Supplementary Information

Rapid formation of a stable boron-nitrogen heterocycle in dilute, neutral aqueous solution for bioorthogonal coupling reactions

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Contents

1. General

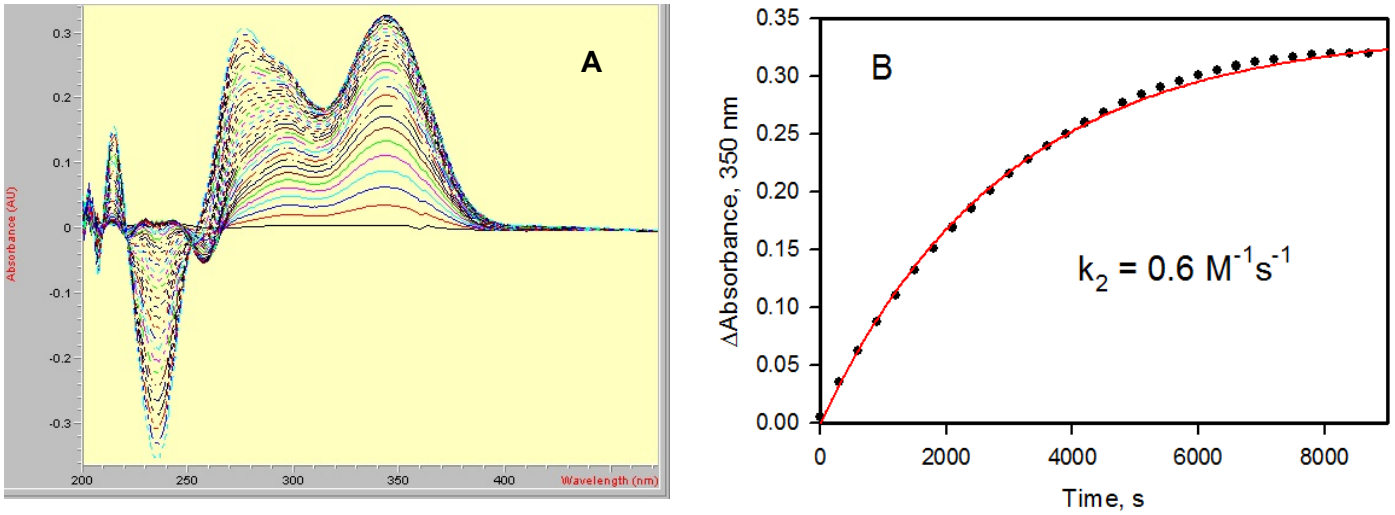
fPBA and Sal were obtained from Aldrich or Acros and used as received. BSA (Bovine serum albumin-fraction V) was purchased from Rockland. All NMR spectra were collected and processed on a Bruker Avance III 600 NMR Spectrometer at 298 K unless otherwise noted. ^{1}H and ^{13}C NMR spectra were referenced to DMSO (2.50 and 39.51 ppm, respectively). ^{11}B was referenced to $BF_3.Et_2O$ (0.00 ppm) by the instrument's software; accuracy of the chemical shift determination was confirmed by measuring a spectrum of **5** with boric acid in D_2O as an internal standard. 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. Multiplicities are indicted by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Chemical shifts are reported in parts per million (ppm). Accurate mass data was obtained using a Waters (Waltham, MA USA) Time-of-Flight mass spectrometer model LCT Premier. The instrument was operated in positive ion W mode with a mass resolution of 10K. Ions were generated in ESI mode using a Z-spray ion source. Samples were flow-injected into a solvent stream consisting of 1:1 water:acetonitrile with 0.2% formic acid. Accurate masses were obtained using internal mass standards and data was processed with MassLynx 4.1 software.

2. Kinetics

Kinetics were measured at 25 °C in 0.1 M sodium phosphate (pH 7.0) using a Hewlett-Packard 8453 diode array absorption spectrophotometer or a Hewlett-Packard 8452A diode array absorption spectrophotometer equiped with Olis GlobalWorks software. A dual chambered cuvette was used to obtain absorption difference spectra as a function of time. Briefly, equal volumes of each solution were placed into the chambers of the cuvette. The instrument was zeroed and collection of kinetics commenced. After checking the baseline the cuvette was removed, rapidly mixed by inversion and replaced in the instrument. Spectra were collected at the indicated time intervals until no additional change in the spectrum was observed. For the reaction of salicylaldehyde (Sal) with *p*-hydrazinylbenzoic acid (HBA), the change in absorption at a particular wavelength as a function of time was extracted from these data and plotted using Sigmaplot. The data of absorbance vs. time for Sal –HBA were fit to a single exponential and an apparent second order rate constant was calculated. The data of absorbance spectra vs. time for fPBA-HBA were collected and analysed using Olis GlobalWorks software.

Figure S1:

A. Absorption difference spectra of 300 μM Sal + 30 μM HBA in 0.1M sodium phosphate buffer, pH 7.0, 25 °C, as a function of time after mixing. Data points were collected at 5 minute intervals. B. Change in absorption at a single wavelength (350 nm) plotted as a function of time (black points). Red line: fit of data to the equation $y = y0 + Ae^{-bx}$. Inset: apparent second order rate constant.



Kinetic data for fPBA-HBA were collected in a similar manner, except that the concentrations of the reagents were lower as noted in the text and data points were collected every 0.5 to 1.2 seconds.

Figure S2:

Absorption spectra of 100 µM fPBA, 100 µM HBA and the product of the reaction of the two components (100 µM final concentration).

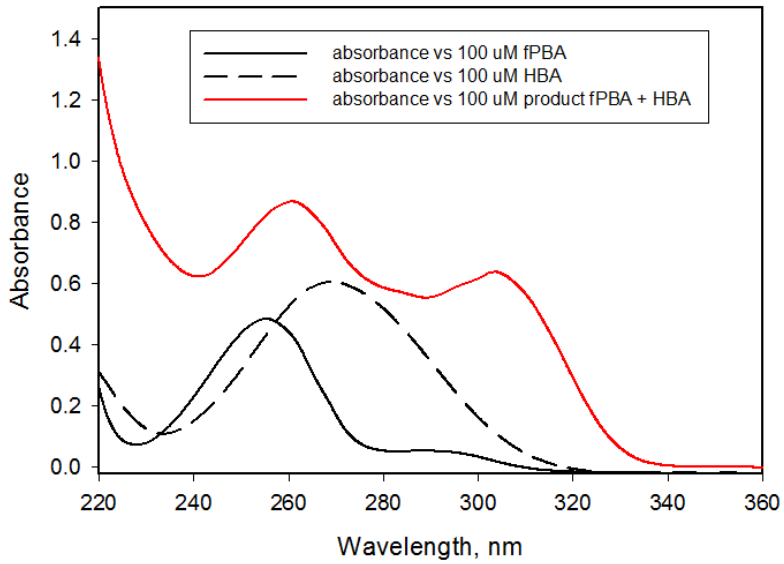


Figure S3:

A. Kinetic data for fPBA-HBA under second order conditions as a function of concentration. fPBA was allowed to react with HBA (25, 50, 75 and 100 µM final concentration) at 25 °C in pH 7.0 buffer. The progress of the reaction was monitored using absorption difference spectroscopy. The change in absorption at 350 nm is plotted as a function of time for each sample. Yellow: 100 µM, green: 75 µM, red: 50 µM, black: 25 µM. B. The data from panel A are normalized to the highest absorption value for the 100 µM trace to illustrate the lack of concentration dependence of the decrease at 350 nm. Note that the initial curvature of the data varies with concentration, indicative of higher order kinetics for the first step of the process.

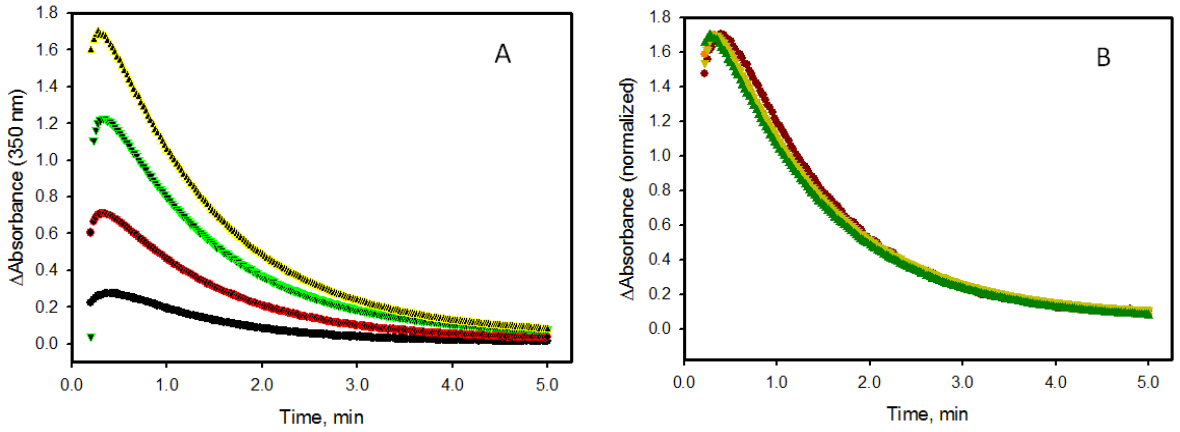
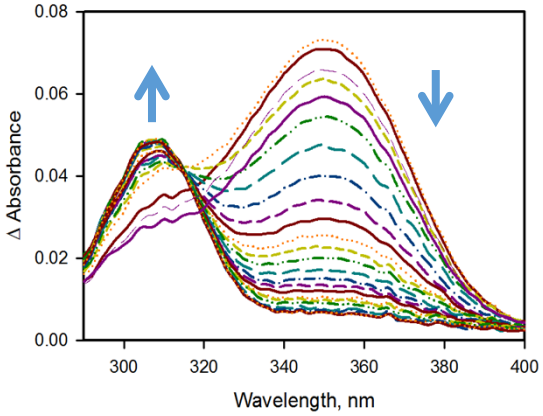


Figure S4:
Absorption difference spectrum of 100 μM HBA + 5 μM fPBA as a function of time in 0.1 M sodium phosphate buffer, pH 7.0, 25 °C. Spectra were collected at 1 s interval; 9 second intervals shown for clarity.

