JOC Article

Hydrolysis of 2′,3′-*O*-methyleneadenos-5′-yl Bis(2′,5′-di-*O*-methylurid-3′-yl) Phosphate, a Sugar *O*-Alkylated Trinucleoside 3′,3′,5′-Monophosphate: Implications for the Mechanism of Large Ribozymes

Tuomas Lönnberg* and Satu Mikkola

Department of Chemistry, University of Turku, Vatselankatu 2, FIN-20014 Turku, Finland

tuanlo@utu.fi

Received July 28, 2003

Hydrolytic reactions of 2′,3′-*O*-methyleneadenos-5′-yl bis(2′,5′-di-*O*-methylurid-3′-yl) phosphate (**1**), a sugar *O*-alkylated trinucleoside 3′,3′,5′-monophosphate, have been followed by RP HPLC over a wide pH range. Under neutral and mildly acidic conditions, the only reaction observed was a pH-independent cleavage of the O−C5′ bond of the 5′-linked nucleoside. Under more alkaline conditions nucleophilic attack by hydroxide ion starts to compete. The reaction is first order in [OH^-] and becomes predominant at pH 10. Each of the 3′-linked nucleosides is displaced 2.9 times as readily as the 5′-linked one. To determine the β_{lg} value for the hydroxide ion catalyzed hydrolysis of **1**, two diesters (**2a**,**b**) having 2′,3′-*O*-methyleneadenosine (**7**) and 2′,5′-di-*O*-methyluridine (**4**) as leaving groups were hydrolyzed under alkaline conditions. Since the β_{lg} value for this reaction is known, ΔpK_a between **4** and **7** could be calculated. The β_{lg} for the hydrolysis of **1** was estimated to be −0.5 with use of this information. The mechanisms of the partial reactions and the role of leaving group properties in ribozyme reactions of large ribozymes are discussed.

Introduction

The naturally occurring catalytic ribonucleic acids, ribozymes, fall according to their size and catalytic mechanism into two categories.[1] The small ribozymes that include hammerhead, hairpin, hepatitis delta virus, and varkud satellite ribozymes catalyze the cleavage of a phosphodiester bond in the target chain by facilitating the intramolecular attack of the neighboring 2′-hydroxy function on the phosphorus atom.[2] In other words, a cyclic phosphorane intermediate (or transition state) preceding the release of the 5′-linked nucleoside and concomitant formation of a 2′,3′-cyclic phosphate is obtained intramolecularly without covalent involvement of the ribozyme. With large ribozymes, viz. group I and II introns and the RNA subunit of RNase P, the 3′-linked nucleoside of the scissile phosphodiester bond is displaced intermolecularly by the 3′(or 2′-)hydroxy function of the entering nucleoside. Accordingly, the reaction proceeds via an acyclic phosphorane intermediate (or transition state) having two nucleosides bonded via a secondary and one via a primary oxygen atom.

While numerous studies with small molecular models have been carried out to clarify the underlying chemical principles of the cleavage of RNA by an intramolecular mechanism,[3] similar to that utilized by small ribozymes, the corresponding data on the intermolecular transesterification mimicking the action of large ribozymes is extremely scanty. In fact such a reaction has been detected only in nonaqueous solutions.[4] The present study is aimed at partly filling this gap. Kinetics for the hydrolysis of a trinucleoside 3′,3′,5′-monophosphate, 2′,3′-*O*-methyleneadenos-5′-yl bis(2′,5′-di-*O*-methylurid-3′-yl) phosphate (**1**), have been studied over a wide pH range. An attack of hydroxide ion on the phosphorus atom of **1** results in a monoanionic pentacoordinated phosphorane intermediate or phosphorane-like transition state. This intermediate/transition state closely resembles the one proposed [5−9] for the ribozyme reaction of group I introns (Figure 1). The main difference is that one of the nonbridged phosphorane oxygens is protonated in the transition state of the nonenzymatic model reaction (**TS1**) but coordinated to one or two magnesium ions in the ribozyme reaction (**TS2**). In addition, the entering and departing oxyanion, i.e. the two 3′-oxygen atoms bonded to the phosphorus atom, in all likelihood interact with a magnesium ion. Despite these differences, an understanding of the factors that affect the relative rates

* Corresponding author.

(1) Takagi, Y.; Warashina, M.; Stec, W. J.; Yoshinari, K.; Taira, K. *Nucleic Acids Res.* **2001**, *29*, 1815−1834.
(2) Zhou, D.-M.; Taira, K. *Chem. Rev.* **1998**, *98*, 991−1026.
(3) Oivanen, M.; Kuusela, S.; Lönnberg, H. *Chem. Rev.* **1998**, *98*, 961−990.
(4) Roussev, C. D.; Ivanova, G. D.; Bratovanova, E. K.; Vassilev, N. G.; Petkov, D. D. *J. Am. Chem. Soc.* **1999**, *121*, 11267−11272.
(5) Piccirilli, J. A.; Vyle, J. S.; Caruthers, M. H.; Cech, T. R. *Nature* **1993**, *361*, 85−88.
(6) Weinstein, L. B.; Jones, B. C.; Cosstick, R.; Cech, T. R. *Nature* **1997**, *388*, 805−808.
(7) Yoshida, A.; Shan, S.; Herschlag, D.; Piccirilli, J. A. *Chem. Biol.* **2000**, *7*, 85−96.
(8) Shan, S.; Yoshida, A.; Sun, S.; Piccirilli, J. A.; Herschlag, D. *Proc. Natl. Acad. Soc. U.S.A.* **1999**, *96*, 12299−12304.
(9) Yoshida, A.; Sun, S.; Piccirilli, J. A. *Nat. Struct. Biol.* **1999**, *6*, 318−321.

10.1021/jo035094k CCC: $27.50
Published on Web 01/10/2004

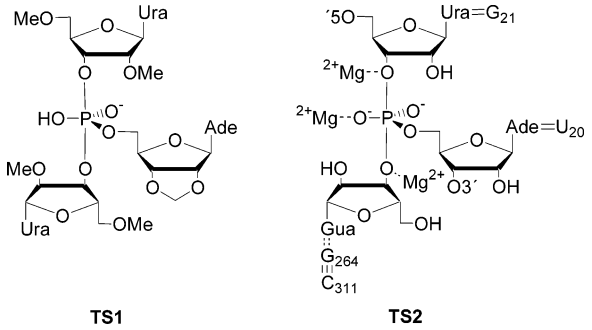


FIGURE 1. Transition state structures for the model (**TS1**) and the ribozyme reaction (**TS2**).

of the breakdown of **TS1** by either P−O3′ or P−O5′ cleavage is of interest.

With group I introns, the intermediate is always decomposed by departure of a 3′-linked nucleoside, leading to formation of either products or starting materials.[10] Both processes have been shown to be subject to a similar catalysis.[11] Alternative explanations may a priori be given for the overwhelmingly preferred cleavage of the P−O3′ bonds: (i) the intermediate is too short-lived to pseudorotate and the initial state geometry dictated by the chain folding determines the course of the reaction, (ii) the apicophilicity and leaving-group property of a secondary alcohol is superior to that of the primary alcohol, and (iii) hydrogen bonding of the neighboring hydroxy group to the developing oxyanion greatly enhances the departure of a 3′-oxygen.[4,7,12,13] Since with our small molecule model (**1**) the nucleophile can attack from any direction and the substrate is free of the constraints present in ribozymes, the differences between rates of cleavage of P−O3′ and P−O5′ bonds should reflect the differences in the apicophilicities and leaving group properties. The possible hydrogen bond stabilization of the leaving 3′-oxyanion by the neighboring 2′-OH is ruled out by alkylating all sugar hydroxy functions of the molecule. Accordingly, the results of the present study serve as necessary reference material for future investigations aimed at elucidating the role of leaving group stabilization by intramolecular hydrogen bonding. In addition, the β_{lg} value for the hydrolysis via the acyclic phosphorane intemediate or phosphorane-like transition state has been estimated by comparing the relative rate of the release of the 3′- and 5′-linked nucleosides, **4** and **7**, from triester **1** to that of the release from diesters **2a**,**b**. The latter compounds react by an intramolecular attack of the 2′-hydroxy group on phosphorus, and the β_{lg} value for such a reaction is known.[14]

