

Supporting Information for

**A highly selective fluorescent ESPT probe for the dual specificity
phosphatase MKP-6**

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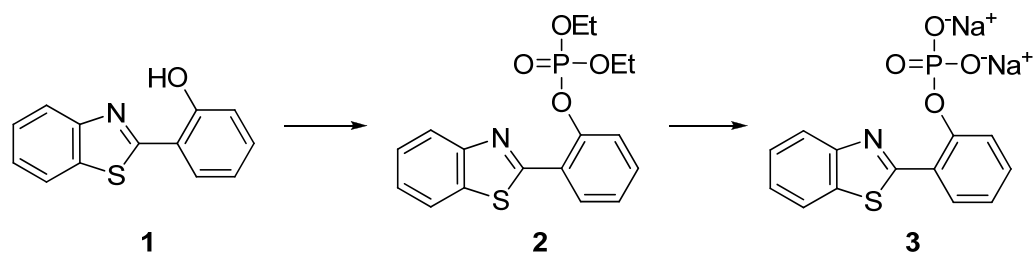
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General Methods**Materials**

All reagents were of the highest commercial quality and used as received without further purification. All solvents were spectral grade unless otherwise noted. Anhydrous dichloromethane was obtained as a sure-seal bottle from Aldrich Co. Inc. (Milwaukee, WI). Silica gel (40 μ m) was obtained from Merck Inc. Aqueous solutions were freshly prepared with deionized water from a water purification system (Human Corp. Korea). The protein tyrosine phosphatase substrate, 6,8-Difluoro-4-methylumbelliferyl phosphate (DiFMUP) was purchased from Invitrogen.

General methods, instrumentation and measurements

Synthetic manipulations that required an inert atmosphere (where noted) were carried out under argon using standard Schlenk techniques. NMR (^1H , ^{13}C , and ^{31}P) spectra were recorded on Bruker Advance 500 MHz spectrometers. The chemical shift data for each signal are given in units of δ (ppm) relative to tetramethylsilane (TMS) where δ (TMS) = 0, and referenced to the residual solvent resonances. Splitting patterns are denoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). ^{31}P NMR chemical shifts are reported relative to H_3PO_4 as the reference. High-resolution mass spectra (HR-MS) were obtained with JMS-700 (JEOL, Japan) using an electron impact source with an ionizing voltage of 70 V or JEOL-JMS-HX 110A/110A High Resolution Tandem Mass Spectrometry. Absorption spectra were obtained on a Shimadzu UV-2501 spectrophotometer. Fluorescence measurements were recorded on a Hitachi F-7000 fluorescence spectrophotometer at 25 $^\circ\text{C}$ using 10 mm quartz cuvettes with a path length of 1 cm. Primer synthesis and DNA sequencing were performed by Bioneer (Daejeon, Korea) and Genotech (Daejeon, Korea), respectively. Protein expression and purified proteins were monitored by 10% SDS-PAGE analysis. PTP activities were measured by monitoring changes in fluorescence intensity upon the substrate hydrolysis using a Wallac Victor Microplate Reader (Perkin Elmer).

Synthesis of Probe 3**2-(Benzo[d]thiazol-2-yl)phenyl diethyl phosphate (2)**

To a stirred solution of 2-(2'-hydroxyphenyl)benzothiazole (100 mg, 0.44 mmol) and NaH (60% dispersion in oil, 21.2 mg, 0.53 mmol) in tetrahydrofuran (8 mL) at room temperature under argon was added diethyl chlorophosphate (76.2 μL , 0.53 mmol). The resulting reaction mixture was allowed to stir at room temperature for 8 hours. The reaction was quenched with H_2O and the solvent was evaporated under reduced pressure. The product was extracted with dichloromethane. The combined organic layer was dried over MgSO_4 , filtered and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using progressively more polar 10:1 to 1:1 hexanes : ethyl acetate as the mobile phase to afford **2** as a colorless liquid (81.3 mg, 51 %); ^1H -NMR (500 MHz, CDCl_3): δ = 8.44 (d, 1H, J = 7.9 Hz), 8.10 (d, 1H, J = 8.2 Hz), 7.95 (d, 1H, J = 7.9 Hz), 7.66 (d, 1H, J = 8.5 Hz), 7.51 (t, 1H, J = 7.2 Hz), 7.47 (t, 1H, J = 7.6 Hz), 7.42 (t, 1H, J = 7.2 Hz), 7.32 (t, 1H, J = 7.6 Hz); ^{13}C -NMR (125 MHz, CDCl_3): δ = 162.3, 152.6, 148.8, 136.1, 131.8, 130.5, 126.4, 125.3, 125.2, 124.9, 123.3, 121.6, 120.1, 65.2, 16.2; ^{31}P -NMR (202 MHz, CDCl_3): δ = -6.51 (s); HR-MS (EI): calcd. for $\text{C}_{17}\text{H}_{18}\text{NO}_4\text{PS}$ m/z 363.0694, found 363.0691.

Sodium 2-(benzo[d]thiazol-2-yl)phenyl phosphate (3)

