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Clues to Dimetallohydrolase Mechanisms from Studies on Pyrazolate-Based Bioinspired Dizinc Complexes – Experimental Evidence for a Functional $Zn–O_2H_3–Zn$ Motif

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Various hydrolytic metalloenzymes contain two adjacent zinc ions within their active site that work synergistically during substrate turnover. While structural biology, biochemical investigations, and quantum chemical calculations have provided much insight into those cocatalytic sites, crucial details of the catalytic mechanisms have remained under debate. Valuable contributions to further our understanding of the functional role of the zinc ions in a specific coordination environment can be obtained from the study of simple biomimetic synthetic complexes that emulate features of the natural systems. To this end, a series of highly preorganized pyrazolate-based dizinc complexes has been developed, which are particularly amenable to fine-tuning of the dimetallic core. These bioinspired dizinc complexes have made possible the investigation of systematic effects of factors such as the zinc–zinc separation on hydrolytic activity and substrate binding. The present microreview summarizes findings relevant to dimetallohydrolases that have been obtained from studies of the pyrazolate-based synthetic analogues, including experimental support for a functional role of the $Zn–O_2H_3–Zn$ motif and clues to the binding and hydrolytic cleavage of phosphodiester and β-lactam substrates.

1. Introduction

Numerous biological functions rely on enzymes that promote the hydrolytic cleavage of biological substrates such as phosphate esters, peptides or amides, and these enzymes often contain two or even three cooperating metal ions within their active site.[1–4] Although specific mechanisms are involved for the individual metalloenzymes, crucial roles of the metal ions are believed to comprise the generation of a hydroxide nucleophile at physiological pH by lowering the pK_A of water, the Lewis acid activation and orientation of the substrate through metal coordination, as well as the stabilization of intermediates and leaving groups.[2,4,5] Since Zn^{2+} is a strong Lewis acid with rapid ligand exchange and a flat coordination hypersurface that allows the coordination number und geometry to be changed easily, it fulfils all these requirements while, at the same time, lacking undesired redox activity.[6] Hence, zinc is found very often in hydrolytic enzymes, although other metals such as Mg^{2+} or Mn^{2+}, or even Ni^{2+} in urease may be preferred in certain cases.

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2. Oligozinc Hydrolases: Phosphatases, Metallo-β-lactamases, and Aminopeptidases as Examples

Prominent hydrolases that incorporate two proximate zinc ions include some metallo-β-lactamases,[7,8] several aminopeptidases,[9] phosphotriesterase,[10] and alkaline phosphatase.[11,12] Similarly, human phosphodiesterase features two divalent metal ions, at least one of which is Zn^{2+} (the second is probably Mg^{2+}).[13,14] Enzymes that hydrolyze phosphodiesters are of prime importance to the manipula-

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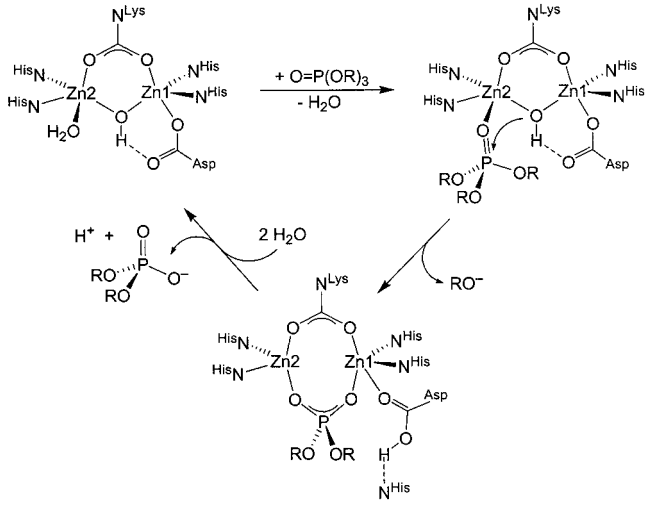
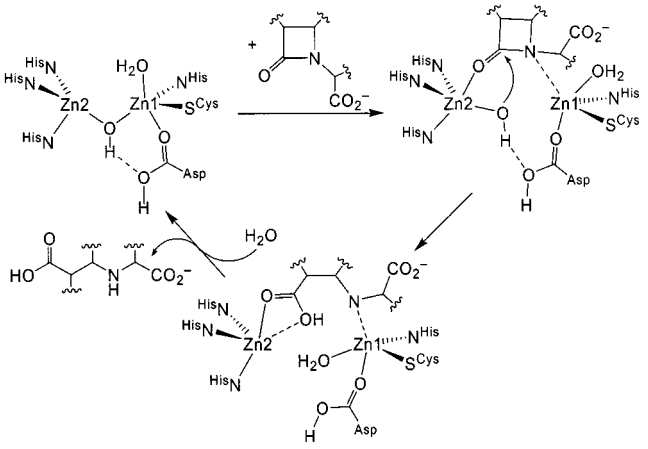
tion of DNA and RNA because of their involvement in replication, transcription, recombination, and DNA repair. It is through the synergistic Lewis acid activation of the substrate, the nucleophile, and the leaving group in these processes that the 10^{16}-fold rate enhancement can be achieved that is needed to hydrolyze DNA on the biological time scale.[3] A representative example illustrating the role of two active-site zinc ions in phosphoester hydrolysis is phosphotriesterase (PTE).[10] PTE efficiently degrades highly toxic organophosphate triesters by the proposed mechanism outlined in Scheme 1.[15] Similar to various other oligozinc enzymes, the crystallographically characterized form of PTE has a bridging O atom, which most likely is a bridging hydroxide in agreement with the experimental p*K*a value of 5.8.[16] It is assumed that the substrate enters the scene to replace a water at Zn2, followed by nucleophilic attack of the μ-OH onto the substrate-P and ejection of the leaving group. Proton transfer away from the active site, assisted by a nearby histidine residue, gives a phosphate anion which spans the two metal ions, and solvent molecules finally move in to regenerate the original state upon product release.



