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ORIGINAL ARTICLE

Novel mono- and dinucleating ligands-containing artificial di- and tetrahistidine and their zinc(II) complexes as a structural *phosphotriesterase* models for the hydrolysis of *p*-nitrophenyl diphenylphosphate (*p*-NPDPP)

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Abstract Two new artificial peptides with histidine side chains, namely *N*-methyl *N*,*N*′-*bis*(Im-bzl-L-histidylmethylestermethyl)amine L1 and *N*,*N*′,*N*″,*N*‴-tetrakis(Im-bzl-L-histidylmethyl-estermethyl)ethylene diamine L2 have been synthesized and were shown to form stable zinc complexes $[L1Zn(H_2O)_2](ClO_4)_2$ **1** and $[L2Zn_2(H_2O)_3](ClO_4)_4$ **2**, respectively. Solution studies (pH–^{1}NMR titrations) of the ligand L2 in the presence of zinc ions were also reported. The catalytic activity of zinc complex species **1** and **2** as structural *phosphotriestrase* models were tested on the hydrolysis/detoxification of *p*-nitrohenl diphenylphosphate (*p*-NPDPP). From the correlation between the pH-rate profiles and the species distribution curves, the catalytically active species could be identified. On the basis of R-dependence as well as the rate acceleration of each complex, the possibility of cooperative action of zincs in dinuclear zinc complex is debated.

Keywords Synthesis · Artificial peptide · *Phosphotriestrase* · Zinc(II) model complexes · Phosphate triester hydrolysis

Introduction

Phosphate esters and related phosphorus(V) materials have been employed for chemical warfare, pest control, and numerous industrial tasks. And most of the phosphates have strong toxicity for human body and animals. So the fast decomposition of toxic P(V) compounds to nontoxic materials is useful from the view point of environmental protection. Many metalloenzymes and proteins contain histidine imidazole, and the imidazole group of histidine plays an important role in stabilizing the active sites of many metalloproteins [1]. In particular, *alkaline phospatase* [2] and *phosphotriesterase* [3, 4] are known to contain several imidazole ligands per metal center. Bimetallic cores exist at the active sites of many metalloenzymes and play essential roles in biological systems by the interplay a pair of metal ions [3, 4]. A typical example is *Pseudomonas diminuta phosphotriesterase* that require at least two metal ions in close proximity to hydrolyse the toxic phosphate triesters. The binuclear zinc(II) site was confirmed by the 2.1 A resolution X-ray crystal structure of the enzyme [5].

One of the challenging problem in studies related to enzymes and enzyme-models is to elucidate the overall catalytic mechanism and to identify the role of metal ions. There are three modes of activation that a metal ion can provide for accelerating the rate of phosphate ester hydrolysis [6]: Lewis-acid activation (coordination of phosphoryl oxygen to the metal), nucleophile activation (coordination of nucleophile such as hydroxide to the metal), and leaving group activation (coordination of the leaving group oxygen to the metal). Additionally, metal coordinated hydroxides could act as an intramolecular general base catalyst and metal coordinated water molecules as an intramolecular general acid catalyst. Dinuclear metal complexes are able to provide double Lewis acid activation for hydrolyzing phosphate esters by initially bridging the two metal centers with the two phosphoryl oxygens. For model studies it became clear that there is considerable cooperativity between the two metal centers

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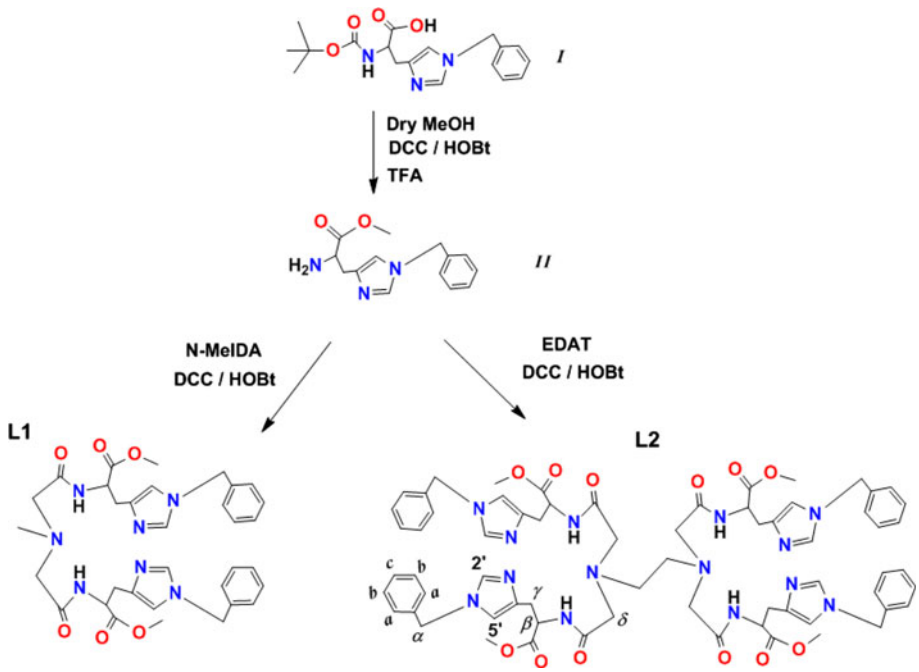
in double Lewis acid activation for phosphodiester hydrolysis.

Recently many studies were conducted related to mono- and polynuclear zinc model complexes [7–13], but critically judging, most of these complexes are not structural models for the native system, do not reflect the structure of the active sites. However, in natural enzymes, proteins exist as being folded, and the amino acid side chains, which provide ligation sites can bind metal ions, and the structure at the active site of these enzymes gives effective catalytic activity. In order to design enzyme model complexes, it is important to reproduce/mimic the structure of the active site of enzymes.

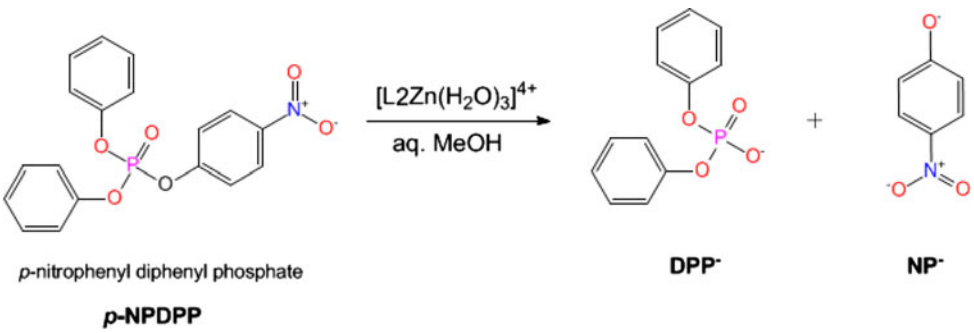
As part of our studies in biomimetic hydrolytic zinc enzymes [14–20] and with the aim to mimic the key features of these metalloenzymes, we designed and synthesized two new ligands-containing di- and tetrahistidines, namely *N*,*N*′-*bis*(Im-bzl-L-histidine(His)-methylestermethyl)amine L1 and *N*,*N*′,*N*″,*N*‴-tetrakis(Im-bzl-L-histidine-(His)methyl-ester-methyletheylene diamine L2 (Scheme 1) and of their mono- and dinuclear zinc complexes. The pk_a values of the ligand L2 as well as the stability constants and deprotonation equilibria of their zinc complexes are reported. Further information about zinc complexes of the ligands is obtained from R-dependent ^{1}H NMR titrations. Finally, the activities of both mon- and dinuclear zinc complex species toward the hydrolysis of *p*-nitrophenyl diphenylphospate (*p*-NPDPP) as a function of R is also reported. The phosphate ester was selected as the principal substrate for this study because: (a) It is an easily handled stimulant of the very dangerous phosphorus(V) compounds such as Soman (Scheme 2), (b) The hydrolysis reaction can be easily monitored spectrophotometrically.

