# Supporting Information for

# A Fluorescent Turn-On Probe for the Detection of Alkaline Phosphatase Activity in Living Cells

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Experimentals 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  $CH_2Cl_2$  and THF were obtained as a sure-seal bottle from Aldrich Co. Inc. (Milwaukee, WI). Silica gel (40  $\mu$ m) was obtained from Merck Inc. Aqueous solutions were freshly prepared with deionized water from a water purification system (Human Corp. Korea). 4-(Diethylamino)-salicylaldehyde, 2,3,6,7-tetrahydro-8-hydroxy-1H,5H-benzo[*ij*]quinolizine-9-carboxaldehyde, (-)-*p*-bromotetramisole oxalate, and levamisole hydrochloride were obtained from Aldrich (Milwaukee, WI). Alkaline phosphatase (ALP, EC 3.1.3.1, 100 U/mg) from bovine kidney and 4-methylumbelliferyl phosphate (4-MUP) were purchased from Sigma. ELF-97 phosphate substrate was obtained from Molecular Probes, Inc (Eugene, OR).

# General methods, instrumentation and measurements

Synthetic manipulations that required an inert atmosphere (where noted) were carried out under argon using standard Schlenk techniques. NMR (<sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P) spectra were recorded on on Varian Oxford NMR 200 MHz or Bruker Advance 500 MHz spectrometers. The chemical shift data for each signal are given in units of  $\delta$  (ppm) relative to tetramethylsilane (TMS) where  $\delta$  (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). <sup>31</sup>P NMR chemical shifts are reported relative to H<sub>3</sub>PO<sub>4</sub> as the reference. High-resolution electrospray ionization (ESI) or Fast atom bombardment (FAB) mass spectra were obtained at national center for inter-university research facilities. Absorption spectra were obtained on a Shimadzu UV-2501 spectrophotometer. Fluorescence measurements were recorded on a Hitachi F-7000 fluorescence quantum yields were determined by standard methods, using fluorescein ( $\Phi_F = 0.95$  in 0.1 N NaOH) as a standard. High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-20A equipped with SPD-20A detector. The reaction with ALP was measured by monitoring changes in fluorescence intensity using a Synergy Mx Microplate Reader (BioTek, USA).

## 1. Synthesis of Compounds

Synthesis of probe 1

Scheme S1. Synthesis of probe 1. a)  $(EtO)_2POCl$ , NaH, THF, 24 h, r.t., 87%; b) 2-benzothiazoleacetonitrile, piperidine, EtOH, 1 h, r.t., 66%; c) i) TMSBr,  $CH_2Cl_2$ , 8 h, r.t. ii) aq. NaOH, 44%.

**4-(Diethylamino)-2-(diethyl phosphoryl)benzaldehyde (5):** Comercially available 4-(diethylamino)-salicylaldehyde (3.0 g, 15.5 mmol) and NaH (60% dispersion in oil, 745 mg, 18.6 mmol) were dissolved in 100 mL of dry THF under an argon atmosphere, and diethyl chlorophosphate (2.7 mL, 18.6 mmol) was added, and the solution was stirred at room temperature for 24 hours. The reaction was quenched with  $H_2O$  (10 mL) and the solvent was evaporated under reduced pressure, and the crude mixture was extracted with  $CH_2Cl_2$ . The combined organic layer was dried over MgSO<sub>4</sub>, 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 **5** as a light-brown liquid (4.58 g, 87 % yield).; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 10.07$  (s, 1H), 7.73 (d, J = 9 Hz, 1H), 6.61 (s, 1H), 6.47 (d, J = 9 Hz, 1H), 4.26-4.20 (m, 4H), 3.43-3.39 (q, J = 7 Hz, 4H), 1.34 (t, J = 7 Hz, 6H), 1.21 (t, J=7 Hz, 6H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>):  $\delta = 186.4$ , 155.5, 155.4, 153.6, 130.7, 115.7, 108.3, 101.8, 65.1, 45.1, 16.4, 12.7; <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>):  $\delta = -5.61$  (s, 1P); HR-MS (ESI): calcd. for  $C_{15}H_{24}NO_5P$  [M+H]<sup>+</sup> 330.1470, found 330.1469.

(*E*)-2-(2-(Benzo[*d*]thiazol-2-yl)-2-cyanovinyl)-5-(diethylamino)phenyl diethyl phosphate (7): To a stirred solution of **5** (1.15 g, 3.48 mmol) and 2-(benzo[*d*]thiazol-2-yl)acetonitrile (0.61 g, 3.48 mmol) in ethanol (20 mL) at room temperature under argon was added one drop of piperidine. The reaction mixture was allowed to stir at room temperature for 1 hour. 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 3:1 hexanes : ethyl acetate as the mobile phase to afford compound **7** as a yellow solid (1.12 g, 66%).; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 8.47$  (d, J = 9 Hz, 1H), 8.45 (s, 1H), 7.99 (d, J = 8.5 Hz, 1H), 7.86 (d, J = 8.5 Hz, 1H), 7.47 (t, J = 15.5 Hz, 1H), 7.37 (t, J = 15.5 Hz, 1H), 6.71 (s, 1H), 6.57 (d, J = 9 Hz, 1H), 4.31-4.27 (q, J = 7 Hz, 4H), 3.47-3.43 (q, J = 7 Hz, 4H), 1.41-1.37 (q, J = 7 Hz, 6H), 1.24 (t, J = 7 Hz, 6H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>):  $\delta = 164.9, 153.8, 152.8, 152.7, 152.0, 140.1, 134.5, 130.1, 126.5, 125.2, 123.0, 121.4, 118.0, 111.2, 108.7, 102.3, 98.2, 65.1, 45.0, 16.3, 12.6; <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>): <math>\delta = -6.80$  (s, 1P); HR-MS (FAB): calcd. for C<sub>24</sub>H<sub>28</sub>N<sub>3</sub>O<sub>4</sub>PS [M+H]<sup>+</sup> 486.1616, found 486.1614.

