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Discrimination in Metal-Ion Binding to RNA Dinucleotides with a Non-Bridging Oxygen or Sulfur in the Phosphate Diester Link[+]

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Abstract: Replacement of a non-bridging oxygen in the phosphate diester bond by a sulfur has become quite popular in nucleic acid research and is often used as a probe, for example, in ribozymes, where the normally essential Mg^{2+} is partly replaced by a thiophilic metal ion to reactivate the system. Despite these widely applied rescue experiments no detailed studies exist quantifying the affinity of metal ions to such terminal sulfur atoms. Therefore, we performed potentiometric pH titrations to determine the binding properties of $pUp_{(S)}U^{3-}$ towards Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}, and compared these data with those previously obtained for the corresponding $pUpU^{3-}$ complexes. The primary binding site in both dinucleotides is the terminal phosphate group. Theoretically, also the formation of 10-membered chelates involving the terminal oxygen or sulfur atoms of the (thio)phosphate bridge is possible with both ligands. The results show that Mg^{2+} and Mn^{2+} exist as open (op) isomers binding to both dinucleotides only at the terminal phosphate group. Whereas $Cd(pUpU)^{-}$ only exists as $Cd(pUpU)^{-}_{op}$, $Cd(pUp_{(S)}U)^{-}$ is present to about 64%

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as the S-coordinated macrochelate, $Cd(pUp_{(S)}U)^{-}_{cl/PS}$. Zn^{2+} forms with $pUp_{(S)}U^{3-}$ three isomeric species, that is, $Zn(pUp_{(S)}U)^{-}_{op}$, $Zn(pUp_{(S)}U)^{-}_{cl/PO}$, and $Zn(pUp_{(S)}U)^{-}_{cl/PS}$, which occur to about 33, 12 (O-bound), and 55%, respectively. Pb^{2+} forms the 10-membered chelate with both nucleotides involving only the terminal oxygen atoms of the (thio)phosphate bridge, that is, no indication of S binding was discovered in this case. Hence, Zn^{2+} and Cd^{2+} show pronounced thiophilic properties, whereas Mg^{2+}, Mn^{2+}, and Pb^{2+} coordinate to the oxygen, macrochelate formation being of relevance with Pb^{2+} only.

1. Introduction

The phosphate group of nucleotides has been altered in many ways (see citations in refs. [1,2]). Probably the most popular alteration is the substitution of one of the oxygens by a sulfur atom, such compounds being first synthesized in 1966.[3] Initially these derivatives were employed in studies regarding the mechanisms of enzymatic reactions[4,5] for

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[+] **Abbreviations and definitions** (see also Figures 1 and 2): *I*, ionic strength; K_a, general acidity constant; L, general ligand; M^{2+}, general divalent metal ion; $MeOPS^{2-}$, methyl thiophosphate; $pUpU^{3-}$ and $pUp_{(S)}U^{3-}$, see Figure 1; $RibMP^{2-}$, D-ribose 5-monophosphate; $R\text{-}PO_3^{2-}$, simple phosphate monoester or phosphonate ligand with R representing a non-interacting residue; UMP, uridine 5′-monophosphate; $UMPS^{2-}$, uridine 5′-*O*-thiomonophosphate. Species written without a charge either do not carry one or represent the species in general (i.e., independent of their protonation degree); which of the two possibilities applies is always clear from the context. A formula like $(pUp_{(S)}U-H)^{4-}$ means that the dinucleotide has lost a proton from one of its (N3)H sites and that it has to be read as "$pUp_{(S)}U$ *minus* H".

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the authors.

which purpose they are still used.[6,7] However, more recently, with the development of the antisense strategy[8,9] and the observation that oligonucleotides with a sulfur modification at the phosphodiester linkage are usually more resistant toward nuclease degradation than natural oligonucleotides,[10,11] their application has widened including also drugs.[12] A further important aspect is the increasing focus on RNA-catalyzed reactions.[13–16] Here, thio analogues are important tools[17] for studying, for example, the role of metal ions in ribozyme folding and activity[18–20] as well as in attempts to understand the mechanisms of ribozyme reactions[21] and to identify those phosphate oxygen atoms which are important for catalysis.[22,23] Similar experiments have also been recently conducted with a thioderivative of the 10–23 DNAzyme to identify a metal-ion binding site within the catalytic core.[24]

Considering the above indicated wide interest in thiophosphate derivatives, surprisingly few investigations have been conducted to determine the basic differences between phosphate and thiophosphate groups, such as the effects of hydrogen bonding to the sulfur on the ribozyme cleavage reaction[25] or the stabilizing effects on DNA–RNA triplex formation.[26,27] In all instances metal ions need to be present at the very least for charge neutralization. With regard to kinetically labile divalent metal ions (M^{2+}) and thio derivatives of nucleotides only a few reports exist, that is, of adenosine 5′-mono-[1,2], di-, and triphosphate.[28] In addition, a more recent study dealt with the interaction of M^{2+} with uridine 5′-*O*-thiomonophosphate ($UMPS^{2-}$) and methyl thiophosphate ($MeOPS^{2-}$).[29] These experiments proved that the uracil residue is not involved in metal-ion binding in M(UMPS) complexes as is also the case with M(UMP) complexes.[30] Concerning the present study with $pUp_{(S)}U^{3-}$, this means that there will be no interference of the uracil moieties.

The phosphate diester bridge, that is, -O-P(O)$_2^-$-O-, is the dominating phosphate group in nucleic acids and corresponding thio substitutions, that is, -O-P(O)(S)$^-$-O-, are widely employed in ribozyme studies.[15,16,19–23,31] Hence, it is surprising to find that, to the best of our knowledge, not a single study exists where the intrinsic affinity of a terminal (non-bridging) sulfur atom in such a bridge towards divalent metal ions was quantified. Considering further our own interests in metal-ion–ribozyme interactions,[32–35] we made now an effort to measure such affinities.

It is very difficult to directly measure the metal-ion affinity of such a -O-P(O)(S)$^-$-O- site in a polymer or even in an oligonucleotide, such as $UpUpU^{2-}$. The reason being that competition for binding between a metal ion and a proton occurs neither within the experimentally accessible nor within the physiological pH range because the primary protons of phosphate or thiophosphate residues are released with pK_a values of about one (or even below).[29,36] Therefore, we measured the metal-ion affinity of such a thiophosphate diester unit by using a dinucleotide containing both a terminal 5′-phosphate group and a 5′→3′-phosphate diester bridge in which one of the two terminal O atoms was replaced by a S atom. The main binding site in such a dinucleotide is the terminal phosphate group, but chelate formation with the neighboring thiophosphate diester bridge is at least theoretically possible and therefore information can be gained on the metal-ion affinity of this second site.

In order to make the interpretation of the experimental data unequivocal, we selected a dinucleotide with nucleobases of low metal-ion affinity. As indicated above, the uracil residue is such a nucleobase and hence we studied now the metal-ion binding properties of $pUp_{(S)}U^{3-}$ towards Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}. Comparison with the experimental data obtained earlier[36] for $pUpU^{3-}$ allowed us to answer questions regarding the selectivity of biologically relevant metal ions like Mg^{2+} or Zn^{2+} for the S atom of the thiophosphate bridge. Here, we quantify the formation degrees of the 10-membered chelates formed by the different metal ions in their complexes with the mentioned two dinucleotides (Figure 1) and determine the extent of O or S coordination, respectively, in those cases where macrochelate formation occurs. Indeed, the observed selectivity and discrimination is considerable in several respects: For example, there is no chelate formation in $Mg(pUp_{(S)}U)^-$ whereas $Zn(pUp_{(S)}U)^-$ exists in total to about 67% in the form of chelates (S and O bound), a formation degree significantly larger than the one observed[36] for $Zn(pUpU)^-$ which amounts to about 26% only.