To a solution of **2** (80 mg, 0.22 mmol) in dry CH_2Cl_2 (5 mL) was added bromotrimethylsilane (170 μL , 1.32 mmol) dropwise at room temperature. The reaction mixture was stirred for 2 days at room temperature under argon, and quenched with MeOH (5 mL). After the mixture was stirred at room temperature for 30 minutes, the solvent was removed under reduced pressure. The reaction mixture was redissolved in H_2O (1 mL), pH was adjusted to 8~9 with 0.1 N NaOH, and evaporated to dryness. The crude solid was purified by 10 g RP C-18 cartridge (Waters, Milford, MA) eluting with increasing concentration of MeOH in H_2O (0 to 20%) to afford **3**

as an yellow solid (61.8 mg, 80%); $^1\text{H-NMR}$ (500 MHz, D_2O): δ = 8.31 (d, 1H, J = 8.2 Hz), 8.16 (d, 1H, J = 8.2 Hz), 8.09 (d, 1H, J = 8.3 Hz), 7.77 (d, 1H, J = 8.3 Hz), 7.63 (t, 1H, J = 7.7 Hz), 7.57 (t, 1H, J = 7.7 Hz), 7.53 (t, 1H, J = 7.2 Hz), 7.28 (t, 1H, J = 7.2 Hz); $^{13}\text{C-NMR}$ (125 MHz, D_2O): δ = 166.2, 153.1, 151.2, 136.0, 132.6, 128.6, 126.8, 125.5, 123.2, 122.6, 122.3, 121.7, 120.5; $^{31}\text{P-NMR}$ (202 MHz, D_2O): δ = 0.04 (s); HR-MS (FAB): calcd. for $\text{C}_{13}\text{H}_8\text{NNa}_2\text{O}_4\text{PS}$ $[\text{M}+\text{H}]^+$ 352.0512, found 352.0182.

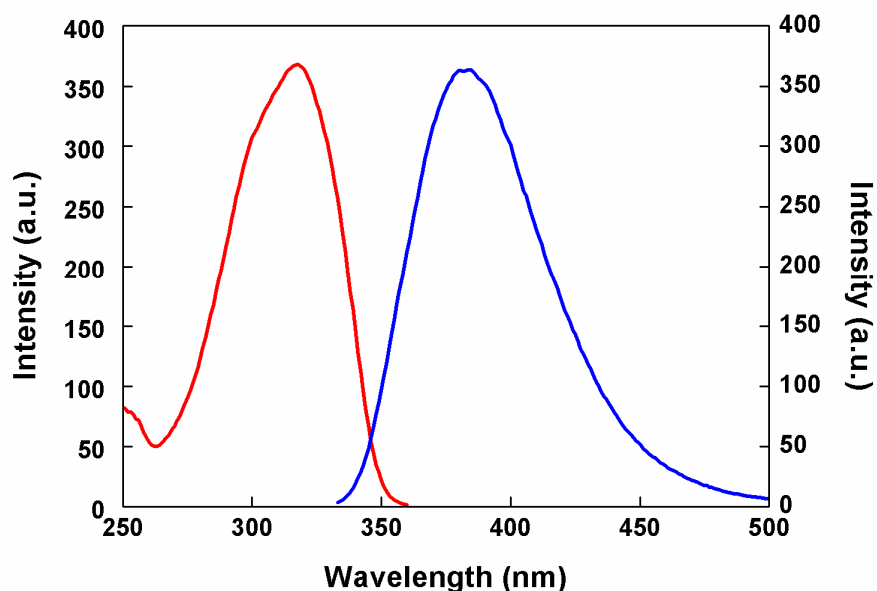


Fig. S1 Excitation and emission spectra of probe **3** in PBS buffer solution (0.1 M, pH 7.4). Emission at 384 nm. Excited at 318 nm. [probe **3**] = 10 μM .

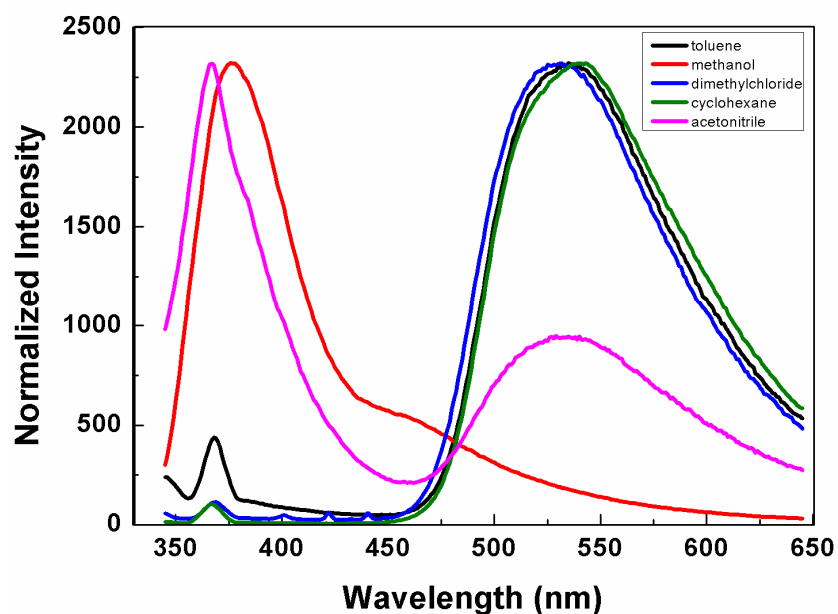


Fig. S2 Emission spectra of **HBT** in various solvents. Excited at 330 nm. The spectra were normalized for clarity.

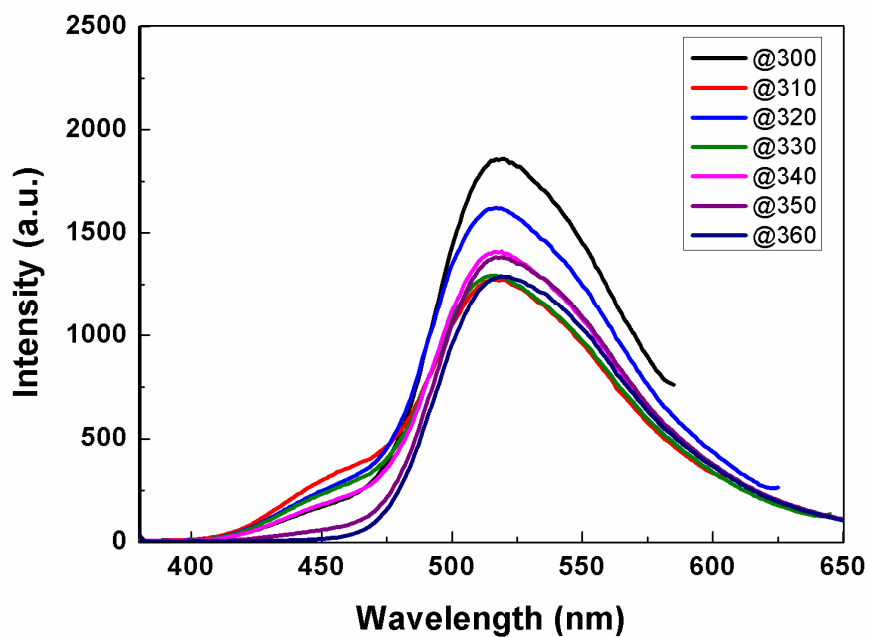


Fig. S3 Emission spectra of **HBT** (20 μ M) in 2% DMSO/water (v/v) at various excitation wavelengths.

