Electronic Supplementary Material (ESI) for ChemComm.

4-Phosphopyrazol-2-yl alanine: A non-hydrolysable analogue of phosphohistidine

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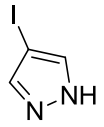
Electronic Supplementary Information

Methods

General methods were as described in the ESI in an earlier paper.[1]

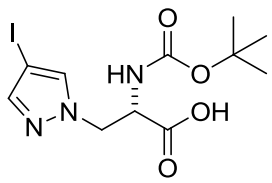
4-iodo-1H-pyrazole



Pyrazole (25.53 g, 375 mmol, 1 eqv) and iodine (47.6 g, 187.5 mmol, 0.5 eqv) were added to a round bottom flask, followed by H_2O (224 mL) and 30 wt% H_2O_2 (26 mL). The reaction was then left to stir over 2 nights, before addition of cold saturated sodium thiosulfate was added until the reaction mixture was colourless. The mixture was then vacuum filtered to give a brown solid, which was recrystallised using hot H_2O to give 4-iodo-1H-pyrazole (39.6 g, 204 mmol, 54% yield).

Colourless needles; m.p. 109-110 °C, lit. m.p. 105-107 °C;[2] δ_H (400 MHz, *CDCl*$_3$) 7.67 (s, 2H), 10.28 (s, 1H); δ_C (101 MHz, *CDCl*$_3$) 56.7 (s), 138.8 (s). Proton and carbon NMR in agreement with the literature.[2]

(2S)-2-{[(tert-Butoxy)carbonyl]amino}-3-(4-iodo-1H-pyrazol-1-yl)propanoic acid, 5

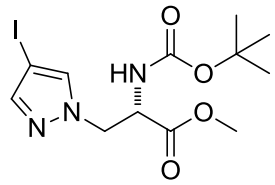


N-Boc-L-serine β-lactone **4** (935 mg, 5 mmol, 1 eqv) in a solution of MeCN (5 mL) was added to a round bottom flask, followed by the addition of 4-iodo-1H-pyrazole (1.164 g, 6 mmol, 1.2 eqv). The reaction mixture was then heated to 50 ^oC and left stirring overnight. The reaction was then concentrated by reduced pressure, and partitioned with Et_2O, followed by washing the aqueous layer with Et_2O (2x). The aqueous layer was then carefully acidified to pH 5, using 1M HCl, before partitioning with EtOAc, followed by a extraction of the aqueous layer with EtOAc (4x) while maintaining the aqueous layer at pH 5. The EtOAc layers were then combined, dried with $MgSO_4$, and the solvent removed via reduced pressure to give compound **5** (991 mg, 2.6 mmol, 52% yield).

Amorphous white solid; m.p. 141-146 ^oC; ν_{max}(film)/cm^{-1} 3336, 3123, 2980, 2922, 2847, 1707, 1513, 1454, 1394, 1368, 1302, 1276, 1251, 1164, 1113, 1063, 1026; δ_H (400 MHz, $CDCl_3$) 1.49 (s, 9H), 4.51-4.56 (m, 1H), 4.70 (dd, J = 4.5, 14.5 Hz, 1H), 4.77 (dd, J = 2.0,

14.5 Hz, 1H), 5.50 (d, J = 5.5 Hz, 1H), 7.47 (s, 1H), 7.65 (s, 1H), carboxylic acid proton not observed; δ_C (101 MHz, $CDCl_3$) 28.3 (s), 52.6 (s), 53.9 (s), 56.6 (s), 80.7 (s), 136.0 (s), 145.2 (s), 155.4 (s), 171.0 (s); $[\alpha]_D^{22}$ +71 (c 1.0, $CHCl_3$); m/z (ES) Found: MH^+ 382.0265 $C_{11}H_{17}N_3O_4I$ requires MH^+ 382.0263.

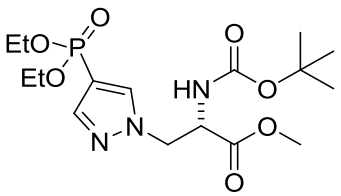
Methyl 2-{[(tert-butoxy)carbonyl]amino}-3-(4-iodo-1H-pyrazol-1-yl)propanoate, 6



Compound **5** (1.143 g, 3 mmol, 1 eqv) in a solution of MeCN was added to a round bottom flask containing K_2CO_3 (636 mg, 4.6 mmol, 1.2 eqv), the reaction was allowed to stir for 30 minutes, before being cooled to 0 ^oC and MeI (852 mg, 374 μL, 6 mmol, 2 eqv) slowly added to the reaction. After complete addition of the MeI the reaction was allowed to warm up to room temperature and left overnight stirring. The reaction mixture was then concentrated via reduced pressure and partitioned between 0.1 M NaOH and EtOAc, the aqueous was then extracted with EtOAc (2x). The organic layers were then combined, dried using $MgSO_4$, and the solvent removed using reduced pressure resulting in the desired product **6** (1.067 g, 2.7 mmol, 90% yield).

Thick colourless oil; ν_{max}(film)/cm^{-1} 3235, 3118, 3056, 3004, 2978, 2943, 1748, 1696, 1551, 1477, 1450, 1431, 1356, 1298, 1282, 1254, 1220, 1201, 1162, 1026; δ_H (400 MHz, $CDCl_3$) 1.47 (s, 9H), 3.79 (s, 3H), 4.51-4.68 (m, 3H), 5.64 (d, J = 5.64, 1H), 7.40 (s, 1H), 7.52 (s, 1H); δ_C (101 MHz, $CDCl_3$) 28.3 (s), 30.9 (s), 52.9 (s), 53.0 (s), 53.9 (s), 80.5 (s), 134.9 (s), 145.3 (s), 155.1 (s), 170.0 (s); $[\alpha]_D^{22}$ +35 (c 1.0, $CHCl_3$); m/z (ES) Found: MH^+ 396.0416 $C_{12}H_{19}N_3O_4I$ requires MH^+ 396.0420.

Methyl 2-{[(tert-butoxy)carbonyl]amino}-3-[4-(diethoxyphosphoryl)-1H-pyrazol-1-yl]propanoate, 7

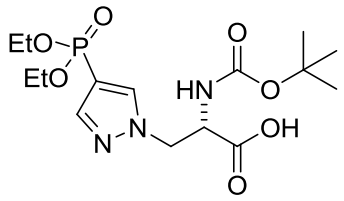


Palladium acetate (16 mg, 0.075 mmol, 0.03 eqv), dppf (83 mg, 0.15 mmol, 0.06 eqv), and sodium acetate (25 mg, 0.3 mmol, 0.12 eqv) were added to a round bottom flask with condenser, before being purged with nitrogen, by applying three cycles of vacuum, followed by nitrogen. Dry THF (5 mL) was then added by syringe followed by $^iPr_2EtN$ (388 mg, 523

μL, 3 mmol, 1.2 eqv). The reaction mixture was then stirred at 60 ^{o}C for 30 minutes, before addition of compound **6** (988 mg, 2.5 mmol, 1 eqv) and diethyl phosphite (345 mg, 322 μL, 2.5 mmol, 1 eqv). The reaction mixture was then brought to reflux and left for 24 hours. The crude reaction mixture was then concentrated by reduced pressure and applied directly to a silica gel column for purification, using EtOAc, to afford the desired compound **7** (648 mg, 1.6 mmol, 64% yield).