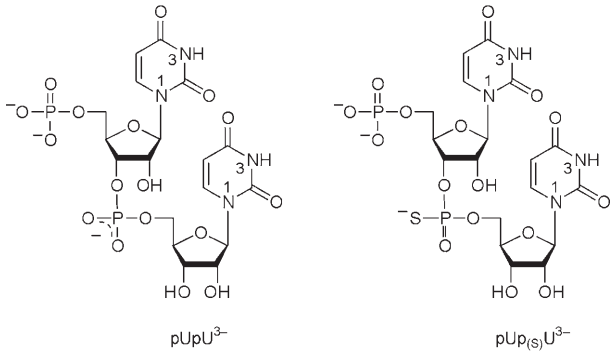

Figure 1. Chemical structures of uridylyl-(5′→3′)-[5′]-uridylate ($pUpU^{3-}$) and of its thio derivative *P*-thiouridylyl-(5′→3′)-[5′]-uridylate ($pUp_{(S)}U^{3-}$). The two uridine units in each structure are shown in their dominating *anti* conformation.[37,38] For the charge distribution in the thiophosphate diester bridge see also Section 2.1.

2. Results and Discussion

2.1. Acid–base properties of $pUp_{(S)}U^{3-}$: This dinucleotide with a terminal S atom at the diester bridge (Figure 1) can accept a total of three protons at its (thio)phosphate groups. Two of these protons are released at very low pH, which follows from $pK_a = 1.0 \pm 0.3$ estimated for the release of a proton from the P(O)(OH)$_2$ group of $H_2(pUpU)^-$.[36] For $H_2(pUp_{(S)}U)^-$ the release of the proton from the bridging thiophosphate group is expected to occur with $pK_a < 1$ in

$H_3(pUp_{(S)}U)$ because substitution of an O atom in a phosphate group by an S atom increases the acidity.[1,29,39] The proton is thereby most likely bound at the terminal oxygen atom of this bridge.[2,39] After deprotonation the negative charge of the thiophosphate bridge is mainly located at the sulfur atom,[39–41] as is depicted in Figure 1.

It follows from the above that for the present study three deprotonation reactions of the $H(pUp_{(S)}U)^{2-}$ species are of relevance; that is, the release of the "second" proton from the terminal phosphate group as well as the two protons from the uracil (N3)H sites. This leads to the following deprotonation equilibria:

$$H(pUp_{(S)}U)^{2-} \rightleftharpoons pUp_{(S)}U^{3-} + H^+ \quad (1a)$$

$$K^{H}_{H(pUp_{(S)}U)} = [pUp_{(S)}U^{3-}][H^+]/[H(pUp_{(S)}U)^{2-}] \quad (1b)$$

$$pUp_{(S)}U^{3-} \rightleftharpoons (pUp_{(S)}U{-}H)^{4-} + H^+ \quad (2a)$$

$$K^{H}_{pUp_{(S)}U} = [(pUp_{(S)}U{-}H)^{4-}][H^+]/[pUp_{(S)}U^{3-}] \quad (2b)$$

$$(pUp_{(S)}U{-}H)^{4-} \rightleftharpoons (pUp_{(S)}U{-}2H)^{5-} + H^+ \quad (3a)$$

$$K^{H}_{(pUp_{(S)}U-H)} = [(pUp_{(S)}U{-}2H)^{5-}][H^+]/[(pUp_{(S)}U{-}H)^{4-}] \quad (3b)$$

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

The acidity constants for the Equilibria (1a), (2a), and (3a) were measured by potentiometric pH titrations (see Experimental Section). The results are listed in Table 1 whereby the site attributions are evident from the given related data.[30,36,42,43]

With regard to the release of the final proton from the phosphate groups, the data show that $H(pUpU)^{2-}$ is more basic than $H(UMP)^-$ by about 0.3 pK units (6.44 compared to 6.15). This increased basicity of the terminal phosphate group of $pUpU^{3-}$ compared to that of UMP^{2-} is clearly a charge effect and in accord with related observations.[44]

Table 1. Negative logarithms of the acidity constants for the deprotonation of the $P(O)_2(OH)^-$ and (N3)H sites in $H(pUp_{(S)}U)^{2-}$ [Eqs. (1–3)], together with some related data, as determined by potentiometric pH titrations in aqueous solution (25 °C; $I = 0.1$ M, $NaNO_3$).[a,b]

Acids	pK_a of the sites		Refs.
	$P(O)_2(OH)^-$	(N3)H	
$H(RibMP)^-$	6.24 ± 0.01		[29,45]
uridine		9.18 ± 0.02	[46]
$H(UMP)^-$	6.15 ± 0.01	9.45 ± 0.02	[29,45]
$H(pUpU)^{2-}$	6.44 ± 0.02	8.99 ± 0.03/9.63 ± 0.08	[34]
$H(pUp_{(s)}U)^{2-}$	6.32 ± 0.03	9.29 ± 0.04/9.98 ± 0.12	–

[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[45] are listed (see also Section 4.3).

The small increase in acidity by about 0.1 pK unit for the terminal $-P(O)_2(OH)^-$ group in going from $H(pUpU)^{2-}$ to $H(pUp_{(S)}U)^{2-}$ is more difficult to explain and probably a solvation effect: The nearby S atom of the thiophosphate bridge is expected to be less effective in hydrogen bonding than it is the case with an O atom.

Interestingly, the difference in acidity between the two (N3)H sites is small and the same within the error limits for the two dinucleotides $pUpU^{3-}$ ($\Delta pK_{a(O)} = (9.63 \pm 0.08) - (8.99 \pm 0.03) = 0.64 \pm 0.09$) and $pUp_{(S)}U^{3-}$ ($\Delta pK_{a(S)} = (9.98 \pm 0.12) - (9.29 \pm 0.04) = 0.69 \pm 0.13$) (see also Table 1). These differences are identical within the error limits to the one expected for symmetrical diprotonic acids (H_2L), where the difference amounts to 0.6 pK units.[36] The observation that the two uracil residues in a given dinucleotide (Figure 1) have practically identical acidic properties means that these residues do not significantly affect each other.

Another interesting aspect is the observation that the presence of the S atom in the diester bridge increases the basicity of all $(N3)^-$ sites as the comparisons of the $pK_{a/2}$ and $pK_{a/3}$ values according to Equilibria (2a) and (3a) shows: $\Delta pK_{a/2} = (9.29 \pm 0.04) - (8.99 \pm 0.03) = 0.30 \pm 0.05$ and $\Delta pK_{a/3} = (9.98 \pm 0.12) - (9.63 \pm 0.08) = 0.35 \pm 0.14$ (Table 1). This effect is most likely due to the larger hydrophobicity of $pUp_{(S)}U$, compared to that of pUpU, as well as to the localization of the negative charge of the diester bridge mainly on the S atom in $pUp_{(S)}U$ whereas in pUpU this charge is equally distributed between two O atoms (Figure 1). Hence, the introduction of a bridging thiophosphate group into a nucleic acid also affects the acid–base properties of the neighboring nucleobases to a certain degree. Interestingly, this effect is only observed with bridging (thio)phosphate groups, but not with a terminal phosphate group (where the charge distribution differs less), as can be seen by comparison of UMP^{2-} ($pK^{H}_{UMP} = 9.45 \pm 0.02$; Table 1) and $UMPS^{2-}$ ($pK^{H}_{UMPS} = 9.47 \pm 0.02$).[29] In the latter case, only the release of the proton from the phosphate group is strongly affected, that is, $pK^{H}_{H(UMP)} = 6.15 \pm 0.01$ versus $pK^{H}_{H(UMPS)} = 4.78 \pm 0.02$.[29]

2.2. Stabilities of $M(pUp_{(S)}U)^-$ complexes: The stability constants of several $M(pUp_{(S)}U)^-$ species were determined by potentiometric pH titrations. All experimental data can be perfectly explained by taking Equilibrium (1a) as well as the following complex-forming Equilibrium (4a) into account:

$$M^{2+} + pUp_{(S)}U^{3-} \rightleftharpoons M(pUp_{(S)}U)^- \quad (4a)$$

$$K^{M}_{M(pUp_{(S)}U)} = [M(pUp_{(S)}U)^-]/([M^{2+}][pUp_{(S)}U^{3-}]) \quad (4b)$$

The data evaluation was restricted to the pH range in which neither hydroxo complexes are formed nor (N3)H is deprotonated, the pH range in which the formation of hydroxo complexes occurs being evident from the titrations in the absence of ligand (see Section 4.4).

The stabilities of the five metal-ion complexes studied are listed for comparison in column 3 of Table 2, along with the corresponding values of the $M(pUpU)^-$ complexes.[36,46] Evidently, the stabilities of the $M(pUp_{(S)}U)^-$ complexes of Mg^{2+} and Mn^{2+} are hardly affected by the presence or absence of an S atom in the diester bridge, whereas the complexes of Zn^{2+} or Cd^{2+} become more stable if S is present and those with Pb^{2+} show a reduced stability.

