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The pK_a of the Internucleotidic 2′-Hydroxyl Group in Diribonucleoside (3′→5′) Monophosphates

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Ionization of the internucleotidic 2′-hydroxyl group in RNA facilitates transesterification reactions in Group I and II introns (splicing), hammerhead and hairpin ribozymes, self-cleavage in lariat-RNA, and leadzymes and tRNA processing by RNase P RNA, as well as in some RNA cleavage reactions promoted by ribonucleases. Earlier, the pK_a of 2′-OH in mono- and diribonucleoside (3′→5′) monophosphates had been measured under various nonuniform conditions, which make their comparison difficult. This work overcomes this limitation by measuring the pK_a values for internucleotidic 2′-OH of eight different diribonucleoside (3′→5′) monophosphates under a set of uniform noninvasive conditions by ^{1}H NMR. Thus the pK_a is 12.31 (±0.02) for ApG and 12.41 (±0.04) for ApA, 12.73 (±0.04) for GpG and 12.71 (±0.08) for GpA, 12.77 (±0.03) for CpG and 12.88 (±0.02) for CpA, and 12.76 (±0.03) for UpG and 12.70 (±0.03) for UpA. By comparing the pK_as of the respective 2′-OH of monomeric nucleoside 3′-ethyl phosphates with that of internucleotidic 2′-OH in corresponding diribonucleoside (3′→5′) monophosphates, it has been confirmed that the aglycons have no significant effect on the pK_a values of their 2′-OH under our measurement condition, except for the internucleotidic 2′-OH of 9-adeninyl nucleotide at the 5′-end (**A**pA and **A**pG), which is more acidic by 0.3−0.4 pK_a units.

Introduction

The 2′-OH group distinguishes[1] RNA from DNA both functionally[2,3] as well as structurally.[3,4] It is involved in recognition,[3,4] processing, and catalytic properties of RNA,[5] such as the stereospecific transesterification reactions involved in the Group I and Group II splicing reactions,[3h,i] self-cleavage in lariat-RNA,[6] and RNA catalysis[5] and in ribonuclease[7] action, as well as in tRNA processing by RNase P RNA.[2c] The biological importance[1−7] of the 2′-OH group makes determination of its pK_a an extremely important issue in terms of understanding the structure and function of RNA in molecular details. It is, however, not possible to measure the pK_a of 2′-OH of a larger RNA than a dimer in an unambiguous and accurate manner, since it decomposes readily under alkaline conditions.[8] Several attempts[9−22] have been

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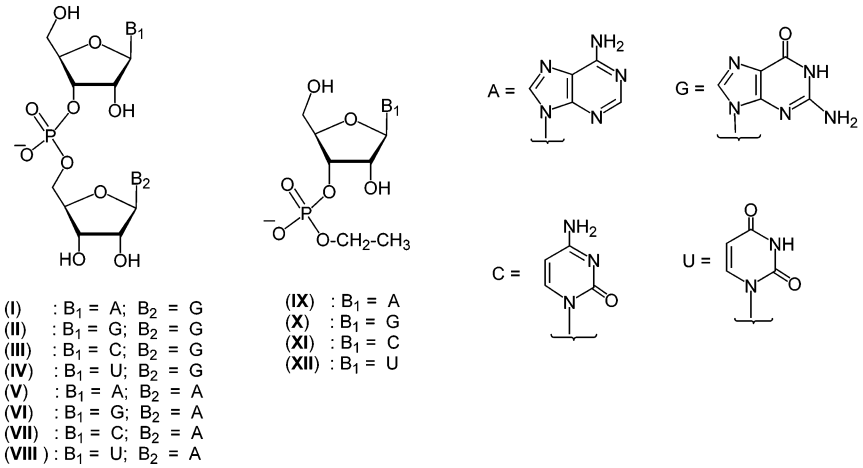