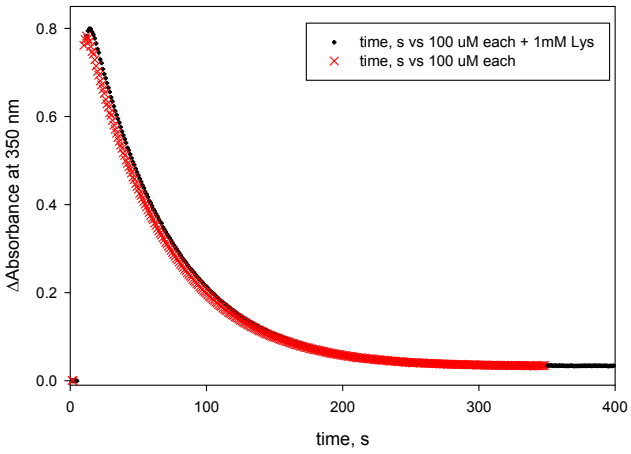


3. Test of stability

Equal volumes of solutions containing 5 mM fPBA amd 5 mM HBA in 0.1 M sodium phosphate buffer, pH 7.0, containing 10% D_2O were mixed and added to the NMR tube. An internal standard (n-butanol in 90% buffer and 10% D_2O) in a capillary tube was inserted into the tube. The proton NMR spectrum of the sample was measured. The sample was allowed to remain in the NMR tube on a lab bench and the spectrum was collected periodically over a period one month. The initial spectrum and the spectrum after one month is illustrated (page S18).

4. Tests of orthogonality

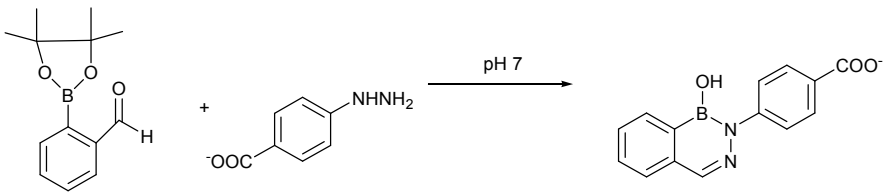
The effect of other functional groups on the kinetics of fPBA + HBA were assessed by making stock solutions of fPBA in pH 7 buffer containing 2x the desired concentration of the additive. [Final concentrations: Lysine, 1 mM; glutathione, 5 mM; glucose, 10 mM] Kinetics of DAB formation was observed by absorption difference spectroscopy as described above. Subsequently, the same stock solutions of fPBA (without additive) were used with same solution of HBA and the reaction was again observed by absorption difference spectroscopy. A typical result is shown below:

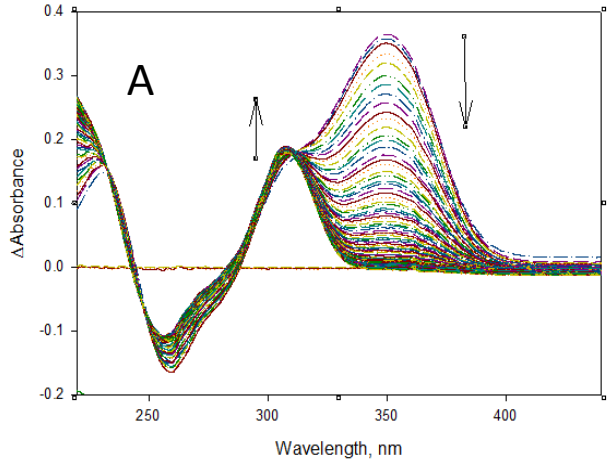


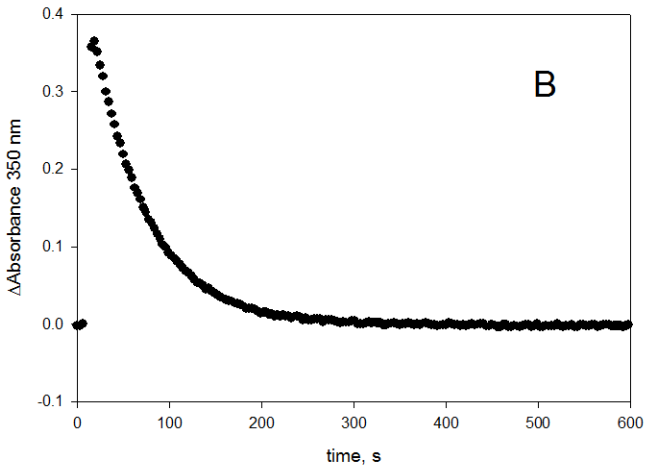
5. Reaction of pinacol ester of fPBA with HBA

Figure S5:

A. Absorption difference spectra of 50 µM pinacol-2fPBA and 50 µM HBA as a function of time after mixing. Data points were collected at 1.5 sec intervals. B. Change in absorption at a single wavelength (350 nm) plotted as a function of time.







6. Synthesis

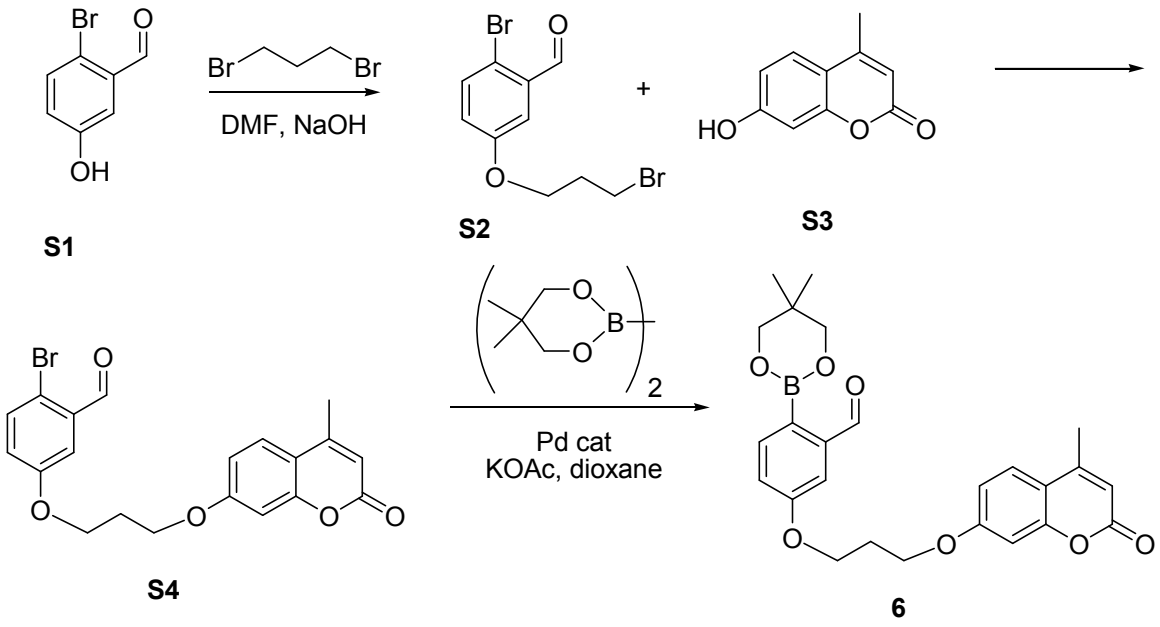
A. fPBA-HBA product (1,2-Dihydro-2-(4'-carboxyphenyl)-1-hydroxy-2,3,1-benzodiazaborine (5))

A round bottom flask was charged with 2fPBA (0.213 mmol), distilled water (2 mL), and a HBA (0.320 mmol). A white solid was formed immediately in the solution. The reaction mixture was stirred at room temperature for 10 min. The solid was collected by centrifugation and was washed once with distilled water. The resulting product was dried by a stream of nitrogen for 18-24 hours.

No attempt was made to optimize the yield of this preparation. The reaction was confirmed to form a single product in aqueous solution by mixing the individual components in a 1:1 molar ratio and obtaining NMR spectra immediately after mixing. This process was performed in both water and 0.1 M phosphate buffer, pH 7.0, with identical results.

1,2-Dihydro-2-(4'-carboxyphenyl)-1-hydroxy-2,3,1-benzodiazaborine (**5**): Yield 41%. **^{1}H NMR:** δ 12.78 (1H, s), 9.27 (1H, s), 8.41 (1H, d, *J* = 7.61 Hz), 8.25 (1H, s), 7.98 (2H, d, *J* = 8.59), 7.83 (1H, s, *J* = 7.73 Hz), 7.77 (3H, m), 7.68 (1H, t, *J* = 7.54 Hz). **^{13}C NMR**: δ 167.06, 150.20, 139.95, 134.80, 131.78, 131.69, 129.52, 127.13, 126.63, 123.64. HRMS (ESI-TOF) m/z: Calcd for C14H11BN2O3 266.0977; Found 266.0979.

B. Coumarin-fPBA product (2-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)-5-(3-(4-methyl-2-oxo-2H-chromen-7-yloxy)propoxy)benzaldehyde (6))



The fluorophore-fPBA conjugate was prepared following the scheme above.

2-Bromo-5-hydoxybenzaldehyde (S1) : To a suspension of 3-hydroxybenzaldehyde (8 g, 65.6 mmol) in methylene chloride (100 mL) in an ice bath was added bromine (1.5eq, 5 ml) slowly over one hour. The solution was allowed to rise to room temperature, and then the mixture was stirred for 4 hours. The resulting solid was collected by filtration and was recrystallized from hexanes/diethyl ether Yellow needle shaped crystals (6 g, 75%) were formed. **^{1}H NMR** ($CDCl_3$): δ 10.3 (1H, s), 7.515 (1H, d, *J* = 8.0 Hz), 7.41 (1H, d, *J* = 3.6 Hz), 7.00 (1H, dd, *J* = 8.99 Hz, *J* = 3.14 Hz). **^{13}C NMR** ($CDCl_3$): δ 192.33, 155.74, 135.14, 134.24, 123.55, 117.98, 115.94.

