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Catalytic promiscuity of a copper(II)-Mannich base complex having unprecedented radical pathway in catecholase activity

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ABSTRACT

A flexible single-side Mannich base ligand namely 4-(*tert*-butyl)-2-((ethyl(2-hydroxyethyl)amino)methyl)phenol (**H_2L**) having O, N, O donor sites has been designed and synthesized using 4-(*tert*-butyl)phenol, formaldehyde and 2-(ethylamino)ethanol as precursors with the view to prepare polynuclear complex(s). In the first attempt a tetranuclear copper (II) complex, **$[Cu_4(L)_2(HL)_2]\cdot(ClO_4)_2\cdot H_2O$** (**1**) has been synthesized employing **H_2L** and copper(II) perchlorate with the aim to explore catalytic promiscuity of the species. Detailed experimental investigations suggest that in solution complex **1** exits as a dinuclear entity. The dinuclear species is a square-planar Cu^{II} complex as is evident from EPR and UV-Vis spectral studies. It exhibits excellent catalytic activities in catalyzing the oxidation of 3,5-di-*tert*-butyl catechol (3,5-DTBC) to 3,5-di-*tert*-butyl quinone (3,5-DTBQ); oxidation of o-aminophenol to phenoxazinone and P-O bond hydrolysis of 4-nitrophenyl phosphate. Amazingly, complex **1** catalyses the aerobic oxidation of 3,5-DTBC not by well established metal centered redox participation, rather via a ligand centered radical pathway, a completely new finding in copper(II) based catechol oxidase modeling as are verified by experiments and seconded by DFT calculations.

Keywords: Copper(II)-Mannich Base complex, Catalytic promiscuity, Catecholase activity, Phenoxazinone Synthase activity, Phosphatase like activity, DFT study

1. Introduction

'Catalytic Promiscuity', a new popular terminology is now widely used by bioinorganic chemists. The term 'Catalytic Promiscuity' of an enzyme is defined as its ability to catalyze multiple catalytic transformations which belong to different classes [1]. Another definition of 'Catalytic Promiscuity' is the capability of a single active site to catalyze more than one chemical transformation as proposed by Romas J Kazlauskas [2]. Chymotrypsin (that catalyzes hydrolysis of amides, esters, thiol esters, acid chlorides and anhydrides) [3], bovine carbonic anhydrase II(catalyzes phosphotriesterase activity, carbon esterase activity and CO_2 hydrates activity)[4], carbonic anhydrase III (catalyzes hydrolysis of phosphomonoester monocation, carbon ester and hydration of carbon dioxide)[5] are familiar enzymes showing catalytic promiscuity.

In recent times activation of dioxygen by dinuclear transition metal complexes has been studied comprehensively and preparation of bio-mimetic models of oxidases, oxygenases, dehydrogenases and other metalloenzymes draws the immense attention of the bioinorganic chemists.[6] Multicopper oxidase belongs to an important class of oxidase enzyme which contains seven kinds of active sites depending on the structural and functional properties of copper centre namely, Type-1, Type-2, Type-3, Type-4, Cu_A, Cu_B and Cu_Z active sites. Catechol oxidase(CO) and phenoxazinone synthase (PHS) belongs to oxidases group of enzyme [7].

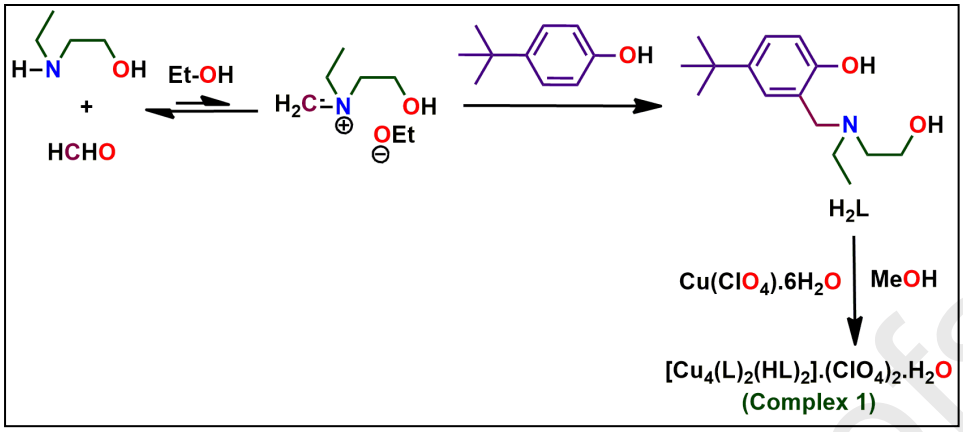
CO, a Type-3 copper protein, contains antiferromagnetically coupled dinuclear copper(II) centers at their active site and catalyzes oxidation of catechol derivative to corresponding quinone [8-14]. This oxidation is worthy for medical diagnosis of the hormonally active catecholamine, adrenaline, noradrenaline and dopa [15,16]. Till date several bio mimicking models of CO have been reported to have an insight about their mechanistic pathways. Generally two kinds of pathways are operative, (i) metal centered redox participation [17-24] and (ii) ligand centered radical pathway [25-32]. The ligand centered radicals till now demonstrated are of three types: (i) imine radical, [25-30] found in Schiff base based models, (ii) arene/benzene anion radical observed in reduced Schiff base complexes [31] and (iii) phenoxy radical for Mannich base complexes[32].

