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Uridine Binding by Zn(II) Macrocyclic Complexes: Diversion of RNA Cleavage Catalysts

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Zn(II) complexes of 1-oxa-4,7,10-triazacyclododecane (**12[ane]N3O**), 1,5,9-triazacyclododecane (**12[ane]N3**), and 1-hydroxyethyl-1,4,7-triazacyclononane (**9[ane]N3OH**) promote cleavage of the RNA analogue, 2-hydroxypropyl-4-nitrophenyl phosphate (**HpPNP**) at pH 8.0, $I = 0.10$ M (NaCl), 25 °C with second-order rate constants of 8.9×10^{-3}, 9.0×10^{-3}, and 3.3×10^{-3} M^{-1} s^{-1}, respectively. Cleavage of **HpPNP** by these catalysts is inhibited by uridine with inhibition constants (K_i) of 1.2, 0.46, and 45 mM, respectively, under these conditions. Binding constants derived from these inhibition constants are 2–200-fold larger than those for binding of related Zn(II) complexes to phosphate diesters under similar conditions, suggesting that uridine sequences in RNA will inhibit Zn(II)-catalyzed cleavage by competing with phosphate diester binding sites. Further studies are carried out that utilize pH–potentiometric titrations to monitor uridine binding to five Zn(II) macrocyclic complexes in aqueous solution at 25 °C, $I = 0.10$ M (NaCl). The data are consistent with binding of the Zn(II) complexes to the N3-deprotonated form of uridine to give log K_{U^-} values of 5.29, 4.57, 4.56, 3.47, and 2.65 for the Zn(II) complexes of **12[ane]N3**, **12[ane]N4**, **12[ane]N3O**, **15[ane]N3O2**, and **9[ane]N3OH**, respectively (**12[ane]N4** = 1,4,7,10-tetraazacyclododecane, **15[ane]N3O2** = 1,4-dioxa-7,10,13-triazacyclopentadecane). For the five Zn(II) complexes studied, there is a linear relationship between uridine anion binding constants and hydroxide binding constants.

Introduction

Metal ion complexes catalyze the hydrolytic cleavage of RNA and RNA analogues by promoting transesterification at the phosphate diester to give a 2′,3′-cyclic phosphate diester and concomitant cleavage of the RNA strand. In the first step of catalysis, the metal ion complex binds to the phosphate diester.[1] This interaction is very weak; binding constants of simple phosphate diesters to mononuclear Zn(II) catalysts are typically on the order of 10 M^{-1}.[2] Sites that bind metal ions more strongly than this will divert the metal ion catalyst from binding to the phosphate diester backbone and will inhibit catalytic cleavage.

Nucleobases of RNA are the most common competing binding site. Quantitative studies of metal ion binding to the nucleobases in nucleosides have been reported[3−6] for first-row transition metal ions. Renewed interest in metal ion binding sites in structurally complex RNA molecules has led to recent additional advances in this area.[7] In contrast, there are few quantitative solution studies of nucleoside binding to first row transition metal ion or Zn(II) *macrocyclic complexes*. This is surprising given that metal ion complexes are employed as RNA cleavage catalysts and nucleobase binding to these complexes has a pronounced effect on the efficiency of RNA cleavage. For example, interaction of Cu(II) and Zn(II) complex catalysts with the nucleobases of RNA leads to base-sequence-selective cleavage even in simple dinucleoside substrates.[8−11]

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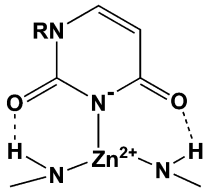
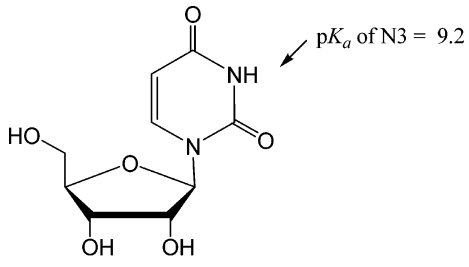
Scheme 1



Scheme 2

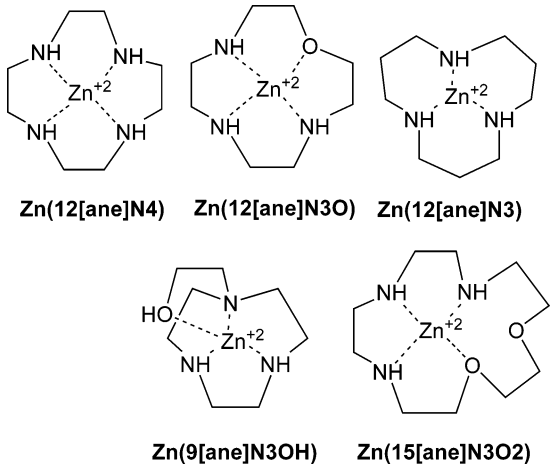


Uridine (U)

Our interest in Zn(II) catalysts for RNA cleavage has lead us to consider nucleobase binding by these catalysts. Reports by Kimura and co-workers on Zn(II) tetraazamacrocyclic complexes as nucleoside receptors serve as a guide for our work. Kimura's Zn(II) complexes bind strongly and specifically to uridine or thymidine nucleosides or dinucleosides in aqueous solution.[12−16] To rationalize the specificity of Zn(II) tetraazamacrocyclic complex binding to uridine or thymidine, a three-point recognition model is proposed.[17] This model accommodates the fact that **Zn(12[ane]N4)** and related macrocyclic complexes bind exclusively to the N3 deprotonated form $(N3)^-$ of uridine or thymidine (Schemes 1 and 2). In this model, the important interactions are Zn(II) coordination to $(N3)^-$ and hydrogen-bond formation between two of the amine groups of the macrocycle and the carbonyl groups of the uridine. A study of compounds that contain an N-deprotonated imide group similar to that of uridine shows a linear correlation between the Zn(II) complex substrate binding and the pK_a of the conjugate acid of the imide group.[17] The observation that **Zn(12[ane]N4)** does not interact strongly with other nucleosides is attributed to the lack of a basic imide site (adenosine or cytidine) or to repulsion between the Zn(II) macrocyclic ring and the amino C(2) group (guanosine) which prevents strong binding of the metal complex to $(N1)^-$.

