Supporting Information

Iminoboronate Formation Leads to Fast and Reversible Conjugation Chemistry of α-Nucleophiles at Neutral pH

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I. General methods

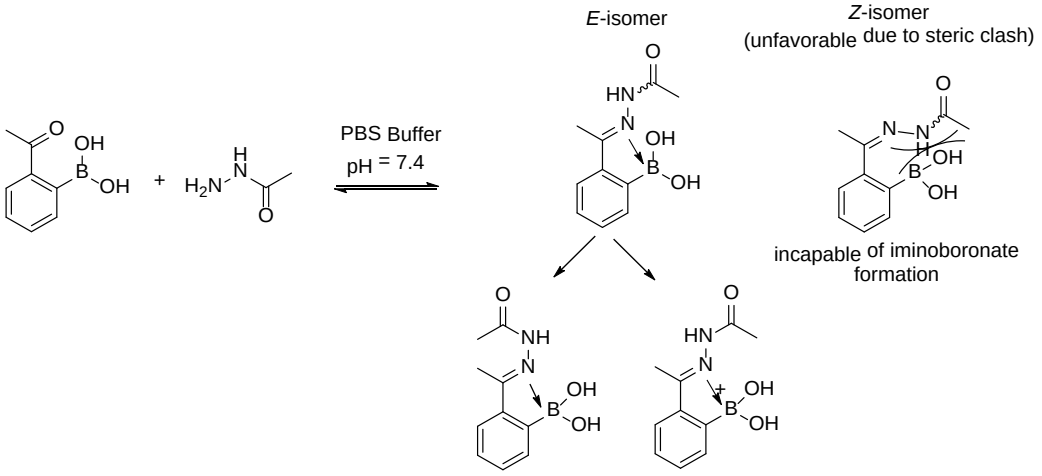
2-acetylphenylboronic acid (2-APBA), acethydrazide, benzhydrazide, 6-aminoxy hexanoic acid (AOHA), phenylhydrazine (Phzn), 4-hydrazinobenzoic acid (HBA), glutathione, fructose and BSA were purchased from Fischer. Rink Amide MBHA resin and HBTU were purchased from Novabiochem (San Diego, CA). Fmoc-protected amino acids were purchased from Advanced Chemtech (Louisville, KY) or Chem-Impex Int'l Inc (Wood Dale, IL). Fmoc-AB1(pin)-OH was synthesized according to the published procedure.[1] Alexa Fluor 488-C_5-maleimide was purchased from Life Technologies. UV spectra were collected on a Nanodrop UV-vis spectrometer. NMR data of the small molecules were collected on a VNMRS 500 MHz NMR spectrometer and titration data were collected in VNMRS 600 MHz. Mass-spec data were generated by an Agilent 6230 LC TOF mass spectrometer. Peptide synthesis was carried out on a Tribute peptide synthesizer from Protein Technologies. Unless specified otherwise, all experiments were performed in a 5x PBS buffer (50 mM Na•Pi, 685 mM NaCl and 13.5 mM KCl, pH = 7.4).

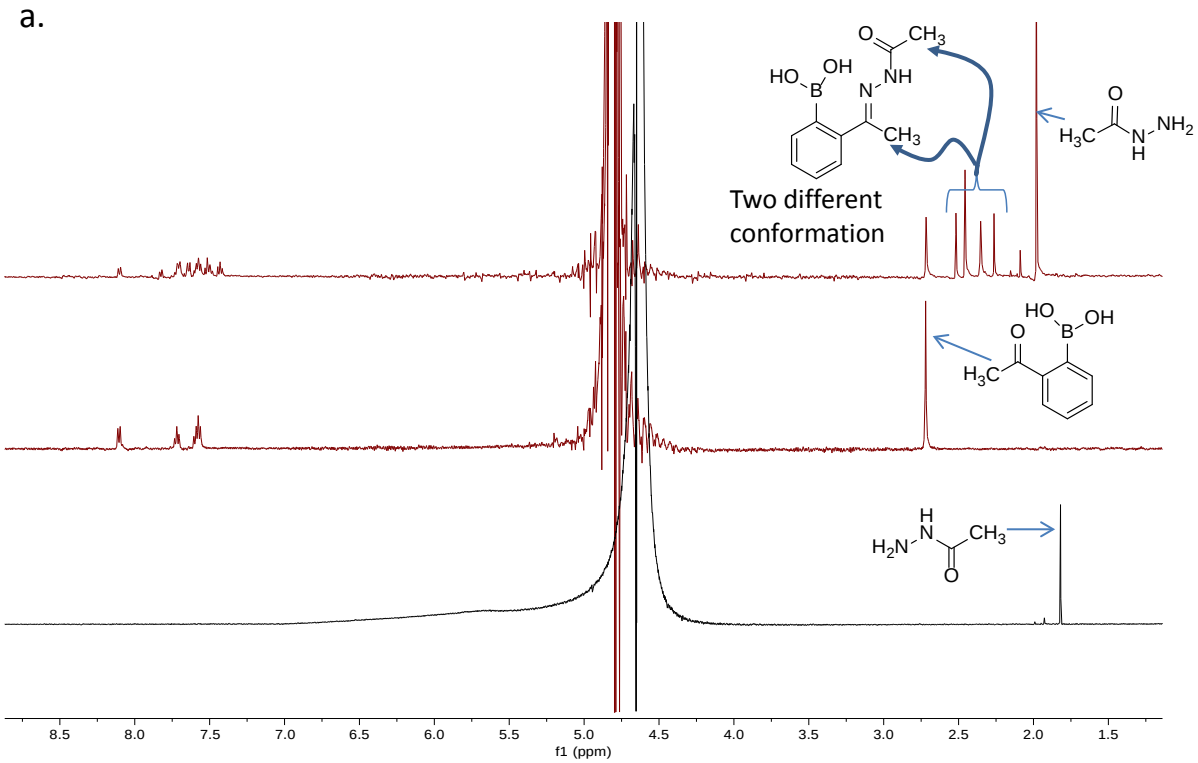
II. General method for K_d determination

K_d values were determined via titration experiments monitored by UV-vis absorption and ^{1}H-NMR. For UV-Vis absorption reactions were performed in quartz cuvettes (10 mm path length; total volume ~ 2 mL) pre-equilibrated at room temperature. 1 mL PBS buffer was pipetted into the cuvette, followed by the addition of 1 µL of the 2-APBA stock (10 mM) in PBS buffer. The solution was mixed rapidly and the spectrum of 2-APBA was recorded. Then titration and dilution experiments were performed using stock solutions of the α-nucleophiles: acethydrazide and benzhydrazide at 40 mM, 6-aminoxy hexanoic acid (AOHA) and Phzn at 10 mM in PBS buffer. The pH of all stock solutions was adjusted to 7.4. All spectra collected during titration were background corrected by subtracting that of the α-nucleophile alone at corresponding concentrations. 2-APBA shows an absorption peak at ~ 254 nm with a small hump at ~ 275-280 nm. Upon titration with α-nucleophile, red shift of absorption was observed. The reactions of the hydrazides and hydrazines were monitored by recording the absorption at the new peak (Figure 1a, 1b, S2). AOHA conjugation with 2-APBA does not produce a distinct new peak, and therefore the ratio of absorption A_{280}/A_{254} was used to generate the titration curves (Figure 1c, S4). The curves were fitted according to a hyperbola equation. All the titration experiments were repeated twice, which gave consistent results.

The NMR titration experiments were performed using the PBS buffer in the presence of 10% D_2O. Percentage of product formation was calculated using the ratio of the integrated area between the acetyl -CH_3 peak of 2-APBA and the –CH_3 of imine product. In our hand, 0.2 mM of 2-APBA (limiting reagent) was found to be the lowest concentration required in NMR (600 MHz) titration with 64 scans (4.3 min) for quantitative measurement. Each titration experiment took 5 minutes including locking time. With 2-APBA at 0.2 mM, titration experiment with

acethydrazide and benzhydrazide was performed and the K_d values determined (Figure 1b, Figure S1, S3). We were unable to measure the K_d of AOHA and Phzn using NMR because their low K_d values require low concentrations of the reactants for analysis. Instead, we performed UV-Vis based titration (Figure S4) for the oxime formation and fluorophore (AF88) assisted measurement for the hydrazone formation (Figure 2, S6).





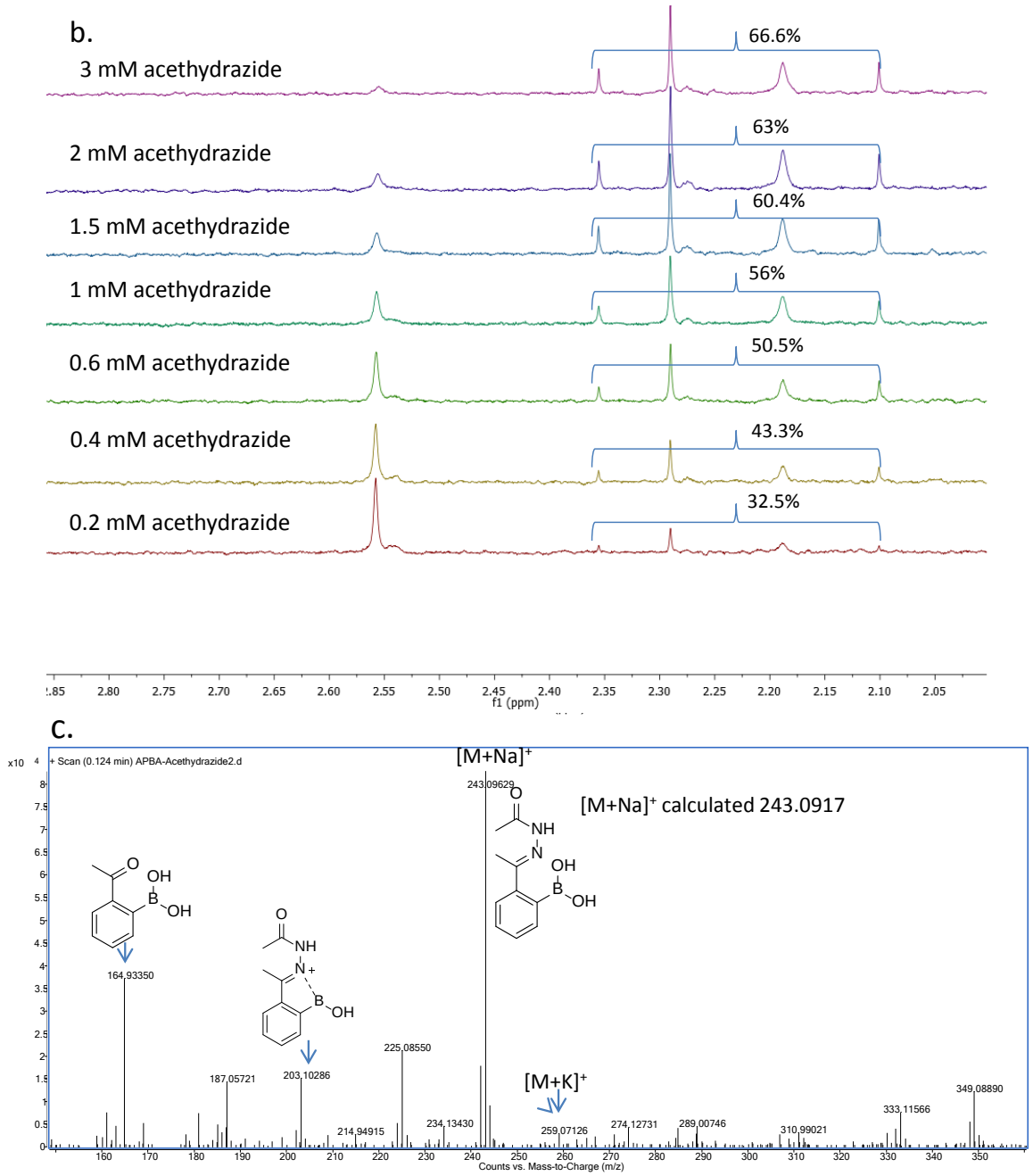


Figure S1: a) ^{1}H-NMR spectra of 2-APBA (2 mM), acethydrazide (3 mM) and their mixture. The spectrum of the acylhydrazone shows two sets of peaks, which indicate formation of two stable isomers, both presenting an iminoboronate as revealed by the ^{11}B-NMR spetrum (Figure 4 of the main text). Possible structures of the acylhydrazone isomers are shown above the NMR spectra. b) Stacked partial NMR spectra of the titration experiment between 2-APBA (0.2 mM) and acethydrazide (0.2-3 mM). Because of two geometrical isomers, the product shows four peaks for the two –CH_3 groups present in the product. c) ESI-MS result of 2-APBA-acethydrazide conjugate, confirming product formation.

