4-Phosphothiophen-2-yl alanine: a new 5-membered analogue of phosphotyrosine

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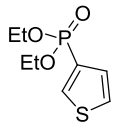
Electronic Supplementary Information

Methods

All reagents used were purchased from commercial sources and used without further purification, unless otherwise stated. Specifically, histidine, phosphoserine, phosphothreonine, and phosphotyrosine were purchased from Sigma-Aldrich, and τ-phosphohistidine was generated using Hultquist's method and purified by chromatography.[1] All moisture/air sensitive reactions were conducted under a positive pressure of nitrogen in flame dried glassware. Water is demineralised water and all solvents used were HPLC grade or distilled. Petroleum ether refers to the fraction which boils in the range 40-60 °C. Unless otherwise stated, nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX-400 NMR spectrometer at room temperature. Chemical shifts were measured relative to residual solvent and are expressed in parts per million (δ). Coupling constants (*J*) are given in Hertz and the measured values are rounded to the nearest 0.5 Hertz and are rationalised. High-resolution mass spectra were recorded using a MicroMass LCT operating in electrospray (ES) mode or Kratos MS 25 or MS 80 for electro impact (EI). MALDI-TOF-MS were recorded on a Bruker Reflex III MALDI-TOF instrument using positive ion linear mode, using sinapinic acid (SA) as the matrix of the protein solutions. Aliquots 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 protein solution (≈ 5 mg / mL) in a 1 to 5 ratio and spotted onto a MALDI-TOF target. Infra-red spectra were recorded on a Perkin Elmer Paragon 100 FTIR spectrophotometer (ν_{max} in cm^{-1}) as thin films using NaCl plates. Melting points were determined using a Linkam HFS91 heating stage, used in conjunction with a TC92 controller and are uncorrected. Optical rotations were recorded on a Perkin Elmer 241 automatic polarimeter at λ 589 nm (Na, D-line). Thin layer chromatography (TLC) was performed on precoated plates (0.2 mm, Merck DC alufolien Kieselgel 60 F254) and compounds visualised by UV light (254 nm), ninhydrin solution (5% in MeOH). Column chromatography was performed using silica gel 60 from BDH Lab. $CFTR_{inh172}$ was obtained from Merck Millipore (UK). The osmolality of the experimental solutions was checked using a Roebling osmometer (Camlab Ltd, Cambridge, UK).

All animals were maintained in accordance with local rules and the Animals and Scientific Procedures Act (ASPA), and the work was carried out under a Personal Home Office Approved Licence for the generation of polyclonal and monoclonal antibodies. Statistical significance was tested using ANOVAs and Student's t-test as appropriate and assumed at the 5% level.

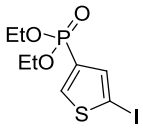
Diethyl (thiophen-3-yl)phosphonate, 4.[2]



Palladium acetate (202 mg, 0.9 mmol, 0.03 eq.), dppf (998 mg, 1.8 mmol, 0.06 eq.), and sodium acetate (295 mg, 3.6 mmol, 0.12 eq.) 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 (60 mL) was then added by syringe followed by $^{i}Pr_2EtN$ (4.65 g, 6.27 mL, 36 mmol, 1.2 eq.). The reaction mixture was then stirred at 60 ^{o}C for 30 minutes, before addition of 3-bromothiophene (4.89g, 2.81 mL, 30 mmol, 1 eq.) and diethyl phosphite (4.14 g, 3.86 mL, 30 mmol, 1 eq.). The reaction mixture was then brought to reflux and left for 24 hours. The crude reaction mixture was then concentrated under reduced pressure and applied directly to a silica gel column for purification, using 40% EtOAc / 60% 40-60 petroleum ether, to afford the desired compound **3** (4.76 g, 21.6 mmol, 72% yield).

Yellow oil; ν_{max}(film)/cm^{-1} 3474, 3103, 2983, 2939, 2905, 1500, 1478, 1444, 1393, 1369, 1208, 1164, 1119, 1098, 1051, 1023; δ_H (400 MHz, *CDCl*$_3$) 1.34 (t, *J* = 7.0 Hz, 6H), 4.05-4.21 (m, 4H), 7.33-7.37 (m, 1H), 7.45 (dt, *J* = 3.0, 5.0 Hz, 1H), 8.01 (ddd, *J* = 1.0, 3.0, 8.0 Hz, 1H); δ_C (101 MHz, *CDCl*$_3$) 16.3 (d, *J* = 6.5 Hz), 62.2 (d, *J* = 5.5 Hz), 127.2 (d, *J* = 18.0 Hz), 129.0 (d, *J* = 16.5 Hz), 129.5 (d, *J* = 195.5 Hz), 135.4 (d, *J* = 18 Hz); δ_P (101 MHz, *CDCl*$_3$) 13.6 (s); m/z (ES) Found: MH$^+$ 221.0391. $C_8H_{14}O_3PS$ requires MH$^+$ 221.0401.

