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Simple but efficient models for nuclease catalysis

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Abstract: The dianions of hydroxyphosphate diesters (**3**) designed to have high effective molarities for intramolecular cyclisations are hydrolysed in water at 50°C with half-lives as short as 40 ms. General acid catalysis is characterised in detail for a methyl ester. An important electrostatic effect, observed for general acids with a suitably positioned second NH^+ group, enhances the rate of the loss of methoxide from the dianion by 100-fold even in water. The data allow an estimate of 10^4 - 10^5 for the factor by which protonation to give the neutral acid activates a phosphate diester anion towards attack by a neighbouring OH group. The results define basic requirements for efficient catalysis of the reaction, which is common to many enzymes which process nucleic acids.

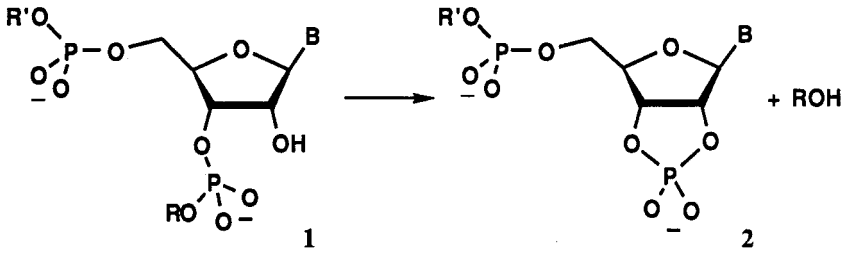
INTRODUCTION

Of the phosphate esters which play a role in living systems, diesters are the most important - and the least reactive. The stability towards hydrolysis of DNA is crucial to the conservation of the genetic information, and even RNA, though readily hydrolysed in alkali, is cleaved only very slowly at pH 7 in the absence of a relevant enzyme. This hydrolysis is in fact so slow that good quality data on catalysis of the reaction are difficult to obtain. This in turn makes it difficult to draw firm conclusions about mechanism, and there is conflicting evidence on the status - transition state or intermediate - of the pentacovalent species involved in such reactions.

Two factors have given new impetus to the field. One is the work of Breslow and his co-workers, who measured the very slow hydrolysis of ribonucleoside derivatives (for example, UpU) by a method based on HPLC separation of the products (ref. 1). This work has been criticised (ref. 2), and the data are necessarily of limited accuracy, and in some cases inconsistent with other published data (ref. 3), so cannot support a unique interpretation. But they break new ground, and lead to interesting suggestions for the mechanism of the hydrolysis reaction which deserve careful consideration. The work described in this paper addresses two of these in particular: the details of general acid-base catalysis of the reaction in which a neighbouring OH group attacks the phosphorus centre of a phosphate diester, and the suggestion of Breslow and his co-workers that the substrate for the initial reaction is the phosphoric acid rather than the anion of the phosphate diester.

The second important new factor is the explosive growth in the number of enzymes known to 'process' nucleic acids, catalysing various sorts of sequence-modifications. These include recombinases (over 2,000 are now known: ref. 4), topoisomerases (ref. 5) and integrases (ref. 6); and even non-enzymes like ribozymes (ref. 7). All appear to involve a common mechanism by which phosphate transfer takes place by a two step process via a phosphoryl enzyme (or ribozyme) intermediate. The catalytic nucleophilic centre is typically a hydroxyl group, of a nucleotide ribose residue in the case of a ribozyme and of a serine or tyrosine side-chain of the case of the enzyme reactions. In none of these cases does reaction involve the formation of a five-

membered ring, as in the ribonuclease reaction (where **1** → **2** is the initial step), so a further question of interest is whether the special structural features of ribonucleotides favour a unique mechanism. (The Breslow mechanism (ref. 1) involves mechanistically unsymmetrical partitioning of a pentacovalent intermediate.)



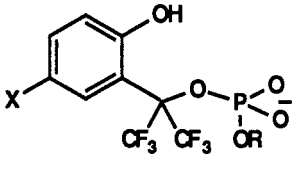
1 **2**

The fixed geometry of the neighbouring 2′-hydroxy group of ribonucleotides enhances but also limits their reactivity to hydrolysis. Our work on the efficiency of intramolecular nucleophilic catalysis (ref. 8) shows that the dominant feature controlling reactivity is the thermodynamic driving force for ring formation. It is to be expected that systems where cyclisation is thermodynamically more favourable than for **1** → **2** will undergo faster cyclisations, so we are looking at a number of phosphate esters designed to show high reactivity in reactions catalysed by neighbouring OH groups. We aim to define and understand the relevant mechanisms, and the factors controlling the efficiency of their catalysis by general acids and bases.

