Supporting Information

Artificial Metalloproteins Containing Co_4O_4 Cubane Active Sites

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Index *Page*

Materials. Distilled deionized water (ddH_2O) (18 MΩ cm^{-1}) was used for all aqueous solutions and other uses of water. All commercially available reagents were obtained of the highest purity and used as received. Biotin, iminobiotin agarose resin, and the codon optimized pET24a-Sav plasmid[1] were received as a generous gift from Professor Thomas R. Ward. Thin-layer chromatography (TLC) was performed on Whatman 250 μm layer 6 Å aluminum-backed silica gel plates or Analtech silica gel and alumina G 250 μm glass backed plates. Eluted plates were visualized using UV or visible light and/or p-DACA stain as indicated.[2] Column chromatography was performed with the indicated solvent system using Fisher reagent silica gel 60 (230-400 mesh) or basic alumina (60-325 mesh, Brockman Activity I). Q5 High-fidelity 2x master mix was purchased from NEB and primers, in purified form, were custom ordered from Invitrogen. Lysozyme from chicken egg white, lyophilized DNase I, and Sephadex G-25 medium-grain gel filtration resin were purchased from Sigma Aldrich.

Preparation of biot-β-Ala-1. The synthesis of biot-β-Ala-**1** was achieved through slight variations to published methods. Biotin pentafluorophenyl ester was prepared as previously described from the condensation of biotin and pentafluorophenyl trifluoroacetate.[3] Biot-β-Ala was synthesized from the reaction of β-alanine with the activated biotin pentafluorophenyl ester in the presence of 1.2 eq. Et_3N and in a mixture of H_2O:DMF (1:6), with water included to aid in β-Ala solubility. Solvent was removed *en vacuo* and the dry crude product triturated with Et_2O. Filtration on a fine glass frit followed by washing with excess Et_2O provided the pure product. The synthesis[4] and purification[5] of $Co_4O_4(OAc)_4(py)_4$ (**1**) was carried out as previously described. Equilibrating **1** with biot-β-Ala in refluxing MeOH for 5 h afforded a mixture of mono-biotinylated (biot-β-Ala-[$Co_4O_4(OAc)_3(py)_4$], hereafter referred to as biot-β-Ala-**1**) and doubly-biotinylated ((biot-β-Ala)$_2$-[$Co_4O_4(OAc)_2(py)_4$]) cubanes. The crude reaction mixture could be purified by two methods: a) Dry-loading the crude material onto silica gel and column chromatography provided the purified products using a solvent

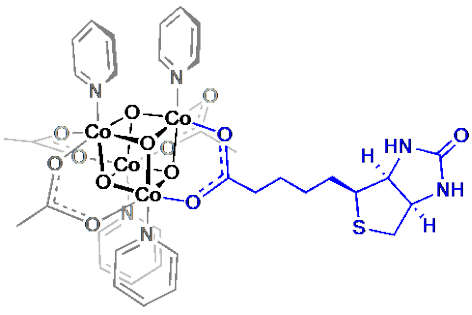
mixture containing 3% Et_3N in acetone and a gradient of 2 to 10% MeOH (R_f with 5% MeOH = 0.36) followed by concentration under reduced pressure to yield the dark green solid in 36%; or b) wet-loading the crude material onto a chromatography column of silica gel with a mobile phase of CH_2Cl_2/MeOH (99:1) and a gradual increase of polarity up to 9:1 followed by further purification on basic alumina with a gradient of CH_2Cl_2/MeOH (99.9:0.1) up to 95:5 provided the purified material in 6.15% yield. ^{1}H NMR (600 MHz, Methanol-d_4, 25 °C) δ 8.43 (d, *J* = 5.2 Hz, 8H, aryl-*H*), 7.68 (t, *J* = 6.5 Hz, 4H, aryl-*H*), 7.20 (t, *J* = 6.1 Hz, 8H, aryl-*H*), 4.48 (dd, *J* = 7.9, 5.0 Hz, 1H, –C*H*), 4.28 (dd, *J* = 7.8, 4.5 Hz, 1H, –C*H*), 3.46 (dt, *J* = 13.3, 6.4 Hz, 1H, –C*H*), 3.17 (ddt, *J* = 20.2, 9.8, 5.3 Hz, 1H, –CH_2), 2.91 (dt, *J* = 12.3, 6.0 Hz, 1H, –CH_2), 2.70 (dd, *J* = 12.8, 2.9 Hz, 1H, –CH_2), 2.57 (t, *J* = 6.6 Hz, 2H, –CH_2), 2.12 (s, 9H, –CH_3), 1.74 – 1.51 (m, 4H, –CH_2), 1.44 – 1.34 (m, 2H, –CH_2). ^{13}C {^{1}H} (151 MHz, MeOD, 25 °C) δ 188.55, 188.52, 188.36, 188.34, 175.67, 166.09, 154.09, 154.06, 139.07, 139.05, 125.33, 125.30, 63.35, 61.64, 61.62, 56.94, 49.85, 49.57, 41.03, 39.98, 39.94, 37.02, 36.82, 29.82, 29.81, 29.48, 26.75, 26.72, 26.47, 26.40. Elem. anal. calcd for [$C_{39}H_{49}Co_4N_7O_{14}S$ · 2.0 CH_2Cl_2 · MeOH]: C: 38.52, H: 4.39, N: 7.49, S: 2.45; found C: 38.87, H: 4.62, N: 7.87, S: 2.59. ESI-HRMS (MeOH) *m/z* calcd for $C_{39}H_{49}Co_4N_7O_{14}S$ [M + H]$^+$ 1108.0464, found 1108.0495; [M + Na]$^+$ 1130.0284, found 1130.0279.

Preparation of additional biot-Z-1 complexes (Z = various linkers). Biot-gaba and biot-py were prepared analogously to the procedure described above. Biot-histamine was prepared by adding isobutyl chloroformate (0.098 mL, 0.740 mmol) to a solution of biotin (0.150g, 0.614 mmol) in DMF (10 mL) containing Bu_3N (0.190 mL, 0.800mmol). After 10 min of stirring at room temperature, the mixture was slowly added to a suspension of histamine (0.136g, 1.230 mmol) in 10 mL of DMF/H_2O (1:1) stirring at 5 °C. The mixture was stirred for 2h at 5 °C, after which the volatiles were evaporated under vacuum at temperatures less than 50 °C. The remaining solid was dissolved in warm aqueous ethanol (1:1, 20 mL) and acidified with 2.0 *N* HCl solution to pH = 4 at –30 °C for 16 h to yield a white precipitate (0.109 g, 53%) that was filtered, washed with

cold water, and dried under vacuum. ^{1}H NMR (600 MHz, DMSO-d_6) δ 14.53 (s, 1H, Imz–N*H*), 9.01 (d, *J* = 1.4 Hz, 1H, Imz-C*H*), 8.04 (t, *J* = 5.7 Hz, 1H, CO–N*H*), 7.41 (s, 1H, Imz–C*H*), 6.40 (d, *J* = 23.2 Hz, 2H, –N*H*), 4.31 (dd, *J* = 7.8, 5.1 Hz, 1H, –C*H*), 4.12 (ddd, *J* = 7.4, 4.6, 1.8 Hz, 1H, –C*H*), 3.08 (ddd, *J* = 8.6, 6.1, 4.4 Hz, 1H, –C*H*), 2.83 (dd, *J* = 12.4, 5.1 Hz, 1H, –CH_2), 2.78 (t, *J* = 6.8 Hz, 2H, –CH_2), 2.59 (s, 1H, –CH_2), 2.04 (t, *J* = 7.4 Hz, 2H, –CH_2), 1.64 – 1.38 (m, 4H, –CH_2), 1.25 (ddt, *J* = 18.9, 13.4, 6.5 Hz, 2H, –CH_2). ^{13}C NMR (75 MHz, DMSO) δ 172.24, 162.73, 131.03, 116.07, 61.06, 59.22, 55.42, 37.26, 35.12, 28.17, 28.05, 25.23, 24.51. ESI-HRMS (MeOH) *m/z* calcd for $C_{15}H_{23}N_5O_2S$ [M+H]$^+$ 338.1651, found 338.1636.

Preparation of biot-1, biot-gaba-1, biot-py-1, and biot-histamine-1. Substitution reactions with **1** were performed in stirred MeOH suspensions (20 mL) at 50 °C during 4 h for acetate derived ligands (biotin and biot-gaba) and for 2 h for pyridine and imidazole donor moieties (biot-py and biot-histamine). These resulted in dark green solutions containing mono- and di-biotinylated derivatives. Volatiles were removed under vacuum and the crude product was wet loaded onto a chromatography column of silica gel with a mobile phase of CH_2Cl_2/MeOH (99:1) with a gradual increase of polarity up to (9:1). Further purification of the mono-biotinylated fraction was performed by wet loading onto basic alumina and elution with a mixture of CH_2Cl_2/MeOH (99.9:0.1) with a gradient to (95:5).

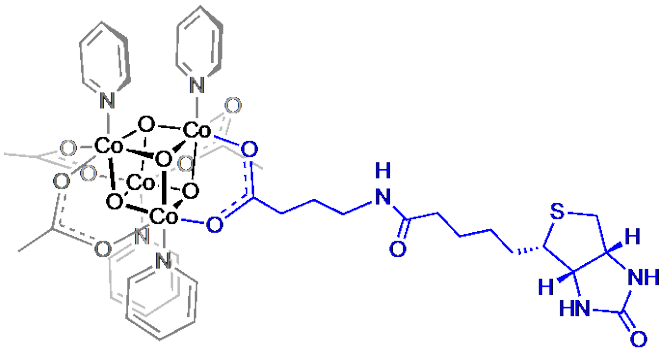
biot-1: Yield: 12.44% from **1** (0.200 g, 0.235 mmol) and biotin (0.057 g, 0.235 mmol). Dark green solid [Rf. in SiO_2 with CH_2Cl_2/MeOH (9:1) = 0.5; Al_2O_3 with CH_2Cl_2/MeOH (95:5) = 0.6]. ^{1}H NMR (500 MHz, Methanol-d_4, 25 °C) δ 8.42 (d, *J* = 5.9 Hz, 8H, aryl-*H*), 7.72 – 7.63 (m, 4H, aryl-*H*), 7.20 (q, *J* = 6.0 Hz, 8H, aryl-*H*), 4.45 (ddd, *J* = 24.3, 7.9, 4.9 Hz, 1H, C*H*), 4.26 – 4.22 (m, 1H, –C*H*), 3.20 – 3.10 (m, 1H, –C*H*), 2.89 (ddd, *J* = 23.5, 12.7, 4.9 Hz, 1H, –CH_2), 2.70 (dd, *J* = 18.5, 12.7 Hz, 1H, –CH_2), 2.45 – 2.31 (m, 2H, –CH_2), 2.11 (s, 9H, –CH_3), 1.73 – 1.36 (m, 6H, –CH_2). ^{13}C {^{1}H }



biot-1

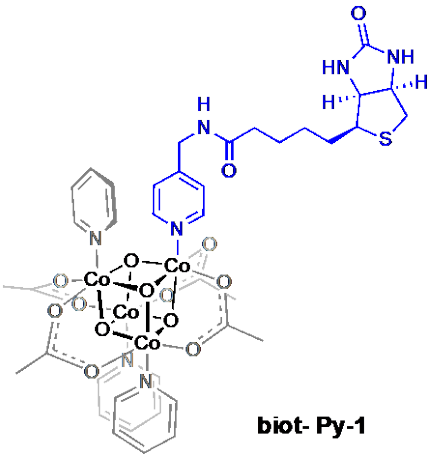
(151 MHz, MeOD, 25 °C) δ 190.53, 188.29, 188.26, 188.22, 154.14, 154.12, 154.09, 138.95, 125.27, 125.23, 63.34, 61.59, 57.27, 56.91, 41.06, 40.30, 40.21, 30.01, 29.80, 29.67, 29.42, 26.95, 26.60, 26.43, 26.40. Elem. anal. calcd for [$C_{36}H_{44}Co_4N_6O_{13}S$ · 1.5 CH_2Cl_2 · MeOH]: C: 38.66, H: 4.30, N: 7.03, S: 2.68; found C: 38.67, H: 4.36, N: 7.36, S: 2.75. ESI-HRMS (MeOH) *m/z* calcd for $C_{36}H_{44}Co_4N_6O_{13}S$ $[M + H]^+$ 1037.0093, found 1037.0070; $[M + Na]^+$ 1058.9913, found 1058.9878.

