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Supporting Information

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for

Self-Cleavable Bioluminogenic Luciferin Phosphates as Alkaline Phosphatase Reporters

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1. Materials and General Methods

2-Cyano-6-hydroxybenzothiazole and 2-cyano-6-aminobenzothiazole were provided by Promega Biosciences, Inc. All other reagents and solvents for chemical syntheses were purchased from Aldrich, Sigma, and Fisher and were used without further purification. Nuclear Magnetic Resonance (NMR) and mass spectra were recorded on a Varian-300 and Fisions VG Platform II spectrometers. UV-Vis absorption spectra were recorded on Beckman DU 650. The final compounds **1-2** were purified on the Beckman HPLC System with Programmable Solvent Module 126. The purity and free luciferin analyses were performed on Agilent 1100 HPLC with monitoring absorbance at 254 nm and 330 nm.

2. Assay Methods and Protocols

2.1 Background comparisons: Since luciferin/aminoluciferin generated from hydrolysis of phosphate or contaminated from organic syntheses might be the major contributors for the luminescent background levels, in order to diminish the background differences caused from luciferin contamination by organic syntheses, a trace of luciferin/aminoluciferin for fresh samples was detected by highly sensitive fluorescence HPLC. The initial free luciferin/aminoluciferin contents for 6-luciferin phosphate, **1** and **2** were controlled in very low levels (<< 0.1%) and with slight differences for this particular case. The bioluminescent background signals were read after incubation with luciferin detection reagent for 30 minutes, while the significantly high background from 6-luciferin phosphate might be attributed to the generation of luciferin from slow hydrolysis of phosphate.

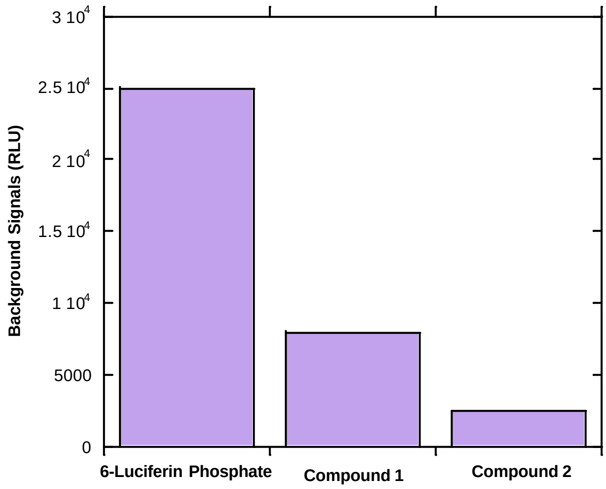


Figure S1. Luminescent background levels for three compounds

2.2. Kinetic parameter determination

Step1: AP reaction. Generally, AP reaction was conducted in a reaction volume of 100 µL containing calf intestinal AP enzyme (Promega) and substrate in 50 mM Tris-HCl pH 9.3, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine and incubated for 30 min.

Step 2: Detection of luciferin. To each Step 1 reaction, 100 µL of a proprietary luciferin detection reagent, comprising luciferase, ATP and buffer, were added and the luminescent signal was measured on a Veritas microplate luminometer (Turner Biosystems).

The apparent K_m values for compounds **1-2** were measured by varying the substrate concentrations while AP enzyme concentration was held constant (14 pg). The values were calculated from the double-reciprocal linear Lineweaver-Burk analysis.

$$\frac{1}{n} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S]} \qquad (1)$$

Where ν is luminescence (RLU), [S] is substrate concentration, V_{max} is the maximum luminescence, and K_m is the Michaelis-Menten constant.