Scheme 1. Proposed mechanism of phosphotriesterase.[15]

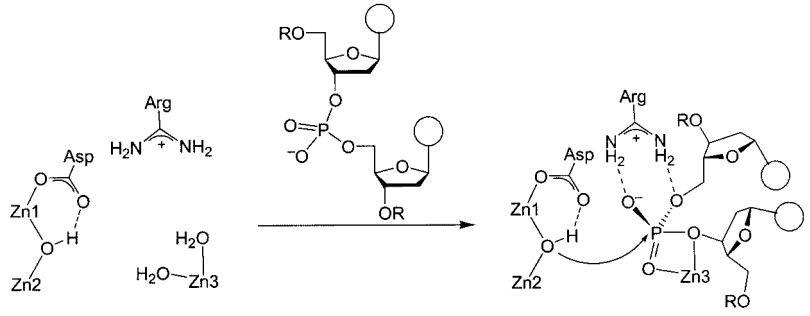
Even more than two metal ions may be involved in phosphoester cleavage; an example is the P1 nuclease, which degrades single-stranded RNA and DNA into 5′-mononucleotides. P1 nuclease features a trinuclear zinc site sketched in Scheme 2, where a hydroxide bridging Zn1 and Zn2 is assumed to attack the phosphate substrate that binds to Zn3 in a bidentate fashion.[17]

Despite manifold investigations, details of the mode of action of these and other metallohydrolases remain controversial. One important aspect under debate is the identity and the exact binding mode of the nucleophile.[18,19] Since a hydroxide spanning two zinc ions has been detected crystallographically in the majority of dimetallohydrolases, this μ-OH is usually considered to be the active nucleophile. Computational evidence for a catalytic bridging hydroxide in a phosphodiesterase site has indeed been reported.[19] On the other hand, the hydroxide can be suspected to exhibit a rather low nucleophilicity in this tightly bridging form. It has thus been suggested in an alternative picture that a shift of the bridging hydroxide to a terminal position occurs upon substrate binding prior to attack on the coordinated substrate.[20] A similar scenario is also assumed for the dizinc-β-lactamase mechanism depicted in Scheme 3.



Scheme 3. Proposed simplified mode of action of dizinc metallo-β-lactamase CcrA from *B. fragilis*.[18,21]

β-Lactamases are enzymes that efficiently hydrolyze and cleave the four-membered ring of β-lactams.[22] This β-lactam motif is a crucial functionality in antibiotics derived from the penicillin, cephalosporin, and carbapenem families. Expression of β-lactamases capable of inactivating this motif and thus inactivating these drugs has become a standard bacterial defense mechanism. Among the four classes (A, B, C, and D),[23,24] of β-lactamases, enzymes of the most recently discovered class B depend on one or two zinc ions

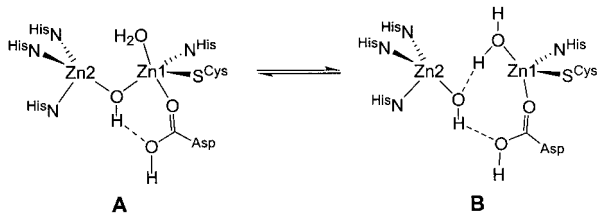


Scheme 2. Proposed mechanism of P1 nuclease.[17]

within their active site.[8,25] Unfortunately, clinically useful inhibitors are not yet available for those metallo-β-lactamases, in contrast to the serine-based β-lactamases of classes A, C, and D.[26] Bacterial resistance caused by metallo-β-lactamases now represents a serious clinical threat, and a spread of these enzymes to pathogenic bacteria has raised the concern of the biomedical community.[27] Current efforts thus focus on gathering a detailed picture of the mechanism of action within the active site of the metallo-β-lactamases,[8,28] which is not only of fundamental interest but may also contribute to the development of effective mechanism-based inhibitors.[29]

Molecular structures of several dizinc metallo-β-lactamases have been determined,[30–36] but mechanistic studies have concentrated on CcrA from *B. fragilis* and BcII from *B. cereus II*. While intimate details of the active-site structures vary, common features of dizinc metallo-β-lactamases comprise one tetrahedral {$(His)_3Zn(\mu\text{-}OH)$} motif (Zn2 in Scheme 3) in which the hydroxide serves as a bridge to the second zinc (Zn1). The latter is found in a distorted trigonal bipyramidal environment as a {$(His)(Asp)(X)Zn(H_2O)(\mu\text{-}OH)$} center, where X is His or Cys. From stopped-flow kinetic investigations in combination with theoretical studies, a mechanism has been postulated[18,21,37] that starts by docking the substrate to Zn2 by means of its ring carbonyl group. This polarizes the lactam unit and allows the N-atom to interact with Zn1, inducing conformational changes with rupture of the Zn–OH–Zn bridge and activation of the hydroxide for nucleophilic attack.[38] Zn1 then stabilizes the negative charge on the nitrogen leaving group, which is released upon rate-determining proton transfer.[18] Bringing in water from the medium finally regenerates the initial active-site state (Scheme 3).

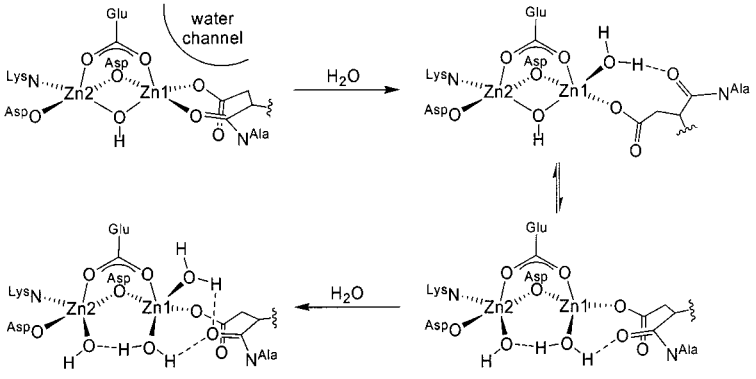
Recent DFT, Hartree–Fock, and molecular dynamics studies have revealed a somewhat refined picture, suggesting that the active site can easily assume different conformations.[39] When the Asp residue is neutral, the dizinc core may readily interchange between a tightly bridged state **A** and a loosely bridged state **B** with changes of coordination number at Zn1 (Scheme 4). The more flexible state **B** can be described as an O_2H_3 unit between the two metal ions. Evidence for different active-site conformation is also available from experimental studies,[37] but details such as the protonation state of the Asp residue remain controversial.[40,41] It is obvious, however, that solid-state structures obtained crystallographically do not provide the whole story.