Amorphous yellow solid; m.p. 103-106 ^{o}C; ν_{max}(film)/cm^{-1} 3273, 2978, 2944, 1725, 1531, 1440, 1390, 1366, 1339, 1301, 1271, 1255, 1224, 1163, 1137, 1103, 1051, 1019, 1000; δ_H (400 MHz, $CDCl_3$) 1.34 (dt, J = 1.5, 7.0 Hz, 6H), 1.46 (s, 9H), 3.79 (s, 3H), 4.01-4.18 (m, 4H), 4.58 (dd, J = 5.5 Hz, 15.0 Hz, 1H), 4.63-4.73 (m, 2H), 5.44 (d, J = 7.0 Hz, 1H), 7.72 (d, J = 2.0 Hz, 1H), 7.74 (s, 1H); δ_C (101 MHz, $CDCl_3$) 16.2 (d, J = 6.5 Hz), 28.2 (s), 52.75 (s), 52.8 (s), 53.7 (s), 61.9 (d, J = 5.5 Hz), 80.3 (s), 108.0 (d, J = 221.0), 136.0 (d, J = 23.5 Hz), 143.0 (d, J = 13.0 Hz), 155.1 (s), 169.9 (s); δ_P (101 MHz, $CDCl_3$) 13.25; $[\alpha]_D^{22}$ +29 (c 1.0, $CHCl_3$); m/z (ES) Found: MH^+ 406.1745 $C_{16}H_{29}N_3O_7P$ requires MH^+ 406.1743.

(2S)-2-{[(tert-Butoxy)carbonyl]amino}-3-[4-(diethoxyphosphoryl)-1H-pyrazol-1-yl]propanoic acid, 8

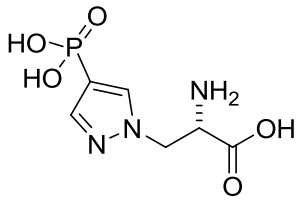


Compound **7** (203 mg, 0.5 mmol, 1 eqv) was added to a round bottom flask followed by the H_2O (1 mL) and THF (1 mL). Once dissolved LiOH (14 mg, 0.6 mmol, 1.2 eqv) was added and the reaction was monitored via TLC until the starting material spot had disappeared. The reaction was then concentrated by reduced pressure, and partitioned with Et_2O, followed by washing the aqueous layer with Et_2O (2x). The aqueous layer was then carefully acidified to pH 5, using 1M HCl, before partitioning with EtOAc, followed by a extraction of the aqueous layer with EtOAc (4x) while maintaining the aqueous layer at pH 5. The EtOAc layers were then combined, dried with $MgSO_4$, and the solvent removed via reduced pressure to give the pure compound **8** (196 mg, 0.5 mmol, 99% yield).

Thick yellow oil, ν_{max}(film)/cm^{-1} 3346, 3119, 2981, 2932, 2571, 1713, 1531, 1443, 1392, 1367, 1339, 1313, 1227, 1165, 1054, 1024; δ_H (400 MHz, $CDCl_3$) 1.30-1.35 (m, 6H), 1.47 (s, 9H), 4.05-4.18 (m, 4H), 4.57-4.72 (m, 2H), 4.77 (d, J = 13.5 Hz, 1H), 5.63 (d, J = 6.5 Hz, 1H), 7.80 (s, 1H), 7.90 (s, 1H), carboxylic acid proton not observed; δ_C (101 MHz, $CDCl_3$) 16.2 (d, J = 7.0 Hz), 28.3 (s), 52.9 (s), 53.8 (s), 62.5 (d, J = 5.0 Hz), 80.3 (s), 107.1 (d, J =

223.0 Hz), 136.6 (d, J = 24.5 Hz), 142.8 (d, J = 13.5 Hz), 155.4 (s), 170.8 (s); δ_P (101 MHz, $CDCl_3$) 13.93; $[\alpha]_D^{22}$ -30 (c 1.0, $CHCl_3$); m/z (ES) Found: MH^+ 392.1592 $C_{15}H_{27}N_3O_7P$ requires MH^+ 392.1587.

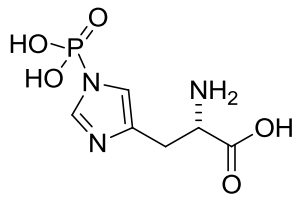
(2S)-2-(Carboxyamino)-3-(4-phosphono-1H-pyrazol-1-yl)propanoic acid, 3



Compound **8** (196 mg, 0.5 mmol, 1 eqv) in a solution of MeCN (5 mL) was added to a round bottom flask, followed by the addition of trimethylsilyl bromide (765 mg, 660 μL, 5 mmol, 10 eqv). The reaction mixture was then brought up to 50 ^oC and left overnight. The solvent was then removed from the reaction mixture via reduced pressure and the resulting residue was triturated using H_2O and isopropanol to give the desired compound **3** (60 mg, 0.26 mmol, 51% yield).

Amorphous cream solid; m.p. 200 ^oC (decomp.); ν_{max}(film)/cm^{-1} 2973, 2159, 1977, 1739, 1613, 1535, 1446, 1387, 1146; δ_H (400 MHz, CD_3OD) 4.55 (dd, J = 3.5, 6.0 Hz, 1H), 4.75 (dd, J = 3.5, 15.0 Hz, 1H), 4.80 (dd, 6.0, 15.0 Hz, 1H), 7.80 (s, 1H), 7.99 (d, J = 2.0 Hz, 1H); δ_C (101 MHz, D_2O) 50.0 (s), 52.7 (s), 111.0 (d, J = 214.5 Hz), 136.3 (d, J = 23.5), 143.2 (d, J = 14.5 Hz), 168.8 (s); δ_P (101 MHz, D_2O) 10.73; δ_P (101 MHz, DMSO) 7.62; $[\alpha]_D^{22}$ +30 (c 1.0, H_2O); m/z (ES) Found: MH^+ 236.0426 $C_6H_{11}N_3O_5P$ requires MH^+ 236.0436.

2-Amino-3-(1-phosphonoimidazol-4-yl) propanoic acid, 2.



2

A solution of histidine (42 mg, 0.27 mmol, 1 eqv) in water (0.75 mL) was added to a round bottom flask followed by potassium aminophosphonic acid **50** (100 mg, 0.74 mmol, 2.7 eqv), and stirred for 3 days, according to the method of Hultquist.[3] The reaction mixture was then directly applied to a silica gel column, eluted with 60% EtOH / 4% 25% $NH_{3(}$aq) / 26 % H_2O. Isopropanol was added to the fractions containing compound **2** and the white precipitate (16 mg) was isolated by filtration off to give material that was ~ 90% pure **2** (as judged by NMR) and contaminated with histidine.

