The Negative Charge of Alkyl Phosphate Diesters and the Slow-Gaited Hydrolysis of RNA and DNA. Catalysis of RNA Hydrolysis through Metal Ion Ligation to the Ester $>PO_2^-$ Moiety

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The catalysis of the hydrolysis of mono- and trialkyl phosphate esters has garnered considerable attention. The mechanisms of hydrolysis of dialkyl phosphate esters has received much less study. This is due to the fact that the lifetime of human investigators is much shorter than the $t_{1/2}$ for hydrolysis of dialkyl phosphate esters near neutrality {estimated[1] k_{obsd} at pH 7 for hydrolysis of dimethyl phosphate is 2×10^{-14} s^{-1}}. The hydrolytic stability of dialkyl phosphate linkages explains their presence as the repeating linkers in biopolymers responsible for the storage (DNA) and transmission (RNA) of genetic information. A keen present interest in the mechanisms for hydrolysis and nucleophilic displacement with phosphodiesters attends the ardent interest in enzymes which readily cleave the phosphodiester bonds of DNA and RNA, as well as the self-cleavage of RNA by ribozymes. Conceptually, enzymatic hydrolysis of RO-(PO_2^-)-OR′ without formation of enzyme covalent intermediates may involve three catalytic processes: (i) general-base catalysis of nucleophilic attack of H_2O on phosphorus; (ii) general-acid catalysis of departure of the leaving R′O$^-$; and (iii) cancellation of the negative charge on the $>PO_2^-$ moiety and stabilization of any pentacovalent intermediate by association of an acid or metal species with one or both oxygens. We report here the catalysis of hydrolysis of an RNA analogue by ligation of Zn^{2+} on the negative oxygen of the $>PO_2^-$ (iii) moiety where it can stabilize a pentacoordinate intermediate (or transition state).

The general procedures of searching for Zn^{2+} adenosine 3′-phosphate ester complexes as suitable substrates was carried out as indicated for **1**. An energy-minimized structure of the Ad-ribose-O-(PO_2^-)-O-CH_2-H portion of the molecule was created on a Silicon Graphics 4D/340GTX work station with the programs Quanta 3.3 and CHARMm 22 (Molecular Simulations, Waltham, MA). The coordinates of various X-ray structures[2] of (H-ligand)$_n$$Zn^{2+}$ were chosen from the Cambridge structural data base and transferred to the 4D/340GTX, where the structure for [Ad-ribose-O-(PO_2^-)-O-CH_2-ligand]Zn^{2+} was created. Covalently linking the 2-position of 8-hydroxyquinoline in its complex with Zn^{2+} {(8-HQ)$Zn(H_2O)_2$} provided (**1**)$Zn(H_2O)_2$. In (**1**)-$Zn(H_2O)_2$, the phosphate oxyanion may replace one of the water molecules on the tetrahedral zinc, thereby neutralizing the negative charge on the phosphate. This would appear to be the only reasonable means of catalysis of the hydrolysis of **1** by Zn^{2+}. In this structure, the bond distance and the angle from Zn–O–P (115°) are reasonable (see stereo presentation of Figure 1). It is not possible for Zn^{2+} to ligate with the oxygen leaving group (caption to Figure 1), and ligation of Zn^{2+} with the 2′-OH would require formation of a 12-membered ring with proper orientation about six single bonds.

The preparation of **1** was accomplished in five steps (Scheme 1), utilizing phosphoramidite chemistry similar to that previously used by Modak et al.[3] The substituted quinoline **2** was prepared by a three-step process, starting with the protection of 8-hydroxyquinoline with the *tert*-butyldiphenylsilyl group. Subsequent oxidation of the 2-methyl group with selenium oxide and reduction of the resulting aldehyde with sodium borohydride led to the alcohol. Coupling of **2** with N^6-benzoyl-5′-(4,4′-dimethoxytrityl)-2′-(*tert*-butyldimethylsilyl)adenosine 3′-[β-cyanoethyl-*N*,*N*-diisopropylphosphoramidate] (**3**) in the presence of tetrazole afforded the intermediate **4** in 71% yield. Oxidation of **4** with *tert*-butyl hydroperoxide in methylene chloride followed by cleavage of the 5′-dimethoxytrityl protecting group with 2% dichloroacetic acid in methylene chloride yielded the diastereomers of intermediate **5**, which could be isolated separately by silica gel column chromatography. The diastereomers present nearly