biot-gaba-1: Yield: 8.3% from **1** (0.100 g, 0.118 mmol) and biotin (0.037 g, 0.118 mmol). Dark green solid [Rf. in SiO_2 with CH_2Cl_2/MeOH (9:1) = 0.36; Al_2O_3 with CH_2Cl_2/MeOH (95:5) = 0.48]. ^1H NMR (500 MHz, Methanol-d_4, 25 °C) δ 8.42 (m, 8H, aryl-*H*), 7.68 (t, *J* = 6.6 Hz, 4H, aryl-*H*), 7.20 (t, *J* = 5.8 Hz, 8H, aryl-*H*), 4.43 (ddd, *J* = 15.0, 7.9, 4.8 Hz, 1H, –C*H*), 4.24 (ddd, *J* = 15.5, 7.9, 4.5 Hz, 1H, –C*H*), 3.16 (m, 2H, –CH_2), 3.14 (m, 1H, –C*H*), 2.87 (ddd, *J* = 24.0, 12.7, 5.0 Hz, 1H, –CH_2), 2.67 (dd, *J* = 12.7, 7.3 Hz, 1H, –CH_2), 2.47 – 2.38 (m, 2H, –CH_2), 2.11 (d, *J* = 3.4 Hz, 9H, –CH_3), 1.78 (p, *J* = 7.3 Hz, 2H, –CH_2), 1.61 (m, 4H, –CH_2), 1.38 (p, *J* = 7.8 Hz, 2H, –CH_2). ^{13}C {^1H } (151 MHz, MeOD, 25 °C) δ 189.91, 189.86, 188.31, 188.28, 188.26, 175.85, 154.14, 154.12, 138.95, 129.04, 125.28, 125.24, 63.31, 61.60, 56.93, 49.43, 41.00, 40.04, 38.03, 36.79, 29.73, 29.45, 26.85, 26.51, 26.45, 26.43, 26.41, 26.39. Elem. anal. calcd for [$C_{40}H_{51}Co_4N_7O_{14}S$ · 2.0 CH_2Cl_2 · MeOH]: C: 38.52, H: 4.39, N: 7.49, S: 2.45; found C: 38.87, H: 4.62, N: 7.87, S: 2.59. ESI-HRMS (MeOH) *m/z* calcd for $C_{39}H_{49}Co_4N_7O_{14}S$ $[M + H]^+$ 1122.0621, found 1122.0683; $[M + Na]^+$ 1144.0440, found 1144.0502.

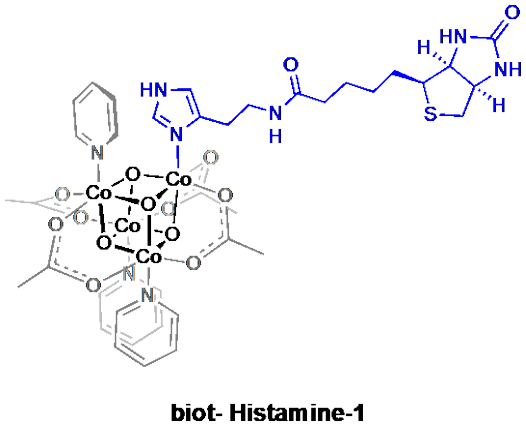


biot-GABA-1

biot-py-1: Yield: 11.15% from **1** (0.200 g, 0.235 mmol) and biotin (0.074 g, 0.235 mmol). Dark green solid [Rf. in SiO_2 with CH_2Cl_2/MeOH (9:1) = 0.31; Al_2O_3 with CH_2Cl_2/MeOH (95:5) = 0.6]. ^{1}H NMR (600 MHz, Methanol-d_4, 25 °C) δ 8.42 (d, *J* = 5.1 Hz, 4H, aryl-*H*), 8.39 (d, *J* = 5.1 Hz, 2H, aryl-*H*), 8.32 (d, *J* = 5.9 Hz, 2H, aryl-*H*), 7.78 (t, *J* = 7.6 Hz, 1H, aryl-*H*), 7.67 (t, *J* = 7.6 Hz, 2H, aryl-*H*), 7.20 – 7.17 (m, 5H, aryl-*H*), 7.06 (d, *J* = 5.6 Hz, 2H, aryl-*H*), 4.50 (dd, *J* = 8.0, 5.0 Hz, 1H, –C*H*), 4.40 (s, 2H, –CH_2), 4.33 (dd, *J* = 7.8, 4.5 Hz, 1H, –CH_2), 3.26 (dt, *J* = 9.8, 5.0 Hz, 1H, –C*H*), 2.95 (dd, *J* = 12.8, 5.0 Hz, 1H, –CH_2), 2.73 (d, *J* = 12.6 Hz, 1H, –CH_2), 2.40 (t, *J* = 7.4 Hz, 2H, –CH_2), 2.11 (s, 12H, –CH_3), 1.84 – 1.64 (m, 4H, –CH_2), 1.55 (q, *J* = 7.8 Hz, 2H, –CH_2). ^{13}C NMR (151 MHz, MeOD, 25 °C) δ 188.31, 188.28, 176.21, 154.11, 154.07, 153.84, 152.04, 138.98, 138.96, 125.34, 125.25, 123.25, 63.47, 61.65, 57.08, 42.38, 41.07, 36.75, 30.02, 29.61, 26.90, 26.43, 26.42, 26.41. Elem. anal. calcd for [$C_{39}H_{49}Co_4N_7O_{14}S$ · 2.0 CH_2Cl_2 · MeOH]: C: 38.52, H: 4.39, N: 7.49, S: 2.45; found C: 38.56, H: 4.76, N: 7.90, S: 2.56. ESI-HRMS (MeOH) *m/z* calcd for $C_{39}H_{49}Co_4N_7O_{14}S$ $[M + H]^+$ 1108.0464, found 1108.0429; $[M + Na]^+$ 1130.0284, found 1130.0221.



biot-histamine-1: Yield: 2% from **1** (0.140 g, 0.164 mmol) and biotin (0.055 g, 0.164 mmol). Dark green solid [Rf. in SiO_2 with CH_2Cl_2/MeOH (9:1) = 0.12; Al_2O_3 with CH_2Cl_2/MeOH (95:5) = 0.30]. ^{1}H NMR (500 MHz, Methanol-d_4, 25 °C) δ 8.51 (d, *J* = 5.5 Hz, 2H, aryl-*H*), 8.42 (d, *J* = 5.5 Hz, 4H, aryl-*H*), 7.87 (t, *J* = 7.6 Hz, 1H, aryl-*H*), 7.66 (t, *J* = 7.6 Hz, 2H, aryl-*H*), 7.50 (s, 1H, Imz–C*H*), 7.33 (t, *J* = 6.8 Hz, 2H, aryl-*H*), 7.18 (t, *J* = 6.7 Hz, 4H, aryl-*H*), 6.66 (s, 1H, Imz–C*H*), 4.49 (dd, *J* = 7.9, 5.0 Hz, 1H, –C*H*), 4.30 (dd, *J* = 7.9, 4.5 Hz, 1H, –C*H*), 3.36 (m, 2H, –CH_2), 3.21 (dt, *J* = 9.7, 5.2 Hz, 1H, –C*H*), 2.93 (dd, *J* = 12.8, 5.0 Hz, 1H, –CH_2), 2.71 (d, *J* = 12.7 Hz, 1H, –



CH_2), 2.63 (t, *J* = 7.3 Hz, 2H, –CH_2), 2.24 (t, *J* = 7.3 Hz, 2H, –CH_2), 2.11 (s, 6H, –CH_3), 2.08 (s, 6H, –CH_3), 1.77 – 1.57 (m, 4H, –CH_2), 1.45 (p, *J* = 7.6 Hz, 2H, –CH_2).^{13}C NMR (176 MHz, MeOD, 25 °C) δ 188.22, 188.04, 176.14, 154.14, 154.11, 139.14, 138.93, 138.85, 129.21, 126.77, 125.28, 125.24, 124.81, 63.38, 61.64, 57.02, 41.05, 39.68, 36.78, 29.83, 29.50, 26.84, 26.43, 26.40, 26.34, 26.14. ESI-HRMS (MeOH) *m/z* calcd for $C_{38}H_{50}Co_4N_8O_{14}S$ $[M + H]^+$ 1111.0573, found 1111.0520; $[M + Na]^+$ 1133.0393, found 1133.0353.

Preparation of Sav variants. Construction of $E_{101}Q/K_{121}A$-Sav and other variants were achieved by site-directed mutagenesis (SDM) using the codon optimized $K_{121}A$-pET24a-Sav plasmid,[1] the following primers and, and Q5 polymerase.

$E_{101}Q$_fwd: 5'-GGTGCACAAGCACGCATTAATACCC-3'

$E_{101}Q$ _rev: 5'-GTGCTTGTGCACCACCAACATACTG-3'

$S_{112}Y$_fwd: 5'-GACCTACGGCACCACCGAAGCAAATGC-3'

$S_{112}Y$_rev: 5'-GTGCCGTAGGTCAGCAGCCACTGG-3'

$S_{112}F$_fwd: 5'-CTGCTGACCTTTGGCACCACCGAAGC-3'

$S_{112}F$_rev: 5'-TGGTGCCAAAGGTCAGCAGCCACTGG-3'

Amplification of pET24a-Sav mutant plasmids was accomplished by transformation of SDM reaction mixtures into DH5α ultracompetent cells. Plasmids were isolated using a Miniprep kit from Qiagen, eluting the final plasmid with distilled deionized-water (ddH_2O, 18 MΩ cm^{-1}). DNA sequencing was performed by Genewiz.

Sav Expression. Transformation of 3 µL amplified plasmids into 50 µL Rosetta cells, was followed by rescue with 450 µL LB media. Of this solution 200 µL were spread aseptically onto LB/Kanamycin agar plates and incubated overnight at 37 °C. Inoculation of a starter culture containing 500 mL LB media and the same antibiotic from a single colony was followed by incubation overnight at 37 °C and shaking at 225 rpm. From this starter culture, 25 mL was used to inoculate each 2L flask containing 500 mL LB media, 25 mL each of 20x sugar (12% glycerol, 1% glucose, 10% lactose) and salt (1 M Na_2HPO_4, 1 M KH_2PO_4, 0.5 M $(NH_4)_2SO_4$) stocks, 1 mL of 1 M $MgSO_4$, 100 µL

5000x trace metal mix (containing 1 M $CaCl_2$, 100 mM $FeCl_3$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM $CoCl_2$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM Na_2MoO_4, and 2 mM H_3BO_3 all in 60 mM HCl), and 250 µL of 100 mg/mL Kanamycin. Incubation at 37 °C and 225 rpm was continued until cells reached OD_{600} = 0.6–0.8, at which point the temperature was dropped to 25 °C and cultures incubated another 24 h.

Sav purification. Cultures were centrifuged at 4000 x g for 10 min at 4 °C. The resulting cell pellet was resuspended in lysis buffer (50 mL per L expressed) containing 20 mM Tris buffer pH 7.4, 1 mM PMSF, 1 mg/mL lysozyme, and a spatula tip of DNase I. The suspension was then incubated at 37 °C and 225 rpm for 45 min followed by one freeze-thaw cycle. Dialysis against 6 M guanidinium hydrochloride pH 1.5 for 24 h was followed by neutralization via dialysis against 20 mM Tris buffer pH 7.4 for 24 h. Dialysis overnight against iminobiotin (IB) buffer containing 500 mM NaCl and 50 mM $NaHCO_3$ at pH 9.8 afforded the crude, biotin-free lysate. This material was centrifuged at 10,000 x g for 45 min at 4 °C and the soluble portion loaded onto an iminobiotin-agarose column pre-equilibrated with IB buffer. The column was washed with 6 CVs of IB buffer or until the absorbance at 280 nm (A_{280}) dropped to zero. Elution with 1% AcOH in ddH_2O, and pooling fractions by A_{280}, provided highly pure (>95%) Sav as assessed by 18% SDS-PAGE. Pooled fractions were dialyzed against 10 mM Tris pH 7.4 for 24 h, followed by dialysis in ddH_2O for an additional 24 h and were then lyophilized. Yields of lyophilized protein were typically 100 mg per L expressed and the solid protein was stored at 4 °C.

Binding titrations. To 2.4 mL of 8 µM Sav in 200 mM NaPi pH 7 was added 300 µL of 10 mM 2-(4'-hydroxyazobenzene)benzoic acid (HABA). After 3 min equilibration the absorbance at 506 nm was recorded. 10 µL aliquots of 1 mM biot-β-Ala-**1** in water were added sequentially, the cuvette inverted, and the absorbance at 506 nm recorded until no further changes in intensity were observed.

Electrochemical methods for ArM samples (biot-β-Ala-1 ⊂ 2xm-S_{112}X-Sav).

Sample preparation. To remove any decomposition products of biot-β-Ala-**1**, or trace amounts of unbound metallocofactor, samples were subjected to size exclusion chromatography as follows. To a known concentration of solution of ~1 mM Sav in ddH_2O (ε_{280} = 167,760 M^{-1} cm^{-1}) was added 8–10 eq. of solid biot-β-Ala-**1** and the solution immediately loaded onto a Sephadex G-25 column. Running the column with water, the lower fraction (that containing bound biot-β-Ala-**1**) was pooled by eye and concentrated using 10,000 kDa MWCO spin filters purchased from Millipore. The final concentration of biot-β-Ala-**1** ⊂ Sav was determined from ε_{350} = 4,742 M^{-1} cm^{-1} reported by Das and coworkers for **1** in water.[4] Samples were allowed to equilibrate with water by storing overnight at 4 °C to emulate crystallography soaking conditions in which ligand exchange between acetate and water was consistently observed. Samples were then diluted to their final concentrations in a final volume of 250 µL containing 100 mM potassium phosphate (KPi) at the indicated pH.