Scheme 4. Interconversion of tightly and loosely bridged active site forms of CcrA from *B. fragilis* as predicted by calculations.[39]

A Zn–(H)OHO(H)–Zn motif reminiscent of the situation in **B** has indeed been observed in bioinspired synthetic model complexes, and from those findings the bridging O_2H_3 unit has been proposed as a structural and possibly functional unit in oligozinc hydrolases.[42,43] Some further corroboration comes from a most recent DFT study on the dizinc active site of bovine lens leucine aminopeptidase (*bl*LAP).[44] This widespread enzyme preferably hydrolyzes the peptide bond of N-terminal leucine in a peptide chain and plays a central role in the degradation and modification of proteins.[9a,45,46] Most proposed catalytic mechanisms have assumed that the terminal amino group of the peptide binds to Zn2 and that the OH acts as the nucleophile,[46,47] but a QM/MM study predicted an unreasonably high calculated barrier for nucleophilic attack.[48] A more recent DFT study then demonstrated that an additional water molecule can be introduced by way of the water channel above Zn1 and may undergo facile insertion into the Zn–OH–Zn unit to result in an O_2H_3 bridge (Scheme 5). Since an additional water molecule may be spontaneously incorporated at Zn1, this scenario has been described as the formation of a water sluice in the *bl*LAP active site, providing a plausible mechanism for the continual generation of an active nucleophile (Scheme 5).[44]

In view of this accumulating body of evidence for the relevance of a bridging O_2H_3 in dizinc hydrolases, it appeared particularly desirable to experimentally test the hydrolytic efficiency of this motif relative to the related Zn–OH–Zn unit.

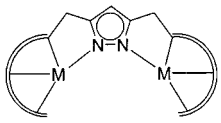


Scheme 5. Generation of a water sluice in the active site of *bl*LAP according to recent DFT calculations.[44]

3. Relevance of Synthetic Model Complexes

Synthetic hydrolase models have been very useful in providing information about basic functional principles and mechanistic aspects of enzyme action.[49,50] This is particularly true for the role of active-site metal ions in a specific biological donor environment, and most recent examples of hydrolase mimics even aim at elucidating second-sphere effects such as hydrogen-bonding patterns at metal-bound substrates.[51,52] As an advantage over their enzyme counterparts, small molecule synthetic analogues allow for easy systematic investigations of substituent effects, and it becomes possible to examine a variety of factors that influence reactivity.[50] Apart from the fundamental insights obtained from those biomimetic studies regarding structural, spectroscopic, and mechanistic features, there is hope that artificial nucleases for biomimetic hydrolysis of DNA or RNA will eventually lead to beneficial applications in biotechnology and medicine.[53] This has stimulated considerable efforts directed towards the development of well-designed metal complexes that mediate the hydrolytic cleavage of DNA or RNA or phosphodiester model substrates.[4,54,55] One should keep in mind, however, that no synthetic models have yet been developed that at the same time mimic all aspects of the natural enzymes, i.e., active-site structure, function and mechanism. As has been emphasized recently,[50] many structural models lack functional equivalence, while many functional models bear little structural resemblance to the enzyme active sites – a more elaborate combination of both properties in new synthetic compounds certainly is a demanding task. This is even more true for the emulation of additional factors provided by the protein matrix, such as regulation of activity, site-specific reactivity, and many more. These aspects are still far from being included in current model systems.

Successful strategies for obtaining (in a targeted manner) stable and well-defined di- and multinuclear complexes generally rely on compartmental ligand scaffolds that provide distinct binding pockets for the metal ions.[4,56] This may be achieved by incorporating proper donor sites within a single macrocyclic framework or by linking two (or more) individual ligand subunits by means of a more or less rigid spacer. The spacer itself may contain built-in donor atoms to reduce the flexibility and increase preorganization of the dinucleating scaffold; phenolate-based systems are certainly the most abundant among these. Attractive metal–metal distances usually range from 3 to 5 Å. This report will highlight a particular class of model complexes for di- and oligonuclear metallohydrolase active sites, where compartmental pyrazolate-based ligands are used as dinucleating scaffolds for nesting two metal ions (**C**).[57] The central pyrazolate unit is a reasonable compromise for mimicking bridging carboxylate donors that are widely found in nature but are difficult to incorporate into a polydentate compartmental ligand framework: just like a bridging carboxylate group, the pyrazolate provides a single negative charge and it supports a similar range of metal–metal distances.



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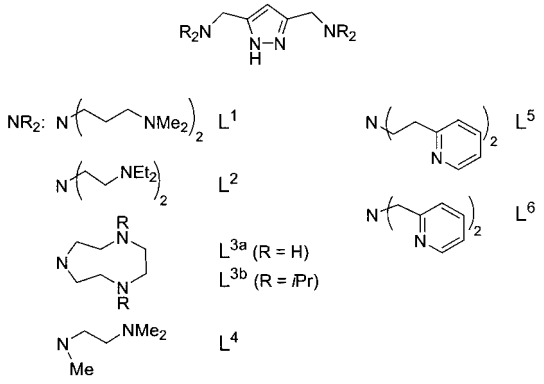
The scope of modulating electronic and geometric characteristics of the dimetallic core by varying the ligand side arms attached to the 3- and 5-positions of the heterocycle is an attractive option for this class of ligands. Important variations comprise the type and number of side-arm donor atoms as well as the lengths of the chelate arms (Scheme 6). While the former allows for the modification of the coordination numbers and electronic structures of the metal ions, the latter controls the properties of the dimetallic pocket, including the metal–metal distance.[58–63]



Scheme 6. Selection of dinucleating pyrazolate ligands.

