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Tripodal, Cooperative, and Allosteric Transphosphorylation Metallocatalysts

Alessandro Scarso,† Giovanni Zaupa, Florence Bodar Houillon, Leonard J. Prins, and Paolo Scrimin*

University of Padova, Department of Chemical Sciences and CNR- ITM, Padova Section, Via Marzolo, 1-35131 Padova, Italy

paolo.scrimin@unipd.it

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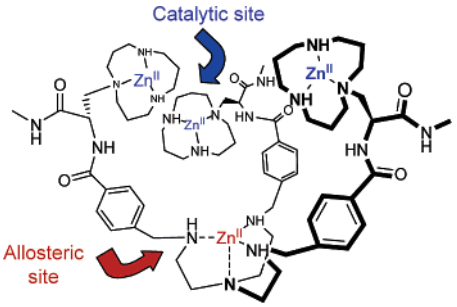

Three artificial amino acids derived from L-serine by replacing the hydroxyl moiety with 1,4,7-triazacyclononane, 1,5,9-triazacyclododecane, and 1,4,7,10-tetraazacyclododecane, respectively, have been connected to the three arms of the tetraamine tris(2-aminoethyl)amine, Tren, to obtain tripodal ligands. They are able to bind up to four metal ions (like Cu^{II} and Zn^{II}), three with the polyazamacrocycles and one with the Tren platform. Some of the Zn^{II} complexes of these tripodal ligands proved to be good catalysts for the cleavage of the RNA model substrate 2-hydroxypropyl-*p*-nitrophenylphosphate (HPNP). Studies of the catalytic activity in the presence of increasing amounts of Zn^{II} show that the complexes represent minimalist examples of metallocatalysts with cooperativity between the metal centers and allosteric control by a metal ion. The Tren binding site constitutes the allosteric regulation unit, while the three Zn^{II}−azacrown complexes provide the cooperative, catalytic site. The allosteric role of the Zn^{II} ion located in the Tren binding site was unambiguously demonstrated by studying the catalytic activity of a derivative unable to complex Zn^{II} in that site. In this case, the cooperativity between the three Zn^{II} ions bound to the peripheral azacrowns was totally suppressed. The kinetic analysis has shown that cooperativity is due to neither the occurrence of general-acid/general-base catalysis nor a decreased binding of the substrate because of the deprotonation of a water molecule bound to the complex but, rather, stabilization of the complexed substrate in its transformation into the transition state.

Introduction

The hydrolytic cleavage of the phosphate bond under physiological conditions is incredibly slow. So slow that, in the case of phosphate diesters, the determination of reliable rate constants has been elusive, with numbers that have been updated to a slower figure almost yearly. The latest values indicate a half-life of 10^{10} years for the cleavage of dimethyl phosphate.[1,2] Not far from this sluggish reactivity should be the time required for hydrolytically cleaving the P−O bond of DNA. RNA is more labile because the nucleophilic attack on phosphorus is performed intramolecularly by the −O(H) in the 2′ position of the ribose. Thus, the half-life is, in this case, only 10^4 years. These processes can be accelerated by several orders of magnitude by metal ions and a considerable number of laboratories has embarked in the challenging goal of preparing powerful, metal ion-based catalysts with, in some cases, interesting results.[3]

* To whom correspondence should be addressed. Tel: +39-049-8275276. Fax: +39-049-8275239.
† Current address: University of Venice, Calle Larga Santa Marta 2137, 30123 Venezia, Italy.

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(2) For an update see: Wolfenden, R. *Chem. Rev.* **2006**, *106*, 3379−3396.

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Chin[4] has estimated that by simply adding up independent contributions to the catalysis by two cooperating metal ions, a catalyst could bring up to about 18 orders of magnitude in rate acceleration. Such a catalyst will, however, remain the Holy Grail for scientists for many years to come. In the meantime, addressing fundamental points pertinent to the metal-catalyzed hydrolytic process may provide key information for its design. One of these issues is cooperativity: indeed, not only the above-mentioned estimates made by Chin but most of the enzymes devoted to the hydrolytic cleavage of the phosphate ester bond point to a key role played by more than one metal ion in the catalytic process.[5] Following these insights, dinuclear and trinuclear complexes of Co^{III}, Cu^{II}, and Zn^{II} have been synthesized and shown to be better catalysts for the cleavage of these substrates.[6] Because of the faster reactivity than that of DNA, RNA and RNA model derivatives are quite popular substrates.

Another important feature present in many enzymes (including metalloenzymes) is the presence of allosteric sites devoted to the control of the structure of the catalyst. They are not involved directly in the catalytic process but, rather, fine-tune the activity of the catalyst by stabilizing its active conformation. An example is provided by *E. coli* alkaline phosphatase, an enzyme that catalyzes the cleavage of phosphate monoesters. In its active site, two Zn^{II} ions are directly involved in the catalytic process, while 6 Å apart there is a Mg^{II} ion that acts as a strong allosteric activator. Thus, all three ions are necessary for catalysis but with rather different roles.

Recently,[7] in search of systems where metal ion cooperativity in a catalytic process could be triggered by appropriate conformational changes induced by a metal ion placed in a remote position, we have reported the catalytic activity of the tripodal polypeptide TP_3 in the transphosphorylation of the RNA model substrate 2-hydroxypropyl-*p*-nitrophenylphopsphate (HPNP). Allosteric synthetic catalysts have also been reported by others.[8] Krämer has reported[9] a 2 + 1 metal ion synthetic phosphodiesterase where a metal ion acts as an allosteric regulator and the remaining two constitute a functional (catalytic) site. A similar example was reported by Shinkai and Takeuchi.[10]

Our control unit was based on tris(2-aminoethyl)amine (Tren), a tripodal tetraamine that has proven to be a very versatile platform for the allosteric control of the activity of rather different synthetic systems ranging from HIV-1 protease inhibitors[11] to membrane permeability affectors.[12] Recent work by Anslyn has also shown that similar systems recognize very efficiently phosphate anions.[13] The tetraamine, upon metal ion binding, changes its conformation from an open one, where the three arms stay far apart in order to minimize charge repulsion between the protonated amines, to a closed one necessary for metal ion coordination (see Figure 1).[14]

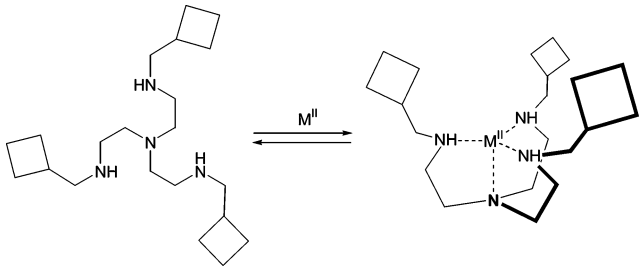

FIGURE 1. Conformational change associated to the binding of a metal ion (M^{II}) to Tren. The diamonds represent hypothetical functional groups linked to the three arms.

In this latter conformation, the three arms get closer, and if properly functionalized with metal complexes, cooperativity between them in an hydrolytic process may be observed. Thus, the three arms must bear suitable ligands for metal ion coordination but should also not get too close to avoid the formation of μ-complexes that are known to be very little active in hydrolytic catalysis.[15,16] The interesting results obtained with our original system based on polypeptide TP_3 prompted us to investigate simpler, synthetically more accessible catalysts where the ligand subunits could be tuned to better enhance their catalytic properties. Furthermore, an unambiguous proof of the allosteric role of the Tren-bound metal ion was still lacking. The tripodal systems we have designed and synthesized are reported in Chart 1. The metal ion coordination units are provided by artificial amino acids derived from L-serine where the −OH has been replaced by a cyclic polyamine. These cyclic ligands are known to coordinate very strongly transition metal ions like Cu^{II} and Zn^{II}. Furthermore, previous work by Kimura has shown that the pK_a of a water molecule bound to these Zn^{II}-polyamine complexes varies within the 7.3−9.8 interval.[17] Accordingly, it should be possible to tune the activity of the system also by modulating the acidity (and nucleophilicity) of this metal-bound water molecule within a physiologically relevant pH interval. A simple 1,4-disubstituted benzene acts as spacer to avoid the collapse of the arms which would result in inactive complexes.

