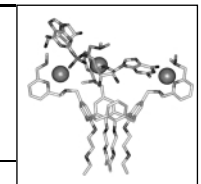
Dinuclear metallo-phosphodiesterase models: application of calix[4]arenes as molecular scaffolds



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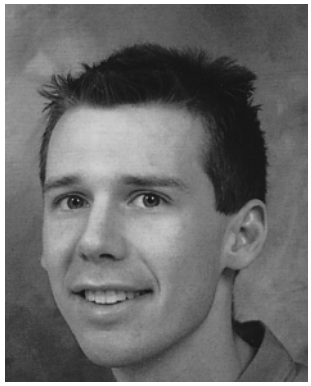
An important goal in supramolecular chemistry is the synthesis of molecules that exhibit catalytic activity analogous to the activity of enzymes. In this respect, studies toward the biomimetic catalysis of phosphate diester cleavage have received particular attention. In nature this process is catalyzed by enzymes that possess often two or three divalent metal ions in their active sites. In order to mimic the active sites of these metallo-phosphodiesterases chemists generally attempt to connect ligated metal ions by a molecular spacer in such a way that the metal–metal distance matches with the anionic pentacoordinate phosphorus transition state. However, in contrast to enzymes, which bind the transition state by multiple contacts *via* an induced fit mechanism, many of the low molecular weight model systems exhibit only minor catalytic activity due to lack of catalytic groups and too much rigidity or flexibility. Our approach is to use calix[4]arenes as a molecular scaffold for the dynamic preorganization of multiple catalytic groups. In this review models for dinuclear metallo-phosphodiesterases and the use of calix[4]arenes in such models are described.

Peter Molenveld was born in Haaksbergen, The Netherlands, in 1969. He graduated at the laboratory technician school on the synthesis of glucuronide metabolites at Organon International. Subsequently, he studied organic chemistry at Nijmegen University. His undergraduate project was carried out in the group of Professor Binne Zwanenburg on the synthesis of unnatural amino acids. He moved to the University of Twente to work for his PhD with Professor David N. Reinhoudt and Professor Johan F. J. Engbersen. His thesis is entitled 'Synthetic metallo-phosphodiesterases based on calix[4]arenes' and part of the work is described in this article. In 1999, he was awarded his PhD; currently he is Research Scientist at MercaChem in Nijmegen, The Netherlands.

Johan F. J. Engbersen (born 1949) studied Chemistry at the University of Groningen, The Netherlands, and received his PhD on a physical organic dissertation in 1976 with Professor J. B. F. N. Engberts. In the period 1976–1990 he was teaching Organic Chemistry and had a research program in Bio-organic Chemistry at Wageningen Agricultural University. In 1990 he moved to the University of Twente as an associate professor in the department of Professor Reinhoudt. Major research topics are in the field of bio-organic chemistry (enzyme models, enzymes in organic media, cyclodextrins), and chemical recognition applied to chemical sensors. He is the author of more than 135 scientific publications. In 1998 he was appointed as full professor and director of education of the Faculty of Chemical Technology of the University of Twente.

David N. Reinhoudt was born in 1942 in The Netherlands. He studied Chemical Technology at the Delft University of Technology and graduated (summa cum laude) in chemistry in 1969 with Professor H. C. Beijerman. In the period 1970–1978 he worked at Shell where he started the crown ether research program. In 1975 he was appointed as a part-time professor (extraordinarius) at the University of Twente followed by appointment as a full professor in 1978. The major part of his research deals with supramolecular chemistry and technology. Nanotechnology, molecular recognition, and non-covalent synthesis are the major fields, together with the application of supramolecular chemistry, e.g. in membrane transport, in the field of electronic or optical sensor systems, catalysis, and molecular materials. He is the author of more than 600 scientific publications, patents, review articles, and books. He has been honored with the Izatt–Christensen award (1995) and the Simon Stevin Mastership (1998). In 1998 he was elected as a member of the Royal Dutch Academy of Sciences. In 1999 he was appointed as the scientific director of the MESA⁺ Research Institute of the University of Twente.



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1 Introduction

The widespread research on molecular recognition of guests by synthetic hosts has stimulated bio(in)organic chemists to design catalysts that mimic the active site of enzymes. This aspect of supramolecular chemistry involves the synthesis of enzyme models or artificial enzymes that have the same catalytic function but are structurally less complex and more stable than enzymes. Such enzyme models can provide information on mechanistic aspects of enzyme action and may find future application as catalysts in industrial synthesis.

1.1 Enzyme models and phosphate diester cleavage

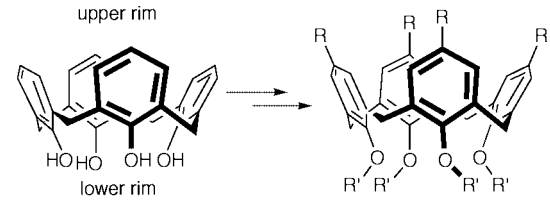
In the past decades many enzyme-catalyzed processes have been mimicked with synthetic molecules.[1–4] Biochemically important processes that have received considerable attention are hydrolytic reactions, *i.e.* hydrolysis of amino acid esters, peptides, and phosphate esters by esterases, peptidases and phosphoesterases, respectively.[5] The abiotic hydrolysis or transesterification of phosphate diesters is of particular interest.[6,7] Under neutral conditions phosphate diesters are negatively charged and resistant toward hydrolysis, which explains their presence in nature as the repeating linkers in DNA and RNA. In this respect, the development of artificial catalysts that can cleave phosphate diester bonds is a fundamental challenge. Moreover, in the future, artificial enzymes that can cleave DNA or RNA at specific sites may find application as therapeutic antisense oligonucleotides in gene technology.[8]

From X-ray analysis, it is known that many phosphodiesterases possess two or three divalent metal ions, such as Zn^{II}, in the active site.[9] Examples include the exonuclease site of DNA polymerase, phospholipase C and nuclease P1. The metal ions act cooperatively as Lewis acid sites in the activation of a nucleophile and the substrate, and in the stabilization of the pentacoordinate phosphorus transition state and the leaving group. In addition, the active sites of phosphoesterases often contain amino acids with acidic, basic and/or positively charged residues. For instance, in alkaline phosphatase two Zn^{II} ions and one Mg^{II} ion are assisted by a histidine and an arginine.[9] In RNase A, a lysine residue activates the phosphoryl group while two histidines cooperate in the deprotonation of the nucleophile and the protonation of the leaving group.[10]

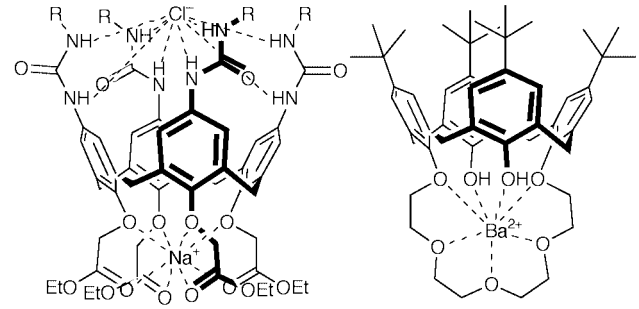
In the past decades many research groups have attempted to catalyze biochemical reactions with synthetic compounds in which appropriate catalytic groups are oriented in such a way that the transition state of the reaction can be stabilized.[1–4] However, compared to the catalytic activity of enzymes a low activity is generally observed. Enzymes exhibit a certain degree of flexibility and can bind the substrate and transition state by an induced fit mechanism,[1,2,11] whereas the biomimetic compounds are often too rigid or too flexible.[3] Designed active site models for dinuclear metallo-phosphoesterases are often dinuclear transition metal complexes in which a molecular spacer orients two catalytic metal ions.[6,7] The catalytic properties are at least in part determined by the choice of this spacer. A very flexible spacer may result in a lack of catalytic cooperativity between the metal centers. In contrast, very rigid molecular scaffolds might not be able to match the geometrical changes of the substrate during the dynamic catalytic process. Thus, in analogy with enzymes, such models require molecular scaffolds that allow both a proper preorganization of multiple catalytic groups and a certain degree of flexibility. Furthermore, a handle for further functionalization, like linkage to recognition sites or a solid support, is strongly favored.

1.2 Calix[4]arenes

Calix[4]arenes (**1**) are cyclic tetramers of phenol that can be functionalized (selectively) at four phenolic hydroxy groups, the lower rim, and at four *para*-phenolic positions, the upper rim (**2**). Calix[4]arenes are versatile molecular scaffolds for the design of host molecules because the functional groups are directionally preorganized.[12] Moreover, these molecular scaffolds create a binding site in which functional groups can



1 **2**



3-NaCl **4**-Ba

dynamically adjust to the guest by low energy conformational changes of the calix[4]arene skeleton. Two opposing aromatic units rapidly move inward to or outward from the hydrophobic cavity, resulting in interconversion between conformations with either two diverged (flattened) or parallel (pinched) aromatic units *via* a cone-shaped symmetrical intermediate (*vide infra*). An excellent example of a dynamic molecular receptor is calix[4]arene **3**.[13] This compound contains a receptor site for a Na^+ ion at the lower rim, and a receptor site for a Cl^- or Br^- ion at the upper rim. Cation binding induces a structural change in the calix[4]arene backbone which is a prerequisite for anion binding.

