General and specific acid/base catalysis of the hydrolysis and interconversion of ribonucleoside 2′- and 3′-phosphotriesters: kinetics and mechanisms of the reactions of 5′-*O*-pivaloyluridine 2′- and 3′-dimethylphosphates

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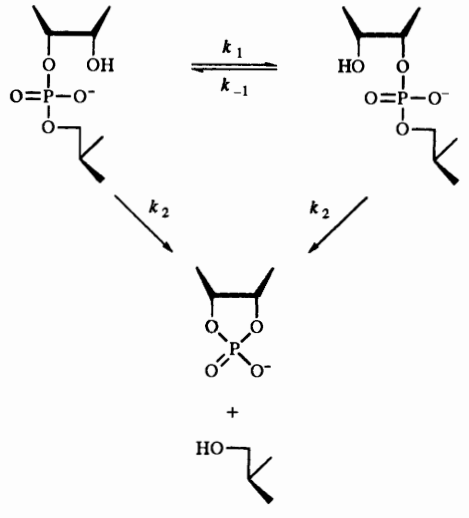
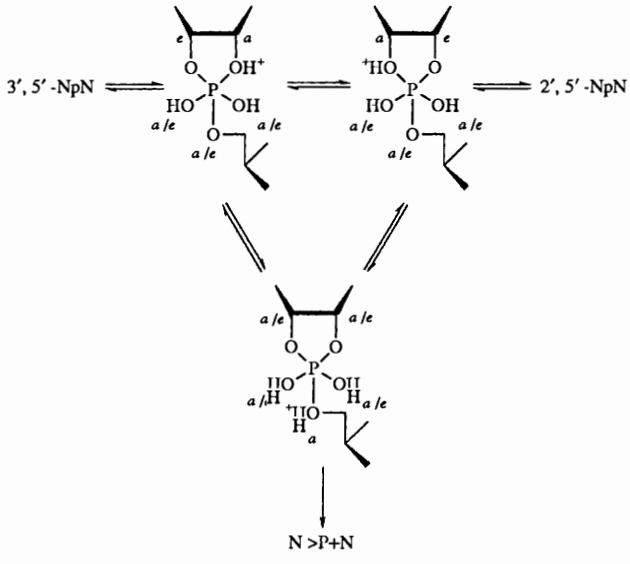
Kinetics of the hydrolysis and interconversion of 5′-*O*-pivaloyluridine 2′- and 3′-dimethylphosphates in the pH range 0–9 have been studied. At pH < 2, both reactions are first order in hydronium ion concentration, the hydrolysis being three times as fast as the interconversion. The interconversion, however, becomes hydroxide-ion-catalysed at pH as low as 2.5, whereas the hydrolysis remains pH independent to pH 7, and becomes then base-catalysed. Both reactions are susceptible to general-base catalysis, the Brønsted β values based on carboxylate ions being 0.75. These observations suggest that the monocationic phosphorane intermediate, obtained by the attack of 2′-OH on the phosphotriester monocation, is decomposed to the hydrolysis and isomerization products at a comparable rate. By contrast, the monoanionic phosphorane, obtained by the attack of 2′-O^- on the neutral phosphotriester, predominantly gives isomerization products; the methoxide ion leaves 10^5 times less readily than the sugar oxyanions, 2′-O^- or 3′-O^-. Accordingly, the pH-independent hydrolysis appears to consist of consecutive specific base/acid catalysis. The buffer-catalysed reactions are suggested to proceed by general-base-catalysed attack of 2′-OH on neutral phosphotriester, followed by general-acid-catalysed decomposition of the phosphorane intermediate to either hydrolysis or isomerization products. The mechanisms of the hydrolysis and isomerization of the internucleosidic phosphodiester bonds are discussed on these bases.

The internucleosidic phosphodiester bonds of polyribonucleotides[1,2] and their dimeric fragments, *viz.*, 3′,5′-dinucleoside monophosphates,[3–7] undergo two concurrent reactions over a wide acidity range: (*i*) hydrolysis to a cyclic 2′,3′-monophosphate with release of the 5′-linked nucleoside, and (*ii*) intramolecular transesterification to a 2′,5′-phosphodiester bond (Scheme 1). It is most likely that both reactions proceed



Scheme 1

via a common pentaco-ordinated phosphorane intermediate that is obtained by an intramolecular nucleophilic attack of the neighbouring 2′-hydroxy group on the tetraco-ordinated phosphorus. Consistent with this assumption, the pH–rate profiles of the hydrolysis and phosphate migration are very similar at pH < 2,[6] indicating that the reactive ionic form is, in both cases, the phosphodiester monocation. In other words, the phosphorane intermediate is formed by the attack of unionized sugar hydroxy function on the monocationic phosphodiester, and decomposed by the departure of protonated oxygen ligand, either 2′-OH, 3′-OH or 5′-OH (Scheme 2). The breakdown of the phosphorane intermediate must be rather symmetrical, since the hydrolysis and phosphate migration proceed under these conditions at a comparable rate;[6] 2′-, 3′- and 5′-hydroxy functions all leave as alcohols approximately as readily. Evidently this, to a large extent, results from the fact that the