To be able to explain the above observations, a more rigorous evaluation procedure is required to elucidate the structures of the $M(pUp_{(S)}U)^-$ and $M(pUpU)^-$ complexes in solution. It is well known that straight lines are obtained for a series of related ligands by plotting $\log K^{M}_{M(L)}$ versus $pK^{H}_{H(L)}$ [1,47] Such correlation lines are available for complexes formed between several divalent metal ions (M^{2+}) and simple phosphate monoester or phosphonate ligands ($R\text{-}PO_3^{2-}$)[42,48] and the parameters according to the straight-line Equation (5), have been listed.[36,42,46]

$$\log K^{M}_{M(R\text{-}PO_3)} = pK^{H}_{H(R\text{-}PO_3)} \cdot m + b \qquad (5)$$

The data pairs of the $M(R\text{-}PO_3)$ complexes for the Mg^{2+}, Zn^{2+}, and Pb^{2+} systems, on which the parameters for Equation (5) are based, are shown together with the corresponding data points for the $M^{2+}/pUp_{(S)}U^{3-}$ and $pUpU^{3-}$ systems in Figure 2. For all six complexes an increased stability is observed. However, this stability varies considerably depending on both the metal ion and the dinucleotide involved in complex formation: In the case of Zn^{2+} the thio derivative forms the more stable complex whereas with Pb^{2+} the $pUpU^{3-}$ complex is more stable.

A more quantitative evaluation is possible by applying Equation (5) with its parameters[36] together with $pK^{H}_{H(pUp_{(S)}U)} = 6.32$ (Table 1). The results for these calculations are listed in column 4 of Table 2 representing the stability constants $\log K^{M}_{M(R\text{-}PO_3)}$ of $M(R\text{-}PO_3)$ complexes in which the metal ion is coordinated solely to a phosphate group that has the basicity of the terminal phosphate group in $pUp_{(S)}U^{3-}$, that is, no additional interaction occurs. Comparison of these data with the measured stabilities demonstrates an enhanced stability for all five $M(pUp_{(S)}U)^-$ complexes studied.

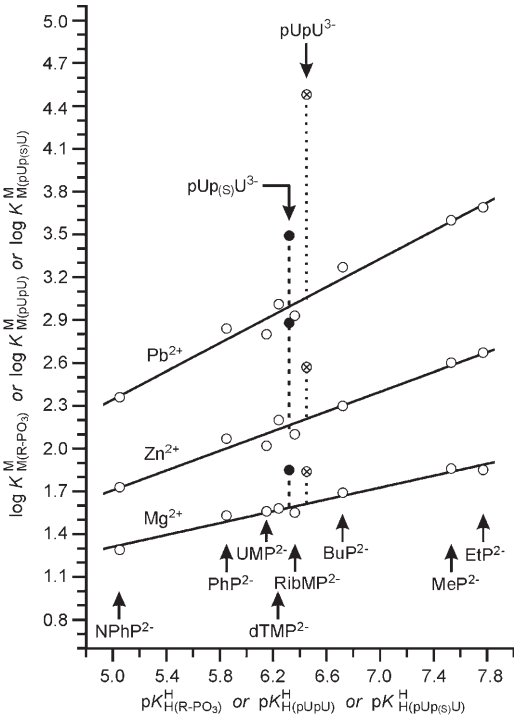

Figure 2. Evidence for an enhanced stability of some $M(pUp_{(S)}U)^-$ (●) and $M(pUpU)^-$ (⊗) complexes, based on the relationship between $\log K^{M}_{M(R\text{-}PO_3)}$ and $pK^{H}_{H(R\text{-}PO_3)}$ for $M(R\text{-}PO_3)$ complexes of some simple phosphate monoester and phosphonate ligands ($R\text{-}PO_3^{2-}$) (○): (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. (5)] are drawn through the corresponding eight data sets (○) taken from ref. [30] for the phosphate monoesters and from ref. [48] for the phosphonates. The corresponding straight-line parameters are listed in refs. [36,46] and [48]. The data points due to the $M^{2+}/H^+/pUpU^{3-}$ systems (⊗) are from ref. [36] and those for the $M^{2+}/H^+/pUp_{(S)}U^{3-}$ systems (●) are based on the constants in Tables 1 and 2. The vertical broken lines emphasize the stability differences from the reference lines as defined by Equation (6) (see also Table 2, column 5). All plotted equilibrium constants refer to aqueous solutions at 25 °C and $I = 0.1$ M ($NaNO_3$).

Table 2. Comparison of the stability constants of the $M(pUp_{(S)}U)^-$ complexes between the measured stability constants [Eq. (4)] and the calculated stability constants for $M(R\text{-}PO_3)$ species, based on the basicity of the terminal phosphate group of $pUp_{(S)}U^{3-}$ ($pK^{H}_{H(pUp_{(S)}U)} = 6.32$) and the reference-line Equation (5) with its corresponding parameters,[36,46] together with the stability differences $\log\Delta_{M/pUp_{(S)}U}$ as defined in Equation (6). For comparison the corresponding data for the $M^{2+}/pUpU^{3-}$ systems are also listed (aqueous solution; 25 °C; $I = 0.1$ M, $NaNO_3$).[a]

Ligand	M^{2+}	$\log K^{M}_{M(pUp_{(S)}U)}$	$\log K^{M}_{M(R\text{-}PO_3)}$	$\log\Delta_{M/pUp_{(S)}U}$
$pUp_{(S)}U^{3-}$	Mg^{2+}	1.85 ± 0.08	1.59 ± 0.03	0.26 ± 0.08
	Mn^{2+}	2.42 ± 0.07	2.19 ± 0.05	0.23 ± 0.09
	Zn^{2+}	2.88 ± 0.07	2.16 ± 0.06	0.72 ± 0.09
	Cd^{2+}	3.16 ± 0.07	2.48 ± 0.05	0.68 ± 0.09
	Pb^{2+}	3.49 ± 0.11	2.99 ± 0.08	0.50 ± 0.14
$pUpU^{3-}$	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 ± 0.20	3.05 ± 0.08	1.40 ± 0.22

[a] For the error limits, see footnote [a] of Table 1. The error limits (3σ) of the derived data in column 5 were calculated according to the error propagation after Gauss.

2.3. Quantification of the enhanced stabilities of the $M(pUp_{(S)}U)^-$ complexes and extent of their chelate formation: The stability differences between the measured values for the $M(pUp_{(S)}U)^-$ complexes

and the calculated values for the M(R-PO_3) species were obtained by using Equation (6):

$$\log\Delta_{M/pUp_{(S)}U} = \log K^{M}_{M(pUp_{(S)}U)} - \log K^{M}_{M(R\text{-}PO_3)} \quad (6)$$

These differences are listed in column 5 of Table 2 together with the results obtained previously[36] for the corresponding $M(pUpU)^-$ complexes.

