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Evidence That Nucleophile Deprotonation Exceeds Bond Formation in the HDV Ribozyme Transition State

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Abstract

Steric constraints imposed by the active sites of protein and RNA enzymes pose major challenges to the investigation of structure—function relationships within these systems. As a strategy to circumvent such constraints in the HDV ribozyme, we have synthesized phosphoramidites from propanediol derivatives and incorporated them at the 5'-termini of RNA and DNA oligonucleotides to generate a series of novel substrates with nucleophiles perturbed electronically through geminal fluorination. In nonenzymatic, hydroxide-catalyzed intramolecular transphosphorylation of the DNA substrates, pH—rate profiles revealed that fluorine substitution reduces the maximal rate and the kinetic pK_a , consistent with the expected electron-withdrawing effect. In HDV ribozyme reactions, we observed that the RNA substrates undergo transphosphorylation relatively efficiently, suggesting that the conformational constraints imposed by a ribofuranose ring are not strictly required for ribozyme catalysis. In contrast to the nonenzymatic reactions, however, substrate fluorination modestly increases the ribozyme reaction rate, consistent with a mechanism in which (1) the 2'-hydroxyl nucleophile exists predominantly in its neutral, protonated form in the ground state and (2) the 2'-hydroxyl bears some negative charge in the rate-determining step, consistent with a transition state in which the extent of 2'-OH deprotonation exceeds the extent of P—O bond formation.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

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The hepatitis delta virus (HDV) ribozyme serves as an outstanding model system for foundational studies of RNA catalysis because of its small size,¹ the availability of high-resolution crystal structures,^{2–4} and the wealth of biochemical and kinetic data.⁵ The RNA genome of the hepatitis delta virus encodes two forms of the HDV ribozyme, genomic and antigenomic, which mediate an essential step of viral replication by *cis* cleavage.⁶ Both forms of the ribozyme adopt a similar overall structure, are likely to employ similar catalytic mechanisms, and can be divided into *trans*-cleaving constructs through separation of the 5'-terminus from the rest of the ribozyme. Like the other small endonucleolytic ribozymes, the HDV ribozyme facilitates cleavage by catalyzing the nucleophilic attack of the 2'-hydroxyl on the adjacent phosphate to form a 2',3'-cyclic phosphate and 5'-hydroxyl termini (Figure 1A).

A significant body of functional,^{7–10} spectroscopic,^{11,12} and crystallographic^{2–4} data support the role of C76 in the antigenomic form as a general acid that stabilizes the leaving group through proton transfer. Perhaps the strongest evidence was generated through observation of C76 mutants with substrates containing a 5'-bridging phosphorothioate at the cleavage site.⁹ In the 5'-phosphorothioate background, activation of the leaving group is not required for cleavage because of the highly labile thiolate leaving group; therefore, the insensitivity of the phosphorothioate to C76 mutation provides evidence of leaving group activation by the mutated group. Subsequently, similar experiments have been employed to interrogate leaving group activation in other ribozymes.^{13–15} However, an analogous experimental approach to investigate nucleophile activation has remained elusive because no chemical system with a preactivated nucleophile has been devised.

Consequently, nucleophilic activation arguably represents the least understood feature of ribozyme (and enzyme) catalysis.¹⁶ Phosphoryl transfer to oxygen nucleophiles shows a strong dependence on the nucleophile pK_a ,^{17–19} which suggests that reactivity is highly sensitive to the protonation state of the nucleophile. Ground state interactions with metal ions, hydrogen bond donors, or solvent can also influence reactivity.²⁰ In the HDV ribozyme, the mechanism of nucleophile activation remains poorly understood. However, crystallographic³ and biochemical^{21,22} data are consistent with a well-positioned magnesium

ion with inner-sphere coordination to the pro- R_p oxygen of the scissile phosphate, which could play a role in nucleophile activation⁷

The features of nucleophile activation are profoundly significant for understanding catalysis, yet limited strategies for probing nucleophile function exist. Analogous to substrates with leaving group modifications, a series of substrates that perturb the nucleophilic 2'-hydroxyl group might provide a complementary strategy for investigating the catalytic mechanism, e.g., identifying charge development on the nucleophilic oxygen through the transition state, estimating the degree of bonding to the nucleophile in the transition state, and identifying possible partners for interaction with the nucleophile. In particular, we aimed to develop substrates that differ in nucleophilicity and basicity from the hydroxyl group in the native ribozyme and uncover ribozyme modifications whose effects on catalysis are suppressed or enhanced in the presence of these mutant substrates. Optimally, these substrates should maintain the 2'-hydroxyl group but modulate its basicity via inductive effects.

A series of oligonucleotides containing modifications (R being CH₃, CH₂F, CHF₂, or CF₃, and the wild type [H]) at the 2'- β -position of the ribose ring were developed previously in our laboratory that perturb the pK_a and nucleophilicity of the 2'-hydroxyl group;¹⁸ Brønsted analysis of these substrates in nonenzymatic, hydroxide-catalyzed cleavage reactions yields an apparent β_{nuc} value of -0.75 , indicating a significant degree of bonding to the nucleophile in the transition state, consistent with recent results from kinetic isotope effect analysis.²³ However, introduction of these modified nucleotides into HDV ribozyme substrates blocked cleavage, presumably because the ribozyme active site could not accommodate substituents at the 2'- β -position, thereby precluding efforts to understand similarities and differences in nucleophile bonding between solution and ribozyme-catalyzed reactions. Because the HDV ribozyme has no strict sequence requirements upstream of the cleavage site,^{24–26} we anticipated that the ribozyme might catalyze cleavage of substrates lacking a nitrogenous base and/or the ribose ring at the cleavage site. Thus, we considered the use of pK_a -perturbing modifications in the context of substrates possessing greater flexibility and occupying less volume at the cleavage site relative to natural nucleotides. Specifically, we were inspired by 2- (hydroxylpropyl)aryl phosphate model compounds (herein linked to oligonucleotides to generate “tethered substrates”) initially developed more than 50 years ago.²⁷ Here we report the incorporation of 1,2-propanediol, 3-fluoro-1,2-propanediol, and 3,3,3-trifluoro-1,2-propanediol into the 5'-terminus of DNA and RNA oligonucleotides and the analysis of their hydroxide-catalyzed and HDV ribozyme-catalyzed cleavage reactions, respectively (Figure 1B).

