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Proton and Cu(II) binding to tren-based tris-macrocycles. Affinity towards nucleic acids and nuclease activity

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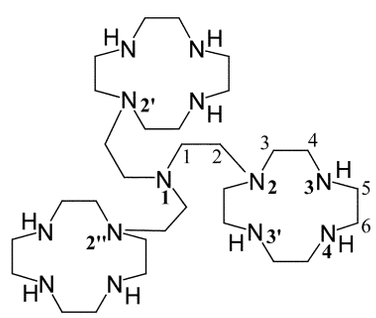
Proton binding by the two tren-based tris-macrocycles **L1** and **L2**, composed, respectively, by three 1,4,7,10-tetrazacyclododecane ([12]aneN$_4$) and three 1-oxa-4,7,10-triazacyclododecane ([12]aneN$_3$O) macrocyclic moieties appended to a "tren" unit (tren = tris(2-aminoethyl)amine), has been analyzed by means of potentiometric and ^{1}H and ^{13}C NMR measurements in aqueous solutions. This study reveals that the ligands form highly charged polyammonium cations at neutral pH, containing six acidic protons equally distributed among the three macrocyclic units. A potentiometric and UV-vis spectrophotometric study shows that both ligands can form stable trinuclear Cu(II) complexes in a wide pH range. In the polynuclear complexes each metal is coordinated to a single macrocyclic unit. While the Cu(II) complexes with **L1** do not show any tendency to form hydroxylated complexes, the mono-, di- and trinuclear **L2** complexes give stable hydroxo-complexes, present in aqueous solutions from slightly acidic to alkaline pH values. Melting point studies indicate that the new tris-macrocyles and their Cu(II) complexes lead to stronger stabilization of double-stranded nucleic acids than those observed earlier with analogous ditopic macrocyclic ligands, again with preference for RNA-type polymers compared to DNA. The copper complexes promote cleavage of plasmid DNA and of bis-*p*-nitrophenyl phosphate (BNPP). Particular rate enhancements for BNPP with some complexes are attributed to the simultaneous action of three metal ions and partially to the formation of hydroxo complexes at neutral pH.

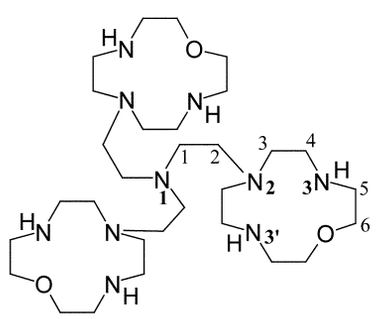
Introduction

There is a current interest in the chemistry of polyamine macrocyclic ligands because of their special ligational properties which give rise to both cation[1–10] and anion complexation.[10–18] Protonation of polyamine macrocycles, in fact, may occur readily, yielding polyprotonated species which are well suited to the study of anion coordination. Anion binding generally occurs by virtue of strong charge–charge and hydrogen bond interactions between the polyammonium functions of the receptor and the anionic sites of the substrate. Structural factors, however, have been shown to play a significant role for the strength of the interactions. Actually, the receptor molecular architecture can be modulated in order to selectively bind different guests, from simple inorganic anions[13–17] to species of biological relevance,[10–12] such as nucleotide phosphates.[18] Considering metal complexation, macrocyclic ligands with a large number of donors and cavities of appropriate shape and dimension may be able to hold two or more metal centers in close proximity, mimicking the multinuclear metal arrays at the active sites of several metallo-enzymes. Examples include P1 nuclease which uses three metal ions to catalyze the cleavage of phosphate ester bonds in nucleotides, such as RNA and DNA.[19] To this purpose, several dinuclear metal complexes with macrocyclic ligands have been used as structural or functional simple models for hydrolytic metallo-enzymes.[20–42] Trinuclear synthetic metal complexes used in biomimetic studies are rarer.[43–47] In one of the approaches, multiple macrocyclic rings, held by covalent linkages, have been used to hold three metal centers at short distances.[44,48] Examples of trinucleating ligands containing three polyazamacrocyclic binding units separated by rigid phenylene spacers have been recently reported.[44,48]

Earlier we described the synthesis of a new series of tren-based tris-macrocycles (tren = tris(2-aminoethyl)amine).[49] Two of the simplest contain, respectively, three 1,4,7,10-tetrazacyclododecane ([12]aneN$_4$) and three 1-oxa-4,7,10-triazacyclododecane ([12]aneN$_3$O) macrocyclic moieties appended to a "tren" unit (**L1** and **L2**).



L1



L2

Compared to previous reported phenylene-spaced tris-macrocyclic systems, the molecular structures of **L1** and **L2** display a higher overall flexibility. Because of the large number of protonable nitrogen donors, these polyamines, in principle, would give highly charged polyammonium cations at neutral

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Table 1 Protonation constants of **L1** and **L2** (0.1 M NMe_4Cl aqueous solution, 298.1 K)

	L1	L2
$\mathbf{L} + H^+ = \mathbf{LH}^+$	10.65(3)	10.10(8)
$\mathbf{LH}^+ + H^+ = \mathbf{LH_2}^{2+}$	9.75(3)	9.35(8)
$\mathbf{LH_2}^{2+} + H^+ = \mathbf{LH_3}^{3+}$	9.15(3)	8.9(1)
$\mathbf{LH_3}^{3+} + H^+ = \mathbf{LH_4}^{4+}$	9.08(9)	8.3(1)
$\mathbf{LH_4}^{4+} + H^+ = \mathbf{LH_5}^{5+}$	8.17(9)	7.8(2)
$\mathbf{LH_5}^{5+} + H^+ = \mathbf{LH_6}^{6+}$	7.88(7)	7.6(2)
$\mathbf{LH_6}^{6+} + H^+ = \mathbf{LH_7}^{7+}$	7.2(1)	5.4(2)
$\mathbf{LH_7}^{7+} + H^+ = \mathbf{LH_8}^{8+}$	4.1(1)	3.4(2)

pH. At the same time, since both the [12]aneN_4 and [12]aneN_3O macrocycles form stable complexes with transition metals,[50] we hoped that also **L1** and **L2** could give trinuclear Cu(II) complexes in aqueous solution. In the **L1** and **L2** complexes the metals should display a coordination sphere not saturated by the ligand donors, offering free binding sites for substrate coordination, as actually observed in the Cu(II) complexes with [12]aneN_4 and [12]aneN_3O.

