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Adenosine/Guanosine-3',5'-Bis-Phosphate as Biocompatible and Selective Zn^{2+}-Ion Chelators. Characterization and Comparison with Adenosine/Guanosine-5'-Di-Phosphate

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Abbreviations: A - adenine, G - guanine, N – Nucleoside, NTP - nucleoside 5'-triphosphate, NDP - nucleoside 5'-diphosphate, AMP – adenosine 5'-monophosphate, GMP – guanosine 5'-monophosphate, ADP – adenosine 5'-diphosphate, GMP – guanosine 5'-diphosphate, ATP – adenosine 5'-triphosphate, GTP – guanosine 5'-triphosphate, dA-3',5'-PO - 2'-deoxy-adenosine 3',5'-bisphosphate, dG-3'5'-PO - 2'-deoxy-guanosine 3',5'-bisphosphate, d(pNp) – 2'-deoxy-nucleoside 3',5'-bisphosphate, M – metal ion, L – ligand, H - proton, ML – complex of metal and ligand, MD – molecular dynamics.

Key Words: nucleoside 5'-diphosphate, nucleoside 3',5'-bisphosphate, NMR, potentiometric pH-titrations, molecular dynamics, conformational analysis, Zn^{2+}, Mg^{2+}.

Abstract

Although involved in various physiological functions, nucleoside bis-phosphate analogues and their metal-ion complexes have been scarcely studied. Hence, here, we explored the solution conformation of 2'-deoxyadenosine- and 2'-deoxyguanosine-3',5'-bisphosphate, **3** and **4**, d(pNp), as well as their Zn^{2+}/Mg^{2+} binding sites and binding-mode (i.e. inner- vs. outer-sphere coordination), acidity constants, stability constants of their Zn^{2+}/Mg^{2+} complexes, and their species distribution. Analogues **3** and **4**, in solution, adopted a predominant *Southern* ribose conformer (ca. 84%), *gg* conformation around C4'-C5' and C5'-O5' bonds, and glycosidic angle in the *anti*-region (213-270°). ^{1}H- and ^{31}P- NMR experiments indicated that Zn^{2+}/Mg^{2+} ions coordinated to P5' and P3' groups of **3** and **4** but not to N7 nitrogen atom. Analogues **3** and **4** formed ca. 100-fold more stable complexes with Zn^{2+} vs. Mg^{2+}-ions. Complexes of **3** and **4** with Mg^{2+} at physiological pH were formed in minute amounts (11% and 8%, respectively) vs. Zn^{2+} complexes (46% and 44%). Stability constants of Zn^{2+}/Mg^{2+} complexes of analogues **3** and **4** (log K^{M}_{ML} = 4.65-4.75/2.63-2.79, respectively) were similar to those of the corresponding complexes of ADP and GDP (log K^{M}_{ML} = 4.72-5.10/2.95-3.16, respectively). Based on the above findings, we hypothesized that the unexpectedly low log*K* values of Zn^{2+}-d(pNp) as compared to Zn^{2+}-NDP complexes, are possibly due to formation of outer-sphere coordination in Zn^{2+}-d(pNp) complex vs. inner-sphere in NDP-Zn^{2+} complex, in addition to loss of chelation to N7 nitrogen atom in Zn^{2+}-d(pNp). Indeed, explicit solvent molecular dynamics simulations of **1** and **3** for 100 ns supported this hypothesis.

Introduction

Nucleoside 5'-diphosphate analogues e.g. adenosine-, and guanosine-5'-diphosphate, ADP, **1**, and GDP, **2**, play numerous biochemical roles.[1-5] Part of the activities of nucleoside-5'-diphosphates involve metal-ion coordination. Hence, these analogues have been studied as metal-ion chelators.[6-8] The effect of the 2'-deoxyribo- vs. ribo-nucleotides on the divalent metal ion (Zn^{2+}, Mg^{2+}, Ni^{2+}, Cu^{2+}) binding properties of the purine nucleotide analogues (dA/GMP, dA/GDP, dA/GTP) has also been described before, showing insignificant enhancement of the stability constants of 2'-deoxyribo-nucleotides complexes vs. the corresponding ribo-nucleotide complexes ($\Delta_{\log K(ML)} \leq 0.2$).[9] Related nucleotides, namely, nucleoside 3',5'-bisphosphate analogues, d(pNp), **3** and **4**, also play physiological[10, 11] and pharmacological roles.[12-14] Yet, their potential as chelators of divalent metal ions has not been evaluated so far.

Previously, we found that nucleotide-Zn^{2+}-complexes, e.g., ATP-γ-S, **5**-Zn^{2+}/ATP-α,β-CH_2-γ-S, **6**-Zn^{2+}, were more stable by 2-3 orders of magnitude than the corresponding dinucleotide-Zn^{2+}-complexes, e.g. Ap_3(β-S)A, **7**-Zn^{2+} (Log*K* = 5.74/6.5 vs. 3.8, respectively).[15] Subsequently, we reported that an additional terminal phosphate group on the AMP/GMP scaffold, as in 2'-deoxy-adenosine/guanosine-3',5'-bisphosphate, resulted in 1.8- and 2.8-fold increased Fe^{2+} chelation as compared to adenosine/guanosine-5'-monophosphate, respectively.[16] Hence, we hypothesized that enhanced Fe^{2+} or Zn^{2+} chelation is due to the presence of two terminal phosphate groups in d(pNp) vs. one terminal phosphate group in NMP analogues[16], or one terminal phosphate in ATP vs. no terminal phosphate in Ap_3A. [15]

Based on these findings we expected that two terminal phosphate groups, as in nucleoside bis-phosphate analogues **3** and **4**, will increase the log*K* values of their M^{2+}

complexes as compared to the corresponding nucleoside 5'-di-phosphate (**1** and **2**) complexes**.**

In addition, we evaluated the selectivity of **3** and **4** to Zn^{2+} vs. Mg^{2+} ions. Hence, we compared binding of Zn^{2+}/Mg^{2+} ions to **3** and **4** vs. ADP and GDP. Specifically, we report on the acidity constants of **3** and **4**, and the stability constants of their Zn^{2+}/Mg^{2+} complexes as determined by potentiometric pH-titrations. Based on these acidity and stability constants, species distribution at physiological pH was predicted. Furthermore, Zn^{2+}/Mg^{2+} coordination sites were identified by ^{1}H-NMR- and ^{31}P-NMR- monitored Zn^{2+}/Mg^{2+} titrations. NMR-based conformational analysis of **3** and **4** and molecular dynamics (MD) simulations allowed the rationalization of the

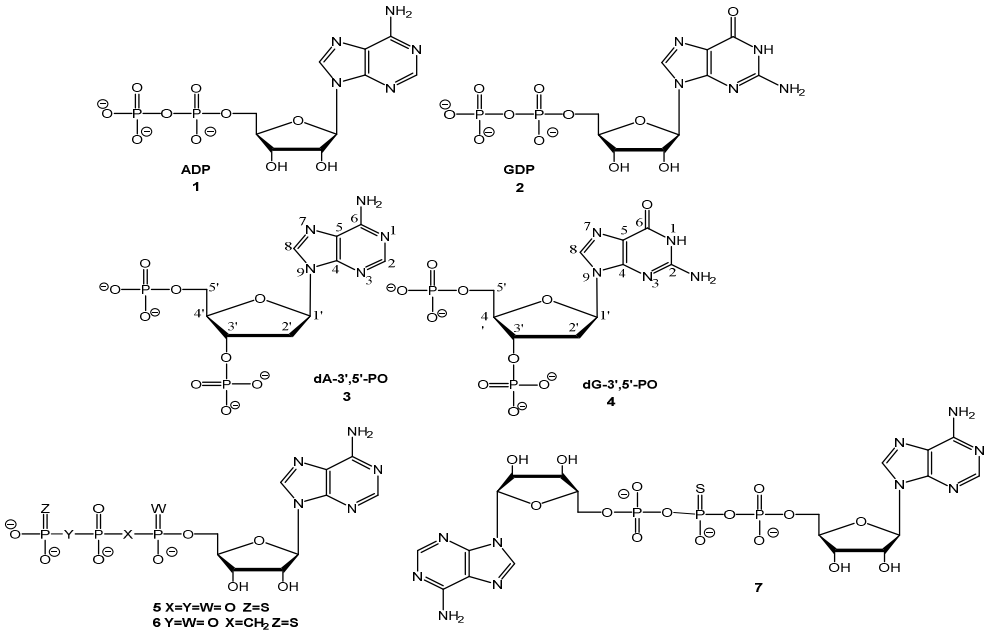


Fig. 1 Nucleotide analogues explored as potential Zn^{2+}-chelators.

binding mode of Zn^{2+}/Mg^{2+} by nucleoside 3',5'-bisphosphate vs. nucleoside-5'-diphosphate analogues.