MATERIALS AND METHODS

Synthesis of Phosphoramidites (4a–c).

All reactions were performed in anhydrous solvents under a positive pressure of argon. Commercially available reagents and anhydrous solvents were used without further purification. ¹H, ¹³C, ¹⁹F, and ³¹P nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 500 or Bruker 400 MHz NMR spectrometer. ¹H chemical shifts are reported in δ (parts per million) relative to tetramethylsilane and ¹³C chemical shifts in δ (parts per

million) relative to the solvent used. High-resolution mass spectra were obtained from the Department of Chemistry of the University of California at Riverside (Riverside, CA).

1,2-Bis(tert-butyldimethylsiloxy)propane (2a).—1*H*-Imidazole (1.97 g, 29.0 mmol) and *t*-BuMe₂SiCl (4.22 g, 28.0 mmol) were added to a solution of **1a** (0.76 g, 10.0 mmol) in dry dimethylformamide (10 mL). The mixture was stirred overnight at room temperature and then evaporated to a syrup under vacuum. The residue was partitioned between CH₂Cl₂ and H₂O; the organic layer was washed with water followed by brine and dried over anhydrous Na₂SO₄. The sodium sulfate was removed by filtration, and the solvent was subsequently removed under vacuum. The residue was purified by silica gel chromatography, eluting with 2% ethyl acetate in hexane, to give product **2a** as an oil: 2.90 g (95% yield); ¹H NMR (CDCl₃) δ 3.80 (m, 1H), 3.52 (m, 1H), 3.33 (m, 1H), 1.11 (d, 3H, *J*=6.1 Hz), 0.90 (s, 9H), 0.89 (s, 9H), 0.07 (s, 3H), 0.06 (s, 3H), 0.05 (s, 3H), 0.04 (s, 3H); ¹³C NMR (CDCl₃) δ 69.6, 69.2, 26.2, 26.1, 20.8, 18.6, 18.4, -4.4, -4.6, -5.1, -5.2; HRMS (FAB⁺) *m/z* calcd for C₁₅H₃₇O₂Si₂ [M + H⁺] 305.2332, found 305.2325.

1,2-Bis(tert-butyldimethylsiloxy)-3-fluoropropane (2b).—Compound **2b** was prepared from **1b** (1.00 g, 10.6 mmol), 1*H*-imidazole (2.95 g, 30.7 mmol), and *t*-BuMe₂SiCl (4.48 g, 29.7 mmol) as described for **2a**. Silica gel chromatography (2% ethyl acetate in hexane) of the residue yielded 3.09 g (90% yield) of **2b** as an oil: ¹H NMR (CDCl₃) δ 4.18–4.46 (m, 2H), 3.79–3.88 (m, 1H), 3.44–3.53 (m, 1H), 0.82 (s, 9H), 0.81 (s, 9H), 0.02 (s, 3H), 0.01 (s, 3H), -0.02 (s, 6H); ¹³C NMR (CDCl₃) δ 85.9, 84.2, 72.4, 72.2, 63.9, 63.8, 26.0, 25.9, 18.4, 18.3, -4.7, -5.30, -5.36, -5.4; ¹⁹F NMR (CDCl₃) δ -109 (t); HRMS (FAB⁺) *m/z* calcd for C₁₅H₃₆FO₂Si₂ [M + H⁺] 323.2238, found 323.2238.

1,2-Bis(tert-butyldimethylsiloxy)-3,3,3-trifluoropropane (2c).—Compound **2c** was prepared from **1c** (1.00 g, 7.7 mmol), 1*H*-imidazole (1.52 g, 22.3 mmol), and *t*-BuMe₂SiCl (3.25 g, 21.5 mmol) as described for **2a**. Silica gel chromatography (2% ethyl acetate in hexane) of the residue yielded 1.99 g (72% yield) of **2c** as an oil: ¹H NMR (CDCl₃) δ 3.97–4.03 (m, 1H), 3.81–3.84 (m, 1H), 3.63–3.66 (m, 1H), 0.91 (s, 9H), 0.90 (s, 9H), 0.12 (s, 3H), 0.11 (s, 3H), 0.072 (s, 3H), 0.071 (s, 3H); ¹³C NMR (CDCl₃) δ 124.6.0 (q), 73.0 (q), 63.3, 26.0, 25.7, 18.5, 18.2, -4.6, -5.2, -5.4, -5.5; ¹⁹F NMR (CDCl₃) δ -77.6 (d); HRMS (FAB⁺) *m/z* calcd for C₁₅H₃₄F₃O₂Si₂ [M + H⁺] 359.2049, found 359.2044.

2-tert-Butyldimethylsiloxy-1-propanol (3a).—HF pyridine (890 μL, 6.84 mmol; Aldrich) was carefully diluted with pyridine (1.0 mL) and then added dropwise to a solution of **2a** (1.53 g, 5.02 mmol) in tetrahydrofuran (10 mL). After the mixture had been stirred overnight at room temperature, thin-layer chromatography of the mixture showed that no starting material remained. The mixture was partitioned between CH₂Cl₂ and H₂O; the organic layer was washed with 5% aqueous NaHCO₃ followed by brine and dried over anhydrous Na₂SO₄. The sodium sulfate removed by filtration and the solvent was subsequently removed under vacuum. The residue was purified by silica gel chromatography, eluting with 17% ethyl acetate in hexane, to give product **3a** as an oil: 516 mg (54% yield); ¹H NMR (CDCl₃) δ 3.91 (m, 1H), 3.49 (m, 1H), 3.37 (m, 1H), 2.02 (m, 1H), 1.12 (d, 3H, *J* = 6.2 Hz), 0.91 (s, 9H), 0.09 (s, 6H); ¹³C NMR (CDCl₃) δ 69.2, 68.3,

25.9, 19.9, 18.2, -4.3, -4.7; HRMS (FAB⁺) m/z calcd for C₉H₂₃O₂Si [M + H⁺] 191.1467, found 191.1467.