PHS contains five different copper centers at active site namely type-1, type-2 and type-3 [33]. PHS which is very important in pharmaceutical industries being involved in the last step of biosynthesis of antibiotic actinomycin D by *Streptomyces antibioticus*[34]. The mechanistic

pathway of phenoxazinone synthase, involves the formation of organic radical with concomitant reduction of either metal or ligand centre [30, 35,36].

On the other hand designing of metal complexes as bio-mimicking models of phosphatase, a class of hydrolysis enzymes became an imperative field to bio-inorganic chemists. These enzymes catalyse the hydrolysis of P-O bond of phosphate monoesters, di-esters, tri-esters, fluorophosphates, fluorophosphonates, phosphoric anhydrides etc. and the hydrolysis reaction plays a significant role in life processes, medical field and agriculture[37-42]. It is well known that bi-valent metal cations play a vital role towards the nucleophilic substitution of naturally occurring phosphate ester bond in most of the cases. It is already well documented in literature that the rate of hydrolysis of phosphate ester bond increases several times in presence of mononuclear model complexes in compare to the uncatalysed reaction and that rate enhance appreciably when dinuclear catalysts are used. Most of the reported models contain zinc(II) as metal centre. However, Sigel *et al.* and Trogler *et al*. reported that copper(II) metal ion can also efficiently catalyse the hydrolysis of P-O bond of triphosphates [43-44]. It is already established that Cu^{2+} and Ni^{2+} metal based models exhibit the highest catalytic activity among the first row transitional metals[44]. Thus in past few decades Cu^{2+} has been used as a good alternative to Zn^{2+} in model complexes[45-47].

In the present work we report a tridentate Mannich base ligand with O, N, O donor set, which is designed and synthesized for the flexibility of the CH_2-NR moiety (Scheme 1), that may help to produce complexes of different nuclearity [48-49]. In the first attempt, a tetranuclear complex, $\mathbf{[Cu_4(L)_2(HL)_2]\cdot(ClO_4)_2\cdot H_2O}$ (**1**) has been synthesized (Scheme 1) purposefully to explore its catalytic promisicuty in terms of catecholase activity, phenoxazinone synthase activity and phosphatase activity. Complex **1** acts as the catalyst precursor to generate dinuclear active catalyst in solution. Complex **1** appears not only as a highly efficient oxidizing catalyst but also as a potential hydrolytic agent. It catalyses the aerobic oxidation of the model substrate 3,5-DTBC to 3,5-DTBQ in excellent rate. The reaction proceeds not via metal centered redox participation but through ligand centered radical pathway. It is a completely new finding in catecholase activity study in dinuclear copper(II) system as is established by combined experimental and theoretical investigations. The mechanistic pathways of phenoxazinone synthase activity and phosphatase activity of complex **1** have also been extensively studied. All those findings have been vividly portrayed in this communication.



Scheme 1. Synthetic Route of Complex **1**.

2. Experimental section.

2.1. Physical methods and materials

Elemental analyses (carbon, hydrogen, and nitrogen) were carried out using a Perkin–Elmer 240C elemental analyzer. Fourier Transform Infrared spectra were recorded on KBr disks and NaCl disks (400-4000 cm^{-1}) and a Perkin-Elmer RXI FTIR spectrophotometer. Electronic spectra and kinetic studies (200-900 nm) were measured at room temperature with a Shimadzu UV 3101PC instrument and dry DMF as solvents. ^{1}H NMR spectra (300 MHz) were recorded in d_6-DMSO as solvents and TMSCl as standard at 25 °C with a Bruker AV300 Supercon NMR spectrometer and use of the solvent signal as the internal standard in a 5 mm BBO probe. Electrospray mass spectra were recorded on a WATERS Xevo G2-S Q Tof mass spectrometer using HRMS-grade acetonitrile as solvent. The cyclic voltammograms were obtained with a Basi C-3 cell voltameter under nitrogen with a glassy carbon electrode as working electrode, Ag/AgCl as reference electrode and tetrabutylammonium perchlorate as supporting electrolyte.

