Selective Seoul-Fluor-based bioprobe, S*f*BP, for vaccinia H1-related phosphatase—a dual-specific protein tyrosine phosphatase

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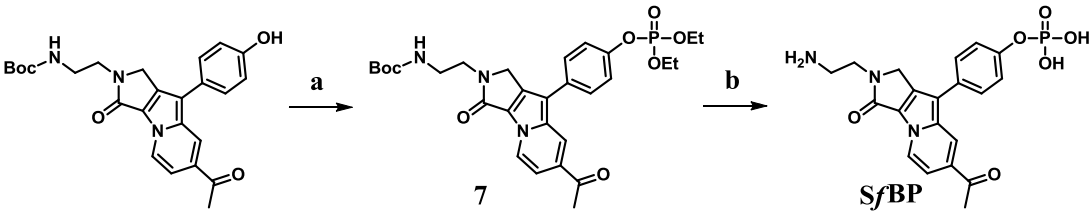
I. General information

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-300 (Bruker Biospin, Germany) and Varian Inova-500 (Varian Assoc., Palo Alto, USA), and chemical shifts were measured in ppm downfield from internal tetramethylsilane (TMS) standard. Multiplicity was indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet); dd (doublet of doublet); dt (doublet of triplet); td (triplet of doublet); br s (broad singlet), etc. Coupling constants were reported in Hz. Routine mass analyses were performed on LC/MS system equipped with a reverse phase column (C-18, 50 x 2.1 mm, 5 μm) and photodiode array detector using electron spray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The identity of desired compounds was confirmed by high-resolution mass spectrometry (HRMS), which was conducted at the Mass Spectrometry Laboratory of Seoul National University by direct injection on a JEOL JMS AX505WA spectrometer using fast atom bombardment (FAB) method. The reaction progress was monitored using thin-layer chromatography (TLC) (silica gel 60 F_{254} 0.25 mm), and individual components were visualized by observation under UV light (254 and 365 nm) or by treating the TLC plates with anisaldehyde, $KMnO_4$, and phosphomolybdic acid followed by heating. All reactions were conducted in oven-dried glassware under dry argon atmosphere, unless otherwise specified. Toluene and THF were dried by distillation from sodium-benzophenone immediately prior to use. CH_2Cl_2 was distilled from CaH_2 and TEA was distilled over KOH. Other solvents and organic reagents were purchased from commercial venders and used without further purification unless otherwise mentioned. Distilled water was polished by ion exchange and filtration. UV absorbances of fluorescence compounds were measured by UV-VIS spectrophotometer (UV-1650PC, Shimatzu, Japan). Excitation and emission maxima were measured by Cary Eclipse Fluorescence spectrophotometer (Varian Assoc., Palo Alto, USA). All the photography images of the fluorescent compounds in each condition (final concentration = 67 μM) were taken under irradiation at 365 nm wavelengths. Reverse phase HPLC analysis was performed on a VPODS C-18 column (150 x 4.6 mm) at a flow rate of 1.0 mL/min for analysis, and PRC-ODS C-18 column (250 x 20 mm) at a flow rate of 10.0 mL/min for preparation, equipped with Shimadzu LC-6AD pump and SPD-10A detector (Japan). HPLC solvents consist of water and acetonitrile, containing 0.1% TFA.

II. Synthetic procedure and compound characterization

General procedure for preparing compound **6** is identical to the procedure described in the previous report.[1] In this study, compound **7**, **8**, and S*f*BP were synthesized from **6**, utilizing a sequence of reactions as following.

a. Preparation of compound 7 and S*f*BP.



Reagents and conditions: (a) I_2, DMAP, $P(OEt)_3$ / DCM; (b) TMSBr / DCM

tert-Butyl 2-(7-acetyl-9-(4-(diethoxyphosphoryloxy)phenyl)-3-oxo-1*H*-pyrrolo[3,4-*b*]indolizin-2(3*H*)-yl)ethylcarbamate (7)

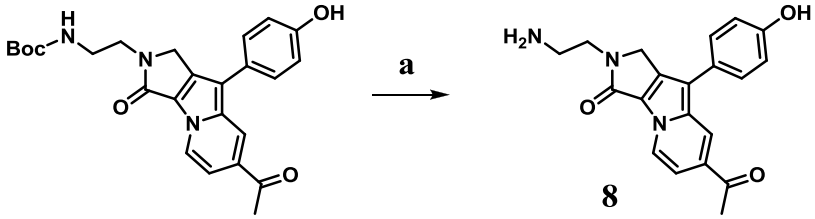
To a stirred solution of iodine (141 mg, 0.556 mmol) in DCM (1.5 mL) at 0 ℃, added a triethylphosphite (114 μL, 0.666 mmol) dropwise. Then, the reaction mixture was added to a stirred solution of **6** (100 mg, 0.222 mmol) and DMAP (68 mg, 0.556 mmol) in DCM (1.5 mL) at room temperature. The resulting mixture was stirred at room temperature overnight. After completion of the reaction monitored by TLC, the reaction mixture was diluted with ddH_2O and extracted with DCM 3 times. The combined organic layer was dried over anhydrous Na_2SO_4(s), and the filtrate was concentrated *in vacuo*. The desired compound **7** was purified by silica-gel flash column chromatography (EA:Hex = 1:3) as a yellow solid (122 mg, 0.208 mmol, yield : 93.8%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55 (d, *J* = 7.5 Hz, 1H), 8.34 (s, 1H), 7.50 (d, *J* = 8.5 Hz, 2H), 7.36 (d, *J* = 8.0 Hz, 2H), 7.28 (dd, *J* = 7.5 Hz, 1.5 Hz, 1H), 4.98 (br s, 1H), 4.56 (s, 2H), 4.32–4.25 (m, 4H), 3.73 (t, *J* = 5.8 Hz, 2H), 3.47–3.43 (m, 2H), 2.61 (s, 3H), 1.41 (t, *J* = 7.2 Hz, 6H), 1.33 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 195.7, 162.2, 156.4, 149.9, 135.4, 134.8, 130.6, 129.6, 129.0, 124.9, 122.9, 121.2, 113.4, 109.9, 65.0, 64.9, 47.2, 43.3, 39.9, 28.5, 26.3, 16.4, 16.3; ^{31}P NMR (202 MHz, $CDCl_3$) δ -6.91(s); LRMS (EI): *m*/*z* calcd for $C_{29}H_{36}N_3O_8P$ $[M+H]^+$ 586.22, found 586.42; HRMS (FAB): *m*/*z* calcd for $C_{29}H_{36}N_3O_8P$ $[M]^+$ 585.2240, found 585.2241.

[1] E. Kim, M. Koh, B. J. Lim, and S. B. Park, *J. Am. Chem. Soc.*, 2011, **133**, 6642–6649.

4-(7-acetyl-2-(2-aminoethyl)-3-oxo-2,3-dihydro-1*H*-pyrrolo[3,4-*b*]indolizin-9-yl)phenyl dihydrogen phosphate (S*f*BP)

A solution of compound **7** (115 mg, 0.197 mmol) in TMSBr and DCM (v:v = 1:1, 1 mL) was stirred at room temperature. After completion of the reaction, TMSBr was removed by evaporation. The resulting residue was redissolved in saturated aqueous $NaHCO_3$ solution, purified by preparative-HPLC, and subsequently freeze-dried to afford the desired compound, **S*f*BP**, as a yellow fluffy solid (57 mg, 0.133 mmol 67.6%). ^{1}H NMR (500 MHz, CF_3CO_2D) δ 8.64–8.60 (m, *2*H), 7.65–7.41 (m, 5H), 4.83 (br s, 2H), 4.24 (br s, 2H), 3.78 (br s, 2H), 2.82 (br s, 3H); ^{31}P NMR (202 MHz, D_2O) δ –0.75 (s); LRMS (EI): *m*/*z* calcd for $C_{20}H_{20}N_3O_6P$ $[M+H]^+$ 430.11, found 430.21.

b. Preparation of compound 8.