2-tert-Butyldimethylsiloxy-3-fluoro-1-propanol (3b).—Compound **3b** was prepared from **2b** (2.89 g, 8.96 mmol) and HF pyridine (1.20 mL, 9.21 mmol) as described for **3a**. Silica gel chromatography (10% ethyl acetate in hexane) of the residue yielded 0.99 g (53% yield) of **3b** as an oil: ¹H NMR (CDCl₃) δ 4.28–4.47 (m, 2H), 3.95–4.00 (m, 1H), 3.56–3.67 (m, 1H), 0.92 (s, 9H), 0.11 (s, 6H); ¹³C NMR (CDCl₃) δ 84.6, 83.3, 71.4, 71.3, 63.3, 63.2, 25.9, 18.2, -4.6, -4.8; ¹⁹F NMR (CDCl₃) δ -106; HRMS (FAB⁺) m/z calcd for C₉H₂₂FO₂Si [M + H⁺] 209.1373, found 209.1367.

2-(tert-Butyldimethylsiloxy)propyl Cyanoethyl N,N-Diisopropylphosphoramidite (4a).—To a solution of **3a** (441 mg, 2.16 mmol) in dry dichloromethane (5 mL) under argon were added *N,N*-diisopropylethylamine (1.85 mL, 10.75 mmol), 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.00 g, 4.23 mmol), and 1-methylimidazole (173 μL, 0.136 mmol). After the mixture had been stirred for 1 h at room temperature, thin-layer chromatography of the mixture showed that no starting material remained. The reaction was then quenched with MeOH (1 mL) and the mixture stirred for an additional 5 min. After the solvent was removed under vacuum, the residue was purified by silica gel chromatography, eluting with 5% ethyl acetate in hexane containing 0.5% triethylamine, to give the corresponding phosphoramidite **4a** as an oil: 689 mg (82% yield); ³¹P NMR (CD₃CN) δ 150.8, 150.3; HRMS (FAB⁺) m/z calcd for C₁₈H₄₀N₂O₃SiP [M + H⁺] 391.2546, found 391.2551.

2-tert-Butyldimethylsiloxy-3-fluoropropyl Cyanoethyl N,N-Diisopropylphosphoramidite (4b).—Compound **4b** was prepared from **3b** (441 mg, 2.12 mmol), *N,N*-diisopropylethylamine (2.29 mL, 13.31 mmol), 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.00 g, 4.23 mmol), and 1-methylimidazole (200 μL, 0.157 mmol) as described for **4a**. Silica gel chromatography (5% ethyl acetate in hexane containing 0.5% triethylamine) purification of the residue yielded 625 mg (72% yield) of **4b** as an oil: ³¹P NMR (CD₃CN) δ 151.9, 151.4; ¹⁹F NMR (CDCl₃) δ -107.7 (d), 108.5 (d); HRMS (FAB⁺) m/z calcd for C₁₈H₃₉N₂O₃FSiP [M + H⁺] 409.2452, found 409.2454.

2-tert-Butyldimethylsiloxy-3,3,3-trifluoropropyl Cyanoethyl N,N-Diisopropylphosphoramidite (4c).—2-tert-Butyldimethylsiloxy-3,3,3-trifluoro-1-propanol (**3c**) was prepared from **2c** (1.81 g, 5.04 mmol) and HF-pyridine (680 μL, 5.18 mmol) as described for **3a**. Silica gel chromatography (10% ethyl acetate in hexane) of the residue yielded 441 mg (36% yield) of **3c** as an oil: ¹H NMR (CDCl₃) δ 4.03–4.07 (m, 1H), 3.77–3.81 (m, 1H), 3.70–3.75 (m, 1H), 0.93 (s, 9H), 0.15 (s, 6H); ¹³C NMR (CDCl₃) δ 124.4 (q), 71.8 (q), 61.9, 25.6, 18.2, -4.9, -5.2; ¹⁹F NMR (CDCl₃) δ -77.4 (d). Compound **4c** was then prepared from **3c** (387 mg, 1.58 mmol), *N,N*-diisopropylethylamine (1.71 mL, 9.94 mmol), 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.0 g, 4.23 mmol), and 1-methylimidazole (160 μL, 0.126 mmol) as described for **4a**. Silica gel chromatography (5% ethyl acetate in hexane containing 0.5% triethylamine) purification of the residue yielded 590 mg (84% yield) of **4c** as an oil: ³¹P NMR (CD₃CN) δ 152.3, 151.5; ¹⁹F NMR

(CDCl₃) δ –77.6 (t); HRMS (FAB⁺) m/z calcd for C₁₈H₃₇N₂O₃F₃SiP [M + H⁺] 445.2263, found 445.2262.

Experiments with Substrates That Perturb the 2'-Hydroxyl Nucleophile pK_a for the Study of Nucleophile Activation by the HdV Ribozyme.

RNA Substrates Were 3'-End-Radiolabeled as Follows.—Ten picomoles of RNA was incubated with 10 pmol of 5'-³²P-pCp (cytidine 3',5'-bisphosphate), 6 μ M ATP, 1 $\times$ RNA ligase buffer, 3.3 mM DTT, 1 μ L of DMSO, and 20 units of T4 RNA ligase in a 10 μ L reaction volume at 4 °C overnight. The reaction was then quenched by the addition of 8 μ L of a stop solution [95% formamide, 15 mM EDTA, 0.01% (w/v) bromophenol blue, and xylene cyanol], gel purified on a 20% denaturing polyacrylamide gel, eluted into 1 mL of TE overnight, and finally precipitated with ethanol with glycogen as a carrier.

DNA Substrates Were 3'-End-Radiolabeled as Follows.—Ten picomoles of DNA was incubated with 10 pmol of [α -³²P]ddATP (dideoxyadenosine triphosphate), 5 μ g of BSA, 1 $\times$ TdT (terminal deoxytransferase) buffer, and 15 units of TdT in a 10 μ L reaction volume at 37 °C for 1 h. The reaction was then quenched by the addition of 8 μ L of the stop solution, and the mixture gel purified on a 20% denaturing polyacrylamide gel, eluted into 1 mL of TE overnight, and finally precipitated with ethanol with glycogen as a carrier.

