Dinuclear Bisimidazolyl-Cu(II) Calix[4]arenes as Metalloenzyme Models. Synthesis and Bifunctional Catalysis in Phosphate Diester Transesterification

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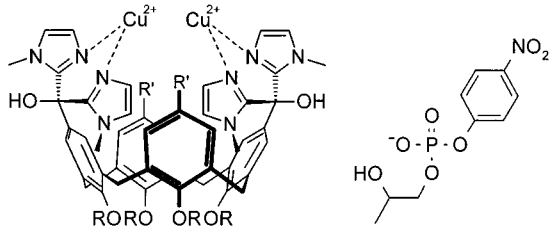
Received March 24, 1999

Calix[4]arenes functionalized with two catalytic bisimidazolyl-Cu(II) centers and two additional hydroxymethyl (**2**-Cu_2) or aminomethyl groups (**3**-Cu_2) were synthesized as models for enzymes that cleave phosphate diester bonds. The kinetics of **2**-Cu_2 and **3**-Cu_2 in the catalysis of the intramolecular transesterification of the RNA model substrate 2-hydroxypropyl-*p*-nitrophenyl phosphate were compared with those of the parent calix[4]arene **1**-Cu_2, lacking the two additional functional groups. Under neutral conditions, all complexes show high rate enhancements due to the cooperative action of the Cu(II) ions. The kinetics indicate for **3**-Cu_2 bifunctional catalytic effects. At the pH optimum of 7.4 at least one amine is protonated, which can assist as a general acid in the binding and activation of the substrate.

Introduction

In nature, phosphate ester hydrolysis is catalyzed by enzymes that often possess two or even three divalent metal ions in the active site.[1] The catalytic role of the metal ions has been studied with model systems consisting of two or three transition-metal complexes.[2−4] It has been shown that the metal ions can act cooperatively as Lewis acids in the activation of substrate and nucleophile and in the stabilization of transition state and leaving group. Generally, in the phosphoesterase metalloenzymes also several amino acid residues are involved in the catalysis. In the hydrolysis of phosphate monoesters by alkaline phosphatase, two Zn(II) ions and one Mg(II) ion are assisted by a serine, a histidine, and an arginine side chain.[1] In RNase A, an ammonium group of a lysine residue activates the phosphoryl group while two histidines cooperate in the deprotonation of the nucleophile and in the protonation of the leaving group.[5] The catalytic role of the amino acid residues in RNase A has been mimicked with general acid−base systems,[5] like bisimidazoles or aqueous solutions of imidazole or diamines.[6] Model systems that combine one or two catalytic metal centers and an additional catalytic group are of particular interest. Whereas in mononuclear models amino functionalities have been incorporated,[7] dinuclear metal complexes with additional functional groups have not been reported. Enzyme mimics that accommodate two metal centers and other functional groups require an appropriate molecular scaffold for spatial organization.

Chart 1



In this respect, calix[4]arenes[8] are suitable candidates since two sets of four directionally preorganized functional groups can be introduced at the upper and lower rim, respectively. We have previously shown that calix[4]arene-based dinuclear complexes[3,4] like **1**-Cu_2 (Chart 1) mimic dinuclear metallophosphodiesterases. They exhibit large rate enhancements in the catalytic transesterification[5a] of the RNA model substrate 2-hydroxypropyl-*p*-nitrophenyl phosphate (HPNP). In this paper, we report (bifunctional) phosphodiesterase models **2**-Cu_2 and **3**-Cu_2 that possess two bisimidazolyl-Cu(II) centers and two hydroxymethyl or aminomethyl groups at the upper rim of the calix[4]arene, respectively. Their synthesis and the catalysis of the transesterification of HPNP are described.

