Hydrolysis and Interconversion of the Dimethyl Esters of 5′-*O*-Methyluridine 2′- and 3′-Monophosphates: Kinetics and Mechanism

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The hydrolysis and isomerization of the phosphodiester bonds of ribonucleic acids (RNA) have recently been studied rather intensively.[1] Studies with dinucleoside monophosphates, *i.e.*, the dinucleoside structural units of RNA, have suggested that the hydrolysis proceeds *via* a pentacoordinated phosphorane intermediate obtained by an intramolecular nucleophilic attack of the 2′-hydroxyl group on phosphorus.[2–4] Under acidic and neutral conditions isomerization of the 3′,5′-phosphodiester bond to the 2′,5′-bond competes with the hydrolysis. Most likely this reaction also proceeds *via* the phosphorane intermediate. The data on the corresponding reactions of phosphotriesters are scarce. However, replacement of the dissociable proton of the phosphodiester bond with an alkyl group would undoubtedly help to distinguish between the kinetically equivalent mechanisms of phosphodiester hydrolysis that differ only in the site of proton in the transition state. Moreover, hydrolysis and isomerization of protected phosphoester bonds are encountered as harmful side reactions of the chemical synthesis of RNA. It has been shown,[5,6] for example, that the 2-chlorophenyl esters of dinucleoside monophosphates yield under slightly acidic conditions a mixture of 2′,5′/3′,5′-phosphodiesters, as soon as the protecting group of the 2′-hydroxyl function is removed. Accordingly, the intramolecular nucleophilic attack on the fully esterified phosphoric acid must be a very facile process. No convincing evidence for the migration of a phosphotriester group has been described. We now report on the kinetics of the hydrolysis and interconversion of the dimethyl esters of nucleoside 2′- and 3′-monophosphates. The dimethyl esters of 5′-*O*-methyl-2′(3′)-*O*-tetrahydropyranyluridine 3′(2′)-monophosphates (**1**, **2**) were used as model compounds. The tetrahydropyranyl protection was removed under acidic conditions (pH < 5.5), and the subsequent reactions were followed by HPLC.

When the tetrahydropyranyl group was removed from **1** at pH 2, the disappearance of the signal of the starting material (t_R = 18.0 min with system A, for the chromatographic conditions see the Experimental Section) was accompanied with appearance of an equally strong signal at a shorter retention time (t_R = 7.7 min). Similarly, deprotection of **2** (t_R = 14.2 min) resulted in a new signal at t_R = 7.1 min. Subsequently, these compounds, assigned as 5′-*O*-methyluridine 3′- (**3**) and 2′-(dimethyl phosphates) (**4**), underwent two parallel reactions, *viz.* mutual isomerization and hydrolysis to a mixture of 5′-*O*-methyluridine 2′/3′-(methyl phosphates) (**5**, **6**), 2′/3′-monophosphates (**7**, **8**) and 2′,3′-(cyclic)monophosphate (**9**) (separation of **5**–**9** achieved by system B). Figure 1 shows the time-dependent product distribution observed on using **2** as starting material. This product distribution strongly suggests that the reactions taking place may be dipicted by Scheme 1.

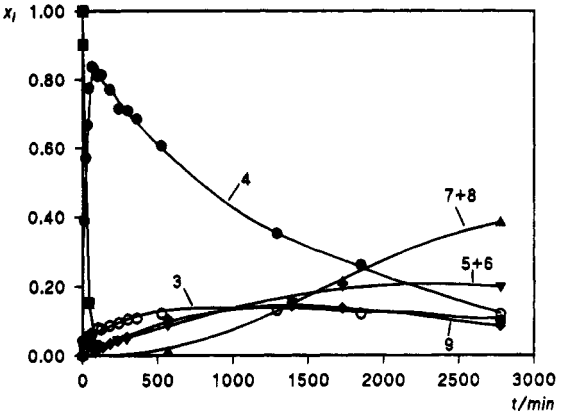

Figure 1. Time-dependent product distribution for the reaction of 5′-*O*-methyl-3′-*O*-tetrahydropyranyluridine 2′-(dimethyl phosphate) (**2**) with aqueous hydrogen chloride at 298.2 K ([H$^+$] = 0.01 mol L^{-1}, I = 0.1 mol L^{-1} with NaCl).