Results and Discussion

Preparation of the Trinucleoside 3′,3′,5′-Monophosphate (1). N^6-Benzoyl-2′,3′-*O*-methyleneadenos-5′-yl bis(2′,5′-di-*O*-methylurid-3′-yl) phosphate (**10**) was obtained by tetrazole-activated stepwise displacement of the dimethylamino groups from tris(dimethylamino)-phosphine with the appropriately protected nucleosides (**3**, **4**) prepared as described in the literature (Scheme 1).[15−19] Removal of the N^6-benzoyl protection with methanolic ammonia, followed by RP-HPLC purification, then gave the desired triester **1**. Similar compounds have previously been prepared with the H-phosphonate method.[20]

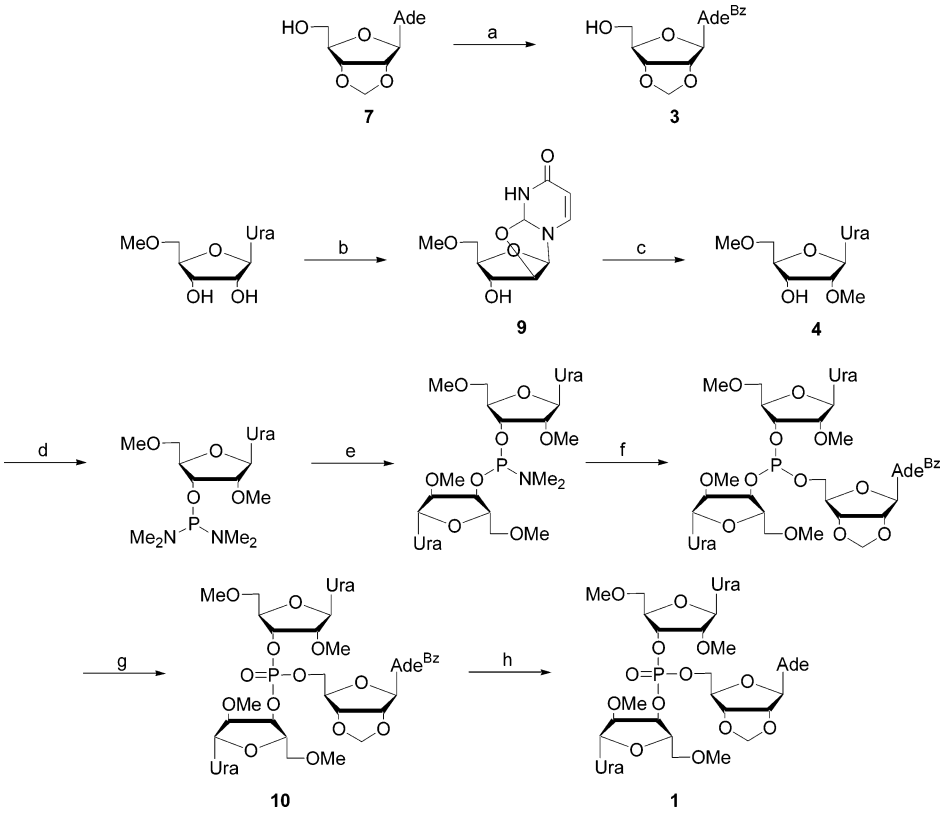
Product Distribution and Reaction Pathways. The hydrolysis of **1** was followed over a wide pH range (5−13) by analyzing the composition of the aliquots withdrawn from the reaction mixture at appropriate time intervals by HPLC. The products were identified by spiking with authentic samples. The time-dependent product distributions obtained under neutral and alkaline conditions were somewhat different. In aqueous alkali, the 2′,5′-di-*O*-methyluridylyl-3′,3′-(2′,5′-di-*O*-methyluridine) [$m_2U(3',3')m_2U$, **5**] and 2′,5′-di-*O*-methyluridylyl-3′,5′-(2′,3′-*O*-methyleneadenosine) [$m_2U(3',5')m<A$, **6**] diesters were formed by the release of 2′,3′-*O*-methyleneadenosine ($m<A$, **7**) and 2′,5′-di-*O*-methyluridine (m_2U, **4**), respectively (Figure 2). In neutral or slightly acidic solutions, the only chromophoric product observed to be accumulated was the uridylyl-3′,3′-uridine diester [$m_2U(3',3')m_2U$] (Figure 3). In other words, no nucleosidic product was accumulated, as could have been expected and as was also detected at high pH. As discussed below in more detail, $m_2U(3',3')m_2U$ was in all likelihood formed by phosphate elimination (route C), not by phosphoester hydrolysis (route B), and the other product of the elimination reaction, vinyl ether **8**, was not accumulated, owing to rapid subsequent decomposition to several products. No nucleoside monophosphates were formed either under neutral or alkaline conditions, indicating that the diesters, $m_2U(3',3')m_2U$ (**5**) and $m_2U(3',5')m<A$ (**6**), were not hydrolyzed under the experimental conditions.

Studies in ^{18}O-enriched water (95%) offered an explanation for the different product compositions. HPLC-ESI-MS analyses of the products revealed that no ^{18}O was incorporated into the $m_2U(3',3')m_2U$ diester (**5**) in neutral or slightly acidic solutions. Accordingly, the reaction in all likelihood proceeded by cleavage of the O−C5′($m<A$) bond. By contrast, in 50 mmol L^{-1} aqueous sodium hydroxide, 78% of $m_2U(3',3')m_2U$ had one ^{18}O atom incorporated and all of the $m_2U(3',5')m<A$ diester (**6**) formed contained one ^{18}O atom. In other words, the trinucleoside monophosphate **1** is decomposed either by nucleophilic attack of hydroxide ion on the phosphorus atom or by cleavage of the O−C5′($m<A$) bond (Scheme 2).

In the pH range 5−9 (T = 50 °C), the cleavage of the O−C5′ bond (route C) is the only reaction detected. As

(10) Cech, T. R.; Zaug, A. J.; Grabowski, P. J. *Cell* **1981**, *27*, 487−496.
(11) Steitz, T. A.; Steitz, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6498−6502.
(12) Tzokov, S. B.; Momtcheva, R. T.; Vassilev, N. G.; Kaneti, J.; Petkov, D. D. *J. Am. Chem. Soc.* **1999**, *121*, 5103−5107.
(13) Bakalova, S.; Siebrand, W.; Fernández-Ramos, A.; Smedarchina, Z.; Petkov, D. D. *J. Phys. Chem. B* **2002**, *106*, 1476−1480.
(14) Kosonen, M.; Youseti-Salakdeh, E.; Strömberg, R.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1997**, 2661−2666.
(15) Hovinen, J. *Helv. Chim. Acta* **1997**, *80*, 851−855.
(16) Legorburu, U.; Reese, C. B.; Song, Q. *Tetrahedron* **1999**, *55*, 5635−5640.
(17) McGee, D. P. C.; Zhai, Y. *Nucleosides Nucleotides* **1996**, *15*, 1797−1803.
(18) Norman, D. G.; Reese, C. B.; Serafinowska, H. T. *Synthesis* **1985**, *8*, 751−754.
(19) Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316−1319.
(20) Garegg, P. J.; Lindh, I.; Stawinski, J. *Bioactive Molecules*; *Vol. 3*, Biophosphates Their Analogues; Elsevier Science: New York, 1987; pp 89−92.

