Phosphodiester Cleavage of Ribonucleoside Monophosphates and Polyribonucleotides by Homo- and Heterodinuclear Metal Complexes of a Cyclohexane-Based Polyamino – Polyol Ligand

Attila Jancsó,*[a] Satu Mikkola,[a] Harri Lönnberg,[a] Kaspar Hegetschweiler,[b] and Tamás Gajda[c]

Abstract: The ability of the dinuclear complexes of tdci [1,3,5-trideoxy-1,3,5-tris(dimethylamino)-*cis*-inositol] to promote the cleavage of the phosphodiester bonds of nucleoside 2′,3′-cyclic monophosphates, dinucleoside monophosphates and polyribonucleotides has been studied. The homodinuclear copper(II) and zinc(II) complexes efficiently promote the hydrolysis of cyclic nucleotides. The second-order rate constant ($k_2 \approx 0.44\ \text{M}^{-1}\text{s}^{-1}$) estimated for the cleavage of 2′,3′-cAMP induced by dinuclear copper(II) complexes is about 107 times greater than that for the hydroxide-ion-catalysed reaction. The complex selectively cleaves the 2′O–P bond of 2′,3′-cUMP and forms the 3′-product in 91% yield. An equimolar mixture of copper(II), zinc(II) and tdci proved to be more efficient than either of the binary systems: a 7 – 20-fold rate enhancement was observed for the cleavage of 2′,3′-cNMP substrates. The half-life for the hydrolysis of 2′,3′-cAMP decreased from 300 days to five minutes at 25 °C when the concentration of each of the three components was 2.5 mM. In contrast to the copper(II) or zinc(II) complexes of tdci, the heterodinuclear species promoted the hydrolysis of several dinucleoside monophosphates. For two ApA isomers, cleavage of the 3′,5′-bond was about 6.5 times faster than cleavage of the 2′,5′-bond. On the basis of the kinetic data, a trifunctional mechanism is suggested for the heterodinuclear-complex-promoted cleavage of the phosphodiester bond. Double Lewis acid activation occurs when the metal ions bind to the phosphate oxygen atoms. In particular, a metal-bound hydroxide ion serves as a general base or a nucleophilic catalyst, and, presumably, a zinc(II)-bound aqua ligand behaves as a general acid and facilitates the departure of the leaving alkoxide group. The effect of the complexes on the hydrolysis of poly(U), poly(A) and type III native RNA was also investigated, and, for the first time, kinetic data on the cleavage of the phosphodiester bonds of polyribonucleotides by a dinuclear complex was obtained.

Keywords: heterometallic complexes · kinetics · phosphodiester cleavage · phosphoesterase models · RNA

Introduction

The cleavage of biomolecular phosphodiester bonds (e.g., RNA, DNA, ATP, etc.) plays a crucial role in various fundamental biochemical processes.[1] In living organisms, such processes are catalysed by phophoesterase enzymes, which contain, in many cases, two or three metal ions in their active sites.[2] The use of dinuclear (or trinuclear) metal complexes of low molecular weight ligands as mimics for these centres has been the focus of bioinorganic/bioorganic research for more than a decade.[3] Numerous homodinuclear species have been proposed as structural or functional models of the bimetallic centres of phosphoesterases,[4, 5] and in many cases, their solid phase and/or solution structure has been determined.[5] A spacer group or a bridging functional group (e.g., alkoxide, phenoxide, phthalazine, etc.) that separates the metal-binding sites in the molecule, is the key structural motif of binucleating ligands. It has been observed that proper positioning of the metal ions and a certain flexibility around the metal centres are both required for efficient acceleration of the hydrolytic processes. The activity of the dinuclear species is also highly dependent upon the metal – metal distance.[6, 7] In a recent report, in which the dinuclear zinc(II) complex efficiently promoted the hydrolytic cleavage of

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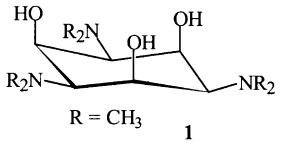
plasmid DNA, the precise distance between the metal centres was provided by the pitch of the tailored heptapeptide helix.[7]. However, questions that concern the optimal structural properties of a ligand, including the ideal donor sets of a metal binding site, still have to be answered.

The advantage of having two metal ions in close proximity has been clearly proven by comparing the activity of bimetallic systems to their mononuclear counterparts.[8] Double Lewis acid activation of the bound phosphoester substrates provided by the two metal ions has been regarded as an important factor in reaction rate enhancement.[4c–e, 8a, 9, 10] Nevertheless, a general base or a nucleophilic catalyst is substantial for the cleavage of phosphoester bonds, as was shown by the comparison of the catalytic activity of successively formed alkoxide-bridged dinuclear zinc(II) complexes of an asymmetric ligand.[10c] The central hydroxy group and the zinc(II)-bound water molecule were deprotonated in well-defined steps; this allowed the authors to unambiguously identify the hydroxo/mixed-ligand dinuclear complex as the catalytically active species. In general, a metal-bound hydroxide can assist cleavage either by acting as a general base,[4d,e, 8f,g, 10] or as a direct nucleophilic catalyst.[4d, 6a, 7, 8c] This has also been suggested to be the case with the hydrolytic reactions of nucleoside phosphoesters promoted by uncomplexed metal–aqua ions.[11] While the hydrolytic transformations of activated esters [e.g., (4-nitrophenyl)phosphate (NPP), bis(4-nitrophenyl)phosphate (BNPP), 2,4-dinitrophenylethyl phosphate (DNEP) and 2-hydroxypropyl-4-nitrophenyl phosphate (HPNP)] do not require stabilisation of the leaving group, stabilisation of the transition state of the reaction by the dinuclear core can strongly enhance the catalytic activity.[8f] On the other hand, the departure of an alkoxide leaving group from an unactivated nucleoside phosphoester is most likely a catalysed process. It has been suggested that metal-bound water molecules in aqua ions or in mononuclear complexes, metal–aqua ions or serve as general acid catalysts that enhance the cleavage of the dinucleoside monophosphate 5′O–P bond.[11a] Indeed, a kinetic study conducted on a series of uridine 3′-alkyl and aryl phosphates proved that this was the case.[12] It has also been proposed that dinuclear copper(II) complexes catalyse the release of the leaving group.[8e, 10b]

The spectacular catalytic activity of dinuclear (or trinuclear) complexes is undoubtedly based on the cooperative behaviour of the metal centres during their catalytic cycles. Therefore, the use of two metal ions that have different characteristics (e.g., size, Lewis acidity, favoured coordination geometry, etc.) may be even more advantageous. A number of hydrolytic metalloenzymes clearly display metal-ion cooperation.[2a] Amongst them, purple-acid phosphatases (PAP) have been extensively studied, and the crystal structures of several different types of PAPs have been determined (e.g. red kidney bean (BBPAP),[13a] and rat (TRAP)[13b]). In turn, the above results have instigated model studies with heterodinuclear Fe^{III} and Zn^{II},[10c, 14] Fe^{III} and Mn^{III},[15] or Fe^{III} and Ni^{II}[16] complexes aimed at mimicking the structure or function of native enzymes. Other groups have demonstrated that dinucleoside monophosphate cleavage is enhanced when the reaction is mediated by heterodinuclear[17a,b] or heterotrinuclear[17c] complexes of different ligands.

Copper(II) and/or zinc(II) complexes of several triamine ligands, amongst them *cis*-1,3,5-triaminocyclohexane and its congener 1,3,5-trideoxy-1,3,5-triamino-*cis*-inositol, have been found to promote the hydrolysis of both activated[18a,c] [2,4-dinitrophenyldiethyl phosphate (DNDEP)] and unactivated (DNA) phosphoesters.[18b,c] In a recent publication, we reported a kinetic and equilibrium study, as well as the structure determination in solution, of the copper(II) complexes of a polyamino–polyol ligand tdci (1,3,5-trideoxy-1,3,5-tris(dimethylamino)-*cis*-inositol).[19] Due to the unique steric orientation of the three amino and three hydroxyl groups, tdci can bind up to three metal ions in aqueous solutions. These studies indicated that a complicated equilibrium exists for the complexes, since differently protonated mono-, di- and trinuclear species are formed depending on the pH and metal-to-ligand ratio. The dinuclear complex Cu_2LH_{-3} showed outstanding catalytic activity towards the activated phosphoester BNPP, the hydrolysis obeying Michaelis–Menten kinetics. As mentioned above, catalysis for the cleavage of activated esters and unactivated nucleoside phosphoesters may be different. Consequently, a species that is able to cleave nitrophenyl phosphates is not necessarily a good catalyst for the cleavage of unactivated esters,[8g] and vice versa.[20]In the present paper, we report on the catalytic activity of dinuclear copper(II) complexes of tdci (**1**) for the cleavage of two cyclic nucleoside monophosphates (2′,3′-cUMP and 2′,3′-cAMP; **2**) and a dinucleoside monophosphate (”3′,5′-UpU, **3a**). For comparison, kinetic studies on the binary tdci–zinc(II) system have also been performed. As an extension of the studies conducted on nucleotide monomers and dimers, the hydrolysis of homopolynucleotides [poly(U) and poly(A)] and native type III RNA was also investigated. To the best of our knowledge, kinetic data on the cleavage of polyribonucleotides by dinuclear metal complexes have not yet been published.



