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1,4,7,10-Tetraazacyclododecane Metal Complexes as Potent Promoters of Phosphodiester Hydrolysis under Physiological Conditions

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Previously reported mono- and dinuclear Zn(II), Cu(II), and Ni(II) complexes of 1,4,7,10-tetrazacyclododecane ([12]aneN$_4$ or cyclen) with different heterocyclic spacers (triazine, pyridine) of various lengths (bi- and tripyridine) or an azacrown-pendant have been tested for the hydrolysis of bis(4-nitrophenyl)phosphate (BNPP) under physiological conditions (pH 7−9, 25 °C). All Zn(II) complexes promote the hydrolysis of BNPP under physiological conditions, while those of Cu(II) and Ni(II) do not have a significant effect on the hydrolysis reaction. The hydrolysis kinetics in buffered solutions (0.05 M Bis/Tris, TRIS, HEPES, or CHES, $I = 0.1$ M, NaCl) at 25 °C were determined by the initial slope method (product conversion <5%). Comparison of the second-order pH-independent rate constants (k_{BNPP}, $M^{-1}\ s^{-1}$) for the mononuclear complexes **ZnL1**, **ZnL3**, and **ZnL6**, which are 6.1×10^{-5}, 5.1×10^{-5}, and 5.7×10^{-5}, respectively, indicate that the heterocyclic moiety improves the rate of hydrolysis up to six times over the parent Zn([12]aneN$_4$) complex ($k_{BNPP} = 1.1 \times 10^{-5}\ M^{-1}\ s^{-1}$). The reactive species is the Zn(II)−OH$^-$ complex, in which the Zn(II)-bound OH$^-$ acts as a nucleophile. For dinuclear complexes **Zn$_2$L2**, **Zn$_2$L4**, and **Zn$_2$L5**, the rate of reaction is defined by the degree of cooperation between the metal centers, which is determined by the spacer length. **Zn$_2$L2** and **Zn$_2$L4** possessing shorter spacers are able to hydrolyze BNPP 1 to 2 orders of magnitudes faster than **Zn$_2$L5**. The second-order rate constants k of **Zn$_2$L4** and **Zn$_2$L2** at pH 7, 8, and 9 are significantly higher than those of previously reported related complexes. The high BNPP hydrolytic activity may be related to π-stacking and hydrophobic interactions between the aromatic spacer moieties and the substrate. Complexes **Zn$_2$L4** and **Zn$_2$L2** show hydrolytic activity at pH 7 and 8, which allows for the hydrolysis of activated phosphate esters under physiological conditions.

Introduction

Phosphate esters exist ubiquitously in nature.[1] They are found in nucleoside phosphates (nucleotides) as components of RNA and DNA, in sugar nucleotides for the glycosylation of oligosaccharides, and in proteins involved in intracellular signaling and regulation.[2] Phosphodiester linkages of DNA are very stable to hydrolysis, with a half-life for spontaneous hydrolysis estimated to be 10^{11} years at pH 7 at 25 °C.[3] In nature, many enzymes that catalyze phosphate ester hydrolysis are activated by two or more metal ions.[4] These include phosphate monoesterases, diesterases, and triesterases. Enzymes that catalyze the replication of DNA and RNA and ribozymes that catalyze the intermolecular transesterification of RNA are also activated by more than one metal ion.

Currently there is considerable interest in understanding the reaction mechanism of such enzymes, especially the role of the metal ions. One goal is developing more reactive chemical systems that efficiently hydrolyze phosphate diester

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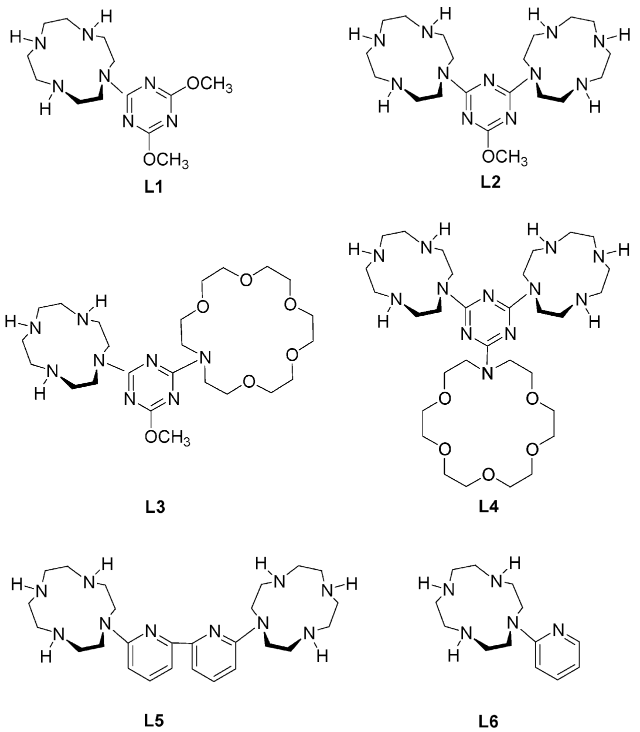
bonds under physiological conditions. Many metal ion based model systems have been reported, generally featuring tridentate or tetradentate ligands with free coordination sites of the metal cation.[5] However, with only a few exceptions these systems are active either at high pH values (pH ≥ 9), elevated temperatures ($T \geq 35$ °C), or show a low solubility in aqueous solutions. For the development of synthetic hydrolases with applications in biotechnology, medicine, or environmental sciences, for example, the detoxification of pesticides like parathion, malathion, and other organophosphorus compounds, artificial systems with activity under physiological conditions are required.

The proposed general mechanism of the hydrolysis reaction promoted by these complexes is based on the Lewis acid character of the metal ion reducing the pK_a of the coordinated water. This provides a metal-bound hydroxide nucleophile at neutral pH and at the same time activates a coordinated substrate toward nucleophilic attack by charge neutralization.[5a,6] For dinuclear species, the two metal ions may act cooperatively in the catalytic process, either one metal ion provides the nucleophile and the other one coordinates the substrate or both metal ions participate in substrate binding, activation, and cleavage.[7] This cooperative action renders dinuclear complexes far more reactive than their mononuclear analogues.

