Electronic Supplementary Material (ESI) for ChemComm.

Supporting information for:

Application of Good's Buffers to pH Imaging Using Hyperpolarized 13C MRI

Robert R Flavell†, Cornelius von Morze†, Joseph E. Blecha†, David Korenchan†, Mark Van Criekinge†, Renuka Sriram†, Jeremy Gordon†, Hsin-Yu Chen†, Sukumar Subramaniam†, Robert Bok†, Zhen J. Wang†, Daniel Vigneron†, Peder Larson†, John Kurhanewicz†, and David M Wilson†

†Department of Radiology and Biomedical Imaging, University of California, San Francisco, California 94158, United States

General methods:

^{13}C, ^{15}N ACES was synthesized as described, or was produced by custom synthesis by Isotec (Miamisburg, OH). 1-^{13}C-Glycine and ^{15}N ammonium chloride were purchased from Cambridge Isotope Labs (Tewksbury, MA). All other compounds were purchased from Sigma-Aldrich. For routine characterization of chemical intermediates and products, ^{1}H, ^{13}C, and ^{19}F spectra were obtained on a Varian 400 or 500 MHz NMR. All spectra were processed offline using ACDLABS 12.0 or VNMRJ. All ^{1}H chemical shifts are reported in parts per million and referenced to the residual solvent peak or to TSP. All ^{13}C chemical shifts are reported in parts per million and referenced to the residual solvent peak of DMSO or to TSP. High resolution mass spectrometry was performed by the Notre Dame mass spectrometry facility, on a microTOF instrument (Notre Dame, IN). Silica gel flash chromatography was performed on a Biotage SP4 system.

Thermal equilibrium 11.7 T NMR Studies and titration curve. NMR studies were performed on an 11.7 T Varian INOVA spectrometer (125 MHz ^{13}C, Varian Instruments) using a 5 mm $^{15}N/^{31}P/^{13}C$ broadband direct detect probe. For screening of compounds, 250 mM solutions of compound and urea in water were prepared, and the pH adjusted to 7.4 or 6.5. For the titration curve, a solution containing 250 mM natural abundance ACES and Urea was prepared, and the pH adjusted to the indicated values. All NMR spectra were obtained at 37° C. Values for the δ_{min}, δ_{max}, and pK_a were solved by iteratively fitting the δ vs. pH data to the following equation in Microsoft excel:

$$pH = pKa - \log_{10}\frac{(\delta - \delta min)}{(\delta max - \delta)}$$

In this equation, δ refers to the measured chemical shift difference between ACES and urea, and δ_{min} and δ_{max} are constants referring to the smallest and largest differences in chemical shift between ACES and urea. This yielded values of -5.52, -13.92, and 6.58 for δ_{max}, δ_{min}, and pK_a, respectively.

Hyperpolarized ADA calcium response studies. Previous data indicated that ADA buffer binds calcium with a log(K_M) of 3.96.[1] At the physiologic blood calcium concentration of approximately 1 mM, this implies that 90% of ADA would be bound to calcium at equilibrium. Thus, we tested the ability of ADA to bind calcium. In initial experiments, we found the calcium – ADA complex to be insoluble in water at the concentrations necessary for thermal equilibrium ^{13}C NMR. Thus, we developed a hyperpolarized ^{13}C method for testing calcium binding. A hyperpolarized preparation of natural abundance ADA was prepared by dissolving 50 mg ADA in 119 µL of DMSO. To this was added 4 mg of OX63 radical. In a typical experiment, 50 µL of the ADA prep was frozen in liquid nitrogen. To this was added 50 µL of natural abundance urea prep, prepared as previously described[2], which was also rapidly frozen to avoid mixing of the materials. The mixture was polarized for 1 h using a Hypersense DNP polarizer (Oxford Instruments, Abingdon, UK). The compound was then dissolved in buffer containing 100 mM TES, at pH 7.6 or 6.7, with or without 10 mM calcium. The solution was mixed manually in a teardrop flask, and injected using a 5 cc syringe into a previously shimmed 5 mm NMR tube at 37 °C. Time-resolved hyperpolarized spectra were obtained as previously described.[3]

HPLC analysis of synthetic products. HPLC analysis of compounds was performed on a Hitachi Elite LaChrome system with L-2455 diode array detector (Hitachi, Tokyo, Japan), and a Phenomenex Luna C18(2) column, 100 Å 250 x 4.6 mm 5 micron. All spectra are reported at 220 nm absorbance. The gradient was from 0-50% acetonitrile over 20 minutes, with a 2 minute isocratic in water at the start of the run, with a flow rate of 1 mL/min.

Temperature and concentration dependence of chemical shift studies. For temperature studies, a 2.5 mM solution of ^{13}C, ^{15}N ACES with 250 mM natural abundance urea at pH 7.4 was prepared. Thermal equilibrium ^{13}C NMR spectra were acquired at the indicated temperatures. For concentration dependence studies, a 2.5 mM solution of ^{13}C, ^{15}N ACES, with 250 mM natural abundance urea, and

variable amounts of natural abundance ACES at pH 7.4 was prepared. Thermal equilibrium ^{13}C NMR spectra were then acquired.

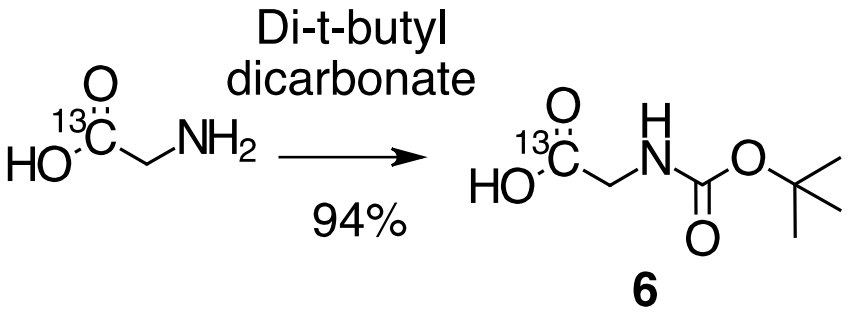
Hyperpolarized 11.7 T NMR Studies. NMR studies were performed on an 11.7 T Varian INOVA spectrometer (125 MHz ^{13}C, Varian Instruments) using a 5 mm $^{15}N/^{31}P/^{13}C$ broadband direct detect probe. ^{13}C, ^{15}N ACES was dissolved in 0.95 equivalents of 10N NaOH, Gd-DOTA was added to 0.5M, and OX63 trityl radical was added to 20 mM. In a typical experiment, 50 µL of the prep was frozen in liquid nitrogen, and polarized for 2 h using a Hypersense DNP polarizer (Oxford Instruments, Abingdon, UK). The solid-state polarization build-up curve was fit to the equation: $P(t) = P_{eq}(1-\exp^{(-t/Tbuildup)})+baseline$, where P_{eq} is the equilibrium polarization achieved for the sample and $T_{buildup}$ is the polarization build-up time constant. For copolarization experiments, 50 µL of ACES prep was frozen with liquid nitrogen. To this was added 20 µL of ^{15}N,^{13}C Urea prep[2], which was also rapidly frozen to avoid mixing of the materials. The sample was subsequently dissolved in 4.0 mL of 40 mM hydrochloric acid in water, which typically yielded a solution with pH 7.4. For experiments where a more acidic pH was desired, a greater concentration of hydrochloric acid was used. The solution was mixed manually in a teardrop flask, and injected using a 5 cc syringe into a previously shimmed 5 mm NMR tube at 37 °C. This process required approximately 15 s. T_1's and signal enhancements were calculated as previously described.[3]

3T phantom experiment: For the phantom experiment, 50 µL of the ACES prep and 20 µL of the urea prep were copolarized and dissolved as above. Following dissolution, the solutions were transferred to 5 tubes containing 50 mM phosphate buffer at various pH values. The hyperpolarized pH phantom was imaged in a clinical 3T MRI scanner (GE Healthcare, Waukesha, WI) using a dual-tuned $^{13}C/^{1}H$ radiofrequency (RF) volume coil designed for imaging rats. Spectroscopic images were acquired using 2D chemical shift imaging (CSI) with the following acquisition parameters: matrix = 10 x 10, spatial resolution = 7.5mm, TR = 105 ms, spectral resolution= 12.2 Hz, spectral bandwidth = 25 kHz, acquisition time = 10.5s. The data was analyzed using open source SIVIC software

(Sourceforge.net).

Synthesis of ^{13}C, ^{15}N ACES (4):

Synthesis of **6**

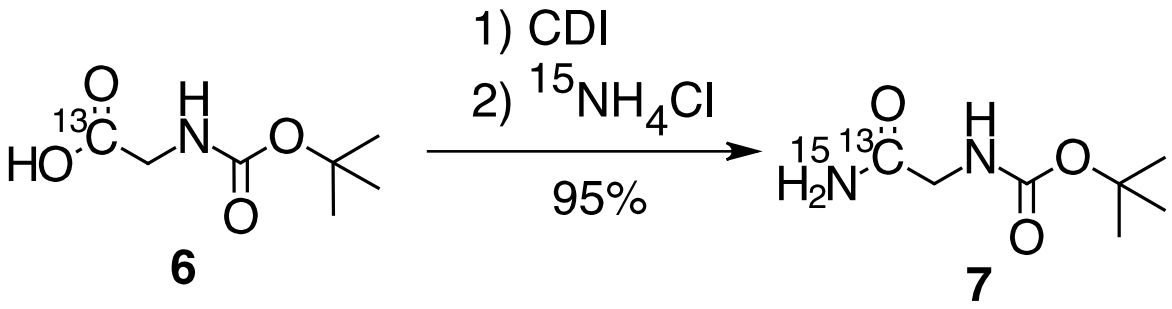


6

2 g (26.3 mmol) of 1-^{13}C-glycine (Cambridge isotope labs) was dissolved in 60 mL 2:1 dioxane:water (vol:vol). To this solution was added 1.064 g (26.6 mmol) sodium hydroxide. The solution was cooled to 0° C. To this solution was added 6.4 g (29.2 mmol) boc anhydride in portions. The solution was allowed to warm to room temperature for 1 hour with stirring. The solvent was removed *in vacuo*. The resulting residue was dissolved in 100 mL water, and washed with 2 portions of 50 mL ethyl acetate. The aqueous solution was acidified using concentrated hydrochloric acid to pH 1-2. The aqueous fraction was extracted with three portions of 75 mL ethyl acetate. The organic fraction was dried over magnesium sulfate, and concentrated to dryness. Yield was 4.37 g (24.8 mmol), 94%.

^{1}H NMR (400 MHz, DMSO-d_6): δ 12.42 (broad, 1H), 7.04 (t, J = 6.2 Hz, 1H), 3.57 (t, J = 5.9 Hz, 2H), 1.38 (s, 9H). ^{13}C NMR (400 MHz, DMSO): δ 171.75 (enriched), 155.80, 78.00, 28.17. HR-MAS – *m/z* (microTOF) $C_6{}^{13}CH_{13}NO_4Na$ (M+ Na^+) found 199.0768, calculated 199.0770. Anal. calcd. for $C_6{}^{13}CH_{13}NO_4$: C, 48.29, H, 7.44; N, 7.95. Found: C, 48.51; H, 7.80; N, 8.04.

