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3,4-Dihydro-3,4,6-trimethyl-2*H*,8*H*-pyrano-[3,2*g*]-1,3-benzoxazin-2,8-dione, a Potential Fluorogenic Reporter Group Reagent for Esterases—Synthesis and Interaction with Chymotrypsin

Trevor M. Kitson and Graham H. Freeman

Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand

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The synthesis of a novel "reporter group" reagent—3,4-dihydro-3,4,6-trimethyl-2*H*,8*H*-pyrano-[3,2*g*]-1,3-benzoxazin-2,8-dione (DTPBD)—is described. This compound has a cyclic carbamate functionality and thus, like the previously used 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one (DMNB), has the potential to label an esterase such as chymotrypsin. In so doing, DTPBD would incorporate into the protein a covalently linked 4-methylumbelliferone derivative, thus providing a sensitive reporter group that is both chromophoric and fluorescent. Experiments show that DTPBD reacts with chymotrypsin in the predicted manner, except that the labeling process is freely reversible (unlike the case with DMNB). 4-Methylumbelliferyl acetate (which is structurally closely related to DTPBD) is a good substrate for chymotrypsin. The rate of acylation of the enzyme is about an order of magnitude faster than with *p*-nitrophenyl acetate (which in turn is structurally related to DMNB), an unexpected observation in view of the relative leaving-group abilities of the groups concerned. The results suggest that these various modifiers and substrates show subtle but significant differences in the way they position themselves in chymotrypsin's binding site.

INTRODUCTION

Many esterases utilize *p*-nitrophenyl acetate as a substrate. In the related compound, *p*-nitrophenyl dimethylcarbamate, the presence of the nitrogen atom greatly attenuates the reactivity, such that the compound is likely to be an inactivator rather than a substrate. That is, although it can still acylate (for example) aldehyde dehydrogenase, albeit slowly, the rate of hydrolysis of the resulting acyl-enzyme is virtually zero (*1*,*2*). A cyclic analogue of *p*-nitrophenyl dimethylcarbamate, namely 3,4-dihydro-3-methyl-6-nitro-2*H*-1,3-benzoxazin-2-one (DMNB, *3–5*), reacts similarly except that now the *p*-nitrophenoxy "leaving group" does not actually leave, but remains covalently attached to the enzyme as a chromophoric reporter group of the type originally envisaged by Koshland (*6*). (See Scheme 1a.) DMNB has been used in our previous work to probe the active sites of chymotrypsin (*3*) and aldehyde dehydrogenase (*4*,*5*). We theorized that a similar cyclic carbamate in which the leaving group is based on 7-hydroxy-4-methylcoumarin (or 4-methylumbelliferone) would label an esterase in a similar way, thus providing it with a highly fluorescent reporter group; see Scheme

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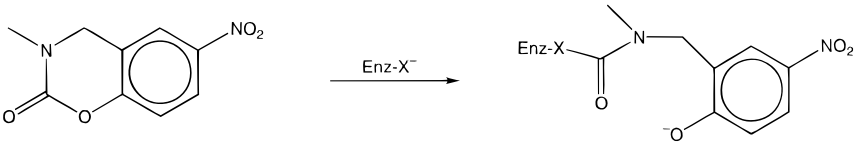


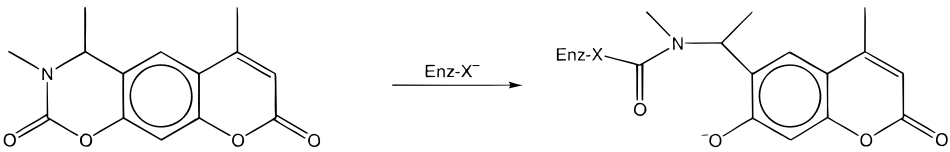
SCHEME 1a. Labeling of an enzymic nucleophile by DMNB.

1b. (Many highly sensitive enzyme substrates and modifying reagents based on 4-methylumbelliferone have been extensively used.) Accordingly, we report here the synthesis of 3,4-dihydro-3,4,6-trimethyl-2*H*,8*H*-pyrano-[3,2*g*]-1,3-benzoxazin-2,8-dione (DTPBD); see Scheme 2. The potential of this compound to act as a reporter group reagent was then tested using chymotrypsin as the enzyme, and the results are discussed below.