It has been demonstrated that additional substrate interactions next to the metal ion center influence the properties of the metal complexes and that the hydrolytic activity may increase by the attachment of functional groups to a chelate ligand,[8] such as a basic or nucleophilic auxiliary group[9] or an NH-acidic group.[10] With the aim to develop more efficient metal complexes possessing hydrolytic activity under physiological conditions, we have previously reported the synthesis of the macrocyclic ligands **L1**−**L6** (Scheme 1) with different heterocyclic spacers of various lengths and determined the hydrolytic properties of their Zn(II), Cu(II), and Ni(II) complexes in aqueous solution with 4-nitrophenyl acetate (NA).[11] We showed that the Zn(II) complexes have a high hydrolytic activity toward NA under physiological conditions compared to other similar macrocyclic compounds. The monohydroxy species [Zn_2−L−OH_2−OH^-] was the active form. The mechanism of reaction, either following Michaelis−Menten type or bimolecular kinetics, depended on the spacer length.

Scheme 1. Structure of the [12]aneN_4 Ligands **L1**−**L6**



This paper presents kinetic studies of the hydrolysis of bis(4-nitrophenyl)phosphate (BNPP) in aqueous solution at 25 °C in the pH range 7 to 9. The influence of type and length of the spacer bridging the two metal complexes, the metal ions Cu(II), Ni(II), or Zn(II), and of mono- and dinuclear complexes on the hydrolytic efficiency were investigated.

Experimental Section

General Information. UV−vis spectra were recorded on a Varian Cary BIO 50 UV−vis−NIR spectrophotometer equipped with a jacketed cell holder using 1-cm cuvettes (quartz or glass) from Hellma and on a Zeiss SPECORD M500 equipped with six cuvette holders using disposable acrylic (PMMA) 1-cm cuvettes from Sarstedt. For all UV−vis measurements the temperature was kept constant at 25 °C (±0.1 °C). ESR spectra in the X-band (9.459 GHz) were recorded with a Bruker System EMX in frozen acetonitrile/butyronitrile (4:1) solution at 110 K.

Materials and Reagents. All reagents and solvents used for the synthesis of the metal complexes were of analytical grade. BNPP (Fluka) was transformed into the disodium salt form by titration with a 2 M NaOH solution. 4-Nitrophenol (4-NP) (Riedel-de Haën), Bis/Tris (bis[2-hydroxyethyl]-imino-tris-[hydroxymethyl]-methane) (Sigma), TRIS (2-amino-2-hydroxymethyl-propane-1,3-diol) (Usb), HEPES (*N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid) (Sigma), CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid) (Sigma), were purchased from commercial sources and used without any further purification. The ligands **L1**−**L6** and their metal complexes were synthesized according to the published procedure.[11]

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Kinetic Measurements. The hydrolysis rate of BNPP promoted by the $ML-OH^-$ species was measured in the pH range 7 to 9 by an initial slope method following the increase in 408 nm-absorption of 4-nitrophenolate in aqueous solution for the Zn(II) complexes and the increase in 400 nm-absorption in aqueous solution for the Cu(II) and Ni(II) complexes (50 mM Bis/Tris, HEPES, TRIS, or CHES buffer, $I = 0.1$ M, NaCl) at 25 °C. The reactions were corrected for the degree of ionization of the 4-nitrophenol at the respective pH and temperature using the molar extinction coefficients for *para*-nitrophenolate at 400 nm[11] and 408 nm[12] previously determined. The kinetic data were collected using the initial slope method (product conversion <5%).[13] The concentration of the reaction product 4-nitrophenyl phosphate is therefore too low to cause any inhibition of the metal complex.[14] All measurements were performed in triplicate. From the slope [produced 4-nitrophenolate]/time and the concentration of BNPP, the pseudofirst-order rate constant k_{obs}(BNPP) (s^{-1}) was determined. A plot of these k_{obs} values versus metal complex concentrations at a given pH gave a straight line, its slope representing the second-order rate constant k(BNPP) (M^{-1} s^{-1}). All correlation coefficients are ≥0.9992. Correction for the spontaneous hydrolysis of the substrate by the solvent was accomplished either by directly measuring the difference between the production of 4-nitrophenolate in the reaction cell and a reference cell containing the same concentration of phosphate diester as in the reaction cell in absence of metal complex or by separate measurement of the spontaneous hydrolysis for BNPP under the given experimental conditions and manual subtraction of the measured absorption from the absorption curve of the catalyzed reaction. In comparison with NA, the spontaneous hydrolysis of BNPP has a much lower reaction rate when compared to the metal catalyzed reaction, and for pH values <7 the rate of spontaneous hydrolysis of BNPP is too low to be taken into account in the kinetic measurements. Therefore, the general rate of spontaneous hydrolysis was not separately determined as previously in the case of NA.[11] The reaction solutions contained 0.04−0.5 mM Cu(II)-, 0.16−0.3 mM Ni(II)-, 0.08−18 mM Zn(II)-complex, 0.002−10.0 mM BNPP, and 50 mM buffer.

Results and Discussion

Table 1. Deprotonation Constants (pK_a) of Metal-Bound H_2O at 25 °C and $I = 0.10$ (TEAP)

metal complex	pK_a
ZnL1	8.35 ± 0.03[11]
NiL1[15]	11.13 ± 0.02
CuL1[a, 16]	
ZnL3	8.28 ± 0.05
ZnL6	7.89 ± 0.05
Zn-[12]aneN_4	8.06 ± 0.01

[a] Complex **CuL1** is not sufficiently soluble under the given experimental conditions to allow a potentiometric pH titration.