To measure the rate of base-catalyzed cleavage of DNA substrates, the following high-pH reaction conditions were employed. First, 19.5 μ L of reaction buffer at the appropriate pH was added to 0.5 μ L of the 3'-end-radiolabeled substrate and incubated at 25 °C. Between pH 12.0 and 14.59, reaction conditions were set by mixing appropriate concentrations of KOH (3.89 M, pH 14.59) and KCl (3.89 M) to achieve the desired hydroxide concentration while maintaining a constant ionic strength, and the pH values of the reaction solutions were confirmed by colorimetric strips. In high-ionic strength solutions, pH, which is defined as the negative log of the activity of hydronium ions, can deviate from the expected pH in terms of hydronium concentration.¹⁰ The pH values reported herein refer to the concentration of hydronium. Reaction aliquots of 1 μ L were removed at appropriate time points, reactions quenched by adding 6 μ L of 1 M Tris-HCl (pH 7.5) (final pH's of quenched reaction aliquots are typically no higher than 8) with 7 μ L of the stop solution, and mixtures rapidly chilled on dry ice. Hydrolysis products were separated from the unhydrolyzed substrate on a denaturing 20% polyacrylamide/7 M urea gel and quantified using a PhosphorImager with ImageQuant software (Molecular Dynamics). Data for cleavage reactions was fit to the equation $y = 1 - y_0 - Ae^{-k_{\text{obs}}t}$ using Origin 7.0 (OriginLab), where k_{obs} is the first-order rate constant for substrate cleavage and A is the extent of substrate cleavage. For reactions that did not reach completion (>80% reacted) after more than 20 days (reaction rate of $<3 \times 10^{-5} \text{ min}^{-1}$), data were fit assuming $A = 0.8$ to obtain k_{obs} . The reaction pK_a was determined by fitting reaction rate data to the equation $k = k_{\text{max}}/(1 + 10^{pK_a - \text{pH}})$.

HdV ribozyme-catalyzed reactions were conducted as previously described.⁹ Briefly, the ribozyme (final concentration of 1 μ M) was preincubated with 10 mM MgCl₂ at 70 °C for 2 min and then at 25 °C for 14 min; buffer was added, and reaction was initiated by the addition of trace radiolabeled substrate (10 μ L reaction volume). Buffers contained 25 mM

acetic acid, 25 mM MES, and 50 mM Tris (pH 4.0–8.0) or 50 mM MES, 25 mM Tris, and 25 mM 2-amino-2-methyl-1-propanol (pH 7.5–10.0). Reaction aliquots were removed at appropriate times, reactions quenched by addition 9 μ L of the stop solution, and mixtures rapidly chilled on dry ice. Cleavage products were separated from the uncleaved substrate on a denaturing 20% polyacrylamide/7 M urea gel and quantified using a PhosphorImager with ImageQuant software (Molecular Dynamics). Data for cleavage reactions were fit to the equation $y = 1 - y_0 - Ae^{-k_{\text{obs}}t}$ using Origin 7.0 (OriginLab), where k_{obs} is the first-order rate constant for substrate cleavage and A is the extent of substrate cleavage. Reaction pK_a values (pK_{a1} and pK_{a2}) were determined by fitting reaction rate data to the following equation:

$$k = \frac{k_{\text{max}}}{1 + 10^{\text{pH} - \text{p}K_{a1}} + 10^{\text{p}K_{a2} - \text{pH}} + 10^{\text{p}K_{a2} - \text{p}K_{a1}}}$$

RESULTS AND DISCUSSION

Synthesis and Incorporation of Phosphoramidites (4a–c) into RNA/DNA Substrates.

Commercially available, racemic 1,2-propanediol (**1a**), 3-fluoro-1,2-propanediol (**1b**), and 3,3,3-trifluoro-1,2-propanediol (**1c**) were converted into the corresponding phosphoramidites (**4a–c**) in three steps, as shown in Figure 2. Both primary and secondary hydroxyl groups of **1** were first protected as *tert*-butyldimethylsilyl (TBS) ethers in 72–95% yield (Figure 2). The primary TBS ethers were selectively cleaved using the HF-pyridine complex to give alcohols **3a–c** in 36–54% yield (Figure 2). Phosphitylation of **3a–c** with 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite gave phosphoramidites (**4a–c**) in good yields [72–84% (Figure 2)]. All phosphoramidites were prepared as racemic mixtures with the exception of the 1,2-propanediol derivative that was prepared as both a racemic mixture and an enantiopure *S* isomer. Ribozyme-catalyzed cleavage assays showed that both enantiopure and racemic versions of the resulting oligonucleotides display identical kinetic behavior (data not shown). All subsequent assays were performed with oligomers derived from racemic phosphoramidites.

To investigate nonenzymatic base-catalyzed cleavage of these modified linkages, we incorporated the phosphoramidites (**4a–c**) into DNA oligonucleotides (5'-Xgg gtc ggc-3'). To investigate HDV ribozyme-catalyzed cleavage of these linkages, we incorporated **4a–c** into RNA oligonucleotides [5'-XGG GUC GGC-3' (Figure 1)]. We used a modified 1 μ mol RNA protocol with double coupling of the phosphoramidites (**4a–c**) (~75 mg) in anhydrous acetonitrile (0.75 mL). After the synthesis, the solid support was treated with a 3:1 (v/v) mixture of concentrated aqueous ammonium hydroxide and ethanol at 55 °C for 4 h and then desilylated with the triethylamine trifluoride complex at 65 °C for 25 min.²⁸ We purified the oligonucleotides on a denaturing polyacrylamide gel and confirmed their identities by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Table S1).

Base-Catalyzed Cleavage of Tethered DNA Oligonucleotides.

Under alkaline conditions, the 5'-propanediol phosphate tethers [5'-Xgg gtc ggc-3'; X = RCH(OH)-CH₂OPO(OH)O-5', and R = CH₃, CH₂F, or CF₃] release themselves from DNA via intramolecular transphosphorylation to the neighboring hydroxyl group, analogous to alkaline cleavage of RNA by 2'-O-transphosphorylation (Figure 1B). To assess the effect of the increasing level of fluorine substitution on the reactivity of the nucleophilic hydroxyl group, we observed the rate of tether release over a range of pH values [25 °C, 3.90 M KCl, pH 12.0–14.6 (Table 1 and Figure S1)]. Our expectation was that the inductive effect from fluorination would reduce both the nucleophilicity and the pK_a of the 2'-hydroxyl nucleophile, causing the fluorinated substrates to react more slowly at pH values above pK_a , when the substrates react from an oxyanionic ground state, but react with a similar, or even enhanced, rate at pH values below the pK_a . For comparison, ethanol and trifluoroethanol, primary alcohols that have -CH₃ and -CF₃ groups geminal to the hydroxyl group, respectively, ionize with pK_a values of 15.2 and 13.2.²⁹ Indeed, at the highest pH tested (14.6), the methyl substrate (R = CH₃) releases itself from the tether ~10-fold faster than the trifluoromethyl (R = CF₃) substrate (Table 1 and Figure S1). At pH 13, the two substrates react at similar rates, and at lower pH values, the trifluoromethyl substrate reacts somewhat faster than its methyl counterpart (Table 1 and Figure S1); however, we emphasize that observations for reactions occurring below pH 13 should be interpreted with caution as they correspond to rates with half-lives on the order of weeks that are thus subject to errors more significant than those seen for faster reactions (Table 1). Together, these observations suggest that the electron-withdrawing effect of fluorination reduces both the pK_a and the intrinsic rate of reaction. Fitting of these data to a single-ionization model suggests a pK_a of ~1 between the methyl substrate (pK_a = 14.5) to the trifluoromethyl substrate (pK_a = 13.5) (Figure S1). Given a pK_a of ~1 between the two substrates, comparison of the maximal, observed rates offers a crude estimate of the Bronsted coefficient (β_{nuc} ~ 1), consistent with the value of ~0.75 measured previously,¹⁸ suggesting that the bond between the 2'-oxygen and the phosphorus is nearly or completely formed in the transition state.

