

UGC-MRP (Completion report) April 2013

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Title : “Biomimicking of Cu-Zn superoxide dismutase enzyme”

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The principal aim of this article was to highlight the diseases associated with expression of Cu-ZnSOD. The significance of this project was to develop novel model compounds of superoxide dismutase which are anti oxidants and that have effective cancer inhibiting properties

As per given methodology we followed the scheme and we are successful to get results of the project work.

Co-investigator herself worked on this project and we have submitted paper to the journal : Res.J.of Chem and Env. And which was accepted and very soon it will be published.

Scheme :

1. Synthesis of Bisacetylacetone ethylenediimine ligand
Synthesis of this ligand using literature*
2. Synthesis of cupric hydroxide which is a precursor prepared by adding NaOH to an aqueous solution of cupric chloride dehydrate
3. Synthesis of asymmetric Schiff bases using literature survey*
4. Synthesis of Cu-Zn Bimetallic complexes

Prepared Cu (Salen) complex, triazole and Zinc chloride anhydrous were mixed in 1:1:1 molar proportion. The mixture was then kept for refluxing for 18 hrs, a red colour complex was formed.



Characterization was done using elemental analysis, TGA, IR, ¹H NMR, ESR and magnetic properties from IIT Mumbai and Department of Chemistry , University of Pune.

Biological activity studies we worked in our college and DNA cleavage Nuclease activity and cell line study in NCL with Dhiman Sarkar Sir. This paper soon we will publish in good journal.

Out come of Project :

1. Publication in journal : 01 (submitting accepted paper) Annexure -I
2. In national conference : 03 (poster presentation) Annexure -II

3. Co-investigator has registered for PhD., in University of Pune.

Annexure -II

1. Antimicrobial and Biological Activity of bimetallic Cu-Zn Complex, Gouri Bhide^a, Vidya Kalyankar^a, , Sharada Dagade^b and **Shobha Waghmode^a**, poster presentation in **national conference**, current trends in chemistry organised by Y.M. college, Bharati Vidyapeet, Pune 12-13 Jan 2013.
 2. Correlation of superoxide dismutase enzyme from *Vetiveria Zizanoides* with its bimetallic (Cu-Zn) model complex, Vikas Jadhav^a, Vidya Kalyankar^a, , Sharada Dagade^b and **Shobha Waghmode**, poster presentation in **national conference**, current trends in chemistry organised by Y.M. college, Bharati Vidyapeet, Pune
 3. Efficient oxidation catalyst by model complex of superoxide dismutase, , Vidya Kalyankar^a, Sudhir Phadtare^a, Priyanka dagade, Sharada Dagade^b and **Shobha Waghmode^a**, , poster presentation in **national conference** on Chalcogenides, organised by DIAT , Pune 14-15 Jan 2013.
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Annexure -I

Synthesis and characterization of novel Bimetallic Cu (II)-Zn (II) complexes: Antimicrobial and Superoxide dismutase activity

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Abstract

A new class of *N-O* donor imine based monomeric Cu(II) (Cu-1, Cu-3) and bimetallic Cu(II)-Zn(II) (Cu-2, Cu-4) complexes have been synthesized using L-1 [(E)-4-(2-aminoethylimino)penta-2-one] and L-2 [(E)-(2-aminoethylimino)methyl]phenol as ligand. These are characterized by elemental analysis, thermogravimetric analysis and infrared spectroscopy. Anhydrous nature of monomeric complexes Cu-1 and Cu-2 confirmed using thermogravimetric and infrared data.

Confirmation of structural features was determined from quantization of activation energies (E_a) of different pyrolytic steps in TG using the rising temperature expression of Coats and Redfern.²⁰ Cu-1, Cu-3 and Cu-4 show two step decomposition and only Cu-2 shows three step decomposition from TG studies. Bimetallic Cu-2 and Cu-4 show SOD activity is confirmed by NBT assay. Cu-2 shows very good antimicrobial activity as it has aliphatic ligation system. The five coordinate geometry of Cu(II) ion in Cu-2 and Cu-4 complexes acts as a good model for active site of superoxide dismutase enzyme.

Keywords: Superoxide dismutase (SOD), NBT-Nitro Blue Tetrazoline, Triazolate bridged bimetallic complexes, CuZnSOD, Antimicrobial activity, Thermal analysis.

