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Hydrolytic Reactions of Diribonucleoside 3′,5′-(3′-*N*-Phosphoramidates): Kinetics and Mechanisms for the P−O and P−N Bond Cleavage of 3′-Amino-3′-deoxyuridylyl-3′,5′-uridine

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Abstract: Hydrolytic reactions of 3′-amino-3′-deoxyuridylyl-3′,5′-uridine (**2a**), an analogue of uridylyl-3′,5′-uridine having the 3′-bridging oxygen replaced with nitrogen, have been followed by RP HPLC over a wide pH range. The only reaction taking place under alkaline conditions (pH > 9) is hydroxide ion-catalyzed hydrolysis (first-order in [OH^-]) to a mixture of 3′-amino-3′-deoxyuridine 3′-phosphoramidate (**7**) and uridine (**4**). The reaction proceeds without detectable accumulation of any intermediates. At pH 6−8, a pH-independent formation of 3′-amino-3′-deoxyuridine 2′-phosphate (**3**) competes with the base-catalyzed cleavage. Both **3** and in particular **7** are, however, rather rapidly dephosphorylated under these conditions to 3′-amino-3′-deoxyuridine (**5**). In all likelihood, both **3** and **7** are formed by an intramolecular nucleophilic attack of the 2′-hydroxy function on the phosphorus atom, giving a phosphorane-like intermediate or transition state. Under moderately acidic conditions (pH 2−6), the predominant reaction is acid-catalyzed cleavage of the P−N3′ bond (first-order in [H^+]) that yields an equimolar mixture of **5** and uridine 5′-phosphate (**6**). The reaction is proposed to proceed without intramolecular participation of the neighboring 2′-hydroxyl group. Under more acidic conditions (pH < 2), hydrolysis to **3** and **4** starts to compete with the cleavage of the P−N bond, and this reaction is even the fastest one at pH < 1. Formation of 2′-*O*,3′-*N*-cyclic phosphoramidate as an intermediate appears probable, although its appearance cannot be experimentally verified. The rate constants for various partial reactions have been determined. The reaction mechanisms and the effect that replacing the 3′-oxygen with nitrogen has on the behavior of the phosphorane intermediate are discussed.

Introduction

Phosphoramidate analogues of oligodeoxyribonucleotides (**1a**), having the 3′-oxygen of each internucleosidic phosphodiester linkage replaced with nitrogen, have been shown to be resistant toward nucleases, and to form stable duplexes with complementary oligonucleotide sequences[1] and stable triplexes with double-stranded DNA.[2] For this reason, they have received attention as potential antisense oligonucleotides.[3] More recently, similarly modified oligoribonucleotides (**1b**) have been synthesized and shown to be, analogously to their 2′-deoxy counterparts, so tolerant toward enzymatic cleavage and to hybridize so efficiently that they also may be regarded as viable candidates for antisense purposes.[4,5] Being RNA analogues, they may also be expected to find applications as aptamers, i.e., oligomeric sequences selected to fold into structures exhibiting high affinity for various proteins or low molecular weight ligands.[6] Despite these attractive properties, the intrinsic chemical reactivity of nucleoside 3′-*N*-phosphoramidates in comparison to their native phosphate ester counterparts has not been studied in detail. Only some semiquantitative data on the stability of 3′-*N*-bridged phosphoramidate RNA dimers in aqueous acetic acid and ammonia have been reported.[4,7] Besides these, a study on the

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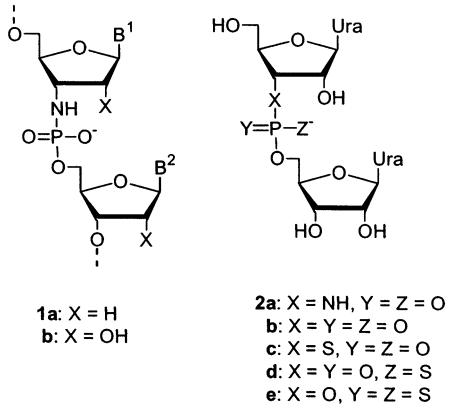
product distributions of the hydrolysis of the 2′-*N*-phosphoramidate analogues of 2′,5′-diribonucleoside monophosphates under acidic, neutral, and alkaline conditions has been published.[8] The present study is aimed at providing a more detailed kinetic analysis of the acid- and base-catalyzed hydrolysis of the nucleoside 3′-*N*-phosphoramidates. Kinetics for the hydrolytic reactions of 3′-amino-3′-deoxyuridylyl-3′,5′-uridine (UnpU, **2a**) have been studied over a wide pH range, and the mechanisms of various partial reactions have been discussed. In addition, the paper has a more general mechanistic aim. The cleavage and concurrent 3′,5′ → 2′,5′ isomerization of internucleosidic phosphodiester bonds proceed via a pentacoordinated phosphorane intermediate.[9] The effects of thiosubstitution on the behavior of this intermediate have been previously elucidated by comparing the kinetic data obtained with unmodified uridylyl-3′,5′-uridine[10] (UpU, **2b**) to those obtained with its phosphoromonothioate analogues having either the 3′-oxygen[11,12] (**2c**) or one[13,14] (**2d**) or both[15] (**2e**) of the nonbridging oxygens replaced with sulfur. The present results together with those reported earlier for the cleavage of diribonucleoside 3′,5′-(5′-*N*-phosphoramidates)[16] allow a similar evaluation of the effects of aza substitutions.



1a: X = H
b: X = OH

2a: X = NH, Y = Z = O
b: X = Y = Z = O
c: X = S, Y = Z = O
d: X = Y = O, Z = S
e: X = O, Y = Z = S

Results and Discussion

Product Distributions. The hydrolysis of **2a** was followed over a wide pH range 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. According to the time-dependent product distributions obtained, the internucleosidic phosphoramidate linkage is cleaved by two basically different pathways, viz., by cleavage of the P−N3′ bond (route B in Scheme 1) or P−O5′ bond (routes A, C, and D in Scheme 1). The competition between these two modes of bond cleavage is strictly pH-dependent. Under mildly acidic conditions (pH 2−6, T = 90 °C), the cleavage of the P−N3′ bond is the only reaction detected: a mixture of 3′-amino-3′-deoxyuridine (**5**) and uridine 5′-phosphate (**6**) is obtained without appearance of any intermediate (route B in Scheme 1). This kind of reaction, where the phosphate group remains bonded to the 5′-esterified nucleoside, has no counterpart among the hydrolytic reactions of either dinucleoside 3′,5′-monophosphates[10] or their 3′-*S*-phosphorothioate analogues.[11,12]

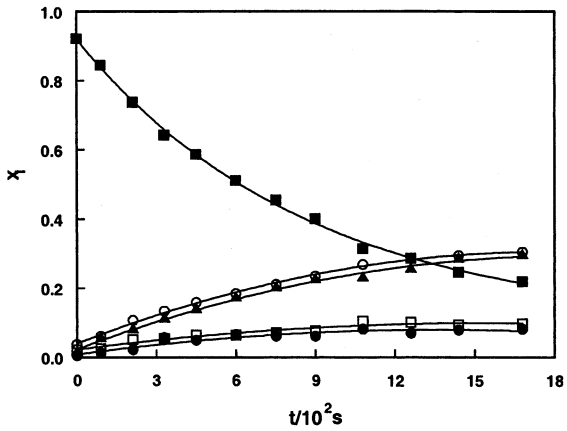


Figure 1. Time-dependent product distribution for the hydrolysis of **2a** in 0.5 mol L^{-1} aqueous hydrogen chloride at 25.0 °C: (■) **2a**, (●) **5**, (□) **6**, (○) **3**, (▲) **4**.