Results and Discussion

Syntheses. Our strategy toward the synthesis of Boc-protected ligand-amino amides **2** and **3** follows the general procedure reported in Scheme 1 and already employed by us for the

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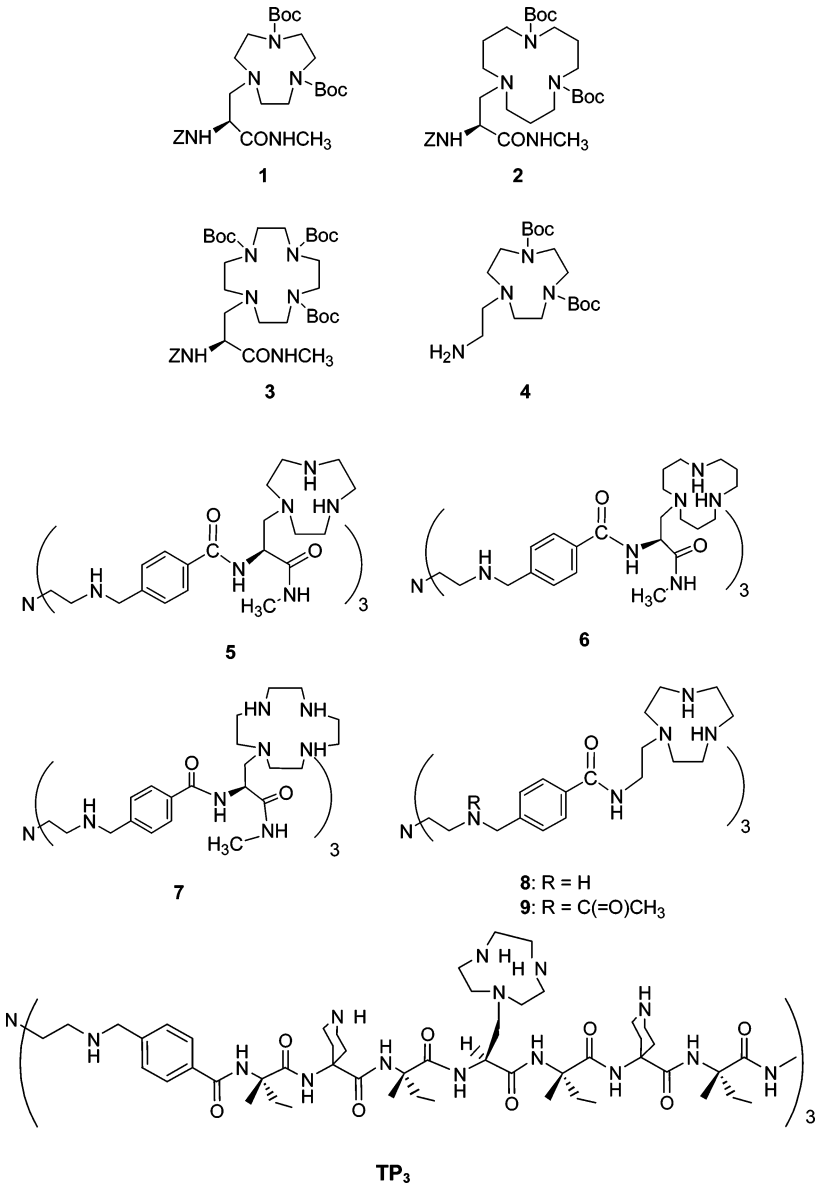
CHART 1



synthesis of **1**.[18] This requires the Boc-protection of all amino groups but one, of the corresponding cyclic polyamine and reaction of the latter with the Z-protected lactone of serine. The reactions give predominantly the desired amino acid and minor amounts of the undesired amide (up to 20%). The amino acids were then converted into the *N*-methyl amides by treatment with methylamine and standard coupling reagents. The Z-protecting group was removed by hydrogenation immediately before coupling to the Tren platform. The most delicate step of this procedure is the protection of the cyclic polyamines for which dichloromethane (contrary to what we had reported previously)[18] must be avoided, and a very slow addition of the required amount of $(Boc)_2O$ is mandatory (see the Experimental Section for details). Additionally, Boc-protected ligand **4**, a structurally simpler analogue of **1**, was synthesized in two steps via alkylation of di-Boc-protected 1,4,9-triazacyclononane with *N*-(2-bromoethyl)phthalimide and subsequent deprotection with hydrazine.

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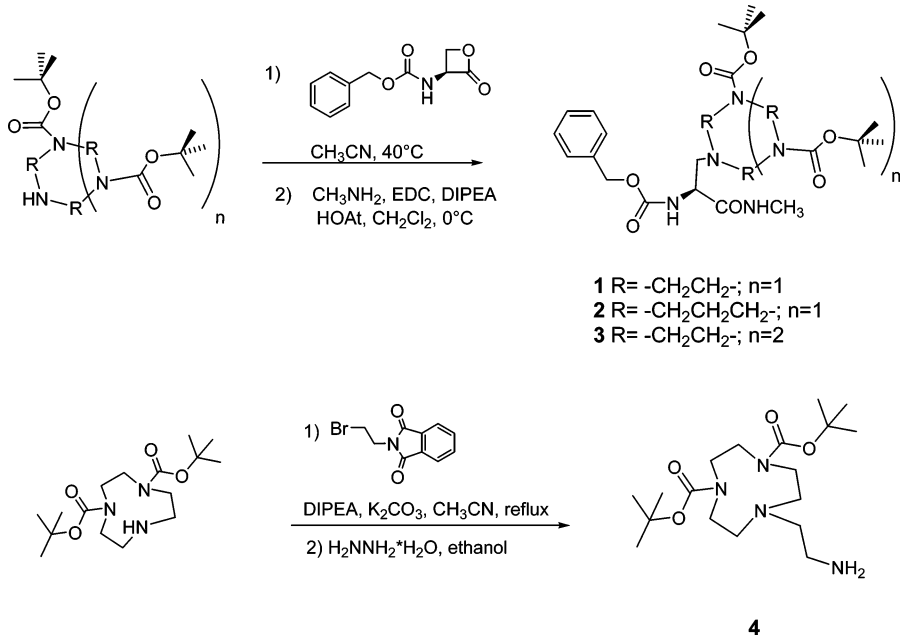
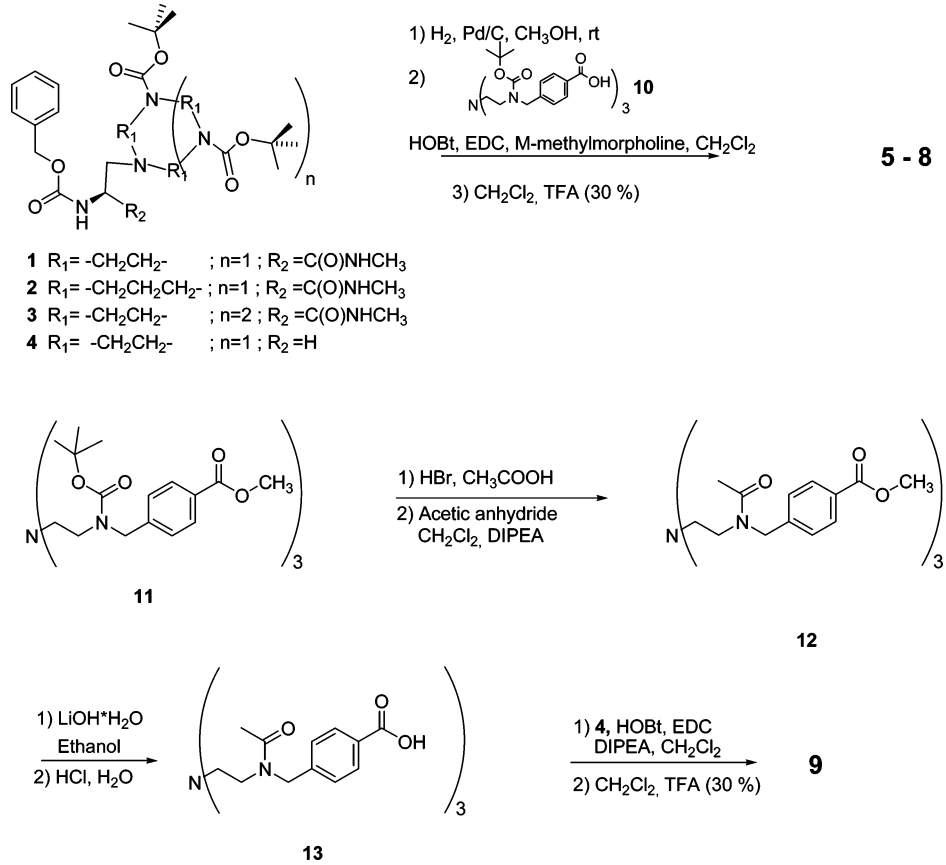
Subsequently, the Boc-protected azacrown ligands **1**−**4** (after removal of the Z-group) were coupled to the modified Tren-platform **10** as reported previously.[12b] After a final deprotection step to remove the Boc-groups, the expected tripodal ligands **5**−**8** were obtained in moderate yield (Scheme 2). The synthesis of the control compound **9**, in which the secondary amines of the Tren-binding site are acylated, required some additional steps. Initially, the Boc-groups of compound **11**[12b] were removed, and the deprotected amines were reacted with acetic anhydride. Next, the methyl ester was hydrolyzed under basic conditions to give **12** which was finally reacted with ligand **4** in a similar manner as discussed before. Final deprotection of the Boc-groups gave compound **9**.