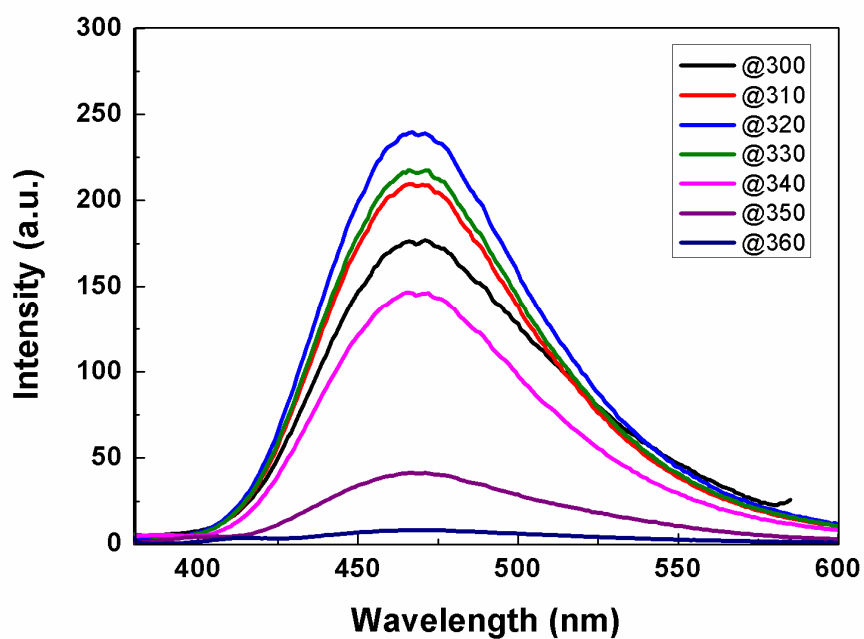


Fig. S4 Emission spectra of **HBT** (2 μ M) in 2% DMSO/water (v/v) at various excitation wavelengths.

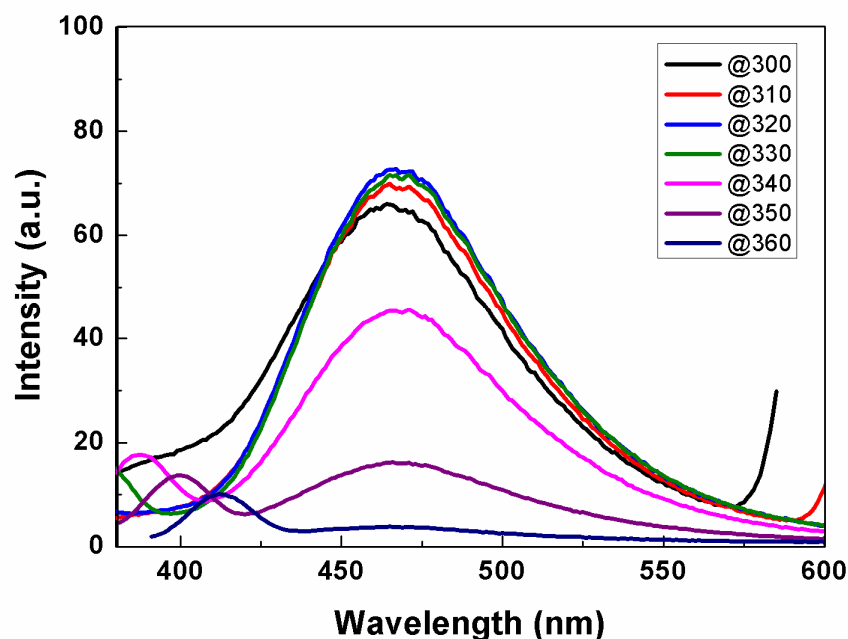


Fig. S5 Emission spectra of HBT (0.2 μM) in 2% DMSO/water (v/v) at various excitation wavelengths.

Construction of plasmid vector for PTP overexpression in *E. coli*.

Genes corresponding to the listed PTP sequences were subcloned to PET 28a vector (Novagen) with N-terminal His-tag: STEP (157-537aa), TMDP (1-198 aa), RPTP κ (879-1440 aa), PTP1B (1-299 aa), TypPTP (149-420 aa), PTP-SL (327-650 aa), PRL-3 (1-161 aa), VHZ (1-150 aa), PTP-BAS (2095-2490 aa), SHP1 (1-595 aa), LAR (1316-1897 aa), TCPTP (1-387 aa), SHP2 (1-593 aa), VH1 (1-243 aa). To increase solubility, maltose-binding protein (MBP)-tag was inserted to the N-termini of the following PTPs: DUSP24 (35-211 aa), PRL-2 (14-167 aa), CDC14B (1-448 aa), MKP-7 (1-331 aa), hVH-3 (11-321 aa), MKP-3 (1-381 aa), BEDP (1-188 aa), MKP-6 (1-198 aa), SSH2 (298-449 aa), hVH-5 (1-314 aa), VHR (1-185 aa), STYX (1-223 aa).