Instrumentation. Experiments were performed in a home built Faraday cage using a Pine WaveDriver 10 potentiostat with the data analyzed using AfterMath software. All potentials were measured against a saturated Ag/AgCl reference electrode using glassy carbon working (3 mm diameter) and counter (5 mm diameter) electrodes and converted to NHE by subtraction of 0.197 V from the recorded value. The working electrode was cleaned with diamond polish (15, 3, then 1 µm granules sequentially) between every measurement.

Square wave voltammetry (SWV). SWV Experiments were performed using a pulse amplitude of 25 mV, an increment of 1 mV, a 5 s period and sampling width of 250 ms. Oxidation potentials were determined by first smoothing the raw data by 10-point FFT filtering to eliminate the presence of minor extrema, followed by differentiation to locate the global peak maxima, all using OriginPro 2017 software. The point at which derivative spectra equal zero were selected for the E_p reported in plots of E_p versus pH.

The raw data are presented in the SWV traces, while the anodic peak potentials (E_p) were determined in this way.

Cyclic voltammetry (CV). The CV experiments provided in Figure S4 were performed at 2 mV/s by scanning anodically from 1 to 1.5 V followed by a reverse segment to 0.9 V and then a final anodic sweep. Samples were prepared in an identical fashion to those used for SWV experiments and stored at 4 °C in between data collection times.

Electrochemical methods with biot-Z-1 samples (no Sav).

CVs were obtained on a BASi Epsilon potentiostat. Experiments were performed at 25 °C in solutions of 100 mM KPi electrolyte in which the pH was adjusted by addition of ortho-phosphoric acid or KOH. The Thermo Orion 2-Star Benchtop pH meter used to determine pH and was calibrated using three points: pH 4, 7, 10 buffer solutions. A platinum wire counter electrode, glassy carbon working electrode (7 mm diameter, polished with 0.05 μm alumina), and saturated Ag/AgCl (for pH 0–11) as reference electrode were used. CV Experiments were performed on samples of 500 μL and addition of aliquots of 5μL - 20μL of acid or base. The small concentration differences created by this method of titration did not appear to affect the electrochemical behavior. Control experiments in which CV scans with buffer were collected at 100 mV/s before and after experimental runs revealed no increase in current or additional peaks. Experimental scans at each pH were performed at a range of scan rates and revealed no change of the value of the $E_{1/2}$ or current. The values of midpoint potentials were calculated from the averaged E_p and E_a values reported by the BASi Epsilon-EC Ver 2.00.71 software.

Protein crystallization. Apo-Sav protein crystals were obtained at 20°C within two days by hanging-drop vapor diffusion technique mixing 1 μL crystallization buffer (2.0 M $(NH_4)_2SO_4$, 0.1 M NaOAc, pH 4.0) and 4 μL protein solution (26 mg/mL lyophilized protein in water). The droplet was equilibrated against a reservoir solution of 100 μL

crystallization buffer. Subsequently, single crystals of Sav were transferred sequentially to pH 5, 6 and then pH 7 soaking buffer containing 2.6 M $(NH_4)_2SO_4$, 0.1 M NaOAc, and then soaked overnight in 9 µL pH 7 soaking buffer, 1 µL 25 mM biot-β-Ala-1, and 0.5 µL of the original protein stock. For the crystal structures shown in Figure 2b, 1 µL of 10 mM Na_2IrCl_6 was also included in this mixture. After 24 h, crystals were transferred for 30 seconds into a cryo-protectant solution consisting of 30% (v/v) glycerol in soaking buffer and then shock-frozen in $N_{2(l)}$.

XRD Data collection and processing. XRD Data were collected at the Advanced Light Source (BL8.2.1 and 8.2.2) and Stanford Synchrotron Radiation Light Source (BL5.0.2 and 9.2) at a wavelength of 1 Å, processed with software iMOSFLM7[6] or XDS[7] and scaled with AIMLESS (CCP4 Suite)[6]. The structure was solved by molecular replacement using program PHASER (CCP4 Suite)[6] and the structure 2QCB from the PDB as input model with ligand and water molecules removed. For structure refinement REFMAC5 (CCP4 Suite)[8] and PHENIX.REFINE[9] were used. Ligand manipulation was carried out with program REEL using the small molecule crystal structures[5] as input models. For water picking, electron density, and structure visualization, the software COOT[10] was used. Figures were drawn with PyMOL (the PyMOL Molecular Graphics System, Version 1.5.0.5, Schrodinger, LLC). Crystallographic details, processing and refinement statistics are given in Table S5.

Structural Refinement. Apo-crystals of Sav variants soaked biot-β-Ala-**1** constituted space group $I4_122$ with virtually identical unit cell parameters (Table S5). A single Sav monomer was obtained per asymmetric unit after molecular replacement. Protein residues 2-12 and 133-159 of the *N*- and *C*-termini, respectively were not resolved in the electron density, presumably due to disorder. Starting from the Sav monomer the biological homotetramer is generated by application of crystallographic C2-symmetry axes along the x-, y- and z-axes of the unit cell. The overall protein structures are virtually identical to structure biotin⊂ S_{112}K-Sav (PDB 2QCB, see Table S5).

General Complex Modeling. For all structures of apo-protein crystals soaked with the corresponding Co-complexes the following general observations were made: i) residual electron density in the F_o-F_c map was observed in the biotin binding pocket, ii) in the biotin vestibule, which is flanked by protein residues of loop-3,4^{A} (the superscript number indicates Sav monomer within tetramer) loop-4,5^{C}, loop-5,6^{A} loop-7,8^{A} and loop-7,8^{B}, and iii), an anomalous dispersion density map indicated a significant peak in the biotin vestibule superimposed with the electron density peak (Figures S2B, S3B, S9B, S11C, S12C). The residual electron density was fit with the corresponding Co-complexes which projected Co atoms to the position of the strong anomalous density peak.

We do not believe that any Co(III) centers exist with a coordination number less than 6 but were not able to confidently assign a ligand to the electron density obtained in some cases. Based on our understanding of ligand substitution processes in solution,[11] it is likely that pyridine ligands remain bound, and rotation about the Co–N reduces the amount of electron density obtained. For acetate ligands, these sites are readily exchanged for water molecules *in crystallo* and in some cases dissociation/association processes may have prevented the observation of sufficient electron density. Each protein variant appears to produce some minor structural changes at the active site. These changes are difficult to predict (e.g. where and when a structurally conserved water molecules will be observed).

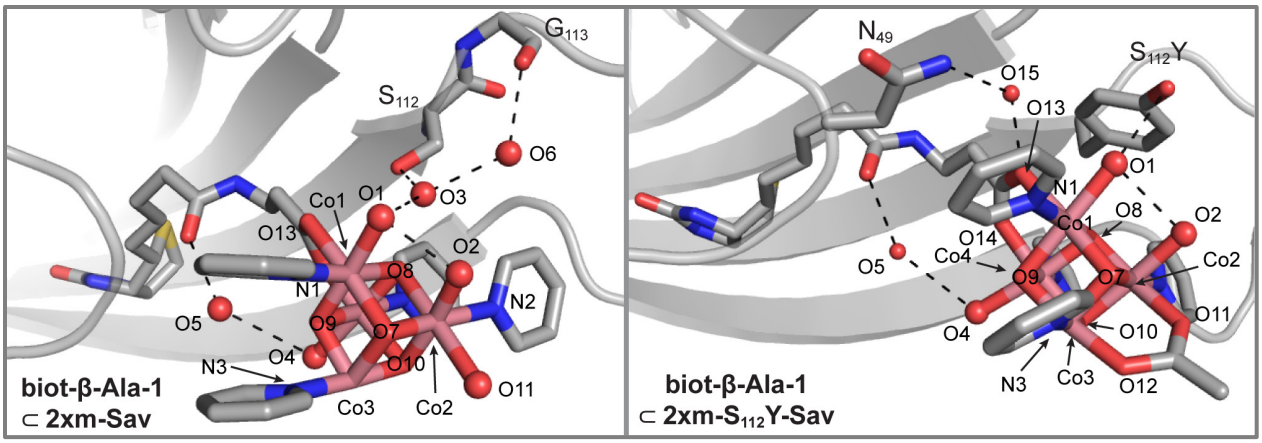


Table S1. Selected Bond (and H-bond) Lengths and Angles

Bond Lengths	biot-β-Ala-**1** ⊂ 2xm-Sav (± 0.04 Å)	biot-β-Ala-**1** ⊂ 2xm-S_{112}Y-Sav (± 0.05 Å)	Angles	biot-β-Ala-**1** ⊂ 2xm-Sav (± 6 °)	biot-β-Ala-**1** ⊂ 2xm-S_{112}Y-Sav (± 6 °)
Co1–O7	2.07	2.03	Co1–O7–Co2	89	89
Co1–O8	2.20	2.10	Co1–O8–Co2	80	91
Co1–O9	1.81	2.06	Co1–O7–Co3	92	94
Co2–O7	1.89	1.98	Co1–O9–Co3	105	92
Co2–O8	2.11	1.86	Co1–O8–Co4	83	86
Co2–O10	1.87	2.06	Co1–O9–Co4	90	86
Co3–O7	2.02	1.87	Co2–O8–Co4	94	99
Co3–O9	1.92	1.89	Co2–O10–Co3	95	84
Co3–O10	1.99	2.05	Co2–O10–Co4	91	93
Co4–O8	1.85	1.93	Co2–O7–Co3	94	91
Co4–O9	2.03	1.96	Co3–O9–Co4	92	97
Co4–O10	2.18	1.90	Co3–O10–Co4	86	94
Co1–O1	1.95	2.17	O1–Co1···Co2	93	93
Co2–O2	1.79	2.08	Co1···Co2–O2	81	90
Co4–O4	2.25	2.32	O14–Co4–O4	88	83
Co2–O11	2.29	2.08			
Co3–O12	-	2.11			
Co1–O13	2.12	2.08			
Co4–O14	1.98	2.03			
O1···O2	2.62	2.96			
O1···O3	3.24	-			
O1···O_{Tyr112}	-	3.07			
O3···O_{Ser112}	3.14	-			
O3···O6	2.74	-			
O6···O_{Gly113}	2.93	-			
O4···O5	3.28	3.49			
O5···O_{biot}	2.80	2.89			
O13···O15	-	2.47			
O12···N_{Asn49}	-	2.73			
Co1–N1	2.09	2.28			
Co2–N2	2.38	2.60			
Co3–N3	2.10	2.46			
Co4–N4	2.33	2.34			

Table S2. Oxidation potentials for biot-β-Ala-1 ⊂ 2xm-S_{112}X-Sav variants

	E_p (V vs NHE)		
pH	**S**	**F**	**Y**
6.0	1.261	1.241	
6.5	1.204	1.216	1.206
7.0	1.194	1.185	1.187
7.5	1.150	1.155	1.181
8.0	1.130	1.113	1.158
8.5		1.115	1.115
9.0	1.126	1.111	1.105
9.5			1.104
10.0	1.128		1.050
10.5			0.953
11.0			0.904

Letter codes S, F, and Y correspond to amino acids at position 112 in 2xm-Sav.

Table S3. **$E_{1/2}$ values for biot-Z-1 complexes in the absence of Sav**

	E (V vs NHE)			
pH	biot-**1**	biot-β-Ala-**1**	biot-GABA-**1**	biot-Py-**1**
1.5	1.299	1.333	1.312	1.337
2.0	1.280	1.300	1.298	1.292
2.5	1.238	1.256	1.248	1.248
3.0	1.223	1.249	1.232	1.238
3.5	1.211	1.245	1.225	1.228
4.0	1.212	1.244	1.228	1.226
5.5	1.210	1.242	1.226	1.227
6.0	1.213	1.240	1.228	1.230
6.5	1.216	1.240		
7.5	1.218	1.250	1.229	1.232
8.0		1.244	1.229	1.231
9.0		1.240	1.232	1.234

Table S4. Normalized SWV peak current for biot-β-Ala-1 ⊂ 2xm-S_{112}X-Sav variants

	I_p (μA/mM)/$I_{p,max}$ (μA/mM)		
pH	**S**	**F**	**Y**
6.0	0.41	0.45	
6.5	0.37	0.60	0.49
7.0	0.35	0.49	0.68
7.5	0.44	0.75	0.43
8.0	0.33	0.60	1.00
8.5		0.55	0.62
9.0	0.49	0.53	0.51
9.5			0.25
10.0	0.53		0.38
10.5			0.13
11.0			0.09

Letter codes S, F, and Y correspond to amino acids at position 112 in 2xm-Sav. $I_{p,max}$ was 1.64 μA/mM, obtained with biot-β-Ala-**1** ⊂ 2xm-S_{112}Y-Sav (1 mM biot-β-Ala-**1**, 0.25 mM 2xm-S_{112}Y-Sav) at pH 8.0.

