Efficient Catalytic Phosphate Diester Cleavage by the Synergetic Action of Two Cu(II) Centers in a Dinuclear *Cis*-Diaqua Cu(II) Calix[4]arene Enzyme Model

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Abstract: A calix[4]arene derivative **2**-$[Cu(II)]_2$ functionalized with two *cis*-diaqua Cu(II) centers at the distal positions of the upper rim was synthesized and investigated as a model for dinuclear metalloenzymes that catalyze chemical transformations of phosphate esters. The flexible dinuclear calix[4]arene efficiently catalyzes the transesterification of the RNA model 2-hydroxypropyl-*p*-nitrophenyl phosphate (HPNP) and the hydrolysis of the DNA model ethyl-*p*-nitrophenyl phosphate (EPNP) with turnover conversion, thereby exhibiting rate enhancement factors of 1.0×10^4 and 2.7×10^4, respectively. The mononuclear reference complex, **3**-Cu(II), lacking the macrocyclic backbone, has a much lower activity, showing that the high catalytic activity of the dinuclear calix[4]arene complex is due to synergetic action of the two Cu(II) centers. Saturation kinetics and pH variation studies point to the formation of a Michaelis−Menten complex in which the phosphate group is doubly Lewis acid activated by coordination to the two Cu(II) centers. In this complex, a Cu(II) bound hydroxide ion, which is present already at pH 6.5, can act as a base in the intramolecular transesterification of HPNP or as a nucleophile in the hydrolysis of EPNP. The remarkably low pK_a of the Cu(II) bound water molecules in the hydrophobic calix[4]arene **2**-$[Cu(II)]_2$ mimics the low pK_a of metal bound water molecules in hydrophobic enzyme active sites, which makes the enzyme (model) active under slightly acidic to neutral conditions. The high catalytic efficiency of this enzyme model is attributed to a dynamic binding of the substrate and (pre)-transition state, possible by rapid low energy conformational changes of the flexible calix[4]arene backbone.

Introduction

In nature, chemical transformations of phosphate esters by metalloenzymes are generally facilitated by the cooperative action of two metal ions, *e.g.*, in P1 nuclease, DNA polymerase I, phospholipase C, and alkaline phosphatase.[1] These metal ions are usually Zn(II), Mg(II), Mn(II), or Fe(III) and are separated by 3−5 Å in the active site of these enzymes. They function as Lewis acids for the activation of the phosphate group, they generate a reactive nucleophile, and they stabilize both the pentacoordinate phosphorous transition state and the leaving group. Artificial models for these dinuclear metalloenzymes are of particular interest because they can increase the understanding of enzymatic cleavage of chemically stable phosphate esters[2] and because of the possible applications in biotechnology.[3] Several artificial active site models have been reported in which two metal centers, such as Zn(II),[4,5] Cu(II),[4b,6,7] Co(III),[8] and lanthanides(III),[9] are held apart by two ligands that are linked to a molecular scaffold.

Nonenzymatic phosphate ester cleavage is effectively facilitated by the strongly Lewis acidic Co(III)[8,10] and lanthanide(III)[9,10a,11] ions. However, for application as artificial nucleases the use of Co(III) is not attractive since it forms substitutional

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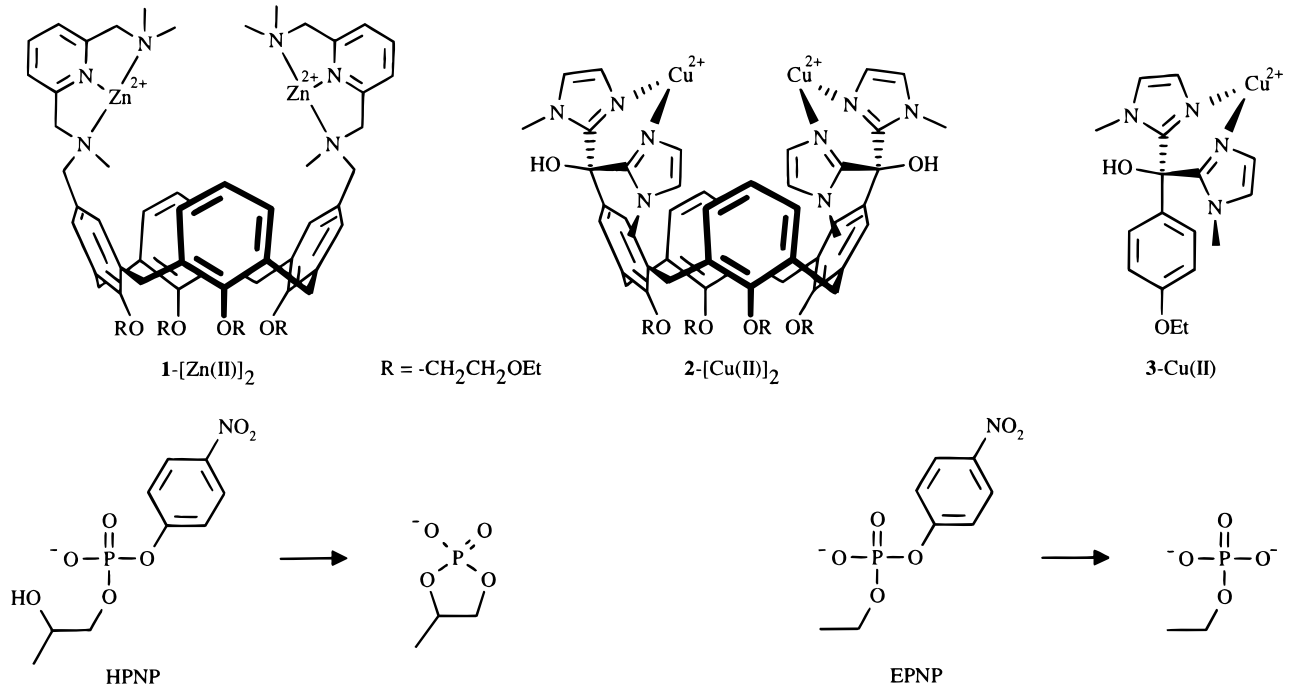
Chart 1



inert complexes with the products, which hamper turnover catalysis at neutral pH. The disadvantage of lanthanide(III) ions is their toxicity and the laborious formation of sufficiently stable complexes. Free lanthanide(III) ions form readily lanthanide-(III)-hydroxide precipitates, and both forms are active in phosphate ester cleavage, which makes kinetic control complicated. *Cis*-diaqua Cu(II) complexes, either mononuclear[12−17] or dinuclear,[6,7] have also been reported as active artificial catalysts for the cleavage of phosphate diesters. Chin has shown that the unique catalyst neocuproine-Cu(II)[14] and a 1,8-naphthalene substituted dinuclear [9]aneN$_3$-Cu(II) complex[6b] cleave RNA rapidly under mild conditions. Burstyn *et al.*[16] and Fujii *et al.*[17] reported that DNA is cleaved hydrolytically by [9]aneN$_3$-Cu(II) and triaminocyclohexane-Cu(II) complexes, respectively.

From the work of Clewley *et al.*,[4b] Czarnik *et al.*,[8d] and of Breslow and Chapman[4c] it appeared that two metal centers which were linked to flexible molecular scaffolds hardly showed any cooperativity in catalytic phosphate ester cleavage. Consequently, attempts were made to design dinuclear complexes on rigid molecular scaffolds which orient the metal centers in such a way that the metal−metal distance matches the distance required for selective recognition and binding of the pentacoordinate phosphorous transition state.[4−9] However, in contrast to enzymes, many of all these designed enzyme models exhibit poor substrate binding and activation and do not show efficient catalytic turnover. This might be due to the fact that the molecular scaffolds used are either too flexible or too rigid in nature, as for substrate binding a certain degree of preorganization of the binding sites is required whereas the substrate activation process involves considerable change in geometry.[18] Imperfect preorganization of the catalytic metal centers and a lack of a certain flexibility can give rise to insufficient capacity to bind dynamically the substrate as well as the transitions state, which is of crucial importance for catalysis.[18]

Modified calix[4]arenes[19,20] have been proposed as promising host molecules due to the directional preorganization of functional binding groups and their capacity to adjust the guest binding site rapidly by low energy conformational changes.[21] They have also been proposed as building blocks for multifunctional enzyme models.[5,19,22] However, despite the many

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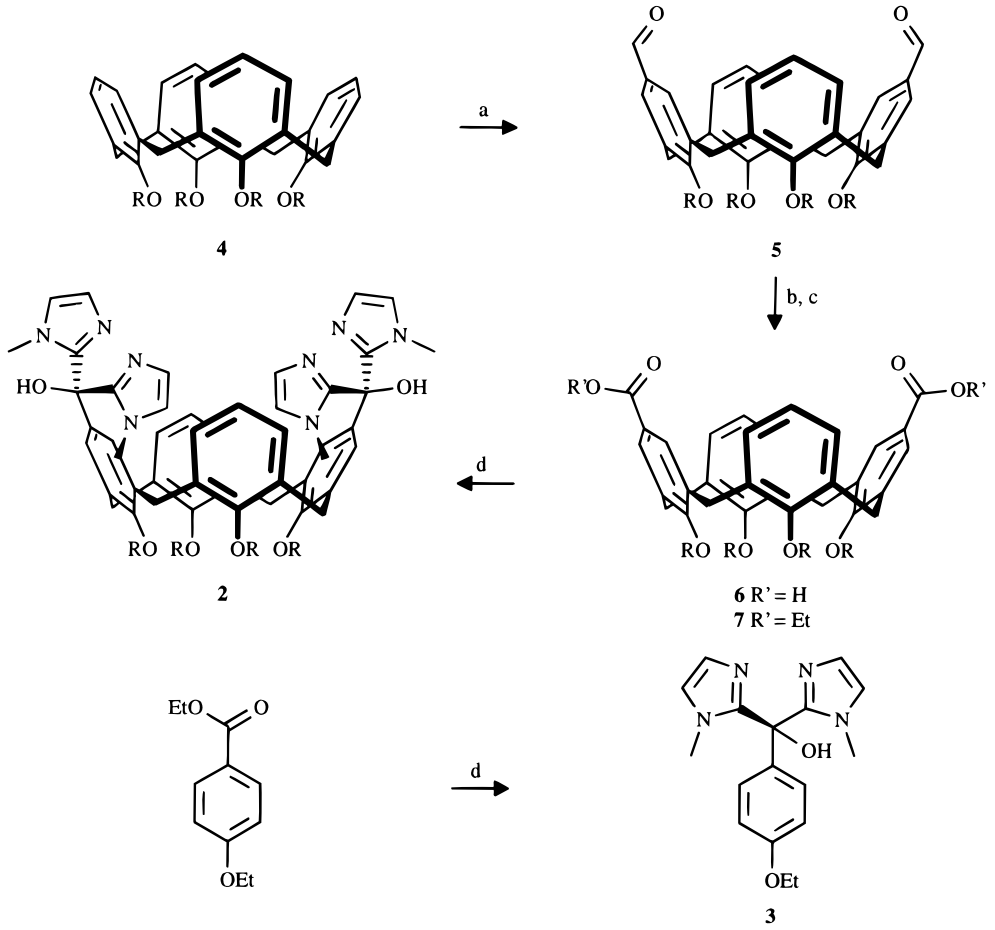
Scheme 1. Synthesis of the Ligands (R = $-CH_2CH_2OEt$)[a]