The mononuclear Zn(II) azamacrocyclic complexes we have studied as catalysts for RNA cleavage[2,18,19] are structurally related to **Zn(12[ane]N4)** and thus are anticipated to bind to uridine. However, our best catalysts contain macrocycle donor groups that include alcohols and ethers (Scheme 3) in addition to amine donors. These groups will modulate the strength of the interaction with uridine by modifying the Lewis acidity and accessibility of the Zn(II) center. In addition, macrocyclic complexes containing both nitrogen and oxygen donors will have different hydrogen-bonding interactions with uridine, and according to Kimura's three point recognition model, this may modulate the strength of the interaction.

Scheme 3



Here we present our studies on the recognition of uridine by the series of Zn(II) macrocyclic complexes shown in Scheme 3. We show that there is a linear relationship between the uridine anion binding constant and the hydroxide binding constant for these complexes. This highlights the importance of the anion affinity of the Zn(II) center in uridine binding and suggests that structural differences in the Zn(II) complexes that influence interactions of the macrocyclic ring with uridine are of secondary importance. Implications for the design of RNA cleavage catalysts are discussed.

Experimental Section

Materials, General Procedures, and Instrumentation. All reagents and solvents were of reagent grade and used without further purification unless otherwise noted. Aqueous solutions for pH− potentiometric titrations were prepared with freshly boiled Millipore MILLI-Q purified water cooled under an argon stream. Aqueous stock solutions (50.0 mM) of the ligands were prepared from their respective salts, and the concentrations were determined by use of ^{1}H NMR using *p*-toluene sulfonic acid as an internal standard. ^{1}H NMR spectra were recorded on a Varian Inova 500 (500 MHz) spectrometer or Varian Inova 400 (400 MHz). Solutions of $ZnCl_2$ were calibrated against a standardized solution of ethylenediaminetetraacetic acid (EDTA) with Eriochrome Black T as the indicator. **12[ane]N3** was purchased from Aldrich as the HBr salt and purified by extraction into chloroform followed by precipitation out of ethanol as the HCl salt. **12[ane]N4** was purchased from Strem as the free base and was used as received. **9[ane]N3OH** was prepared as previously reported.[2] *N*,*N*′,*N*″-tris(*p*-tolylsulfonyl)diethylene triamine,[20] 1,5-bis(*p*-tolylsulfonyloxy)-3-oxapentane,[21] and 1,8-bis-(*p*-tolylsulfonyloxy)-3,6-dioxaoctane[20] were prepared as reported.

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The syntheses of **12[ane]N3O** and **15[ane]N3O2** were modified from published procedures[22−24] as described below.

Potentiometric pH Titrations. Potentiometric pH titrations were conducted on a Brinkmann Metrohm 702 SM Titrino autotitrator using an Orion Research Ross Combination pH Electrode 8115BN. The glass electrode was calibrated as a hydrogen concentration probe by titrating known amounts of HCl, standardized with sodium tetraborate, with CO_2-free NaOH prepared from dilution of a J. T. Baker dilute-it ampule and standardized using potassium hydrogen phthalate. The program GLEE was used to determine the standard electrode potential and the carbonate content of the NaOH solution. The pH−potentiometric titrations were carried out with $I = 0.10$ M (NaCl) at 25 °C, and at least two independent titrations were performed. Equilibrium binding constants were reproducible to ±20%. The Zn(II) complexes of the macrocycles were prepared in aqueous solution by mixing $ZnCl_2$ and the corresponding HCl salt of the ligand in a 1:1.05 molar ratio. Aqueous solutions (50 mL) of the Zn(II) macrocyclic complexes in the presence and absence of 1.00 mM uridine were titrated with carbonate-free 0.10 M NaOH. The protonation constants of the macrocyclic ligands including **12[ane]N3**, **12[ane]N4**, **12[ane]N3O**, **15[ane]N3O2**, and **9[ane]N3OH** were taken from the literature (Supporting Information, Table S1), as was the protonation constant for uridine.[17] The $K_w = ([H^+][OH^-])$ value used under our experimental conditions was 1.660×10^{-14}. The program Hyperquad 2000 was used to obtain equilibrium constants. The pH sigma values defined in the program are smaller than 2 for all data fits of these pH−potentiometric titrations. Speciation diagrams, plotted as percent formation against pH, were obtained by use of the program HYSS available at http://www.hyperquad.co.uk/.