On going to strongly acidic conditions (pH < 2), cleavage of the P−O5′ bond starts to compete with the P−N3′ cleavage. In addition to **5** and **6**, 3′-amino-3′-deoxyuridine 2′-phosphate (**3**) and uridine (**4**) are formed (route A in Scheme 1). Under these conditions, the hydrolysis products **3**−**6** are not markedly hydrolyzed further during the kinetic runs. Accordingly, the rate constants of the hydrolysis of **2a** could be bisected to the rate constants of the parallel first-order reactions, i.e., to k_1 and k_2, on the basis of the product distribution (see eqs 1 and 2 in the Experimental Section). The reactions at pH < 2 were followed at lower temperature (25 °C). The proportion of P−O5′ cleavage was somewhat increased with decreasing temperature. Figure 1 shows the time-dependent product distribution in 0.5 mol L^{-1} aqueous hydrogen chloride at 25 °C. Again no intermediate is accumulated, but in all likelihood the initial product is the 2′-*O*,3′-*N*-cyclic phosphoramidate. One might also expect 3′-amino-3′-deoxyuridylyl-2′,5′-uridine to occur among the reaction products, since the acid-catalyzed cleavage of intranucleosidic phosphodiester bonds, which also proceeds by the P−O5′ bond cleavage, is accompanied by almost as fast 3′,5′ → 2′,5′ isomerization.[10] This does not, however, seem to be the case with 3′-*N*-phosphoramidates; the 2′,5′-isomer of **2a** is not formed.

The cleavage of the P−O5′ bond also becomes favored over that of the P−N3′ bond on going from acidic to neutral and alkaline solutions (pH > 6). At pH 6−8, **3** and **4** are accumulated, and the former is subsequently dephosphorylated to **5** (route C in Scheme 1). At still higher pH (pH > 7), hydroxide ion-catalyzed hydrolysis to 3′-amino-3′-deoxyuridine 3′-*N*-phosphoramidate (**7**; route D in Scheme 1) with concomitant release of **4** starts to compete with formation of **3**, becoming the predominating reaction at pH > 8. At pH > 7, the dephosphorylation of **3** to **5** becomes slow and routes C and D

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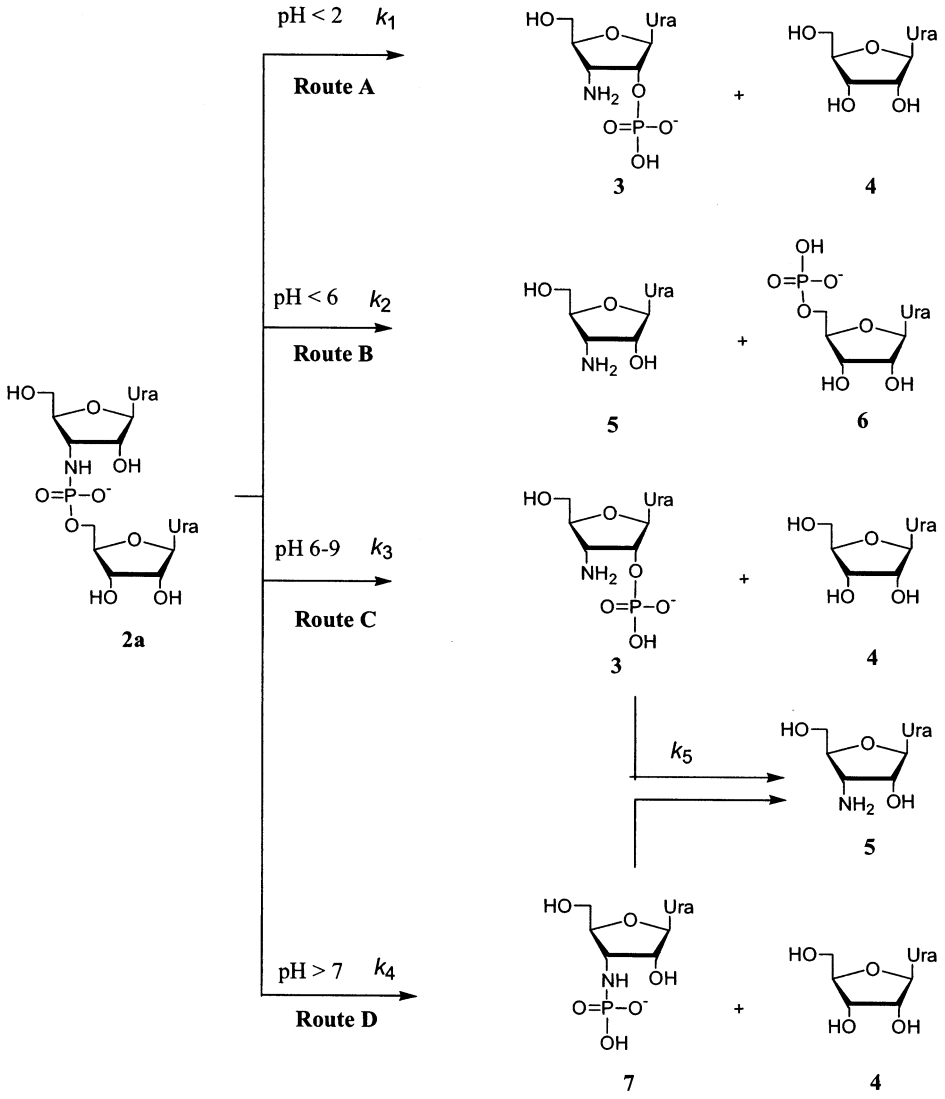
Scheme 1



may be distinguished on the basis of the accumulation of **3** (see eqs 4 and 5 in the Experimental Section). The 3′-*N*-phosphoramidate is accumulated at pH > 9, but at lower pH it is rapidly dephosphorylated to **5**. This was verified by independent measurements with **7**. Both **3** and **7** may be expected to be formed via 2′-*O*,3′-*N*-cyclic phosphoramidate, but no evidence for the intermediary appearance of this species has been obtained. Figure 2 shows as an example the time-dependent product distribution at pH 8.0.

pH−Rate Profiles. Figure 3 shows the pH−rate profile for the hydrolysis of UnpU. As discussed above, the hydrolysis proceeds, depending on pH, by four different routes (routes A−D in Scheme 1). The rate profiles of these partial reactions are indicated in Figure 4. Comparison of these rate profiles with that of the cleavage of UpU (**2b**),[10] also included in Figure 3, reveals that, under alkaline conditions, where UnpU is hydrolyzed to a mixture of **7** and uridine **4**, UnpU is decomposed about 2 times as fast as UpU, which yields a mixture of uridine 2′- and 3′-phosphates and uridine.[10] In acidic solutions (pH 2−6), where UnpU and UpU react via entirely different pathways, viz., by the cleavage of the P−N3′ and P−O5′ bonds, respectively, UnpU is up to 300-fold less stable than UpU.