In this paper we report on proton and Cu(II) binding features of the two ligands. We have also analyzed the affinity for double-stranded nucleic acids of **L1** and **L2** and their Cu(II) complexes, and their potential use as chemical nucleases.

Results and discussion

Ligand protonation in aqueous solution

The protonation equilibria of **L1** and **L2** have been studied in 0.1 mol dm^{-3} NMe_4Cl aqueous solution at 298.1 ± 0.1 K by means of potentiometric pH ($-\log[H^+]$) measurements and the results are reported in Table 1. Fig. 1 shows the distribution diagrams of the protonated species of **L1** and **L2** as a function of pH. Both ligands can bind up to eight protons in the pH range investigated; in the case of **L2**, a marked grouping of the first six protonation constants, with a sharp decrease in basicity between the sixth and the seventh protonation step (more than 2 logarithm units), is also observed. This behavior, common in polyamine compounds,[51] is generally explained in terms of minimization of the electrostatic repulsion between protonated ammonium groups. The rather complex structure of the present molecules, however, does not allow inferring of hypotheses on the proton distribution in the $[H_xL]^{x+}$ species. To clarify the stepwise protonation of both ligands, we recorded ^1H and ^{13}C NMR spectra on aqueous solutions containing **L1** or **L2** at different pH values. Both ligands display only six resonances in their ^1H and ^{13}C NMR spectra all over the pH range investigated, indicating a time averaged C_{3v} ternary symmetry for both ligands in all their protonation forms. The analysis of the pH dependence of the ^1H and ^{13}C resonances of **L2** (Fig. 2) allows us to deduce the protonation pattern for this ligand. In fact, in the pH range 11–7, where the first six protons bind to the ligand, a remarkable downfield shift is observed for the signals of protons H4 and H5, adjacent to N3 and N3′. Minor shifts are observed for the remaining proton signals. In ^{13}C NMR spectroscopy, the upfield shift of the signals of the carbons in β-position to the amine groups involved in proton binding can be used as diagnostic tool to identify the protonation site.[52] In the present case, the formation of the $[H_6\mathbf{L2}]^{6+}$ species is also accompanied by a predictable upfield shift of the resonances of the carbon atoms C3 and C6, in β-position with respect to N3 and N3′. These observations indicate that the first six protonation steps take place on the N3 and N3′ secondary amine groups. Therefore, in $[H_6\mathbf{L2}]^{6+}$ species, which is prevalent in aqueous solution at neutral pH, each macrocyclic unit contains two protonated nitrogens, separated one from each other by an

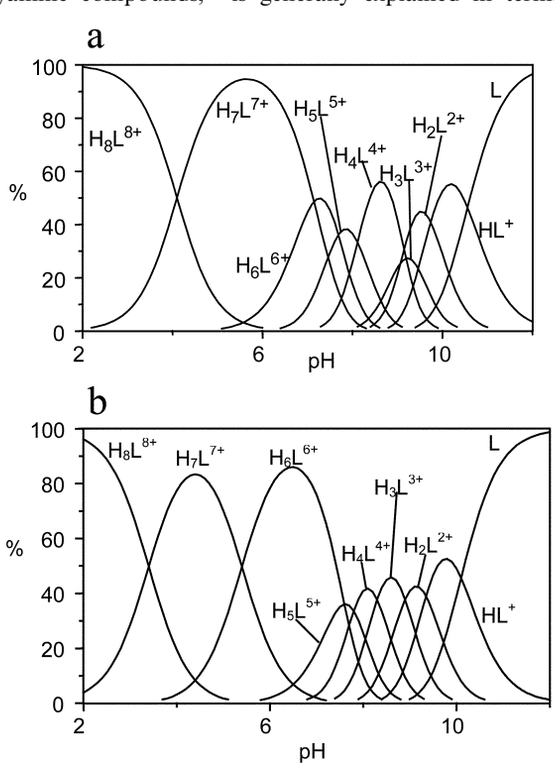


Fig. 1 Distribution diagrams of the protonated species of (a) **L1** and (b) **L2** ([**L1**] = [**L2**] = 1×10^{-3} M).

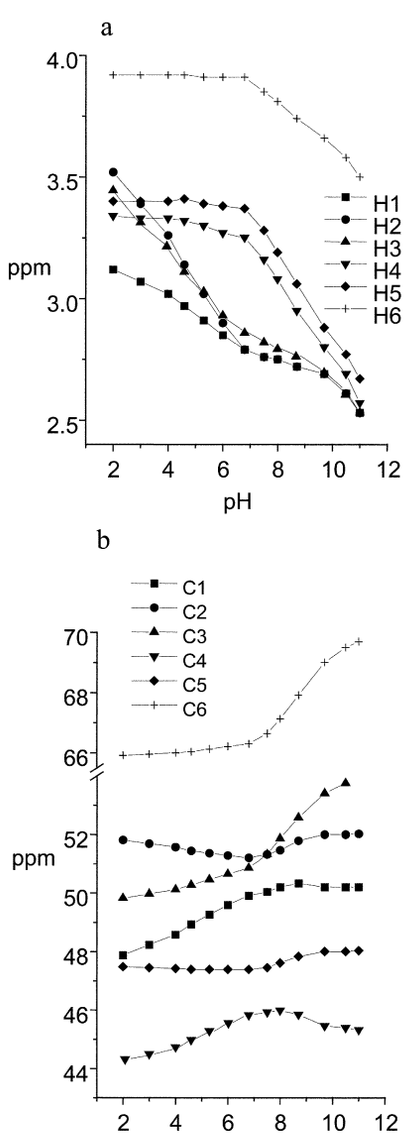


Fig. 2 pH Dependence of (a) ^1H and (b) ^{13}C NMR signals of **L2**.

