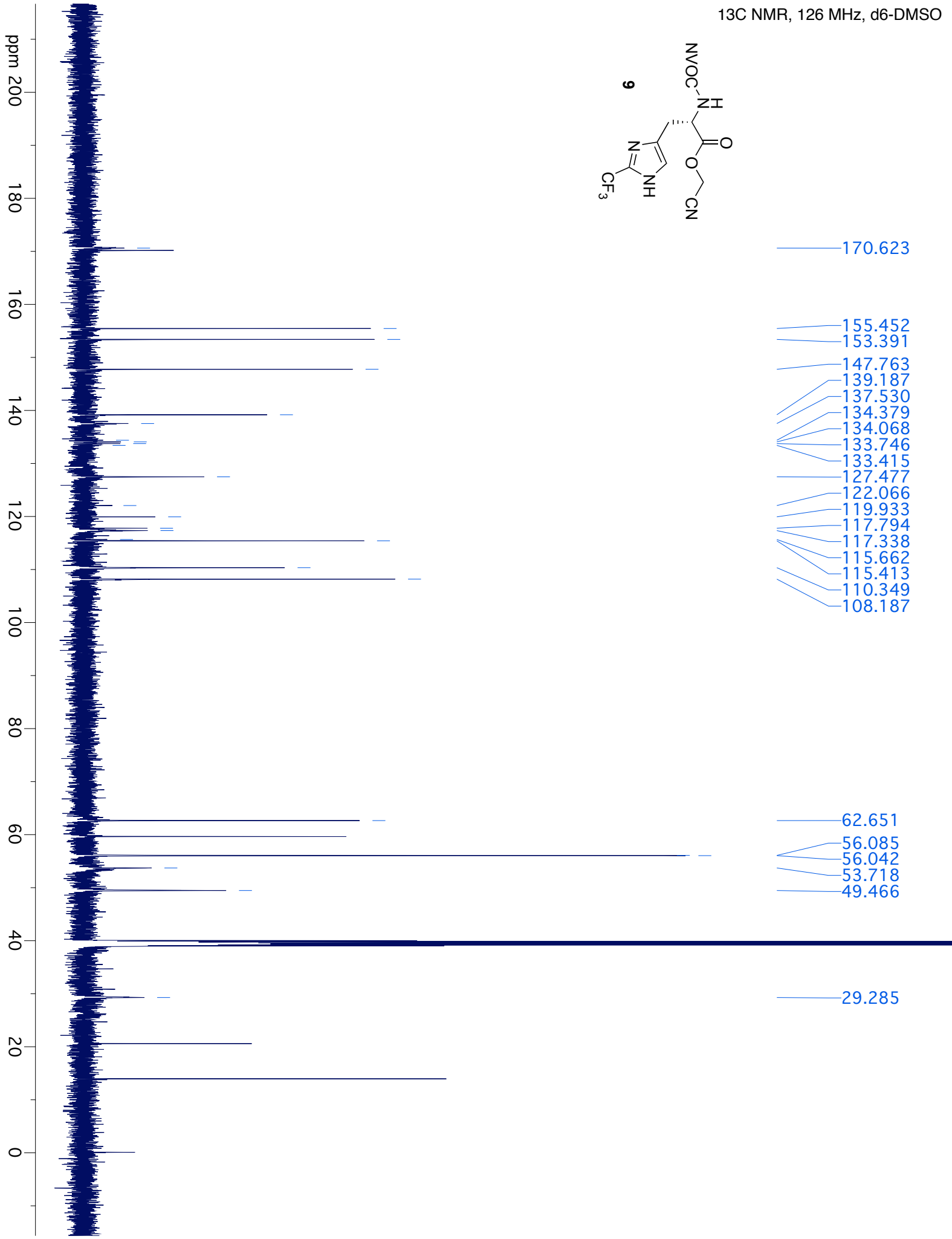
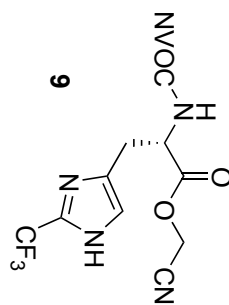
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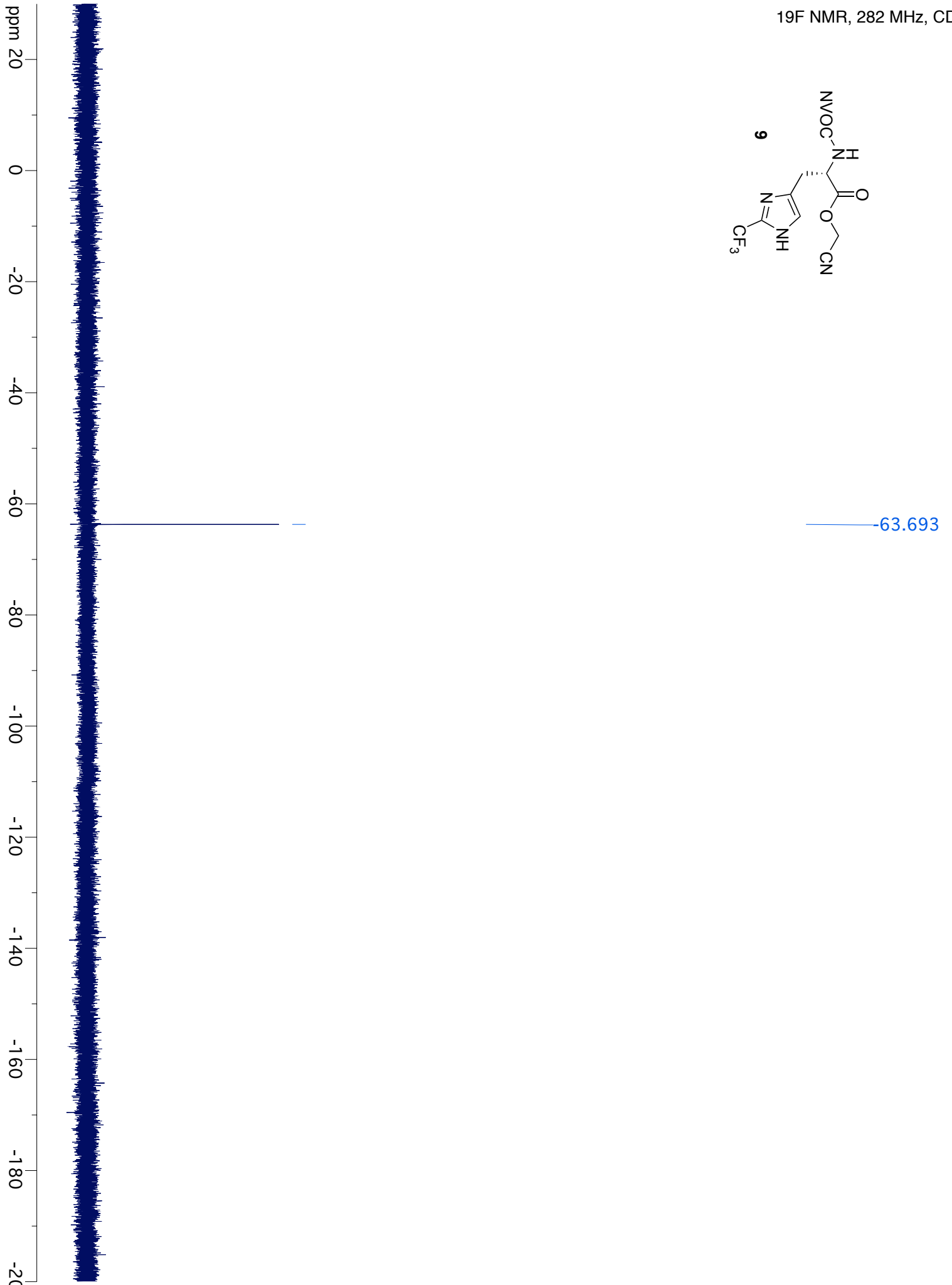
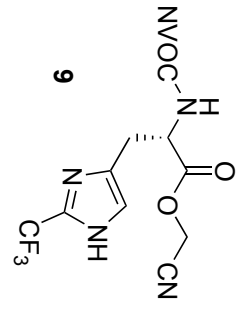


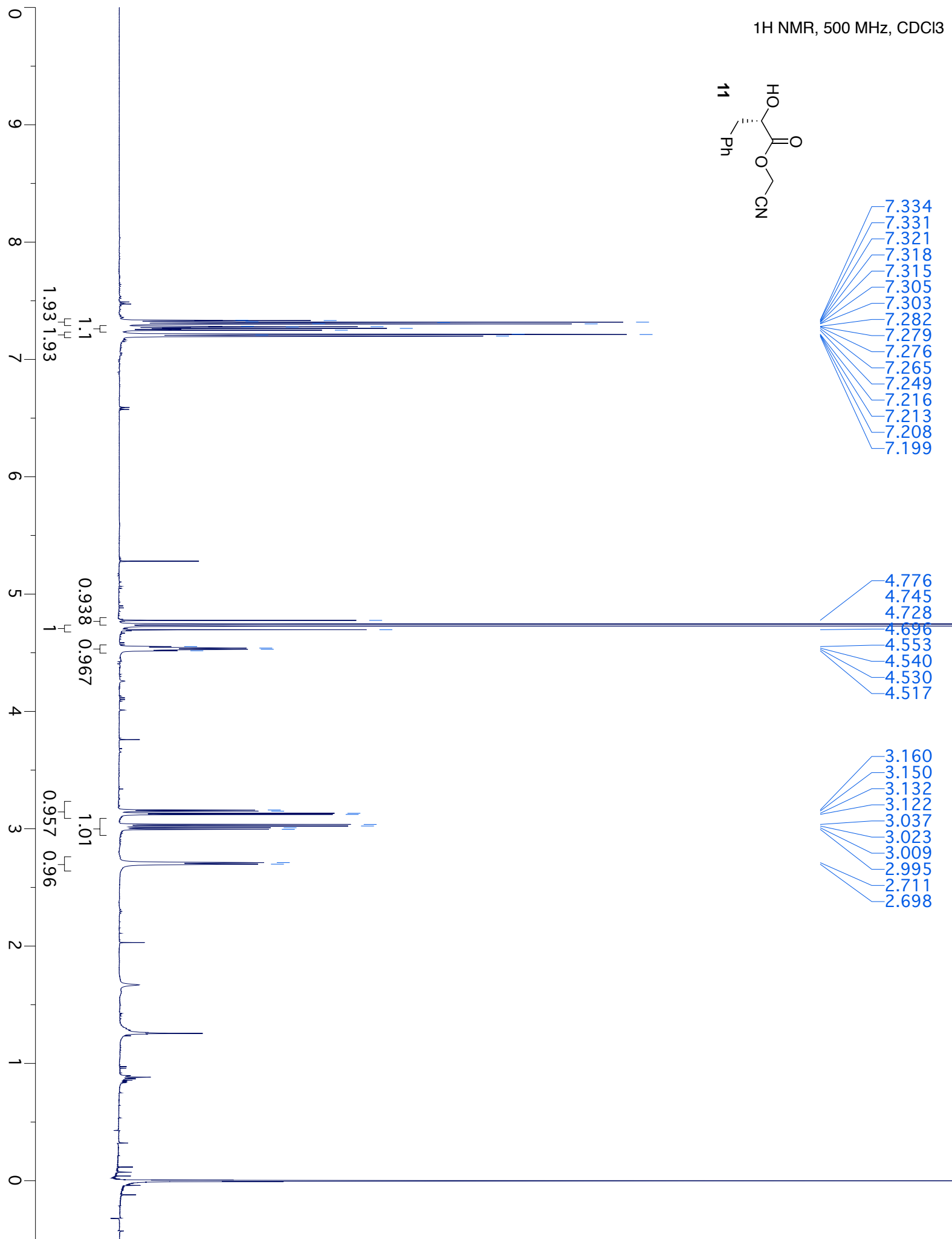
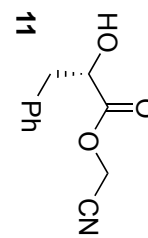


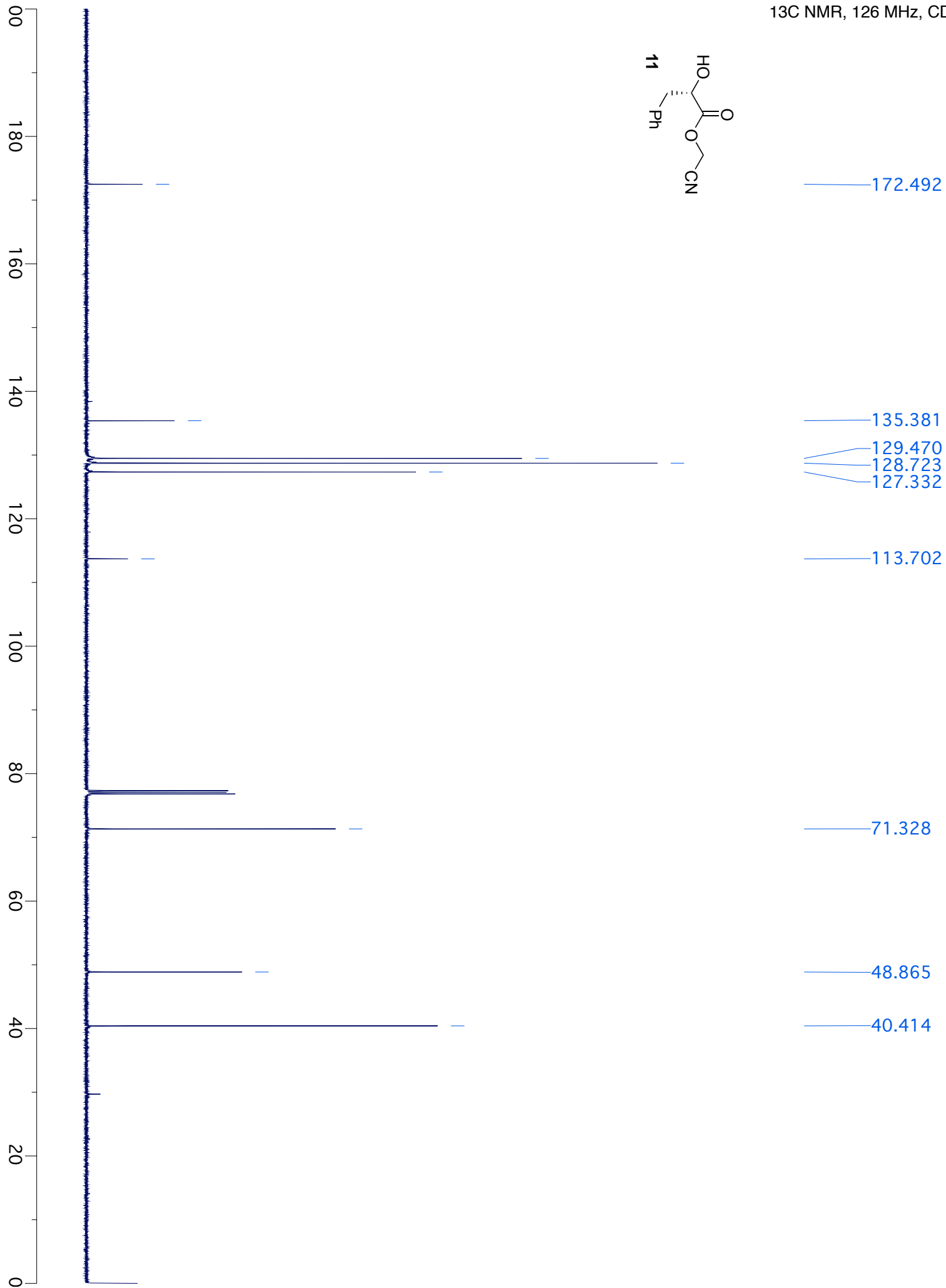
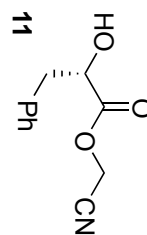












