Supporting Information

Enzyme-activatable Probe with a Self-immolative Linker for Rapid and Sensitive Alkaline Phosphatase Detection and Cell Imaging through Cascade Reaction

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1) Synthesis of probe 1

Synthesis of 3: A solution of 4-hydroxybenzyl alcohol (500 mg, 4.03 mmol), imidazole (1.10 g, 16.11 mmol), and tert-butylidemethylsilyl chloride (1.52 g, 10.07 mmol) in dry methylene chloride (20 mL) was heated at reflux overnight. The solution was then cooled to room temperature, diluted with methylene chloride, washed with brine, dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure to afford the product as colorless oil in a yield of 90%. $^1$H NMR (400 MHz, DMSO-d$_6$): δ 7.19 (d, 2H), 6.82 (d, 2H), 4.63 (s, 2H), 0.95 (s, 9H), 0.89 (s, 9H), 0.17 (s, 6H), 0.05 (s, 6H). MS: calcd M$^+$=352.7, obsd (M+H)$^+$=353.1.

Fig. S1 $^1$H NMR spectrum of 3 in DMSO-d$_6$.

Synthesis of 4: A mixture of 3 (1 g, 2.84 mmol), and Cs$_2$CO$_3$ (0.46 g, 1.42 mmol) in DMF–H$_2$O (10:1, v/v, 11 mL) was stirred at room temperature until the reaction was finished as indicated by thin-layer chromatography (TLC). The reaction mixture was then diluted with Et$_2$O (100 mL), washed with brine, and dried over anhydrous Na$_2$SO$_4$. The solvent was removed in vacuum, and the residue was purified by flash chromatography using hexane/ethyl acetate as eluent in a yield of 88%. $^1$H NMR (400 MHz, DMSO-d$_6$): δ 7.06 (d, 2H), 6.71 (d, 2H), 5.26 (s, 1H), 4.55 (s, 2H), 0.93 (s, 9H), 0.02 (s, 6H). MS: calcd M$^+$=238.4, obsd (M+H)$^+$=239.2.
Synthesis of 5: A mixture of 4 (0.75 g, 3.15 mmol), freshly distilled triethylamine (2.19 mL, 15.73 mmol) and chlorophosphoric acid diethyl ester (0.91 mL, 6.29 mmol) in dry chloroform (20 mL) was stirred at room temperature under N₂ overnight. The reaction mixture was then concentrated under reduced pressure, and purified by flash chromatography using hexane/ethyl acetate as eluent in a yield of 35%. ¹H NMR (400 MHz, DMSO-d6): δ 7.35 (d, 2H), 7.17 (d, 2H), 4.48 (s, 2H), 4.15 (q, 4H), 1.27 (t, 6H). MS: calcd M⁺=260.2, obsd (M+H)⁺=261.1.

Fig. S2 ¹H NMR spectrum of 4 in DMSO-d6.

Fig. S3 ¹H NMR spectrum of 5 in DMSO-d6.
**Synthesis of 6:** To the solution of carbon tetrabromide (150.2 μL, 1.55 mmol) and 5 (0.34 g, 1.29 mmol) in 20 mL of methylene chloride was added triphenylphosphine (0.41 g, 1.55 mmol) at 0 °C. The resultant mixture was stirred for 2 h. The compound was purified by flash chromatography using hexane/ethyl acetate as eluent in a yield of 45%. $^1$H NMR (400 MHz, DMSO-d$_6$): δ 7.50 (d, 2H), 7.21 (d, 2H), 4.72 (s, 2H), 4.16 (q, 4H), 1.27 (t, 6H). MS: calcd M$^+$=323.1, obsd (M+H)$^+$ =323.0.