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 *Bam*HI or *Nde*I and *Xho*I, and subcloned into *Nde*I-*Bam*HI or *Nde*I-*Xho*I digested *pET* 28a(+) vector (Novagen). The resulting N-terminal His-tagged human PTPs and N-terminal MBP-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 (3570 g, at 4 °C for 10 min), washed with buffer A (50 mM Tris pH 7.5, 250 mM NaCl, 5% glycerol, and 0.05% 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. Proteins were eluted from the metal affinity resin with buffer A containing 100 mM imidazole. N-terminal MBP-tagged human PTPs were purified with similar method except using MBP affinity resin (Amylose resin®, NEB) and buffer A containing 100 mM maltose for elution. Following dialysis against 20 mM Tris buffer (pH 7.5) containing 250 mM NaCl, 5% glycerol, and 1 mM DTT, PTPs were concentrated to 2 mg/ml and stored at -80°C. PTPs tested in this study was listed in Table S1.

Table S1. PTPs tested in this study

Classical PTPs	STEP
	RPTP κ
	PTP1B
	TypPTP
	PTP-SL
	PTP-BAS
	SHP1
	LAR
	TCPTP
	SHP2
Dual Specific PTPs	TMDP
	VHZ
	VHY
	DUSP24
	MKP-7
	hVH3
	MKP-3
	BEDP
	MKP-6
	SSH2
	hVH5
	VHR
	PRL-3
	CDC14B
	CDC14B
	STYX

Measurement of enzymatic activities of cloned PTPs with DiFMUP, a known substrate

The enzymatic reaction mixtures were prepared by the addition of each PTP (100 nM) to aqueous solution of DiFMUP (50 μ M) in 20 mM Tris buffer (pH 8.0, 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μ M BSA). Reaction mixtures were incubated at 25 $^{\circ}$ C for 5 min and their fluorescence intensities were measured at 460 nm when excited at 355 nm (Fig S6). MKP-6 showed about 20% activity compared to that of TCPTP, which showed the best catalytic activity for hydrolysis of DiFMUP.

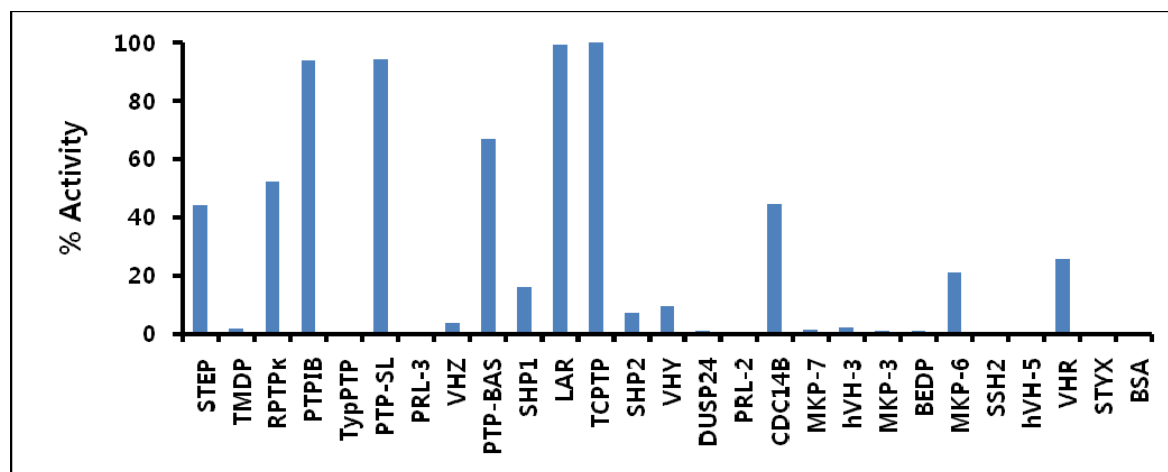


Fig. S6 Fluorescence response of DiFMUP to cloned PTPs. PTP activities for DiFMUP hydrolysis were monitored for 5 min, and normalized based on the activity of TCPTP, which showed the strongest activity.

Fluorescence assays of probe 3 with various PTPs

The enzymatic reaction mixtures were prepared by the addition of each PTP (100 nM or 1 μ M) to aqueous solution of probe **3** (50 μ M) in 20 mM Tris buffer (pH 8.0, 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μ M BSA). Reaction mixtures were incubated at 25 $^{\circ}$ C for 60 min and their fluorescence intensities were measured at 535 nm when excited at 355 nm (Fig 4).

Determination of the correlation of fluorescence intensity and concentration of HBT at enzyme assay condition

In order to determine the correlation of fluorescence intensity and concentration of **HBT** at enzyme assay condition, fluorescence intensities of various concentrations of HBT (0.0625 ~ 64 μ M) at 535 nm were measured. Different concentrations of **HBT** solutions were prepared in 20 mM Tris (pH 8.0) containing 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μ M BSA, 100 nM MKP-6, and 10% DMSO. Fluorescence intensity at 535 nm was measured on a 96-well plate using a Wallac Victor Microplate Reader (PerkinElmer). (Fig. S7) The extinction coefficient of fluorescence intensity and concentration of HBT was calculated from the slope of a plot of the fluorescence intensity versus **HBT** concentration. The calculated coefficient was used in determining kinetic constants of probe **3** hydrolysis by MKP-6.

