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An improved fluorogenic substrate for the detection of alkaline phosphatase activity

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ABSTRACT

We designed a new alkaline phosphatase (ALP)-sensitive fluorogenic probe in which a self-immolative spacer group, *p*-hydroxybenzyl alcohol, is linked to a profluorogenic compound to improve substrate specificity. Enzymatic hydrolysis converts the fluorogenic substrate **1** to a highly fluorescent reporter **3**, thus allowing for the fast and quantitative analysis of ALP activity with greatly increased affinity for the enzyme.

Alkaline phosphatases (ALPs), which catalyze the hydrolysis of phosphate esters, are widely distributed in mammalian tissues, and are present in high concentration in the bones, intestines, kidneys, placenta, and liver.[1,2] ALP is not only the most commonly used conjugated enzyme for enzyme immunoassays in molecular biology,[3,4] but is also routinely used as a diagnostic marker for several human diseases, including hepatobiliary and bone disorders.[5–7] Accordingly, the development of convenient and sensitive molecular probes capable of reporting ALP expression and its dynamic activity in various biological systems will be extremely valuable for studying detailed mechanisms of ALP activity regulation during pathogenesis, and for the development of efficient diagnostic and therapeutic agents. Several optical assays in combination with chemiluminescent,[8] chromogenic,[9] or fluorescent probes[10] have been employed for the detection of ALP activity. Among them, fluorescence-based detection methods are generally superior in terms of sensitivity, spatial and temporal resolution, and ease of use. In that context, nonpeptidic ALP fluorogenic substrates such as 4-methyl-7-hydroxycoumarinyl phosphate (MUP), 3,6-fluorescein diphosphate (FDP), and 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) are in widespread use.[11]

Recently, we reported a sensitive ALP probe (**4**) in which P–O bond cleavage is triggered by ALP-catalyzed hydrolysis, and a subsequent intramolecular nucleophilic reaction results in the highly emissive cyclized product, the benzothiazolyl iminocoumarin (**3**).[12] As a continuation of our research program aimed at the development of efficient ALP fluorogenic probes that have higher enzyme affinity, as well as the modulation of fluorogenic substrates for enzyme specificity, we newly designed probe **1** that incorporates a reactive *p*-hydroxybenzyl linker, as a spacer unit between the phosphate moiety and profluorescent compound. The presence of a spacer between the recognition unit and the bulky profluorescent compound has the benefit of lower steric hindrance that increases its accessibility to the enzyme's active site, consequently improving the substrate's affinity for the enzyme (i.e., lower the Michaelis constant, K_M) while maintaining a dye platform as signal transducing element. Herein, we report the synthesis and photophysical characterization of **1**. The hydrolysis of **1** by ALP was monitored in fluorescence-based assays to assess its utility as an indicator of ALP activity. In addition, the kinetic parameters of the enzymatic hydrolysis of **1** were determined, and its potential for the screening of ALP inhibitors was investigated.

The fluorescence of probe **1** is effectively quenched through internal free rotation about the vinylene linker. ALP-catalyzed P–O cleavage in probe **1** causes a chemical breakdown of the linker via 1,6-elimination of the *p*-hydroxybenzylether derivative, followed by a spontaneous intramolecular cyclization, leading to the release of benzothiazolyl iminocoumarin **3**, which is water-insoluble and highly emissive upon photoexcitation (Scheme 1).

Probe **1** was easily prepared in a five-step synthesis as shown in Scheme 2, and the structural identification of **1** was confirmed by ^{1}H NMR, ^{13}C NMR, and HR-MS. It is also noted that probe **1** is highly soluble in water. Dye **3,** the expected product resulting from the enzymatic reaction was also independently synthesized according to a previously reported method.[12]