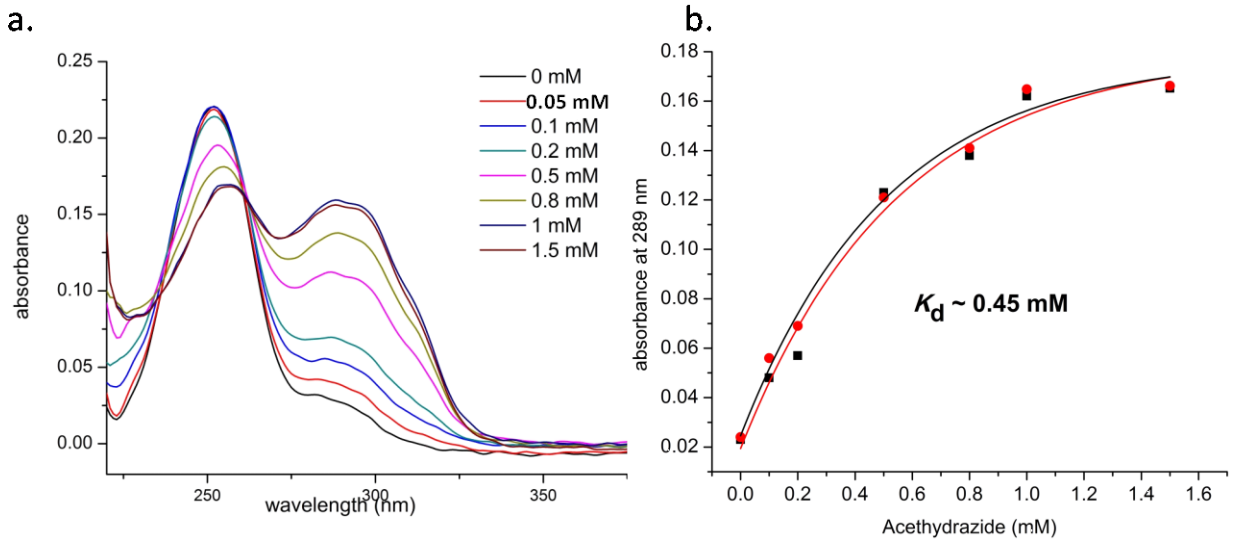
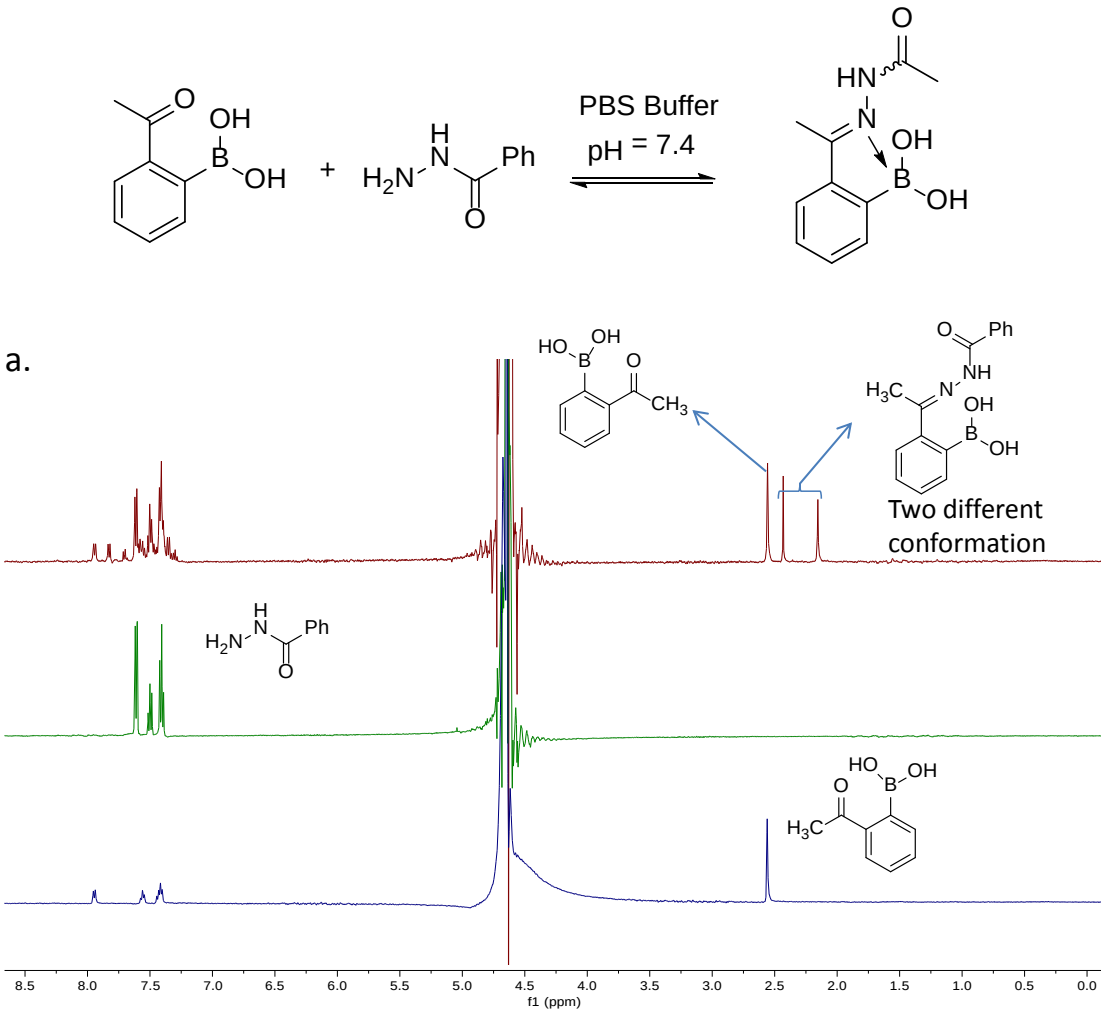


Figure S2: a) UV-vis spectral changes with titration of aethydrazide into 25 µM of 2-APBA. b) Overlay of two titration curves that show consistent results.



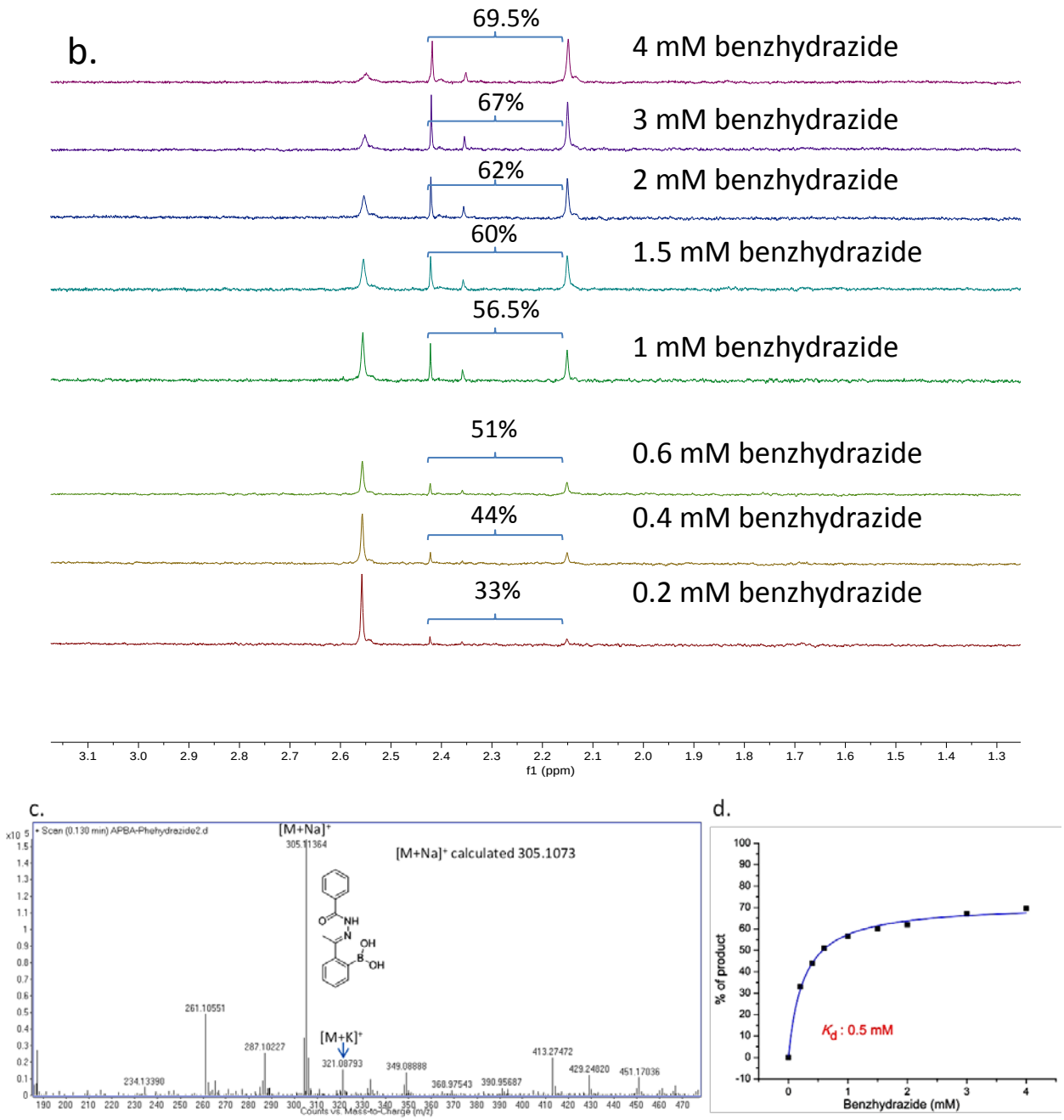


Figure S3: a) NMR spectra of 2-APBA (2 mM), benzhydrazide (3 mM) and their mixture. b) Stacked partial NMR spectra of the titration experiment between 2-APBA (0.2 mM) and benzhydrazide (0.2-4 mM). Because of two geometrical isomers, the product shows two peaks for $-CH_3$ group. c) ESI-MS result of 2-APBA-benzhydrazide conjugate confirming product formation. d) Titration curve for 2-APBA-benzhydrazide conjugation generated by using NMR data in b.

