VIP RNA Cleavage

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The Mechanism of RNA Strand Scission: An Experimental Measure of the Brønsted Coefficient, β_{nuc}**

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Ribonucleic acids play essential informational, structural, catalytic, and regulatory roles in living systems.[1] A central facet of RNA biology emanates from the capacity of the ribose phosphate backbone to undergo enzymatic and non-enzymatic cleavage through internal transphosphorylation to yield products bearing 2′,3′-cyclic phosphate and 5′-hydroxy termini.[2] Interest in this cyclizing reaction, first established in the 1950s, has generated an enormous body of literature[3] pertaining to the pathway, chemical mechanism, and catalytic agents (ribozymes, protein enzymes, and small molecules) for the reaction. Attempts to develop a physical organic description of the reaction have relied primarily on modifications of the 5′-oxygen leaving group[3b,d–f] and the nonbridging phosphorus oxygen atoms.[3h] In contrast, little information exists about the nature of the bond-making process to the 2′-oxygen nucleophile. To address this fundamental aspect of RNA cleavage, we incorporated into oligonucleotides a series of nucleoside analogues bearing 2′-*C*-β-branched substituents with increasing degree of fluorine substitution (Figure 1).[4] Analysis of the cleavage rates under alkaline conditions shows that the substituents perturb the pK_a value and nucleophilicity of the ribose 2′-hydroxy group in a systematic manner, which allows an experimental measure of the Brønsted coefficient, β_{nuc}, for the classic RNA strand scission reaction.

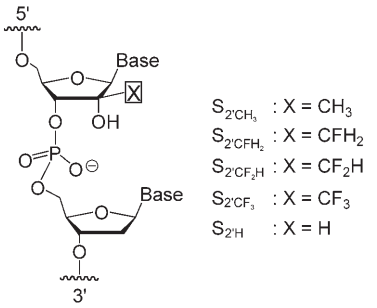

Figure 1. Chimeric oligonucleotides bearing modifications at the 2′-β-position. X indicates the substituents at the modification site in the chimeric RNA. Sequence: 5′-d(CTGTCACCGAAA)$U_{2'X}$d(ACACGCAAGATG)-3′.

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To simplify the kinetic analysis of ribose phosphate cleavage, we adopted the approach of Li and Breaker and incorporated the modified ribonucleotides into the middle of a DNA strand.[5a] The chimeric DNA/RNA oligonucleotide serves as an excellent model substrate to study RNA strand scission.[5] To minimize possible complications with the cleavage kinetics, we chose a sequence (Figure 1) with no apparent propensity for intramolecular or intermolecular interactions (as predicted by MFOLD[6]). Because of difficulties with phosphoramidite synthesis, we developed a reliable enzymatic method to incorporate the 2′-*C*-β-branched analogues into chimeric oligonucleotides using two successive enzymatic ligations (see the Supporting Information).[4c,7] The final products (Figure 1) were ^{32}P-labeled at the 5′ terminus and exhibited the same electrophoretic mobility as the corresponding synthetic 25-mer, $S_{2'H}$, which contains only natural nucleotides.

Li and Breaker characterized the cleavage of an unmodified chimeric RNA over a range of alkaline pH values.[5a] To allow comparison with that study, we monitored the cleavage rates of our oligonucleotides under analogous conditions at 23 °C with similar buffer composition (Supporting Information) and ionic strength (maintained with KCl, $[K^+] = 3.16$ M). Like the parent oligonucleotide, $S_{2'H}$, the modified chimeric DNA/RNA substrates undergo specific base-catalyzed cleavage at the ribonucleotide linkage. We determined the cleavage rate constants at different pH values ranging between 9.0 and 14.5 (Figure 2). For each substrate, cleavage rates increase log-linearly with unit slopes in the low pH range and become pH-independent under more alkaline conditions. These pH dependencies suggest that the modified substrates react by the same pathway as natural RNA: internal transphosphorylation whereby the 2′-oxyanion nucleophilically attacks the adjacent phosphodiester linkage (Scheme 1).

