External Transesterification of Ribonucleotide Esters Naturally Catalyzed by Large Ribozymes

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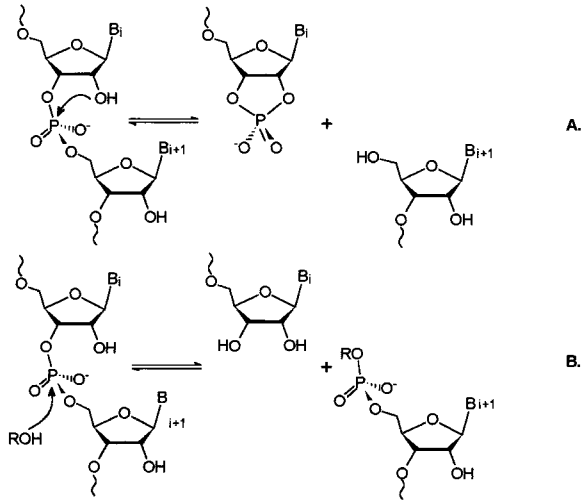
Abstract: The intriguing chemical mechanism of the external transesterification by which large ribozymes (group I, group II, and spliceosomal introns) splice RNA has been found to operate in the methanolysis of ribonucleoside 2′-/3′-dimethyl phosphates in non-hydrogen-bonding organic solvents. Besides needing aprotic organic media this mechanism requires a high concentration of the attacking alcohol accounting for the binding of an external guanosine by group I introns and the use of a non-adjacent internal 2′-OH by group II and spliceosomal introns. This finding is the first example of an external non-ribozymic transesterification of ribonucleotide esters and the means by which this crucial biochemical reaction can be accelerated.

Introduction

Nuclear mRNA splicing is central to the transmission of genetic information in human and other eucaryotic cells. It consists of two consecutive, ribozyme-mediated phosphotransesterifications during which two specific ribonucleotide bonds exchange diols on the spliceosome.[1] The corresponding non-ribozymic (spontaneous) exchange reaction has never been observed in neutral aqueous solutions. Under these physiological conditions RNA undergoes rapid hydrolytic cleavage due to the nucleophilic participation of the adjacent internal 2′-OH.[2] This simply leads to phosphate migration from the 3′- to the 2′-oxygen or expulsion of the 5′-oxygen of the next nucleotide leaving the phosphate at the 3′-position (Scheme 1, reaction **A**), whereas Cech's ribozyme (group I intron) yields a free 3′-hydroxyl and leaves a phosphate at the 5′-position (Scheme 1, reaction **B**).

The difference in the products of these two reactions suggests that a mechanism different from that of reaction **A** must be operating in reaction **B**.[3,4] The internal transesterification reaction **A** is catalyzed by acids, bases, ribonuclease **A**, and small ribozymes, and its mechanism involving nucleophilic assistance by the adjacent 2′-OH is well understood.[5] This is not, however, the case with the external transesterification reaction **B**, and the unusual mechanistic feature of the large ribozymes (group I, group II, and spliceosomal introns) poses an interesting problem for the mechanism of catalysis that cannot be resolved without mechanistic information for the corresponding noncatalyzed reaction.

Scheme 1



Since nucleophilic participation of the 2′-OH would lead to rearrangement rather than 3′-P−O strand scission,[3b] one must assume that this vicinal hydroxyl has a catalytic function in the transesterification reaction **B**, but this cannot be as a nucleophile to phosphorus as in reaction **A**. We tentatively assumed that if an aqueous medium favors the nucleophilic assistance of the 2′-OH, we should study the non-ribozymic ribonucleotide transesterification in aprotic organic media where electrophilic assistance of this group should be favored. Thus, our previous observations[4] suggest that 2-hydroxyalkyl alkyl H-phosphonates and phosphates exchange diols rather than mono-ols in aprotic organic solvents such as pyridine or dioxane. We now report on a change of the phosphoryl transfer mechanism of the ribonucleotide transesterification due to the change of the nature of the 2′-OH participation in aprotic organic media. This finding is the first example of external non-ribozymic (spontaneous)

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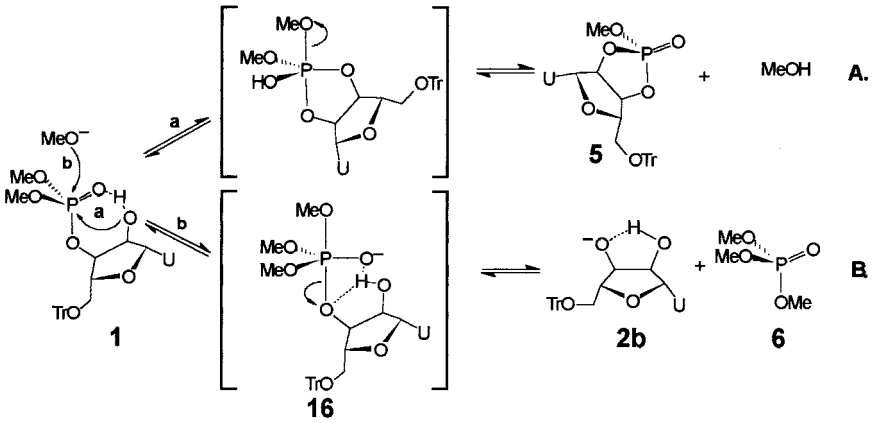
Scheme 2



transesterification of ribonucleotide esters and the means by which this crucial biochemical reaction can be accelerated.

