Zinc(II) complexes as hydrolytic catalysts of phosphate diester cleavage: from model substrates to nucleic acids

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The development of synthetic agents able to hydrolytically cleave phosphate diester bonds with high efficiency is a fascinating challenge, which will ultimately open the way to artificial nucleases able to compete with the natural enzymes. This Perspective highlights the progress reported in the realization of hydrolytic catalysts based on the Zn^{2+} ion, a metal ion which, due to its peculiar properties, is a very promising candidate. The review critically examines the reactivity of such catalysts toward model substrates and nucleic acids, paying particular attention to the strategies that can be pursued to improve efficiency and sequence selectivity.

Introduction

The ability of metal ions to promote the hydrolytic cleavage of esters has been well known for decades. Nature learned a long time ago how to take advantage of such an ability and, as a matter of fact, many hydrolytic enzymes contain metal ions in their active sites.[1] However, the development of synthetic agents capable of accelerating the hydrolysis of phosphate esters, and in particular phosphate diesters, has only attracted substantial research efforts in the last twenty years.[1a,2] There are several reasons that justify interest in such systems. Firstly, mechanistic information obtained in the study of artificial agents could lead to a better understanding of the chemistry of the corresponding hydrolytic enzymes. Secondly, they could be employed for the detoxification of pesticides and chemical weapons, which often have phosphate ester-like structures. Thirdly, efficient phosphate diester hydrolytic agents could be employed as artificial restriction enzymes for molecular biology. Such systems would be highly valuable because they could, in principle, be designed to cleave DNA with a sequence selectivity different from that of natural enzymes, which are often inadequate for the manipulation of DNA from higher organisms. Finally, the realization of anti-DNA drugs can be envisaged in the more distant future. The exceptional resistance of phosphate diesters to hydrolytic cleavage at neutral pH has, so far, slowed down research in the field, but very promising results, and even the first studies on DNA manipulation with artificial agents, have been described in the last few years.[3]

Hydrolysis of a phosphate diester involves the nucleophilic attack of a water (or hydroxide ion) oxygen at the phosphorus to give a five-coordinate phosphate intermediate (or transition state); the subsequent expulsion of one alcoholic (or alkoxide) fragment provides the products.[2c,h,k,n] Depending on the

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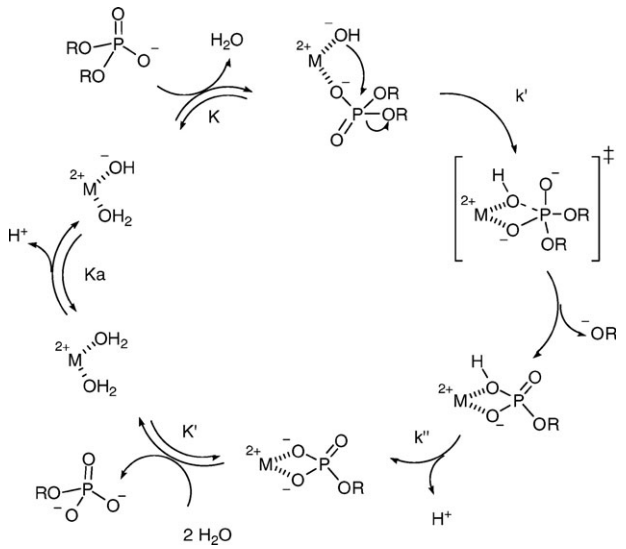

Fig. 1 Reaction pathway for phosphate diester hydrolysis catalyzed by a metal ion.

substrate structure, either the nucleophilic attack or the breakdown of the intermediate can be rate limiting. The hydrolysis is assisted by metal ions in different ways. In the case of monometallic complexes, the commonly accepted mechanism is reported in Fig. 1. The main steps are the acid dissociation of a metal coordinated water molecule, the coordination of the substrate to the metal ion and the nucleophilic attack of the metal-bound hydroxide on the substrate.

Acting as a Lewis acid, the metal ion activates the phosphate group towards attack of the nucleophile and promotes the deprotonation of a water molecule to form the nucleophile. Moreover, it brings together the two reactants, neutralizing the electrostatic repulsion between the two negatively charged species. Other catalytic roles have been proposed, such as stabilizing the developing negative charge of the transition state and assisting the departure of the leaving group.[2m]

The requirements for a metal ion to be an effective hydrolytic catalyst are high Lewis acidity and rapid ligand exchange.[2n] Two free binding sites in a *cis* disposition on the metal ion are needed to grant the coordination of both reactants at the right distance for the reaction. On the other hand, redox activity should be avoided, since it can lead to the activation of concurrent oxidative cleavage reactions, which are particularly relevant in the case of nucleic acids. Nature's choices fall mainly on Mg^{2+}, Zn^{2+}, Ca^{2+} and Fe^{2+}, the latter being the only redox active metal ion found in hydrolytic enzymes. Among the bioavailable metal ions, these are the ones that best fulfil the above criteria. Chemists have the freedom to pick their candidates from the whole periodic table, and artificial systems are usually based on Zn^{2+}, Cu^{2+}, Co^{3+}, Fe^{3+} and lanthanide ions (Eu^{3+}, Ce^{4+}). Such ions are, in fact, better Lewis acids than those used by enzymes and can potentially achieve better reactivities.

Zn^{2+} is the only metal frequently encountered in both natural and artificial agents. This is due to a variety of factors: Zn^{2+} is a good Lewis acid, exchanges ligands rapidly, is not toxic and is not redox active. Moreover, it has no ligand field stabilization energy and, as a consequence, it can easily adapt its coordination geometry to best fulfil the structural requirement of a reaction.[2n] For all of these reasons, the development of Zn^{2+}-based artificial enzymes would be highly valuable. Following some pioneering studies on the cleavage of RNA by Zn^{2+} alone, and of simple phosphate esters by Zn^{2+} complexes, the first report of a true zinc-based artificial nuclease was a 1987 study by J. K. Barton and co-workers,[4] who reported plasmid DNA cleavage by a rhodium intercalator conjugated with two metal binding groups. After this first example, several other groups studied the reactivity of Zn^{2+} complexes toward nucleic acids and phosphate diesters. In this Perspective, we aim to critically review the Zn^{2+} based synthetic agents reported so far for the hydrolytic cleavage of phosphate diesters.[5] Particular attention will be devoted to a comparison of the activity of the different systems in order to elucidate the most promising strategies that can be employed to obtain effective catalysts.

DNA and its models

Substrates. DNA is obviously the most interesting substrate for hydrolytic agents. Due to its polyanionic nature, DNA is tremendously resistant to hydrolysis; half-life times of a single P–O bond cleavage at 25 °C and pH 7 have been estimated to range between hundreds of thousands and hundreds of millions of years.[2m,6] Clearly, this scarce reactivity makes it difficult to perform any mechanistic investigation on this substrate. Supercoiled plasmid DNA is a more accessible substrate and, because of this, it is very popular for such studies. This particular form of DNA, which is commonly found in bacteria cells, is a cyclic supercoiled double strand made of several thousand base pairs. One single strand scission (over ten thousand base pairs) unravels the supercoiled DNA (form I) to a relaxed circular one (nicked, form II), while a second scission on the complementary strand, within about twelve base pairs from the first one, generates a linear DNA form (form III). These three DNA forms can be easily separated and quantified by gel electrophoresis (Fig. 2), thus allowing easy detection of even a small number of scission events. Moreover, supercoiled DNA is somehow more reactive than a short linear DNA fragment because the internal strain results in ground state destabilization.[2q]

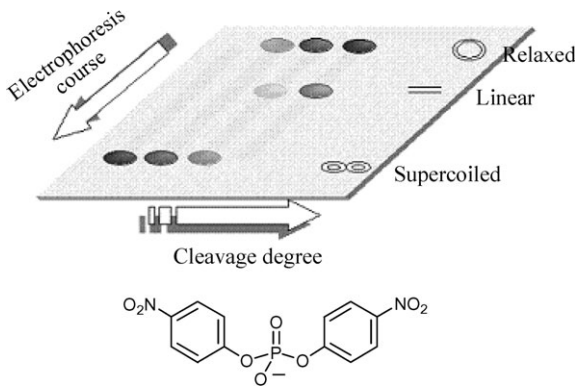

Fig. 2 Typical electrophoresis gel appearance in the cleavage of plasmid DNA and the structure of the model substrate BNP.

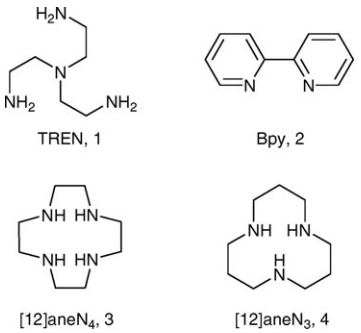

Fig. 3 Polyamine ligands used for Zn^{2+} complexation in early examples of BNP cleaving agents.

Model substrates, *i.e.* phosphate diesters with good leaving groups, are also very popular. Mechanistic studies are much easier with such small molecules, and their greater reactivity allows shorter reaction times. The most popular DNA model is bis *para*-nitrophenyl phosphate (BNP, Fig. 2) which, notwithstanding the presence of two good leaving groups, is still quite resistant to hydrolytic cleavage; the rate of spontaneous hydrolysis in water at pH 7 and 25 °C has been estimated to be 1.6×10^{-11} s^{-1},[7] which corresponds to a half-life of more than 1300 years. However, there are significant mechanistic differences in the reaction pathway with respect to non-activated substrates; the rate-determining step is probably nucleophile attack and not leaving group departure. The reaction does not proceed through an intermediate and is more likely to be a bimolecular substitution.[8] For this reason, the information obtained with this substrate can be extended to natural DNA with some precaution.

Monometallic agents. In 1990, Trögler and De Rosch studied the hydrolysis of the model phosphate diester BNP catalyzed by zinc complexes for the first time.[9] TREN (**1**) and Bpy (**2**, Fig. 3) were used as ligands and the results were quite unimpressive; at pH 7.0 and 75 °C (1 mM catalyst, 0.1 M $NaNO_3$) the pseudo-first order rate of BNP cleavage was only 5.1×10^{-8} s^{-1} for the TREN complex, which represented only a two-fold acceleration over the background reaction. Bpy complexes were found to be more reactive, leading to a 53-fold rate acceleration. The authors attributed the different activity of the two ligands to the availability of free binding sites in the complexes; the tetradentate TREN leaves no space for the simultaneous binding of the substrate and the hydroxide to the metal ion, while this is possible with the bidentate coordination mode of Bpy.