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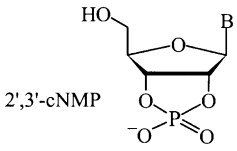

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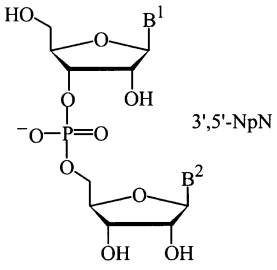

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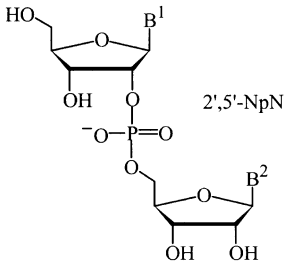
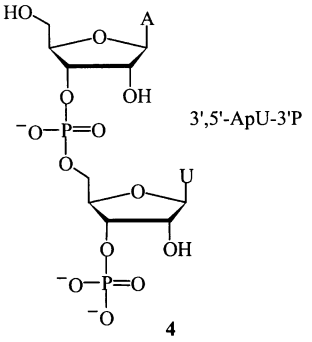
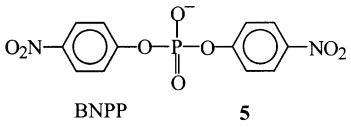


3b

Besides binary systems that contain the ligand and one type of metal ion, solutions of tdci and two different metal ions, copper(II) and zinc(II) (ternary system), have also been investigated. The effect of the ternary system on the hydrolysis of four cyclic ribonucleoside monophosphates **2**, several dinucleoside mono- and diphosphates, **3a**,**b** and **4**, respectively, as well as polyribonucleotides, has been studied in detail. For a comparison the results of kinetic measurements for the activated ester BNPP **5** are also reported. The





rates of the reactions were followed as a function of pH, metal-to-ligand ratio, copper(II)-to-zinc(II) ratio and catalyst concentration. The aim of replacing one of the copper(II) ions by zinc(II) was to provide a more flexible coordination environment with similar Lewis acid properties. The combination of the two metal ions may facilitate the binding of the catalyst to the substrate, generate an attacking nucleophilic reactant and promote departure of the leaving group. Hence, markedly enhanced cleavage of the phosphoester bond is expected.

Results and Discussion

Spectrophotometric measurements and matrix rank analysis (MRA) of the absorbance matrices: The composition and stability of dinuclear copper(II) complexes of tdci **1** are well established,[19] but data on the system that contains both a copper(II) and a zinc(II) ion are scarce. The dinuclear copprer(II) complexes of tdci have been characterised by means of EPR measurements.[19] Partial replacement of copper(II) ions by zinc(II) ions in an equimolar tdci – Cu^{II} solution at pH 11 resulted in a significant increase in the intensity of the EPR signal relative to that obtained for the dinuclear copper(II) complexes. This indicated the presence of a mixed dinuclear complex that contained one copper(II) and one zinc(II) ion at pH 11. pH-Metric studies at lower pH would not show conclusively that a heterodinuclear species had been formed, due to the complexity of the species distribution equilibria. To elucidate the possible formation of a heterodinuclear complex, spectrophotometric measurements on the tdci – copper(II) system, in the absence (Figure 1A) and presence (Figure 1B) of zinc(II), were performed. A continuous blue shift of the d – d transition band from 722 to 654 nm was observed on decreasing the copper(II)-to-tdci ratio; this is

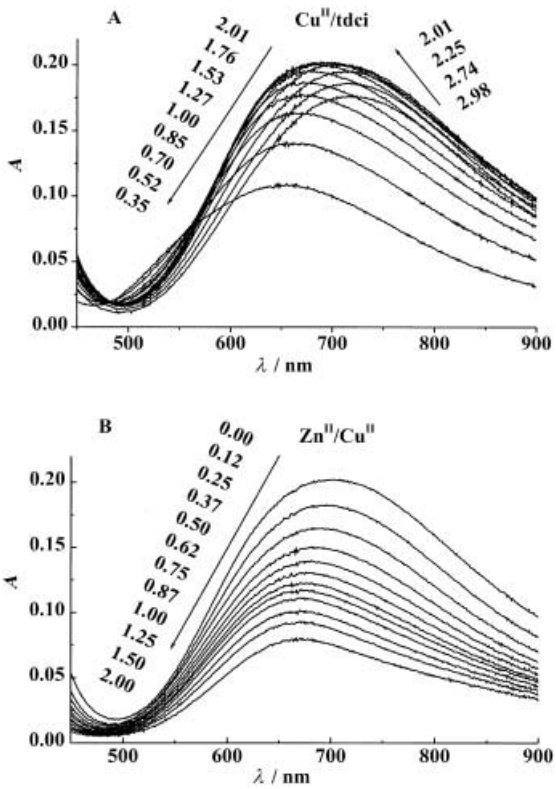


Figure 1. Visible spectra measured for the tdci – Cu^{II} (A) and tdci – Cu^{II} – Zn^{II} (B) systems in which the metal-to-ligand ratio for A is 2.98:1 – 0.35:1, while the Zn^{II}/Cu^{II} ratio is 0:2 – 2:1 for B. (A, $[tdci] = 1.35 \times 10^{-3} - 7.49 \times 10^{-3}$ M, $[Cu^{II}] = 4.02 \times 10^{-3} - 2.65 \times 10^{-3}$ M; B, $2[tdci] = [Cu^{II}] + [Zn^{II}] = 4.00 \times 10^{-3}$ M).

consistent with transformations of the trinuclear complexes into di- and mononuclear species. Figure 2A shows the species distribution (% Cu^{II}) as a function of the copper(II)/tdci ratio at pH 8.6.[19] Matrix rank analysis (MRA), according to Peintler et al.,[21] was performed on the spectral data, and the results are summarised in Table 1. Standard deviations at the corresponding wavelength (rows) and copper(II)/tdci ratio values (columns) are also given. In general, a diagonal element can be considered to deviate from zero when it is significantly larger than the reproducibility of the spectra (i.e., the accuracy of the equipment), and if it is at least twice as large than the standard deviation. However, experimental accuracy and the starting values used for the error propagation calculations remarkably influence the number of elements detected, especially when the minor components are concerned. Calculation of the residual absorbance curves (RAC) was used to verify the existence of ambiguous species, as these curves represent features of the spectra that cannot be explained by the presence of the $(n-1)$th species. The simple MRA calculation clearly showed the presence of four linearly independent complexes. This was consistent with the species distribution studies, and was also supported by the wavelength-dependent RAC curves (Figure S1 A in the Supporting Information). The rows and columns of the non-zero diagonal elements are generally very informative with respect to the absorbing species. Unfortunately, in the present case, the individual spectra are relatively similar, which significantly reduces the information that can be obtained from such

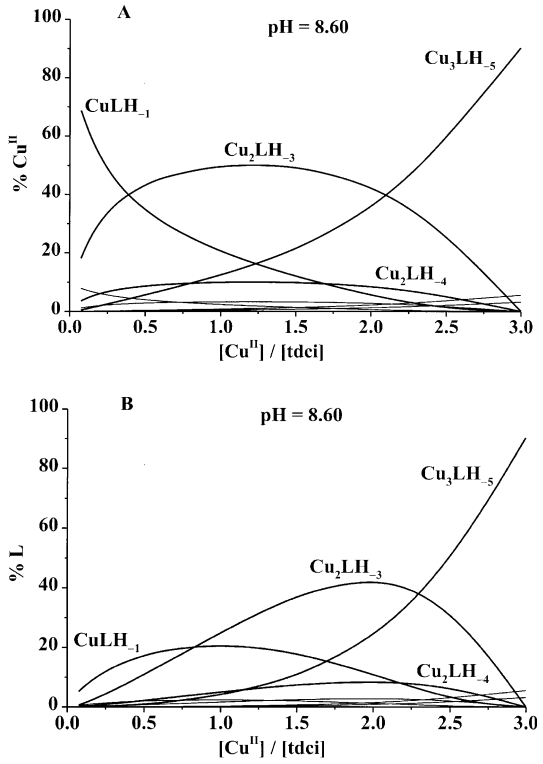


Figure 2. Cu^{II} (A) and ligand (B) distribution at pH 8.60 for the tdci – Cu^{II} binary system as a function of the metal-to-ligand ratio ([tdci] = 0.002 M, stability constants from reference [19] were used for the calculations).

