Metal ion-promoted cleavage of mRNA 5′-cap models: hydrolysis of the triphosphate bridge and reactions of the N^7-methylguanine base

Satu Valakoski, Suvi Heiskanen, Sanna Andersson, Mari Lähde and Satu Mikkola*

University of Turku, Department of Chemistry, FIN-20014 Turku, Finland

Reactions of mRNA 5′-cap model compounds were studied to evaluate the potential of these reactions in the development of artificial RNases. Diadenosine triphosphate was used as a model for the triphosphate bridge, and its hydrolysis was studied in the presence of several Cu^{2+} complexes. The results of the kinetic experiments show that bifunctional catalysis by phosphate bound Cu^{2+} complexes is involved. The most efficient catalysis is achieved with complexes with acidic aqua ligands, and a metal ion-bound hydroxo ligand most probably acts as a nucleophile in the reaction. A detailed mechanism cannot, however, be suggested on the basis of the data. N^7-methylguanosine and its 5′-monophosphate and diphosphate were used to study the reactions of the N^7-methylguanine base of the mRNA 5′-cap moiety. While Cu^{2+} complexes efficiently enhance the hydrolysis of the triphosphate bridge, little effect on the reactions of the N^7-methylguanine base was observed: neither the cleavage of the imidazole ring or the depurination of the nucleoside were enhanced to any significant extent.
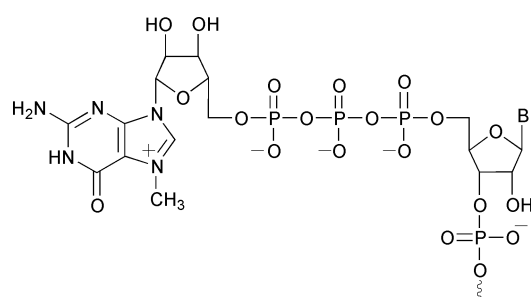
Introduction

Selective elimination of intracellular mRNA molecules by conjugates that recognise their target and actively enhance a chemical modification within it is generally believed to form a novel method to treat viral or bacterial infections or to prevent the propagation of tumor cells.[1] The 5′-terminal cap-structure of eukaryotic mRNA molecules, which is essential for stability[2] and intracellular processing of the molecule,[3–7] is an attractive target for chemical modification. Even a modest structural alteration in the 5′-cap moiety has been shown[8] to prevent the recognition of the mRNA molecule by the cap-binding enzymes involved and hence the biosynthesis of the corresponding protein molecule. Specific inhibition of protein biosynthesis resulting from the Eu^{3+} complex promoted cleavage of the 5′-cap structure has recently been reported.[9]

The 5′-cap structure consists of N^7-methylguanosine attached to the terminal nucleotide of the RNA strand via a 5′,5′-triphosphate bridge (Scheme 1).[5–7] While the hydrolysis

Scheme 1

of nucleoside triphosphates has been extensively studied,[10–14] studies on the hydrolysis of dialkyl triphosphates are less abundant. Results obtained with 5′-cap models have shown that similarly to the hydrolysis of nucleoside triphosphates, the hydrolysis of dinucleoside triphosphates is promoted by metal ions and their complexes.[15–20] There appears, however, to be a difference between these two classes of compounds: while
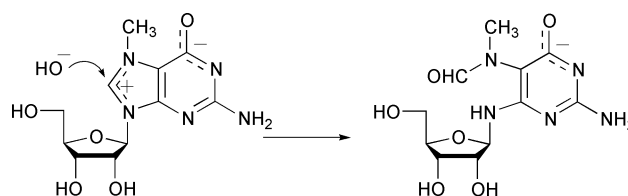
a large variety of metal ions promote the hydrolysis of nucleotides,[10–14] such as ATP, only a few metal ions enhance the reaction of dialkyl triphosphates. In the case of 5′-cap models, the most efficient catalysis has been achieved with complexes of trivalent lanthanide ions[16,20] and Cu^{2+}.[15,17–19] Information about the catalytic properties of metal ion complexes other than those of lanthanide ions or Cu^{2+} is scarce. Monometallic Zn^{2+} species have been mentioned as clearly less active than Cu^{2+} complexes.[15] In all cases constructs that contain more than one metal ion generally are more efficient catalysts than mononuclear metal ion complexes.[17,18]

Another potential cleavage site within the 5′-cap structure is the N^7-methylguanine base. The base is electrophilic, and under alkaline conditions its imidazole ring undergoes a rapid cleavage as a result of a nucleophilic attack by a hydroxide ion at C8 (Scheme 2).[21] An attack by a nucleophile other than the
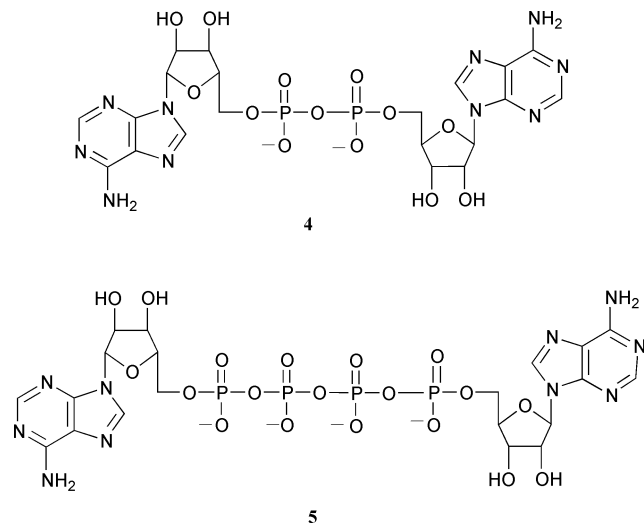

Scheme 2

hydroxide ion has not been reported, even though it could be presumed that the positively charged form of the base present under neutral conditions is even more electrophilic than the zwitterion ion predominating under alkaline conditions (pK_a 7.3[22] at 25 °C).