The monofluoromethyl substrate (R = CH₂F) reacted with both rates and apparent pK_a similar to the methyl substrate, suggesting minimal perturbation from the single fluorine substitution (Figure S1). However, on the basis of our prior experience synthesizing an analogous β -branched ribonucleo-ribonucleotide,³⁰ we hypothesized that the monofluoromethyl substrate may release itself through a more complex mechanism (Figure 3). Possibly, the oxyanion initially attacks the vicinal carbon to displace fluoride, generating the corresponding epoxide (Figure 3). Subsequent base-catalyzed opening of the epoxide would result in conversion to a glycerol tether that could undergo release to form the product by attack at the phosphorus (Figure 3). Formation of the glycerol tether could account for the modestly higher reactivity of the monofluoromethyl substrate relative to that of the methyl substrate at high pH values (Figure S1). Further work is necessary to determine whether this alternative pathway exists; therefore, our subsequent analysis largely focuses on the methyl and trifluoromethyl substrates, which likely follow the simpler, expected reaction path. We do not expect this alternative mechanism to be operative in the ribozyme-catalyzed reaction where the pH is closer to neutral.

For comparison with the tethered substrates, we also determined the relationship between pH and rate for DNA oligonucleotides containing either a uridine or abasic residue upstream of the cleavage site under alkaline conditions. The 2'-hydroxyl of the uridine substrate ionized with a pK_a of ~ 13.5 and exhibited a maximum cleavage rate of 0.090 min^{-1} (Figure S2), consistent with previous observations.³¹ The abasic ribose ionized with a higher pK_a of ~ 14.5 and a maximal rate of 0.84 min^{-1} , ~ 10 -fold faster than uridine, presumably reflecting the electron-withdrawing effect of the uracil heterocycle versus a hydrogen atom at position 1' (Figure S2). Compared to the cleavage rate of the abasic ribose under similar conditions, the methyl-tethered ($R = \text{CH}_3$) substrate, which should have a comparably nucleophilic 2'-hydroxyl, reacts ~ 500 -fold slower (Table 3), reflecting the structural preorganization imparted by the ribose ring. This comparison suggests that conformational restriction around the C2'-C3' bond, which is freely rotatable in the tethered substrates but restricted in the ribofuranosyl context, imparts a rate enhancement of 2–3 orders of magnitude.

HDV Ribozyme-Catalyzed Cleavage of RNA Oligonucleotides.

Next, we tested the tethered RNA oligonucleotides [$5'\text{-XGG GUC GGC-3'}$; $X = \text{RCH(OH)CH}_2\text{OPO(OH)O}5'$; $R = \text{CH}_3, \text{CH}_2\text{F}, \text{or CF}_3$ (Figure 1)] as substrates for HDV ribozyme-catalyzed cleavage at various pH values. To ensure that differences in reactivity between tethered and ribonucleotide substrates arose from differences in the cleavage step rather than substrate binding, we examined rate as a function of ribozyme concentration. The rate was insensitive to ribozyme concentration over the range of 50–2000 nM (Figure S3). These data set an upper limit of 100 nM for K_m and suggest that at the ribozyme concentrations employed in our subsequent experiments (1000 nM) the reaction proceeds exclusively from a bound ES complex ground state for both substrates (Figure S3).

Qualitatively, the tethered substrates reacted from the ES complex with a pH dependence similar to that of the wild-type substrate, showing a log-linear increase with pH in the acidic limb of the profile that undergoes a transition to a pH-independent regime and finally to an inverse dependence at $\text{pH} > 9.0$ (Figure 4 and Table 2). The log-linear increase with pH reflects a rate-stimulating deprotonation. The identity of the titrating group remains uncertain but has been attributed to deprotonation of a water molecule coordinated to an active site metal ion to generate the catalytic base.³² The transition to the pH-independent regime reflects the onset of deprotonation of the general acid.^{9,11,33} In the log-linear region of pH, the ES complexes for the tethered substrates react ~ 2 orders of magnitude slower than the wild-type ribonucleotide substrate, consistent with the importance of the conformational constraint imposed by the ribose ring (Table 3).

The tethered substrates react more slowly than the standard, ribose-containing substrates in the presence and absence of ribozyme (Table 3). However, the difference in reactivity between the two sets of substrates is reduced in the presence of the ribozyme, presumably because of the conformational restriction imposed by the ribozyme active site on the tethered substrates (Table 3). Comparison between substrate pairs with similar observed solution pK_a 's (WT vs CF_3 and abasic vs CH_3) allows for evaluation of the energetic contribution from conformational restriction while controlling for differences in nucleophile

pK_a . For each pair of substrates, there is an ~5-fold difference between the ratio of rates of the solution reaction and those of the ribozyme reaction (Table 3).

While it remains possible that a nonchemical step masks the true rate of the ribofuranosyl substrates, a more compelling explanation is that the constrained environment of the ribozyme active site modestly accelerates the reactions of the tethered substrates by reducing their conformational entropy. Ribozyme docking possibly confers some restriction around the C2'-C3' bond but not as much as the ribose ring. Moreover, this result provides a plausible explanation for our prior observation that 2'- β -substituted ribonucleotide substrates were inactive. The same features within the active site that restrict rotation around the C2'-C3' bond, conferring a rate enhancement on the reactions of the tethered substrates, might clash sterically with a substituent in the 2'- β -position preventing catalysis.