White amorphous solid; m.p. 230 ^{o}C decomp.; ν_{max}(film)/cm^{-1} 3282, 2797, 2610, 2466, 2160, 2031, 1977, 1713, 1619, 1453, 1417, 1131, 1018; δ_H (400 MHz, *D_2O*) 2.88 (dd, *J* = 9.5, 15.0 Hz, 1H), 3.08 (dd, *J* = 4.0 Hz, 15.0 Hz, 1H), 3.76 (dd, *J* = 4.0, 9.5 Hz, 1H), 6.98 (s, 1H), 7.64 (s, 1H); δ_C (101 MHz, *D_2O*) 29.5 (s), 55.0 (s), 118.5 (s), 134.9 (s), 139.5 (s), 175.5 (s); δ_P (101 MHz, *D_2O*) -4.69 (s); enriched $[\alpha]_D^{22}$ +20 (c 1.0, H_2O); m/z (ES) Found: MH^+ 236.0428 $C_6H_{11}N_3O_5P$ requires MH^+ 236.0436.

General coupling procedure of (2S)-2-(carboxyamino)-3-(4-phosphono-1H-pyrazol-1-yl)propanoic acid, 3 to a carrier protein using glutaraldehyde.

Carrier protein, KLH or BSA, (50 mg) and (2S)-2-(carboxyamino)-3-(4-phosphono-1H-pyrazol-1-yl)propanoic acid, **3** (5 mg) were added to a vessel followed by the addition of pH 7.4 phosphate buffered saline (1 mL, 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl). Glutaraldehyde (50 wt% in H_2O) was then added to the reaction mixture to result in a final concentration of 0.1 wt% glutaraldehyde and was then left overnight at 4 ^{o}C, rotating gently. $NaBH_4$ (5 mg) was added to the solution and was then left overnight at 4 ^{o}C, rotating gently. The reaction mixture was then diluted to 2.5 mL using pH 7.4 phosphate buffered saline and then subjected to dialysis overnight at 4 ^{o}C in pH 7.4 phosphate buffered saline (1 L), to give the desired protein-amino acid conjugate in solution (20 mg / mL).

Maldi-TOF Mass spectrometry of BSA-Amino acid conjugates.

Sinapinic acid was used as the matrix for MS analysis of peptide or protein solutions, respectively. Aliquots (1.3 μL) of the matrix solution (10 mg SA in 1 mL aqueous solution of 66% [v/v] acetonitrile containing 0.1% [v/v] formic acid) were mixed with the peptide or protein solution, made by procedure A, and spotted onto a MALDI-TOF target. A Bruker Reflex III with a nitrogen laser was used to analyze the samples. Protein/peptide mass was measured using the positive-ion linear mode.

BSA: 66441.8

(2S)-2-(Carboxyamino)-3-(4-phosphono-1H-pyrazol-1-yl)propanoic acid, **3** conjugated by glutaraldehyde to BSA: 71702.2.

Each glutaraldehyde-conjugated residue **3** adds 303 to the MW, so the average number of residues conjugated to BSA is 17.3.

Procedure for the generation of (2S)-2-(carboxyamino)-3-(4-phosphono-1H-pyrazol-1-yl)propanoic acid, 3 antibodies.

Protein-(2S)-2-(carboxyamino)-3-(4-phosphono-1H-pyrazol-1-yl)propanoic acid, **3** solution (100 μL - ~ 1 mg/mL), made by the coupling procedure above, was added to an equal volume of Freunds complete adjuvant. The solution was then passed through a 23G needle until an emulsion, which does not separate on standing, forms. Each rat was then injected subcutaneously with the emulsion (200 μL). After 2 weeks an additional injection was performed, using the same procedure as above but with Freunds incomplete adjuvant, and was then repeated again after an additional 2 weeks. After 10 days, test bleeds were collected from the rats for assays. If the immunoresponse was too low, a further injection was performed using the solution made with Freunds incomplete adjuvant, and test bleeds collected for assays. After 3 weeks, post the last injection, one final injection is performed with the solution of antigen and Freunds incomplete adjuvant, then the terminal bleed is collected after 10 days. The terminal bleeds are then allowed to clot overnight at 4 ^{o}C, before centrifuging at 13000 rpm in a benchtop microfuge, to give the serum as the top clear layer.

General dot blot procedure.

Solutions of the BSA-amino acid conjugates of histidine, τ-phosphohistidine, and the analogue **3**, were first diluted 1000 fold. These solutions as well as the commercially available solutions of BSA conjugated to phosphoserine, phosphothreonine, and phosphotyrosine were spotted (2 μL, ~ 1 mg/mL) onto a PVDF membrane. Once the spots were dry, the membrane was rinsed with methanol before being blocked in 5% non-fat dry milk in 10 mM Tris-HCl pH 8.0, 17 mM NaCl (TBS), tween (0.05%) (5% NFDM/TBST) for 1 hour and then incubated with the rat sera raised against analogue **3** at 1/3000 dilution in 5% NFDM/TBST overnight at 4 °C. The membrane was then washed using TBS-tween (0.05%) 3 times for 15 minutes, before being incubated with a 1/2000 fold dilution of goat anti-rat IgG-HRP conjugate in 5% NFDM/TBST for 60 minutes at room temperature. Membranes were then washed 3 times for 15 minutes using TBS-tween (0.05%) and then treated with SuperSignal West Dura Chemiluminescent Substrate for 3 minutes. The images were then captured on a Chemidoc XRS^{+} CCD imaging system.

General affinity column preparation (for rat antisera).

AminoLink coupling resin (4 mL) was added to a column and the storage solution drained off to the level of the resin. The column is then washed with 1M pH 7.4 PBS (12 mL) and the

contents then drained. A solution of histidine conjugated to KLH (4 mL)), prepared using the method highlighted above, was added to the resin followed by a solution of 5M $NaCNBH_3$ in 1M NaOH (40 μL). The reaction was then mixed by end-over-end rocking overnight at 4 ^oC. The solution is then drained from the column and washed with 1M pH 7.4 PBS (12 mL) and drained. 1M Tris.HCl pH 7.4 was then added to the resin followed by 5M $NaCNBH_3$ in 1M NaOH (40 μL), and mixed gently at room temperature for 30 minutes by end over end rocking, before draining off the solution. The column is then washed with 1M NaOH (40 mL) and then 1M pH 7.4 PBS containing 0.05 wt% sodium azide for storage.

Protocol for affinity depletion of the rat polyclonal antibody.

The storage solution was drained from the affinity column containing the histidine conjugated to KLH, and washed with 1M pH 7.4 PBS solution (10 mL). The solution was then drained to the level of the resin, before gentle addition of the 4-phosphopyrazole-2-yl alanine polyclonal serum (0.5 mL) to the top of the resin. The serum was then allowed to flow into the resin, before being left overnight at 4 ^oC, the resin was the washed with 1M pH 7.4 PBS solution collecting 500 μL fractions. The fractions were then tested *via* ELISA to establish the quality of the antibodies collected.