As seen from Figure 4, the P−O5′ cleavage becomes favored over the P−N3′ cleavage only under very acidic conditions (pH < 1). While the P−N3′ cleavage is first-order in [H^+] over the entire acidic pH range studied, the P−O5′ cleavage appears to be a second-order reaction in [H^+] at pH > 1, leveling off

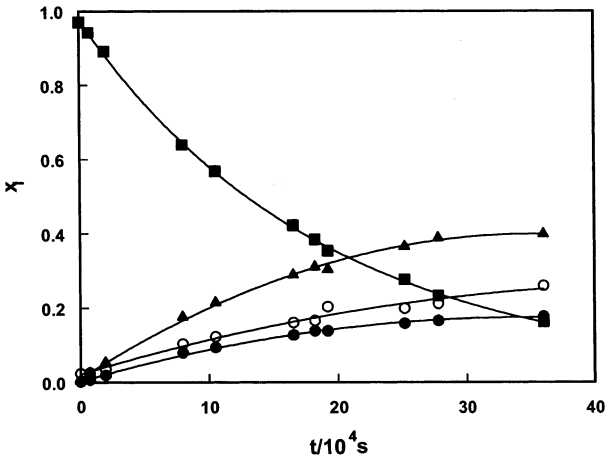


Figure 2. Time-dependent product distribution for the hydrolysis of **2a** at pH 8.0 and 90.0 °C: (■) **2a**, (●) **5**, (○) **3**, (▲) **4**.

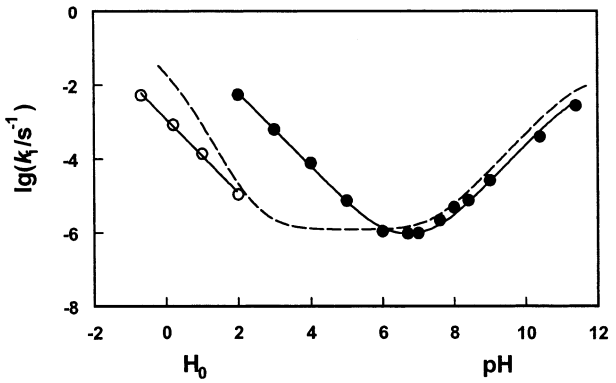


Figure 3. pH−rate profile for the hydrolysis of **2a** at 90.0 (●) and 25.0 (○) °C. The ionic strength of the solutions was adjusted to 0.1 mol L^{-1} with sodium chloride. The dotted line shows the corresponding curve for the decomposition of UpU at 90 °C.[10]

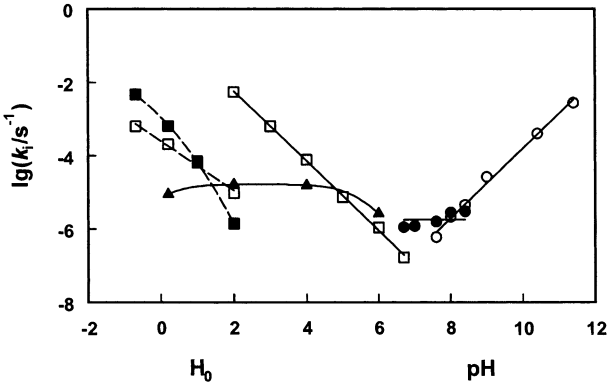
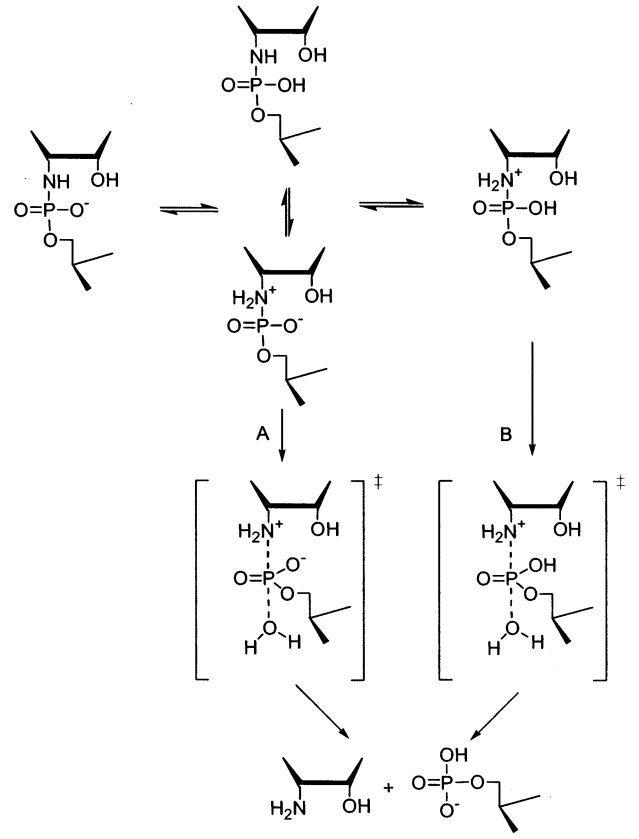


Figure 4. pH−rate profiles for the partial reactions involved in the hydrolysis of **2a** at 90.0 (solid lines) and 25.0 (dotted lines) °C: (■) route A, (□) route B, (●) route C, (○) route D in Scheme 1, (▲) dephosphorylation of **3**.

toward a first-order dependence at still higher acidity. Accordingly, both reactions take place at comparable rates at pH < 1, but in less acidic solutions the P−N3′ bond cleavage gradually becomes the only detectable reaction. It is worth noting that the hydrolysis of UpU also exhibits a second-order dependence of rate on [H^+] at pH 1−3 and a first-order dependence on the acidic side of the pK_a value of the phosphodiester linkage at pH 0.7.[10] The mechanistic explanation in this case is that the predominant reaction consists of an intramolecular nucleophilic attack of the 2′-hydroxy function on the doubly protonated (monocationic) phosphodiester.[9a,b] By analogy, the P−O5′ cleavage of UnpU may be assumed to proceed by an attack of the 2′-hydroxy function on the phosphoramidate monocation. In other words, while neutral phosphoramidate is decomposed solely by the P−N3′ bond cleavage, the phosphoramidate monocation appears to be hydrolyzed by two concurrent routes, viz., by the attack of the 2′-OH followed by the P−O5′ bond cleavage (route A in Scheme 1) in addition to the P−N3′ fission (route B in Scheme 1). The present data do not allow accurate determination of the pK_a of the phosphoramidate, but it appears to be around unity. For comparison, a pK_a value of *N*-carboxymethyl-*O*-phenylphosphoramidate has been reported to be 1.9 at 35.0 °C (I = 0.2 mol L^{-1})[17] and that of *O*-methylphosphoramidate 2.5 at 36.8 °C (I = 0.2 mol L^{-1}).[18]

Scheme 2



The hydrolysis of UnpU turns from the hydronium ion-catalyzed P−N3′ bond cleavage to a hydroxide ion-catalyzed P−O5′ bond cleavage in the pH region from 6 to 8 (Figures 3 and 4). Over this narrow pH range, the fastest reaction, however, is pH-independent formation of **3** with concomitant release of the 5′-linked **4** (route C in Scheme 1). At pH > 8, the hydroxide ion-catalyzed hydrolysis of UnpU to **7** then becomes the predominant reaction (route D in Scheme 1).