One year later, Kimura and Koike[10] compared the reactivity of the Zn^{2+} complexes of 1,4,7,10-tetraazacyclododecane ([12]aneN$_4$, cyclen, **3**) and 1,5,9-triazacyclododecane ([12]aneN$_3$, **4**); the pseudo-first order rate constants[11] for BNP cleavage at pH 7 and 35 °C (0.2 M $NaClO_4$) were 2.8×10^{-9} s^{-1} and 3.3×10^{-8} s^{-1}, respectively, corresponding to 46- and 550-fold acceleration over the background reaction. Again, the greater reactivity of the tridentate ligand compared with that of the tetradentate example was attributed to the occupation of less binding sites on the metal ion. Moreover, the most important feature of the Zn^{2+} complex of the [12]aneN$_3$ ligand is the remarkably low acidity of the metal-bound water molecule, which also makes the system active at physiological pH, where a significant fraction of metal–hydroxide species are already present. Subsequently, the influence of ligand structure on the reactivity of complexes has been investigated in detail by us.[12] The reactivity of ten Zn^{2+} complexes with different tri- and tetradentate polyamine ligands towards BNP was determined. The main results obtained are summarized in the Brønsted plot reported in Fig. 4, which relates the logarithms of the second order rate constants (k_2) for the reaction of metal alkoxide species with the pK_a values of their metal-bound water molecules. Inspection of the plot confirms the results of the pioneering studies of Trögler and Kimura; complexes of tetradentate ligands are ineffective catalysts, and this behavior is closely related to the occupation of the binding sites on the metal ion by the ligand donor atoms. The three-fold reactivity difference between TREN (**1**) and cyclam ([14]aneN$_4$, **12**) is particularly diagnostic of this effect. Indeed, both ligands are tetradentate, and the pK_a of the water molecule is almost identical in the two complexes (this indicates that the metal centres have similar Lewis acidity) but the TREN ligand wraps around the metal in a trigonal bipyramidal geometry, leaving less space for additional ligands than the facial coordination mode of cyclam. Other interesting

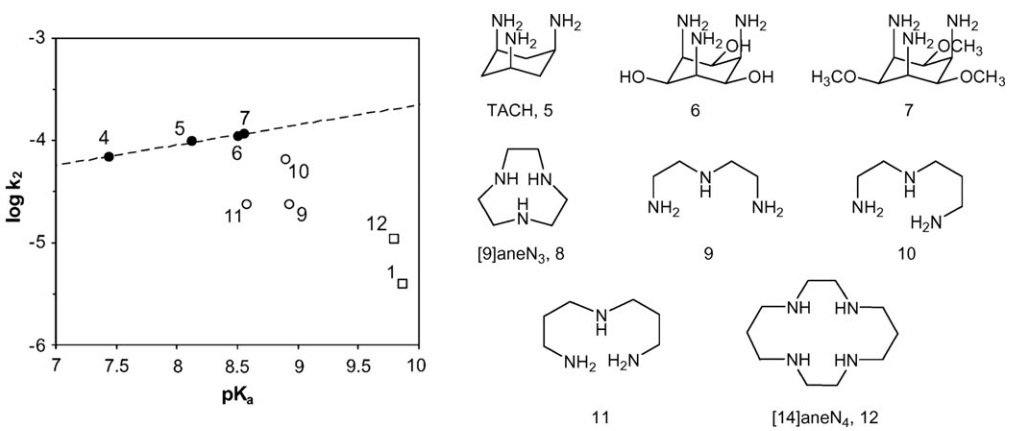

Fig. 4 Log k_2 *vs.* pK_a for the hydrolysis of BNP catalyzed by Zn^{2+} complexes in water at 25 °C. The dashed line shows the linear fit of the reactivity data for the complexes of cyclic ligands **4** and **5–7**.

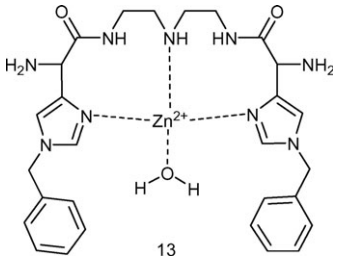

Fig. 5 Proposed structure of the Zn^{2+} complex of the pseudopeptide ligand **13**.

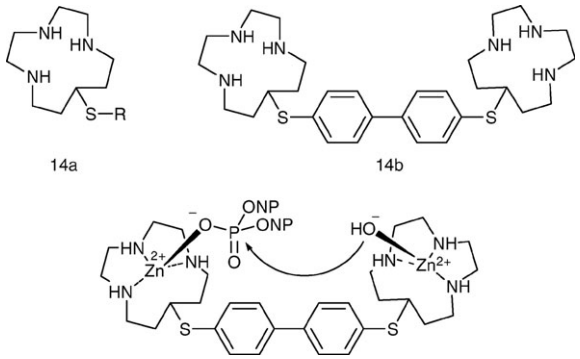

Fig. 6 Structure of the macrocyclic ligands **14a** and **14b** and proposed reaction mechanism for the bimetallic complex.

information can be deduced from Fig. 4. Among tridentate ligands, tripodal examples appear to be more reactive than linear ligands. Such different reactivities cannot be ascribed to the Lewis acidities of the metal ions, since the pK_a values of the coordinated water molecules are very similar. Moreover, the reactivity of tripodal ligands shows a clear correlation with the acidity of the metal-bound water molecule. A linear Brønsted correlation is obtained with a slope value (β_{nuc}) of 0.20. The Lewis acidity of the Zn^{2+} ion has two opposite effects on the reactivity of the complexes; on one hand, a strongly acidic metal ion greatly activates the substrate toward nucleophilic attack, but on the other, the correlated increase in acidity of the metal-bound water molecule decreases its nucleophilicity. The low β_{nuc} value obtained reflects such a balance of opposite effects, with the reactivity of the nucleophile slightly prevailing over the activation of the substrate. However, considering the reactivity at pH 7, the picture is reversed, since the small reactivity differences cannot overcome the availability of a larger amount of reactive complex for agents with the most acidic water molecules, and Kimura's Zn·[12]aneN$_3$ (**4**) complex still appears as the most reactive Zn-based monometallic agent.

One exception is the Zn^{2+} complex of a histidine-containing pseudopeptide ligand (**13**, Fig. 5) reported by Ichikawa and co-workers.[13] At pH 7.0 and 50 °C, BNP is cleaved by 3.6 mM **13**·Zn^{2+} at a rate of 1.1×10^{-5} s^{-1}, which represents a 36 000-fold acceleration over the background reaction. On the basis of the pH–reactivity profiles, the active species appears to be a monohydroxo species (but the exact identification is made difficult by the possible deprotonation of the ligand amino groups), which form with the very low pK_a value of 6.1.

Studies explicitly devoted to elucidation of the reactivity of simple monometallic systems on DNA are rare, and the activities reported are usually low. However, they have sometimes been reported as reference compounds in the study of more complex systems. Complexes of TREN[9] and a methylated derivative of [12]aneN$_3$ (**4**)[14] do not produce any cleavage of plasmid DNA. On the other hand, complexes of triaminocyclohexane (TACH, **5**)[15] and 1,4,7-triazacyclononane ([9]aneN$_3$, **8**)[16] derivatives show a moderate activity (the pseudo-first order rate constant for the cleavage of plasmid DNA by TACH·Zn^{2+} complexes at pH 7, 37 °C and 45 μM complex concentration is 2.0×10^{-6} s^{-1}).[15] Ichikawa's complex **13**·Zn^{2+} is also very reactive towards plasmid DNA;[13] at pH 7, 50 °C and 3 μM complex, the plasmid DNA is completely cleaved after 24 h, leading to both nicked and linear products. From this data, a rate constant of around 4×10^{-5} s^{-1} can be roughly estimated. The same complex is also active in the cleavage of the DNA-type dinucleotide TpT, which at pH 8.5, 50 °C and 0.7 mM complex is cleaved with a rate constant of 2.0×10^{-7} s^{-1}, corresponding to an acceleration of 300 times over the background reaction. This reduced activity with respect to plasmid DNA emphasizes the apparent lower stability of the phosphate ester bond in plasmid DNA with respect to an isolated example.

More recently, the Zn^{2+} complexes of dipeptides,[17] tripeptides[18] and cyclotriphospazenes[19] have been shown to be active in the cleavage of plasmid DNA with rate constants in the 10^{-5}–10^{-6} s^{-1} range. However, these results have been obtained using high concentrations of metal complex (1–2 mM), making rather complicated comparisons with other systems.

Bimetallic agents. A promising strategy in order to increase the efficiency of hydrolytic agents is the use of bimetallic systems. Also, in this case, Nature is the source of inspiration. In fact, the extraordinary catalytic efficiency of natural metallonucleases most often relies on the cooperative action of two or more metal ions. The use of two metal ions not only increases the effect of the Lewis acid catalysis exerted on the substrate and transition state, but also makes possible other concomitant activation modes, such as leaving group activation.[2m] On the basis of these guidelines, a series of bimetallic Zn^{2+} complexes have been synthesized and investigated as catalysts for the hydrolytic cleavage of DNA models and DNA itself.

The first example was reported from the group of Breslow in 1995.[20a] They prepared a series of ditopic ligands, made by connecting the tridentate macrocyclic ligand **14a** (Fig. 6) with different spacers, and studied the reactivity of their Zn^{2+} complexes towards several phosphate esters, including BNP. The results obtained showed that when a flexible alkyl linker was used, the reactivity of the bimetallic system was the same as that of the monometallic system. The reactivity increased in the case of complexes with rigid spacers and reached the optimum with ligand **14b**, featuring a 4,4′-biphenyl linker. In this case, BNP was cleaved at 55 °C and pH 8.36 (20% DMSO) with a pseudo-first order rate of 6.4×10^{-6} s^{-1}, which corresponds to an acceleration of about 2000 times the background reaction. Moreover, the bimetallic complex is about 5 times more effective than the corresponding

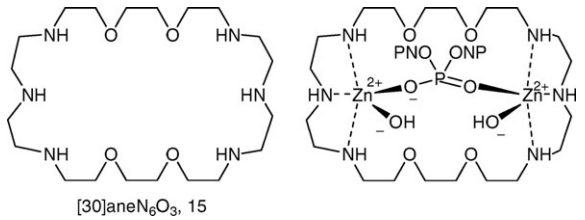

Fig. 7 Structure of the macrocyclic ligand [30]aneN$_6$O$_3$ (**15**) and proposed reaction mechanism for the bimetallic complex.

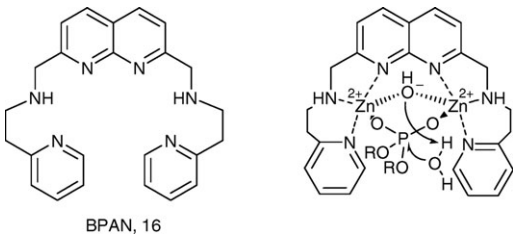

Fig. 8 The BPAN ligand (**16**) and proposed reaction mechanism for the bimetallic complex.

monometallic system. The reactivity–pH profile show a bell-shaped behavior diagnostic of two deprotonation events, with pK_a values of 8 and 12, respectively. The mechanism proposed by the authors (Fig. 6) involved binding of the substrate to one of the zinc ions, while the second delivered the attacking hydroxide. In this view, deprotonation of the first metal-bound water molecule lead to the formation of the nucleophile and hence to acceleration, while deprotonation of the second water molecule, bound to the other metal ion, prevented the binding of the substrate, leading to retardation.

