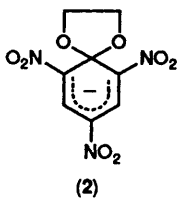
1-(2-Acetoxyethoxy)-2,4,6-trinitrobenzene. A New Substrate for Enzyme Catalysed Hydrolysis

Michael R. Crampton,* Karen E. Holt, and Jonathan M. Percy
Chemistry Department, Durham University, Durham DH1 3LE, UK

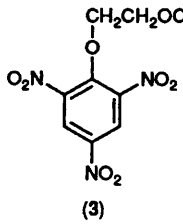
The preparation of the title compound (**3**) is described. Hydrolysis of the ester group of (**3**) is readily detected spectrophotometrically since the 1-(2-hydroxyethoxy)-2,4,6-trinitrobenzene produced spontaneously cyclises in water at pH > 7 to give a strongly coloured spiro-adduct. The pH–rate profile of the hydrolysis has been determined. The kinetics of the hydrolysis of (**3**) catalysed by porcine liver esterase have been examined and are compared with those for the hydrolysis of 4-nitrophenyl acetate.

The hydrolysis of 4-nitrophenyl esters yields the yellow-coloured product 4-nitrophenol which is conveniently monitored spectrophotometrically. Such esters, particularly 4-nitrophenyl acetate, are frequently used as substrates for kinetic studies of enzyme action.[1] The use of these substrates has the disadvantage that it involves hydrolysis of an ester group directly attached to an aromatic ring—this is not a usual situation in natural enzymic reactions where hydrolysis of ester groups in a more aliphatic environment usually occurs. A further disadvantage is that spontaneous hydrolysis of these substrates in alkaline buffers may be sufficiently rapid to compete effectively with the enzyme catalysed pathway.

It is known that ring-activated 2-hydroxyethoxyarenes will cyclise to give strongly coloured spiro-adducts[2] and we had the idea that esters prepared from these compounds might provide alternative substrates for investigating hydrolytic enzymes. A suitable parent appeared to be 1-(2-hydroxyethoxy)-2,4,6-trinitrobenzene (**1**) since this cyclises spontaneously[3,4] in water at pH ≥ 7 to give (**2**) with λ_{max} 416 nm (ε 2.5 × 10^4 dm^3 mol^{-1} cm^{-1}) and 470 nm (ε 1.8 × 10^4). We report here the preparation of the title compound (**3**) by acetylation of (**1**), and kinetic studies of the pH dependence of its non-enzymatic hydrolysis and of its reactions in the presence of esterase (porcine liver) and α-chymotrypsin.



(**2**)



(**3**)

Results and Discussion

The ester (**3**) was prepared by acetylation of (**1**) using excess acetyl chloride in dichloromethane. The ^1H NMR spectrum in deuteriochloroform showed a singlet due to ring protons δ 8.92 (2-H), a multiplet due to methylene protons δ 4.45 (4-H) and a singlet due to acetyl protons δ 2.10 (3-H).

We first examined the pH dependence of the hydrolysis of (**3**). A stock solution was prepared in dioxane, in which (**3**) was indefinitely stable, and test solutions in water contained 2% (v/v) of dioxane. The UV spectrum of (**3**) shows a maximum at 230 nm (ε 1.8 × 10^4 dm^3 mol^{-1} cm^{-1}) but there is negligible absorbance above 350 nm. This hydrolysis could be measured using the increase in absorbance in the visible region. First order rate coefficients in solutions of hydrochloric acid or sodium hydroxide in the range 0 < pH < 13 fitted equation (1); with

