Peptide-based activity-based probes (ABPs) for target-specific profiling of protein tyrosine phosphatases (PTPs)

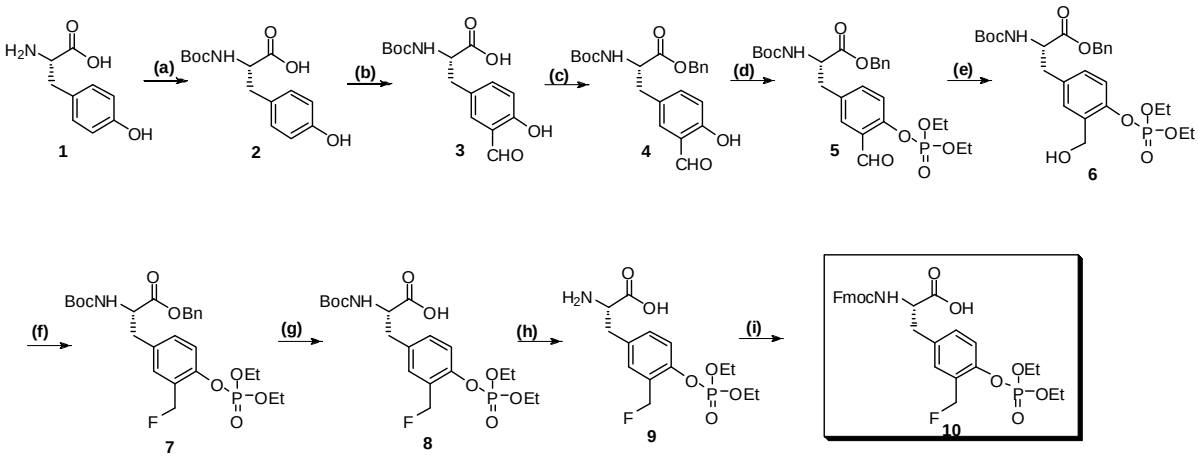
Karunakaran A. Kalesh,[‡] *Lay Pheng Tan,*[§] *Kai Liu,* [§] *Liqian Gao,*[¶] *Jigang Wang* [§] *and Shao Q. Yao* [‡§¶*]

[‡]Department of Chemistry, [§]Department of Biological Sciences and [¶]NUS MedChem Program of the Office of Life Sciences, 3 Science Drive 3, National University of Singapore, Singapore 117543
*correspondence author (chmyaosq@nus.edu.sg)

1. General

All chemicals were purchased from commercial vendors and used without further purification, unless indicated otherwise. All proteins used are either recombinantly expressed in house, or purchased from commercial sources. All NMR spectra (^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR and ^{31}P-NMR) were recorded on a Bruker 300 MHz NMR spectrometer. Chemical shifts are reported in parts per million referenced with respect to appropriate internal standards. The following abbreviations were used in reporting spectra, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets. HPLC-grade solvents were used for all reactions. The following abbreviations for chemicals were used: Boc (*tert*-butyl carbamate), DAST (diethylaminosulfurtrifluoride), TFA (trifluoroacetic acid), Fmoc (9-fluorenylmethyl carbamate), BnOH (benzyl alcohol), DIEA (diisopropylethyl amine), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, DMAP (N,N-dimethylaminopyridine), DBU (1,8-diazabicyclo[5.4.0]undec-7-ene), FmocOSu (N-(9-fluorenylmethoxycarbonyloxy)-succinimide and DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate). All LC-MS analysis were performed with Shimadzu LC-MS (IT-TOF) system. For enzyme activity measurements and kinetic experiments, Tecan microplate reader (Multimode Reader, Infinite®200) in fluorescence mode with i-control™ software was used. General PTP activity assays with DiFMUP were carried out as previously described.[1] The semilogarithmic and double-reciprocal plots were generated using the GraphPad Prism software v.4.03 (GraphPad, San Diego, USA). Fluorescence scanning of the SDS-PAGE gels was carried out with Typhoon 9200 fluorescence gel scanner (Amersham Biosciences), and where applicable, the bands were quantified with ImageQuant 3.3 (Molecular Dynamics) software installed on the scanner

2. Synthesis of the Unnatural Amino Acid



Scheme S1: Synthesis of unnatural amino acid: (a) $(Boc)_2O$, Et_3N, Dioxane-Water (1:1), 0 ^oC to RT, 18 h, 90%. (b) $CHCl_3$, NaOH, H_2O, Reflux, 2 h, 39%. (c) BnOH, EDC, DMAP, DCM, 0 ^oC to RT, 3 h, 80%. (d) Chlorodiethylphosphate, DBU, DCM, 0 ^oC to RT, 6 h, 70%. (e) $NaBH_4$, EtOH-THF (4:1), 0 ^oC, 15 min, 69%. (f) DAST, DCM, 0 ^oC 30 min, 74%. (g) H_2, Pd-C, MeOH, 20 min, 96%. (h) TFA-DCM (1:1), 3 h, 90%. (i) FmocOSu, $NaHCO_3$, THF-Water (1:1), 4 h, 76%.

2-(tert-butoxycarbonylamino)-3-(4-hydroxyphenyl) propanoic acid (2)

To a solution of L-Tyrosine (Aldrich, 40 g, 220.8 mmol) in 1/1 dioxane/water (250 mL each) was added triethylamine (46 mL, 1.5 eq, and 331.2 mmol). The reaction flask was cooled to 0 ^oC with an ice bath and di-*tert*-butyl-dicarbonate (53 g, 1.1 eq, 331.2 mmol) was added. After 1 h, the cold bath was removed and the reaction mixture was stirred at ambient temperature for 20 h. The reaction mixture was then concentrated on a rotary evaporator and the residue diluted with water and ethyl acetate. The aqueous layer was acidified to pH 1 with 1 N HCl and back extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to give the protected amino acid, N-Boc-L-tyrosine **1**, as a white solid (56 g, 90%) which was used without further purifications. ^1H- NMR (300 MHz, $CDCl_3$) δ 6.95 (2H, d, J = 8.22 Hz), 6.70 (2H, d, J = 7.41 Hz), 5.92 (1H, bs, OH), 5.16 (1H, bs, NH), 4.53 (1H, unresolved m, α H), 3.01 (2H, unresolved m, βH), 1.40 (9H, s, Boc); ESI-MS: m/z = 280.2 $[M-H]^-$

2-(tert-butoxycarbonylamino)-3-(3-formyl-4-hydroxyphenyl) propanoic acid (3)

N-Boc-L-tyrosine **2** (40 g, 142.3 mmol) in 600 mL of $CHCl_3$ was heated to 65 ^oC. To this was added a hot (65 ^oC) solution of NaOH (22.8 g, 4 eq, 569 mmol) in 80 mL of water

and the mixture was refluxed for 1.5 h. After cooling, $CHCl_3$ was evaporated off and the reaction mixture was diluted with water and EtOAc, and the aqueous layer was acidified to pH 1 with 1 N HCl and back extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated. Flash column chromatography (silica gel, 12/1 $CHCl_3$/MeOH with 1% acetic acid eluent) afforded **2** as a pale yellow oil (17.1 g, 39%).Unreacted starting material **1** was also recovered. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.9 (1H, s, OH), 9.8 (1H, s, CHO), 7.36 (1H,s C2-H), 7.32 (1H,d, J = 8.37 Hz), 6.91 (1H, d, J = 8.37 Hz, C5-H), 5.27 (1H, bs, NH), 4.57 (1H, unresolved m, α H), 3.15 (1H, unresolved m, β H), 3.02 (1H, unresolved m, βH), 1.38 (9H, s, Boc) ; ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 196.52, 177.03, 160.52, 155.29, 138.08, 134.23, 127.62, 120.37, 117.72, 80.43, 54.16, 36.83, 28.16; ESI-MS: m/z = 308.1 $[M-H]^-$

