# Phosphodiester Cleavage Promoted by an Asymmetric Dinuclear Zinc Complex: Synthesis, Structure, and Catalytic Activity

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**Abstract.** The dinuclear  $Zn^{II}$  complex  $[Zn_2L(DNBA)_2]BPh_4$ ·EtOH (1) (DNBA = 3,5-dinitrobenzonic acid) with an asymmetric dinuclear ligand, *N*-4-methyl-homopiperazine-*N'*-[*N*-(2-pyridylmethyl)-*N*-2-(2pyridylethyl)amine]-1,3-diamino-propan-2-ol (HL), was synthesized and characterized. Single crystal X-ray crystallographic analysis shows that the coordination around the two  $Zn^{II}$  ions in **1** is significantly asymmetric, and the distance between both atoms is 3.426 Å, which is close to the Zn···Zn distance in related natural dinuclear metalloenzymes. Phosphodiesterase activity of Zn<sub>2</sub>L in situ formed from a 2:1 mixture of Zn<sup>2+</sup> ion and HL was investigated using bis(4-

## **1** Introduction

Hydrolases are a class of ubiquitous enzymes that catalyze the hydrolytic cleavage of target bond in a substrate-specific manner. Most of these enzymes contain metal ions, typically Zn<sup>II</sup>, Mg<sup>II</sup>, and Fe<sup>III</sup> in their active centers.<sup>[1–3]</sup> For example, in many natural phosphoesterase, such as nuclease P1, purple acid phosphatase and alkaline phosphatases, the two or three zinc ions are bridged by a carboxyl group from an aspartate residue and a water molecule. The catalytic role of the central zinc atom in these enzymes is ascribed to the orientation and activation of substrates; the bridging groups keep the ions at a suitable distance and give a fundamental contribution to substrate activation.<sup>[4]</sup>

Functional mimics of phosphoesterases are important for understanding the role of metal ions in the hydrolytic mechanisms and for developing artificial nucleases for biochemical and medicinal applications.<sup>[5]</sup> Dinuclear transition metal complexes are of particular interest, and the divalent zinc ion is the preferred metal ion for both natural enzymes and hydrolase models. This is due to a variety of factors: Zn<sup>2+</sup> is a good Lewis acid, exchanges ligands rapidly, redox inactivity, and admittedly physiological relevancy. Moreover, it has no ligand field stabilization energy and as a consequence, it can easily adapt its coordination arrangement to best fulfil the structural requirement of a reaction. Over the past years, many dinuclear Zn<sup>II</sup> complexes derived from phenol-based, alkoxide-based, carboxylate-based, phthalazine-based, pyrazolyl-based ligands

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Guangzhou University of Chinese Medicine Guangzhou, 510006, P. R. China nitrophenyl) phosphate (BNPP) as substrate. The pH dependence of the BNPP cleavage in aqueous buffer media reveals a bell-shaped pH- $k_{obs}$  profile with an optimum at about pH 7.9, which is parallel to the formation of the dinuclear species Zn<sub>2</sub>L-OH obtained from the potentiometric titration. The catalytic rate constant ( $k_{cat}$ ) is  $6.30 \times 10^{-4}$  s<sup>-1</sup> at pH 7.9 and 25 °C, which is approx. 10<sup>8</sup>-fold higher than that of the uncatalyzed reaction. The homopiperazine bound deprotonated Zn-OH group is responsible for the hydrolysis reaction. The possible mechanism for the BNPP cleavage promoted by Zn<sub>2</sub>L is proposed on the basis of kinetic and spectral analysis.