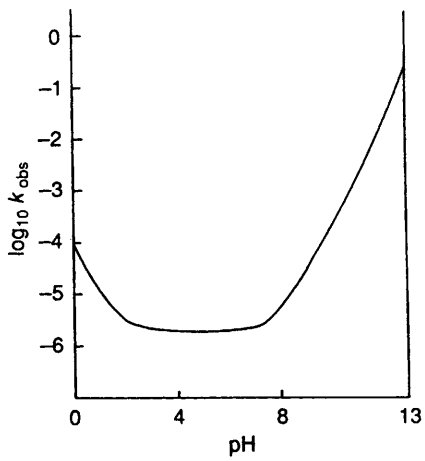
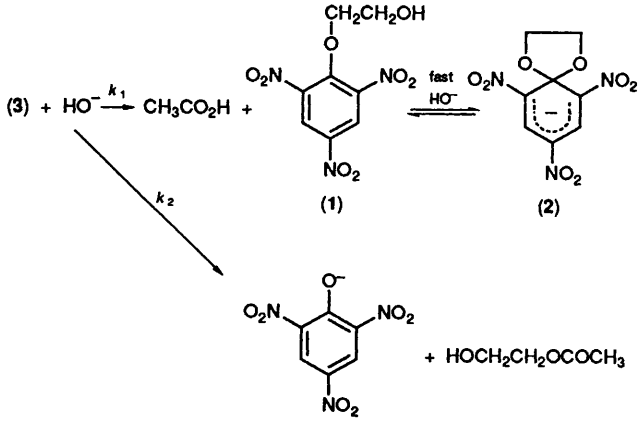


Figure 1. pH profile for hydrolysis of (**3**) in water at 25 °C.

$$k_{obs} = k_W + k_H[H^+] + k_{OH}[OH^-] \qquad (1)$$

the values $k_W(2 \pm 0.5) \times 10^{-6}$ s^{-1}, $k_{H^+}(9 \pm 2) \times 10^{-5}$ dm^3 mol^{-1} s^{-1} and $k_{OH^-}(2.2 \pm 0.2)$ dm^3 mol^{-1} s^{-1}. These values lead to the pH profile shown in Figure 1.

In acid solutions the only reaction observed was hydrolysis of the ester grouping leading quantitatively to formation of the glycol ether (**1**); the reaction was sufficiently slow to allow the withdrawal of aliquots which were buffered at pH 9 for measurement of the absorbance at 416 nm due to the spiro-adduct (**2**). In alkaline solutions the two possible hydrolytic pathways shown in Scheme 1 were observed. These result from nucleophilic



Scheme 1.

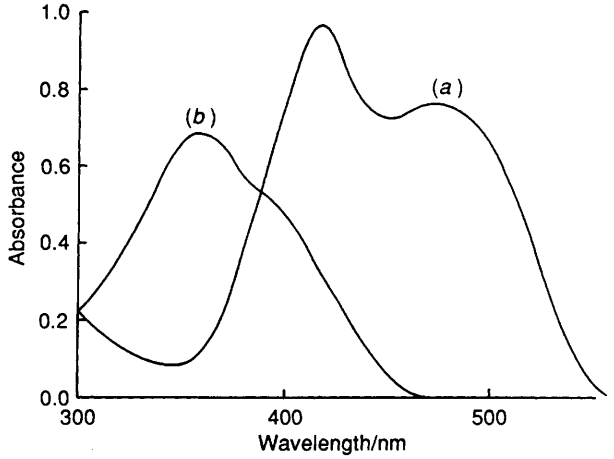
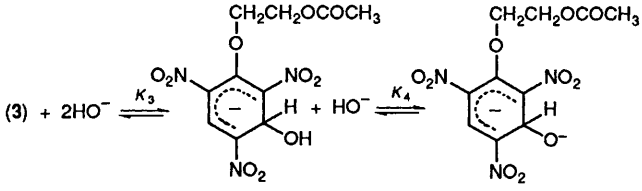


Figure 2. Visible spectra in water at pH 9 for 4×10^{-5} mol dm^{-3} concentrations of: (*a*) spiro adduct (**2**); (*b*) picrate.

attack at the ester group or at the aromatic ring.[5,6] The visible spectra of the products, the spiro-adduct (**2**) and the picrate ion, are quite distinct, as shown in Figure 2, allowing quantitative determination of the relative importance of these pathways. We were able to calculate that ester hydrolysis accounted for 30% of the observed reaction so that the overall value for k_{OH^-} can be split to yield values for k_1 0.66 dm^3 mol^{-1} s^{-1} and k_2 1.54 dm^3 mol^{-1} s^{-1}.