Table S5. XRD Data processing and refinement statistics

Identification					
Sav variant	2xm	2xm-$S_{112}Y$	2xm-$S_{112}Y$	2xm-$S_{112}Y$	2xm-$S_{112}F$
ligand	biot-β-Ala-**1**	biot-β-Ala-**1**	biot-β-Ala-**1**	biotin	biot-β-Ala-**1**
PDB code	6AUC	6AUE	6AUH	6AUL	6AUO
3-letter ligand code	OLS	OL3	OL5	BTN	OL4
Data Processing					
Unit cell	$I4_122$	$I4_122$	$I4_122$	$I4_122$	$I4_122$
Space group	a, b = 57.58, c = 183.79 α, β, γ = 90 °	a, b = 57.94, c = 184.57 α, β, γ = 90 °	a, b = 57.70, c = 183.77 α, β, γ = 90 °	a, b = 57.75, c = 184.09 α, β, γ = 90 °	a, b = 57.56, c = 183.72 α, β, γ = 90 °
Resolution (Å)	54.95 − 1.46	37.45 − 1.36	37.29 − 1.60	37.33 − 1.36	45.93 – 1.70
Highest resolution shell (Å)	1.49 – 1.46	1.39 – 1.36	1.63 – 1.60	1.39 – 1.36	1.73 – 1.70
R_{merge} (%)	8.3 (38.8)	6.3 (64.4)	18.0 (305.5)	6.3 (86.9)	55.1 (1673)
No. of unique reflections	226791 (7959)	348106 (11567)	224511 (9593)	33716 (1485)	435110 (21112)
Multiplicity	8.3 (6.0)	10.2 (7.0)	10.6 (9.7)	10.2 (7.2)	24.8 (23.3)
I/sig(I)	13.2 (2.9)	14.2 (1.6)	8.9 (1.7)	14.9 (1.7)	16.5 (6.2)
Completeness	99.9 (100.0)	99.8 (96.1)	99.9 (98.2)	99.4 (90.9)	100 (100)
CC(½)	0.995 (0.873)	0.999 (0.691)	0.944 (0.625)	0.996 (0.665)	0.995 (0.947)
Structure refinement					
R_{work}	0.1779	0.2026	0.1951	0.1798	0.1895
R_{free}	0.2006	0.2290	0.2194	0.1952	0.2218
Rmsd bond length (Å)	0.0440	0.0508	0.0317	0.0300	0.0331
Rmsd bond angle (°)	5.9154	6.3149	5.7007	2.6220	5.0735
Rmsd compd to biotin ⊂ wt-Sav (1STP, Å)	0.620	0.652	0.627	0.365	0.645
Ligand model					
Avg. anomalous dispersion density at Co (σ)	9.8	9.5	5.1	-	6.3
B factor (Å²)					
overall protein	17.3	20.3	22.3	19.7	20.5
$S_{112}X$	13.2	27.1	29.7	20.1	22.9
biot-β-Ala-**1**	34.5	39.0	33.6	-	44.6
Co (Avg)	41.3	53.6	46.1	-	60.3
nearest Co—Co distance (Å)	5.7	4.7	6.2	-	5.5

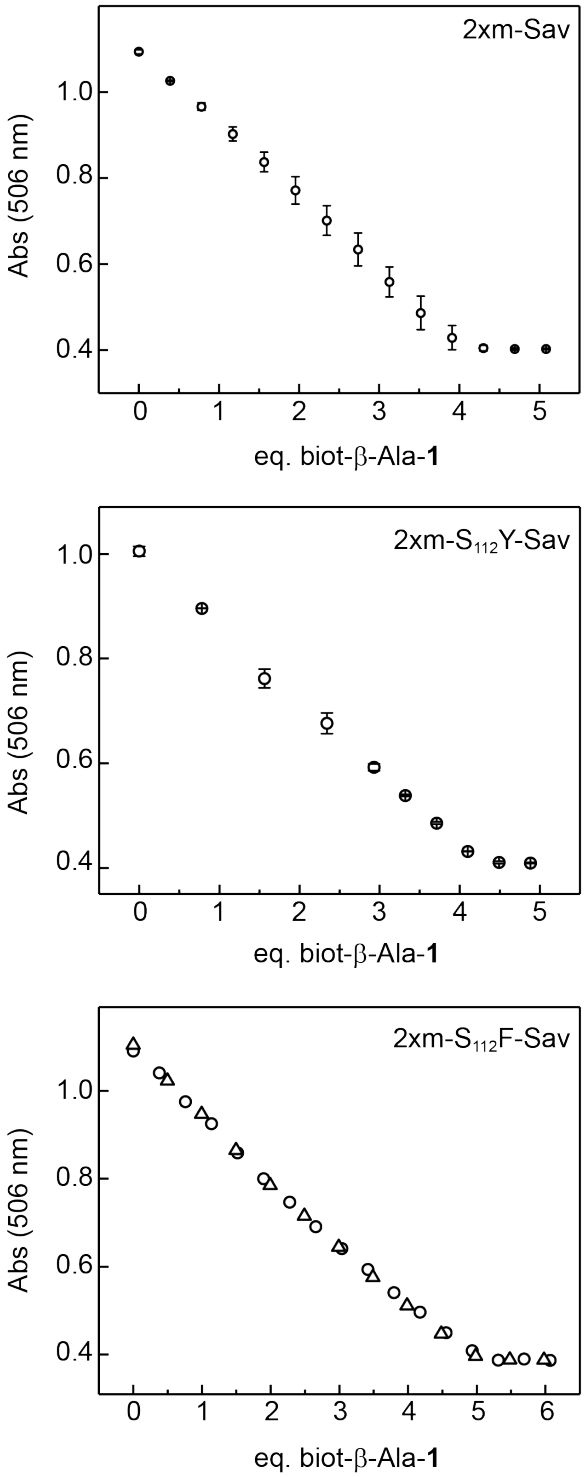


Figure S1. Binding titrations performed using 2-(4'-hydroxyazobenzene)benzoic acid as described above for each of the three Sav variants used in these studies, as indicated. All experiments were performed in duplicate with independently prepared samples with standard deviations indicated with error bars (2xm-Sav and 2xm-S_{112}Y-Sav) or replicate trials represented by different shapes (2xm-S_{112}F-Sav).

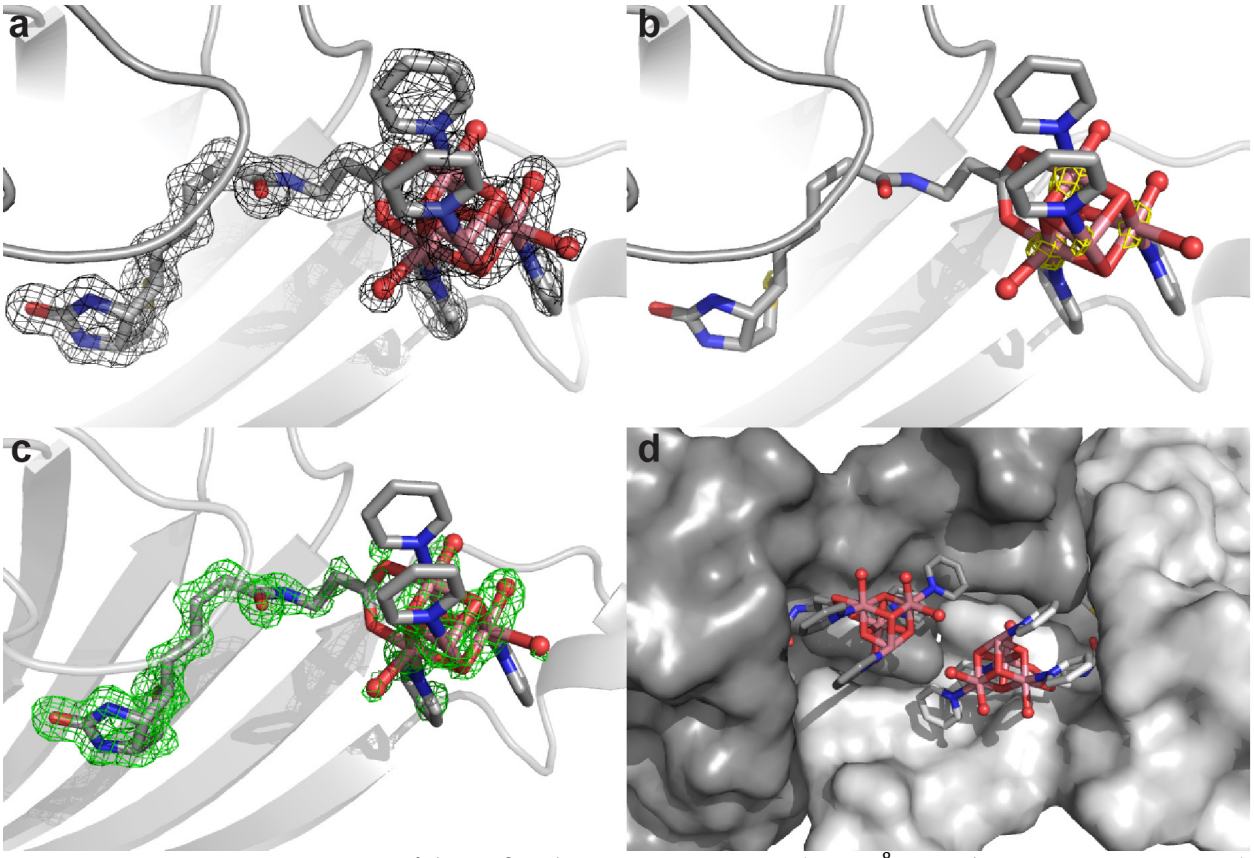


Figure S2. XRD structure of biot-β-Ala-**1** ⊂ 2xm-Sav (1.46 Å resolution, PDB 6AUC) overlain with $2F_o-F_c$ electron density map contoured at 1 σ (black mesh, **a**), with anomalous density map contoured to 6 σ (yellow mesh, **b**), and with F_o-F_c omit map centered around biot-β-Ala-**1** contoured at 3 σ (green mesh, **c**) and a space filling model showing the dimer interface with in the Sav vestibule (**d**).

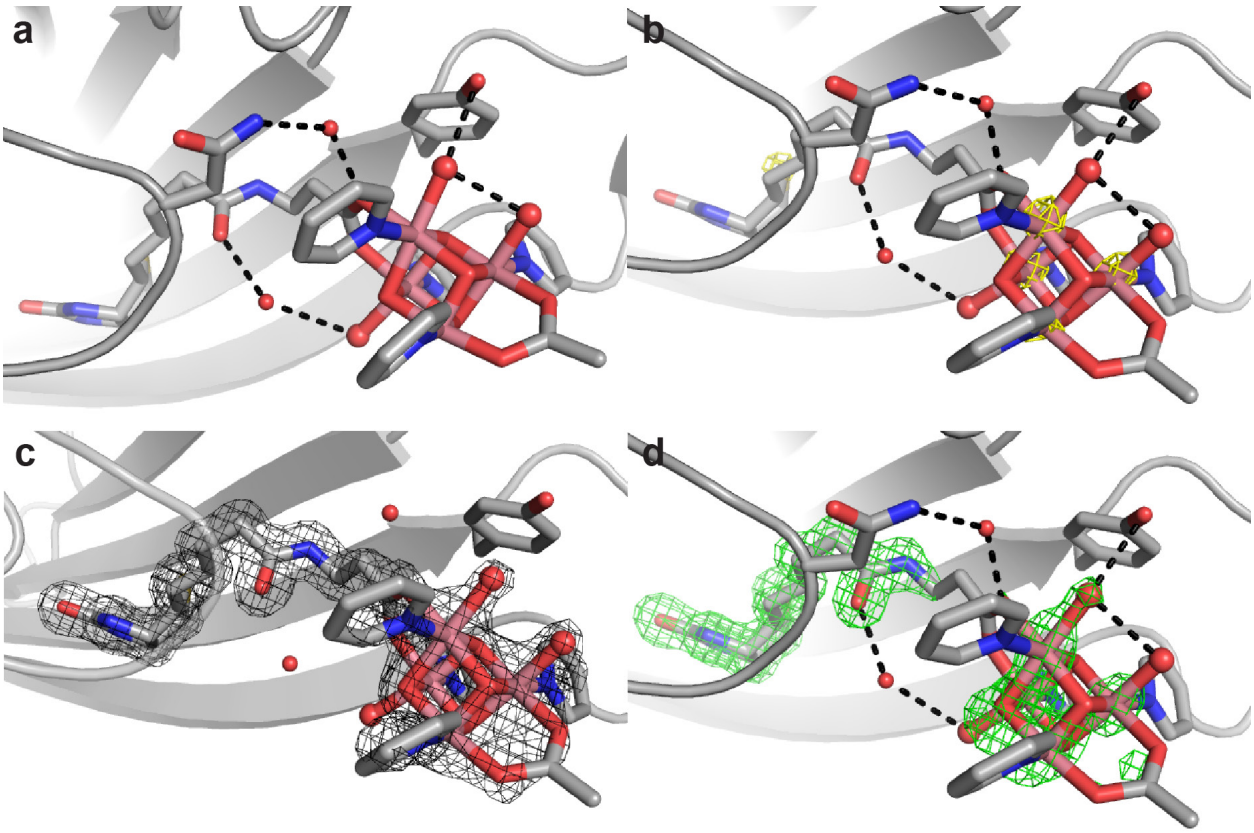


Figure S3. XRD structure biot-β-Ala-**1** ⊂ 2xm-S_{112}Y-Sav (1.36 Å resolution, PDB 6AUE, **a**); overlain with anomalous density map contoured at 5 σ (yellow mesh, **b**), $2F_o-F_c$ electron density map contoured at 1 σ (black mesh, **c**), and with F_o-F_c omit map centered around biot-β-Ala-**1** contoured at 3 σ (green mesh, **d**).