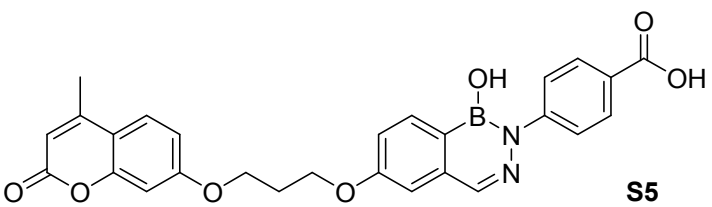
2-Bromo-5-(3-bromopropoxy)benzaldehyde (S2): To a solution of **S1** (0.5 g, 2.5 mmol) in DMF (10 mL) was added NaOH (1 eq, 0.1 g, 2.5 mmol) at room temperature. The mixture was stirred until all the NaOH was dissolved (1 hour). This solution was added slowly into 1,3-dibromopropane (2.8 eq, 0.7 mL, 6.9 mmol) in DMF (15 ml). The reaction was stirred for 12 hours at room temperature. Aqueous HCl was added to the solution (1 M, 5 mL),, which was then (1 M, 5 mL), diluted with water (10 mL), and extracted with diethyl ether. After column chromatography (silica gel, CH_2Cl_2/hexanes 3:1), a white solid was collected (0.341g, 42.5% yield). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 10.20 (1H, s), 7.435 (1H, d, *J* = 8.8 Hz), 7.25 (1H, d, *J* = 3.0 Hz), 6.98 – 6.94 (1H, dd, *J* = 3.2Hz, *J* = 8.80 Hz), 4.09 – 4.07 (2H, t, *J* = 5.8Hz), 3.56 – 3.52 (2H, t, *J* = 6.4Hz), 2.31 – 2.18 (2H, m). **^{13}C NMR** (300 MHz, $CDCl_3$): δ 189.5, 156.3, 132.61, 131.95, 121.16, 116.05, 111.57, 63.91, 30.08, 27.74.

7-Hydroxy-4-methylcoumarin (S3): Compound **S3** was prepared by the Pechmann condensation using resorcinol and ethyl acetoacetate. The ^{1}H and ^{13}C NMR spectra matched those published for the commercially available material.

2-Bromo-5-(3-(4-methyl-2-oxo-2H-chromen-7-yloxy)propoxy)benzaldehyde (S4): At room temperature, to a solution of bromide **S2** (0.8 g, 2.5 mmol) in DMF (10 mL) was added NaOH powder (1eq, 0.102 g, 2.55 mmol) and the mixture was stirred for 1 hour until all NaOH was dissolved in DMF. This mixture was added to a solution of coumarin **S3** (0.448 g, 2.54 mmol) in DMF (20 mL) slowly over a period of 1 hour. After

stirring for another 6 hours, the reaction was quenched by addition of aqueous HCl (4 M, 10 mL), diluted with distilled water (10 mL), and extracted with diethyl ether. The crude oil product was purified by column chromatography (silica gel, ethyl acetate:hexanes 2:3) and a white solid was isolated (0.24 g, 39% yield). **^{1}H NMR** ($CDCl_3$): δ 10.29 (1H, s), 7.51 (1H, d, *J* = 8.82 Hz), 7.48 (1H, d, *J* = 8.76 Hz), 7.41 (1H, d, *J* = 3.18Hz), 7.05 (1H, dd, *J* = 8.89 Hz, *J* = 3.22 Hz), 6.87 (1H, dd, *J* = 9.02 Hz, *J* = 2.59 Hz), 6.12 (1H, s), 4.21 – 4.19 (4H, m), 2.38 (1H, s), 2.33 – 2.29 (2H, m). **^{13}C NMR** ($CDCl_3$): δ 192.0, 162.0, 161.5, 158.6, 155.5, 152.7, 134.9, 134.2, 125.8, 123.7, 118.4, 114.0, 113.5, 112.8, 112.3, 101.7, 65.0, 64.9, 29.1, 18.9.

fPBA-coumarin probe [2-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)-5-(3-(4-methyl-2-oxo-2H-chromen-7-yloxy)propoxy)benzaldehyde (6)]: To a dry three neck flask under argon was added compound **5** (0.164 g, 0.395 mmol), bis(neopentyl glycolato)diboron (0.268 g, 1.19 mmol), KOAc (0.206 g, 1.19 mmol), SPhos Pd G2 catalyst (0.0085 g, 0.0119 mmol) and sphos ligand (0.113 g, 0.0237 mmol). The flask was sealed with rubber stopper, connected with argon and vaccum, evacuated and backfilled with argon. Next, freshly distilled, oxygen free 1,4-dixoane (20 mL) was added via syringe. The reaction was stirred at 110 °C for 30 min. After cooling to room temperature, water was added (10 mL), and the solution was extracted with ethyl acetate and concentrated in vaccum. After column chromatography (silica gel, hexanes:ethyl acetate 1:1), a white solid was isolated (60 mg, 40% yield). **^{1}H NMR** ($CDCl_3$): δ 10.6 (1H, s), 7.83 (1H, d, *J* = 8.56 Hz), 7.49 (1H, d, *J* = 6.43 Hz), 7.47 (1H, s), 7.12 (1H, dd, *J* = 8.50 Hz, *J* = 2.58 Hz), 6.87 (1H, dd, *J* = 9.24 Hz, *J* = 2.52 Hz), 6.83 (1H, d, *J* = 2.51 Hz), 6.13 (1H, s), 4.25 (2H, t, *J* = 6.36 Hz*),* 4.22 (2H, t, J = 6.15 Hz), 3.80 (1H, s), 2.39 (3H, s), 2.31 (2H, m), 1.05 (6H, s). **^{13}C NMR** ($CDCl_3$): δ δ195.08, 161.87, 161.28, 160.49, 155.29, 152.48, 143.30, 136.96, 125.55, 120.19, 113.70, 112.58, 112.05, 111.13, 101.53, 72.51, 64.88, 64.25, 28.96, 21.88, 18.66. **^{11}B NMR** ($CDCl_3$): δ 27.98. **HRMS** (ESI-TOF) m/z: Calcd for C25H2710BO7 450.1964; Found 450.1958. **UV-Vis:** λ_{max} = 320 nm (log ε =3.38, calc) in DMSO.



S5

C. DAB-coumarin product (S5) [4-(1-hydroxy-6-(3-(4-methyl-2-oxo-2H-chromen-7-yloxy)propoxy)-benzo-[d][1,2,3]-diazaborinin(1H)-yl)benzoic acid]: The model compound for the expected product of the coumarin-fPBA probe with HBA was prepared in aqueous methanol owing to the low water solubility of the coumarin-containing molecule. To a solution of compound **6** (20 mg, 0.044 mmol) in a mixture of water (1 mL) and methanol (3 mL) was added excess HBA (40 mg, 0.214 mmol). The solution was stirred at room temperature for 3 hours. A white solid formed, which was collected and washed with distilled water. 100% conversion was obtained base on NMR. **^{1}H NMR** (600MHz, DMSO-d_6, 298K): δ 12.76 (1H, s), 9.12 (1H, s), 8.33 (1H,d, *J* = 8.64Hz), 8.19 (1H, s), 7.98 (2H, d, *J* = 8.70Hz), 7.78 (2H, d, *J* = 8.70Hz), 7.69 (1H, d, *J* = 8.70Hz), 7.39 (1H,s, *J* = 3.02Hz), 7.30 (1H, dd, *J* = 9.49 Hz, *J* = 1.95 Hz), 7.01 (2H, m), 6.22 (1H, s), 4.32 (4H, m, *J* = 6.37 Hz), 2.40 (3H, s), 2.30 (2H, m, *J* = 6.71 Hz). **^{13}C NMR** (600MHz, DMSO-d_6, 298K): δ 167.08, 161.53, 161.09, 160.10, 154,.73, 153.36, 153.36, 150.27, 139.76, 136.90, 133.74, 129.51, 126.47, 123.45, 118.35, 113.18, 112.40, 111.16, 109.84, 101.29, 65.05, 64.38, 28.36, 18.08. **^{11}B NMR** ($CDCl_3$): δ27.9 (broad). **HRMS** (ESI-TOF) m/z: Calcd for C27H23BN2O7 498.1713; Found 498.1715.

D. Bifunctional coupling reagent for protein/fPBA-coumarin

4-(2-(Propan-2-ylidene)hydrazinyl)benzoic acid, N-hydroxy succimidyl ester (7) The bifunctional coupling reagent was synthesize from HBA by first forming the acetone hydrazone and then the NSH ester through carbodiimide coupling with N-hydroxy succinamide. **^{1}H NMR** (300 mHz NMR, $CDCl_3$): 8.01 (2H, d, *J* = 8.6 Hz), 7.35 (1H, s, broad), 7.06 (2H, d, *J* = 8.7 Hz), 2.91 (4H, s), 2.09 (1H, s), 1.93 (1H, s). **^{13}C NMR** (600 mHz NMR, $CDCl_3$ 77.03 ppm): δ 169.65, 161.63, 150.39, 150.36, 132.71, 112.07, 25.65, 25.22, 16.01**.**

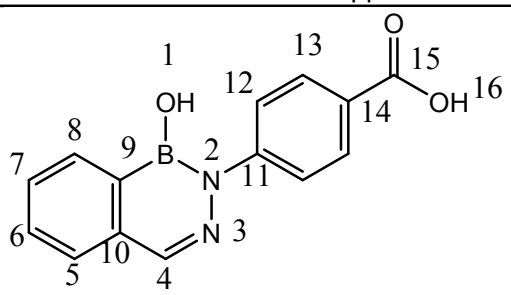
7. Protein labeling

BSA (final concentration 40 μM) was allowed to react with ~100 fold excess of the crosslinker **7** for 1 h 45 min at room temperature in HEPES buffer (25 mM, pH 7.2) containing DMSO (5% (v/v)). The protein solution was then subjected to rapid gel filtration into phosphate buffer (10 mM, pH 7.0) to remove excess **7**. Aliquots of the protein solution was snap frozen in liquid nitrogen, stored at -80 °C until use and were not subjected to an additional freeze-thaw cycle. No decrease in activity was detected in protein

samples stored for at least one month. The fluorophore, fPBA-coumarin **6,** was dissolved in DMSO and its concentration was determined spectrophotometrically using an extinction coefficient of 24000 M^{-1} cm^{-1} at 320 nm. The hydrazine-BSA or unmodified BSA (final concentration 25 μM) was allowed to react by continuous mixing with 75 μM of **6** for 15 min at room temperature in phosphate buffer (10 mM, pH 7.0). The final concentration of DMSO in the samples was 2 % (v/v). The protein samples were subjected to rapid gel filtration chromatography using spin columns to remove unreacted fluorophore. Absorption and emission spectra of each sample (equal protein concentration) were obtained using a BioTek Synergy Mx plate reader. The absorption and emission spectra of protein samples (BSA and BSA-hydrazine) that were not treated with fluorophore were also obtained as additional controls. The fluorophore treated protein samples were subjected to SDS-PAGE analysis. The gel was photographed using a handheld long wavelength UV lamp for illumination. The same gel was Coomassie stained for protein, destained using acetic acid/methanol/water and then photographed under white light.