In the case of the $M(pUpU)^-$ complexes of Mg^{2+}, Mn^{2+}, and Cd^{2+}, the stability enhancements are identical within the error limits (see Table 2, column 5 in the lower part). Considering the different coordinating properties[49–51] of these three metal ions it is evident that their 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. This charge effect is represented by the average of the $\log\Delta_{M/pUpU}$ values, defined in analogy to Equation (6), for the Mg^{2+}, Mn^{2+}, and Cd^{2+} systems, $\log\Delta_{M/pUpU/charge} = 0.24 \pm 0.04$. It is reasonable to assume that exactly the same charge effect is present in the corresponding $M(pUp_{(S)}U)^-$ complexes. Hence, any further stability increase in $M(pUp_{(S)}U)^-$ and $M(pUpU)^-$ complexes must be attributed to an additional interaction of the metal ion already coordinated to the terminal phosphate group of $pUp_{(S)}U^{3-}$ or $pUpU^{3-}$. This increase is defined for $M(pUp_{(S)}U)^-$ complexes by Equation (7):

$$\log\Delta^* = \log\Delta_{M/pUp_{(S)}U} - \log\Delta_{M/pUpU/charge} \quad (7a)$$

$$\log\Delta^* = \log\Delta_{M/pUp_{(S)}U} - (0.24 \pm 0.04) \quad (7b)$$

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

The values for $\log\Delta^*$ according to Equation (7) are listed in column 4 of Table 3 for the $M(pUp_{(S)}U)^-$ and $M(pUpU)^-$ complexes. As expected, these values are zero within the error limits for the $M(pUpU)^-$ complexes of Mg^{2+}, Mn^{2+}, and Cd^{2+}. This also holds for the Mg^{2+} and Mn^{2+} complexes of the thio analogue $pUp_{(S)}U^{3-}$, illustrating that Mn^{2+} does not remarkably coordinate the sulfur atom. In all other instances the $\log\Delta^*$ values are clearly positive. The different stability enhancements for the various $M(pUp_{(S)}U)^-$ and $M(pUpU)^-$ complexes mean that the position of the intramolecular Equilibrium (8) of the $M(pUp_{(S)}U)^-$ species, between an open (op) and a closed (cl) or chelated isomer, varies depending on the metal ion involved.

$$M(pUp_{(S)}U)^-_{op} \rightleftharpoons M(pUp_{(S)}U)^-_{cl} \quad (8)$$

The position of Equilibrium (8) is defined by the dimensionless intramolecular equilibrium constant K_I [Eq. (9)]:

$$K_I = [M(pUp_{(S)}U)^-_{cl}]/[M(pUp_{(S)}U)^-_{op}] \quad (9)$$

which is related to the stability enhancement $\log\Delta^*$ [Eq. (7)] by Equation (10):[1,47]

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

Knowledge of K_I allows calculation of the formation degree of the closed species in Equilibrium (8) by using Equation (11):

$$\%\ M(pUp_{(S)}U)^-_{cl} = 100 \cdot K_I/(1 + K_I) \quad (11)$$

The results for K_I and $\%\ M(pUp_{(S)}U)^-_{cl}$ are listed in Table 3 in columns 6 and 7, respectively.

As already stated, in the case of several $M(pUp_{(S)}U)^-$ and $M(pUpU)^-$ systems, the values of $\log\Delta^*$ are zero within the error limits. However, within these error limits, traces of chelated species might form with the corresponding upper limits as given in parenthesis in Table 3 (column 6). In contrast, chelates for the $M(pUp_{(S)}U)^-$ species with Zn^{2+}, Cd^{2+}, and Pb^{2+} definitely exist in remarkable amounts. It is interesting to note that the formation degree for $Cd(pUpU)^-_{cl}$ is (close to) zero but reaches about 64% for $Cd(pUp_{(S)}U)^-_{cl}$. Similarly, $Zn(pUp_{(S)}U)^-_{cl}$ reaches a formation degree of about 67% which is much larger than the approximately 26% determined earlier[36] for $Zn(pUpU)^-_{cl}$. This proves the known high thiophilicity of these two metal ions.[49–51] The situation with Pb^{2+} is more surprising because % Pb-

Table 3. Extent of chelate formation in $M(pUp_{(S)}U)^-$ complexes [Eq. (8)], as calculated from the stability enhancement $\log\Delta^*$ [Eq. (7)] and quantified by the dimensionless equilibrium constant K_I [Eqs. (9, 10)], and the percentage of the chelated isomer [Eq. (11)]. The corresponding data of the $M(pUpU)^-$ complexes are also listed for comparison (aqueous solution; 25 °C; $I = 0.1$ M, $NaNO_3$).[a]

Ligand	M^{2+}	$\log\Delta_{M/pUp_{(S)}U}$[b]	$\log\Delta^*$	K_I	% $M(pUp_{(S)}U)^-_{cl}$
$pUp_{(S)}U^{3-}$	Mg^{2+}	0.26 ± 0.08	0.02 ± 0.09	≈ 0	≈ 0 (<22)
	Mn^{2+}	0.23 ± 0.09	-0.01 ± 0.10	≈ 0	≈ 0 (<19)
	Zn^{2+}	0.72 ± 0.09	0.48 ± 0.10	2.02 ± 0.68	67 ± 8
	Cd^{2+}	0.68 ± 0.09	0.44 ± 0.10	1.75 ± 0.62	64 ± 8
	Pb^{2+}	0.50 ± 0.14	0.26 ± 0.15	0.82 ± 0.61	45 ± 18
$pUpU^{3-}$	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 2. [b] These values are from column 5 of Table 2.

$(pUpU)^-_{cl}$ > % $Pb(pUp_{(S)}U)^-_{cl}$ (Table 3; see also discussion in Section 2.4), that is, macrochelate formation is diminished upon introduction of the sulfur atom.

2.4. A more detailed appraisal of the $M(pUp_{(S)}U)^-$ chelates: Considering that the thiophosphate diester bridge involved in chelate formation as discussed in Section 2.3 has not only a terminal S atom but also a terminal O atom (see Figure 1), it is evident that the situation is ambiguous with regard to which of the two atoms participates in the chelate. In this context the following three points need to be noted: i) The stacking tendency of uracil residues is very small[52] and therefore, no preferred stacked conformer of $pUp_{(S)}U^{3-}$ is expected to occur in aqueous solution. ii) In accord herewith, the acidity constants of the (N3)H sites indicate that the two uracil residues in $pUp_{(S)}U^{3-}$ do not "feel" each other (see Section 2.1). iii) The thiophosphate diester bridge in $pUp_{(S)}U^{3-}$ involves five single bonds which means that the two nucleotide residues may rotate rather freely around these five bonds. Hence, one has to conclude that both, the S or the O atom of the thiophosphate bridge can be involved in chelate formation and that the right part of the following intramolecular Equilibrium (12) needs to be considered:

$$M^{2+} + pUp_{(S)}U^{3-} \rightleftharpoons M(pUp_{(S)}U)^-_{op} \begin{cases} \xrightarrow{K_{I/PO}} M(pUp_{(S)}U)^-_{cl/PO} \\ \xrightarrow{K_{I/PS}} M(pUp_{(S)}U)^-_{cl/PS} \end{cases} \quad (12)$$

In this equilibrium $M(pUp_{(S)}U)^-_{op}$ designates the "open" complex [see also Eq. (8)], in which the metal ion is only bound to the terminal phosphate group. The chelated or "closed" isomers can involve either the O or S atom of the thiophosphate diester bridge, giving the species $M(pUp_{(S)}U)^-_{cl/PO}$ or $M(pUp_{(S)}U)^-_{cl/PS}$, respectively.

As a consequence the charge-corrected stability enhancements $\log\Delta^*$ [Eq. (7)] (see also Section 2.3 and column 4 of Table 3) reflect all possible additional interactions that a metal ion coordinated to the terminal phosphate group in $pUp_{(S)}U^{3-}$ may experience according to Equilibrium (12). Or, in other words, $\log\Delta^*$ encompasses the total (tot) amount of all chelated complexes and the definition given in Equation (13) holds.

$$[M(pUp_{(S)}U)^-_{cl/tot}] = [M(pUpU_{(S)}U)^-_{cl/PO}] + [M(pUp_{(S)}U)^-_{cl/PS}] \quad (13)$$

In analogy to Equation (9), one can then define Equation (14):

$$K_{I/tot} = \frac{[M(pUp_{(S)}U)^-_{cl/tot}]}{[M(pUp_{(S)}U)^-_{op}]} \quad (14a)$$

$$= \frac{[M(pUp_{(S)}U)^-_{cl/PO}] + [M(pUp_{(S)}U]^-_{cl/PS}]}{[M(pUp_{(S)}U)^-_{op}]} \quad (14b)$$

$$= 10^{\log\Delta^*} - 1 \quad (14c)$$

As $K_{I/tot}$ equals K_I [as defined in Eq. (10)] and Equation (11) providing % $M(pUp_{(S)}U)^-_{cl}$ is still valid, this means that in accord with Equilibrium (12) the following definitions can be written:[53]

$$K_{I/PO} = [M(pUp_{(S)}U)^-_{cl/PO}]/[M(pUp_{(S)}U)^-_{op}] \quad (15)$$

$$K_{I/PS} = [M(pUp_{(S)}U)^-_{cl/PS}]/[M(pUp_{(S)}U)^-_{op}] \quad (16)$$

$$K_{I/tot} = K_{I/PO} + K_{I/PS} \quad (17)$$

By subtracting % $M(pUp_{(S)}U)^-_{cl/tot}$, which follows from Equations (14a) and (14b), from 100% the formation degree of the open species, $M(pUp_{(S)}U)^-_{op}$, can be calculated. The results for $K_{I/tot}$, % $M(pUp_{(S)}U)^-_{cl/tot}$, and % $M(pUp_{(S)}U)^-_{op}$ are listed in Table 4 in columns 2, 3, and 4, respectively. In those cases where one of the closed isomers, for example, $M(pUp_{(S)}U)^-_{cl/PO}$, is not formed, $K_{I/PO}$ [Eq. (15)] becomes zero and Equation (17) reduces to a two-isomer problem as discussed in Section 2.3.