Metal Ion Binding. We have studied complex formation of ligands **5**−**7** with two metal ions, Cu^{II} and Zn^{II}. Tripodal ligands **5**−**7** have two different types of binding sites: the Tren-templating unit and the three cyclic polyamines. Accordingly, each of them can bind up to four metal ions with different binding strength. We were not particularly interested in the determination of the binding constants, which, on the basis of

SCHEME 1. Synthesis of the Z-Protected Amino Acids 1−3 and of the Free Base 4



SCHEME 2. Synthesis of the Tripodal Ligands 5−9



the values reported in the literature[19] should be large enough to ensure the complete formation of the 4:1 complex even under the most diluted reaction conditions. We were, on the contrary, extremely interested in knowing exactly the sequence of binding of the metal ions in the two different sites as the tripodal ligands are progressively loaded with increasing amounts of Cu^{II} or Zn^{II}. Luckily enough, due to the different maximum of the absorption bands of the complexes with Cu^{II} ions of the cyclic polyamines (ca. 650 nm) and Tren (ca. 850 nm), this information could be easily obtained for this metal via spectrophotometric titrations. Figure 2a−c reports the number of ions bound to each site during the titration of each tripodal ligand **5**−**7** with Cu^{II}. Analysis of this curve reveals that in all cases the Cu^{II} ions

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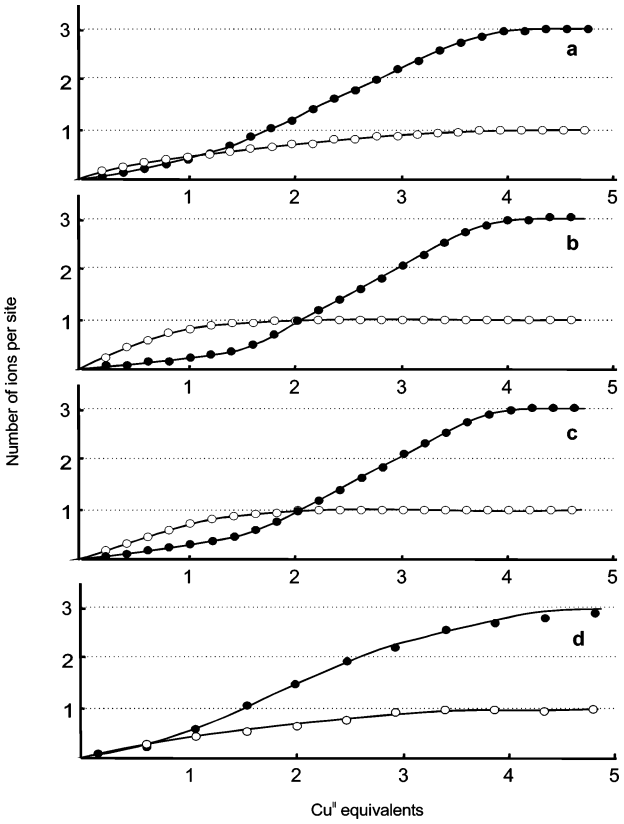

FIGURE 2. Binding of Cu^{II} ions to the two different binding sites present in the tripodal ligands **5**−**8**: (a) **5**, (b) **6**, (c) **7**, (d) **8**. In all cases, the open circles refer to the binding to the Tren site, while the filled circles refer to the binding to the azacrown subunits. Conditions: [ligand] = 1.0×10^{-3} M, pH = 6.3 (0.1 M MES buffer). The solid lines are drawn to guide the eye.

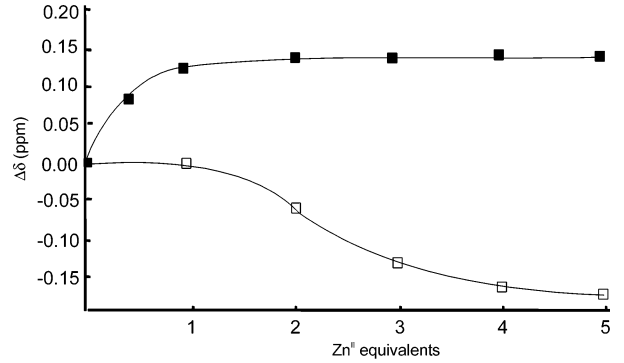

FIGURE 3. Shifts of the ^{1}H NMR signals of selected protons of ligand **6** upon addition of Zn^{II}: $CHCH_2N-$ signal close to the triazacyclododecane (open symbols) and ortho protons of the aromatic spacer close to the Tren arms (filled symbols).

first bind to the Tren site and only subsequently to the cyclic amines. However, this selectivity of binding is modest in the case of triazacyclononane (after the first equivalent of copper is added to **5** only 50% of the Tren subsite is occupied by the metal) and excellent in the case of both triazacyclododecane and cyclen (more than 80% of the first equivalent of Cu^{II} binds to Tren with these polyamines present in ligands **6** and **7**, respectively). Thus, upon progressively adding Cu^{II} ions to the tripodal ligands, the metal first binds to Tren changing the conformation of the system and, subsequently, the remaining three metal ions bind to the cyclic polyamines defining the