Although many calix[4]arene-based molecular receptors have been reported,[12,13] calix[4]arene-based enzyme models are little developed.[14–19] A notable example is reported by the group of Mandolini,[20] who modified the calix[4]arene lower rim with a crown ether (**4**). Upon the addition of Ba^{II} ions a crown ether–Ba^{II} complex **4**–Ba is formed that exhibits transacylase activity in the methanolysis of *p*-nitrophenyl acetate. In this review, it will be shown that calix[4]arenes are suitable molecular scaffolds for the design of catalytically active enzyme models. Recently, we have developed calix[4]arene-based dinuclear complexes that efficiently catalyze phosphate diester cleavage with a high degree of cooperativity between two Zn^{II} or Cu^{II} centers.[14–16] By synthetic modification of the calix[4]arene scaffold the catalytic metal centers in these enzyme models can be combined with other functional groups like amino functionalities.[17,18] Extension to systems with a third metal ion binding site afforded a trinuclear Zn^{II} complex that mimics trinuclear Zn^{II} phosphodiesterases.[15,19] First, a short overview of other models for dinuclear metallo-phosphodiesterases is given.

2 Dinuclear metallo-phosphodiesterase models

Over the years many model systems for *mononuclear* hydrolytic metallo-enzymes have been designed and studied for the

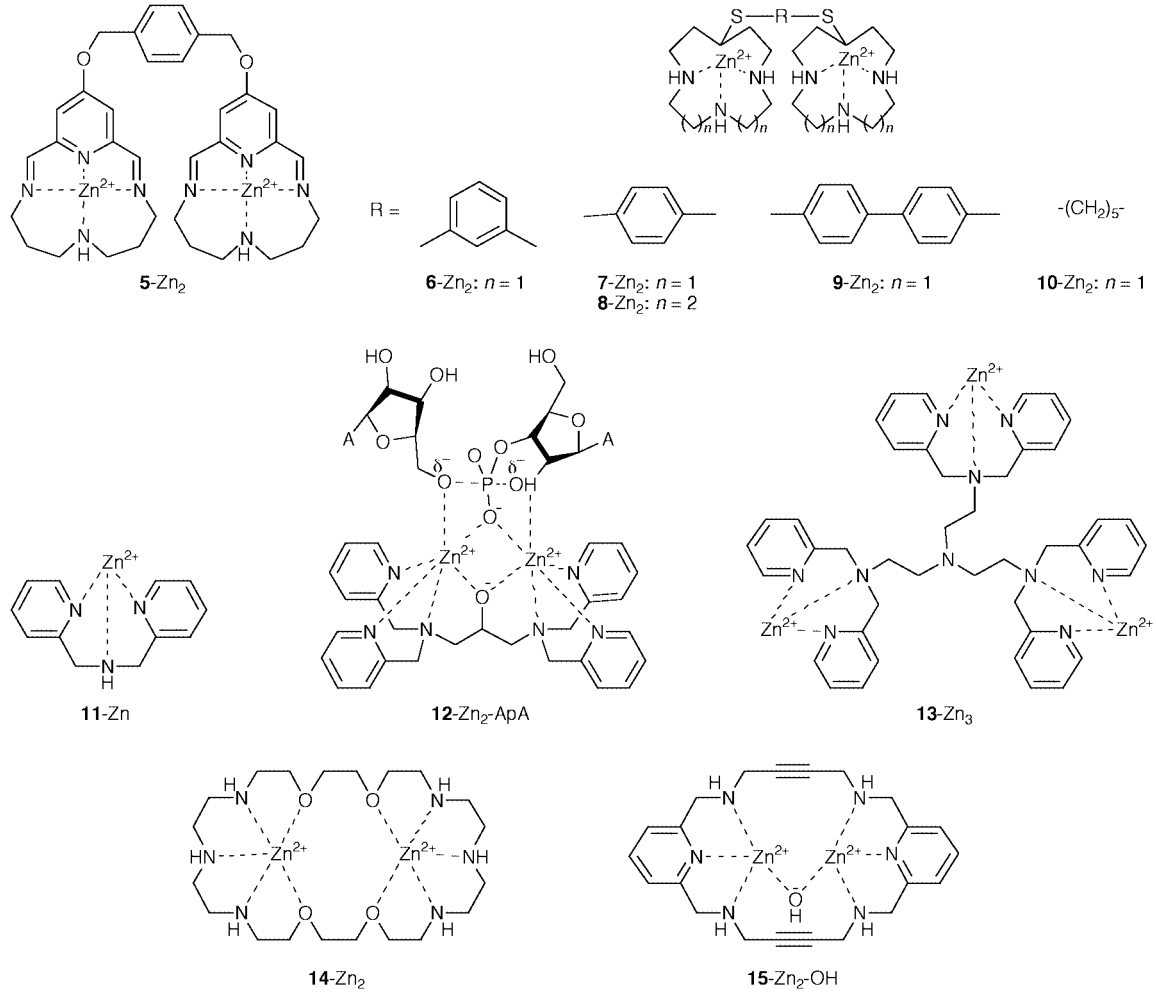
hydrolysis of amides, nitriles, carboxylate esters, and phosphate esters.[5] These studies have provided information about the fundamental role of metal ions in promoting hydrolytic reactions. Whereas trivalent metal ions like Co^{III} and lanthanides(III) generally exhibit a high catalytic activity due to their high Lewis acidity,[6,7] they are less interesting from a mechanistic point of view since they are not biomimetic. In this respect, studies with divalent metal ions like Zn^{II} are more interesting.[21] In contrast to Zn^{II} ions, Cu^{II} ions have so far not been observed in the active sites of hydrolytic enzymes. However, in abiotic systems Cu^{II} exhibits a high catalytic activity in hydrolytic reactions.[16]

Model systems for *dinuclear* hydrolytic metallo-enzymes are less developed and they are currently investigated actively in biomimetic chemistry.[6,7] The mode of catalysis of phosphate diester cleavage, either by hydrolysis or transesterification,[10] has been studied with various model compounds in which two metal ions are held apart by ligands that are linked to a molecular spacer. The presence of two metal ions gives rise to many possible modes of Lewis acid catalysis. The ultimate goal is to obtain catalysis with a high degree of cooperative action between two (biomimetic) metal centers. In the following section a selection of dinuclear complexes with various molecular spacers is reviewed.

2.1 Dinuclear Zn^{II} complexes

One of the early reports on hydrolytic dinuclear metal complexes describes two Zn^{II}– or Cu^{II}–aminomethylimidazole complexes held in proximity by a flexible *n*-butyl spacer.[22] Although the dinuclear complexes display rate enhancements in the hydrolysis of the triester tris(*p*) nitrophenyl phosphate (TPNP), the activities are only two times higher than their mononuclear analogs, suggesting a statistical enhancement of catalysis. The lack of cooperative catalysis originates from a poor substrate binding, probably caused by the formation of a hydroxide-bridged dinuclear complex with diminished Lewis acidity.

The first report on catalytic phosphate ester cleavage by cooperating metal ions came from the group of Breslow.[23] They used a phenyl spacer to orient two macrocyclic ligands known to form extremely stable Zn^{II} complexes, preventing precipitation of polymeric Zn^{II}–hydroxide species. At pH 8.0, the resulting dinuclear Zn^{II} complex **5**–Zn_2 is 4.4 and 7 times as effective as its monomer in the hydrolysis of the neutral esters diphenyl *p*-nitrophenyl phosphate (DPNP) and *p*-nitrophenyl acetate, respectively. A detailed study of the effect of spacer lengths and geometry on cooperative catalysis was published in 1995.[24] The investigated model compounds consisted of two Zn^{II} ions ligated by tridentate macrocyclic ligands which are linked to different phenyl or alkyl spacers (**6**–**10**). The substrates examined were the monoester *p*-nitrophenyl phosphate (PNP), the diester bis(*p*-nitrophenyl) phosphate (BPNP), the RNA model 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP), and the RNA dinucleotide UpU. The flexible complex **10**–Zn_2 is a poor catalyst. It appeared that hydrolysis of the monoester PNP is most effective with complex **6**–Zn_2 containing the short 1,3-phenyl spacer. However, the overall rate acceleration is only 4.7. The long dimer **9**–Zn_2 is the most reactive toward the diesters BPNP, HPNP, and UpU, exhibiting rate accelerations of 1937, 1072, and 39, respectively, and being approximately 10 times more active than its monomeric analog. The different preferences of the phosphate monoesters and diesters with respect to catalyst geometry can originate from



electronic and steric factors. PNP is highly basic and may react *via* double Lewis acid activation, which requires a close proximity of the Zn^{II} centers. BPNP, HPNP, and UpU are less basic and sterically more hindered and favor therefore a larger Zn^{II}–Zn^{II} separation, resulting in single Lewis acid activation. The authors expect that structures more rigid than **5**–**10** would be more effective for bifunctional catalysis, and also more substrate selective.

Cleavage of ribonucleotide dimers NpN by intramolecular transesterification to 2′,3′-cNMP was studied by Komiyama and coworkers.[25] In contrast to the mononuclear complex **11**–Zn, the similar dinuclear complex **12**–Zn_2 efficiently cleaves ApA for which the authors propose a mechanism comprising Lewis acid activation of the phosphoryl group, the nucleophilic 2′–OH and the leaving group. In order to mimic trinuclear Zn^{II} phosphodiesterases they have prepared the trinuclear analog **13**–Zn_3.[25] The activity is much larger than that of **12**–Zn_2. Moreover, **13**–Zn_3 shows a considerable dependence on the structure of the substrate. However, no explanation is given for the reactivity CpA > ApA > GpA > UpA ≈ ApG > GpC. In the subsequent hydrolysis of 2′,3′-cNMP they observed preferential formation of 3′-NMP over 2′-NMP. This is not observed for **12**–Zn_2 and for the uncatalyzed reaction.