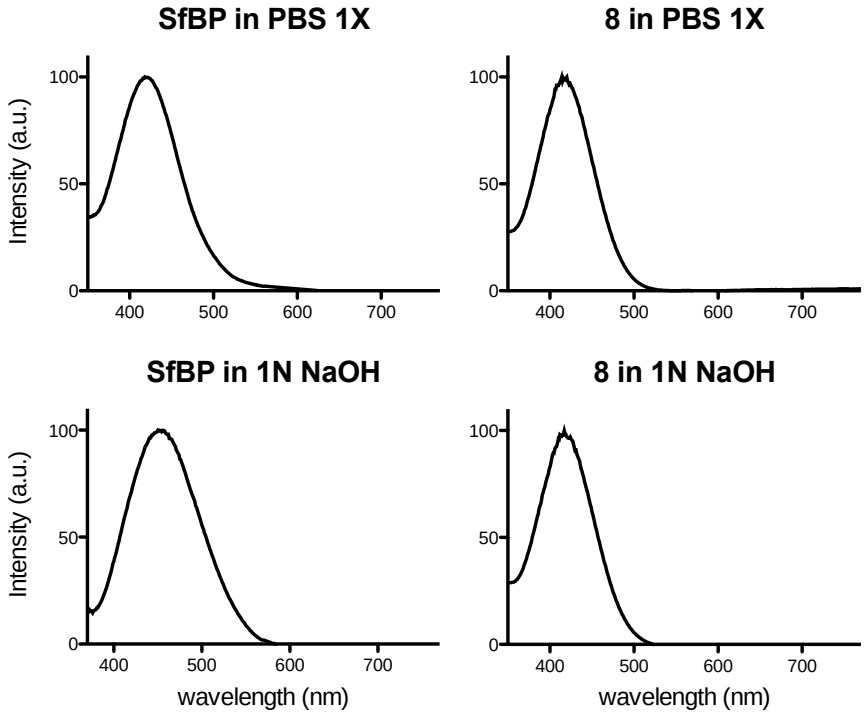
Reagents and conditions: (a) TFA:DCM (v:v = 1:1)

7-acetyl-2-(2-aminoethyl)-9-(4-hydroxyphenyl)-1*H*-pyrrolo[3,4-*b*]indolizin-3(2*H*)-one (8)

A solution of compound **6** (30 mg, 0.067 mmol) in TFA and DCM (v:v = 1:1) was stirred at ambient temperature. After completion of the reaction, TFA was removed by evaporation. After basification with aqueous $NaHCO_3$(sat), crude product was purified by preparative-HPLC and subsequently freeze-dried to afford the desired compound **8** as a yellow solid (27 mg, 0.058 mmol, yield : 87.2%) ^{1}H NMR (500 MHz, DMSO-d_6) 9.71 (br s, 1H), 8.50 (d, *J* = 7.0 Hz, 1H), 8.40 (s, 1H), 7.89 (br s, 3H), 7.51 (d, *J* = 8.5 Hz, 2H), 7.27 (d, *J* = 7.0 Hz, 1H), 6.94 (d, *J* = 8.5 Hz, 2H), 4.68 (s, 2H), 3.77 (br s, 2H), 3.14 (br s, 2H), 2.63 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) 195.7, 161.1, 158.0 (q, $J_{C,F}$ = 30.6 Hz), 156.6, 135.1, 133.1, 128.6, 128.3, 124.1, 123.8, 122.0, 121.6, 116.2, 113.6, 109.3, 45.4, 37.8, 26.2; LRMS (EI): *m*/*z* calcd for $C_{20}H_{20}N_3O_3$ $[M+H]^+$ 350.15, found 350.07; HRMS (FAB): *m*/*z* calcd for $C_{20}H_{20}N_3O_3$ $[M+H]^+$ 350.1511, found 350.1505.

III. Supporting figures and procedures for photophysical properties, enzymatic kinetics study and docking analysis.

A.



B.

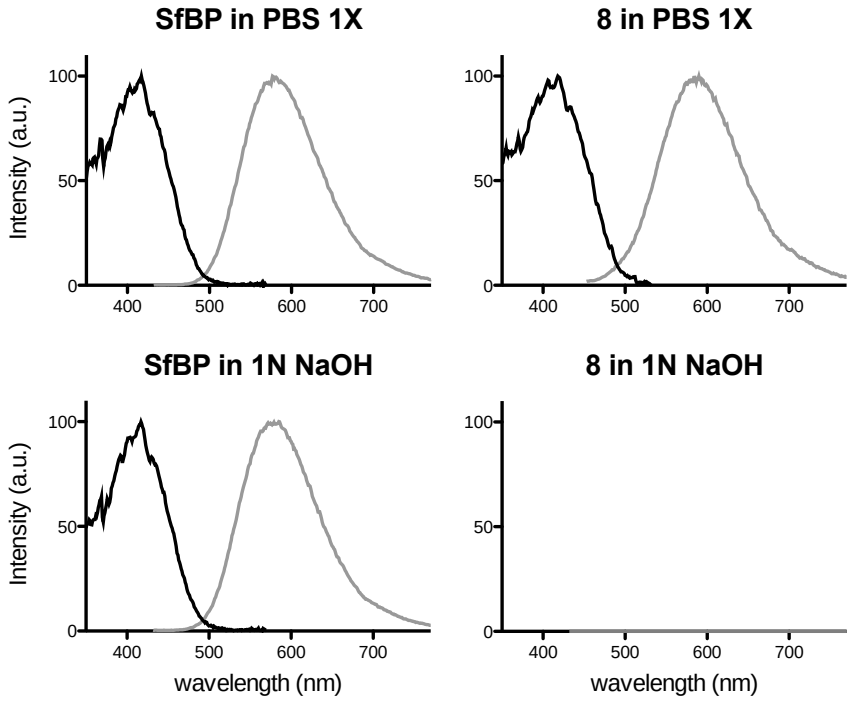


Fig. S1. The normalized absorption (A) spectra and excitation (black) & emission (grey) spectra (B) of S*f*BP and **8**.

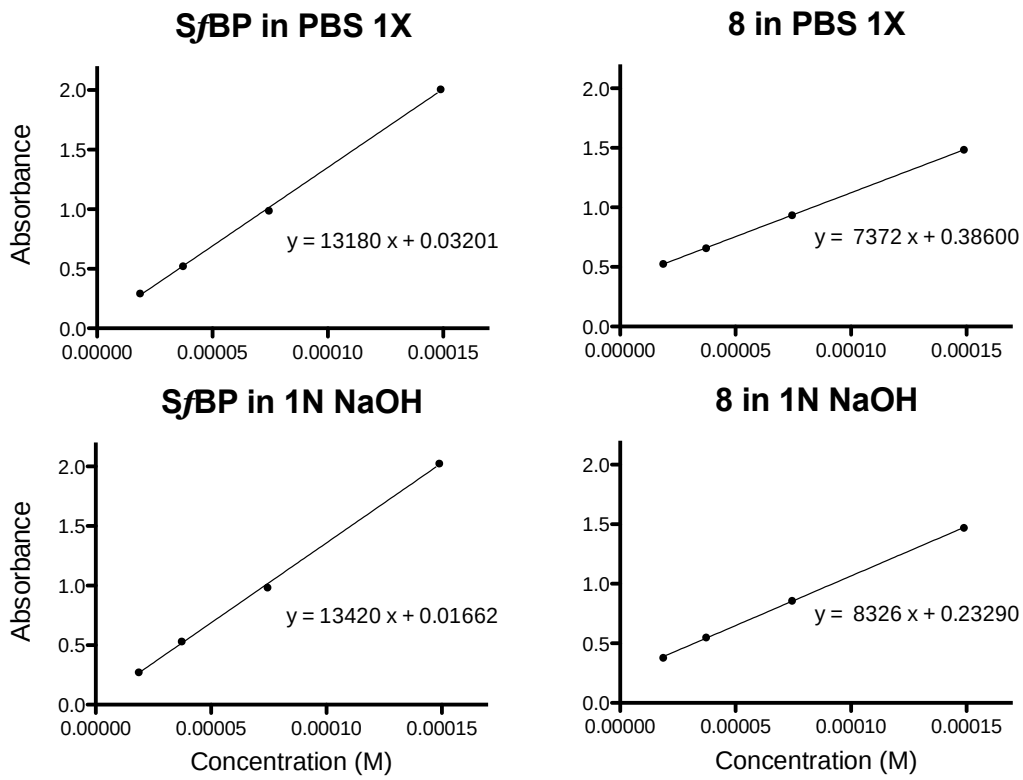


Fig. S2. Calculation of the molar extinction coefficient (ε) for S*f*BP (left) and **8** (right) in PBS 1X (up) and 1N NaOH (down) solution at the absorption maximum in each condition.