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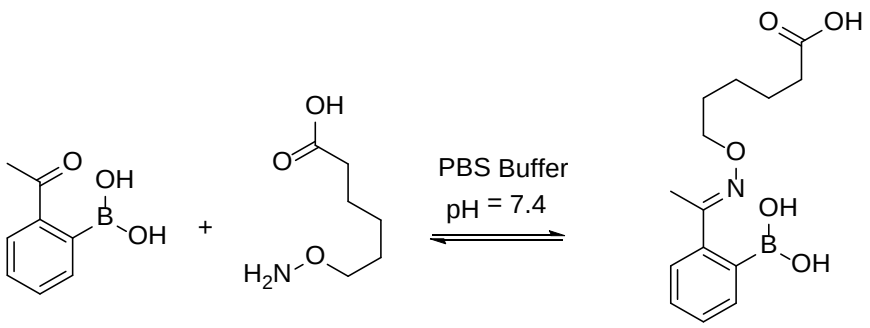
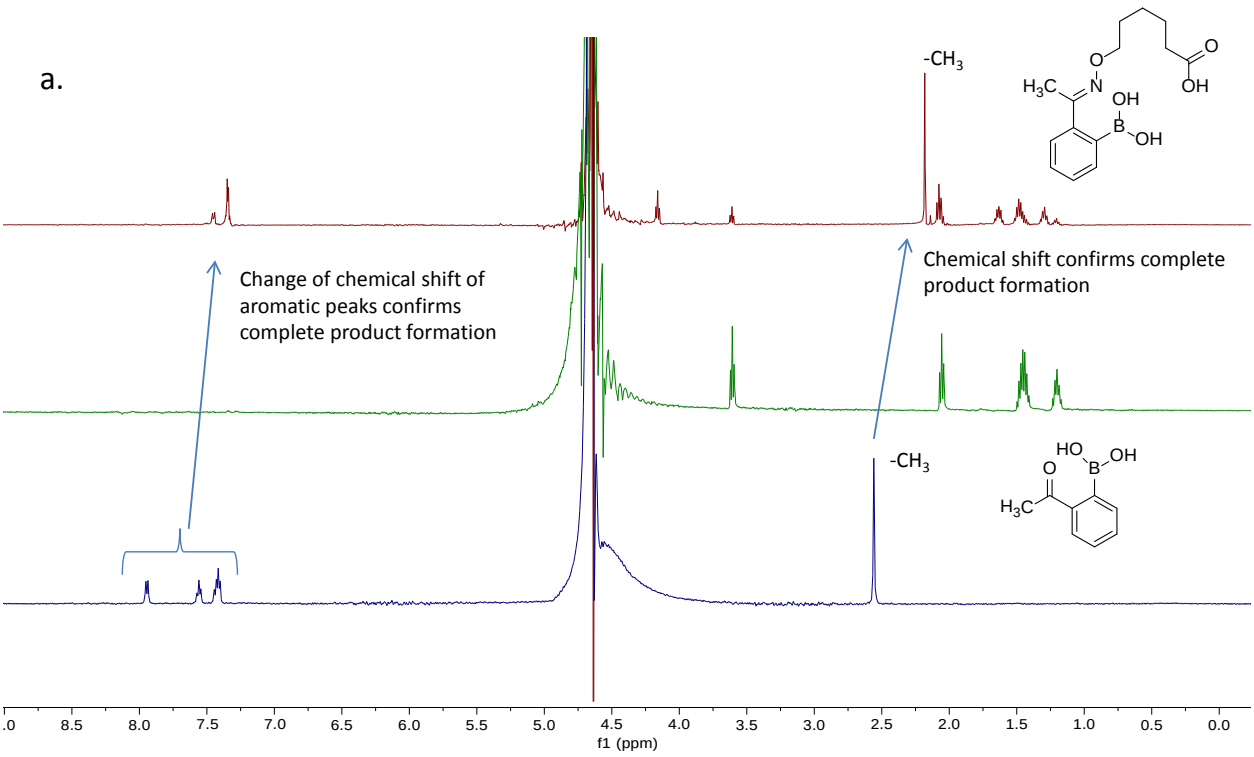
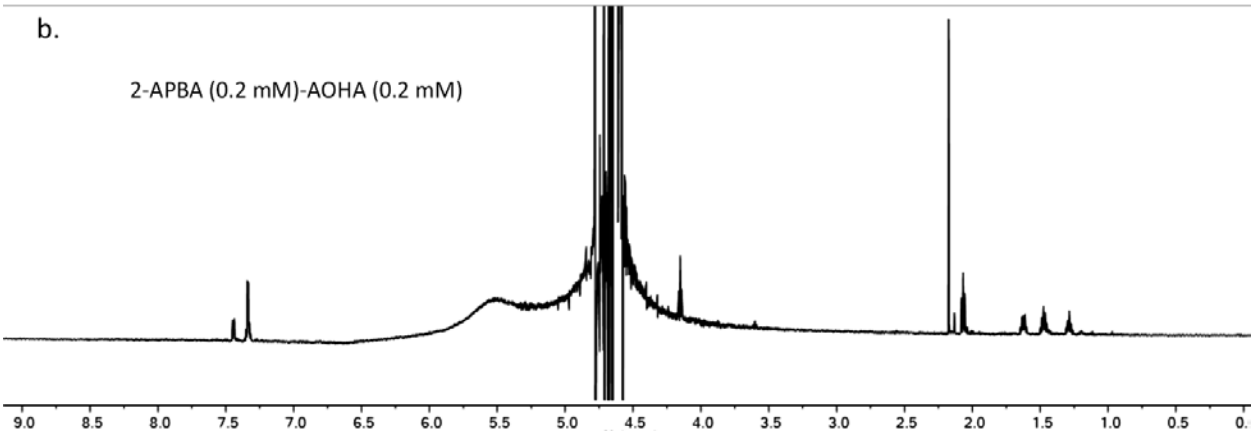
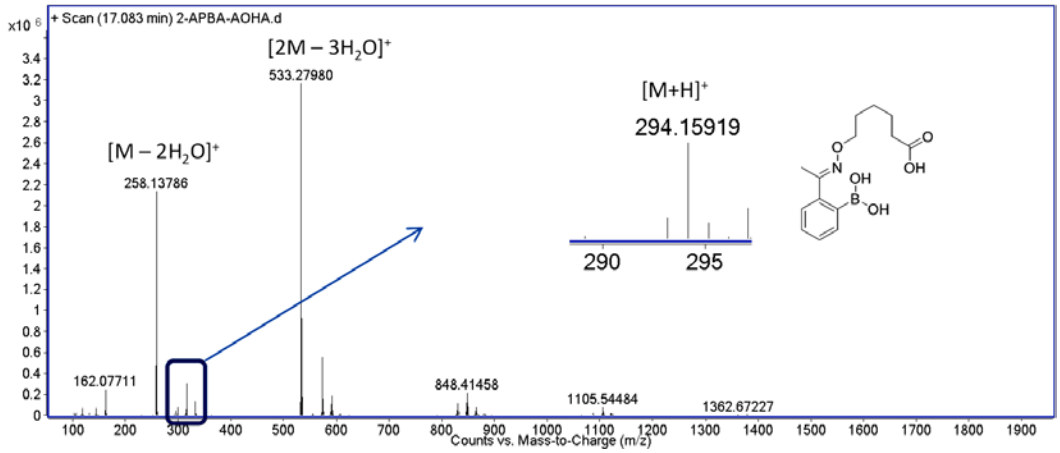

PBS Buffer
pH = 7.4

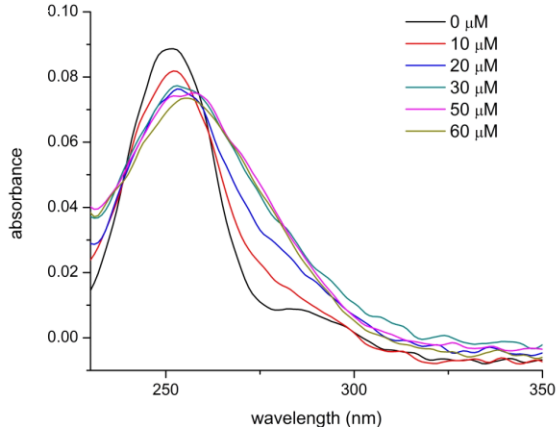

a.
-CH3
Change of chemical shift of aromatic peaks confirms complete product formation
Chemical shift confirms complete product formation
-CH3
f1 (ppm)


b.
2-APBA (0.2 mM)-AOHA (0.2 mM)

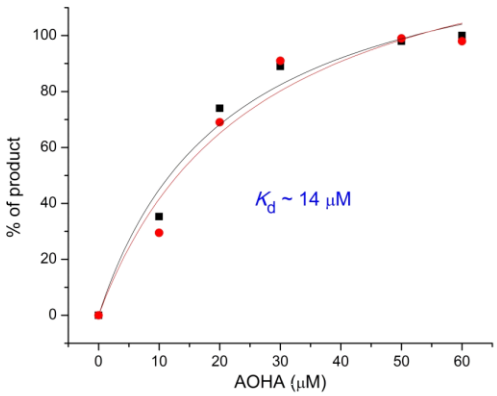
c.


+ Scan (17.083 min) 2-APBA-AOHA.d
[2M – 3H2O]+
533.27980
[M+H]+
294.15919
[M – 2H2O]+
258.13786
290
295
162.07711
848.41458
1105.54484
1362.67227
Counts vs. Mass-to-Charge (m/z)

d.


0 μM
10 μM
20 μM
30 μM
50 μM
60 μM
absorbance
wavelength (nm)

e.


% of product
AOHA (μM)
Kd ~ 14 μM

f.