SCHEME 1. Preparation of the Trinucleoside 3′,3′,5′-monophosphate 1[a]



[a] Reagents and conditions: (a) (1) TMSCl, Py, (2) BzCl, (3) H_2O, NH_3; (b) $(PhO)_2C{=}O$, $NaHCO_3$, DMA; (c) $Mg(OMe)_2$, DMF; (d) $P(NMe_2)_3$, tetrazole, MeCN; (e) **4**, tetrazole, MeCN; (f) **3**, tetrazole, MeCN; (g) I_2, H_2O, THF; (h) NH_3, MeOH.

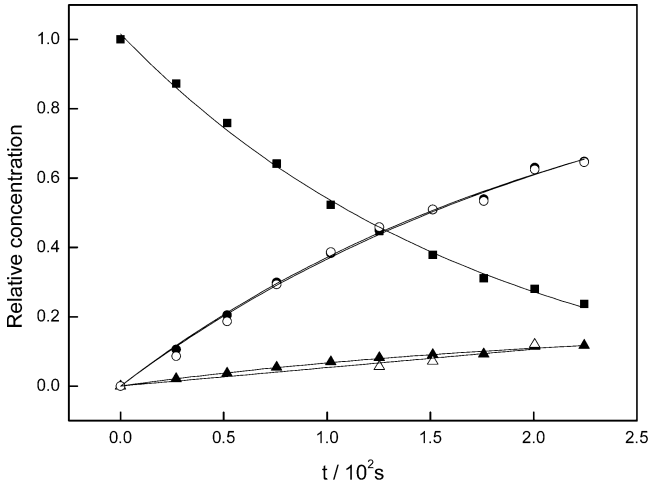


FIGURE 2. Time-dependent product distribution for the hydrolysis of **1** in 0.5 mol L^{-1} aqueous NaOH at 50.0 °C: (■) **1**, (●) **6**, (○) **4**, (▲) **5**, and (△) **7**.

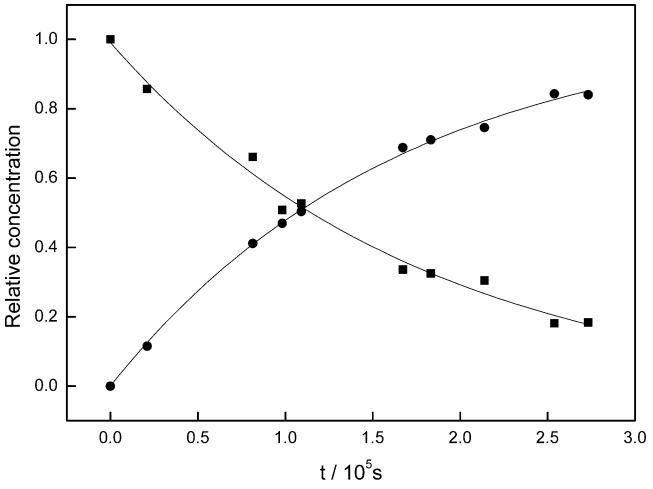
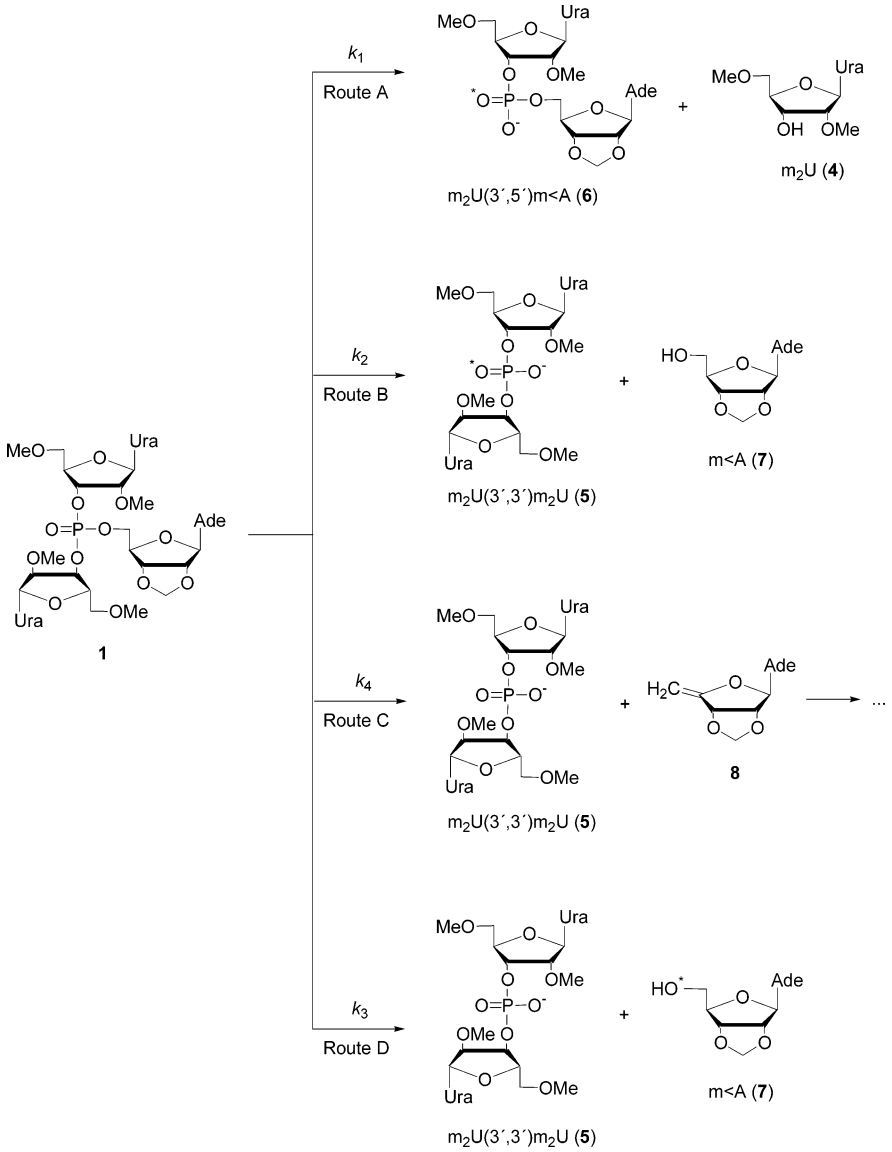


FIGURE 3. Time-dependent product distribution for the hydrolysis of **1** at pH 4.8 and 50.0 °C: (■) **1** and (●) **5**.

mentioned above, only the $m_2U(3',3')m_2U$ diester product can be detected. One may tentatively assume that the 2′,3′-*O*-methyleneadenosyl moiety departs as a vinyl ether type elimination product **8**, and this subsequently reacts to a variety of products, the concentration of each being too low to be detected. Many organic phosphates, including phosphotriesters, are known to undergo *β*-elimination processes.[21,22] In addition, 2-methylenetetrahydrofuran, mimicking the probable elimination product of **1**, has been reported to hydrolyze rapidly under neutral conditions ($t_{1/2}$ = 48 s, pH 6.87, T = 25 °C, 0.00333 mol L^{-1} phosphate buffer, I = 0.07 mol L^{-1}), presumably to 4-hydroxy-2-pentanone.[23] The phenyl ester of thymidine 3′,5′-cyclic phosphate has previously been shown to undergo a pH-independent cleavage of the O−C5′ bond, but in that case the 5′-linked nucleoside departs as an

(21) Cox, J. R.; Ramsey, O. B. *Chem. Rev.* **1964**, *64*, 317−352.
(22) Lapidot, A.; Samuel, D.; Silver, B. *Chem. Ind.* **1963**, 468−471.
(23) Kankaanperä, A.; Taskinen, E.; Salomaa, P. *Acta Chem. Scand.* **1967**, *21*, 2487−2494.

SCHEME 2



alcohol, not as a vinyl ether.[24] Evidently the carbocation developing by cleavage of the C−O5′ bond may be stabilized either by deprotonation to a vinyl ether or by attack of hydroxide ion to give an alcohol. Which one of these mechanisms prevail may depend on the detailed structure of the starting material. Since the C−O5′ bond cleavage was not of interest from the point of view of the ribozyme action, the course of the elimination reaction was not studied in more detail.