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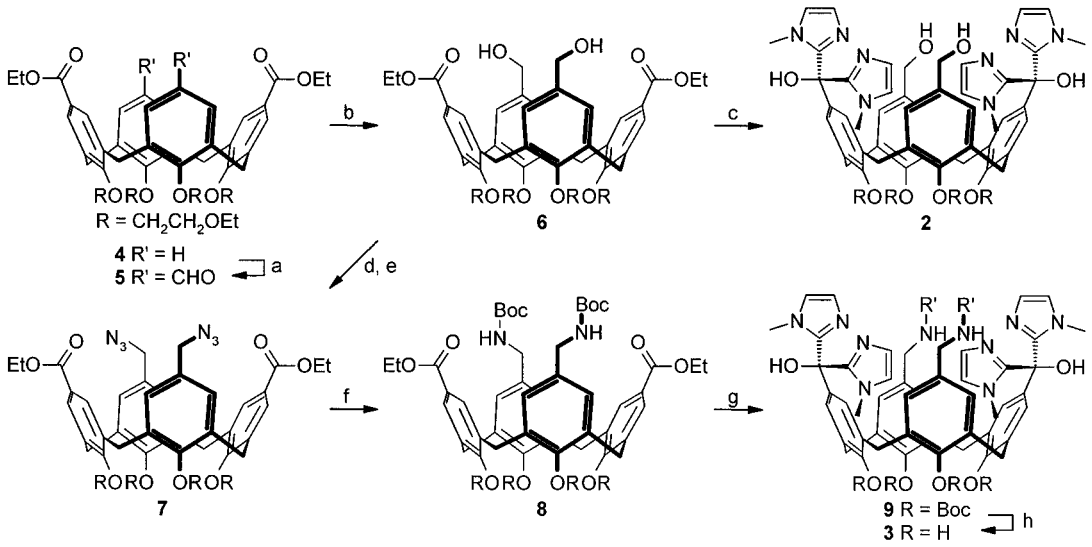
10.1021/jo9905266 CCC: $18.00
Published on Web 08/03/1999

Scheme 1[a]



[a]Key: (a) HMTA, TFA, 79%; (b) $NaBH_4$, EtOH, 87%; (c) *N*-methylimidazole, *n*-BuLi, THF, 66%; (d) $SOCl_2$, CH_2Cl_2; (e) NaN_3, DMF, 82%; (f) 10% Pd−C, H_2, Boc_2O, EtOH, 83%; (g) *N*-methylimidazole, *n*-BuLi, THF, 45%; (h) TFA, CH_2Cl_2, 81%.

Results and Discussion

The synthesis of the dinucleating ligand **1** was described previously.[4] Here, the synthetic modification of the two remaining positions on the upper rim of calix[4]arene **1** with hydroxymethyl (**2**) and aminomethyl groups (**3**) is described. The catalytic activities of the corresponding dinuclear Cu(II) complexes **2**-Cu_2 and **3**-Cu_2 and, in particular, the contribution of the additional functional groups in **3**-Cu_2, is discussed.

Synthesis. The bisimidazolyl ligands in **1** were introduced by reaction of 2-lithio-*N*-methylimidazole with an ester or an acid chloride.[4] Thus, the synthesis of the dinucleating ligands **2** and **3** requires calix[4]arene starting materials that possess at the upper rim two ester or acid chloride groups and two additional functional groups that are stable toward organolithium compounds. The synthetic route to these bisimidazolyl ligands that are extended with hydroxyl (**2**) and amino groups (**3**) is depicted in Scheme 1. The diester **4**[4] was used as the starting material for electrophilic aromatic substitution of the two remaining calix[4]arene upper rim positions. Reaction with hexamethylenetetraamine in refluxing trifluoroacetic acid[9] afforded, after hydrolysis, aldehyde **5** in 79% yield. The aldehyde groups were reduced to hydroxymethyl groups using sodium borohydride in ethanol at room temperature. Under these mild conditions, the ester groups stay intact, giving rise to 87% yield of **6**, which was reacted with excess 2-lithio-*N*-methylimidazole.[4,10] Protection of the hydroxyl groups in **6** was not necessary; they are deprotonated during the reaction and do not hamper the double nucleophilic attack of 2-lithio-*N*-methylimidazole at the two carbonyl carbons. Calix[4]arene **2**, with two Cu(II) binding sites and two hydroxymethyl groups at the upper rim, was obtained in 66% yield. To introduce aminomethyl groups, the hydroxyl groups in **6** were reacted with thionyl chloride to chlorides that were immediately converted to azides via reaction with sodium azide. The azide groups in **7** were reduced[11] to amines and in situ[12] protected with Boc groups by a catalytic hydrogenation with 10% Pd−C/H_2 in the presence of Boc_2O. This method prevents (intramolecular) amination of the esters during the reduction reaction and allows purification of the protected amine **8** by column chromatography. Ester **8** was reacted with excess 2-lithio-*N*-methylimidazole[4,10] affording the dinucleating ligand **9** in 45% yield. The Boc groups in compound **9** were cleaved by reaction with trifluoroacetic acid to give the crude TFA ammonium salt of **3**. The free amine **3** was obtained in 81% yield by elution over a strongly basic anion-exchange column and subsequent recrystallization.

