Acid–Base and Metal-Ion Binding Properties of the RNA Dinucleotide Uridylyl-(5′→3′)-[5′]uridylate ($pUpU^{3-}$)

Bernd Knobloch,[a] Danuta Suliga,[b] Andrzej Okruszek,[b] and Roland K. O. Sigel*[a]

Abstract: It is well known that Mg^{2+} and other divalent metal ions bind to the phosphate groups of nucleic acids. Subtle differences in the coordination properties of these metal ions to RNA, especially to ribozymes, determine whether they either promote or inhibit catalytic activity. The ability of metal ions to coordinate simultaneously with two neighboring phosphate groups is important for ribozyme structure and activity. However, such an interaction has not yet been quantified. Here, we have performed potentiometric pH titrations to determine the acidity constants of the protonated dinucleotide $H_2(pUpU)^-$, as well as the binding properties of $pUpU^{3-}$ towards Mg^{2+}, Mn^{2+}, Cd^{2+}, Zn^{2+}, and Pb^{2+}. Whereas Mg^{2+}, Mn^{2+}, and Cd^{2+} only bind to the more basic 5′-terminal phosphate group, Pb^{2+}, and to a certain extent also Zn^{2+}, show a remarkably enhanced stability of the $[M(pUpU)]^-$ complex. This can be attributed to the formation of a macrochelate by bridging the two phosphate groups within this dinucleotide by these metal ions. Such a macrochelate is also possible in an oligonucleotide, because the basic structural units are the same, despite the difference in charge. The formation degrees of the macrochelated species of $[Zn(pUpU)]^-$ and $[Pb(pUpU)]^-$ amount to around 25 and 90%, respectively. These findings are important in the context of ribozyme and DNAzyme catalysis, and explain, for example, why the leadzyme could be selected in the first place, and why this artificial ribozyme is inhibited by other divalent metal ions, such as Mg^{2+}.

Keywords: dinucleotides · magnesium · metal-ion binding properties · ribozymes · RNA

Introduction

The observation that RNA molecules can perform enzymatic functions,[1,2] once thought to be unique to proteins, has stimulated interest in understanding the structure and mechanism used by these catalytic RNAs, that is, ribozymes.[3–6] This interest has been further spurred by the discovery that the ribosome is also a ribozyme, a consequence of the former's peptidyltransferase site being comprised exclusively of RNA.[7,8] Another remarkable example is the spliceosome, a huge ribonucleoprotein complex of more than 70 proteins and five RNA molecules, which catalyzes the removal of introns by two consecutive transesterification reactions.[9–11] The exact make-up of the spliceosome's active site is not known; however, two of the RNA components of the spliceosome (the small nuclear U2 and U6 RNAs) are considered to be the main candidates constituting the catalytic center.[10] Furthermore, there is evidence for intrinsic metal-ion binding in the intramolecular stem-loop of U6.[9,11,12] Based on various experiments using thiophosphate derivatives of U6, it was concluded that Mg^{2+} binds to the phosphate group of uridine 80 within the stem-loop of U6.[11,12]

For most of the nine classes of catalytic RNAs known today, there is now considerable evidence for the requirement of divalent metal ions, especially Mg^{2+},[13] in ribozymal activity.[3,4,6,12–17] However, until now, very little quantitative information concerning the binding strength of metal ions to the individual sites of a nucleic acid has been reported.[18–20] Evidently, the most frequently repeated individual site is the phosphate–diester bridge, $-O-P(O)_2^-O-$, in which the two terminal oxygen atoms together carry one negative charge. It is difficult to measure directly the metal-ion affinity of such a site in a polymer or even in an oligonucleotide, such as $UpUpU^{2-}$. This is because no competition for binding occurs between a metal ion and a proton within the experi-

[a] Dr. B. Knobloch, Prof. Dr. R. K. O. Sigel
Institute of Inorganic Chemistry, University of Zürich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)
Fax:(+41) 44-635-6802
E-mail: roland.sigel@aci.unizh.ch

[b] D. Suliga, Prof. Dr. A. Okruszek
Polish Academy of Sciences
Center of Molecular and Macromolecular Studies
Department of Bioorganic Chemistry, 90–363 Lódz (Poland)

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mentally accessible pH range, as well as within the physiological pH range, because the primary protons of phosphate residues are released with pK_a values of about one.

We measured the metal-ion affinity of such a phosphate–diester unit by using a dinucleotide containing both a terminal 5′-phosphate group and a 5′→3′-phosphate–diester bridge. The main binding site in such a dinucleotide is the terminal phosphate group; however, chelate formation with the neighboring phosphate–diester bridge is at least theoretically possible and, therefore, information can be gained about the metal-ion affinity of this second site. Furthermore, the following questions arise: Is there selectivity? Can all biologically meaningful metal ions, for example, Mg^{2+} or Zn^{2+}, form such a chelate? Here, we address these questions and quantify the formation of macrochelates with different metal ions in dinucleotide complexes. Notably, the steric demands of a dinucleotide are comparable to those of neighboring phosphate groups in a larger oligomer.

To focus on the interaction between metal ions and the phosphate–diester bridge, and to make the interpretation of the results unequivocal, a dinucleotide with nucleobases of low metal-ion affinity must be selected. We selected the dinucleotide uridylyl-(5′→3′)-[5′]uridylate ($pUpU^{3-}$, Figure 1).[21,22] This molecule has previously been synthesized,[23–26] although no data are available regarding its metal-ion binding properties,[27–29] and to the best of our knowledge, its acid–base properties have also not yet been studied.

