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Structure and Dynamics of the Lincomycin–Copper(II) Complex in Water Solution by ^{1}H and ^{13}C NMR Studies

Elena Gaggelli,[†] Nicola Gaggelli,[†] Daniela Valensin,[†] Gianni Valensin,*[,†] Małgorzata Jeżowska-Bojczuk,[‡] and Henryk Kozłowski[‡]

Department of Chemistry, University of Siena, Via Aldo Moro, 53100 Siena, Italy, and Faculty of Chemistry, University of Wrocław, Joliot-Curie 14, 50-383 Wrocław, Poland

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The copper(II) complex of lincomycin in water solution at pH = 7.15 was characterized by ^{1}H and ^{13}C NMR and UV–vis spectroscopy. A 1:1 complex is formed in these conditions. The temperature dependence of spin–lattice relaxation rates was measured, showing that all protons behave in a similar fashion and slow exchange conditions prevail. The spin–lattice relaxation rate enhancements were interpreted by the Solomon–Bloembergen–Morgan theory. Reorientational dynamics of the complex was approximated by evaluating the motional correlation time of free lincomycin in water solution. The observed proton and carbon relaxation rate enhancements allowed us to calculate copper–proton and copper–carbon distances that were used for building a molecular model of the complex. The obtained data provide an interpretation of the relatively high stability constant.

Introduction

Lincomycin (LNCM; Figure 1), a clinically important natural antibiotic synthesized by *Streptomyces lincolnensis*,[1] is particularly active against Gram-positive bacteria. It is widely used in human and veterinary applications.[2,3]

Despite its biological relevance, few reports have appeared on conformational and structural features of the drug and of its metal complexes in solution.[4−6] The structural similarity to amino-glycoside antibiotics and the occurrence of a peptide linkage make LNCM a potentially strong ligand for copper(II) and other biologically relevant metals, as verified in the case of many naturally occurring aminoglycosides[7−10] and amikacin, a semisynthetic antibiotic.[11,12]

As a matter of fact, it has been recently shown that copper binds LNCM strongly and that the complex is active in mediating oxidation of deoxyguanosine by H_2O_2.[13] The most stable complex in water solution at pH ≈ 7.0 was suggested to be a $CuH_{-2}L$ species with copper(II) bound by the two deprotonated nitrogen donors and by the deprotonated 4-OH group.[13]

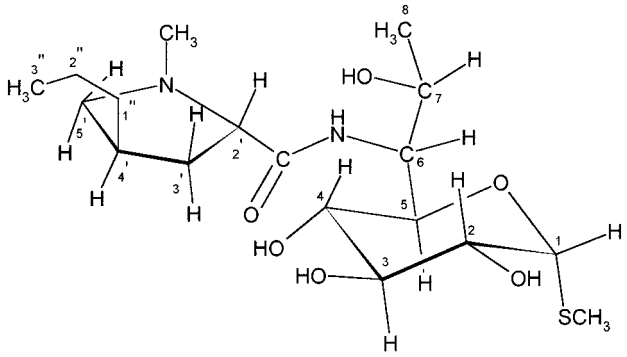

Figure 1. Molecular formula of lincomycin.

* Corresponding author. Phone: +39-0577-234231. Fax: +39-0577-234233. E-mail: valensin@unisi.it.

[†] University of Siena.

[‡] University of Wrocław.

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600 MHz ^{1}H and ^{13}C NMR studies are herein reported that allow a comprehensive delineation of structure and dynamics of the LNCM−Cu(II) complex in water solution at pH = 7.15. The previously suggested structure was verified, and a model of the copper complex was provided by molecular mechanics and dynamics.

Experimental Section

Lincomycin hydrochloride was obtained from Fluka Chemie AG. The purity of the compound was confirmed by NMR spectra, and no further purification was required. Solutions were made in deuterium oxide (99.95% from Merck) buffered at pH 7.15 with NaD_2PO_4 and Na_2DPO_4 and carefully deoxygenated through a freezing/vacuum pumping/sealing/thawing procedure. The desired concentration of copper ions was achieved by using a stock solution of copper nitrate (Sigma Chemical Co.) in deuterium oxide. TSP-d_4, 3-(trimethylsilyl)-[2,2,3,3-d_4] propansulfonate, sodium salt, was used as internal reference standard.