Results and Discussion

Our chosen bioorganic model reaction is the transesterification of the isomeric mixture of 5′-*O*-trityluridine 2′- and 3′-dimethyl phosphates **1** (Scheme 2). The substrate is designed to be soluble in organic media and to undergo transesterification with a measurable rate. These requirements can be met by substituting the rapidly exchangeable proton at the nonbridging oxygen of the phosphodiester group with a methyl group. The resulting phosphate triesters mimic the neutral ionic form of phosphodiesters;[5a] they are 10^3-10^5-fold more reactive than the ionized phosphodiesters,[6] and although questioned, a triester-like mechanism has been proposed as a possible catalytic route for the enzymic cleavage of RNA.[7] Moreover, the background information on the methanolysis of dibenzyl *trans*-2-hydroxycyclohexyl phosphate is available.[8]

The time-dependent product distribution, obtained by RP-HPLC for the reaction of phosphotriester **1** in methanolic sodium methoxide, is shown in Figure 1a. As seen, the disappearance of the starting material is accompanied by the formation of 5′-*O*-trityluridine **2a**, 5′-*O*-trityluridine 2′-/3′-methyl phosphate **3** and 5′-*O*-trityluridine 2′-/3′-phosphate **4** (Chart 1). Since the latter two phosphates are hydrolytic products of the cyclic phosphate **5** (Scheme 2) in the aqueous HPLC buffer, we looked further for the origin of the diol **2a** and the cyclic phosphate **5**.

Ten minutes after the reaction was started only trimethyl phosphate **6** and phosphate diester **3** were detected by ^{31}P NMR spectroscopy (Figure 2b). Therefore, the diol **2a** and the phosphotriester **6** are products of external transesterification **B** (Scheme 2). Under the conditions used (0.6 M NaOMe in dried methanol or dichloromethane), the cyclic phosphate triester **5** is very sensitive to moisture and underwent hydrolysis even when experiments were carried out under argon and in oven-dried NMR tubes. This is to be expected since as a five-membered cyclic ester it should be 10^7-10^8-fold more reactive than its acyclic analogue **1**[5c] that is extremely unstable at high pH.[9] Thus, the incomplete exclusion of moisture accounts for the occurrence of the phosphodiester **3** instead of the cyclic phosphotriester **5**, a product of the internal transesterification **A** (Scheme 2). This interpretation is further supported by the decomposition of the phosphodiester **3** to a cyclic phosphate **7** 45 min later (Figure 2c).

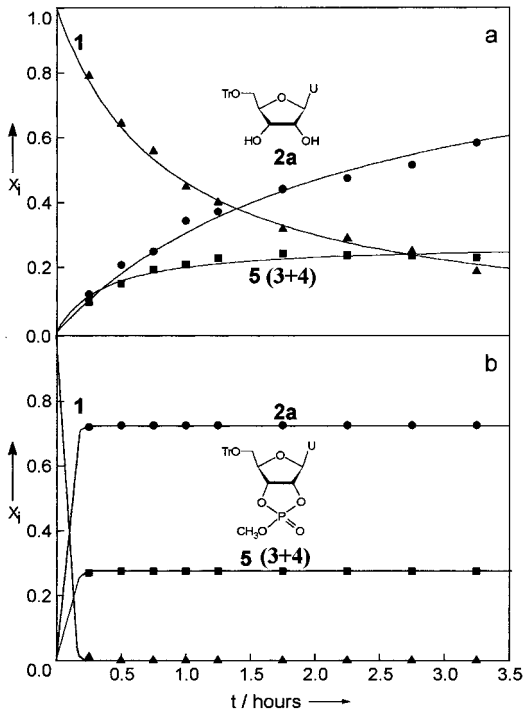
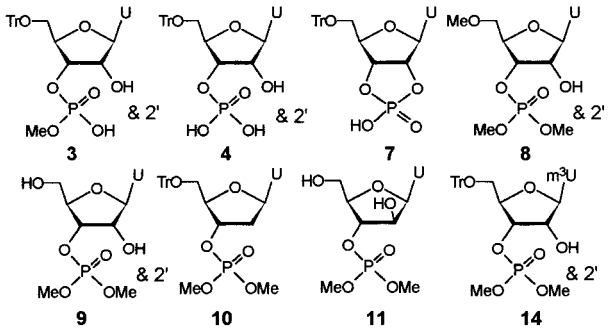