### ***Immunofluorescence Experiments***

Following injection and incubation, oocytes were fixed in a 3.7% solution of paraformaldehyde in phosphate buffered saline (PBS) buffer (0.01 M, pH 7.4) for 3 h. The cells were then washed in a 3% solution of bovine serum albumin (BSA) in the same buffer (3 x 10 min) at room temperature, and then labeled overnight at 4 °C with monoclonal mouse-anti-HA (1:1000 in 3% BSA/PBS). Cells were washed in 3% BSA/PBS at room temperature (3 x 10 min), then incubated 3 h at room temperature with anti-mouse IgG secondary antibody conjugated with Alexa Fluor 555 (1:500 in 3% BSA/PBS). Cells were then washed in PBS buffer (3 x 5 min) and embedded in blocks of 3% low melting agarose in PBS buffer before sectioning into 50 µm slices with a vibratome. The slices were then mounted in 70% glycerol in PBS on glass slides. Slices were imaged at room temperature using a an Eclipse C1si laser-scanning confocal microscope (Nikon Instruments) equipped with a 10x, 0.45 NA, Plan Apo objective and 32 photomultiplier tubes. Alexa Fluor 555 was excited at 561 nm. Fluorescence data was collected from 570-730 nm, and images were averaged over three scans. Images were unmixed using standard spectra acquired from slices of mock-injected cells and Alexa Fluor 555 alone.

### *Supplemental References*

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