This work studies the hydrolysis of simple mRNA 5′-cap model compounds in the presence of metal ion complexes to find efficient catalysts for their cleavage, and to evaluate the potential of the catalysts as constituents of artificial nucleases targeted towards the 5′-cap moiety of mRNA. Hydrolysis of diadenosine triphosphate (ApppA; **1**,) to AMP (**2**) and ADP (**3**) (Scheme 3), and the hydrolysis of diphosphate and tetraphos-
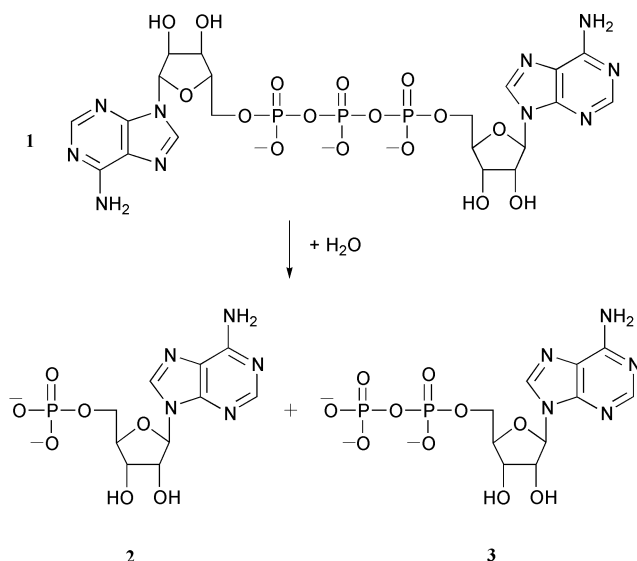
Scheme 3

phate analogues of **1** (**4** and **5**, respectively) was studied to obtain detailed mechanistic information about the metal ion-promoted hydrolysis of the triphosphate bridge. These compounds can be regarded as relevant models, since metal ion complexes most probably interact with these substrates only through the triphosphate moiety. A bipyridine ligand has been reported to prevent an interaction of Cu²⁺ with the base moiety[23] and a bidentate binding of Cu²⁺-terpyridine to two phosphate groups of adenosine diphosphate has been shown by an X-ray structure.[24] The effect of the metal ion complexes on the ring-opening reaction of the N^7-methylguanine base (Scheme 2) was studied by using the corresponding nucleoside (N^7-mGuo; **6**) and its 5′-monophosphate (N^7-mGMP; **7**) and diphosphate (N^7-mGDP; **8**) as model compounds.


Results

Hydrolysis of the triphosphate bridge

The hydrolysis of P¹,P³-diadenosine triphosphate (**1**) (Scheme 3), P¹,P²-diadenosine diphosphate (**4**) and P¹,P⁴-diadenosine tetraphosphate (**5**) was studied in the presence of different metal ion complexes (**9**–**14**) at 60 °C under neutral and slightly alkaline conditions. Aliquots withdrawn from the reaction solutions were analysed either by capillary zone electrophoresis (CZE) or reversed-phase HPLC. In both analysis methods, the starting material and all the products could be separated under the conditions described in the Experimental section. Pseudo-first-order rate constants of the disappearance of the starting
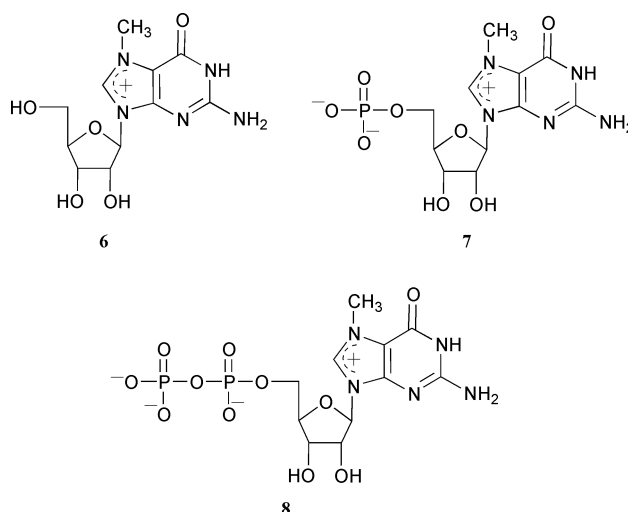
material were calculated by using an integrated first-order rate law. Products were identified by spiking with authentic samples. In reactions of **1**, in the presence of complexes **10**–**14**, the only products formed were adenosine 5′-monophosphate (AMP, **2**) and adenosine 5′-diphosphate (ADP, **3**). Reactions of the tetraphosphate **5** additionally produced adenosine 5′-triphosphate (ATP, **15**). AMP was the only product in reactions of **4**. Formation of adenosine (**16**) or adenine (**17**) was not observed.
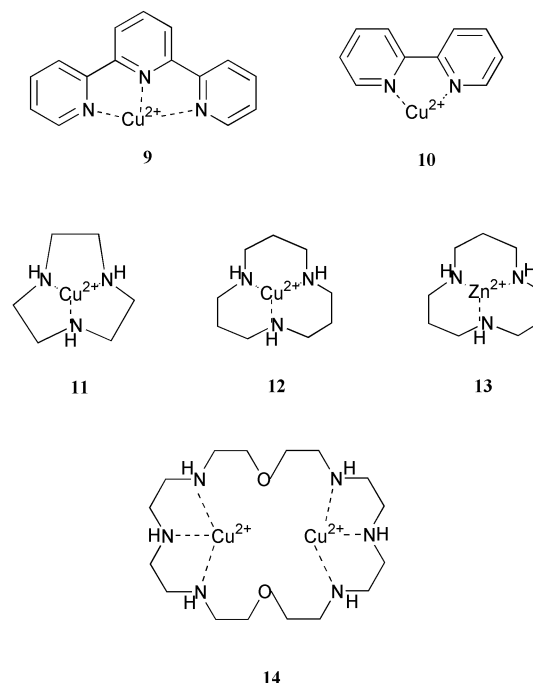
In HEPES [HEPES = N-(2-hydroxyethyl)piperazine-N'-ethanesulfonic acid] buffers the Cu²⁺TerPy promoted hydrolysis of **1**, **5** and **15** was accompanied by a formation of a by-product that most probably is a HEPES adduct of 5′-AMP. Consistent with the assignment, reactions of Cu²⁺TerPy with **1** in CHES [CHES = 2-(N-cyclohexylamino)ethanesulfonic acid] buffers of comparable basicity only gave AMP and ADP. In the CHES buffers these products were formed in an equimolar concentration, while in HEPES buffers the concentration of AMP observed was only 60% of the concentration of ADP. The rate constant of disappearance of **1** in the presence of 10 mM Cu²⁺–terpyridine in HEPES buffer is 20 % larger than a rate constant of the same reaction in a CHES buffer. Results of HPLC-MS/MS characterisation ($M + 1$ value of 567), the UV-spectrum obtained by a diode array detector and the electrophoretic behaviour of the product are also consistent with the suggestion.