NMR experiments were carried out at 14.1 T (Bruker Avance 600) operating at a controlled temperature (±0.1 K) and equipped with a Silicon Graphics workstation. A 5 mm triple broad-band inverse probe was used for all 2D and ^{1}H experiments. Standard ^{1}H NMR spectra were acquired with a FID composed of 32768 points over a spectral width of 6000 Hz (acquisition time 2.730 s), a 90° pulse of 9.2 μs, a relaxation delay of 1.0 s, and 16 transients.

^{1}H NMR spectra were assigned by COSY experiments, recorded in the phase-sensitive mode. The COSY spectrum was collected over a spectral width of 6000 Hz (acquisition time 0.085 s); a total of 1024 points were used in F2 dimension, and 256 in F1 dimension which was zero filled once before processing. The spectrum was acquired with 4 transients for each of the 256 increments, with a relaxation delay of 1.0 s. Phase-sensitive NOESY experiments were carried out at different values of the mixing time with standard pulse sequences. NOESY spectra were acquired over a spectral width of 6600 Hz; a total of 2048 points were used in F2 dimension, and 512 in F1 dimension which was zero filled once before processing. The spectrum was acquired with 8 transients for each of the 512 increments, with a relaxation delay of 3.0 s.

2D ^{1}H−^{13}C shift correlation methods were used to detect and assign ^{13}C NMR spectra at relatively low concentration. HMBC (multiple-bond heteronuclear multiple-quantum coherence) spectra were obtained with standard pulse sequences, over a spectral width of 6000 Hz in F2 dimension and 31700 Hz in F1 dimension; a total of 1024 points were used in F2, and 256 in F1 which was zero filled once before processing. Eight transients were accumulated with a relaxation delay of 2.0 s for each of the 256 increments.

A 5 mm broad-band probe was used to obtain the ^{13}C NMR spectra. They were acquired with a FID composed of 32768 data points over a spectral width of 28700 Hz (acquisition time 0.570 s), a 90° pulse of 8.5 μs, and a relaxation delay of 5.0 s; WALTZ16 broadband decoupling method was used.[14,15]

Proton and carbon spin−lattice relaxation rates were measured with inversion recovery pulse sequences and calculated with regression analysis of the initial recovery curves of longitudinal magnetization components, leading to errors in the range ±3%. A DANTE pulse train was used to measure single-selective proton spin−lattice relaxation rates.[16]

Electronic absorption (UV−vis) spectra were recorded at 25 °C on a HP 8453 spectrophotometer (Hewlett-Packard) over a spectral range 200−1000 nm, by using quartz cells. Samples with lincomycin/Cu(II) 100/1 molar ratios were used. The lincomycin concentration in UV−vis spectroscopic measurements was 0.092 mol dm^{-3}.

Molecular structures were generated by the HYPERCHEM software package[17] implemented on a Pentium 120 MHz PC by using the ZINDO-1 semiempirical method for charge calculations and the MM+ force field for molecular mechanics and dynamics calculations.

Results and Discussion

The ^{1}H- and ^{13}C NMR spectra of LNCM are shown in Figures 2 and 3. The NMR assignments are summarized in Tables 1 and 2 and agree well with previous reports.[4,6]

When examining the temperature dependence of ^{1}H NMR chemical shifts, a relatively high-temperature coefficient of the amide ^{1}H chemical shift ($\Delta\delta/\Delta T = -2.8$ ppb/K; Figure 4) was observed, typical of a proton engaged in intramolecular direct or water-mediated hydrogen bonding. In light of the dipole−dipole connectivities previously measured in NOESY spectra[6] and also observed in our experimental conditions (data not shown), occurrence of an intramolecular H-bond with the C_7-hydroxyl was concluded.

Upon addition of copper(II) ions, sizable effects on line width and spin−lattice relaxation rates were only observed at relatively high concentrations of the metal (Figures 2 and 3). When compared with other copper-binding molecules, metal concentrations 10−100-fold higher were required with LNCM for measuring similar effects on NMR parameters (Tables 1 and 2).

Consideration of exchange of the ligand between the metal-bound state and the bulk yields the well-known equation[18]

$$R_{1p} = R_{1obs} - R_{1f} \cong \frac{p_b}{R_{1b}^{-1} + k_{off}^{-1}} \qquad (1)$$

where the labels b and f refer to the bound and free environments, R_1 is the spin−lattice relaxation rate, p_b is the fraction of metal-bound ligand, and k_{off} is the kinetic constant for dissociation of the ligand.

