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Self-Cleavable Bioluminogenic Luciferin Phosphates as Alkaline Phosphatase Reporters

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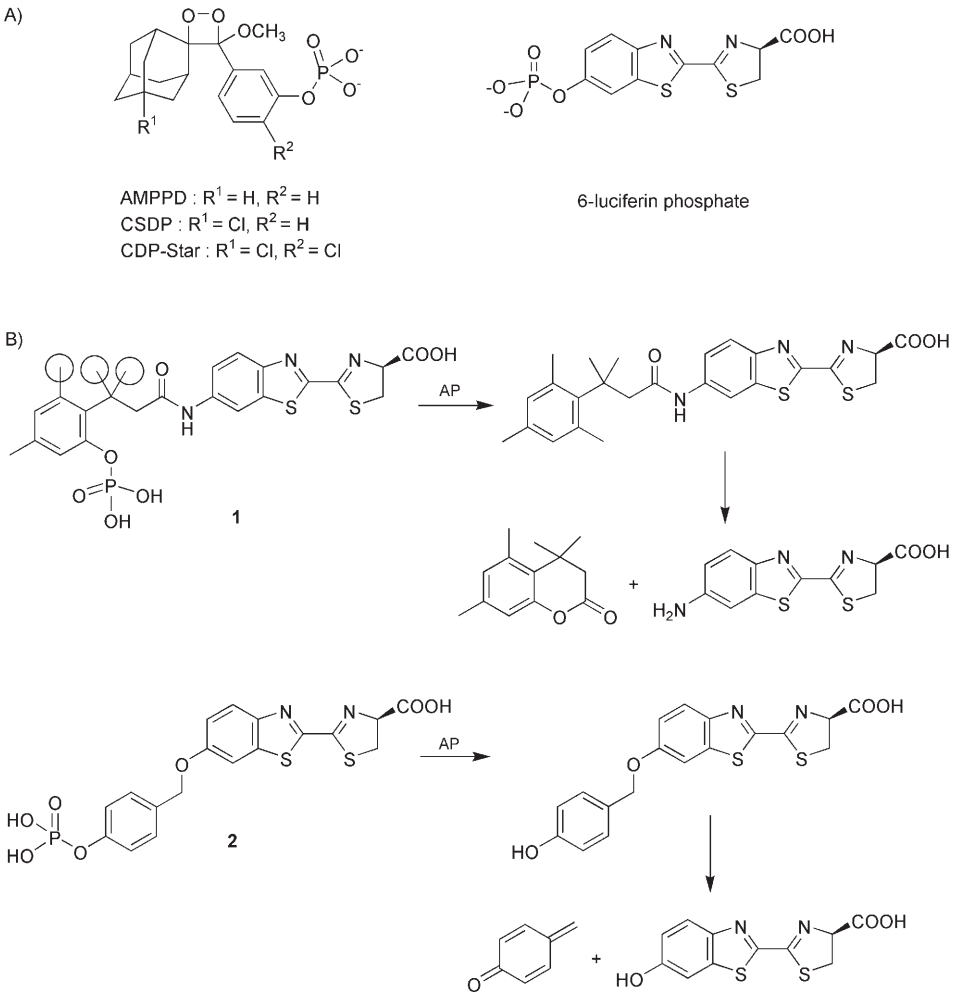
Alkaline phosphatase (AP)—a stable enzyme with high specific activity for the hydrolysis of phosphate esters—is widely used as a conjugated enzyme label in enzyme-linked immunosorbent assays (ELISA)[1] and DNA hybridization assays.[2] It is also used as an in situ probe to monitor the expression and translocation of fusion proteins from the cytoplasm[3] and for visualization of the spatial distribution of target biomolecules, such as cognate ligands or receptors in cells, tissues, and embryos.[4] Among the many methods for detecting AP activity, there are various phosphate substrates, such as the colorimetric *p*-nitrophenyl phosphate,[5] the fluorescent AttoPhos®,[6] and the chemiluminescent adamantyl 1,2-dioxetane AMPPD derivatives[7] (Scheme 1). It is the ultrasensitivity of chemiluminescence, specifically with 1,2-dioxetane AMPPD derivatives, that has made this the overwhelming choice for monitoring AP activity.



