Sulfonate/nitro bearing methylmalonyl-thioester isosteres applied to methylmalonyl-CoA decarboxylase structure-function studies

Lee M. Stunkard, Austin D. Dixon, Tyler J. Huth and Jeremy R. Lohman*

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907, United States

Purdue Center for Cancer Research, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

Table of Contents:

I. Materials and Methods

Chemicals and solvents: All chemicals were purchased from Acros, Aldrich, Alfa Aesar, Fluka, Oakwood or TCI America and used without further purification. For reactions, technical grade solvents were used without further purification and dried over molecular sieves (3Å) when applicable. HPLC grade solvents were used for flash chromatography, analytical and preparative HPLC. Deuterated solvents were purchased from Acros or Sigma-Aldrich.

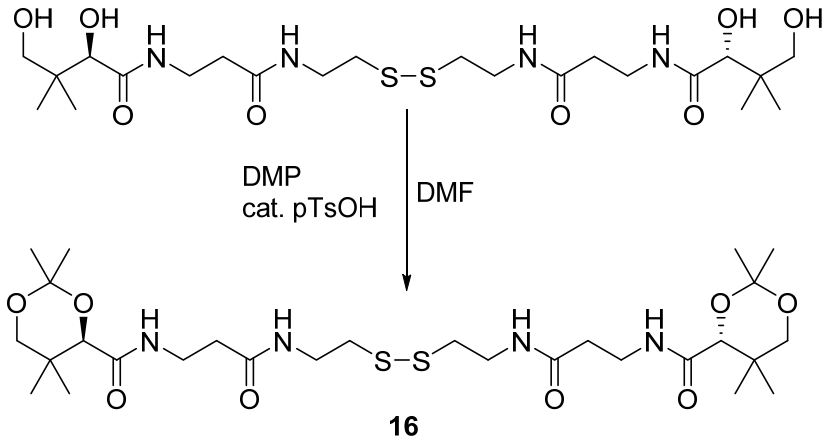
Reactions: All organic synthesis reactions were performed under normal atmosphere at room temperature unless otherwise noted. The reactions and purifications were not optimized. Reactions were magnetically stirred with Teflon coated stirbars. Flash chromatography was performed on a CombiFlash Rf200 (Teledyne ISCO) with 24 or 40 gram silica Flash Columns. Preparative HPLC chromatography was performed on an Agilent 1100 preparative HPLC with diode array UV/Vis detection over a Luna 5µ C18(2) 100 Å 250 x 21.2 mm column (Phenomenex). The reported yields are post purification and spectroscopically pure unless previously reported or otherwise indicated.

Analysis: Reactions and products were characterized by HPLC-MS on an Agilent 1100 HPLC with diode array UV/Vis detection over a Luna 5 µm C18(2) 100 Å 50 x 2 mm (Phenomenex) or Luna 5 µm C18(2) 100 Å 250 x 4.6 mm (Phenomenex) with low resolution mass spectrometry (LRMS) analysis in positive and negative modes by an Agilent 1100 G1946D quadrupole with electrospray ionization (ESI). NMR spectra were collected on a Bruker AV500HD equipped with a 5mm BBFO Z-gradient cryoprobe in the solvents indicated. ^{1}H and ^{13}C NMR spectra are referenced using the signals of the residual undeuterated solvent ($CDCl_3$ ^{1}H-7.27 ppm and ^{13}C-77.14 ppm, DMSO ^{1}H-2.48 ppm and ^{13}C-39.5 ppm and D_2O ^{1}H-4.68 ppm) and where applicable tetramethylsilane 0-ppm. All spectra were collected at 298 K. Chemical shifts are reported in parts per million (ppm) and multiplicities are abbreviated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). Coupling constants (*J*) are reported in Hertz (Hz).

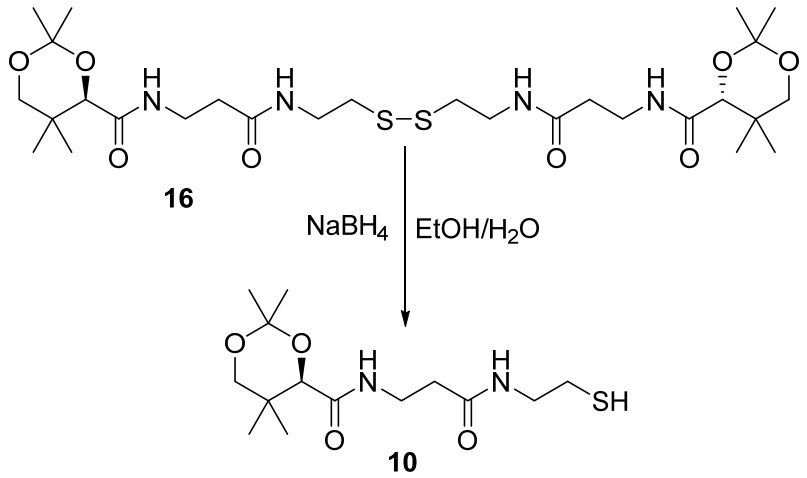
II. List of Abbreviations

ACN	acetonitrile
ATP	adenosine triphosphate
CF	chloroform
DCM	dichloromethane
DMF	dimethylformamide
DMP	2,2-dimethoxypropane
ECF	ethylchloroformate
EtOAc	ethyl acetate
EtOH	ethanol
pTsOH	p-toluenesulfonic acid
TEA	triethylamine
TFA	trifluoroacetic acid

III. Experimental Procedures and Characterization Data

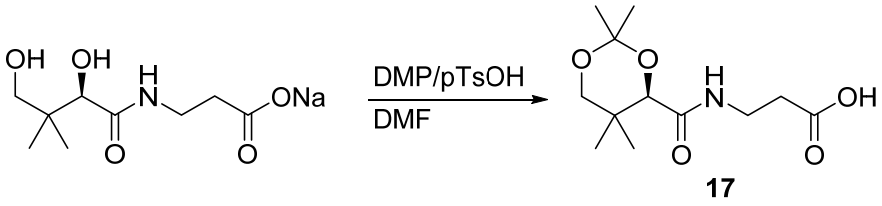


***D*-pantethine acetonide (16):**[1]
To a solution of DMF containing 380 mg (2.00 mmol) of pTsOH·H_2O and 10 grams (18.03 mmol) of *D*-pantethine syrup, 360 mL (2.9 mol) of DMP was slowly added.[1] The reaction was allowed to stir for 12 hours at room temperature and was quenched with solid sodium bicarbonate. The solvent was removed leaving a white precipitant which was suspended in DCM and filtered. The solvent from the flow through was removed yielding **16** as an oil that slowly crystallized (10 g, 15.75 mmol 87.4%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.67 (br, 2H, NH), 7.12 (br, 2H, NH), 4.00 (s, 2H), 3.62 (d, *J* = 11.6 Hz, 2H), 3.57 – 3.38 (m, 8H), 3.20 (d, *J* = 11.7 Hz, 2H), 2.76 (t, *J* = 6.9 Hz, 4H), 2.43 (t, *J* = 6.5 Hz, 4H), 1.37 (s, 6H), 1.39 (s, 6H), 0.96 (s, 6H), 0.91 (s, 6H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.38, 169.57, 98.70, 76.83, 71.00, 38.27, 37.42, 35.27, 34.72, 32.63, 29.20, 21.90, 18.70, 18.48. LRMS (ESI) *m/z* calculated for C28H50N4O8S2H ($[M+H]^+$) 635.31, found 635.3.



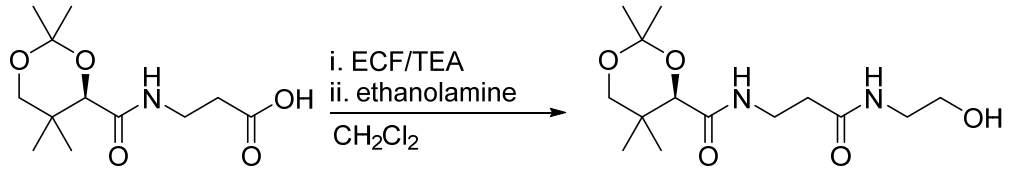
***D*-pantetheine acetonide (10):**[2]
To a solution of **16** (10 grams, 15.75 mmol) in ethanol, sodium borohydride (15.7 grams, 415 mmol) was added. The reaction was allowed to stir for 6 hours at room temperature. The reaction was carefully quenched with acetic acid to pH 5.0. The solvent was removed, re-suspended in DCM and filtered. The flow through solvent was removed and the remaining residue was

subjected to flash chromatography (0 → 100% gradient of hexanes → EtOAc) to afford **10** (transparent oil, 8.5 g, 26.69 mmol, 84.7%).
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.05 (br, 1H, NH), 6.42 br, 1H, NH), 4.08 (s, 1H), 3.67 (d, *J* = 11.8 Hz, 1H), 3.62 – 3.48 (m, 2H), 3.48 – 3.33 (m, 2H), 3.27 (d, *J* = 11.7 Hz, 1H), 2.70-2.59 (m, 2H), 2.51-2.44 (m, *J* = 6.8, 5.7, 3.8 Hz, 2H), 1.45 (s, 3H), 1.41 (s, 3H), 1.02 (s, 3H), 0.96 (s, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.24, 170.50, 99.14, 76.78, 71.39, 42.47, 36.12, 34.99, 32.99, 29.46, 24.51, 22.11, 18.92, 18.70. Spectroscopic data are consistent with previously reported data.[2-3] LRMS (ESI) *m/z* calculated for C14H26N2O4SH ($[M+H]^+$) 319.16 and ($[M-H]^-$) 317.16, found 319.2, 317.1, respectively.



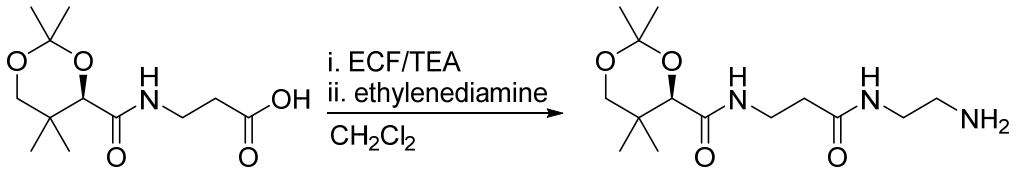
17

O,O'-isopropylidene-*D*-pantothenic acid (17):[2]
To a solution of DMF containing 3.94 g (20.7 mmol) of $pTsOH \cdot H_2O$ and 5 g (20.7 mmol) of sodium *D*-pantothenate, 200 mL (1.6 mol) of DMP was slowly added.[4] The reaction was allowed to stir for 12 hours at room temperature. The solvent was removed leaving a white precipitant that was suspended in DCM and filtered. The solvent was removed yielding **17** (crystalline oil, 5.4 g, 20.83 mmol 100%). Spectroscopic data are consistent with previously reported data.[2, 4] LRMS (ESI) *m/z* calculated for C12H21NO5 ($[M+H]^-$) 258.14, found 258.1.

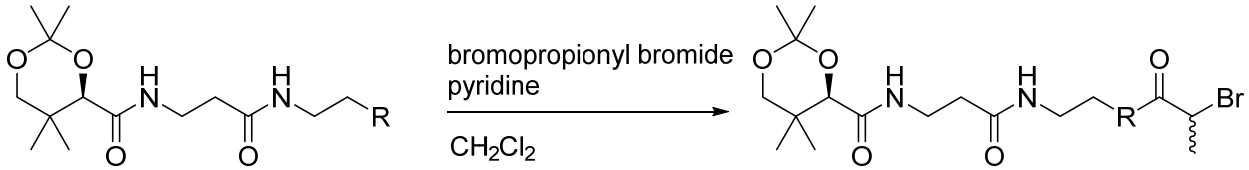


17 **11**

oxa(dethia)pantetheine acetonide (11):[4]
To a solution of DCM containing **17** (5.9 g, 22.75 mmol) and TEA (4 mL, 28.68 mmol) at 4°C, ECF (2.5 mL, 26.27 mmol) was added slowly. The reaction was allowed to stir for 15 minutes at 4°C. Then ethanolamine (2 mL, 33.14 mmol) was added to the reaction dropwise. The reaction was allowed to stir for 12 hours. The solution was transferred to a separatory funnel. The mixture was diluted with CF and then washed with brine. The organic layer was collected. The brine mixture was back extracted with CF. The organic layers were pooled and dried with anhydrous sodium sulfate. The solvent was removed yielding **11** (off white powder, 5.72g, 18.9 mmol 83.1%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.04 (br, 1H, NH), 6.54 (br, 1H, NH), 4.07 (s, 1H), 3.75 – 3.63 (m, 3H), 3.61-3.51 (m, 2H), 3.48 – 3.36 (m, 2H), 3.28 (d, *J* = 11.7 Hz, 1H), 2.48 (t, *J* = 6.3 Hz, 2H), 1.46 (s, 3H), 1.42 (s, 3H), 1.03 (s, 3H), 0.97 (s, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.94, 170.61, 99.17, 76.77, 71.39, 62.20, 42.53, 36.38, 35.00, 32.98, 29.45, 22.12, 18.87, 18.68. LRMS (ESI) *m/z* calculated for C14H26N2O5H ($[M+H]^+$) 303.19, found 303.1.



amino(dethia)pantetheine acetonide (12):[5]
To a solution of DCM containing **17** (5 g, 19.28 mmol) and TEA (6 mL, 28.68 mmol) at 4°C, ECF (4 mL, 28.68 mmol) was added slowly. The reaction was allowed to stir for 15 minutes at 4°C. Then the reaction was slowly added to a solution of DCM containing ethylenediamine (20 mL, 300 mmol). The reaction was allowed to stir for 12 hours. The solvent was removed and the remaining oil was subjected to flash chromatography (0 → 100% gradient of DCM → MeOH) affording **12** (oil, 5.2 g, 17.25 mmol 89.5%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.06 (br, 1H, NH), 6.50 (br, 1H, NH), 4.06 (s, 1H), 3.66 (d, *J* = 11.7 Hz, 1H), 3.63 – 3.43 (m, 2H), 3.30 – 3.22 (m, 3H), 2.80 (t, J = 12.7, 7.7 Hz, 2H), 2.44 (t, J = 6.3 Hz, 2H), 1.45 (s, 3H), 1.40 (s, 3H), 1.02 (s, 3H), 0.95 (s, 3H). Spectroscopic data are consistent with previously reported data.[5] **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.29, 170.22, 99.09, 76.79, 71.42, 42.07, 41.34, 36.17, 35.01, 32.96, 29.47, 22.13, 18.88, 18.69. LRMS (ESI) *m/z* calculated for C14H27N3O4H ($[M+H]^+$) 302.20, found 302.2.

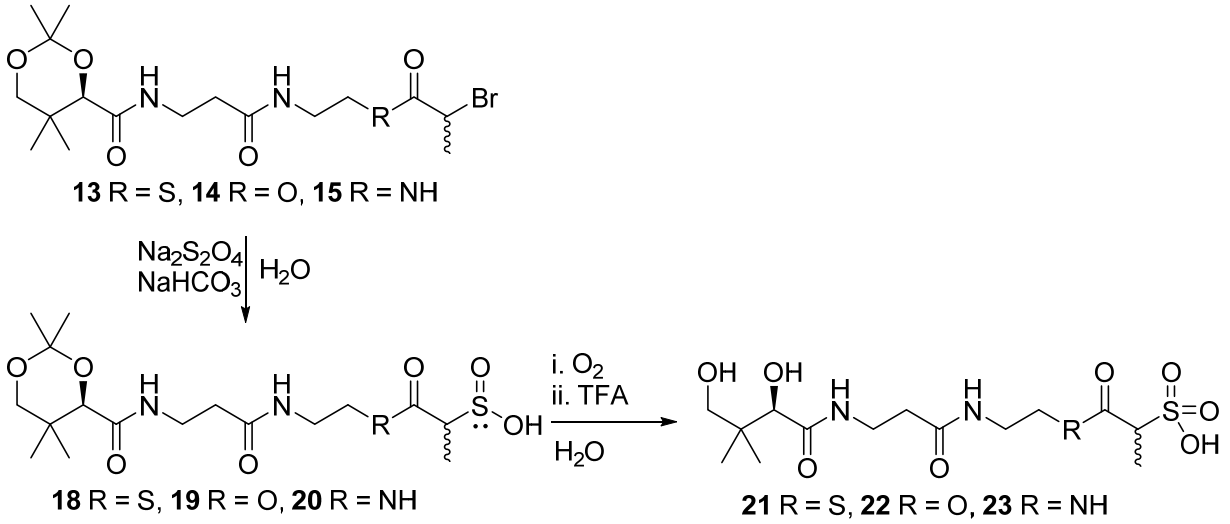


Preparation of 13-15 via 10-12:
General procedure
A solution of DCM containing **10-12** pyridine was added slowly to a solution of DCM containing bromopropionyl bromide at 4°C. The reaction was allowed to stir for 12 hours while warming to room temperature. The solution was transferred to a separatory funnel. The solution was washed with brine, copper sulfate, and sodium thiosulfate, repeatedly. The solvent was removed and the remaining red/orange oil was subjected to flash chromatography (0 → 100% gradient of DCM → acetone or MeOH) affording **13-15**.

2-bromopropionyl-*S*-pantetheine acetonide (13):
10 (9.0 g, 28.26 mmol) was reacted with pyridine (19 mL, 235.8 mmol) and bromopropionyl bromide (9.5 mL, 90.7 mmol) according to the general procedure above affording **13** (oil, 1.5 g, 3.31 mmol 11.7%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34 (br, 1H, NH), 7.14 (br, 1H, NH), 4.55 (q, *J* = 6.9 Hz, 1H), 4.08 (s, 1H), 3.69 (d, J = 11.7 Hz, 1H), 3.64-3.52 (m, 2H), 3.52 – 3.38 (m, 2H), 3.27 (d, J = 11.7 Hz, 1H), 3.08 (t, 2H), 2.49 (t, J = 15.4, 6.4 Hz, 2H), 1.84 (d, J = 6.9 Hz, 3H), 1.46 (s, 3H), 1.43 (s, 3H), 1.02 (s, 3H), 0.97 (s, 3H). **^{13}C NMR** (126 MHz, CDCl3) δ 196.20, 171.36, 169.90, 98.89, 76.97, 71.17, 47.81, 38.70, 35.45, 34.81, 32.79, 29.36, 29.10, 22.04, 21.88, 18.85, 18.63. LRMS (ESI) *m/z* calculated for C17H29BrN2O5SH ($[M+H]^+$) 453.10 and 455.10, found 453.1 and 455.1.

2-bromopropionyl-oxa(dethia)pantetheine acetonide(14):
11 (2.5 grams, 8.27 mmol) was reacted with pyridine (2 mL, 24.83 mmol) and bromopropionyl bromide (1.5 mL, 14 mmol) according to the general procedure above affording **14** (oil, 830 mg, 1.90 mmol 22.9%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 (br, 1H, NH), 7.20 (br, 1H, NH), 4.43 (q, J = 6.8 Hz, 1H), 4.25 (t, *J* = 6.2, 5.7 Hz, 6H), 4.07 (s, 1H), 3.69 (d, J = 11.7 Hz, 1H), 3.60 – 3.47 (m, 4H), 3.27 (d, J = 11.7 Hz, 1H), 2.48 (t, J = 6.5 Hz, 2H), 1.82 (d, *J* = 7.0 Hz, 3H), 1.46 (s, 3H), 1.43 (s, 3H), 1.02 (s, 3H), 0.97 (s, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 171.46, 169.99, 169.94, 98.86, 76.90, 71.08, 64.13, 39.91, 37.98, 35.47, 34.80, 32.72, 29.27, 21.97, 21.39, 18.75, 18.57. LRMS (ESI) *m/z* calculated for C17H29BrN2O6H ($[M+H]^+$) 437.12 and 439.12, found 437.1 and 439.1.

2-bromopropionyl-amino(dethia)pantetheine acetonide (15):
12 (5 g, 16.59 mmol) was reacted with pyridine (5.5 mL, 68.3 mmol) and bromopropionyl bromide (3.6 mL, 34.4 mmol) according to the general procedure above affording **15** (oil, 510 mg, 1.17 mmol 7.1%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 (br, 1H, NH), 7.32 (br, 1H, NH), 7.08 (br, 1H, NH), 4.34 (q, *J* = 6.9, 4.0 Hz, 1H), 4.00 (s, 1H), 3.60 (d, *J* = 11.7 Hz, 1H), 3.52 – 3.38 (m, 2H), 3.33 – 3.24 (m, 6H), 3.19 (d, *J* = 11.8 Hz, 2H), 2.39 (t, *J* = 6.4, 3.3 Hz, 2H), 1.74 (d, *J* = 6.9 Hz, 2H), 1.38 (s, 3H), 1.35 (s, 3H), 0.93 (s, 3H), 0.88 (s, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) δ 172.34, 170.66, 170.35, 99.08, 76.89, 71.27, 60.87, 43.95, 40.14, 35.87, 35.17, 32.89, 29.39, 22.44, 22.07, 18.87, 18.67. LRMS (ESI) *m/z* calculated for C17H30BrN3O5H ($[M+H]^+$) 436.14 and 438.14, found 436.1 and 438.1.



Preparation of 21-23 via 18-20 via 13-15:
General procedure
A solution of water containing equimolar sodium dithionite and sodium bicarbonate is allowed to stir for 5 minutes, followed by an addition of a solution of water containing 2-bromopropionyl pantetheine acetonide (**13-15**). The reaction was allowed to stir at room temperature. After stirring for 30 minutes, HPLC-MS analysis indicated complete conversion of bromides (**13-15**) to sulfinates (**18-20**). After stirring for 18 hours, sulfinates (**18-20**) oxidize to sulfonates (**21**-**23**)

as indicated by HPLC-MS analysis. The acetonides were deprotected with 15% TFA in water. The resulting solution was subjected to preparative HPLC with a 0 → 40% gradient of 0.1% TFA in water → methanol over 30 minutes. Solvent was removed affording **18**-**20**.

2-sulfiniatepropionyl-S-pantetheine acetonide (18):
13 (600 mg, 1.32 mmol) was reacted with sodium dithionite (1.22 g, 7.00 mmol) and sodium bicarbonate (588 mg, 7.00 mmol) according to the general procedure above affording **18** (off white powder which was used directly in the next reaction). MS (ESI) m/z calculated for $C_{17}H_{30}N_2O_7S_2$ ($[M-H]^-$) 437.15, found 437.1.

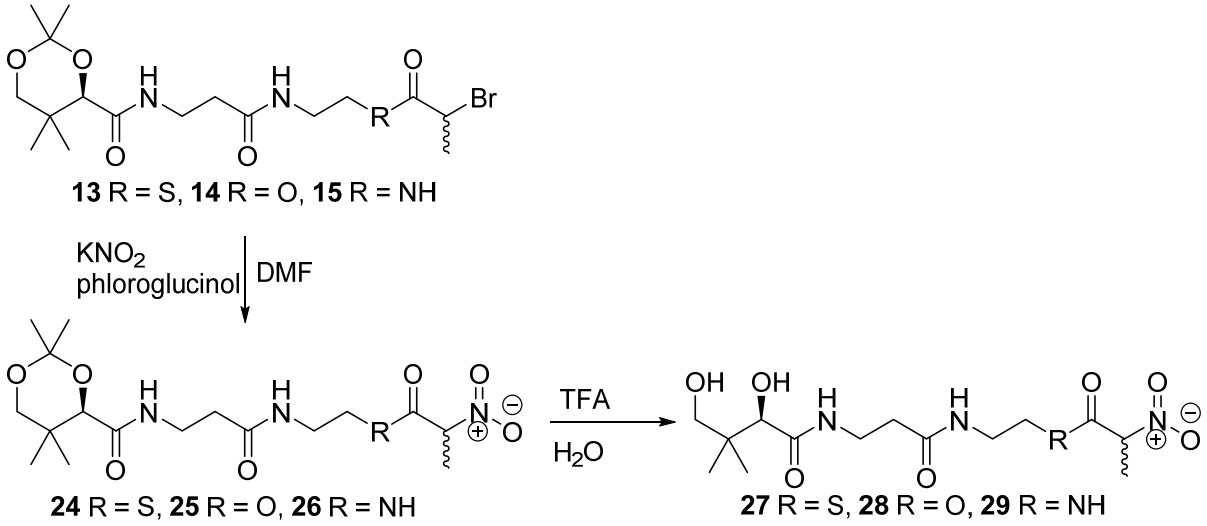
2-sulfonatepropionyl-S-pantetheine (21):
The deprotection of **18** yielded **21** (off white powder, 50 mg, 0.12 mmol 9.1%, **13** → **21**). **^{1}H NMR** (500 MHz, D_2O) δ 4.35 (s, 1H), 4.08 (m, 1H), 3.97 (d, J = 2.4 Hz, 1H), 3.49 – 3.43 (m, 3H), 3.25 (t, J = 6.7 Hz, 3H), 2.67 (t, J = 6.7 Hz, 2H), 2.51 – 2.39 (m, 3H), 1.15 (s, 3H), 0.87 (s, 3H), 0.84 (s, 3H). **^{13}C NMR** (126 MHz, D_2O) δ 180.08, 174.94, 174.00, 77.12, 75.93, 75.61, 40.72, 35.93, 35.48, 35.34, 32.05, 21.48, 20.62, 18.24. MS (ESI) m/z calculated for $C_{14}H_{26}N_2O_8S_2$ ($[M-H]^-$) 413.11, found 413.0.

2-sulfinatepropionyl-oxa(dethia)pantetheine acetonide (19):
14 (800 mg, 1.83 mmol) was reacted with sodium dithionite (1.92 g, 11.00 mmol) and sodium bicarbonate (924 mg, 11.00 mmol) according to the general procedure above affording **19** (off white powder which was used directly in the next reaction). MS (ESI) m/z calculated for $C_{17}H_{30}N_2O_8S$ ($[M-H]^-$) 421.17, found 421.1.

2-sulfonatepropionyl-oxa(dethia)pantetheine (22):
The deprotection of **19** yielded **22** (off white powder, 140 mg, 0.35 mmol 19.1%, **14** → **22**). **^{1}H NMR** (500 MHz, D_2O) 4.24 – 4.10 (m, 2H), 3.87 (s, 1H), 3.46 – 3.31 (m, 5H), 3.27 (d, *J* = 11.2 Hz, 1H), 2.39 (t, *J* = 6.5 Hz, 2H), 1.40 (d, 3H), 0.80 (s, 3H), 0.76 (s, 3H). **^{13}C NMR** (126 MHz, D_2O) δ 175.01, 174.08, 170.33, 75.76, 68.37, 64.28, 60.50, 38.53, 38.10, 35.35, 35.17, 20.44, 19.03, 12.72. MS (ESI) m/z calculated for $C_{14}H_{26}N_2O_9S$ ($[M-H]^-$) 397.17, found 397.0.

2-sulfinatepropionyl-amino(dethia)pantetheine acetonide (20):
15 (1.0 g, 2.29 mmol) was reacted with sodium dithionite (2.1 g, 12.0 mmol) and sodium bicarbonate (1.0 g, 12.0 mmol) according to the general procedure above affording **20** (off white powder which was used directly in the next reaction). MS (ESI) m/z calculated for $C_{17}H_{31}N_3O_7S$ ($[M-H]^-$) 420.19, found 420.1.

2-sulfonatepropionyl-amino(dethia)pantetheine (23):
The deprotection of **20** yielded **23** (off white powder, 800 mg, 2.01 mmol 87.8%, **15** → **23**). **^{1}H NMR** (500 MHz, D_2O) δ 8.36 (br, 1H, NH), δ 7.99 (br, 1H, NH), 3.92 (s, 1H), 3.75 (q, *J* = 7.0 Hz, 1H), 3.50 – 3.37 (m, 3H), 3.35 – 3.19 (m, 5H), 2.43 (t, *J* = 6.6 Hz, 2H), 1.41 (d, *J* = 7.0 Hz, 3H), 0.85 (s, 3H), 0.81 (s, 3H). **^{13}C NMR** (126 MHz, D_2O) δ 174.99, 174.07, 170.50, 75.81, 68.45, 61.10, 38.74, 38.69, 35.52, 35.43, 29.26, 20.68, 19.30, 13.02. MS (ESI) m/z calculated for $C_{14}H_{27}N_3O_8S$ ($[M-H]^-$) 396.15, found 396.1.



Preparation of 27-29 via 24-26 via 13-15:
General procedure
To a solution of DMF containing 2-bromopropionyl pantetheine acetonide (**13-15**), anhydrous phloroglucinol and KNO_3 were added.[6] The mixture was allowed to stir for 12 hours at room temperature, over which time a bright yellow-orange color formed. Solvent was removed yielding a yellow oil affording 2-nitropropionyl-pantetheine acetonide (**24-26**). The acetonides are deprotected with 15% TFA in water. The resulting solution is subjected to preparative HPLC with a gradient of 28 → 45% of 0.1% TFA in water → methanol or ACN over 30 minutes. Pooled fractions solvent were removed and dissolved in water. The resulting solution is subjected to preparative HPLC with a gradient of 0 → 35% of 0.1% TFA in water → methanol or ACN over 30 minutes. Pooled fractions solvent were removed and dissolved in water. The solvent is removed affording 2-nitropropionyl-pantetheine (**27-29**)

2-nitropropionyl-S-pantetheine acetonide (24):
13 (600 mg, 1.32 mmol) was reacted with anhydrous phloroglucinol (441 mg, 3.50 mmol) and KNO_3 (255 mg, 3.00 mmol) according to the general procedure above. The solvent was removed and the remaining orange oil was subjected to flash chromatography (0 → 100% gradient of DCM → acetone or MeOH). Solvent was removed affording **24** (orange oil which was used directly in the next reaction). MS (ESI) m/z calculated for $C17H29N3O7S$ ($[M+H]^+$) 420.17 and ($[M-H]^-$) 418.17, found 420.1, 418.1, respectively.

2-nitropropionyl-S-pantetheine (27):
The deprotection of **24** yielded **27** (light yellow oil, 110 mg, 0.29 mmol 22.0%, **13** → **27**).
^{1}H NMR (500 MHz, D_2O) δ 3.85 (s, 1H), 3.42 – 3.22 (m, 6H), 3.06 (t, J = 6.2 Hz, 2H), 2.33 (t, J = 6.5 Hz, 2H), 1.65 (s, 3H), 0.79 (s, 3H), 0.75 (s, 3H). **^{13}C NMR** (126 MHz, D_2O) δ 194.44, 175.03, 174.04, 75.72, 68.34, 42.24, 38.57, 38.08, 35.33, 35.14, 28.84, 20.44, 19.06, 15.52. MS (ESI) m/z calculated for $C14H25N3O7S$ ($[M+H]^+$) 380.14 and ($[M-H]^-$) 378.14, found 380.2, 378.1, respectively.

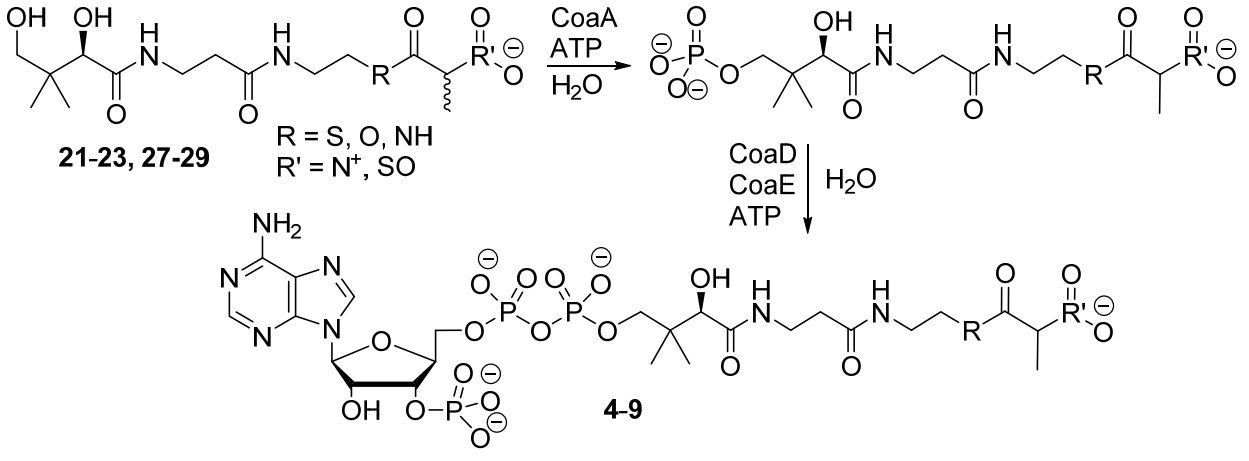
2-nitropropionyl-oxa(dethia)pantetheine acetonide (25):

14 (650 mg, 1.49 mmol) was reacted with anhydrous phloroglucinol (188 mg, 1.49 mmol) and KNO_3 (254 mg, 2.98 mmol) according to the general procedure above. The solvent was removed and the remaining orange oil was subjected to flash chromatography (0 → 100% gradient of DCM → acetone or MeOH). Solvent was removed affording **25** (orange oil which was used directly in the next reaction). MS (ESI) m/z calculated for C17H29N3O8 ($[M+H]^+$) 404.20 ($[M-H]^-$) 402.20, found 404.2, 402.1, respectively.

2-nitropropionyl-oxa(dethia)pantetheine (28):
The deprotection of **25** yielded **28** (light yellow oil, 80 mg, 0.22 mmol 14.8%, **14** → **28**).
^{1}H NMR (500 MHz, D_2O) δ 4.30 – 4.13 (m, 2H), 3.86 (s, 1H), 3.38 (m, 5H), 3.26 (d, 1H) 3.08 (q, J = 7.3 Hz, 1H), 2.38 (t, J = 7.0, 5.8, 3.0 Hz, 2H), 1.16 (t, J = 7.3 Hz, 3H), 0.79 (s, 3H), 0.76 (s, 3H). **^{13}C NMR** (126 MHz, D_2O) δ 175.06, 174.12, 167.07, 83.58, 75.70, 68.33, 65.40, 46.63, 37.87, 35.37, 35.15, 20.42, 19.01, 8.18. MS (ESI) m/z calculated for C14H25N3O8 ($[M+H]^+$) 364.16 and ($[M-H]^-$) 362.16, found 364.2 and 362.1, respectively.

2-nitropropionyl-amino(dethia)pantetheine acetonide (26):
15 (510 mg, 1.17 mmol) was reacted with anhydrous phloroglucinol (151 mg, 1.20 mmol) and KNO_3 (230 mg, 2.70 mmol) according to the general procedure above. The solvent was removed and the remaining orange oil was subjected to flash chromatography (0 → 100% gradient of DCM → acetone or MeOH). Solvent was removed affording **26** (orange oil which was used directly in the next reaction). MS (ESI) m/z calculated for C17H30N4O7 ($[M+H]^+$) 403.21 and ($[M-H]^-$) 401.21, found 403.2, 401.1, respectively.

2-nitropropionyl-amino(dethia)pantetheine (29):
The deprotection of **26** yielded **29** (light yellow oil, 18.7 mg, 0.05 mmol 4.3%, **15** → **29**).
^{1}H NMR (500 MHz, D_2O) δ 5.28 (q, J = 6.9, 1.0 Hz, 1H), 3.86 (s, 1H), 3.42 – 3.34 (m, 3H), 3.33 – 3.24 (m, 5H), 2.36 (t, J = 6.6 Hz, 2H), 1.59 (d, J = 6.9, 1.0 Hz, 3H), 0.80 (s, 3H), 0.76 (s, 3H).
^{13}C NMR (126 MHz, D_2O) δ 175.08, 174.15, 167.60, 84.22, 75.74, 68.31, 48.82, 39.02, 38.55, 38.32, 35.26, 20.42, 19.02, 15.04. MS (ESI) m/z calculated for C14H26N4O7 ($[M+H]^+$) 363.18 and ($[M-H]^-$) 361.18, found 363.1, 361.0, respectively.



Chemoenzymatic preparation of 4-9:

General procedure[7]
A solution containing 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 10 mM TCEP (pH 8.0) and 20 μM ATP was used to dissolve the malonyl-pantethine analogs **21-23** or **27-29** at a final concentration of 5.5 mM, ~60-150 mL total. Then CoaA was added to a final concentration of 2.7 μM and the reaction allowed to mix at room temperature for 2 hours. Then CoaD was added to a concentration of 5.6 μM and allowed to mix at room temperature for 1 hour. Then CoaE was added to a final concentration of 13.1 μM and allowed to mix at room temperature overnight. The reaction was quenched with 10% TFA, precipitating the protein out of solution, which was removed by filtration. Reverse phase HPLC was used to purify the final products using a 0 → 20% gradient of 0.1% TFA in water → methanol or ACN over 30 minutes. Fractions were pooled, rotary evaporated and lyophilized.

2-sulfonatepropionyl-S-CoA (4):
21 (50 mg, 0.12 mmol) was used as starting material to afford **4** (off white powder, 5 mg, 0.01 mmol 8.3%). **^{1}H NMR** (500 MHz, D_2O) δ 8.49 (s, 1H), 8.33 (s, 1H), 6.12 (d, J = 5.2 Hz, 1H), 4.83 – 4.74 (m, 2H), 4.47 (s, 1H), 4.13 – 4.03 (m, 2H), 3.90 (s, 1H), 3.74 (dd, J = 9.7 Hz, 1H), 3.55 (dd, J = 9.7 Hz, 1H), 3.40 – 3.35 (m, 2H), 3.26 (m, 2H), 3.01 – 2.93 (m, 2H), 2.35 (t, J = 6.5, 3.1 Hz, 2H), 1.41 (d, J = 7.0 Hz, 3H), 0.91 – 0.69 (m, 6H). **^{13}C NMR** (126 MHz, D_2O) δ 197.55, 174.76, 174.05, 149.86, 148.39, 144.56, 142.39, 118.60, 87.80, 74.34, 73.77, 73.72, 71.64, 66.95, 64.29, 38.37, 38.30, 38.24, 35.37, 35.16, 28.48, 20.53, 18.59, 13.38. MS (ESI) m/z calculated for $C_{24}H_{40}N_7O_{20}P_3S_2$ ($[M-H]^-$) 902.10, found 902.2.

2-sulfonatepropionyl-oxa(dethia)CoA (5):
22 (100 mg, 0.25 mmol) was used as starting material to afford **5** (off white powder, 16.7 mg, 0.02 mmol 8.0%). **^{1}H NMR** (500 MHz, D_2O) δ 8.53 (s, 1H), 8.33 (s, 1H), 6.11 (d, J = 5.5, 1.9 Hz, 1H), 4.86 – 4.75 (m, 2H), 4.50 (s, 1H), 4.22 – 4.07 (m, 4H), 3.89 (s, 1H), 3.87 – 3.81 (m, 1H), 3.80 – 3.75 (m, 1H), 3.55 – 3.51 (m, 1H), 3.41 – 3.30 (m, 4H), 2.36 (t, J = 7.0, 3.4 Hz, 2H), 1.38 (dd, J = 7.0, 4.9, 1.6 Hz, 3H), 0.91 – 0.68 (m, 6H). **^{13}C NMR** (126 MHz, D_2O) δ 174.67, 174.05, 170.19, 149.84, 148.47, 144.62, 142.49, 118.58, 87.52, 83.19, 74.33, 74.06, 73.70, 72.21, 64.19, 60.49, 52.57, 38.27, 38.08, 35.35, 35.18, 20.72, 18.07, 12.68. MS (ESI) m/z calculated for $C_{24}H_{40}N_7O_{21}P_3S$ ($[M-H]^-$) 886.12, found 886.0.

2-sulfonatepropionyl-amino(dethia)CoA (6):
23 (600 mg, 1.51 mmol) was used as starting material to afford **6** (off white powder, 56.9 mg, 0.06 mmol 4.0%). **^{1}H NMR** (500 MHz, D_2O) δ 8.49 (s, 1H), 8.30 (s, 1H), 6.06 (d, J = 5.8 Hz, 1H), 4.83 – 4.78 (m, 2H), 4.47 (s, 1H), 4.22 – 4.08 (m, 2H), 3.87 (s, 1H), 3.75 (m, 1H), 3.64 (m, 1H), 3.59 – 3.50 (m, 2H), 3.33 (m, 4H), 2.37 – 2.28 (m, 2H), 1.32 (d, J = 6.7, 2.8 Hz, 3H), 0.81 (s, 3H), 0.70 (s, 3H). **^{13}C NMR** (126 MHz, D_2O) δ 174.65, 174.08, 170.39, 149.74, 148.37, 144.67, 142.39, 118.43, 87.46, 83.15, 74.16, 73.69, 72.18, 65.12, 61.04, 38.60, 38.54, 38.29, 35.40, 35.30, 35.26, 20.68, 18.16, 12.70. MS (ESI) m/z calculated for $C_{24}H_{41}N_8O_{20}P_3S$ ($[M-H]^-$) 885.14, found 885.0.

2-nitropropionyl-S-CoA (7):
27 (110 mg, 0.29 mmol) was used as starting material to afford **7** (off white powder, 63.6 mg, 0.07 mmol 24.1%). **^{1}H NMR** (500 MHz, D_2O) δ 8.48 (s, 1H), 8.30 (s, 1H), 6.06 (d, J = 5.9, 1.2 Hz, 1H), 4.81 (t, J = 5.1, 2.6 Hz, 1H), 4.75 (t, J = 5.7 Hz, 1H), 4.47 (s, 1H), 4.17 (q, J = 11.7, 5.9

Hz, 2H), 3.88 (s, 1H), 3.77 (d, J = 9.7, 4.3 Hz, 1H), 3.54 (d, J = 9.7 Hz 1H), 3.32 (t, J = 6.7 Hz, 2H), 3.26 (t, 2H), 3.01 (t, J = 6.3 Hz, 2H), 2.30 (t, J = 6.6 Hz, 2H), 1.60 (d, J = 1.2 Hz, 3H), 0.81 (s, 3H), 0.70 (s, 3H). **^{13}C NMR** (126 MHz, D_2O) δ 194.37, 174.61, 174.02, 149.74, 148.37, 144.68, 142.37, 118.42, 87.47, 83.08, 74.43, 74.14, 73.67, 72.23, 65.13, 38.37, 38.31, 38.02, 35.33, 35.17, 28.82, 20.68, 18.29, 15.51. MS (ESI) m/z calculated for C24H39N8O19P3S ($[M+H]^+$) 869.13 and ($[M-H]^-$) 867.13, found 869.1, 867.0, respectively.

2-nitropropionyl-oxa(dethia)CoA (8):
28 (80 mg, 0.22 mmol) was used as starting material to afford **8** (off white powder, 46 mg, 0.05 mmol 22.7%). **^{1}H NMR** (500 MHz, DMSO) δ 8.59 (s, 1H), 8.33 (s, 1H), 8.13 (br, 1H, NH), 7.74 (br, 1H, NH), 5.97 - 5.83 (m, 1H), 5.65 – 5.51 (m, 1H), 4.37 (s, 1H), 3.86 – 3.76 (m, 1H), 3.72 (s, 1H), 3.59 – 5.46 (m, 1H), 3.35 – 3.28 (m, 4H), 3.24 – 3.15 (m, 1H), 2.54 – 2.43 (m, 2H), 2.32 – 2.18 (m, 2H), 1.61 (d, J = 7.0 Hz, 3H), 0.86 (s, 3H), 0.70 (s, 3H). **^{13}C NMR** (126 MHz, DMSO) δ 173.14, 172.06, 166.27, 150.90, 148.76, 146.28, 141.84, 118.74, 87.51, 83.64, 74.59, 73.89, 73.46, 72.46, 65.38, 39.50, 39.33, 39.17, 37.70, 35.45, 35.23, 21.44, 19.28, 15.64. MS (ESI) m/z calculated for C24H39N8O20P3 ($[M+H]^+$) 853.15 and ($[M-H]^-$) 851.15, found 853.1, 851.1, respectively.

2-nitropropionyl-amino(dethia)CoA (9):
29 (18.7 mg, 0.05 mmol) was used as starting material to afford **9** (off white powder, 23.1 mg, 0.03 mmol 60%). **^{1}H NMR** (500 MHz, D_2O) δ 8.51 (s, 1H), 8.31 (s, 1H), 6.13 – 6.04 (m, 1H), 5.32 – 5.20 (m, 1H), 4.50 (s, 1H), 4.23 – 4.11 (m, 2H), 3.90 (s, 1H), 3.77 (d, J = 9.5, 1H), 3.54 (d, J = 9.5, 1H), 3.35 (t, J = 6.7 Hz, 2H), 3.28 – 3.17 (m, 4H), 2.34 (t, J = 6.7 Hz, 2H), 1.56 (d, J = 6.8 Hz, 3H), 0.83 (s, 3H), 0.72 (s, 3H). **^{13}C NMR** (126 MHz, D_2O) δ 174.66, 174.16, 167.57, 149.77, 148.39, 144.67, 142.40, 118.44, 87.47, 84.19, 83.22, 74.20, 73.76, 72.10, 65.10, 39.03, 38.35, 38.29, 38.26, 35.38, 35.31, 20.68, 18.31, 15.05. MS (ESI) m/z calculated for C24H40N9O19P3 ($[M+H]^+$) 852.17 and ($[M-H]^-$) 850.17, found 852.7 and 852.6, respectively.