DESIGN OF A MODEL PHOSPHODIESTER

We report here some results of a detailed study of the hydrolysis of one such ester (**3**) and its three derivatives shown (**3a-c**). **3** is designed to show an effective molarity (ref. 8) large enough to allow us to observe the displacement of a simple alkoxy group from phosphorus in the absence of complications from the ionisation of the nucleophilic hydroxyl; which in the case of a phenol can be fully ionised well within the pH range. The fully ionised phenolate models a partially deprotonated alcohol OH group, and allows us to study the reaction in the absence of complications from an initial deprotonation step. The phenol chromophore makes it possible to follow the reaction continuously, while the two CF_3 groups are included to minimise competition from the loss of phosphate by C—O cleavage (i.e. an S_N1 reaction).

The design was successful: we see no evidence of C—O cleavage products under any conditions. **3** (the least reactive compound we have studied) is hydrolysed over 50,000 times faster than UpU in imidazole-buffered solution, and its hydrolysis is strongly catalysed by general acids and bases. The dianion of **3** has a half-life of 2.4 minutes at 50°C, while that of **3c**, with a better leaving group (the pK_a of trifluoroethanol is 12.43) can be estimated at less than 40 ms (ref. 9). This discussion will be limited to the reactions of the simplest methyl ester **3**.

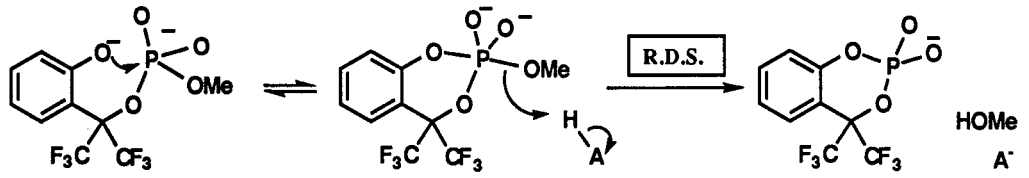


3, X = H, R = CH_3
3a, X = OMe, R = CH_3
3b, X = H, R = CH_2CH_2F
3c, X = H, R = CH_2CF_3

3 and its derivatives are also designed to ensure that the elimination of the leaving group (RO^-) should be the rate determining step of the reaction for both reactions of interest near pH 7, the hydrolyses of the mono and the dianions. From the kinetic evidence (ref. 9) this appears to be the case.

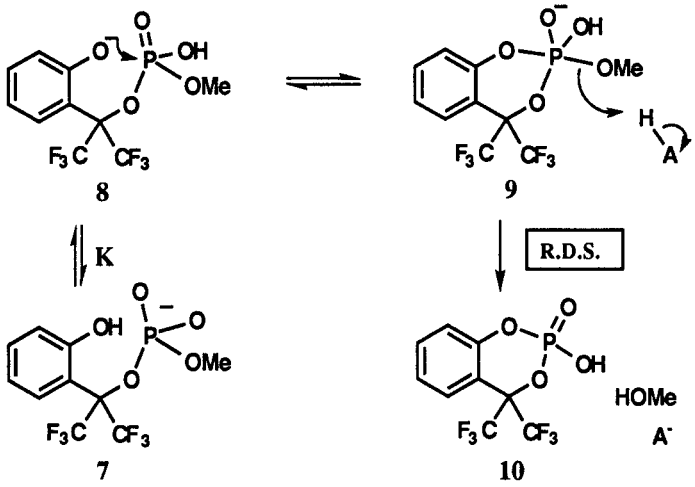
HOW EFFECTIVELY DOES PROTONATION ACTIVATE A DIESTER?

The reaction of the dianion 4 involves simple intramolecular nucleophilic catalysis:



4 5 6

while the reaction of the monoanion 7 is expected to involve the minor tautomeric form 8, with the mobile proton on the phosphate group.