made to determine the pK_a values for 2′-OH in mononucleos(t)ides[15−21] and internucleotidic 2′-OH in diribonucleoside (3′→5′) monophosphates[9,14] as well as in an oligo-DNA with incorporation of a single diribonucleoside (3′→5′) monophosphates unit.[22] All of these studies have been performed at different temperatures and salt concentration conditions, producing a wide range of pK_a values. Due to the varied experimental conditions, these values cannot be compared directly. For every change in 10 K, the pK_a values for acids and bases differ approximately by 0.1 to 0.3 units.[23,24] Furthermore, a change in K^+ ion concentration from 0.5 to 3.0 M produces a change in pK_a values for 2′-OH by 0.6 pK_a units.[22] Moreover, the procedures used in the separation and quantification of the reaction components in all earlier works varied from case to case for determination of the pH-dependent first-order rate constants[9−14,22] of the alkaline hydrolysis, which also produced anomalous pK_a values, making any comparison very difficult and compromising. For example, kinetically determined pK_a values of internucleotidic 2′-OH has been measured to be 12.24 for ApA, 12.55 for UpU, 12.52 for UpA, and 12.04 for ApU at 333.2 K with ionic strength 0.1 M NaCl, where HPLC technique has been used[9] for separation of all reaction components (actual rates measured up to pH 13.04). On the other hand, diribonucleoside (3′→5′) monophosphates, ApA, CpG, UpG, ApG, GpG, when inserted in a 22mer DNA[22] gave uniform pK_a of 13.1 for all internucleotidic 2′-OH at the K^+ strength of 3.16 M at 296 K by PAGE analysis (actual rates measured up to pH 14.5). In another set of dimers[14] with the phosphorothioate backbone, p*IspU, and the natural counterpart, p*IpU, the pK_a values for the internucleotidic 2′-OH were reported to be 12.99 and 13.0 respectively, in which the alkaline cleavage rates were determined by PAGE analysis at 283K at ionic strength of 1 M NaCl (compare with those measured at 3.16 M K^+ strength[22]). So, the variable pK_a values found for the internucleotidic 2′-OH in the dimers show clearly that they are not in agreements. These uncertainties necessitated that the pK_a values of internucleotidic 2′-OH for a set of diribonucleoside (3′→5′) monophosphates should be determined under an uniform condition of temperature and salt concentration using the same quantification technique and calibration method for measuring the reaction kinetics.

Result and Discussion

We report here the pK_a values for internucleotidic 2′-OH for eight different diribonucleoside (3′→5′) monophosphates **I**−**VIII**. The pK_a values were determined from pH dependent first-order cleavage rate analysis (pH 10.7−13.6). The first-order cleavage rates were obtained at different pH by integrating the volumes of the proton NMR absorptions resulting from the diribonucleoside (3′→5′) monophosphates and the products (error: ±0.5%) at each pH and plotting against the corresponding reaction time (see Experimental Section for details). The kinetics were followed at 500 or 600 MHz proton NMR. These gave adequate resolution for baseline separations for all signals, which were used for volume integration purpose. The main advantage of the NMR method is that it allows accurate pH-dependent quantification of the starting diribonucleoside (3′→5′) monophosphate and the products in the same NMR tube in a *noninvasive* manner to give the first-order rate constants, leading to very small error in the experimental pK_a values. In sharp contrast, the HPLC or PAGE techniques produce considerable errors by involving at least two separate operations: one is for the separation of the unreacted substrate from the products, and the second is for quantification.

The diribonucleoside (3′→5′) monophosphates (5′-**N**^{1}p**N**^{2}-3′) **I**−**VIII** were chosen such that while we varied the 5′-**N**^{1}p (**N**^{1} = A, G, C and U), the p**N**^{2}-3′ have been kept constant, either to G or A, to examine if the nature of base−base stacking had any influence on the pK_a value of internucleotidic 2′-OH. The pH-dependent internucleotide cleavage rates were plotted to give typical sigmoidal plots (Figure 1), which were analyzed by the Hill plot (Figure S2 and S3 in the Supporting Information) to give the pK_as of internucleotidic 2′-OH within an error limit varying from ±0.02 to ±0.08 pK_a unit. The resulting pK_a of the internucleotidic 2′-OH in all eight