Results and discussion

Characterization of Zn^{2+}/Mg^{2+} complexes of 2'-deoxynucleoside-3',5'-bisphosphate analogues by potentiometric pH-titrations

Compounds **3** and **4** were prepared as we recently described[16] and were pH-titrated in the absence or presence of Zn^{2+}/Mg^{2+}-ions in order to determine the acid-base equilibria of the nucleotides, as well as the stability of the corresponding Zn^{2+}/Mg^{2+}-complexes. The acidity constants and log*K* values obtained were then used to deduce the species distribution of nucleotides under the titration conditions. Log*K* data helped to establish the selectivity of nucleotides **3** and **4** to Zn^{2+} vs. Mg^{2+} ions.

pK_a values, stability constants, and species distribution of complexes of analogues 3 or 4 with Zn^{2+}/ Mg^{2+}

We performed potentiometric pH-titrations of Na^+ salts of nucleotides **1** or **2** and of the **3** or **4** - Zn^{2+}/Mg^{2+} 1:1 complexes, as compared to ADP and GDP and their Zn^{2+}/Mg^{2+} complexes. Stability constants of Zn^{2+}/Mg^{2+} complexes were determined at a 1:1 M^{2+}:L ratio according to literature.[17, 18]

Acidity constants of analogues 3, 4 as compared to ADP and GDP

In the pH titration range of 2.7-10 we expect the following protonation equilibria to occur for 2'-deoxynucleoside-3',5'-bisphosphates (d(pNp)'s):

(1a) $H_3[d(\mathrm{pNp})]^- \rightleftarrows H_2[d(\mathrm{pNp})]^{2-} + H^+$

(1b) $K^H_{H_3[d(\mathrm{pNp})]} = [H_2[d(\mathrm{pNp})]^{2-}][H^+]/[H_3[d(\mathrm{pNp})]^-]$

(2a) $H_2[d(\mathrm{pNp})]^{2-} \rightleftarrows H[d(\mathrm{pNp})]^{3-} + H^+$

(2b) $K^H_{H_2[d(\mathrm{pNp})]} = [H[d(\mathrm{pNp})]^{3-}][H^+]/[H_2[d(\mathrm{pNp})]^{2-}]$

(3a) $H[d(\text{pNp})]^{3-} \rightleftarrows \text{d(pNp)}^{4-} + H^+$

(3b) $K^{H}_{H[d(\text{pNp})]} = [d(\text{pNp})^{4-}][H^+]/[H[d(\text{pNp})]^{3-}]$

(4a) $\text{d(pNp)}^{4-} \rightleftarrows d(\text{pNp}-H)^{5-} + H^+$

(4b) $K^{H}_{\text{d(pNp)}} = [(d(\text{pNp}-H)^{5-}][H^+]/[d(\text{pNp})^{4-}]$

H_3(2'-dA-3',5'-bisphosphate)$^-$, **3**, and H_3(2'-dG-3',5'-bisphosphate)$^-$, **4**, release their first proton (Eq. 1a-b) from the N1(H^+) and (N7)H^+ sites[19], respectively, and the second and the third one (Eq. 2a-b & 3a-b) from the $-P(O)_2(OH)$ groups. For analogue **4**, the fourth deprotonation (Eq. 4a-b) occurs at the N1H position of the nucleobase.[20] The acidity constants for equilibria (1a) through (4b) and the deprotonation sites of analogues **3** and **4**, as well as ADP and GDP, are listed in Table 1.[6, 7]

Acidity constants of nucleoside 3',5'-bisphosphate analogues were compared to those of ADP and GDP to evaluate the effect of two terminal phosphate groups vs. one terminal and one bridging phosphate groups. The pK_a values of ADP and GDP, which we determined, were consistent with the literature.[6, 7]

There were no significant changes in N1(H^+)/N7(H^+)-pK_a values of **3** or **4** vs. the corresponding values of ADP and GDP (4.21, 3.10 vs. 4.07, 3.23, respectively). The resemblance between the pK_a values of N1(H^+) in analogue **3**, and ADP is expected since the proton on the remote purine N1 site (in the *anti*-region) has no interaction with any phosphate group in **3** which could affect the pK_a value. Moreover, the pK_a values of both 3'- and 5'- phosphate groups in analogues **3** and **4** are very similar (6.01, 7.06 vs. 5.99, 7.04, respectively). These results indicate that both 3',5'-phosphate groups and the bases (A/G) do not affect acid-base equilibria of each other.

The first pK_a value of the terminal phosphate groups in **3** and **4** (6.01 and 5.99, respectively) is more acidic than that of P_β of NDP's (ca. 6.40). The reason for this

phenomenon could be the "basicity-enhancing effect" of a second phosphate group (P_β) in NDP's.[7] Specifically, one of the two primary protons of the β-phosphate group is first released (pK_a<1). This effect suggests that the next proton released from H_3(ADP) (pK_a≈1.02) is largely located at the α-phosphate group. Therefore, the last proton released from the β-phosphate group is more basic than the proton released from **3** or **4** (pK_a 6.40 vs. 6.01/5.99, respectively) due to charge repulsion between adjacent negatively charged phosphate groups. Yet, the pK_a value of the second terminal phosphate in **3** and **4** (7.06 and 7.04) is more basic than P_β of NDP's (ca. 6.40), probably due to a larger charge repulsion between the phosphate groups in **3** or **4** bearing three negative charges vs. two negative charges in NDP. Furthermore, the enhanced acidity of the first phosphate group of nucleotide **3** may be due to enhanced stabilization of the 3'/5'- phosphate anion by intra-molecular H-bonding with the other (5'/3') protonated phosphate group (Fig. 2). In addition, analogue **3** undergoes a third deprotonation of the second phosphate moiety at pK_a 7.04. The latter is higher than that of dAMP (pK_a 6.21),[21] possibly due to hydrogen bonds between the phosphate groups through a bridging H_2O molecule (Fig. 2).[22, 23] The relatively large $\Delta pK_{a2,3}$, (at pH range 2.7-10) of *ca.* one log unit, between the two phosphate moieties of analogue **3,** is possibly due to repulsion between the negative charges of the phosphate groups, which occurs upon the ionization of the second phosphate group.

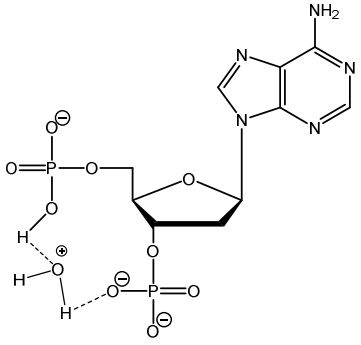
Table 1. pK_a values of d(pNp)s, **3, 4,** compared to ADP and GDP as determined by potentiometric pH-titrations in aqueous solution at 24° C and I = 0.1 M (background electrolyte - $NaNO_3$)

Acid	$pK_a^{H}{}_{LH_4}$	$pK_a^{H}{}_{LH_3}$	$pK_a^{H}{}_{LH_2}$	$pK_a^{H}{}_{LH}$
$\mathbf{H_2(ADP)^{-}}$ [7]	--------	--------	3.92±0.02(N1)	6.40±0.01(Pβ)
$\mathbf{H_2(ADP)^{-}}$	--------	--------	4.07±0.01(N1)	6.40±0.01(Pβ)
$\mathbf{H_3(GDP)^{-}}$ [6]	--------	2.67±0.02(N7)	6.38±0.01(Pβ)	9.56±0.03(N1)
$\mathbf{H_3(GDP)^{-}}$	--------	3.23±0.03(N7)	6.41±0.01(Pβ)	9.47±0.01(N1)
$\mathbf{H_3(2'\text{-}dA\text{-}3'5'\text{-}PO)^{-}}$	--------	4.21±0.01(N1)	6.01±0.01(P-5'/3')	7.06±0.01(P-5'/3')
$\mathbf{H_3(2'\text{-}dG\text{-}3'5'\text{-}PO)^{-}}$	3.10±0.08(N7)	5.99±0.01(P-5'/3')	7.04±0.01(P-5'/3')	9.50±0.01(N1)

Literature data[6, 7]

Deprotonation sites appear in brackets.

Fig. 2 Hypothetic hydrogen bond in 2′-deoxyadenosine-3′,5′-bisphosphate



The second deprotonation of nucleotide **4** (pK_a 5.99) in the pH range 2.7-10 occurs at the phosphate group (either P3' or P5') with a pK_a value lower than that of GDP (6.41). The third deprotonation occurs at the second phosphate group (pK_a 7.04). The last deprotonation (pK_a 9.50) takes place at the guanine N1-H site and is similar to that in GDP (pK_a 9.47). The reasons for these similarities/differences are as discussed above for the corresponding adenine analogue, **3**.

Stability constants of complexes of nucleotides 3, 4 with Zn^{2+}/Mg^{2+} as compared to complexes of nucleotides 1, 2 with Zn^{2+}/Mg^{2+}

Stability constants of complexes of nucleotides **3, 4** with Zn^{2+}/Mg^{2+} were determined at 1:1 Zn^{2+}/Mg^{2+}:L ratio. The pH range of the titration was 2.7-10. The equations describing the formation of 1:1 d(pNp): M^{2+} (N = A, G, M^{2+} is Zn^{2+} or Mg^{2+}) complex are:

(5a) $M^{2+} + H_2[d(\text{pNp})]^{2-} \overrightarrow{\leftarrow} M(H_2;d(\text{pNp})$

(5b) $K^{M}_{M(H_2;d(\text{pNp}))} = [M(H_2;d(\text{pNp}))]/([M^{2+}][H_2[d(\text{pNp})^{2-}]])$

(6a) $M^{2+} + H[d(\text{pNp})]^{3-} \overrightarrow{\leftarrow} M(H;d(\text{pNp}))^{-}$

(6b) $K^{M}_{M(H[d(\text{pNp})])} = [M(H;d(\text{pNp}))^{-}]/([M^{2+}][H[d(\text{pNp})]^{3-}])$

(7a) $M^{2+} + d(\text{pNp})^{4-} \overrightarrow{\leftarrow} M(d(\text{pNp}))^{2-}$

(7b) $K^{M}_{M(d(\text{pNp}))} = [M(d(\text{pNp}))^{2-}]/([M^{2+}][d(\text{pNp})^{4-}])$

Deprotonation of the complex is represented in equations 8a/9a:

(8a) $M(H;d(\text{pNp}))^{-} \overrightarrow{\leftarrow} M(d(\text{pNp}))^{2-} + H^{+}$

(8b) $K^{H}_{M(H[d(\text{pNp})])} = [H^{+}][M(d(\text{pNp}))^{2-}]/[M(H;d(\text{pNp})^{-}]$

(9a) $M(H_2;d(\text{pNp})) \overrightarrow{\leftarrow} M(H;d(\text{pNp}))^{-} + H^{+}$

(9b) $K^{H}_{M(H_2;d(\text{pNp}))} = [H^{+}][M(H;d(\text{pNp}))^{-}]/[M(H_2;d(\text{pNp}))]$

Calculation of the acidity constants of the complexes:[18]

(10) $pK^{H}_{M(H;d(\text{pNp}))} = pK^{H}_{H[d(\text{pNp})]} + \log K^{M}_{M(H;d(\text{pNp})\cdot} - \log K^{M}_{M(d(\text{pNp}))}$

(11)

$$pK^{H}_{M(H_2;d(\text{pNp}))} = pK^{H}_{H[d(\text{pNp})]} + pK^{H}_{H_2[d(\text{pNp})]} + \log K^{M}_{M(H_2;\text{pdNp})\cdot} - \log K^{M}_{M(\text{pdNp})} - pK^{H}_{M(H;d(pNp))}$$

The stability constants for equilibria (5a) through (11) and the acidity constants of **3, 4, 1**, and **2 -** Zn^{2+} /Mg^{2+} complexes are listed in Tables 2 and 3.