Determination of kinetic constants for probe 3 and DiFMUP, a known substrate

To determine the kinetic constants, probe **3** at a series of the final concentrations (32~2000 μ M) was hydrolyzed by MKP-6 (50 nM). In this experiments, 10% DMSO was necessarily added to the reaction buffer due to the limited solubility of the probe **3**. As a standard, hydrolysis of DiFMUP, a commercially available PTP probe, were also measured under the same conditions except the final concentration of DiMUP (1.5~50 μ M). The reaction was monitored in a 45 sec interval for 15 min by measuring fluorescence change at 535 nm for probe **3** (excited at 355 nm) and 460 nm for DiFMUP (excited at 355 nm) at 25 $^{\circ}$ C. The initial velocity was calculated from the slope of the each progress curve (Fig S8(a)). The parameters such as K_m and k_{cat} with MKP-6 for probe **3** and DiFMUP were determined by Lineweaver-Burk plot. Fig. S8(b) showed the mean value of the relative initial velocity of probe **3** hydrolysis by MKP-6. K_m , k_{cat} , and k_{cat}/K_m values for Probe **3** were 535 μ M, 0.15 s^{-1} , and 0.28 $mM^{-1}s^{-1}$ respectively. K_m , k_{cat} , and k_{cat}/K_m values for DiFMUP were 186.4 μ M, 1.51 s^{-1} , and 8.1 $mM^{-1}s^{-1}$ respectively.

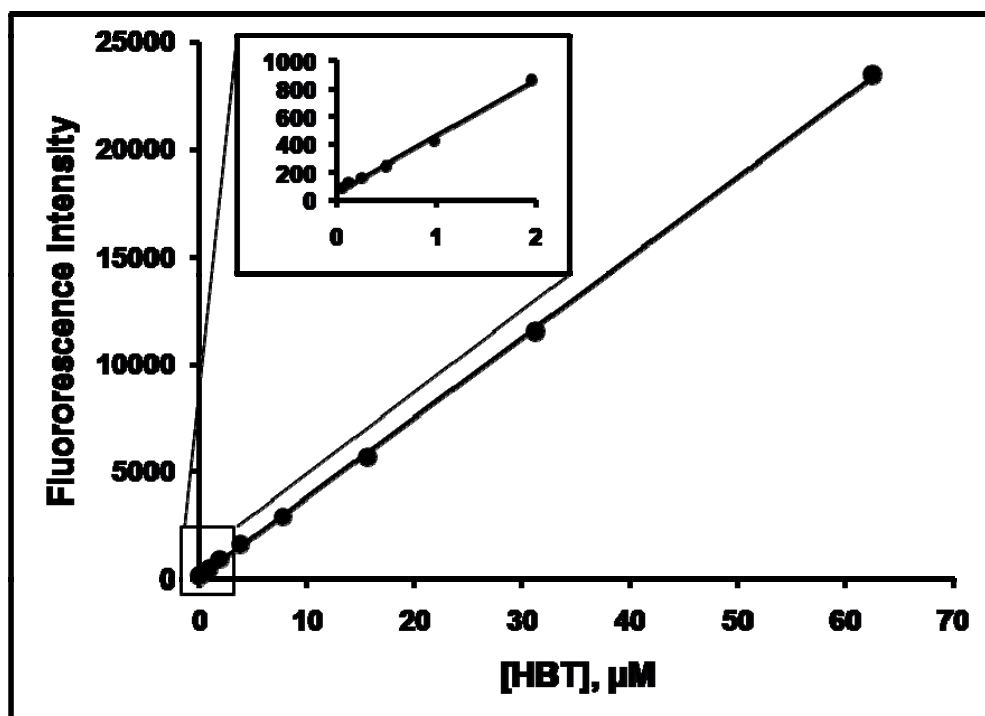


Fig. S7 Fluorescence intensity at various concentrations of **HBT** in 20 mM Tris (pH 8.0) containing 150 mM NaCl, 0.01% Triton X-100, 5 mM DTT, 5 μ M BSA, 100 nM MKP-6, and 10% DMSO. Fluorescence intensity at 535 nm was measured. $[\text{HBT}] = 0.0625 \sim 64 \mu\text{M}$. Insert shows linear increase of fluorescence intensity at low concentrations of **HBT**.

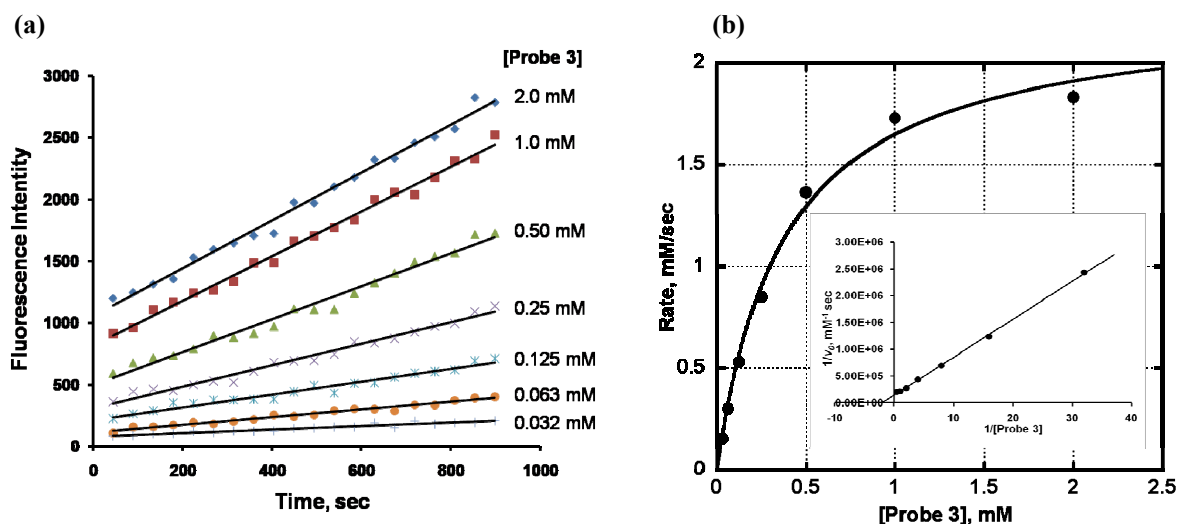


Fig. S8 Catalytic hydrolysis of probe **3** by MKP-6. (a) Progress curves of hydrolysis of probe **3** at a series of concentrations. Referring Fig S7, the final fluorescence intensity of each linear curve demonstrates that less than 5% of substrate was consumed to give linear curves during the experiments, meaning that the slope of each curve represents the initial velocity. (b) A plot of the initial velocity v_0 of the hydrolysis of probe **3** by MKP-6 versus concentration of probe **3**. The insert represents a double reciprocal (Lineweaver-Burk) plot.