Kinetics of Transesterification of HpPNP (2-Hydroxypropyl-4-nitrophenyl Phosphate). The concentration of the Zn(II) complexes examined in these experiments ranged from 0.500 to 4.00 mM. Stock solutions of the Zn(II) complexes were prepared in aqueous solution by mixing $ZnCl_2$ and the corresponding standardized solution of the ligand in 1:1.1 molar ratio and adjusting the pH to 6.5−7.0. In a typical experiment, a solution of the Zn(II) complex in 20.0 mM EPPS buffer at $I = 0.10$ M (NaCl) was adjusted to pH 8.0, transferred to a cuvette, and equilibrated at 25 °C in a thermostated spectrophotometer. In these experiments, the electrode was calibrated for proton *activity* (a_{H^+}; $pH = -\log a_{H^+}$) by using standard buffers. Our pH values in these experiments are 0.1 units lower than those in pH−potentiometric measurements where the electrode was calibrated for proton concentration ($[H^+]$, $p[H^+] = pH + \log \gamma_H$ where γ_H is 0.78 for our electrode). For inhibition experiments, the concentration of uridine was varied from 1.00 to 40.0 mM in solutions containing 1.00 mM Zn(II) complex, with all other conditions the same as above. The reaction was initiated by injection of a stock solution of **HpPNP** to give a final concentration of 20 μM. Transesterification of **HpPNP** was monitored by following the increase in absorbance at 400 nm due to the release of 4-nitrophenolate. The pH of these solutions was measured at the end of each experiment and was within 0.03 pH units of the initial value.

The reactions catalyzed by Zn(II) complexes at concentrations of 1.00 mM or greater were monitored for three half-lives and pseudo-first-order rate constants, k_{obsd}, were determined as the slopes of the semilogarithmic plots of reaction progress against time. For samples with low concentrations of catalyst or with high concentrations of uridine, the cleavage of **HpPNP** was monitored for the disappearance of the first 5−10% of the substrate, and the reaction was heated to 60 °C until the endpoint was reached. Values of k_{obsd} (s^{-1}) were determined as $k_{obsd} = v_i/[S]_o$, where v_i is the initial reaction velocity and $[S]_o$ is the initial substrate concentration. Second-order rate constants (k_{Zn} (M^{-1} s^{-1})) for the reactions catalyzed by the different Zn(II) complexes were determined as the slope of linear plots of k_{obsd} against Zn(II) complex concentration (correlation coefficients > 0.997). For uridine inhibition experiments, the pseudo-first-order rate constants in the presence of uridine are normalized by dividing them by the pseudo-first-order rate constant in the absence of uridine (k_o) (correlation coefficients > 0.995, standard deviations from the curve fit are ≤10%). Rate constants were reproducible to ±7%. The pseudo-first-order rate constants for the transesterification of **HpPNP** in the absence of the catalyst k_{uncat} (s^{-1}) were determined by the method of initial rates (5% conversion). At pH 8.0, the background rate constant was 1.5×10^{-7} s^{-1}, and this value was subtracted from the pseudo-first-order rate constant k_{obsd} (s^{-1}) for inhibition studies where background cleavage was not negligible.

Synthesis of 4,7,10-Tris(*p*-tolylsulfonyl)-1-oxa-4,7,10-triazacyclododecane. *N*,*N*′,*N*″-tris(*p*-tolylsulfonyl)diethylene triamine (10 mmol, 5.61 g), cesium carbonate (25 mmol, 8.15 g), and 150 mL of *N*,*N*-dimethylformamide were stirred for 1 h under argon. 1,5-Bis(*p*-tolylsulfonyloxy)-3-oxapentane (10 mmol, 4.14 g) in 50 mL of *N*,*N*-dimethylformamide was then added dropwise over a period of 5 h by means of a syringe pump. The reaction was stirred for an additional 3 days. The solution was poured into an Erlenmeyer flask, and ice was added slowly with stirring to form a precipitate. When all the ice was almost melted, the solution was placed in the refrigerator overnight. The resulting precipitate was filtered and recrystallized from a hot solution of 80% *N*,*N*-dimethylformamide in water. Yield: 6.36 g (88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, $^3J = 8$ Hz, 2 H, Ar), 7.62 (d, $^3J = 8$ Hz, 4 H, Ar), 7.34 (d, $^3J = 8$ Hz, 2 H, Ar), 7.30 (d, $^3J = 8$ Hz, 4 H, Ar), 3.65 (t,$^3J = 4.2$, 4 H, CH_2), 3.51 (t, $^3J = 6.4$, 4 H, CH_2), 3.19 (m, 8H, CH_2), 2.45 (s, 3 H, CH_3), 2.43 (s, 6 H, CH_3). ESI *m*/*z* (relative intensity): 658.2 (MNa^+, 31%), 1292.7 (M_2Na^+, 100%).

The protected macrocycle 4,7,10-tris(p-tolylsulfonyl)-1-oxa-4,7,10-triazacyclododecane (15.34 mmol, 9.75 g) and 340 mL of 30% HBr in acetic acid were refluxed under argon for 48 h. The reaction volume was reduced to 20% by vacuum distillation, and absolute ethanol was added to give a white solid that was filtered and dried. The white solid was dissolved in 50 mL of water, the pH was adjusted to ∼13 with sodium hydroxide, and the solution was extracted five times with chloroform. The organic layer was evaporated to produce a white solid. The solid was dissolved in ethanol and filtered; precipitation was induced by addition of hydrobromic acid to give the pure HBr salt. Yield: 4.79 g (75%). ^{1}H NMR (400 MHz, D_2O): δ 3.77 (t, $^3J = 4.8$, 4 H, CH_2), 3.32 (m, 8 H, CH_2), 3.20 (m, 4 H, CH_2). ESI *m*/*z* (relative intensity): 174.2 (MH^+, 100%).