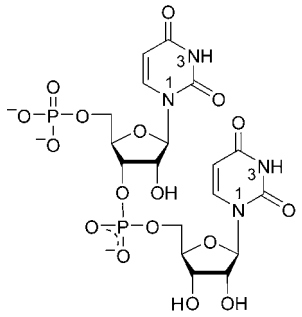

Figure 1. Chemical structure of uridylyl-(5′→3′)-[5′]-uridylate ($pUpU^{3-}$). The two uridine units are shown in their dominating *anti* conformation.[21,22]

The uracil residue is known to be a poor ligating site, as long as the (N3)H site is not deprotonated.[30] This does not mean that carbonyl oxygen atoms cannot interact with metal ions; indeed, such interactions in RNA molecules are known for the solid state.[31–35] However, such an interaction occurs only under sterically most favorable conditions, that is, a suitable directing primary binding site must be close by.[36–38] If such a neighboring binding site is not present, in aqueous solution water molecules dominate the situation by forming hydrogen bonds to the carbonyl oxygen atoms. For $pUpU^{3-}$, it seems feasable that a metal ion bound to the terminal 5′-phosphate residue might reach (C4)O. However, (C4)O is not in a sterically favored position, and more importantly, the metal-ion affinity of a carbonyl oxygen atom in aqueous solution is too low for the formation of such a macrochelate.[39] The second carbonyl group, (C2)O, cannot be reached by a phosphate-bound metal ion for steric reasons, because in the dominating *anti* conformation this acceptor group is directed away,[21,22] and the energy barrier for a switch into the *syn* conformation is too high.[40] Therefore, the metal-ion binding sites to be considered for $pUpU^{3-}$ in aqueous solution are the terminal phosphate group and the phosphate–diester bridge. Indeed, the stability studies performed with Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} presented here confirm this reasoning. In addition, our experiments show that discrimination occurs; for example, Mg^{2+} coordinates in only a monodentate fashion to the terminal phosphate group, whereas Zn^{2+} forms a chelate that also involves the phosphate–diester bridge.

Results and Discussion

Acid–base properties of $pUpU^{3-}$: This dinucleotide (Figure 1) can accept a total of three protons at its phosphate groups; however, two of these protons are released at a very low pH, and for one of these protons, a pK_a value can be estimated (see below). Therefore, the species $H_2(pUpU)^-$ must be considered, as the two uracil residues can also be deprotonated at their (N3)H sites. This then leads to the following four deprotonation reactions:

$$H_2(pUpU)^- \rightleftharpoons H(pUpU)^{2-} + H^+ \quad (1a)$$

$$K^H_{H_2(pUpU)} = [H(pUpU)^{2-}][H^+]/[H_2(pUpU)^-] \quad (1b)$$

$$H(pUpU)^{2-} \rightleftharpoons pUpU^{3-} + H^+ \quad (2a)$$

$$K^H_{H(pUpU)} = [pUpU^{3-}][H^+]/[H(pUpU)^{2-}] \quad (2b)$$

$$pUpU^{3-} \rightleftharpoons (pUpU-H)^{4-} + H^+ \quad (3a)$$

$$K^H_{pUpU} = [(pUpU-H)^{4-}][H^+]/[pUpU^{3-}] \quad (3b)$$

$$(pUpU-H)^{4-} \rightleftharpoons (pUpU-2H)^{5-} + H^+ \quad (4a)$$

$$K^H_{(pUpU-H)} = [(pUpU-2H)^{5-}][H^+]/[(pUpU-H)^{4-}] \quad (4b)$$

Notably, in Equilibrium (3a), $(pUpU-H)^{4-}$ should be read as "pUpU minus H", meaning that one of the two (N3)H sites has lost a proton, without defining which one. Analogously, in the species $(pUpU-2H)^{5-}$, both (N3)H sites are deprotonated.

For Equilibrium (1a), an acidity constant could be only estimated (see below), whereas the values for the other three equilibria were measured by performing potentiometric pH titrations. The results are listed in Table 1, and the site attributions are evident from the given related data.[41–46] The data show that, regarding the release of the final proton from the phosphate groups, $H(pUpU)^{2-}$ is more basic than

Table 1. Negative logarithms of the acidity constants for the deprotonation of the $P(O)(OH)_2$ and (N3)H sites in $H_2(pUpU)^-$ [Eqs. (1)–(4)], together with related data determined by performing potentiometric pH titrations in aqueous solution (25 °C; $I = 0.1$ M, $NaNO_3$).[a,b]

Acids[c]	pK_a of the sites			Reference
	$P(O)(OH)_2$	$P(O)_2(OH)^-$	(N3)H	
$H_2(pUpU)^-$	1.0 ± 0.3[d]	6.44 ± 0.02	8.99 ± 0.03/ 9.63 ± 0.08	–
$H(RibMP)^-$		6.24 ± 0.01		[43,44]
uridine			9.18 ± 0.02	[30]
$H_2(UMP)$	0.7 ± 0.3	6.15 ± 0.01	9.45 ± 0.02	[43,44]
$H_2(UDP)^-$	1.26 ± 0.20	6.38 ± 0.02	9.47 ± 0.02	[45]
$H_2(UTP)^{2-}$	2.0 ± 0.1[e]	6.48 ± 0.02	9.57 ± 0.02	[41]

[a] The errors given are three times the standard error of the mean value or the sum of the probable systematic errors, whichever is larger. [b] So-called practical, mixed, or Brønsted acidity constants[46] are listed (see also Experimental Section). [c] $RibMP^{2-}$, ribose 5′-monophosphate; UMP^{2-}, UDP^{3-}, or UTP^{4-}, uridine 5′-mono-, 5′-di-, or 5′-triphosphate. [d] Estimate based on the other values in this column (see also text). [e] From reference [42].

$H(UMP)^-$ by around 0.3 pK units (6.44 compared to 6.15). Because experience shows[47,48] that the same difference also holds for the release of the primary phosphate proton, a value for $H_2(pUpU)^-$ could be estimated based on the data for $H_2(UMP)$, that is, $pK^H_{H_2(pUpU)} = 0.7 + 0.3 = 1.0$.