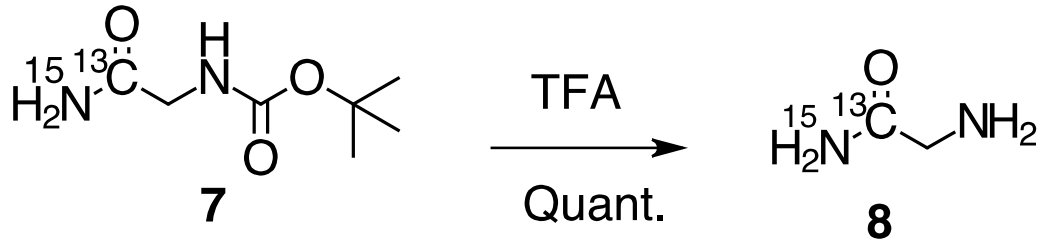
Synthesis of **7**



2.5 g of **6** (14.2 mmol) was dissolved in 5 mL of tetrahydrofuran. To this was added 2.32 g (14.2 mmol) carbonyldiimidazole, portion wise, under nitrogen atmosphere. 1.54 g of ^{15}N ammonium chloride was dissolved in 4 mL water, and 3.95 mL of triethylamine was added to this solution. The ammonium chloride solution was diluted with 5 mL THF, and this solution was then added to the first vial. The reaction was stirred at room temperature for 3 hours, and the solvent removed *in vacuo*. The resulting solution was dissolved in a minimum volume of methanol. The compound was purified by flash chromatography using 9:1 dichloromethane:methanol as an eluent. Yield was 2.4 g (13.5 mmol), 95%.

^{1}H NMR (400 MHz, DMSO-d_6): δ 7.30 (s, 1H), δ 7.06 (d, 1H, J = 13.6 Hz), δ 6.83 (s, 1H), 3.46 (t, J = 5.3 Hz, 2H), 1.37 (s, 9H). ^{13}C NMR (400 MHz, DMSO): δ 171.33 (enriched, d, J_{C-N} = 19.2 Hz), 155.71, 77.90, 28.18. HR-MAS – *m/z* (microTOF) $C_6{}^{13}CH_{14}N^{15}NO_3Na$ (M+ Na^+) found 199.0888, calculated 199.0901. Anal. calcd. for $C_6{}^{13}CH_{13}N^{15}NO_3$: C, 48.28; H, 8.01; N, 16.46. Found: C, 48.21; H, 8.48; N, 15.97.

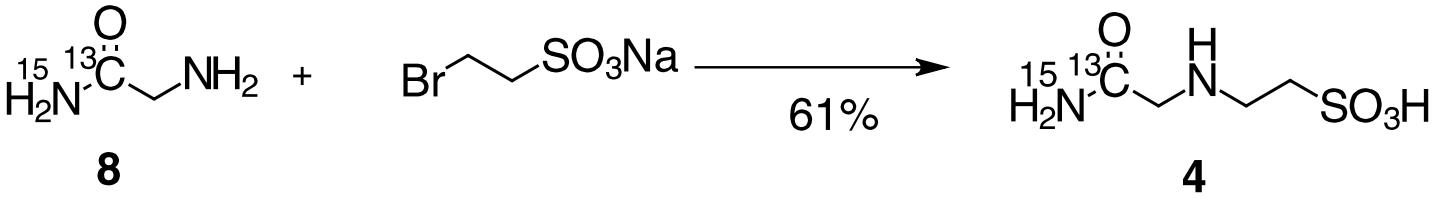
Synthesis of **8**



2 g of **7** was dissolved in 5 mL of trifluoroacetic acid. The resulting solution was stirred for one hour at room temperature. The solvent was removed *in vacuo* and the resulting solid (TFA salt) was dried azeotropically with acetonitrile. Yield: 2.16 g, quantitative.

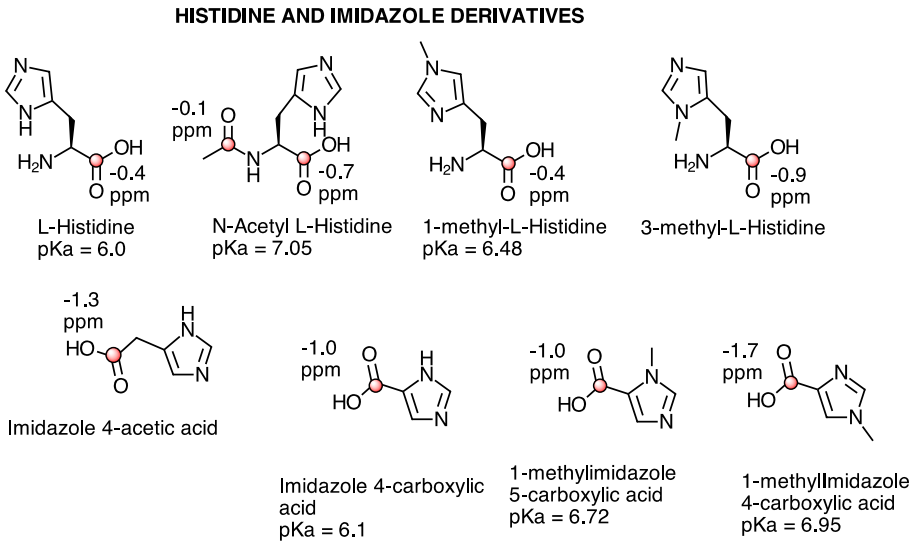
^{1}H NMR (400 MHz, D_2O): δ 3.85 (s, 2H). ^{13}C NMR (500 MHz, D_2O): δ 172.16 (enriched, d, $J_{C\text{-}N}$ = 18.1 Hz), 42.75 (d, $J_{C\text{-}C}$ = 10.1 Hz). HR-MAS – *m/z* (microTOF) $C^{13}CH_6N^{15}NO$ (M+ H^+) found 99.0357, calculated 99.0376. Anal. calcd. for $C_3{}^{13}CH_6F_3N^{15}NO_3$: C, 25.93; H,3.20; N,15.34. Found: C, 26.30; H, 3.62; N, 12.77.

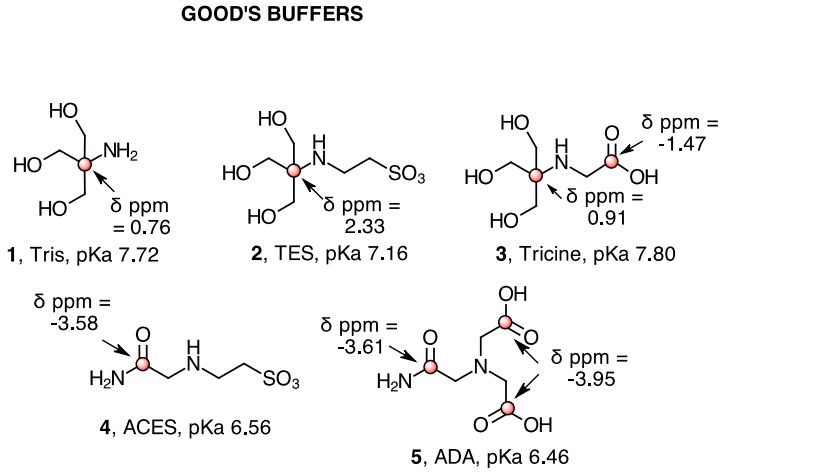
Synthesis of **4**.

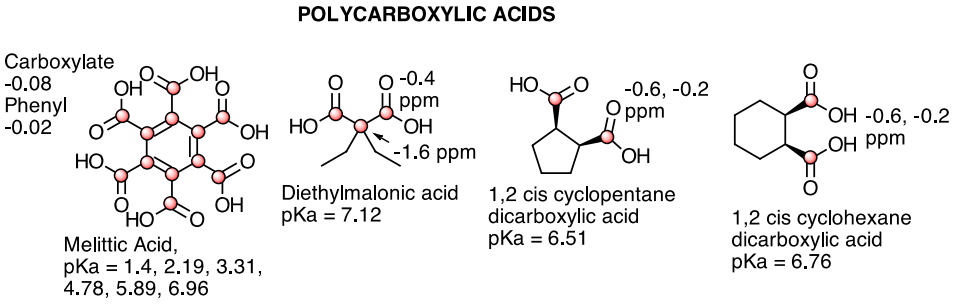


To 1g (5.6 mmol) of 8 in 20 mL water was added 1.12 g (5.3 mmol) sodium 2-bromoethanesulfonate. The pH was kept at approximately 8 - 9 with the addition of 500 uL of 50% NaOH, and the solution was refluxed for 3 hours. The reaction was cooled and acidified to pH 3 with concentrated hydrochloric acid. 20 mL ethanol was added to the solution, and precipitate formed upon standing overnight. The resulting compound was recrystallized from water:ethanol. Final yield: 633 mg (3.4 mmol), 61%.

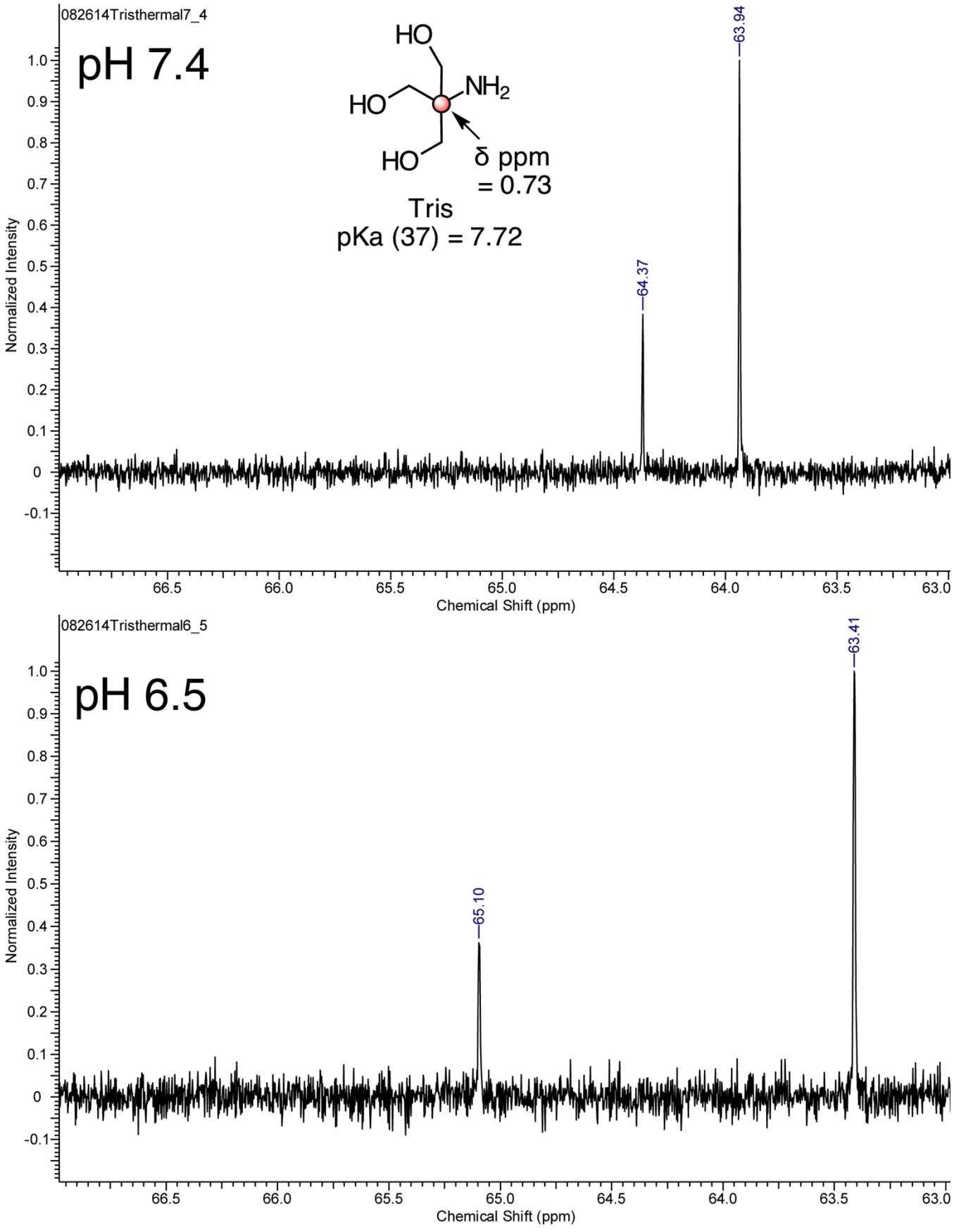
^{1}H NMR (400 MHz, D_2O): δ 3.92 (d, J = 4.7 Hz, 2H), 3.48 (t, J = 7.0 Hz), 3.31 (t, J = 7.4 Hz). ^{13}C NMR (500 MHz, D_2O + NaOH): δ 179.26 (enriched, d, $J_{C\text{-}N}$ = 16.6 Hz), 53.14 (d, $J_{C\text{-}N}$ = 7.2 Hz), 53.00, 46.48. HR-MAS – *m/z* (microTOF) $C_3{}^{13}CH_{11}N^{15}NO_4S$ (M+ H^+) found 185.0419, calculated 185.0438. Anal. calcd. for $C_3{}^{13}CH_{10}{}^{15}NNO_4S$: C, 26.62; H, 5.47; N, 15.75. Found: C, 26.39; H, 5.68; N, 15.20.