Mechanism of the Acid-Catalyzed Cleavage of the P−N3′ Bond. As discussed above, the internucleosidic phosphoramidate linkage of UnpU is cleaved at pH 2−6 predominantly via the acid-catalyzed cleavage of the P−N3′ linkage (route B in Scheme 1), as also observed previously with both ribonucleoside[4,7,8] and deoxyribonucleoside[19,20] phosphoramidates. Under these conditions the predominant ionic form of *N*,*O*-disubstituted phosphoramidates, such as UnpU, is the monoanion.[17] Since the reaction is first-order in [H^+], the species that actually undergoes the P−N3′ bond cleavage is the neutral phosphoramidate. The phosphoramidate monoanion may in principle be protonated either at the phosphoryl oxygen or at the amide nitrogen (Scheme 2). No conclusive evidence for the preferred site of protonation exists, and indirect evidence in favor of both the oxygen[21−23] and nitrogen[24,25] protonation has been reported. In any case, it seems obvious that the reactive tautomer is the *N*-protonated zwitterionic species, since the leaving group may

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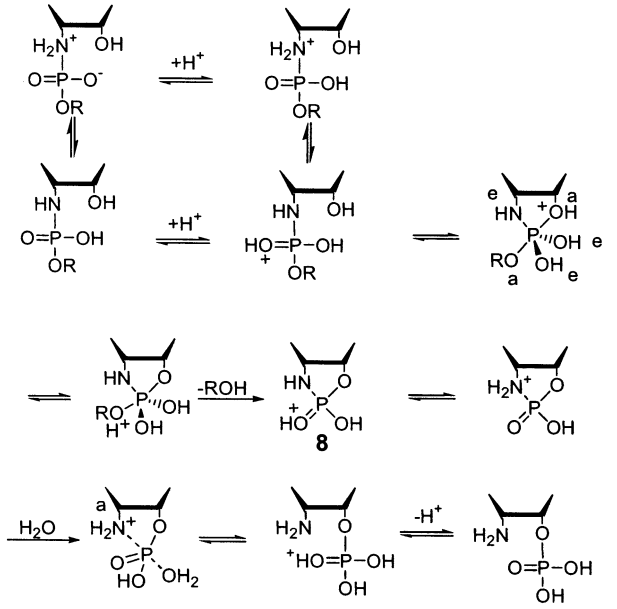
depart from this tautomer as neutral amine. The equilibration between various tautomers most likely takes place in a rapid preequilibrium step, the rate-limiting step being either a nucleophilic attack of a water molecule on the phosphorus atom or a unimolecular rupture of the P−N3′ bond. For the entropy of activation of this reaction, a value of -65 ± 5 J K^{-1} mol^{-1} was obtained at pH 2 (four experimental points at $25 < T < 90$ °C). While this clearly negative value argues against a unimolecular rate-limiting step, an even more negative value could be expected for a typical bimolecular nucleophilic displacement. Evidently the reaction represents a borderline case between an associative and dissociative mechanism. Both the entering and leaving nucleophiles are present in the transition state, the departure of the leaving group being considerably more advanced than the bond formation between the entering nucleophile and the phosphorus atom. The involvement of water as a nucleophile in the transition state receives support from previously published studies on the hydrolysis of simple phosphoramidates. The solvolyses of the neutral zwitterionic forms of phosphoramidic acid,[26] *O*-alkylphosphoramidates,[18] and *N*-alkylphosphoramidates[27] in aqueous alcohol all favor the alcoholysis products over the hydrolysis products. This kind of selectivity toward the entering nucleophile has been taken as an indication of the involvement of the nucleophile in the transition state. For comparison, the hydrolysis of aryl phosphates utlizing a dissociative (preassociation) mechanism[28] does not show a similar selectivity toward the nucleophile. Also consistent with the suggested mechanism, the hydrolysis of *N*-alkylphosphoramidates has been shown to exhibit high sensitivity to the basicity of the leaving group ($\beta_{lg} = -0.9$)[29] but only modest susceptibility to the nature of the entering nucleophile (β_{nuc}).[29,30] The entropy of activation reported only for the hydrolysis of phosphoramidic acid, -76 J K^{-1} mol^{-1},[26] is within the limits of experimental errors equal to that obtained for the cleavage of UnpU in the present work. Evidently the neighboring 2′-hydroxy group does not play any role in the P−N3′ bond cleavage of UnpU; the rate is of the same order of magnitude as that of the simple phosphoramidates indicated above.

The cleavage of the P−N3′ bond of UnpU does not show any marked deviation from the first-order dependence of the rate on [H$^+$] in the pH range 0−4, i.e., on passing the pK_a value of the phosphoramidate. The cleavage rate is increased with increasing hydronium ion concentration even under conditions where the phosphoramidate is almost entirely in the neutral form. Evidently the predominant pathway is gradually changed from the water-catalyzed hydrolysis of the neutral zwitterionic phosphoramidate at pH > pK_a (route A in Scheme 2) to hydronium ion-catalyzed hydrolysis of the same species at pH < pK. The latter reaction probably involves a nucleophilic attack

Scheme 3



of water on the phosphoramidate monocation obtained in a rapid preequilibrium step (route B in Scheme 2).

Mechanism of the Acid-Catalyzed Cleavage of the P−O5′ Bond. While the neutral ionic form of UnpU is hydrolyzed only by cleavage of the P−N3′ bond, the monocation having both the amide nitrogen and phosphoryl oxygen protonated seems to be decomposed by two concurrent pathways. Either the P−N3′ bond is cleaved (route B in Scheme 2), or the 5′-linked **4** is released and **3** is obtained. Formation of the latter product clearly indicates that the reaction must at some stage involve an intramolecular attack of the 2′-hydroxy function on the phosphorus atom. A plausible mechanism is depicted in Scheme 3. The 2′-hydroxy group is assumed to attack on a phosphoramidate tautomer having both of the nonbridging oxygen atoms protonated. The 2′-oxygen takes as the entering nucleophile an apical position in the phosphorane intermediate (or transition state), and hence, the nitrogen atom as a member of the same five-membered ring is forced to an equatorial position.[31] This is possible only if the nitrogen atom does not bear a positive charge. The 5′-linked uridine may occupy the remaining apical position and hence depart after protonation of the 5′-oxygen. The present data do not unambiguously reveal whether this is an "in-line" displacement via a pentacoordinated transition state, or whether a pentacoordinated intermediate having a finite lifetime is formed. The latter is considered to be the case with the acid-catalyzed hydrolysis of phosphodiesters,[31] including the cleavage and isomerization of dinucleoside 3′,5′-monophosphates[9a,b] and their thioate analogues.[11,13] With these compounds, the interconversion between the 3′,5′- and 2′,5′-diesters that competes with the acid-catalyzed phosphodiester cleavage may be taken as strong evidence for the existence of a pseudorotating phosphorane intermediate. However, no evidence for a similar isomerization with UnpU was obtained; the 2′,5′-isomer of UnpU was not detected among the products. It is known[16,32] that replacement of the 2′-hydroxy group of a nucleoside 3′-phosphodiester with an amino function markedly