Introduction

In biological systems balance between superoxide generation and its dismutation, there are some compound present named antioxidant substances which take care of toxic reactive species¹. 'Antioxidants' cover the area of several organic substances, vitamins – C, E, A, selenium, metalloenzymes (superoxide dismutases), carotenoids. Present work is restricted only on synthesis of superoxide dismutase mimics².

Superoxide dismutase is only one metalloenzyme converting superoxide radicals to hydrogen peroxide and

molecular oxygen and thus forming a crucial part of the cellular part of the antioxidant defence mechanism. Among various types of SODs, Cu – Zn superoxide dismutase (SOD1) is widely distributed and comprised 90% of the total SOD³. This ubiquitous enzyme requires Cu and Zn for biological activity. The ideal SOD-mimic should be a low molecular weight metal complex that possesses high membrane permeability, however, very few Cu-carboxylate complexes had been reported⁴.

In an attempt to obtain small molecule-based metal complexes, we now report the synthesis of novel copper-zinc complexes as potent SOD mimics. Binuclear triazole-bridged copper (II) – Zinc (II) complexes have attracted much attention as models for metalloenzymes which contain the same structural units⁵. It has been found in the cytosol of the eukaryotic cells and it protects cells from the toxic effects of superoxide ion by catalysing the dismutation reaction. In the enzymatic reaction, O_2^- reaches the bottom of the protein channel where Cu (II) resides and O_2^- reduces Cu(II) to Cu(I) producing O_2 .

As soon as the Cu(I) centre is produced, the bridging triazole group (His-61) is dislodged from the copper center but remains coordinated with Zn(II) and water protonates the partially dislodged imidazole group. At the next step, another O_2^- anion approaches and oxidizes Cu(I) to Cu(II) and is reduced to H_2O_2 which is further protonated by water to give H_2O_2 .

Thus, the Cu-centre is reversibly reduced and oxidized by successive encounters with superoxide giving rise to O_2 and H_2O_2 at the reactive steps. On the other hand, the Zn (II) centre primarily plays a supportive role and it indirectly helps the formation and stabilisation of the active Cu-site in the course of reaction. Triazolate bridged heterobinuclear Cu(II) Zn(II) complexes are still very rare to date⁶. Therefore, in the course of our studies on synthetic dinuclear transition-metal complexes,^{7, 8, 9} we have synthesized and characterized two hetero dinuclear complexes.

Biological activities of superoxide dismutase enzyme of these complexes have been measured. Results indicated

that the calculated physicochemical parameters were well correlated with the experimental SOD activities. Such theoretical parameters included energies of HOMO and LUMO as well as Ea. Furthermore, to confer value-added benefits to the copper complexes as well as explore the possibility of using such compounds as a multifaceted drug, the antimicrobial activity of the novel complexes was also determined⁹.

Material and Methods

Chemicals: All the chemicals used in the preparation of ligand as well as complexes were of analytical grade and used without further purification. Triazole (Tz) was purchased from S.D.Fine chemicals. Potassium superoxide (KO₂) was obtained as a yellow powder from Aldrich (45.04% O by weight) and used as received. 18-crown-6 ether was obtained from Aldrich (99.9% purity).

Physical measurements: The Perfit electrical melting point apparatus is used to record melting points of the synthesized complexes and are uncorrected. Carbon, hydrogen and nitrogen were estimated using Perkin-Elmer 2400 Elemental Analyzer. IR spectra were recorded on a Shimadzu spectrometer in KBr.

Thermal analysis for Cu-1 to Cu-4 was performed on laboratory built thermobalance under air atmosphere with heating rate 3-5°C per min, 30-35mg sample and 200-250 mesh size.

Synthesis of metal complexes

L-1[(E)-4-(2-aminoethylimino) penta-2-one] and L-2[2-((E)-(2-aminoethylimino) methyl) phenol] were synthesized using literature procedure.^{10a,b}

Cu-1 [Cu(L-1)Cl] : To the warm methanolic solution of ligand L-1 (0.142gm, 1mmole), freshly prepared aqueous solution of CuCl₂·2H₂O (0.170g, 1mmole) was added with constant stirring. The mixture was continuously stirred for 6 hours heating at 50-60°C on water bath. Precipitate of blue coloured complex was separated immediately. The product was then washed with cold water and ether. The complex was dried under vacuum at room temperature. An elemental analysis of this complex gave: C, 35.29; H, 5.82; N, 11.66. Calc.: C, 34.01; H, 6.02; N, 10.96.