Scheme 1. Chemical structures of substrates for AP enzyme. A) Known chemiluminescent substrate AMPPD derivatives and bioluminescent substrate 6-luciferin phosphate; B) proposed self-cleavable luciferin phosphates, aminoluciferin trimethyl lock phosphate **1**, and luciferin *p*-hydroxymethylphenyl phosphate **2**.

Although a luciferase-coupled bioluminescent assay is not only generically similar to the chemiluminescent assay and could show similar sensitivity, it also has the additional potential of creating recombinant luciferase to AP protein fusions, which might be preferable for the detection of AP activity in situ. The development of a suitable substrate to reach this ultrasensitivity is needed in order to promote the bioluminescent AP assay for practical applications. Chemical modification of the 6-hydroxyl group of luciferin (or the 6-amino group of aminoluciferin) is an effective means to approach bioluminescent assays for enzymes of interest,[8] and 6-luciferin phosphate (Scheme 1) has been previously shown to detect AP activity.[9] However, the detection limit of 10^{-19} mol of AP was 2–3 orders of magnitude lower than that for the AMPPD assay.[7] Since the hydrolysis of phosphate monoesters is highly dependent on the pK_a of the leaving group[10] and the lower pK_a 8.5[11] of the luciferin phenol compared to a pK_a ~9.0[7] of the adamantyl dioxetane phenol favors both nucleophilic attack and P–O bond fission

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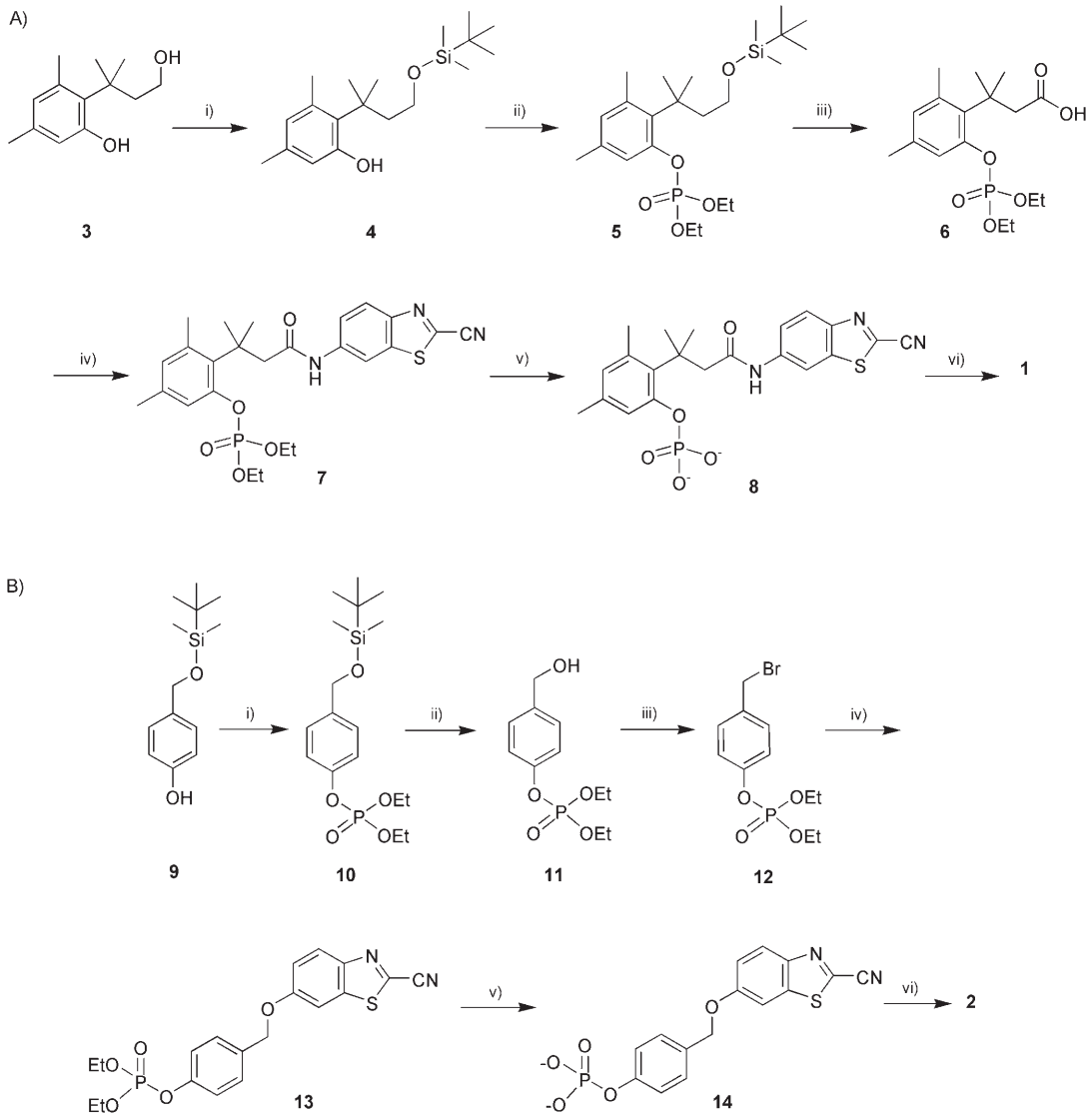
for phosphate hydrolysis, we reasoned that the high background from nonenzymatic hydrolysis of 6-luciferin phosphate might be responsible for the low sensitivity in this particular case. We, therefore, hypothesized that the use of a self-cleavable chemical adaptor that is bound to luciferin at one end and phosphate at the other, could allow us to optimize the pK_a of the hydroxyl group at the phosphate-bonding site. This could lead to enhanced stability, but still allow the release of free luciferin upon AP activity.