Hydrolysis of bis(4-Nitrophenyl)phosphate (BNPP) Promoted by the Mononuclear Metal Complexes ZnL1, CuL1, NiL1, ZnL3, and ZnL6. The reaction rates of ester bond cleavage of BNPP (0.002−10 mM) in aqueous solutions in the pH range 7−9 (50 mM HEPES or TRIS buffer, $I = 0.1$ M, NaCl) at 25 °C were measured by an initial slope method following the increase in 408 nm-absorption of 4-nitrophenolate for the Zn(II) complexes and the increase in 400 nm-absorption of 4-nitrophenolate for the Cu(II) and Ni(II) complexes. The reactions were corrected for the degree of ionization of the 4-nitrophenol at the respective pH and temperature. The absorption increase was recorded immediately after mixing and then monitored until 5% reaction conversion. The second-order dependence of the rate constant k on the concentration of BNPP and metal complex is described by kinetic eq 1.

$$v_{cat} = \frac{d(Abs)}{d(t)\,\varepsilon_{obs}} = k_{obs}[BNPP] = k[M-L][BNPP] \qquad (1)$$

In eq 1, k is the observed BNPP hydrolysis rate caused by the metal complex. The term $[M-L]$ describes the total concentration of metal complex and is composed of the concentration of the metal-hydroxy species $[M-L-OH^-]$ and the concentration of the water coordinated complex $[M-L-H_2O]$, depending on the corresponding pK_a of the metal complex. Equation 2 describes this equilibrium:

$$M-L-OH_2 \xrightleftharpoons{K_a} M-L-OH^- + H^+$$

$$K_a = \frac{[M-L-OH][H^+]}{[M-L-OH_2]} \qquad (2)$$

with

$$[M-L] = [M-L-OH_2] + [M-L-OH^-]$$

The corresponding pK_a values of the mononuclear complexes are given in Table 1.

Among the mononuclear Zn complexes **ZnL6** forms more of the catalytically active species at lower pH values because of its smaller pK_a value. The Lewis acid character of Zn(II) and Ni(II) are reflected in the pK_a values of their cyclen complexes.

Kimura and Norman have shown for the hydrolysis of BNPP and other activated phosphoester derivates with various Zn[12]aneN_3 and Zn[12]aneN_4 complexes that the active nucleophilic species attacking the phosphate ester is in fact the Zn-L-OH^- species.[17–19] The same has been shown

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(14) The association constant of **Zn2L2** with phenylphosphate in neutral aqueous solution was determined by an indicator displacement assay using pyrocatechol violet to be $K = 104$ L/mol. The association constant of 4-nitrophenylphosphate to **Zn2L2** is expected to be even lower in the millimolar range because of the reduced basicity of this phosphate. This excludes a significant product inhibition under the experimental conditions used (less than 5% conversion; typical initial concentrations of BNPP 0.1−1 mM).

(15) (a) The UV and IR spectra of **NiL1** coincide with the described structure of Ni[12]aneN_4: Fabrizzi, L.; Micheloni, M.; Paoletti, P. *Inorg. Chem.* **1980**, *19*, 535–538. (b) Fabrizzi, L. *Inorg. Chem.* **1977**, *16*, 2667–2668. (c) Bencini, A.; Bianchi, A.; Garcia-Espana, E.; Jeannin, Y.; Julve, M.; Marcelino, V.; Philoche-Levisalles, M. *Inorg. Chem.* **1990**, *29*, 963–970.

(16) (a) The comparison of UV and IR spectra with literature values indicate a square pyramidal complex with one molecule of water as the fifth ligand: Styka, M. C.; Smierciak, R. C.; Blinn, E. L.; DeSimone, R. E.; Passariello, J. V. *Inorg. Chem.* **1978**, *17*, 82–86. (b) Thöm, V. J.; Hosken, G. D.; Hancock, R. D. *Inorg. Chem.* **1985**, *24*, 3378–3381. (c) Thöm, V. J.; Fox, C. C.; Boeyens, J. C. A.; Hancock, R. D. *J. Am. Chem. Soc.* **1984**, *106*, 5947–5955.

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for Cu[9]aneN$_3$ complexes by Burstyn and Spiccia.[20,21] To our knowledge there are no reported mononuclear macrocyclic Ni(II) complexes which promote the hydrolysis of phosphoresters. However, the reactive species of the dinuclear complex of Ni[9]aneN$_3$, developed by Kaden et al., is the monohydroxy compound.[22] Thus eq 1 can be written as follows:

$$v_{\mathrm{cat}} = k_{\mathrm{BNPP}}[\mathrm{M{-}L{-}OH^-}][\mathrm{BNPP}] \quad \text{with}$$

$$k_{\mathrm{BNPP}} = \frac{[\mathrm{M{-}L}]k}{[\mathrm{M{-}L{-}OH^-}]} \qquad (3)$$

Equations 2 and 3 give eq 4 which describes the relationship between the rate constant k, the pH independent reaction rate constant k_{BNPP}, and the pH value.

$$k = \frac{k_{\mathrm{BNPP}}K_{\mathrm{a}}}{K_{\mathrm{a}} + [\mathrm{H}^+]} \qquad (4)$$

The value of the second-order rate constant k ($\mathrm{M^{-1}\ s^{-1}}$) is equal to the slope of the line obtained in the plot of k_{obs} versus the metal complex concentration at a given pH. The rate constants k_{obs} ($\mathrm{s^{-1}}$) are determined by an initial slope method ([produced 4-nitrophenolate]/time) using the log ε values determined previously.[11,12] All correlation coefficients are ≥0.9992. The use of the pH independent second-order reaction rate constant k_{BNPP} ($\mathrm{M^{-1}\ s^{-1}}$) enables a comparison with previous reports from the literature.

Complex **CuL1** showed poor solubility under the given experimental conditions and could not be used for the hydrolysis experiments. **NiL1** did not show a significant effect on the hydrolysis, with pH dependent k_{obs} values in the range of 10^{-8} to 10^{-7} $\mathrm{s^{-1}}$.[23]

A typical plot of k_{obs} versus Zn(II) complex concentration for the mononuclear Zn(II) complexes is presented in the Supporting Information. The obtained second-order rate constants k ($\mathrm{M^{-1}\ s^{-1}}$) and the pH-independent hydrolysis rate constants k_{BNPP} ($\mathrm{M^{-1}\ s^{-1}}$) for the mononuclear Zn(II) complexes are presented in Table 2.

