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NOVEL FLUOROGENIC SUBSTRATES FOR ACID PHOSPHATASE

Kyle R. Gee

Molecular Probes, Inc., 4849 Pitchford Ave., Eugene, OR 97402, U.S.A.

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Abstract: Fluorinated versions of fluorescein diphosphate (FDP) can provide significantly enhanced fluorescence upon hydrolysis by acid phosphatase, as compared with FDP, when measured at the reaction pH.

Fluorogenic enzyme substrates have found extensive usage for the detection and quantitation of enzymes in live cells, cell lysates, and immunohistochemistry.[1] Before enzymatic reaction, fluorogenic substrates exhibit very low signal, but afterward yield bright fluorescence. Fluorescein diphosphate (FDP, **1**) has been widely used for the detection of alkaline phosphatase; the substrate is readily hydrolyzed by the enzyme, and after cleavage of the phosphate(s) fluorescein (**2**) is liberated.[2] Fluorescein is extremely powerful as a fluorophore because of its high absorptivity, high fluorescence quantum yield, and spectral match to the argon-ion laser. However, fluorescein's relatively high pKa of ~6.5 means that its use in fluorogenic substrates for enzymes with pH optima in the moderately acidic range is limited.

We recently demonstrated that some fluorinated derivatives of fluorescein retain the positive attributes of fluorescein, while improving upon its pKa deficiency.[3] Conversion of four of these fluorinated fluoresceins (**3–6**) into their corresponding diphosphates (**7–10**) was accomplished using standard phosphoramidite chemistry, followed by purification on Sephadex® LH-20.[4] These diphosphates were then examined for susceptibility to reaction with alkaline and acid phosphatases *in vitro*.

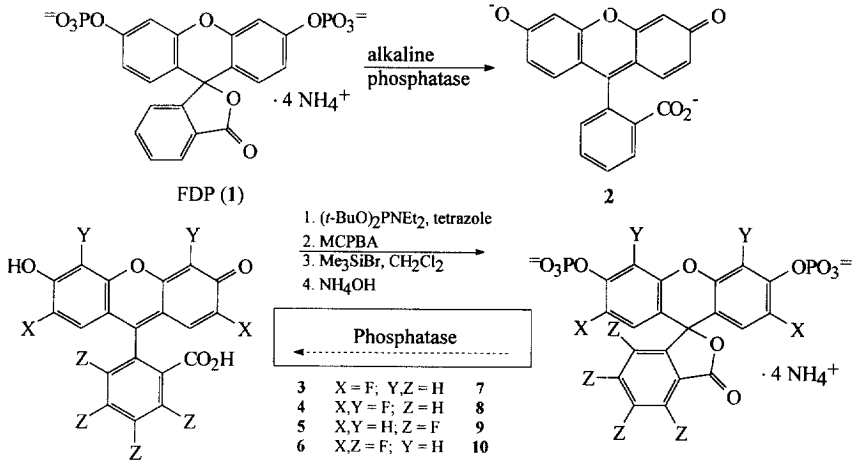

Table 1 shows the fluorescence yields upon reaction of equivalent amounts of FDP and **7–10** with excess alkaline phosphatase.[5] The reaction pH (10.5) is well above the pKa of each parent fluorophore (**2–6**), ensuring that after cleavage each fluorophore is fully ionized and thus maximum fluorescence signal is obtained. While the fluorinated analogs **7, 8,** and **10** provided comparable signals to that obtained from FDP, **9** gave only about a tenth of FDP's signal and was thus eliminated from further consideration.

Table 1 also shows the fluorescence yields obtained upon reaction of equimolar amounts of FDP and **7, 8,** and **10** with excess prostatic acid phosphatase.[5] At the reaction pH (5.0), the fluorinated FDP's provided much greater fluorescence. Raising the pH to 10 (after complete enzyme reaction), essentially equalized the fluorescence signals from FDP and **7**, which demonstrated that the signal increase from the fluorinated substrate

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resulted from the lower pKa (4.8) of the parent fluorophore **3**.[3] The lower pKa of fluorinated FDP's should allow for continuous assay of acid phosphatases.

Table 1. Relative Fluorescence Yield[a]

Substrate	Alkaline Phosphatase	Acid Phosphatase	Acid Phosphatase[b]
FDP (1)	100	100	100
7	90	400	85
8	79	500	-
9	<10	-	-
10	85	380	-

[a] Measured at the reaction pH; ±10% [b] After raising the reaction pH to 10

Figure 1 shows the enhanced sensitivity of the difluorofluorescein diphosphate **7** towards acid phosphatase, as compared with FDP. Reaction of 100 μM substrate with increasing enzyme concentrations shows a better signal from **7** over the entire concentration range. The background signals from each substrate were subtracted from the fluorescence measurements at all time points. Although the background of **7** was consistently 2–3 times higher than that of FDP, presence of the background does not account for the increased response of **7** to the enzyme.

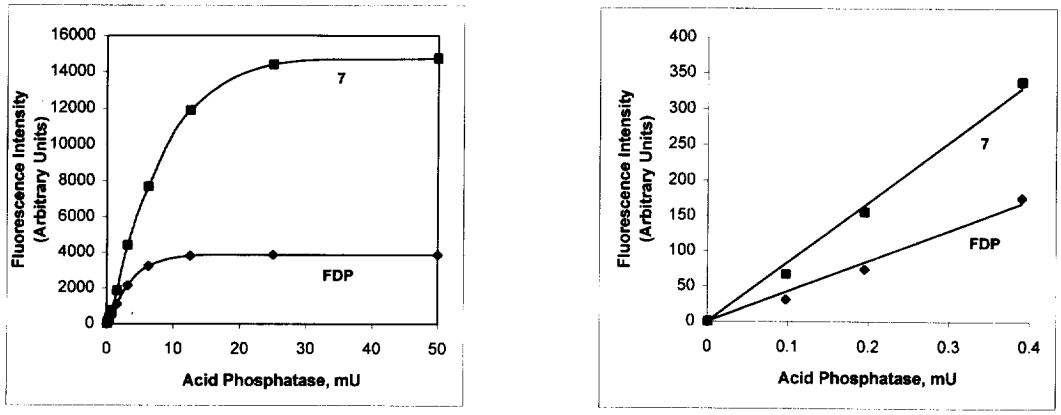

Figure 1. Fluorescence (measured at pH 5.0) liberated from 100 μM substrate after a 60 minute incubation with increasing amounts of enzyme.

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4. For example, **8**: ^{1}H NMR (D_2O) δ 8.18 (d, J = 7.7 Hz, 1H), 7.9 (m, 2H), 7.42 (d, J = 7.7 Hz, 1H), 6.68 (dd, J = 10.4, 1.9 Hz, 2H); ^{19}F NMR Φ 128.9 (s, 2F), 143.9 (s, 2F); ^{31}P NMR δ 4.55 (s).
5. Reactions contained 3 μM substrate and either 0.75 U/mL alkaline phosphatase (pH 10.5 buffer, 100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$) or 0.1 U/mL prostatic acid phosphatase (pH 5.0, 100 mM NaOAc), and were incubated for 1 h. Fluorescence signals were measured with a Cytofluor 2300 microplate flourometer using excitation at 485 ± 20 nm and emission detection at 520 ± 20 nm.