Diethyl (5-iodothiophen-3-yl)phosphonate, 5. [2]

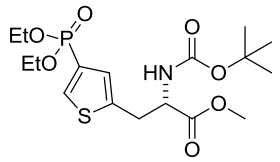


A solution of compound **3** (1.10 g, 5 mmol) in EtOH (10 mL) was added to a round bottom flask with condenser containing H_5IO_6 (0.57 g, 2.5 mmol, 0.5 eq.) and I_2 (0.54 g, 2.15 mmol, 0.43 eq.). The reaction mixture was then brought to reflux and left overnight stirring. The reaction mixture was then concentrated under reduced pressure, and purified by silica gel column using 30% EtOAc / 70% 40-60 petroleum ether to afford the desired compound **4** (1.09 g, 3.15 mmol, 63% yield), and the 2,5-diiodo derivative (0.425 g, 0.9 mmol, 18% yield).

Monoiodide 4 was isolated as a thick yellow oil; ν_{max}(film)/cm^{-1} 3468, 3092, 2981, 2931, 2900, 1495, 1476, 1442, 1391, 1327, 1292, 1342, 1181, 1164, 1099, 1050, 1022; δ_H (400 MHz, *CDCl$_3$*) 1.34 (t, *J* = 7.0 Hz, 6H), 4.03-4.22 (m, 4H), 7.44 (dd, *J* = 1.5, 4.0 Hz, 1H), 7.99 (dd, *J* = 1.5, 9.0 Hz, 1H); δ_C (101 MHz, *CDCl$_3$*) 16.3 (d, *J* = 6.5 Hz), 62.4 (d, *J* = 5.5 Hz), 75.4 (d, *J* = 23.0 Hz), 132.0 (d, *J* = 195.5 Hz), 138.1 (d, *J* = 16.0 Hz), 141.1 (d, *J* = 17.0 Hz); δ_P (101 MHz, *CDCl$_3$*) 10.56 (s); m/z (ES) Found: MH$^+$ 346.9383. $C_8H_{13}O_3PSI$ requires MH$^+$ 346.9368.

The 2,5-diiodo derivative was isolated as a yellow amorphous solid; m.p. 129-130 ^{o}C; ν_{max}(film)/cm^{-1} 3451, 3082, 2980, 2933, 2903, 2866, 1725, 1484, 1477, 1441, 1390, 1374, 1292, 1239, 1161, 1150, 1096, 1050, 1022; δ_H (400 MHz, *CDCl$_3$*) 1.31 (t, *J* = 7.0 Hz, 6H), 4.01-4.17 (m, 4H), 7.27 (d, *J* = 4.0 Hz, 1H); δ_C (101 MHz, *CDCl$_3$*) 16.3 (d, *J* = 6.5 Hz), 62.7 (d, *J* = 5.5), 77.9 (d, *J* = 20.5), 86.2 (d, *J* = 12.0), 137.6 (d, *J* = 197 Hz), 142.1 (d, *J* = 15.0 Hz); δ_P (101 MHz, *CDCl$_3$*) 7.65; m/z (ES) Found: MH$^+$ 472.8335. $C_8H_{12}O_3PSI_2$ requires MH$^+$ 472.8334.

(*S*)-Methyl-2-(*tert*-Butoxycarbonylamino)-3-[4-(diethoxyphosphoryl)thiophen-2-yl] propanoate, 7.