[a] (a) $SnCl_4$, Cl_2CHOCH_3, $CHCl_3$, 87%. (b) H_2NSO_3H, $NaClO_2$, H_2O, $CHCl_3$, acetone, 61%. (c) EtOH, *p*-TosOH, 98%. (d) 1-methylimidazole, *n*-BuLi, THF, 85% (**2**), 75% (**3**).

calix[4]arene-based supramolecular receptors that are known, calix[4]arene-based enzyme models have been hardly developed.[23] A notable example is the calix[4]arene modified with a crown ether Ba(II) complex that exhibits transacylase activity, reported by Mandolini *et al.*[22a] Recently, we have shown that the calix[4]arene dinuclear Zn(II) complex **1**-$[Zn(II)]_2$ acts as an efficient catalyst, with turnover, for the intramolecular transesterification of the RNA model substrate 2-hydroxypropyl-*p*-nitrophenyl phosphate (HPNP).[5] The synergetic action of the two Zn(II) centers on the flexible calix[4]arene moiety resulted in a rate enhancement over the uncatalyzed reaction of 2.3×10^4. The advantage of a calix[4]arene as a molecular scaffold for two catalytic transition metals is that the calix[4]arene moiety has sufficient flexibility to breathe, *i.e.*, it can dynamically adopt flattened/pinched conformations by the simultaneous movement of the two facial aromatic units toward (pinched) and away (flattened) from each other.[21] For application in catalytic phosphate diester cleavage possibilities arise for binding of the substrate by molecular recognition, subsequently followed by conversion of the substrate via dynamic binding of the (pre)-transition state(s), and release of the products.

The favorable properties of a flexible calix[4]arene platform for the preorganization of functional groups and the generally high catalytic activity of *cis*-diaqua Cu(II) complexes in phosphate diester cleavage led us to study the effect of combining these features in a novel catalytic dinuclear metalloenzyme model **2**-$[Cu(II)]_2$. This dinuclear complex is based on the calix[4]arene ligand **2**, which contains chelating bisimidazolyl groups that mimic the natural histidine residues (Scheme 1).[24−28] A number of related dinuclear metal complexes with

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either very flexible or very rigid scaffolds have been studied by Trogler and co-workers.[25] They observed that the dinuclear complexes have a higher affinity for DNA binding than the corresponding mononuclear complexes. However, no notable rate enhancements were observed for the dinuclear compounds in the catalytic cleavage of (activated) phosphate diesters.

Results and Discussion

In this paper we describe the synthesis, the characterization, and the catalytic activity of the dinuclear calix[4]arene complex **2**-$[Cu(II)]_2$ and the mononuclear reference complex **3**-Cu(II). The formation of these Cu(II) complexes from the corresponding ligands **2** and **3** was studied by spectrophotometric and potentiometric titrations and X-ray crystallography. The dinuclear complex **2**-$[Cu(II)]_2$ shows rate enhancement factors of more than 10^4 in the transesterification and hydrolysis of phosphate diesters. The rate of phosphate diester cleavage was studied as a function of pH and the concentration catalyst and substrate, respectively. Comparison with the mononuclear reference compound **3**-Cu(II) shows that the high catalytic activity of **2**-$[Cu(II)]_2$ is the result of efficient synergetic action of the two preorganized but still dynamic, Cu(II) centers in phosphate diester binding, activation, and conversion.

Synthesis. In order to confine the calix[4]arene moiety in the flexible cone conformation[19−21] we alkylated the calix[4]-arene lower rim with four ethoxyethyl groups (**4**, Scheme 1).[29] These ethoxyethyl groups increase the solubility in polar solvents and render the direct regioselective Gross formylation of the diametrical positions of the calix[4]arene upper rim possible. This method, published by Ungaro *et al.*,[21c] was improved by using a large excess of both tin tetrachloride and 1,1-dichloromethyl ether to give the diformyltetrakis(ethoxethyl)calix[4]-arene **5** in 87% yield. Oxidation of the formyl groups to carboxylic acids (**6**)[30] and acid catalyzed esterification with ethanol afforded diethyl ester **7**. The bis(1-methylimidazol-2-yl) ligand groups were introduced[25,26] by reacting diethyl ester **7** with excess of lithiated 1-methylimidazole, to give the calix-[4]arene-based dinucleating bidentate ligand **2** in 85% yield. The mononucleating reference ligand **3** is an ethoxy substituted analog of a known ligand[26a] and was prepared from 4-ethoxy-benzoic acid ethyl ester.

Cu(II) Complex Formation. Complex formation was determined spectrophotometrically by monitoring the increase in absorbance at 274 nm of the bisimidazolyl groups upon titration of the ligands **2** and **3** with $Cu(ClO_4)_2 \cdot 6H_2O$ in 35% EtOH/20 mM aqueous buffer (v/v). Experiments at pH 6 (aqueous buffer part of the reaction mixture before dilution with EtOH)[31] with 0.075 mM of the ligands show titration curves corresponding to the formation of a 1:2 ligand:Cu(II) complex **2**-$[Cu(II)]_2$ for dinucleating calix[4]arene ligand **2** and a 1:1 complex **3**-Cu(II) for mononucleating ligand **3**. When calix-[4]arene **2** is titrated with excess Cu(II), an isosbestic point appears at 266 nm after 1 equiv of Cu(II) with respect to ligand **2** has been added. This indicates that the desired 1:2 complex **2**-$[Cu(II)]_2$ is readily formed from the 1:1 complex **2**-Cu(II) and that almost no intra- or intermolecular bischelated Cu(II) complexes are formed under conditions with 2 equiv of Cu(II) present. When the mononucleating ligand **3** is titrated with excess Cu(II), an isosbestic point is observed at 266 nm after 0.7 equiv of Cu(II) with respect to ligand **3** has been added. This indicates that when less than 0.7 equiv of Cu(II) are present, the mononuclear 1:1 complex **3**-Cu(II) may be in equilibrium with the bischelated 2:1 complex $(\mathbf{3})_2$Cu(II).[24,25,27] Both complexes could be isolated by crystallization from 1:1 mixtures of ligand **3** and Cu(II) salts and were analyzed by X-ray crystallography (*vide infra*).

Table 1. Protonation Constants of the Ligands and the Corresponding Cu(II) Complexes Determined by Potentiometric pH Titrations

	2 in 35% EtOH[a,b]	**3** in 35% EtOH[a,c]	**3** in 0.1 M KNO_3[c]
log K_1	1.97 ± 0.06	2.12 ± 0.1	2.61 ± 0.05
log K_2	5.92 ± 0.02		
log K_3	6.64 ± 0.01	6.32 ± 0.25	6.22 ± 0.15
log K_4 ($CuOH_2$)	6.53 ± 0.04	6.96 ± 0.15	7.03 ± 0.15
log K_5 ($CuOH_2$)	6.59 ± 0.06		
log K_6 ($CuOH_2$)	9.69 ± 0.11	10.04 ± 0.40	9.93 ± 0.35
log K_7 (Cu)		>6	>6

[a] In 0.1 M KNO_3 in 35% EtOH/H_2O (v/v), at 20 °C. [b] $K_1 = [H_3\mathbf{2}]/[H_2\mathbf{2}][H^+]$; $K_2 = [H_2\mathbf{2}]/[H\mathbf{2}][H^+]$; $K_3 = [H\mathbf{2}]/[\mathbf{2}][H^+]$; $K_4 = [\mathbf{2}Cu_2(H_2O)_n]/[\mathbf{2}Cu_2(OH)(H_2O)_{n-1}][H^+]$; $K_5 = [\mathbf{2}Cu_2(OH)(H_2O)_{n-1}]/[\mathbf{2}Cu_2(OH)_2(H_2O)_{n-2}][H^+]$; $K_6 = [\mathbf{2}Cu_2(OH)_2(H_2O)_{n-2}]/[\mathbf{2}Cu_2(OH)_3(H_2O)_{n-3}][H^+]$. [c] $K_1 = [H_2\mathbf{3}]/[H\mathbf{3}][H^+]$; $K_3 = [H\mathbf{3}]/[\mathbf{3}][H^+]$; $K_4 = [\mathbf{3}Cu(H_2O)_n]/[\mathbf{3}Cu(OH)(H_2O)_{n-1}][H^+]$; $K_6 = [\mathbf{3}Cu(OH)(H_2O)_{n-1}]/[\mathbf{3}Cu(OH)_2(H_2O)_{n-2}][H^+]$; $K_7 = [\mathbf{3}Cu]/[\mathbf{3}][Cu]$; at 20 °C.

Fitting of the titration curves by a nonlinear least squares method[32] gave association constants larger than 10^6 M^{-1} for binding of Cu(II) to the bisimidazolyl groups in both the dinucleating calix[4]arene **2** and mononucleating ligand **3** (see also section Potentiometric Titrations). Such a value for the stability constants is in the same range as those found for transition metal(II) complexes of other bidentate bisimidazolyl and bipyridyl ligands reported in the literature[24,26b,33] and indicates that more than 95% of the Cu(II) ions are bound during the kinetic studies (*vide infra*).