High-purity 4-(*tert*-butyl)phenol, 2-(ethylamino)ethanol and 37% (w/v) formalin solution, copper(II) perchlorate hexahydrate were purchased from commercial sources (Alfa Aesar, Sigma-Aldrich, Merck) and used as received. Solvents were dried by standard procedures and distilled prior to use.

Caution! Perchlorate salts are explosive. They should be handled with great care and in small amounts.

2.2. Synthesis of ligand (H_2L)

To an ethanolic solution of 4-(*tert*-butyl)phenol (25 mmol, 3.75 g), ethanolic solution of 2-(ethylamino)ethanol (25 mmol, 2.22 g) is added. Then 37% (w/v) formalin solution (25 mmol, 2.1 mL) has been added and the resulting yellow colored solution is refluxed for 10 hours. The reaction mixture is evaporated under reduced pressure. The oil-like residue is extracted with brine solution and dichloromethane for three times. The organic layer has been collected and dried with anhydrous $MgSO_4$. The ligand has been collected after complete evaporation of dichloromethane.Yield: 90%; FT-IR (NaCl): ν (C-N) 1583 cm^{-1}; ν (skeletal vibration) 1472 cm^{-1} (Fig. S1). ^{1}H NMR (300 MHz, d_6-DMSO, 25 °C) : δ 1.124 (s, 9H, $C(C\underline{H}_3)_3$); δ 3.630 (s, 2H, Ar-$C\underline{H}_2$-N); δ 2.463 (q, 2H, N-$C\underline{H}_2$-CH_3 and 2H, N-$C\underline{H}_2$-CH_2-OH); δ 3.449 (q, 2H, N-CH_2-$C\underline{H}_2$-OH); δ 0.899 (t, 3H, N-CH_2-$C\underline{H}_3$); δ 3.630 (q, 1H, N-CH_2-CH_2-O$\underline{H}$); δ 7.078 (d,1H,Ar) ; δ 6.973 (d,1H,Ar); δ 6.598 (d,1H,Ar) (Fig. S2). ^{13}C NMR (75 MHz, d_6-DMSO, 25 °C): δ 31.85 (3C,$C(\underline{C}H_3)_3$); δ 34.01(1C, $\underline{C}(CH_3)_3$); δ 155.47, 141.29, 126.32, 124.99, 122.48, 115.25 (6C,Ar) ; δ 54.95 (1C,Ar-$\underline{C}H_2$-N); δ 47.20 (1C,N-$\underline{C}H_2$-CH_3); δ 11.26 (1C,N-CH_2-$\underline{C}H_3$); δ 58.79 (1C,N-$\underline{C}H_2$-CH_2-OH); δ 56.96 (1C,N-CH_2-$\underline{C}H_2$-OH) (Fig. S3). ESI-MS (ES^+, Acetonitrile) calcd. *m/z* value for $C_{15}H_{25}NO_2$ $[M+H^+]$: 252.1964 amu, found 252.1986 amu (Fig. S4).

*2.3. Synthesis of $[Cu_4(L)_2(HL)_2]\cdot(ClO_4)_2\cdot H_2O$ (**1**)*

To a methanolic solution of **H_2L** (0.251 g, 1 mmol) copper(II) perchlorate hexahydrate (0.365, 1 mmol) is added and resulting green colored solution is stirred very slowly for 15 min. The solution is filtered and kept in desiccators. Green colored needle shaped crystals suitable for X-Ray diffraction is obtained after three days. Yield: 85%, Elemental analysis calcd. (%) for $C_{60}H_{96}N_4O_{17}Cl_2Cu_4$: C, 49.01; H, 6.58, N 3.81; found: C 49.05, H 6.62, N 3.82; FT-IR (KBr): ν(C-N) 1606 cm^{-1}; ν(skeletal vibration) 1495 cm^{-1}; ν(perchlorate) 1090 cm^{-1}; ν(tertiary amine) 2960 cm^{-1} (Fig. S5); UV-Vis (DMF): $\lambda_{max}(\varepsilon)$ = 421 nm(716 L mol^{-1} cm^{-1}), 710 nm (170 L mol^{-1} cm^{-1}) (Fig. S6). ESI-MS (ES^+, Acetonitrile) calcd. *m/z* for $C_{30}H_{47}Cu_2N_2O_4^+$: 625. 2128 amu, found 625. 2158 amu (Fig. S7).