Inhibition of the MKP-6-catalyzed hydrolysis of probe 3 by sodium pervanadate, a PTP inhibitor

50 μ M of probe **3** was hydrolyzed by 50 nM MKP-6 in the presence and absence of 2 mM sodium pervanadate, a PTP inhibitor. The mixture of MKP-6 and probe **3** failed to increase the fluorescence intensity in the presence of 2 mM sodium pervanadate. This explains that the fluorescence increase of probe **3** results from the catalytic hydrolysis of probe **3** by MKP-6.

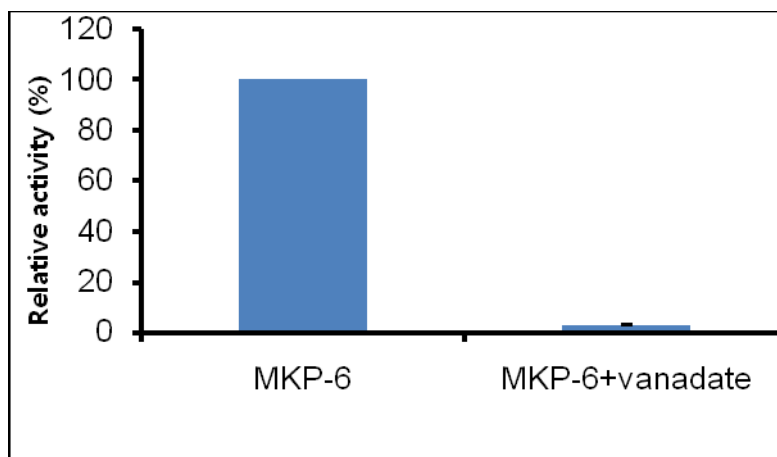
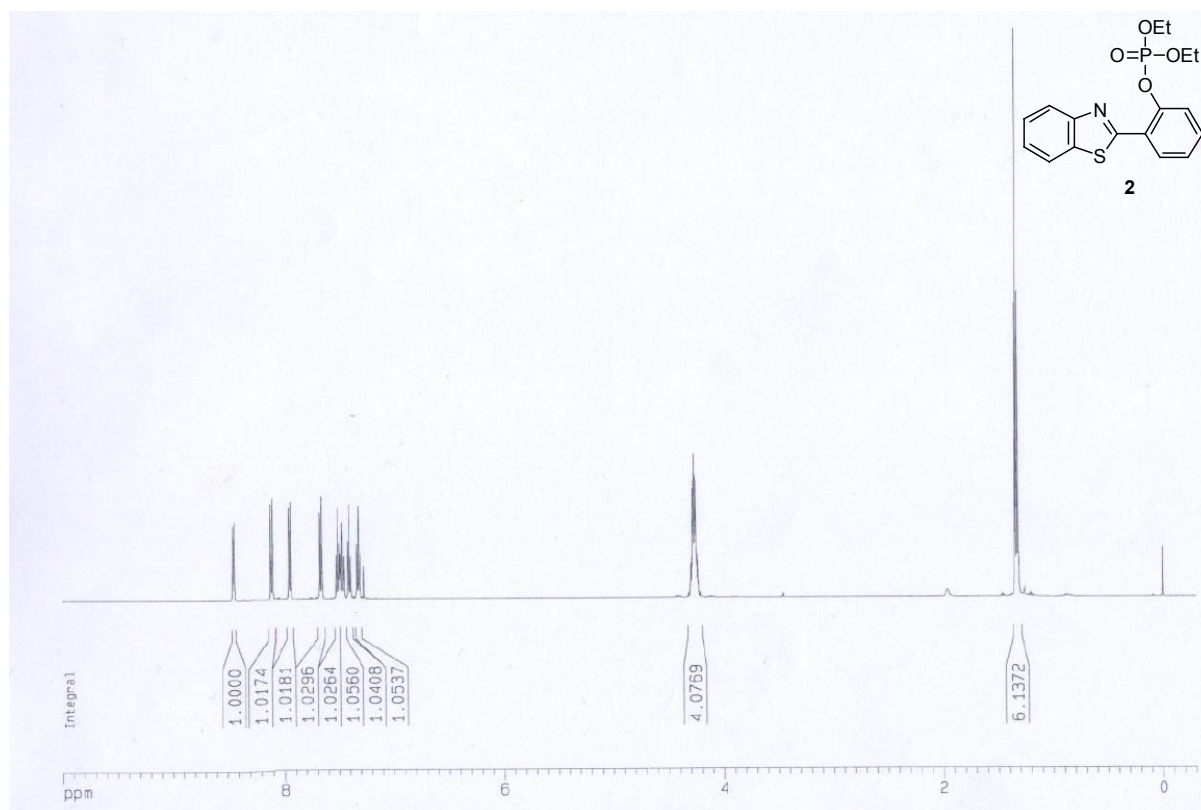
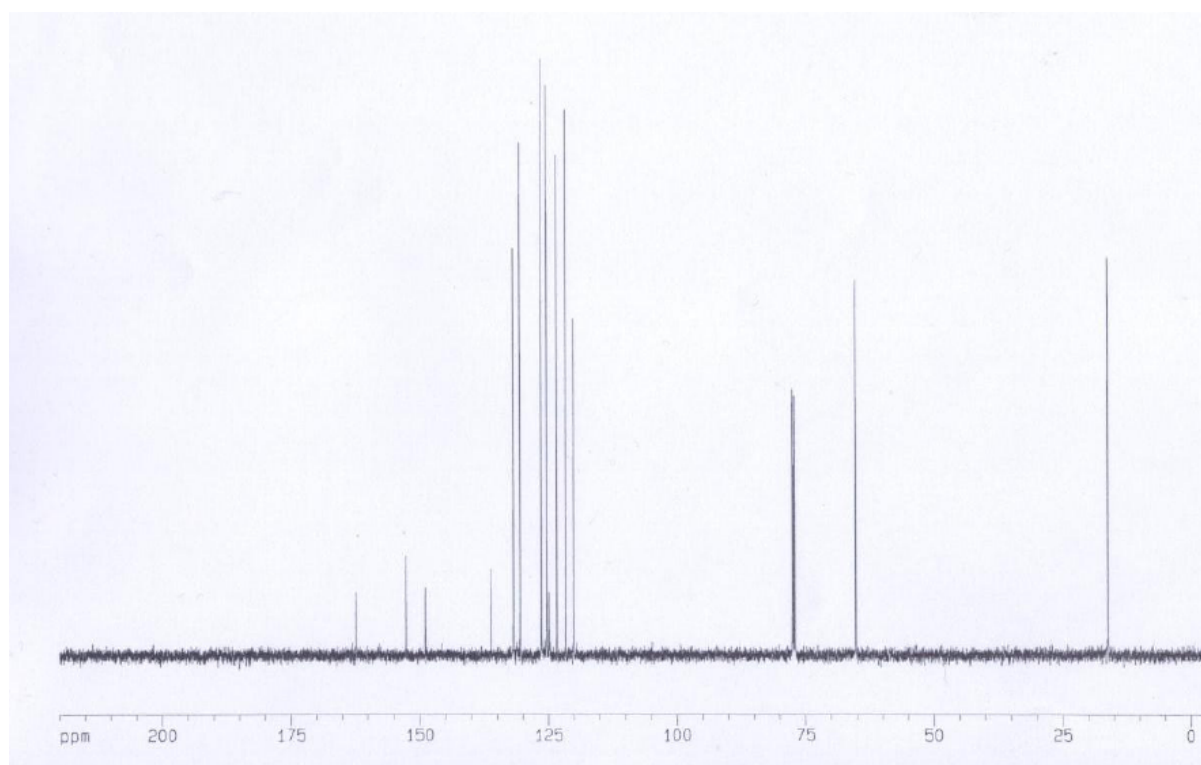


