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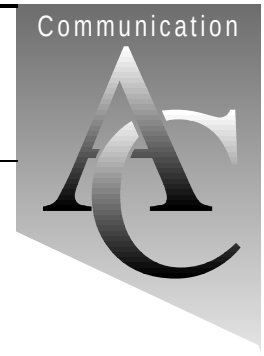
A new long-wavelength fluorigenic substrate for alkaline phosphatase: synthesis and characterisation



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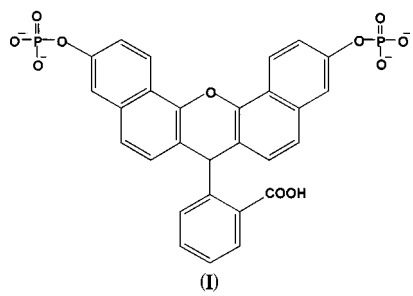
Naphthofluorescein diphosphate has been synthesised from the parent dye, and shown to be an attractive long-wavelength alternative to other fluorigenic substrates for the determination of alkaline phosphatase. Its application to the determination of theophylline, an inhibitor of this enzyme, has been demonstrated. The optimum excitation wavelength of the hydrolysis product naphthofluorescein has been found to depend on the presence of additives such as cyclodextrins and (3-[3-cholamidopropyl]-dimethylamino)-1-propane sulfonate (CHAPS): such effects can be used to raise the excitation wavelength to match the output of a 635 nm diode laser in a simple and sensitive fluorescence detector.

Amongst many recent developments in fluorescence spectrometry, the increased use of long wavelength probes and labels is one of the most important.[1,2] The major benefits include the much reduced 'autofluorescence' background obtained from many biological samples if the excitation wavelength exceeds *ca*. 550 nm and the emission wavelength *ca*. 600 nm, and the ease with which long wavelength fluorescence is detected by using diode laser sources, simple fibre optics and solid state detectors in small, low-cost and robust instruments.

The application of fluorigenic substrates, which are non- or weakly fluorescent but are converted by an appropriate enzyme to a highly fluorescent product, is widespread in biochemical analysis.[3] Such methods combine the amplification effects of the catalytic enzyme action with the high sensitivity of fluorescence spectrometry. Some applications are straightforward enzyme assays, while in others the enzyme is a label through which other reactions such as immunoassays are monitored.[4] Alkaline phosphatase, which hydrolyses phosphate esters in alkaline solution, is one of the most widely used labels in such indirect assays. The most commonly used fluorigenic substrates are those based on a number of umbelliferyl(7-hydroxycoumarin) derivatives,[5] but the reaction products fluoresce at *ca*. 460 nm, a wavelength at which there is much background fluorescence from biosamples. Assays based on fluorescein (emission at *ca*. 520 nm)[6] and resorufin (emission at *ca*. 590 nm)[7] conjugates have also been described. Here we describe the synthesis and properties of naphthofluorescein diphosphate (**I**), a new long-wavelength fluorigenic substrate for alkaline phosphatase, and show how the fluorescence properties of its hydrolysis product can be red-shifted by appropriate choice of the solvent environment, facilitating analysis using diode laser light sources. Previous studies have demonstrated the applicability of carboxynaphthofluorescein as a solution pH probe,[8] and a naphthofluorescein calcein derivative as an intracellular pH probe.[9] Naphthofluorescein has also been investigated as a possible acceptor for use in fluorescence energy transfer assays.[10]



(I)

