Hydrolysis of the *cis*-Phenyl Ester of Thymidine 3′,5′-Cyclic Monophosphate: pH-Dependent Competition between Depyrimidination and Phosphotriester Hydrolysis *via* CO and PO Bond Ruptures

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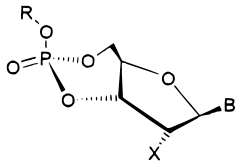
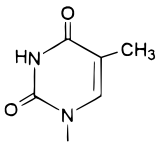
Hydrolytic reactions of the *cis*-phenyl ester of thymidine 3′,5′-cyclic monophosphate (**1a**) have been followed by HPLC over a wide pH range. Under acidic conditions (pH < 4) two reactions compete: depyrimidination (cleavage of the *N*-glycosidic bond) and phosphotriester hydrolysis to a mixture of three phosphodiesters, *viz.* thymidine 3′,5′-cyclic monophosphate (**2**) and thymidine 3′- and 5′-(phenyl phosphates) (**3** and **4**). Depyrimidination predominates (>80%) at pH < 1 and shows first-order dependence on acidity. The reaction is 4.5 to 5 times slower than with **2**. The phosphotriester hydrolysis of **1a** is acid catalyzed at pH < 2, giving all three phosphodiester products (**2**−**4**). Over a broad acidity range from pH 2 to 7, the reaction is pH-independent. In this pH region, the predominant product is **3** (up to 85%). At pH > 10, the hydrolysis is hydroxide-ion-catalyzed, yielding the three phosphodiester products in a 42:42:16 ratio ([**2**]:[**3**]:[**4**]). From pH 7 to 10, the pH−rate profile is nonlinear, possibly due to N3H deprotonation of the thymine moiety. In the same pH range, the site of bond cleavage appears to be changed. The product analyses of the corresponding methanolysis reactions suggest that the pH-independent reaction predominantly takes place *via* cleavage of the C5′O bond, while the alkaline reaction proceeds by rupture of one of the PO bonds. Consistent with this proposal, the pH-independent hydrolysis yields at high concentrations of sodium chloride 5′-chloro-5′-deoxythymidine 3′-(phenyl phosphate) and in concentrated acetate buffers 5′-*O*-acetylthymidine 3′-(phenyl phosphate). Accordingly, the hydrolytic reactions of **1a** markedly differ from those of more simple 2-aryloxy-2-oxo-1,3,2-dioxaphosphorinanes.

Introduction

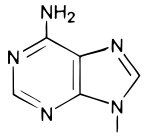
The alkaline hydrolysis of aryl and alkyl esters of nucleoside 3′,5′-cyclic monophosphates **1a**−**d** has previously been studied[1,2] as a model of the enzyme-catalyzed hydrolysis of adenosine 3′,5′-cyclic monophosphate (3′,5′-cAMP), a regulator of a variety of cellular processes. The



1a: B = Thy, X = H, R = Ph
1b: B = Thy, X = H, R = 4-NO_2-Ph
1c: B = Ade, X = OMe, R = Ph
1c: B = Ade, X = OMe, R= Me



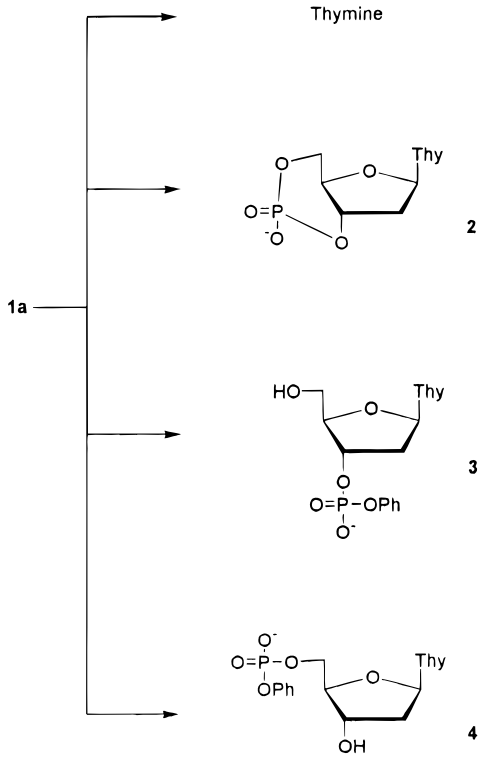
Thy



Ade

reactions were carried out in alkaline D_2O/1,4-dioxane-d_8 mixtures and followed by ^{31}P NMR. With the aryl esters **1a**−**c**, nucleoside 3′,5′-cyclic monophosphate was observed to be the main product, but the acyclic aryl esters of nucleoside 3′- and 5′-monophosphates also accumulated in a constant molar ratio throughout the kinetic runs (see Scheme 1).[1] Studies with phosphoryl ^{18}O-labeled analogues[1] revealed that the major part of the hydrolysis proceeds by retention of configuration at

Scheme 1



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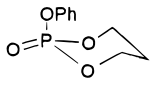
phosphorus, while the alkaline hydrolysis of nucleoside 3′,5′-cyclic phosphodiesters is known[3] to take place with complete inversion of configuration. Accordingly, the hydrolysis of the phosphotriesters was suggested[1] to involve formation of a pseudorotating phosphorane intermediate,[4] whereas in the alkaline hydrolysis of phosphodiesters the leaving group departs "in-line" with the attack of a nucleophile.