Catalysis. The bifunctional calix[4]arenes **2** and **3** were mixed with 2 equiv of $Cu(ClO_4)_2$, and the in situ formed[4] dinuclear complexes **2**-Cu_2 and **3**-Cu_2 were tested for their catalytic activity in the transesterification of the RNA model substrate HPNP[13] in 35% EtOH/20 mM buffer.[14] The catalytic activities, represented as observed pseudo-first-order rate constants, are based on initial rates (<4% conversion) and were determined by measurement of the increase in absorbance at $\lambda = 400$ nm due to the release of *p*-nitrophenol. The catalytic activities of the bifunctional complexes, in particular that of **3**-Cu_2, were compared with the activity of **1**-Cu_2.[4] The observed pseudo-first-order rate constants are collected in Table 1.

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(14) Because the calix[4]arene dinuclear complexes are insoluble in pure water, EtOH was added 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 (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 (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.

Table 1. Observed Pseudo-First-Order Rate Constants for the Transesterification of HPNP Catalyzed by 0.48 mM of 1-Cu_2, 2-Cu_2, and 3-Cu_2[a]

catalyst	pH	k_{obs} (10^{-4} s^{-1})	catalyst	pH	k_{obs} (10^{-4} s^{-1})
1-Cu_2	6.2[b]	2.9	**3**-Cu_2	6.2	0.26
1-Cu_2	7.4	1.4	**3**-Cu_2	7.4[b]	1.7
2-Cu_2	6.2[b]	2.0	none	6.2[c]	0.00029
2-Cu_2	7.4	0.94	none	7.4[c]	0.00054

[a] In 35% EtOH/20 mM buffer at 25 °C; MES buffer pH 6.2 or HEPES buffer pH 7.4. [b] Optimum pH. [c] Measured from a 2.0 mM HPNP solution (see ref 4).

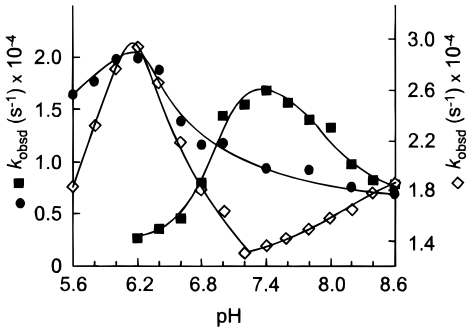

Figure 1. Dependence of k_{obs} on pH for the transesterification of HPNP (0.19 mM) catalyzed by **1**-Cu_2 (◇), **2**-Cu_2 (•), and **3**-Cu_2 (■, 0.48 mM) in 35% EtOH/20 mM buffer (lines are drawn for clarity).

Dinuclear complex **2**-Cu_2 shows at pH 6.2 a catalytic activity that is in the same range as for **1**-Cu_2, i.e., a rate acceleration of a factor 6.9×10^3 over the uncatalyzed reaction. Dinuclear complex **3**-Cu_2, with two additional amino groups, exhibits at pH 7.4 a rate acceleration of 3.1×10^3 but is not an efficient catalyst at pH 6.2. Complete conversion of a 4-fold excess of the substrate over the catalysts **2**-Cu_2 or **3**-Cu_2 was observed (pH 7.4), demonstrating that these calix[4]arene complexes are genuine catalysts. When we varied the ratio [**3**]/[Cu(II)] we found an optimum rate at 2.5 equiv of Cu(II) (not shown), which indicates that most likely both Cu(II) ions are involved in the catalysis.

The pH−rate profiles for HPNP cleavage catalyzed by **1**-Cu_2, **2**-Cu_2, and **3**-Cu_2 are depicted in Figure 1. The hydroxymethyl calix[4]arene **2**-Cu_2 and the reference calix[4]arene **1**-Cu_2 exhibit the highest catalytic activity at pH 6.2. However, their pH dependency is different at high pH, where **2**-Cu_2 is hardly active and **1**-Cu_2 is reasonably active.[4] pH-dependent catalysis that might be affected by the hydroxymethyl groups in **2**-Cu_2 are the deprotonation of Cu(II)-bound water molecules and the orientation of water molecules or hydroxide ions near the catalytic center. The low activity of **2**-Cu_2 at alkaline pH indicates a weak binding of the substrate to the catalyst.[4] This might be caused by tightly bound Cu-hydroxides and steric hindrance by the hydroxymethyl groups.[15] The two aminomethyl groups in **3**-Cu_2 shift the optimum pH from 6.2 for **1**-Cu_2 to pH 7.4 for **3**-Cu_2. The pH−rate profile for compound **3**-Cu_2 is bell shaped, displaying acid−base transitions with kinetic pK_a's of approximately 6.9 and 8.0 (Figure 1). These acid−base transitions may correspond to the deprotonation of one or two Cu(II)-bound water molecules and ammonium groups, respectively. The pK_a values of the water molecules bound to **1**-Cu_2 are 6.5 and 6.6,[4] and the pK_a values of the primary amines in the dinuclear complex **3**-Cu_2 are expected to be around 8.[6,7a] The pH−rate profile for **3**-Cu_2 indicates that in the catalytically most active form, **3**-Cu_2 bears one or two ammonium groups and at both Cu(II) centers a hydroxide ion. Presumably, the low activity below pH 6.9 is due to the absence of sufficient general base that can assist in the deprotonation of the nucleophilic hydroxyl group of HPNP. The low activity above pH 8.0 is most likely originating from a weak affinity of the catalyst for the substrate, due to the presence of tightly bound Cu(II)-hydroxides.