Optimization of the complex geometry and the distance between the two metal ions in order to increase the reactivity has been the goal of almost all the following examples reported. A few years after the Breslow study, Bencini, Bianchi, Paoletti and co-workers[21a] investigated the reactivity of the macrocyclic ligand [30]aneN$_6$O$_3$ (**15**, Fig. 7), which binds two Zn ions in aqueous solution, towards BNP. The bimetallic complex is about 10 times more reactive that its monometallic counterpart, but the overall reactivity is not exceptional, particularly at physiological pH. Different to the previous example, the substrate binds to both the metal ions (this justifies the remarkable cooperative effect of the two metal ions) but the reactive species is the dihydroxo complex (Fig. 7), which forms with a pK_a value of 9.2. In fact, the monohydroxo complex is substantially inactive because of the formation of an unreactive μ-hydroxo bridge; the addition of the second hydroxide breaks the μ-OH bridge, leading to a substantial reactivity increase. A subsequent attempt by the same group to enlarge the macrocyclic ligand led to a reactivity decrease.[21b]

A different system was reported in 2000 by Lippard and co-workers.[22b] They used a rigid linker also capable of providing metal binding sites and obtained a very pre-organized system. At pH 7 and 40 °C, the dizinc complex of 2,7-bis[2-pyridylethyl)aminomethyl]-1,8-naphthyridine (BPAN**16**, Fig. 8) cleaved BNP with a pseudo-first order rate of 3.7×10^{-7} s^{-1}, which represents a 9200-fold acceleration over the background reaction but only a 1.8-fold acceleration over the monometallic model. Concentration *vs.* reactivity profiles show a saturation profile diagnostic of the binding of the substrate to the bimetallic complex, but the binding constant is quite small (K_{ass} = 86 M^{-1}). The reactive species is the monohydroxo complex, with the hydroxide ion bound to both the metal ions (μ-hydroxo bridge). In the mechanism proposed by the authors (Fig. 8), this bridging hydroxide acts as a general base catalyst to assist the nucleophilic attack of a solvent water molecule on the substrate.

The structure of the catalysts studied by Meyer and co-workers[23] is somewhat similar; the ligands used are based on a pyrazolate bridge connecting different polyamino ligands (**17a** and **17b**, Fig. 9), which allows the tuning of the intermetallic distance. At pH 8.28 and 50 °C, the two complexes **17a** and **17b** cleave BNP at similar rates (respectively, 2.6×10^{-7} and 8.5×10^{-7} s^{-1}). The acceleration of the reactions does not appear dramatic, but the authors could observe saturation concentration-dependent reactivity profiles. Analysis of the Michaelis–Menten parameters highlighted that the complex with the greater intermetallic distance, where formation of a μ-hydroxo bridge is prevented or at least disfavored, is five-fold more reactive than the system where a μ-hydroxo bridge is present. Interestingly, prevention of the formation of the μ-hydroxo bridge does not affect the pK_a (7.6) of the first deprotonation of the metal-bound species in the complex. This is attributed to the formation of intracomplex hydrogen bonds between the metal-bound water molecules, which assist their deprotonation.

The dizinc complex of related ligand **18** (Fig. 9), based on two [9]aneN$_3$ units connected by a pyrazolate bridge, was studied by Kaden and Vichard.[24] However, the complex was scarcely reactive, cleaving BNP at pH 7 and 35 °C with a pseudo-first order rate constant of 1.2×10^{-8} s^{-1},[11] which represents only a 200-fold acceleration over the background reaction.

More flexible systems were studied by Bencini, Lippolis and co-workers,[25] who connected two [9]aneN$_3$ (**8**) units using semi-rigid spacers such as 2,2′-dimethylbipyridine, 1,10-bimethylphenantroline and 2,3-bimethylquinoxaline. The complex of ligand **19** (Fig. 10), featuring the quinoxaline spacer,

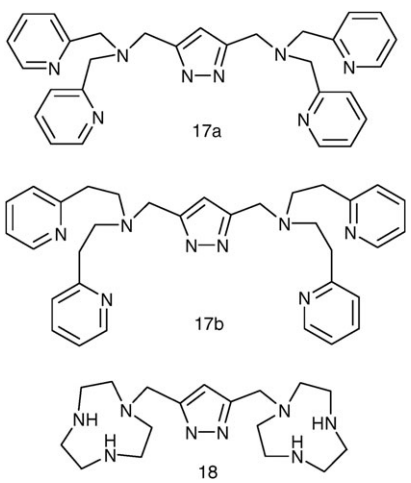

Fig. 9 Binuclear Zn^{2+}-binding ligands based on pyrazolate bridge.

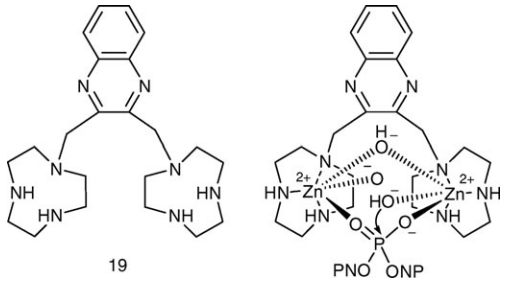

Fig. 10 Structure of the binuclear Zn^{2+}-binding ligand **19** and proposed reaction mechanism for the bimetallic complex.

was found to be the most reactive among the systems studied, cleaving BNP with a pseudo-first order rate of 2.1×10^{-8} s^{-1} (350-fold acceleration)[11] at pH 7 and 35 °C. Surprisingly, the active species is the trihydroxo complex (which forms above pH 9.2), with one hydroxide ion bridging the two metals and the other two bound to a single metal centre. In the mono- and dihydroxide complexes, the hydroxide ions bridge the two metals and, as a consequence, are unreactive as nucleophiles. When the third hydroxide ion is added, at least one μ(OH) bridge is weakened, and this makes available a good nucleophile for substrate cleavage (Fig. 10).

Surprisingly, examples of DNA-cleaving bimetallic systems based on Zn^{2+} are scarce and show a disappointingly low reactivity.[14,26] An important exception is the binuclear Zn^{2+}-binding heptapeptide **20** (Fig. 11) described by Scrimin and co-workers,[16] which cleaves plasmid DNA with a first order rate constant of 1.0×10^{-5} s^{-1} at pH 7.0, 37 °C and 3.6 μM complex concentration. Due to presence of several α-disubstituted amino acids, the peptide is folded to a relevant extent in a 3_{10}-helical conformation, and the two metal chelating [9]aneN_3 moieties face each other at a distance apart of about 6 Å (the pitch of the 3_{10}-helix). Even if such a distance is larger than that found in bimetallic hydrolytic enzymes, the system is about 20 times more reactive than its monometallic counterpart, and the cooperativity between the two metal centres has been clearly demonstrated with Zn^{2+} concentration-dependent experiments. To justify the high cooperativity observed, the authors proposed that the bimetallic catalyst binds to DNA by inserting the two macrocycles within three adjacent phosphate groups. Such an arrangement forces the central phosphate to interact with both metal centres, thus taking full advantage of their complementary roles in its hydrolytic cleavage.

An impressively high reactivity was reported very recently by Yu and co-workers, who studied dimetallic Zn^{2+} complexes made by linking two cyclen ligands with different spacers bearing alcoholic, phenolic or pyridinic moieties.[27a] The authors claim that at pH 7.1, 37 °C and 25 μM complex concentration, the plasmid DNA pUC18 is completely nicked to give form II after only 5 min. A longer incubation time results in further fragmentation and DNA smearing on the electrophoresis gel. However, the complexes appear active only in the presence of the reducing agent vitamin C, and therefore the cleavage mechanism could be non-hydrolytic with an oxidative pathway activated by the reducing agent, probably involving the ligand backbone or adventitious redox active metal ions. The same type of reactivity was also observed in the case of the Zn^{2+} complexes of uracil–cyclen conjugate ligands.[27b] These results, although impressive if the mechanism proves to really be hydrolytic, stress the importance of using caution when studying the mechanism of DNA cleavage, even when Zn^{2+} is employed as the metal ion. Indeed, this natural substrate is very stable towards hydrolysis but is also very delicate from other points of view, and competitive cleavage pathways are always possible.

Organic group assistance. The second strategy used to increase the reactivity of metal-based hydrolytic agents is again inspired by hydrolytic enzymes. Functional groups belonging to the amino acid side chains that participate in the reaction with fundamental catalytic roles are present in the active sites of these natural catalysts. They can provide more powerful nucleophiles, activate the substrate by means of hydrogen bonds or electrostatic interactions, stabilize developing charges or assist the departure of the leaving groups. Hence, the addition of strategically placed organic groups on the structure of the ligand may lead to the realization of more reactive artificial systems.

The first example of hydrolytic agents featuring the co-operation between Zn^{2+} ions and organic functional groups was described by Krämer and Kovari,[28] who prepared derivative **21** (Fig. 12) of Bpy bearing two ammonium groups at the end of two rigid propyne spacers. The ammonium groups are in the right position to donate a hydrogen bond to one of the phosphate diester oxygen atoms not bound to the zinc ion and to provide either stabilization of the developing negative charge or leaving group activation. Unfortunately, the metal ion binding ability of **21** was very low and the reactions were performed in acetonitrile/water 19 : 1. Under these conditions, the presence of the two dimethylammonium groups lead to a five-fold reactivity increase with respect to the Zn complex of Bpy.[28a] Much better results (acceleration up to 4×10^7 times over the background reaction at pH 7 and 20 °C) were

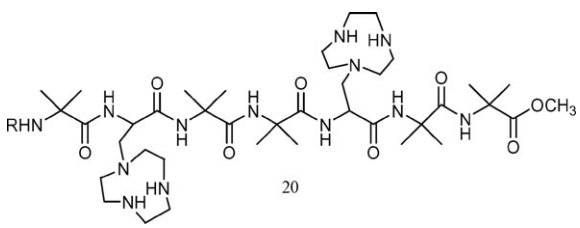

Fig. 11 Structure of the binuclear Zn^{2+}-binding heptapeptide ligand **20**.

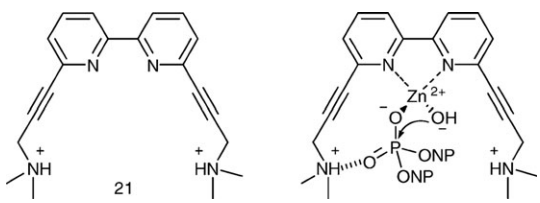

Fig. 12 Structure of ligand **21** and proposed reaction mechanism for the Zn^{2+} complex.

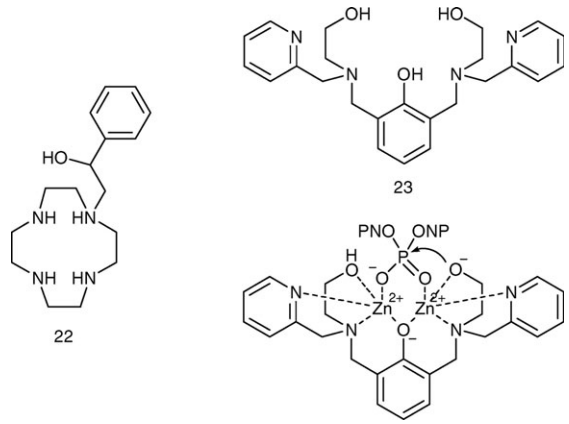

Fig. 13 Structure of alcohol group-containing ligands **22** and **23**, and proposed reaction mechanism for the dizinc complex of **23**.

obtained using Cu^{2+} instead of Zn^{2+}, probably because the square planar coordination geometry of this metal placed the substrate in a better position to interact with the ammonium groups.[28b]

Kimura and co-workers[29] appended a benzyl alcohol to a [12]aneN_4 ligand in order to provide a better nucleophile with respect to the metal-bound water molecule. The complex of **22** (Fig. 13) cleaves BNP 125 times faster than the simple [12]ane$N_4 \cdot Zn^{2+}$ complex. Observation of the formation of a complex phosphorylated at the alcohol group clearly indicates that the active nucleophile is the alcohol moiety (activated by complexation to the Zn^{2+} ion). Unfortunately, under the reaction conditions used, the phosphorylated arm did not undergo further hydrolysis and the reaction stopped after one turnover cycle.