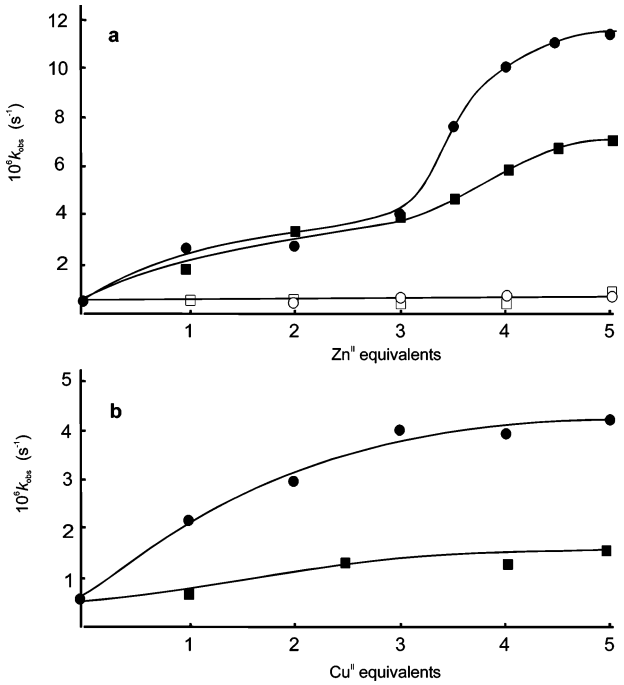

FIGURE 4. Kinetic effect on the observed rate constant for the cleavage of HPNP exerted by ligands **5** (filled squares), **6** (filled circles), and **7** (open circles) in the presence of increasing amounts of (a) Zn^{II} and (b) Cu^{II} ions. The open squares in (a) show the behavior of a mixture of the trismethylamide of deprotected **10** (1 equiv) and fully deprotected **1** acetylated at the α-amino group (3 equiv).[7] (Conditions: [catalyst] = 2×10^{-4} M, [HPNP] = 2×10^{-5} M, 40 °C, pH = 7). The solid lines are drawn to guide the eye.

putative catalytic site (see Scheme 3). In this site, the three metals form a cluster where they may cooperate in the catalytic process. The observed site-selectivity of Cu^{II} binding for **6** follows the relative strength of Cu^{II} complexation to Tren[20] and triazacyclododecane.[21] We would have expected a similar selectivity for **5**, too, as the reported binding affinity of triazacyclononane[22] for Cu^{II} is more than 3 orders of magnitude smaller than that for Tren, while in the case of **7** the inverse should have occurred (as the affinity of cyclen for Cu^{II} is higher than that for Tren[23]). Clearly, the substituents on the ligands (particularly Tren or cyclen) decrease their affinity for the metal ion in a way which is dependent on the final structure of the tripodal catalyst.

A direct spectroscopic investigation could not be performed with Zn^{II} because of the lack of distinct absorption bands for the complexes with this metal ion. However, competition experiments with the Cu^{II} complexes revealed a quite similar pattern in terms of selectivity (data not shown). Furthermore, with Zn^{II}, by following the shift of the signals of selected protons of the ligands in the ^{1}H−NMR spectrum we were able to unambiguously confirm that the binding first occurs with the Tren site and only subsequently with the azamacrocycles. Figure 3 reports this behavior for ligand **6**. Only in the case of tripodal ligand **5** does the selectivity appear to be slightly lower than that observed with Cu^{II}. In general, we may conclude that the

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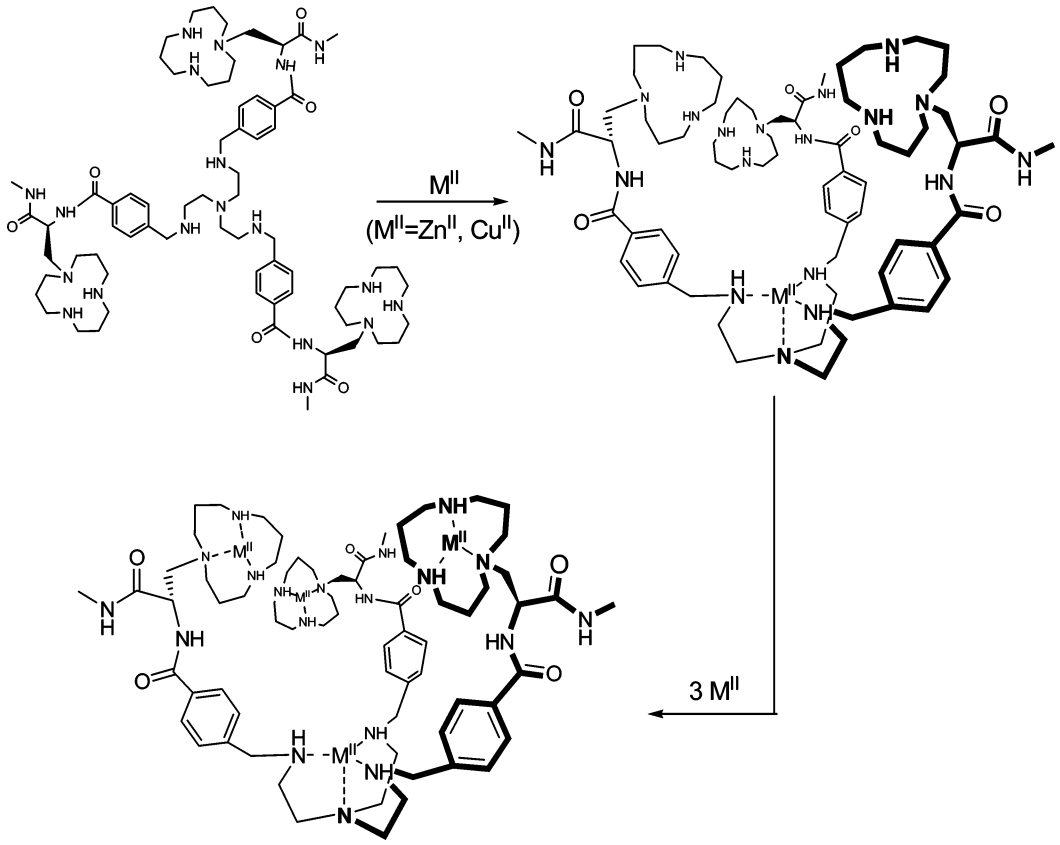
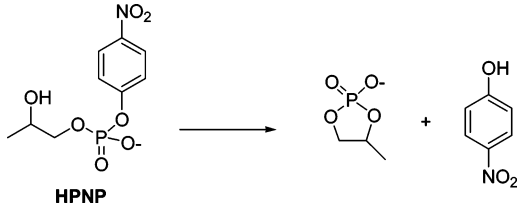
SCHEME 3. Proposed Scheme for the Sequential Occupation of the Metal-Binding Binding Sites in Tripodal Ligand 6 by Cu^{II} and Zn^{II}



pattern described in Scheme 3 for Cu^{II} and Zn^{II} binding to **6** is also valid for the other ligands.

Transphosphorylation Catalysis. We have tested our tripodal metallocatalysts in the cleavage of HPNP, an activated phosphate ester frequently used as a model for RNA. The release of *p*-nitrophenol (or *p*-nitrophenolate, depending on pH) is accompanied by the formation of a cyclic phosphate[24] (Scheme 4) and can be easily followed spectrophotometrically. In order to assess what is the best tripodal ligand and the nuclearity of the most active complex, we have run kinetics at a fixed ligand concentration and pH ([ligand] = 2×10^{-4} M, pH = 7, 40 °C) in the presence of increasing amounts of metal ions (Zn^{II} or Cu^{II}). The results are reported in Figure 4. They reveal the following: (a) the Zn^{II} complexes are far better catalysts than the Cu^{II} complexes; (b) in all cases the best catalyst is the tetranuclear complex;[25] (c) the best ligand appears to be **6**, while the poorest one is **7**; (d) the sigmoidal shape of the curves for the Zn^{II} complexes may be indicative of allosteric control by a metal ion or cooperativity between several metal centers. Noteworthy, a mixture of the Tren platform and 3 equiv of Boc-deproteced **2** showed almost no activity.[7] Hence, it is a peculiar behavior of the entire ligand (and not of the unassembled parts) that we observe in the plots of Figure 4a. Because of the structure of the ligands, it is conceivable that both allosteric control and cooperativity are present with these systems. The above data, however, do not give a clear-cut answer to this question. Accordingly, this point will be examined in more detail in a separate section.

