Inorganic Chemistry

Article

pubs.acs.org/IC

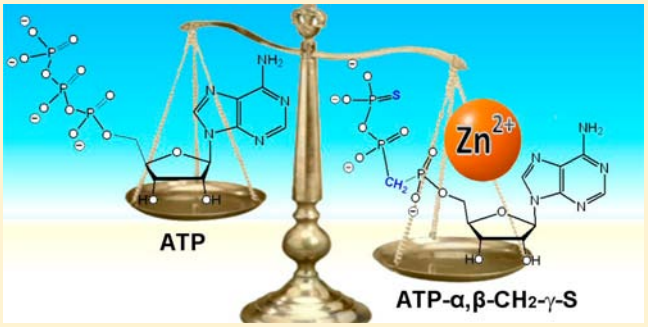
Characterization of Complexes of Nucleoside-5′-Phosphorothioate Analogues with Zinc Ions

Alon Haim Sayer, Yehudit Itzhakov, Noa Stern, Yael Nadel, and Bilha Fischer*

Department of Chemistry, Bar Ilan University, Ramat-Gan 52900, Israel

Supporting Information

ABSTRACT: On the basis of the high affinity of Zn^{2+} to sulfur and imidazole, we targeted nucleotides such as GDP-β-S, ADP-β-S, and AP_3(β-S)A, as potential biocompatible Zn^{2+}-chelators. The thiophosphate moiety enhanced the stability of the Zn^{2+}-nucleotide complex by about 0.7 log units. ATP-α,β-CH_2-γ-S formed the most stable Zn^{2+}-complex studied here, log K 6.50, being ∼0.8 and ∼1.1 log units more stable than ATP-γ-S-Zn^{2+} and ATP-Zn^{2+} complexes, and was the major species, 84%, under physiological pH. Guanine nucleotides Zn^{2+} complexes were more stable by 0.3−0.4 log units than the corresponding adenine nucleotide complexes. Likewise, AP_3(β-S)A-zinc complex was ∼0.5 log units more stable than AP_3A complex. ^{1}H- and ^{31}P NMR monitored Zn^{2+} titration showed that Zn^{2+} coordinates with the purine nucleotide N7-nitrogen atom, the terminal phosphate, and the adjacent phosphate. In conclusion, replacement of a terminal phosphate by a thiophosphate group resulted in decrease of the acidity of the phosphate moiety by approximately one log unit, and increase of stability of Zn^{2+}-complexes of the latter analogues by up to 0.7 log units. A terminal phosphorothioate contributed more to the stability of nucleotide-Zn^{2+} complexes than a bridging phosphorothioate.



1. INTRODUCTION

Nucleoside 5′-diphosphates (NDPs) and nucleoside 5′-triphosphates (NTPs) form stable complexes with Zn^{2+} with log K values between 4 and 4.5[1,2] (Zn^{2+}-NDPs complexes), and 5−5.5[3] (Zn^{2+}-NTPs complexes). Zn^{2+}-nucleotide complexes are ∼1−1.5 log units more stable than their corresponding Mg^{2+}-nucleotide and Ca^{2+}-nucleotide complexes, but are ∼1−2 log units less stable than their corresponding Cu^{2+}-nucleotide complexes.[1−4] Crystal structure data on Zn^{2+}-nucleotide complexes is limited.[5] A crystal structure of dimeric [Zn-(H_2ATP)(bpy)]$_2$ complex exhibits distorted octahedral geometry,[6] where Zn^{2+} coordinates with ATP through Pγ, Pβ, and Pα oxygen atoms. Yet, Zn−O (Pα) bonding is relatively weak as indicated by the long bond distances.[6]

Zn^{2+} is classified as "intermediate" Lewis-acid, according to Hard-Soft Acid−Base theory,[7] and coordinates preferentially atoms such as nitrogen and sulfur.[8] Moreover, Zn^{2+} has affinity for thiolate second only to Cu^{2+} throughout biological systems.[9,10] For instance, Zn^{2+}-dithiothreitol complex exhibits log K of 11.06,[11] where a 7-membered ring is formed because of Zn^{2+} coordination with two thiol groups. The "thiophilic" character of Zn^{2+} is also exemplified in Zn^{2+}-2-thiocytidine complex with log K of 2.53, being ∼2.3 log units more stable than the parent complex, Zn^{2+}-cytidine.[12]

Yet, the Zn^{2+} vs Mg^{2+}-selectivity of phosphorothioate analogues was barely reported. Stability constants of several nucleoside 5′-phosphorthioate analogues with Mg^{2+} and Cd^{2+} were determined by ^{31}P NMR,[13,14] indicating that Cd^{2+} affinity to ATP-α-S, ATP-β-S, and ADP-α-S is higher than that of Mg^{2+} by ∼0.5−1.4 log units (log K 4.47, 4.04, and 3.66 vs 4.92, 5.44, and 4.95, respectively).[14] Later, stability constants of adenosine 5′-monothiophosphate (AMP-α-S) with Zn^{2+} ion and Mg^{2+} ion were determined by potentiometric titrations (log K 2.52 vs 1.28, respectively).[15]

The limited data on nucleoside 5′-phosphorothioate analogues as Zn^{2+} chelators prompted us to explore the properties of nucleoside-phosphorothioate-Zn^{2+} complexes. Specifically, we report on their acidity constants and stability constants as determined by potentiometric titrations. These constants were compared to those of the natural nucleotides, and species distribution at physiological pH was predicted. In addition, Zn^{2+} coordination sites were identified by ^{1}H NMR- and ^{31}P NMR- monitored Zn^{2+} titrations.

2. RESULTS AND DISCUSSION

We targeted the evaluation of purine-nucleotide analogues **1**−**6** (Figure 1) as potentially promising biocompatible Zn^{2+}-chelators. We chose purine nucleotides **1**−**6**, because of their imidazole moiety, a borderline base, and their "soft" sulfur atom, both of which preferably coordinate Zn^{2+}.[16,17]

2.1. NMR Monitored Zn^{2+} Titrations. To determine Zn^{2+}-coordination sites in nucleoside 5′-phosphorothioate analogues,

Received: April 9, 2013
Published: September 19, 2013

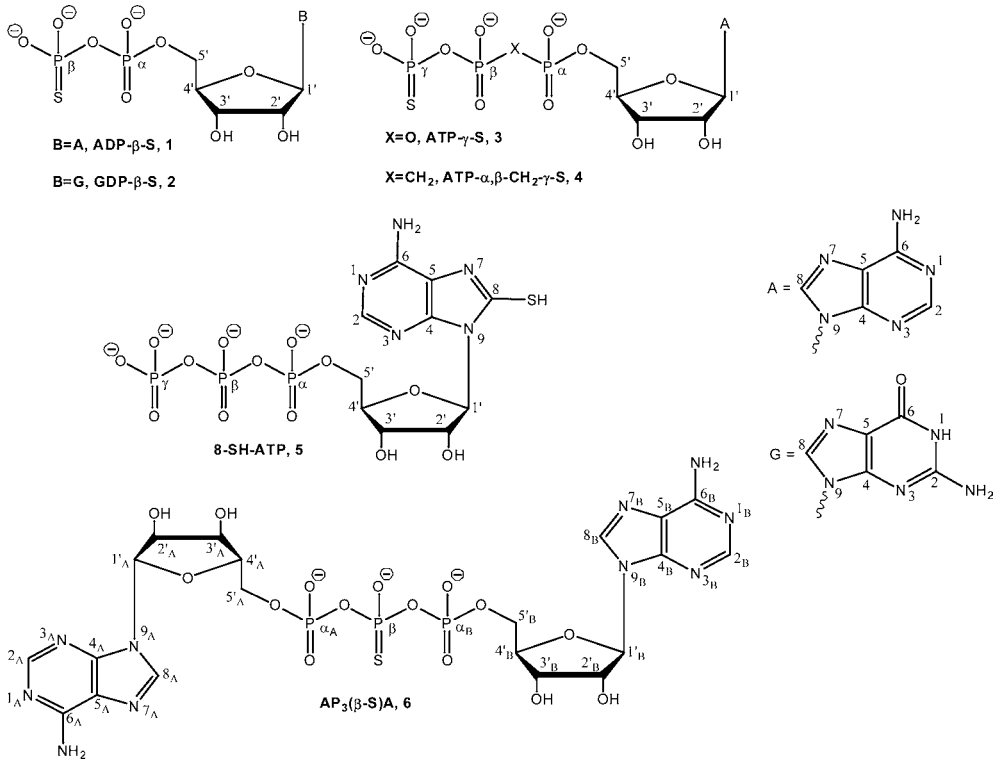


Figure 1. Nucleoside 5′-phosphorothioate analogues studied here.

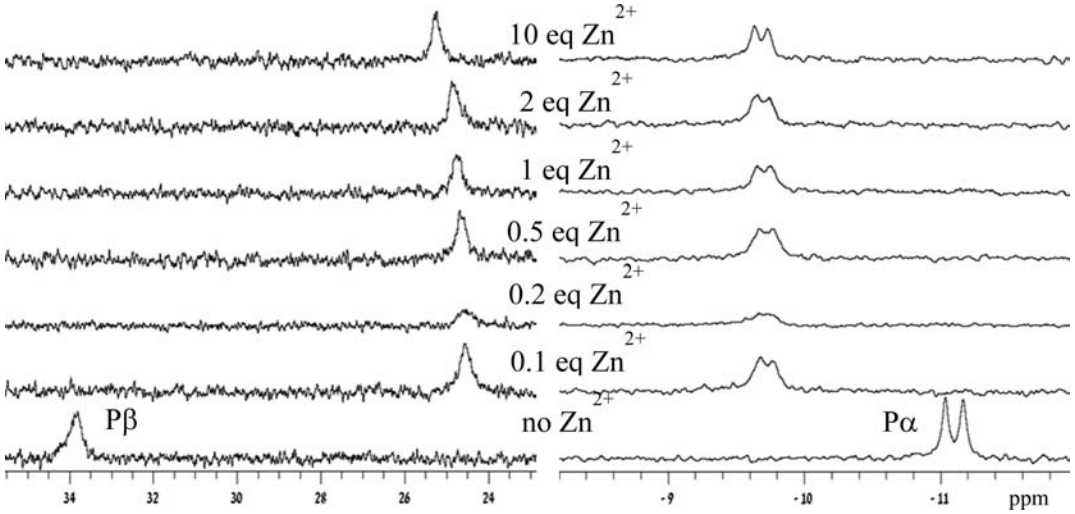


Figure 2. Zn^{2+}-titration of 6 mM GDP(β-S) in D_2O at pD 7.34 at 300 K. ^{31}P NMR spectrum was measured at 243 MHz.

we performed Zn^{2+}-titration of analogues **1**−**3** and **6**, monitored by ^{1}H and ^{31}P NMR spectroscopy.