Absolute quantum yield measurement

Absolute quantum yield was measured with QE-1000, Otsuka Electronics Co., LTD, Japan. Absolute quantum yields of known fluorescent dyes in various wavelengths were measured to confirm the reliability of the measurement system. [Anthracene: 0.275 (reported: 0.27)[2] in EtOH; fluorescein: 0.831 (reported: 0.79)[3] in 1N NaOH solution; rhodamine 6G: 0.933 (reported: 0.95)[4] in EtOH]

[2] W. H. Melhuish, *J. Phys. Chem.*, 1961, 65, 229–235.
[3] J. Q. Umberger, V. K. LaMer, *J. Am. Chem. Soc.*, 1945, **67**, 1099–1109.
[4] R. F. Kubin and A. N. Fletcher, *J. Luminescence*, 1982, **27**, 455–462.

Purification of recombinant PTPs

Human PTP genes were amplified by PCR using appropriate primers and corresponding cDNAs as templates. The PCR products were digested with *Nde*I and *BamH*I or *Nde*I and *Xho*I, and subcloned into *Nde*I-*BamH*I or *Nde*I-*Xho*I digested *pET* 28a(+) vector (Novagen). The resulting *N*-terminal MBP, *C*-terminal-His_6-tagged human PTPs were transformed into *E. coli* Rosetta (DE3) (Novagen). Expression of the recombinant human PTPs was induced by adding 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) at 291 K for 16 h. Cells were harvested by centrifugation (3,570*g*, at 4 °C for 10 min), washed with buffer A (50 mM Tris pH 7.5, 250 mM NaCl, 5% glycerol, and 0.025% β-mercaptoethanol), and lysed by ultrasonication. After centrifugation (29,820*g* for 30 min), the supernatant was incubated with a cobalt affinity resin (TALON®, Clontech) on a rocker at 4 °C for 1 h, and then resin was washed with buffer A containing 10 mM imidazole. PTPs were eluted with buffer A containing 100 mM imidazole, concentrated to 2 mg/mL, and stored at –70 °C. PTPs tested in this study were listed in **Fig. S3**.

	Classical PTPs			Dual specific PTPs			
	A	B	C	D	E	F	G
1	PTPRA	PTPRN2	PTPN6	DUSP1	DUSP11	DUSP25	TENC1
2	PTPRC	PTPRR	PTPN7	DUSP3	DUSP12	DUSP26	MTMR1
3	PTPRE	PTPRS	PTPN11	DUSP4	DUSP13B	EPM2A	MTMR3
4	PTPRF	PTPRT	PTPN12	DUSP5	DUSP14	SSH1	MTMR7
5	PTPRG	PTPRU	PTPN13	DUSP7	DUSP18	SSH2	MTMR8
6	PTPRJ	PTPRZ	PTPN14	DUSP8	DUSP19	PTP4A1	MBP
7	PTPRK	PTPN1	PTPN18	DUSP9	DUSP21	PTP4A2	
8	PTPRM	PTPN2	PTPN21	DUSP10	DUSP22	PTP4A3	
9	PTPRN	PTPN3	PTPN22	DUSP16	DUSP23	CDC14B	
10	PTPRO	PTPN5	PTPN23	MK-STYX	DUSP24	TPTE	

Fig. S3. PTPs used in this study. Class I human PTPs, consisting of 30 classical PTPs and 35 dual specific PTPs, were tested for S*f*BP catalysis.

Measurement of enzymatic activities of cloned PTPs with DiFMUP at p*H* 8.0

The enzymatic reactions were started by addition of each PTP (1 μM) to a solution of DiFMUP (100 μM) in 100 μL of reaction buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μM BSA) on a 96 well-plate. Reaction mixtures were incubated at 25 °C for 10 min. The fluorescence intensities were measured at Ex/Em = 355/460 nm on a Wallac Victor Microplate Reader (Fig. S4).

Fluorescence assay of S*f*BP hydrolysis by various PTPs at p*H* 8.0

The enzymatic reactions were started by addition of each PTP (1 μM) to a solution of S*f*BP (100 μM) in the reaction buffer (100 μL, 20 mM Tris, p*H* 8.0, 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μM BSA) on a 96 well-plate. Reaction mixtures were incubated at 25 °C for 30 min and followed by addition of 2 N NaOH (100 μL). The fluorescence intensities were measured at Ex/Em = 405/570 nm on a Wallac Victor Microplate Reader (Fig. S4). In addition, the three DUSPs (DUSP3, DUSP13B, DUSP14), which showed meaningful activity for SfBP in the primary screening, were tested for SfBP hydrolysis at the same conditions described above except that the enzymes (100 nM) were used (Fig. 3).

DiFMUP

	Classical PTPs			Dual Specific PTPs			
	A	B	C	D	E	F	G
1	75	0	74	3	1	14	1
2	71	98	100	45	1	3	69
3	2	98	80	4	1	95	1
4	97	57	63	1	24	11	1
5	97	3	88	8	41	1	4
6	95	0	6	25	4	1	0
7	1	96	97	12	22	1	
8	90	59	66	1	8	1	
9	2	95	96	1	1	96	
10	91	1	1	0	1	0	

Activity (%)

76-100
51-75
26-50
0-25

S*f*BP

	Classical PTPs			Dual Specific PTPs			
	A	B	C	D	E	F	G
1	8	4	5	6	16	0	9
2	10	13	10	100	14	9	9
3	11	8	11	11	53	12	13
4	18	8	11	1	52	10	11
5	8	10	8	10	8	12	9
6	12	7	10	17	18	12	0
7	9	10	8	24	10	13	
8	10	9	7	5	10	9	
9	11	10	10	8	8	12	
10	22	4	14	6	7	19	

Fig. S4. Normalized percentage activity of DiFMUP and S*f*BP for 65 class I human PTPs. The data were normalized as percentage activity relative to that of the PTP with highest activity: PTPRC for DiFMUP, and VHR (DUSP3) for S*f*BP, respectively.