Benzyl 2-(tert-butoxycarbonylamino)-3-(3-formyl-4-hydroxyphenyl) propanoate (4)

To a solution of **3** (10 g, 32.36 mmol) in CH_2Cl_2 was added benzyl alcohol (6.73 mL, 64.72 mmol, 2 eq) followed by DMAP (0.6 g, 0.15 eq) and the solution was cooled to 0 ^oC with an ice-water bath while stirring. EDC (7.45 g, 38.83 mmol, 1.2 eq) was added and after 30 min the ice bath was removed, and continued stirring at room temperature for 3 h. The reaction mixture was then extracted with water, brine, dried over Na_2SO_4 and concentrated. Flash column chromatography (silica gel, $CHCl_3$/MeOH, 12/1) afforded **3** as a pale yellow oil (13.8 g, 80%) ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.84 (1H, s, OH), 9.59 (1H, s, CHO), 7.33-7.24 (5H, unresolved m), 7.12 (2H, m), 6.8 (1H, d, J = 8.4 Hz) 5.27 (1H, bs, NH), 5.10 (2H, m, CH_2-benzyl), 4.57 (1H, unresolved m, α H), 3.15 (1H, unresolved m, βH), 3.02 (1H, unresolved m, βH), 1.38 (9H, s, Boc) ; ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 196.37, 171.37, 160.40, 154.99, 137.99, 135.04, 134.07, 128.57, 127.56, 120.26, 117.58, 79.95, 67.08, 54.34, 37.03, 28.21

Benzyl-2-(tert-butoxycarbonylamino)-3-(4-(diethoxyphosphoryloxy)-3-formylphenyl) propanoate (5)

Compound **4** (8 g, 20 mmol) was dissolved in CH_2Cl_2 (90 mL). To this was added DBU (6 mL, 40 mmol, 2 eq) and the reaction mixture was cooled to 0 ^oC with an ice-water bath while stirring under N_2 gas. After 15 min, diethylchlorophosphate (6.4 mL, 44 mmol, 2.2 eq) was added and the ice bath was continued for 45 min, and after that the ice bath was removed and stirring continued at room temperature for 8 h. The reaction was extracted with 5% citric acid solution, brine, dried over Na_2SO_4 and concentrated. Flash column chromatography (silica gel, Hexane/EtOAc, 10/2) afforded **4** as a pale yellow oil (7.5 g, 70%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.29 (1H, s, CHO), 7.35-7.28 (5H,unresolved m), 7.27-7.23 (2H,m C2-H & C6-H), 7.21 (1H, d, J = 8.37 Hz, C5-H), 5.10 (2H, m, CH_2-benzyl), 4.55 (1H, unresolved m, α H), 4.22 (4H, q, CH_2 -phosphate) 3.14 (1H, unresolved m, βH), 2.98 (1H, unresolved m, βH), 1.33 (9H, s, Boc), 1.28 (6H, t, phosphate) ; ^{31}P-NMR (121.5 MHz, $CDCl_3$) δ = -6.0902 (s); ESI-MS: m/z = 558.2 $[M+Na]^+$

Benzyl-2-(tert-butoxycarbonylamino)-3-(4-(diethoxyphosphoryloxy)-3-(hydroxymethyl) phenyl) propanoate (6)

To a solution of the aldehyde **5** (6 g, 11.2 mmol) in absolute EtOH (90 mL), THF (20 mL) and water (900 μL) was added $NaBH_4$ (0.43 g, 11.2 mmol) at 0 ^oC and stirred for 15 min. The reaction mixture was then diluted with acidic water (pH = 4; 80 mL) and stirring continued for 10 min. The mixture was extracted with EtOAc (2 × 90 mL) and concentrated under reduced pressure. The oily compound obtained was then coevaporated with MeOH three times, again dissolved in EtOAc, extracted with brine, dried over Na_2SO_4 and concentrated at reduced pressure. It was then purified by silica gel flash chromatography (DCM/ MeOH 10/1) to provide **5** as a pale yellow oil (4.2 g, 69.7%) ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.35-7.27 (5H,unresolved m), 7.15 (1H,s) 7.05 (1H, d, J = 8.22 Hz), 6.92 (1H, d, J = 8.04 Hz), 5.1 (2H, m, CH_2-benzyl), 4.62 (2H, s, CH_2-alcohol), 4.55 (1H, m, α H), 4.21 (4H, m, CH_2-phosphate) 3.14 (1H, unresolved m, βH), 2.98 (1H, unresolved m, βH), 1.33 (9H, s, Boc), 1.28 (6H, t, CH_3-phosphate) ; ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 171.72, 155.31, 146.97, 135.35, 133.66, 130.56, 129.26, 128.53, 120.11, 79.63, 66.98, 64.85, 59.33, 54.70, 37.27, 28.23, 16.07; ^{31}P-NMR (121.5 MHz, $CDCl_3$) δ = -5.1757(s)

Benzyl-2-(tert-butoxycarbonylamino)-3-(4-(diethoxyphosphoryloxy)-3-(fluoromethyl) phenyl) propanoate (7)

A solution of the alcohol **6** (3.23 g, 6mmol) in dry CH_2Cl_2 (40 mL) was cooled to 0 ^oC and diethylaminosulfurtrifluoride (2.36 mL, 18 mmol, 3 eq) was added and stirred for 30 min to complete the reaction. Saturated $NaHCO_3$ solution (40 mL) was added to quench the DAST and stirring continued for another 10 min. The reaction was then extracted with water, brine, dried over Na_2SO_4 and concentrated on a rotary evaporator. The yellow oily compound obtained was purified by flash column chromatography on silica gel (Hexane/ EtOAc 70/30) to obtain **6** as pale yellow oil (2.4 g, 74.1%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.33-7.27 (5H,unresolved m), 7.25 (1H,s) 7.03 (1H, d, J = 8.22 Hz), 7.05 (1H, d, J = 8.22 Hz), 5.46-5.30 (2H, d, J = 47.50 Hz, CH_2F), 5.12 (2H, m, CH_2-benzyl), 4.58 (1H, m, α H), 4.18 (4H, m, CH_2-phosphate), 3.1-2.97 (2H, unresolved m, βH), 1.39 (9H, s, Boc), 1.30 (6H, t, CH_3-phosphate); ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 171.35, 154.99, 147.21, 135.11, 133.24, 130.76, 130.19, 128.23, 127.54, 119.70, 80.54, 78.32, 66.83, 64.62, 54.40, 36.96, 28.02, 15.87; ^{31}P-NMR (121.5 MHz, $CDCl_3$) δ = -5.8829 (s); ^{19}F-NMR (288.2 MHz, $CDCl_3$) δ = -138.41 (t, J= 47.45 Hz); ESI-MS: m/z = 562.2 $[M+Na]^+$

2-(tert-butoxycarbonylamino)-3-(4-(diethoxyphosphoryloxy)-3-(fluoromethyl) phenyl) propanoic acid (8)