The pH–rate profile for $S_{2'H}$ gives an apparent pK_a value[8a] of 13.8 ± 0.1, similar to that obtained by Li and Breaker (13.1).[5a] For the modified oligonucleotides,[8b] $S_{2'CH_3}$, $S_{2'CFH_2}$, $S_{2'CF_2H}$, and $S_{2'CF_3}$, the pH–rate profiles give apparent pK_a values of 14.3 ± 0.3, 13.2 ± 0.1, 12.2 ± 0.1, and 10.9 ± 0.1, respectively. Previous reports attributed the apparent pK_a value derived from the pH–rate profile to the ionization of the 2′-hydroxy group.[5a,9] Our results for the modified substrates strongly support this interpretation, as the apparent pK_a values decrease with increasing fluorine substitution (and therefore electron-withdrawing power) in accord with our experimental design. The observed pK_a values span a range greater than three units and encompass those of the 2′-

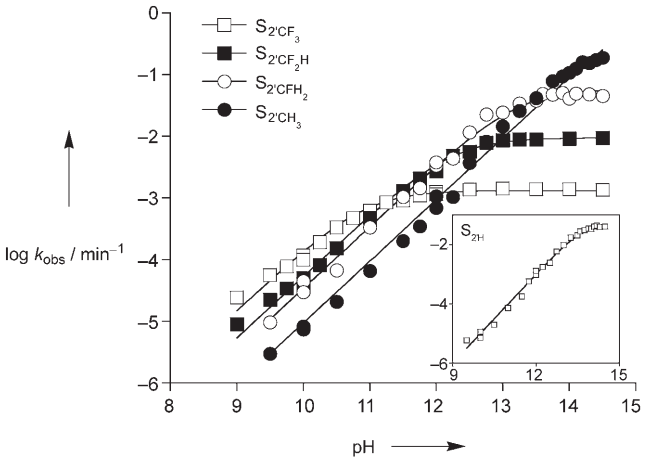


Figure 2. The pH dependence of the ribonucleotide cleavage for the chimeric oligonucleotides. Plots were fit to the equation: $\log k_{obs} = \log k_{max} - \log(1+10^{pK_a-pH})$. Inset: For the wild-type substrate ($S_{2'H}$), values of pK_a and k_{max} were obtained as 13.77 and 0.057 min^{-1}, respectively, which are comparable to the reported values (13.1 and 0.04 min^{-1}) for the cleavage of a UpG linkage in the chimeric oligonucleotide.[5a]

hydroxy groups for the natural nucleotides.[5a] We also determined pK_a values of the 2′-hydroxy groups for the free nucleosides by monitoring the appropriate NMR chemical shift versus pH (Table S1 in the Supporting Information). These NMR-derived pK_a values are consistent with those obtained from the kinetic profiles in Figure 2.

In the plateau region of the pH–rate profiles, in which the 2′-hydroxy group of the substrate predominantly populates the deprotonated, oxyanion state, the apparent first-order rate constant k_{max} decreases with increasing fluorine substitution. Here, the fluorine substitution is closely related to the electron-withdrawing ability of the β substituent, causing a shift in the pK_a value of the 2′-hydroxy group. We plotted $\log k_{max}$ against the apparent pK_a value of the corresponding 2′-hydroxy group (Figure 3).[8b] The resulting Brønsted-type plot gives a good linear correlation, $\log k_{max} = 0.75 \times pK_a - 10.85$ ($n = 4$; $r = 0.99$) with slope $\beta_{nuc}^{app} = 0.75 \pm 0.15$.