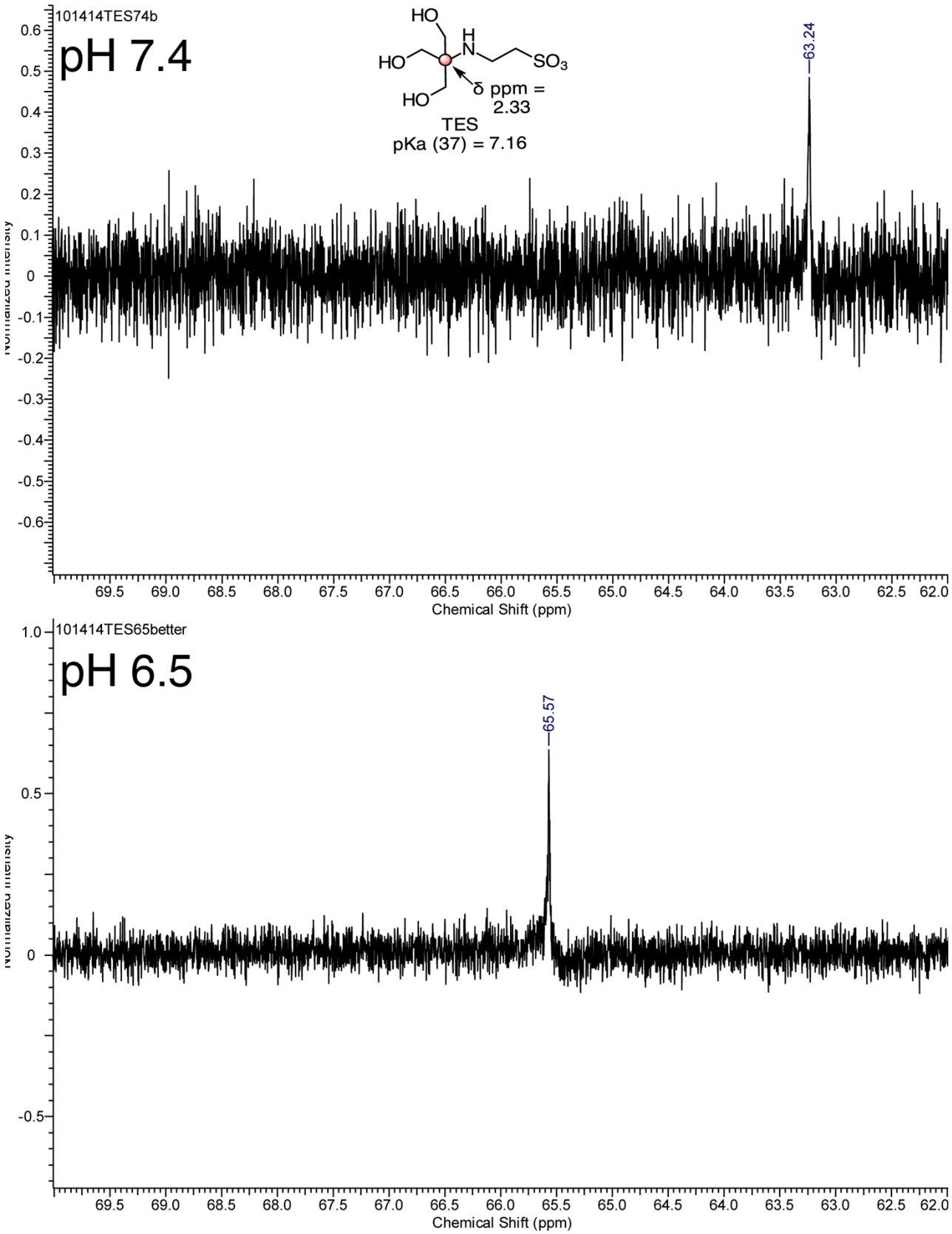




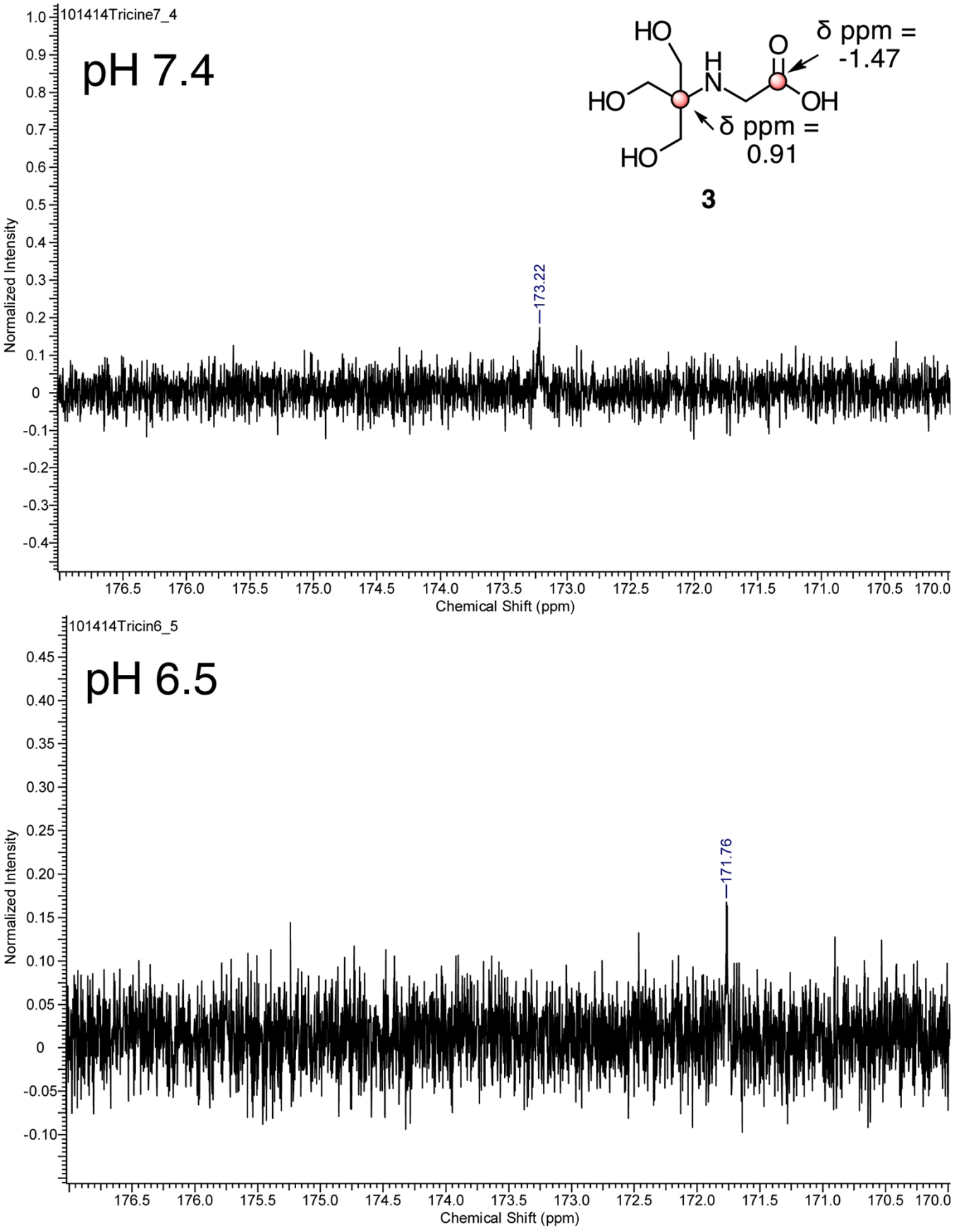
Supplemental figure 1. Structures of compounds investigated by thermal equilibrium NMR. Long T_1 nuclei which were easily amenable to enriched synthesis are indicated in red. The difference in chemical shift between pH 6.5 and 7.4 is shown for long T_1 nuclei. A positive δ□ppm indicates a downfield ^{13}C chemical shift.