SCHEME 4. Cleavage of HPNP



Very surprisingly, the Cu^{II} complexes are poor catalysts, but also the kinetic profiles do not indicate neither cooperativity or allosteric control (Figure 4b). Our best explanation for the observed behavior is that the substrate binds to the free apical position of the metal ion of the Tren platform instead of to those of the azamacrocycles. This is supported by the fact that the maximum rate acceleration with this metal is observed when, according to the plots of Figure 2, there is complete saturation of the Tren subunit of ligands **5** and **6** (compare Figure 4b with Figure 2a,b). It has been shown[24] that metal complexes of Tren are rather poor transphosphorylation catalysts in accord with the modest rate accelerations we observe here. The reason why HPNP interacts with the Tren-bound Cu^{II} rather than with the metal ions bound to the azamacrocycles could be related to the formation of inactive dimeric complexes of the latter.[16] A summary of the rate accelerations observed with these catalysts is reported in Table 1.

The kinetics of Figure 4a were performed at pH 7, and under those conditions, the best catalyst appears to be the tetranuclear Zn^{II} complex of ligand **6**. In order to determine the kinetic pK_a

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(25) Noteworthy saturation is attained after at least 5 equiv of Zn^{II} ions are added. This indicates that the apparent binding constant for Zn^{II} at this pH is not particularly high because of the competition with the protons.

TABLE 1. Transesterification of HPNP Catalyzed by the Tetranuclear Complexes of Ligands 5−7 and TP_3 with Zn^{II}

catalyst[a]	$10^5 k_{obs}$[b] (s^{-1})	k_{obs}/k_o[c]	$k_{obs}^{tripodal}/k_{obs}^{unassembled\ mixture}$
5−4Zn^{II}	0.55	140	23[d]
5−4Zn^{II}	1.35[e]	n.d.	n.d.
6−4Zn^{II}	1.1	275	30[f]
6−4Zn^{II}	1.1[e]	n.d.	n.d.
7−4Zn^{II}	0.07	17	n.d.
TP_3−4Zn^{II}	1.2	300	50[d]

[a] [catalyst] = 2×10^{-4} M. [b] At pH = 7.0 and 40 °C. [c] Estimated k_o from initial rate measurements is 4.0×10^{-8} s^{-1}. [d] $k_{obs}^{unassembled\ mixture}$ is 2.2×10^{-7} s^{-1}. [e] At pH = 8.5 and 40 °C. [f] $k_{obs}^{unassembled\ mixture}$ is 3.7×10^{-7} s^{-1}.

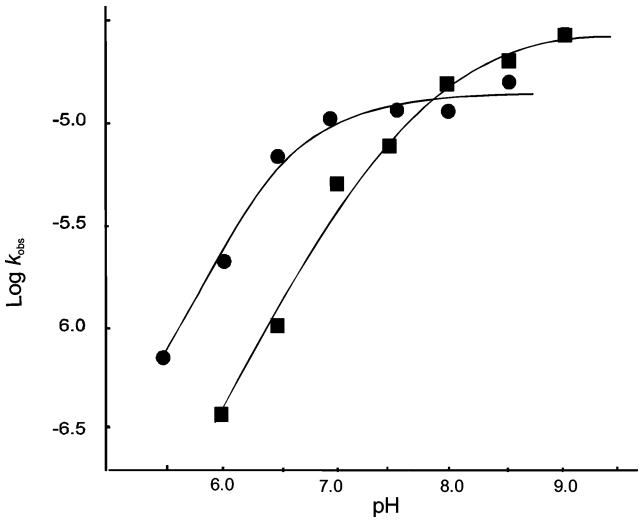

FIGURE 5. Effect of pH on the observed rate constants measured for the cleavage of HPNP by catalysts **5**−4Zn^{II} (filled squares) and **6**−4Zn^{II} (filled circles). Conditions: [catalyst] = 2×10^{-4} M; [HPNP] = 2×10^{-5} M.

of the catalytically active species, we performed a series of experiments at different pH for the two most active complexes, **5**−4Zn^{II} and **6**−4Zn^{II}. The results, reported in Figure 5, reveal that the kinetic pK_a for the **6**−4Zn^{II} complex is ca. 1.2 units lower than that of **5**−4Zn^{II} (6.8 and 8.0, respectively, for the two complexes).

Accordingly, the difference in activity measured at pH 7 simply reflects the different amount of active species present at that pH, and in fact, at pH 7.8 the two catalysts become equally active and, above this pH, **5**−4Zn^{II} is slightly better so that the intrinsic reactivity of this latter catalyst appears to be superior to the one based on the triazacyclododecane macrocycle. The apparent second-order rate constants for the fully deprotonated complexes **5**−4Zn^{II} and **6**−4Zn^{II} are 0.125 and 0.063 M^{-1} s^{-1}, respectively. This finding appears to be in full accord with what has been recently reported in the literature[24] where it has been shown that, in the case of HPNP, the dependence of the reactivity on the acidity of the nucleophile is rather pronounced (Brønsted $\beta_{nuc} = 0.75$) and the most active nucleophile is the least acidic. Interestingly, the kinetic pK_a's we find for the tripodal complexes are ca. 0.5 units lower than those reported[26] for the Zn^{II} complexes of the parent cyclic amines. This provides an indication that in the catalytic site of

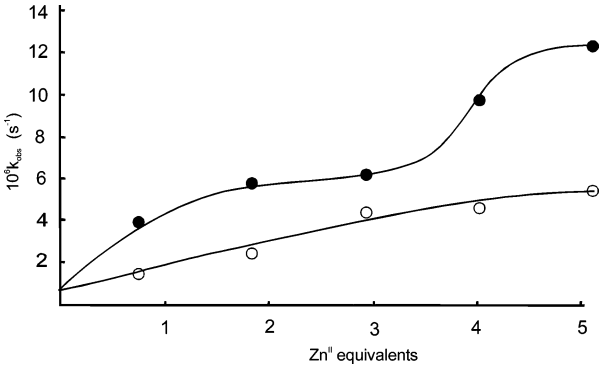

FIGURE 6. Kinetic effect on the observed rate constant for the cleavage of HPNP exerted by ligands **8** (filled circles) and **9** (open circles) upon addition of Zn^{II} ions. Conditions: [catalyst] = 2×10^{-4} M, [HPNP] = 2×10^{-5} M, 40 °C, pH = 7.

our complexes, likely because of the presence of three metal ions, the acidity of the effective nucleophile becomes slightly higher. Morrow and Richard[27] have reported a decrease of the pK_a of a Zn^{II}-bound water molecule of 1.4 on moving from a mononuclear to a dinuclear complex.

Cooperativity and Allosteric Control. In order to unambiguously prove the allosteric role of the Tren-bound Zn^{II} metal, we have synthesized tripodal ligands **8** and **9** and investigated their catalytic behavior in the presence of Zn^{II}. The only difference between **8** and **9** is the fact that, in the latter, the secondary amines of Tren are acetylated, thus preventing binding of a metal ion to this site. In fact, a spectrophotometric titration of ligand **8** with Cu^{II} shows the appearance of two new absorption bands at 650 and 850 nm corresponding to the complexes of Cu^{II} with the cyclic polyamines and Tren, respectively (Figure 2d) in accord with the behavior of ligands **5**−**7**. At variance with the previous ligands, the selectivity for Tren in binding is almost lost and both binding sites are more or less simultaneously occupied upon addition of Cu^{II}. On the other hand, the same titration with ligand **9** only gives an increase of the absorption band at 650 nm, indicating that acetylation of Tren indeed suppresses Cu^{II} binding at this site. Next, the kinetics were repeated using ligands **8** and **9** in the presence of increasing amounts of Zn^{II}. The results are given in Figure 6. For compound **8**, a curve was obtained that was very similar to those obtained for compounds **5** and **6**, with the characteristic jump in activity upon the addition of the fourth Zn^{II}, indicating a similar behavior in terms of cooperativity. Interestingly, compound **8** is significantly more reactive with respect to compound **5** having identical azacrowns. For the acetylated compound **9**, on the other hand, a low activity is observed, with a smooth increase of rate until the addition of 3 equiv of Zn^{II} after which the curve flattens. This clearly proves the allosteric role of the Zn^{II} ion in the Tren binding unit.