With those metal ions, where all three isomers are formed according to Equilibrium (12), $K_{I/tot}$ and the concentration fractions of $M(pUp_{(S)}U)^-_{cl/tot}$ and $M(pUp_{(S)}U)^-_{op}$ can still be determined [Eqs. (14) and (11)] as shown above. $K_{I/PS}$ values can be calculated by assuming that the extent of chelate for-

Table 4. Formation degrees of the isomeric species $M(pUp_{(S)}U)^-_{op}$, $M(pUp_{(S)}U)^-_{cl/PO}$, and $M(pUp_{(S)}U)^-_{cl/PS}$, see [Eq. (12)], expressed in percentages in which the isomers occur in aqueous solution of 25 °C and $I = 0.1$ M ($NaNO_3$).[a]

M^{2+}	$K_{I/tot}$ [Eq. (14)][b]	% $M(pUp_{(S)}U)^-_{cl/tot}$ [Eq. (13)][c]	$M(pUp_{(S)}U)^-_{op}$ [Eq. (12)][d]	$K_{I/PO}$ [Eq. (15)][e]	$K_{I/PS}$ [Eqs. (16, 17)]	% $M(pUp_{(S)}U)^-_{cl/PO}$ [Eq. (15)]	% $M(pUp_{(S)}U)^-_{cl/PS}$ [Eq. (16)]
Mg^{2+}	≈0	≈0	100	≈0	≈0	≈0	≈0
Mn^{2+}	≈0	≈0	100	≈0	≈0	≈0	≈0
Zn^{2+}	2.02±0.68	67±8	33±8	0.35±0.25	1.67±0.72	12	55
Cd^{2+}	1.75±0.62	64±8	36±8	≈0	1.75±0.62	≈0	64
Pb^{2+}	0.82±0.61	45±18	55±18	(13.45±8.65)[f]	≈0	45[g]	0

[a] See footnote [a] of Table 2. [b] Values from column 5 of Table 3 (upper part); see text. [c] From column 6 in Table 3 (upper part). [d] These values follow from 100−% $M(pUp_{(S)}U)^-_{cl/tot}$. [e] These values are from column 5 of Table 3 (lower part); see text. [f] This value is larger than $K_{I/tot} = 0.86$; hence, no meaningful calculation is possible. [g] See discussion in Section 2.4.

mation involving the oxygen of the bridging (thio)phosphate group is the same in $M(pUpU)_{cl}^{-}$ and $M(pUp_{(S)}U)_{cl/PO}^{-}$ species. Values for $K_{I/PS}$ (Table 4) are then obtained from Equation (17) by using $K_{I/PO}$ values of the $M(pUpU)_{cl}^{-}$ species (see lower part in Table 3).

Our experimental data described in Section 2.3 shows that Mg^{2+} and Mn^{2+} form only $M(pUp_{(S)}U)_{op}^{-}$ isomers. The same is true for $Cd(pUpU)^{-}$ where no closed isomer has been discovered.[36] Hence, the complete stability enhancement observed for $Cd(pUp_{(S)}U)^{-}$ is to be attributed to the formation of the $Cd(pUp_{(S)}U)_{cl/PS}^{-}$ isomer, or in other words, in this case only the lower pathway of Equilibrium (12) operates. For $Zn(pUp_{(S)}U)^{-}$ clearly both pathways of Equilibrium (12) are in action and the three isomers $Zn(pUp_{(S)}U)_{op}^{-}$, $Zn(pUp_{(S)}U)_{cl/PO}^{-}$, and $Zn(pUp_{(S)}U)_{cl/PS}^{-}$ occur with formation degrees of about 33, 12, and 55%, respectively (Table 4), confirming the pronounced preference for S over O, that is, the thiophilicity of Zn^{2+}.

The situation with Pb^{2+} is considerably more complicated because from the entries in Table 3 it follows that the stability enhancement $\log\Delta^*$ is larger for $Pb(pUpU)^{-}$ compared to that for $Pb(pUp_{(S)}U)^{-}$. Hence, for this metal ion no meaningful calculation is possible because $K_{I/PO} > K_{I/tot}$. From this observation it follows that in these complexes the affinity of Pb^{2+} for O is higher than for S. Considering that in $Pb(pUp_{(S)}U)^{-}$ only one terminal O in the thiophosphate bridge is available for chelate formation compared to two in $Pb(pUpU)^{-}$, the stability enhancement of the latter, $\log\Delta^* = 1.16 \pm 0.26$ (Table 3, column 4) may be reduced by the statistical factor of one half, that is, the expected $\log\Delta^*_{exp}$ for $Pb(pUp_{(S)}U)^{-}$ amounts then to 0.86 ± 0.26. However, this value is still much larger than the determined value, $\log\Delta^* = 0.26 \pm 0.15$ (Table 3), for $Pb(pUp_{(S)}U)^{-}$. The reason for this discrepancy can be found in the fact that the negative charge of the thiophosphate bridge is mainly located at the terminal S atom and not on the terminal O atom (see also Section 2.1). Consequently, the affinity of this O atom towards Pb^{2+} is lower than that of the two non-bridging oxygen atoms in $pUpU^{3-}$ which each carry a charge of -0.5. Hence, in all likelihood Pb^{2+} forms with $pUp_{(S)}U^{3-}$ a 10-membered chelate involving only oxygen atoms.

Based on this finding in the $Pb(pUp_{(S)}U)^{-}$ system, one may conclude that also in the above discussed $Zn(pUp_{(S)}U)^{-}$ species the affinity of Zn^{2+} towards the neutral non-bridging oxygen of the thiophosphate is reduced (compared to $Zn(pUpU)^{-}$). Hence, the formation degree of 12% given for $Zn(pUp_{(S)}U)_{cl/PO}^{-}$ in Table 4 (column 7) may be considered an upper limit and consequently the value for $Zn(pUp_{(S)}U)_{cl/PS}^{-}$ a lower limit.

3. Conclusion

From this study it follows that in so-called thio rescue experiments,[15,16] being often conducted in ribozyme chemistry,[18–23] only Zn^{2+} and Cd^{2+} can effectively be applied as rescuing agents due to their pronounced affinity towards sulfur. Mn^{2+} is not suitable because it does not own a pronounced thiophilicity.[1,15,16] In the few cases, where Mn^{2+} has an effect in such experiments, this is most likely due to its larger global stability constants observed for phosphate complexes compared to those of the corresponding Mg^{2+} complexes (see also Table 2). These conclusions are confirmed by the metal-ion-promoted hydrolytic cleavage reaction of the S_p and R_p diastereomers of the phosphoromonothioate analogues of uridylyl(3′→5′)uridine ($Up_{(S)}U$).[54] The cleavage (to 2′,3′-cUMPS) is significantly accelerated by Zn^{2+} and Cd^{2+}, the rate enhancements being almost equal with the S_p and R_p diastereomers. The effect of Mn^{2+} and Mg^{2+} on the cleavage rate is, in turn, very modest.[54]

The high affinity of Pb^{2+} towards O sites is well known[49–51,55] and, for example, exhibited in the large stability of the G-quadruplex formed by guanine residues into which a Pb^{2+} ion is inserted.[56] In the resulting $(G)_8$–Pb^{2+} coordination pattern Pb^{2+} sits between two G-quartets being coordinated to eight (C6)O carbonyls.[56] As a consequence, Pb^{2+} also shows a pronounced ability to interact strongly with two neighboring phosphate sites and it is not surprising to find that leadzymes could be isolated[57,58] and that Pb^{2+}-dependent DNAzymes[59] exist. Because Pb^{2+} is a well known mimic of Ca^{2+},[49,50,60] one may add that for this alkaline earth metal ion it is not expected that it has any affinity towards sulfur, but due to its size it could be that, in contrast to Mg^{2+}, it may bind favorably to two neighboring phosphate groups in a nucleic acid, just like Pb^{2+} does.

