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Spectroscopic and polarographic investigations of copper(II)-azithromycin interactions under equilibrium conditions

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Abstract

Interactions in the copper(II) ions-azithromycin system were studied by the use of spectroscopic and polarographic methods under equilibrium conditions. Two coordinative compounds, soluble in non-aqueous solvents (methanol, acetonitrile, chloroform, tetrachlorcarbon, etc.), were formed in the system. The molar metal to ligand compositions of compounds were 1: 1 and 1: 4. The compounds are relatively stable, stability constants K_{st} being 7.94×10^{-5} ($pK_{St} = 4.1$) and 2.51×10^{-14} ($pK_{St} = 13.6$) in methanol solution, and solubility product constants K_{sp} being 3.98×10^{-13} ($pK_{St} = 12.4$) and 2.50×10^{-27} ($pK_{St} = 26.6$) in aqueous solution, respectively. The structure of the coordinative compounds CuL and CuL_4 were computed by the Hyperchem program.

Keywords: Azalide; Azithromycin; Copper; Interaction; Complex formation; Coordination compound

1. Introduction

Copper(II) ions as 'biometal' ions are crucial to normal biological activity. Physiologically, they act as constituents of enzymes and other biologically active molecules (Helmut, 1985). They are present in healthy humans in relatively low, distinct concentrations (Schwarz, 1977). Moreover, the levels of copper ions undergo changes in human fluids during serious bacterial infections (Marolt-Gomiscek et al., 1985). On the other side, a high degree of correlation was observed between the resistance of bacteria to particular chemotherapeutics in the presence of metal ions (Groves and Young, 1975).

Azithromycin (10-Dihydro-10-deoxo-11-methyl-11-azaerythromycin A, DCH3; CP-62; 993; XZ-450) (Fig. 1) is the first representative of a new class of very effective antibiotics, known as azalides, which is widely used in medical practice (Djokic et al., 1986; Bright et al., 1988). Azithromycin contains a methyl substituted nitrogen in the 15-membered macrolide aglycone ring and has greater stability than macrolide antibiotics in the presence of acids, leading to good absorption in the digestive tract (Djokic et al.,

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1988; Fiese and Steffen, 1990; Foulds et al., 1990; Shepard and Falkner, 1990). The antibiotic also has an enhanced spectrum of activity, with significantly greater activity against Gram-negative microorganisms whilst retaining activity against Gram-positive organisms (Djokic et al., 1987; Retsema et al., 1987). Furthermore, azithromycin has a long half-life in serum, and the tissue concentrations of azithromycin are greatly in excess of serum concentrations (Girard et al., 1987; Retsema et al., 1990).

In an organism, equilibria between metal ions and bioligands (i.e. amino acids, proteins, etc.) are established. The addition of antibiotics can change these equilibria by the formation of new coordinative compounds which result from interactions between metal ions and antibiotics. Thus, these interactions may be responsible for the alteration of metal ion and/or antibiotic levels in human serum and tissue. These changes and the formation of new compounds can effect the antibacterial activity of azithromycin as well as the physiological activity of copper ions.

The purpose of the present work was to study interactions in the copper(II) ions-azithromycin system as well as to determine and characterize the possible species present in the solution or precipitate. The integrated analyses, i.e. the simultaneous use of electrochemical and spectroscopic methods, were applied in order to obtain reliable information.

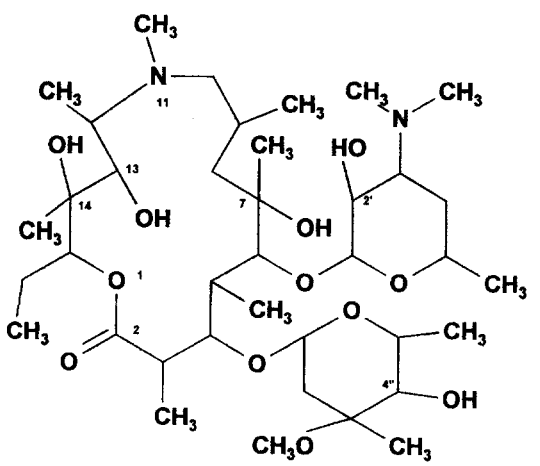

Fig. 1. Azithromycin. Atomic positions after Djokic et al., 1986 and Djokic et al., 1988.