IV. Extinction Coefficient Determination
The extinction coefficients of **4**-**6** were assumed to be the same as acetyl-CoA (15.4 $mM^{-1}cm^{-1}$ at A_{259})[8], as there was no appreciable absorption at 259 nm for the intermediates **18**-**23**. The nitro bearing analog intermediates **24**-**29** have an absorption with a peak at 318 and 326 nm with some absorption at 259 nm due to the tail of the 318 peak in 10 mM potassium phosphate buffer (KH_2PO_4/K_2HPO_4) at pH 6.5. Calculation of an extinction coefficeient was performed at 259 nm for **27** and use to adjust the extinction coefficient of **7**-**9**. Compound **27** was measured to 5.0379 mg and dissolved in 1 mL of water (100mM) buffered to pH 7.0 with Tris:HCl. Measurement of the UV spectrum for a serial dilution of **27** gave an extinction coefficient at 259 nm of 0.997±0.022 $mM^{-1}cm^{-1}$. Addition of the adenine and nitro extinction coefficients yields overall extinction coefficients at 259 nm for **7**, **8** and **9** of 16.4 $mM^{-1}cm^{-1}$.

V. Cloning of *coaA*, *coaD*, *coaE* and *ygfG* from *Escherichia coli*
The pBS3080 expression plasmid was used to generate gene fustions with N-terminal hexahistidine tags (His-tag) and Tobacco etch virus protease (TEV) sites as previously described.[9] Briefly, pBS3080 was digested with BsmFI and purified by gel electrophoresis and the linearized vector was treated with T4 DNA polymerase in the presence of dGTP at 20 °C for 30 min and then heated at 75 °C for 20 min to denature the polymerase, affording overhangs

with complementary sequences to clone the PCR-amplified genes. The *coaA/D/E* and *ygfG* genes were amplified by PCR from *E. coli* DH5α genomic DNA using the following primers:
coaA-forward 5′-AAAACCTCTATTTCCAG TCGATAAAAGAGCAAACGTTAATGAC-3′,
coaA-reverse 5′-TACTTACTTAAATG TTATTTGCGTAGTCTGACCTCTTC-3′,
coaD-forward 5′-AAAACCTCTATTTCCAG TCGCAAAAACGGGCGATTTATCCGG-3′,
coaD-reverse 5′-TACTTACTTAAATG TTACGCTAACTTCGCCATCAGC-3′,
coaE-forward 5′-AAAACCTCTATTTCCAG TCGAGGTATATAGTTGCCTTAACGGG-3′,
coaE-reverse 5′-TACTTACTTAAATG TTACGGTTTTTCCTGTGAGACAAAC-3′,
ygfG-forward 5′-AAAACCTCTATTTCCAG TCGTATCAGTATGTTAACGTTGTC-3′,
ygfG-reverse 5′- TACTTACTTAAATG TTAATGACCAACGAAATTAGGTTTAC-3′.
The PCR products were purified by gel electrophoresis, and similarly treated with T4 DNA polymerase in the presence of dCTP at 20 °C for 30 min and then heated at 75 °C. The T4 DNA polymerase-treated pBS3080 vector and gene fragments were then mixed at room temperature, annealed on ice for 5 min, and transformed into *E. coli* DH5α. Plasmids containing the appropriate genes were isolated and confirmed by DNA sequencing, yielding expression plasmids for CoaA (pJLHis6T-ecCoaA), CoaD (pJLHis6T-ecCoaD), CoaE (pJLHis6T-ecCoaE) and MMCD-His6 (pJLHis6T-ecYgfG).

Due to incomplete his-tag removal from expressed MMCD in the above construct, we subcloned *ygfG* as follows. The pBS3080 plasmid was digested with NcoI and XhoI enzymes and purified by gel electrophoresis. The *ygfG* gene was amplified by PCR from the His-tagged version with the following primers (start/stop codons are bold, NcoI and XhoI cut sites are underlined):
ygfG-his6-forward 5′-GTACCC**ATGG**CGTATCAGTATGTTAACGTTGTC-3′,
ygfG-his6-reverse 5′-ATCGGAGCTCA **TTA**ATGACCAACGAAATTAGGTTTAC-3′.
The PCR product was treated with NcoI and XhoI and purified by gel electrophoresis. The purified product was then ligated into the respectively cut pBS3080 with T4 DNA ligase and transformed into *E. coli* DH5α. Plasmids containing the appropriate genes were isolated and confirmed by DNA sequencing, yielding an expression plasmid for MMCD (pJL-ecYgfG(S2A)) that generates a protein with a Ser2→Ala mutation.

VI. Expression and Purification of CoaA, CoaD, CoaE and YgfG (MMCD)

CoaA (pJLHis6T-ecCoaA), CoaD (pJLHis6T-ecCoaD), CoaE (pJLHis6T-ecCoaE) and MMCD (pJLHis6T-ecYgfG) were transformed into *E. coli* BL21 (DE3), and the resultant recombinant strains were grown overnight in 50 mL of LB and 50 μg/mL kanamycin. A 5 mL aliquot of the overnight culture was used to inoculate 1 L of LB containing 10 mM $MgCl_2$ and 50 μg/mL kanamycin, which was incubated at 37 ˚C while being shaken at 180 rpm. Once the OD_{600} reached ~0.5-0.6, the temperature was reduced to 18 ˚C. Once the cultures reached thermal equilibrium, gene expression was induced by the addition of isopropyl β-d-thiogalactopyranoside with a final concentration of 500 μg/mL, with incubation for an additional 16 hours. *E. coli* cells were harvested by centrifugation at 6300 rpm and 4 ˚C for 30 min.

E. coli cell pellets, carrying CoaA, CoaD, CoaE and MMCD were re-suspended in lysis buffer [1 μg/mL DNase, 300 mM NaCl, 20 mM imidazole, 10% glycerol, and 20 mM Tris-HCl (pH 8.0)], sonicated (60 × 1 s on ice), and clarified by centrifugation at 11000 rpm and 4 ˚C for 30 min. The supernatant was filtered applied to a 5 mL HisTrap HP (GE Healthcare,) and washed with lysis buffer using an Äkta pure fast-performance liquid chromatography system (GE Healthcare,).

Wash buffer [300 mM NaCl, 40 mM imidazole and 20 mM Tris-HCl (pH 8.0)] was used to remove additional contaminants, and proteins were eluted with wash buffer containing 500 mM imidazole. At this point the purity of CoaA, CoaD and CoaE from the fractions was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Pure fractions were pooled, concentrated, buffer-exchanged into 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl, frozen in small aliquots with liquid nitrogen, and stored at −80 °C. MMCD-His6 was buffer-exchanged into 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl and further purified using size exclusion chromatography HiLoad 26/600 Superdex 200pg (GE Healthcare) in the same buffer. The purity of the protein from the fractions was analyzed by SDS–PAGE. Pure fractions were pooled, concentrated via filtration and final concentration determined using calculated extinction coefficients at 280 nm. The proteins were frozen in small aliquots with liquid nitrogen, and stored at −80 °C.

MMCD(S2A) expressed from pJL-ecYgfG(S2A) was subjected to similar cell lysis, sonication, and clarity by centrifugation as above. The supernatant was slowly mixed with 15% w/v ammonium sulfate for 30 minutes at 4 ˚C. The sample was clarified by centrifugation at 11000 rpm and 4 ˚C for 30 min. The supernatant was slowly mixed with an additional 20% w/v (35% w/v total) ammonium sulfate for 30 minutes at 4 ˚C. The sample was clarified by centrifugation at 11000 rpm and 4 ˚C for 30 min. The supernatant was filtered, buffer-exchanged into 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl and loaded onto a 5 mL HiTrap Q HP (GE Healthcare,). A linear gradient over 12 column volumes from 0 to 70% buffer B [50 mM Tris-HCl (pH 8.5) and 1.0 M NaCl] was used to elute the proteins. The pooled fractions were buffer-exchanged into 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl and further purified using size exclusion chromatography HiLoad 26/600 Superdex 200pg (GE Healthcare). The purity of the protein from the fractions was analyzed by SDS–PAGE. Pure fractions were pooled, concentrated via filtration and final concentration determined using calculated extinction coefficients at 280 nm. The protein was frozen in small aliquots with liquid nitrogen, and stored at −80 °C.

VII. Crystallization, X-ray Crystallographic Collection and Refinement of MMCD

MMCD(S2A) was screened against 384 crystallization conditions in 500 nL sitting drops at 20 ˚C, set up with a Mosquito (TTPlabtech, Melbourne, Australia) to find initial conditions. MMCD(S2A) [23 mg/mL in 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl], 10 mM **4-9** and 10 μM $NiSO_4(H_2O)_6$ (Ni omitted in solution with compound **5**) were screened by the hanging drop method over 1.0 mL wells containing 0-5% PEG 400, 50-200 mM NaCl, 0.4 M NaH_2PO_4/1.6 M K_2HPO_4, and 0.1 M imidazole (pH 8.0) in 4 μL drops (1:1, protein:well and 3:1, protein:well), which produced crystals in conditions with less than or equal to 4% PEG 400.

Crystals were looped and frozen directly out of the drops with liquid nitrogen. X-ray diffraction data for all datasets were collected at Advanced Photon Source LS-CAT beamline 21-ID-G (PDB: 6N92, 6N93, and 6N94) at a wavelength of 0.97856, beamline 21-ID-F (PDB: 6N95 and 6N97) at a wavelength 0.97872, and beamline 21-ID-D (PDB: 6N96) at a wavelength 0.97849. Diffraction intensities were integrated, reduced, and scaled using HKL2000,[10] with data collection and refinement statistics listed in Table 1. Molecular replacement with the program Phaser was used to phase our initial structure of MMCD with **7** (PDB: 6N92) based off PDB 1EF8 coordinates. The remaining structures of MMCD with **4-6** (PDB: 6N95, 6N96, and 6N97) and **8-9** (PDB: 6N93 and 6N94) were solved by isomorphous replacement from **7** (PDB: 6N92).

Refinement was conducted using Refmac[11] in the CCP4i package[12] with automated model building performed with ARP/wARP[13] and manual model building with Coot.[14]

VIII. MMCD Enzymatic Assays, pH rate profile and K_i determination

General procedure for MMCD-His6 catalyzed activity assays. The reactions were performed in a 550 µL reaction mixture which contained 50 mM potassium phosphate buffer (KH_2PO_4/K_2HPO_4) at pH 6.5 unless otherwise noted, 10 mM $MgCl_2$, methylmalonyl-CoA and the assay was initiated by the addition of MMCD. Reaction mixtures were incubated at 25 ˚C, 65 µL aliquots taken at times noted below were quenched with 25 µL of 50% TFA v/v, precipitating the protein. Centrifugation at 2000 rpm at 25 ˚C for 10 minutes was used to pellet the protein and the supernatant was analyzed via the procedure outlined below.

General procedure for determination of methylmalonyl-CoA, propionyl-CoA and CoA concentrations in MMCD catalyzed assays. Substrate and product concentrations were determined using HPLC with detection at A_{254} over the 250 x 4.6 mm C18(2) column. The analytes were separated with a 2 → 25% gradient of 0.1% TFA in water → ACN over 20 min. Peak areas of substrate and products were converted to concentration by summing their areas and dividing each peak by this total to give relative percentages that were converted to concentration by adjusting to the starting concentration of methylmalonyl-CoA. This procedure gave essentially the same values as using a standard curve to generate concentrations for each peak, but enhanced reproducibility due to small differences in recovery from the reaction quenching step outlined above.

The pH rate profile was performed according to the general procedure above with 50 mM potassium phosphate buffer (KH_2PO_4/K_2HPO_4) at pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5, 500 µM methylmalonyl-CoA, and reactions were initiated by the addition of 5 nM MMCD. Aliquots were taken at the times 30s, 50s, 70s, 100s, 130s, 190s, 310s, and 910s.

Inhibition assays of MMCD by **4**-**9** were performed according to the general procedure above with 200 µM methylmalonyl-CoA, **4**-**9** (0 µM, 2 µM, 5 µM, 20 µM, and 50 µM) and the assay was initiated by the addition of 1 nM MMCD. Aliquots were taken at the times 20s, 40s, 60s, 150s, 300s, 600s, 900s, and 1800s.

The initial rates (V_i) of decomposition of methylmalonyl-CoA were determined by fitting the progress curve data to equation (1) which describes a simple exponential decay with rate (k), time (t) and initial substrate concentration ($[S]_t$), see Figure S5 for a representative fit. The rate of formation of propionyl-CoA or CoA could be fit to equation (2). The derivative at time 0 for Equation (1) divided by enzyme concentration gives the initial rates V_i as shown in equation (3). Using this method rather than linear estimation from the early data points gave similar values. However, it allowed more accurate determination of V_i at early time points.

Equation (1) $$[S] = [S]_t \cdot e^{-k \cdot t}$$

Equation (2) $$[S] = [S]_t \cdot (1 - e^{k \cdot t})$$

Equation (3) $$V_i = ([S]_t \cdot k)/[E]$$

Initial rate data for the pH rate profile was fit to equation (4) to determine the pK_a of a single titratable group for the decomposition of methylmalonyl-CoA and appearance of propionyl-CoA. Equation (5) describes two titratable groups and was used to fit appearance of CoA.

$$\text{Equation (4)} \quad V_i = \frac{V_{max}}{1+10^{pH-pK_a}}$$

$$\text{Equation (5)} \quad V_i = \frac{V_{max}}{(1+10^{pK_{a1}-pH}+10^{pH-pK_{a2}})}$$

Inhibition of MMCD by **4**-**9** was determined by fitting initial rate data to equation (6), which describes competitive inhibition and V_{ii} describes the V_i in the presence of inhibitor.[15] The values of $(V_{ii} \cdot V_{is})/(V_{ii} - V_{is})$ and K_m/k_{cat} were determined from the initial slopes of V_i or V_{ii} versus substrate concentration rather than from the full assay, see Figures S5 and S9. A minimum of two concentrations were used, 20 or 50 μM of each **4**-**9** and the experiments were repeated at least twice.

$$\text{Equation (6)} \quad K_i = \frac{V_{ii} \cdot V_{is}}{V_{ii} - V_{is}} \cdot \frac{[I]}{[E] \cdot [S]} \cdot \frac{K_m}{k_{cat}}$$

SI References

1. Ma, M.; Lohman, J. R.; Liu, T.; Shen, B., C-S bond cleavage by a polyketide synthase domain. *Proc. Natl. Acad. Sci. U.S.A.* **2015,** *112* (33), 10359-64.
2. Crawford, J. M.; Dancy, B. C.; Hill, E. A.; Udwary, D. W.; Townsend, C. A., Identification of a starter unit acyl-carrier protein transacylase domain in an iterative type I polyketide synthase. *Proc. Natl. Acad. Sci. U.S.A.* **2006,** *103* (45), 16728-33.
3. Agarwal, V.; Diethelm, S.; Ray, L.; Garg, N.; Awakawa, T.; Dorrestein, P. C.; Moore, B. S., Chemoenzymatic Synthesis of Acyl Coenzyme A Substrates Enables in Situ Labeling of Small Molecules and Proteins. *Org. Lett.* **2015,** *17* (18), 4452-5.
4. Tosin, M.; Spiteller, D.; Spencer, J. B., Malonyl carba(dethia)- and malonyl oxa(dethia)-coenzyme A as tools for trapping polyketide intermediates. *Chembiochem* **2009,** *10* (10), 1714-23.
5. Li, H. J.; Li, X.; Liu, N.; Zhang, H.; Truglio, J. J.; Mishra, S.; Kisker, C.; Garcia-Diaz, M.; Tonge, P. J., Mechanism of the intramolecular Claisen condensation reaction catalyzed by MenB, a crotonase superfamily member. *Biochemistry* **2011,** *50* (44), 9532-44.
6. Kornblum, N.; Blackwood, R. K.; Powers, J. W., A New Synthesis of α-Nitroesters. *J. Am. Chem. Soc.* **1957,** *79* (10), 2507-2509.
7. Strauss, E.; Begley, T. P., The antibiotic activity of N-pentylpantothenamide results from its conversion to ethyldethia-coenzyme a, a coenzyme a antimetabolite. *J. Biol. Chem.* **2002,** *277* (50), 48205-9.
8. The National Academies Press. *Specifications and Criteria for Biochemical Compounds,* Third ed.: Washington, DC, 1972.

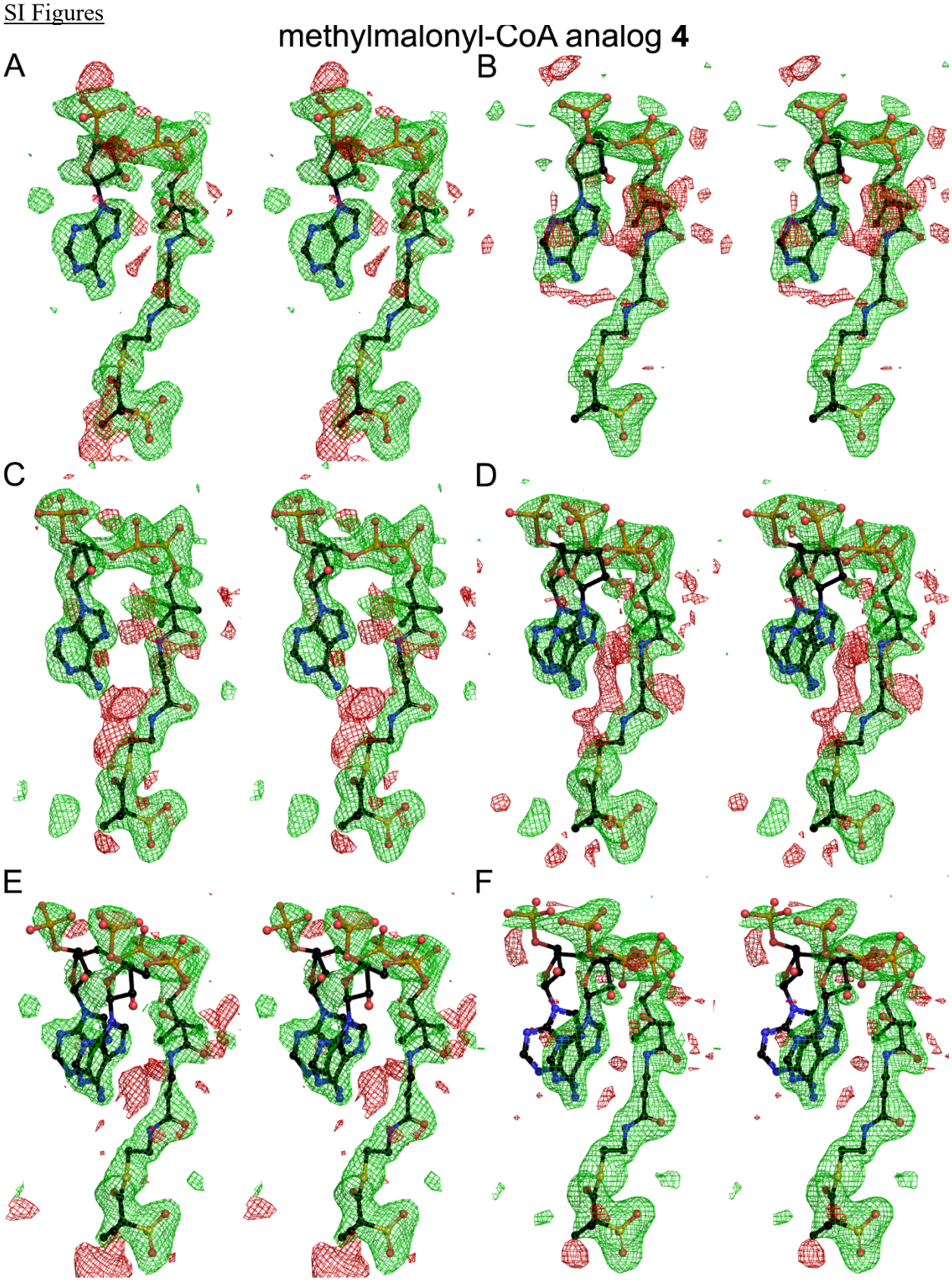
9. Lohman, J. R.; Bingman, C. A.; Phillips, G. N., Jr.; Shen, B., Structure of the bifunctional acyltransferase/decarboxylase LnmK from the leinamycin biosynthetic pathway revealing novel activity for a double-hot-dog fold. *Biochemistry* **2013,** *52* (5), 902-11.
10. Otwinowski, Z.; Minor, W., Processing of X-ray diffraction data collected in oscillation mode. *Meth. Enzymol. Macromolecular Crystallography, Pt A* **1997,** *276*, 307-326.
11. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J., Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D* **1997,** *53* (Pt 3), 240-55.
12. Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S., Overview of the CCP4 suite and current developments. *Acta Crystallogr. D* **2011,** *67* (Pt 4), 235-42.
13. Langer, G.; Cohen, S. X.; Lamzin, V. S.; Perrakis, A., Automated macromolecular model building for X-ray crystallography using ARP/wARP version 7. *Nat. Protoc.* **2008,** *3* (7), 1171-9.
14. Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K., Features and development of Coot. *Acta Crystallogr. D* **2010,** *66* (Pt 4), 486-501.
15. Lu, J.; Dong, Y.; Ng, E. C.; Siehl, D. L., Novel form of the Michaelis-Menten equation that enables accurate estimation of (kcat/KM)*KI with just two rate measurements; utility in directed evolution. *Protein Eng. Des. Sel.* **2017,** *30* (5), 395-399.

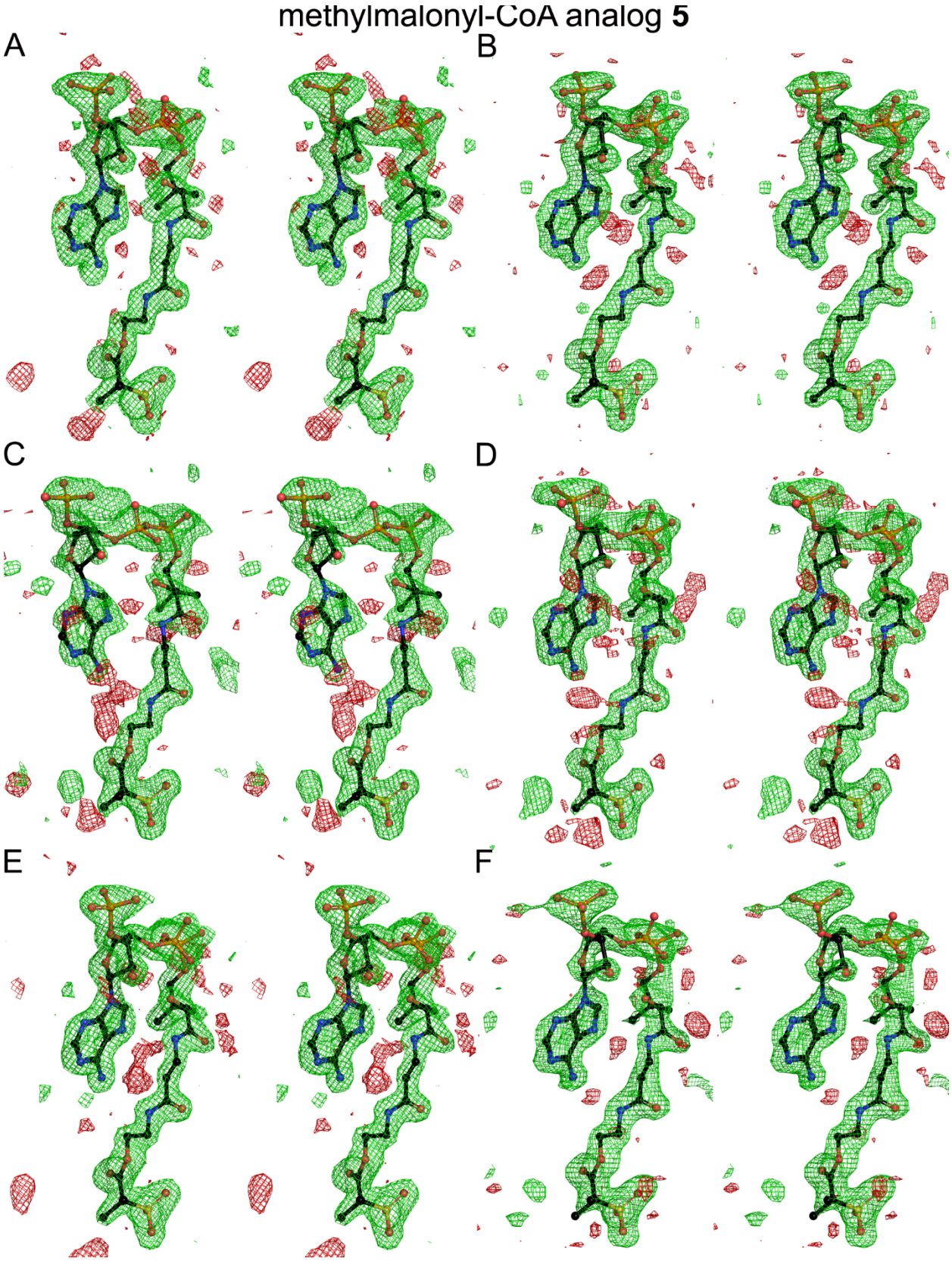
SI Tables

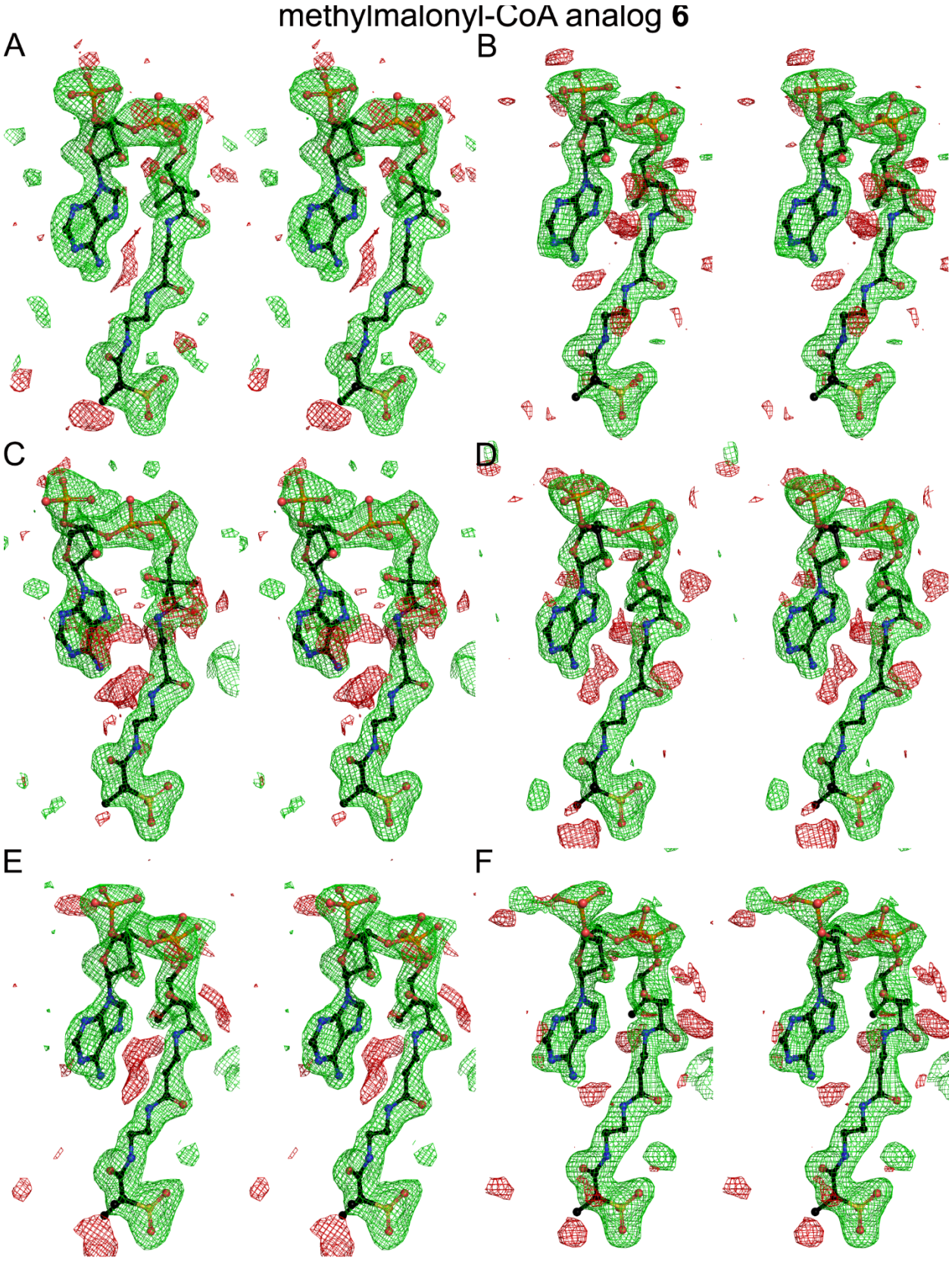
Table S1. Statistics of Data Collection, Processing and Refinement

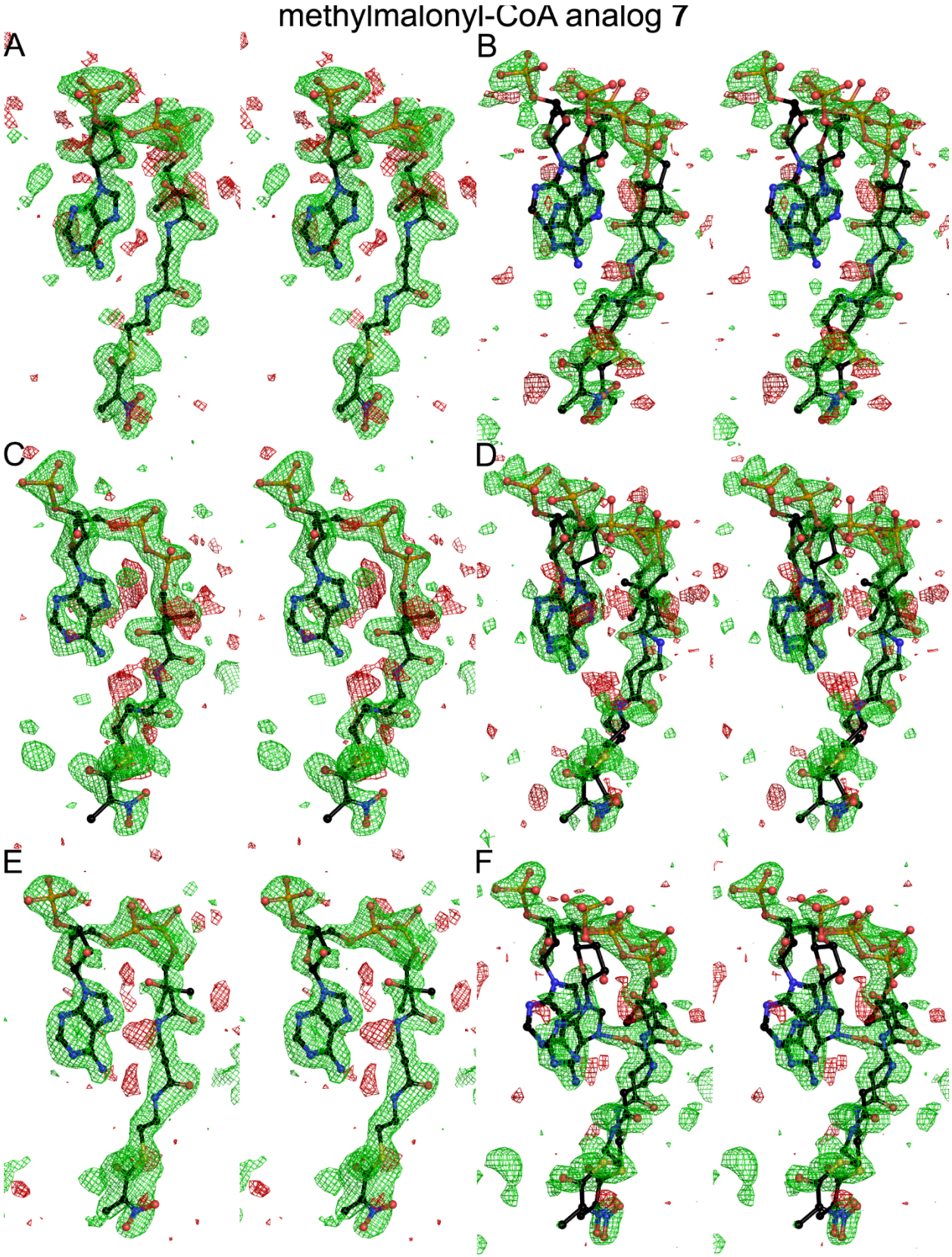
MMCD +	**4**	**5**	**6**	**7**	**8**	**9**
Data collection						
Wavelength	0.97872	0.97849	0.97872	0.97856	0.97856	0.97856
Total reflections	1311297	1174981	1242089	1473722	1256192	1422149
Unique reflections	178862	191624	189024	213244	203268	194971
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions						
a, *b*, *c* (Å)	86.93, 114.42, 193.43	87.15, 114.66, 192.70	86.96, 114.61, 192.34	87.01, 114.32, 194.35	87.18, 114.65, 194.55	87.05, 114.82, 193.73
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	30.00-1.80	30.00- 1.70	30.00- 1.75	30.00-1.70	30.00-1.70	30.00- 1.75
R_{merge}	0.165 (1.395)	0.105 (0.840)	0.093 (1.018)	0.117 (0.813)	0.110 (1.192)	0.125 (1.489)
R_{meas}	0.177 (1.503)	0.108 (0.914)	0.101 (1.102)	0.122 (0.874)	0.119 (1.303)	0.135 (1.604)
$I/\sigma(I)$	46.7 (3.5)	185.7 (20.1)	121.8 (7.4)	69.5 (4.1)	34.5 (2.1)	39.9 (2.1)
$CC_{1/2}$	0.991 (0.617)	0.995 (0.724)	0.997 (0.788)	0.994 (0.793)	0.992 (0.544)	0.995 (0.599)
CC*	0.998 (0.873)	0.999 (0.917)	0.999 (0.939)	0.999 (0.941)	0.998 (0.839)	0.999 (0.865)
Completeness (%)	100.0 (100.0)	90.8 (90.7)	97.5 (94.9)	99.7 (100.0)	94.0 (93.8)	100.0 (100.0)
Redundancy	7.3 (7.2)	6.1 (5.7)	6.6 (6.5)	6.9 (6.7)	6.2 (5.9)	7.3 (7.3)
Wilson B-factor	22.2	10.8	19.9	11.4	10.9	17.8
Refinement						
Resolution (Å)	20.0-1.80	29.96-1.70	30.0- 1.75	29.4-1.70	28.68-1.70	29.29-1.75
No. reflections	178638	177529	179523	199274	177818	175192
R_{work}	0.1593	0.1509	0.1475	0.1509	0.1545	0.1457
R_{free}	0.1922	0.1839	0.1794	0.1841	0.1838	0.1777
No. atoms						
Protein	12715	12692	12940	12856	12854	12823
Ligands	470	415	448	585	472	550
B-factors						
Protein	25.5	15.7	25.4	16.6	16.6	22.1
Ligands	39.0	26.4	40.2	32.0	41.2	39.5
Water	36.7	27.6	36.2	29.1	28.9	33.8
Ramachandran outliers	6	10	6	9	10	6

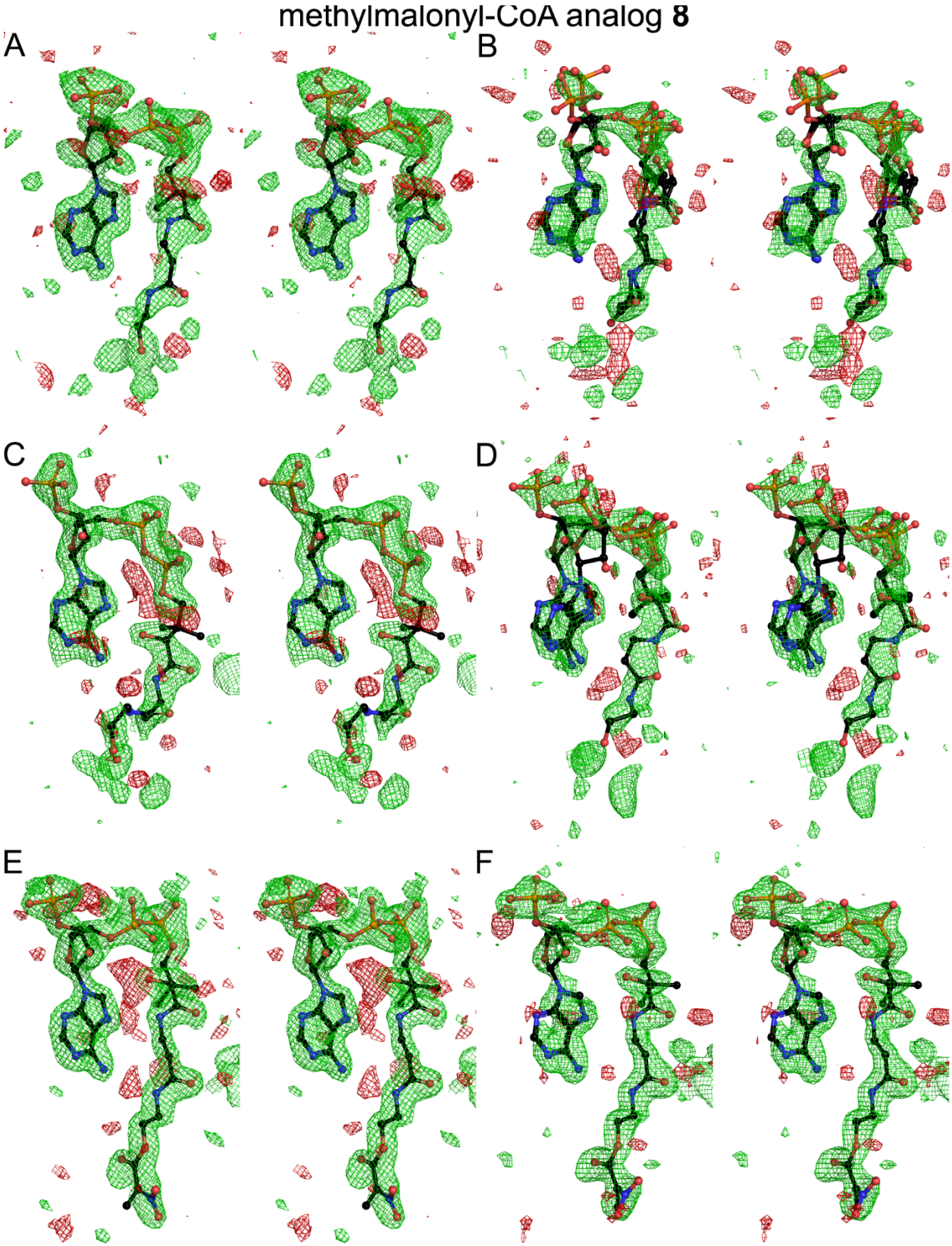
SI Figures




methylmalonyl-CoA analog **5**
A
B
C
D
E
F


methylmalonyl-CoA analog 6
A
B
C
D
E
F


methylmalonyl-CoA analog 7
A
B
C
D
E
F


methylmalonyl-CoA analog 8
A
B
C
D
E
F

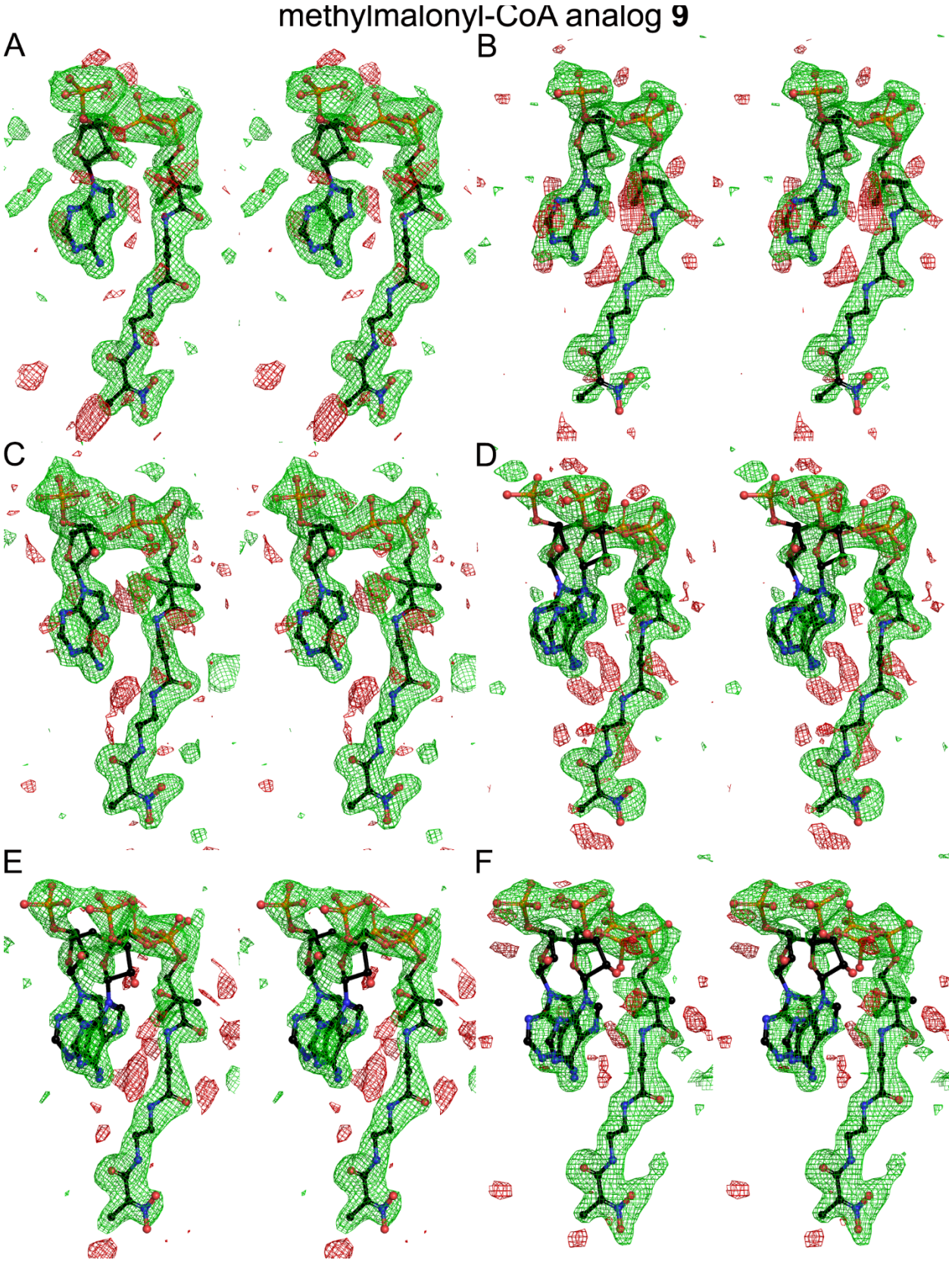

methylmalonyl-CoA analog 9
A
B
C
D
E
F

Figure S1. Omit maps for **4**-**9** bound to MMCD. Stereoimages of sigmaA weighted mF_o-DF_c omit maps. The green mesh is contoured at +3σ an the red mesh is at -3σ shown for a 5Å region around the ligand. The ligand is denoted in each title and the letters correspond to each monomer in the deposited PDBs.

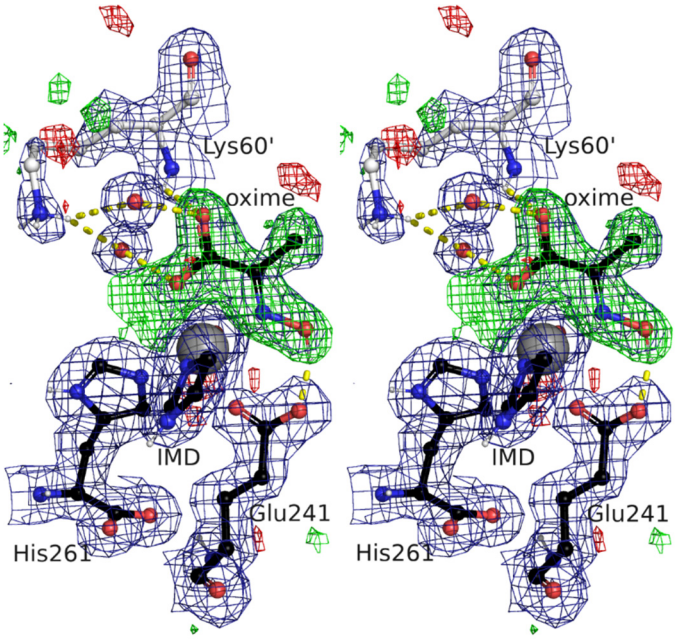


Figure S2. Pyruvate oxime degradation product of **7** and **8**. Stereoimage of the pyruvate oxime degradation product from the structure of **8** bound to MMCD in chain F. The same ligand is found in the structure of MMCD bound to **7** in chain F. SigmaA weighted mF_o-DF_c omit map for the oxime shown in green at +3σ and red at -3σ within 4Å, with the sigmaA weighted $2mF_o$-DF_c map shown in blue at 1σ for the surrounding ligands. Residues from chain F are in black and the Ni ion is shown as a gray sphere. The Lys60' residue in white is from a symmetry mate.