General Western blotting and sample preparation

The human bronchial epithelial cells (16HBE14o-) were obtained from Dr. Gruenert[4] and cultured as described previously.[5] Whole cell lysates were prepared by lysing the cell using lysis buffer - 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0, with protease inhibitors (Protease inhibitors cocktail Roche Tablets) and 1mM PMSF. Additionally, a membrane and cytosol fraction was prepared from cells as previously described.[6] Briefly, the cells were scraped and dislodged into homogenisation buffer (250 mM sucrose, 10 mM Triethanolamine pH 7.6) containing NaF 50 mM, DTT 1 mM, benzamidine 1mM, EDTA 1 mM and complete protease inhibitor cocktail (1 tablet/50 ml, Roche). Pooled scrapings were homogenised and spun at 600 **g** for 15 min. The post-nuclear supernatant was re-spun at 100 000 **g** for 60 min. The pellet was re-suspended in homogenisation buffer and spun for 30 min at 100 000 **g** (this procedure was repeated 3 times). All procedures were conducted at 4 °C. Aliquots of cytosol and membrane pellet were stored at -80 °C. Protein concentration was determined according to the method of Bradford[7] using bovine albumin serum as standard.

Proteins (50-100 μg), separated by SDS-PAGE, were transferred to PVDF membrane (Millipore). Pre-stained markers were used to confirm transfer. The blots were probed with antibodies to anti-pPza 3 (1:500), anti-phosphotyrosine (clone 4G10, 1:1000), and Horseradish Peroxidase (HRP) conjugated secondary antibody (1:10,000) followed by supersignal™ West Pico chemiluminescence detection (Pierce).

Western blot for BSA-pHis

Western blots were performed with τ-phosphohistidine-BSA conjugate to establish the utility of the antibody in a standard biological technique. Denaturing of the τ-phosphohistidine-BSA conjugate using Laemmli buffer at 90 °C for 3 min yielded no signal (not shown). Denaturing was therefore repeated at room temperature using 10x Laemmli buffer for 30 min; although the target protein could now be detected, denaturing was still incomplete (Figure S3, A). Lithium dodecyl sulphate (LDS) is an alternative denaturing reagent that is used for more efficient denaturing of proteins at lower temperatures.[8] Use of Laemmli buffer containing LDS instead of SDS allowed for a more efficient denaturing, leaving a single band at the expected molecular weight (Figure S3, B).

Immunoprecipitation

Cell lysate from 16HBE14o- cells was incubated with protein G Sepharose beads for 1 hour at 4°C to pre-clear the lysate. Following incubation, the mixture was spun down and the supernatant incubated with 2μg – 6μg of the respective immunoprecipitating antibody for 2 hours with rotation at 4°C. Then fresh protein G Sepharose beads were added and incubated overnight with rotation at 4°C. The immunoprecipitation solution was spun down and the supernatant discarded. The pellet, consisting the immunoprecipitate, was washed with lysis buffer and aliquoted.

For phosphohistidine containing proteins, aliquots of the immunoprecipitate (from above) were re-suspended in protein loading buffer containing LDS (1X Protein Loading Buffer (EC-887, National Diagnostics) and 1% 2-mercarptoethanol for 30 minutes at room temperature to denature the proteins.

Affinity Columns

To prepare columns for affinity purification, BSA conjugates of His and pHis were each immobilized on NHS-activated agarose (Pierce Biotechnology) according to the

manufacturer's instructions. Reactions were carried out at ambient temperature in PBS pH 7.4, at a starting concentration of 3 mg/mL BSA conjugate. The resin was then stored at 4 °C in PBS until use. Analysis of column flow-through from the coupling reactions, according to the provided protocol, indicated coupling efficiencies of 75% (BSA-His) and 90% (BSA-pHis).

Affinity Purification

The storage solution (1M PBS pH 7.4) was drained from the affinity column containing BSA-His (histidine conjugated to BSA via glutaraldehyde linker), and washed with 10mM Tris pH 7.5 solution. The solution was then drained to the level of the resin. The rabbit serum was diluted with 10mM Tris pH 7.5 at 1:1 volume ratio before addition to the resin. The serum was incubated in the resin for 1 hour at room temperature with rotation. The resin was allowed to drain and the flow through collected. The collected flow through was applied to Sepharose BSA-pHis (phosphohistidine conjugated to BSA via glutaraldehyde) column and was incubated for 1 hour at room temperature with rotation. The flow through and wash fractions were collected in column volumes. Elution with 100mM glycine pH 2.2 was carried out and the pH of the eluate immediately adjusted with 1M Tris pH 10 (10% of the final volume of each fraction). All the fractions were then analysed by ELISA and western blot to establish the efficiency of the purification process and the quality of the antibodies.

Elisa

A 96- well plate was coated with 2µg/ml BSA-pHis (phosphohistidine conjugated to BSA), 100µL per well and incubated overnight at 4 °C. The wells were then emptied and washed 3 times with 1M pH 7.4 PBS/Tween-20 (0.05%), and then dried. 1M pH 7.4 PBS/Tween-20 + 0.2% gelatin (150 µL) was added into each well and the plate was incubated for 2 hours at 37 °C, before being washed 3 times with 1M pH 7.4 PBS/Tween-20 (0.05%), and dried. 1M pH 7.4 PBS/Tween-20 + 0.2% gelatin (100µL) was added to each well, before adding the primary antibody (100 µL), diluted 200 fold using 1M pH 7.4 PBS/Tween-20 + 0.2% gelatin. The wells were washed 3 times with 1M pH 7.4 PBS/Tween-20 (0.05%), and dried. 50µL of the anti-rabbit-HRP labelled secondary antibody, diluted 2000 fold in 1M pH 7.4 PBS/Tween-20 + 0.2% gelatin, was added to each well and incubated for 1 hr at 37 °C, before being washed 3 times with 1M pH 7.4 PBS/Tween-20 (0.05%), and 2 times with distilled water. The 96-well plate was then dried and TMB was added (50 µL) to each well,

before being allowed to develop for 10 minutes at room temperature. The development was then stopped with 2M H_2SO_4 (50 µL) and read with plate reader at a wavelength of 450nm.

