Revised: 26–November–2002

Alkaline and Acid Phosphatase Substrates: FDP, MUP, DiFMUP and DDAO Phosphate

D-6487 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate, diammonium salt (DDAO phosphate)
D-6567 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP)
D-22065 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) *packaged for high-throughput screening*
F-2999 fluorescein diphosphate, tetraammonium salt (FDP)
M-6491 4-methylumbelliferyl phosphate, free acid (MUP)
M-8425 4-methylumbelliferyl phosphate, dicyclohexylammonium salt, trihydrate (MUP DCA salt)

Quick Facts

Storage upon receipt:
- ≤–20°C
- Desiccate

Ex/Em of reaction products: *See Table 1*

Introduction

Fluorescein diphosphate (FDP) is a fluorogenic alkaline phosphatase substrate that was first described in 1963[1] and has been isolated in pure form and made commercially available by Molecular Probes. Molecular Probes' researchers have used the substrate in an alkaline phosphatase–based enzyme-linked immunosorbent assay (ELISA), finding that it provided a detection limit 50 times lower than that obtained with the chromogenic substrate, *p*-nitrophenyl phosphate.[2] The high pH used to monitor alkaline phosphatase activity is advantageous as it also produces optimal absorptivity and fluorescence of fluorescein ($\epsilon \sim 90{,}000\ cm^{-1}M^{-1}$, quantum yield ~ 0.92). FDP is colorless and nonfluorescent. Sequential alkaline phosphatase-mediated hydrolysis of the two phosphate substituents (Figure 1) yields weakly fluorescent fluorescein monophosphate followed by strongly fluorescent fluorescein (excitation/emission ~490/514 nm).

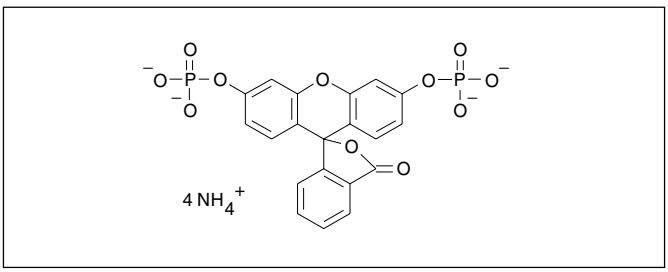

Figure 1. *Structure of fluorescein diphosphate (FDP).*

4-Methylumbelliferyl phosphate (MUP) has been in widespread use in fluorescence-detected ELISAs for many years.[3] In addition, Dr. Hans Tanke and co-workers have described a hybridization assay for PCR products with a detection limit of 0.15 femtomoles in which immunoenzymatic analysis using MUP was found to be advantageous among a number of nonisotopic detection formats in terms of dynamic range and simplicity of instrumentation.[4]

The reaction product of MUP is optimally fluorescent at alkaline pH, making MUP ideal for detecting alkaline phosphatase activity, but not practical for the continuous assay of acid phosphatases. Molecular Probes has developed a fluorinated MUP derivative, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP). The reaction product of DiFMUP has a lower pK_a than that of MUP (4.7 versus 7.9, Table 1), making DiFMUP an excellent substrate for continuously assaying acid phosphatases at low pH. The reaction products of MUP and DiFMUP have excitation/emission maxima ~358/450 nm.

The phosphatase substrate derived from the red-fluorescent dimethylacridinone (DDAO) contains only a single hydrolysis-sensitive moiety, thereby avoiding the biphasic kinetics of fluorescein-based substrates. DDAO-phosphate yields a hydrolysis product that is efficiently excited by the 633 nm spectral line of the He-Ne laser to produce bright red fluorescence with absorption/emission maxima of ~646/659 nm. Although the substrate itself is fluorescent, the difference between the substrate's excitation maximum and that of the product is over 200 nm, allowing the two species to be easily distinguished. DDAO phosphate has good water solubility, a low K_M and a high turnover rate.