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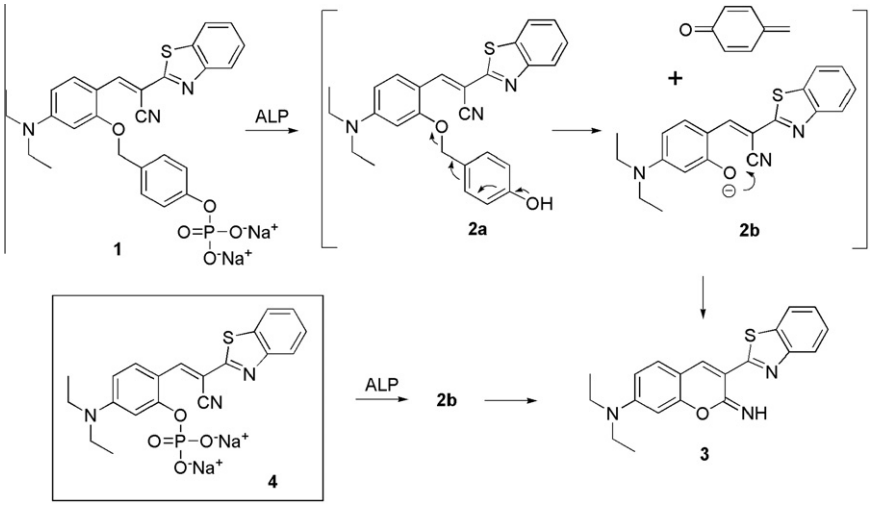
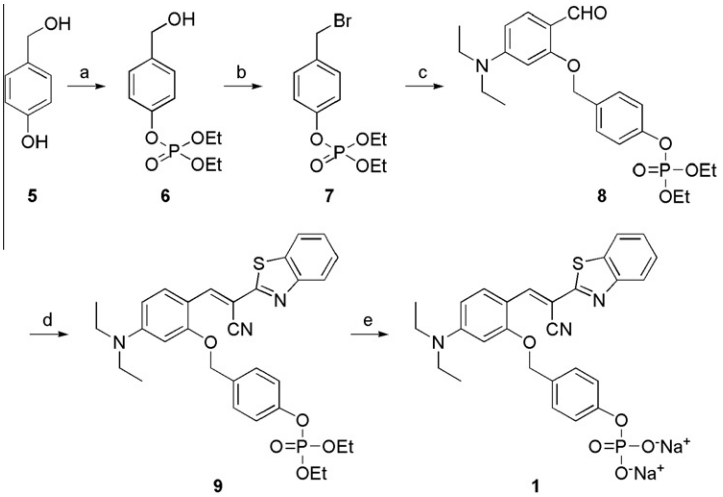


Scheme 1. Chemical structures of fluorogenic substrates **1** and **4**, and the proposed sensing mechanism.



Scheme 2. Synthesis of probe **1**: Reagents and conditions: (a) $(EtO)_2POCl$, NaH, THF, room temperature, 24 h, 81%; (b) CBr_4, PPh_3, CH_2Cl_2, room temperature, 1 h, 88%; (c) 4-(diethylamino)salicylaldehyde, K_2CO_3, DMF, 60 °C, overnight 80%; (d) 2-benzothiazoleacetonitrile, iperidine, EtOH, room temperature, 6 h, 84%; (e) (i) TMSBr, CH_2Cl_2, 24 h, rt (ii) aq NaOH, 27%.

The photophysical properties of probe **1** and dye **3** were initially investigated to confirm their suitability as substrate for fluorogenic reaction in aqueous buffer solution (10 mM Tris–HCl, pH 8). Probe **1** exhibited a maximum absorbance band centered at 470 nm ($\varepsilon = 3.3 \times 10^4\ M^{-1}\ cm^{-1}$) and a fluorescence maximum at λ_{max} = 530 nm with weak green emission ($\Phi_{fl} = 0.0035$). The absorption and fluorescence emission maxima of **3** were displayed at 470 nm ($\varepsilon = 2.8 \times 10^4\ M^{-1}\ cm^{-1}$) and 530 nm, respectively, with a fluorescence quantum yield (Φ_{fl}) of 0.10, which was about 30 times greater than that of fluorogenic substrate **1**.