15

16   **17**
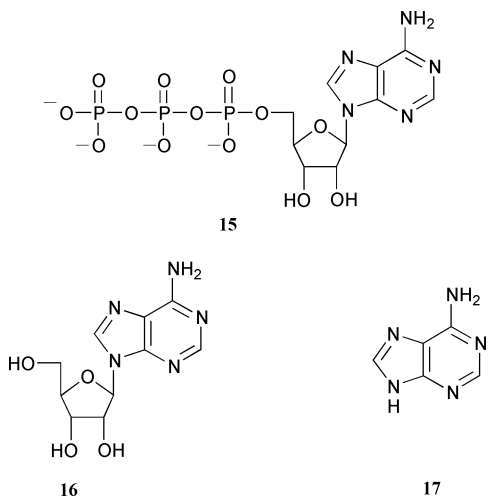
The effect of pH on the rate constants of the Cu^{2+}–terpyridine (Cu^{2+}TerPy, **9**) promoted hydrolysis of diadenosine triphosphate (**1**), diphosphate (**4**) and tetraphosphate (**5**), and of the Cu^{2+}–bipyridine (Cu^{2+}BiPy; **10**) and Zn^{2+}–1,5,9-triazacyclododecane (Zn^{2+}[12]aneN$_3$, **13**) promoted reaction of **1** over a limited pH range from 5.5 to 8.5 is shown in Fig. 1.
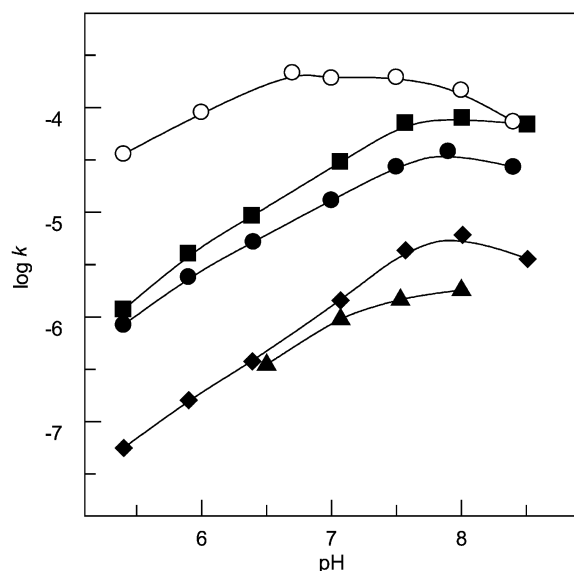
Fig. 1 The effect of pH on the metal ion promoted hydrolysis of the phosphate bridge of 5′-cap model compounds. Legend: (○) ApppA (**1**)–2mM Cu^{2+}BiPy; (■) AppppA (**5**)–2 mM Cu^{2+}TerPy; (●) ApppA (**1**)–2 mM Cu^{2+}TerPy; (◆) AppA (**4**)–2 mM Cu^{2+}TerPy; (▲) ApppA (**1**)–2 mM Zn^{2+}[12]aneN$_3$. pH adjusted with 0.1 M HEPES buffer, ionic strength 0.1 M adjusted with NaNO$_3$.

Under these conditions the hydrolysis of **1** and **5** exhibited clear first-order kinetics, the plots ln *A* *vs. t* being linear. In reactions of **4** some curvature was observed on a longer reaction time. This, however, most probably results from slow dissociation of the complexes rather than a second-order dependence of the reaction rate on [**4**]. As the reactions of **4** are slower than hydrolysis of **1** or **5**, the inactivation of the catalysts is only observed in this case.

As can be seen in Fig. 1, the hydrolysis of **1**, **4** and **5** in the presence of Cu^{2+}TerPy exhibits a first-order dependence on hydroxide ion concentration at pH below 8, but the rate-increase levels off to zero-order dependence as the pH is further increased. The optimal pH of the Cu^{2+}BiPy-promoted cleavage of **1** is lower than that of the corresponding reaction in the presence of Cu^{2+}TerPy: The maximal rate is reached around pH 7. Under these conditions the Cu^{2+}BiPy -promoted reactions are faster than those promoted by the Cu^{2+} TerPy-

complex: the maximal rate constants obtained differ by a factor of 7. The pH–rate profile of the Zn^{2+}[12]aneN$_3$ (**13**) promoted reaction consists only of four points, but it appears that the maximal rate is obtained at the same pH as with Cu^{2+}TerPy, but the levelling off may be slightly less pronounced.

Even though the shape of the pH–rate profiles of the cleavage of **4** and **5** seem to be similar to those determined for the cleavage of **1** under the same conditions, the absolute values of the rate constants are different. The cleavage of diadenosine tetraphosphate (**5**) by the Cu^{2+}–TerPy complex is two to four times faster than the Cu^{2+}TerPy-promoted cleavage of **1**, which may well result from the stronger electrostatic attraction by the more anionic tetraphosphate moiety. The rate constants of the cleavage of diadenosine diphosphate (**4**) are approximately one order of magnitude smaller than those of the cleavage of the triphosphate **1**. The reactivity difference in the presence of Cu^{2+}BiPy appears to be of the same magnitude. The rate constant of the Cu^{2+}BiPy (2.0 mM) promoted hydrolysis of **4** at pH 7.5 and at 60 °C is $(1.09 \pm 0.02) \times 10^{-5}$ s^{-1}.

The rate-enhancement by the complexes is rather significant. The spontaneous hydrolysis of **1** was followed over a pH range from 6.0 to 10.0 for more than four months. A rate constant could only be obtained at pH 6.0, where a value of 7.5×10^{-8} s^{-1} was determined. At pH higher than that, less than 5% of the starting material was cleaved in four months at 60 °C, suggesting that the rate constants are of order of 5×10^{-9} s^{-1} or less. At least a 20000-fold rate-enhancement can hence be obtained with 2 mM Cu^{2+}BiPy complex under neutral and slightly alkaline conditions. The bimetallic $Cu^{2+}{}_2$–bisdien complex (**14**) is a slightly more efficient catalyst, but rate constants could only be obtained under neutral conditions. The rate constant obtained in the presence of 1 mM complex at pH 5.7 and 60 °C was $(7.4 \pm 0.2) \times 10^{-5}$ s^{-1}. At a higher pH a precipitate was formed during the kinetic run. The complex also appears to be rapidly inactivated under the experimental conditions, which has previously been attributed to irreversible formation of strong phosphate complexes.[25] Consistent with previously reported results,[14] Zn^{2+}[12]aneN$_3$ is a rather poor catalyst in comparison to Cu^{2+} complexes. Over the pH range studied, Cu^{2+}BiPy promoted reactions, for example, are approximately a hundred times more efficient than those promoted by **13**. An efficient reaction of the bisdien ligand with ATP has previously been reported[13,26] but no such activity was observed with **1** : bisdien in 2 mM concentration did not induce any reaction to any significant extent in three weeks at pH 7.0 and 60 °C.