Experimental section

CAUTION: Perchlorate salts of amine ligands and their metal complexes are potentially explosive and should be handled in small quantities.



Scheme 1 The synthesis of the artificial peptides L1 and L2



Scheme 2 The hydrolysis of *p*-NDPP by using zinc(II) complex **2**

Materials

All reagents and solvents used were of analytical grade. *N*(α-*t*-Butoxycarbonyl-*N*-(π)-benzyl-L-histidine was purchased from Sigma. *P*-Nitrophenyl diphenylphosphate (*P*-NPDPP) was prepared as previously described [21]. The buffer: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), was purchased from Sigma.

Syntheses

Synthesis of Im-bzl-L-histidylmethylester ***I***

Dry MeOH (83 mL, 2.61 mmol) was added to an ice-cold solution of dicyclohexylcarbodiimide (DCC, 0.59 g, 2.82 mmol), 1-hydroxybenzotriazole (HOBt, 0.4 g, 2.60 mmol) and *N*(α)-*t*-butoxycarbonyl-*N*(π)-benzyl-L-histidine (0.89 g, 2.63 mmol) in dimethylformide (DMF, 5 mL). pH was adjusted to 7.0 by *N*-methylmorpholine (NMM). The reaction mixture was stirred at 0 °C for 2 h and at room temperature for 12 h, filtered, and evaporated to give a solid which was suspended in dichloromethane (30 mL), extracted with 10% sodium carbonate(10 mL), dried which was dissolved in ethylacetate (EtOAc, 5 mL) and aside at 4 °C overnight. Further dicyclohexylurea (DCU) was then filtered off and solvent was evaporated to give an hygroscopic powder, yield 0.90 g, 96%, FAB-MS, M^+ 359. ^{1}H NMR ($CDCl_3$): 7.36 (s, 1H; im *2′*), 7.30–7.22 (m, 3H; H-*b,c*), 7.05–7.03 (d, $J = 9.0$ Hz, 2H; H-*a*), 6.58 (s, 1H; im *5′*), 5.83 (br, s, 1H, amide-NH), 4.93 (s, 2H, $-C^{\alpha}H_2$), 4.49–4.44 (m, 1H, His-$C^{\beta}H$) 3.57 (s, 3H, OCH_3), 3.02–2.93 (m, 2H, His-$C^{\delta}H_2$), 1.34 (s, 9H, BOC).

To 0.90 g (2.51 mmol) of ***I***, 5 mL of 98% formic acid was added; the removal of BOC group was monitored by TLC. After 15 h the formic acid was removed in vacuo. The residue was taken in water (10 mL) and washed with EtOAc (4 × 15 mL). The pH was then adjusted to 8 with solid sodium bicarbonate and extracted with EtOAc (3 × 20 mL). The extracts were pooled, washed with saturated brine, dried over sodium sulfate, and concentrated to dryness. ^{1}H NMR ($CDCl_3$): 7.48 (s, 1H; im *2′*), 7.39–7.27 (m, 3H; H-*b,c*), 7.11–7.09 (d, $J = 8.4$ Hz, 2H; H-*a*), 6.66 (s, 1H; im *5′*), 5.04 (s, 2H, $-C^{\alpha}H_2$), 4.90–4.86 (m, 1H, His-$C^{\beta}H$), 3.64 (s, 3H, OCH_3), 3.13–3.10 (m, 2H, His-$C^{\delta}H_2$).

Synthesis of N,N′-bis(Im-bzl-L-histidylmethylestermethyl)amine L1

N-Methyliminodiacetic acid, MIDA (0.188 g, 0.3 mmol) was added to an ice cold solution of dicyclohexyl carbodiimide (DCC) (0.078 g, 0.38 mmol), hydroxyl-benzotriazole (HOBt) (0.53 g, 0.34 mmol), and histidine(His) methylester ***I*** (0.155 g, 0.6 mmol) in dimethylformamide (DMF) (8 mL) and the pH was adjusted to 7.0 using *N*-methyl morpholine (NMM). The reaction mixture was stirred at 0 °C for 2 h and at room temperature for 12 h, filtered and evaporated to produce a solid which was suspended in CH_2Cl_2 (30 mL), extracted with 10% aqueous sodium carbonate (10 mL) and dried. The remaining solid was dissolved in EtOAc (5 mL) and kept at 4 °C overnight. Dicyclohexylurea (DCU) was then filtered off and the solvent was evaporated to give a white powder. Found: C 58.89, H 6.44, N 14.57. Anal. calcd for $C_{33}H_{39}N_7O_6{\cdot}2H_2O$: C 59.59, H 6.52, N 14.75. ^{1}H NMR (CD_3OD:D_2O, 3:1): 7.74 (s, 2H; im *2′*), 7.40–7.331 (m, 6H; H-*b,c*), 7.27–7.22 (d, $J = 8.0$ Hz, 4H; H-*a*), 6.97 (s, 4H; im *5′*), 5.17 (s, 4H; $-C^{\alpha}H_2$), 4.83–4.80 (m, 2H; His-$C^{\beta}H$), 3.58 (s, 6H; OCH_3), 3.47 (s, 4H; N-CH_2), 3.13–3.07 (m, 4H; His-$C^{\delta}H_2$), and 2.44 pm (s, 3H; N-CH_3). FAB$^+$-MS: *m/z*: 629 $[M+H]^+$.

Synthesis of N,N′,N″,N‴-tetrix(Im-bzl-L-histidine-(His)methylestermethyl)-ethylene diamine L2

Ethylenediamine tetraacetic acid, EDTA (0.117 g, 0.4 mmol) was added to an ice cold solution of dicyclohexylcarbodiimide (DCC) (0.103 g, 0.5 mmol), hydroxyl-benzotriazole (HOBt) (0.07 g, 0.45 mmol), and histidine(His) methylester ***I*** (0.413 g, 1.6 mmol) in dimethylformamide (DMF) (8 mL) and the pH was adjusted to 7 using *N*-methyl morpholine (NMM). The reaction mixture was stirred at 0 °C for 2 h and at room temperature for 12 h, filtered and evaporated to produce a solid which was suspended in CH_2Cl_2 (30 mL), extracted with 10% aqueous sodium carbonate (10 mL) and dried. The remaining solid was dissolved in EtOAc (5 mL) and kept at 4 °C overnight. Dicyclohexylurea (DCU) was then filtered off and the solvent was evaporated to give a white powder. Found: C 63.67, H 6.34, N 15.59. Anal. calcd for $C_{66}H_{76}N_{14}O_{12}$: C 62.68, H 6.07, N 15.55. ^{1}H NMR (CD_3OD:D_2O, 3:1): 7.71 (s, 4H; im *2′*), 7.42–7.31 (m, 12H; H-*b,c*), 7.27–7.25 (d, $J = 8.0$ Hz, 8H; H-*a*), 6.96 (s, 4H; im *5′*), 5.15 (s, 8H; $-C^{\alpha}H_2$), 4.85–4.81 (m, 4H; His-$C^{\beta}H$), 3.58 (s, 12H; OCH_3), 3.48 (s, 8H; N-$C^{\gamma}H_2$), 3.14–3.09 (m, 8H; His-$C^{\delta}H_2$), and 2.51 (m, 4H; N-C H_2-CH_2-N-). FAB$^+$-MS: *m/z*: 1257 $[M+H]^+$.