2. Experimental

2.1. Materials and methods

2.1.1. Chemicals

Azithromycin (98.9%, Pfizer, USA), commercial azithromycin 'Sumamed' (Pliva, Croatia) and metal salts $CuCl_2$, $CuSO_4$ and/or $CuSO_4.5H_2O$ of analytical grade were used in the investigation. 'Sumamed' was purified in the following way: the entire sample was suspended in methanol, the solids were removed by filtration, and H_2O was added until the methanol solution became slightly turbid. Upon standing overnight, azithromycin crystallized from solution. Azithromycin methanol solutions were prepared just before the experiments were performed. The methanol, water-methanol and acetonitrile solutions with metal to azithromycin molar ratio from 10:1 to 1:10 as well as with constant metal plus ligand concentrations from 1.25×10^{-3} to 1.25×10^{-2} mol/l were prepared by combining antibiotic and metal salt solutions.

2.1.2. Differential pulse polarography

Differential pulse polarographic measurements were carried out using a Polarecord 626, Metrohm analyzer. The measuring system consisted of a platinum wire auxiliary and a silver/silver chloride reference electrode for aqueous solutions (or silver/silver chloride reference electrode for acetonitrile solutions) together with a dropping mercury electrode as a working electrode. For polarographic investigations, 0.1 mol/l KNO_3 solution was used to maintain the ionic strength, and phosphate buffers (1/15 mol/l KH_2PO_4 and 1/15 mol/l $Na_2HPO_4 \cdot H_2O$) were used for all measurements in aqueous solutions where an accurate pH value was required. In the case of methanol and/or acetonitrile solutions of 0.1 mol/l, Et_4NClO_4 solutions were utilized. Solutions in the polarographic cell were deoxygenated by bubbling with oxygen-free argon.

2.1.3. Spectroscopic methods

The spectroscopic measurements were carried out using a Hewlett Packard 8452A diode array spectrophotometer and Cary 4E spectrophotometer in UV and Vis regions, and by a Perkin Elmer 1330 infrared spectrophotometer in the IR region. KBr pellets were prepared from solid precipitates or sample residues obtained after rapid evaporation and drying (air stream, room temperature) of sample solutions. A Perkin Elmer 4000 atomic absorption spectrometer was used for the determination of the total copper concentration in aqueous solutions.

^{1}H NMR spectra were recorded at 250 MHz on a Bruker WM250 spectrometer in $CDCl_3$ solutions and chemical shifts are given in ppm relative to TMS as an internal standard.

3. Results and discussion

3.1. Polarographic investigations

The differential pulse polarographic measurements were performed at constant concentrations of Cu(II) ions (2.5, 6.25 or 7.5 x 10^{-4} mol/l and 2.5 x 10^{-3} mol/l, and increasing azithromycin (L) concentrations (as well as the reverse in the case of acetonitrile solutions). The polarograms were measured in phosphate buffer solutions (pH 5.0–7.35) as well as in non-aqueous acetonitrile solutions. In aqueous solutions only the peak corresponding to copper (II) ions reduction was observed. The values of currents for Cu(II) ions reduction (at initial constant concentration of copper (II) ions in the system) steadily decrease with increasing of ligand concentration. The reason for this can be explained by the very slight solubility of azithromycin as well as its compounds in water.

Another behavior was observed in the case of non-aqueous solutions. The polarograms obtained for the acetonitrile solutions at constant copper concentration (6.25 x 10^{-4}) are presented in Fig. 2. The height of peak I which corresponds to the reduction of Cu(II) to Cu(O) decreases with increasing amounts of azithromycin. The initial presence of the ligand is demonstrated in the