have been studied extensively in the context of the hydrolysis of nucleic acid backbones or phosphate ester models,[6-17] however, in which less attention has been paid to asymmetric ligands and their metal complexes. Synthesis and comparison of asymmetric dinuclear model complexes will investigate the different roles of the two ions in the catalytic hydrolysis and provide deeper insight into the structure and mechanism of phosphoesterases. Recently, we have developed a convenient and versatile procedure for the synthesis of a new asymmetric alkoxide-based dinucleating ligand, N-4-methyl-homopiperazine-N'-[N-(2-pyridylmethyl)-N-2-(2-pyridylethyl)amine]-1,3-diaminopr-opan-2-ol (HL).<sup>[18]</sup> This ligand can force two nickel ions into proximity with different chemical environments, as in related natural dinuclear metalloenzymes. At 25 °C, the Ni<sub>2</sub>L species formed in situ from a 2:1 mixture of Ni<sup>2+</sup> and HL in aqueous solution at pH 8.4 shows efficient phosphodiesterase activity. As a continuing study, in this paper, synthesis and structure of a dinuclear zinc complex are reported. The cleavage activity of the in situ prepared complex Zn<sub>2</sub>L on the phosphate diester bond was examined using bis(4nitrophenyl) phosphate (BNPP) as the substrate, and a possible cleavage mechanism is proposed on the basis of kinetic, pH titration, and spectral analysis.

# **2** Experimental Section

#### 2.1 Methods and Materials

The FT-IR spectrum was recorded with a PE Spectrum-One infrared spectrophotometer using KBr pellets (4000–400 cm<sup>-1</sup>). <sup>31</sup>P NMR spectra were measured with a Bruker 400 spectrometer. Elemental analysis was performed with a Vario EL CHNS-O elemental analyzer. Mass spectra were measured with a Bruker HCT-plus mass spectrometer.

The potentiometric titration and pH measurements were carried out with a Orion 420A pH meter with an Aldrich combination pH electrode, the electrode was calibrated with standard buffers (pH = 4.01, 7.00, and 10.01). Kinetic measurements were recorded using quarts cuvettes (1 cm) with Teflon stopper on a U-3100 UV/Vis spectrophotometer. The buffers were prepared with MES (2-[*N*-morpholino]ethanesulfonic acid,  $pK_a = 6.1$  at 25 °C), HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic],  $pK_a = 7.5$  at 25 °C] and adjusted to desired pH by adding freshly prepared NaOH solution. Ligand HL was synthesized as described in previous literature.<sup>[18]</sup> HBNPP purchased from Aldrich was recrystallized from ethanol/water before use. Other regents were reagent grade and were used without further purification, unless otherwise noted. Zn<sup>II</sup> stock solution was titrated against EDTA using standard procedures.

## 2.2 Synthesis of [Zn<sub>2</sub>L(DNBA)<sub>2</sub>]BPh<sub>4</sub>·EtOH (1)

A solution of Zn(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O (58.2 mg, 0.156 mmol) in methanol (1 mL) was added to a stirred solution of HL (30 mg, 0.078 mmol) in methanol (1 mL). Afterwards, a solution of Et<sub>3</sub>N (11 µL, 0.078 mmol) in methanol (1 mL) was added and the mixture was stirred at room temperature for 1 h. A solution of 3,5-dinitrobenzonic acid (DNBA) (33 mg, 0.156 mmol) and Et\_3N (22  $\mu L,$  0.146 mmol) in methanol (1 mL) was added to the reaction solution, and this solution was stirred for 1 h. A solution of NaBPh<sub>4</sub> (107 mg, 0.312 mmol) in methanol (1 mL) was added and then slight yellow precipitate was formed. The crude product was collected by filtration and then washed with methanol and diethyl ether. Colorless block shaped crystals suitable for single-crystal X-ray study were obtained by recrystallization from 3:1 MeCN-EtOH solution (4 mL) with a yield of 72 %. C<sub>62</sub>H<sub>64</sub>BN<sub>9</sub>O<sub>14</sub>Zn<sub>2</sub>: found (calcd.): C, 57.89 (57.23); H, 4.61 (4.92); N, 10.05 % (9.69) %. **IR** (KBr):  $\tilde{v}$  = 3447, 3104, 3054, 2867, 1619, 1580, 1541, 1479, 1459, 1446, 1426, 1400, 1345, 1267, 1121, 1067, 1030, 919, 742, 790, 730, 706, 649, 613, 556, 475 cm<sup>-1</sup>.