The increased basicity of the terminal phosphate group of $pUpU^{3-}$ compared to that of UMP^{2-} is clearly a charge effect, and is in accordance with the observations made for UDP^{3-} and UTP^{4-}. Considering the competition between H^+ and Na^+ ions for binding,[49] which has the effect of slightly lowering the pK_a in the case of di- and triphosphates,[49] the binding tendency of monophosphates[50] toward Na^+ ions is very low under the experimental conditions.

Interestingly, the difference in acidity between the two (N3)H sites in $pUpU^{3-}$ amounts to only $\Delta pK_a = (9.63 \pm 0.08) - (8.99 \pm 0.03) = 0.64 \pm 0.09$ (Table 1). Within the error limits, this difference is identical to the one expected for *symmetrical* diprotonic acids (H_2L). In such instances, the formation of HL^- is favored, because its formation by the deprotonation of H_2L can occur by two ways. Similarly, there are two ways to protonate L^{2-} to yield HL^-. As a consequence, the formation of HL^- is doubly favored by a factor of two, corresponding to $\Delta pK_a = 0.6$.[51] The observation that the two uracil residues in $pUpU^{3-}$ have identical acidic properties is somewhat surprising, as it could be expected that the uracil residue close to the 5′-terminal phosphate group, which carries two negative charges, is less acidic. However, our result is in line with the recently published[52] acidity constants determined by ^1H NMR shift experiments for two uridine derivatives carrying singly negatively charged phosphate–ethyl ester groups at either the 3′ position or at both the 3′ and 5′ positions. The acidity constants $pK_{a(3')} = 9.21 \pm 0.05$ and $pK_{a(3'/5')} = 9.26 \pm 0.05$[52] are identical within their error limits, revealing that the effect of a single negative charge in this case is small.

Nevertheless, these observations cannot be interpreted as evidence that there are no charge effects at all: Comparison of the average acidity constant, $pK_{a/av} = 9.31 \pm 0.09$ (Table 1) for the release of the (N3)H protons from $pUpU^{3-}$ with the value for uridine itself ($pK^H_{Urd} = 9.18 \pm 0.02$) reveals an inhibition of the deprotonation reaction by $\Delta pK_a = 0.13 \pm 0.09$ (3σ), due to the three negative charges present in $pUpU^{3-}$. Nevertheless, this effect is relatively small compared to the ΔpK_a values between pairs of uridine and UMP^{2-}, UDP^{3-}, or UTP^{4-}, in which the differences amount to about 0.3 and 0.4 pK units (Table 1). To conclude, two neighboring uracil residues show an increased acidity relative to that of "isolated" uracil groups, which is significant in the context of biological systems.

Stability constants of $[M(pUpU)]^-$ complexes: Stability constants of several $[M(pUpU)]^-$complexes were determined by performing potentiometric pH titrations. All experimental data can be perfectly explained by taking Equilibrium (2a), as well as the following complex-forming Equilibrium (5a) into account:

$$M^{2+} + pUpU^{3-} \rightleftharpoons [M(pUpU)]^- \quad (5a)$$

$$K^M_{[M(pUpU)]} = [[M(pUpU)]^-]/([M^{2+}][pUpU^{3-}]) \quad (5b)$$

Our evaluation was not extended into the pH range in which either hydroxo complexes are formed or (N3)H is deprotonated. The pH range in which the formation of hydroxo complexes occurs was evident from the titrations in the absence of ligand (see below).

The stabilities of the five metal-ion complexes studied are listed in Table 2, along with the corresponding values of the

Table 2. Logarithms of the stability constants of $[M(pUpU)]^-$ complexes [Eq. (5)] as determined by potentiometric pH titrations in aqueous solution, in comparison with the corresponding values for [M(UMP)] and $[M(UDP)]^-$ complexes determined under the same conditions (25 °C; $I = 0.1$ M, $NaNO_3$).[a]

M^{2+}	$\log K^M_{[M(UMP)]}$	$\log K^M_{[M(pUpU)]}$	$\log K^M_{[M(UDP)]}$
Mg^{2+}	1.56 ± 0.02	1.84 ± 0.04	3.32 ± 0.05
Mn^{2+}	2.11 ± 0.02	2.49 ± 0.05	4.07 ± 0.05
Zn^{2+}	2.02 ± 0.07	2.57 ± 0.03	4.07 ± 0.05
Cd^{2+}	2.38 ± 0.04	2.75 ± 0.03	4.22 ± 0.05
Pb^{2+}	2.80 ± 0.04	4.45 ± 0.25	5.30 ± 0.15

[a] For the error limits, see footnote [a] of Table 1. The stability constants of the [M(UMP)] complexes are from reference [43], except that for [Pb(UMP)], which is from reference [44]. The values for the $[M(UDP)]^-$ species are from reference [45], the constant for $[Pb(UDP)]^-$ is an estimate taken from reference [19].

[M(UMP)] and $[M(UDP)]^-$ complexes for comparison. Evidently, the stability of the $[M(pUpU)]^-$ complexes is generally closer to that of the [M(UMP)] species than to the $[M(UDP)]^-$ species. The only exceptions are the Pb^{2+} complexes, in which the stability of $[Pb(pUpU)]^-$ is closer to that of $[Pb(UDP)]^-$ than to that of [Pb(UMP)]. Therefore, a more rigorous evaluation procedure is required to elucidate the structures of the $[M(pUpU)]^-$ complexes in solution.