uncharged heteroatom, thus achieving an optimal minimization of the electrostatic repulsion between charged ammonium groups. Binding of the seventh and eighth protons below pH 6 is accompanied by the downfield shift of the resonances of the H2 and H3 protons in the ^{1}H NMR spectra and by the upfield shift of C1 and C4 signals in the ^{13}C spectra. These spectral features indicate that the last protonation steps occur on the N2 amine groups, adjacent to the two already protonated N3 nitrogens. Such a proton disposition leads to an increase of the electrostatic repulsions, thus explaining the markedly lower values of the seventh and eighth protonation constants.

L1 shows a somewhat different behavior. As shown in Fig. 3(a), binding of the first three protons in the pH range 11–9.5 leads to relevant downfield shifts of the ^{1}H NMR resonances of the H4 and H5, adjacent to N3 and N3′. Minor shifts are observed for the other signals. In the same pH range, the ^{13}C NMR signals of C3 and C6, in β-position with respect to N3 and N3′ show a marked upfield shift (Fig. 3(b)). As in **L2**, the first three protonation steps occur on these secondary nitrogens, as sketched in Scheme 1.

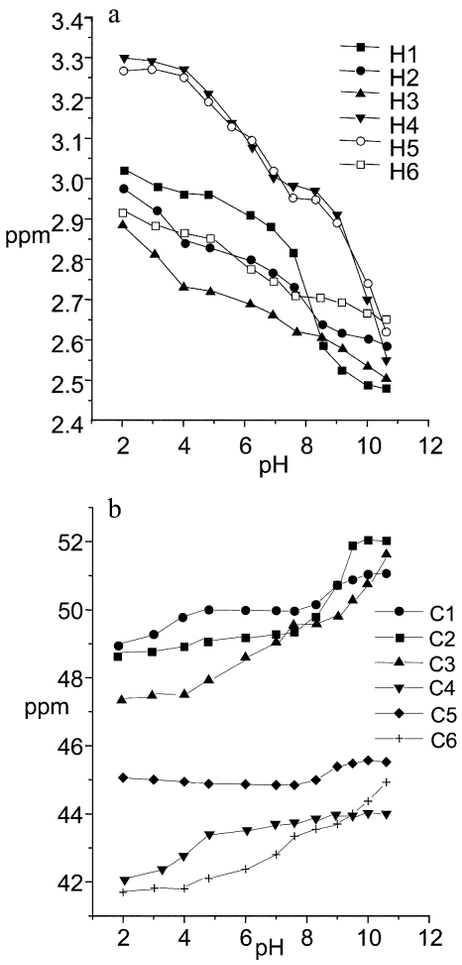


Fig. 3 pH dependence of (a) ^{1}H and (b) ^{13}C NMR signals of **L1**.

In the pH range 9.5–8.0, the tetraprotonated $[H_4\mathbf{L1}]^{4+}$ ligand is the prevalent species in solution. Fig. 3(a) shows that a marked downfield shift of the H1 signal, adjacent to N1, occurs in this pH range. At the same time, the other ^{1}H signals display only minor shifts. Furthermore, a marked upfield shift of the resonance of C2, in β-position with respect to N1, can be observed in the ^{13}C spectra (Fig. 3(b)). These data suggest that the fourth protonation step occurs on the bridgehead tertiary nitrogen N1. The further three protonation steps in the pH range 8.0–4.0 take place, once again, on the N3 and N3′ nitrogens, as demonstrated by the downfield displacement of the ^{1}H resonances of H4 and H5. Accordingly, the ^{13}C NMR signals of C3 and C6, in β-position with respect to N3 and N3′, are upfield shifted. Finally, the eighth proton, bound at strongly acidic pH values, is probably shared between the N2, N2′ and N2″ nitrogens, as shown by the remarkable downfield shift exhibited by the H2 and H3 signals.

Table 2 Stability constants (log K) of the Cu(II) complexes with **L1** and **L2** (0.1 M NMe_4Cl aqueous solution, 298.1 K)