The studies discussed above were limited only to alkaline hydrolysis. To learn more about the reactions of these model compounds under various conditions, the hydrolysis of the *cis*-phenyl ester of thymidine 3′,5′-cyclic monophosphate (**1a**) has now been studied over the entire pH range (pH 0−14). Previously, the hydrolysis of non-nucleosidic counterparts of **1a**−**d**, *viz.* 2-(aryloxy)-2-oxo-1,3,2-dioxaphosphorinanes **5** and their *trans*-4,5-tetramethylene derivatives **6**, has been extensively studied.[5,6]



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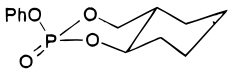

6

The results of the present paper, however, reveal marked differences in the behavior of nucleoside 3′,5′-cyclic phosphotriesters and their non-nucleosidic models **5** and **6**. Above all, **1a** undergoes over a wide pH range (pH 2−7) an unexpectedly rapid pH-independent hydrolysis, which appears to proceed predominantly by C5′O bond cleavage. This kind of a reaction has not been reported for either **5** or **6**. Its existence is, however, important to bear in mind on using this type of compounds as models for elucidation of mechanistic aspects of enzyme catalysis under neutral conditions. Under acidic conditions, depyrimidination of **1a** (hydrolysis of the *N*-glycosidic bond) efficiently competes with the phosphotriester hydrolysis.

Some previous publications provide useful background information for the present study. The benzyl esters of adenosine[7] and guanosine[8] 3′,5′-cyclic monophosphates have been shown to react under neutral and moderately acidic conditions by the attack of water on the benzyl carbon. It is also known[9] that acyclic trialkyl phosphates are hydrolyzed in alkaline solutions by PO bond cleavage, whereas the neutral hydrolysis proceeds by CO bond rupture. No acid catalysis could be observed on going to 3 M perchloric acid. All these findings underline the importance of CO bond cleavage in phosphotriester hydrolysis.

Results and Discussion

Product Distributions and pH−Rate Profiles. The pH−rate profiles for the depyrimidination and phosphotriester hydrolysis of the *cis*-phenyl ester of thymidine 3′,5′-cyclic monophosphate (**1a**) were obtained by following the decomposition of **1a** by HPLC. The reactions at pH > 8 were carried out at 298.2 K (Figure 1), whereas in the lower pH range the experimental temperature was 363.2 K (Figure 2). Under basic conditions (pH > 10), the hydrolysis of **1a** to cyclic (**2**) and acyclic phosphodiesters (**3** and **4**) is first order in hydroxide ion concentration. The product distribution remains constant, **2**, **3**, and **4** being formed in parallel in a 42:42:16 ratio (Figure 3). No depyrimidination takes place. The same product distribution, within experimental errors, was also observed by ^{31}P NMR spectroscopy for hydrolysis in 0.1 mol dm^{-3} sodium hydroxide. For comparison, the previous ^{31}P NMR spectroscopic study[1] in an alkaline D_2O/1,4-dioxane-d_8 mixture (2:3, v/v) showed formation of the same products but in a 58:20:22 ratio.

At pH 7−10, the pH−rate profile shows a broad nonlinear part. The proportion of the 3′-(phenyl phosphate) **3** in the product mixture is slightly more pronounced than in more alkaline solutions (Figure 3). The curvature in the rate profile in all likelihood refers to deprotonation of the N3H site of the thymine base (the

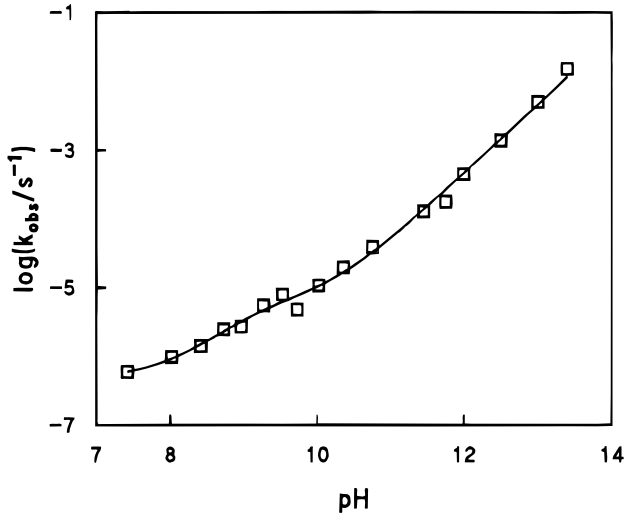

Figure 1. pH−rate profile for the phosphotriester hydrolysis of the *cis*-phenyl ester of thymidine 3′,5′-cyclic monophosphate (**1a**) at 298.2 K. I = 0.1 mol dm^{-3}, adjusted with sodium chloride. The curve is calculated by least-squares fitting to eq 1.

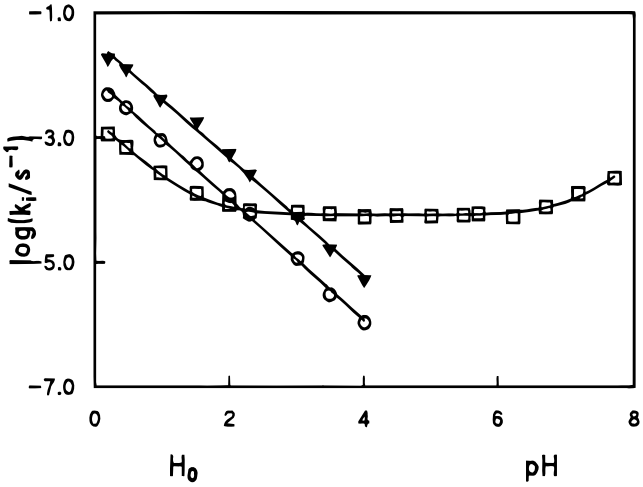

Figure 2. The pH-rate profiles for the hydrolytic reactions of thymidine 3′,5′-cyclic monophosphate (**2**) and its *cis*-phenyl ester (**1a**) at 363.2 K. I = 0.1 mol dm^{-3}, adjusted with sodium chloride. Notation: (▼) depyrimidination of **2**, (○) depyrimidination of **1a**, (□) phosphotriester hydrolysis of **1a**.

