Supplementary Information

Formation of hydrazones and stabilized boron-nitrogen heterocycles in aqueous solution from carbohydrazides and *ortho*-formylphenylboronic acids

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Contents

1. General

2-Formylphenylboronic acid (2fPBA), 2-acetylphenylboronic acid, acethydrazide, propanoic acid hydrazide and N,N-dimethylglycine ethyl ester were obtained from Aldrich or Acros and used as received. Glycine ethyl ester was purchased from J.T.Baker. Glycine hydrazide and N,N-dimethylglycine ethyl ester were synthesized from the ethyl ester with hydrazine hydrate.[1] All NMR spectra were collected and processed on a Bruker Avance III 600 NMR spectrometer at 298 K unless otherwise noted. ^{1}H and ^{13}C NMR spectra were referenced to DMSO (2.50 and 39.51 ppm, respectively). NOESYGPPR1D (water suppression ^{1}H spectrum) in 90% H_2O (aqueous buffer) and 10% D_2O was referenced to TSP-d_4 (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt) at 0.00 ppm. NOE measurements were obtained from 2D NOESY. One-bond ^{1}H-^{13}C correlations were determined by HSQC, and two-three- bond were measured by HMBC experiments. ^{1}H-^{15}N connectivities were obtained by HMBCGP-^{15}N. The ME_mod of ^{11}B spectra was set to LPbr. Background spectra to compensate for the glass in the probe and in the NMR tube were collected and subtracted from the sample spectrum. Multiplicities are indicted by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Chemical shifts are reported in parts per million (ppm).

Crystal structure determination:

Compound **5a** was crystallized by dissolving the solid in DMSO at room temperature. A small amount of water (~ 5% v/v) was added to the solution. The solution was cooled to 4 °C and left undisturbed until crystals were observed. Compounds **5b**, **7c** and **7e** were dissolved in a minimal amount of DMSO at ~ 70 °C. Solutions allowed to cool at room temperature and were kept undisturbed at room temperature until crystals were observed.

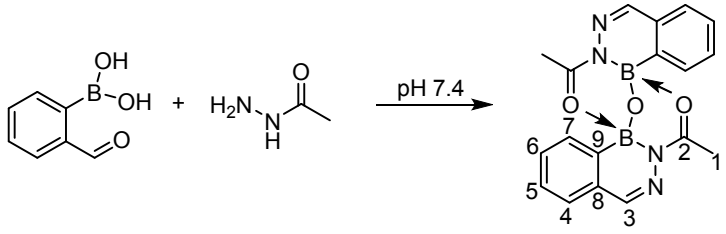
Crystals were mounted on a nylon loop with paratone oil on a Bruker APEX-II CCD diffractometer. The crystal was kept at T = 173(2) K during data collection. Using **Olex2,** the structure was solved with the **ShelXS** structure solution program, using the Direct Methods solution method. The model was refined with version 2014/6 of **XL** using Least Squares minimization.

COSMO-V1.61 •Software for the CCD Detector Systems for Determining Data Collection Parameters, Bruker axs, Madison, WI (2000).
O.V. Dolomanov and L.J. Bourhis and R.J. Gildea and J.A.K. Howard and H. Puschmann, Olex2: A complete structure solution, refinement and analysis program, *J. Appl. Cryst.*, (2009), **42**, 339-341.
SAINT-8.34A-2013 - Software for the Integration of CCD Detector System Bruker Analytical X-ray Systems, Bruker axs, Madison, WI (2013).
Sheldrick, G.M., A short history of ShelX, *Acta Cryst.*, (2008), **A64**, 339-341.

2. Synthesis

2-Acetyl-1-hydroxy-2,3,1-benzodiazaborine (anhydro dimer) (5a)

CA name: 1,1-oxybis[2-acetyl-1,2-dihydro-2,3,1-benzodiazaborine] (CAS 157619-14-2)

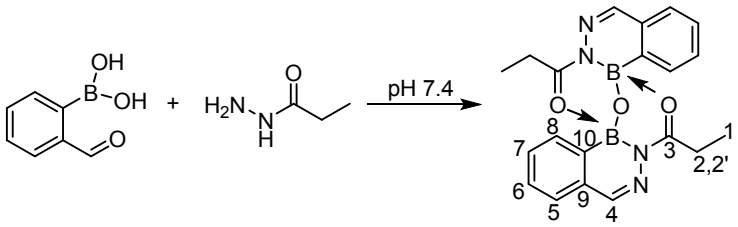


Acethydrazide (37 mg, 0.5 mmol) was dissolved in 0.5 ml pH 7.4 sodium phosphate buffer (PB), and then mixed with 2fPBA (75.0 mg, 0.5 mmol) in 3.5 ml PB. The final concentration of each component was 125 mM. A white precipitate formed immediately, which was filtered and washed with 3 ml distilled water. After drying with a stream of nitrogen, followed by several hours under vacuum at room temperature, 77.2 mg of a white solid was obtained. Yield: 86 %. Melting point: 227.0˚C. ^{1}H and ^{13}C NMR spectra were previously collected in $CDCl_3$.[2]

^{1}H NMR *(600 MHz, DMSO-d_6, 298K): δ 8.14 (1H, s,* H3*), 7.63 (1H, d, J=7.42 Hz,* H4*), 7.59 (1H, d, J=7.58 Hz,* H7*), 7.50 (2H, m,* H5/H6*), 2.39 (3H, s,* H1*).* ***^{13}C NMR*** *(600 MHz, DMSO-d6, 298 K): δ177.29(*C2*), 151.28(*C3*), 131.28(*C6*), 130.32(*C4*), 130.30(*C8*), 128.52(*C7*), 128.42(*C5*), 18.72(*C1*).* ***^{11}B NMR*** *(600 MHz, DMSO-d_6, 298 K): δ 0.62.* ***HRMS*** *(TOF MS ES$^+$): calculated for $C_{18}H_{17}B_2N_4O_3$ = [M+H]$^+$: m/z = 357.1559, found: m/z =357.1557.*

2-Propanoyl-1-hydroxy-2,3,1-benzodiazaborine (anhydro dimer) (5b)

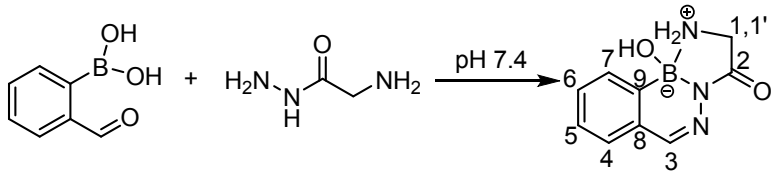
CA name: 1,1'-oxybis[2-propyl-1,2-dihydro-2,3,1-benzodiazaborine] (CAS number not yet assigned)



Propanoic acid hydrazide (44.2 mg, 0.5 mmol) was dissolved in 0.5 ml PB, and then mixed with 2fPBA (75.0 mg, 0.5 mmol) in 3.5 ml PB. The final concentration of each component was 125 mM. The precipitate was filtered and washed with 3 ml distilled water, dried at room temperature in vacuo, yielding a white solid (80 mg). Yield = 83%. Melting point: 146.9˚C.

^{1}H NMR *(600 MHz, DMSO-d6, 298K): δ 8.15 (1H, s,* H4*), 7.62 (1H, d, J=7.54 Hz, H5/H8), 7.59 (1H, d, J=7.59 Hz, H5/H8), 7.51(2H, m, H6/ H7) 2.90 (1H, dq, J=15.08, 7.59 Hz,* H2*), 2.79 (1H, dq, J=15.13, 7.54 Hz,* H2'*), 0.98 (3H, t, J=7.58 Hz,* H1*); (600 MHz, $CDCl_3$, 298K): δ 7.91 (1H, s,* H4*), 7.85 (1H, d, J=7.19 Hz, H5/H8), 7.51 (1H, t, J=7.22 Hz, H6/H7), 7.44(2H, m, H5/H8, H6/H7) 2.96 (1H, dq, J=14.80, 7.60 Hz,* H2*), 2.78 (1H, dq, J=14.75, 7.54 Hz,* H2'*), 1.11 (3H, t, J=7.65 Hz,* H1*); (600 MHz, C_6H_6, 298K): δ 8.28 (1H, d, J= 7.36 Hz, H5/H8), 7.71 (1H, s, H4), 7.33 (1H, t, J= 7.45 Hz, H6/H7), 7.16(1H, overlap with solvent peak, H6/H7), 7.01 (1H, d, J= 7.70 Hz, H5/H8), 2.57 (2H, m,* H2 and H2'*), 0.81 (3H, t, J=7.57 Hz*, H1*).* ***^{13}C NMR*** *(600 MHz, DMSO-d6, 298 K): δ179.94(*C3*), 151.30(*C4*), 131.31(*C6/C7*), 130.26(*C9*), 130.24(*C5/C8*), 128.53(*C6/C7*), 128.41(C5/C8), 24.39(C2), 9.28(*C1*).* ***^{11}B NMR*** *(600 MHz, DMSO-d6, 298 K): δ 0.35.* ***HRMS*** *(TOF MS ES$^+$): calculated for $C_{20}H_{21}B_2N_4O_3$ = [M+H]$^+$: m/z = 385.1872, found: m/z =385.1867. Crystal was made by DMSO under heat.*

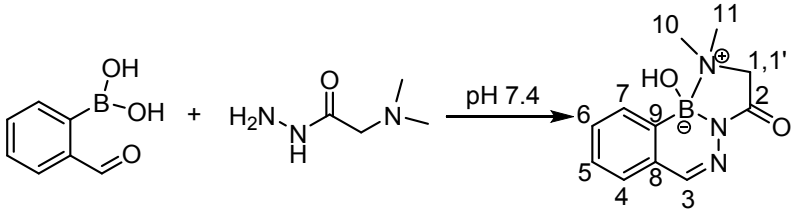
2fPBA-Glycine hydrazide product (7c)



Glycine hydrazide (44.5 mg, 0.5 mmol) was dissolved in 0.5 ml pH 7.4 PB buffer, and then mixed with 2fPBA (75.0 mg, 0.5 mmol) in 3.5 ml PB. The precipitate was filtered and washed with 3 ml distilled water, dried at room temperature in vacuo, yielding a white solid (92.9 mg). Yield =91.5%. Melting point: > 260˚C.

*^{1}**H NMR** (600 MHz, DMSO-d6, 298K): δ 7.76 (1H, s,* H3*), 7.46(1H, d, J=7.13 Hz, H4/H7), 7.42(1H, m, H4/H7 and H5/H6), 7.34(1H, t, J=7.26 Hz, H5/H6), 6.64 (2H, s, N**H$_2$**), 3.71 (1H, dt, J=15.14, 7.52 Hz,* H1*), 3.38 (1H, s, B-O**H**), 3.14 (1H, dd, J=15.38, 4.50 Hz,* H1'*). ^{13}**C NMR** (600 MHz, DMSO-d6, 298 K): δ168.25(*C2*), 148.19(*C3*), 131.63(*C8*), 129.64 (*C5/C6*), 129.33 (*C4/C7*), 126.79(C5/C6), 126.75(C4/C7), 43.80(C1). ^{11}**B NMR** (600 MHz, DMSO-d6, 298 K): δ 0.54. **HRMS** (TOF MS ES$^+$): calculated for $C_9H_{11}BN_3O_2$ = [M+H]$^+$: m/z = 204.0944, found: m/z =204.0941. Crystal was made by DMSO under heat.*

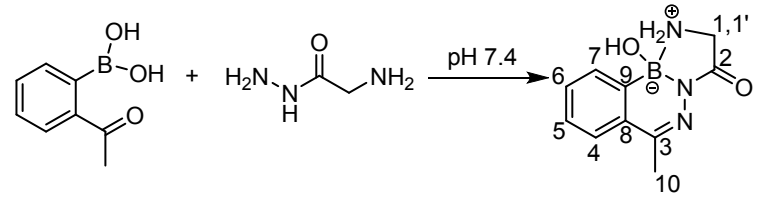
2fPBA-N, N-Dimethylglycine hydrazide product (7e)



N, N-Dimethylglycine hydrazide (29.3 mg, 0.25 mmol) was dissolved in 1 ml pH 7.4 PB, and then mixed with 2fPBA (37.5 mg, 0.25 mmol) in 3 ml PB. D_2O was added to a final volume 10% (v/v), after which ^{1}H NMR spectra were collected.

*^{1}**H NMR** (600 MHz, 90% 10mM phosphate buffer+10% D_2O, 298K): δ 7.82(1H, s,* H3*), 7.60(1H, d, J=7.35 Hz, H4/H7), 7.51-7.48(2H, m, H4/H7 and H5/H6), 7.43(1H, t, J= 7.63 Hz, H5/H6), 4.04(1H, d, J=15.35 Hz, H1), 3.76(1H, d, J=15.33 Hz, H1'), 3.01(3H, s,* H10*), 2.47(3H, s, H11). ^{11}**B NMR** (600 MHz, 90% H_2O+10% D_2O, 298 K): δ 3.62.*

2AcPBA-Glycine hydrazide product (7d)



Glycine hydrazide (22.3 mg, 0.25 mmol) and 2-acetylphenylboronic acid (41.0 mg, 0.25 mmol) were mixed in 5ml MeOH. The reaction was stirred for 30 min at room temperature. Solvent was removed using a stream of dry nitrogen. The resulting white residue was dissolved in DMSO-d6 to be characterized on NMR.

*^{1}**H NMR** (600 MHz, DMSO-d6, 298K): δ 7.53(1H, d, J=7.92 Hz, H4/H7), 7.46(1H, d, J=7.28 Hz, H4/H7), 7.41(1H, t, J=7.16 Hz, H5/H6), 7.35(1H, t, J=7.63 Hz, H5/H6), 6.57 (2H, s, N**H$_2$**), 3.67 (1H, dt, J=7.92 Hz, J=15.12, 7.28 Hz,* H1*), 3.10 (1H, dd, J=15.45, 3.97 Hz,* H1'*), 2.36(3H, s, H10). ^{13}**C NMR** (600 MHz, DMSO-d6, 298 K): δ167.19(*C2*), 152.54(*C3*), 132.13(*C8*), 129.36 (*C5/C6*), 129.25 (*C4/C7*), 126.68(C5/C6), 124.89(C4/C7), 43.74(C1), 20.50(C10). ^{11}**B NMR** (600 MHz, DMSO-d6, 298 K): δ 0.64. **HRMS** (TOF MS ES$^+$): calculated for $C_{10}H_{13}BN_3O_2$ = [M+H]$^+$: m/z = 217.1137, found: m/z =217.1140. Crystal was made by DMSO under heat.*

3. Preparation of NMR samples at varying pH.

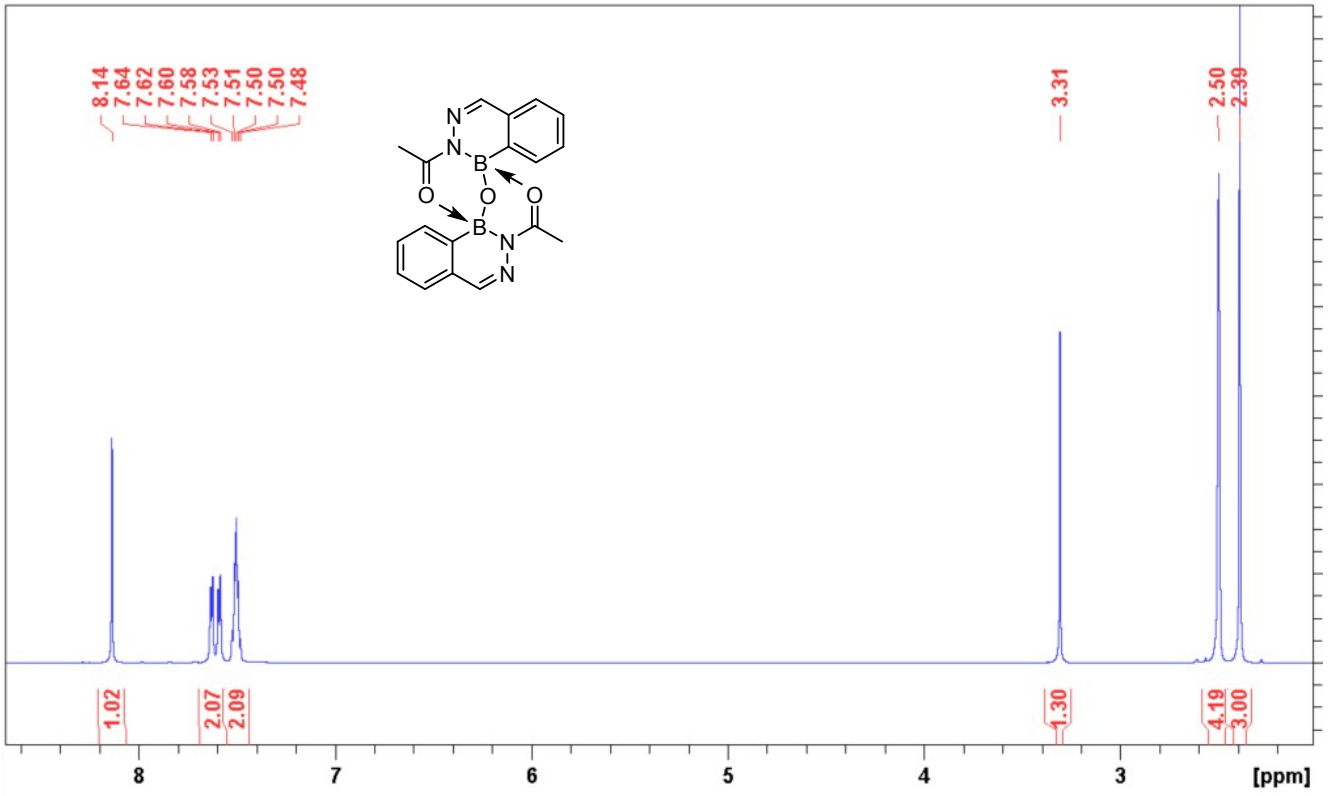
Method A: Equal volumes of solutions containing 4.44 mM 2fPBA and 4.44 mM hydrazide in 0.01 M sodium phosphate buffer, pH 7.0, were mixed to a total volume of 9.0 ml D_2O (1.0 ml containing 10 mM TSP-d_4) was added. The final concentration of the solution was 2 mM 2fPBA, 2 mM hydrazide and 1 mM TSP-d_4. The solution was separated into 1 ml aliquots. The pH of each was adjusted to the selected pH value (4 to 9) using small volumes (up to 20 μl) of 1 M HCl or 1 M NaOH measured on a pH meter.

Method B:Solids of **5a, 5b**, **7c** were directly dissolved in 10 ml of in 0.01 M sodium phosphate (pH 8) containing 10% (v/v) D_2O to get a 2 mM of final concentration. The solution was separated into 1 ml aliquots. The pH of each was adjusted to the selected pH value (4 to 9) using small volumes (up to 20 µl) of 1 M HCl or 1 M NaOH.