Cu-2 [CuZn(L-1)TzCl₃H₂O] : To the warm methanolic solution of Cu-1 (0.240g, 1 mmole), was added freshly prepared aqueous solution of triazole (0.069g, 1 mmole). The reaction was carried on water bath with constant stirring and, then aqueous solution of ZnCl₂ (0.136g, 1mmole) was added to the reaction mixture. Total reaction mixture was refluxed for 5 hours heating at 50-60°C to get blue coloured complex precipitate. It was filtered on Buchner funnel and dried under vacuum at room temperature. An elemental analysis of this complex gave: C, 23.37; H, 15.14; N, 3.92. Calc.: C, 22.89; H, 15.50; N,

3.58.

Cu-3 [Cu(L-2)Cl] : To the warm methanolic solution of ligand L-2 (1mmole, 0.164gm), was added freshly prepared aqueous solution of CuCl₂·2H₂O (1mmole, 0.170g) with constant stirring. The mixture was continuously stirred for 6 hours heating at 50-60°C on water bath. Precipitate of blue colored complex was separated immediately. The product was then washed with cold water and ether. The complex was dried under vacuum at room temperature. An elemental analysis of this complex gave: C, 41.40; H, 4.59; N, 10.64. Calc.: C, 42.01; H, 5.02; N, 9.89.

Cu-4 [CuZn(L-2)TzCl₃H₂O] : To the warm methanolic solution of Cu-3 (0.264g, 1 mmole), was added freshly prepared aqueous solution of triazole (0.069g, 1 mmole). The reaction was carried on water bath with constant stirring and then aqueous solution of ZnCl₂ (0.136g, 1mmole) was added to the reaction mixture. Total reaction mixture was refluxed for 5 hours heating at 50-60°C to get blue coloured complex precipitate. It was filtered on Buchner funnel and dried under vacuum at room temperature. An elemental analysis of this complex gave: C, 29.69; H, 2.90; N, 14.42. Calc.: C, 30.01; H, 3.20; N, 14.95.

Bioactivity: Antimicrobial (antibacterial) and superoxide dismutase (SOD) activities were evaluated using the following methods. In vitro antimicrobial (antibacterial) activities of these complexes were tested using paper disc diffusion method^{11,12a,b}. The chosen strains were *E.coli*, *Pseudomonas*, *S.paratyphi B*, *Proteus*, *Klebsiella* and *S.Aureus*.

Liquid media:

1. Sterile Muller Hinton (MH) broth tubes were prepared (9 ml in each tube).
2. 300 µl of each compound was added into the broth using sterile pipette.
3. 200 µl of inoculum (bacterial suspension) was then added into the broth using sterile pipette.
4. A control was prepared for each organism, by only adding the inoculum and no compound.
5. Another control tube was prepared by adding the inoculum and only DMSO to the broth.
6. The tubes were incubated at 37°C for 18-24 hours.
7. After incubation the tubes were observed for presence of turbidity (bacterial growth) or its absence (no bacterial growth) by comparing with the control and uninoculated tubes.

Solid media:

1. Sterile Muller-Hinton (MH) agar plates were prepared.
2. Using sterile forceps a trough was dug at the top of the agar plate.
3. Using sterile inoculation loop each of the six organisms were streaked (straight line) on a single plate, beginning from the trough.

- Using sterile pipettes, the troughs were filled with the compound to be tested. For each compound there was a separate plate.
- A control plate was prepared by filling the trough with DMSO only.

The width of the growth inhibition zone was measured after 24 h incubation. Four replicas were made for each treatment.

SOD activity: In order to determine SOD activity, the indirect method using nitroblue tetrazolium (NBT) is used^{13,14}.

Materials: Tris base buffer 12.1gm in 500ml of H₂O; pH of solution maintained in between 8-9. If the desired pH is not achieved then 0.2 conc. HCl is added. Super oxide solution: 0.528gm of crown ether and 10.8mg KO₂ were added in 20ml of DMSO. NBT (Nitro Blue Tetrazolium) Solution: 0.0114gm of in 250ml of DMSO to give 56µM of NBT. Sample preparation: 1 mM concentration of test samples was prepared.

Procedure: 1ml tris buffer was taken in a test tube. To this 0.5ml of NBT solution was added. Solution was mixed well. Then 0.2ml (200µl) of sample solution under study was added and mixed well. Whole assembly was kept in ice for 15 min. After 15 min. 0.75ml (750µl) of KO₂ was added and incubated for 10 min. Absorbance was measured at 560 nm. Blank reading was taken without sample addition.