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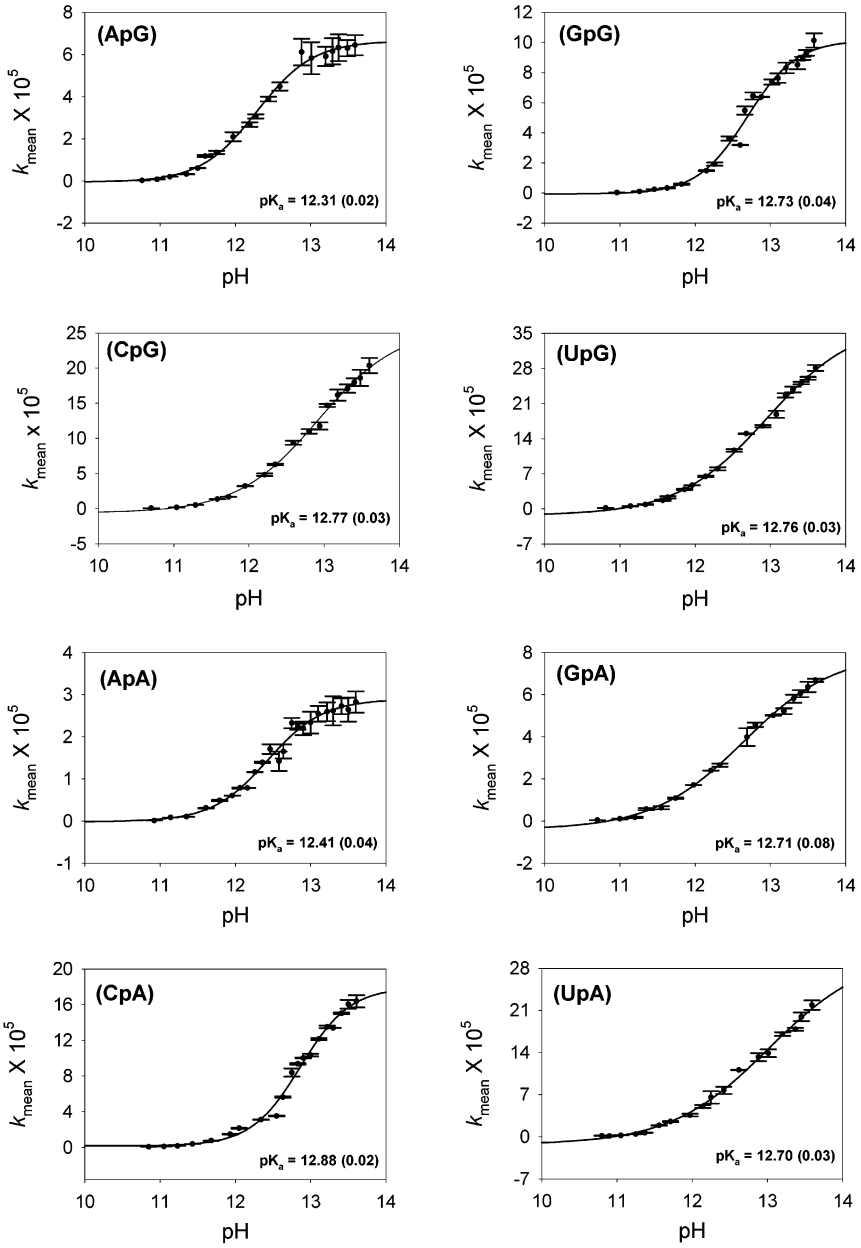

FIGURE 1. Plot of pH-dependent (pH 10.7−13.6) mean rate constants (k_{mean}) for alkaline cleavage of dimers **I**−**VIII** to give the pK_a at the inflection point. The error bars in red show the standard deviation of the rate constants obtained from different marker protons (see Experimental Section and Supporting Information for details).

TABLE 1. The pK_a Values of Diribonucleoside (3′→5′) Monophosphates (I−VIII) by Determination of the pH-Dependent First-Order Rate Constants by ^{1}H NMR at 298 K and Ionic Strength of 1 M NaCl

dimer	pK_a
ApG (**I**)	**12.31 (±0.02)**
GpG (**II**)	12.73 (±0.04)
CpG (**III**)	12.77 (±0.03)
UpG (**IV**)	12.76 (±0.03)
ApA (**V**)	**12.41 (±0.04)**
GpA (**VI**)	12.71 (±0.08)
CpA (**VII**)	12.88 (±0.02)
UpA (**VIII**)	12.70 (±0.03)

diribonucleoside (3′→5′) monophosphates are shown in Table 1. It can be seen that the pK_a values remain almost the same for all internucleotidic 2′-OH for 5′-**N**^{1}p (**N**^{1} = G, C, and U) in dimers **I**−**VIII**, except for the internucleotidic 2′-OH with 9-adeninyl as the 5′-aglycon [12.31 ± 0.02 in ApG (**I**), and 12.41 ±0.04 in ApA (**V**)] which are 0.3 to 0.4 pK_a units more acidic compared to the others shown in Table 1.