Experimental

Naphthofluorescein diphosphate (NFDP) was synthesised by reacting naphthofluorescein (NF, Molecular Probes, Oregon; 0.25 mmol) with 10.7 mmol $POCl_3$ in 4 ml dry pyridine under nitrogen gas at 0 °C.[11] The reaction was monitored by thin layer chromatography on silica gel, using ethyl acetate : methanol : water : acetic acid (7 : 1 : 1 : 1 v/v/v/v) to develop the chromatogram: NF and NFDP had R_f values of 0.8 and 0.2 respectively. This method showed that 30 min reaction was sufficient, after which the reaction mixture was quenched by pouring into 40 ml of cold water and neutralising to pH 7.0 with ammonia. The pyridine was extracted with excess chloroform and the aqueous layer lyophilised. The resulting pink solid was characterised using IR spectrometry (phosphate absorption band at 1402 cm^{-1}) and mass spectrometry (NFDP molecular ion at m/z = 593). Fluorescence spectra were measured at room temperature using a Perkin-Elmer LS-50B spectrometer fitted with an R928 red-sensitive photomultiplier tube and a spectral bandwidth of 10 nm. Alkaline phosphatase (from calf intestine, 4.3 units mg^{-1}) was obtained from Sigma (Poole, Dorset) and was immobilised on 200–400 mesh amino-propyl controlled pore glass beads using glutaraldehyde prior to incorporation in a flow injection solid phase reactor of volume *ca*. 100 μl.[12] Cyclodextrins and (3-[3-cholamidopropyl]-dimethylamino)-1-propane sulfonate (CHAPS) were also products from Sigma. Fluorescence detection in flow injection analysis experiments was performed using a small laboratory constructed spectrometer incorporating a 635 nm diode laser light source (Power Technology Inc.), a 640 nm cut-off filter in the emission beam, and a silicon photodiode. Flow tubing of 0.8 mm diameter and a Hellma flow cell of 45 μl volume were used. The flow injection manifold used in the estimation of theophylline is shown in Fig. 1: the buffer flow rate was 1.5 ml min^{-1}, the injected sample and substrate volumes were 25 μl in each case, and the substrate concentration was 10 μg ml^{-1}.

Results

Fig. 2 shows that NFDP is effectively non-fluorescent, but is hydrolysed by alkaline phosphatase to yield the fluorescent naphthofluorescein, with excitation and emission wavelengths

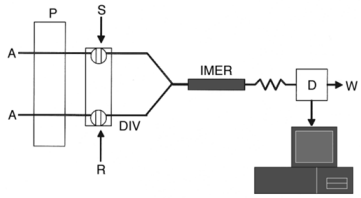


Fig. 1 Flow injection manifold for the determination of theophylline. S = sample, A = buffer, R = substrate injection, P = peristaltic pump, DIV = dual injection valve, IMER = immobilised enzyme reactor; D = detector, W = waste.

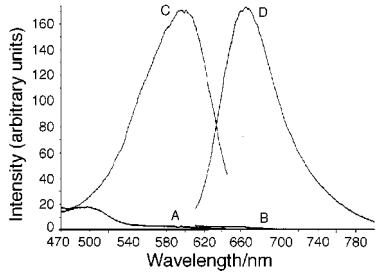


Fig. 2 Excitation and emission spectra in 0.1 M NaOH of (1) naphthofluorescein diphosphate (A and B, respectively) and (2) naphthofluorescein (C and D, respectively).

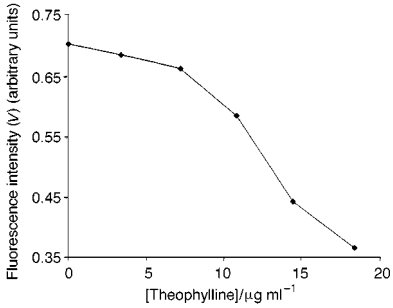


Fig. 3 Inhibition of immobilised alkaline phosphatase by theophylline, determined with the use of naphthofluorescein diphosphate using the solid state fluorescence spectrometer.