Measurement of enzymatic activities of cloned DUSPs for DiFMUP hydrolysis at p*H* 6.0

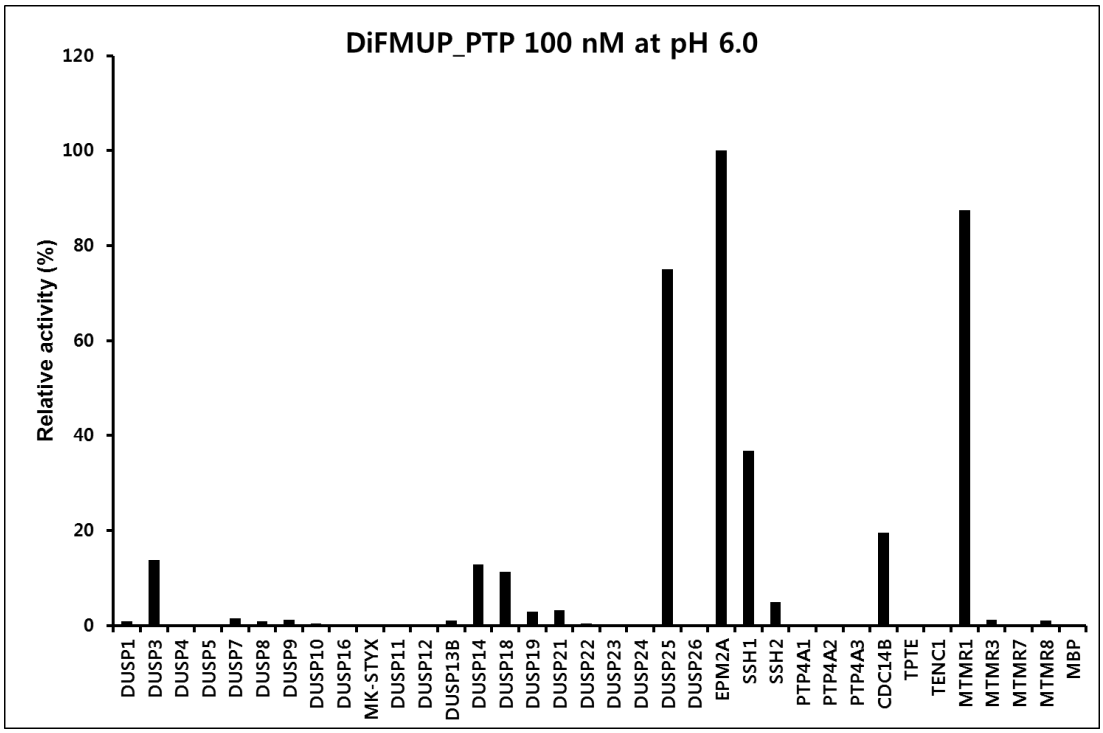
Since most DUSPs show the optimal catalytic activity the catalytic activity of DUSPs for DiFMUP was tested at p*H* 6. The enzymatic reaction mixtures were prepared by the addition of each PTP (10 nM or 100 nM) to aqueous solution of DiFMUP (100 μM) in 100 mM Bis-Tris p*H* 6.0 reaction buffer with 1 mM dithiothreitol (DTT) present.[5] Reaction mixtures were incubated at 25 °C for 10 min and the fluorescence intensities were measured at Em/Ex = 355/460 nm on a 96-well plate using Wallac Victor Microplate Reader (Fig. S5A).

Fluorescence assay of S*f*BP hydrolysis with various DUSPs at pH 6.0

Since most DUSPs show the optimal catalytic activity the catalytic activity of DUSPs for S*f*BP was tested at p*H* 6. The enzymatic reaction mixtures were prepared by the addition of each PTP (10 nM or 100 nM) to aqueous solution of S*f*BP (100 μM) in 100 mM Bis-Tris pH 6.0 reaction buffer with 1 mM dithiothreitol (DTT) present.[5] Reaction mixtures were incubated at 25 ℃ for 30 min and followed by addition of 2 N NaOH solution to mixture solutions. The fluorescence intensities were measured at Ex/Em = 405/570 nm on a 96-well plate using a Wallac Victor Microplate Reader (Fig. S6).

[5] S. Wu, S. Vossius, S. Rahmouni, A. V. Miletic, T. Vang, J. Vazquez-Rodriguez, F. Cerignoli, Y. Arimura, S. Williams, T. Hayes, M. Moutschen, S. Vasile, M. Pellecchia, T. Mustelin, L. Tautz, *J. Med. Chem.*, 2009, **52**, 6716–6723.

A. DiFMUP hydrolysis at pH 6 by 100 nM DUSPs



B. DiFMUP hydrolysis at pH 6 by 10 nM DUSPs

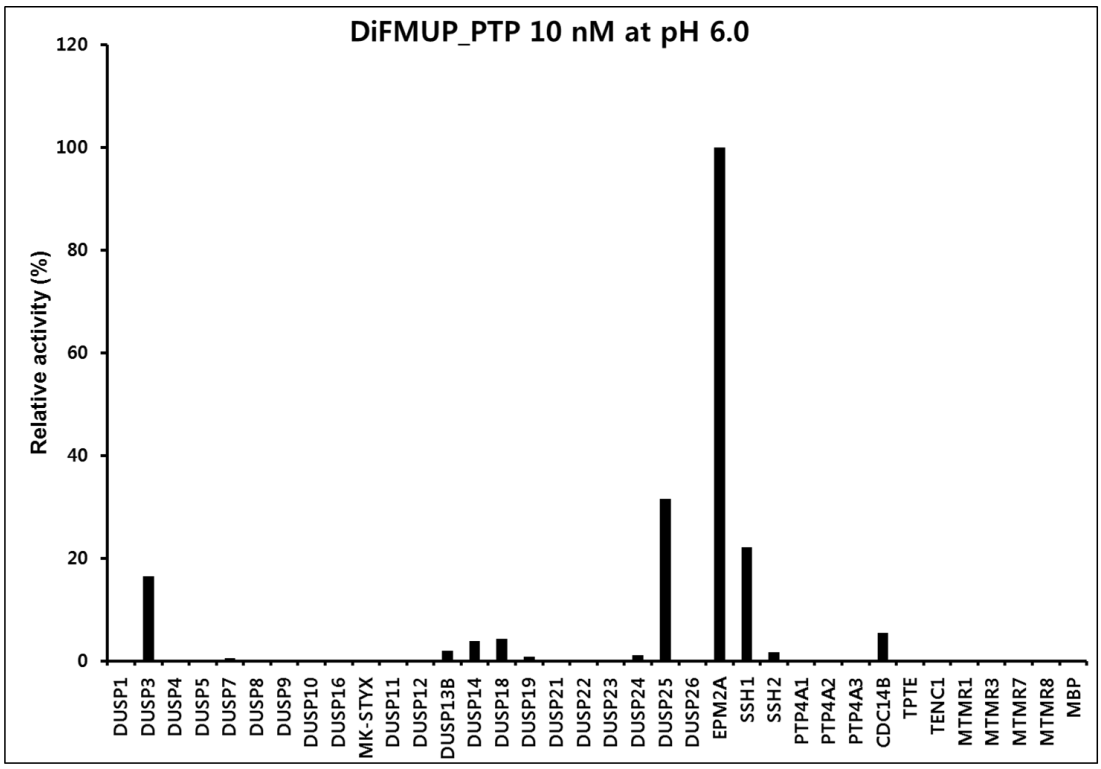
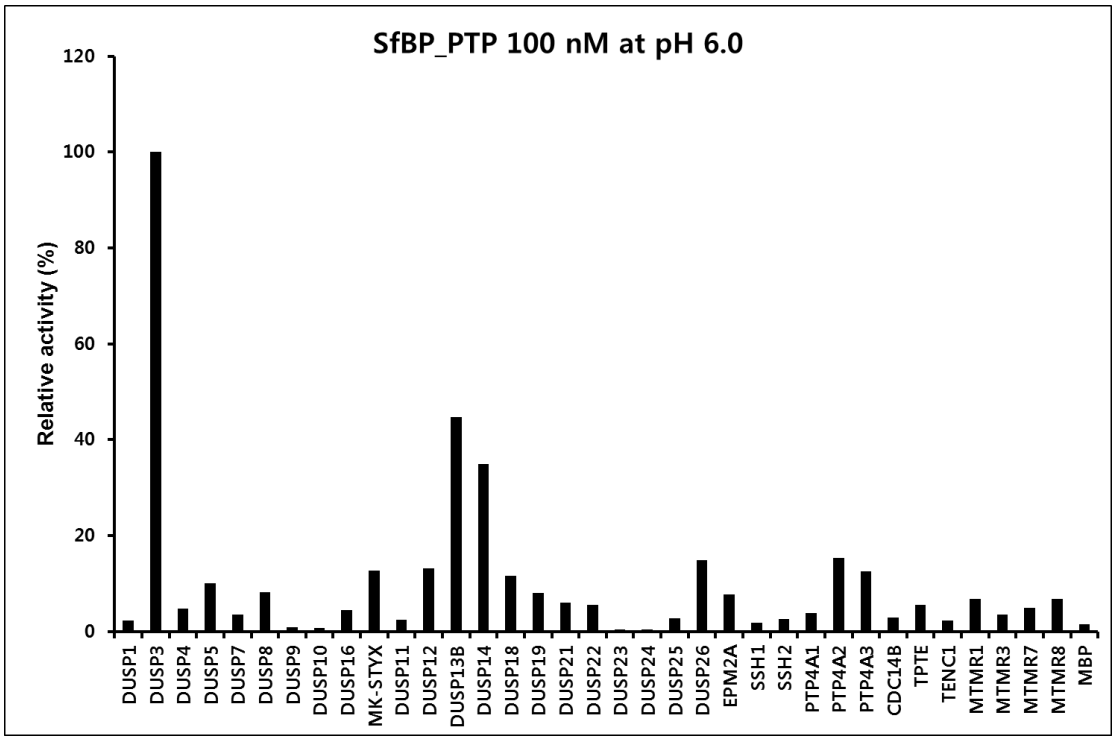