To confirm that the aglycones have no significant effect on the pK_as of the respective 2′-OH in comparison with the internucleotidic 2′-OH in diribonucleoside (3′→5′) monophosphates, we have also determined the pK_a values of 2′-OH of monomeric nucleoside 3′-ethyl phosphates **IX**−**XII**. It is noteworthy that these monomeric phosphodiesters, **IX**−**XII**, are the simplest models of a dimer since it does not have any intramolecular base−base stacking. Additionally, the pK_a of their 2′-OH is only affected by the vicinal phosphodiester function, hence will show only the effect of its aglycone. Thus, the plot of

TABLE 2. The pK_a Values of Nucleoside 3′-Ethyl Phosphates (IX−XII) by pH-Dependent ^{1}H Chemical Shift Measurements at 298 K and Ionic Strength of 1 M NaCl

nucleoside 3′-ethyl phosphates	pK_a
ApEt (**IX**)	**12.51 (±0.05)**
GpEt (**X**)	12.74 (±0.06)
CpEt (**XI**)	12.81 (±0.04)
UpEt (**XII**)	12.85 (±0.03)

chemical shifts for the sugar protons (δH1′, δH2′, and δH3′) as a function of pH gave the pK_a values of 2′-OH in nucleoside 3′-ethyl phosphates **IX**−**XII** (Table 2 and Figures S4−S6 in the Supporting Information). Note that the pK_a of their 2′-OH are very comparable to those obtained by the alkaline first order cleavage kinetics for the corresponding dimeric counterparts (Table 1). The only exception is, however, found for 2′-OH in ApEt (**IX**), which falls by ∼0.2 pK_a units compared to the pK_a of the internucleotidic 2′-OH in ApG (**I**) and ApA (**V**) (compare the results in Tables 1 and 2).

Conclusion

The pK_a measurement of internucleotidic 2′-OH in mono- and diribonucleoside (3′→5′) monophosphates (phosphorothioate) had been earlier performed[9,14−22] under various nonuniform experimental conditions showing clear disagreements with each other; hence, they cannot be compared to gain understanding how the sequence-dependent generation of the 2′-oxyanion (with or without a cofactor) could aid the process of ubiquitous transesterification reaction in the RNA processing in biology. Surprisingly, none of these reports show any error bars in the pK_a values, which are expected as a result of intrinsic error generated from the separation and quantification technique used such as HPLC or PAGE. Hence, we have employed a uniform temperature and ionic strength at various pHs for measuring the pK_a values of 2′-OH in eight diribonucleoside (3′→5′) monophosphates by ^{1}H NMR, which is noninvasive in nature, and thereby minimizing the intrinsic error in the quantification of products and the residual reactant at various pH.

It has thus emerged that the presence of adenin-9-yl as an unique aglycon at the 5′-end of diribonucleoside (3′→5′) monophosphates [ApG: 12.31 ±0.02 and ApA: 12.41 ±0.04] causes the pK_a of its internucleotidic 2′-OH to be more acidic by 0.3−0.4 pK_a units compared to that of guanin-9-yl [**G**pG: 12.73 ±0.04 and **G**pA: 12.71 ±0.08], uracil-1-yl [**U**pG: 12.76 ±0.03 and **U**pA: 12.70 ±0.03] or cytosin-1-yl [**C**pG: 12.77 ±0.03 and **C**pA: 12.88 ±0.02] counterparts.

The reason that the internucleotidic 2′-OH of 5′-**A**p moiety of **A**pA or **A**pG is more acidic than the 5′-**G**p counterpart in **G**pA or **G**pG lies in the unique aromatic characters of their respective aglycones, 9-adeninyl vis-à-vis 9-guaninyl groups. Note that the N7 of guanin-9-yl system can be easily protonated (pK_a 1.7), whereas the counterpart in adenin-9-yl system does not act as a proton acceptor under a similar condition because of its poor basic character. This is mainly because of the fact that the imidazolyl moiety in adenin-9-yl drains its π charge successfully to the fused fully aromatic electron-deficient pyrimidine moiety, which is only partly possible in the pseudoaromatic pyrimidine part in guanin-9-yl. This is the reason the imidazolyl moiety in guanin-9-yl system retains a considerable basic character (although rather poorly compared to the basicity of 1-methylimidazole, pK_a 7.25[27]) by poorly conjugating its π charge to the fused 2-amino-6-pyrimidone moiety. Thus, the relatively high electron-deficient character of the imidazolyl moiety in adenin-9-yl allows it to stabilize its 2′-oxyanion more efficiently compared to that of guanin-9-yl, hence causing an increased acidity of its 2′-OH group (compare also with the pK_a of 2′-OH of 3-deaza adenosine, 12.38, to compare the effect on the pK_a of 2′-OH of the fused pyrimidine in adenosine vis-a-vis fused pyridine in 3-deaza adenosine). It is also noteworthy that owing to differential stabilization of the internucleotidic 2′-oxyanions in nucleos(t)ides, one would expect a differential hydration behavior, which may also contribute to the oxyanion stability.