**Sodium** (*E*)-2-(2-(benzo[*d*]thiazol-2-yl)-2-cyanovinyl)-5-(diethylamino)phenyl phosphate (1): To a solution of 7 (102 mg, 0.21 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was added bromotrimethylsilane (135 µL, 1.04 mmol) dropwise at room temperature. The reaction mixture was stirred for 8 hours at room temperature under argon, and quenched with MeOH (3 mL). After the mixture was stirred at room temperature for 30 min, the solvent was removed under reduced pressure. The reaction mixture was redissolved in H<sub>2</sub>O (1 mL), pH was adjusted to 9 ~ 10 with 0.01 N NaOH, and evaporated to dryness. The crude solid was subjected to reverse-phase C-18 column chromatography (Waters 10g RPC 18 cartridge) eluting with increasing concentrations of MeOH (0 to 20 %) in H<sub>2</sub>O to afford **1** as a red solid (42 mg, 44 %).; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  = 8.11 (s, 1H), 7.80 (d, J = 9 Hz, 1H), 7.65 (d, J = 7.5 Hz, 1H), 7.55 (d, J = 8 Hz, 1H), 7.19 (t, J = 7.5, 1H), 7.06 (t, J = 8 Hz, 1H), 6.62 (s, 1H), 6.09 (d, J = 9 Hz, 1H), 3.16-3.14 (q, J = 7 Hz, 4H), 0.99-0.96 (t, J = 7 Hz, 6H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>):  $\delta$  = 167.9, 157.5, 153.3, 152.4, 143.6, 133.6, 129.5, 126.8, 125.2, 121.9, 121.3, 119.2, 11.5, 107.1, 102.0, 92.6, 44.6, 12.2; <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>):  $\delta$  = -4.31 (s, 1P); HR-MS (FAB): calcd. for C<sub>20</sub>H<sub>18</sub>N<sub>3</sub>Na<sub>2</sub>O<sub>4</sub>PS [M+2H-Na]<sup>+</sup> 452.0810, found, 452.0811.

Synthesis of probe 2

Scheme S2. Synthesis of probe 2. a)  $(EtO)_2POCl$ , NaH, THF, 24 h, r.t., 53%; b) 2-benzothiazoleacetonitrile, piperidine, EtOH, 1 h, r.t., 54%; c) i) TMSBr,  $CH_2Cl_2$ , 8 h, r.t. ii) aq. NaOH, 45%.

**2,3,6,7-Tetrahydro-8-(diethyl phosphoryloxy)-1H,5H-benzo**[*ij*]**quinolizine-9-carboxaldehyde** (6): To a stirred solution of comercially available 2,3,6,7-tetrahydro-8-hydroxy-1H,5H-benzo[*ij*]**quinolizine-9-carboxaldehyde** (500 mg, 2.30 mmol) and NaH (60% dispersion in oil, 110 mg, 2.76 mmol) in THF (40 mL) at room temperature under argon was added diethyl chlorophosphate (400  $\mu$ L, 2.76 mmol). The resulting reaction mixture was allowed to stir at room temperature for 24 hours. The reaction was quenched with H<sub>2</sub>O (5 mL) and the solvent was evaporated under reduced pressure. The mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub>. The combined organic layer was dried over MgSO<sub>4</sub>, filtered and the solvent was evaporated under reduced pressure. The roude 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 6 as a colorless liquid (435 mg, 53%).; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 10.00$  (s, 1H), 7.35 (s, 1H), 4.21-4.16 (m, 4H), 3.26 (t, J = 5.8 Hz, 4H), 2.85 (t, J = 6.3 Hz, 2H), 2.69 (t, J = 6.3 Hz, 2H), 1.93-1.90 (m, 4H), 1.33 (t, J = 7.0 Hz, 6H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>):  $\delta = 187.5$ , 150.2, 149.1, 126.5, 118.4, 116.3, 112.3, 65.2, 50.3, 27.6, 21.9, 21.4, 16.4; <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>):  $\delta = -5.04$ (s); HR-MS (FAB): calcd. for C<sub>17</sub>H<sub>24</sub>NO<sub>5</sub>P [M]<sup>+</sup> 353.1392, found 353.1391.

**8-Diethylphosphoryloxy-9-[2-(2-benzothiazolyl)-2-cyanoethenyl]-2,3,6,7-tetrahydro-1H,5Hbenzo[***ij***]<b>quinolizine (8):** To a stirred solution of **6** (1.34 g, 3.79 mmol) and 2-(benzo[*d*]thiazol-2yl)acetonitrile (660 mg, 3.48 mmol) in ethanol (20 mL) at room temperature under argon was added one drop of piperidine. The reaction mixture was allowed to stir at room temperature for 1 hour. 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 3:1 hexanes : ethyl acetate as the mobile phase to afford compound **8** as a red solid (1.05 g, 54%).; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta = 8.34$  (s, 1H), 8.10 (s, 1H), 8.01 (d, J = 10 Hz, 1H), 7.84 (d, J = 10 Hz, 1H), 7.46 (m, 1H), 7.35 (m, 1H), 4.28-4.23 (q, J = 10 Hz, 4H), 3.33-3.30 (m, 4H), 2.89 (t, J = 5 Hz, 2H), 2.78 (t, J = 5 Hz, 2H), 1.98-1.95 (t, J = 5 Hz, H), 1.34 (t, J = 5 Hz, 6H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>):  $\delta =$ 166.7, 153.9, 147.9, 141.9, 134.4, 126.5, 126.4, 125.0, 123.4, 122.9, 121.4, 118.7, 118.1, 112.9, 111.9, 97.3, 65.1, 50.2, 49.6, 27.6, 22.2, 21.2, 20.7, 16.2; <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>):  $\delta =$  -6.11 (s, 1P); HR-MS (FAB): calcd. for C<sub>26</sub>H<sub>28</sub>N<sub>3</sub>O<sub>4</sub>PS [M+H]<sup>+</sup> 510.1616, found 510.1615.