Synthesis of 7,10,13-Tris(*p*-tolylsulfonyl)-1,4-dioxa-7,10,13-triazacyclopentadecane. *N*,*N*′,*N*″-tris(*p*-tolylsulfonyl)diethylene triamine (10 mmol, 5.61 g), cesium carbonate (25 mmol, 8.15 g), and 150 mL of *N*,*N*-dimethylformamide were stirred under argon for 1 h. 1,8-Bis(*p*-tolylsulfonyloxy)-3,6-dioxaoctane (10 mmol, 4.58 g) in 50 mL of *N*,*N*-dimethylformamide was then added dropwise

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over a period of 5 h by means of a syringe pump. The reaction mixture was stirred for an additional 3 days. The solution was poured into an Erlymeyer flask, and ice was added slowly with stirring to form a precipitate. When almost all the ice was melted, the solution was placed in the refrigerator overnight. The precipitate was filtered and recrystallized from a hot solution of 80% *N*,*N*-dimethylformamide in water. Yield 5.59 g (83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.80 (d, $^3J = 8$ Hz, 2 H, Ar), 7.70 (d, $^3J = 8$ Hz, 4 H. Ar), 7.30(d, $^3J = 8$ Hz, 6 H, Ar), 3.55 (t, $^3J = 4$, 4 H, CH_2), 3.47 (s, 4 H, CH_2), 3.38 (m, 4H, CH_2), 3.26 (m, 8H, CH_2), 2.41 (m, 9 H, CH_3). ESI *m*/*z* (relative intensity): 702.3 (MNa^+, 98%), 1380.8 (M_2Na^+, 100%).

The protected macrocycle 7,10,13-tris(*p*-toylsulfonyl)-1,4-dioxa-7,10,13-triazacyclopentadecane (1.1 mmol, 0.746 g), disodium phosphate (2.2 g), 3% sodium amalgam (46.2 g), and methanol were refluxed under argon for 48 h. Upon completion, the resulting solution was decanted from the amalgam. The amalgam was washed with water, and the solutions were combined. The solvent was removed under reduced pressure to form a white solid. The solid was dissolved in 50 mL of water, the pH was adjusted to 13, and the solution was extracted five times with chloroform. The chloroform layer was retained, and solvent was removed under reduced pressure to give a solid. The solid was dissolved in ethanol, filtered, and precipitated by addition of hydrochloric acid. Yield: 0.248 g (69%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.70 (t, $^3J = 5$, 4 H, CH_2), 3.61 (s, 4 H, CH_2), 3.22 (t,$^3J = 5$, 4 H, CH_2), 3.19 (t,$^3J = 5.8$, 4 H, CH_2), 3.01 (m, 4 H, CH_2). ESI *m*/*z* (relative intensity): 218.2 (MH^+, 100%).

Results

Syntheses. All N-tosylated macrocycles were prepared by employing a modification of the Richman−Atkins procedure[25] by using cesium carbonate[26] to deprotonate the tosylamines (Figure S1). For the preparation of **15[ane]N3O2**, removal of the tosyl protecting groups with 3% sodium amalgam in methanol gave an improved yield compared to the more traditional HBr/acetic acid deprotection method.[22−24] The macrocycles prepared here, isolated as either hydrogen bromide or hydrogen chloride salts, are typically a mixture of salts because the diprotonated, as well as triprotonated, macrocycles precipitate from solution. It was thus necessary to standardize aqueous solutions of the macrocycles by use of ^{1}H NMR spectroscopy prior to preparation of the Zn(II) macrocyclic complexes.

Plots of the pH−potentiometric titrations of macrocycles **15[ane]N3O2**, **12[ane]N3O**, **12[ane]N4**, **12[ane]N3**, and **9[ane]N3OH** in the presence of 1 equiv of $ZnCl_2$, *(I* = 0.10 M (NaCl), 25 °C) reveal two inflection points corresponding to the formation of the Zn(II) complex and the loss of a proton from the Zn(II) complex as shown in Figure 1 for **Zn(12[ane]N3O)**. Fitting of these data gives equilibrium binding constants for complexation of the Zn(II) ion to the neutral macrocyclic ligand (L) and ionization constants for the Zn(II) complexes (pK_a) (Table 1; Supporting Information, Table 1 and Seqs 1−14).

All macrocycles bind Zn(II) strongly, and all complexes have an ionizable group with a pK_a ranging from 7.88 to 9.25 (Table 1). This ionization is assigned as a Zn(II)-bound water ligand that converts to a Zn(II)-bound hydroxide. This assignment also holds for the **Zn(9[ane]N3OH)** complex since it has been shown that the water ligand ionizes prior to the alcohol group of the macrocycle.[2] The pK_a values reported here are approximately 0.1−0.4 units higher than those reported previously under similar conditions in solutions of the same ionic strength but with weakly coordinating anions such as nitrate and perchlorate.[2,27−29,30] The higher Zn(II) water pK_a values here are consistent with competition of the chloride anion for binding to the Zn(II) center. Such chloride competition produces a smaller effective binding constant for the hydroxide ligand and, correspondingly, a higher Zn(II) water pK_a value.

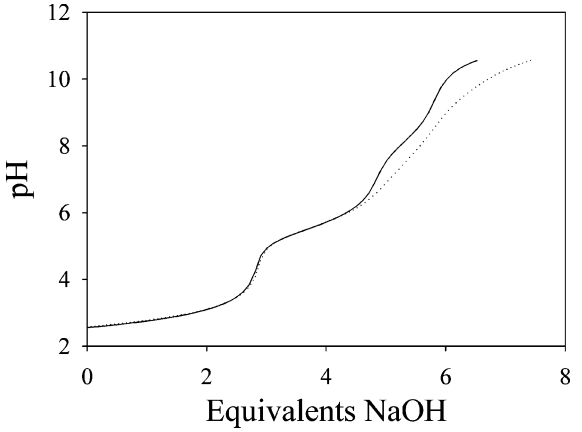

Figure 1. pH−potentiometric titration diagram for **Zn(12[ane]N3O)** in the presence and absence of uridine, (25 °C, I = 0.10 M (NaCl)). **Zn(12[ane]N3O)** (solid line), **Zn(12[ane]N3O)**−uridine (dashed line).