Experimental Section

(A) Sample Preparation. Solutions of diribonucleoside (3′→5′) monophosphates **I**−**VIII** and nucleoside 3′-ethyl phosphates **IX**−**XII** (1 mM) were made in NaOD solutions supplemented with NaCl to keep the ionic strength of Na^+ constant at 1 N at 298 K. No chemical shift change was found to take place up to 3 mM concentration of the dimer, suggesting that our measurements at 1 mM concentration of the dimers involved no aggregation. DSS (3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt, δ = 0.015 ppm) was used as an internal standard for measurement of chemical shifts for compounds **I**−**XII**. pH measurements (pH range 10.7 to 13.1) were made by a pH meter equipped with a calomel microelectrode (see below for calibration). In the pD range of 13.2−14, Na^+ ion supplemented NaOD (0.158 N to 1 N) solutions were standardized by titration with standard solution of 1.0 N sulfuric acid. All solutions were freshly prepared for every experiment. D_2O was made free of dissolved carbon dioxide by flushing with a stream of nitrogen and transferring to a stoppered bottle.

(B) pH Measurements. The pD measurements were made on a Denver instrument AP15pH/mV/FET meter with a Ag/AgCl calomel microelectrode. The electrode was standardized against standard buffers of pH = 7.0 and pH = 10.00. The pH was calculated using the correction of deuterium effect.[25] Each pD of the sample was measured twice, before and after each experiment, and the average value of two successive measurements was used for the titration plots.

(C) NMR Measurements. ^{1}H NMR spectra were recorded at 298 K at 500 or 600 MHz (Bruker AMX) for **I**−**XII**. For compounds **I**−**VIII**, the NMR signals of reactants and products were integrated to calculate the first-order rate constants for cleavage. For compounds **IX**−**XII**, chemical shifts were measured for H1′, H2′, and H3′. Signal volume measurement for the compound **I** and compounds **III**−**VIII** and chemical shift measurements for **IX**−**XII** were done in 500 MHz except for the compound GpG (**II**), where signals of anomeric protons from the reactant dimer GpG and the signals from the anomeric protons of the products of cleavage (δH1′G of 2′GMP, δH1′G of 3′GMP, and δH1′G of guanosine) had considerable overlap, hence spectra were collected at 600 MHz to obtain improved resolution. Error estimation in integration: ±0.3% error for two successive measurements of the same peak.

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(D) Determination of Rate Constant for Decomposition of Dinucleotides I−VIII. The first-order rate constants for internucleotidic 2′-OH cleavage was calculated by plotting $\ln(C/C_0)$ as a function of time (t) where C = amount of dinucleoside (3′→5′) monophosphates left unreacted at time t and C_0 = initial concentration of diribonucleoside (3′→5′) monophosphates (Figure S1 in the Supporting Information). C and C_0 were determined by integration of the volume of the ^{1}H NMR signals of the dimer and the hydrolysis products. For determining the quantity of the hydrolysis products at a time t, we have used either the sum of H1′ of all monophosphates (2′-NMP + 3′-NMP + 2′,3′-cNMP) or H1′ of 5′-OH containing nucleoside generated as a result of hydrolysis, see Supporting Information. Rate constants at 17−23 different pH values were used to calculate the pK_a of the internucleotidic 2′-OH for each dimer with the pH difference between two successive solutions being 0.1−0.3 pH units. Rate constant of cleavage has been estimated using different marker protons for calculating the fraction of reactant left at time t. Average values of the rate constants obtained from different marker protons were plotted as a function of pH to calculate the pK_a for dinucleotides. Details of the mean rate constant calculations using different marker proton resonances at each pH for each dimer along with their respective standard deviations are shown in the Supporting Information. Product peaks in the reaction mixture in each case of dimers **I**−**VIII** were identified by comparing the NMR spectra of the reaction mixture at a particular pH with the NMR spectra of the products, 2′-monophosphates, 3′-monophosphates, 2′,3′-cyclic phosphates, and the ribonucleosides separately at that pH.