**Sodium** 8-phosphoryl-9-[2-(2-benzothiazolyl)-2-cyanoethenyl]-2,3,6,7-tetrahydro-1H,5Hbenzo[*ij*]quinolizine (2): To a solution of 8 (90 mg, 0.17 mmol) in dry CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was added bromotrimethylsilane (110  $\mu$ L, 0.86 mmol) dropwise at room temperature. The reaction mixture was stirred for 8 hours at room temperature under argon, and quenched with MeOH (3 mL). After the mixture was stirred at room temperature for 30 min, the solvent was removed under reduced pressure. The reaction mixture was redissolved in H<sub>2</sub>O (1 mL), pH was adjusted to 9 ~ 10 with 0.01 N NaOH, and evaporated to dryness. The crude solid was subjected to reverse-phase C-18 column chromatography (Waters 10g RPC 18 cartridge) eluting with increasing concentrations of MeOH (0 to 20 %) in H<sub>2</sub>O to afford **2** as an red solid (38 mg, 45 %).; <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  = 8.95 (s, 1H), 8.06 (s, 1H), 7.90 (d, J = 15 Hz, 2H), 7.43 (t, J = 15 Hz, 1H), 7.33 (t, J = 15 Hz, 1H), 3.03 (t, J = 5Hz, 2H), 2.73 (t, J = 5 Hz, 2H), 1.96-1.91 (m, 4H); <sup>13</sup>C-NMR (125 MHz, CDCl<sub>3</sub>):  $\delta$  = 169.3, 154.5, 149.4, 135.4, 126.9, 126.2, 125.5, 122.7, 122.3, 119.6, 116.9, 115.2, 114.8, 106.3, 50.6, 50.4, 28.4, 22.9, 22.4, 21.7; <sup>31</sup>P-NMR (202 MHz, CDCl<sub>3</sub>):  $\delta$  = -1.05 (s, 1P); HR-MS (FAB): calcd. for C<sub>22</sub>H<sub>18</sub>N<sub>3</sub>Na<sub>2</sub>O<sub>4</sub>PS [M+H-Na]<sup>+</sup> 475.0810, found. 475.0812. Synthesis of compounds 3 and 4

**Compound 3**<sup>1</sup>: To a solution of 4-(diethylamino)-salicylaldehyde (220 mg, 1.15 mmol) and 2-(benzo[*d*]thiazol-2-yl)acetonitrile (200 mg, 1.15 mmol) in dry MeOH (20 mL) at room temperature under argon was added piperidine (1.14 mL, 11.5 mmol). The resulting solution was stirred at room temperature for 3 hours. The precipitate was collected by filtration. The crude solid 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 compound **3** as a yellow solid (200 mg, 50%).; <sup>1</sup>H-NMR (500 MHz, CD<sub>3</sub>OD):  $\delta$  = 8.01 (br, 1H), 7.90 (d, J = 0.5 Hz, 1H), 7.87 (d, J = 0.5 Hz, 1H), 7.41 (t, J = 7.5 Hz, 1H), 7.33-7.31 (t, J = 5 Hz, 1H), 6.56 (d, J = 5 Hz, 1H), 6.40 (t, J = 2.5 Hz, 1H), 3.43-3.38 (q, J = 7 Hz, 4H), 1.14 (t, J = 7 Hz, 6H); <sup>13</sup>C-NMR (125 MHz, CD<sub>3</sub>OD and CDCl<sub>3</sub>):  $\delta$  = 155.5, 153.1, 152.1, 138.4, 130.1, 126.2, 125.2, 122.4, 121.1, 108.4, 107.5, 96.4, 44.7, 12.0.; HR-MS (FAB): calcd. for C<sub>20</sub>H<sub>19</sub>N<sub>3</sub>OS [M+H]<sup>+</sup> 350.1327; found, 350.1327.

**4**<sup>1</sup>: 2,3,6,7-tetrahydro-8-(diethyl Compound То a solution of phosphoryl)-1H,5Hbenzo[ij]quinolizine-9-carbox aldehyde (200 mg, 0.92 mmol) and 2-(benzo[d]thiazol-2yl)acetonitrile (160 mg, 0.92 mmol) in dry MeOH (20 mL) at room temperature under argon was added piperidine (0.9 mL, 9.2 mmol). The resulting solution was stirred at room temperature for 5 hours. The precipitate was collected by filtration. The crude solid was purified by column chromatography on silica gel using progressively more polar 10:1 to 1:1 hexanes : ethvl acetate as the mobile phase to afford compound **4** as a vellow solid (260 mg, 76%).; <sup>1</sup>H-NMR (200 MHz, CDCl<sub>3</sub>):  $\delta = 8.01$  (d, J = 8 Hz, 1H), 7.89 (d, J = 8 Hz, 1H), 7.46 (t, J = 7 Hz, 1H), 7.33 (t, J = 1H), 6.87 (s, 1H), 3.3-2.9 (m, 4H), 2.86 (t, J = 6 Hz, 2H), 2.73 (t, J = 6 Hz, 2H), 2.00-1.96 (m, 4H); HR-MS (FAB): calcd. for  $C_{22}H_{19}N_3OS [M+H]^+ 374.1327$ , found, 374.1328.

#### 2. Studies of photophysical properties

Absorption and emission spectra of compounds



**Figure S1.** Absorption (dashed lines) and emission spectra (solid lines) of probe **1** (5  $\mu$ M) in Tris-HCl buffer (10 mM, pH 7.4) at 25 °C. Excited at 460 nm.



**Figure S2.** Absorption (dashed lines) and emission spectra (solid lines) of probe 2 (5  $\mu$ M) in Tris-HCl buffer (10 mM, pH 7.4) at 25 °C. Excited at 460 nm.

