Dinuclear Metal Complexes Based on *all-cis*-2,4,6-Triaminocyclohexane-1,3,5-triol as Catalysts for Cleavage of Phosphate Esters

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Two dimetallic ligands **2** and **3** for transition metal ions were obtained by connecting two *all-cis*-2,4,6-triamino-cyclohexane-1,3,5-triol (TACI, **1**) subunits via 1,3- or 1,4-xylyl linkers. Their dimetallic Cu^{II} and Zn^{II} complexes were investigated as catalysts for the cleavage of the phosphate diesters 2,4-dinitrophenyl ethyl phosphate (DNPEP) and 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP) and the triester 2,4-dinitrophenyl diethyl phosphate (DNPDEP). The results of a comparative kinetic study using the monometallic complexes of TACI as a reference indicate that the Cu^{II} complexes of **2** and **3** are virtually inert; this finding is ascribed to the formation of intra-complex μ-hydroxo bridges that prevent the required interactions with the substrate. On the other hand, the dimetallic Zn^{II} complexes produce remarkable accelerations, particularly in the case of the HPNP transesterification. The dimetallic systems are more efficient than the TACI·Zn^{II} complex in promoting the hydrolysis of HPNP at pH values close to neutrality (7.0–7.8). In this case, the effects of cooperativity between the two metal centers were highlighted in a detailed kinetic study; the catalytic efficiency seems to be related to the stronger binding of the substrate to the dimetallic Zn^{II} complexes. Additionally, in this case, although to a much lesser extent than in the case of the Cu^{II} counterparts, the formation of μ-hydroxo bridges apparently hampers the catalytic efficiency, as indicated also by the observation that the activity of the dimetallic complexes increases as the distance between the two metal centers increases, which, thus, thwarts the formation of intermetallic bridges.

Introduction

Enzymes that catalyze the hydrolytic or nucleophilic cleavage of phosphate esters and nucleic acids are normally activated by more than one metal ion.[1,2] The search for simple, yet effective, synthetic catalysts is being addressed by the study of systems that somehow mimic natural metalloenzymes and may exploit the additivity and, possibly, the synergistic effects of two or more metal ions bound into the structure.[3,4,5] The large interest in the realization of such biomimetic systems is stimulated by their potential applications, such as the development of artificial restriction enzymes for molecular biology and biotechnology and of antiviral and antitumor drugs.[6] Extensive mechanistic studies of the metal ion-promoted hydrolysis of phosphates are now available[3,7−10] so that the design of effective binuclear metal ion complexes has apparently been made easier.[3] As pointed out by Chin, however, when considering the efficiency (up to 10^{17}-fold accelerations) of metalloenzymes toward the cleavage of the phosphate ester bonds of DNA, the development of synthetic catalysts that may compete with their natural counterparts is a formidable problem.[3] As a matter of fact, the reactivities of the synthetic models that have been reported, as either mono-, di- or tri-nuclear complexes, are far from the target of approaching those of enzymes.

In such artificial enzymes, very large catalytic effects could be achieved, at least in principle, if the direct modes of activation that metal ions can provide for hydrolyzing phosphate esters (at least in the case of diesters) are simply combined even without relying on cooperative effects. Such "direct" activation effects are essentially (i) Lewis acid activation, (ii) intramolecular nucleophile activation, and (iii) leaving group activation.[3] Even if identifying and evaluating these catalytic roles provides encouraging perspectives, combining the estimated benefits that result from them still faces quite a number of problems that make the design of catalysts very difficult and quite challenging. For instance, a few variables that play crucial roles, and that must be solved mostly in the absence of established guidelines, are the nature of the metal ion involved, the distance between the metal ions, the degree of conformational freedom allowed by the linker of the two ligand subunits, the nature of the ligand with regard to the coordinating atoms, and the coordination geometry. Moreover, solubility in aqueous solutions is another property that adds to the above prob-

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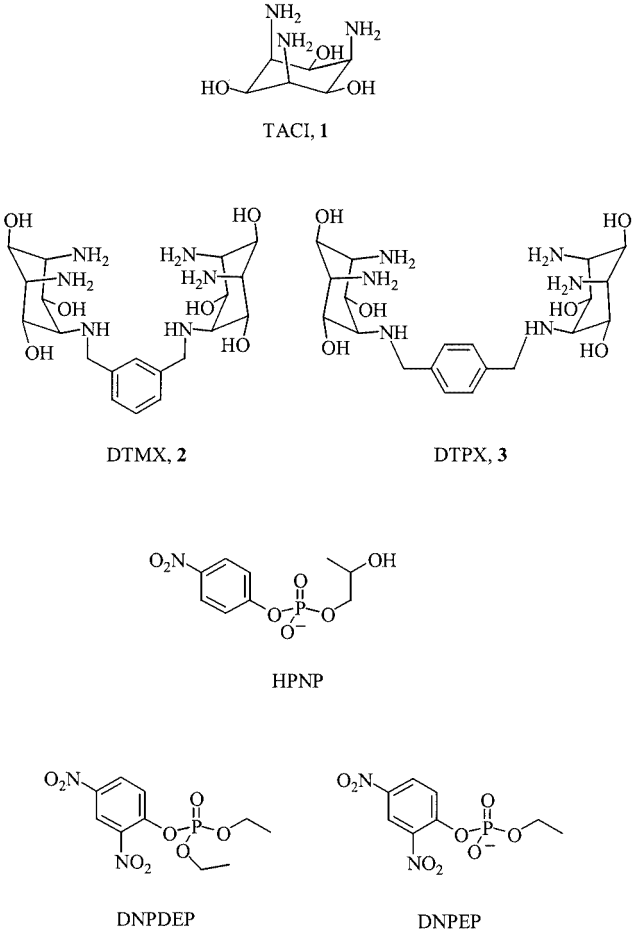
lems if the systems are intended for biological applications.[11]

Recently, we reported the results of a study, on the hydrolytic reactivity of Zn^{II} complexes of polyamino structures toward phosphodiesters, that highlighted, among several interesting mechanistic features, the role played by the ligand in modulating the catalytic efficiency of the complexes.[10] Among the several polyamino ligands investigated, we found that *all-cis*-2,4,6-triamino-cyclohexane-1,3,5-triol (TACI, **1**), is a suitable structure for preparing reactive Zn^{II} complexes. As a matter of fact, complexes of TACI are not only good catalysts towards model phosphate triesters and diesters, but, more interestingly, toward supercoiled DNA: TACI·Cu^{II}, in particular, ranks among the most effective of the simple DNA-cleaving agents.[12] Furthermore, a dicopper complex of a hexamethyl derivative of TACI is quite effective in promoting phosphodiester cleavage.[13] These findings stimulated studies now in progress on the reactivity of derivatives of TACI and related ligands that are aimed at the realization of binuclear catalysts.