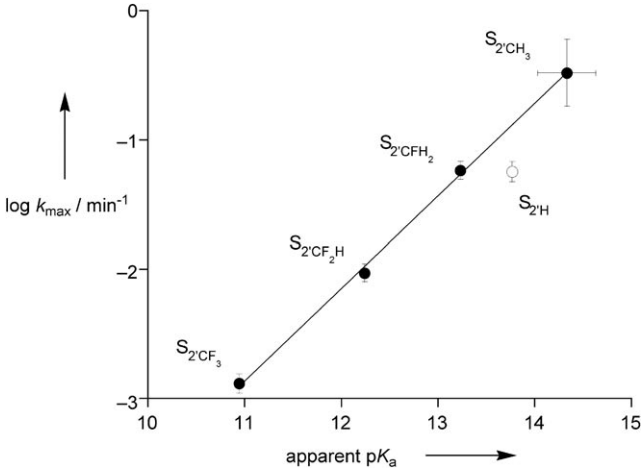
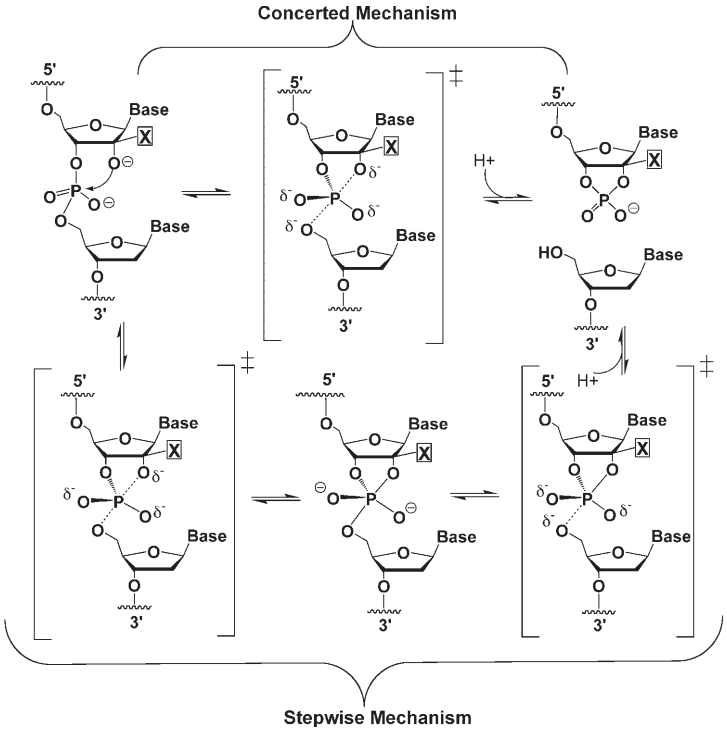


Figure 3. Brønsted plot ($\log k_{max}$ versus pK_a of the nucleophile) for 2′-*O*-transphosphorylation. The plot gives the Brønsted coefficient β_{nuc}^{app} as 0.75 ± 0.15.



Scheme 1. Possible mechanisms of RNA strand scission under alkaline conditions. Both pathways proceed from the 2′-oxyanion.

The Brønsted coefficient β_{nuc} provides a measure of the change in effective charge on the nucleophile en route to the transition state. Several factors could obscure the β_{nuc}^{app} value as a true measure of the effective charge change. First, the decrease in reactivity with increasing fluorine substitution could reflect the concomitant increase in substituent volume rather than the reduction of electron density on the hydroxy group. However, $S_{2'CFH_2}$ reacts as fast as $S_{2'H}$ in accord with the pK_a values of the 2′-hydroxy groups at the reaction site, despite more than 20 Å^3 difference in the solvent-excluded volume of the fluoromethyl group compared to a hydrogen atom (Figure S2B in the Supporting Information). As an additional test for volume effects, we synthesized the $S_{2'CH_2OCH_3}$ substrate bearing a CH_2OCH_3 substituent at the 2′-β-position and examined the pH dependence of base-catalyzed cleavage. This substituent has a larger volume than other substituents, but $S_{2'CH_2OCH_3}$ reacts in accord with its 2′-OH pK_a value (Figure S2 in the Supporting Information). These observations provide no evidence that the β_{nuc}^{app} value reflects changes in substituent volume.