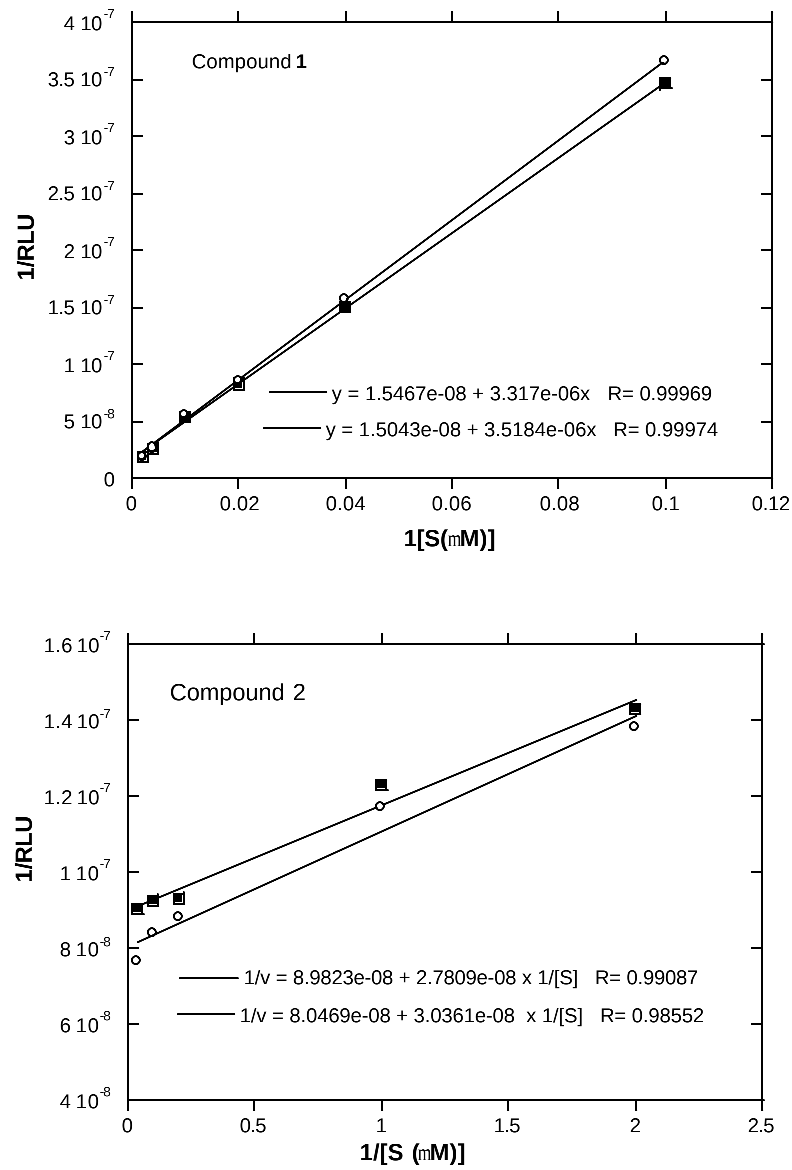


Figure S2. Determination of K_m values from the Lineweaver-Burk plots for compounds **1-2** with calf intestinal AP enzyme.

The K_m values obtained from **Figure S2** are 214 μM and 234 μM for compound **1**, and 0.31 μM and 0.38 μM for compound **2**, respectively.

2.3 Protocols for AP immunoassay measurements: General procedures for immunoassay on rabbit anti-caspase-3 polyclonal antibody as an antigen and goat anti-rabbit IgG AP conjugate as an antibody with compound **2** as a bioluminescent reporter for AP activities.

1) Serially dilute Anti-active Caspase-3 pAb in 1XTBST (0.1%) from 0.19mg/mL to 19pg/mL.

2) Place 100ul of each Caspase antibody dilution into wells of an Immulon4 microtiter plate.

3) Place 100ul of 1xTBST (0.1%) into wells as a negative control.

4) Incubate 1 hr. at RT.

5) Wash 3x with 1xTBST (0.1%).

6) Block the plate by adding 200ul of 3%BSA/1xTBST (0.1%) to each well.

7) Incubate 1 hr. at RT.

8) Wash 3x with 1xTBST (0.1%)

9) Dilute Anti-rabbit IgG AP-conjugate 1:7500 in 1x TBST(0.1%).

10) Place 100ul of the Anti-rabbit IgG Ab into each well.

11) Incubate 1 hr. at RT.

12) Assemble the following master mix:1uM Luc-P4, 2 mM ATP, 10 mM $MgCl_2$, 50 mM Tris pH 8.5, 100uM $ZnCl_2$ and100ug/mL UltraGlo luciferase

13) Incubate the master mix for 1 hr. at RT.

14) Wash plates 3x with 1x TBST(0.1%).

15) Add 100 µL of Promega Master Mix to each well.

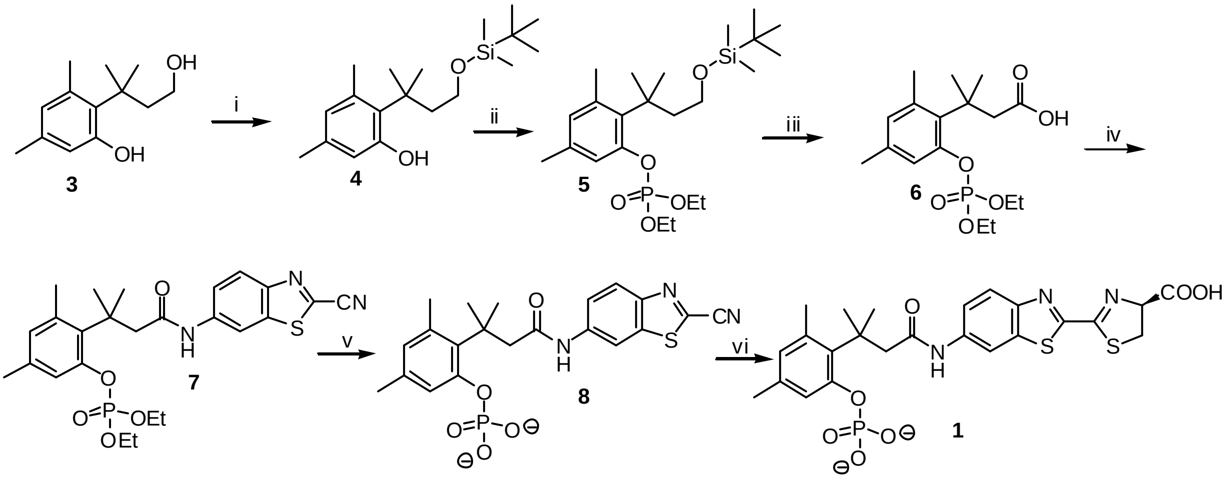
16) Incubate 30 min. at RT.