On passing pH 10, m$_2$U(3′,5′)m<A (**6**) started to appear as a product besides m$_2$U(3′,3′)m$_2$U (**5**) and both nucleosidic products, m$_2$U (**4**) and m<A (**7**), accumulated. On the basis of ^{18}O incorporation studies, m$_2$U(3′,5′)m<A was formed solely by a P−O bond cleavage (route B). m$_2$U(3′,3′)m$_2$U in turn exhibited only 78% incorporation of ^{18}O (route B). Since under the experimental conditions (50 mmol L^{-1} of sodium hydroxide) the elimination reaction (route C) accounts for only 5% of the overall formation of m$_2$U(3′,3′)m$_2$U, the remaining 17% is in all likelihood formed by a hydroxide ion catalyzed cleavage of the C−O5′ bond, probably by an attack of hydroxide ion on C5′(m<A) concerted with the bond rupture (route D).

pH−Rate Profiles. Figure 4 shows the pH−rate profile for the overall disappearance of **1**. From pH 5 to pH 9 the overall reaction is pH independent. Over this relatively wide pH range the cleavage of the C−O bond by route C predominates. On going to more alkaline conditions (pH >10) the overall reaction becomes first order in hydroxide ion concentration. This refers to the cleavage of P−O3′ (route A) and P−O5′ (route B) bonds, as well as the cleavage of the C−O5′ bond by route D.

The rate constants for the partial reactions were calculated from the observed pseudo-first-order rate constants as follows: since the m$_2$U(3′,5′)m>A diester is only formed by one route, namely by attack of hydroxide ion, the rate constant for this reaction (route A) is obtained directly from the observed rate constant (eq 1).

$$k_1' = k_{obs}\frac{[m_2U(3',5')m<A]}{[m_2U(3',5')m<A] + [m_2U(3',3')m_2U]} \quad (1)$$

(24) Varila, J.; Hankamäki, T.; Oivanen, M.; Koole, L. H.; Lönnberg, H. *J. Org. Chem.* **1997**, *62*, 893−898.

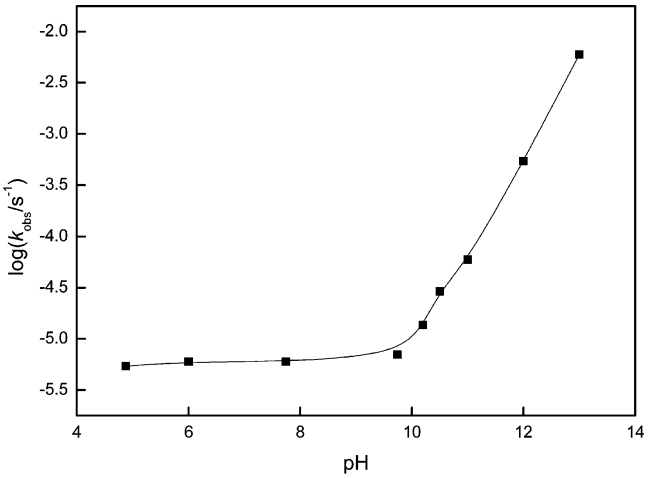


FIGURE 4. pH−rate profile for the decomposition of **1** at 50 °C.

There are, however, two similar P−O3′ bonds present and, hence, k_1' must be divided by 2 to obtain the actual rate constant k_1 for the bond cleavage. Routes B and D (Scheme 2) lead to identical products. ^{18}O incorporation studies reveal that in 50 mmol L^{-1} of aqueous sodium hydroxide the attack on phosphorus is more rapid by a factor of 4.6. Accordingly, under these particular conditions the overall rate constant for the formation of m_2U-(3′,3′)m_2U and m<A may be broken down to the rate constants for reactions B and D (eqs 2 and 3). Finally, since the elimination is the only reaction detected over a wide pH range, its rate constant is simply the observed rate constant under those conditions (eq 4). The following partial rate constants were obtained: $k_1/[OH^-] = 2.3 \times 10^{-2} \pm 1 \times 10^{-4}$ L mol^{-1} s^{-1}, $k_2/[OH^-] = 8.0 \times 10^{-3} \pm 2 \times 10^{-4}$ L mol^{-1} s^{-1}, $k_3/[OH^-] = 1.8 \times 10^{-3} \pm 2 \times 10^{-4}$ L mol^{-1} s^{-1}, $k_4 = 5.6 \times 10^{-6} \pm 1 \times 10^{-8}$ s^{-1}.

$$k_2 = 0.82k_{obs}\frac{[\text{m<A}]}{[m_2\text{U}(3',5')\text{m<A}] + [\text{m}_2\text{U}(3',3')\text{m}_2\text{U}]} \quad (2)$$

$$k_3 = 0.18k_{obs}\frac{[\text{m<A}]}{[\text{m}_2\text{U}(3',5')\text{m<A}] + [\text{m}_2\text{U}(3',3')\text{m}_2\text{U}]} \quad (3)$$

$$k_4 = k_{obs} \qquad (\text{pH } 5-9) \quad (4)$$

Reactions A and B are first order in hydroxide ion concentration over the entire pH range where they can be detected, while reaction C is pH independent at pH <10, i.e. under conditions where it can be clearly detected. Rate constants k_1 and k_2 are in reasonably good agreement with that measured for methyl thymid-3′-yl thymid-5′-yl phosphate.[25] On the other hand, the hydroxide ion catalyzed cleavage of trimethyl phosphate is about 2 orders of magnitude slower.[26] The difference is expected, since both 3′- and 5′-linked nucleoside oxyanions are much better leaving groups than methoxide ion.

Comparison of the rate constants obtained for reactions A and B in 50 mmol L^{-1} of aqueous sodium hydroxide indicates that the 3′-linked m_2U nucleoside (**4**) departs the phosphorane intermediate 2.9 times as readily as the 5′-linked m<A nucleoside (**7**). Since all of the hydroxy functions in triester **1** are methylated, this difference cannot be attributed to a hydrogen bond stabilization of the leaving 3′-oxyanion by the neighboring 2′-OH, but it reflects the higher inherent leaving group ability of a 3′-linked nucleoside. The difference is, however, smaller than that observed with group I intron and gives, hence, only a partial explanation for the course of the ribozyme reaction.

