Convergent Functional Groups. 16. Hydrolysis of Phosphate Triesters by a Novel Cleft. Influence of Binding on Overall Rate Acceleration

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Received June 23, 1994[⊗]

Abstract: The rates of hydrolysis of two activated phosphate triesters, bis(*p*-nitrophenyl) methyl phosphate **3** and adenosine 5′-*O*,*O*-bis(*p*-nitrophenyl) phosphate **4** have been studied using two copper-containing catalysts, Cu(Kemp-bpy) **1** and Cu(bpy) **2**. The catalytic activities of these two species have been compared within the pH range of 6−9.5. The rates of hydrolysis of both substrates, **3** and **4**, have been compared to the uncatalyzed reaction. This rate-enhancement is dependent on pH. In both cases, the monohydroxo $H(OH^-)^+$ species is more catalytically active than the diaqua species, $H(H_2O)^{2+}$. The effect of binding on overall rate acceleration was evaluated; the maximum contribution was found to be 44.

Introduction

As part of an effort toward designing a site-specific chemical nuclease, we intend to combine both recognition and catalytic elements on a novel receptor.[1] Previous work in this laboratory has shown that cleft-like receptors can be built efficiently and incrementally by convergent modular synthesis from Kemp's triacid.[2−4] High-affinity adenine receptors have been developed using two convergent imide surfaces for simultaneous Watson−Crick and Hoogsteen base pairing in addition to π-stacking to a carbazole scaffold. The carbazole nitrogen has provided a convenient handle for further functionalization. Here we report the incorporation of a metal ligand complex in order to evaluate the effect of substrate binding on phosphate ester hydrolysis.

The hydrolysis of phosphate triesters is known to be promoted by transition metals, such as Cu^{2+},[5−8] Zn^{2+},[5,6,9−12] and lanthanides.[13] The activity of the metal−ligand complex depends on not only its exchange properties and its geometry but also the acidity of the coordinated water molecules. Promoting intracomplex hydrolysis of phosphate esters requires a geometry for the reaction complex in which the coordinated hydroxide ion or water is *cis* with respect to the bound phosphate.[10] To achieve such geometry, the metal must have adjacent coordination sites unoccupied. This geometry can be achieved using a Cu(II) catalyst. Accordingly, we have chosen to incorporate the well-studied Cu(II) bipyridine system into our adenine receptor. If the receptor is able to bring the catalyst and substrate together in the correct orientation, then enhanced rates of hydrolysis should result. The design, synthesis, and binding studies of this cleft have been described earlier.[1] Here, we present mechanistic studies of the catalytic hydrolysis of phosphate triesters with these systems.

Experimental Section

General. All chemicals were purchased from Aldrich Chemical Co. and were used without further purification. MES, HEPES, TAPS, and CHES buffers were purchased from Sigma Chemical Co. Ultraviolet−visible spectra were taken on a Perkin-Elmer Lambda II spectrophotometer equipped with a Haake D1 circulating water bath. Data were collected using the program PECSS, Perkin Elmer Computer Software System. Nonlinear regressions were performed using the program Systat 5.2. pH titrations were performed with a SPER scientific Digital pH meter. The concentration of NaOH was determined by titration with standardized HCl. UV cuvettes, supracil grade with 10 mm path length, were purchased from Hellma Cells, Inc. ^1H and ^{31}P NMR spectra were obtained on Varian XL-300 or Varian UN-300 spectrometers. All ^1H chemical shifts were reported in ppm and were referenced to residual solvents. ^{31}P chemical shifts were reported in ppm with an external reference of 85% H_3PO_4 in DMSO-d_6 at 0 ppm.

Synthesis. Synthesis of receptor Kemp-bpy has been described previously.[1] The syntheses of bis(*p*-nitrophenyl) chlorophosphate and adenine 5′-*O*,*O*-bis(*p*-nitrophenyl) phosphate (**4**) were according to established procedures.[14,15]

Kinetics. Buffers used were within a pH range of 6−9.5. Concentrations of buffers were 0.01−0.09 M with an ionic strength of 0.1 M ($NaNO_3$). Stock solutions of $Cu(NO_3)_2$ (1.6×10^{-3} M) and bipyridine (3.5×10^{-2} M) in respective buffers were used; receptor **1** (0.01 M), bis(*p*-nitrophenyl) methyl phosphate (**3**) (4.2×10^{-3} M), and adenosine 5′-*O*,*O*-bis(*p*-nitrophenyl) phosphate (**4**) (2.433×10^{-3} M) in anhydrous methanol were prepared.