# 2.3 X-ray Crystallography

The colorless single crystal of complex **1** was mounted on glass fibers and used for data collection. Single X-ray diffraction data were obtained at 100 K with a Bruker Smart Apex CCD area detector using graphite monochromated Mo- $K_{\alpha}$  radiation ( $\lambda = 0.71073$  Å). The structure was solved by direct methods and refined on  $F^2$  by full-matrix least-squares techniques with SHELXTL-97. All non-hydrogen atoms were refined anisotropically, and all hydrogen atoms of the ligands were located and included at their calculated positions. The crystal data and structure refinement results are summarized in Table 1, and selected bond lengths and angles are listed in Table 2.

Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Centre, CCDC, 12 Union Road, Cambridge CB21EZ, UK. Copies of the data can be obtained free of charge on quoting the depository number CCDC-1027685 (Fax.: +44-1223-336-033; E-Mail: deposit@ccdc.cam.ac.uk, http://www.ccdc.cam.ac.uk)

#### 2.4 Potentiometric Titration

The protonation and coordination equilibria of the dinuclear zinc complex ( $Zn_2L$ ) synthesized in situ in water solution were investigated by potentiometric titration at 25 °C. The cell contained HL (1.0 mM), HClO<sub>4</sub> (0.5 mM), and Zn(ClO<sub>4</sub>)<sub>2</sub> (2.0 mM) in a water solution (10 mL)

Table 1.	Crystal	data	and	structure	refinement	for	1.
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	[Zn <sub>2</sub> L(DNBA) <sub>2</sub> ]BPh <sub>4</sub> •EtOH (1)
Empirical formula	$C_{62}H_{64}B N_9O_{14}Zn_2$
Formula weight	1300.77
Crystal size /mm	$0.40 \times 0.25 \times 0.20$
T /K	100(2)
Wavelength /Å	0.71073
Crystal system	triclinic
Space group	ΡĪ
a/Å	14.1342(9)
b /Å	14.3285(9)
c /Å	16.2746(10)
a /°	106.4460(10)
β /°	98.5390(10)
γ /°	104.1030(10)
$V/Å^3$	2980.9(3)
Ζ	2
Density /g·cm <sup>-1</sup>	1.449
F(000)	1352
Absorption coefficient /mm <sup>-1</sup>	0.880
Data / restraints / parameters	11419/0/793
$\theta$ range for data collection /°	1.53 to 26.00
Reflections collected	17382
Independent reflections	11419 ( $R_{int} = 0.0270$ )
$GOF$ on $F^2$	0.976
Final <i>R</i> indices $[I > 2\sigma(I)]$	$R_1 = 0.0437, wR_2 = 0.0971$
<i>R</i> indices (all data)	$R_1 = 0.0654, wR_2 = 0.1043$
Largest diff. peak and hole /e·Å <sup>-3</sup>	0.950 and -0.393

Table 2. Selected bond lengths /Å and angles /° for 1.

Zn(1)–O(1)	1.923(2)	O(1)–Zn(1)–N(10)	126.56(9)
Zn(1)-N(10)	2.045(2)	O(1)-Zn(1)-O(41)	92.53(7)
Zn(1)–O(41)	2.051(2)	N(1)-Zn(1)-N(10)	97.75(9)
Zn(1)-N(20)	2.069(2)	O(1)-Zn(1)-N(1)	80.69(8)
Zn(1)-N(1)	2.213(2)	N(1)-Zn(1)-N(20)	79.26(9)
Zn(2)-O(1)	1.988(2)	O(1)-Zn(2)-O(51)	99.62(8)
Zn(2)–O(51)	1.995(2)	O(1)-Zn(2)-O(42)	92.99(7)
Zn(2)–O(42)	2.026(2)	O(42)-Zn(2)-O(51)	96.34(8)
Zn(2)-N(34)	2.128(2)	O(1)-Zn(2)-N(30)	79.22(8)
Zn(2)-N(30)	2.153(2)	N(30)-Zn(2)-N(34)	74.88(9)
Zn(1)···· $Zn(2)$	3.426	Zn(1)-O(1)-Zn(2)	122.33(10)
Zn(2)–O(42) Zn(2)–N(34) Zn(2)–N(30) Zn(1)•••Zn(2)	2.026(2) 2.128(2) 2.153(2) 3.426	O(42)-Zn(2)-O(51) O(1)-Zn(2)-N(30) N(30)-Zn(2)-N(34) Zn(1)-O(1)-Zn(2)	96.34(8) 79.22(8) 74.88(9) 122.33(10)