Similar results were obtained a few years later by Bencini, Bianchi, Paoletti and co-workers,[30] who appended a hydroxyethyl arm to their [30]aneN_6O_3 bimetallic complex; the authors witnessed a 7-fold reactivity increase due to the presence of the metal-bound alkoxide, but again the transesterification product did not undergo any further cleavage. However, in this example, the reactive species was the alkoxide hydroxo bis-deprotonated species, since the alkoxide species formed after the first deprotonation bridged the two metal ions and hence was unreactive.

Bimetallic ligands with phenolate bridges are very popular, but there is only one reported example of dizinc complexes featuring such linker that cleaves BNP. Guo and co-workers[31] studied the reactivity of dizinc complexes of the phenolate-based ligand **23** (Fig. 13), equipped with two pyridylmethylamine binding units and two hydroxyethyl arms. At pH 7 and 50 °C, the rate of BNP cleavage in the presence of this complex was about 1×10^{-6} s^{-1}, which represents an acceleration of about 2500 times over the background reaction. The reactive species was the monodeprotonated complex (Fig. 13), which formed with a pK_a of 7.1. As usual with bimetallic systems, the concentration *vs.* reactivity profiles showed a saturation profile but substrate binding was very weak.

All of the examples examined so far deal with the use of a single type of functional group to increase the reactivity of the hydrolytic complex. In order to obtain better performance, we recently studied the reactivity of a derivative (**24**, Fig. 14) of

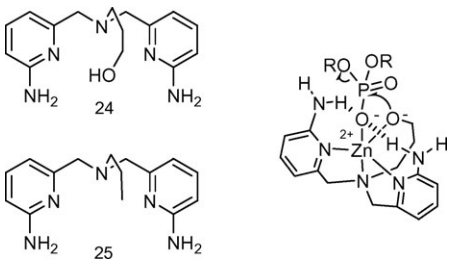

Fig. 14 Structure of ligands **24** and **25** containing hydrogen bond-donating groups, and proposed reaction mechanism for the Zn^{2+} complex of **24**.

bis(pyridylmethyl)amine, featuring both a hydroxypropyl arm as the nucleophile and two hydrogen bond-donating amino groups.[32] The Zn^{2+} complex of this ligand cleaved BNP at pH 7 and 25 °C with a rate of 1.1×10^{-5} s^{-1}, representing an almost one million-fold acceleration over the background reaction. The reactive species was the monodeprotonated (alkoxide) complex, which formed with a pK_a of 7.9, while further deprotonation of a metal-bound water molecule (pK_a = 10.2) lead to an activity decrease because of saturation of the available binding sites on the metal ion. Dissection of the contribution of the organic functional groups indicated that the alkoxide nucleophile and the two hydrogen bond donating groups brought about 17- and 230-fold reactivity increases, respectively. Such effects, combined with the relative low pK_a of the reactive species, lead to the observed acceleration. As in the previous examples, the phosphorylated complex formed in the reaction did not undergo any further cleavage, and hence the system is not catalytic. However, the analogue complex, without the hydroxypropyl arm (**25**), was still very effective and produced a true hydrolytic cleavage of the substrate.

Conjugation with DNA binder subunits. There is a further approach to more active and possibly sequence-selective artificial nucleases that is peculiar to natural substrates (DNA and RNA) that cannot be modelled using simple phosphodiesters. This approach is based on the conjugation of hydrolytically-active metal complexes to elements with a high affinity for natural polymeric substrates. As a consequence of the formation of a tight complex, the local concentration of the catalyst should increase, leading to an acceleration of the reaction. Moreover, these binding subunits may also provide sequence specificity, thus opening the way to real artificial restriction enzymes.

Taking inspiration from the excellent results obtained with oxidative DNA cleaving agents,[33] the obvious choice among the different families of compounds able to increase DNA affinity is the utilization of intercalators. Quite surprisingly, examples of metal complexes appended to intercalating groups as DNA hydrolytic agents are rare, and the effect of such elements on the reactivity of systems is not always straightforward. For example, in the case of the Scrimin *et al.*'s peptide discussed above,[16] the introduction of an acridine moiety at the N-terminus of the heptapeptide resulted in a slightly higher activity at low catalyst concentration, compensated for by a decrease at higher concentrations.

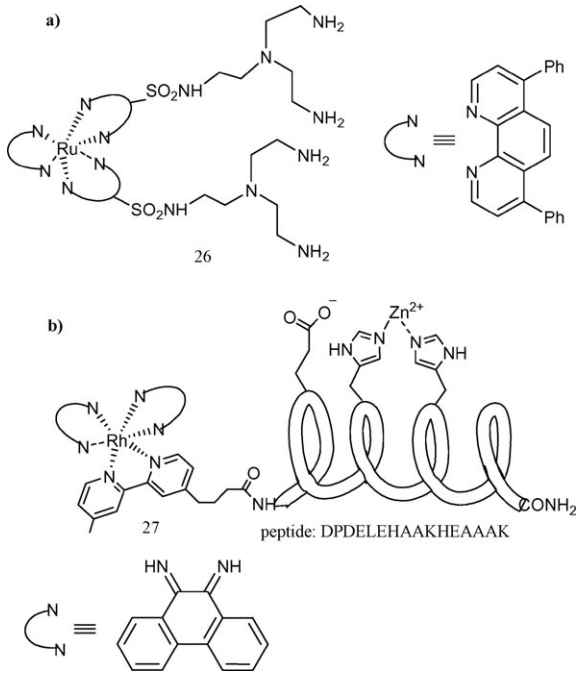

Fig. 15 DNA hydrolytic agents containing ruthenium or rhodium intercalator studied by Barton and co-workers.

In 1987, Barton and co-workers published the first example of an artificial system with DNA hydrolytic cleavage activity.[4] It was based on a ruthenium intercalator with two arms having the role of metal binding moieties (**26**, Fig. 15a). Among the different metal ions tested, the Zn^{2+} binuclear complex of this ligand cleaved plasmid DNA with high efficiency. At 37 °C and pH 8.5, 40% of the supercoiled form was degraded in the presence of a 7 μM concentration of complex after 5 h of incubation (a first order rate constant of 3×10^{-5} s^{-1} could be roughly estimated). Re-ligation experiments showed that the hydrolysis occurred randomly at the P–O3′ and P–O5′ bonds. However, the affinity of the ligand for the metal ions seemed to be quite low and a large excess of metal ion was required. Unfortunately, no evidence concerning the role played by the intercalator was reported.

Ten years later, Barton and co-workers again studied the reactivity of a mononuclear Zn^{2+} binding peptide tethered to a rhodium complex as a major groove intercalator (**27**, Fig. 15b).[34] The system promoted plasmid DNA cleavage with a rate constant of 2.5×10^{-5} s^{-1} at pH 6, 37 °C and in the presence of 5 μM complex concentration, with a similar activity also towards linear double strand DNA. Analysis of the fragments produced showed that the cleavage occurred only at the P–O3′ bonds, with a modest sequence selectivity for 5′-Pu-*Py*-Pu-Py-3′ sites (with cleavage at *Py*), presumably the result of the Rh complex binding selectivity, and the reactivity decreased as the pH approached neutrality. The peptide was scarcely structured and this was probably the source of the weak binding of the Zn^{2+} ion. On the other hand, although the presence of the rhodium complex intercalator was essential for the activity of the complex, its substitution with a different rhodium based intercalator, which preferentially binds at mismatches or with the ethidium intercalator, led to unreactive systems.[34b]

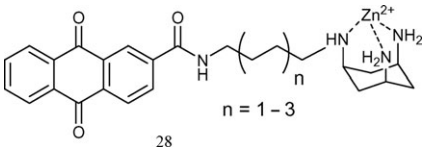

Fig. 16 Anthraquinone Zn^{2+} · TACH complex conjugates.

Geometrical requirements are important for the reactivity of these conjugate systems, and this has been further highlighted by us in a study that focused attention on the length of the spacer that tethers the intercalating unit to the catalytic group as a key element of cleavage activity. We investigated a series of *cis*,*cis*-triaminocyclohexane (TACH) Zn^{2+} complex anthraquinone intercalator conjugates linked by alkyl spacers of different lengths (**28**, Fig. 16).[35] At a concentration of 5 μM, the complex of the derivative with a C_8 alkyl spacer cleaved supercoiled DNA with a rate of 4.6×10^{-6} s^{-1} at pH 7 and 37 °C. Saturation kinetics were observed with a binding constant of about 1×10^{-4} M^{-1}, in agreement with the reported DNA affinity of anthraquinone. The conjugation of the metal complex with the intercalating group led to a 15-fold increase in the cleavage efficiency compared to the Zn triaminocyclohexane complex lacking the anthraquinone moiety. Comparison of the reactivity of the different complexes showed a remarkable increase of DNA cleaving efficiency due to the increased length of the spacer. In the case of the shortest spacer (C_4), no cleavage was observed, indicating that the advantages derived from the increased DNA affinity may be cancelled out by incorrect positioning of the reactive group.

A recent example of a DNA intercalating Zn^{2+} complex has been described by Yang and co-workers, who studied the reactivity of the Zn[(phen)(dione)Cl]ClO_4 complex **29** toward pBR322 plasmid DNA (Fig. 17).[36] In this case, the ligand itself was able to intercalate DNA with a binding constant of 2.4×10^4 M^{-1}, and the cleavage proceeded with a rate constant of 5.8×10^{-5} s^{-1} at pH 8.1, 37 °C and a 3.0 mM complex concentration. The observed rate constant was relatively high, but it has to be taken in account that it had been obtained at a high concentration of complex. Due to the nature of the ligand, a direct comparison with a non-binding complex is not possible, but the authors reported that other complexes made from Zn^{2+} and phen were not active.

The last example is taken from the work of Nomura and Sugiura, and it is probably the only known system to show DNA hydrolytic cleavage with clear sequence selectivity.[37] The authors have succeeded in preparing zinc finger peptides with hydrolytic abilities toward phosphate esters by mutating amino acid residues coordinated to the zinc ion. The zinc finger

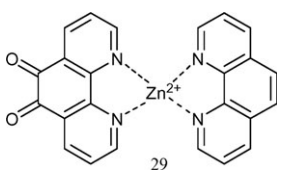

Fig. 17 Schematic structure of Zn[(phen)(dione)]$^{2+}$ complex **29**.

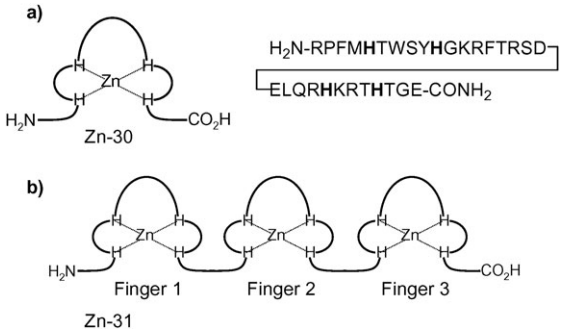

Fig. 18 (a) Schematic representation and amino acid sequence of the Zn finger peptide mutant prepared by Nomura and Sugiura. (b) The three-tandem zinc finger alignment.