(15) The formation of catalytically inactive μ-hydroxy-bridged complexes **2**-[Cu(OH)$_2$Cu] (or kinetic equivalents) was not investigated but cannot be ruled out.

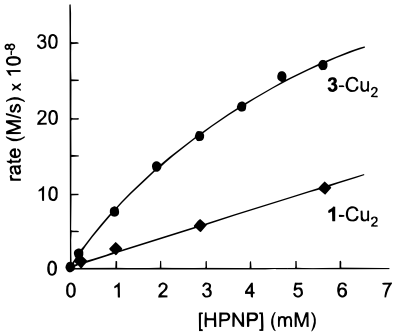

Figure 2. Rate as a function of the substrate concentration for the transesterification of HPNP catalyzed by 0.24 mM of **1**-Cu_2 and **3**-Cu_2 in 35% EtOH/20 mM HEPES pH 7.4. The experimental data points for **3**-Cu_2 are fitted to the Michaelis−Menten equation with $K_m = 7.1$ mM and $k_{cat} = 2.6 \times 10^{-3}$ s^{-1}. The experimental data points for **1**-Cu_2 are fitted to a second-order rate law with $k_2 = 0.079$ M^{-1} s^{-1}.

Table 2. Kinetic Data for the Transesterification of HPNP Catalyzed by 1-Cu_2 and 3-Cu_2[a]

catalyst	pH[a]	k_2 ($M^{-1}s^{-1}$)[b]	k_{cat}[c] (s^{-1})	K_m[c] (mM)	K_{assoc}[d] (M^{-1})
1-Cu_2	6.2	0.52	2.1×10^{-3}	4.0	2.5×10^2
3-Cu_2	6.2	0.083	0.33×10^{-3}	4.0	2.5×10^2
3-Cu_2	7.4	0.36	2.6×10^{-3}	7.1	1.4×10^2
1-Cu_2	7.4	0.079[e]			

[a] In 35% EtOH/20 mM buffer at 25 °C (see ref 14). [b] At low concentrations of HPNP $k_2 = k_{cat}/K_m$. [c] Determined by least-squares analysis of an Eady−Hofstee plot. [d] $K_{assoc} = 1/K_m$. [e] Based on pseudo-first-order rate constants for catalyst concentrations of 0.24 mM (see ref 4).

To elucidate the binding of the substrate and the rate of the conversion of HPNP within the catalyst−substrate complex, the transesterification rate was measured as a function of the substrate concentration. In contrast to the reference complex **1**-Cu_2, lacking the extra amino groups, the bifunctional catalyst **3**-Cu_2 shows a saturation curve at pH 7.4 (Figure 2). This Michaelis−Menten kinetics demonstrates that **3**-Cu_2 forms relatively much catalyst−substrate complex at pH 7.4. The first-order dependency exhibited by **1**-Cu_2 indicates a weak affinity for the substrate at this pH. Both complexes **1**-Cu_2 and **3**-Cu_2 show a saturation curve at pH 6.2 (not shown).[4] The Michaelis−Menten curves were analyzed by means of Eady−Hofstee plots, and the kinetic data are summarized in Table 2. From the data at pH 6.2 and 7.4, it is clear that the binding of the substrate (K_{assoc}) is more efficient at pH 6.2 and the conversion of the substrate within the catalyst−substrate complex (k_{cat}) is more efficient at pH 7.4. This is in agreement with the presence of weakly bound water molecules at low pH and the higher concentrations general base at high pH. At pH 6.2, the binding constants are the same for **3**-Cu_2 and **1**-Cu_2 (2.5×10^2 M^{-1}), indicating no additional effect of