Table 2. Stability constants of d(pNp)s, **3, 4**-Zn^{2+} complex compared to ADP and GDP-Zn^{2+} complexes, together with the negative logarithms of the acidity constants of the corresponding Zn(H;L) and Zn(H_2;L) complexes as determined by potentiometric pH titrations in aqueous solution at 24° C and I = 0.1 M (background electrolyte - $NaNO_3$)**.**

Nucleotide	$\log K^{Zn}_{Zn(L)}$	$\log K^{Zn}_{Zn(H;L)}$	$\log K^{Zn}_{Zn(H;LH)}$	$pK_a{}^{H}_{Zn(H;L)}$	$pK_a{}^{H}_{Zn(H;LH)}$
ADP^{3-} *7*	4.28±0.05	2.31±0.20	----------	4.43±0.21	----------
ADP^{3-}	4.72±0.03	3.19±0.02	----------	4.87±0.02	----------
GDP^{3-} *8*	4.52±0.03	2.60±0.07	----------	4.46±0.08	----------
GDP^{3-}	5.10±0.03	3.45±0.01	----------	4.76±0.03	----------
2'-dA-3',5'-PO^{4-}	4.65±0.09	3.74±0.13	3.13±0.10	6.15±0.13	5.40±0.10
2'-dG-3',5'-PO^{4-}	4.75±0.07	3.91±0.08	3.15±0.05	6.19±0.08	5.24±0.05

Literature data[7, 8]

Stability constants of Zn^{2+} complexes of both nucleoside 2'-deoxy-3',5' bisphosphate analogues, **3** (log K^{M}_{ML} = 4.65), and **4** (log K^{M}_{ML} = 4.75), are slightly lower than those of the corresponding complexes of nucleoside 5'-diphosphates, ADP (log K^{M}_{ML} = 4.72) and GDP (log K^{M}_{ML} = 5.10) (Table 2). This was a rather unexpected finding which contradicted our working hypothesis, namely, that additional terminal phosphate groups on a nucleoside scaffold (i.e. nucleoside-bisphosphate vs. nucleoside-diphosphate) will increase log K^{M}_{ML} value of the corresponding complex with M(II)-ion.

We considered that these unexpected logK values for Zn^{2+} complexes of **3** and **4**, may be due to formation of unstable 10-14-membered rings, Fig. 3A-B, [24] while, in NDP both P_α and P_β are involved in Zn^{2+} coordination,[25] forming a stable six-membered ring (inner-sphere coordination), Fig. 3C.[26, 27]

We based this hypothesis on a study on the formation of lactones (ring sizes from 3 to 23) from ω-bromoalkanoates. A 10^6- and 10^4-fold decreased rate of cyclization was observed for the formation of medium sized rings, 8- and 9-membered rings vs. 5- and 6-membered rings, respectively.[24, 28] However, an increase of ca. 10- and 17- fold of the cyclization rate was reported for formation of 11- and 14- membered ring, respectively, vs. 8- or 9-membered ring.[24]

In the presence of Zn^{2+} the acidity constants of the terminal phosphate groups (P3' and P5') of nucleotides **3** and **4** decreased by 0.6-0.9 log units (6.15, 5.40 vs. 7.06, 6.01 and 6.19, 5.24 vs. 7.04, 5.99, respectively), while for ADP and GDP, the acidity constants of the terminal phosphate (Pβ) decrease by 1.5-1.6 log units (6.40 vs. 4.87, and 6.41 vs. 4.76, respectively). This observation may also support our hypothesis that Zn^{2+} interacts with 3'/5'-phosphate group through a bridging water molecule. Likewise, the stability constants of protonated P3' or P5' group nucleotides, **3** and **4,** with Zn^{2+} ($\log K^{Zn}_{Zn(H;L)}$) decrease by 0.8-0.9 log units as compared to the stability constants of fully ionized analogues **3** or **4** with Zn^{2+} ($\log K$ 3.74 vs. 4.65, and 3.91 vs. 4.75, respectively). However, stability constants of protonated ADP and GDP complexes decrease by 1.5-1.6 log units vs. fully ionized A(G)DP complexes ($\log K$ 3.19 vs. 4.72, and 3.45 vs. 5.10, respectively), which also support the above hypothesis.

Additionally, the stability constants of Zn^{2+}-complexes of P3' and P5' di-protonated nucleotides, **3** and **4**, ($\log K^{Zn}_{Zn(H;LH)}$) decreased only by 0.61 and 0.76 log

units, respectively (log*K* 3.13 and 3.15), as compared to the stability constants of mono-protonated nucleotides, **3** and **4,** with Zn^{2+} ($\log K^{Zn}_{Zn(H;L)}$).

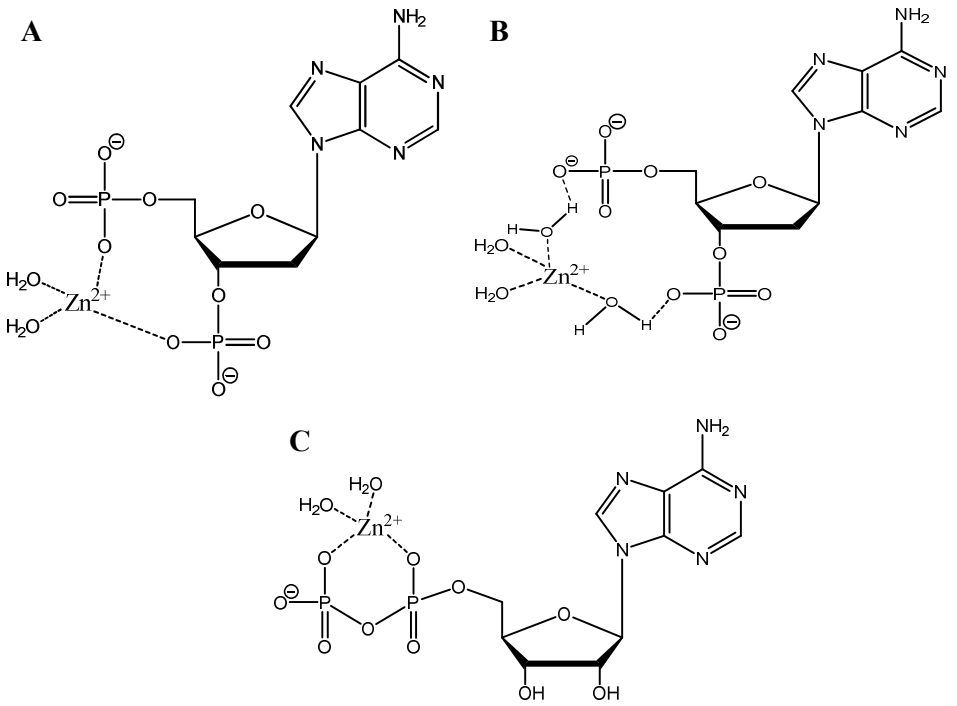


Fig. 3 A. A 10-membered ring may be formed in d(pAp)-Zn^{2+} inner sphere complex. **B.** A 14-membered ring may be formed in d(pAp)-Zn^{2+} outer sphere complex. **C.** A stable 6-membered ring is formed in ADP - Zn^{2+} inner sphere complex.[26, 27]

Table 3 Stability constants of d(pNp)'s, **3, 4**-Mg^{2+} complexes as compared to ADP and GDP- Mg^{2+} complexes**,** where L = ligand (Nucleotide), together with the negative logarithms of the acidity constants of the corresponding Mg(H;L) complexes. These values were determined by potentiometric pH titrations in aqueous solution at 24° C and I = 0.1 M (background electrolyte - $NaNO_3$)

Nucleotide	$\log K^{Mg}_{Mg(L)}$	$\log K^{Mg}_{Mg(H:L)}$	$pKa^{H}_{Mg(H:L)}$
ADP^{3-}[7]	3.36±0.03	1.68±0.1	4.72±0.1
ADP^{3-}	2.95±0.03	----------	----------
GDP^{3-}[8]	3.39±0.04	1.6±0.3	4.59±0.3
GDP^{3-}	3.16±0.06	----------	----------
2'-dA-3',5'-PO^{4-}	2.79±0.09	----------	----------
2'-dG-3',5'-PO^{4-}	2.63±0.07	----------	----------

Literature data[7, 8]

Next, we determined log*K* and acidity constant of Mg^{2+} complexes of analogues **3** and **4**. Log $K^{Mg}_{Mg(H;L)}$ constants could not be determined due to their very low values which could not be detected in our system, where titration started at ca. pH 2.7. Hence, we could also not obtain $pK_a{}^{H}_{Mg(H;L)}$ values since these are determined by Equation (8), using log $K^{Mg}_{Mg(H;L)}$ value.