Material

Fluorescein Diphosphate (F-2999)

Samples of fluorescein diphosphate contain a variable amount of water of hydration and a small amount of inorganic salts that do not affect the use of the product. The lot-specific

Table 1. *Soluble fluorescent phosphatase substrates.*

Catalog #	Substrate	Fluorogenic Product	Abs *	Em *	pK_a	Phosphatases Detected †
D-6487	DDAO-phosphate	Dimethylacridinone (DDAO)	646	659	~6.0	Protein phosphatase 2A (PP2A) [1]
F-2999	FDP	Fluorescein	490	514	6.4 ‡	Protein tyrosine phosphatases [2,3] Alkaline phosphatase [4,5]
M-6491 M-8425	MUP	4-methylumbelliferone (7-hydroxy-4-methylcoumarin)	360	449	7.8 §	Alkaline phosphatase [6,7]
D-6567 D-22065	DiFMUP	6,8-difluoro-4-methylumbelliferone	358	455	4.7 §	Acid phosphatase [8] Protein phosphatase 1 (PP1) [9,10] Protein phosphatase 2A (PP2A) [9,10]

* Absorption and emission maxima were taken from Molecular Probes' data tables and reported in nm.† Phosphatase substrates can be detectedby phosphatases that may not be specifically listed. ‡ Toxicon 38, 1833 (2000). § Bioorg Med Chem Lett 8, 3107 (1998). **1.** Toxicon 38, 1833 (2000); **2.** Biochem J 337, 219 (1999); **3.** Biochim Byophys Acta 1431, 14 (1999); **4.** Nature Biotech 18, 847 (2000); **5.** Science 283, 1892 (1999); **6.** J Microbiol Methods 40, 147 (2000); **7.** Micron 31, 41 (2000); **8.** Anal Biochem 273, 41 (1999); **9.** Anal Biochem 248, 258 (1997); **10.** Anal Biochem 269, 289 (1999).

weight purity of substrate in the vial is indicated on the product label. Thus, the total amount of material in the container is:

$$\text{Total material (mg)} = \left(\frac{(5 \text{ mg} \times 100)}{\text{weight purity (\%)}} \right)$$

We recommend preparation of a 10 mM stock solution of FDP (MW = 560) by dissolving the entire contents of the bottle in 0.89 mL of a dilute pH 7–8 buffer (not phosphate buffer). Background fluorescence of FDP samples is primarily due to fluorescein monophosphate. If not used immediately, this stock solution should be promptly divided into aliquots containing sufficient substrate for a single set of experiments and stored frozen at -20°C.

4-Methylumbelliferyl phosphate (MUP) (M-6491, M-8425)

A 10 mM stock solution of MUP can be prepared by dissolving the free acid (M-6491; MW = 256.2) in either dimethylsulfoxide (DMSO) or dilute pH 7–8 buffer (not phosphate buffer). The MUP dicyclohexylammonium salt (M-8425; MW [5] = 508.6) should be dissolved in dilute pH 7–8 buffer. For best results, we recommend preparing stock solutions just prior to use. If not used immediately, stock solutions should be promptly divided into single-use aliquots and stored frozen at -20°C.

6,8-Difluoro-4-methylumbelliferyl phosphate (DiFMUP) (D-6657, D-22065)

A 10 mM stock solution of DiFMUP (MW = 292.1) can be prepared by dissolving the entire contents of the vial in 1.71 or 3.42 mL of DMSO for the 5 and 10 mg unit sizes respectively. If not used immediately, this stock solution should be promptly divided into single-use aliquots and stored frozen at -20°C.

9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate, diammonium salt (DDAO-phosphate) (D-6487)

A 5 mM stock solution of DDAO phosphate (MW = 422.2) can be prepared by dissolving the entire contents of the vial in 2.37 mL of deionized water (dH_2O) or dimethylformamide (DMF). If not used immediately, this stock solution should be promptly divided into single-use aliquots and stored frozen at -20°C. When the solution turns a blue color, the substrate has broken down and is no longer usable.

References

1. Proc Natl Acad Sci USA 50, 1 (1963); **2.** J Immunol Methods 149, 261 (1992); **3.** J Immunol Methods 150, 23 (1992); **4.** Anal Biochem 205, 1 (1992); **5.** The molecular weight of M-8425 is for the hydrated form.

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
D-6487	9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate diammonium salt (DDAO phosphate)	5 mg
D-6567	6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP)	5 mg
D-22065	6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) *packaged for high-throughput screening*	10 x 10 mg
F-2999	fluorescein diphosphate, tetraammonium salt (FDP)	5 mg
M-8425	4-methylumbelliferyl phosphate, dicyclohexylammonium salt, trihydrate (MUP DCA salt)	1 g
M-6491	4-methylumbelliferyl phosphate, free acid (MUP)	1 g

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