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Characterization of the Transition-State Structures and Mechanisms for the Isomerization and Cleavage Reactions of Uridine 3′-*m*-Nitrobenzyl Phosphate

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Abstract: The transition-state structures and mechanisms of the isomerization to the 2′-isomer and cleavage reactions of uridine 3′-*m*-nitrobenzyl phosphate to *m*-nitrobenzyl alcohol and a 2′,3′-cyclic UMP at 86 °C and at pH 2.5, 5.5, and 10.5 have been characterized through kinetic isotope effects. The ^{18}O primary isotope effect of 1.0019 ± 0.0007 and the secondary isotope effect of 0.9904 observed for the cleavage reaction at pH 2.5 are consistent with a neutral phosphorane-like transition-state structure. The cleavage and isomerization reactions at pH 2.5 proceed through a neutral phosphorane intermediate. The $^{18}k_{bridge}$ and $^{18}k_{nonbridge}$ of unity measured for the pH-independent isomerization reaction at neutral pH support a stepwise mechanism with a monoanionic phosphorane intermediate. The primary and secondary isotope effects of 1.009 ± 0.001 and of 0.9986 ± 0.0004 observed for the pH-independent cleavage reaction are consistent with either a stepwise mechanism through a monoanionic phosphorane intermediate or with an A_ND_N reaction with a transition state resembling a monoanionic phosphorane intermediate. The absolute requirement of a water-mediated proton transfer for the formation of a phosphorane intermediate is proven by the absence of the isomerization reaction in anhydrous *tert*-butyl alcohol. The primary isotope effect of 1.0272 ± 0.0001 for the cleavage reaction at pH 10.5 is consistent with a concerted reaction through a transition state in which the leaving group departs with almost a full negative charge.

Introduction

The central role played by phosphate esters as building blocks of DNA and RNA requires a thorough understanding of the mechanisms of their reactions. By studying the hydrolysis of five-member cyclic and acyclic phosphates such as ethylene phosphate and dimethyl phosphate, Westheimer set the foundation for comprehending the reactions of phosphate esters.[1] Phosphate diesters undergo nucleophilic substitution reactions by an addition−elimination mechanism.[2] In the concerted $S_N2(P)$ (A_ND_N)[3] reaction, the pentacoordinated transition state will resemble a trigonal bipyramid (TBP), assuming a phosphorane-like structure. In the TBP geometry the ligands may occupy two different positions, the apical or the equatorial. Both the leaving-group departure and the nucleophile entry must occur at the apical position. If the phosphorane intermediate formed in the two-step mechanism (A_N+D_N) has a sufficient lifetime, pseudorotation may occur to form a new TBP structure with the ligand positions exchanged. This polytopal rearrangement accounts for the different stereochemistry and product distribution of nucleophilic substitution at phosphorus.[1]

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Model compounds of RNA such as dinucleoside 3′,5′-phosphates, ribonucleoside 3′-(alkyl phosphates) and polyribonucleotides in acidic or neutral pH undergo two transesterification reactions, a reversible one, which gives the 2′-isomer, and the cyclization reaction with 2′,3′-cyclic phosphate as product, which is then hydrolyzed to 2′- and 3′-phosphates.[4] The similar kinetics of the cyclization and isomerization of analogues of RNA under acidic conditions indicate that these reactions proceed through the same neutral phosphorane intermediate.[4] The neutral phosphorane is sufficiently stable to pseudorotate to give either the 2′,3′-cyclic monophosphate or the 2′-isomer.[5] Further evidence for the existence of a polytopal rearrangement in the transesterification reaction is the overall retention of configuration at the phosphorus in the reaction of phosphoromonothioate analogues of 3′,5′-uridylyluridine.[6]

At neutral pH, although the isomerization and cleavage reactions are of particular interest due to their biological relevance, very little is known about their mechanisms. The isomerization reaction is pH-independent from pH 4.0 to 8.0 and is believed to proceed through a phosphorane intermediate, while the cyclization reaction shows pH-independence over a smaller range around pH 5.0.[4d] The uncatalyzed cleavage reaction may be concerted with a phosphorane-like transition state or be stepwise with a phosphorane intermediate which may or may not pseudorotate.[4b,c] In aqueous alkali, RNA molecules are cleaved by intramolecular nucleophilic attack by the 2′-oxyanion. The 2′,3′-cyclic phosphate product is then hydrolyzed to 2′- and 3′-phosphates.[7] Evidence for concerted cyclization is that no isomerization reaction has been observed with model compounds of RNA,[4b−d] ab initio calculations indicate that a dianionic phosphorane is too unstable to exist as an intermediate,[8] and the stereochemistry of the cleavage reaction is inversion.[6]

Heavy atom kinetic isotope effects are a powerful method to study the mechanism and the transition-state structure of enzymatic and nonenzymatic reactions of phosphate diesters.[9] The magnitude of the primary isotope effect, $^{18}k_{\text{bridge}}$, is an indication of the degree of bond breaking measured at the leaving group oxygen atom in the transition state. The partial loss or increase of bond order between the nonbridge oxygen atoms and the phosphorus atom in the transition state is revealed by the secondary kinetic isotope effects, $^{18}k_{\text{nonbridge}}$ (Figure 1). We have measured the heavy atom kinetic isotope effects in the cleavage and isomerization reactions of uridine 3′-*m*-nitrobenzyl phosphate at different pHs. The isotope effects reported shed light on the mechanism of the isomerization and cyclization reactions of RNA and of model compounds of RNA in acidic, neutral, and basic conditions and fully characterize their phosphorane-like transition states.

