Mycobiology 34(4): 214-218 (2006)

Antifungal Activities of Biorelevant Complexes of Copper(II) with Biosensitive Macrocyclic Ligands

N. Raman*, J. Joseph, A. Senthil Kumara Velan[1] and C. Pothiraj[1]

Research Department of Chemistry, VHNSN College, Virudhunagar-626 001
[1]Department of Microbiology, VHNSN College, Virudhunagar-626 001

(Received September 5, 2006)

Four copper(II) complexes have been prepared using macrocyclic ligands. The macrocyclic ligands have been synthesized by the condensation reaction of diethyl phthalate with Schiff bases derived from *o*-phenylene diamine and Knoevenagel condensed *β*-ketoanilides (obtained by the condensation of acetoacetanilide and substituted benzaldehydes). The ligands and copper complexes have been characterized on the basis of Microanalytical, Mass, UV-Vis., IR and CV spectral studies, as well as conductivity data. On the basis of spectral studies, a square-planar geometry for the copper complexes has been proposed. The *in vitro* antifungal activities of the compounds were tested against fungi such as *Aspergillus niger*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Rhizoctonia bataicola* and *Candida albicans*. All the synthesized copper complexes showed stronger antifungal activities than free ligands. The minimum inhibitory concentrations (MIC) of the copper complexes were found in the range of 8~28 *μg/ml*. These compounds represent a novel class of metal-based antifungal agents which provide opportunities for a large number of synthetic variations for modulation of the activities.

KEYWORDS: Antifungal, Knoevenagel, Macrocyclic ligands, Square planar

Infectious diseases like diarrhea, dysentery, tuberculosis, acute respiratory tract infections, AIDS and recently SARS are global threat and their incidences are increasing significantly day by day. Although a number of chemotherapeutic agents are available in market places, the pathogenic organisms are developing resistance to these agents. So, it is important to find out safer, more effective and inexpensive chemotherapeutic agents. An extensive literature has developed in recent years in the field of chelate compounds with special reference to their antimicrobial activities. Metal coordination complexes have been widely studied for their antimicrobial (Kamalakannan *et al.*, 2002) and anticancer (Treshchalina *et al.*, 1979) properties. Many drugs possess modified pharmacological and toxicological properties when administered in the form of metallic complexes. Platinum anticancer drugs are now the widely used anticancer drugs in the world e.g. cisplatin, carboplatin, oxaliplatin, tetraplatin etc. This inspires synthetic chemists to search for new metal complexes for bioactive compounds and copper in particular has attracted the researchers. Probably the most widely studied cation in this respect is Cu^{2+}, since a host of low-molecular-weight copper complexes have been proven beneficial against several diseases such as tuberculosis, rheumatoid, gastric ulcers and cancers (Sorenson, 1976; Brown *et al.*, 1980). Metal complexes of copper containing nitrogen and oxygen donor ligands are found to be effective catalysts for oxidation of olefins, etc. The coordination environment around copper plays the key role in stabilizing its different oxidation states and hence dictates the redox properties of the central atoms.

It is well known that various organic ligands possess strong antibacterial, herbicidal, insecticidal and fungicidal properties. It has also been reported that the activity of biometals is very often altered through the formation of chelates with different bioligands. It is suggested that the compounds having antimicrobial activity may act either by killing the microbe or by inhibiting multiplicity of the microbe or blocking their active site. In addition to this, the antimicrobial activity of the compounds also depends upon the nature of the microorganisms.

Schiff bases form stable complexes with metals that perform important role in biological systems. They find also wide applications in analytical chemistry since they allow simple and inexpensive determinations of several organic and inorganic substances. Furthermore, many Schiff bases exhibit antiviral, anticancer and antibacterial activity and can also be regarded as mimetic systems for enzyme models. Therefore, metal complexes of Schiff bases attained a prominent place in coordination chemistry, which was shown over many years by the large number of publications and by the comprehensive reviews.

This prompted us to investigate copper(II) complexes formed by using the series of macrocyclic ligands. Further insight into the bonding and possible geometrical structure was made by elemental, Mass, IR, Electronic and CV spectral studies, as well as conductivity data. The *in vitro* antifungal activities of the investigated compounds

*Corresponding author <E-mail: drn_raman@yahoo.co.in>

were tested against fungi like *Aspergillus niger*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Rhizoctonia bataicola* and *Candida albicans*.