Table 2. Hydrolysis Rate Constants k ($\mathrm{M^{-1}\ s^{-1}}$) and pH-Independent Hydrolysis Rate Constants k_{BNPP} ($\mathrm{M^{-1}\ s^{-1}}$) for the Mononuclear Zn(II) Complexes at 25 °C in Aqueous Solution[a]

Zn(II)-Cyclen (pK_{a} = 7.9)		**ZnL1** (pK_{a} = 8.35)	
pH	10^6 k ($\mathrm{M^{-1}s^{-1}}$)	pH	10^6 k ($\mathrm{M^{-1}s^{-1}}$)
7.51[b]	3.1	7.12[b]	3.1
8.00[b]	6.3	7.53[b]	7.2
8.52[b]	8.7	8.00[b]	18.4
9.00[b]	9.9	8.49[b]	32.3
		9.00[b]	48.4
10^5 k_{BNPP} ($\mathrm{M^{-1}\ s^{-1}}$)[b] = 1.1 ± 0.3		10^5 k_{BNPP} ($\mathrm{M^{-1}\ s^{-1}}$)[b] = 6.1 ± 0.2	

ZnL3 (pK_{a} = 8.28)

pH	10^6 k ($\mathrm{M^{-1}s^{-1}}$)	pH	10^5 k ($\mathrm{M^{-1}s^{-1}}$)	pH	10^5 k ($\mathrm{M^{-1}s^{-1}}$)
6.83[c]	1.7	8.00[b]	1.70	9.24[e]	4.60
7.02[c]	2.3	8.26[b]	2.31	9.58[e]	4.89
7.26[b,c,f]	4.2	8.50[b]	3.13	9.91[e]	4.99
7.52[b]	7.7	8.69[b]	3.66	10.22[e]	5.05
7.73[b]	10.3	9.00[b,e,f]	4.38		

10^5 k_{BNPP} ($\mathrm{M^{-1}\ s^{-1}}$)[b,c,e] = 5.1 ± 0.1

ZnL6 (pK_{a} = 7.89)

pH	10^6 k ($\mathrm{M^{-1}s^{-1}}$)	pH	10^5 k ($\mathrm{M^{-1}s^{-1}}$)	pH	10^5 k ($\mathrm{M^{-1}s^{-1}}$)
6.49[d]	2.1	7.83[b]	2.42	9.24[e]	5.35
6.83[c,d,f]	4.2	8.09[b]	3.42	9.58[e]	5.60
7.15[c]	8.2	8.31[b]	3.93	9.91[e]	5.58
7.36[b,c,f]	11.4	8.55[b]	4.61	10.22[e]	5.65
7.59[b]	18.7	8.87[b,e,f]	4.98		

10^5 k_{BNPP} ($\mathrm{M^{-1}\ s^{-1}}$)[b,c,d,e] = 5.7 ± 0.1

[a] [BNPP] = 0.5−10 mM, Δ pH = ±0.005, Δk_{obs} = 0.3−6.8%, Δk = 1.1−4.1%, ΔT = ±0.1 °C. [**Zn(II)-Cyclen**] = 0.8−14 mM, [**ZnL1**] = 0.5−6 mM, [**ZnL3**] = 0.5−15 mM, [**ZnL6**] = 0.5−8 mM. [b] Tris/HCl [50 mM], I = 0.1 M (NaCl), T = 25 °C. [c] HEPES [50 mM], I = 0.1 M (NaCl), T = 25 °C. [d] Bis/Tris [50 mM], I = 0.1 M (NaCl), T = 25 °C. [e] CHES [50 mM], I = 0.1 M (NaCl), T = 25 °C. [f] k and k_{obs} measured at the same pH value in different buffers are in the given error margins and are listed as average values.

Kimura et al. have described the hydrolysis of BNPP promoted by the Zn(II)-cyclen complex but using different reaction conditions (T = 35 °C, pH = 9.2).[16] Kinetic measurements with this complex under our reaction conditions were performed for comparison. The kinetic measurements with **Zn(II)-cyclen** and **ZnL1** were performed in the pH range 7−9 in TRIS buffer while the hydrolysis of BNPP promoted by **ZnL3** and **ZnL6** was measured in the extended pH range 6.5−10.3 (50 mM Bis/Tris, HEPES, TRIS or CHES buffer, I = 0.1 M, NaCl). The derived sigmoidal pH-rate profiles[24] (Figure 1) are characteristic of a kinetic process controlled by an acid−base equilibrium and exhibit inflection points corresponding to the pK_{a} values of the coordinated water molecules of **ZnL3** (pK_{a} = 8.28) and **ZnL6** (pK_{a} = 7.89). This indicates that the Zn(II)−OH$^-$ complex is the reactive species. The Zn(II)-bound OH$^-$ acts as a nucleophile to attack the phosphate atom of the phosphodiester hydrolyzing the bis(4-nitrophenyl)phosphate to 4-nitrophenolate and 4-nitrophenyl phosphate. This mechanism of BNPP hydrolysis has been reported for other Zn(II) cyclen complexes, too.[17−19,25,26] A subsequent hydrolysis of the phosphate monoester to inorganic phosphate was not detected.

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(23) The reactivity increases from pH 7 to pH 9 because of increasing amounts of the active species in solution (0.007% at pH 7, 0.07% at pH 8, and 0.7% at pH 9). Previously reported azamacrocyclic Ni(II) complexes (see ref 22) were also active only at pH > 9.

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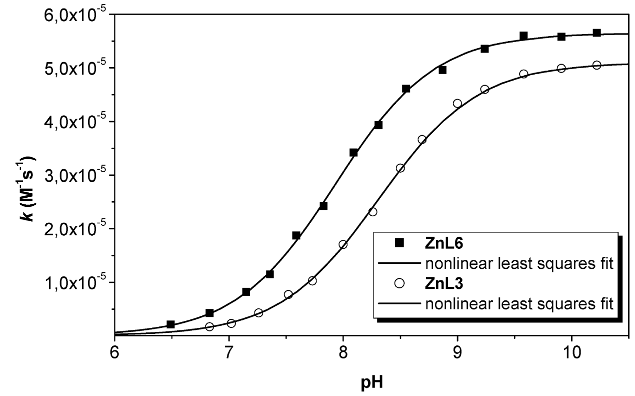
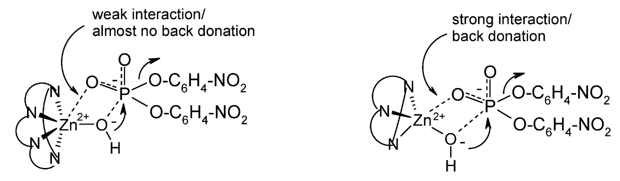


Figure 1. pH-Rate profile for the second-order rate constants of BNPP hydrolysis of **ZnL3** and **ZnL6** at 25 °C and $I = 0.1$ (NaCl) in aqueous solution.