Fig. S5. Normalized percentage activity of DiFMUP hydrolysis at pH 6.0 by 100 nM (A) and 10 nM (B) of 35 DUSPs, respectively. The data were normalized as percentage activity relative to that of EPM2A.

A. S*f*BP hydrolysis by 100 nM DUSPs.



B. S*f*BP hydrolysis by 10 nM DUSPs.

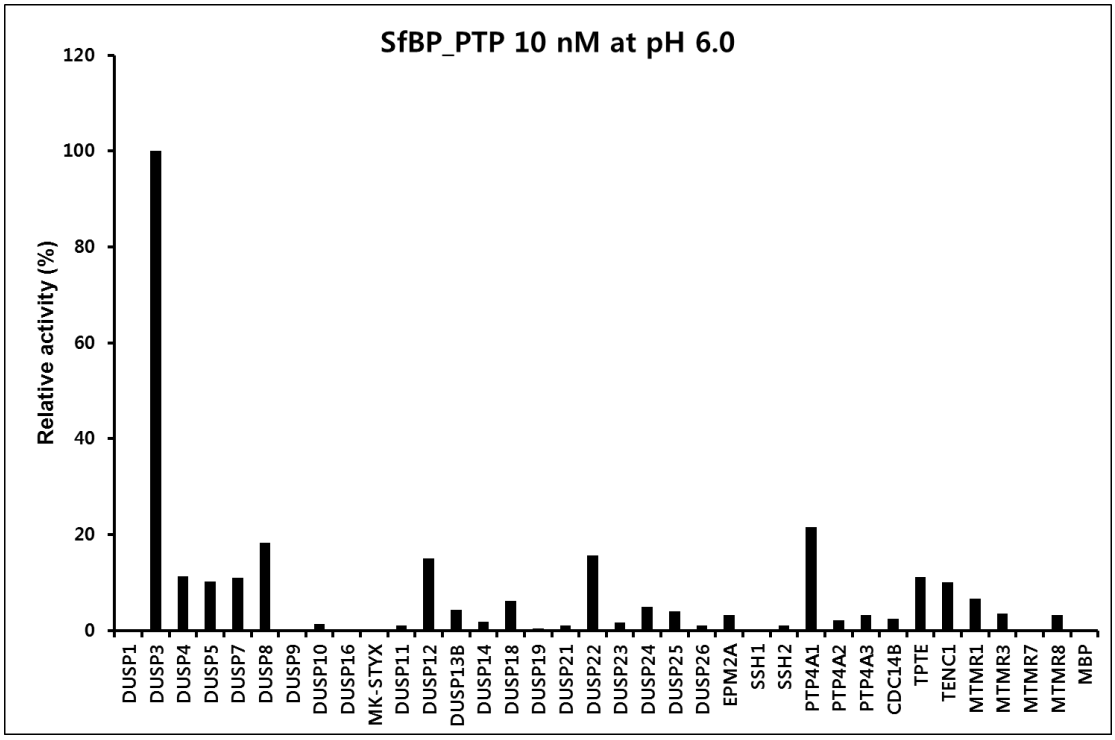


Fig. S6. Normalized percentage activity of S*f*BP hydrolysis at pH 6.0 by 100 nM (A) and 10 nM (B) of 35 DUSPs, respectively. The data were normalized as percentage activity relative to that of VHR (DUSP3). When 10 nM of DUSPs were tested, only DUSP3 showed the strong activity for SfBP catalysis.

Determination of kinetic constants for S*f*BP hydrolysis at p*H* 8.0 by DUSP3

To determine the kinetic constants, S*f*BP at a series of the final concentrations (12.5–200 μM) was hydrolyzed by DUSP3 (1 μM). The reaction (100 μL), in the reaction buffer (20 mM Tris, p*H* 8.0, 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μM BSA), was aliquated and quenched by adding 2 N NaOH (100 μL) in a 5 min interval over 30 min, and measured fluorescence intensity at Ex/Em = 405/570 nm at 25 ℃. The initial velocity was calculated from the slope of the each progress curve (**Fig. S7** (left)). The parameters such as K_m and k_{cat} for S*f*BP catalysis by DUSP3 were determined by Lineweaver-Burk plot. **Fig. S7** (right) showed the mean value of the relative initial velocity of S*f*BP hydrolysis by DUSP3. K_m, k_{cat}, and k_{cat}/K_m values for S*f*BP catalysis by DUSP3 were determined to be 100.8 μM, 2.45 min^{-1}, and 0.02 $\mu M^{-1} \cdot min^{-1}$, respectively.

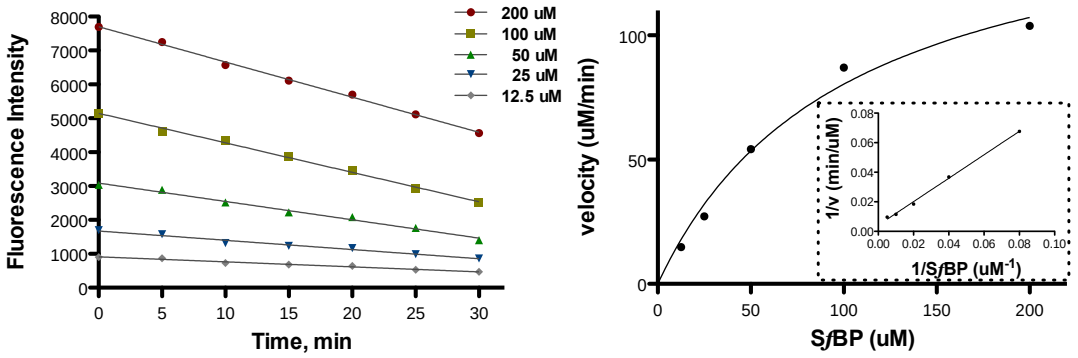


Fig. S7. Catalytic hydrolysis of S*f*BP by DUSP3. Progress curves of S*f*BP hydrolysis at a series of concentrations (left) and Michaelis-Menten curve of S*f*BP by DUSP3 (right). The insert represents Lineweaver-Burk plot.

Determination of kinetic constants for DiFMUP at pH 8.0

To determine the kinetic constants, DiFMUP at a series of the final concentrations (25–800 μM) was hydrolyzed by DUSP3 (0.1 μM). The reaction (100 μL), in the reaction buffer (20 mM Tris, p*H* 8.0, 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μM BSA in a 1 min interval over 10 min, and measured fluorescence intensity at

Ex/Em = 355/460 nm at 25 ℃. The initial velocity was calculated from the slope of the each progress curve. The parameters such as K_m and k_{cat} for DiFMUP catalysis by DUSP3 were determined by Lineweaver-Burk plot. K_m, k_{cat}, and k_{cat}/K_m values for S*f*BP catalysis by DUSP3 were determined to be 199.6 μM, 7.43 min^{-1}, and 0.37 $\mu M^{-1} \cdot min^{-1}$, respectively.

