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Susceptibility Determination of Candida Species against Binuclear complexes derived from Copper Surfactants with Azole ring compounds

Neha Mathur [1], V.K. Mathur [2],
[1]Department of Chemistry, Govt.P.G.College, Dausa (Raj.) INDIA.
[2]Rajasthan Rajya Vidhyut Prasaran Nigam Ltd. Jaipur, India.

Abstract. Nitrogen containing aromatic ligands are ubiquitous components both for physiologically active products and important pharmaceuticals. The study of binuclear complexes of these ligands with transition metal Copper (II) is highly interesting due to their significance in physical and bioinorganic chemistry, material science and multi-electron redox chemistry[1]. Some of these complexes are highly effective anticancer agents, blocking carcinogenic before they reach their cellular targets and eliminating DNA damage in cell nuclei. Their applications in medicinal and industrial field have stimulated our interest to construct the new libraries of hybrid binuclear complexes of nitrogen donor aromatic ligands.
Owing to the above biological importance of these binuclear macrocycles of benzothiazole moiety we have synthesised a novel series of these antifungal agents, their molecular modelling and geometry were characterized by spectroscopic data as IR, NMR, MASS and elemental analysis. Their Biological activities against Candida species have been done to continue the new research era.

Keywords: Binuclear complexes, Benzothiazole moiety, anticancer agent, blocking carcinogenic, Spectroscopic data.

1. Introduction

Literature survey reveals that azoles are well known for their various pharmacological and antimicrobial activities. They have been used as tranquilizers, antiinflammotry, diuretics, and antimalarials. Presence of, nitrogen and sulphur with their electron donor property makes them useful as intermediates for much organic synthesis such as agrochemicals and pharmaceuticals. Apart from these ligands copper is one of the heavy transition metal ions which are capable of being toxic when present beyond the tolerance level and thus copper complexes act as insecticides and fungicides. The toxicity and biological activities often enhanced by complexation of metal with nitrogen ligands, so the study of binuclear complexes of copper (II) has much significance in bioinorganic chemistry, Metal complexes of the ligands containing nitrogen and sulphur are known to possess antimicrobial[4], antiviral[5], antifungal[6], and anticonvulsant[7] activities. . Among the nitrogen donor ligands, benzothiazoles usually react with metallic cation giving complexes in which the ligand behaves as chelating agent bonding through the primary nitrogen atom. Thus it is worthwhile to carry out the spectral and biocide study of transition metal complexes with such ligands[2-4].

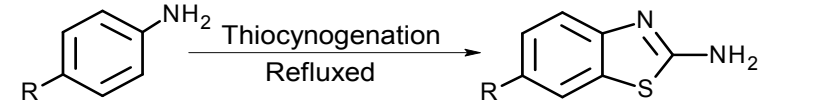
2. Experimental

All chemicals used were of A.R. Grade, solvents were purified by standard procedures[5-6]. Micro analytical data of the compounds were recorded at RSIC, CDRI. Luck now. T.L.C. was used to access the purity of the compounds. The IR spectra of the complexes were obtained on Perkin Elmer spectrophotometer at CDRI, Luck now. ^{1}H NMR spectra were recorded at CDRI, Luck now using $CDCl_3$ as reference.

2.1 Ligand Preparation

In the Thiocynogenation method[6-7] substituted aniline (0.1 moles) was treated with a mixture of 7.6 gm NH4CNS and 80 ml glacial CH3COOH and it refluxes at room temperature for one and half hour. The thiocynogenation takes place in the presence of thiocynogen gas, generated insitu by the reaction of Cu2Cl2and NH4CNS. After cooling, 100 ml of concentrated HCl (6N) is added to the mixture and heat again for half an hour, cool and saturated solution of (Na_2CO_3) is added to neutralize it, till the solid was formed. Filter the solid separated out,wash with cold water, dried and recrystallised with ethanol. (Scheme-1).

Benzothiozole of sustituted aniline

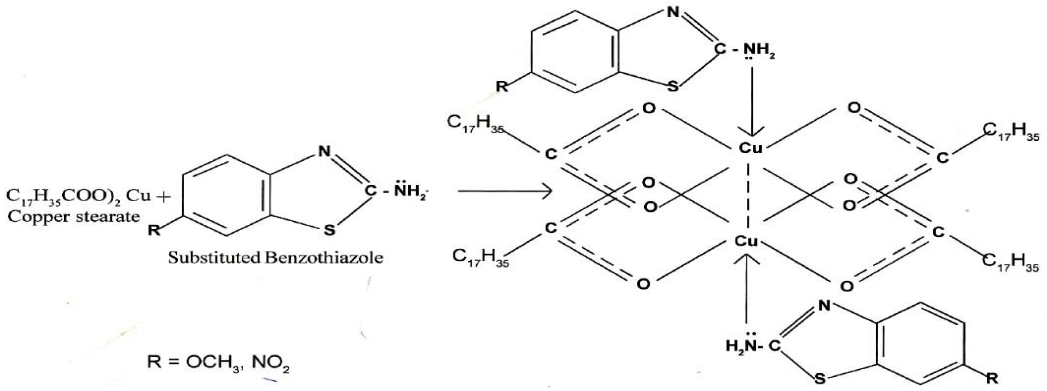


p-subsituted Aniline Substituted 2-amino benzothiazole

Scheme -1

2.2 Complex Preparation[8]

Prepare the complexes of copper palmitate and benzothiazoles by adding 0.001 mole copper palmitate with 0.002 mole benzothiazoles in 25–30 ml C2H5OH and reflux the mixtures for about two hours with constant stirring. Cool and filter the solid separated out, dried and recrystallised with hot benzene. The formation of complexes was confirmed by using IR, NMR techniques and elemental analysis. (Scheme-2)



Scheme-2 Complex formation betweem copper stearate and substituted benzothiozole

3. Results and Discussion

In the present study the synthesised complexes are abbreviated as follows.

- Complex of Copper Palmitate with 2-amino-6-methylbenzothiazole CP[BTA]CH_3
- Complex of Copper Palmitate with 2-amino-6-chlorobenzothiazole CP[BTA]$_{Cl}$
- Complex of Copper Palmitate with 2-amino-6-methoxybenzothiazole. CS[BTA]OCH_3
- Complex of Copper Palmitate with 2-amino-6-nitrobenzothiazole. CS[BTA]NO_2

3.1 IR Spectral Studies[9]

The IR spectra provide valuable information regarding coordination site of the ligand attached to the metal ion. From IR spectral data, it is evident that ligand acts as a monodentate, bonded to copper ion through primary nitrogen atom of NH_2.The strong band at 1602 cm^{-1} is due to the N-H bending vibration of NH_2 group in all four free ligands but in the complexes it is shifted to lower frequency at 1592 and 1589 cm^{-1} indicating that the primary nitrogen is the coordinating site in the complexes. This is further supported by the formation of new band at 548 cm^{-1} and 487 cm^{-1}, which are due to υ_{M-N} band in all the complexes

Table – 1 IR Spectral Data for Copper (II) Complexes

Absorption Bands	CP[BTA]CH_3	CP[BTA]OCH_3	CP[BTA]Cl	CP[BTA]NO_2
CH_3 & CH_2, C-H antisym. Stretching	2920	2919.5	2920	2919.0
CH_3 and CH_2 C-H sym. Stretching	2852	2850.6	2852	2851.1
N-H bending	1575	1592.7	1560	1589.1
COO^-, C-O antisym. Stretching	1511	1509.4	1550.2	1558.4
COO^-, C-O sym. Stretching	1462	1466.2	1470	1468.0
CH_2, C-H Bending (δ)	1365.0	1360.9	1390.2	1395.2
CH_3, C-H rocking	1116.8	1114.8	1110.2	1111.6
CH_2, C-H rocking	720.4	722.4	745.0	721.1
Cu-N stretching	540.2	548.1	558.3	513.9
Cu-O stretching	495.0	495.2	490.3	487.5
NH_2, N-H stretching	3420.4	3422.3	3456.8	3448.2
Ar-C-NO_2 /Cl	-	-	760	1442.4
N-C=S stretching	1304.2	1301.7	1310.0	1306.0
C=S stretching	1182.6	1180.1	1190.2	1182.5
Ar-C-OCH_3 asym. Stretching	-	1251.1	-	-
Ar-C-OCH_3 sym. Stretching	-	1026.9	-	-
C-H, Deformation ("oop")	830.2	832.2	855.2	843.7

The following bands were also corresponds to ligand moiety. Asymmetrical and symmetrical stretching vibrations at 1210cm^{-1} -1010cm^{-1},1251 cm^{-1}-1026cm^{-1},due to –$CH_3$and -OCH_3groups, 1442 cm^{-1} for –NO_2 and1036$cm^{-1}$for –Cl group. C-O stretching bond at 495-503 cm^{-1} The IR data suggest that the copper is bound to its ligand through the nitrogen of NH_2 group.

3.2 NMR Spectral Studies

The ^1H NMR spectra of ligands and complexes have been compared and. The signals were assigned on the basis of chemical shifts, spin-spin interaction and their effect on substitution.

Table – 2 NMR Spectral Data for Copper (II) Complexes

Peak/Signal	CP[BTA]CH_3	CP[BTA]OCH_3	CP[BTA]Cl	CP[BTA]NO_2
-CH_3-CH_2-R	0.84	0.89	0.96	0.90
-CH_2-CH_2-R	1.256	1.254	1.260	1.250
-NH_2	3.80-3.88	3.80-3.88	3.85-3.88	3.81-3.88
Tautomeric -NH_2	7.12	7.16	7.38	7.26

Aliphatic -CH_3 and -CH_2 proton attached to -CH_2-R group show signal at -0.89 and δ-1.25 respectively. A broadened peak is observed at δ-3.80-3.88 corresponding to -NH_2 proton. This peak indicates the co-ordination through -NH_2 group of benzothiazole segment to the metal. Broadening of the observed peak is suggestive to be a slow exchange because the electrical quadripole moment of nitrogen nucleus induces a moderately efficient spin relaxation.

4. Biocidal Study

In the present study, we investigated the applicability of the Clinical Laboratory Standards Institute CLSI M44-A disc diffusion method for determining the susceptibility of Candida species against synthesized complexes, for this purpose some standard antifungal compounds were used as references.

These antifungal research powders were stored at -20°C until they were used. They are

(1) Amphotericine (2) Ketoconozole

4.1 Materials and Methods

DD method was performed on Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg of methylene blue per ml due to the ability of that medium to produce enhanced definition of growth margins.

To prepare the medium, stock solutions of MB (0.1gm/20 ml) were made 100 µl of this solution per litre of agar suspension was added then 20 Gms of glucose per litre of agar suspension were added.

The GMB stock solution was filter, sterilized and cooled in a 45 to 50 ^{0}C water bath. Mueller-Hinton agar was prepared by pouring stock solution into petridishes on a level horizontal surface to give a uniform depth of 4 mm and absorb it for 4 to 6 hour. If excess surface moisture was present in the petridishs after gel setting they were dried in an incubator (10-30 minutes) with the lids ajar until the excess moisture has evaporated. The surface should be moist, but with no droplet on the agar surface or the petridish cover. Petridishs were stored in refrigerator (4 ^{0}C).The agar medium had pH between 7.2 and 7.4 at room temperature. These petridishes were kept for 24 hours in other incubator at 37^{0}C for sterility test.

4.2 Reference Strains

To Control the precision and accuracy of the results obtained by this method several quality control stains were obtained from reliable source.

(1) Candida albicans ATCC 90028 (2) Candida Krusei ATCC6258

The quality control strains were tested by the standard D. D.test procedure using the same materials and methods that are used to test clinical isolates. Quality control strains were stored in a way that minimizes the possibility of mutation in the organism.

4.3 Inoculum preparation

Inoculum was prepared by picking five distinct colonies of approximately 1 mm in diameter from a 24-hour-old culture of Candida species. Colonies were suspended in 5 ml of sterile 0.145 mol/litre saline. (8.5 gm/litre NaCl; 0.85 % saline).

4.4 Inoculum suspensions

The inoculum suspensions were prepared as described for the CLSI M44-A, USA method. The turbidity was measured with a spectrophotometer at 625 nm and was adjusted to match a 0.5 McFarland density standard, resulting in a concentration of 1 x 10^6 to 5 x 10^6 cells/ml. This inoculum was used directly for inoculation of agar plate's growth. To standardize the inoculum density for a susceptibility test, a $BaSO_4$ suspension with turbidity equivalent to a 0.5 McFarland standard or its optical equivalent was used as turbidity standard for inoculum.

4.5 Preparation of McFarland nephlometry Standard

(a) Required Reagents

I. $BaCl_2.2H_2O$ 0.048M/Lit or 1.175% [W/v] II. H_2SO_4 0.18M/Lit or 1%

III. Distilled Water 200ml

(b) Procedure

100 ml distil water is taken in each two 250ml capacity sterile flask, from the other flask 1ml of DW is discarded and 1ml pure H_2SO_4 is added to make a 1% [v/V] solution. From the H_2SO_4 solution, 0.5 ml is discarded and to the remaining 99.5 ml, 0.5 ml $BaCl_2.2H_2O$ solution is added drop by drop with a constant stirring. The solution thus prepared is now consisting of 0.5 standards of McFarland standards which is equivalent to 1.5 X 10 ^{8} cell/ml. and distributed into 2-3 large [18 X 150 mm] test tubes in approximately 4-6 ml amount. The transmittance of the solution at 625 nm was between 0.08-0.1 and is standard.

4.6 Inoculation of Test Plates

A sterile cotton swab was dipped into the suspension. Dipping a sterile cotton swab into the dried surface of inoculum and evenly streaking the swab in three directions (60^0 each) over the entire surface of the plate inoculated the agar plates. The plates were allowed to dry for at least 15 minutes before the disks were applied to the surface.

4.7 Preparation of Dispensing Disks

For disk diffusion test we require two types of antimicrobial disks.

1. Antimicrobial disk :-

They were used as standard for quality control. Here we use Amphotericine and ketoconozole antifungal agents for this purpose.

2. Testing {for complexes and ligands} disks:-

To prepare the disk of testing samples we required Watmann filter paper No.2 and vials. First of all 6 mm diameter disks of Watmann filter paper were punched.100 disks were kept in each vial. These vials were sealed with cotton lug and sterilized by heating in oven. The different concentration testing sample solutions were made by adding the sample compound and required amount of benzene methanol mixture as solvents.

All the requirements used in dispensing were sterile. Sterile vials containing 100 disks, which absorbed all the solution. This dispensing was done in sterile hood / chamber that was already cleaned with methanol and exposed to U, V. light and blower; solutions were dispensed in the vials near the lighted sprit lamp kept in the hood. The testing compounds of concentration 0.5 mg/disk are listed as follows:-

(1) CP [BTA]CH_3—A-1 (2) CP{ BTA}OCH_3---A-2
(3) CP[BTA]Cl----A—3 (4)CP[BTA]NO_2---A--4
(5) [BTA]CH_3----L-5 (6) [BTA]OCH_3----L-6
(7) [BTA]Cl----L-7 (8) [BTA]---L-8
(9) Standard-Amphotericine---S-1 (10) Standard-Ketoconozole-S-2

5. Conclusion

The antifungal activities of the ligands and complexes have been evaluated by the D.D. test .The results are expressed in millimetre. The two antimicrobial disks Amphotericine and ketoconozole were taken as standards and the sample disks are compared with it.

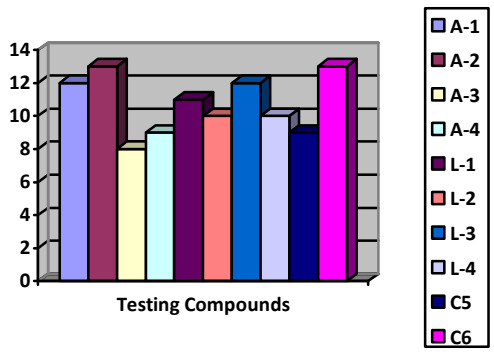
A scrutiny of Table 4 revels that all complexes show higher activities than pure ligands suggesting that complexes are more powerful agents and presence of Nitrogen and Sulphur are able to enhance the performance of copper surfactants. The organic compounds containing amino group play a significant role in biology, as it constitute the repeating unit of polypeptide macromolecules. It was observed that enhanced activity of complexes was due to synergistic mechanism, i.e. free ligand and pure soap show less activity but on complexation they show enhanced activity. These results support our studies where pure soaps and ligands show less inhibition but on complexation the inhibition is enhanced.

The studies also suggest that against Candida albicans the methoxy and methyl groups show higher activity than nitro and chloro group, but in case of Candida Krusei the results were just opposite. This may be attributed to the fact that the atom introduced into the complex through the ligand also plays an important role in enhancing the effectiveness of fungicidal molecule. On the basis of our results, it is suggested that the DD test is a useful method for testing the activity of synthesized compounds against Candida species.

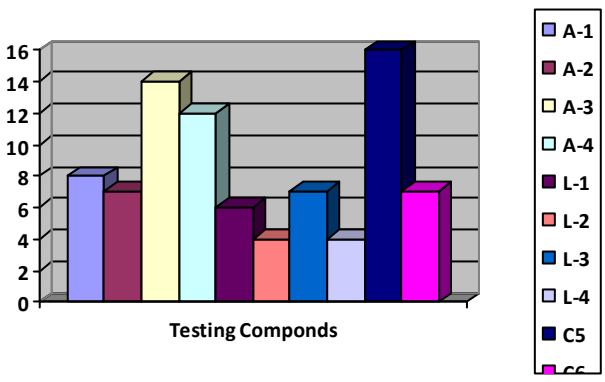
TABLE-4 Antimicrobial activity of Compounds (Zone inhibition size in mm)

Fungi	CP[BTA]CH_3	CP[BTA]OCH_3	CP[BTA]Cl	CP[BTA]NO_2	Amphotericine	Ketoconozole
C.albicans	12	13	8	9	9	13
C.krusei	8	7	14	12	16	7
	[BTA]CH_3	[BTA]OCH_3	[BTA]Cl	[BTA]NO_2	Amphotericine	Ketoconozole
C.albicans	11	10	12	10	9	13
C.krusei	6	4	7	4	16	7

Data for Candida Krusei



Data for Candida Albicans



Which conclude that. Inhibition zone size value for free ligand (substituted benzothiazole) show difference but the trend remains same as their corresponding complexes.

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