Comparison of the stabilities of [M(pUpU)]$^-$ complexes with those of M[phosph(on)ate] species: For series of related ligands,[53,54] straight lines are obtained if $\log K^{M}_{M(L)}$ is plotted versus $pK^{H}_{H(L)}$. Such correlation lines are available[36,55] for complexes formed between several divalent metal ions (M^{2+}) and simple phosphate monoester or phosphonate ligands ($R–PO_3^{2-}$). The straight-line parameters for the five metal ions studied here, along with Cu^{2+} (see Experimental Section), are listed in Table 3.

Table 3. Straight-line correlations for M^{2+}–phosphate monoester or –phosphonate complex stabilities, and phosph(on)ate group basicities (aqueous solutions; 25 °C; $I=0.1$ M, $NaNO_3$).[a]

M^{2+}	m	b	SD
Mg^{2+}	0.208 ± 0.015	0.272 ± 0.097	0.033
Mn^{2+}	0.238 ± 0.022	0.683 ± 0.144	0.051
Zn^{2+}	0.345 ± 0.026	−0.017 ± 0.171	0.060
Cd^{2+}	0.329 ± 0.019	0.399 ± 0.127	0.045
Pb^{2+}	0.493 ± 0.033	−0.122 ± 0.213	0.076
Cu^{2+}	0.465 ± 0.025	−0.015 ± 0.164	0.057

[a] Slopes (m) and intercepts (b) for the straight-reference-line plots of $\log K^{M}_{[M(R-PO_3)]}$ versus $pK^{H}_{H(R-PO_3)}$ (Figure 2), as calculated by using the least-squares procedure from the equilibrium constants for simple $R–PO_3^{2-}/H^+/M^{2+}$ systems (R = noncoordinating residue); the $R–PO_3^{2-}$ ligands are listed in the legend of Figure 2. The straight-line equations are defined by $y=mx+b$, in which x represents the $pK^{H}_{H(R-PO_3)}$ value of any monoprotonated phosph(on)ate group, and y the calculated stability constant ($\log K^{M}_{[M(R-PO_3)]}$) of the corresponding [M(R–PO$_3$)] complex; the given errors of m and b correspond to one standard deviation (1σ).[36,44,55] Column 4 lists three times the standard deviations (SD), resulting from the differences between the experimental and calculated values for the various $R–PO_3^{2-}$ ligands employed.[36,44,55] The above parameters are taken from reference [55], except those for the Pb^{2+} systems, which are from reference [44].

For the Mg^{2+}, Zn^{2+}, and Pb^{2+} systems, the data pairs of the [M(R–PO$_3$)] complexes, on which the parameters in Table 3 are based, are shown in Figure 2, together with the corresponding data points for the $pUpU^{3-}$ systems. For all three $[M(pUpU)]^-$ complexes, an increased stability is observed. However, this stability varies quite considerably from metal ion to metal ion; for example, for $[Pb(pUpU)]^-$, the stability enhancement is clearly above one log unit!

A more quantitative evaluation was possible by applying the parameters of Table 3 together with $pK^{H}_{H(pUpU)}=6.44=pK^{H}_{H(R-PO_3)}$ (Table 1) to the straight-line Equation (6):

$$\log K^{M}_{[M(R-PO_3)]} = pK^{H}_{H(R-PO_3)} \times m+b \qquad (6)$$

The results of these calculations (Table 4) represent the stability constants of [M(R–PO$_3$)] complexes in which no additional interaction occurs, that is, the metal ion is coordinated only to a phosphate group that has the basicity of the terminal phosphate group in $pUpU^{3-}$. Comparison of these data with the measured stability constants demonstrates an enhanced stability for all five complexes studied.

Quantification of the enhanced stability of the [M(pUpU)]$^-$ complexes and the extent of chelate formation: The stability

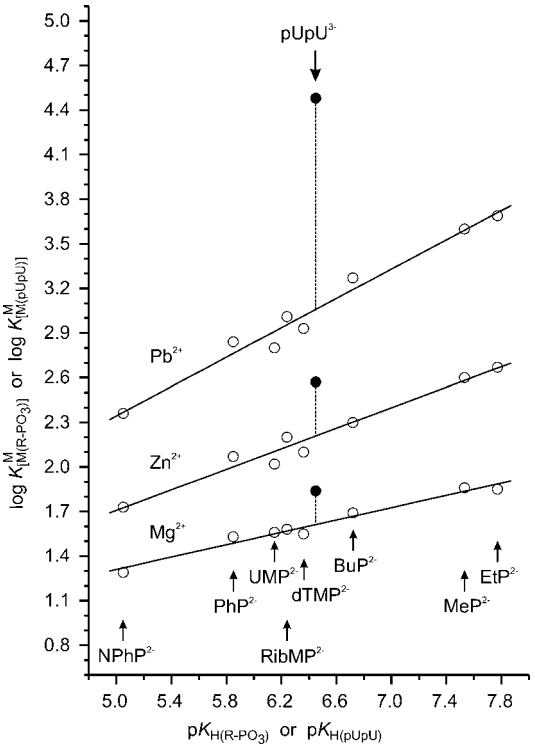

Figure 2. Evidence for an enhanced stability of the $[Mg(pUpU)]^-$, $[Zn(pUpU)]^-$, and $[Pb(pUpU)]^-$ complexes (●), based on the relationship between $\log K^{M}_{[M(R-PO_3)]}$ and $pK^{H}_{H(R-PO_3)}$ for [M(R–PO$_3$)] complexes of some simple phosphate monoester and phosphonate ligands ($R–PO_3^{2-}$) (○): (from left to right) 4-nitrophenyl phosphate ($NPhP^{2-}$), phenyl phosphate (PhP^{2-}), uridine 5′-monophosphate (UMP^{2-}), D-ribose 5-monophosphate ($RibMP^{2-}$), thymidine [1-(2′-deoxy-β-D-ribofuranosyl)thymine] 5′-monophosphate ($dTMP^{2-}$), *n*-butyl phosphate (BuP^{2-}), methanephosphonate (MeP^{2-}), and ethanephosphonate (EtP^{2-}). The least-squares lines [Eq. (6)] are drawn through the corresponding eight data sets (○) taken from reference [43] for the phosphate monoesters, and from reference [55] for the phosphonates; the corresponding straight-line parameters are listed in Table 3. The data points due to the $M^{2+}/H^+/pUpU^{3-}$ systems (●) are based on the constants listed in Tables 1 and 2. The vertical broken lines emphasize the stability differences from the reference lines, $\log\Delta_{M/pUpU}$, as defined in Equation (7) (see also Table 4, column 4). All plotted equilibrium constants refer to aqueous solutions at 25 °C and $I=0.1$ M ($NaNO_3$).