Potentiometric Titrations. The protonation constants (Table 1) for the bisimidazolyl ligands **2** and **3** and the corresponding dinuclear and mononuclear complexes **2**-$[Cu(II)]_2$ and **3**-Cu-(II) were determined by potentiometric pH titrations[34] in 0.1 M KNO_3 in 35% EtOH/H_2O (**2** and **3**) and in 0.1 M aqueous KNO_3 (**3**). Typical titration curves of acidified mixtures of the ligands in the absence (curve a) and in the presence (curve b) of stoichiometric amounts of $Cu(NO_3)_2$ revealed the formation of the ligand-Cu(II) complexes (Figure 1). A remarkable observation is the extremely low protonation constant of the imidazole nitrogens, which causes Cu(II) complex formation to start already at pH 3.5 and to be complete at pH 6. Calculation of the exact association constants for Cu(II) complexation with the bisimidazolyl ligands is hampered by the low value of the first protonation constant of the ligands, but the data indicate that the association constants are at least larger than 10^6 M^{-1}, which means that the Cu(II) complexes are very stable (see also UV spectrophotometric titrations). Upon titration of the di-

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(31) Because the calix[4]arene dinuclear complex **2**-$[Cu(II)]_2$ is insoluble in pure water, we selected EtOH as a cosolvent. The pH discussed in the text refers to the pH of the aqueous portion of the reaction mixture before dilution with EtOH up to 35% (v/v). The corresponding pH value in 100% aqueous solution can be determined by adding 0.09 units, according to the method of Bates (ref a). The pK_a values in 35% EtOH are almost the same as in 100% aqueous solution as was confirmed by the potentiometric titrations, see also ref b. (a) Bates, R. G.; Paabo, M.; Robinson, R. A. *J. Phys. Chem.* **1963**, *67*, 1833. (b) Koike, T.; Kimura, E. *J. Am. Chem. Soc.* **1991**, *113*, 8935.

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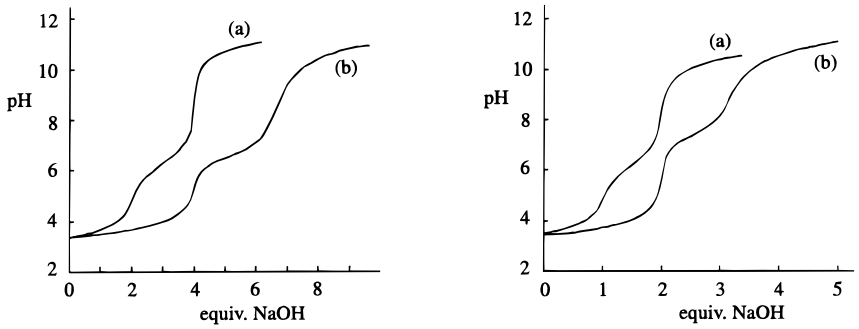

Figure 1. Experimental titration curves for dinucleating ligand **2**·4HNO_3 (left) in 0.1 M KNO_3 in 35% EtOH/H_2O and mononucleating ligand **3**·2HNO_3 (right) in 0.1 M KNO_3, at 20 °C, in the absence (a) and in the presence (b) of 2 equiv (**2**) and 1 equiv (**3**) of $Cu(NO_3)_2$, respectively.

nuclear calix[4]arene complex **2**-$[Cu(II)]_2$ with base, two Cu(II) bound water molecules deprotonate with pK_a's of 6.5 and 6.6, respectively. These pK_a's are lower than the pK_a of the first dissociating Cu(II) bound water molecule in the mononuclear reference complex **3**-Cu(II). This pK_a of 7.0 is already lower than those found for Cu(II) bound waters in bypiridine-Cu(II) (7.8)[14,33a] and phenanthroline-Cu(II) (>7.9),[33a] and the same as found for the highly active phosphate transesterification catalyst neocuproine-Cu(II).[14] The very low pK_a's for Cu(II) bound waters in the dinuclear calix[4]arene complex **2**-$[Cu(II)]_2$ could be due to a stabilizing interaction of the neighboring Cu(II) center and the result of the hydrophobic environment created by the calix[4]arene moiety, mimicking the hydrophobic cavity of a hydrolytic enzyme active site.[5,35]

X-ray Crystallography. X-ray structures in which a phosphate group is bound to a transition metal complex[4eg,6a,8be,13d,15,36] can give more insight in the role of metal ions in catalytic phosphate diester cleavage. However, crystallization from mixtures of **2** or **3**, Cu(II) salts, and the inert substrate diphenyl phosphate did not give suitable crystals for X-ray structure analysis. In order to demonstrate the mode of Cu(II) binding by this type of bisimidazolyl ligands we have prepared crystalline Cu(II) complexes of **3**. Crystallization from a 1:1 mixture of ligand **3** and $Cu(ClO_4)_2$·$6H_2O$ in EtOH/Et_2O afforded unexpectedly the (**3**)$_2$$Cu(ClO_4)_2$ complex, in which the Cu(II) ion is chelated by two bisimidazolyl ligands (Figure 2, Table 2).[24,25,27] The Cu(II) coordination geometry in (**3**)$_2$$Cu(ClO_4)_2$ is not square-planar but is severely distorted toward a tetrahedral geometry, as is indicated by the dihedral angle of 44.63(19)° between the N−Cu−N planes of the two ligands.[27]

The crystallization of ligand:Cu(II) 2:1 complexes could be avoided by using acetate as the counter ion and CH_3CN as the solvent. Crystals of the desired ligand:Cu(II) 1:1 complex (**3**)-$Cu(CH_3COO)_2$ were grown by slow evaporation of a solution of a 1:1 mixture of ligand **3** and $Cu(CH_3COO)_2$·H_2O in MeOH/CH_3CN. We found two pseudopolymorphs of (**3**)$Cu(CH_3COO)_2$ with the Cu(II) complex (Figure 2, Table 2) in essentially equally distorted octahedral geometries: an anhydrate and a trihydrate. In both modifications the hydrogen bonds formed by the hydroxyl group connect the molecules into infinite chains. In the trihydrate these chains are interlinked through extensive hydrogen bonding involving water molecules.

The X-ray structures demonstrate that Cu(II) is bound by bidentate chelation of both the bisimidazolyl nitrogens and that the tertiary hydroxyl group of the ligand is not involved in Cu(II) coordination. In (**3**)$Cu(CH_3COO)_2$ the two acetate ions occupy the remaining coordination sites at the Cu(II) center by bidentate chelation. This is different from $Cu(CH_3COO)_n$ bisimidazolyl complexes reported by Lippard *et al.*,[28] where the acetate ions bridge two Cu(II) centers, resulting in dinuclear complexes. Unfortunately, we were not successful in growing suitable crystals of similarly bridged dinuclear Cu(II) calix[4]arene-based complexes.

Catalysis. The catalytic activity of the Cu(II) complexes **2**-$[Cu(II)]_2$ and **3**-Cu(II), generated *in situ* by adding stoichiometric amounts of $Cu(ClO_4)_2$·$6H_2O$ to the ligands, was studied in the transesterification of the RNA model substrate[37] 2-hydroxypropyl-*p*-nitrophenyl phosphate[38] (HPNP) and the hydrolysis of the DNA model substrate ethyl-*p*-nitrophenyl phosphate[12] (EPNP, Chart 1) in 35% EtOH/20 mM aqueous buffer at 25 °C.[31] The observed pseudo-first-order rate constants, calculated from the extinction coefficient of the released *p*-nitrophenolate at 406 nm by the initial rate method, are summarized in Tables 3 and 4. In the absence of the metallocatalysts HPNP is very stable (half-life *ca.* 280 days), but in the presence of 0.48 mM dinuclear Cu(II) complex **2**-$[Cu(II)]_2$ it is rapidly cleaved at pH 6.2 (half-life 40 min). This corresponds to a rate acceleration[39] of 1.0×10^4. For the extremely stable EPNP (half-life 20 years), lacking a β-hydroxyl group for intramolecular transesterification, the rate acceleration is even larger, *i.e.*, 2.7×10^4 at pH 6.4 (half-life 6.4 h). The catalytic activity of the mononuclear reference complex **3**-Cu(II) is 22 times lower in the case of HPNP and even 330 times lower in the case of EPNP. This shows that the high catalytic activity of **2**-$[Cu(II)]_2$ must be attributed to synergetic action of the two Cu(II) centers.

In experiments with HPNP present in 4-fold excess over dinuclear catalyst **2**-$[Cu(II)]_2$, we observed turnover conversion to completion with almost no loss of catalytic activity. Also a 4-fold excess of EPNP is cleaved with turnover, but for this substrate the rate strongly decreases in the course of the reaction. The product of HPNP transesterification is a cyclic phosphate diester (monoanion), whereas the product of EPNP hydrolysis is a phosphate monoester (dianion). In order to establish whether differences in product binding to the catalyst could account for the decrease in rate, we have performed catalysis experiments in which catalyst **2**-$[Cu(II)]_2$ was preequilibrated with either the monoanion diethyl phosphate or the dianion α-glycerol phosphate. Addition of an equimolar amount diethyl phosphate with respect to the substrates HPNP or EPNP caused only 5% of rate inhibition, whereas addition of α-glycerol phosphate caused 50% of rate inhibition. This shows that the product of **2**-$[Cu(II)]_2$-catalyzed EPNP hydrolysis acts as a competitive inhibitor by relatively strong binding to the catalyst.