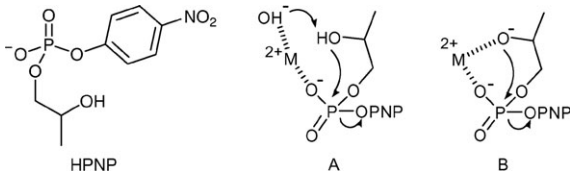

Fig. 19 Structure of HPNP and proposed mechanisms for its Zn-assisted cleavage.

mutant Zn-**30** (Fig. 18a), whose sequence is based on the second finger of the three-tandem zinc finger protein Sp1, is indeed able to cleave, with moderate activity, BNP and plasmid DNA pUC19GC, which contains the GC-rich consensus sequence (GC box), to which Sp1 binds specifically. In the case of plasmid DNA after 96 h at 37 °C, pH = 7.5 and 2 μM complex concentration, all the supercoiled form had been nicked, and the cleavage efficiency increased linearly with the zinc concentration, reaching a plateau at one equivalent of metal ion, demonstrating that the zinc complex is the reactive species. However, the cleavage is not sequence selective, and the binding of Zn-**30** to DNA appears to be mainly electrostatically driven. A sequence selective system has been obtained by the tandem alignment of three zinc finger mutants in a way similar to natural proteins, where repeated zinc finger motifs ensure a drastic enhancement in DNA affinity compared to that of a single zinc finger motif Zn-**31** (Fig. 18b). The system indeed shows an enhanced cleavage activity for pUC19GC and, more importantly, at high ionic strength, the non-specific electrostatic binding to DNA is suppressed in favor of specific interactions, such as hydrogen bonding, with DNA bases of the GC box. The selective cleavage at the GC box was finally demonstrated with a 37 bp DNA duplex containing a GC box.

RNA and its models

Substrates. RNA is less resistant to hydrolysis than DNA, with an estimated half-life at 25 °C and pH 7 of 110 years,[38] due to the presence of the 2′-hydroxyl group on the ribose ring, which acts as internal nucleophile in the cleavage reaction. This enhanced reactivity makes it easier to perform kinetic investigations directly on RNA sequences, dinucleotides or nucleotide esters. Notwithstanding, the RNA model 2-hydroxypropyl-*para*-nitrophenyl phosphate (HPNP, Fig. 19) is perhaps the most employed substrate in studies on phosphate ester hydrolytic agents. There are probably two reasons behind this fact. Firstly, HPNP reactions are reasonably fast, even at room temperature, thus allowing detailed investigations of reactivity. Secondly, the requirements for effective catalysts are less rigid, since the substrate itself contains the reactive nucleophile. As a consequence, the design of effective hydrolytic agents may be simpler.

However, the great number of studies on HPNP cleavage has still not led to a definitive assessment of the mechanism of the reaction assisted by metal ions. Two kinetically indistinguishable mechanisms are generally proposed, and apply, with some modifications, to both mono- and bimetallic systems.[2e] The first involves deprotonation of the substrate hydroxyl group by a metal-bound hydroxide (A in Fig. 19), which acts as a general base catalyst. The second possible pathway implies (B in Fig. 19) the direct coordination of the substrate's alcoholic function to the metal ion and subsequent nucleophilic attack on the phosphorous centre. Evidence in favour of both pathways has been reported. Recently, the absence of a relevant solvent kinetic isotopic effect has been observed in the cleavage of HPNP and related substrates by Zn^{2+} complexes. This finding should be taken as strong evidence in favour of the nucleophilic (B in Fig. 19) mechanism.[12,39]

In the case of RNA and non-activated esters of nucleosides, the mechanism is similar; the transfer of the phosphate ester to the 2′-oxygen forms a pentacoordinated phosphorane species, which, depending on the conditions, may be considered as an intermediate or a transition state (Fig. 20). The cleavage of the exocyclic P–O bond in the intermediate leads to the 2′,3′-cyclic phosphate and the release of the 5′-linked nucleoside as a reaction product (route **a** in Fig. 20).[2e] The departure of the leaving group is generally assumed to be the rate-determining step of the reaction. In the metal ion-catalyzed reaction, the phosphorane species is probably better viewed as a transition state or a very short living intermediate, as indicated by the absence of isomerization products in the Zn^{2+}-promoted reaction.[40] Indeed, a pseudorotation around the phosphorus atom in the phosphorane intermediate, followed by cleavage of the endocyclic 3′-O–P bond, yields the 2′-5′-isomer of the starting material (route **b** in Fig. 20). This competitive reaction, usually observed under neutral and acidic conditions in the absence of metal ions, is one of the more compelling pieces of evidence for the existence of the intermediate itself.[40] Analogously to HPNP, metal ions may accelerate the cleavage reaction by following different mechanisms, such as general base or nucleophilic catalysis.[2e] However, assisting the departure of the leaving group probably plays an important role, as suggested in the case of the Zn^{2+}-promoted cleavage of different RNA substrates with alkyl leaving groups of different pK_a (box in Fig. 20).[41] The reactivity of RNA is further complicated by the dependence of the stability of the RNA phosphodiester bond on the secondary structure of the biopolymer. Within double helical structures, intra-strand base stacking around cleavage sites prevents the departing 5′-linked nucleoside from adopting an apical position in the phosphorane intermediate, which is a prerequisite for the departure of

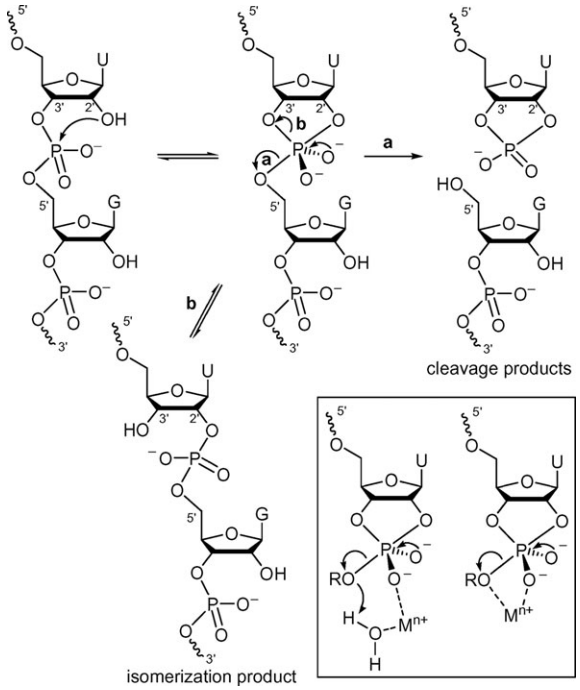

Fig. 20 Schematic mechanism for RNA cleavage (route **a**) and isomerization (route **b**). The box shows mechanisms for metal ion-assisted leaving group departure.

the leaving group. Even within a single strand, base stacking around the cleavage site is rate-retarding, and this fact must be taken in account in the design of artificial nucleases.[2a] Moreover, when using RNA, great attention should be paid to avoid contamination from natural ribonucleases, which are widely present and may lead to artefacts.[42]

The design and development of artificial ribonucleases has been extensively reviewed recently,[2a,e] and for this reason, the following sections will deal more with HPNP cleavage, and less with RNA and non-activated esters of nucleosides, for which the reader may refer to the cited reviews. Nonetheless, some selected examples will be discussed in order to outline the modern strategy currently under investigation.

Monometallic systems. RNA cleavage by free Zn^{2+} ions was first described in 1965.[43] More than twenty years later, Breslow and co-workers compared the mechanism of the cleavage of a dinucleoside monophosphate (3′,5′-UpU) and HPNP promoted by Zn^{2+} and imidazole mixtures.[44] At pH 7 and 37 °C, transesterification of HPNP was accelerated 850 times in the presence of the catalytic system and 150 times in the presence of Zn^{2+} alone. The reactivity reached its optimum at an imidazole/Zn^{2+} ratio of 24 : 1, while higher amounts of the organic base decreased the reactivity due to saturation of the binding sites on the metal. Also, the addition of strong ligands lead to a substantial reactivity decrease. The pH dependence of the reaction rate is consistent with a mechanism that involves Lewis acid catalysis by the metal ion and base catalysis by the imidazole, which promote the deprotonation of the substrate hydroxyl group. A similar mechanism was proposed for UpU (in this case, Zn^{2+} alone and Zn^{2+}/imidazole produced 32-fold and 103-fold rate accelerations, respectively), but in this

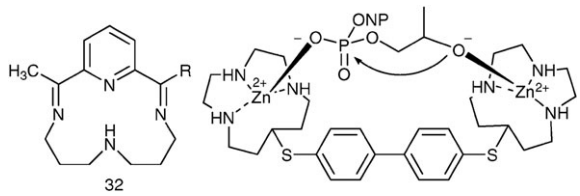

Fig. 21 Structure of tetraazamacrocycle **32** and proposed mechanism of the HPNP cleavage by the dizinc complex of **14b**.

case, a kinetically invisible protonation of the leaving group by imidazolium was postulated.

When they turned their attention to complexes of macrocyclic ligands, Breslow and co-workers found that the complex of tetraazamacrocycle **32** (Fig. 21, R = CH_3)[45] was less reactive than Zn^{2+} alone in promoting HPNP transesterification, while complexes of tridentate ligands [12]aneN_3[45] and **14a**[20a] produced, respectively, 403-fold (pH 7, 37 °C) and 194-fold (pH 8.36, 30 °C) accelerations of the transesterification reaction of HPNP. The same Zn^{2+} complex of **32** was also active toward the dinucleotide UpU, but the acceleration of the cleavage was only 9-fold (pH 8.36, 41 °C) over the background reaction.[20a]

These early results suggested that, as in the case of BNP, there are precise requirements of the ligand's structure to obtain efficient cleaving agents for RNA and its models. The investigations we have performed on the reactivity of different polyamino ligands toward HPNP confirmed these indications.[12] As is the case for BNP, the most reactive complexes are those featuring three donor atoms in a tripodal arrangement, while tetradentate ligands are substantially inactive. However, with this substrate, a β_{nuc} value of 0.75 was found in the Brønsted correlation drawn for the complexes of tripodal ligands, indicating that either the basicity or the nucleophilicity of the metal-bound species plays a more important role in the reaction of HPNP with respect to substrate Lewis acid activation.