Finally, Pb^{2+} exhibits one more fascinating characteristic that is revealed by its coordination chemistry to thiophosphate derivatives. Pb^{2+} appears to be a chameleon-like metal ion because its binding properties seem to depend on the first strongly coordinating site, that is, a directing effect of the first ligand is observed: In such cases, where only one binding site is present, Pb_{aq}^{2+} shows a preference for sulfur, as evident from the comparison of, for example, $UMPS^{2-}$ with UMP^{2-}.[2,29] In contrast, when two binding sites are present in a ligand enabling macrochelate formation, as is the case with $pUpU^{3-}$ or $pUp_{(S)}U^{3-}$, no such thiophilic behavior is observed anymore: If coordination occurs first to a phosphate group then oxygen binding is further favored despite the presence of the neighboring sulfur in the bridging thiophosphate group of $pUp_{(S)}U^{3-}$.

Experimental Section

4.1. Synthesis of *P*-thiouridylyl-(5′→3′)-[5′]-uridylate, $pUp_{(S)}U^{3-}$ (1): This dinucleotide was synthesized in three different ways with all three compounds showing the same metal-ion binding properties. Dinucleotide **1** was obtained as a mixture of two diastereomers in ca. 2:1 ratio (synthetic route I) and 1:1 ratio (synthetic routes II and III), having opposite configuration at the phosphorus atom.

4.1.1. Synthetic route I: The trisodium salt of $pUp_{(S)}U^{3-}$ was prepared by a multistep synthesis (see Scheme S1, Supporting Information) using the phosphoramidite methodology, with the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) group for protection of the 2′-hydroxy functionality.[61,62] Thus, 5′-*O*-dimethoxytrityl-2′-*O*-Fpmp uridine-3′-*O*-(2-cyanoethyl-

N,N-diisopropylphosphoramidite) (**2**) was prepared as previously described[62] and characterized by ^{31}P NMR (Bruker Avance spectrometer, 200 MHz) showing two singlets at $\delta = 150.24$ and 152.13 ppm (in CD_3CN) (ratio of diastereomers of 3:2). Compound **2** was reacted with 2′,3′-di-*O*-acetyluridine in the presence of 1*H*-tetrazole in CH_2Cl_2 solution to yield the fully protected dinucleoside phosphorothionate triester (**3**) upon addition of elemental sulfur to the intermediate phosphite (^{31}P NMR of **3** (in CD_3CN); $\delta = 68.40$ and 68.77 ppm (ratio 2:3 of phosphorothionate diastereomers)). After selective removal of the dimethoxytrityl (DMT) group with 2% dichloroacetic acid in CH_2Cl_2, the resulting 5′-hydroxyl compound was 5′-*O*-phosphorylated with bis-*O,O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite reagent and the phosphite intermediate subsequently oxidized with I_2/pyridine/H_2O.[63] The resulting fully protected 5′-*O*-phosphorylated dinucleoside-phosphorothionate triester (**4**) was purified by silica gel chromatography (^{31}P NMR of **4** (CD_3CN); $\delta = -1.32$ ppm (phosphotriester group) 67.85 and 68.22 ppm (ratio 2:3, phosphorothionate diastereomers)). Compound **4** was then subjected to stepwise deprotection, including 16 h incubation in 30% aqueous ammonia at 55 °C (removal of 2-cyanoethyl and acetyl groups) followed by 12 h treatment with 0.01 M HCl (pH 2.0) at room temperature (removal of the Fpmp group).

The crude dinucleotide **1** was purified by ion-exchange chromatography on 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 through Dowex 50Wx8 (Na^+ form) and lyophylized to give a white solid in 17% overall yield (based on 2′,3′-di-*O*-acetyluridine). The structure of **1** was confirmed by spectroscopic methods: ^{31}P NMR (D_2O), $\delta = 0.74$ ppm (phosphate group), 56.45, and 56.69 ppm (ratio ca. 2:1, phosphorothioate diastereomers); FAB MS (Finnigan MAT 95): *m/z*: 645.2 (negative ions), calculated M_W 646.41 for the free acid. Analytical RP HPLC of the product **1** showed two peaks with retention times of 13.32 and 14.05 min in a ca. 2:1 ratio.

4.1.2. Synthetic route II (see also Scheme S2, Supporting Information): Anhydrous 2′,3′-di-*O*-acetyluridine (**6**) (93 mg, 0.28 mmol) was dissolved in anhydrous acetonitrile (1.5 mL) and mixed with ethylthio-1*H*-tetrazole (50 mg, 0.38 mmol), to which a solution of 2-cyanoethyl-*N,N*-diisopropylphosphoramidite of 2′-*O*-*tert*-butyldimethylsilyl-5′-*O*-dimethoxytrityluridine (**5**) (270 mg, 0.32 mmol; Glen Research, Sterling VA, USA) in anhydrous acetonitrile (1.5 mL) was added dropwise. After stirring the reaction mixture for 2 h anhydrous sulfur (13.5 mg, 0.053 mmol S_8) was added. The reaction mixture was left overnight, the solvent evaporated and the residue subsequently chromatographed on a silica gel 60H short column with chloroform elution. 270 mg (95%) of pure **7** were obtained. This compound (200 mg, 0.18 mmol) was treated with 50% acetic acid (15 mL) for 30 min and after concentration purified by means of silica gel column chromatography. Dimer **8** was obtained in 58% yield (80 mg; spectral data: FAB MS: *m/z*: 763.3 $[M-H]^+$; M_W 764).

Anhydrous **8** (0.05 mmol, 40 mg) and ethylthio-1*H*-tetrazole (9 mg, 0.07 mmol) were dissolved in anhydrous acetonitrile (1.0 mL). Then a solution of the chemical phosphorylation reagent 2-[2-4,4′-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N,N*-diisopropyl)-phosphoramidite[64] (35 mg, 0.053 mmol; Glen Research, Sterling VA, USA) in anhydrous acetonitrile (0.25 mL) was added. After stirring the reaction mixture for 30 min, a 5 M solution of *tert*-butylperoxide in decane (80 μL, 0.4 mmol) was added. The reaction was chromatographed on a silica gel 60H short column with chloroform/methanol (0→3%) elution yielding 45 mg (64%) of pure **9**. The structure of **9** was confirmed by MALDI-TOF MS: *m/z*: 1334.2 (M_W 1334) and ^{31}P NMR: $\delta = -2.21$, -2.26, 67.75, 67.29 ppm.

For deprotection, compound **9** (45 mg, 0.03 mmol) was treated with a mixture of 20% ammonia in ethanol (1 mL) and mercaptoethanol (50 μL, 0.72 mmol).[65] After 1 h, more ethanolic ammonia (1 mL) and aqueous ammonia (28%, 3 mL) were added to the reaction mixture and left overnight at +4 °C. A precipitate was filtered off and the remaining solution concentrated, the residue dissolved in water and purified over a DEAE Sephadex A-25 column. Two fractions were isolated: The slower fraction was the main product **10** giving the expected MALDI-TOF MS *m/z* signal at 759.2 whereas the faster eluting fraction exhibited a MALDI-TOF signal at *m/z* 812 corresponding to incompletely deprotected **9**. The latter product was again treated with aqueous ammonia (28%, 2 mL) for 48 h at +4 °C and purified as before to give also **10**. Both fractions of **10** were combined and, after concentration to dryness, treated with $(C_2H_5)_3N{\cdot}3HF$ (0.2 mL, 1.2 mmol; Aldrich, Steinheim, Germany) for 24 h at room temperature. The reaction mixture was dissolved in water and HPLC purified on a C18 reverse phase column (PRP-1, Hamilton). Pure thio-dinucleotide **1** was isolated by elution with buffer A (1 M TEAB buffer, pH 7.4) with a gradient of buffer B (40% acetonitrile in 1 M TEAB buffer, pH 7.4), 0–100% in 30 min. The obtained yield was 5.5 mg (25%). MALDI-TOF MS gave a single signal of *m/z* 645.3 in negative ions (M_W 646.41).