The rate constants of the cleavage of **1** as a function of the Cu^{2+} complex concentration are shown in Fig. 2. The concentration of the complexes ranges from 2 to 10 mM. The narrow concentration range is determined by practical reasons: to maintain pseudo-first-order conditions, the concentration of the catalyst had to be at least 10 times higher than that of the substrate. On the other hand, the complexes tend to precipitate if the concentration is too high. It can be seen in Fig. 2 that there is a major difference between the complexes studied: reactions catalysed by different complexes show a dissimilar dependence of the rate on the metal ion concentration. The Cu^{2+}TerPy promoted reaction shows a higher than first-order dependence on the complex concentration. The slopes of the log (k/s^{-1}) *vs.* log (c/M) plots were 1.5 and 2.0 at pH 6.0 and 8.0, respectively (Table 1). In contrast, values of 0.9 and 0.8 were obtained for the Cu^{2+}BiPy promoted hydrolysis. A similar difference was observed between Cu^{2+}[12]aneN$_3$ (**12**) and Cu^{2+}[9]aneN$_3$ (**11**) ([9]aneN$_3$ = 1,4,7-triazacyclononane). The slopes of the plots log (k/s^{-1}) *vs.* log (c/M) were 1.9 and 0.8, respectively (Table 1). The slope also seems to be dependent on the substrate; in Cu^{2+}[12]aneN$_3$ promoted reaction of AppA (**4**), the slope is clearly smaller than in reaction of **1** (Table 1).

The steep dependence of the rate constants on the concentration of Cu^{2+}TerPy and Cu^{2+}[12]aneN$_3$ shows that more than one metal ion probably is involved in the rate-limiting step of

Table 1 The effect of the copper complex concentration on the hydrolysis of the phosphoanhydride bridge mRNA 5′-cap models **1** and **4** at 60 °C

Substrate	Catalyst	pH[a]	Slope of the plot[b] log (k/s^{-1}) *vs.* log (c/M)
ApppA (**1**)	Cu²⁺TerPy	6.0	1.5 ± 0.1
ApppA (**1**)	Cu²⁺TerPy	8.0	2.0 ± 0.1
ApppA (**1**)	Cu²⁺BiPy	6.0	0.9 ± 0.1
ApppA (**1**)	Cu²⁺BiPy	8.0	0.8 ± 0.1
ApppA (**1**)	Cu²⁺[12]aneN₃	7.5	1.9 ± 0.1
ApppA (**1**)	Cu²⁺[9]aneN₃	8.0	0.8 ± 0.1
AppA (**4**)	Cu²⁺[12]aneN₃	7.5	1.3 ± 0.1

[a] pH adjusted with 0.1 M HEPES buffer, $I = 0.1$ M with NaNO₃. The pH values reported refer to 20 °C. [b] Plots are shown in Fig. 2.
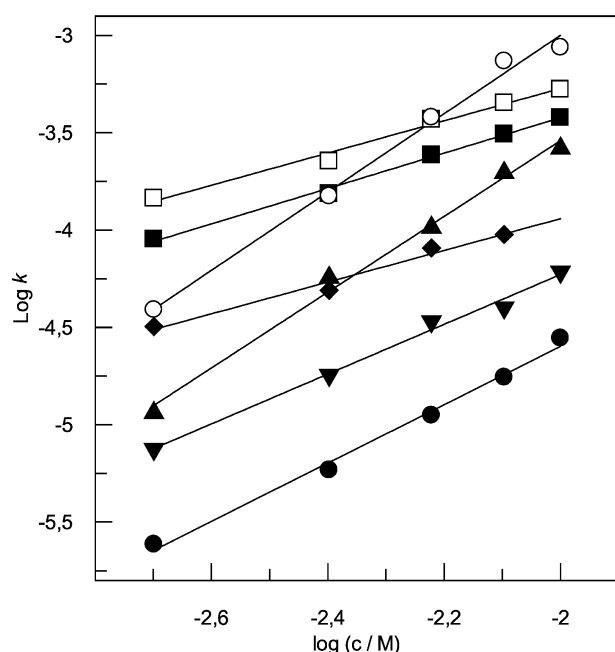
Fig. 2 The effect of catalyst concentration of the metal ion promoted hydrolysis of the triphosphate bridge of 5′-cap model compounds. Legend: (□) ApppA (**1**)–Cu²⁺BiPy, pH 8.0; (■) ApppA (**1**)–Cu²⁺BiPy, pH 6.0; (○) ApppA (**1**)–Cu²⁺TerPy, pH 8.0; (◆) ApppA (**1**)–Cu²⁺[9]aneN₃, pH 8.0; (▲) ApppA (**1**)–Cu²⁺[12]aneN₃, pH 7.5; (▼) AppA (**4**)–Cu²⁺[12]aneN₃, pH 7.5; (●) ApppA (**1**)–Cu²⁺TerPy, pH 6.0. pH adjusted with HEPES buffer. The values reported refer to 20 °C. I adjusted to 0.1 M with NaNO₃. Slopes of the plots are collected in Table 1.

the reaction. To study the role of individual metal ions in the catalysis, experiments were carried out where a replacement of a metal ion by a co-catalyst was attempted. When ethylamine was added as a potential external nucleophile in Cu²⁺TerPy promoted reaction at pH 8.0, it was observed that an increasing concentration of ethylamine slightly decreased the rate of the hydrolysis of **1**. The rate constants obtained were $(3.0 \pm 0.1) \times 10^{-5}$, $(2.4 \pm 0.1) \times 10^{-5}$ and $(2.1 \pm 0.1) \times 10^{-5}$ s^{-1}, at an ethylamine concentration of 5, 25 and 50 mM, respectively. A rate constant of $(3.8 \pm 0.1) \times 10^{-5}$ s^{-1} was obtained in the absence of ethylamine.

Table 2 shows the results of experiments where Mg²⁺ ions were added into reaction solutions to study whether the catalytic efficiency of Cu²⁺ complexes could also be enhanced by another metal ion. The pK_a of the Mg²⁺-bound aqua ligand is 12.8[27] (30 °C and $I = 0.1$ M) and consequently Mg²⁺ cannot provide hydroxide ion catalysts in the reaction, but it can enhance the reaction by coordinating to the phosphate and hence making it more electrophilic. Mg²⁺ was added at a concentration equal to or less than that of Cu²⁺TerPy, and in ten-fold excess. The results in Table 2 show that under these conditions Mg²⁺ itself is inactive as a catalyst and the addition of Mg²⁺ ion slightly retards the Cu²⁺TerPy promoted hydrolysis rather than enhances it.

Table 2 The effect of Mg²⁺ ions (added as the nitrate salt) on the Cu²⁺TerPy (2.0 mM) promoted reaction of **1** at pH 7.5[a] and 60 °C

[Mg(NO₃)₂]/M	$k/10^{-5}$ s^{-1}
None	No reaction in ten days
0.5	2.6 ± 0.1
1.0	2.04 ± 0.04
2.0	1.78 ± 0.05
5.0	1.28 ± 0.03
20	0.80 ± 0.02

[a] Adjusted with 0.1 M HEPES buffer. pH refers to 20 °C. Ionic strength was adjusted to 0.1 M with NaNO₃.