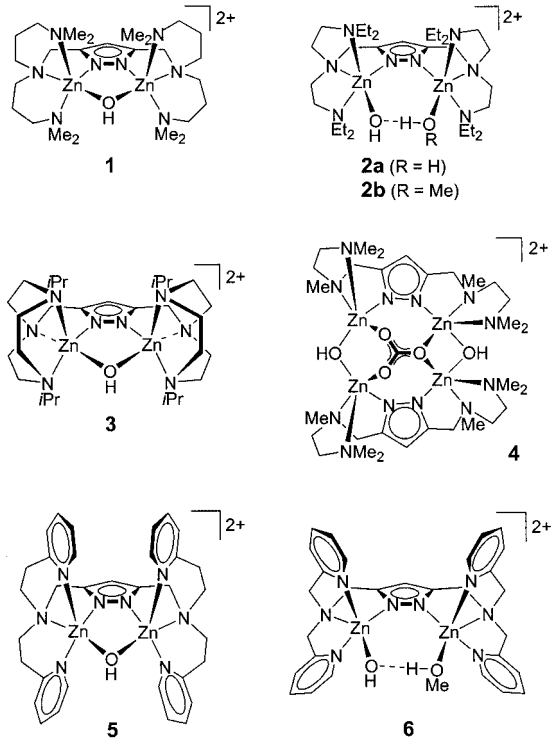
The comparative experimental studies summarized in this microreview focus on dizinc complexes of ligands L^1–L^6 that provide two metal-ion-binding pockets each, either tridentate (L^4) or tetradentate (L^1–L^3, L^5, L^6). The primary intention behind this approach is to elucidate how the mutual arrangement of two metal ions determines their cooperative reactivity. It is not aimed at exactly reproducing any biological coordination environment, but emphasis is laid on the question how two (or more) metals may work in concert in order to bind and transform biorelevant substrates. Inter alia, studies on this assortment of ligands have provided experimental evidence for the less favorable reactivity of a tightly bridging hydroxide in the clamp of a dimetal site as compared with a more loosely bridging O_2H_3 unit. The results also reveal some general structure–activity correlations for bioinspired di- and oligozinc hydrolase mimics. In the long term, it is anticipated that such findings will contribute to the development of two-center catalysis not only in the biomimetic but also in a broader sense.

4. Zinc Coordination Chemistry of the Pyrazolate-Based Scaffolds

(i) Dizinc Complexes with OH versus O_2H_3 Bridges

As outlined above, a dimetallic arrangement with a bridging $O_2H_3^-$ group can be described as a combination

of both a $Zn–OH_2$ and a Zn–OH function, and hence as a hydrated form of an active terminal Zn–OH. A comparative evaluation of the hydrolytic activity of a Zn–O(H)–Zn relative to that of a Zn–(H)OHO(H)–Zn species in synthetic model chemistry was achieved with the highly preorganized dizinc complexes **5** and **6** (Scheme 7), where the metal–metal separation is controlled by the lengths of the chelating side arms.[64] Complementing information was obtained from complexes **1** and **2** that are based on the related aliphatic ligand scaffolds, but in this case the system with long side arms (forming six-membered chelate rings in **1**) exhibits rather weak Zn^{2+}-binding capabilities in aqueous solution, which precludes any conclusive evaluation of its hydrolytic efficiency under the appropriate conditions.[65]



Scheme 7. Assortment of crystallographically characterized pyrazolate-based zinc complexes.[64,65]

In **1** and **5** the zinc ions may come rather close together (≈3.5 Å) and allow for a bridging position of a hydroxide coligand within the dimetallic pocket. In contrast, the shorter ligand side arms in **2a/b** and **6** pull the two zinc ions back and apart, thus enforcing much longer Zn···Zn distances (>4 Å). This prevents the small hydroxide from spanning the two metal ions and induces incorporation of an additional solvent molecule, water or MeOH, to give an O_2H_3 (**2a**) or O_2H_2Me (**2b**, **6**) bridging unit, respectively. Differences in reactivity for these species are apparent, since in acetone solution **2a** gradually absorbs CO_2 from air to give a carbonato-bridged complex while **1** does not.[43] Stopped-flow studies on the dinickel(II) analogues of **2a/2b** have revealed that exchange of water and methanol in these complexes is very rapid with $k_{obs} > 10^3\ s^{-1}$ at room temperature.[66] The lability of the $Zn–H_3O_2–Zn$ bridge has been corroborated by extensive DFT calculations on the mechanism of the replacement of water by methanol in pyrazolate-based complexes of types **2** and **6** to successively give the Zn-bound HO–H–OMe and MeO–H–OMe functionalities (by an endothermic process).[67] The replacement can occur through two fundamentally different substitution mechanisms: an addition-elimination, controlled by the metal ions, and a direct exchange in which the metal ions serve as a template. Since the steric bulk and the stiffness of the ligands in **2a** and **6** prevent an easy expansion of the coordination sphere, only the direct exchange mechanism is possible for these systems. Nevertheless, barriers for the conversion of the HO–H–OH, HO–H–OMe, and MeO–H–OMe bridges are low enough (lower for **6** than for **2**) to enable a rapid equilibrium under experimental conditions. Comparing the different functionalities, the HO–H–OH form is clearly less nucleophilic than the methoxide form MeO–H–OMe, but the hydroxide in the mixed species HO–H–OMe is more nucleophilic than the methanol component.[67]

Fewer short side arms provided by the ligand in **4** allow for greater flexibility of the Zn···Zn distance and offer additional coordination sites for substrate binding. At neutral to basic pH, the dizinc complex **4′** formed in situ from L^4 and $Zn(ClO_4)_2$ was found to readily absorb CO_2 from air to give tetranuclear **4**, where two $\{L^4H_{-1}Zn_2\}$ subunits are linked by two hydroxide bridges and the resulting rectangle of four zinc ions is capped by a μ_4-carbonate.[68]

Unexpectedly, a bridging hydroxide was detected crystallographically in **3**,[65] despite the short ligand side arms that were assumed to induce a situation similar to the corresponding dinickel(II) complex **2a**. This can be traced to the ability of zinc(II) to adopt a {4+1} coordination with two significantly elongated backside bonds between zinc and the bridgehead-N in **3**. However, the dinuclear scaffold in **3** appears to be somewhat strained and it is assumed that the bridging hydroxide in **3** more easily adopts a semibridging or even nonbridging position in solution than the tightly bound hydroxide in **1** and **5**. Unfortunately, no crystallographic information is yet available for $[L^{3a}Zn_2(OH)(H_2O)_x]^{2+}$.

(ii) pK_a of the Zn-Bound Water

While the X-ray crystallographic results provide structural insight for the various complexes, knowledge of the species distribution in aqueous solution is crucial for understanding their hydrolytic reactivity. Species distributions of ligands L^1–L^6 in the presence of Zn^{2+} have been determined by potentiometric titrations and have revealed the existence of various mononuclear and dinuclear complexes depending on the pH, except for the case of L^1, where only mononuclear species are formed in the pH range 4–7 and precipitation of zinc hydroxide is observed at higher pH.[64,65,69,70] As expected, ligands that form six-membered chelate rings generally provide lower complex stability than the corresponding systems that form five-membered chelate

rings, and complexes with pyridyl-containing (L^5, L^6) or triazacyclononane-derived ligands (L^{3a}, L^{3b}) give more stable complexes than the corresponding open-chain aliphatic scaffolds (L^1, L^2, L^4). The species distribution diagram of L^{3a}/Zn^{2+} differs somewhat from that of the system L^{3b}/Zn^{2+}, supporting a significant effect of the outer side-arm substituents.[65,70] For all ligands except L^1, the dominant species above pH ≈ 8 is $[(LH_{-2})Zn_2]^{2+}$, which most likely represents the complex structures **2a**, **3**, **5**, or **6** observed in the solid state. The species $[(LH_{-2})Zn_2]^{2+}$ is thus better described as $[(LH_{-1})Zn_2(OH)]^{2+}$ or $[(LH_{-1})Zn_2(O_2H_3)]^{2+}$. The pK_A of the zinc-bound water can be extracted from the titration data, and values obtained for $[(LH_{-1})Zn_2(OH_2)_x]^{3+}$ (L = L^2 – L^6) are collected in Table 1.