Experimental Section

Materials and Methods. Methanol-^{18}O, $H_2{}^{18}O$,$^{14}NH_4{}^{14}NO_3$, and $^{15}NH_4{}^{15}NO_3$ were purchased from Isotec. All synthetic reagents and anhydrous *tert*-butyl alcohol were from Aldrich. Buffers were from Sigma. The Microsorb-MV C18 HPLC column was purchased from Varian Analytical Instruments. Econosil C18 HPLC column was from Alltech.

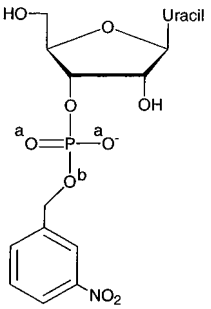

Figure 1. Uridine 3′-*m*-nitrobenzyl phosphate, showing the positions of the isotope effect measurements. (a) $^{18}k_{\text{nonbridge}}$ measured at the nonbridge oxygen atoms. (b) $^{18}k_{\text{bridge}}$ measured at the bridge oxygen atom.

The synthesis of uridine 3′-*m*-nitrobenzyl phophate was carried out as previously reported.[9] [^{14}N], [^{15}N, bridge-^{18}O], and [^{15}N, nonbridge-$^{18}O_2$]-uridine 3′-*m*-nitrobenzyl phosphate compounds were synthesized as previously described.[10] Mass spectrometry (FAB) showed [^{15}N, nonbridge-$^{18}O_2$]-uridine 3′-*m*-nitrobenzyl phosphate to have 64% incorporation of oxygen-18 at nonbridge oxygens. Mass spectrometry (FAB) showed [^{15}N, bridge-^{18}O]-uridine 3′-*m*-nitrobenzyl phosphate to have 93% incorporation of oxygen-18 at bridge oxygen. NMR spectrometry was employed to check the purity and identity of the compounds. ^{1}H NMR spectra were recorded at 500 MHz in D_2O with TSP as an internal reference. ^{13}C NMR spectra were recorded at 125 MHz in D_2O with TSP as an internal reference. ^{31}P NMR spectra (^{1}H decoupled) were recorded at 202 MHz in D_2O unless otherwise specified with 85% H_3PO_4 as an external reference.

Kinetic Measurements. Uridine 3′-*m*-nitrobenzyl phosphate sodium salt (30 μmoles) was heated at 92 °C in a oil bath in 10 mL of 50 mM buffer, and the temperature of the solution was 86 °C. Citric acid was used as a buffer for the reaction run at pH 2.0, sodium acetate for pH 4.0 and 5.0, MES for pH 6.0, MOPS for pH 7.0, CHES for pH 9.0,and CAPS for pH 11.5. Reactions were run at least in duplicate. Each reaction was followed by periodically assaying withdrawn aliquots with a Microsorb-MV C18 HPLC column (5 μm, 4.6 mm i.d. × 250 mm) equilibrated with 6 mM NaH_2PO_4 pH 6.8, in 27% methanol. Detection was accomplished by absorption at 254 nm. Uridine 3′-*m*-nitrobenzyl phosphate elutes at 9.2 min, uridine 2′-*m*-nitrobenzyl phosphate at 4.6 min, UMP at 2.4 min, and *m*-nitrobenzyl alcohol at 18.2 min. The rates were determined by integrating peak areas. The pH-rate profiles were fitted to the following equations: $k_{\text{obs}} = k_o + [H^+]/K_1$ for the isomerization reaction and $k_{\text{obs}} = k_o + [H^+]/K_1 + K_2/[H^+]$ for the cleavage reaction where k_o is the pH independent rate.

Reaction in *tert*-Butyl Alcohol. The sodium salt of uridine 3′-*m*-nitrobenzyl phosphate (10 μmol) was loaded on a Dowex 50W-X8 column (1.2 × 27 cm) in the proton form. The column was eluted with distilled H_2O, and the pooled fractions were concentrated to 5 mL and titrated to pH 6.5 with 1.0 M tetrabutylammonium hydroxide in water. The solution was lyophilized and then dried over P_2O_5 in vacuo for 2 days. The tetrabutylammonium salt of uridine 3′-*m*-nitrobenzyl phosphate was dissolved in 5 mL of anhydrous *tert*-butyl alcohol in a glovebox. The boiling point of *tert*-butyl alcohol is 83 °C, and its melting point is 25 °C; thus, the reaction needed to be run with a condenser with running water at 37 °C to prevent crystallization of the alcohol on the coils of the condenser. The reaction was run at 86 °C under nitrogen for 5 days. After removal of *tert*-butyl alcohol, each time point aliquot was analyzed by HPLC as already described. The solution mixture was analyzed after 5 days by ^{31}P NMR (80% *tert*-butyl alcohol and 20% acetone-d_6).