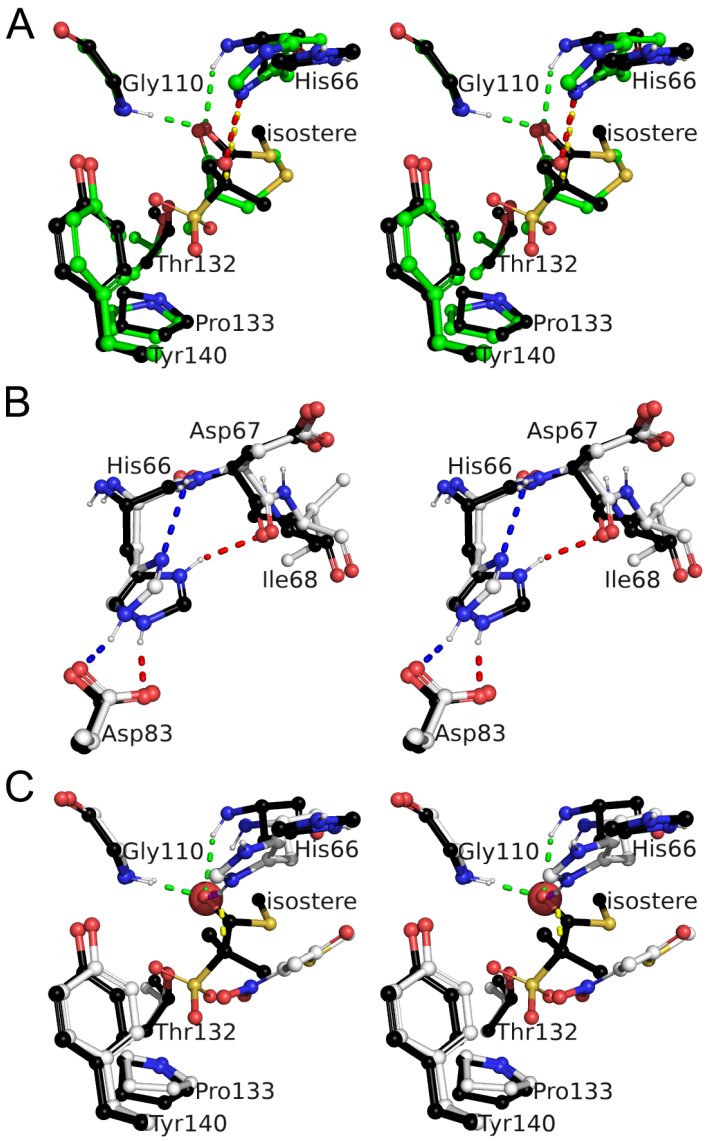


Figure S3. Alternative His66 sidechain conformations. Stereoimages of MMCD active site comparisons. A) Binding of **2** from PDB 1EF9 in green compared to **4** from chain A in black revealing rotation of His66 towards the putative enolate as mimicked by the carboxylate of **2**. B) The position of His66 has two positions dependent on protonation state as revealed by an overlay of MMCD with **4** bound in chain A in black (protonated His66, interactions shown in red dashes) and chain C in white (neutral His66, interactions shown in blue dashes). Notice that Ile68 changes conformation, which likely changes the affinity for CoA between the protonation states. C) Interaction of His66 with a water (shown as a large sphere, with the His66 interaction shown with magenta dashes) in the active site and alternative positions of the isosteres from MMCD with **7** bound in chain A in black and chain C in white. Notice that the His66 backbone changes significantly between the two states. Not shown is that Ile68 and Leu71 also change position significantly to accommodate the **7** in the orientation in chain C.

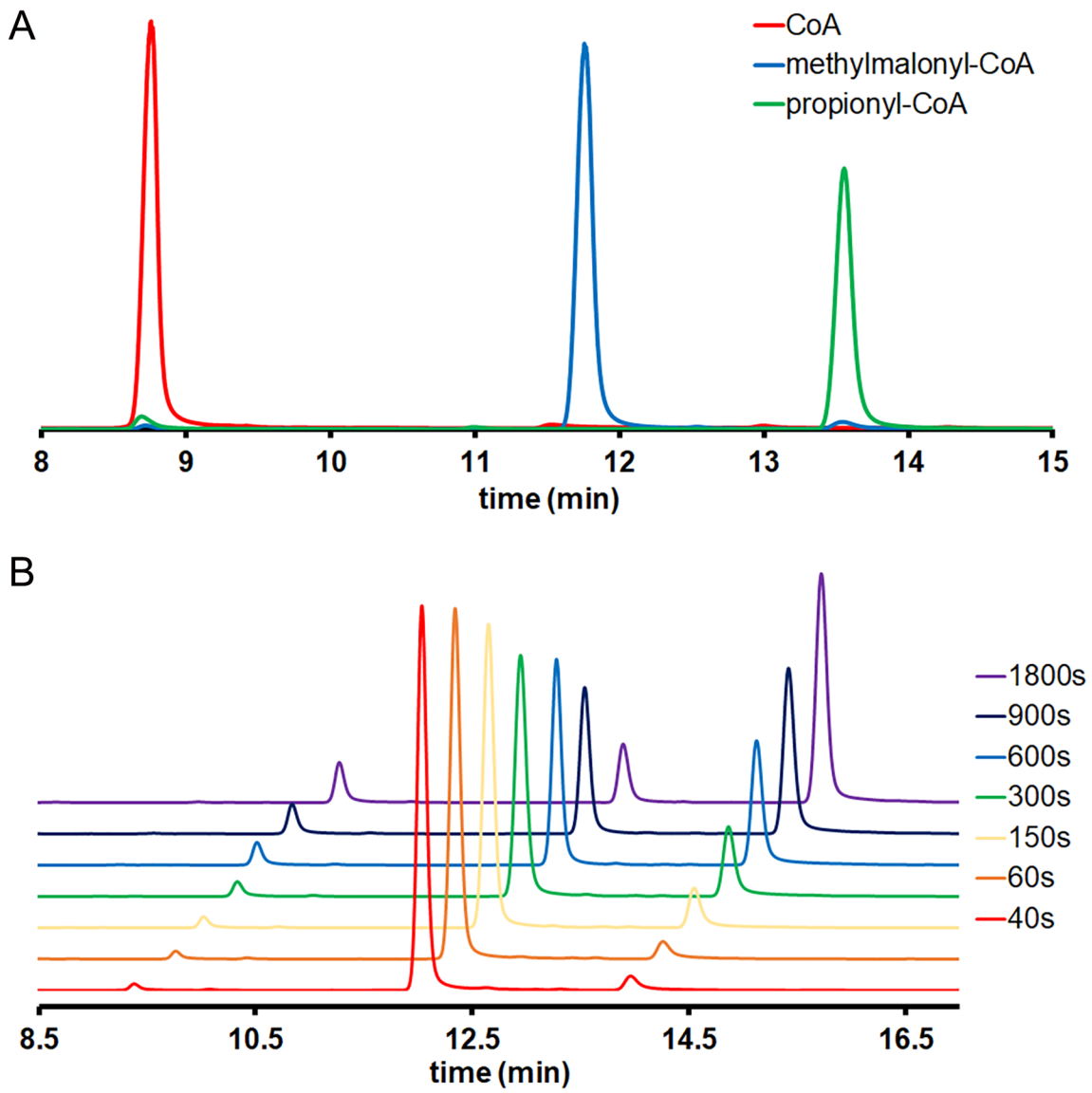


Figure S4. Example HPLC traces of **1** and MMCD products. HPLC chromatograms of A) standards and B) an experiment showing 5 nM MMCD activity on 200 µM (*R*/*S*)-methylmalonyl-CoA at pH 6.5 over time.

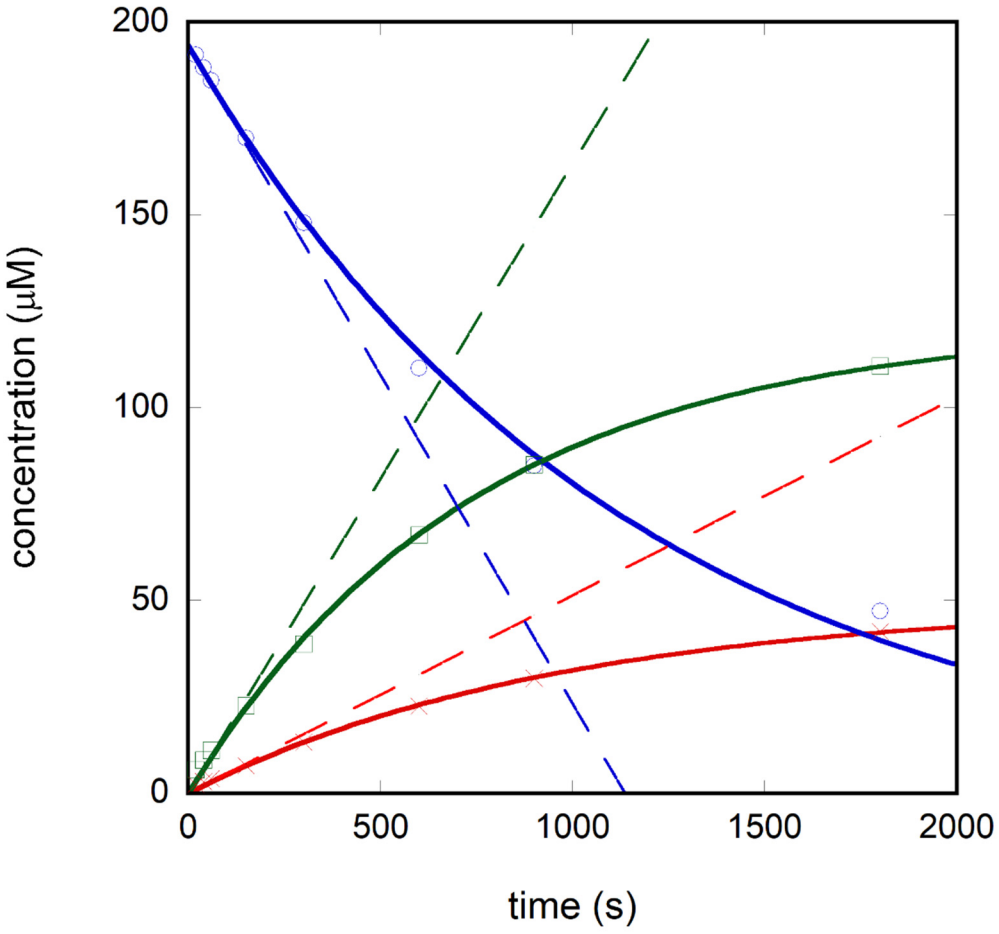


Figure S5. Kinetic trace for (*R*/*S*)-methylmalonyl-CoA decomposition by MMCD. Data is from Figure S4B. Substrate (*R*/*S*)-methylmalonyl-CoA data is shown in blue and circles, decarboxylation product propionyl-CoA is in dark green squares and hydrolysis produdct CoA is in red Xs. Solid lines represent fitting of the data to equations (1) or (2). The blue dashes represent a V_i of 0.171 μM s^{-1} for (*R*/*S*)-methylmalonyl-CoA decomposition, green dashes represent a V_i of 0.163 μM s^{-1} for propionyl-CoA production and red dashes represent a V_i of 0.051 μM s^{-1} for CoA production.

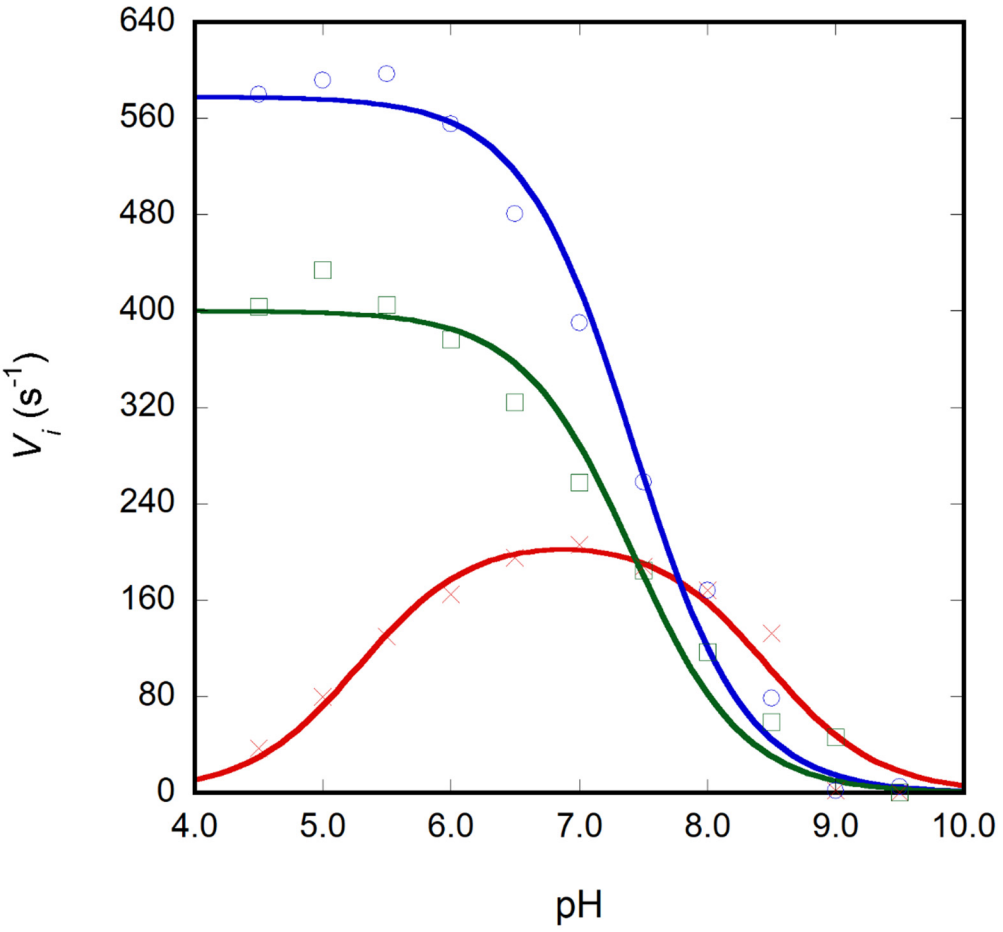


Figure S6. MMCD pH rate profile. Initial rates of methylmalonyl-CoA decomposition is designated by blue circles for data points and blue line is fit to data. Initial rate of propionyl-CoA formation is designated by green squares and dark green line is fit to data. Initial rate of CoA formation is designated by red Xs and red line is fit to data. Starting methylmalonyl-CoA substrate concentration is 500 μM, and enzyme concentration is 5 nM.

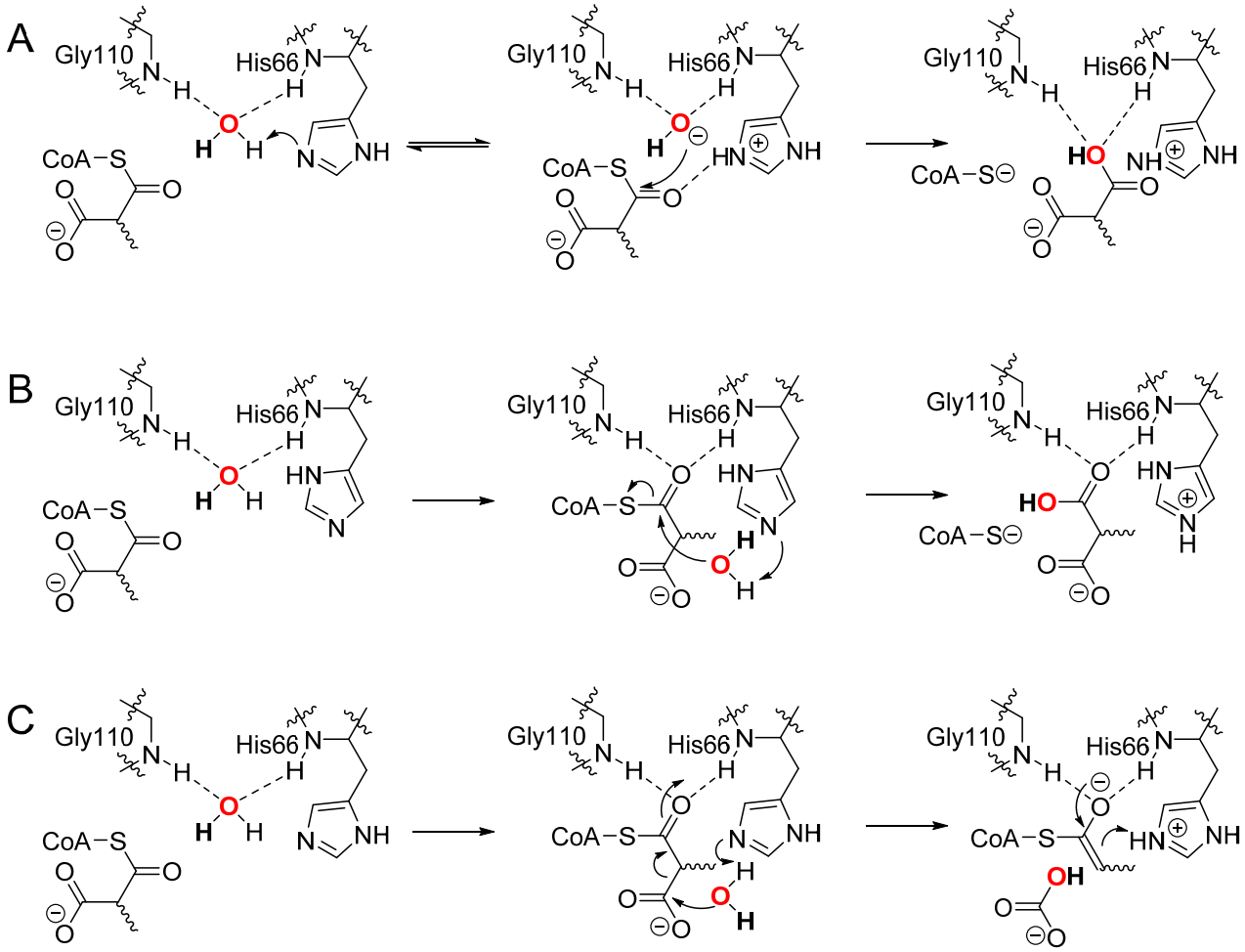


Figure S7. Alternative mechanisms for hydrolysis and decarboxylation. A) Hydrolysis via activation of water in the oxyanion hole of His66 and Gly110. The thioester tetrahedral intermediate is stabilized by the protonated His66 side chain. B) Hydrolysis via rearrangement of water and the methylmalonyl group. The water is deprotonated by His66 allowing attack on the activated thioester carbonyl. C) Alternative mechanism for decarboxylation via bicarbonate formation. Rearrangement of the water and methylmalonyl group leads to activation of the water for attack on the carboxylate. This mechanism is disfavored, as there was little evidence for a water molecule being retained in the active site upon binding of **9**.

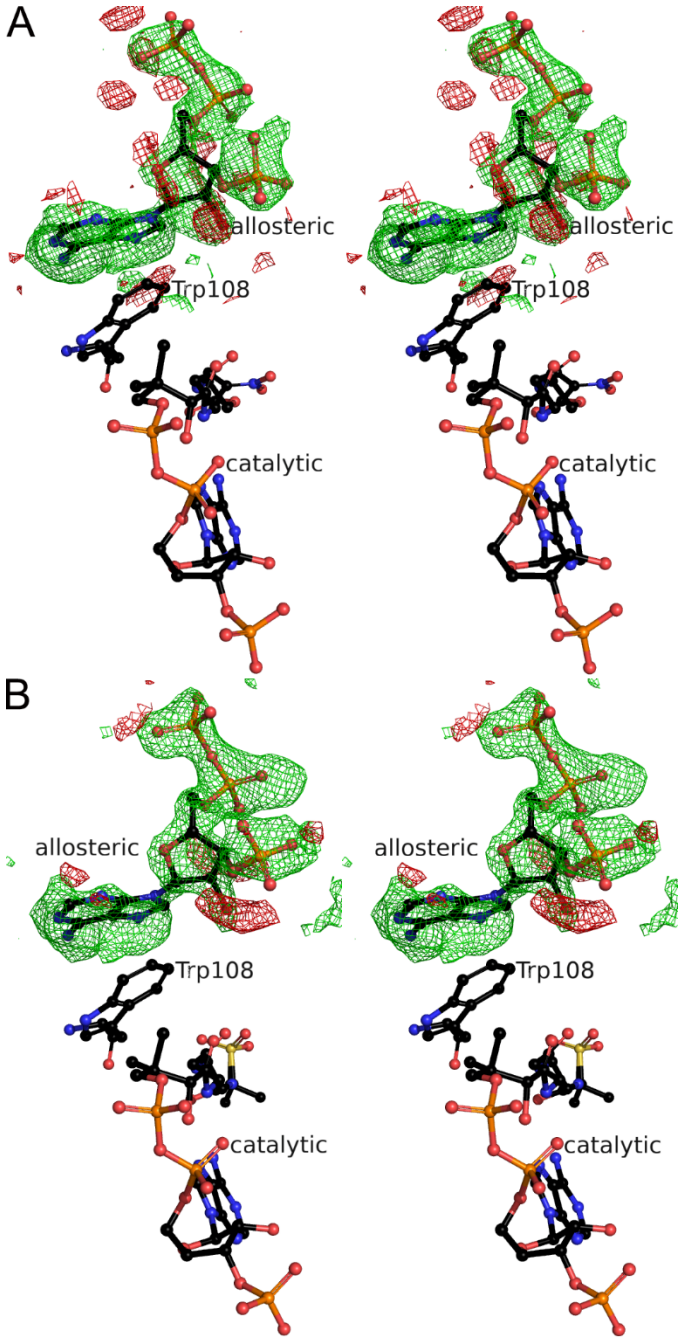


Figure S8. Potential allosteric site with partial binding of phospho-adenosine. Stereoimages of sigmaA weighted mF_o-DF_c omit maps contoured at +2.5σ in green or -2.5σ in red in a within 3Å of the partial ligand. The ligand in the catalytic site is designated along with a Trp108 which interacts with both. A) Chain F of MMCD with **8** bound. B) Chain C of MMCD with **6** bound. The partial allosteric ligand is also found in MMCD with **5** bound (chains C and F), **6** bound (chains C, E and F), **7**/**8**/**9** bound (chains C, E and F) with variable occupancy.

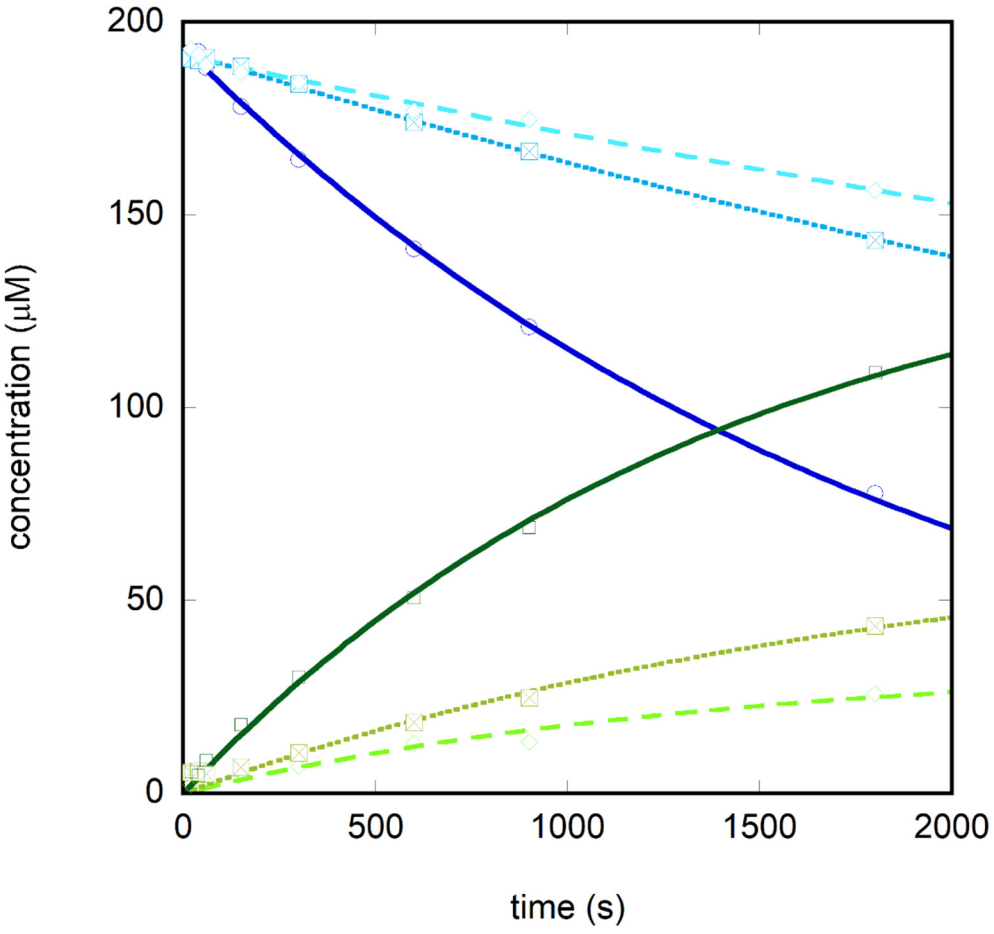
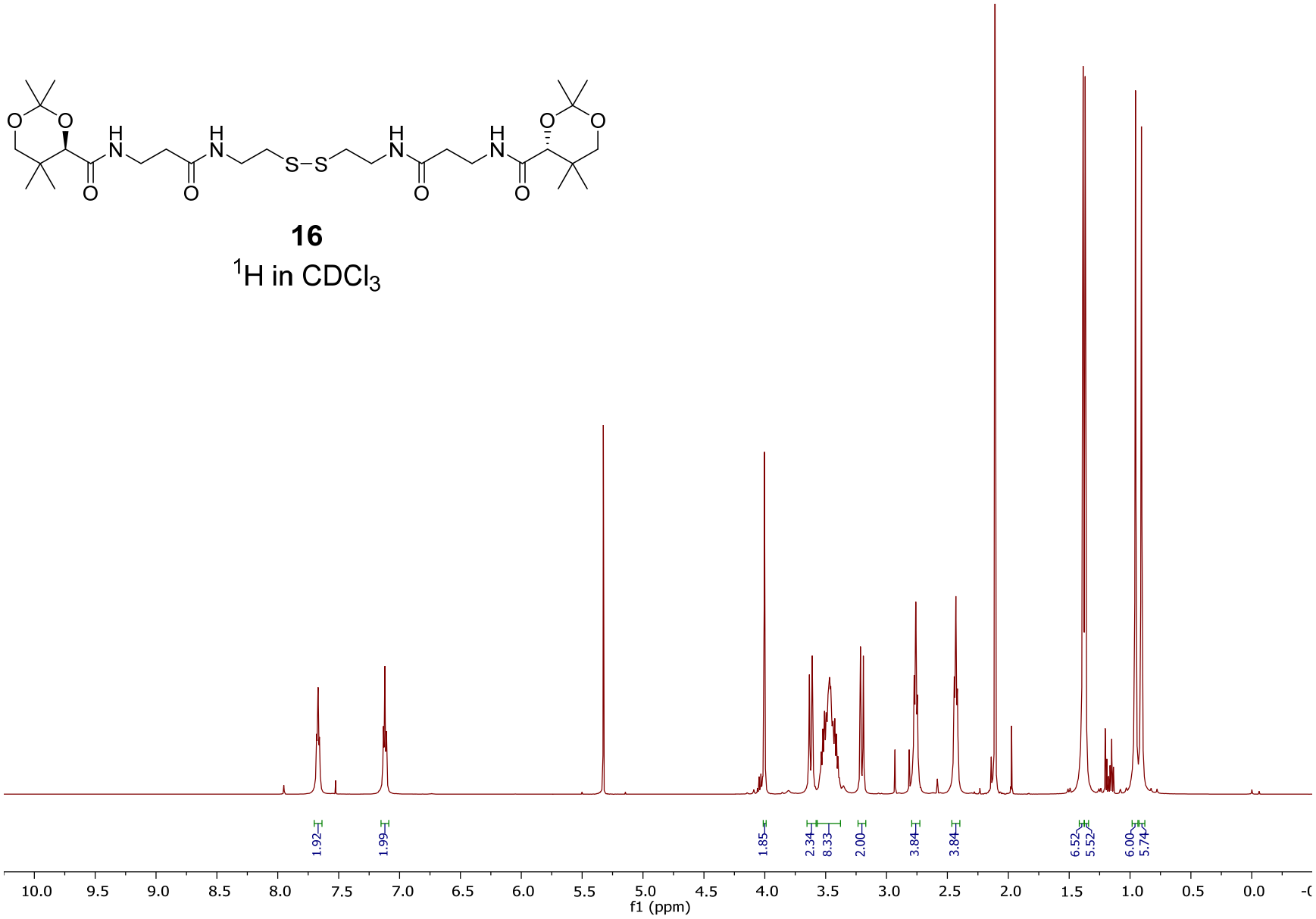
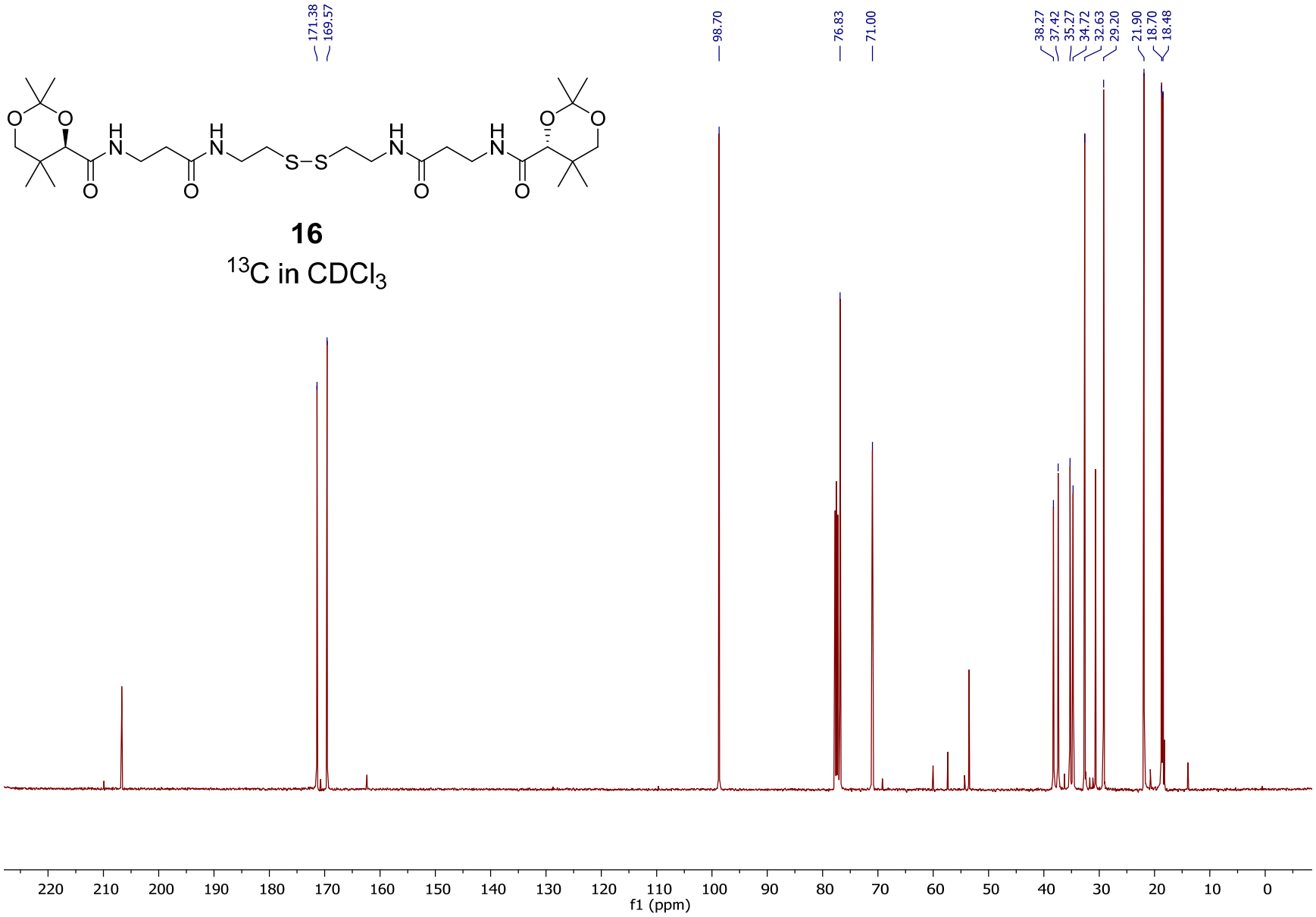


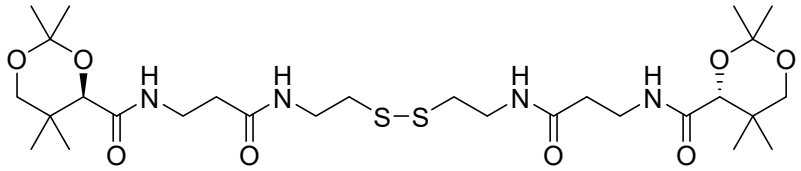
Figure S9. Representative data for inhibition of MMCD activity by **4**-**9**. Initial rate analysis to determine K_i of **4**. Solid lines are fit of data without **4**, dotted lines (boxed Xs are data) are in the presence of 20 μM **4** and dashed lines (open diamonds are data) are for 50 μM **4**, and all experiments were done at 200 μM (2*R*/*S*)-methylmalonyl-CoA. The blue lines are the fit of equation (1) for the disappearance of (2*R*/*S*)-methylmalonyl-CoA (open circles for no **4**) and the green lines are the fit of equation (2) for the appearance of propionyl-CoA (open squares for no **4**).

16

^{1}H in $CDCl_3$

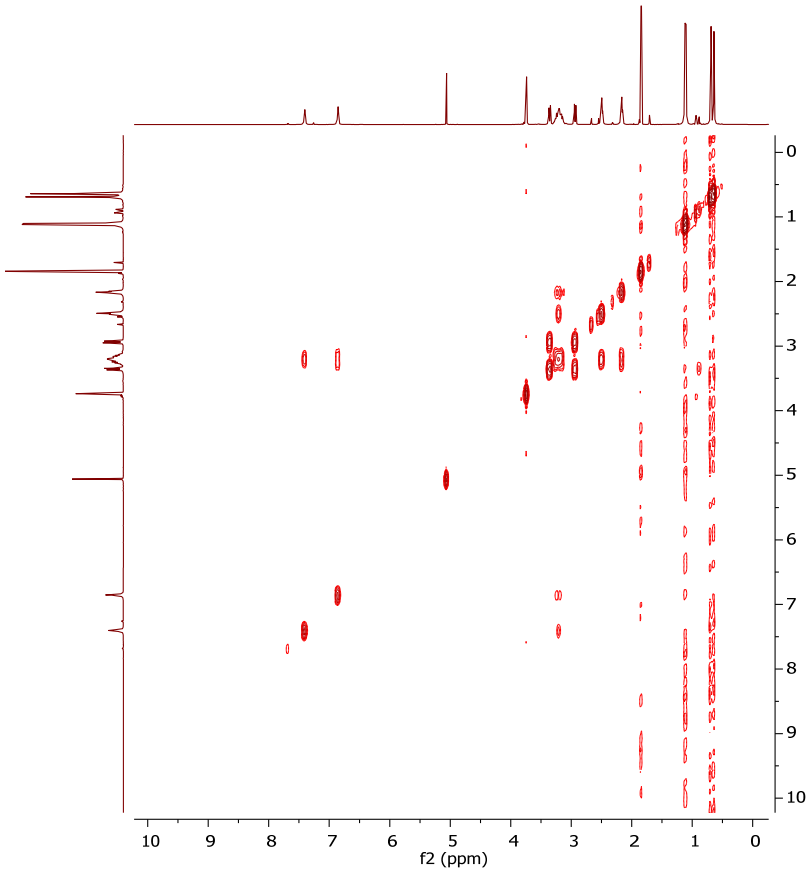


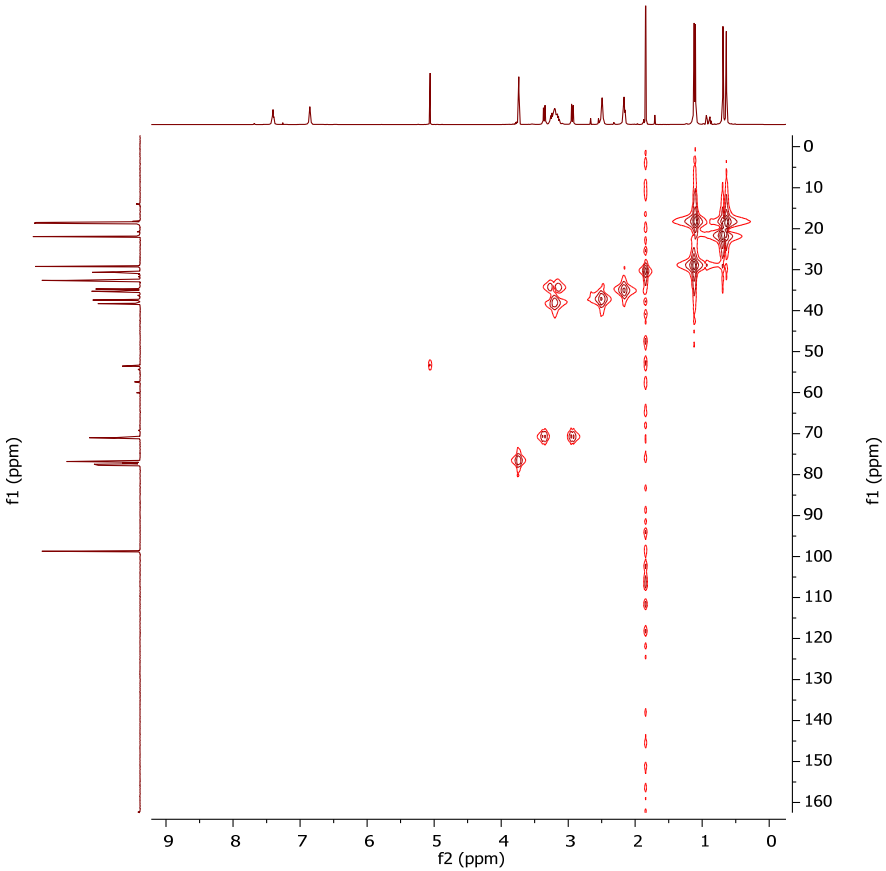

171.38
169.57
98.70
76.83
71.00
38.27
37.42
35.27
34.72
32.63
29.20
21.90
18.70
18.48
16
13C in CDCl3
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)