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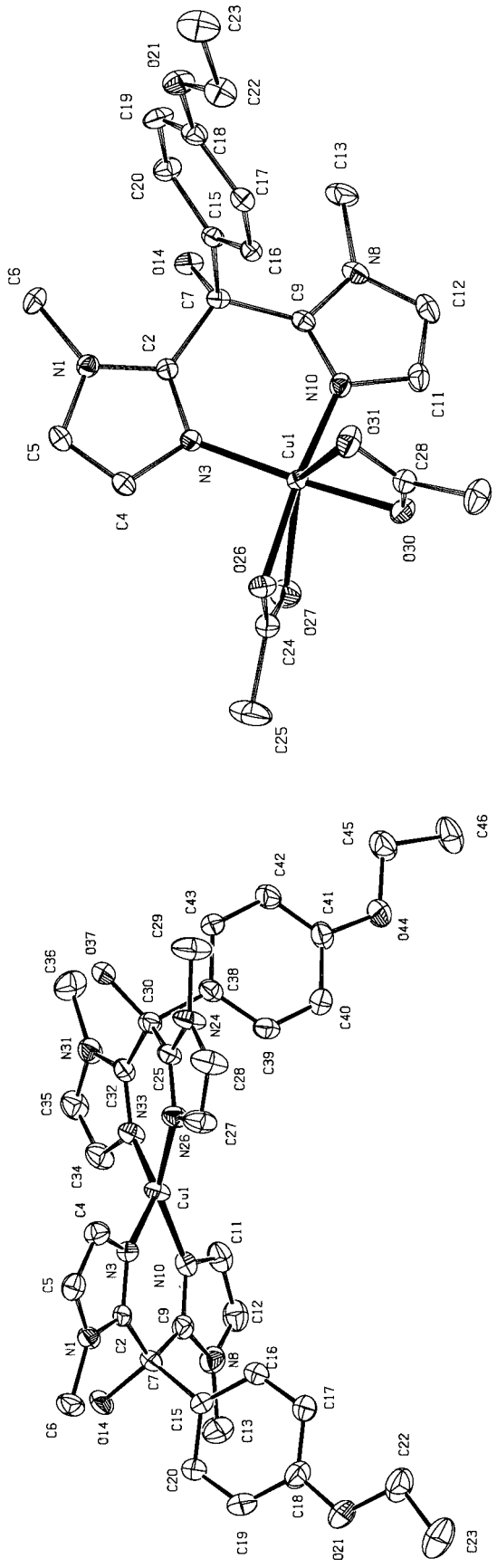


Figure 2. X-ray crystal structure of (**3**)$_2$Cu(ClO$_4$)$_2$ (left) and (**3**)Cu(CH$_3$COO)$_2$ (right) drawn as an atomic displacement ellipsoid plot (at 50% probability level). Hydrogen atoms and counter ions are omitted for clarity.

The product of the transesterification reaction only slightly reduces the turnover rate of HPNP since it only binds with similar binding constant than the substrate does.

Previously we have shown that dinuclear Zn(II) catalyst **1**-[Zn(II)]$_2$ efficiently catalyzes the transesterification of HPNP and that it was not active in the hydrolysis of EPNP.[5] The Cu(II) analog **1**-[Cu(II)]$_2$, however, is much less active in the HPNP cleavage than **1**-[Zn(II)]$_2$ and is also not active in EPNP hydrolysis.[5] Conversely, we have here also measured the catalytic activities of the Zn(II) complexes of **2** and **3**, *i.e.*, **2**-[Zn(II)]$_2$ and **3**-Zn(II). For the mononuclear complex **3**-Zn(II) almost the same catalytic activity is observed as for **3**-Cu(II). In contrast to **2**-[Cu(II)]$_2$ the dinuclear complex **2**-[Zn(II)]$_2$ shows no synergism in the catalytic cleavage of HPNP and EPNP but a catalytic activity which corresponds to twice the activity of the mononuclear complex **3**-Zn(II). The reason for the lack of synergism might be the different coordination geometries for Zn(II) and Cu(II) in these calix[4]arene compounds.

Effect of pH. The catalytic activity of complexes **2**-[Cu(II)]$_2$ and **3**-Cu(II) is strongly influenced by the pH of the reaction mixture.[31] The dinuclear Cu(II) complex **2**-[Cu(II)]$_2$ shows for HPNP cleavage a remarkable pH-rate profile, *viz.*, a bell shaped curve between pH 5.4 and 7.2 with a rate optimum at pH 6.2 and a linear rate increase at pH > 7.2 (Figure 3). The pH-rate profile for the cleavage of EPNP is also bell shaped, with an optimum at pH 6.4 (Figure 4). The absence of such a rate increase in the alkaline region shows that the mechanisms for EPNP cleavage and HPNP cleavage must be different. EPNP lacks a β-hydroxyl group and is 8−40 times less reactive than HPNP in the pH region 5.6−8.8. This must be due to the entropic advantage of intramolecular transesterification in HPNP compared to hydrolysis in EPNP.

Bell shaped pH-rate profiles[4c,5,6b,7] for phosphate diester cleavage by transition metal catalysts result from opposing pH effects on formation of the catalyst−substrate complex (K_{ass}) and conversion of the substrate within this complex (k_{cat}). When we compare the pH-rate profiles of calix[4]arene **2**-[Cu(II)]$_2$ with the pK_a values for the Cu(II) bound water molecules obtained by the potentiometric titrations (pK_a 6.5 and 6.6), we can conclude that the most active form of this dinuclear complex must have a hydroxide ion at one of its Cu(II) centers. This Cu(II) bound hydroxide ion can contribute to the catalysis by acting as a nucleophile[12,13,15−17] or as a base.[7,13i] On the other hand, hydroxide ions bind tightly to the Cu(II) center and consequently inhibit the coordination of the anionic phosphate ester to the Cu(II) catalyst. In the pH region of 6.0−6.5, the formation of catalyst−substrate complexes should proceed easily via the displacement of one or two weakly bound water molecules by the phosphate moiety. At this pH there is sufficient (general) base or Cu(II) bound hydroxide for conversion of the substrates within the complex, either by intramolecular transesterification (HPNP) or by hydrolysis (EPNP). Between pH values 6.5 and 7.2, the formation of catalyst−substrate complexes is increasingly inhibited, because the catalyst **2**-[Cu(II)]$_2$ possesses two tightly bound Cu(II) hydroxides. The increase in HPNP transesterification rate at pH ≥ 7.2 may be explained by the increasing contribution of hydroxide as a general base in the proton abstraction of the β-hydroxyl group (Chart 2).

The mononuclear complex **3**-Cu(II) displays for HPNP cleavage a bell shaped pH-rate profile over the pH region 6.2−8.8 with an optimum at pH 7.4 (Figure 3). The increase of k_{obs} in the pH region 6.2−7.4 corresponds to the deprotonation of

Table 2. Crystallographic Data for (**3**)$_2$Cu(ClO$_4$)$_2$, (**3**)Cu(CH$_3$COO)$_2$, and (**3**)Cu(CH$_3$COO)$_2$·3H$_2$O

compound	(**3**)$_2$Cu(ClO$_4$)$_2$	(**3**)Cu(CH$_3$COO)$_2$	(**3**)Cu(CH$_3$COO)$_2$·3H$_2$O
		Crystal Data	
formula	$C_{34}H_{40}CuN_8O_4$·2ClO$_4$	$C_{21}H_{26}CuN_4O_6$	$C_{21}H_{26}CuN_4O_6$·3H$_2$O
molecular weight	887.19	494.01	548.05
crystal system	triclinic	monoclinic	monoclinic
space group	$P\bar{1}$ (no. 2)	$P2_1/c$ (no.14)	$C2/c$ (no. 15)
a, Å	8.4355(5)	12.645(3)	23.562(8)
b, Å	14.0011(8)	19.003(4)	12.434(8)
c, Å	17.6161(9)	9.577(3)	16.948(3)
α, deg	92.713(4)		
β, deg	102.107(4)	103.890(17)	91.18(2)
γ, deg	103.352(5)		
V, Å^3	1969.25(19)	2234.0(10)	4964(4)
D_{calc}, g cm^{-3}	1.496	1.469	1.467
Z	2	4	8
F(000)	918	1028	2296
μ, cm^{-1}	7.6 [Mo Kα]	10.2 [Mo Kα]	9.4 [Mo Kα]
crystal color	blue-green	blue	dark blue
crystal size, mm	0.2 × 0.4 × 0.4	0.05 × 0.2 × 0.3	0.3 × 0.7 × 0.8
		Data collection	
θ_{min}, θ_{max} deg	1.2, 27.5	1.1, 25.0	1.2, 25.4
SET4 θ_{min}, θ_{max} deg	10.03, 13.96	10.56, 13.85	11.39, 13.94
$\Delta\omega$, deg	0.91 + 0.35 tanθ	0.50 + 0.35 tanθ	0.63 + 0.35 tanθ
hor, ver aperture, mm	2.17 + 1.08 tanθ, 4.00	3.00 + 1.50 tanθ, 4.00	2.15 + 1.07 tanθ, 4.00
X-ray exposure, h	27	13	22
linear instability, %	3	1	3
reference reflcns	$\bar{2}\,\bar{1}\,\bar{2}$, $\bar{3}\,\bar{2}\,2$, $\bar{2}\,3\,\bar{2}$	3 0 2, $\bar{5}\,\bar{2}\,3$, $2\,\bar{2}\,3$	2 2 6, $\bar{1}\,3\,\bar{2}$, 4 4 2
data set	−10:7, −17:18, −22:22	−15:14, −22:0, −5:11	−28:13, −13:14, −20:20
total data	9959	5744	9518
total unique data	9005	3931	4554
R_{int}	0.0573	0.0555	0.0451
		Refinement	
no. of refined params	550	295	342
final $R1$[a]	0.0584 [6007$I > 2\sigma(I)$]	0.0706 [2409$I > 2\sigma(I)$]	0.0306 [4057$I > 2\sigma(I)$]
final $wR1$[b]	0.1461	0.2044	0.0818
goodness of fit	1.008	1.028	1.045
w^{-1} [c]	$\sigma^2(F^2) + (0.0624P)^2 + 2.14P$	$\sigma^2(F^2) + (0.0811P)^2 + 11.66P$	$\sigma^2(F^2) + (0.0389P)^2 + 6.23P$
$(\Delta/\sigma)_{av}$, $(\Delta/\sigma)_{max}$	0.000, 0.002	0.000, 0.001	0.001, 0.107
min. and max. residual density, e Å^{-3}	−0.45, 0.71	−0.72, 0.72	−0.57, 0.41

[a] $R1 = \sum||F_o| - |F_c||/\sum|F_o|$. [b] $wR2 = [\sum[w(F_o^2 - F_c^2)^2]/\sum[w(F_o^2)^2]]^{1/2}$. [c] $P = (\mathrm{Max}(F_o^2,0) + 2F_c^2)/3$.