To a degassed solution of **7** (2 g, 3.7 mmol) in dry MeOH (40 mL) was added 10% Pd/C (0.2 g) and the solution was stirred under hydrogen atmosphere for 20 min to complete

the reaction. The Pd/C was removed by filtration through a celite and the filtrate was concentrated under reduced pressure to afford **7** as pale yellow oil (1.6 g, 96%). It was used in the next reaction without further purification ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.32 (1H, m), 7.27 (1H, s) 7.19 (1H, d, J = 8.22 Hz), 5.51-5.35 (2H, d, J = 47.50 Hz, CH_2F), 4.52 (1H, m, α H), 4.22 (4H, m, CH_2-phosphate), 3.22-3.01 (2H, unresolved m, βH), 1.41 (9H, s, Boc), 1.34 (6H, t, CH_3-phosphate); ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 173.55, 155.41, 147.31, 133.84, 131.20, 130.75, 130.66, 119.79, 80.82, 78.61, 65.23, 54.19, 37.16, 28.28, 15.97; ^{31}P-NMR (121.5 MHz, $CDCl_3$) δ = -6.1999 (s); ^{19}F-NMR (288.2 MHz, $CDCl_3$) δ = -138.25 (t, J= 47.41 Hz); ESI-MS: m/z = 448 $[M-H]^-$

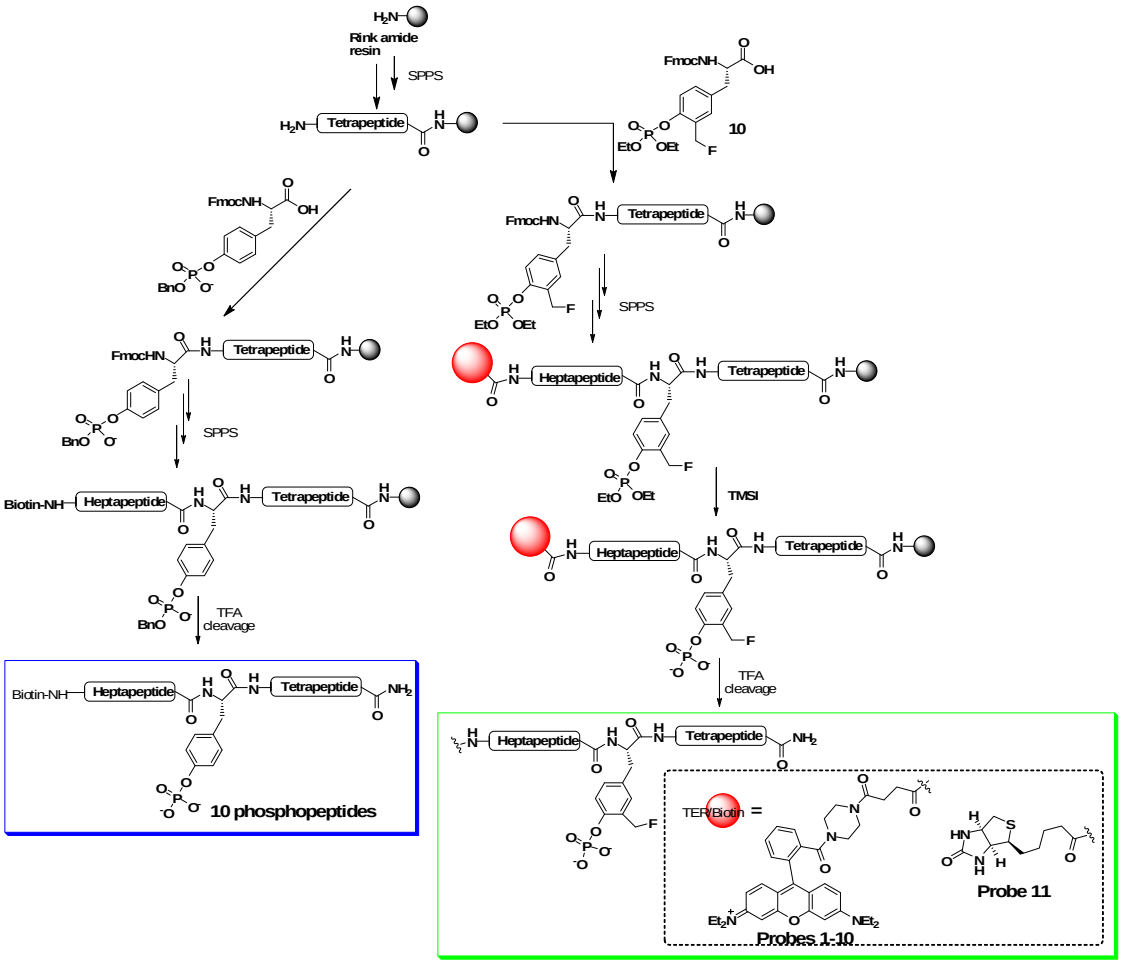
2-amino-3-(4-(diethoxyphosphoryloxy)-3-(fluoromethyl) phenyl) propanoic acid (9)

The N-Boc unnatural amino acid **8** (1.6 g, 3.56 mmol) was dissolved in a mixture of TFA/DCM (1/1; 40 mL each) and the solution was stirred for 3 h in an open RBF. TFA and DCM are evaporated off under reduced pressure to obtain **8** as pale yellow oil which was used in the next reaction without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 12.90 (1H, COOH), 7.57 (2H, bs, NH_2), 7.37 (2H, m), 7.15 (1H, m), 5.47-5.31 (2H, d, J = 48 Hz, CH_2F), 5.27 (1H, m, α H), 4.25 (4H, t, CH_2-phosphate), 3.32-3.04 (2H, unresolved m, βH), 1.37 (6H, t, CH_3-phosphate); ^{31}P-NMR (121.5 MHz, $CDCl_3$) δ = -7.2485 (s)

2-(((9H-fluoren-9-yl) methoxy) carbonyl amino)-3-(4-(diethoxyphosphoryloxy)-3-(fluoromethyl) phenyl) propanoic acid (10)

Compound **9** (1.6 g, 3.45 mmol) was dissolved in a mixture of THF/Water (1/1; 30 mL each) and the solution was adjusted to pH = 9 by adding $NaHCO_3$ (0.87 g, 10.35 mmol, 3.3 eq). To this was added FmocOSu (1.28 g, 3.8 mmol, 1.1 eq) and stirring continued for 4 h. THF from the reaction mixture was evaporated off and the solution was diluted with EtOAc. The aqueous layer was then acidified to pH = 2 by 1 N HCl and back extracted with EtOAc. The organic layer was then extracted with brine, dried over Na_2SO_4 and concentrated. Flash column chromatography (silica gel, 90/10 hexane/EtOAc to 97/3 EtOAc/MeOH with 0.1% acetic acid) afforded the N-Fmoc unnatural amino acid **9** as an off white foam (1.51 g, 76.5%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.38 (1H, s, COOH), 7.72 (2H, d, J = 7.38 Hz), 7.56 (2H, t, J = 7.08 Hz), 7.36 (1H, s), 7.31 (1H, s), 7.28 (4H, m), 7.16 (1H, d, J = 7.89 Hz), 6.93 (1H, m), 5.48-5.33 (2H, d, J = 47.00 Hz, CH_2F), 4.69 (1H, unresolved m, α H), 4.20 (4H, m, CH_2-phosphate), 3.23-3.06 (2H, unresolved m, βH), 1.31 (6H, t, CH_3-phosphate); ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 173.11, 163.32, 155.77, 147.33, 143.69, 141.20, 133.39, 131.18, 130.53, 127.31, 127.00, 125.04, 119.61, 80.73, 78.53, 66.97, 65.04, 47.03, 36.77, 15.97; ^{31}P-NMR (121.5 MHz, $CDCl_3$) δ = -6.2853 (s); ^{19}F-NMR (288.2 MHz, $CDCl_3$) δ = -137.57 (t, J = 47.43 Hz); ESI-MS: m/z = 570.3 $[M-H]^-$

3. Peptide Probe Synthesis



Scheme S2. Solid-phase synthesis of the 10 phosphopeptides and 11 peptide-based ABPs. See **Table S1** for sequence information.