R_{1b}, the spin−lattice relaxation rate of the metal-bound ligand, is accounted for by the following simplified equation reporting the dipole−dipole interaction, as provided by the Solomon−Bloembergen−Morgan theory:[19−23]

$$R_{1b} = \frac{1}{10}\left(\frac{\mu_o}{4\pi}\right)^2 \frac{\hbar^2 \gamma_I^2 \gamma_S^2}{r^6}\left\{\frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right\} \qquad (2)$$

where γ_I and γ_S are the nuclear and electron magnetogyric ratios, ω_I is the proton Larrmor frequency, r is the proton−Cu(II) distance, and τ_c is the effective correlation time.

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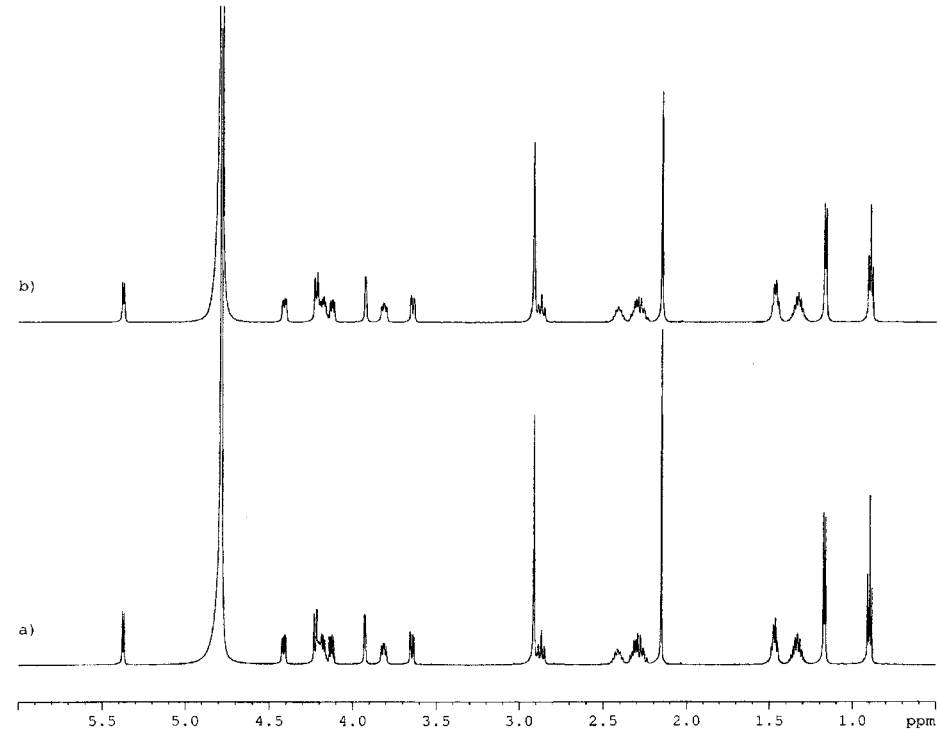

Figure 2. Superimposed ^{1}H NMR spectra of (a) lincomycin 9.3 mM and (b) lincomycin 9.3 mM in the presence of Cu(II) ions at a concentration of 0.093 mM in D_2O (pH = 7.15; T = 298 K).