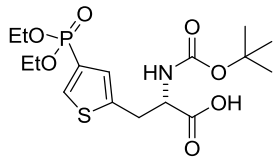


Zinc dust (588 mg, 9 mmol, 6 eq.) was added to a flame dried nitrogen purged side arm round bottom flask. DMF (3 mL) was added via syringe followed by a sub-stoichiometric amount of iodine (120 mg, 0.45 mmol, 0.15 eq.). Once the reaction mixture had turned colourless, *N*-(*tert*-butoxycarbonyl)-3-iodo-L-alanine methyl ester (987 mg, 3 mmol, 1.5 eq.) was added. The reaction mixture was then monitored via TLC to check for complete zinc insertion. Once the spot for *N*-(*tert*-butoxycarbonyl)-3-iodo-L-alanine methyl ester had disappeared, the reaction mixture was transferred to another flame dried nitrogen purged side arm round bottom flask. $Pd_2(dba)_3$ (46 mg, 0.05 mmol, 0.025 eq.), SPhos (46 mg, 0.10 mmol, 0.05 eq.) and compound **4** (700 mg, 2.0 mmol, 1.0 eq.) were then added to the flask. The reaction was heated to 50 ^{o}C and left stirring overnight, under a positive pressure of nitrogen. The reaction mixture was then partitioned between EtOAc and brine, followed by extraction of the aqueous layer with EtOAc (2 x 10 mL). The organic layers were then combined, dried with $MgSO_4$, and concentrated under reduced pressure. The crude compound was then purified by

silica gel column using 50% EtOAc / 50% toluene to afford the desired compound **7** (494 mg, 1.17 mmol, 59% yield).

Thick yellow oil; ν_{max}(film)/cm^{-1} 3272, 3100, 2980, 2930, 2896, 1747, 1713, 1529, 1436, 1392, 1366, 1289, 1245, 1166, 1094, 1052, 1023; δ_H (400 MHz, *CDCl$_3$*) 1.31 (t, J = 7.0 Hz, 6H), 1.44 (s, 9H), 3.31 (dd, J = 5.0, 15.0 Hz, 1H), 3.40 (dd, J = 5.0, 15.0 Hz, 1H), 3.74 (s, 3H), 4.01-4.19 (m, 4H), 4.55-4.63 (m, 1H), 5.19 (d, J = 7.5 Hz, 1H), 6.98-7.03 (m, 1H), 7.84 (d, J = 9.0 Hz, 1H); δ_C (101 MHz, *CDCl$_3$*) 16.3 (d, J = 6.5 Hz), 28.2 (s), 32.3 (s), 52.5 (s), 54.2 (s), 62.3 (d, J = 4.5 Hz), 80.23 (s), 128.2 (d, J = 16.5 Hz), 129.6 (d, J = 109.0 Hz), 135.2 (d, J = 17.0 Hz), 140.1 (d, J = 19.5 Hz), 155.0 (s), 171.3 (s); δ_P (101 MHz, *CDCl$_3$*) 12.73 (s); $[\alpha]_D^{22}$ +54 (c 1.0, *CHCl$_3$*); m/z (ES) Found: MH$^+$ 422.1414 $C_{17}H_{29}NO_7PS$ requires MH$^+$ 422.1402.

(*S*)-2-(*tert*-Butoxycarbonylamino)-3-[4-(diethoxyphosphoryl)thiophen-2-yl]propanoic acid 8.

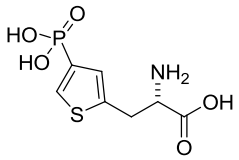


Compound **7** (316 mg, 0.75 mmol, 1 eq.) was added to a round bottom flask followed by the addition of H_2O (1.5 mL) and THF (1.5 mL). Once dissolved, LiOH (22 mg, 0.9 mmol, 1.2 eq.) was added and the reaction was monitored *via* TLC until the starting material had disappeared. The reaction was then 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 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 Boc-protected acid acid **8** (306 mg, 0.75 mmol, 99% yield).

Thick pale yellow oil; ν_{max}(film)/cm^{-1} 3425, 3330, 3093, 2979, 2931, 2906, 2842, 2549, 1712, 1500, 1435, 1392, 1367, 1333, 1231, 1164, 1094, 1051, 1021; δ_H (400 MHz, *CDCl$_3$*) 1.28 (t, *J* = 7.0 Hz, 6H), 1.42 (s, 9H), 3.34 (dd, *J* = 5.0, 15.0 Hz, 1H), 3.45 (dd, *J* = 5.0, 15.0 Hz, 1H), 3.98-4.17 (m, 4H), 4.56 (m, 1H), 5.37 (d, *J* = 7.5 Hz, 1H), 7.04 (d, *J* = 3.5 Hz, 1H), 7.83 (d, *J* = 9.0 Hz, 1H), carboxylic acid proton not observed; δ_C (101 MHz, *CDCl$_3$*) 16.2 (d, *J* = 6.5 Hz), 28.3 (s), 32.0 (s), 54.0 (s), 62.6 (d, *J* = 4.5 Hz), 80.0 (s), 128.0 (d, *J* = 196.7 Hz), 128.1 (d, *J* = 17.0 Hz), 135.3 (d, *J* = 17.4 Hz), 140.8 (d, *J* = 19.5 Hz), 155.2 (s), 172.4 (s); δ_P (101

MHz, *CDCl*$_3$) 13.34; $[\alpha]_D^{22}$ +42 (c 1.0, $CHCl_3$); m/z (ES) Found: MH^+ 408.1246 $C_{16}H_{27}NO_7PS$ requires MH^+ 408.1246.