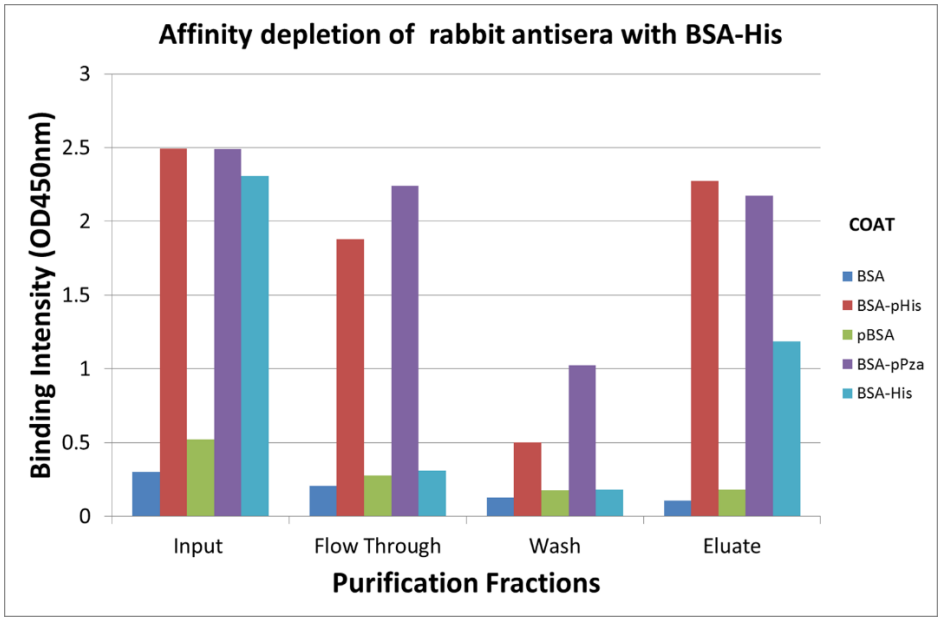
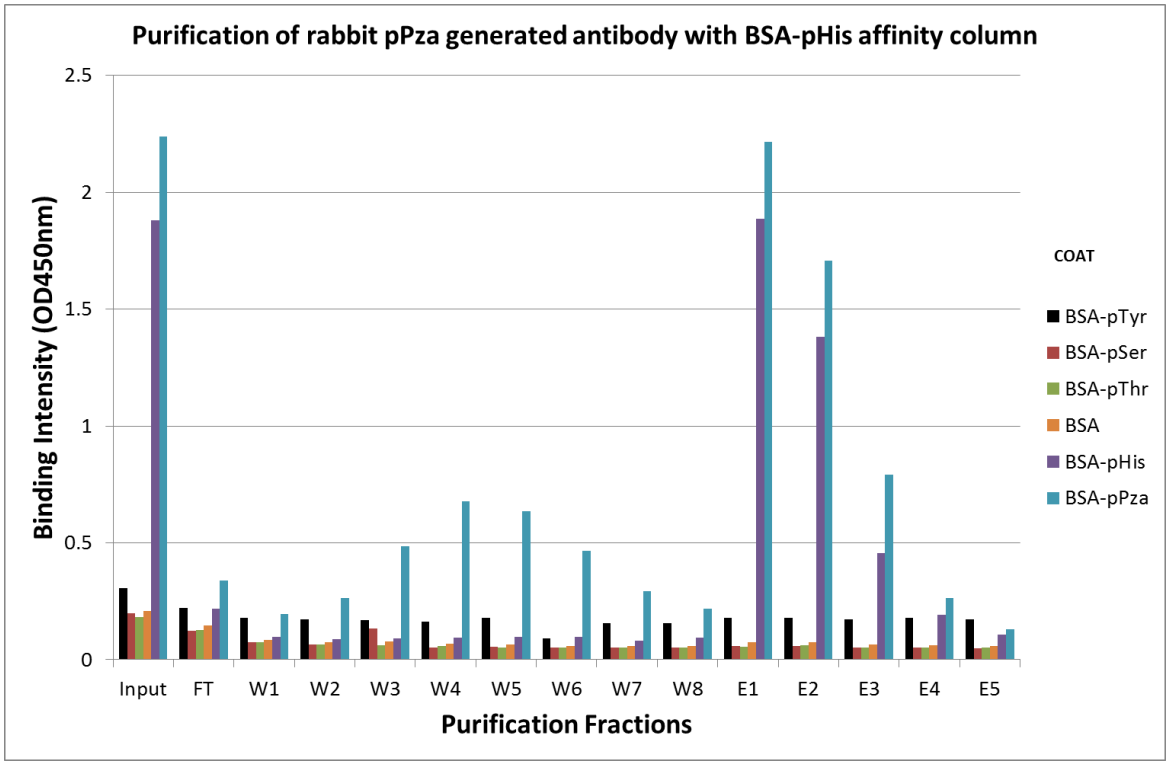


Figure S1. Affinity depletion of pPza generated rabbit antisera. Rabbit polyclonal antibody was affinity depleted using a Agarose-BSA-His affinity column: ELISA analysis of fractions – Input, Flow through, Washes and Eluate. Plate was coated with BSA, BSA-His, pBSA, BSA-pHis, BSA-pPZa. (pBSA – BSA chemically phosphorylated using phosphoramidate)

a)



b)

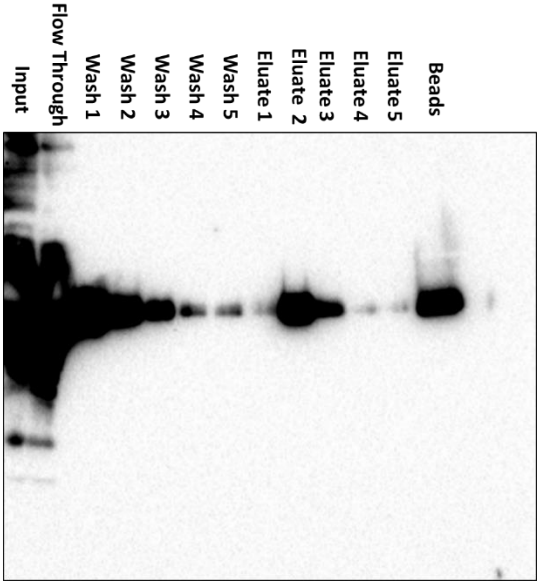


Figure S2. Affinity purification of pPza generated rabbit antisera. a) Rabbit polyclonal antibody affinity depleted using an agarose-BSA-His affinity column was purified on an agarose-BSA-pHis affinity column: ELISA analysis of fractions – ELISA analysis shows binding intenstity of purified pPza antibody in various fractions to phosphoamino acids measured at 450nm - Input, Flow through (FT), Washes and Eluate. Plate was coated with BSA, BSA-His, pBSA, BSA-pHis, BSA-pPZa. (pBSA – BSA chemically phosphorylated using phosphoramidate). b) Western blot of the purification profile of pPza rabbit antisera on an agarose BSA-pHis (phosphohistidine conjugated to BSA via glutaraldehyde) column.

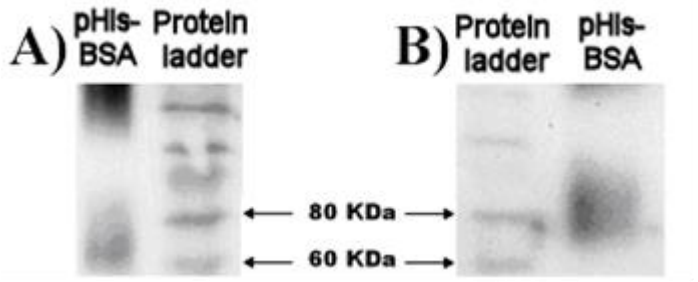


Figure S3. Western blot of τ-phosphohistidine conjugated to BSA denatured by Laemmli buffer containing either A) SDS or B) LDS, for 30 min at RT, and probed with **pPza** rat polyclonal antibodies.