Here, we report the synthesis of two ditopic ligand structures, DTMX **2** and DTPX **3** (Scheme 1), that bear two TACI subunits held together at a convenient distance by 1,3- or 1,4-xylyl linkers, and a study of the reactivity of their Cu^{II} and Zn^{II} complexes towards two phosphodiesters, 2,4-dinitrophenyl ethyl phosphate (DNPEP) and 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP), and the triester 2,4-dinitrophenyl diethyl phosphate (DNPDEP).



Scheme 1. Structures of ligands and substrates used in this work

Results and Discussion

Design of the Ditopic Ligands 2 and 3

Our target catalytic species was a binuclear complex that could operate in neutral aqueous solutions as an artificial phosphatase and could somehow mimic its natural counterparts.

As anticipated, the choice of TACI as the ligand subunit, notwithstanding the synthetic problems concerning modifications of its basic structure, whose chemistry is rather unpredictable, was mainly motivated by the known reactivities of its complexes and by the hydrophilic properties of its three hydroxy groups that could contribute to the overall solubility of the species in water. Moreover, the same hydroxy groups could play a relevant role in increasing the reactivity of the complexes toward DNA.

The linkers were selected among those that have been used successfully to realize other binuclear complexes.[14−17] We employed preliminary force field calculations (MM2)[18] to choose the most suitable structures; we examined the structures of the ternary complexes between dimetallic Zn^{II} complexes having different spacers and dimethylphosphate (dmp), which was chosen as a model of the phosphodiesters investigated. Structures featuring *p*-xylyl and *m*-xylyl spacers turned out to have the proper geometries to act as predicted or, at least, they do not hint at any adverse features, and are shown in Figure 1.

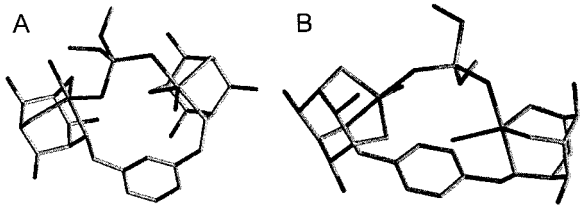


Figure 1. Computed structures of (A) $[Zn_2(DTMX)(dmp)(H_2O)_2]^{2+}$ and (B) $[Zn_2(DTPX)(dmp)(H_2O)_2]^{2+}$. Hydrogen atoms omitted for clarity

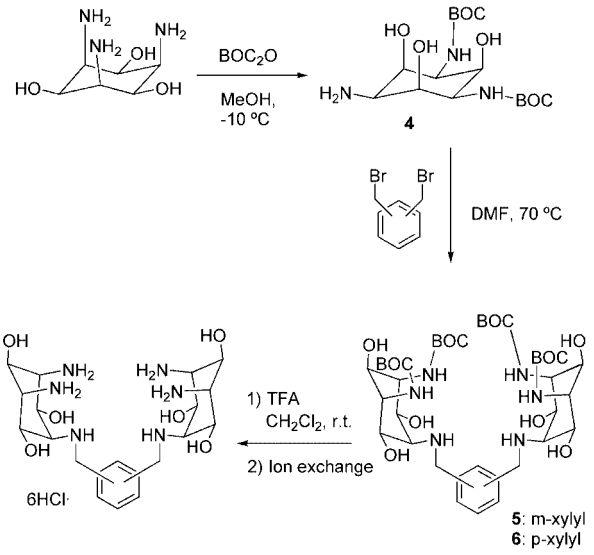
The intermetallic distances calculated for the two complexes are 5.0 Å in the case of DTMX and 5.2 Å in DTPX; such distances are within the range of those reported for metalloenzymes. The same kind of calculations for TACI-based dimetallic complexes having different, but apparently suitable, spacers, such as 1,8- or 2,7-dimethylnaphthalene, led to strained structures and greater inter-metal distances. These results directed our choice to the two *meta*- and *para*-xylyl linkers, which was also guided in view of their decreased hydrophobic character.

As for the substrates, HPNP, which bears a 2-hydroxypropyl residue, is the activated diester usually taken as an

RNA model and it may be compared with the diester DNPEP, a DNA model, and with the triester DNPDEP.

Synthesis and Characterization of DTMX and DTPX

Handling the TACI molecule to realize its desired *N*-monoalkylation is a difficult task, as is predictable in view of its multifunctionality.[19] Furthermore, the number of accessible synthetic pathways is greatly reduced because the solubility of TACI is limited to water and methanol. Early attempts aimed at achieving the BOC protection of two of the three amino groups failed to give reasonable yields of the desired intermediate since the reaction of TACI with even less than one equivalent of $(BOC)_2O$ yielded, in each case, only the triprotected product. Then, was uncovered a successful route (Scheme 2) by the synthesis of the di-*N*-BOC-protected precursor **4**. This compound was obtained in reasonable yield only by an extremely slow (1 day) addition of $(BOC)_2O$ to a very dilute (1−2 mM) solution of TACI in methanol at low temperature, followed by column chromatography. Compound **4**, once purified, was treated with the requisite *meta*- or *para*-α,α′-dibromoxylene to give **5** and **6** and these compounds were deprotected to give the final product(s) (as hexahydrochloride salts) in fair yields.