	Log K	
Reaction	**L1**	**L2**
$Cu^{2+} + \mathbf{L} = Cu\mathbf{L}^{2+}$	21.9(1)	15.38(8)
$Cu\mathbf{L}^{2+} + H^+ = Cu\mathbf{L}H^{3+}$	10.6(1)	9.48(8)
$Cu\mathbf{L}^{2+} + 2H^+ = Cu\mathbf{L}H_2^{4+}$	9.8(1)	8.40(8)
$Cu\mathbf{L}H_2^{4+} + H^+ = Cu\mathbf{L}H_3^{5+}$	8.7(1)	7.95(6)
$Cu\mathbf{L}H_3^{5+} + H^+ = Cu\mathbf{L}H_4^{6+}$	8.6(1)	7.61(6)
$Cu\mathbf{L}H_4^{6+} + H^+ = Cu\mathbf{L}H_5^{7+}$	4.2(1)	
$Cu\mathbf{L}^{2+} + OH^- = Cu\mathbf{L}(OH)^+$		3.2(1)
$Cu\mathbf{L}^{2+} + Cu^{2+} = Cu_2\mathbf{L}^{4+}$	16.3(4)	10.12(8)
$Cu_2\mathbf{L}^{4+} + H^+ = Cu_2\mathbf{L}H^{5+}$	10.8(8)	8.9(1)
$Cu_2\mathbf{L}H^{5+} + H^+ = Cu_2\mathbf{L}H_2^{6+}$	9.2(1)	7.3(1)
$Cu_2\mathbf{L}^{4+} + OH^- = Cu_2\mathbf{L}(OH)^{3+}$		4.9(1)
$Cu_2\mathbf{L}^{4+} + Cu^{2+} = Cu_3\mathbf{L}^{6+}$	11.5(1)	7.0(1)
$Cu_3\mathbf{L}^{6+} + H^+ = Cu_3\mathbf{L}H^{7+}$	8.8(2)	7.1(1)
$Cu_3\mathbf{L}^{6+} + OH^- = Cu_3\mathbf{L}(OH)^{5+}$		6.8(1)
$Cu_3\mathbf{L}(OH)^{5+} + OH^- = Cu_3\mathbf{L}(OH)_2^{4+}$		5.5(1)
$Cu_3\mathbf{L}(OH)_2^{4+} + OH^- = Cu_3\mathbf{L}(OH)_3^{3+}$		4.0(1)

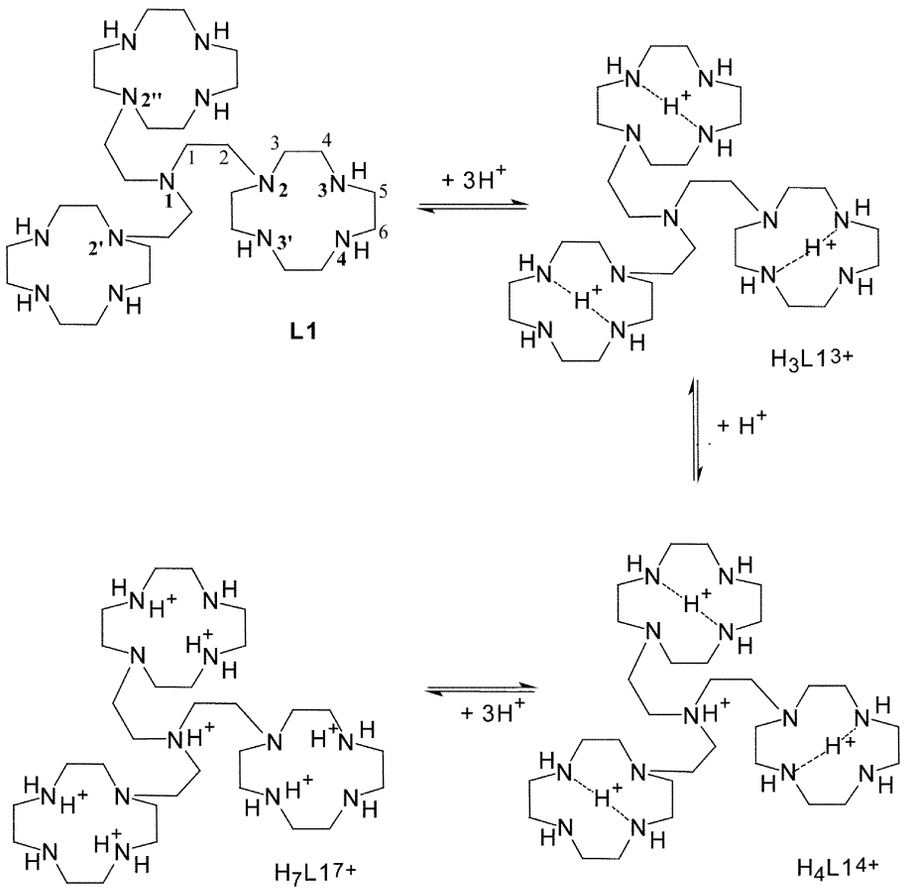
The most interesting finding, however, is the formation, in both **L1** and **L2**, of highly protonated (hexa- or hepta-protonated) species at neutral pH, where the protons are equally distributed on the three macrocyclic units of the ligands. These features are necessary requirements to achieve strong interaction with RNA or DNA, through hydrogen bond and electrostatic interactions.

Cu(II) coordination in aqueous solution

The coordination characteristics of **L1** and **L2** toward Cu^{2+} have been studied in 0.1 mol dm^{-3} NMe_4Cl aqueous solutions at 298.1 K and the stability constants of their complexes are reported in Table 2. Both ligands form mononuclear, binuclear and trinuclear metal complexes with Cu(II).

Considering the mononuclear complexes, the stability of the $[Cu\mathbf{L1}]^{2+}$ and $[Cu\mathbf{L2}]^{2+}$ complexes are just somewhat lower than those of the corresponding complex with [12]aneN$_4$ and [12]aneN$_3$O, respectively (log K = 21.9 and 23.4 for the equilibrium $Cu^{2+} + \mathbf{L} = Cu\mathbf{L}^{2+}$, with **L** = **L1** and [12]aneN$_4$,[53] respectively, and log K = 15.38 and 15.63 for **L2** and [12]aneN$_3$O,[54] respectively). The UV-vis spectra of the mononuclear complexes with **L1** and **L2** are also similar to those with [12]aneN$_4$ and [12]aneN$_3$O (for instance, the $[Cu\mathbf{L1}]^{2+}$ and $[Cu[12]aneN_4]^{2+}$ complexes display an absorption band at 595 and 580 nm with ε = 230 and 215 dm^3 mol^{-1} cm^{-1}, respectively). These thermodynamic and spectral characteristics suggest that in the $[Cu\mathbf{L}]^{2+}$ complexes (**L** = **L1** and **L2**), the metal is lodged inside one of the N$_4$ or N$_3$O macrocyclic cavities. The other two cyclic units are not involved in metal coordination and facile protonation may occur on their nitrogen atoms. Actually, the $[Cu\mathbf{L}]^{2+}$ complexes present a high tendency to undergo protonation and several protonated $[Cu\mathbf{L}H_n]^{(n+2)}$ species are formed in aqueous solution (Table 2). The first four protonation constants are only 1–2 logarithmic units lower than the corresponding basicity constants of the free amines, indicating that protonation occurs on nitrogen atoms not bound to the metal.