Acknowledgements

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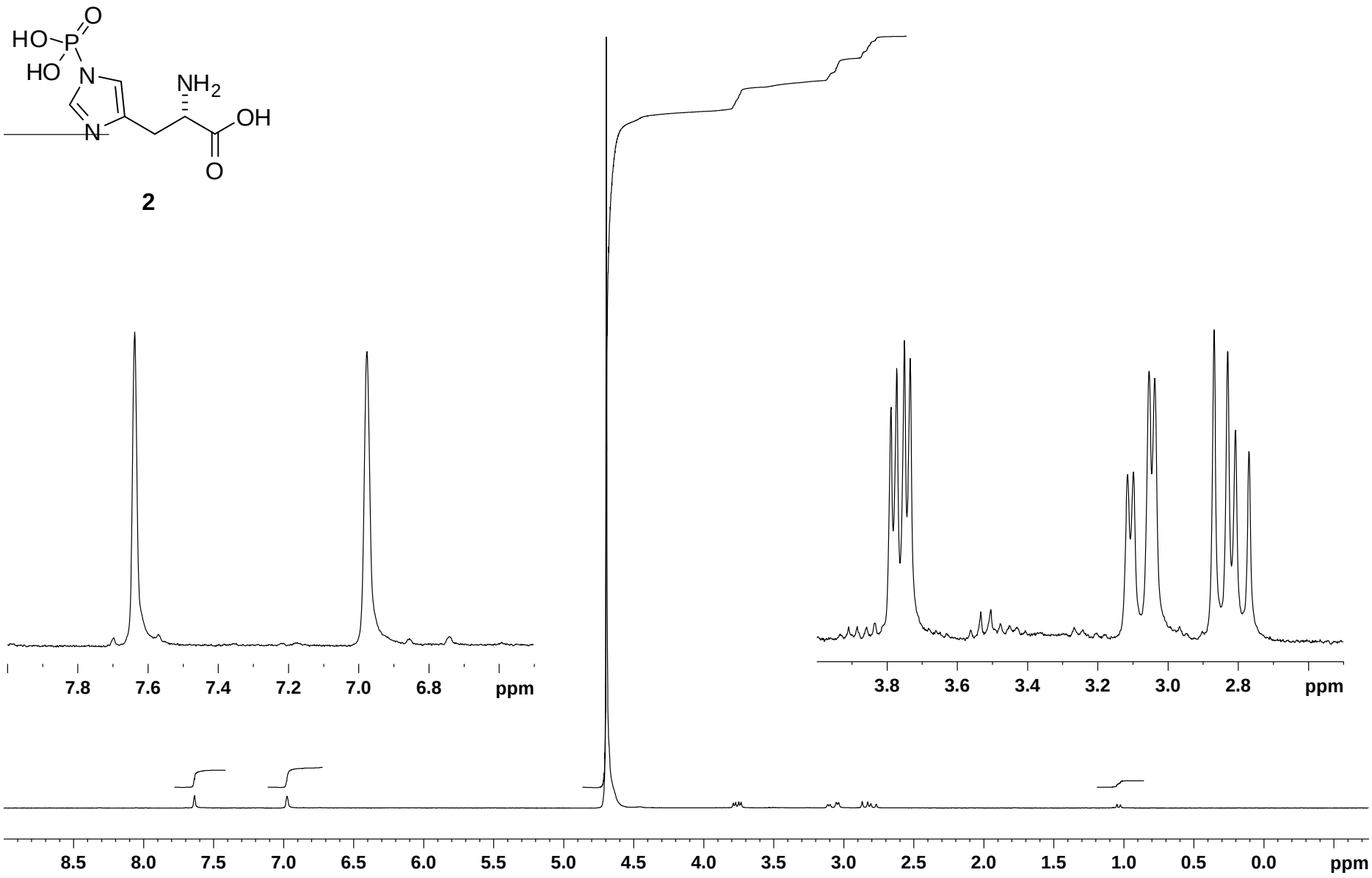
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ppm
3.8
3.6
3.4
3.2
3.0
2.8
ppm
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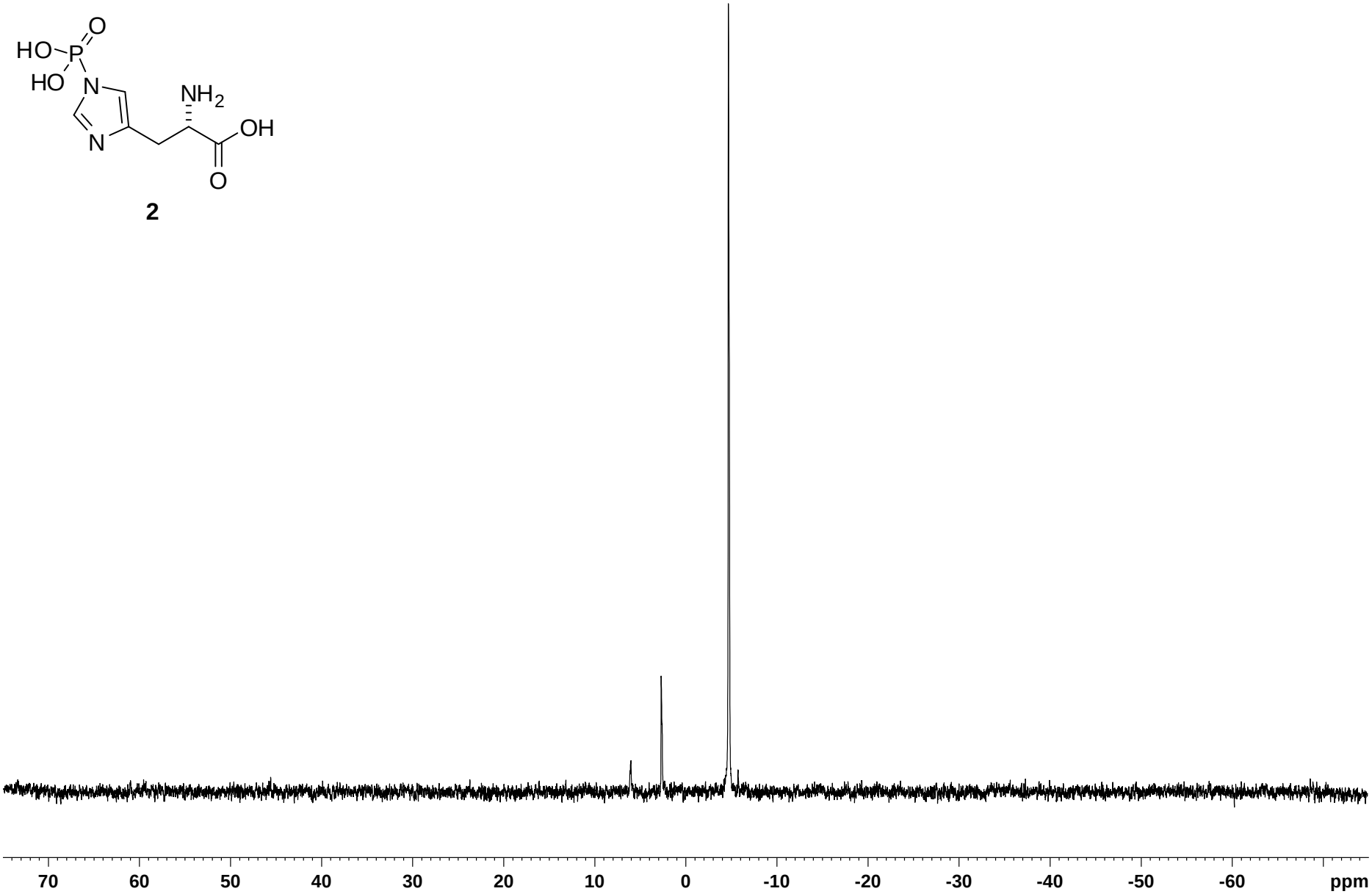