Nucleotides **3** and **4** showed ca. 100-fold higher affinity to Zn^{2+}-ion vs. Mg^{2+}-ion ($\log K^{M}_{ML}$ = 4.65, 4.75 vs. 2.79, 2.63, respectively) (Tables 2 and 3). Additionally, $\log K^{M}_{ML}$ values of these complexes with Mg^{2+} were lower than those of the corresponding ADP and GDP complexes (up to 0.5 log unit) (Table 3). The log stability constants ($\log K^{M}_{ML}$) of **3** with Zn^{2+} and Mg^{2+} were slightly lower than those of the corresponding ADP complexes (0.07-0.16 log unit), whereas the stability constants of **4** with Zn^{2+} and Mg^{2+} were significantly lower than those of the corresponding GDP complexes (0.35-0.53 log unit), possibly because Zn^{2+} and Mg^{2+}

may form additional outersphere interaction with the guanine O^6 of GDP via a hydrogen bond.[19]

Species distribution of nucleotides 3 or 4 under physiological pH

The titration mixture consists of various equilibrating species: L, LH, LH_2, LH_3, ML, MLH and MLH_2, where M = Mg^{2+}, Zn^{2+} and L= ligand, **3** or **4**. To determine the ligands' population under physiological pH (7.4) (and under the titration conditions), we used the acidity and stability constants of the related species (Tables 1-3) and simulated the population of ligand species with the HYSS program (Fig. 4).[29] In the presence of Zn^{2+} the major species of ligand **3** is ML (46 %, Fig. 4A). Yet, in the presence of Mg^{2+} the major species is free L (59%, Fig. 4C) and ML species is present in only 11%. In addition, the major species of ligand **4** with Zn^{2+} and Mg^{2+} are 44% of ML (Fig. 4B) and 62% of free L (only 8% ML, Fig. 4D), respectively. These results are expected considering the high log K^{M}_{ML} values of **3** or **4**-Zn^{2+} vs. Mg^{2+}-complexes discussed above. To summarize, we found a high percentage of the Zn^{2+}-bound ligands **3** and **4** under physiological pH, in contrast to extremely low percentage of Mg^{2+}-bound **3** or **4**. These results clearly indicate selectivity of nucleotides **3** or **4** to Zn^{2+} vs. Mg^{2+}-ion, with up to 22-fold preference for Zn^{2+} - ion under physiological pH.

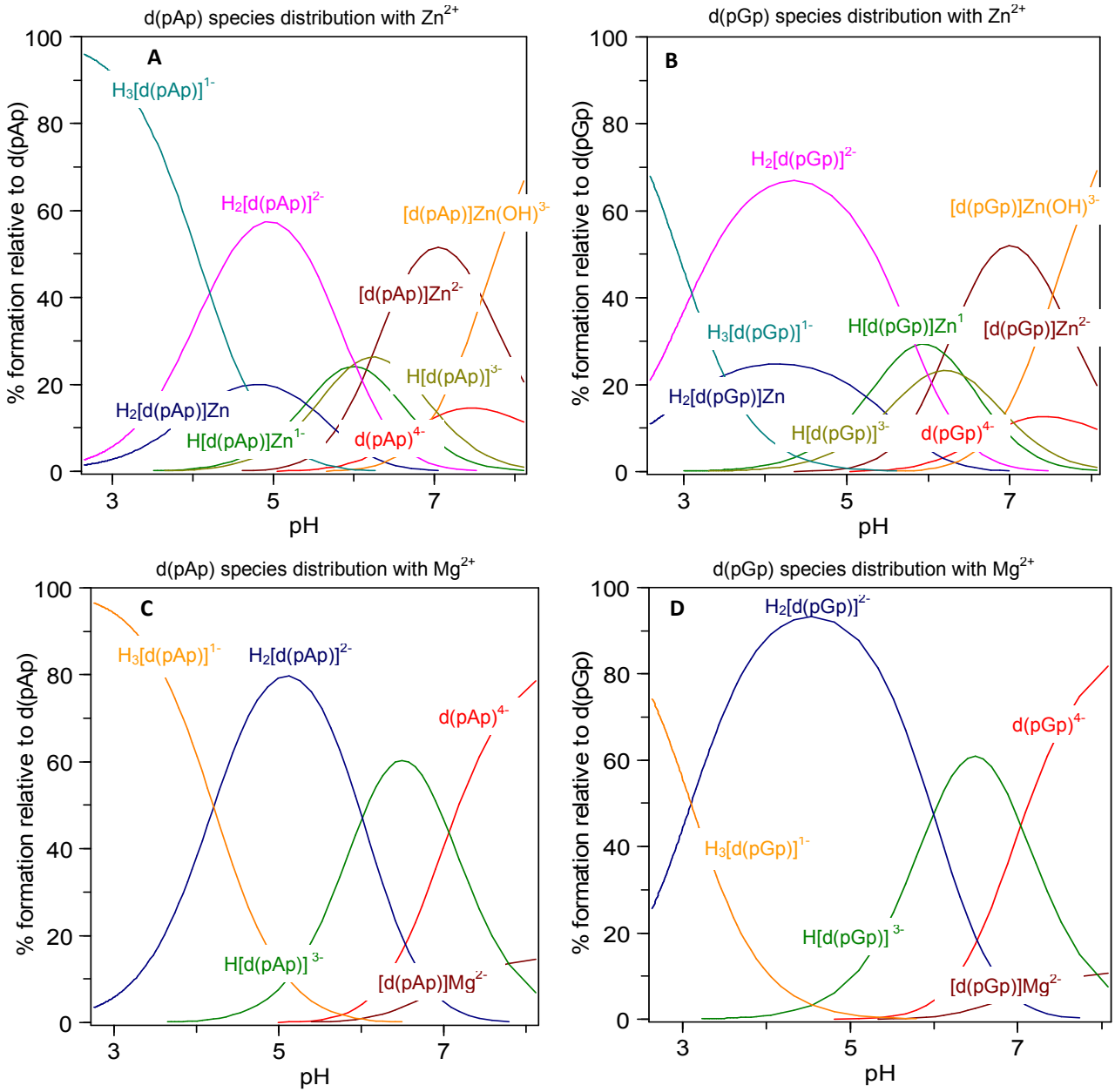


Fig. 4 Simulations of potentiometric titrations obtained with HYSS program of d(pAp)/ d(pGp)-Mg^{2+}/Zn^{2+} 1:1 complex (sodium salt). **A**: pH titration of d(pAp)-Zn^{2+} showing eight species: H_3[d(pAp)], H_2[d(pAp)], H[d(pAp)], free d(pAp), H_2[d(pAp)]-Zn^{2+}, H[d(pAp)]-Zn^{2+}, d(pAp)-Zn^{2+} and d(pAp)-Zn^{2+}(OH). **B**: pH titration of d(pGp)-Zn^{2+} showing eight species: H_3[d(pGp)], H_2[d(pGp)], H[d(pGp)], free d(pGp), H_2[d(pGp)]-Zn^{2+}, H[d(pGp)]-Zn^{2+}, d(pGp)-Zn^{2+} and d(pGp)-Zn^{2+}(OH). **C**: pH titration of d(pAp)-Mg^{2+} showing five species: H_3[d(pAp)], H_2[d(pAp)], H[d(pAp)], free d(pAp) and d(pAp)-Mg^{2+}. **D**: pH titration of d(pGp)-Mg^{2+} showing five species H_3[d(pGp)], H_2[d(pGp)], H[d(pGp)], free d(pGp) and d(pGp)-Mg^{2+}.

Determination of Zn^{2+}/Mg^{2+} binding sites in analogue 3 by $^{1}H/^{31}P$-NMR

Metal-ion coordination sites in nucleotide **3** were elucidated by titrations of nucleotide **3** with Zn^{2+} or Mg^{2+}-ions, which were monitored by ^{1}H and ^{31}P- NMR.

We focused on four sites in the molecule: H2, H8 (on the base), and P3', P5' (on the ribose), and determined $^{1}H/^{31}P$ $\Delta\delta$ values, ($\Delta\delta = \delta_{\text{free ligand}} - \delta_{\text{M-ligand complex}}$) between analogue **3** Na^{+} salt and the corresponding Zn^{2+}/Mg^{2+}-complexes.

Relatively low analogue **3** concentration was used to avoid inter-molecular base stacking (π stacking interactions) in aqueous solutions.[30] The Na^{+} -salt of analogue **3** at *ca.* 6 mM was titrated with Zn^{2+} / Mg^{2+} solutions (0.1-5 eq) at pD 7.4, at 300K, and titrations were monitored by NMR. The spectra and data obtained for these titrations are depicted in Figures 5-6, S3-S4 (Supplementary Material) and Tables 4-5.