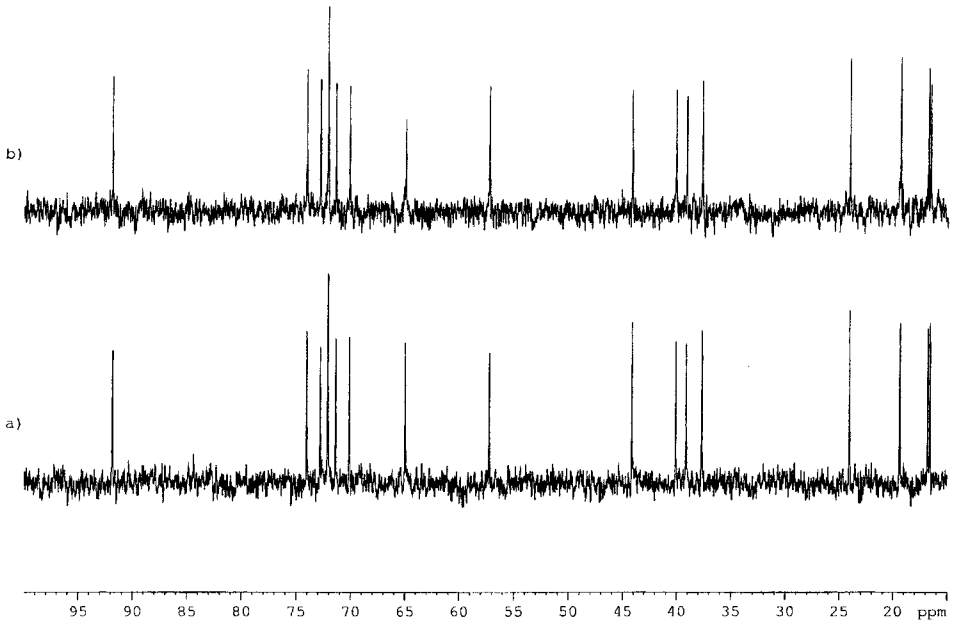

Figure 3. Superimposed ^{13}C NMR spectra of (a) lincomycin 9.3 mM and (b) lincomycin 9.3 mM in the presence of Cu(II) ions at a concentration of 0.093 mM in D_2O (pH = 7.15; T = 298 K).

R_{1b} is the structure-sensitive parameter, from which metal−nucleus distances can be calculated. However, R_{1b} can be obtained from R_{1p} only in cases where $k_{off} \gg R_{1b}$, that is, within the so-called fast exchange region.

The motional correlation time in eq 2 is generally determined by the rotation of the metal complex. Reorientational dynamics of the complex can be approximated by evaluating the motional correlation time of LNCM in water solution. This was calculated from spin−lattice relaxation rates of protonated carbons[24] at $\tau_c = 0.10 \pm 0.05$ ns at 298 K. This value was in reasonable agreement with that calculated by measuring the ratio between nonselective and selective relaxation rates[25] of selected protons of LNCM (also reported in Table 1) yielding $\tau_c = 0.21 \pm 0.06$ ns at 298 K.

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Table 1. ^{1}H NMR Parameters for Lincomycin 9.3 mM in H_2O/D_2O, Buffered at pH = 7.15, in the Presence of Cu(II) 0.093 mM, T = 298 K

	δ (ppm)	R_{nsel}/R_{sel}	R_{1p} (s^{-1})	R_{1b} (s^{-1})	k_{off} (s^{-1})	$\Delta G^{\#}$ (kcal mol^{-1})	r_{H-Cu} (nm)
$H_{(1)}$	5.37	1.208	0.041				
$H_{(2)}$	4.13		0.101				
$H_{(3)}$	3.64	1.094	0.049				
$H_{(4)}$	3.92	1.105	0.309	15632	30.960	6.10	0.286
$H_{(5)}$	4.22		0.341	10218	34.200		0.307
SCH_3	2.15		0.002				
$H_{(6)}$	4.41	1.110	0.733	3444	74.627	10.6	0.368
$H_{(7)}$	4.17		0.271				
$CH_{3(8)}$	1.17		0.139				
$H_{(2')}$	4.20		0.271				
$CH_{2(3')}$	2.29		0.773	891	74.627		0.461
$H_{(4')}$	2.41		1.126	990	126.58	8.11	0.453
$H_{(5'a)}$	3.81	1.141	1.126	25269	112.99	9.71	0.264
$H_{(5'b)}$	2.86		0.899	9274	90.909		0.312
NCH_3	2.91		0.964	8757	98.039	7.20	0.315
$CH_{2(1'')}$	1.47		0.283				
$CH_{2(2'')}$	1.33		0.229				
$CH_{3(3'')}$	0.90		0.098				

Table 2. ^{13}C NMR Chemical Shifts, Paramagnetic Relaxation Rates, and C−Cu Distances for Lincomycin (9.3 mM) in H_2O/D_2O in the Presence of Cu(II) (0.093 mM), pH = 7.15, T = 298 K

	δ (ppm)	R_{1p} (s^{-1})	R_{1b} (s^{-1})	r_{C-Cu} (nm)
$C_{(1)}$	91.89	0.006	28.0	0.518
$C_{(2)}$	71.37	0.066	29.3	0.514
$C_{(3)}$	73.93	0.188	134	0.399
$C_{(4)}$	72.18	0.206	1634	0.263
$C_{(5)}$	73.03	0.226	820	0.295
SCH_3	16.42			
$C_{(6)}$	57.31	0.062	1075	0.282
$C_{(7)}$	70.31	0.303	221	0.367
$C_{(8)}$	19.46	0.302	33.4	0.503
$C_{(2')}$	72.18	0.206	1492	0.267
$C_{(3')}$	39.40	0.124	142	0.395
$C_{(4')}$	40.25	0.302	130	0.401
$C_{(5')}$	64.93	0.894	1365	0.271
NCH_3	44.27	0.423	1365	0.271
$C_{(1'')}$	37.74	0.159	28.3	0.517
$C_{(2'')}$	24.13	0.162	25.2	0.527
$C_{(3'')}$	17.13			
CO	173.21			

Substituting the τ_c value into eq 2 provides $\omega_I^2 \tau_c^2 \ll 1$ such that R_{1b} becomes directly proportional to τ_c, which is expected to decrease with raising the temperature. On the contrary, k_{off}, is obviously getting faster and faster at increasing temperatures. Measuring the temperature dependence of R_{1p} is therefore the method of choice for ascertaining the exchange regime experienced by the ligand.