The formation of the dinuclear and trinuclear Cu(II) complexes do not alter significantly the shape and positions of the absorption band in the visible spectra (λ_{max} = 600 and 604 nm for $[Cu_2\mathbf{L1}]^{4+}$ and $[Cu_3\mathbf{L1}]^{6+}$, respectively). A *ca.* two-fold and a



Scheme 1

three-fold increase of the molar absorbance, however, is observed passing from the mononuclear **L1** complex to the binuclear $[Cu_2\mathbf{L1}]^{4+}$ (ε = 440 dm^3 mol^{-1} cm^{-1}) and trinuclear $[Cu_3\mathbf{L1}]^{6+}$ (ε = 640 dm^3 mol^{-1} cm^{-1}) complexes. An almost equivalent behavior is also found for the **L2** complexes, which display a band at *ca.* 620 nm, with a molar absorbance increasing almost linearly from the mononuclear complexes (ε = 280 dm^3 mol^{-1} cm^{-1}) to the trinuclear ones (ε = 920 dm^3 mol^{-1} cm^{-1}). These spectral features indicate that in the dinuclear and trinuclear complexes each metal is hosted, almost independently, in a macrocyclic N_4 or N_3O moiety. The equilibrium constants for the addition of a second Cu(II) ion to the $[Cu\mathbf{L}]^{2+}$ complexes (**L** = **L1** and **L2**) and of a third to the $[Cu_2\mathbf{L}]^{4+}$ ones are obviously lower than the formation constant of the $[Cu\mathbf{L}]^{2+}$ (Table 2), due to the electrostatic repulsions between the metal ions, and, to a lesser extent, to statistical effects.

Interestingly, both ligands give rise to the formation of a stable protonated trinuclear complex, $[Cu_3\mathbf{L}H]^{7+}$. Fig. 4(a) clearly shows that, in the case of **L1**, the $[Cu_3\mathbf{L1}H]^{7+}$ complex is prevalent in a wide pH range and deprotonation to give $[Cu_3\mathbf{L1}]^{6+}$ occurs only at alkaline pH values. The observed high value of the constant for proton addition to the $[Cu_3\mathbf{L}]^{6+}$ complexes may be ascribed to protonation of a nitrogen not bound to the metal, most likely the bridgehead nitrogen of the "tren" unit. Actually, protonation of the trinuclear complexes does not affect their UV-vis spectra, confirming that protonation occurs on a nitrogen donor not involved in metal coordination.

Comparing the binding features of **L1** and **L2**, the data in Table 2 reveal that the formation constants of mono-, di- and trinuclear Cu(II) complexes with **L1** are remarkably higher than the corresponding complexes with **L2**, due to replacement of the ethereal oxygen in **L2** with a better σ-donor amine group in **L1**. Most likely, as often observed in oxa-aza macrocycles, the ethereal oxygen is only weakly involved in metal coordination and, therefore, gives a minor contribution to complex stability. The increase in stability from the **L2** complexes to the corresponding **L1** ones is also in accord with the higher stability of

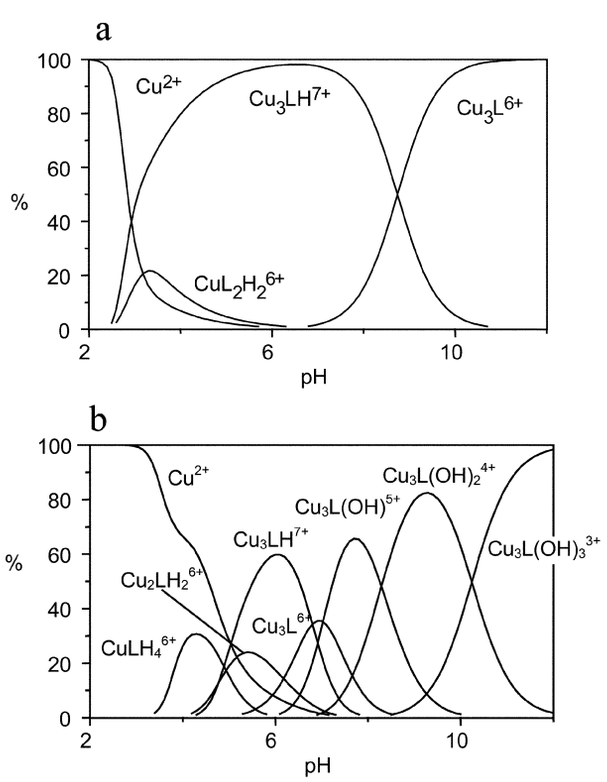


Fig. 4 Distribution diagrams for the systems **L1**/Cu^{2+} (a) and **L2**/Cu^{2+} (b) with a ligand to metal molar ratio of 1 : 3 ($[Cu^{2+}]$ = 3 × 10^{-3} M; [**L1**] = [**L2**] = 1 × 10^{-3} M).