Fig. 5 ^{31}P-NMR monitored titration of 6 mM **3** with $Zn(NO_3)_2$, pD ≅ 7.4 in D_2O at 243 MHz, at 300K

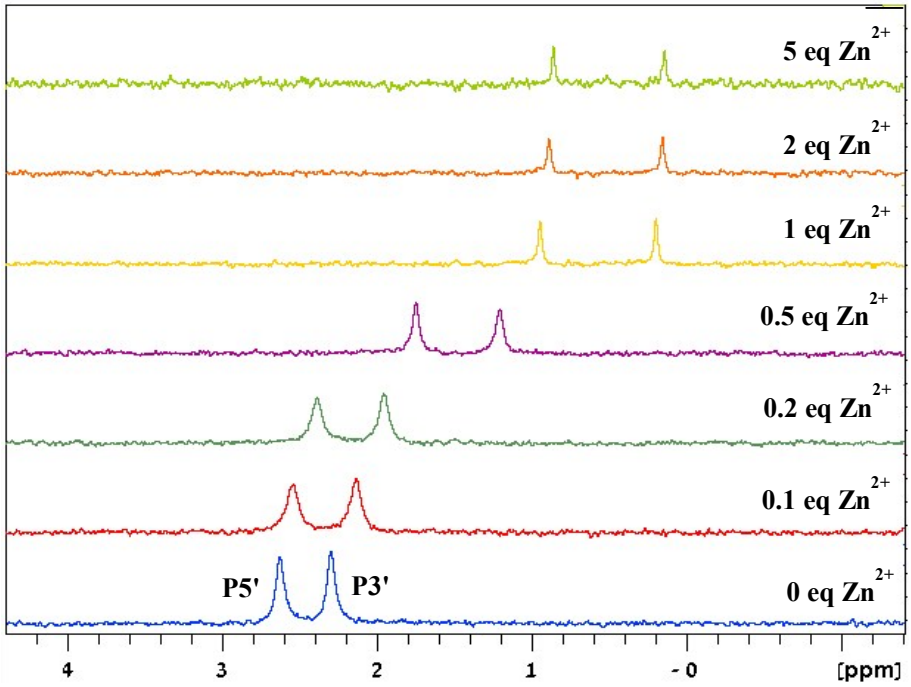


Fig. 6 ^{1}H-NMR monitored titration of 6 mM **3** with $Zn(NO_3)_2$, pD ≅ 7.4 in D_2O at 600 MHz, at 300K

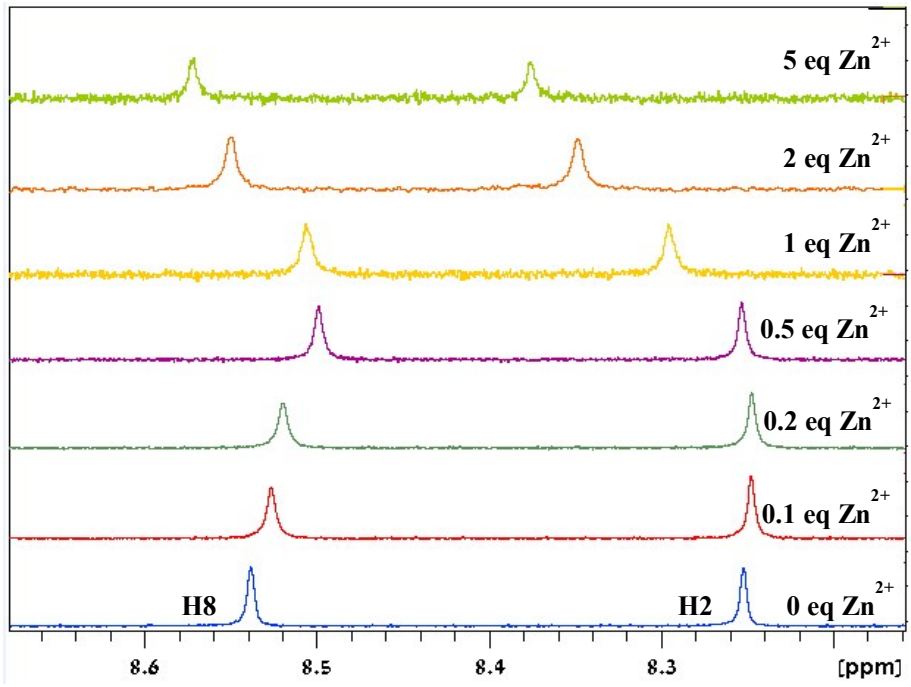


Table 4 ^{31}P-NMR shifts of the chemical shifts (Δδ) of analogue **3** obtained upon titration with 0.1 / 0.2 / 0.5 / 1 / 2 / 5 equivalents of Zn^{2+} / Mg^{2+} [a] at 300 K

Cmp	Ion	Phosphate group	Shift of chemical shift upon the addition of M^{2+} (ppm)[b]					
			0.1 eq	0.2 eq	0.5 eq	1 eq	2 eq	5 eq
d(pAp)	Zn^{2+}	P–3'	-0.16	-0.34	-1.00	-2.10	-2.14	-2.15
	Mg^{2+}	P–3'	-0.05	-0.10	-0.12	-0.22	-0.32	-0.39
d(pAp)	Zn^{2+}	P–5'	-0.08	-0.24	-0.88	-1.68	-1.74	-1.76
	Mg^{2+}	P–5'	-0.04	-0.04	-0.10	-0.17	-0.25	-0.31

[a]Titrations were monitored at 243 MHz.

[b]Negative Δδ indicates upfield shifts and positive Δδ indicates downfield shift

Table 5 ^{1}H NMR shifts of the chemical shifts (Δδ) of analogue **3** obtained upon titration with 0.1 / 0.2 / 0.5 / 1 / 2 / 5 equivalents of Zn^{2+} / Mg^{2+} [a] at 300 K

Cmp	Ion	Base proton position	Shift of chemical shift upon the addition of M^{2+} (ppm)[b]					
			0.1 eq	0.2 eq	0.5 eq	1 eq	2 eq	5 eq
d(pAp)	Zn^{2+}	H2	0	0	0	0.04	0.10	0.12
	Mg^{2+}	H2	0.01	0.01	0.01	0.01	0	0
d(pAp)	Zn^{2+}	H8	-0.01	-0.02	-0.04	-0.03	0.01	0.03
	Mg^{2+}	H8	0.02	0.02	0.01	0.01	0	0

[a]Titrations were monitored at 600 MHz.

[b]Negative Δδ indicates upfield shifts and positive Δδ indicates downfield shift

^{31}P-NMR spectrum (Fig. 5) showed clearly coordination of Zn^{2+}-ion to P3' and P5' sites of analogue **3** ($\Delta\delta = -2.1$ and -1.68 ppm with 1 eq Zn^{2+}, respectively) (Table 4). Similarly, analogue **3** coordinated with Mg^{2+}-ion (Fig. S3, Supplementary Material), yet, $\Delta\delta$ value was much smaller than that of the corresponding Zn^{2+}-complex (-0.22 and -0.17 ppm with 1 eq Mg^{2+} for P3' and P5', respectively). This expected observation is based on the larger log*K* value of **3**-Zn^{2+} vs. **3**-Mg^{2+} complex. Finally, there is no significant change in $\Delta\delta$ upon addition of 2 or 5 eq of Zn^{2+}/Mg^{2+} vs. 1 eq (-2.14, -2.15 vs. -2.10 / -0.32, -0.39 vs. -0.22, respectively) (Table 3). Therefore, we conclude that the stoichiometry of the Zn^{2+} or Mg^{2+}-nucleotide **3** complexes is 1:1.

Based on the larger $\Delta\delta$ of P3' vs. P5' in Zn^{2+}-complexes, we hypothesize that the coordination of Zn^{2+} to P3'/P5' groups occurs possibly through direct coordination with P3' and coordination through H_2O molecule to P5'.

^{1}H-NMR data (Table 5, Figs. 6 and S4) indicated that downfield and upfield $\Delta\delta$ values of H2 and H8, respectively, during Mg^{2+}/Zn^{2+}-titration (up to 1 eq) were rather small.

Bock has previously reported small upfield shifts of both adenine proton resonances (H8 and H2) of ATP upon coordination with various M(II)-ions.[31] These effects were explained as possibly due to a direct metal-adenine binding, but it was also suggested that it can be explained more readily by enhanced π-stacking in the presence of the metal ions. Bock has demonstrated formation of a specific bis(ATP) complex for Sn(II), which exerted the largest upfield shift on the H8 resonance, but little effect on the H2 resonance, and attributed the effect on H8 possibly to an interaction of the two adenine rings in the complex. Zn(II)-ion induced a *downfield* shift of the H8 resonance along with an *upfield* shift of the H2 resonance, and Bock suggested again that the latter effect is probably a result of stacking, but the effect on

H8 was due to direct metal coordination to N7 of the adenine ring. This observation was also made by Sigel, who suggested that Zn(II)-ions bridge the phosphate and N7 sites of nucleoside-*5'-phosphate* analogues forming a dimer.[32, 33] Sigel suggested that charge neutralization at the phosphate group of a nucleotide facilitates its self-association.

Here, increased Zn(II)-concentration resulted in *downfield* shift of H2 of analogue **3**, whereas for H8 an *upfield* shift was observed at up to 1 eq Zn(II). Above 1 eq (at 2 and 5 eq), a *downfield* shift was observed (up to 0.03 ppm), Table 5. Therefore, we assume that the formation of π-stacked Zn:d(pAp) species is possible, yet, direct metal coordination to N7 of the adenine ring is less likely (see below).

Conformational analysis of analogues 3, 4 by NMR

To elucidate the mode of coordination of Zn^{2+} by analogues **3** and **4**, we first analyzed the conformation of the free nucleotides in solution. Our methods of choice were NMR techniques including ^{1}H-, ^{13}C-, and ^{31}P-1D NMR and COSY, HMQC, HMBC and NOESY 2D - experiments. Chemical shifts (δ_H, δ_C, δ_P) and coupling constants (J_{HH}, J_{CH}, J_{PH}, J_{CP}) of d(pNp)s spectra were measured. NMR data were analyzed by Eq. 12-21 (Experimental Section).[34-36] The following conformational features of d(pNp)s analogues were determined: the value of χ (glycosidic angle), dominant conformers around C4'-C5' and C5'-O5' bonds, and dominant ribose conformer.