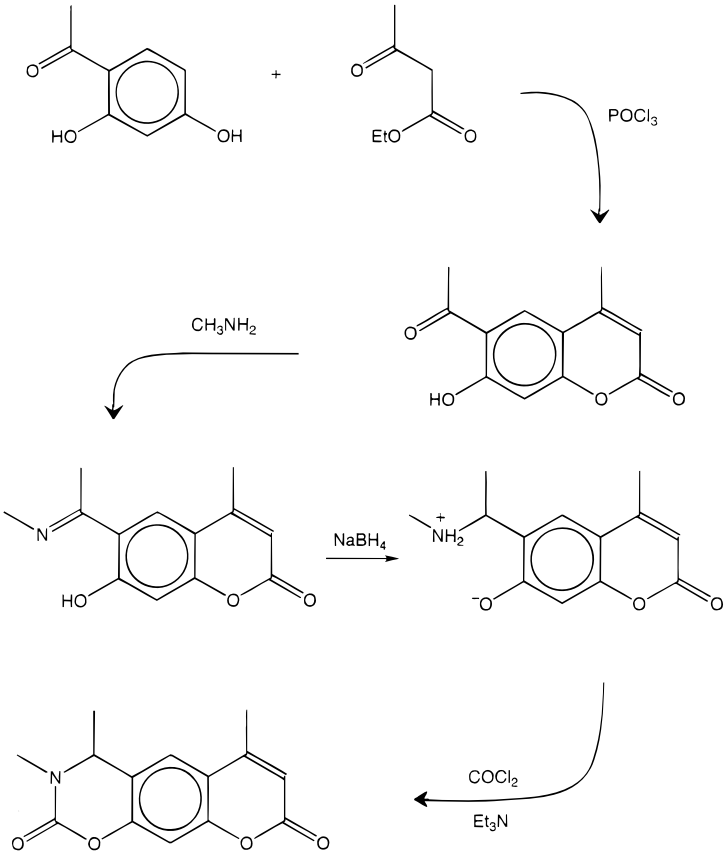
MATERIALS AND METHODS

Preparation of DTPBD (see Scheme 2)

2,4-Dihydroxyacetophenone (8 g, 0.053 mol) was dissolved in dry benzene (80 ml) and then ethyl acetoacetate (0.053 mol) and phosphorus oxychloride (0.022 mol) were added. The mixture was refluxed gently for 20 h with a steady flow of nitrogen through the apparatus. The solution was decanted from a dark oily residue which was then extracted with three portions (30 ml) of boiling benzene; the combined benzene extracts were evaporated leaving a dark brown oil. This oil was extracted with hexane (4×40 ml) to remove any unreacted ethyl acetoacetate, resulting in a brown solid. Recrystallization from ethanol gave a rust-colored powdery solid (6-acetyl-7-hydroxy-4-methylcoumarin) (1.01 g; in several repeats of this step we were unable to achieve more than 12% yield, although the literature preparation (*7*) quotes 40%). m.p. 212°C (lit. 212°C). NMR in $CDCl_3$ showed the phenol resonance at 12.699 ppm, the three other expected singlets (1H each) at 8.020, 6.896, and 6.222 ppm and the two expected singlets (3H each) at 2.721 and 2.467 ppm. This material was dissolved in boiling ethanol (100 ml) and methylamine (0.6 ml of a 40% aqueous solution) was added. The mixture was allowed to cool to room temperature over 2 h and then cooled in ice. The fine yellow crystals of the methyl imine of 6-acetyl-7-hydroxy-4-methylcoumarin were filtered off (0.90 g, 84%). Its identity was confirmed by NMR; the spectrum was very similar to the precursor (above) except for the presence of an extra singlet (3H) due to the methyl imine group. This material was suspended in ethanol (100 ml) and sodium borohydride (0.3 g) was added; the mixture was stirred



SCHEME 1b. Labeling of an enzymic nucleophile by DTPBD.