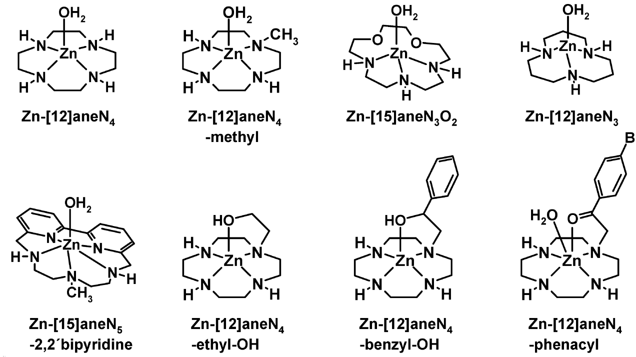
The k_{BNPP} values of our Zn(II) cyclen complexes show a 5 to 6-fold higher hydrolysis rate than the simple Zn[12]aneN$_4$ system because of the aromatic substituent. π-π interactions of the heterocycle with the aromatic ring of the bis(4-nitrophenyl)phosphate leads to a tighter substrate binding. The more hydrophobic environment may result in a higher reactivity of the hydroxyl species and a better substrate preorganization for hydrolysis.[27] Tang et al. previously reported on the influence of an aromatic substituent, emphasizing on its positive influence on the substrate orientation and stabilization of the leaving group in the transition state.[28]

We have previously reported the X-ray structure of **Zn$_2$L2** showing that the zinc ion is coordinated by only three out of four N-atoms of cyclen. The bond to the fourth N-atom is significantly longer (2.6 Å).[11] Kimura describes the same effect for other Zn cyclen derivates with electron-poor aromatic substituents.[29] The structure of the Zn(II) complexes here reported is in between that of [12]aneN$_4$[30] and of [12]aneN$_3$, which explains the enhanced hydrolysis rates for BNPP. Complexes of the [12]aneN$_3$ type promote phosphate diester hydrolysis by coordination of the phosphoester to the metal ion (Scheme 2).

Scheme 2. Proposed Transition States of Phosphate Diesters Hydrolysis by Azamacrocyclic Zn(II) Complexes with Tetraza- (left) and Triaza-Ligands[17]



Scheme 3. Structures of Previously Studied Mononuclear Azamacrocyclic Zn(II) Complexes[a]



[a] Charges and counter ions are not represented for clarity reasons.

The structures of previously studied mononuclear azamacrocyclic Zn(II) complexes are depicted in Scheme 3, and their reported second-order reaction rates k_{BNPP} and pK_a values are summarized in Table 3. Because of the high number of publications in the field of metalloenzyme-promoted phosphate ester hydrolysis, only mononuclear azamacrocyclic Zn(II) complexes promoting the hydrolysis of BNPP have been selected.

Zn[12]aneN$_3$ complexes show higher reaction rates of BNPP hydrolysis than Zn[12]aneN$_4$ derivates.[34] The complexes with an ethyl- and benzylhydroxyl pendant arm show significantly higher hydrolytic activity because of a different reaction mechanism with the alcoholate as the reactive species: The first reaction step is a transphosphorylation followed by intramolecular nucleophilic attack of the Zn(II) bound hydroxide ion.[9g,33] Zn-[15]aneN$_5$-2,2′-bipyridine has the highest reaction rate hydrolyzing BNPP (1.1×10^{-4} M^{-1} s^{-1}), which is 5-fold higher than the simple Zn[12]aneN$_4$ system. Complexes **ZnL1**, **ZnL3**, and **ZnL6** show k_{BNPP} values in the same range as Zn[12]aneN$_3$. Because of their lower pK_a values, their activity under physiological condi-

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(26) As shown by previous reports, the mechanism of BNPP hydrolysis by macrocyclic metal complexes implies the following steps: deprotonation of a metal bound water molecule (K_a), binding of the substrate to the metal complex (K), intracomplex nucleophilic attack of the hydroxide (k') with simultaneous departure of the leaving group and, eventually, deprotonation and decomplexation of the substrate to restore the catalyst. The reaction rate is pH dependent and saturation behavior by formation of the substrate-catalyst complex is expected. However, the affinities of phosphate diesters to Zn(II) complexes are very low and the curvature of the plot is not detectable in the exploitable concentration range. This is common to all reports on metal complexes hydrolyzing phosphate diesters.

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(34) The higher Lewis acidic character of the metal ion in Zn[12]aneN3 complexes leads to a stronger interaction between the metal complex and the phosphodiester, as proposed by Kimura. In the carboxylic ester hydrolysis promoted by mononuclear complexes, the simple nucleophilic mechanism is predominant and the Zn(II)-bound hydroxides act as nucleophile to the carbonyl group. Therefore, the Zn[12]aneN3 complexes with higher Lewis acidic character of the metal ion, but also lower nucleophilic character of the Zn-L-OH- species, have lower reaction rates in the hydrolysis of carboxyesters than the Zn[12]aneN4 system.