of *ca*. 595 and 660 nm respectively in 0.1 M NaOH. Similarly, UV–visible absorption spectrometry shows that NF has a strong absorption band near 600 nm, while NFDP has only a weak band at *ca*. 500 nm. The effects of a range of shift reagents on the spectroscopic properties of NF were studied (Table 1). Addition of α-, β- and γ-cyclodextrins (5% w/v) had relatively little effect on the absorption wavelength, while methyl-β-cyclodextrin and 2-hydroxy-β-cyclodextrin at the same concentration produced shifts to 609 and 615 nm respectively. The most dramatic wavelength shifts were obtained with CHAPS, which produced shifts to *ca*. 630 nm (excitation) and 680 nm (fluorescence) when present at the 5% level. Lower levels of this reagent produced only slightly less significant shifts while 10% CHAPS produced a small further shift in the excitation wavelength, but had little effect on the fluorescence emission wavelength. Modest but useful increases in fluorescence intensity (*ca*. 2-fold) were also obtained by the use of CHAPS (Table 1). The combined effects of the wavelength shift and intensity enhancement facilitated the use of NFDP as a fluorigenic substrate. This was demonstrated by the determination of the anti-asthmatic drug theophylline by using its inhibitory effect (Fig. 3) on alkaline phosphatase immobilised in the flow injection microreactor system. The solid state fluorescence instrument could detect NF at levels of *ca*. 10^{-9} M: although the inhibition of the enzyme by theophylline in the flow injection system was modest, it sufficed to determine this drug at therapeutic (μg ml^{-1}) levels.

Table 1 Effect of additives on the fluorescence properties of naphthofluorescein

Additive	Excitation maximum/nm	Emission maximum/nm	Intensity enhancement factor (%)
None	595	660	—
α-Cyclodextrin (5% w/v)	596	660	11
β-Cyclodextrin (5% w/v)	603	660	43
γ-Cyclodextrin (5% w/v)	599	660	37
Methyl β-cyclodextrin (5% w/v)	609	660	—
2-Hydroxypropyl-β-cyclodextrin (5% w/v)	616	660	—
CHAPS (5% w/v)	630	680	47

Conclusions

The results presented here demonstrate the development of a new fluorigenic substrate for alkaline phosphatase, and show that the hydrolysis product of this and other NF based substrates can be used for trace analyses using diode laser based fluorescence detection. The novel use of CHAPS to shift the fluorescence, and especially the excitation wavelength of NF is crucial in matching the characteristics of this fluorophore to those of the lowest wavelength diode lasers currently available at low cost. Further new NF-based substrates for the determination of esterase and other enzymes are currently being developed in our laboratory, and are expected to be of importance in high throughput screening applications. Preliminary studies also suggest that naphthofluorescein monophosphate might be an even better substrate, with a lower Michaelis constant, than NFDP; and that the wavelength shift effect of CHAPS might be extended to other long-wavelength and near-IR fluorophores. The origin of this interesting phenomenon is also under further study.

Acknowledgements

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References

1 J. N. Miller, M. B. Brown, N. J. Seare and S. Summerfield, in *Fluorescence Spectroscopy, New Methods and Applications*, ed. O. S. Wolfbeis, Springer-Verlag, Berlin, 1993.
2 M. B. Brown, T. E. Edmonds, J. N. Miller, D. P. Riley and N. J. Seare, *Analyst*, 1992, **118**, 407.
3 D. Robinson, *Biochem. J.*, 1956, **62**, 39
4 J. F. Bard, R. J. Carrico, M. C. Fetter, R. T. Buckle, R. D. Johnson, R. C. Boguslaski and J. E. Christner, *Anal. Biochem.*, 1977, **77**, 56.
5 D. H. Leaback, *Biochem. J.*, 1961, **78**, 22P.
6 A. Plovins, A. M. Alvarez, M. Ibanez, M. Molina and C. Nombela, *Appl. Environ. Microbiol.*, 1994, **60**, 4638.
7 J. Hofmann and M. Sernetz, *Anal. Chim. Acta*, 1984, **67**, 163.
8 A. Song, S. Parus and R. Kopelman, *Anal. Chem.*, 1997, **69**, 863.
9 Y. Zhou, E. M. Marcus, R. P. Haugland and M. Opas, *J. Cell Physiol.*, 1995, **164**, 9.
10 A. P. Weir, J. N. Heron and D. A. Christensen, *ACS Symposium Series*, 1992, **511**, 105.
11 H. D. Hill, G. K. Summer and M. D. Water, *Anal. Biochem.*, 1968, **24**, 9.
12 M. Masoom and A. Townshend, *Anal. Chim. Acta*, 1984, **166**, 111.

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