4.1.3. Synthetic route III: Compound **9** was alternatively synthesized by routine automated solid phase synthesis[66] using standard 2′-TBDMS phosphoramidite CE monomers and a chemical phosphorylating reagent (Glen Research, Sterling VA, USA). Deprotection of **9** to give pure **1** was then done according to the procedure described for Synthetic route II.

4.2. Other materials: Nitric acid (HNO_3), the nitrate salts of Na^+, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}, disodium ethylenediamine-*N,N,N′,N′*-tetraacetate dihydrate ($Na_2H_2EDTA{\cdot}2H_2O$), potassium hydrogen phthalate (all pro analysi), and sodium hydroxide (NaOH) solution (Titrisol) were purchased from Merck, Darmstadt, Germany. The buffer solutions used (pH 4.00, 7.00, 9.00) were traceable to standard reference materials (SRM) of the US National Institute of Science and Technology (NIST) and purchased from Metrohm, Herisau, Switzerland. All solutions were prepared using deionised, ultra pure (Milli-Q185 Plus; from Millipore, Molsheim, France) CO_2-free water.

The concentrations of the NaOH solutions were determined with potassium hydrogen phthalate, those of the stock solutions of divalent metal ions by potentiometric pH titrations via their EDTA complexes. The stock solutions of $(pUp_{(S)}U)^{3-}$ were freshly prepared daily and the pH of the solutions was adjusted close to 8.0 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).

4.3. Potentiometric pH titrations: The pH titrations were performed with a E536 potentiograph connected to a E665 dosimat and a 6.0253.100 Aquatrode-plus combined macro glass electrode (all from Metrohm, Herisau, Switzerland). The instruments were calibrated using the buffer solutions mentioned above. The acidity constants determined at $I = 0.1$ M ($NaNO_3$) and 25 °C are so-called practical, mixed or Brønsted constants,[45] which may be converted into the corresponding concentration constants by subtracting 0.02 from the measured pK_a values.[45] The ionic product of water (K_w) is not included in our calculations because the differences in NaOH consumption between solutions with and without ligand are evaluated.[45,67] The stability constants of the $M(pUp_{(S)}U)^-$ complexes are, as usual, concentration constants.

4.4. Determination of the equilibrium constants: The acidity constants $K^H_{H(pUp_{(S)}U)}$, $K^H_{pUp_{(S)}U}$, and $K^H_{(pUp_{(S)}U-H)}$ of $H(pUp_{(S)}U)^{2-}$ [Eqs. (1–3)] 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 0.02 M NaOH in the presence and absence of 0.20 mM $(pUp_{(S)}U)^{3-}$. Due to the scarceness of the ligand a second set of titrations was performed using a $(pUp_{(S)}U)^{3-}$ concentration of 0.07 mM only. It should be emphasized that the calculated acidity constants showed no dependence on the dinucleotide concentration.

The experimental data were evaluated with a curve-fitting procedure using a Newton–Gauss non-linear least-squares program by employing every 0.1 pH unit the difference in NaOH consumption between the mentioned pair of titrations, that is, with and without ligand. The acidity constants of $H(pUp_{(S)}U)^{2-}$ were calculated within the pH range 5.2 to 10.4, corresponding to about 17% neutralization (initial) for the equilibrium $H(pUp_{(S)}U)^{2-}/(pUp_{(S)}U)^{3-}$ and about 72% (final) for $(pUp_{(S)}U-H)^{4-}/(pUp_{(S)}U-2H)^{5-}$. The final result for $K^H_{H(pUP_{(S)}U)}$ [Eq. (1)], is the average of eight independent pairs of titrations; those for $K^H_{pUp_{(S)}U}$ [Eq. (2)], and $K^H_{(pUP_{(S)}U-H)}$ [Eq. (3)] are the averages of four independent

pairs of titrations only because the low concentrations (0.07 mM) were titrated only up to pH 7.

At the end of each titration a small volume (about 0.8 mL or less) of 0.1 M HNO_3 was added to the solutions to restore the initial pH of about 3.3. A further comparatively small volume of a $M(NO_3)_2$ solution (M^{2+} = Mg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}) was subsequently added and the titrations were repeated. The total volume of these solutions was approximately 35 mL with an ionic strength I varying between 0.095 and 0.13 M. This small variation in I has no effect on complex stability[36] as is also evident from the following titrations. This means that the stability constants of the Mg^{2+}, Mn^{2+}, Zn^{2+}, and Pb^{2+} systems were additionally determined under the same conditions used for the acidity constants, but $NaNO_3$ was partly (Mn^{2+}, Zn^{2+}, Pb^{2+}) or fully (Mg^{2+}) replaced by $M(NO_3)_2$ (25 °C; I = 0.1 M).

The metal-to-ligand ratios in the various titrations, with [$pUp_{(S)}U$] being about 0.07 or 0.20 mM, were 233:1 and 170:1 for Mg^{2+}; 285:1 and 113:1 for Mn^{2+}; 116:1, 36:1, and 25:1 for Zn^{2+}; 285:1, 27:1, and 24:1 for Cd^{2+}, and 58:1, 22:1, and 15:1 for Pb^{2+}. Even though the metal-to-ligand ratios vary widely, the calculated stability constants for the M^{2+} complexes showed no dependence on the excess of M^{2+} used.

The titration data were evaluated with 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 an apparent acidity constant K_a'. Depending on the metal ion, the evaluation commenced at a formation degree of the $M(pUp_{(S)}U)^-$ species of about 4 to 12%, while the upper limit was given either by the hydrolysis of $M(aq)^{2+}$ or by the formation of complexes, where the (N3)H sites of the uracil residues lost a proton, which was evident from the titrations without ligand or by the deviation of the experimental data from the calculated curves. Representative examples for the pH ranges employed are in the case of the $M(pUp_{(S)}U)^-$ complexes 4.7–6.8 (Mg^{2+}), 4.7–6.6 (Mn^{2+}), 4.4–6.0 (Zn^{2+}), 3.8–5.9 (Cd^{2+}), and 3.9–5.3 (Pb^{2+}). These pH ranges correspond to variations in the formation degrees of about 5–63% for $Mg(pUpU)^-$, 12–79% for $Mn(pUpU)^-$, 4–55% for $Zn(pUpU)^-$, 3–73% for $Cd(pUpU)^-$, and 4–50% for $Pb(pUpU)^-$, respectively. The stability constants of the complexes were calculated as described previously.[1,53,68]

The final results for the stability constants of the $M(pUp_{(S)}U)^-$ complexes are the averages of two independent titrations in the case of the Mg^{2+} and Mn^{2+} systems, whereas for the Zn^{2+}, Cd^{2+}, and Pb^{2+} systems three independent pairs of titrations were performed.