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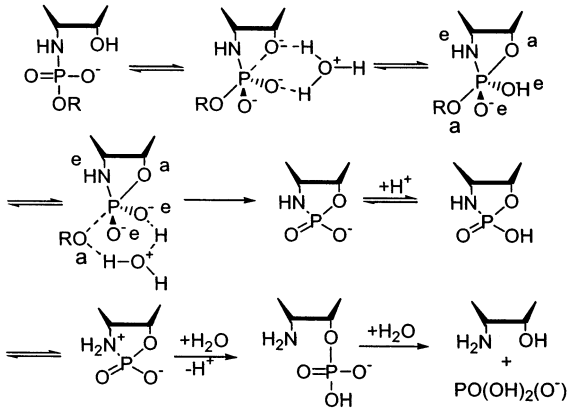
Scheme 4

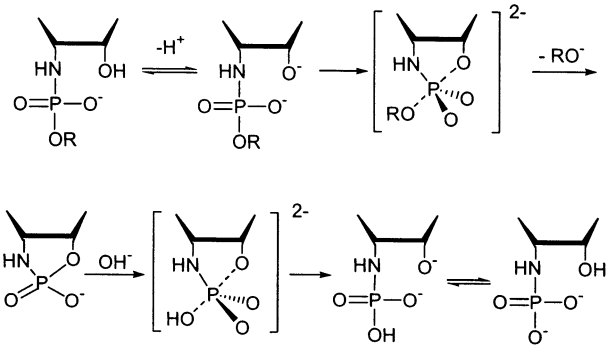


increases the hydrolytic stability of the compound, since the 2′-amino function is a much less effective nucleophile than the 2′-hydroxy group toward the neighboring phosphodiester. Accordingly, the 2′,5′-isomer, if formed, should be sufficiently stable to accumulate. The absence of this isomer among the hydrolysis products does not strictly exclude the existence of a pentacoordinated intermediate, but shows that the intermediate does not undergo pseudorotation that is a prerequisite for the cleavage of the P−N3′ bond. In cases where the pentacoordinated intermediate has a finite lifetime, the pseudorotation barrier seems to be considerably higher than that for the departure of the 5′-linked nucleoside.

The mechanism proposed requires formation of the 2′-*O*,3′-*N*-cyclic phosphoramidate (**8** in Scheme 3) as an intermediate, even though **8** does not accumulate, and its fast hydrolysis exclusively to the 2′-*O*-monophosphate. When a molecule of water attacks protonated **8** obtained by cleavage of the 5′-linked nucleoside, the departure of the 3′-amino ligand is favored over that of the 2′-oxy ligand. The reactive tautomer is the *N*-protonated species, and the amino group departs from the apical position of the pentacoordinated intermediate (or transition state), which is too short-lived to pseudorotate.[33] Conversion of **8** to **3** under acidic conditions is consistent with the results[8,16] according to which the 2′-*N*,3′-*O*-cyclic phosphoramidate is hydrolyzed under acidic conditions exclusively to the 3′-phosphate, i.e., by cleavage of the P−N bond. On the basis of the same results, **8** also appears to be too unstable to accumulate under acidic conditions.

pH-Independent Hydrolysis. At pH 6−8, the hydrolysis of UnpU undergoes a change from the acid-catalyzed cleavage of the P−N3′ bond to the base-catalyzed cleavage of the P−O5′ bond. Within this narrow pH range, the fastest reaction, however, is a pH-independent cleavage to **3** and **4** (Figure 4), **3** being further dephosphorylated to **5**. By analogy with the pH-independent cleavage of ribonucleoside 3′-phosphodiesters, one might assume that an intramolecular attack of the 2′-oxyanion on neutral phosphoramidate gives a monoanionic phosphorane intermediate (Scheme 4).[9a,b,34] The proton transfer from the 2′-hydroxy group to the phosphoryl oxygen takes place either prior to or concerted with the nucleophilic attack. Cleavage of the P−O5′ bond concerted with the proton transfer from the phosphoryl oxygen to the leaving group[35] may then be expected

Scheme 5



to give the 2′-*O*,3′-*N*-cyclic phosphoramidate **8**. The pseudorotation barrier appears to be much higher than that for the cleavage of the exocyclic P−O bond, since no sign of the migration of uridine 5′-phosphate from N3′ to O2′ can be detected. As under acidic conditions, the cyclic phosphoramidate **8** is too unstable to accumulate, and it is rapidly hydrolyzed to **3**, probably by the attack of a water molecule on the *N*-protonated zwitterionic tautomer of **8**. For comparison, 2′-amino-2′-deoxyuridine 2′,3′-cyclic phosphoramidate has been shown to react exclusively by cleavage of the P−N2′ bond at pH 6.[16]

Mechanism of the Hydroxide Ion-Catalyzed Phosphoramidate Hydrolysis. The hydroxide ion-catalyzed hydrolysis of UnpU becomes the predominant reaction at pH > 8. By analogy with the corresponding reaction of ribonucleoside 3′-phosphodiesters,[9a,b,36] the reaction may be assumed to involve a rapid initial deprotonation of the 2′-hydroxy function and a subsequent rate-limiting attack of the resulting oxyanion on the monoanionic phosphoramidate group (Scheme 5). This gives a dianionic phosphorane transition state, or marginally stable intermediate, from which the 5′-linked uridine departs as an alkoxide ion, yielding a 2′-*O*,3′-*N*-cyclic phosphoramidate (**8**), which is immediately hydrolyzed to **7**. It should be noted that intermolecular nucleophilic attack on *O*,*N*-disubsituted phosphoramidate monoanion is a slow reaction. For example, the only reaction that *O*-phenyl-*N*-carboxymethylphosphoramidate undergoes in aqueous alkaline solution is pH-independent intamolecular attack of the carboxylate group on the phosphorus atom.[17] Accordingly, it appears quite clear that the hydroxide ion-catalyzed hydrolysis of UnpU proceeds by intramolecular participation of the 2′-oxyanion.