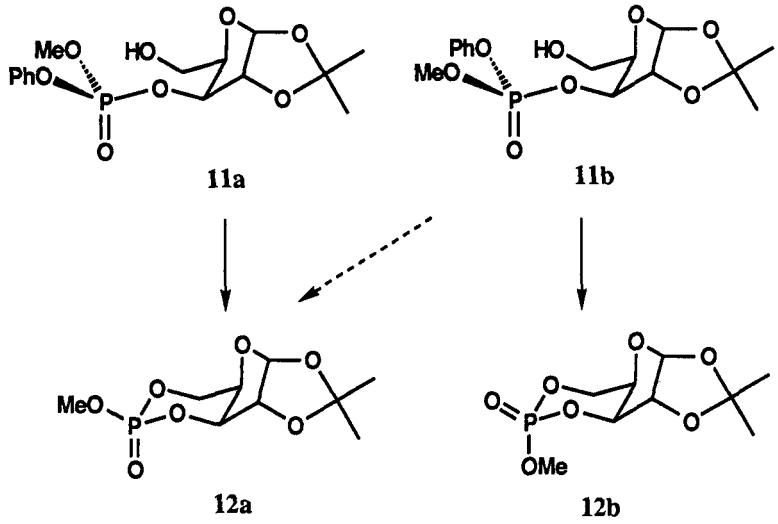


8 9 7 10

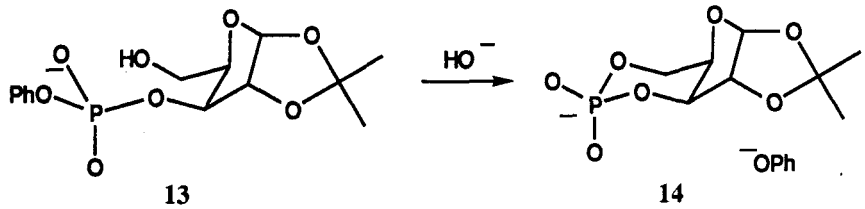
If this mechanism is correct, the rate constants for the reactions of the two ionic forms allow a simple estimate of the effect of protonation on the reactivity of the phosphate diester anion, since the pK_a's of the phosphoric acid and the phenol OH groups are known. The magnitude of the effect depends on the general acid involved (HA in 5 and 9), and lies in the range 490 (HA = H_2O) to 5.5×10^5 (HA = H_3O^+), with a figure of 8×10^4 for imidazolium.

These figures are significantly larger than rate ratios estimated previously for series' of phosphate di- and triesters of 2,4-dinitrophenol (Table), which provided early evidence for electrostatic repulsion as a factor decreasing the reactivity of diester anions towards negatively charged nucleophiles (ref. 10). The new calculation depends on a reasonable but not infallible interpretation of the kinetic ambiguity of the position of the proton in the reaction, and the result is important enough for us to look for independent confirmation of the result. The earlier comparison involved the kinetically unambiguous neutralisation of the diester anion by an alkyl group, so we have applied the same test to an intramolecular reaction with a nucleophilic hydroxyl group (ref. 11). Triesters based on the diesters 3 would be inconveniently reactive, so we chose the xylose-based system (11) for this investigation. As for 3 the reaction of 11 involves the formation of a six-membered cyclic ester.

The separate diastereoisomers **11a** and **11b** give the products of inversion at phosphorus, **12a** and **12b**, almost exclusively (only **11b** gives a few percent of the retention product **12a**).



As expected, these reactions are general base catalysed, and catalysis of this reaction will be described in a later paper. For the present purpose only the hydroxide-catalysed reaction is relevant, as this is the only reaction that is fast enough to measure for the corresponding diester **13**.



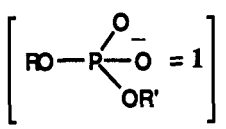
The second order rate constants for the cyclisations of the two triesters **11** are over 10^5 times greater than for **13** (Table), confirming the figure estimated from the reactions of the two ionic forms of **3**. Since the dissociation constants of a phosphate diester and a general base with a pK_a near 7 differ by 10^5 - 10^6 this indicates that the kinetic advantage of protonating a diester anion is marginal in free solution, though local environmental effects in an enzyme active site could act to make it significant.

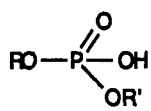
CATALYSIS BY GENERAL ACIDS OF THE DISPLACEMENT OF METHOXIDE.

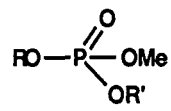
One of the most important results of this work is that it allows us to observe and study the step in which a poor leaving group departs from the phosphorus centre of a phosphate diester, without complications from other reactions. We have therefore carried out a detailed study of the reaction of **3**. The displacement of methoxide, whether direct or by way of a pentacovalent intermediate as in (**5**), is expected to be assisted by protonation, and strong catalysis is observed for a wide range of general acids HA.