A 1:1 mixture of $Cu(NO_3)_2$ and receptor **1** or bipyridine was used to give the corresponding Cu(Kemp-bpy) **1** or Cu(bpy) **2**, respectively. Binding of Cu^{2+} to ligand **1** was confirmed by UV titrations.[1] Upon addition of Cu^{2+} to a solution of ligand **1**, a characteristic decrease in intensity at 282 nm accompanied by the appearance of shoulders at

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[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1995.

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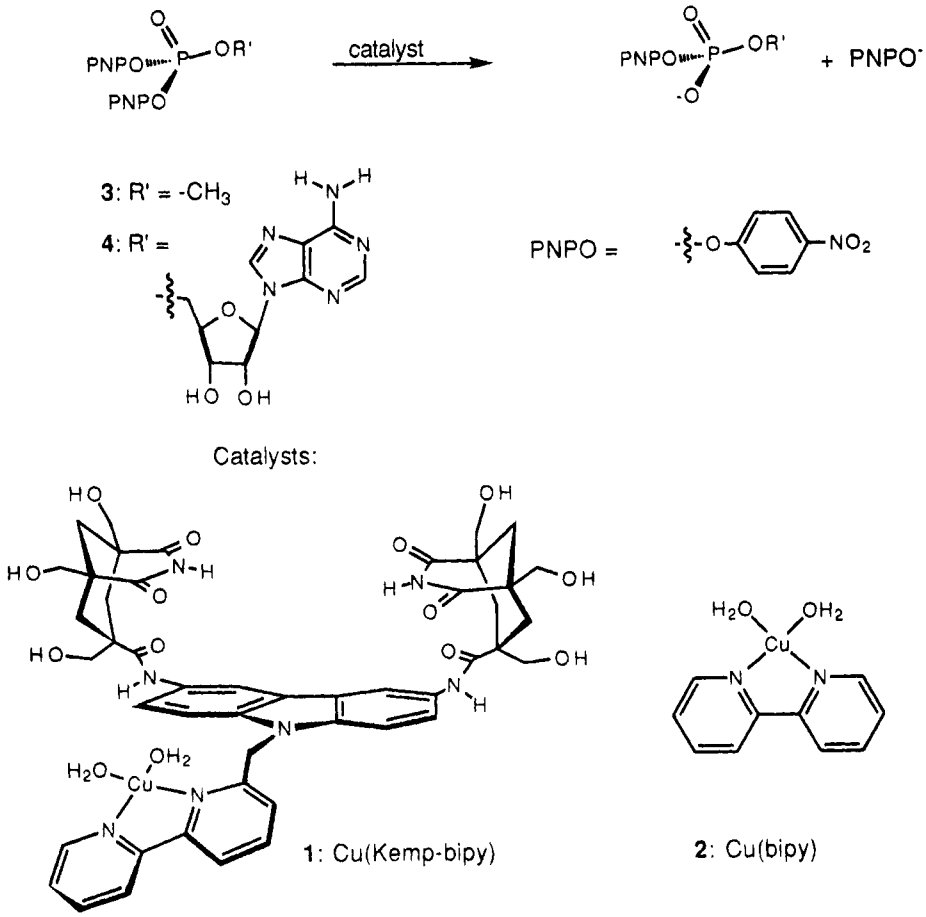

Figure 1. The overall hydrolysis reaction.

297 and 318 nm was observed. These observations are consistent with coordination of Cu^{2+} to bipyridine.[16] To a cuvette containing 0.1−1 equiv of $Cu(Kemp\text{-}bpy)^{2+}$ **1** or $Cu(bpy)^{2+}$ **2** in the appropriate buffers was added 1 equiv of phosphate solution, **3** or **4**. The total volume in the cuvette was 1 mL, and the total concentration of phosphate was kept constant at 7.0×10^{-5} or 3.5×10^{-5} M. The total concentrations of catalysts varied between 3.5×10^{-6} and 7.0×10^{-5} M. The rate of hydrolysis was determined spectrophotometrically at 400 nm, which corresponds to the release of *p*-nitrophenolate. Rate constants were determined by fitting to first-order rate equations.