with the ionic strength maintained at 0.1 M by NaClO<sub>4</sub>. After stirring for 30 min, the titration was initiated by adding 20  $\mu$ L of fresh NaOH solution (0.040 M) with a syringe to the titrated solution. Three titration experiments were performed in the pH rage 3.5–10.0. The species formed in the system were characterized by the following equilibrium process of Equation (1), Equation (2), and Equation (3). The corresponding dissociation constants were calculated using BEST program.<sup>[19]</sup>

$\Sigma \Pi_2 \Pi \Sigma = \Sigma \Pi_2 \Sigma \Pi_1 \Pi_1 \Pi_1 \Pi_2 \Pi_2 \Sigma \Pi_2 \Pi_2 \Pi_2 \Pi_2 \Pi_2 \Pi_2 \Pi_2 \Pi_2 \Pi_2 \Pi_2$
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 $Zn_2L = Zn_2L-OH + H^+; K_{a2} = [Zn_2L-OH][H^+]/[Zn_2L]$  (2)

 $Zn_2L-OH = Zn_2L-(OH)_2+H^+; K_{a3} = Zn_2L-(OH)_2][H^+]/[Zn_2L-OH]$  (3)

#### 2.5 Kinetic Measurements

The cleavage of BNPP was monitored in a buffered water solution ( $I = 0.1 \text{ M} \text{ NaClO}_4$ , T = 25 °C) by detecting the increase in the maximum absorbance at 400 nm of the 4-nitrophenolate anion (NPat), using an extinction coefficient of 18,700 L mol<sup>-1</sup>.<sup>[20]</sup> In a typical experiment, the solution of Zn<sub>2</sub>L was prepared by thoroughly mixing 40 µL of stock solution of HL (50 mM in water) and Zn(ClO<sub>4</sub>)<sub>2</sub> (100 mM in

water) in containing 1.92 mL of 0.05 M buffer solution (HEPES and CHEM) for 30 min at 25 °C. 50 µL of BNPP (40 mM in water) was added to the above complex solution in a cuvette and mixed well. The final concentration of Zn<sub>2</sub>L and BNPP were all 1 mM. In the determination of the pH-dependent cleavage reaction in an aqueous solution, pH values vary in the region of 6.5-9.5. After addition, the mixture was allowed to equilibrate for 5 min and the reaction was followed up to 5% of BNPP cleavage. The absorbance values were converted into the concentration of the NPat ion, and the total analytic 4-nitrophenol product was calculated using the buffer pH and the  $pK_a$ of 4-nitrophenol (7.15).<sup>[21]</sup> The pseudo-first-order rate constant  $k_{obs}$  (s<sup>-1</sup>) for cleavage of BNPP were generally determined from the slopes of semilogarithmic plots of reaction progress against time by the method of initial rates which was linear with R > 0.995. In all cases, the standard deviation for the values of  $k_{obs}$  was <6%. Secondorder rate constants  $k_{Zn}$  (M<sup>-1</sup> s<sup>-1</sup>) for the catalyzed reactions were determined as the slope of the linear plots of  $k_{obs}$  vs. Zn<sub>2</sub>L concentration. All experiments were run in triplicate, and the average values were adopted. To correct for the spontaneous cleavage of BNPP, each reaction was measured against a reference cell, which was identical to the sample cell in composition except for the absence of Zn<sub>2</sub>L.