Scheme 2. Synthetic pathway to DTMX and DTPX

Both ditopic ligands are soluble in water at up to 2 mM at room temperature. They can complex two Cu^{II} or Zn^{II} metal ions. In the case of the copper complex, this binding is clearly indicated by changes in the absorbance at 647 nm (the region of the d−d transition of the ion), as shown in Figure 2 for DTMX and DTPX following the addition of $Cu(NO_3)_2$ at pH 6.3. The titration profiles of both ligands display a linear increase up to a ratio $[Cu^{II}]$/[ligand] of 2:1, which indicates rather large formation constants for the mono- and di-nuclear complexes.

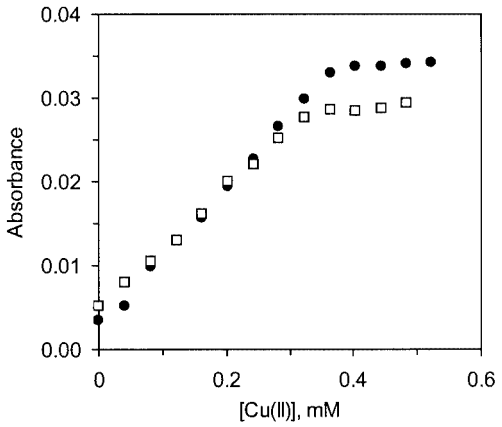


Figure 2. UV/Vis titration of DTMX (squares, 0.18 mM) and DTPX (black circles, 0.19 mM) with $Cu(NO_3)_2$ at 647 nm. (MES buffer 0.05 M, pH 6.3, 25 °C)

In the case of the Zn^{II} complex, the binding of the metal ion was investigated by potentiometric titrations, which allowed us to evaluate the values of pK_a of the amino groups (pK_n), the formation constant of the Zn^{II} complexes (log β_n), and the values of pK_a of the acid dissociation of the water molecule(s) bound to the metal ion(s) (pK_a^n) (Table 1). The values of pK_a of the ammonium ions are reasonably similar to those of TACI when taking into account the electrostatic effects at play in polyacidic compounds. These effects are likely responsible for the lower complex-formation constants, particularly the second one, for DTMX. The relatively small binding constant of the second Zn^{II} ion in this dinuclear complex was a matter of concern; under the conditions used for the reactivity experiments (see below), it can be evaluated that at values of pH larger than 8−9, ca. 80% of the ligand is in the form of dimetallic complexes.

Kinetic Studies

We carried out our first preliminary kinetic measurements in water at pH 9 and 25 °C, employing DTMX and DTPX and either Cu^{II} or Zn^{II} in a 1:2 molar ratio, for the cleavage of the three substrates of choice, namely DNPEP, DNPDEP, and HPNP. Table 2 shows the apparent pseudo-first-order rate constants (k_ψ) together with those measured for TACI under the same conditions.

The main findings are the following. The effects of the metal ion complexes depend strongly on the structure of the complex and, in several cases, the efficiency of the dimetallic ones turns out to be undeniably disappointing when the control complexes of TACI are taken as a reference. In fact, the dinuclear Cu^{II} complexes are remarkably less effective than the mononuclear TACI analog, particularly in the transesterification of HPNP (by two orders of magnitude) and in that of DNPEP for which a measurable reactivity (after reasonable observation times) was recorded only for Cu^{II}·TACI. On the other hand, when using the Zn^{II} complexes, the effects of all the complexes used (including that of TACI) are comparable and larger that those of the

Table 1. Ligand deprotonation constants (K_n), stepwise Zn^{II} complex-formation constants (β_n), and deprotonation constants of Zn^{II}-bound H_2O [K_a^n (H_2O)] for TACI, DTMX, and DTPX at 25 °C, in 0.1 M NaCl (estimated errors in the values of these thermodynamic constants are ±5%)

Ligand[a][b]	pK_1	pK_2	pK_3	pK_4	pK_5	pK_6	log β_1	log β_2	pK_a^1 (H_2O)	pK_a^2 (H_2O)
TACI[c]	8.86	7.40	6.02				8.39		8.56	
DTMX	8.98	8.39	7.33	6.73	5.67	4.99	7.75	4.71	7.66	8.44
DTPX	9.30	8.87	7.65	6.95	5.65	5.26	8.05	7.21	8.68	—[d]

[a] $K_n = [H_{n-1}L][H_3O^+]/[H_nL]$. [b] $\beta_n = [Zn_nL]/[Zn^{II}][Zn_{n-1}L]$. [c] Ref.[10]. [d] Not accessible.

Table 2. Rates for the transesterification of HPNP and cleavage of DNPEP and DNPDEP in the presence of Zn^{II} and Cu^{II} complexes of DTMX, DTPX, and TACI at 25 °C and pH 9 (CHES buffer, 0.05 M). The estimated errors in the vales of the kinetic constants are ±1%

Ligand	Substrate	k_ψ (Cu^{II}), s^{-1}	k_ψ (Zn^{II}), s^{-1}
DTMX[a]	HPNP	5.0×10^{-6}	4.2×10^{-5}
DTPX[b]	HPNP	6.1×10^{-6}	7.8×10^{-5}
TACI[c]	HPNP	1.7×10^{-4}	1.8×10^{-4}
DTMX[a]	DNPDEP	4.9×10^{-5}	8.9×10^{-5}
DTPX[b]	DNPDEP	5.2×10^{-5}	1.4×10^{-4}
TACI[c]	DNPDEP	6.0×10^{-4}	2.1×10^{-4}
DTMX[a]	DNPEP	—[d]	—[d]
DTPX[b]	DNPEP	—[d]	—[d]
TACI[c]	DNPEP	4×10^{-5}	—[d]

[a] [DTMX] = 0.44 mM, [Metal] = 0.88 mM. [b] [DTPX] = 0.47 mM, [Metal] = 0.94 mM. [c] [TACI] = 0.98 mM, [Metal] = 0.98 mM. [d] No reaction observed.

Cu^{II} analogs, at least in the cases of DNPDEP and HPNP.