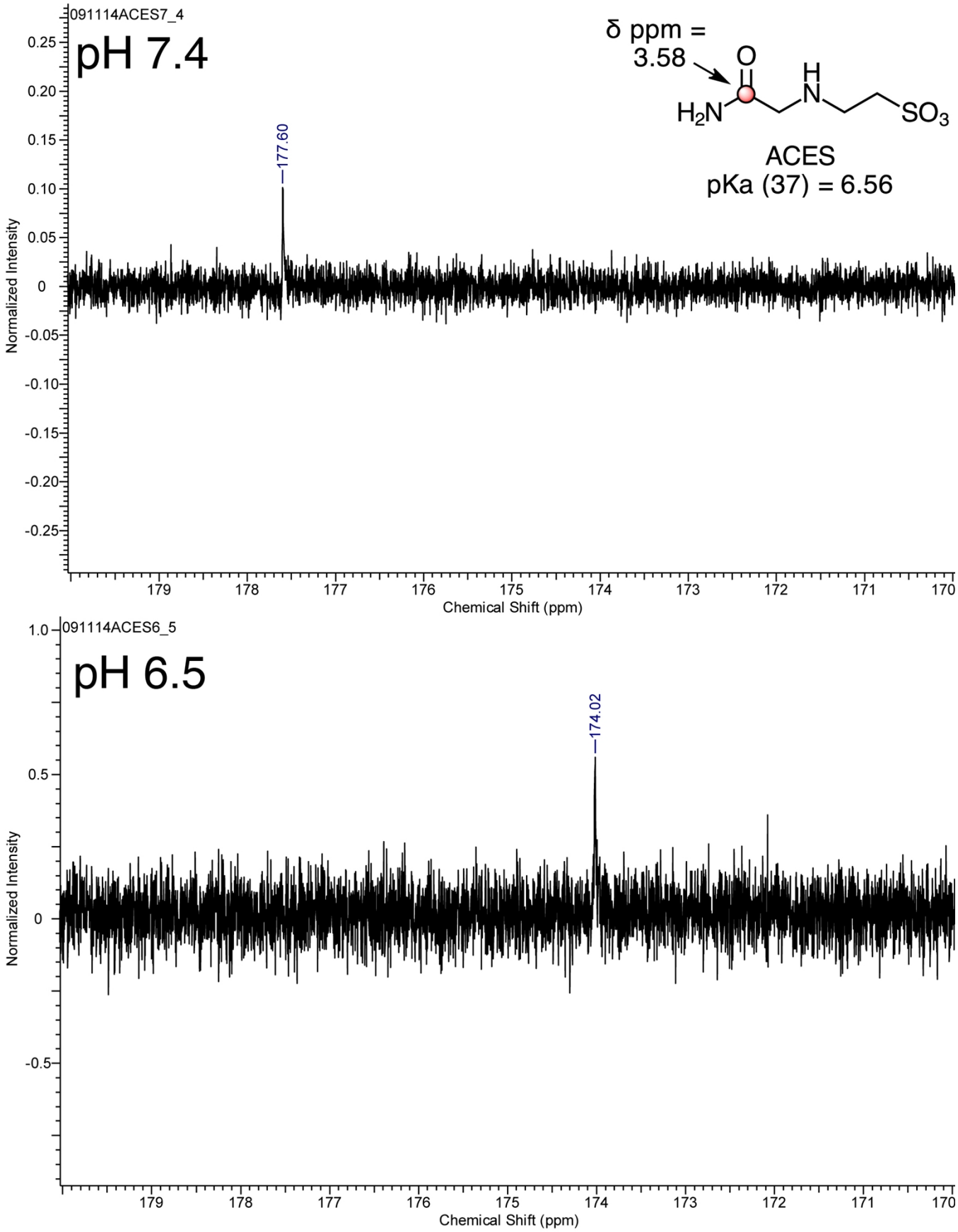
Supplemental figure 2. ^{13}C NMR of Tris at pH 7.4 (top) and pH 6.5 (bottom).



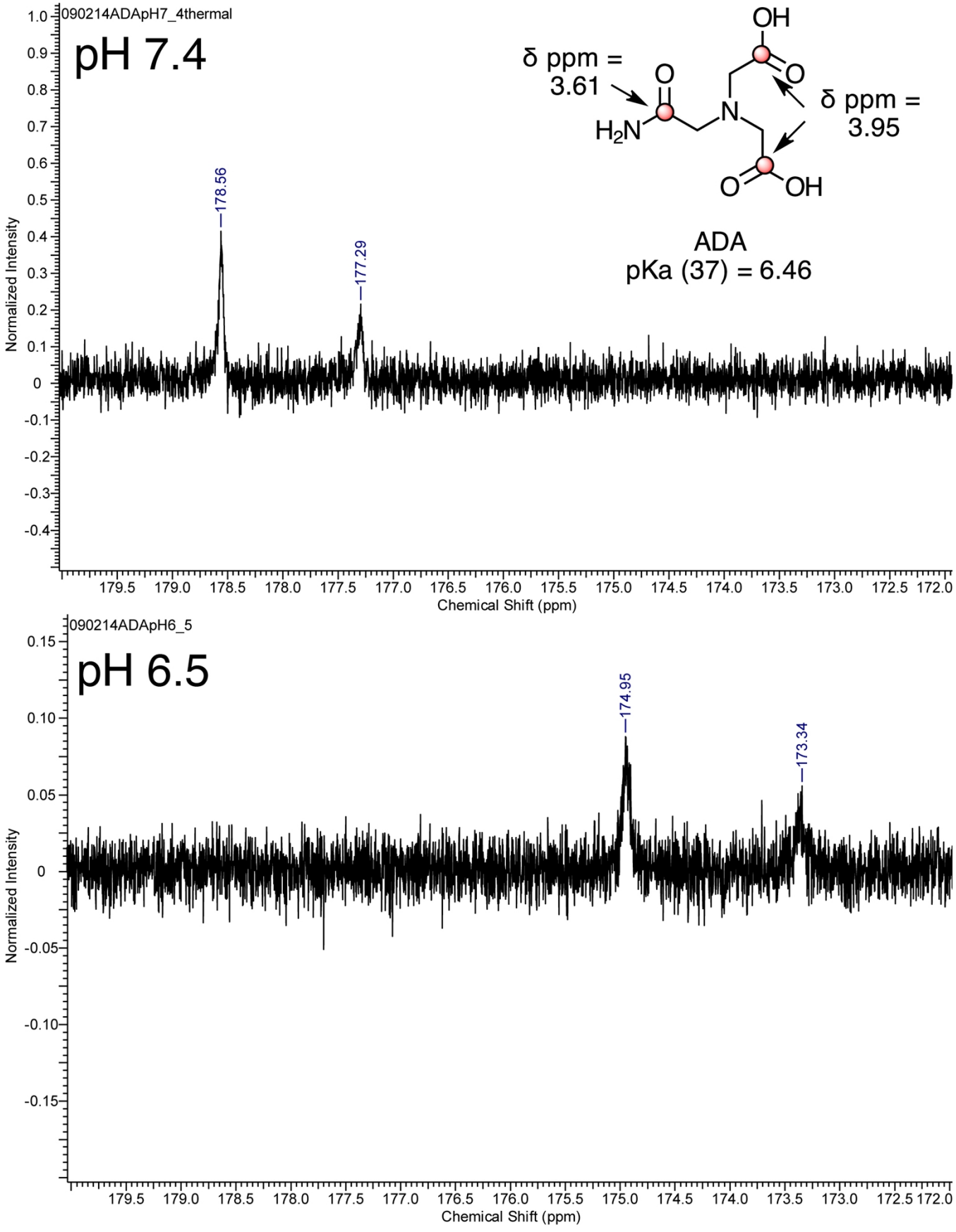
Supplemental figure 3. ^{13}C NMR of TES at pH 7.4 (top) and pH 6.5 (bottom).



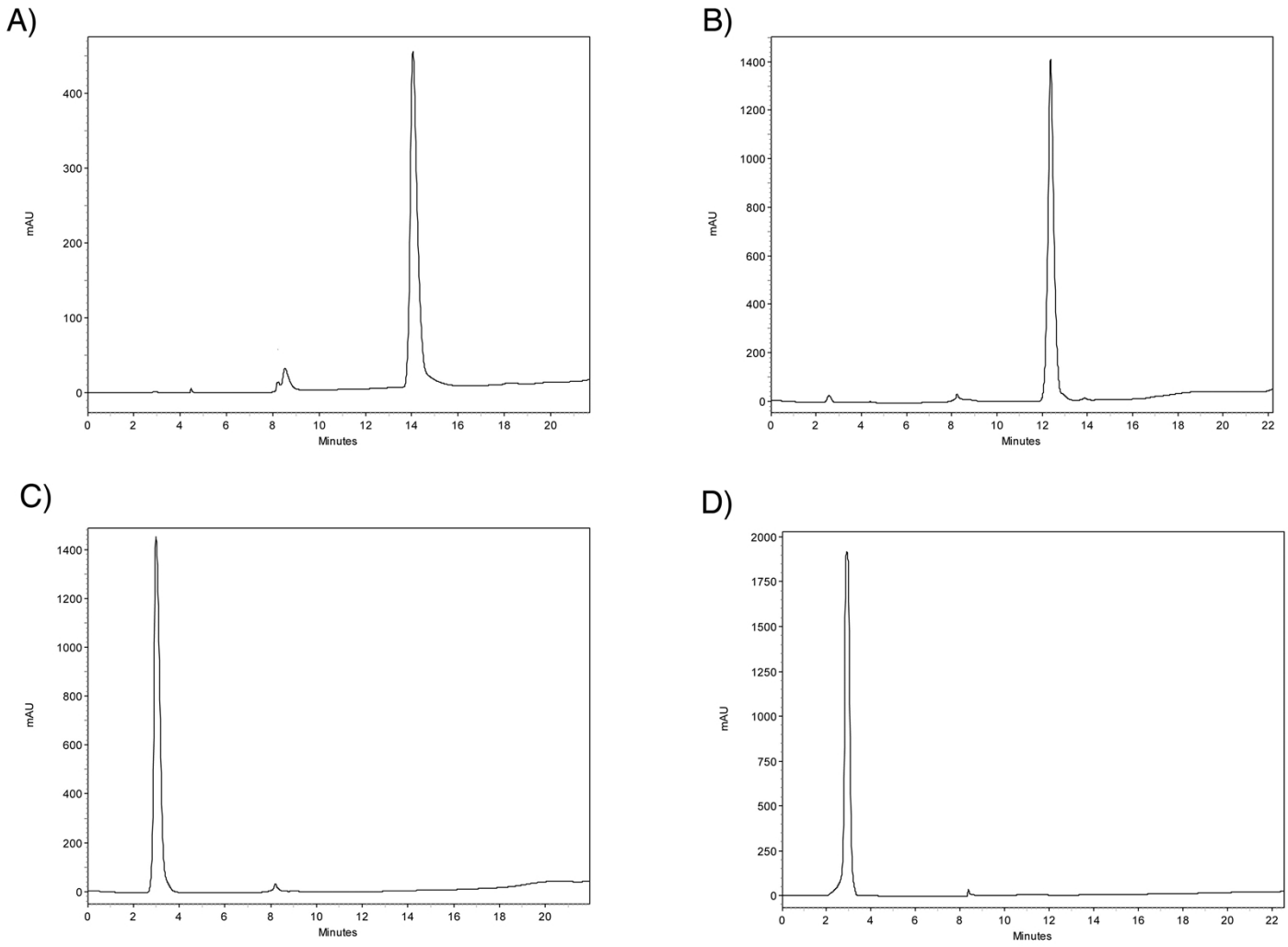
Supplemental figure 4. ^{13}C NMR of Tricine at pH 7.4 (top) and pH 6.5 (bottom).