SCHEME 2. Synthetic route leading to DTPBD.

at room temperature for 3 h. The solvent was removed by rotary evaporation and the resulting mixture was used directly in the next step without separation. (This is because 6-(1-methylaminoethyl)-7-hydroxy-4-methylcoumarin evidently exists as a water-soluble zwitterion—the basic amino group being protonated by the acidic phenol group—and our attempts at separating it from the other components of the borohydride reduction were unsuccessful.) The complete residue from the previous step was added to toluene (50 ml) and triethylamine (0.81 g). After the addition of phosgene (as a 20% solution in toluene, 2.1 ml), the reaction mixture was stirred overnight at room temperature. The toluene solution was extracted with 0.5 M HCl (2×50 ml), dried over $MgSO_4$, and evaporated to dryness. The resulting material was recrystallized from ethanol to give a pale cream powdery solid (3,4-dihydro-3,4,6-trimethyl-2*H*,8*H*-pyrano-[3,2g]-1,3-benzoxazin-2,8-dione, DTPBD, 0.11 g, 11% over the last two steps). m.p. 229–231°C. The identity was established by NMR. δ ($CDCl_3$): 7.271 (s, 1H), 6.950 (s, 1H), 6.190 (s, 1H), 4.496 (q, 1H), 3.090 (s, 3H), 2.376 (s, 3H), 1.486 (d, 3H). ^{13}C NMR showed only the expected fourteen peaks. Mass

spectrometry confirmed the identity of the product; $m/z = 259.085609$ (calculated for $C_{14}H_{13}NO_4$: 259.084458).

Instrumentation and Enzyme Assays

All UV/visible spectra were recorded using a Varian Cary 1 spectrophotometer. Chymotrypsin was assayed in 50 mM Tris buffer, pH 8.5, at 25°C using 0.1 mM *p*-nitrophenyl acetate as substrate. Fluorescence was monitored using a Perkin–Elmer LS 50 B luminescence spectrometer. Stopped-flow studies were carried out with a Hi-Tech Scientific instrument and the data were analyzed using software supplied with the instrument. One syringe contained chymotrypsin in 50 mM Tris buffer, pH 8.4; the other syringe contained substrate in a mixture of 4.5 ml of the same buffer and 0.5 ml of acetonitrile. The concentrations in the reaction mixture after mixing were enzyme 13.3 μM and substrate 0.08–0.2 mM.

RESULTS AND DISCUSSION

Synthesis and Hydrolysis of DTPBD

The overall yield of the route we used for the synthesis of DTPBD (Scheme 2) was, in our hands, very low, but the starting materials are readily available and only small amounts of the final product are required for enzymological experiments. DTPBD would be a closer structural analogue of DMNB if it lacked the methyl group at position 4, but we could not get the first step of the synthesis to occur at all using 2,4-dihydroxybenzaldehyde in place of 2,4-dihydroxyacetophenone.

As a test of the likelihood that DTPBD would react with an enzymic nucleophile in the manner envisaged in Scheme 1, we studied its reaction with hydroxide ion. We first examined the spectrophotometric and fluorometric properties of 4-methylumbelliferone, as we anticipated that this would be a simple model for the product of nucleophilic cleavage of the carbamate ring of DTPBD. Figure 1 shows the UV absorption of the anionic form of 4-methylumbelliferone (λ_{max} 361-2 nm, ε 18,800 L mol^{-1} cm^{-1}); the undissociated form absorbs maximally at 321 nm and evidently has a pK_a of 7.92. Figure 1 also shows that both forms of the compound are highly fluorescent, the undissociated form more so than the anion, and the experimental

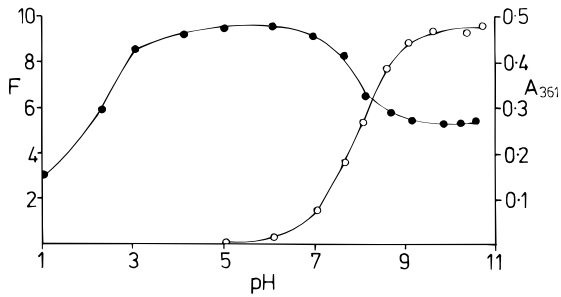


FIG. 1. The closed symbols show the fluorescence (in arbitrary units) of 4-methylumbelliferone as a function of pH. The open symbols show the absorbance at 361 nm, due to the anionic form of 4-methylumbelliferone.

value for the pK_a in this case is 7.86. An excitation wavelength of 325 nm was used and the maximal emission was at 446-7 nm over most of the pH range, rising slightly to 449 nm at pH 2.3 and 476 nm at pH 1.0. (DTPBD itself is not significantly fluorescent.) At very high pH (i.e., in 0.1 M NaOH) the anion of 4-methylumbelliferone is unstable; both the fluorescence and the A_{362} decay with a half-life at 25°C of about 90 min. It is assumed that this is due to alkaline hydrolysis of the ester group in the pyranone ring (*8*).