The hydrolysis of probe **1** by ALP was monitored by fluorimetry (Fig. 1). In these experiments, probe **1** (5 μM) was incubated with ALP (30 nM) in Tris–HCl buffer (10 mM, pH 8) at 37 °C, and the emission spectra of the resulting solution were measured at 4 min intervals over 40 min with excitation at λ_{ex} = 440 nm. The fluorescence emission intensity at 530 nm gradually increased with the incubation time from 0 to 40 min and reached a plateau after 30 min (Fig. 1A). An approximately 30-fold fluorescence enhancement was obtained upon incubation with ALP (30 nM) for 30 min. In contrast, it was observed that the fluorescence intensity of probe **1** without addition of ALP displayed no noticeable fluorescence change under the same conditions, implying insignificant nonenzymatic hydrolysis of probe **1** (Fig. 1B). The nonemissive nature of probe **1** and the significant fluorescence turn-on of ALP-treated probe **1** are clearly visualized (Fig. 1A, inset).

In order to confirm the origin of the fluorescence increase during the enzymatic reaction, we monitored the assay solution by HPLC–MS. Incubation of probe **1** (10 μM) with ALP (100 nM) gave rise to complete **1** → **3** conversion in 30 min. The formation of intermediates (**2a** and/or **2b**) was not detected in our HPLC–MS experiments throughout the duration of the **1** → **3** conversion process (Fig. 2). This reveals that the fluorescence increase is attributed to the formation of expected product **3** through the sequence of rapid self-immolative reaction after catalytic cleavage of the P–O bond upon treatment of probe **1** with ALP, with a concomitant increase in fluorescence.

Encouraged by these results, we further investigated the ALP-catalyzed hydrolysis of probe **1** as a function of incubation time at different ALP concentrations. Figure 3A shows the increase in fluorescence intensity at 530 nm for solutions of probe **1** (5 μM) incubated with ALP concentrations ranging from 0 to 30 nM, in which fluorescence intensity was measured every 2 min. As

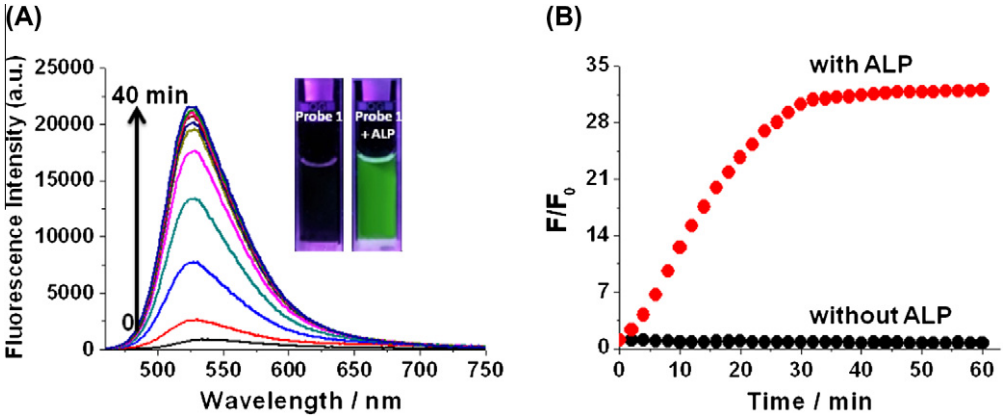


Figure 1. (A) Time-dependent emission spectra (λ_{ex} = 440 nm) of probe **1** (5 μM) upon treatment with ALP (30 nM) in Tris–HCl buffer (10 mM, pH 8.0) at 37 °C. The spectra were obtained every 4 min (0–40 min). Inset: Photographs of probe **1** (10 μM) in the absence (left) and presence (right) of ALP (30 nM) after incubation for 40 min at 37 °C under UV light (365 nm) illumination. (B) Relative fluorescence intensity (F/F_0) at 530 nm with and without ALP (30 nM) as a function of incubation time.