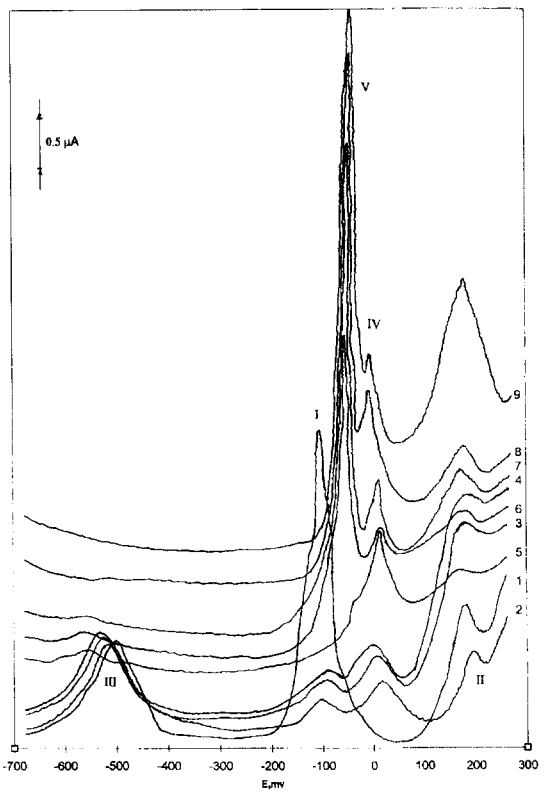

Fig. 2. DPP curves of the copper(II)–azithromycin system in acetonitrile solutions at metal/ligand ratios: (1) Cu^{2+}, (2) 5:1, (3) 4:1, (4) 3:1, (5) 1:1, (6) 1:2, (7) 1:3, (8) 1:4, (9) 1:10. [Cu^{2+}] = 2.5 x 10^{-4} mol/l.

appearance of two new peaks which can be ascribed to the one-electron reductions of Cu(II) → Cu(I) and Cu(I) → Cu(O) (peaks II and III, respectively). These results are rather similar to observations reported previously for cephalosporins and penicillins, where the stabilization of intermediate Cu(I) ions also took place (Ogorevc et al., 1985; Sher et al., 1993). Increasing amounts of azithromycin give rise to an additional peak IV, which probably corresponds to the reduction of the copper bonded in the coordinative compound. After the attainment of a 1:2 metal to ligand molar ratio a new peak (V) appears. This peak is connected with the formation of a new coordinative compound of copper with a higher content of ligand. All these peaks were observed in the polarograms of the acetonitrile solutions, both immediately after mixing and under equilibrium conditions.

3.2. Spectroscopic investigations

The differential pulse polarography method was complemented by spectroscopic measurements.

Spectroscopic determinations in the ultraviolet and visible regions were performed at constant concentration of copper(II) ions (1.25×10^{-3} or 1.25×10^{-4} mol/l) and increasing ligand concentrations as well as at constant (1.25×10^{-2} or 1.25×10^{-3} mol/l) metal plus ligand concentrations. The spectroscopic study of methanol solutions containing Cu(II) ions and azithromycin in the visible region indicates the presence of two compounds with absorption maxima at 510 and 760 nm, respectively. The absorbance at 760 nm increases with increasing ligand concentration up to a copper(II) to ligand molar ratio 1:1 and subsequently falls rapidly and simultaneously a new peak with an absorption maximum at 510 nm appears. This is proportional to increasing ligand concentration and, after reaching a molar ratio 1:4, the absorbance at 510 nm remains practically constant. The simultaneous presence of two peaks can be observed in the spectra of solutions with different copper(II) to ligand molar ratios, i.e. 2:1, 1:1, 1:2 (Fig. 3). This is due to the formation of a new compound at higher ligand concentration. Analogous behavior is seen in the UV region with wavelength maxima at 255 and 290 nm corresponding to the formation of two compounds with metal to ligand molar ratio 1:1 and 1:4, respectively. Similar results were observed for both $CuCl_2$-azithromycin and $CuSO_4$-azithromycin systems. Consequently, on the basis of the molar ratio methods, it can be concluded that the molar copper to azithromycin ratios of the stoichiometric numbers of these compounds are 1:1 and 1:4.

Additionally, we studied the interactions between metal ions and azithromycin in aqueous-methanol solutions under different H_2O: CH_3OH volume ratios. In accordance with spectroscopic investigations, the analogous spectra of samples in these solutions with wavelength maxima at 760 and 290 nm as well as at 510 and 255 nm confirm the formation of coordinative compounds CuL and CuL_4, respectively. Dilution of methanol solutions of the Cu(II) ions-antibiotic system with water results in precipitation due to the poor aqueous solubility of azithromycin and its copper-containing compounds. The total copper concentration in the supernatant solutions of the copper (II) ion-azithromycin system under equilibrium conditions has two maxima at metal to ligand molar ratio 1:1 and 1:4 (Fig. 4). The reason for this is the decreasing of metal concentration in the solution due to the formation of slightly soluble basic salts of copper(II) in the region of metal excess. Also, the poor aqueous solubility of azithromycin leads to a decrease in the formation of coordinative compounds in the region of ligand excess. Simultaneously, the formation of soluble coordinative compounds takes place in the intermediate region and, as a result, the highest concentration of copper bonded in compounds can be observed.