a = apical ligand; *e* = equatorial ligand; *a/e* = apical or equatorial ligand

Scheme 2

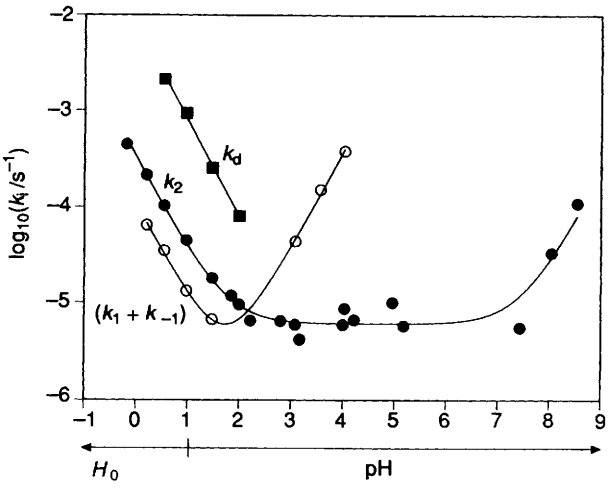


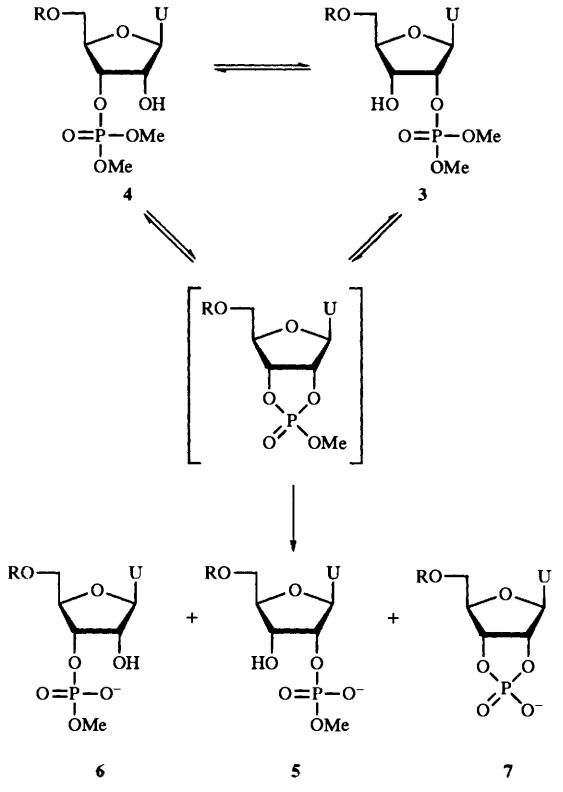
Fig. 1 pH–rate profiles for the hydrolysis (filled circles) and interconversion (open circles) of 5′-*O*-pivaloyluridine 2′- and 3′-dimethylphosphate at 298.2 K. The points at pH > 2 refer to rate constants at zero buffer concentration and ionic strength 1.0 mol dm^{-3} (adjusted with sodium chloride). The points at pH < 2 refer to rate constants obtained in aqueous hydrogen chloride, the ionic strength of which was not adjusted. Filled squares refer to removal of the 2′-*O*-tetrahydropyranyl group from the protected starting material.

leaving-group effects on the protonation and heterolysis steps are opposite: the less basic a particular esterified oxygen is, the more polarized, and hence weaker, the respective PO bond is.

Somewhat unexpectedly, the partition of the phosphorane intermediate to hydrolysis and migration products becomes markedly unsymmetrical in less acid solutions. While the hydrolysis exhibits a first-order dependence on the hydronium ion concentration in the pH range 2.5–4.5, the migration is almost pH-independent at pH > 3.[6] At pH 4.5, the migration is 30 times as fast as hydrolysis. A plausible mechanistic explanation for this dissimilarity of the pH–rate profiles appears necessary to maintain the concept of a common phosphorane intermediate. To learn more about the mechanistic details of the reactions of phosphodiester bond under mildly acidic conditions, where the reactive ionic form may be neutral or monoanionic phosphodiester instead of monocation, we have studied the hydrolysis and intramolecular transesterification of nucleoside 2′- and 3′-phosphotriesters. These compounds may be regarded as mimics of the neutral ionic form of phosphodiesters; the rapidly exchangeable proton has been replaced by an alkyl group. In addition to specific acid/base catalysis, attention has been paid to general acid/base catalysis, since these studies may, in part, be useful in attempting to solve the existing controversy on imidazole-catalysed hydrolysis of RNA: according to Breslow[3–5] the formation of the phosphorane intermediate and its decomposition are general-acid-catalysed reactions, whereas the breakdown of the intermediate to hydrolysis products is general-base-catalysed. This sequential bifunctional mechanism has been recently criticized,[8,9] but also defended[7] by additional measurements. The previous investigations on intramolecular transesterification of nucleoside 2′/3′-phosphotriesters, accompanying their hydrolysis, include the early work of Brown *et al.*[10] with uridine 3′-dimethylphosphate, the semiquantitative observations of Sekine *et al.*[11] on migration of the di-*tert*-butoxyphosphoryl group, and our preliminary note on the kinetics of the hydrolysis and interconversion of 5′-*O*-methyluridine 2′- and 3′-dimethylphosphates.[12] In addition, de Rooji *et al.*,[13] Reese and Skone,[14] and Pathak and Chattopadhyaya[15] have reported on the hydrolytic stability of the aryl esters of dinucleoside 3′,5′-monophosphates towards deblocking of the 2′-hydroxy function.