(1) Broeders, N. L. H. L.; van der Heiden, A. P.; Peeters, I.; Janssen, H. M.; Koole, L. H. *J. Am. Chem. Soc.* **1992**, *114*, 9624−9633.
(2) Broeders, N. L. H. L.; van der Heiden, A. P.; Koole, L. H.; Kanters, J. A.; Schouten, A. *Can. J. Chem.* **1993**, *71*, 855−863.
(3) Mehdi, S.; Coderre, J. A.; Gerlt, J. A. *Tetrahedron* **1983**, *39*, 3483−3492.
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(5) Khan, A. J.; Kirby, A. J. *J. Chem. Soc. B* **1970**, 1172−1182.
(6) Rowell, R.; Gorenstein, D. G. *J. Am. Chem. Soc.* **1981**. *103*, 5894−5902.
(7) Engels, J. *Z. Naturforsch.* **1977**, *32b*, 807−809.
(8) Engels, J. *Bioorg. Chem.* **1979**, *8*, 9−16.
(9) Barnard, P. W. C.; Bunton, C. A.; Llewellyn, D. R.; Vernon, C. A.; Welch, V. A. *J. Chem. Soc.* **1961**, 2670−2676.

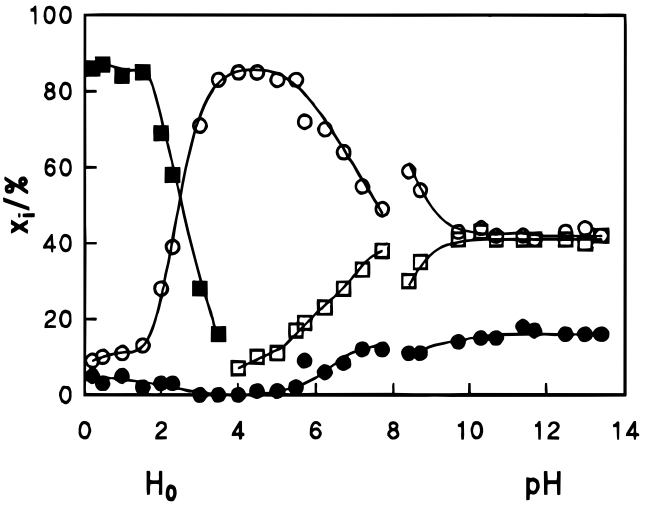
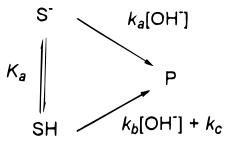


Figure 3. Product distribution for the hydrolysis of the *cis*-phenyl ester of thymidine 3′,5′-cyclic monophosphate. Points at pH > 8 refer to 298.2 K and those at pH < 8 to 363.2 K. Notation: thymidine 3′,5′-cyclic monophosphate (**2**, □), thymidine 3′-(phenyl phosphate) (**3**, ○), thymidine 5′-(phenyl phosphate) (**4**, ●), and thymine (■).

Scheme 2



pK_a of thymidine N3H is 9.6−9.8).[10] The curve indicated in Figure 1 was obtained by fitting the observed first-order rate constants (k_{obs}) of degradation of the starting compound to eq 1

$$k_{obs} = [k_a(K_a/K_w)[OH^-]^2 + k_b[OH^-] + k_c]/[(K_a/K_w)[OH^-] + 1] \quad (1)$$

where the rate and equilibrium constants are those depicted in Scheme 2. The best fit was obtained with the following values of the adjustable parameters: $K_a = 6.3 \times 10^{-10}$ mol dm^{-3}, $k_a = 0.029$ dm^3 mol^{-1} s^{-1}, $k_b = 0.30$ dm^3 mol^{-1} s^{-1}, and $k_c = 4.8 \times 10^{-7}$ s^{-1} ($K_w = 1.58 \times 10^{-14}$ mol dm^{-3} [11]). Accordingly, deprotonation of the thymine moiety appears to retard the nucleophilic attack of hydroxide ion by 1 order of magnitude, possibly due to electrostatic repulsion between the two negatively charged species.

At pH 2−7, the phosphotriester hydrolysis of **1a** is pH-independent. The product distribution differs in this pH range significantly from that observed at pH > 10 (Figure 3); up to 85% of the hydrolysis yields thymidine 3′-(phenyl phosphate) (**3**). At pH 3−5, the proportion of the 5′-(phenyl phosphate) **4** even remains below the limit of detection.

At pH < 4, the acid-catalyzed depyrimidination (*i.e.*, release of thymine from **1a**) competes with the phosphotriester hydrolysis. The depyrimidination shows first-order dependence on the acidity, and at pH < 1 more than 80% of the starting material is degraded *via* this route. For this reason, the acid-catalyzed hydrolysis of phosphotriester to phosphodiesters is difficult to quantitate. The depyrimidination of thymidine 3′,5′-cyclic monophosphate (**2**) is considerably faster than the phosphoester hydrolysis of **1a** (see Figure 2), and hence, it is not appreciably accumulated as an intermediate. The acyclic phosphodiesters, **3** and **4**, appear to be accumulated in about the same mutual ratio as in the alkaline hydrolysis.

PO *vs.* CO Bond Cleavage in the Phosphotriester Hydrolysis and Methanolysis of 1a. As seen from Figure 3, the product distribution of the pH-independent phosphotriester hydrolysis of **1a** differs dramatically from that of the hydroxide-ion-catalyzed reaction. To find a possible explanation for this, the site of bond cleavage was elucidated by product analysis of the methanolysis of **1a** under acidic, neutral, and alkaline conditions.