The reactivity of the methyl-tethered substrate relative to that of the trifluoromethyl-tethered substrate provides insight into the protonation state of the nucleophilic hydroxyl group in the ground state of the ES complex. In the nonenzymatic reactions, when the reaction starts from an oxyanionic ground state ($pH > pK_a$), the electron-withdrawing effect of $-CF_3$ manifests in the nucleophilic attack step, resulting in a lower maximal rate. However, when the reaction starts from the protonated ground state ($pH < pK_a$), the electron-withdrawing effect on the pK_a increases the fraction of oxyanion present, offsetting the reduced nucleophilicity. In the HDV reaction, the trifluoromethyl substrate reacts moderately faster than the methyl substrate does (1.6-fold) and the monofluoromethyl substrate reacts with an intermediate rate (Table 2). This effect of fluorine substitution more closely resembles the effect observed for nonenzymatic reactions at low pH rather than at high pH, consistent with the ES complex starting from the protonated ground state rather than the deprotonated oxyanion ground state. Moreover, the positive correlation between reaction rate and fluorination suggests that the effective charge on the 2'-oxygen increases as the reaction progresses toward the transition state, implying that the extent of O-H bond cleavage exceeds the extent of O-P bond formation. The pathway to achieve this condition could involve a concerted process, in which deprotonation and nucleophilic attack occur simultaneously, or a stepwise process involving pre-equilibrium deprotonation of the 2'-OH to form the corresponding oxyanion followed by O-P bond formation to an extent that a significant degree of the effective charge on the 2'-oxyanion is lost in the transition state.

Comparison between HDV-catalyzed reactions of the abasic and wild-type ribonucleotide substrates reinforces the trend observed for the tethered substrates that the substrate bearing the higher- pK_a nucleophile reacts more slowly. The abasic substrate reacts ~4-fold slower than the wild-type substrate (Table 3), consistent with prior observations.²⁶ Given that HDV is known to be largely insensitive to the identity of the sequence upstream of its cleavage site, it seems plausible that an increase in pK_a upon removal of the electron-withdrawing nucleobase could explain some, if not all, of the difference in reactivity between the abasic and wild-type substrates.

Several alternative explanations could account for the rate stimulation from fluorine substitution observed in the ribozyme reaction. (I) The transition state for the HDV ribozyme reaction has relatively little bonding between the nucleophilic oxygen and the

phosphorus atom, so perturbation of the geminal hydroxyl's nucleophilicity by fluoride substitution has a negligible impact on reactivity. However, the nonenzymatic reaction occurs with a large effective change in charge on the nucleophile.¹⁸ Moreover, the established importance of general acid catalysis in the HDV ribozyme implicates significant breaking of bonds to the leaving group in the transition state,⁹ which, in the case of phosphodiester, accompanies significant formation of bonds to the nucleophile.³⁴ (II) Differential ground state interactions such as solvation of the nucleophile could attenuate the relative reactivity of the methyl-tethered substrate relative to that of the trifluoromethyl-tethered substrate. Stronger solvation of the former substrate in the ground state could engender a greater energetic penalty for nucleophile desolvation en route to the chemical transition state. It is important to note, however, that the tethered substrates react with nearly identical K_m values in kinetic experiments, suggesting that the substrates bind similarly in the ground state. Lastly, we cannot rule out the possibility that a nonchemical step limits the reaction rates of the tethered substrate and masks the true chemical effect of fluorine substitution. However, the significantly lower reactivity of the tethered substrates compared to that of the wild-type substrate argues against this possibility.

CONCLUSIONS AND IMPLICATIONS

In an effort to obtain substrates that subtly perturb the basicity of the 2'-hydroxyl nucleophile in the HDV ribozyme reaction, we prepared the phosphoramidite derivatives of 1,2-propanediol, 3-fluoro-1,2-propanediol, and 3,3,3-trifluoro-1,2-propanediol and successfully incorporated them into the 5'-terminus of RNA and DNA oligonucleotides. These oligonucleotides undergo hydroxide- and HDV ribozyme-catalyzed transphosphorylation to release the tethers. The hydroxide-catalyzed reactions show that the nucleophilic hydroxyl group ionizes ~ 1 pK_a unit lower and reacts ~ 10 -fold slower for the trifluoromethyl substrate than for the methyl substrate. This relative reactivity suggests an apparent β_{nuc} value of ~ 1 , consistent with almost complete loss of negative charge from the oxyanion nucleophile in the transition state due to substantial formation of bonds between the oxygen nucleophile and the scissile phosphate.¹⁸ Comparison between the base-catalyzed and RNA-catalyzed reactions of these suggests that (I) the ribozyme's catalytic apparatus engages without the cleavage site nucleobase or the conformational constraints imposed by the ribofuranose ring, (II) the nucleophilic hydroxyl group predominantly populates the neutral form in the starting ground state for the enzyme-substrate complex, and (III) in the transition state, the 2'-hydroxyl bears some negative charge consistent with a transition state in which the extent of proton loss from the 2'-OH exceeds the extent of nucleophilic attack.