7 is accumulated at high alkalinity (pH > 9), but under less basic conditions dephosphorylation to **5** takes place. No more **3** is obtained at pH > 8.5. This compound is under alkaline conditions sufficiently stable to become detected when formed. The formation of 3′-*N*-phosphoramidate **7** as a sole product under alkaline conditions is consistent with the earlier observations,[8,16] according to which the alkaline hydrolysis of the 3′-*O*,2′-*N* analogue of **8** proceeds exclusively by the P−O bond cleavage. Since nitrogen is a less electronegative element than oxygen, the 2′-*O* takes an apical position and the 3′-*N* an equatorial position upon the attack of hydroxide ion on the phosphorus atom of **8**, and hence only the P−O2′ bond is

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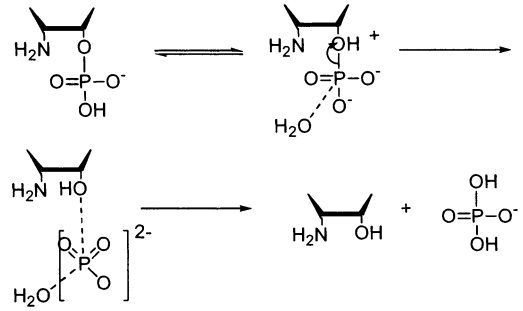
(34) (a) Kosonen, M.; Oivanen, M.; Lönnberg, H. *J. Org. Chem.* **1994**, *59*, 3704. (b) Kosonen, M.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1995**, 1203.
(35) (a) Kosonen, M.; Hakala, K.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1998**, 663. (b) Kosonen, M.; Yousefi-Salakdeh, E.; Strömberg, R.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1998**, 1589. (c) Kosonen, M.; Seppänen, R.; Wichmann, O.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1999**, 2433.
(36) Kosonen, M.; Yousefi-Salakdeh, E.; Strömberg, R.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1997**, 2661.

Scheme 6

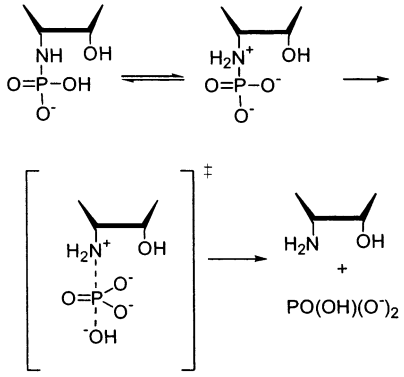


cleaved. This explanation receives support from the results of quantum chemical calculations on the attack of hydroxide ion on methyl aminoethylenephosphonate.[37] In other words, a prerequisite for nitrogen to adopt an apical position is protonation that renders the nitrogen ligand apicophilic. The P−N3′ bond is hence cleaved in acidic and even neutral solutions, but not at high pH.

Dephosphorylation of 3′-Amino-3′-deoxyuridine 2′-Phosphate and 3′-*N*-Phosphoramidate. As mentioned above, **3** is dephosphorylated to **5** under neutral and mildly acidic conditions. This is the only reaction observed to take place. No sign of interconversion of **3** to **7** could be detected under any conditions. The pH−rate profile for dephosphorylation of **3** (Figure 4) is similar to that observed earlier[38] for 2′- and 3′-phosphates of unmodified nucleosides. The "bell-shaped" rate profile shows a rate maximum between pH 2 and pH 4, indicating that the reactive ionic form is the phosphate monoanion. The first-order rate constant for the dephosphorylation of the monoanion is 1.7×10^{-5} s^{-1} at 90 °C ($I = 0.1$ mol L^{-1}), while a value of 1.4×10^{-5} s^{-1} has been reported for uridine 2′-phosphate under the same conditions.[38b] In all likelihood, the reaction proceeds by a unimolecular departure of a metaphosphate monoanion preassociated with a water molecule (Scheme 6), as described previously for phosphomonoesters.[28]

7 is at high pH more labile than **3**. It is accumulated only at pH > 9, and still at pH 10.4 the first-order rate constant for the dephosphorylation is 8.1×10^{-5} s^{-1} (90 °C, $I = 0.1$ mol L^{-1}), thus up to 5 orders of magnitude greater than that estimated for the dephosphorylation of **3** or unmodified nucleoside 2′- and 3′-phosphates[38] under these conditions. The main reason for the more facile dephosphorylation of **7** under alkaline conditions is the lower acidity of monoanionic *N*-alkylphosphoramidates compared to monoanions of phosphomonoesters. The pK_a values are on the order of 9[27] and 6,[39] respectively. Since the reactive ionic form is, as discussed above, the monoanion, the dianion being virtually stable, the rate retardation on going to alkaline solutions is with monoesters, e.g., **3**, 1000-fold compared to that observed with phosphoramidates, e.g., **7**. Mechanistically the reaction has been suggested to represent a borderline case between a unimolecular elimination (formation of metaphosphate ion) and rate-limiting nucleophilic attack on the phosphorus atom (Scheme 7).[26,40]

Scheme 7



In summary, two major differences are detected between the behaviors of 3′-*N*-phosphoramidate and phospodiester oligoribonucleotides. First, the P−N3′ bond cleavage makes the phosphoramidate oligomers up to 300 times more labile at pH 2−6 than the phosphodiester oligomers. Second, the phosphorane intermediate derived from 3′-amino-3′-deoxyribonucleoside 3′-*N*-phosphoramidates appears to be too unstable to pseudorotate, and hence no isomerization of the internucleosidic linkages takes place.

Experimental Section

Methods. The NMR spectra were recorded on a Bruker AM 200 or JEOL JNMA 500 spectrometer. The ^{1}H NMR chemical shifts (at 300 K) were referred to internal TMS, and the ^{31}P NMR shifts (202 MHz, 300 K) to external orthophosphoric acid. The mass spectra were acquired using a Perkin-Elmer Sciex API 365 triple quadrupole LC/MS/MS or ZabSpec-oa TOF spectrometer.

Materials. Uridine, uracil, and uridine 5′-phosphate were products of Sigma. 3′-Azido-3′-deoxyuridine was prepared as described previously.[41] 3′-Amino-3′-deoxyuridylyl-3′,5′-uridine was obtained by oxidative amination of appropriately protected uridine 5′-(*H*-phosphonate) 2-cyanoethyl ester with 3′-amino-3′-deoxyuridine (Scheme 8), a method that has previously been used for the synthesis of oligoribonucleotide phosphoramidates on a solid support.[42] 3′-Amino-3′-deoxyuridine 2′-phosphate was prepared essentially as described by Eckstein et al.[16,43] The trifluoroacetyl group was used as an amino protecting group, and the phosphorylation was performed with phosphoryltris(triazole)[44] in acetonitrile.

3′-Azido-2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyuridine (10). 3′-Azido-3′-deoxyuridine (**9**)[41] (0.60 g, 2.2 mmol) and *tert*-butyldimethylsilyl chloride (2.5 g, 16.3 mmol) were dissolved in 50 mL of anhydrous pyridine. After the solution was stirred for 7 days at 45 °C, the crude product was isolated by a conventional aqueous workup, and purified on a silica gel column eluted with a mixture of dichloromethane and methanol (95:5, v/v). ^{1}H NMR (δ_H) (200 MHz, $CDCl_3$): 8.03 (d, 1H, $J = 8.1$ Hz), 7.96 (s, 1H), 5.86 (d, 1H, $J = 3.2$ Hz), 5.67 (d, 1H), 4.38 (dd, 1H, $J = 4.4$, 3.2 Hz), 4.17 (m, 1H), 4.07 (d, 1H, $J = 12.0$ Hz), 3.86 (d, 1H, $J = 6.3$ Hz), 3.80 (d, 1H), 0.95 (s, 9H), 0.93 (s, 9H), 0.14 (s, 12H).