Figure 1. Time-dependent product distribution for the reaction of 0.012 M 5′-*O*-trityluridine 2′/3′-dimethyl phosphate **1** (▲) in 0.6 M NaOMe in CH_3OH (a) and in CH_2Cl_2/MeOH (70:30 v/v) solution (b) at 298.2 K. The mole fraction of cyclic phosphate **5** (■) is a sum of the mole fractions of its hydrolytic products **3** and **4**.

Chart 1



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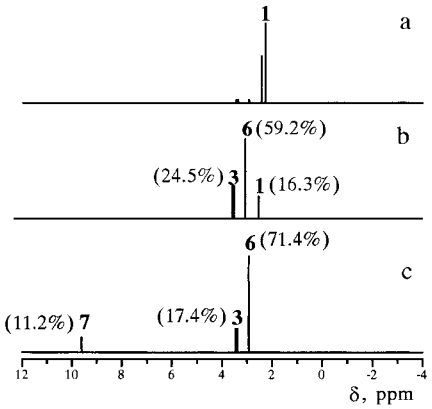


Figure 2. Phosphate product distribution as judged by ^{31}P NMR spectra of the reaction mixture for the reaction of 0.012 M 5′-*O*-trityluridine 2′/3′-dimethyl phosphate **1** in 0.6 M NaOMe in CH_2Cl_2/MeOH (70:30 v/v) solution and 298.2 K at 0′ (a), 10′ (b), and 50′ (c) after the reaction was started. Signals for the 2′- and 3′- isomers are seen on (a).

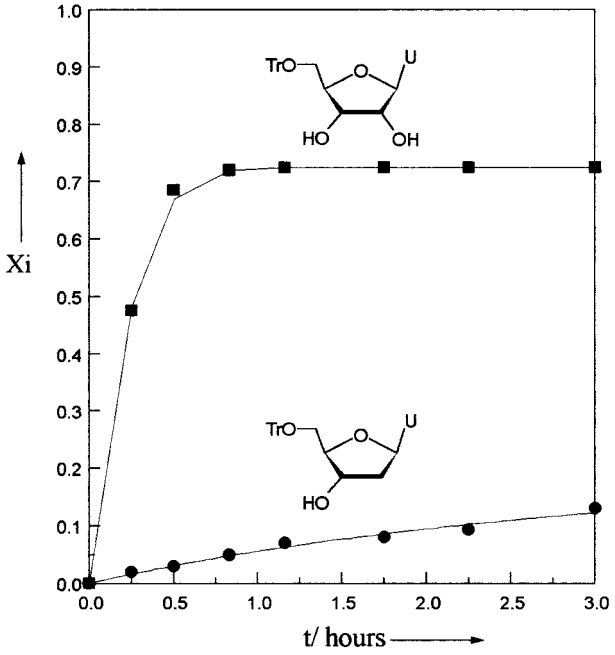
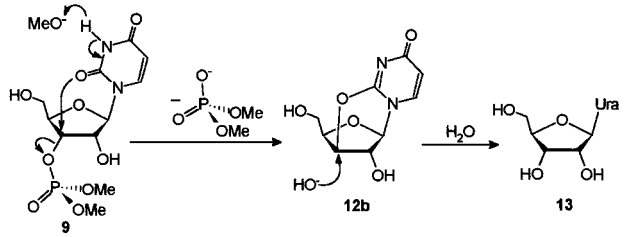


Figure 3. Kinetic curves for the appearance of 5′-*O*-trityluridine (■) and 5′-*O*-trityl-2′-deoxyuridine (●) in the methanolysis of the corresponding nucleoside dimethyl phosphate in 0.6 M NaOMe in CH_2Cl_2/MeOH (70:30 v/v).

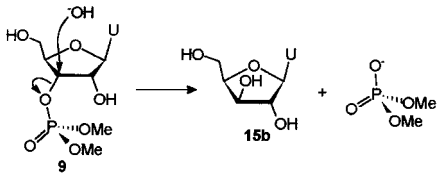
Neither substitution of trityl by methyl (compound **8**), nor detritylation (compound **9**)[9] of the model substrate **1** had any effect on its transformation in methanolic sodium methoxide. The 2′-deoxy derivative, 5′-*O*-trityl-2′-deoxyuridine 3′-dimethyl phosphate **10** and arabinouridine 3′-dimethyl phosphate **11**[10] (Chart), however, undergo more than a 30-fold slower methanolysis (Figure 3) to 5′-*O*-trityl-2′-deoxyuridine or arabinouridine respectively and trimethyl phosphate **6**, indicating that the presence of the 2′-hydroxyl group accelerates the external transesterification (route **B** in Scheme 2).