Compound	$\lambda_{abs, max}$	$\epsilon^b$	$\lambda_{\rm em,\ max}^{c}$	$\Phi_{ ext{FL}}{}^d$
	[nm]	$[M^{-1}cm^{-1}]$	[nm]	
1	472	$1.7  imes 10^4$	542	0.002
2	493	$2.2  imes 10^4$	545	0.03
3	485	$2.9  imes 10^4$	529	0.10
4	500	$3.2  imes 10^4$	542	0.63
"Doto for 1 and	7 wore obtained in	10 mM Tria U(1) (nH 7	1) and data for 2 and	A ware obtained in 10

**Table S1.** Photophysical properties of compounds  $1-4^a$ 

"Data for 1 and 2 were obtained in 10 mM Tris-HCl (pH 7.4) and data for 3 and 4 were obtained in 10 mM Tris<sub>7</sub>HCl (pH 7.4) containing 0.5% DMSO. Measured at each absorption maximum. Excited at 460 nm. Quantum yields vs. fluorescein in 0.1 N NaOH ( $\Phi_F = 0.95$ )."



**Figure S3.** Absorption (dashed lines) and emission spectra (solid lines) of compound **3** (5  $\mu$ M) in Tris-HCl buffer (10 mM, pH 7.4) containing 0.5% DMSO as a cosolvent at 25 °C. Excited at 460 nm.



**Figure S4.** Absorption (dashed lines) and emission spectra (solid lines) of compound **4** (5  $\mu$ M) in Tris-HCl buffer (10 mM, pH 7.4) containing 0.5% DMSO as a cosolvent at 25 °C. Excited at 460 nm.



**Figure S5.** Absorption (dashed lines) and emission spectra (solid lines) of probe **1** (red) in Tris-HCl buffer (10 mM, pH 7.4) and compound **3** (black) in Tris-HCl buffer (10 mM, pH 7.4) containing 0.5% DMSO as a cosolvent at 25 °C. Excited at 460 nm.  $[1] = [3] = 5 \,\mu M$ 



**Figure S6.** Absorption (dashed lines) and emission spectra (solid lines) of probe **2** (red) in Tris-HCl buffer (10 mM, pH 7.4) and compound **4** (black) in Tris-HCl buffer (10 mM, pH 7.4) containing 0.5% DMSO as a cosolvent at 25 °C. Excited at 460 nm. [**2**] = [**4**] = 5  $\mu$ M



**Figure S7.** Absorption (dashed lines) and emission spectra (solid lines) of probe 1 (black) and 2 (red) in Tris-HCl buffer (10 mM, pH 7.4) at 25 °C. Excited at 460 nm.  $[1] = [2] = 5 \,\mu M$ 



**Figure S8.** Absorption (dashed lines) and emission spectra (solid lines) of compound **3** (black) and **4** (red) in Tris-HCl buffer (10 mM, pH 7.4) containing 0.5% DMSO as a cosolvent at 25 °C. Excited at 460 nm.  $[3] = [4] = 5 \,\mu\text{M}$ 

#### 3. Stability Studies and pH effect of probe





**Figure S9.** (left) Absorption and (right) emission spectra of probe **1** (10  $\mu$ M) in different pH buffer systems as a cosolvent at 25 °C. 10 mM citrate buffer for pH 3 and 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.



**Figure S10.** (left) Absorption and (right) emission spectra of compound **3** (10  $\mu$ M) in different pH buffer systems containing 1% DMSO as a cosolvent at 25 °C. 10 mM citrate buffer for pH 3 and 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.



Figure S11. Effect of pH on the fluorescence intensity of probe 1 (red) and compound 3 (blue) at 25 °C.  $[1] = [3] = 10 \ \mu M$ 

#### Chemical stability test of probe 1 and compound 3 in aqueous solution

The chemical stability of probe **1** was examined in Tris-HCl buffer (10 mM, pH 7.4). As shown in Figure 12, a slight decrease in the fluorescence intensity (< 5%) of probe **1** at 542 nm was detected following incubation for 30 min, but a new emission signal at 529 nm, indicative of **3**, is not observed. Therefore, background fluorescence due to competing non-enzymatic hydrolysis would be minimal.



**Figure S12.** Chemical stability of probe 1 (10  $\mu$ M) in 10 mM Tris-HCl buffer (pH = 7.4) at 37 °C. Excited at 460 nm. The spectra were obtained every 2 min (0 – 30 min). Inset shows the fluorescence intensity versus incubation time. Fluorescence intensity was measured at 542 nm.



**Figure S13.** Chemical stability of compound **3** (10  $\mu$ M) in 10 mM Tris-HCl buffer (pH = 7.4, 1% DMSO) at 25 °C. Excited at 460 nm. The spectra were obtained every 2 min (0 – 30 min). Inset shows the fluorescence intensity versus incubation time. Fluorescence intensity was measured at 529 nm.

#### Photostability test of probes 1-2 and compounds 3-4 in aqueous solution

We have investigated photostabilities of 1, 2, 3 and 4. The photooxidation studies were performed by continuous UV irradiation of each compound in Tris-HCl buffer (10 mM, pH 7.4, 0.5% DMSO) at 25 °C using a 150 W steady-state Xe lamp as the irradiation source under aerobic conditions. The photoinduced degradation was quantified by monitoring the decrease of fluorescence intensity of 1, 2, 3 and 4 as a function of elapsed photolysis time (Figure S14). Negligible changes in the fluorescence intensity of 1, 2, 3 and 4 were observed after 2 hours of irradiation at 460 nm.



**Figure S14.** Photostability of probes **1-2** and compounds **3-4** in Tris-HCl buffer (10 mM, pH 7.4, 25 °C) containing 0.5% DMSO as a cosolvent. The remaining fluorescence emission intensities of the each compound as a function of irradiation time. Irradiated at 460 nm.  $[1] = [2] = [3] = [4] = 10 \,\mu$ M.

### 4. Enzymatic hydrolysis of probes by alkaline phosphatase (ALP)

# 4.1. Optimization conditions for ALP assays

Enzyme assay with probe 1 in various buffer systems



**Figure S15.** Fluorescence response of probe **1** (10  $\mu$ M) upon incubation with ALP (350 nM) at 37 °C in various buffer systems. Excited at 460 nm. Fluorescence intensity was measured at 525 nm. The spectra were obtained every 2 min (0 – 30 min). A: Tris-HCl buffer (10 mM, pH 7.4), B: HEPES buffer (10 mM, pH 7.4), C: Tris-HCl buffer (10 mM, pH 7.4, 2 mM MgCl<sub>2</sub>), D: PBS buffer (10 mM, pH 7.4).