# **3 Results and Discussion**

#### 3.1 Crystal Structure of Complex 1

Crystal structure analysis reveals that 1 crystallizes in a triclinic  $P\overline{1}$  space group with a mono-cation of  $[Zn_2L(DNBA)_2]^+$ , and one BPh<sub>4</sub><sup>-</sup> acting as the counter anion. There is one ethanol molecule in the lattice for each complex. This is also confirmed by the result of element analysis. An ellipsoid drawing for the crystal structure of the cationic core in 1 with numbering scheme is depicted in Figure 1.



**Figure 1.** ORTEP drawing of the cationic core of **1**. Thermal ellipsoids for the non-hydrogen atoms are drawn at the 30% probability level. All hydrogen atoms are omitted for clarity.

In 1, the two zinc atoms are bridged by the ionized alkoxide oxygen of L and one carboxyl from DNBA in  $\mu^{1,2}$  bridging mode. Both zinc atoms are five-coordinate. In the bipyridyl pendant, the five-coordinate environment around Zn(1) is comprised of the N<sub>3</sub>O donor box from L and one oxygen donor form the bridging carboxyl. The calculated structural index,<sup>[22]</sup>  $\tau$ , is 0.51, indicating that the arrangement around the central Zn(1) atom is somewhat intermediate between trigonal-bipyramidal and square-pyramidal. In the homopiperazine pendant, the N<sub>2</sub>O donor box from L, one oxygen donor from the bridging carboxyl, and one oxygen donor from the monodentate carboxyl of another DNBA molecule finish a distorted squarepyramidal arrangement around Zn(2) ( $\tau = 0.26$ ) with the atom O(51) occupying the apical position in the coordination sphere. This difference in the environment around each zinc atom can be ascribed to the asymmetry of the ligand. The Zn-O distances [Zn(1)–O(1), 1.923 (2) Å; Zn(2)–O(1), 1.988 (2) Å; Zn(1)-O(41), 2.051(2) Å; and Zn(2)-O(42), 2.026(2) Å] for the bridging alkoxide and carboxyl groups indicate the bridges in 1 are asymmetric. The bond length of Zn(1)-O(1) is obviously shorter than that of Zn(2)–O(1), partly because the coordination potency of the bipyridyl pendant is stronger than that of the homopiperazine pendant. The distance between the two zinc atoms is 3.426 Å, and the ionized alkoxide bridging angle [Zn(1)-O(1)-Zn(2)] is  $122.33(10)^{\circ}$ . In order to chelate a metal ion, the seven-membered homopiperazine ring has to adopt a less stable boat conformation. The bond angle N(30)-Zn(2)-N(34) is 74.88(9)°. This value deviates significantly from the normal bond angle observed in complexes with square-pyramidal arrangement, suggesting that the homopiperazine pendant is in a strained fashion.<sup>[18]</sup>

#### 3.2 Potentiometric Studies of Zn<sub>2</sub>L

The potentiometric titration was carried out in a pH range of 3.5–10.0. The solution started to turn turbid when the pH value was above pH 10.0 because of the precipitation of zinc hydroxide which impeded the data processing. The pH titration of HL (1 mM), HClO<sub>4</sub> (0.5 mM), and Zn(ClO<sub>4</sub>)<sub>2</sub> (2 mM) in water at 25 °C and I = 0.1 M (NaClO<sub>4</sub>) gave three well-defined inflections and revealed the formation of stable zinc complexes at pH > 4 with simultaneous deprotonation of the alcohol OH (i.e., Zn<sub>2</sub>HL to Zn<sub>2</sub>L). A conclusion derived from the observation of the neutralization break at eq(OH<sup>-</sup>) = 3.5 (see Figure 2) can be drawn that the zinc complex Zn<sub>2</sub>L-(OH)<sub>2</sub> will form and remains stable up to pH 10. Moreover, this specie was also proved by the ESI-MS spectra, in which one peak at m/z565.2 can be ascribed to the positively charged ion of [Zn<sub>2</sub>L(OH)<sub>2</sub>(H<sub>2</sub>O)]<sup>+</sup>.