17) Read plate on luminometer.

The same protocol was employed for immunoassay on *S. japonicum* GST as an antigen and rabbit anti-GST AP conjugate as antibody with compound **2** as a bio-luminescent reporter for AP activities, while a series of GST in 1x TBST (0.1%) dilutions from 0.82 mg mL to 82 pg mL were used in step 1 and the solution of 1:400 for rabbit anti-GST AP conjugate in TBST (0.1%) was used in step 9.

3. Syntheses of Compounds 1-2

3.1 Synthesis of compound 1



i. TBDMS-Cl/TEA/CH_2Cl_2, 73%; ii. $ClPO(OEt)_2$/KO-*t*-Bu/THF/60^{o}C, 83%; iii. Jone's/KF/Acetone, 87%; iv. Isobutylchloroformate/N-methyl-morpholine/2-cyano-6-aminobenzothiazole, 60%;v. $(CH_3)_3SiI$/CH_2Cl_2, 75%; vi. D-cysteine/TEA

Synthesis of 2-(3-hydroxy-1,1-dimethyl-propyl)-3,5-dimethyl-phenol (3). The known compound **3** was prepared by lactonization of the commercially available 3,5-dimethylphenol and methyl 3,3-dimethylacrylate in methanesulfonic acid followed by reduction with lithium aluminum hydride. (M. G. Nicolaou, C.-S. Yuan, R. T. Borchardt, *J. Org. Chem.* **1996**, 61, 8636-8641)

Syntheis of 2-[3-(*tert*-butyl-dimethyl-silanyloxy)-1,1-dimethyl-propyl]-3,5-dimethyl-phenol (4). To the solution of **3** (22.26 g, 0.108 mol) and *tert*-butyldimethyl-silane chloride (17.89 g, 0.119 mol) in 250 mL of CH_2Cl_2 was added TEA (44.34 g, 0.438 mol) at 0 ° C. The resultant mixture was stirred overnight at room temperature. The solution was washed with water and dried over anhydrous sodium sulfate. After removal of the solvent, the product was purified by flash chromatography using heptanes/ethyl acetate (95/5 to 90/10) as eluent in a yield of 73%.

Synthesis of diethyl 2-[3-(*tert*-Butyl-dimethyl-silanyloxy)-1,1-dimethyl-propyl]-3,5-dimethyl-phenylphosphate (5). To the solution of **4** (10.0 g, 31.2 mmol) and potassium *tert*-butoxide (3.85 g, 34.3 mmol) in 30 mL of anhydrous THF was heated to 60 °C for 5 minutes and then diethyl chlorophosphate (5.86 g, 33.96 mmol) was added. The resultant mixture was heated at 60-70 °C for 2.5 hours. Upon cooling to room temperature, the insoluble solid was removed by filtration. The solvent of fil-trate was removed and the residue was purified by flash chromatography using hep-tane and ethyl acetate (90/10 to 80/20) as eluent in an average yield of 83%. ^{1}H NMR (CD_2Cl_2): 7.10 (s, 1H), 6.78 (s, 1H), 4.21 (q, 4H, OCH_2), 3.51 (t, 2H, CH_2OSi), 2.58 (s, 3H, CH_3), 2.25 (s 3H, CH_3), 2.13 (t, 2H, CH_2), 1.57 (s, 6H, CH_3), 1.37 (t, 6H, CH_3), 0.85 (s, 9H, CH3), 0.02 (s, 6H, $SiCH_3$). MS (ES): m/e (M+2), 460.