The much greater efficiencies of the dinuclear Zn^{II} complexes relative to those of the Cu^{II} complexes, which is a finding that is at variance with those commonly reported for mononuclear complexes, is not unprecedented. The main reason for this finding is quite likely to be ascribed to the easier formation of μ-hydroxo bridges between two copper ions than between two zinc ions. Such bridged species are known to be poorly active also in monometallic systems,[20,21] as in the case of Cu^{II}·TACI complexes, which have a greater tendency to form dimeric forms than do complexes of other tripodal ligands.[22,23] In the dimetallic complex, the two Cu^{II} ions may face each other readily so that the formation of intramolecular bridges (Figure 3) is further favored and, hence, their disruption, which is a prerequisite for the productive binding of a phosphate ester, is more difficult. The combination of the formation of bridges and the limited affinity of phosphate esters to the metal ion leads to the poor reactivity displayed by the Cu^{II} complexes of DTMX and DTPX.

The Zn^{II} Complexes as Catalysts of HPNP Transesterification

The preliminary results led us to restrict our further kinetic studies to the dinuclear Zn^{II} complexes as catalysts of HPNP transesterification. The attention devoted to this substrate not only was due to the fact that it can be taken as an RNA model but also because we have recently investigated in detail the mechanism of its transesterification promoted by Zn^{II}·TACI.[10]

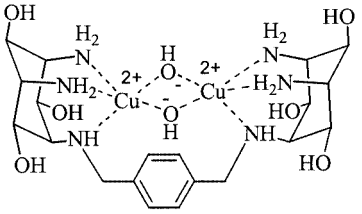


Figure 3. Proposed structure of a μ-hydroxo-bridged dimetallic complex

First we analyzed the effect of pH on the reaction rate using the same concentrations and ratios employed in the preliminary experiments. Figure 4, A, displays the rate−pH profiles obtained with the two dimetallic Zn^{II} complexes.

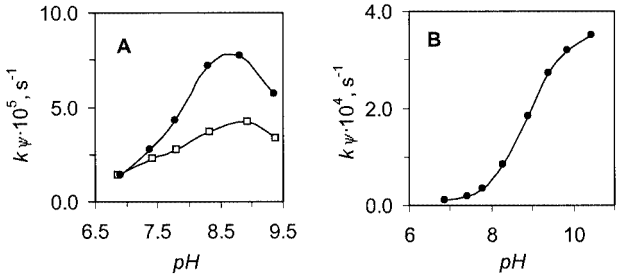


Figure 4. (a) The pH dependence of the rate constant for HPNP transesterification promoted by the Zn^{II}_2·DTMX (squares) and Zn^{II}_2·DTPX complexes (black circles). Conditions: [ligand] = 5.0×10^{-4} M, [Zn^{II}] = 1.0×10^{-3} M, [Buffer] = 0.05 M (see Exp. Sect.), 25 °C. (b) The pH dependence of the rate constant for HPNP transesterification promoted by the Zn^{II}·TACI complex. Conditions: [ligand] = 1.0×10^{-3} M, [Zn^{II}] = 1.0×10^{-3} M, [Buffer] = 0.05 M, 25 °C

In both cases, we obtained bell-shaped curves having maxima at ca. pH 8.7−9. The two complexes exhibit slightly different behavior and the DTPX system is more reactive by a factor of ca. 2 than is DTMX in the pH range 8−9.5. This behavior is diagnostic of the deprotonation of two acidic functions of the complex: the first deprotonation event leading to a reactivity increase and the second to the opposite effect. At variance with the rate profile of the dimetallic complexes, that of Zn^{II}·TACI describes a clean sigmoidal curve (Figure 4, B) that is diagnostic of a single favorable deprotonation. A comparison of the data at differ-

ent values of pH indicates that the dinuclear complexes are slightly more efficient (1.5-fold) than the mononuclear one under neutral conditions (pH 7−7.8), but the opposite is true at higher values of pH. More interestingly, the rate−pH profiles obtained for the monometallic TACI complex and for the dimetallic catalysts have quite different shapes, which is diagnostic of different mechanistic patterns, as is expected for the dimetallic systems assuming cooperativity of the two reactive sites.

In the concentration range 0.1−0.6 mM, the profiles of reaction rate versus complex concentration at pH 9 were linear, which indicates that no intramolecular reaction occurs. The apparent second-order rate constants determined for these profiles are 0.19, 0.12, and 0.060 M^{-1} sec^{-1} for Zn^{II}·TACI, $(Zn)_2$·DTPX, and $(Zn)_2$·DTMX, respectively.

To evaluate the possible benefits or disadvantages of such cooperativity in terms of reactivity, we carried out kinetic experiments using a fixed concentration of ditopic ligand and increasing amounts of the metal ion (pH 8.0, 25 °C). The results are shown in the diagram (Figure 5) of the rate vs. the $[Zn^{II}]/[DTPX]$ ratio.

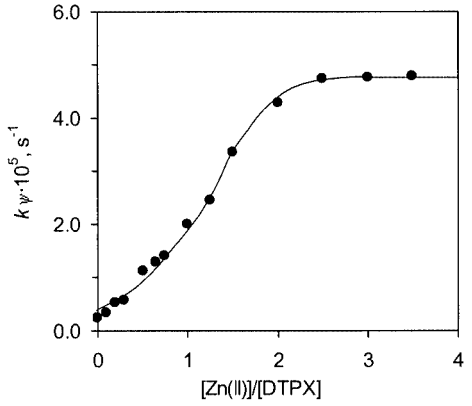


Figure 5. Kinetic profile for the transesterification of HPNP as a function of the Zn^{II}/DTPX ratio, in HEPES buffer (0.05 M) at pH 8. [DTPX] = 5.0×10^{-4} M, 25 °C

The rate profile describes a smooth sigmoidal curve that reaches a plateau for ratios between 2 and 2.5. The reaction rate observed after the introduction of the second metal ion is ca. 2.3-times larger than that observed in the presence of one equivalent of Zn^{II}, which indicates that the effect of the two complexed subunits is more than additive and is diagnostic of synergistic effects. A different profile (not shown) was observed with the ligand DTMX; in this case, the kinetic data describe an almost linear curve up to 2 equiv. of added metal ion that then proceeds smoothly to saturation such that a plateau of reactivity is not reached even after addition of 3 equiv. This behavior can probably be ascribed to the low formation constant of the dimetallic complex of DMTX (see Table 1), such that the transition from the mono- to dinuclear complex is rather difficult and may mask the synergistic effects at play in the case of DPTX.