We have measured second order rate constants for catalysis of the cyclisation of **3** by over 30 general acids, and find general acid catalysis of the reactions of both mono - and dianions (the

Table. Relative Rates of Nucleophilic Attack on Phosphate Diester Anions and Acids







(Intermolecular attack by) Nucleophile (ref. 10)

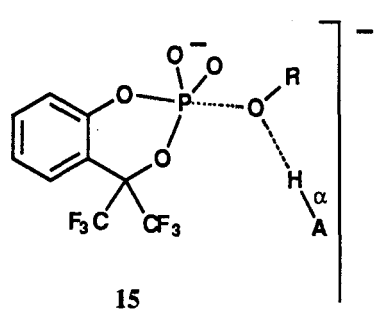
H_2O	26	Leaving group R'O⁻ = 2,4-dinitrophenoxy
Acetate	2000	
Phosphate dianion	4000	
Pyridines	2 - 40	
Hydroxide	2300	

Intramolecular attack by OH (This work)

This work: general acid	H_3O^+	490	Leaving group methoxy
	HOAc	2×10^5	
	H_2O	5×10^5	
This work, hydroxide reaction		2×10^5	Leaving group phenoxy

latter observed as the kinetically equivalent general base catalysis at lower pH where the monoanion is the predominant ionic form). Reactivity depends simply on the concentration and the degree of ionisation of the catalytic species, and in no case do we observe curved buffer plots.

Some of the data are collected in the Brönsted plot shown (Figure 1). The plot of all the data shows extensive scatter. This is somewhat unusual for general species catalysis, and indicates that the effectiveness of a general acid depends not only on its pK_a, but on other factors also. The most significant of these factors appears to be electrostatic. The data for 8 primary alkylammonium cations, spanning a range of almost 6 pK_a units, are correlated by a good straight line (with a slope, corresponding to a Brönsted α of -0.33. This is consistent with the Brönsted β of 0.67 obtained by Davis, Hall and Williams (ref. 12) for the hydrolysis of 4-nitrophenyl uridine 3'-phosphate catalysed by amine general bases, where the rate determining step (**16**) is mechanistically the microscopic reverse of that for the reaction of **3**)



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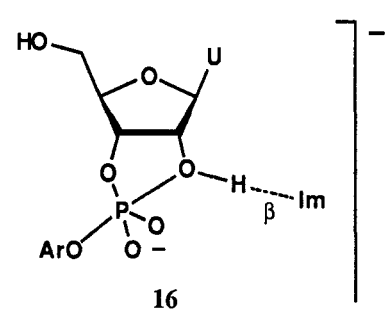