Under the conditions used (room temperature, 0.1−1 M NaOMe in MeOH or CH_2Cl_2/MeOH (70:30 v/v)) there are two additional routes leading to the dephosphorylation of **9**. Thus, a nucleophilic attack of the nucleobase−carbonyl−oxygen on 2′-C/3′-C would produce 2,3′-/2,2′-anhydro uridine **12a/b** that

Scheme 3



Scheme 4



after hydrolysis would give uridine **13**. No presence of **12a/b**, however, was detected by HPLC using an authentic sample of the anhydro nucleoside as reference. Furthermore, the latter compound proved to be stable under the conditions used: HPLC analysis gave no indication of the presence of **13** after 24 h; when 3-*N*-methyl 5′-*O*-trityluridine 2′/3′-dimethyl phosphate **14** was used instead of **9**, no change in the rate and the product distribution was observed. Therefore, the anhydro route (Scheme 3) is not followed.

A similar but external nucleophilic attack on 2′-C/3′-C (Scheme 4) would displace dimethyl phosphate from **9** and, as a result of the stereochemical inversion, 5′O-tritylarabino- and xylo-uridine **15a/b** (Scheme 4) would be obtained. This reaction, however, is evidently very much slower than the nucleophilic displacement at phosphorus (Scheme 2) since no uridine epimers **15a/b** were detected in the reaction mixture by HPLC using an authentic samples of the *trans*-1,2-diols as references.

As recently suggested[4,11] the vicinal hydroxyl could function as an electrophilic catalyst of the nucleophilic attack by hydrogen bonding either to the former ether and/or phosphoryl oxygen(s) in the corresponding pentacoordinated phosphorane intermediate/transition state **16** (Scheme 2), instead of acting as a nucleophilic catalyst (route **B**, Scheme 2). Such a catalytic mechanism is used by proteolytic enzymes, an oxyion hole being the hydrogen-bond donor.[12] Actually, we observed a downfield shift ($\Delta\,\delta = 1.66$ ppm) of the 2′-/3′-hydroxyl proton in $CDCl_3$ on ^{1}H NMR of the phosphotriester **1** indicative of weak intramolecular hydrogen bonding even in the ground state.

The medium effect on the supposed electrophilic catalytic activity of the adjacent hydroxyl must follow the medium effect on hydrogen bonding. Thus, in hydrogen-bond-donor solvents such as water, the adjacent hydrogen donor and acceptor are better solvated separately. Actually, hydrolysis to uridine (route **B**, Scheme 2) did not occur in aqueous solutions.[9] The change from water to methylated water (MeOH), promotes the dephosphorylation reaction (Figure 1a). Furthermore, the addition of a non-hydrogen-bond-donor solvent like CH_2Cl_2 results in the partitioning of the triester **1** between competing external (MeO^-) and internal (2′-/3′-O^-) nucleophiles faster than the mixing and analysis time (5 min for ^{31}P NMR and 15 min for HPLC) (Figure 1b).

Since external (route **B**) and internal (route **A**) transesterifications (Scheme 2) are parallel reactions, the partitioning ratio

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$$\alpha = \frac{v_B}{v_A} = \frac{k_B}{k_A}[\mathrm{NaOMe}] = \frac{[\mathrm{TrU}]}{\sum_i [\mathrm{TrUp}_i]}$$

where [TrU] and $\sum[\mathrm{TrUp}_i]$ are the concentrations of the diol **2a** and the diol phosphates (**3** + **4**) respectively at fixed intervals of time, k_B is the second-order rate constant of the diol-exchange reaction **B** and k_A, the first-order rate constant of the intramolecular reaction **A**. The linear α/[NaOMe] plot (Figure 4) supports this interpretation and indicates that a high methoxide concentration is a necessary condition for the operation of the phosphoryl transfer mechanism **B**. Furthermore, it is a reason for the rejection of a mechanism involving simple deprotonation of the 2′-OH which in turn leads exclusively to the usual cyclization pathway observed in aqueous solutions.