Finally, we performed kinetic experiments using HPNP concentrations that largely exceed those of the Zn^{II}·TACI, Zn^{II}_2·DTMX, and Zn^{II}_2·DTPX complexes. Under these conditions ([complex] = 0.05 mM; [substrate] ranging from 0.2 to 3 mM; pH 9), complete cleavage of the substrate was achieved and we observed no detectable rate effects that are due to product formation. Thus, the complexes employed act as real catalysts (i.e., with turnover). The profiles of rate versus substrate concentration possess a moderate curvature as a result of the modest degree of binding of the substrate to the catalyst; at any rate, the analysis of data allowed us to estimate (albeit with a rather large error) the binding constant of the substrate to the catalyst, K_b, and the limiting rate constant, k_{lim}, which are reported in Table 3.[24]

Table 3. Kinetic and thermodynamic parameters for the transesterification of HPNP promoted by the Zn^{II} complexes of TACI, DTMX, and DTPX. Estimated errors are within 10%

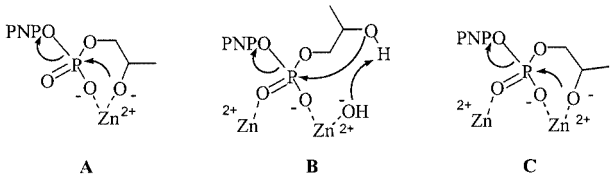
Complex[a]	K_b (M^{-1})	k_{lim} (s^{-1})
TACI·Zn^{II} [b]	33	9.7×10^{-3}
DTMX·Zn^{II}_2 [c]	107	1.5×10^{-3}
DTPX·Zn^{II}_2 [c]	67	2.6×10^{-3}

[a] Conditions: CHES buffer 0.05 M, pH 9.0, 25 °C. [b] [ligand] = 5.0×10^{-4} M, $[Zn^{II}] = 5.0 \times 10^{-4}$ M. [c] [ligand] = 2.5×10^{-4} M, $[Zn^{II}] = 5.0 \times 10^{-4}$ M.

It appears that, on one hand, the intrinsic reactivity of Zn^{II}·TACI is larger than that of the dimetallic complexes and, on the other, that they have a larger affinity (the absolute values are in each case rather small) for the substrate; on the whole, the balance of these parameters still makes the TACI system the most effective catalyst.

The cleavage of HPNP promoted by Zn^{II}·TACI occurs by a transesterification process that results in the formation of a cyclic phosphate ester and *p*-nitrophenol. In this case, the suggested mechanism involves direct coordination to the metal ion of the substrate alcoholic function and its subsequent nucleophilic attack on phosphorus leading to the cyclic phosphate (Scheme 3, **A**).[10] There are no reasons to assume that the outcome of the reaction catalyzed by the dimetallic Zn^{II} complexes investigated here are different. The mechanistic details, however, are substantially different insofar as the reported results point to the fact that the two reacting sites somehow cooperate, as is indicated clearly by the pH−rate profiles, vis-à-vis that of TACI in Figure 4 and by the kinetic titration diagram of Figure 5. Bell-shaped pH profiles are often observed in the cleavage of phosphodiesters by dimetallic complexes.[15,16] In principle, the cooperative effects of the two Zn^{II} ions and the increased affinity of the dimetallic complexes for the substrate may be explained by two main reasonable, and kinetically indistinguishable, mechanistic hypotheses (Scheme 3, **B**−**C**): case **B** indicates that the bound substrate is activated by the two metal ions for a nucleophilic attack and that a Zn^{II}-coordinated water molecule, in turn, acts as a base to deprotonate the substrate's hydroxy group that subsequently acts as the nucleophile; case **C** involves substrate activation again by the two metal ions and concomitant coordination

to one of them to the substrate's hydroxy group, which is, thus, activated as the nucleophile. The bell-shaped profile can be explained by assuming that each metal ion weakly binds a water molecule and the substrate in a competitive way up to the pH value at which deprotonation of the water molecule occurs to give a stronger base, but also a stronger ligand. Thus, there are two opposite effects at play in the bell-like region: (i) acid dissociation of a metal-bound water molecule (or of the substrate hydroxy group) to give a better base or nucleophile that still does not impair substrate binding; (ii) acid dissociation of the second water molecule to generate a bound hydroxide ion that weakens the association of the substrate. In the region of low pH of the "bell," the balance of these effects is favorable to an enhanced reactivity while the opposite effect occurs in more alkaline solutions. The mechanistic hypotheses above offer a rationale for the bell-shaped behavior, but not for the rather modest reactivity, at least when compared to that of Zn^{II}·TACI. This complex is slightly less reactive than the dinuclear complexes only below pH 7.5, but is more reactive by a factor of ca. 2 at pH 8.7, at the top of the bell-like profile, and is remarkably more reactive at higher values of pH. Clearly, other factors at play defy a simple explanation. One of them, supported by the behavior of the Cu^{II} complexes, could imply the formation, also in the case of Zn^{II}, of μ-hydroxo bridges that have a severe adverse effect on the reactivity. A similar effect has been suggested for a related di-Zn^{II} complex that features a *m*-xylyl spacer.[25] In addition, the greater reactivity of the DTPX complex, relative to that of DTMX, supports this hypothesis; in fact, inspection of molecular models indicates that the increased intermetallic distance in the "*para*" complex makes the formation the μ-hydroxo bridges more difficult.