Table 1. Equilibrium Constants for Complexation, Water Ligand Ionization, and Uridine Binding

macrocycle	log K_{Zn}[a]	log K_{OH}[b]	pK_a[c]	log K_{U^-}[d]
12[ane]N3O	10.83(1)	5.60(3)	8.18	4.56(6)
12[ane]N4	15.74(2)	5.50(4)	8.28	4.57(1)
12[ane]N3	9.41(1)	5.90(1)	7.88	5.29(2)
15[ane]N3O2	8.91(3)	4.93(5)	8.85	3.47(2)
9[ane]N3OH	11.54(2)	4.53(1)	9.25	2.65(6)

[a] K_{Zn} = [ZnL]/([Zn][L]). [b] K_{OH} = [ZnL(OH^-)]/([ZnL][OH^-]). [c] pK_a = log K_w − log K_{OH}. [d] K_{U^-} = [ZnL(U^-)]/([ZnL][U^-]), I = 0.10 M (NaCl), T = 25 °C.

Analysis of pH−potentiometric data suggests that uridine binds to all Zn(II) macrocyclic complexes. An example of a titration with an equivalent of uridine is shown in Figure 1 for **Zn(12[ane]N3O)** and in Figures S2−S6 for the other macrocyclic complexes. The data are fit to a model that has binding of the Zn(II) complexes exclusively to the N3 deprotonated uridine and not to neutral uridine (Scheme 4). Uridine anion binding constants are listed in Table 1. Speciation diagrams generated from these data have three major Zn(II) species, including Zn(L), Zn(L)(U^-) and Zn(L)(OH^-). A typical speciation diagram is given in Figure 2 for **Zn(12[ane]N3O)**, and diagrams for other complexes are in the Supporting Information (Figures S7−S11). Examination of the speciation diagrams shows that the amount of bound uridine initially increases with pH, consistent with binding to the deprotonated $(N3)^-$ form of uridine. At higher pH values, hydroxide competes with uridine for binding to

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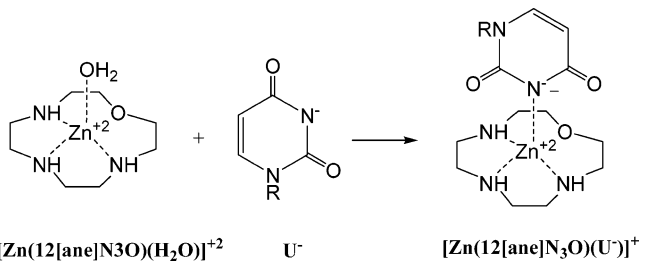
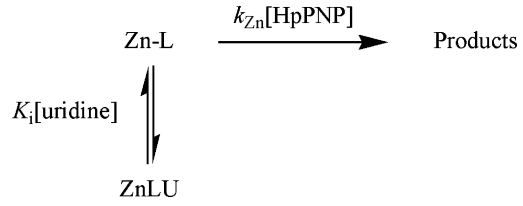
Scheme 4



$[Zn(12[ane]N3O)(H_2O)]^{+2}$ U^- $[Zn(12[ane]N_3O)(U^-)]^+$

Scheme 5



the Zn(II) macrocycle complex. The consequence of this competition is that there is an optimal pH for each complex to bind to uridine.

Zn(12[ane]N3O), **Zn(12[ane]N3)**, and **Zn(9[ane]N3OH)** were studied as catalysts for the cleavage of an RNA analogue. The transesterification of 2-hydroxypropyl-4-nitrophenyl phosphate, **HpPNP**, catalyzed by the three Zn(II) complexes was monitored by following the increase in absorbance at 400 nm due to the release of 4-nitrophenolate. The cyclic phosphate diester was identified by ^{31}P NMR as the sole phosphorus-containing product for all metal-ion catalyzed reactions, consistent with cleavage occurring by phosphate diester transesterification. Second-order rate constants (k_{Zn}, M^{-1} s^{-1}) at pH 8.0, 20 mM buffer, 25 °C, I = 0.10 M (NaCl) for the cleavage of **HpPNP** catalyzed by the Zn(II) complexes, determined as the slopes of the plots of k_{obsd} against catalyst concentration are 9.0×10^{-3}, 8.9×10^{-3}, and 3.3×10^{-3} M^{-1} s^{-1} for **Zn(12[ane]N3)**, **Zn(12-[ane]N3O)**, and **Zn(9[ane]N3OH)**, respectively.

For the catalysts **Zn(12[ane]N3O)**, **Zn(12[ane]N3)**, and **Zn(9[ane]N3OH)**, uridine binding was monitored by competitive inhibition of **HpPNP** cleavage at pH 8.0, (I = 0.10 M (NaCl), 25 °C). Normalized plots of the rate constants at varying concentrations of uridine are shown in Figure 3. The data were fit to eq 1,

$$\frac{k_{obsd}}{k_o} = \left(\frac{[Zn_t] - [U_t] - K_i + \sqrt{[Zn_t]^2 + [U_t]^2 + K_i^2 - 2[Zn_t][U_t] + 2[Zn_t]K_i + 2K_i[U_t]}}{2[Zn_t]}\right) \quad (1)$$

derived from Scheme 5. In these studies, binding to uridine is sufficiently strong such that the concentration of free uridine cannot be approximated by the concentration of added uridine, leading to the use of eq 1.[31] A nonlinear least-squares fit of the data to eq 1 for competitive inhibition yields values of K_i = 1.2, 0.46, and 45 mM for **Zn(12[ane]N3O)**, **Zn(12[ane]N3)**, and **Zn(9[ane]N3OH)**, respectively. These inhibition constants are inversely related to the effective binding constants of uridine to the macrocyclic complexes under these conditions and correspond to uridine binding constants obtained by pH−potentiometry as discussed below.