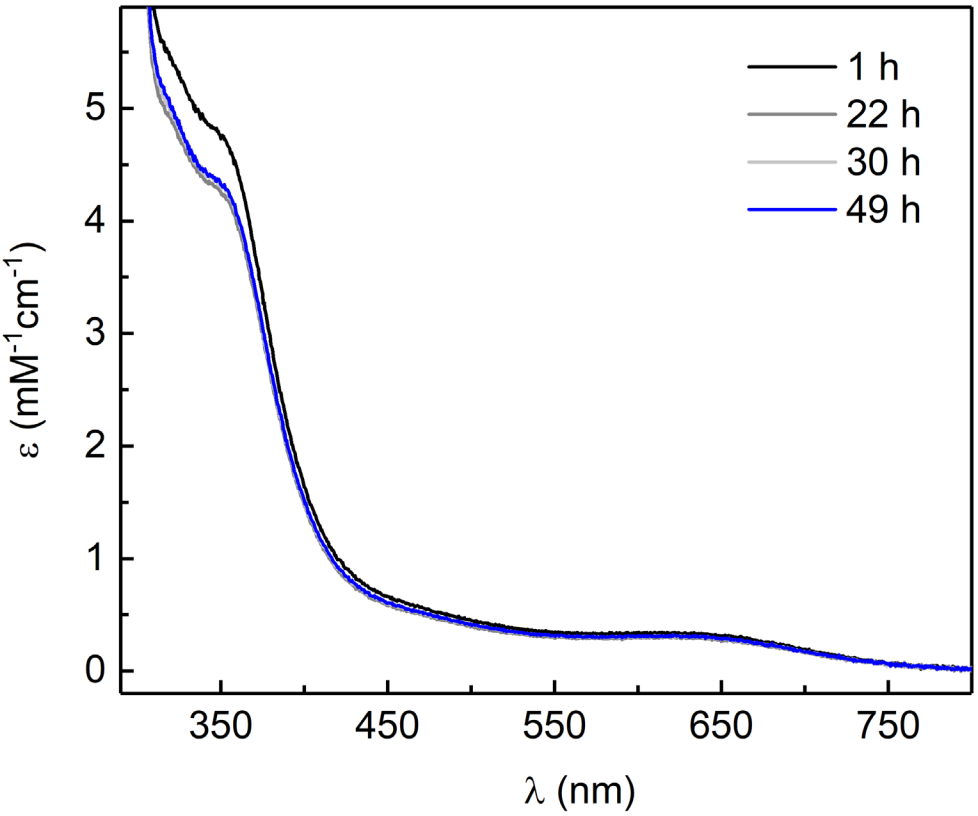


Figure S4. Absorption spectra of biot-β-Ala-**1** ⊂ 2xm-Sav in water at the indicated time points after purification by size exclusion chromatography (samples were prepared in an identical fashion to those used for protein electrochemistry). When not in use samples were stored at 4 °C.

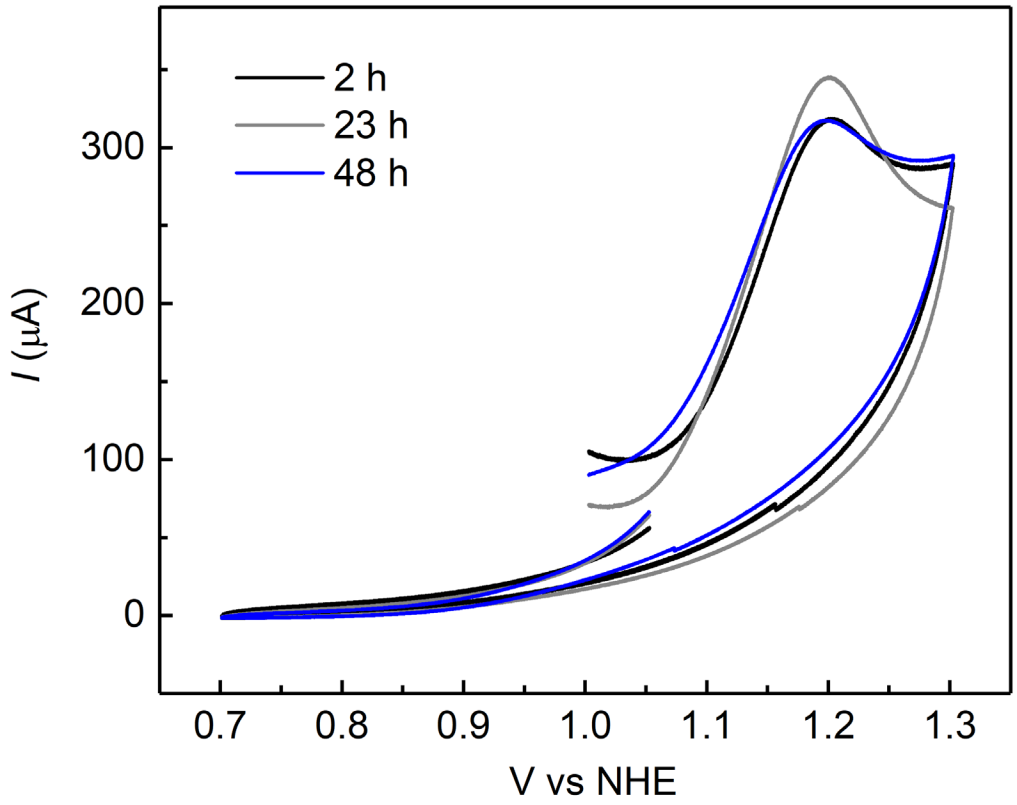


Figure S5. Cyclic voltammograms of biot-β-Ala-**1** ⊂ 2xm-Sav in 100 mM KPi at pH 7 scanning at 2 mV/s. Each trace represents a fresh aliquot taken from a single concentrated sample stored in water at 4 °C over the 48 h period.

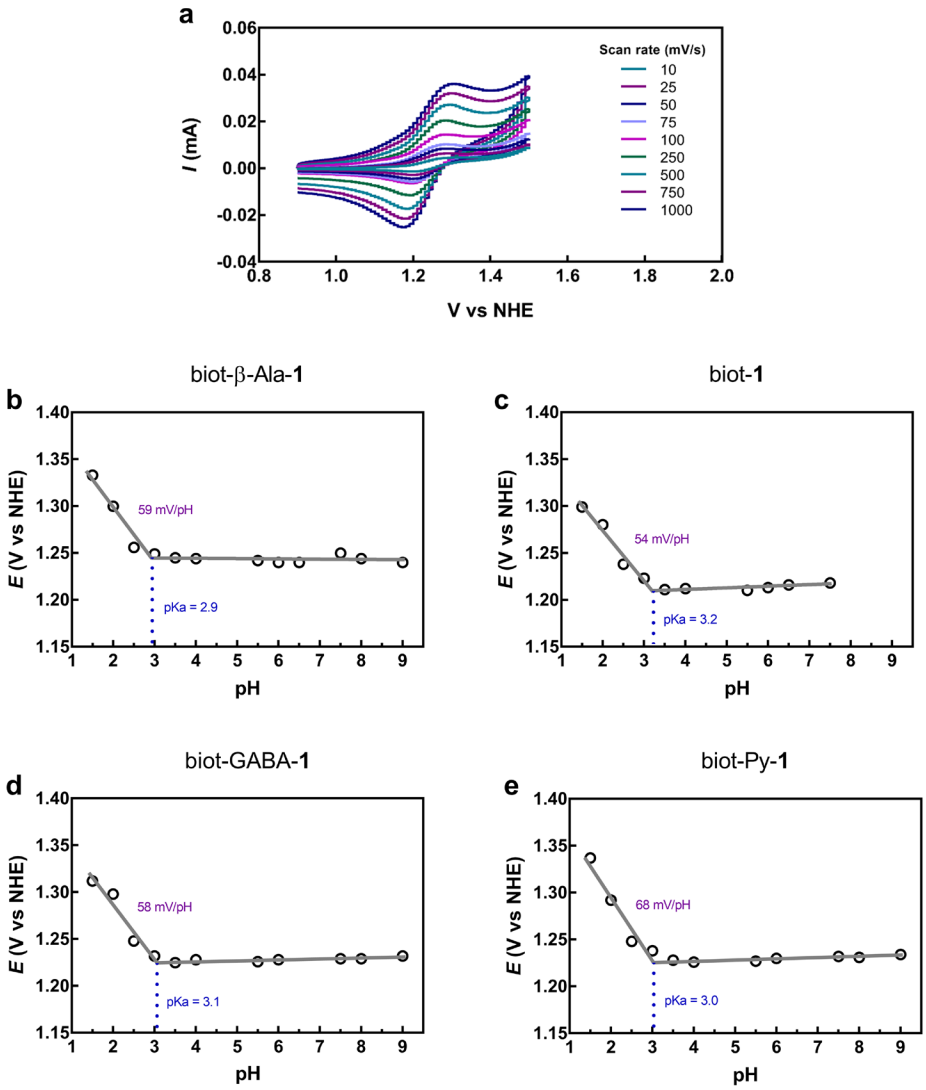


Figure S6. CV Data collected at multiple scan rates in the presence of 1 mM biot-β-Ala-**1** (no protein) in 100 mM KPi at pH 7 (**a**) and the corresponding $E_{1/2}$ vs pH plot for biot-β-Ala-**1** (**b**), biot-**1** (**c**), biot-gaba-**1** (**d**), biot-py-**1** (**e**). Gray lines represent the linear regression fits for these data obtained using GraphPad Prism 7.00 software.

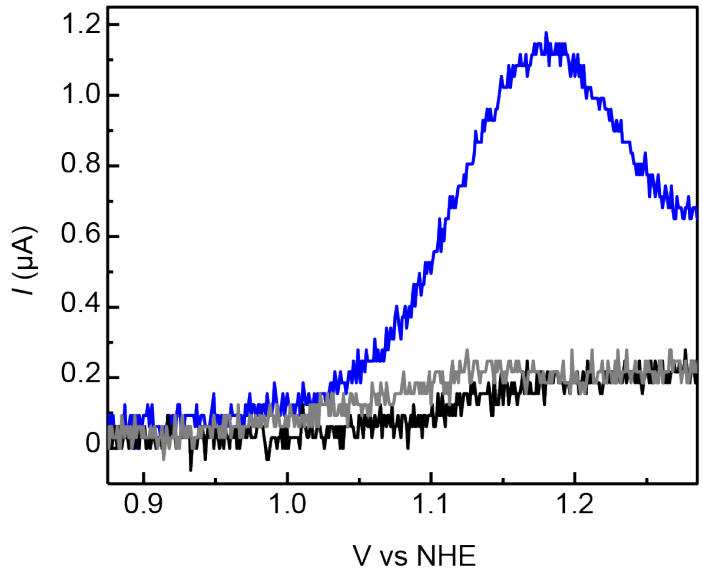


Figure S7. SWV performed at pH 7 with biot-β-Ala-**1** ⊂ 2xm-Sav (blue). After the run, the working electrode was rinsed lightly with H_2O, placed in a fresh solution containing only buffer, and the SWV experiment was re-run (black). The sample solution from the original SWV experiment shown in blue was centrifuged in a 10 kDa MWCO spin filter to separate protein-bound species from bulk solution and the flow-through was again subjected to the SWV experimental conditions (gray).