Table 4. Comparison of the stability constants of the $[M(pUpU)]^-$ complexes between the measured stability constants [Eq. (5)] and the calculated stability constants for [M(R–PO$_3$)] species, based on the basicity of the terminal phosphate group of $pUpU^{3-}$ ($pK^{H}_{H(pUpU)}=6.44$) and the reference-line equation [Eq. (6)] defined in Table 3, together with the stability differences $\log\Delta_{M/pUpU}$, as defined in Equation (7) (25 °C; $I=0.1$ M, $NaNO_3$).[a]

M^{2+}	$\log K^{M}_{[M(pUpU)]}$ [b]	$\log K^{M}_{[M(R-PO_3)]}$	$\log\Delta_{M/pUpU}$
Mg^{2+}	1.84 ± 0.04	1.61 ± 0.03	0.23 ± 0.05
Mn^{2+}	2.49 ± 0.05	2.22 ± 0.05	0.27 ± 0.07
Zn^{2+}	2.57 ± 0.03	2.20 ± 0.06	0.37 ± 0.07
Cd^{2+}	2.75 ± 0.03	2.52 ± 0.05	0.23 ± 0.05
Pb^{2+}	4.45 ± 0.25	3.05 ± 0.08	1.40 ± 0.26

[a] For the error limits, see footnote [a] of Table 1. The error limits (3σ) of the derived data in column 4 were calculated according to the error propagation of Gauss. [b] From column 3 in Table 2.

differences between the measured values for the [M(pUpU)]$^-$ complexes and the calculated values for the [M(R–PO$_3$)] species were obtained by using Equation (7), and are listed in Table 4.

$$\log \Delta_{M/pUpU} = \log K^{M}_{[M(pUpU)]} - \log K^{M}_{[M(R-PO_3)]} \quad (7)$$

The stability enhancements are identical within the error limits for the complexes of Mg^{2+}, Mn^{2+}, and Cd^{2+}. Considering the different coordinating properties[56–58] of these three metal ions, it becomes clear that this increased stability is simply due to the charge effect by going from [M(R–PO$_3$)] to [M(pUpU)]$^-$. In other words, the metal ion coordinated to the terminal phosphate group in pUpU^{3-} "feels" the presence of the negative charge located on the neighboring phosphate–diester bridge.

The average of the $\log \Delta_{M/pUpU}$ values for the Mg^{2+}, Mn^{2+}, and Cd^{2+} systems, $\log \Delta_{M/pUpU/charge} = 0.24 \pm 0.04$, represents this charge effect. Hence, any further stability increase must be attributed to an *additional* interaction of the metal ion already coordinated to the terminal phosphate group of pUpU^{3-}. This increase is defined by Equation (8):

$$\log \Delta^* = \log \Delta_{M/pUpU} - \log \Delta_{M/pUpU/charge} \quad (8)$$

As discussed in the Introduction, the only other available binding site in pUpU^{3-} is the phosphate–diester bridge, which allows the formation of a 10-membered chelate (Figure 1) involving both phosphate groups.

The values for $\log \Delta^*$ according to Equation (8) are listed in Table 5. As expected, these values are zero within the error limits for the [M(pUpU)]$^-$ complexes of Mg^{2+}, Mn^{2+}, and Cd^{2+}. In contrast, for the $\log \Delta^*$ values of Zn^{2+} and Pb^{2+}, this is clearly not the case. The different stability enhancements for the [Zn(pUpU)]$^-$ and [Pb(pUpU)]$^-$ complexes mean that the position of the intramolecular Equilibrium (9), between an open (op) and a closed (cl) or chelated isomer, varies.

Table 5. Extent of chelate formation in [M(pUpU)]$^-$ complexes [Eq. (9)], as calculated from the stability enhancement $\log \Delta^*$ [Eq. (8)] and quantified by the dimensionless equilibrium constant K_I [Eqs. (10) and (11)], and the percentage of the chelated isomer [Eq. (12)] in aqueous solution (25 °C; $I = 0.1$ M, NaNO$_3$).[a,b]

M^{2+}	$\log \Delta_{M/pUpU}$	$\log \Delta^*$	K_I	% [M(pUpU)$_{cl}$]$^-$
Mg^{2+}	0.23 ± 0.05	-0.01 ± 0.06	~0	~0 (<11)
Mn^{2+}	0.27 ± 0.07	0.03 ± 0.08	~0	~0 (<22)
Zn^{2+}	0.37 ± 0.07	0.13 ± 0.08	0.35 ± 0.25	26 ± 14
Cd^{2+}	0.23 ± 0.05	-0.01 ± 0.06	~0	~0 (<11)
Pb^{2+}	1.40 ± 0.26	1.16 ± 0.26	13.45 ± 8.65	93 ± 4

[a] For the error limits, see footnote [a] of Table 4. [b] The values given in column 2 are from column 4 in Table 4.

$$[M(pUpU)_{op}]^- \rightleftharpoons [M(pUpU)_{cl}]^- \quad (9)$$

The position of Equilibrium (9) is defined by the dimensionless intramolecular equilibrium constant K_I [Eq. (10)]:

$$K_I = [[M(pUpU)_{cl}]^-]/[[M(pUpU)_{op}]^-] \quad (10)$$

As shown previously[36,53,54] the stability enhancement $\log \Delta^*$ [Eq. (8)] is related to K_I by Equation (11):

$$K_I = 10^{\log \Delta^*} - 1 \quad (11)$$

Knowledge of K_I facilitates calculation of the formation degree of the closed species in Equilibrium (9) by using Equation (12):

$$\% \ [M(pUpU)_{cl}]^- = 100 \times [K_I/(1+K_I)] \quad (12)$$

The results for K_I and % [M(pUpU)$_{cl}$]$^-$ are listed in Table 5.