Synthesis of the mononuclear zinc(II) complex ***1***

L1 (50 mg, 0.04 mmol) was dissolved in 3 mL aqueous metanol (75%, v/v) and the pH was adjusted to 7.0 using 1 M HNO_3. Aqueous methanolic solution of $Zn(ClO_4)_2{\cdot}6H_2O$ (30 mg, 0.08 mmol) was then added. A white precipitate was produced, filtered off by membrane filter, washed out by ether, and dried up in vacuo. Found: C, 42.34; H, 4.68; N, 10.54; Cl, 7.32%. Anal. Calcd. For $C_{33}H_{45}Cl_2N_7O_{17}Zn$ ($[LZn(OH_2)_2](ClO_4)_2{\cdot}H_2O$: C, 41.81; H, 4.78; N, 10.34; Cl,

7.47%. ^{1}H NMR (CD_3OD:D_2O, 3:1): 7.81 (s, 2H; im *2′*), 7.45–7.37 (m, 6H; H-*b,c*), 7.33–7.25 (d, $J = 8.2$ Hz, 4H; H-*a*), 7.07 (s, 4H; im *5′*), 5.24 (s, 4H; $-C^{\alpha}H_2$), 4.95–4.86 (m, 2H; His-$C^{\beta}H$), 3.66 (s, 6H; OCH_3), 3.56 (s, 4H; N-CH_2), 3.18–3.10 ppm (m, 4H; His-$C^{\delta}H_2$) and 2.52 pm (s, 3H; N-CH_3).

Synthesis of the dinuclear zinc(II) complex **2**

L2 (50 mg, 0.04 mmol) was dissolved in 3 mL aqueous metanol (75%, v/v) and the pH was adjusted to 7.0 using 1 M HNO_3. Aqueous methanolic solution of $Zn(ClO_4)_2 \cdot 6H_2O$ (30 mg, 0.08 mmol) was then added. A white precipitate was produced, filtered off by membrane filter, washed out by ether, and dried up in vacuo. Found: C, 42.22; H, 4.77; N, 10.45; Cl, 7.51%. Anal. Calcd. For $C_{66}H_{76}N_{14}O_{12}$ $C_{66}H_{88}Cl_4N_{14}O_{34}Zn_2$ ($[LZn_2(OH_2)_3](ClO_4)_4 \cdot 3H_2O$): C, 41.85; H, 4.68; N, 10.36; Cl, 7.49%. ^{1}H NMR (CD_3OD:D_2O, 3:1): 7.77 (s, 4H; im *2′*), 7.46–7.35 (m, 12H; H-*b,c*), 7.29–7.27 (d, $J = 8.4$ Hz, 8H; H-*a*), 6.98 (s, 4H; im *5′*), 5.16 (s, 8H; $-C^{\alpha}H_2$), 4.86–4.81 (m, 4H; His-$C^{\beta}H$), 3.58 (s, 12H; OCH_3), 3.52 (s, 8H; N-$C^{\gamma}H_2$), 3.15–3.11(m, 8H; His-$C^{\delta}H_2$), and 2.57 (m, 4H; N-C H_2-CH_2-N-).

Potentiometric titration measurements

The potentiometric titrations were carried out with a TOA AUT-501 automatic titrator connected to a TOA ABT-511 automatic burette with a combined glass electrode. The temperature was kept constant at 25 °C. The combined glass electrode was calibrated using standard aqueous buffers (acetate buffer: pH 4.01; phosphate buffer: pH 6.86; borate buffer: pH 9.18). Solutions were made up with MeOH:H_2O (75%, v/v). The ionic strength was adjusted to 0.1 M by addition of appropriate amounts of analytical grade $NaNO_3$. The ligand concentration was 0.5 mM and it was acidified by 0.1 M HNO_3. The initial volume of solutions at the beginning of titrations were set to 50 mL. Dropwise addition of NaOH solution was regulated automatically depending on the pH-change after each addition. From these titrations, ligand deprotonation constants have been determined. To get information about the stability constants of the zinc complexes of each ligand and about the deprotonation constants of Zn^{2+}-bound water molecule, titrations in the presence of zinc ions were also carried out. In each case, appropriate amounts of $Zn(ClO_4)_2 \cdot 6H_2O$ were added to the solutions, and were acidified and titrated the same way as it was mentioned above. Equilibrium constants have been calculated using the program BEST and species distributions were derived using the program SPE [22].

^{1}H NMR titration measurements

To get deeper insight into complex formation behavior of the ligand, an additional technique, ^{1}H NMR titration experiments have been performed. There are several advantages of ^{1}H NMR compared to pH-potentiometric titration, since this method can reveal the kinetic behavior of complexation, and can confirm the stoichiometry of complexes determined before by pH-titration. ^{1}H NMR has the advantage that kinetic exchange processes can be followed, the kinetic stability/lability of the complex can be decided from these kinds of studies. From the R-dependence of chemical shifts we can get more insight about that which protons are effected (thus shifted) most, i.e. gives evidence about the location of protonation/complexation sites [23–27]. To confirm stoichiometry of zinc complexes of the ligand, ^{1}H NMR analyses were undertaken in order to investigate the effect of the added zinc on the shape and the chemical shift of ligand peaks. The concentration used were analogous to that was used in pH-titration; ionic strength was kept constant at 0.1 M by using $NaNO_3$. 75% v/v MeOH-d_4/D_2O was used as solvent for the titration. R (= $[Zn^{2+}]/[L]$) was increased by addition of concentrated D_2O solution of $Zn(ClO_4)_2$ while pD was kept constant (was adjusted to 7.0 or to 7.5). Samples were measured at 30 °C. Chemical shifts are reported relative to the resonance of the resonance signal of sodium 2,2-dimethyl-2-silapentane (DSS).

Experimental for kinetic measurements

The histidine units attached to EDTA through peptide bond model the histidine side chains of the active site of *phosphohydrolase* enzymes. The purpose of using zinc as a complexing ion was to keep the structural similarity between the native system and our model compounds. In order to show that these zinc complexes do not only serve as structural mimics, kinetic studies have been carried out to investigate the effect of the presence of histidine-containing peptide–zinc complexes on the hydrolysis rate of a phosphotriester substrate, *p*-nitrophenyl diphenyl-phosphate, *p*-NPDPP. In this part we report the results of a series of experiments carried out at different pH and zinc-to-ligand ratio. From these profiles we make suggestion about the active species, and from the comparison of activities of different complexes we attempt to propose reaction mechanism and some comments on the structure of zinc complexes are also made.