Bi- and trimetallic complexes. In the same paper where they had investigated the reactivity of Zn^{2+} complexes of **14a** (Fig. 6),[20a] Breslow and Chapman, Jr found that the bimetallic complex **14b**, with a long 4,4′-biphenyl spacer, produced the best accelerations (1072-fold for HPNP and 39-fold for UpU), being five-fold more reactive than the monometallic example. In the mechanism proposed, they supposed that one metal ion binds the phosphate oxygen, providing Lewis acid activation, while the second coordinates to the substrate hydroxyl group, promoting its deprotonation and subsequent nucleophilic attack (Fig. 21, right). More recently, however, Breslow and Leivers partly reconsidered this work, and from experiments conducted at different metal-to-ligand ratios in the cleavage of HPNP, they concluded that at least part of the higher reactivity observed in the case of ligand **14b** was due to an increased interaction with the substrate, probably a consequence of π-stacking between the aromatic substrate and the aromatic ligand.[20b]

Around the same time, Komiyama and co-workers studied the reactivity of mono-, di- and trimetallic complexes of ligands featuring bis(pyridinylmethyl)amine units as metal

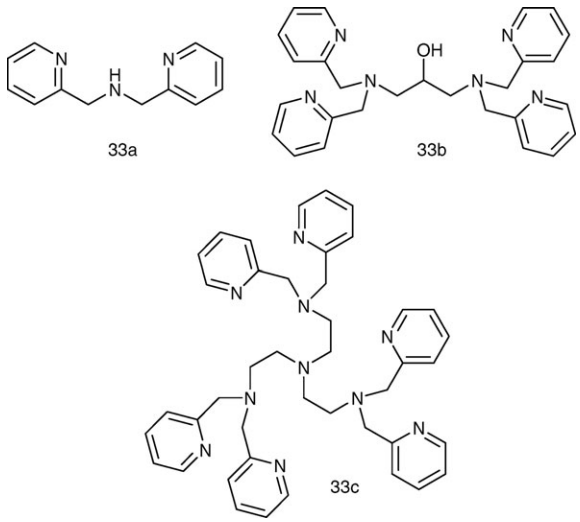

Fig. 22 Komiyama *et al.*'s bis(pyridinylmethyl)amine-containing ligands.

binding moieties (Fig. 22).[46] They found clear evidence of three metal ion cooperativity (the trimetallic complex was about three times more reactive than the bimetallic one) but accelerations over the background reaction were not reported.[47] Lippard's BPAN bimetallic complex[22a] (**16**, Fig. 8) cleaved HPNP at 25 °C and pH 7.0 with a rate of 1.5×10^{-5} s^{-1} (2900-fold acceleration[48] over the background reaction at 0.2 mM complex concentration); the system was 6 times more reactive than its mononuclear counterpart but bound the substrate very weakly (K_{ass} = 100 M^{-1}). The pH–rate profile showed a sigmoidal behavior, indicating the deprotonation of a single metal-bound species with a p$K_a$of 6.97 (formation of the bridging hydroxide).

A beautiful example of a cooperative system was reported at the end of the 1990s by Engbertsen, Reinhoudt and co-workers.[49] They modified a calix[4]arene by appending one, two or three 2,6-bis(aminomethyl)pyridine units on the upper rim. The zinc complexes of this ligand provided spectacular rate accelerations of HPNP transesterification, the most active being the trimetallic systems (**34**, Fig. 23), which reached a 32 000-fold acceleration over the background reaction at pH 7 (optimum reactivity pH) and 25 °C (50% water/CH_3CN).[49b] This system was, respectively, 35-fold more reactive than its monometallic counterparts (however, the reactivity increase with respect to the 1,3-bimetallic system was very small). The source of such efficiency must be envisaged in the peculiarity of the calixarene scaffold.[50] On the one hand, it provides a hydrophobic pocket for the binding of the nitrophenyl moiety of the substrate. On the other hand, its flexibility allows the adjustment of the intermetallic distance in order to better fulfil the requirements of the reaction. As a matter of fact, a rigidified bimetallic system, obtained by modification of the lower rim with an ether bridge, is about 8 times less reactive than its flexible counterpart.[49b] Saturation profiles were observed in each case; substrate binding constants were particularly remarkable in the bimetallic complex (5.5×10^4 M^{-1}) and decreased with the insertion of the third metal chelating unit. Interestingly, the catalyst was not active towards a

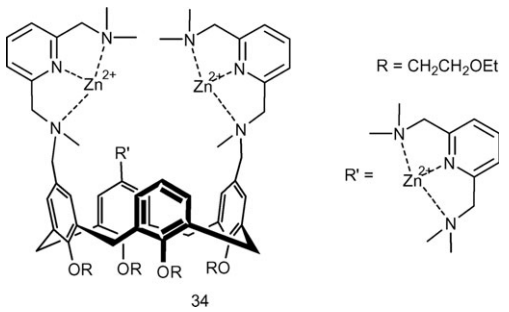

Fig. 23 Calixarene-based trimetallic complex **34**.

phosphate triester, diester or monoester.[49b] For this reason, the authors proposed a mechanism similar to that indicated by Breslow, with one Zn^{2+} ion activating the phosphate and the second promoting the deprotonation of the hydroxyl group.[51] The same complexes were also active towards diribonucleotides; UpU cleavage was accelerated 8600-fold at pH 8 and 50 °C (35% EtOH/H_2O) by the trinuclear complex.[49c] With these substrates, a remarkable difference was observed in the reactivity of the trinuclear complex compared to the binuclear complex. Moreover, activity towards dinucleoside monophosphate UpU, and particularly GpG, was much greater than that towards ApA. One of the three Zn^{2+} ions acts as a recognition unit by binding the acidic amide group on U or G. This resulted in a greater affinity of the catalytic complex for the substrate and an enhanced reactivity.

Base selective cleavage of dinucleotides has also been recently described by Wang and Lönnberg, who investigated the reactivity of Zn^{2+} dinuclear and trinuclear complexes obtained from a [12]aneN_3 derivative and spacers of different length (Fig. 24a).[52] The dinuclear complexes cleave the dinucleotides containing one uracil base (ApU and UpA) up to 100-fold more readily than those containing either two uracil bases (UpU) or two adenine bases (ApA), while the trinuclear complex cleaves without selectivity UpU, ApU and UpA, and is less active toward ApA. The observed selectivity has been explained on the basis of the formation of complexes between the metal ion catalyst and the N3 deprotonated thymidine. In the case of substrates containing a single uracil, one of the Zn^{2+}-azacrowns anchors the cleaving agent to the uracil base while the other azacrown complex acts as a catalyst for the transesterification reaction (Fig. 24b). When two uracil bases are present, both azacrown Zn^{2+} complexes are engaged in binding to the nucleobases, and this leads to kinetically unproductive complexes (Fig. 24c). However, if a third azacrown arm is present, as in **35f**, this can interact with the phosphate group, restoring the catalytic activity. On the other hand, the inability of all the metal complexes to accelerate the cleavage of ApA, regardless to the number of azacrowns present, is related to their low affinity for the adenine nucleobase.

Another highly reactive bimetallic complex has been proposed by Richard, Morrow *et al.*[53] The ligand connects two [12]aneN_3 units with a 2-propanol spacer (**36a**, Fig. 25). On the basis of the kinetic data reported, a HPNP cleavage rate of 9.7×10^{-5} s^{-1} at pH 7 and 25 °C could be calculated, which represents an acceleration of 19 000 times[48] over the

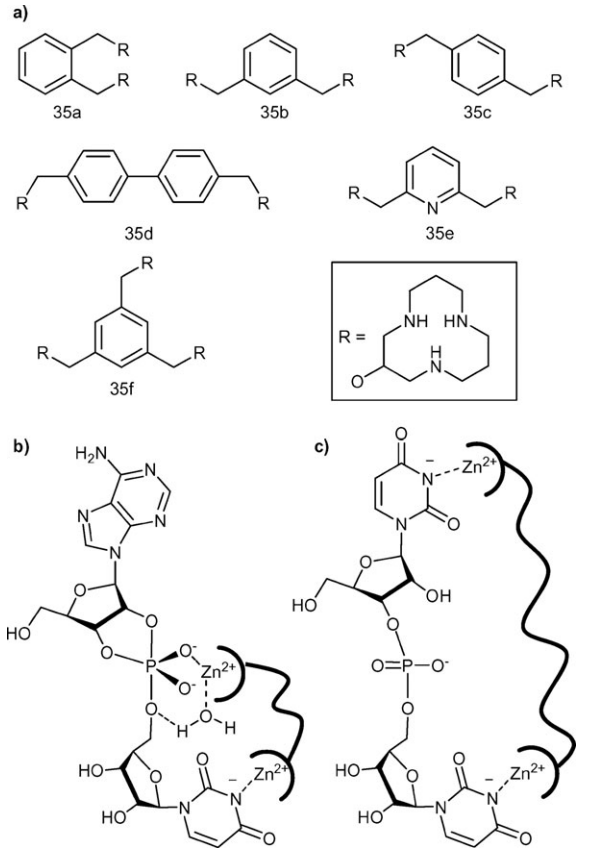

Fig. 24 (a) Polynuclear complexes investigated by Wang and Lönnberg, and a schematic representation of the mode of binding of a binuclear complex with (b) ApU and (c) UpU.

background reaction.[53d] The bimetallic complex is from 12 to 290 times more reactive than the monometallic model (**36b**), depending on the pH. Kinetic analysis also indicated in this case a weak binding of the substrate to the complex (K_{ass} = 62 M^{-1}), and that the monohydroxo complex, which forms with a pK_a value of 7.8, is the reactive species. The mechanism proposed by the authors involves a double Lewis acid activation on the substrate bridging the two metal ions and general base catalysis by the metal-bound hydroxide to assist the deprotonation of the substrate hydroxyl group. The authors also investigated a series of bimetallic complexes made by connecting the same [12]aneN_3 units with both flexible alkyl or rigid aromatic groups, finding that any system other than the one featuring the 2-propanol spacer was much less effective.[53c] The system was also active towards UpU and brought about a three order of magnitude rate increase at neutral pH towards an oligoribonucleotide.[53c,54]

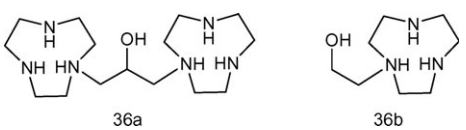

Fig. 25 Binuclear Zn^{2+} binding ligands **36a**, based on a 2-propanol bridge, and its mononuclear analogue **36b**.

However, the positioning of two or more metal ions at a distance compatible with that usually found in bimetallic nucleases (in the order of 4 Å) is not a warranty of cooperation between the metal centres in the cleavage reaction.[55] The reasons for failure vary and can be ascribed to an incorrect geometry, excessive flexibility of the spacer, formation of μ-hydroxo bridges between the metal ions and, in the case of natural substrates, the presence of more stringent steric requirements and of several donor atoms that may result in the formation of unproductive complexes. For example, this last effect has been invoked to justify the behavior observed in the case of a bimetallic Zn^{2+} complex,[55b] where the intermetallic distance is fixed at 4.5 Å by steric gearing, which accelerates the cleavage of HPNP by 80 times with respect to the mononuclear complex but is ineffective toward the dinucleoside UpU, or in the case of the bimetallic complex **36a**, which is 60-fold less active in the cleavage of uridine 3′-*para*-nitrophenyl phosphate (UpU) with respect to HPNP.[53e] However, the balance between these effects is quite delicate, as shown by the fact that **36a** is more active in the cleavage of UpU with respect to HPNP (and of course UpPNP), and this has been attributed to the stabilization of the transition state by interaction of the metal complex with the C-5′-oxyanion of the basic alkoxy leaving group, which is less favoured in the case of the more acidic *para*-nitrophenol leaving group.[53a]

In the search for more exotic spacers to obtain a better control of the reactivity of bimetallic systems, both Scrimin *et al.*[56] and Kawai *et al.*[57] turned their attention to peptides. We have already examined the 3_{10}-helix-forming peptide investigated by Pasquato, Scrimin and co-workers, and DNA cleaving agents. In the case of HPNP, the same bimetallic complex produces a 50-fold acceleration of the substrate cleavage at pH 7 and 40 °C over the background reaction, and is three-fold more reactive than the monometallic model.[16] The pH–reactivity profiles show that the monodeprotonated complex is the reactive species and, as in several previous examples, there is a moderate affinity of the substrate for the complex (K_{ass} = 250 M^{-1}). The mechanism proposed by the authors is the same that indicated in the previous examples by Breslow and Engbersen.

