

# A Novel Fluorogenic Coumarin Substrate for Monitoring Acid Phosphatase Activity at Low pH Environment

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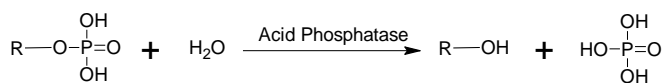
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**Abstract:** This article described the synthesis and application of 6-chloro-8-fluoro-4-methylumbelliferone phosphate (CF-MUP) in analyzing acid phosphatase activity. Compared to the existing MUP, the new coumarin phosphate, CF-MUP, demonstrated much higher sensitivity and was more robust for detecting the activity of acid phosphatase than the classic substrate 4-methylumbelliferone phosphate (MUP). The product of enzyme reaction, 6-chloro-8-fluoro-4-methylumbelliferone (CF-MU) possesses strong fluorescence at ~450 nm with low pKa (4.7), high fluorescence quantum yield and pH independence in the physiological pH range. This new fluorescence dye, CF-MU, is a convenient tool for assays with buffer pH between 4.5 and 8.

## INTRODUCTION

Acid phosphatases are family of enzymes ubiquitous in nature and have been found in many tissues in animals and plants. The function of acid phosphatases is catalyzing the hydrolysis of orthophosphate monoesters under acidic condition that regulates a variety of cellular functions. Human prostatic acid phosphatase was used as a surrogate biomarker for monitoring prostate cancer before the detection of prostate-specific antigen (PSA) became available [1-8]. Acid phosphatase has also been reportedly associated with Gaucher's disease in which patients exhibit unique peaks in their electrophoresed sera [9-11]. The acid phosphatase catalyzes the following reaction at an optimal pH, usually under pH 7:



Clinically, the measurement of acid phosphatase activity has been used for diagnosing cancers and for monitoring cell viability. Both para-nitrophenol phosphate (pNPP) and 4-methylumbelliferyl phosphate (MUP) have been widely used for measuring acid phosphatase activity [12-15]. Although MUP is more sensitive for detecting acid phosphatase activity than pNPP, its sensitivity is still quite limited [12, 13]. The detection of fluorescence product, MU (pKa = ~8.0) requires an optimal pH greater than 8.0 for its maximum sensitivity while the enzyme reaction of acid phosphatase is usually performed at an optimal pH under 7.0. Thus, the buffer pH has to be raised to 8.5 to 10 after the enzyme reaction by an addition of the stop solution with high pH in order to detect acid phosphatase activity with MUP. This two-step procedure is not convenient for automated assay environ-

ments such as clinical settings or high throughput screening labs.

This requirement of an additional step to increase in buffer pH after enzyme reaction with MUP substrate is due to the high pKa of enzymatic reaction product, MU (a 7-hydroxycoumarin analog). This fluorescent dye is typically not fully deprotonated (and therefore not maximally fluorescent) unless it is present in an environment having a pH of 9 or higher. Therefore, the detection sensitivity of assays using 7-hydroxycoumarin-based enzyme substrates such as MUP suffers at lower pH. On the other hand, the rate of enzyme reaction for acid phosphatases is very limited at neutral or higher pH. In addition to acid phosphatases, a numbers of lipid hydrolases and glycosidases such as beta-glucosidase, alpha-glucosidase and alpha-galactosidase also have optimal pH between 4 and 6. Therefore the glycoside derivatives of CF-MU should be useful for detecting the enzyme activity of these lipid hydrolases and glycosidases in a continuous assay format without a need to raise pH after the enzyme reaction.