Table 1. pK_A values for the deprotonation of Zn-bound water in dizinc complexes $[(LH_{-1})Zn_2(OH_2)_x]^{3+}$ to give $[(LH_{-1})Zn_2(OH)(OH_2)_{x-1}]^{2+}$.[64,65,69,70]

Species	L^1	L^2	L^{3a}	L^{3b}	L^4	L^5	L^6
pK_A	–	7.57	8.29	8.04	7.66	7.96	7.60

All pK_A values are clearly lower than the pK_a of $[Zn(H_2O)_6]^{2+}$ (8.96)[71] and lower than most pK_A values of zinc-bound water in five-coordinate mononuclear zinc complexes with tetradentate tripodal ligands. The pK_A values 8.29, 8.04, and 7.96 for $[(L^{3a}H_{-1})Zn_2]^{3+}$, $[(L^{3b}H_{-1})Zn_2]^{3+}$, and $[(L^5H_{-1})Zn_2]^{3+}$, respectively, appear to be relatively high for bridging water in dinuclear zinc(II) complexes, which is often found to have pK_a values below 8. However, a similar pK_A of 8.0 for metal-bound water has been determined for the dizinc complex of the dinucleating ligand comprising two tacn subunits tethered by a bridging alkoxide.[72] The mononuclear complex $[(Me_3tacn)Zn(OH_2)_3]^{2+}$ (Me_3tacn = 1,4,7-trimethyl-1,4,7-triazacyclononane) is much less acidic (pK_A ≈ 10.8), which has to be attributed (at least in part) to its higher coordination number.[73] Comparison is more straightforward for the system L^6/Zn^{2+}, since $[(tpa)Zn(OH_2)]^{2+}$ {tpa = tris(pyridylmethyl)amine} can be viewed as a mononuclear analogue of $[(L^6H_{-1})Zn_2(OH_2)_x]^{3+}$.[60] The pK_a of $[(tpa)Zn(OH_2)]^{2+}$ is 8.03,[74] clearly indicating a certain increase in acidity due to the dinuclear arrangement in **6** that induces formation of the O_2H_3 bridge.

As the most interesting result of these considerations, it should be noted that involvement in strong hydrogen bonding (such as in the O_2H_3 bridge) can cause an even more drastic decrease in the pK_A of Zn-bound water than incorporation of the resulting hydroxide in a bridging position between two zinc ions, as is evident from a comparison of the values of pK_A = 7.60 for $[(L^6H_{-1})Zn_2(OH_2)_x]^{3+}$ and pK_A = 7.96 for $[(L^5H_{-1})Zn_2(OH_2)_x]^{3+}$.[64] The same holds true for the corresponding pyrazolate systems with aliphatic N-donor side arms, where pK_A = 7.57 for $[(L^2H_{-1})Zn_2(OH_2)_x]^{3+}$, pK_A = 8.29 for $[(L^{3a}H_{-1})Zn_2(OH_2)_x]^{3+}$, and pK_A = 8.04 for $[(L^{3b}H_{-1})Zn_2(OH_2)_x]^{3+}$.[65,69,70] These findings clearly demonstrate that a bridging position of water (or the resulting hydroxide) is *not* required for sufficiently lowering its pK_A to give a zinc-bound hydroxide at physiological pH, since this increase in acidity may well be achieved by the appropriate hydrogen-bonding pattern such as that found in the intramolecular O_2H_3 bridge.[64,65] Similar conclusions have also been reached for the influence of hydrogen bonding in mononuclear zinc hydroxide systems.[75] This observation supports the view that the O_2H_3 unit is an attractive structural and possibly functional motif in oligozinc enzyme chemistry.[42,43,64]

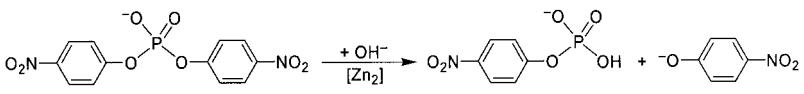
5. Models for Oligozinc Phosphatases

Despite the manifold studies on zinc-containing model phosphatases, there is still only limited knowledge about the intrinsic factors that govern hydrolytic activity and a lack of general structure–reactivity correlations.[72,76] The assortment of dizinc complexes described above appeared to be a promising set of systems for gaining further clues on the cooperative action of two proximate zinc atoms in biomimetic hydrolytic reactions.

(i) Structure–Activity Correlations in Phosphate Diester Hydrolysis

Phosphatase activities of the various pyrazolate-based dizinc complexes have been evaluated in 1:1 DMSO/aqueous buffer (or pure water in the case of the L^{3a}/Zn^{2+} system[77]), by using sodium bis(4-nitrophenyl) phosphate (sodium bnpp) as a test substrate (Scheme 8). Differences among the series of complexes gave valuable insights into structure–activity correlations and allowed for the experimental assessment of the hydrolytic efficiency of a bridging versus pseudo-terminal Zn-bound hydroxide.[64,65] Instead of crystallographically characterized **4**, dinuclear species $\{L^4H_{-1}Zn_2\}$ (**4′**) prepared in situ from L^4 and 2 equiv. $Zn(ClO_4)_2 \cdot 6H_2O$ was used without isolation of the complex in order to avoid any possible active-site inhibition by the carbonate.