Product Analysis. Reaction mixtures from 10 to 20 kinetic runs were combined. Cu^{2+} was removed by cation exchange using a Sephadex SP-C25 column, Na^+ form, 1.5 × 10 cm, with H_2O as eluant. Water was then removed by rotary evaporation and products were analyzed by ^{31}P NMR. ^{31}P NMR (DMSO-d_6, reference to 85% H_3PO_4 in DMSO-d_6): δ −12.306 (bis(*p*-nitrophenyl) methyl phosphate), −12.928 (mono(*p*-nitrophenyl) methyl phosphate sodium salt), −123.859 (adenosine 5′-*O*,*O*-bis(*p*-nitrophenyl) phosphate), −93.066 (adenosine 5′-*O*-(*p*-nitrophenyl) phosphate sodium salt).

Molecular Modeling. All molecular modeling was performed on a Silicon Graphics 4D30G+ with MacroModel 3.5X. The conformations of the complexes were derived by minimization using the MULT routine and TNCG algorithm, all-atom AMBER* force field, and GB/SA continuum water solvation.

Results and Discussion

Mechanism. Hydrolyses of adenosine 5′-*O*,*O*-bis(*p*-nitrophenyl) phosphate (**4**) or bis(*p*-nitrophenyl) methyl phosphate (**3**) have been studied using both our receptor, Cu(Kemp-bpy) **1**, and Cu(bpy) **2** as catalysts. The overall reaction is shown in Figure 1. The products of the reactions were analyzed by ^{31}P NMR. The chemical shifts of the products were compared to authentic samples of diesters (see Experimental Section). In both cases, no further hydrolysis to the corresponding phosphate monoesters took place.

All relevant association processes and reaction steps are presented in Figure 2.

Under our reaction conditions, the Cu(bpy) unit of catalysts **1** or **2** exists in two forms. Both the acidic, $H(H_2O)_2^{2+}$, and basic forms, $H(OH^-)^+$, are kinetically active.[8] First of all, bimolecular reactions between the substrates, **3** or **4**, and the acidic, $H(H_2O)_2^{2+}$, or basic, $H(OH^-)^+$, components of the catalysts are described by routes 1 and 2. Routes 3 and 4 are operational only if formation of association complexes between the catalyst and the substrate is possible. In this case, the substrate can bind to either the acidic, $H(H_2O)_2^{2+}$, or basic, $H(OH^-)^+$, form of the catalyst. This would give the respective host−guest complexes with association constants K_b^1 and K_b^2. These host−guest complexes can turn over to the respective host−product complexes with catalytic rate constants of k_3 and k_4. Finally, there remains the specific-base-catalyzed hydrolysis of the substrate as described by route 5.

The overall rate law can be written as follows:

$$\frac{dP}{dt} = (k_1[H(H_2O)_2^{2+}] + k_2[H(OH^-)^+])[G] + k_3[H(H_2O)_2^{2+}\text{-}G] + k_4[H(OH^-)^+\text{-}G] + k_{un}[OH^-][G] \quad (1)$$

Substituting the equilibrium expressions and mass balance equation into (1) and reexpressing it in terms of the total concentrations of host and guest gives after some simplification:

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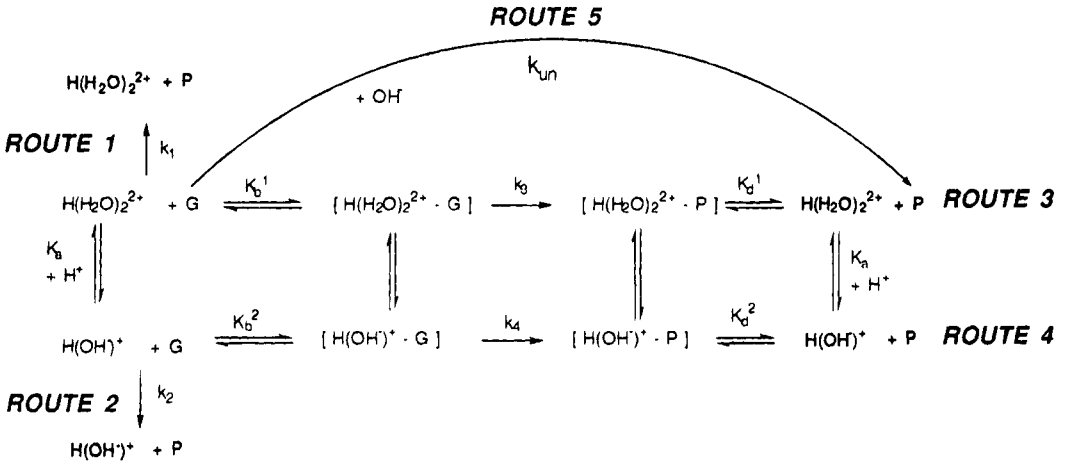