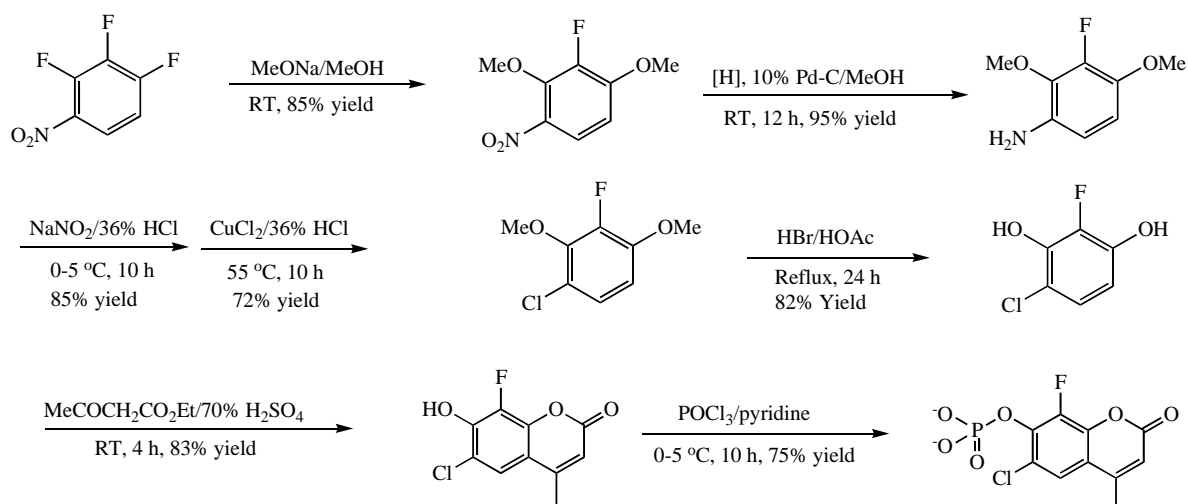
## MATERIALS AND METHODS

CF-MU and CF-MUP are commercially available from ABD Bioquest and was synthesized as shown in Scheme 1. CF-MUP is readily soluble in water and all the aqueous buffers while CF-MU is readily soluble in DMSO. Wheat germ acid phosphatase (5 U/mg) was purchased from Calzyme. All the other reagents are from Sigma Chemical Company.

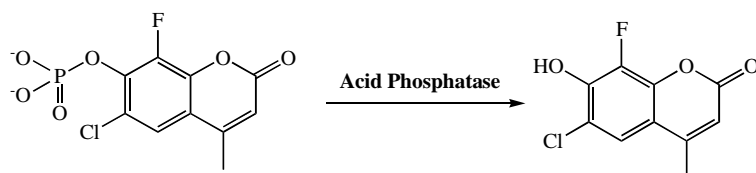
### The pH Titration of Coumarins

CF-MU was first dissolved in a series of buffers that were each calibrated using a pH meter. Acetate buffers were typically used in the range of pH 4-6, and phosphate buffers in the pH range 6-8. Absorption measurements were made using solutions that were approximately 5 μM in concentration, and fluorescence measurements were made using solu-

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**Scheme 1.** The synthesis of CF-MUP.



**Fig. (1).** Assay principle of CF-MUP for detecting acid phosphatase activity.

tions that were approximately 1  $\mu\text{M}$  in concentration. The absorption or emission data was then plotted versus pH to determine pKa value using equation:  $\text{pH} = \text{pKa} + c[\log(F_{\text{min}})/(F_{\text{max}} - F)]$  [16].

#### Detection of Acid Phosphatase Activity with CF-MUP

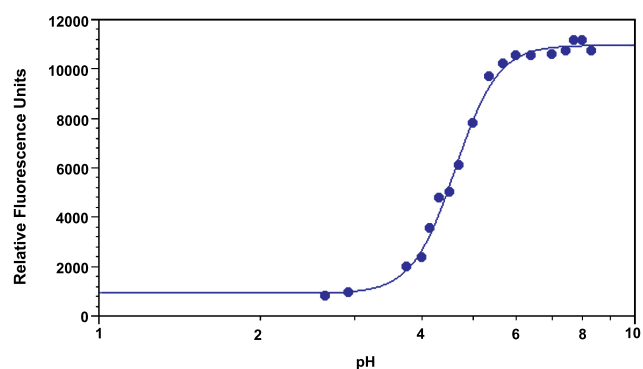
The utility of CF-MUP as a substrate for acid phosphatase was compared with MUP. For the accurate comparison, the concentrations of the two substrates (initially approximately 1 mM) were matched by normalizing the absorbance of each substrate solution at 319 nm (pH 10) to a value of 0.52 (assuming the extinction coefficient of each substrate was approximately equivalent). The matched samples were then diluted 1:10 into enzyme buffer (10 units/mL), and resulted solution was incubated at room temperature for 30 minutes at pH 5.5. The resulting fluorescence signal was recorded using excitation at 360 nm and emission at 450 nm.