Table 1. Diagonal elements from MRA calculations[a] performed on the spectral data matrices of the tdci – Cu^{II} (pH = 8.63), tdci – Cu^{II} – Zn^{II} (pH = 8.67) and tdci – Cu^{II}/tdci – Cu^{II} – Zn^{II} systems (evaluation range (λ): 450–900 nm).

Diagonal element →	1	2	3	4	5	6
tdci – Cu^{II}	*0.2016*	*0.0400*	*– 0.0187*	*0.0164*	– 0.0044	– 0.0035
σ[b]	*0.0020*	*0.0023*	*0.0038*	*0.0058*	0.0041	0.0073
λ [nm][c]	695	553	629	450	897	892
R[d]	1.76	0.35	2.98	2.50	0.70	
tdci – Cu^{II} – Zn^{II}	*0.2022*	*0.0143*	*– 0.0068*	– 0.0036	0.0029	– 0.0023
σ[b]	*0.0020*	*0.0030*	*0.0037*	0.0051	0.0078	0.0067
λ [nm][c]	709	642	843	450	755	864
R [d]	0.00	1.00	0.25	2.00	0.12	0.37
tdci – Cu^{II}/tdci – Cu^{II} – Zn^{II}	*0.2022*	*0.0418*	*– 0.0205*	*0.0177*	*0.0075*	– 0.0048
σ[b]	*0.0020*	*0.0023*	*0.0029*	*0.0041*	*0.0042*	0.0075
λ [nm][c]	709	558	450	647	874	658
R(s)[d]	0[f]	0.35[e]	2.98[e]	1.25[f]	1.53[e]	2.74[e]

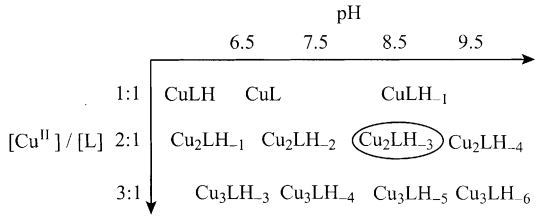
[a] The elements considered to be non-zero and their standard deviations are marked with italics. [b] Standard deviation of the given element. [c] Wavelength of the chosen element. [d] Cu^{II}/tdci (tdci – Cu^{II} system) or Zn^{II}/Cu^{II} (tdci – Cu^{II} – Zn^{II} system) ratio belonging to the column from which the element was chosen. [e] tdci – Cu^{II} system. [f] tdci – Cu^{II} – Zn^{II} system.

data. However, the columns of the original matrix, from which the first three diagonal elements are chosen, are characteristic for the three main complexes present in solution at pH 8.6.

In a parallel experiment, a solution of tdci and zinc(II) in a 1:2 ratio, was gradually added to a similar solution of tdci and copper(II) at pH 8.6 (Figure 1B). In this way, the concentration of the ligand and the total metal-ion/ligand ratio remained constant, while the copper(II) ions were gradually replaced with zinc(II). As seen in Figure 1B, a blue shift similar to the one observed for the tdci – copper(II) binary system occurred, but the change was not as great. After the zinc(II)-to-copper(II) ratio is greater than about 1.00 – 1.25 (λ_{max} = 671 nm), the shape of the spectrum remains unchanged. If addition of the zinc(II) – tdci solution did not have any influence on the copper(II) – tdci complex speciation, a simple dilution effect, that is, a decrease in intensity without other spectral changes, should be observed. This is clearly not the case. Indeed, spectral changes are observed that cannot be explained by a shift of equilibrium from tri- and dinuclear complexes to mononuclear species. Such processes would result in the occurence of free zinc(II) and consequently a zinc(II) hydroxide precipitate would be formed. As it did not happen, a plausible explanation for the blue shift is that the homodinuclear species are transformed to give heterodinuclear complexes. MRA was performed on the latter spectral data set and on the matrices of both experiments. In drawing conclusions on the basis of the data evaluated, it should be kept in mind that: 1) if heterodinuclear complexes are only formed to a small extent, they may only have a slight effect on the spectra and 2) if the presence of zinc(II) causes only a slight disturbance in the coordination environment of the copper(II) ion(s) in the heterodinuclear species, the number of non-zero diagonal elements may not increase when the two sets of experiments are evaluated together. The simple MRA of the spectra recorded for the solution of zinc(II) and copper(II) in different ratios shows the existence of at least two, but not more than three, linearly independent absorbing species (Table 1). The first diagonal element, which was chosen from the spectrum that did not contain zinc(II), corresponds to the same complex as the first element of the MRA of the binary system. On the other hand, the second diagonal element, which was chosen from the spectrum that contained the two metal ions in an equimolar ratio, probably corresponds to a heterodinuclear complex. The wavelength-dependent residual spectra suggest that three absorbing species exist in solution (Figure S1 B in the Supporting Information). In fact, the fourth residual curve (number of independent absorbing species (NIAS) = 3) depicted a non-random shape in certain wavelength ranges, but these values are within the experimental error of the measurements. Then the two data sets were evaluated together, and, on this basis, four or five absorbing species were suggested (Table 1). Calculated residual curves indicated the presence of a fifth absorbing species (Figure S1 C in the Supporting Information), as the residuals obtained on the assumption that four species (NIAS = 4) exist, contained non-random variations larger than the experimental error of the data. Although the similarities of the individual spectra do not allow a detailed analysis, the increased number of non-zero diagonal elements, as well as all the other data available about the ternary system

(EPR, kinetics—see below), strongly suggests the presence of heterodinuclear complexes.

Hydrolysis of nucleoside 2′,3′-cyclic monophosphates in the tdci–copper(II) system: According to formerly published potentiometric results on the binary system,[19] several differently protonated mono-, di- and trinuclear species exist in a complicated equilibrium that is dependent both on pH and the metal-to-ligand ratio. The main species formed between pH 6 and 10 are depicted in Scheme 1. Detailed kinetic



Scheme 1. The main species present in the tdci–Cu^{II} binary system according to reference [19] for the pH range marked.

measurements for the hydrolysis of BNPP (**5**) indicated outstanding activity of the dinuclear Cu_2LH_{-3} species. On the basis of these results, kinetic experiments for two ribonucleoside 2′,3′-cyclic monophosphates (2′,3′-cUMP, 2′,3′-cAMP; **2**) have been performed. Pseudo-first-order rate constants for the hydrolysis of the two substrates in the presence of a twofold metal excess were determined as a function of pH (Figure 3). The observed bell-shaped pH–rate profiles and

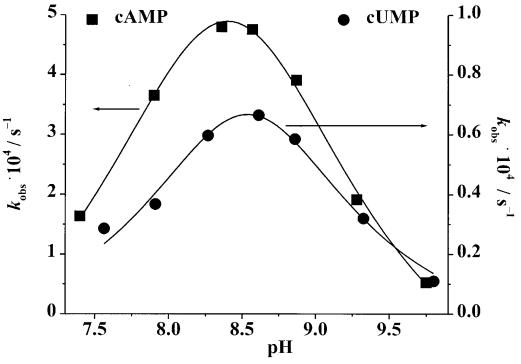


Figure 3. pH–Rate profiles for 2′,3′-cAMP and 2′,3′-cUMP hydrolysis in the tdci–Cu^{II} 1:2 system ($T=25\,^{\circ}C$, [2′,3′-cAMP] and [2′,3′-cUMP] $\sim 1\times10^{-4}$ M, $2\,[\text{tdci}]=[Cu^{II}]=5.0\times10^{-3}$ M).

the pH maxima of the curves ($pH_{max}\sim8.5$) are very similar to those found earlier for the activated esters.[19] Since the higher total concentrations used in the present study have only a slight effect on the speciation the observed kinetic activity can also be attributed to the above mentioned Cu_2LH_{-3} complex. To further support this assignment, the hydrolysis of 2′,3′-cAMP was also studied as a function of the ligand-to-metal ratio at pH 8.5 (Figure 4). The maximum of the resultant