The shift of NMR signals as well as their line-broadening indicates which atoms in the ligands are involved in Zn^{2+}-chelation. Solutions of analogues **1**−**3** and the corresponding parent nucleotides were titrated by 2 M $Zn(NO_3)_2$ solution (0.1−10 equiv of Zn^{2+}) at pD 7.4 and 300 K, and monitored by ^{1}H and ^{31}P NMR at 500 or 600, and 202.5 or 243 MHz, respectively. Relatively low nucleotide concentrations (3 or 6 mM) were used to avoid intermolecular base stacking (π-stacking interactions) in aqueous solutions.[18] Chemical shifts (δ_H, δ_p) of these compounds were measured, at different Zn^{2+} concentrations. Zn^{2+} coordination sites in nucleoside phosphorothioate analogues and the corresponding natural nucleotides were identified by the difference, $\Delta\delta = \delta_{\text{free ligand}} - \delta_{\text{Zn-ligand complex}}$ between the chemical shifts of nucleotide Na^+ salt and the corresponding Zn^{2+} complex.

The data are reported in Supporting Information, Table S1 (^{31}P NMR) and Supporting Information, Table S2 (^{1}H NMR). Significant $\Delta\delta$ values of terminal thiophosphate in ATP-γ-S, ADP-β-S and GDP-β-S (Figure 2) were observed only after addition of 0.1 equiv or 0.2 equiv of Zn^{2+}. Addition of 0.1 equiv of Zn^{2+} caused upfield shifts of Pβ of GDP-β-S (9.33 ppm) and of Pγ of ATP-γ-S (6.95 ppm). In contrast the $\Delta\delta$ of Pβ of ADP-β-S could not be measured after addition of 0.1 equiv of Zn^{2+} because of line-broadening of Pβ, resulting from dynamic equilibrium between the free ADP-β-S and the Zn^{2+}-ADP-β-S complex. However for GDP-β-S this equilibrium is less

dx.doi.org/10.1021/ic400878k | Inorg. Chem. 2013, 52, 10886−10896

dynamic possibly because of outersphere coordination of Zn^{2+} with O^6 of the guanine oxygen.[19] After addition of 0.2 equiv of Zn^{2+}, the signal of Pβ sharpened and shifted upfield to 8.92 ppm. Pα of GDP-β-S and Pβ of ATP-γ-S also showed a large change upon addition of 0.1 equiv of Zn^{2+} resulting in downfield shifts of 1.37 ppm and 2.13 ppm, respectively. This effect was observed also for Pα signal of ADP-β-S, yet was smaller (shift of 0.75 ppm), and further shifted by 1.36 ppm upon addition of 0.2 equiv of Zn^{2+}.

Additional equivalents of Zn^{2+} (0.5, 1, 2, 10 equiv) did not shift any further the Pα signal of ADP-β-S and GDP-β-S, and Pβ signal of ATP-γ-S (Figure 2). Pα of ATP-γ-S almost did not shift during the titration implying that Zn^{2+} did not coordinate with Pα of ATP-γ-S, similar to ATP ($\Delta\delta \cong 0.5$ ppm upon addition of 1 equiv of Zn^{2+}). Addition of Zn^{2+} to ADP and GDP resulted in gradual shifts, as the number of equivalents was increased (Supporting Information, Table S1). Unlike nucleoside 5′-phosphorthioate, addition of 0.1 equiv of Zn^{2+} to ADP, GDP, and ATP affected immediately the $\Delta\delta$ value (Supporting Information, Table S1).

Previously, ATP was found to coordinate metal ions mainly through Pβ and Pγ.[20−22] This observation supports our finding that Pα of ATP/ATP-γ-S did not coordinate with Zn^{2+}. In addition, our findings are consistent with the crystal structure of $[Zn(H_2ATP)(bpy)]_2$ complex showing that Zn−O (Pα) bonding is relatively weak.[6] Whereas Pα and Pβ of ADP were found as the coordination sites of Mg^{2+} based on ^{25}Mg-[21] and ^{17}O NMR spectroscopy,[22] here, we found that nucleoside 5′-phosphorthioate analogues chelate Zn^{2+} mainly through the terminal thiophosphate group (Pγ of ATP-γ-S and Pβ of ADP-β-S and GDP-β-S) based on their large $\Delta\delta$ values (∼7−9 ppm) after addition of only 0.1−0.2 equiv of Zn^{2+}.

Signals of terminal phosphate of natural nucleotides (ATP, ADP, and GDP) were less affected even after addition of 10 equiv of Zn^{2+} ($\Delta\delta_{P\beta\ (ADP)} = +1.22$, $\Delta\delta_{P\beta\ (GDP)} = +0.48$, $\Delta\delta_{P\gamma\ (ATP)} = 2.23$). Similar data were found for phosphate groups adjacent to the terminal thiophosphate (Pα of ADP-β-S and GDP-β-S) and for corresponding phosphate group of the natural nucleotides after addition of 10 equiv of Zn^{2+} ($\Delta\delta_{P\alpha\ (ADP)} = +1.23$ vs $\Delta\delta_{P\alpha\ (ADP\text{-}\beta\text{-}S)} = +1.16$ and, $\Delta\delta_{P\alpha\ (GDP)} = +1.48$ vs $\Delta\delta_{P\alpha\ (GDP\text{-}\beta\text{-}S)} = +1.42$). While after addition of 1 equiv of Zn^{2+}, $\Delta\delta$ of Pβ of ATP was slightly larger than $\Delta\delta$ of Pβ ATP-γ-S (+2.89 vs +1.61). This may indicate that the major chelation of ATP-γ-S with Zn^{2+} occurs through Pγ, unlike ATP where Zn^{2+}-chelation occurs through Pγ and Pβ.

While addition of Zn^{2+} to nucleoside phosphorothioate analogues resulted in downfield shift of Pα or Pβ adjacent to thiophosphate, δ of the terminal thiophosphate group shifted upfield. These observations are supported by previous reports on ^{31}P NMR Zn^{2+}-monitored titrations of adenosine thiophosphate analogues at a much lower magnetic field (24.3 MHz) and basic conditions.[23] 1:1 Zn^{2+}:ADP-β-S and Zn^{2+}:ATP-γ-S terminal thiophosphate shifted upfield of the δ of the terminal thiophosphate by 8.2 and 9 ppm, respectively.[23]

Titration with Ca^{2+} and Mg^{2+} ions shifted downfield the chemical shift of the terminal thiophosphate of ADP-β-S and ATP-γ-S by ∼1−2 ppm.[23] This phenomenon is probably because of coordination of Ca^{2+} and Mg^{2+} through the oxygen atom of the terminal thiophosphate of adenosine phosphorothioate analogues, whereas Zn^{2+} is bound through the sulfur atom.[24]

Related NMR-monitored Mg^{2+}-titrations of nucleoside diphosphates/triphosphates were reported before,[25,26] indicating that Mg^{2+} binds only with Pβ of nucleoside triphosphates and Pα of nucleoside diphosphates at pD ≅ 8. Another report on NMR monitored Mg^{2+} titration at pD ≅ 5−7 indicated that ATP binds Mg^{2+} through Pβ and Pγ, and ADP through Pα and Pβ, due to the $\Delta\delta$ of their respective chemical shifts in the presence and absence of Mg^{2+}.[20] In this pH range deprotonation of the terminal phosphate (Pβ/Pγ) occurs, and NMR data may result from a mixture of protonated and nonprotonated species.

Here, the ^{31}P NMR monitored Zn^{2+} titration of nucleoside 5′-thiophosphate analogues was performed at pD ≅7.4. Hence, the terminal thiophosphate was not protonated ($pK_a \cong 5.5$), especially in presence of Zn^{2+} ions which further lower the pK_a value of the terminal thiophosphate. Namely, the observed shift of chemical shift of thiophosphate moiety was due to coordination with zinc ions and not acid−base equilibrium of the terminal thiophosphate.

Dinucleotides, AP_3A and $AP_3(\beta\text{-S})A$ Zn^{2+}-complexes also exhibited a $\Delta\delta$ difference due to the sulfur modification. ^{31}P NMR spectra of $AP_3(\beta\text{-S})A$ with and without Zn^{2+} ions are shown in Supporting Information, Figure S1. $AP_3(\beta\text{-S})A$ spectrum showed two Pα signals (Pα_A and Pα_B) due to the sulfur modification in Pβ and the chirality of the ribose, removing symmetry through S−P−O plane in Pβ.

For both dinucleotides, AP_3A and $AP_3(\beta\text{-S})A$, a gradual shift of phosphate signals was observed due to Zn^{2+} addition, yet, a more significant shift was observed for the modified dinucleotide, **6**. After addition of 10 equiv of Zn^{2+} to $AP_3(\beta\text{-S})A$, its Pβ signal shifted downfield by ∼3 ppm (Supporting Information, Figure S1), unlike Pβ of AP_3A which shifted downfield by only ∼1 ppm. The enhanced binding of Zn^{2+} to **6** is further supported by the similar $\Delta\delta$ of Pα in AP_3A and $AP_3(\beta\text{-S})A$ upon addition of Zn^{2+} ($\Delta\delta$ +0.67 and 0.5 ppm respectively).

Data of ^{1}H NMR monitored Zn^{2+}-titrations are presented in Supporting Information, Table S2 and in Figure 3 and

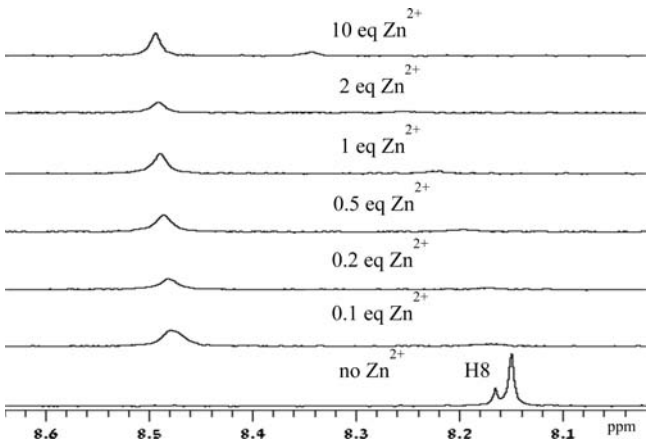


Figure 3. Zn^{2+}-titration of 6 mM GDP(β-S) in D_2O at pD 7.34 at 300 K. ^{1}H NMR spectrum was measured at 600 MHz.