Table 3. Comparison of Hydrolysis Rate Constants k_{BNPP} (M^{-1} s^{-1}) and pK_a Values for Previously Reported Mononuclear Zn(II) Complexes

complex	10^5 k_{BNPP} (M^{-1} s^{-1})	reaction conditions[a]	pK_a[b]	lit.
Zn-[12]aneN_4	2.1 ± 0.2	$I = 0.2$ ($NaClO_4$), pH-stat method	7.9	17
Zn-[12]aneN_4-methyl	0.52 ± 0.2	$I = 0.1$ ($NaNO_3$), buffer (20 mM)[c]	7.68	9g
Zn-[15]aneN_3O_2	1.37[d]	$I = 0.15$ (NaCl), buffer (50 mM)[e]	8.8	31
Zn-[12]aneN_3	8.5 ± 0.2	$I = 0.2$ ($NaClO_4$), pH-stat method	7.2	17
Zn-[15]aneN_5-2,2′bipyridine	11 ± 0.1	$I = 0.1$ (NMe_4NO_3), buffer (50 mM)[e]	10.73	32
Zn-[12]aneN_4-ethyl-OH	50 ± 0.1	$I = 0.1$ ($NaNO_3$), buffer (20 mM)[c]	7.6	9g
Zn-[12]aneN_4-benzyl-OH	65 ± 0.1	$I = 0.1$ ($NaNO_3$), buffer (20 mM)[c]	7.5	9g
Zn-[12]aneN_4-phenacyl	4.3 ± 0.2	$I = 0.1$ ($NaClO_4$), buffer (20 mM)[c]	8.4	33

[a] All values were determined in aqueous solutions at 35 °C. [b] pK_a value determined at 25 °C. [c] Goods buffer. [d] k_{BNPP} was calculated from the value of k up to pH 10 ($1.3 \pm 0.1 \cdot 10^{-5}$ M^{-1} s^{-1}). [e] MOPS, TAPS, CHES, CAPS buffer, depending on the pH range.

tions is high, whereas Zn-[15]aneN_5-2,2’bipyridine is active only at pH > 9.

Hydrolysis of BNPP Promoted by the Dinuclear Metal Complexes Zn_2L2, Cu_2L2, Ni_2L2, Zn_2L4, and Zn_2L5. The reaction rates of ester bond cleavage of BNPP (0.002−10 mM) in aqueous solutions in the pH range 7−9 (50 mM HEPES or TRIS buffer, $I = 0.1$ M, NaCl) at 25 °C were measured by an initial slope method. The reaction parameters and method of evaluation of data are identical to those described for the mononuclear complexes.

The dependence of the reaction rate on the concentrations of the metal complex, the substrate, k_{obs}, and k is given in eq 5.

$$v_{cat} = \frac{d(Abs)}{d(t)\, \varepsilon_{obs}} = k_{obs\,1,\,2}[BNPP] = k[M-L]^{Total}[BNPP] \tag{5}$$

The reaction rate v_{cat} reflects in this case the contribution of two reactive species, the monohydroxy and the dihydroxy species. Therefore eq 6 can be derived from eq 5.

$$k[M-L]^{Total} = k_1[M_2-L-(OH_2)(OH)] + k_2[M_2-L-(OH)_2] \tag{6}$$

The contribution of the two reactive species, the monohydroxy and the dihydroxy species depends on the corresponding pK_a of the metal complex.

We have previously shown that for the dinuclear complexes **Zn_2L4** (in aqueous solution) and **Zn_2L2** (in aqueous solution and in MeOH/H_2O 9:1 solution) two distinct buffer regions were determined, one around pH 6 and the other in the pH range 8−10, corresponding to three distinct p*K_a* values.[11] The proposed chemical model is based on an equilibrium in solution between the μ-hydroxo-bridged species $Zn_2-L-(\mu OH_2)(OH_2)$, analogous to the obtained crystal structure of **Zn_2L2**,[11] and an open form corresponding to the species where each Zn(II) ion is coordinating a water molecule, $Zn_2-L-(OH_2)_2$. This model is supported by a good match with the calculated and the measured pH profiles and by reports from literature where a similar equilibrium between open and closed species was postulated.[35] The proton independent equilibrium K_{D1} can be determined indirectly. The p*K_a* values of **Zn_2L2** and **Zn_2L4** are summarized in Table 4.

Table 4. Deprotonation Constants (pK_a) of Metal-Bound H_2O at 25 °C and $I = 0.10$ (TEAP)

metal complex	pK_a[a]			log K_{D1}
	pK_{a1}	pK_{a2}	pK_{a3}	
Zn_2L2		9.72 ± 0.03[b]		
Zn_2L4	8.27 ± 0.02	9.42 ± 0.06	5.96 ± 0.02	0.47 ± 0.04

[a] From ref 11. [b] The titration curve does not permit a determination of pK_{a1} and pK_{a3} values because of the insufficient solubility of the complex in water and/or MeOH/water 9:1.

Table 5. Deprotonation Constants (pK_a) of Metal-Bound H_2O at 25 °C and $I = 0.10$ (TEAP)[11]

metal complex	pK_{a1}	pK_{a2}
Ni_2L2	9.75±0.02	10.10±0.02
Cu_2L2	8.34±0.03	8.68±0.03
Zn_2L5	7.45±0.03	8.85±0.03

The p*K_a*value of the μ-hydroxo-coordinated water molecule, pK_{a3}, is smaller than those reported for similar compounds,[35c] indicating enhanced acidity and stability of the μ-hydroxo-bridge because of the close proximity of the two Zn(II) cyclen moieties. For the dinuclear complexes, **Cu_2L2**, **Ni_2L2**, and **Zn_2L5**, the previously described pH profiles[11] correspond to the general model[36] with each metal ion coordinating a water molecule and two successive deprotonation steps leading to the species $M_2-L-(OH^-)_2$. Electron spin resonance measurements (see Supporting Information for data) indicate a typical square-pyramidal geometry for **Cu_2L2** (and **CuL1**), with the Cu(II) ion coordinating to four N-atoms of the macrocycle and one H_2O molecule.

The p*K_a* values of these complexes are summarized in Table 5.