Kinetic Isotope Effect Measurements. The kinetic isotope effects were measured using the remote label method.[11] In this method substrate labeled at both the position of interest and the remote label position is

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mixed with the substrate depleted in the heavy atom at the remote label position to reconstitute the natural abundance at the remote position. In the reactions of uridine 3′-*m*-nitrobenzyl phosphate the nitrogen atom of the nitro group is the remote label. A 4 mM solution of the appropriate substrate mixture (80 μmol) was prepared in 20 mL of 50 mM acetate buffer for reaction at pH 5.5, 50 mM of citric acid for pH 2.5, and 50 mM of CAPS for pH 10.5. Reactions run at 86 °C were stopped by cooling on ice before the isomerization reaction of the uridine 3′- and uridine 2′-*m*-nitrobenzyl phosphate reached equilibrium. Analytical HPLC was used to determine the fraction of the cyclization and isomerization reactions as already described. The solution was titrated to pH 7.0 with 1 N KOH. *m*-Nitrobenzyl alcohol, the product of the cleavage reaction, was separated by extraction of the solution three times with freshly distilled diethyl ether. The ether layers were washed once with 10 mM NaH_2PO_4, pH 4.5, and the acidic aqueous layer was back-extracted three times with diethyl ether. The combined ether layers were dried over anhydrous Mg_2SO_4 and concentrated by rotary evaporation. The residue was sublimed at 95 °C for 20 min.[9] After removal of the diethyl ether, the aqueous layer containing the 2′ and 3′ isomers was concentrated to 3 mL, filtered with 0.45 μm filters, and chromatographed on an Econosil C18 HPLC column (22 mm i.d. × 250 mm) equilibrated with 6 mM NaH_2PO_4, pH 6.8, in 15% methanol. Detection was accomplished by absorption at 254 nm. Two major peaks with retention times of 28−35 min, corresponding to the 3′ isomer, and of 13−16 min, corresponding to the 2′ isomer, eluted with a flow rate of 14 mL/min. After removal of the methanol, each peak was concentrated to ∼25 mL. Each solution was made 1 N in HCl, and the uridine 3′- and uridine 2′-*m*-nitrobenzyl phosphate were hydrolyzed to *m*-nitrobenzyl alcohol at 95 °C for 48−72 h. Hydrolysis was followed by analytical HPLC as described. When the hydrolysis was completed, the cooled solution was titrated to pH 12.5 with 1 N KOH. The *m*-nitrobenzyl alcohol was extracted and treated as described above. Control experiments were run to ensure that no fractionation occurred in the separation of the two isomers by preparative HPLC. An elemental analyzer (Carlo Erba-NA 1500 Series 2) coupled with a Europa Tracermass 20−20 isotope ratio mass spectrometer was used to analyze the isotopic composition of each sample.

Data Analysis. The isotope effects were calculated from the following equations:

$$^{18}k_{\text{cleavage}} = \ln(1 - f_c)/\ln[1 - (R_c f_c/R_o)] \quad (1)$$

$$^{18}k_{\text{isomerization}} = \ln[(1 - f_c - 2f_i)/(1 - f_c)]/\ln\{1 - [(2R_i f_i/R_o)/(1 - R_c f_c/R_o)]\} \quad (2)$$

where f_c is the fraction of the cleavage reaction, f_i is the fraction of the isomerization reaction, R_o is the isotopic ratio in the starting material, R_c is the isotopic ratio in the cleavage product *m*-nitrobenzyl alcohol, and R_i is the isotopic ratio in the isomerization product uridine 2′-*m*-nitrobenzyl phosphate. These equations were derived as described in the appendix on the assumption that the K_{eq} of the isomerization reaction is unity, as it is for uridylyl (3′,5′)uridine,[4d] and that the rates of cleavage of the 3′ isomer and the 2′ isomer are the same.[12]

The kinetic isotope effects calculated with eqs 1 and 2 were corrected for incomplete isotopic incorporation in the starting material using the remote label equations.[11]

Results

Uridine 3′-*m*-nitrobenzyl phosphate at 86 °C undergoes a reversible isomerization to uridine 2′-*m*-nitrobenzyl phosphate and a cleavage reaction with *m*-nitrobenzyl alcohol and 2′,3′-cyclic UMP as products. The 2′,3′-cyclic UMP is readily hydrolyzed to 2′-UMP and 3′-UMP. The pH rate profile of the isomerization and cleavage reaction of uridine 3′-*m*-nitrobenzyl phosphate at 86 °C is shown in Figure 2. In the acidic region the rates of isomerization and cleavage reactions are very similar. The isomerization reaction is pH-independent from pH 5.0 to 9.0. The cleavage reaction is pH-independent in a smaller range of pHs from 5.0 to 7.0. In this region the rates of isomerization and cleavage reactions are almost identical. Under alkaline conditions the cleavage reaction becomes first order with respect to hydroxide ion and the isomerization product uridine 2′-*m*-nitrobenzyl phosphate is not formed at pHs higher than 9.0.

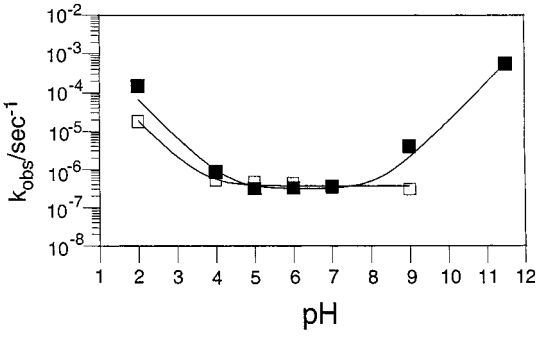

Figure 2. pH-rate profile for the isomerization (□) and cleavage (■) reactions of 3 mM uridine 3′-*m*-nitrobenzyl phosphate in 50 mM buffer at 86 °C.