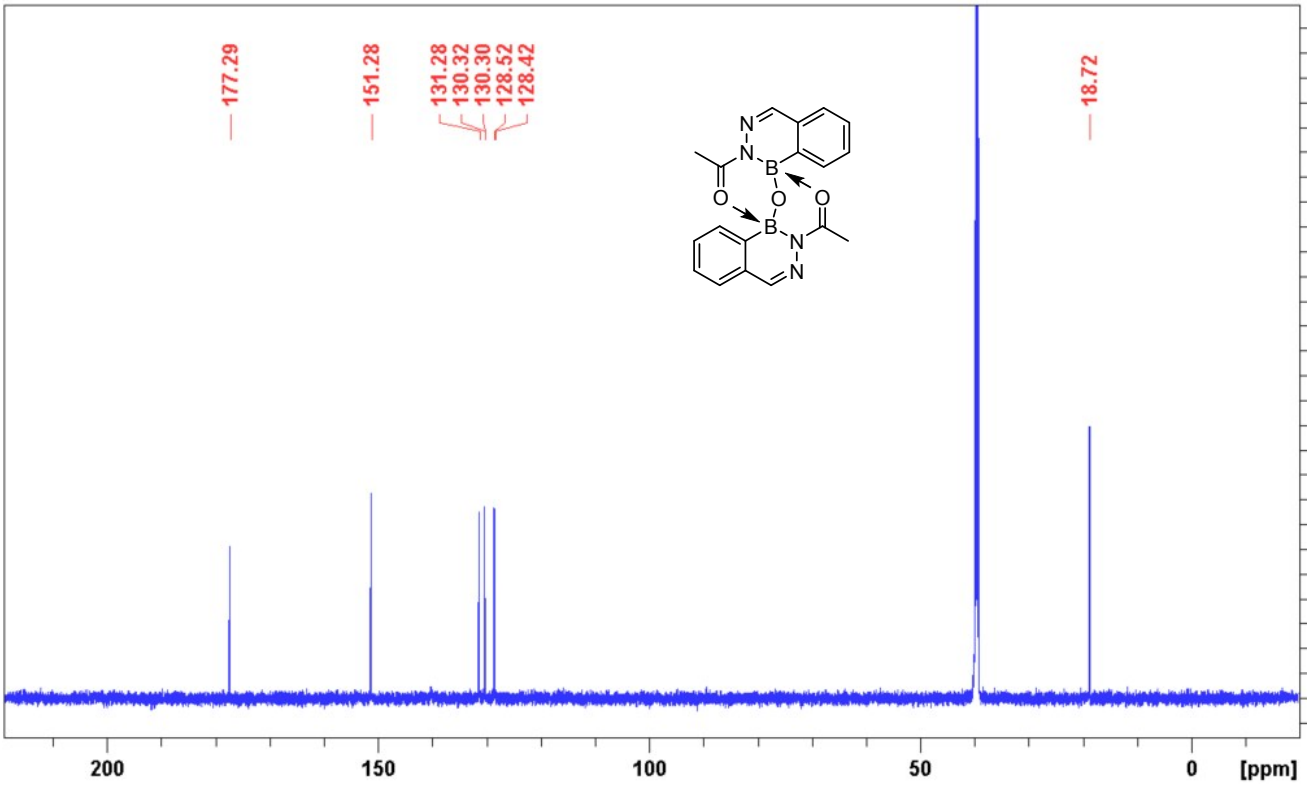
4. NMR spectra

2-Acetyl-1-hydroxy-2,3,1-benzodiazaborine (5a)

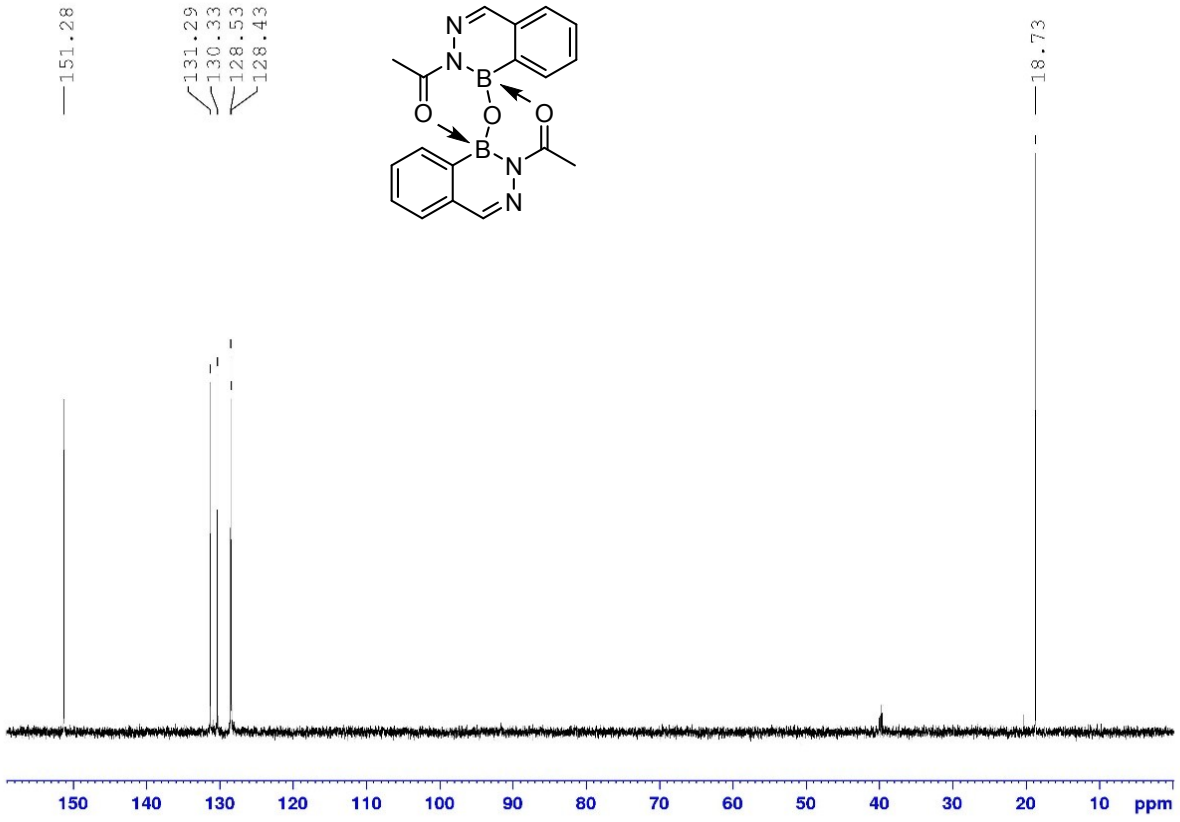
^{1}H NMR (DMSO-d6)



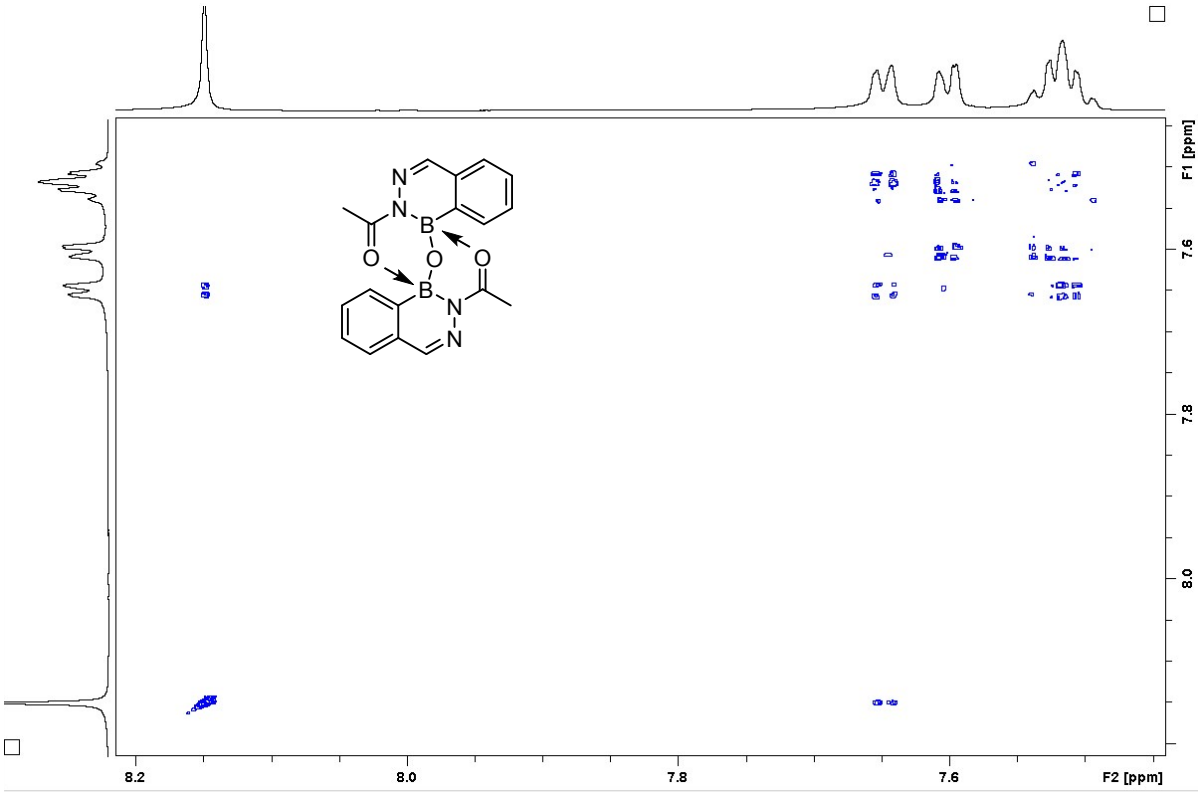
^{13}C NMR (DMSO-d6)



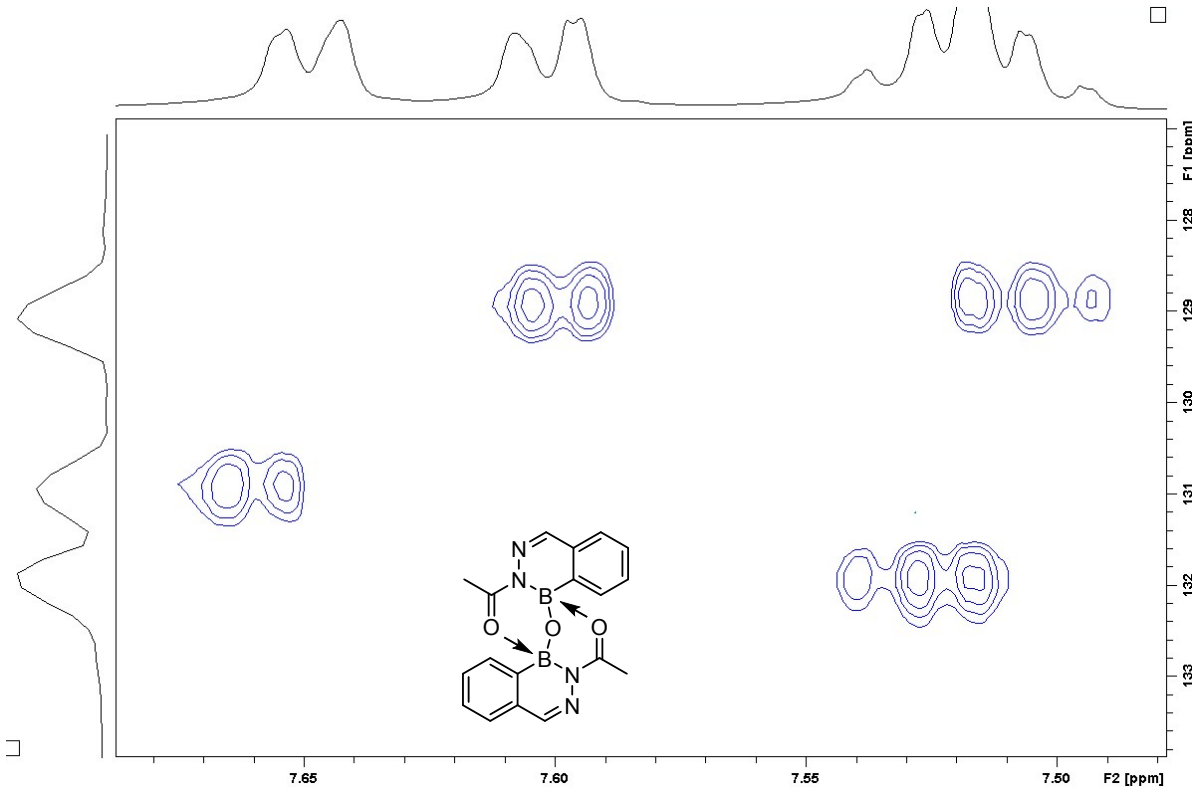
Dept135 (DMSO-d6)



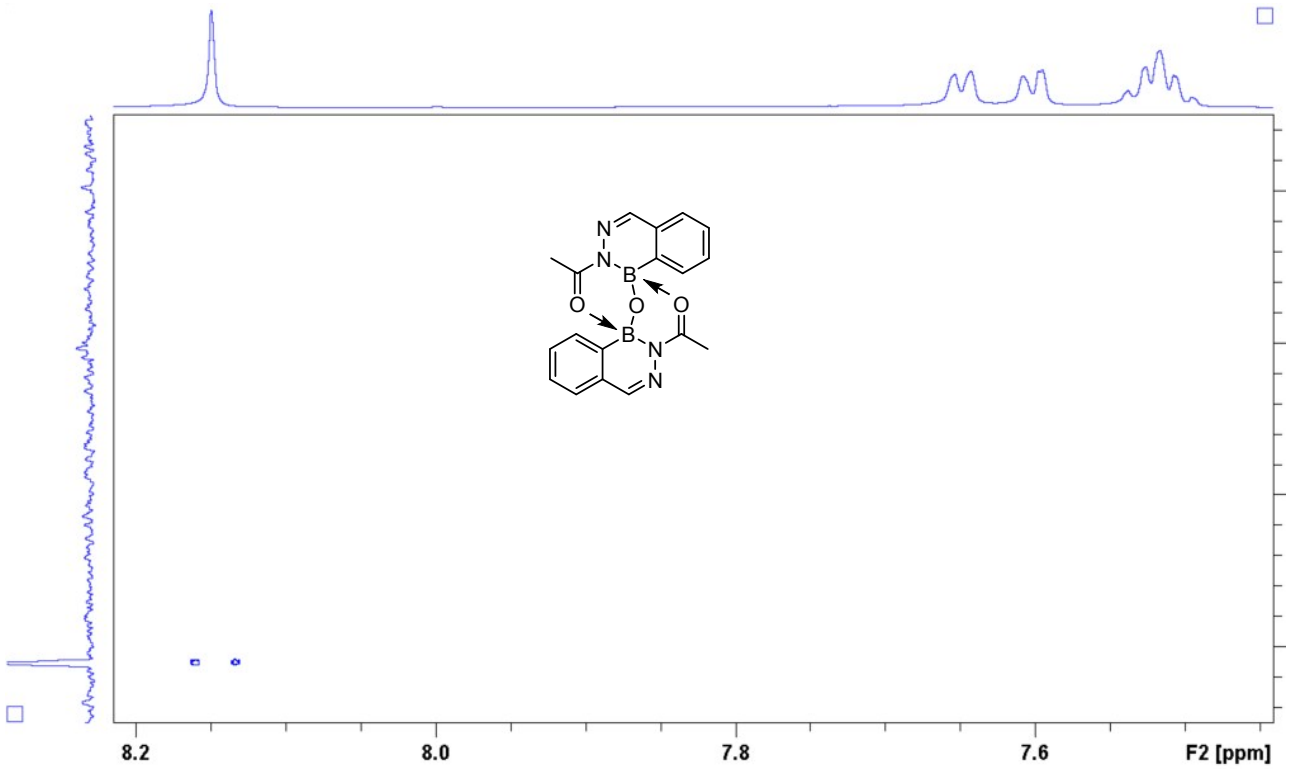
^{1}H-^{1}H COSY (DMSO-d6)



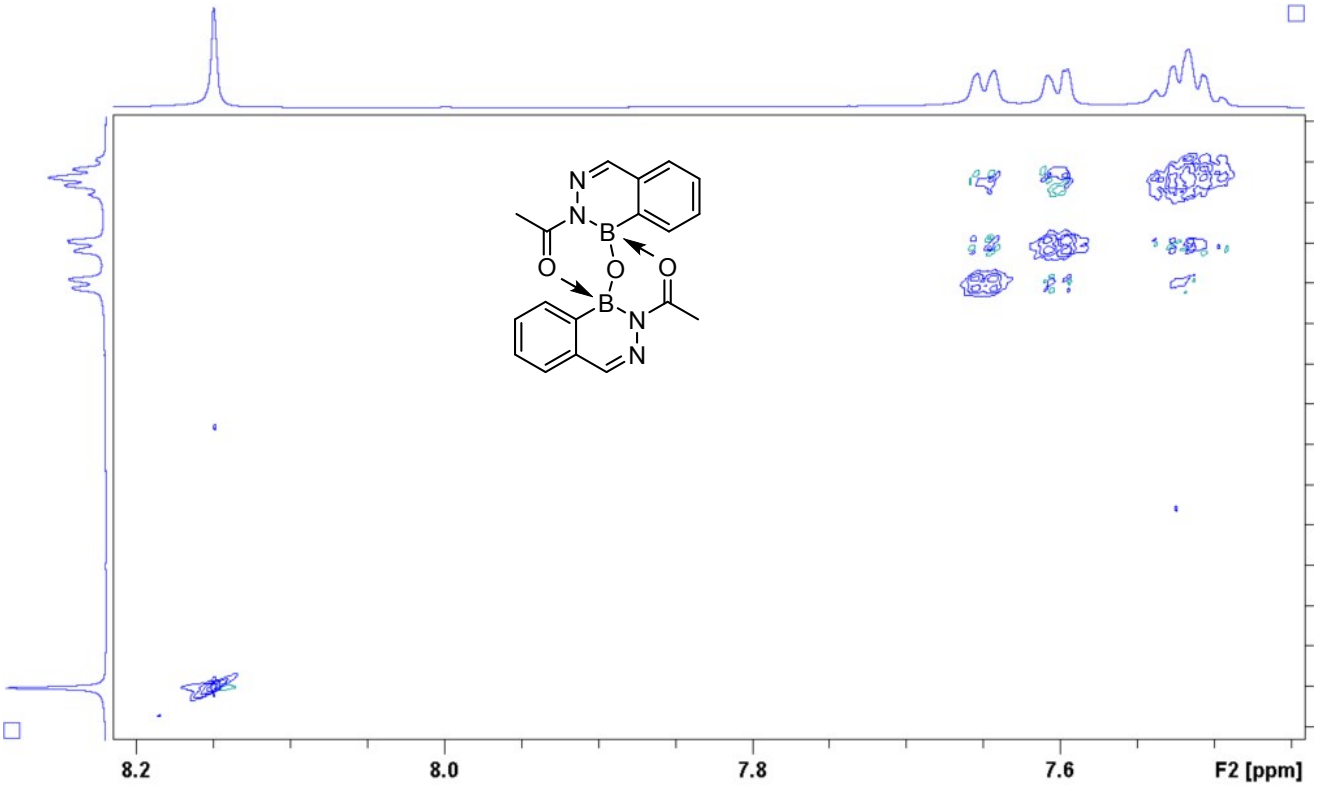
^{1}H-^{13}C HSQC (DMSO-d6)



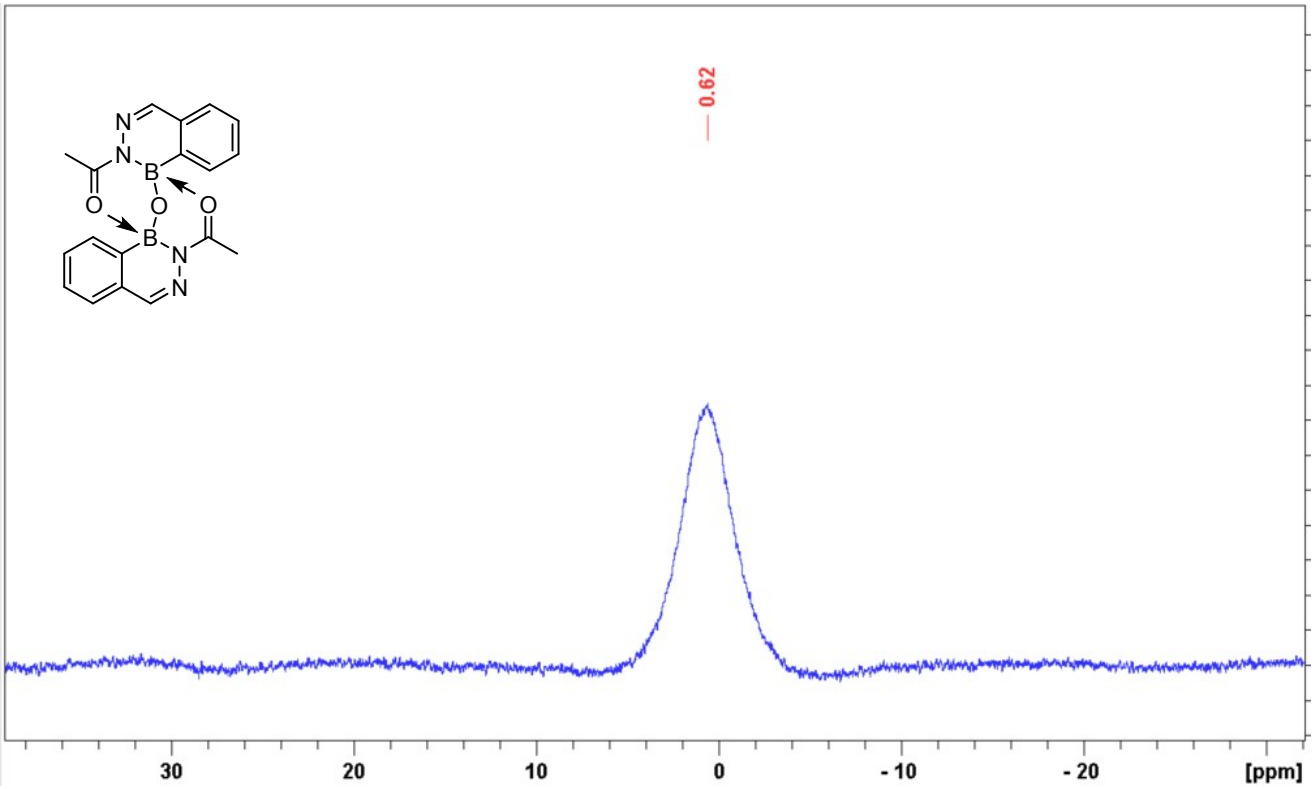
^{1}H-^{15}N HMBCGP (DMSO-d6)



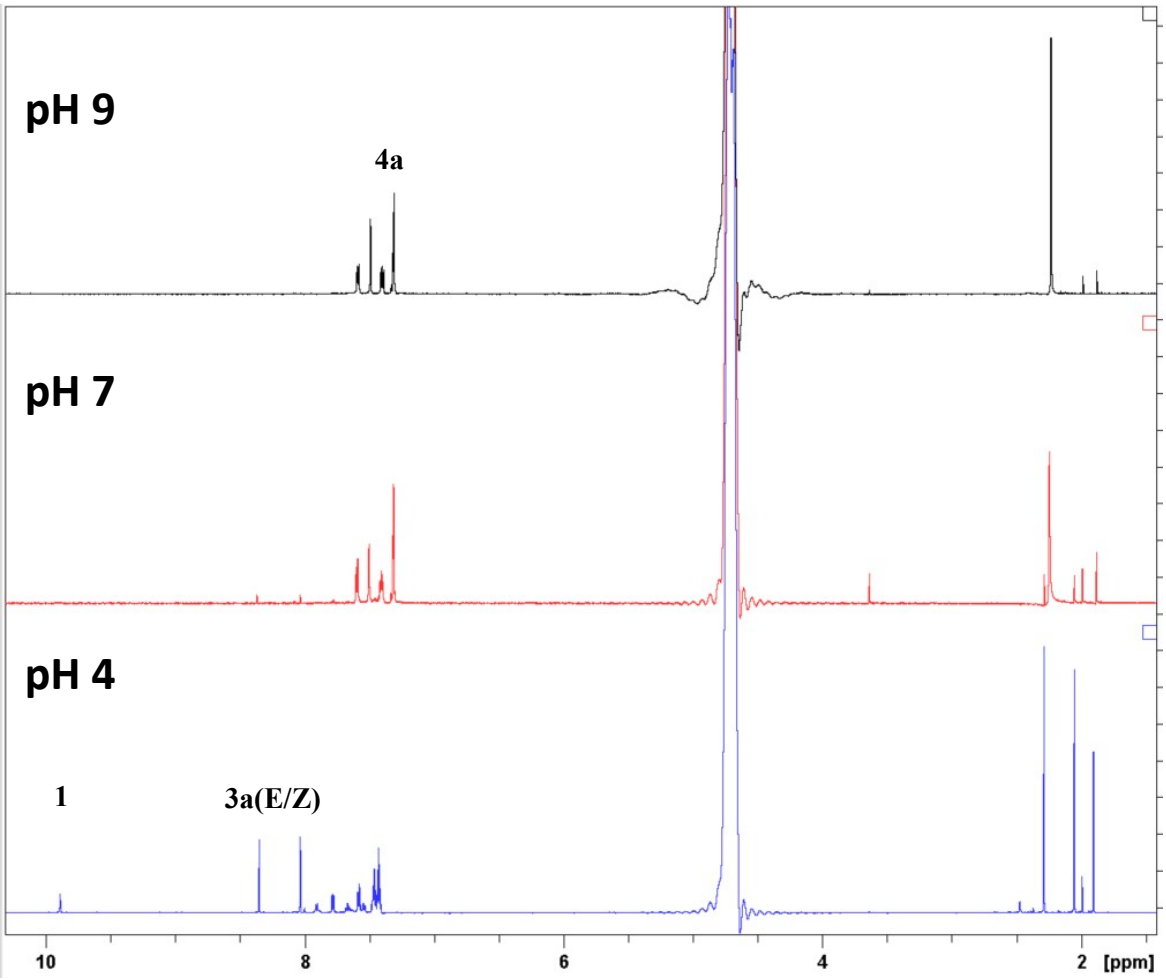
^{1}H-^{1}H NOESY (DMSO-d6)