The first report of a dinuclear Zn^{II} complex applied in an antisense oligonucleotide[8] was published recently by the group of Komiyama.[21] They coupled two equivalents of **11** to *m*-xylene and tethered the dinucleating ligand to the 5′ ends of DNA oligomers. Incubation with a complementary RNA oligonucleotide resulted in sequence-selective cleavage of the RNA target. The cleavage is due to cooperative action of the Zn^{II} centers within the DNA–RNA duplex and even at high concentrations of free Zn^{II} ions the cleavage is selective. The dinuclear complex is also active in the cleavage of the RNA dinucleotide ApA.

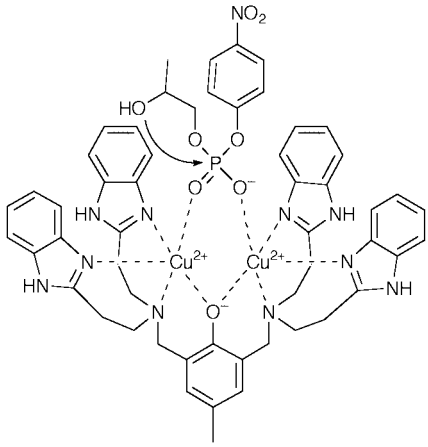
A dinuclear Zn^{II} complex based on a large oxa-aza-macrocycle (**14**–Zn_2) was reported by the group of Bianchi.[26] Whereas this complex shows no cooperative character in the hydrolysis of *p*-nitrophenyl acetate it is 10 times more active than its mono analog in the hydrolysis of the phosphate diester BPNP. A reaction pathway in which the phosphoryl group bridges the two Zn^{II} centers and is cleaved by attack of a Zn^{II} bound hydroxide is supported by an X-ray structure of the substrate bound to the catalyst.

Krämer and coworkers investigated the hydrolytic cleavage of *p*-nitrophenyl acetate catalyzed by a dinuclear Zn^{II} complex based on the rigid ligand **15**.[27] The Zn^{II} ions are bridged by a hydroxide ion and do not show cooperativity in the catalysis. The substrate is cleaved by nucleophilic attack of a terminal Zn^{II} bound hydroxide ion.

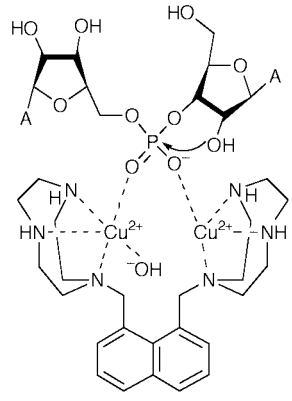
2.2 Dinuclear Cu^{II} complexes

The group of Chin was one of the first to report on the high catalytic activity of Cu^{II} complexes in phosphate diester cleavage. In complex **16**–Cu_2, the two Cu^{II} centers are bridged by a phenoxide and display a high cooperativity in the intramolecular transesterification of the RNA model HPNP.[28] Despite an enormous rate acceleration of approximately 2×10^4 in the cleavage of HPNP the cleavage of RNA dinucleotides was not catalyzed, probably because of steric hindrance. As an extension of this research they developed a 1,8-naphthalene substituted with two [9]aneN_3–Cu^{II} complexes **17**–Cu_2.[29] At pH 6.0 **17**–Cu_2 is 300–500 times more reactive per metal center than its mononuclear analog in the cleavage of ApA and 2′,3′-cAMP, by transesterification and hydrolysis, respectively. Bell-shaped pH–rate profiles indicate that the monohydroxide form of **17**–Cu_2 is the active species that *(i)* binds the phosphoryl group by double Lewis acid coordination, and (*ii*) uses the Cu^{II} bound hydroxide as a general base or as a nucleophile.

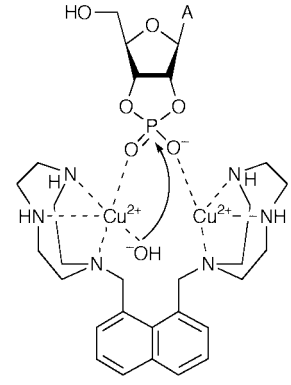
Since mononuclear Cu^{II} complexes of bipyridine and terpyridine are individually efficient hydrolytic catalysts, the group of Hamilton has connected these with a simple amide spacer to obtain dinuclear complexes with higher catalytic activity.[30–32] In the transesterification of the RNA model HPNP both



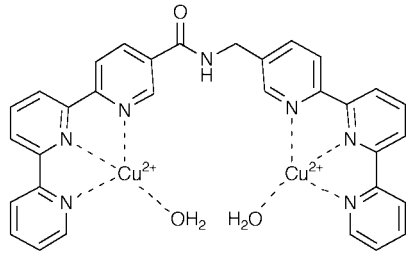
16-Cu_2-HPNP



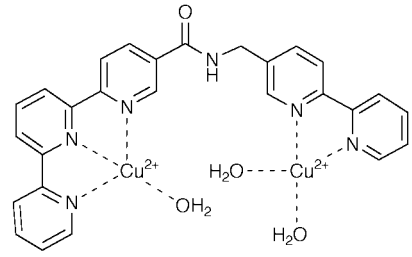
17-Cu_2-ApA(OH)



17-Cu_2-cAMP(OH)



18-$Cu_2(H_2O)_2$
p$K_{a1,2}$ = **7**.4, 8.2



19-$Cu_2(H_2O)_3$
p$K_{a1,2}$ = 5.3, **7**.4

dinuclear complexes **18**–Cu_2 and **19**–Cu_2 are superior to the mononuclear analogs.[30] The lower pK_a values of the Cu^{II} bound water molecules in **19**–Cu_2 (5.3 and 7.4) compared to **18**–Cu_2 (7.4 and 8.2) cause a higher activity of **19**–Cu_2 at pH 7.0. The different bell shaped pH–rate profiles point to Cu^{II}–OH general base catalysis in combination with double and single Lewis acid activation for **18**–Cu_2 and **19**–Cu_2, respectively. The stability of the amide bond in close proximity to the hydrolytic Cu^{II} centers as well as both the possible involvement of the amide functionality in the catalysis and the low pK_a in **19**–Cu_2 are not discussed.

At pH 7.5, the complexes **18**–Cu_2 and **19**–Cu_2 are also effective in the hydrolysis of ribonucleoside 2′,3′-cyclic monophosphates (2′,3′-cNMP) showing some base- and regioselectivity.[31] Whereas natural RNases produce only 3′-NMP, **18**–Cu_2 produces 2′-NMP a factor of 10 and 18 faster than 3′-NMP when the nucleobase is uracil or cytosine, respectively. Furthermore, **19**–Cu_2 hydrolyzes 2′,3′-cAMP 31 times faster than 2′,3′-cGMP. The nucleobase- and regioselectivities do not correlate with the size or shape of the nucleobases, nor do they correlate with the metal ion affinity of the nucleobases. The selectivities may arise from specific interactions of the nucleobases with the ligands, either by hydrogen bonding or π–π stacking. The high catalytic activity is proposed to be the collective result of double Lewis acid activation, Cu^{II} bound hydroxide attack, and leaving group stabilization *via* protonation by a Cu^{II} bound water molecule.

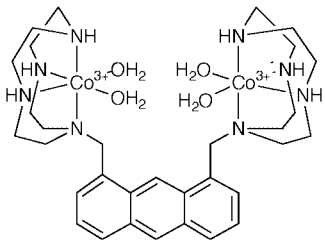
Scrimin *et al.* prepared a rigid macrocyclic aminomethylpyridine ligand (not shown) that forms a dinuclear Cu^{II} complex and compared the catalytic activity in the hydrolysis of amino esters with Cu^{II} complexes based on monomeric and polymeric ligands.[33] In the hydrolysis of β-AlaPNP the dinuclear complex is a factor of 10 more efficient than the mononuclear complex, exhibiting a rate acceleration of 80-fold at pH 6.3. The metallo-polymer is twice as active as the macrocyclic dinuclear complex. This enhanced catalysis is attributed to cooperativity between two neighboring Cu^{II} centers which is more favorable in the conformationally flexible metallo-polymer.

2.3 Dinuclear complexes of trivalent metal ions

Non-enzymatic phosphate ester cleavage is effectively facilitated by the strongly Lewis acidic Co^{III} and lanthanide(III) ions. However, for application in artificial nucleases the use of Co^{III} is not attractive since it forms substitutional inert complexes with the products, which hamper turnover catalysis at neutral pH. The disadvantages of lanthanide(III) ions are their toxicity and the generally low stability of the complexes with regular ligands. Free lanthanide(III) ions readily form lanthanide(III) hydroxide precipitates and both forms are active in phosphate ester cleavage, which makes kinetic control complicated. However, some stable La(III) complexes have been successfully applied as RNA cleavers in antisense oligonucleotides.[8] The chemistry of Co^{III}- and lanthanide(III)-mediated phosphate ester cleavage has been reviewed recently.[5–8] Some examples, among others the use of designed dinucleating ligands are given below.

Dinuclear Co^{III} complexes. Czarnik *et al.* have reported dinuclear cyclen–Co^{III} complexes based on both a very rigid anthracene spacer (**20**)[34] and a very flexible alkyl spacer (**21**).[34] The flexible complex **21** promotes the hydrolysis of phosphate diester BPNP a factor of 6.4 faster than the parent cyclen–Co^{III} complex (no turnover). In the hydrolysis of phosphate monoester PNP the rigid complex **20** is 20 times more active, whereas no metal ion cooperativity is observed for the hydrolysis of the diester BPNP.

