Research of new mixed-chelate copper complexes with Quinoxaline N1,N4-dioxide derivatives and Alanine as ligands, potential antimycobacterial agents

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RESEARCH OF NEW MIXED-CHELATE COPPER COMPLEXES WITH QUINOXALINE N^1,N^4-DIOXIDE DERIVATIVES AND ALANINE AS LIGANDS, POTENTIAL ANTIMYCOBACTERIAL AGENTS

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Abstract

Three new mixed-chelate copper complexes with 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives and alanine as ligands were synthesized in solid state. The spectroscopic

characterization (FTIR, EPR, UV-Vis) showed that copper coordinated through the amine and the *N*-oxide groups of the quinoxaline derivatives and the amine and carboxylate moieties from alanine forming a dimeric species. The tree complexes showed *in vitro* activity against *M. tuberculosis* H_{37}Rv (ATCC 27294) similar to that of ethambutol while they are inactive against *E. coli* and *S. aureus*.

Keywords: antimycobacterial agents, copper complexes, quinoxaline derivatives.

Resumen

Tres nuevos complejos mixtos de cobre con derivados de 3-aminoquinoxalina-2-carbonitrilo N^1,N^4-dióxido y alanina como ligandos fueron sintetizados en estado sólido. La caracterización espectroscópica (FTIR, EPR, UV-Vis) mostró que el Cu coordina con el grupo amino y el *N*-óxido del derivado quinoxalínico y con el amino y el carboxilato de la alanina formando especies diméricas. Los tres complejos mostraron actividad frente al *M. tuberculosis* H_{37}Rv (ATCC 27294) similar a la del etambutol, mientras que fueron inactivos frente a *E. coli* y *S. aureus.*

Palabras clave: agentes antimicobacterianos, complejos de Cu, derivados quinoxalínicos.

Introduction

Tuberculosis is an infectious disease caused by mycobacteria, mainly *Mycobacterium tuberculosis*. It can lead to serious complications and even death, especially if it is accompanied by other bacterial infections and if the patients have their immune systems compromised by immunosuppressive drugs, abuse substances, or HIV/AIDS, among others [1].

The World Health Organization (WHO) estimates that about 30 million people will be infected in the next 20 years, not only in the third world but also in the developed countries [1]. In view of the importance of this disease and the emergence of multi-drug resistant strains, the investigation and development of new drugs is a leading area of research.

The study of quinoxaline derivatives has become of much interest in recent years due to their antibacterial, antiviral, anticancer, antifungal, antihelminthes and insecticidal activities [2]. In particular, the di-*N*-oxide derivatives of quinoxaline show a dramatically increase of the diversity of biological properties. For example, some of these organic derivatives have shown hypoxia-selective citotoxicity and they could be potentially useful for the treatment of solid tumors. Besides, some derivatives have presented excellent *M. tuberculosis* growth inhibition values, leading generally the lack of the two *N*-oxide groups to the loss of the antimycobacterial activity [3-6]. Although excellent *in vitro* biological results have been obtained with some 3-amino-2-carbonitrile-quinoxaline N^1,N^4-dioxide derivatives, they were not useful for therapy owing to too short *in vivo* half lives and low solubility in physiological media [7-10].

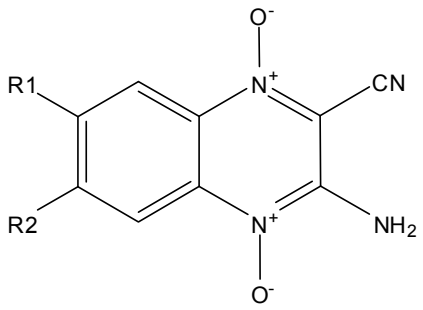
As an effort to improve bioavailability and pharmacological and toxicological properties of quinoxaline N^1,N^4-dioxide derivatives, we have focused our current research on the synthesis, characterization and biological evaluation of a large amount of metal complexes with this family of organic compounds [11-15]. These studies lead to several complexes with higher pharmacological activity than the free ligands, especially iron complexes bearing anti-*Mycobacterium tuberculosis* activity. Nevertheless, in many cases the lipophilicity and solubility of the developed complexes still remained non adequate for therapeutically purposes. Therefore we decided to take advantage of a well known strategy in the search for more active metal-based drugs that involves the incorporation of a second ligand in the metal coordination sphere, like an aminoacid, with the aim of obtaining different levels of hydrophobicity [16]. Taking into account these antecedents and