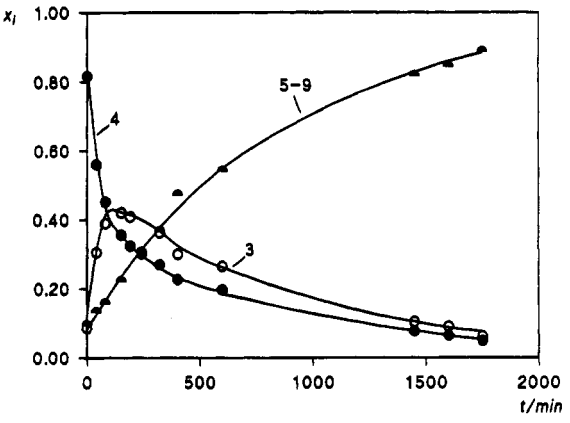

Figure 2. Time-dependent product distribution for the reactions of 5′-*O*-methyluridine 2′-(dimethyl phosphate) (**4**) in an acetic acid/sodium acetate buffer at pH 4.0 and 298.2 K (I = 0.1 mol L^{-1} with NaCl).

As seen from Figure 1, the interconversion and hydrolysis of the triesters **3** and **4** proceed at pH 2 at comparable rates, and under more acidic conditions the hydrolysis becomes faster than the interconversion. However, the hydrolysis product, *viz.* the cyclic monophosphate **9** is not accumulated at pH < 1, owing to its rapid hydrolysis to the 2′- and 3′-monophosphates **7** and **8**. At pH > 3, the interconversion of **3** and **4** becomes faster than their hydrolysis, leading to an equilibrium mixture that contains 58% of the more abundant 3′-isomer (**3**). Under these conditions, the rate of interconversion and hydrolysis was studied by removing the tetrahydropyranyl protection rapidly with 0.1 mol L^{-1} aqueous hydrogen chloride. Addition of the resulting reaction mixture to the prethermonstated solution of sodium formate or acetate then formed the desired buffer system. An illustrative example of the competition between the isomerization and hydrolysis of the deprotected phosphorotriester **4** is given in Figure 2. The reactions of the hydrolysis products **5**–**9** were not studied in detail, since the kinetics and mechanisms for the interconversion and

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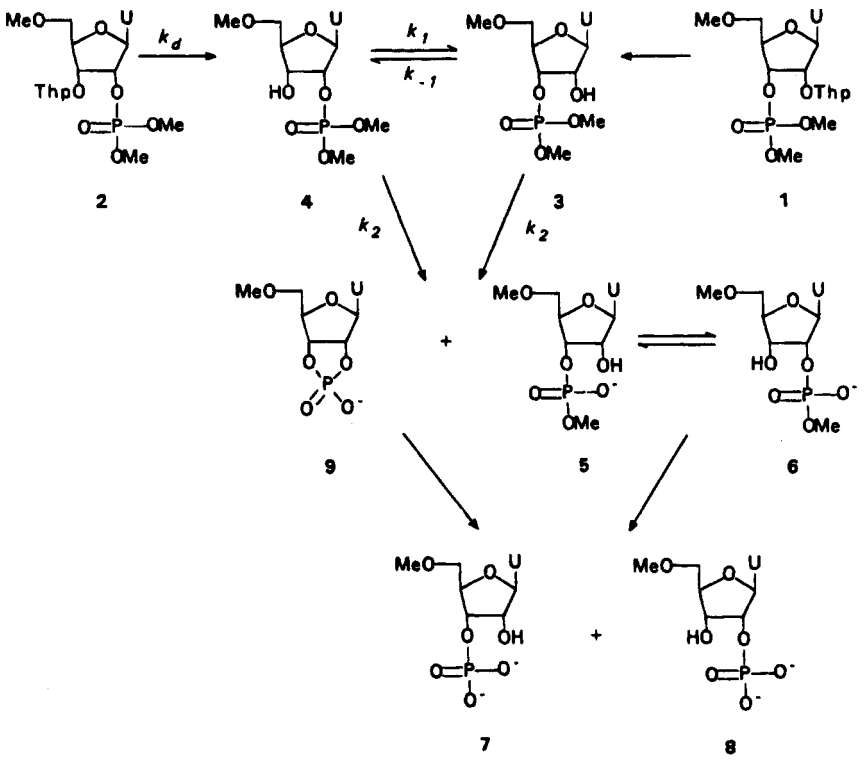
Scheme 1