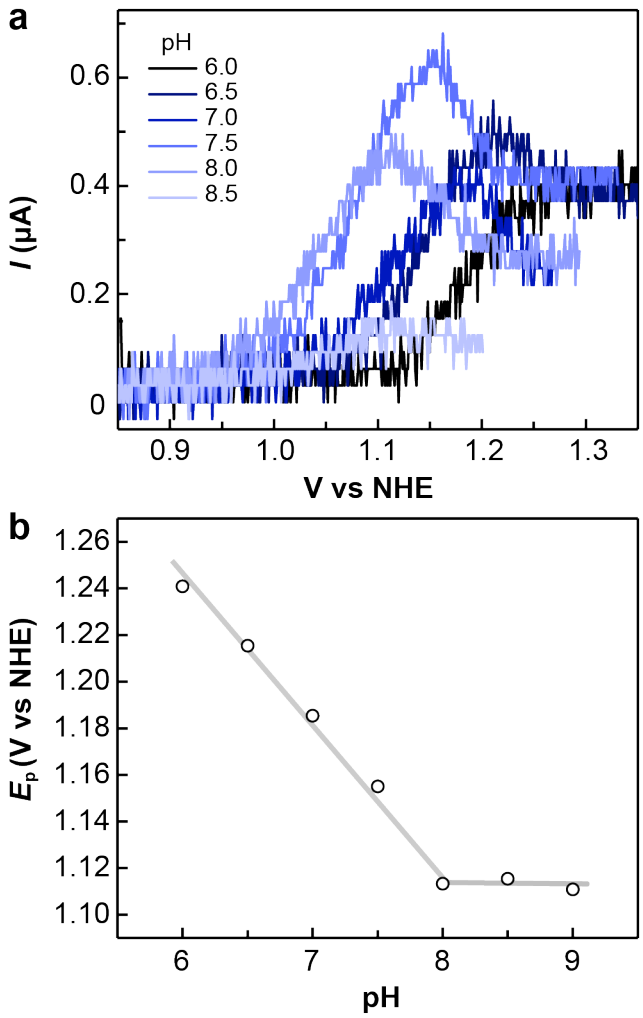


Figure S8. SWV data collected with 0.5 mM biot-β-Ala-**1** ⊂ 2xm-S$_{112}$F-Sav (**a**),[1] and a plot of the resulting peak potentials as a function of pH (**b**).

[1] Owing to the reduced solubility of this Sav variant, expression yields in the soluble fraction, and thus, total purified protein yields were low. The lower yield required a reduced concentration of protein to be used in SWV experiments and accordingly gave rise to lowered current.

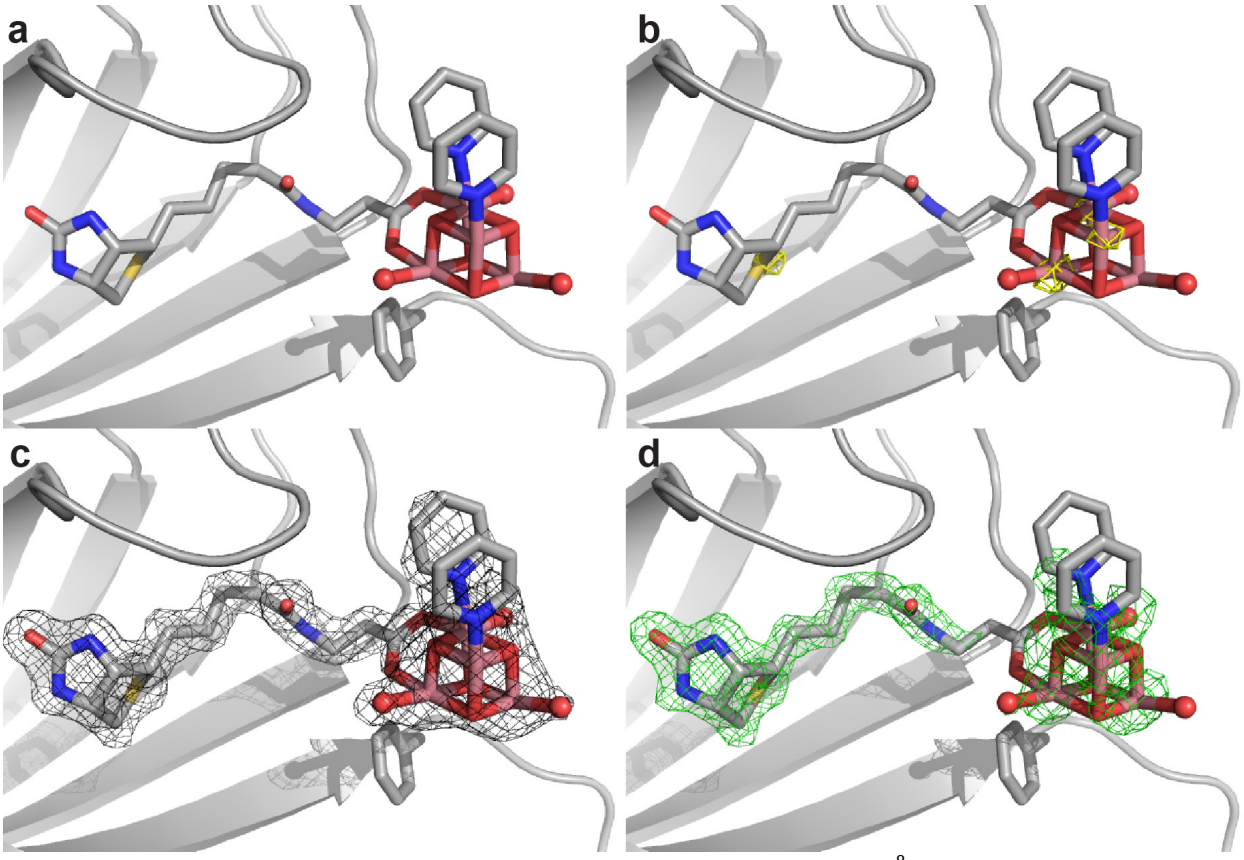


Figure S9. XRD structures biot-β-Ala-**1** ⊂ 2xm-S_{112}F-Sav (1.42 Å resolution, PDB 6AUO, **a**); overlain with anomalous density map contoured at 3.5 σ (yellow mesh, **b**), $2F_o-F_c$ electron density map contoured at 1 σ (black mesh, **c**), and with F_o-F_c omit map centered around biot-β-Ala-**1** contoured at 3 σ (green mesh, **d**).

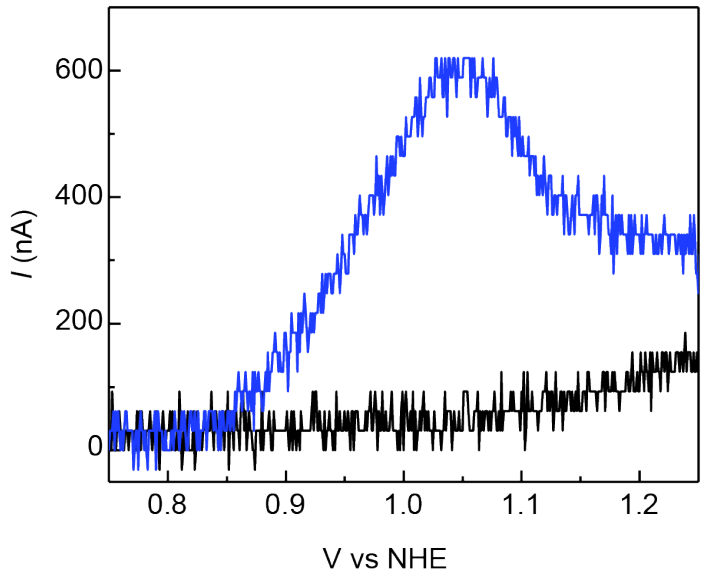


Figure S10. SWV data collected at pH 10 in the presence of biot-β-Ala-**1** ⊂ 2xm-S_{112}Y-Sav (blue) and biotin ⊂ 2xm-S_{112}Y-Sav (black).

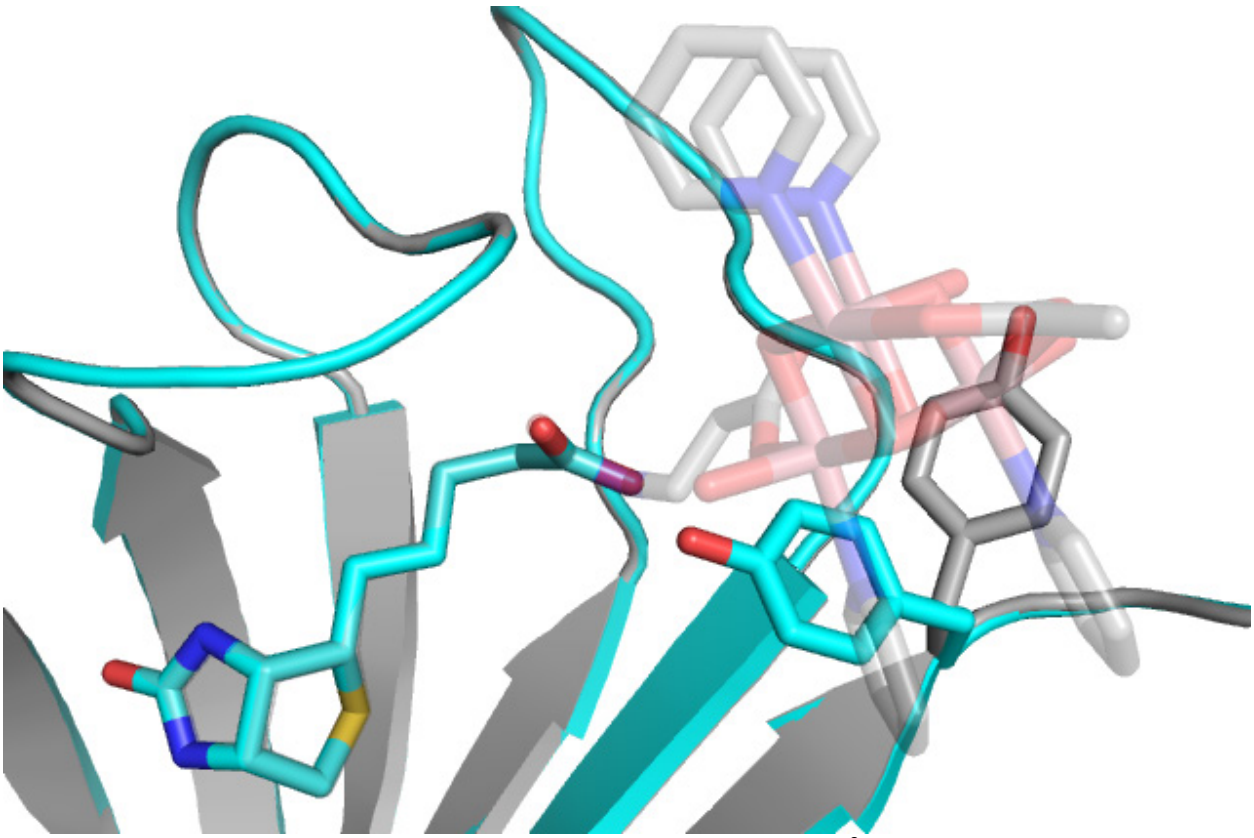


Figure S11. XRD structure of biotin ⊂ 2xm-S_{112}Y-Sav (1.36 Å resolution, turquoise, PDB 6AUL) overlaid with that of biot-β-Ala-**1** ⊂ 2xm-S_{112}Y-Sav (1.49 Å resolution, gray).