The key feature of the external transesterification **B** is the selective microsolvation of the product-like transition state **16** and the oxyanion **2b** produced by the adjacent hydroxyl. This effect, recognized as the Henbest−Kupcham effect in acyl transfer reactions,[13] favors the solvolysis of carboxylate esters possessing a neighboring hydroxyl group. The stabilization of the ribonucleotide monoanion **2b** via intramolecular hydrogen bonding is supposed to be the major factor responsible for the enhanced acidity of sugar *cis*-hydroxyls in ribonucleotides.[14] The strength of such a homonuclear negative-charge-assisted, low-barrier hydrogen bonding (LBHB) in the transition state is currently the subject of debates on whether it can be used as a rationale for the exceptional catalytic abilities of biocatalysts.[15]

Unlike protein−enzyme mechanisms, the large ribozyme mechanism has been studied up to now directly, without preliminary elucidation of the mechanism of the non-ribozymic reaction and the means by which this reaction can be accelerated.[11,16] When such a model reaction is now available (route **B**, Scheme 2), it is intriguing to see how this mechanistic family of ribozymes meets the major requirements derived from the non-ribozymic diol phosphotransesterification. First of all, to use this mechanism the ribozyme must provide a high concentration of the external attacking alkoxide. Large ribozymes (group I, group II, and spliceosomal introns) perform this job perfectly using the non-adjacent internal 2′-OH of **A** or the 3′-OH of G, specifically bonded in its guanosine-binding pocket.[16] The binding increases the effective molarity (EM)[17] of the external alkoxide resulting in its effective competition with the internal alkoxide (2′-, 3′-O^-).

The increased concentration of the external nucleophile in the non-ribozymic reaction, however, is not the only parameter that imparts a diol phosphotransesterification. It has been estimated that the EM of the 2′-OH group of RNA is $\sim 3 \times 10^7$ M,[17] a value that could not be reached simply by increasing the nucleophile concentration. Therefore, the nucleophilic participation of the vicinal hydroxyl seems to be suppressed and its electrophilic participation-favoured. Actually, unlike water, aprotic organic solvents suppress ionization of weak acids as 2′-OH ($\Delta pK_a \sim 17$ in acetonitrile[18]) and favor formation of stronger intramolecular hydrogen bonds as judged by the better solvation of *cis*-1,2-diols in non-aqueous solutions.[4c,18c]

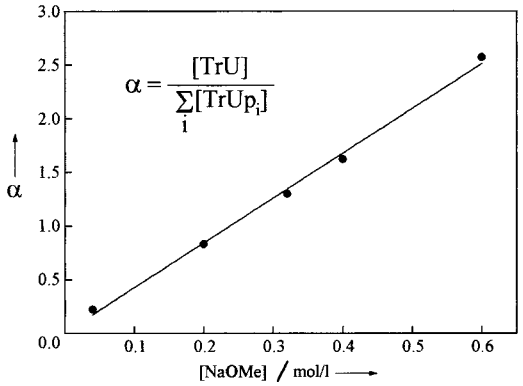

Figure 4. Plot of the partitioning ratio α vs methoxide concentration for the methanolysis of 5′-*O*-trityluridine 2′/3′-dimethyl phosphate **1** in CH_2Cl_2/MeOH (40:60 v/v) solution. α is the ratio of the peak areas of **2a** and (**3**+**4**) in HPLC chromatograms at different methoxide concentrations, 20 min after the reaction was started.

The observed distinct medium effect on the rate of the diol phosphotransesterification **B** (Scheme 2) predicts effective control of the local environment of the substrate 2′-hydroxyl in the ribozyme active site. As shown here using a bioorganic model reaction (route **B**, Scheme 2), only a medium with a low dielectric constant and non-hydrogen-bonding properties can provide effective differential microsolvatation of the phosphotransesterification transition state **16**. Moreover, since only unionized phosphodiesters are energetically compatible with such a medium, the large ribozymes are expected to take advantage of the triester-like mechanism.

The present study implies the important role of metal ions in the ribozymic diol phosphotransesterification as well. In the non-ribozymic reaction **B** (Scheme 2) Na^+ ions activate the attacking nucleophile MeOH[19] and assist the departure of the leaving diol **2a** as an ion pair. Since according to the Westheimer's guidelines,[3b,20] nucleophiles will enter at and leaving groups depart from apical positions of the oxyphosphorane intermediate/transition state **16**, at least two Na^+ ions must participate in the elementary act. Ribozymes use two Mg^{2+}.[21] Moreover, alkali-metal alkoxides exist in nonpolar organic solvents as clusters competent to catalyze the transesterification reactions.[22] This suggests that metal ions must be associated as they are in the ribozymic active site.

Conclusion

The reported diol phosphotransesterification in organic media is the first example of an external non-ribozymic phosphotransesterification of ribonucleotide esters and the means by which this reaction can be accelerated. Similar reactivity in a non-hydrogen-bonding medium of RNA as ribozyme substrate is thus determined by the inherent property of the *cis*-1,2-diol system of its constituent ribonucleosides, containing hydrogen bonded catalytic and leaving groups in the same molecule. The conformation of RNA enzyme provides specificity, external

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nucleophile, metal ion binding sites and medium for the ribozyme reaction, i.e., a catalysis of the catalysis, observed in the non-ribozymic reaction.