HPLC analyses of the aliquots withdrawn at different intervals from a solution of **1a** in methanolic sodium methoxide (0.1 mol dm^{-3}, 298.2 K) showed that the alkaline methanolysis proceeds by parallel formation of 3 initial products, eluting at 18, 29, and 33 min on an RP column (for conditions see the Experimental Section). The slowly migrating products (t_R 29 and 33 min) exhibited, in addition to the ^{1}H NMR signals of the sugar and base moiety, the proton resonances of the phenoxy group (at 7.2−7.4 ppm) and a doublet of three protons at 3.79 ppm (J = 11.3 Hz) and 3.82 ppm (J = 11.5 Hz), respectively. Both the chemical shifts and coupling constants ($J_{H,P}$) of the latter signals are typical for a methyl ester of phosphoric acid.[2] By comparison to the ^{1}H NMR spectra of thymidine 3′- and 5′-monophosphates, these products were assigned as thymidine 3′-(methyl phenyl phosphate) (**10**, t_R 33 min) and its 5′-counterpart (**11**, t_R 29 min). Neither of these compounds could be shown to accumulate as two diastereomers (R_P and S_P). Only one signal in the ^{31}P NMR spectrum and one doublet in the ^{1}H NMR spectrum at 3.8 ppm were observed. However, the existence of two diastereomers cannot be strictly excluded. The rapidly migrating product (t_R 18 min) was subsequently decomposed to two products (t_R 17.5 and 19 min), the latter one of which (predominating in 86:14 ratio) was isolated. The ^{1}H NMR spectrum of this compound exhibited a six-proton doublet (J = 11.2 Hz) at 3.69 ppm, whereas only the signal of H6 of the thymine moiety was observed in the aromatic region. The products at t_R 17.5 and 19 min were hence assigned as thymidine 3′- (**9**) and 5′-(dimethyl phosphates) (**8**), formed *via* thymidine 3′,5′-cyclic methylphosphate (**7**). The course of methanolysis of **1a** in methanolic sodium methoxide may thus be described by Scheme 3. The initial product distribution was observed to be 25:61:14 for **7**:**10**:**11**. Accordingly, the alkaline methanolysis of **1a** proceeds by the attack of methoxide ion on phosphorus, consistent with the previous findings, according to which **1a**[1] and its non-nucleosidic counterparts, *viz.* 2,4-dinitrophenyl analogs of **5** and **6**,[5,6] are hydrolyzed in aqueous alkali by PO bond rupture.

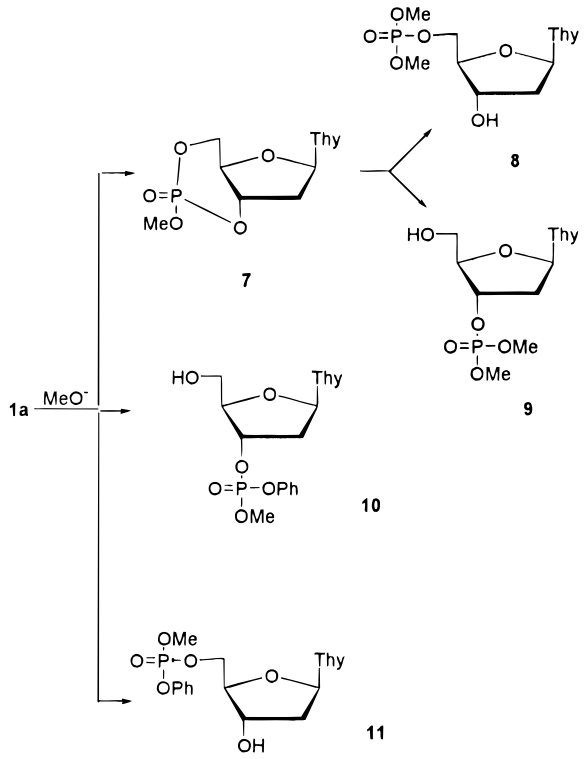
Under acidic and neutral conditions, methanolysis of **1a** also gave 3 nucleosidic products, but they all differed chromatographically from the products obtained in methanolic sodium methoxide.[12] Neither 3′,5′-cyclic methylphosphate triester nor acyclic methyl phenyl phosphate

(10) Lönnberg, H. In *Biocoordination Chemistry: Coordination Equilibria in Biologically Active Systems;* Burger, K., Ed.; Ellis Horwood: Chichester, 1990; Chapter VII.

(11) Harned, H. S.; Owen, B. B. *Physical Chemistry of Electrolyte Solutions*; Reinold: New York, 1943; p 752.

(12) A trace of hydrolysis products, mainly **3**, was also formed due to incomplete exclusion of moisture, when the reactions were followed at 363.2 K for 5 d in sealed tubes. No sign of hydrolysis products was observed when the alkaline methanolysis was followed at 298.2 K for 1 h.

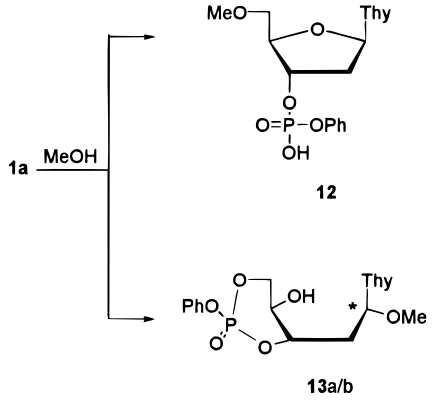
Scheme 3



triesters were detected to accumulate. The experiments were carried out in methanolic trifluoroacetic acid (1.0 and 0.1 mol dm^{-3}) and in methanolic triethanol amine/triethanolammonium buffers (0.005/0.05 and 0.05/0.005 mol dm^{-3}) at elevated temperature (363 K). The product that was the fastest migrating on HPLC (t_R 24 min; for conditions see the Experimental Section) was predominating (70−80%) under neutral conditions, whereas in 1 M acid solution it formed only 20% of product mixture. The other two products, the formation of which was thus favored under acidic conditions, accumulated always in a constant 10:70 molar ratio with each other, the slower eluted (t_R 37 min) product predominating over the other (t_R 32 min). All three products were isolated by RP HPLC and characterized by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of the fastest migrating product showed the presence of the phenoxy group and appearance of a three-proton singlet at 3.26 ppm (in DMSO-d_6 from TMS). The latter signal was assigned as the 5′-*O*-methyl resonance of the sugar moiety, and while the other resonances were comparable to those obtained with thymidine 3′-monophosphate and its phenyl ester (**3**), the product was assigned as 5′-*O*-methylthymidine 3′-(phenyl phosphate) (**12**). This finding strongly suggests that the rupture of the phosphotriester moiety under neutral conditions proceeds, in contrast to the alkaline methanolysis, by cleavage of the CO bond, as depicted in Scheme 4.