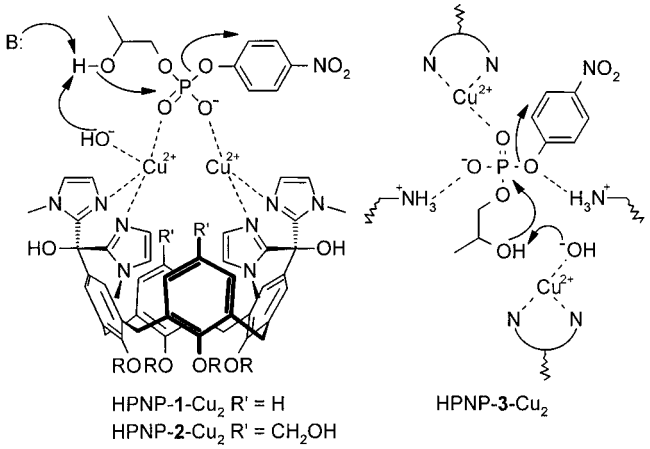

Figure 3. Schematic representations of possible mechanisms for the cleavage of HPNP catalyzed by **1**-Cu_2 and **2**-Cu_2 (left) and **3**-Cu_2 (right).

the amino groups at low pH. Although at the pH optimum (**1**-Cu_2, pH 6.2; **3**-Cu_2, pH 7.4) the binding constant is lower for **3**-Cu_2, the catalytic rate constant k_{cat} is higher for **3**-Cu_2. This is due to the higher concentration of (general) base at pH 7.4. The data indicate that the amino groups, probably present as ammonium groups at pH 7.4, assist in the substrate binding and activation process.

Mechanism of Catalysis. In analogy with the catalysis by **1**-Cu_2,[4] catalysis by **2**-Cu_2 proceeds presumably via a double Lewis activation mechanism[2] in which both Cu(II) centers bind the phosphoryl group and where a Cu(II) bound hydroxide ion can assist as a general base in the deprotonation of the substrate (Figure 3, left). Although for catalyst **3**-Cu_2 a similar mechanism can be proposed in which the primary amines act as the general base, it is likely that at the optimum pH of 7.4 the amines in **3**−Cu_2 are in the protonated form. The increased substrate binding affinity of **3**-Cu_2 can then be due to cooperative action of one or both Cu(II) centers with one or two ammonium groups (Figure 3, right). Two ammonium groups and one of the Cu(II) centers may bind and activate the negatively charged phosphoryl group of the substrate. The remaining Cu(II) center can then contribute to the catalysis by deprotonation of the substrate hydroxyl group via a bound hydroxide ion. Moreover, the ammonium groups can stabilize the transition state and possibly the leaving group by electrostatic interactions and hydrogen bonding.

Conclusions

We have shown that calix[4]arenes are suitable building blocks for the design of multifunctional enzyme models. Methods have been developed for the synthesis of calix[4]arenes that possess besides bisimidazolyl ligands also hydroxymethyl (**2**) or aminomethyl groups (**3**) at the upper rim. The dinuclear complexes **2**-Cu_2 and **3**-Cu_2 mimic metalloenzymes that cleave phosphate diester bonds. The aminomethyl calix[4]arene **3**-Cu_2 exhibits interesting bifunctional catalysis in the intramolecular transesterification of the RNA model substrate HPNP, e.g., the protonated aminomethyl groups can assist in the formation of a catalyst-substrate complex and in the stabilization of the transition state. In addition to the fact that the additional functional groups in calix[4]-arenes **2** and **3** can contribute in the catalysis, they can be a handle for further functionalization with, for instance, RNA- or DNA-binding compounds. This opens the way to antisense oligonucleotides[16,17] with phosphodiesterase activity.

Experimental Section

THF was freshly distilled from Na/benzophenone and 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). Melting points are uncorrected. ^{1}H NMR (250 MHz) and ^{13}C NMR spectra were recorded in $CDCl_3$ with Me_4Si as internal standard. FAB-MS spectra were recorded with *m*-NBA as a matrix. Compounds **1**[4] and **4**[4] and HPNP[13] were synthesized according to literature procedures. The pH meter used for adjustment of buffered solutions was calibrated daily. UV−vis spectra were measured on a diode array spectrophotometer equipped with a thermostated cuvette holder (seven cuvettes, 1.0 cm path length) and a sample transport accessory.