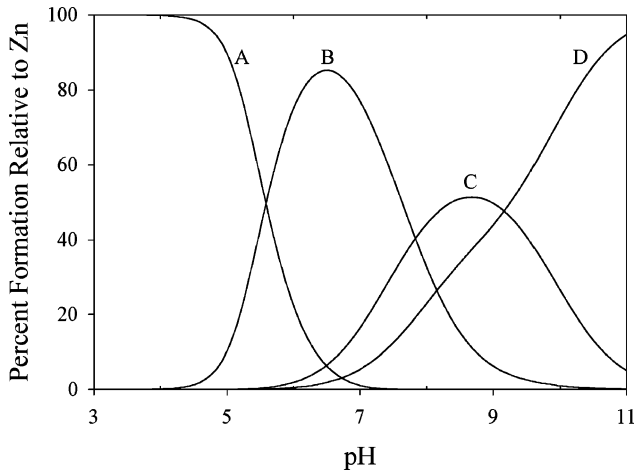


Figure 2. Speciation diagram for **Zn(12[ane]N3O)** binding to uridine as a function of pH, (25 °C, I = 0.10 M (NaCl), 1.0 mM **Zn(NO$_3$)$_2$**, 1.0 mM **12[ane]N3O**, and 1.0 mM uridine). (A) Zn^{2+}, (B) $[Zn(12[ane]N3O)]^{2+}$, (C) $[Zn(12[ane]N3O)(U^-)]^+$, (D) $[Zn(12[ane]N3O)(OH^-)]^+$.

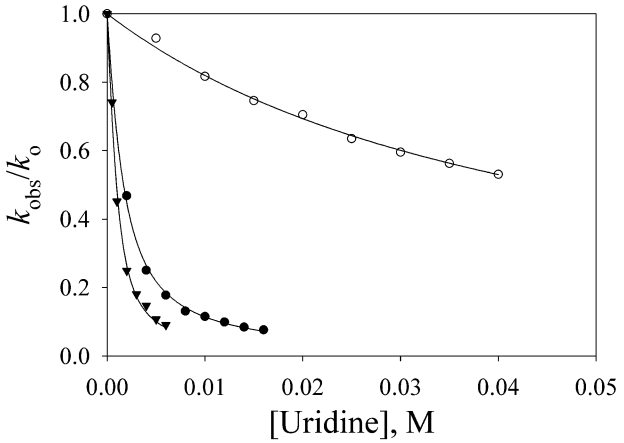


Figure 3. Uridine inhibition of the macrocyclic Zn(II) complex catalyzed cleavage of **HpPNP** at 25 °C, I = 0.10 M (NaCl), pH 8.0 with 1.00 mM EPPS buffer Zn(II) complex. ●, **Zn(12[ane]N3O)**; ○, **Zn(9[ane]N3OH)**; ▼, **Zn(12[ane]N3)**.

Discussion

Phosphate Diester Cleavage. Mononuclear Zn(II) complexes have been studied as catalysts for the cleavage of RNA and RNA analogues in our laboratory[2,18,19] and in several other groups.[8,32−39] Among the most active of these mononuclear Zn(II) catalysts are **Zn(12[ane]N3O)**, **Zn(9[ane]-N3OH)**, and **Zn(12[ane]N3)**. As shown here, all three complexes promote cleavage of the RNA analogue, **HpPNP**,

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at 25 °C, pH 8.0. Under these conditions, second-order rate constants range from 9.0×10^{-3} to 3.3×10^{-3} M^{-1} s^{-1}. Two of these complexes catalyze the cleavage of RNA as well. **Zn(12[ane]N3O**) promotes cleavage of the oligoribonucleotide A_6 at 37 °C.[40] Both **Zn(12[ane]N3)** and a **Zn(12[ane]N3)** conjugate of an antisense oligonucleotide promote the cleavage of RNA.[33,34,41]

Prior to conducting further studies with different RNA substrates, we decided to investigate uridine binding by our Zn(II) catalysts. There are several reports of related Zn(II) macrocyclic complexes that bind to uridine,[17,42−44] suggesting that this is a common property of these complexes. Zn(II) catalyst binding to uridine sequences would affect the rate and sequence specificity of RNA cleavage and would ultimately influence our choice of substrate. For example, uridine-containing sequences are not cleaved effectively by a Zn(II) macrocyclic catalyst,[41] and it is suspected that uridine binding to the Zn(II) catalyst is involved.

Cleavage of **HpPNP** by all three Zn(II) catalysts is inhibited by uridine. The inhibition constants obtained in these studies are useful as estimates of the effective binding constants for uridine to the Zn(II) macrocyclic complexes at pH 8.0. The reciprocal of the inhibition constant (K_i = 0.46, 1.2, and 45 mM) gives effective binding constants (K_f) of 2200, 830, and 22 M^{-1} for **Zn(12[ane]N3)**, **Zn(12[ane]-N3O)**, and **Zn(9[ane]N3OH)**, respectively. Note that these inhibition constants are pH dependent because they are a function of the concentration of Zn(II) aqua complex ($Zn(L)(OH_2)$), as well as uridine anion in solution. This is true because the aqua species is the active catalyst[45] and it is also the major Zn(II) complex species that binds to uridine. Similarly, binding constants from pH−potentiometric titrations derive from a model with the Zn(II) aqua species binding to uridine anion. Effective binding constants for uridine at pH 8.0 as obtained by pH−potentiometric measurements, calculated as described (Supporting Information, Seq 15), are 4900, 1400, and 25 M^{-1} for **Zn(12[ane]N3)**, **Zn(12[ane]N3O)**, and **Zn(9[ane]N3OH)**, respectively. These two sets of values are in reasonable agreement given the experimental errors in these measurements and differences in experimental conditions. For example, kinetic experiments contain buffer that may bind to catalyst to give lower formation constants compared to pH−potentiometric experiments which do not contain buffer. Other factors contributing to differences in the two values include pH−potentiometric binding constants (reproducible to ±20%), inhibition constants (reproducible to ±10%), and measurement of pH values (±0.1 pH unit) due to differences in electrode calibration for the two methods (see Experimental Section).