The rate of the hydrolysis of *p*-NPDPP catalyzed by zinc complex **2** was measured in aqueous MeOH (MeOH:H_2O, v/v). The hydrolysis rates were measured by following the increase of absorption at 402 nm of the released

p-nitrophenolate as a reaction product. The pH of solution was adjusted by using HEPES buffer (20 mM) (20 mM, pH = 7.5). Zinc complex **2** (0.5 mM) was incubated at 50 °C, while the applied *p*-NPDPP concentration was 20 mM. All reactions showed first-order characteristics. Reactions rates are corrected by blank experiments which are made up similarly but without the addition of zinc complex **2**. The first-order decrease of substrate concentration can be expressed by the differential equation: $d[p\text{-NPDPP}]/dt = -k_{obsd} \cdot [p\text{-NPDPP}]$ and $\ln([p\text{-NPDPP}]_t/[p\text{-NPDPP}]_o) = -k_{obsd} \cdot t$.

Results and discussion

As it was emphasized in the object of this work, the aim is to mimic the structure of the *phosphohydrolase* active site by not only utilizing zinc as natural ion in the artificial model complexes, but also to incorporate peptides as the building blocks of large proteins. On the other hand, these peptides serve as donor sites by mostly histidine imidazoles, so our target is to synthesize artificial histidine-containing peptides. Most of the model studies do not deal with peptides or peptide-like compounds, so the synthesis is rather connected to the well-established solution phase methods of peptide synthesis [28]. An approach to the formation of the peptide bond is the use of coupling reagents which compounds can be added in stoichiometric amount to the mixture of C-protected amino acid/peptide. The benzyl groups attached to the pyrrolic nitrogens of histidine imidazoles will represent a bulky substituent thus will prevent octahedral complex formation, and at the same time will serve as hydrophobic hindering, modelling the hydrophobic environment around the active site of the hydrolytic enzymes. The purity of all isolated compounds were checked by TLC, then characterized by elemental analysis and both ^{1}H NMR and FAB-mass spectroscopies.

Protonation constants (pK_a) of the artificial tetrapeptide ligand, stability constants (log*K*) of zinc(II) complexes and deprotonation constants ($pK_a(H_2O)$) of zinc-bound water molecules

The newly obtained ligands provided an opportunity to examine the effect of changing the number of histidine side chains on the ligand deprotonation and complexation constants, and nevertheless on the pK_a of the coordinated water molecule. Typical set of pH titration and species distribution curves for the ligand in the absence and in the presence of stoichiometric amounts of Zn^{2+} are shown in Fig. 1. The obtained protonation constants (pK_a) are summarized in Table 1.

In L2, four deprotonation steps were found, these four acidic deprotonation constants are assigned to imidazole nitrogens (5.82, 5.06, 4.92, and 4.17). On the course of titration of the ligand, four equivalents of base were consumed. These four acidic pK_a's were assigned to the histidine imidazole nitrogens, while the two tertiary nitrogens are too basic to be determined. One interesting pattern related to the imidazole pK_a's is that their deprotonation takes place in almost equal steps which is indication about the relative symmetry of the ligand. The effect of the presence of these basic tertiary amines gives rise in the acidity of imidazole nitrogens (Figs. 2, 3, 4).

It is interesting to compare our pK_a values to other related histidine-containing peptides. In dipeptides, where the amino group of histidine is unprotected, the pK_a of the imidazole nitrogens is lower than in histidine itself: e.g. 5.39 in His–Gly, 5.65 in His–Leu while this value is 5.87 in histidine [29, 30]. In structurally similar peptides, *N*,*N*′ di-L-histidylethane-1,2-diamine (dhen), pK_a values of 4.62 and 5.32 were reported for the imidazole nitrogens [31], while these values in another compound im-bzl *N*,*N*″ dihistidyldiethylenetriamine are 4.3 and 4.9, respectively [32].

The potentiometric pH titration curve for ligand in the presence of equimolar Zn^{2+} revealed complex formations until [OH]/[L2] = 4. But Zn(II) complexes reacted with more OH^- than what was needed to fully deprotonate the ligand. This means each complex contains one extra deprotonable group which is not present in the ligands themselves, providing evidence for the deprotonation of an excessive group which was not reacting when titration of ligand itself was carried out.

The extra deprotonation can be assigned to two different processes: (i) deprotonation of the coordinated water molecule; or (ii) metal-promoted deprotonation of the amides or the basic tertiary amino nitrogens. Rabenstein et al. [23] showed that zinc(II)-promoted deprotonation of amide nitrogens in imidazole-containing peptides generally results in kinetically stable complexes. For thae case of L2, the ^{1}H NMR spectra showed that, the chemical shifts of the methylene protons ($C^{\gamma}H_2$ and N-*C* H_2-CH_2-N-) near to the amide nitrogen and tertiary nitrogens are considerably altered. In order to clarify whether the amide nitrogens and the tertiary nitrogens are coordinated to zinc or not, we have conducted ^{1}H NMR titration measurements for similar artificial imidazole-containing peptides namely, *N*,*N*′,*N*″-tris(*N*-benzyl-L-histidinyl)tri(2-aminoethyl)amine and *N*,*N*′,*N*″-tris[(1S)-2-methoxy-2-oxy-1-(1-benzylimidazol-4-ylmethyl)]nitrilotriacetamide in the presence of equimolar amount of Zn^{2+} ion in the same solvent system [14, 33]. Through this, we found out that the methylene protons of the tri(2-aminoethyl)amine part, directly linked to both amide nitrogens and tertiary nitrogens were weakly