$H(H_2O)_2^{2+}$ is Cu(Kemp-bipy)$(H_2O)_2^{2+}$ **1a** or Cu(bipy)$(H_2O)_2^{2+}$ **2a**, $H(OH^-)^+$ is Cu(Kemp-bipy)$(OH^-)^+$ **1b** or Cu(bipy)$(OH^-)^+$ **2b**, G is bis(p-nitrophenyl) methyl phosphate **3** or adenosine 5'-O,O-bis(p-nitrophenyl) phosphate **4** and P is the product.

Figure 2. The mechanism of hydrolysis of bis(*p*-nitrophenyl) methyl phosphate (**3**) or adenosine 5′-*O*,*O*-bis(*p*-nitrophenyl) phosphate (**4**) catalyzed by receptor **1** or catalyst **2**.

$$k_{obs} = \frac{(k_1 + k_3K_b^1)[H^+] + (k_2 + k_4K_b^2)K_a}{[H^+] + K_a}[H]_t + k_{un}[OH^-] \quad (2)$$

Assuming that the binding constants to both $H(H_2O)_2^{2+}$ and $H(OH^-)^+$ are the same, that is, $K_b^1 = K_b^2 \equiv K_b$, then, eq 2 simplifies to eq 3.

$$k_{obs} = \frac{(k_1 + k_3K_b)[H^+] + (k_2 + k_4K_b)K_a}{[H^+] + K_a}[H]_t + k_{un}[OH^-] \quad (3)$$

Since Cu(bpy) **2** possesses no recognition element, both routes 3 and 4 in Figure 2 can be neglected and the rate expression simplifies to:

$$k_{obs} = \frac{k_1[H^+] + k_2K_a}{[H^+] + K_a}[H]_t + k_{un}[OH^-] \quad (4)$$

The acid dissociation constant, K_a, for the catalyst **1** was determined by titration with known concentrations of NaOH ($pK_{a_1} = 6.1 \pm 0.5$). The acid dissociation constant for Cu(bpy) at 25 °C ($pK_{a_1} = 7.9$) determined by Gustafson and Martell[17] was used for calculating various rate constants for Cu(bpy). Unlike Cu(bpy) which dimerizes with K_d of 1×10^5 M^{-1} at 25 °C, titrations of Cu^{2+} with ligand **1** indicated a 1:1 stoichiometry. The dimerization pathway was inhibited sterically. However, in the case of Cu(bpy), at concentrations higher than 10^{-5} M, dimerization decreases the amount of active catalyst. This has been taken into account in calculating various rate constants.

Catalytic Hydrolysis. To assure that the U-shaped cleft does not interfere with the catalytic Cu(bpy) group, both receptor **1** and Cu(bipy) **2** were evaluated as catalysts toward substrate **3**. The effect of binding was then investigated using substrate **4**.

According to eq 1, linear regression of a plot of k_{obs} vs $[H]_t$ gives $\{(k_1 + k_3K_b[H^+] + (k_2 + k_4K_b)K_a\}/\{[H^+] + K_a\}$ as the slope and the background first-order rate constant, $k_{un}[OH^-]$, as intercept. Then a plot of the slope vs $[H^+]$ gives the catalytic rate constants, k_3 and k_4, and the binding constant, K_b. The various rate constants are presented in Table 1.