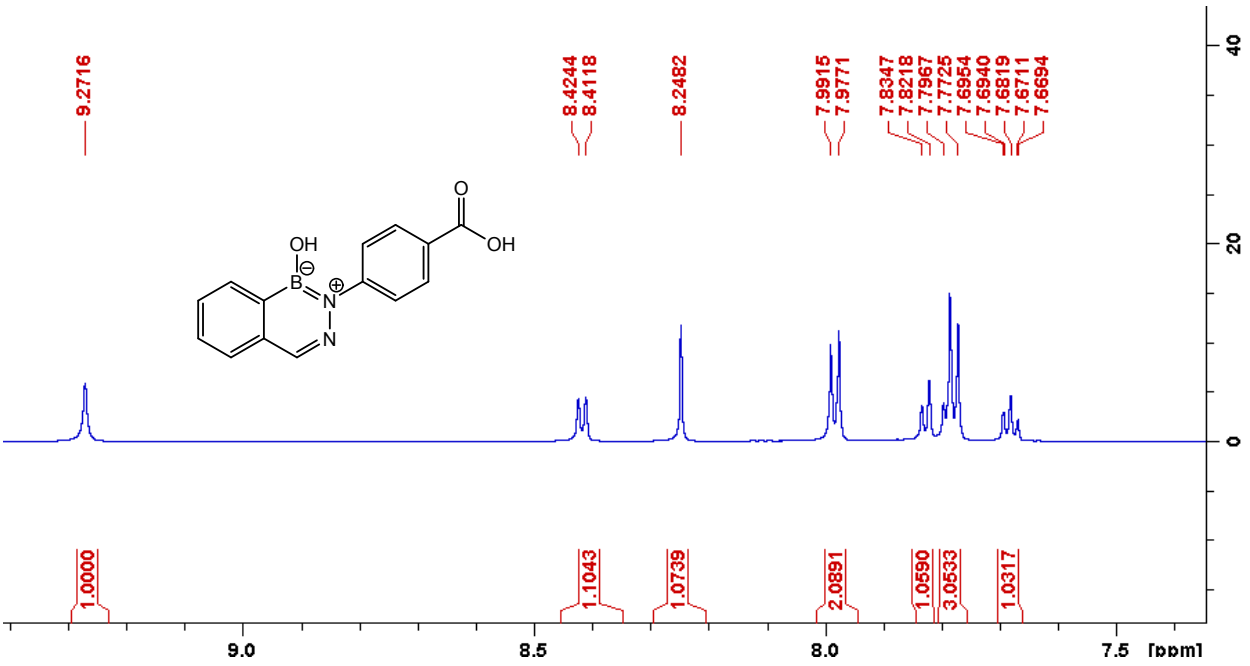
8. NMR spectra

1,2-Dihydro-2-(4'-carboxyphenyl)-1-hydroxy-2,3,1-benzodiazaborine (5)

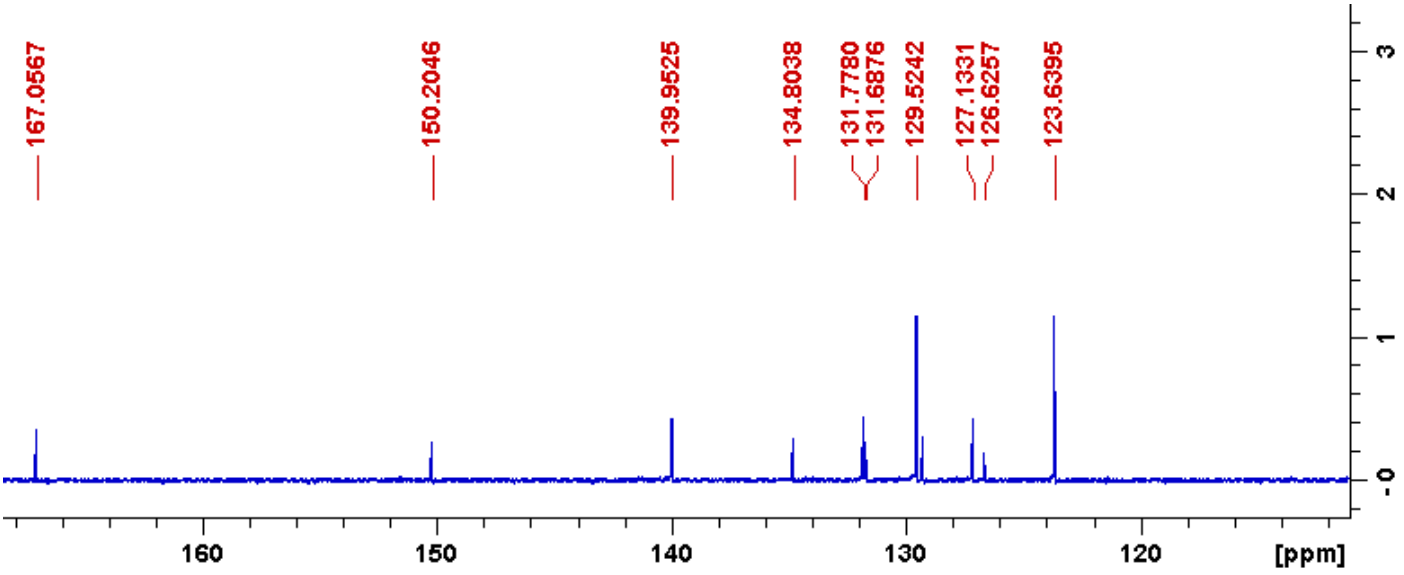
Nucleus	Chemical shift/ ppm	Nucleus	Chemical shift/ ppm
			
H4	8.25	H12	7.78
H5	7.83	H13	7.99
H6	7.79		
H7	7.69		
H8	8.42		
B-OH1	9.27	OH16	12.75
N-H3			
C4	139.95	C11	126.63
C5	127.17	C12	123.64
C6	131.70	C13	129.3
C7	129.52	C14	150.34
C8	131.79	C15	167.12
C9	ND		
C10	134.81		
N3	330.76		
N2	183.29		
B1	28.61		

Spectra in DMSO-d6

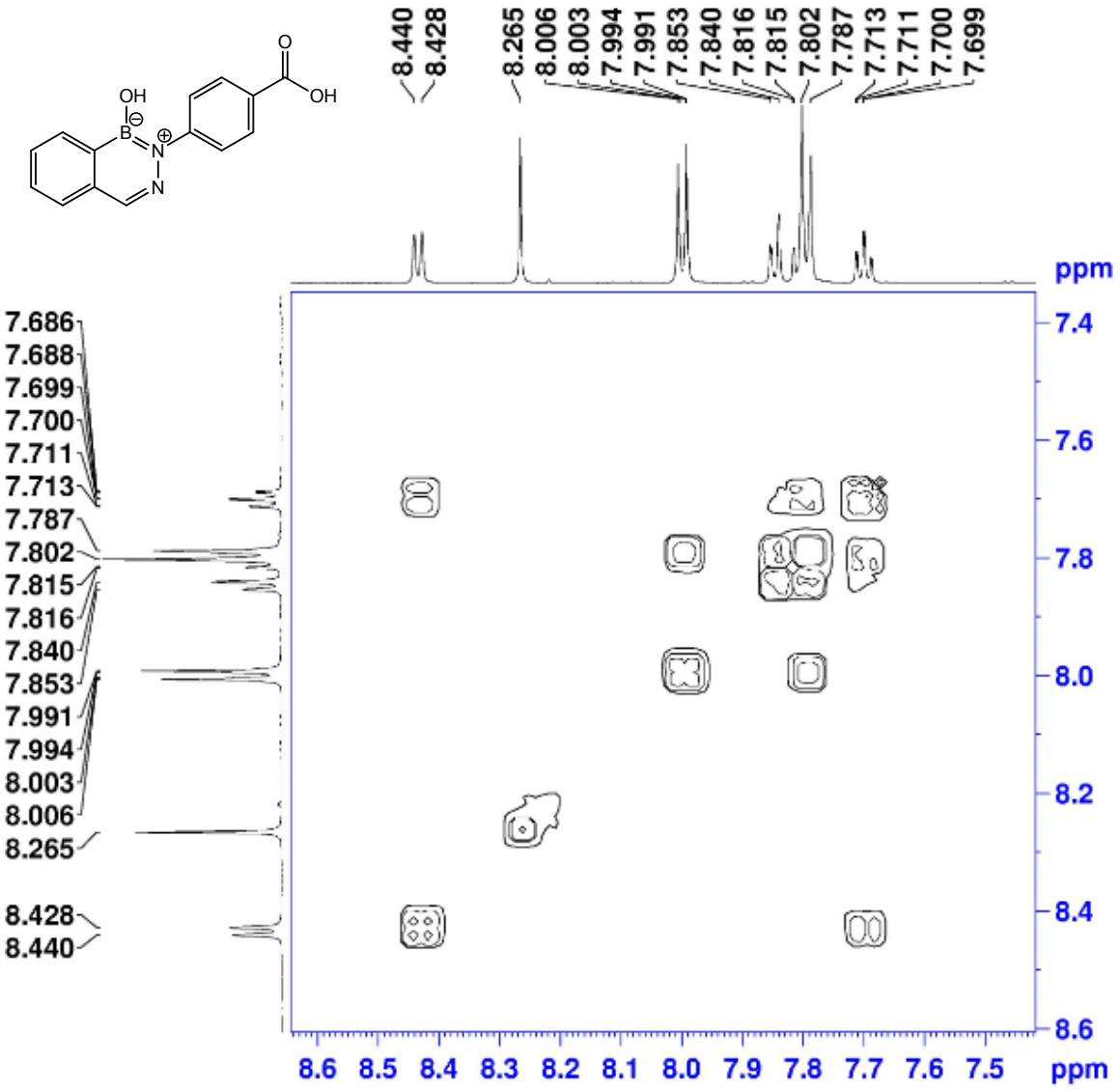
^{1}H NMR (DMSO-d6)