Table 3. Observed Pseudo-First-Order Rate Constants and Relative Rate Accelerations for the Transesterification of HPNP Catalyzed by **2**-[Cu(II)]$_2$ and **3**-Cu(II) in 35% EtOH/20 mM Aqueous Buffer, at 25 °C[a]

pH	$k_{uncat} \times 10^5$ s^{-1} [b]	**2**-[Cu(II)]$_2$ $k_{obs} \times 10^5$ s^{-1}	**3**-Cu(II) $k_{obs} \times 10^5$ s^{-1}	synergism k_{obs}/k_{obs}	**2**-[Cu(II)]$_2$ k_{obs}/k_{uncat}
6.2[c]	0.0029	29	1.3	22	1.0×10^4
7.4[d]	0.0054	14	1.9	7.4	2.6×10^3
8.8[e]	0.0083	20	1.4	14	2.4×10^3

[a] Catalyst concentration 0.48 mM. [b] Measured from a 2.0 mM HPNP solution. [c] MES buffer. [d] HEPES buffer. [e] EPPS buffer.

Table 4. Observed Pseudo-First-Order Rate Constants and Relative Rate Accelerations for the Hydrolysis of EPNP Catalyzed by **2**-[Cu(II)]$_2$ and **3**-Cu(II) in 35% EtOH/20 mM Aqueous Buffer, at 25 °C[a]

pH	$k_{uncat} \times 10^5$ s^{-1} [b]	**2**-[Cu(II)]$_2$ $k_{obs} \times 10^5$ s^{-1}	**3**-Cu(II) $k_{obs} \times 10^5$ s^{-1}	synergism k_{obs}/k_{obs}	**2**-[Cu(II)]$_2$ k_{obs}/k_{uncat}
6.4[c]	0.00011	3.0	0.009	330	2.7×10^4
7.4[d]	0.00026	1.5	0.024	62	5.8×10^3
8.4[e]	0.00091	0.5	0.011	45	5.5×10^2

[a] Catalyst concentration 0.48 mM. [b] Measured from a 2.0 mM EPNP solution. [c] MES buffer. [d] HEPES buffer. [e] EPPS buffer.

a Cu(II) bound water molecule with an apparent pK_a of approximately 7. This observed pK_a agrees with the pK_a calculated from the potentiometric pH titrations (Table 1, Figure 1) and reveals that the Cu(II)-monohydroxide form of complex

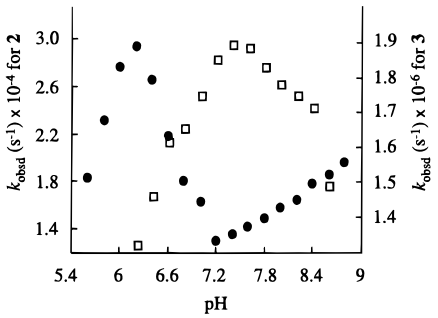


Figure 3. Dependence of k_{obs} on pH for the transesterification of HPNP (0.19 mM), catalyzed by **2**-[Cu(II)]$_2$ (●) and **3**-Cu(II) (□, 0.48 mM) in 35% EtOH/20 mM buffer (v/v), at 25 °C. Buffers: pH 5.6−6.8, MES; pH 7.0−8.0, HEPES; pH 8.2−8.8, EPPS.

3-Cu(II) is the key catalyst in HPNP cleavage. Remarkably, whereas the mononuclear reference complex **3**-Cu(II) displays for EPNP cleavage a similar pH-rate profile (not shown) it cleaves EPNP with a much lower absolute rate acceleration (0.9×10^2) than HPNP (4.5×10^2). This is in sharp contrast to dinuclear complex **2**-[Cu(II)]$_2$ which cleaves both EPNP and HPNP with enormous rate accelerations (2.7×10^4 and 1.0×10^4).

In order to investigate whether general base catalysis by the buffer[37] plays a role in the Cu(II) complex catalyzed cleavage of HPNP we have conducted experiments at different buffer

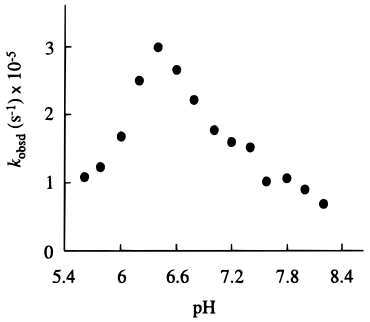


Figure 4. Dependence of k_{obs} on pH for the transesterification of EPNP (0.19 mM), catalyzed by **2**-[Cu(II)]$_2$ (0.48 mM) in 35% EtOH/20 mM buffer (v/v), at 25 °C. Buffers: pH 5.6−6.8, MES; pH 7.0−8.0, HEPES; pH 8.2−8.8, EPPS.

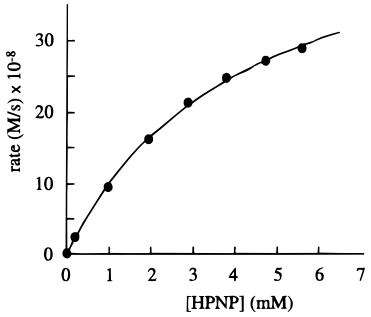


Figure 5. Saturation kinetics curve for the transesterification of HPNP by **2**-[Cu(II)]$_2$ (0.24 mM) in 35% EtOH/20 mM MES pH 6.2 (v/v), at 25 °C. The experimental data points are fitted to the Michaelis−Menten equation with $K_m = 4.0$ mM, and $k_{cat} = 2.1 \times 10^{-3}$ s^{-1}, determined by least-squares analysis of an Eady-Hofstee plot (correlation coefficient 0.990).

concentrations at different pH values. Increase of the buffer concentration from 20 mM (adjusted to an ionic strength of 100 mM with Et_4NClO_4) to 100 mM at pH 5.8 resulted in a more than 3-fold rate enhancement in HPNP transesterification catalyzed by **2**-[Cu(II)]$_2$ and a 2-fold enhancement when catalyzed by **3**-Cu(II). At pH 6.6, a slight decrease in rate was observed for the dinuclear complex **2**-[Cu(II)]$_2$. These buffer effects reveal that phosphate diester transesterification is subject to general base catalysis under slightly acidic to neutral conditions, *i.e.*, in the part of the bell shaped pH-rate curves where k_{obs} increases. No buffer effect was observed for complexes **2**-[Cu(II)]$_2$ and **3**-Cu(II) at pH 8. This indicates that at alkaline conditions the catalytic reaction proceeds most predominantly by the assistance of a hydroxide ion, either free, or bound to one of the Cu(II) centers.

Effect of Catalyst Concentration. The rate of HPNP cleavage is over the pH region 5.4−8.8 first-order dependent on the concentration dinuclear catalyst **2**-[Cu(II)]$_2$, up to 2 mM catalyst. For mononuclear complex **3**-Cu(II) a first-order dependency up to 2 mM complex is observed at pH 6.2 and 8.6. However, at pH 7.4, the order of the reaction decreases below unity already at 0.3 mM of **3**-Cu(II). This might be due to partial dimerization of **3**-Cu(II) to a catalytically inactive Cu(II)−Cu(II) bis-μ-hydroxy bridged complex. Such a dimerization of *cis*-diaqua Cu(II) complexes generally takes place at relatively high concentrations of Cu(II) complex at the pH equal to the pK_a of the Cu(II) bound water molecule.[7,12,13cdh,14,33,40] Although no minimum in the rate profile is observed at this particular pH, the observed pseudo-first-order rate constants (0.48 mM of **3**-Cu(II), Tables 3 and 4) might be approximately 20% lower[41] due to partial dimerization of **3**-Cu(II). For calix[4]arene **2**-[Cu(II)]$_2$ there are no indications that Cu(II)−Cu(II) μ-hydroxy bridged complexes are formed.[42]

Effect of Substrate Concentration. Previously[5] we have reported the high efficiency of dinuclear Zn(II) calix[4]arene **1**-[Zn(II)]$_2$ in the catalysis of the phosphate diester transesterification of HPNP, displaying a bell shaped pH dependency and saturation kinetics. The most remarkable feature of **1**-[Zn(II)]$_2$ is its very high affinity for HPNP (Table 5). In order to determine the affinity of Cu(II) catalysts **2**-[Cu(II)]$_2$ and **3**-Cu(II) for HPNP, the rate of transesterification was measured as a function of the HPNP concentration (up to 25 equiv HPNP). The calix[4]arene **2**-[Cu(II)]$_2$ (0.24 mM) displays at pH 6.2 saturation kinetics with a Michaelis−Menten constant $K_m = 4.0$ mM and a turnover rate constant $k_{cat} = 2.1 \times 10^{-3}$ s^{-1} (Figure 5). This means that HPNP is efficiently bound to the catalyst and that at low concentrations of HPNP the second-order rate constant k_2 ($= k_{cat}/K_m$) is 0.52 M^{-1}•s^{-1}. At pH 7.4 a first-order dependency on HPNP concentration is observed ($k_2 = 0.25$ M^{-1}•s^{-1}). This means that HPNP is relatively weakly bound at this pH, which was already concluded from the pH-rate profiles. For the mononuclear complex **3**-Cu(II) the rate is first-order at least to 6 mM HPNP (25 equiv) at pH 6.2 and 7.4, with second-order rate constants $k_2 = 1.3 \times 10^{-2}$ M^{-1}•s^{-1} and 2.5×10^{-2} M^{-1}•s^{-1}, respectively (Table 5).

Table 5. Kinetic Data for the Transesterification of HPNP Catalyzed by **1**-[Zn(II)]$_2$, **2**-[Cu(II)]$_2$, and **3**-Cu(II) at 25 °C

catalyst	pH[a]	k_2, M^{-1} s^{-1}	k_{cat}, s^{-1}	K_m, mM	K_{ass}, M^{-1}
1-[Zn(II)]$_2$	7.0[b]	43[c]	7.7×10^{-4} [d]	0.018[e]	5.5×10^4 [d]
1-[Zn(II)]$_2$	7.4[b]	17[c]	10×10^{-4} [d]	0.059[e]	1.7×10^4 [d]
2-[Cu(II)]$_2$	6.2[f]	0.52[c]	2.1×10^{-3} [g]	4.0[g]	2.5×10^2 [h]
2-[Cu(II)]$_2$	7.4[i]	0.25[j]			
3-Cu(II)	6.2[f]	0.013[j]			
3-Cu(II)	7.4[i]	0.025[j]			

[a] See ref 31. [b] In 50% CH_3CN/20 mM HEPES, see ref 5. [c] At low concentrations of HPNP $k_2 = k_{cat}/K_m$. [d] Determined by nonlinear least squares curve fitting (see ref 32) according to pseudo-first-order kinetics. [e] Calculated from K_{ass}. [f] In 35% EtOH/20 mM MES. [g] Determined by least-squares analysis of an Eady-Hofstee plot. [h] Calculated from K_m. [i] In 35% EtOH/20 mM HEPES. [j] Based on pseudo-first-order rate constants for catalyst concentrations of 0.24 mM.