Effect of pH on enzyme assay with probe 1



**Figure S16.** Fluorescence response of probe **1** (10  $\mu$ M) upon incubation with ALP (350 nM) at 37 °C in 10 mM Tris-HCl buffer with various pH conditions. Excited at 460 nm. Fluorescence intensity was measured at 525 nm. The spectra were obtained every 2 min (0 – 30 min).

#### Enzyme assay with probe 1 as a function of concentration of ALP

ALP was dissolved in Tris-HCl buffer (10 mM, pH 7.4) and diluted with the buffer solution to make different ALP concentrations. ALP solution (20  $\mu$ L) was mixed with the substrate (20  $\mu$ L) solution in Tris-HCl buffer (10 mM, pH 7.4, 160  $\mu$ L). Final probe concentration in the solution was 10  $\mu$ M, and the ALP concentration was 0, 0.35, 1.75, 3.5, 17.5, 35, 87.5, 175, 262, 350 nM, respectively. Fluorescence intensity at 525 nm was recorded every 2 min for 30 min at 37 °C using a computer-controlled fluorescence plate reader.



**Figure S17.** Fluorescence response of probe **1** (10  $\mu$ M) upon incubation with different amounts of ALP (from 0 to 350 nM) in Tris-HCl buffer (10 mM, pH = 7.4, 37 °C). Excited at 460 nm. Fluorescence intensity was measured at 525 nm. The spectra were obtained every 2 min (0 – 30 min).



**Figure S18.** Increase of fluorescence intensity of probe **1** (10  $\mu$ M) as a function of amounts of ALP (from 0 to 350 nM) in Tris-HCl buffer (10 mM, pH = 7.4, 37 °C). Incubation time: 2 min. Excited at 460 nm. Fluorescence intensity was measured at 525 nm.

Enzyme assay with probe 2 as a function of concentration of ALP



**Figure S19.** Fluorescence response of probe 2 (10  $\mu$ M) upon incubation with different amounts of ALP (from 0 to 350 nM) in Tris-HCl buffer (10 mM, pH = 7.4, 3°C). Excited at 460 nm. Fluorescence intensity was measured at 540 nm. The spectra were obtained every 2 min (0 – 60 min).

## 4.2. Kinetic Studies of probes

To determine the kinetic constants, probe **1** and probe **2** at a series of the final concentrations (0.1 - 25  $\mu$ M) were hydrolyzed by ALP (50 nM). As standards, hydrolysis of 4-MUP and ELF-97, commercially available ALP probes, was also measured under the similar conditions. The reaction was monitored by measuring fluorescence change at 525 nm for probe **1** (excited at 460 nm), at 540 nm for probe **2** (excited at 460 nm), 440 nm for 4-MUP (excited at 360 nm) and 530 nm for ELF-97 (excited at 345 nm) at 37 °C. The initial velocity was calculated from the slope of the each progress curve. The parameters such as  $K_{\rm M}$  and  $k_{\rm cat}$  with ALP for probe **1**, probe **2**, 4-MUP, and ELF-97 were determined by Lineweaver-Burk plot and listed in Table S2. For example, Figure S21(b) showed the mean value of the relative initial velocity of probe **1** for the hydrolysis by ALP.  $K_{\rm M}$ ,  $k_{\rm cat}$ , and  $k_{\rm cat}/K_{\rm M}$  values for probe **1** were 19.3  $\mu$ M, 0.27 s<sup>-1</sup>, and 1.4 x 10<sup>4</sup> M<sup>-1</sup>s<sup>-1</sup> respectively.

Determination of kinetic constants for probe 1 at pH 7.4



**Figure S20.** Standard fluorescence curve of probe 1 at different concentrations with excess amounts of ALP in 10 mM Tris-HCl (10 mM, pH = 7.4,  $37^{\circ}$ C). Fluorescence intensity at 525 nm was measured. Excited at 460 nm.



**Figure S21.** Catalytic hydrolysis of probe **1** by ALP. (a) Progress curves of hydrolysis of probe **1** at a series of concentrations  $(0.1 - 25 \,\mu\text{M})$  upon incubation with ALP (50 nM) in Tris-HCl buffer (10 mM, pH 7.4, 37 °C). The spectra were obtained every 2 min (0 – 30 min). Fluorescence intensity was measured at 525 nm with excitation at 460 nm. (b) A plot of the initial velocity  $v_0$  of the hydrolysis of probe **1** by ALP versus concentration of probe **1**. Insert represents a double reciprocal (Lineweaver-Burk) plot

Determination of kinetic constants for probe 1 at pH 9.0



**Figure S22.** Standard fluorescence curve of probe 1 at different concentrations with excess amounts of ALP in 10 mM Tris-HCl (10 mM, pH = 9.0, 37  $^{\circ}$ C). Fluorescence intensity at 525 nm was measured. Excited at 460 nm.



**Figure S23.** Catalytic hydrolysis of probe **1** by ALP. (left) Progress curves of hydrolysis of probe **1** at a series of concentrations  $(0.1 - 25 \,\mu\text{M})$  upon incubation with ALP (50 nM) in Tris-HCl buffer (10 mM, pH 9.0, 37 °C). The spectra were obtained every 2 min (0 – 30 min). Fluorescence intensity was measured at 525 nm with excitation at 460 nm. (right) A plot of the initial velocity  $v_0$  of the hydrolysis of probe **1** by ALP versus concentration of probe **1**. Insert represents a double reciprocal (Lineweaver-Burk) plot

Determination of kinetic constants for probe 2



**Figure S24.** Standard fluorescence curve of probe **2** at different concentrations with excess amounts of ALP in 10 mM Tris-HCl (10 mM, pH = 7.4,  $37^{\circ}$ C). Fluorescence intensity at 540 nm was measured. Excited at 460 nm.