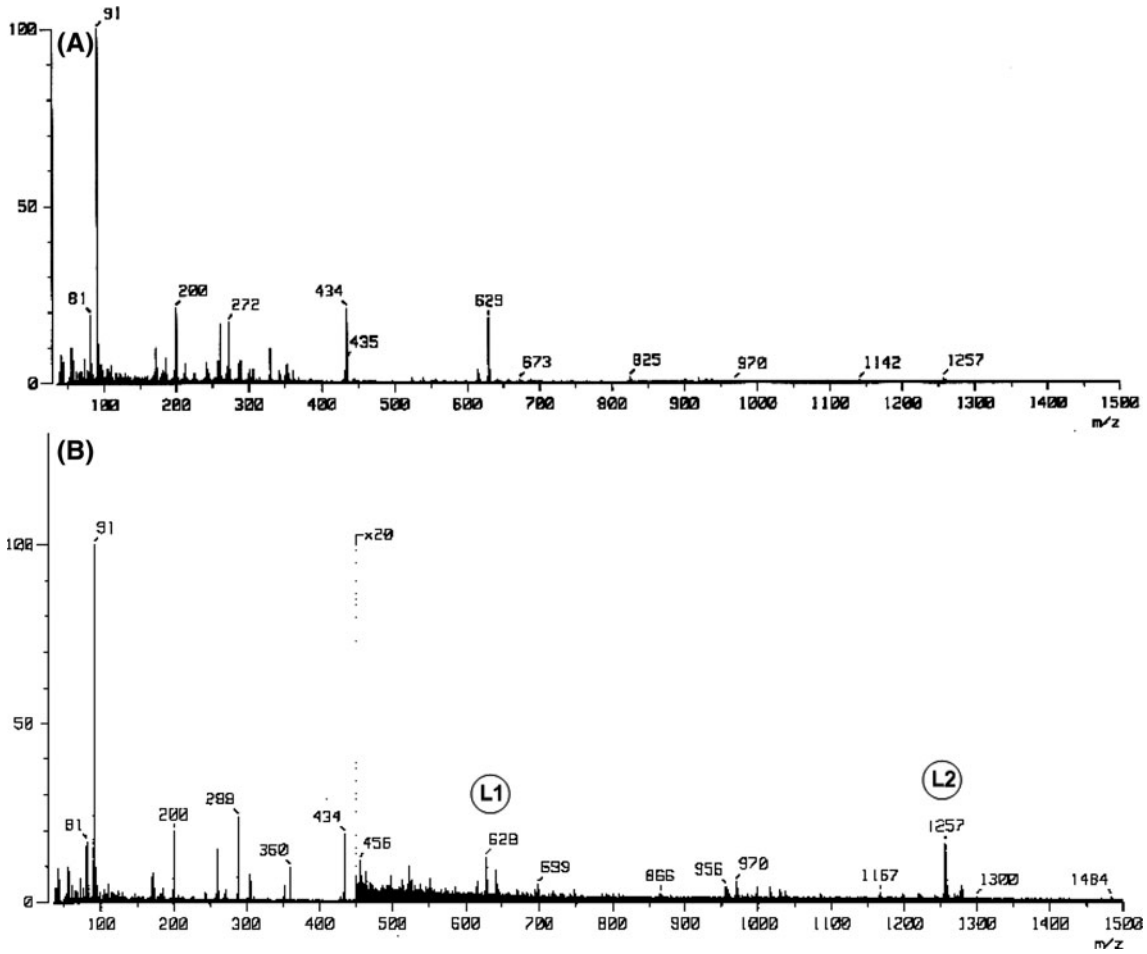


Fig. 1 FAB-mass spectra of the ligand L1 (**a**) and ligand L2 (**b**)

Table 1 Acidity constants (pk_a) of the ligand L2, stability constants ($\log k_{st}$), and deprotonation constants (pk_a) of the indicated species

Species/reactions	pk_a	$\log k_{st}$	pk_a (H_2O)
$H_4L2^{4+} = H_3L2^{3+} + H^+$	5.83		
$H_3L2^{3+} = H_2L2^{2+} + H^+$	5.08		
$H_2L2^{2+} = HL2^{+} + H^+$	4.93		
$HL^{+} = L2 + H^+$	4.17		
$Zn^{2+} + L2 = ZnL^{2+}$		3.62	
$ZnL2^{2+} + H^+ = ZnL2H^{3+}$		5.25	
$ZnL2H^{3+} + H^+ = ZnL2H_2{}^{4+}$		5.04	
$ZnL2^{2+} - H^+ = ZnL2(OH)^{+}$			7.15
$ZnL2^{2+} + Zn^{2+} = Zn_2L2^{4+}$		6.49	
$Zn_2L2^{4+} - H^+ = Zn_2L2(OH)^{3+}$			6.74
$Zn_2L2(OH)^{3+} - H^+ = Zn_2L2(OH)_2{}^{3+}$			13.72

influenced (0.03 ppm). The above result strongly indicates that the extra deprotonation is only related to the formation of zinc-bound hydroxo species.

The ligand showed a markedly different pH-titration curve when 2:1 zinc-to-ligand ratio was utilized. This is a clear indication about formation of different complexes between the ligand and zinc ion. In the presence of two equivalents of zinc, excess consumption of hydroxide was observed: two more base was reacted than that was found in the absence of zinc ion. This indicates that two extra deprotonable groups are present in L–Zn complexes formed in 2:1 zinc-to-ligand ratio.

The obtained stability constants log*K* and deprotonation constants $pK_a(H_2O)$ of zinc complexes are listed in Table 1. The potentiometric data indicated that titrimetrically detected complexes with different protonation states can exist as a function of pH. The low $\log k_{st}$ for $[L2Zn]^{2+}$ is consistent with a related complex bearing only imidazole donor functions [34]. *N*-Alkylation of the pyrrolic nitrogen in imidazole units reduces the metal binding ability of the ligand. For example in the case of tripodal *N*-methyl imidazole derivatives, log*K* decreases about 3–4 units, with a log*K* value of around 4, which is consistent with the analogous value of $[HL2Zn]^{3+}$ species.

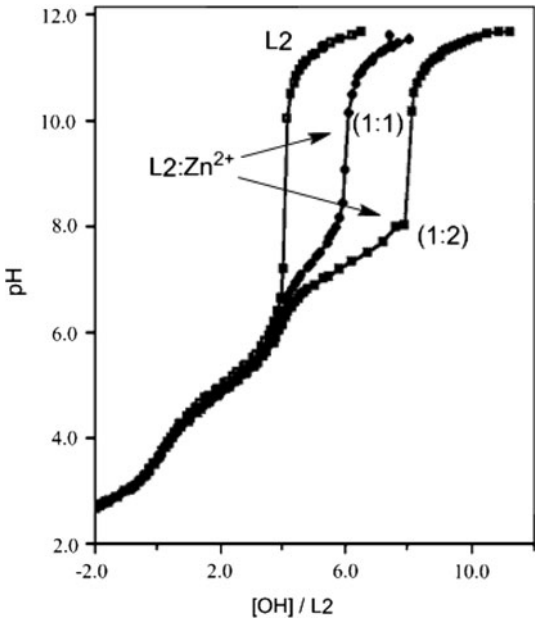


Fig. 2 pH titration curves (*filled circle* observed, *filled square* calculated) of $0.5 \times 1^{0-3}$ M L2 (i) in the absence of Zn^{2+} ion, (ii) in the presence of equivalent amount of Zn^{2+} ions, and (iii) in the presence of twice equivalent amount of Zn^{2+} ions in aqueous methanol (75%, v/v) at $I = 0.1$ M $NaNO_3$ and 25 °C

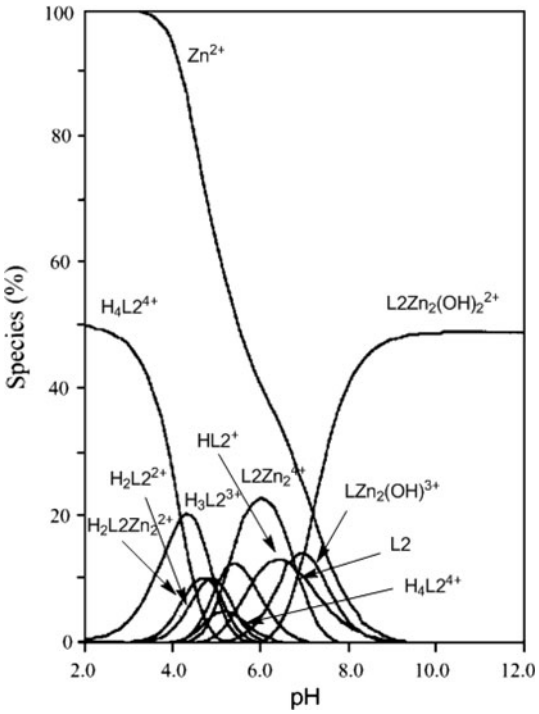


Fig. 3 Distribution curve of zinc-containing species of 0.5×10^{-3} M of L2 in the presence of equivalent amount of Zn^{2+} ions in aqueous methanol (75%, v/v) at $I = 0.1$ M $NaNO_3$ and 25 °C