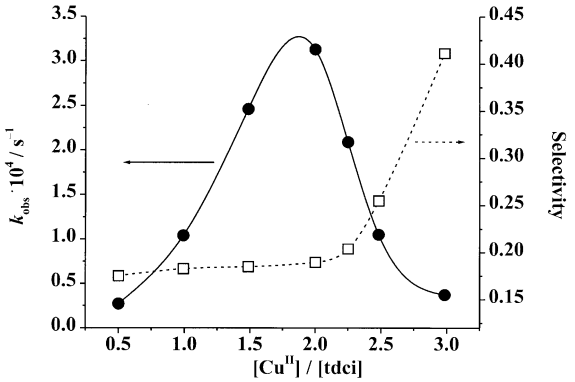


Figure 4. Pseudo-first-order rate constants and regioselectivity for the hydrolysis of 2′,3′-cAMP at pH 8.45 as a function of the Cu^{II}–tdci ratio ($T=25\,^{\circ}C$, $[\text{tdci}]=1.85\times10^{-3}$ M, [2′,3′-cAMP] $\sim 1\times10^{-4}$ M, Selectivity: [2′-AMP]/[3′-AMP]).

curve occurred when there was a twofold excess of metal to ligand. Remarkable catalytic activity was also observed at the equimolar ligand-to-metal ion ratio[19]; this can be attributed to the presence of the dinuclear species even in the equimolar solution (see Figure 2). At higher metal ion to ligand ratios, trinuclear complexes are formed, and these become the predominant species at $[Cu^{II}]:[\text{tdci}]>2.3$ (Figure 2B). These trinuclear complexes also enhance the hydrolytic process, but their catalytic activity is lower than that of the dinuclear species; the observed rate constants decrease as the metal-ion-to-ligand ratio increases (Figure 4).

Although the shapes of the k_{obs} versus pH plots are similar for both 2′,3′-cUMP and 2′,3′-cAMP, the reactions of 2′,3′-cAMP are seven times faster than those of 2′,3′-cUMP (Figure 3). Nevertheless, the active complexes significantly enhance the reaction rates for both compounds. In the absence of metal complexes, hydroxide-ion catalysis contributes to the observed rate constant, k_{uncat}, but hydronium-ion catalysis is negligible $\{k_{uncat}=k_{H_2O}+(k_{OH}[OH^-])\}$.[22a–c] At 25 °C, data is available for the uncatalysed reaction of 2′,3′-cUMP[22a,d] ($k_{uncat}(pH=8.57)\sim1.3\times10^{-8}\,s^{-1}$), but on the basis of the data obtained at different temperatures,[22b–d] a slightly higher value may be estimated for 2′,3′-cAMP at the 25 °C ($k_{uncat}(pH=8.57)\sim1.9\times10^{-8}\,s^{-1}$). The maximum observed k_{obs} values at 2.5 mM ligand concentration represent approximately a 5000- and 25000-fold rate enhancement for the hydrolysis of 2′,3′-cUMP and 2′,3′-cAMP, respectively. The proportion of active Cu_2LH_{-3} species present can be calculated from the stability constants,[19] and at the pH of the maximum k_{obs} values (Table 2) was calculated to be 1.1 mM. The second-order rate constants that can be estimated from the data at pH 8.57 ($k_{2,cUMP}\sim0.061\,M^{-1}s^{-1}$, $k_{2,cAMP}\sim0.44\,M^{-1}s^{-1}$)[22c,d] are approximately 23 and 107 times higher than the rate constants of the hydroxide-ion-catalysed reactions of 2′,3′-cUMP and 2′,3′-cAMP, respectively.

Protein nucleases selectively cleave the 2′O–P bond of cyclic nucleoside monophosphates, but base-catalysed reactions produce comparable amounts of two possible products, the 3′-*O*-phosphorylated compound generally being slightly favoured.[22c, 23] Only a few metal complexes that have been studied as nuclease mimics are able to hydrolyse nucleoside

Table 2. Observed rate constants[a] and product distribution for the hydrolysis of nucleoside 2′,3′-cyclic monophosphates at $T = 25\,^{\circ}C$ ([substrate] $= 5-10\times10^{-5}$ M).

System	Substrate	$k_{obs}\times10^{5}$ [s^{-1}]	Selectivity[b]
tdci–Cu^{II} 1:2 [c]	2′,3′-cUMP	6.6 ± 0.1	0.10 ± 0.01
	2′,3′-cAMP	48 ± 1	0.19 ± 0.01
tdci–Zn^{II} 1:2 [d]	2′,3′-cUMP	2.3 ± 0.1	0.51 ± 0.01
	2′,3′-cAMP	5.2 ± 0.1	0.58 ± 0.02
tdci–Cu^{II}–Zn^{II} 1:1:1[e]	2′,3′-cUMP	47 ± 1	0.66 ± 0.03
	2′,3′-cAMP	230 ± 5	0.33 ± 0.03
	2′,3′-cCMP	62 ± 1	1.10 ± 0.02
	2′,3′-cGMP	87 ± 2	0.47 ± 0.01

[a] The errors given refer to the standard deviaton of the k_{obs} values determined from the slope of the curves obtained after applying the integrated first-order rate law for the data points of each kinetic run. The values are the average of duplicate measurements with a reproducibility $\leq 15\,\%$. [b] Ratio 2′-NMP/3′-NMP. [c] [tdci] $= 2.5\times10^{-3}$ M, pH = 8.57. [d] [tdci] $= 2.5\times10^{-3}$ M, pH = 8.26, [e] [tdci] $= 2.5\times10^{-3}$ M, pH = 8.75.

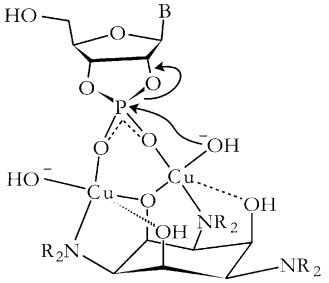
cyclic monophosphates regioselectively.[8e, 24] The dashed curve in Figure 4 represents the ratio of the two products, 2′-AMP and 3′-AMP, as a function of the metal-to-ligand ratio for the tdci–copper(II) system at pH 8.50. The reaction exhibits a clear preference for 2′O–P over 3′O–P bond cleavage (see Table 2). Product distribution varies only slightly up to a Cu^{II}:tdci ratio of about two, and suggests that the observed activity and regioselectivity can be attributed mostly to one species (Cu_2LH_{-3}).[19] Further increases of the metal-ion excess results in a decrease in the selectivity (Figure 4). Although the observed selectivity is lower than for the dinuclear complex, cleavage of the 2′O–P bond is still preferred. The difference in selectivity between the cAMP cleavage catalysed by di- and trinuclear complexes may arise from differences in the coordination of the substrate to the catalysts.

Cleavage of the 2′O–P bond is even more favoured for 2′,3′-cUMP than for 2′,3′-cAMP (see Table 2), as the mole fraction of 2′-UMP is only 0.091. In the past, interactions (hydrogen bonding or stacking) between the base moiety of the substrate and the complexed ligand were believed to account for the observed selectivities,[8e] because there did not appear to be any clear correlation between the regioselectivity and the base sizes and the known metal-ion affinities[25] for the bases. Hydrogen-bond formation, which would promote a certain orientation of the substrate in the substrate–catalyst adduct, is also possible in the present system; however, there is no proof to support this suggestion. Such interactions may also play a role in the observed base selectivity (see Figure 3 and Table 2), although this may originate from metal–base interactions (see below).

Hydrolysis of 3′,5′-UpU (**3a**) was also studied for the tdci–copper(II) binary system. The reaction in the presence of 2.5 mM tdci and 5 mM Cu^{II} at pH 8.57 and 35 °C turned out to be very slow, since less than 5 % of the substrate was cleaved in 15 days ($k_{obs}\leq 4\times10^{-8}\ s^{-1}$). At a higher pH (9.5), the reaction was somewhat faster, but the observed rate constant ($k_{obs}\sim 2\times10^{-7}\ s^{-1}$) was only about four times greater than that observed for the uncatalysed cleavage.[26]

The shape of the pH–rate profiles and the pH at the maximum rate acceleration suggests that a metal-bound hydroxide is involved in the nucleophilic attack of the phosphorous, either as a general base or as a nucleophile. A metal-bound alkoxide group from the ligand could be the nucleophilic reactant, but this would result in the phosphorylation of the ligand and subsequent formation of a ligand–nucleotide adduct: this was not observed by high-performance liquid chromatography (HPLC). The second-order rate constants estimated for the dinuclear-complex-promoted cleavage of the cyclic nucleotides are much higher than those for the hydroxide-ion-catalysed reactions, in spite of the fact that the nucleophilicity of a metal-bound hydroxide ion is undoubtedly lower than that of a free hydroxide ion. These findings suggest that direct intracomplex nucleophilic attack on the phosphorous atom by a hydroxo ligand takes place during the cleavage of the cyclic phosphodiesters. Furthermore, the reaction is probably enhanced by double Lewis acid activation induced by the phosphate-coordinated metal centres. The proposed mechanism depicted in Scheme 2 explains the experimental observations, apart from the regioselectivity.