Fig. S9 Effect of sodium pervanadate probe **3** hydrolysis by MKP-6. The mixture of MKP-6 and probe **3** failed to increase the fluorescence in the presence of 2 mM sodium pervanadate, a PTP inhibitor, meaning that the fluorescence increase of probe **3** results from the catalytic hydrolysis of probe **3** by MKP-6.

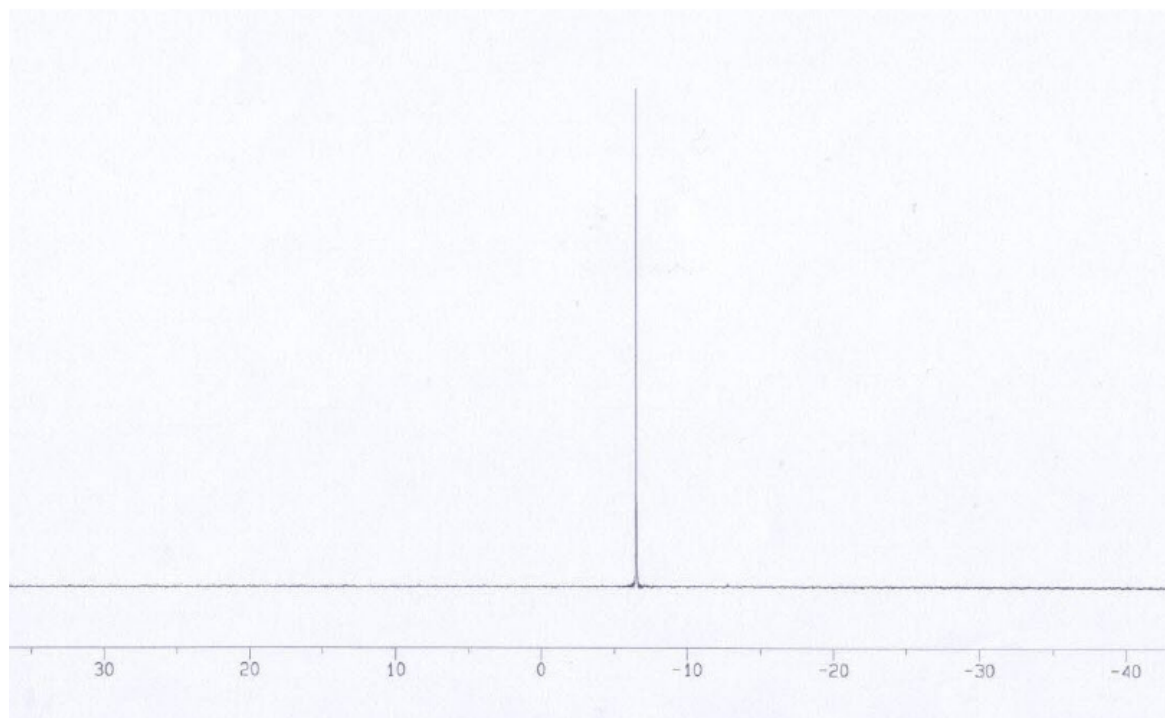
^1H -NMR Spectrum of **2** in CDCl_3 (500 MHz):