(*S*)-2-Amino-3-(4-phosphonothiophen-2-yl)propanoic acid, 2a.



Compound **8** (204 mg, 0.5 mmol, 1 eq.) 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 eq.). 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 4-phosphothiophen-2-yl alanine **2a** (55 mg, 0.22 mmol, 44% yield).

Amorphous cream solid; m.p. 260 ^oC decomp.; ν_{max}(film)/cm^{-1} 3403, 2991, 2892, 1971, 1713, 1603, 1586, 1519, 1447, 1427, 1305, 1253, 1191, 1140, 1093, 1041; δ_H (400 MHz, *D$_2$O*) 3.34-3.47 (m, 2H), 4.26 (t, *J* = 6.0 Hz, 1H), 7.06 (d, *J* = 4.0 Hz, 1H), 7.73 (d, *J* = 9.0 Hz, 1H); δ_C (101 MHz, *D$_2$O*) 29.6 (s), 53.9 (s), 129.4 (d, *J* = 16.5 Hz), 133.3 (d, *J* = 17.0 Hz), 134.6 (d, *J* = 187.0 Hz), 137.3 (d, *J* = 18.5 Hz), 171.0 (s); δ_P (101 MHz, *D$_2$O*) 8.19 (s); $[\alpha]_D^{22}$ -18 (c 1.0, H_2O); m/z (ES) Found: MH^+ 252.0098 $C_7H_{11}NO_5PS$ requires MH^+ 252.0096.

General coupling procedure of 4-phosphothiophen-2-yl alanine 2a to a carrier protein using glutaraldehyde.

Carrier protein, KLH or BSA, (50 mg) and 4-phosphothiophen-2-yl alanine (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 ^oC, rotating gently. $NaBH_4$ (5 mg) was added to the solution and was then left overnight at 4 ^oC, 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 ^oC 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

4-Phosphothiophen-2-yl alanine conjugated by glutaraldehyde to BSA: 70259.5.

Each glutaraldehyde-conjugated residue **2a** adds 319 to the MW, so there are twelve **2a** residues conjugated to BSA (the calculated value on the basis of the masses is 11.98).

Procedure for the generation of 4-phosphothiophen-2-yl alanine antibodies.

Protein-4-phosphothiophen-2-yl alanine 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 **2a**, 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 **2a** 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.

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 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-phosphothiophen-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.

ELISA studies of purified sera.

A solution of the BSA-phosphotyrosine conjugate (10 µL, ~ 1 mg/mL) was first diluted 1000 fold using 0.2 M pH 9.6 carb/bicarb buffer. The solution was then used to coat a 96 well plate with 100 µL per well and incubated overnight at 4 °C. The wells were then emptied and washed 3 times with 1 M pH 7.4 PBS/tween-20 (0.05%), and then dried. 1 M 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 1 M pH 7.4 PBS/tween-20 (0.05%), and dried. 1 M pH 7.4 PBS/tween-20 + 0.2% gelatin (100 µL) was added to each well, before adding the primary antibody (100 µL), diluted 12.5 fold using 1M pH 7.4 PBS/tween-20 + 0.2% gelatin, wells A1 and A2. 100 µL of the solution from wells A1 and A2 were used to perform a serial dilution of the antibody until wells G3 and G4, to give the following dilutions in each well (Figure S1). The wells were washed 3 times with 1 M pH 7.4 PBS/tween-20 (0.05%), and dried. 50 µL of the anti-rat, HRP labelled secondary antibody, diluted 2000 fold in 1 M pH 7.4 PBS/tween-20 + 0.2% gelatin, was added to each well and incubated for 1 hour at 37 °C, before being washed 3 times with 1 M 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 2 M H_2SO_4 (50 µL) and read with plate reader.

	1	2	3	4	5	6	7	8	9	10	11	12
a	1:25		1:6400									
b	1:50		1:12800									
c	1:100		1:25600									
d	1:200		1:51200									
e	1:400		1:102400									
f	1:800		1:202800									
g	1:1600		1:405600									
h	1:3200		0									

Figure S1: General dilution gradient used to determine the required concentration of antibody for competition ELISAs on a 96 well plate. Each cell shows the dilution fold of the primary antibody.