Reactive molecules capable of spontaneous intramolecular cyclization or 1,4- and 1,6-elimination have been utilized in the field of prodrugs for enzyme-triggered drug delivery, and in solid-phase synthesis for the release of synthetic molecules from solid supports.[12] Specifically, substituted phenol propionic acid derivatives, referred to as "trimethyl lock",[13] upon unmasking of the phenol group either undergo a facile spontaneous intramolecular lactonization due to the steric interaction of three methyl groups to form hydroxylcoumarin and release the moieties attached to the carboxyl functional group (Scheme 1 B, **1**); or spontaneous 1,6-elimination of *p*-hydroxy benzylether derivatives[12,14] triggers the liberation of the moieties at the ether end by the formation of a quinone methide (Scheme 1 B, **2**). Given that the adaptors of trimethyl lock and *p*-hydroxy benzylether are alkylsubstituted phenoles, we estimated that the pK_a values of the hydroxyl group in adaptors were close to the pK_a 9.95[15] of phenol, and consequently, we predicted that their corresponding phosphates would be more stable than 6-luciferin phosphate. Therefore, we designed aminoluciferin trimethyl lock phosphate (**1**) and luciferin *p*-hydroxymethyl phenyl phosphate (**2**; Scheme 1).

The synthesis of trimethyl lock phosphate **1** was accomplished as shown in Scheme 2 A. The known 2-(3-hydroxy-1,1-



Scheme 2. A) Synthesis of luciferin trimethyl lock phosphate **1**. i) TBDMS-Cl/TEA/CH_2Cl_2, 73 %; ii) $ClPO(OEt)_2$/KO-*t*Bu/THF, 60 °C, 83 %; iii) Jones'/KF/acetone, 87 %; iv) isobutylchloroformate/*N*-methyl-morpholine/2-cyano-6-aminobenzothiazole, 60 %; v) $(CH3)_3SiI/CH_2Cl_2$, 75 %; vi) D-cysteine/TEA. B) Synthesis of luciferin *p*-hydroxylmethyl phenol phosphate **2**. i) $ClPO(OEt)_2$/TEA/CH_2Cl_2, room temperature, 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.