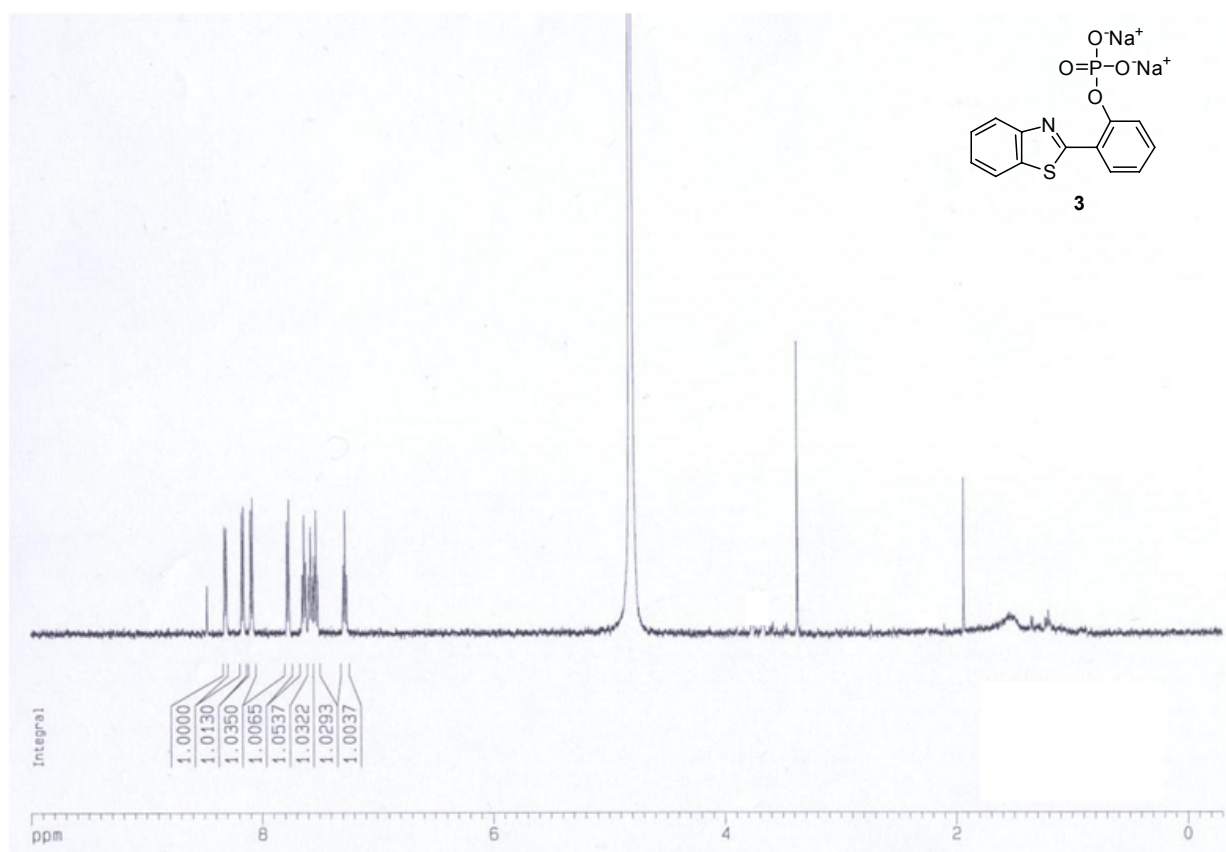
^{13}C -NMR Spectrum of **2** in CDCl_3 (125 MHz):



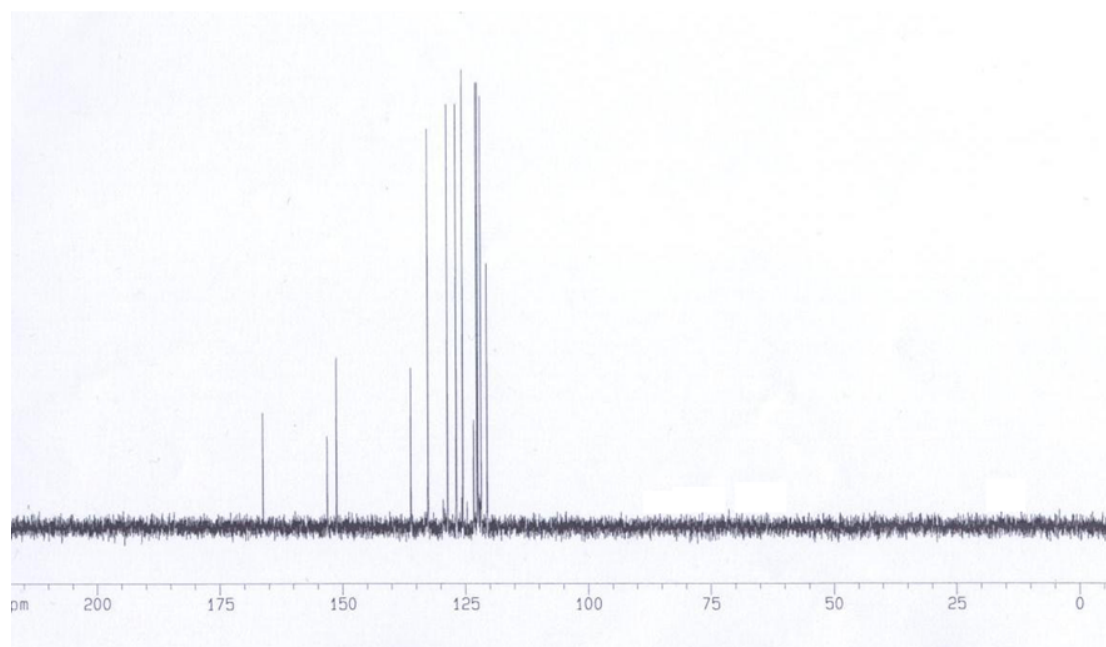
^{31}P -NMR Spectrum of **2** in CDCl_3 (202 MHz):



^1H -NMR Spectrum of **3** in D_2O (500 MHz):



^{13}C -NMR Spectrum of **3** in D_2O (125 MHz):



^{31}P -NMR Spectrum of **3** in D_2O (202 MHz):