Ribose puckering

To determine the ribose North/South, *N/S*, populations of analogues **3** or **4,** $J_{2''3'}$, $J_{1'2''}$ H-H couplings and NOESY data were analyzed by previously reported equations

12-13.[36] In both analogues **3** and **4**, the predominant conformation was South (*S*) (ca. 84%), (Figs. 7A, and S5 (Supplementary Material), Table 6).

Conformation around the ribose -CH_2O- exocyclic group

Coupling constants, $J_{H4'-H5'}$, $J_{H4'-H5''}$, $J_{P-H5'}$, $J_{P-H5''}$, were used for analysis of the conformation around the exocyclic ribose group**.** The percentage of *gg, gt*, and *tg* conformer populations around the ribose –CH_2O- exocyclic group in d(pNp)s, **3, 4,** were obtained from Eqs. 14-19. Both **3** and **4** exhibited *gg* the conformation around C4'-C5' (68% and 47%, respectively, Figs. 7B and S5, Supplementary Material). Moreover, the conformation around C5'-O5' for both compounds is also *gg* (68% and 71%, respectively, Fig. 7C). All the conformational data are presented in Table 6.

Table 6. Conformational data obtained for analogues **3** and **4** in solution

No.	Comp.	χ degrees ($^3J_{C8-H1'}$)	χ degrees ($^3J_{C4\-H1'}$)	% *S* Ribose puckering	C4'-C5'			C5'-O5'		
					% *gg*	% *tg*	% *gt*	% *gg*	% *tg*	% *gt*
3	d(pAp)	216 / 264	213 / 267	84	68	18	14	68	18	14
4	d(pGp)	210 / 270	230 / 270	84	47	31	22	71	17	13

Glycosydic angle

We calculated the glycosydic angle of d(pNp)s based on $^3J_{C8-H1'}$ and $^3J_{C4-H1'}$ values (Eqs. 20-21).

Each equation gives four solutions. One solution is in the *anti*-region, the second one is in the *high-anti*-region, the third one is in the *syn*-region, and the last one is *syn* or *anti*. We selected the solutions that are in the *anti*-range as the most reasonable

possibility, considering NOESY results (Fig. S1, Supplementary Material). The range of glycosydic angles for analogues **3, 4** was 213-270° (*anti-region*, Fig. 7A).

This determination of *anti*-conformation of the nucleotides **3** and **4** is also supported by H2' and H2" chemical shifts, 2.82 and 2.68 for **4** and 2.85 and 2.76 ppm for **3**.[21, 37]

The bond-length between the Zn^{2+} ion and phosphate oxygen atoms in most known inner sphere Zn^{2+}-nucleotide complexes is *ca.* 2 Å and the O-Zn-O angle is *ca.* 90°.[26, 38] In the most stable solution conformation of **3** or **4** (Fig. 7), these geometrical requirements for inner sphere Zn^{2+} coordination could not be fulfilled. Hence, solution conformation of **3** or **4,** log*K* values of Zn^{2+}-complexes of **3** or **4**, as well as Zn^{2+} coordination-sites in **3** or **4**, led us to hypothesize that the coordination of the P3'/P5' phosphate groups to Zn^{2+} occurs through direct binding to one of the phosphate groups, P3' phosphate (see above discussion), and indirect binding to P5' through H_2O molecule or possibly outer-sphere coordination with both P3' and P5'. Additional molecular dynamics simulations and X-ray crystal structure of Zn^{2+} complexes of nucleotides **3** or **4** may help sort out the fine-details of Zn^{2+} coordination.

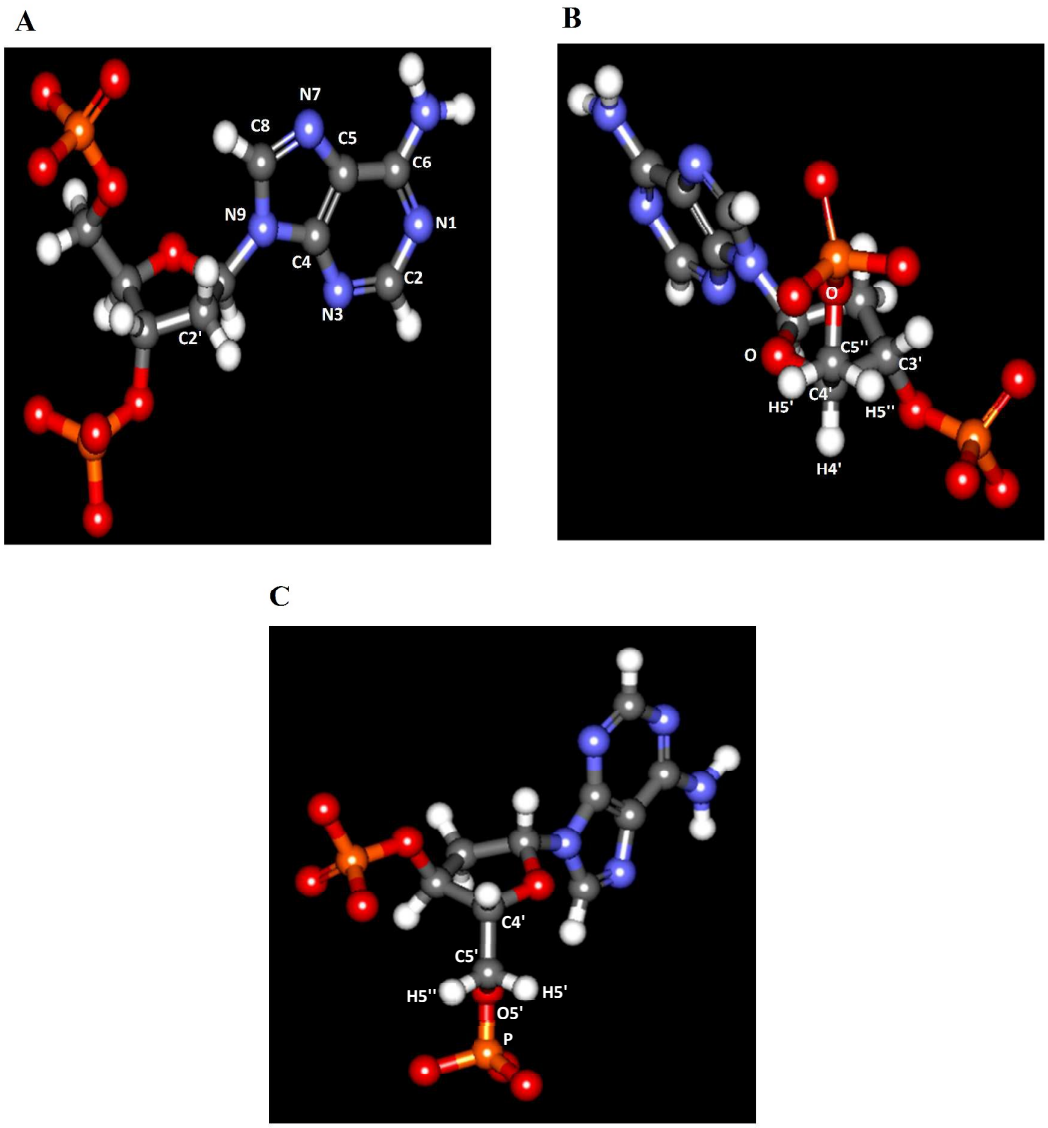


Fig. 7 NMR-based conformational analysis of analogue **3** in an aqueous solution**: View A**. *South* ribose conformation and *anti*-χ angle, **View B.** *gg*-conformer around C4'-C5' bond, **View C.** *gg*-conformation around C5'-O5' bond

Dynamics of coordination and mode of binding of zinc ion to ADP and d(pAp)

The above pK_a and log K values of Zn^{2+} complexes of d(pAp) and ADP implied that Zn^{2+} is bound via outer-sphere coordination to d(pAp), and inner-sphere

coordination to ADP (Fig. 3A-C). To further explore the mode of Zn^{2+} coordination (i.e. outer- vs. inner-sphere coordination) we have performed MD simulations.