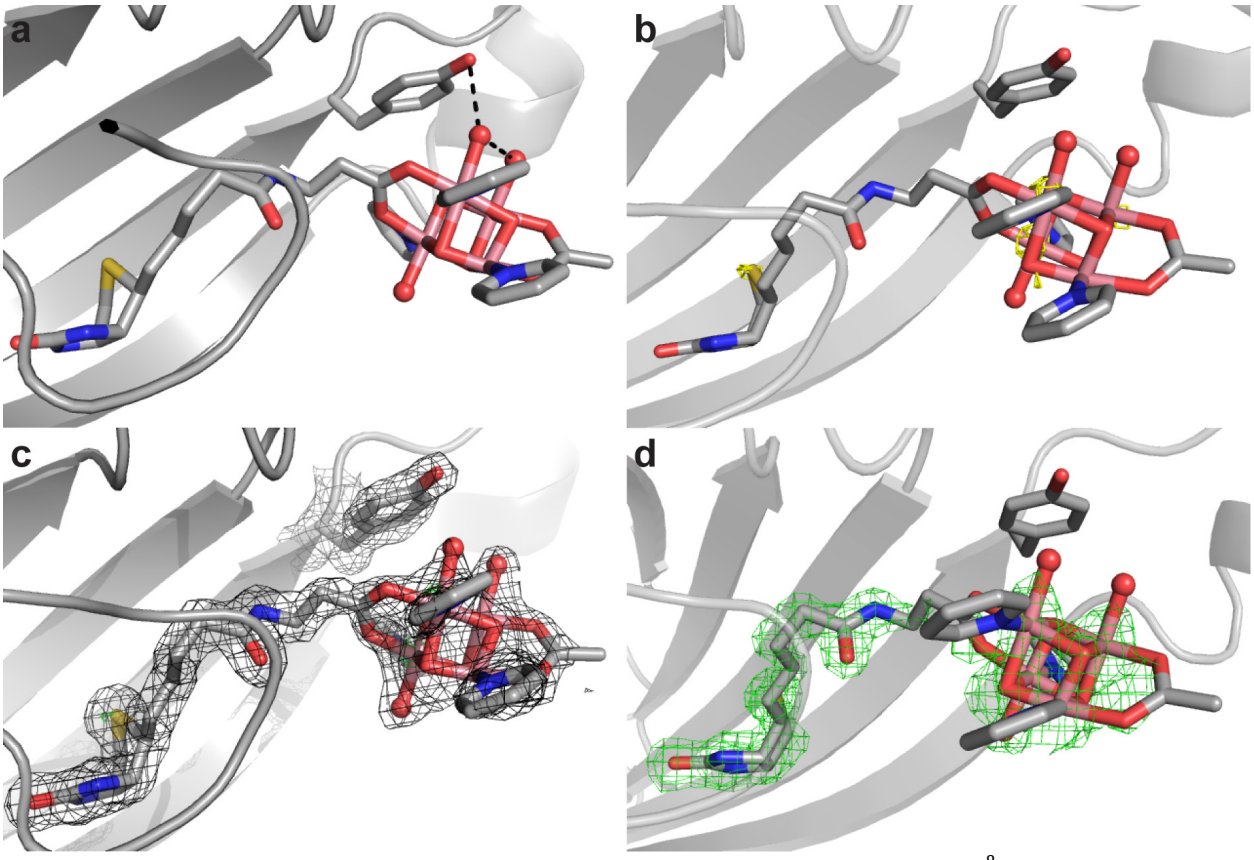


Figure S12. XRD structures biot-β-Ala-**1** ⊂ 2xm-S$_{112}$Y-Sav (1.60 Å resolution, PDB 6AUH, **a**), overlain with anomalous density map contoured at 5 σ (yellow mesh, **b**), 2F_o−F_c electron density map contoured at 1 σ (black mesh, **c**), and with F_o−F_c omit map centered around biot-β-Ala-**1** contoured at 3 σ (green mesh, **d**).

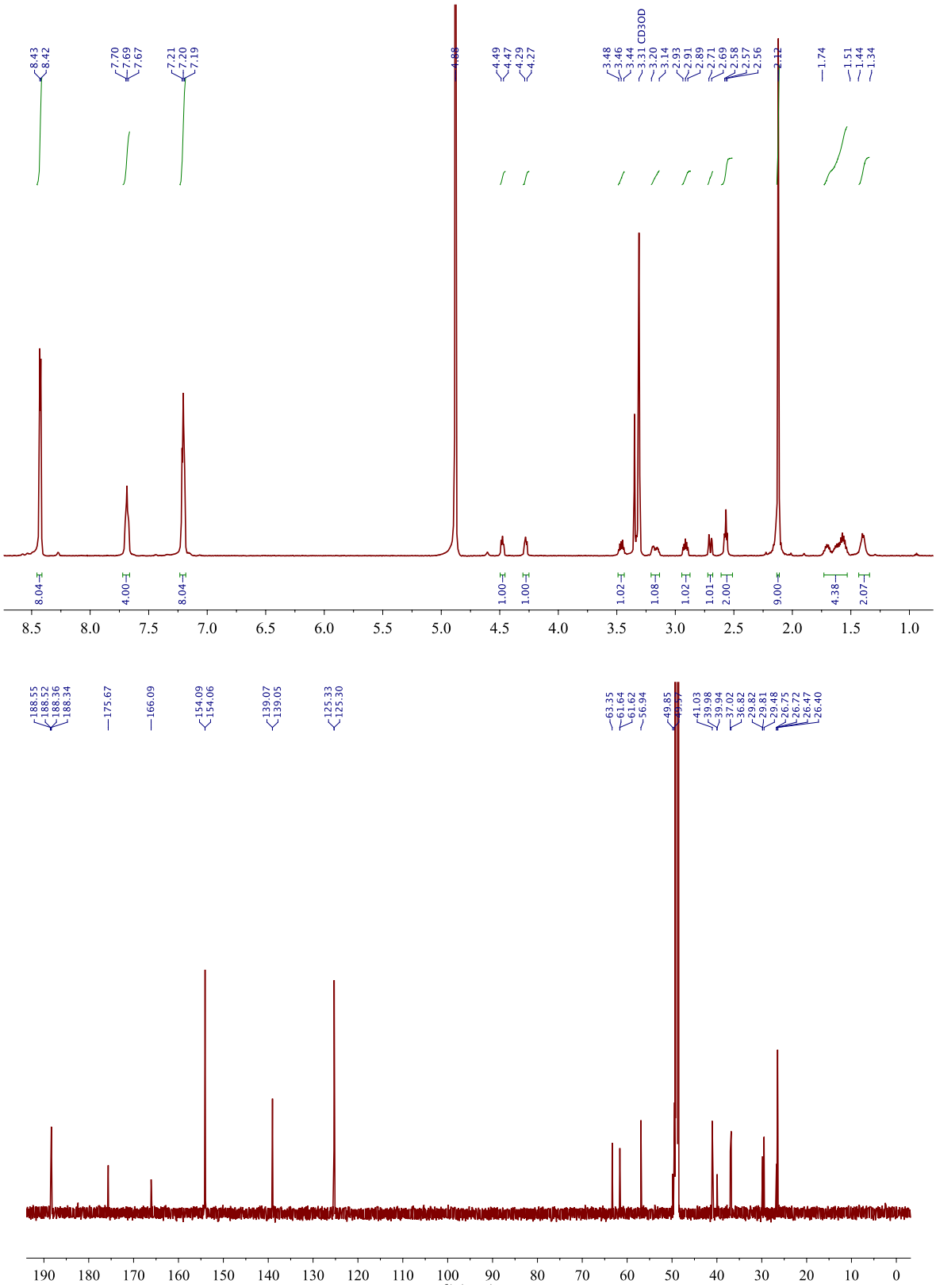


Figure S13. ^{1}H (top) and ^{13}C (bottom) NMR spectra of biot-β-Ala-**1**.

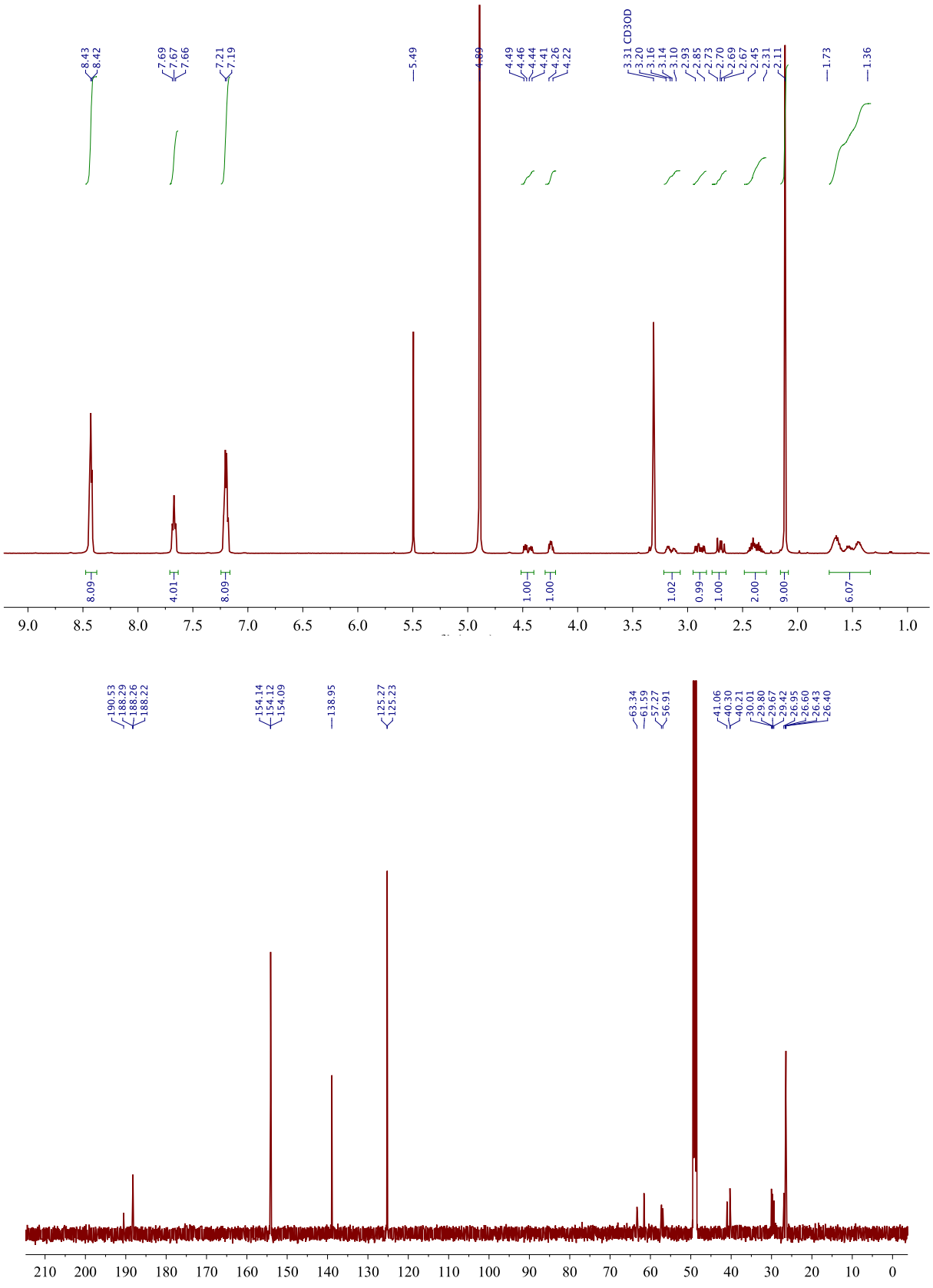


Figure S14. ^{1}H (top) and ^{13}C (bottom) NMR spectra of biot-**1**.

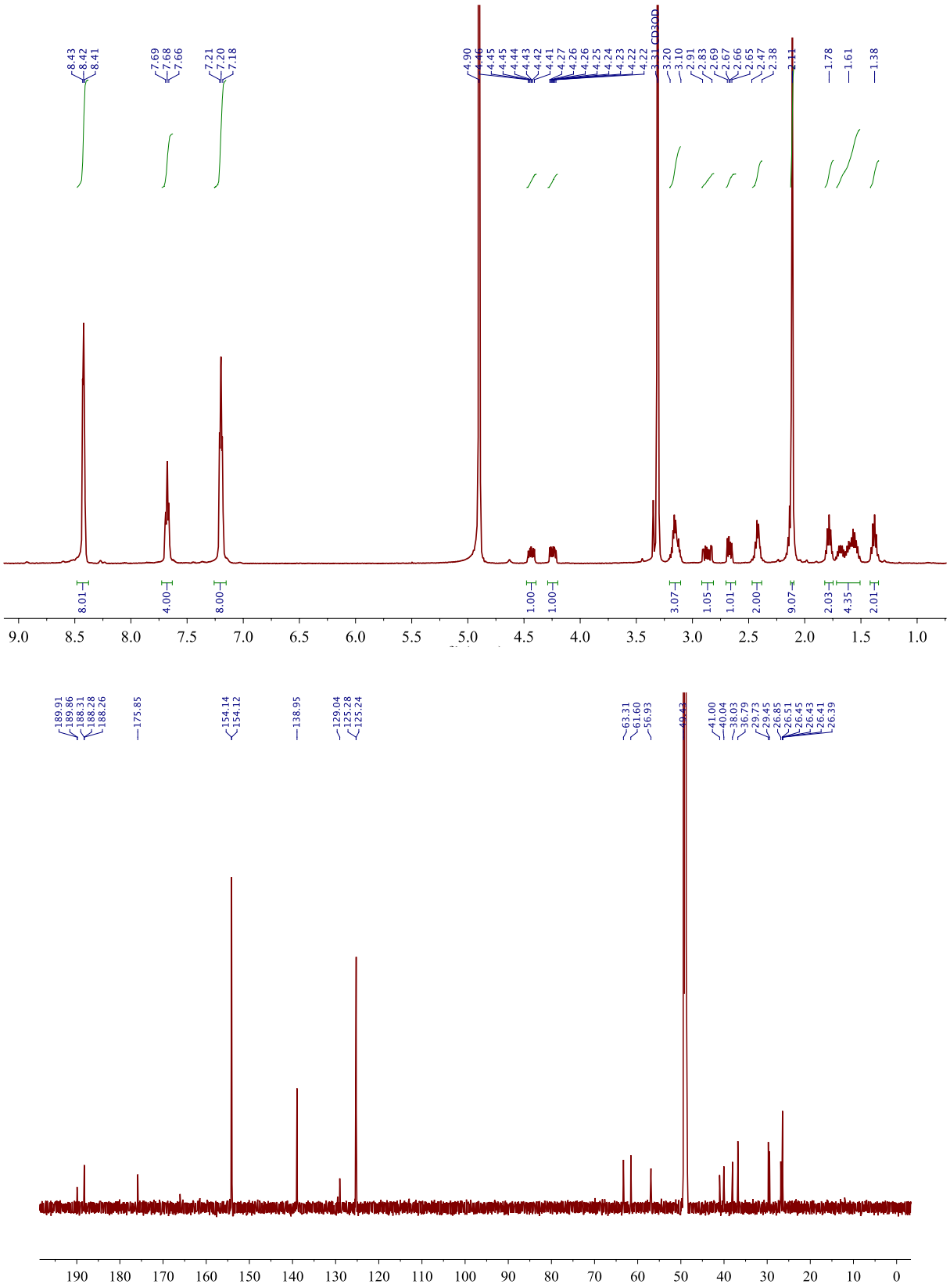


Figure S15. ^{1}H (top) and ^{13}C (bottom) NMR spectra of biot-gaba-**1**.