Cleavage of the N^7-methylguanine base

The effect of Cu²⁺ complexes on the cleavage of the N^7-guanine base appears to be very modest (Table 3). Only a 5 to 10-fold rate-enhancement of the cleavage of the nucleoside **6** was observed in 10 mM Cu²⁺TerPy and Cu²⁺BiPy solutions. 5′-Phosphorylation that might allow complexation with the Cu²⁺ catalyst and hence an intramolecular attack of the hydroxo ligand on C8, does not seem to enforce the catalysis. Increasing metal ion concentration only slightly enhances the reaction of N^7-methylguanosine 5′-monophosphate (**7**) (Table 3). Rate-enhancement of the same magnitude was observed also with N^7-methylguanosine 5′-diphosphate (**8**). The maximal rate enhancement by 10 mM metal ion complexes is approximately 10-fold.

The products of these reactions were identified by comparing them with products formed in well-known reactions of N^7-methylguanine derivatives. Under alkaline conditions a hydroxide ion attacks the base at C8, which results in a rapid cleavage of the imidazole ring (Scheme 2).[21] Mixing aliquots from alkaline reaction solution and a neutral solution containing Cu²⁺ complex, and analysing the mixture by RP-HPLC shows that the same products are formed in both reactions. In the presence of Cu²⁺ complexes, the formation of the initial ring-opening product (**I** in Scheme 2) is apparently slow and a significant amount of subsequent products is formed during the reaction. In contrast to this, in the alkaline cleavage **I** is the predominant product and its subsequent reactions are slow in comparison to its formation. Under the experimental conditions, N^7-methylguanosine is also depurinated (Scheme 4), which is shown by a corresponding comparison analysis using acid catalysed depurination[28] as a reference reaction. Metal ion complexes do not however seem to enhance the depurination, but the rate appears to be comparable to that of the spontaneous reaction under neutral conditions. In contrast, a slight enhancement by Cu²⁺ aqua ions of depurination of N^7-methylguanosine was previously observed under slightly acidic conditions.[19]

Discussion

Cu²⁺ complexes efficiently promote the hydrolysis of the triphosphate bridge in dialkyl oligophosphates. More than 20 000-fold rate-enhancement by these complexes was observed under

Table 3 The effect of $Cu^{2+}TerPy$ and $Cu^{2+}BiPy$ on the cleavage of N^7-methylguanine base at 60 °C

Substrate	Catalyst	Conditions[a]	$k/10^{-6}\ s^{-1}$
N^7-mGuo (**6**)	None	pH 7.9, $I = 0.15$ M	5.8 ± 0.8
N^7-mGuo (**6**)	2 mM $Cu^{2+}TerPy$	pH 7.9, $I = 0.15$ M	14 ± 2
N^7-mGuo (**6**)	10 mM $Cu^{2+}TerPy$	pH 7.9, $I = 0.15$ M	32 ± 2
N^7-mGMP (**7**)	None	pH 7.0, $I = 0.10$ M	2.2 ± 0.1
N^7-mGMP (**7**)	2 mM $Cu^{2+}TerPy$	pH 7.0, $I = 0.15$ M	6.3 ± 0.3
N^7-mGMP (**7**)	5 mM $Cu^{2+}TerPy$	pH 7.0, $I = 0.15$ M	9.0 ± 0.4
N^7-mGMP (**7**)	10 mM $Cu^{2+}TerPy$	pH 7.0, $I = 0.10$ M	10.1 ± 0.3
N^7-mGMP (**7**)	10mM $Cu^{2+}BiPy$	pH 7.0, $I = 0.15$ M	8.5 ± 0.7

[a] pH adjusted with 0.1 M HEPES buffer. The values reported refer to 20 °C. Ionic strength adjusted with $NaNO_3$.
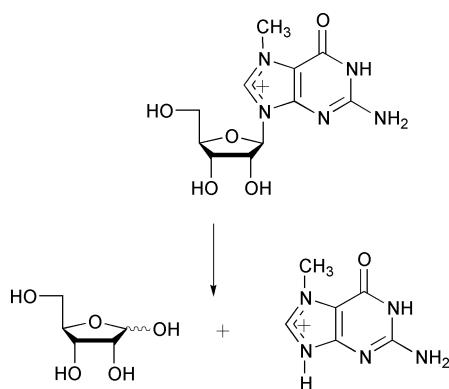
Scheme 4

neutral conditions. $Zn^{2+}[12]aneN_3$ is clearly less active a catalyst and Mg^{2+} does not enhance the reaction to any significant extent. The catalytic activity of bimetallic Cu^{2+}_2bisdien (**14**), with a hydroxo bridge between the two Cu^{2+} ions,[29] is slightly higher than that of monometallic complexes. Further studies on this complex were, however, prevented by the instability of the complex. The bisdien ligand, which has previously[20] been shown to react efficiently with ATP through a nucleophilic attack of an amino function to a phosphate group, did not enhance the reaction of **1**.

The rate-enhancement by $Cu^{2+}TerPy$ and $Cu^{2+}BiPy$ in this work appears to be larger than in the cleavage of RNA model compounds. However, the difference strongly depends on the source of the data and the choice of the model compound. A meaningful comparison is also made difficult by the fact that the reaction conditions have different effects on different reactions. The hydrolysis of RNA model compounds typically shows a first-order dependence on the concentration of monomeric Cu^{2+} complexes[30,31] whereas a second-order dependence is seen in the present work. Results of Stern *et al.*[32] show that at pH 7.1, 0.16 mM $Cu^{2+}TerPy$ and $Cu^{2+}BiPy$ promote the cleavage of oligo-A by factors of 40 and 15, respectively. Rate-enhancement of the cleavage of a chimeric oligonucleotide by 1 mM $Cu^{2+}TerPy$ at pH 8.1 appears to be even more modest.[33] The cleavage of 2′,3′-cAMP, a simpler model compound, is promoted to a more significant extent: using the rate constants of the spontaneous cleavage reported by Ora *et al.*[34] and the temperature dependence of the rate constants reported by Eftinkt and Biltonen,[35] an approximately 1000-fold rate-enhancement by 2 mM $Cu^{2+}TerPy$ and $Cu^{2+}BiPy$ at pH 7.5 can be estimated from the rate constants reported by Liu *et al.*[36] Rate-enhancement of the cleavage of dinucleoside monophosphate 3′,5′-ApA by 10 mM $Cu^{2+}TerPy$ at pH 8.2 appears to be of the same order, whereas catalysis by $Cu^{2+}BiPy$ under these conditions is prevented by dimerisation.[30]

The results of the present work suggest that metal ion catalysts play more than one role in the hydrolysis of a triphosphate bridge, and more than one metal ion may be required for an efficient hydrolysis. The reaction order of the cleavage **1** on the

concentration of $Cu^{2+}TerPy$ and $Cu^{2+}[12]aneN_3$ complexes ranged from 1.5 to 2. The mixed reaction order most probably suggests that different metal ion–substrate complexes are formed, and the most efficient catalysis is achieved with complexes where more than one metal ion is involved in the rate-limiting step of the reaction. The results in Table 1 also show that the slope of the plot log (k/s^{-1}) *vs.* log (c/M) for reaction of **4** in the presence of $Cu^{2+}[12]aneN_3$ is clearly less than in reaction of **1** under the same conditions suggesting that in the former case the two-metal pathway is of less importance. This may be due to steric factors: binding of two metal ion catalysts to a diphosphate function may be less favourable than trinuclear complexing of **1**.