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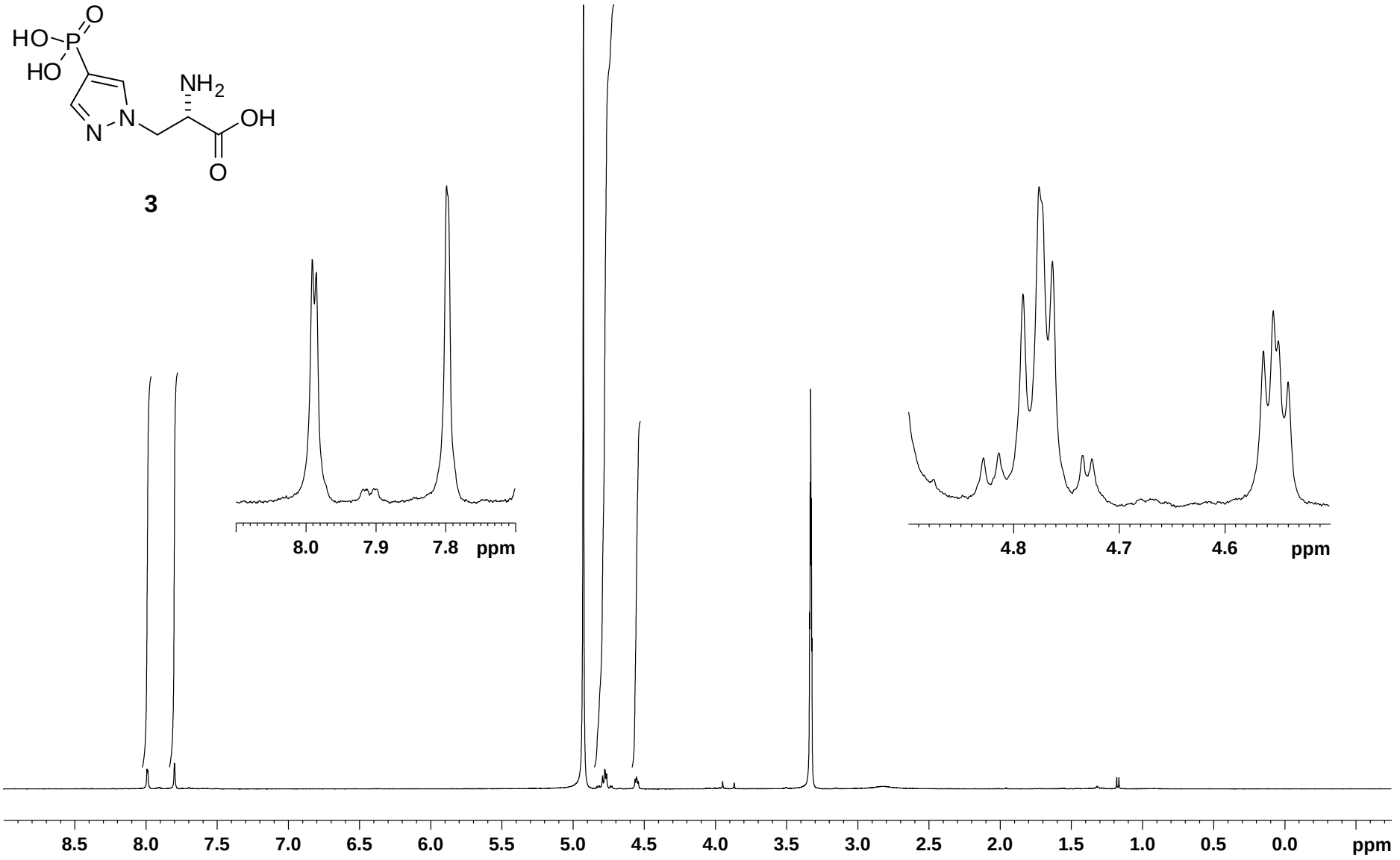
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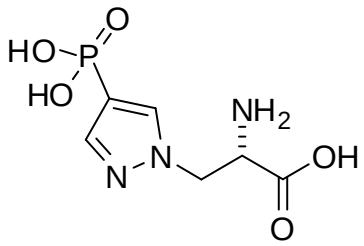
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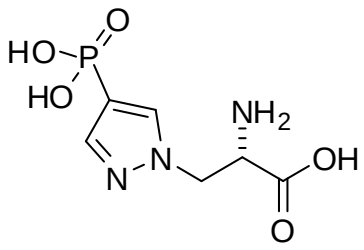