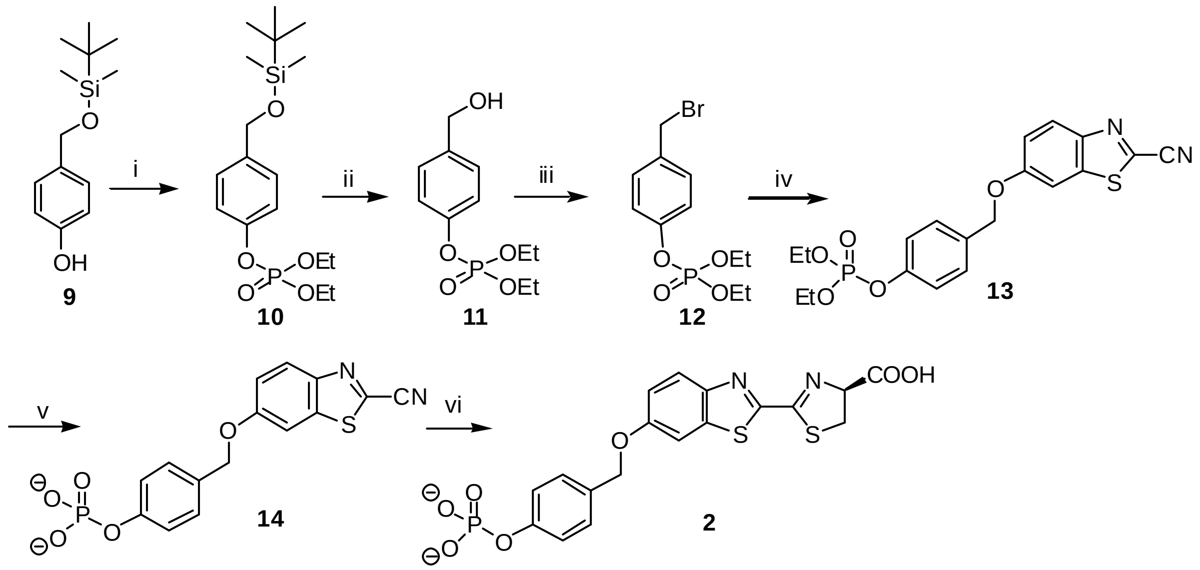
Synthesis of 3-[2-(Diethoxy-phosphoryloxy)-4,6-dimethyl-phenyl]-3-methyl-butyric acid (6). To the solution of **5** (3.0 g, 6.57 mmol) in 50 mL of acetone was added potassium fluoride (0.41 g, 7.0 mmol). The mixture was cooled at 0 °C and then Jone's reagent containing 2.7 g CrO_3, 2.3 mL H_2SO_4 in 10 mL water was added over 30 minutes till the solution became deep orange. The resultant mixture was allowed to warm up to room temperature and stirred for 3 hours. The insoluble solid was removed by filtration and washed several times with acetone. After removal of the solvent of the filtrate, the product was purified by flash chromatography using heptane/ethyl acetate (70/30 to 40/60) as eluent in a yield 87%. ^{1}H NMR (CD_2Cl_2): 7.02 (s, 1H), 6.79 (s, 1H), 4.25 (q, 4H, OCH_2), 2.90 (s, 2H, CH_2), 2.52 (s, 3H, CH_3), 2.22 (s 3H, CH_3), 1.63 (s, 6H, CH_3), 1.38 (t, 6H, CH_3). MS (ES): *m/e* [*M*+1], 359.

Synthesis of diethyl {2-[2-(2-cyano-benzothiazol-6-ylcarbamoyl)-1,1-dimethyl-ethyl]-3,5-dimethyl-phenyl} phosphate (7). To the solution of **6** (0.91 g, 2.36 mmol) in 20 mL of anhydrous THF was added isobutylchloroformate (0.36 g, 2.60 mmol) and N-methylmorpholine (0.26 g, 2.60 mmol) at 0 °C. The mixture was then stirred at room temperature for 30 minutes (the reaction should be checked by TLC to ensure acid is completely converted into anhydride). 6-Aminobenzothiazole (0.275 g, 1.57 mmol) and N-methylmorpholine (0.53 g, 5.20 mmol) were added and the mixture was stirred for 5 days at room temperature. After removal of the solvent, the compound was purified by flash chromatography using heptane/ethyl acetate (80:20 to 70:30) as eluent in a yield of 60%. ^{1}H NMR (CD_2Cl_2): 9.58 (s, 1H, NH), 8.55 (d, 1H), 7.98 (d, 1H), 7.38 (dd, 1H), 7.0 (s, 1H), 6.69 (s, 1H), 4.36 (q, 4H, OCH2), 2.80 (s, 2H, CH_2), 2.42 (s, 3H, CH_3), 2.20 (s, 3H, CH_3), 1.78 (s, 6H, CH_3), 1.48 (t, 6H, CH_3). MS (ES): *m/e* [*M*+1], 516.

Synthesis of {2-[2-(2-cyano-benzothiazol-6-ylcarbamoyl)-1,1-dimethyl-ethyl]-3,5-dimethyl-phenyl} phosphoric acid (8). To the solution of **7** (0.355 g, 0.66 mmol) in CH_2Cl_2 was added iodotrimethylsilane (0.31 g, 1.52 mmol) at 0 °C. The resultant mixture was stirred for 40 minutes and then added to the solution of *p*-toluidine (1.50 g, 14.0 mmol) in 20 mL. After removal of solvent, the product was purified by flash chromatography using methylene chloride/methanol (90/10-80/20) as eluent in a yield of 75%. ^{1}H NMR (d6-DMSO): 8.62 (d, 1H), 8.03 (d, 1H), 7.50 (dd, 1H), 7.11 (s, 1H), 6.98 (d, 4.5 H), 6.75 (d, 4.5 H), 6.48 (s, 1H), 2.86 (s, 2H, CH_2), 2.38 (s, 3H, CH_3), 2.19 (s, ~6.75H, CH_3), 2.07 (s 3H, CH_3), 1.65 (s, 6H, CH_3). (Containing 2.25 eqvalent molar toluididne as counter-ions) MS (ES): *m/e* [*M*+1], 459.