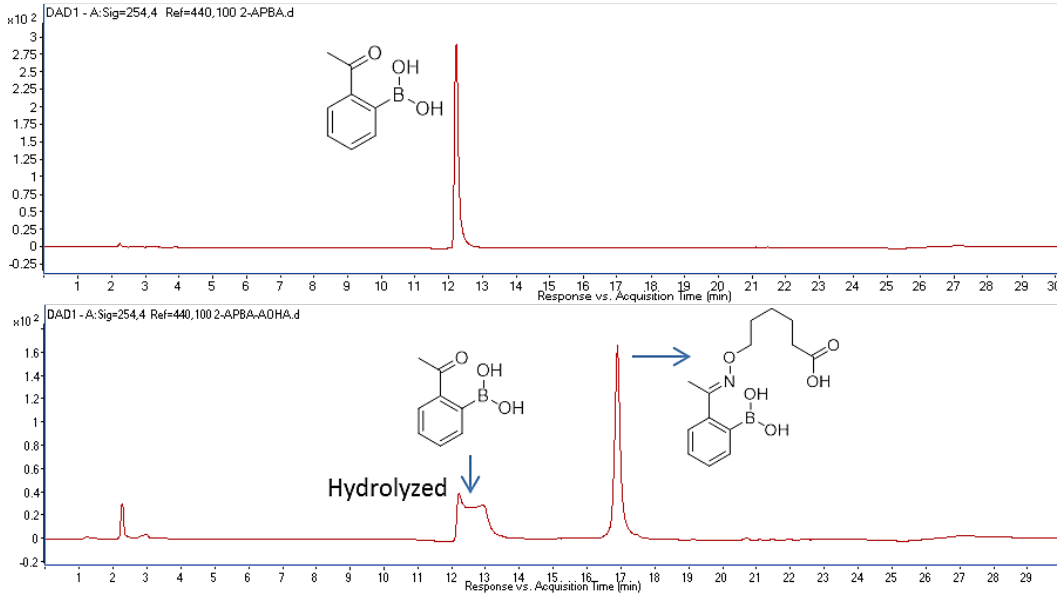

DAD1 - A:Sig=254,4 Ref=440,100 2-APBA.d
Response vs. Acquisition Time (min)
DAD1 - A:Sig=254,4 Ref=440,100 2-APBA-AOHA.d
Hydrolyzed
Response vs. Acquisition Time (min)

Figure S4: a) NMR spectra of 2-APBA (2 mM), 6-aminoxy hexanoicacid (AOHA) (3 mM) and their mixture. b) The NMR spectrum of a mixture of 2-APBA (0.2 mM)-AOHA (0.2 mM), which confirms the complete product formation. c) ESI-MS result of the 2-APBA-AOHA conjugate. d) UV-vis spectral changes with titration of AOHA into 10 µM of 2-APBA. e) Overlay of two titration curves that show consistent results. The titration curves were generated by plotting A_{280}/A_{254} against AOHA concentration and then normalized. f) HPLC analysis of the 2-APBA-AOHA conjugate (0.2 mM) with the mobile phase composed of pH 7.4 buffer and MeCN. The presence of free 2-APBA confirms the quick hydrolysis of the oxime under neutral conditions.

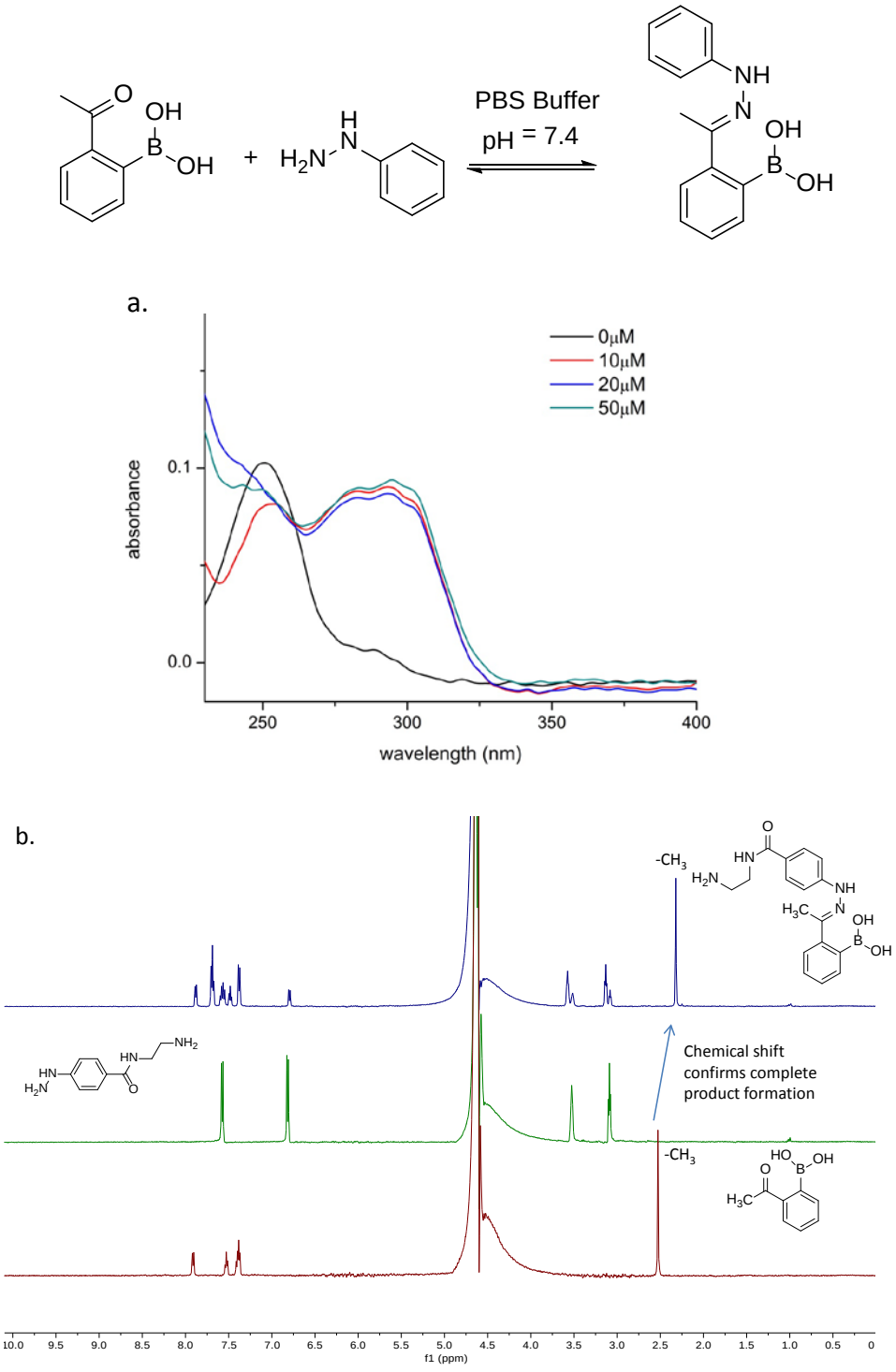


Figure S5: a) UV-vis spectral changes upon titration of Phzn into 10 μM of 2-APBA. The data indicate complete conversion of the starting material, even with 10 μM phenylhydrazine. b) Complete and clean formation of the 2-APBA-Phzn conjugate was confirmed by NMR using a mixture of 2-APBA (1 mM) and a Phzn (1.5 mM).

III. Kinetic study of the hydrazine conjugation with AB1-presenting peptides

The kinetics of conjugation between the peptides and Phzn were studied by performing HPLC (Waters) analysis and the product formation was confirmed by LC-MS. Water/acetonitrile in the presence of 0.1% of formic acid was used as mobile phase. HPLC analysis of product formation was done at multiple time points. The experiment was repeated three times and consistent results were obtained. The kinetic data were fitted using an exponential function, which is expected for a reversible reaction that is pseudo-first order for the forward reaction and the first order for the backward reaction. The assumption that the forward reaction is pseudo-first order is made with the use of Phzn in excess (5 equivalents). We note that, rigorously speaking, the Phzn concentration does change by up to 20% with our experimental setup. However, this 20% variation is unlikely to change the order of magnitude of the determined rate constant, although it may give slightly higher error associated with the rate constant.

If we consider a reversible reaction

$$A + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AB$$

For pseudo first order forward reaction we assume $[A]_0 << [B]_0$. Then,

$$-\frac{d[A]}{dt} = (k_1[B]_0 + k_{-1})[A] - k_{-1}[A]_0 \quad (1)$$

$$\text{or, } -\frac{d([A]/[A]_0)}{dt} = (k_1[B]_0 + k_{-1})[A]/[A]_0 - k_{-1} \quad (2)$$

We define, $x = [A]/[A]_0$ (3) remaining concentration of A, $a = k_1[B]_0 + k_{-1}$ (4) and $b = -k_{-1}$ (5)
The equation (2) simplifies to the following

$$\frac{dx}{ax + b} = -dt \quad (6)$$

Integration on both sides gives

$$\frac{1}{a} \ln\frac{ax + b}{a + b} = -t \quad (7)$$

Rearranging the equation (7) gives

$$x = \frac{k_1[B]_0}{a} e^{-at} + \frac{k_{-1}}{a} \quad (8)$$

If we define Y as fraction of product formation. Then, $y = 1-x$ (9)
Combining (8) and (9) gives,

$$y = -\frac{k_1[B]_0}{a} e^{-at} + \frac{k_1[B]_0}{a} \quad (10)$$

Equation 10 is used to fit the curve determining pseudo first order kinetics.