(E) pK_a Determination. The pK_as of the internucleotidic 2′-OH groups of **I**−**VIII** were determined by plotting first-order rate constants at 17−23 different pHs (0.2−0.3 pH intervals) at 298 K. The pK_as of the 2′-OH group of nucleoside 3′-ethyl phosphates **IX**−**XII** were determined by plotting pH-dependent chemical shifts (H1′, H2′, and H3′) at 298K (19−20 different pHs) as shown in Figure S4. The pH differences of 0.1−0.3 were used between two successive solutions for NMR measurements. pK_a values were estimated by Hill plot analyses (vide infra) (Figure S3 for **I**−**VIII** and Figure S6 for **IX**−**XII** in the Supporting Information). The 2′-OH pK_a in **IX**−**XII** was estimated from the average of the pK_a values obtained from Hill plot analyses of δH1′, δH2′, and δH3′ for each compound. The curves fitted through the experimental points using nonlinear least-squares fitting procedure to the Henderson−Hasselbalch eq 1.[26]

$$\mathrm{pH} = \mathrm{p}K_a + \log [A^-]/[AH] = \mathrm{p}K_a + \log(1-\alpha)/\alpha \quad (1)$$

where α is a mole fraction of the protonated state. The end point (or plateau) at high pH could not be measured for all compounds because the [Na^+] concentration was restricted in all cases to 1 M (note, one requires at least 3 M [Na^+] to reach pH 14.5). Hence, the first-order rate constants (for **I**−**VIII**) and chemical shifts (for **IX**−**XII**) at pH value above our maximum pH of 13.5 was determined using the modified Henderson equation (2a and 2b):[26,28]

$$k_{obsd} = k_h + 1/K_a(k_l - k_{obsd})a(H^+) \quad (2a)$$

$$\delta_{obsd} = \delta_h + 1/K_a(\delta_l - \delta_{obsd})a(H^+) \quad (2b)$$

where, k_h is the first-order rate constant at high pH for **I**−**VIII**, δ_h is chemical shift at high pH for **IX**−**XII**; $a(H^+)$ is the concentration of proton, k_l is the first-order rate constant for **I**−**VIII**, δ_l is the chemical shift for **IX**−**XII** at low pH, k_{obsd} is the first-order rate constant for **I**−**VII** and δ_{obsd} is the chemical shift for **IX**−**XII** at an observed pH. Equation 2a[26,28] gives a straight line with a slope $1/K_a$ and k_h value at intercept (Figure S2 in the Supporting Information). Equation 2b gives a straight line with a slope $1/K_a$ and δ_h value at intercept (Figure S5 in the Supporting Information). The values of intercepts (k_h and δ_h) have been used in the calculation of respective Hill plots. The pK_a determination is based on the Hill plot analysis using equation: $\mathrm{pH} = \log((1-\alpha)/\alpha) + \mathrm{p}K_a$, where α represents fraction of protonated species. The value of α is calculated from the change of first order k for **I**−**VIII** and δ for **IX**−**XII** relative to the deprotonated (D) state at a given pH ($\Delta_D = k_D - k_{obsd}$ for deprotonation, where k_{obsd} is the experimental first-order rate constant for **I**−**V111** and $\Delta_D = \delta_D - \delta_{obsd.}$ for deprotonation, where δ_{obsd} is the experimental chemical shift for **IX**−**XII** at a particular pH), divided by the total change in first order k for **I**−**VIII** and δ for **IX**−**XII** between neutral and deprotonated (D) state (Δ_T). So the Henderson−Hasselbalch type equation can then be written as $\mathrm{pH} = \log((\Delta_T - \Delta_D)/\Delta_D) + \mathrm{p}K_a$. The p$K_a$ is calculated from the linear regression analysis of the Hill plot (Figure S3 for **I**−**VIII** and Figure S6 for **IX**−**XII** in the Supporting Information).

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Supporting Information Available: Rate constants for alkaline cleavage at various pHs (17−23 pHs) for dimers **I**−**VIII** (Figure S1), pH-dependent chemical shifts for nucleoside ethyl phosphates **IX**−**XII** (Figure S4), graphical determination of rate constants for alkaline cleavage of dimers **I**−**VIII** (Figure S2), and for chemical shifts for nucleoside ethyl phosphates **IX**−**XII** at a highest pH (Figure S5), Hill plot analysis for the pH-dependent rate constants for dimers **I**−**VIII** (Figure S3), and for nucleoside ethyl phosphates **IX**−**XII** (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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