The group of Schneider has increased the DNA binding affinity of cyclen–Co^{III} by modification with positively charged peralkylammonium groups which can interact with the anionic phosphate ester linkages in DNA. The presence of these groups leads to lower activities in the hydrolysis of the activated phosphate esters PNP and BPNP but to higher activities in the transition of (strained) supercoiled form RFI to open circular form RFII of plasmid DNA. The highest activity was observed for complex **22** in which two Co^{III} centers can act simultaneously on the DNA grooves.



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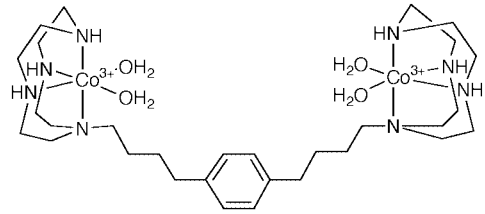

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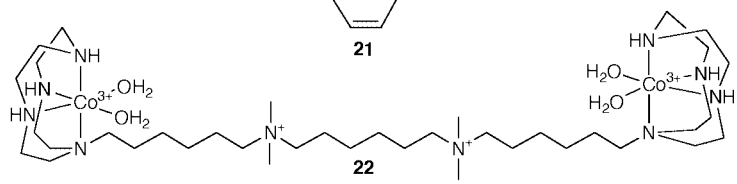

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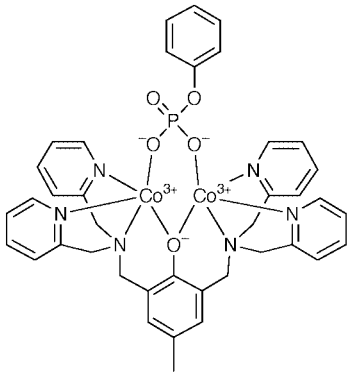

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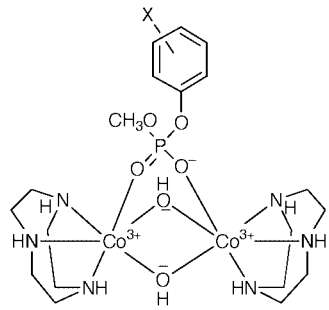
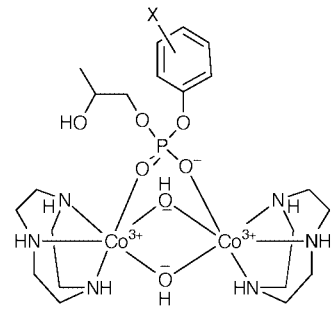


24a: X = *m*-F
24b: X = *p*-NO_2
24c: X = *m*-NO_2
24d: X = H



25a: X = *p*-NO_2
25b: X = H

Substitutionally inert Co^{III} complexes consisting of a phosphate bound to one or two Co^{III} centers can be considered as models for enzyme–substrate Michaelis–Menten complexes and can be unambiguously analyzed. In this way the group of Chin has investigated the importance of structural binding features and Lewis acidity with respect to phosphate ester cleavage.[7] For instance, in analogy with the work of Czarnik *et al.*,[34] they have prepared a dinuclear Co^{III} complex (**23**) demonstrating phosphate monoesterase activity.[35] An X-ray structure of a dinuclear Co^{III} complex bridged with a phosphonate supports a mechanism in which the phosphate is doubly Lewis acid activated and subsequently cleaved by attack of a Co^{III} bound hydroxide ion.

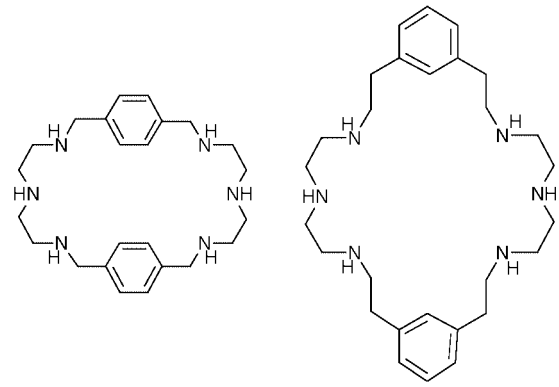
In a more detailed study[36] the group of Chin has investigated the dependence of the rate acceleration for phosphate diester cleavage on the basicity of the leaving group by studying the reactivities of **24** and **25**. Complexes **24** and **25a** cleave by nucleophilic attack of a Co^{III}–Co^{III}-bridged oxide that is formed by deprotonation of the bridged hydroxide. However, **25b** cleaves by intramolecular transesterification. While double Lewis acid activation alone provides comparable rate accelerations for cleaving phosphate diesters with good or poor leaving groups, the oxide nucleophile provides increasingly larger rate accelerations for phosphate diesters with better leaving groups. The bridging oxide shifts the transition state to larger P–O bond cleavage compared with the transition state for the hydrolysis of the unbound phosphates. This study indicates that there will be an enormous cooperative effect between nucleophile activation and leaving group activation. A potential way to increase the rate accelerations would be to make the leaving group better by coordination to another metal ion, or through general acid catalysis.

Dinuclear lanthanide(III) complexes. Schneider *et al.* described dinuclear Pr^{III} and Eu^{III} complexes based on the macrocyclic ligands **26**–**29** that accelerate the cleavage of supercoiled DNA by almost two orders of magnitude over that with metal ions alone.[37] The relative activity order of the complexes is similar for the cleavage of plasmid DNA and BPNP. The metal centers bound in ligand **29** are at a larger distance, which appeared to be more effective in this case.

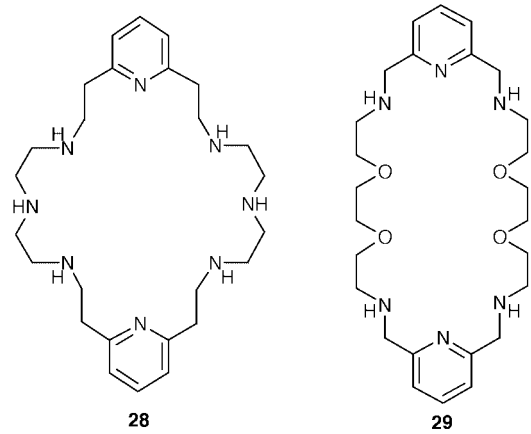
Miscellaneous dinuclear M^{II} complexes. Trogler *et al.*[38] observed that dinuclear complexes like **30**–M_2 and **31**–M_2 have a higher affinity for DNA binding than the corresponding mononuclear complexes. However, dinuclear Ni^{II}, Cu^{II}, and Zn^{II} complexes of the flexible and rigid ligands **30** and **31** did not show notable rate enhancements in the catalytic cleavage of the phosphate diester BPNP.

The group of Canary has synthesized a heterodinucleating ligand **32** that can bind an alkaline earth metal ion and a Zn^{II} ion at addressed sites.[39] At pH 8.5, the Ba^{II}–Zn^{II} couple **32**–BaZn gave a rate enhancement of 1120-fold over background hydrolysis of BPNP, the Zn^{II} complex and Ba^{II} complex alone a 570- and 150-fold enhancement, respectively. From saturation kinetics studies it appeared that the dinuclear Ba^{II}–Zn^{II} complex has a higher affinity for the substrate and that the mononuclear Zn^{II} complex exhibits a higher turnover rate. The low cooperativity in catalysis is attributed to a non-ideal geometrical relationship between the metal centers.

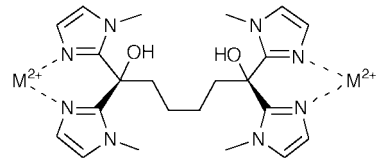
Besides the many functional enzyme models for hydrolytic metallo-enzymes, many structural active site models have also been reported.[6,40] An example is the dinuclear complex **33**–Zn_2 in which the hydrated form of a Zn–OH function is coordinated by a second Zn^{II} ion.[40] This structural Zn–O_2H_3–Zn motif is less established than the Zn–OH_2, Zn–OH, and Zn–OH–Zn functions and may reproduce the connection between two Zn^{II} ions in the trinuclear enzymes phospholipase C and nuclease P1.