$[Cu([12]aneN_4)]^{2+}$ than $[Cu([12]aneN_3O)]^{2+}$. In other words, in the **L2** complexes the metal displays a coordination environment less saturated by the ligand donors than in **L1** complexes. This feature may affect the acidity of the coordinated water molecules in the trinuclear **L1** and **L2** complexes, since less coordinately saturated coordination spheres generally favor the dissociation of metal-bound water molecules. Actually, the trinuclear **L1** complex does not form hydroxylated species of the type $[Cu_3\mathbf{L1}(OH)_n]^{(6-n)+}$, at least in the pH range investigated. Once again, this parallels the behavior of the $[Cu([12]aneN_4)]^{2+}$ complex, which does not show any tendency to give the monohydroxo $[Cu([12]aneN_4)(OH)]^{+}$ species.[53] By contrast, the trinuclear **L2** complex gives stable hydroxylated species. From the equilibrium constants reported in Table 2, we calculated the pK_a of the water-bound molecules in the trinuclear complex, (pK_a = 7.1 and 8.2 and 9.8, respectively, for the equilibria (1)–(3):

$$[Cu_3\mathbf{L2}]^{6+} + H_2O = [Cu_3\mathbf{L2}(OH)]^{5+} + H^+ \quad (1)$$

$$[Cu_3\mathbf{L2}(OH)]^{5+} + H_2O = [Cu_3\mathbf{L2}(OH)_2]^{4+} + H^+ \quad (2)$$

$$[Cu_3\mathbf{L2}(OH)_2]^{4+} + H_2O = [Cu_3\mathbf{L2}(OH)_3]^{3+} + H^+ \quad (3)$$

In consequence of these pK_a values, hydroxylated species are present in solution at neutral or slightly alkaline pH values (Fig. 4b). A similar behavior is also shown by the $[Cu([12]aneN_3O)]^{2+}$ complex (pK_a = 8.8).[54]

Interestingly, the pK_a values for the first two deprotonation steps of metal-bound water molecules in the $[Cu_3\mathbf{L2}]^{6+}$ complex are rather low in comparison with the analogous pK_a value for water deprotonation observed for the mononuclear $[Cu\mathbf{L2}]^{2+}$ complex (pK_a = 10.5). This behavior indicates a strong binding of the hydroxide ion in $[Cu_3\mathbf{L2}(OH)]^{5+}$ and $[Cu_3\mathbf{L2}(OH)_2]^{4+}$ and is generally ascribed to the a bridging coordination mode of OH^- between two metal centers.[32]

Although both **L1** and **L2** form stable trinuclear complexes in aqueous solutions and in both cases only trinuclear species are present in neutral solution containing ligand and metal in 1 : 3 molar ratio, the formation of hydroxide complexes makes the **L2** complexes more promising hydrolytic systems, since M–OH functions generally play an important role as nucleophiles in the hydrolysis of the phosphate ester bonds.

Affinity of L1 and L2 and their Cu(II) complexes toward DNA/RNA and nuclease activity of the Cu(II) complexes

Table 3 collects the results on the interaction of the ligands **L1** and **L2** and their Cu(II) complexes with double stranded nucleic acids polyA.polyU (corresponding to RNA) and polydA.polydT (a model for DNA). Results previously obtained with the saturated polyamine macrocycles 1,4,7,16,19,22-hexaaza-10,13,25,28-tetraoxacyclotriacontane (**L3**) and 4-hydroxyethyl-1,4,7,16,19,22-hexaaza-10,13,25,28-tetraoxacyclotriacontane (**L4**), which contain two separated triamine units as proton or metal binding sites, are also shown for comparison.[18a] As for all polyamines, the ligands as well as their Cu(II) complexes lead to stabilization of the double-stranded nucleic acids as is obvious from the considerable increase of melting temperature in each case (Table 3). Therefore, both the protonated amines and their Cu(II) complexes can act as multipoint binding sites for the phosphate groups of RNA and DNA. Compared to the analogous ligands **L3** and **L4**, **L1** and **L2** and their Cu(II) complexes show strong effects on the double strand stability due to the presence of three protonated moieties, suggesting that all three macrocyclic units participate in the binding to the groove. At neutral pH, **L1** and **L2** are prevalent in solution in their heptaprotonated and hexaprotonated forms, respectively; compared to corresponding open chain polyamines with same number of charges. The T_m increase is, however, smaller than with other polyamines bearing the same number of charges, which, as in the case of **L3** and **L4**, can be explained by a more restricted contact of all positively charged N sites and the groove phosphate residues.[18b] At higher r ratios such as r = 0.2 or 0.3 precipitation occurred, in contrast to experiments with the analogous ligands **L3** and **L4**. As with the other polyamines[18] the stabilization is more pronounced with RNA-type polymers than with DNA; as shown recently, only with specifically designed bulky amines can this preference be reversed in favour of DNA stabilization.[18c]

As discussed above, ligands **L1** and **L2** form quite stable complexes with the Cu(II) ion (Table 2) and there is no free copper ion under the conditions employed for the present study of nuclease activity. The T_m values of Cu(II) complexes (Table 3) show that the affinity of the free ligands and the metallated ligands are not dramatically different, and that the behavior is similar to those observed earlier.[18a,b] Thus these complexes are also promising candidates for interaction with nucleic acids.