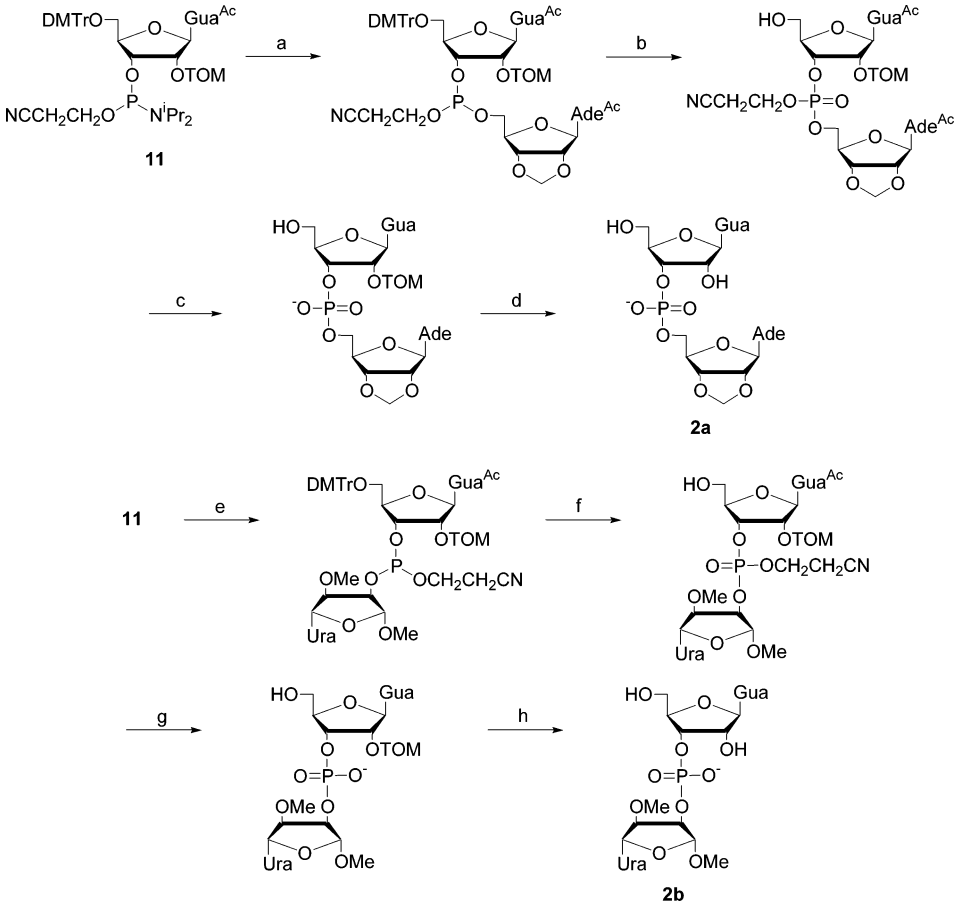
The observed difference between the cleavage rates of the P−O3′ and P−O5′ bonds allows one to estimate the β_{lg} value for the reaction via an acyclic trialkylated phosphorane intermediate and, hence, to estimate the extent of P−O bond cleavage in the transition state. For this purpose, the pK_a values of the departing m_2U and m<A alcohols are required. Since the β_{lg} value for the cleavage of ribonucleoside 3′-alkyl phosphates is known to be -1.28 ± 0.05,[14] this piece of information may be exploited to determine the ΔpK_a value for m_2U and m<A. The justification of this approach receives support from the fact that the correlation line based on the pK_a values of alcohols that are either secondary or primary and sterically very different, such as ethanol and 2,2,2-trichloroethanol, exhibits practically no scattering. Accordingly, m_2U and m<A were inserted as leaving groups in guanosine 3′-diesters **2a** and **2b** (Scheme 3) and the rate constants for the alkaline cleavage of these compounds were determined under the same conditions used previously for the determination of the β_{lg} value for uridine 3′-alkyl phosphates, i.e. at 25.0 °C in 0.1 mol L^{-1} of aqueous sodium hydroxide the ionic strength of which was adjusted to 1.0 mol L^{-1} with sodium chloride. The P−O3′(m_2U) bond (**2b**) was cleaved 19.2 times as rapidly as the P−O5′(m<A) bond (**2a**), the observed first-order rate constants obtained with **2a** and **2b** being 9.92×10^{-5} and 1.90×10^{-4} s^{-1}, respectively. Accordingly, the β_{lg} value for the hydrolysis of triester **1** can be obtained by (eq 5).

$$\beta_{lg}(\text{triester}) = \beta_{lg}(\text{diesters})\frac{\log(k_1/k_2, \text{ triester})}{\log(k_1/k_2, \text{ diesters})} = -0.46 \quad (5)$$

The value of approximately −0.5, although susceptible to marked uncertainty, represents a relatively early transition state, where the leaving group is still largely bound to the phosphorus atom. For comparison, the β_{lg} value for the cleavage of ribonucleoside 3′-aryl phosphates is of the same magnitude and this reaction is generally assumed to be a one-barrier process where both the formation of the P−O2′ bond and the cleavage of the P−OAr bond are only moderately advanced.[27,28] The cleavage of ribonucleoside 3′-phosphotriesters and 3′-phosphodiesters, suggested to be cleaved via a very late transition state, in turn, both exhibit markedly negative β_{lg} values, -1.38 ± 0.18 [29] and -1.28 ± 0.05,[14] respec-

(25) Conrad, J.; Müller, N.; Eisenbrand, G. *Chem.-Biol. Interact.* **1986**, *60*, 57−65.
(26) Barnard, P. W. C.; Bunton, C. A.; Llewellyn, D. R.; Vernon, C. A.; Welch, V. A. *J. Chem. Soc.* **1961**, 2670−2676.
(27) Davis, A. M.; Hall, A. D.; Williams, A. *J. Am. Chem. Soc.* **1988**, *110*, 5105−5108.
(28) Ba-Saif, S. A.; Davis, A. M.; Williams, A. *J. Org. Chem.* **1989**, *54*, 5483−5486.
(29) Kosonen, M.; Seppänen, R.; Wichmann, O.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1999**, 2433−2439.

SCHEME 3. Preparation of Diesters 2a and 2b[a]



[a] Reagents and conditions: (a) **3**, tetrazole, MeCN; (b) (1) I_2, H_2O, THF, 2,6-lutidine, (2) HOAc, H_2O; (c) NH_3, MeOH; (d) $Et_3N{\cdot}3HF$, THF; (e) **4**, tetrazole, MeCN; (f) (1) I_2, H_2O, THF, 2,6-lutidine, (2) HOAc, H_2O; (g) NH_3, MeOH; (h) TBAF, THF.

tively. We therefore presume the reaction to be rather of $S_n2(P)$ type than to proceed via a well-developed phosphorane intermediate.

In summary, the results of the present study suggest that a nucleoside esterified through its 3′-hydroxy departs upon nucleophilic attack of hydroxide ion on the phosphorus atom of a phosphotirester only 3 times as fast as its 5′-esterified counterpart, as far as stabilization of the leaving group by intramolecular hydrogen bonding is prevented. Accordingly, additional factors, such as hydrogen-bonding stabilization of the leaving group and noncovalent interactions steering the nucleophilic attack to take place opposite the 3′-linked nucleoside, must be considered in future studies.

Experimental Section

Materials. Nucleosides and protected nucleoside phosphoramidites were commercial products that were used as received after checking the purity by HPLC. The ^{18}O atom fraction of $H_2{}^{18}O$ was 95%. The buffer constituents were of reagent grade.

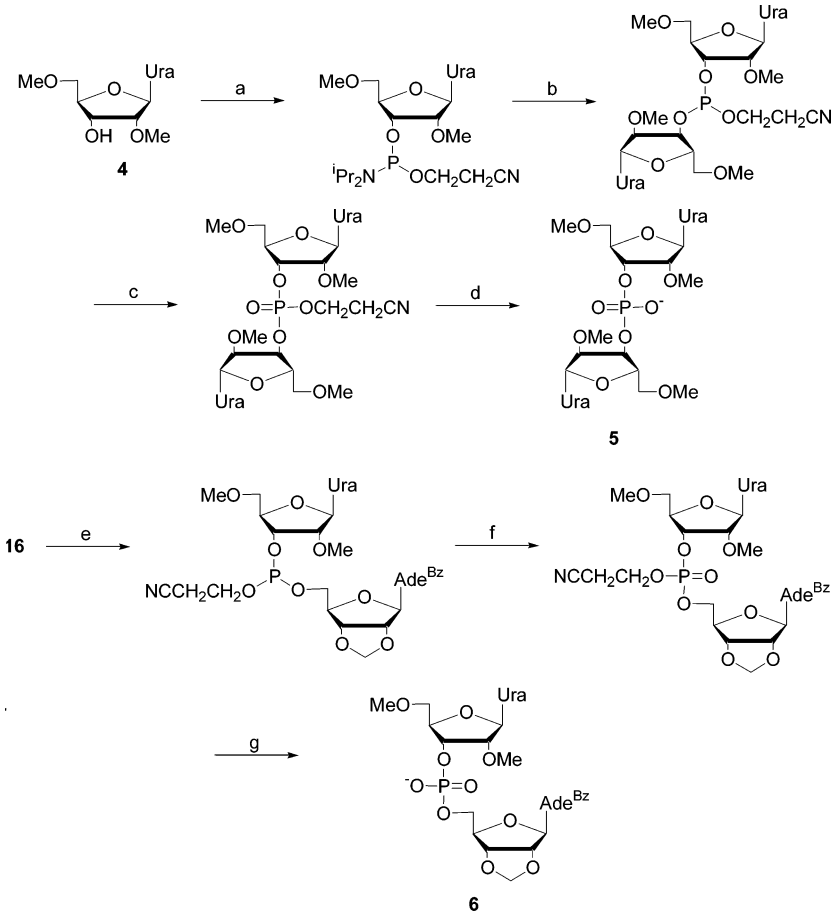
5′-*O*-Methyl-2,2′-anhydro-1-*β*-D-arabinofuranosyluracil (9). 9 was prepared from 5′-*O*-methyluridine[11] by a method previously described for 2,2′-anhydro-1-*β*-D-arabinofuranosyluracil.[12] Yield 4.3 g (86%). ^1H NMR (δ_H) (400 MHz, DMSO-d_6) 7.82 (d, 1H, $J = 7.5$ Hz), 6.30 (d, 1H, $J = 5.8$ Hz), 5.83 (d, 1H, $J = 7.5$ Hz), 5.18 (d, 1H, $J = 5.8$ Hz), 4.32 (s, 1H), 4.19 (m, 1H), 3.30−3.18 (m, 2H), 3.06 (s, 3H). ESI^+-MS *m*/*z* 241.1 $[M + H]^+$.