Results and discussion

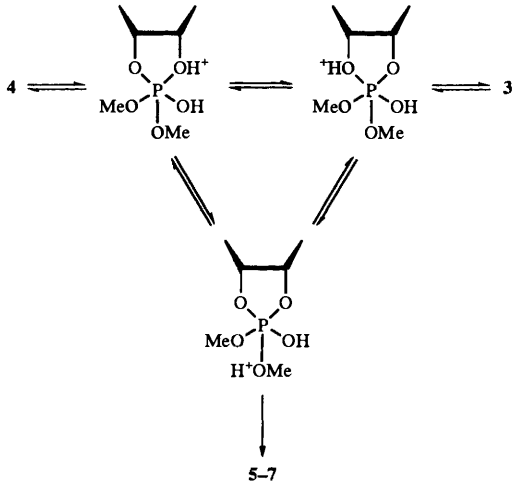
We have shown previously[12] that the dimethyl esters of 5′-*O*-methyluridine 2′- and 3′-monophosphates (**3** and **4**, R = Me) undergo at pH < 5 concurrent mutual isomerization and hydrolysis to the corresponding monomethyl esters (**5**, **6**) and a 2′,3′-cyclic monophosphate (**7**) (Scheme 3). In aqueous hydrogen chloride at pH < 2, the hydrolysis was observed to be approximately three times as fast as the phosphate migration, while in carboxylate buffers around pH 4 the situation was the opposite: the rate of migration was more than ten times that of hydrolysis. However, the rate constants obtained in the buffer solutions were not determined as a function of buffer concentration. Accordingly, the values obtained contained a contribution of buffer catalysis, and hence firm mechanistic conclusions concerning the pH-independent and base-catalysed reactions could not be drawn on the basis of the pH–rate profile.



Scheme 3

Fig. 1 shows the pH–rate profile obtained in the present work for the hydrolysis and interconversion of 5′-*O*-pivaloyluridine 2′- and 3′-dimethylphosphates (**3** and **4**, R = pivaloyl). As described previously,[12] the reactions were initiated by acid-catalysed hydrolysis of the 2′-acetal protection (2′-*O*-tetrahydropyranyl group), and the pH was then rapidly adjusted by adding calculated amounts of the desired buffer constituents. All the rate constants indicated in Fig. 1 refer to zero buffer concentration. Kinetic data for the deprotection step are also included. The rate constants obtained for the hydronium-ion-catalysed hydrolysis and interconversion of 5′-*O*-pivaloyluridine 2′- and 3′-dimethylphosphates are to within 20% of those reported previously[12] for the corresponding 5′-*O*-methyl protected phosphotriesters. In other words, the 5′-*O*-protecting group does not affect the reactions of the phosphoester moiety.

The hydrolysis and phosphate migration are both first order in acidity at pH < 1, and the reactions most likely proceed *via* a monocationic phosphorane intermediate (Scheme 4), as suggested earlier for the acid-catalysed reactions of both



Scheme 4

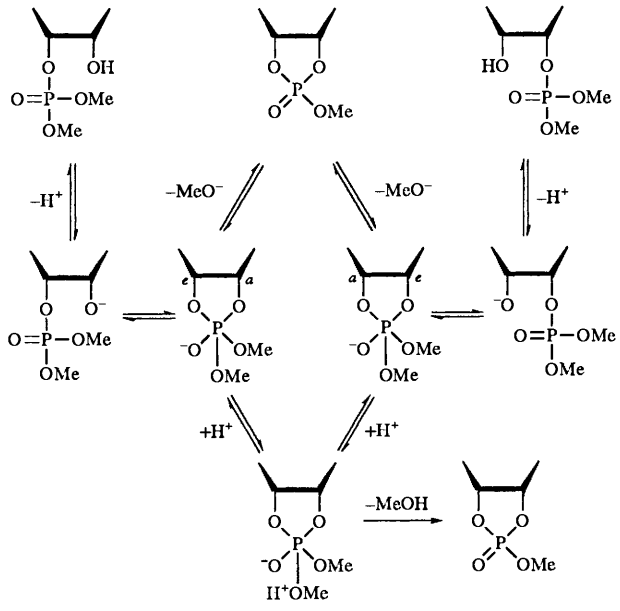
Table 1 Second-order rate constants, k_H, and the enthalpy and entropy of activation for the hydronium-ion-catalysed hydrolysis of 5′-*O*-pivaloyluridine 3′-dimethylphosphate[a]

T/K	$k_H/10^{-4}$ dm^3 mol^{-1} s^{-1}	$\Delta S^{\ddagger}$/J mol^{-1} K^{-1}[b]	$\Delta H^{\ddagger}$/kJ mol^{-1}
298.2	4.47 ± 0.09	−107 ± 9	60.1 ± 2.9
303.2	7.37 ± 0.08		
313.2	12.9 ± 0.3		
323.2	31.5 ± 0.8		
333.2	66.3 ± 2.8		
363.2[c]	406 ± 41		