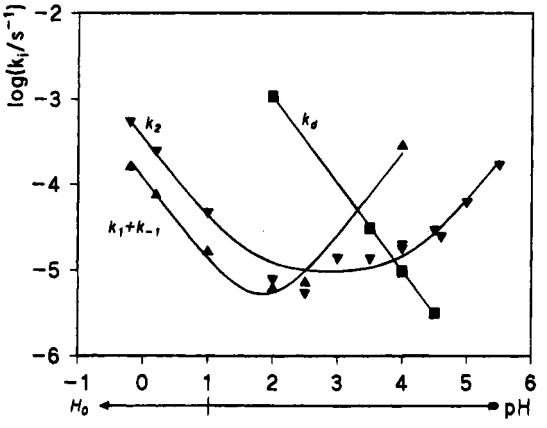


Figure 3. pH–rate profiles for the deprotection of 5′-*O*-methyl-3′-*O*-tetrahydropyranyluridine 2′-(dimethyl phosphate) (**2**; k_d), interconversion of 5′-*O*-methyluridine 2′- and 3′-(dimethyl phosphates) (**3** and **4**; $k_1 + k_{-1}$), and hydrolysis of the latter compounds (k_2) at 298.2 K (I = 0.1 mol L^{-1} with NaCl).

hydrolysis of nucleoside 2′/3′-(methyl phosphates),[7] 2′/3′-monophosphates,[8,9] and 2′,3′-(cyclic)monophosphates[8,10] have been described previously.

Figure 3 shows the pH–rate profiles for the following reactions: (i) the removal of the tetrahydropyranyl group from 5′-*O*-methyl-3′-*O*-tetrahydropyranyluridine 2′-(dimethyl phosphate) (**2**), (ii) the interconversion of 5′-*O*-methyluridine 2′- and 3′-(dimethyl phosphates) (**3** and **4**), and (iii) the hydrolysis of the latter compounds to phosphorodiesters (**5**, **6**, **9**). As seen, the interconversion of **3** and **4** and their hydrolysis are both first-order in hydronium ion concentration at pH < 1.5, the hydrolysis being five times as fast as the interconversion. On going to less acidic solutions, both reactions become hydroxide-ion-catalyzed, and at pH > 3 the interconversion is more than 1 order of magnitude faster than the hydrolysis. Since the rate constants obtained at pH > 2 may contain a contribution of buffer catalysis, no firm conclusions concerning the significance of pH-independent hydrolysis or interconversion near the minima of the rate profiles can be drawn.

It has been suggested previously[7] that the acid-catalyzed hydrolysis and interconversion of the corresponding phosphorodiesters, *i.e.*, adenosine 2′- and 3′-(monomethyl phosphates), proceed *via* a common phosphorane intermediate obtained by the attack of the neighboring hydroxyl group on monocationic phophorodiester group. The present data may be interpreted analogously (Scheme 2). A rapid initial protonation of the phosphorotriester group is followed by the nucleophilic attack of of the neighboring hydroxyl group on phosphorus, giving a monocationic phosphorane intermediate that is rapidly converted to the more stable neutral form. According to the Westheimer concept of pseudorotating phosphorane intermediates,[11] the attacking sugar-oxygen (O3′) initially adopts an apical position and the ring strain of the resulting five-membered ring forces O2′ to an equatorial position. The other apical ligand may be either methoxy or hydroxy, since the electronegativity of these groups is comparable. Pseudorotation of the phosphorane intermediate and subsequent protonation of the apical O2′ results in migration of the dimethyl phosphate group. Alternatively, protonation of an apical methoxy group leads to cleavage of the PO bond and, hence, hydrolysis to methanol and a cyclic triester. The latter compound, which is too unstable to be detected, is rapidly hydrolyzed to the 2′- and 3′-(methyl phosphates) and the 2′,3′-cyclic monophosphate. As seen from Figure 1, the formation of all these products (**5**, **6**, **9**) is parallel. Most probably this reaction also