Synthesis of luciferin trimethyllock phosphate (1). To the solution of **8** (0.25 g, 0.34 mmol) and D-cysteine (0.0847 g, 0.48 mmol) in methanol/water (8:3) was added TEA (0.087 g, 0.86 mmol). The resultant mixture was stirred for 30 min. The product was purified by HPLC using 5 mM ammonium acetate buffer/ acetonitrile as solvent. ^{1}H NMR (D_2O): 7.80 (s, 1H), 7.77 (d, 1H), 7.21 (s, 1H), 7.06 (d, 1H), 6.53 (s, 1H), 5.12 (t, 1H, C**H**-COOH), 3.5-3.8 (m, 2H, CH_2), 2.85 (s, 2H, CH_2), 2.28 (s, 3H, CH_3), 2.07 (s 3H, CH_3), 1.60 (s, 6H, CH_3). MS (ES): *m/e* [*M*+1], 564. λ_{max} 322 nm, ε_{max} 16,700 $cm^{-1}M^{-1}$ in water. (Containing 0.2 equiv ammonium acetate and 0.8 equiv triethylammonium acetate).

3.2 Synthesis of compound 2



i. $ClPO(OEt)_2$/TEA/CH_2Cl_2/RT, 83%; ii. HCl/MeOH, 98%; iii. CBr_4/Ph_3P/CH_2Cl_2, 92%; iv. 6-hydroxylbenzothiazole/K_2CO_3/Acetone, 77%; v. $(CH_3)_3SiI$/CH_2Cl_2, 63%; vi. D-cysteine/TEA

Synthesis of 4-(*tert*-butyl-dimethyl-silanyloxymethyl)-phenol (9). Compound **9** was synthesized by employing the reported procedures. (Z.-Y**.** Jiang, Y.-G. Wang, Yan-Guang, *Tetrahedron Lett.* **2003**, 44, 3859-3861.)

Synthesis of diethyl 4-(TBDMS-oxymethyl)phenyl phosphate (10). To the solution of **9** (5.53 g, 23.2 mmol) and TEA (3.79 g, 37.4 mmol) was added diethyl phosphornyl chloride (4.31 g, 24.9 mmol). The resultant mixture was stirred overnight. The compound was purified with flash chromatography in a yield of 63%. ^{1}H NMR (CD_2Cl_2): 7.23 (d, 2H), 7.05 (d, 2H), 4.60 (s, 2H, OCH_2), 4.09 (q, 4H, $POCH_2$), 1.15 (t, 6H, CH_3), 0.92 (s, 9H, CH_3), 0.12 (t, 6H, $SiCH_3$). MS (ES): *m/e* [*M*+2], 376.

Synthesis of diethyl 4-(hydroxymethyl)phenyl phosphate (11). The solution of conc. HCl (8 mL) in ethanol (100 mL) was added to **10** (5.44g, 14.5 mmol). The resultant solution was stirred for 20 minutes and neutralized with sodium bicarbonate. The mixture was extracted three times with ether and the combined organic layer was dried over magnesium sulfate. After removal of solvent, the compound was obtained in a yield of 98%. Without further purification, it was directly used in next step. ^{1}H NMR (CD_2Cl_2): 7.38 (d, 2H), 7.10 (d, 2H), 4.65 (s, 2H, OCH_2), 4.09 (q, 4H, $POCH_2$), 1.18 (t, 6H, CH_3). MS (ES): *m/e* [*M*+1], 261

Synthesis of diethyl 4-(bromomethyl)phenyl phosphate (12). To the solution of carbon tetrabromide (5.73 g, 17.3 mmol) and **11** (3.74 g, 14.4 mmol) in 50 mL of methylene chloride was added triphenylphosphine (4.54 g, 17.3 mmol) at 0 °C. The resultant mixture was stirred for 2 h. The compound was purified by flash chromatography using heptane/ethyl acetate (80:20 to 70:30) as eluent in a yield 92%. ^{1}H NMR (CD_2Cl_2): 7.41 (d, 2H), 7.20 (d, 2H), 4.53 (s, 2H, OCH_2), 4.20 (q, 4H, $POCH_2$), 1.17 (t, 6H, CH_3). MS (ES): *m/e* [*M*+1], 323