2′,5′-Di-*O*-methyluridine (4). 4 was prepared from 5′-*O*-methyl-2,2′-anhydro-1-*β*-D-arabinofuranosyluracil (**9**) as previously described for 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-methyluridine.[13] Yield 2.4 g (89%). ^1H NMR (δ_H) (400 MHz, DMSO-d_6) 7.71 (d, 1H, $J = 8.1$ Hz), 5.82 (d, 1H, $J = 4.3$ Hz), 5.67 (d, 1H, $J = 8.1$ Hz), 5.26 (d, 1H, $J = 6.0$ Hz), 4.06 (q, 1H, $J = 5.6$ Hz), 3.91 (dd, 1H, $J_1 = 10.9$ Hz, $J_2 = 3.0$ Hz), 3.48 (dd, 1H, $J_1 = 11.1$ Hz, $J_2 = 4.1$ Hz), 3.34 (s, 3H), 3.31 (s, 3H). HRMS (FAB) M^+ calcd 273.1087, obsd 273.1089.

***N*^6^-Benzoyl-2′,3′-*O*-methyleneadenosine (3). 3** was prepared from 2′,3′-*O*-methyleneadenosine[14] as previously described for N^6-benzoyl-2′-deoxyadenosine.[15] Yield 3.6 g (83%). ^1H NMR (δ) (400 MHz, DMSO-d_6) 11.3 (s, 1H), 8.83 (s, 1H), 8.74 (s, 1H), 8.11 (d, 2H, $J = 7.5$ Hz), 7.70 (t, 1H, $J = 7.5$ Hz), 7.61 (t, 2H, $J = 7.5$ Hz), 6.34 (d, 1H, $J = 3.0$ Hz), 5.45 (dd, 1H, $J_1 = 2.8$ Hz, $J_2 = 6.4$ Hz), 5.25 (s, 1H), 5.21 (s, 1H), 5.19 (t, 1H, $J = 5.6$ Hz), 5.01 (dd, 1H, $J_1 = 3.4$ Hz, $J_2 = 6.4$ Hz), 4.28 (dd, 1H, $J_1 = 4.72$ Hz, $J_2 = 8.36$ Hz), 3.64 (m, 2H). HRMS (FAB) M^+ calcd 384.1308, obsd 384.1307.

***N*^6^-Benzoyl-2′,3′-*O*-methyleneadenos-5′-yl Bis(2′,5′-di-*O*-methylurid-3′-yl) Phosphate (10).** 2′,5′-Di-*O*-methyluridine (**4**, 0.583 mmol, 0.1587 g) was dissolved in anhydrous MeCN (2.1 mL). Tris(dimethylamino)phosphine (0.644 mmol, 0.117 mL) and a solution of tetrazole (0.571 mmol, 40 mg) in anhydrous MeCN (2 mL) was added and the reaction mixture was stirred for 21 h at 50 °C. A solution of 2′,5′-di-*O*-methyluridine (**4**, 0.551 mmol, 0.15 g) in anhydrous MeCN (2 mL) was then added, followed by a solution of tetrazole (0.571 mmol, 40 mg) in anhydrous MeCN (1.5 mL). After the mixture was stirred for 45 h at 60 °C, a solution of N^6-benzoyl-2′,3′-*O*-methyleneadenosine (**3**, 0.548 mmol, 0.21 g) in anhydrous

SCHEME 4. Preparation of the Reference Diesters 5 and 6[a]



[a] Reagents and conditions: (a) $NCCH_2CH_2OP(N^iPr_2)_2$, tetrazole, MeCN; (b) **4**, tetrazole, MeCN; (c) I_2, H_2O, THF, 2,6-lutidine; (d) NH_3, MeOH; (e) **3**, tetrazole, MeCN; (f) I_2, H_2O, THF, 2,6-lutidine; (g) NH_3, MeOH

MeCN (2 mL) was added. The reaction mixture was stirred for an additional 23 h at 60 °C, after which iodine (1.74 mmol, 0.45 g) in a mixture of THF (12 mL) and water (6 mL) was added. After the solution was stirred for 19 h at room temperature, 50 mL of CH_2Cl_2 was added. The resulting mixture was washed with saturated aqueous $NaHSO_3$ (3 × 10 mL) and the aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL). The organic phase was dried with Na_2SO_4 and evaporated to dryness. No attempt was made to optimize the synthesis. The product was purified by HPLC on a LiChrospher RP-18 column (10 × 250 mm^2, 10 μm) eluting with a mixture of 0.05 mol L^{-1} of aqueous NH_4OAc and MeCN (76:24, v/v). The main purpose was to obtain a highly pure sample for the kinetic measurements and, hence, no attention was paid to the quantitativeness of the isolation. Yield 11.5 mg (2%). ^{31}P NMR (δ_H) (202 MHz, DMSO-d_6) 0.75. ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6) 11.43 (d, 1H, J = 2.2 Hz), 11.42 (d, 1H, J = 2.2 Hz), 8.76 (s, 1H), 8.68 (s, 1H), 8.03, (d, 2H, J = 7.2 Hz), 7.7−7.4 (m, 6H), 6.36 (d, 1H, J = 2.9 Hz), 5.89 (d, 1H, J = 6.3 Hz), 5.85 (d, 1H, J = 6.4 Hz), 5.71 (d, 1H, J = 8.1 Hz), 5.71 (d, 1H, J = 8.1 Hz), 5.42 (dd, 1H, J_1 = 2.8 Hz, J_2 = 6.4 Hz), 5.20 (s, 1H), 5.19 (s, 1H), 5.09 (dd, J_1 = 4 Hz, J_2 = 6.4 Hz), 4.92 (m, 1H), 4.86 (m, 1H), 4.44 (m, 1H), 4.38 (m, 1H), 4.26 (m, 1H), 4.22 (dd, 1H, J_1 = 6.6 Hz, J_2 = 3.2 Hz), 4.13 (dd, 1H, J_1 = 6.7 Hz, J_2 = 3.5 Hz), 4.08 (dd, 2H, J_1 = 5.8 Hz, J_2 = 11.8 Hz), 3.58−3.43 (m, 4H), 3.34 (s, 3H), 3.31 (s, 3H), 3.30 (s, 3H), 3.24 (s, 3H). ESI$^-$-MS m/z 970.7 [M − H]$^-$.

