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A new long-wavelength fluorogenic 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 fluorogenic 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 fluorogenic 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 analysis. 3 Such methods combine the amplification effects of fluorescence energy transfer assays. 10

Experimental
Naphthofluorescein diphosphate (NFDP) was synthesised by reacting naphthofluorescein (NF, Molecular Probes, Oregon; 0.25 mmol) with 10.7 mmol POCI 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

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...
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 fluorogenic 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

Table 1 Effect of additives on the fluorescence properties of naphthofluorescein

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

flow injection system was modest, it sufficed to determine this drug at therapeutic (µg ml⁻¹) levels.

Conclusions

The results presented here demonstrate the development of a new fluorogenic 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