^{13}C NMR



^{1}H-^{1}H COSY


OH
B
N
N
O
OH
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7.991
7.853
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7.815
7.802
7.787
7.713
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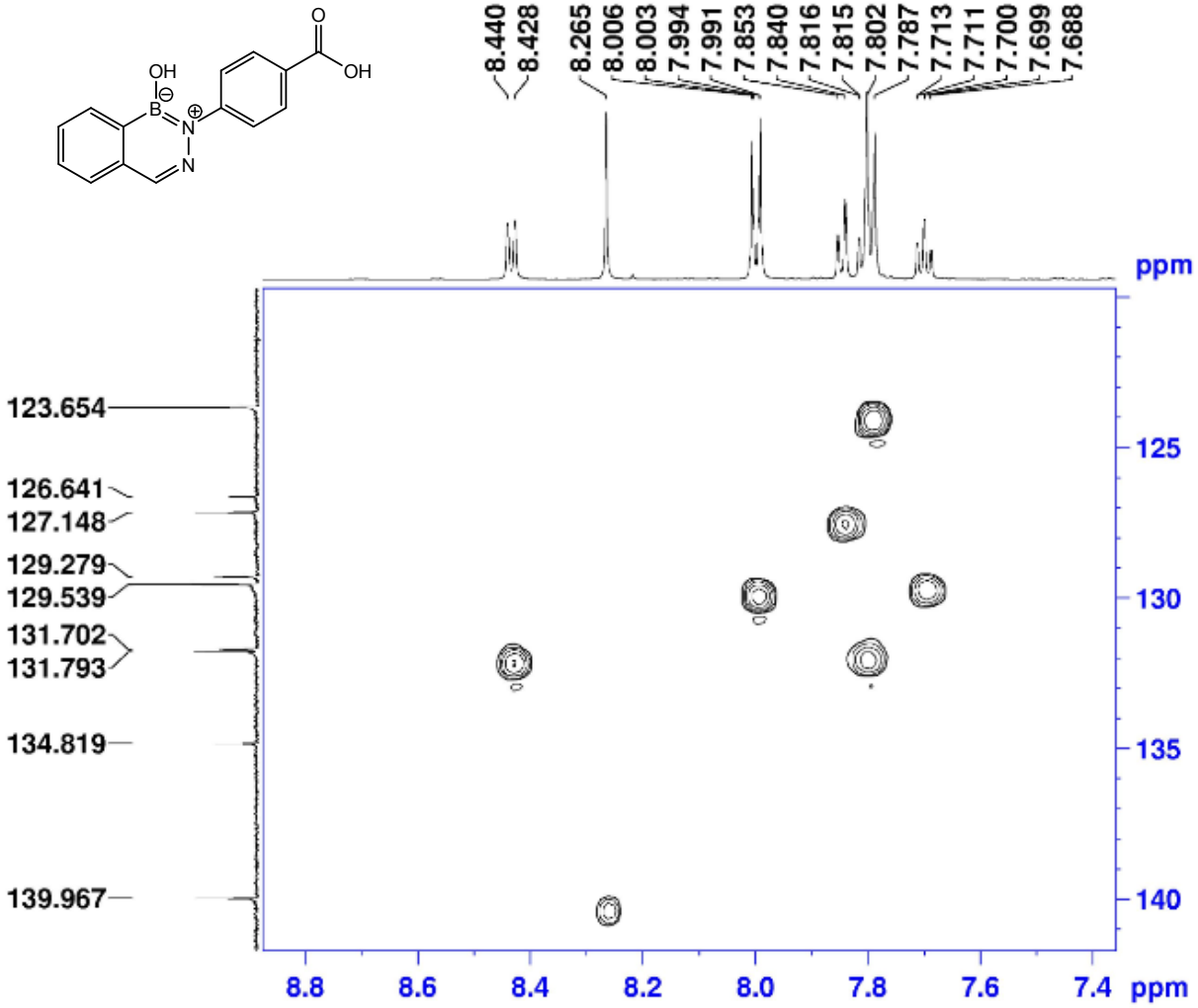
7.713
7.787
7.802
7.815
7.816
7.840
7.853
7.991
7.994
8.003
8.006
8.265
8.428
8.440
7.4
7.6
7.8
8.0
8.2
8.4
8.6
8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5
ppm

^{1}H-^{13}C HSQC


OH
B
N
N
O
OH
8.440
8.428
8.265
8.006
8.003
7.994
7.991
7.853
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7.816
7.815
7.802
7.787
7.713
7.711
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7.688
ppm
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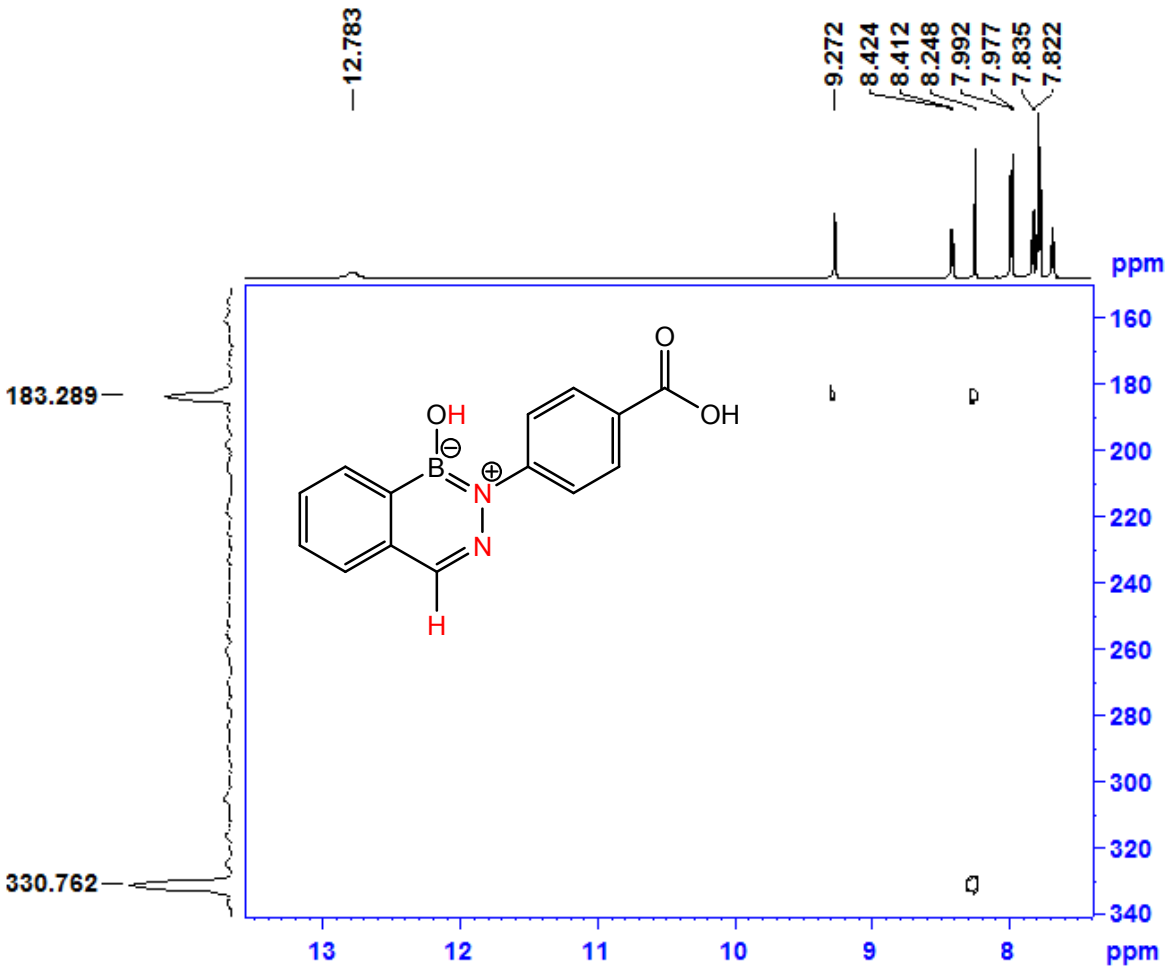
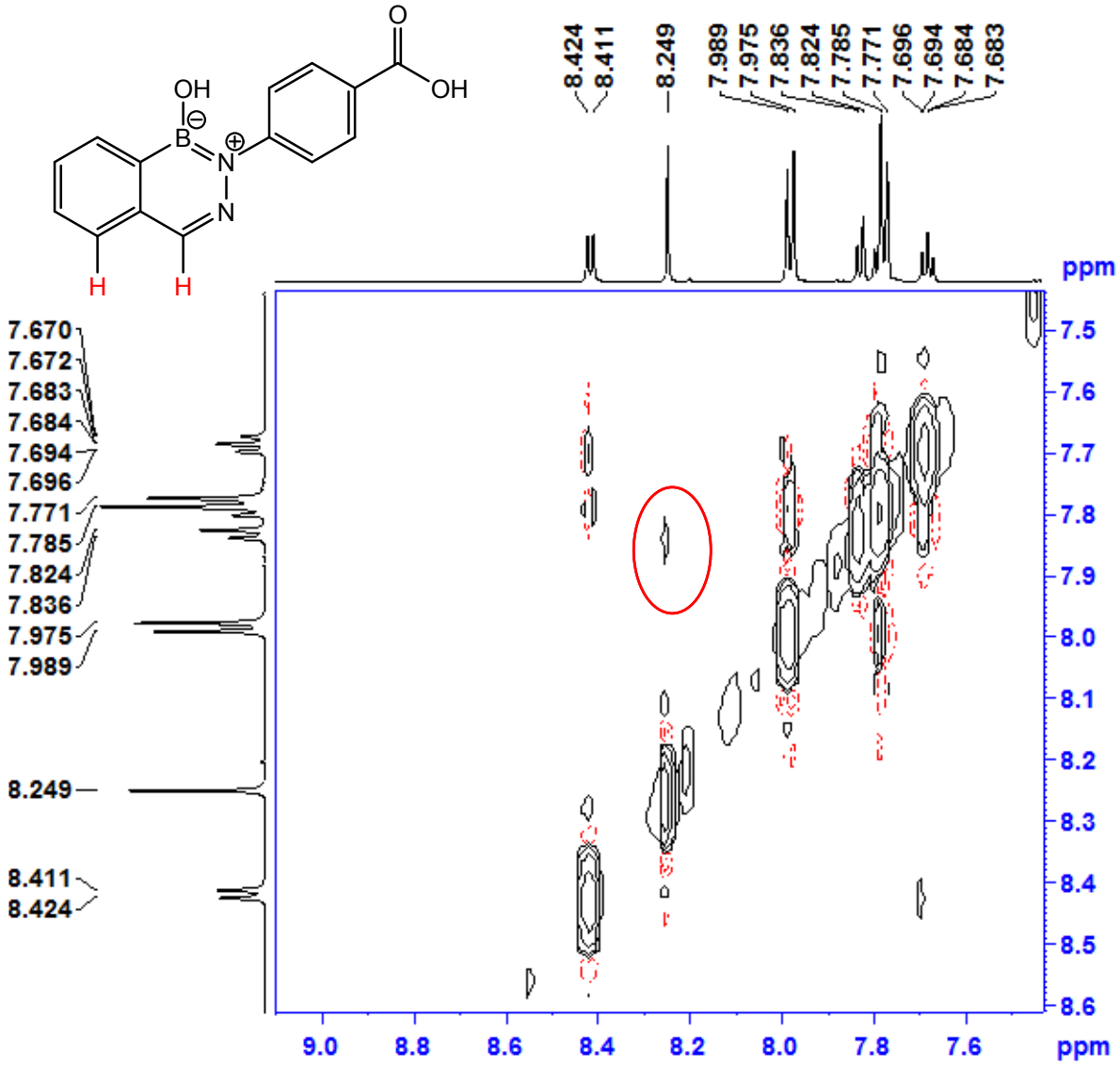
126.641
127.148
129.279
129.539
131.702
131.793
134.819
139.967
125
130
135
140
8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
ppm