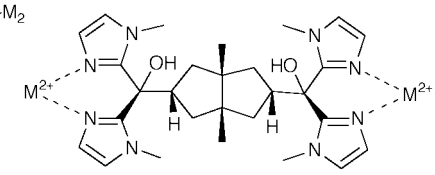
26 **27**



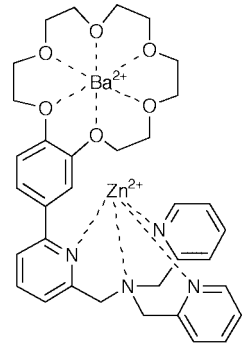
28 **29**



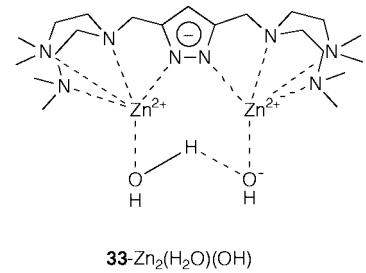
30-M_2 M = Ni, Cu, Zn



31-M_2



32-BaZn



33-$Zn_2(H_2O)(OH)$

3 Calix[4]arene-based models for metallo-phosphodiesterases

In order to study catalytic cooperativity between two metal centers on a calix[4]arene-based molecular scaffold we have compared the phosphodiesterase activity of dinuclear calix[4]arene metal complexes with that of mononuclear calix[4]arene complexes and mononuclear reference complexes that lack the calix[4]arene backbone (Section 3.1).[14–16] Subsequently, we enlarged the dinuclear metal site with additional catalytic

groups like amino groups (Section 3.2)[17,18] or a third metal center (Section 3.3).[15,19] Initially we used *p*-nitrophenyl ester substrates to screen for phosphodiesterase activity. The substrate 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP, Fig. 1a) is an RNA model that reacts *via* intramolecular transesterification by nucleophilic attack of the β-hydroxy group.[14–18] Ethyl *p*-nitrophenyl phosphate (EPNP, b) can be considered as a DNA model substrate that reacts *via* intermolecular nucleophilic attack of a hydroxide ion.[14–16] Furthermore, we have tested the catalysis of intramolecular transesterification of RNA dinucleotides (NpN, c).[16,19]

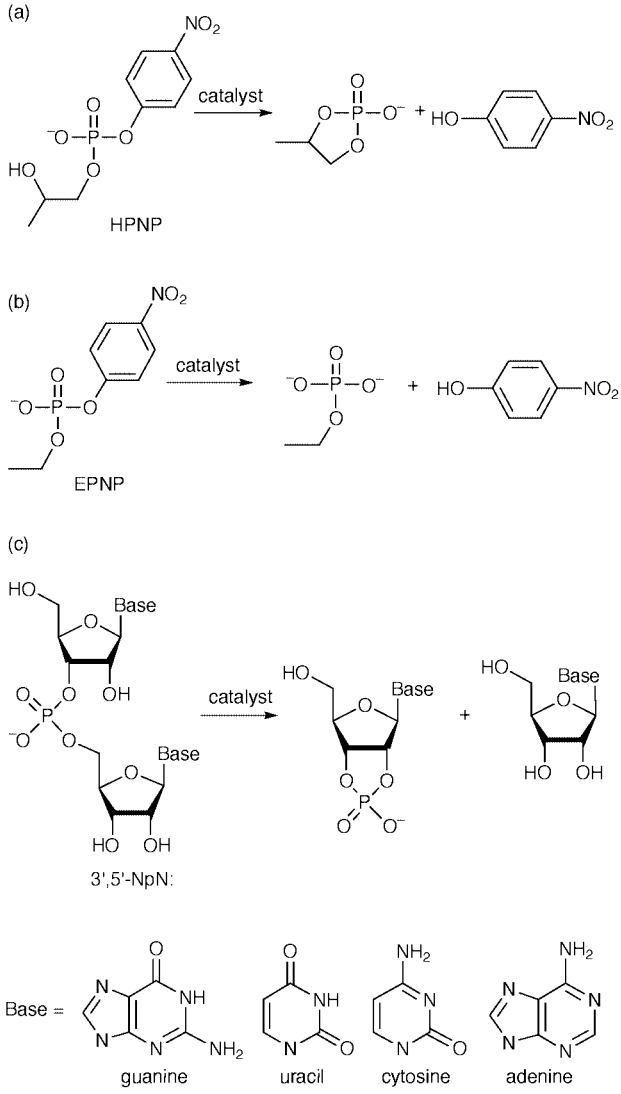
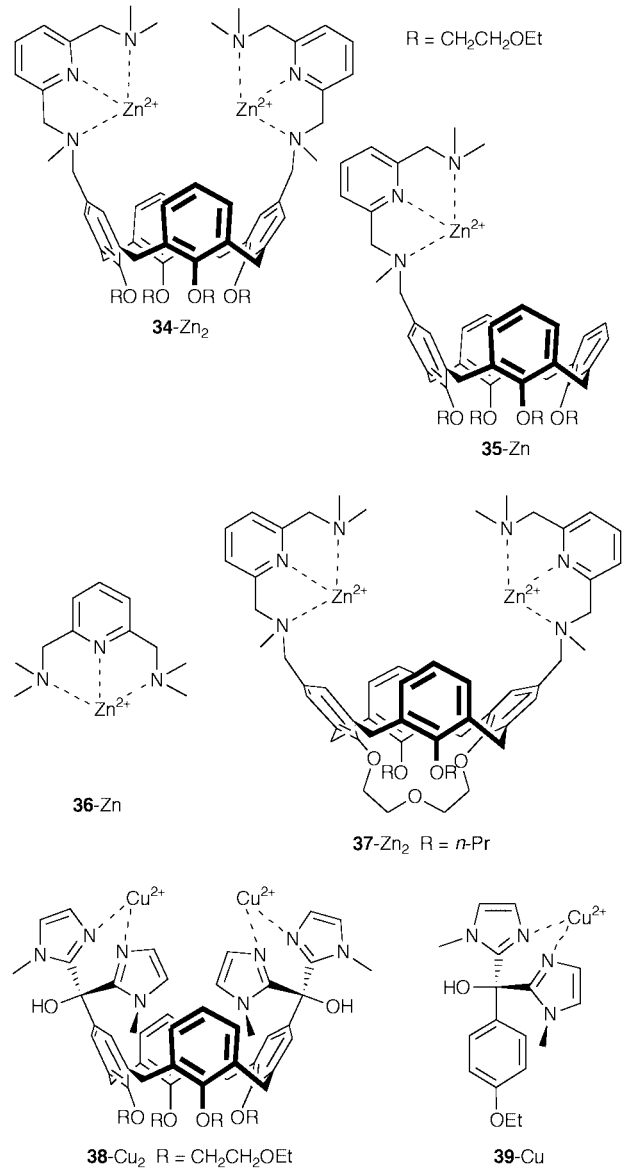


Fig. 1 Catalytic cleavage of an RNA model substrate (a), a DNA model substrate (b), and RNA dinucleotides (c).

The introduction of two transition metal binding sites on the calix[4]arene unit requires the functionalization of two positions at either the lower or upper rim. Although generally calix[4]arenes can easily be functionalized at the lower rim,[12,20] functionalization of the upper rim seems to be more appropriate from the perspective of preorganization and steric requirements of the substrates. CPK-models indicate that when two metal binding sites are present at diametrical positions, at least a one-atom spacer between the calix[4]arene moiety and the first metal-coordinating atom is required to allow sufficient space for substrate binding. A longer alkyl spacer will probably induce too much flexibility. In our studies we have investigated two types of ligands, *i.e.* a tridentate ligand derived from 2,6-bis(aminomethyl)pyridine (**34**)[14,15,18,19] and a bidentate ligand derived from bis(*N*-methylimidazol-2-yl)methane (**38**).[16,17] Both ligands are synthetically readily accessible and can bind a variety of divalent transition metal ions. Furthermore, binding of Zn^{II} or Cu^{II} ions affords complexes with two vacant *cis*-oriented coordination sites which is ideal for catalytic action.



3.1 Dinuclear Zn^{II} and Cu^{II} calix[4]arenes

Calix[4]arene ligand **34**, possessing two bis(dimethylaminomethyl) pyridine ligands at the upper rim, was designed as the starting point for the exploration of the concept regarding preorganization and flexibility in catalytically active calix[4]arenes. The four ethoxyethyl groups at the lower rim prevent inversion of the aromatic units through the annulus of the macrocycle, and bring the calix[4]arene skeleton in a rapid equilibrium between conformations with two diverged (flattened) or parallel (pinched) opposing aromatic units, *via* a symmetrical cone-shaped intermediate (Fig. 2). Moreover, these ethoxyethyl groups increase the solubility in polar solvents and allow the selective functionalization of two opposing upper rim positions.

The ligand system in **34** was built up stepwise starting from the diformylcalix[4]arene.[15] The addition of 2 equiv. $Zn(ClO_4)_2$ to **34** in 50% CH_3CN–20 mM aqueous buffer results in the rapid formation of the dinuclear complex **34**–Zn_2, according to potentiometric and UV–Vis titrations and X-ray crystallog-

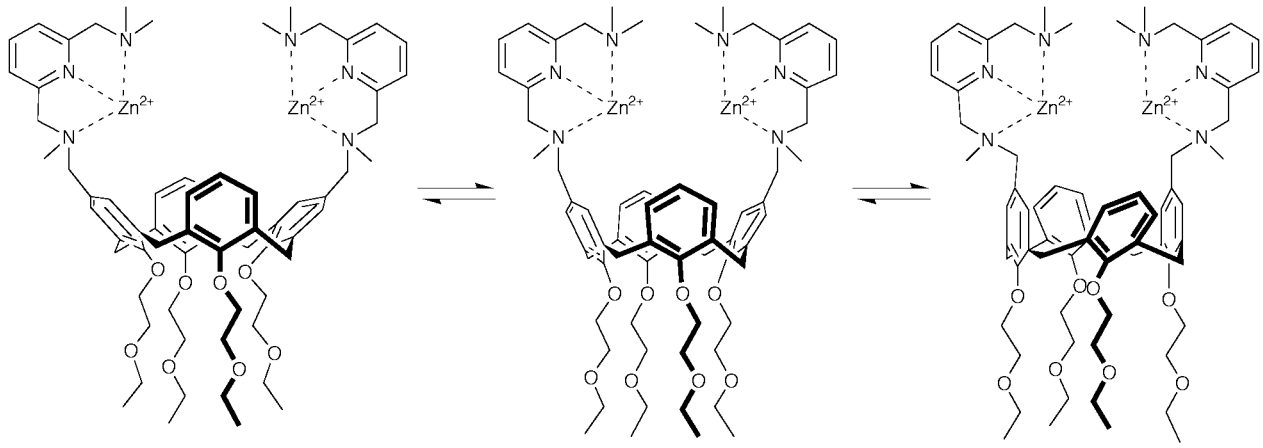


Fig. 2 Conformational flexibility of dinuclear calix[4]arene **34**–Zn_2.

raphy. The catalytic activity of the corresponding dinuclear complex **34**–Zn_2 in the cleavage of phosphate esters was examined.