Within the pH range under investigation, the copper–hydroxo species, $H(OH^-)^+$, is always more active than the copper–diaqua species, $H(H_2O)_2^{2+}$, that is, $H(OH^-)^+$-dependent terms, k_2 and k_4, are always larger than $H(H_2O)_2^{2+}$-dependent terms, k_1 and k_3. The second-order rate constants for the hydrolysis of adenosine 5′-*O*,*O*-bis(*p*-nitrophenyl) phosphate (**4**) catalyzed by Cu(Kemp-bpy) **1** and calculated association constants at different pH's are listed in Table 2.

It is seen from Table 1 that the second-order rate constants, k_1 and k_2, describing routes 1 and 2 are similar for both our receptor **1** and Cu(bpy) **2**. Therefore, it may be assumed that

Table 1. List of Catalytic Rate Constants for Hydrolysis of Bis(*p*-nitrophenyl) Methyl Phosphate (**3**) and Adenosine *O*,*O*-Bis(*p*-nitrophenyl) Phosphate (**4**) Catalyzed by Cu(Kemp-bpy) **1** and Cu(bpy) **2** at 25 °C[a]

substrate	catalyst	k_1 (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})	k_3 (s^{-1})	k_4 (s^{-1})
3	Cu(bpy) **2**	0.75	1.2		
3	Cu(Kemp-bpy) **1**	0.75	1.76		
4	Cu(bpy) **2**	0.5 ± 0.09	1.2 ± 0.7		
4	Cu(Kemp-bpy) **1**	0.5 ± 0.09	1.2 ± 0.7	0.08 ± 0.002	0.7 ± 0.05

[a] Where substrate **3** is MeO-P(O)-$OPNP_2$, substrate **4** is Ad-OP(O)-$OPNP_2$, k_{un} is 1 M^{-1} s^{-1}; $pK_{a_1} = (6.2 \pm 0.5)$ for Cu(Kemp-bpy) **1**; $pK_{a_1} = (7.9)$ for Cu(bpy) **2**. Studies were performed at 25 °C from pH 6 to 9.5 with buffer concentrations ranging from 0.01 to 0.09 M.

Table 2. List of Second-Order Rate Constants and Calculated Association Constants for the Hydrolysis of Adenosine 5′-*O*,*O*-Bis(*p*-nitrophenyl) Phosphate (**4**) Catalyzed by Cu(Kemp-bpy) **1** at Various pH at 25 °C[a]

				ref rate	
pH	K_b (M^{-1})	k_3K_b (M^{-1} s^{-1})	k_4K_b (M^{-1} s^{-1})	k_3K_b/k_1	k_4K_b/k_2
6	75	6	52.5	12	44
7.5	24	1.9	16.8	3.8	14
8.5	13	1.0	9.1	2.0	7.6
9.5	9.4	0.8	6.6	1.6	5.5

[a] Where substrate is Ad-OP(O)-$OPNP_2$ **4** and catalyst is Cu(Kemp-bpy) **4**. Studies were performed at 25 °C with buffer concentrations ranging from 0.01 to 0.09 M and ionic strength 0.1 M.

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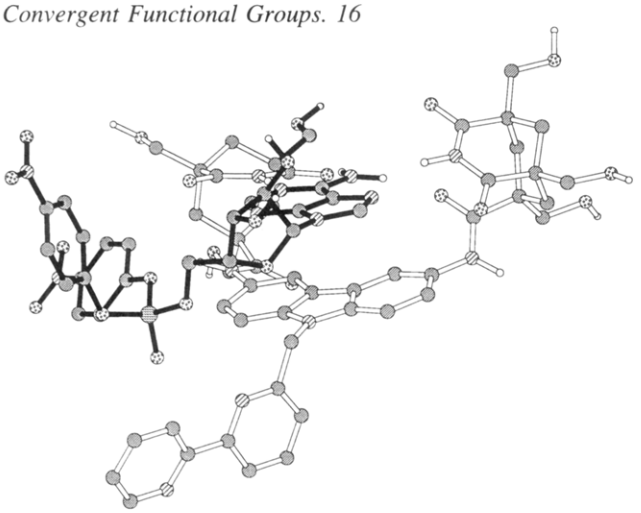

Figure 3. Minimized structure of the host-guest complex between **4** (darkened structure) and metal free **1** using the program "MacroModel".

receptor **1** is as catalytically active as Cu(bpy) **2** and that incorporation of a rigid binding domain does not affect its catalytic ability. This also allowed us to substitute values for k_1 and k_2 obtained using Cu(bpy) **2** into eq 1 in order to determine rate constants, k_3 and k_4, describing routes 3 and 4.