Solvation of the nucleophile also can obscure the value of β_{nuc} as a measure of bonding in the transition state. Alkoxide ions frequently react with smaller β_{nuc} values than do phenoxide ions because the former must overcome a more significant energetic penalty

for partial desolvation before nucleophilic attack can occur.[10] Desolvation attenuates the value of β_{nuc}, particularly for reactions in which little bonding to the nucleophile has occurred in the rate-limiting transition state.[10] However, we expect that the β_{nuc}^{app} value observed here requires little correction for desolvation for the following reasons. First, tertiary alkoxide ions such as those studied herein experience weaker solvation than do primary alkoxide ions.[11] Second, a large β_{lg} (-1.28)[3f] and ^{18}O primary kinetic isotope effect (1.027)[12] indicate a significant degree of bond breaking to the leaving group (lg) in the transition state. This and the value of β_{nuc}^{app} itself imply a late transition state with significant bonding to the nucleophile (see below), conditions under which β_{nuc} requires little correction for solvation.[10b]

The value of β_{nuc}^{app} agrees with estimates from three other observations. 1) The estimated 10^{10}-fold difference[13] in reactivity for transphosphorylation to the neutral 2′-hydroxy group versus the 2′-oxyanion gives $\beta_{nuc} = 0.59$.[14] 2) Herschlag and Jencks[16] estimated the β_{nuc} value as 0.53 for the reaction of oxygen nucleophiles with 2,4-dinitrophenyl phosphate monoanion (2,4-dinitrophenol, $pK_a = 4.1$). Using $\beta_{nuc} = 0.53$ and the interaction coefficient $p_{xy} = 0.016$,[15] which reflects how β_{nuc} changes as a function of the pK_a value of the leaving group, we estimate that $\beta_{nuc} = 0.72$ for the reaction of oxygen nucleophiles with phosphate diesters bearing alkoxide leaving groups ($pK_a \approx 16$), within the error of our experimentally obtained value. 3) A relatively large value of β_{nuc} for intramolecular transphosphorylation is not unprecedented. Dalby et al[16] showed that $\beta_{nuc} = 0.95$ for phenolate-mediated intramolecular displacement of methoxide from a phosphate diester. These observations support β_{nuc}^{app} as a reliable measure of the change in effective charge on the 2′-oxyanion as the reaction progresses from the ground state to the transition state.[17] Thus, the β_{nuc} value of 0.75 suggests that the 2′-oxygen bears an effective charge of -0.25 in the transition state of the rate-limiting step, implying significant bonding between the phosphorus center and the 2′-oxygen. The leaving group undergoes an even more significant change in effective charge ($\beta_{lg} = -1.28$),[3f] though the extent to which this reflects bond breaking depends on solvation of the developing oxyanion in the transition state. Jencks showed that for acyl-transfer reactions, β_{lg} for alkoxide anion leaving groups requires a correction of approximately 0.5 to reflect accurately the bonding in the transition state.[11b]

Knowledge of the changes in effective charge on the nucleophile and leaving group for the overall reaction β_{eq}^{nuc} and β_{eq}^{lg}, respectively, would allow calibration of β_{nuc} and β_{lg}, such that the ratios $\beta_{nuc}/\beta_{eq}^{nuc}$ and $\beta_{lg}/\beta_{eq}^{lg}$ give measures of bonding to the nucleophile and leaving group in the transition state (eq: equilibrium). β_{eq} values for RNA strand scission remain unknown, but we may estimate these using data reported in the literature. To obtain a crude measure of the extent of bonding in the transition state, we use the value of 1.56 for both β_{eq}^{nuc} and β_{eq}^{lg} to calibrate β_{nuc} and β_{lg} (Supporting Information). Using the calibrated values, we constructed an effective charge map (Figure 4), which suggests that in the transition state the nucleophile has undergone about half of the overall effective charge change and the leaving group has undergone about half to four-fifths of its overall effective charge change (depending on whether the reported value of β_{lg} requires correction for solvation). Thus, in the rate-limiting transition state, bond breaking has progressed at least as far as bond making and little or no negative effective charge has built up on the phosphoryl group.