^{1}H-^{15}N HMBCGP



^{1}H-^{1}H NOESY


O
OH
OH
B
N
N
H
H
8.424
8.411
8.249
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7.975
7.836
7.824
7.785
7.771
7.696
7.694
7.684
7.683
ppm
7.670
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7.683
7.684
7.694
7.696
7.771
7.785
7.824
7.836
7.975
7.989
8.249
8.411
8.424
7.5
7.6
7.7
7.8
7.9
8.0
8.1
8.2
8.3
8.4
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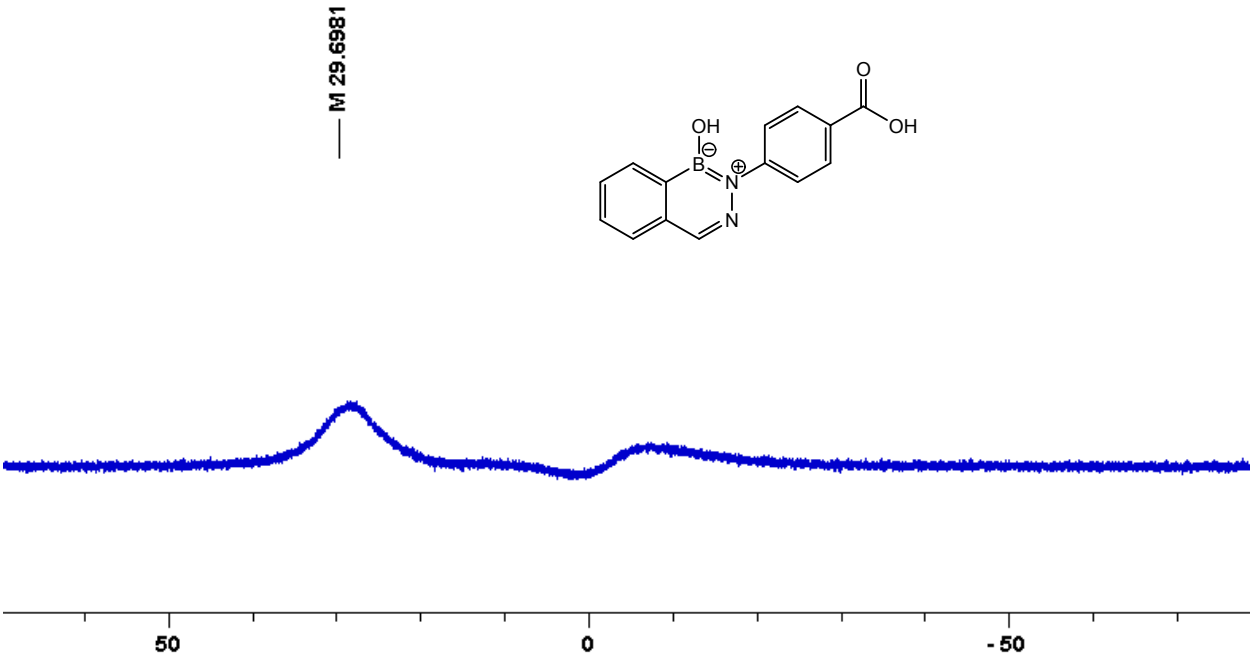
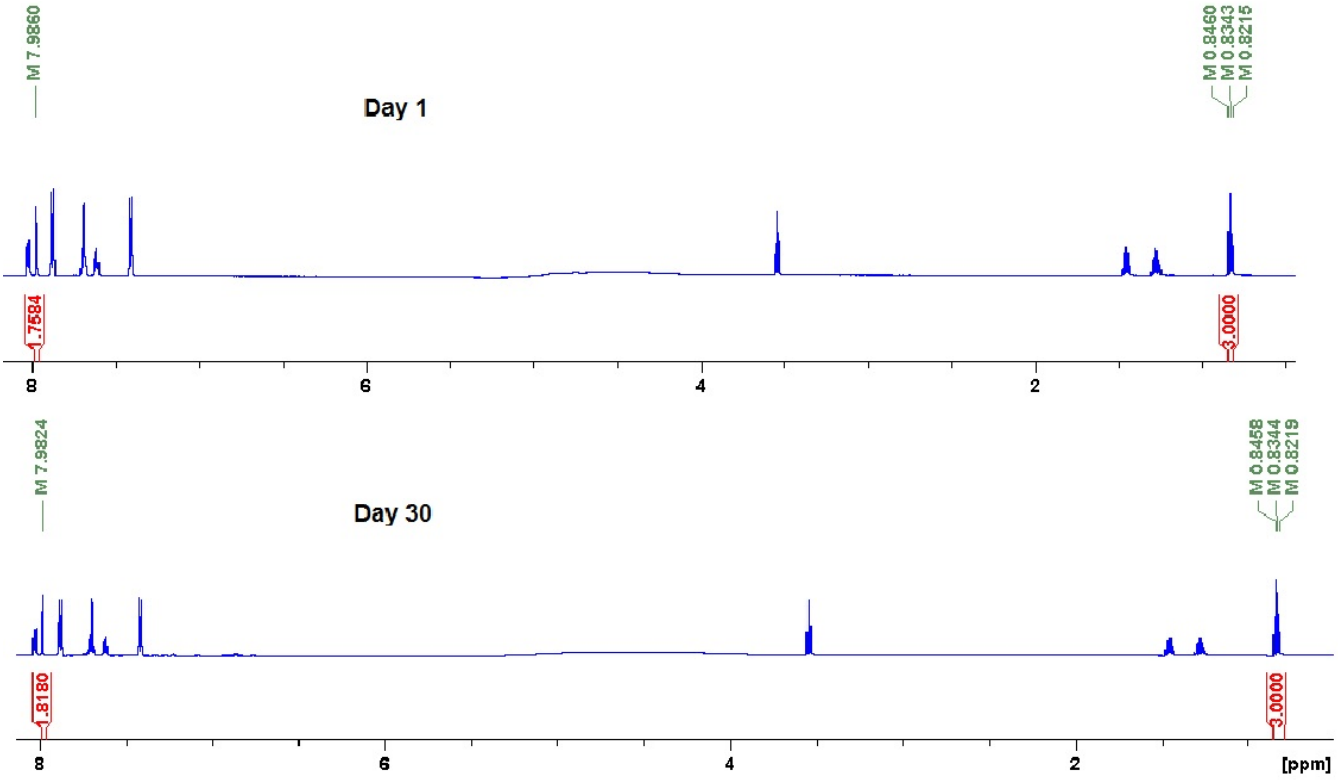
8.4
8.2
8.0
7.8
7.6
ppm