Supporting Information, Figure S2. Two sets of protons are observed for $AP_3(\beta\text{-S})A$ in ^{1}H NMR spectrum (Supporting Information, Figure S2), as for ^{31}P NMR (Supporting Information, Figure S1), because of asymmetry of the molecule as mentioned above. The data in Supporting Information, Table S2 show a similar trend to that in Supporting Information, Table S1. H8/H2 signals of analogues **1**−**3**

shifted substantially after addition of only 0.1/0.2 equiv of Zn^{2+}, unlike for H8/H2 signals of the corresponding parent nucleotides. For instance, H8 of ATP-γ-S and ATP shifted downfield by 0.12 ppm and 0.01 ppm, respectively, upon addition of 0.2 equiv of Zn^{2+} (Supporting Information, Table S2). On the other hand, the H2 signals of ADP, ADP-β-S, and ATP-γ-S moved upfield upon addition of Zn^{2+}, whereas the chemical shift of H2 of ATP did not change significantly. The upfield shifts of H2 in the presence of zinc ions possibly result from stacking interactions[27] due to formation of dimeric species, ($[ML]_2$), as reported for $[Cd(ADP)_2]$, $[Zn(ADP)_2]$[27] and $[M^{2+}(ATP)]_2$ or $[M^{2+}(ATP)]_2(OH^-)$[28,29] (M^{2+} = Zn^{2+}, Cd^{2+}, Ni^{2+}, and Cu^{2+}). It was also reported that self-stacking decreases in the series, N > NMP^{2-} > NDP^{3-} > NTP^{4-} because of repulsion of the increasing number of phosphate negative charges.[29] This evidence may explain the higher upfield shifts of H2/H8 in ADP-β-S ($\Delta\delta$= −0.14/−0.20 ppm upon addition of 0.2 equiv of Zn^{2+}) as compared to upfield shifts of H2 in ATP-γ-S ($\Delta\delta$ = −0.03 ppm upon addition of 0.2 equiv of Zn^{2+}).

The chemical shifts of H8 of dinucleotides AP_3A and AP_3(β-S)A shifted in a similar way, whereas their H2 chemical shifts did not change (Supporting Information, Table S2). The downfield shifts of H8 imply that N7 is a coordination site of Zn^{2+}.

In addition to shift of signals, line-broadening was observed as well as during zinc titration. This phenomenon was more significant for nucleoside phosphorothioate analogues as compared to the corresponding parent compounds (see Supporting Information, Table S3). For example, the H8 signal of GDP-β-S broadened 3-fold as compared to H8 of GDP, upon addition of 0.1 equiv of Zn^{2+}. Likewise, the H2 signal of ATP-γ-S broadened 5-fold more than ATP. On the other hand, signals of adenine base protons of dinucleotides, AP_3A and AP_3(β-S)A (Supporting Information, Figure S2), did not broaden significantly as compared to nucleotides and nucleoside phosphorothioate analogues. The major broadening effect was observed for the terminal phosphate groups. This fact may indicate that Zn^{2+} has a stronger affinity to terminal phosphate or thiophosphate groups.

Purine-nucleotides can form macrochelates with metal ions like Zn^{2+} or Cd^{2+} through the interaction of the phosphate-coordinated metal ion with N7.[30−32] In solution purine-nucleotide complexes can form two species: an "open" form in which only the phosphate chain coordinates the metal ion, and a "closed" form in which the phosphate chain and N7 of the purine nucleobase coordinate simultaneously the metal ion.[3] Furthermore, there are at least two types of macrochelates of M^{2+}-(purine nucleotide) complexes: one in which the metal ion binds directly to N7 of the purine residue (innersphere coordination), and one in which M^{2+}-coordination involves a water molecule between N7 and M^{2+}, an outersphere interaction.[3]

Zn(ADP) exists mostly (80%) as the "open" form. The "closed" form, ($Zn(ADP)_{cl}$), is composed of 33% of the outersphere and 67% of innersphere form.[1] Zn(ATP) exists mostly (70%) as the "open" form. The minor "closed" species consists of 50:50 outersphere: innersphere coordinated complexes. Hence, adenine nucleotide-Zn^{2+} complexes adopt mainly the "open" form, implying that the major species of Zn(ADP-β-S) or Zn(ATP-γ-S) complexes probably adopt the "open" form as well. Since the basicity of N7 site in guanosine is higher[33−35] than that of adenosine, larger amounts of the macrochelate are expected for guanine over adenine nucleotides. Indeed, $M^{2+}(GMP)$[19,36] species were found to form substantially larger amounts of macrochelates than $M^{2+}(AMP)$[32,36] species. Zn(GTP) and Zn(GMP) species include 68% and 72% "closed" conformation. Macrochelates of $Zn(GTP)_{cl}$ and $Zn(GMP)_{cl}$ species are mainly innersphere.[19,30] Hence, we assume that Zn(GDP-β-S) complex adopts a "closed" macrochelate form which is mainly innersphere.

3. CHARACTERIZION OF NUCLEOSIDE 5′-PHOSPHOROTHIOATE-Zn^{2+} COMPLEXES BY POTENTIOMETRIC PH-TITRATIONS

To characterize nucleoside 5′-phosphorothioate-Zn^{2+} complexes we determined the pK_a values of nucleotide analogues **1**−**6**, their stability constants with Zn^{2+}, log K^{Zn}_{ZnL} and log K^{Zn}_{ZnLH}, and the acidity constants of their complexes, $pK_a{}^{H}{}_{ZnLH}$. In addition, we predicted the species distribution of nucleoside phosphorothioate complexes under physiological pH. Potentiometric pH-titrations were performed for compounds **1**, **2**, and **4**−**6** as Na^+ salts, and compound **3** Li^+ salt. The determination of the stability constants of Zn^{2+} complexes were performed at a 1:1 Zn^{2+}:L ratio.[4,37]

3.1. Acidity Constants of Nucleoside 5′-Phosphorothioate Analogues 1−6. The protonation equilibria of nucleoside-5′-phosphorothioate (NP_nS) and nucleoside-5′-phosphate (NP_n) analogues are expected to occur in the pH range 2.5−10. 8-SH-ATP, **5**, and GDP-β-S, **2**, undergo three protonation equilibria. Thiophosphate analogues, **1**, **3**, **4**, and **6**, and the corresponding nucleotides, undergo only two protonation equilibria. The following protonation equilibria apply for NP_nS and the corresponding NP_n analogues where the charge of their protonated species is $m = n - 1$ (n = 2−3):

$$H_3(NP_nS)^{m-} \rightleftarrows H_2(NP_nS)^{(m+1)-} + H^+ \quad (1a)$$

$$K^{H}_{H_3(NP_nS)} = \frac{[H_2(NP_nS)^{(m+1)-}][H^+]}{[H_3(NP_nS)^{m-}]} \quad (1b)$$

$$H_2(NP_nS)^{(m+1)-} \rightleftarrows H(NP_nS)^{(m+2)-} + H^+ \quad (2a)$$

$$K^{H}_{H_2(NP_nS)} = \frac{[H(NP_nS)^{(m+2)-}][H^+]}{[H_2(NP_nS)^{(m+1)-}]} \quad (2b)$$

$$H(NP_nS)^{(m+2)-} \rightleftarrows (NP_nS)^{(m+3)-} + H^+ \quad (3a)$$

$$K^{H}_{H(NP_nS)} = \frac{[(NP_nS)^{(m+3)-}][H^+]}{[H(NP_nS)^{(m+2)-}]} \quad (3b)$$

For guanosine 5′-phosphate or phosphorothioate, the first deprotonation occurs at the guanine N7−H^+ position (eqs 1a, 1b), the second deprotonation occurs at the terminal phosphate group (eqs 2a, 2b), and the third deprotonation occurs at guanine N1−H position (eqs 3a, 3b). For adenosine 5′-phosphate or phosphorothioate, the first deprotonation occurs at the adenine N1−H^+ position (eqs 2a, 2b), and the second deprotonation occurs at the terminal phosphate group (eqs 3a, 3b). For 8-SH-ATP the first two deprotonation steps occur at N1−H^+ and the terminal phosphate position (eqs 1a, 1b, 2a, 2b). The third deprotonation occurs at 8-SH position (eqs 3a, 3b).

The following protonation equilibria for the dinucleotide, AP_3(β-S)A, are expected to occur in the pH range 3−5:

dx.doi.org/10.1021/ic400878k | Inorg. Chem. 2013, 52, 10886−10896

Table 1. Acidity Constants of Nucleoside 5′-Phosphate and Phosphorothioate Analogues, 1−6[b]

compound no.	compound	$pK_a^H{}_{LH3}$	$pK_a^H{}_{LH2}$	$pK_a^H{}_{LH}$
	ADP[1]		3.92 ± 0.02(N1)	6.40 ± 0.01(Pβ)
1	ADP		4.07 ± 0.01(N1)	6.40 ± 0.01(Pβ)
	ADP-β-S		4.00 ± 0.05(N1)	5.56 ± 0.03(Pβ)
	GDP[38]	2.67 ± 0.02(N7)	6.38 ± 0.01(Pβ)	9.56 ± 0.03(N1)
	GDP	3.23 ± 0.03(N7)	6.41 ± 0.01(Pβ)	9.47 ± 0.01(N1)
2	GDP-β-S	3.14 ± 0.03(N7)	5.49 ± 0.02(Pβ)	9.53 ± 0.01(N1)
	ATP[4]		4.00 ± 0.01(N1)	6.47 ± 0.01(Pγ)
	ATP		4.00 ± 0.02(N1)	6.52 ± 0.01(Pγ)
3	ATP-γ-S		4.14 ± 0.01(N1)	5.63 ± 0.01(Pγ)
4	ATP-α,β-(CH_2)-γ-S[a]		4.26 ± 0.04(N1)	5.86 ± 0.02(Pγ)
5	8-SH-ATP	3.27 ± 0.02(N1)	6.36 ± 0.01(Pγ)	7.28 ± 0.01(SH)
6	AP_3(β-S)A		3.41 ± 0.01(N1)	4.42 ± 0.01(N1)

[a]Titrations were performed in triplicates or quadruplicates, except for compound **4**, the titration of which was performed in duplicate. [b]The deprotonation positions appear in brackets.