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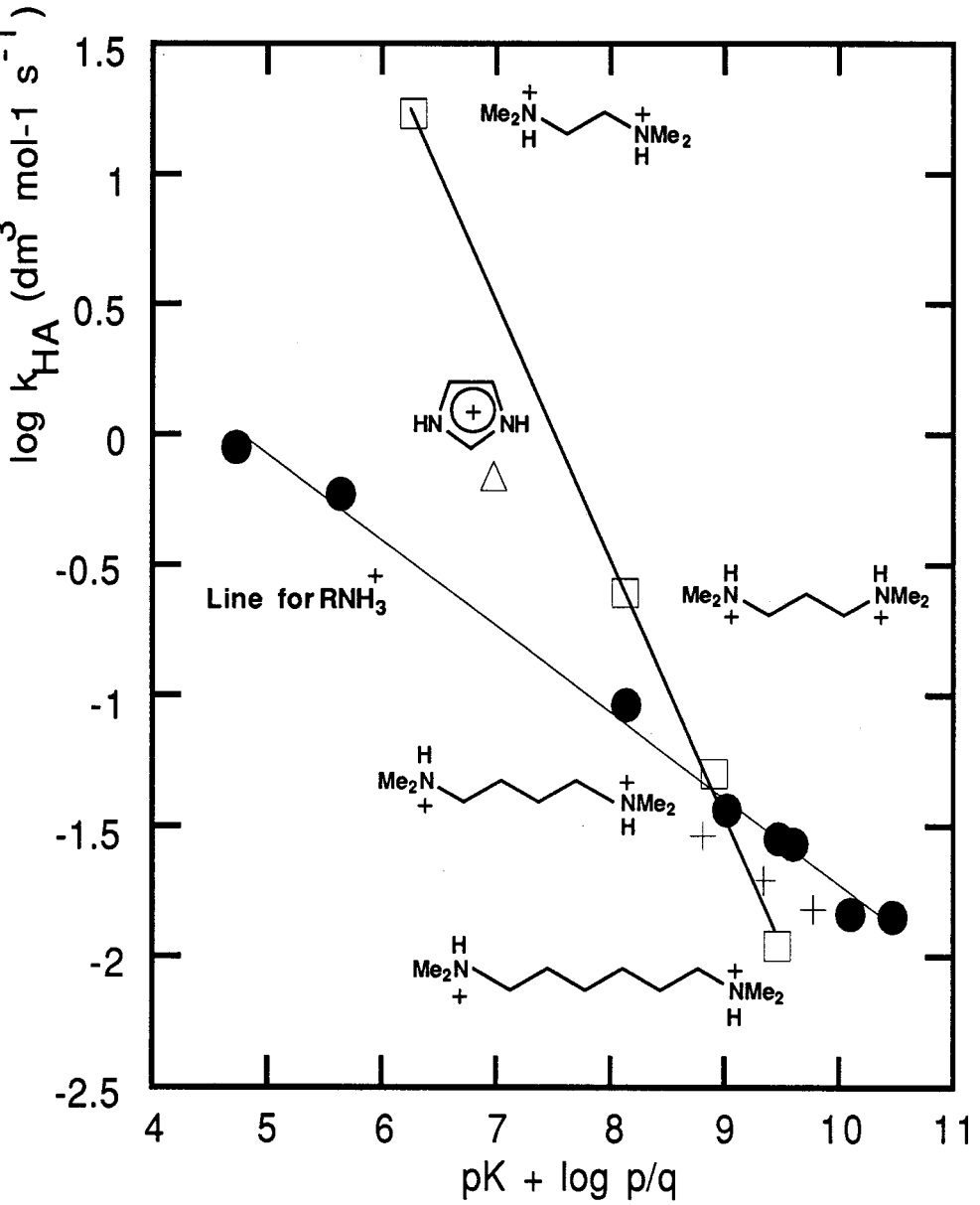


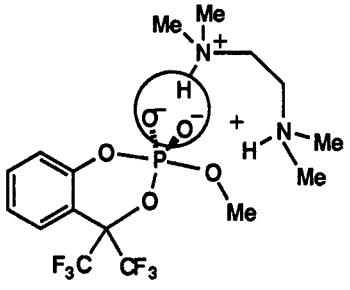
Figure 1. Statistically corrected Brønsted plot for the general acid catalysed cyclisation of **3** at 50° and ionic strength 1.0M (KCl) in H_2O, by: primary and secondary amines (●), tertiary amines (+) and tetramethylalkylylene diamines (open squares).

The marked deviations from this line appear to be associated primarily with the charge on the general acid. Neutral and especially anionic oxyacids show negative deviations, while the largest positive deviations are shown by diamine dications and by hydroxylammonium. This is

most dramatically demonstrated by the direct correlation (Figure 1) between the rate enhancement (the postive deviation) and the distance between the second positive charge and the general acid centre. For the series of dications $Me_2NH^+(CH_2)_nNH^+Me_2$, with n = 2,3,4 and 6, the rate enhancement increases with decreasing n, to such an extent that the apparent Brönsted α for the series is 0.99. (This value, equal within experimental error to unity, is consistent with a rate determining diffusion-controlled proton transfer, but this possibility was ruled out by an experiment in which the viscosity of the solvent was increased without a proportionate decrease in the rate of the reaction catalysed by the TMEDA dication.) The effect on α of increasing the proximity of the positive charge is thus twice that of increasing the strength of the general acid.

The monocations derived from these bases define normal behaviour for tertiary ammonium cations: they are correlated by a line of slope similar to that drawn (Figure) for alkylammonium cations, and the positive deviation from this line for the tetramethylethylenediamonium cation corresponds to a rate factor of over 100. The effect in terms of free energy of activation is thus worth up to 12.3 kJ dm^3 mol^{-1} even in water. Assuming a simple point charge model for electrostatic stabilisation, an effective active site dielectric constant half that of bulk water would double this figure, equivalent to a factor of 10^4 in rate.