These inhibition studies suggest that all three Zn(II) catalysts bind uridine despite their differences in structure. To more fully explore this relationship, pH−potentiometric studies were used to quantify binding of the three catalysts in addition to two other Zn(II) macrocyclic complexes to uridine.

Uridine and Hydroxide Binding Studies. Five Zn(II) macrocyclic complexes were studied with the goal of assessing the effect of water ligand pK_a and the number and orientation of the amine donor groups on binding to uridine. Despite differences in macrocycle ring size and different donor atoms, these Zn(II) complexes have certain similarities in coordination sphere and in solution properties. The solid-state structures of four of the complexes in Scheme 3 have five-coordinate Zn(II) centers with four sites occupied by macrocycle donor groups and one site by an anionic ligand such as bromide or carbonate.[2,29,40,46,47] The only exception to this is the **12[ane]N3** ligand, which can accommodate both four- and five-coordinate Zn(II) centers.[46,48] However, the coordinative flexibility of Zn(II) makes it difficult to ascertain the coordination number and geometry of the complexes in solution on the basis of crystal structure data. For example, a related dinuclear Zn(II) complex of a ligand with two linked, symmetrical **9[ane]N3** derivatives has one five-coordinate and one six-coordinate Zn(II) in the solid state.[2] Nonetheless, each Zn(II) macrocyclic complex binds to hydroxide in solution, consistent with each complex having at least one site available for binding an anion. Binding of anionic ligands such as uridine to the Zn(II) complexes competes with hydroxide binding, suggesting that there is a single strong anion binding site in these complexes.

Water ligand pK_a values for the five Zn(II) macrocyclic complexes vary by about one pK_a unit. There is no correlation between Zn(L) binding constant (Table 1) and water ligand pK_a as has been reported previously,[49] suggesting that other factors are important. The most likely factor is a change in coordination number within the series of Zn(II) aqua complexes. Although the coordination number of each of these aqua complexes is not known, there are trends within the series that can be deduced from crystal structures. For example, Zn(II) complexes of **12[ane]N3** generally have low coordination numbers of four or five, whereas Zn(II)

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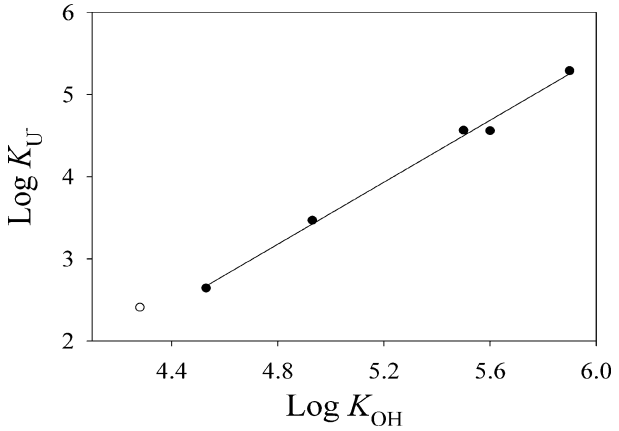

Figure 4. Correlation between Zn(II) complex binding to hydroxide and Zn(II) complex binding to uridine anion at 25 °C, $I = 0.10$ M (NaCl). The point with the open circle is for free Zn(II) ion.

complexes of **9[ane]N3** derivatives have higher coordination numbers of five or six.[2,50] Zn(II) complexes with lower coordination numbers typically have higher hydroxide ion affinity and correspondingly lower water ligand pK_a values than those with higher coordination numbers,[51,52] consistent with the data presented here.

The uridine anion binding affinity varies by nearly 3 orders of magnitude for the five Zn(II) macrocycles studied here. There is no straightforward correlation between binding strength and the types of donor groups, the number of amine groups, nor the size of the macrocycle ring as might be expected if the three-point recognition model of Kimura were important. Instead, there is a good correlation between uridine anion binding constants and hydroxide binding constants (Figure 4) for these Zn(II) complexes. As the binding constant for hydroxide anion (corresponding to a lower water ligand pK_a) increases, so does the binding constant for the uridine anion. This is consistent with the dominant interaction being that of the Zn(II) center with the uridine anion. The correlation does not support a substantial binding contribution from hydrogen-bonding interactions of the amine donors with the carbonyl groups. If the macrocyclic ligands were involved directly in binding (Scheme 1), each Zn(II) complex would have different hydrogen-bonding interactions due to structural variations, leading to a nonlinear plot.

Zn(II) complexes are more discriminating toward binding of uridine than hydroxide, as demonstrated by the slope of 1.9 in Figure 4. This may be due to the greater steric requirements of uridine anion compared to hydroxide and the increased steric crowding in complexes with reduced log K_{OH}. For example, the lower coordinate complexes formed by **Zn(12[ane]N3)** have a more open coordination sphere to allow stronger uridine anion binding whereas the higher-coordinate complexes formed by **Zn(9[ane]N3OH])** have a more crowded coordination sphere and the weakest binding constant for uridine. In addition, electronic effects are likely to be important.[53] **Zn(12[ane]N3)** is a softer Lewis acid than **Zn(12[ane]N4),** as determined by its ligand binding properties.[54] Softer Zn(II) centers with increased covalent interactions may interact more strongly with the softer uridine $(N3)^-$ anion compared to higher-coordinate, harder Zn(II) centers such as those in **Zn(12[ane]N4)** or **Zn(9[ane]N3OH))**.