^{11}B NMR (DMSO-d6)

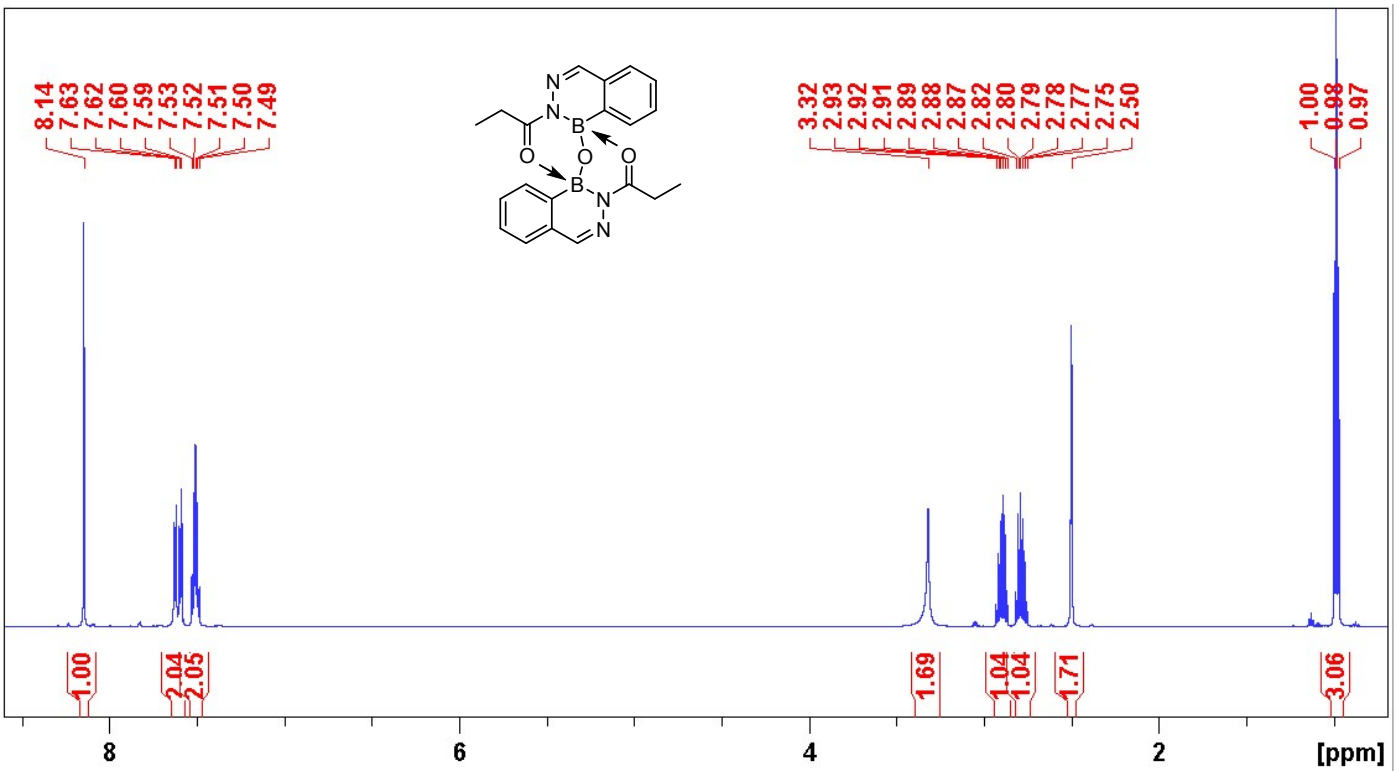


^{1}H NMR spectra in pH 4 (bottom), pH 7 (middle) and pH 9 (top) aqueous solutions (90% 10 mM phosphate buffer + 10% D_2O) (Method B)

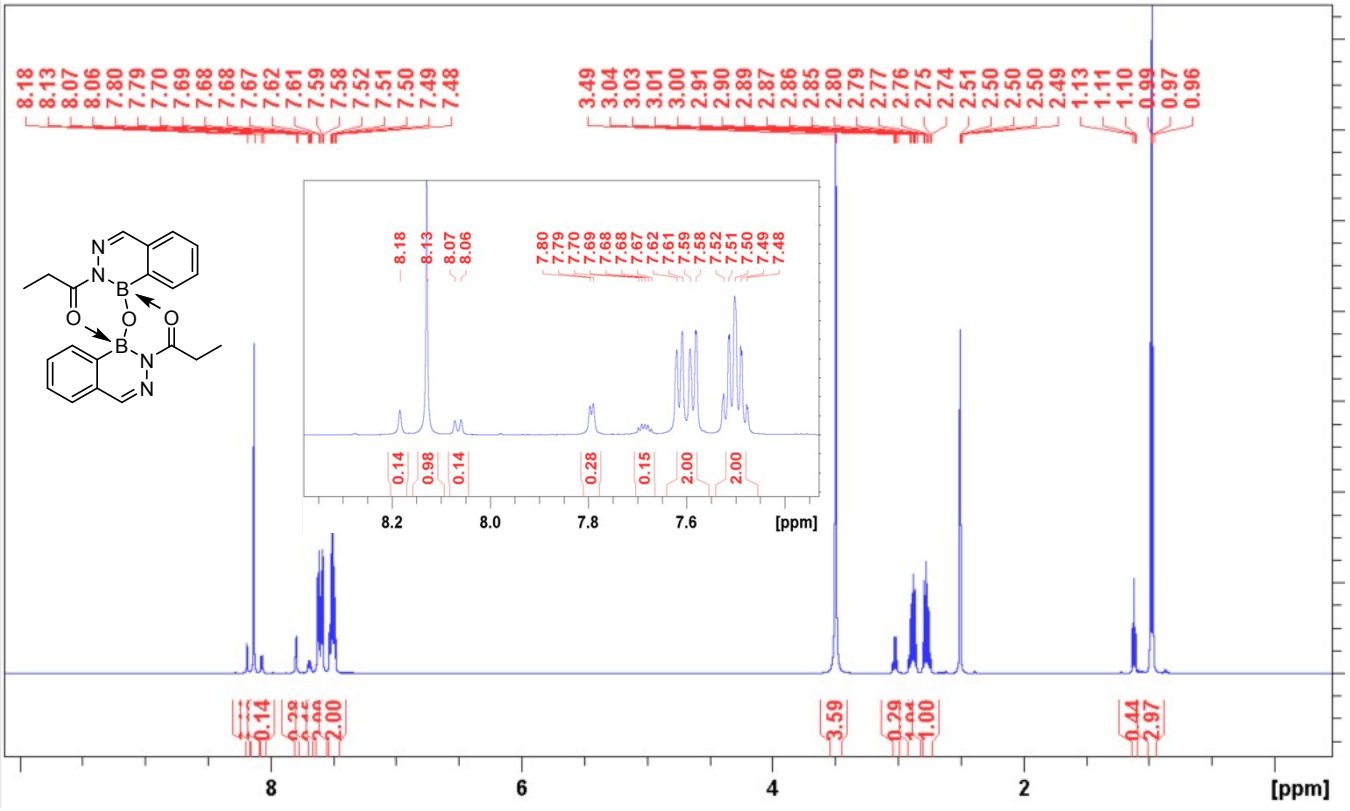


2-Propanoyl-1-hydroxy-2,3,1-benzodiazaborine (5b)

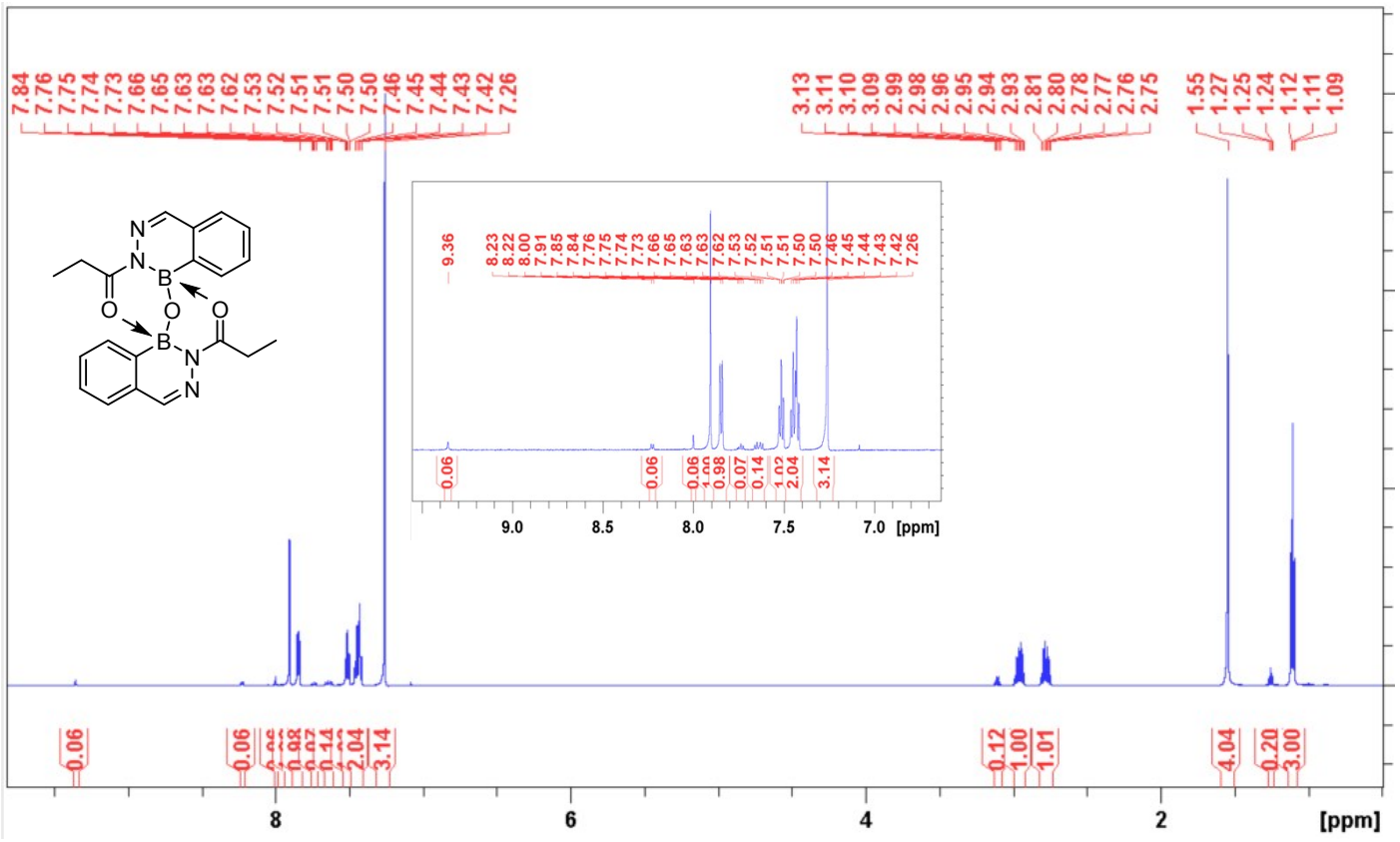
^{1}H NMR (DMSO-d6)



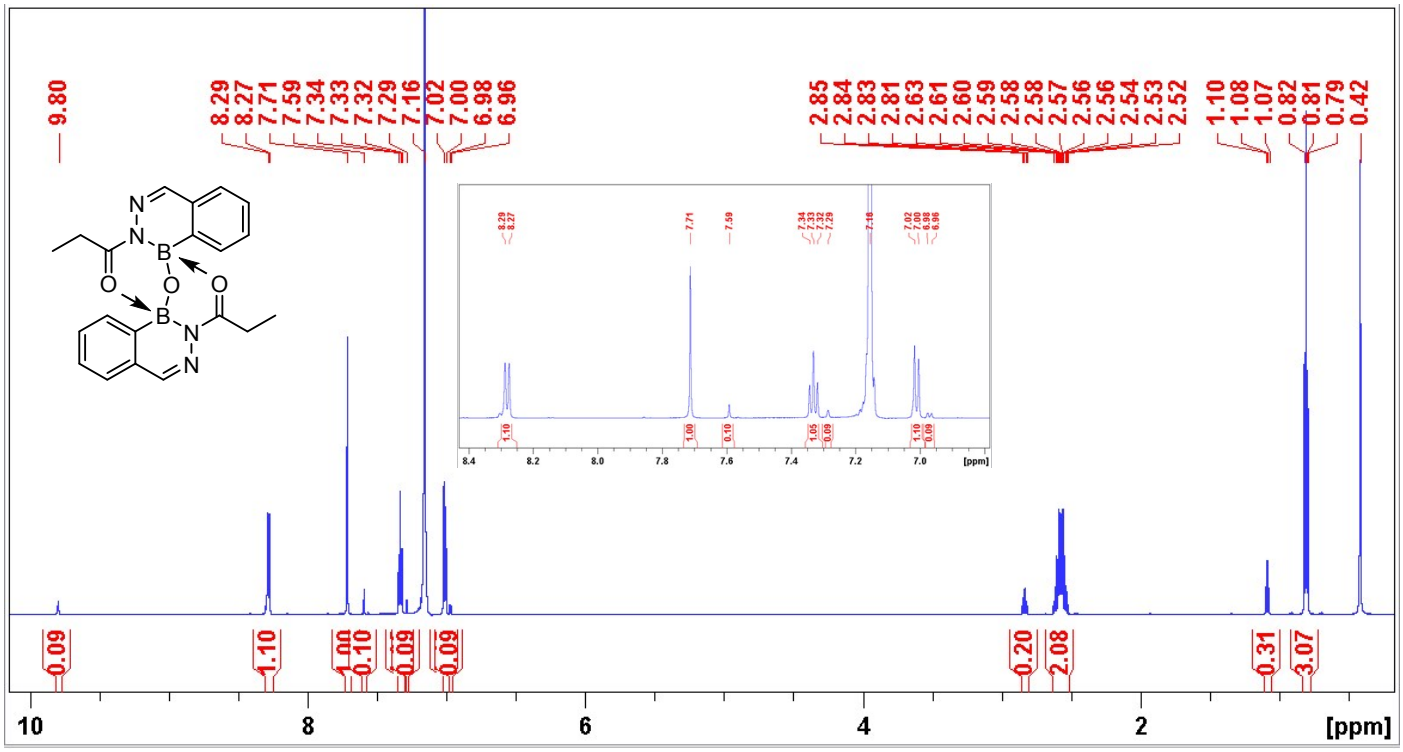
^{1}H NMR (DMSO-d6 + 1 drop of D_2O)



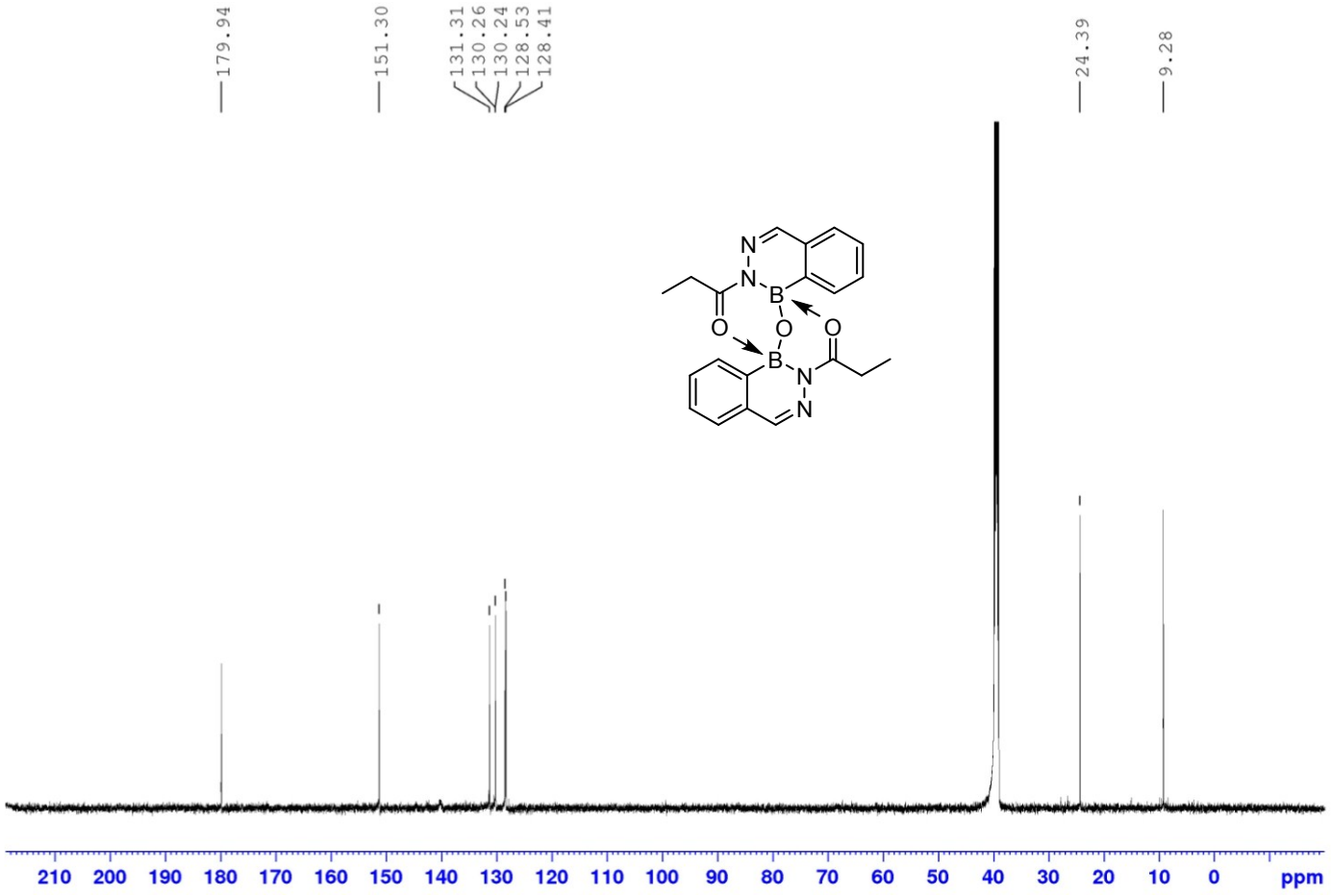
^{1}H NMR ($CDCl_3$)



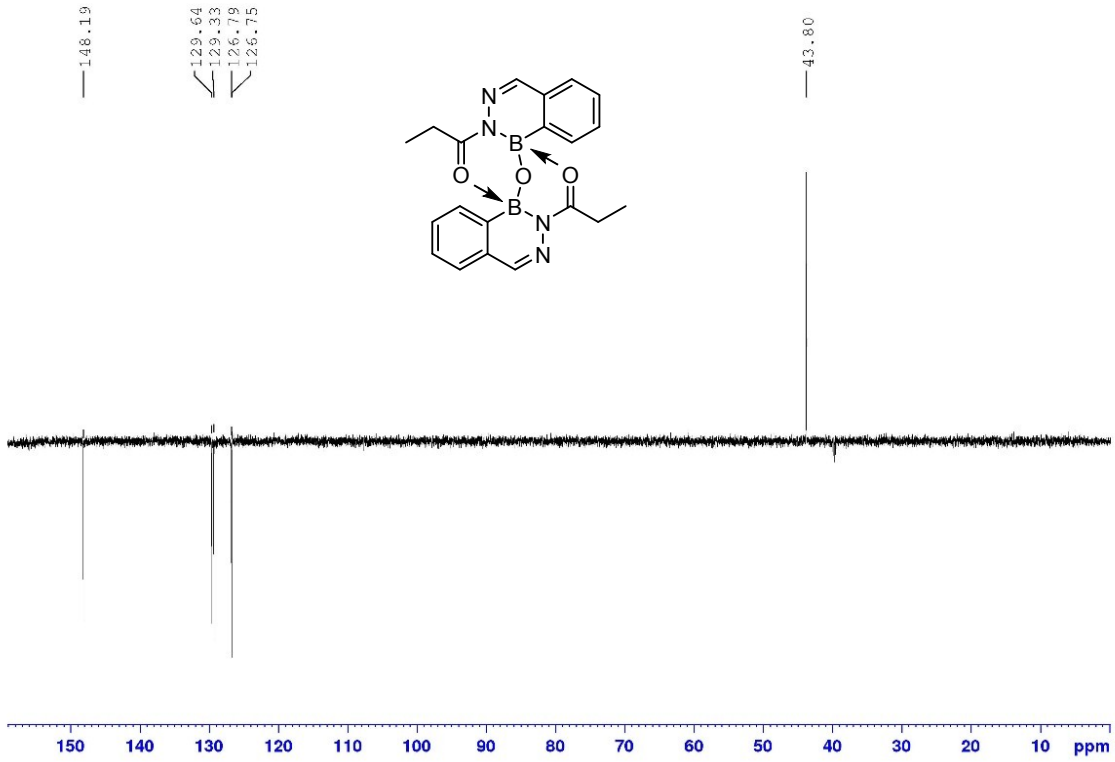
^{1}H NMR (C_6D_6)