The ability of the Cu(II) complexes of the ligands to cleave supercoiled plasmid DNA is summarized in Table 4. A very slow cleavage was observed with the Cu(II) complex of **L1** at neutral pH. Unfortunately, for the Cu(II) complexes of **L2** no

Table 3 Interaction of ligands and their Cu(II) complexes with PolydA.PolydU and PolydA.PolydT[a]

		ΔT_m/°C	
	r[b]	PolyA.PolyU	PolydA.PolydT
Ligand			
L1[c]	0.1	>50.0	Broad[d]
	0.2	–[e]	–[e]
	0.3	–[e]	–[e]
L2	0.1	>50.0	Broad[d]
	0.2	–[e]	–[e]
	0.3	–[e]	–[e]
L3[f]	0.1	28.8	11.1
	0.2	16.6/42.1[g]	22.0
	0.3	14.1/44.6[g]	Broad[d]
L4[f]	0.1	27.0	10.7
	0.2	16.6/39.0[g]	16.6
	0.3	13.4/41.0[g]	29.1
Cu(II) complex			
L1·3$CuCl_2$[h]	0.1	39.4/46.8[g]	7.0/broad[g,d]
	0.2	–[e]	–[e]
	0.3	–[e]	–[e]
L2·3$CuCl_2$	0.1	47.0	Broad[d]
	0.2	–[e]	–[e]
	0.3	–[e]	–[e]
L3·2$CuCl_2$[f]	0.1	25.2	12.0
	0.2	34.4	17.9/26.7[g]
	0.3	9.0/34.5[g]	37.2/42.4[g]
L4·2$CuCl_2$[f]	0.1	20.5	11.9
	0.2	26.4	28.2
	0.3	5.3/34.0[g]	34.7/42.2[g]

[a] *Conditions*: 0.01 M MES (2-(morpholino)ethanesulfonic acid) buffer; pH 6.25, I = 0.01 M; error in ΔT_m = ± 0.5 °C. [b] r = Molar ratio of ligand/nucleic acid phosphate. [c] Protonated species (calculated from the protonation constants values reported in Table 1 (**L1** and **L2**) and from ref. 18*a* (**L3**–**L4**)): **L1**: $[H_7\mathbf{L1}]^{7+}$ = 85%, $[H_6\mathbf{L1}]^{6+}$ = 15%; **L2**: $[H_6\mathbf{L2}]^{6+}$ = 85%, $[H_7\mathbf{L2}]^{7+}$ = 10%, $[H_5\mathbf{L2}]^{5+}$ = 5%; **L3**: $[H_4\mathbf{L3}]^{4+}$ = 90%, $[H_3\mathbf{L3}]^{3+}$ = 10%; **L4**: $[H_4\mathbf{L4}]^{4+}$ = 90%, $[H_3\mathbf{L4}]^{3+}$ = 10%. [d] Broad phase transition (no clear inflection is observed in the melting profile) [e] Precipitation of the complex. [f] Values from ref. 18*a*. [g] Two phase transitions observed in the melting profile. [h] Complexed species in solution at pH 6.25 (calculated from the stability constants values in Table 2 for ligands **L1** and **L2** and from ref. 18*a* for **L3** and **L4**; **L1**: $[Cu_3\mathbf{L1}H]^{7+}$ = 100%, **L2**: $[Cu_3\mathbf{L2}H]^{7+}$ = 85%, $[Cu_3\mathbf{L1}]^{6+}$ = 5%, $[Cu_2\mathbf{L2}H_2]^{6+}$ = 10%, **L3**: $[Cu_2\mathbf{L3}]^{4+}$ = 100%, **L4**: $[Cu_2\mathbf{L4}]^{4+}$ = 100%.

Table 4 Cleavage of pBR322 plasmid DNA[a]

Cu(II) complex	RF I (%)	RF II (%)[b]
L1·3$CuCl_2$	99	1
L2·3$CuCl_2$	–[c]	–[c]
L3·2$CuCl_2$	88	12
L4·2$CuCl_2$	–[c]	–[c]

[a] *Conditions*: [metal complex] = 1×10^{-3} M; [DNA] = 1.9×10^{-5} M (bp); 0.01 M EPPS (*N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid) buffer; pH 7.0; 37 °C; incubation time 2 h. [b] RF I is the open form of plasmid DNA; RF II is the supercoiled form; amount RF in %, corrected for the decreased stability of RF I by a factor of 1.22; corrected for the impurity of RF II in starting material and background noise; double runs, error in calculation is ± 2.5%. [c] No bands could be located.

Table 5 Cleavage of BNPP

Cu(II) complex	$10^6 k_{obs}/s^{-1}$
L1·$CuCl_2$[a]	2.3
L1·2$CuCl_2$	1.8
L1·3$CuCl_2$	3.5
L2·$CuCl_2$	2.6
L2·2$CuCl_2$	2.8
L2·3$CuCl_2$	9.7
L3·2$CuCl_2$	2.3
L4·2$CuCl_2$	4.0

[a] *Reaction conditions*: [metal complex] = 1×10^{-3} M; [BNPP] = 3.76×10^{-5} M; 0.01 M EPPS buffer; pH 7.0; 75 °C.

data could be obtained as the bands of DNA were not moving in the gel even after treatment of ion exchange resin which was successfully used with other polyamine ligands.[55] Earlier experiments with Cu(II) complexes from **L3** and **L4** in the presence of radical scavengers[18a,b] suggest that the observed nucleic acid cleavages are at least partially due to non-hydrolytic pathways.