Complex **Ni_2L2** did not show a significant effect on the hydrolysis (k_{obs} is in the range of 10^{-8} to 10^{-7} s^{-1}, depending on the pH value of the solution) because of the weak Lewis acid character of the metal ion. At physiological pH no active species is present in solution.[22,37] For **Cu_2L2**, the k_{obs} values were in the range of 10^{-5} s^{-1}.[38,39]

For the dinuclear Zn(II) complexes **Zn_2L2**, **Zn_2L4**, and **Zn_2L5** the reaction rates k [from eq 6] of ester bond cleavage of BNPP (0.01−8 mM) were measured in aqueous solutions in the pH range 7−9 (50 mM TRIS buffer, $I = 0.1$ M, NaCl)

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Table 6. Second-Order Rate Constants k (M^{-1} s^{-1}) at pH 7, 8, and 9 for the Dinuclear Zn(II) Complexes at 25 °C in Aqueous Solution[a]

metal complex	10^3 k (M^{-1} s^{-1}) at pH 7[b]	10^3 k (M^{-1} s^{-1}) at pH 8[b]	10^3 k (M^{-1} s^{-1}) at pH 9[b]
Zn_2L2[c]		1.28	1.35
Zn_2L4	1.03	2.45	2.63
Zn_2L5	0.049	0.244	0.406

[a] [BNPP] = 0.01−8 mM, ΔpH = ±0.005, Δk_{obs} = 2.2−9.3%, Δk = 0.8−4%, ΔT = ±0.1 °C. [**Zn_2L2**] = 0.3−8 mM, [**Zn_2L4**] = 0.4−10 mM, [**Zn_2L5**] = 0.6−8 mM. [b] Tris/HCl [50 mM], I = 0.1 M (NaCl), T = 25 °C. [c] The measurements with **Zn_2L2** at pH 7 entered the error margin of the apparatus despite numerous attempts, thus making the evaluation of the kinetic measurements inaccurate.

at 25 °C following the increase in 408 nm absorption of 4-nitrophenolate. The reaction parameters and method of data evaluation are identical to those described for the mononuclear complexes.[26] The plots of k_{obs} versus Zn(II) complex concentration for the dinuclear Zn(II) complexes are presented in the Supporting Information. All complexes show second-order kinetics.[40] The obtained second-order rate constants k (M^{-1} s^{-1}) for **Zn_2L2**, **Zn_2L4**, and **Zn_2L5** are summarized in Table 6.

From the obtained k values it is obvious that **Zn_2L2** and **Zn_2L4** possessing shorter spacers are able to hydrolyze BNPP 1 to 2 orders of magnitudes faster than **Zn_2L5**. It has been previously shown that dinuclear Zn(II) complexes with short spacers are able to coordinate the phosphate diester bidentate, thus increasing the activation of the substrate and bringing it closer to the attacking nucleophile.[39] A short spacer leads to a higher degree of cooperation between the metal centers for the hydrolysis of BNPP. The second-order rate constants increase with pH as expected. For **Zn_2L2** and **Zn_2L4** the increase from pH 8 to pH 9 is very small compared to **Zn_2L5** where the value of k at pH 9 is almost double the value of k at pH 8. The high cooperativity of the two metal centers in **Zn_2L4** leads to faster BNPP hydrolysis, although it is the weaker Lewis acid if compared to **Zn_2L5**.

The hydrolytic activity of the dinuclear Zn(II) complexes is 2 to 3 orders of magnitude higher than that of the corresponding monomers **ZnL1** and **ZnL3**. A direct comparison between **Zn_2L5** and **ZnL6** is not possible because of the different spacer length. The increased rates indicate the importance of cooperativity of the two metal centers for hydrolysis activity.[6,7,31,32,41]

From the pK_a values and species distribution diagram of **Zn_2L4** (see Supporting Information) we can assert that the monohydroxy species $Zn_2-L-(OH_2)(OH^-)$ in its opened form is the catalytically active species at pH 7 and 8. The bis-hydroxy form $Zn_2-L-(OH^-)_2$ is present in very low concentration at this pH (less than 3% at pH 8). Even for pH 9 the concentration of the dihydroxy species is only around 25%. Complex **Zn_2L2** behaves similarly. For **Zn_2L5** with pK_{a1} and pK_{a2} values of 7.45 and 8.85, the mono- and dihydroxy species are both present at pH 7−9 and contribute to the BNPP hydrolysis (species distribution diagram, see Supporting Information).

The second-order rate constants k (M^{-1} s^{-1}) at pH 7, 8, and 9 and pK_a values of previously reported di- and trinuclear metal complexes for BNPP hydrolysis, which showed good activity, are summarized in Table 7. (see Supporting Information for structures of the complexes). For all the reported complexes an "associative" mechanism is postulated in which the substrate approaches the Zn(II) complex and the oxygens of BNPP coordinate to the two electrophilic Zn(II) cations forming a bidentate complex. A zinc-bound hydroxide nucleophile attacks the phosphorus atom. Strong interaction of the BNPP ester with the electrophilic metal centers and a highly nucleophilic character of the Zn−OH enhances the hydrolytic activity of a complex. The lack of hydrolytic activity of the monohydroxy species of the complexes in Table 7 is attributed to a bridged coordination of the hydroxide anion to two metal centers which reduces the nucleophilicity of the generated hydroxide. This applies similarly to **Zn_2L2** and **Zn_2L4**: the open form monohydroxy species $Zn_2-L-(OH_2)(OH^-)$ is the catalytically active form and not the closed form with the μ-hydroxo bridge. The high activity of the $Zn_2-(OH^-)_2$ complexes in Table 7 is explained by a cooperative role of the two metal ions in the hydrolytic process.

For Zn_2-[18]aneN_6-bis(2,2′-dipyridine)[31] the inverse behavior is observed (the monohydroxy species is the active form, while the dihydroxy species is inactive) because each metal ion is coordinated to five N-atoms of the macrocycle, leaving only one coordination site available on each metal ion. In the monohydroxy complex, one Zn(II) ion coordinates to the OH^- anion and the second metal center to the substrate BNPP, whereas in the dihydroxy complex, both Zn(II) complexes are occupied by hydroxide anions leaving no binding site available for substrate interaction. The high hydrolytic activity of the monohydroxy complex is attributed to π-stacking and hydrophobic interactions between the

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(37) The k_{obs} values of **Ni_2L2** are about twice the ones of **NiL1**. This may indicate similar mechanisms of hydrolysis of BNPP by the monohydroxy complex **Ni_2-L-$(OH_2)(OH^-)$**, as previously reported for other dinuclear Ni(II) complexes. However, we refrain from any mechanistic interpretation for the hydrolysis mediated by **Ni_2L2** or **Cu_2L2**. Their low activity and the concentrations of metal complex and substrate required to reach acceptable experimental errors lead to kinetic data that do not allow a more extended analysis.