We also examined, using stopped-flow spectrophotometry, reactions in more basic media, $[OH^-] \geqslant 0.1$ mol dm^{-3}. At 393 nm, a wavelength at which (**2**) and picrate have equal absorbance, we observed a further rapid reaction preceding the hydrolytic reactions. Also at these higher basicities the values of k_{obs} for the hydrolysis ceased to show a linear dependence on base concentration (Table 1). These observations are best explained by the rapid reversible attack of hydroxide at the unsubstituted 3-position of (**3**) with subsequent ionisation of the added hydroxy group (Scheme 2). There is precedence for these



Scheme 2.

processes in related systems,[7] and they lead to a revised expression, [equation (2)], for the rate coefficient for the

$$k_{obs} = \frac{k_{OH^-}[OH^-]}{1 + K_3[OH^-](1 + K_4[OH^-])} \qquad (2)$$

hydrolytic process. Values obtained previously with 2,4,6-trinitroanisole as the substrate are K_3 1.4 dm^3 mol^{-1} and K_4 6 dm^3 mol^{-1} and these values also give an acceptable fit (Table 2) for the reaction of (**3**).

Enzyme-catalysed Hydrolysis.—There have been many kinetic studies of the hydrolytic action of porcine liver esterase and it has been shown that at high substrate concentrations deviations from Michaelis–Menten kinetics may occur with the observation of enhanced reactivity.[1d,e,8] Also, it is possible to separate the enzyme into several fractions having different catalytic properties.[8c,9] Despite these drawbacks we examined the effects of this enzyme on the hydrolysis of (**3**) and for comparison also report measurements on the hydrolysis of 4-nitrophenyl acetate obtained under identical reaction conditions.

Table 1. Rate coefficients for hydrolysis of (**3**) at higher base concentrations.

[NaOH][a]/mol dm^{-3}	k_{obs}/s^{-1}	k_{calc}
0.10	0.18	0.18
0.20	0.29	0.27
0.30	0.32	0.30
0.40	0.32	0.30

[a] Ionic strength 1 mol dm^{-3} with NaCl. [b] Calculated from equation (2) with k_{OH} 0.22 dm^3 mol^{-1} s^{-1}, K_3 1.4 dm^3 mol^{-1} and K_4 6 dm^3 mol^{-1}.

All measurements were made at 25 °C and pH 8.1 ± 0.1 using a 0.0125 mol dm^{-3} borax buffer, and with the condition $[S] \gg [E]_0$. Stock solutions of the substrates were prepared in dioxane and test solutions contained ⩽2% dioxane by volume. In the presence of the esterase the ester (**3**) was smoothly and quantitatively hydrolysed to give glycol ether (**1**) which spontaneously cyclised to the spiro-adduct (**2**). Values of the initial velocity, V_0, measured over the first 10% of reaction, were calculated from linear plots of the absorbance at 416 nm *versus* time using a measured extinction coefficient of 2.5×10^4 dm^3 mol^{-1} cm^{-1}. The data in Table 2 (entries a–e) show that with increasing substrate concentration the values of V_0 increase to reach a maximum value, and a satisfactory fit is obtained using equation (3) with the values $V_{max}(7 \pm 1) \times 10^{-8}$ mol dm^{-3} s^{-1}

$$V_0 = \frac{V_{max}[S]}{K_M + [S]} \qquad (3)$$

and K_M $(4 \pm 0.5) \times 10^{-5}$ mol dm^{-3}. Entries c and f–h in Table 2 show that V_0 increases linearly with enzyme concentration. Dividing V_{max} by $[E]_0$ we obtain a value, V'_{max}, of 1.8×10^{-8} mol s^{-1} (mg protein)$^{-1}$ or 1.1 μmol min^{-1} (mg protein)$^{-1}$. In this system there was negligible interference from spontaneous hydrolysis of (**3**) in the buffer, this reaction accounting for <1% of the enzymic reaction.