The dependence of the rate constants of the cleavage of **1** promoted by $Cu^{2+}BiPy$ and $Cu^{2+}[9]aneN_3$ on the catalyst concentration is different: The slopes of the plots log (k/s^{-1}) *vs.* log (c/M) suggest that only one metal ion complex participates in the rate-limiting step of the reaction. The difference most probably results from dimerisation: Both $Cu^{2+}BiPy$[37] and $Cu^{2+}[9]aneN_3$[38] are known to dimerise under the experimental conditions, whereas no report on such behaviour for $Cu^{2+}TerPy$ or $Cu^{2+}[12]aneN_3$ exists. The effect of dimerisation has clearly been seen in kinetic studies on RNA hydrolysis. At a low concentration $Cu^{2+}TerPy$ and $Cu^{2+}BiPy$ are approximately equally good catalysts in hydrolysis of RNA model compounds.[36] At a higher catalyst concentration, the catalytic activity of $Cu^{2+}BiPy$ dramatically drops in comparison to that of $Cu^{2+}TerPy$: 10 mM $Cu^{2+}TerPy$ at pH 7.0 and 20 °C is a hundred times as good a catalyst as $Cu^{2+}BiPy$.[30] The dimer of $Cu^{2+}[9]aneN_3$, which at pH 7.2 and 50 °C has been reported to be the predominant species, is also inactive as a catalyst in phosphodiester hydrolysis.[38] In this case a half-order dependence of the rate of the phosphodiester cleavage on the metal ion complex concentration has been observed.

Whether or not the dimer is catalytically active in the hydrolysis of a triphosphate bridge cannot be concluded on the basis of the present data, since the stability constants of the dimers, and therefore the proportions of monomeric and dimeric species under the experimental conditions, are not accurately known. This being the case, the slopes of around 0.8 and 0.9 can be explained in three alternative ways. Firstly, if the dimerisation is complete under the experimental conditions, a first-order dependence of rate on the complex concentration shows that the dimer is the catalytically active species. Secondly, a first-order dependence could also be observed if the dimerisation is not quite complete under the experimental conditions and if the dimer is not catalytically active, but two separate complexes are required for the catalysis. In this case a second-order dependence on the complex concentration may be compensated by the effect of dimerisation, and over the narrow concentration range utilised, an approximately first-order dependence is observed. Alternatively, it can also be suggested that the structure of $Cu^{2+}BiPy$ and $Cu^{2+}[9]aneN_3$ allows a single complex to provide the bifunctional catalysis and an assistance by another complex is not required, as in the case of

Cu^{2+}TerPy and Cu^{2+}[12]aneN$_3$. If this were the case, the proportion of the catalytically inactive dimer would have to be low under the experimental conditions, since only a slight deviation (if any) from a first-order dependence is observed.

The results in Fig. 1 show that the catalytic activity of the complexes increases as the pH increases. The increase also seems to level off at a higher pH. This suggests that a metal ion co-ordinated hydroxide ion plays a role in the catalysis. Also the fact that Mg^{2+} aqua ions that are not markedly deprotonated under the experimental conditions,[27] and are therefore inactive, supports a mechanism that involves a metal ion bound hydroxo ligand. No clear correlation can, however, be seen between the catalytic activity (or the pH optimal for the catalysis) of a given metal ion complex and the pK_a of the metal ion bound water ligand. The pK_a of a Zn^{2+}[12]aneN$_3$ bound aqua ligand (7.3 reported by Zompa[39]) is clearly smaller in than those in Cu^{2+}BiPy and Cu^{2+}TerPy (pK_a values of 7.8 and 8.2, respectively, have been reported[30]), yet the complex is approximately 100-fold poorer a catalyst than Cu^{2+}BiPy, for example. Among Cu^{2+} complexes, Cu^{2+}[9]aneN$_3$ (pK_a value of 7.3 reported by Deal and Burstyn[38] and a value of 7.7 by Itoh *et al.*[40]) is more acidic than Cu^{2+}TerPy or Cu^{2+}BiPy. The lack of correlation has been reported before[18] and it has been suggested that it results from the effect of the highly charged triphosphate moiety on the deprotonation of an aqua ligand, even though no experimental data on this has been reported.

In the absence of quantitative data on the strength of the metal ion–phosphate complexes, and on the acidity of the metal bound water ligands in these complexes, no firm mechanistic conclusions can be drawn on the basis of comparisons between different metal ion complexes. The results of the present work are, however, consistent with previous suggestions that metal ions assist the hydrolysis as bifunctional catalysts and a nucleophilic attack by a metal ion-bound hydroxo ligand is assisted by co-ordination of another metal ion. This mechanism is very similar to that one often suggested for the Cu^{2+} complex-promoted cleavage of RNA model compounds, where a single metal ion may provide both the electrophilic assistance and the attacking hydroxide nucleophile. It may, however, be suggested that in the case of the cap-model compounds studied in the present work, the catalysis is more complex than that. The catalysis by Cu^{2+} complexes appears to be a very specific process, and external nucleophiles or electrophiles cannot replace Cu^{2+} complexes as catalysts. Addition of an amine as external nucleophile did not enhance the catalytic activity of Cu^{2+}TerPy, even though a nucleophilic attack by an amino group on a phosphate function has previously been reported.[14,26] It should be borne in mind, though, that the slight effect observed may also be a sum of two opposite effects that compensate each other, and that while the amine is actually a nucleophile, it also prevents the co-ordination of the substrate by strongly binding the metal ion catalysts. The fact that Mg^{2+} ion could not assist the Cu^{2+}TerPy promoted reaction by binding to the phosphate lends, however, additional support to the suggestion that the catalysis specifically requires two Cu^{2+}TerPy complexes, which cannot be replaced.