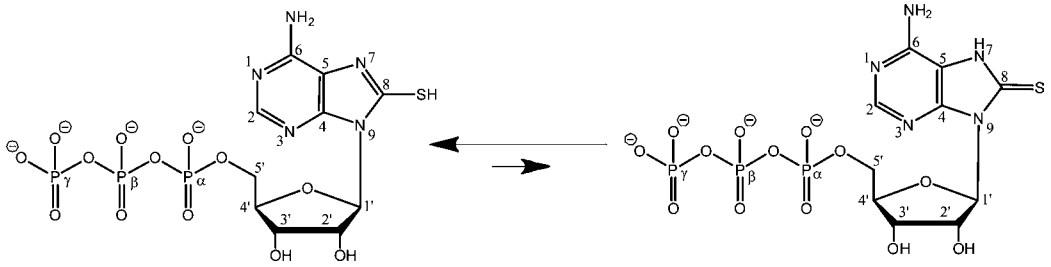


Figure 4. Keto−enol tautomerism of 8-SH-ATP.

$$H_2(AP_3(\beta - S)A)^- \rightleftarrows H(AP_3(\beta - S)A)^{2-} + H^+ \quad (4a)$$

$$K^H_{H_2(AP_3(\beta-S)A)} = \frac{[H(AP_3(\beta - S)A)^{2-}][H^+]}{[H_2(AP_3(\beta - S)A^-)]} \quad (4b)$$

$$H(AP_3(\beta - S)A)^{2-} \rightleftarrows (AP_3(\beta - S)A)^{3-} + H^+ \quad (5a)$$

$$K^H_{H(AP_3(\beta-S)A)} = \frac{[(AP_3(\beta - S)A)^{3-}][H^+]}{[H(AP_3(\beta - S)A)^{2-}]} \quad (5b)$$

For dinucleotide, AP_3(β-S)A, there are two deprotonation steps: the first deprotonation occurs at one of the N1−H^+ positions in the adenine bases (eqs 4a, 4b), and the second deprotonation occurs at the N1−H^+ position of the second nucleobase (eqs 5a, 5b).

Acidity constants of nucleoside 5′-phosphorothioate analogues, determined as described above, were compared to those of the corresponding nucleotide analogues. The pK_a values of ADP, GDP, and ATP, which we determined, were consistent with literature (Table 1).[1,4,38] pK_a values of the protons in the guanine and adenine nucleobases at N7−H^+ and N1−H^+ protons, respectively were not affected by the sulfur modification at the phosphate chain (Table 1). Likewise, pK_a values of AP_3(β-S)A were highly similar to the parent dinucleotide, AP_3A.[39] However, pK_a values of the thiophosphate group in ADP-β-S, GDP-β-S, and ATP-γ-S were more acidic by about 1 log unit as compared to those of their parent nucleotides (Table 1), because of the higher polarizibility of the sulfur atom as compared to oxygen, inducing greater stability of the thiophosphate anion. These results are consistent with the literature.[13,40] Increased acidity of at least 1 log unit was reported for AMP-α-S and UMP-α-S vs A(U)MP.[41] The acidity constant of the thiophosphate group in ATP-α,β-CH_2-γ-S was lower than that of ATP (5.86 vs 6.52), but was more basic as compared to ATP-γ-S (by 0.23 log units). The replacement of the bridging oxygen atom between Pα and Pβ with an electron donating methylene group, reduces the acidity of the Pγ-thiophosphate. The pK_a value of Pγ of 8-SH-ATP is similar to that of Pγ of ATP (Table 1).

pK_a 7.28 is assigned to SH moiety of 8-SH-ATP (Table 1), involved in keto−enol tautomerism (Figure 4). This assignment is supported by ^{13}C NMR experiments of Mercaptobenzimidazole (MBI) and its sodium salt (MBI^-Na^+),[42] suggesting that MBI and its Na^+ salt exist as enol and enolate forms respectively, and not as the keto forms. 8-SH substitution affects also the pK_a value of N1−H^+, which is more acidic by about 0.7 log units than that of N1−H^+ in ATP. A similar trend was reported for the pK_a value of N1−H^+ position in 8-OH-adenosine (2.9).[43]

3.2. Stability Constants of Zn^{2+}-Nucleoside 5′-Phosphorothioate Complexes, 1−6. The equations describing the formation of 1:1 Zn^{2+}-complexes with NP_nS (n = 2−3, m = n − 1) are as follows.

$$H(NP_nS)^{(m+1)-} + Zn^{2+} \rightleftarrows Zn(H{\cdot}NP_nS)^{(m-1)-} \quad (6a)$$

$$K^{Zn}_{Zn(H\cdot NP_nS)} = \frac{[Zn(H{\cdot}NP_nS)^{(m-1)-}]}{[Zn^{2+}][(H{\cdot}NP_nS)^{(m+1)-}]} \quad (6b)$$

$$(NP_nS)^{(m+2)-} + Zn^{2+} \rightleftarrows Zn(NP_nS)^{m-} \quad (7a)$$

$$K^{Zn}_{Zn(NP_nS)} = \frac{[Zn(NP_nS)^{m-}]}{[Zn^{2+}][(NP_nS)^{(m+2)-}]} \quad (7b)$$

The deprotonation of the complex is represented by: eq 8a

$$Zn(H{\cdot}NP_nS)^{m-1} \rightleftarrows Zn(NP_nS)^{m-} + H^+ \quad (8a)$$

dx.doi.org/10.1021/ic400878k | Inorg. Chem. 2013, 52, 10886−10896

$$K^{H}_{Zn(H\cdot NP_nS)} = \frac{[Zn(NP_nS)^{m-}][H^+]}{Zn(H\cdot NP_nS)^{m-1}]} \quad (8b)$$

The acidity constant of the complex is calculated by the following equation:[4]

$$pK^{H}_{Zn(H\cdot NP_nS)} = pK^{H}_{H(NP_nS)} + \log K^{Zn}_{Zn(H\cdot NP_nS)} - \log K^{Zn}_{Zn(NP_nS)} \quad (9)$$

The equations describing the formation of 1:1 Zn^{2+}-complexes with dinucleotide analogues, $AP_3(\beta\text{-S})A$ and AP_3A are the following.

$$H(AP_3(\beta - S)A)^{2-} + Zn^{2+} \rightleftarrows Zn(H\cdot AP_3(\beta - S)A) \quad (10a)$$

$$K^{Zn}_{Zn(H\cdot AP_3(\beta-S)A)} = \frac{[Zn(H\cdot AP_3(\beta - S)A)]}{[Zn^{2+}][(H\cdot AP_3(\beta - S)A)^{2-}]} \quad (10b)$$

$$(AP_3(\beta - S)A)^{3-} + Zn^{2+} \rightleftarrows Zn(AP_3(\beta - S)A)^{-} \quad (11a)$$

$$K^{Zn}_{Zn(AP_3(\beta-S)A)} = \frac{[Zn(AP_3(\beta - S)A)^{-}]}{[Zn^{2+}][(AP_3(\beta - S)A)^{3-}]} \quad (11b)$$

The deprotonation of the complex is represented by eq 12a.

$$Zn(H\cdot AP_3(\beta - S)A) \rightleftarrows Zn(AP_3(\beta - S)A)^{-} + H^+ \quad (12a)$$

$$K^{H}_{Zn(AP_3(\beta-S)A)} = \frac{[Zn(AP_3(\beta - S)A)^{-}][H^+]}{[Zn(H\cdot AP_3(\beta - S)A)]} \quad (12b)$$

The acidity constant of the complex is calculated by the following equation.

$$pK^{H}_{Zn(H\cdot AP_3(\beta-S)A)} = pK^{H}_{H(AP_3(\beta-S)A)} + \log K^{Zn}_{Zn(H\cdot AP_3(\beta-S)A)} - \log K^{Zn}_{Zn(AP_3(\beta-S)A)} \quad (13)$$

All titrations were performed on 1:1 ligand:Zn^{2+} mixtures, and the resulting stability constants of nucleoside 5′-phosphorothioate-Zn^{2+} complexes were compared to those of natural nucleotides (Table 2).[1,4,44] We found that ADP-β-S, GDP-β-S, and ATP-γ-S complexes with Zn^{2+} were more stable than the parent nucleotide complexes at least by 0.4−0.7 log units (Table 2). Likewise, the stability constant of $AP_3(\beta\text{-S})A$-Zn^{2+} complex was approximately 0.5 log units higher than that of AP_3A (3.8 vs 3.32). Furthermore, the stability constant of the complex $AP_3(\beta\text{-S})A$ with zinc was less stable than of the Zn^{2+}-complexes of nucleoside 5′-phosphorthioate analogues (3.8 for $AP_3(\beta\text{-S})A$ vs 5.42 and 5.74 for ADP-β-S and ATP-γ-S), indicating the role of a terminal thiophosphate group in stabilizing the complex.

Furthermore, Zn^{2+} forms a more stable complex with ATP-γ-S than ADP-β-S (5.74 vs 5.42), as observed for ATP vs ADP (5.37 vs 4.72), implying that a longer phosphate chain increases the stability constant of Zn^{2+} complexes. These results are supported by literature.[1]

GDP-β-S complex with Zn^{2+} is more stable than the corresponding ADP-β-S complex (5.75 vs 5.42), as observed also for GDP-Zn^{2+} complex vs ADP-Zn^{2+} complex (5.10 vs 4.72). This may be a result of outersphere interaction of Zn^{2+} with the guanine O^6 via a hydrogen bond.[19]

ATP-α,β-(CH_2)-γ-S was found to form the most stable Zn^{2+}-complex studied here (log K 6.50) being approximately 0.8 and 1.1 log units more stable than ATP-γ-S-Zn^{2+} and ATP-Zn^{2+}-complexes, respectively. The high stability of this complex is due to both its terminal thiophosphate moiety and the electron donating methylene group. The latter makes the phosphate chain more electron rich, and hence, a better chelator.

C8-SH substitution of ATP did not increase significantly the stability constant of the complex with Zn^{2+} (log K 5.54). To explain this phenomenon, the conformations of 8-SH-ATP and its Zn^{2+} complex were determined by NOESY 2D-experiment (Figure 5). *syn* Conformation of the free nucleotide was indicated by cross peaks between H2 and H2′, and H2 and H3′ (Figure 5A). Addition of Zn^{2+} shifted the purine ring vs the ribose ring in such a way, that H2 is closer to H1′ as indicated by cross peaks between H2 and H1′ (Figure 5B). *syn* Conformation implies that the major contribution to Zn^{2+} chelation occurs through the phosphate chain. Moreover, the signal of H2 of 8-SH-ATP shifted downfield by 0.02 ppm upon addition of 1 equiv of Zn^{2+} which may imply that N1 is another coordination site of Zn^{2+} in 8-SH-ATP.