16

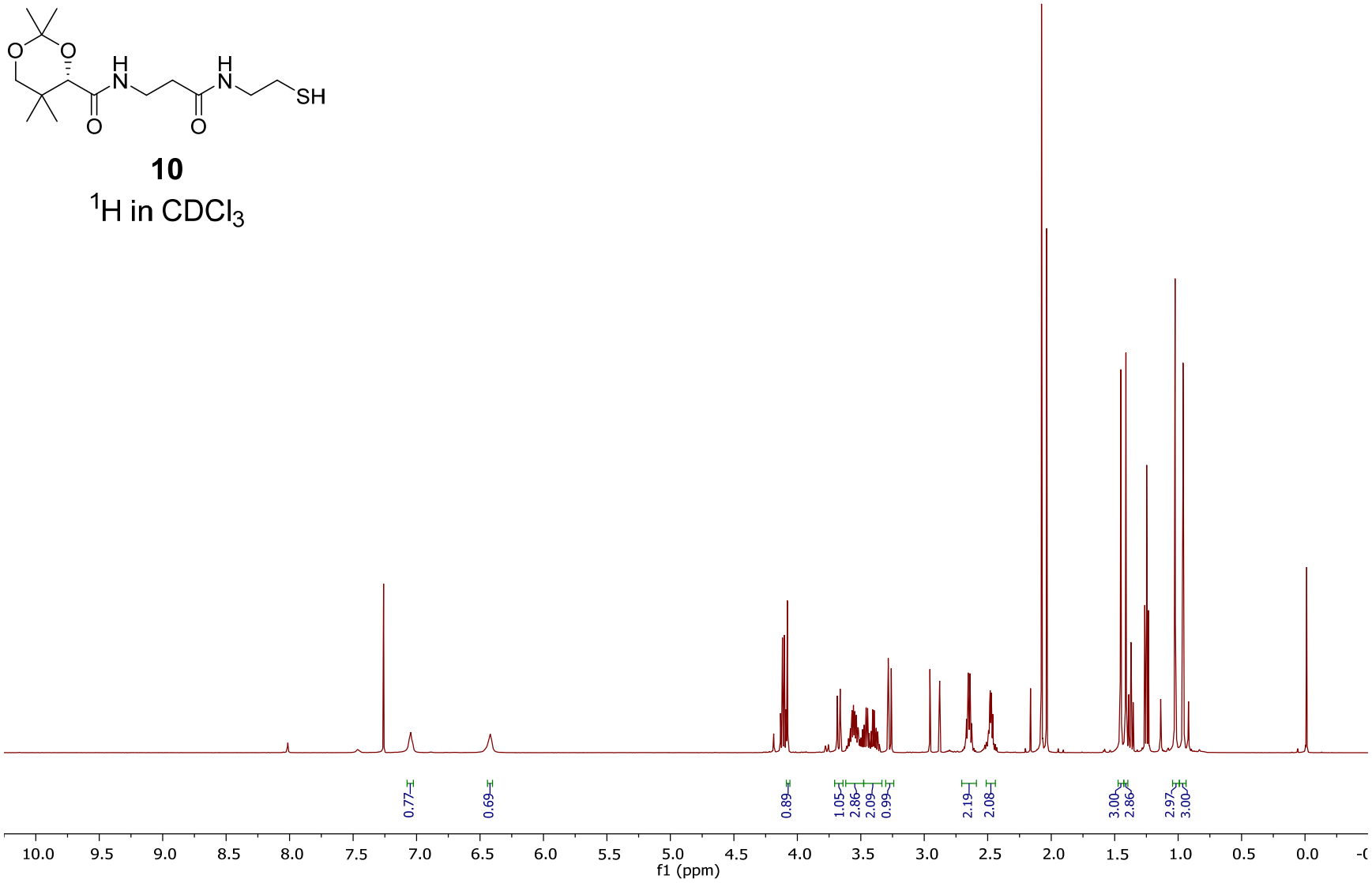
COSY and HMQC in $CDCl_3$

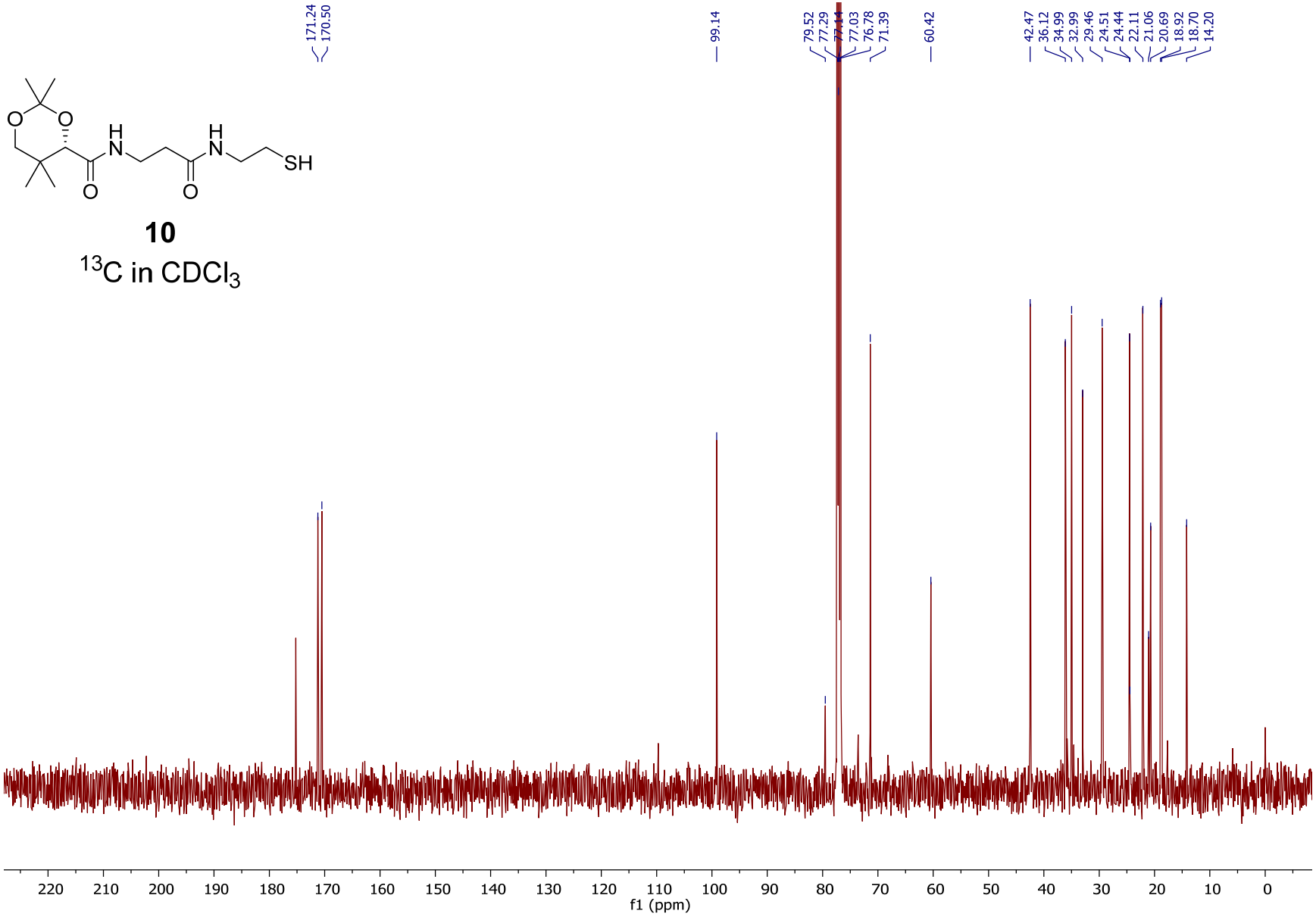


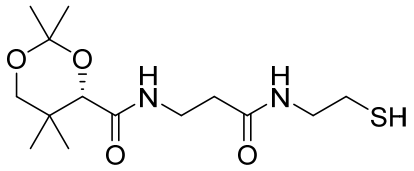


10

^{1}H in $CDCl_3$

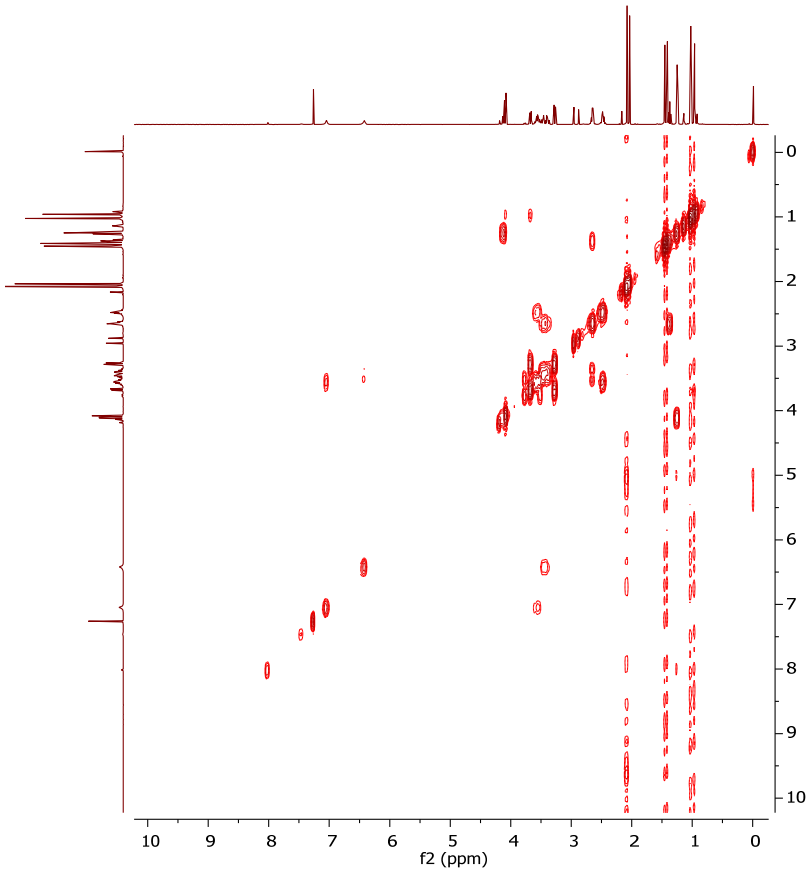


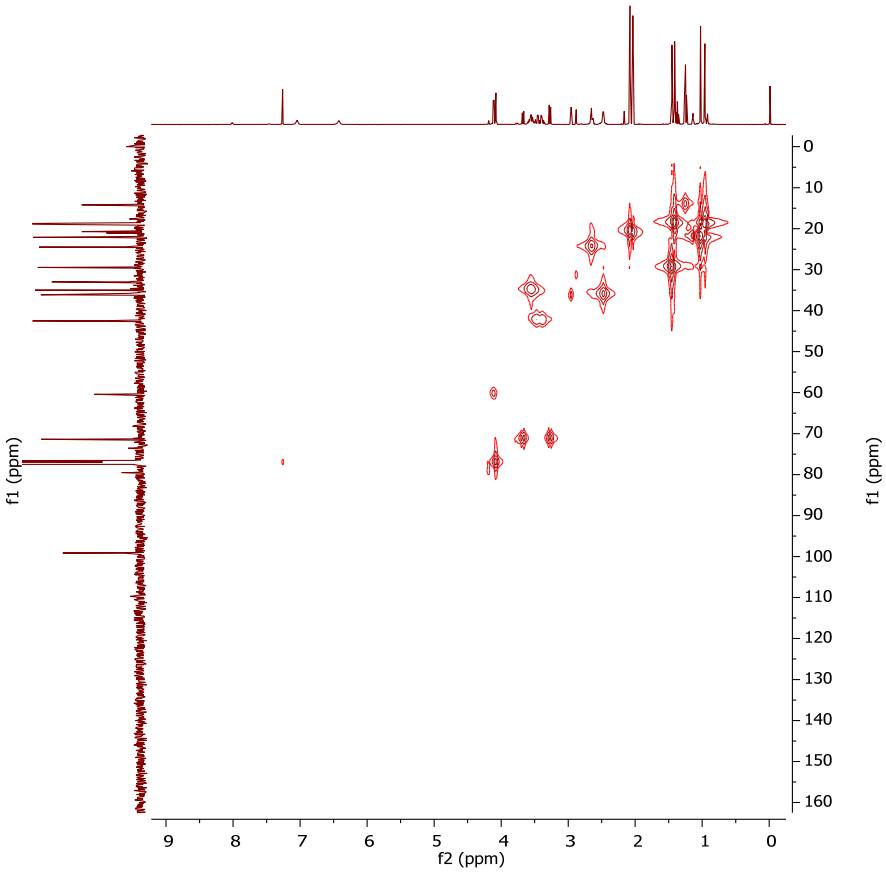

171.24
170.50
99.14
79.52
77.29
77.14
77.03
76.78
71.39
60.42
42.47
36.12
34.99
32.99
29.46
24.51
24.44
22.11
21.06
20.69
18.92
18.70
14.20
O
O
H
N
O
H
N
SH
O
10
13C in CDCl3
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



10

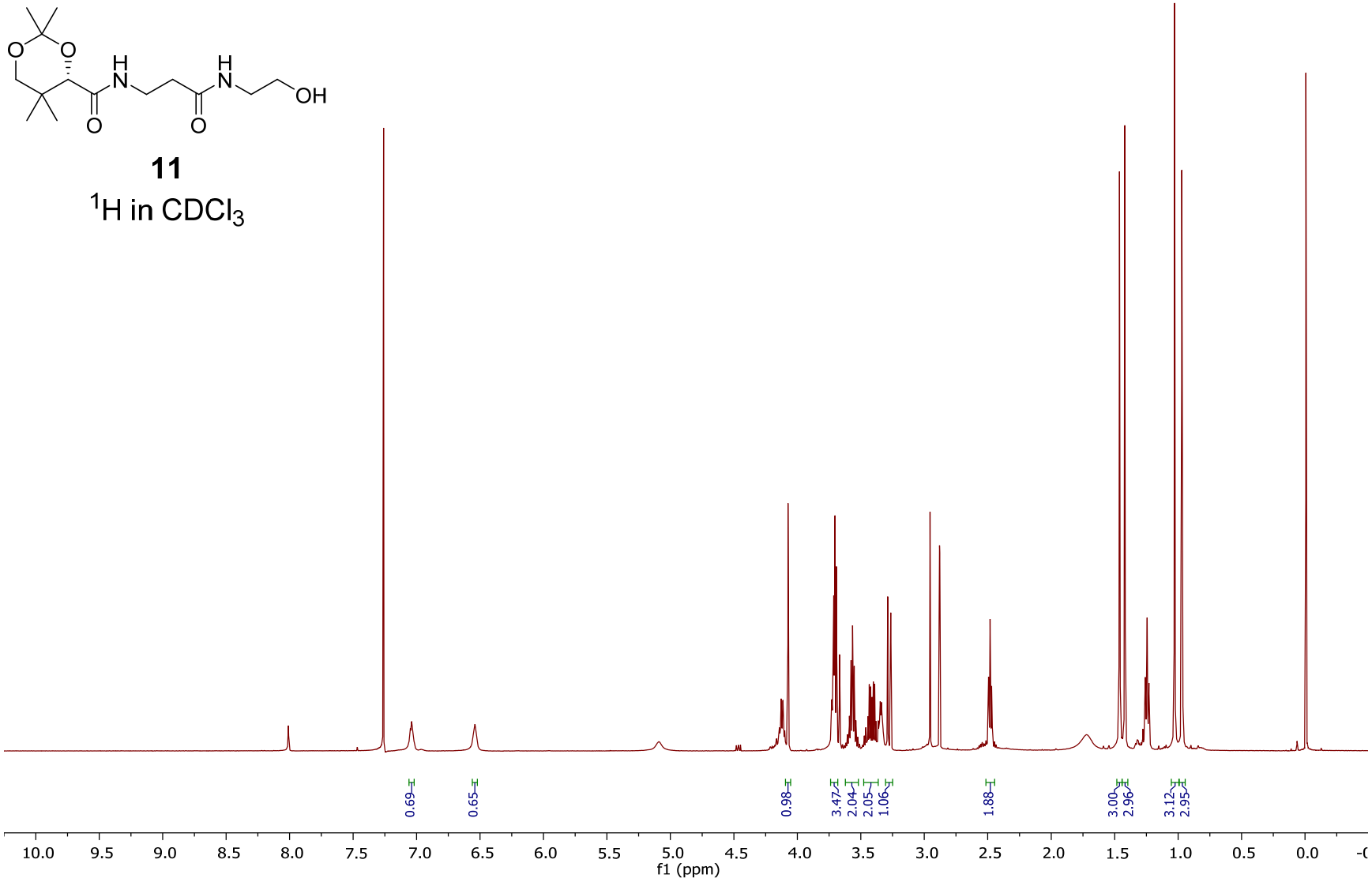
COSY and HMQC in $CDCl_3$

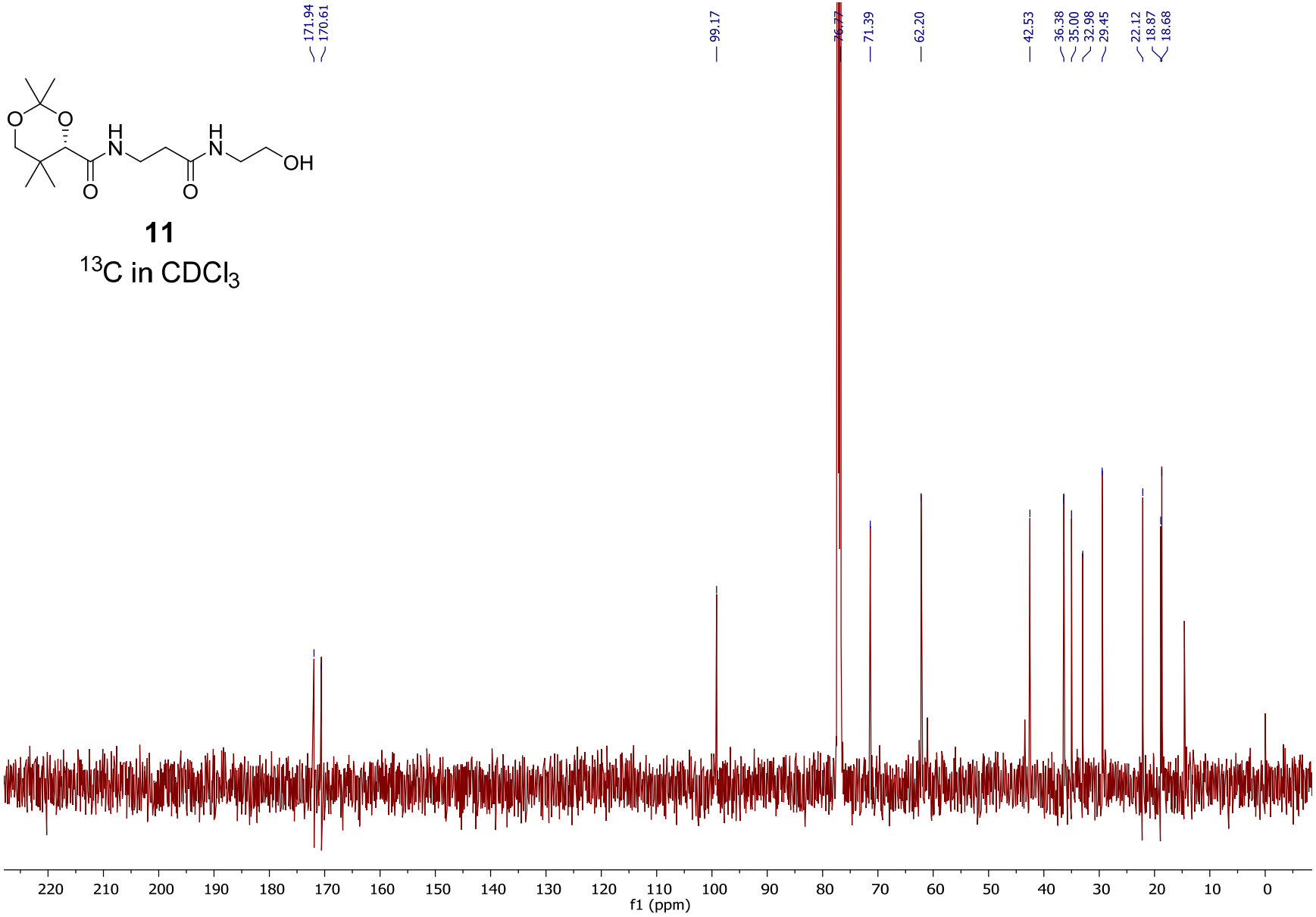




11

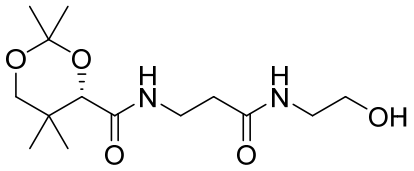
^{1}H in $CDCl_3$





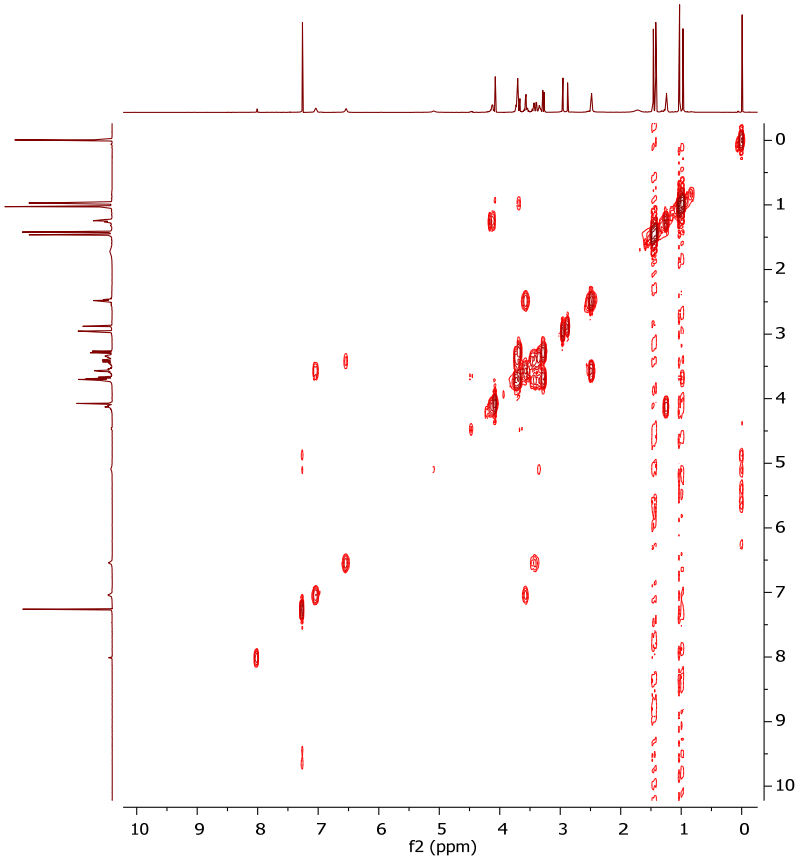
11

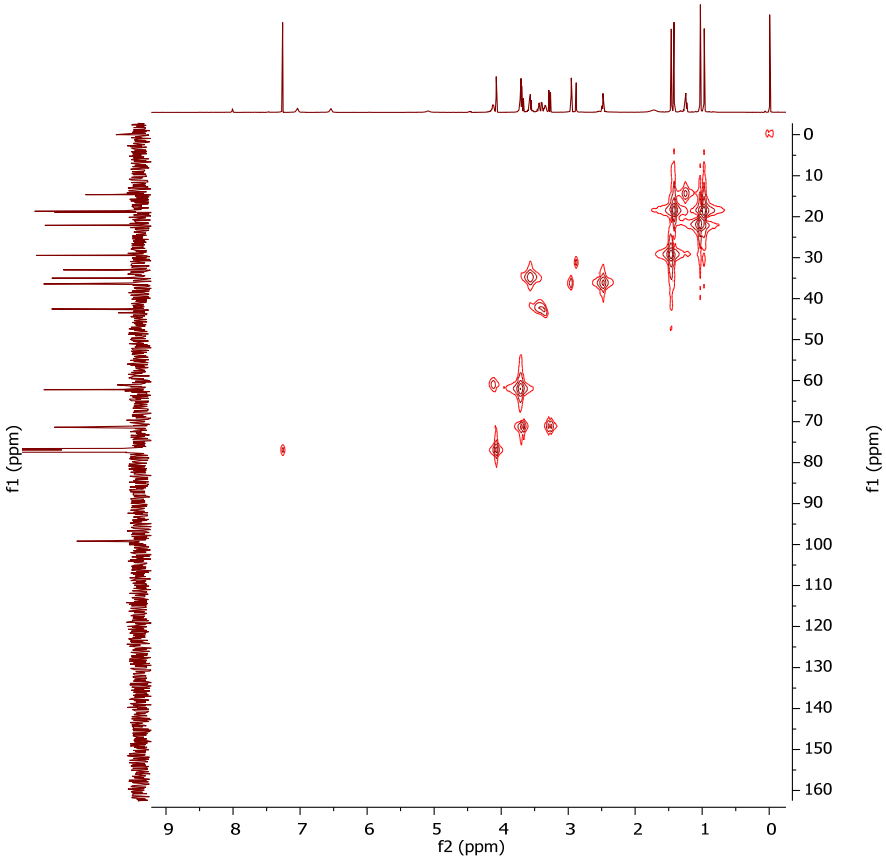
^{13}C in $CDCl_3$

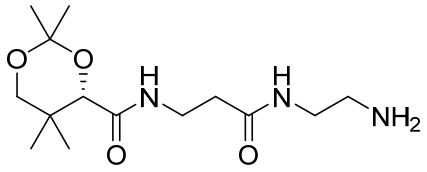


11

COSY and HMQC in $CDCl_3$

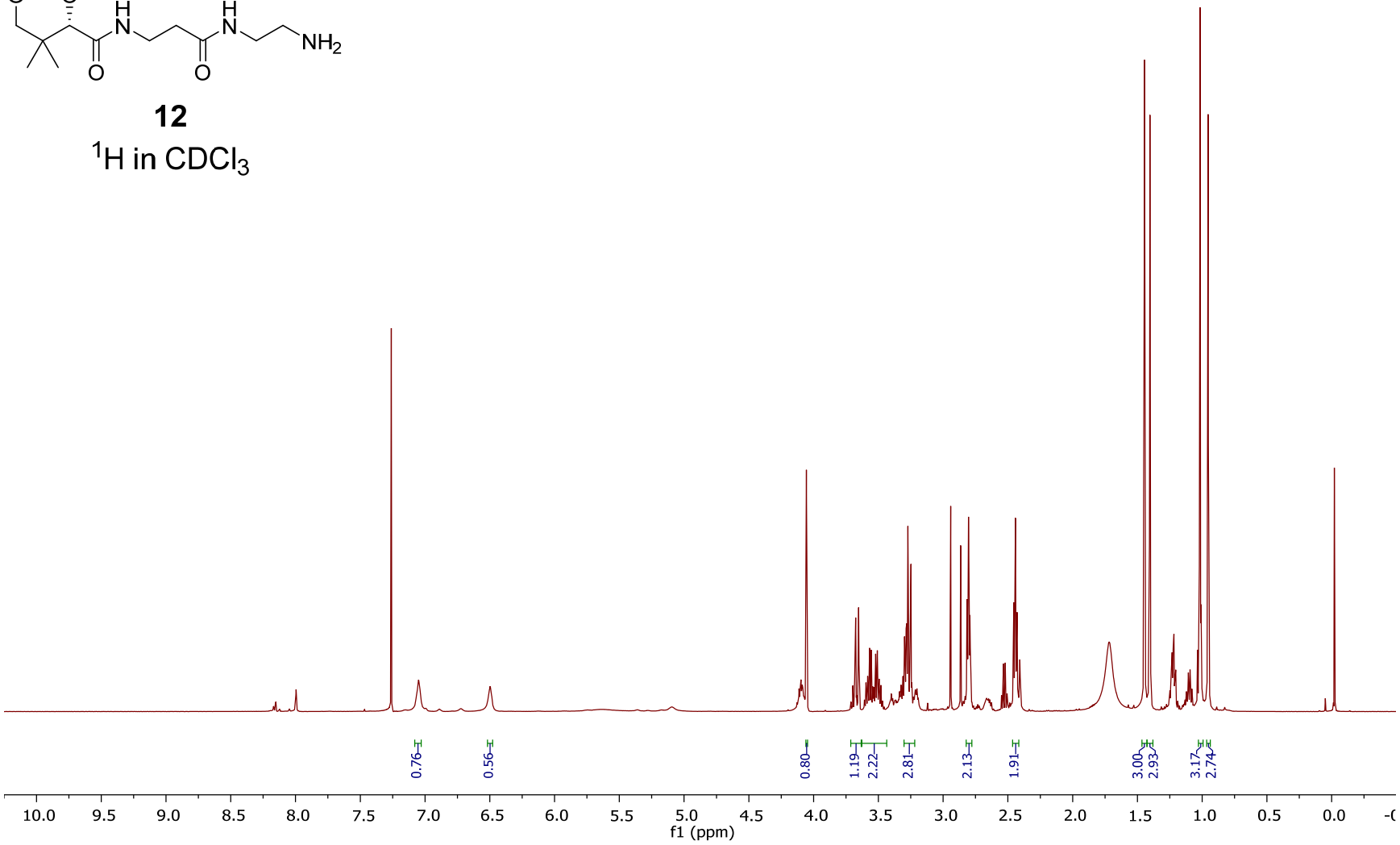


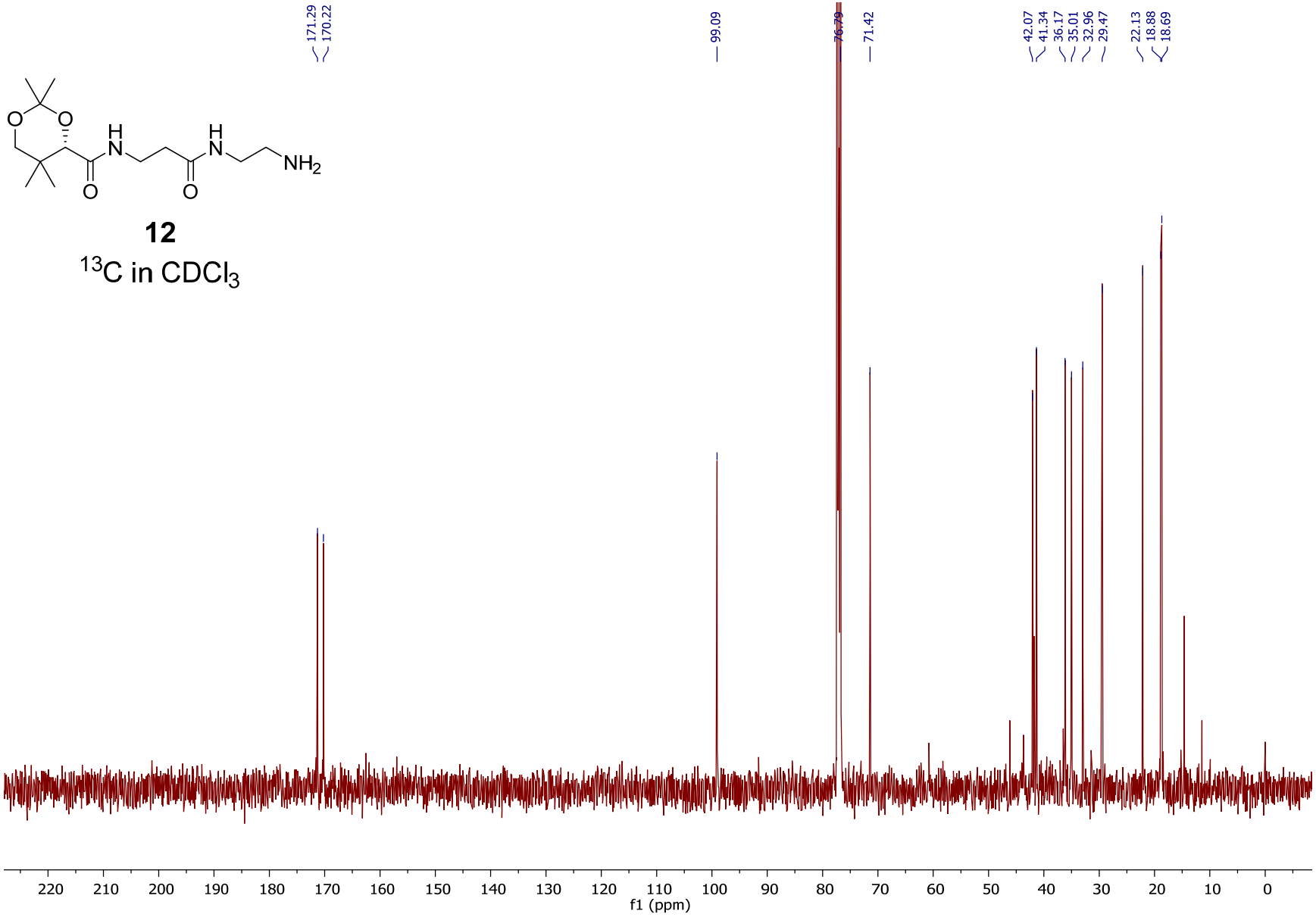


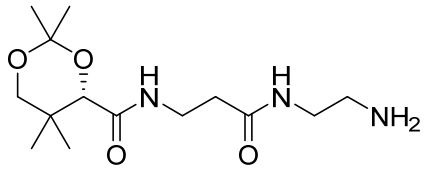


12

^{1}H in $CDCl_3$

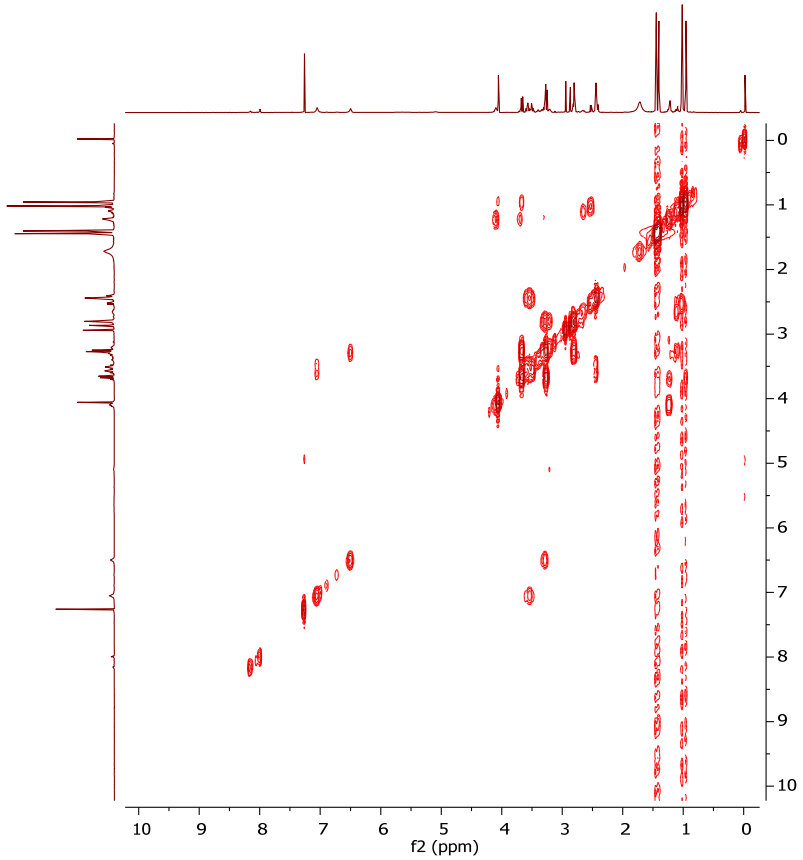


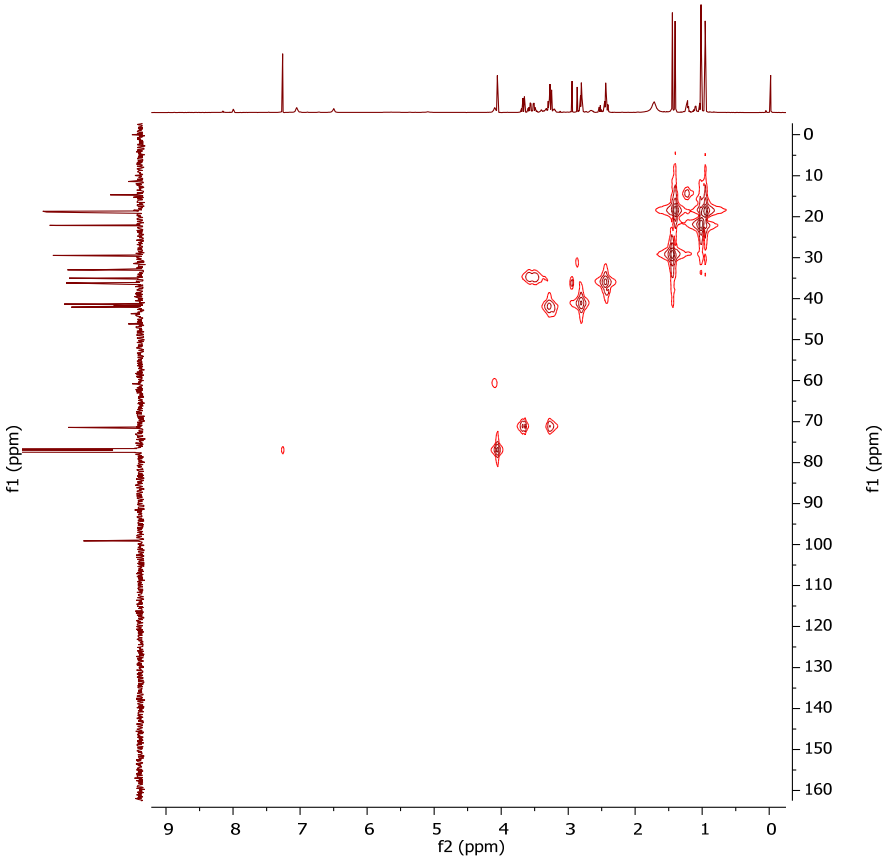

171.29
170.22
99.09
76.79
71.42
42.07
41.34
36.17
35.01
32.96
29.47
22.13
18.88
18.69
O
O
H
N
O
H
N
NH2
O
12
13C in CDCl3
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

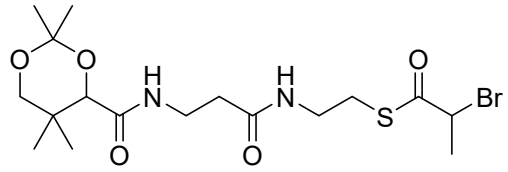


12

COSY and HMQC in $CDCl_3$

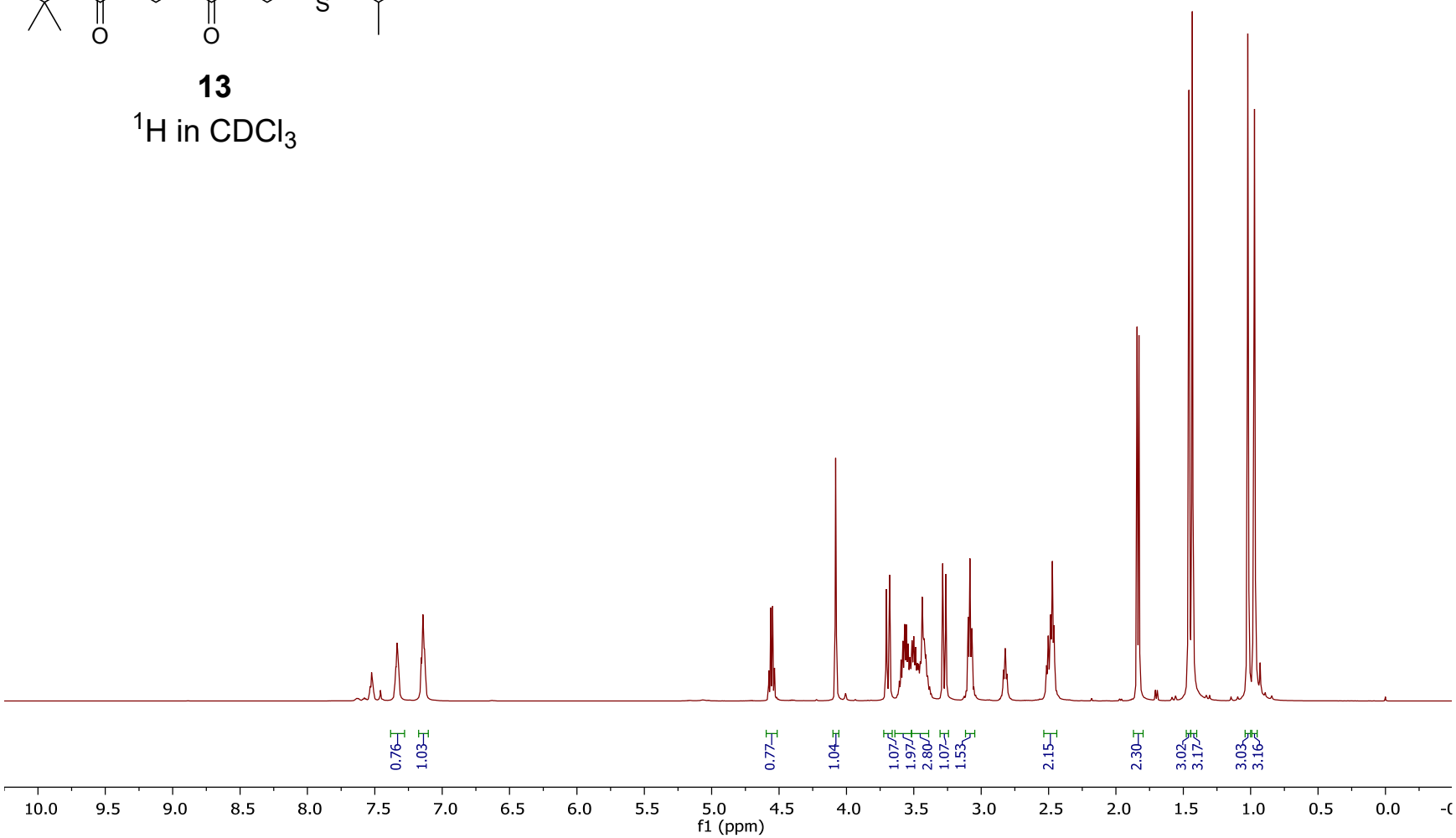


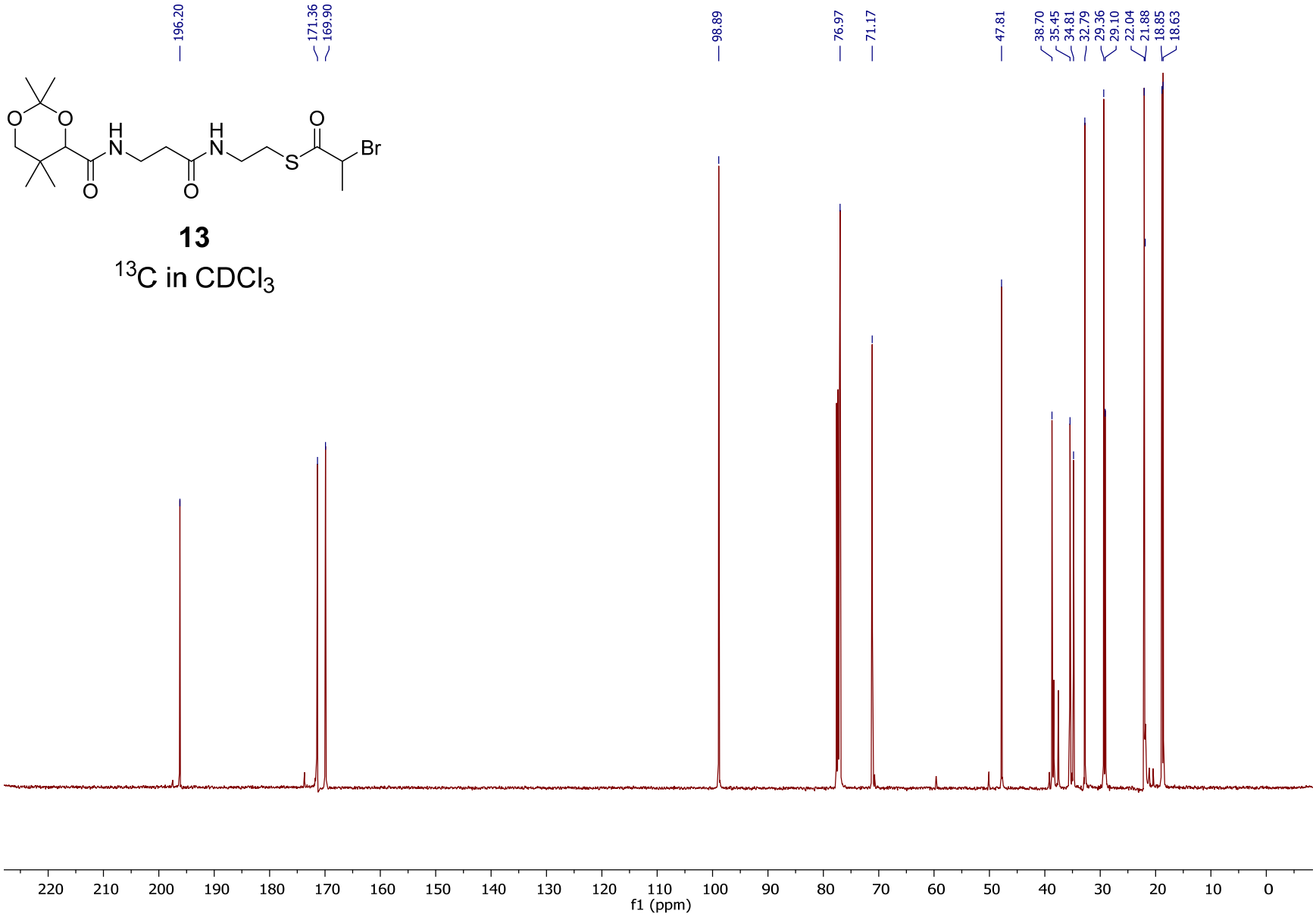


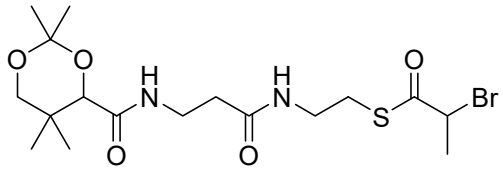


13

^{1}H in $CDCl_3$

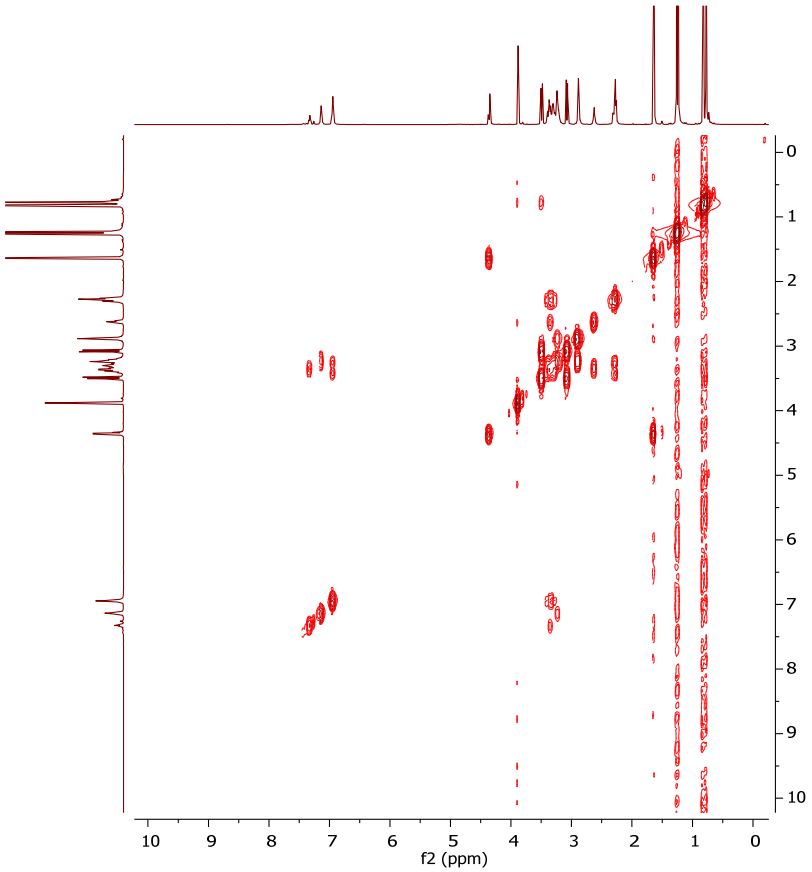


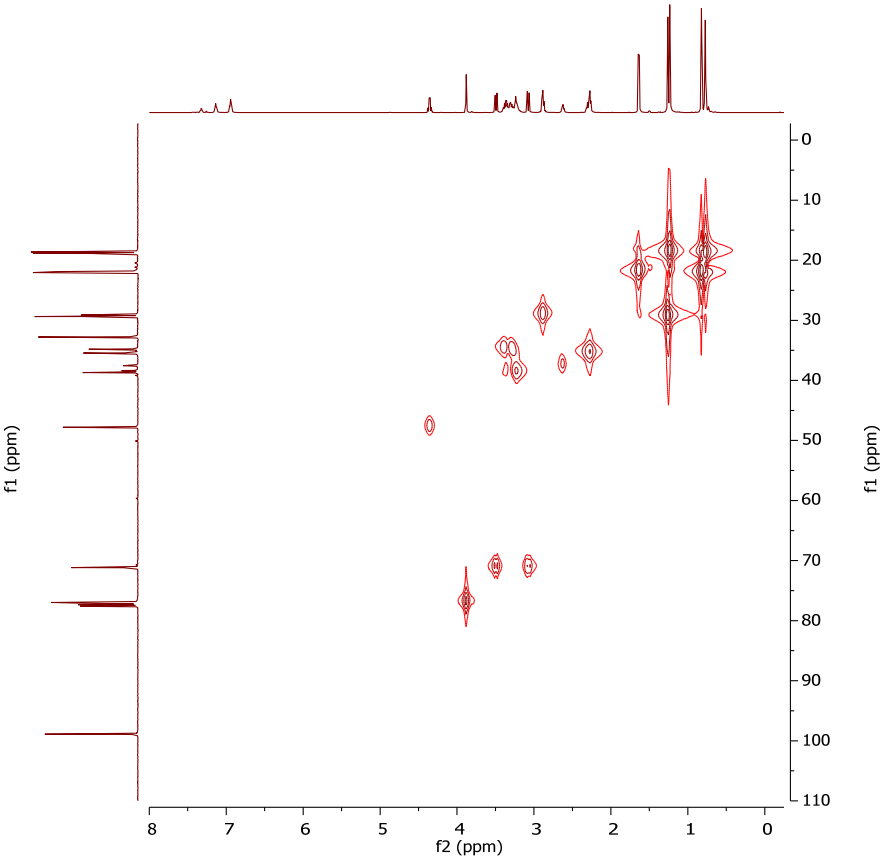

196.20
171.36
169.90
98.89
76.97
71.17
47.81
38.70
35.45
34.81
32.79
29.36
29.10
22.04
21.88
18.85
18.63
O
O
H
N
O
H
N
O
S
O
Br
13
13C in CDCl3
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