Results and Discussion

Synthesis: Triazolate bridged bimetallic CuZnSOD model complexes have been synthesized by adding monomeric complex of Cu with triazole and ZnCl₂ solution and resultant was bimetallic CuZn complex with triazole bridge and aqua ligation. Water molecule is essential in functioning of SOD hence in our synthesis our dimers are with aqua ligation. Here we are reporting the relevance of energetic of SOD model compounds from the pyrolytic reactions of Cu-1 to Cu-4 complexes.

Infrared spectral studies: The absence of absorption band in 3600 to 3200cm⁻¹ range in compounds Cu-1 and Cu-3 indicates its anhydrous nature (Table 1); however a strong broad absorption band ~3425cm⁻¹ in Cu-4 is assigned to γ (OH) frequency of either intra/intermolecular H bonded water molecules¹⁵. γ C=O vibrational band in all four complexes shows reduced form¹⁶ of ligand as the frequency is lower than 1600cm⁻¹ except in Cu-3. Typical γ (N-N) vibration are seen ~1171cm⁻¹ in Cu-2 and Cu-4 at ~1176cm⁻¹. In all complexes as well in both ligands γ (NH₂) bonded vibration can be seen ~3000cm⁻¹ with very strong band¹⁷. Vibrational frequency for γ C=N vibration suggests coordination through nitrogen which is seen in all complexes. γ (C=C) aromatic bands are seen in Cu-3 and Cu-4 complexes only¹⁸.

Non -isothermal TG studies: The simultaneous non-isothermal TG curves for the solid phase thermal decomposition of Cu-1 to Cu-4 at different steps are ascertained in fig.1. The dynamic TG data with percentage mass loss and energy of activation at different steps in the pyrolytic reactions for Cu-1 to Cu-4 are presented in table 2.

The kinetic parameters were calculated¹⁹ from the dynamic TG curves using the computer program developed in our laboratory with the help of rising temperature expression of Coats and Redfern²⁰. Activation energies (E_a) quantified from kinetic plot was obtained at two different heating rates with best fit correlation coefficient. The E_a value for two rates is, within an accuracy range of ± 2.2kJ/mole. Table 2 represents best fit kinetic parameters for one heating rate only.

The pyrolytic decomposition of Cu-2 is in three steps (Fig.1b) viz. loss of coordinated water molecule^{21a, b} and one chloride in step-I and bridging triazole in step-II and in step-III loss of ligand (L-1) and two chlorides.

Although the decomposition is in two steps and similar infrared bands are observed for Cu-1, Cu-3, Cu-4, (Fig.1a,c,d) but fractional mass losses are different (Table.2), indicating their different individual coordinating nature is in dimer and monomer. Using additive property of activation energies the energy required for release of ligand in all complexes is calculated and for L-1 it is 16.9kJ/mole. Expulsion of L-2 is 28.9kJ/mole. One chloride coordination is different in both dimers i.e. Cu-2 and Cu-4 which can be well explained in TG studies.

First step is release of Cl⁻ and easy to quantities E_a for both dimers. And that E_a for Cl⁻ is 98.8+96.9 =195.712=97.85kJ/mole. Similarly for L-1 and L-2 we can directly quantize the E_a value from second step. In Cu-2 and Cu-4 triazole(Tz) is bridging ligand and its expulsion is in second step of Cu-2 well as Cu-4. From Cu-4 we can get direct E_a value for Tz and that is 32.2kJ/mole.

Presence of anhydrous nature of monomeric Cu-1 and Cu-3 complexes can be explained from temperature range and weight loss, where as in dimeric complexes Cu-2 and Cu-4, water is present in coordinating sphere²². Thermal stabilizes of complexes are indicated from their decomposition temperature. Anhydrous Cu-1 and Cu-3 decomposed at ~200°C while Cu-2 and Cu-4 show decomposition at ~110°C. The fractional decomposition of H₂O molecule in Cu-2 and Cu-4 are similar using additives property of E_a²³, the expulsion of water molecule is calculated and value is 6.68kJ/mole for 1H₂O.