Since **1** is not stable in aqueous solution, results for this complex are not meaningful. For all other systems (**2**, **3**, **4′**, **5**, **6**), the rate of BNPP hydrolysis was linearly dependent on complex concentration, in agreement with pyrazolate-based dizinc active species. Pseudo-first-order rate constants k_{obs} (defined by $v_0 = k_{obs} \cdot [complex]_0$) at pH 8.28 are collected in Figure 1, showing drastic differences between the complexes. Except for **2**, inspection of the pH-depen-



Scheme 8. Phosphodiesterase model reaction with BNPP test substrate.

dence of k_{obs} revealed that the sigmoidal increase in hydrolysis rate at higher pH generally coincides with the formation of species $[LH_{-1}Zn_2(OH)(H_2O)_x]^{2+}$ featuring the Zn-bound hydroxide. This clearly established that deprotonation of the $Zn–OH_2$ function is required for hydrolytic activity, in accordance with findings for other phosphatase model systems. Only in the case of **2** did the species $[LH_{-1}Zn_2(OH)(H_2O)_x]^{2+}$ turn out to be inactive, which was interpreted in terms of a bidentate substrate binding within the dimetallic pocket that replaces the Zn-bound hydroxide and leaves no accessible coordination sites for activation of the water nucleophile (compare also results for phosphate ester binding summarized below).[65] Taking into account the individual species distributions and the predominance of the active species at the relevant pH, a more reliable comparison with reference to the true second-order rate constants k_{bim} that included the percentage factor for the $[LH_{-1}Zn_2(OH)(H_2O)_x]^{2+}$ species at pH 8.28 is also given (Figure 1). Trends are qualitatively similar to those for the k_{obs} values, with the order of activity **4′** > **6** > **5** > **3**.

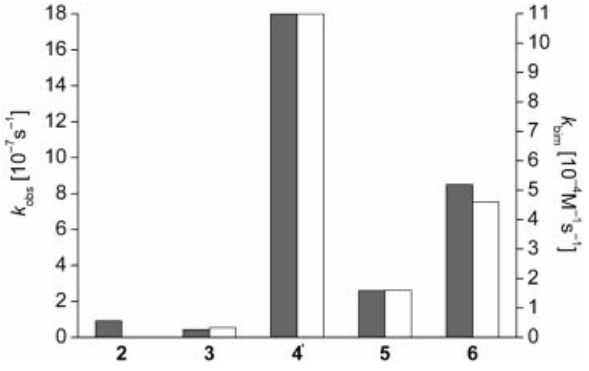


Figure 1. Comparison of rate constants k_{obs} (dark) and k_{bim} (light) for BNPP hydrolysis at pH 8.28 mediated by the different pyrazolate-based dizinc complexes.

Direct comparison of **5** and **6** proved most insightful and allowed us to draw valuable conclusions regarding the relative hydrolytic efficiencies of the Zn–OH–Zn and Zn–O_2H_3–Zn functions, since both **5** and **6** are fully stable under the experimental conditions and have closely related dimetallic core structures. The effect of substrate concentration on the initial hydrolysis rate suggested a substrate-binding pre-equilibrium, and a Michaelis–Menten type analysis gave almost identical K_M values of 51 ± 5 mM and 56 ± 4 mM, but very distinct k_{cat} values of $(0.49 \pm 0.04) \times 10^{-5}$ s^{-1} and $(2.3 \pm 0.1) \times 10^{-5}$ s^{-1} for **5** and **6**, respectively. Evidently, it is not the initial substrate binding, but the subsequent intrinsic reactivity at the dizinc core that makes the difference, leading to an almost fivefold higher hydrolytic activity for **6**. Although any further interpretation was hampered by the lack of structural information for the intermediate {complex–substrate} adducts, these data strongly support the view of the Zn–O_2H_3–Zn moiety as a favorable functional unit in dizinc enzyme chemistry.

After a single turnover, BNPP hydrolysis by any of the dizinc complexes **3**–**6** became much slower or even stopped completely. This has to be attributed to inhibition by the formed phosphate monoester (which is a much stronger ligand), and structural insight for the phosphate inhibited dizinc sites was obtained from isolated product-bound complexes (see below). In methanol, however, catalytic transesterification was observed to sequentially give the mixed diester methyl 4-nitrophenyl phosphate and dimethyl phosphate (dmp).[64] While the first transesterification step again occurred much faster when mediated by **6** as compared to **5**, the opposite was true for the second step. Apparently, complex **6** is intrinsically more active, but on the other hand, inhibition is also more pronounced for the system with the larger Zn···Zn distance. On the basis of structural insight for the two dizinc complexes with bound DMP as well as NMR studies, the differences in inhibition could be rationalized and traced to stronger DMP binding in the dimetallic clamp of **6**: while the larger Zn···Zn separation enforced by the ligand scaffold L^6 is perfectly suited to accommodate the bridging O–P–O moiety of DMP, ligand L^5 favors much shorter Zn···Zn distances.[64] Hence the pyrazolate-based dizinc arrangement in **5** has to be distorted drastically to host the DMP bridge (Figure 2), resulting in a much decreased DMP binding affinity. This may hint towards a suitable strategy to avoid the latent problem of product inhibition in synthetic dizinc hydrolase models.

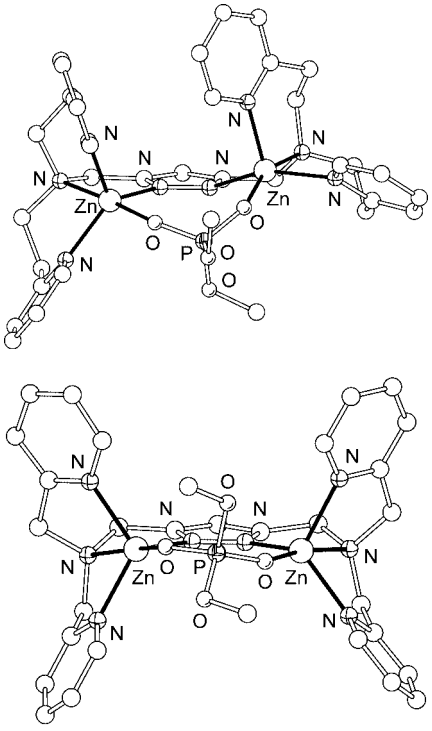


Figure 2. Molecular structures of $[L^5H_{-1}Zn_2(dmp)]^{2+}$ (top) and $[L^6H_{-1}Zn_2(dmp)]^{2+}$ (bottom) illustrating the much greater distortion of the dizinc scaffold in the former case.