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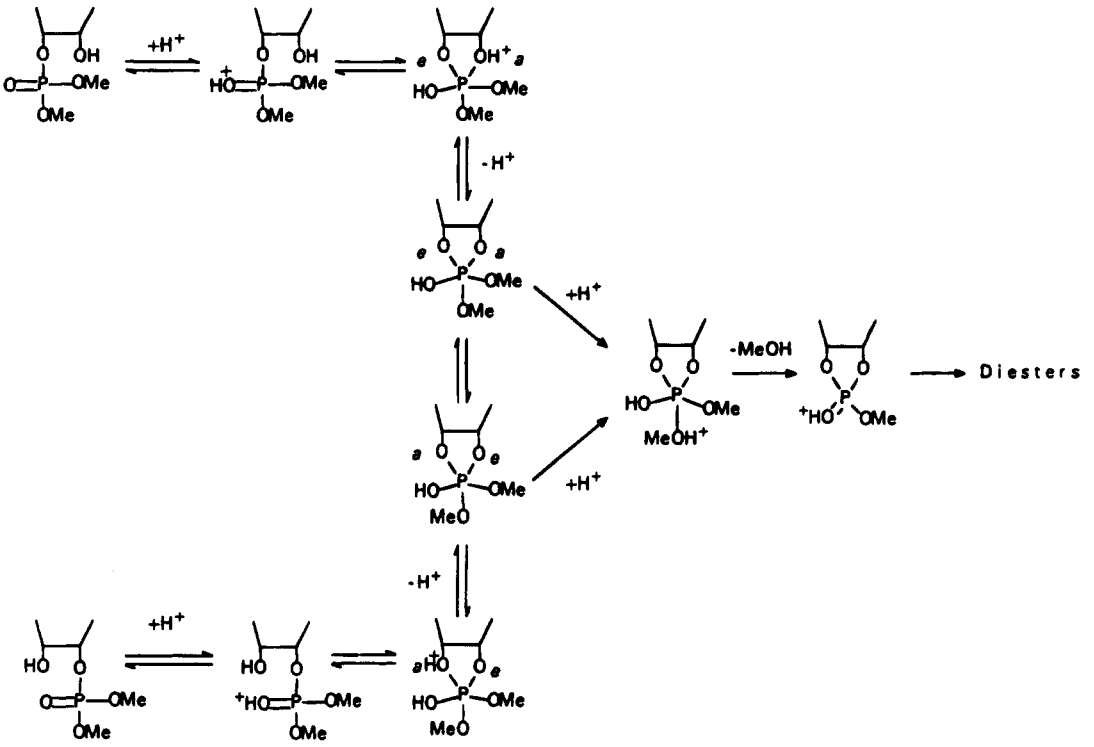
Scheme 2



proceeds *via* a phosphorane intermediate, obtained now by an attack of water on the protonated phosphorotriester.

The base-catalyzed hydrolysis and interconversion proceed in all likelihood *via* a substrate monoanion. The unprotected sugar hydroxyl is initially deprotonated, and it subsequently attacks on the phosphorotriester function, giving a monoanionic phosphorane intermediate (Scheme 3). Departure of methoxide ion from the apical position leads to hydrolysis, while pseudorotation and departure of the originally phosphorylated sugar hydroxyl as oxyanion results in phosphate migration. It is worth noting that the monoanionic phosphorane preferably undergoes endocyclic PO bond rupture, whereas the monocationic species prefers exocyclic cleavage. A possible explanation is that the leaving group is an alkoxide ion in the former case and alcohol in the latter. Sugar hydroxyls are more acidic than methanol,[12] and hence the oxyanions derived from them may be expected to be better leaving groups than the methoxide ion. By contrast, unionized sugar hydroxyls are not necessarily superior to methanol as a leaving group. Now the polar effects on protonation of the leaving oxygen ligand and rupture of the PO bond are opposite, and hence, the polar nature of the leaving group plays a less decisive role.