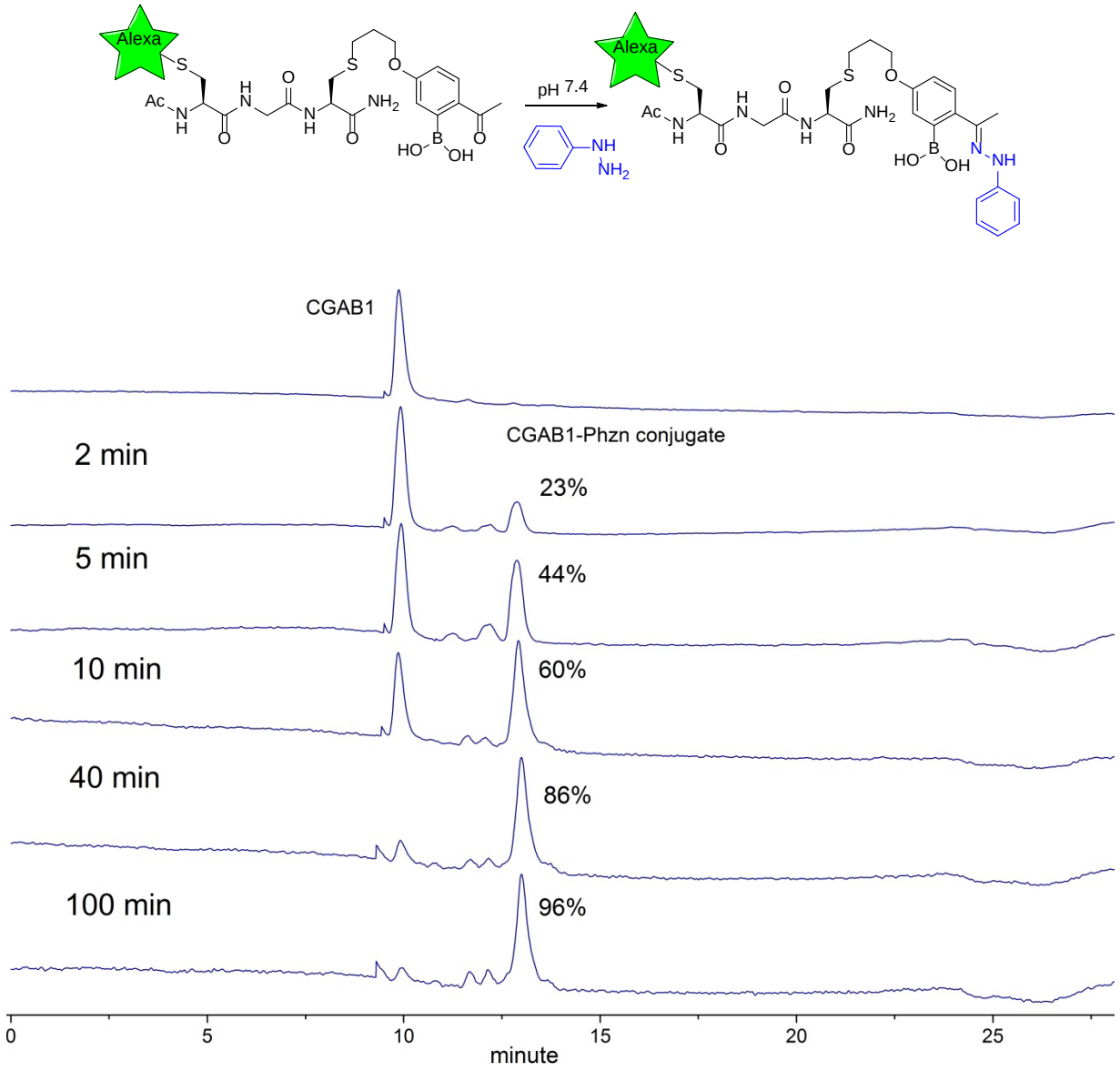


Figure S6: Kinetic analysis of the hydrazine conjugation. The reaction was set up with 1 µM of Phzn and 0.2 µM Ac-C*G(AB1)-NH_2, where C* denotes a Alexa Fluor 488 (AF488) labelled cysteine. The peptide is referred to as CGAB1. The HPLC traces were obtained by monitoring AF488 absorption at 488 nm. The percentage of product formation was calculated based on the peak areas of the labeled and unlabelled peptides. The two small peaks before the product originate from impurities from Phzn. The experiment was performed on Agilent Extend C18 (1.8 µm, 2.1× 50 mm) analytical column using mobile phase of water-MeCN-(0.1% formic acid) with a flow rate 0.3 mL/min. The gradient starts with 5% MeCN for 2 minutes, followed by MeCN ramping up to 95% over 20 minutes. Further, MeCN is kept at 95% for 2 minutes before returning back to 5% over 3 minutes. Then the column is equilibrated with 5% MeCN for 5 minutes to be ready for the next injection.

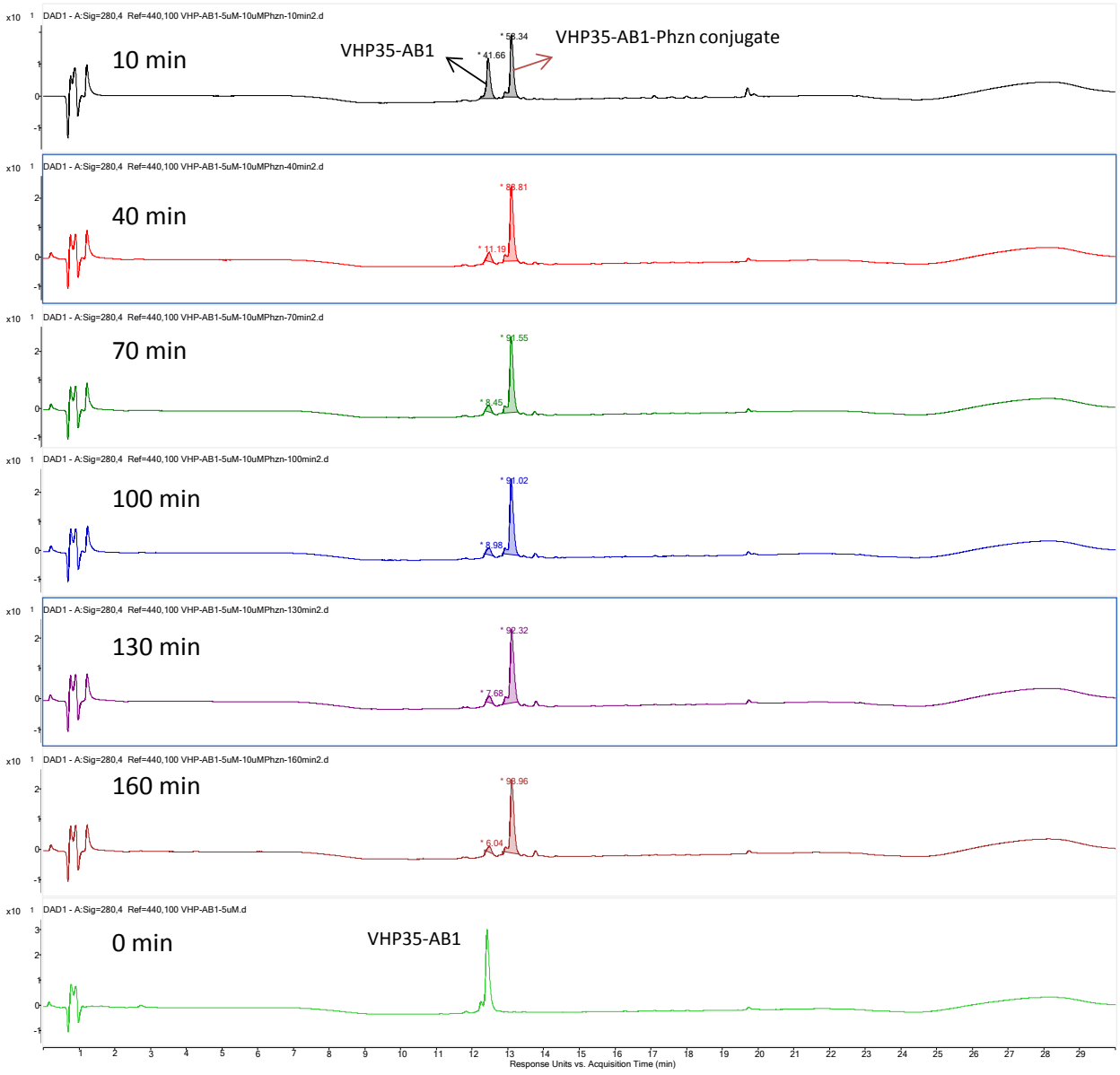


Figure S7: Kinetic analysis of VHP35-AB1 labeling by Phzn. The reaction was set up with 2 µM peptide and 10 µM Phzn. The traces were recorded by monitoring Trp absorption at 280 nm. The time point for respective chromatogram was displayed in the left corner. The 0 min trace was recorded with VHP35-AB1 alone. The percentage of product formation was calculated based on the peak areas of the labelled and unlabelled protein. The LC analysis was performed by following the same method described in the legend of Figure S6.

IV. Assessing potential inhibitors of protein labeling by AB1-Phzn conjugation

The potential inhibitors at specified concentrations were mixed with VHP35-AB1 (5μM) respectively in PBS buffer. Further 1 μL of the Phzn stock (10 mM) was added into the mixture. After 1 hour, LC-MS analysis was performed to quantify the Phzn labelled protein.

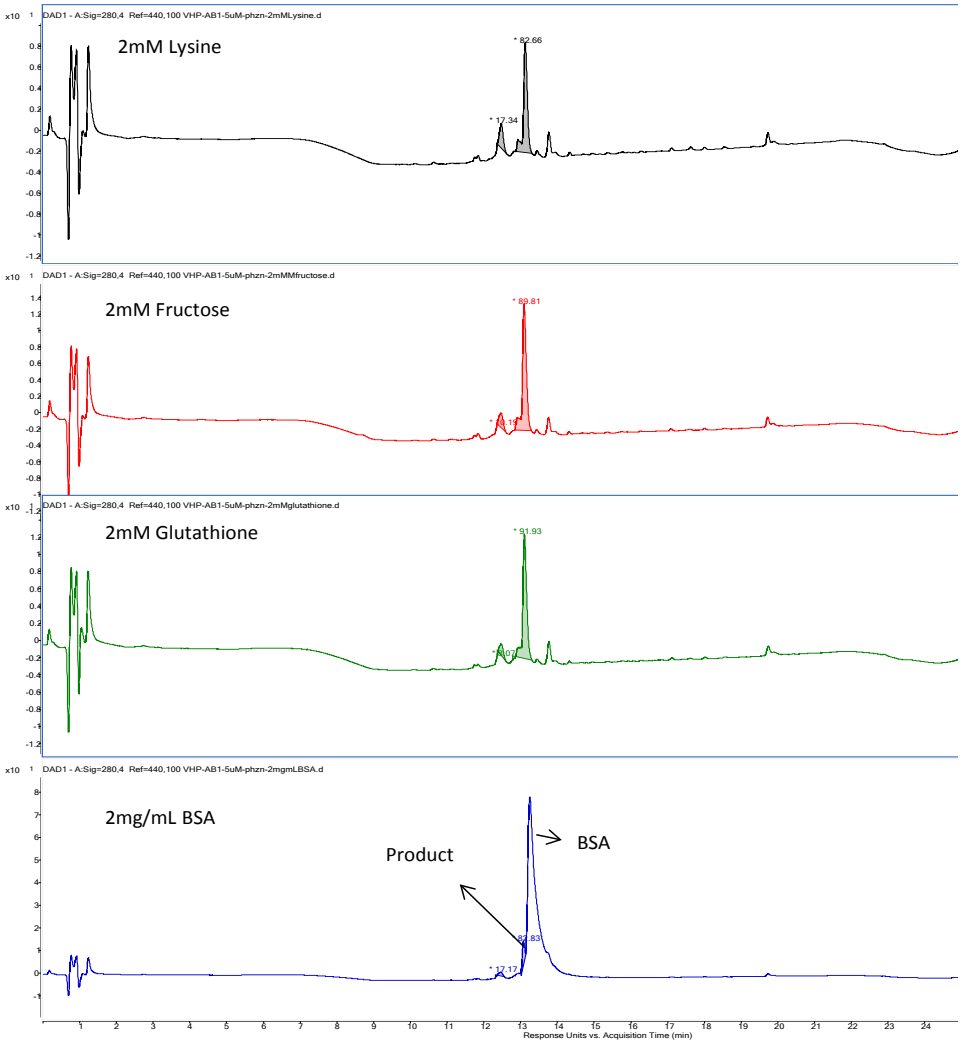


Figure S8: Assessing the interference of Phzn labeling of VHP35-AB1 by small molecules and proteins. All samples were prepared with 5 μM VHP35-AB1 and 10 μM Phzn. The concentration of the inhibiting reagents is shown in the left corner of individual chromatogram. The percentage of inhibition by small molecules (lysine, fructose, glutathione) was assessed by comparing the integrated peak areas of the labelled protein in the presence and absence of inhibitors. For the BSA, the peak of the labelled protein overlaps with that of the BSA, which makes it difficult to integrate. Therefore, the inhibitory effect of BSA was assessed by using the peak height values instead of the areas. The LC analysis followed the same protocol as described for Figure S6 and S7.