2′,3′-*O*-Methyleneadenos-5′-yl Bis(2′,5′-di-*O*-methylurid-3′-yl) Phosphate (1). Compound **10** was dissolved in saturated methanolic ammonia. After being stirred for 5.5 h the solution was evaporated to dryness and the product was purified by HPLC on a LiChrospher RP-18 column (10 × 250 mm^2, 10 μm) eluting with a mixture of water and MeCN (75:25, v/v). Yield 9.85 mg (99%). ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6) 0.76. ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6) 8.34 (s, 1H), 8.17 (s, 1H), 7.67 (d, 1H, J = 8.2 Hz), 7.66 (d, 1H, J = 8.1 Hz), 7.37 (s, 2H), 6.23 (d, 1H, J = 2.8 Hz), 5.90 (d, 1H, J = 6.3 Hz), 5.87 (d, 1H, J = 6.3 Hz), 5.71 (d, 1H, J = 8.1 Hz), 5.71 (d, 1H, J = 8.1 Hz), 5.36 (dd, 1H, J_1 = 2.9 Hz, J_2 = 6.6 Hz), 5.18 (s, 1H), 5.18 (s, 1H), 5.06 (dd, 1H, J_1 = 2.9 Hz, J_2 = 6.6 Hz), 4.92 (m, 1H), 4.86 (m, 1H), 4.38 (m, 2H), 4.22 (m, 2H), 4.13 (m, 1H), 4.07 (m, 2H), 3.54 (dd, 2H, J_1 = 3.2 Hz, J_2 = 11.1 Hz), 3.48 (dd, 2H, J_1 = 3.75 Hz, J_2 = 11.0 Hz), 3.45 (dd, 1H, J_1 = 3.6 Hz, J_2 = 11.0 Hz), 3.39 (dd, 1H, J_1 = 3.6 Hz, J_2 = 11.0 Hz), 3.35 (s, 3H), 3.32 (s, 3H), 3.31 (s, 3H), 3.26 (s, 3H). HRMS (FAB) M$^-$ calcd 866.2358, obsd 866.2362.

2′,5′-Di-*O*-methyluridylyl-(3′,3′)-(2′,5′-di-*O*-methyluridine) (5). 2′,5′-Di-*O*-methyluridine (**4**, 1.14 mmol, 0.3111 g) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (1.43 mmol, 0.455 mL) were dissolved in a solution of tetrazole (1.11 mmol) in MeCN (3.46 mL). After being stirred for 40 min at room temperature, the reaction mixture was evaporated to dryness and a conventional CH_2Cl_2/aq $NaHCO_3$ workup was carried out. The organic phase was evaporated to dryness, coevaporated from anhydrous MeCN, and dried in a vacuum desiccator overnight. A solution of 2′,5′-di-*O*-methyluridine (**4**, 0.646 mmol, 0.1756 g) and tetrazole (0.608 mmol) in acetonitrile (2.350 mL) were added and the reaction mixture was stirred for 3.5 h at room temperature. Iodine (1.37 mmol, 0.354 g) in a mixture of water (4 mL), THF (8 mL), and 2,6-lutidine (2 mL) was added and the reaction mixture was stirred for an additional 2.5 h at room temperature. CH_2Cl_2 (50 mL) was

added and the resulting mixture was washed with saturated aqueous $NaHSO_3$ (2 × 20 mL). The aqueous phase was extracted with dichloromethane (2 × 50 mL). The combined organic phases were dried with Na_2SO_4 and evaporated to dryness. The product was purified on a silica gel column with a mixture of CH_2Cl_2 and MeOH as an eluent (90:10, v/v). The purified product was then dissolved in saturated methanolic ammonia. After being stirred for 2 h at room temperature the reaction mixture was evaporated to dryness. Yield 0.11 g (34%). ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6) −0.02. ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6) 7.72 (d, 2H, J = 8.1 Hz), 5.83 (d, 2H, J = 5.8 Hz), 5.69 (d, 2H, J = 8.1 Hz), 4.47 (m, 2H), 4.20 (m, 2H), 3.84 (m, 2H), 3.60−3.48 (m, 4H), 3.33 (s, 6H), 3.32 (s, 6H). HRMS (FAB) M^- calcd 605.1496, obsd 605.1496.

2′,5′-Di-*O*-methyluridylyl-(3′,5′)-(2′,3′-*O*-methyleneadenosine) (6). 2′,5′-Di-*O*-methyluridine (**4**, 0.763 mmol, 0.2076 g) was dried by coevaporating from anhydrous pyridine and anhydrous MeCN. To the residue were added a solution of tetrazole (0.916 mmol) in MeCN (2.035 mL) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (0.916 mmol, 0.291 mL). After being stirred for 60 min at room temperature, the reaction mixture was evaporated to dryness. A conventional CH_2Cl_2/aq $NaHCO_3$ workup was performed and the combined aqueous phases were evaporated to dryness. To the residue was added N^6-benzoyl-2′,3′-*O*-methyleneadenosine (**3**, 0.787 mmol, 0.3015 g), and the resulting mixture was dried by coevaporating from anhydrous pyridine and anhydrous MeCN. The residue was dissolved in a solution of tetrazole (0.916 mmol) in MeCN (3.035 mL). The reaction mixture was stirred for 67 h at room temperature, after which iodine (1.43 mmol, 0.37 g) in a mixture of THF (8 mL), water (4 mL), and 2,6-lutidine (2 mL) was added. After being stirred for an additional 7 h at room temperature, the reaction mixture was concentrated under reduced pressure and a CH_2Cl_2/aq $NaHSO_3$ workup was carried out. The combined organic phases were evaporated to dryness and the product was purified on a silica gel column with a mixture of CH_2Cl_2 and MeOH as an eluent (93:7, v/v). The purified product was then dissolved in saturated methanolic ammonia. After being stirred for 7 h at room temperature the reaction mixture was evaporated to dryness. The product was purified by HPLC on a LiChrospher RP-18 column (10 × 250 mm^2, 10 μm) eluting with a mixture of 0.06 mol L^{-1} of aqueous NaOAc and MeCN (91.5:8.5, v/v). Finally the buffer salts were removed on the same column by eluting with a mixture of water and MeCN. Yield 55.5 mg (11%). ^{31}P NMR (δ_P) (162 MHz, DMSO-d_6) −0.36. ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6) 11.36 (s, 1H), 8.44 (s, 1H), 8.14 (s, 1H), 7.68 (d, 1H, J = 8.4 Hz), 7.31 (s, 2H), 6.12 (d, 1H, J = 3.0 Hz), 5.81 (d, 1H, J = 5.6 Hz), 5.68 (dd, 1H, J_1 = 8.1 Hz, J_2 = 2.1 Hz), 5.30 (dd, 1H, J_1 = 6.4 Hz, J_2 = 3.2 Hz), 5.16 (s, 1H), 5.11 (s, 1H), 4.95 (dd, 1H, J_1 = 6.4 Hz, J_2 = 3.2 Hz), 4.41 (m, 1H), 4.28 (m, 1H), 4.08 (m, 1H), 3.77 (m, 3H), 3.45−3.36 (m, 2H), 3.27 (s, 3H), 3.23 (s, 3H). HRMS (FAB) M^- calcd 612.1455, obsd 612.1472.