Furthermore, the formation of two methanol soluble compounds under equilibrium conditions also were confirmed using the continuous variation (Job's) method (Fig. 5). The peak with absorption maximum at 760 nm appears with excess

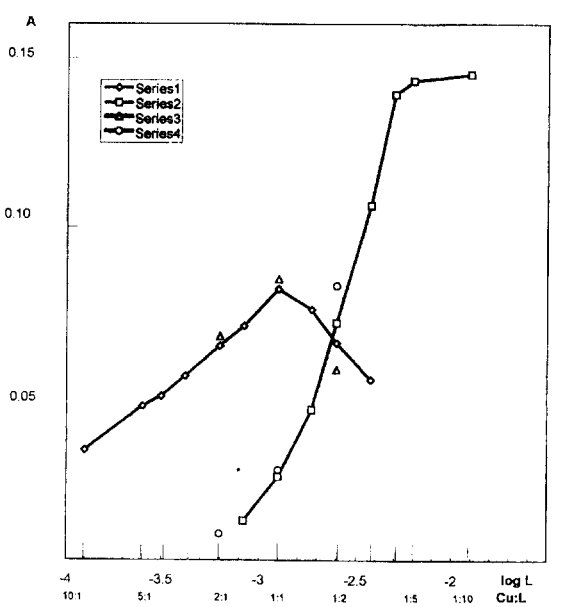

Fig. 3. Dependence of absorbance of coordinative compounds in the copper(II)–azithromycin system under constant metal concentration in $CuSO_4$ solutions on different metal/ligand ratios at 760 nm (1) and at 510 nm (2), and in $CuCl_2$ solutions at 760 nm (3) and at 510 nm (4).

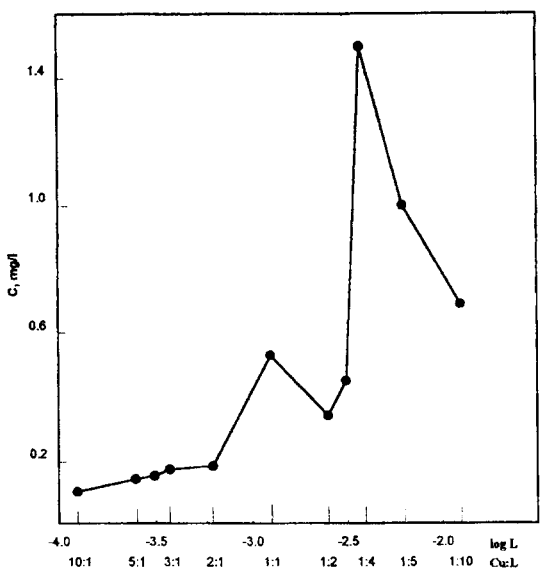

Fig. 4. Total copper concentrations in the H_2O: methanol solutions (1:1) of the copper(II)–azithromycin system under equilibrium conditions.

of metal ions concentration and the peak with absorption maximum at 510 nm appears with excess of ligand concentration. In the intermediate region, the formation of both coordinative compounds with the above-mentioned absorption maxima can be seen. The molar ratio of the first compound formed in the system is close to 1:1, but the ratio of the compounds with higher content of ligand is between 1:3 and 1:4. The last result is probably related to the effect of the first compound on the formation of the second one. In order to obtain the accurate composition of the compound with a higher content of ligand, we determined the copper concentration of the precipitate isolated from the system previously diluted by water in the region of ligand excess. It was found to be 1.95 $\pm$ 0.20% Cu, corresponding closely to the theoretical value of 2.08% Cu for the 1:4 ratio of Cu:L. Therefore, it may be concluded that the compositions of the two coordinative compounds formed in the copper(II)-azithromycin system correspond to the formula CuL and CuL_4.

The stability constants for these complexes have been calculated from the continuous variation method data. It was estimated that the coordinative compounds CuL and CuL_4, with absorption maxima at 760 and 510 nm, are relatively stable, pK_{st} being 4.1 and 13.6, respectively.

The solubilities of CuL and CuL_4 were studied in aqueous solutions over a broad pH region. The solubility of the compounds isolated from Cu(II)-azithromycin methanol solutions was minimal in the region of pH 5.0–9.0 under equilibrium conditions (Fig. 6). The solubility product constant K_{sp} calculated on the basis of determination of total copper concentrations at pH 7.0 and constant ionic strength was estimated to be 3.98 x 10^{-13} and 2.50 x 10^{-27} (0.1 M KNO_3) for the precipitate CuL and CuL_4, respectively.