From the analysis of the pH titration data, the deprotonation constants  $pK_{a1}$  of 5.45,  $pK_{a2}$  of 7.02, and  $pK_{a3}$  of 8.73 were obtained [see Equations (1)–(3)]. The extremely small  $pK_{a1} < 6$  observed for alcohol linker in Zn<sub>2</sub>L is attributed to double coordination of the alkoxide to two zinc(II) ions. Facile deprotonation of an alcohol upon binding of two Zn<sup>II</sup> was also observed by *Valtancoil*<sup>[23]</sup> and *Morrow*<sup>[24]</sup> for similar alkoxide-bridged dinuclear Zn<sup>II</sup> complexes. The  $pK_{a2}$  of Zn<sub>2</sub>L corresponds to the



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Figure 2. pH titration curve of HL (1 mM) and  $Zn(ClO_4)_2$  (2 mM) in water (10 mL).

deprotonation of zinc coordinated water in the homopiperazine pendant. Although a value of 7.02 is somewhat like the usual  $pK_a$  (<8) of a bridge water in dinuclear zinc(II) complexes,<sup>[25]</sup> it is still comparable with the  $pK_a$  value of 6.7 determined for the unbridged zinc-coordinated water in an alcohol-linked 1,3bis(bis-pyridin-2-ylmethylamino)propan-2-ol dizinc complex.<sup>[26]</sup> The theoretical calculations on the relationships of  $pK_a$ , coordination numbers, and nucleophilicity among Zn<sup>II</sup> complexes indicate that lower coordination numbers at the central metal atom lower  $pK_a$  values of bound water.<sup>[27]</sup> Therefore, the relatively lower  $pK_{a2}$  value may be partly the results of the lower coordination number of zinc ion in the homopiperazine pendant. The higher  $pK_{a3}$  of  $Zn_2L$  corresponds to the further deprotonation of another zinc-coordinated water in bipyridyl pendant to form  $Zn_2L^{3+}-(OH^{-})_2$ , and it is comparable with that observed for some dizinc(II) complexes containing dinclear compartmental ligands.<sup>[6]</sup> A typical diagram for the species distribution as a function of pH (5.5-10) at [total zinc(II)] = 2 mM and [HL] = 1 mM is displayed in Figure 3.



**Figure 3.** Species distribution curves (solid line) and the effect of pH on the hydrolysis of BNPP (filled square) for zinc(II)-HL system (T = 25 °C, [HL] = 1 mM, [Zn<sup>2+</sup>] = 2 mM, [BNPP] = 1 mM).

#### 3.3 Phosphodiesterase Activity of Zn<sub>2</sub>L

BNPP is often used as a DNA model compound in the investigation of phosphodiesterase activity. The test was carried out in buffers in order to mimic biological conditions. The reaction was monitored by following the increase of NP from BNPP. The dependence of the reaction rate on pH for the BNPP cleavage promoted by Zn<sub>2</sub>L (1 mM) in aqueous solution at 25 °C shows a bell-shaped profile with an optimum at about pH 7.9 (Figure 3). Notable cleavage activity  $(k_{obs})$  was observed at pH > 6, parallel with the formation of the dinuclear species Zn<sub>2</sub>L-OH. These results strongly suggest that the monohydroxo form of the complex is the activity species responsible for cleaving BNPP. Based on the pH/rate constant profile, the successively formed Zn<sub>2</sub>L-(OH)<sub>2</sub> complex is also able to promote the cleavage, and its activity is almost half of the former Zn<sub>2</sub>L-OH species partly due to the leaving tendency of the OH group of Zn-OH in bipyridyl pendant becomes lower, which reduce the binding ability of BNPP to the central metal atoms.

Figure 4 shows the effect of Zn<sub>2</sub>L concentrations on the  $k_{obs}$  for the cleavage of BNPP at pH 7.9 and 25 °C. The rate of BNPP cleavage initially increases linearly with the increase of Zn<sub>2</sub>L, indicating the reaction is first-order for Zn<sub>2</sub>L concentration. The calculated pseudo-second-order rate constant for Zn<sub>2</sub>L determined from the plot is  $7.75 \times 10^{-2} \text{ m}^{-1} \text{ s}^{-1}$ .