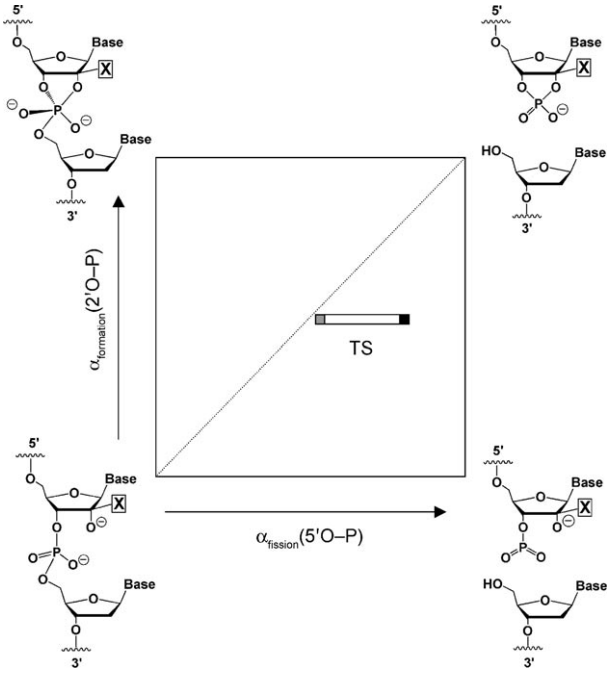


Figure 4. More–O'Ferrall–Jencks diagram showing the relative degrees of 2′-O—P bond formation and 5′-O—P bond fission at the transition state (TS). The diagram illustrates a range of $\alpha_{fission}$ values, reflecting the possibility that β_{lg} requires correction for solvation. The black square represents no solvation correction. The open rectangle represents a range of solvation correction to β_{lg} from 0 to 0.5 (gray square).

In summary, the incorporation of nucleotide analogues modified with the CH_3, CFH_2, CF_2H, and CF_3 series of substituents at the 2′-β-position into oligonucleotides enables systematic variation of the pK_a and nucleophilicity of the 2′-hydroxy group. These nucleotide analogues permit the use of physical organic approaches to investigate bonding to the nucleophile in the classic RNA strand scission reaction. Brønsted analysis of the internal 2′-*O*-transphosphorylation reaction using these nucleotide analogues gives $\beta_{nuc}^{app} = 0.75 \pm 0.15$. This measurement advances our understanding of the transition-state structure of this biologically important reaction and may help to provide insight into the function of enzymes and ribozymes that catalyze it.

Experimental Section

For typical assays, 19.5 μL of reaction buffer at appropriate pH (between 9.0 and 14.5) was added to 0.5 μL of 5′-^{32}P-labeled substrates (final concentration around 5 nM) and incubated at 23 °C in a circulating water bath. Reaction aliquots were removed at various time points, quenched with aqueous HCl, and loaded on 20% denaturing polyacrylamide gel. The radioactive gel was dried and

visualized by phosphor imaging, and the reaction yield was quantified using ImageQuant software (Molecular Dynamics).

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[14] Using the measured pK_a value for the 2′-hydroxy group and the pK_a of hydronium ion as an estimate for the pK_a of the 2′-oxonium ion, we estimate β_{nuc} as 0.59. This value likely underestimates β_{nuc} for two reasons: 1) the neutral hydroxy group would experience less electrostatic repulsion with the reaction center than the oxyanion; 2) the nucleotidyl context of the hydroxy group likely decreases the pK_a value of the 2′-oxonium ion relative to a hydronium ion. For example, ammonium ion has a pK_a value of 10, but the 2′-nucleotidyl ammonium ion ionizes with p$K_a \approx 6.2$.[18]

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[17] Sequence and structure can influence the acidity of the 2′-hydroxy group, underscoring the need for caution in extrapolating these results to other contexts. Nevertheless, recent nucleobase pK_a measurements[19] suggest that within unstructured RNAs, sequence context exerts only small effects on acidity (≈ 0.2 pK_a units). These context effects likely would produce similar pK_a shifts for all analogues in the series, resulting in only minimal effects on β_{nuc}.

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