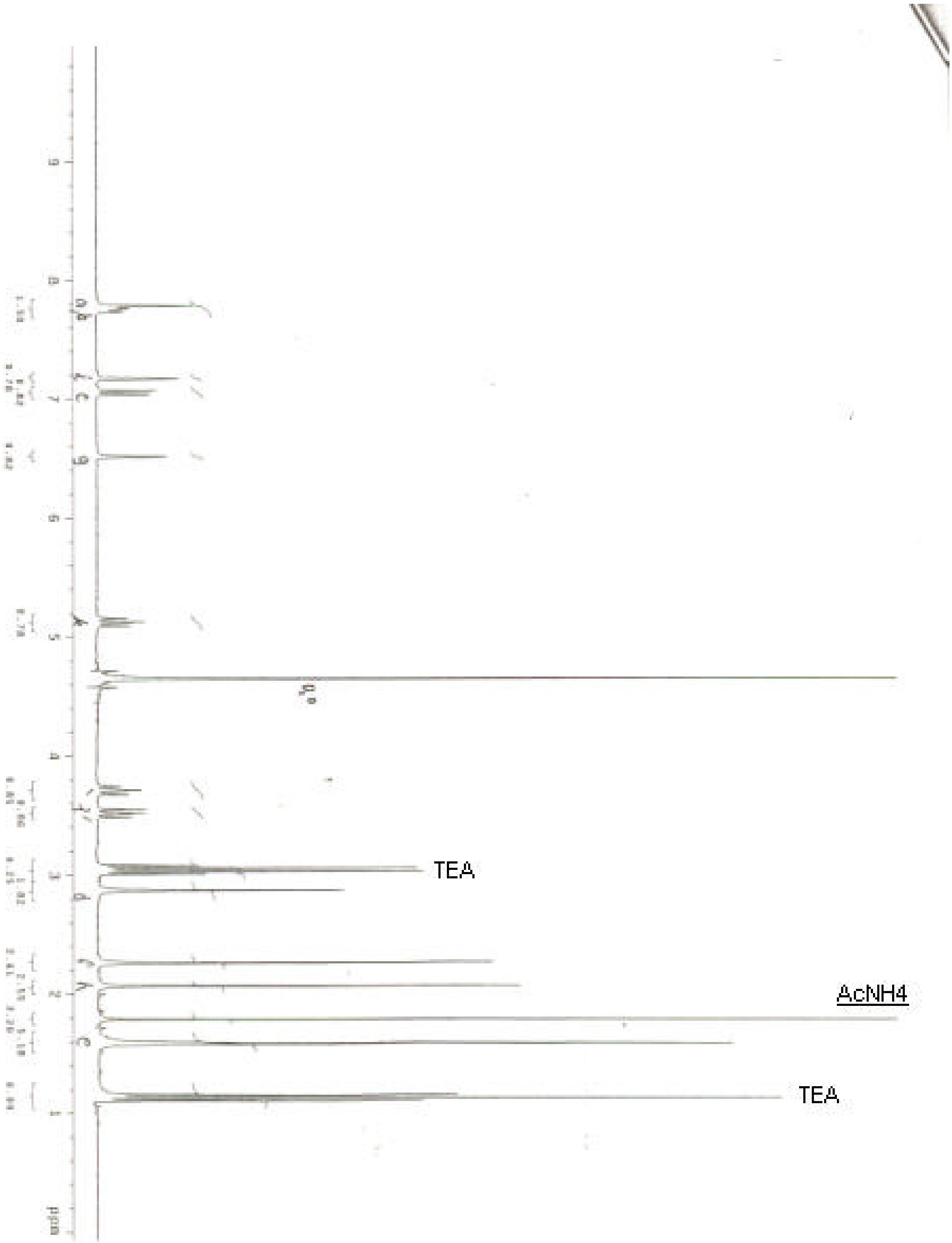
Synthesis of phosphoric acid 4-(2-cyano-benzothiazol-6-yloxymethyl)-phenyl diethyl ester (13). The mixture of **12** (0.39 g, 1.20 mmol), 2-cyano-6-hydroxybenzothiazole (0.21 g, 1.20 mol) and K_2CO_3 (0.166 g, 1.20 mmol) in 30 mL of acetone was refluxed overnight. The insoluble solid was removed by filtration and the product was purified by flash chromatography using heptanes/ethylacetate (70:30 to 60:40) as eluent in a yield of 77%. ^{1}H NMR (CD_2Cl_2): 8.12 (d, 1H), 7.49 (s, 1H), 7.46 (d, 2H), 7.33 (d, 1H), 7.26 (d, 2H), 5.18 (s, 2H, OCH_2), 4.20 (m, 4H, $POCH_2$), 1.34 (t, 6H, CH_3). MS (ES): *m/e* [*M*+1], 420