Superoxide dismutase activity: The superoxide dismutase activity of this compound was studied and presented in table 3. Bimetalic complexes of Cu-Zn showed good

superoxide dismutase activity. Low molecular weight complex of superoxide dismutase containing transition metal ions, have been proposed²⁴ to overcome the limitations of the use of SOD enzyme as therapeutic agent and pharmaceutical tool²⁵.

Table 3 indicates that heterobimetallic Cu-(2)-Tz-Zn(2) system with simple bidentate imine were more active than the Cu(2)-Tz-Zn(2) i.e. Cu-4 complex. Both bimetallic complexes were more active than monomeric Cu-2 complex of same ligation. The IC₅₀ value for Cu was less than Cu-4 dimeric complex due to macro cyclic ligands in Cu-4²⁶. Although the superoxide activity of these complexes is in the same order²⁷, less SOD activity is due to macro cyclic ligation than the simple ligand which gives more flexibility and steric effect to the complex.

Antimicrobial activity of compounds: The susceptibility of the contain strains of bacteria towards the present copper (II) complexes was determined by measuring the size of inhibition diameter (Table 3). The disc diffusion technique was employed. Cu-2 bimetallic complexes were highly effective against *E. coli* and *pseudomonas*²⁸. It was also observed that antibacterial activity of these complexes for removing bacteria was fairly good.

Monomeric complexes showed resistance against all species. Cu-4 showed less activity against all species. Due to small ligation system Cu-2 bimetallic complex showed good SOD as well as antimicrobial activity.

Conclusion

Two new type of complexes viz. [CuZn(L-1)Cl₃H₂O] and [CuZn(L-2)Cl₃H₂O] have been synthesized and appear to be good models for active site in CuZnSOD. Similar to natural origin these complexes show similar structural behaviour. Water ligation is considered in synthesis and confirmed. As zinc has supporting role in CuZnSOD active site, so coordination surrounded by zinc is not important. In particular, elemental analysis, thermal studies and IR studies are close to those of proteins. The triazole bridge in these complexes has been found to be stable in the pH range 7.5-10.5; it is similar to protein stability.

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Table 1
Selected Infrared frequencies (in cm^{-1}) in L-1, L-2 and Cu-1 to Cu-4

	L-1	L-2	Cu-1	Cu-2	Cu-3	Cu-4
$\gamma(\text{OH})$ of H_2O	–	–	–	3425(br), 3650(br)	–	3425(sbr)
$\gamma(\text{NH}_2)$ bonded	3200(br)	3035(sbr)	2926(w)	3134(br)	3049(mbr)	3107(s)
$\gamma(\text{C}=\text{O})$	1608(s)	1633(s)	1582(s)	1577(w)	1687(w)	1590(br.)
$\gamma(\text{C}=\text{N})$	1575(mbr)	1577(s)	1556(s)	1516(s)	1546(s)	1531(s)
$\gamma(\text{C}=\text{C})$ aromatic	-	1601(sh)	-	-	1600(s)	1626(br)
$\gamma(\text{C}-\text{N})$ $\gamma(\text{C}-\text{C})$	-	-	1483(m)	1495(m)	1469(s)	1498(s)
$\gamma(\text{C}-\text{O})$	1220(s)	-	1193(m)	1171(s)	1201(s)	1220(sh)
$\gamma(\text{N}-\text{N})$	-	-	-	1171(s)	-	1176(s)
$\gamma(\text{Cu}-\text{N})$	-	-	457(s)	460(s)	462(s)	447(w)

Table 2
Data on activation energies (kJmol^{-1}) from TGA in air for Cu-1 to Cu-4 complexes

Comp	Step No.	Temp. range $^{\circ}\text{C}$	%mass loss	Probable comp. of group lost	Residue	Order	Ea/kJ
Cu-1	I	200-320	15.0	Cl^-	Cu(L-1)	1.05	98.8
	II	380-680	58.0	(L-1)	CuO	1.85	16.9
Cu-2	I	120-170	10.0	$\text{Cl}^- + \text{H}_2\text{O}$	CuZn(L-1)TzCl_2	2.1	105.1
	II	180-270	14.0	Tz	CuZn(L-1)Cl_2	0.4	32.2
	III	280-520	49.0	(L-1)+2 Cl^-	$\text{CuO} + \text{ZnO}$	1.6	102.3
Cu-3	I	250-320	13.0	Cl^-	Cu(L-2)	1.4	96.9
	II	412-655	59.92	(L-2)	CuO	1.9	28.9
Cu-4	I	80-200	11.0	$\text{Cl}^- + \text{H}_2\text{O}$	CuZn(L-2)TzCl_2	1.9	103.9
	II	240-700	62.59	(L-2)+Tz+2 Cl^-	$\text{CuO} + \text{ZnO}$	2.0	108.1