ATP coordinates Zn^{2+} ions mainly by the phosphate chain and in addition by N7-nitrogen atom. However, 8-SH-ATP coordinates Zn^{2+} by the phosphate chain and possibly N1-nitrogen atom. Yet, the contribution of N7−Zn^{2+} binding in ATP is relatively small[36] since the adenosine N1 site is more basic than N7 by 5 orders of magnitude. This may explain the minor increase of log K of 8-SH-ATP-Zn^{2+} as compared to ATP-Zn^{2+} complex (5.54 vs 5.37). The shift of the purine ring vs the ribose ring of 8-SH-ATP-Zn^{2+} complex (Figure 5B), possibly reduced slightly the pK_a of the complex as compared to that of the free ligand (6.80 vs 7.28, C-8-SH).

The log K^{Zn}_{ZnLH} values of the protonated zinc complexes were lower than log K^{Zn}_{ZnL} by 1.7−2.3 log units because of protonation on the phosphate chain, whereas log K^{Zn}_{ZnLH} of 8-SH-ATP (5.06) and $AP_3(\beta\text{-S})A$ (3.03) were less stable by only ∼0.5−0.8 log units because of protonation on the nucleobase. This finding also supports the notion that the major contribution to coordination is the phosphate chain. As expected, the acidity constants of the Zn^{2+}-complexes were more acidic by at least 1.5 log units as compared to the free ligand. However, the acidity constants of 8-SH-ATP and $AP_3(\beta$-

Table 2. Stability Constants of Zn^{2+}-NP_n, Zn^{2+}-NP_nS, and Zn^{2+}-NP_nN Complexes

compound	log K^{Zn}_{ZnL}	log K^{Zn}_{ZnLH}	pK^{H}_{ZnLH}
ADP[1]	4.28 ± 0.05	2.31 ± 0.20	4.43 ± 0.21
ADP	4.72 ± 0.03	3.19 ± 0.02	4.87 ± 0.02
ADP-β-S	5.42 ± 0.02	3.49 ± 0.08	3.62 ± 0.06
GDP[44]	4.52 ± 0.03		
GDP	5.10 ± 0.03	3.45 ± 0.01	4.76 ± 0.03
GDP-β-S	5.75 ± 0.02	3.54 ± 0.04	3.28 ± 0.02
ATP[4]	5.16 ± 0.06	2.86 ± 0.11	4.17 ± 0.13
ATP	5.37 ± 0.03	3.14 ± 0.04	4.29 ± 0.01
8-SH-ATP	5.54 ± 0.09	5.06 ± 0.07	6.80 ± 0.02
ATP-γ-S	5.74 ± 0.03	4.05 ± 0.01	3.94 ± 0.01
ATP-α,β-(CH_2)-γ-S	6.50 ± 0.05	4.31 ± 0.05	3.67 ± 0.01
$AP_3(\beta$-S)A	3.80 ± 0.02	3.03 ± 0.03	3.66 ± 0.02
AP_3A	3.32 ± 0.02	n.o.[a]	

[a]n.o. = not observed.

dx.doi.org/10.1021/ic400878k | *Inorg. Chem.* 2013, 52, 10886−10896

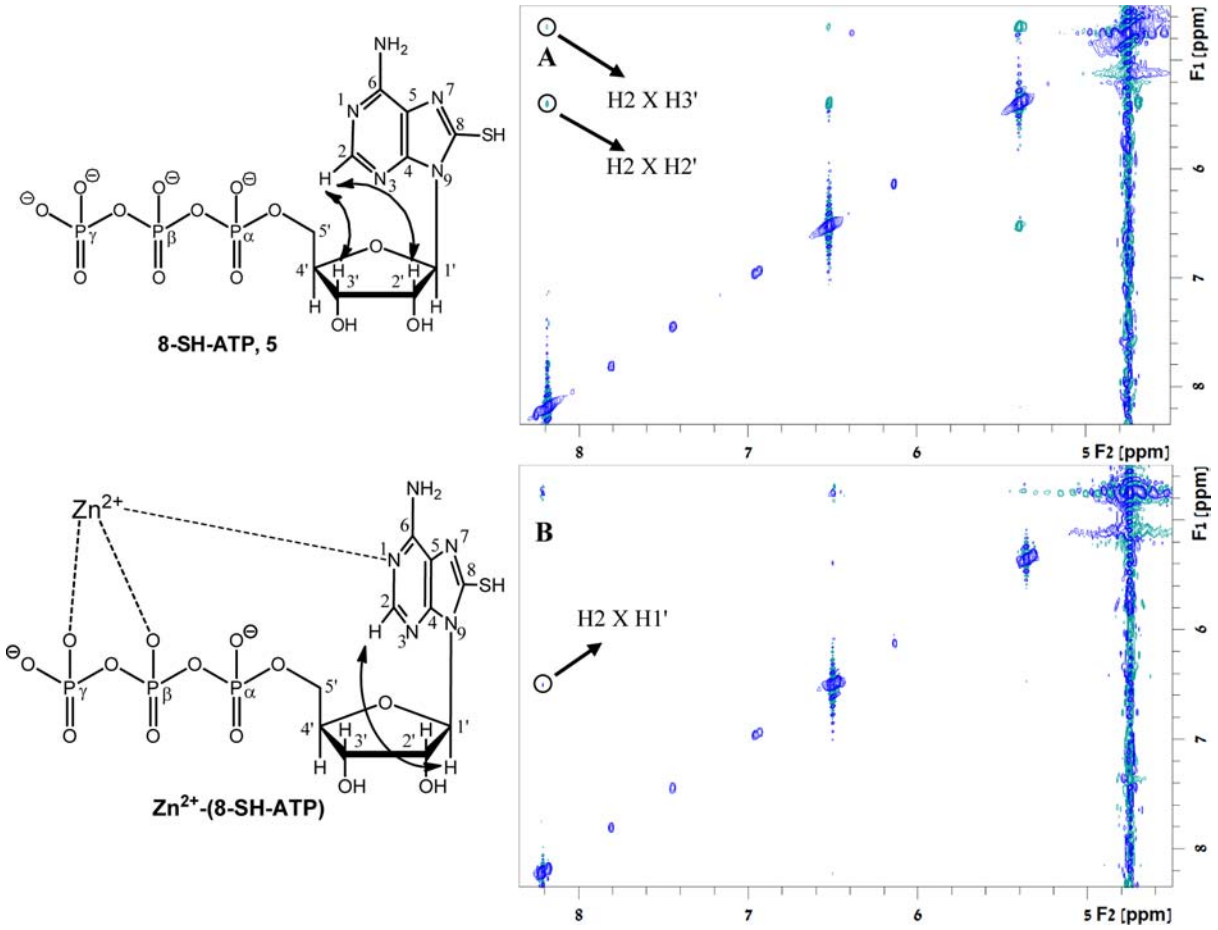


Figure 5. Determination of the conformation of **5**. (A) $^{1}H,^{1}H$-NOESY spectrum of compound **5** Na^{+} salt. (B) $^{1}H,^{1}H$-NOESY spectrum of compound **5** in presence of 1 equiv of Zn^{2+}.

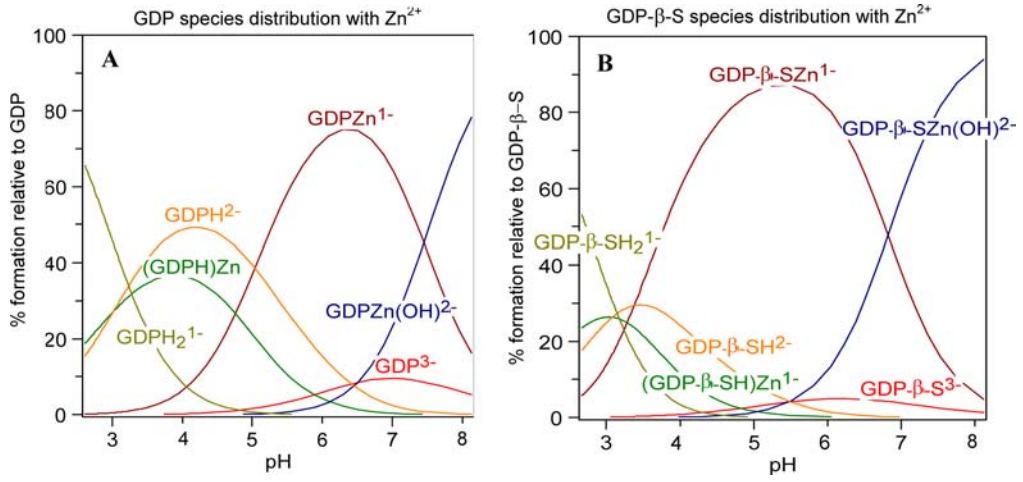


Figure 6. Simulation of pH titration of 1:1 GDP and GDP-β-S complexes with Zn^{2+}. (A) GDP species distribution with Zn^{2+}. (B) GDP-β-S species distribution with Zn^{2+}.

S)A complexes with zinc were more acidic only by 0.48 and 0.76 log units, respectively, as compared to the free ligands.

To analyze the composition of the nucleoside 5′-phosphorothioate complexes with Zn^{2+} in titration solutions, as compared to their parent nucleotide complexes, we performed titration simulations using the Hyss program[45] (Figures 6 and 7).

Under physiological pH (7.4) the major species is $GDPZn^{-}$ (~48%, Figure 6A) and $[GDP\text{-}\beta\text{-}SZn(OH)]^{2-}$ (~76%, Figure 6B), whereas both GDP^{3-} (Figure 6A) and GDP-β-S^{3-} (Figure 6B) are approximately 9% and 3% of all species, respectively.