Scheme 3. Proposed mechanisms for the transesterification of HPNP promoted by the Zn^{II} complexes

Conclusion

The results of this present investigation, on the use of dimetallic complexes based on TACI ligand subunits as catalysts for the cleavage of phosphate esters in water, offer a mixed picture. The Zn^{II} complexes rank among the good artificial Zn^{II}-based catalysts for the transesterification of HPNP;[5,11,15,26–28] the kinetic enhancements over the rate of spontaneous hydrolysis recorded at pH 9 are in the range of two order of magnitude and compare well to those reported for other binuclear catalytic complexes. Moreover, they indicate cooperativity of the two reactive sites. On the other hand, the results obtained also highlight that the difficulties implicit in the design of the dimetallic hydrolytic catalysts cannot be underestimated. As a matter of fact, comparison of the efficacy of the Zn^{II} complexes of TACI and of DTMX or DTPX, and the inefficacy of the Cu^{II} complexes, could simply suggest that the synthetic effort expended to obtain the ditopic ligands did not pay off, at least it has not so far. Much work is needed to understand all the factors at play to design and realize a really potent catalyst. Placing two metal ions at a short distance in not a sufficient requirement for obtaining great rate accelerations. Factors such as the formation of intracomplex μ-hydroxo bridges must be taken in the account as they can lead to significant reactivity losses. Unexpected, modest accelerations obtained by other dimetallic catalyst, at least in aqueous solutions, are likely to meet similar problems.[26,28]

Experimental Section

General Remarks: Solvents were purified by standard methods. All commercially available reagents and substrates were used as received. TLC analyses were performed using Merck 60 F_{254} precoated silica gel glass plates. Column chromatography was carried out on Macherey–Nagel silica gel 60 (70–230 mesh). Melting points were determined with a Buchi 510 capillary melting point apparatus and are uncorrected. NMR spectra were recorded using a Bruker AC250F (250 MHz). Chemical shifts are reported relative to internal Me_4Si. Multiplicity is given as usual. Elemental analyses were performed by the Laboratorio di Microanalisi of the Inorganic and Analytic Chemistry Department of the University of Padova. UV/Vis absorption measurements were performed with a Perkin–Elmer Lambda 16 spectrophotometer equipped with a thermostatted cell holder. Potentiometric titrations were performed using a Metrohom 716 DMS Titrino dynamic titrator. $Cu(NO_3)_2$ and $Zn(NO_3)_2$ were analytical grade products. Metal ion stock solutions were titrated against EDTA following standard procedures. The buffer components were used as supplied by the manufacturers: acetic acid (Aldrich), 2-morpholinoethanesulfonic acid (MES, Fluka), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma), 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPES, Sigma), 2-[cyclohexylamino]ethanesulfonic acid (CHES, Sigma), and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS, Sigma). The 2,4-dinitrophenyl ethyl phosphate (DNPEP) lithium salt,[29] the 2,4-dinitrophenyl diethyl phosphate (DNPDEP),[29] and the 2-hydroxypropyl *p*-nitrophenyl phosphate barium salt (HPNP)[30] were prepared and purified following literature methods. *all-cis*-2,4,6-Triamino-cyclohexane-1,3,5-triol (TACI, **1**) was prepared as reported.[31] DTMX and DTPX were prepared starting from TACI according to Scheme 2.

***all-cis*-2-Amino-4,6-bis(*tert*-butoxycarbonylamino)cyclohexane-1,3,5-triol (4):** A solution of $(BOC)_2O$ (1.16 g, 5.32 mmol) in dry CH_2Cl_2 (950 mL) was added dropwise (ca. 60 mL/min) to a solution of TACI (0.42 g, 2.4 mmol) in dry methanol (950 mL) that was cooled in a ice/brine bath. At the end of the addition, the reaction mixture was stirred at room temperature for 12 h with monitoring by TLC [CH_2Cl_2/MeOH/NH_3(aq), 10:2:0.2; R_f (**4**) = 0.36]. The solvent was evaporated under reduced pressure and purification of the crude product was performed by flash column chromatography [SiO_2; CH_2Cl_2 /MeOH/NH_3(aq), 10:1:0.1] to yield **4** as a white solid (0.36 g, 41%). ^1H NMR (250 MHz, CD_3OD, 25 °C, TMS): δ = 1.46 (s, 18 H, *t*Bu), 2.78 (s, 1 H, $-CH-NH_2$), 3.60 (s, 2 H,

−C*H*−NH−BOC), 3.84−3.97 (m, 3 H, −C*H*−OH) ppm. ^{13}C NMR (63 MHz, CD_3OD, 25 °C, TMS): δ = 27.7, 52.6, 52.9, 71.8, 73.6, 79.6, 156.3 ppm.

***all-cis*-4,4′,6,6′-Tetrakis(*tert*-butoxycarbonylamino)-6,6′-*m*-xylylidenebis(cyclohexane)-1,3,5-triol (5):** α,α′-Dibromo-*p*-xylene (0.103 g, 0.390 mmol) was added to a solution of **4** (0.303 g, 0.804 mmol) in dry DMF (4 mL) in the presence of diisopropylethylamine (0.50 mL, 2.9 mmol). The reaction mixture was heated at 70 °C for 48 h with monitoring by TLC [$CHCl_3$/MeOH/NH_3(aq), 10:2:0.2; R_f (**5**) = 0.51]. After this time, CH_2Cl_2 (150 mL) was added and the organic layer was extracted with 10% Na_2CO_3 solution (3 × 50 mL) and brine (3 × 50 mL). The organic phase was dried (Na_2SO_4) and the solvent was evaporated. Purification of the crude product was performed by flash column chromatography [SiO_2; $CHCl_3$/MeOH/NH_3(aq), 10:1.5:0.15] to obtain **5** as a pale-yellow solid (0.128 g, 39%). ^{1}H NMR (250 MHz, CD_3OD/$CDCl_3$ 4:1, 25 °C, TMS): δ = 1.47 (s, 36 H, *t*Bu), 2.77 (s, 2 H, Ar-CH_2-NH-C*H*), 3.59 (s, 4 H, −C*H*−NH−BOC), 3.93 (m, 6 H, −C*H*−OH), 4.13 (s, 4 H, arom.−CH_2−NH−), 7.34−7.45 (m, 3 H, H_{Ar}), 7.69 (s, 1 H, H_{Ar}) ppm. ^{13}C NMR (63 MHz, CD_3OD/$CDCl_3$, 4:1, 25 °C, TMS): δ = 28.5, 52.0, 58.0, 69.1, 72.3, 80.0, 129.4, 155.7 ppm.