**Figure S25.** Catalytic hydrolysis of probe **2** by ALP. (left) Progress curves of hydrolysis of probe **2** at a series of concentrations  $(0.1 - 25 \,\mu\text{M})$  upon incubation with ALP (50 nM) in Tris-HCl buffer (10 mM, pH 7.4, 37 °C). The spectra were obtained every 2 min (0 – 30 min). Fluorescence intensity was measured at 540 nm with excitation at 460 nm. (right) A plot of the initial velocity  $v_0$  of the hydrolysis of probe **2** by ALP versus concentration of probe **2**. Insert represents a double reciprocal (Lineweaver-Burk) plot

Determination of kinetic constants for 4-MUP



**Figure S26.** Standard fluorescence curve of 4-MUP at different concentrations with excess amounts of ALP in 10 mM Tris-HCl (10 mM, pH = 7.4,  $37^{\circ}$ C). Fluorescence intensity at 440 nm was measured. Excited at 360 nm.



**Figure S27.** Fluorescence response of 4-MUP (10  $\mu$ M) upon incubation with different amounts of ALP (from 0 to 350 nM) in Tris-HCl buffer (10 mM, pH = 7.4, 3°C). Excited at 360 nm. Fluorescence intensity was measured at 440 nm. The spectra were obtained every 2 min (0 – 30 min).



**Figure S28.** Catalytic hydrolysis of 4-MUP by ALP. (a) Progress curves of hydrolysis of 4-MUP at a series of concentrations  $(0.1 - 25 \,\mu\text{M})$  upon incubation with ALP (50 nM) in Tris-HCl buffer (10 mM, pH 7.4, 37 °C). The spectra were obtained every 2 min (0 – 30 min). Fluorescence intensity was measured at 440 nm with excitation at 360 nm. (b) A plot of the initial velocity  $v_0$  of the hydrolysis of 4-MUP by ALP versus concentration of 4-MUP. Insert represents a double reciprocal (Lineweaver-Burk) plot

Determination of kinetic constants for ELF-97



**Figure S29.** Standard fluorescence curve of ELF-97 at different concentrations with excess amounts of ALP in 10 mM Tris-HCl (10 mM, pH = 7.4,  $37^{\circ}$ C). Fluorescence intensity at 530 nm was measured. Excited at 345 nm.



**Figure S30.** Fluorescence response of ELF-97 (10  $\mu$ M) upon incubation with different amounts of ALP (from 0 to 350 nM) in Tris-HCl buffer (10 mM, pH = 7.4, 3°C). Excited at 345 nm. Fluorescence intensity was measured at 530 nm. The spectra were obtained every 20 sec (0 – 300 sec).



**Figure S31.** Catalytic hydrolysis of ELF-97 by ALP. (a) Progress curves of hydrolysis of ELF-97 at a series of concentrations  $(0.25 - 25 \,\mu\text{M})$  upon incubation with ALP (50 nM) in Tris-HCl buffer (10 mM, pH 7.4, 37 °C). The spectra were obtained every 20 sec (0 – 300 sec). Fluorescence intensity was measured at 530 nm with excitation at 345 nm. (b) A plot of the initial velocity  $v_0$  of the hydrolysis of ELF-97 by ALP versus concentration of ELF-97. Insert represents a double reciprocal (Lineweaver-Burk) plot

Table S2. Kinetic Data for substrates probes 1-2, 4-MUP, and ELF-97

Substrate	<i>K</i> <sub>M</sub> (μM)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_{\rm m} ({\rm M}^{-1}{\rm s}^{-1})$	
Probe <b>1</b> (pH 7.4)	19.2	0.27	$1.4  imes 10^4$	
Probe <b>1</b> (pH 9.0)	29	0.44	$1.5  imes 10^4$	
Probe 2 (pH 7.4)	9.0	0.10	$1.1  imes 10^4$	
4-MUP (pH 7.4)	18.3	0.14	$7.7  imes 10^3$	
ELF-97 (pH 7.4)	20.0	0.60	$3.0  imes 10^4$	

#### 4.3. Inhibition assay of ALP activity

For inhibition assay of enzyme activity, the final concentrations of ALP and probe **1** were fixed to 350 nM and 10  $\mu$ M, respectively. Two Potent ALP inhibitors, (-)-*p*-bromotetramisole oxalate (*p*-BTO) and levamisole were dissolved in 20  $\mu$ L Tris-HCl buffer (10 mM, pH 7.4) at different concentrations, and then mixed with 20  $\mu$ L of ALP solution in Tris-HCl buffer (10 mM, pH 7.4), respectively. Final concentrations of *p*-BT in the mixed solution were 0 ~ 1000  $\mu$ M and final concentrations of levamisole in the mixed solution were 0 ~ 5000  $\mu$ M. The mixed solutions were incubated at room temperature for 20 min to inhibit enzyme activity. To the inhibitor-treated ALP solution was then added 20  $\mu$ L of probe **1** solution. Enzymatic reaction was performed by incubating the solution as previously described. Fluorescence at 525 nm was recorded every 2 min for 30 min at 37 °C using a computer-controlled fluorescence plate reader. IC<sub>50</sub> value (the inhibitor concentration at 2 min time point.



**Figure S32.** Inhibition assay of ALP activity using probe **1** in Tris-HCl buffer (10 mM, pH 7.4, 37 °C). Kinetic profiles of probe **1** (10  $\mu$ M) after addition of the ALP solution, which was pretreated with (a) *p*-BT at different concentrations (0-1000  $\mu$ M) and (b) levamisole at different concentrations (0- 5000  $\mu$ M). Excited at 460 nm. Fluorescence intensity at 525 nm was recorded every 2 min.