These binding studies confirm that the strength of substrate binding to the catalyst **2**-[Cu(II)]$_2$ is decreased upon increase of the pH of the reaction medium. They also show that the two Cu(II) centers preorganized on the flexible calix[4]arene platform are more efficient in substrate binding and activation than the single Cu(II) center in the mononuclear complex. The

(40) (a) Courtney, R. C.; Gustafson, R. L.; Chaberek, S., Jr.; Martell, A. E. *J. Am. Chem. Soc.* **1959**, *81*, 519. (b) Arenare, E.; Paoletti, P.; Dei, A.; Vacca, A. *J. Chem. Soc., Dalton Trans.* **1972**, 736.

(41) Estimated by comparison of the measured k_{obs} with the k_{obs} obtained by extrapolation of the observed first-order dependency at 0−0.2 mM **3**-Cu(II) to 0.48 mM **3**-Cu(II).

(42) We could not detect μ-hydroxy bridged Cu(II) complexes by EPR spectroscopy. Attempts to prepare crystalline μ-hydroxy bridged complexes from slightly alkaline solutions or μ-chloride bridged complexes from $CuCl_2$ solutions were not successful. Glassy EPR solutions of complexes **2**-[Cu(II)]$_2$ and Cu(II)-**3** (1 or 5 mM, 77 K) in 35% EtOH/20 mM aqueous buffer (pH 6.4, 7.0, 8.8) did not exhibit an isotropic $\Delta M_s = \pm 2$ half-field transition caused by the triplet state of dimeric Cu(II) complexes. This half-field transition is theoretically forbidden and thus often of low intensity, whereas it is sometimes not observed at all. Lack of this EPR signal is therefore not a guarantee for the absence of dimeric Cu(II) species. For further information about EPR spectroscopy with Cu(II) complexes, see refs 13h, 15, and the following: (a) Chaudhuri, P.; Oder, K. *J. Chem. Soc., Dalton Trans.* **1990**, 1597. (b) Siddiqui, S.; Shepherd, R. E. *Inorg. Chem.* **1986**, *25*, 3869. (c) Chaudhuri, P.; Ventur, D.; Wieghardt, K.; Peters, E. M.; Peters, K.; Simon, A. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 57. (d) Barnes, J. A.; Hodgson, D. J.; Hatfield. W. E. *Inorg. Chem.* **1972**, *11*, 144. (e) Walker, A.; Sigel, H.; McCormick, D. B. *Inorg. Chem.* **1972**, *11*, 2756.

Michaelis−Menten constant of 4.0 mM at pH 6.2 corresponds to a binding constant K_{ass} for HPNP binding to calix[4]arene catalyst **2**-$[Cu(II)]_2$ of 250 M^{-1}. This binding constant is significantly higher than those reported for phosphate diester binding to mononuclear bipyridine Cu(II) complexes.[12,15] The rate constants k_{obs}, k_{cat}, and k_2 for phosphate diester cleavage are also considerably higher than those reported for *cis*-diaqua bipyridine Cu(II) complexes,[7,12,15] emphasizing the efficiency of synergetic action of the Cu(II) centers in catalyst **2**-$[Cu(II)]_2$. Comparison of dinuclear Cu(II) calix[4]arene **2**-$[Cu(II)]_2$ (in 35% EtOH) with dinuclear Zn(II) calix[4]arene **1**-$[Zn(II)]_2$ (in 50% CH_3CN, Table 5)[5] shows that the high catalytic activity of **2**-$[Cu(II)]_2$ 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 **1**-$[Zn(II)]_2$ is primarily due to strong substrate−catalyst affinity combined with a moderate turnover rate.[5]

RNA Cleavage Experiments. Chin *et al.* have shown that a 1,8-naphthalene substituted dinuclear [9]aneN_3−Cu(II) complex[6b] and especially neocuproine-Cu(II)[14] cleave the RNA dinucleotide ApA rapidly in neutral aqueous buffer solution. The incubation of ApA with complexes **1**-$[Zn(II)]_2$, **2**-$[Cu(II)]_2$, or **3**-Cu(II) in 35% EtOH/20 mM buffer or 50% CH_3CN/20 mM buffer, at pH 6.2, 7.4, or 8.8 at 25 °C for 24 h, however, showed no cleavage products (HPLC reversed phase detection). We found that, under the same reaction conditions, also the activity of neocuproine-Cu(II) is much lower than in aqueous solution. Kövári and Krämer[15] reported that neocuproine-Cu(II) is even completely inactive in an EtOH/water 19/1 medium. This shows the importance of water as a medium in the cleavage of this RNA dinucleotide. Since our calix[4]arene catalysts are only poorly soluble in pure water we have solubilized **1**-$[Zn(II)]_2$ and **2**-$[Cu(II)]_2$ (1 mM) in an aqueous solution of the inert neutral surfactant Brij35 (4 mM). Although neocuproine-Cu(II) is still active in cleaving ApA under these conditions, only traces of cleavage products could be detected with our calix[4]arene complex.[43]

These results show that the mononuclear complex neocuproine-Cu(II) displays a rather unique activity in ApA cleavage. On the basis of the rate enhancements observed for the cleavage of HPNP and EPNP we expected dinuclear complexes **1**-$[Zn(II)]_2$ and **2**-$[Cu(II)]_2$ also to cleave ApA. Besides the mentioned restricted solubility of our calix[4]arene complexes in pure water also steric reasons may cause the lack of activity with ApA.[44]

Mechanism of Catalysis. The generally high activity of mononuclear *cis*-diaqua Cu(II) complexes in intramolecular phosphate diester *transesterifications* proceeds via the formation of catalyst−substrate complexes by bidentate coordination of the anionic phosphate to the Cu(II) center.[14] This so-called double Lewis acid activation of the phosphate group enables the nucleophilic β-hydroxyl group in RNA type substrates to attack intramolecularly at phosphorous. In contrast, *hydrolysis* of phosphate diesters by mononuclear Cu(II) complexes proceeds via monodentate coordination to the Cu(II) center (single Lewis acid activation) followed by nucleophilic attack of a hydroxide ion which is *cis* bound to the same Cu(II) center, *i.e.*, bifunctional catalysis.[6b,12,13,15−17] The high synergetic effect observed in the transesterification of HPNP and particularly in the hydrolysis of EPNP indicates that the dinuclear calix[4]arene complex **2**-$[Cu(II)]_2$ catalyzes phosphate diester cleavage via double Lewis acid activation in combination with bifunctional catalysis (Chart 2, top).[6b,7,13c]

The substrate affinity (K_{ass}) and the rate constants (k_{obs}, k_2, k_{cat}) of phosphate diester cleavage are higher for **2**-$[Cu(II)]_2$ than for both the reference compound **3**-Cu(II) and bipyridine Cu(II) complexes.[7,12,15] Moreover, the pH-rate profiles, the substrate binding studies, and the potentiometric titrations reveal that substrate binding is most efficient when the dinuclear catalyst comprises only Cu(II) bound water molecules. This suggests that calix[4]arene **2**-$[Cu(II)]_2$ binds the phosphate group of the substrate by coordination to both Cu(II) centers, *i.e.*, by double Lewis acid activation. The catalyst **2**-$[Cu(II)]_2$ has the highest reactivity at a pH near the pK_a of the first dissociating Cu(II) bound water molecule. Moreover, the large rate enhancements by dinuclear calix[4]arene complex **2**-$[Cu(II)]_2$ in the cleavage of EPNP under slightly acidic to neutral conditions show that for high catalytic activity in phosphate diester cleavage a nucleophilic β-hydroxyl group in the substrate is not necessary. This indicates that the catalyst **2**-$[Cu(II)]_2$ can promote a reactive nucleophile, probably a Cu(II) bound hydroxide ion, to attack the Cu(II) coordinated phosphate (Chart 2, top right). This Cu(II) bound hydroxide ion may, like buffer ions at low pH, also act as a general base,[37] which deprotonates the β-hydroxyl group in HPNP before intramolecular transesterification (Chart 2, top left).[7,13i]

The catalysis of EPNP cleavage by the reference compound **3**-Cu(II) is extremely low when compared to HPNP cleavage, which must be due to a lack of Lewis acid activation and the absence of a sufficiently reactive nucleophilic. In contrast to double Lewis acid activation in **3**-Cu(II) catalyzed HPNP transesterification (Chart 2, bottom left), this mode of activation by the single Cu(II) center[14] in **3**-Cu(II) is a nonproductive mode for the hydrolysis of EPNP. When the two *cis* coordination sites are occupied by the phosphate group of EPNP, there is no *cis* bound Cu(II) hydroxide ion available for nucleophilic attack at phosphorous. Thus, **3**-Cu(II) must cleave EPNP via the generally accepted mechanism of single Lewis acid activation and bifunctional catalysis (Chart 2, bottom right).[6b,12,13,15−17]

The mechanism of catalysis by **2**-$[Cu(II)]_2$ is different from the mechanism by dinuclear calix[4]arene complex **1**-$[Zn(II)]_2$, which only works for the transesterification of HPNP.[5] This was attributed to synergetic action of the Zn(II) centers via single Lewis acid activation of both the phosphate group and the hydroxyl group of HPNP. Compared with **1**-$[Zn(II)]_2$ (Table 5) is the dinuclear Cu(II) complex **2**-$[Cu(II)]_2$ less effective in substrate binding (K_{ass}, K_m) and more effective in conversion of the catalyst-substrate complex (k_{cat}). The lower binding constant reflects the nature of Cu(II), being a weaker acceptor for anionic phosphate ligands compared to Zn(II).[45] The higher k_{cat} for **2**-$[Cu(II)]_2$ can be attributed to the double Lewis acid activating mode of phosphate binding which stabilizes the pentacoordinate phosphorous transition state better.[14]