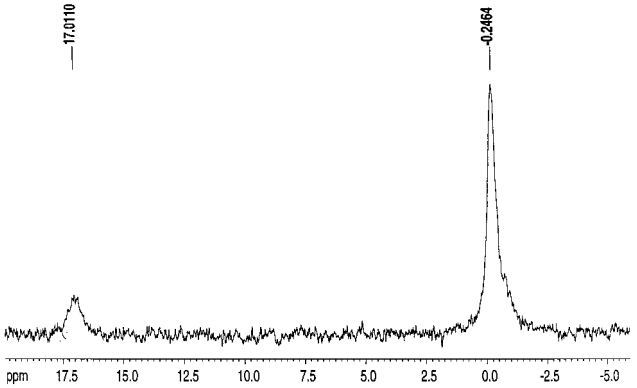

Figure 3. ^{31}P NMR spectrum of the reaction mixture of 2 mM uridine 3′-*m*-nitrobenzyl phosphate in anhydrous *tert*-butyl alcohol after 5 days at 86 °C. The ^{31}P NMR spectrum (^{1}H decoupled) was recorded at 202 MHz in 80% *tert*-butyl alcohol and 20% acetone-d_6 with 85% H_3PO_4 as an external reference.

Table 1. Kinetic Isotope Effects of the Cleavage and Isomerization Reaction of Uridine 3′-*m*-Nitrobenzyl Phosphate at 86 °C

pH	reaction	$^{18}k_{\text{bridge}}$	$^{18}k_{\text{nonbridge}}$[a]	$^{18}k_{\text{nonbridge}}$[b]
2.5	cleavage	1.0019 ± 0.0007	0.9904	0.9885
5.5	cleavage	1.009 ± 0.001	0.9986 ± 0.0004	0.9983
5.5	isomerization	1.0004 ± 0.0002	0.9990 ± 0.0007	0.9988
10.5	cleavage	1.0272 ± 0.0001		

[a] Kinetic isotope effect for ^{18}O in both nonbridge oxygen atoms. [b] Isotope effects corrected to 27 °C using the equation: ln(IE at 27 °C) = (359 K/300 K) ln(IE at 86 °C).

Kinetic isotope effects have been measured with the remote label method[11] at pH 2.5, 10.5, and 5.5 to study the transition-state structures of the hydronium- and hydroxide-dependent reactions and of the pH-independent reactions, respectively. The isotope effects are shown in Table 1 with their standard errors. The reactions at pH 5.5 were run in triplicate, while the measurements at pH 2.5 and 10.5 were run in duplicate. Although the $^{18}k_{\text{nonbridge}}$ reported at pH 2.5 has been calculated from a single measurement, the calculated R_o value from the experimental R_i, R_c, and f_c values agrees with the measured R_o.

(12) While the p*K*'s of the 2′- and 3′-hydroxyl groups differ by 0.29 pH units, this will cause little difference in the reaction rates of the 3′- and 2′-phosphodiesters because both participate as protonated species. The Brønsted value is ∼0.3 for such reactions,[13] which would cause only a ∼20% difference in the rates of cleavage of the 3′- and 2′-isomers.

Scheme 1

$$S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} X \xrightarrow{k_3} P$$

The ^{18}O isotope effects have been corrected for the isotopic incorporation and for the ^{15}k effect (1.0004 ± 0.0007).[9a,11]

The reaction in anhydrous *tert*-butyl alcohol was run at the same temperature as the aqueous reactions. Only the cleavage reaction was observed with a rate constant of 1.11×10^{-6} sec^{-1}. The presence of 2′,3′-cyclic UMP and not its hydrolytic products 2′- and 3′-UMP was shown by the existence of a peak at 17.01 ppm in the ^{31}P spectrum of the reaction mixture (Figure 3). This observation is consistent with the ^{31}P spectrum of cyclic phosphates such as ethyl ethylene phosphate which show a peak in the 15 ppm region.[14] The peak at -0.24 ppm is assigned to the tetrabutylammonium salt of uridine 3′-*m*-nitrobenzyl phosphate. The tetrabutylammonium salt of 3′-UMP in 80% *tert*-butanol and 20% acetone-d_6 shows a peak at 2.4 ppm (data not shown).

Discussion

The p*K* of the hydroxyl group of the leaving group *m*-nitrobenzyl alcohol is 14.9,[9a] close to the p*K*'s of both the 3′- and 2′-hydroxyl groups of the ribose ring which are respectively 12.84 and 12.55.[4b] The similarity in the leaving groups p*K*'s is reflected in the almost identical pH rate profiles for the cleavage and isomerization reactions of polyuridylic acid, uridylyl (3′,5′)-uridine[4b,c] and uridine 3′-*m*-nitrobenzyl phosphate. Thus uridine 3′-*m*-nitrobenzyl phosphate can be considered a good model compound to study the kinetics and mechanism of the isomerization and cleavage reactions of RNA. In addition there is only one nitrogen atom in the leaving group, *m*-nitrobenzyl alcohol, making uridine 3′-*m*-nitrobenzyl phosphate an ideal candidate to measure the kinetic isotope effects of the isomerization and cleavage reactions with the remote isotope-labeling method.[11]

The minimal mechanism for the cleavage and isomerization reactions is shown in Scheme 1, where k_3 is the irreversible step for the cleavage reaction and k_1 and k_2 represent the rates of formation from and breakdown of a phosphorane to a phosphodiester (uridine 3′- or 2′-*m*-nitrobenzyl phosphate). X, S, and P represent, respectively, all of the possible phosphorane intermediates that can be formed through pseudorotation, the sum of the 3′- and 2′-isomers, and the cleavage products. In the reaction with the bridge-labeled uridine 3′-*m*-nitrobenzyl phosphate, the only isotope-sensitive step is k_3 because no significant change in bond order occurs at the bridge oxygen in the previous steps. Therefore, the apparent ^{18}k isotope effect is given by eq 3 where $^{18}k_3$ is the intrinsic isotope effect and k_3/k_2 is the forward commitment. The forward commitment represents the propensity of the phosphorane to go forward to the cleavage products as opposed to reforming one of the phosphodiesters.

$$^{18}k_{obs} = (^{18}k_3 + k_3/k_2)/(1 + k_3/k_2) \qquad (3)$$

An approximate value of the intrinsic primary isotope effect can be calculated from this equation by assuming a reasonable value for the forward commitment as described later.