It would seem, on the basis of the discussion above, that the different roles of a metal ion-catalyst cannot be separated, but the reaction rather requires two phosphate-bound metal ions that have acidic water ligands. While a metal ion bound hydroxo ligand acts as a nucleophile, it could be suggested that another acidic aqua ligand also has a role in the catalysis. Consistent with this suggestion, it has been shown that Zn^{2+}, but not Mg^{2+}, enhances the lanthanum complex promoted hydrolysis of a cap-model compound.[20] An unexpectedly high catalytic activity of a bimetallic Zn^{2+} complex with no hydroxo ligands under experimental conditions, has also been reported.[20] The most logical role of an acidic water ligand is that of a general acid catalyst, and it could be possible that an appropriately positioned metal ion enhances the nucleophilic attack by pro-

tonating the phosphate group. While this kind of catalysis by metal ions has actually never been observed in RNA cleavage, Zn^{2+} aqua ions have been shown[41] to act as general acid-catalysts that protonate the poor leaving group in the cleavage of RNA model compounds. It is also possible that the other apparent role of the metal ion complexes is not catalytic, but structural. This is the case in Zn^{2+} promoted cleavage of short 3′-phosphorylated oligonucleotides that are folded in a structure that is stabilised by a co-ordination of another Zn^{2+} ion.[42,43] Similarly to the results of the present work, higher than first-order dependence of the rate constants on the catalyst concentration was observed,[43] and addition of Mg^{2+} ion resulted in a slower cleavage.

The formation of a HEPES–AMP adduct, observed in the presence of Cu^{2+}TerPy complexes, is not inconsistent with the discussion above, even though the formation of such a product implies that nucleophilic attack of a HEPES ion on the phosphate group may take place during the reaction. The adduct formation does not seem to compete with the Cu^{2+}TerPy promoted hydrolysis, but the proportion of the adduct remains the same when the Cu^{2+}TerPy concentration is changed. It can tentatively be suggested that the by-product is formed through an additional reaction pathway of **1** that binds two Cu^{2+}TerPy complexes. The adduct formation only takes place with Cu^{2+}-TerPy in HEPES buffers, which suggests that there may be a specific interaction between the buffer constituent and Cu^{2+}-TerPy complex, and an entropically favoured intracomplex nucleophilic attack by the HEPES ion on the phosphate group takes place.

Conclusions

Cu^{2+} complexes significantly enhance the hydrolysis of the triphosphate bridge in an mRNA 5′-cap model. The catalysis may involve a single catalyst species, two metal ion complexes as a dimer or two separate metal ion complexes as in the case of Cu^{2+}TerPy or Cu^{2+}[12]aneN$_3$. Independent of the composition of the catalyst, bifunctional catalysis by phosphate-bound metal ion complexes is involved. Active catalysts contain acidic aqua ligands, and when deprotonated, a hydroxo ligand most probably acts as a nucleophile that attacks the phosphate. An acidic aqua ligand may play another role in the catalysis, possibly as a general acid catalyst. Alternatively, the other role of a metal ion catalyst may be structural. In any case more data is still required to draw any final conclusions on the details of the mechanism. In contrast to the triphosphate hydrolysis, Cu^{2+} complexes do not enhance the cleavage of N^7-methylguanine.

Experimental

Materials

Diadenosine 5′,5′-triphosphate (**1**), diadenosine 5′,5′-diphosphate (**4**), diadenosine 5′,5′-tetraphosphate (**5**), adenosine 5′-diphosphate (**3**), adenosine 5′-monophosphate (**2**) and adenosine 5′-triphosphate (**15**) were products of Sigma and they were used as received. N^7-methylguanosine (**6**) was prepared from guanosine by methylating with methyl iodide as described before.[44] Dimethyl sulfate was used as methylating agent in the synthesis of N^7-methylguanosine 5′-monophosphate (**7**)[45] and N^7-methylguanosine 5′-monophosphate (**8**).[46] The compounds were characterised by ^{1}H and ^{31}P NMR spectroscopy (**7** and **8**) and EI-MS. Buffer constituents, metal salts and bipyridine, terpyridine, 1,4,7-triazacyclononane and 1,5,9-triazacyclododecane ligands were of reagent grade. The synthesis of the bisdien ligand has been reported elsewhere.[25]

Preparation of reaction solutions

Metal ion complexes were prepared by mixing the metal ion (as nitrate) and 1.1 equivalents of the ligand. The pH of the

solution was adjusted with HEPES buffer [*N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid; pK_a 7.56], with MES buffer (2-morpholinoethanesulfonic acid; pK_a 6.15) or with CHES buffer [CHES = 2-(*N*-cyclohexylamino)ethanesulfonic acid; pK_a 9.3]. The pH of the reaction solutions was checked with a pH-meter either at 25 °C or at 60 °C. Ionic strength was adjusted with NaNO$_3$. Sterilised water was used to prepare the reaction solutions, and they were handled with sterilised equipment.

Kinetic measurements

Reactions were carried out in Eppendorf tubes immersed in a water bath, the temperature of which was thermostated at 60 °C. The reactions were initiated by adding substrate stock solution (a few microlitres) to give a concentration of 0.1 mM. The total reaction volume was 1.5 ml. Aliquots (12) of 100 μl were withdrawn at appropriate intervals to cover approximately two half-lives of the reaction. The aliquots were immediately cooled down on an ice-bath, and stored in the freezer until analysed.

Analysis of aliquots

Aliquots from reactions of **1**, **4** and **5** were analysed either by capillary zone electrophoresis (CZE) or HPLC. Capillary electrophoretic analysis was carried out in a fused silica capillary (50 μm id, 77 cm) with 0.1 M boric acid, pH 8.0 as the run buffer. A voltage of 30 kV was applied. The compounds were detected at 260 nm (diode array detector). Alternatively, an HPLC analysis was utilised, particularly in case of **4**. The column used was Thermo Quest, Hypersil HS RP-18 (250 × 4.6 mm, 5 μm particle size), and the eluent a 0.1 M formic acid buffer, pH 3.0, containing 0.1 M tetrabutylammonium bromide. UV-analysis at 260 nm was utilised. Aliquots from reactions of **6** and **7** were analysed by RP-HPLC using the Thermo Quest, Hypersil HS column mentioned above. The eluent was a mixture of 0.1 M triethyl amine–phosphoric acid buffer and methanol. In case of **6**, the pH of the buffer was 6.8 and the methanol content was 0.5 %. With **7**, the pH was 3.0 and methanol content 1.0 %. Compounds were detected at wavelength of 274 nm (**6**) or 256 nm (**7**). Aliquots from reactions of **8** were analysed by CZE. The run buffer was 25 mM boric acid, pH 9.0, and a voltage of 30 kV was applied. The capillary dimensions were as described above.

Calculation of rate constants

The rate constants of the disappearance of the starting material (either decrease of mole fraction or decrease of the signal area) were calculated by using the integrated first-order rate-law. In CZE analysis the signal areas were divided by the respective migration times to normalise the areas.