Solid-phase peptide synthesis (SPPS) was performed on an automated peptide synthesizer (Chemspeed Technologies ASW2000) utilizing standard Fmoc chemistry on rink amide resin (ChemPep Inc.). All the natural Fmoc amino acids (4 eq) (Advanced ChemTech) were activated by DIC/HOBt (4 eq each) (Advanced ChemTech) and each coupling reaction was performed for 2 h in the presence of 8 eq of DIEA. For coupling of the unnatural amino acid, pre-activation of the unnatural amino acid with HOBt/HBTU (4 eq each) in the presence of 8 eq of DIEA for 10 min was performed before being added to the growing peptide chain on the resin. After washing with DMF (6X), the Fmoc-protecting group was removed using 20% piperidine in DMF, followed by another washing with DMF (6X). The N-terminus of each peptide was coupled to a Rhodamine dye containing an acid linker (or biotin) employing the same pre-activation method as earlier described for the unnatural amino acid. Finally deprotection of the phosphate ethyl ester groups was performed by treating the resin-bound peptide with 10 eq of TMSI in dry DCM, followed by washings with DMF (6X) and DCM (6X). Prior to cleavage of the

peptide, the resin was washed with MeOH (6X), DCM (6X), and MeOH (6X), and dried *in vacuo*. Peptide cleavage was carried out with an 1-mL cleavage cocktail (95:2.5:2.5 of TFA:H_2O:TIS) at RT for 2 h. The synthesis and cleavage of the phosphopeptides were done as previously described.[1] After filtration and precipitation with cold ether, the precipitated peptides were washed twice with cold ether, followed by drying *in vacuo* (Genevac). Each peptide was purified by HPLC and characterized by LC-MS (IT-TOF) (Shimadzu). Appropriate standard solutions were made in DMSO and stored at -80 ^{o}C freezer prior to use.

Table S1. MS characterizations of peptides

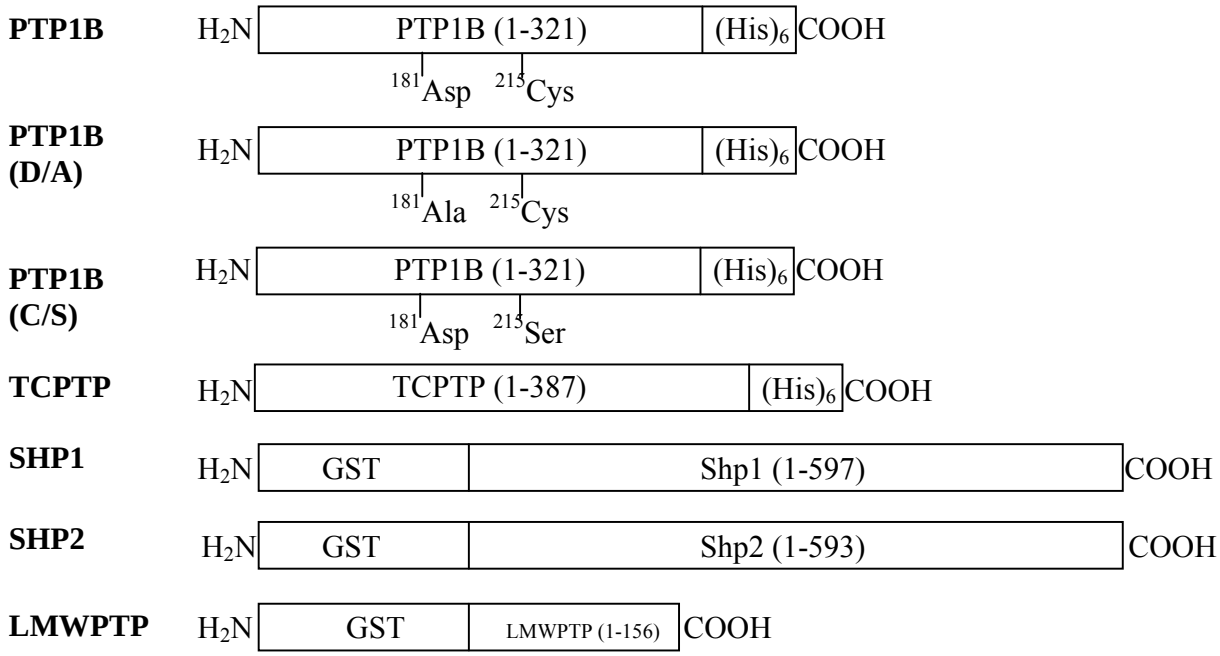
Peptide No.	Peptide Sequence	Expected MW	Observed MW
1	Biotin-GGDEGIH-**pY**-SELIQ	1721.05	861.79 (M+2)
2	Biotin- GGGSAAP-**pY**-LKTKF	1601.58	801.33 (M+2)
3	Biotin- GGKAVDG-**pY**-VKPQI	1636.61	818.84 (M+2)
4	Biotin- GGLNSDG-**pY**-TPEPA	1581.37	791.76 (M+2)
5	Biotin- GGLPPEG-**pY**-VVVVK	1618.63	810.35 (M+2)
6	Biotin- GGNSDVQ-**pY**-TEVQV	1698.50	850.31 (M+2)
7	Biotin- GGPEGHE-**pY**-pYRVRE	1933.20	967.81 (M+2)
8	Biotin- GGPQDKE-**pY**-YKVKE	1845.76	923.86 (M+2)
9	Biotin- GGVDADE-**pY**-LIPQQ	1709.54	855.81 (M+2)
10	Biotin- GGELEF-**pY**-MDYE	1798.66	900.33 (M+2)
1	Rhodamine dye- GGDEGIH-**X**-SELI	1992.37	997.38 (M+2)
2	Rhodamine dye- GGGSAAP-**X**-LKTK	1852.31	617.94 (M+3)
3	Rhodamine dye- GGKAVDG-**X**-VKPQ	1921.36	641.28 (M+3)
4	Rhodamine dye- GGLNSDG-**X**-TPEP	1909.24	955.36 (M+2)
5	Rhodamine dye- GGLPPEG-**X**-VVVV	1888.38	944.43 (M+2)
6	Rhodamine dye- GGNSDVQ-**X**-TEVQ	1999.31	1000.87 (M+2)
7	Rhodamine dye- GGPEGHE-**X**-pYRVR	2204.53	735.28 (M+3)
8	Rhodamine dye- GGPQDKE-**X**-YKVK	2116.67	705.30 (M+3)
9	Rhodamine dye- GGVDADE-**X**-LIPQ	1979.37	990.90 (M+2)
10	Rhodamine dye- GGELEF-**X**-MDE	1892.28	946.64 (M+2)
11	Rhodamine dye-K(Biotin)- KAVDG-**X**-VKPQI	2276.14	758.71 (M+3)