We have previously[4,7] tried to distinguish the two kinetically equivalent mechanisms for the pH-independent interconversion of 2′,5′- and 3′,5′-dinucleoside monophosphates, *viz.* the attack of unionized sugar hydroxyl on the monoanionic phosphorodiester and the attack of sugar hydroxyl oxyanion on the neutral phosphorodiester. For the following reasons the latter alternative appears more attractive (in contrast to our previous conclusion). Firstly, with phosphorotriesters, the attacking nucleophile is oxyanion even under relatively acidic conditions. Secondly, the pH-dependence of the competition between the hydrolysis and phosphate migration is similar with phosphorodiesters and phosphorotriesters; under neutral conditions migration prevails, while the rates of the acid-catalyzed reactions are comparable, or the hydrolysis may even be faster.

Experimental Section

Materials. 5′-*O*-Methyl-2′-*O*-tetrahydropyranyluridine 3′-(dimethyl phosphate) (**1**) and 5′-*O*-methyl-3′-*O*-tetrahydropyranyluridine 2′-(dimethyl phosphate) (**2**). 5′-*O*-Methyluridine[13] (2.3 mmol) was dissolved in dry acetonitrile (35 mL) containing 60 μL of trifluoroacetic acid, and a slight excess of 3,4-dihydro-2*H*-pyran was added in four portions. The progress of the reaction was followed by RP HPLC. After 3 h the reaction mixture was neuralized with sodium methoxide, and the resulting 2′- and 3′-*O*-tetrahydropyranyl derivatives of 5′-*O*-methyluridine (two diastereomers of each) were separated on a RP column (Bondesil, 40 μm, 3.5 × 45 cm), using 15% aqueous acetonitrile (v/v) as eluant. Only two diastereomers (one 2′-*O*-alkylated and one 3′-*O*-alkylated) were obtained in pure form. No attempt was made to determine the absolute configuration of the tetrahydropyranyl group of these diastereomers. Both products were phosphorylated with phosphoryltris(triazolide) in dry acetonitrile[14] and converted to the dimethyl esters **1** and **2** with a large excess of methanol. After conventional workup, the products were purified by RP chromatography using 25% aqueous acetonitrile (v/v) as eluant. The compounds were assigned as the 2′- and 3′-(dimethyl phosphates) by ^{1}H NMR spectroscopy (400 MHz) as follows. The irradiation technique, starting from the anomeric proton, was applied to assign the signals of the sugar protons, and the assignment was verified by the Raccoon simulation routine. The site of phosphorylation was clearly indicated by the coupling of phosphorus with either H2′ or H3′ (coupling constants 8.7 Hz and 7.6 Hz, respectively). ^{1}H NMR (ppm from internal TMS in C^2HCL_3) for **1**: 8.68 (br s, 1H); 7.71 (d, 1H, J = 8.2 Hz); 6.23 (d, 1H, J = 7.3 H); 5.76 (d, 1H, J = 8.2 Hz); 4.95 (ddd, 1H, J_1 = 7.6 Hz, J_2 = 5.0 Hz, J_3 = 1.8 Hz); 4.77 (m, 1H), 4.41 (ddd, 1H, J_1 = 7.3 Hz, J_2 = 5.0 Hz, J_3 = 1.3 Hz); 4.33 (ddd, 1H, J_1 = 2.4 Hz, J_2 = 2.3 Hz, J_3 = 1.8 Hz); 3.79 (d, 3H, J = 11.2 Hz); 3.82 (d, 3H, J = 11.2 Hz); 3.66 (dd, 1H, J_1 = 10.7 Hz, J_2 = 2.4 Hz); 3.57 (dd,