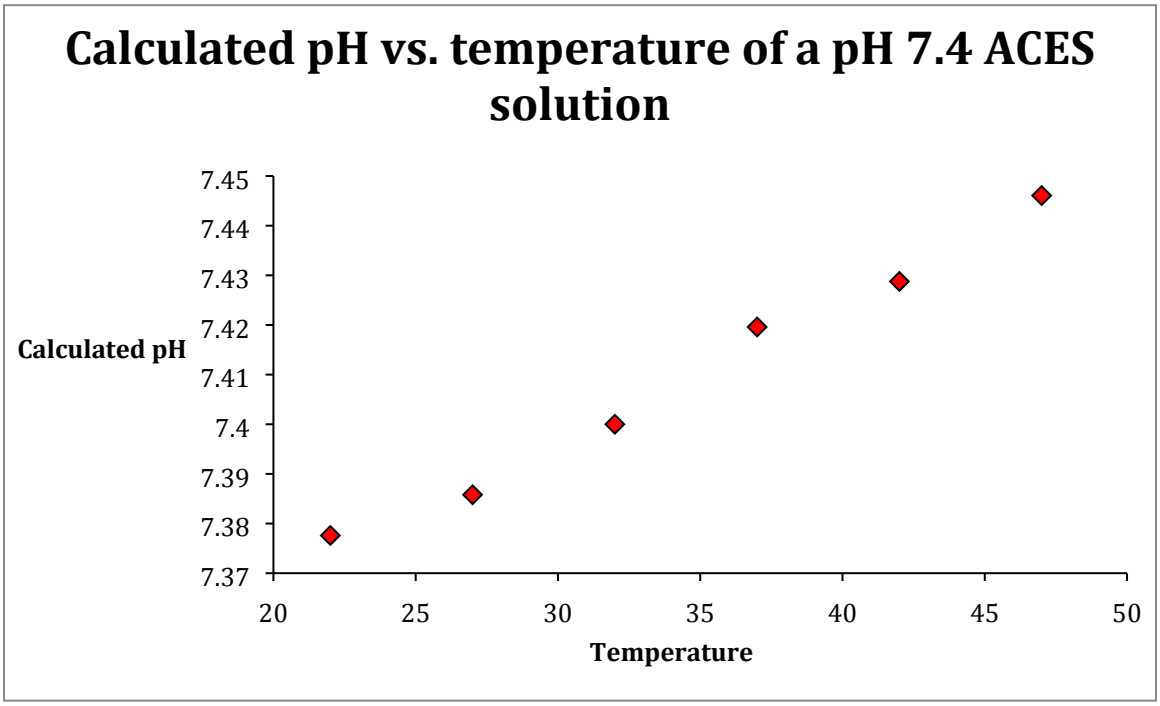
Supplemental figure 5. ^{13}C NMR of ACES at pH 7.4 (top) and pH 6.5 (bottom).



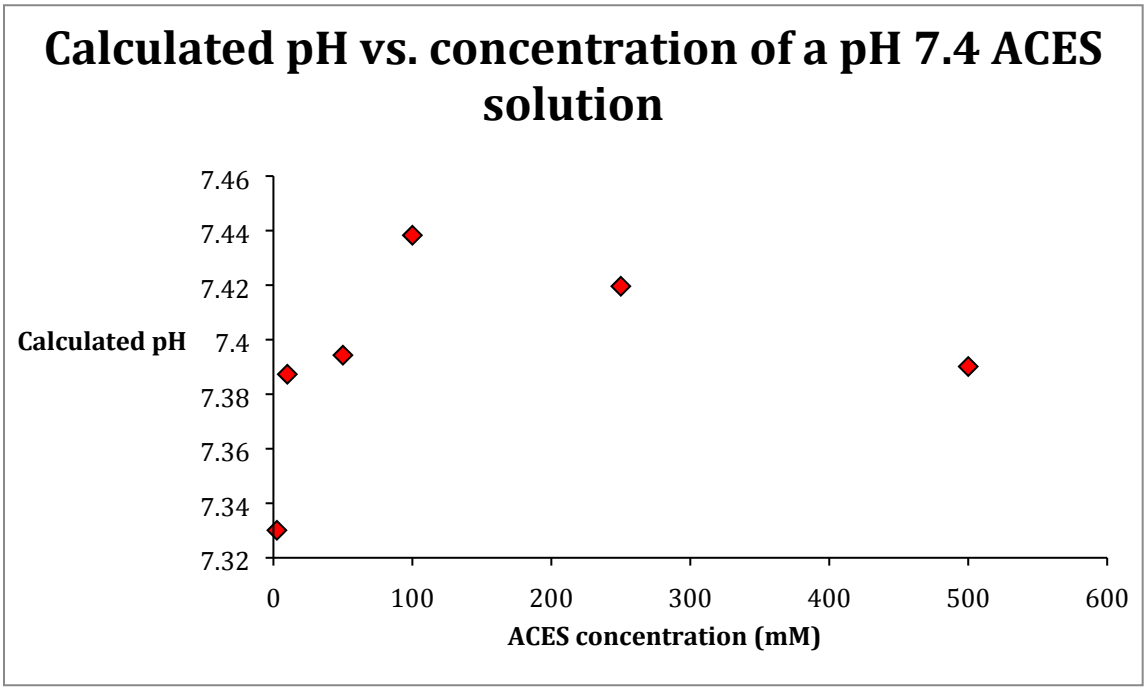
Supplemental figure 6. ^{13}C NMR of ADA at pH 7.4 (top) and pH 6.5 (bottom).



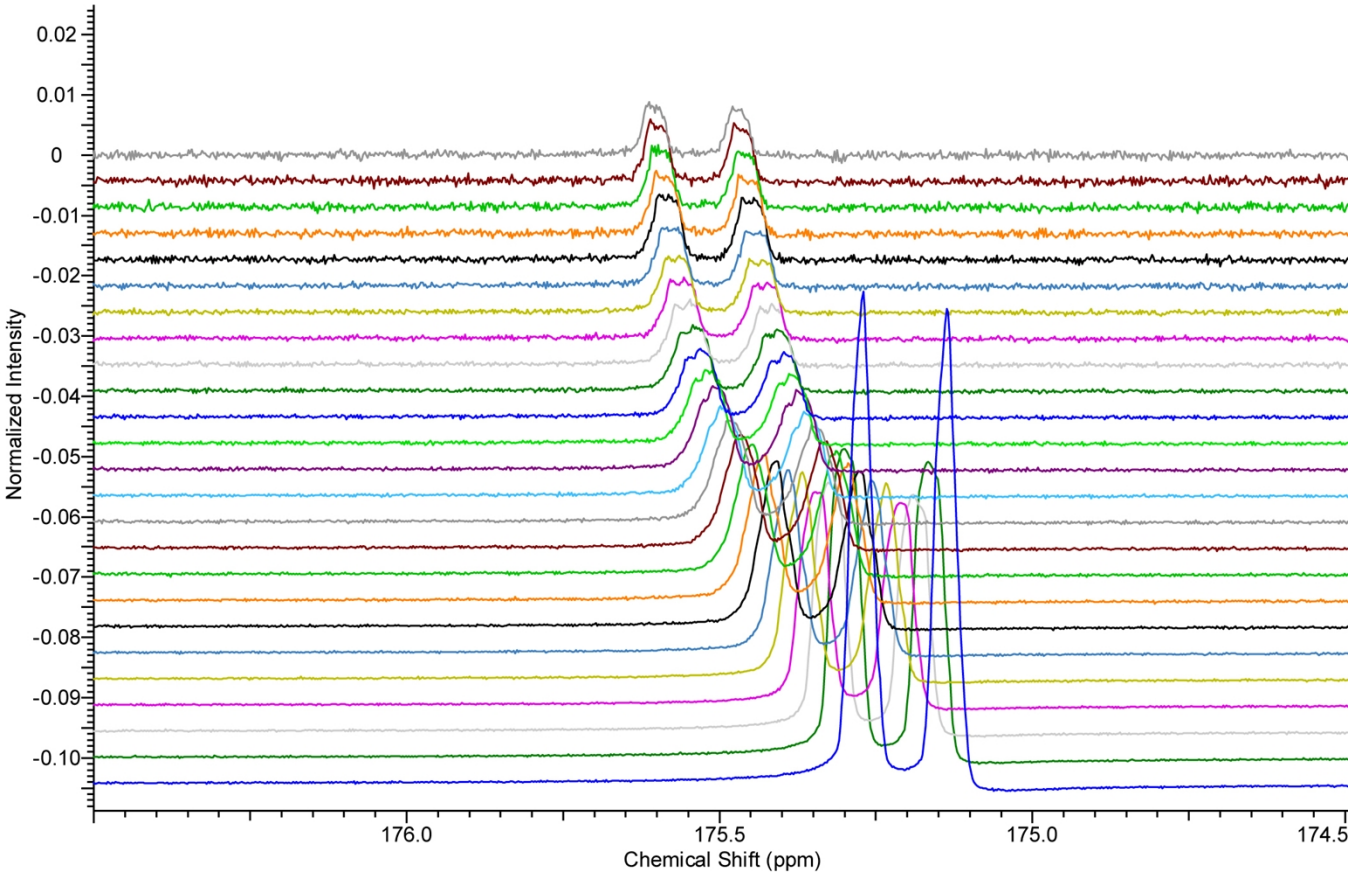
Supplemental figure 7. HPLC analysis of synthetic products. A) HPLC analysis of **6**. B) HPLC analysis of **7**. C) HPLC analysis of **8**. D) HPLC analysis of $^{13}C,^{15}N$ ACES.



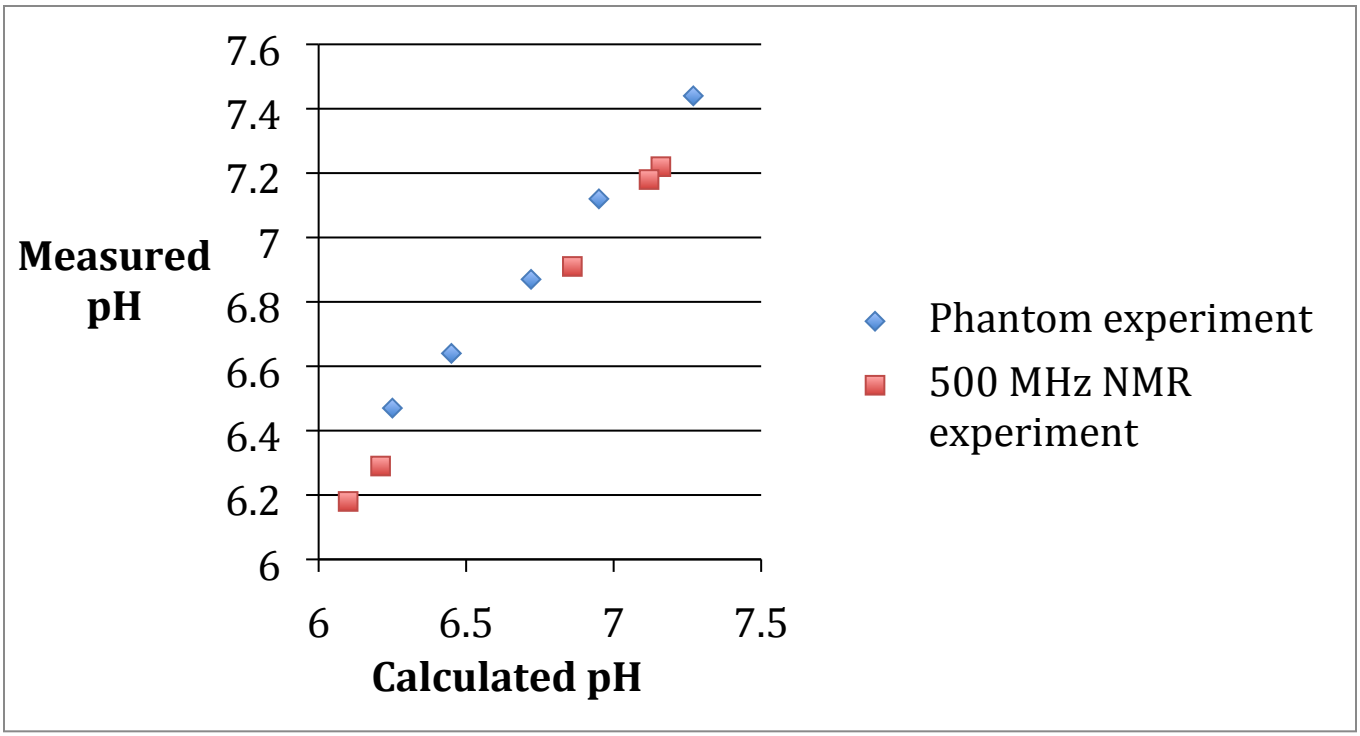
Supplemental figure 8. Change in pH calculated by NMR experiments as a function of temperature.