Guanosyl-(3′,5′)-(2′,3′-*O*-methyleneadenosine) (2a). N^6-Benzoyl-2′,3′-*O*-methyleneadenosine (**3**, 0.561 mmol, 0.2147 g) and N^2-acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(triisopropylsilyloxymethyl)guanosine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (**11**, 0.5 mmol, 0.5 g) were dissolved in a solution of tetrazole (0.63 mmol) in MeCN (1.4 mL). The reaction mixture was stirred for 1 h at room temperature, after which iodine (1.82 mmol, 0.47 g) in a mixture of THF (8 mL), water (4 mL), and 2,6-lutidine (2 mL) was added. After being stirred for an additional 1 h, the reaction mixture was concentrated under reduced pressure and a CH_2Cl_2/aq $NaHSO_3$ workup was performed. The organic phase was evaporated to dryness and the residue was dissolved in a mixture of acetic acid and water (25 mL, 80:20, v/v). After being stirred for 50 min at room temperature the reaction mixture was evaporated to dryness, followed by a conventional CH_2Cl_2/aq $NaHCO_3$ workup. The organic phase was evaporated to dryness and the product was purified on a silica gel column eluting with a mixture of CH_2Cl_2 and MeOH (98:2, v/v). The purified product was then dissolved in saturated methanolic ammonia. After being stirred for 7.5 h at room temperature the reaction mixture was evaporated to dryness. The residue was dissolved in anhydrous THF (1 mL) and triethylamine trihydrofluoride (0.27 mL) was added. The reaction mixture was stirred for 45 min at 45 °C, after which 10 mL of 0.1 mol L^{-1} of aqueous NaOAc (10 mL) was added. The mixture was evaporated to dryness and a CH_2Cl_2/aq NaOAc workup was performed. The aqueous phase was concentrated under reduced pressure and the product was purified by HPLC on a LiChrospher RP-18 column (10 × 250 mm^2, 10 μm) eluting with a mixture of 0.06 mol L^{-1} of aqueous NaOAc and MeCN (91:9, v/v). Finally the buffer salts were removed on the same column by eluting with a mixture of water and MeCN. Yield 67.7 mg (21%). ^{31}P NMR (δ_P) (162 MHz, DMSO-d_6) 0.64. ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6) 8.40 (s, 1H), 8.16 (s, 1H), 7.89 (s, 1H), 7.32 (s, 2H), 6.53 (s, 2H), 6.14 (d, 1H, J = 5.6 Hz), 5.72 (d, 1H, J = 6.8 Hz), 5.28 (dd, 1H, J_1 = 6.0 Hz, J_2 = 3.4 Hz), 5.17 (s, 1H), 5.12 (s, 1H), 4.96 (dd, 1H, J_1 = 8.1 Hz, J_2 = 4.1 Hz), 4.34 (m, 2H), 4.28 (m, 1H), 3.86 (m, 1H), 3.44−3.41 (m, 4H). HRMS (FAB) M^- calcd 623.1364, obsd 623.1376.

Guanosyl-(3′,3′)-(2′,5′-di-*O*-methyluridine) (2b). 2′,5′-Di-*O*-methyluridine (**4**, 0.551 mmol, 0.15 g) and N^2-acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(triisopropylsilyloxymethyl)guanosine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (**11**, 0.5 mmol, 0.5 g) were dissolved in a solution of tetrazole (0.63 mmol) in MeCN (1.4 mL). The reaction mixture was stirred at room temperature for 30 min, after which iodine (1.67 mmol, 0.43 g) in a mixture of water (4 mL), tetrahydrofuran (8 mL), and 2,6-lutidine (2 mL) was added. After being stirred for an additional 3 h at room temperature, the reaction mixture was concentrated under reduced pressure. A CH_2Cl_2/aq $NaHSO_3$ workup was carried out, the organic phase was evaporated to dryness, and the residue was dissolved in a mixture of acetic acid and water (25 mL, 80:20, v/v). After being stirred for 3 h at room temperature, the reaction mixture was evaporated to dryness, followed by a conventional CH_2Cl_2/aq $NaHCO_3$ workup. The organic phase was evaporated to dryness and the product was purified on a silica gel column eluting with a mixture of CH_2Cl_2 and MeOH, the MeOH content of which was increased stepwise from 5% to 10%. The purified product was then dissolved in saturated aqueous ammonia. After being stirred for 4 h at room temperature the reaction mixture was evaporated to dryness. The residue was dissolved in a solution of tetrabutylammonium fluoride (1.62 mmol, 0.422 g) in anhydrous MeCN (1.6 mL), and the solution was stirred for 3 h at room temperature. The reaction mixture was then evaporated to dryness and a CH_2Cl_2/aq NaOAc workup was carried out. The aqueous phase was concentrated under reduced pressure and the product was purified by HPLC on a LiChrospher RP-18 column (10 × 250 mm^2, 10 μm) eluting with a mixture of 0.06 mol L^{-1} of aqueous NaOAc and MeCN (88:12, v/v). The purified product was passed through a Na^+-form Dowex 50-W (100−200 mesh) cation exchange column. Finally, the buffer salts were removed by HPLC on the same column as previously eluting with a mixture of water and MeCN (88:12, v/v). Yield 64.7 mg (20%). ^{31}P NMR (δ_P) (162 MHz, DMSO-d_6) 0.64. ^{1}H NMR (δ_H) (400 MHz, DMSO-d_6) 7.87 (s, 1H), 7.70 (d, 1H, J = 8.1 Hz), 7.35 (d, 1H, J = 7.3 Hz), 5.68 (d, 1H, J = 8.1 Hz), 4.57−4.51 (m, 2H), 4.40 (m, 1H), 4.18 (dd, 1H, J_1 = 6.2 Hz, J_2 = 3.0 Hz), 3.94 (m, 1H), 3.87 (t, 1H, J = 5.6 Hz), 3.60−3.49 (m, 4H), 3.34 (s, 3H), 3.30 (s, 3H). HRMS (FAB) M^- calcd 616.1404, obsd 616.1403.

Kinetic Measurements. The reactions were carried out in sealed tubes immersed in a thermostated water bath, the temperature of which was adjusted to 50 °C within ±0.1 °C. The hydronium ion concentration of the reaction solutions was adjusted with sodium hydroxide and acetate, triethanolamine, glycine, and triethylamine buffers. The pH values of the buffers were calculated from the literature data of the pK_a values of the buffer acids under experimental conditions. The ionic strength of the solutions was adjusted to 1.0 mol L^{-1} with

$NaNO_3$, except with experiments on diesters **2a**,**b**, in which case NaCl was used. The effect of the buffer concentration on the reaction rate was determined at pH 8 by carrying out two runs at different buffer concentrations. No buffer catalysis was observed. Otherwise, a buffer concentration of 0.1 mol L^{-1} was used.

The initial substrate concentration in the kinetic runs was ca. 10^{-4} mol L^{-1}. The composition of the samples withdrawn at appropriate intervals was analyzed by HPLC on a Supelcosil LC-18-S column (4.6 × 150 mm^2, 5 μm) or a Hypersil-Keystone Aquasil C18 column (4 × 150 mm^2, 5 μm), using 0.05 mol L^{-1} of aqueous NH_4OAc and MeCN as an eluent. The amount of MeCN was linearly increased from 8 to 30%. The observed retention times (t_R, min) for the hydrolytic products of **1** (the flow rate was 1.5 mL min^{-1}) were as follows: 14.3 (**1**), 12.2 (**5**), 11.5 (**6**), 5.4 (**7**), and 4.6 (**4**). The products were identified by spiking with authentic reference samples (Scheme 4), and the characterization was further verified by LC/MS analysis.

Mass Spectrometric Analysis of the Products of Hydrolysis in ^{18}O-Enriched Water. Compound **1** (ca. 35 μg) was dissolved in a mixture of $H_2{}^{18}O$ (95 atom % ^{18}O, 98 μL) and 2.375 mol L^{-1} of aqueous sodium hydroxide (2 μL). The vial was sealed and immersed in a water bath at 50 °C for 2 h, after which the solution was neutralized by adding 1 mol L^{-1} of aqueous nitric acid (4.76 μL). For reference purposes, an analogous experiment was carried out by using a corresponding sodium hydroxide solution prepared in ^{16}O water. Composition of the product mixture was analyzed by HPLC/MS.

Acknowledgment. We thank Prof. Harri Lönnberg for fruitful discussions.

Note Added after ASAP Posting. Due to a production error, the wrong figures were published in the version posted ASAP January 10, 2004; the corrected version was posted January 13, 2004.

Supporting Information Available: ^{13}C NMR spectra of compounds **9**, **4**, **3**, **10**, **1**, **5**, **6**, **2a**, and **2b**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO035094K