The other two products of the acidic methanolysis of **1a** appeared to have been formed *via* opening of the sugar ring without reaction of the phosphotriester moiety. Both the ^{1}H and ^{13}C NMR spectra of these two compounds very closely resembled each other and showed marked similarities to the spectra of the starting material. In comparison to the ^{1}H NMR spectrum of **1a**, two new resonances appeared in the spectra of these products. Three-proton singlets at 3.18 and 2.99 ppm, corresponding most likely to methoxide proton resonances and doublets typical for the resonance of a secondary OH

Scheme 4

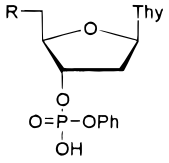


proton at 5.79 and 5.85 ppm, were observed in the spectra of the faster (t_R 32 min) and slower (t_R 37 min) eluted products, respectively. At elevated temperature (353 K), the latter resonances (OH) were shifted 0.2 ppm upfield and collapsed to broad singlets. On the basis of COSY spectra, the OH protons were coupled with the H4′ of the sugar moiety. The products were assigned as the two diastereomers of the structure **13**. The mechanism of the formation of these from **1a** is discussed below in comparison with the acid-catalyzed hydrolysis of the *N*-glycosidic bond (depyrimidination).

The fact that methoxide ion attacks the phosphorus atom of **1a** and methanol the C5′ atom suggests that the alkaline and pH-independent hydrolysis of **1a** may well exhibit a similar mechanistic difference. The observed marked difference between the product distributions of these two hydrolysis reactions rather supports than argues against this assumption. The predominant formation of the 3′-phosphodiester (**3**) under neutral conditions may be attributed to a more facile reaction of solvent molecule at the sterically less hindered primary carbon atom. One may, however, also argue that the reaction may be initiated by the attack of the thymine O^2 atom at C5′. Possibly the hydrolysis partially proceeds by a nucleophilic attack on phosphorus even under neutral conditions, since the accumulation of thymidine 3′,5′-cyclic monophosphate (**2**) as a minor product (< 10%) is better explained by this competing mechanism than by cleavage of the PhO bond. For comparison, the 2,4-dinitrophenyl analogs of **5** and **6** have been shown to undergo pH-independent hydrolysis by PO bond cleavage,[5,6] giving the cyclic phosphodiester.

The effects that electrolytes exert on the product distribution of the pH-independent hydrolysis of **1a** lend some additional support to the assumed CO bond cleavage. The hydrolysis was followed in a dilute acetic acid/sodium acetate buffer (0.02/0.02 mol dm^{-3}), the ionic strength of which was adjusted to 1.0 mol dm^{-3} with either sodium perchlorate or sodium chloride. With sodium perchlorate, the product distribution obtained at this high ionic strength was similar to that observed at I = 0.1 mol dm^{-3}: **2** and **3** were accumulated in a 6:94 ratio (compare Figure 3). The first-order rate constant for disappearance of **1a** was $k_d = 4.1 \times 10^{-5}$ s^{-1}, *i.e.*, approximately equal to the value at I = 0.1 mol dm^{-3}. When the ionic strength was adjusted with sodium chloride, the reaction was somewhat faster ($k_d = 8.7 \times 10^{-5}$ s^{-1}) and an additional product was observed at t_R slightly longer than those of **2** and **3**. This product was isolated by HPLC, and its ^{1}H and ^{13}C NMR spectra were recorded. The presence of phenyl proton resonances,

together with spectral similarity with **3** and **12**, suggests that the additional product is 5′-chloro-5′-deoxythymidine 3′-(phenyl phosphate) (**14**), formed *via* the attack of chloride ion on C5′ of **1a**. The assignment was verified by the FAB mass spectrum of the product. Molecular ion signals at 416 and 418 mass units in intensity ratio 3:1 were observed, referring to compounds containing ^{35}Cl and ^{37}Cl, respectively. When the hydrolysis was carried out in concentrated acetic acid/sodium acetate buffer (1.0/1.0 mol dm^{-3}) in the absence of chloride ion, another product was accumulated. This product was isolated and assigned as 5′-*O*-acetylthymidine 3′-(phenyl phosphate) (**15**) by ^{1}H and ^{13}C NMR and FAB mass spectroscopy. It appears clear that **15** was formed by the attack of acetate ion on C5′ of the starting material.



14: R = Cl
15: R = OAc

At pH < 2, acid-catalyzed phosphotriester hydrolysis takes place, and the product distribution resembles that observed for the base-catalyzed reaction. One may speculate that preequilibrium protonation of the phosphotriester moiety makes the attack of water molecule on phosphorus again favored over the attack on carbon. Under these conditions, replacement of sodium perchlorate with sodium chloride as the background electrolyte affected neither the competition between the acid-catalyzed rections, *viz*. depyrimidination and phosphotriester hydrolysis, nor the product composition.