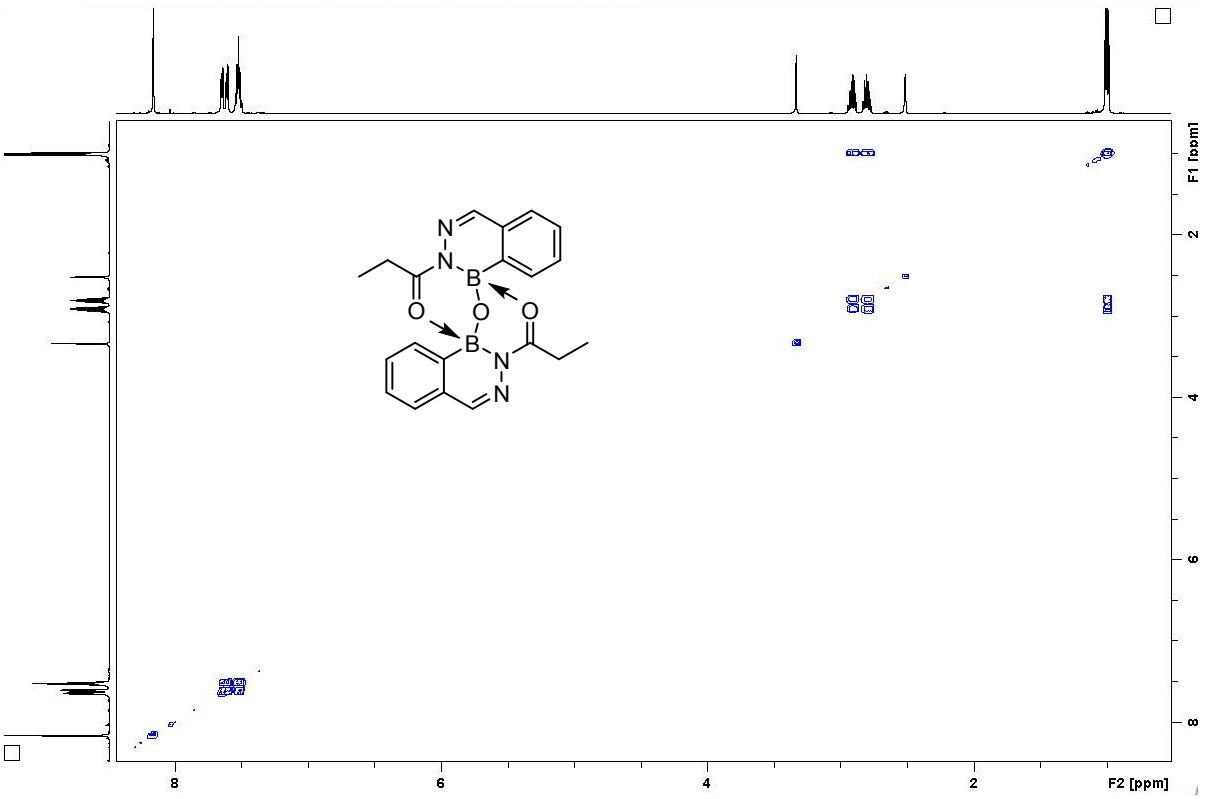
^{13}C NMR (DMSO-d6)



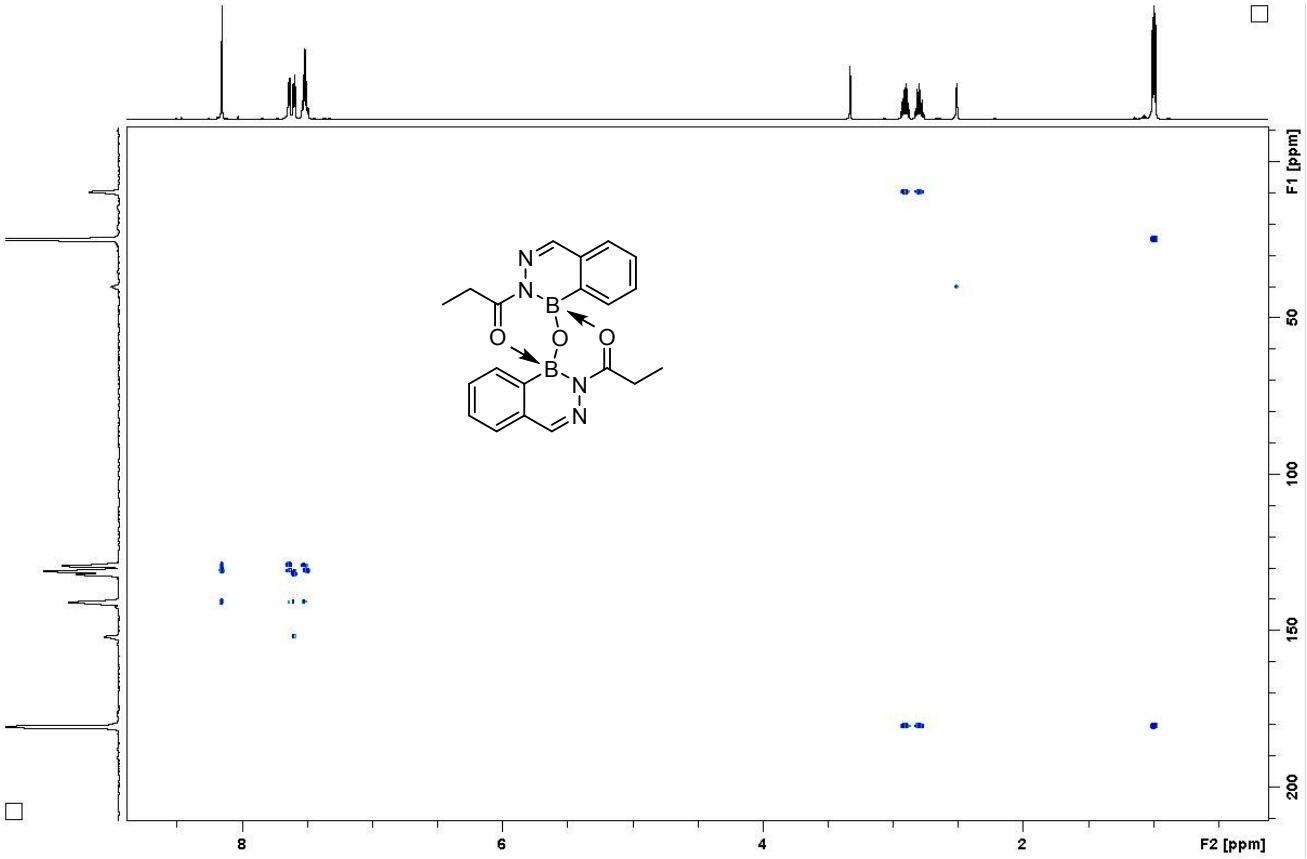
Dept 135 (DMSO-d6)



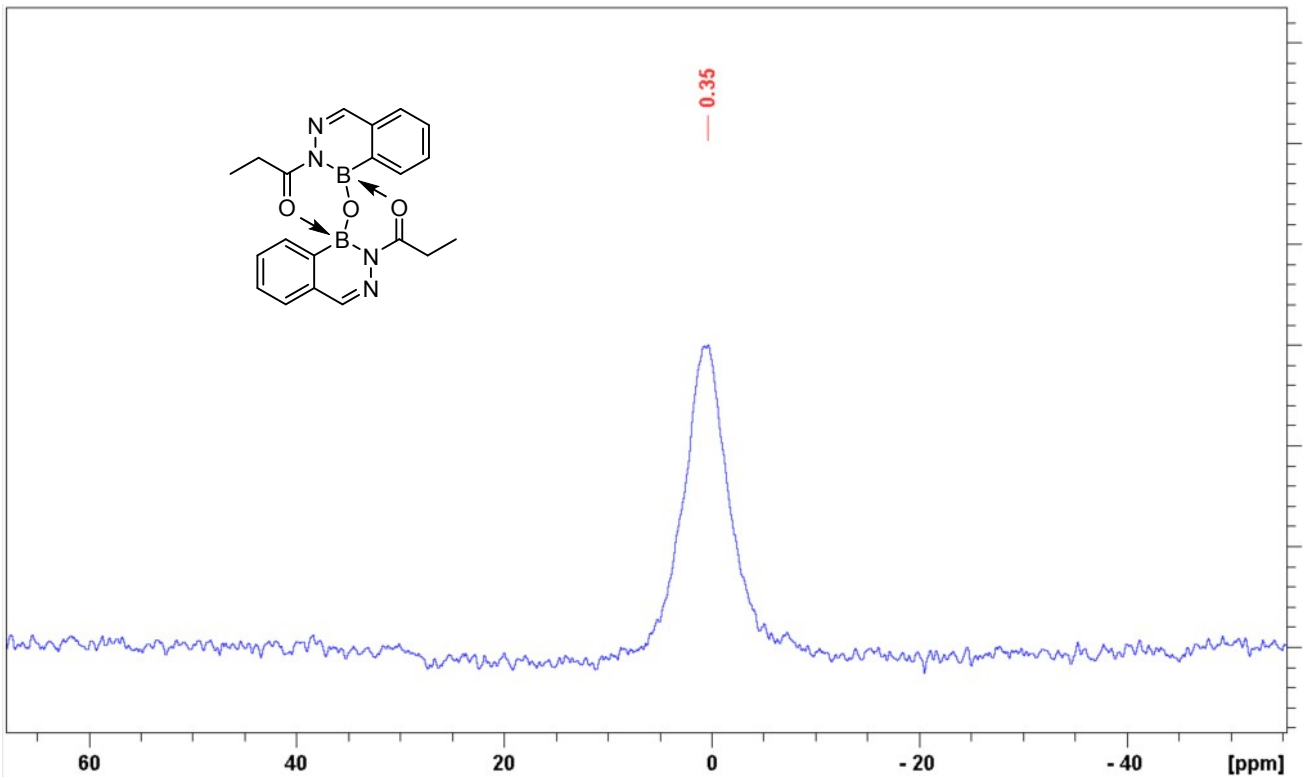
^{1}H-^{1}H COSY (DMSO-d6)



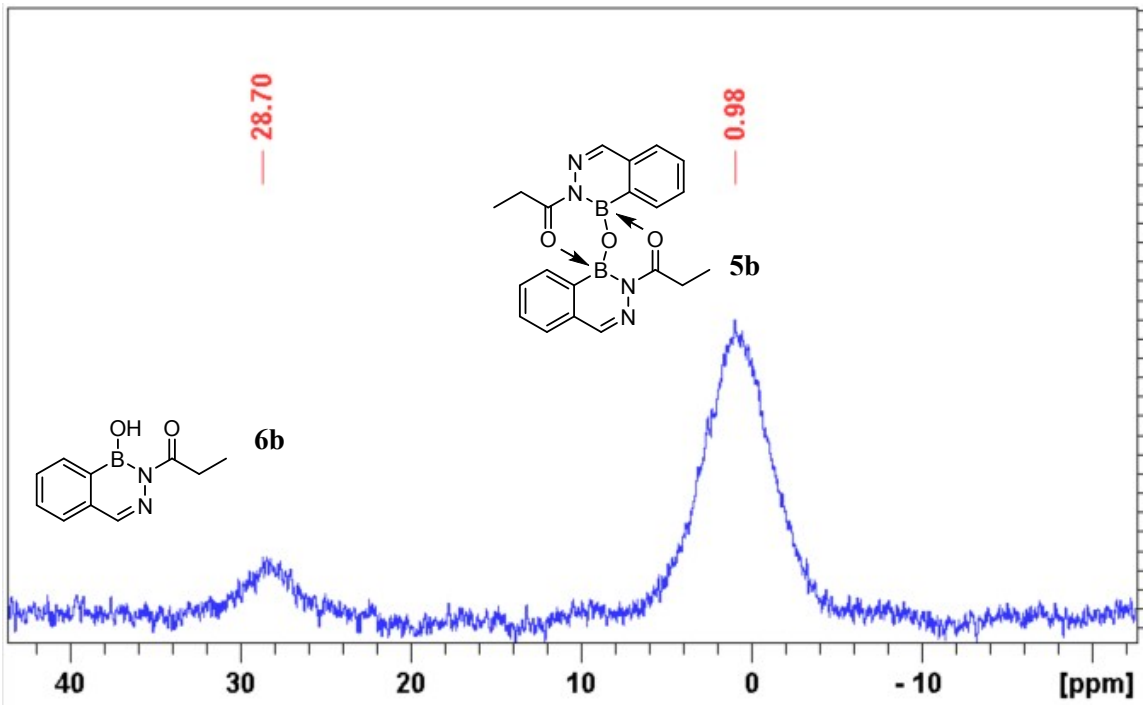
^{1}H-^{13}C HMBC (DMSO-d6)



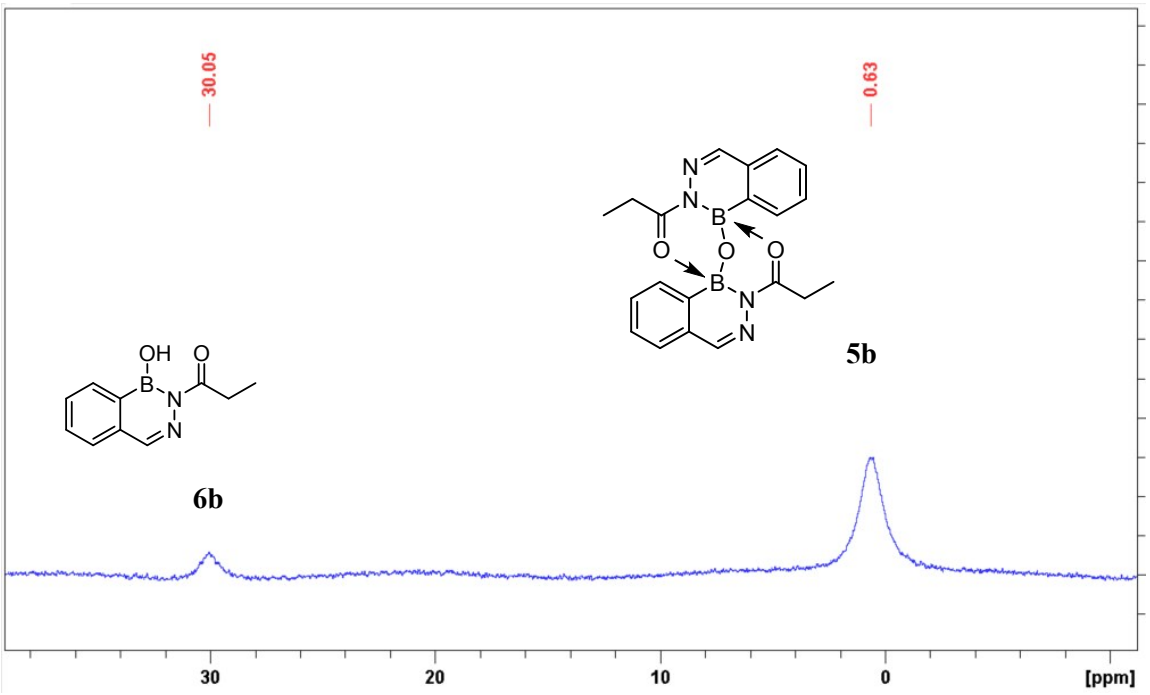
^{11}B NMR (DMSO-d6)



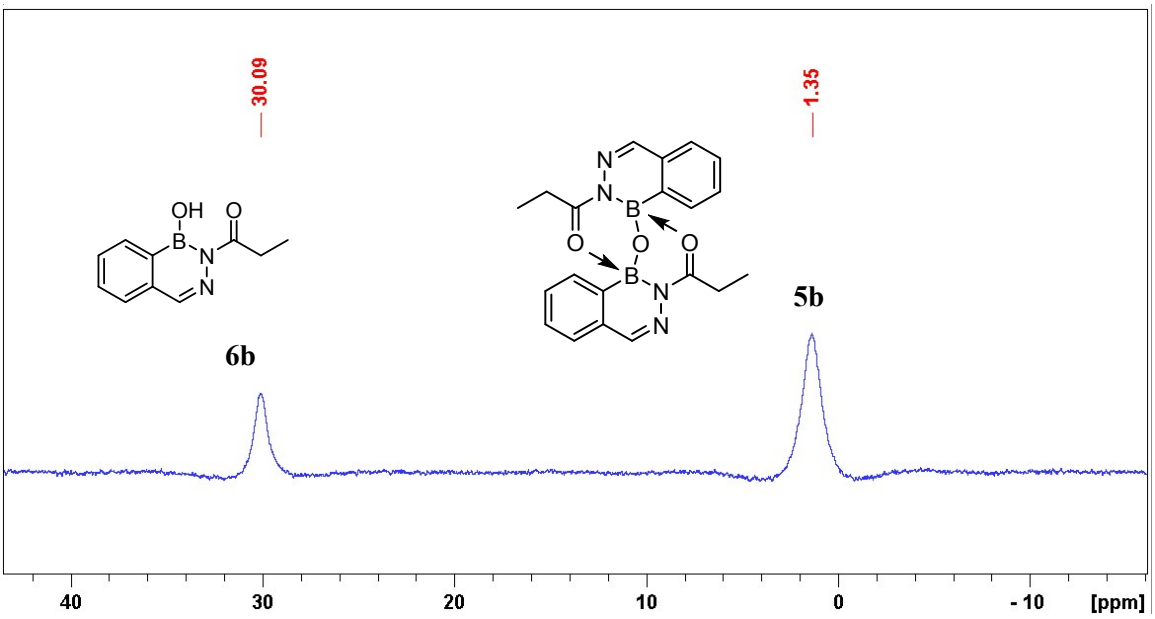
^{11}B NMR (DMSO-d6 + 1 drop of D_2O)



^{11}B NMR ($CDCl_3$)

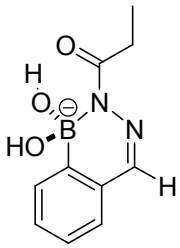


^{11}B NMR (C_6D_6)

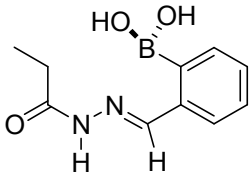


2-Propanoyl-1-hydroxy-2,3,1-benzodiazaborine (4b)

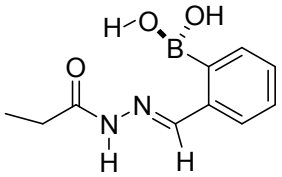
Structures



4b

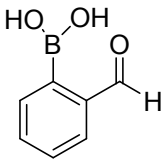


E-C=N
E- amide

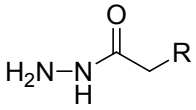


E-C=N
Z- amide

3b(E/Z)