### RESULTS AND DISCUSSION

CF-MUP has good water solubility and weak fluorescence. It is readily converted to highly fluorescent CF-MU upon enzymatic hydrolysis by acid phosphatases. CF-MU exhibits much stronger fluorescence in aqueous solutions at low pH (Fig. 1), making CF-MUP a sensitive probe for monitoring the activity of acid phosphatase.

The CF-MUP substrate overcomes the problems of non-continuous assay mode and high assay background since its reaction product, CF-MU, has a pKa of  $\sim 4.8$  (Fig. 2) with the absorption peak at  $\sim 360$  nm and emission peak at 450 nm, that are available in many detection instruments including microplate readers, fluorescence microscopes and flow cytometers. We found that CF-MUP is about 100 times more sensitive than MUP for the detection of acid phosphatase at

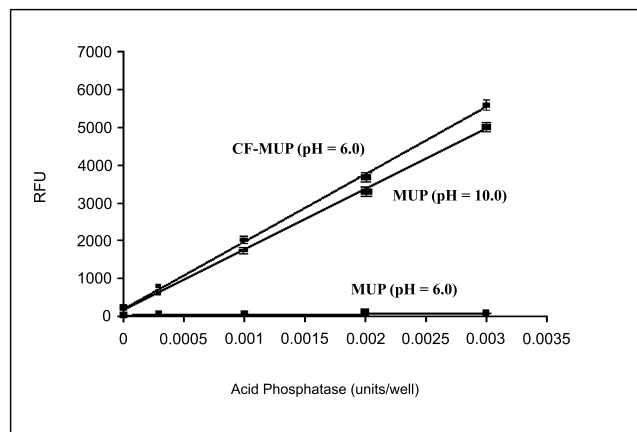
pH 5.5 (Fig. 3). This higher sensitivity is resulted from both the higher turn-over rate of CF-MUP by acid phosphatase and lower pKa of CF-MU. The direct comparison of initial hydrolysis CF-MUP and MUP by acid phosphatase at pH 5.5 followed by raising pH to 10 indicated that CF-MUP is still slightly more sensitive than MUP for acid phosphatase detection (see Fig. 3).



**Fig. (2).** Determination of pKa of CF-MU. A series of CF-MU solutions with different pH were made with the constant concentration of 1  $\mu\text{M}$  CF-MU. Their fluorescence intensities were measured, and plotted against pH.

Because the enzyme reaction product of CF-MUP does not require addition of base to the reaction mixture prior to measuring the fluorescence, it can be used for the continuous assay of acid phosphatases with a wide range of pH. As seen in Fig. (2), CF-MU reaches its maximal fluorescence at pH 6.0, a slightly lower pKa derivative of CF-MU might further enhance acid phosphatase detection sensitivity. In addition, this CF-MU fluorophore can also be used for labeling the

substrates to develop continuous assays for lipid hydrolases and glycosidases such as beta-glucosidase, alpha-glucosidase and alpha-galactosidase that have optimal pH between 4 and 6.



**Fig. (3).** Detection of acid phosphatase activity with CF-MUP and MUP. For the top and bottom curves MUP and CF-MUP were incubated with acid phosphatase buffer at pH 6.0, and the resulting fluorescence signal was recorded with Ex/Em=360 nm/450 nm without pH adjustment. For the middle curve, MUP was incubated with acid phosphatase buffer at pH 6.0, and the enzyme reaction solutions were adjusted to have pH = 10 by 1 M NaOH alkalization. The resulting fluorescence signal was recorded with Ex/Em=360 nm/450 nm.

## CONCLUSION

In conclusion, this new substrate CF-MUP demonstrated its advantage over the classic MUP for detecting the activity of acid phosphatase. The enzyme reaction product, CF-MU, possesses substantially lower pKa than MU and can be directly detected in the low pH assay condition that is usually between 4.5 and 7. This property is particularly useful for studying enzyme activity in the kinetic assay mode in low pH condition, such as acid phosphatases, lipid hydrolases and glycosidases. The excitation at 360 nm and emission at 460 nm of this new dye enables the enzyme assays compatible with the existing fluorescence detection instruments that have been set up for the MUP assays.

## ACKNOWLEDGEMENT

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