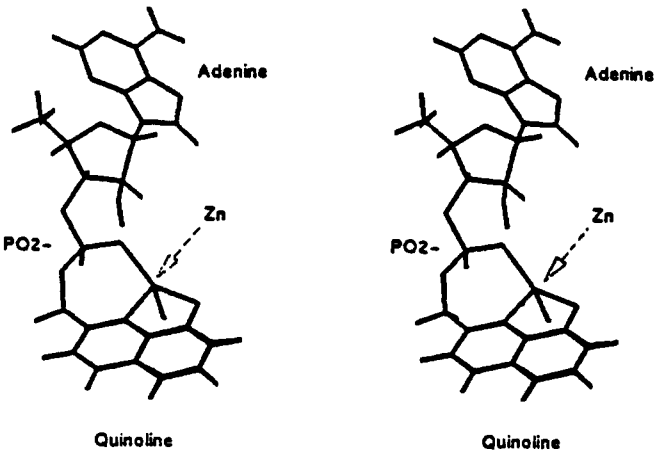
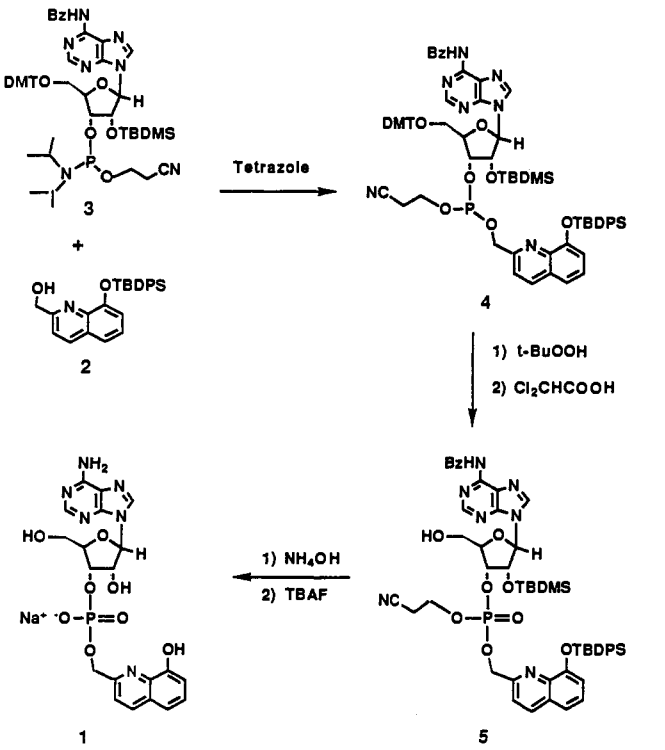


Figure 1. Stereoview of the structure of (**1**)Zn with the phosphate oxyanion ligated to the zinc at a bond length of 1.98 Å. The distance between zinc and the leaving oxygen (3.36 Å) is too far for interaction, and any bonding would require distortion of the zinc tetrahedral structure by creation of a N···Zn^{2+}···O angle of 60°. Thus, catalysis of hydrolysis with zinc assisting in the departure of the leaving group is unlikely. As proposed for the cleavage site of a hammerhead RNA,[6] the conformation of the ribose is C′-2 endo, such that the 2′-OH is in position for an in-line displacement of the leaving aliphatic hydroxyl oxygen of the 2″-(hydroxymethyl)-8″-hydroxyquinoline moiety.

Scheme 1



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(3) Modak, A. S.; Gard, J. K.; Merriman, M. C.; Winkeler, K. A.; Bashkin, J. K.; Stern, M. K. *J. Am. Chem. Soc.* **1991**, *113*, 283.

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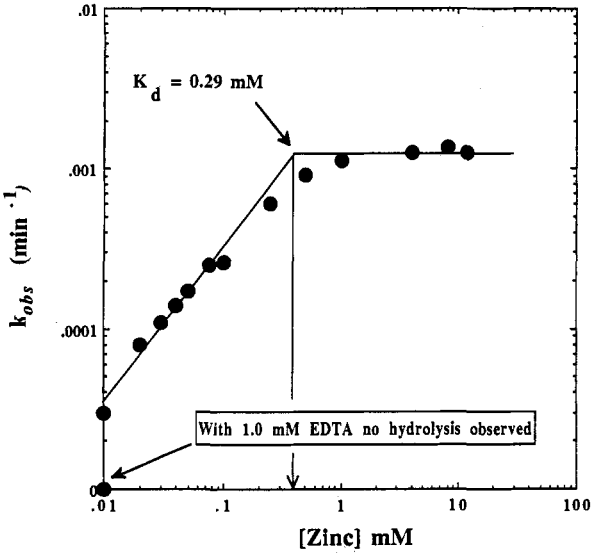
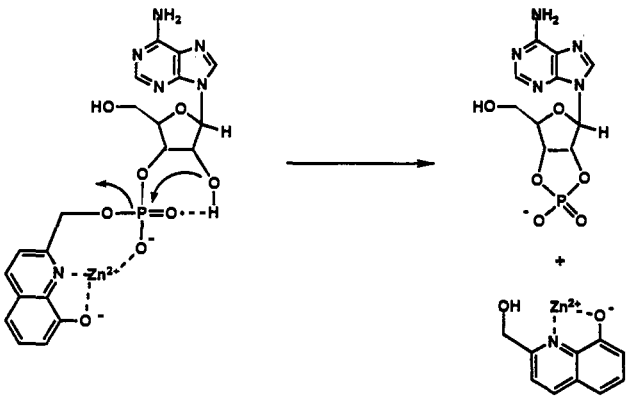