trying to know more about the effect of metal coordination on the bioactivity of selected 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives, in this work we present the synthesis in solid state and the analytical and spectroscopic characterization of a new series of mixed-chelate Cu(II) complexes with different non- and mono-substituted 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives (Figure 1) and alanine (ala) as ligands. Besides, their antibacterial activities against *E. coli*, *S. aureus* and *M. tuberculosis* are reported.

Experimental

Materials and methods

All starting materials were commercially available research-grade chemicals and were used without further purification. The quinoxaline derivatives (see Figure 1) were synthesized in CIFA, Navarra University, Pamplona (Spain) by Dr. Monge's group.



Ligand	R1	R2
L1	H	H
L2	Cl	H
L3	F	H

Figure 1. 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxides used as ligands.

Synthesis of the complexes

The complexes were synthesized through a solid state technique [17] by mixing $CuCO_3Cu(OH)_2$ (11 mg), alanine (9 mg) and L (20.0 mg of L1 or 23.5 mg of L2 or 22.0 mg of L3), adding some drops of water and heating the mixture at 80 ºC during 6 h. In all the cases red complexes were formed. The yields (%) were 95.5, 77.6 and 90.0, respectively.

The stoichiometries obtained were [Cu(L-H)(ala-H)] for the three complexes where L-H and ala-H are the deprotonated ligands.

The results of the elemental analysis (%) obtained with a Carlo Erba EA 1108 analyzer were: for [Cu(L1-H)(ala-H)] (Code CuL1ala) $C_{12}H_{12}N_5O_4Cu$ Found/ Calc.: C, 40.48/ 40.97; N, 19.74/ 19.91; H, 3.05/ 3.15; for [Cu(L2-H)(ala-H)] (Code CuL2ala) $C_{12}H_{11}N_5O_4ClCu$ Found/ Calc.: C, 36.89/ 37.06; N, 17.58/ 18.02; H, 3.02/2.83 and for [Cu(L3-H)(ala-H)] (Code CuL3ala) $C_{12}H_{11}N_5O_4FCu$ Found/ Calc.: C, 38.72/ 38,76; N, 18.61/ 18.84; H, 3.15/ 2.96.

The electronic spectra of the three complexes showed in DMF and ethanol a broad band near 500 and a shoulder near 570 nm, in agreement with the red-purple colour of the solutions.

Spectroscopic measurements

IR spectra, in the range between 4000 and 400 cm^{-1}, were recorded on a Bomem M 102 FTIR spectrophotometer using the KBr pellet technique. Electronic spectra of the complexes were registered on a Shimadzu UV-1603 spectrophotometer.

Electron Paramagnetic Resonance (EPR) experiments were carried out in a Bruker ELEXSYS E580 spectrometer operating at 9.5 GHz. EPR spectra of solid- (polycrystalline) and in DMSO solution samples of the three compounds were measured at 30 K using experimental parameters such that signal saturation and distortion was avoided. Temperature was controlled by means of an ITC503 Oxford cryostat system.

Microbiological assays

Agar dilution tests were used to determine the minimal concentration of the antimicrobial agent required to inhibit or kill the microorganisms (MIC). Every step in the procedure for performing the agar dilution susceptibility test [18] was based on recommendations from the National Committee for Clinical Laboratory Standards [19].

A suspension in sterile physiological serum of the complexes was incorporated in 20 mL of Mueller-Hinton agar and poured into Petri dishes. The pH of each batch of medium was in the range 7.2 to 7.4. The dilution scheme for each antimicrobial agent covered a 400-12.5 μg of complex/mL range. The same process was carried out with a water dilution of $CuSO_4 \cdot 5H_2O$.