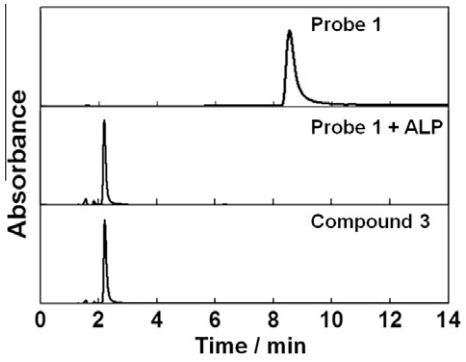


Figure 2. HPLC chromatograms of probe **1** without ALP treatment (top); with ALP treatment for 30 min at 37 °C (middle); and **3** only (bottom). The samples were analyzed by HPLC–MS (eluent A/B = 50:50, (A) deionized water with 1% acetic acid, (B) acetonitrile, flow rate 0.3 mL/min, UV: 340 nm). The MW at the retention time of 8.5 min is 536.1, which corresponds to $[M+H]^+$ for probe **1** and the MW at the retention time of 2.2 min is 350.1, which corresponds to $[M+H]^+$ for **3**. [**1**] = [**3**] = 10 μM, [ALP] = 100 nM.

expected, the fluorescence intensity increased with increasing ALP concentration within the measured range (Fig. 3B). Subsequent data analysis revealed a good linear relationship (R^2 = 0.98) between the normalized fluorescence signal at 530 nm and the ALP concentrations from 0 to 5 nM (Fig. 3B, inset), where the emission intensity was obtained with the ALP incubation time of 30 min. These observations indicate that **1** can be utilized as a probe to assay ALP concentration quantitatively, thus achieving real-time monitoring of enzyme activity. The detection limit for ALP using probe **1** under optimized conditions was determined to be 1×10^{-10} M on the basis of the signal-to-noise ratio (S/N = 3), which is impressively sensitive compared with existing fluorescent probes for ALP detection.[11]

To directly compare enzyme specificity and kinetics between probe **1** and the previously described **4**, kinetic parameters for the enzymatic hydrolysis reaction of **1** and **4** were determined under the same assay conditions (see the Supplementary data). By using Lineweaver–Burk analysis, we obtained the kinetic value of K_M = 0.90 μM for probe **1**, which is smaller compared to probe **4** (K_M = 20.3 μM), indicating that ALP has a higher affinity for probe **1**. Thus, even the lower turnover number of **1** (k_{cat} = 0.06 s^{-1}) translated to the higher k_{cat}/K_M value of 6.2×10^4 M^{-1} s^{-1} relative to the k_{cat}/K_M of 4.5×10^4 M^{-1} s^{-1} for **4**. These results confirmed that the incorporation of a spacer linker not only maintained, but actually enhanced efficiency and specificity of fluorogenic substrate **1** by the targeted enzyme ALP compared to that of **4**, which has a direct linkage between the profluorescent compound and phosphate moiety. Better affinity of ALP for probe **1** is advantageous for in vivo imaging as it allows the use of lower overall doses of probe, thus preventing extended circulation that can possibly cause cross-reactivity with other biomolecules.

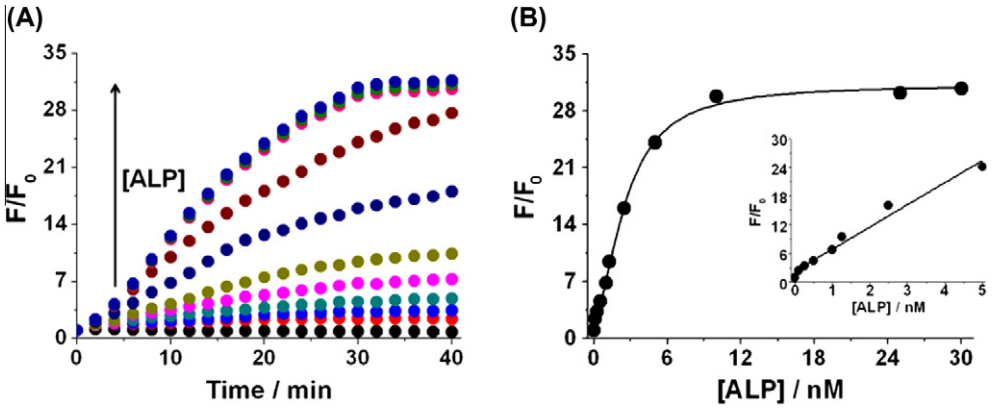


Figure 3. (A) Increase in fluorescence intensity at 530 nm recorded every 2 min (0–40 min) during real-time turn-on assay of probe **1** (5 μM) under varying ALP concentrations (0–30 nM) in Tris–HCl buffer (10 mM, pH 8.0) at 37 °C. λ_{ex} = 440 nm. (B) Plot of relative fluorescence intensity (F/F_0) at 530 nm versus ALP concentration (0–30 nM). Inset shows the linear relationship between fluorescence intensity and ALP concentration (0–5 nM). Incubation time = 30 min. F_0 and F correspond to the fluorescence intensity of probe **1** in the absence and the presence of ALP, respectively. Data points are averages of three independent measurements.