Depyrimidination. We have shown previously[13] that the *N*-glycosidic bond of thymidine 3′,5′-cyclic monophosphate (**2**) is 800-fold more labile in aqueous acid than that of thymidine and its 5′-monophosphate, whereas 2′-deoxyadenosine 3′,5′-cyclic monophosphate is depurinated 3 orders of magnitude less readily than 2′-deoxyadenosine or its 5′-monophosphate. Thymidine has been suggested[14] to be hydrolyzed, in striking contrast to purine nucleosides,[15] by a mechanism involving protonation of the sugar O$^{4'}$-atom and subsequent opening of the sugar ring, leading to formation of a Schiff-base intermediate, possibly preceded by hydration of the 5,6-double bond.[16] The facile depyrimidination of **2** was hence interpreted[13] to result from the strongly puckered high-energy conformation of the sugar ring, caused by the fused 1,3,2-dioxaphosphorinane ring in fixed chair conformation.[17] The depyrimidination of the phenyl ester, **1a**, is only 4.5−5 times slower than that of the corresponding diester **2** and proceeds in all likelihood by the same Schiff base mechanism.

Formation of **13** in acidic methanolysis of **1a** also shows that opening of the sugar ring may take place in acidic media. In contrast to the course of hydrolytic depyrimidination, however, the attack of the solvent methanol to the Schiff-base intermediate does not lead to departure of the thymine moiety, but the diastereomers of **13** accumulate as stable products.

Structural Effects in the Phosphotriester Hydrolysis. The mechanism of the hydroxide-ion-catalyzed hydrolysis of **1a** has been discussed previously in detail.[1] The reaction has been suggested to proceed *via* a pentacoordinated phosphorane intermediate (obtained by the attack of hydroxide ion on phosphorus) that is stable enough to undergo pseudorotation. The product distribution of this reaction appears to be rather sensitive to both the solvent and the substitution of the 2-phenoxy-2-oxo-1,3,2-dioxaphosphorinane ring. According to the results of the present study, alkaline hydrolysis of **1a** gives **2**, **3**, and **4** in a 42:42:16 ratio, while in a mixture of D_2O and dioxane (2:3, v/v) the product distribution has been reported[1] to be 58:20:22. With the non-nucleosidic model compound **6**, the only hydrolysis product was the cyclic diester.[6] In even more striking contrast, no indication of pH-independent hydrolysis was obtained with **6**, not *via* PO or CO bond cleavage.[6] The reaction *via* PO bond cleavage occured only when the phenoxy group was replaced with a much better leaving group, the 2,4-dinitrophenoxy group.[6] Under acidic conditions, **6** yielded 52% cyclic diester, 14% acyclic diester esterified to the primary carbon, and 35% acyclic diester esterified to the secondary carbon. The product distribution of the acid-catalyzed hydrolysis of **1a** is difficult to determine accurately, since more than 80% of the starting material is depyrimidinated under acidic conditions. In 0.5 mol dm^{-3} aqueous perchloric acid, the product mixture consisted of 90% thymine, 8% **3**, and 2% **4**. Accordingly, it appears quite clear that fusion of the 2-phenoxy-2-oxo-1,3,2-dioxaphosphorinane ring with a nucleosidic ribofuranosyl moiety markedly alters its chemical reactivity, resulting in not only increased hydrolytic instability but also changes in the competition between PO and CO bond ruptures. That is why cautiousness should be exercised on drawing conclusions concerning biomolecules on the basis of experimental data obtained with simpler models.

Experimental Section

Materials. Preparation of the *cis*-phenyl ester of thymidine 3′,5′-cyclic monophosphate (**1a**) has been described.[1] Thymine, thymidine, and thymidine monophosphates were purchased from Sigma.

Kinetic Measurements. Reactions were followed by the HPLC method described previously.[15] Separations were carried out on a Hypersil ODS5 column (4 × 250 mm, 5 μm) using mixtures of acetic acid buffer (pH 4.2, containing ammonium chloride 0.1 mol dm^{-3}) and acetonitrile as eluent. Thymine and thymidine 3′,5′-cyclic monophosphate (**2**) detected as hydrolysis products were assigned chromatographically by spiking the signals with authentic commercial samples. The signals of thymidine 3′- and 5′-(phenyl phosphates) were identified on the basis of the known ^{31}P NMR shifts.[1] The retention times reported in text refer to elution with a linear gradient from 5% to 30% acetonitrile in 25 min at a flow rate 1.0 cm^3 min^{-1}. Signals were recorded on a UV-detector at wavelength 267 nm. Signal areas were converted to concentrations with the aid of calibration samples of known concentrations.

The hydronium ion concentrations of the reaction solutions were adjusted with hydrogen chloride and sodium hydroxide and formate, acetate, triethanolamine and triethylamine buffers. The pK_a values of the buffer acids under the experimental conditions were calculated from literature data.[18−21]

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First-order rate constants, k_{obs}, for the hydrolysis of **1a** were calculated by applying the integrated first-order rate equation to the diminution of the signal area of the starting material. First-order rate constants for the parallel reactions in acidic solutions, *viz.* depyrimidination and phosphotriester hydrolysis, were determined by bisecting the values of k_{obs} on the basis of product distribution during the early stages of the reaction. Under very acidic conditions, however, **2** is depyrimidinated too rapidly to be accumulated as an intermediate, and hence the rate rate constants of the parallel reactions could not be determined accurately at pH < 2. The molar ratio of the products accumulated, *viz.* **3**, **4**, and thymine, remained constant at pH < 1, suggesting that all the reactions are first order in hydronium ion concentration.

Methanolysis. The products of methanolysis were separated on a semipreparative LiChrospher RP-18 column (10 × 250 mm, 5 μm), using mixtures of the above-mentioned acetic acid buffer and acetonitrile as eluent. The ^{1}H, ^{13}C, and ^{31}P NMR spectra of the isolated compounds were recorded on a JEOL GX 400 or a JEOL JNMA 500 spectrometer in DMSO-d_6 at 300 K. The proton chemical shifts were measured from internal TMS and the ^{31}P shifts from external H_3PO_4.