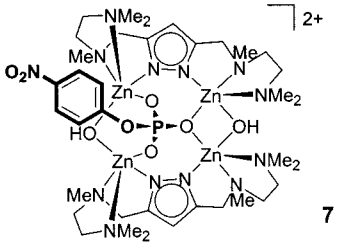
As was also stated for the related complex $[L^{3a}H_{-1}Zn_2(OH)(H_2O)_x]^{2+}$,[77] it can be concluded that the pyrazolate-based dizinc complexes show many typical features of a metalloenzyme, such as formation of a substrate complex, activation of the substrate through coordination, preorganiza-

tion of the substrate and the nucleophile, and inhibition by the product.

(ii) Dizinc Scaffolds with Bound Phosphate

The same *O*,*O*′-bridging DMP coordination mode shown in Figure 2 has been detected crystallographically in complexes $[L^2H_{-1}Zn_2(dmp)]^{2+}$, $[L^4H_{-1}Zn_2(dmp)(NO_3)_2]^{2+}$, $[L^5H_{-1}Zn_2(dmp)]^{2+}$, and $[L^6H_{-1}Zn_2(dmp)]^{2+}$, with Zn···Zn distances between 4.2 and 4.4 Å.[64,65] A similar binding mode was also observed for diphenyl phosphonate (dpp) in the related dicopper(II) complex $[L^{3a}H_{-1}Cu_2(dpp)]^{2+}$, while a μ_4-κ*O*:κ*O*′:κ*O*′′:κ*O*′′′ phosphate was found in $[(L^{3a}H_{-1}Cu_2)_2(PO_4)]^{3+}$.[70] It should be noted that structures with bidentate bridging DMP are reminiscent of the proposed phosphate ester binding in the dizinc active sites of various metallohydrolases[78] and mimic a crucial intermediate in the phosphotriesterase mechanism (compare Scheme 1).[15]

ESI mass spectrometry confirmed the weak binding of BNPP, but significantly stronger coordination of the first hydrolysis product 4-nitrophenyl phosphate (npp), as well as of DMP. Active-site inhibition of the most active complex **4**′ by NPP was visualized in the structure of the complex $[(L^4H_{-1}Zn_2)_2(OH)_2(npp)]^{2+}$ (**7**; Scheme 9), where the NPP caps a rectangle of four zinc ions and blocks all accessible coordination sites.[65] Accordingly, only 0.5 equiv. BNPP is hydrolyzed per $\{L^4H_{-1}Zn_2\}$ (**4**′).



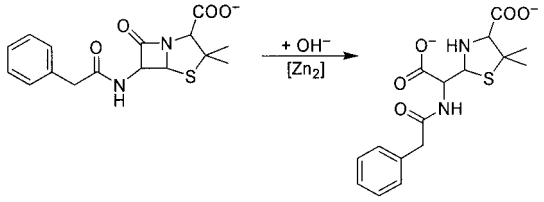
Scheme 9. Complex derived from inhibition of **4**′ by 4-nitrophenyl phosphate.[65]

6. Models for Dizinc Metallo-β-lactamases

Despite the extensive work on synthetic metallohydrolase models, very few studies have employed preorganized dinuclear zinc(II) complexes as functional mimics of metallo-β-lactamases.[79–81] Such model studies may contribute valuable insights into the mechanistic roles and cooperative effects of the proximate metal ions in that important class of enzymes and the identity of the active nucleophile. Furthermore, isolated adducts between synthetic dizinc scaffolds and β-lactam compounds or their ring-opened derivatives should provide clues to possible substrate binding modes.

(i) Penicillin G Hydrolysis

Several pyrazolate-based dizinc complexes turned out to be active mediators for the hydrolytic cleavage of penicillin G. Following the time course of penicillin G hydrolysis promoted by **1**, **2a**, **5**, and **6** with in situ FTIR spectroscopy revealed drastic differences in activity (Scheme 10, Figure 3).[80]



Scheme 10. Hydrolytic cleavage of penicillin G mediated by the dizinc complexes.

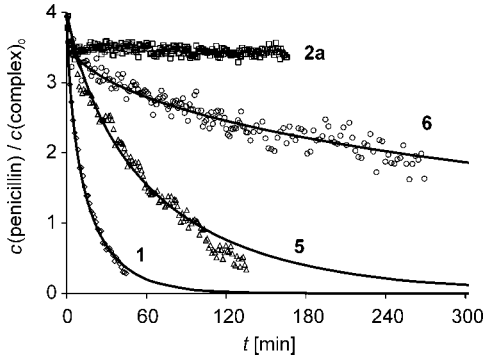


Figure 3. Time course of penicillin G hydrolysis in DMSO/water (9:1) in the presence of the different dizinc complexes.[80]

Unexpectedly, hydrolytic efficiency is much greater for systems **1** and **5**, which feature a shorter Zn···Zn separation and a bridging hydroxide in their resting state, while complex **2a** is not active at all, and **6** is still less efficient than free Zn^{2+}. Mass spectrometry in combination with NMR and IR spectroscopy indicated predominant coordination of the β-lactam antibiotics through their carboxylate group, whereas involvement of the β-lactam C=O in metal coordination could not be unambiguously deduced from the spectroscopic data, in agreement with findings for other dizinc metallo-β-lactamase model systems.[79] NMR experiments of the reaction mixtures suggested an explanation for the much greater hydrolytic activity of **1** and **5** relative to **2a** and **6**: after substrate binding through the carboxylate anchor with replacement of the Zn-bound hydroxide (compare the structure of **9** shown below), only in the case of the six-membered chelate rings in **1** and **5** do the labile side-arm N-donors tend to dissociate to generate an accessible coordination site and to allow for the binding and activation of the water nucleophile. In contrast, the five-membered chelate rings in **2a** and **5** impart much higher stability, rendering any detachment of the ligand side arms less likely. Hemilability of the longer side arms in related pyrazolate-based dimetal complexes could also be confirmed structurally in several cases.[59a,62a,80] These observations underline the necessity for the activation of both the substrate and the nucleophile through zinc coordination, which requires suitably designed dimetallic scaffolds that offer sufficient and properly oriented binding sites.