**Figure S33.** Relative activity of ALP versus concentration of *p*-BT and levamisole at 2 min time point. [ALP] = 350 nM. IC<sub>50</sub> value was determined as 36  $\mu$ M for *p*-BT and 75  $\mu$ M for levamisole. **5. Analysis of reaction product by LC-MS** 



**Figure S34.** HPLC chromatograms of probe **1** (top) without and (middle) with ALP treatment for 1 hour and (bottom) compound **3**. The samples were analyzed by LC-MS with a linear gradient elution (from 0 to 50% B, A: deionized water with 1% formic acid, B: acetonitrile, flow rate 0.3 mL/min, UV: 340 nm). MW of the retention time at 5.5 min is 430.1, which corresponds to  $[M+H]^+$  for probe **1** and MW of the retention time at 3.4 min is 350.1, which corresponds to  $[M+H]^+$  for **3**.  $[\mathbf{1}] = [\mathbf{3}] = 10 \,\mu\text{M}$ ,  $[ALP] = 350 \,\text{nM}$ 



**Figure S35**. ESI-MS spectra of probe **1** (a) without enzyme treatment, (b) after treating with ALP for 1 h, (c) compound **3**. MW of the retention time at 5.5 min is 430.1, which corresponds to  $[M+H]^+$  for probe **1** and MW of the retention time at 3.4 min is 350.1, which corresponds to  $[M+H]^+$  for **3**. [**1**] = [**3**] = 10  $\mu$ M, [ALP] = 350 nM

# 6. Cell studies

# **Cell culture**

HeLa (human cervical carcinoma) and HT-29 (human colon adenocarcinoma) cell lines were obtained from the American Type Culture Collection (Rockville, MD), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% antibiotic-antimycotic in a humidified incubator containing 5% CO<sub>2</sub> at 37 °C. The stock solution of probe **1** in DMSO (10 mM) was diluted with cell culture medium. Unless otherwise mentioned, final concentration of probe **1** applied to cells is 10  $\mu$ M.

## **Confocal fluorescence microscopy**

For confocal fluorescence imaging study, HeLa cells were plated at a density of  $1 \times 10^5$  cells/well into 4well LabTek-II chambered cover glass (Nalge Nunc International Corp., USA) and incubated for 24 h for cell attachment. After 24 h of incubation, the cells were washed and treated with the fresh media containing probe **1** for 2~10 min. For inhibition assay, additional cells were preincubated with ALP inhibitor levamisole (10 mM) for 15 min and then treated with probe **1** for 2 min. All cells were then washed 3 times with pre-warmed phosphate buffered saline solution (100 mM, pH 7.4, 138 mM NaCl), and fresh culture medium was added into the wells. Confocal fluorescence images (Ex. 488 nm, Em. 505-530 nm) were observed with a confocal laser scanning microscope (C-Apochromat 40×/1.2 W, and 2× scan zoom, Zeiss LSM 510 META, Zeiss, Germany). In case of HT-29 cells, cells were treated with probe **1** for 30 min, washed 3 times, and observed with a confocal laser scanning microscope.

# **FACS** analysis

Cells were seeded at a density of  $1 \times 10^6$  cells/well into 12-well plate, and incubated for 24 h for cell attachment. After 24 h of incubation, HeLa cells were washed and treated with the media containing probe **1** for 2, 5, and 10 min, respectively. For inhibition assay, three additional sets of HeLa cells were preincubated with levamisole (10 mM) and then treated with probe **1** for 2, 5, and 10 min, respectively. For unstained control groups, the cells were treated with fresh media without probe **1** for 2, 5, and 10 min, respectively. For unstained control groups, the cells were treated from the plate using Trypsin/EDTA, centrifuged, and washed 3 times with PBS solution. Cell pellet was resuspended in PBS solution and transferred into the test tube for FACS analysis (BD, FACSCalibur, Flow Cytometer, USA). Then fluorescence intensity (Ex. 488 nm, Em. 515-545 nm) was measured. In case of HT-29 cells, cells were treated with the fresh media or the media containing probe **1** for 30 min, detached from the plate using Trypsin/EDTA, centrifuged, and washed 3 times with PBS solution. Then cell pellet was resuspended in PBS solution and transferred into the tube for FACS analysis (BD, FACSCalibur, Flow Cytometer, USA).

## Cell viability test

Cells were seeded in each well of 96-well plates at a density of  $1 \times 10^4$  cells/well and incubated for 24 h for cell attachment. The stock solution of probe **1** was diluted with a cell culture medium containing 10% FBS to have a final concentration of 0, 10, 20, and 50 µM, respectively. Existing culture medium was replaced with 100 µL of the fresh one containing probe **1**. HeLa cells were treated with probe **1**-containing media for 5 and 10 min, respectively. To investigate the effect of incubation time with probe **1**, additional HeLa cells were treated with 10 µM probe **1** for 0.5, 1, and 4, 24 h, respectively. In case of HT-29 cells, the cells were treated with probe **1**-containing media for 24 h. After washing the cells twice, cell viability was measured using a cell counting kit-8 (Dojindo Laboratories, Japan). The cells were incubated with CCK-8 containing media and incubated for additional 4 h. The absorbance was measured at 450 nm (reference = 650 nm) by using a microplate reader (Tecan Safire 2, Switzerland). Cell viability was calculated as a percentage compared to media-treated control cells. Data are expressed as a mean ± standard deviation. Statistical significance compared to the media-treated control cells was calculated by determining *p* values using the *t*-test. From cell viability assays, no cytotoxic effect was detected in HeLa cells treated with probe **1** up to a concentration of 50 µM after 5 min of treatment time and 87.5% of cells were viable at the same concentration for 10 min incubation.

However, no cytotoxic effect in HT-29 cells was observed up to 50  $\mu$ M concentration of probe 1 even at longer incubation of probe 1 (i.e., 24 h).