Data are presented in Table 3 for the analogous hydrolysis of 4-nitrophenylacetate. Absorbances due to the 4-nitrophenolate ion were measured at 400 nm and initial velocities were calculated using ε 1.7×10^4 dm^3 mol^{-1} cm^{-1}. Here there was quite severe interference from spontaneous decomposition of the substrate in the buffer, this reaction accounting for *ca.* 50% of the observed reaction at the highest substrate concentration used. The velocities of the enzyme catalysed decomposition give a reasonable fit in equation (3) with the values V_{max} $(1.3 \pm 0.2) \times 10^{-7}$ mol dm^{-3} s^{-1} and K_M $(2.0 \pm 0.2) \times 10^{-4}$ mol dm^{-3}. Division of V_{max} by $[E]_0$ gives a value of V'_{max} 1.6×10^{-6} mol s^{-1} (mg protein)$^{-1}$ which is equivalent to 97 μmol min^{-1} mg^{-1}. Our values for K_M and V'_{max} are in the range expected for this system[8c] (K_M 4.3×10^{-5}–2.2×10^{-4} mol dm^{-3}; V'_{max} 73–109 μmol min^{-1} mg^{-1}).

We also examined the reaction of (**3**) with α-chymotrypsin[1h] within the concentration ranges $2.5 \times 10^{-5} < [S] < 5 \times 10^{-4}$ mol dm^{-3} and $1 \times 10^{-5} < [E]_0 < 4 \times 10^{-5}$ mol dm^{-3}. These reactions were less satisfactory than those using the esterase. Colour formation was inconveniently slow and resulted in the formation of one or more unidentified species showing λ_{max} 342 nm (ε 1.3×10^4 dm^3 mol^{-1} cm^{-1}) and 416 nm (ε 0.6×10^4). The visible spectra did not correspond either to the spiro-adduct (**2**) or picrate ion which, in the same medium, shows λ_{max} 357, 400 (sh) nm. The velocity of the formation of the observed species increased with increasing enzyme concentration but quantitative measurements were not made.

Table 2. Kinetics of the hydrolysis of (**3**) by esterase at 25 °C and pH 8.

	$[S]/10^{-4}$ mol dm^{-3}	$[E]_0$[a]/mg dm^{-3}	$V_0/10^{-8}$ mol dm^{-3} s^{-1}	V_0(calc.)/10^{-8} mol dm^{-3} s^{-1} [b]
a	0.25	4.0	3.0	2.7
b	0.50	4.0	3.7	3.9
c	1.0	4.0	4.7	5.0
d	2.0	4.0	5.9	5.8
e	3.0	4.0	5.8	6.2
f	1.0	2.0	2.3	
g	1.0	8.0	10.1	
h	1.0	12.0	15.5	

[a] M 1.63 × 10^5, ref. 1(*e*). [b] Calculated from equation (3) with V_{max} 7 × 10^{-8} mol dm^{-3} s^{-1} and K_M 4 × 10^{-5} mol dm^{-3}.

Table 3. Kinetics of the hydrolysis of 4-nitrophenyl acetate by esterase at pH 8 and 25 °C.

$[S]/10^{-4}$ mol dm^{-3}	$[E]_0$[a]/mg dm^{-3}	V_0[b]/10^{-8} mol dm^{-3} s^{-1}	V_0(calc.)/10^{-8} mol dm^{-3} s^{-1} [c]
0.25	0.08	1.7	1.5
0.50	0.08	2.4	2.6
1.0	0.08	4.0	4.3
3.0	0.08	7.5	7.8
5.0	0.08	9.7	9.3
10.0	0.08	10	10.8

[a] M 1.63 × 10^5, ref. 1(*e*). [b] After subtraction of contribution from buffer catalysis. [c] Calculated from equation (3) with V_{max} 1.3 × 10^{-7} mol dm^{-3} s^{-1} and K_M 2.0 × 10^{-4} mol dm^{-3}.

Table 4. Comparison of data for hydrolyses of (**3**), 4-nitrophenyl acetate and ethyl acetate in water at 25 °C.