[a] The first-order rate constants, k_2, were obtained in 0.1 mol dm^{-3} aqueous hydrogen chloride. [b] At 298.2 K. [c] Extrapolated value.

nucleoside phospho-diesters[6,16] and -triesters.[12] The second-order rate constant for the hydronium-ion-catalysed hydrolysis of the neutral ionic form of adenosine 3′-methylphosphate [3′-Ado-O-PO(OH)-OMe] has been reported to be 1.2×10^{-2} dm^3 mol^{-1} s^{-1} at 363.2 K.[16] With 5′-*O*-pivaloyluridine 3′-dimethylphosphate the corresponding value, when extrapolated to the same temperature, is 4×10^{-2} dm^3 mol^{-1} s^{-1} (Table 1). Accordingly, replacement of the hydroxy proton of phosphodiester with a methyl group does not seem to retard the attack of the 2′-OH on phosphorus, and hence the dimethyl ester, **3**, appears to be a reasonably good model for the neutral phosphodiester.

As seen from Fig. 1, the phosphate migration becomes hydroxide-ion-catalysed even from pH 2. The simplest mechanistic explanation is initial deprotonation of the 2′-OH and subsequent intramolecular attack of the resulting oxyanion on phosphorus (Scheme 5). On the basis of microscopic reversibility, the breakdown of the monoanionic phosphorane intermediate obtained would thus proceed by departure of 2′-O$^-$, or, after pseudorotation, 3′-O$^-$ which leads to phosphate migration. However, the data in Fig. 1 also show that the phosphoester hydrolysis remains pH-independent until pH 7, and becomes then hydroxide-ion-catalysed. If the migration and hydrolysis proceed *via* the same intermediate, which appears to be a reasonable assumption, the sugar hydroxyl groups as oxyanions are 10^5 times better leaving groups than methoxide ion. The dissimilar pH-dependence at pH 2–7 thus means that, under these conditions, the departure of the methoxy ligand is hydronium-ion-catalysed, while the better leaving groups, *i.e.*, 2′- and 3′-oxygen ligands, depart spontaneously as oxyanions (Scheme 5). Accordingly, the hydrolysis is pH-independent, and the migration exhibits an inverse dependence on acidity.



Scheme 5

The fact that the methoxide ion appears to depart from the phosphorane intermediate as much as 10^5 times less readily than the sugar hydroxy groups is somewhat unexpected, although the sugar hydroxy groups are certainly more acidic than methanol (pK_a 15.5),[17] and hence, as oxyanions, they are better leaving-groups. One might argue that breakdown of the phosphorane intermediate to a strained cyclic triester (hydrolysis) is a more difficult process than breakdown to an acyclic triester (migration). However, this hardly can be of major importance, since the partition of the phosphorane intermediate to hydrolysis and migration products is almost symmetrical under acidic conditions, where the oxygen ligands depart as alcohols. Anyway, the reluctant hydrolysis of nucleoside phosphotriesters compared with their intramolecular transesterification helps to understand the dissimilar pH-dependence of the corresponding reactions of internucleosidic phosphodiester bonds under mildly acidic conditions: migration is pH-independent and hydrolysis first-order in hydronium-ion concentration.[6] The preceding discussion suggests that the phosphorane intermediate is obtained as low as pH 2 by the attack of 2′-O$^-$ on the neutral phosphotriester centre, in spite of the fact that the mole fraction of this ionic form is extremely low under such conditions, probably of the order of 10^{-10}. This fact together with the observations of Chandler *et al.*,[18] according to which a neutral phosphoester is attacked by an anionic nucleophile more than 10^5 times faster than a monoanionic phosphate, strongly suggests that the pH-independent interconversion (and probably also hydrolysis) of dinucleoside 2′,5′- and 3′,5′-monophosphates proceeds analogously. In other words, the phosphorane intermediate is obtained by the attack of 2′-O$^-$ on neutral phospodiester, and not by the attack of 2′-OH on anionic phosphodiester. The dissimilar pH-dependence of the hydrolysis and migration at pH 2.5–4.5 would result from the fact (as with the phosphotriesters) that the 5′-oxygen ligand is unable to depart without protonation, while 2′-O$^-$ and 3′-O$^-$ can do so.