We can now dissect the different contributions of the metal complexes in the kinetic profiles of Figure 4a for ligands **5** and **6**. Since the first metal binds to the Tren platform, the first event is a conformational change of the ligand while the putative catalytic site remains metal free. Accordingly, very little rate acceleration is observed, likely due to the interaction of the substrate with the protonated cyclic amines[28] or with the Zn^{II}

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ion bound to Tren. The following metal ions start binding to the triazamacrocycle units but a significant rate acceleration is observed only when at least two Zn^{II} complexes are formed with these ligands. The complexation of the last metal ion is required in order to observe the maximum catalytic efficiency of the system. Thus, these experiments indicate that the first metal is a structural (allosteric) ion, while the remaining three are functional (catalytic) ions, which cooperate in the acceleration of the transesterification process.

Conclusions

While the plots of Figure 4a strongly support the cooperativity between the metal centers in the catalytic sites, the kinetic profiles of Figure 5 indicate that the role of the metal ions is that of stabilizing the complexed substrate toward the transition state where a further negative charge develops and in facilitating deprotonation of the nucleophilic species. We do not observe a bell-shaped profile for these plots indicating that the cooperativity is not due to the occurrence of general-acid/general-base catalysis;[29] neither there is a decreased binding of the substrate because of the deprotonation of a water molecule bound to the complex.[30] This might imply that the binding of the substrate to Zn^{II} does not require the displacement of this metal-bound hydroxide.

If we compare the efficiency of the peptide-based catalyst TP_3-4Zn^{II} with its simpler sibling **5**$-4Zn^{II}$, based on the same ligand subunits (see Table 1), we note that the polypeptide-based system is just 2-fold faster. This is not a particularly exciting result in view of the synthetic effort put into its synthesis. This different reactivity does not come from a difference in pK_a of the effective nucleophilic species (they are the same within the experimental error), but from a better binding of the substrate due to a slightly more hydrophobic environment present in the catalytic site of the peptide-based catalyst. This is, obviously, a reasonable speculation in the absence of experimental binding data. On the contrary, the effect of cooperativity is immediately evident if one compares the activity of the tripodal systems with that observed when three copies of the *N*-acetylamino amide **1** (after Boc-deprotection) and the Tren platform are mixed together: 25-fold with respect to **5**$-4Zn^{II}$ and ca. 50-fold with respect to the peptide-based catalyst. Allowing for the difference in pK_a of the nucleophile between the monomeric and tripodal, peptide-based catalyst the cooperative gain is of ca. 1 order of magnitude. Morrow and Richard have recently reported[27] a 12-fold gain in comparing the activity against HPNP transphosphorylation of a fully deprotonated mononuclear catalyst with that of a dinuclear catalyst.

In conclusion, we have shown in this paper that the conjugation of three arms of a Tren derivative with ligands units for Zn^{II} ions provides an entry to simple, cooperative catalysts where the Tren platform acts as a regulation site: only when a metal ion is bound to it cooperativity between the metal ions in the three arms in the cleavage of a RNA model substrate is observed.

Experimental Section

Synthesis. Synthetic procedures for **1**,[18] tris[4-(carboxy)phenylmethyl-2-(*tert*-butyloxycarbonylamino)ethyl]amine **10**,[12a] and the corresponding methyl ester derivative **13**[12a] have already been reported by us.

(*S*)-2-Benzyloxycarbonylamino-3-[1-(5,9-bis(*tert*-butyloxycarbonyl)-1,5,9-triazacyclododecane)] *N*-Methyl Propanamide, 2. To a solution of 1,5,9-triazacyclododecane (462 mg, 2.7 mmol) and Et_3N (1.10 mL, 7.86 mmol) in 30 mL of dry $CHCl_3$ was added di-*tert*-butyl dicarbonate (886 mg, 4.02 mmol) dissolved in 15 mL of dry $CHCl_3$ over a period of 4 h using a syringe pump. The solution was stirred for 24 h, the solvent removed under reduced pressure, and the crude product purified by column chromatography (SiO_2, CH_2Cl_2/AcOEt) to yield 498 mg of di-Boc-protected 1,5,9-triazacyclododecane. This material was dissolved in 8 mL of dry CH_3CN and added to a solution of the β-lactone of Z-(L)-serine (333 mg, 1.51 mmol) in CH_3CN. The solution was kept at 40 °C for 7 days under stirring under a N_2 atmosphere. After evaporation of the solvent, the crude product was dissolved in 20 mL of CH_2Cl_2, di-*tert*-butyl dicarbonate (255 mg, 1.31 mmol) was added, and the solution was stirred for 24 h. This procedure allowed the conversion of the excess of diprotected azacrown into the triprotected material that could be separated more easily from the product. Evaporation of the solvent under reduced pressure and purification of the crude product by flash chromatography (SiO_2, AcOEt/CH_3OH) gave 220 mg (28% yield) of the intermediate free acid of **2**: ^{1}H NMR ($CDCl_3$) δ (ppm): 1.46 (s, 18H), 1.7−2.1/2.8−3.6 (br, 20H), 4.20 (br, 1H), 5,06 (s, 2H), 6.13 (br, 1H), 7.31 (br, 5H); ^{13}C NMR ($CDCl_3$) δ (ppm): 171.1, 156.3, 136.4, 128.4, 128.0, 79.4, 67.7,66.3, 60.3, 49.7, 46.8, 43.8, 29.7, 28.4, 26.2, 21.0, 14.2, 13.8; ESI-MS (CH_3OH, *m*/*z*) calcd 592.7, found 593 $[M + H]^+$, 615 $[M + Na]^+$. The acid (0.48 mmol) was dissolved in a 5 mL CH_2Cl_2 solution, and at 0 °C under a N_2 atmosphere were added EDC·HCl (206 mg, 1.05 mmol) and, subsequently, HOAT (133 mg, 0.958 mmol). After 20 min, CH_3NH_2·HCl (100 mg, 1.48 mmol) was added, and the reaction mixture was made basic (pH ca. 10) with 900 μL of diisopropylethylamine (5.14 mmol). The reaction was then stirred at room temperature for 6 days. Next, the solvent was evaporated, and the residue was taken up in 25 mL of AcOEt, washed with $KHSO_4$ 10% (2 × 10 mL), $NaHCO_3$ 5% (2 × 10 mL), and H_2O (2 × 10 mL), and dried over Na_2SO_4. Evaporation of the solvent gave a crude that was purified by chromatography (SiO_2, light petroleum/AcOEt) yielding **2** (260 mg, 90% yield): ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm) 1.46 (s, 18H), 1.7−2.1/2.8−3.6 (br, 20H), 2.76 (d, 3H), 4.20 (br, 1H), 5.06 (s, 2H), 6.13 (br, 1H), 7.31 (br, 5H); ESI-MS (CH_3OH, *m*/*z*) calcd 605.8, found 606 $[M + H]^+$, 628 $[M + Na]^+$.

(S)-2-Benzyloxycarbonylamino-3-[1-(4,7,10-tris(*tert*-butyloxycarbonyl)-1,4,7,10-tetraazacyclododecane)] *N*-Methyl Propanamide, 3. The procedure followed for the preparation of this material was essentially the same as for the preparation of **2**. The intermediate acid was obtained in 25% yield: ^{1}H NMR ($CDCl_3$) δ (ppm) 1.35−1.48 (m br, 27 H), 2.67−3.70 (br, 18H), 4.25−4.65 (br, 1H), 5.05 (br, 2H), 7.27 (m, 5H); ESI-MS (CH_3OH, *m*/*z*) calcd 693.8, found 694 $[M + H]^+$, 716 $[M + Na]^+$. Coupling with CH_3NH_2 in analogous conditions as before gave **3** (119 mg, 35% yield): ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm) 1.39−1.45 (s br, 27H), 2.0−3.7 (br, 18H), 2.76 (d, 3H), 4.27 (br, 1H), 5.05 (AB, 2H), 6.32 (br, 1H), 6.69 (br, 1H), 7.32 (s br, 5H); ESI-MS (CH_3OH, *m*/*z*) calcd 706.9, found 707 $[M + H]^+$, 729 $[M + Na]^+$.