Hydrolysis of the cyclic nucleotides was found to be significantly different than hydrolysis of the dinucleoside monophosphate 3′,5′-UpU; cleavage of the former compounds being much more efficient. Hydrolytic cleavage of dinucleoside monophosphates is a complicated process, and, according to the results discussed above, catalysis by the homodinuclear copper(II) complex of tdci is not enough to efficiently cleave the phosphodiester bond of 3′,5′-UpU.



Scheme 2. Proposed mechanism for the hydrolysis of 2′,3′-cNMP promoted by the Cu_2LH_{-3} complex.

Kinetic studies with the tdci–zinc(II) system: In light of the solution equilibrium properties and catalytic behaviour of the tdci–copper(II) system, kinetic studies of the tdci–zinc(II) system were mainly focused on solutions that contained tdci and zinc(II) in a 1:2 ratio. Hydrolysis of 2′,3′-cUMP **2** was monitored as a function of pH. A bell-shaped pH–rate profile was obtained with a maximum at around pH 8.25. At a ligand concentration of 2.5 mM, the maximum first-order rate constant is about 2600 times greater than for the uncatalysed reaction. The rate acceleration is slightly higher (ca. 4600) for 2′,3′-cAMP **2** under the same conditions (see Table 2). Cleavage of 3′,5′-UpU **3a** was also studied, but similarly to the copper(II) complexes, only a modest rate acceleration was observed.

Hydrolysis of nucleoside 2′,3′-cyclic monophosphates in the tdci–copper(II)–zinc(II) ternary system: Since efficient hy-

drolysis was observed in the binary system when the copper(II)-to-tdci ratio was 2:1, the first experiments were performed in solutions that contained the ligand, copper(II) and zinc(II) ions in a ratio of 1:1:1. Several salts were tested to adjust the ionic strength, but they caused precipitate formation at about pH 8. Therefore, the constant ionic strength (0.05 M) was adjusted by the buffers (HEPES, CHES). All the four natural ribonucleoside 2′,3′-cyclic monophosphates **2** were studied, and the catalytic properties of the ternary system were investigated in detail with 2′,3′-cUMP as the substrate. The rate constants and product distributions are summarised in Table 2.

The pH dependence of the k_{obs} values for the hydrolysis of 2′,3′-cUMP is depicted in Figure 5. The rate profile is symmetrically bell-shaped and has a maximum around pH 8.7. The rate constants are approximately seven and 20 times

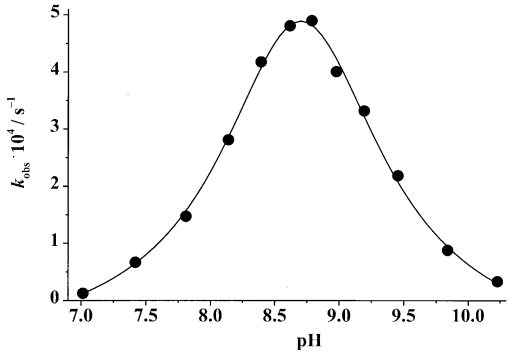


Figure 5. pH–rate profile for the hydrolysis of 2′,3′-cUMP in the tdci–Cu^{II}–Zn^{II} 1:1:1 system ($T = 25\,^{\circ}C$, $[2',3'\text{-cUMP}] \sim 1 \times 10^{-4}$ M, $[tdci] = [Cu^{II}] = [Zn^{II}] = 2.5 \times 10^{-3}$ M).

larger than the values determined for the copper(II)–tdci and zinc(II)-tdci binary systems, respectively (see Table 2). This indicates that the presence of two different transition-metal ions has a favourable effect on catalysis and that the increased activity very likely results from the formation of heterodinuclear complexes. As described above, the existence of such species is further supported by spectrophotometric and EPR experimental data.

Several other investigations of the catalytic properties of the heterodinuclear complexes were performed in order to determine the ideal metal-to-ligand and copper(II)-to-zinc(II) ratio for the cleavage of 2′,3′-cUMP. Figure 6 shows the result of an experiment that applied Job's method at the pH maximum of the pH–rate profile. The concentration of the ligand was kept at 2.5 mM, while the concentration of the two metal ions was varied between 0.0–5.0 mM, maintaining a constant 2:1 total metal-to-ligand ratio (and a constant 5.0 mM metal ion concentration); meanwhile the ratio of the two metal ions themselves was varied from 0:2 to 2:0. The pseudo-first-order rate constants as a function of $[Cu^{II}]:([Cu^{II}] + [Zn^{II}])$ ratios afforded a nearly symmetric bell-shaped curve that has a maximum when the components are equimolar. The end points of the curve represent the kinetic activity of the homodinuclear complexes at the same pH and are in a good

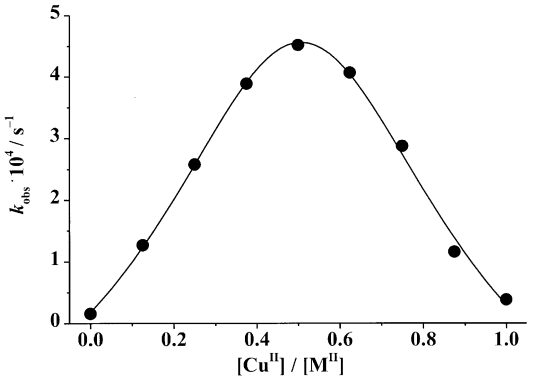


Figure 6. Pseudo-first-order rate constants at pH 8.75 for the hydrolysis of 2′,3′-cUMP as a function of the Cu^{II}–total metal-ion ratio ($T = 25\,^{\circ}C$, $2 \times [tdci] = [Cu^{II}] + [Zn^{II}] = 5.00 \times 10^{-3}$ M, $[cUMP] \sim 1 \times 10^{-4}$ M).

agreement with those determined for the binary systems. The maximum activity displayed at a Cu^{II}/Zn^{II} ratio of 1:1 suggests that a heterodinuclear complex is responsible for the cleavage of the cyclic phosphodiester. As trimetallic species were detected in the copper(II)–tdci binary system, the hydrolysis rate for 2′,3′-cUMP was also determined as a function of the metal-to-ligand ratio, Figure S2 A and Figure S2 B in the Supporting Information). The kinetic maximum was found at a ratio of 2:1 ($[Cu^{II}] = [Zn^{II}] = [tdci] = 2.5$ mM), though notable cleavage was also observed at a 3:1 ratio, especially for the solution that contained a twofold excess of zinc(II) over tdci and copper(II) (Figure S2 A in the Supporting Information). A trinuclear species must be responsible for this activity, since the presence of dinuclear complexes would also require the presence of free metal ions, which in turn would result in the precipitation of a metal hydroxide under the experimental conditions applied. Kinetic measurements for solutions that contained a threefold excess of the metal ions were also obtained as a function of pH. The highest k_{obs} values occurred around pH 8.5 when the Cu^{II}/Zn^{II} ratio was 1:2 and between pH 7.5–8.5 for a 2:1 Cu^{II}/Zn^{II} ratio (data not shown). The notable shift in pH relative to the maximum pH observed in the equimolar solution of the three components also indicates that trinuclear complexes possess catalytic activity.

Cleavage of 2′,3′-cUMP to give phosphomonoesters was also monitored as a function of the total concentration of the ligand and the two metal ions. The concentration of the three components was varied from $7.8 \times 10^{-5} - 2.4 \times 10^{-3}$ M, while the tdci, copper(II) and zinc(II) ratio was kept at 1:1:1. As depicted in Figure 7, the observed rate constants are linearly related to the concentration of the ligand and metal ions, and the intercept occurs close to the origin. Moreover, a straight line with a slope of 1.12 is obtained when a logarithmic plot is used; this means that the reaction is first-order with respect to the complex concentration. Since the mole fraction of the active complex in the ternary system is not known, only a lower limit for the second-order rate constant may be estimated based on the assumption that the active complex is the only species present. In this manner, a value of $0.19\ M^{-1}s^{-1}$ was obtained; this is more than 70 times greater than the second-order rate constant for the hydroxide-ion-catalysed reaction. This again suggests a mulitfunctional

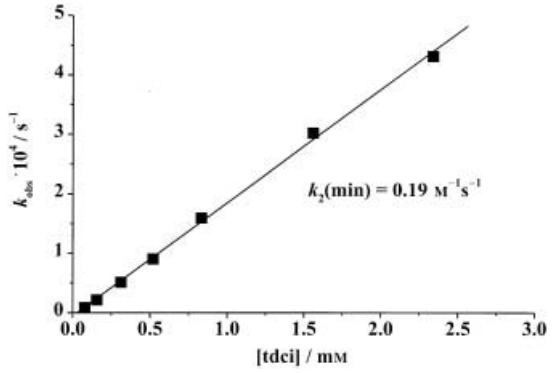


Figure 7. Pseudo-first-order rate constant at pH 8.75 for the hydrolysis of 2′,3′-cUMP as a function of the total concentration of tdci, Cu^{II}, and Zn^{II} ($T = 25\,^{\circ}C$, tdci:Cu^{II}:Zn^{II} = 1:1:1, [2′,3′-cUMP] $\sim 1 \times 10^{-4}$ M. The value determined from the slope of the curve refers to the lower limit of the second-order rate constant).

mechanism in which the (double) Lewis acid activation most likely plays a significant role.