As evidenced from the pH-titration curves, and from their fitting, the ligand can form dinuclear zinc complexes as well. Formation of dinuclear species is not that much

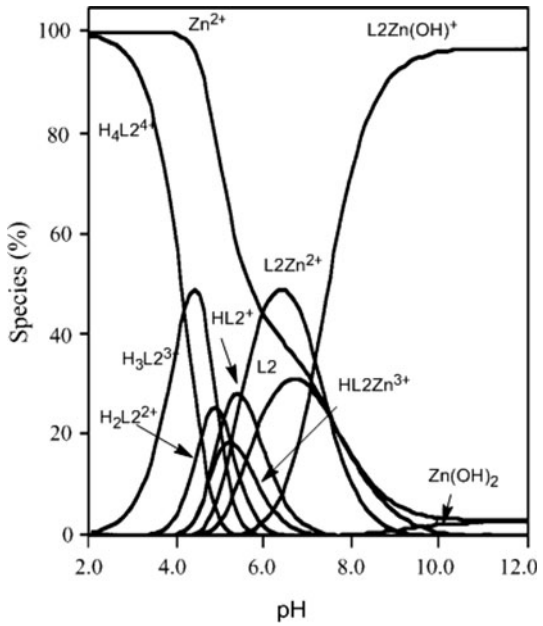


Fig. 4 Distribution curve of zinc-containing species of 0.5×10^{-3} M of L2 in the presence of twice equivalent amount of Zn^{2+} ions in aqueous methanol (75%, v/v) at $I = 0.1$ M $NaNO_3$ and 25 °C

stabilizing force (i.e. the formation constants for the reaction to produce dinuclear zinc complexes from the mononuclear ones, are not too high). In spite of this, dinuclear species are stable enough to be major ones in 1:2 solution. Interestingly, the log$K$ value for LZn_2^{4+} is almost exactly the double than that of the mononuclear complex (log$K$ 3.62), which can be related to the structural similarity of the two species: formally $L2Zn_2^{4+}$ can be considered as a "dimer" of the mononuclear ones. From this, it seems to be evident that the dihistidyl units in L2 operate individually during complexation with zinc (in the dinuclear case). Similarly, what was found in the mononuclear complexes, dinuclear ones can also be protonated to form species of lower stability. This fact, again explains the importance of all possible donor sites in the coordination with zinc ions.

As it was mentioned above, during the titration of ligands in the presence of zinc, more OH^- was consumed than was necessary for the ligands themselves. This showed the evidence for the presence of coordinated water molecule(s) being bound to the central zinc ion(s). The pK_a value of this water is quite high in the aquated zinc ion $[Zn(H_2O)_6]^{2+}$ with pK_a of 9.0 [35]. Both mono- and dinuclear zinc complexes showed extra deprotonation, in other words, presence of coordinated water molecule(s) in these complexes was evidenced. The proton loss in $[L2Zn(H_2O)]^{2+}$ is characterized with a pK_a of 7.15. It is well known that there is a direct correlation between the coordination number of the central zinc ion and the acidity/nucleophilicity of the zinc-bound water molecule. The

lower coordination number enhances deprotonation, thus reduces the pK_a of the bound water. The dinuclear zinc complexes shows presence of two water molecules bound to each zinc centers, respectively. The proton loss of these waters takes place in two steps and show relatively low pK_a values (6.74 and 13.72, respectively). This large difference in the pK_a's is related to the nature of binding these water molecules and it was in consistence with Bazzicalupi et al. [36], who this large pK_a-variation by the different binding mode of waters: one of them is monocoordinated to one zinc, while the other is bridged between the two metals. Since in our case, the difference is much higher, so bridging between the zinc centers is favored in the dinuclear complexes of the ligand.

R-dependent ^{1}H NMR titration

The R-dependence for the ligand are presented in Fig. 5. Effect of addition of zinc(II) for the peak positions and peak shapes clearly gave indication about the chemical exchange processes. As shown in Fig. 5, in case of free ligand (without addition of zinc), all peaks are well resolved. When increasing R from 0 to 1.0, almost all peaks show broadening with the exception of the peaks related to the benzyl group (the aromatic and the benzyl methylene peaks). At R = 1.0, both imidazolyl protons show extreme broadening, which is evidence for a fast chemical exchange process between different species, most probably between the structural isomers of mononuclear zinc complexes of the ligand. From the analysis of pH-metric titration, formation of mononuclear complexes was strongly indicated. The broadening of the imidazole protons suggest that the donor sites which take part in zinc ligation are the imidazole nitrogens which are close to the broadened peaks. These structures (mono- and dinuclear species) coexisting in solution are in rapid chemical equilibrium, thus the protons close to the donor sites (imidazole protons) show significant broadening. This indicates the formation of dinuclear complexes, in analogy with the results of pH-titration. Kinetic liability of binding zinc through imidazole nitrogens was evidenced for all ligands since no separate set of peaks representing the free and bound form was observed, instead, an averaged peak of the complexed and uncomplexed species was found.

R-dependent kinetic studies on the hydrolysis of *p*-NPDPP

R-dependence study was carried out to demonstrate the effect of the degree of complexation on the catalytic activity on the hydrolysis reaction. The applied range of R for the ligand was decided on the basis of the R-dependent ^{1}H NMR titration results. The plot of the rate constants as a function of zinc-to-ligand ratio (R) is shown in Fig. 6. The values are tabulated in Table 2. It can be generally concluded that increase in the degree of complexation has resulted in gradual acceleration in the hydrolysis process. The dinuclear zinc complex which is the possible maximum stoichiometry as it was demonstrated from pH- and NMR titrations. As shown in Fig. 7, the R-dependence of rate constants showed extremely low activity below R = 1. This can be explained by the higher coordination number of zinc, more precisely the larger variety of donor groups in the ligand. The highly coordinated central zinc in the mononuclear complex might reduce the nucleophilicity of the bound-water molecule, and at the same time, may leave

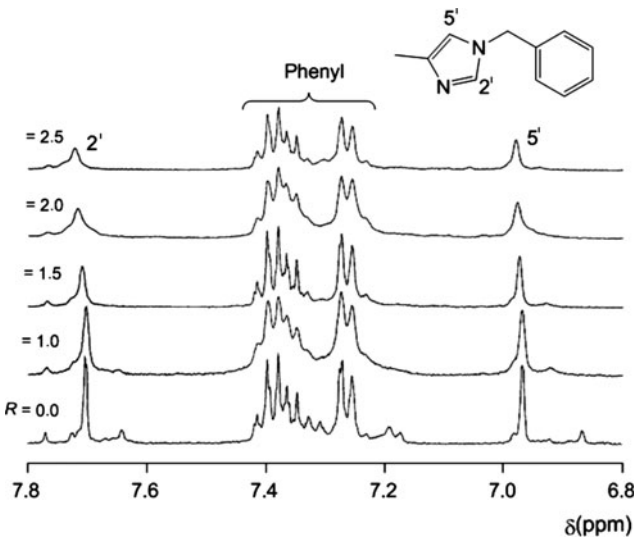


Fig. 5 ^{1}H NMR titration of L2 as a function of R = $[Zn^{2+}]_o/[L2]_o$ in $CD_3OD:D_2O$ (3:1) at 2.0×10^{-3} M of L2 at $I = 0.1$ M $NaNO_3$, pH = 7.5, and 25 °C