Both the formation and breakdown of the phosphorane intermediates are isotope-sensitive steps in the reaction with the nonbridge labeled uridine 3′-*m*-nitrobenzyl phosphate, and thus the observed isotope effect is described by eq 4.

$$^{18}k_{obs} = {}^{18}k_1[(^{18}k_3/{}^{18}k_2 + k_3/k_2)/(1 + k_3/k_2)] \qquad (4)$$

This equation is simplified to $^{18}k_{obs} = {}^{18}k_1$ by assuming that $^{18}k_3$ and $^{18}k_2$ are the same due to the similarity of the chemical steps and of the p*K*'s of the leaving groups. Thus, the observed isotopic discrimination is determined by the isotope effect on the rate of formation of the phosphorane intermediate. This secondary kinetic isotope effect gives information on the transition-state structure for the formation of the phosphorane intermediate.

Hydronium Ion-Catalyzed Reactions.[15] The primary and secondary ^{18}O isotope effects were measured for the cleavage reaction at pH 2.5 and at 86 °C. The primary isotope effect represents the loss of bond order with the *m*-nitrobenzyl group during the breakdown of the phosphorane intermediate. The magnitude of the observed isotope effect is decreased by the presence of the forward commitment (eq 3). The observed primary isotope effect is 0.19%.[16] Calculation of the intrinsic primary isotope effect requires assumption of a value for the forward commitment in eq 3. The neutral phosphorane intermediate can break down in three ways to give cleavage to *m*-nitrobenzyl alcohol and a 2′,3′-cyclic phosphate, or to the 2′- or 3′-isomers of uridine *m*-nitrobenzyl monophosphate. If these rates are equal because of the similarity of p*K*'s of the leaving groups,[12] the forward commitment would be 0.5, and the intrinsic primary isotope effect at 86 °C is 1.0028. The isomerization reactions produce single products, while the cleavage reactions produce two products but one of these is a less stable cyclic phosphate. The pK_a of the *m*-nitrobenzyl alcohol is also 2 pH units greater than those of the 2′- and 3′-hydroxyl groups, which might suggest a faster rate of cleavage than that of isomerization if the proton shift to the leaving group determines the rate. The balance between these factors is not clear, and thus we can only say that the primary ^{18}O isotope effect on cleavage is greater than 0.19% at 86 °C. The small normal intrinsic primary isotope effect results from the combination of two opposite effects, the loss of the P−O stretch and the formation of an O−H bond. If protonation of the leaving group is so far advanced that it largely compensates for the loss of the P−O stretch, a slightly normal primary isotope effect would be expected.

The inverse secondary ^{18}O isotope effect of 1.15% (corrected to 27 °C) indicates an increase in bond strength with the

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(15) An inverse secondary isotope effect of ~1.5% can be expected from the protonation of the second nonbridge oxygen when forming a neutral phosphorane from a monoanionic phosphorane (Scheme 2) based on secondary isotope effects of 1.54 and of 1.9%, respectively, measured for the deprotonation of glycerol-3-phosphate and of phosphoric acid (see ref 17). If we assume that the strength of a P=O and a P−OH bond are similar, based on the ^{18}O fractionation factors of 1.033 for the hydroxyl of L-malate and of 1.031 for the carbonyl group of ketoglutarate (see ref 18), then the secondary isotope effect for the reaction from an anionic phosphodiester to a monoanionic phosphorane can be predicted to be close to unity and that for forming a neutral phosphorane should be ~1.5% inverse. A second model uses the normal secondary isotope effect of 2.5% measured for the base-catalyzed hydrolysis of the triester diethyl(4-carbamoylphenyl) phosphate (see ref 19) to determine the change from a P=O to a P−O$^-$ bond. With this model a secondary isotope effect of ~0.7% inverse is predicted in the formation of a neutral phosphorane and of ~0.9% normal for the formation of a monoanionic phosphorane from an anionic phosphodiester. It is not clear which model is more correct, but the isotope effects we observed are closer to those predicted by the first model.

(16) The value of the intrinsic primary isotope effect calculated at 86 °C cannot be corrected to room temperature without knowing how much of the value is temperature-independent, but it cannot be larger than 1.0033 at 27 °C.