Likewise, the most stable complex studied here, ATP-α,β-(CH_2)-γ-SZn^{2-}, is the dominant species under physiological pH (~84%), and only ~2% of ATP-α,β-(CH_2)-γ-S^{4-} exist as a free ligand (Figure 7C).

dx.doi.org/10.1021/ic400878k | Inorg. Chem. 2013, 52, 10886−10896

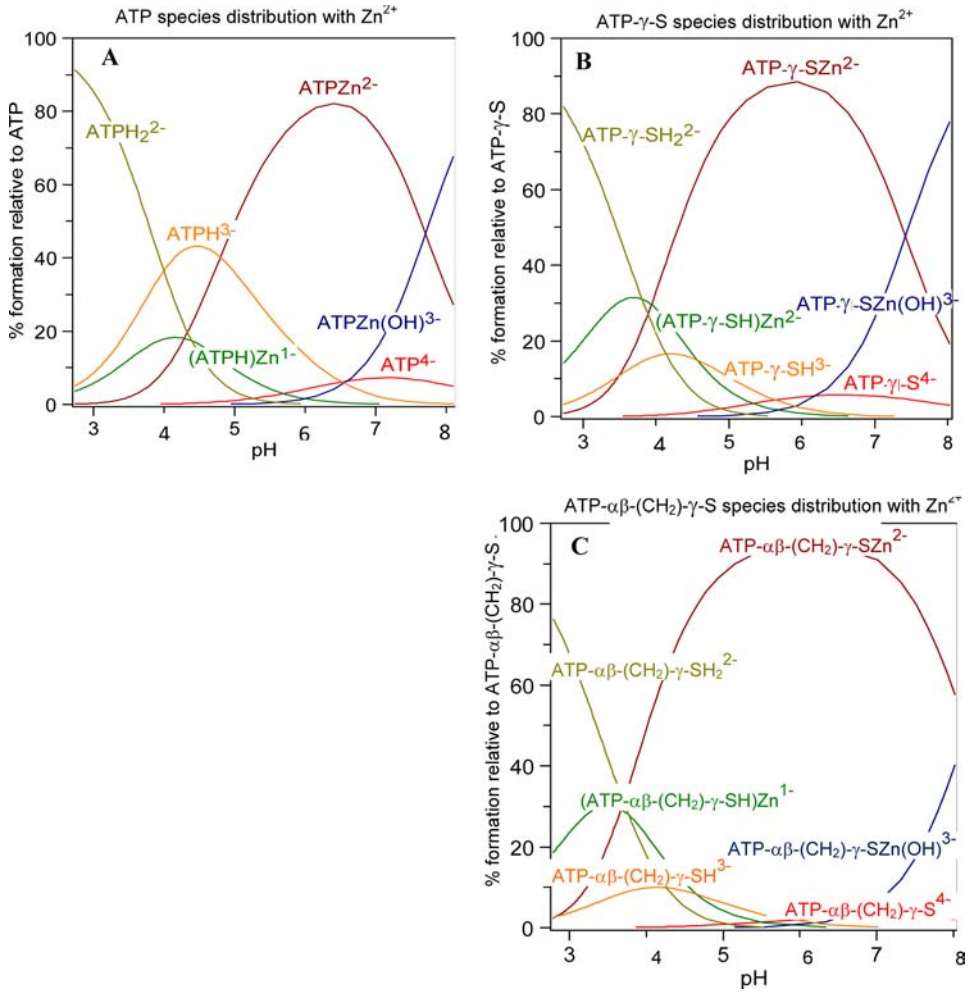


Figure 7. Simulation of pH titration of 1:1 ATP, ATP-γ-S, and ATP-α,β-(CH_2)-γ-S Zn^{2+}complexes species distribution of (A) ATP, (B) ATP-γ-S, and (C) ATP-α,β-CH_2-γ-S.

The major species of ATP-γ-S and ATP in the titration solution of pH 7.4 are also $[ATP\text{-}\gamma\text{-}SZn]^{2-}$ (~49%) and $[ATPZn]^{2-}$ (~62%), yet they are less dominant than ATP-α,β-(CH_2)-γ-SZn^{2-} (Figure 7). Nucleoside 5′-phosphorothioate-Zn^{2+} complexes form hydroxo species much more than their parent nucleotide. For instance, $[GDP\text{-}\beta\text{-}S\text{-}Zn\text{-}(OH)]^{2-}$ is ~76% of all species under physiological pH, whereas GDP-Zn-$(OH)^{2-}$ is ~42%. Similarly, $[ATP\text{-}\gamma\text{-}S\text{-}Zn\text{-}(OH)]^{3-}$ vs [ATP-Zn-$(OH)]^{3-}$ make ~47% and ~31%, respectively of all species. The same trend was reported before for [AMP-α-S-Zn] complex.[46]

4. CONCLUSIONS

Replacement of a nonbridging oxygen atom in the phosphate chain of purine nucleotides by sulfur increased the affinity to Zn^{2+} as we expected according to HSAB theory. Thus, ADP-β-S and GDP-β-S complexes with Zn^{2+} were ~0.7 log unit more stable than the parent nucleotide complexes. Likewise, the stability constant of the AP_3-(β-S)A-Zn^{2+} complex was higher by ~0.5 log units than that of AP_3A. In addition to sulfur substitution; an electron donating methylene group replacing a bridging oxygen atom rendered ATP-α,β-CH_2-γ-S the most stable Zn^{2+}complex studied here (log K = 6.50). Additionally, Zn^{2+}-GDP-β-S and Zn^{2+}-GDP were found to be more stable complexes than ADP-β-S and ADP complexes possibly resulting from an outersphere interaction of Zn^{2+} with the guanine O^6 via hydrogen bond. We concluded that a terminal thiophosphate group is required to achieve a highly stable Zn^{2+}-complex. Indeed, Zn^{2+}-AP_3(β-S)A is a less stable complex than Zn^{2+}-complexes of nucleoside phosphorothioate analogues.

Furthermore, replacement of an α,β-bridging oxygen in nucleoside-5′-phosphorothioate by a CH_2 group further increase affinity to Zn^{2+} ions. The pK_a values of the thiophosphate group in ATP-γ-S, ADP-β-S, and GDP-β-S decreased by ~1 log unit as compared to natural nucleotides. Furthermore, pK_a value of the thiophosphate group in ATP-α,β-CH_2-γ-S was more acidic by ~0.7 log units as compared to ATP, but was more basic as compared to ATP-γ-S because of the positive inductive effect of the methylene group. C8-SH substitution of ATP did not increase significantly the stability constant of the nucleotide-Zn^{2+}complex, because of *syn* conformation of this nucleotide. Shift of the purine ring vs the ribose ring of 8-SH-ATP in its Zn^{2+} complex enabled Zn^{2+} coordination by the N1 site, unlike ATP, where the Zn^{2+} coordination site in adenine ring is the N7 nitrogen atom. Zn^{2+} ion was found to coordinate with two phosphates, Pα and Pβ, or Pβ and Pγ, in nucleoside-5′-di/tri phosphate/thiophosphate, respectively. In AP_3(β-S)A, Pβ is the major coordination site of Zn^{2+}, in addition to, Pα and N7. In summary, sulfur-modified nucleotides are promising Zn^{2+}-chelators. Specifically, we propose ATP-α,β-CH_2-γ-S as a promising zinc-ion-chelator

dx.doi.org/10.1021/ic400878k | *Inorg. Chem.* 2013, 52, 10886−10896

forming a highly stable Zn^{2+}-complex, log K 6.50, being ~0.8 and ~1.1 log units more stable than ATP-γ-S-Zn^{2+} and ATP-Zn^{2+} complexes. Furthermore, [ATP-α,β-CH_2-γ-S-Zn]$^{2-}$ is the major species, 84%, under physiological pH.

5. EXPERIMENTAL SECTION

General Information. All reactions were carried out in flame-dried, argon-flushed, two necked flasks sealed with rubber septa, and the reagents were introduced with a syringe. The progress of reactions was monitored by thin-layer chromatography (TLC) on precoated Merck silica gel plates (60F-254) applying elution of iPrOH/H_2O/NH_4OH (55%:35%:10%). Nucleotide starting materials were dried overnight in a vacuum oven with P_2O_5. Mass spectra were measured on a Q-TOF Micro Mass Spectrometer instrument (Micromass-Waters, United Kingdom). Purification of the nucleotides was achieved on a liquid chromatography (LC) (Isco UA-6) system with a Sephadex DEAE-A25 column, which was swelled in 1 M $NaHCO_3$ in the cold for 1 day. Conditions for LC separation are described below. Final purification of the nucleotides was achieved on an HPLC (Hitachi Elite LaChrome) system, using a semipreparative reverse-phase column (Gemini 5u C-18 110A, 250 × 10.00 mm, 5 μm, Phenomenex, Torrance, U.S.A.). The purity of the nucleotides was evaluated with an analytical reverse-phase column system (Gemini 5u C-18 110A, 150 mm × 4.60 mm; 5 μm; Phenomenex, Torrance, CA) using the following solvent systems: solvent system I, (A) 100 mM triethylammonium acetate (TEAA), pH 7:(B) CH_3CN; solvent system II, 10 mM PBS buffer, pH 7.4:(B) CH_3CN. The details of the solvent system conditions used for the separation of each product are given below. To obtain the nucleotides as sodium salts, they were passed, following final purification, through a column of Sephadex-CM C25 (Na^+-form). The purity of the nucleotides was generally ≥95%. ATP-γ-S, **3**, tetralitium salt (≥90% pure) was obtained from Enzo Life Sciences (Lausen, Switzerland). ADP, GDP, ATP, and AP_3A sodium salts were purchased from Sigma (Steinheim, Germany). Nucleotide **5** was synthesized according to literature.[47] AMP·H_2O (free acid), GDP (sodium salt), sodium thiophosphate tribasic hydrate were purchased from Sigma-Aldrich Co. GMP (disodium salt) was purchased from Acros Organics.

Adenosine 5′-[Beta-thio]diphosphate, 1. CDI (0.83 g, 5 mmol, 5 equiv) was added to AMP (Bu_3NH^+, $Octyl_3NH^+$) salt (1 mmol, 1 equiv) and anhydrous DMF (10 mL) were stirred in a two-necked flask to form a colorless solution. The reaction was stirred at room temperature overnight. Next, dry MeOH (0.22 mL, 5 mmol, 5 equiv) was added to the reaction flask, and the solution was stirred for 8 min. Subsequently, $[PSO_3H]^{2-}(Bu_3NH^+)_2$ salt (2.91 g, 6 mmol, 6 equiv) dissolved in anhydrous DMF (7 mL) and anhydrous $ZnCl_2$ (1.05 g, 7.7 mmol, 7.7 equiv) were added to form a colorless solution. After 3 h, the reaction was quenched by addition of EDTA solution (3.16 g, 8.5 mmol in 127.5 mL deionized water). The neutral solution was then freeze-dried. The resulting white solid was separated on a Sephadex DEAE-A25 column applying a buffer gradient of water (1000 mL) to 0.4 M NH_4HCO_3 (1000 mL). The relevant fraction was freeze-dried at least 4 times to yield a yellowish solid. The LC separation was repeated applying a buffer gradient of water (800 mL) to 0.3 M NH_4HCO_3 (800 mL). The solution was freeze-dried at least 4 times to yield a yellowish solid. Product **1** was obtained in 34% yield (169.3 mg) after LC separation as a yellowish solid. The final purification was achieved by HPLC applying an isocratic elution of 0.1 M TEAA (pH 7)/CH_3CN, 96:04, in 10 min (flow rate 5 mL/min). Retention time of **1** was 7.6 min. The spectral data are consistent with literature.[48] ESI-MS (negative) m/z: calcd for $C_{10}H_{14}N_5O_9P_2S^-$: 442.26 found: 442(M^-).