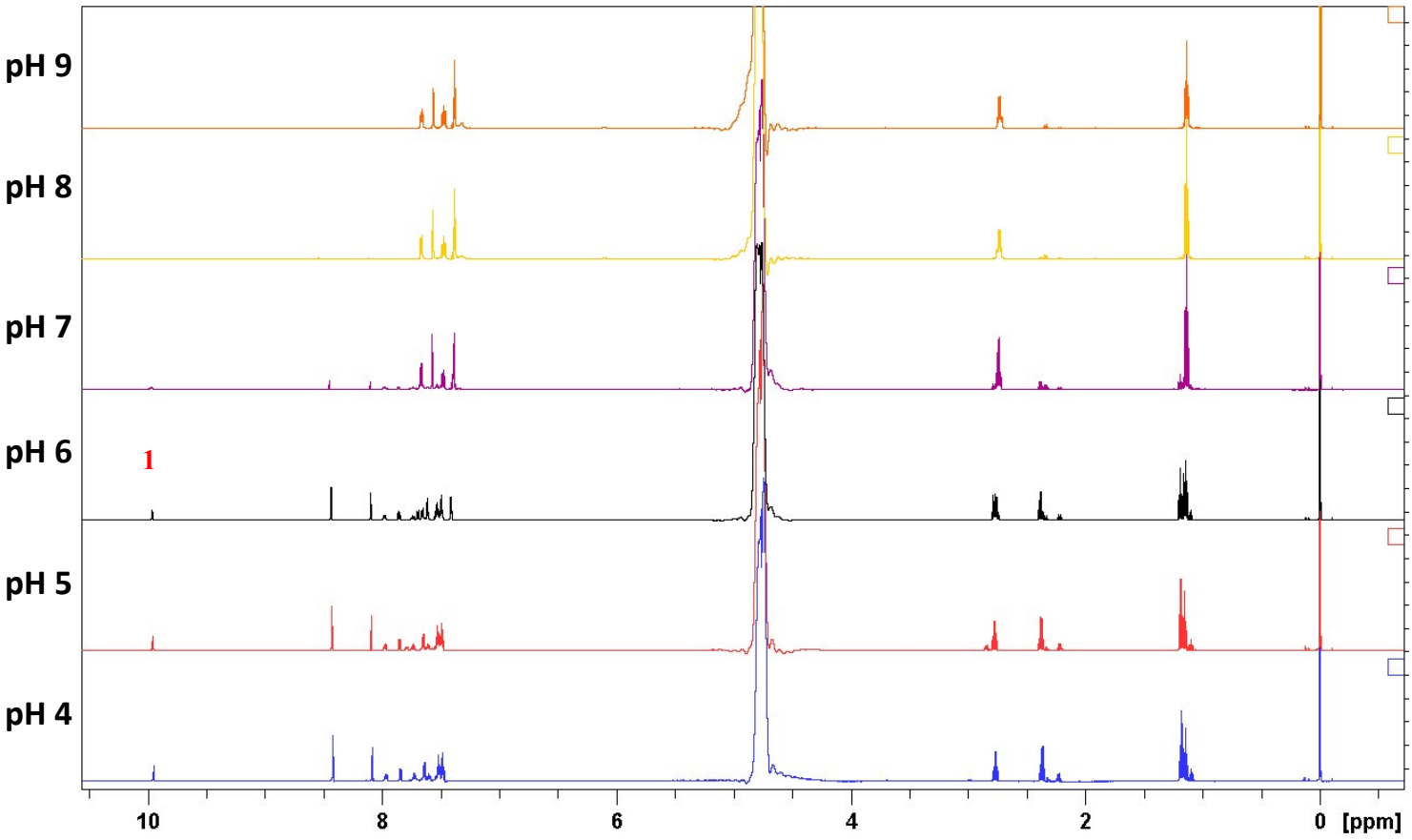
1

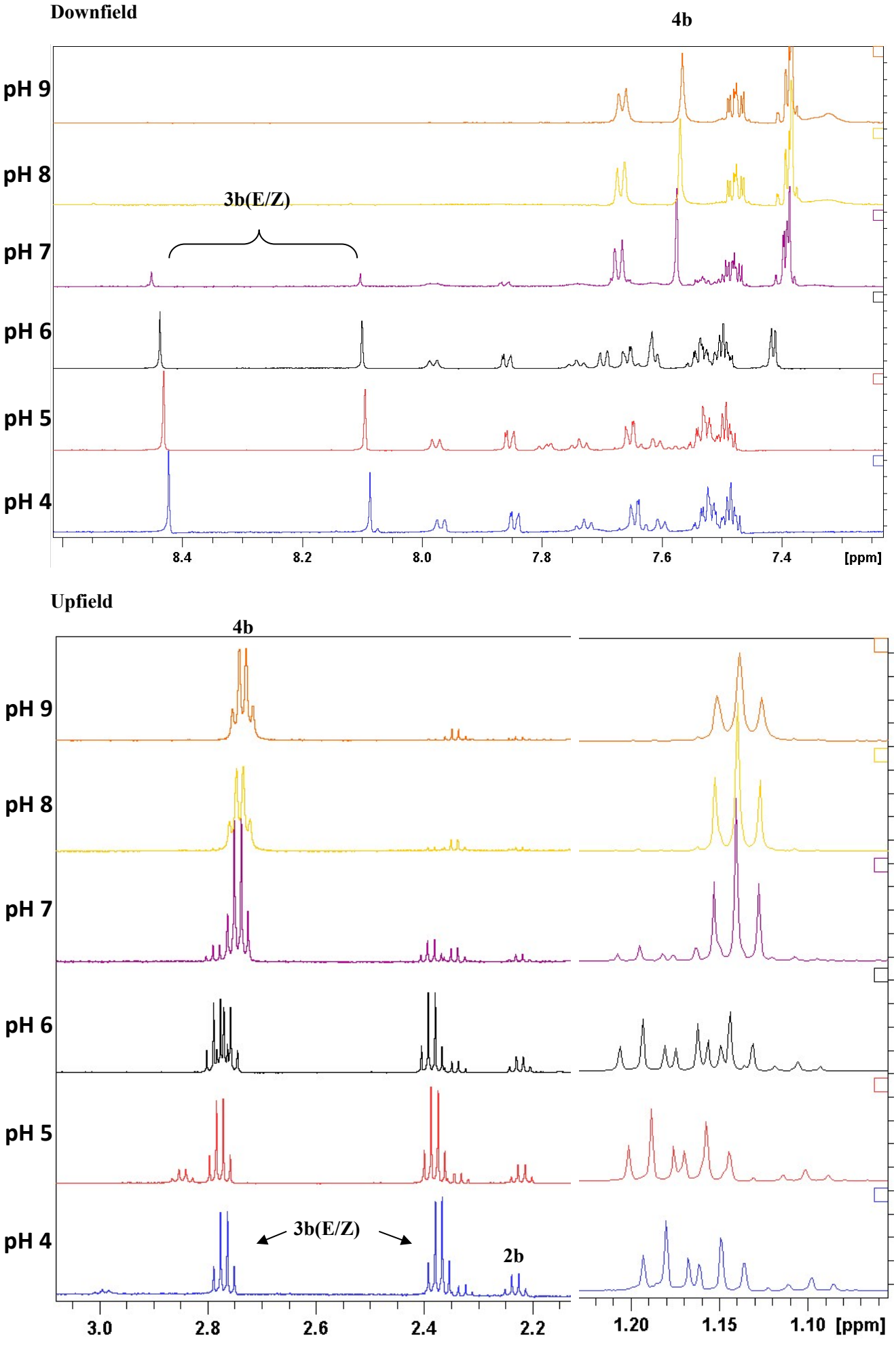


2b

^{1}H NMR spectra in pH 4 (bottom) to pH 9 (top) aqueous solutions (90% 10 mM phosphate buffer + 10% D_2O, internal standard-TSP-d_4) (Method A)

Full spectra

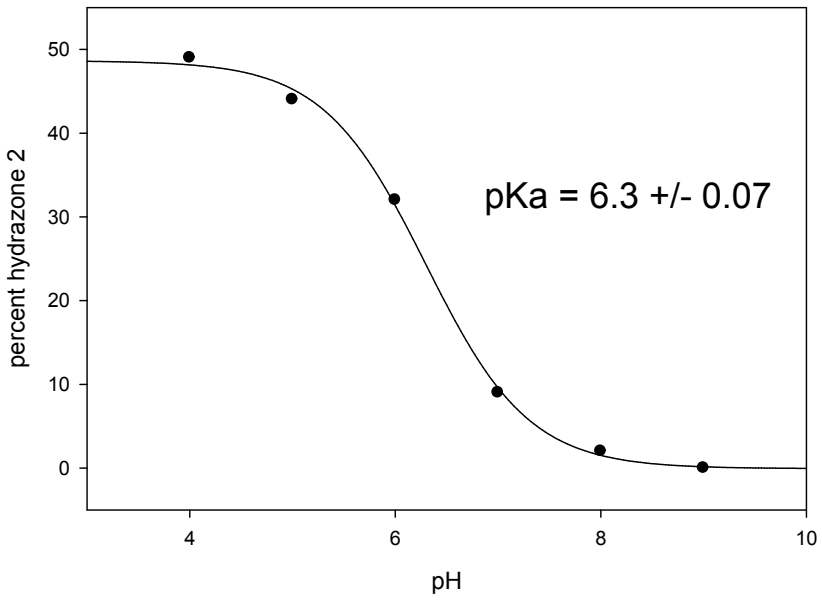



Downfield
4b
pH 9
pH 8
3b(E/Z)
pH 7
pH 6
pH 5
pH 4
8.4
8.2
8.0
7.8
7.6
7.4
[ppm]
Upfield
4b
pH 9
pH 8
pH 7
pH 6
pH 5
pH 4
3b(E/Z)
2b
3.0
2.8
2.6
2.4
2.2
1.20
1.15
1.10
[ppm]

Solution composition as a function of pH

pH	DAB	Hydrazone E	Hydrazone Z	Hydrazide
9	100%	0	0	0
8	96%	1%	2%	1%
7	82%	8%	9%	2%
6	61% (overlapping peaks)		32%	7%
5	11%	35%	44%	10%
4	4%	41%	49%	6%

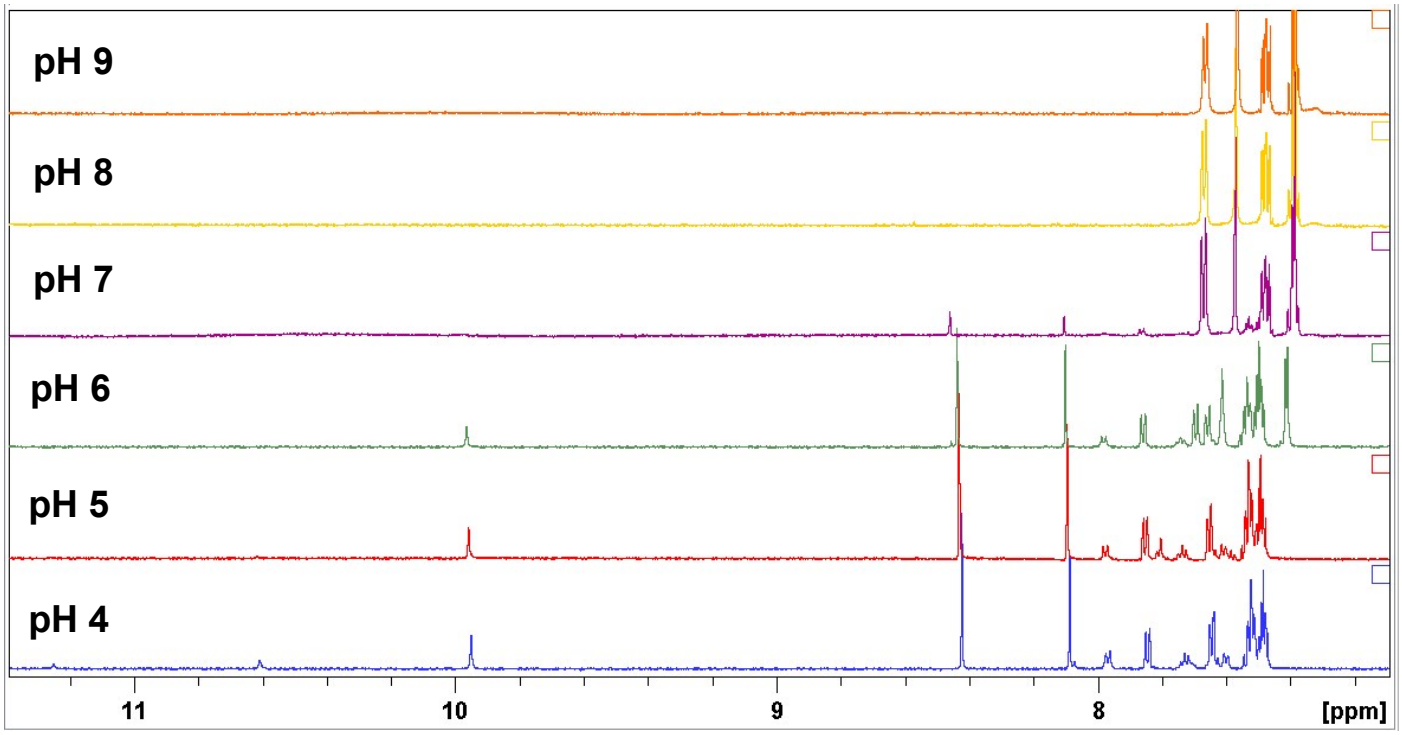
Integrals for the methylene protons were used for this table, which were normalized using TSP-d4 as the internal standard.



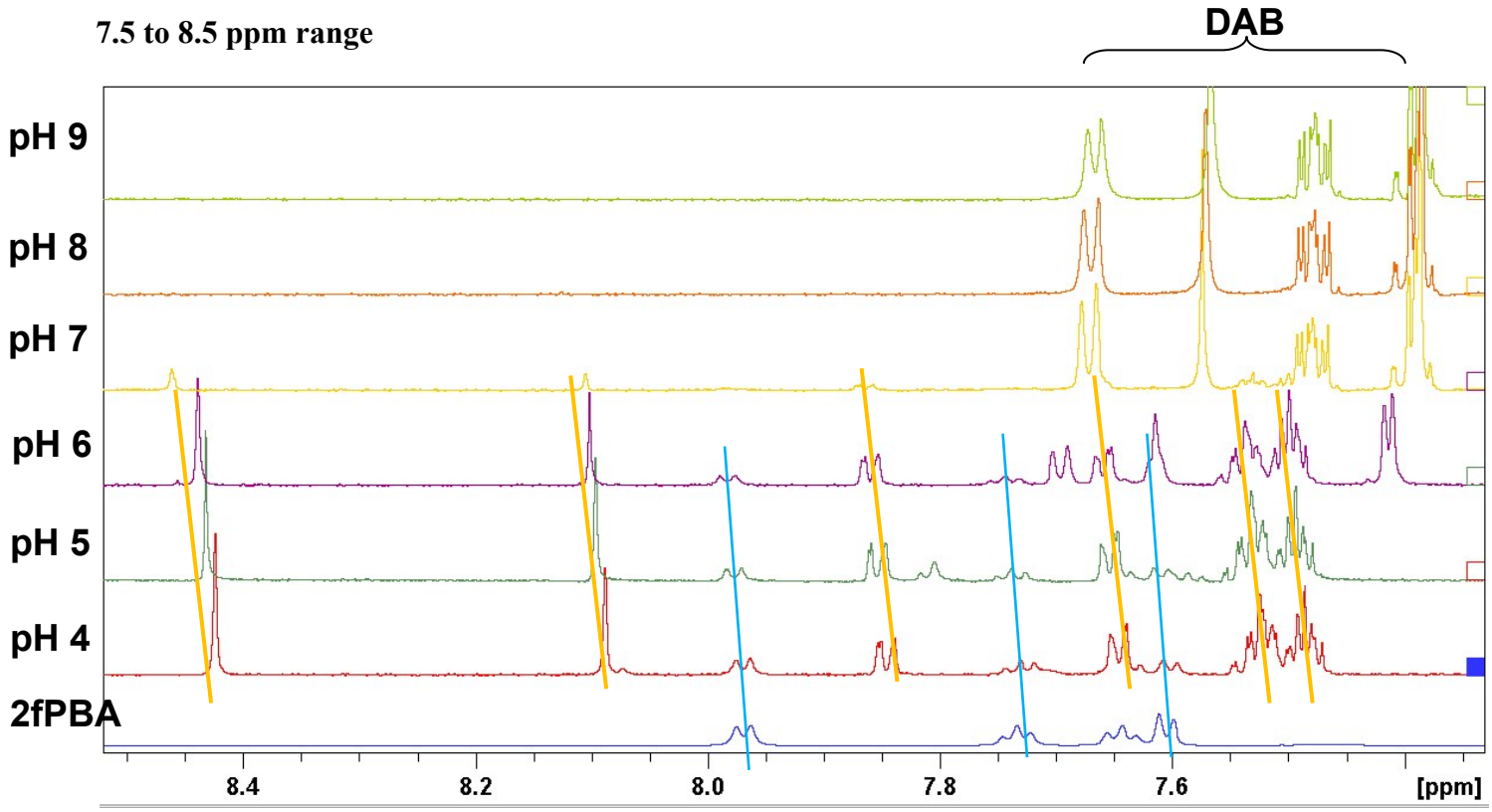
Data for hydrazone Z are plotted as a function of pH. The data were fit to a 4 parameter sigmoidal equation (r^2=0.9986). The pH at the midpoint of the curve is hypothesized to approximate the pKa of the hydrazone.

^{1}H NMR spectra in pH 4 (bottom) to pH 9 (top) aqueous solutions (90% 10 mM phosphate buffer + 10% D_2O, Method B)

Downfield

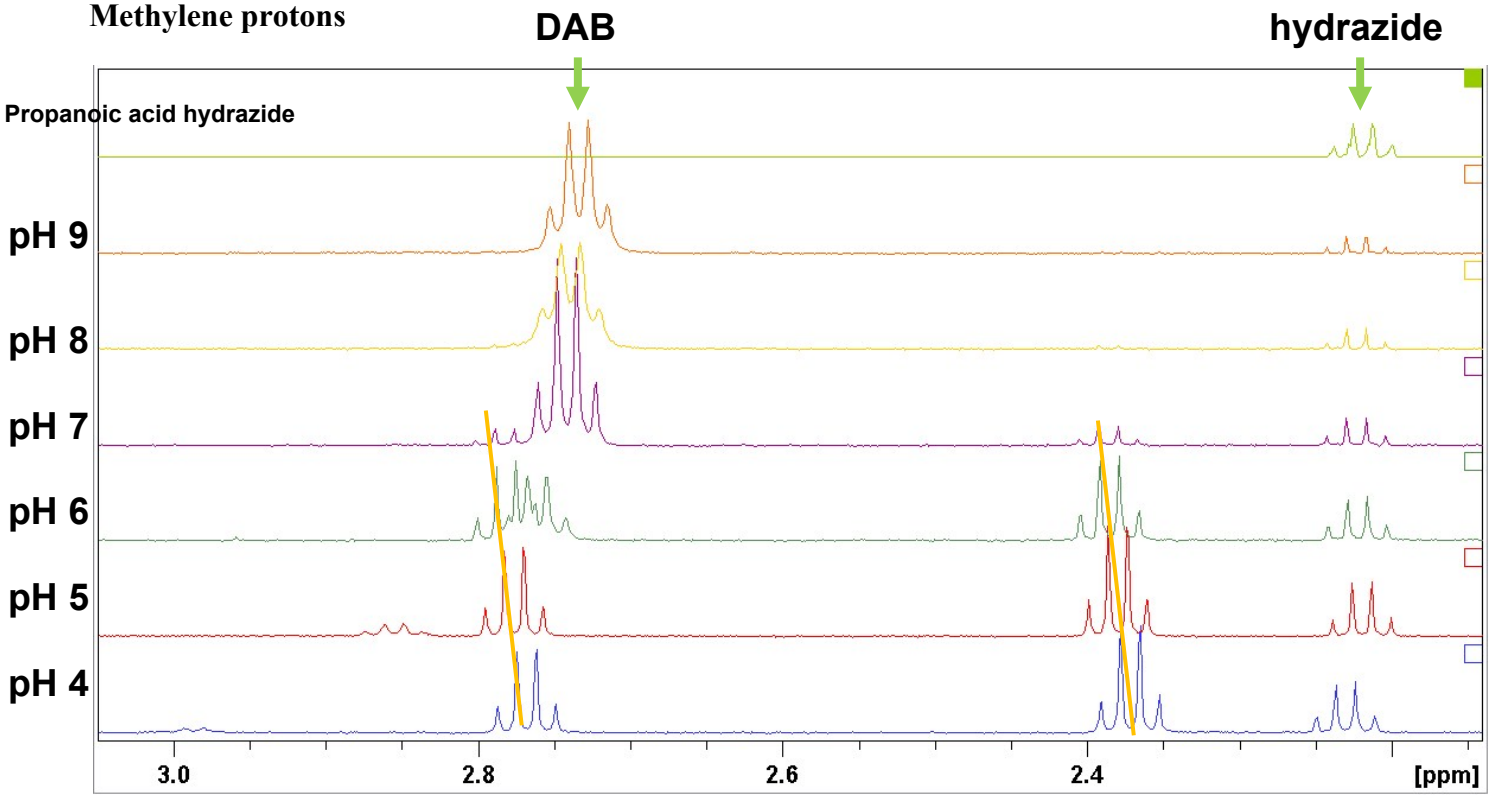


7.5 to 8.5 ppm range



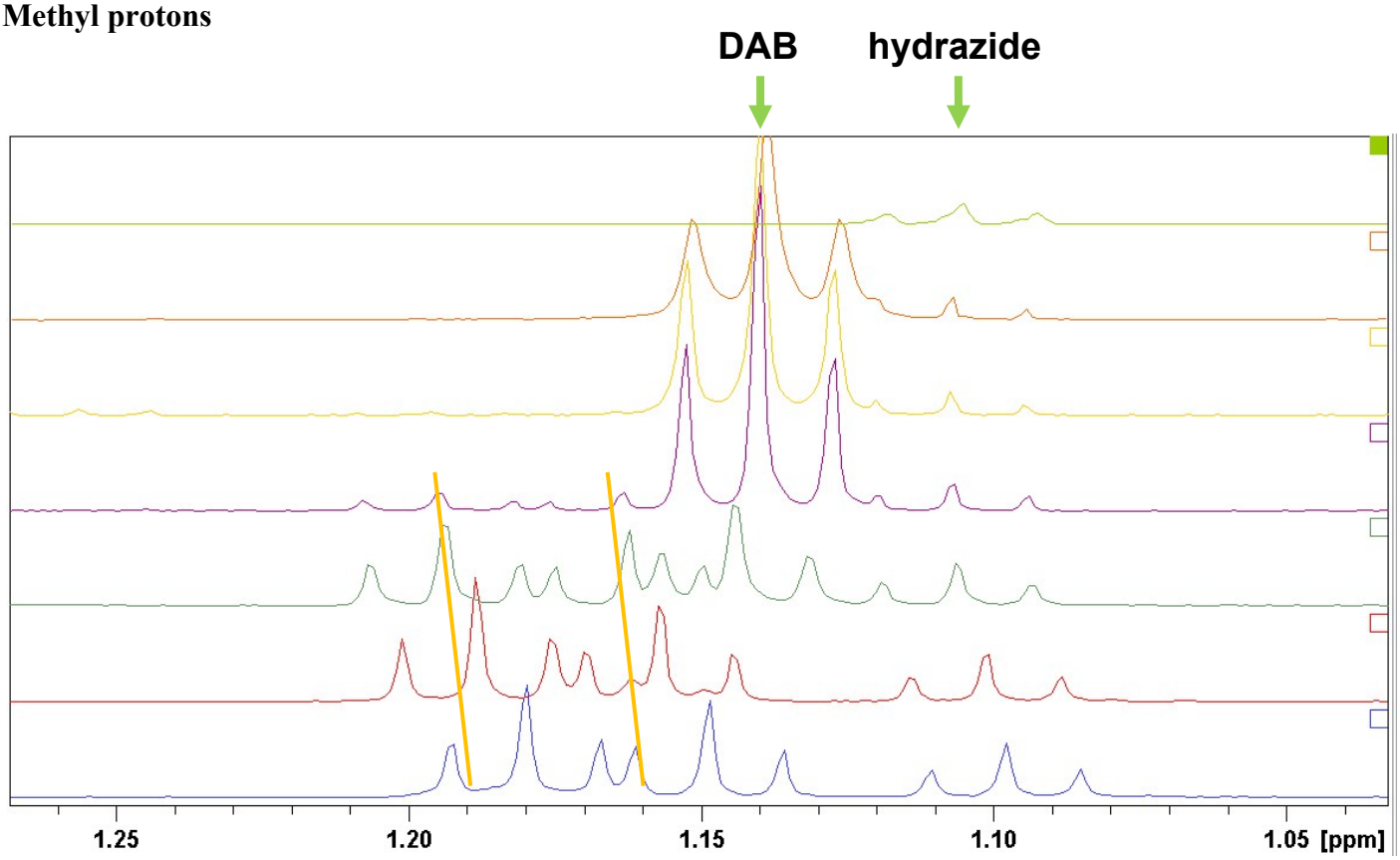
Orange: E/Z hydrazone (shift to downfield from pH 4 to pH 7), blue: 2fPBA (shift to downfield from pH 4 to pH 7),