Cleavage of diribonucleotides and oligoribonucleotides: The hydrolysis of six different dinucleoside monophosphates (**3a**,**b**) and one dinucleoside diphosphate (**4**) was investigated in equimolar solutions of tdci, copper(II) and zinc(II). Since the reaction rates were slower[26] than those observed for the cyclic nucleotides, the experiments were performed at a slightly higher temperature (35 °C). A full pH–rate profile for the metal-complex-dependent hydrolysis of 3′,5′-UpU was measured (Figure 8). The shape of the curve is distorted

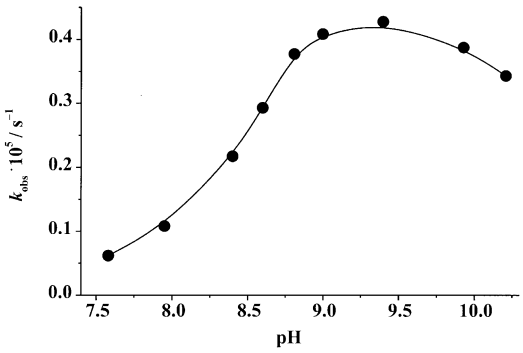


Figure 8. pH–rate profile for the hydrolysis of 3′,5′-UpU in the tdci–Cu^{II}–Zn^{II} 1:1:1 system ($T = 35\,^{\circ}C$, [3′,5′-UpU] $\sim 8 \times 10^{-5}$ M, [tdci] = [Cu^{II}] = [Zn^{II}] = 2.5×10^{-3} M).

relative to the symmetric profile obtained for 2′,3′-cUMP at 25 °C. Instead of a sharp maximum at pH 8.7, a relatively wide plateau in the pH range of 9–9.5 is observed; this suggests a speciation change, and/or that hydrolysis is promoted by at least one more species beside those already considered. The observed rate constant at about pH 9 is approximately 50- and 10-fold higher than the values obtained at the same pH for the copper(II)–tdci and zinc(II)–tdci binary systems, respectively. Hydrolysis of five other dinucleoside monophosphates and a dinucleoside diphosphate was also studied at this pH, and the k_{obs} values obtained are presented in Table 3. The differences in the rate constants for the cleavage of various dinucleoside

Table 3. Observed rate constants[a] for the hydrolysis of dinucleoside mono- and diphosphates ($T = 35\,^{\circ}C$) and polyribonucleotides ($T = 60\,^{\circ}C$) in the tdci–Cu^{II}–Zn^{II} 1:1:1 system at pH = 9.0: [substrate] $\sim 1 \times 10^{-4}$ M, [tdci] = 2.5×10^{-3} M (dinucleoside mono- and diphosphates) and 1.0×10^{-3} M (polynucleotides).

Substrate	$k_{obs} \times 10^6$ [s^{-1}]	$k_{obs,uncat} \times 10^6$ [s^{-1}][b]
3′,5′-ApA	15 ± 1	0.0053
2′,5′-ApA	2.3 ± 0.1	0.016
3′,5′-UpU	4.1 ± 0.1	0.017
2′,5′-UpU	5.0 ± 0.1	0.025
3′,5′-UpA	11 ± 1	0.018
3′,5′-ApU	6.6 ± 0.1	0.0056
3′,5′-ApU-3′p	0.56 ± 0.02	0.0042 [c]
poly(U)	2.1 ± 1	0.57 [d]
poly(A)	12 ± 1	0.18 [d]
RNA	8 [e]	–

[a] The errors given refer to the standard deviaton of the k_{obs} values determined from the slope of the curves obtained after applying the integrated first-order rate law for the data points of each kinetic run. [b] Extrapolated from the data in reference [26]. [c] Calculated on the basis of reference [28]. [d] The rate of the uncatalysed reactions were assumed to be equal to that of 3′,5′-UpU or 3′,5′-ApA[31] and k_{uncat} was extrapolated from the data given in reference [26]. [e] Due to precipitate formation data has to be considered as rough estimation.

monophosphates are rather modest. As with the cyclic phosphodiesters, the presence of an adenine base in the substrate results in faster cleavage. Surprisingly, the cleavage rate of 2′,5′-ApA is only 15% of that determined for the 3′,5′-ApA isomer. As a result, it can be speculated that the base moiety somehow participates in binding the catalyst, although the nature of the interaction (direct coordination, water-mediated coordination, or hydrogen bonding) is not clear. This interaction appears to place the catalyst in a position in which it is better able to cleave the 3′,5′-bond than the 2′,5′-bond. Direct coordination of the N3 uracil nitrogen atoms of 3′,5′-UpU to the copper(II) centres of a trinuclear complex was recently assumed to be a key factor in the observed substrate specifity.[27]

The 3′-phosphorylated dinucleoside diphosphate (3′,5′ApU-3′p, **4**) is considerably less susceptible to cleavage by the metal complexes discussed above. The rate increase for the catalysed system over the uncatalysed system is only 11% of that observed for 3′,5′-ApU (see Table 3).[28] Evidently, the active complex is unable to bind the substrate through two adjacent phosphate groups or this type of binding mode is not advantageous for cleavage. Exclusive coordination to the dianionic 3′-phosphate is believed to predominate; this in turn retards the cleavage of the 3′,5′-phosphodiester bond. In this respect, the above cleavage differs from the metal–aqua ion promoted cleavage of di- and oligoribonucleotides that have a 3′-terminal monophosphate group[29] because the 3′-phosphate group markedly accelerates hydrolysis even when the scissile phosphodiester bonds are situated several nucleoside units apart.[29e,f]

The metal-ion-induced hydrolysis of polyribonucleotides (poly(U) and native RNA) and a number of different oligonucleotides has previously been studied.[29–31] Metal ions and some of their tri- and tetraazamacrocyclic chelates have been shown to enhance the cleavage of poly(U) more efficiently than the cleavage of UpU.[30a, 32] To our knowledge,

data on the cleavage of oligoribonucleotides by dinuclear metal complexes is not available. Studies that involved poly(U), poly(A), or type III RNA in tdci – copper(II) – zinc(II) ternary systems were carried out at 60 °C in order to reduce the influence of base-stacking interactions within the polymer chains.[33] The data obtained are presented in Table 3. Notable activity was found, especially with poly(A); this is consistent with the base selectivity observed for mono- and dinucleotides. The rate constants determined indicate approximately a 66- and 4-fold rate increase ([tdci] = 1 mM) relative to the metal-complex-independent reaction of poly(A) and poly(U), respectively. Taking into account the different conditions (temperature, pK_w, concentration of the catalyst), these data indicate that the cleavage of polynucleotides is significantly slower than the hydrolysis of dinucleoside monophosphates. In agreement with the low catalytic activity observed for the ternary system towards the cleavage of 3′,5′-ApU-3′p, the presence of neighbouring phosphodiester groups does not increase the efficiency of the catalyst, as with metal – aqua ions.[30a] Furthermore, the 3′-terminal cyclic phosphate groups that are rapidly hydrolysed to give dianionic monophosphates exert a rate-retarding effect similar to that observed for 3′,5′-ApU-3′p. Bridging coordination of the active complex to two neighbouring phosphate diester moieties presumably does not predominate or cannot increase the rate of hydrolysis. Kinetic studies conducted on native RNA resulted in the formation of a precipitate. Though first-order kinetics were obeyed, the reliability of the data is questionable. Measurements were also taken for the tdci – copper(II) (1:2) binary system ([tdci] = 1.0 mM). The observed rate constants are only slightly higher than the extrapolated values determined for the uncatalysed reactions (poly(U): $k_{obs} \sim 8.0 \times 10^{-7}\ s^{-1}$, poly(A): $k_{obs} \sim 1.3 \times 10^{-6}\ s^{-1}$).