Figs. 2 and 3 show the rate constants obtained for the hydrolysis 5′-*O*-pivaloyluridine 3′-dimethylphosphate and its isomerization to the corresponding 2′-phosphotriester in various formic acid buffers. Both reactions are susceptible to buffer catalysis, the rate accelerations being more marked in 1 : 3 ([HCOOH]/[HCOO$^-$]) than in 3 : 1 buffers. In fact, a break down of the catalytic rate constant to the contributions of general-acid and -base catalysis by eqn. (1) suggests that the

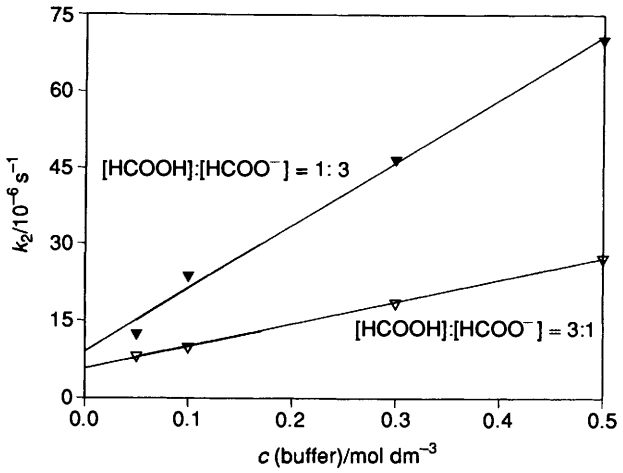


Fig. 2 First-order rate constants for the buffer-catalysed hydrolysis of 5′-*O*-pivaloyluridine 2′/3′-dimethylphosphate in formic acid/sodium formate buffers at 298.2 K. The ionic strength was adjusted to 1.0 mol dm^{-3} with sodium chloride.

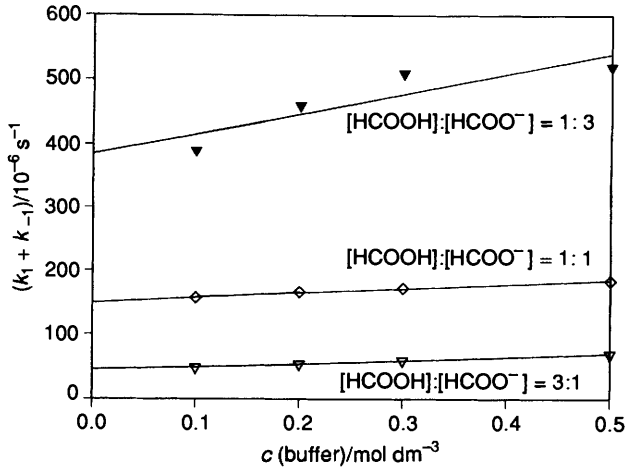
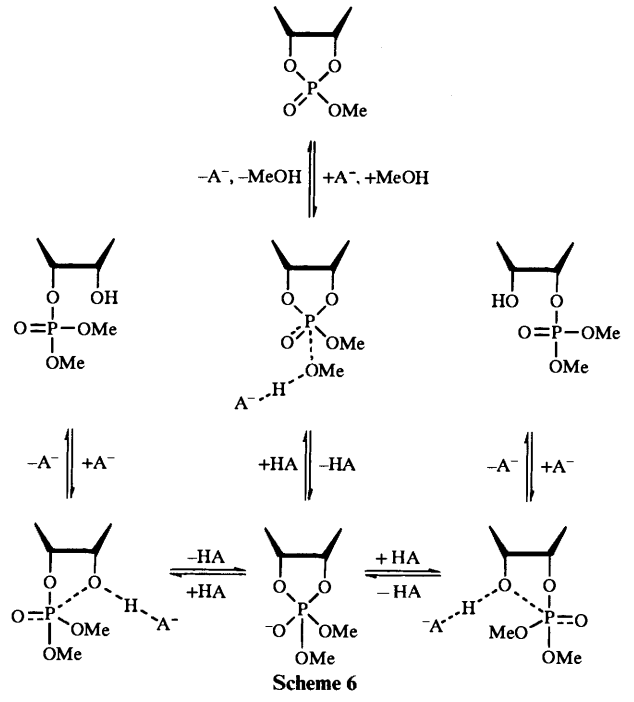


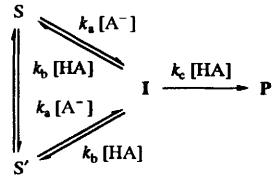
Fig. 3 First-order rate constants for the buffer-catalysed interconversion of 5′-*O*-pivaloyluridine 2′- and 3′-dimethylphosphates in formic acid–sodium formate buffers at 298.2 K. The ionic strength was adjusted to 1.0 mol dm^{-3} with sodium chloride.

$$k_{cat}([HA] + [A^-]) = k_{HA}[HA] + k_A[A^-] \quad (1)$$

formate ion is the catalytically active species. Catalysis by formic acid is much weaker, if present at all. In other words, both reactions appear to be general-base-catalysed, the k_A values being 1.65 10^{-4} dm^3 mol^{-1} s^{-1} and 8.3 10^{-5} dm^3 mol^{-1} s^{-1} (at 298.2 K) for the hydrolysis and migration, respectively. Since the rate constants extrapolated to buffer concentration zero (when [HCOOH]/[HCOO$^-$] = 1:3) are 8.8 10^{-6} s^{-1} for the hydrolysis and 3.9 10^{-4} s^{-1} for the migration, it is clear that the buffer constituents do not only catalyse the formation of the phosphorane intermediate, but also affect the product distribution, and hence the breakdown of the intermediate to the hydrolysis and migration products. Interestingly, the product distribution of the buffer-catalysed reaction closely resembles that of the hydronium-ion-catalysed reaction: hydrolysis is twice as fast as migration. A logical explanation is that the buffer-catalysed reactions also proceed by departure of the oxygen ligands as alcohols, not as alkoxide ions. Accordingly, the mechanism depicted in Scheme 6 is proposed. Attack of 2′-OH on the phosphotriester centre is general-base-catalysed, and the breakdown of the resulting monoanionic phosphorane is general-acid-catalysed. For the following reason only the general-base catalysis is observed experimentally. Application