The mechanism of this effect is of great interest. It can be presumed to stabilise the high concentration of negative charge on the pentacovalent transition state, and intermediate if one is involved, either electrostatically (through space) or by hydrogen bonding. The evidence suggests that both factors may be involved: when the second proton of the tetramethylethylenediammonium dication is replaced by a methyl group much but not all of the rate enhancement disappears. Thus the rate constant for catalysis by the pentamethylethylenediammonium cation is 28 times smaller than that for the tetramethylethylenediammonium dication, though still some 6-fold greater than expected for a tertiary ammonium cation of the same pK_a. The simplest explanation of these results is that the two effects - both normally weak in water - reinforce each other, possibly as in **17**: here (circled) intramolecular hydrogen bonding is strengthened by ion-pair association, which in turn is favoured by the close approach of the opposite charges made possible by the formation of the (possibly bifurcate) hydrogen bond (impossible for the $^+NMe_3$ group).



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This substantial effect on the rate of the combination of general acid and positive charges gives us specific leads for the development of more efficient catalysts for this important reaction, and we are currently extending the work to more complex polycation catalysts. Many groups are currently working on metal-catalysed cleavage reactions of diesters, particularly DNA, and it will be interesting to combine the two functionalities: as may well happen in some newly emerging phosphodiesterases which appear to use zinc in the three-metal active site motif found in alkaline phosphatase (ref. 13). In preliminary experiments at pH 4.7 we have shown substantial catalysis of the hydrolysis of **3** even by the hydrated zinc cation (Figure 2). If, as seems likely, this catalysis involves the dianion, which is present in only minute concentration at this pH, it is very efficient indeed.

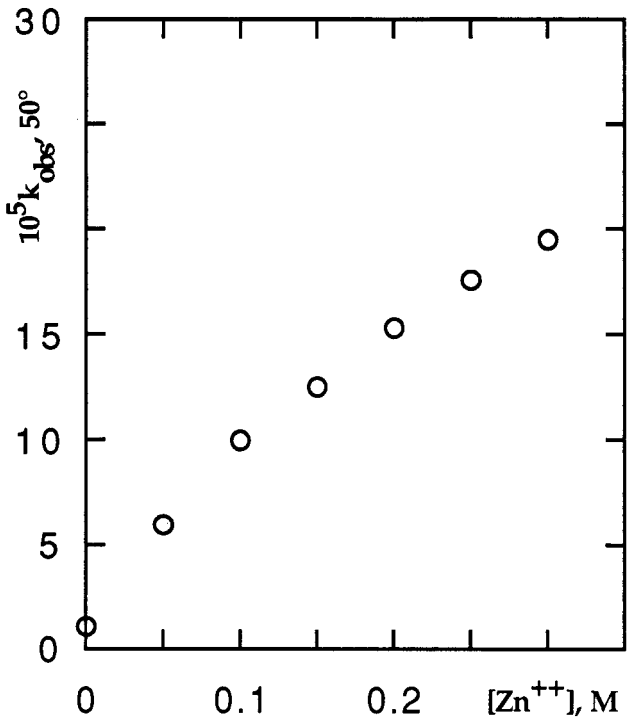

Figure 2. Catalysis by the hydrated zinc cation of the hydrolysis of the diester **3**, in 0.2M acetate buffer, pH 4.7, at 50° and ionic strength 1.0M.

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References

1. R. Breslow, E. Anslyn and D.-L. Huang, *Tetrahedron*, **47**, 2365 (1991).
2. F. Menger, *J. Org. Chem.* **56**, 6251 (1991). A. Haim, *J. Am. Chem. Soc.* **114**, 8384 (1992).
3. K. N. Dalby. Thesis, Cambridge, 1991.
4. S. E. Halford, pesonal communication.
5. A. Landy, *Ann. Rev. Biochem.* **58**, 913 (1989).
6. J. C. Wang, *Ann. Rev. Biochem.* **54**, 665 (1985).
7 T. R. Cech and B. L. Bass, *Ann. Rev. Biochem.* **55**, 599 (1986).
8. A. J. Kirby, *Adv. Phys. Org. Chem.* **17**, 183 (1980).
9. K. N. Dalby, A. J. Kirby and F. Hollfelder, *J. Chem. Soc., Perkin Trans. 2*, (1993). *In press*. A preliminary report of some of this work appeared in *J. Chem. Soc., Chem. Commun.*, **1992**, 1770.
10. A. J. Kirby and M. Younas, *J. Chem. Soc., Sect. B* 1165 (1970).
11. Unpublished work with F. Hollfelder, F. O'Carroll and K. R. Strömberg.
12. A.M. Davis, A.D. Hall and A. Williams, *J. Am. Chem. Soc.*, **110**, 5105 (1988).
13. J. E. Coleman, *Ann. Rev. Biochem.* **61**, 897 (1992).