13

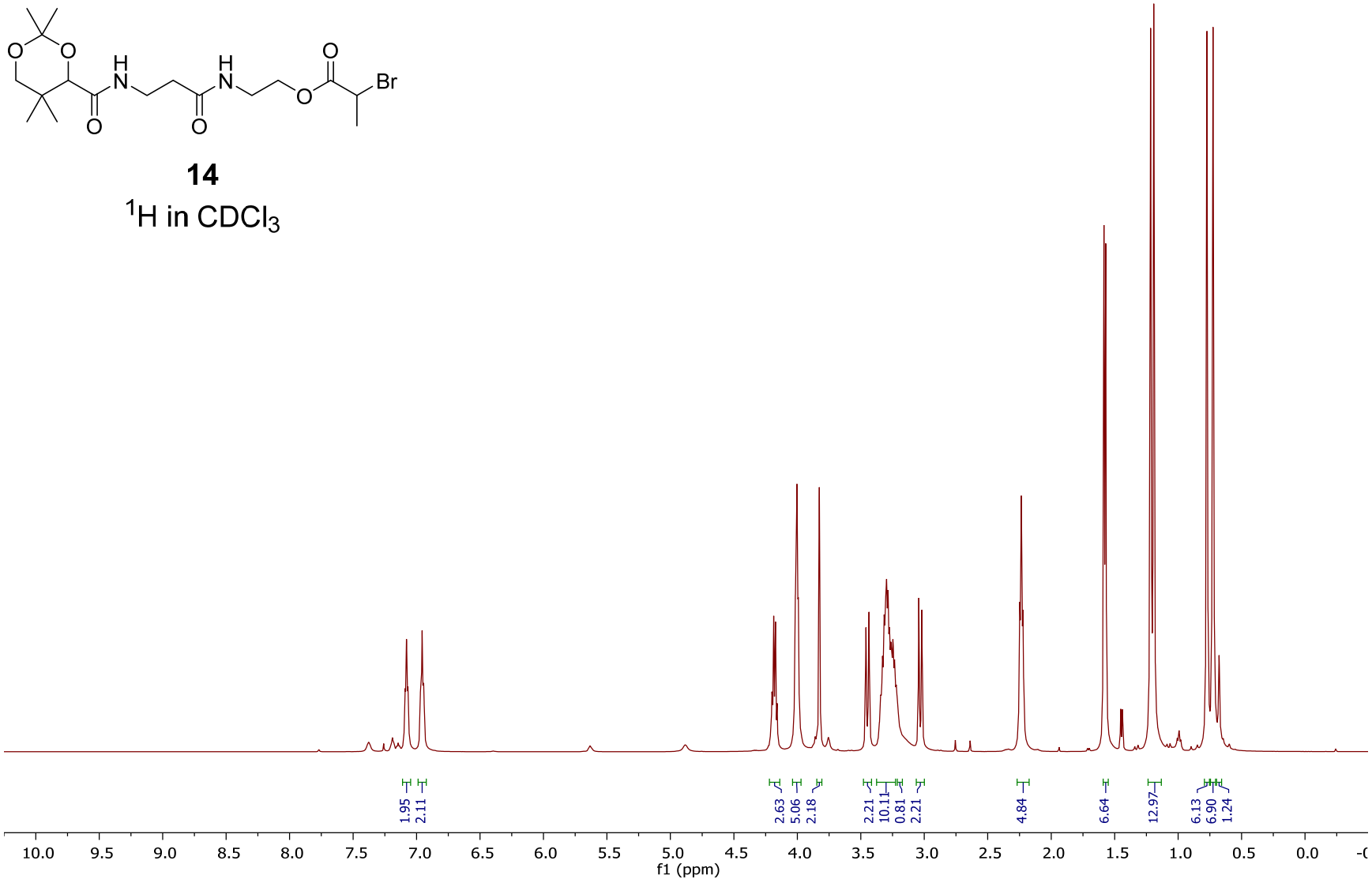
COSY and HMQC in $CDCl_3$

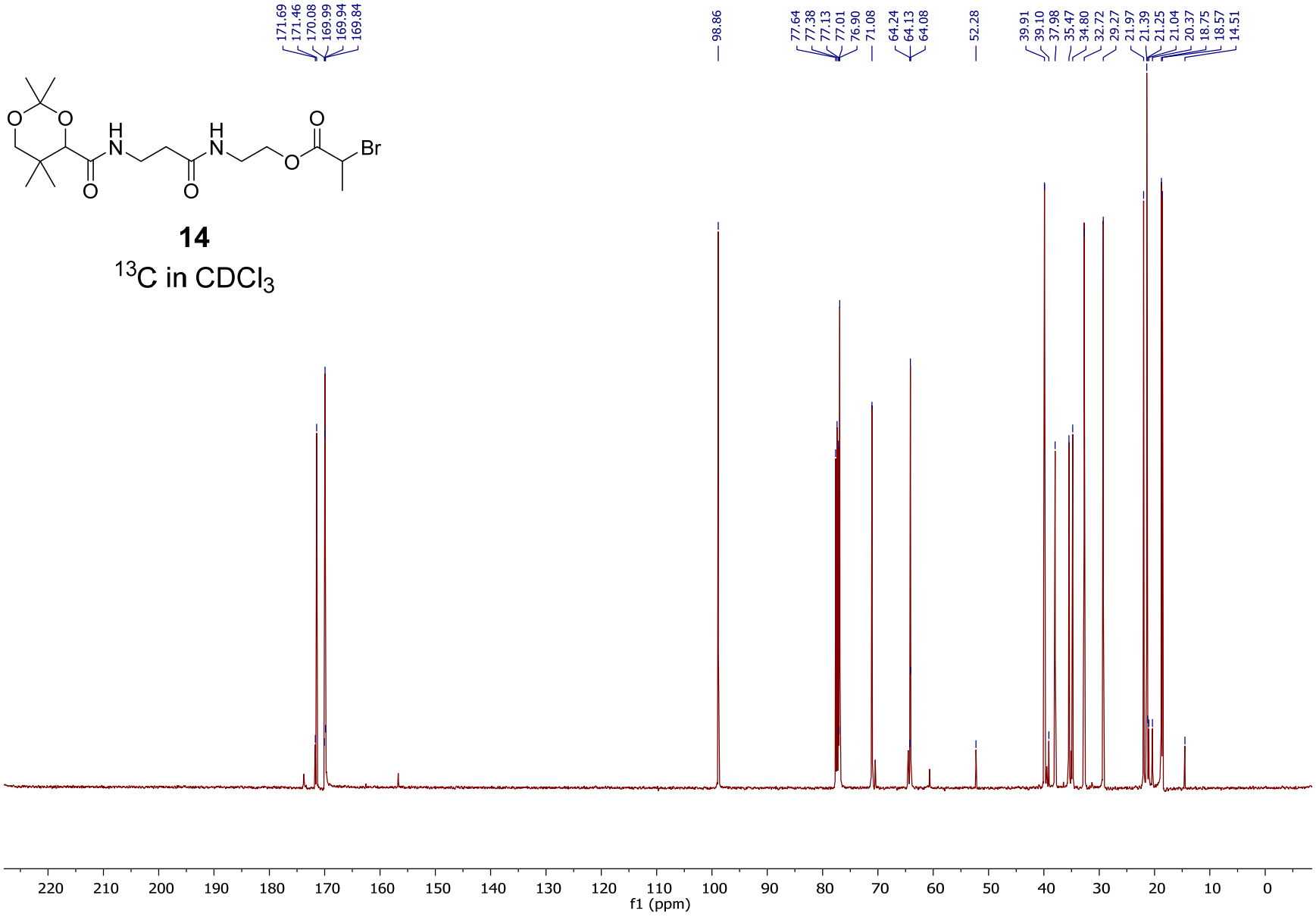


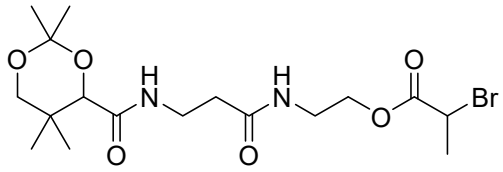


14

^{1}H in $CDCl_3$

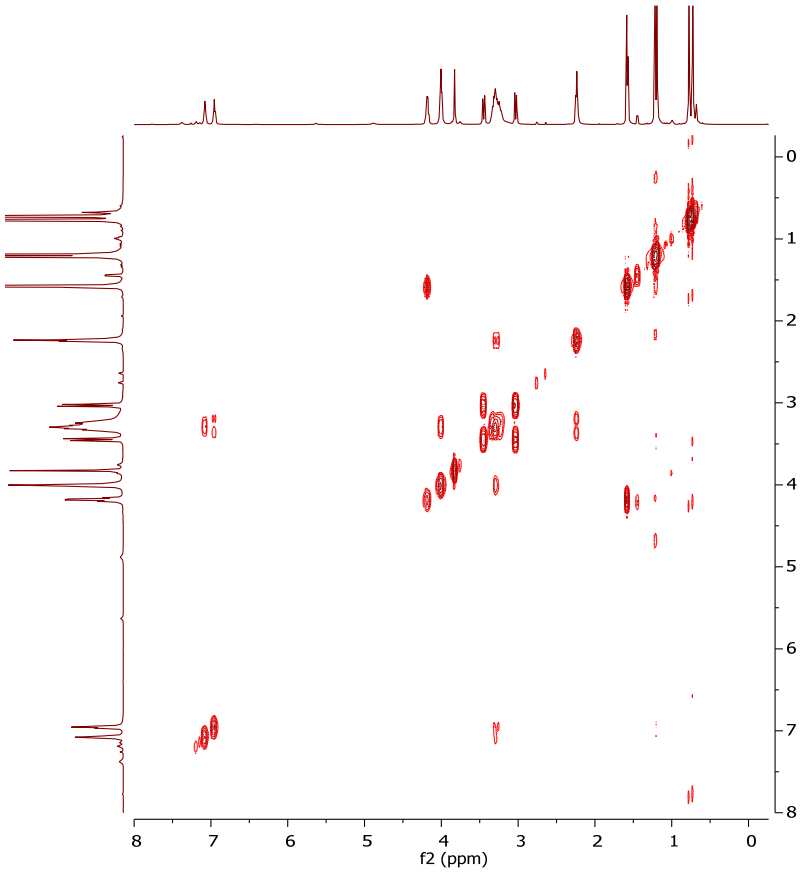


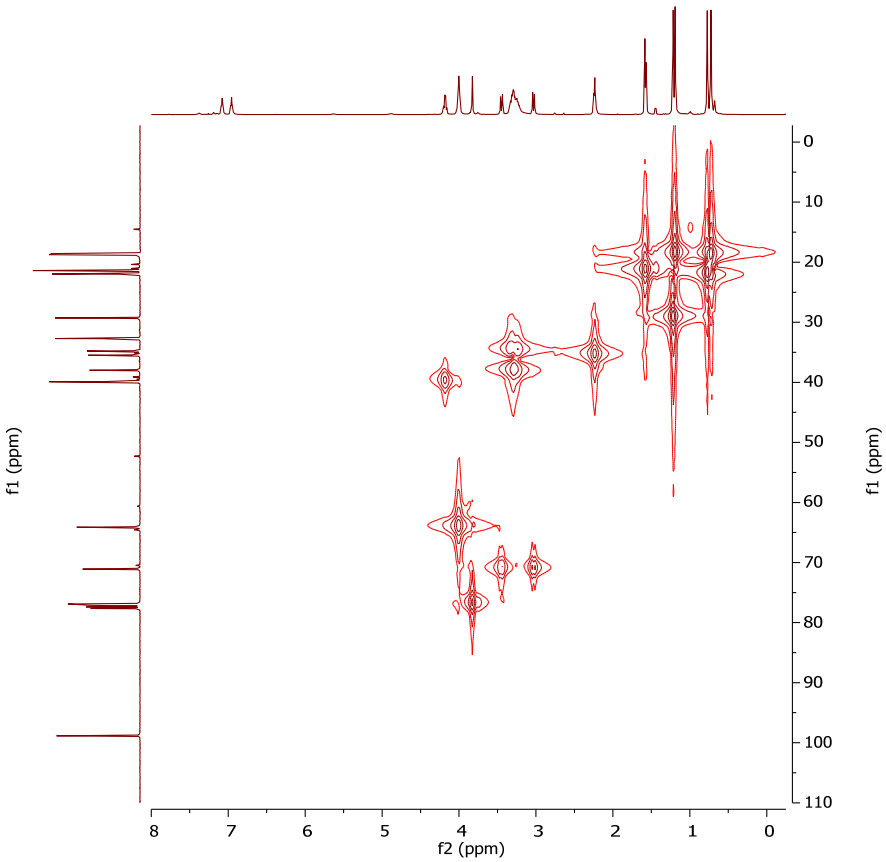

171.69
171.46
170.08
169.99
169.94
169.84
98.86
77.64
77.38
77.13
77.01
76.90
71.08
64.24
64.13
64.08
52.28
39.91
39.10
37.98
35.47
34.80
32.72
29.27
21.97
21.39
21.25
21.04
20.37
18.75
18.57
14.51
O
O
H
N
O
H
N
O
O
Br
14
13C in CDCl3
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



14

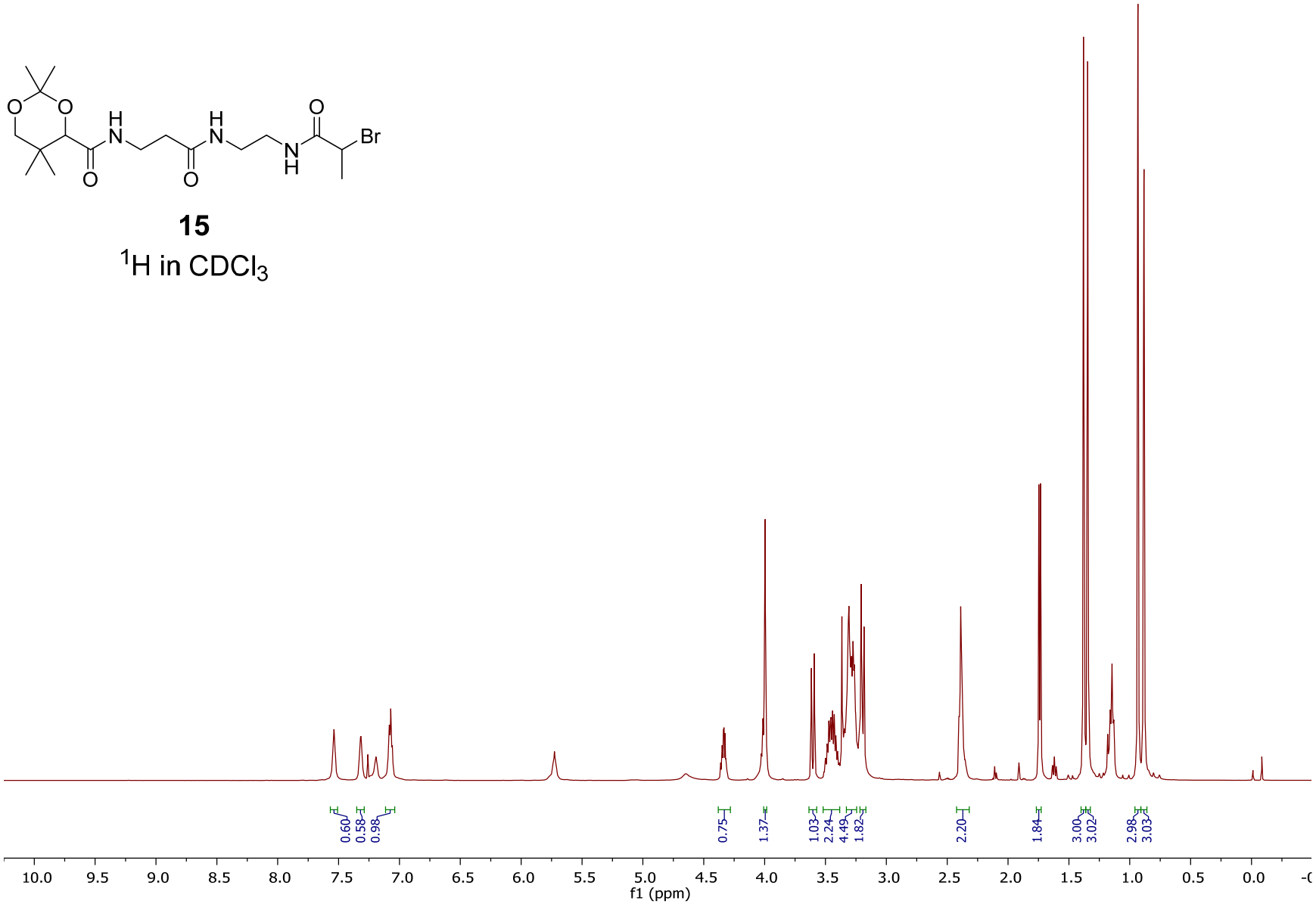
COSY and HMQC in $CDCl_3$

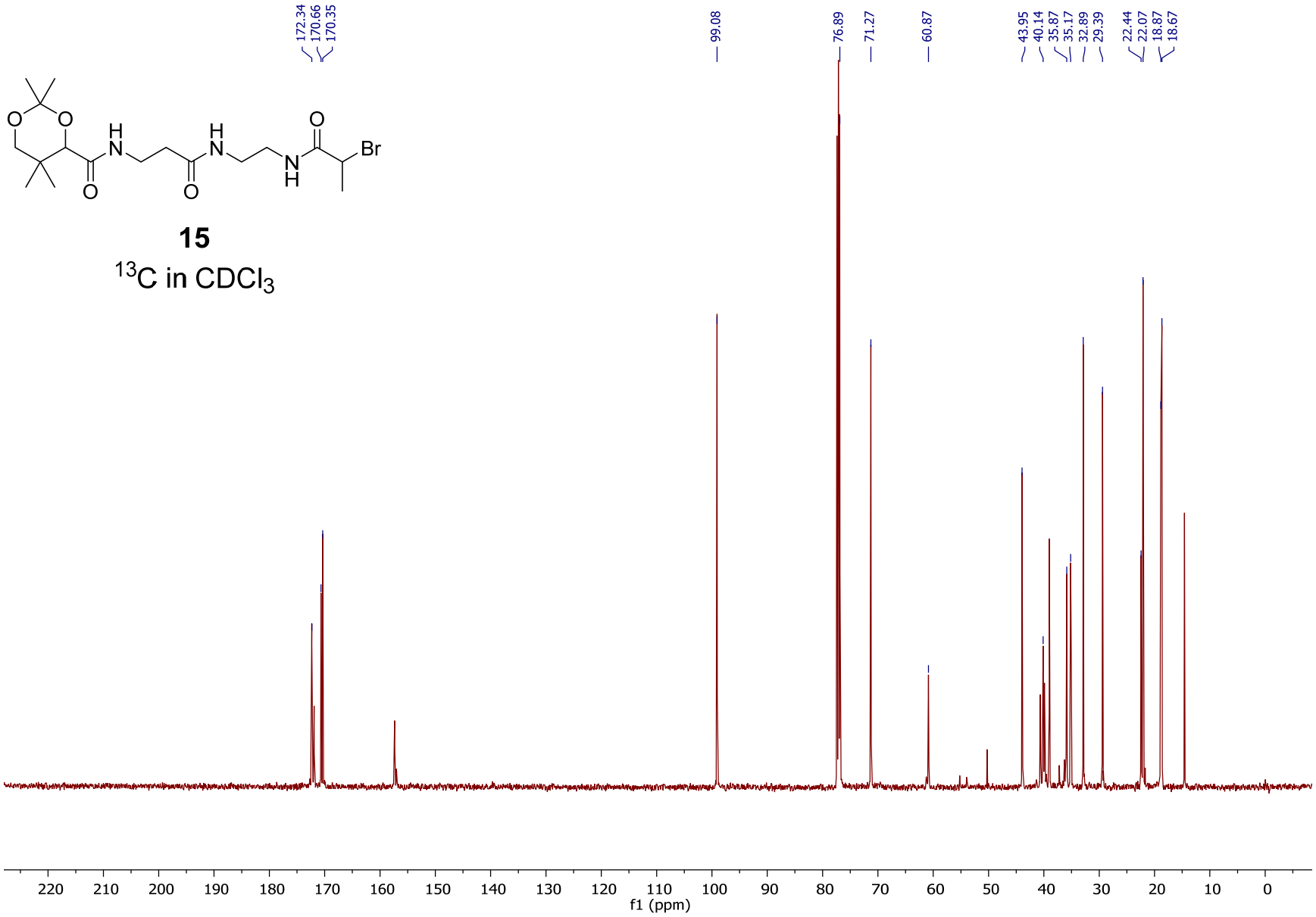


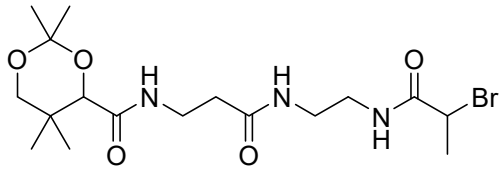


15

^{1}H in $CDCl_3$

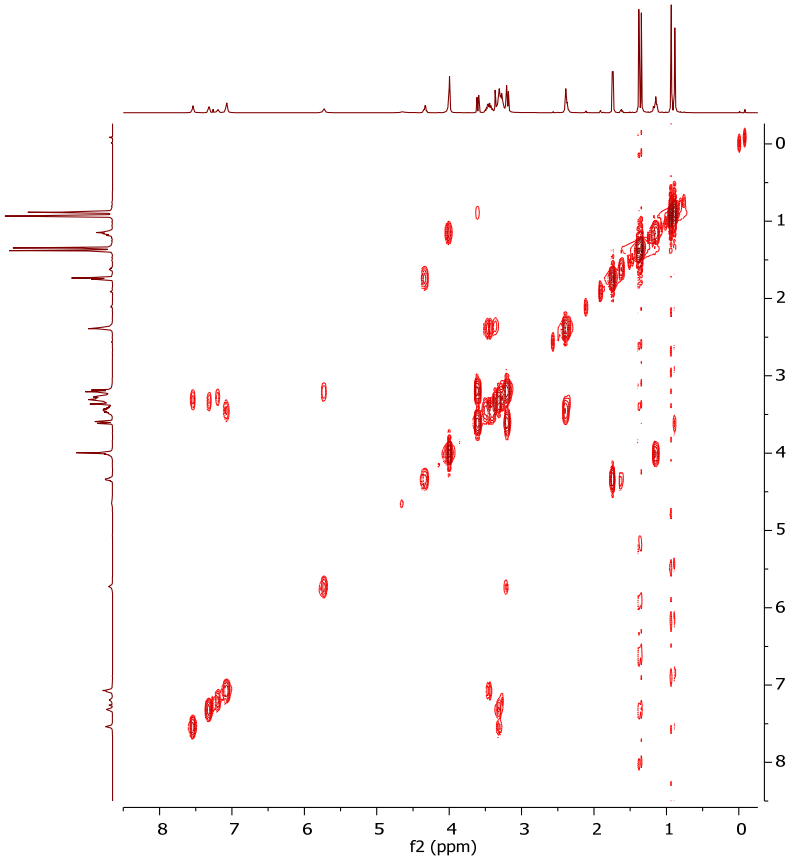


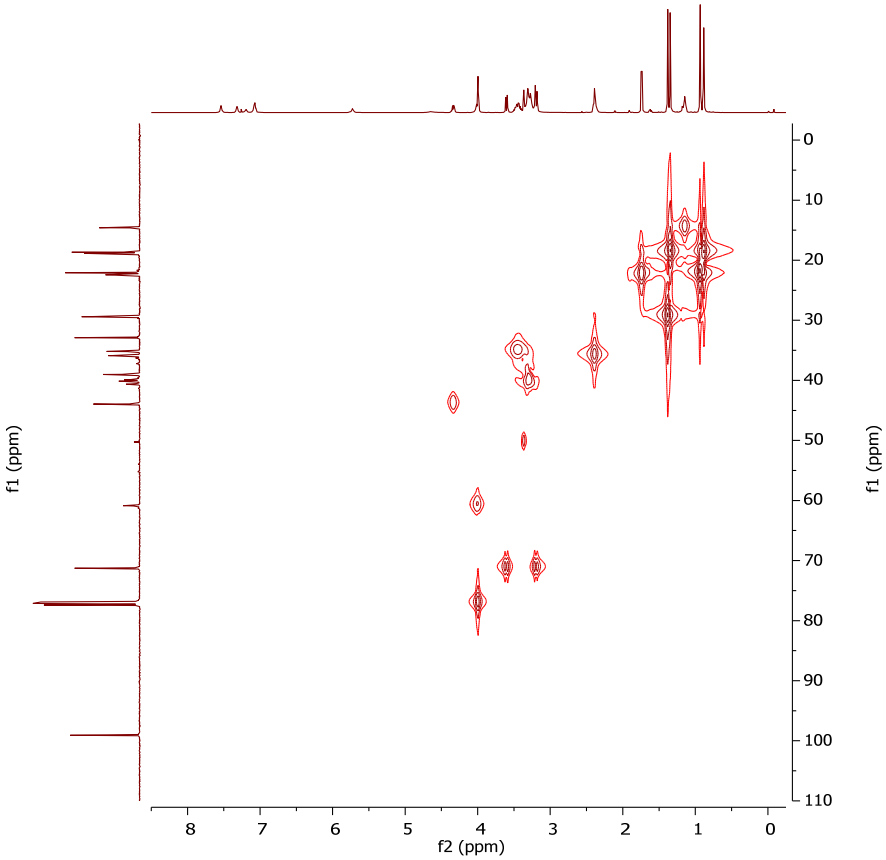

172.34
170.66
170.35
99.08
76.89
71.27
60.87
43.95
40.14
35.87
35.17
32.89
29.39
22.44
22.07
18.87
18.67
O
O
H
N
O
H
N
O
N
H
Br
15
13C in CDCl3
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



15

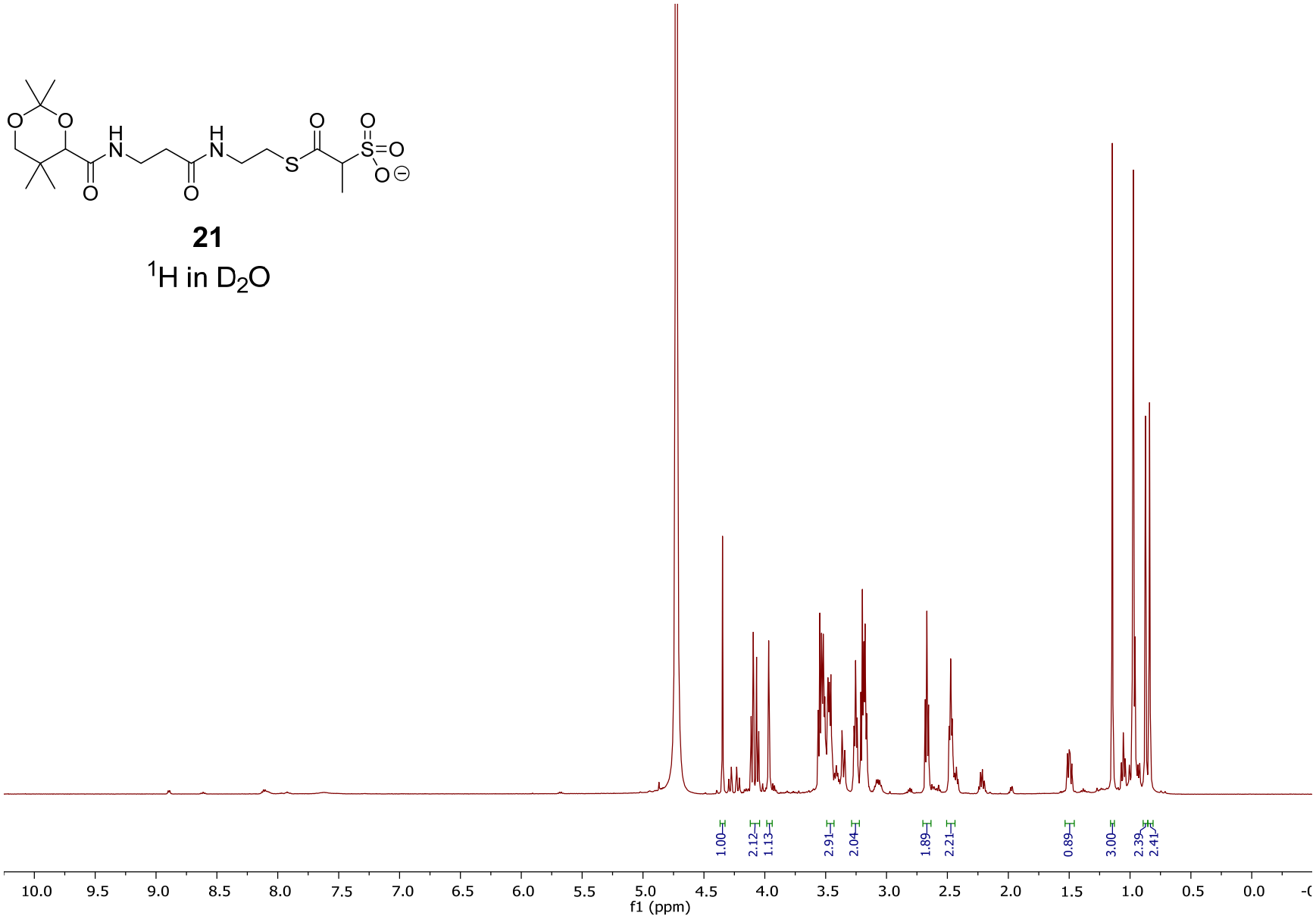
COSY and HMQC in $CDCl_3$

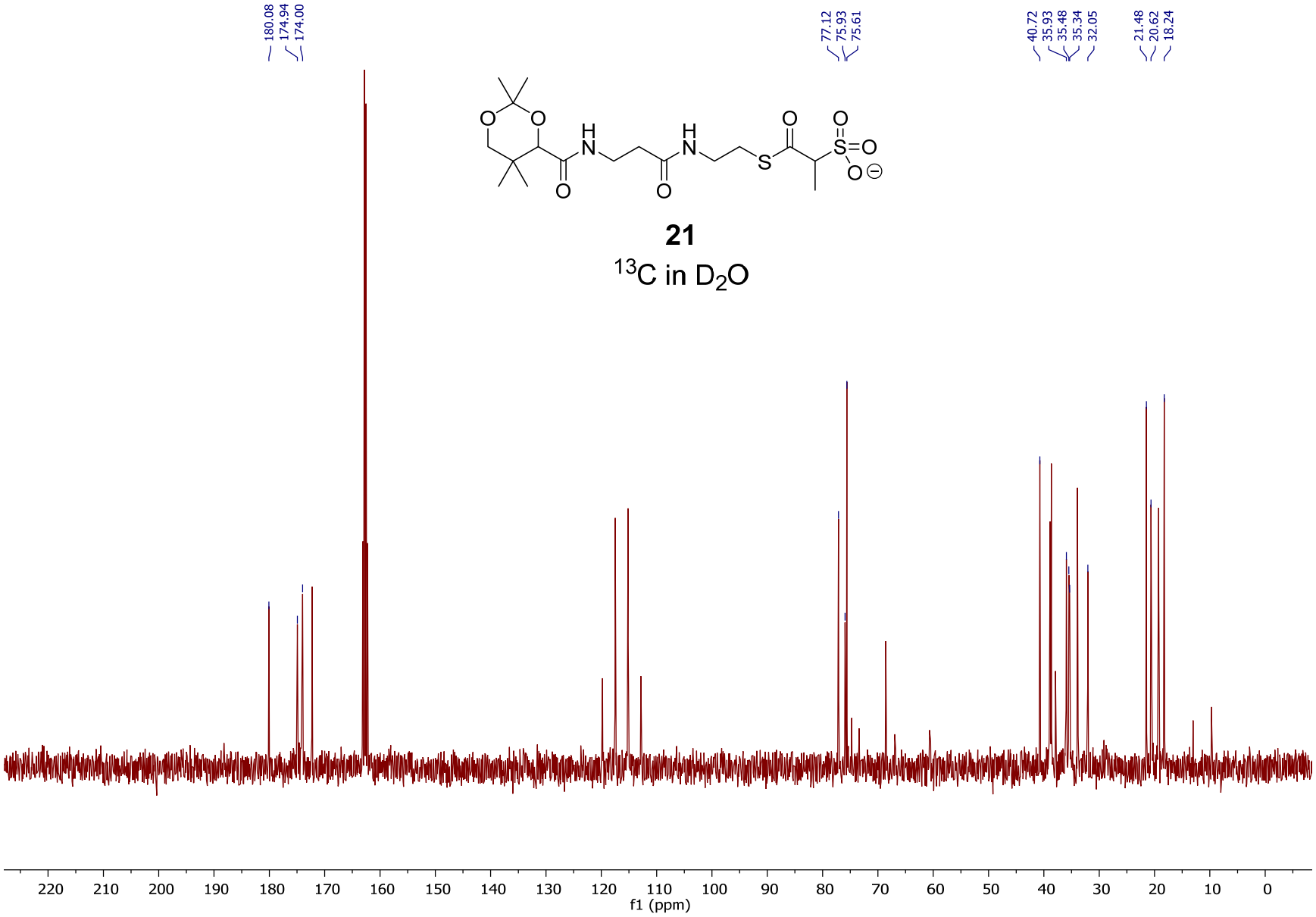


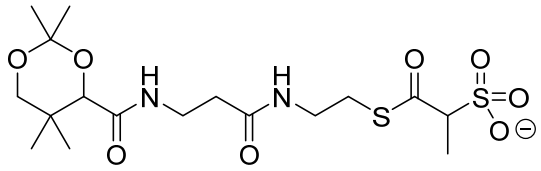


21

^{1}H in D_2O

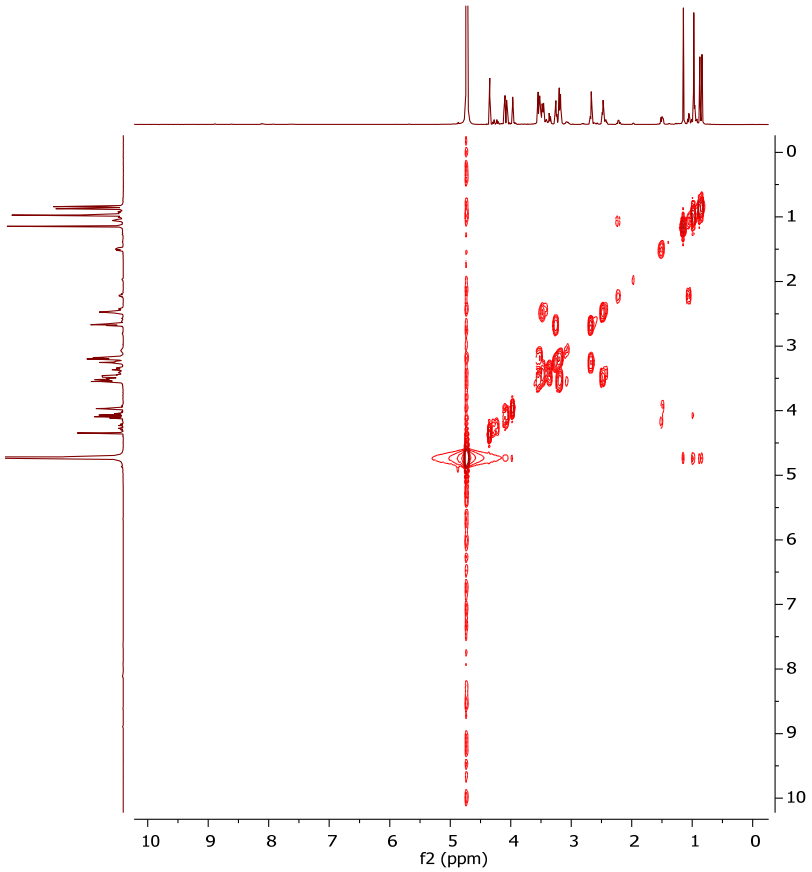

1.00
2.12
1.13
2.91
2.04
1.89
2.21
0.89
3.00
2.39
2.41
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

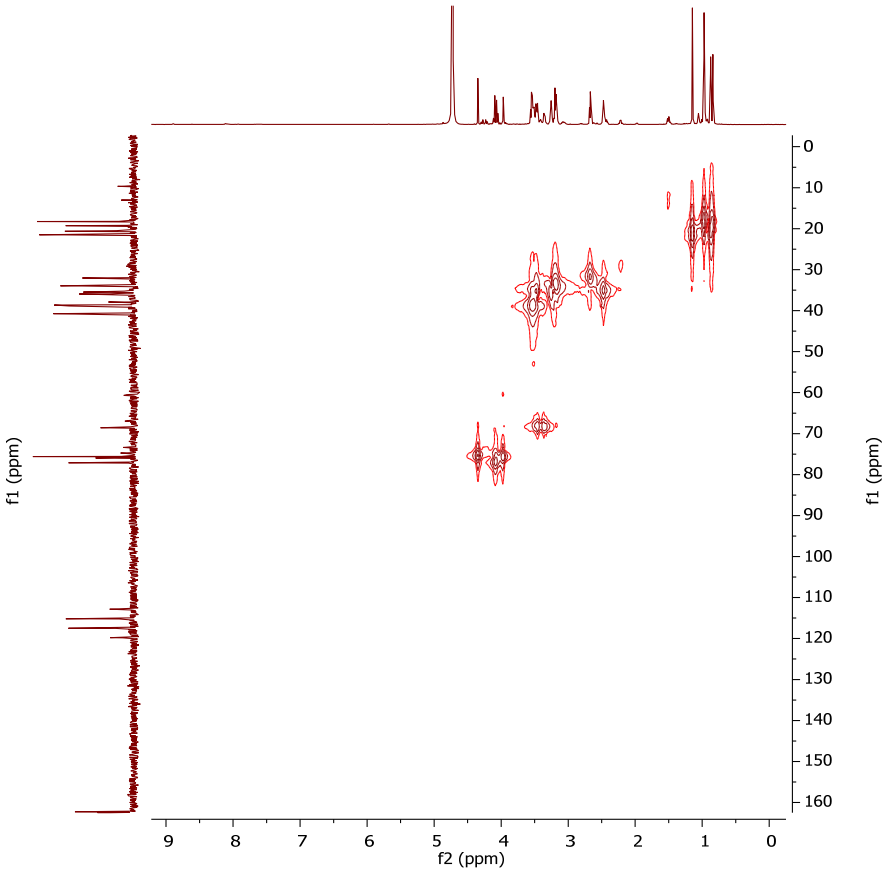

180.08
174.94
174.00
77.12
75.93
75.61
40.72
35.93
35.48
35.34
32.05
21.48
20.62
18.24
21
13C in D2O
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