(ii) Structural Characterization of Dizinc β-Lactam Adducts

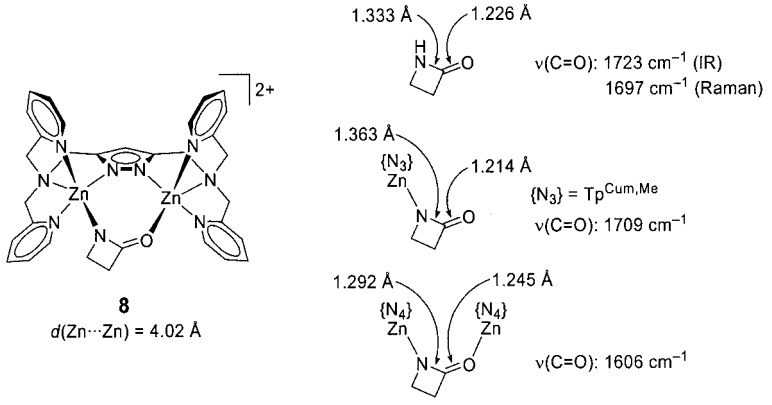
Unfortunately, it was not possible to isolate any adduct between a dizinc complex and the penicillin substrate or its ring-opened product in crystalline form suitable for X-ray diffraction. In order to obtain structural insight into β-lactam binding at dizinc sites, we therefore turned to smaller substrate analogues featuring different levels of structural simplification as compared to penicillin. The most simple choice is 2-azetidinone, which is readily incorporated into the dimetallic pocket of some of the pyrazolate-based dizinc complexes, giving lactamide-bridged complexes such as **8** (Scheme 11).[82]

Comparison of the IR spectroscopic and metric characteristics of the N-deprotonated lactamide with those of free azetidinone,[83,84] and the β-lactamide subunit of a mononuclear $Tp^{Cum,Me}$Zn(β-lactamido) complex [Scheme 11; $Tp^{Cum,Me}$ = tris(3-cumyl-5-methylpyrazolyl)borate][85] was informative: in the latter system the β-lactamide is solely bound by its N atom to a single zinc ion, which reduces conjugation within the amide moiety and hence leads to lengthening of the C–N bond and a slight shortening of the C=O bond. Binding of a second zinc ion to the β-lactamide-O as observed in complex **8** reverses this trend, i.e. the C–N bond is shortened and the C=O bond is clearly elongated. In line with these structural findings, the β-lactam ν(C=O) stretch in $Tp^{Cum,Me}$Zn(β-lactamido) at 1709 cm^{-1} is barely shifted from those of free 2-azetidinone, while the ν(C=O) vibration of **8** appears at much lower frequency. These findings underline the particular ability of highly preorganized dinuclear scaffolds to accommodate and polarize small substrate molecules within their dimetallic binding pocket, which is quite distinct from activation at a single metal ion.

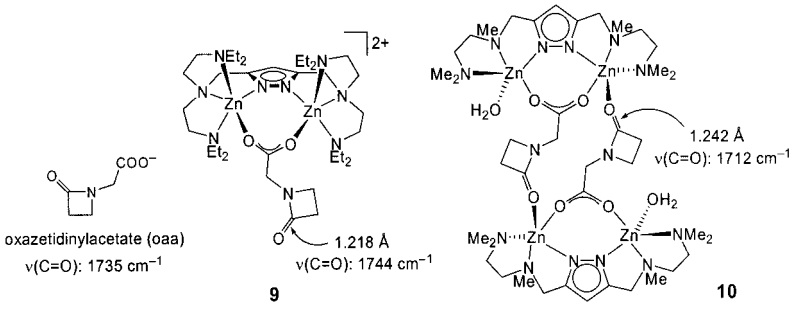
2-Azetidinone is apparently unsuited as a model substrate with regard to penicillin binding at a dizinc site because coordination of the deprotonated β-lactam-*N* is undesired. N-substituted oxazetidinylacetate (oaa; Scheme 12) proved more appropriate, since that also incorporates the carboxylate group in an α position to the β-lactam-*N*. The carboxylate of penicillin G was identified spectroscopically as the primary anchoring group for substrate binding at the dizinc scaffolds, and the same applies to complexes of oaa, e.g. in structurally characterized **9**.[80]

Interestingly, additional coordination of the β-lactam-*O* does occur if the ligand scaffold provides fewer side arms and hence leaves accessible sites at the metal ion. The tetrametallic complex **10** represents the first system for which binding of a β-lactam-*O* has been verified crystallographically. The C=O bond in **10** is markedly lengthened relative to **9**, and the ν(C=O) stretch is found at a significantly lower frequency (Scheme 12). Such polarization is usually assumed to activate the amide moiety for nucleophilic attack.

While the structural findings confirm that for β-lactam antibiotics (or related β-lactam derivatives) binding of zinc to the carboxylate group is favored over binding to the β-lactam function, coordination of the β-lactam amide-O may still be induced under suitable circumstances or upon proper orientation of the substrate within the binding



Scheme 11. (β-lactamido)zinc systems.



Scheme 12. Binding of oxazetidinylacetate (oaa).[80]

pocket, and this leads to significant changes of the internal geometry of the β-lactam moiety, which are likely to facilitate subsequent hydrolytic ring cleavage. It is plausible that electrostatics contributes significantly to the preferential binding of an anionic carboxylate to the highly charged pyrazolate-based dizinc scaffolds $\{LZn_2\}^{3+}$, but a lower positive charge of the dizinc arrangement or involvement of the carboxylate in interactions with positively charged protein residues or in strong hydrogen bonding within the enzyme active site may well alter the binding preference in favor of the neutral β-lactam amide moiety.

Concluding Remarks

The studies summarized above demonstrate how the investigation of well-designed synthetic model complexes may afford clues to the understanding of substrate transformations at metalloenzyme active sites. The compartmental pyrazolate-based ligand scaffolds have the distinct advantage of easy tuning of essential parameters in the dimetallic core – such as the metal–metal separation – which have a decisive effect on reactivity. A bridging position of water (or the resulting hydroxide) between the two zinc ions is not required for sufficiently lowering its pK_a, but proper hydrogen bonding of the pseudo-terminal Zn–OH, such as the one in the $Zn–O_2H_3–Zn$ function, does the job and at the same time may result in higher nucleophilicity of the Zn-bound hydroxide. Comparative functional studies on the phosphodiesterase activity of an assortment of pyrazolate-based dizinc complexes provided experimental support for a possible functional role of the $Zn–O_2H_3–Zn$ motif and structural insight into phosphate ester binding at dimetal sites. Similarly, some structure–activity correlations could be deduced for penicillin hydrolysis at bioinspired dizinc sites, and first crystallographic evidence for coordination and activation of a β-lactam could be obtained. Future directions in the development of synthetic analogues of dizinc enzymes will comprise, inter alia, the incorporation of functional groups that emulate mechanistically important protein residues in the vicinity of the active site.

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