3′-Amino-2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyuridine (11). Compound **10** (0.32 g, 0.64 mmol) was dissolved in 8 mL of anhydrous pyridine, and triphenylphosphine (0.90 g, 3.44 mmol) was added. After

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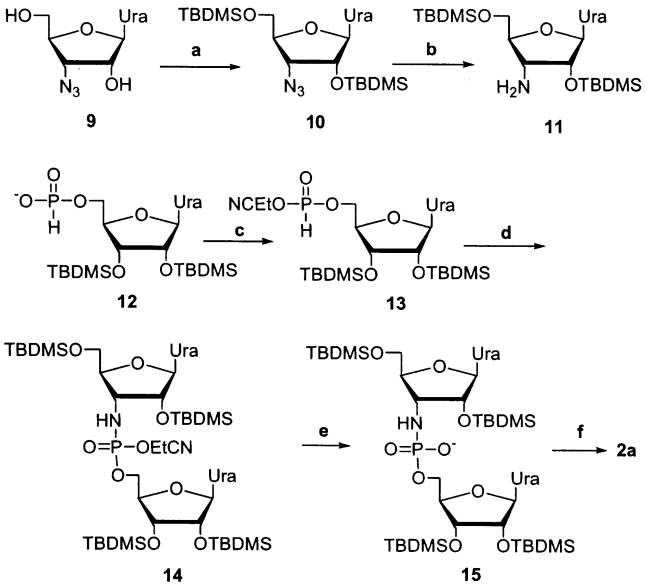
Scheme 8 [a]



[a] Reagents and conditions: (a) *t*-$BuMe_2SiCl$, (b) (1) $(Ph)_3P$, (2) aqueous NH_3, (c) $CNCH_2CH_2OH$, PivCl, (d) **11**, CCl_4, Et_3N, (e) NH_3/MeOH, (f) TBAF.

the solution was stirred for 6 h at 50 °C, heating was stopped, and a mixture of aqueous NH_3 (33%, 6 mL) and pyridine (10 mL) was added. After being stirred for 16 h, the solution was concentrated in vacuo to a syrup, which was dissolved in 100 mL of dichloromethane, washed with water, and concentrated in vacuo. The product was purified on a silica column eluted with a mixture of ethyl acetate and hexane (70:30, v/v). ^{1}H NMR (δ_H) (400 MHz, $CDCl_3$): 8.15 (d, 1H, J = 8.1 Hz), 5.68 (s, 1H), 5.57 (d, 1H), 4.02 (dd, 1H, J = 5.3 Hz), 4.00 (m, 1H), 3.82 (s, 1H), 3.79 (s, 1H), 3.31 (m, 1H), 1.35 (m, 2H), 0.86 (s, 18H), 0.03 (s, 12H). ESI$^-$-MS: m/z 470.4 [M − H]$^-$.

3′-Amino-2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyuridylyl-3′,5′-[2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)uridine] 2-Cyanoethyl Ester (14). The triethylammonium salt of 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-uridine 5′-hydrogenphosphonate (**12**) was prepared as described earlier,[45] and 0.26 g (0.49 mmol) of the product was dissolved in a mixture of anhydrous pyridine (2.0 mL) and acetonitrile (2.0 mL). 2-Cyanoethanol (24 μL, 0.34 mmol) and pivaloyl chloride (63 μL, 0.51 mmol) were added. The cyanoethyl ester **13** was not isolated, but after the solution was stirred for 2 h at room temperature, 3′-amino-3′-deoxyuridine 3 (0.185 g, 0.393 mmol), carbon tetrachloride (2.5 mL), and triethylamine (0.17 mL) were added, and stirring was continued for 75 min. The mixture was then poured into 35 mL of dichloromethane and washed with saturated aqueous NaCl (3 × 30 mL). The organic layer was dried with Na_2SO_4 and concentrated. The product was purified on a silica gel column using a mixture of dichloromethane and methanol as eluent (70:30, v/v). ESI$^+$-MS: m/z 1081.9 [M + Na]$^+$.

3′-Amino-2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyuridylyl-3′,5′-[2′,3′-bis-*O*-(*tert*-butyldimethylsilyl)uridine] (15). Compound **14** was dissolved in saturated methanolic ammonia (3 mL). After being stirred for 2 h, the solution was evaporated to dryness, and the product was purified on a silica gel column eluting with a mixture of dichloromethane and methanol, the methanol content of which was increased stepwise from 0% to 60%. ^{31}P NMR (δ_P) (202 MHz, DMSO-d_6): 8.23. ^{1}H NMR (δ_H) (500 MHz, DMSO-d_6): 11.37 (s, 1H), 11.30 (s, 1H), 8.21 (d, 1H, J = 7.9 Hz), 7.79 (d, 1H, J = 8.1 Hz), 5.85 (d, 1H, J = 4.9 Hz), 5.79 (d, 1H, J = 6.0 Hz), 5.62 (d, 1H, J = 8.1 Hz), 5.58 (d, 1H, J = 8.1 Hz), 4.32 (dd, 1H, J = 4.9 Hz), 4.17 (dd, 1H), 4.12 (m, 1H), 3.94 (m, 1H), 3.86 (m, 1H), 3.84 (m, 1H), 3.77 (m, 1H), 3.72 (m, 1H), 3.70 (m, 1H), 3.45 (m, 1H), 0.87 (s, 9H), 0.87 (s, 9H), 0.84 (s, 9H), 0.81 (s, 9H), 0.06 (s, 6H), 0.05 (s, 6H), 0.04 (s, 6H), 0.01 (s, 6H). ESI$^-$-MS: m/z 1004.7 [M − H]$^-$.

3′-Amino-3′-deoxyuridylyl-3′,5′-uridine (2a). The *tert*-butyldimethylsilyl-protected phosphoramidate **15** was dissolved in 1 mol L^{-1} tetrabutylammonium fluoride (0.265 g, 1.02 mmol) in tetrahydrofuran (1 mL), and the solution was stirred for 16 h at room temperature. The mixture was evaporated to dryness, and the product was purified by reversed-phase chromatography on a Lobar RP-18 column (37 × 440 mm, 40−63 μm) eluting with a mixture of 0.1 mol L^{-1} aqueous ammonium acetate and acetonitrile, the acetonitrile content of which was linearly increased from 0% to 20%. The buffer salts were removed on the same column by eluting with a mixture of water and acetonitrile. Finally, the product was passed through a Na^+-form Dowex 50-W (100−200 mesh) cation exchange column. ^{31}P NMR (δ_P) (202 MHz, D_2O): 8.85. ^{1}H NMR (δ_H) (500 MHz, D_2O): 7.93 (d, 1H, J = 8.1 Hz), 7.90 (d, 1H, J = 8.1 Hz), 5.80 (d, 1H, J = 3.4 Hz), 5.71 (d, 1H, J = 8.1 Hz), 5.65 (d, 1H, J = 8.1 Hz), 5.59 (s, 1H), 4.22 (dd, 1H, J = 5.5 Hz), 4.17 (m, 1H), 4.14−4.09 (m, 2H), 3.95−3.86 (m, 4H), (m, 1H), 3.78 (m, 1H). ESI$^-$-MS: m/z 321.9 [M − H]$^-$.