[1]**X** denotes the unnatural amino acid as shown in **Scheme 2**

4. Expression and Purification of PTPs

The catalytic domain (1-321) of PTP1B was amplified by polymerase chain reaction and subcloned using Nde1 and BamH1 restriction sites into a pET-28b vector to give PTP1B with a C-terminal hexahistidine tag. The two PTP1B mutants (D/A and C/S mutants) were obtained by site-directed mutagenesis using the QuikChange Mutagenesis Kit (Stratagene) following manufacturer's instructions. The final constructs were verified by DNA sequencing and transformed into *Escherichia coli* BL21 DE3 cells for protein expression. A single colony was grown overnight at 37 ^{o}C in Luria Bertani media that contain kanamycin at a final concentration of 50 μg/ml. Subsequently, the overnight

culture was diluted 1:100 and grown at 37 ^{o}C to an optical density of 0.6-0.8 at 600 nm. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM and grown for a further 4 hours at 30 ^{o}C. The culture was harvested and lysed by sonication on ice, in the presence of complete proteases inhibitor (Roche). The histidine-tagged PTP1B proteins were purified from the clarified lysates on Nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices (Qiagen) according to manufacturer's instructions. The plasmid encoding TC-PTP was a kind gift of Prof Harry Charbonneau and was modified to insert a C-terminal hexahistidine tag in-frame with the TCPTP gene. The mutagenesis was carried out using the QuikChange Mutagenesis Kit (Stratagene) following manufacturer's instructions. The final construct was verified by DNA sequencing and were transformed into BL21 DE3 cells for protein expression. TC-PTP-$(His)_6$-protein was expressed and purified following the same protocol as for the PTP1B proteins, with the only difference being that IPTG was added to a final concentration of 50 μM and grown for a further 12-16 h at 18 ^{o}C. The plasmids encoding Shp1 and Shp2 were obtained from Addgene.org (Plasmid 8578, Plasmid 8322), while the plasmid encoding LMW-PTP was a kind gift of Prof. Tomas Mustelin. All purification steps were performed at 4 ^{o}C following the same protocol described above. Briefly, the pelleted cells were resuspended in lysis buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 0.2 mM DTT and 1X complete proteases inhibitors. Sonication was done on ice and lysate was clarified by centrifuging at 12000 g for 30 min. The supernatant was then incubated with Gluthione Sepharose 4B beads (Amersham Pharmacia Biotech) for 30 min, with rotation. Subsequently, beads were washed 4X 5 min with 1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) wash buffer, complemented with 0.2 mM DTT and 0.2% Tween 20. Elution was carried out using 20 mM Tris, pH 8.0, and 10 mM reduced gluthione.



Scheme S3. Schematic showing of different PTP constructs used in this study.

5. Labeling Experiments with Purified Proteins

All the labeling reactions with PTPs (e.g. PTP1B, TCPTP, YOPH, LMWPTP) and non-PTP proteins (14-3-3 protein, Sortase, BSA & CnA), as shown in **Figure S1**, were initiated by adding 1 μL of the probe **7** to a pre-equilibrated enzyme/protein solution in an appropriate buffer at 25 ^{o}C. The final probe concentrations in all the reactions were 10 μM and the protein amounts were 1 μg. Tris buffer (50 mM, pH = 7.5) with ionic strength adjusted to 150 mM with NaCl was employed for PTP1B and TCPTP and YOPH. Sodium acetate buffer (50 mM, pH = 5.2) was used for LMWPTP. Labeling reactions with non-PTP proteins were performed in Tris buffer (50 mM, pH = 7.5, 150 mM NaCl).

All labeling reactions were run for 1 h and subsequently quenched by the addition of 4 μL of 6X SDS loading dye followed by heating at 95 ^{o}C for 10 min. The proteins were resolved on a 10% SDS-PAGE gel and the labeled bands were visualized by in-gel fluorescence scanning with Typhoon 9200 fluorescence gel scanner with green (533 nm) laser with optimum emission filters. The scanned images were quantified with the ImageQuant 3.3 (Molecular Dynamics) software.

(a) Fluorescence **(b) Coomassie**

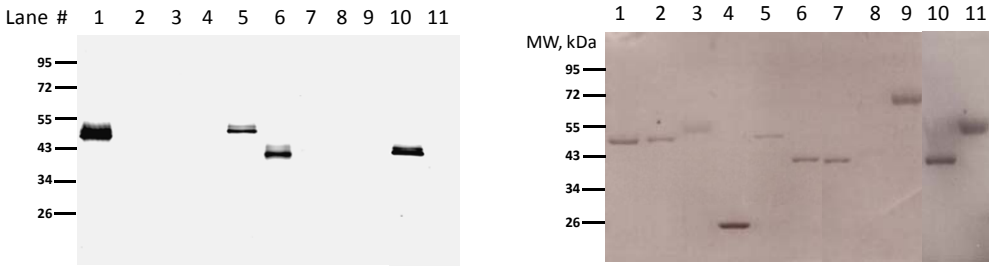


Figure S1. Activity-based labeling of proteins using probe **7**. lane 1- TCPTP; lane 2- Boiled & cooled TCPTP; lane 3- CnA; lane 4- Sortase; lane 5- YOPH; lane 6- PTP1B; lane 7- Boiled & Cooled PTP1B; lane 8- empty; lane 9- BSA; lane 10- LMWPTP; lane 11- 14-3-3 protein.

6. Detection Limit of the Probes

In order to determine the fluorescence detection limit of the probes, 200 ng of PTP1B was incubated with probe 3 (one of the highly selective probes for PTP1B) at a final probe concentration of 2 mM in the reaction buffer (50 mM Tris buffer, pH 7.5, 150 mM NaCl) for 1 h. After quenching the reaction, the labeled PTP1B was serially diluted with the same buffer and separated from excess of the probe on SDS-PAGE and the labeled bands were visualized by in-gel fluorescence scanning as described above

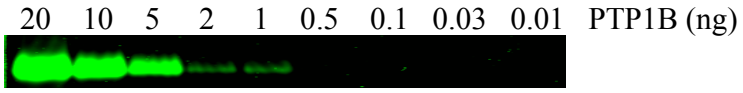


Figure S2. a) Detection limit of PTP1B with **Probe 3.**

7. Labeling with Mutant PTPs

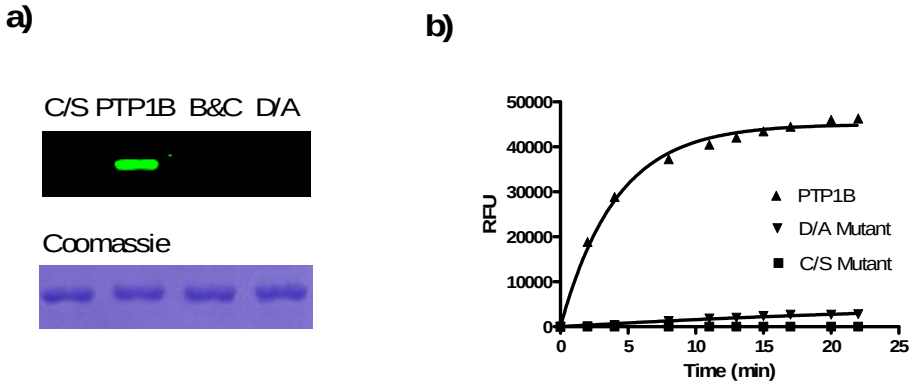


Figure S3. a) Labeling experiment with mutant PTPs. lane 1. PTP1B C/S mutant; lane 2. PTP1B; lane 3. Boiled and Cooled PTP1B; lane 4. PTP1B D/A mutant. b) Microplate-based enzymatic assay of PTP1B and mutants used with DiFMUP as the fluorogenic enzyme substrate.

PTPs possess a highly conserved Cys residue in the active site that plays a key role in initiating a nucleophilic attack on the phosphotyrosine of the substrate, leading to the formation of a thiophosphoryl enzyme intermediate which eventually collapses to give the dephosphorylated product. PTPs with mutations at this position (e.g. ^{215}Cys→Ser mutant of PTP1B) are catalytically inactive. The substrate binding into the active site of PTPs is followed by a well characterized conformational change in the highly conserved "WPD" loop in which it flips over the phosphotyrosine residue such that the aspartic acid in this loop gets close enough to the phosphoryl-cysteine intermediate for effective general acid mechanism (protonating the leaving phenolic oxygen). For PTP1B, mutation of this Asp in the WPD motif to Ala (e.g. ^{181}Asp→Ala mutant shown in **Scheme S3**) was shown to significantly reduce the k_{cat} of the enzyme. In order to get more insight into the activity-based labeling of PTP1B using our probes, we performed the labeling experiments with both C/S and D/A mutants of PTP1B (**Figure S3a**). The mutant PTPs were expressed and purified as reported elsewhere.[1, 2] As anticipated, the labeling result showed a complete lack of labeling with the mutants as well as the heat-denatured PTP1B, showing that the probes are indeed activity-based. Microplate-based enzymatic assay of the mutants using DiFMUP as substrate, revealed no catalytic activity for the C/S mutant while the D/A mutant retained a very low activity (**Figure S3b**).