Materials and Methods

Apparatus and Reagents. All reagents were of Merck products and used as supplied. For the voltammetric experiments, tetrabutylammoniumperchlorate (TBAP) used as supporting electrolyte, was purchased from Sigma. Anhydrous grade methanol and DMSO were obtained from Fisher Scientific Company. Micro analytical data and FAB Mass spectra of the compounds were recorded at the Regional Sophisticated Instrumentation Center, Central Drug Research Institute (RSIC, CDRI), Lucknow. The FAB mass spectrum of the complex was recorded on a JEOL SX 102/DA-6000 mass spectrometer/data system using argon/xenon (6 kV, 10 mA) as the FAB gas. The accelerating voltage was 10 kV and the spectra were recorded at room temperature using *m*-nitrobenzylalcohol (NBA) as the matrix. The IR spectra of the samples were recorded on a Shimadzu FTIR-8400S spectrophotometer in 4000~200 cm^{-1} range in a KBr pellet. The UV-Vis. spectra were recorded on a Shimadzu UV-1601 spectrophotometer. Magnetic susceptibility measurements of the complexes were carried out by Guoy balance using copper sulphate as the calibrant. The values were corrected for diamagnetism by applying Pascal's constants. Electrochemical studies were carried out using EG&G Princeton Applied Research Potentiostat/ Galvanostat Model 273A, controlled by M270 software. CV measurements were performed using a glassy carbon working electrode, platinum wire auxiliary electrode and an Ag/AgCl reference electrode. All solutions were purged with N_2 for 30 min prior to each set of experiments. The molar conductance of the complexes was measured using a Systronic conductivity bridge.

***In vitro* antifungal activity.** *In vitro* antifungal assay was performed by disc diffusion method. Both positive (nystatin for fungi) and negative (solvent, DMSO) controls were used in the technique. The complexes and ligands were tested against fungi such as *Aspergillus niger*, *Rhizopus stolonifer*, *Aspergillus flavus*, *Rhizoctonia bataicola* and *Candida albicans*, cultured on potato dextrose agar as medium. In a typical procedure, a well was made on the agar medium inoculated with the fungi. The well was filled with the test solution using a micropipette and the plate was incubated at 30°C for 72 h. During this period, the test solution diffused and the growth of the inoculated fungi was affected. The inhibition zone developed on the plate was measured. The MIC of the complexes was determined by serial dilution technique (Reiner, 1982).

Synthesis of macrocyclic ligands.

Synthesis of Knoevenagel condensate *β*-ketoanilide: Condensation of acetoacetanilide with *p*-methoxybenzaldehyde (L^1)/*o*-chlorobenzaldehyde (L^2)/benzaldehydes (L^3)/ *m*-nitrobenzaldehyde (L^4) was performed by heating equimolar amounts (10 mmol) under reflux in 50 *ml* ethanol, in the presence of 5 drops of piperidine as the catalyst for 5 h. The solution was then cooled and the condensed product was separated by adding 5 *ml* of toluene and 30 *ml* of petroleum ether (40~60°C). The yellow colour solid Knoevengel condensate b-ketoanilide was isolated by filtration, washed and recrystallised from ethanol.

Synthesis of Schiff bases: Knoevenagel condensate *β*-ketoanilide (10 mmol) was dissolved in ethanol (30 *ml*) and refluxed with *o*-phenylene diamine (20 mmol) in ethanol (20 *ml*) with the addition 1 g of anhydrous K_2CO_3 for about 6 h. The solvent was reduced to one-third and the pasty mass so obtained was treated with hot water and set aside in refrigerator for 10 h. The solid material formed was removed by filtration and recrystallised from ethanol.

Synthesis of macrocyclic ligands: An ethanolic solution of Schiff base (10 mmol) was added to the ethanolic solution of diethyl phthalate (10 mmol) and refluxed for 3 h. Then the solution was reduced to one-third on a water bath. The solid complex precipitated was filtered and washed thoroughly with ethanol and dried *in vacuo*.

Synthesis of metal complexes: A solution of macrocyclic ligand (5 mmol) in ethanol (20 *ml*) was added to a solution of MCl_2 (5 mmol) in ethanol (10 *ml*) and the mixture was refluxed for 6 h and concentrated to one-third volume and kept at 0°C for 2 h. The solid product formed was filtered, washed with ethanol and dried *in vacuo*.

Results and Discussion

All the complexes are stable at room temperature, insoluble in water but soluble in DMF, DMSO and chloroform. The physical properties and analytical data of the complexes are enlisted in Table 1. The elemental analysis data of the complexes are in good agreement with theoretical values. These complexes showed high conductance values (95~106 ohm^{-1} cm^2 mol^{-1}) indicating their electrolytic nature. The magnetic moments (Table 1) of all the Cu(II) complexes under the present study were found to be in the range of 1.79~1.85 B.M. at room temperature, suggesting a square-planar geometry around the copper ion (Harikumaran Nair *et al.*, 2005).