Experimental Section

General Procedures, Methods and Materials. ^{1}H and ^{31}P NMR spectra were taken on a BRUKER Avance-DRX 250 spectrometer at 300 K with tetramethylsilan as internal and 85% H_3PO_4 as external standard, respectively. Chemical shifts are reported in δ (ppm). Reverse phase HPLC analyses were performed on Waters Liquid Chromatograph equipped with absorbance detector model 441 set at 280 nm and column Nucleosil 100-5C_{18} (12.5 cm × 4.6 mm) or aminocolumn Spherisorb 5m NH_2 (25 cm × 5 mm) for analytical runs, or Nucleosil 100-5C_{18} (25 cm × 10 mm) for semi-preparative runs. Commercial solvents and reagents were used as received unless otherwise noted. Pyridine was dried over sodium hydroxide and distilled over calcium hydride before use. Dichloromethane and methanol were dried using standard procedures and distilled before use. A 2 M stock solution of sodium methoxide in methanol was prepared prior to use by dissolving metallic sodium in the dried methanol under anhydrous conditions. 5′-*O*-Trityluridine,[23] 5′-*O*-methyluridine,[24] 5′-*O*-trityl-2′-deoxyuridine[25] and 2,2′-anhydrouridine[26] were prepared as described.

Synthesis of Nucleoside Dimethyl Phosphates. Uridine 2′-/3′-dimethyl phosphates **9** and arabinouridine 3′-dimethyl phosphate **11** were prepared from the corresponding nucleoside phosphates as described previously.[9,10]

General Procedure for the Preparation of 5′-O-Protected Nucleoside Dimethyl Phosphates. To a cooled (5 °C) solution of $POCl_3$ (1 mmol) in dry CH_2Cl_2 (4 mL) pyridine (4 mmol) was added dropwise. After little initial fuming the resulting clear solution was added to a precooled mixture (5 °C) of 5′-*O*-protected nucleoside (0.2 mmol) and pyridine (4 mmol) in 4 mL dry CH_2Cl_2 and stirred at room temperature for 5−10 min. Methanol (4 mmol) was added, and the stirring continued for another 20−40 min (**1**, **8**, **14**) or overnight (**10**), when the reactions were completed as judged by RP-HPLC (isocratic elution with 40% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0 for **1**, **14**, and **10** or with 15% CH_3CN in the same buffer for **8**) and ^{31}P NMR. Then the reaction mixture was evaporated to dryness under reduced pressure, dissolved in acetonitrile, and applied on a semi-preparative RP-HPLC column (isocratic elution with 50% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0 for **1**, **14**, and **10** or with 25% CH_3CN in the same buffer for **8**). Appropriate fractions (2′- and 3′-isomers can be collected separately) were evaporated immediately (reduced pressure, 40 °C), dried several times by coevaporation with dry acetonitrile or dichloromethane, and kept in a desiccator. The analytically pure samples of the triesters were prepared by dry extraction in acetonitrile or dichloromethane from the inorganic buffer salts. Total yields of the isolated isomeric mixtures: 80−90%.

5′-*O*-Trityluridine 2′-/3′-dimethyl phosphate 1: Yield 82%, 3′-isomer: ^{1}H NMR ($CDCl_3$, 25 °C, 250 MHz) δ = 3.45 (dd, $J_{4',5'}$ = 2.6 Hz, $J_{5',5''}$ = 9.9 Hz, 1H, H-5′), 3.48 (s, 1H, HO), 3.56 (dd, $J_{4',5'}$ = 2.6 Hz, $J_{5',5''}$ = 9.9 Hz, 1H, H-5″), 3.72 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 3.78 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 4.33 (dt, $J_{4',5'}$ = 2.6 Hz, $J_{3',4'}$ = 4.7 Hz, 1H, H-4′), 4.48 (dd, $J_{2',3'}$ = 4.6 Hz, $J_{3',4'}$ = 4.7 Hz, 1H, H-3′), 4.88 (1H, H-2′), 5.34 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-5), 5.98 (d, $J_{1',2'}$ = 4.3 Hz, 1H, H-1′), 7.26−7.41 (m, 15H, C_6H_5), 7.75 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-6), 9.35 (s, 1H, H−N^3); ^{31}P NMR (CH_2Cl_2, 25 °C, 101 MHz) δ = 0.92 ppm; analytical RP-HPLC (40% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0; flow 0.8) t_R = 12 min. 2′-Isomer: ^{1}H NMR ($CDCl_3$, 25 °C, 250 MHz) δ = 3.45 (dd, $J_{4',5'}$ = 2.6 Hz, $J_{5',5''}$ = 9.9 Hz, 1H, H-5′), 3.54 (s, 1H, HO), 3.56 (dd, $J_{4',5'}$ = 2.6 Hz, $J_{5',5''}$ = 9.9 Hz, 1H, H-5″), 3.82 (d, $^3J_{P,H}$ = 11.4 Hz, 3H, H_3COP), 3.84 (d, $^3J_{P,H}$ = 11.4 Hz, 3H, H_3COP), 4.16 (dt, $J_{4',5'}$ = 2.6 Hz, $J_{3',4'}$ = 6.7 Hz, 1H, H-4′), 4.59 (dd, $J_{2',3'}$ = 4.7 Hz, $J_{3',4'}$ = 6.7 Hz, 1H, H-3′), 4.88 (1H, H-2′), 5.34 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-5), 6.06 (d, $J_{1',2'}$ = 2.7 Hz, 1H, H-1′), 7.26−7.41 (m, 15H, C_6H_5), 7.88 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-6), 9.13 (s, 1H, H−N^3); ^{31}P NMR (CH_2Cl_2, 25 °C, 101 MHz) δ = 1.11 ppm; analytical RP-HPLC (40% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0; flow 0.8) t_R = 13 min.