Hydrolysis of BNPP: To compare the results obtained for the cleavage of BNPP **5** catalysed by the dinuclear copper(II) complex, studies were performed on the tdci – copper(II) – zinc(II) ternary system. The pH – rate profiles for equimolar solutions of the components at two different complex concentrations (Figure 9) are bell-shaped and exhibit a maximum around pH 8.5. The pH maximum is somewhat lower than that observed for the cyclic nucleotides ($pH_{max} \sim 8.70$), but is almost identical to the one observed for the dinuclear copper(II) complex when either unactivated (2′,3′-cUMP, 2′,3′-cAMP) or activated (BNPP, NPP, DNEP) esters were used. In contrast to the results obtained for the unactivated esters under similar conditions, the pseudo-first-order rate constant for hydrolysis of BNPP, at the pH maximum, is only about one quarter of the k_{obs} value measured for the solution of tdci and copper(II). The dependence of the reaction rate on the total metal-to-ligand ratio (Figure S3 in the Supporting Information) indicates that the dinuclear species plays an essential role. The shape of the curve is again similar to that observed for the binary system.[19] Pseudo-first-order rate constants were found to be proportional to the total concentration of the complex mixture (using equimolar amounts of the ligand and the metal ions; Figure S4 in the Supporting Information); this indicates that the reaction is first-order with respect to the concentration of the active species. In the absence of information about the concentration of the active complex in solution, only a lower limit for the second-order rate constant could be calculated ($k_2 = 7.6 \times 10^{-2}\ M^{-1} s^{-1}$) from the slope of the linear correlation. This value is less than 10% of the second-order rate constant determined for the Cu_2LH_{-3} complex, but 3800-fold higher than the second-order rate constant calculated for the hydroxide-ion-catalysed reaction.[34] It can be concluded that the ternary system is less efficient than the copper(II) complex in promoting the cleavage of BNPP, which has a good leaving group. Furthermore, the homodinuclear Cu_2LH_{-3} complex may contribute to the observed rate acceleration and, hence, an even larger difference between the catalytic activity of the heterodinuclear species and Cu_2LH_{-3}, in favour of the latter complex, is possible.

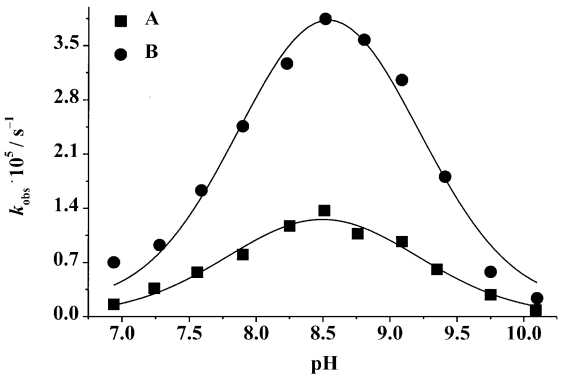
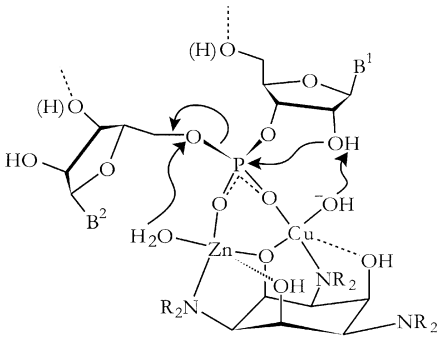


Figure 9. pH – rate profiles for the hydrolysis of BNPP in the tdci – Cu^{II} – Zn^{II} 1:1:1 system (T = 25 °C, [BNPP] = 5×10^{-4} M; **A**: [tdci] = 2.5×10^{-4} M, **B**: [tdci] = 7.1×10^{-4} M).

Mechanism of the heterodinuclear-complex-promoted hydrolytic cleavage: On the basis of the results obtained for the four different types of substrates, some general conclusions can be drawn with regard to the mechanism of the cleavage of the phosphodiester bond promoted by the heterodinuclear – tdci complex. As summarised in a recent review,[35] dinuclear metal complexes can promote the cleavage of phosphodiester bonds by participating at several different stages of the process. As described for the binary systems of tdci, the pH for maximum catalytic activity and the relative magnitude of the rate enhancement observed for cyclic phosphodiesters and diribonucleoside monophosphates suggests that a metal-bound hydroxide ion serves either as a direct nucleophile, which attacks the phosphorous atom (cyclic nucleotides), or as a general base, which deprotonates the attacking 2′-hydroxy group (dinucleotides, polynucleotides). Moreover, the magnitude of the second-order rate constants, relative to the corresponding values of the hydroxide-ion-catalysed reactions, strongly suggests that Lewis acid activation by the metal – phosphate coordination contributes to the rate acceleration. The metal-to-ligand ratio dependent experiments indicate that the second metal ion is essential for hydrolysis. However, questions arise as to the role the second metal ion plays. Although cooperation between the metal ions in dinuclear complexes has recently been disputed,[36] many

findings on the beneficial role of a dinuclear core in which two metal centres can perform a concerted action on the phosphate (double Lewis acidactivation) exist.[4c–e, 8a, 9, 10, 19] In the present case, double Lewis-acid activation may occur, but the remarkable differences between the catalytic activity of the hetero- and homodinuclear complexes suggests that the zinc(II) ion, in the mixed dinuclear complex, assists the departure of the alkoxide leaving group. This is further supported by the findings that in the hydrolysis of the activated ester BNPP, the catalytic efficiency of the ternary system is significantly lower than that found for the copper(II)–tdci binary system. Taking into account that the pK_a of a water molecule bound to a copper(II) ion is generally lower than that of a zinc(II)-bound water molecule, the latter may act as a general acid catalyst in the hydrolytic process promoted by a heterodinuclear tdci complex. Double Lewis acid activation, induced by the copper(II) ions of the dinuclear copper(II) species, plays a crucial role in the cleavage of BNPP (and cyclic nucleotides), but is not capable of enhancing the cleavage of the phosphodiester bonds of di- and polynucleotides that contain poor leaving groups. The mechanism of the heterodinuclear complex catalysed reaction is presumably trifunctional because the catalyst promotes three steps of the process (Scheme 3): 1) (double) Lewis acid activation of the



Scheme 3. Proposed mechanism for the phosphodiester bond cleavage catalysed by the heterodinuclear species.

substrate by coordination to the phosphate group, 2) general base or nucleophile catalysis by a metal-bound hydroxide ion and 3) general acid catalysis by a zinc(II)-bound water molecule for the departure of the leaving alkoxide group.

Conclusion

The homodinuclear copper(II) and zinc(II) complexes of tdci promote the cleavage of cyclic ribonucleoside 2′,3′-monophosphates by several orders of magnitude. The active copper(II) complex shows remarkable base selectivity in favour of substrates that contain an adenine base and a high regioselectivity that favours formation of the 3′-phosphorylated product. For the system that contains copper(II) and zinc(II) in an equimolar ratio with respect to tdci, the reaction rate increases 7–20 times to that observed for the homodinuclear complexes. The ternary system, in contrast to the homodinuclear complexes, was also found to effectively promote the hydrolysis of dinucleoside monophosphates. Interestingly, 3′,5′-ApA is cleaved 6.5 times faster than the 2′,5′-isomer. This, together with the observed preference for the cleavage of substrates that contain adenine, suggests that the base is involved in the catalyst–substrate interaction. When a dinucleoside diphosphate (3′,5′-ApU-3′p) is used as a substrate, a significantly lower catalytic activity is observed.

On the basis of all the kinetic data, the catalytic activity of the ternary system can be attributed to a heterodinuclear complex, and the presence of this species is also supported by the spectrophotometric measurements and MRA of the spectral data. The mechanism proposed for the heterodinuclear complex catalysed phosphodiester bond cleavage is trifunctional: 1) (double) Lewis-acid activation of the substrate by coordination to the phosphate group, 2) general base or nucleophilic catalysis (depending on the substrate) of a metal-bound hydroxide ion and 3) general acid catalysis of a zinc(II)-bound water molecule that promotes the departure of the leaving alkoxide group.

For the first time, kinetic data for the cleavage of polyribonucleotides by dinuclear complexes have been obtained. In contrast to metal–aqua ions, the heterodinuclear complexes of tdci exert a smaller rate increase for the cleavage of 3′,5′ApU-3′p and polynucleotides in comparison to the cleavage of dinucleoside monophosphates. In summary, the copper(II)–zinc(II)–tdci ternary system provides a new example of cooperative behaviour of bimetallic centres in hydrolytic reactions and also provides evidence for the beneficial influence of the presence of two different metal ions in the complex.