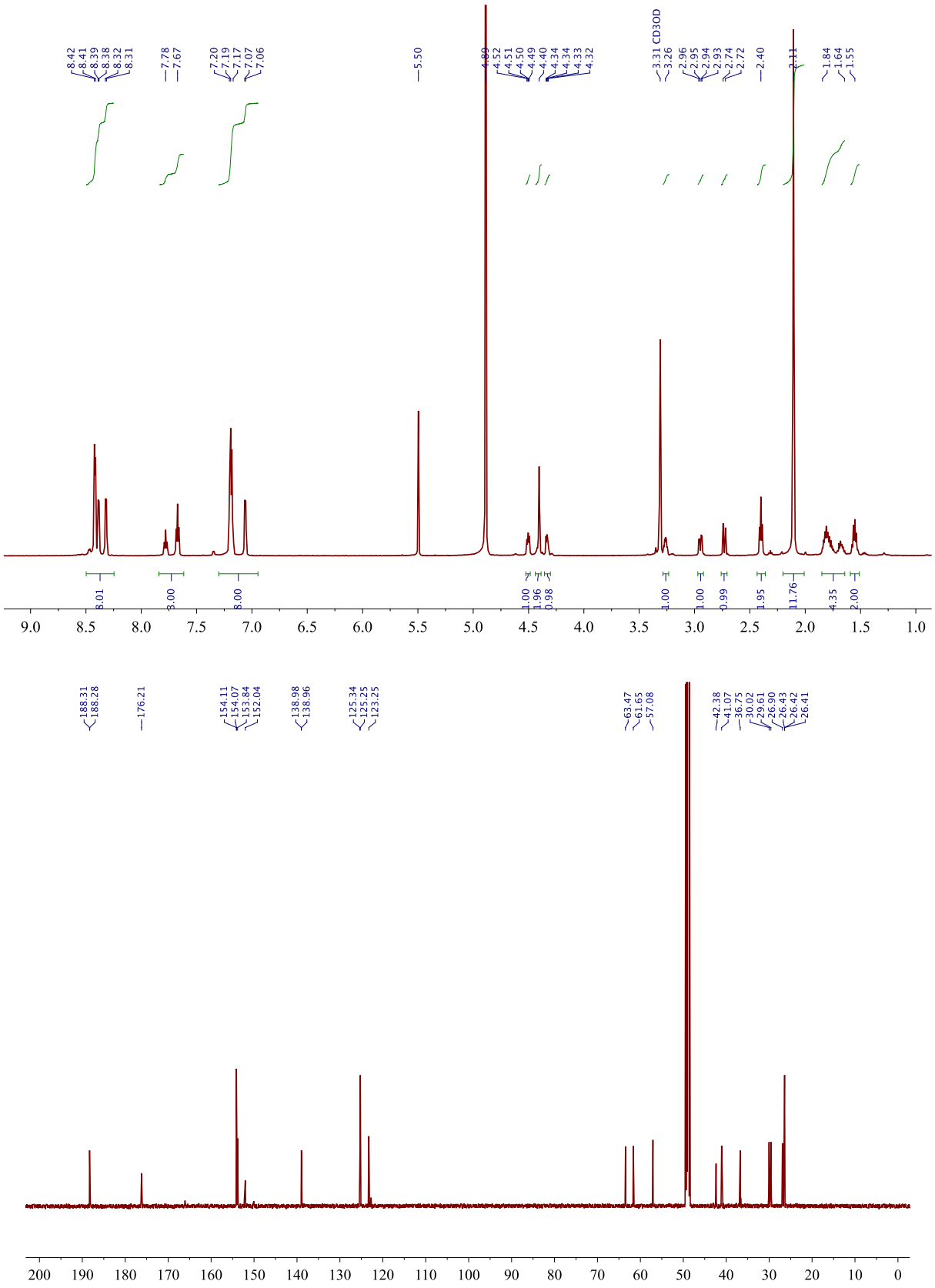


Figure S16. ^{1}H (top) and ^{13}C (bottom) NMR spectra of biot-py-**1**.

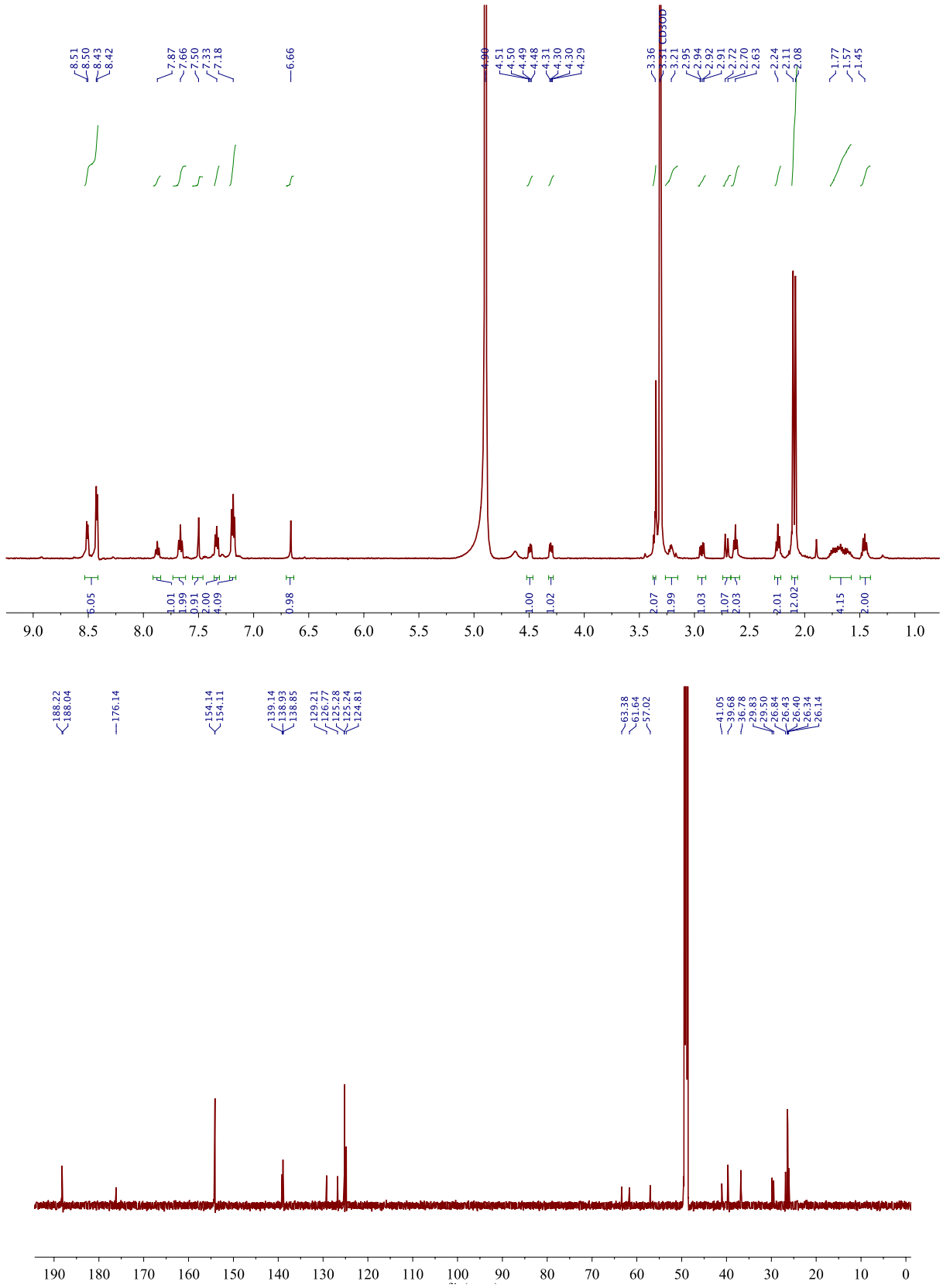


Figure S17. ^{1}H (top) and ^{13}C (bottom) NMR spectra of biot-histamine-**1**.

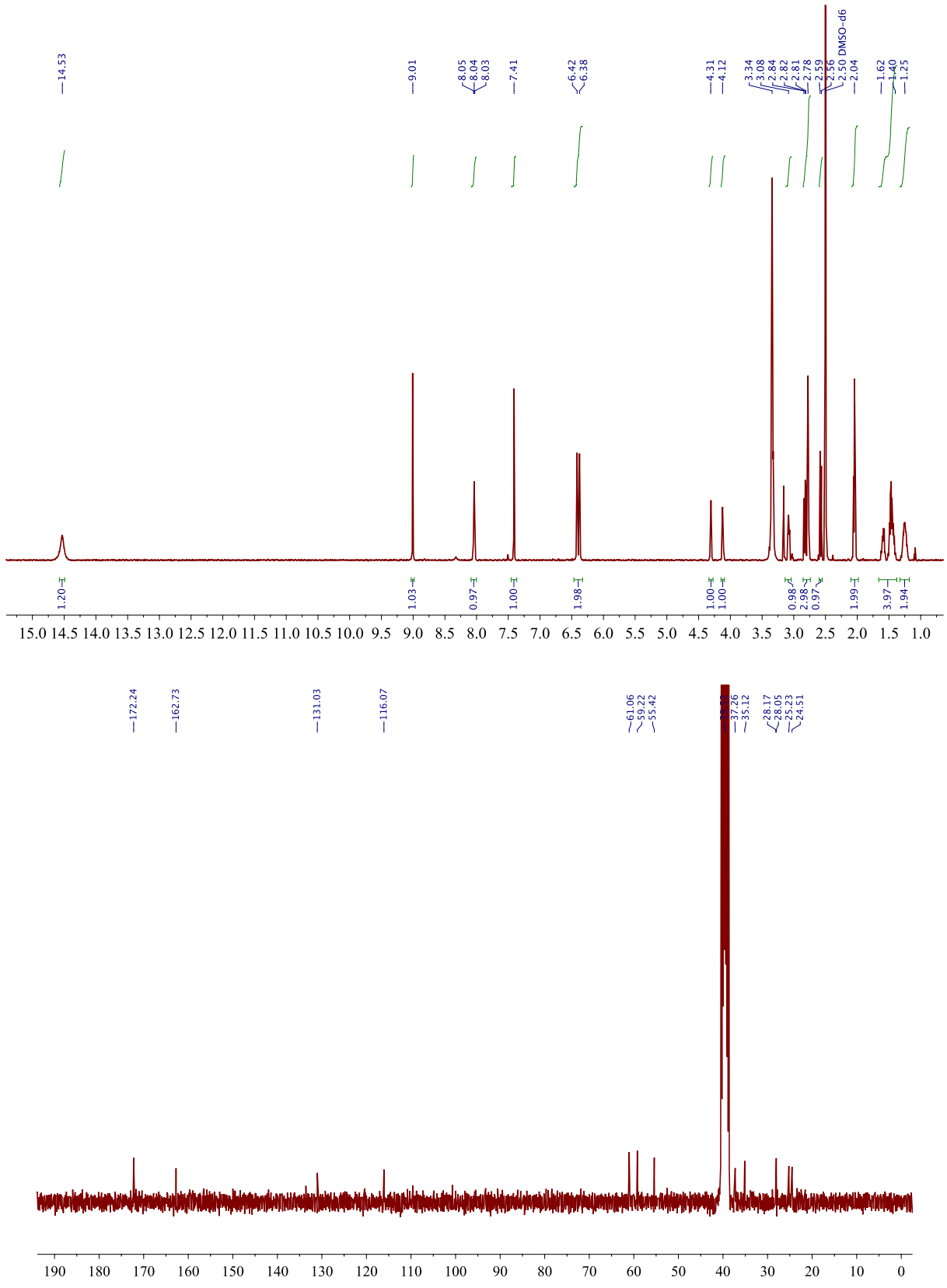


Figure S18. ^{1}H (top) and ^{13}C (bottom) NMR spectra of biot-histamine.

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