DTPBD (50 μM) in 0.2 M phosphate buffer, pH 8.0, gives the UV spectrum shown in Fig. 2, and this is perfectly stable for at least an hour at 25°C. However, adding the same concentration of the compound to 0.1 M NaOH gives evidence of rapid reaction as shown by the other spectra in the figure. It is clear, though, that hydrolysis of the carbamate ring is not the only reaction that occurs, since if this were so, it would result in the peak at 360-70 nm having an absorbance of about 0.94 (based on the ε value of 4-methylumbelliferone). We conclude, therefore, that the pyranone ring of DTPBD also undergoes rapid hydrolysis (and this reaction is likely to be faster than the alkaline hydrolysis of 4-methylumbelliferone referred to above, since that is an anion and DTPBD is neutral). Any DTPBD that does undergo hydrolysis in the carbamate ring subsequently also has its pyranone ring cleaved (at a slow rate similar to that with 4-methylumbelliferone) as shown by the slowly declining peak at 360-70 nm in the figure.

Reaction of Chymotrypsin with DTPBD

The preceding results raise the possibility that chymotrypsin could utilise DTPBD as a substrate (hydrolysing its pyranone ester group) instead of, or as well as, becoming inactivated by the chemistry shown in Scheme 1b. However, when DTPBD (70 μM) and chymotrypsin (20 μM, α-chymotrypsin, type II from bovine pancreas, Aldrich) were incubated for 2 h at 25°C in 50 mM Tris buffer, pH 8.5, there was no change in the magnitude of the maximal absorbance of DTPBD (which occurs at 317 nm), showing that the pyranone ring is not being cleaved. Some coumarin derivatives are

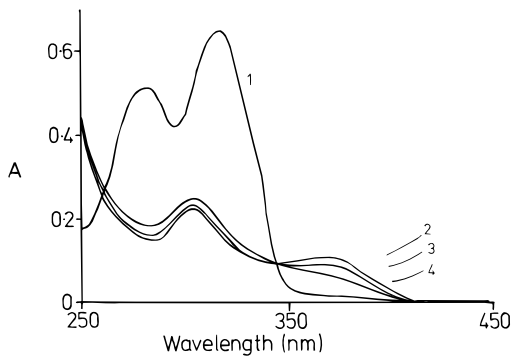


FIG. 2. Spectrum 1 is that of DTPBD (50 μM) in phosphate buffer, pH 8.0. The spectra labeled 2, 3, and 4 are the result of adding the same concentration of DTPBD to 0.1 M NaOH and scanning 1, 15, and 45 min, respectively, after mixing.

cleaved by chymotrypsin (*9*), but in these the phenoxy "leaving group" carries a *p*-halomethyl substituent, which loses halide ion (resulting in a reactive quinone methide derivative) and this presumably furnishes the driving force for the initial cleavage.

Although there was no change in A_{317} in the experiment just referred to, there was a small increase in A_{365}, which is consistent with some cleavage of the carbamate ring (as in Scheme 1b). This was monitored over a range of pH (see Fig. 3, for example) and in all cases it appeared that the reaction was over in less than about 15 min. Plotting the size of the increase in A_{365} against pH gave an apparent pK_a value of 7.92, the same as that of free 4-methylumbelliferone. However, using the ε value for 4-methylumbelliferone, it is calculated that only about 16% of the enzyme is labeled under these conditions.

Further information was sought using the assumed fluorescence characteristics of the enzyme adduct in Scheme 1b. DTPBD (0.25 mM) and chymotrypsin (50 μM) were incubated at 25°C in 50 mM Tris buffer, pH 8.5, as were a control solution containing no modifier and one containing no enzyme. At intervals, small samples of the reaction mixture and of the enzyme-free control were taken, diluted into 50 mM phosphate buffer, pH 7.4, and monitored for fluorescence. The results are shown in Fig. 4, where it can be seen that there is an increase in fluorescence in the reaction mixture, which is complete within the first half-hour of the incubation. The limiting difference in fluorescence between the reaction mixture and the control would be equivalent to about 27% of the enzyme being labeled (if the enzyme adduct has the same fluorescence properties as free 4-methylumbelliferone). Aliquots were also taken at intervals for assay of chymotryptic activity; the Figure shows that there was virtually no more loss of activity in the reaction mixture than that due to spontaneous denaturation or autolysis (as seen in the modifier-free control reaction). After the full 7-h incubation period, the reaction mixture was passed down a gel filtration column and the protein-containing fraction was assayed for fluorescence. The results showed that 98% of the fluorescence in the reaction mixture was removed from the protein by gel filtration. Based on our experience with DMNB and chymotrypsin (*3*), we had anticipated that DTPBD would label about 70% of the protein and cause major loss of enzyme activity and that the label would be permanently covalently attached to the protein.