Figure 2. Plot of the dependence of the log of the pseudo-first-order rate constant (k_{obsd}) for the hydrolysis of **1** on the log of the concentration of Zn^{2+} (pH 6.5 in cacodylate buffer, μ = 1.0 with KCl, 30 °C). The ascending slope of +1 establishes the stoichiometry of the reactive complex to be (**1**)Zn. The kinetic apparent equilibrium constant for (**1**)Zn formation (K_d = 0.29 mM) is identical to the thermodynamic value obtained by spectrophotometric titration.

identical ^{1}H NMR spectra, with the greatest difference in the chemical shifts noted for the dimethylsilyl protons. The diastereomers of **5** were converted to the convergent product **6** by treatment with ammonium hydroxide for 1.0 h at 60 °C, which cleaved the cyanoethyl group in addition to the N^6-benzoyl and the *tert*- butyldiphenylsilyl protecting groups in good yield. The target nucleotide **1** was obtained by cleaving the 2′-*tert*-butyldimethylsilyl group with tetrabutylammonium fluoride in THF, followed by conversion to the sodium salt form of the nucleotide by cation-exchange chromatography. Purification of **1** was performed by reverse-phase chromatography followed by recrystallization from ethyl acetate–hexane. Titration of **1** with $ZnCl_2$ at pH 6.4 shows that the formation of a complex with Zn^{2+} is accompanied by a change in λ_{max} of **1** from 301 to 366 nm. By spectral titration of **1** with Zn^{2+} and monitoring of A_{366}, a dissociation constant (K_d) of 3.0 × 10^{-4} M was determined for the 1:1 complex (**1**)Zn.

Hydrolysis of (**1**)Zn was followed by HPLC, using an Altima C18 analytical column (Alltech) and eluting with 20% acetonitrile in 50 mM pH 3.4 aqueous potassium phosphate buffer at a flow rate of 1.0 mL/min. The concentrations of (**1**)Zn and products were monitored at 260 nm. In a typical reaction, aliquots were withdrawn at intervals from a solution of **1** (10 μM) and $ZnCl_2$ (up to 15 mM) in cacodylate buffer (pH 6.5, μ = 1.0 with KCl) maintained at 30 °C. The first-order disappearance of **1** was independent of buffer concentration. Hydrolysis of **1** is accompanied by the appearance of four new peaks with retention times identical with those of standards of 2′,3′-cyclic AMP, 3′-AMP, 2′-AMP, and 2-(hydroxymethyl)-8-hydroxyquinoline. The major immediate products of the reaction were 2′,3′-cyclic AMP

Scheme 2



plus 2-(hydroxymethyl)-8-hydroxyquinoline (Scheme 2). The minor species 3′-AMP and 2′-AMP were produced in roughly equal concentrations and must result from the hydrolysis of 2′,3′-cyclic AMP.

Pseudo-first-order rate constants (k_{obsd}, s^{-1}) were obtained by fitting the plots of the integrated areas of the substrate peak vs time to the first-order rate law. No buffer catalysis was observed. At saturation in [Zn^{2+}], the value of k_{obsd} is pH-independent between pH 5 and 6.2 and increases with an increase in pH from 6.2 to 7.0, and precipitation of zinc hydroxide is noted at higher pH values. These observations are consistent with participation of both 2′-OH and 2′-O^- as intramolecular nucleophiles. Plots of k_{obsd} vs added [$ZnCl_2$] exhibit saturation. Results obtained at pH 6.5 are shown in Figure 2. The kinetically determined constant (K_d = 2.9 × 10^{-3} M) for dissociation of Zn^{2+} from (**1**)Zn is identical to the thermodynamic values determined by spectral titration. Hydrolysis of **1**, therefore, takes place through the formation of (**1**)Zn, which decomposes to 2′,3′-cyclic AMP plus the zinc complex of 2-(hydroxymethyl)-8-hydroxyquinoline with a rate constant (k_{obsd}) equal to 2.34 × 10^{-5} s^{-1} at saturation (pH 6.5) (Scheme 2). At nonsaturating concentrations, the rate of hydrolysis of **1** shows a strong dependence on [Zn^{2+}]. Without added $ZnCl_2$, and in the presence of 1.0 mM EDTA to sequester trace heavy metal contaminants, no measurable hydrolysis of **1** was detected at pH 6.5 over a 3-month period. A 1% hydrolysis of **1** in 4 months would provide a first-order rate constant of ~1 × 10^{-9} s^{-1}, which would be the maximum possible rate of hydrolysis of **1** in the absence of metal (in actuality, no hydrolysis has been observed). The minimal rate enhancement for hydrolysis of **1** by complexation to Zn^{2+} is then >10^4. Though not previously established, enhancement of the rate of hydrolysis of phosphate esters by interaction of the PO_2^- moiety with positive metal ions is not unexpected.[4,5]

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