The temperature dependencies of R_{1p} for some LNCM protons are shown in Figure 5, with all other protons behaving in a similar fashion. The plots suggest that the exchange rate contributes to the observed relaxation effects, thus justifying the relatively small paramagnetic effects experienced by LNCM. This is most likely the reason nonselective broadening of all NMR lines at 300 MHz was previously observed.[13] However, the same plots also indicate that R_{1p} is not far from its maximum where intermediate exchange conditions ($k_{off} \approx R_{1b}$) dominate. As a matter of fact, should k_{off} exclusively determine R_{1p}, similar R_{1p} values would be measured for all ^{1}H or ^{13}C NMR lines, or

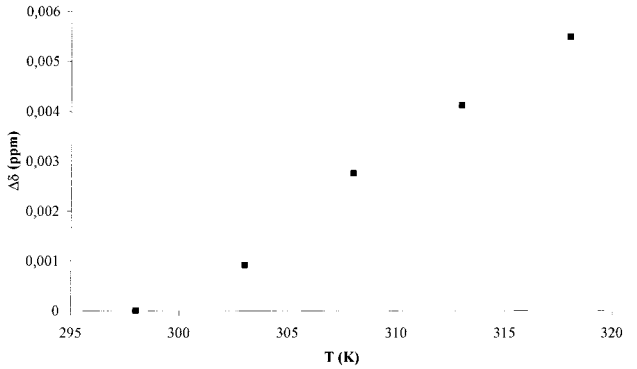

Figure 4. $\Delta\delta$ vs T for the amide proton of lincomycin 10 mM in H_2O/D_2O (9:1) at pH = 7.15.

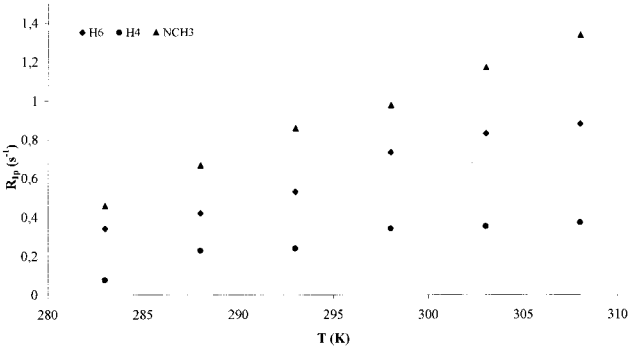

Figure 5. Temperature dependence of paramagnetic relaxation rates (R_{1p}) of selected protons of lincomycin 9.3 mM in D_2O (pH = 7.15), in the presence of Cu(II) at a concentration of 0.093 mM.

alternatively, the differential values would imply a troublesome exchange mechanism. It can be therefore concluded that both R_{1b} and k_{off} contribute to R_{1p} and that the dominant contribution arises from the exchange rate.

The exchange rates were calculated by fitting the initial part of temperature-dependent R_{1p} data (where $R_{1p} \approx p_b k_{off} = p_b/\tau_M$, τ_M being the exchange lifetime) with the Eyring equation:[26]

$$\tau_M^{-1} = A(T) \exp\left\{-\frac{\Delta G^{\#}}{RT}\right\}$$

$$\ln\{R_{1p}\} = \ln\{p_b A(T)\} - \frac{\Delta G^{\#}}{RT} \qquad (3)$$

where $\Delta G^{\#}$ is the free energy of activation for the dissociation process. By approximating p_b with the $[Cu^{2+}]/[LNCM]$ ratio, the values reported in Table 1 were obtained, indicating that the ligand exchange occurs in two steps, with the pyrrolidine ring being released at a rate twice faster. The relatively slow rates obtained (reported in Table 1) may account for the high value of the binding constant of the $CuH_{-2}L$ species.[13]

The k_{off} values were introduced in eq 1 in order to evaluate R_{1b} for each affected proton and carbon and, consequently, the proton−copper and carbon−copper distances reported in Tables 1 and 2. An average τ_c value of 0.15 ns was used for the calculations.