s-strong, w-weak, sh-shoulder, br-broad, m medium

Table 3
SOD antimicrobial activity of Cu -1 to Cu-4 complexes

Comp.	IC_{50} (μM)	Diameter of inhibition zone (in mm)					
		<i>E.coli</i>	<i>Pseudomonas</i>	<i>S.paratyphi B</i>	<i>Proteus</i>	<i>Klebsiella</i>	<i>S.aureus</i>
Cu-1	93	R	R	R	R	R	R
Cu-2	15	31.4	30.1	28.7	25.3	22.6	34.7

Cu-3	89	R	R	R	R	R	R
Cu-4	21	8.3	7.1	9.9	15.4	11.0	10.1

R-Resistant

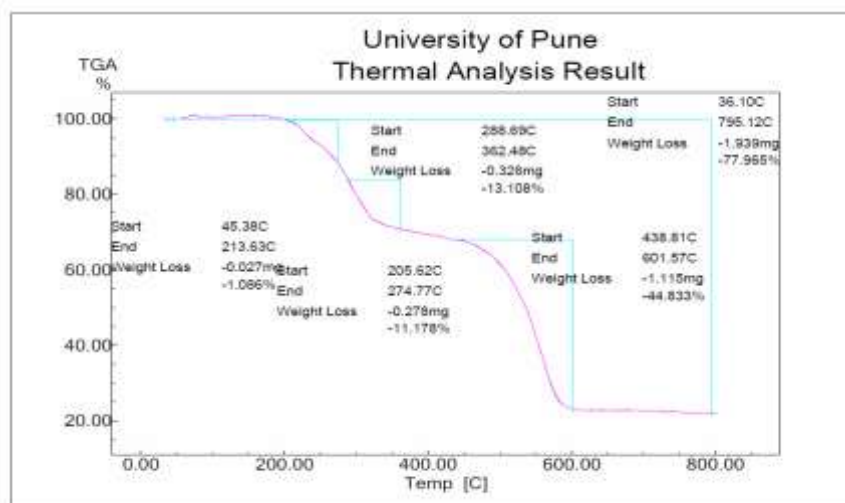