Supplemental figure 9. Change in pH calculated by NMR experiments as a function of ACES concentration.



Supplemental figure 10. Serial 3 s spectra of hyperpolarized ^{13}C, ^{15}N ACES demonstrating a slight change in chemical shift over time. At the first transient, the calculated pH is 6.77, while at the last the calculated pH is 6.86. The pH measured on a pH meter following the experiment was 6.91.



Supplemental figure 11. Summary of all data correlating calculated with measured pH, from both 500 MHz NMR experiments (Red squares) and in the 3T phantom experiment (Blue diamonds).

		Calcium concentration (mM)	
		0	10
pH	7.4	178.56	179.61
	6.5	174.95	179.49

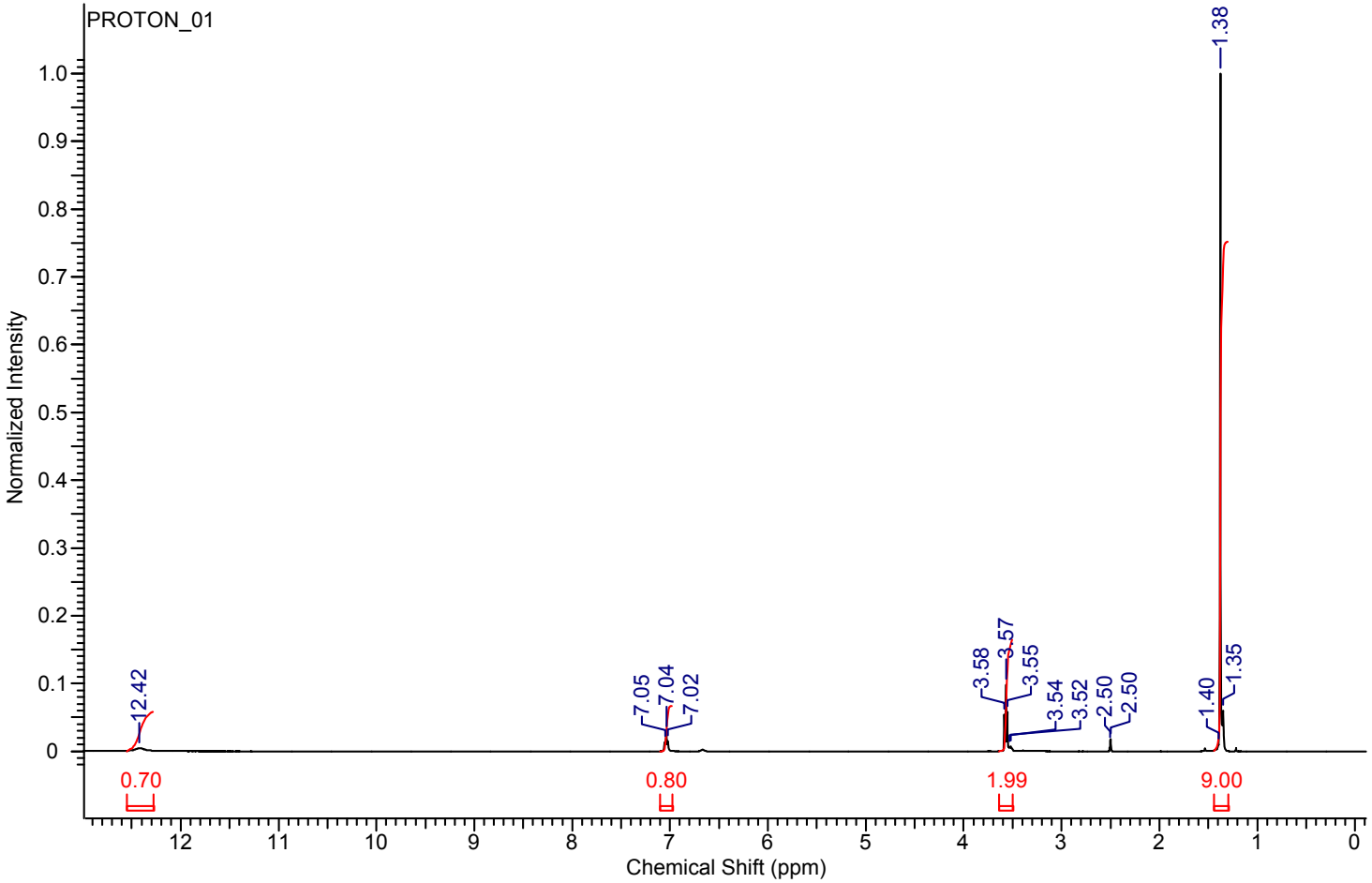
Supplemental table 1. Chemical shift of ADA buffer in response to pH and calcium.

Gd-DOTA concentration (mM)	Time constant (s)	Max polarization (a.u.)
0.00	1182	2755
0.50	1660	5533
1.00	1430	5100

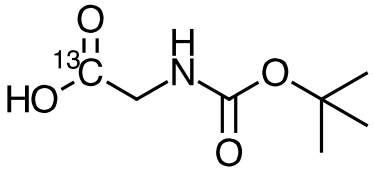
Supplemental table 2. Optimization of Gd-DOTA concentration for ^{13}C, ^{15}N ACES hyperpolarization

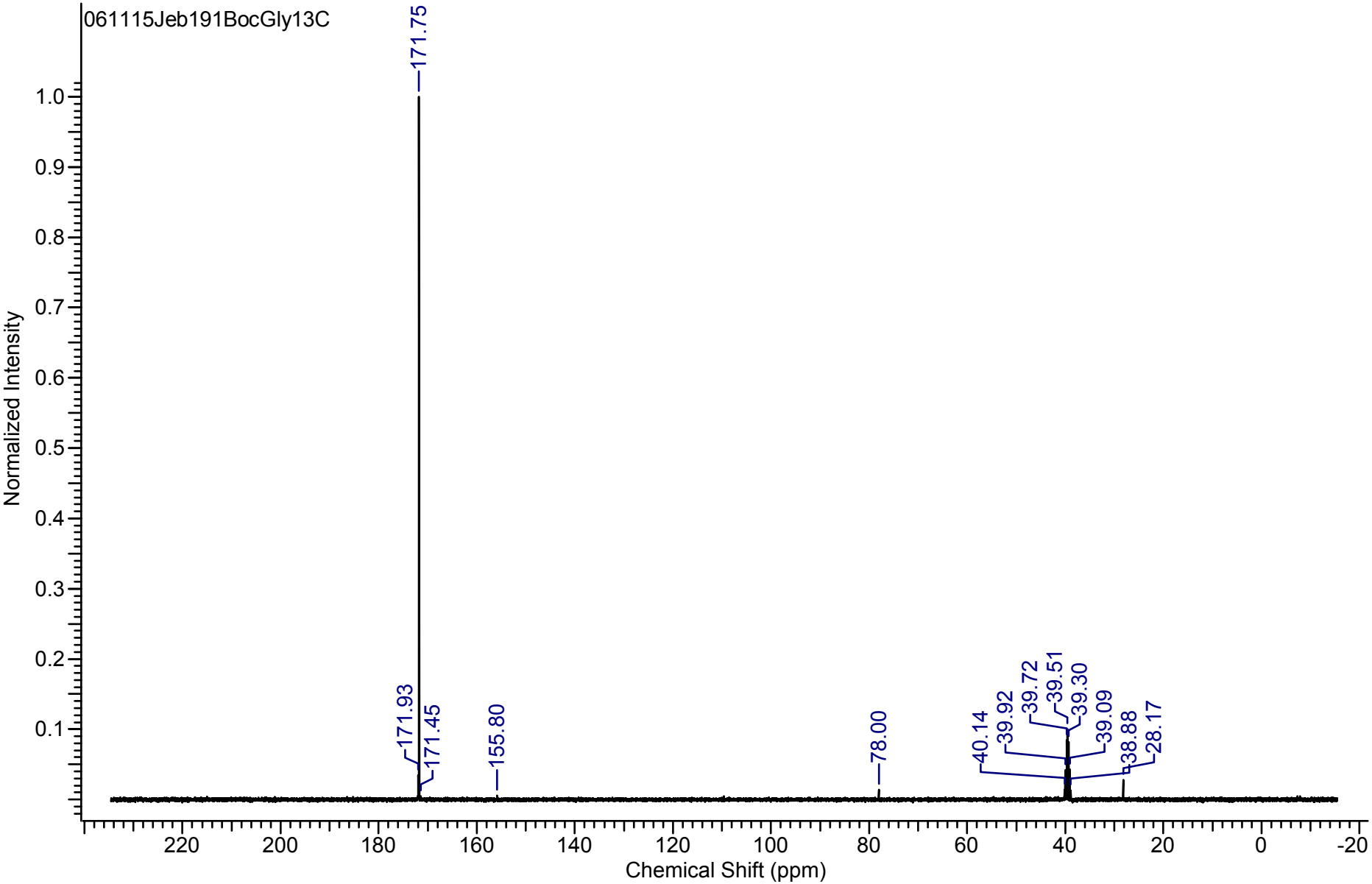
	[Phosphate buffer] (mM)	
	0	50
δ ppm	-12.65	-12.50
Calculated pH	7.33	7.27

Supplemental table 3. Effect of phosphate buffer on calculated pH. Both solutions contained 2.5 mM ^{13}C,^{15}N ACES, with or without 50 mM phosphate buffer. Both solutions measured pH 7.40 on the pH meter.