2.4. X-ray crystallography

Intensities data for crystal structure analysis of complex **1** are collected at room temperature with Mo-Kα radiation (λ = 0.71073 Å) on a Bruker Smart Apex diffractometer equipped with CCD.

Cell refinement, indexing, and scaling of the data set were performed using the Bruker Smart Apex, and Bruker Saint packages [51]. The structure is solved by direct methods and subsequent Fourier analyses [52] and refined by the full-matrix least-squares method based on F^2 with all observed reflections[52]. Hydrogen atoms are placed at calculated positions and included in final cycles of refinement. A perchlorate anion was found disordered over two positions of refined occupancies 0.574/0.426(16). All calculations are performed using the WinGX System, Ver2013.3[53]. Crystal data and details of refinements are given in Table S1.

*2.5. Catecholase activity and kinetics study of complex **1***

The catecholase activity of complex **1** is performed using 3,5-DTBC as model substrate because the presence of two bulky *tert*-butyl groups lowers the catechol-quinone redox potential and it also inhibits polymerization of oxidized product. The catalytic reaction is monitored by adding 1×10^{-4} (M) complex solution to 1×10^{-2} (M) 3,5-DTBC solution under aerobic condition at 25 °C in *N,N*-dimethylformamide(DMF) medium. The progress of the reaction is monitored by time dependent wavelength scan for 2 hours at 10 min interval of time.

The kinetic study of the oxidation of 3,5-DTBC by complex **1** have been performed by initial rate method at 25 °C. Solutions of concentration varying from 0.001 to 0.05 (M) have been prepared from a freshly prepared concentrated substrate solution. The dependence of the initial rate on the concentration of the substrate is monitored by UV-Vis spectroscopy at the respective wavelength by maintaining the fixed catalyst concentration [1×10^{-4}(M)] for each set. The complexes show saturation kinetics, and a treatment based on the Michaelis-Menten found to be the most appropriate. The binding constant (K_M), maximum velocity (V_{max}), and rate constant for dissociation of the substrates (*i.e.* turn over number, k_{cat}) are calculated for the complexes using the Lineweaver-Burk graph of $\frac{1}{V}$versus $\frac{1}{[S]}$, with the equation (2),

$$\frac{1}{V} = \left(\frac{K^M}{V^{max}}\right) \times \frac{1}{[S]} + \frac{1}{V^{max}} \qquad (2)$$

2.6.Detection of hydrogen peroxide in the catalytic reaction

In order to detect the formation of hydrogen peroxide during the catalytic cycle reaction mixtures have been prepared as in kinetic experiments. After 1 hour of reaction equal volume of water is added and 3,5-DTBQ formed is extracted with dichloromethane. pH of the aqueous layer is fixed

at 2 with H_2SO_4. 1 mL of a 10% solution of KI and three drops of 3% solution of ammonium molybdate are added. In the presence of hydrogen peroxide, the reaction, $H_2O_2 + 2I^- + 2H^+ \rightarrow 2H_2O + I_2$ occurs, and with an excess of iodide ions, the triiodide ion is formed according to the reaction $I_2(aq) + I^- = I_3^-$ .The reaction rate is slow but increases with increasing concentrations of acid, and the addition of an ammonium molybdate solution renders the reaction almost instantaneous. The formation of I_3^- can be monitored spectrophotometrically due to the development of the characteristic I_3^- band (λ_{max}= 353 nm, ε = 26000 L mol^{-1} cm^{-1}).

2.7. Computational methods

To further understand a possible mechanistic pathway for catalytic catechol oxidation by dinuclear copper complex (**1b**), density functional theoretical (DFT) computations are performed using Gaussian09 program package [54]. The Minnesota functional, M06-2X, in conjugation with 6-31G (d) basis set, is employed for ground state geometry optimizations [55]. M06-2X functional is found to estimate energy of metal complex catalyzed catechol oxidations, satisfactorily [31]. For triplet state optimizations, unrestricted broken spin symmetry approach is utilized to break the spin and spatial symmetries by mixing frontier molecular orbitals. Grimme's d3 dispersion is incorporated to consider dispersive interactions in all computations[56]. The solvent effect (DMF) is also introduced in computations by polarizable continuum model. Calculated minima have been confirmed by the absence of imaginary frequency (NIMAG=0) in harmonic vibrational frequency analysis. Gibbs free energies including thermally corrected unscaled zero point vibrational energies are reported in kcal mol^{-1} relative to the corresponding starting materials the dinuclear copper complex (**1b**). In computations, perchlorate/perchloric acid was used for proton acceptor/donor.