Therefore, to compare the efficiency of the **L1** and **L2** complexes in phosphate ester bond cleavage, we analyzed the hydrolysis of bis-*p*-nitrophenyl phosphate (BNPP) in the presence of the ligands and different amounts of Cu(II) (neutral pH, 75 °C). All complexes promote BNPP hydrolysis to give mono-*p*-nitrophenyl phosphate and *p*-nitrophenolate, with a pseudo-first order behaviour; the rate constants are collected in Table 5. Similarly to DNA cleavage, the **L1** complexes are not particularly efficient in BNPP hydrolysis at pH 7. Their ability in promoting hydrolysis is similar to that found for the dinuclear Cu(II) complexes with **L3** and **L4**. In the present case, however, a slight rate constant increase is observed from **L1**·2$CuCl_2$ to **L1**·3$CuCl_2$. On the other hand, the **L1** complexes do not show any tendency to form hydroxo-complexes (see Table 2), which are often the active species in BNPP hydrolysis. A somewhat different behavior is found in the case of **L2**. Similarly to **L1**, a slow cleavage is observed in the presence of 1 and 2 eq. of $CuCl_2$ at pH 7. Under these conditions mononuclear and dinuclear complexes are the only species present in solution. A remarkable promotion of the hydrolytic process is instead found in the presence of 3 eq. of $CuCl_2$. The different hydrolytic ability of the mono-, di- and trinuclear **L2** complexes would indicate that the formation of trinuclear complexes can significantly accelerate the hydrolytic cleavage, in line with the few literature studies with trinuclear catalysts.[43–47] The higher ability in BNPP hydrolysis displayed by the **L2** trinuclear complexes compared to the **L1** complex is likely due to a coordinatively less saturated coordination sphere of Cu(II) in the complexes, which can lead to a stronger substrate interaction and water activation. At the same time, differently from **L1**, ligand **L2** in the presence of 3 eq. of Cu(II) may form, even at neutral pH, relevant amounts of the monohydroxo complex $[Cu_3\mathbf{L2}(OH)]^{5+}$, which contains a Cu(II)–OH function. This group is a better nucleophile than Cu–OH_2 and its presence in solution would also lead to a promotion of the cleavage process.

Experimental

General procedures

Ligand **L1** and **L2** were obtained as previously reported.[34] 300.07 MHz ^{1}H and 75.46 MHz ^{13}C NMR spectra in D_2O solutions at different pH values were recorded at 298.1 K on a Varian Unity 300 MHz spectrometer. In ^{1}H NMR spectra peak positions are reported relative to HOD at 4.75 ppm. Dioxane was used as reference standard in ^{13}C NMR spectra (δ = 67.4 ppm). ^{1}H–^{1}H and ^{1}H–^{13}C 2D correlation experiments were performed to assign the signals. Small amounts of 0.01 mol dm^{-3} NaOD or DCl solutions were added to a solution of **L1**·12HCl or **L2**·8HBr to adjust the pD. The pH was calculated from the measured pD values using the relationship: pH = pD − 0.40:[56]

UV-vis spectra were recorded on a Shimadzu UV-2101PC spectrophotometer.

PolyA.polyU, polydA.polydT, MES and EPPS were obtained from Sigma while pBR322 plasmid DNA was acquired from Pharmacia. The Cu(II) complexes used in RNA and DNA affinity and cleavage experiments were obtained by combining appropriate amounts of a standardized solution of $CuCl_2$, ligand and buffer. The pH was adjusted with a Knick digital pH-meter 646.

Potentiometric measurements

Equilibrium constants for protonation and complexation reactions with **L1**, and **L2** were determined by means of potentiometric measurements (pH = −log[H^+]), carried out in 0.1 mol dm^{-3} NMe_4Cl at 298.1 ± 0.1 K, in the pH range 2.5–11, by using the equipment that has been already described.[32a] The reference electrode was an Ag/AgCl electrode in saturated KCl solution. The glass electrode was calibrated as a hydrogen concentration probe by titrating known amounts of HCl with CO_2-free NaOH solutions and determining the equivalent point by Gran's method:[57] this allows one to determine the standard potential $E°$, and the ionic product of water (pK_w = 13.83 ± 0.01). The ligand concentration was about 1×10^{-3} M, while the metal concentration was in the range 3×10^{-3}–5×10^{-4} M. At least three measurements (about 100 experimental points in each) were performed for each system. The computer program HYPERQUAD[58] was used to calculate the protonation constants and the stability constants of Cu(II) complexes from e.m.f. data. The titration curves for each system were treated either as a single set or as separated entities without significant variations in the values of the protonation or metal complexation constants.

DNA/RNA Melting experiments

Thermal melting curves were obtained with a Cary 1 Bio UV-Vis spectrophotometer. The melting curves were recorded at different compound : nucleic acid phosphate ratios (r) by following the absorption change at 260 nm as a function of temperature. T_m values were determined from the graphs at the mid point of the transition curves. ΔT_m values were calculated by subtracting T_m of the free nucleic acid from T_m of the complex.

DNA Cleavage experiments

Samples were incubated at 37 °C for 2 h in 10 μL samples, as described earlier.[55] The reactions were quenched by addition of 2 μL of a loading buffer containing 40 wt% saccharose, 0.89 M

TRIS, 0.89 M boric acid, 1.0 M EDTA and a little Bromophenol Blue. The macrocyclic polyamines inhibit the migration of DNA in the gel electrophoresis; this was overcome by using ion exchange resin as previously reported.[55] Electrophoresis was conducted on 0.9% agarose in a horizontal gel apparatus at 70 V for 2 h. The electrophoresis buffer contains 0.89 M TRIS, 0.89 M boric acid, 2 mM EDTA and 0.5 μg ml^{-1} ethidium bromide. Quantification after electrophoresis was performed with an "Eagle Eye II" densitometry system using the "Zero-Dscan" software from Scanlytics.

BNPP Cleavage experiments

The rate of *p*-nitrophenolate release was monitored at 404 nm (ε = 6430 M^{-1} cm^{-1}) with a Shimadzu UV-2101PC spectrophotometer at 75 °C. The required amount of BNPP solution was added to 1 ml of the reaction solution in a quartz semi-microcuvette of 1 cm path length. The reaction was monitored for a period of 1000 min. The rate constants k_{BNPP} were calculated from non-linear least square fitting to a first order rate law.

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