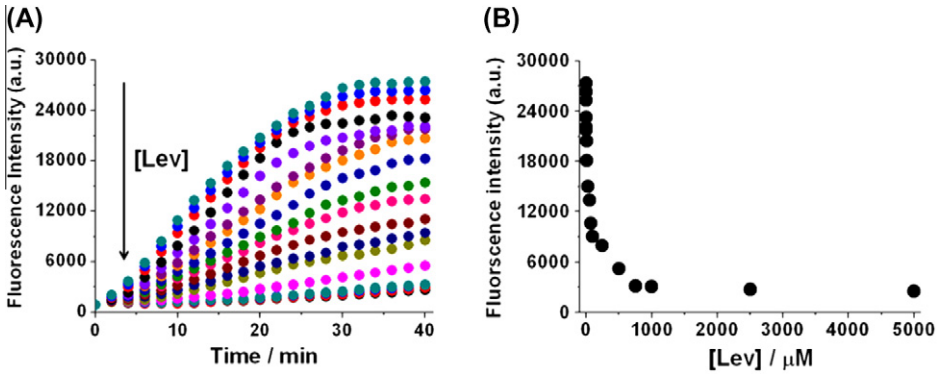


Figure 4. (A) Inhibition assay of ALP activity by levamisole showing emission intensity of probe **1** as a function of inhibitor concentration (0–5 mM). ALP (30 nM) was incubated with the inhibitor for 120 min at 25 °C before addition of probe **1** (5 μM). After 30 min of incubation at 37 °C, emission intensity at 530 nm was recorded (λ_{ex} 440 nm). (B) Relative activity of ALP versus concentration of levamisole at 38 min time point. IC_{50} value was determined as 71 μM for levamisole.

Next, the potential utility of probe **1** for the screening of ALP inhibitor was investigated. The inhibition of ALP activity was tested with levamisole—a known ALP inhibitor—in Tris–HCl buffer (10 mM, pH 8).[13] ALP was preincubated with different concentrations of inhibitor for 120 min at 25 °C. The solution of ALP pretreated with the inhibitor was then added to probe **1** in aqueous solution and the enzyme reaction was monitored by measuring the fluorescence intensity at 530 nm. As shown in Figure 4, the enhancement of fluorescence intensity of probe **1** was inhibited in a dose-dependent manner. In particular, addition of 5000 μM of levamisole entirely blocked the fluorescence increase, providing confirmation that the enzymatic activity of ALP was required for hydrolysis of probe **1**. The IC_{50} was calculated to be 71 μM for levamisole, which is in good agreement with the reported literature value determined by other methods.[12] These results emphasize that probe **1** can be a useful tool for ALP activity assays, as well as the screening of inhibitors.

In summary, we have designed and synthesized an efficient fluorogenic probe **1**, suitable for real-time dynamic monitoring of ALP activity. In this probe, a self-immolative spacer was employed to improve enzyme's affinity for the substrate. Fluorogenic assays proved that probe **1** exhibited high enzyme affinity with the ability to detect ALP at concentrations as low as 0.1 nM. In addition, the potential utility of **1** in the screening of ALP inhibitors was demonstrated with levamisole. This work demonstrates the utility of self-immolative linkers in the design of fluorogenic substrates for enzymes possessing a comparatively difficult to access active catalytic site and opens the possibility for the improvement of existing fluorogenic substrates for various enzymes.

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Supplementary data

Supplementary data (detailed experimental procedures, additional UV-visible and emission spectra, enzyme assay studies) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.063.

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