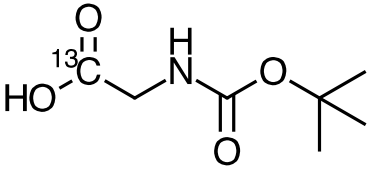


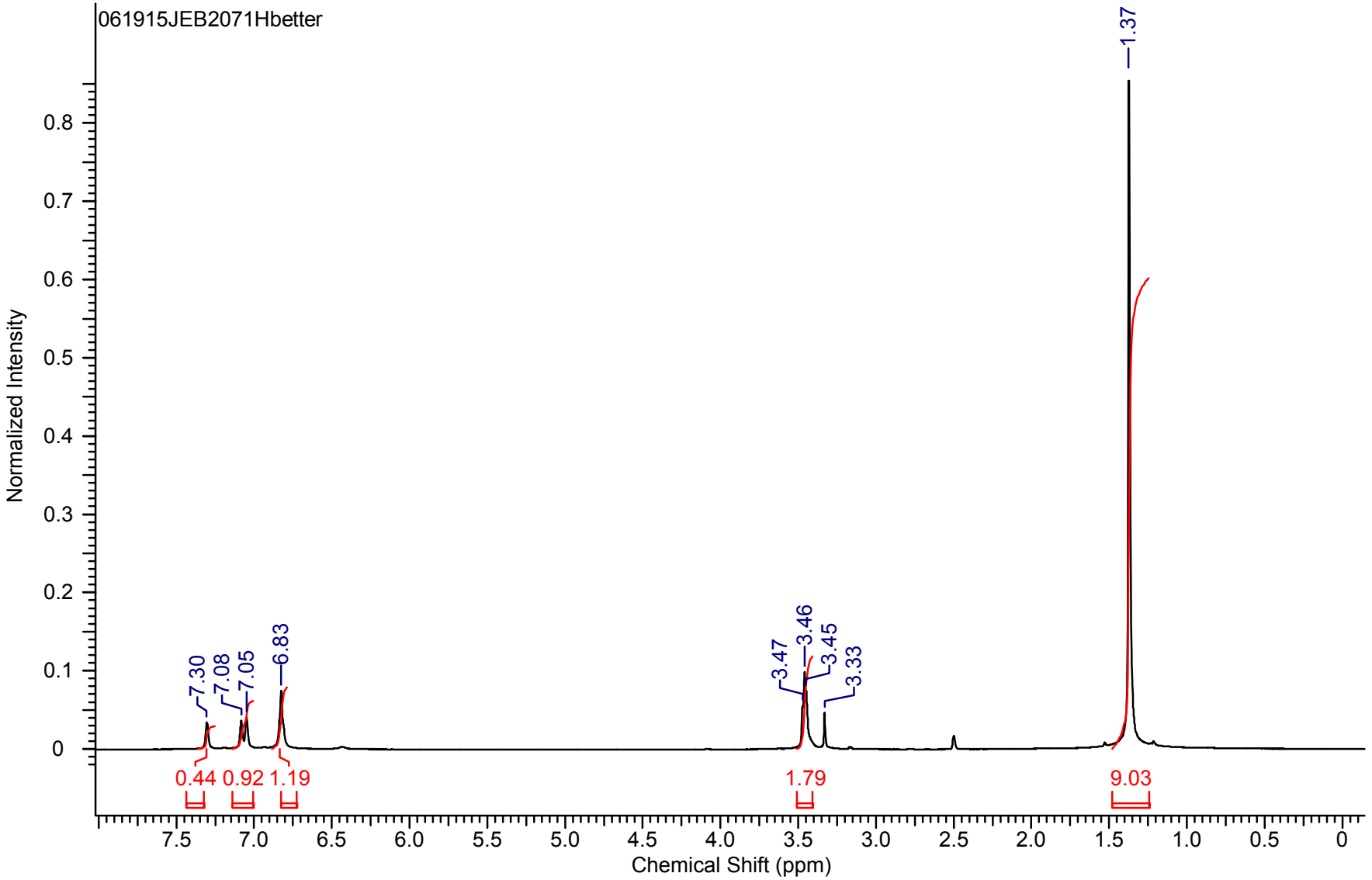
1H NMR, Compound **6**, DMSO-d_6, 400 MHz



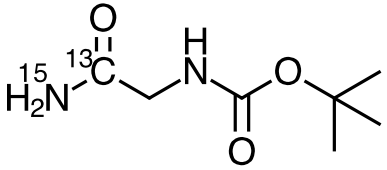


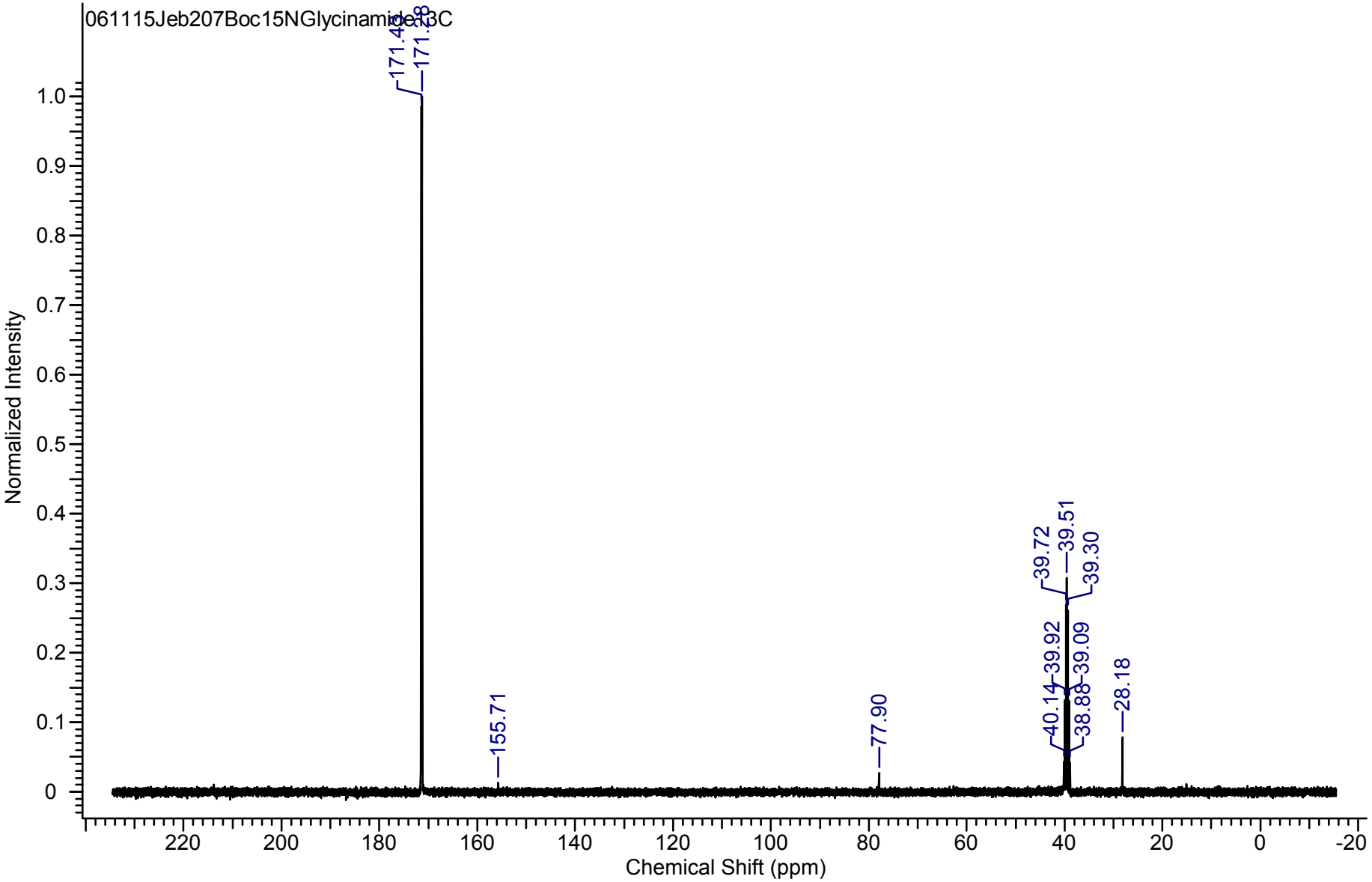
13C NMR, Compound **6**, DMSO-d_6, 400 MHz



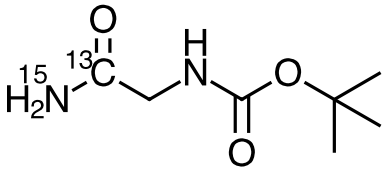


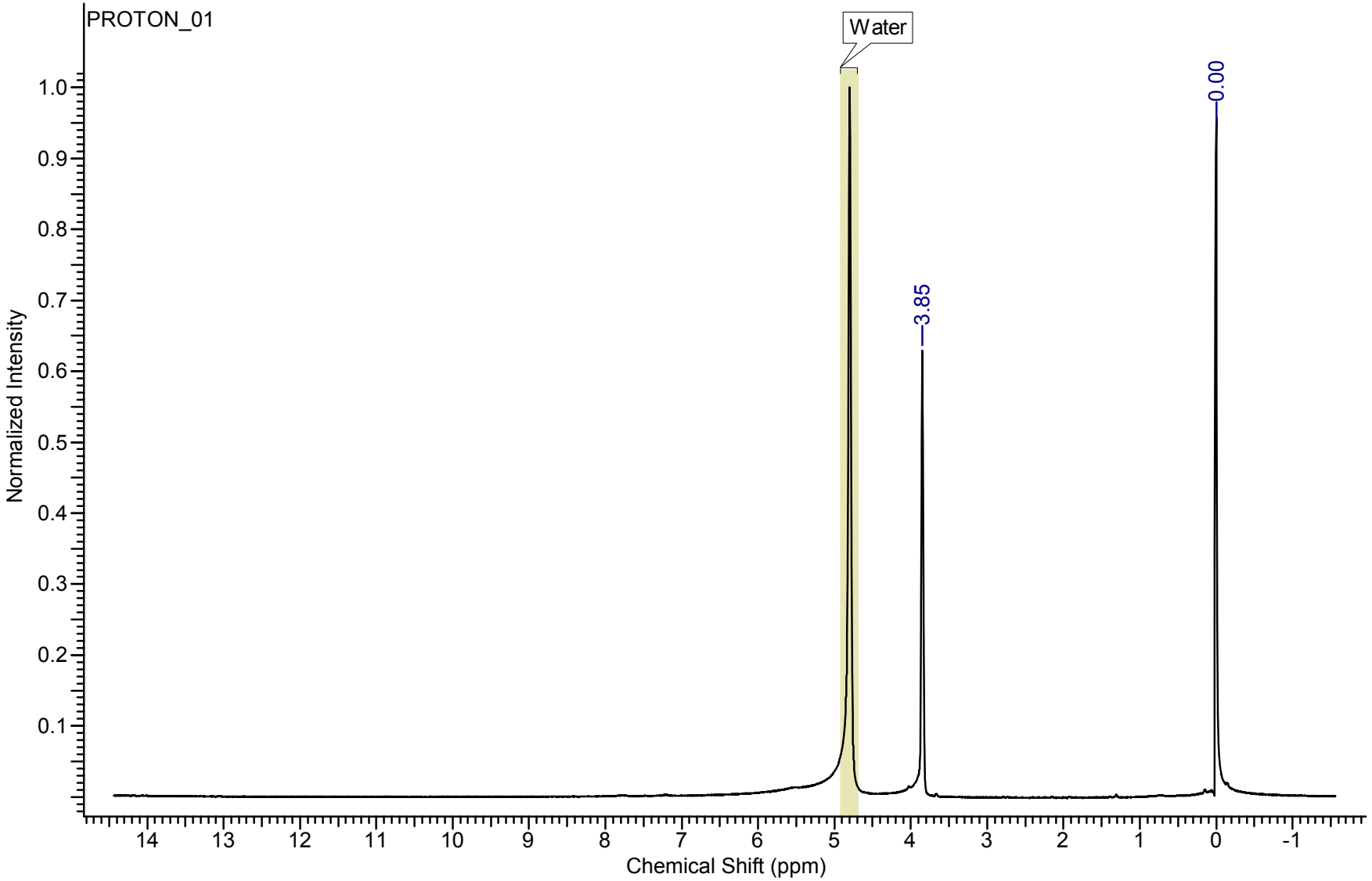
1H NMR, Compound **7**, DMSO-d_6, 400 MHz