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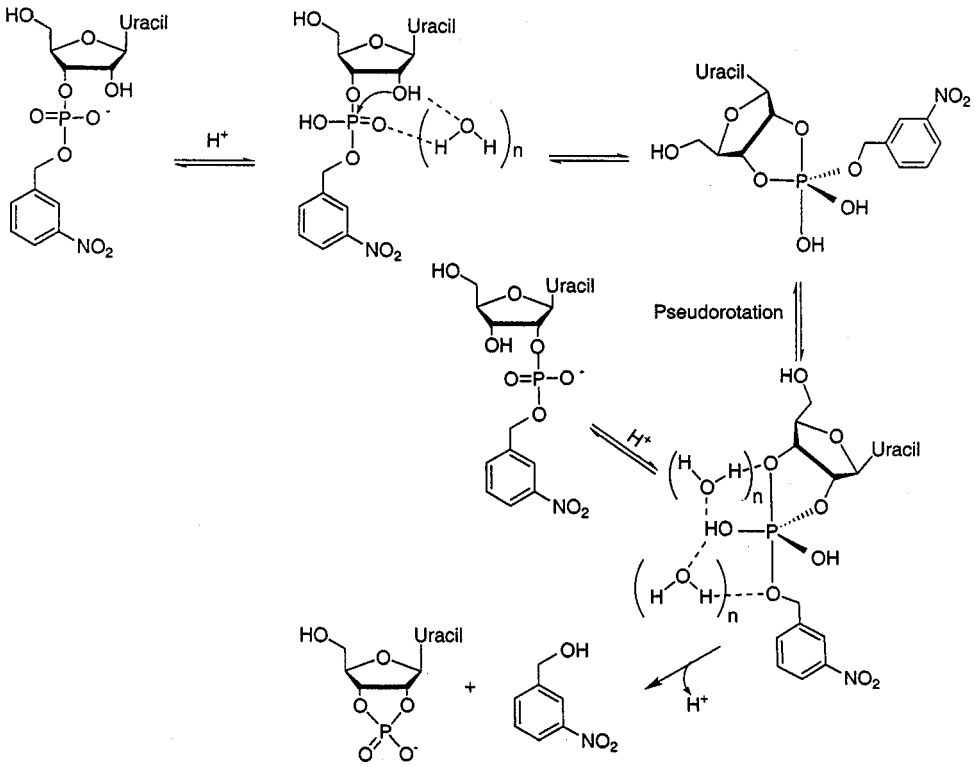
Scheme 2



nonbridge oxygens in the formation of the phosphorane intermediate. The inverse effect is due to the formation of a neutral phosphorane which has an additional O−H stretch and bending motions with respect to the monoanionic phosphodiesters. Since the ^{18}O equilibrium isotope effect for protonation of a phosphate nonbridge oxygen is 1.6% inverse,[17] the magnitude of the secondary ^{18}O isotope effect represents a somewhat late transition state, which is not surprising, considering the relative instability of the phosphorane intermediate.

The primary and secondary kinetic isotope effects observed are consistent with the mechanism described in Scheme 2. An initial preequilibrium protonation step is followed by the formation of a neutral phosphorane intermediate with a concomitant water-mediated proton transfer from the 2′-hydroxyl group to the second nonbridge oxygen. Models suggest the number of water molecules needed to mediate this proton transfer is two or three. After the nucleophile 2′-hydroxyl attack at an apical position, the phosphorane intermediate formed is in fast equilibrium with all of the possible phosphoranes formed by polytopal rearrangements. The *m*-nitrobenzyl alcohol departs from a phosphorane in which it is located in the apical position. The isotope effects measured also agree with a mechanism of the cleavage reaction where the neutral phosphorane formed with both the *m*-nitrobenzyl group and the 2′-hydroxyl in apical positions. This phosphorane may give the cleavage products without pseudorotating.

Westheimer's rules dictate that both the leaving group and the nucleophile must respectively depart or enter in the apical position of a phosphorane.[1] It is thus impossible for the 2′-hydroxyl to attack in a concerted manner with the departure of the 3′-hydroxyl of the ribose moiety. Thus, the isomerization of uridine 3′-*m*-nitrobenzyl phosphate to uridine 2′-*m*-nitrobenzyl phosphate must occur through a phosphorane intermediate. Since the rates of isomerization and cleavage of uridine 3′-*m*-nitrobenzyl phosphate are similarly dependent on the hydronium ion concentration (Figure 2) and the cleavage reaction proceeds through a neutral phosphorane intermediate, it is likely that the isomerization reaction in acidic pHs proceeds through the same neutral phosphorane intermediate which after pseudorotation may undergo cleavage of the apical bond with the *m*-nitrobenzyl moiety (cleavage reaction) or of the apical bond with the 3′-hydroxyl of the ribose ring (isomerization reaction) as shown in Scheme 2.

pH-Independent Reactions.[15] The isomerization reaction must proceed through a phosphorane intermediate, while the cleavage reaction may be either concerted or stepwise. The stepwise mechanisms for the isomerization and cleavage reactions are shown in Scheme 3. The putative phosphorane may exist in several ionization states. At acidic pH an inverse secondary isotope effect was observed, indicating the formation of a neutral phosphorane. A monoanionic phosphorane intermediate is proposed for the isomerization reaction at neutral pH based on the secondary isotope effect close to unity and the pH-independent rate (Figure 2). A secondary isotope effect close to unity was also measured for the cleavage reaction, indicating that the two reactions may proceed through the same monoanionic phosphorane intermediate as illustrated in Scheme 3. In the breakdown of the phosphorane intermediate to give the 2′-isomer, the bond order with the bridge oxygen should remain essentially unchanged, which is consistent with the observed bridge oxygen isotope effect close to unity. In the cleavage reaction the bond between the bridge oxygen and the phosphorus is broken in the degradation of the phosphorane intermediate. The observed primary ^{18}O isotope effect is 0.9%. Calculation of the intrinsic primary isotope effect requires assumption of a value for the forward commitment in eq 3. If we assume a forward commitment of 0.5, as we did earlier for the hydronium-catalyzed reaction, the intrinsic primary isotope effect is 1.35%. The same uncertainties in the forward commitment value apply here, and thus we can only say that the primary ^{18}O isotope

(19) Caldwell, S. R.; Raushel, F. M.; Weiss, P. M.; Cleland, W. W. *J. Am. Chem. Soc.* **1991**, *30*, 7444−7450.