dimethyl-propyl)-3,5-dimethyl-phenol (**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 aluminium hydride.[13i] Selective protection of the alkylhydroxyl group of **3** with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) in methylene chloride under basic conditions gave **4** in 73% yield. Compound **4** was then phosphorylated with diethyl chlorophosphate under strong basic potassium *tert*-butoxide conditions to produce **5** in a yield of 83%. We found no phosphorylation of **4** under the more common base conditions, such as with TEA. Compound **5** was converted into acid **6** by in situ deprotection with KF followed by oxidation with Jones' reagent in acetone; the oxidation was sensitive to temperature. The aldehyde intermediate, instead of acid **6**, was often found as a dominant product at low temperature (0 °C), whereas at room temperature we could drive the formation of **6** to completion with an average yield of 87%. Compound **6** was activated with isobutylchloroformate, and then coupled to 2-cyano-6-aminobenzothiazole to give **7**. The coupling reaction had to be conducted for 4–5 days at room temperature in the presence of excess base in order to gain moderate yields (40–60%). We ascribe this slow reactivity to the lack of nucleophility of the amine group of the electron deficient benzothiale ring. Deethylation of **7** with trimethyliodosilane, followed by treatment with toluidine gave **8** in a yield of 75%. The cyclization of **8** with D-cysteine under basic conditions[8b,c] gave the desired luciferin trimethyl lock, **1**.

A similar method was employed for the synthesis of luciferin *p*-hydroxymethyl phenylphosphate **2** (Scheme 2B). Selective protection of the alkyl hydroxyl group of *p*-hydroxymethyl phenol followed by phosphorylation of the phenol group with diethyl chlorophosphate yielded **10**. Upon removal of the protecting group alcohol **11** was produced and then converted into bromide **12** with CBr_4/Ph_3P in methylene chloride. The subsequent alkylation of 2-cyano-6-hydroxybenzothiazole with **12** under basic conditions,[8b,c] followed by deethylation with trimethyliodosilane and ring cyclization with D-cysteine yielded the final compound **2**.

The reactivity of **1** and **2** as substrates was examined in a two-step assay format. In the alkaline phosphatase reaction (step 1), calf intestinal AP (Promega) was incubated with the substrate for 30 min at room temperature in a reaction (100 μL) containing Tris-HCl (50 mM, pH 9.3), $MgCl_2$ (10 mM), $ZnCl_2$ (0.10 M), and spermidine (1 mM). In the luciferin-detection reaction (step 2), luciferin detection reagent (100 μL), which comprised of luciferase, ATP, and buffer, was added to the AP reaction, and the luminescent signal was measured by using a Veritas 96 microplate luminometer. The resulting luminescence with **1** and **2** in the presence of AP enzyme above control background signal suggested that the compounds were substrates for AP and produced aminoluciferin or luciferin either by spontaneous intramolecular cyclization or 1,6-elimination upon dephosphorylation. Only the starting compounds and free luciferin or aminoluciferin were observed by HPLC, and no intermediates were detected; this confirms that the luminescent signal indeed reflected the oxidation of luciferin by luciferase. The background controls for **1** and **2** yielded low net signals without noticeable change upon increasing incubation time in the absence of AP; this implies insignificant non-enzymatic hydrolysis for either compound. The apparent K_m values for **1** and **2** were measured by varying the substrate concentrations while AP enzyme concentration was maintained constant (Figure 1). Compounds **1** and **2** exhibited K_m values of (224 ± 10) and (0.34 ± 0.04) μM for AP, respectively. This indicates that relative to the K_m of 43 μM reported for 6-*O*-luciferin phosphate, **1** is a poor substrate for AP, whereas **2** appeared to be a good substrate.