21

COSY and HMQC in D_2O

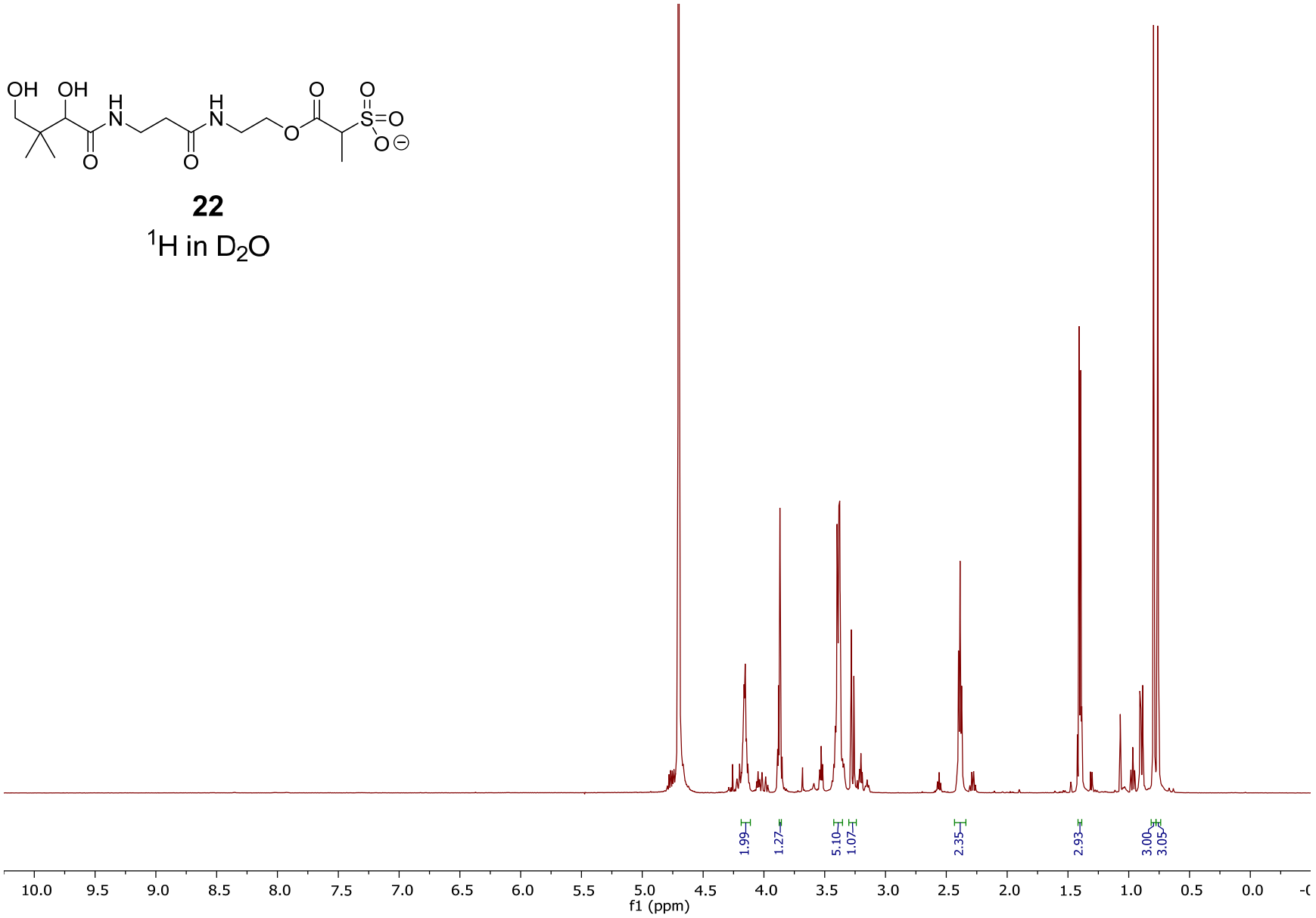


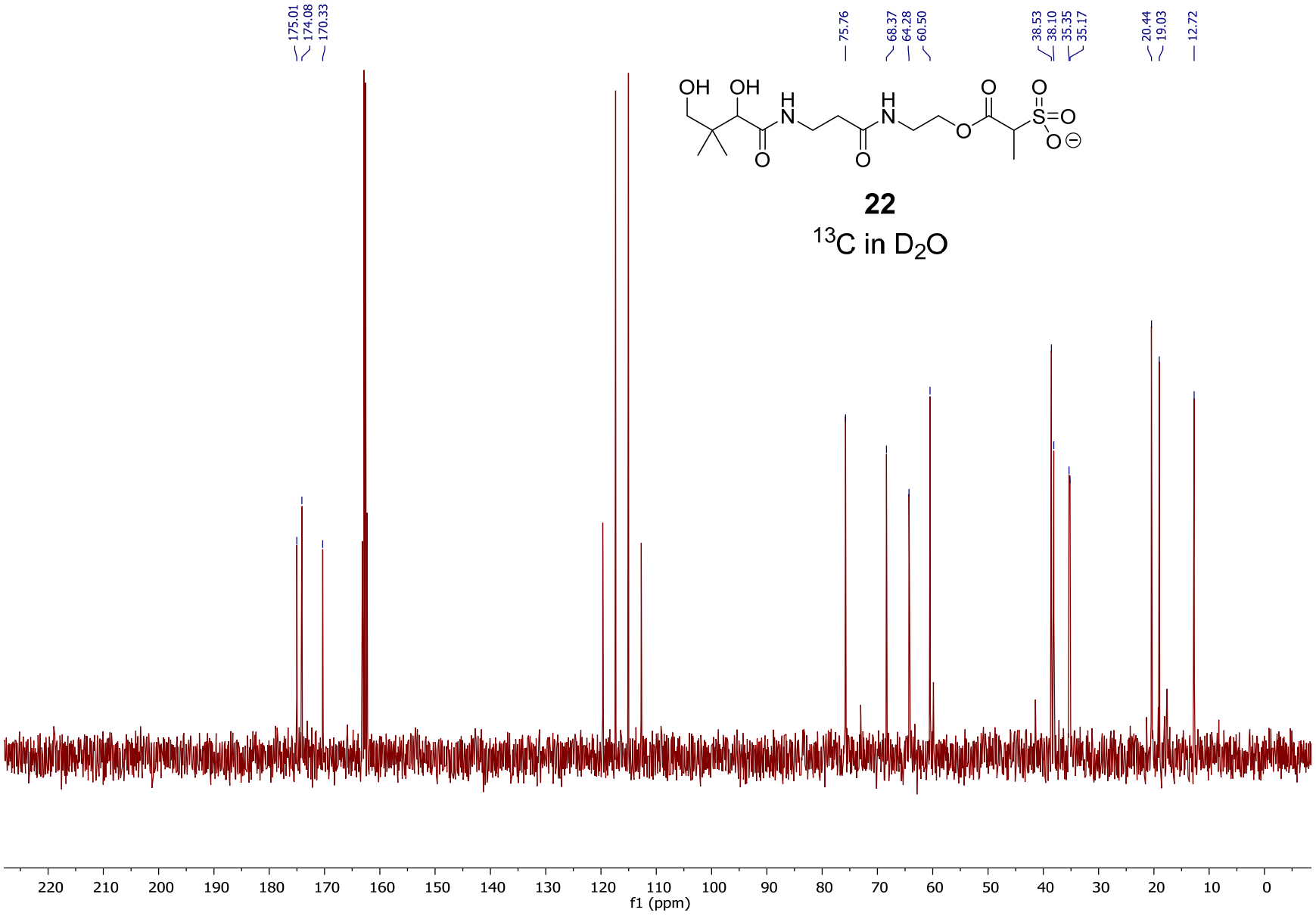


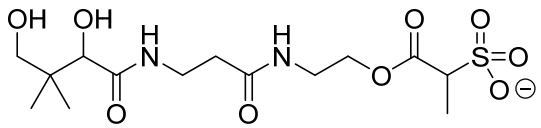
OH
OH
H
N
O
H
N
O
O
O
S=O
O

22

^{1}H in D_2O

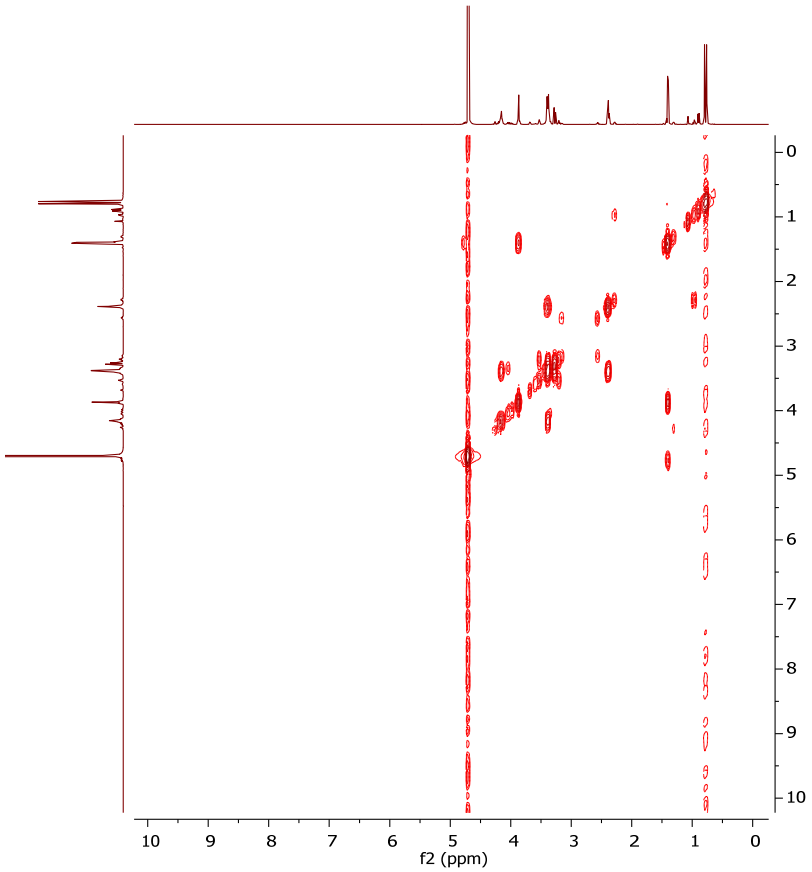

1.99
1.27
5.10
1.07
2.35
2.93
3.00
3.05
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

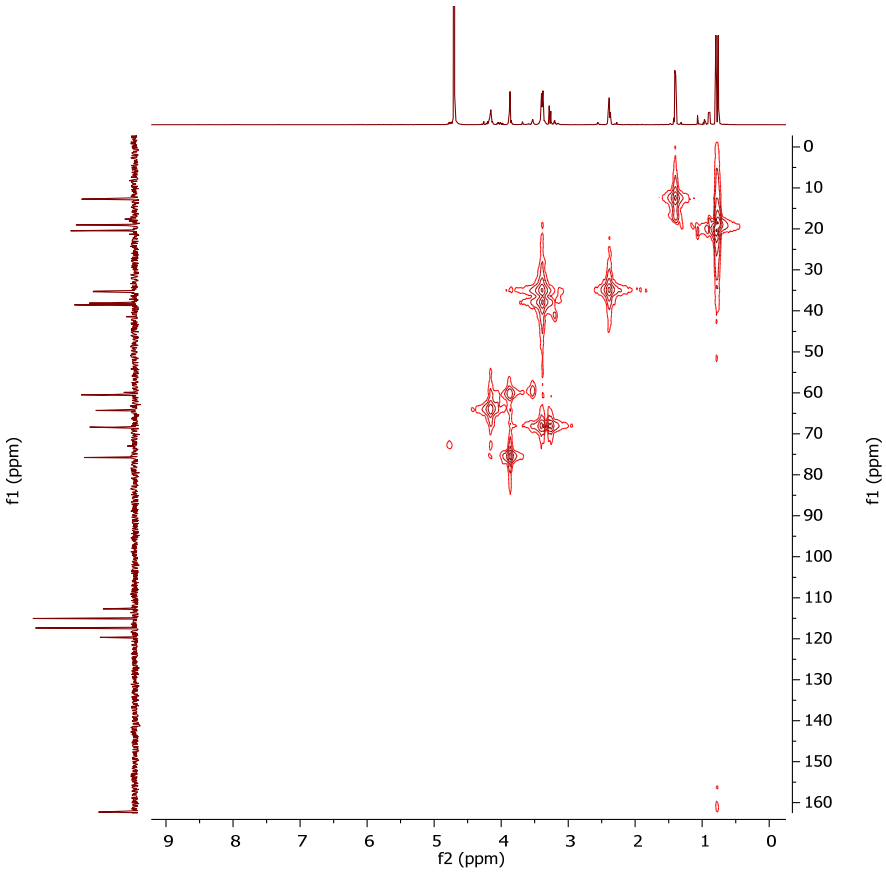

175.01
174.08
170.33
75.76
68.37
64.28
60.50
38.53
38.10
35.35
35.17
20.44
19.03
12.72
OH
OH
H
N
O
H
N
O
O
O
S=O
O
22
13C in D2O
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



22

COSY and HMQC in D_2O

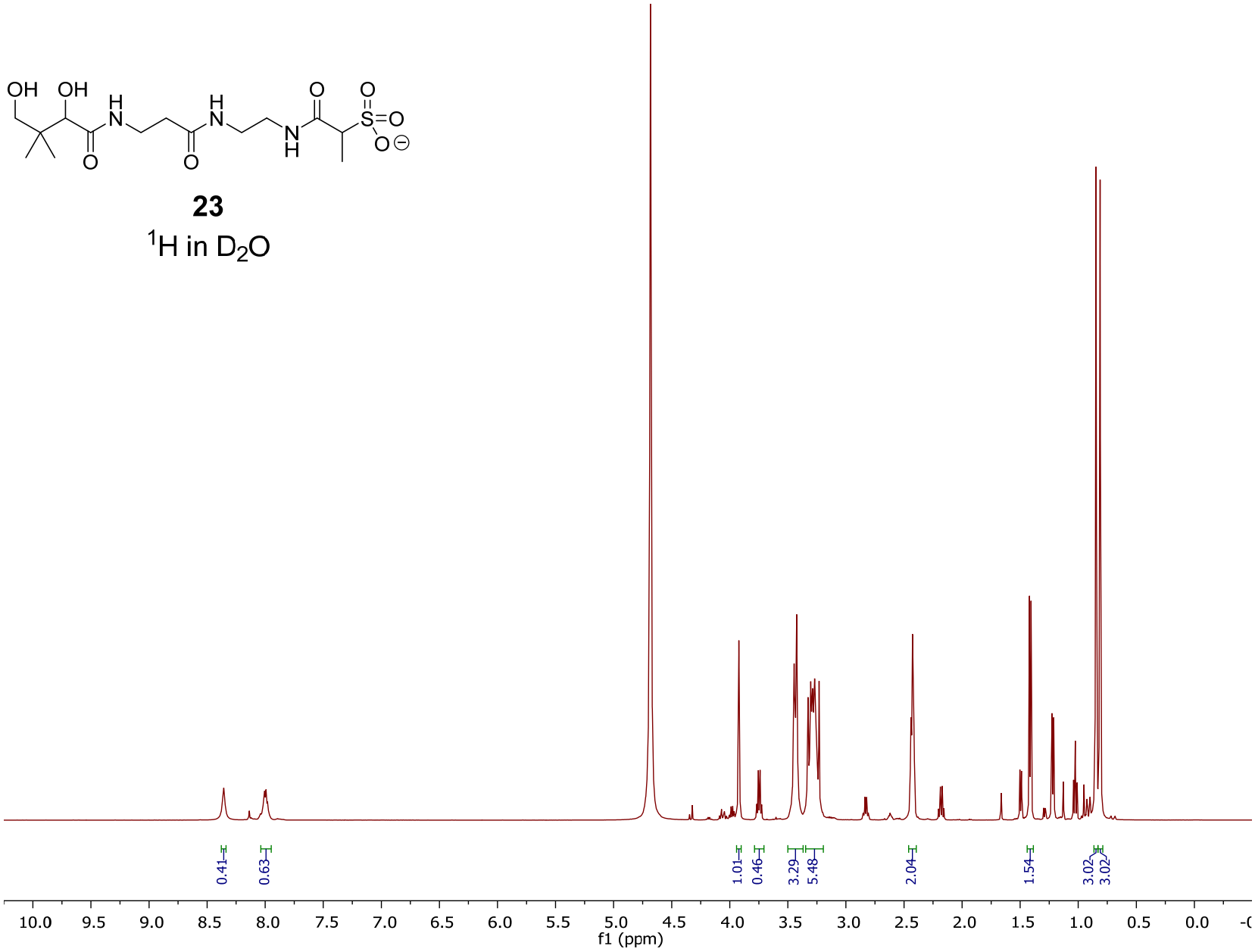


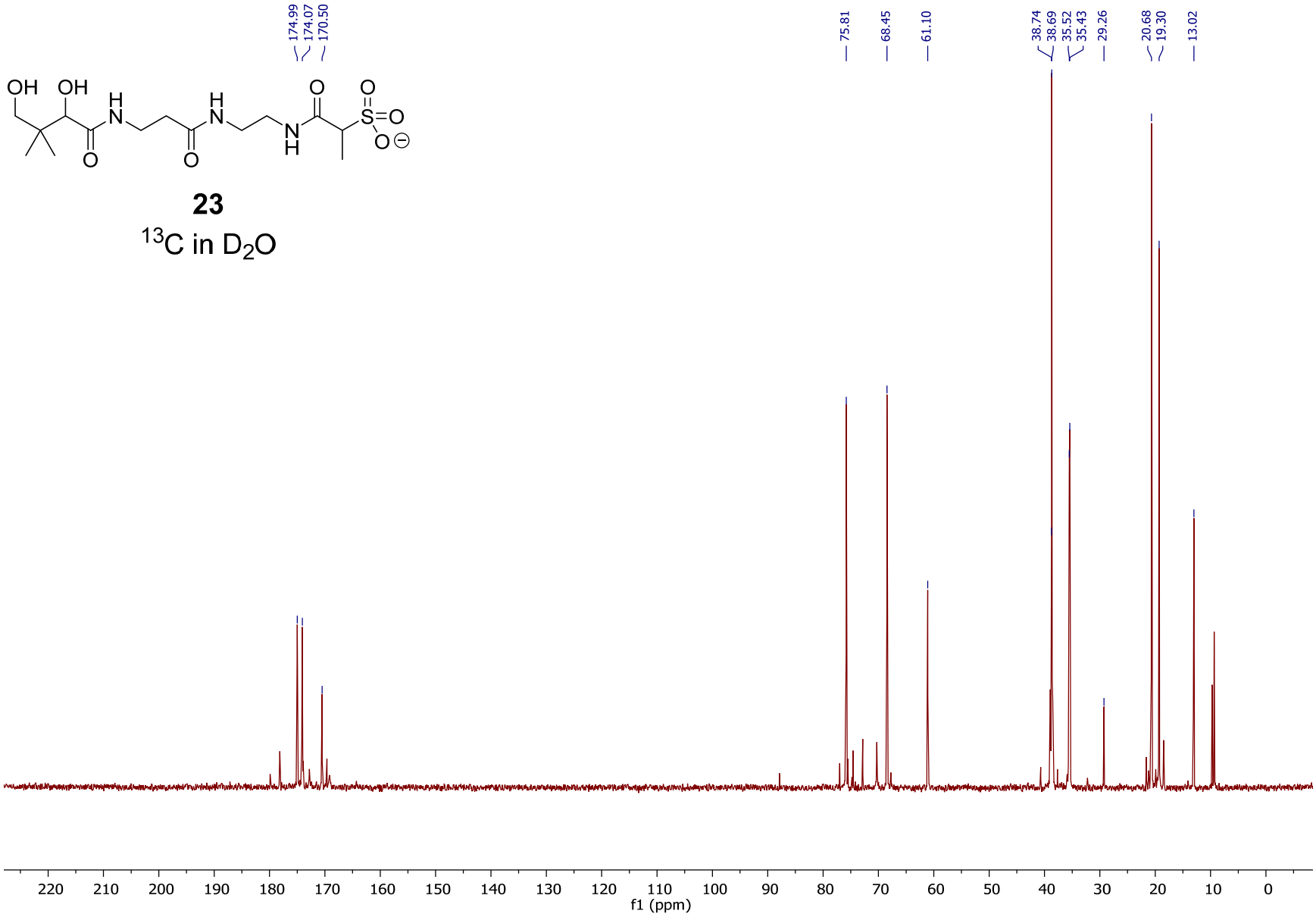


OH
OH
H
N
O
H
N
O
O
N
H
O
S=O
O⊖

23

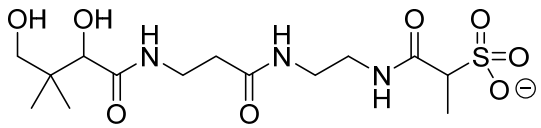
^{1}H in D_2O


0.41
0.63
1.01
0.46
3.29
5.48
2.04
1.54
3.02
3.02
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


174.99
174.07
170.50
75.81
68.45
61.10
38.74
38.69
35.52
35.43
29.26
20.68
19.30
13.02
OH
OH
H
N
O
H
N
O
N
H
O
O
S=O
O⊖
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

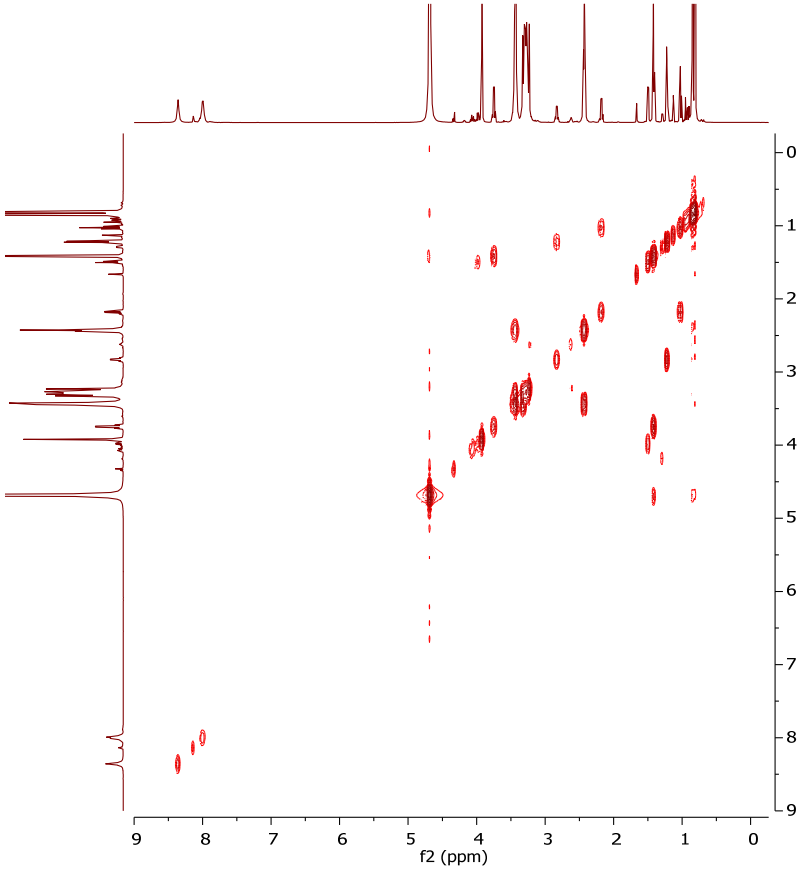
23

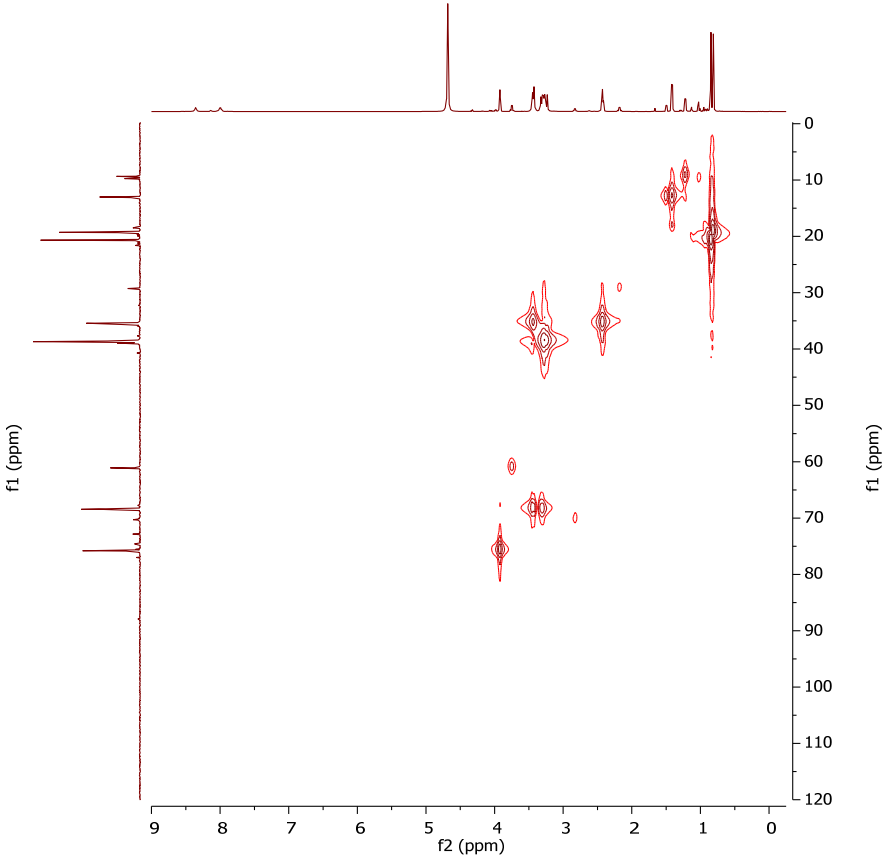
^{13}C in D_2O



23

COSY and HMQC in D_2O

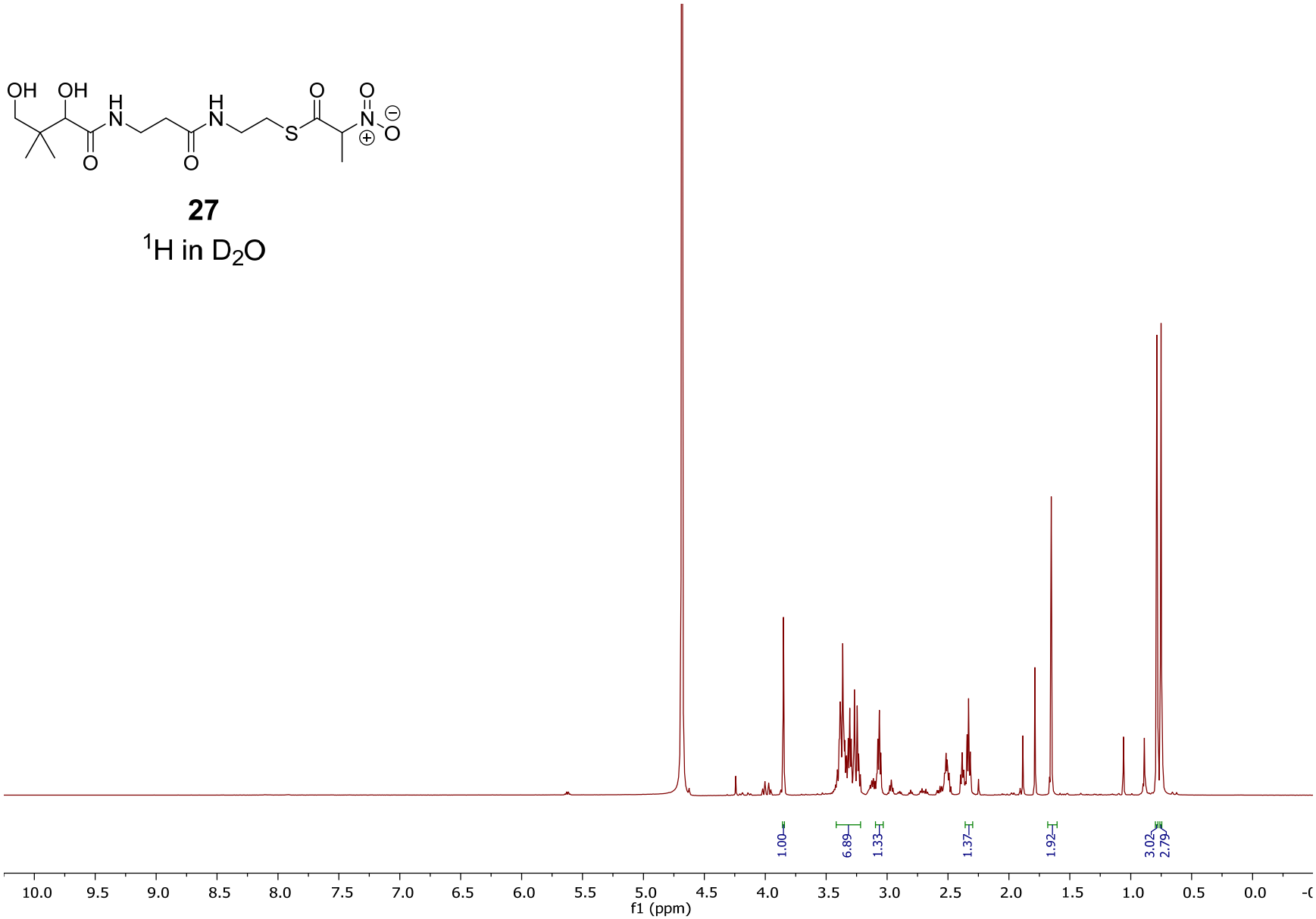


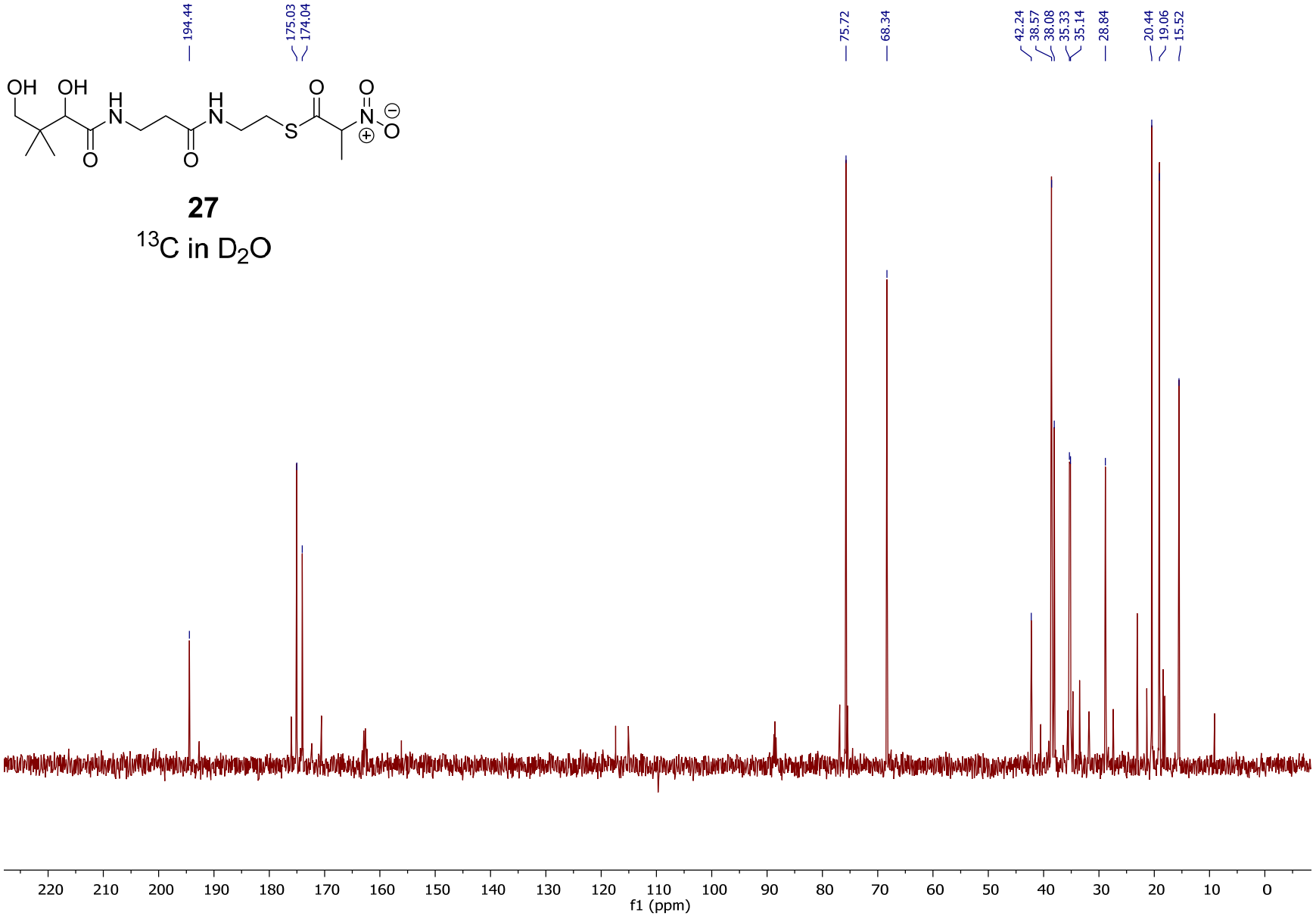


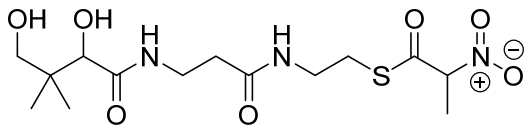
OH
OH
H
N
O
H
N
O
O
S
O
O
N
O

27

^{1}H in D_2O

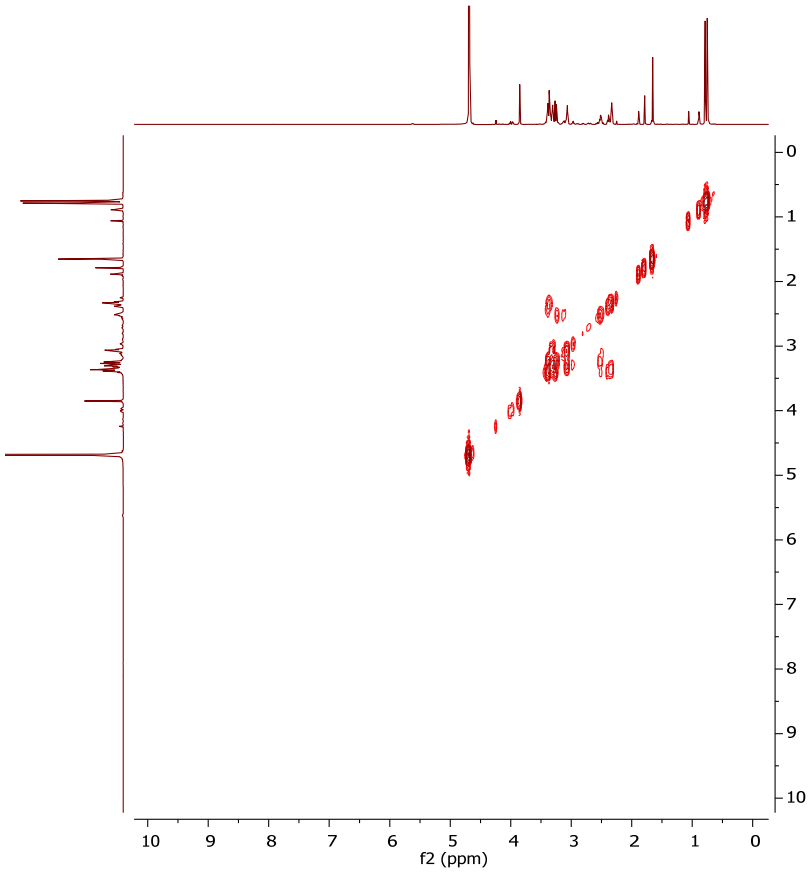

1.00
6.89
1.33
1.37
1.92
3.02
2.79
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

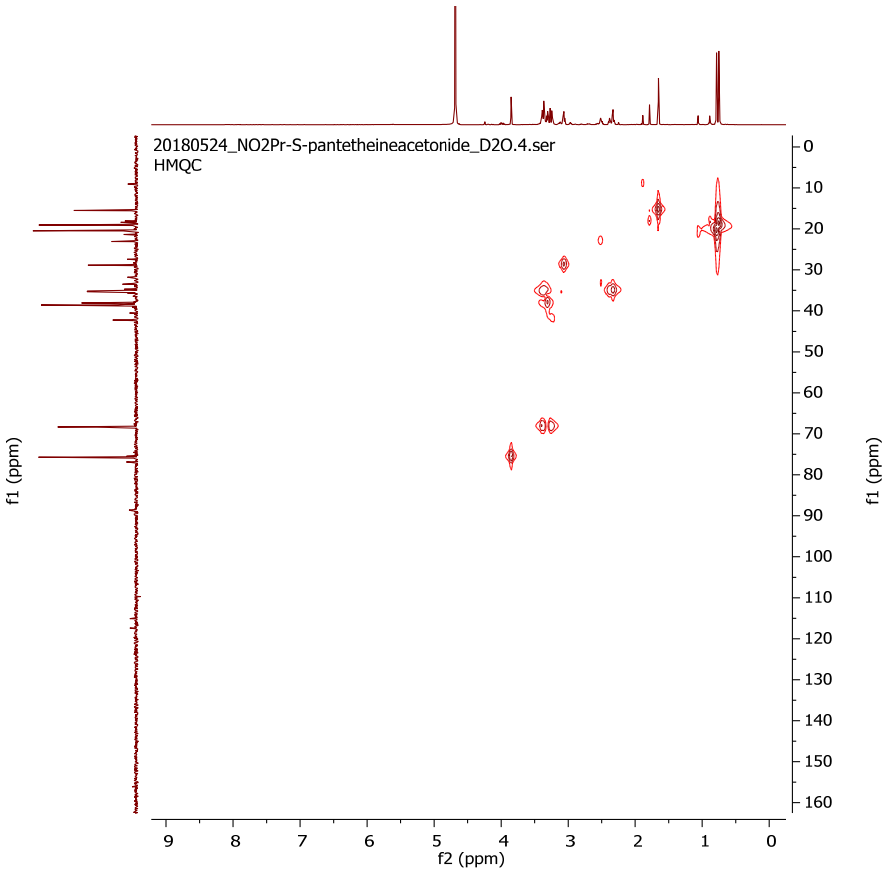

194.44
175.03
174.04
75.72
68.34
42.24
38.57
38.08
35.33
35.14
28.84
20.44
19.06
15.52
OH
OH
H
N
O
H
N
O
S
O
O
N
O
O
27
13C in D2O
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



27

COSY and HMQC in D_2O

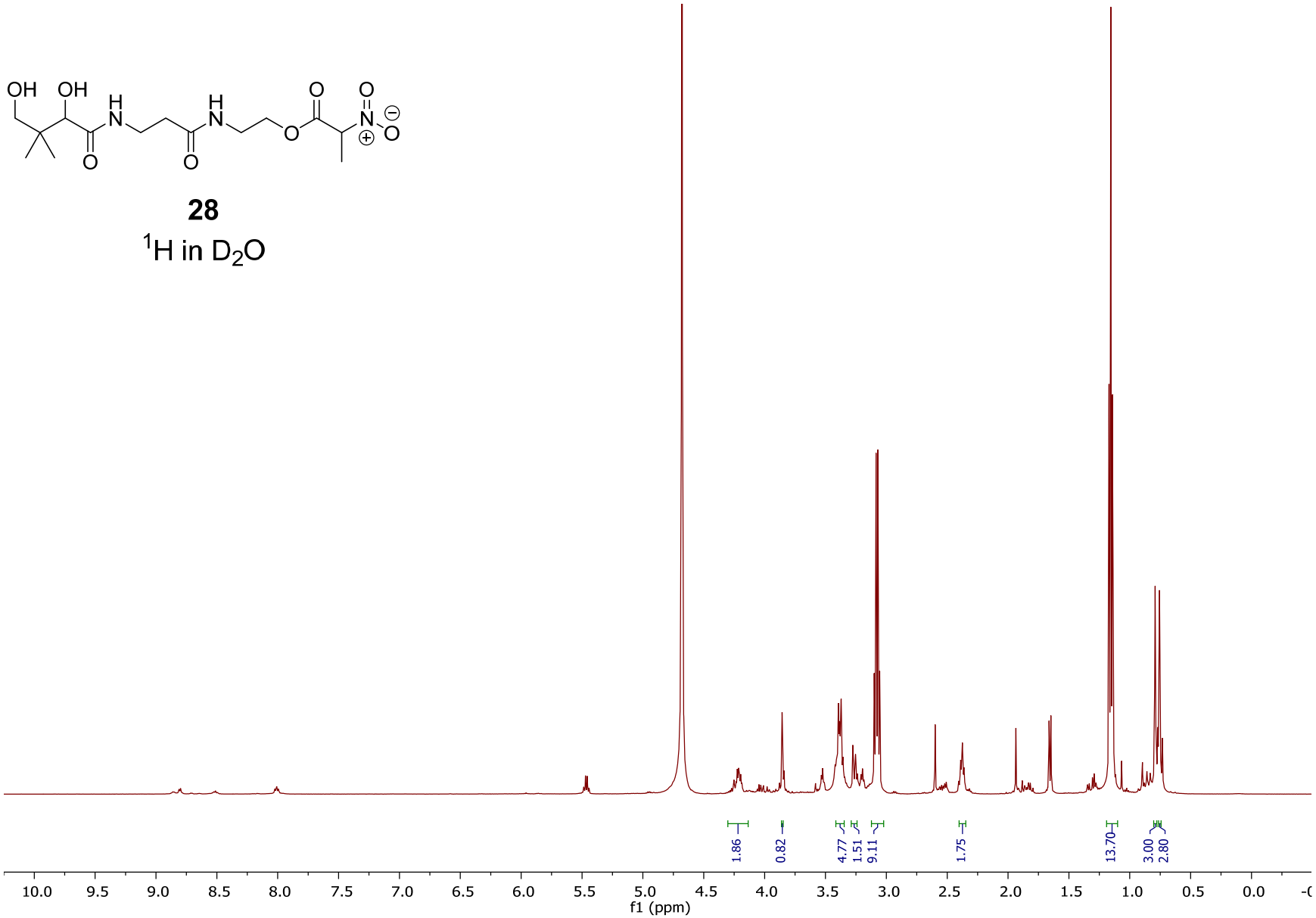


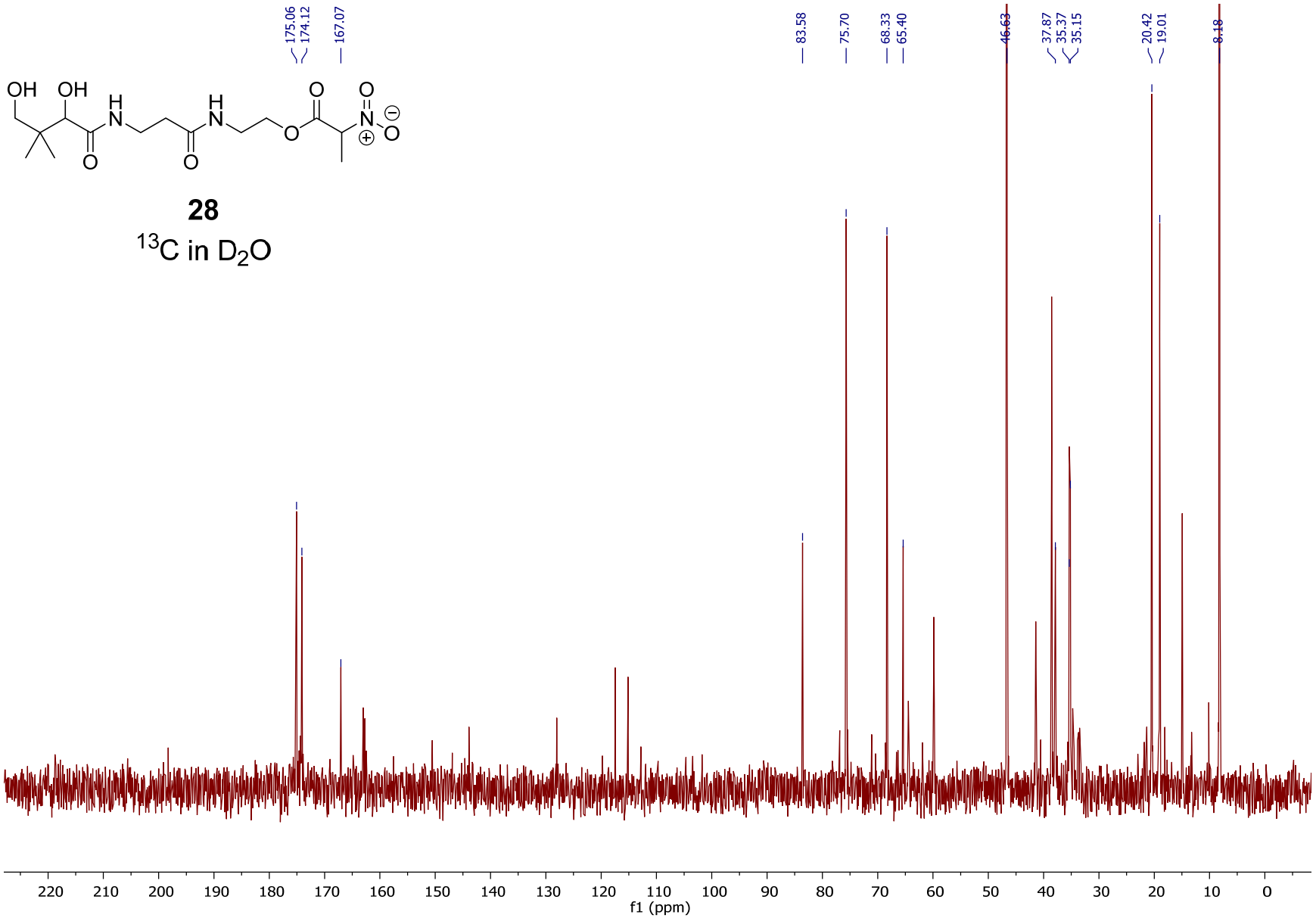


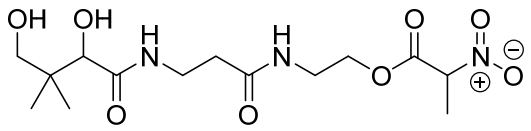
OH
OH
H
N
O
H
N
O
O
O
N
O

28

^{1}H in D_2O

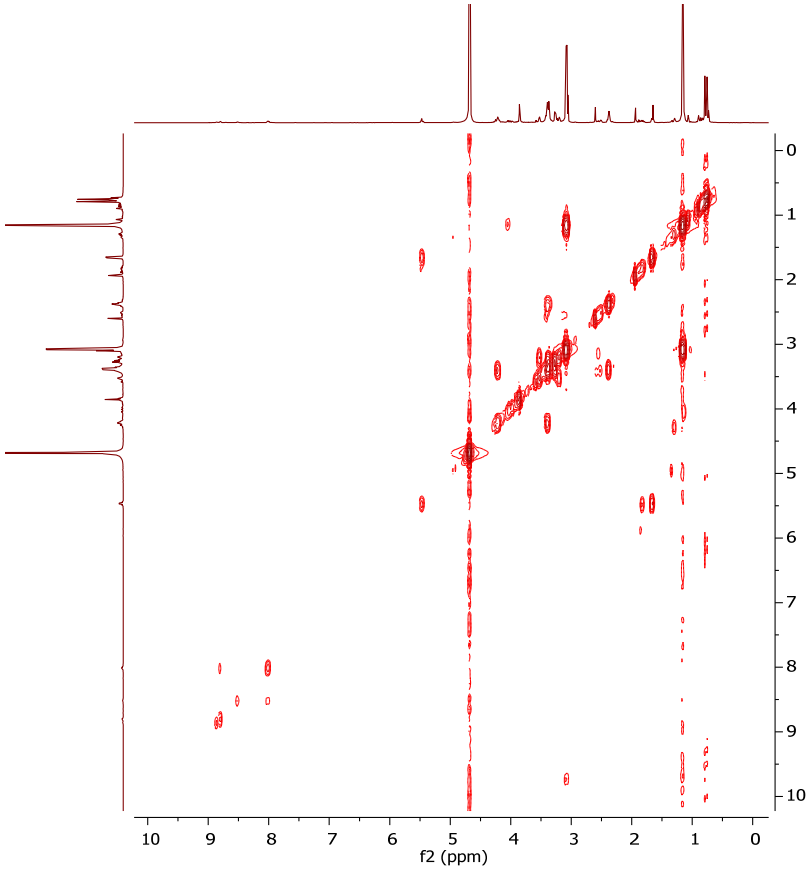

1.86
0.82
4.77
1.51
9.11
1.75
13.70
3.00
2.80
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