Conclusion

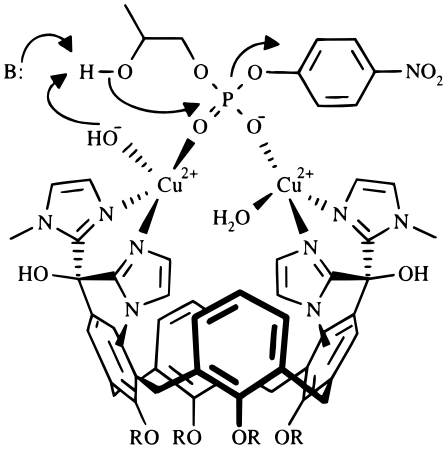
The novel calix[4]arene dinuclear complex **2**-$[Cu(II)]_2$ binds and cleaves the phosphate diesters HPNP and EPNP far more efficiently than the mononuclear reference complex **3**-Cu(II). This is due to efficient synergetic action of the two Cu(II) centers in **2**-$[Cu(II)]_2$, which are well preorganized on the flexible calix[4]arene scaffold. An important, hitherto underestimated, role of the calix[4]arene moiety in catalysis might be that rapid low energy conformational changes of the macrocyclic backbone[21]

(43) Control experiments show that complexes **1**-$[Zn(II)]_2$ and **2**-$[Cu(II)]_2$ keep their catalytic activity in the transesterification of the RNA model substrate HPNP in aqueous Brij35 solution.

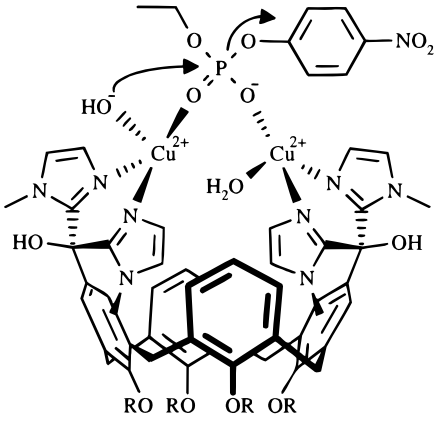
(44) This was also concluded for one of the dinuclear Cu(II) complexes of Chin, see ref 6.

(45) *Critical Stability Constants*; Martell, A. E., Smith, R. M., Eds.; Plenum: New York, 1974; Vol. 2.

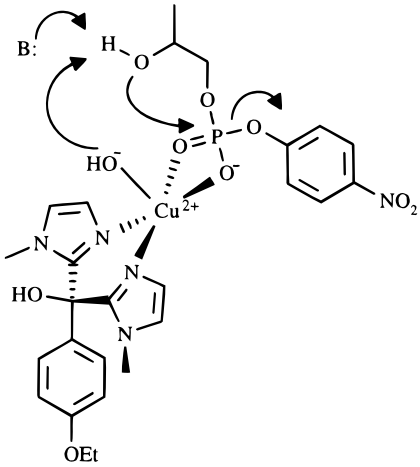
Chart 2



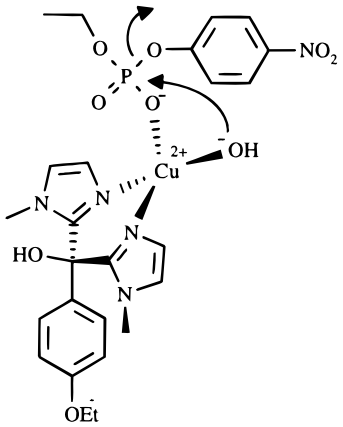
2-HPNP catalysis



2-EPNP catalysis



3-HPNP catalysis



3-EPNP catalysis

compensate for otherwise energetically unfavorable geometric transitions at the catalytic reaction center, mimicking in this way one of the functions of the enzyme peptide backbone.[18] This flexibility in the catalyst seems to be essential for facilitating a dynamic binding of the substrate and the pentacoordinate phosphorous (pre)transition state, as Trogler *et al.*[25] have not observed notable effects of cooperative catalysis by neither flexible nor by rigid dinuclear bisimidazolyl−transition metal complexes. Another interesting similarity of our calix[4]arene-based artificial metalloenzymes with natural metalloenzymes is the remarkably low pK_a of the metal(II) bound water molecules, which makes the (artificial) enzymes active under slightly acidic to neutral conditions. The low pK_a is the result of the local hydrophobic environment, in the natural enzymes[35] created by hydrophobic amino acid residues and in our artificial enzymes probably in part be created by the aromatic surface of the calix[4]arene moiety.[5] The use of the calix[4]arene building block in the design of new biomimetic catalysts has the advantage of providing preorganization of catalytic groups which can bind dynamically the substrate and (pre)-transition state(s), possibly resulting in fast turnover and fast release of products.

In summary, the flexible calix[4]arene **2**-$[Cu(II)]_2$ functionalized with two *cis*-diaqua Cu(II) centers at the distal positions of the upper rim exhibits some characteristic features present in hydrolytic dinuclear metalloenzymes. Under very mild conditions, this calix[4]arene-based artificial enzyme catalyzes the transesterification of the RNA model HPNP (rate enhancement 1.0×10^4) and the hydrolysis of the DNA model EPNP (rate enhancement 2.7×10^4) by efficient synergetic action of the Cu(II) centers in the formation of catalyst−substrate complexes and conversion of the substrate within these complexes.

Experimental Section

General information. THF was freshly distilled from Na/benzophenone, CH_2Cl_2 from $CaCl_2$. Other solvents and chemicals were of reagent grade and were used as received from commercial sources. Column chromatography was performed with silica gel (SiO_2, 0.040−0.063 mm, 230−400 mesh) from Merck. Melting points were determined with a Reichert melting point apparatus and are uncorrected. ^1H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ with Me_4Si as internal standard on a Bruker AC 250 spectrometer. FAB-MS spectra were recorded with a Finnigan MAT 90 spectrometer using *m*-NBA as a matrix. Elemental analyses were performed using a Carlo Erba EA1106. Tetrakis(2-ethoxyethoxy)calix[4]arene **4**,[29] tetrakis(2-ethoxyethoxy)calix[4]arene-dicarboxylic acid **6**,[30] and 4-ethoxybenzoic acid ethyl ester[46] were synthesized according to literature procedures. The pH meter used for adjustment of buffered solutions was a Metrohm 691 equipped with a glass electrode and was calibrated daily. UV−vis spectra were measured on a Hewlett Packard 8452A diode array spectrophotometer equipped with a thermostated cuvet holder (7 cuvettes, 1.0 cm path length) and a sample transport accessory.

(46) Node, M.; Nishide, K.; Sai, M.; Fuji, K.; Fujita, E. *J. Org. Chem.* **1981**, *46*, 1991.

5,17-Diformyl-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]-arene (5). This compound was synthesized by an improved literature procedure.[21c] To a solution of tetrakis(2-ethoxyethoxy)calix[4]arene **4** (8.00 g, 11.2 mmol) in $CHCl_3$ (250 mL) cooled at −10 °C was added 1,1-dichlorodimethyl ether (34.0 mL, 333 mmol). The solution was vigorously stirred, while tin tetrachloride (40.0 mL, 342 mmol) was added rapidly. The reaction mixture was stirred at −10 °C for 30 min, quenched carefully with water (600 mL), and vigorously stirred at room temperature for an additional 30 min. The organic layer was separated, washed with saturated Na_2CO_3 solution (300 mL) and water (2 × 300 mL), and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the crude product was purified by column chromatography (hexane/EtOAc, 3/2) to give **5** as a colorless oil (7.62 g, 87%). Spectroscopic data were the same as reported.

25,26,27,28-Tetrakis(2-ethoxyethoxy)calix[4]arene-5,17-dicarboxylic Acid Diethyl Ester (7). A solution of calix[4]arenedicarboxylic acid **6**[30] (3.67 g, 4.58 mmol) and *p*-toluenesulfonic acid (174 mg, 0.92 mmol) in EtOH (150 mL) was refluxed for 5 days. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (250 mL) and washed twice with saturated $NaHCO_3$ solution (150 mL) and water (150 mL). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by column chromatography (CH_2Cl_2/EtOAc, 95/5) to give **7** as a colorless oil (3.85 g, 98%). ^{1}H NMR ($CDCl_3$, 250 MHz): δ (ppm) 7.77 (s, 4 H), 6.33−6.21 (m, 6 H), 4.54 and 3.23 (AB q, 8 H, J = 13.5 Hz), 4.41−4.33 (m, 8 H), 3.97 (t, 4 H, J = 4.8 Hz), 3.88 (t, 4 H, J = 5.8 Hz), 3.78 (t, 4 H, J = 4.8 Hz), 3.58 (q, 4 H, J = 7.0 Hz), 3.49 (q, 4 H, J = 7.0 Hz), 1.41 (t, 6 H, J = 7.1 Hz), 1.24 (t, 6 H, J = 7.0 Hz), 1.15 (t, 6 H, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$, 250 MHz): δ (ppm) 166.9, 162.2, 154.8, 136.6, 132.9, 130.2, 124.1, 122.6, 73.9, 72.9, 69.9, 69.6, 66.5, 66.2, 60.6, 30.8, 15.3, 15.2, 14.5. FAB-MS *m/z* 827.4 ([M − CH_2CH_3]$^+$, calcd 827.4), 856.4 ([M]$^-$, 856.4), 811.4 ([M − OCH_2CH_3]$^-$, 811.4).