	(**3**)	4-Nitrophenyl acetate	Ethyl acetate
k_{H^+}/dm^3 mol^{-1} s^{-1}	9 × 10^{-5}	3.6 × 10^{-5} [b]	8 × 10^{-5} [b]
k_{OH^-}/dm^3 mol^{-1} s^{-1}	2.2[c]	9.5[d]	0.11[d]
k_{H_2O}/s^{-1}	2 × 10^{-6}	8.5 × 10^{-7} [e]	2.5 × 10^{-10} [f]
K_M/mol dm^{-3} [a]	4 × 10^{-5}	2 × 10^{-4} [g]	
V'_{max}/μmol min^{-1} mg^{-1} [a]	1.1	97[g]	

[a] Catalysis by porcine liver esterase. [b] K. Yates and R. A. McClelland, *J. Am. Chem. Soc.*, 1967, **89**, 2686. [c] Value for attack at the ester group is 0.66 dm^3 mol^{-1} s^{-1}. [d] W. P. Jencks and J. F. Kirsch, *J. Am. Chem. Soc.*, 1964, **86**, 837. [e] V. Gold, D. G. Oakenfull, and T. Riley, *J. Chem. Soc. B*, 1968, 525. [f] A. Skrabal and A. Zahorka, *Monatsh. Chem.*, 1929, **53–54**, 562. [g] Ref. 8(*c*).

Comparisons.—Data for the hydrolysis of (**3**) are compared in Table 4 with those for 4-nitrophenylacetate and for ethyl acetate. Rate coefficients for catalysis by protons and hydroxide ions correspond more closely with those for ethyl acetate than with 4-nitrophenyl acetate indicating that the inductive effect of the picrate group attenuated by two methylene groups is not particularly large. The relatively high rate coefficient for the neutral hydrolysis of (**3**) can be accounted for by solvent attack at the 1-position of the aromatic ring (the corresponding value [10] for 2,4,6-trinitroanisole is 5 × 10^{-6} s^{-1}). In the esterase catalysed pathway the value of K_M is lower for (**3**) than for 4-nitrophenyl acetate which may indicate the tighter binding of (**3**). That the value of V'_{max} is *ca.* 100 times smaller for (**3**) than for 4-nitrophenyl acetate might be attributed to the better leaving group ability of the 4-nitrophenoxide ion than of the picrylglycolate anion.

The ester (**3**) has been shown to be a useful alternative to 4-nitrophenyl esters for the study of enzymic hydrolyses.

Experimental

1-(2-Hydroxyethoxy)-2,4,-6-trinitrobenzene (**1**) was prepared as before [4] by reaction of picryl chloride with 1 mol equiv. of base in ethane-1,2-diol. Recrystallisation from water gave yellow needles, m.p. 60 °C (lit.,[4] 61 °C).

Acetylation of (**1**) produced the ester (**3**) in the following way. Compound (**1**) (1.1 g) was dissolved in the minimum quantity of dichloromethane and excess acetyl chloride (0.8 cm^3) was added. The mixture was stirred at 20 °C and the progress of the reaction was periodically monitored by TLC. After 12 h no starting material remained. Methylene dichloride (20 cm^3) was then added and the mixture was washed (×4) with small volumes of water until the washings were neutral. The organic layer was dried ($MgSO_4$) and the solvent removed under reduced pressure to give a pale yellow solid (0.8 g, 63%). Recrystallisation from methylene dichloride–pentane gave pale yellow crystals, m.p. 102 °C (Found: C, 37.9; H, 2.9; N, 12.8%. Calc. for $C_{10}H_9N_3O_9$: C, 38.1; H, 2.86; N, 13.3%).

4-Nitrophenyl acetate was a commercial specimen, and porcine liver esterase (EC 3.1.1.1) and α-chymotrypsin (EC 3.4.21.1) type II were obtained from the Sigma Chemical Co. Enzymes and enzyme solutions were stored under refrigeration. UV-vis. spectra and kinetic measurements were made using Perkin-Elmer Lambda 2 or Lambda 3 instruments or, for rapid reactions, with a Hi-Tech SF-3L spectrophotometer. First order rate coefficients and initial velocities are precise to ± 5%.

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