2-(1-(4,7-Bis(*tert*-butyloxycarbonyl)-1,4,7-triazacyclonane)-ethylamine, 4. To a solution of di-Boc-protected triazacyclononane (150 mg, 0,45 mmol) in CH_3CN (10 mL) were added *N*-(2-bromoethyl)phthalimide (115 mg, 0,45 mmol), diisopropylethylamine (250 μL), and a small amount of K_2CO_3. The solution was stirred at 60 °C and followed by TLC (SiO_2, $CHCl_3$/diethyl ether 9/1, UV/ninhydrin), R_f 0.0, diBoc-TACN; 0.3, product; 0.8, *N*-(2-bromoethyl)phthalimide. After completion of the reaction (7 days), the solvent was evaporated and the residue purified by column

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chromatography (SiO_2, $CHCl_3$). The intermediate (153 mg, 0.3 mmol) was dissolved in ethanol (5 mL), and hydrazine monohydrate (100 μL, 2 mmol) was added. The solution was stirred at room temperature, and after 3 h, a white precipitate formed. After 6 h, the solvent was evaporate under reduced pressure and the residue treated with CH_2Cl_2 (15 mL). The precipitate was filtered off and the solution dried on Na_2SO_4. After evaporation, compound **4** (67 mg, 60%) was obtained as a colorless oil: ^{1}H NMR ($CDCl_3$) δ (ppm) 1.47 (s, 18H), 2.63 (br m, 8H), 3.28 (br m, 4H), 3.40−3.48 (m, 4H); ESI-MS (CH_3OH, *m*/*z*) calcd 372.3, found 373.2 [M + H^+].

Tris{4-[(*S*)-2-yl-3-(1-(1,4,7-triazacyclononane))-*N*-methylpropanamide]carboxyphenylmethyl-2-aminoethyl}amine, 5. Compound **1**[16] was dissolved in 6 mL of CH_3OH to which, after purging with N_2, 35 mg of Pd/C 10% was added. H_2 was fluxed through the solution and the reaction flask saturated with H_2. After 18 h, the reaction mixture was filtered through a Celite pad. The Celite was washed several times with methanol and the solvent removed to give an oil, which was used as such for the coupling. Compound **10**[12a] (100 mg) was dissolved in 15 mL of CH_2Cl_2 and the solution cooled to 0 °C. Subsequently, HOBt (100 mg, 0.74 mmol) followed by EDC (120 mg, 0.62 mmol) were added, and the reaction mixture was stirred under a N_2 atmosphere. For the coupling, 210 mg of deprotected **1** was added to the above solution that was stirred at room temperature for 2 days with occasional addition of *N*-methylmorpholine to adjust the pH to ca. 9. The solvent was then removed under reduced pressure and the residue was taken up with AcOEt (25 mL) and extracted with 10% aqueous $KHSO_4$ (25 mL), H_2O (25 mL), 5% aqueous $NaHCO_3$ (25 mL), and H_2O (25 mL). After the organic solvent was dried over Na_2SO_4 and evaporated, 230 mg of crude material was obtained, which was purified by chromatography (SiO_2, CH_2Cl_2/AcOEt) to yield 65 mg (26%) of Boc-protected **5**: ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.35−1.45 (81 H), 2.54−3.72 (m, 54 H), 2.82 (d, 9H), 4.41 (br, 6H), 4.58 (m br), 7.59 (d br, 3H), 7.20−7.79 (AA"BB", 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 171.9, 171.1, 166.9, 156.3, 155.8, 132.8, 127.6, 126.8, 80.2, 80.0, 77.2, 62.0, 60.4, 55.6, 54.9, 52.7, 52.5, 51.4, 51.1, 50.4, 50.0, 49.3, 49.1, 45.6, 28.5, 28.4, 26.3, 21.0, 14.2; ESI-MS (CH_3OH, *m*/*z*) calcd 2083, found 1064 [M + 2Na]$^{2+}$. This compound was dissolved in 3 mL of a 33% solution of HBr/CH_3-COOH and stirred at room temperature for 1 h. Addition of 4 mL of ethyl ether precipitated the HBr salt of **5** (60 mg) that was filtered and dried: ^{1}H NMR (300 MHz, D_2O) δ (ppm) 2.69 (s, 9H), 2.85−3.58 (m, 54H), 4.26 (s, 6H,), 7.54−7.57−7.80−7.83 (AA"BB", 12H).

Tris{4-[(*S*)-2-yl-3-(1-(1,5,9-triazacyclododecane))-*N*-methylpropanamide]carboxyphenylmethyl-2-aminoethyl}amine, 6. A procedure similar to that described for the preparation of **5** was followed but using 67.1 mg (79.1 μmol) of **10**[12a] as starting material. The Boc-protected compound **6** was obtained with a yield of 50%: ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm) 1.36−1.44 (br, 81H), 1.7−1.9/2.3−3.5 (br, 81H), 4.40 (br, 9H), 7.16−7.97 (m br, 12H); ^{13}C NMR ($CDCl_3$) δ (ppm) 171.1, 156.3, 127.1, 80.0, 79.7, 79.6, 79.2, 63.7, 60.4, 46.0, 44.7, 44.0, 28.4, 21.0, 14.1.

The above material was dissolved in 3 mL of a 33% solution of HBr/CH_3COOH and stirred at room temperature for 1 h. Addition of 4 mL of ethyl ether gave a white precipitate of the HBr salt of **6** (92 mg) that was filtered and dried: ^{1}H NMR (250 MHz, D_2O) δ (ppm) 1.75−2.20/2.55−3.25 (m br, 72H), 2.62 (d, 9H), 4.20 (s, 9H), 7.48−7.79 (m br, 12H); ^{13}C NMR (D_2O) δ (ppm) 170.0, 156.3, 127.0, 124.9, 124.4, 47.2, 46.3, 45.3, 40.5, 38.4, 37.8, 37.4, 37.3, 35.3, 14.5; ESI-MS (CH_3OH, *m*/*z*) calcd 1308.8, found 655 [M + 2H]$^{2+}$, 437 [M + 3H]$^{3+}$, 677 [M + 2Na]$^{2+}$, 459 [M + 3Na]$^{3+}$.

Tris{4-[(*S*)-2-yl-3-(1-(1,4,7,10-tetraazacyclododecane))-*N*-methylpropanamide]carboxyphenyl methyl-2-aminoethyl}amine, 7. A procedure similar to that described for the preparation of **5** was followed but using 13.1 mg (15.4 μmol) of **10**[12a] as starting material. The Boc-protected compound **7** was obtained with a 27% yield: ^{1}H NMR (250 MHz, CDCl3) δ (ppm) 1.43 (s, 108H), 2.4−3.6 (br, 66H), 2.77 (d, 9H), 4.40 (br, 9H), 7.05−7.85 (m br, 12H). The above material was dissolved in 3 mL of a 33% solution of HBr/CH_3COOH and stirred at room temperature for 1 h. Addition of 4 mL of ethyl ether gave a white precipitate of the HBr salt of **7** (8 mg) that was filtered and dried: ^{1}H NMR (300 MHz, D_2O) δ (ppm) 2.67 (d, 9H), 2.75−3.25 (m br, 66H), 4.21 (m br, 6H), 4.55 (tr, 3H), 7.52−7.81 (m br, 12H); ^{13}C NMR (D_2O) δ (ppm) 173.1, 148.2, 130.5, 128.3, 50.8, 50.6, 48.4, 44.4, 42.1, 41.5, 26.0; ESI-MS (CH_3OH, *m*/*z*) calcd 1311.8, found 656 [M + 2H]$^{2+}$, 437 [M + 3H]$^{3+}$, 678 [M + 2Na]$^{2+}$, 459 [M + 3Na]$^{3+}$.