^{11}B NMR

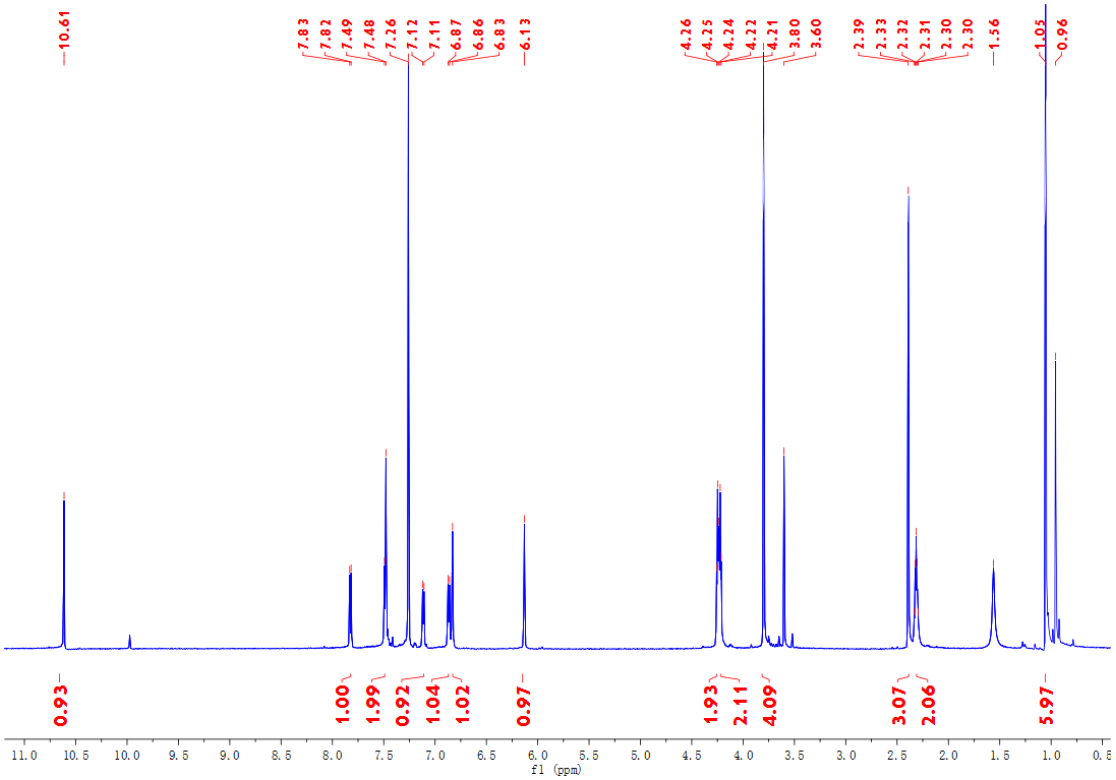


^{1}H NMR spectra in pH 7 buffer, showing internal standard

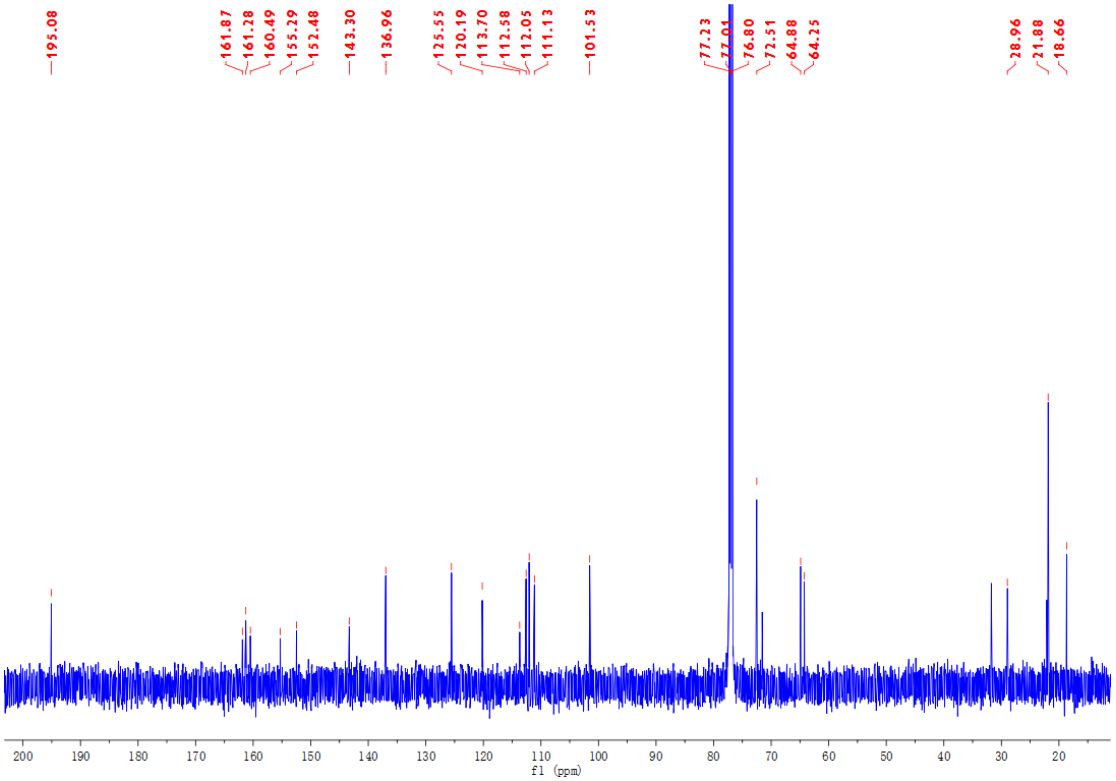


Coumarin-fPBA product (2-(5,5-dimethyl-1,3,2-dioxaborinan-2-yl)-5-(3-(4-methyl-2-oxo-2H-chromen-7-yloxy)propoxy)benzaldehyde (6))

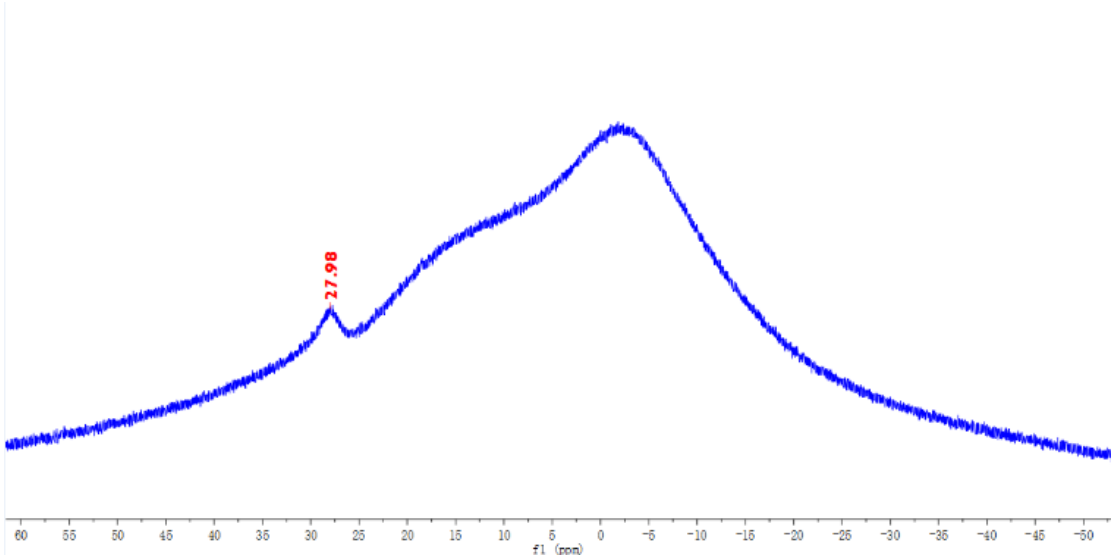
^{1}H NMR ($CDCl_3$)