Kawai and co-workers[57] appended two bis(pyridinylmethyl) amine metal binding units to a cyclic peptide, assuming a stable antiparallel β-sheet conformation (**37**, Fig. 26). The complex accelerated HPNP transesterification by a factor of 6500 at pH 7 (the authors did not report the temperature of the experiments) in 80% CH_3CN/water solutions and was 300-fold more reactive than the mononuclear complex of the same peptide bearing only one bis(pyridinylmethyl)amine unit. Interestingly, a bimetallic complex made using the same binding

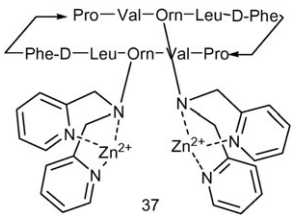

Fig. 26 Schematic structure of cyclic peptide catalyst **37**.

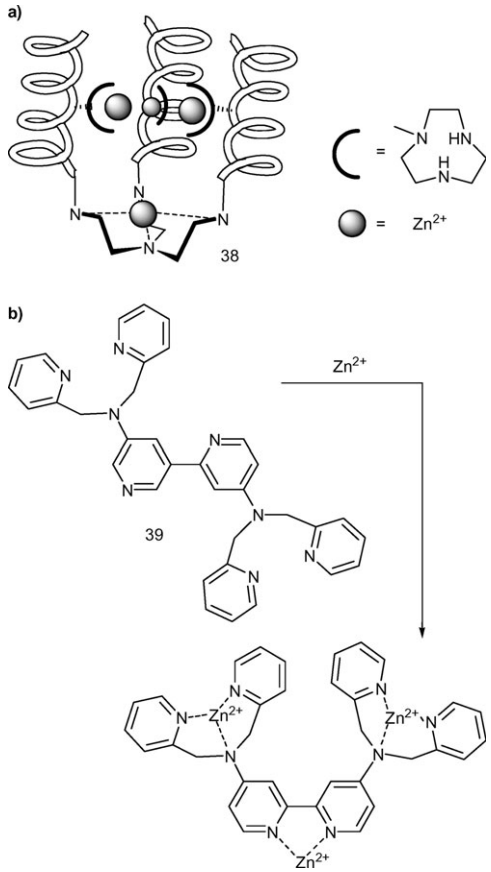

Fig. 27 Two allosteric catalysts of HPNP cleavage described by Scrimin (a) and Shinkai (b). The active conformations are shown.

units but linked by a propane spacer was found to produce only an 80-fold acceleration of the reaction, underlining the importance of the peptide scaffold.

A different approach to cooperative catalysis involves the allosteric regulation of nuclease activity by a metal ion that does not directly participate in the cleavage reaction. There are two important examples of such systems that use Zn^{2+} as a catalytic metal ion. The first came from the group of Scrimin[58] and used an allosteric TREN ligand subunit functionalized at the three primary amines with three copies of a Zn^{2+} binding heptapeptide (due to the presence of a synthetic amino acid bearing a [9]aneN_3 azamacrocycle in the lateral chain, Fig. 27a). The complex with four Zn^{2+} ions was the most active catalyst towards HPNP and cleaved the substrate 50 times more rapidly with respect to a mixture of the different monomeric ligands (rate constant = 1.2×10^{-5} s^{-1}, pH 7, 40 °C, 0.2 mM catalyst concentration) but was inactive towards an oligoribonucleotide. With the aid of UV-vis binding studies with Cu^{2+}, the authors were able to assess the role played by the different metal ion binding sites present in the catalyst and showed that the binding of the Zn^{2+} ion to the TREN subunit exerted a positive allosteric effect on the reactivity, presumably by favoring a more productive conformation of the system.[58b] More recently, the same group has reported the investigation of other allosteric systems, in which the TREN subunit has been decorated with three copies of an amino acid bearing different metal ion binding azacrowns.[58a] The mechanism of action was similar to that described above, and the Zn^{2+} bound to the TREN subunit again played the role of positive allosteric effector. The most active complex was that bearing the [12]aneN_3 ligand, cleaving HPNP with a rate constant of 1.1×10^{-5} s^{-1} at pH 7, 40 °C and 0.2 mM catalyst concentration, with a corresponding 30-fold acceleration with respect to an unassembled mixture of the different binding subunits.

The second example was reported by Shinkai and co-workers,[59] and used a bipyridine ligand as a regulatory binding site. The bipyridine was symmetrically substituted with two bis (pyrindinylmethyl)amine (DPA) ligands (Fig. 27b) as catalytic sites, and the system could complex up to three metal ions. Binding of a Zn^{2+} ion to the central bipyridine ligand induced a conformational change from transoid to cisoid, enforcing an alignment of the DPA sites at a distance of *ca.* 5 Å and favouring their cooperation in the cleavage reaction. As in the previous case, the kinetic effects were not really impressive (acceleration of about five times with respect to a system lacking the bipyridine subunit, and rate constant in the order of 2×10^{-4} s^{-1} at pH 7, 25 °C and 0.4 mM complex concentration), but these systems clearly and elegantly illustrate the concept of allosteric regulation of catalytic activity, a phenomenon frequently observed in natural systems.

Cooperativity between different active subunits can be achieved, as in the examples discussed above, by their covalent linking to a molecular scaffold or, at least in principle, by their self-assembly onto a proper template. While this approach has been used successfully for the realization of other devices, such as chemosensors,[60] its application to the development of hydrolytic catalysts remained elusive until the work of Pasquato, Scrimin *et al.*[61] The authors prepared gold nanoparticles (diameter 2.5 nm) protected on the surface by a monolayer made from a mixture of an alkyl thiol and a [9]aneN_3 azamacrocycle-functionalized thiol in the ratio 1 : 1.2 (Fig. 28). Each nanoparticle carried about 45 [9]aneN_3 subunits, and the activity towards HPNP increased, following the

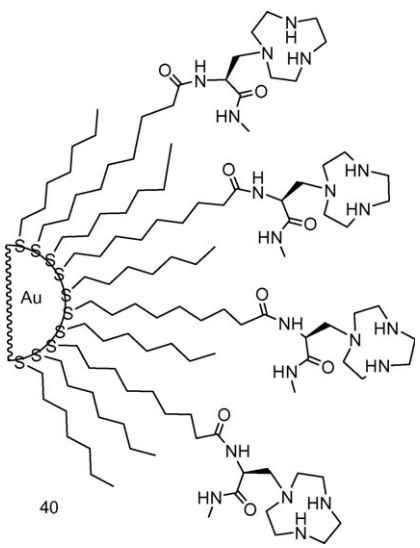

Fig. 28 Pasquato, Scrimin *et al.*'s "nanozyme", obtained by the self-assembly of [9]aneN_3 azamacrocycle-functionalized thiols on the surface of a gold nanoparticle.

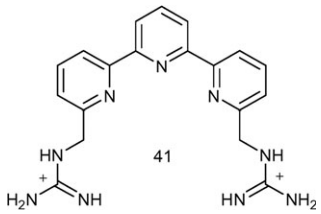

Fig. 29 Guanidinium-containing ligand **41**.

loading of the Zn^{2+} ion, reaching a maximum when all the ligands had bound one Zn^{2+} ion. Also, the substrate bound to the nanoparticle, and at pH 7.4 and 40 °C, $k_{cat} = 4.2 \times 10^{-3}$ s^{-1} and a Michaelis–Menten analysis gave $K_M = 0.93$ mM, values which are comparable to that obtained in the case of the calixarene-based ligands discussed above, ranking the system among the most active ever described. The catalyst showed a clear cooperativity, with an acceleration of over 600-fold with respect to the mononuclear complex and over 160-fold with respect to an analogous metal complex in a micellar environment. The cleavage of dinucleotides was also accelerated, although to a lesser extent, with some selectivity for UpU. The self-assembling nature of the catalyst and the high activity obtained in a true catalytic process induced the authors to call the system ''nanozyme'', in analogy to the nomenclature of catalytic polymers (synzymes).

Organic group assistance. The first example of the use of organic functional groups to increase the reactivity of metal complexes was again the pioneering work of Breslow and co-workers.[45] They studied the effect of inserting thiophenol or imidazole subunits into Zn^{2+} complexes of the tetradentate macrocyclic ligand **32** (Fig. 21), reporting an increase in the rate of cleavage of HPNP by one order of magnitude, probably *via* general base catalysis. However, the maximum acceleration reached was only 130-fold greater than the background reaction, presumably because of the use of a scarcely active tetradentate ligand.

Later on, Anslyn and co-workers studied the reactivity of the Zn^{2+} complex of the terpyridine-like ligand **41** (Fig. 29) bearing two guanidinium groups.[62] They found a six order of magnitude acceleration of the hydrolysis rate of the RNA dinucleotide ApA at pH 7.4 and 37 °C compared to the background reaction. The system was 3300-fold more reactive than the corresponding complex devoid of guanidinium groups, while substitution of the latter with ammonium groups lead to a 9-fold reactivity decrease. The active species was the monohydroxo complex, which formed with a pK_a value of 7.3. Such remarkable reactivity was partly because of the two orders of magnitude decrease of the metal-bound water pK_a due to the presence of the positively charged guanidinium groups. However, the authors also suggested that the presence of a combination of hydrogen bonding, electrostatic interactions and acid catalysis by the two guanidinium groups lead to additional substrate activation, transition state stabilization and possibly leaving group assistance.

Very recently, Mareque-Rivas, Williams *et al.*[63c] found that the presence of three hydrogen bond donating amino groups in the Zn^{2+} complex of tris(2-amino-6-pyridinylmethyl)amine

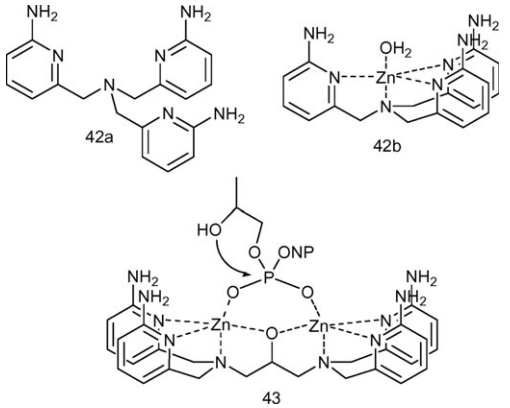

Fig. 30 Mono and bimetallic complexes containing hydrogen bond donating groups studied by Mareque-Rivas *et al.*

(**42a**, Fig. 30) increased the rate of HPNP transesterification by almost three orders of magnitude with respect to the effect of the corresponding complex devoid of amino groups. At pH 7 and 25 °C, the rate of HPNP cleavage was 7.5×10^{-5} s^{-1},[11] which corresponds to a 15 000-fold acceleration[47] over the background reaction. According to the authors, the active species is the aqua complex (**42b**), which binds the substrate through water displacement. Within this ternary complex, the substrate was activated, simultaneously by the metal ion and by three hydrogen bonds, resulting very reactive toward the base-assisted intramolecular nucleophilic attack of the hydroxyl group.[64] The authors estimated that the activation effect of the three hydrogen bonds was similar to that of a second metal ion.[63b] At pH values greater than the pK_a of formation of the hydroxo complex, the reactivity levelled off due to competition by the hydroxide with the substrate for binding to the catalyst.[65]

Such remarkable rate enhancements became spectacular when the authors combined the effect of hydrogen bond substrate activation with the use of a bimetallic complex (**43**, Fig. 30).[63a] Two bis(2-amino-6-pyridinylmethyl)amine metal binding units were connected by the well studied 2-propanol spacer to obtain a dizinc complex capable of forming four hydrogen bonds with the substrate. The half-life of HPNP in the presence of this catalyst at pH 7.4 and 25 °C was about one minute, which corresponds to almost a one million-fold acceleration over the background reaction. Saturation profiles were observed by increasing the substrate concentration, and Michaelis–Menten analysis afforded a K_{ass} value of 3100 M^{-1}. The presence of the four hydrogen bond donating groups brought about a 726-fold reactivity increase at low substrate concentrations (second order rate constant comparison). The system had an identical reactivity towards non-activated substrates; acceleration of UpU cleavage was again six orders of magnitude.