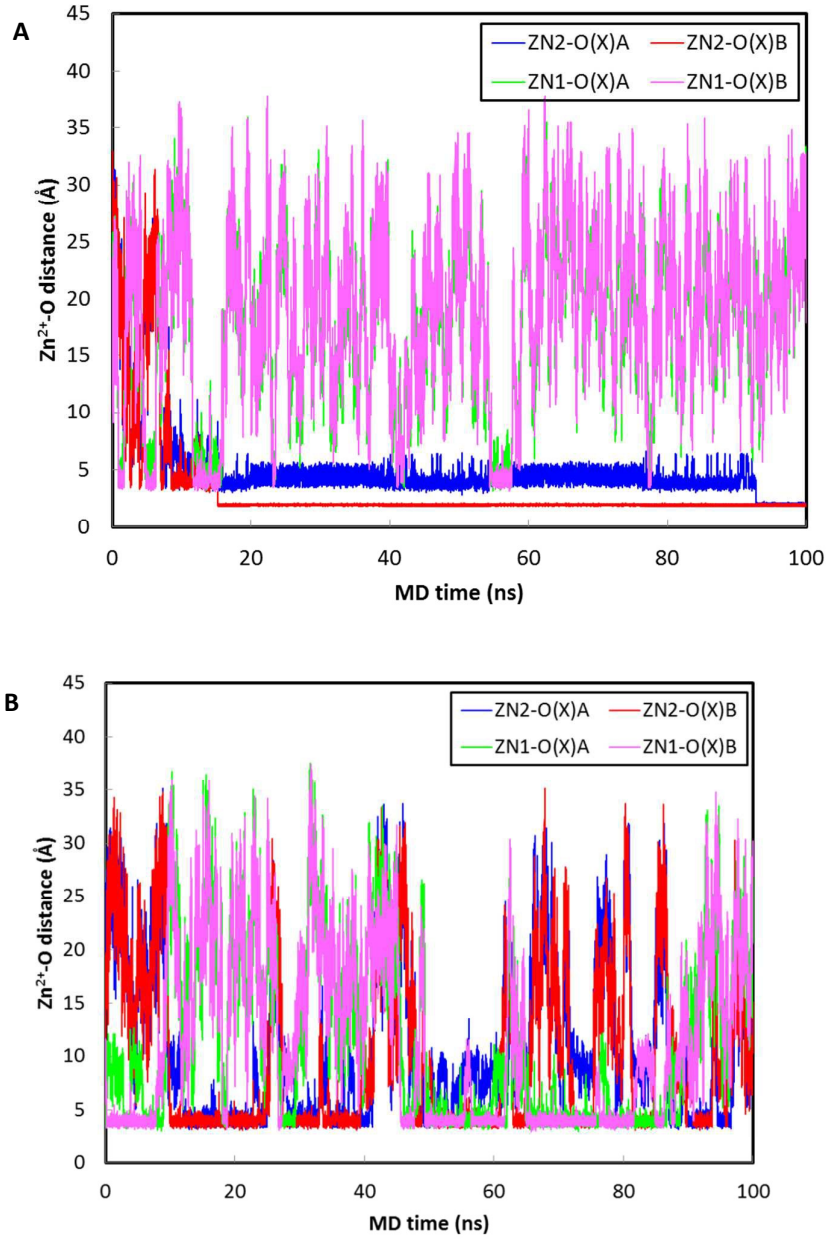
The ADP and d(PNP) systems were subjected to 100 ns of MD simulations in an explicit water and ion environment to probe the mode of complexation. The results of these simulations are presented in Fig. 8.

At the outset of the production stage of the MD simulations of ADP, the nucleotide is un-complexed, although initial Zn^{2+}-binding occurs within the first ns of the simulation. Subsequently, several metal-binding and unbinding events occur until complete binding takes place at ca. 8 ns. At this early binding stage, the binding mode is outer sphere via bridging water molecules. Specifically, Zn^{2+}-ion binds to the oxygen atoms of Pα and Pβ via bridging water to each of the phosphate moieties. At ca. 16 ns the water molecule bridging the terminal β-phosphate is displaced in favor of inner-sphere coordination. This binding mode, which has a Zn^{2+}-O distance of 2.0 Å, remains stable throughout the 100 ns MD simulation. At ~92 ns the second bridging water molecule is displaced to form a complete inner sphere complexation. Based on our earlier free energy simulations of metal ion binding in dinucleotides we expect a significant barrier to metal-ion dissociation from the nucleotide.[39] Therefore, we consider the final complexation state of the simulation to be representative of the preferred binding mode.

The Zn^{2+}-binding in the case of d(pAp) is markedly different than that of ADP (Fig. 8B). At the outset of the MD simulation, the metal ion is bound in an outer-sphere mode to a single phosphate moiety. Within approximately 4 ns the Zn^{2+} ion shifts to a position allowing outer-sphere coordination with both phosphate moieties. Numerous unbinding and rebinding events occur throughout the simulation. However, the coordination mode remains outer-sphere for the entire 100 ns, suggesting weaker

nucleotide-metal ion association in the case of d(pAp) than for ADP. Both nucleotides were found to be predominantly in the *Southern* conformation.

Fig. 8 Shortest instantaneous distance between the Zn^{2+} ions and oxygen atoms of the α and β phosphate moieties of (A) ADP and (B) d(pAp) over the course of 100 ns of MD simulations.



Based on the coordination observed during the MD simulations of ADP and d(pAp) in explicit water-ion environments, we conclude that Zn^{2+}-ion binding is tighter in the case of ADP. Indeed, ADP coordinates Zn^{2+} in an inner sphere coordination mode, with direct metal-ion and phosphate oxygen contact. In contrast, in the case of d(pAp) we do not observe inner-sphere coordination. The difference in the Zn^{2+}-binding modes between ADP and d(pAp) may be ascribed to a combination of enthalpic and entropic considerations, which are consistent with previous observations on the stability of a 6-membered ring vs. 14-membered ring[24] (here it applies to ADP-Zn^{2+} complex and d(pAp)-Zn^{2+} outer-sphere complex, 6- vs. 14-membered ring, respectively). In the case of ADP, the phosphate charge density is greater than that in d(pAp), and thus forms stronger interaction with the Zn^{2+}-ions. In the case of d(pAp) there is both lower charge density per phosphate site (i.e. -2 charge per phosphate site vs. -3 in ADP, as well as a greater entropic penalty for inner sphere coordination (i.e. bringing together two separate phosphate moieties for coordination, as opposed to two covalently linked groups in ADP).

Conclusions

Based on our previous characterization of nucleoside phosphorothioate-Zn^{2+} complexes by potentiometric pH-titrations, and the finding that a terminal phosphate group plays an important role in stabilizing the nucleotide-Zn^{2+} complex,[15] we expected that two terminal phosphate groups, as in bis-phosphate analogues **3** and **4**, will increase log*K* values of nucleotide-Zn^{2+} complexes as compared to the corresponding di-phosphate analogues **1** and **2**. Therefore, here we explored the coordination of Zn^{2+}/Mg^{2+} by chelators based on a nucleoside bis-phosphate scaffold.

We found that log K^{M}_{ML} of Zn^{2+} complexes with analogues **3** and **4** is 100-fold larger than that of the corresponding complexes with Mg^{2+}-ion. This finding was

exemplified by the species distribution at physiological pH, by the high percentage of the Zn^{2+}-complexes of **3** and **4** (46% and 44%, respectively) as opposed to the corresponding Mg^{2+}-complexes (11% and 8%).

Surprisingly, the stability constants of Zn^{2+}/Mg^{2+}-complexes with nucleoside-bisphosphate **3** and **4**, were slightly lower than those of the corresponding nucleoside 5'-diphosphate complexes ($\Delta \log K^{M}_{LM} \leq 0.5$).

In order to explain these results we performed conformation analysis of the free nucleotides **3** and **4** by solution NMR. We found that their glycosidic angle was in the *anti-region*, the sugar pucker was *South* and the conformation around C4'-C5' and C5'-O5' was *gg*; namely, Zn^{2+}/Mg^{2+} ions cannot coordinate with N1 or N7 nitrogen atom of 2'-deoxy-adenosine 3',5'-bisphosphate in addition to P3'/P5'-coordination.

Indeed, ^{1}H- and ^{31}P-NMR monitored Zn^{2+}/Mg^{2+}-titrations showed that Zn^{2+}/Mg^{2+} coordinated with the terminal phosphate groups of 2'-deoxy-adenosine 3',5'-bisphosphate, P5' and P3', and not with N1 or N7 nitrogen atoms of the adenine residue.

Our NMR data also imply that the coordination of P3'/P5' phosphate groups to Zn^{2+} or Mg^{2+} occurs through a bridging H_2O molecule. Indeed, this interpretation was supported by MD simulations showing clearly outer-sphere coordination of a Zn^{2+}-ion by both P3'- and P5'-phosphate groups.

We suggest that the unexpected similarity of logK values of Zn^{2+}-d(pNp) and Zn^{2+}-NDP complexes is due to formation of large and relatively unstable 14-membered ring in outer-sphere Zn^{2+}-d(pNp) complexes, vs. a stable 6-membered ring in an inner-sphere NDP-Zn^{2+} complex,[24, 26, 27] and also due to the loss of Zn^{2+} chelation through N7 nitrogen atom, unlike in NDP-Zn^{2+} complexes. The loss of N7-coordination and outer-sphere-type coordination in Zn^{2+}-d(pNp) complexes may be

due to the solution conformation of nucleoside 3',5'-bisphosphate (*anti* glycosidic angle*; S* sugar pucker*; gg* C4'-C5' bond*; gg* C5'-O5' bond conformation), the distance between P3' and P5' groups, and the constraint of 90° O-Zn-O angle in Zn^{2+}-nucleotide complexes.[26, 38]

In summary, we found that indeed nucleoside 3',5'-bisphosphate analogues are biocompatible and Zn^{2+}-selective chelators. Surprisingly, they have similar affinity to Zn^{2+}, as that of nucleoside-diphosphate analogues. Based on NMR and molecular dynamics data, we found that this is due to outer- vs. inner-sphere coordination of Zn^{2+} by d(pNp) vs. NDP analogues.

Experimental:

Materials

ADP, GDP sodium salts were purchased from Sigma (Steinheim, Germany). Nucleotides **3** and **4** were synthesized according to the literature.[24, 40, 41] The titrated nucleotides were ≥95 % pure. 5 M NaOH, 2 M HNO_3, and potassium biphthalate were purchased from Merck (Darmstadt, Germany). $NaNO_3$ salt (background electrolyte) and $Zn(NO_3)_2$, and $Mg(NO_3)_2$ standard solutions 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 vacuum before use for NaOH calibration. The concentration of the nucleotide stock solutions was determined by titration with NaOH.

NMR monitored Zn^{2+}/Mg^{2+}-titrations of nucleotide 3

The pD of the D_2O samples was adjusted to *ca.* 7.4 with NaOD and DCl D_2O solutions. 6 mM analogue **3** Na^+ salt in D_2O was titrated with 0.1, 0.2, 0.5, 1, 2, and 5 eq of 0.75 M $Zn(NO_3)_2$ or 0.15 M $MgSO_4$ D_2O solution and ^{31}P NMR and ^{1}H NMR spectra were measured at 243, 600 MHz, respectively.