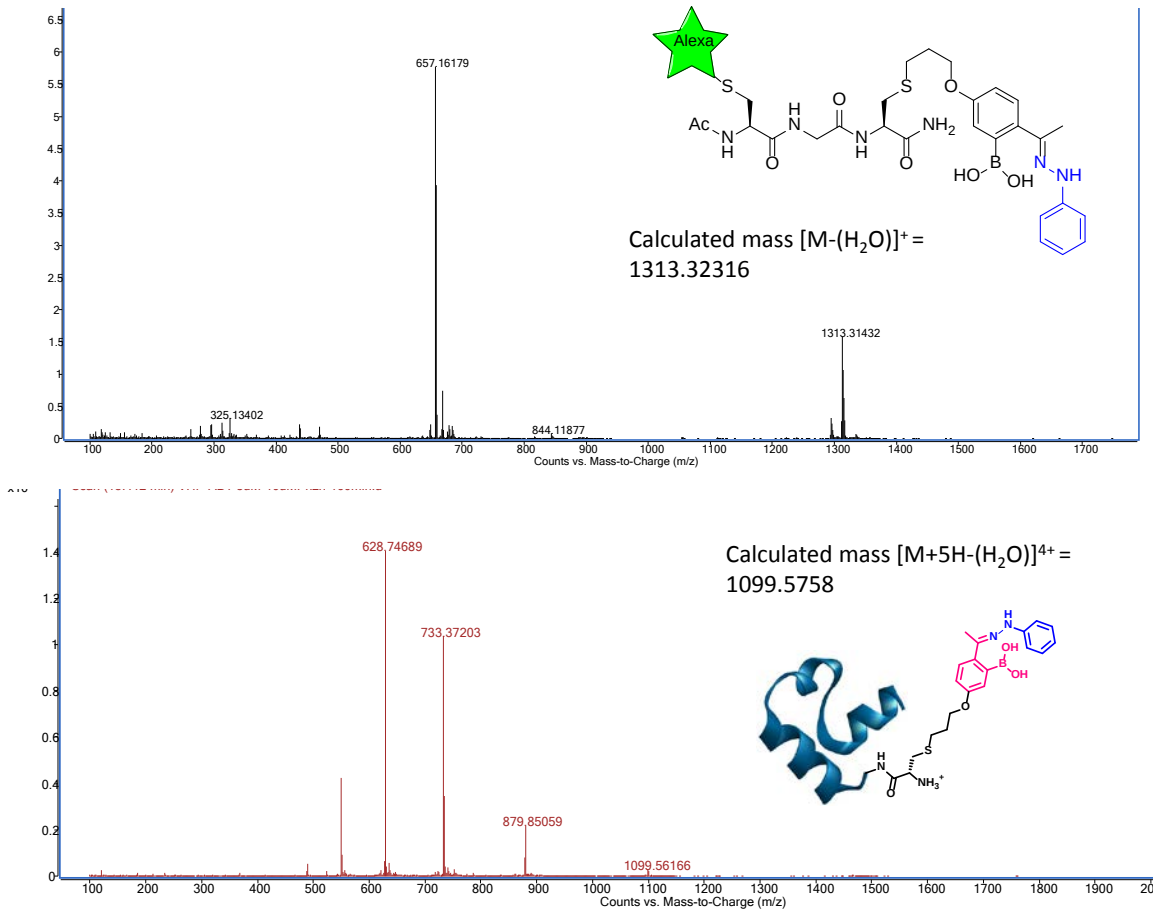


Figure S9: Mass-spec characterization of peptide (top) and protein (bottom) labeling via the hydrazone chemistry of AB1.

V. Hydrazone-hydrazine exchange with AB1 containing peptide

Two different phenylhydrazone conjugates of the peptide Ac-C*K(AB1)-NH_2 (0.2 µM, referred to as CKAB1) was prepared by incubating the peptide with Phzn (1 µM) and HBA (1 µM). The product formation was confirmed by LC-MS. The exchange experiments were performed by mixing the preformed hydrazone with the other hydrazine (1 µM). The kinetics of imine exchange were monitored via HPLC analysis.

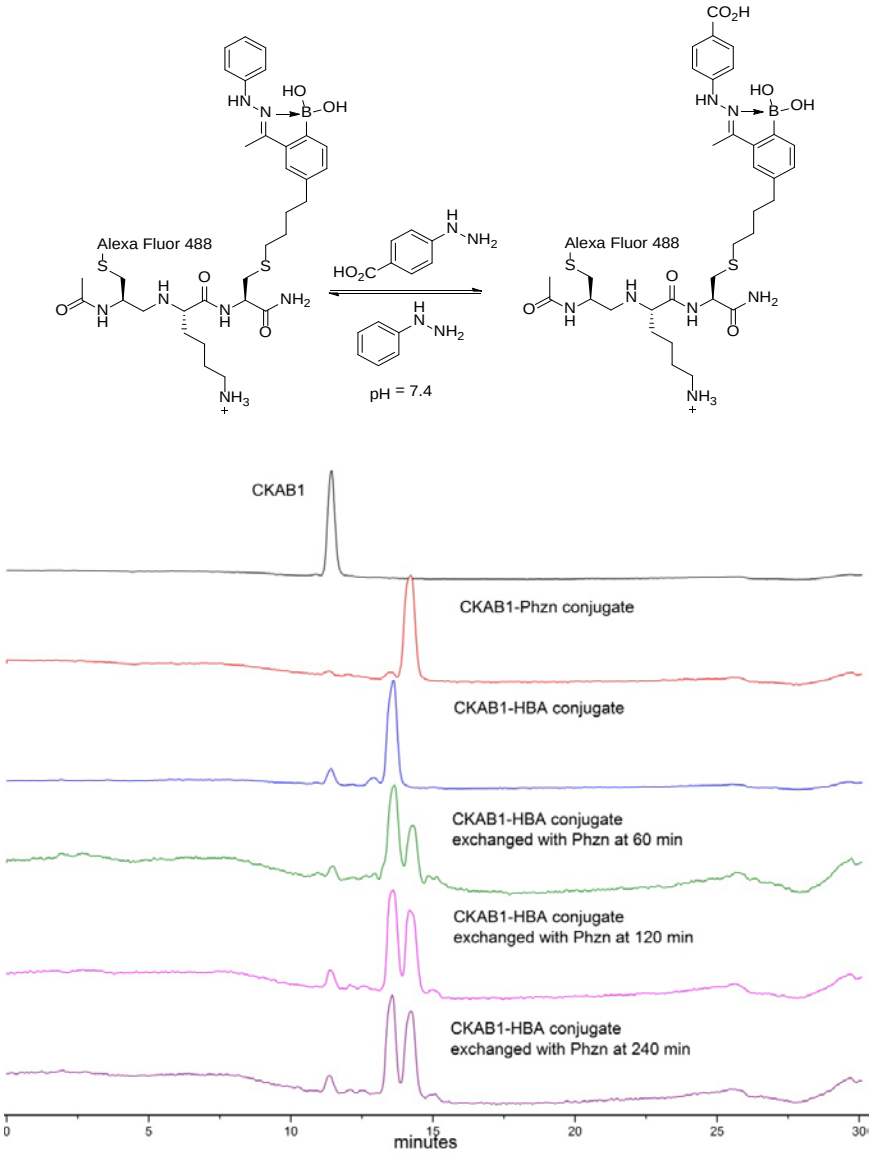


Figure S10: Hydrazone-hydrazine exchange probed by HPLC analysis. The results show the exchange completes within 2 hours under the experimental conditions. The LC setup followed the same protocol as described for Figure S6. The chromatograms were monitored at 488 nm. The small peak of the unlabeled peptide (~11.5 min) indicates a small extent of hydrolysis of the hydrazone, presumably due to the presence of 0.1% formic acid during LC analysis.

VI: Hydrazone-hydrazine exchange in absence of the boronic acid substituent

Two hydrazone derivatives of acetophenone were synthesized using Phzn and 4-hydrazinobenzoic acid (HBA) using previously published protocol.[2] A stock solution of the acetophenone-Phzn (1 mM, 20 µL) conjugate and HBA (1 mM, 20 µL) were mixed in 960 µL PBS buffer (pH = 7.4) to give the final concentration of 20 µM for each reactant. The mixture was incubated for 24 hours and then subjected to LC-MS analysis, which revealed no detectable exchange. The same result was obtained for the other combination with Phzn and the acetophenone-HBA conjugate as the starting materials.

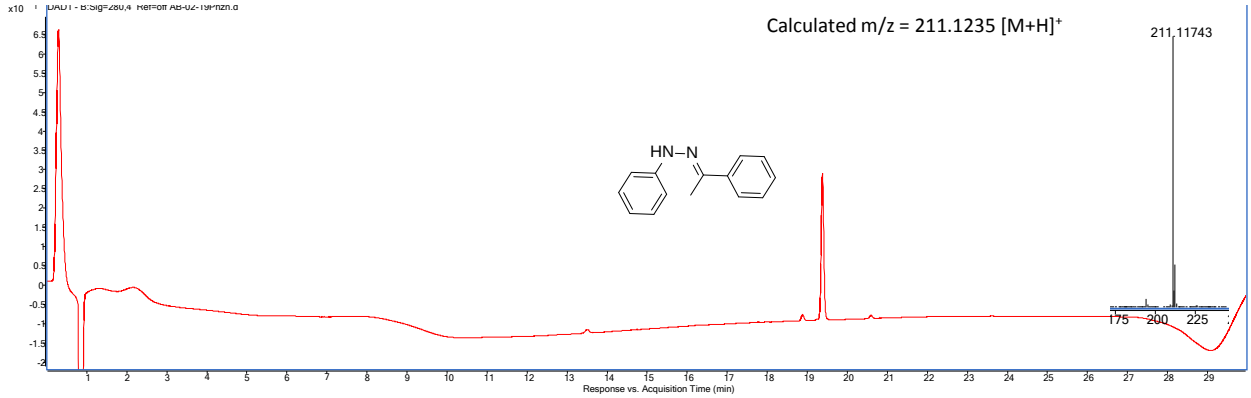


Figure S11: HPLC analysis of the acetophenone-Phzn adduct subjected for HBA exchange. The analysis setup followed the same protocol as described for Figure S6 with the trace monitored at 280 nm. The single peak at 19.4 corresponds to the hydrazone. The lack of hydrazone-hydrazine exchange for the acetophenone derivatives highlights the importance of the boronic acid moiety of 2-APBA in catalyzing the exchange reactions.

VII: Peptide synthesis and labeling

Peptide synthesis and characterization

The two tripeptides were synthesized with the Rink Amide MBHA resin. VHP35-AB1 was synthesized on Fmoc-Phe-Wang resin. The syntheses were carried out on 0.05 mmole scale via Fmoc chemistry. Five equivalents of the commercially available amino acids and HBTU were used for the coupling reaction. The incorporation of the unnatural amino acid, Fmoc-AB1(pin)-OH[1] was accomplished by using four equivalents of the amino acid and an extended coupling time (1 hour). The peptides were cleaved off resin and globally deprotected with reagent K (82.5% TFA : 5% phenol : 5% H_2O : 5% thioanisole : 2.5% EDT). The crude peptides were obtained through ether precipitation and purified by RP-HPLC (Waters Prep LC, Jupiter C18 Column). The peptides were characterized with LC-MS to confirm their identities and excellent purities

(>95%). The peptide sequences and corresponding observed mass are listed in Table 1. The LC-chromatograms of peptides are shown in Figure S12.

The amino acid sequence of VHP35-AB1 is:
AB1-βAGLSDEDFKAVFGMTRSAFANLWKQQHLKKEKGLF
βA stands for beta alanine,which is used as a linker to separate AB1 from the rest of the peptide. The stock concentration of the peptide is determined by monitoring the Trp absorption at 280 nm with the extinction coeffient of 5,690 M^{-1} cm^{-1}.