The tethered substrates described here provide a simple and accessible context in which to test structure-activity relationships for both solution- and ribozyme-catalyzed transphosphorylation. Insights gleaned from these substrates could help in the interpretation of additional biochemical data gathered on ribozyme transition states, including linear free energy relationships for general acid-base catalysis and kinetic isotope effects. Specifically, the qualitative information derived here regarding the protonation of the ribozyme substrate ground state could inform future heavy atom kinetic isotope measurements for the 2'-O nucleophile.³⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Riccitelli N, and Luptak A (2013) HDV Family of Self-Cleaving Ribozymes. *Prog. Mol Biol. Transl* 120, 123–171.
- (2). Ke A, Zhou K, Ding F, Cate JH, and Doudna JA (2004) A conformational switch controls hepatitis delta virus ribozyme catalysis. *Nature* 429, 201–205. [PubMed: 15141216]
- (3). Chen JH, Yajima R, Chadalavada DM, Chase E, Bevilacqua PC, and Golden BL (2010) A 1.9 angstrom Crystal Structure of the HDV Ribozyme Precleavage Suggests both Lewis Acid and General Acid Mechanisms Contribute to Phosphodiester Cleavage. *Biochemistry* 49, 6508–6518. [PubMed: 20677830]
- (4). Kapral GJ, Jain S, Noeske J, Doudna JA, Richardson DC, and Richardson JS (2014) New tools provide a second look at HDV ribozyme structure, dynamics and cleavage. *Nucleic Acids Res.* 42, 12833–12846. [PubMed: 25326328]
- (5). Shih IH, and Been MD (2002) Catalytic strategies of the hepatitis delta virus ribozymes. *Annu. Rev. Biochem.* 71, 887–917. [PubMed: 12045114]
- (6). Sharmeen L, Kuo MY, Dinter-Gottlieb G, and Taylor J (1988) Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *J. Virol.* 62, 2674–2679. [PubMed: 2455816]
- (7). Nakano S, Chadalavada DM, and Bevilacqua PC (2000) General acid-base catalysis in the mechanism of a hepatitis delta virus ribozyme. *Science* 287, 1493–1497. [PubMed: 10688799]
- (8). Nakano S, Proctor DJ, and Bevilacqua PC (2001) Mechanistic characterization of the HDV genomic ribozyme: Assessing the catalytic and structural contributions of divalent metal ions within a multichannel reaction mechanism. *Biochemistry* 40, 12022–12038. [PubMed: 11580278]
- (9). Das SR, and Piccirilli JA (2005) General acid catalysis by the hepatitis delta virus ribozyme. *Nat. Chem. Biol.* 1, 45–52. [PubMed: 16407993]
- (10). Cerrone-Szakal AL, Siegfried NA, and Bevilacqua PC (2008) Mechanistic characterization of the HDV genomic ribozyme: solvent isotope effects and proton inventories in the absence of divalent metal ions support C75 as the general acid. *J. Am. Chem. Soc.* 130, 14504–14520. [PubMed: 18842044]
- (11). Gong B, Chen JH, Chase E, Chadalavada DM, Yajima R, Golden BL, Bevilacqua PC, and Carey PR (2007) Direct measurement of a pK(a) near neutrality for the catalytic cytosine in the genomic HDV ribozyme using Raman crystallography. *J. Am. Chem. Soc.* 129, 13335–13342. [PubMed: 17924627]
- (12). Chen JH, Gong B, Bevilacqua PC, Carey PR, and Golden BL (2009) A catalytic metal ion interacts with the cleavage Site G.U wobble in the HDV ribozyme. *Biochemistry* 48, 1498–1507. [PubMed: 19178151]
- (13). Thomas JM, and Perrin DM (2009) Probing general acid catalysis in the hammerhead ribozyme. *J. Am. Chem. Soc.* 131, 1135–1143. [PubMed: 19154176]

- (14). Wilson TJ, Li NS, Lu J, Frederiksen JK, Piccirilli JA, and Lilley DM (2010) Nucleobase-mediated general acid-base catalysis in the Varkud satellite ribozyme. *Proc. Natl. Acad. Sci. U. S. A.* 107, 11751–11756. [PubMed: 20547881]
- (15). Kath-Schorr S, Wilson TJ, Li NS, Lu J, Piccirilli JA, and Lilley DM (2012) General acid-base catalysis mediated by nucleobases in the hairpin ribozyme. *J. Am. Chem. Soc.* 134, 16717–16724. [PubMed: 22958171]
- (16). Anderson VE, Ruszczycky MW, and Harris ME (2006) Activation of oxygen nucleophiles in enzyme catalysis. *Chem. Rev.* 106, 3236–3251. [PubMed: 16895326]
- (17). Oivanen M, Kuusela S, and Lonnberg H (1998) Kinetics and Mechanisms for the Cleavage and Isomerization of the Phosphodiester Bonds of RNA by Bronsted Acids and Bases. *Chem. Rev.* 98, 961–990. [PubMed: 11848921]
- (18). Ye JD, Li NS, Dai Q, and Piccirilli JA (2007) The mechanism of RNA strand scission: an experimental measure of the Bronsted coefficient, beta nuc. *Angew. Chem., Int. Ed.* 46, 3714–3717.
- (19). Kirby AJ, and Younas M (1970) Reactivity of Phosphate Esters - Reactions of Diesters with Nucleophiles. *J. Chem. Soc. B*, 1165.
- (20). Mikkola S, Stenman E, Nurmi K, Yousefi-Salakdeh E, Stromberg R, and Lonnberg H (1999) The mechanism of the metal ion promoted cleavage of RNA phosphodiester bonds involves a general acid catalysis by the metal aquo ion on the departure of the leaving group. *J. Chem. Soc. Perkin Trans. 2* 2, 1619–1625.
- (21). Chen J, Ganguly A, Miswan Z, Hammes-Schiffer S, Bevilacqua PC, and Golden BL (2013) Identification of the Catalytic Mg²⁺ Ion in the Hepatitis Delta Virus Ribozyme. *Biochemistry* 52, 557–567. [PubMed: 23311293]
- (22). Thaplyal P, Ganguly A, Golden BL, Hammes-Schiffer S, and Bevilacqua PC (2013) Thio Effects and an Unconventional Metal Ion Rescue in the Genomic Hepatitis Delta Virus Ribozyme. *Biochemistry* 52, 6499–6514. [PubMed: 24001219]
- (23). Harris ME, Dai Q, Gu H, Kellerman DL, Piccirilli JA, and Anderson VE (2010) Kinetic isotope effects for RNA cleavage by 2'-O- transphosphorylation: nucleophilic activation by specific base. *J. Am. Chem. Soc.* 132, 11613–11621. [PubMed: 20669950]
- (24). Perrotta AT, and Been MD (1990) The self-cleaving domain from the genomic RNA of hepatitis delta virus: sequence requirements and the effects of denaturant. *Nucleic Acids Res.* 18, 6821–6827. [PubMed: 2263447]
- (25). Kellerman DL, Simmons KS, Pedraza M, Piccirilli JA, York DM, and Harris ME (2015) Determination of hepatitis delta virus ribozyme N(–1) nucleobase and functional group specificity using internal competition kinetics. *Anal. Biochem.* 483, 12–20. [PubMed: 25937290]
- (26). Shih IH, and Been MD (2001) Energetic contribution of non-essential 5' sequence to catalysis in a hepatitis delta virus ribozyme (vol 20, pg 4884, 2001). *EMBO J.* 20, 5302.
- (27). Brown DM, and Usher DA (1965) Hydrolysis of Hydroxyalkyl Phosphate Esters - Effect of Changing Ester Group. *J. Chem. Soc.* 6558.
- (28). Wincott F, DiRenzo A, Shaffer C, Grimm S, Tracz D, Workman C, Sweedler D, Gonzalez C, Scaringe S, and Usman N (1995) Synthesis, deprotection, analysis and purification of RNA and ribozymes. *Nucleic Acids Res.* 23, 2677–2684. [PubMed: 7544462]
- (29). Mock WL, and Zhang JZ (1990) Concerning the Relative Acidities of Simple Alcohols. *Tetrahedron Lett.* 31, 5687–5688.
- (30). Dai Q, and Piccirilli JA (2003) Synthesis of 2'-C-beta- fluoromethyluridine. *Org. Lett.* 5, 807–810. [PubMed: 12633077]
- (31). Li YF, and Breaker RR (1999) Kinetics of RNA degradation by specific base catalysis of transesterification involving the 2'-hydroxyl group. *J. Am. Chem. Soc.* 121, 5364–5372.
- (32). Thaplyal P, Ganguly A, Hammes-Schiffer S, and Bevilacqua PC (2015) Inverse thio effects in the hepatitis delta virus ribozyme reveal that the reaction pathway is controlled by metal ion charge density. *Biochemistry* 54, 2160–2175. [PubMed: 25799319]
- (33). Perrotta AT, Wadkins TS, and Been MD (2006) Chemical rescue, multiple ionizable groups, and general acid-base catalysis in the HDV genomic ribozyme. *RNA* 12, 1282–1291. [PubMed: 16690998]