Tris{4-[2-(1-(1,4,7-triazacyclononane))ethanamide]-carboxyphenylmethyl-2- aminoethyl}amine, 8. A procedure similar to that described for the preparation of **4** was followed but using 20.0 mg (24 μmol) of **10**[12a] as starting material. The Boc-protected compound **8** was dissolved in CH_2Cl_2 (2 mL) and TFA (1 mL) was added. The mixture was stirred for 4 h after which the volatiles were removed under reduced pressure. Compound **8** (28.0 mg, 27%) was obtained as TFA salt: ^{1}H NMR (250 MHz, CD_3-OD) δ (ppm) 2.76 (br, 4H), 2.91 (br, 4H), 3.07 (br, 2H), 3.30 (br, 4H), 3.45 (br, 6H), 4.16 (s, 2H), 7.47 (d, 2H), 7.76 (m, 2H); ESI-MS (CH_3OH, *m*/*z*) calcd 1010.9; found 503.2 [M + 2H]$^{2+}$, 337.9 [M + 3H]$^{3+}$, 253.7 [M + 4H]$^{4+}$; RP-HPLC (Phenomenex Jupiter 4 μm, 90 Å, 230 nm) 26.5 min.

Tris[4-carboxymethylphenylamino(*N*-acetyl)ethyl]amine, 12. Compound **11**[12a] (700 mg, 0.78 mmol) was dissolved in 10 mL of HBr (33% in CH_3COOH) and 7 mL of acetic acid and stirred for 2 h at room temperature. Diethyl ether (40 mL) was added, and the formed precipitate was filtered off and washed with diethyl ether. The resulting BOC-deprotected compound **11** was dissolved in CH_2Cl_2 (15 mL) and DIPEA (2 mL) and cooled to 0 °C. Acetic anhydride (15 mL) was added, and the mixture was stirred at room temperature for 20 h. Next, the mixture was washed three times with an aqueous solution of NaOH (1 M, 50 mL), dried over Na_2-SO_4, and evaporated to dryness. Compound **12** was obtained as a reddish oil (320 mg, 57%): ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm) 2.10 (m, 3H), 2.54 (m, 2H), 2.33 (m, 2H), 3.92 (d, 3H), 4.56 (m, 2H), 7.25 (m, 2H), 7.98 (m, 2H); ESI-MS (CH_3OH, *m*/*z*) calcd 716.3, found 739.2 [M + Na]$^+$.

Tris{4-[2-(1-(1,4,7-triazacyclononane))ethanamide]-carboxyphenylmethyl-2-amino(*N*-acetyl)ethyl}amine, 9. Compound **12** (320 mg, 0.4 mmol) was dissolved in ethanol (5 mL), and a solution of LiOH·*x*H_2O (165 mg, 7 mmol) in H_2O (1 mL) was added. The mixture was stirred overnight, after which time solvent was removed via liophilization. The residue was taken in H_2O (10 mL), and 1 M HCl was added until the formation of a precipitate was observed. The precipitate was isolated via filtration and dried under over P_2O_5. Compound **13** (270 mg) was analyzed by ^{1}H NMR to verify the disappearance of the methyl ester and used directly for the coupling step: ^{1}H NMR (250 MHz, DMSO) δ (ppm) 2.07 (s, 3H), 3.37 (m br, 2H), 3.61 (m br, 2H), 3.92 (d, 3H), 4.60 (m, 2H), 7.35 (m, 2H), 7.96 (m, 2H). Compound **13** (17 mg, 2.5 μmol) was dissolved in anhydrous CH_2Cl_2 (4 mL) and DIPEA (100 μL) and cooled to 0 °C. Subsequently, HOBt (20 mg, 0.15 mmol) and EDC (27 mg, 0.14 mmol) were added, and the mixture was stirred for 45 min at 0 °C, after which time a solution of compound **4** (38 mg, 0.1 mmol) in anhydrous CH_2Cl_2 (2 mL) was added. The mixture was stirred at room temperature for 7 days, after which time the solvent was removed under reduced pressure. The residue was taken in EtOAc (5 mL), and the product was extracted in 10% $KHSO_4$ (6 mL). The aqueous phase was basified to pH 10 using NaOH (1 M) and extracted with $CHCl_3$ (4 × 15 mL). The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The crude product was purified using preparative TLC (Al_2O_3, $CHCl_3$/MeOH/aq NH_3 = 92.5/7/0.5), and 17 mg of a yellowish oil was obtained. This compound was dissolved in CH_2Cl_2 (2 mL), and TFA (1 mL) was added, after which the mixture was stirred for 4 h. The volatiles were removed under reduced pressure to give compound **9** (15 mg) as its TFA salt: ^{1}H NMR (250 MHz, CD_3OD) δ (ppm) 2.20 (s, 3H), 2.90 (br,

4H), 3.08 (m br, 4H), 3.30 (br, 4H), 3.50−3.65 (m br, 6H), 3.76 (br, 2H), 4.83 (s, 2H), 7.44 (d, 2H), 7.94 (m, 2H); ESI-MS (CH_3-OH, *m*/*z*) calcd 1136.8, found 569 $[M + 2H]^{2+}$, 380 $[M + 3H]^{3+}$; RP-HPLC (Phenomenex Jupiter 4 μm, 90 Å, 230 nm) 14.0 min.

Metal Ion Binding Experiments. Via Spectrophotometry. To a cuvette containing 200 μL of H_2O and 1000 μL of buffer solution MES (4-morpholineethanesulfonic acid) 0.2 M pH 6.3 were added 800 μL of a stock solution of the ligands (1.51 mM). The increase in absorbance at 845 and 605 nm at 25 °C were followed upon the stepwise addition of 1 μL of 50 mM $Cu(NO_3)_2$ in water up to 7 equiv of metal. The absorbance readings were corrected for the dilution upon titration.

Via ^{1}H NMR. Compound **6**·13HBr (44.7 mg, 18,7 μmol)) was dissolved in D_2O (5 mL). From this stock solution 2.5 mL was transferred into a 5 mL volumetric flask to which 6 equiv of $Zn(NO_3)_2$ was added. The pD of both solutions was adjusted to 7.0 using a solution of NaOD in D_2O, after which both volumes were adjusted to 5 mL. The resulting stock solutions **A** and **B** had a final concentration of 1.90 mM in compound **6**, and stock solution **B** had additionally a 11.4 mM concentration of Zn $Zn(NO_3)_2$. NMR samples were prepared by mixing solutions **A** and **B** in the following ratios (6:0; 5:1; 4:2; 3:3; 2:4; 1:5; 0:6) resulting in six NMR tubes with 0−6 equiv of Zn^{II}, respectively.

Kinetics. In a typical experiment, the ligand (120 μL, 1.51 mM in water) and $Zn(NO_3)_2$ (50 mM) were added to a cuvette containing 500 μL of a 0.2 M buffer solution (adjusted with NaOH to the desired pH, pH 6−6.5 MES; pH 7−7.5 HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pH 8−8.5 EPPS, 4-(2-hydroxyethyl)piperazine-4-(3-propansulfonic acid); pH 9÷9.5 CHES, 2-(cyclohexylamino)ethanesulfonic acid; pH 10 CAPS, 2-(cyclohexylamino)-1-propanesulfonic acid), 80 μL of methanol (spectrophotometric grade), and the proper volume of water to reach a final volume of 880 μL and then thermostated at 40 °C. After a couple of minutes of equilibration time, HPNP (3.6 μL, 5 mM in water) was injected, and the increase in UV absorbance at $\lambda = 400$ nm due to the release of *p*-nitrophenolate (or 317 nm at low pH) was recorded. All of the solutions remained clear during the kinetic measurements (at least 4 half-lives). The pseudo-first-order rate constants k_{obs} (s^{-1}) were calculated by fitting of the data by standard methods.

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Supporting Information Available: ^{1}H NMR spectra of compounds **2**−**9** and **12** and materials and methods used in the syntheses. This material is available free of charge via the Internet at http://pubs.acs.org.

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