5,17-Diformyl-11,23-bis(ethoxycarbonyl)-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (5). A solution of calix[4]arene **4** (2.98 g, 3.48 mmol) and hexamethylenetetraamine (4.87 g, 34.7 mmol) in TFA (200 mL) was refluxed for 3 days. The mixture was cooled to room temperature, poured into ice−water, and extracted with CH_2Cl_2. The combined extracts were washed with saturated $NaHCO_3$ and water and were dried over $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by column chromatography (CH_2Cl_2/EtOAc, 9/1) to give **5** as a colorless oil (2.51 g, 79%). ^{1}H NMR: δ (ppm) 9.27 (s, 2 H), 7.80 (s, 4 H), 6.72 (s, 4 H), 4.59 and 3.30 (AB q, 8 H, J = 13.8 Hz), 4.40 (q, 4 H, J = 7.1 Hz), 4.32 (t, 4 H, J = 5.4 Hz), 4.04 (t, 4 H, J = 4.7 Hz), 3.82−3.75 (m, 8 H), 3.55 (q, 4 H, J = 7.0 Hz), 3.46 (q, 4 H, J = 7.0 Hz), 1.44 (t, 6 H, J = 7.1 Hz), 1.22 (t, 6 H, J = 7.0 Hz), 1.14 (t, 6 H, J = 7.0 Hz). ^{13}C NMR: δ (ppm) 191.4, 166.4, 161.7, 160.4, 136.0, 134.6, 131.4, 130.5, 129.6, 124.7, 74.1, 73.1, 69.7, 69.4, 66.5, 66.2, 60.8, 30.7, 15.2 (2×), 14.4. FAB-MS *m*/*z*: 935.4 ([M + Na]$^+$, calcd 935.4), 867.8 ([M − OCH_2CH_3]$^+$, calcd 867.4).

5,17-Bis(hydroxymethyl)-11,23-bis(ethoxycarbonyl)-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (6). To a solution of calix[4]arene **5** (580 mg, 0.635 mmol) in EtOH (40 mL) was added $NaBH_4$ (96 mg, 2.5 mmol). After 2 h of stirring at room temperature, acetic acid was carefully added until hydrogen gas formation stopped. The solvent was removed under reduced pressure, and the residue was taken up in CH_2Cl_2, washed with saturated $NaHCO_3$ and water, and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by column chromatography (CH_2Cl_2/EtOAc 8/2) to give **6** as a white foam (507 mg, 87%). ^{1}H NMR: δ (ppm) 7.80 (s, 4 H), 6.22 (s, 4 H), 4.54 and 3.23 (AB q, 8 H, J = 13.5 Hz), 4.43−4.32 (m, 8 H), 4.04 (s, 4 H), 3.95−3.85 (m, 8 H), 3.79−3.75 (m, 4 H), 3.58 (q, 4 H, J = 7.0 Hz), 3.46 (q, 4 H, J = 7.0 Hz), 2.74 (s, 2H), 1.43 (t, 6 H, J = 7.1 Hz), 1.24 (t, 6 H, J = 7.0 Hz), 1.13 (t, 6 H, J = 7.0 Hz). ^{13}C NMR: δ (ppm) 166.9, 162.1, 154.4, 136.5, 135.2, 132.9, 130.3, 126.4, 124.1, 74.1, 72.8, 69.8, 69.6, 66.5, 66.1, 64.2, 60.7, 30.9, 15.3, 15.2, 14.4. FAB-MS *m*/*z*: 915.2 ([M − H]$^-$, calcd 915.5), 939.3 ([M + Na]$^+$, calcd 939.5).

5,17-Bis(hydroxymethyl)-11,23-bis(bis(1-methylimidazol-2-yl)hydroxymethyl)-25,26,27,28-tetrakis(2-eth-

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oxyethoxy)calix[4]arene (2). To a solution of 1-methylimidazole (0.35 mL, 4.47 mmol) in THF (20 mL) at −78 °C under Ar was added *n*-BuLi (1.79 mL, 2.5 M, 4.48 mmol). After the solution was stirred for an additional 1 h at −78 °C, calix[4]arene **6** (410 mg, 0.447 mmol) in THF (20 mL) was added dropwise. The reaction mixture was allowed to warm to room temperature and was stirred overnight. Brine was added, and the solution was extracted with CH_2Cl_2. 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, 9/1) to give **2** (340 mg, 66%) as a white foam. ^{1}H NMR: δ (ppm) 6.92 (s, 8 H), 6.80 (s, 4 H), 6.17 (s, 4 H), 4.47 and 3.05 (AB q, 8 H, J = 13.0 Hz), 4.33 (t, 4 H, J = 6.2 Hz), 3.95 (t, 8 H, J = 6.2 Hz), 3.87 (t, 4 H, J = 4.6 Hz), 3.72 (t, 4 H, J = 4.6 Hz), 3.59−3.47 (m, 8 H), 3.44 (s, 12 H), 1.23 (t, 6 H, J = 7.0 Hz), 1.16 (t, 6 H, J = 7.0 Hz). ^{13}C NMR: δ (ppm) 157.5, 153.9, 148.6, 136.6, 135.9, 135.2, 132.9, 128.1, 126.6, 125.8, 123.6, 74.9, 74.2, 72.6, 69.8, 69.5, 66.5, 66.2, 64.2, 35.0, 30.8, 15.4, 15.3. FAB-MS *m/z:* 1053.7 ([M + H]$^+$, calcd 1053.6).