# Western blot analysis

Cells were seeded into 100-pi plates at a density of  $5 \times 10^6$  and further incubated for 2-3 days. When the cell confluency was reached up to 70-80%, cells were detached by trypsinization and centrifuged at 3,000 rpm for 10 min. Whole-cell lysates were prepared by sonication using Misonix Sonicator 3000 (Misonix, Inc., USA) after resuspending the cells in 100 µL lysis buffer. 32 µg proteins determined by quantitative analysis using BCA assay (Micro BCA<sup>TM</sup> Protein Assay Kit, Thermo scientific, USA) were mixed with  $4 \times$  SDS sample buffer, heating at 70 °C for 10 min and run by NuPAGE® 4-12% gradient Bis-Tris gels (Invitrogen, USA) at 200 V for 1 h. SDS-PAGE gels were electro-transferred onto nitrocellulose membranes at 170 mA for 1 h, blocked with 5% (wt/vol) BSA for 1h at 4 °C. The blot was washed with 1× PBST (1% Tween 20) for 5×5 min after incubating overnight at 4 °C in the presence of the human intestinal alkaline phosphatase antibody (Rabbit polyclonal Ig G antibody, 1:10,000 dilutions, Abcam, England). Goat anti-rabbit IgG-HRP antibody (Santa Cruz Biotechnology, Inc., USA) was used as the secondary antibody at a 1 : 5000 ratio for 1 h at RT, and ALP was detected using ECL kit (SuperSignal® West Pico Chemiluminescent Substrate, Thermo scientific, USA).



**Figure S36.** Confocal fluorescence images of HeLa cells treated with probe **1** for 2, 5, and 10 minutes, respectively (magnification:  $80\times$ ). Imaging acquisition condition of these samples was adjusted to the cells treated for 10 min to avoid saturation of fluorescence signals, and also for qualitative comparison of the fluorescence generation between samples. Ex. 488 nm, Em. 505~530 nm.



**Figure S37.** Flow cytometric detection of ALP activity in HeLa cells (Ex. 488 nm, Em. 515~545 nm). (a) Histogram of cell counts vs. fluorescence intensity for the samples of different treatment time of probe **1**. *Black line*: unstained control cells, *red line*: cells treated with inhibitor and probe **1**, *green line*: probe **1**-treated cells for 2, 5, or 10 minutes. (b) Graph summarizing Geo mean fluorescence intensities of the samples.  $4 \times 10^5$  cells were seeded into 12 well plate and then treated 10 µM probe **1** for 2, 5, 10 min, respectively. For inhibition assay, 10 mM levamisole were pre-treated for 15 min before adding probe **1**.



**Figure S38.** ALP-negative control cell studies. (a) Representative confocal fluorescence images of live HT-29 cells incubated with probe **1** for 30 min (magnification:  $80\times$ ). Ex. 488 nm, Em. 505-530 nm. (b) FACS study of ALP activity in HT-29 cells. Ex. 488 nm, Em. 515-545 nm. (c) Western blot analysis of ALP expression in HeLa and HT-29 cell lines.



**Figure S39.** Cell viability of HeLa cells treated with probe **1** for 5 min (**n**) or 10 min (**n**) at different concentration. Statistically significant difference in cell viability compared with media-treated control group is denoted as asterisk (\*p < 0.05). No cytotoxic effect was detected in HeLa cells treated with probe **1** up to a concentration of 50  $\mu$ M after the treatment for 5 min and 87.5% of cells were viable at the same concentration for 10 min incubation.



**Figure S40.** Cell viability of HeLa cells treated with 10  $\mu$ M probe 1 for different time periods. No cytotoxic effect was observed for the cells incubated with probe 1 for 1 h and 92.8 % of cells were viable even after the treatment for 4 h. When the cells were treated for 24 h, cell viability was decreased to 28.7 %. Statistically significant difference in cell viability compared with media-treated control group is denoted as asterisk (\*p < 0.05, \*\*p < 0.01).



**Figure S41.** Cell viability depending on the concentration of probe **1** treated. Cell viability was measured after treating HT-29 cells with probe **1** for 24 h. No cytotoxicity was observed up to the concentration of 50  $\mu$ M even upon treatmentfor 24 hr.

**7.** <sup>1</sup>H-NMR and <sup>13</sup>C-NMR Spectra

# <sup>1</sup>H-NMR Spectrum of **5** in $CDCl_3$ (500 MHz):



<sup>13</sup>C-NMR Spectrum of **5** in CDCl<sub>3</sub> (125 MHz):



<sup>31</sup>P-NMR Spectrum of **5** in CDCl<sub>3</sub> (202 MHz):



<sup>1</sup>H-NMR Spectrum of **7** in CDCl<sub>3</sub> (500 MHz):





<sup>13</sup>C-NMR Spectrum of **7** in CDCl<sub>3</sub> (125 MHz):



<sup>31</sup>P-NMR Spectrum of **7** in CDCl<sub>3</sub> (202 MHz)



<sup>1</sup>H-NMR Spectrum of **1** in D<sub>2</sub>O (500 MHz):



 $^{13}$ C-NMR Spectrum of **1** in D<sub>2</sub>O (125 MHz):



 $^{31}$ P-NMR Spectrum of **1** in D<sub>2</sub>O (202 MHz)



<sup>1</sup>H-NMR Spectrum of **3** in CD<sub>3</sub>OD (500 MHz):





<sup>13</sup>C-NMR Spectrum of **3** in CD<sub>3</sub>OD+CDCl<sub>3</sub> (125 MHz):



<sup>1</sup>H-NMR Spectrum of **6** in CDCl<sub>3</sub> (500 MHz):



<sup>13</sup>C-NMR Spectrum of **6** in CDCl<sub>3</sub> (125 MHz):



<sup>31</sup>P-NMR Spectrum of **6** in CDCl<sub>3</sub> (202 MHz):



<sup>1</sup>H-NMR Spectrum of **8** in CDCl<sub>3</sub> (500 MHz):





<sup>13</sup>C-NMR Spectrum of **8** in CDCl<sub>3</sub> (125 MHz):



<sup>31</sup>P-NMR Spectrum of **8** in CDCl<sub>3</sub> (202 MHz)



<sup>1</sup>H-NMR Spectrum of **2** in CDCl<sub>3</sub> (500 MHz):





<sup>13</sup>C-NMR Spectrum of **2** in CDCl<sub>3</sub> (125 MHz):



<sup>31</sup>P-NMR Spectrum of **2** in CDCl<sub>3</sub> (202 MHz)



<sup>1</sup>H-NMR Spectrum of **4** in CDCl<sub>3</sub> (200 MHz):





## 8. References

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