5,17-Bis(bis(1-methylimidazol-2-yl)hydroxymethyl)-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (2). To a solution of 1-methylimidazole (0.17 mL, 2.1 mmol) in THF (20 mL) at −78 °C under Ar was added *n*-BuLi (1.31 mL, 1.6 M, 2.1 mmol). After stirring for 1 h at −78 °C, calix[4]arene diethyl ester **7** (300 mg, 0.35 mmol) in THF (20 mL) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 18 h. Brine (50 mL) was added, and the solution was extracted with CH_2Cl_2 (3 × 50 mL). The combined extracts were dried over Na_2CO_3, and the solvent was removed under reduced pressure. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 95/5) to give **2** as a white foam (325 mg, 85%). ^{1}H NMR ($CDCl_3$, 250 MHz): δ (ppm) 6.94 (d, 4 H, J = 1.0 Hz), 6.84 (d, 4 H, J = 1.0 Hz), 6.44 (m, 6 H), 6.53 (s, 4 H), 6.20 (br s, 2 H), 4.48 and 3.11 (AB q, 8 H, J = 13.0 Hz), 4.14 (t, 4 H, J = 5.7 Hz), 4.09 (t, 4 H, J = 5.5 Hz), 3.85 (t, 4 H, J = 5.5 Hz), 3.83 (t, 4 H, J = 5.7 Hz), 3.53 (q, 4 H, J = 7.0 Hz), 3.52 (q, 4 H, J = 7.0 Hz), 3.14 (s, 12 H), 1.20 (t, 6 H, J = 7.0 Hz), 1.18 (t, 6 H, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$, 250 MHz): δ (ppm) 156.6, 155.6, 148.8, 135.3, 135.2, 134.6, 128.1, 127.8, 125.7, 123.4, 122.3, 74.5, 73.5, 73.2, 69.7, 69.5, 66.4, 66.3, 34.8, 30.8, 15.3. FAB-MS *m/z* 1093.8 ([M + H]$^+$, calcd 1093.6). Anal. Calcd for $C_{62}H_{76}N_8O_{10}\cdot 2.5H_2O$: C, 65.42; H, 7.11; N, 9.84. Found: C, 65.39; H, 6.88; N, 9.75.

Bis(1-methylimidazol-2-yl)-4-ethoxy-phenylhydroxymethane (3). To a solution of 1-methylimidazole (5.18 mL, 65.0 mmol) in THF (300 mL) at −78 °C under Ar was added *n*-BuLi (40.6 mL, 1.6 M, 65.0 mmol). After stirring for 1 h at −78 °C, 4-ethoxybenzoic acid ethyl ester[46] (4.21 g, 21.7 mmol) in THF (50 mL) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 6 h. Brine (300 mL) was added, and the solution was extracted with CH_2Cl_2 (3 × 200 mL). The combined extracts were dried over Na_2CO_3, the solvent was removed under reduced pressure, and the residue was triturated with diisopropyl ether. The resulting white powder was recrystalized from CH_2Cl_2/diisopropyl ether to yield **3** (5.11 g, 75%). Mp 150−152 °C. ^{1}H NMR ($CDCl_3$, 250 MHz): δ (ppm) 6.96 (s, 2 H), 6.89 (s, 2 H), 6.83 (d, 2 H, J = 8.6 Hz), 6.99 (d, 2 H, J = 8.6 Hz), 6.50 (br s, 1 H), 4.01 (q, 2 H, J = 6.9 Hz), 3.38 (s, 6 H), 1.39 (t, 3 H, J = 6.9 Hz). ^{13}C NMR ($CDCl_3$, 250 MHz): δ (ppm) 158.8, 134.1, 128.7, 125.8, 123.4, 114.1, 74.6, 63.4, 34.7, 14.8. FAB-MS *m/z* 313.2 ([M + H]$^+$, calcd 313.2). Anal. Calcd for $C_{17}H_{20}N_4O_2$: C, 65.37; H, 6.45; N, 17.94. Found: C, 65.04; H, 6.33; N, 17.70.

Spectrophotometric Titrations. To a cuvet containing 2 mL of 35% EtOH/20 mM aqueous MES solution pH 6.0 (v/v, see kinetics)[31] was added 3 μL of a 50 mM ligand (**2** or **3**) stock solution in EtOH. The increase in absorbance at 274 nm at 25 °C by the ligand (ϵ_{274} = 2.8 × 10^3) was followed upon the stepwise addition of 0.5 μL of 50 mM $Cu(ClO_4)_2$ in EtOH up to 4 equiv for dinuclear complex **2**-[Cu(II)]$_2$, and up to 2 equiv for formation of the mononuclear Cu(II) complex **3**-Cu(II). The concentration of the ligand before titration was 0.0749 mM. The absorbance was corrected for both dilution upon titration and for the weak absorbance of free $Cu(ClO_4)_2$ (ϵ_{274} = 1.4 × 10^2). Stability constants for Cu(II) complexation were estimated by nonlinear least squares fitting[32] of the titration curves to a 1:1 model using the absorption end values at 274 nm.

Potentiometric pH Titrations. Solutions (50 mL) of calix[4]arene ligand **2** (0.2 mM) in 0.1 M KNO_3 in 35% EtOH/H_2O (v/v), acidified with HNO_3 (1.2 mM), were titrated under N_2 at 25.0 °C in the presence and absence of 2 equiv of $Cu(NO_3)_2$. Solutions (50 mL) of ligand **3** (0.4 mM) in 0.1 M aqueous KNO_3, or in 0.1 M KNO_3 in 35% EtOH/H_2O (v/v), acidified with HNO_3 (1.6 mM), were titrated under N_2 at 20.0 °C in the presence and absence of equimolar amounts $Cu(NO_3)_2$. The titrations were carried out with a Schott TPC2000 Titration System at fixed titrant increments of 10 μL of NaOH solution (0.100 M). During the titration the pH was measured with a Metrohm 6.0702.100 glass electrode and a 6.0101.102 (NF) reference electrode. The titration apparatus was calibrated with appropriate buffers immediately before use and checked by the pK_a determination of acetic acid. At least two independent pairs of titrations were always made for pK_a determinations. For calculation of deprotonation constants and Cu(II) association constants from the titration data a multiparameter curve fitting program based on SUPERQUAD was used.[34] The resulting equilibrium constants could be interpreted as conditional constants at I = 0.1 M. The values for K_w (= [H$^+$][OH$^-$]) at 25 °C were $10^{-13.78}$ and $10^{-14.26}$ in 0.1 M aqueous KNO_3 and in 0.1 M KNO_3 in 35% EtOH/H_2O (v/v), respectively.

X-ray Crystallography. (**3**)$_2$Cu(ClO$_4$)$_2$: To a solution of **3** (100 mg, 0.32 mmol) and $Cu(ClO_4)_2\cdot 6H_2O$ (118 mg, 0.32 mmol) in EtOH (4 mL) was added Et_2O (3 mL). The obtained blue crystals were recrystallized by slow vapor diffusion of diisopropyl ether to a methanolic solution (4 mL), resulting in the formation of crystals suitable for X-ray crystal structure analysis. (**3**)Cu(CH$_3$COO)$_2$: To a suspension of **3** (100 mg, 0.32 mmol) and $Cu(CH_3COO)_2\cdot H_2O$ (64 mg, 0.32 mmol) in acetonitrile (3 mL) was added MeOH (1 mL), yielding a clear solution. Slow evaporation of the solution resulted in the formation of blue crystals suitable for X-ray crystal structure analysis.

Pertinent data for the structure determinations are collected in Table 2. All data were collected on an Enraf-Nonius CAD4T diffractometer on rotating anode (ω scan, T = 150 K, Mo Kα radiation, graphite monochromator, λ = 0.71073 Å). Accurate unit-cell parameters and an orientation matrix were determined by least-squares fitting of the setting angles of 25 well-centered reflections (SET4).[47] The unit-cell parameters were checked for the presence of higher lattice symmetry.[48] Data were corrected for *Lp* effects and for the observed linear decay of the reference reflections. Structures were solved with automated Patterson and subsequent Fourier methods using SHELXS86[49] and refined on F^2 using SHELXL-96.[50] No observance criterion was applied during refinement on F^2. The hydroxyl hydrogen atoms were located on a difference Fourier map, and their coordinates were included as parameters in the refinement. (For (**3**)Cu(CH$_3$COO)$_2$ restraints were introduced to ensure a correct valence angle of the hydroxyl oxygen atom.) All other hydrogen atoms were included in the refinement on calculated positions riding on their carrier atoms. The non-hydrogen

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atoms of all structures were refined with anisotropic thermal parameters. The hydrogen atoms were refined with a fixed isotropic displacement parameter related to the value of the equivalent isotropic displacement parameter of their carrier atoms. Neutral atom scattering factors and anomalous dispersion corrections were taken from the International Tables for Crystallography.[51] Geometrical calculations and illustrations were performed with PLATON;[52] all calculations were performed on a DEC Alpha 255 station.

Kinetics. Solutions for spectrophotometric titrations and kinetic measurements were made by adding EtOH (spectrophotometric grade) up to 35% (v/v) to a 20 mM aqueous buffer solution adjusted with NaOH to the desired pH.[31] Buffers (MES, pH 5.6−7.0; HEPES, pH 7.0−8.2; EPPS pH 8.2−8.8) were obtained from commercial sources and used without further purification in deionized (Millipore) distilled water. HPNP[38] and EPNP[12] were prepared according to literature procedures. Stock solutions were freshly prepared before performing the kinetic measurements.

In a typical experiment, the ligand **2** (20 μL, 50 mM in EtOH) and $Cu(ClO_4)_2$ (40 μL, 50 mM in EtOH) were added to a cuvet containing 2 mL of 35% EtOH/20 mM buffer solution (v/v) and thermostated at 25 °C. After a couple of minutes equilibration time, HPNP (4 μL, 100 mM in water) was injected, and the increase in UV absorption at 406 nm was recorded every minute. Final concentrations were 0.48 mM in Cu(II) complex, 0.19 mM in substrate (HPNP or EPNP), and 13 mM in buffer. All solutions remained clear during the time of the kinetic measurements. In the absence of ligand precipitation of polymeric Cu(II) hydroxide took place. The observed pseudo-first-order rate constants k_{obs} (s^{-1}) were calculated with the extinction coefficient of *p*-nitrophenolate at 406 nm by an initial slope method (<4% conversion). Second-order rate constants were obtained from plots of first-order rate constants against catalyst concentration. All rate constants were obtained by averaging three kinetic measurements. The pseudo-first-order rate constants for uncatalyzed reactions (k_{uncat}, s^{-1}) were measured by following the increase in absorbance from a 2.0 mM HPNP or EPNP solution for 1 week.

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Supporting Information Available: Further details of the X-ray structure determinations, including atomic coordinates, bond lengths and angles, and thermal parameters (21 pages, print/PDF). See any current masthead page for ordering and Internet access instructions.

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