of the steady-state approximation to the kinetic system depicted in Scheme 7 gives expression (2) for the concentration of the



$$[I] = \frac{k_a[A^-]([S] + [S'])}{(k_b + k_c)[HA]} \quad (2)$$

phosphorane intermediate, I. Here S and S′ denote the isomeric triesters, and P stands for the hydrolysis products. The rate of hydrolysis, for example, may hence be calculated from eqn. (3).

$$d[P]/dt = k_c[HA][I] = \frac{k_a k_c[A^-]([S] + [S'])}{(k_b + k_c)} \quad (3)$$

In other words, it depends only on [A$^-$]. Similar reasoning applies to the breakdown of I to the isomeric triesters, S and S′.

Table 2 summarizes the results obtained for the hydrolysis of the isomeric mixture of 5′-*O*-pivaloyluridine 2′/3′-dimethylphosphates in various carboxylic acid and amine buffers. The migration was, in most of these buffers, too fast to be followed, and that is why buffer-catalysed migration was not studied more extensively than indicated above. In acetate, propionate, triethanolamine and diethanolamine buffers the hydrolysis was catalysed only by the basic buffer constituent, as in formate buffers (Table 3). In most acidic buffers, *viz.*, in chloroacetate and cyanoacetate buffers, catalysis by the acidic buffer constituent also contributed. Application of the Brønsted catalysis law to the catalysis by carboxylate anions gave a β value of 0.75 (Fig. 4). Accordingly, the proton transfer from the 2′-OH to the carboxylate ion appears to be rather advanced in the transition state.

Table 2 First-order rate constants for the hydrolysis of 5′-*O*-pivaloyluridine 2′/3′-dimethylphosphates in buffer solutions at 298.2 K (I = 1.0 mol dm^{-3} with NaCl)

Buffer acid	[HA]/mol dm^{-3}	[A$^-$]/mol dm^{-3}	k_2/10^{-4} s^{-1}
Cyanoacetic acid	0.075	0.025	0.130 ± 0.004
	0.150	0.050	0.154 ± 0.005
	0.225	0.075	0.171 ± 0.004
	0.375	0.125	0.197 ± 0.006
	0.0125	0.0375	0.071 ± 0.002
	0.025	0.075	0.079 ± 0.003
	0.075	0.225	0.114 ± 0.002
	0.125	0.375	0.139 ± 0.003
Chloroacetic acid	0.0375	0.0125	0.067 ± 0.002
	0.075	0.025	0.082 ± 0.002
	0.225	0.075	0.113 ± 0.002
	0.375	0.125	0.135 ± 0.002
	0.0125	0.0375	0.056 ± 0.001
	0.025	0.075	0.065 ± 0.002
	0.075	0.225	0.112 ± 0.002
	0.125	0.375	0.146 ± 0.002
Formic acid	0.0375	0.0125	0.083 ± 0.002
	0.075	0.025	0.100 ± 0.004
	0.225	0.075	0.185 ± 0.004
	0.375	0.125	0.271 ± 0.006
	0.0125	0.0375	0.126 ± 0.004
	0.025	0.075	0.237 ± 0.009
	0.075	0.225	0.467 ± 0.014
	0.125	0.375	0.702 ± 0.008
Acetic acid	0.0375	0.0125	0.140 ± 0.002
	0.075	0.025	0.205 ± 0.004
	0.225	0.075	0.518 ± 0.015
	0.375	0.125	0.825 ± 0.020
	0.0125	0.0375	0.378 ± 0.005
	0.025	0.075	0.558 ± 0.013
	0.075	0.225	1.53 ± 0.04
	0.125	0.375	2.55 ± 0.02
Propionic acid	0.0375	0.0125	0.155 ± 0.004
	0.075	0.025	0.250 ± 0.006
	0.225	0.075	0.613 ± 0.012
	0.375	0.125	0.973 ± 0.012
	0.0125	0.0375	0.305 ± 0.014
	0.025	0.075	0.702 ± 0.030
	0.075	0.225	1.76 ± 0.04
	0.125	0.375	2.96 ± 0.04
Triethanolammonium ion	0.040	0.010	0.182 ± 0.009
	0.060	0.015	0.245 ± 0.008
	0.080	0.020	0.310 ± 0.018
	0.100	0.025	0.372 ± 0.013
	0.025	0.025	0.598 ± 0.016
	0.0375	0.0375	0.745 ± 0.007
	0.0500	0.0500	0.823 ± 0.016
	0.0625	0.0625	1.02 ± 0.01
Diethanolammonium ion	0.040	0.010	5.47 ± 0.35
	0.060	0.015	7.92 ± 0.14
	0.080	0.020	11.6 ± 0.2
	0.100	0.025	12.0 ± 0.3

The amine-based buffers catalyse the phosphotriester hydrolysis two to three orders of magnitude less efficiently than the Brønsted plot based on the carboxylate buffers suggests. The reason for this remains obscure. One should note, however, that these buffers are already so basic that the hydrolysis tends to proceed by the departure of methoxide ion, which makes the determination of the rate constants of buffer catalysis susceptible to errors arising from medium effects.