Figure 4. Dependence of the pseudo-first-order rate constants on the concentration of  $Zn_2L$  at pH 7.9 and 25 °C. I = 0.10 M (NaClO<sub>4</sub>), [BNPP] = 1 mM, and [HEPES buffer] = 50 mM.

The initial rate of BNPP cleavage was also determined as a function of the substrate concentration at pH 7.9. The data depicted in Figure 5 reveals saturation kinetics with Michaelis-Menten-like behavior in the system, typical for native metalloenzymes. The experiment data were fitted to the Michaelis-Menten model by evaluating from the Lineweave-Burk double-reciprocal plot, which resulted in a Michaelis constant ( $K_{\rm M}$ ) of  $7.22 \times 10^{-3}$  M and a catalytic rata constant ( $k_{\rm cat} = V_{\rm max}/$  [Zn<sub>2</sub>L]) of  $6.30 \times 10^{-4}$  s<sup>-1</sup>. The second order rate constant  $K_{\rm BNPP}$  ( $k_{\rm cat}/K_{\rm M}$ ) for BNPP is  $8.73 \times 10^{-2}$  m<sup>-1</sup>. The reciprocal of the Michaelis-Menten constant could be treated as the substrate binding constant, that is,  $K_{\rm b} = 1/K_{\rm M} = 138.5$  m<sup>-1</sup>. The

low  $K_{\rm b}$  is fit for the observations that BNPP is a weak ligand for  $Zn_2L^{[28,29]}$  At pH 7.9 ([OH<sup>-</sup>] =  $6.31 \times 10^{-7}$  M]) and 25 °C, the first-order rate constant ( $k = k_{OH}[OH^{-}]$ ) of automatic hydrolysis of BNPP is calculated to be  $3.66 \times 10^{-12}$  s<sup>-1</sup> with  $k_{OH}$ =  $5.8 \times 10^{-6} \text{ m}^{-1} \cdot s^{-1}$  for BNPP ( $V_{\text{auto}} = k_{\text{OH}}[\text{BNPP}][\text{OH}^{-}]$ ).<sup>[30]</sup> Therefore, it can be concluded that in pH 7.9 buffer solution of 50 mM HEPES, 0.1 M NaClO<sub>4</sub> in water and at 25 °C, complex Zn<sub>2</sub>L can promote the hydrolysis of BNPP by a factor of  $1.7 \times 10^8$ . Under the same conditions, the catalytic activity of complex 1 was also measured, and it can promote the hydrolysis of BNPP by a factor of  $5.6 \times 10^5$ , indicating that the bridging carboxyl group is an inhibitor of the catalytic species and reduce the catalytic hydrolysis rate. These results further interpret that BNPP coordinates the two central zinc atoms by a bidentate bridging mode in the catalytic reaction. In general, since different reaction conditions such as catalysts loading, pH value of reaction system, reaction medium, and models of phosphodiester were used, it is hard to critically evaluate and compare the reactivity of the reported di-Zn<sup>II</sup> based systems. Nevertheless, complex Zn<sub>2</sub>L reported upon herein are competitive with most synthetic dinuclear metallohydrolase models.[31-33]



**Figure 5.** Saturation kinetic experiments for the cleavage of BNPP by  $Zn_2L$  at pH 7.9 and 25 °C. I = 0.10 M (NaClO<sub>4</sub>), [Zn<sub>2</sub>L] = 0.02 mM, and buffer solution (50 mM of HEPPS). Inset: Lineweaver-Burk double-reciprocal plot.