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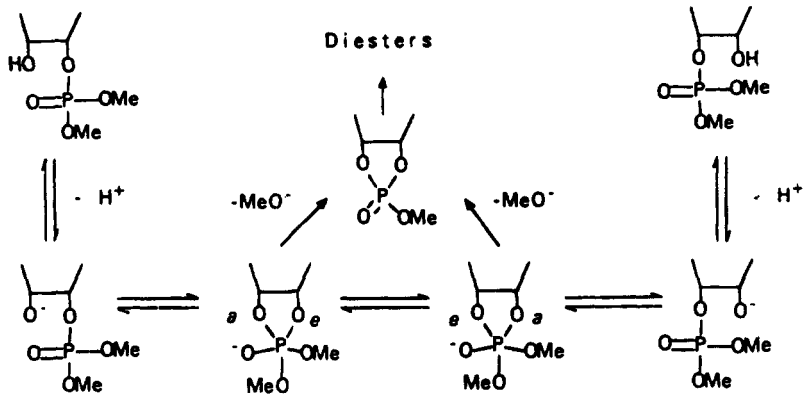
Scheme 3



1H, J_1 = 10.7 Hz, J_2 = 2.3 Hz); 3.43 (s, 3H); 1.4–1.8 (m, 8H). For **2**: 8.55 (br s, 1H); 7.77 (d, 1H, J = 8.2 Hz); 6.14 (d, 1H, J = 4.1 Hz); 5.76 (d, 1H, J = 8.2 H); 4.95 (ddd, 1H, J_1 = 8.7 Hz, J_2 = 4.4 Hz, J_3 = 4.1 Hz); 4.75 (m, 1H), 4.40 (dd, 1H, J_1 = 5.3 Hz, J_2 = 4.4 Hz); 4.21 (ddd, 1H, J_1 = 5.3 Hz, J_2 = 2.4 Hz, J_3 = 2.2 Hz); 3.80 (d, 3H, J = 11.2 Hz); 3.81 (d, 3H, J = 11.2 Hz); 3.75 (dd, 1H, J_1 = 11.0 Hz, J_2 = 2.2 Hz); 3.51 (dd, 1H, J_1 = 11.0 Hz, J_2 = 2.4 Hz); 3.43 (s, 3H); 1.5–1.9 (m, 8H).