Alexa Fluor 488 (AF488) labeling of peptides

A stock solution of AF488-C_5-maleimide (1.3 mM in DMF, 100 μL) and the peptide (3 mM in DMF, 50 μL) were mixed together in a small vial. Then, NMM (30 mM in DMF, 6 μL) and TCEP (15 mM in DMF, 5 μL) were added into the reaction mixture sequentially. The reaction was stirred at room temperature for 1 hour to complete. The crude products were purified by RP-HPLC (Waters Prep LC, Jupiter C18 Column). All the labelled peptides were characterized by LC-MS (Table 1) to confirm their identities and excellent purities (> 93%). The labelled peptides were dissolved in PBS (pH 7.4) and the stock concentration was determined by using a UV-vis spectrometer with ε (495 nm) = 71,000 $M^{-1}cm^{-1}$. The LC-chromatograms of pure AF488 labelled peptide CGAB1 is shown in Figure 6 and CKAB1 is shown in Figure S10.

Table 1.

Peptides	Chemical formula	Calculated m/z	Observed m/z
Ac-CG(AB1)-NH_2	$C_{21}H_{31}BN_4O_8S_2$	525.1649 for $[M-(H_2O)+H]^+$	525.1644
Ac-CK(AB1)-NH_2	$C_{25}H_{40}BN_5O_8S_2$	614.2490 for $[M+H]^+$	614.2495
Ac-C*G(AB1)-NH_2	$C_{51}H_{58}BN_8O_{20}S_4$	1223.2635 for $[M-(H_2O)]^+$	1223.2633
Ac-C*K(AB1)-NH_2	$C_{55}H_{67}BN_9O_{20}S_4$	1294.3426 for $[M-(H_2O)]^+$	1294.3442
Villin headpiece35-AB1	$C_{197}H_{299}BN_{50}O_{55}S_2$	1077.07325 for $[M+5H-(H_2O)]^{4+}$	1077.0560

C*: cysteine labelled with AF488-C5-maleimide.

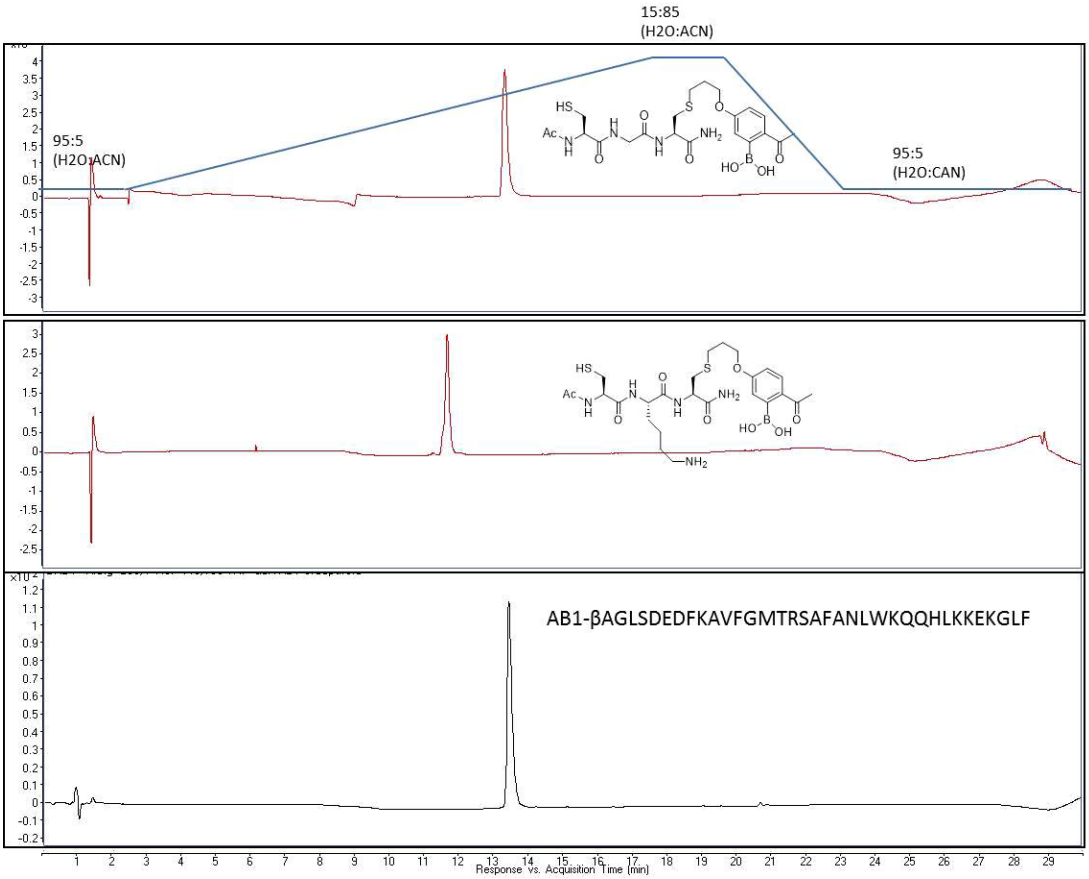


Figure S12: LC-trace of peptide CGAB1 (top), CKAB1(middle) and Villin headpiece35-AB1 (bottom). All the peptide traces were monitored at 280 nm. The peptide purity was checked through analytical column Phenomenex C18 (5 µm, 2.0 × 150 mm) using mobile phase water-acetonitrile-(0.1% formic acid) with a flow rate 0.2 mL/min. The MeCN gradient of the mobile phase is mentioned in top LC-trace, which was followed for another two peptides.

VIII. ^{11}B-NMR experiment

All the boron NMR was recorded in the PBS buffer in the presence of 10% D_2O using 160 MHz Avance NMR spectrometer. The spectra were obtained at 25 ^{o}C with 5,000 scans (~ 17 min). The instrument was calibrated with BF_3 (chemical shift at 0 ppm) before measuring every sample. The ^{11}B-NMR data were collected to confirm the interaction between imine nitrogen and the boron atom in the conjugates of 2-APBA and the data is shown in Figure 4 of the main text. We further compared the ^{11}B NMR chemical shift between the oxime products of 2-formylphenyl boronic acid (2-FPBA) and 2-APBA to rationalize the different observations reported by this work and the recent paper from the Gillingham group (Figure S13).[3]

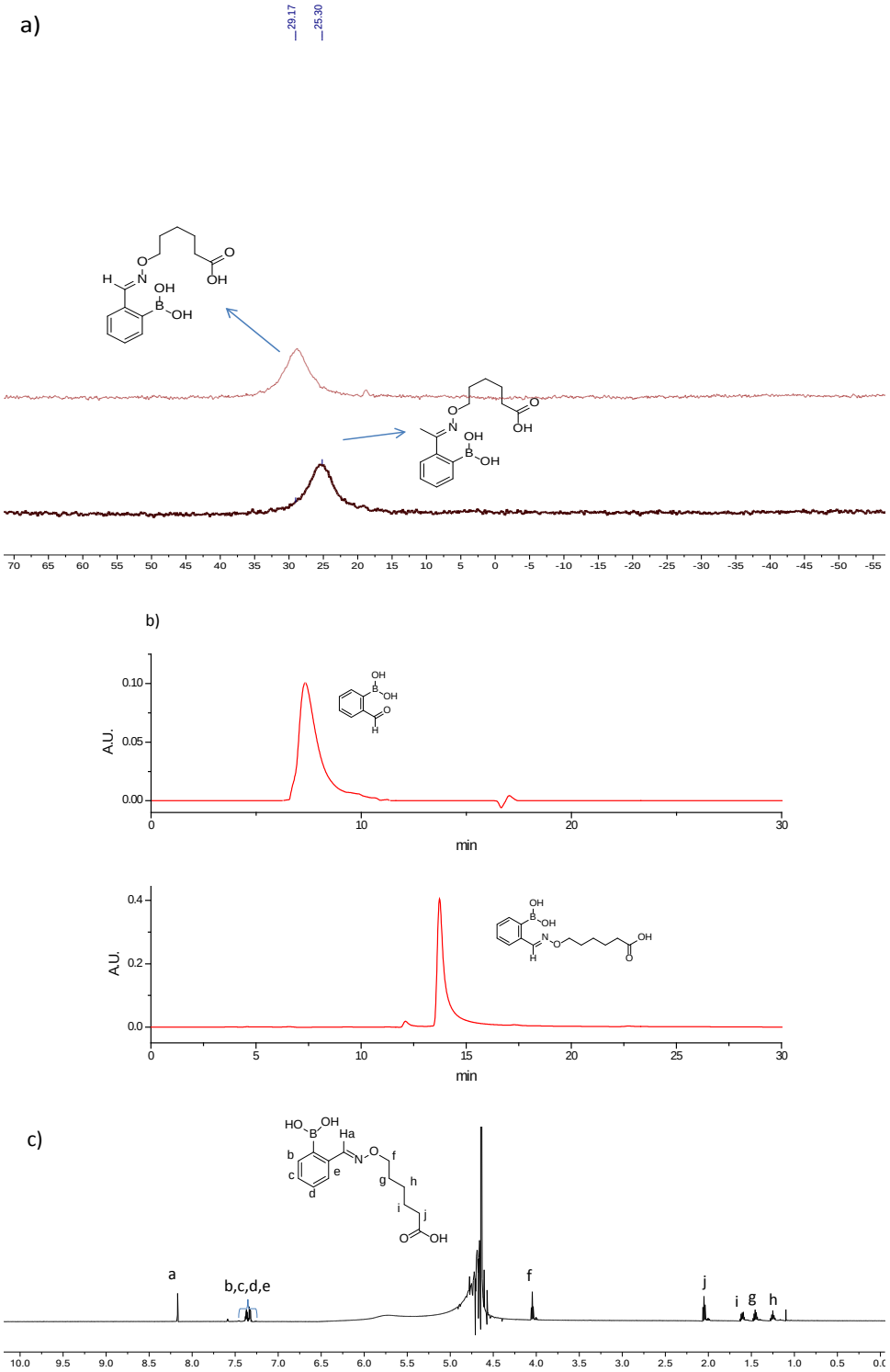
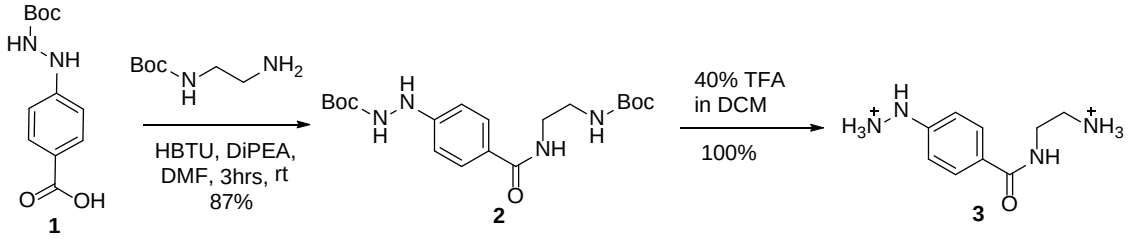


Figure S13: a) ^{11}B-NMR spectra of the oxime products of 2-FPBA (4 mM) and 2-APBA (4 mM). The little upfield shift of the 2-FPBA conjugate indicates minimal iminoboronate formation, which explains why the oxime of 2-FPBA displays higher stability as reported by Gillingham and coworkers.[3] b) HPLC analysis of the 2-FPBA and 2-FPBA-AOHA conjugate (0.2 mM) with the mobile phase composed of pH 7.4 buffer and MeCN. In the chromatogram of 2-FPBA-AOHA conjugate, no peak was found that

corresponds to free 2-FPBA, confirmimg the stability of the 2-FPBA oxime. c) Complete oxime formation was confirmed by the ^{1}H-NMR spectrum of 2-FPBA (0.2 mM) and AOHA (0.2 mM) mixed in PBS (10% D_2O). The spectrum was recorded at 25 ^oC with 96 scans (~ 7 min).