8. Effect of H_2O_2 on PTP activity assessed with the Probes

The catalytic Cys residue in the active site of PTPs exhibit an unusually low pKa value which makes it an efficient nucleophile and at the same time makes the enzyme susceptible to oxidative inactivation. H_2O_2 generated during cellular insulin stimulation is shown to cause inactivation of PTPs via oxidizing the catalytic Cys to cysteine sulfenic acid (Cys-SOH) or a cyclic sulfenamide species [4]. Since cysteine sulfenic acid and the cyclic sulfenamide species can be reduced back to free cysteine by various cellular reductants, this mode of oxidative inactivation of PTPs may have a significant role in controlling the tyrosine phosphorylation-dependant signal transduction events. If the PTP is exposed to higher levels of oxidants the catalytic Cys residue gets irreversibly oxidized to either sulfinic acid (Cys-SO_2H) or sulfonic acid (Cys-SO_3H) which results in abrogation of enzymatic activity [4]. Thus in order to assess the feasibility of employing our activity-based probes for monitoring the H_2O_2-mediated PTP inactivation, PTP1B (1 μg) was treated with 1 mM H_2O_2 in 20 μL reaction at 25 ^oC for 5 min followed by treatment with the **Probe 3** for 1 h. The reaction was quenched with 6X SDS loading dye followed by heating at 95 ^oC for 10 min. A control experiment without H_2O_2 treatment was also performed simultaneously. As shown in **Figure S4**, H_2O_2-treated PTP1B didn't not show any trace of labeling showing the effectiveness of the probe in distinguishing the active versus oxidatively inactivated forms of the enzyme.

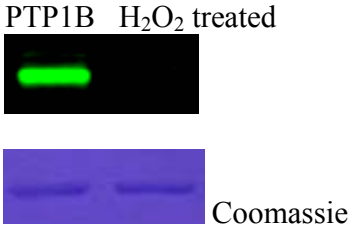


Figure S4. Effect of H_2O_2 on PTP1B activity assessed with the probe

9. Kinetic characterizations and substrate specificities of the probes and the corresponding phosphopeptides

All kinetic experiments were performed at 25 ^oC in HEPES buffer (50 mM, pH = 7.5, 150 mM NaCl). Time- and concentration-dependant inactivation kinetics of PTP1B with all the 10 probes (**Probes 1-10**) was investigated. Briefly, appropriate dilutions of the probes (0.2 mM to 2 mM) in the reaction buffer (45 μL) were treated with 5 μL aliquots of PTP1B stock solution. From this 2 μL was withdrawn at appropriate time intervals (1 to 60 min) and added into 48 μL of a 20 μM solution of DiFMUP in the same reaction buffer in a black 384 well flat bottom plate. The fluorescence intensity in relative fluorescence units (RFU) were continuously measured for 15 min (e.g. until a consistent trend is observed) using Tecan fluorescence plat reader at excitation wavelength of 360 nm and emission wavelength of 460 nm at a gain of 65. Background corrections for non-enzymatic hydrolysis of the DiFMUP were made. The experiments were performed in triplicate and the data for each probe were averaged and used to derive the kinetic parameters. The inactivation reaction follows Kitts and Wilson kinetics[5] and the pseudo-

first order inactivation constants (k_{obs}) at various concentrations of the probes were obtained from the initial slope of the semilogarithmic curves of $\ln(v_t/v_0)$ versus preincubation time, where v_t and v_0 are the enzyme activities at time t and zero, respectively. A non-linear regression fit of the double-reciprocal plot of k_{obs} versus probe concentration gives the maximal inactivation rate constant (k_i) and dissociation constant K_i according to the following equation and results are summarized in **Table S2**.

$$k_{obs} = k_i \times [I] / K_i + [I] \qquad \text{Eq. 1}$$

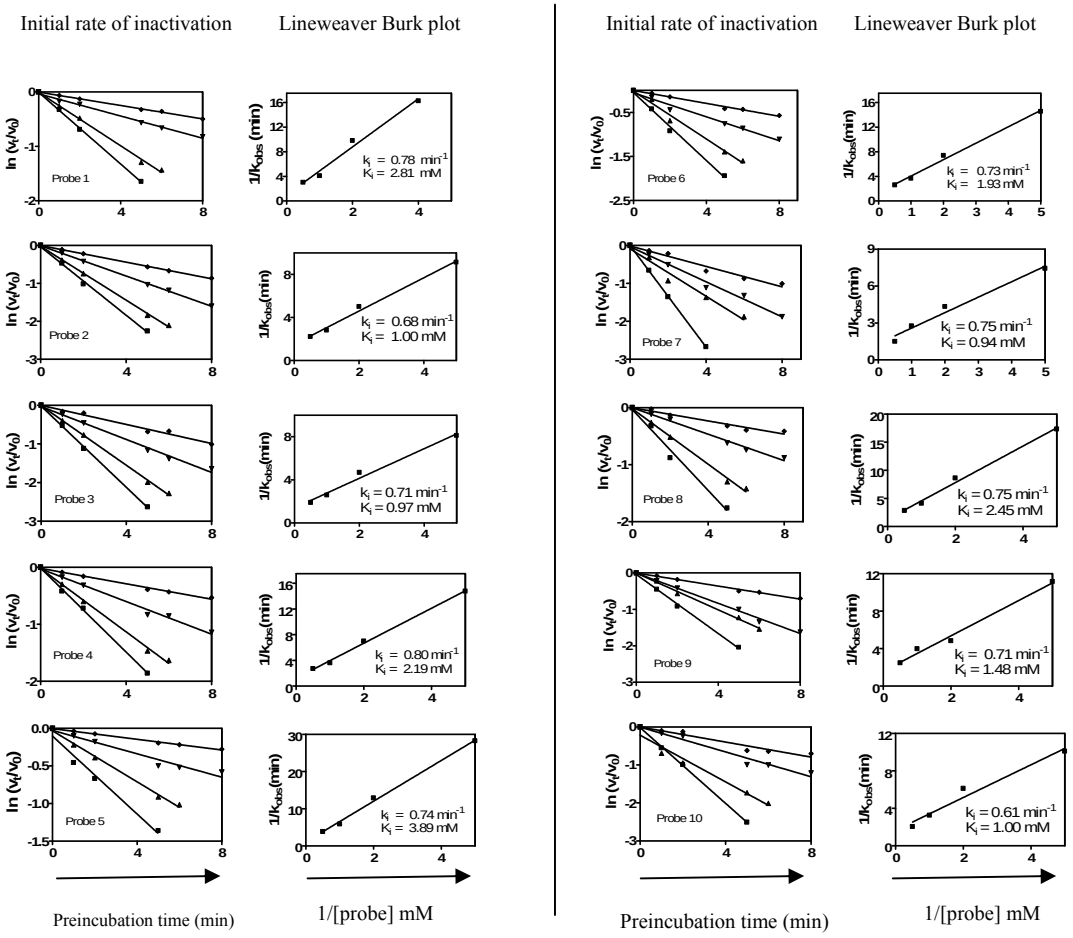


Figure S5. Determination of the kinetics of inactivation for the 10 probes on PTP1B.