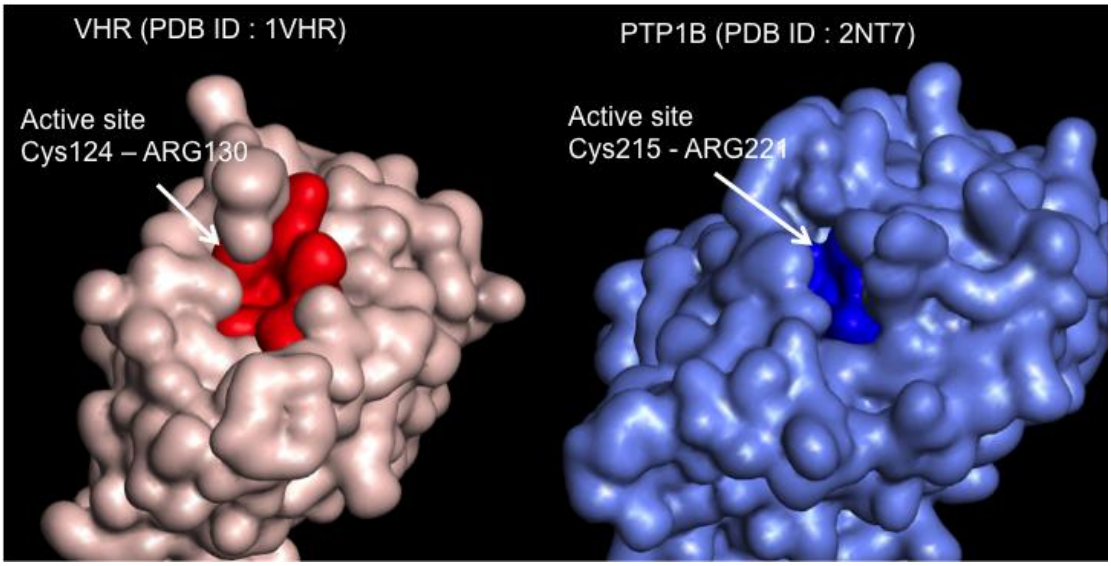
Determination of kinetic constants for S*f*BP at p*H* 6.0

To determine the kinetic constants at pH 6.0, S*f*BP and DiFMUP at a series of the final concentrations (SfBP: 6.25–200 μM, DiFMUP: 6.25-800 μM) were hydrolyzed by VHR (DUSP3), DUSP14, and DUSP13B. The reactions (100 μL) with SfBP, in the reaction buffer (20 mM Bis-Tris, pH 6.0, 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μM BSA), were aliquoted and quenched by adding 2 N NaOH (100 μL) in a 5 min interval over 30 min, and measured fluorescence intensity at Ex/Em = 405/570 nm at 25 ℃. Another reactions (100 μL) with DiFMUP, in the reaction buffer (20 mM Bis-Tris, pH 6.0, 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μM BSA) were measured fluorescence intensity at Ex/Em = 355/460 nm at 25 ℃ in a 1 min interval over 10 min. The initial velocity was calculated from the slope of the each progress curve. The parameters such as K_m and k_{cat} for S*f*BP and DiFMUP catalysis by VHR (DUSP3), DUSP14 and DUSP13B were determined by Lineweaver-Burk plot. Kinetic constants (K_m, k_{cat}, and k_{cat}/K_m values) for S*f*BP and DiFMUP hydrolysis by three DUSPs were listed in Table. 2.

Docking analysis

All possible conformers of S*f*BP were virtually generated with Vconf Interface program of Verachem, LLC. All generated conformers were analyzed with computer-aided docking simulation, which was performed by the Discovery Studio 1.7® program. The detailed docking calculation was performed using LigandFit module implemented in the Receptor-Ligand Interaction protocol. We utilized the crystal structure of PTP1B (PDB ID: 2NT7) and DUSP3 (PDB ID: 1VHR) for our docking simulation. The binding sites for S*f*BP were defined from their receptor cavities.

A.



B.

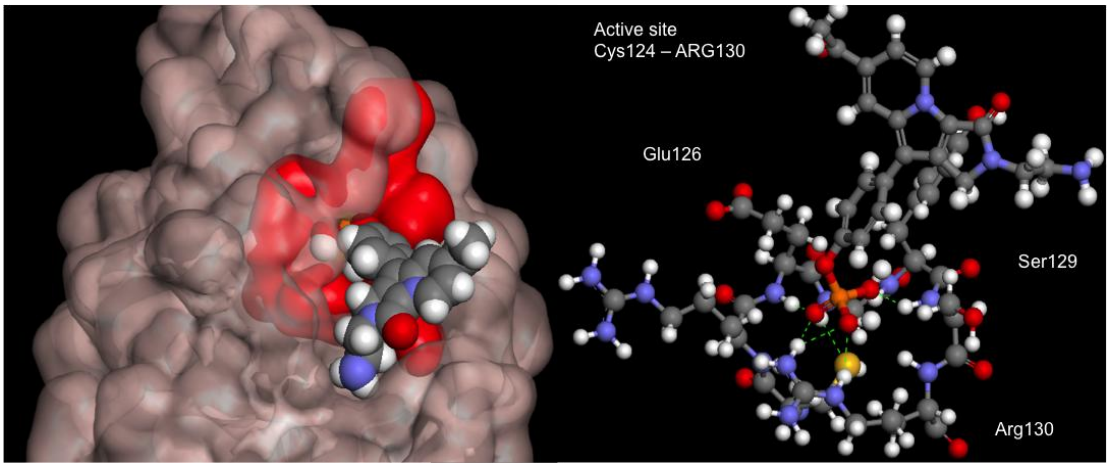
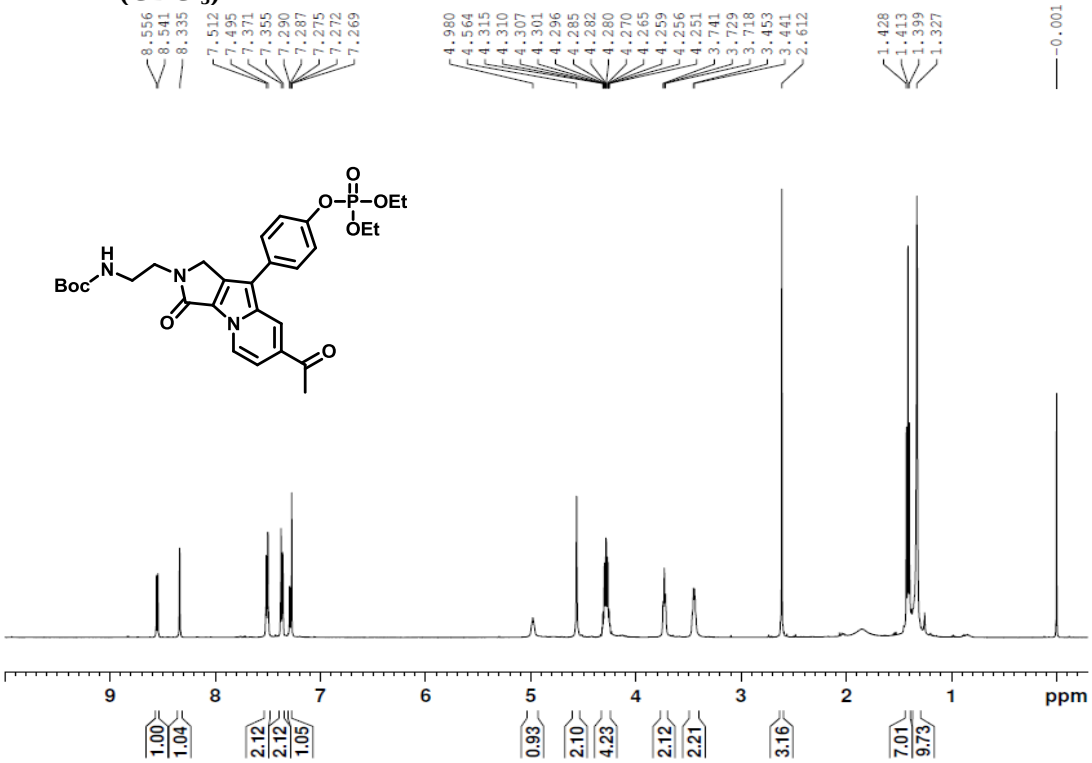


Fig. S8. *In silico* docking analysis. A) Crystal structures of VHR and PTP1B. Active sites (Cys124–Arg130 for VHR and Cys215-Arg221 for PTP1B) were highlighted in red and blue (for VHR and PTP1B, respectively). B) The docking mode of S*f*BP at the active site of VHR and its specific interaction are presented. Phosphate group of S*f*BP potentially involves 3 hydrogen bonding interactions with Glu126, Ser129 and Arg130 in active site. We failed to identify the meaning docking of S*f*BP with PTP1B.