Also included in the plot shown in Figure 4 is a data point for free Zn(II) ion from work recently published by Sigel.[4] Although there is a larger error associated with the determination of the pK_a value for free Zn(II) ion[55] compared to the pK_a of our Zn(II) macrocyclic complexes, this data point falls within the linear correlation reasonably well and supports the notion that anion binding strength, not macrocycle−uridine interactions, is the most important feature in uridine binding. This suggests that the primary effect of the macrocycle is to increase the Lewis acidity of the Zn(II) center in part through a decrease in coordination number, and this leads to stronger binding of anions such as hydroxide or deprotonated uridine.

Implications for the Design of RNA Cleavage Catalysts. The properties that make Zn(II) complexes effective catalysts for RNA cleavage include an accessible coordination sphere for binding substrate and strong interactions between the positively charged catalyst and the anionic phosphorane transition state.[2,45] Recent solvent deuterium isotope studies on the cleavage of an RNA analogue suggest that it is the Zn(II) aqua complex (i.e., $ZnL(H_2O)$) that stabilizes the phosphorane transition state in the rate-limiting step for cleavage of RNA analogues with good leaving groups.[45] This result highlights the importance of strong electrostatic interactions of cationic catalyst with the anionic phosphorane for good RNA cleavage catalysts. Related to this observation is a correlation between increasing catalytic rate constants for **HpPNP** cleavage and increasing hydroxide ligand affinity (or decreasing water ligand pK_a) in structurally similar Zn(II) macrocyclic complexes.[56] Effective RNA cleavage catalysts interact strongly with anionic oxygen donor ligands.

For the Zn(II) azamacrocyclic complexes, strong hydroxide binding correlates to strong uridine anion binding, giving these complexes dual properties as uridine recognition agents and as phosphate diester cleavage catalysts. The effective constants for uridine binding by **Zn(12[ane]N3O)** or **Zn(12[ane]N3)** or **Zn(9[ane]N3OH)** at pH 8.0, ranging from 22 to 2200 M^{-1} in kinetic inhibition experiments, are larger than typical binding constants for phosphate diesters to mononuclear Zn(II) complexes ($\sim$10 M^{-1} for diethyl phosphate).[2] Uridine bases are thus potential sites for competitive binding, and their presence will inhibit catalytic cleavage of RNA if they interfere with interaction of the catalyst with the phosphate diester.

Competitive binding of catalyst to uridine in RNA sequences has two major consequences. First, binding of

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uridine may decrease catalytic cleavage rates through nonproductive interactions such as that observed for cleavage of UpU by multinuclear Cu(II) or Zn(II) complexes.[9,57] Similarly, a **Zn(12[ane]N3)**−oligonucleotide conjugate promotes cleavage of a RNA bulge lacking uridine but cannot cleave a bulge containing a uridine sequence.[41] These examples are consistent with competitive inhibition of phosphate diester interactions by the uridine nucleobase. Second, binding of Zn(II) azamacrocycles to RNA-containing uridine perturbs RNA structure by disrupting double-stranded regions of the RNA.[14,58] Such strong nucleobase binding will make it more challenging to employ this class of cleaving agent for the site- and structure-specific cleavage of RNA.

Our work here suggests that uridine binding is a property that is common to mononuclear Zn(II) azamacrocyclic complexes. While it will be difficult to redesign these macrocyclic complexes such that they do not bind uridine but retain catalytic activity for RNA cleavage, it may be possible to alter substrate binding modes by elaboration of these catalysts. Enzymes are effective catalysts in part because the substrate is oriented within the active site, optimizing interactions with respect to the enzyme catalytic groups. While such binding specificity is challenging to engineer into small molecule catalysts, there are a few examples of metal ion catalysts being tethered to an RNA recognition unit in order to increase substrate binding and to direct the catalyst toward a specific site on the RNA.[41,59−62] The majority of these examples have an antisense oligonucleotide as a recognition agent tethered to a metal ion complex catalyst to effect sequence-specific cleavage of RNA.[41,58−61] A flexible tether, however, does not deliver the complex catalyst precisely to the phosphate diester backbone and uridine sequences in the neighborhood of the catalyst may still inhibit cleavage.[41] However, this approach may succeed if the RNA sequences to be cleaved do not contain uridine in close proximity to the catalyst or if the structure of the tether is optimized to facilitate specific interactions with the RNA substrate.

Alternately, there are other classes of metal ion complex catalysts that do not bind to uridine as strongly as the complexes studied here. A dinuclear Zn(II) catalyst binds uridine groups more weakly than would be anticipated from its hydroxide anion binding constant, most likely because the accessibility of each Zn(II) center is decreased by the presence of a bridging alkoxide group and a second Zn(II).[57] Another alternative is to use more oxophilic metal ion complex catalysts that do not bind strongly to nitrogen donor atoms in the pyrimidines, such as lanthanide ion complexes. Finally, it may be feasible to exploit Zn(II) macrocyclic complex interactions with uridine groups to increase the interaction of a tethered metal ion catalyst with RNA and to orient the catalyst with respect to the phosphate diester. This approach is currently being studied in our laboratory.

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Supporting Information Available: Speciation diagrams and potentiometric data. This material is available free of charge via the Internet at http://pubs.acs.org.

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