Synthesis of 4-(2-cyano-benzothiazol-6-yloxymethyl)-phenyl phosphoric acid (14). To the solution of **13** in 15 mL of methylene chloride was added iodotrimethylsilane (0.46 g, 2.31 mmol) at 0 °C. The resultant mixture was stirred for 40 min and then added to the solution of *p*-toluidine (1.50 g, 14.0 mmol) in 20 mL. After the solvent was partially removed, the light yellow precipitate was collected by filtration and washed with ethanol and ether to give a yield of 63%. ^{1}H NMR ([D_6]DMSO): 8.18 (d, 1H), 8.0 (s, 1H), 7.43 (d, 2H), 7.38 (d, 1H), 7.19 (d, 2H), 6.82 (d, 3.75H), 6.45 (d, 3.75H), 5.19 (s, 2H, OCH_2), 2.16 (s, ~6 H, CH3). (Containing ~3.3 molar equivalent toluidine). MS (ES): *m/e* [*M*+1], 361

Synthesis of luciferin p-hydroxymethylphenyl phosphate (2). To the solution of **14** (0.22 g, 0.40 mmol) and D-cysteine (0.074 g, 0.42 mmol) in methanol/water (8:3) was added TEA (0.081 g, 0.80 mmol). The resultant mixture was stirred for 30 min. The product was purified by HPLC using 5 mM ammonium acetate buffer/acetonitrile as solvent. ^{1}H NMR (D_2O): 7.82 (d, 1H), 7.56 (s, 1H), 7.39 (d, 2H), 7.15 (d, 1H), 7.10 (d, 2H), 5.11 (t, 1H, C$\underline{H}$-COOH), 5.06 (s, 2H, OCH_2), 3.45-3.80 (m, 2H, CH_2). MS (ES): *m/e* [*M*+1], 467. λ_{max} 329 nm, ε_{max} 19,100 $cm^{-1}M^{-1}$ in water. (Containing 0.6 equiv ammonium acetate and 0.4 eq triethylammonium acetate).

3.3 Copies of NMR and HPLC spectra for compounds 1-2.

Compound 1:



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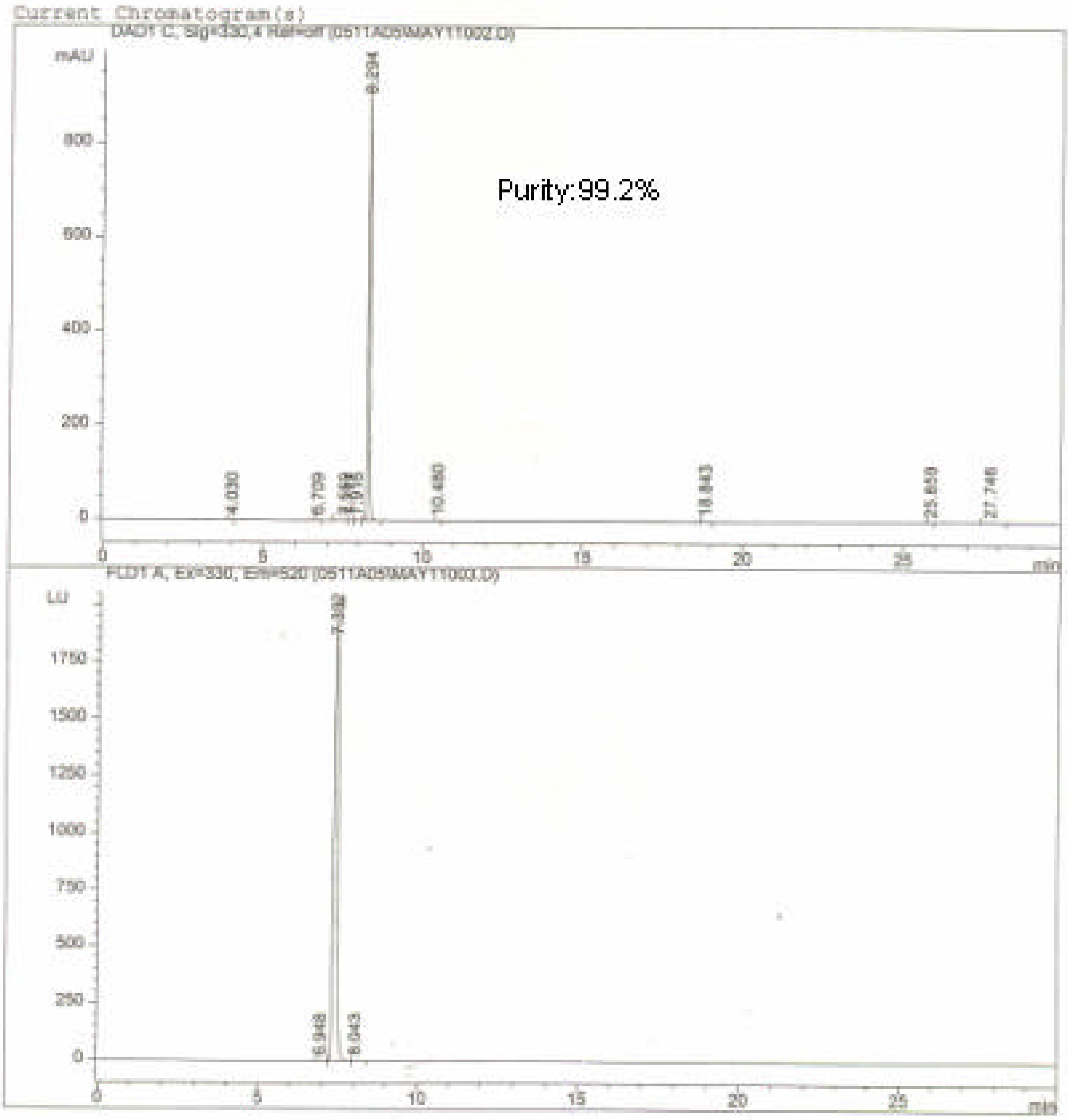
Acq. Operator : GR Inj : 1
Inj Volume : 10 µl