Scheme 3

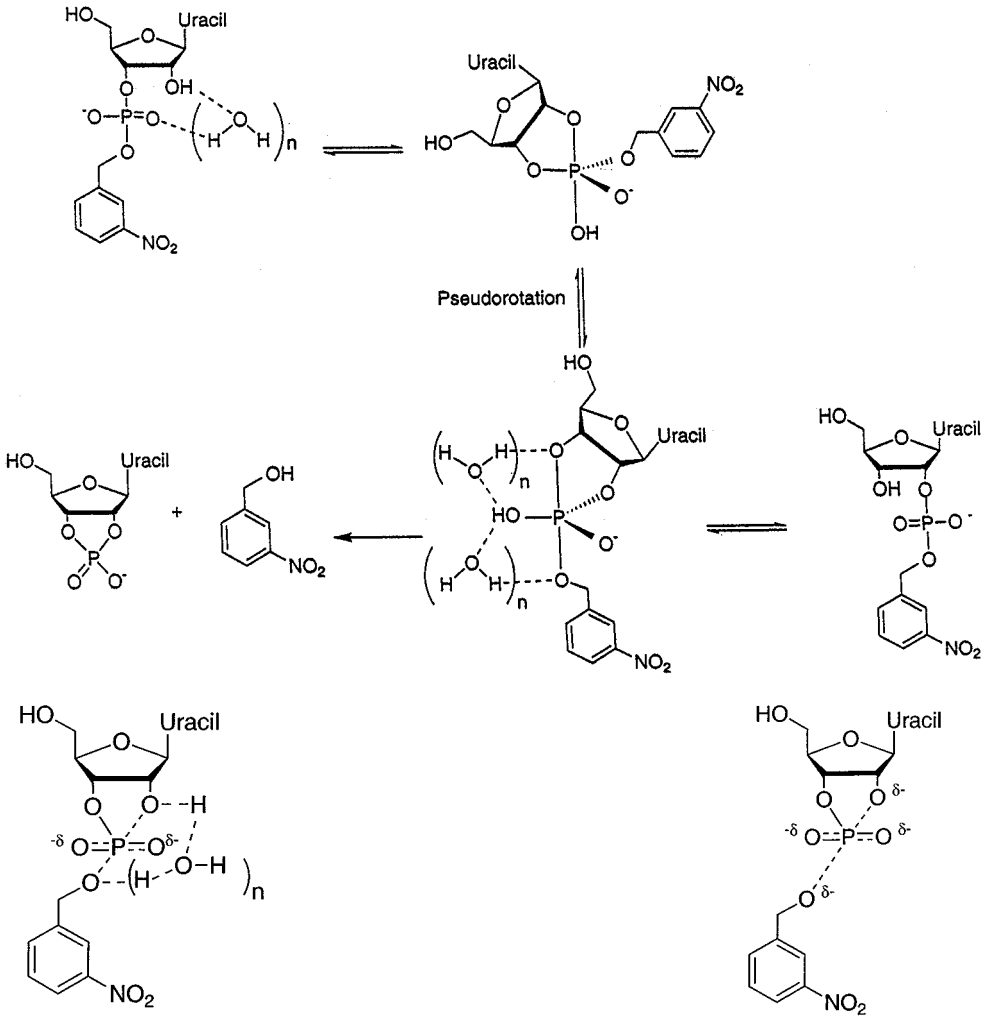


Figure 4. Proposed transition-state structure for the concerted cleavage reaction at neutral pH.

Figure 5. Proposed transition-state structure for the concerted cleavage reaction in alkaline conditions.

effect on cleavage is greater than 0.9%. The size of the primary effect indicates that the bond between the phosphorus and the bridge oxygen is considerably cleaved in the transition state. Thus, the secondary and primary isotope effects measured for the cleavage reaction are consistent with a stepwise mechanism with a monoanionic phosphorane intermediate. As for the reaction in acidic conditions, a water-mediated proton transfer from the 2′-hydroxyl group to the second nonbridge oxygen is required for the formation of the phosphorane intermediate as shown by the absence of the isomerization reaction in anhydrous conditions in *tert*-butanol.

The isotope effects observed for the cleavage reaction do not invalidate an A_ND_N mechanism with a transition state resembling a phosphorane (Figure 4) in which the bond with the *m*-nitrobenzyl group has been weakened and the strength of the bonds between the phosphorus and the nonbridge oxygens has not changed significantly from the ground state. This transition-state structure differs from the structure of the transition state for the ribonuclease A reaction in which the secondary isotope effect of 0.5% and the primary isotope effect of 1.6% were consistent with a concerted mechanism with a slightly associative transition state.[9a]

Reactions in Anhydrous *tert*-Butanol. The need for water catalysis of the pH-independent isomerization and cleavage reactions was tested by running the reaction in anhydrous *tert*-butanol. The isomerization, which must proceed through a phosphorane intermediate, did not occur in anhydrous conditions. (In aqueous solution at pH 9.0, the isomerization was readily detected under conditions where the cleavage reaction was three times faster than in *tert*-butyl alcohol.) This result proves the absolute requirement of a water-mediated proton transfer for the formation of a phosphorane intermediate. The cleavage reaction was observed in anhydrous conditions with a rate 3-fold faster than the cleavage rate at pH 6.0. We believe that a small level of base present in the solution catalyzed the cleavage reaction. This base-catalyzed cleavage is an A_ND_N reaction and does not proceed through a phosphorane intermediate.