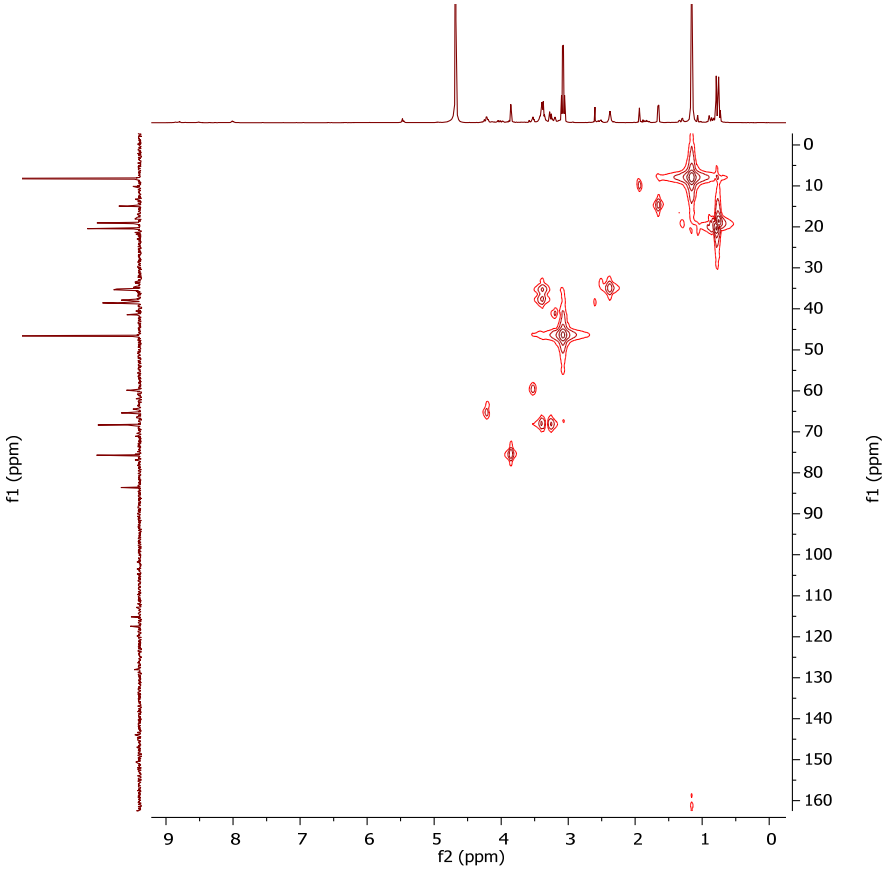

175.06
174.12
167.07
83.58
75.70
68.33
65.40
46.63
37.87
35.37
35.15
20.42
19.01
8.18
OH
OH
H
N
O
H
N
O
O
O
N
O
O
28
13C in D2O
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



28

COSY and HMQC in D_2O

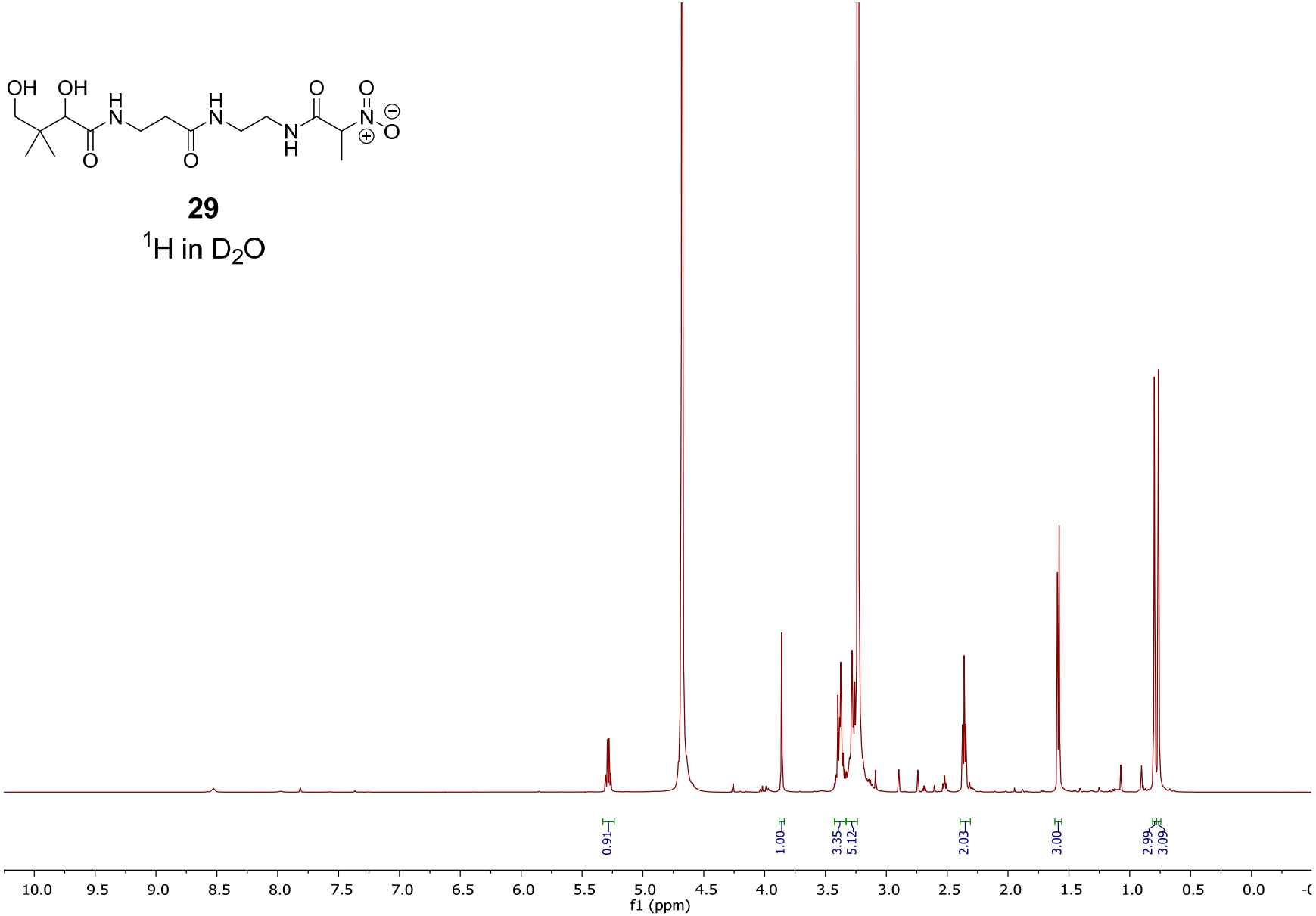


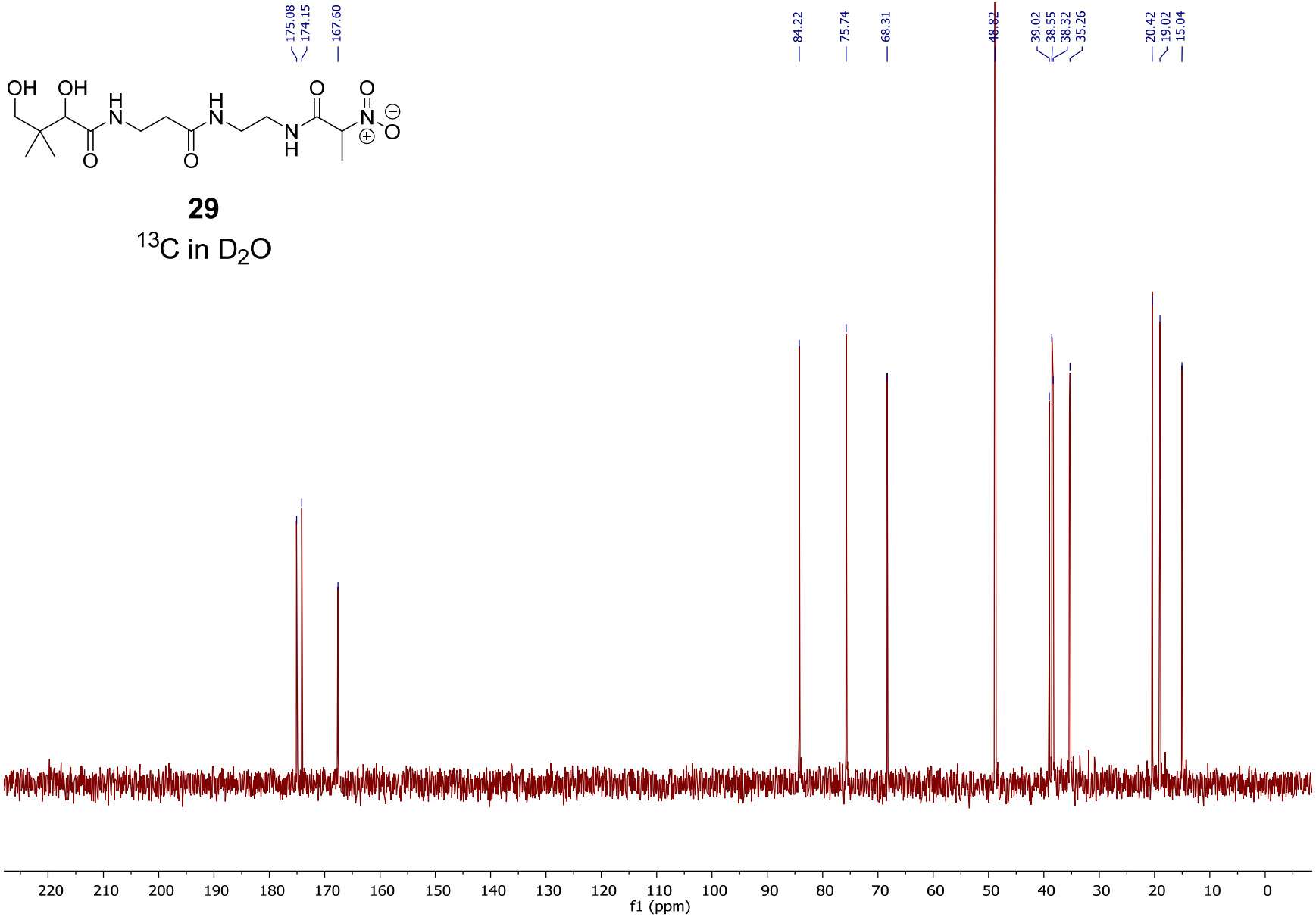


OH
OH
H
N
O
H
N
O
O
N
H
O
O
N
O

29

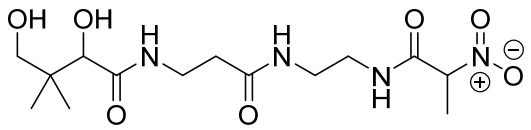
^{1}H in D_2O


0.91
1.00
3.35
5.12
2.03
3.00
2.99
3.09
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


175.08
174.15
167.60
84.22
75.74
68.31
48.82
39.02
38.55
38.32
35.26
20.42
19.02
15.04
OH
OH
H
N
O
H
N
O
N
H
O
O
N
O
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

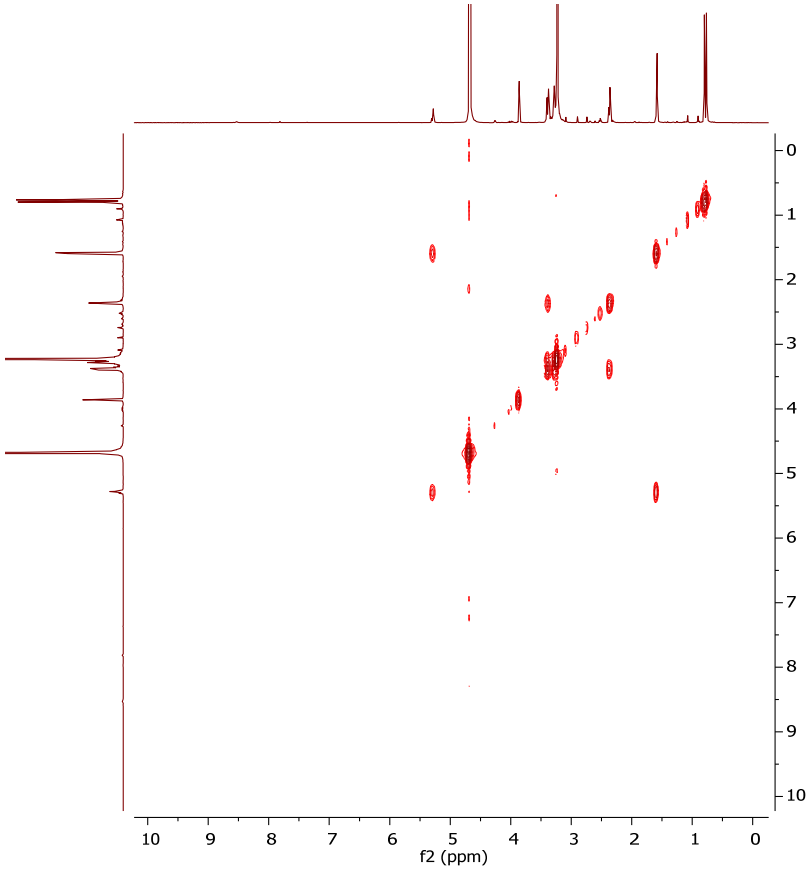
29

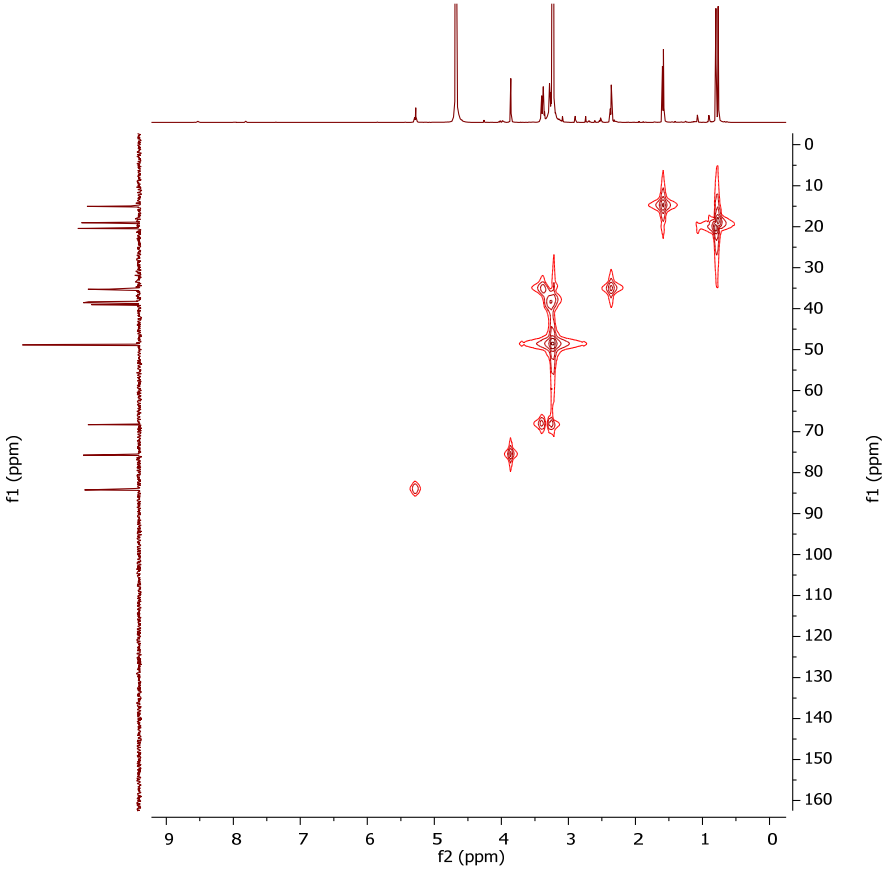
^{13}C in D_2O

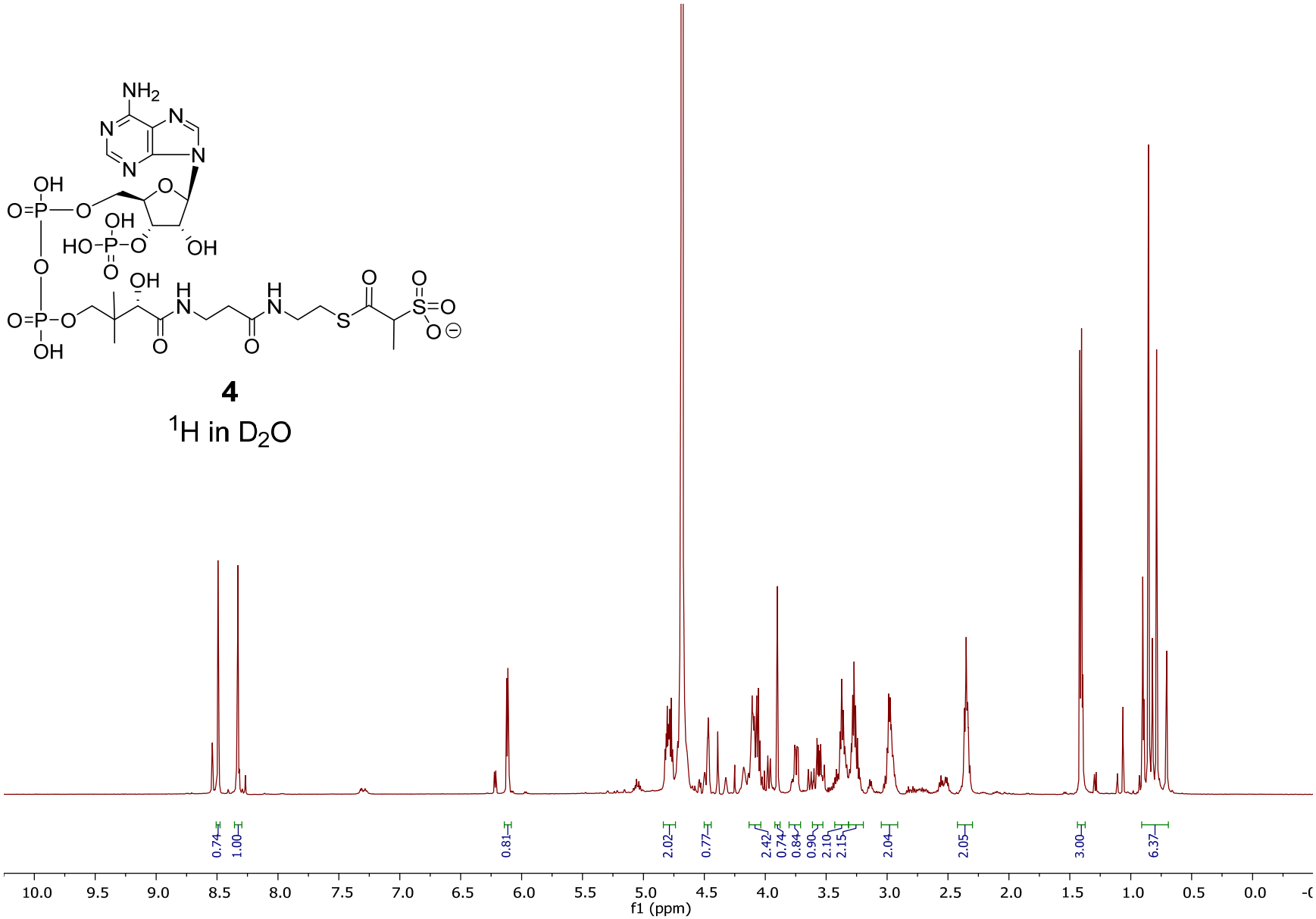


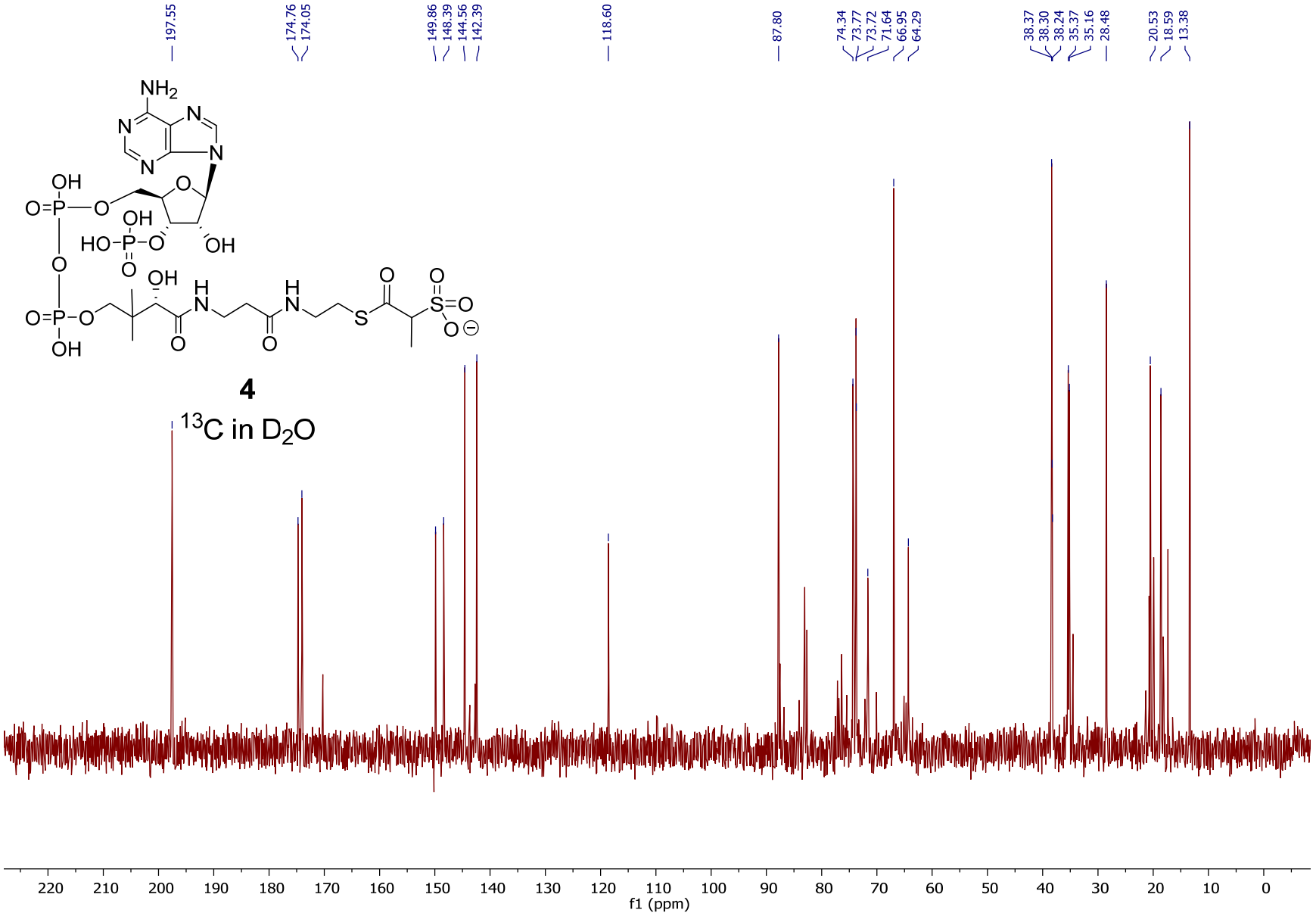
29

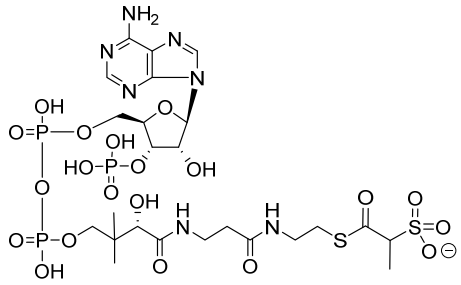
COSY and HMQC in D_2O





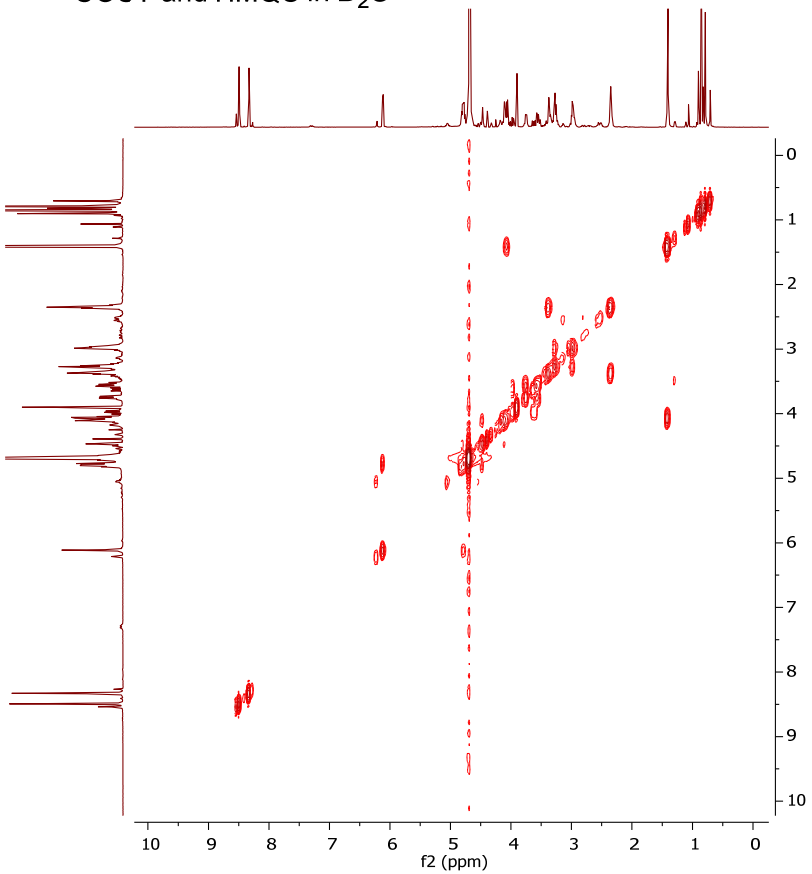

4
^{1}H in D_2O
0.74
1.00
0.81
2.02
0.77
2.42
0.74
0.84
0.90
2.10
2.15
2.04
2.05
3.00
6.37
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

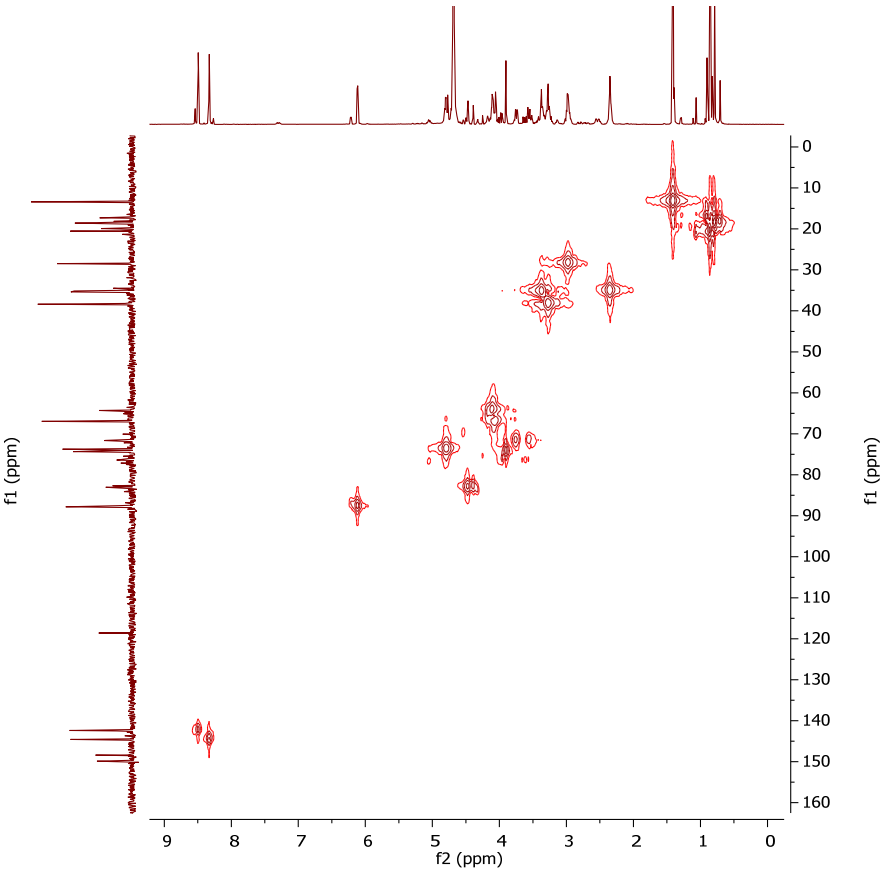

197.55
174.76
174.05
149.86
148.39
144.56
142.39
118.60
87.80
74.34
73.77
73.72
71.64
66.95
64.29
38.37
38.30
38.24
35.37
35.16
28.48
20.53
18.59
13.38
4
13C in D2O
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

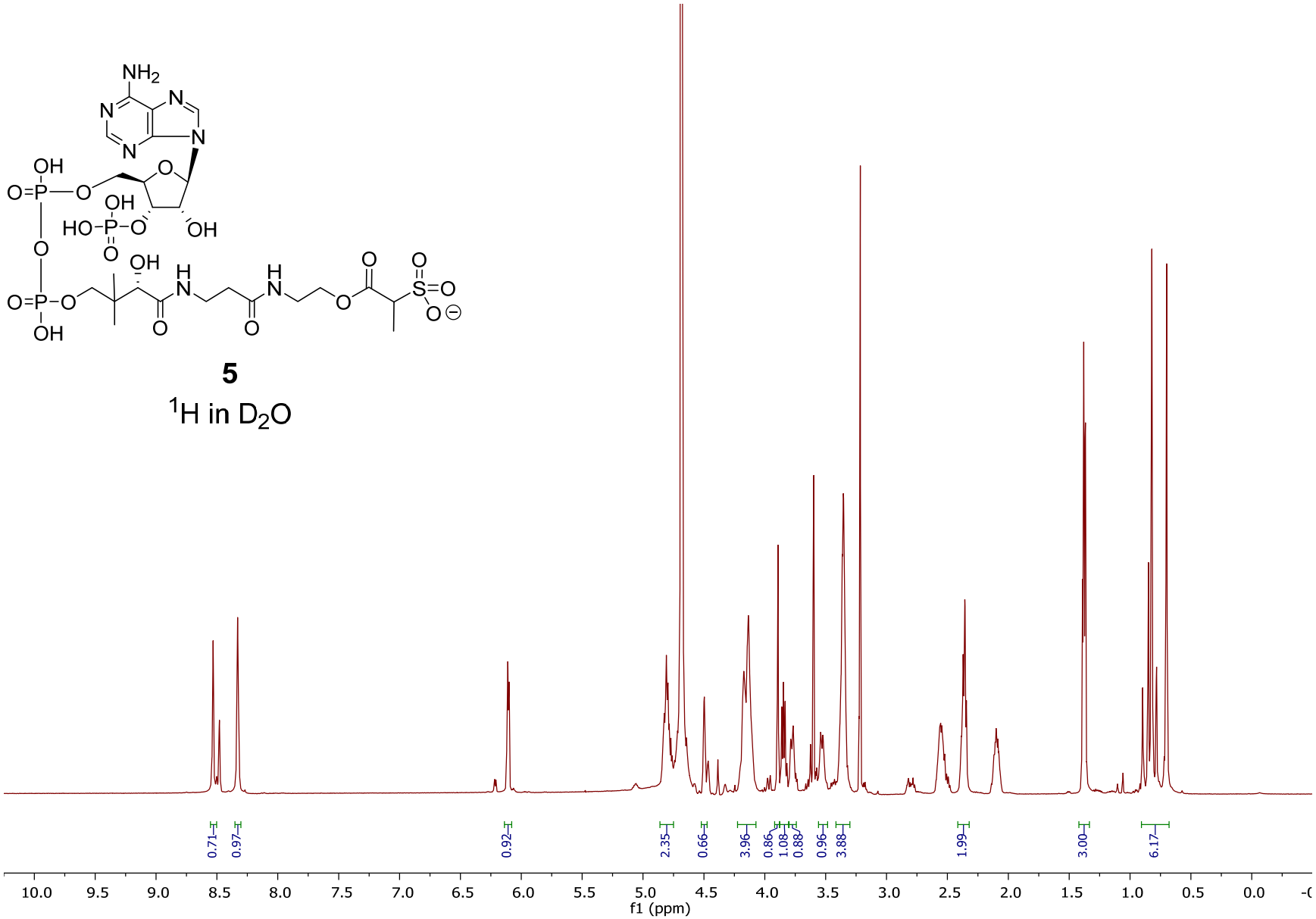


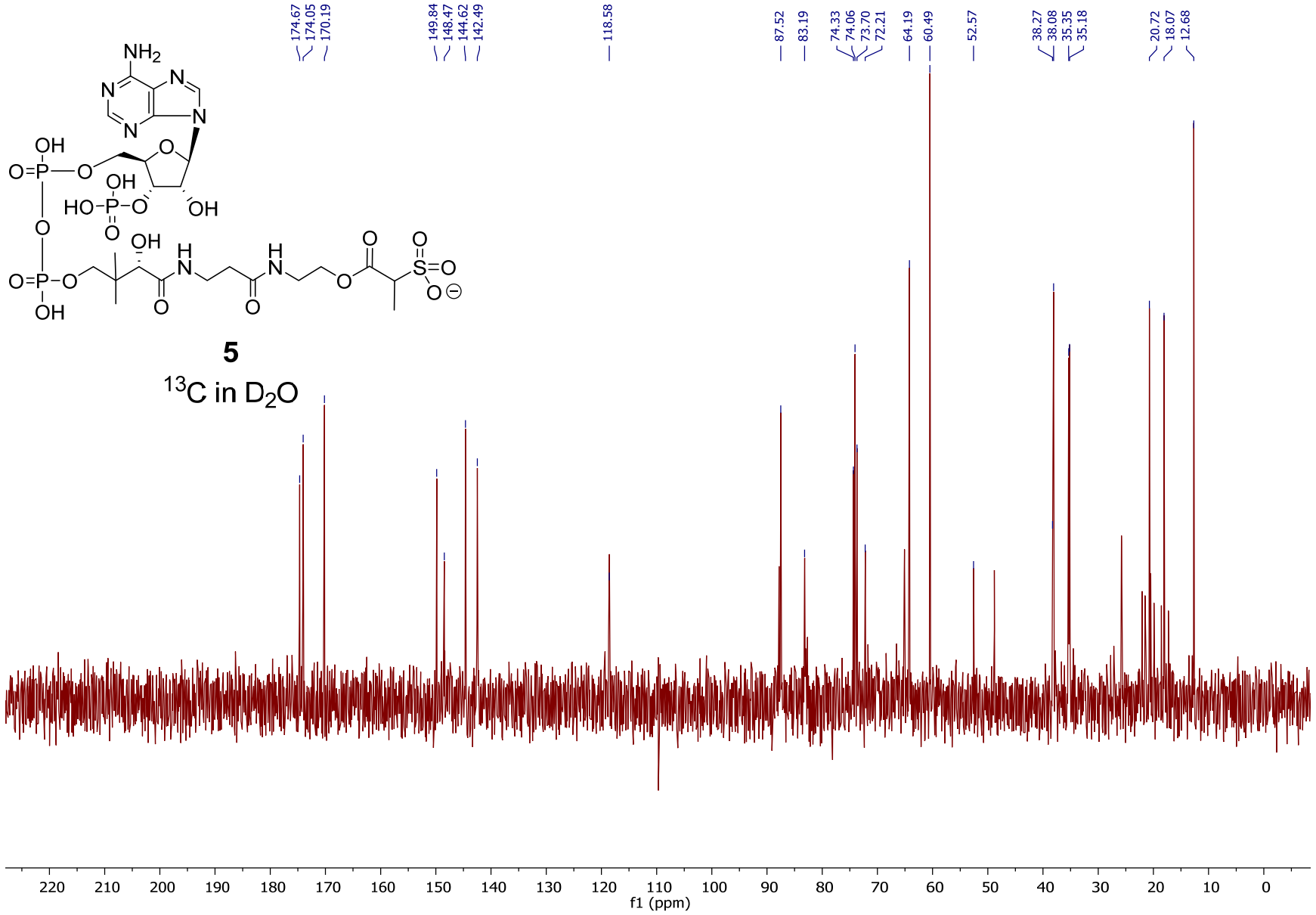
4

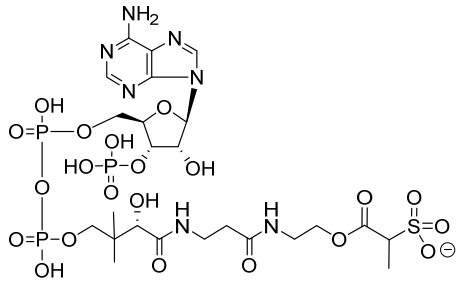
COSY and HMQC in D_2O





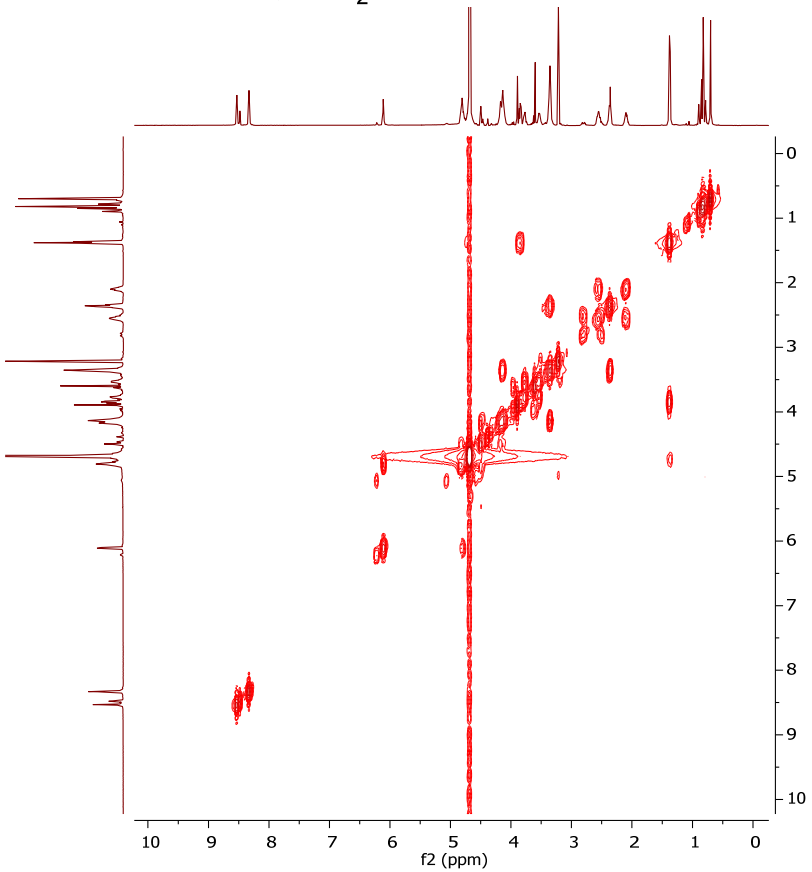

NH2
N
N
N
N
OH
O=P—O
O
OH
HO–P–O
O
OH
O
OH
O=P–O
OH
H
N
H
N
O
O
O
O
S=O
O⊖
5
1H in D2O
0.71
0.97
0.92
2.35
0.66
3.96
0.86
1.08
0.88
0.96
3.88
1.99
3.00
6.17
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

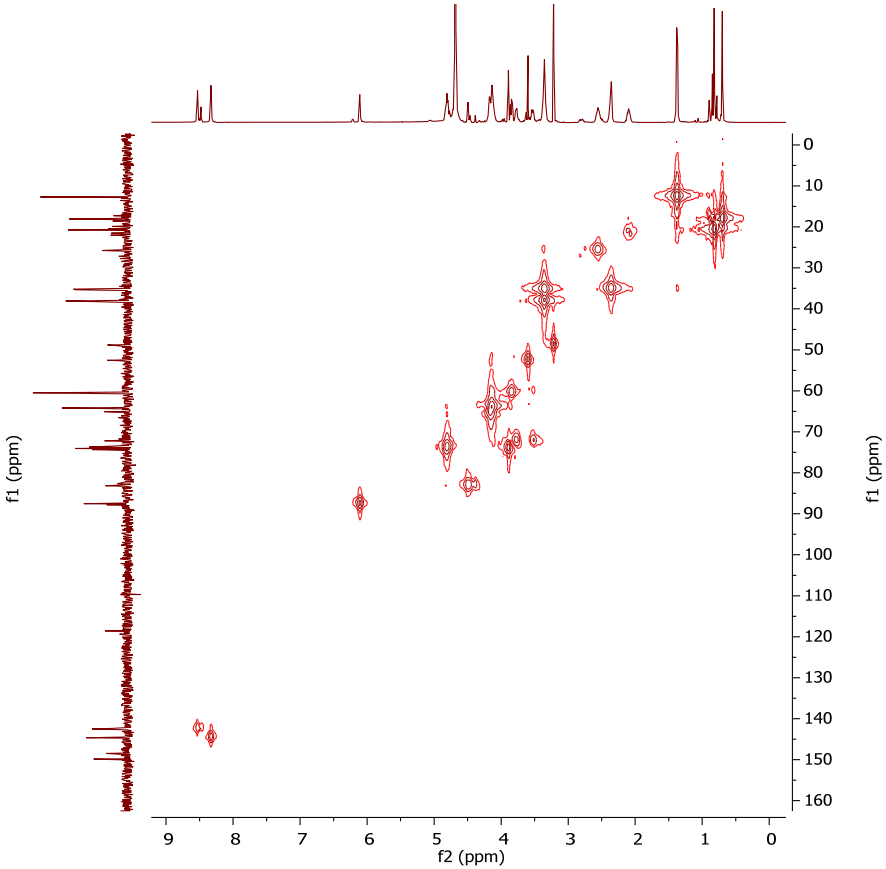

174.67
174.05
170.19
149.84
148.47
144.62
142.49
118.58
87.52
83.19
74.33
74.06
73.70
72.21
64.19
60.49
52.57
38.27
38.08
35.35
35.18
20.72
18.07
12.68
5
13C in D2O
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