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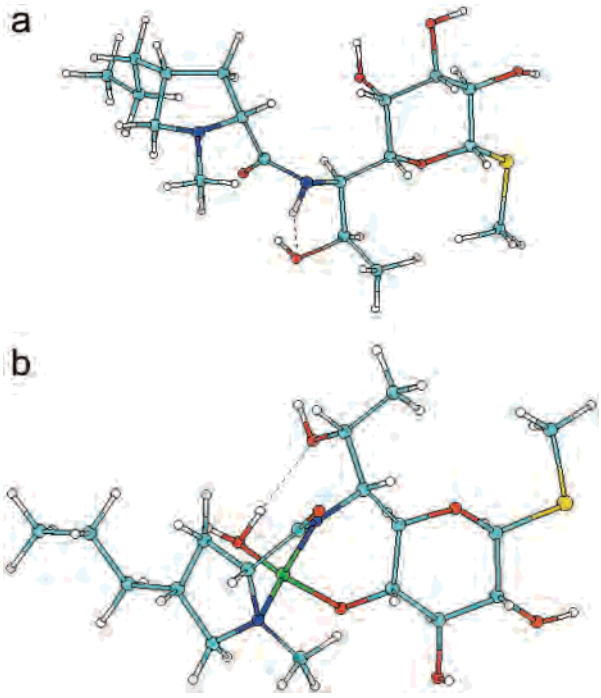

Figure 6. (a) Structural model of lincomycin in water solution. (b) Structural model of the copper(II)−lincomycin 1:1 complex in water solution.

To obtain a molecular model of the copper−LNCM complex, the following evidence on the complex stoichiometry was collected:

(i) As compared with free LNCM in solution, addition of copper determines a very small R_{1p} on the residual water signal ($R_{1p} = 0.042\ s^{-1}$). The spin−lattice relaxation rate of a HDO proton bound to copper ion can be calculated at $9.7 \times 10^4\ s^{-1}$ when the dipole−dipole H−Cu interaction is modulated by $\tau_c = 0.15$ ns [eq 2]. It is consequent that no fast exchanging water is retained in the metal coordination sphere.

(ii) UV−vis spectra were recorded under the same experimental conditions as those for NMR measurements. A relatively strong ($\epsilon = 40\ M^{-1}\ cm^{-1}$) d−d absorption band centered at 656 nm was observed in agreement with amide and amino donations to Cu(II)[27] and also with observations at higher copper concentrations.[13]

(iii) The measured and calculated ^1H and ^{13}C R_{1p} values suggest that all molecular moieties are brought at a relatively close distance from the paramagnetic ion.

It can, therefore, be concluded that the structure of the metal complex is the one corresponding to the already suggested $CuH_{-2}L$ species;[13] however, besides the donation of both nitrogens to copper and binding of the deprotonated C_4-OH, evidence was provided of the C_7-OH approaching the metal coordination sphere through an intervening water molecule. Such water is not, of course, expected to freely exchange with the bulk and therefore does not contribute to the observed spin−lattice relaxation rate of HDO.

The suggested structure was verified by molecular mechanics and dynamics. As a first step, an energy-minimized molecular model of LNCM was obtained using the HYPERCHEM graphics package.[17] Occurrence of the experimentally detected hydrogen bond was apparent (Figure 6a). A hydrated copper ion was then added and linked to the amino and to the deprotonated amide groups. Charges were calculated by the ZINDO-1 semiempirical method, and the model was then subjected to 25 ps unrestrained molecular dynamics at 300 K with the MM+ force field. There were 125 structures sampled over the MD run. It came out that both hydroxyls were approaching the two coordinated water molecules in the low-energy conformations. The C_4−O−Cu bond was then formed by elimination of a water molecule. The hydrogen−copper and carbon−copper distances, as obtained by NMR experiments, were introduced as restraints for the final 25 ps MD run at 300 K. Five structures per picosecond were sampled, and the occurrence of the C_7-OH−water hydrogen bond was verified in the minimum conformational energy structures (Figure 6b).

Insertion of the metal ion and of one of its coordinated water molecules between the hydrogen-bonded amide and C_7-OH may consistently explain the relatively slow rate of dissociation of the involved ligand moieties.

The present results not only ratify what had been previously inferred about the structure of the complex but also provide a precise structural model that may be helpful in explaining the redox properties of copper−aminosugar complexes toward nucleobases and nucleic acids.

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