The <sup>31</sup>P NMR spectroscopic method was used to further investigate the cleavage reaction at 25 °C with a solution of BNPP and Zn<sub>2</sub>L complex formed in situ from ligand L and Zn<sup>2+</sup> in a molar ratio of 1/2 in D<sub>2</sub>O (Figure 6). The increase of the <sup>31</sup>P resonance for the cleavage product (NP) and the decrease of the <sup>31</sup>P resonance for BNPP were monitored with time. After 15 h, a new signal attributed to the cleavage product NP was abserved at –6.2 ppm, and this signal became more intense after 2 d. No further hydrolysis of NP producing free phosphate could be detected even after 2 weeks. The observation suggested that the phosphate monoester NP produced by the hydrolysis of BNPP binding to the dinuclear zinc complex, and that no further hydrolysis occurs.



**Figure 6.** <sup>31</sup>P NMR spectra recorded during the cleavage reaction of BNPP by the  $Zn_2L$  formed by mixing  $Zn^{2+}$  ion and ligand HL in a 2/1 molar ratio at 25 °C as a function of time: (a) t = 0; (b) t = 15 h; (c) t = 2 d.

### 3.4 Mechanism of Phosphodiester Hydrolysis Promoted by Zn<sub>2</sub>L

On the basis of the kinetics data, pH potentiometric titration studies and crystal structure of 1, a double Lewis acid activation mechanism is proposed for the catalytic hydrolysis of BNPP by Zn<sub>2</sub>L, which is similar to the reported cases.<sup>[34]</sup> As shown in Scheme 1, the catalytic hydrolysis cycle may contain the following steps: firstly, the active species  $\mathbf{B}$  is formed through the dissociation of a zinc-bound water in homopiperazine pendant at pH 7.9, and the phosphodiester BNPP coordinates to the two central zinc atoms as a bidentate ligand by replacing two coordination water molecules, forming the BNPP adduct C. This species was observed in the ESI-MS spectrum of mixture solution of in situ prepared complex Zn<sub>2</sub>L and HBNPP, in which one positively charged peak at m/z 869.4 corresponds to [Zn<sub>2</sub>L(OH)(BNPP)]<sup>+</sup> (C); secondly, the zincbound deprotonated water molecule group acts as a nucleophile and attacks BNPP, forming a transition complex D; thirdly, the P-O bond in D breaks to release p-nitrophenolate followed by reestablishment of the starting complex A, accomplished by the replacement of NPP with water. In the first step, an equilibrium may exist between **B** and **C** since BNPP is a weak ligand for Zn<sub>2</sub>L.

The intrinsic activity of dinuclear catalyst is highly dependent upon the metal-metal distance. The appropriate intermetallic separation (3.426 Å in 1) can facilitate the cooperative action for the phosphate bridging, and thus BNPP is activated by the zinc atoms as in other dinuclear metallohydrolases.<sup>[1-3]</sup> In addition, acting as Lewis acid, one zinc ion of complex  $Zn_2L$  can promote the deprotonation of a water molecule to form nucleophile to attack phosphate group. Furthermore, the two zinc ions connect the two reactants (substrate BNPP and nucleophile OH<sup>-</sup>), neutralizing the electrostatic repulsion between the two negatively charged species.





Scheme 1. Proposed mechanism for hydrolysis of BNPP by Zn<sub>2</sub>L.

# **4** Conclusions

The asymmetric dinuclear complex Zn<sub>2</sub>L holds essential physical features in common with the nuclease P1, phospholipase C, and purple acid phosphatases. The complex can efficiently catalyze the hydrolysis of BNPP with a rate approximately 10<sup>8</sup>-fold higher than that of the uncatalyzed reaction. The hydrolysis of BNPP promoted by Zn<sub>2</sub>L may be triggered by the phosphodiester coordination to two central zinc atoms in a bidentate bridging mode, followed by an attack of the deprotonated Zn-bound water (Zn-OH-) to the phosphorus atom, and finally resulting in cleavage of the P-O bond. Since the correlation between the phosphatase activity and the coordination environment of a complex is an important factor to be considered in designing efficient artificial phosphatases, this research provides some insights into this issue and affords us the knowledge to obtain more efficient phosphodieserase mimics in the future.

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