10 phosphopeptides with the same amino acid sequence as those in the probes were also synthesized (with the only difference being that in the 10 phosphopeptides, phosphotyrosine was used in place of the unnatural amino acid, and biotin in place of the rhodamine dye) and the dephosphorylation preference of these peptides by PTP1B was measured in a microplate-based enzymatic assay using the malachite green phosphatase assay. First, a standard phosphate assay was performed in order to quantify the absorbance values (**Figure S6a**). Thus, for the phosphate standard curve, 20 μL aliquots

of different concentrations of phosphate (0.02 to 120 μM) prepared in TETBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Tween20) were treated with 5 μL of malachite green reagent in a 384-well plate (transparent flat bottom) at 25 ^{o}C for 15 min and the absorbance at 620 nm was measured using the Tecan plate reader. The absorbance was found to be linear in the range of 30 to 800 picomoles of phosphate (inset in **Figure S6a**). Next, the phosphatase assay was performed with 3 μg/mL of PTP1B and a 100 μM solution of each of the 10 phosphopeptides in TETBS buffer. The reaction was incubated for 10 min at 25 ^{o}C in a final reaction volume of 20 μL. The phosphatase reaction was quenched by adding 5 μL of malachite green reagent containing hydrochloric acid and the absorbance at 620 nm was measured after 15 min. The experiments were carried out in duplicate and averaged values were used to derive the number of nanomoles of inorganic phosphate released per min per mg of the enzyme (**Table S2**). Corrections were made for the non enzymatic hydrolysis of the peptides. In order to further verify the substrate specificity, the k_{obs} values for the dephosphorylation reaction were obtained via following the time course of dephosphorylation (**Figure S6b** & **Table S2**). Briefly, the Absorbance values were measured at 620 nm over 60 min and fitted into the following equation,

$$A = A_{\infty} [1 - \exp (- k_{obs} . t)] \qquad \text{Eq. 2}$$

where $k_{obs} = (k_{cat}/K_M)$. E, represents the observed k_{cat}/K_M at a given enzyme concentration (E). The assay was run under conditions where the enzyme and peptide substrate concentrations were well below the K_M, such that the relative rates of dephosphorylation (k_{obs}) truly reflect the k_{cat}/K_M.

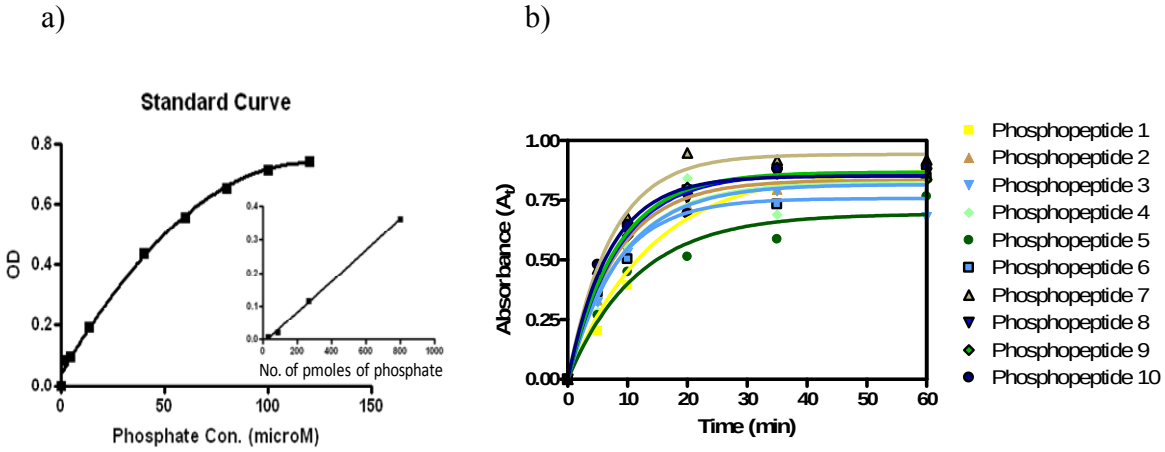


Figure S6. a) Standard curve of phosphate detection using the Malachite green assay, with inset showing the linear portion (30 to 800 pmoles of phosphate). b) Time-dependant variation in the absorbance value for the 10 phosphopeptides with PTP1B. The k_{obs} values were derived from the curves using eq.2.

Table S1. Comparison of the substrate specificity of PTP1B obtained from the probe mediated inactivation of the enzyme (K_i values) and that from the standard Malachite green phosphatase assay of the corresponding phosphopeptides. Red indicates preferred substrate, Yellow for substrate and Blue indicates no substrate or very week substrate.

Probe	K_i (mM)	Phosphopeptide	Phosphate released (nmol/min/mg)	k_{obs}
1	2.81	1	6741	0.072
2	1.00	2	10524	0.121
3	0.97	3	13549	0.127
4	2.19	4	9083	0.109
5	3.89	5	7528	0.086
6	1.93	6	8707	0.111
7	0.94	7	14419	0.137
8	2.48	8	11180	0.107
9	1.48	9	12397	0.123
10	1.00	10	15749	0.143

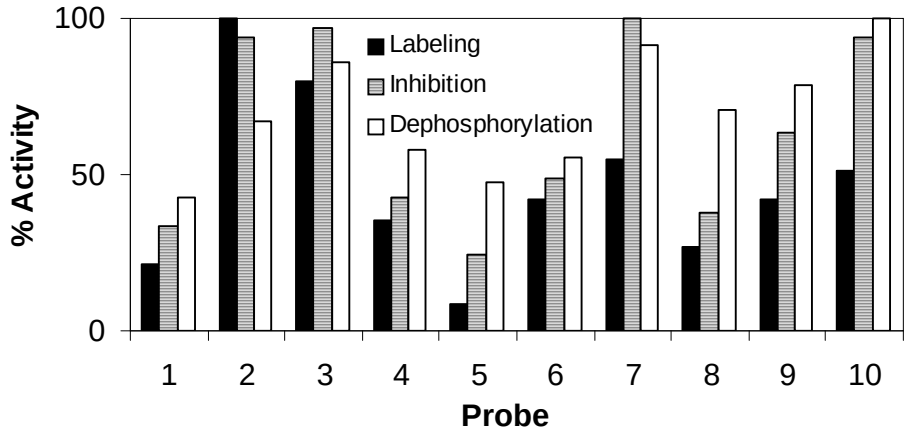


Figure S7. Comparison of relative activity of the 10 probes against PTP1B as determined from (■) quantitative analysis of the fluorescent gel shown in Figure 2 (main text), (⊞) $1/K_i$ values obtained from inactivation kinetic experiments and (□) data obtained from the dephosphorylation of ten corresponding phosphopeptides catalyzed by PTP1B. All three sets of data were internally normalized. % Activity: relative extent of labeling/inactivation/dephosphorylation for each probe/peptide (100%: most active probe/peptide within each set).

10. Labeling Experiments with Bacterial Lysate

To evaluate the performance of the probes in a complex proteome, we set up the reaction with spiked PTP1B in a bacterial lysate. Cell lysates from *E. coli* strain BL21 (DE3) were used as there is no known PTP present in this bacterial genome. Bacterial lysates were prepared following procedures mentioned elsewhere [3] with minor modifications. Briefly, an overnight culture of LB (100 ml) inoculated with a single colony of the bacteria, and grown at 37 ^{o}C with shaking (300 rpm), was harvested by centrifugation at 4000 rpm for 10 min at 4 °C. The resulting Pellets were suspended in the lysis buffer (50 mM Tris at pH 7.5, 150 mM NaCl) and sonicated (to complete lysis, 20 rounds of 1s on and 5s off, at 25 % amplitude), before centrifugation for 10 min (1000 rpm at 4 ^{o}C). The resulting supernatant was further centrifuged for 15 min (13000 rpm at 4 $^{\circ}$C). The total protein concentration of this lysate was then quantified by Bradford's Assay (Biorad), and adjusted to 6.0 mg/ml, aliquoted and stored in -20 °C, and used for all subsequent labeling experiments.