![Fig. S4 $^1$H NMR spectrum of 6 in DMSO-d6.](image)

**Synthesis of 7:** To the solution of 6 (0.082 g, 0.2528 mmol) in 10 mL of dry acetone, resorufin (0.054 g, 0.2528 mol) and K$_2$CO$_3$ (0.070 g, 0.5056 mmol) was added and the mixture was refluxed overnight. The insoluble solid was removed by filtration and the product was purified by flash chromatography using hexane/ethyl acetate as eluent in a yield of 50%. $^1$H NMR (400 MHz, DMSO-d$_6$): δ 7.76 (d, 1H), 7.52 (m, 3H), 7.23 (d, 2H), 7.18 (m, 1H), 7.10 (dd, 1H), 6.76 (m, 1H), 6.25 (d, 1H), 5.25 (s, 2H), 4.14 (q, 4H), 1.26 (t, 6H). MS: calcd M$^+$=455.4, obsd (M+H)$^+$ =456.1.

**Synthesis of 1:** To the solution of 7 (20 mg, 0.04392 mmol) in 10 mL of dry methylene chloride was added ioddotrimethylsilane (62.5 μL, 0.4392 mmol) at 0 °C. The mixture was stirred at room temperature for 3 h under N$_2$ atmosphere. The solvent was removed and 10 ml of methanol was added, the mixture was stirred for another 2 hours. The solid obtained after evaporating the solvents was purified by HPLC to afford title compound 1 in a yield of 40%. $^1$H NMR (400 MHz, DMSO): δ 7.76 (d, 1H), 7.51 (d, 1H), 7.39 (d, 2H), 7.16 (m, 3H), 7.11 (dd, 1H), 6.75 (m, 1H), 6.25 (d, 1H), 5.19 (s, 2H). $^{31}$P NMR (400 MHz, DMSO) δ -5.67 (s). MS: calcd M$^+$=399.3, obsd (M+H)$^+$ =400.0.
Fig. S5 $^1$H NMR spectrum of 7 in DMSO-$d_6$.

Fig. S6 $^1$H NMR spectrum of 1 in DMSO-$d_6$. 
Fig. S7 $^{31}$P NMR spectrum of 1 in DMSO-$d_6$.

2) Optical images of probe 1 solution for colorimetric ALP assay

![Optical images](image)

Fig. S8 Time evolution of color change of the solution of probe 1 (30 μM) before and after the addition of ALP (1.0 U/mL).
Fig. S9 (A) HPLC chromatograms of 1 (top) and 2 (bottom) before and after dephosphorylation by alkaline phosphatase, (B) Mass spectrum analysis for the generation of 2 from 1 through enzymatic reaction triggered by alkaline phosphatase.

3) Enzyme assay with probe 1 as a function of concentration of ALP

A series of solutions in pH=7.4 Tris-HCl buffer, which contained 10 μM of probe 1 and different concentrations of ALP (0, 0.01, 0.1, 0.2, 0.5 and 1.0 U/mL), were prepared and incubated at 37°C. The fluorescent intensities of the solutions were collected at different periods of time, and the relationships between fluorescent intensity and ALP concentration was analysed by calibration curve. Based on the good linear relationship between the relative fluorescence intensity (F/F₀) and ALP concentration in the range between 0.01 and 1.0 U/mL within the period of time at 2 min, a regression equation was obtained: Y=2.892X+0.031 (where Y is the relative fluorescence intensity, and X is the concentration of ALP, U/mL) with a correlation coefficient of 0.9978. The limit of detection (LOD) was calculated from the standard deviation of the response (S₀=1.05×10⁻³) and the slope of the calibration curve (b=2.892), by following the formula: LOD = 3(S₀/b).\(^1\)
4) Kinetic studies of dephosphorylation process of probe 1 by ALP hydrolysis

For determination of the kinetic parameters of hydrolysis reaction by ALP, we dissolved 1 in Tris-HCl buffer (pH=7.4) to prepare 1 solution in different concentrations (0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10 μM), followed by the addition of ALP (1.0 U/mL) for dephosphorylation reaction. The enzymatic reaction was monitored by the fluorescence change at 585 nm at different time scales. The kinetic parameters (e.g., $K_M$ and $k_{cat}$) of the hydrolysis reaction of 1 were determined from Lineweaver-Burke plot.