IX. Synthesis of *N*-(2-aminoethyl)-4-hydrazinylbenzamide (3)



Synthesis of 2: 1 (4 mmol, 1.08 g) and Boc-NH-$(CH_2)_2$-NH_2 (4.4 mmol, g) were dissolved together in DMF (5 mL), followed by HBTU (4.04 mmol, 1.55 g) was added to the reaction mixture and cooled to 0 ^oC for 5 min. Then, DiPEA (6 mmol, 1.07 mL) was added with stirring and the reaction mixture was allowed to come to the room temperature. The progress of the reaction was monitored by TLC. After completion (roughly 4 hrs), the reaction mixture was diluted with 250 mL of ethyl acetate and washed with 1N HCl (2 ×150 mL) followed by 10 % sodium carbonate solution in water (2 × 150 mL). Further organic layer was washed with brine (150 mL) and dried over anhydrous Na_2SO_4. The organic layer was evaporated under reduced pressure to give solid pink crude product, which was purified through silica gel using EtOAc/hexane to yield white crystalline product (1.37g, 87%). ^{1}H NMR (DMSO d_6) δ: 8.85 (s, 1H), 8.10-8.08 (d, J = 5.2, 1H), 8.00 (s, 1H), 7.66-7.64 (d, J = 8.7, 2H), 6.87-6.85 (d, J = 8.2, 1H), 6.64-6.63 (d, J = 6 Hz, 1H), 3.26-3.23 (q, J = 5.3 Hz, 2H), 3.09-3.06 (q, J = 5.1 Hz, 2H), 1.37 (s, 18H); ^{13}C NMR (DMSO d_6) δ: 166.23, 155.71, 152.06, 128.38, 123.89, 110.37, 78.99, 77.63, 38.72, 28.07; MS-ESI$^+$: *m/z* calculated for $C_{19}H_{31}N_4O_5$ $[M+H]^+$ 395.2294, found 395.2257.

Synthesis of 3: 2 (2 mmol, 0.793g) was dissolved in 6 mL in DCM and the solution was cooled to 0 ^oC for 5 min. TFA (4 mL) was added slowly to solution during 1 minute in stirring condition. Then reaction mixture was allowed to come in room temperature and it was further stirred for 30 minutes. Then TFA was completely evaporated from the reaction mixture by triturating several times using DCM. The yellow gummy residue was precipitated using ether (50 mL) and white precipitate was collected by centrifugation. Further the white solid product was used directly without further purification. Purity of product was confirmed by NMR and LC-MS. ^{1}H NMR (10% D_2O) δ: 8.57 (s, 1H), 7.81-7.79 (d, J = 7.9, 2H), 7.06-7.04 (d, J = 8.7, 2H), 3.71-3.68 (q, J = 4.8 Hz, 2H), 3.26 (b, 2H); ^{13}C NMR (10% D_2O) δ: 170.60, 147.46, 128.91, 126.91, 113.81, 39.58, 37.49; MS-ESI$^+$: *m/z* calculated for $C_9H_{15}N_4O$ $[M+H]^+$ 195.1240, found 195.1293.

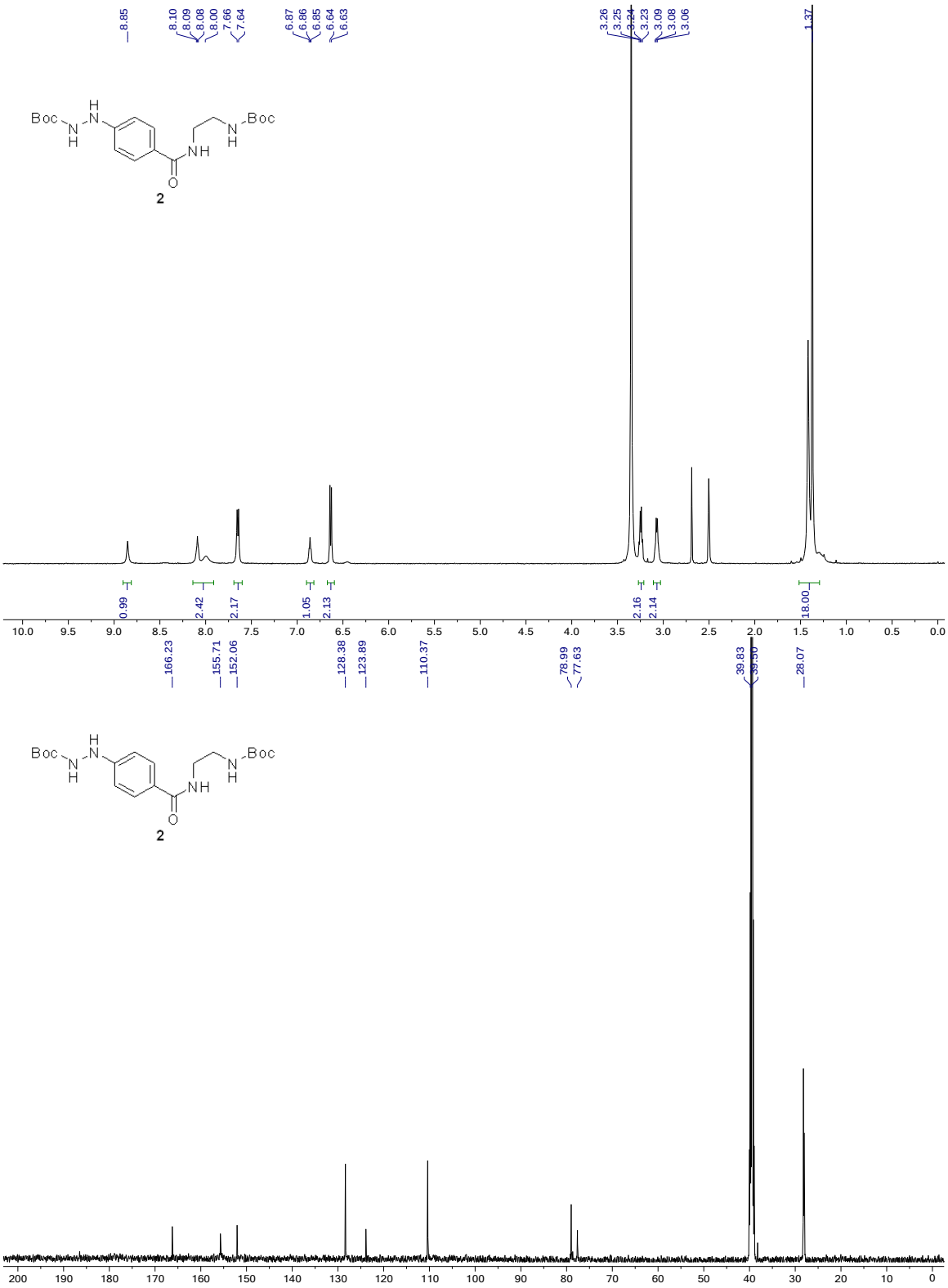


Figure S14: ^{1}H and ^{13}C NMR spectra of compound **2** in DMSO (d_6).

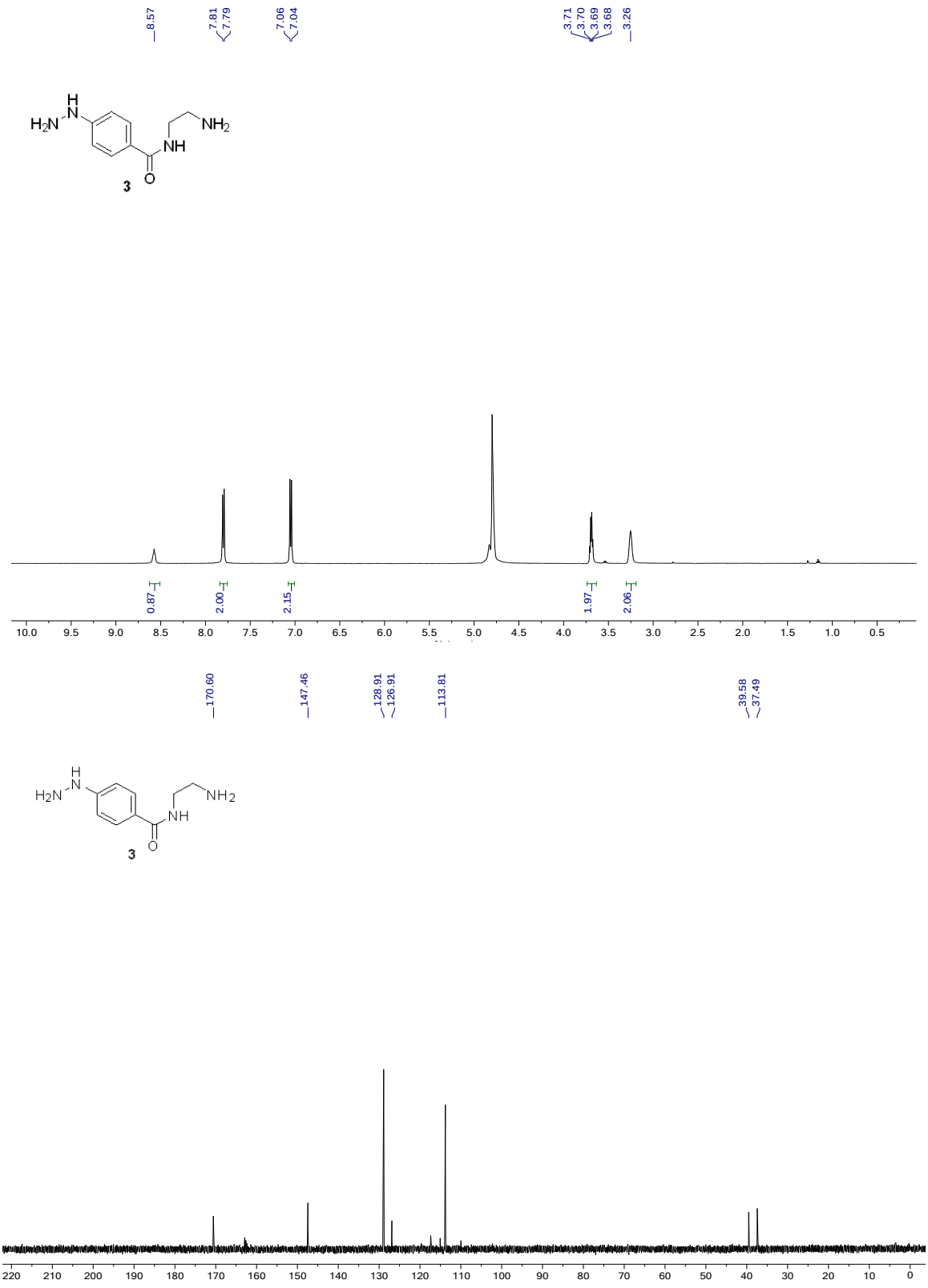


Figure S15: ^{1}H and ^{13}C NMR spectra of compound 3 in D_2O.

X. Reference

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