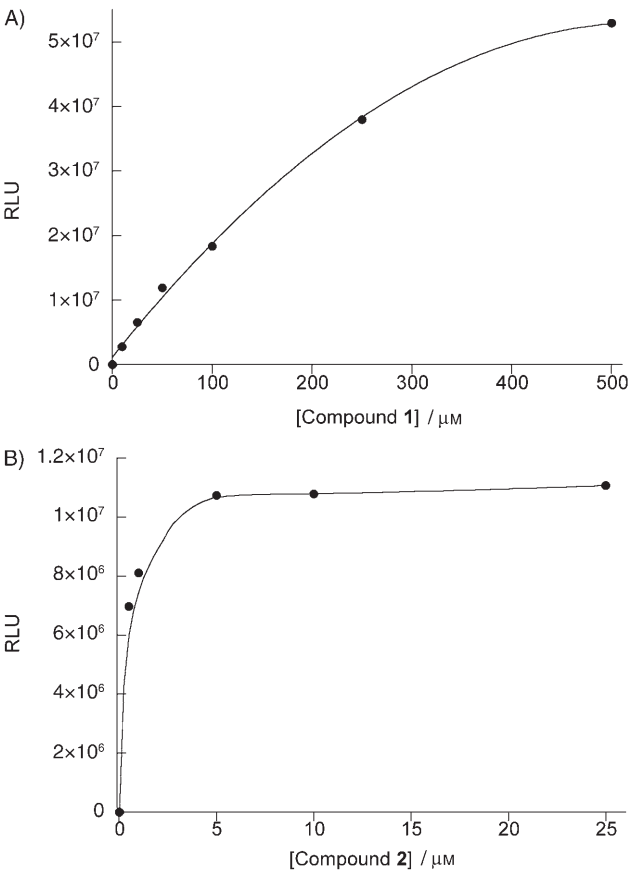


Figure 1. Michaelis–Menten kinetics of **1** and **2** with AP enzyme. The AP reaction was conducted in a volume of 100 μL, and contained AP (14 pg), $MgCl_2$ (10 mM), $ZnCl_2$ (0.10 M), spermidine (1 mM), and Tris-HCl (50 mM, pH 9.3). After 30 min incubation, luciferin detection reagent (100 μL) was added and the luminescent signal was measured. A) **1**; B) **2**.

We also performed the assay in a one-step format, similar to that described above but with incubation of AP in the presence of luciferin-detection reagent. We found insignificant differences between the one-step and two-step assay formats; this suggested that the cleavage of the adaptors was not a rate-limiting step, and thus we employed the one-step method for evaluating the sensitivity of our assay. The sensitivity of **1** and **2** for AP was assessed by measuring the limit of detection, which was defined as the amount of enzyme necessary to give a net luminescent signal equal to three times the standard deviation of the background control. Compounds **1** and **2** exhib-

ited a 100000-fold linear dynamic range with limits of detection of $\sim 10^{-20}$ and $\sim 10^{-22}$ mol of AP enzyme, respectively. The lower sensitivity of **1** was most likely due to its approximately three-times higher background than that of **2**.[16] However, both compounds showed poor linearity at low concentration of AP (< 10000 molecules; Figure 2). The same trend was observed for the chemiluminescent assay with the commercially available AMPPD derivative (CDP-Star, Tropix). We postulate that at low concentration alkaline phosphatase might dissociate into monomers that show higher activity than the dimeric species.[17]

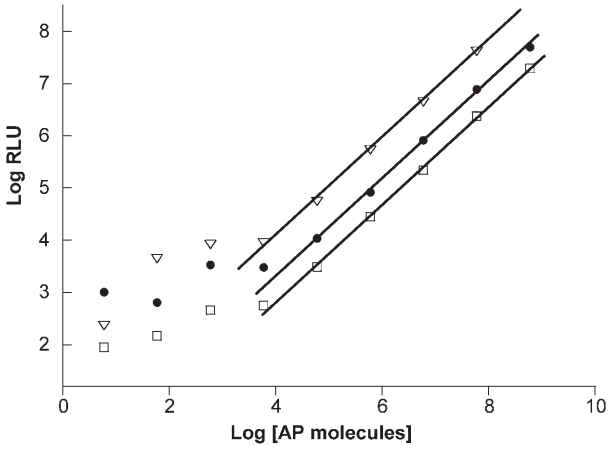


Figure 2. Comparison of the sensitivity of bioluminescent assays with **1**, **2**, and CDP-Star for AP activity with variable amounts of enzyme. Compound **1** (●), **2** (□), and CDP-Star (△). A series of AP enzyme dilutions (10 μL) from 1.4 fg mL^{-1} to 140 ng mL^{-1} in Tris-HCl (50 mM, pH 9.3), $MgCl_2$ (10 mM), $ZnCl_2$ (0.1 mM), and spermidine (1 mM) were placed into wells of a microtiter plate. Luciferin detection reagent (100 μL) was added to each well, which contained AP substrate **1** (400 μM) or **2** (1 μM), ATP (2 mM), $MgCl_2$ (10 mM), Tris-HCl (50 mM, pH 8.5), $ZnCl_2$ (0.1 mM), and UltraGlo luciferase (100 μg mL^{-1}); after 30 min incubation at room temperature the luminescent signal was measured by using a Veritas microplate luminometer.