To identify the hydrolysis products (**5**–**9**) the following authentic samples were prepared. Monomethyl esters of 5′-*O*-methyluridine 3′- (**5**)- and 2′-monophosphates (**6**). 5′-*O*-Methyluridine[13] (0.20 mmol, 56 mg) was silylated with *tert*-butyldimethylsilyl chloride (0.25 mmol, 37 mg) in dry pyridine as described earlier.[15] The obtained mixture of 2′- and 3′-silylated nucleosides was purified on a silica gel 60 column eluted with dichloromethane containing 2% methanol and phosphorylated with 7 equiv of phosphoryltris(triazolide) in acetonitrile.[14] After 45 min of stirring at room temperature, 5 mL of methanol was added. After 20 min, the mixture was poured in water (15 mL) and extracted with dichloromethane (4 × 15 mL). The organic layers were dried with sodium sulfate and evaporated to dryness. The silyl protecting groups were removed with tetrabutylammonium fluoride (0.45 mmol, 142 mg) in dry tetrahydrofuran (1 h). The mixture was evaporated to dryness and eluted through a cation exchange column (Dowex 50 WX8 in pyridinium form) with a mixture of pyridine and water (50/50 v/v). The product was evaporated to dryness, coevaporated with toluene and ethanol, dissolved in water, and washed with dichloromethane. **5** and **6** were separated by HPLC on a LiChrospher RP-18 column (10 × 250 mm, 5 μm) eluted with an acetic acid buffer (pH 4.2) containing 0.1 mol L^{-1} ammonium chloride and 1.4% (v/v) acetonitrile. At a flow rate 3 mL min^{-1} **6** and **5** eluted in 70 and 82 min, respectively. The buffer salts were removed by eluting the products through the same column with pure water. Ten mg of each isomer was obtained. A small amount of 5′-*O*-methyluridine 3′-(**7**) and 2′-monophosphates (**8**) (1 mg of each) were also isolated from the same mixture. ^{1}H NMR (ppm from external TMS in ^2H_2O) for **5**: 7.72 (d, 1H, J = 8.3 Hz); 5.82 (d, 1H, J = 4.9 Hz); 5.80 (d, 1H, J = 8.3 Hz); 4.40 (m, 1H); 4.29 (dd, 1H, J_1 = J_2 = 5.0 Hz); 4.15 (m, 1H); 3.77 (dd, 1H, J_1 = 13.0 Hz, J_2 = 2.8 Hz); 3.67 (dd, 1H, J_1 = 13.0 Hz, J_2 = 4.3 Hz); 4.46 (d, 3H, J = 11.2 Hz); 3.13 (s, 3H). For **6**: 7.65 (d, 1H, J = 7.8 Hz); 5.90 (d, 1H, J = 5.4 Hz); 5.81 (d, 1H, J = 7.8 Hz); 4.60 (ddd, 1H, J_1 = 8.8 Hz, J_2 = 5.4 Hz, J_3 = 5.4 Hz); 4.21 (dd, 1H, J_1 = 5.4 Hz, J_2 = 5.4 Hz); 3.98 (m, 1H); 3.72 (dd, 1H, J_1 = 12.7 Hz, J_2 = 2.9 Hz); 3.64 (dd, 1H, J_1 = 12.7 Hz, J_2 = 4.4 Hz); 3.36 (d, 3H, J = 10.7 Hz); 3.13 (s, 3H). For **7**: 7.73 (d, 1H, J = 7.8 Hz); 5.80 (2d, 2H, J = 5 and 8 Hz); 4.41 (ddd, 1H); 4.29 (dd, J_1 = J_2 = 5.0 Hz); 4.14 (m, 1H); 3.77 (dd, 1H, J_1 = 12.0 Hz, J_2 = 3.0 Hz); 3.68 (dd, 1H, J_1 = 12.0 Hz, J_2 = 4 Hz); 3.13 (s, 3H). For **8**: 7.64 (d, 1H, J = 8.3 Hz); 5.89 (d, 1H, J = 5.4 Hz); 5.80 (d, 1H, J = 7.8 Hz); 4.6 (ddd, 1H, J_1 = 8.8 Hz, J_2 = 5.4 Hz, J_3 = 5.4 Hz); 4.21 (dd, 1H, J_1 = J_2 = 5.4 Hz); 3.98 (m, 1H); 3.71 (dd, 1H, J_1 = 12.7 Hz, J_2 = 2.9 Hz); 3.64 (dd, 1H, J_1 = 12.7 Hz, J_2 = 4.4 Hz); 3.12 (s, 3H). It is worth noting that the spectra of **5** and **7** closely resemble that of commercially available 3′-UMP and those of **6** and **8** that of 2′-UMP. Actually, the only differences are the additional signals of 5′-*O*-CH_3 and P-*O*-CH_3 (with **5** and **6**). Moreover, **5** and **7** were UV spectroscopically identical with 3′-UMP and **6** and **8** with 2′-UMP.

5′-*O*-Methyluridine 2′,3′-(Cyclic)monophosphate (9). 5′-*O*-Methyluridine[13] was phosphorylated with 1.5 equiv of phosphoryltris(triazolide) in dry acetonitrile.[14] After 4 h treatment at room temperature, the reaction was quenched with water. After workup, the desired cyclophosphate could be isolated by the HPLC system described above, although in very low yield (<10%). Additionally, small amounts of **7** and **8** could be isolated. ^{1}H NMR (ppm from esternal TMS in ^2H_2O): 7.54 (d, 1H, J = 7.8 Hz); 5.77 (d; 1H, J = 8.3 Hz); 5.72 (d, 1H, J = 2.9 Hz); 5.02 (ddd, 1H, J_1 = J_2 = 6.5 Hz, J_3 = 2.8 Hz); 4.79 (ddd, 1H, J_1 = 12.3 Hz, J_2 = 6.5 Hz, J_3 = 5.7 Hz); 4.14 (m, 1H); 3.75 (dd, 1H, J_1 = 11.3 Hz, J_2 = 3.8 Hz); 3.67 (dd, 1H, J_1 = 12.9 H, J_2 = 5.9 Hz); 3.10 (s, 3H). The spectrum closely resembled that of commercially available 2′,3′-cUMP, the only difference being the presence of the additional 5′-*O*-CH_3 resonance. The compound was UV spectroscopically identical with 2′,3′-cUMP.