Experimental Section

Materials: Copper(II) and zinc(II) perchlorate or nitrate (Fluka) solutions were standardised complexometrically. HEPES, CHES, 2′,3′-cUMP, 2′,3′-cAMP, 2′,3′-cCMP, 2′,3′-cGMP, 3′,5′-UpU, 2′,5′-UpU, 3′,5′-ApA, 2′,5′-ApA, 3′,5′-UpA, 3′,5′-ApU, 3′,5′-ApU-3′p, polyuridylic acid, polyadenylic acid, ribonucleic acid type III from baker's yeast, and BNPP (all Sigma products) were used without further purification. Phosphodiesterase I (lyophilised powder) was purchased from USB, and alkaline phosphatase (a concentrated solution) was obtained from Boehringer, Mannheim. Ligand tdci (tdci·3 HCl·2 H_2O) was prepared as described earlier.[37]

UV-Visible spectroscopic measurements and evaluation of the absorbance matrices by MRA: UV-Visible spectra were measured on a Hewlett Packard 8453 diode array spectrophotometer. Analyses of the absorbance matrices obtained for the tdci–copper(II) binary system and tdci–copper(II)–zinc(II) ternary system were performed by a recently described algorithm based on the application of MRA.[21] The simple MRA method and the calculation of the wavelength-dependent residual absorbance curves (RAC) were also used to evaluate the NIAS. The initial standard deviation of the actual matrix element applied for the computation of error propagation was set to 0.002 absorbance units; this was in accordance with the reproducibility of the spectra.

Kinetic studies for the hydrolysis of nucleoside 2′,3′-cyclic monophosphates and transesterification of dinucleoside monophosphates: The pH of the reaction solutions was adjusted with HEPES (0.03 M), CHES (0.03 M), and by adding an appropriate amount of sodium hydroxide stock solution. The ionic strength was adjusted to 0.1 M with $NaClO_4$ or $NaNO_3$. After measuring the pH of the reaction solutions at the temperature of the kinetic measurements, the Eppendorf tubes (1.0 mL of reaction solution) were immersed in a water bath, the temperature of which was maintained at 25.0 ± 0.1 °C (cNMP) or 35.0 ± 0.1 °C (NpN). The reactions were initiated by adding 30–40 μL of the stock solution of the substrate. The initial

substrate concentration was 0.05 – 0.1 mM. The ligand (tdci) and metal-ion concentrations varied from 0.0 – 6.0 mM. In a typical experiment, the concentration of tdci was 2.5 mM, while that of the metal ion was 5.0 mM. Aliquots (7 – 10) were withdrawn at appropriate intervals from the reaction solutions. The reactions were quenched by mixing a 0.1 mL aliquot with an equal amount of the eluent used in the HPLC analyses. The samples were kept in a freezer until analysis.

The aliquots were analysed by RP-HPLC (Perkin – Elmer) by using a Hypersil ODS RP-18 column (250 × 4 mm, 5 μm particle size). The flow rate was 1 mL min^{-1}. The composition of the eluents was as follows: A) 0.06 mol dm^{-3} acetate buffer, pH = 4.3, I = 0.1 mol dm^{-3} (NH_4Cl); and B) eluent A with 10 % acetonitrile. The elution programs (proportion of buffer A and duration time) used for the different substrates were: 2′,3′-cUMP, 0 – 10 min, 100 % A; 2′,3′-cAMP, 0 – 12 min, 65 % A; 2′,3′-cCMP, 0 – 10 min, 100 % A; 2′,3′-cGMP, 0 – 12 min, 85 % A; 3′,5′-UpU and 2′,5′-UpU, 0 – 5 min, 100 % A; 5 – 17 min, 60 % A; 3′,5′-ApA and 2′,5′-ApA, 0 – 8 min, 65 % A; 8 – 18 min, 25 % A; 3′,5′-UpA, 0 – 8 min, 100 % A; 8 – 17 min, 30 % A; 3′,5′-ApU, 0 – 9 min, 65 % A; 9 – 19 min, 40 % A; 3′,5′-ApU-3′p, 0 – 3 min, 90 – 65 % A (linear gradient); 3 – 16 min, 65 % A; 16 – 18 min, 30 % A. The products (nucleosides, nucleoside 2′,3′-cyclic monophosphates, nucleoside 2′-monophosphates, and nucleoside 3′-monophosphates) were identified by spiking with authentic samples. The UV detection wavelength was 260 nm. The reactions were followed for about three half-lives. In all cases, the disappearance of the starting material obeyed pseudo-first-order kinetics. The pseudo-first-order rate constants (k_{obs}) for the cleavage of the starting materials were calculated by application of the integrated first-order rate law. For nucleoside 2′,3′-cyclic-monophosphates, the reported data are the average of duplicate measurements with a reproducibility ≤ 15 %.

Kinetic measurements of polynucleotides and native RNA: The temperature and pH of the reaction mixtures were adjusted as described above (T = 60.0 ± 0.2 °C). Aliquots from the solutions were taken periodically, the reactions were quenched by the addition of an excess of EDTA solution with respect to the metal ions (pH = 9.1), and the samples were cooled in an ice bath. The uncleaved polynucleotides were digested by treatment with phosphodiesterase I, as described earlier.[30, 38] Accordingly, phosphodiesterase I in a TRIS:HCl buffer, which contained NaCl and $MgCl_2$ (pH = 9.1), was added to the quenched aliquots. The final concentrations of TRIS, NaCl and $MgCl_2$ were 0.05 M, 0.1 M, and 0.01 M, respectively. To ensure that the enzymatic digestion was complete, the aliquots were left to stand for 20 – 24 h, at which time they were filtered through a syringe-driven Hydrophobic Fluoropore (PTFE) membrane filter unit (20 μm, Millipore), which removed the proteins. Prior to HPLC analysis, the samples were neutralised with acetic acid. The eluent buffers employed for separation of the homopolymer product mixtures were those described above for the mono- and dinucleotide substrates. The elution programs applied were: poly(U), 0 – 12 min, 100 % A; poly(A), 0 – 1 min, 80 % A, 1 – 6 min, 80 % – 65 % A (gradient); 6 – 16 min, 65 % A. For separation of the RNA cleavage products, buffers with 0.3 M NH_4Cl were used to improve the separation. The elution program was: 0 – 7 min, 100 % A, 7 – 22 min, 100 % – 35 % A (gradient); 22 – 30 min, 35 % A.

The method applied for the determination of the rate constants has previously been described in detail.[30a] The reactions were followed for 10 – 20 % transformation of the starting materials, and the aliquots withdrawn (6 – 7) were treated with phosphodiesterase I to cleave all the 3′O–P bonds of the starting material and oligomeric cleavage products. As a result, the 5′-terminal nucleosides formed upon the chemical cleavage of the RNA chain were detected as nucleosides, and the intrachain nucleosides were detected as 5′-phosphates. Small amounts of the 2′,3′-cyclic monophosphates and the hydrolysis products (2′- and 3′-monophosphates) that are formed upon chemical cleavage of the 3′-terminal phosphodiester bond were also released. The amount of these products was added to the nucleoside yields in order to obtain the total amount of phosphodiester bonds cleaved chemically. Aliquots withdrawn from the reaction solutions were also treated with a mixture of phosphodiesterase I and alkaline phosphatase in order to determine the amount of 3′-terminal nucleoside and 2′,3′-cyclic phosphates formed upon chain cleavage.[30b] However, only nucleosides were detected after the enzymatic digestion; this indicates that chemical cleavage of the 3′-terminal cyclic phosphate groups was rapid. The rate constants were calculated by determining the mole fraction of 5′-terminal nucleosides (i.e., the number of cleaved bonds) at time t with respect to all the nucleoside units of the starting material (i.e., the total number of phosphodiester bonds).

Hydrolysis of bis(4-nitrophenyl)phosphate (BNPP): The samples were prepared as described above and kept at 25.0 ± 0.1 °C prior to analysis. The reactions were initiated by injecting 100 μL of a 0.0103 M solution of BNPP (solid substrate dissolved in a 50 w/w % ethanol/water mixture) into an efficiently stirred, pre-thermostated sample solution. The reactions were followed by the increase of the absorption band for the p-nitrophenoxide ion at 400 nm (ε = 18900 M^{-1}cm^{-1}, pK_a = 6.98). The initial slope method (≤ 4 % conversion) was used to determine the pseudo-first-order rate constants. The increase in absorbance at 400 nm was followed immediately after injection of the substrate. The reported data are the average of duplicate measurements with a reproducibility of better than 10 %. The initial concentration of BNPP was 0.5 mM. In a typical experiment, the concentration of tdci, Cu^{II} and Zn^{II} was 0.25, 0.25 and 0.25 mM, respectively.

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