Methylene protons



Orange: E/Z hydrazone (shift to downfield from pH 4 to pH 7)

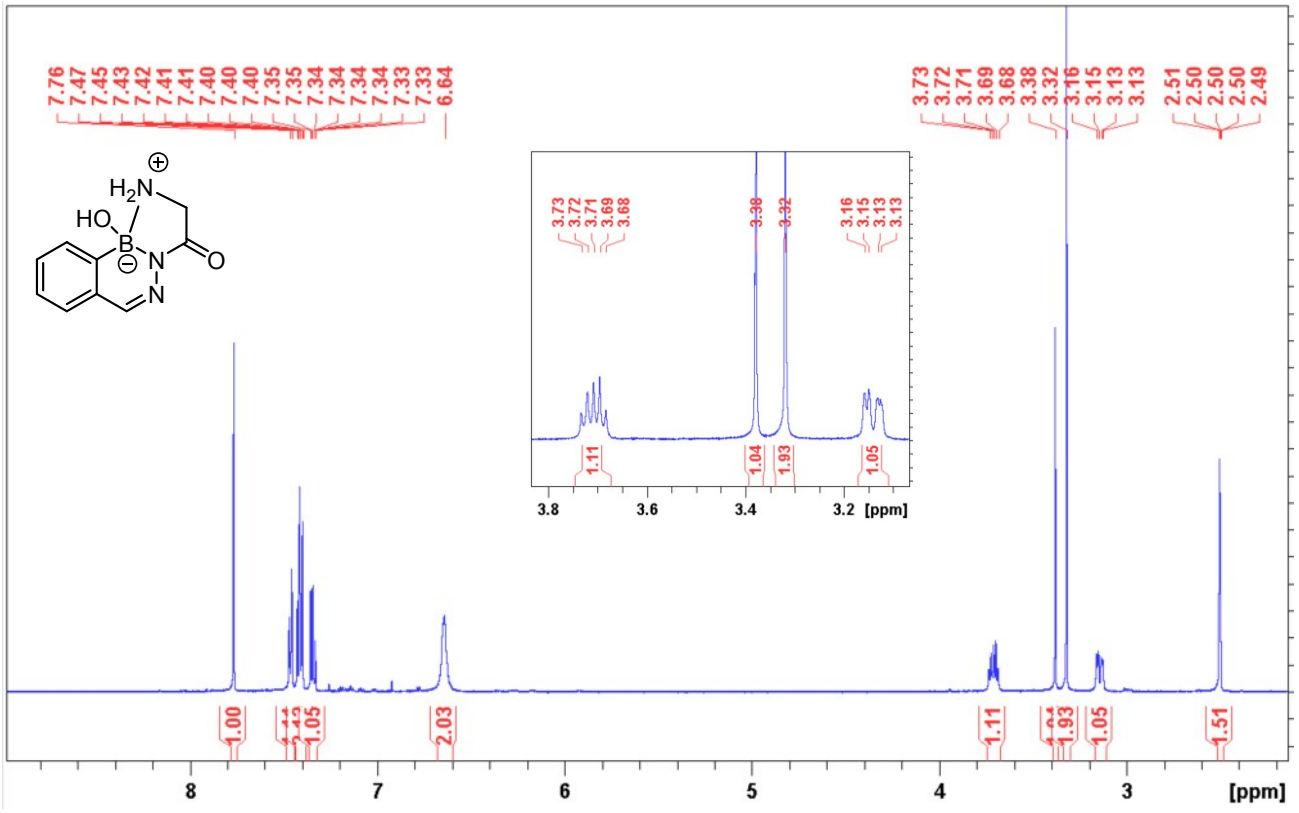
Methyl protons



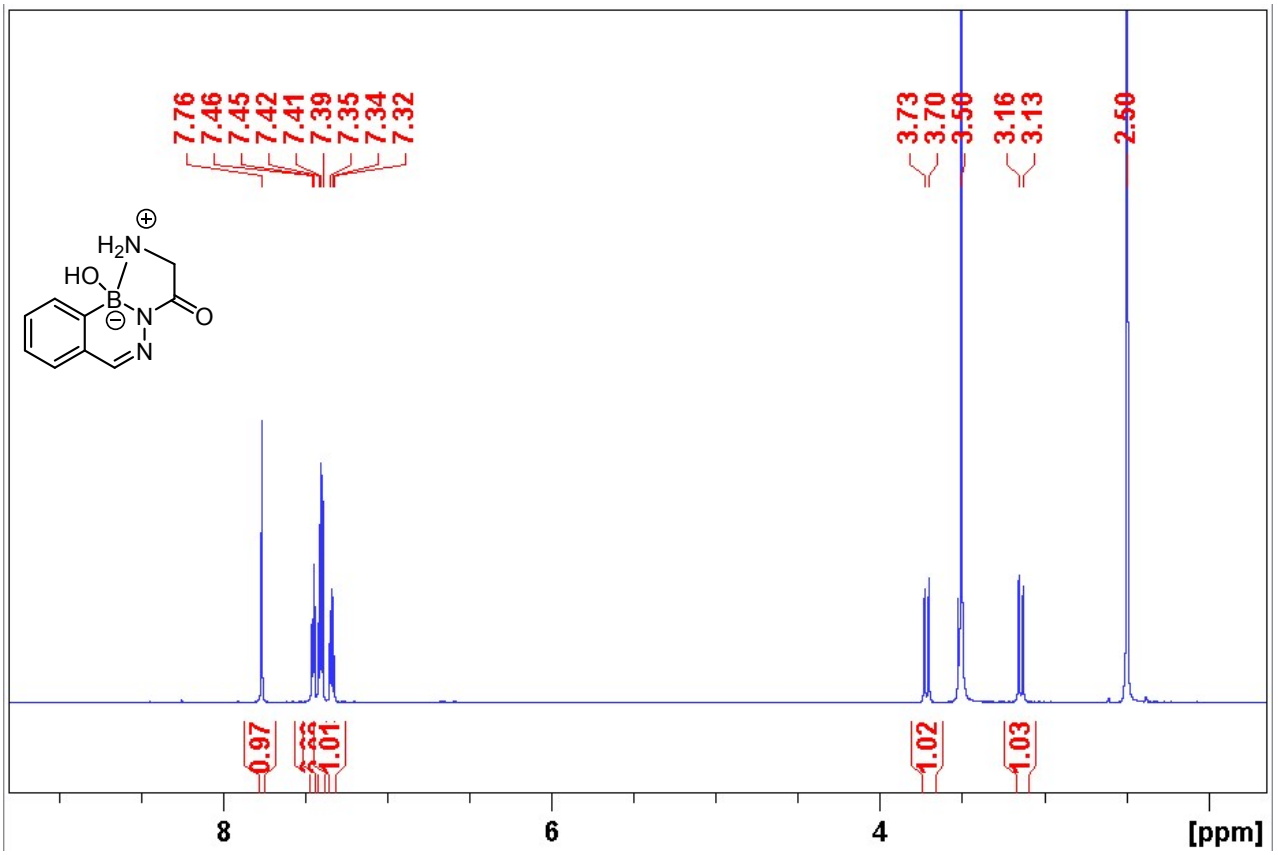
Orange: E/Z hydrazone (shift to downfield from pH 4 to pH 7)

2fPBA-Glycine hydrazide product (7c)

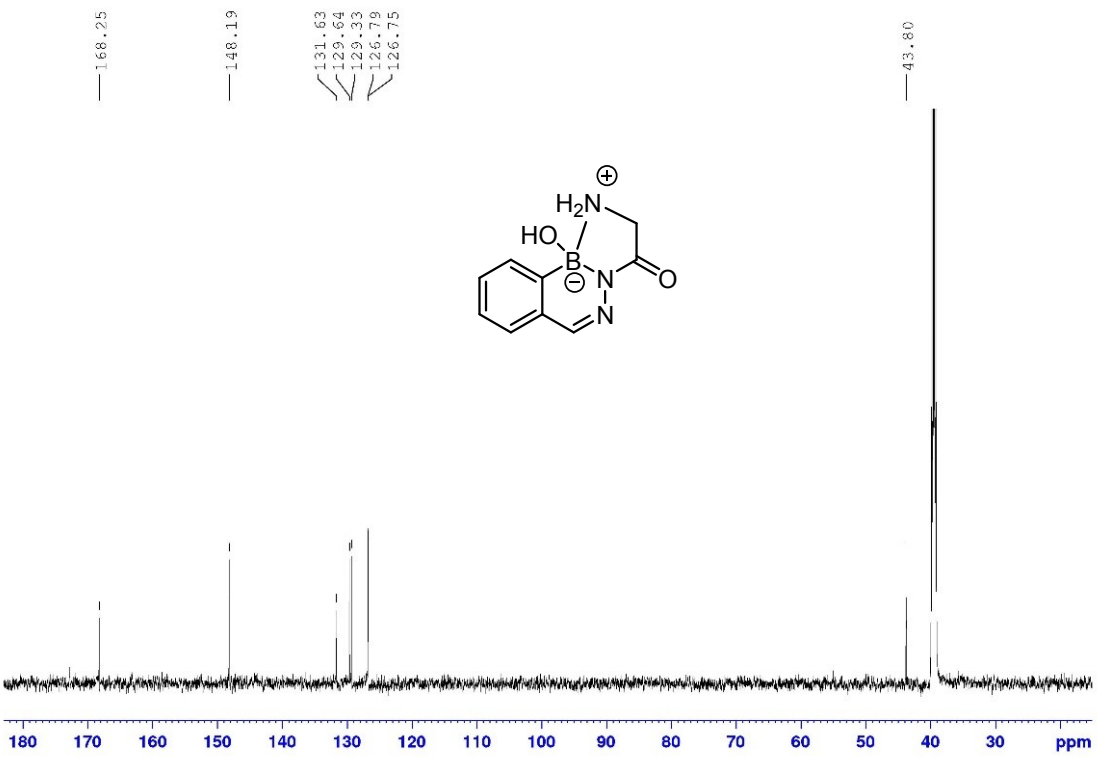
^{1}H NMR (DMSO-d6)



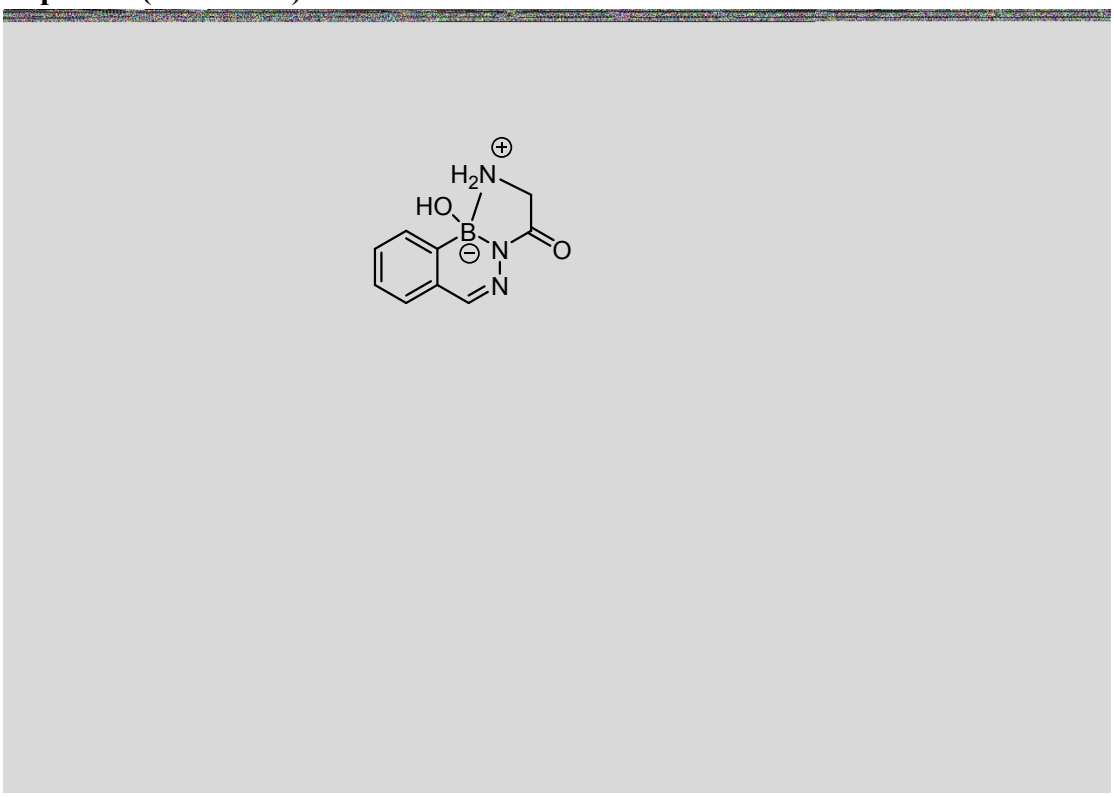
^{1}H NMR (DMSO-d6+1 drop of D_2O)



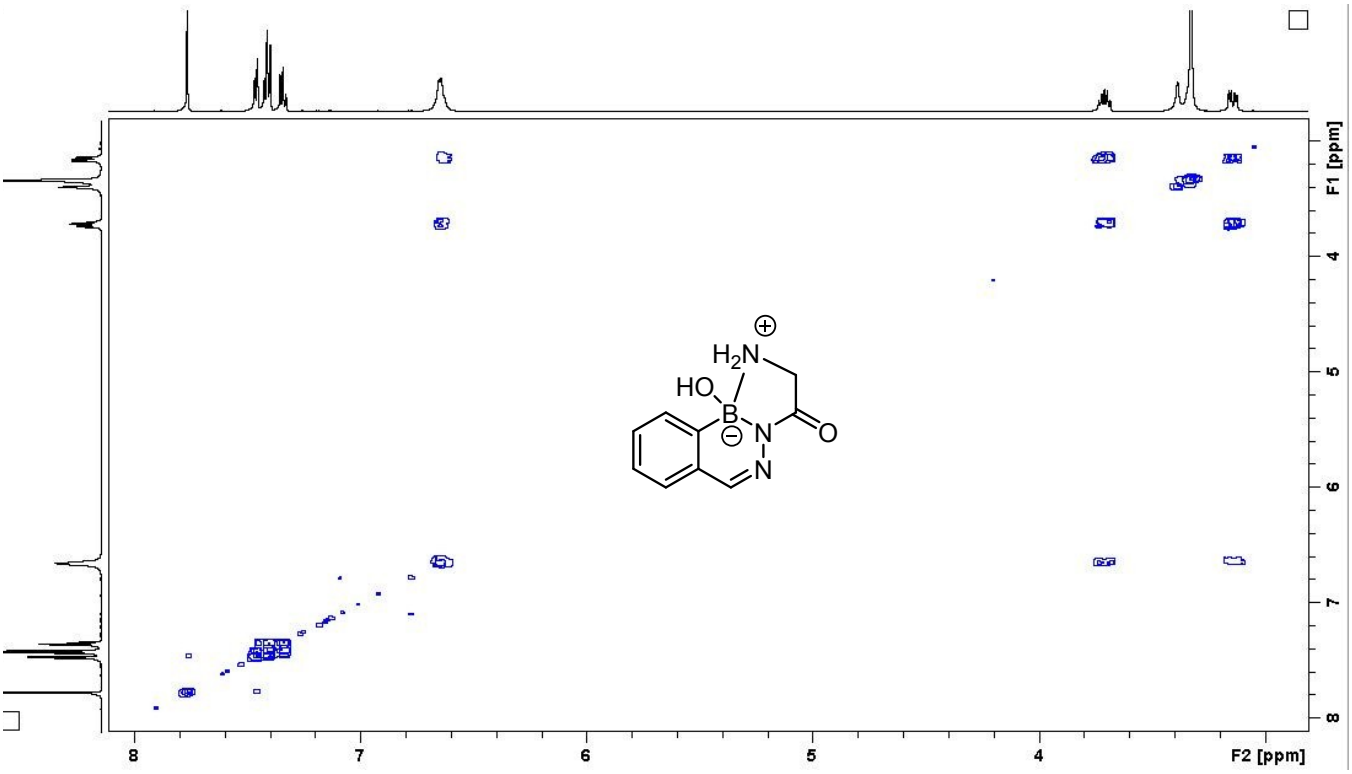
^{13}C NMR (DMSO-d6)



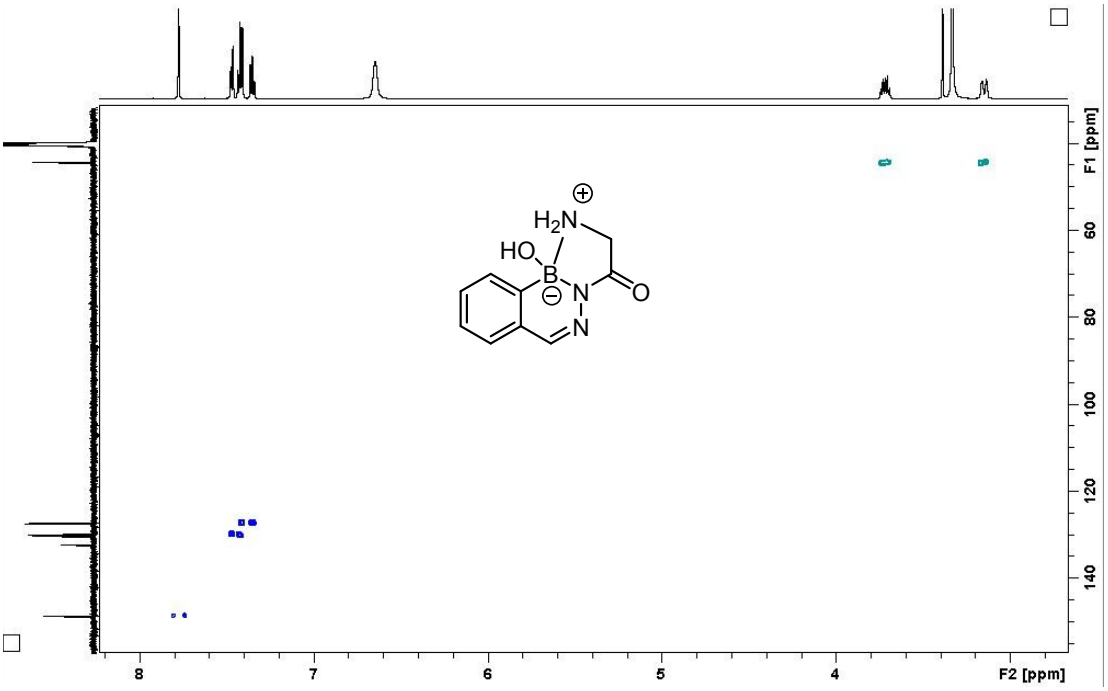
Dept 135 (DMSO-d6)



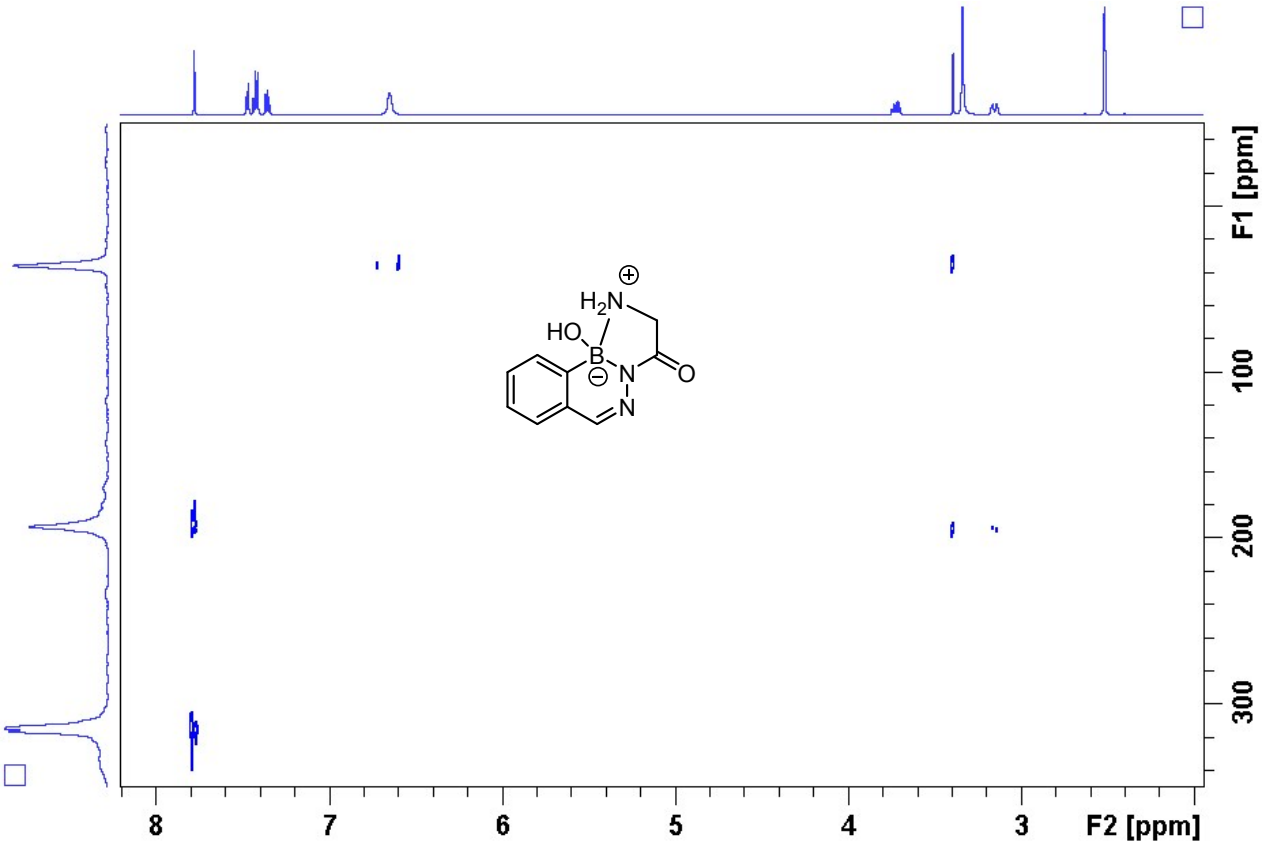
^{1}H-^{1}H COSY (DMSO-d6)