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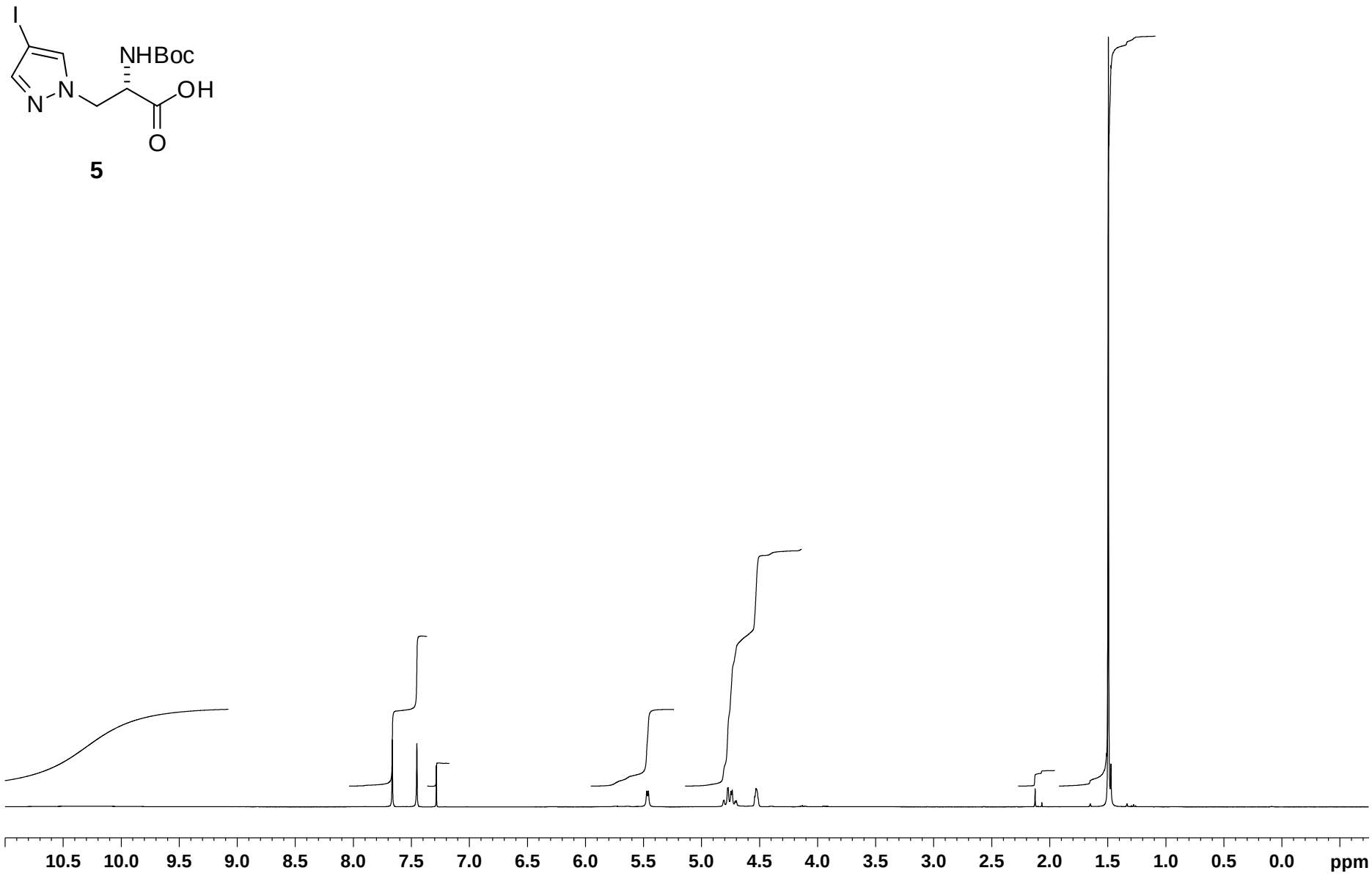
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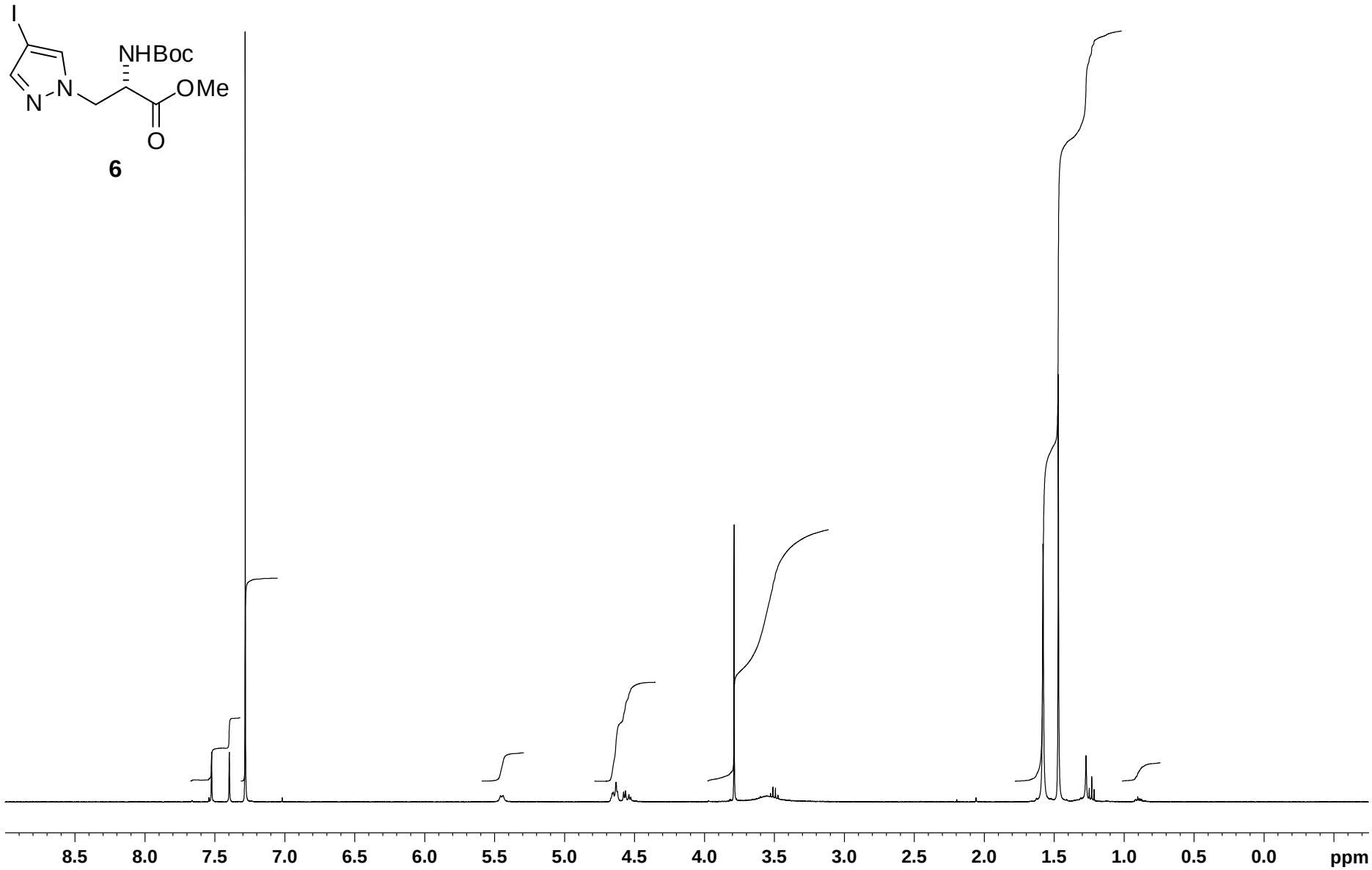
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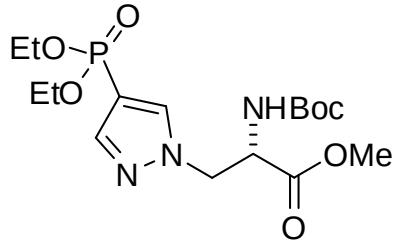
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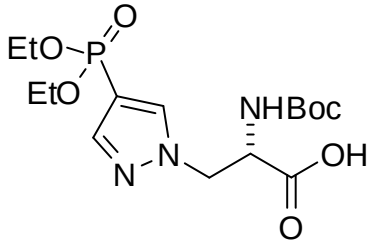
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