Mass spectra. A fast atom bombardment mass spectrum was obtained for the macrocyclic Schiff base (L^1). This spectrum showed a peak at m/z 606[M^+], as expected for a monomeric formulation of the respective ring. Also the fast atom bombardment mass spectrum of its

Table 1. Physical characterization, analytical, molar conductance and magnetic susceptibility data of the complexes

S.No	Compound	Found (calc)%				Λ_m mhocm^2 mol^{-1}	Magnetic moment μ_{eff} (BM)
		M	C	H	N		
1	L^1	–	77.4 (77.2)	5.5 (5.1)	12.3 (12.4)	–	–
2	[CuL^1]	12.7 (12.9)	69.7 (69.5)	4.9 (4.6)	11.4 (11.0)	115	1.81
3	L^2	–	72.9 (72.8)	4.9 (4.6)	11.8 (11.5)	–	–
4	[CuL^2]	11.3 (11.8)	65.8 (65.6)	4.5 (4.2)	10.5 (10.4)	136	1.85
5	L^3	–	71.9 (71.6)	4.6 (4.6)	13.8 (13.5)	–	–
6	[CuL^3]	11.8 (12.2)	64.7 (64.9)	4.4 (4.1)	12.5 (12.3)	106	1.79
7	L^4	–	75.8 (75.4)	5.3 (5.1)	11.8 (11.6)	–	–
8	[CuL^4]	9.7 (9.5)	68.5 (68.2)	5.0 (4.7)	10.6 (10.5)	95	1.83

[$Cu(L^1)$]Cl_2 complex exhibited a peak at m/z 740[M^+], which confirms the stoichiometric composition of the complex formation.

IR spectral. The infrared spectra gave some important information regarding to the skeleton of the complexes. The IR spectra of the macrocyclic ligands show characteristic bands for n(N-H) at 3320 cm^{-1}, n(C=O) at 1635 cm^{-1} and n(C-N) at 1590 cm^{-1}. In all the complexes, n(N-H) bands were shifted by 122~140 cm^{-1} to lower frequencies, due to coordination of the NH groups. The n(C=N) bands were also shifted by 19-48 cm^{-1} to lower frequencies, due to participation of the azomethine groups in coordination. On the other hand, the stretching vibration of n(C=O) was not affected in all the complexes, which indicates that the carbonyl groups are not involved in coordination to the copper cation. The coordination of nitrogen to the metal atom is supported by the appearance of a new band in the region 430~478 cm^{-1} assignable to n(M-N) vibration.

Electronic spectra. The electronic absorption spectra of $L^1/L^2/L^3/L^4$ and their copper complexes were recorded at 300 K using suitable solvent. The solvent, absorption region, assignment of the absorption bands and the proposed geometry of the complexes are given in Table 2. From the table, we concluded that all the complexes are having square-planar geometry around the copper atom.

Based on the above spectral data, the proposed structure of the macrocyclic ligands and its copper complexes are shown in Fig. 1 and 2.

Electrochemical behaviour. The expansion of bioinorganic chemistry in the last decades gave a strong impetus to the development of copper coordination chemistry, and an enormous number of new complexes, with very interesting structures and properties, have been prepared. As a rule, their redox properties have been investigated by electrochemical techniques, especially the cyclic voltammetry of solution in appropriate solvents.

The cyclic voltammogram of the [$Cu(L^1)$]Cl_2 complex in DMSO at 300 K in the potential range +0.8 to –0.4 V. It shows a well-defined redox process corresponding to the formation of the quasi-reversible couple copper(II)/copper(III). The anodic peak at Ep_a = 0.52 V versus Ag/AgCl and the associated cathodic peak at Ep_c = 0.35 V

Table 2. Electronic absorption spectral data of the complexes at 300 K

S.No	Compound	Solvent	Absorption (cm^{-1})	Band assignment	Geometry
1	L^1	$CHCl_3$	28560	INCT	--
2	L^2	$CHCl_3$	27536	INCT	--
3	L^3	$CHCl_3$	30150	INCT	--
4	L^4	$CHCl_3$	29750	INCT	---
			29875	INCT	
5	[$Cu(L^1)$]Cl_2	DMSO	22345	$^2B_{1g} \rightarrow {}^2E_g$	Square-planar
			18691	$^2B_{1g} \rightarrow {}^2A_{1g}$	
			30250	INCT	
6	[$Cu(L^2)$]Cl_2	DMSO	21248	$^2B_{1g} \rightarrow {}^2E_g$	Square-planar
			18126	$^2B_{1g} \rightarrow {}^2A_{1g}$	
			32258	INCT	
7	[$Cu(L^3)$]Cl_2	DMSO	21670	$^2B_{1g} \rightarrow {}^2E_g$	Square-planar
			17426	$^2B_{1g} \rightarrow {}^2A_{1g}$	
			30145	INCT	
8	[$Cu(L^4)$]Cl_2	DMSO	21735	$^2B_{1g} \rightarrow {}^2E_g$	Square-planar
			17650	$^2B_{1g} \rightarrow {}^2A_{1g}$	