The standard strains used were *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. In each case, a suspension of about 10^4 colony-forming units / drop (CFU/drop) in Trypticase soy broth was prepared and diluted (1:20) in physiological serum. One drop of this suspension was inoculated on the agar surface containing the indicated dilutions of the antimicrobial agent, and on the control dish containing no antimicrobial agent.

All tests and inoculations on each dish were run in duplicate.

The anti-*M. tuberculosis* activities of the compounds were determined using the MABA as analytical method [20]. Stock solutions of copper complexes were prepared in dimethyl sulfoxide and diluted in broth medium Middlebrook 7H9 (Difco), supplemented with oleic acid, albumin, dextrose, and catalase (OADC enrichment - BBL/Becton-Dikinson, Sparks, MD, USA), to obtain final compounds concentration ranges of 0.15 to 50 μg/mL. Isoniazid was solubilized with distilled water according to the manufacturers' recommendations (Difco laboratories, Detroit, MI, USA) and used as standard drug. *M. tuberculosis* $H_{37}Rv$ ATCC 27294 was grown for 7 to 10 days in Middlebrook 7H9 supplemented with OADC added of 0.05 % Tween 80 to avoid clumps. Suspensions were prepared and their turbidities matched to a McFarland no. 1 (turbidity standard). After further dilution of 1:25 in Middlebrook 7H9 supplemented with OADC, the inoculum was added to each well of the 96 well microtiter plate (Falcon 3072; Becton Dickinson, Lincoln Park, NJ) together with the compounds. Samples were set up in triplicate. Cultures were incubated for 7 days at 37 ^oC, and Alamar Blue was added for the reading. The minimum inhibitory concentration (MIC) was defined as the lowest concentration resulting in 90 % inhibition of growth of *M. tuberculosis* determined by measuring the fluorescence (excitation/emission of 530/590 nm filters respectively) in a SPECTRAfluor Plus (Tecan) instrument. For standard test, the MIC value of isoniazid was determined each time. The acceptable MIC of isoniazid ranged from 0.015 to 0.05 μg/mL [21].

Results and discussion

Infrared spectra

The IR spectra of the complexes were compared with those of the free ligands and of previously reported complexes [11-15] [22] [23]. The results obtained are shown in Table 1. For the sake of clarity data of alanine IR spectrum were also presented in Table 1. Figure 2 shows as an example the IR spectra of L3, ala and the corresponding complex.

All the complexes showed a similar spectral pattern. After the coordination the ligand´s bands corresponding to $\nu_{as}(NH_2)$ (in the 3336-3436 cm^{-1} range) and $\nu_s(NH_2)$ (in the 3255-3295 cm^{-1} range) of the amino group of quinoxaline derivatives disappeared and only one band was observed (in the 3298-3385 cm^{-1} range). This is in accordance with the presence of a secondary amine formed by deprotonation of the primary amine as a consequence of the coordination with copper ion, as previously observed for vanadyl and copper complexes with this family of ligands. This behavior was also observed in metal chelates with aromatic ligands involving the amino group in *ortho* position to the *N*-oxide [11-15] [22].

Table 1. Main bands and proposed assignments (cm^{-1}) of copper complexes IR spectra and free ligands

Compound	$\nu_{as}(NH_2)$	$\nu_s(NH_2)$	ν(NH)	ν(N-O)	$\nu_{as}(C\equiv N)$	$\delta(NH_3^+)$	$\nu_{as}(COO^-)$	$\nu_s(COO^-)$
L1	3353	3262	-	1343	2237	-	-	-
CuL1ala	-*	-*	3385	-	2226	-	nd	1395
L2	3436	3295	-	1335	2237	-	-	-
CuL2ala	-*	-*	3298	-	2221	-	nd	1389
L3	3336	3255	-	1345	2231	-	-	-
CuL3ala	-*	-*	3373	-	2229	-	nd	1397
ala	3082	2991-2936	-	-	-	2113	1590	1413

*:Only the symmetric and antisymmetric stretchings of the quinoxaline ligands were included ; nd = not determined.