Although **34**–Zn_2 is not active in the hydrolysis of the phosphate triester diethyl *p*-nitrophenyl phosphate (DEPNP), the diester EPNP and the monoester *p*-nitrophenyl phosphate (PNP), it exhibits a very high activity in the transesterification of the RNA model substrate HPNP. At pH 7.0 0.48 mM of **34**–Zn_2 induces a rate acceleration of a factor 23 000 over the uncatalyzed reaction (Table 1). The catalysis takes place with turnover and a high degree of cooperativity between the Zn^{II} centers. The catalytic activities of mononuclear calix[4]arene **35**–Zn and the reference complex **36**–Zn, lacking the flexible calix[4]arene backbone, are a factor of 50 and 300 lower, respectively.[14,15] The factor of 6 higher activity of calix[4]arene **35**–Zn over reference **36**–Zn indicates a favorable role of the calix[4]arene moiety. This shows that calix[4]arenes are more than simple molecular scaffolds. Besides providing a dynamic preorganization of catalytic groups the calix[4]arene moiety itself can assist in the catalysis *via* its hydrophobic aromatic surface that can lower the pK_a of a nearby metal bound water molecule (*vide infra*)[15,16] and can interact with the substrate.

Saturation studies demonstrate the efficiency of the two nearby Zn^{II} centers in **34**–Zn_2. Both the binding of the substrate (K_{ass}) and the conversion of the substrate (k_{cat}) are far more efficient in **34**–Zn_2 compared to **35**–Zn. The unusually high binding constant of 5.5×10^4 M^{-1} and the high metal ion cooperativity in **34**–Zn_2 may be the result of the directional preorganization of the Zn^{II} centers on the calix[4]arene and the capacity of the calix[4]arene scaffold to adjust the receptor site by low-energy conformational changes.[12,13] The importance of a certain conformational flexibility in the catalyst is shown by comparison of the flexible calix[4]arene **34**–Zn_2 with the rigid calix[4]arene **37**–Zn_2.[15] In **37**–Zn_2 the calix[4]arene lower rim is modified with a crown ether bridge, inhibiting the common interconversion between cone and flattened/pinched cone conformations (Fig. 2). As a consequence, the rigidified complex **37**–Zn_2 is a factor of 8 less efficient than **34**–Zn_2 in the catalysis of transesterification of HPNP. Generally, binding interactions of a substrate and a transition state with the active site of an enzyme involve an induced fit, in which the enzyme undergoes conformational changes. A similar dynamic process might explain the enzyme-like binding affinity of the flexible enzyme model **34**–Zn_2, since the rigidified analog **37**–Zn_2 demonstrates a lower substrate binding strength as well as a lower catalytic rate.

The dinuclear Zn^{II} complex of the bis(aminomethyl)pyridine ligand **34** is superior to its Cu^{II} analog **34**–Cu_2. This is in contrast with the dinucleating bisimidazolyl ligand **38**, which forms a highly catalytically active Cu^{II} complex **38**–Cu_2 and a weakly active Zn^{II} complex **38**–Zn_2.[16] Moreover, the Cu^{II} complex **38**–Cu_2 is, besides being active in the transesterification of HPNP, also active in the hydrolysis of EPNP. In 35% EtOH–20 mM aqueous buffer pH 6.4 0.48 mM of **38**–Cu_2

Table 1 Kinetic data for the transesterification of HPNP catalyzed by Zn^{II} and Cu^{II} complexes

Catalyst	pH	k_{obs}[a]/10^{-4} s^{-1}	k_2/M^{-1} s^{-1}	k_{cat}/10^{-4} s^{-1}	K_{ass}/10^3 M^{-1}	K_m/mM	Ref.
34–Zn_2	7.0[b]	6.3	43	7.7	55	0.018	14, 15
34–Cu_2	7.0[b]	0.18	[c]	[c]	[c]	[c]	15
34–Zn_2	7.4[b,d]	8.0	17	10	17	0.059	14, 15
35–Zn	7.0[b]	0.12	0.015	0.19	0.75	1.3	14, 15
36–Zn	7.0[b]	0.022	[c]	[e]	[e]	[e]	14, 15
37–Zn_2	7.0[b,d]	0.82	0.68	0.95	7	0.14	15
45–Zn_2	6.8[b,d]	2.0	0.68	3.6	1.9	0.53	18
46–Zn_3	7.0[b,d]	8.6	2.9	24	1.2	0.83	15
46–Cu_3	7.0[b]	0.16	[c]	[c]	[c]	[c]	15
None	7.0[b]	2.7×10^{-4}	—	—	—	—	14, 15
38–Cu_2	6.2[d,f]	2.9	0.52	21	0.25	4.0	16
38–Cu_2	7.4[f]	1.4	0.079	[e]	[e]	[e]	16
38–Cu_2	8.8[f]	2.0	[c]	[e]	[e]	[e]	16
39–Cu	6.2[e]	0.13	0.013	[e]	[e]	[e]	16
39–Cu	7.4[d,f]	0.19	0.025	[e]	[e]	[e]	16
39–Cu	8.8[f]	0.14	[c]	[e]	[e]	[e]	16
42–Cu_2	6.2[f]	0.26	0.083	3.3	0.25	4.0	17
42–Cu_2	7.4[d,f]	1.7	0.36	26	0.14	7.1	17
None	6.2[f]	2.9×10^{-4}	—	—	—	—	16
None	7.4[f]	5.4×10^{-4}	—	—	—	—	16
None	8.8[f]	8.3×10^{-4}	—	—	—	—	16

[a] At 0.48 mM catalyst and 0.19 mM HPNP. [b] In 50% CH_3CN–20 mM HEPES at 25 °C. [c] Not determined. [d] Optimum pH. [e] No saturation kinetics observed up to 25 equiv. HPNP. [f] In 35% EtOH–20 mM buffer at 25 °C.

induces a rate acceleration of 27 000. Also in this case a strong cooperativity between the metal centers is observed. The mononuclear complex **39**–Cu exhibits a factor of 330 lower activity.

The pH–rate profile of the dinuclear complex **38**–Cu_2 is bell shaped with the optimum at pH 6.4. Potentiometric titrations reveal that in the most active form of **38**–Cu_2 a Cu^{II} bound water molecule (pK_a 6.5) is deprotonated to a hydroxide ion. The absence of such a very acidic Cu^{II} bound water molecule in the mononuclear complex **39**–Cu (pK_a 7.0) causes its low activity. Furthermore, the only Cu^{II} center in **39**–Cu lacks Lewis acidity to activate and cleave the substrates EPNP and HPNP efficiently.

Comparison of dinuclear Cu^{II} calix[4]arene **38**–Cu_2 (in 35% EtOH) with dinuclear Zn^{II} calix[4]arene **34**–Zn_2 (in 50% CH_3CN, Table 1) shows that the high catalytic activity of **38**–Cu_2 in HPNP transesterification is mainly the result of a high turnover rate (k_{cat}), combined with a moderate substrate–catalyst binding constant (K_{ass}), whereas the activity of **34**–Zn_2 is primarily due to strong substrate–catalyst affinity combined with a relatively moderate turnover rate. This is in agreement with the reported higher phosphate affinity of Zn^{II}. The differences in reactivity between the Zn^{II} complexes **34**–Zn_2 and **38**–Zn_2 and the Cu^{II} complexes **34**–Cu_2 and **38**–Cu_2 can be explained by different coordination geometries of the metal centers.[15,16]

In line with the statement of Chapman and Breslow,[24] we observe that the length and geometry of the ligands determine the catalytic activity and the mechanism of catalysis. Although the distance between the metal centers in **34**–Zn_2 and **38**–Cu_2 changes constantly due to the rapid equilibrium between two extreme calix[4]arene conformations, the average distance in the (aminomethyl)pyridine complex **34**–Zn_2 is longer than in the bis(imidazolyl) complex **38**–Cu_2. Consequently, **34**–Zn_2 is selective for the long RNA model substrate HPNP. Catalyst **34**–Zn_2 is proposed to bind HPNP by a two-point coordination, one Zn^{II} center activating the phosphoryl group and the other Zn^{II} activating the β-hydroxy group. Subsequent intramolecular transesterification can then be promoted by deprotonation of the β-hydroxy group by a general base (Fig. 3). The close proximity of the Cu^{II} centers in **38**–Cu_2 allows the phosphoryl group of EPNP and HPNP to bridge between the Cu^{II} centers, resulting in double Lewis acid activation. Subsequent in-line nucleophilic attack by either a (metal bound) hydroxide ion in the case of EPNP or the β-hydroxy group of HPNP results in leaving group expulsion (Fig. 3).