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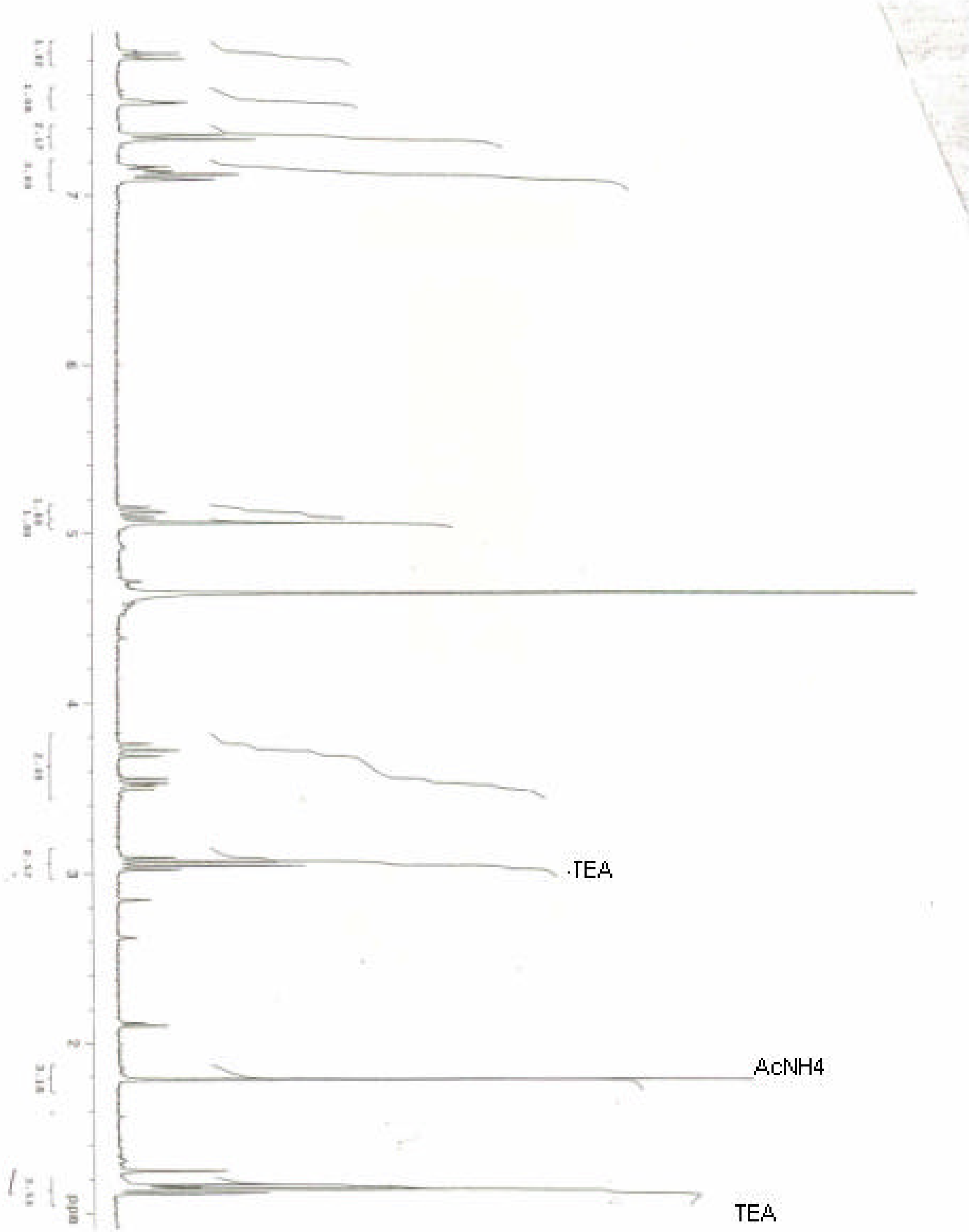

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Synergi 142334-2, 10mM Ammonium Acetate pH 7, ACN.
HPLC 70281. 0.3mg/mL Ammonium Acetate.

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