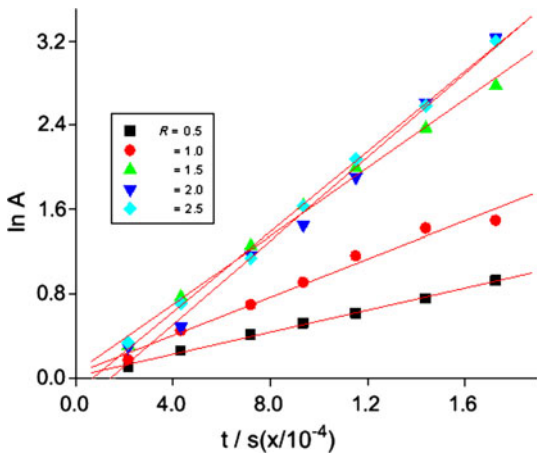


Fig. 6 Semilogarythmic plot for the cleavage of *p*-NPDPP (8.0×10^{-5} M) at different Zn^{2+}/L2 ratios in aqueous methanol (75%, v/v) at pH 7.5, in 0.05 M HEPES at 25 °C

Table 2 The observed pseudo-first order rate constants (k_{obs}, s^{-1}) for the cleavage of *p*-NPDPP (8.0×10^{-5} M) by at different Zn^{2+}/L2 ratios in aqueous methanol (75%, v/v) at pH 7.5, in 0.05 M HEPES buffer at 25 °C

R = Zn^{2+}/L2	k_{obs} ($\times 10^{-5}$ s^{-1})
0.5	0.522
1.0	0.908
1.5	1.616
2.0	1.979
2.5	1.897

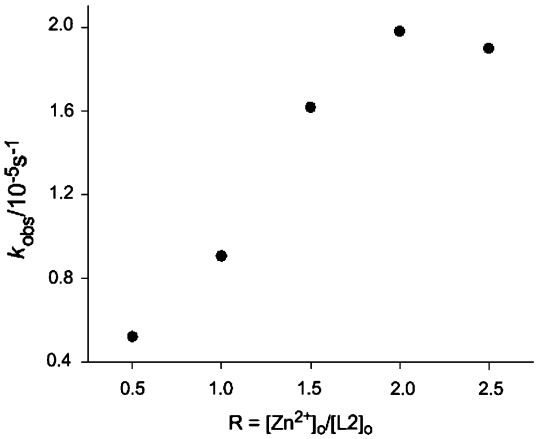


Fig. 7 Plot of k_{obs} versus R = $[Zn^{2+}]_o/[L2]_o$. [*p*-NPDPP] = 8.0×10^{-5} M in aqueous acetone (75%, v/v) at pH 7.5 (0.05 M HEPES), I = 0.1 M $NaNO_3$ and 25 °C

no sufficient free space around the coordinated water to interact with the phosphotriester substrate. The rate constants increase sharply between 1/1 and 2/1 metal-to-ligand ratio which indicates the high catalytic activity of the formed dinuclear complex. Since the rate acceleration is eightfold and 31-fold when considering the rate constants of R = 2 in comparison with R = 1, cooperation between the catalytic zinc centers is indicated. If the two zincs in the complex acted individually in the hydrolysis reaction, only a twofold statistical enhancement would be seen, since the concentration of the complexed catalytic zinc centers becomes double in 2/1 metal-to-ligand ratio in comparison with the equimolar case. The rate constants level off above R = 2, which is in consistence with the NMR-titration results that showed 2/1 as maximum complex stoichiometry. A number of related dinuclear metal complexes have been studied by Trogler and us [18–20, 27]. They observed that the dinuclear complexes have a higher affinity to DNA and RNA binding than the corresponding mononuclear complexes. However, no notable rate enhancements were observed for the dinuclear compounds in the catalytic cleavage of activated phosphate esters.

Conclusions

The present study was aimed at the design and synthesis of histidine-containing artificial tetrapeptide as ligand and of its zinc complex as structural and functional models for the active site of *phosphotriestrase* enzyme. The molecular architecture as well as the positioning of binding sites can make it possible to incorporate zinc with an open ligation site which site could be occupied by a water molecule or hydroxide ion. Maximum complex stoichiometries and the pH dependent formation of different complex species were investigated by pH-metric-^{1}H NMR titrations and comments about exchange processes (e.g. between free and complexed species; between different complex entities) are made on the basis of the degree of peak shape distortion. The activity of the mono- and dinuclear zinc complex species were tested on the hydrolysis/detoxification of *p*-NPDPP. From the correlation between the pH-rate profiles and the species distribution curves, the catalytically active species could be identified. On the basis of the trend of R-dependence as well as the rate acceleration of each complex, possibility of cooperative action of zincs in dinuclear zinc complex is debated.

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References

1. Branden, C.I., Jornvall, H., Eklund, H., Furugren, B.: The Enzymes. Academic Press, New York (1975)
2. Eklund, H., Jones, A.A., Schneider, G., Bertini, I., Luchinat, C., Maret, W.: Zinc Enzymes. Birkhauser, Boston (1986)
3. Fenton, D.E., Okawa, H.: Perspectives on Bioinorganic Chemistry. JAI Press, London (1993)
4. Karlin, K.D.: Metalloenzymes, structural motifs, and inorganic models. Science **261**, 701–708 (1993)
5. Vanhooke, J.L., Benning, M.M., Raushel, F.M., Hoden, H.M.: Three- dimensional structure of the zinc-containing phosphotriesterase with the bound substrate analog diethyl 4-methylbenzylphosphonate. Biochemistry **35**, 6020–6025 (1996)
6. Williams, N.H., Takasaki, B., Wall, M., Chin, J.: Structure and nuclease activity of simple dinuclear metal complexes: quantitative dissection of the role of metal ions. Acc. Chem. Res. **32**, 485–493 (1999)
7. Kondo, S., Shinbo, K., Yamaguchi, T., Yoshida, K., Yano, Y.: Cooperativity of binuclear Zn(II) complexes of bisimidazolyl ligands in the hydrolysis of bis(2,4-dinitrophenyl) phosphate in aqueous solution. J. Chem. Soc. Perkin Trans. **2**, 128–131 (2001)
8. Yamada, K., Takahashi, Y., Yamamura, H., Araki, S., Kawai, M., Saito, K.: Phosphodiester bond cleavage mediated by a cyclic β-sheet peptide-based dinuclear zinc(II) complex. Chem. Commun. 1315–1316 (2000)
9. Gajda, T., Krämer, R., Jancso, A.: Structure, equilibrium and ribonuclease activity of copper(II) and zinc(II) complexes formed with a dinucleating bis-imidazole ligand. Eur. J. Inorg. Chem. **7**, 1635–1644 (2000)