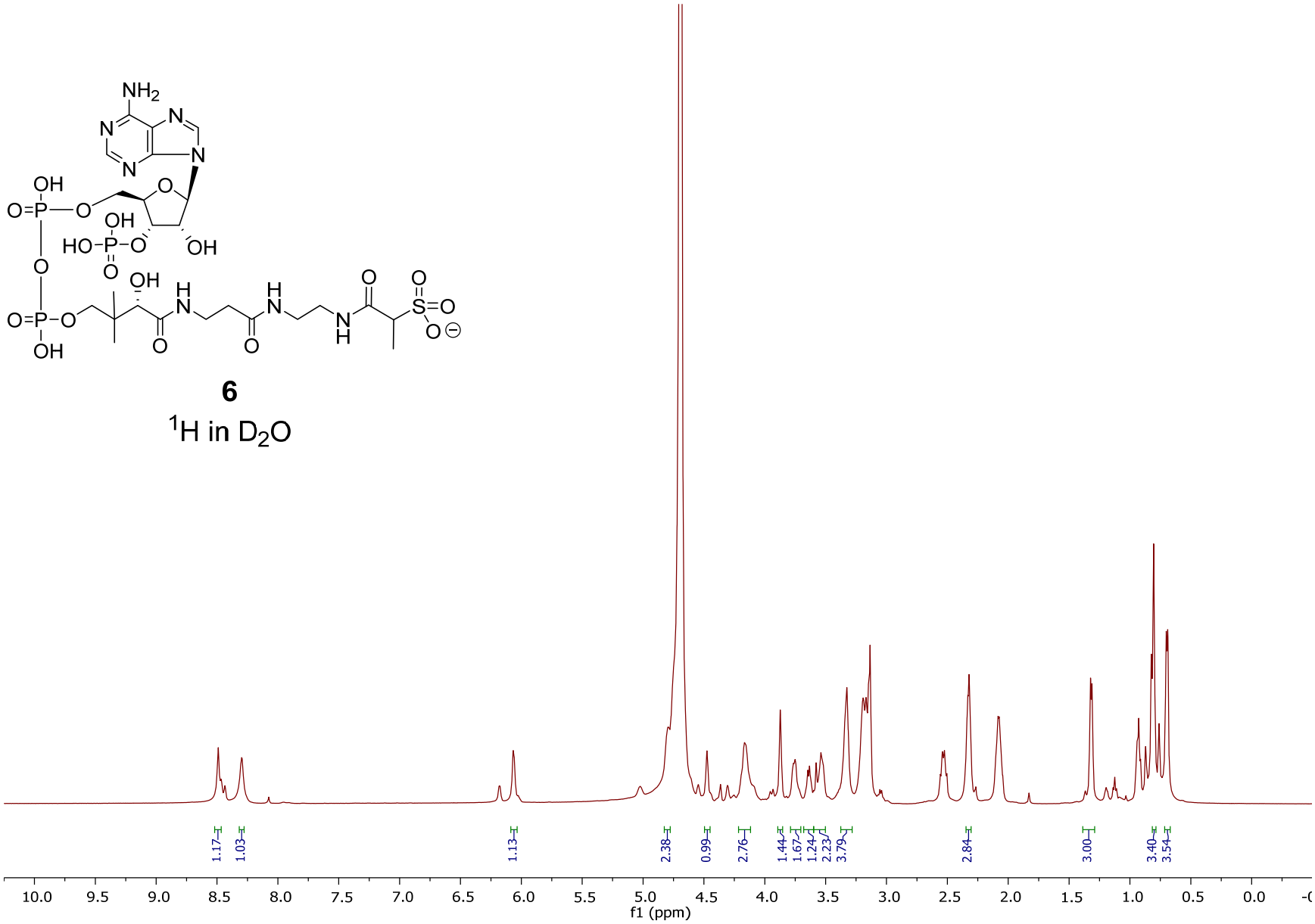


5

COSY and HMQC in D_2O

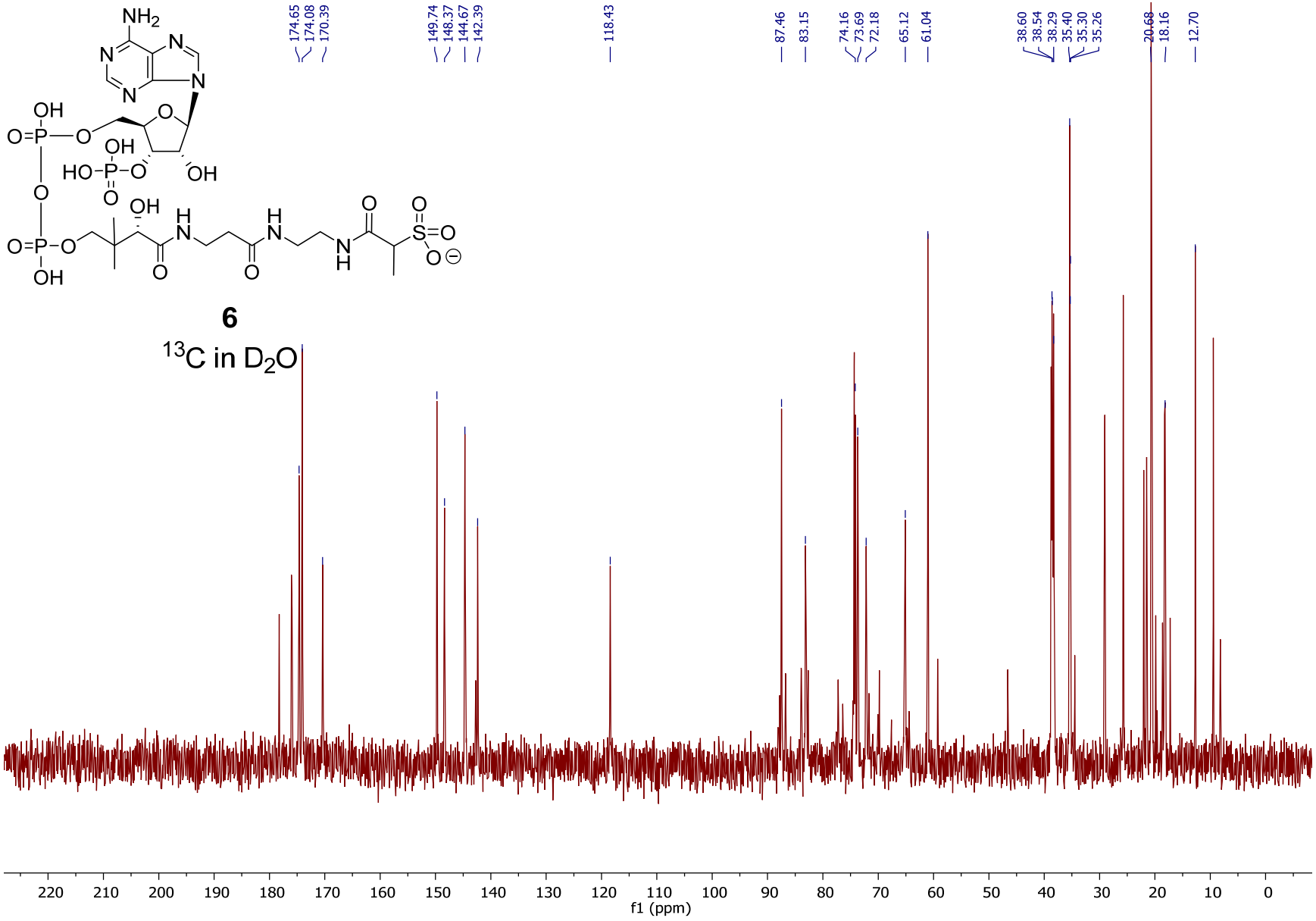


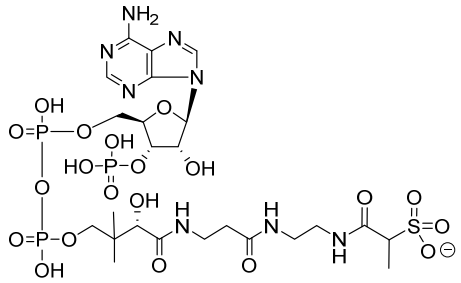



NH2
N
N
N
N
OH
O=P—O
O
OH
HO—P—O
OH
O
O
OH
O=P—O
H
N
H
N
N
H
O
O
O
O
S=O
O
OH
1.17
1.03
1.13
2.38
0.99
2.76
1.44
1.67
1.24
2.23
3.79
2.84
3.00
3.40
3.54
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

6

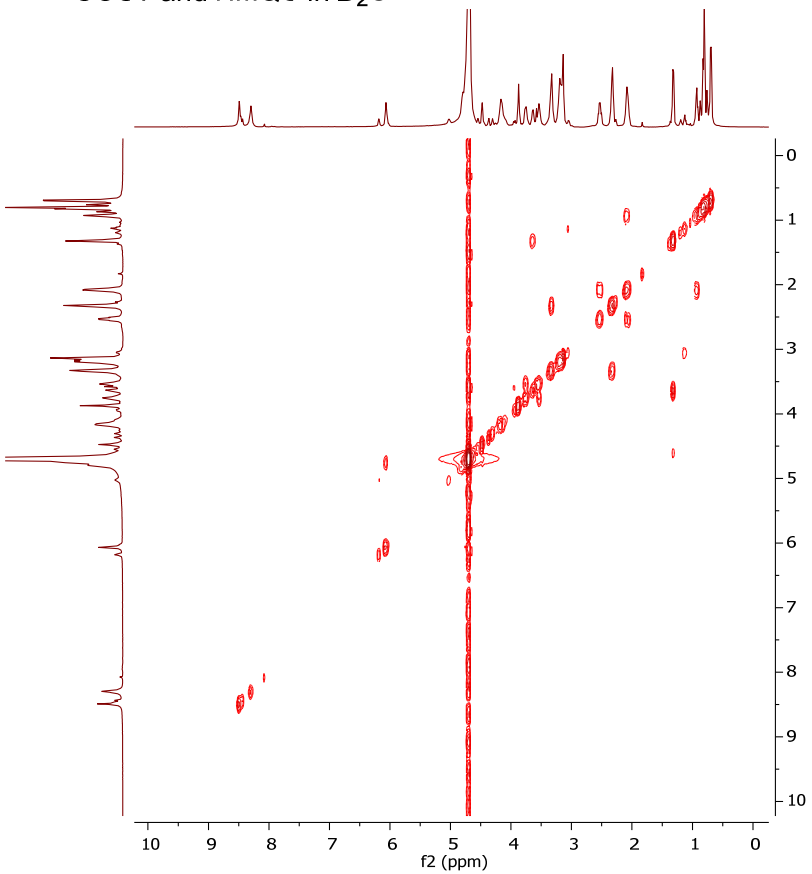
^{1}H in D_2O

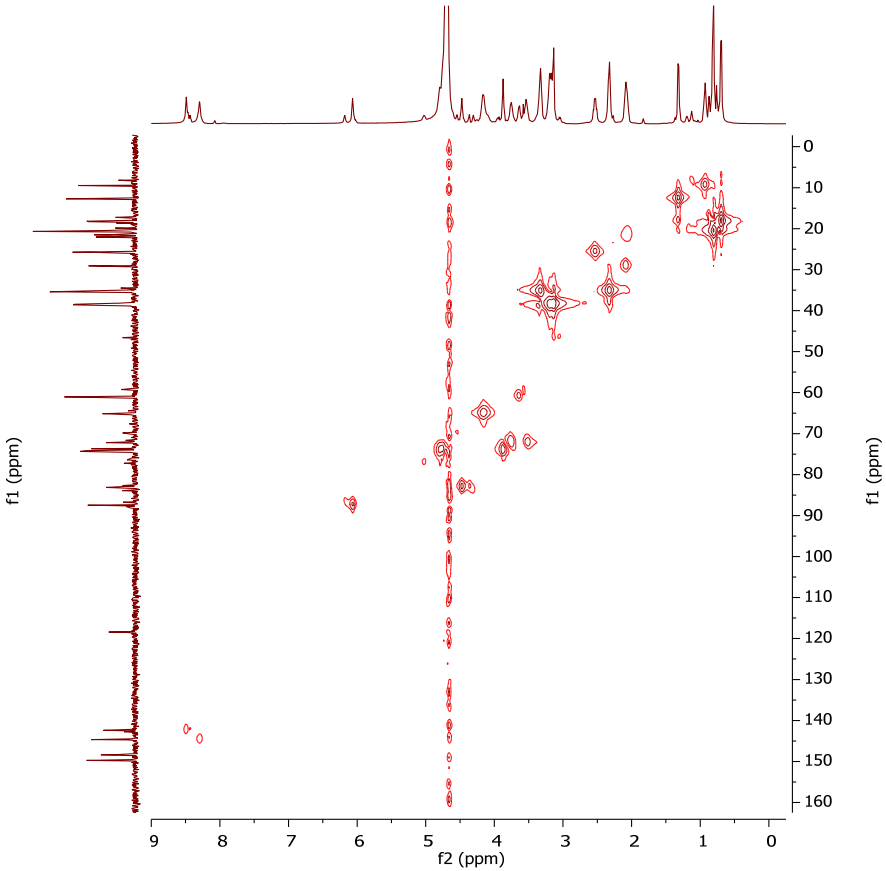

174.65
174.08
170.39
149.74
148.37
144.67
142.39
118.43
87.46
83.15
74.16
73.69
72.18
65.12
61.04
38.60
38.54
38.29
35.40
35.30
35.26
20.68
18.16
12.70
6
13C in D2O
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

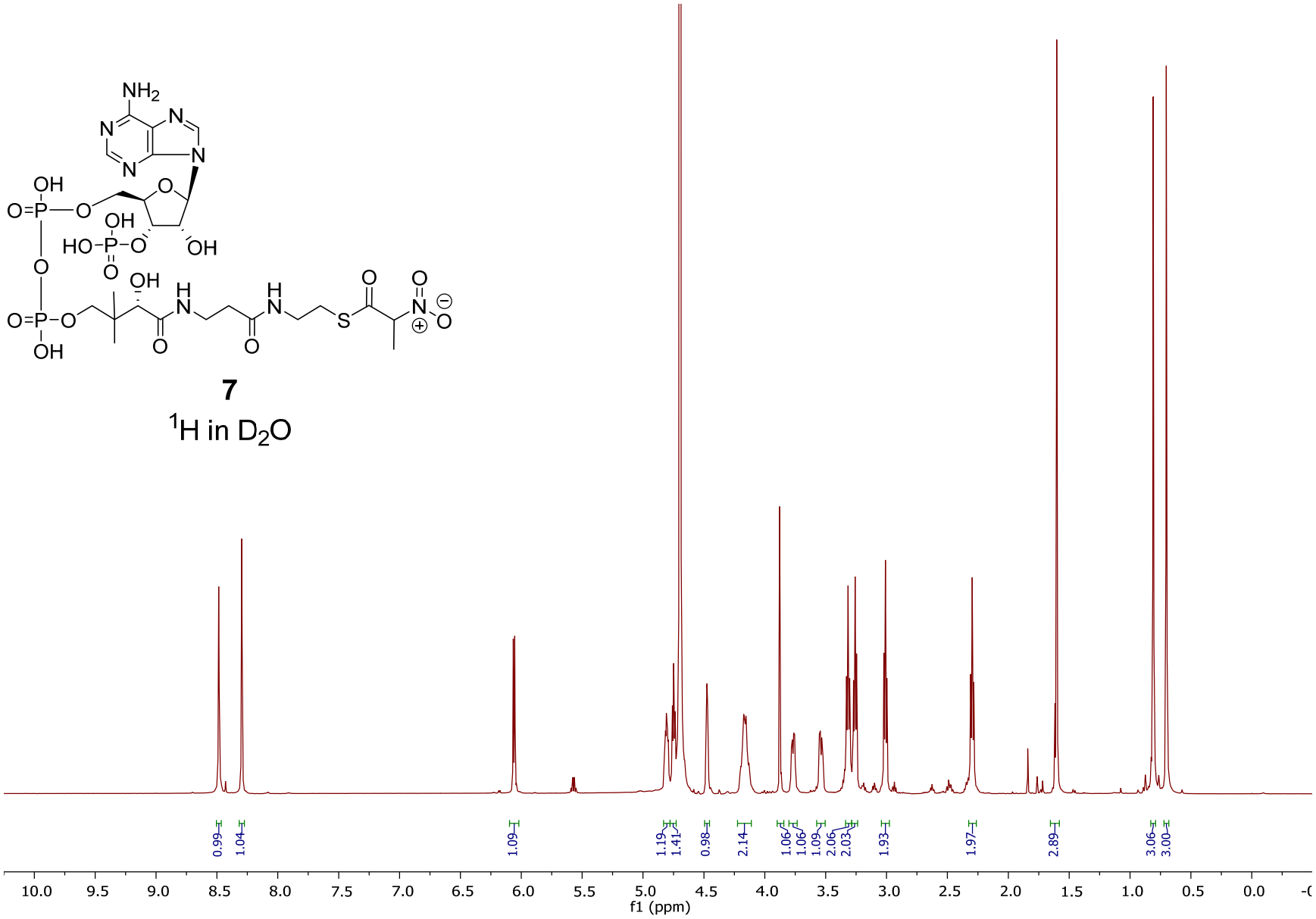


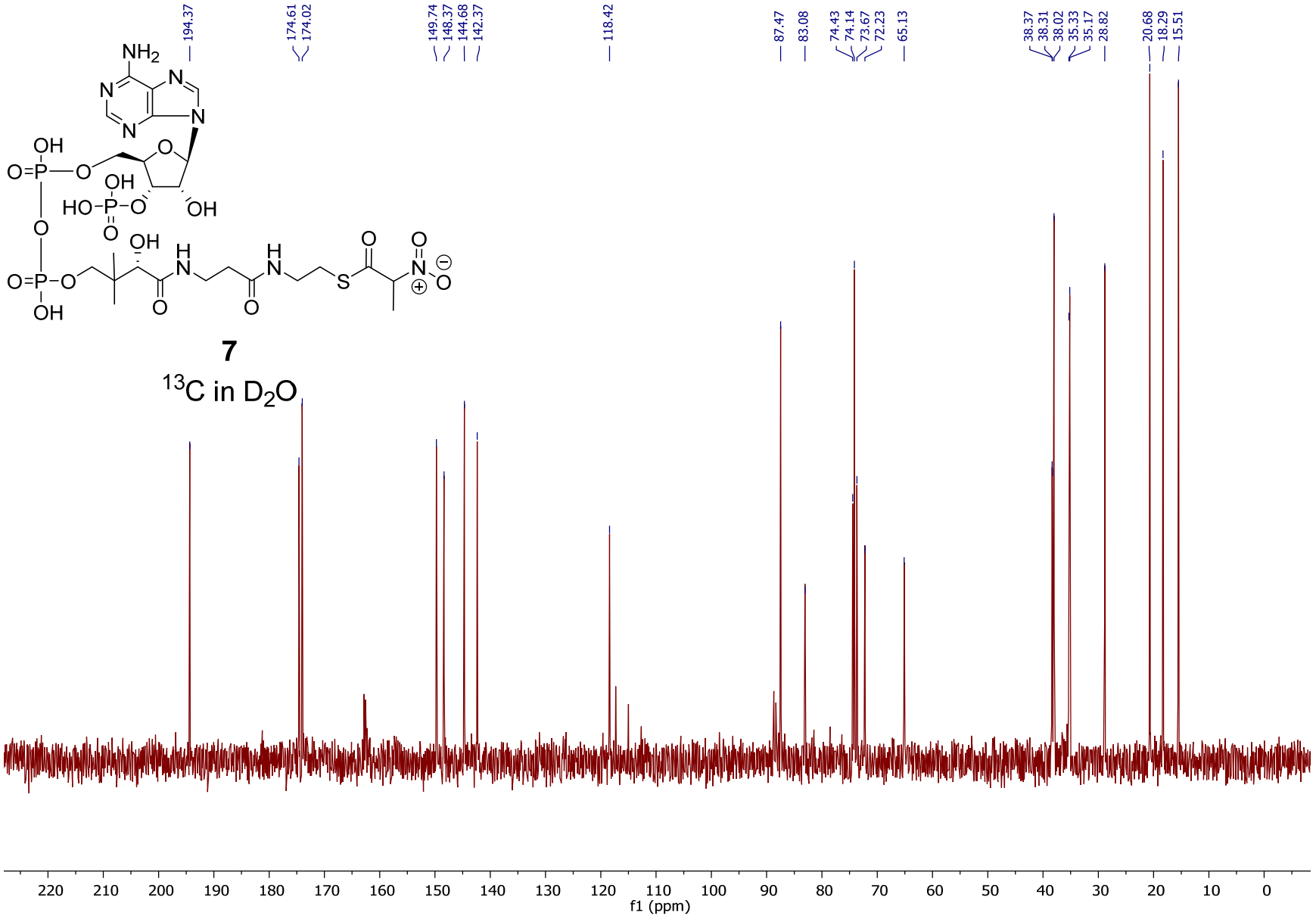
6

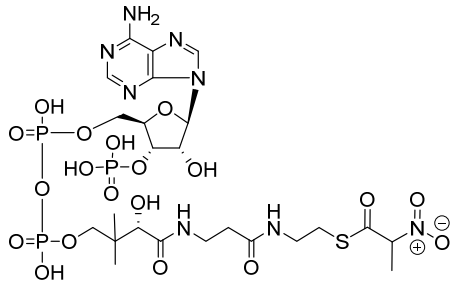
COSY and HMQC in D_2O





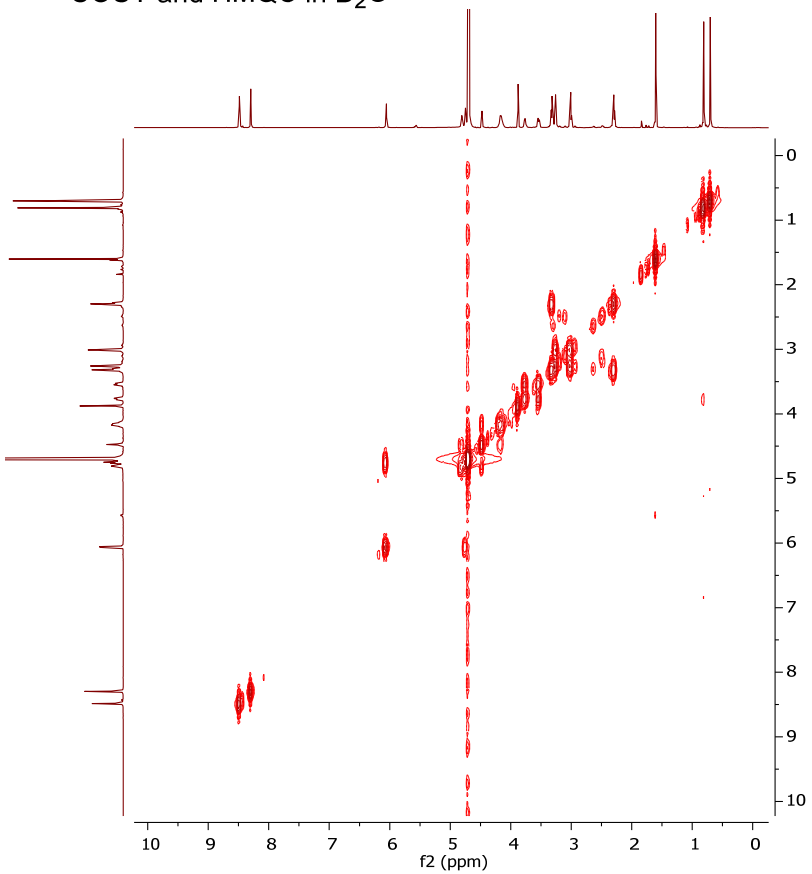

7
1H in D2O
0.99
1.04
1.09
1.19
1.41
0.98
2.14
1.06
1.06
1.09
2.06
2.03
1.93
1.97
2.89
3.06
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

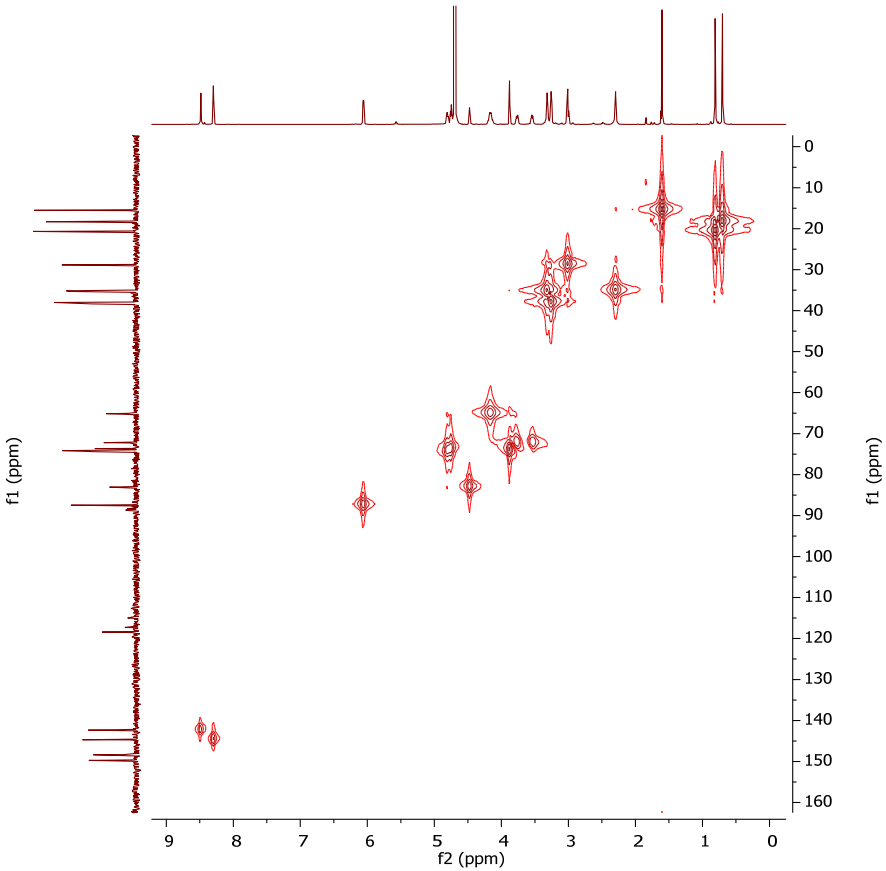

194.37
174.61
174.02
149.74
148.37
144.68
142.37
118.42
87.47
83.08
74.43
74.14
73.67
72.23
65.13
38.37
38.31
38.02
35.33
35.17
28.82
20.68
18.29
15.51
7
13C in D2O
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)



7

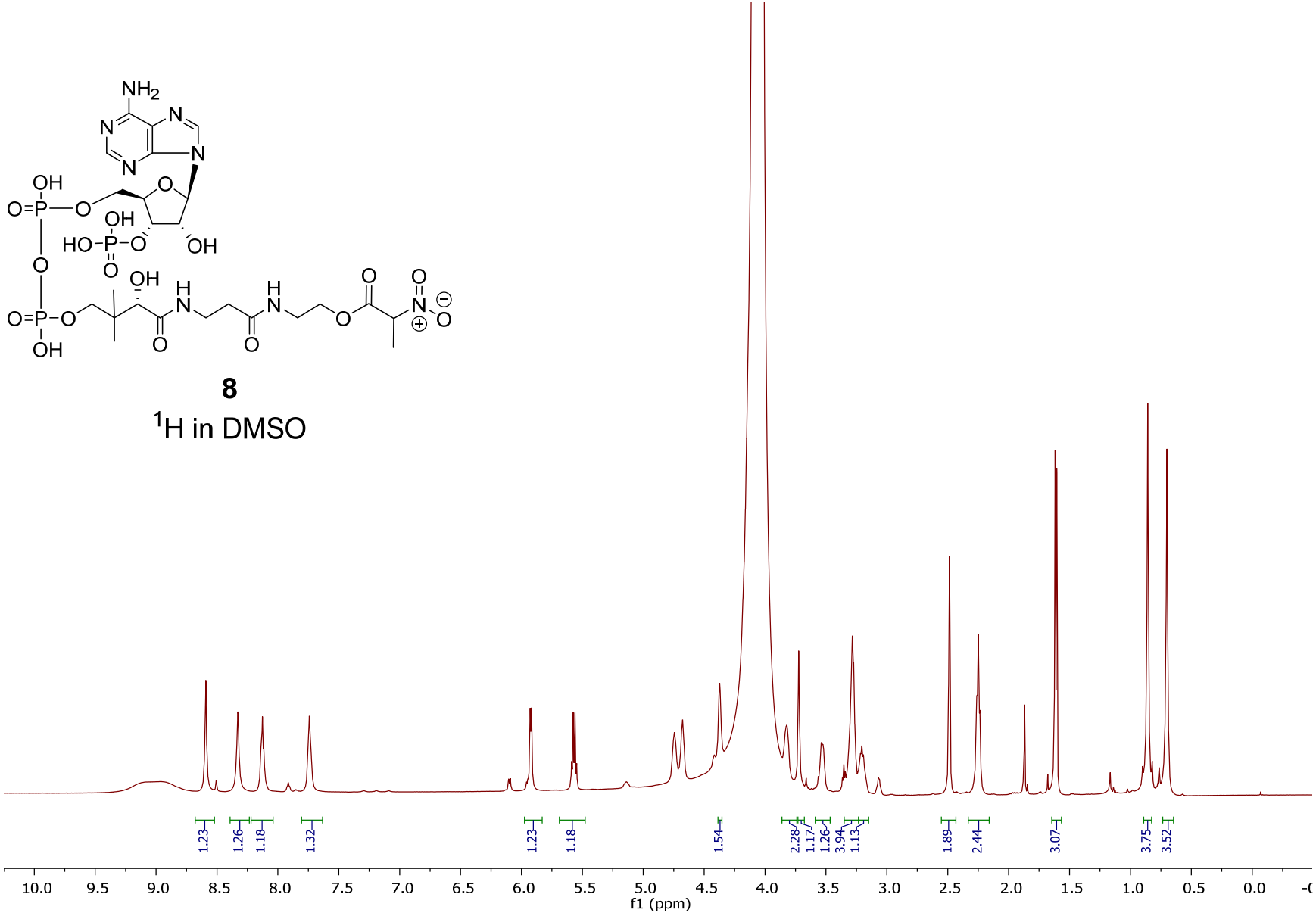
COSY and HMQC in D_2O

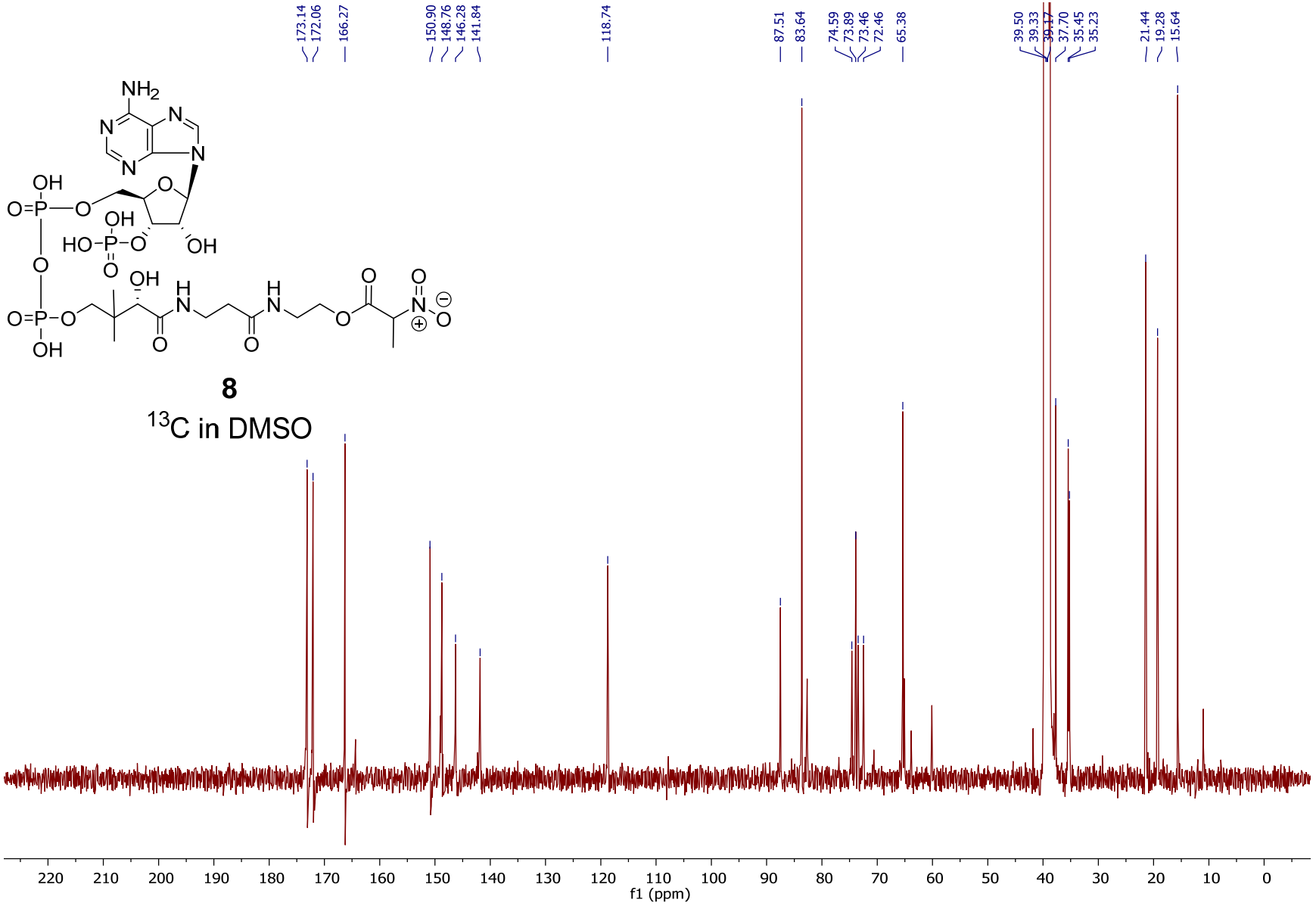


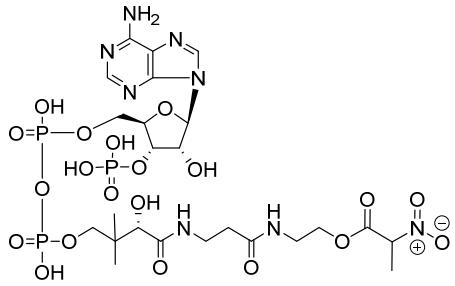


8

^{1}H in DMSO

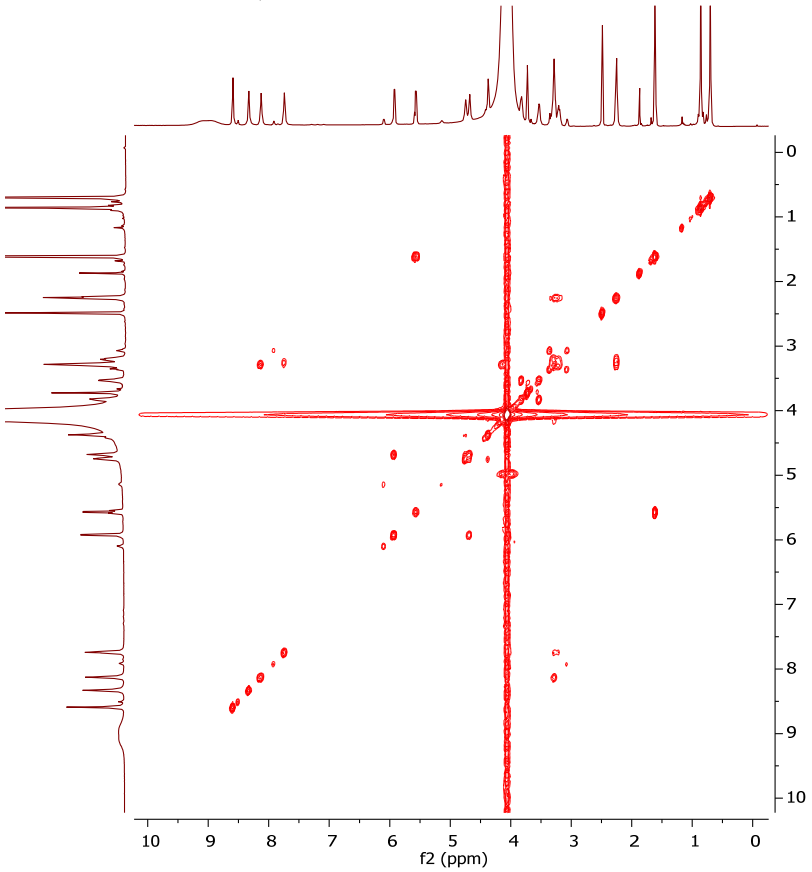

1.23
1.26
1.18
1.32
1.23
1.18
1.54
2.28
1.17
1.26
3.94
1.13
1.89
2.44
3.07
3.75
3.52
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

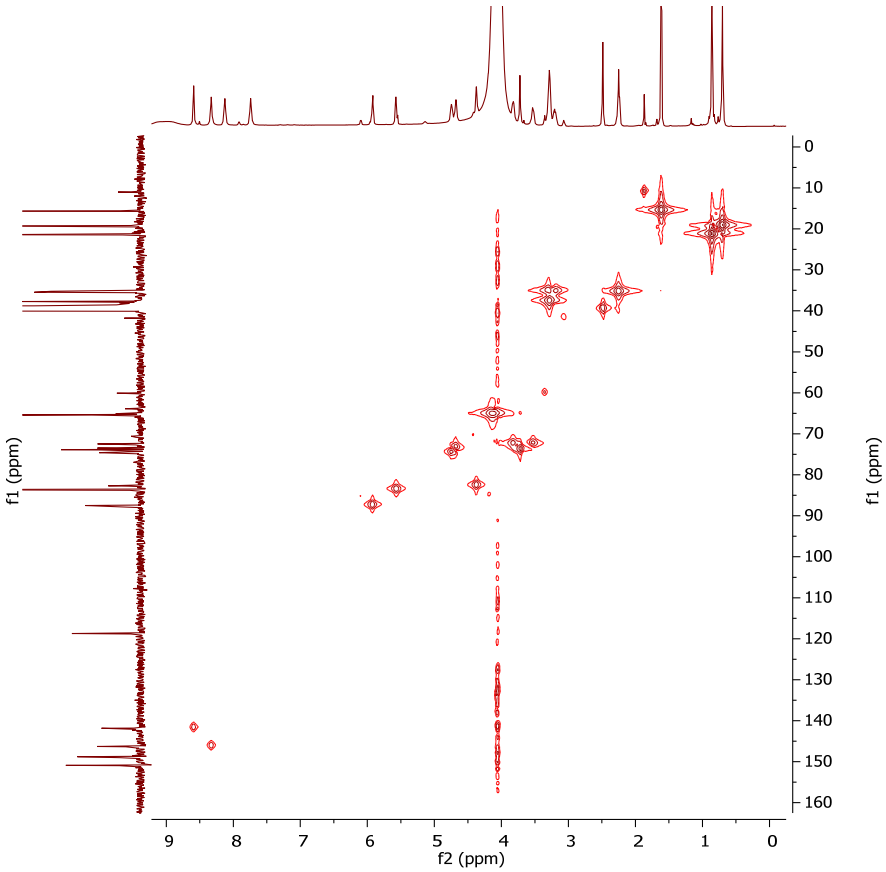

173.14
172.06
166.27
150.90
148.76
146.28
141.84
118.74
87.51
83.64
74.59
73.89
73.46
72.46
65.38
39.50
39.33
39.17
37.70
35.45
35.23
21.44
19.28
15.64
8
13C in DMSO
f1 (ppm)

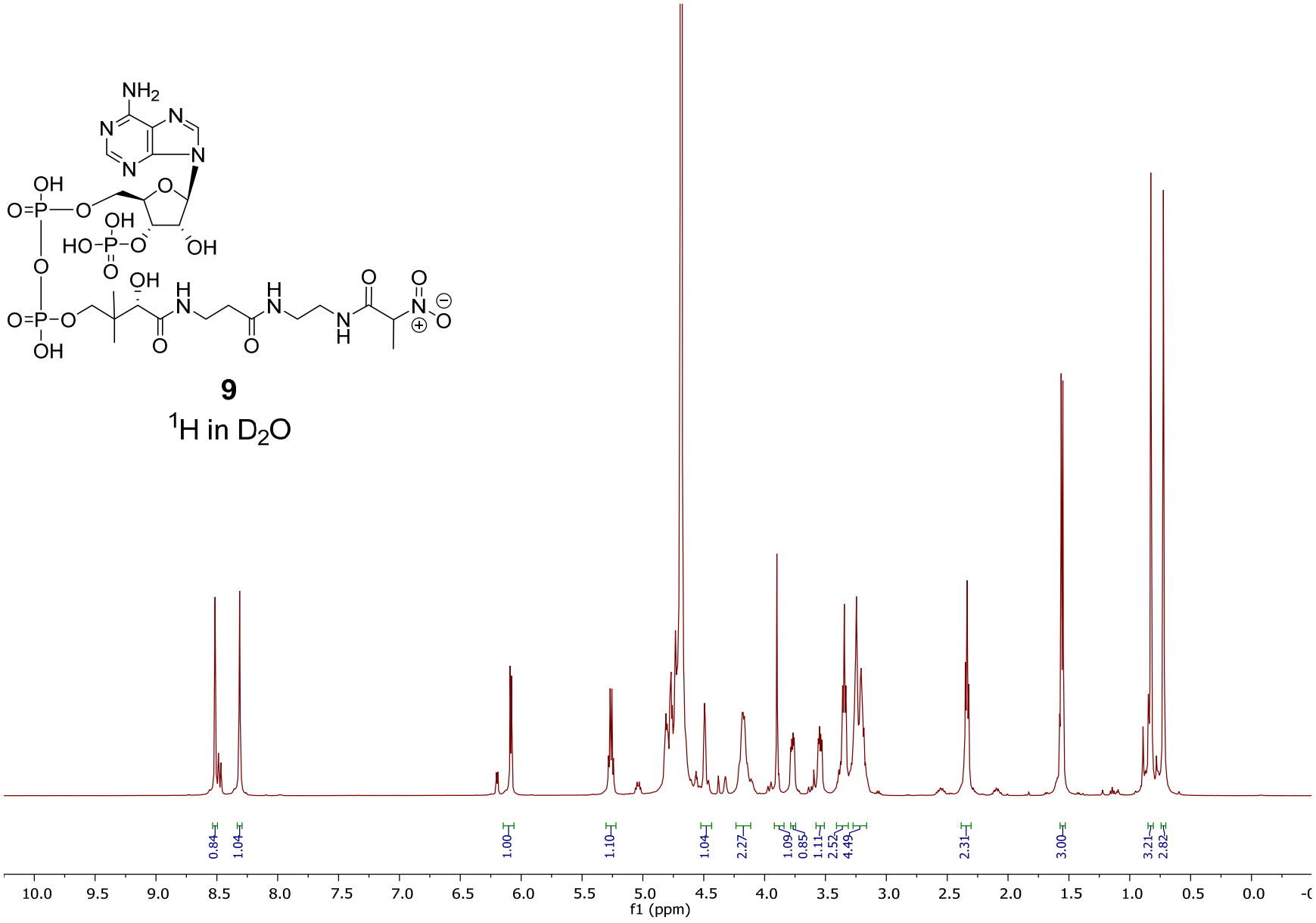


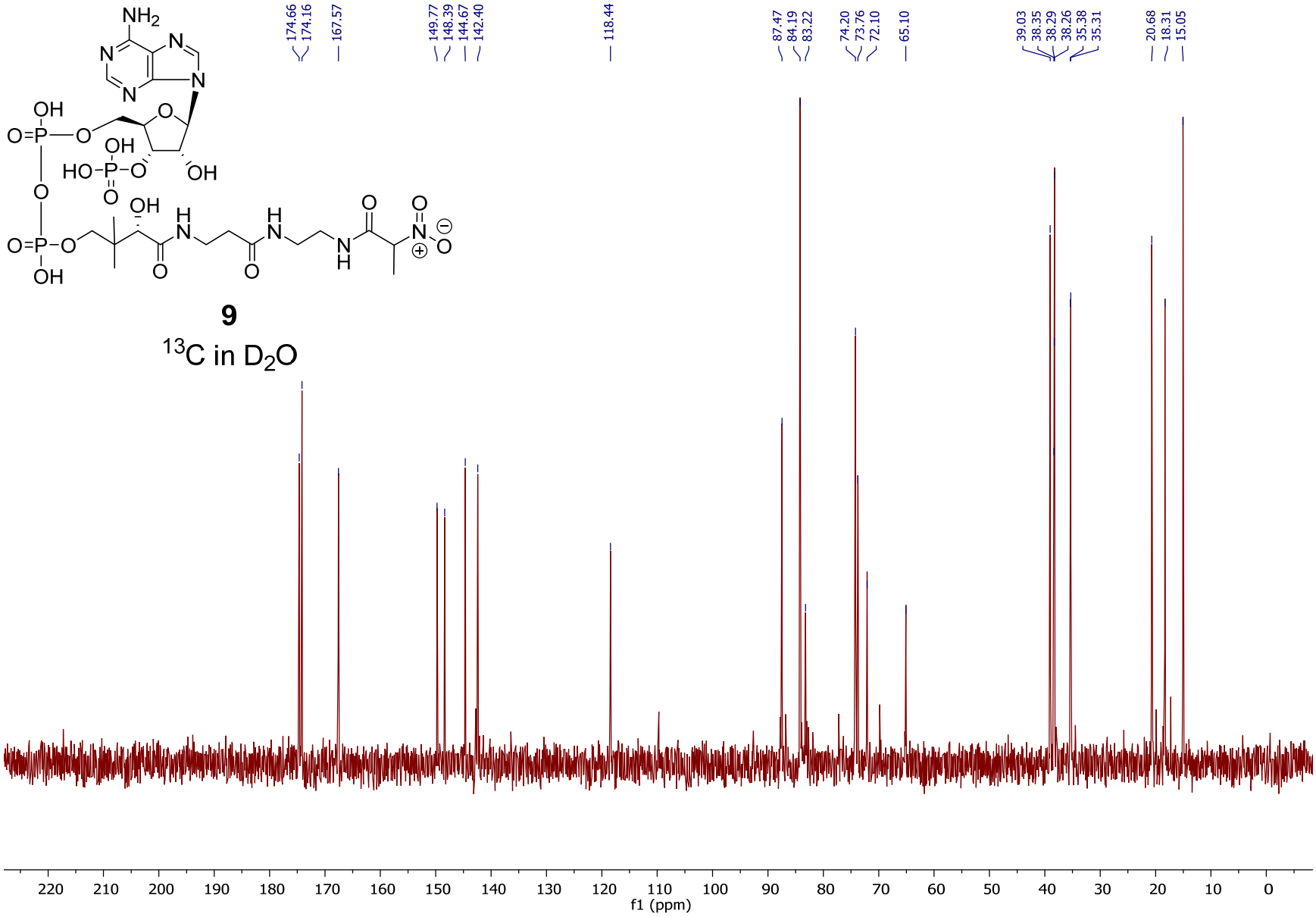
8

COSY and HMQC in DMSO



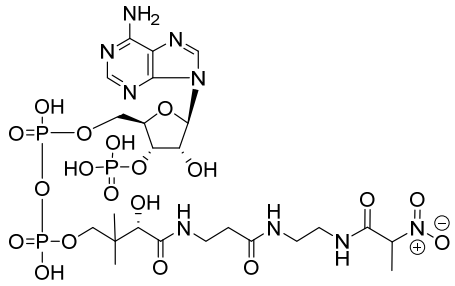



NH2
N
N
N
N
OH
O=P—O
O
OH
HO–P–O
O
OH
OH
O
O=P–O
OH
OH
H
N
O
H
N
O
O
H
N
O
O
N
O
9
1H in D2O
0.84
1.04
1.00
1.10
1.04
2.27
1.09
0.85
1.11
2.52
4.49
2.31
3.00
3.21
2.82
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


174.66
174.16
167.57
149.77
148.39
144.67
142.40
118.44
87.47
84.19
83.22
74.20
73.76
72.10
65.10
39.03
38.35
38.29
38.26
35.38
35.31
20.68
18.31
15.05
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

9

^{13}C in D_2O



9

COSY and HMQC in D_2O