Furthermore, the strong $\nu(N\rightarrow O)$ band for the free ligands (1335-1345 cm^{-1}) shifted in the complexes being overlapped with other bands, showing the coordination through this group. The $\nu(C\equiv N)$ (2221-2237 cm^{-1}) suffered only minor changes upon complexation in agreement with the fact that this group did not coordinate to copper ion.

Moreover, data show the copper coordination through the alanine ligand in the three complexes. The free aminoacid exists as zwitterion in the crystalline state. This is demonstrated by the existence of the $\delta(NH_3^+)$ band at 2113 cm^{-1}. This band disappeared in the complexes showing the copper coordination through the amine terminal group of the alanine ligand. Besides, two vibrations for the COO- moiety ($\nu_{as}(COO^-)$ at 1590 cm^{-1} and $\nu_s(COO^-)$ at 1413 cm^{-1}) are observed in the zwitterion. After coordination a lowering of the frequency of one of these bands and the

increase of the other are expected. In our complexes, $\nu_s(COO^-)$ shifted to low frequencies (1389-1397 cm^{-1}). This behavior is similar to that of $[Cu(ala)_2]$ that presents one band at 1400 cm^{-1} [23]. The $\nu_{as}(COO^-)$ was overlapped by quinoxaline bands and consequently could not be assigned.

Besides, the strong band assigned as $\nu_{as}(NH_2)$ in the alanine spectrum (3082 cm^{-1}) shifted to higher wavelengths in the mixed-chelate complexes (around 3244 cm^{-1}). This behavior is in agreement with previously reported data for $[Cu(ala)_2]$ (3245 cm^{-1}) [23-24].