10. Abe, K., Izumi, J., Ohba, M., Yokoyama, T., Okawa, H.: Dinuclear zinc complexes of phenol-based "end-off" compartmental ligands: synthesis, structures and phosphatase-like activity. Bull. Chem. Soc. Jpn. **74**, 85–95 (2001)
11. Aka, F.N., Akkaya, M.S., Akkaya, E.Y.: Remarkable cooperative action of two zinc centers in the hydrolysis of plasmid DNA. J. Mol. Catal. A **165**, 291–294 (2001)
12. Kaminskaia, N.V., He, C., Lippard, S.J.: Reactivity of μ-hydroxodizinc(II) centers in enzymatic catalysis through model studies. Inorg. Chem. **39**, 3365–3373 (2000)
13. Kurosaki, H., Tawada, T., Kawasoe, S., Ohashi, Y., Goto, M.: A model for ZnII-containing-β-lactamase: synthesis, X-ray crystal structure of a zinc(II) complex bearing thiol group and hydrolysis of phosphate diester. Bioorg. Med. Chem. Lett. **10**, 1333–1337 (2000)
14. Ibrahim, M.M., Shimomura, N., Ichikawa, K., Shiro, M.: Phosphoester hydrolysis using structural phosphatase models of tren based zinc(II) complexes and X-ray crystal structures of $[Zn(tren)(H_2O)](ClO_4)_2$ and $[Zn(tren)(BNPP)]ClO_4$. Inorg. Chim. Acta **213**, 125–136 (2001)
15. Ibrahim, M.M., Ichikawa, K., Shiro, M.: Solution studies of tris(3-aminopropyl)amine-based zinc(II) complexes and X-ray crystal structures of $[Zn(trpn)](ClO_4)_2$ and $[Zn(trpn)(DETP)]$-ClO_4, DETP−=O,O-diethyl thiophospate. Catalytic activity of the complexes in the hydrolysis of the phosphotriester 2,4-dinitrophenyl diethyl phosphate. Inorg. Chim. Acta **353**, 187–196 (2003)
16. Ibrahim, M.M., Ichikawa, K., Shiro, M.: Structural characterization of $(\{[tren]Zn(HOMe)\}\cdot ClO_4\cdot BPh_4)$(tren=tris(2-aminoethyl)amine) and CO_2 fixation into monomethyl carbonato zinc(II) complex. Inorg. Chem. Commun. **6**, 1030–1034 (2003)
17. Ibrahim, M.M.: Phosphate triester hydrolysis promoted by S_3–zinc(II) complexes with a bridged hydroxide: the crystal structure of $TtiZn–OP(O)(OC_6H_4\text{-}p\text{-}NO_2)_2$, Tti = hydrotris(N-xylyl-2-thioimidazolyl)borate. Inorg. Chem. Commun. **9**, 1215–1218 (2006)
18. Ibrahim, M.M., Shaban, S.Y., Ichikawa, K.: A promising structural zinc enzyme model for CO_2 fixation and calcification. Tetrahedron Lett. **49**, 7303–7306 (2008)
19. Ibrahim, M.M.: A novel dinucleating ligand-containing hexabenzimidazoles and its dinuclear zinc complex bridged by a carboxylate group for the hydrolysis of 2-hydroxypropyl-p-nitrophenyl phosphate. J. Inorg. Organomet. Polym. **19**, 532–538 (2009)
20. Ibrahim, M.M., Mersal, G.A.M.: Solution studies of tris(2-aminoethyl)amine with metal ions of biological interest: the electroanalytical determination of the phosphate triester hydrolysis. J. Inorg. Organomet. Polym. **19**, 549–557 (2009)
21. Fawcett, T.G., Bernarducci, E.E., Krogh-Jespersen, K., Schugar, H.J.: Charge-transfer absorptions of copper(II)-imidazole and copper(II)-imidazolate chromophores. J. Am. Chem. Soc. **102**, 2598–2604 (1980)
22. Martell, E.A., Motekaitis, R.J.: The Determination and Use of Stability Constants. VCH, New York (1992)
23. Rabenstein, D.L., Daignault, S.A., Isab, A.A., Arnold, A.P., Shoukry, M.M.: Nuclear magnetic resonance studies of the solution chemistry of metal complexes. The complexation of zinc by glycylhistidine and alanylhistidine peptides. J. Am. Chem. Soc. **107**, 6435–6439 (1985)
24. Harding, M.M., Sargeant, B., Bilyk, A., Augoloupis, S.: Binuclear complexes from tetraimidazole ligands and zinc(II) ions. Aust. J. Chem. **47**, 1133–1142 (1994)
25. Ichikawa, K., Uddin, M.K., Nakata, K.: Zinc complexes of artificial histidine-containing dipeptides as catalysts of hydrolyses of *p*-nitrophenyl phosphate. Chem. Lett. **28**, 115–116 (1999)
26. Kimura, E., Aoki, S., Koike, T., Shiro, M.: A tris(ZnII–1,4,7,10-tetraazacyclododecane) complex as a new receptor for phosphate dianions in aqueous solution. J. Am. Chem. Soc. **119**, 3068–3076 (1997)
27. Kesicki, E.A., DeRosch, M.A., Freeman, L.H., Walton, C.L., Harvey, D.F., Trogler, W.C.: Interaction of binuclear transition-metal complexes with DNA. Inorg. Chem. **32**, 5851–5867 (1993)
28. Bodanszky, M.: Principles of Peptide Synthesis. Springer, New York (1993)
29. Rainer, M.J.A., Rode, B.M.: The complex formation of zinc(II) with GHL and related peptides. Inorg. Chim. Acta **93**, 109–115 (1984)
30. Agarwal, R.P., Perrin, D.D.: Stability constants of complexes of zinc and cobalt(II) ions with some histidine-containing peptides. J. Chem. Soc. Dalton Trans. 1045–1048 (1975)
31. Török, I., Gajda, T., Gyurcsik, B., Tóth, G.K., Péter, A.: Metal complexes of imidazole ligands containing histamine-like donor sets: equilibrium, solution structure and hydrolytic activity. J. Chem. Soc. Dalton Trans. 1205–1212 (1998)
32. Ichikawa, K., Tarnai, M., Uddin, M.K., Nakata, K., Sato, S.: Hydrolysis of natural and artificial phosphoesters using zinc model compound with a histidine-containing pseudopeptide. J. Inorg. Biochem. **91**, 437–450 (2002)
33. Ibrahim, M.M.: Novel znc(II) tweezers containing artificial peptides: the catalyzed hydrolysis of the toxic organophosphate parathion (unpublished results)
34. Jairam, R., Potvin, P.G., Balsky, S.: Zn^{2+} inclusion complexes of endodentate tripodands as carbonic anhydrase-inspired artificial esterases. J. Chem. Soc. Perkin Trans. **2**, 363–368 (1999)
35. Sigel, H., Martin, R.B.: The colourless 'chameleon' or the peculiar properties of Zn^{2+} in complexes in solution. Quantification of equilibria involving a change of the coordination number of the metal ion. Chem. Soc. Rev. **23**, 83–91 (1994)
36. Bazzicalupi, C., Bencini, A., Bianchi, A., Fusi, V., Giorgi, C., Paoletti, P., Valtancoli, B., Zanchi, D.: Carboxy and phosphate esters cleavage with mono- and dinuclear zinc(II) macrocyclic complexes in aqueous solution. Crystal structure of [Zn2L1(μ-PP)2(MeOH)2](ClO4)2 (L1 = [30]aneN6O4, PP^- = diphenyl phosphate). Inorg. Chem. **36**, 2484–2790 (1997)