5,17-Diazido-11,23-bis(ethoxycarbonyl)-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (7). To a solution of calix[4]arene **6** (1.55 g, 1.69 mmol) in CH_2Cl_2 (100 mL) was added $SOCl_2$ (1.23 mL, 16.9 mmol). After being stirred for 2 h at room temperature, the solution was evaporated in vacuo. The residue was dissolved in DMF, NaN_3 (330 mg, 5.08 mmol) was added, and the solution was stirred for 18 h at room temperature. The solvent was removed under reduced pressure, CH_2Cl_2 was added, and the suspension was washed with 0.1 N HCl solution and water. The solution was dried with $MgSO_4$, filtered, and evaporated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/EtOAc 95/5) to give **7** (1.34 g, 82%) as a colorless oil. ^{1}H NMR: δ (ppm) 7.73 (s, 4 H), 6.27 (s, 4 H), 4.56 and 3.23 (AB q, 8 H, J = 13.4 Hz), 4.41−4.33 (m, 8 H), 3.99 (t, 4 H, J = 4.8 Hz), 3.87 (t, 4 H, J = 5.6 Hz), 3.77 (t, 4 H, J = 4.8 Hz), 3.74 (s, 4 H), 3.56 (q, 4 H, J = 7.0 Hz), 3.47 (q, 4 H, J = 7.0 Hz), 1.42 (t, 6 H, J = 7.1 Hz), 1.23 (t, 6 H, J = 7.0 Hz), 1.15 (t, 6 H, J = 7.0 Hz). ^{13}C NMR: δ (ppm) 166.7, 161.7, 155.2, 136.1, 133.7, 130.2, 129.2, 128.0, 124.4, 74.0, 73.0, 69.8, 69.5, 66.5, 66.1, 60.7, 54.0, 30.8, 15.3, 15.2, 14.4. FAB-MS *m/z*: 989.7 ([M + Na]$^+$, calcd 989.5), 896.5 ([M − N_3 − N_2]$^+$, calcd 896.5).

5,17-Bis(*N*-Boc-aminomethyl)-11,23-bis(ethoxycarbonyl)-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (8). A suspension of 10% Pd−C (120 mg) in EtOH (50 mL) was stirred for 30 min under a hydrogen atmosphere. A solution of calix[4]arene **7** (1.22 g, 1.26 mmol) and Boc_2O (1.10 g, 5.05 mmol) in EtOH (500 mL) was added, and the reaction mixture was stirred under a hydrogen atmosphere at room temperature for 20 h. The suspension was filtered over Hyflo and evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 and washed subsequently with 0.1 N HCl solution, water, saturated $NaHCO_3$ solution, and water. The solution was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by column chromatography (CH_2Cl_2/EtOAc, 9/1) and crystallization from EtOH to give **8** as white crystals (1.17 g, 83%). Mp 163−164 °C. ^{1}H NMR: δ (ppm) 7.75 (s, 4 H), 6.16 (s, 4 H), 4.52 and 3.20 (AB q, 8 H, J = 13.4 Hz), 4.95 (br s, 1 H), 4.40−4.32 (m, 8 H), 3.94 (t, 4 H, J = 4.5 Hz), 3.86 (t, 4 H, J = 5.6 Hz), 3.78−3.75 (m, 8 H), 3.56 (q, 4 H, J = 7.0 Hz), 3.46 (q, 4 H, J = 7.0 Hz), 1.46−1.40 (m, 24 H), 1.23 (t, 6 H, J = 7.0 Hz), 1.13 (t, 6 H, J = 7.0 Hz). ^{13}C NMR: δ (ppm) 166.7, 161.9, 155.8, 154.1, 136.3, 133.1, 132.8, 130.2, 126.1, 124.1, 74.0, 72.9, 69.8, 69.5, 66.5, 66.1, 60.6, 30.9, 28.3, 15.3, 15.2, 14.4. FAB-MS *m/z*:, 1041.6 ([M − O-*t*-Bu]$^-$, calcd 1041.5), 1137.8 ([M + Na]$^+$, calcd 1137.6). Anal. Calcd for $C_{62}H_{86}N_2O_{16}$: C, 66.77; H, 7.77; N, 2.51. Found: C, 66.87; H, 7.90; N, 2.51.