As already stated, in the case of the [M(pUpU)]$^-$ systems with Mg^{2+}, Mn^{2+}, and Cd^{2+}, the values of $\log \Delta^*$ are zero within the error limits. However, this means that within these error limits, traces of chelated species might form; the corresponding upper limits are given in parentheses in Table 5. Indeed, for [Mn(pUpU)]$^-$, it appears that a small percentage of a closed species might occur. This is possible because Mn^{2+} is known[45] for its relatively pronounced affinity for phosphate groups, and the given error limits correspond to 3σ. In any case, chelates for [Zn(pUpU)]$^-$ and [Pb(pUpU)]$^-$ definitely exist, with formation degrees of approximately 25 % and greater than 90 %, respectively.

Interestingly, our results are in good agreement with the *Stability Ruler* proposed by Martin.[56–58] With regard to interactions with oxygen donors, such as oxalate, Cd^{2+} is placed significantly below Zn^{2+} and Pb^{2+}, to give the order $Cd^{2+} \ll Zn^{2+} < Pb^{2+}$. In the case of pUpU^{3-} discussed here, Pb^{2+} may be especially favored due to its larger size, which should facilitate binding of two neighboring phosphate groups in a nucleic acid.

Conclusions

An important result from this study with regard to RNAs and ribozymes is the observation that neighboring uracil residues lead to a depression of the pK_a value for the deprotonation of the (N3)H site by about half a log unit compared to UMP^{2-}. This deprotonation may be further promoted by metal ions, such as Mg^{2+} and Ca^{2+}, which have a relatively selective affinity for the carbonyl oxygen atoms of nucleobases, as shown recently[30,40] for the cytosine and uracil residues. As a consequence of such a facilitated deprotonation of the (N3)H site, possibly further enhanced by the indicated coordination of alkaline earth ions, the pK_a for this site approaches the physiological pH range. Uracil residues are, therefore, important factors to be considered in general acid–base catalyzed reactions.

By considering the stability enhancement of the different [M(pUpU)]$^-$ complexes, it is notable that Pb^{2+}, and to a certain extent also Zn^{2+}, differ considerably in their binding properties towards phosphate groups. Firstly, the Pb^{2+}–phos-

phate complexes have a rather high stability compared to, for example, Mg^{2+}, and secondly, Pb^{2+} is the only one of the ions investigated here that shows a very pronounced ability to interact strongly with two neighboring phosphate sites. Therefore, it is not surprising that leadzymes,[59,60] as well as Pb^{2+}-dependent DNAzymes,[61] that is, RNA or DNA oligonucleotides with a structural motif that selectively binds Pb^{2+} to promote hydrolytic cleavage of a second nucleic acid, could be selected by conducting in vitro selection experiments. Our results also explain why some leadzymes are strongly inhibited[62–65] by the presence of Mg^{2+} and other divalent ions; such ions inhibit chelate formation of Pb^{2+}. A difference in RNA binding between Pb^{2+} and Mg^{2+} has also been observed in hydrolytic cleavage experiments of a group II intron ribozyme[15] and other large RNAs.[66] Furthermore, despite its higher affinity, Pb^{2+} showed fewer (and/or different) cleavage sites than was observed with Mg^{2+} and Tb^{3+}. These discrepancies in binding patterns are explained by our results, namely that Pb^{2+} has a high tendency to coordinate two neighboring phosphate groups on its own, whereas Mg^{2+} can only do so if the binding is further stabilized by surrounding nucleotides from other parts of the nucleic acid.

In the context of Pb^{2+}, two further points should be emphasized: Firstly, Pb^{2+} is a well-known mimic of Ca^{2+},[57,58,67] and the alkaline earth metal ion itself is a potent inhibitor of ribozyme activity.[68,69] One may, therefore, assume, though this is still to be proven, that in a nucleic acid, Ca^{2+} can bind simultaneously, like Pb^{2+} as discussed above, to two neighboring phosphate groups, whereas Mg^{2+} can not. Secondly, the straight-line parameters in Table 3 for the Pb^{2+} and Cu^{2+} systems are identical within their error limits. Indeed, the stability constants calculated with a $pK_a = 6.44$ for the two complexes [Pb(R–PO_3)] and [Cu(R–PO_3)], that is, $\log K^{Pb}_{[Pb(R-PO_3)]} = 3.05 \pm 0.08$ and $\log K^{Cu}_{[Cu(R-PO_3)]} = 2.98 \pm 0.06$, are also identical within the error limits. Based on this observation and on the *Stability Ruler* of Martin,[56–58] it can be concluded that the properties regarding stability and structure of the $[Pb(pUpU)]^-$ and $[Cu(pUpU)]^-$ complexes are similar.