In order to obtain more information about the character of binding between copper(II) ions and azitromycin, the IR spectra of the antibiotic and its compounds were recorded. Bands in the region 1300–1800 cm^{-1}, assigned to the C = O, C = N and C-O vibrations, were observed. In the spectra of compounds, only insignificant shifts of these bands (in comparison with IR spectrum of azithromycin) take place. Therefore, it can be

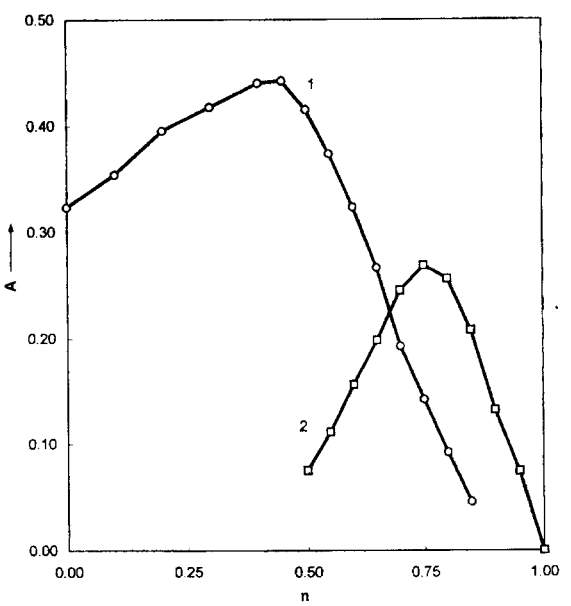

Fig. 5. Dependence of absorbance of coordinative compounds in the copper(II)–azithromycin system under constant metal plus ligand concentration on molar ratios: (1) at 760 nm, and (2) at 510 nm.

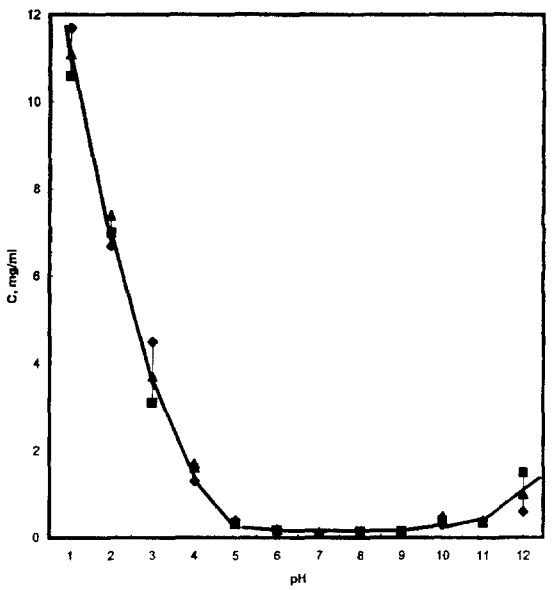

Fig. 6. pH dependence of the solubility of CuL_4 in water under equilibrium conditions.

concluded that copper does not influence the ligand structure and the structure of the compounds entirely take the shape of the ligand. Further characterization of the coordinative compound was accomplished by NMR measurements. The NMR spectra are presented in Fig. 7. The ^1H NMR spectra show well defined resonances at δ_H 3.352 (3 H, s), 2.316 (3 H, s) and 2.288 (6 H, s) assigned to the 3"-OCH_3 absorption of cladinose, the 9a-NCH_3 group of the 15-membered aglycone ring, and the 3'-$N(CH_3)_2$ group of desosamine, respectively. The same chemical shifts (3.352, 2.316 and 2.287) are also observed in the ^1H NMR spectra of copper containing compounds. Additionally, the intense signal at 2.881 ppm observed in the spectrum of azithromycin is absent in the spectra of coordinative compounds. Moreover, in the region δ_H 3.00–3.15, a triplet in the spectra of the coordinative compounds is observed instead of a quadruplet seen in the spectrum of azithromycin. It can be concluded that the hydroxide groups of azithromycin are bonded to copper (II) ions.

