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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

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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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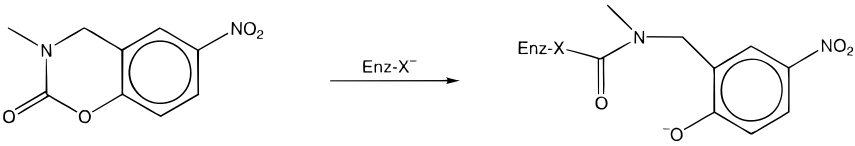


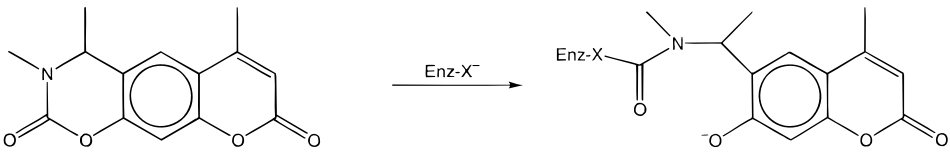
SCHEME 1a. Labeling of an enzymic nucleophile by DMNB.

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.

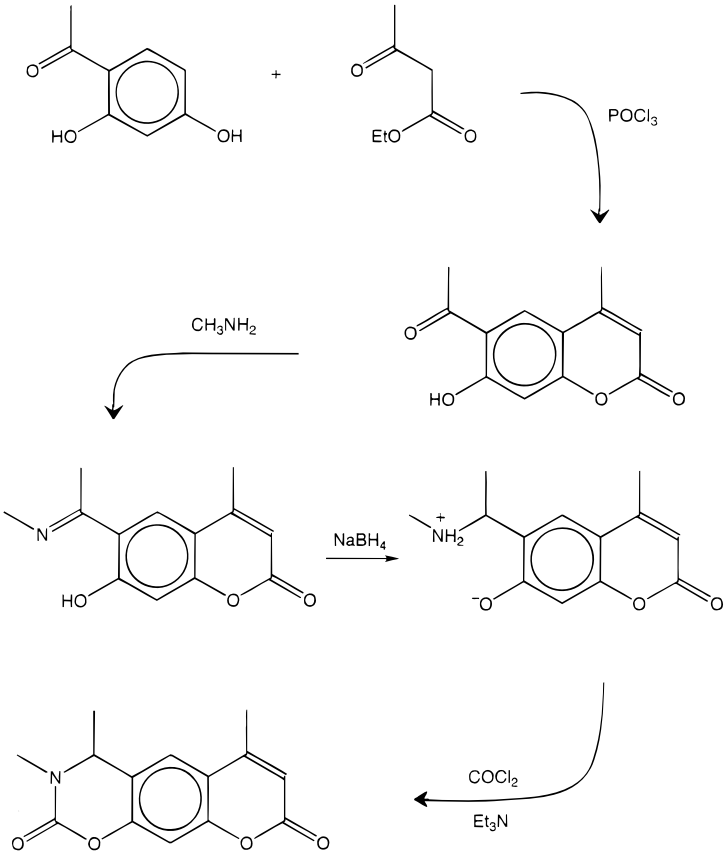
MATERIALS AND METHODS

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

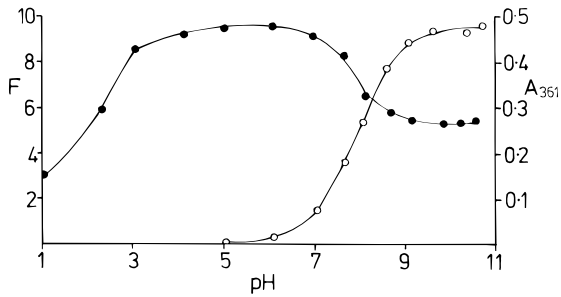


FIG. 1. The closed symbols show the fluorescence (in arbitrary units) of 4-methylumbelliferone as a function of pH. The open symbols show the absorbance at 361 nm, due to the anionic form of 4-methylumbelliferone.

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

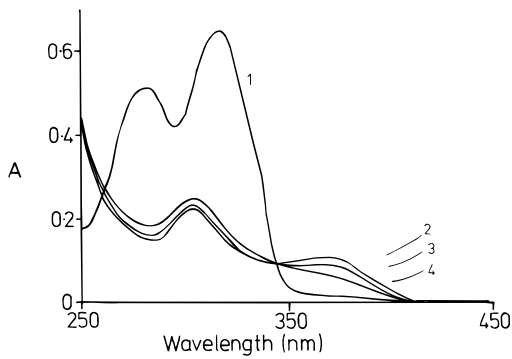


FIG. 2. Spectrum 1 is that of DTPBD (50 μM) in phosphate buffer, pH 8.0. The spectra labeled 2, 3, and 4 are the result of adding the same concentration of DTPBD to 0.1 M NaOH and scanning 1, 15, and 45 min, respectively, after mixing.