***N*^{3}-Methyl-5′-*O*-trityluridine 2′-/3′-dimethyl phosphate 14:** Yield 88%, 3′-isomer: ^{1}H NMR ($CDCl_3$, 25 °C, 250 MHz) δ = 3.29 (s, 3H, H_3C-N^3), 3.45 (dd, $J_{4',5'}$ = 2.6 Hz, $J_{5',5''}$ = 10.0 Hz, 1H, H-5′), 3.48 (s, 1H, HO), 3.56 (dd, $J_{4',5''}$ = 2.6 Hz, $J_{5',5''}$ = 10.0 Hz, 1H, H-5″), 3.72 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 3.78 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 4.33 (dt, $J_{4',5'}$ = 2.6 Hz, $J_{3',4'}$ = 4.7 Hz, 1H, H-4′), 4.48 (dd, $J_{2',3'}$ = 4.6 Hz, $J_{3',4'}$ = 4.7 Hz, 1H, H-3′), 4.88 (1H, H-2′), 5.34 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-5), 5.98 (d, $J_{1',2'}$ = 4.3 Hz, 1H, H-1′), 7.26−7.41 (m, 15H, C_6H_5), 7.75 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-6); ^{31}P NMR (CH_2Cl_2, 25 °C, 101 MHz) δ = 0.95 ppm; analytical RP-HPLC (40% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0; flow 0.8) t_R = 16 min. 2′-Isomer: ^{1}H NMR ($CDCl_3$, 25 °C, 250 MHz) δ = 3.28 (s, 3H, H_3C-N^3), 3.45 (dd, $J_{4',5'}$ = 2.6 Hz, $J_{5',5''}$ = 10.0 Hz, 1H, H-5′), 3.54 (s, 1H, HO), 3.56 (dd, $J_{4',5''}$ = 2.6 Hz, $J_{5',5''}$ = 10.0 Hz, 1H, H-5″), 3.82 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 3.84 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 4.16 (dt, $J_{4',5'}$ = 2.6 Hz, $J_{3',4'}$ = 6.7 Hz, 1H, H-4′), 4.59 (dd, $J_{2',3'}$ = 4.7 Hz, $J_{3',4'}$ = 6.7 Hz, 1H, H-3′), 4.88 (1H, H-2′), 5.34 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-5), 6.06 (d, $J_{1',2'}$ = 2.7 Hz, 1H, H-1′), 7.26−7.41 (m, 15H, C_6H_5), 7.88 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-6); ^{31}P NMR (CH_2Cl_2, 25 °C, 101 MHz) δ = 1.2 ppm; analytical RP-HPLC (40% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0; flow 0.8) t_R = 16.9 min.