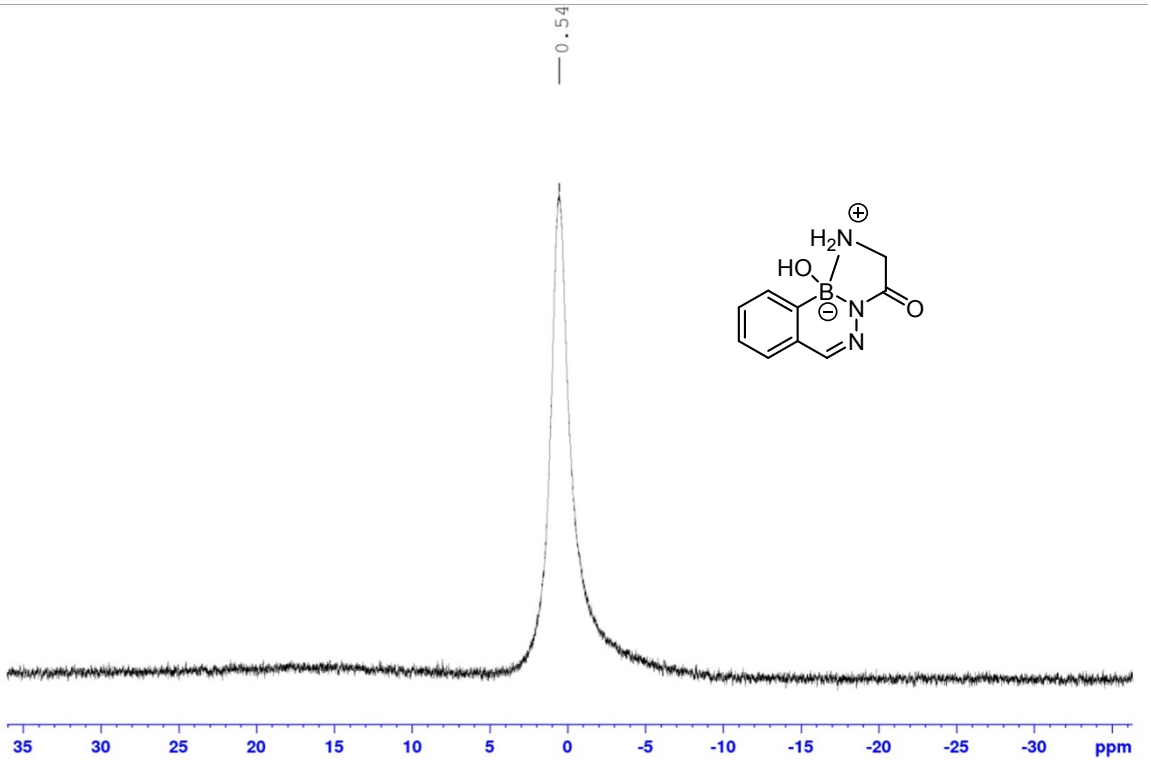
^{1}H-^{13}C HSQC (DMSO-d6)



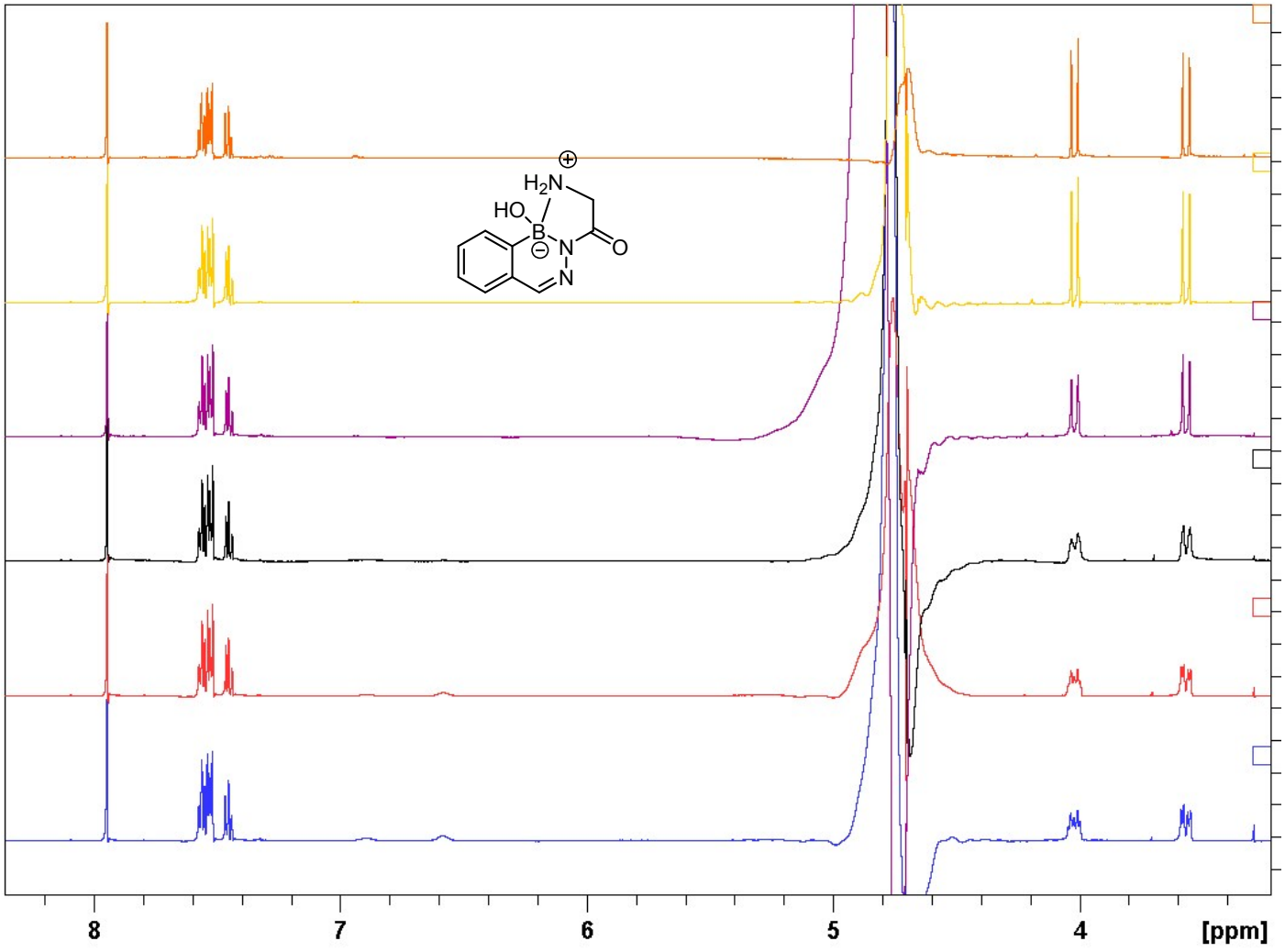
^{1}H-^{15}N HMBCGP (DMSO-d6)



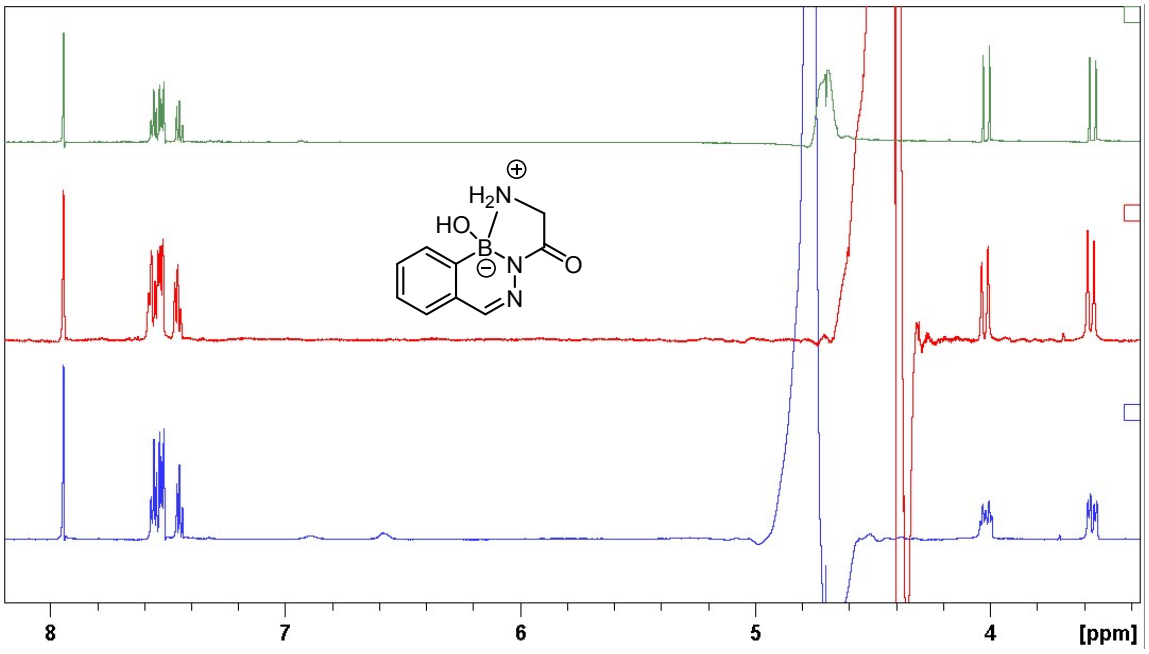
^{11}B NMR (DMSO-d6)



^{1}H NMR spectra in pH 4 (bottom) to pH 9 (top) aqueous solution (90% 10 mM phosphate buffer + 10% D_2O) (Method B)



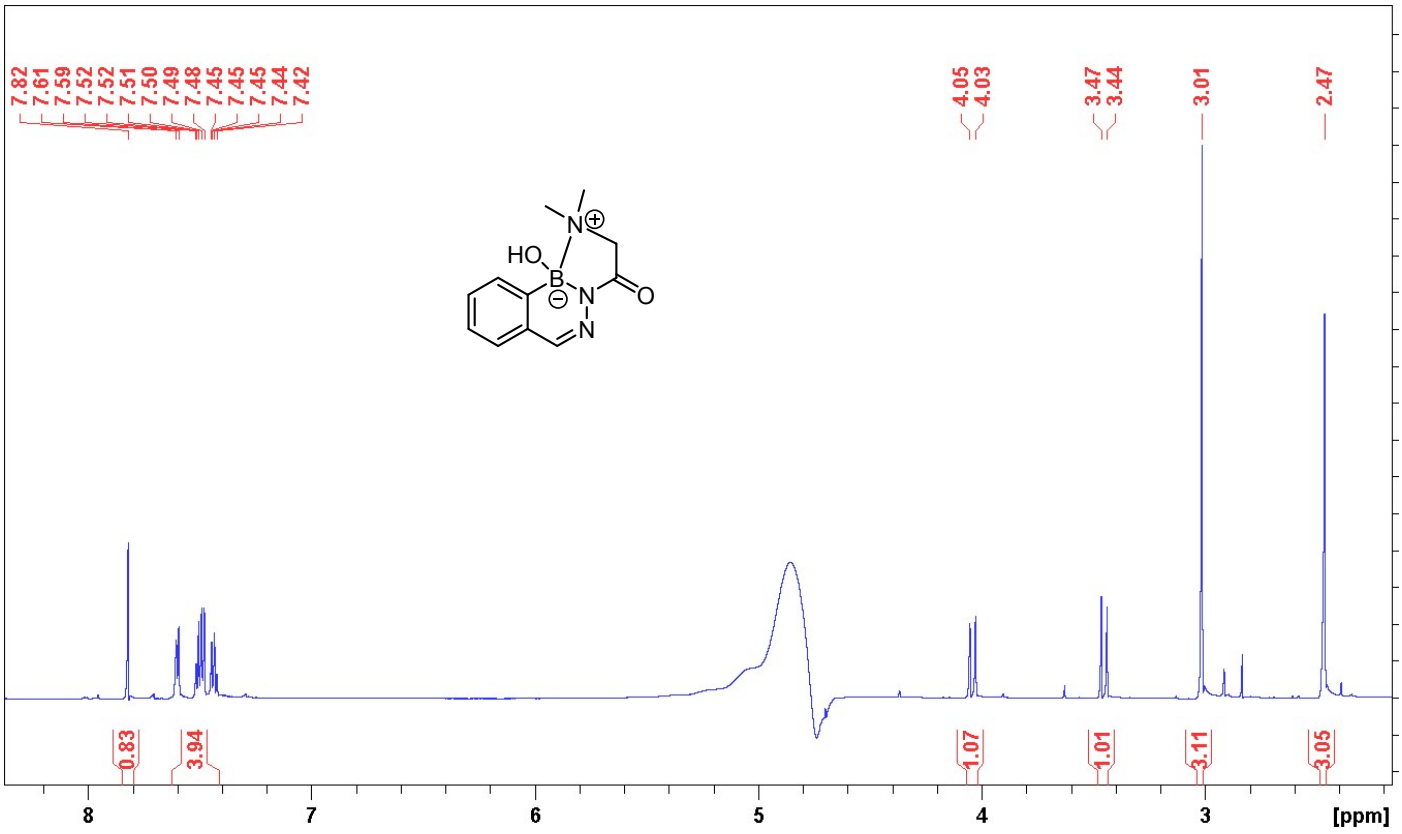
^{1}H NMR spectra in pH 4 at 25° C (298 K) (bottom), pH 4 at 60° C (333 K) (middle) and pH 9, 298 K (top) (90% 10mM phosphate buffer + 10% D_2O)



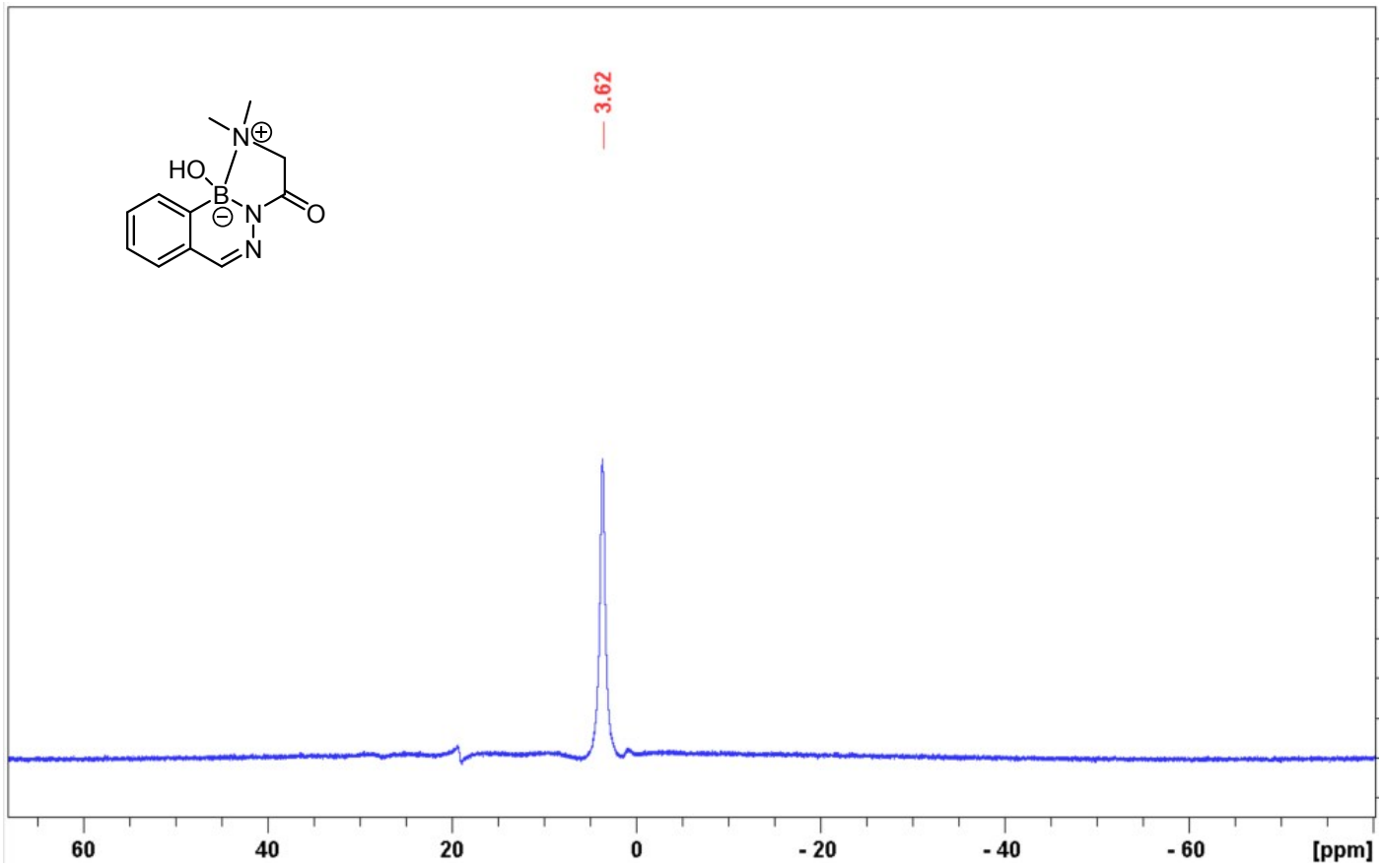
The spectrum at 60° C is aligned with the spectra at 25° C based on the singlet at 7.95.

2fPBA-N, N-Dimethylglycine hydrazide product (7e)

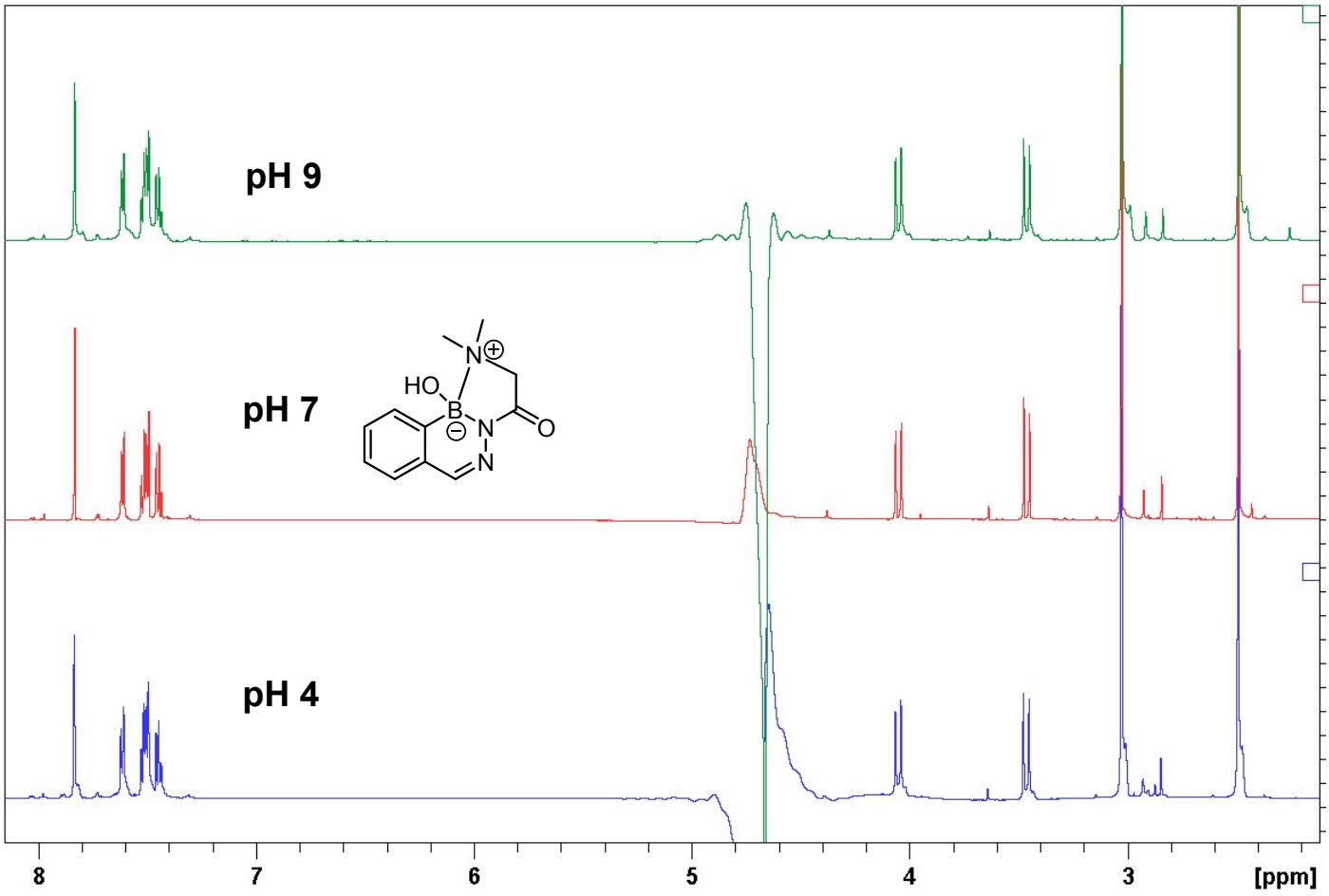
^{1}H NMR (90% 10 mM phosphate buffer + 10% D_2O)