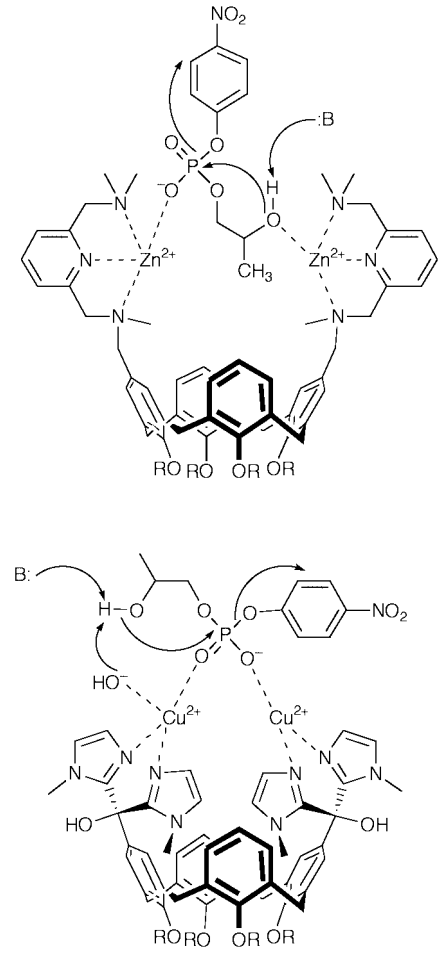


Fig. 3 Proposed mechanisms for HPNP cleavage by calix[4]arene complexes **34**–Zn_2 (top) and **38**–Cu_2 (bottom).

3.2 Dinuclear calix[4]arenes extended with amino functionalities

The active sites of hydrolytic enzymes often contain, besides two transition metal ions, additional functional groups like general acids and bases.[9,10] For the design of enzyme models that combine two catalytic metal ions with other catalytic groups a ligand or molecular scaffold that can be functionalized at multiple positions is required. For this purpose calix[4]arenes are suitable since four directionally preorganized functional groups can be introduced at either the upper or lower rim.[12,13] Hence, we have enlarged the dinucleating ligands **34** and **38** with two amino groups which can provide general base catalysis or, when protonated, general acid catalysis.

The calix[4]arenes **42**[17] and **45**[18] are analogues of ligands **38** and **34**, respectively, and were obtained *via* a multistep synthesis for which the key intermediates are shown in Fig. 4. The formyl groups in calix[4]arene **40**, obtained by formylation of the diester, were converted to azides by, sequential reduction with $NaBH_4$, chlorination with $SOCl_2$, and nucleophilic substitution with NaN_3. Reduction of the azide groups and *in situ* Boc protection gave intermediate **41**, which was reacted with an excess of 2-lithio-*N*-methylimidazole to form the bis(imidazolyl) ligand system. Deprotection afforded calix[4]arene **42**, which has two metal ion binding sites and, in addition to **38**, two primary amines.[17] Similarly, calix[4]arene **45** has, besides two (aminomethyl)pyridine ligands like in **34**, two additional tertiary amines.[18] In the synthesis hydroxymethyl derivative **43** was protected with TBDMS groups. Subsequently, formyl groups were introduced by a bromo–lithium exchange and reaction with DMF. Reductive amination with methylamine and deprotection afforded intermediate **44**. Reaction with 2-(bromomethyl)-6-(hydroxymethyl)pyridine gave the precursor of the ligand. The four hydroxymethyl groups were converted to chloromethyl groups which were immediately substituted by reaction with dimethylamine to give the ligand **45**.

The addition of two equivalents of $Cu(ClO_4)_2$ or $Zn(ClO_4)_2$ to the ligands **42** or **45** results in the formation of dinuclear complexes **42**–Cu_2 and **45**–Zn_2. Both complexes are catalytically active in the transesterification of HPNP, exhibiting efficient cooperativity between the metal centers. Comparison with the parent complexes **38**–Cu_2 and **34**–Zn_2, which lack the amino functionalities, shows a different kinetic behavior. The optimum pH shifts from 6.2 for **38**–Cu_2 to 7.4 for **42**–Cu_2 and from pH 7.5 for **34**–Zn_2 to 6.8 for **45**–Zn_2. Furthermore, at pH 7.4 the amino complex **42**–Cu_2 exhibits Michaelis–Menten kinetics whereas **38**–Cu_2 catalyzes *via* a first-order dependency.[17] Thus, at this pH **42**–Cu_2 has a higher substrate affinity than **38**–Cu_2. The kinetics indicate that at pH 7.4, the amino groups in **42**–Cu_2 are present as ammonium groups that can assist the Cu^{II} centers as general acids in the substrate binding (Fig. 5). In contrast, the substrate affinity of **45**–Zn_2 has dropped by almost a factor of 30 compared to **34**–Zn_2. It is likely that the bulky tertiary dimethylamino groups in **45**–Zn_2 hinder the binding of the substrate to form a catalyst–substrate complex.

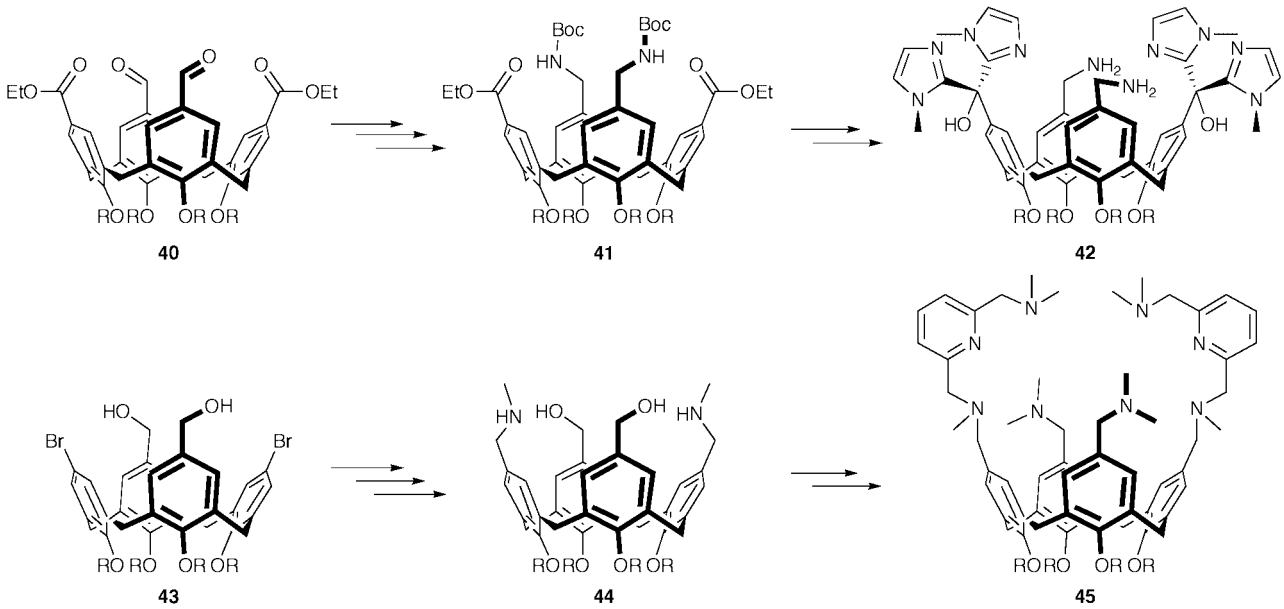


Fig. 4 Key intermediates in the synthesis of calix[4]arene ligands with additional amino functionalities.

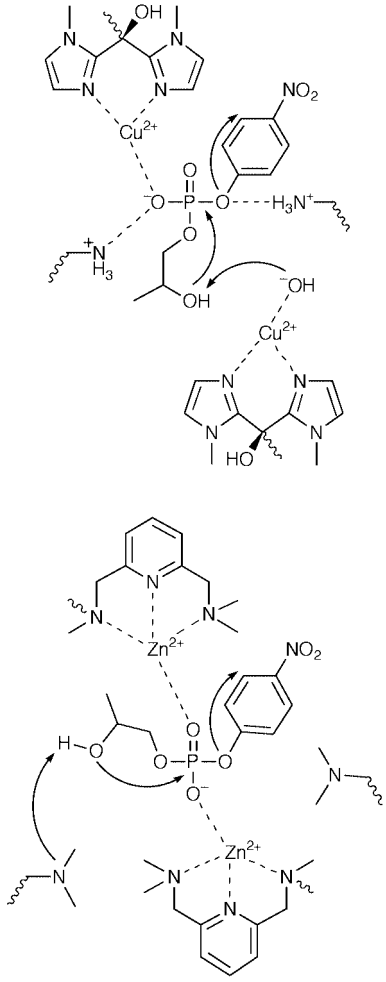


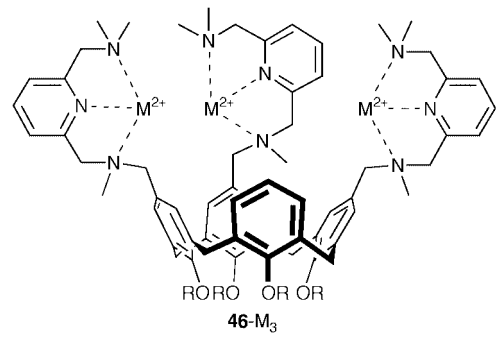
Fig. 5 Schematic representation of possible mechanisms for HPNP cleavage by aminocalix[4]arene complexes **42**–Cu_2 (top) and **45**–Zn_2 (bottom).

Also the rate constant k_{cat} is reduced by a factor of 2 for **45**–Zn_2, suggesting a less favorable binding orientation for conversion of the substrate and a different catalysis mechanism. The pH shift indicates a possible mechanism comprising bifunctional catalysis in which two Zn^{II} centers activate the phosphoryl group and one of the two dimethylamino groups acts as a general base in the deprotonation of the substrate (Fig. 5).