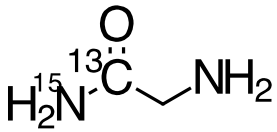


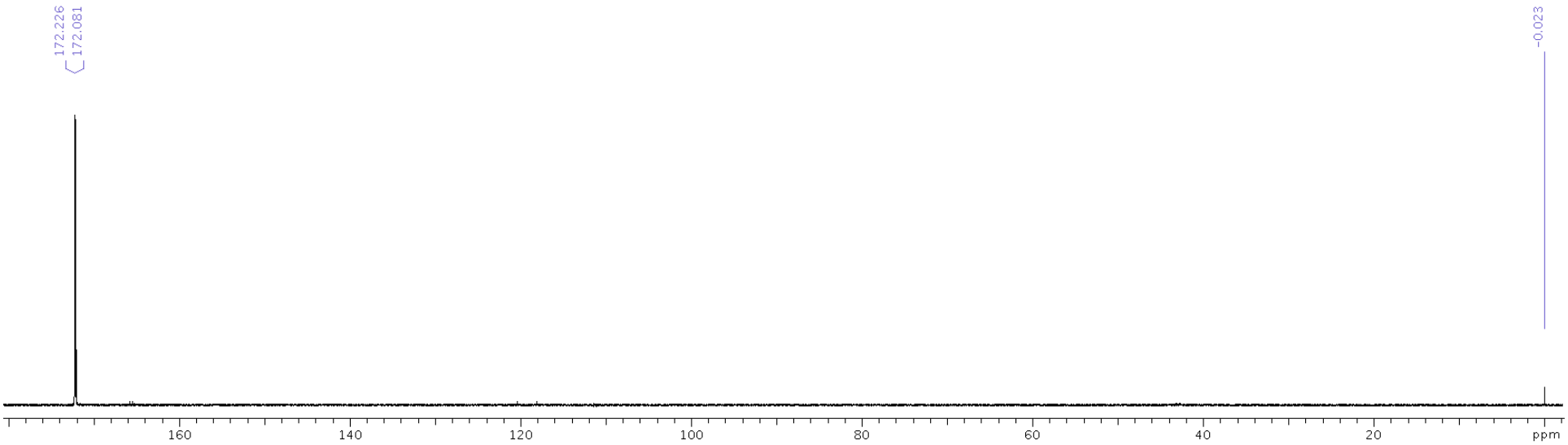
13C NMR, Compound **7**, DMSO-d_6, 400 MHz



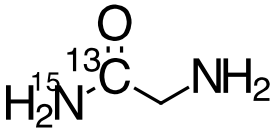


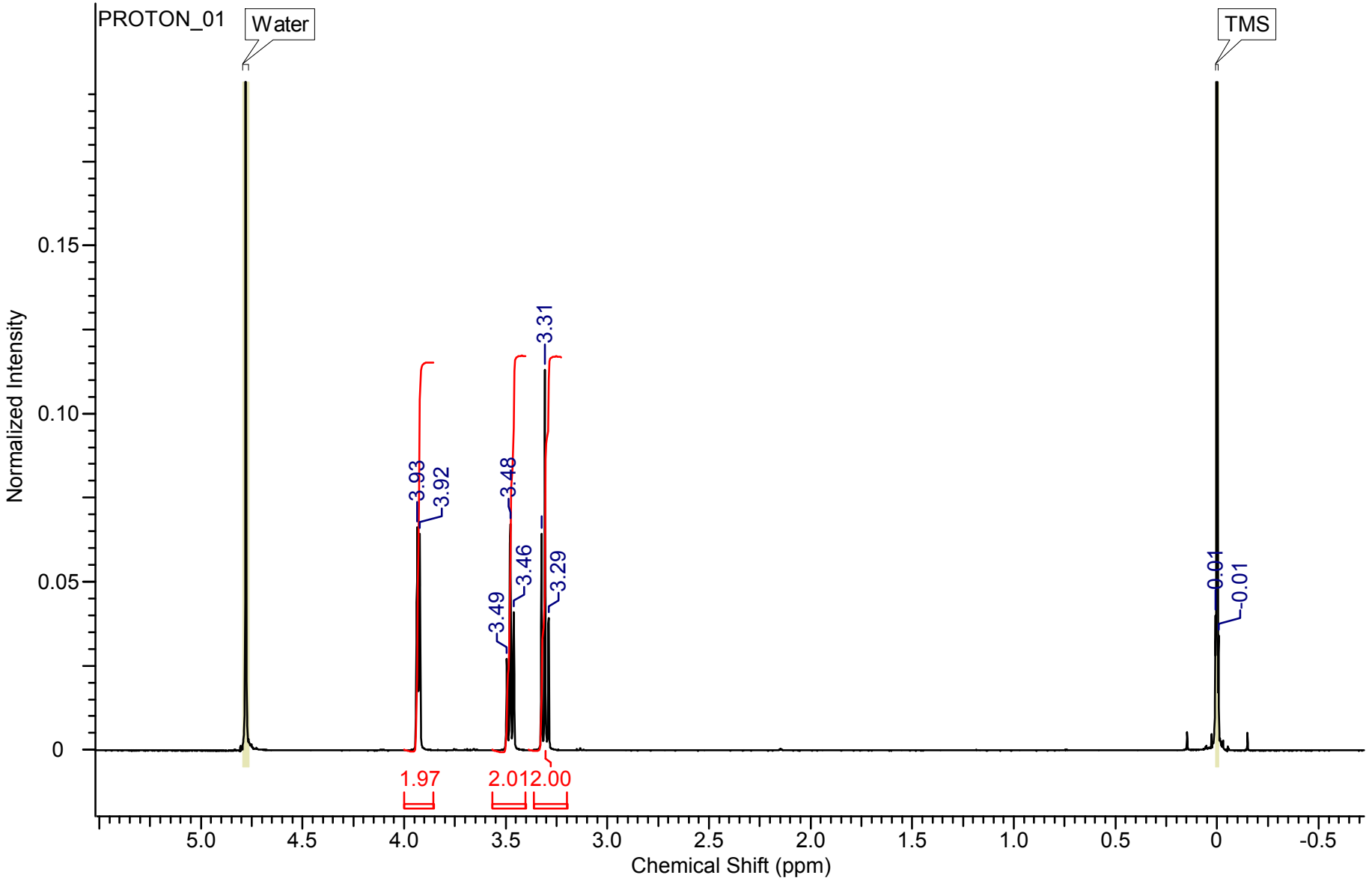
1H NMR, Compound **8**, D_2O, 400 MHz



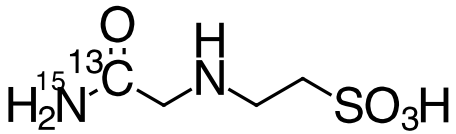


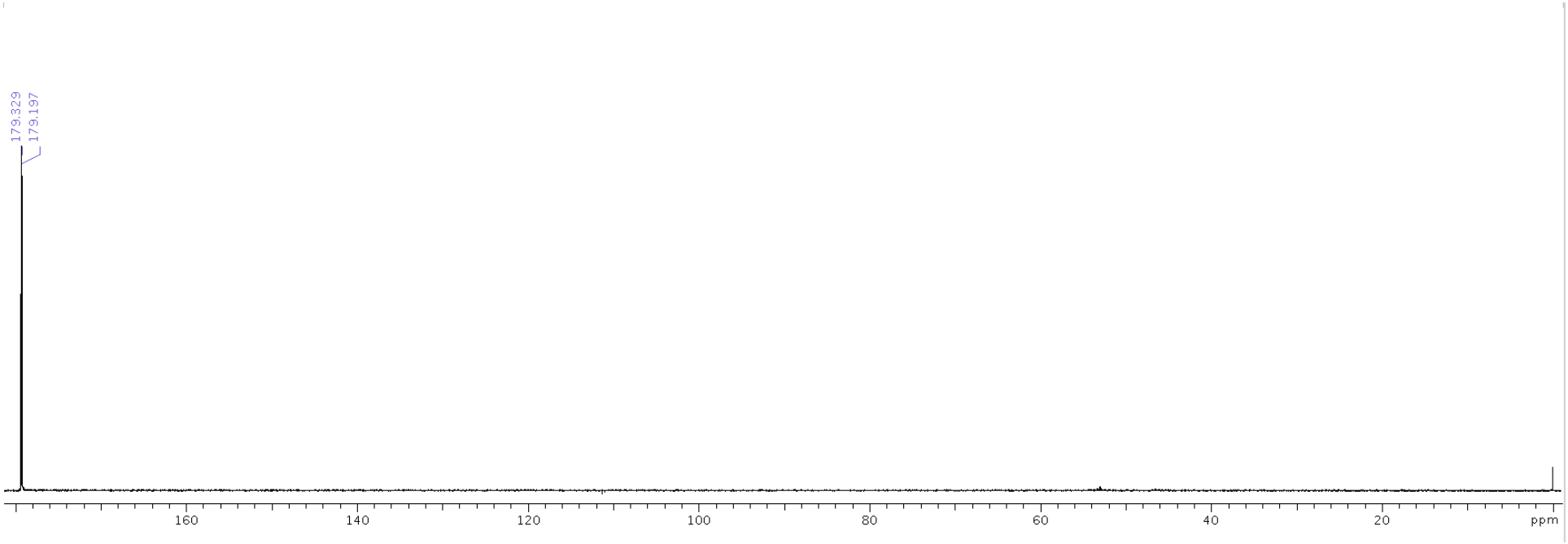
13C NMR, Compound **8**, D_2O, 400 MHz



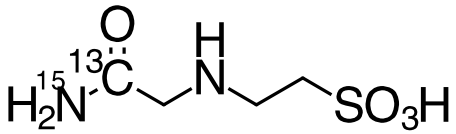


1H NMR, Compound **4**, D_2O, 400 MHz





13C NMR, Compound **4**, D_2O, 500 MHz



1 Good, N. E. *et al.* Hydrogen ion buffers for biological research. *Biochemistry* **5**, 467-477 (1966).
2 von Morze, C. *et al.* Imaging of Blood Flow Using Hyperpolarized [C-13] Urea in Preclinical Cancer Models. *Journal of Magnetic Resonance Imaging* **33**, 692-697, doi:Doi 10.1002/Jmri.22484 (2011).
3 Keshari, K. R. *et al.* Hyperpolarized [2-C-13]-Fructose: A Hemiketal DNP Substrate for In Vivo Metabolic Imaging. *J Am Chem Soc* **131**, 17591-17596, doi:Doi 10.1021/Ja9049355 (2009).