Fig.1a: TG Curve of Cu-1

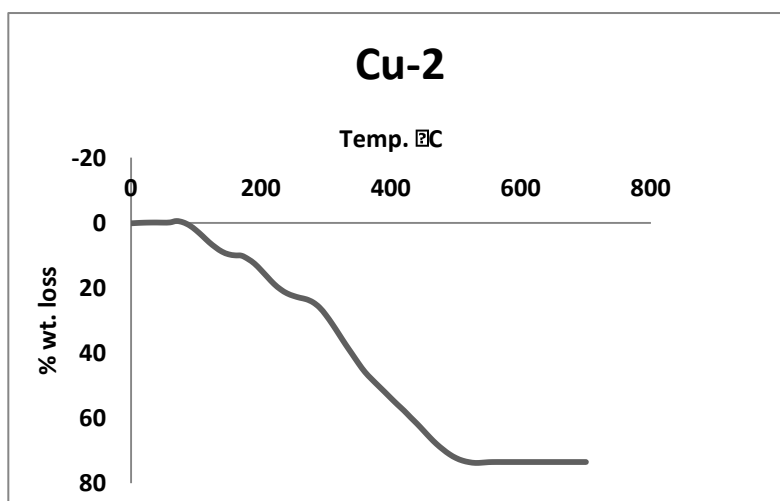


Fig.1b: TG Curve of Cu-2

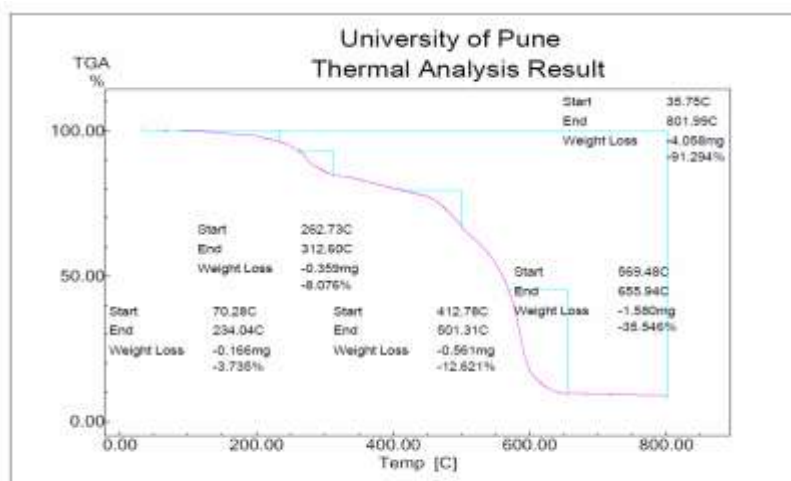


Fig.1c: TG Curve of Cu-3

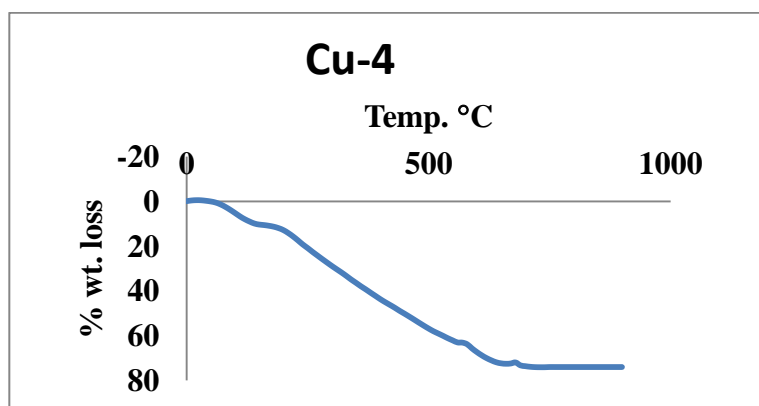


Fig.1d: TG Curve of Cu-4

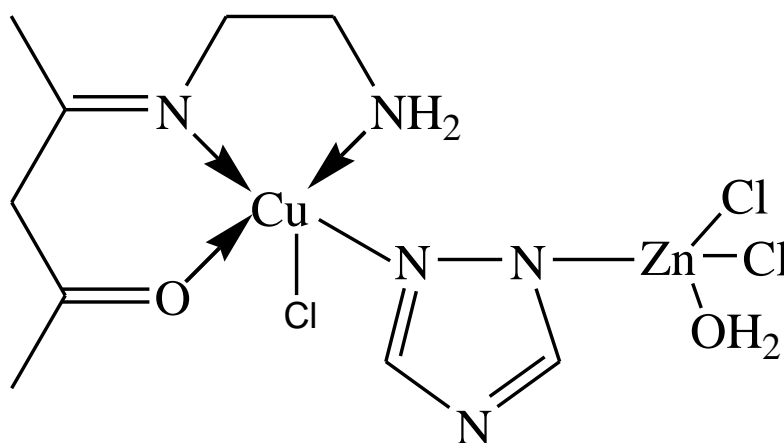


Fig. 2a: Tentative structure of Cu-2

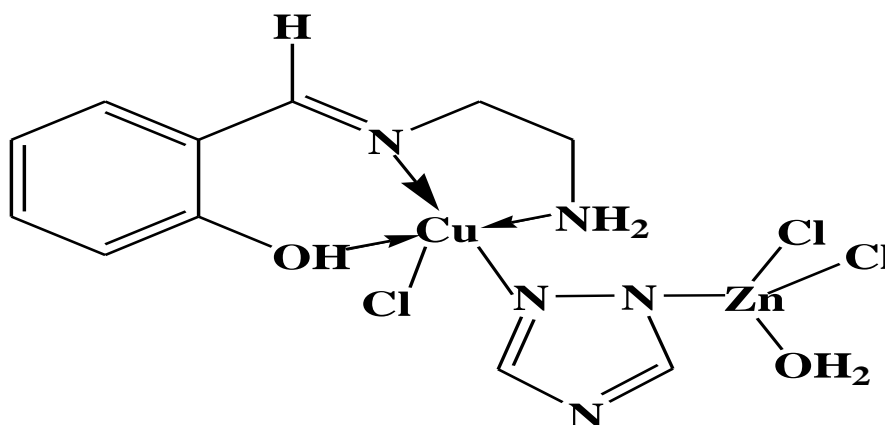


Fig.2b: Tentative structure of Cu-4

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