The selective coordination of Mg^{2+} to only a single phosphate group is also revealing with regard to this metal ion's crucial role in ribozyme catalysis. Although structures are known in which Mg^{2+} bridges several phosphate groups,[70,71] this occurs in a structurally enforced way. Many more examples are known that involve a Mg^{2+} ion coordinated to a single phosphate unit.[3,4,70–74] Among the latter group, Mg^{2+} binding to uridine 80 within U6 of the spliceosome,[11,12] or to adenosine 2 within domain 5 of the group II intron ai5γ[16,75] are only two prominent examples. For cases in which two or more neighboring phosphate groups bind to Mg^{2+} ions, these then tend to cluster close together, as observed in the loop E motif,[14] the active site of group I introns,[73] as well as at a single nucleobase.[76] The findings presented here are in good agreement with all of these observations, and furthermore, they assign the first quantitative evaluation to these phenomena.

Experimental Section

Synthesis of uridylyl-(5′→3′)-[5′]uridylic acid, H_3(pUpU): Although salts of $pUpU^{3-}$ have already been synthesized by using either the phosphodiester[23,24] or phosphotriester[25,26] approach, we used a new method. The trisodium salt of $pUpU^{3-}$ (**1**) was prepared by a multistep synthesis (Scheme 1, Supporting Information) using the phosphoramidite methodology, with the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group for protection of 2′-hydroxy functions.[77,78] 5′-*O*-dimethoxytrityl-2′-*O*-Fpmp uridine-3′-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (**2**) was prepared as previously described.[77] Compound **2** was then reacted in CH_2Cl_2 solution with 2′,3′-di-*O*-acetyluridine in the presence of 1-H-tetrazole to give, after subsequent I_2/pyridine/H_2O oxidation of the intermediate phosphite, the fully protected dinucleosidephosphate (**3**). After selective removal of the dimethoxytrityl (DMT) group with 2% dichloroacetic acid in CH_2Cl_2, compound **3** was 5′-*O*-phosphorylated by using the bis-*O,O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite reagent, and the phosphite intermediate was oxidized with I_2/pyridine/H_2O.[79] The fully protected 5′-*O*-phosphorylated dinucleotide (**4**) obtained was purified by silica gel chromatography and subjected to stepwise deprotection by heating to 55 °C for 16 h in the presence of 30% aqueous ammonia (removal of 2-cyanoethyl and acetyl groups) followed by treatment with 0.01 M HCl (pH 2.0) (12 h at RT; removal of the Fpmp group). The crude product **1** was purified by ion-exchange chromatography using DEAE Sephadex A-25 (elution with a linear gradient of triethylammonium bicarbonate from 0.1 to 0.6 M). Purified **1** was then transformed into its trisodium salt by passing it through Dowex 50Wx8 (Na^+ form) and lyophylized to yield a white solid in 12% overall yield (based on 2′,3′-di-*O*-acetyluridine). The structure of **1** was confirmed by using spectroscopic methods: proton-decoupled ^{31}P NMR (D_2O) two singlets at $\delta = -0.14$ and 0.61 ppm [Bruker Avance, 200 MHz]; FAB MS: m/z: 629.2 (negative ions) (Finnigan MAT 95) (calculated MW 630.4 for free acid). Analytical RP-HPLC of product **1** showed a single peak.

Materials: The disodium salt of ethylenediamine-*N,N,N′,N′*-tetraacetic acid ($Na_2H_2EDTA \cdot 2H_2O$), potassium hydrogen phthalate, nitric acid, sodium hydroxide (Titrisol), and the nitrate salts of Na^+, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} (all pro analysi) were from Merck, Darmstadt, Germany. The buffer solutions (pH 4, 7, 9), all based on the NBS scale (now U.S. National Institute of Science and Technology, NIST), were from Metrohm, Herisau, Switzerland. All solutions were prepared by using deionized, ultrapure (Milli-Q185 Plus; from Millipore S.A., 67120 Molsheim, France) CO_2-free water.

The titer of the NaOH solution used for the titrations was established by using potassium hydrogen phthalate, and the exact concentrations of the stock solutions of Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+} were determined by performing potentiometric pH titrations using their EDTA complexes. The aqueous stock solutions of pUpU were freshly prepared daily and the pH of the solution was adjusted to 8.3 with sodium hydroxide. The exact concentration of the ligand solutions was determined in each experiment by evaluation of the corresponding titration pair, that is, the differences in NaOH consumption between solutions with and without ligand (see below).

Potentiometric pH titrations: The pH titrations were conducted by using a Metrohm E536 potentiograph connected to a Metrohm E665 dosimat and a Metrohm 6.0253.100 Aquatrode-plus combined double-junction macro glass electrode (all from Metrohm, Herisau, Switzerland). The equipment was calibrated by using the buffer solutions mentioned above. The direct pH meter readings were used in the calculations of the acidity constants of $H(pUpU)^{2-}$; thus, the constants determined at $I = 0.1$ M ($NaNO_3$) and 25 °C are so-called practical, mixed, or Brønsted constants,[46] and may be converted into the corresponding concentration constants by subtracting 0.02 from the listed pK_a values.[46] This conversion term includes both the junction potential of the glass electrode and the hydrogen ion activity.[46,80] It should be emphasized that the ionic product of water (K_W) and the conversion term mentioned do not enter into our calculation procedures, because the differences in NaOH consumption between solutions with and without ligand (see below) are evaluated.[46,81] No conversion is necessary for the stability constants of the

$[M(pUpU)]^-$ complexes because these are, as usual, concentration constants.

Determination of equilibrium constants: The acidity constants $K^H_{H(pUpU)}$, $K^H_{(pUpU)}$, and $K^H_{(pUpU-H)}$ of $H(pUpU)^{2-}$ [Eqs. (2)–(4)] were determined by titrating 30 mL of aqueous 0.5 mM HNO_3 (25 °C; $I = 0.1$ M, $NaNO_3$) under N_2 with up to 3.0 mL of 0.03 M NaOH in the presence and absence of 0.27 mM $H(pUpU)^{2-}$. Additional titrations were performed with a ligand concentration of 0.18 mM, and in this case, 3.0 mL of 0.02 M NaOH was used. Notably, no difference between the two conditions was observed.