References

1 For a recent review, see B. N. Trawick, A. Daniher and J. K. Bashkin, *Chem. Rev.*, 1998, **98**, 939–960.
2 K. G. K. Murthy, P. Park and J. L. Manley, *Nucleic Acids Res.*, 1991, **19**, 2685–2692.
3 M. M. Konarska, R. A. Padgett and P. A. Sharp, *Cell*, 1984, **38**, 731–736.
4 J. Hamm and I. W. Mattaj, *Cell*, 1990, **63**, 109–118.
5 A. J. Shatkin, *Cell*, 1985, **40**, 223–224.
6 R. E. Rhoads, in *Progress in Molecular and Subcellular Biology Vol. 9*, H. F. Hahn, D. J. Kopecho and W. E. Muller (Eds.), Springer, Berlin, 1985, pp. 104–155.
7 N. Sonenberg, *Prog. Nucleic Acids Res. Mol. Biol.*, 1988, **35**, 173–207.
8 E. Darzynkiewicz, J. Antosiewicz, I. Ekiel, M. A. Morgan, S. M. Tahara and A. J. Shatkin, *J. Mol. Biol.*, 1981, **153**, 451–458.
9 B. F. Baker, S. S. Lot, J. Kringel, J. S. Cheng-Flournoy, P. Villiet, H. M. Sasmor, A. M. Siwkowski, L. L. Chappell and J. R. Morrow, *Nucleic Acids Res.*, 1999, **27**, 1547–1551.
10 R. M. Milburn, M. Gautam-Basak, R. Tribolet and H. Sigel, *J. Am. Chem. Soc.*, 1985, **107**, 3315–3321.
11 F. Tafesse, S. S. Massoud and R. M. Milburn, *Inorg. Chem.*, 1985, **24**, 2591–2593.
12 S. H. McClauherty and C. M. Grisham, *Inorg. Chem.*, 1982, **21**, 4133–4138.
13 R. J. Geue, A. M. Sargeson and R. Wijesekara, *Aust. J. Chem.*, 1993, **46**, 1021–1040.
14 D. A. Nation, Q. Lu and A. E. Martell, *Inorg. Chim. Acta*, 1997, **263**, 209–217.
15 B. F. Baker, *J. Am. Chem. Soc.*, 1993, **115**, 3378–3379.
16 B. F. Baker, H. Khalili, N. Wei and J. R. Morrow, *J. Am. Chem. Soc.*, 1997, **119**, 8749–875.
17 K. P. McCue, D. A. Voss, Jr., C. Marks and J. R. Morrow, *J. Chem. Soc., Dalton Trans.*, 1998, 2961–2963.
18 K. P. McCue and J. R. Morrow, *Inorg. Chem.*, 1999, **38**, 6136–6142.
19 Z. Wieczorek, E. Darzynkiewicz, S. Kuusela and H. Lönnberg, *Nucleosides Nucleotides*, 1999, **18**, 11–21.
20 D. M. Epstein, L. L. Chappel, H. Khalili, R. M. Supkowski, W. D. Horrocks and J. R. Morrow, *Inorg. Chem.*, 2000, **39**, 2130–2134.
21 E. Darzynkiewicz, J. Stepinski, S. M. Tahara, R. Stolarski, I. Ekiel, D. Haber, K. Neuvonen, P. Lehikoinen, I. Labadi and H. Lönnberg, *Nucleosides Nucleotides*, 1990, **9**, 599–6118.
22 E. Darzynkiewicz, L. Labadi, D. Haber, K. Burger and H. Lönnberg, *Acta Chem. Scand.; Ser. B*, 1988, 86–92.
23 H. Sigel and P. E. Amsler, *J. Am. Chem. Soc.*, 1976, **98**, 7390–7400.
24 R. Cini and C. Pifferi, *J. Chem. Soc., Dalton Trans.*, 1999, 699–710.
25 S. Mikkola, Q. Wang, Z. Jori, H. Helkearo and H. Lönnberg, *Acta Chem. Scand.*, 1999, **53**, 453–456.
26 M. W. Hosseini, J.-M. Lehn and M. P. Mertes, *Helv. Chim. Acta*, 1983, **66**, 2454–2466.
27 *Ionisation Constants of Inorganic Acids and Bases in Aqueous Solution, Second Edition*, D. D. Perrin (Ed.), Pergamon Press, Oxford, England, 1982, p. 67.
28 J. A. Zoltewicz, D. F. Clarc, T. W. Sharpless and G. Grahe, *J. Am. Chem. Soc.*, 1973, **92**, 1741–1750.
29 R. J. Motekaitis, A. E. Martell, J.-P. Lecomte and J.-M. Lehn, *Inorg. Chem.*, 1983, **22**, 609–614.
30 B. Linkletter and J. Chin, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 472–474.
31 J. K. Bashkin and L. A. Jenkins, *J. Chem. Soc., Dalton Trans.*, 1993, 3631–3632.
32 M. K. Stern, J. K. Bashkin and E. D. Sall, *J. Am. Chem. Soc.*, 1990, **112**, 5357–5359.
33 L. A. Jenkins, L. A. Bashkin and M. E. Autry, *J. Am. Chem. Soc.*, 1996, **118**, 6822–6825.
34 M. Ora, M. Oivanen and H. Lönnberg, *J. Org. Chem.*, 1996, **61**, 3951–3955.
35 M. R. Eftinkt and R. L. Biltonen, *Biochemistry*, 1983, **22**, 5134–5140.
36 S. Liu, Z. Luo and A. D. Hamilton, *Angew. Chem., Int. Ed.*, 1997, **36**, 2678–2680.
37 J. R. Morrow and W. C. Trogler, *Inorg. Chem.*, 1988, **27**, 3387–3394.
38 K. A. Deal and J. N. Burstyn, *Inorg. Chem.*, 1996, **35**, 2792–2798.
39 L. J. Zompa, *Inorg. Chem.*, 1978, **17**, 2531–2536.
40 T. Itoh, H. Hisada, Y. Usui and Y. Fujii, *Inorg. Chim. Acta*, 1998, **283**, 51–60.
41 S. Mikkola, E. Stenman, K. Nurmi, E. Yousefi-Salakdeh, R. Strömberg and H. Lönnberg, *J. Chem. Soc., Perkin Trans. 2*, 1999, 1619–1625.
42 S. Kuusela and H. Lönnberg, *Nucleosides Nucleotides*, 1998, **17**, 2417–2427.
43 S. Kuusela, A. Guzaev and H. Lönnberg, *J. Chem. Soc., Perkin Trans. 2*, 1996, 1895–1899.
44 J. W. Jones and R. K. Robins, *J. Am. Chem. Soc.*, 1963, **95**, 193–201.
45 K. Kamiichi, M. Doi, M. Nabae, T. Ishida and M. Inoue, *J. Chem. Soc., Perkin Trans. 2*, 1987, 1739–1745.
46 S. Hendler, E. Fürer and P. R. Srinivasan, *Biochemistry*, 1970, **9**, 4141–4153.