In summary, the hydrolysis and mutual isomerization of nucleoside 2′- and 3′-phosphotriesters proceed under acidic conditions (pH < 1.5) *via* a monocationic phosphorane intermediate obtained by the attack of the neighbouring hydroxy function on the phosphotriester monocation. The intermediate is decomposed to the hydrolysis and isomerization products at a comparable rate. In buffer solutions (pH > 2.5), the attack of the hydroxy function on the neutral phosphotriester centre is susceptible to general-base catalysis, the Brønsted β value based on carboxylate ions being 0.75. Again the sugar hydroxy groups and the esterified alcohol leave the intermediate at a comparable rate, and the departure of all the oxygen ligands is general-acid-catalysed. In the absence of buffer constituents, the migration becomes much faster than the hydrolysis. The

Table 3 Catalytic constants for the buffer-catalysed hydrolysis of 5′-*O*-pivaloyluridine 2′/3′-dimethylphosphates at 298.2 K (I = 1.0 mol dm^{-3} with NaCl)

Buffer acid	$k_{HA}/10^{-4}$ dm^3 mol^{-1} s^{-1}	$k_A^-/10^{-4}$ dm^3 mol^{-1} s^{-1}
Cyanoacetic acid	0.170	0.147
Chloroacetic acid	0.109	0.263
Formic acid	0.015	1.65
Acetic acid	—	6.02
Propionic acid	—	7.77
Triethanolammonium ion	—	11.7
Diethanolammonium ion	—	465

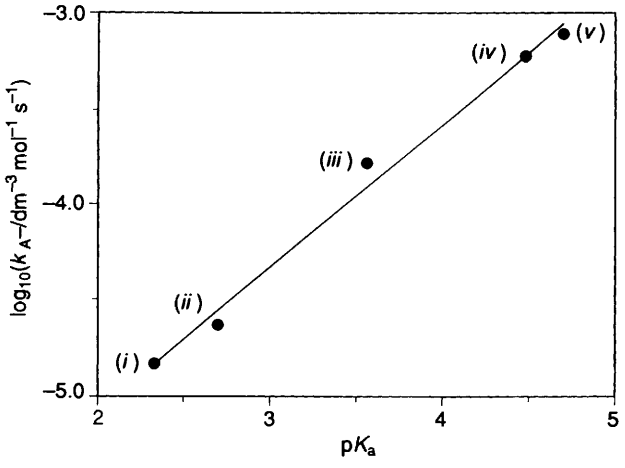


Fig. 4 Brønsted plot for the carboxylate-ion-catalysed hydrolysis of 5′-*O*-pivaloyluridine 2′-/3′-dimethylphosphate at 298.2 K (I = 1.0 mol dm^{-3} with NaCl): (*i*) cyanoacetate, (*ii*) chloroacetate, (*iii*) formate, (*iv*) acetate and (*v*) propionate ion

sugar hydroxy groups appear to leave the monoanionic phosphorane as oxyanions 10^5 times as readily as methoxide ion. Accordingly, while the phosphate migration becomes specific-base-catalysed at pH > 2.5, the hydrolysis rate remains pH-independent at pH 2.5–7, which may be interpreted mechanistically by consecutive specific base/acid catalysis.

Experimental

5′-*O*-Pivaloyl-2′-*O*-tetrahydropyranyluridine 3′-dimethylphosphate

3′,5′-*O*-Tetraisopropyldisiloxyluridine[19] was converted into its 2′-*O*-tetrahydropyranyl derivative by treatment with 3,4-dihydro-2*H*-pyran in acetonitrile in the presence of a catalytic amount of toluene-*p*-sulfonic acid,[20] and the tetraisopropyldisiloxyl group was removed by tetrabutylammonium fluoride in tetrahydrofuran.[19] The two diastereoisomeric forms of the product were separated on Silica gel 60, using a 1:9 (v/v) mixture of ethanol and dichloromethane as eluent [R_F(TLC) 0.43 and 0.34]. The overall yield starting from uridine was 33%.