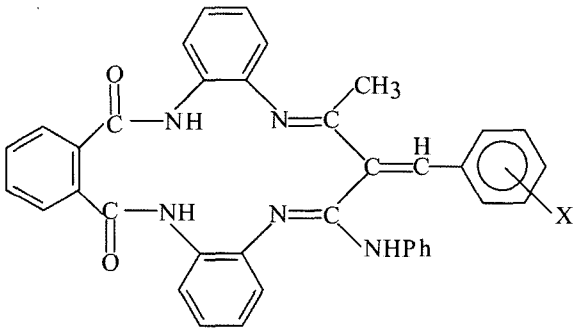

Fig. 1. Structure of macrocyclic ligands.
where X = -Cl, -H, -OCH_3, -NO_2, L^1 = -OCH_3; L^2 = Cl; L^3 = -H; L^4 = -NO_2,

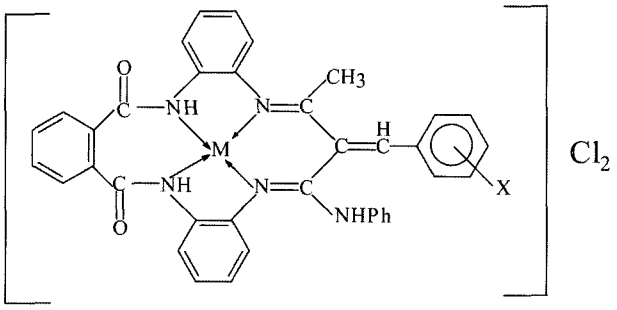

Fig. 2. Structure of macrocyclic complexes.

correspond to the copper(II)/copper(III). The $[Cu(L^1)]Cl_2$ complex exhibits a quasi-reversible behaviour as indicated by the non-equivalent current intensity of cathodic and anodic peaks and also shows large peak separation indicates quasireversible behaviour.

Antifungal activity. The *in vitro* antifungal activities of the compounds were tested against *Aspergillus niger*, *Rhizopus stolinifer*, *Aspergillus flavus*, *Rhizoctonia bataicola* and *Candida albicans* by the serial dilution method. The minimum inhibitory concentration (MIC) values of the compounds are summarized in Table 3. A comparative study of the ligand and its complexes (MIC values) indicates that complexes exhibit higher antifungal activity than the free ligand. From the MIC values (Table 3), it was found that the compound 8, $[Cu(L^4)]Cl_2$ was more potent among the other investigated complexes and standard. Further studies are required to explore these complexes as drugs.

Such increased activity of the complexes can be explained on the basis of Overtone's concept (Anjaneyula *et al.*, 1986) and Tweedy's Chelation theory (Dharamaraj *et al.*, 2001). According to Overtone's concept of cell permeability, the lipid membrane that surrounds the cell favours the passage of only the lipid-soluble materials due to which liposolubility is an important factor, which controls the antifungal activity. On chelation, the polarity of the metal ion will be reduced to a greater extent due to the overlap of the ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Further, it increases the delocalization π-electrons over the whole chelate ring and enhances the lipophilicity of the complexes. This increased lipophilicity enhances the penetration of the complexes into lipid membranes and blocking of the metal binding sites in the enzymes of microorganisms. These complexes also disturb the respiration process of the cell and thus block the synthesis of the proteins that restricts further growth of the organism. Furthermore, the mode of action of the compound may involve formation of a hydrogen bond through the azomethine group with the active centre of cell constituents, resulting in interference with the normal cell process.

Ackowledgements

The authors express their sincere thanks to the Managing Board and Principal of VHNSN College, Virudhunagar for providing research facilities. NR thanks to the UGC for financial assistance.

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Table 3. Minimum inhibition of concentration of the synthesized compounds against growth of five fungi (mg/*ml*)

S.No	Compound	*A. niger*	*R. stolinifer*	*A.flavus*	*R. bataicola*	*C.albicans*
1	L^1	65	50	85	42	36
2	L^2	28	30	68	56	38
3	L^3	45	74	27	22	42
4	L^4	24	14	22	18	15
5	$[Cu(L^1)]Cl_2$	14	17	20	18	16
6	$[Cu(L^2)]Cl_2$	18	28	12	16	14
7	$[Cu(L^3)]Cl_2$	15	18	14	20	19
8	$[Cu(L^4)]Cl_2$	19	8	18	15	10
9	Nystatin	10	16	8	14	12

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