^{11}B NMR (90% 10 mM phosphate buffer + 10% D_2O)

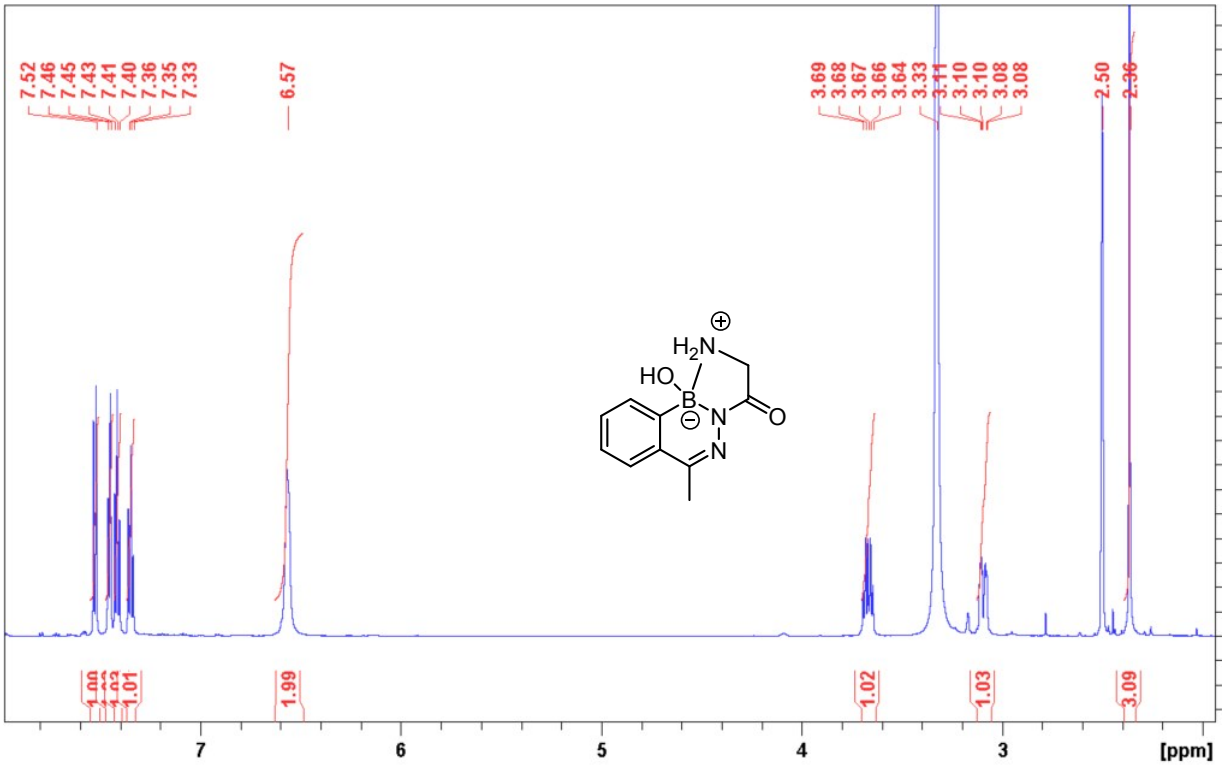


^{1}H NMR spectra in pH 4, 7 and 9 (90% 10 mM phosphate buffer + 10% D_2O) (Method A)

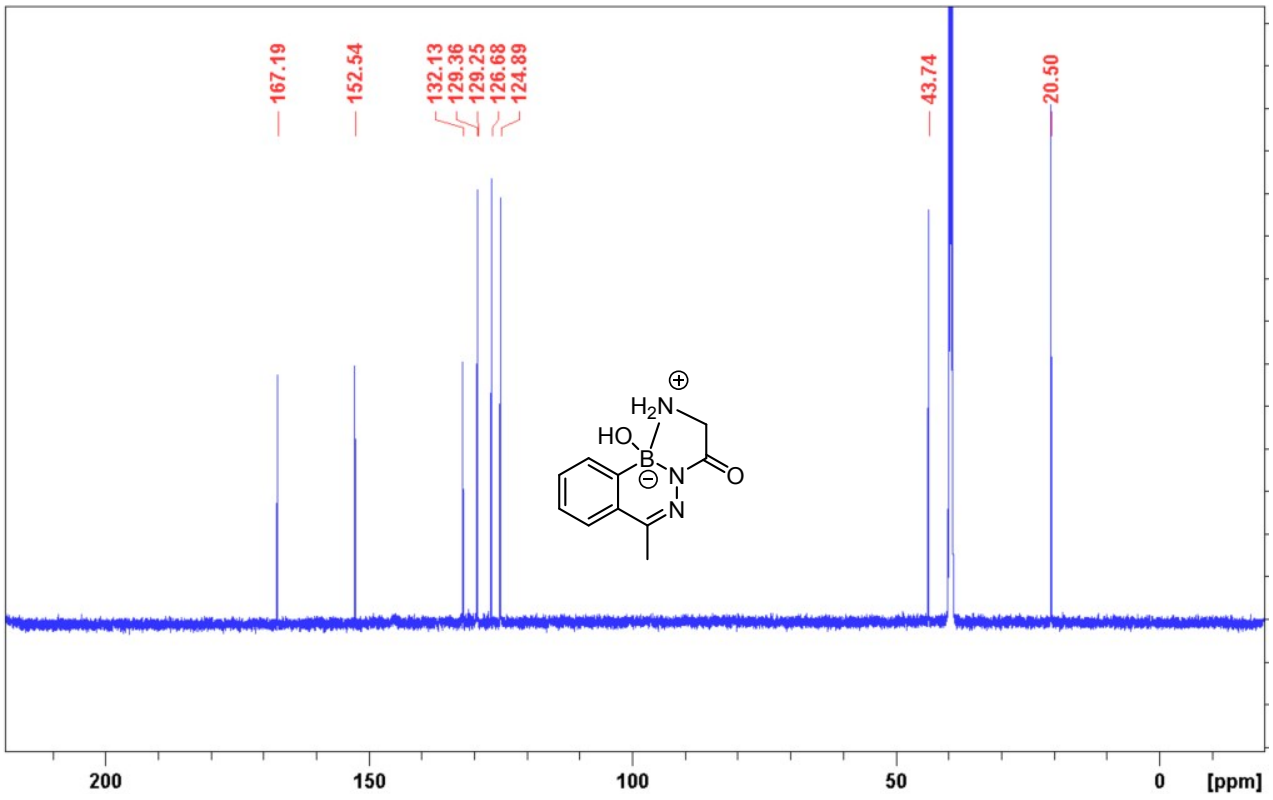


2AcPBA-Glycine hydrazide product (7d)

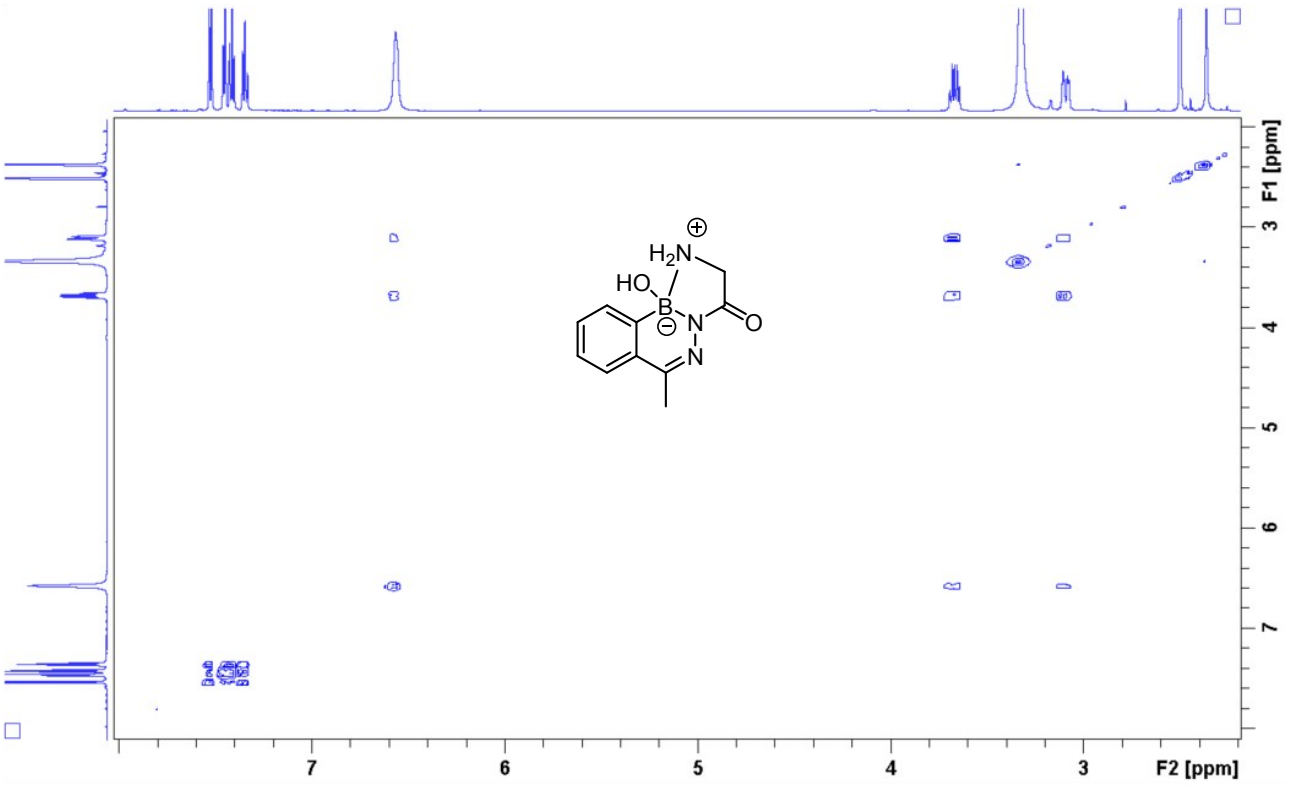
^{1}H NMR (DMSO-d6)



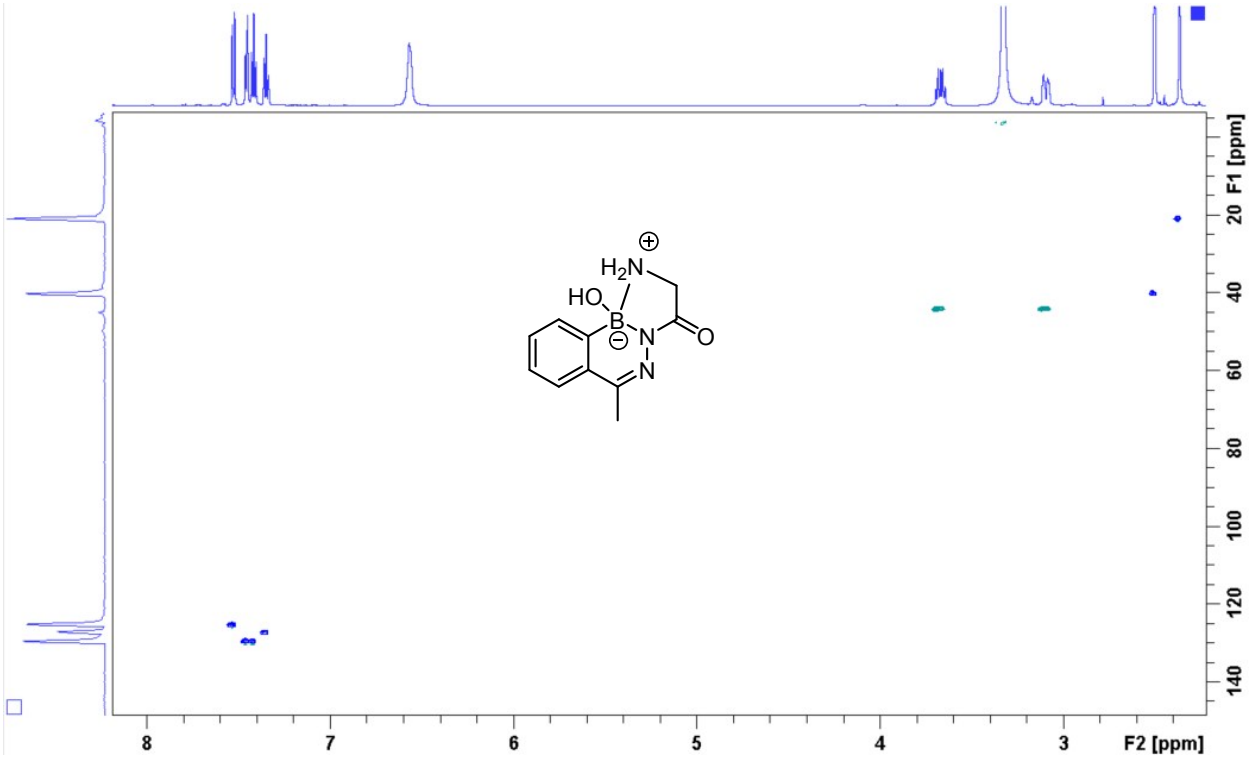
^{13}C NMR (DMSO-d6)



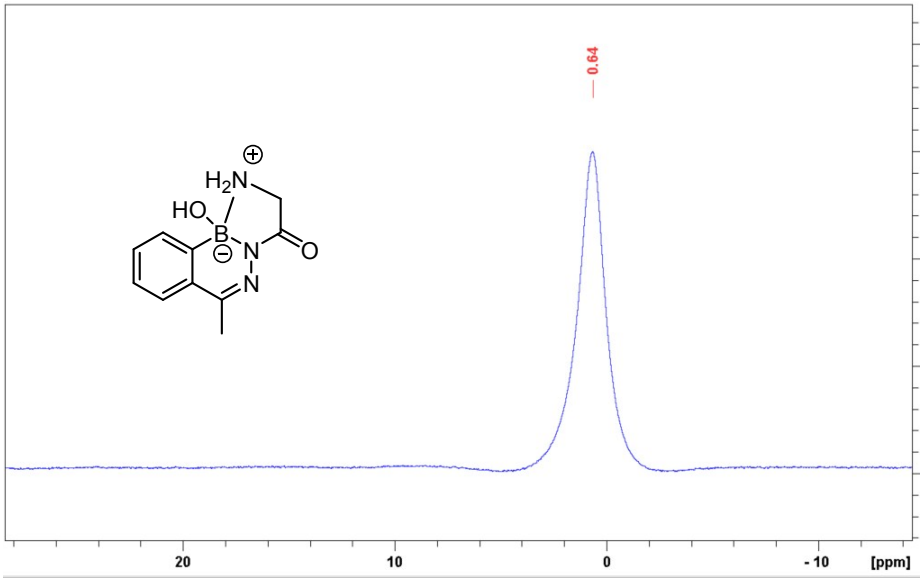
^{1}H-^{1}H COSY (DMSO-d6)



^{1}H-^{13}C HSQC (DMSO-d6)



^{11}B NMR (DMSO-d6)



5. Protein labeling with fPBA-fluorophore

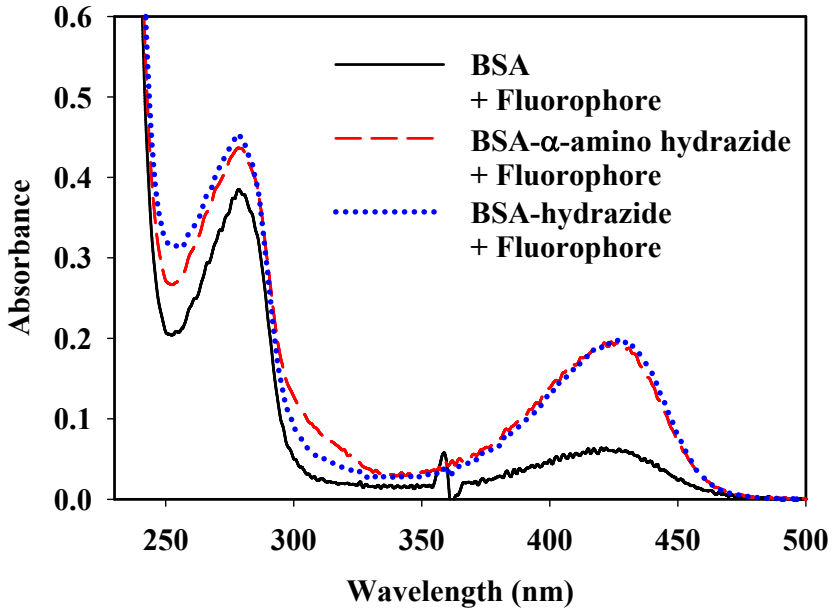
Preparation of BSA-hydrazide and BSA-α-amino hydrazide

BSA-hydrazide was prepared by allowing BSA (final concentration: 60 μM) to react with 1.5 equivalent of ε-maleimidocaproic acid hydrazide trifluoroacetic acid (Chem-Impex International, Inc., 23096) in PB7 (10 mM sodium phosphate buffer, pH 7.0) containing 1 mM EDTA, 0.1 mM TCEP, and 2% DMSO (v/v) for ~1 h at RT. The reaction mixture was then subjected to rapid gel filtration chromatography using Sephadex G-25 equilibrated in PB7 to remove excess crosslinker. Aliquots of the functionalized protein were snap-frozen and stored at -50 °C until use.

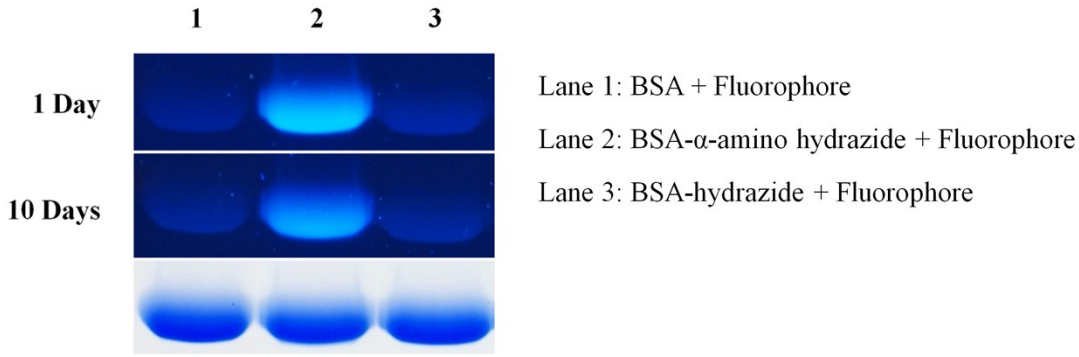
BSA-α-amino hydrazide was prepared by first allowing BSA (final concentration: 80 μM) to react with 1.5 equivalent of 1,11-bismaleimido-triethyleneglycol (ThermoFisher Scientific, 22337) in PB7 containing 1 mM EDTA and 2% DMSO (v/v) for 1 h at RT. The reaction mixture was then subjected to rapid gel filtration chromatography using Sephadex G-25 in PB7 to remove excess crosslinker. The subsequent maleimide-functionalized BSA was then allowed to react with cysteine hydrazide (final concentration: 240 μM) in PB7 containing 0.2 mM TCEP for 1 h at RT. The reaction mixture was again subjected to rapid gel filtration using Sephadex G-25 in PB7 to remove unreacted cysteine hydrazide. Aliquots of the functionalized protein were snap-frozen and stored at -50 °C until use.

Protein labeling

~25 μM BSA-hydrazide or BSA-α-amino hydrazide was allowed to react with ~50 μM coumarin-2fPBA[2] in PB7 containing 1% DMSO (v/v) for 1 h at RT. BSA, treated the same way, was included as control. Samples were then subjected to rapid gel filtration chromatography using Sephadex G-25 in PB7 to remove excess fluorophore. Absorption spectra were subsequently taken using a Hewlett-Packard 8453 spectrophotometer. The remaining protein samples were subjected to two additional rapid gel filtrations followed by SDS-PAGE analysis. The gel was kept in destaining solution (50/40/10 v/v/v methanol/water/acetic acid) and was imaged under a long wavelength UV lamp to observe fluorescence labeling. The same gel was stained with Coomassie to check for protein loading and was imaged under white light.



Absorption spectra of BSA, BSA-α-amino hydrazide, or BSA-hydrazide reacted with a coumarin-labeled fPBA fluorophore. Spectra were taken after rapid gel filtration of the reaction mixtures. The lower energy peak (λ_{max} = ~425 nm) represents absorption from the conjugated coumarin. Note the equal labeling of BSA-α-amino hydrazide and BSA-hydrazide prior to SDS-PAGE.



Stability of the conjugate over time. The SDS-PAGE gel was stored in destaining solution (50/40/10 v/v/v methanol/water/acetic acid) and was imaged after 1 day (top panel) and again after 10 days (middle panel) under a long wavelength UV lamp. Fluorescence images were taken under the same exposure time. Bottom panel: Coomassie stained gel.

References

(1) Jansen, M.; Rabe, H.; Strehle, A.; Dieler, S.; Debus, F.; Dannhardt, G.; Akabas, M. H.; Lüddens, H. Synthesis of GABAA Receptor Agonists and Evaluation of their α-Subunit Selectivity and Orientation in the GABA Binding Site. *J. Med. Chem.* **2008**, *51*, 4430-4448.

(2) Dilek, O.; Lei, Z.; Mukherjee, K.; Bane, S. Rapid formation of a stable boron-nitrogen heterocycle in dilute, neutral aqueous solution for bioorthogonal coupling reactions. *Chem. Commun.* **2015**, *51*, 16992-16995.