3′-Amino-3′-deoxyuridine 2′-Phosphate (3). 5′-*O*-(4,4′-dimethoxytrityl)-3′-deoxy-3′-azidouridine was reduced to the corresponding 5′-*O*-(4,4′-dimethoxytrityl)-3′-amino-3′-deoxyuridine as described above for the 2′,5′-bis-*O*-TBDMS-protected analogue **10**. The 3′-amino group was then acylated[43] with *S*-ethyl trifluorothioacetate, and the 2′-hydroxyl group was phosphorylated[44] with phosphoryltris(triazole). The 5′-*O*-(4,4′-dimethoxytrityl) group was removed with a mixture of trifluoroacetic acid, dichloromethane, and methanol (3:1:3, v/v/v, 15 min at 22 °C). The volatile components were evaporated. The residue was dissolved in water and washed with dichloromethane, and the product was purified on a Lobar RP-18 column (37 × 440 mm, 40−63 μm) using 2% aqueous acetonitrile as an eluent. The 3′-*N*-trifluoroacetyl group was removed with methanolic ammonia within 5 h at 22 °C. The mixture was concentrated, and the product was purified on the Lobar RP-18 column using water as an eluent. ^{31}P NMR (δ_P) (162 MHz, D_2O): 3.47. ^{1}H NMR (δ_H) (400 MHz, D_2O): 7.59 (d, 1H, J = 8.1 Hz), 5.76 (d, 1H, J = 3.36 Hz), 5.73 (d, 1H, J = 8.1 Hz), 4.90 (dd, 1H, J = 5.50 Hz), 4.19 (m, 1H), 3.99 (m, 1H), 3.78 (dd, 1H), 3.68 (dd, 1H). ESI$^-$-MS: m/z 321.9 [M − H]$^-$.

Kinetic Measurements. The reactions were carried out in sealed tubes immersed in a thermostated water bath, the temperature of which was adjusted within ±0.1 °C. The hydronium ion concentration of the reaction solutions was adjusted with hydrogen chloride, sodium hydroxide and formate, acetate, triethanolamine, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), and glycine buffers. The pH values of the buffer solutions were calculated from the literature data of the pK_a values of the buffer acids under the experimental conditions.[46] A low buffer concentration was used (<0.06 mol L^{-1}) to minimize the effects of possible buffer catalysis on the reactions. At pH 3 and 8 the effect of the buffer concentration was determined by carrying out two runs at different buffer concentrations. The effect on the reaction rate was in both cases less than 30% when the total buffer concentration was 0.2 mol L^{-1}.

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 Hypersil ODS 5 column (4 × 250 mm, 5 μm) using acetic acid/sodium acetate buffer (0.045/0.015 mol L^{-1}) containing 0.1 mol L^{-1} ammonium chloride and 1.0% acetonitrile as an eluent. The observed retention times (t_R, min) for the hydrolytic products of **2a** (the flow rate was 1 mL min^{-1}) were as follows: 18.0 (**2a**), 5.4 (**4**), 4.2 (**5**), 4.2 (**7**), 3.1 (**3**), and 2.5 (**6**). **5** and its 3′-*N*-phosphoramidate (**7**) could be separated from each other by

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eluting with the same buffer in the absence of acetonitrile; $t_R = 5.8$ and 6.2 min for **5** and **7**, respectively. The products were identified by spiking with authentic reference samples, and the characterizations were further ascertained by LC/MS analysis. In the MS analysis, a mixture of acetonitrile and 5 mmol L^{-1} aqueous ammonium acetate (1.2:98.8, v/v) was used as an eluent.

Calculation of the Rate Constants. The pseudo-first-order rate constants (k_{dec}) for the decomposition of **2a** were obtained by applying the integrated first-order rate equation to the time-dependent diminution of the concentration of the starting material.

The first-order rate constants of the cleavage of the P−N bond (k_2), under acidic conditions (pH < 6, route B in Scheme 1), were calculated by eq 1, where $[UMP]_t$, $[aminourd]_t$, $[aminourd\ 2'\text{-phosphate}]_t$, and $[urd]_t$ stand for the concentrations of **6**, **5**, **3**, and **4**, respectively, at moment *t*. Equation 2 was then applied to obtain the rate constants (k_1) for the acid-catalyzed hydrolysis of UnpU to a mixture of **3** and **4** (route A in Scheme 1).

$$k_2 = \frac{[UMP]_t + [aminourd]_t}{[UMP]_t + [aminourd]_t + [aminourd\ 2'\text{-phosphate}]_t + [urd]_t} k_{dec} \quad (1)$$

$$k_1 = k_{dec} - k_2 \quad (2)$$

At pH 6−7, where **3** is further dephosphorylated to **5**, the first-order rate constants for the pH-independent hydrolysis of starting material (k_3) (route C in Scheme 1) were obtained by least-squares fitting to eq 3, where k_{dec} is the first-order rate constant for the disappearance of UnpU and k_5 the first-order rate constant for the dephosphorylation of **3**. $[UnpU]_0$ stands for the initial concentration of the starting material.

$$\frac{[aminourd\ 2'\text{-phosphate}]_t}{[UnpU]_0} = \frac{k_3}{k_5 - k_{dec}}[\exp(-k_{dec}t) - \exp(-k_5 t)] \quad (3)$$

At pH > 7, where the dephosphorylation of **3** is exceedingly slow compared to its formation, the first-order rate constants k_3 were calculated by eq 4. Equation 5 was then applied to obtain the rate constant (k_4) for the competing hydroxide ion-catalyzed cleavage of the starting material.

$$k_3 = \frac{[aminourd\ 2'\text{-phosphate}]_t}{[aminourd\ 2'\text{-phosphate}]_t + [aminourd]_t} k_{dec} \quad (4)$$

$$k_4 = k_{dec} - k_3 \quad (5)$$

The first-order rate constant for the dephosphorylation of the monoanionic **3** ($k_d = (1.7 \pm 0.02) \times 10^{-5}\ s^{-1}$) at 90.0 °C and $I = 0.1$ mol L^{-1} was determined by a least-squares fitting of the observed rate constants k_5 to eq 6.

$$k_5 = k_d/([H^+]K_{a1}{}^{-1} + 1 + K_{a2}[H^+]^{-1}) \quad (6)$$

Acknowledgment. We thank Dr. Kari Neuvonen for skillful assistance in the NMR spectrometric analyses and Dr. Mohamed Elzagheid for the preparation of 3′-azido-3′-deoxyuridine.

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