The experimental data were evaluated by employing a curve-fitting procedure using a Newton-Gauss non-linear least-squares program, in which the difference in NaOH consumption between such a pair of titrations at every 0.1 pH unit was used. The acidity constants of $H(pUpU)^{2-}$ were calculated within the pH range 4.8 to 9.7, corresponding to 2% neutralization (initial) for the equilibrium $H(pUpU)^{2-}/(pUpU)^{3-}$ and around 54% (final) for $(pUpU-H)^{4-}/(pUpU-2H)^{5-}$. The final results for $pK^H_{H(pUpU)}$, $pK^H_{(pUpU)}$, and $pK^H_{(pUpU-H)}$ are the averages of the values from six independent pairs of titrations.

After each of these titrations, the solutions were adjusted to the initial pH of around 3.3 by adding a small volume (about 1 mL) of 0.1 M HNO_3. Subsequently, a comparatively small volume of a solution of $M(NO_3)_2$ ($M^{2+} = Mn^{2+}$, Zn^{2+}, Cd^{2+}) was added and the titration was repeated. From the data obtained in the presence of M^{2+} (with and without ligand), the stability constants $K^M_{[M(pUpU)]}$ of the $[M(pUpU)]^-$ complexes [Eq. (5)] were calculated. The total volume of these solutions was around 35 mL, and the ionic strength I varied between 0.1 and 0.13 M. This small variation in I had no effect on complex stability, as evident from the experiments with $M^{2+} = Mn^{2+}$ and Zn^{2+} in which $I = 0.1$ M (see below).

In addition, the stability constants of the $[M(pUpU)]^-$ complexes with $M^{2+} = Mn^{2+}$ and Zn^{2+} were determined under the same conditions used for the acidity constants, although $NaNO_3$ was partly replaced by $M(NO_3)_2$ (25 °C; $I = 0.1$ M). For the corresponding complexes with Mg^{2+} and Pb^{2+}, the same conditions were used, however, in some experiments with Mg^{2+}, $NaNO_3$ was fully replaced by $Mg(NO_3)_2$. The metal-to-ligand ratios in the various titrations were 130:1, 87:1, and 84:1 for Mg^{2+}; 87:1, 65:1, and 63:1 for Mn^{2+}; 65:1, 42:1, and 32:1 for Zn^{2+}; 33:1 and 21:1 for Cd^{2+}; and 14:1, 2.4:1, and 1.8:1 for Pb^{2+}. For all systems, the calculated stability constants showed no dependence on the excess of M^{2+} used.

The titration data, except those for the determination of $K^{Pb}_{[Pb(pUpU)]}$ of the $[Pb(pUpU)]^-$ complex with a very small metal-to-ligand ratio (2.4:1 and 1.8:1), were evaluated by employing a curve-fitting procedure using a Newton-Gauss non-linear least-squares program for each titration pair (i.e., with and without ligand), by calculating the apparent acidity constant K'_a. Depending on the metal ion under consideration, the evaluation commenced at a formation degree of the $[M(pUpU)]^-$ species of about 2 to 10% (see below), and the upper limit was given by either the onset of the hydrolysis of $M(aq)^{2+}$, which was evident from the titrations without ligand, or by the formation of the $[M(pUpU-H)]^{2+}$ or $[M_2(pUpU-H)]$ species, which was evident by the deviation of the experimental data from the calculated curve. Representative examples for the pH ranges employed in the case of the $[M(pUpU)]^-$ complexes are 4.6–6.8 (Mg^{2+}), 4.2–6.3 (Mn^{2+}), 3.9–5.6 (Zn^{2+}), 4.1–5.8 (Cd^{2+}), and 3.6–4.3 (Pb^{2+}). These pH ranges correspond to variations in the formation degrees of about 3–56% for $[Mg(pUpU)]^-$, 4–72% for $[Mn(pUpU)]^-$, 2–39% for $[Zn(pUpU)]^-$, 2–43% for $[Cd(pUpU)]^-$, and 10–26% for $[Pb(pUpU)]^-$. The stability constants of the complexes were calculated as described previously.[30,53,82,83] Notably, the buffer depression $\Delta pK_a = pK^H_{H(pUpU)} - pK'_a$ was satisfactory in all titrations, i.e., $\Delta pK_a \geq 0.35$.

The titration pairs of Pb^{2+} with a small metal-to-ligand ratio (2.4:1 and 1.8:1) were typically evaluated within the pH range 3.8 to 4.2, corresponding to a formation degree of the $[Pb(pUpU)]^-$ complex of about 2.6 to 6.2% (M^{2+}:ligand = 2.4:1). The corresponding stability constant was calculated by taking into account the species H^+, $H(pUpU)^{2-}$, $(pUpU)^{3-}$, Pb^{2+}, and $[Pb(pUpU)]^-$.[84] Note that in the $Pb^{2+}/(pUpU)^{3-}$ system, a precipitation forms at relatively low pH and, therefore, the pH range accessible for the evaluation is small, as is the formation degree of the $[Pb(pUpU)]^-$ species. Hence, only an estimate for the stability constant could be obtained.

The final results for the stability constants of all $[M(pUpU)]^-$ complexes, $K^M_{[M(pUpU)]}$, are the averages of three independent titrations in the case of the Mg^{2+}, Mn^{2+}, and Zn^{2+} systems, whereas for Cd^{2+} and Pb^{2+}, two and four independent pairs of titrations were performed, respectively. There was no indication of a metal-ion-promoted hydrolysis of $pUpU^{3-}$ during the time required for a titration experiment (about 30 min).

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