5,17-Bis(*N*-Boc-aminomethyl)-11,23-bis(bis(1-methylimidazol-2-yl)hydroxymethyl-25,26,27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (9). To a solution of 1-methylimidazole (0.76 mL, 9.53 mmol) in THF (50 mL) at −78 °C under Ar was added *n*-BuLi (3.85 mL, 2.5 M, 9.63 mmol). After the solution was stirred for an additional 1 h at −78 °C, calix[4]arene **8** (1.07 g, 0.959 mmol) in THF (50 mL) was added dropwise. The reaction mixture was allowed to warm to room temperature and was stirred overnight. Brine was added, and the solution was evaporated under reduced pressure. The residue was taken up in CH_2Cl_2/brine, and the aqueous phase was adjusted to pH 8 with saturated Na_2CO_3 solution and extracted with CH_2Cl_2. 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, 9/1) to give **9** as a colorless oil (585 mg, 45%). ^{1}H NMR: δ (ppm) 6.94 (s, 4 H), 6.91 (s, 4 H), 6.61 (s, 4 H), 6.43 (br s, 2 H), 6.28 (s, 4 H), 4.47 and 3.05 (AB q, 8 H, J = 13.0 Hz), 4.20 (t, 4 H, J = 5.7 Hz), 4.00 (t, 4 H, J = 5.3 Hz), 3.88−3.80 (m, 12 H), 3.53 (q, 4 H, J = 7.0 Hz), 3.50 (q, 4 H, J = 7.0 Hz), 3.24 (s, 12 H), 1.46 (s, 18 H), 1.21 (t, 6 H, J = 7.0 Hz), 1.17 (t, 6 H, J = 7.0 Hz). ^{13}C NMR: δ (ppm) 156.7, 155.7, 154.7, 148.7, 135.7, 135.5, 134.3, 127.8, 127.0, 125.8, 123.4, 74.6, 73.5, 73.2, 69.6, 69.5, 66.4, 66.2, 34.7, 30.9, 28.5, 15.3 (2×). FAB-MS *m/z* 1351.8 ([M + H]$^+$, calcd 1351.7).

5,17-Bis(aminomethyl)-11,23-bis(bis(1-methylimidazol-2-yl)hydroxymethyl)-25,26, 27,28-tetrakis(2-ethoxyethoxy)calix[4]arene (3). To a solution of calix[4]arene **9** (270 mg, 0.185 mmol) in CH_2Cl_2 (30 mL) was added TFA (0.85 mL, 11.1 mmol). After being stirred for 18 h at room temperature, the solution was concentrated under reduced pressure. The residue was freed from TFA by elution with MeOH over a strongly basic anion-exchange column (Fluka IRA-400, activated with 0.1 N NaOH solution). The obtained white powder was recrystallized from MeOH/diisopropyl ether to give free amine **3** (173 mg, 81%). Mp 108−111 °C. ^{1}H NMR: δ (ppm) 6.94 (s, 4 H), 6.87 (s, 4 H), 6.64 (s, 4 H), 6.36 (br s, 2 H), 6.27 (s, 4 H), 4.47 and 3.05 (AB q, 8 H, J = 13.0 Hz), 4.21 (t, 4 H, J = 5.7 Hz), 4.01 (t, 4 H, J = 5.1 Hz), 3.89−3.79 (m, 8 H), 3.54 (q, 4 H, J = 7.0 Hz), 3.51 (q, 4 H, J = 7.0 Hz), 3.32 (s, 4 H), 3.25 (s, 12 H), 1.40 (br s, 4 H), 1.24−1.12 (m, 12 H). ^{13}C NMR: δ (ppm) 156.6, 154.6, 148.8, 135.7, 135.5, 134.1, 127.8, 126.6, 125.9, 123.3, 77.3, 74.6, 69.7, 69.5, 66.4, 66.2, 45.7, 34.8, 30.8, 15.4, 15.3. FAB-MS *m/z* 1151.6 ([M + H]$^+$, calcd 1151.6). Anal. Calcd for $C_{64}H_{82}N_{10}O_{10}\cdot 2H_2O$: C, 64.74; H, 7.30; N, 11.80; Found: C, 64.96; H, 7.01; N, 11.79.

Kinetics. Solutions for the 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.[14] 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 was prepared according to a literature procedure.[13] Stock solutions were freshly prepared before performing the kinetic measurements. In a typical experiment, the ligand **3** (20 μL, 50 mM in EtOH) and Cu(ClO_4)$_2$ (40 μL, 50 mM in EtOH) were added to a cuvette 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 λ = 406 nm due to the release of *p*-nitrophenol was recorded every minute. Final concentrations were 0.48 mM in Cu(II) complex, 0.19 mM in substrate, 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*-nitrophenol at λ = 406 nm by an initial slope method (<4% conversion). All rate constants were obtained by averaging three kinetic measurements.

Acknowledgment. This work was supported by the council for Chemical Sciences of The Netherlands Organization for Scientific Research (CW-NWO).

Supporting Information Available: Copies of ^{1}H NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

JO9905266