The data of Table 2 show that the affinity of adenosine phosphate **4** toward our receptor **1** is strongly pH dependent. At lower pH, where binding is more significant, routes 3 and 4 in Figure 2 dominate. As binding becomes weaker at high pH, the receptor begins to behave like Cu(bipy) **2** itself. The decrease of binding affinity upon increasing pH can be attributed to the deprotonation of the imide on receptor **1**. The pK_a of the imide is 9.6 at 25 °C.[18,19] As pH approaches the pK_a of the imide, complex formation is discouraged.

Influence of Binding. Productive binding involves the correct positioning of the reactive groups and enhances rates of reactions. Molecular modeling using MacroModel suggested that binding of the adenine portion of **4** to the receptor of **1** would position the 5′-phosphate in close proximity to the bpy unit (see Figure 3). The proximity of the phosphate moiety to the Cu(bpy) unit allowed facile coordination to the metal. The hydrolysis reaction presumably followed the double-activation pathway proposed by Trogler and Morrow.[7] A coordinated phosphate intermediate is formed, followed by intramolecular hydrolysis by reaction with cis-coordinated water or hydroxide.

Contributions of binding toward rates of hydrolysis of adenosine phosphate **4** can be interpreted using the relative rates shown in Table 2. A maximum of 44-fold was found when comparing the intracomplex versus the un-bound rate constants. It is also apparent that the optimum pH for maximal rate enhancement is at pH 6. The limit of rate enhancement depends on not only the affinity of Cu(Kemp-bpy) **1** for adenosine phosphate **4** but also the concentration of the copper−hydroxo species. This is implicated as the active catalyst at alkaline pH.[7] While the concentration of the copper−hydroxo species increases with increasing pH, the binding affinity of the imide cleft decreases. These opposing effects, thus, determine the optimum pH for rate acceleration to be 6.

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Conclusion

We have described here our first attempt to use a molecular receptor based on directional hydrogen bonds in aqueous solution as a substrate binder for a transition metal catalyst. Hydrogen bonding in the competitive solvent water is weak but can be achieved when combined with hydrophobic binding to aromatic surfaces.[1,20] Although the adenine affinity of the receptor used in this work ranks among the highest known in organic solvents, in water only moderate affinity ($K_b \sim 100$ at 10 °C) remains.[1] Water is the most biorelevant solvent and therefore a successful enzyme model should exhibit substrate recognition (involving also H-bonding) and catalysis in this solvent. We developed here an assay to evaluate the influence of recognition on the catalytic hydrolysis of phosphotriesters. Although phosphotriesters as models for RNA are of limited value,[21] they do allow the evaluation of the geometric constraints involved in metal catalyzed hydrolysis reactions and their higher reactivity compared to phosphodiesters allows for kinetics within a more convenient time window.

In this work a modest rate enhancement was obtained from the kinetic analysis. The results are supported by the magnitude and pH dependence of the kinetic association constants, which are in good agreement with values obtained from binding studies.[1] Moreover, the geometry similar to the one required for intracomplex catalysis here has resulted in 10^3-fold rate enhancements in aminolysis reactions.[22] We do feel that interpreting kinetic data from a system with many equilibria and reaction pathways requires caution. Due to the complexity of the system several uncertainties remain. The lower pK_a of the copper bound water molecules of Cu(Kemp-bpy) as compared to Cu(bpy) may give the former a certain advantage. In addition the degree of Cu(bpy) dimerization, although accounted for in the analysis, possibly complicates matters. The most serious limitation of the system is the low substrate affinity of the receptor, which in several of our experiments is less than 1% saturated with the adenosine phosphotriester. Accordingly, we have been unable to access its true catalytic potential.

The observation that high association constants are not required for use of artificial systems as RNA-hydrolysis catalysts[23] leaves us with optimism about the potential application of our system. It even has the advantage of specific (adenine) recognition, and Cu(bpy) has already been shown to hydrolyze RNA at neutral pH.[16] Efforts toward this goal are currently underway.

Acknowledgment. We are grateful to the National Institutes of Health for support of this research.

JA9420252

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