The base-catalyzed methanolysis of **1a** was carried out in methanolic sodium methoxide (0.1 mol dm^{-3}). The reaction was followed for 1 h at 298.2 K. Three products were initially accumulated with HPLC retention times of 18.0, 29, and 33 min. The product at t_R 18.0 min was subsequently degraded to two products (t_R 17.5 and 19.0 min). The products eluted at 19, 29, and 33 min were isolated by HPLC and characterized by ^{1}H and ^{31}P NMR spectroscopy. They were assigned as thymidine 3′-(dimethyl phosphate) (**9**, t_R 19 min), thymidine 3′-(methyl phenyl phosphate) (**10**, t_R 33 min), and thymidine 5′-(methyl phenyl phosphate) (**11**, t_R 29 min). **10** and **11** may be mixtures of R_P and S_P diastereomers, but no NMR spectral evidence for this was obtained.

Thymidine 3′-(Dimethyl Phosphate) (9). δ_H (400 MHz): 11.3 (1H, s NH), 7.67 (1H, s, H6), 6.18 (1H, dd, $J_{1',2'}$ and $J_{1',2''}$ = 6.4, 4.8 Hz, H1′), 4.91 (1H, m, H3′), 4.04 (1H, m, H4′), 3.69 (6H, d, $J_{P,H}$ = 11.2 Hz, 2 × $POCH_3$), 3.51−3.22 (2 H5′, overlaps with the HDO resonance), 2.32 (2 H, m, H2′ and H2″), 1.77 (3 H, s, 5-CH_3). δ_P 0.3 ppm.

Thymidine 3′-(Methyl phenyl phosphate) (10). δ_H (400 MHz): 11.4 (1 H, NH), 7.66 (1H, H6), 7.4−7.2 (5 H, Ph), 6.18 (1 H, t, $J_{1',2'} = J_{1',2''}$ = 6.7 Hz, H-1′), 5.06 (1 H, H3′), 4.08 (1 H, m, H4′), 3.82 (3 H, d, $J_{H,P}$ = 11.5 Hz, $POCH_3$), 3.6 (2 H, m, H5′ and H5″), 2.34 (2 H, m, H-2′ and H2″), 1.76 (3 H, 5-CH_3). δ_P −5.0 ppm.

Thymidine 5′-(Methyl phenyl phosphate) (11). δ_H (400 MHz): 11.3 (1 H, NH), 7.4−7.2 (6 H, H6 and Ph), 6.19 (1 H, t, $J_{1',2'} = J_{1'2''}$ = 6.6 Hz, H1′), 4.4−4.2 (3 H, H3′,H5′, and H5″), 3.93 (1 H, H4′), 3.79 (3 H, d, $J_{H,P}$ = 11.3 Hz, $POCH_3$), 2.05 (2 H, m, H2′ and H2″), 1.71 (3 H, 5-CH_3). δ_P: −5.0 ppm.

Three products, having retention times of 24, 32, and 37 min, were formed in methanolic trifluoroacetic acid (1.0 and 0.1 mol dm^{-3}) and in methanolic triethanol amine/triethanolammonium buffers (0.005/0.05 and 0.05/0.005 mol dm^{-3}) upon 5 d incubation in sealed tubes at 363.2 K. The first one (t_R 24 min) was assigned as 5′-*O*-methylthymidine 3′-(phenyl phosphate) (**12**). The other two products were most likely formed by opening of the sugar ring, and they were assigned as the two diastereomers of structure **13**, *i.e.*, the phenyl esters of (2-deoxy-L- and -D-*glycero*-1-*O*-methyl-1-*C*-(1-thyminyl)-D-*erythro*-pentitol) 3′,5′-cyclic monophosphate.

5′-*O*-Methylthymidine 3′-(Phenyl phosphate) (12). δ_H (500 MHz): 11.2 (1 H, NH), 7.54 (1 H, s, H6), 7.22−6.94 (5 H, Ph), 6.11 (1 H, dd, $J_{1',2'}$ and $J_{1',2''}$ = 6.3, 7.9 Hz, H1′), 4.64 (1 H, m, H3′), 4.02 (1 H, m, H4′), 3.48 (1 H, dd, $J_{5',5''}$ = 10.6 Hz, $J_{5',4'}$ = 3.2 Hz, H5′), 3.42 (1 H, dd, $J_{5'',4'}$ = 4.4 Hz, H5″), 3.27 (3 H, s, OCH_3), 2.20 (1 H, m, H2′), 2.08 (1 H, m, H2″), 1.77 (3 H, s, 5-CH_3). δ_C (125.6 MHz, BCM irradiation): 12.2, 38.1, 58.4, 72.4, 74.8 (d, $J_{C,P}$ = 5.6 Hz), 84.0, 84.1 (d, $J_{C,P}$ = 5.5 Hz), 109.5, 119.9 (d, $J_{C,P}$ = 4.5 Hz), 121.7, 128.8, 135.9, 150.4, 154.0 (d, $J_{C,P}$ = 6.4 Hz), 163.5. δ_P: −6.7 ppm.