Hydroxide Ion-Catalyzed Reactions. The transition-state structure for the alkaline hydrolysis of uridine 3′-*m*-nitrobenzyl phosphate is shown in Figure 5. The primary isotope effect of 2.72% suggests that the $P-O_{bridge}$ bond is largely broken in the transition state and that the *m*-nitrobenzyl group mainly leaves as an alkoxide. A mechanism with a phosphorane intermediate is unlikely based on ab initio calculations suggesting that a dianionic phosphorane is too unstable to exist as an intermediate[8] and considering that the isomerization reaction which requires a phosphorane intermediate is not observed. The hydroxide ion-catalyzed cleavage reaction is thus a concerted S_N2 reaction.

Conclusions

The kinetic isotope effects measured at pH 2.5 and the pH rate profile in the acidic range support a mechanism involving a neutral phosphorane intermediate for the isomerization and cleavage reactions of uridine 3′-*m*-nitrobenzyl phosphate. Both a concerted transition state with very little change in bond order with the nonbridge oxygens and a stepwise mechanism employing a monoanionic phosphorane intermediate are consistent with the reported isotope effects for the pH-independent cleavage reaction. The pH-independent isomerization reaction proceeds through the monoanionic phosphorane. We have shown the absolute requirement of water molecules to mediate a concerted proton transfer for the formation of the phosphorane intermediate by running the reaction in anhydrous *tert*-butyl alcohol. The alkaline hydrolysis of uridine 3′-*m*-nitrobenzyl phosphate is a concerted reaction with a transition state in which the leaving group departs with almost a full negative charge.

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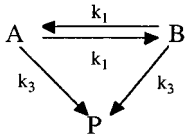
Appendix

The scheme for reaction of uridine 3′-*m*-nitrobenzyl monophosphate (A) to give the 2′-isomer (B) or to give the cleavage products *m*-nitrobenzyl alcohol and 2′,3′-cyclic UMP (P) can be diagrammed:



When the differential equations for this scheme are solved, the concentrations of *A*, *B*, and *C* are given by:

$$A = (A_o/2)(e^{-(2k_1+k_3)t} + e^{-k_3t}) \quad (5)$$

$$B = (A_o/2)(e^{-k_3t} - e^{-(2k_1+k_3)t}) \quad (6)$$

$$P = A_o(1 - e^{-k_3t}) \quad (7)$$

To derive the isotope effects for cleavage (k_3) and isomerization (k_1), one first writes a similar set of equations for the ^{18}O-labeled species, adding an asterisk to *A*, *B*, *P*, and A_o to indicate the label, and dividing each rate constant by the isotope effect on this rate constant. Thus k_1 is divided by $^{18}k_1$ and k_3 by $^{18}k_3$. The mass ratio in the initial 3′-isomer is then:

$$R_o = A_o^*/A_o$$

while the mass ratio in P at a fraction of cleavage, f_c, is:

$$R_c = P^*/P$$

The ratio of R_c/R_o is then:

$$R_c/R_o = (1 - e^{-k_3t/^{18}k_3})/(1 - e^{-k_3t}) \quad (8)$$

The fraction of cleavage is given by:

$$f_c = P/A_o = (1 - e^{-k_3t})$$

and:

$$(1 - f_c) = -e^{-k_3t}$$

so:

$$\ln(1 - f_c) = -k_3t$$

Replacing $-k_3t$ in eq 8:

$$R_c/R_o = (1 - e^{-k_3t/^{18}k_3})/f_c$$

or:

$$1 - (R_c f_c/R_o) = e^{-k_3t/^{18}k_3}$$

Then:

$$\ln[1 - (R_c f_c/R_o)] = -k_3t/^{18}k_3$$

and, replacing $-k_3t$

$$^{18}k_3 = \ln(1 - f_c)/(\ln[1 - (R_c f_c/R_o)]) \quad (1)$$

For the isotope effect on isomerization, we define the mass ratio in B as:

$$R_i = B^*/B$$

and the fraction of isomerization as:

$$f_i = B/A_o = (1/2)(e^{-k_3t} - e^{-(2k_1+k_3)t})$$

Then:

$$R_i/R_o = (1/2)(e^{-k_3t/^{18}k_3} - e^{-[(2k_1/^{18}k_1)+(k_3/^{18}k_3)]t})$$

and:

$$R_i f_i/R_o = (1/2)[e^{-k_3t/^{18}k_3} - (e^{-2k_1t/^{18}k_1})(e^{-k_3t/^{18}k_3})] = (1/2)(1 - R_c f_c/R_o)(1 - e^{-2k_1t/^{18}k_1})$$

Rearranging and taking logs:

$$^{18}k_1 = -2k_1t/\ln[1 - (2R_i f_i/R_o)/[1 - (R_c f_c/R_o)]] \quad (9)$$

Rearranging the equation for f_i and substituting for e^{-k_3t} gives:

$$e^{-2k_1t} = (1 - f_c - 2f_i)/(1 - f_c)$$

Taking logs and replacing $-2k_1t$ in eq 9:

$$^{18}k_1 = \ln[(1 - f_c - 2f_i)/(1 - f_c)]/\ln\{1 - [(2R_i f_i/R_o)/[1 - (R_c f_c/R_o)]]\} \quad (2)$$

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