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 within 0.1 K. The reactions were started by adding the starting material dissolved in methanol (30 μL) to the prethermostated reaction solution (3 mL), the initial substrate concentration being 2 × 10^{-4} mol L^{-1}. Aliquots of 200 mL were withdrawn at appropriate intervals, and their pH was adjusted to about 3 by adding a calculated amount of formic acid or sodium formate. The composition of each sample was analyzed immediately by HPLC. The separations were carried out on a Hypersil ODS column (4 × 250 mm, 5 μm) at the flow rate 1 mL min^{-1}. When the formation and decomposition of compounds **3** and **4** were followed, isocratic elution with an acetic acid/sodium acetate buffer (0.030/0.015 mol L^{-1}, pH 4.3), containing 15% of acetonitrile (v/v) and 0.1 mmol L^{-1} of ammonium chloride, was applied (system A). The retention times observed were as follows: **1**, 18.0 min; **2**, 14.2 min; **3**, 7.7 min; and **4**, 7.1 min. Under these conditions the signals of the phosphorodiester and monoester products (**5**–**9**) partially overlapped (t_R 2–4 min). The fact that the early aliquots of the kinetic runs performed in highly acidic solutions contained only **3** (or **4**) in addition to traces of **1** (or **2**) excludes the possibility of marked hydrolysis or isomerization of **3** and **4** during the chromatographic separation. Separation of all the compounds was achieved as follows: isocratic elution with the acetic acid/sodium acetate buffer containing 5% of acetonitrile was followed by a 15-min gradient elution ending up to 25% of acetonitrile in the same buffer (system B). The retention times were as follows: **1**, 31 min; **2**, 24 min; **3**, 17.9 min; **4**, 17.6 min; **5**, 11.3 min; **6**, 10.8 min; **7**, 6.4 min; **8**, 7.5 min; and **9** 5.7 min (identified by spiking with authentic samples, except **3** and **4** on the basis of their formation from **1** and **2**, respectively). This chromatographic system was unsatisfactory, however, since the separation of the key compounds **3** and **4** was not complete and their slow migration in the column increased the risk of hydrolysis and interconversion during the analysis.

Calculation of the Rate Constants. The first-order rate constants, k_1 and k_{-1}, for the interconversion of 5′-*O*-methyluridine 2′- (**4**) and 3′-(dimethyl phosphates) (**3**) (see Scheme 1) were calculated by eqs 1 and 2, where x_t and x_e stand for the mole

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fraction of the starting nucleoside **4** in the mixture of **3** and **4** at moment t and at equilibrium, respectively. The first-order rate

$$k_1 + k_{-1} = (1/t) \ln [(1 - x_e)/(x_t - x_e)] \quad (1)$$

$$k_1/k_{-1} = (1 - x_e)/x_e \quad (2)$$

constant, k_2, for the hydrolysis of the phosphorotriesters **3** and **4** were obtained by eq 3 using least-squares fitting. Rate constant

$$([\mathbf{3}] + [\mathbf{4}])/[\mathbf{2}]_o = [k_d/(k_2 - k_d)][\exp(-k_d t) - \exp(-k_2 t)] \quad (3)$$

k_d refers to the hydrolysis of the tetrahydropyranyl protecting group. It was calculated by the integrated first-order rate equation from the disappearance of the protected starting material **2**. $[\mathbf{2}]_o$ denotes the initial concentration of the starting material. In all calculations integrated areas of the HPLC signals were assumed to be proportional to concentrations, since no changes in the UV absorbing base moiety took place in the course of the reactions studied.

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Supplementary Material Available: ^{1}H NMR spectra for **1**, **2**, and **5**–**9** (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.