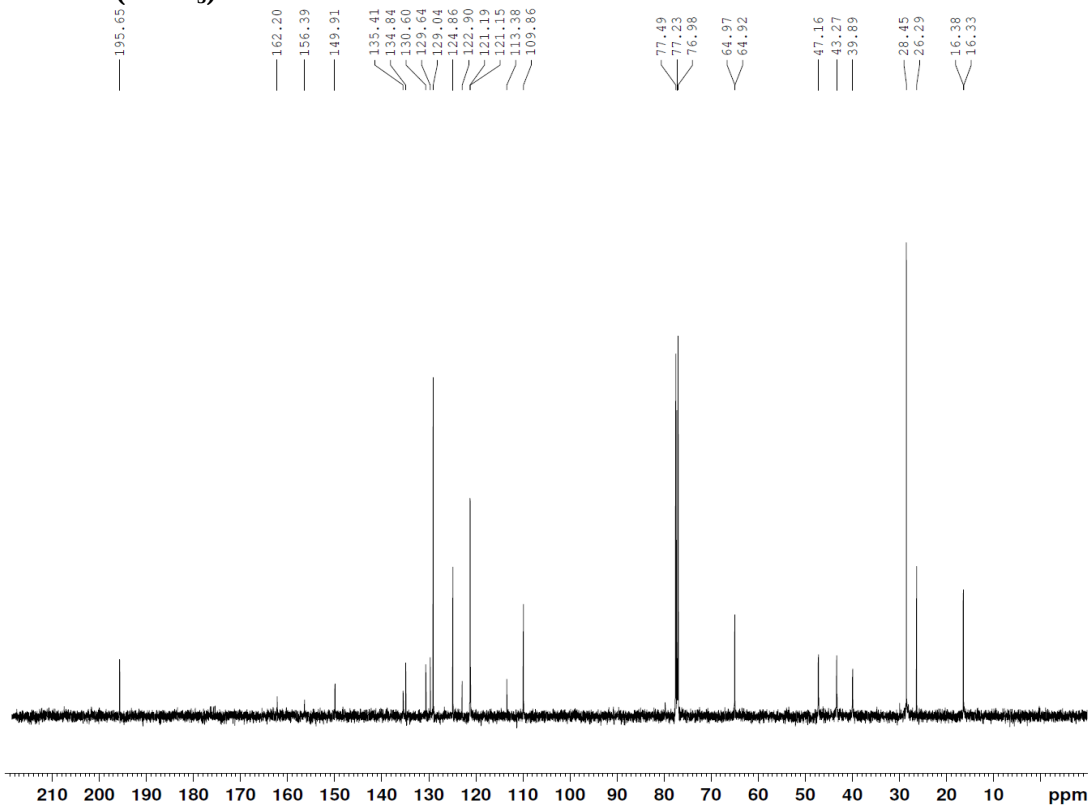
IV. Copies of NMR spectra of all new compounds

tert-Butyl 2-(7-acetyl-9-(4-(diethoxyphosphoryloxy)phenyl)-3-oxo-1*H*-pyrrolo[3,4-*b*]indolizin-2(3*H*)-yl)ethylcarbamate

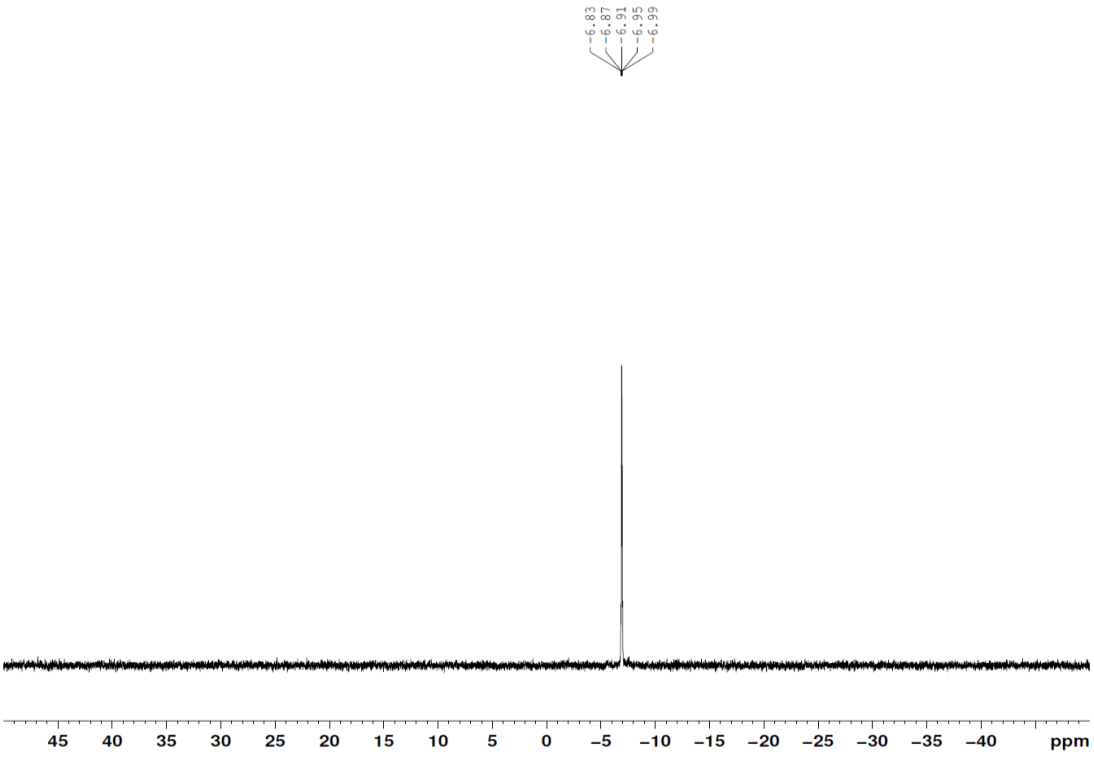
^{1}H NMR ($CDCl_3$)



^{13}C NMR ($CDCl_3$)