The immunobioluminescence assay with **2** as a reporter for AP activity was further evaluated by employing rabbit anti-caspase-3 polyclonal antibody (Promega) and purified *Schistomsoma japonicum* glutathione-*S*-transferase (GST; Sigma) as antigens, and goat anti-rabbit IgG–AP conjugate (Promega) and rabbit anti-GST–AP conjugate (Sigma) as enzyme labels, respectively. As a comparison, standard immunoassay protocols were performed.[18] Figure 3 shows that the bioluminescent immunoassay exhibited a 10000-fold linear dynamic range from 1.9 ng to 1.9 μg for anti-caspase-3 polyclonal antibody and 80 ng to 80 μg for *S. japonicum* GST, with limits of detection of 190 and 650 pg, respectively.

In summary, this report describes the use of a reactive chemical adaptor to stabilize bioluminogenic phosphates for reporting alkaline phosphatase activity. Trimethyl lock and 1,6-elimination based latent luciferin phosphates were successfully prepared. The 1,6-elimination based luciferin phosphate exhibited the ability to detect $\sim 10^{-22}$ mol of AP enzyme in a homogeneous solution, and picograms of protein in an immunoassay;

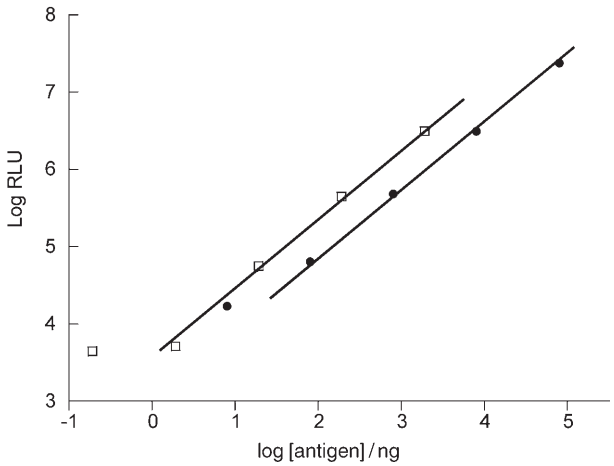


Figure 3. Immunoassay with **2** as a bioluminescent reporter for AP activity; □: rabbit anti-caspase-3 polyclonal antibody as antigen, and goat anti-rabbit IgG–AP conjugate as antibody; ●: *S. japonicum* GST as antigen, and rabbit anti-GST–AP conjugate as antibody. A series of antigen dilutions (100 μL) were incubated in wells of an Immulon4 microtiter plate for 1 h at room temperature, washed three times with TBST (0.1%), and then treated with blocking reagent (200 μL) containing bovine serum albumin (BSA; 3.0%) and TBST (0.1%); the plates were incubated for 1 h at room temperature, and washed three times with TBST (0.1%). AP-conjugate solutions were diluted (1:7500 for goat anti-rabbit IgG–AP conjugate and 1:400 for rabbit anti-GST–AP conjugate) in TBST (0.1%), and 100 μL was added to each well; the plates were incubated for 1 h, and washed three times with TBST (0.1%). The detection reagent (100 μL) containing **2** (1 μM), ATP (2 mM), $MgCl_2$ (10 mM), $ZnCl_2$ (100 mM), Tris-HCl (50 mM), and UltraGlo luciferase (100 μg mL^{-1}), pH 8.5, was added, and plates were incubated for 30 min before the light output was measured.

this indicates that the bioluminescent reporter could be an alternative to the ultrasensitive chemiluminescence assay for monitoring AP activity. This chemical-adaptor strategy could be applied to the development of other fluorogenic or bioluminogenic substrates with an additional means of tuning chemical structures and functionalities for other enzyme assays. The luciferase-coupled bioluminescent assay also provides a unique potential for in situ probing of enzyme activities in combination with protein fusions.

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Keywords: alkaline phosphatase · immunoassays · luminescence · self-cleavable adaptors

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