***all-cis*-4,4′,6,6′-Tetrakis(*tert*-butoxycarbonylamino)-6,6′-*m*-xylylidenebis(cyclohexane)-1,3,5-triol (DTMX, 2) Hexahydrochloride Salt: 5** (0.128 g, 0.149 mmol) was treated with CH_2Cl_2/TFA (2:1, 9 mL) for 4 h with monitoring by TLC [$CHCl_3$/MeOH/NH_3(aq), 10:2:0.2]. The solvent was evaporated under reduced pressure and then water (a few mL) was added to the resulting solid. The crude product was eluted through an ion-exchange column (DOWEX 50WX2, Cl^- form) and **2** (white solid) were obtained as its hexahydrochloride salt (0.082 g, 70%). ^{1}H NMR (250 MHz, D_2O, 25 °C, TMS): δ = 3.62−3.74 (m, 6 H, −C*H*−NH and −C*H*−NH_2), 4.45 (m, 6 H, −C*H*−OH), 4.59 (s, 4 H, Ar−CH_2−NH−), 7.44−7.67 (m, 4 H, H_{Ar}) ppm. ^{13}C NMR (63 MHz, D_2O, 25 °C, TMS): δ = 50.1, 52.3, 58.4, 66.3, 67.6, 132.1, 132.7, 133.4, 133.7 ppm. $C_{20}H_{42}Cl_6N_6O_6{\cdot}6H_2O$ (783.4): calcd. C 30.67, H 6.89, N 10.73; found C 30.76, H 6.11, N 10.04.

***all-cis*-4,4′,6,6′-Tetrakis(*tert*-butoxycarbonylamino)-6,6′-*p*-xylylidenebis(cyclohexane)-1,3,5-triol (6):** α,α′-Dibromo-*p*-xylene (0.061 g, 0.23 mmol) was added to a solution of **4** (0.18 g, 0.48 mmol) in dry DMF (2 mL) in the presence of diisopropylethylamine (0.5 mL, 2.9 mmol). The reaction mixture was heated at 70 °C for 48 h with monitoring by TLC [$CHCl_3$/MeOH/NH_3(aq), 10:2:0.2; R_f(**6**) = 0.56]. After this time, CH_2Cl_2 (150 mL) was added and the organic layer was extracted with 10% Na_2CO_3 solution (3 × 50 mL) and brine (3 × 50 mL). The organic phase was dried (Na_2SO_4) and the solvent evaporated. Purification of the crude product was performed by flash column chromatography [SiO_2; $CHCl_3$/MeOH/NH_3(aq), 10:1.5:0.15] to obtain **6** as a pale-yellow solid (0.088 g, 45%). ^{1}H NMR (250 MHz, CD_3OD/$CDCl_3$ 4:1, 25 °C, TMS): δ = 1.46 (s, 36 H, *t*Bu), 2.67 (s, 2 H, Ar−CH_2−NH−C*H*−), 3.55 (s, 4 H, −C*H*−NH−BOC), 3.83−3.96 (m, 6 H, −C*H*−OH), 4.10 (s, 4 H, arom.−CH_2−NH−), 7.33−7.37 (m, 4 H, H_{Ar}) ppm. ^{13}C NMR (63 MHz, CD_3OD/$CDCl_3$, 4:1, 25 °C, TMS): δ = 28.7, 53.5, 58.3, 71.1, 72.8, 80.4, 129.4, 139.4, 156.9 ppm.

***all-cis*-4,4′,6,6′-Tetrakis(*tert*-butoxycarbonylamino)-6,6′-*p*-xylylidenebis(cyclohexane)-1,3,5-triol (DTMX, 2) Hexahydrochloride Salt: 6** (0.88 g, 0.10 mmol) was treated with CH_2Cl_2/TFA (2:1, 15 mL) for 4 h with monitoring by TLC [$CHCl_3$/MeOH/NH_3(aq), 10:2:0.2]. The solvent was evaporated under reduced pressure and then water (a few mL) was added to the resulting solid. The crude produce was eluted through an ion-exchange column (DOWEX 50WX2, Cl^- form) and **3** (white solid) was obtained as its hexahydrochloride salt (0.062 g, 89%). ^{1}H NMR (250 MHz, D_2O, 25 °C, TMS): δ = 3.55 (t, *J* = 3 Hz, 2 H, −C*H*−NH−), 3.61 (t, *J* = 3 Hz, 4 H, −C*H*−NH_2), 4.35 (m, 6 H, −C*H*−OH), 4.46 (s, 4 H, Ar−CH_2−NH−), 7.51 (s, 4 H, H_{Ar}) ppm. ^{13}C NMR (63 MHz, D_2O, 25 °C, TMS): δ = 50.0, 52.4, 58.4, 66.4, 67.7, 132.9, 133.5 ppm. $C_{20}H_{42}Cl_6N_6O_6{\cdot}6H_2O$ (783.4): calcd. C 30.67, H 6.89, N 10.73; found C 30.71, H 6.35, N 10.23.

Potentiometric Titrations: Protonation constants and Zn^{II} complex-formation constants for ligands **1**−**3** were determined by pH potentiometric titrations (25 °C, 0.10 M NaCl). Solutions containing ca. 1 × 10^{-3} M of the hydrochloride salts of the ligands and, when necessary, $Zn(NO_3)_2$ were titrated using a 0.1 M sodium hydroxide solution. The electrode system was calibrated by titrating a 0.01 M solution of HCl so that the value of pK_w was 13.78. The pH and the volume of added NaOH data were fitted with the computer program BEST[32] to obtain the desired protonation and complex-formation constants.