On the basis of the above mentioned data and literature data concerning the structure and stereochemical configuration of azithromycin, we have computed the structure of the CuL and CuL_4 coordinative compounds using the Hyperchem program. Our calculation of the structure of the copper-containing compounds is based on a ligand structure determined by X-ray and NMR data (Djokic et al., 1986; Djokic et al., 1987; Bright et al., 1988). For the geometry optimization the Polak-Ribiere optimizer (Molecular Mechanics) was used. The results of the MM^+-calculations for these compounds are pre-

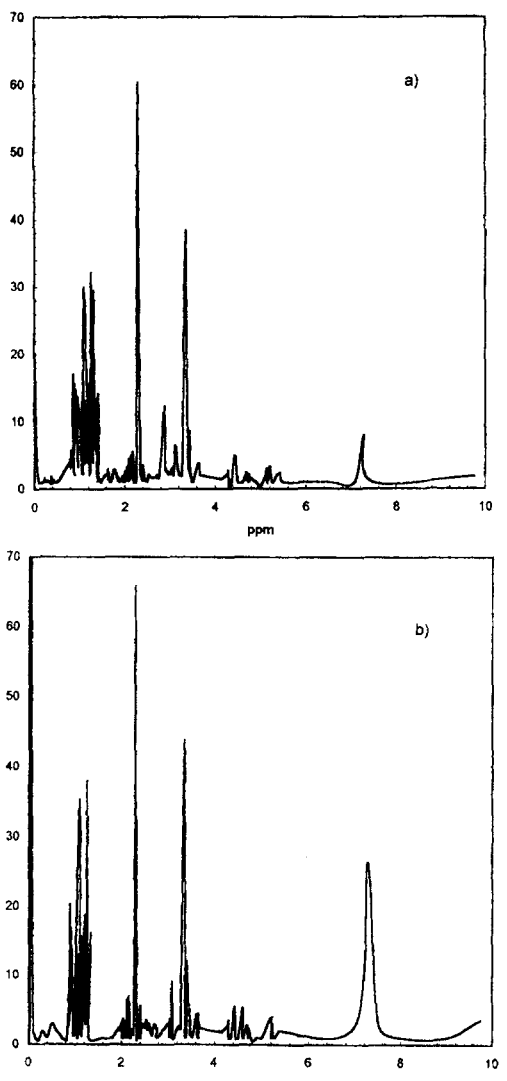

Fig. 7. 250-MHz ^1H NMR spectra of azithromycin (a) and CuL_4 (b).

Table 1
Data of MM$^+$-calculations for copper(II)-azithromycin coordinative compounds

No	Atomic position (as in Fig. 1)	Energy, kcal/mol	Bond	Angle	Dihedral	Stretch-bend	Electrostatic	Compound
1	C(2')-O-	107.6	6.63	32.23	42.00	2.89	0	CuL
2	C(13)-O-	113.8	6.52	36.96	40.21	2.98	0	CuL
3	C(14)-O-	128.4	8.10	44.02	44.32	3.60	0	CuL
4	C(7)-O-	120.0	7.42	38.19	46.15	3.19	0	CuL
5	C(4'''')-O-	125.2	7.89	43.12	42.26	3.57	0	CuL
6	C(2'')-O-, C(4'''')-O-	126.6	6.87	48.23	44.82	3.23	−0.53	CuL
7	C(2'')-O-, C(7)-O-	129.0	8.05	44.25	45.82	3.59	−0.97	CuL
8	C = O, C(13)-O-	205.5	12.6	90.35	47.08	3.71	−0.94	CuL
9	C = O, C(14)-O-	183.8	13.43	90.46	46.79	5.12	−0.63	CuL
10	C = O, C(13)-O-	619.7	36.07	288.2	190.0	11.29	−0.74	CuL_4
11	C = O, C(14)-O-	504.8	31.13	209.1	176.4	12.97	−2.06	CuL_4
12	C = O, C(7)-O-	524.6	34.33	241.6	165.9	11.45	−3.08	CuL_4
13	C = O, C(2'')-O-	476.1	29.78	208.2	169.7	12.05	−1.17	CuL_4
14	C = O, C(14)-O	533.3	31.79	235.2	179.1	11.53	−1.78	CuL_4

sented in Table 1. The positions of the OH-groups to which the Cu(II) is bonded are listed in column 2. The most stable configuration seems to be the one with 107.6 kcal/mol for CuL and 476.1 kcal/mol for CuL_4. It can be concluded that the most stable configuration of the CuL coordinative compound is the one with the same bonding to the C(2')-O-position as in the most stable configuration of CuL_4.

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