The explanation we put forward for the unexpected results just referred to is that the reaction between DTPBD and chymotrypsin is *reversible*. Clearly there is some

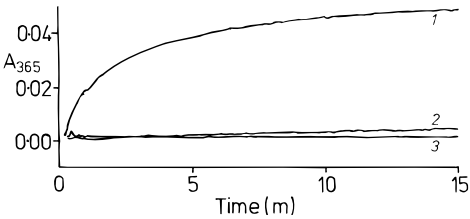


FIG. 3. The reaction of DTPBD (70 μM) and chymotrypsin (20 μM) in 50 mM Tris buffer, pH 8.41, was monitored at 365 nm (trace 1). Trace 2 was obtained with a control solution containing no enzyme and trace 3 with a control solution containing no DTPBD.

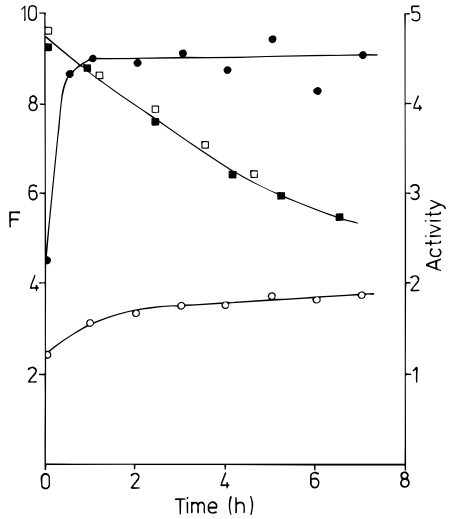


FIG. 4. DTPBD (250 μM) and chymotrypsin (50 μM) were incubated at 25°C in 50 mM Tris buffer, pH 8.5. At intervals, small samples were taken and the fluorescence (closed circles) and enzymic activity (closed squares) were measured. A control solution without enzyme gave the fluorescence shown as open circles. A control solution without DTPBD gave the enzymic activity shown as open squares.

reaction in which the carbamate ring is opened, as this explains the UV absorbance and fluorescence that are produced. However, this cannot be due to the enzyme merely catalysing the hydrolysis of the carbamate in some way (without becoming labeled) as then there would be no reason why the reaction should not continue indefinitely rather than soon reaching a plateau. On the other hand, any labeling of the enzyme that occurs must rapidly reverse in the 5 to 10 min that it takes to carry out the gel filtration. Furthermore, this proposed reversal could also happen when a sample of reaction mixture is added to an assay solution containing excess *p*-nitrophenyl acetate and thus account for the observed lack of effect on enzyme activity. A combination of forward and reverse reactions would result in an equilibrium position in which only a fraction of the enzyme is labeled, consistent with the results discussed above. This equilibrium would shift upon removal of the modifier by gel filtration and also on the addition of a high competing concentration of the enzyme's substrate. The position of equilibrium might well vary with pH, and thus the apparent pK_a value of the enzyme-linked 4-methylumbelliferone reporter group (see above) could be spurious.

It appears therefore that after the reaction shown in Scheme 1b, the phenoxy anion must remain poised in a suitable position for reversal of the individual chemical steps, whereas evidently with DMNB (Scheme 1a), the corresponding phenoxy anion occupies a sufficiently different position within the enzymic binding site that the reversal is not favourable. Subtle differences such as this are not without precedent in reactions involving chymotrypsin; the acyl enzyme derived from 6-nitrodihydrocoumarin undergoes normal deacylation (as with other ester substrates), whereas that derived from the only slightly different lactone, 5-nitrocoumaranone, undergoes

reversal of its formation (liberating the starting material; *10*) as we are proposing here for DTPBD.