Kinetic Measurements: The kinetic traces were recorded on a Perkin−Elmer Lambda 16 spectrophotometer equipped with a thermostatted cell holder. The reaction temperature was maintained at 25 ± 0.1 °C. The reactions were started by adding a solution of substrate (1−2 × 10^{-3} M; 20 μL) to a solution of metal complex in the appropriate buffer (2 mL) and monitored by following the absorption of *p*-nitrophenoxide at 400 nm. The initial concentration of substrate was 1−2 × 10^{-5} M and the kinetics were in each case first-order up to 90% of the reaction. The pseudo-first-order rate constants were obtained by non-linear regression analysis of the data from a plot of absorbance vs. time and the fit error on the rate constant was always less than 1%.

In the case of the experiments performed in the presence of an excess of HPNP, the reactions were started by adding a solution of the substrate (2.57 × 10^{-2} M; 30 μL) to a solution of the metal complex in CHES buffer (2 mL) at pH 9.0 and were monitored by following the absorption of *p*-nitrophenoxide. Reactions were followed up to ca. 1% of substrate hydrolysis (ca. 10 h). The pseudo-first-order rate constants were obtained from the slope of the plot of absorbance versus time (the fit error was always less than 1%) divided by the absorbance of *p*-nitrophenoxide and the concentration of the substrate.

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[1] D. E. Wilcox, *Chem. Rev.* **1996**, *96*, 2345−2458.
[2] M. J. Jedrzejas, P. Setlow, *Chem. Rev.* **2001**, *101*, 608−618.
[3] N. H. Williams, B. Takasaki, M. Wall, J. Chin, *Acc. Chem. Res.* **1999**, *32*, 485−493.
[4] J. R. Morrow, *Met. Ions Biol. Syst.* **1997**, *33*, 561−592.
[5] P. Molenveld, J. F. J. Engbersen, D. N. Reinhoudt, *Chem. Soc. Rev.* **2000**, *29*, 75−86.
[6] E. L. Hegg, J. N. Burstyn, *Coord. Chem. Rev.* **1998**, *173*, 133−165.
[7] P. Hendry, A. M. Sargeson, *Prog. Inorg. Chem.* **1990**, *38*, 201−258.
[8] T. Koike, E. Kimura, *J. Am. Chem. Soc.* **1991**, *113*, 8935−8941.

[9] T. Itoh, Y. Fujii, T. Tada, Y. Yuzo, H. Hisada, *Bull. Chem. Soc. Jpn.* **1996**, *69*, 1265−1274.
[10] L. Bonfà, M. Gatos, F. Mancin, P. Tecilla, U. Tonellato, *Inorg. Chem.* **2003**, *42*, 3943−3949.
[11] For an example of efficient binuclear catalyst operative in water/organic solvent mixtures, see: K. Yamada, Y. Takahashi, H. Yamamura, S. Araki, K. Saito, M. Kawai, *Chem. Commun.* **2000**, 1315−1316. See also ref. [5].
[12] C. Sissi, F. Mancin, M. Palumbo, P. Scrimin, P. Tecilla, U. Tonellato, *Nucleotides, Nucleosides and Nucleic Acids* **2000**, *19*, 1265−1271.
[13] T. Gajda, Y. Dupre, I. Torok, J. Harmer, A. Schweiger, J. Sander, D. Kuppert, K. Hegetschweiler, *Inorg. Chem.* **2001**, *40*, 4918−4927.
[14] D. H. Vance, A. W. Czarnik, *J. Am. Chem. Soc.* **1993**, *115*, 12165−12166.
[15] W. H. Chapman Jr., R. Breslow, *J. Am. Chem. Soc.* **1995**, *117*, 5462−5469.
[16] M. J. Joung, J. Chin, *J. Am. Chem. Soc.* **1995**, *117*, 10577−10578.
[17] F. N. Aka, M. S. Akkaya, E. U. Akkaya, *J. Mol. Cat. A* **2001**, *165*, 291−294.
[18] MM2 Force Field, Spartan 4.0, Wavefunction Inc., Irvine (California), **1993**.
[19] K. Hegetschweiler, *Chem. Soc. Rev.* **1999**, *28*, 239−249.
[20] K. A. Deal, J. N. Burstyn, *Inorg. Chem.* **1996**, *35*, 2792−2798.
[21] M. K. Deck, T. A. Tseng, J. N. Burstyn, *Inorg. Chem.* **2002**, *41*, 669−677.
[22] K. Hegetschweiler, V. Gramlich, M. Ghisletta, H. Samaras, *Inorg. Chem.* **1992**, *31*, 2341−2346.
[23] F. Mancin, PhD Dissertation, Univ. of Padova, **2000**.
[24] A Michaelis−Menten-like equation was applied: $k_{\psi} = k_{cat}[complex]/(K_b^{-1} + [complex])$.
[25] Y. Gultneh, A. Raza Khan, D. Blaise, S. Chaundhry, B. Ahvazi, B. B. Marvey, R. J. Butcher, *J. Inorg. Biochem.* **1999**, *75*, 7−18.
[26] P. Rossi, F. Felluga, P. Tecilla, F. Formaggio, M. Crisma, C. Toniolo, P. Scrimin, *J. Am. Chem. Soc.* **1999**, *121*, 6948−6947.
[27] H. Chuan, S. J. Lippard, *J. Am. Chem. Soc.* **2000**, *122*, 184−185.
[28] K. Worm, F. Chu, K. Matsumoto, M. D. Best, V. Linch, E. V. Anslyn, *Chem. Eur. J.* **2003**, *9*, 741−747.
[29] R. A. Moss, Y. Ihara, *J. Org. Chem.* **1983**, *48*, 588−592.
[30] D. A. Brown, D. A. Usher, *J. Chem. Soc.* **1965**, 6558−6564.
[31] K. Hegetschweiler, I. Erni, W. Schneider, H. Schmalle, *Helv. Chim. Acta* **1990**, *73*, 97−105.
[32] A. E. Martell, R. J. Motekaitis, *Determination and Use of Stability Constants*, 2nd ed., VCH Publishers, New York, **1992**.

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