Guanosine 5′-[Beta-thio]diphosphate, 2. CDI (0.461 g, 2.75 mmol, 5 equiv) was added to GMP (Bu_3NH^+, $Octyl_3NH^+$) salt (0.55 mmol, 1 equiv) and anhydrous DMF (5 mL) were stirred in a two-necked flask to form a colorless solution. The reaction was stirred at room temperature overnight. Next, dry MeOH (0.12 mL, 2.75 mmol, 5 equiv) was added to the reaction flask, and the solution was stirred for 8 min. Subsequently, $[PSO_3H]^{2-}(Bu_3NH^+)_2$ salt (3.3 mmol, 6equiv) dissolved in anhydrous DMF (6.5 mL) and anhydrous $ZnCl_2$ (0.6 g, 4.4 mmol, 8 equiv) were added to form a colorless solution. After 3 h, the reaction was quenched by addition of EDTA solution (1.73 g, 4.4 mmol in 66 mL of deionized water). The neutral solution was then freeze-dried. The resulting white solid was separated on a Sephadex DEAE-A25 column applying a buffer gradient of water (1000 mL) to 0.4 M NH_4HCO_3 (1000 mL). The relevant fraction was freeze-dried at least 4 times to yield a yellowish solid. The LC separation was repeated applying a buffer gradient of water (600 mL) to 0.3 M NH_4HCO_3 (600 mL). The solution was freeze-dried at least 4 times to yield a yellowish solid. Product **2** was obtained in 55% yield (152.7 mg) after LC separation as a yellowish solid. The final purification was achieved by HPLC applying an isocratic elution of 0.1 M TEAA (pH 7)/CH_3CN, 96:04, in 10 min (flow rate 5 mL/min). Retention time of **2** was 6.9 min. The spectral data are consistent with literature.[48]

P^2-Thio P^1, P^3-Di(adenosine 5′-)triphosphate, 6. A mixture of $PSCl_3$ (26 μL, 0.24 mmol, 1.2 equiv) and pyridine (0.57 mL) was stirred in a two-necked flask for 30 min at 0 °C, while AMP (Bu_3NH^+, $Octyl_3NH^+$) salt (0.2 mmol, 1 equiv) was dissolved in dry pyridine (0.68 mL) for 25 min at 0 °C. Subsequently, the pyridine solution of AMP (Bu_3NH^+, $Octyl_3NH^+$) salt was added to the reaction flask and a turbid solution was obtained. The AMP salt was washed with another portion of dry pyridine (0.5 mL), and the solution was added to the reaction flask. Stirring continued for 5 min, then 1 M TEAB (4 mL, pH = 7.5) was added resulting in a clear solution. After 1 h the reaction pH was 8−9. The resulting solution was freeze-dried, to give a white solid residue which was separated on a Sephadex DEAE-A25 column applying a buffer gradient of water (200 mL) to 0.2 M NH_4HCO_3 (200 mL) and then from 0.2 M NH_4HCO_3 (200 mL) to 0.4 M NH_4HCO_3 (200 mL). The solution was freeze-dried for at least 4 freeze-drying cycles to yield a white solid. Product **6** was obtained in 24% yield (19.6 mg) after LC separation as a white solid. ^{1}H NMR (700 MHz, D_2O) δ: 8.33/8.32 (2s, $H8_A/H8_B$), 8.11/8.10 (2s, $H2_A/H2_B$), 6.01/6.01 (2d, J = 4.9 Hz, $H1'_A/H1'_B$), 4.65/4.62 (2t, J = 4.9 Hz, $H2'_A/H2'_B$), 4.56/4.50 (2t, J = 4.6 Hz, $H3'_A/H3'_B$), 4.39/4.32 (2m, $H4'_A/H4'_B$), 4.35/4.27 (4m, $H5''_A$, $H5''_B$, $H5'_A$, $H5'_B$) ppm. ^{31}P NMR (243 MHz, D_2O) δ: 31.04 (t, J = 26.2 Hz, Pβ), −11.55/−11.61 (2d, J = 26.2 Hz $P\alpha_A/P\alpha_B$), ppm. ^{13}C NMR (176 MHz, D_2O) δ: 155.09 ($C6_A/C6_B$), 152.73 ($C2_A/C2_B$), 148.42/148.38 ($C4_A/C4_B$), 139.32/139.28 ($C8_A/C8_B$), 118.02/118.00 ($C5_A/C5_B$), 87.29/87.19 ($C1'_A/C1'_B$), 83.37/83.26 (d, J_{CP} = 9.2 Hz, $C4'_A/C4'_B$), 74.99/74.94 ($C2'_A/C2'_B$), 69.97/69.85 ($C3'_A/C3'_B$), 64.87/64.72 (d, J_{CP} = 5.1 Hz, $C5'_A/C5'_B$) ppm. HRMS (MALDI) m/z calcd for $C_{20}H_{26}N_{10}O_{15}P_3S_1^-$: 771.051, found: 771.051. Purity data obtained on an analytical column-retention time: 5.83 min (97% purity) using solvent system I (isocratic elution of 94:6 A:B over 12 min at a flow rate of 1 mL/min). Retention time: 6.59 min (96% purity) using solvent system II (isocratic elution of 98:2 A:B over 12 min at a flow rate of 1 mL/min).

Typical Procedure for the Preparation of Nucleoside 5′-Thiophosphate Derivatives. AMP (Bu_3NH^+, $Octyl_3NH^+$) salt was prepared from the corresponding free acid, Bu_3N (1 equiv) and $Octyl_3N$ (1 equiv) in EtOH. GMP (Bu_3NH^+, $Octyl_3NH^+$) salt was prepared from its corresponding sodium salt. The nucleotide sodium salt was passed through a column of activated Dowex 50WX-8 200 mesh, H^+ form. The column eluate was collected in an ice-cooled flask containing tributylamine (1 equiv), trioctylamine (1 equiv), and EtOH. The resulting solution was freeze-dried to yield the salt as white solid. GMP (Bu_3NH^+, $Octyl_3NH^+$) salt was dried by repeated coevaporation (three times) with EtOH, followed by coevaporation with anhydrous DMF (three times).

Preparation of $[PSO_3H]^{2-}(Bu_3NH^+)_2$ Salt. $[PSO_3H]^{2-}(Bu_3NH^+)_2$ salt was prepared from the corresponding sodium salt (tribasic hydrate). The thiophosphate sodium salt was passed through a column of activated Dowex 50WX-8 200 mesh, H^+ form. The column eluate was collected in an ice-cooled flask containing tributylamine (1 equiv) and EtOH. The resulting solution was freeze-dried to yield the salt as a colorless oil. The oil was dried by repeated coevaporation with absolute EtOH (three times), followed by

dx.doi.org/10.1021/ic400878k | Inorg. Chem. 2013, 52, 10886−10896

coevaporation with anhydrous DMF (three times) to form a colorless oil. $[PSO_3H]^{2-}(Bu_3NH^+)_2$ salt was stored in a desiccator at −20 °C.

NMR Experiments. Compounds were characterized by nuclear magnetic resonance (^{1}H NMR and ^{31}P NMR) on Bruker AC-200 (200 and 81 MHz for ^{1}H and ^{31}P, respectively), Bruker DPX-300 (300 MHz for ^{1}H), Avance III-500 (500 and 202.5 MHz for ^{1}H and ^{31}P, respectively), DMX-600 (600 and 243 MHz for ^{1}H and ^{31}P, respectively), or Avance III-700 (700 and 176 MHz for ^{1}H, and ^{13}C, respectively) spectrometers. ^{1}H NMR spectra were measured in D_2O, and the chemical shifts are reported in parts per million (ppm) relative to DSS and D_2O (4.8 ppm) as an internal standard. Nucleotides were characterized also by ^{31}P NMR in D_2O, with 85% H_3PO_4 as an external reference. The pD values of the NMR samples were adjusted with diluted NaOD/DCl D_2O solutions. Measurements of pD were performed with a Knick (Berlin, Germany) pH meter equipped with a Hamilton (Bonaduz, Switzerland) biotrode 238140 or tiptrode 238080 electrode.

Preparation of NMR Samples. The samples were prepared by dissolving the nucleotide sodium salts in 99.9% D_2O, and the pD was adjusted to a physiological pH (pD = 7.4 ± 0.2, pD = pH + 0.4). NMR spectra were measured for 6 mM nucleotide or 3 mM dinucleotide samples at 600 or 500 MHz (^{1}H NMR), and 243 or 202.5 MHz (^{31}P NMR) at 300 ± 0.5 K using DSS as an internal reference. The sample concentration was less than 20 mM but more than 2 mM to avoid intermolecular base stacking, but to obtain spectra with sufficient intensity.[4,31,49]

NMR-Monitored Zn^{2+} Titrations of Nucleotides. Nucleoside 5′-phosphorothioate analogues **1**−**3**, **6** and their corresponding parent nucleotides (ADP, GDP, ATP, and AP_3A) in D_2O were titrated with 0.1, 0.2, 0.5, 1, 2, and 10 equiv of 2 M $ZnNO_3$ D_2O solution and measured by ^{31}P NMR spectroscopy at 202.5 or 243 MHz, and ^{1}H NMR spectroscopy at 500 or 600 MHz. The pD of the samples was adjusted to approximately 7.4 with NaOD and DCl D_2O solutions. Conformational analysis (including NOESY studies) of analogue **5**, Na^+ salt, and its Zn^{2+} complex was performed at 700 MHz in D_2O (pD = 7.4), using a 10 mM solution of analogue **5**, with or without $Zn(NO_3)_2$.