The more polar diastereoisomer was acylated with 1.2 equiv. of pivaloyl chloride in pyridine,[21] and the desired product, 5′-*O*-pivaloyl-2′-*O*-tetrahydropyranyluridine, was isolated in 47% yield by column chromatography on Silica gel 60 (EtOH–CH_2Cl_2 1:9 v/v). The 3′-hydroxy function was phosphorylated with phosphoryltris(1,2,4-triazole) in acetonitrile,[22] and the remaining triazole ligands were replaced by treatment with an excess of methanol. The product was first isolated by adsorption chromatography on Silica gel 60 (EtOH–CH_2Cl_2 1:9 v/v) and then further purified by RP chromatography on a Lobar RP-18 C column, using a 1:3 (v/v) mixture of acetonitrile and water as the eluent. The yield was 25% (Found: C, 47.8; H, 6.0; N, 5.8. $C_{21}H_{33}N_2O_{11}P$ requires: C, 48.5; H, 6.4; N, 5.4%); $\delta_H(C^2HCl_3)$ 7.88 (1 H, s, H3), 7.38 (1 H, d, H6, $J_{H6,H5}$ = 8.2), 5.72 (1 H, dd, H5), 6.06 (1 H, d, H1′, $J_{H1',H2'}$ = 6.3), 4.44 (2 H, m, H2′ and H4′), 4.89 (1 H, m, H3′), 4.35 (1 H, dd, H5′, $J_{H4',H5'}$ = 3.9, $J_{H5',H5''}$ = 12.5), 4.27 (1 H, dd, H5″, $J_{H4',H5''}$ = 3.2 Hz), 3.78 and 3.81 (3 H, d, P–OCH$_3$, $J_{P,H}$ = 11,4 Hz), 1.24 (9 H, s, the pivaloyl group), and 4.77, 3.64, 3.44 and 1.4–1.8 ppm (9 H, m, the 2′-tetrahydropyranyl group); $\delta_P(^2H_2O)$ 0.62 ppm from phosphoric acid.

Kinetic measurements

The reactions were carried out in stoppered bottles immersed in a water bath, the temperature of which was adjusted to 298.2 K to within 0.1 K. The reactions, when carried out in aqueous hydrogen chloride, were started by adding the starting material in methanol (30 μl) to the prethermostatted reaction solution (3 cm^3), the initial substrated concentration being 2 × 10^{-4} mol dm^{-3}. On studying the reactions in buffer solutions, the methanolic starting material was first added to 0.5 cm^3 of 0.1 mol dm^{-3} aqueous hydrogen chloride. After 80 min, during which period the tetrahydropyranyl group was completely hydrolysed, the solution was converted into the desired buffer solution by adding calculated quantities of water, aqueous sodium chloride and 2 mol dm^{-3} stock solutions of the buffer constituents. The buffer solutions employed were prepared from formic, acetic, chloroacetic, cyanoacetic and propionic acids, and also di- and tri-ethanolamines. The ionic strength of the buffer solutions was adjusted to 1.0 mol dm^{-3} with sodium chloride.

The HPLC samples were withdrawn directly from the reaction solution and analysed immediately. The separations were carried out on a *Hypersil* ODS (254 × 4 mm, 5 μm) column. When the hydrolysis reaction was followed, isocratic elution with a formic acid–sodium formate buffer (0.05/0.05 mol dm^{-3}, pH 3.6) containing 30% (v/v) acetonitrile and 0.1 mol dm^{-3} ammonium chloride was applied (system A). The retention times observed were as follows: starting material 23 min, unprotected phosphotriesters (mixture of 2′- and 3′-isomers) 6.2 min and hydrolysis products (diesters and monoesters) 2–3 min. When the 2′/3′-isomerization was followed, isocratic elution with a formic acid–sodium formate buffer (0.025/0.025 mol dm^{-3}, pH 3.6) containing 22% (v/v) ethanol and 0.05 mol dm^{-3} tetramethylammonium chloride was applied (system B). The isomeric phosphotriesters were detected at retention times 22 min (3′-phosphotriester) and 24 min (2′-phosphotriester). The latter product was verified as the 2′-isomer of the deblocked starting material by following the reaction by ^{1}H and ^{31}P NMR spectroscopy. Accordingly, removal of the 2′-*O*-tetrahydropyranyl group with 0.1 mol dm^{-3} $^2HClO_4$ in ^2H_2O shifted the anomeric proton signal from 6.04 to 5.84 ppm. Subsequent treatment with a deuterioacetate buffer ([$C^2H_3COO^2H$]/[C^2H_3COONa] = 0.025/0.025 mol dm^{-3}) resulted in the appearance of an additional anomeric proton signal at 6.06 ppm. Simultaneously the pattern of the P–OCH$_3$ proton signals (originally 2 d at 3.78 and 3.81 ppm) became more complicated, while its intensity remained constant. The ^{31}P NMR spectrum showed two signals at 0.91 and 0.95 ppm from phosphoric acid. During this isomerization process the hydrolysis was detected only as a side reaction, as indicated by the appearance of a small amount of methanol. Similar spectral changes also took place when the reaction was followed in 0.1 mol dm^{-3} $^2HClO_4$ in ^2H_2O, but the isomerized product did not accumulate as markedly, consistent with the chromatographic observations. Moreover, it should be noted that previously[12] both 2′- and 3′-dimethylphosphates of 5′-*O*-methyl-2′-*O*-tetrahydropyranyluridine were prepared, and the migration was demonstrated by using both of these in the deblocked form as a starting material. Additionally, the identity of the phospho-diesters and -monoesters appearing as reaction products was then verified by

spiking the product with authentic samples. Since the present reaction system is identical with that above, only the 5′-*O*-protecting group being changed, this was not repeated.

The rate constants were calculated as described previously.[12]

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Paper 5/00032G
Received 4*th January* 1995
Accepted 31*st January* 1995