Conjugation with RNA binder subunits. As in the case of DNA, conjugation of a metal ion catalytic site with a RNA recognition subunit should increase the binding to the substrate and, possibly, confer sequence selectivity. The most explored strategy is the use of an antisense oligonucleotide (ODN), which forms a duplex with the target RNA,

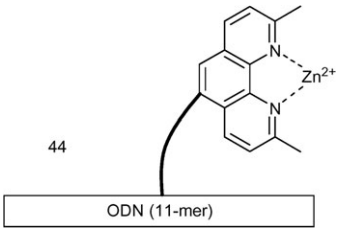

Fig. 31 Schematic representation of a catalyst–antisense oligonucleotide conjugate.

positioning the metal ion complex in proximity to the phosphodiester backbone.[2a] A typical example is reported in Fig. 31, and uses 2,9-dimethylphenanthroline as a metal ion ligand.[66] The Zn^{2+} complex of conjugate **44** cleaves nucleotides in a bulge formed upon dimerization of the ODN with the RNA substrate. Extensive studies have been carried out in order to evaluate the effects of the linker structure and site of tethering on the cleavage activity, but usually these effects are small.[2a] Due to the low formation constant of the Zn^{2+} complex with the phenanthroline ligand, a large excess of metal ion is needed to ensure the formation of the complex. The cleavage activity is rather modest, and at a catalyst concentration of 4 μM, T = 37 °C and pH = 7.4, the half life of the complementary RNA substrate is about 30 h, to which corresponds an acceleration of *ca.* 100-fold with respect to the non-conjugated metal complex. The phenanthroline ligand has also been conjugated with a peptide nucleic acid oligomer (PNA) with similar results.[67] An improved ODN–Zn complex conjugate has been obtained by using the [12]aneN_3 azamacrocycle attached at the 3′- or 5′-terminus or within the oligonucleotide as the ligand.[68] The conjugates bind Zn^{2+} much more strongly, and the cleavage efficiency depends on the site of attachment and chemical nature of the linker. However, the overall rate constants are not really different to those reached by the conjugates reported above (half-life around 20 h under similar conditions). In this case, the acceleration with respect to the non-conjugated metal complex is also two orders of magnitude.

Discussion and perspectives

Notwithstanding the huge research effort devoted to the study of zinc-based artificial nucleases, the identification of a clear pathway that could lead to the development of real applications is still not an easy task, and the results obtained so far may appear in some cases discouraging. The different levels of achievement obtained in the realization of RNA and DNA cleaving agents is clearly evident. In fact, while Zn^{2+} complexes capable of promoting the hydrolysis of DNA or its models are relatively scarce and poorly efficient with both the substrates, the situation is very different in the case of RNA cleaving agents. Here, model HPNP is cleaved by the most recent systems in timescales of seconds[63a] at room temperature and under physiological pH conditions, and cleaving agents with sequence selectivity and reasonable efficiency are already available for RNA hydrolysis.[2a]

The reasons for such differences are probably many. First of all, RNA cleavage is intrinsically easier than DNA hydrolysis. As a consequence, better results are obtained, even with less efficient catalysts. Secondly, RNA has his own nucleophile, the 2′-hydroxyl group, built into the substrate structure, and this simplifies the design of the catalyst. In fact, Lewis acid activation of the substrate is sufficient to guarantee efficient catalysis, even in the absence of reactive nucleophiles on the metal complex due, for example, to binding site saturation or μ-hydroxo bridge formation. Lastly, realization of sequence selective agents simply requires DNA conjugation, since this provides substrate selective recognition without the risk of self-cleavage.

Quite surprisingly, the large majority of studies reported focus on a single substrate category, either RNA or DNA, and their models. As a consequence, in the case of RNA cleaving agents, reactivity towards DNA is rarely assessed and reported, and *vice versa*. Clearly, we do not know if this is due to the scarce reactivity toward the other substrate category or simply because the investigation has not been performed. However, the result is that, notwithstanding the great similarity of DNA and RNA, both being phosphate diesters, it is not clear if the indication obtained for one substrate category can be applied to the other, and it appears more appropriate to separately discuss the results obtained for the two categories.

In the case of RNA model HPNP, examination of the large number of cleaving agents reported allows the identification of some structural features that the complex must contain in order to achieve high efficiency. Tridentate ligands are needed in order to grant enough solvent-occupied binding sites on the metal ion to allow the reaction to occur.[12] Furthermore, bimetallic systems are mandatory, since accelerations obtained even by the most efficient monometallic complexes are quite scarce.

In the development of bimetallic systems, the spacer between the two metal binding units plays a fundamental role, since it determines the distance between the two metal centres and consequently the possibility of their productive participation in the cleavage event. Two different linker structures appear to better fulfil the reaction requirements: calixarene[49] and 2-propanol.[46,53,54,63] Strength points for the first are flexibility, allowing the reactive complex to adjust the intermetallic distance in the substrate binding and cleavage process, and hydrophobic substrate binding. However, this latter favorable effect may be limited to the nitrophenol-containing model substrates. In fact, much lower accelerations have been observed in diribonucleotide cleavage.

The use of 2-propanol (and related 2,6-dimethylphenol) bridges provides cleaving agents with reactivities similar to those of calixarene-based examples. It seems likely that the alkoxide bridge formed by this spacer with the two metal centres plays a fundamental role in keeping them at the right distance for the reaction, while at the same time, the formation of a second, probably unreactive, μ-hydroxo bridge is somehow disfavoured. It is interesting to note that the use of different metal binding units, namely [9]aneN_3 and bis(pyridinylmethyl)amine connected by the same 2-propanol linker, leads to dizinc complexes of similar reactivity, both towards HPNP and diribonucleotides.[63a] On the contrary, connection of two [9]aneN_3 units with different spacers, even the

conceptually similar pyrazolate bridge, leads to dramatic reactivity decrements.[53c]

RNA cleaving agents of reasonable efficiency have also been reported, but their reactivity is at best 10-fold lower than that of the most active lanthanide-based systems.[2a] The general structure of such artificial Zn ribonucleases is based on the conjugation of DNA oligonucleotides with a metal binding unit through an appropriate linker. Surprisingly, the nature of the metal binding unit does not appear to affect systematically the reactivity, but only the amount of metal ion needed to reach optimum reactivity. However, even if reaction mechanisms for HPNP and RNA cleavage have some important differences, which do not allow the direct extrapolation of reactivity information obtained from one to the other, it is important to note that none of the most reactive ligands developed for the *para*-nitrophenyl model have, so far, been tested in the cleavage of RNA. This makes possible the hope that substantial benefits could be obtained by the use of more reactive metal complexes conjugated with appropriate RNA recognition elements.

As already mentioned, results obtained with BNP and DNA are much less attractive. In the case of activated BNP, both mono and bimetallic complexes achieve quite unimpressive rate accelerations at physiological pH, values being in the range one to ten thousand-fold. Moreover, very scarce improvements have been obtained since the earlier work of Breslow, whose biphenyl-based bimetallic complex of ligand **14b** had already achieved a 5000-fold rate acceleration. It seems that bimetallic complexes quite invariably form unreactive μ-hydroxo bridges, which are replaced by nucleophilic metal-bound hydroxide ions only at very high pH values.[21,25] Incrementing the intermetallic distance may help disfavour μ-hydroxo bridge formation, but benefits for the reactivity are again quite scarce, since larger metal separations make double substrate Lewis acid activation of the substrate more difficult to obtain and hence producing less efficient catalysis.[23]

None of the most reactive bimetallic systems used for HPNP cleavage has apparently been tested with BNP, the only exception being Engbertsen, Reinhoudt and co-workers' calixarene-based catalysts, which are reported to be unreactive towards activated phosphate diesters.[49b] This may be insignificant, but since the two substrates may require different catalytic properties, it could also indicate that the successful strategies employed to obtain the very reactive systems toward HPNP cleavage may not be transferred to BNP cleavage. This also seems to be suggested by the quite low reactivity displayed by the dizinc catalyst toward BNP reported by Guo *et al.*,[31] which contains the 2,6-dimethylphenolate bridge, a typical structural element of the most reactive HPNP cleaving agents.

It is also surprising that the reactivity towards plasmid DNA is somewhat more satisfying, probably because of the intrinsic reactivity amplification properties of this substrate, and cleavage rates in the order of $2–4 \times 10^{-5}$ s^{-1} have been achieved, corresponding to half life times of 6–8 h. All the most active examples reported, with the exception of Scrimin *et al.*'s peptide-based system,[16] benefit from conjugation with DNA affinity elements, such as intercalating moieties. In this way, the reactive complex is brought into close proximity with the phosphate groups, and this favours the reaction. However, identifying the right DNA affinity unit and the appropriate way of conjugating it to the reactive metal complex is not trivial, and in many examples, the simple appending of a metal complex to a high DNA affinity molecule has led to the realization of an unreactive system. Moreover, in the case of bimetallic complexes, this approach has been scarcely investigated, and this may suggest that there is still hope for the future realization of more reactive systems.

In the case of both DNA and RNA models, the ultimate strategy to obtain highly reactive systems appears to be cooperation with organic groups. Examples reported by us[32] and particularly the group of Mareque-Rivas[63] demonstrate that the effect of two intracomplex hydrogen bonds is similar to that of one additional metal ion. Even if more detailed mechanistic investigations are needed, the highly reactive systems reported by Kovari and Krämer,[28] Anslyn *et al.*[62] and probably also Ichikawa *et al.*[13] clearly confirm such indications. This allows the multiplication of activation effects while still using quite simple and synthetically accessible systems. The final result is the spectacular acceleration of HPNP cleavage obtained by Mareque-Rivas by combining the effect of four hydrogen bonds and two metal ions. It is very stimulating to note that such reactivity is maintained with RNA dinucleotides, opening the way to the realization of highly efficient artificial ribonucleases.

Finally, "nanozymes"[61] represent a completely new approach to the realization of phosphate diester cleavage catalysts. Although it is too early to draw any clear indications, the potential of such systems appear to be very promising. Indeed, in this case, cooperativity between the reactive functions is achieved *via* a self-assembly process that reduces dramatically the synthetic complexity and opens up the way to a combinatorial optimization of the reactive system. Moreover, the presence of the "hard" gold core may be very useful for biological applications because it allows the use of delivery techniques, such as gene-gun, that are not suitable for "molecular-sized" catalysts. In the next few years, it will be interesting to see if this approach really competes with the more traditional "covalent" one.

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