Potentiometric Titrations. Materials. The titrated nucleotides were mostly ≥95% pure. ATP-γ-S, **3**, tetralitium salt, was ≥90% pure. Five M NaOH, 2 M HNO_3, and potassium biphthalate were purchased from Merck (Darmstadt, Germany). $NaNO_3$ salt (background electrolyte) and $Zn(NO_3)_2$ standard solution were purchased from Sigma (Steinheim, Germany). All solutions for the titrations were prepared with deionized water. The concentration of the titer of NaOH was determined with potassium biphthalate, which was kept in an oven at 130 °C and cooled to room temperature under a vacuum before use for NaOH calibration. The concentration of the nucleotide stock solutions was determined by titration with NaOH.

Potentiometric pH Titrations. The pH titrations were performed with a Metrohm 794 basic Titrino potentiometer and a Metrohm Viscotrode glass electrode. The buffers (pH 4.00 and 7.00, and 9.00)[2] used for calibration were purchased from Metrohm (Herisau, Switzerland). All titrations were performed at 24 °C under argon atmosphere.

Determination of the acidity constants, K^H_{HL}, K^H_{LH2}, and K^H_{LH3} (L -ligand), was made by the titration of 4.5 mL of aqueous 4 mL of HNO_3 in $NaNO_3$ (from a 3 mM $HNO_3/NaNO_3$ stock solution, I = 0.1 M) and 0.5 mL of ligand (from a 4.5 mM nucleotide stock solution, I = 0.1 M). Acidity constants were calculated with the HYPERQUAD software.[50] Determination of the stability constants, K^{Zn}_{ZnL}, K^{Zn}_{ZnH}, and $pK_a{}^{H}_{ZnLH}$, was achieved by the titration of 3−3.5 mL of aqueous HNO_3 in $NaNO_3$ (from a 3 mM HNO_3 stock solution, I = 0.1 M), 0−0.5 mL $NaNO_3$ (I = 0.1 M), 0.5 mL of ligand (from a 4.5 mM nucleotide stock solution, I = 0.1 M), and 0.5 mL of $Zn(NO_3)_2$ (from a 4.5 mM stock solution, I = 0.1 M). 1:1 Zn^{2+}:ligand ratio was used. The pH range of all titrations was about 2.6−10.3. The end points of nucleotide-complex titrations were obtained by the second derivative method.[51] Each titration was repeated up to 4 times. Stability constants were calculated with the HYPERQUAD software. Speciation and titration simulations were achieved with the HYSS2009 software.[45]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Further details are given in Tables S1−S3 and Figures S1−S2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: bilha.fischer@biu.ac.il. Fax: 972-3-6354907. Phone: 972-3-5318303.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS:

A - adenine; G - guanine; N - Nucleoside; NTP - nucleoside 5′-triphosphate; NDP - nucleoside 5′-diphosphate; M - metal ion; L - ligand; H - proton; ML - complex of metal and ligand; AMP - adenosine 5′-monophosphate

■ REFERENCES

(1) Bianchi, E. M.; Sajadi, S. A. A.; Song, B.; Sigel, H. *Chem.—Eur. J.* **2003**, *9*, 881−892.
(2) Sajadi, S. A. A.; Song, B.; Gregan, F.; Sigel, H. *Inorg. Chem.* **1999**, *38*, 439−448.
(3) Sigel, H.; Griesser, R. *Chem. Soc. Rev.* **2005**, *34*, 875−900.
(4) Sigel, H.; Tribolet, R.; Malinibalakrishnan, R.; Martin, R. B. *Inorg. Chem.* **1987**, *26*, 2149−2157.
(5) Aoki, K.; Murayama, K. *Met. Ions Life Sci.* **2012**, *10*, 43−102.
(6) Orioli, P.; Cini, R.; Donati, D.; Mangani, S. *J. Am. Chem. Soc.* **1981**, *103*, 4446−4452.
(7) Pearson, R. G. *Chemical hardness: Applications from Molecules to Solids*, 1st ed.; John Wiley & Sons: Weinheim, Germany, 1997.
(8) Harding, M. M. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2006**, *62*, 678−682.
(9) Berthon, G., Ed.; *Handbook of Metal-Ligand Interactions in Biological Fluids: Bioinorganic Medicine*; Marcel Dekker: New York, 1995; Vol *1*.
(10) Liebeskind, L. S.; Srogl, J.; Savarin, C.; Polanco, C. *Pure Appl. Chem.* **2002**, *74*, 115−122.
(11) Krezel, A.; Lesniak, W.; Jezowska-Bojczuk, M.; Mlynarz, P.; Brasun, J.; Kozlowski, H.; Bal, W. *J. Inorg. Biochem.* **2001**, *84*, 77−88.
(12) Brasun, J.; Matera, A.; Sochacka, E.; Swiatek-Kozlowska, J.; Kozlowski, H.; Operschall, B. P.; Sigel, H. *J. Biol. Inorg. Chem.* **2008**, *13*, 663−674.
(13) Jaffe, E. K.; Cohn, M. *Biochemistry* **1978**, *17*, 652−657.
(14) Pecoraro, V. L.; Hermes, J. D.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5262−5271.
(15) Sigel, R. K. O.; Song, B.; Sigel, H. *J. Am. Chem. Soc.* **1997**, *119*, 744−755.
(16) Andersson, M.; Hedin, J.; Johansson, P.; Nordstrom, J.; Nyden, M. *J. Phys. Chem. A* **2010**, *114*, 13146−13153.
(17) Sundberg, R. J.; Martin, R. B. *Chem. Rev.* **1974**, *74*, 471−517.
(18) Stern, N.; Major, D. T.; Gottlieb, H. E.; Weizman, D.; Fischer, B. *Org. Biomol. Chem.* **2010**, *8*, 4637−4652.
(19) Sigel, H.; Massoud, S. S.; Corfu, N. A. *J. Am. Chem. Soc.* **1994**, *116*, 2958−2971.
(20) Cohn, M.; Hughes, T. R., Jr. *J. Biol. Chem.* **1962**, *237*, 176−181.
(21) Cowan, J. A. *Inorg. Chem.* **1991**, *30*, 2740−2747.
(22) Huang, S. L.; Tsai, M. D. *Biochemistry* **1982**, *21*, 951−959.
(23) Jaffe, E. K.; Cohn, M. *J. Biol. Chem.* **1979**, *254*, 10839−10845.
(24) Jaffe, E. K.; Cohn, M. *J. Biol. Chem.* **1978**, *253*, 4823−4825.
(25) Son, T. D.; Roux, M.; Ellenberger, M. *Nucleic Acids Res.* **1975**, *2*, 1101−1110.

(26) Tran-Dinh, S.; Neumann, J. M. *Nucleic Acids Res.* **1977**, *4*, 397−403.
(27) Bock, J. L. *J. Inorg. Biochem.* **1980**, *12*, 119−130.
(28) Sigel, H. *Inorg. Chim. Acta* **1992**, *198*, 1−11.
(29) Sigel, H. *Pure Appl. Chem.* **1998**, *70*, 969−976.
(30) Scheller, K. H.; Hofstetter, F.; Mitchell, P. R.; Prijs, B.; Sigel, H. *J. Am. Chem. Soc.* **1981**, *103*, 247−260.
(31) Scheller, K. H.; Sigel, H. *J. Am. Chem. Soc.* **1983**, *105*, 5891−5900.
(32) Sigel, H.; Massoud, S. S.; Tribolet, R. *J. Am. Chem. Soc.* **1988**, *110*, 6857−6865.
(33) Martin, R. B. *Metal Ions Biol. Syst.* **1996**, *32*, 61−89.
(34) Sigel, H.; Corf, N. A.; Ji, L.-N.; Martin, R. B. *Comments Inorg. Chem.* **1992**, *13*, 35−59.
(35) Kapinos, L. E.; Holy, A.; Gunter, J.; Sigel, H. *Inorg. Chem.* **2001**, *40*, 2500−2508.
(36) Sigel, H.; Song, B. *Metal Ions Biol. Syst.* **1996**, *32*, 135−205.
(37) Sigel, H.; Bianchi, E. M.; Corfu, N. A.; Kinjo, Y.; Tribolet, R.; Martin, R. B. *Chem.—Eur. J.* **2001**, *7*, 3729−3737.
(38) Bianchi, E. M.; Griesser, R.; Sigel, H. *Helv. Chim. Acta* **2005**, *88*, 406−425.
(39) Stern, N.; Major, D. T.; Gottlieb, H. E.; Weizman, D.; Sayer, A. H.; Blum, E.; Fischer, B. *J. Biol. Inorg. Chem.* **2012**, *17*, 861−879.
(40) Rosch, P.; Kalbitzer, H. R.; Goody, R. S. *FEBS Lett.* **1980**, *121*, 211−214.
(41) Da Costa, C. P.; Krajewska, D.; Okruszek, A.; Stec, W. J.; Sigel, H. *J. Biol. Inorg. Chem.* **2002**, *7*, 405−415.
(42) Sutter, E. m. m.; Ammeloot, F.; Pouet, M. J.; Fiaud, C.; Couffignal, R. *Corros. Sci.* **1999**, *41*, 105−115.
(43) Cho, B. P.; Evans, F. E. *Nucleic Acids Res.* **1991**, *19*, 1041−1047.
(44) Sigel, R. K. O.; Sigel, H. *Met. Ions Life Sci.* **2007**, *2*, 109−180.
(45) Alderighi, L.; Gans, P.; Ienco, A.; Peters, D.; Sabatini, A.; Vacca, A. *Coord. Chem. Rev.* **1999**, *184*, 311−318.
(46) Song, B.; Sigel, R. K. O.; Sigel, H. *Chem.—Eur. J.* **1997**, *3*, 29−33.
(47) Maurizi, M. R.; Ginsburg, A. *Biochemistry* **1986**, *25*, 131−140.
(48) Kowalska, J.; Lewdorowicz, M.; Darzynkiewicz, E.; Jemielity, J. *Tetrahedron Lett.* **2007**, *48*, 5475−5479.
(49) Mayo, K. H.; Mvele, O. M.; Puri, R. N. *FEBS Lett.* **1990**, *265*, 97−100.
(50) Gans, P.; Sabatini, A.; Vacca, A. *Talanta* **1996**, *43*, 1739−1753.
(51) Vogel, A. I.; Jeffery, G. H. *Vogel's textbook of quantitative chemical analysis*, 5th ed.; Longman Scientific & Technical: Essex, U.K., 1989.