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(39) (a) Morrow and Bursty (ref. 6a and 20) have demonstrated that artificial metallohydrolases, mono- as well as dinuclear metal complexes, must possess two cis-oriented labile coordination sites to simultaneously bind the substrate and the nucleophile. The two metal centers of the **Cu_2L2** complex have only one coordination site available each and do not act cooperatively. Iranzo, O.; Richard, J. P.; Morrow, J. P. *Inorg. Chem.* **2004**, *43*, 1743–1750. (b) Iranzo, O.; Elmer, T.; Richard, J. P.; Morrow, J. P. *Inorg. Chem.* **2003**, *42*, 7737–7746.

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Table 7. Comparison of Second-Order Rate Constants k ($M^{-1}\ s^{-1}$) at pH 7, 8, and 9 and pK_a Values for Previously Reported Di- And Trinuclear Zn(II) Metal Complexes

complex/nucleophile	$10^5\ k\ (M^{-1}\ s^{-1})^a$				
	pH 7	pH 8	pH 9	pK_a[b]	lit.
Zn_2-[30]aneN_6O_4/Zn_2-$(OH^-)_2$[c]	*d*	1.1[e]	5.11[f]	9.2	31
Zn_2-[18]aneN_6-bis(2,2′-dipyridine)/Zn_2-(OH^-)[g]	*d*	53	137	8.88	31
Zn_2-[36]aneN_8O_4/Zn_2-$(OH^-)_2$[c]	*d*	*d*	1.6	9.88	39
Zn_2-[33]aneN_7O_4/Zn_2-$(OH^-)_2$[c]	*d*	1.6[e]	6.4[h]	9.38	39
Zn_3-Tren-([14]aneN_4])$_3$/Zn_3-$(OH^-)_2$/Zn_3-$(OH^-)_3$[c,i]	3.8	19.8	30	7.90/8.83[i]	38b
Zn_3-Tren-([12]aneN_4])$_3$/Zn_3-$(OH^-)_2$/Zn_3-$(OH^-)_3$[c,i]	1.02	3.9	9.4	8.21/9.6[i]	38b

[a] Various buffer systems [50 mM], $I = 0.1-0.15$ M (NaCl or NMe_4NO_3), 35.1 °C. The values of k were either taken directly from the literature (if reported) or derived from the reported k_{BNPP} ($M^{-1}\ s-1$) values and concentrations. The errors are <5%. [b] 35.1 °C, water. [c] k_{BNPP} values of the monohydroxy species were not determined because of their negligible effect on the hydrolysis. [d] No active species present in solution at the respective pH. [e] pH = 8.1. [f] pH = 9.1. [g] k_{BNPP} values of the dihydroxy species were not determined because of their negligible effect on the hydrolysis. [h] pH = 8.9. [i] For these trinuclear complexes it has been shown that the di- and trihydroxy species are the kinetically active species and the pK_as of the species are given accordingly.

nitrophenyl groups and the dipyridine moieties which strengthen the association in the transient state of the hydrolysis.[32]

The monohydroxy species of **Zn_2L4** and **Zn_2L2** hydrolyze BNPP at pH 7, 8, and 9 with rates that are 1 to 2 orders of magnitude higher than the once previously reported for complexes given in Table 7. Zn_2-[18]aneN_6-bis(2,2′-dipyridine) reaches the same range but only at higher pH values (pH 9). Furthermore, most of the complexes reported in Table 7 do not possess hydrolytically active species at pH < 9 and show no hydrolytic activity at physiological pH. **Zn_2L5** has comparable hydrolytic activity to Zn_3-Tren-([14]aneN_4])$_3$.[42]

Conclusion

The k_{BNPP} values of mononuclear Zn(II) cyclen complexes reported in this paper show a 5 to 6-fold higher hydrolysis rate than the parent Zn[12]aneN_4 complex, most likely because of the aromatic substituent, which provides a more hydrophobic environment.[28,30] The reactive species is the Zn(II)$-OH^-$ complex, in which the Zn(II)-bound OH^- acts as nucleophile to attack the coordinated phosphate diester. The k_{BNPP} values are in the same range as for previously reported mononuclear complexes but have the advantage of activity under physiological conditions.

For dinuclear complexes the rate of reaction is defined by the degree of cooperation between the metal centers, which is influenced by the spacer length. For all the reported dinuclear complexes an “associative” mechanism is postulated, in which the substrate is coordinated to the Zn(II) complex. The phosphorus atom of the diester is intramolecularly attacked by the zinc-bound hydroxide nucleophile. Second-order rate constants k of **Zn_2L4** and **Zn_2L2** at pH 7, 8, and 9 are 1 to 2 orders of magnitude higher than those of previously reported complexes. In contrast to most previously reported compounds of this type, our complexes possess catalytically active species at pH < 9. Their high activity is explained by a cooperative role of the two metals in the hydrolytic process. The analogous Cu(II) and Ni(II) complexes show no significant hydrolytic activity.

In conclusion, complexes **Zn_2L4** and **Zn_2L2** exceed the BNPP hydrolytic activity of previously reported compounds at pH 7−9 and 25 °C and are better suited for the hydrolysis of phosphate esters under physiological conditions.

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Supporting Information Available: Plots of k_{obs} versus Zn(II) complex concentration for the mono- and dinuclear Zn(II) complexes, species-distribution diagrams for **Zn_2L4** and **Zn_2L5**, ESR study on complexes **CuL1** and **Cu_2L2**, and structures of previously studied diand trinuclear Zn(II) complexes for the hydrolysis of BNPP (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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[42] The hydrolytic activity of the complexes given in Table 7 were determined at 35.1 °C, whereas the hydrolytic activity of the dinuclear complexes reported in this paper was determined at 25 °C.