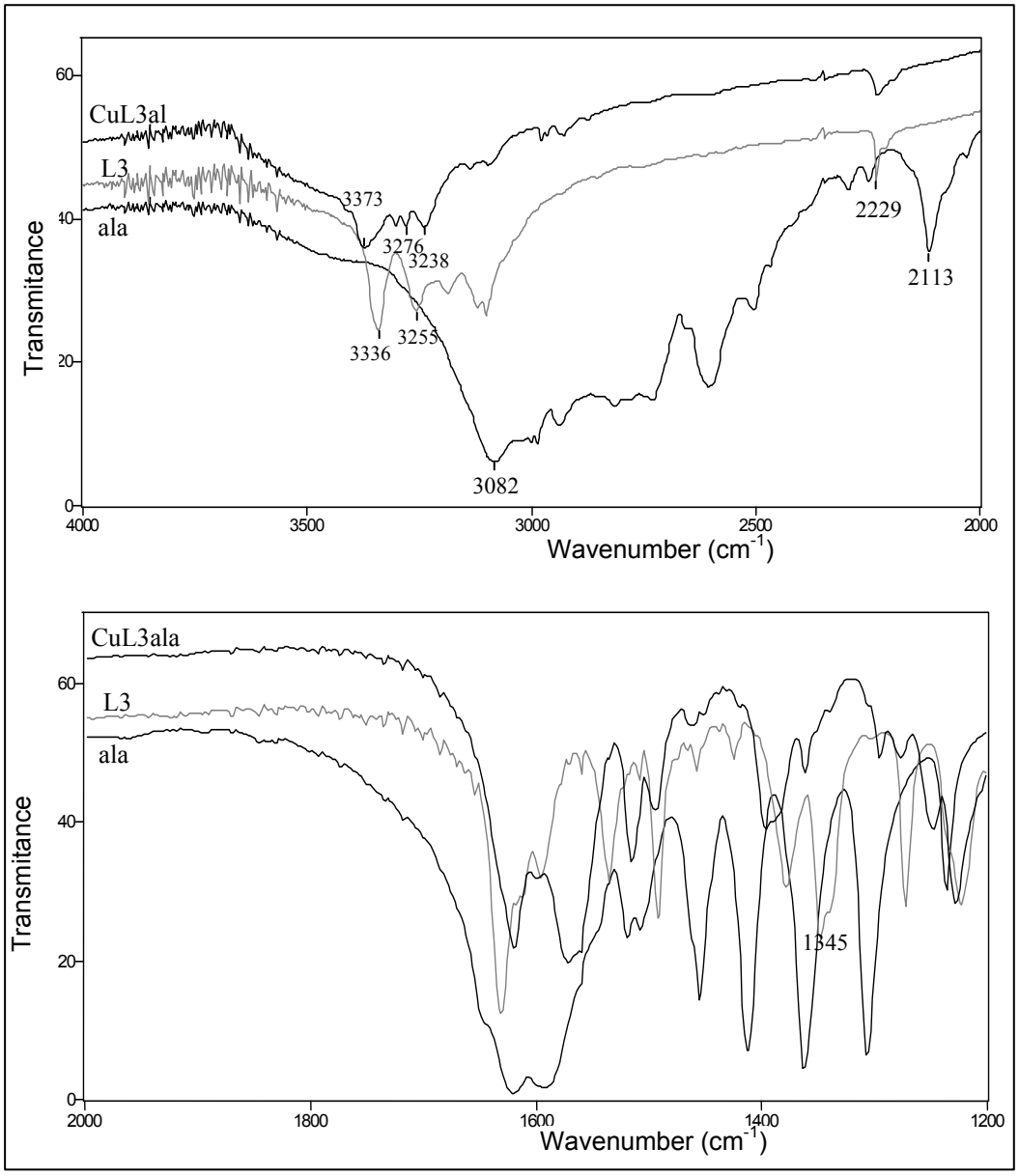


Figure 2. IR spectra of ligands (quinoxaline derivative and ala) and the corresponding complex CuL3ala

EPR spectra

Polycrystalline samples of the three complexes presented EPR spectra (Figure 3) characteristic of coupled pairs with a distorted resonance around 330 mT (g~2) and a half-field

transition around 165 mT (inset in Figure 3A). This half-field line is only observed in S=1 spin systems and is the result of the "forbidden" $\Delta M_S=\pm 2$ transition [25]. Hence, in the solid state, the complexes exist as dimmers containing exchange-coupled pairs of S=1/2 Cu(II) ions as previously observed for other copper compounds in the same conditions [12, 13, 26]. Upon dilution in DMSO, the spectra change to the usual EPR spectra of Cu(II) ions (Figure 3B) showing features assigned to the hyperfine interaction between the S=1/2 electron spin and the I=3/2 nuclear spin [27, 28]. The frozen-solution spectra are characterized by the following parameters: $g_{//}=2.2513$, $A_{//}=17.5$ mT, $g_{\perp}=2.0590$. Our EPR results thus show that, in solution, the complexes are in a tetragonally monomeric form with ground state d_{x2-y2}. The EPR spectrum at room temperature shows the same behavior (data not shown).