5) Synthesis of resorufin-7-O-phosphate

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\begin{align*}
\text{probe 1} & \xrightarrow{\text{i}} \text{EtO}_2\text{PO} \xrightarrow{\text{ii}} \text{resorufin-7-O-phosphate} \\
\text{EtO}_2\text{P} & \text{Et}
\end{align*}
\]

\text{i) } \text{CIP(OEt)2/TEA/Dry/DMF}; \text{ ii) } (\text{CH}_3)_2\text{SiI/Dry/DCM}

Fig. S11 (A) Michaelis-Menten plot and (B) Lineweaver-Burke plot of the hydrolysis of probe 1 by ALP in Tris buffer at 25 °C.
Scheme S1. The synthetic routes for the preparation of fluorogenic probe resorufin-7-O-phosphate.

Synthesis of resorufin-7-O-phosphate: A mixture of resorufin (30 mg, 0.14 mmol), freshly distilled triethylamine (156.91 μL, 1.1257 mmol) and chlorophosphoric acid diethyl ester (121.9 μL, 0.8442 mmol) were dissolved in dry dimethylformamide (20 mL) and stirred overnight at room temperature under N₂ atmosphere. Then the reaction mixture was concentrated under reduced pressure to afford 8. To the solution of 8 (40.14 mg, 0.1407 mmol) in 20 mL of dry methylene chloride was added iodos(trimethyl)silane (201 μL, 1.407 mmol) at 0 °C. The mixture was stirred at room temperature for 3 h under N₂ atmosphere. The solvent was removed and 10 ml of methanol was added, followed by stirring for another 2 hours at room temperature. After evaporation of the solvent, the solid obtained was purified by HPLC and afforded the final product, resorufin-7-O-phosphate, in a yield of 41%. ¹H NMR (400 MHz, DMSO): δ7.76 (d, 1H), 7.53 (d, 1H), 7.38 (s, 1H), 7.22 (d, 1H), 6.81 (d, 1H), 6.27 (s, 1H). ³¹P NMR (400 MHz, DMSO): δ-5.82 (s). MS: calcd M⁺=293.2, obsd (M-H)⁻=292.0.

Fig. S12 ¹H NMR spectrum of resorufin-7-O-phosphate in DMSO-d6.
6) UV absorption spectra and fluorescence emission spectra of resorufin-7-O-phosphate

Fig. S15 (A) UV absorption spectra and (B) fluorescence emission spectra of resorufin-7-O-phosphate (10 μM, pH=7.4, λ_{ex}=550 nm) before (a, black) and after (b, red) the addition of ALP (1.0 U/mL). Inset: The optical images of the corresponding solutions of resorufin-7-O-phosphate (10 μM, pH=7.4) in the absence (a, left) or presence (b, right) of ALP (1.0 U/mL) under (A) visible and (B) UV light irradiation.
(365 nm).

7) Fluorescence emission spectra of resorufin-7-O-phosphate

![Fluorescence emission spectra](image)

**Fig. S16** Time evolution of fluorescence emission spectra ($\lambda_{ex}=550$ nm) of (A) the mixture solution containing resorufin-7-O-phosphate (10 μM) and ALP (1.0 U/mL); (B) Time evolution of fluorescence emission at 585 nm of the mixture solution containing varied amount of resorufin-7-O-phosphate (0.5, 1.0, 2.0, 5.0, 10 and 20 μM) and 1.0 U/mL of ALP.

8) Kinetic studies of dephosphorylation reaction of resorufin-7-O-phosphate by ALP

![Kinetic plots](image)

**Fig. S17** (A) Michaelis-Menten plot and (B) Lineweaver-Burke plot of the hydrolysis of resorufin-7-O-phosphate by ALP in Tris-HCl buffer at 25 °C.