13a. t_R: 32 min. δ_H (500 MHz): 11.3 (1 H, s, NH), 7.45−7.20 (6 H, H6 and Ph), 5.98 (1 H, d, $J_{4',O4'}$ = 6.9 Hz), 5.73 (1 H, dd, $J_{1',2'}$ and $J_{1',2''}$ = 7.3, 5.8 Hz, H1′), 4.35−4.20 (2 H, m, H3′ and H5′), 4.17 (1 H, td, $J_{5',5''} = J_{H,P}$ = 11.3 Hz, $J_{4',5''}$ = 1.5 Hz, H5″), 3.79 (1 H, m, H4′), 3.18 (3 H, s, OCH_3), 2.37 (1 H, m, H2′), 2.22 (1 H, m, H2″), 1.78 (3 H, s, H5). δ_C (125.6 MHz, BCM irradiation): 12.1, 36.5 (d, $J_{C,P}$ = 8.2 Hz), 55.6, 64.3 (d, $J_{C,P}$ = 5.5 Hz), 70.4 (d, $J_{C,P}$ = 6.4 Hz), 79.8 (d, $J_{C,P}$ = 7.3 Hz), 82.3, 110.3, 119.7 (d, $J_{C,P}$ = 4.6 Hz), 125.2, 130.0, 135.5, 149.8 (d, $J_{C,P}$ = 6.4 Hz), 151.1, 163.7.

13b. t_R: 37 min. MS: *m*/*z* 413. δ_H (500 MHz): 11.3 (1 H, NH), 7.5−7.2 (6 H, H6 and Ph), 5.85 (1H, d, $J_{4',O4'}$ = 6.7 Hz, 4′OH; at 353 K, this resonance turned into a broad singlet at 5.65 ppm), 5.54 (1 H, dd, $J_{1',2'}$ and $J_{1',2''}$ = 12, 2.1 Hz, H1′), 4.38 (2 H, m, H3′ and H5′), 4.19 (1 H, t, J = 11.3 Hz, H5″), 3.73 (1 H, m, H4′), 2.99 (3 H, s, OCH_3), 2.5 (1H, m, H 2′, overlaps with the DMSO resonance), 1.87 (1H, H2″), 1.77 (3 H, s, 5-CH_3). δ_C (125.6 MHz, BCM irradiation): 12.0, 36.9 (d, $J_{C,P}$ = 9.0 Hz), 55.7, 64.4 (d, $J_{C,P}$ = 4.6 Hz), 70.7 (d, $J_{C,P}$ = 7.3 Hz), 80.0 (d, $J_{C,P}$ = 7.4 Hz), 81.3, 110.3, 119.5 (d, $J_{C,P}$ = 4.6 Hz), 125.3, 130.1, 135.1, 149.8 (d, $J_{C,P}$ = 6.4 Hz), 150.9, 163.7. δ_P −12.3 ppm.

Products at High Cl^- or AcO^- Concentration. When the hydrolysis of **1a** was followed in an acetic acid/sodium acetate buffer (0.02/0.02 mol dm^{-3}) containing 0.98 mol dm^{-3} sodium chloride, accumulation of thymidine 3′,5′-cyclic monophosphate (**2**), thymidine 3′-(phenyl phosphate) (**3**), and thymine was found to be accompanied by formation of an additional product, having a slightly longer retention time (24 min). This product was isolated by HPLC. It exhibited the following molecular ion signals on FAB MS and ^{1}H, ^{13}C and ^{31}P resonances in D_2O at 300 K and was on these bases assigned as 5′-chloro-5′-deoxythymidine 3′-(phenyl phosphate) (**14**).

5′-Chloro-5′-deoxythymidine 3′-(Phenyl phosphate) (14). MS *m*/*z*: 416 (100), 418 (35%). δ_H (400 MHz): 7.41 (1 H, H6), 7.3−7.05 (5 H, phenyl protons), 6.17 (1 H, t, $J_{1',2'} = J_{1',2''}$ = 7.2 Hz, H-1′), 4.80 (1 H, m, H-3′), 4.22 (1 H, dd, H-4′), 3.70 (1 H, dd, $J_{5',5''}$ = 12.7 Hz, $J_{4',5''}$ = 3.9 Hz, H-5″), 3.63 (1 H, dd, $J_{4',5'}$ = 5.2 Hz, H-5′), 2.40 (1 H, ddd, $J_{2',2''}$ = 16 Hz, $J_{2'',3'}$ = 4.0 Hz, H2″), 2.32 (1 H, dd, $J_{2',3'}$ = 0 Hz, H-2′), 1.73 (1 H, 5-CH_3). δ_C (125.6 MHz): 12.4, 37.9, 44.8, 76.7, 84.8, 85.9, 112.6, 120.9, 125.3, 130.7, 138.2, 152.5, 167.3. δ_P: −5.9 ppm.

When the hydrolysis of **1a** was followed in a concentrated acetic acid/sodium acetate buffer (1.0/1.0 mol dm^{-3}) in the absence of additional electrolytes, another product having t_R of 27 min was accumulated. It was isolated by HPLC and assigned as 5′-*O*-acetylthymidine 3′-(phenyl phosphate) (**15**) by FAB mass spectroscopy and ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy.

5′-*O*-Acetylthymidine 3′-(phenyl phosphate) (15). MS *m*/*z*: 440. δ_H (400 MHz): 7.35 (1 H, H6), 7.3−7.0 (5 H, Ph), 6.14 (1 H, t, $J_{1',2'} = J_{1',2''}$ = 6.8 Hz, H1′), 4.77 (1 H, m, H3′), 4.19 (1 H, dd, H4′), 4.14 (1 H, dd, $J_{5',5''}$ = 12.3 Hz, $J_{4',5''}$ = 3.3 Hz, H5″), 4.03 (1 H, dd, $J_{4',5'}$ = 4.5 Hz, H5′), 2.42 (1 H, ddd, $J_{2',2''}$ = 16 Hz, $J_{2'',3'}$ = 4.1 Hz, H2″), 2.33 (1 H, dd, $J_{2',3'}$ = 0 Hz, H2′), 1.93 (3 H, s, OAc), 1.72 (3H, s, 5-CH_3). δ_C (125.6 MHz): 12.4, 21.2, 38.4, 61.9, 76.4, 85.9, 86.4, 112.4, 120.9, 125.3, 130.7, 138.4, 152.5, 167.4, 171.3. δ_P: −6.0 ppm.

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