Action of Chymotrypsin on 4-Methylumbelliferyl Acetate

Since DMNB and DTPBD affect chymotrypsin in different ways, we thought it might be interesting to compare a similarly related pair of compounds (namely *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate) as substrates for the enzyme. Using a range of concentrations of each ester (0.067–0.30 mM) at pH 8.43 and 25°C, and taking care to use the appropriate ε values for the products at this pH, we determined spectrophotometrically the k_{cat} values as 0.0079 and 0.0063 s^{-1} for *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate, respectively. As both substrates show a pre-steady-state burst of product release (see below), we know k_{cat} represents the rate of acyl-enzyme hydrolysis, and (in the absence of other factors) this should of course be exactly the same for both substrates since they have a common acyl group. Our experimental values are close but not identical; it is possible that the rate of acyl-enzyme hydrolysis is affected by other substrate molecules binding in chymotrypsin's extended substrate-binding domain (*11*).

The pre-steady-state phase of chymotrypsin-catalysed hydrolysis of the two acetates was also examined (at pH 8.40 and 25°C, a substrate concentration range of 0.08 to 0.20 mM and an enzyme concentration of 13 μM). Typical results are shown in Fig. 5. Both substrates show a burst; that from *p*-nitrophenyl acetate is equivalent to 67% of the enzyme concentration (similar to previous results, *11*), whereas that from 4-methylumbelliferyl acetate is slightly smaller (as with the respective k_{cat} values above)

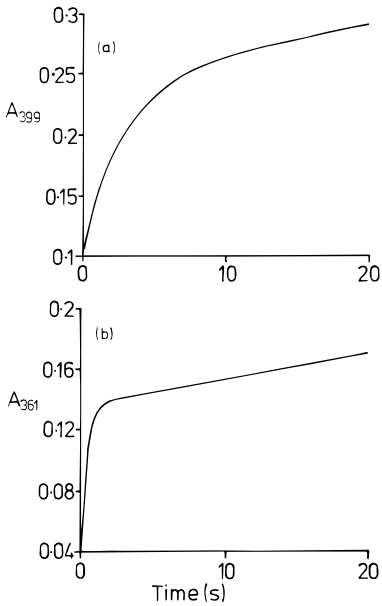


FIG. 5. Stopped-flow absorption traces obtained with chymotrypsin (13.3 μM) and substrate (0.2 mM) at 25°C in 50 mM Tris buffer, pH 8.4. (a) *p*-Nitrophenyl acetate; (b) 4-methylumbelliferyl acetate.

at 55%. Figure 5 makes it clear that the burst with the latter substrate is considerably faster than with the former. Plots of the reciprocal of the rate constant versus the reciprocal of the substrate concentration intercept the y axis rather close to the origin; thus there is considerable error in our estimates of the limiting value of the rate constant at infinite substrate concentration (which are 2.2 ± 0.5 and $12.6 \pm 1.5\ s^{-1}$, for *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate, respectively).

Previous work showed that the burst with resorufin acetate is relatively slow (*11*). For example, at 0.2 mM substrate concentration, the rate constants for resorufin acetate, *p*-nitrophenyl acetate and 4-methylumbelliferyl acetate are in the ratio 1:1.5:12. The pK_a values for the corresponding hydrolysis products are 5.8 (*11*), 7.1 (*3*), and 7.9 (this work). Thus it is interesting that the trend in speed of the burst runs counter to that expected from the acidity data. If the rate of the burst is controlled by nucleophilic attack on the ester group giving a tetrahedral intermediate, then this process should be fastest for the most electron-withdrawing moiety (i.e., resorufin). If, on the other hand, the burst is controlled by the collapse of the tetrahedral intermediate, this should be fastest for the best leaving group, again the resorufin anion. Similarly, from the electronic aspect alone, we should expect the burst with 4-methylumbelliferyl acetate to be relatively slow. Clearly, therefore, some other factor is important here, and we suggest that subtle positional differences of the esters as they bind in the enzyme's active site result in the carbonyl group being held either favourably close to the attacking nucleophile (in the case of the umbelliferyl compound) or in a relatively unfavourable position (in the case of the resorufin compound). This idea, of the importance of positioning factors, ties in well with our proposal above that similar slight differences of position within the active site could explain why reaction with DTPBD, but not with DMNB is reversible. There remains the possibility that the extra methyl group that DTPBD has on the carbamate ring may be responsible for the different action that it displays compared to DMNB.

CONCLUSION

This work shows that DTPBD has the potential to be a sensitive chromogenic and fluorogenic reporter group reagent for esterases. Its usefulness in the particular case of chymotrypsin is limited by the evident reversibility of the modification reaction, but we have no reason to believe at this stage that the same problem will necessarily arise in our future work with other esterases.

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