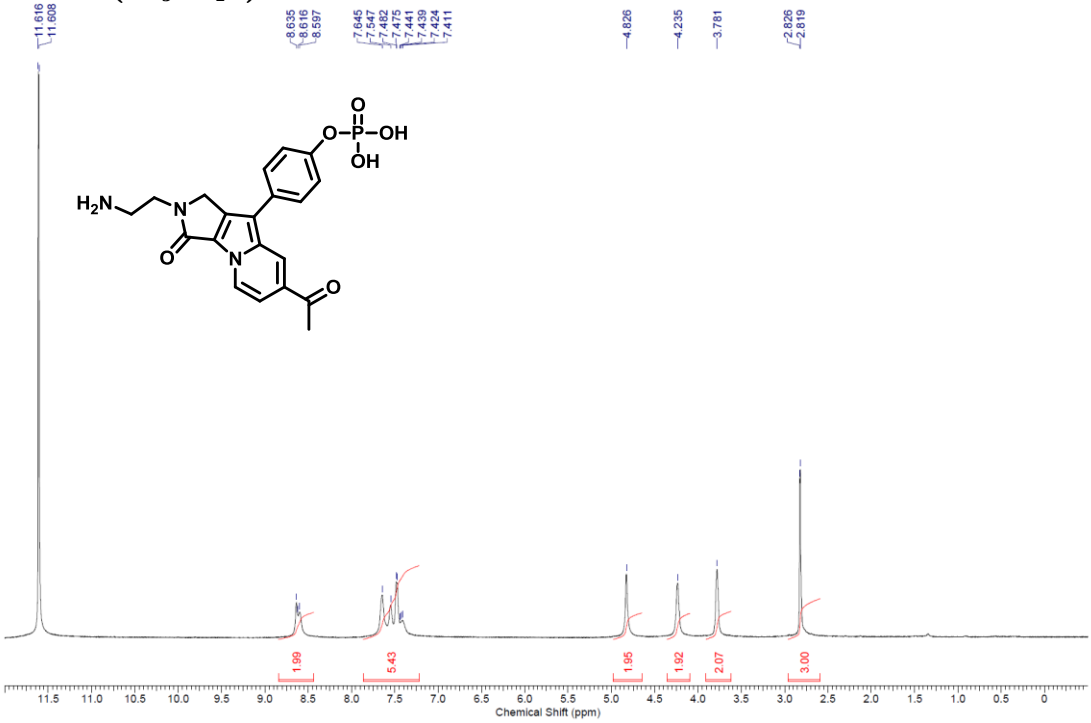


^{31}P NMR ($CDCl_3$)

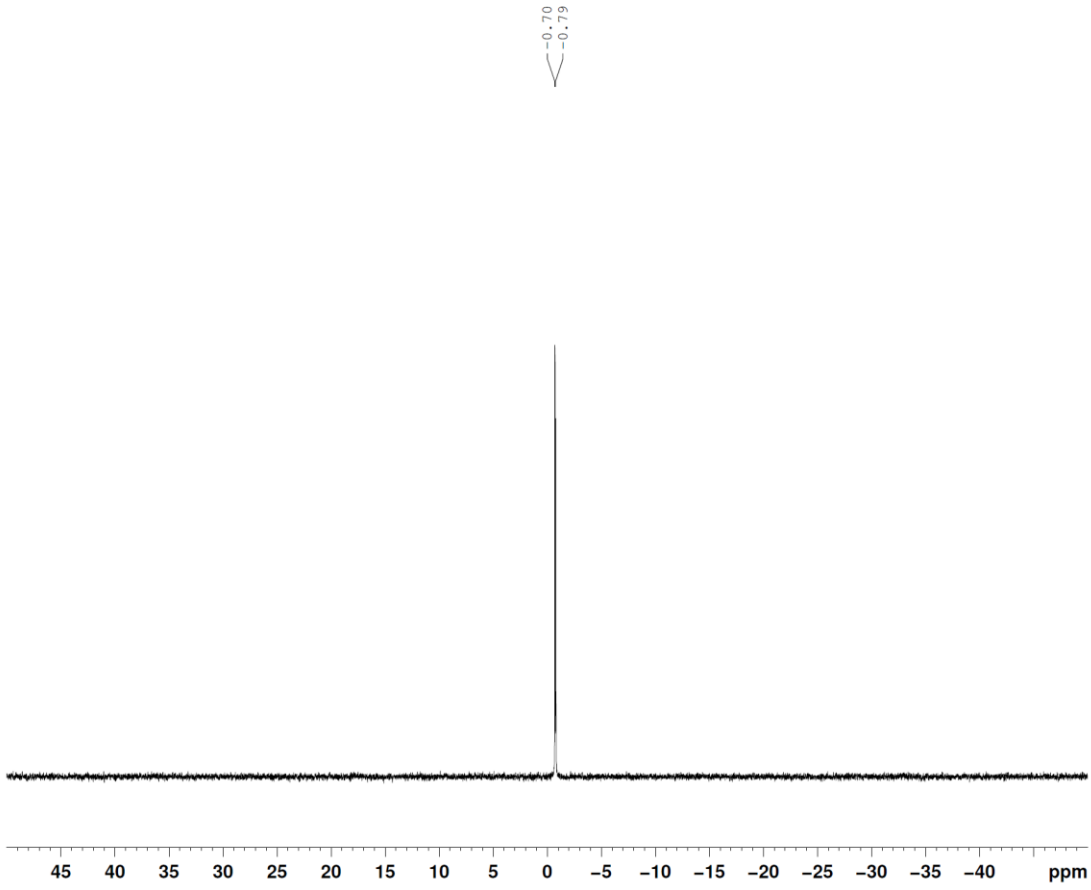


4-(7-Acetyl-2-(2-aminoethyl)-3-oxo-2,3-dihydro-1*H*-pyrrolo[3,4-*b*]indolizin-9-yl)phenyl dihydrogen phosphate

^{1}H NMR (CF_3CO_2D)

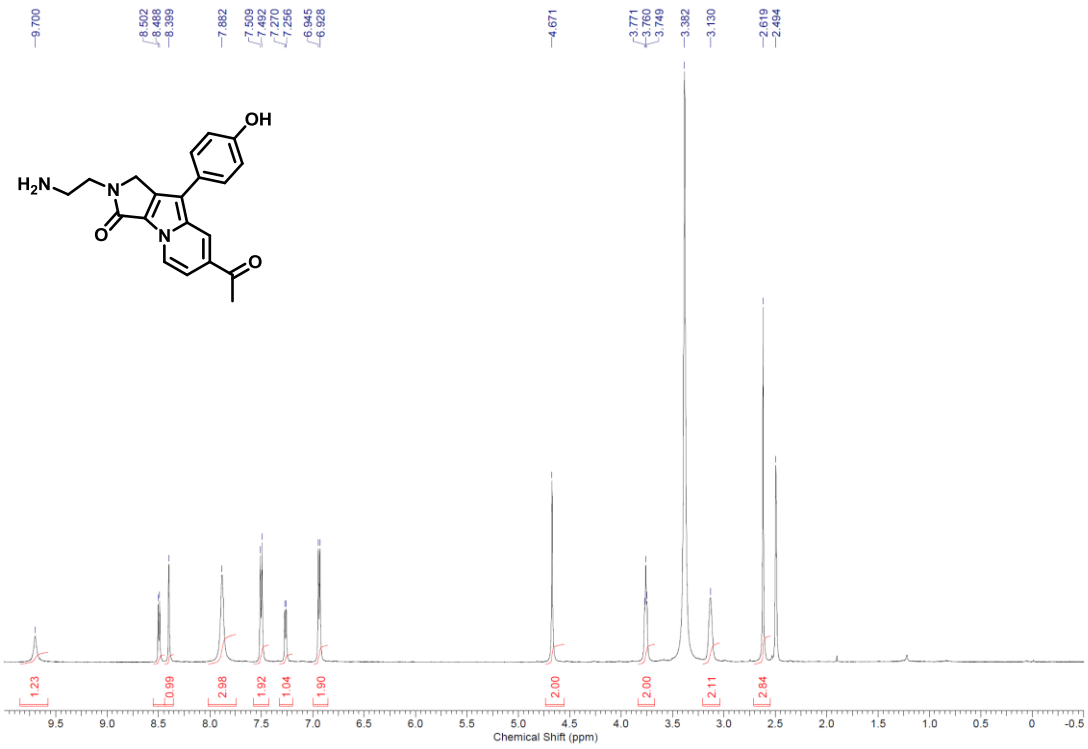


^{31}P NMR (D_2O)



7-Acetyl-2-(2-aminoethyl)-9-(4-hydroxyphenyl)-1*H*-pyrrolo[3,4-*b*]indolizin-3(2*H*)-one

^{1}H NMR ($CDCl_3$)



^{13}C NMR ($CDCl_3$)