3.3 RNA dinucleotide cleavage by a trinuclear Zn^{II} calix[4]arene

The previous sections described dinuclear metallo(II)-calix[4]arenes that mimic the dinuclear metallo-phosphodiesterases in nature. High rate enhancements were achieved in the catalytic cleavage of activated phosphate diesters, like HPNP and EPNP, due to the efficient cooperative action of two Zn^{II} or Cu^{II} ions. However, these dinuclear enzyme models appeared to be hardly active in the cleavage of natural substrates, like RNA dinucleotides.[16,19] Extension of the catalytic metal centers with amino groups as potentially general acid/base catalysts delivered compounds that showed an interesting catalytic behavior, but did not result in a strong increase of catalytic activity. A specific subclass of the dinuclear metallo-phosphodiesterases possesses even a third metal ion in the active site.[9] Examples include phospholipase C and nuclease P1, which use three Zn^{II} ions to catalyze the hydrolytic cleavage of phosphate diester bonds in phosphatidylcholine and phosphatidylinositol and in nucleotides like RNA and DNA, respectively.

In order to elucidate the catalytic effect of three proximal metal ions on phosphate ester hydrolysis[25] we have enlarged the dinuclear Zn^{II} complex **34**–Zn_2 with an additional metal center to obtain trinuclear complex **46**–Zn_3.[15,19] First, catalysis studies



46-M_3

M = Zn, Cu
R = CH_2CH_2OEt

with the activated substrate HPNP showed cooperative action of three Zn^{II} centers resulting in an enhanced reactivity of **46**–Zn_3 compared to **34**–Zn_2.[15] Whereas the HPNP binding constant decreased **46**–Zn_3 showed a three-fold increase of the turnover rate (k_{cat}). This enhanced catalytic power was confirmed by

catalysis experiments with the RNA dinucleotide substrates UpU and GpG (Fig. 1).[19] In 35% EtOH–20 mM HEPES pH 8.0 at 0.9 mM of **46**–Zn_3 we observed rate accelerations in the order of 10^4–10^5 and catalytic turnover, whereas the dinuclear complex **34**–Zn_2 (0.9 mM) and the mononuclear reference complex **36**–Zn (2.7 mM) showed only minor activity.[19] Highest activity is observed at pH 8.0, where at least one of the Zn^{II} centers of **46**–Zn_3 coordinates a hydroxide ion.[15] The rate as a function of the Zn^{II} concentration shows an optimum at three equivalents Zn^{II} with respect to the ligand **46**, supporting a three-metal ion cooperativity. The trinuclear Cu^{II} analog **46**–Cu_3 is hardly active. However, studies with mixtures of Cu^{II} and Zn^{II} demonstrate that a statistical mixture with the heterotrinuclear complex **46**–Zn_2Cu as the main species is more active than **46**–Zn_3. This demonstrates that the combination of metal ions with different properties can lead to complexes with unexpected catalytic activity.[19,39] This approach is also found in natural metallo-phosphoesterases, for example, the active site of alkaline phosphatase contains two Zn^{II} centers and one Mg^{II} center.[9]

Studies with a series of RNA dinucleotides, *i.e.* GpG, UpU, CpC, GpA, ApG, and ApA, revealed for **46**–Zn_3 a significant nucleobase specificity. For instance, GpG (k_{obs} = 72 × 10^5 s^{-1}) and UpU (k_{obs} = 8.5 × 10^5 s^{-1}) are cleaved a factor of 160 and 19 faster than ApA (k_{obs} = 0.44 × 10^5 s^{-1}), respectively. Remarkably, in the cleavage of ApA the three Zn^{II} centers are not cooperatively involved in the catalysis. In substrate saturation studies GpG (K_m = 1.3 mM, k_{cat} = 18 × 10^{-4} s^{-1}) and UpU (K_m = 0.34 mM, k_{cat} = 1.1 × 10^{-4} s^{-1}) show Michaelis–Menten kinetics whereas ApA shows second-order kinetics (k_2 = 4.3 × 10^{-3} M^{-1} s^{-1}). This indicates that, in contrast to ApA, GpG and UpU form a relatively large amount of a reactive substrate–catalyst complex. The molecular basis for the enhanced binding is found in the presence of an acidic amide in guanosine and uridine. At the pH optimum of 8.0, one of the deprotonated nucleobases of GpG and UpU may coordinate to a Zn^{II} center to form a **46**–Zn_3-nucleobase complex. We propose that the two remaining Zn^{II} centers provide double Lewis activation of the phosphoryl group and stabilization of the leaving group. Such a mechanism is supported by a computer-generated model of **46**–Zn_3–GpG^- (Fig. 6).

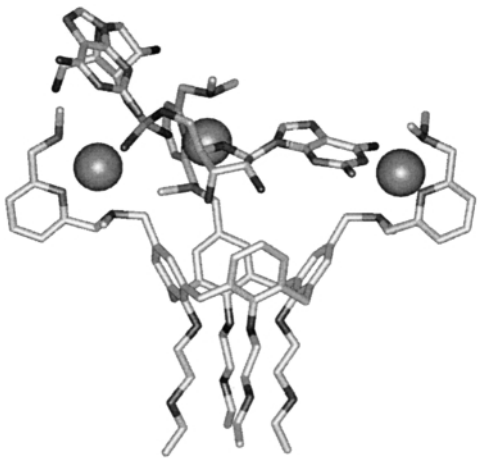


Fig. 6 Computer-generated model of **46**–Zn_3 complexed with deprotonated GpG (reprinted from ref 19. Copyright 1999 Wiley-VCH).

3.4 Outlook for calix[4]arene-based metallo-phosphodiesterases

Our studies have shown that calix[4]arenes are suitable molecular scaffolds for the design of dinuclear phosphodiesterase models. Whereas efficient catalysis by cooperative action of the metal centers is observed, optimal bifunctional catalysis has not been achieved. Future studies should focus on variation in the ligands and additional catalytic groups in order to diminish steric hindrance and find ideal cooperative effects. Besides this fine tuning the solubility in pure water should be increased. In addition, functionalization of the calix[4]arenes with RNA recognition sites, like a complementary strand of DNA, can afford artificial nucleases that cleave RNA oligonucleotides sequence-selectively. In this respect, further investigation of the trinuclear calix[4]arene complexes is promising since these complexes are shown to be active in RNA cleavage[19] and still have one vacant upper rim position for further functionalization.

4 Conclusions

Recently, the elucidation of the three-dimensional structure of enzymes that cleave phosphate ester bonds has initiated novel research in bio(in)organic chemistry. The active sites of many of these phosphoesterases appear to contain two or three divalent metal ions like Zn^{II} and Mg^{II}. In the first part of this article, functional enzyme models that mimic these dinuclear metallo-phosphoesterases have been reviewed. Many of these enzyme models are dinuclear metal complexes in which two Lewis acidic metal centers are oriented in close proximity by either very rigid or very flexible molecular spacers. Compared to the natural enzymes, the catalytic efficiency of these early developed models is moderate. Major drawbacks are poor substrate binding and inefficient catalytic turnover, caused by a low cooperativity between the metal centers. Furthermore, the models cannot compete with the enzymes since they lack multiple catalytic groups that can bind and activate the substrate and can stabilize the transition state and leaving group. However, the enzyme models have given insight into possible catalysis mechanisms employed by the natural dinuclear phosphodiesterases and have provided a basis for the future design of sequence specific RNA cleaving agents. The first artificial nucleases capable of cleaving RNA oligonucleotides in a sequence-selective manner were DNA conjugates of mononuclear complexes of the strongly Lewis acidic, but toxic, lanthanide(III) ions. The next step is to incorporate real biomimetic compounds that use *in vivo* available metal ions like Zn^{II} so that they can be applied in antisense therapeutics. In this respect, progress is now being made.

In the second part of this article we have reviewed our own studies on synthetic metallo-phosphodiesterases based on calix[4]arenes. Our motivation to explore calix[4]arenes in enzyme models was that in the search for new catalysts, molecular scaffolds that exhibit a certain flexibility are preferred over rigid preorganized scaffolds. Furthermore, the ease of functionalization is attractive and opens the way to enzyme models in which multiple functional groups are dynamically preorganized, similar to the amino acid residues and cofactors in the active sites of enzymes. In the past decades, calix[4]arenes have been successfully used for the design of molecular receptors, but remarkably, calix[4]arene-based enzyme models were scarce.

We have shown that calix[4]arenes functionalized with two or even three divalent metal complexes can mimic the active site of dinuclear metallo-phosphodiesterases. The cleavage of phosphate diester substrates *via* intramolecular transesterification or hydrolysis is efficiently catalyzed, with turnover, by the cooperative action of two (or three) Zn^{II} or Cu^{II} centers that are preorganized on the upper rim of the semi-flexible calix[4]arene backbone. The binding of the substrate to the catalyst and conversion of the substrate within the substrate–catalyst complex are more efficient in the calix[4]arene dinuclear

complexes compared to the mononuclear reference compounds which lack the calix[4]arene backbone. Furthermore, we have provided synthetic methodologies to functionalize calix[4]arenes with multiple catalytic groups. Although optimal bifunctional catalysis of two metal centers and two general acid/base groups has not been achieved yet, the approach is promising. The work shows that calix[4]arenes are suitable building blocks for the design of catalytically active enzyme models. The results on metallo-phosphodiesterase mimics may be extrapolated in the future to sequence-selective RNA cleavers and to mimics of other enzymes and the design of industrially applicable catalysts.

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