9) Confocal fluorescence microscopy of resorufin-7-O-phosphate

![Confocal images](image)

**Fig. S18** Fluorescence microscopy images of HeLa cell incubated with resorufin-7-O-phosphate (10 μM) for ALP imaging. Scale bar = 25 μm.

10) Stability studies of probe 1
Fig. S19 (A) Absorption intensities of probe 1 (10 μM) at 550 nm and (B) their stability profiles over the course of 60 min in different pH buffer solutions (10 mM citrate buffer for pH 4; 10 mM sodium acetate buffer for pH 5 and pH 6; 10 mM HEPES buffer for pH 7 and pH 8; 10 mM carbonate buffer for pH 9) at 25°C.

Fig. S20 (A) Emission intensities of probe 1 (10 μM) at 585 nm and (B) their stability profiles over the course of 60 min in different pH buffer solutions (10 mM citrate buffer for pH 4; 10 mM sodium acetate buffer for pH 5 and pH 6; 10 mM HEPES buffer for pH 7 and pH 8; 10 mM carbonate buffer for pH 9) at 25°C.

11) Cell viability measurement with HeLa cells

HeLa cells were cultured in a 96-well plate at a density of 1x10^4 cells/well at 37°C, and exposed to varying concentrations of probe 1 for 24 h in 100 μL of DMEM medium containing 10 % FBS. Cells treated with medium alone served as a negative control group. After removal of the supernatant in each well and washing twice with PBS buffer, 20 μL of MTT solution (5 mg ml⁻¹ in PBS) and 100 μL of medium were then added. After incubation for another 4 hr, the resultant formazan crystals were dissolved in 100 μL of dimethyl sulfoxide and the absorbance was measured by a microplate reader (Thermo Fisher Scientific, Finland) at 570 nm with a reference wavelength at 650 nm. All experiments were performed in triplicate, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells.
12) Confocal fluorescence microscopy

For confocal fluorescence imaging study, HeLa cells were seeded onto glass coverslips at a density of 1x10^5 cells/well in 4-well plates and incubated for 24 h prior to treatment. After 24 h of incubation, cells were washed and treated with the fresh media containing probe 1 (10 μM) for 5 min. For inhibition assay, additional cells were preincubated with ALP inhibitor levamisole (10 mM) for 15 min and then treated with probe 1 for 5 min. At treatment endpoints, cells were washed three times with cold phosphate buffered saline solution (PBS, pH 7.4), and then fixed with 4% paraformaldehyde for 20 min at room temperature. After quenching with 50 mM ammonium chloride and permeabilization with 0.3% Triton X-100, the coverslips were then incubated with DAPI for nucleus staining. After washing with PBS, the coverslips were mounted for confocal fluorescence microscopy. Confocal fluorescence images (Ex: 561 nm, Em: 570-600 nm) were obtained with a Leica TCS SP5 II confocal laser scanning microscope.

13) Flow cytometry analysis

HeLa and HEK 293T cells were seeded onto a 6-cm plate at a density of 5x10^6 cells/plate, and incubated for 24 h for cell attachment. After 24 h of incubation, HeLa and HEK 293T cells were washed and treated with probe 1 in the fresh culture media for 5 min at 37°C, respectively. For inhibition assay, HeLa cells were preincubated with levamisole (10 mM) for 15 min and then treated with probe 1 for 5 min. For unstained control groups, HeLa and HEK 293T cells were treated with fresh media.
without probe 1 for 5 min, respectively. All the cells were washed three times with cold PBS solution, then detached from the plate using cell scraper. Collected cell pellets were resuspended in cold PBS and transferred into the test tube for flow cytometry analysis. (Beckman coulter, FC 500, Flow Cytometer, USA). The fluorescence intensity was measured (Ex: 488 nm, Em: 550-600 nm).

References: