Research Article

In-Vitro Antioxidant And Antimicrobial Studies Of Mixed Ligand Copper(II) Complexes Of Benzimidazolyl Terpyridine

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Abstract

Mixed ligand copper(II) complexes having tridentate NNN-donor benzimidazolyl terpyridine base and planar NN-donor heterocyclic bases are prepared and characterized by various physico-chemical techniques. The biocidal efficiency of the complexes has also been evaluated and found that the Cu(II) complexes show better biological activity when compared with the ligand. The synthesized ligand and its metal complexes were screened for reduction of DPPH. Based on the results obtained, the free ligand was found to be good antioxidant. However, its antioxidant activity can be further confirmed by *in vivo* methods.

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Keywords: Copper Complex, Benzimidazolyl terpyridine base, Biological Activity

Introduction

Transition-metal chemistry, both in the cell and in the chemist's test tube, provides a valuable tool both to accomplish and to explore many biological processes involving nucleic acids structurally and functionally.[1-4] The reactions of transition-metal complexes with polynucleotides generally fall into two categories: (i) those involving a redox reaction of the metal complex that mediates oxidation of the nucleic acid; and (ii) those involving coordination of the metal center to the sugar-phosphate backbone so as to mediate hydrolysis of the polymer. Both redox and hydrolytic reactions of metal complexes with nucleic acids have been exploited with much success in the development of tools for molecular biology. Over several decades the study of artificial nucleases has received attention for their diverse applications not only as chemical therapeutic agents but also in genomic research. [5-9] On comparing the conventional enzymatic nucleases with chemical nucleases, chemical nucleases have some advantages. This is because chemical nucleases being smaller in size can reach more sterically hindered regions of a macromolecule. By utilizing the redox properties of the metal and dioxygen, chemical nucleases produce reactive oxygen species that oxidize DNA, yielding direct strand scission or base modification. [10] In this regard, due to their structural diversity, copper complexes are capable of cleaving DNA by both the hydrolytic and oxidative cleavage modes. The best studied copper complex which induces direct strand damage in the presence of H_2O_2 is $[Cu(phen)_2]^{2+}$.[11] In some of these complexes, the copper-bound hydroxyl is the active species that hydrolyzes the phosphate backbone of the nucleic acid.[12-15] Mixed ligand imidazolyl terpyridine copper(II) complexes with diimines as coligands were shown to exhibit cytotoxic and nuclease activities.[16] Though there are many copper complexes showing DNA cleavage and cytotoxic activities, a number of parameters such as mode of action, distribution inside the cells and possible cause effect need optimization. In addition, some copper metal complexes have been shown interesting biological activities as model systems that artificial metallopeptidases, enzyme inhibitors, and free radical scavengers. In continuation of our efforts to develop Cu(II)-based chemical nuclease, we have chosen the tridentate nitrogen donor ligand benzimidazolyl terpyridine aiming to understand the importance of benzimidazole moiety. Here we report the synthesis and characterization of two mixed ligand copper(II) complexes **1** and **2** and their interaction with microbes to explore their potential biological activity.

Materials and Methods

2-acetyl pyridine, copper(II) perchlorate hexahydrate, 2,2'-bipyridine, 1,10-phenanthroline, agarose (molecular biology grade) and ethidium bromide were procured from Sigma Aldrich, USA and used as received. Other materials like sodium hydroxide, ammonium acetate and solvents like methanol, acetonitrile were of reagent grade. The ligand, bitpy (benzimidazolyl terpyridine) was prepared using published procedure.[17] UV–visible

spectra of the complexes were recorded on a Shimadzu UV 3101PC spectrophotometer at 25°C. Electron paramagnetic resonance spectra of the copper(II) complexes were obtained on a Varian E 112 EPR spectrometer. IR spectra were recorded as KBr pellets (1 % w/w) in the region of 400 - 4000 cm^{-1} using a Perkin-Elmer Spectrum GX-FT-IR spectrophotometer. Positive ion electron spray ionization mass spectra of the complexes were obtained by using Thermo Finnigan LCQ 6000 advantage max ion trap mass spectrometer.

Synthesis of [Cu(bitpy)(bpy)]$(ClO_4)_2$ (1)

The ligand benzimidazolyl terpyridine (bitpy) was prepared by slight modification of the reported procedure and the authenticity was confirmed from ESI-MS, m/z = 350 [bitpy+H^+]. The compound was prepared in high yield. To a solution of $Cu(ClO_4)_2$. $6H_2O$ (0.53 g, 1.4 mmol) in methanol, a hot solution of bitpy (0.5 g, 1.4 mmol) and 2,2'-bipyridine (0.22 g, 1.4 mmol) was added slowly and the reaction mixture was stirred for about 3 hours. The resulting solution was filtered and kept aside. Green solid that separated out upon slow evaporation of the solvent was filtered and washed with diethyl ether. Yield: 0.89 g (81 %). Anal. Calc. for $C_{32}H_{23}CuN_7O_8Cl_2$: C, 50.04; H, 3.02; N, 12.77; Cu, 8.27. Found: C, 50.01; H, 2.98; N, 12.73; Cu, 8.24. ESI-MS: m/z = 567.1, $[M-2ClO_4]^+$.

Synthesis of [Cu(bitpy)(phen)]$(ClO_4)_2$ (2)

This complex was synthesized by adding a hot methanol (5 mL) solution of 1,10-phenanthroline (0.27 g, 1.4 mmol) and bitpy (0.47 g, 1.4 mmol) to a methanol solution of copper(II) perchlorate (0.5 g, 1.4 mmol) and then stirring the solution at room temperature for 3 hours. The resulting solution was filtered and kept aside. Green crystalline product that separated out upon slow evaporation of the solvent was filtered and dried. Yield: 0.86 g (78 %). Anal. Calc. for $C_{34}H_{23}CuN_7O_8Cl_2$: C, 51.56; H, 2.93; N, 12.38; Cu, 8.02; Found: C, 51.53; H, 2.91; N, 12.34; Cu, 7.98. ESI-MS: *m/z* = 591.2 $[M-2ClO_4]^+$.

DPPH free radical scavenging activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical having maximum optical absorbance at 517 nm. A stock solution (1 mg/1 mL) was diluted to final concentrations of 20-100 µg/mL. An ethanolic DPPH solution was added to sample solutions in DMSO at a concentration of 0.1 mM. Test tubes were kept at an ambient temperature for 30 minutes. Using UV-Visible spectrophotometer, the absorbance of the ligands and their copper complexes were measured at 517 nm. These measurements were run in triplicate. The percentage of scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [(A_{DPPH} - A_{TEST})/A_{DPPH}] \text{ X100}$$

where A_{DPPH} is the absorbance of DPPH without test sample (control) and A_{TEST} is the absorbance of DPPH in the presence of test sample.

Antimicrobial Assay
Micro-organisms used

Five species of bacteria, two gram - positive (Streptococcus *faecalis* & Bacillus *subtilis*) and three gram negative (Escherichia coli, Klebsiella *pneumonia* & Salmonella *paratyphi*) were obtained from KMCH, Coimbatore.

Preparation of Inoculum

A loopful of strain was inoculated in 30 mL of nutrient broth in a conical flask and incubated on a rotary shaker at 37°C for 24 hours to activate the strain.

Bioassay

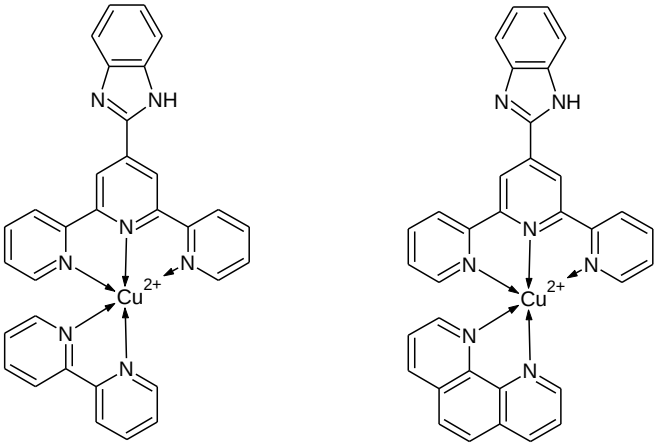
The bioassay used was the standard Agar Disc Diffusion assay. Mueller Hinton Agar was prepared for the study. Mueller Hinton agar plates were swabbed with a suspension of each bacterial species, using a sterile cotton swab. Subsequently, the sterilized filter paper discs were completely saturated with the test compound. The impregnated dried discs were placed on the surface of each inoculated plate. The plates were incubated overnight at 37°C. Each

organism was tested against each organism in triplicate. Methanol was used as negative control. Standard discs of Ampicillin served as positive antibacterial control. The test materials having antimicrobial activity inhibited the growth of the micro organisms and a clear, distinct zone of inhibition was visualized surrounding the disc. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition in mm.

Results and Discussion

Synthesis and Characterization

The complexes with general formula $[Cu(bitpy)(L)](ClO_4)_2$, where bitpy is the tridentate ligand 4'-(benzimidazolyl)-2,2':6',2"-terpyridine and L is 2,2'-bipyridine (**1**) and 1,10-phenanthroline (**2**) have been isolated from methanolic solution containing hexahydrated copper(II) perchlorate as the starting material.(Figure 1) Both the complexes were obtained in good yield and characterized by using elemental analysis, UV-Vis, ESI-MS and EPR spectral techniques. The analytical data obtained for the new complexes agree well with the proposed molecular formula.



1 **2**

Figure 1 Copper(II) complexes

The ESI mass spectra of $[Cu(bitpy)(bpy)](ClO_4)_2$ and $[Cu(bitpy)(phen)](ClO_4)_2$ displayed the molecular ion peak at m/z 567.1 and 591.2 respectively. These peaks are reliable with the proposed molecular formula of the corresponding copper(II) complexes.

Electronic Spectral Analysis

The electronic spectra of both the complexes in acetonitrile-dimethyl sulphoxide mixture showed two bands in the region of 270-343 nm and a broad band in the 684-686 nm region.(Figure 2) For an unsubstituted terpyridine complex, these bands are in the region of 228-324 nm. These bands have also been shifted in the spectra of the new complexes indicating the involvement of the lowering of the LUMO (Lowest Unoccupied Molecular Orbital) (π^*) energy in the bitpy ligand owing to a more extended conjugation relative to the terpy ligand. Both the complexes are paramagnetic, indicating the presence of copper in the +2 oxidation state. Three d–d transitions are possible for copper(II) complexes. They are d_{xz},d_{yz}- $d_{x^2-y^2}$, d_{z^2}-$d_{x^2-y^2}$ and d_{xy}-$d_{x^2-y^2}$. However, only a single broad band is observed for both the copper(II) complexes. This indicates the total sum of all the above transitions. The broadness associated with the d–d bands is generally taken as an indication of the geometrical distortion of the complex from perfect planar symmetry. Spectrum of **1** show a band at 297.6 nm which can be attributed to intra ligand transitions of the bitpy ligand.

Broad metal to ligand charge transfer (MLCT) transition has been observed at 343.4 nm for complex **1**. Complex **2** shows the π-π^* transition of phenanthroline at 270 nm and a sharp band at 294.2 nm due to intra ligand transition of bitpy. Broad MLCT band has been observed at 342.4 nm for complex **2**. Complexes **1** and **2** show their ligand field transitions as broad bands at 684 nm and 686 nm respectively.

IR and EPR Spectral Analysis

IR spectra provide the valuable information about the nature of the binding mode and functional group attached to the metal ion. Presence of perchlorate ion in the IR spectra of complex **1** and **2** were confirmed by the appearance

of a band at 1089 and 1083 cm^{-1} respectively. In complex **2**, the IR peaks observed at 1520 and 1429 cm^{-1} have been attributed to the C=C and C=N ring stretching frequencies of 1, 10-phenanthroline. For an uncoordinated phenanthroline, these bands have been observed at 1519 and 1427 cm^{-1} respectively. This indicates the coordination of heterocylic N-atoms of phenanthroline to metal ion.[18] Upon complexation of metal ion, the characteristic out-of-plane H-bonding modes of uncoordinated phenanthroline observed at 852 and 730 cm^{-1} have been shifted to 846 and 725 cm^{-1} respectively.[19] Medium intensity bands appeared at 3074 and 3076 cm^{-1} for complexes **1** and **2** respectively were attributed to C-H stretching vibration.

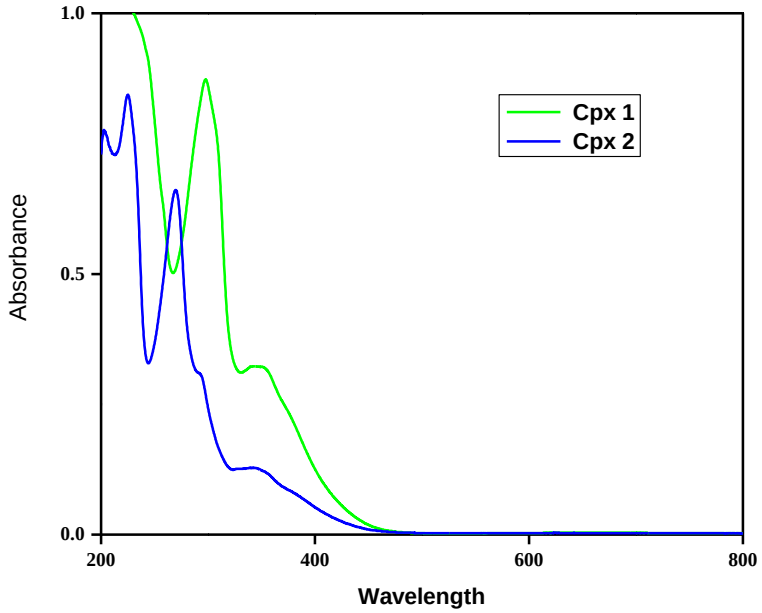


Figure 2 UV-Visible spectra of copper complexes

The EPR spectrum of **1** and **2** shows axial signals at 300 K from a static copper (II) centre with dx^2-y^2 as the ground state. And also the spectra of both the copper complexes at 300 K show one intense band in the high field region, which are isotropic due to tumbling motion of the molecules. The g value for complex **1** and **2** are 2.07 and 2.1 respectively. The broad EPR spectra and their g values confirm the formation of the copper(II) complexes.

Investigation of Antioxidant Activity

For antioxidant activity test we have used DPPH free radical scavenging assay which is one of the short methods for investigation of the hydrogen donating potency.[20] DPPH is a purple-colored stable free radical that becomes reduced to the yellow-colored diphenyl picryl hydrazine. Ascorbic acid (AA) has been used as control standard. AA is a common reference of antioxidant activity test due to its high antioxidant activity.[21] It was expected that AA showed excellent antioxidant activity in DPPH radical scavenging assay.

The DPPH results are shown at Figure 3 and compared with the control standard AA. Lower absorbance values of the reaction mixture indicated higher free radical scavenging activity. It was observed that the test compounds scavenged free radicals in a concentration dependent manner. Both the copper complexes **1** and **2** (58.28-99.19 %) showed significant DPPH scavenging activity compared to that of standard ascorbic acid. On the other hand radical scavenging activity of the ligands was found to be greater than that of the corresponding metal complexes. Of the two complexes, **2** showed a better antioxidant activity than **1**. This might be due to the presence of highly conjugated phenanthroline moiety in the complex. Copper(II) complex has many interest and its antioxidant activity contains experimental and theoretical results, in which the antioxidant activity is related to stable coordination ability of copper(II). A graph may be plotted with % scavenging effects on the y- axis and concentration (µg/mL) on the x- axis. The radical scavenging activity of the present complexes follows the order **2** > **1**.

Antimicrobial Activity

Many coordination compounds have been studied for their antitumor, [22] antiviral [23] and antimalarial activity, [24] which has been related to the ability of metal ions to form stable complexes.[25] The results have led to an

understanding of coordination sphere and electronic properties of the metal ions several workers have reported that heterocyclic rings containing sulfur, nitrogen, and/or oxygen are responsible for the biological activity of ligands and their metal complexes.[26]

Table 1 Antimicrobial activity of copper complexes

S. No	Bacteria	Control Ampicillin (mm)	Zone of inhibition (mm)		
			L1	3	4
1	Streptococcus *faecalis*	12.14±0.31	10±0.21	14.33±1.21	14.33±1.21
2	Bacillus *subtilis*	14.17±1.89	8.3±0.14	13.6±0.29	13.6±0.29
3	Klebsiella *pneumonia*	11.01±0.41	4.2±0.31	07±0.32	9±0.38
4	Salmonella *paratyphi*	14.11± 0.18	5.3±0.49	08±0.13	8±0.13
5	Escherichia *coli*	15.36±1.37	4.4±0.51	05±0.18	6±0.26

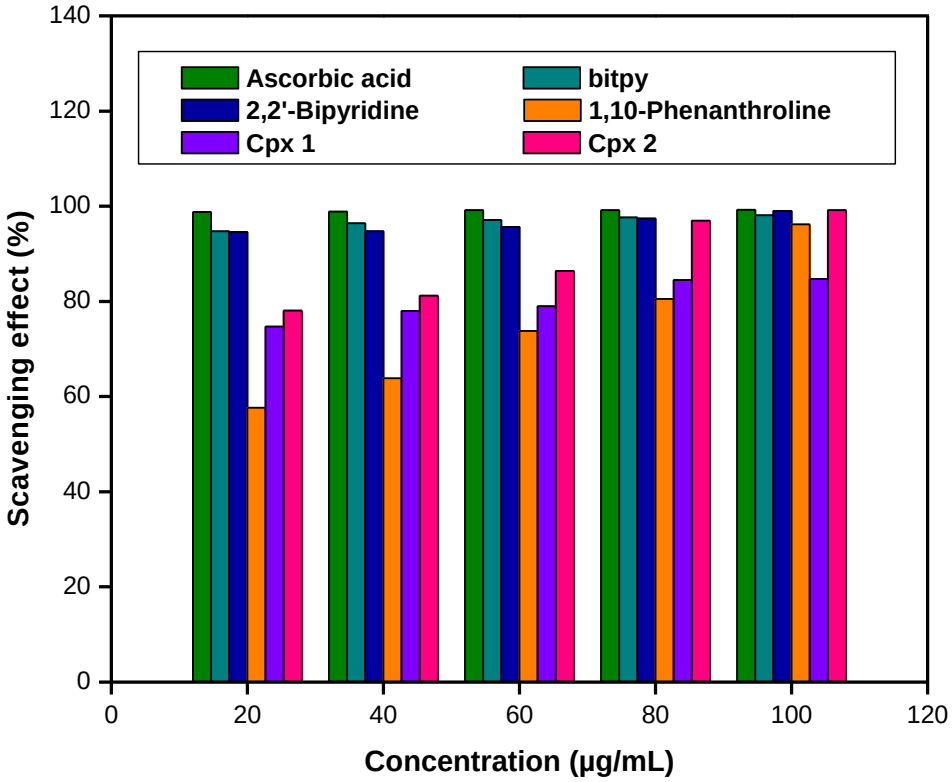


Figure 3 Antioxidant activity of copper complexes

The series of complexes bearing benzimidazolyl terpyridine ligands **1** and **2** were tested for their *in vitro* antimicrobial activity against a number of standard microorganisms. Ampicillin (commercial antibiotic) was used as control. Out of the five bacterial pathogens three were found to be negative (Escherichia *coli*, Klebsiella *pneumonia*, Salmonella *paratyphi*) and two were positive (Bacillus *subtilis*, Streptococcus *faecalis*). Disc diffusion method was used to evaluate the antibacterial activity of taken samples. After twenty-four hours the minimum inhibitory zone of methanolic solution of complexes and control were measured. In complex **1**, the maximum activity (14.33±1.21) was observed against Streptococcus *faecalis*, gram positive bacteria (Table 1). The control ampicillin showed 14.3±0.02. The least activity (6±0.26) was observed against Escherichia *coli*, gram negative bacteria. The control against Escherichia *coli* showed 15.3±0.37. Also in complex **2**, the maximum activity was observed against Streptococcus *faecalis* and the zone of inhibition value was 13.12±0.21. Results clearly indicate that the inhibition is much larger by metal complexes as compare to the metal free ligand. A comparative study of

the ligand and their metal complexes indicates that the metal complexes exhibited higher antimicrobial activity than that of the free ligand. Hence complexation increases antimicrobial activity. The enhanced activity of the complexes can be explained on the basis of Overtone's concept [27] and Tweedy's Chelation theory.[28] Chelation tends to make the ligands act as more powerful and potent bacterial agents, thus killing of the more bacteria than the ligand. It is observed that in complexes the positive charge of the metal partially shared with the donor atoms present in the ligand and there may be π-electron delocalization over the whole chelate ring. The variation in the effectiveness of the different compounds against different organisms depends on their impermeability of the microbial cells or on the difference in the ribosome of the microbial cells.[29]

Conclusions

Mixed ligand copper(II) complexes having tridentate NNN-donor benzimidazolyl terpyridine base and planar NN-donor heterocyclic bases are prepared and characterized by various physico-chemical techniques. The biocidal efficiency of the complexes has also been evaluated and found that the Cu(II) complexes show better biological activity when compared with the ligand. The synthesized ligand and its metal complexes were screened for reduction of DPPH. Based on the results obtained, the free ligand was found to be good antioxidant. However, its antioxidant activity can be further confirmed by *in vivo* methods.

Acknowledgement

The authors thank the Head, Department of Chemistry, UDC, Trichy for providing Laboratory facilities.

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Publication History
Received 28nd Sep 2013
Revised 05th Dec 2013
Accepted 20th Dec 2013
Online 05th Jan 2014