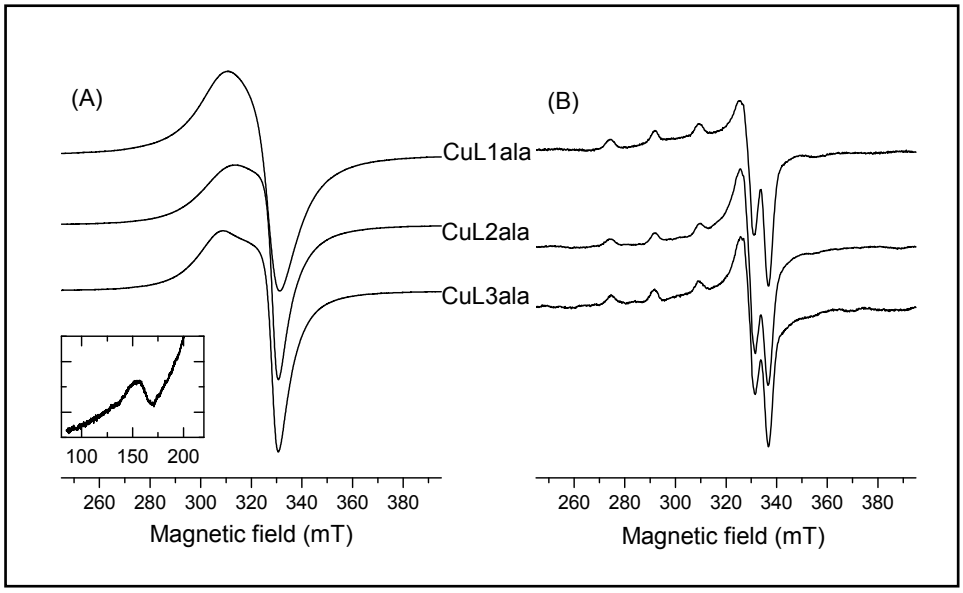


Figure 3. Low-temperature (30 K) EPR spectra from: (A) polycrystalline samples and (B) DMSO solutions of CuL1ala, CuL2ala, and CuL3ala. The inset shows the half-field transition discussed in the text.

Microbiological assays

The minimum inhibitory concentration (MIC) values of Cu complexes against *E.coli* (ATCC 25922), *S. aureus* (ATCC 29213) and *M. tuberculosis* H_{37}Rv (ATCC 27294) are shown in Table 2.

The three complexes were inactive against *E.coli* and *S. aureus*, while they were active against *M. tuberculosis*. The MIC values of all the complexes are of the same order than that of ethambutol (MIC = 5.62 μg/mL), an antimycobacterial agent in clinical use. Nevertheless, they are less active than other drugs like p-amino salicylic (PAS) (MIC = 1.25 μg/mL) and isoniazid (MIC = 0.03-0.015μg/mL).

Taking into account the analytical and spectroscopic results and our antecedents with Cu complexes of this kind of ligands, a possible structure for the complexes is proposed in Figure 4. This proposal is supported by our previous studies where homoleptic copper-quinoxaline complexes

[12-13] showed the presence of bridges that permitted to explain the dimeric molecules shown by EPR. Besides, a similar IR pattern was observed by the compounds presented in this work.

Table 2. MIC values (μg/mL) of Cu complexes against *E. coli*, *S. aureus* and *M. tuberculosis*

Compound	*E. coli*	*S. aureus*	*M. tuberculosis*
CuL1ala	> 400	> 400	7.8
CuL2ala	> 400	> 400	7.8
CuL3ala	>400	> 400	7.8

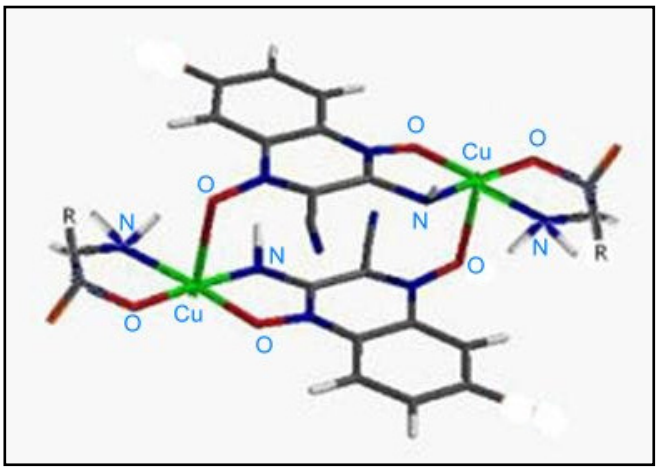


Figure 4. Scheme of the proposed structure for the copper complexes (R=-CH_3).

Conclusions

Three new mixed-chelate copper complexes with 3-aminoquinoxaline-2-carbonitrile N^1,N^4-dioxide derivatives as ligands were synthesized in solid state. The spectroscopic characterization showed that copper coordinated with both ligands: quinoxaline derivative and alanine, forming dimeric species in the solid state. The three complexes showed *in vitro* activity against *M. tuberculosis* H37Rv (ATCC 27294) similar to that of ethambutol. These results have encouraged us to continue this research further.

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