Competition ELISA of the purified polyclonal antibodies.

Primary antibody solutions

5 mM solutions of the amino acid of histidine, τ-phosphohistidine, phosphoserine, phosphothreonine and phosphotyrosine were made using of 1 M pH 7.4 PBS/Tween-20 + 0.2 % gelatine to give the desired initial concentration of 5 mM. 100 µL of the appropriate amino acid solution were added to the top row of the 96 well cell culture plate, and 1 M pH 7.4 PBS/tween-20 + 0.2 % gelatin (50 µL) was added to the remaining wells. The amino acid solutions were then used to make 2.5 mM, 1.25 mM, 0.63 mM, 0.32 mM, 0.16 mM, 0.08 mM, and 0.04 mM solutions in the remaining wells, to give the following 96 well plate (Figure S2).

	Histidine		pHistidine		pSerine		pThreonine		pTyrosine		Controls	
	1	2	3	4	5	6	7	8	9	10	11	12
a	5.00		5.00		5.00		5.00		5.00		0	
b	2.50		2.50		2.50		2.50		2.50		Blocked	
c	1.25		1.25		1.25		1.25		1.25		Primary	
d	0.63		0.63		0.63		0.63		0.63		Secondary	
e	0.32		0.32		0.32		0.32		0.32			
f	0.16		0.16		0.16		0.16		0.16			
g	0.08		0.08		0.08		0.08		0.08			
h	0.04		0.04		0.04		0.04		0.04			

Figure S2: General 96 well plate for the competition ELISA using the purified polyclonal antibodies. Each cell shows the concentration of free amino acid in mM.

The purified antibody of interest was then was diluted in 1 M pH 7.4 PBS/tween-20 + 0.2% gelatin to half the dilution that gave an optical density (OD) of 1.0 at 450 nm according to ELISA. The primary antibody solution (100 µL) was then added to each of the wells, apart from cells B11-12 and D11-12 (Figure S2). The 96 well plate was then incubated overnight at 4 °C. Another 96 well plate was also prepared following the procedure laid out in Elisa, using the inhibited antibodies solutions as the primary antibodies.

Western blotting

The human bronchial epithelial cells (16HBE14o-) were obtained from Dr. Gruenert [3] and cultured as described previously [4]. 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 [5]. 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 [6] using bovine albumin serum as standard.

Proteins (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 **2a** (1:500), anti-phosphotyrosine (pY99, 1:1000), and Horseradish Peroxidase (HRP) conjugated secondary antibody (1:10,000) followed by supersignal™ West Pico chemiluminescence detection (Pierce).

Acknowledgements

The 16HBE14o− cells were provided by Dr. D. Gruenert (California Pacific Medical Center, CA).

References

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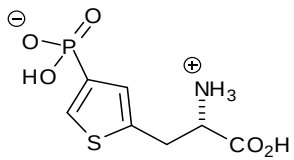
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P
HO
NH3
S
CO2H

2a

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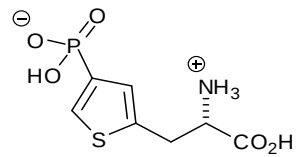
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HO
NH3
S
CO2H

2a

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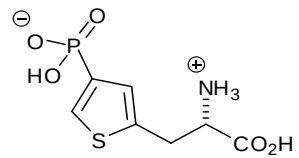

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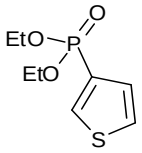
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O
EtO
S

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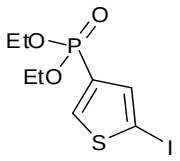
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EtO
S
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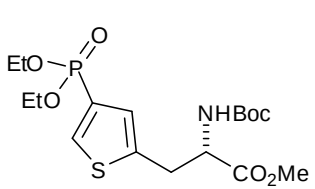
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NHBoc
S
CO2Me

7

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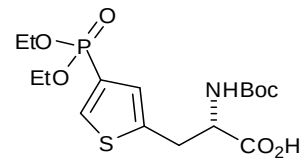
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O
EtO
NHBoc
S
CO2H

8

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7.0
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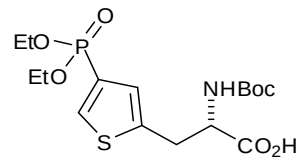
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P
O
EtO
NHBoc
S
CO2H

8

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200
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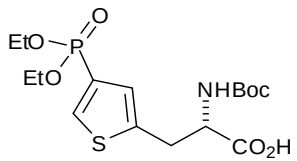

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