^{13}C NMR

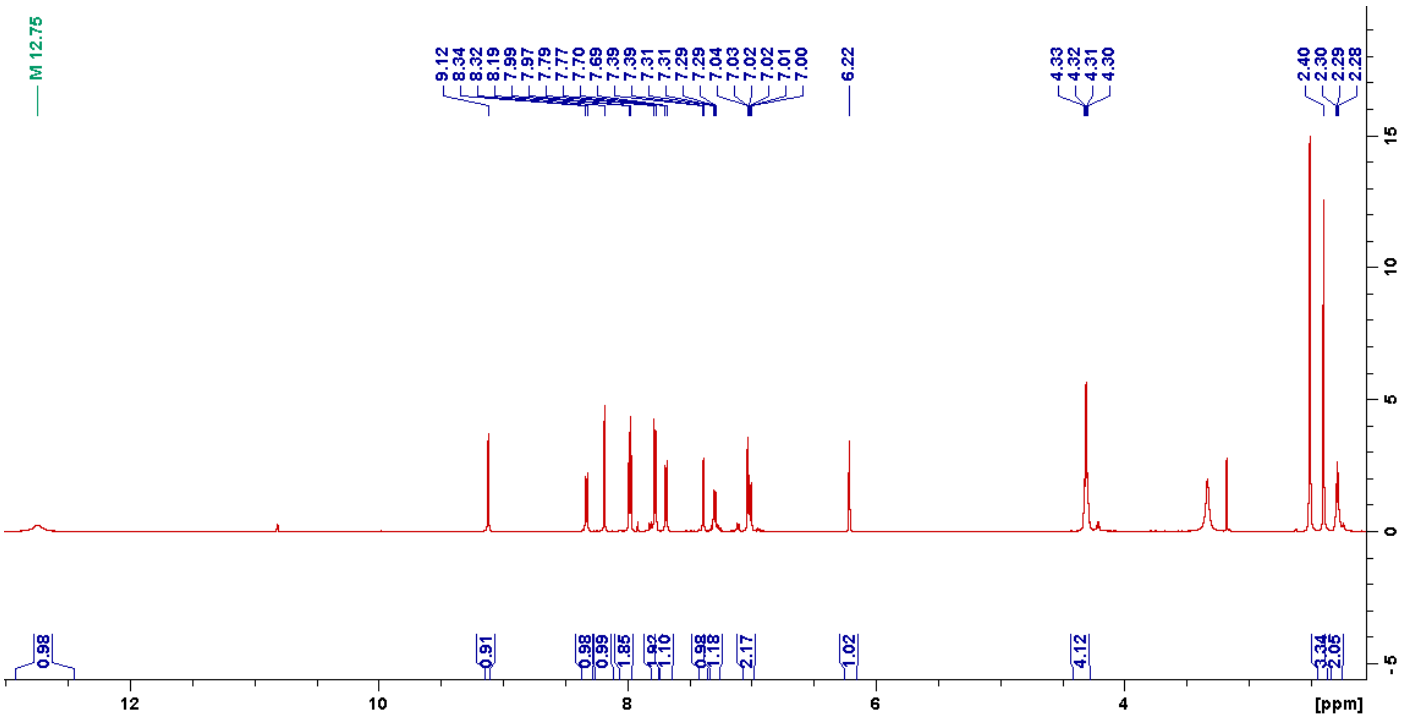


^{11}B NMR

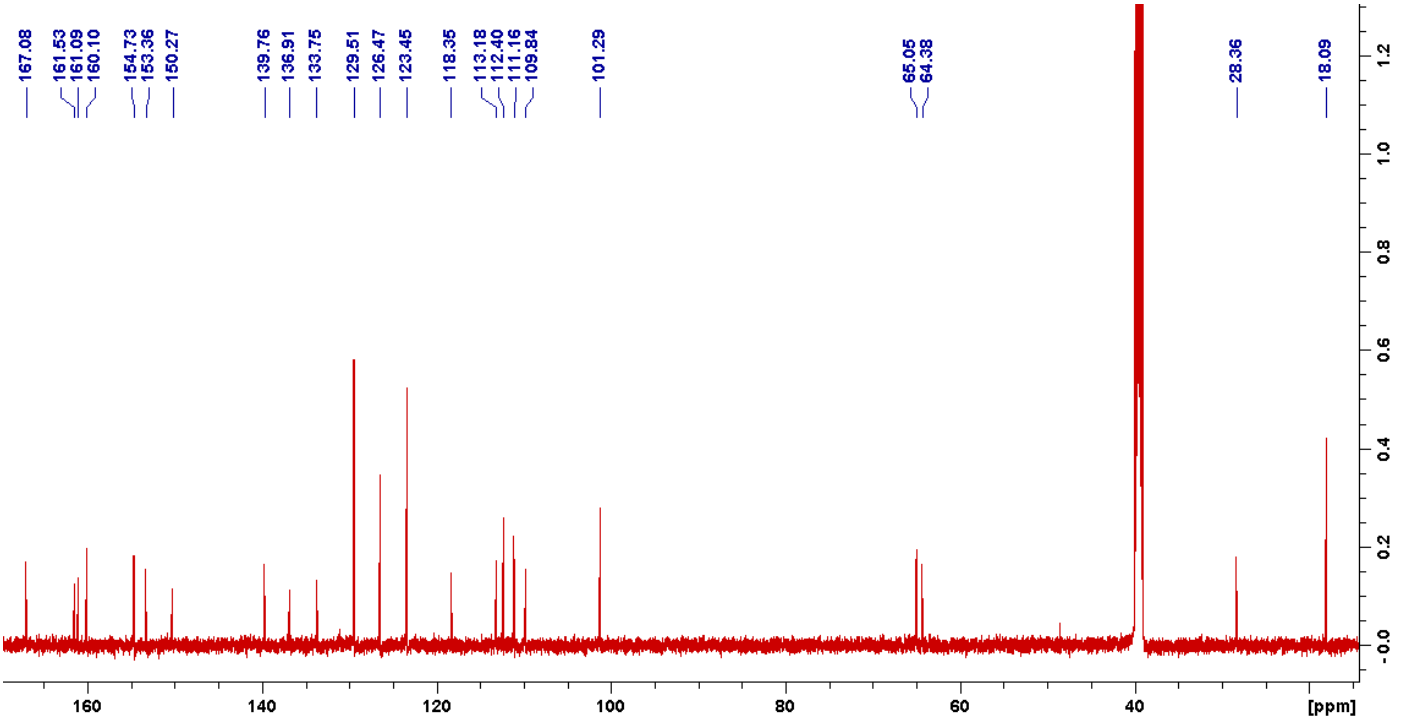


C. DAB-coumarin product (S5) [4-(1-hydroxy-6-(3-(4-methyl-2-oxo-2H-chromen-7-yloxy)propoxy)-benzo-[d][1,2,3]-diazaborinin(1H)-yl)benzoic acid]:

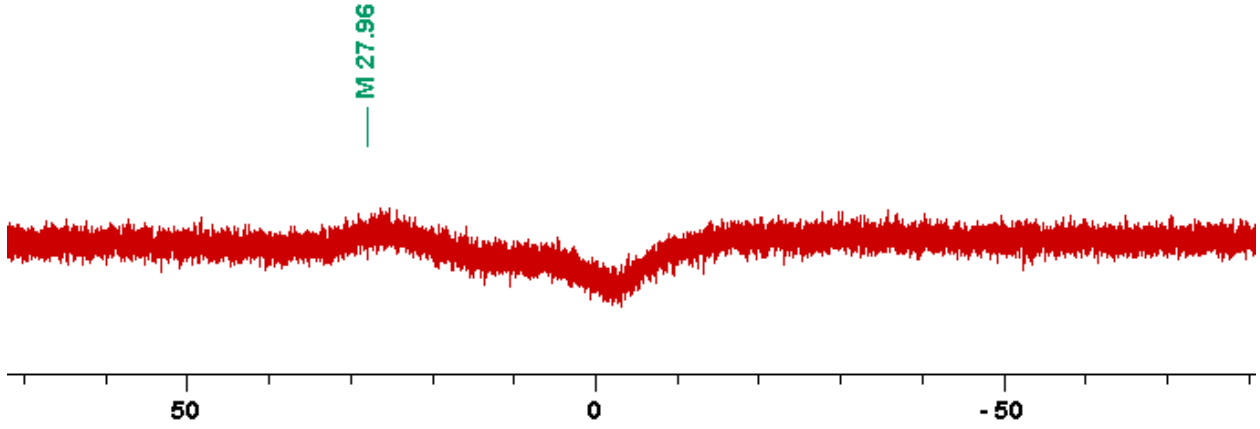
^{1}H NMR



^{13}C NMR

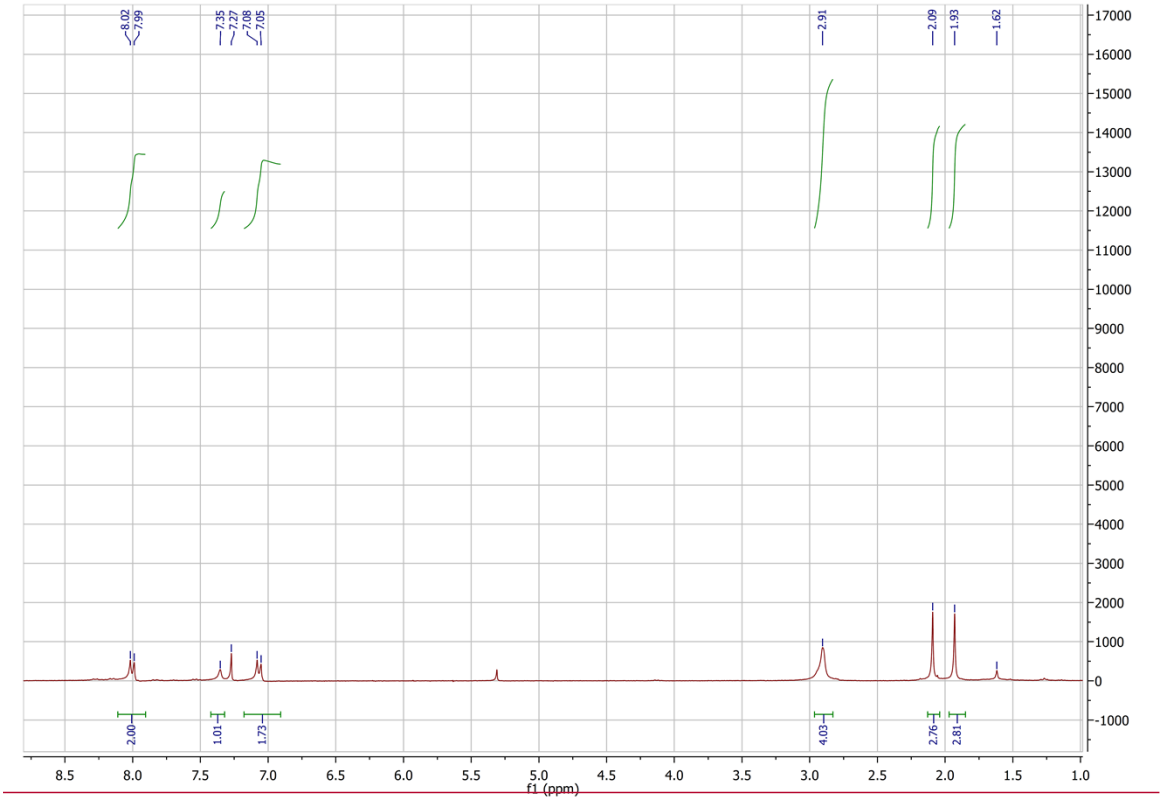


^{11}B NMR



Bifunctional coupling reagent for protein/fPBA-coumarin 4-(2-(Propan-2-ylidene)hydrazinyl)benzoic acid, N-hydroxy succimidyl ester (7)

^{1}H NMR ($CDCl_3$, 300 mHz NMR)



^{13}C NMR ($CDCl_3$, 600 mHz NMR)