In a typical labeling reaction, different amounts of PTP1B (1 µg to 0.05 ng) was spiked into 10 µg of the bacterial proteins in Tris buffer (50 mM Tris, pH 7.5, 150 mM NaCl) at a reaction volume of 19 µL at 25 ^{o}C. To this was added 1 µL of the probe 3 (10 µM in the reaction) and incubated for 1h at 25 ^{o}C before protein separation on SDS-PAGE and in-gel fluorescence scanning. The probe selectively labeled the spiked PTP1B even in the presence of large excess of other proteins in the bacterial proteome, demonstrating the highly selective nature of the probe's PTP trapping reaction. Next, we carried out the labeling reaction with the panel of the 10 probes (10 µM in reaction) in the bacterial proteome (10 µg total protein) with 0.1 µg of spiked PTP1B. As shown in Figure S7, the labeling fingerprint obtained was identical with the fingerprint obtained using pure PTP1B demonstrating the usefulness of the probes in a complex proteome.

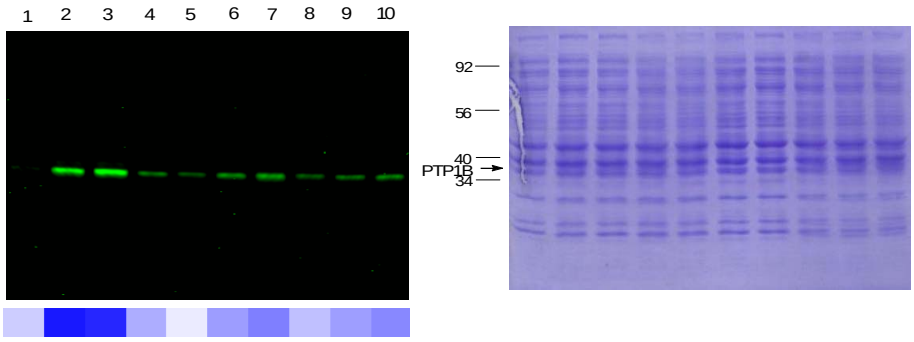


Figure S8. Labeling fingerprint of PTP1B spiked in the bacterial proteome using the panel of the 10 probes.

11. Labeling Experiments with Mammalian Cell Lysates

Total cell lysates from the mammalian cell lines HEK293T and NIH3T3 were prepared as mentioned elsewhere [6]. Briefly, the cell lines maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics (100 units/ml penicillin and 100 units/ml streptomycin) at 37 ^{o}C in 5% CO_2, upon confluence, were washed with 1X PBS, and trypsinized. The cell pellets were stored at -80 ^{o}C until lysis. The pellets were suspended in the lysis buffer (50 mM Tris at pH 7.5, 150 mM NaCl) and sonicated to complete lysis (40 rounds of 1s on and 5s off, at 25 % amplitude) at 4 ^{o}C. The total protein concentration of the lysate was quantified by Bradford's Assay (Biorad), and adjusted to 3.0 mg/ml, aliquoted and stored in -20 °C freezer, and used for all subsequent labeling experiments.

11.1. Labeling of PTPs in mammalian cells using the fluorescent probe

The mammalian cell lysates prepared from HEK293T and NIH3T3 cells (20 µg total protein in each reaction) were treated with the PTP1B selective fluorescent probe 3 (25 µM in reaction) in Tris buffer (50 mM Tris, pH=7.5) at 25 ^{o}C for 1h. The proteins were separated on SDS-PAGE and labeled bands were visualized by in-gel fluorescence scanning.

11. 2. Western Blot Analysis for endogenous PTP1B

The proteins in the gel after in-gel fluorescence scanning were electrotransferred overnight at 4 ^{o}C to a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-Buffered Saline with 0.1% Tween 20 (TBS-T) for 1 h at 25 ^{o}C. After two quick washes with TBS-T, the membrane was treated with PTP1B antibody (ab**cam**, product code: ab52650, EP1837Y) in 5% BSA in TBS-T (1:10^5 dilution) for 2 h at 25 ^{o}C. The membrane was then washed three times (5 min for each wash) with TBS-T and subsequently incubated with goat anti-rabbit HRP antibody (1:2000 dilution in TBS-T) for 1 h. The secondary antibody treated blot was then washed three times (10 min for each wash) with TBS-T, rinsed with TBS and subsequently treated with HRP substrate. Excess chemiluminescence reagent was removed and the image was acquired using dark room development facilities.

11.3. Pull-down of biotinylated probe labeled endogenous PTP1B

To further confirm the identity of the fluorescently labeled band (~ 52 kDa) from HEK293T cell lysate as PTP1B, we performed pull-down of the labeled lysate. We first synthesized the biotinylated probe **11** which has the same sequence as that of probe **3** but equipped with a biotin tag making the probe suitable for pull-down with NeutrAvidine beads. The pull-down followed by immunoblot experiments were carried out as described below. HEK293 cell lysates were prepared as previously described. Before PTP probe labeling, lysates was incubated with washed NeutrAvidin Agarose beads (Pierce) to remove endogenously biotinylated proteins. This flow-through fraction was used for PTP probe labeling. After 1 h reaction at RT, proteins were precipitated from the lysate by acetone and re-dissolved in solubilizing buffer (PBS with 1% SDS). Before incubation

with NeutrAvidin Agarose Beads, samples volumes were adjusted by adding PBS to dilute SDS to 0.1%. After incubation with washed beads for 1 h at RT, beads were washed with 10 bed volumes of PBS with 0.1%SDS for 3 times. Bounded protein was eluted by boiling in SDS sample buffer. For immunoblot analysis, this pull down sample was then separated on 12% SDS-PAGE gel, transferred to PVDF membrane and probed with anti-PTP1B antibody. 52 kDa PTP1B band was observed in the pull-down elute fraction while not in the negative control (lysate without probe treatment) pull-down fraction.

12. References

[1] (a) H. Sun, C.H.S. Lu, M. Uttamchandani, Y. Xia, Y-C. Liou, Shao Q. Yao, *Angew. Chem. Int. Ed*. **2008**, 47, 1698; (b) R. Srinivasan, L.P. Tan, H. Wu, Shao Q. Yao, *Org. Lett.* **2008**, *10*, 2295. (c) H. Sun, L. P. Tan, L. Gao, Shao Q. Yao, *ChemComm*. **2009**, 677.

[2] C. Blanchetot, M.Chagnon, N. Dubé, H. Hallé, M.L. Tremblay, *Methods* **2005**, *35*, 44.

[3] D. Greenbaum, K.F. Medzihradszky, A. Burlingame, M. Bogyo, *Chem. Biol*. **2000**, *7*, 569.

[4] (a) P. Klatt, S. Lamas, *Eur. J. Biochem.* **2000**, *267*, 4928. (b) A. Salmeen, J.N. Andersen, M.P. Myers, T.-C. Meng, J.A. Hinks, N.K. Tonks, D. Barford, *Nature* **2003**, *423*, 769. (c) R.L.M. van Montfort, M. Congreve, D. Tisi, R. Carr, H. Jhoti, *Nature* **2003**, *423*, 773.

[5] R. Kitz, I.B. Wilson, *J. Biol. Chem.* **1962**, *237*, 3245.

[6] T.Florio, S. Arena, A. Pattarozzi, S.Thellung, A. Corsaro, V. Villa, A. Massa, F. Diana, G. Spoto, S. Forcella, G. Damonte, M. Filocamo, U. Benatti, G. Schettini, *Mol. Pharmacol.* **2003**, *63*, 297.