Determination of the conformation of nucleotides 3, 4 by ^{1}H and ^{13}C-NMR

The conformational analysis of 10 mM analogues **3** and **4** Na^+ salts was performed at 700 MHz (^{1}H-NMR) and 176 MHz (^{13}C-NMR) in D_2O (pD 7.4). The ribose conformation of the nucleotides was analyzed in terms of a dynamic equilibrium between two favored puckered conformations *North* (*N*) conformer and *South* (*S*)

conformer. *N* and *S* equilibrium populations were calculated from the observed $J_{1'2'}$ and $J_{3'4'}$ couplings as reported previously (Eqs. 12-13).[36]

(12) $\mathbf{X_N} = [(J^{1'2''} + J^{2''3'}) - 6.9]/10.9$

(13) $\mathbf{X_S} = [17.8 - (J^{1'2''} + J^{2''3'})]/10.9$

The torsional angles around C4'-C5' (γ-angle) and C5'-O5' (β-angle), were obtained by measuring $J_{4'5'}$ and $J_{4'5''}$, $J_{P5'}$ and $J_{P5''}$ values and using previously reported equations Eqs. 14-16 and 17-19, respectively.[34]

(14) $\rho_{gg} = [(J_t + J_g) - (J_{4'5'} + J_{4'5''})]/(J_t - J_g)$

(15) $\rho_{tg} = (J_{4'5'} - J_g)/(J_t - J_g)$

(16) $\rho_{gt} = (J_{4'5''} - J_g)/(J_t - J_g)$

where Jg = 2.04 Hz, Jt = 11.72 Hz.

(17) $\rho_{gg} = [(J_t + J_g) - (J_{P5'} + J_{P5''})]/(J_t - J_g)$

(18) $\rho_{tg} = (J_{P5'} - J_g)/(J_t - J_g)$

(19) $\rho_{gt} = (J_{P5''} - J_g)/(J_t - J_g)$

Where J_t = 20.9 Hz, J_g = 1.8 Hz.

The glycosidic bond was obtained by monitoring the vicinal coupling constants $^3J_{C8/6\text{-}H1'}$ and $^3J_{C4/2\text{-}H1'}$, which were extracted from ^{13}C NMR spectra for each nucleotide (Eqs. 20-21):[35]

(20) $^3J_{C6/8\text{-}H1'} = 4.5\cos^2(\chi\text{-}60°) - 0.6\cos(\chi\text{-}60°) + 0.1$

(21) $^3J_{C2/4\text{-}H1} = 4.7\cos^2(\chi\text{-}60°) + 2.3\cos(\chi\text{-}60°) + 0.1$

Potentiometric pH-titrations

Potentiometric pH-titrations procedure

pH titrations were performed with a Metrohm 794 basic Titrino potentiometer and a Metrohm Viscotrode glass electrode. The buffers (pH 4.00, 7.00, and 9.00)[42]

used for calibration were purchased from Metrohm (Herisau, Switzerland). All titrations were performed at 24°C under argon atmosphere and lasted for 30-45 min. Determination of the acidity constants, $K^{H}_{HL,}$ $K^{H}_{LH2,}$ K^{H}_{LH3} and K^{H}_{LH4} (L -ligand), was done by the titration of 4.5 mL composed of aqueous 4 mL HNO_3 in $NaNO_3$ (from a 3-4.4 mM $HNO_3/NaNO_3$ stock solution, I = 0.1 M) and 0.5 mL ligand (from a 4.5 mM nucleotide stock solution, I = 0.1 M). Acidity constants were calculated with HYPERQUAD software.[43] The determination of the stability constants, $K^{M}_{ML,}$ $K^{M}_{MLH,}$ K^{M}_{MLH2} and $pK_a{}^{H}_{MLH}$ (M-metal), was achieved by the titration of 3-3.5 mL of aqueous HNO_3 in $NaNO_3$ (from a 3-4.4 mM HNO_3 stock solution, I = 0.1 M), 0-0.5 mL $NaNO_3$ (I = 0.1M), 0.5 mL ligand (from a 4.5 mM ligand stock solution, I = 0.1 M), and 0.5 mL $Zn(NO_3)_2$ or $Mg(NO_3)_2$ (from a 4.5 mM stock solution, I = 0.1 M). The metal-ion:ligand ratio was 1:1. The pH range of all titrations was *ca.* 2.5-10.3. The endpoints of the nucleotide-complex titrations were obtained by the second derivative method.[44] Each titration was repeated up to 4 times. Stability constants were calculated with HYPERQUAD software. Speciation and titration simulations were achieved with HYSS2009 software.[29]

Computational Methods

ADP and d(pAp) molecules were build using Discovery Studio 3.5 (Accelrys Inc.). Each dinucleotide was placed at the center of a 41 x 41 x 41 $Å^3$ pre-equilibrated box of 2267 TIP3P water molecules.[45] ADP and d(pAp) were treated at the molecular mechanics (MM) level of theory using the CHARMM27 force-field.[46, 47] In the ADP model system two Zn^{2+} ions and one Cl^- ion were added in order to neutralize the system. In the d(pAp) system two Zn^{2+} ions were added in order to neutralize the system. The counter ions were placed at random positions in the box. Water

molecules overlapping with the solute or counter ions were deleted (<2.5 Å heavy atom distance). The initial systems were minimized by 150 cycles of the Adopted Basis Newton-Raphson (ABNR) method.[48] Periodic boundary conditions were applied. The hydrogen atom–heavy atom bond lengths were constrained by the SHAKE algorithm[49] The Particle Mesh Ewald method was employed to treat long-range electrostatics.[50]The number of grid point for the Ewald summation in each dimension was 40, while the k parameter was set to 0.34 $Å^{-1}$. A spherical cutoff scheme was employed for van der Waals and real-space electrostatic interactions with a value of 12.0 Å.

The ADP and d(pAp) systems were subjected to extensive molecular dynamics (MD) simulations. The isothermal-isobaric ensemble (NPT) was employed at 1 atm and 298 K using the extended system pressure/temperature (CPT) algorithm of Andersen.[51]with an effective mass of 500 amu and the Hoover thermostat[52] with an effective mass of 1,000 kcal/mol·$psec^2$. The system was gradually heated up from 48 to 298 K during five sessions of 5 psec for a total of 25 psec, and thereafter equilibrated at the target temperature (298 K) over the course of 100 nsec at the MM level of theory. The MD trajectories were analyzed and structural data were collected. All the minimizations and simulations were performed with the CHARMM program.[48, 53]

Endocyclic torsion angles were defined as: $\nu 0$: C4'–O4'–C1'–C2', $\nu 1$: O4'–C1'–C2'–C3', $\nu 2$: C1'–C2'–C3'–C4', $\nu 3$: C2'–C3'–C4–O4', $\nu 4$:C3'–C4–O4'–C1'.

The pseudorotation was calculated according to Altona and Sundaralingam:[54]

$$P = \frac{[(v4 + v1) - (v3 + v0)]}{2v2(sin36 + sin72)}$$

where we add 180 to the calculated P if $\nu 2 < 0$. Here $P = 0$ is for the *Northern* conformer and $P = 180$ is for the *Southern* conformer.

Table of Contents Graphics

Adenosine/Guanosine-3',5'-Bis-Phosphate as Biocompatible and Selective Zn^{2+}-Ion Chelators. Characterization and Comparison with Adenosine/Guanosine-5'-Di-Phosphate

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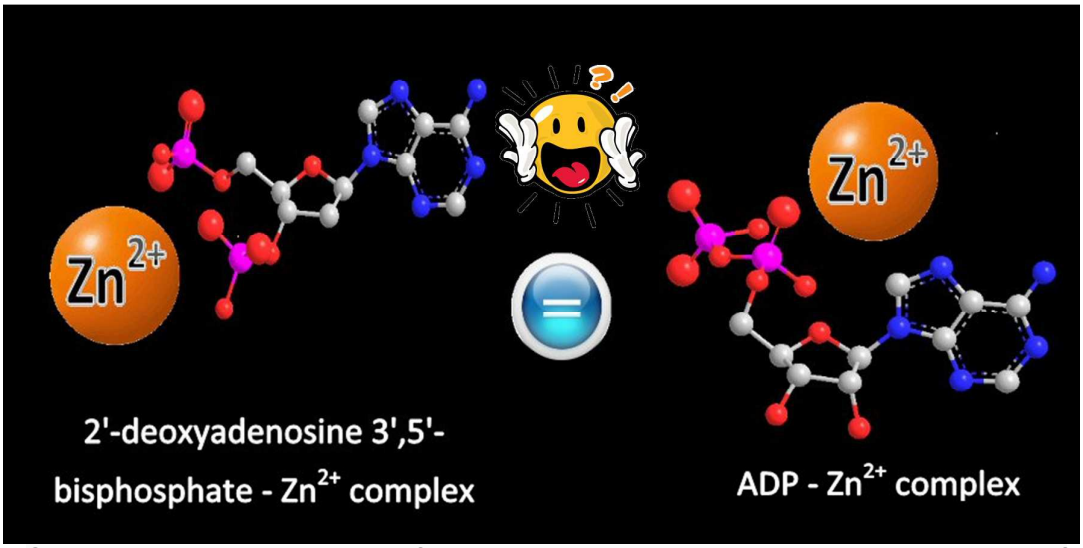

Credit (surprise icon): http://www.fromladtodad.com/wp-content/uploads/2014/04/surprise.gif

Nucleoside-bisphosphates are Zn^{2+}-selective chelators, sharing similar affinity to Zn^{2+} as nucleoside-diphosphates due to their respective outer- vs. inner-sphere Zn^{2+}-coordination.

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