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[1] R. K. O. Sigel, B. Song, H. Sigel, *J. Am. Chem. Soc.* **1997**, *119*, 744–755.
[2] C. P. Da Costa, D. Krajewska, A. Okruszek, W. J. Stec, H. Sigel, *J. Biol. Inorg. Chem.* **2002**, *7*, 405–415.
[3] F. Eckstein, *J. Am. Chem. Soc.* **1966**, *88*, 4292–4294.
[4] J. R. Knowles, *Annu. Rev. Biochem.* **1980**, *49*, 877–919.
[5] F. Eckstein, *Annu. Rev. Biochem.* **1985**, *54*, 367–402.
[6] J. Liu, M. D. Tsai, *Biochemistry* **2001**, *40*, 9014–9022.
[7] M. Koziolkiewicz, A. Owczarek, M. Wojcik, K. Domanski, P. Guga, W. J. Stec, *J. Am. Chem. Soc.* **2002**, *124*, 4623–4627.
[8] W. S. Marshall, M. H. Caruthers, *Science* **1993**, *259*, 1564–1569.
[9] A. DeMesmaeker, R. Haner, P. Martin, H. E. Moser, *Acc. Chem. Res.* **1995**, *28*, 366–374.
[10] M. Gilar, A. Belenky, Y. Budman, D. L. Smisek, A. S. Cohen, *Antisense Nucleic Acid Drug Dev.* **1998**, *8*, 35–42.
[11] F. Eckstein, *Antisense Nucleic Acid Drug Dev.* **2000**, *10*, 117–121.
[12] K. L. Warfield, R. G. Panchal, M. J. Aman, S. Bavari, *Curr. Opin. Mol. Ther.* **2006**, *8*, 93–103.
[13] R. F. Gesteland, T. R. Cech, J. F. Atkins, *The RNA World*, Cold Spring Harbor Press, **2006**, pp. 1–768.
[14] R. K. O. Sigel, A. M. Pyle, *Met. Ions Biol. Syst.* **2003**, *40*, 477–512.
[15] R. K. O. Sigel, *Eur. J. Inorg. Chem.* **2005**, 2281–2292.
[16] R. K. O. Sigel, A. M. Pyle, *Chem. Rev.* **2007**, *107*, 97–113.
[17] R. K. O. Sigel, H. Sigel, *Met. Ions Life Sci.* **2007**, *2*, 109–180.
[18] P. M. Gordon, J. A. Piccirilli, *Nat. Struct. Biol.* **2001**, *8*, 893–898.
[19] M. Boudvillain, A. de Lencastre, A. M. Pyle, *Nature* **2000**, *406*, 315–318.
[20] M. Boudvillain, A. M. Pyle, *EMBO J.* **1998**, *17*, 7091–7104.
[21] T. E. Horton, M. Maderia, V. J. DeRose, *Biochemistry* **2000**, *39*, 8201–8207.
[22] S.-O. Shan, A. V. Kravchuk, J. A. Piccirilli, D. Herschlag, *Biochemistry* **2001**, *40*, 5161–5171.
[23] S.-O. Shan, A. Yoshida, S. Sun, J. A. Piccirilli, D. Herschlag, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12299–12304.
[24] B. Nawrot, K. Widera, M. Wojcik, B. Rebowska, G. Nowak, W. J. Stec, *FEBS J.* **2007**, *274*, 1062–1072.
[25] T. Lönnberg, M. Ora, S. Virtanen, H. Lönnberg, *Chem. Eur. J.* **2007**, *13*, 4614–4627.
[26] P. Guga, M. Boczkowska, M. Janicka, A. Maciaszek, B. Nawrot, S. Antoszczyk, W. J. Stec, *Pure Appl. Chem.* **2006**, *78*, 993–1002.
[27] P. Guga, M. Boczkowska, M. Janicka, A. Maciaszek, S. Kuberski, W. J. Stec, *Biophys. J.* **2007**, *92*, 2507–2515.
[28] V. L. Pecoraro, J. D. Hermes, W. W. Cleland, *Biochemistry* **1984**, *23*, 5262–5271.
[29] C. P. Da Costa, A. Okruszek, H. Sigel, *ChemBioChem* **2003**, *4*, 593–602.
[30] S. S. Massoud, H. Sigel, *Inorg. Chem.* **1988**, *27*, 1447–1453.
[31] N. J. Reiter, L. J. Nikstad, A. M. Allmann, R. J. Johnson, S. E. Butcher, *RNA* **2003**, *9*, 533–542.
[32] M. C. Erat, O. Zerbe, T. Fox, R. K. O. Sigel, *ChemBioChem* **2007**, *8*, 306–314.
[33] R. K. O. Sigel, D. G. Sashital, D. L. Abramovitz, A. G. Palmer III, S. E. Butcher, A. M. Pyle, *Nat. Struct. Mol. Biol.* **2004**, *11*, 187–192.
[34] R. K. O. Sigel, A. Vaidya, A. M. Pyle, *Nat. Struct. Biol.* **2000**, *7*, 1111–1116.
[35] E. Freisinger, R. K. O. Sigel, *Coord. Chem. Rev.* **2007**, *251*, 1834–1851.
[36] B. Knobloch, D. Suliga, A. Okruszek, R. K. O. Sigel, *Chem. Eur. J.* **2005**, *11*, 4163–4170.
[37] K. Aoki, *Met. Ions Biol. Syst.* **1996**, *32*, 91–134.
[38] D. B. Davies, P. Rajani, H. Sadikot, *J. Chem. Soc. Perkin Trans. 2* **1985**, 279–285.
[39] B. Song, R. K. O. Sigel, H. Sigel, *Chem. Eur. J.* **1997**, *3*, 29–33.
[40] P. A. Frey, R. D. Sammons, *Science* **1985**, *228*, 541–545.
[41] P. A. Frey, *Adv. Enzymol. Relat. Areas Mol. Biol.* **1989**, *62*, 119–201.
[42] C. P. Da Costa, H. Sigel, *J. Biol. Inorg. Chem.* **1999**, *4*, 508–514.
[43] B. Knobloch, W. Linert, H. Sigel, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7459–7464.
[44] B. Knobloch, H. Sigel, A. Okruszek, R. K. O. Sigel, *Org. Biomol. Chem.* **2006**, *4*, 1085–1090.
[45] H. Sigel, A. D. Zuberbühler, O. Yamauchi, *Anal. Chim. Acta* **1991**, *255*, 63–72.
[46] H. Sigel, L. E. Kapinos, *Coord. Chem. Rev.* **2000**, *200–202*, 563–594.
[47] R. B. Martin, H. Sigel, *Comments Inorg. Chem.* **1988**, *6*, 285–314.
[48] H. Sigel, D. Chen, N. A. Corfù, F. Gregan, A. Holý, M. Strǎak, *Helv. Chim. Acta* **1992**, *75*, 2634–2656.
[49] R. B. Martin in *Encyclopedia of Inorganic Chemistry, Vol. 4* (Ed.: R. B. King), Wiley, Chichester, **1994**, pp. 2185–2196.
[50] R. B. Martin in *Encyclopedia of Molecular Cell Biology and Molecular Medicine, Vol. 1* (Ed.: R. A. Meyers), VCH, Weinheim, **1996**, pp. 125–134.
[51] R. B. Martin, *Met. Ions Biol. Syst.* **1986**, *20*, 21–65.

[52] H. Sigel, R. Griesser, *Chem. Soc. Rev.* **2005**, *34*, 875–900.
[53] B. Knobloch, H. Sigel, A. Okruszek, R. K. O. Sigel, *Chem. Eur. J.* **2007**, *13*, 1804–1814.
[54] M. Ora, M. Peltomaki, M. Oivanen, H. Lönnberg, *J. Org. Chem.* **1998**, *63*, 2939–2947.
[55] H. Sigel, C. P. Da Costa, R. B. Martin, *Coord. Chem. Rev.* **2001**, *219–221*, 435–461.
[56] F. W. Kotch, J. C. Fettinger, J. T. Davis, *Org. Lett.* **2000**, *2*, 3277–3280.
[57] T. Pan, O. C. Uhlenbeck, *Biochemistry* **1992**, *31*, 3887–3895.
[58] T. Pan, O. C. Uhlenbeck, *Nature* **1992**, *358*, 560–563.
[59] A. K. Brown, J. Li, C. M. B. Pavot, Y. Lu, *Biochemistry* **2003**, *42*, 7152–7161.
[60] R. B. Martin in *Handbook on Toxicity of Inorganic Compounds* (Eds.: H. G. Seiler, H. Sigel, A. Sigel), Marcel Dekker, New York, **1988**, pp. 9–25.
[61] M. V. Rao, C. B. Reese, V. Schehlmann, P. S. Yu, *J. Chem. Soc. Perkin Trans. 1* **1993**, 43–55.
[62] B. Beijer, I. Sulston, B. S. Sproat, P. Rider, A. I. Lamond, P. Neuner, *Nucleic Acids Res.* **1990**, *18*, 5143–5151.
[63] E. Uhlmann, J. Engels, *Tetrahedron Lett.* **1986**, *27*, 1023–1026.
[64] M. S. Urdea, T. Horn, *Tetrahedron Lett.* **1986**, *27*, 2933–2936.
[65] L. Zhang, L. Sun, Z. Cui, R. L. Gottlieb, B. Zhang, *Bioconjugate Chem.* **2001**, *12*, 939–948.
[66] M. H. Caruthers, *Science* **1985**, *230*, 281–285.
[67] M. Bastian, H. Sigel, *J. Coord. Chem.* **1991**, *23*, 137–154.
[68] C. A. Blindauer, T. I. Sjåstad, A. Holý, E. Sletten, H. Sigel, *J. Chem. Soc. Dalton Trans.* **1999**, 3661–3671.

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