- (34). Gu H, Zhang S, Wong KY, Radak BK, Dissanayake T, Kellerman DL, Dai Q, Miyagi M, Anderson VE, York DM, Piccirilli JA, and Harris ME (2013) Experimental and computational analysis of the transition state for ribonuclease A- catalyzed RNA 2'-O-transphosphorylation. *Proc. Natl. Acad. Sci. U. S. A.* 110, 13002–13007. [PubMed: 23878223]
- (35). Humphry T, Iyer S, Iranzo O, Morrow JR, Richard JP, Paneth P, and Hengge AC (2008) Altered transition state for the reaction of an RNA model catalyzed by a dinuclear zinc(II) catalyst. *J. Am. Chem. Soc.* 130, 17858–17866. [PubMed: 19053445]

Figure 1. General mechanism of ribozyme-mediated cleavage of standard RNA oligonucleotides (A) and propanediol-tethered RNA substrates (B). Cleavage occurs with attack of a 2'-hydroxyl group on the adjacent phosphate to give 2',3'-cyclic phosphate and 5'-hydroxyl-containing products. A-H represents a general acid, which protonates the 5'-leaving group; B⁻ represents a general base, which deprotonates the 2' nucleophile. For the sake of simplicity, we show the reaction occurring via a concerted mechanism.

Figure 2.

Synthesis of propanediol phosphoramidites from corresponding diols.

Figure 3.

Proposed mechanism of cleavage by the monofluoromethyl propanediol-tethered DNA oligonucleotide under alkaline conditions.

Figure 4. pH-rate profiles for HDV ribozyme-catalyzed cleavage of tethered substrates. Filled squares and the solid line correspond to $R = \text{CH}_3$. Half-filled triangles and the dashed line correspond to $R = \text{CH}_2\text{F}$. Empty circles and the dotted line correspond to $R = \text{CF}_3$. Data were fit to a double-ionization model (see Materials and Methods).

Table 1.Rates for Uncatalyzed Tether Release under Basic Conditions^a

pH	R= CH ₃	R= CF ₃	<i>k</i> _{rel}
	rate (min ⁻¹)	rate (min ⁻¹)	
14.6	$(8.4 \pm 0.2) \times 10^{-4}$	$(1.1 \pm 0.4) \times 10^{-4}$	0.1
14.0	$(3.7 \pm 0.5) \times 10^{-4}$	$(1.0 \pm 0.2) \times 10^{-4}$	0.3
13.5	$(1.3 \pm 0.4) \times 10^{-4}$	$(5.0 \pm 0.3) \times 10^{-5}$	0.4
13.0	$(5.0 \pm 3.0) \times 10^{-5}$	$(4.0 \pm 2.0) \times 10^{-5}$	0.8
12.5	$(6.8 \pm 4.7) \times 10^{-6}$	$(2.1 \pm 1.5) \times 10^{-5}$	2.9

^aRelative rates compared to that of the R = CH₃ substrate at the given pH. The pH values reported here reflect the calculated concentrations of hydronium rather than the hydronium activity (see Materials and Methods), whereas the pH values in Figure S1 reflect hydronium activity.

Table 2.

Summary of Fits to pH Titration Curves for HDV Ribozyme-Catalyzed Cleavage of Tethered Substrates (Figure 4)

	$k_{\max}$ (min ⁻¹)	pK_{a1}	pK_{a2}
R= CH ₃	$(1.27 \pm 0.06) \times 10^{-3}$	9.5 ± 0.1	5.3 ± 0.1
R = CH ₂ F	$(1.59 \pm 0.07) \times 10^{-3}$	10.1 ± 0.1	5.2 ± 0.2
R= CF ₃	$(2.07 \pm 0.07) \times 10^{-3}$	10.1 ± 0.1	5.1 ± 0.1

Table 3.

Comparison of the Cleavage Rates of the Modified DNA/RNA Ribonucleotide and Tethered Substrates in the Presence of the Ribozyme (pH 5) or Absence of the Ribozyme (pH 14)

	pK_a	base-catalyzed ^a	ribozyme-catalyzed
ribonucleotide, WT	13.5 ± 0.1 ^b	6.9×10^{-2}	1.7×10^{-1}
ribonucleotide, abasic	14.5 ± 0.1	2.0×10^{-1}	4.4×10^{-2}
R = CF ₃	13.5 ± 0.1	3.7×10^{-4}	1.3×10^{-3}
R = CH ₃	14.5 ± 0.2	1.1×10^{-4}	4.9×10^{-4}
abasic/CH ₃ ^c		540	90
WT/CF ₃ ^c		627	133

^aRates extrapolated from the best-fit curve to pH 5.

^bBase-catalyzed reactions measured with N₋₁ = U and ribozyme-catalyzed reaction observed with N₋₁ = C. A previous investigation suggested that the nucleophile pK_a is insensitive to the identity of the N₋₁ nucleobase.³¹

^cRatios of rates given for sterically distinct substrates with similar nucleophile pK_a 's.