5′-*O*-Methyluridine 2′-/3′-dimethyl phosphate 8: Yield 85%, 3′-isomer: ^{1}H NMR ($CDCl_3$, 25 °C, 250 MHz) δ = 1.77 (s, 3H, $H_3COC^{5'}$), 3.45 (dd, $J_{4',5'}$ = 2.6 Hz, $J_{5',5''}$ = 9.9 Hz, 1H, H-5′), 3.48 (s, 1H, HO), 3.56 (dd, $J_{4',5''}$ = 2.6 Hz, $J_{5',5''}$ = 9.9 Hz, 1H, H-5″), 3.72 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 3.78 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 4.33 (dt, $J_{4',5'}$ = 2.6 Hz, $J_{3',4'}$ = 4.7 Hz, 1H, H-4′), 4.48 (dd, $J_{2',3'}$ = 4.2 Hz, $J_{3',4'}$ = 4.7 Hz, 1H, H-3′), 4.89 (1H, H-2′), 5.37 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-5), 5.95 (d, $J_{1',2'}$ = 4.2 Hz, 1H, H-1′), 7.75 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-6); ^{31}P NMR (CH_2Cl_2, 25 °C, 101 MHz) δ = 0.9 ppm; analytical RP-HPLC (15% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0; flow 0.8) t_R = 8.5 min. 2′-Isomer: ^{1}H NMR ($CDCl_3$, 25 °C, 250 MHz) δ = 1.77 (s, 3H, $H_3COC^{5'}$), 3.45 (dd, $J_{4',5'}$ = 2.6 Hz, $J_{5',5''}$ = 9.9 Hz, 1H, H-5′), 3.54 (s, 1H, HO), 3.56 (dd, $J_{4',5''}$ = 2.6 Hz, $J_{5',5''}$ = 9.9 Hz, 1H, H-5″), 3.82 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 3.84 (d, $^3J_{P,H}$ = 11.3 Hz, 3H, H_3COP), 4.18 (dt, $J_{4',5'}$ = 2.6 Hz, $J_{3',4'}$ = 6.5 Hz, 1H, H-4′), 4.58 (dd, $J_{2',3'}$ = 4.2 Hz, $J_{3',4'}$ = 6.5 Hz, 1H, H-3′), 4.89 (1H, H-2′), 5.31 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-5), 6.04 (d, $J_{1',2'}$ = 2.5 Hz, 1H, H-1′), 7.88 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-6); ^{31}P NMR (CH_2Cl_2, 25 °C, 101 MHz) δ = 1.02 ppm; analytical RP-HPLC (15% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0; flow 0.8) t_R = 9.2 min.

5′-*O*-Trityl-2′-deoxyuridine 3′-dimethyl phosphate 10: Yield 90%: ^{1}H NMR ($CDCl_3$, 25 °C, 250 MHz) δ = 2.36 (ddd, $J_{1',2'}$ = 7.5 Hz, $J_{2',2''}$ = 14.2 Hz, $J_{2',3'}$ = 6.6 Hz, 1H, H-2′), 2.66 (ddd, $J_{1',2''}$ = 5.9 Hz, $J_{2',2''}$ = 14.2 Hz, $J_{2'',3'}$ = 3.0 Hz, 1H, H-2″), 3.48 (d, $J_{4',5'}$ = 3.0 Hz, 2H, H-5′), 3.72 (d, $^3J_{P,H}$ = 11.2 Hz, 3H, H_3COP), 3.76 (d, $^3J_{P,H}$ = 11.2 Hz, 3H, H_3COP), 4.26 (dt, $J_{4',5'}$ = 3.0 Hz, $J_{3',4'}$ = 5.8 Hz, 1H, H-4′), 5.13 (ddd, $J_{2',3'}$ = 6.6 Hz, $J_{2'',3'}$ = 3.0 Hz, $J_{3',4'}$ = 5.8 Hz, 1H, H-3′), 5.32 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-5), 6.34 (dd, $J_{1',2'}$ = 7.5 Hz, $J_{1',2''}$ = 5.9 Hz, 1H, H-1′), 7.18−7.41 (m, 15H, C_6H_5), 7.69 (d, $J_{5,6}$ = 8.2 Hz, 1H, H-6), 7.97 (s, 1H, H−N^3); ^{31}P NMR (CH_2Cl_2, 25 °C, 101 MHz) δ = 0.78 ppm; analytical RP-HPLC (40% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0; flow 0.8) t_R = 15 min.

Methanolysis Reactions. To a solution of 0.12 mmol of nucleoside dimethyl phosphate in 7 mL of dry organic solvent (CH_2Cl_2 or MeOH) 3 mL of 0.13−2 M NaOMe in MeOH was added at 298.2 K. The reaction mixture was divided into two samples, and the progress of the reaction was followed simultaneously both by ^{31}P NMR spectroscopy and analytical HPLC.

^{31}P NMR Kinetic Studies. One of the samples was transferred to the NMR tube, and the ^{31}P NMR spectra were recorded every 5 min during 1 h. The time-dependent phosphate product distribution was determined from the signal intensities at different reaction times.

HPLC Kinetic Studies. Aliquots were withdrawn from the other sample at appropriate time intervals, diluted with the mobile phase and

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subjected to HPLC analysis (using Spherisorb 5m NH_2 (25 cm × 5 mm) and isocratic elution with 70% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0 for **9** and **11** or Nucleosil 100-5C_{18} (12.5 cm × 4.6 mm) and isocratic elution with 40% CH_3CN in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.0 for **1**, **14** and **10** or with 15% CH_3CN in the same buffer for **8**). The time-dependent mole fractions of the reaction products were calculated from their peak area fractions. The partitioning ratio α for the methanolysis of **1** was calculated from the peak area ratio of 5′-*O*-trityluridine and the 5′-*O*-trityluridine phosphates at fixed reaction times.

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