*2.8. Phenoxazinone synthase like activity and kinetics study of complex **1***

The phenoxazinone synthase like activity of complex **1** has been monitored by the reaction of 1×10^{-4} (M) solution of the complex with 1×10^{-2} (M) solution of o-aminophenol (OAP) in aerobic condition in DMF at 25°C. The course of the reaction is followed by time dependent wavelength scan. An absorption band at λ_{max} ~ 433 nm (ε = 7400 M^{-1} cm^{-1}) signature of phenoxazinone (APX) chromophore is monitored with time. The progress of the reaction is observed by time dependent wavelength scan for 30 min at 5 min interval of time.

The kinetic study of phenoxazinone synthase activity has been performed by adopting the same procedure as followed in catecholase activity.

*2.9. Phosphatase like activity and kinetics study of complex **1***

The phosphatase like activity of complex **1** is studied by monitoring hydrolysis of phosphate-ester bond of model substrate Disodium (4-nitrophenyl)phosphate (4-NPP) hexahydrate. The hydrolytic property of complex **1** is studied by monitoring the hydrolysis of a solution in 97.5%(v/v) DMF-H_2O containing 1×10^{-3} (M) 4-NPP and 5×10^{-5} (M) complex for 2 h with the help of UV-Vis spectroscopy at 25 °C. The course of the reaction has been observed by increment of a peak at λ_{max} ~ 425 nm characteristics of 4-nitrophenolate anion which is hydrolyzed product of 4-NPP.

The Kinetics study is performed in seven sets having a catalyst of 5×10^{-5} (M) concentration and substrate concentrations of 0.5 (10 equiv), 0.7 (14 equiv), 1.0 (20 equiv), 1.2 (24 equiv), 1.5 mmol (30 equiv), 1.7 mmol (34 equiv) and 2.0 mmol (40 equiv). The reactions were initiated by addition of 0.06 mL of metal complex (2.5×10^{-3} M) into 2.94 mL of a 4-NPP solution, and the spectrum is recorded only after complete mixing at 25 °C. The rates of reaction for various substrate concentrations are interpreted with the help of Michaelis-Menten approach and the kinetic parameters are calculated from Lineweaver-Burk plot.

3. Results and discussions.

*3.1. Synthesis of ligand and complex **1***

A single-side Mannich product 4-(*tert*-butyl)-2-((ethyl(2-hydroxyethyl)amino)methyl)phenol (**H_2L**) having O, N, O donor sites has been purposefully synthesized using 4-(*tert*-butyl)phenol (an activated phenol), 2-(ethylamino)ethanol (secondary amine), 37% (w/v) formalin solution as precursors. The method is similar as reported earlier.[50] H_2L has been characterized by FT-IR, UV-Vis, ^{1}H and ^{13}C NMR, and ESI-MS spectral studies (data are in experimental section, Fig. S1-S4). A tetranuclear copper(II) complex has been prepared by treating methanolic solution of copper(II) perchlorate hexahydrate with the methanolic solution of H_2L.

*3.2. FT-IR and UV-Vis spectra of complex **1***

The FT-IR spectrum of complex **1** contains a sharp band at 1606 cm^{-1} due to C-N stretching. The bands at 1495 cm^{-1} and 2960 cm^{-1} are observed due to skeletal vibration and tertiary amine respectively. The broad band at 1090 cm^{-1} is the signature of perchlorate ion (Fig. S5)[57]. The UV-Vis spectral study has been performed in DMF medium. The bands at λ_{max} = 421 nm (ε = 716 L mol^{-1} cm^{-1}) is observed due to ligand to metal charge transfer but its intensity is relatively lower than Schiff base analogue since tertiary amine is present in this case. The band at λ_{max} = 710 nm (ε = 170 L mol^{-1} cm^{-1}) is observed due to d-d transition (Fig. S6)[58]. Cu^{II} is a d^9 system and if it is in an octahedral coordination environment the electronic transition $^2E_g \rightarrow {}^2T_{2g}$ is expected to take place at around 800 nm and this band will undergo a significant blue shift on distortion of octahedral coordination to a square-pyramidal and square-planar geometry. Thus, coordination environment around copper centres is square-pyramidal or square-planar rather than octahedral.

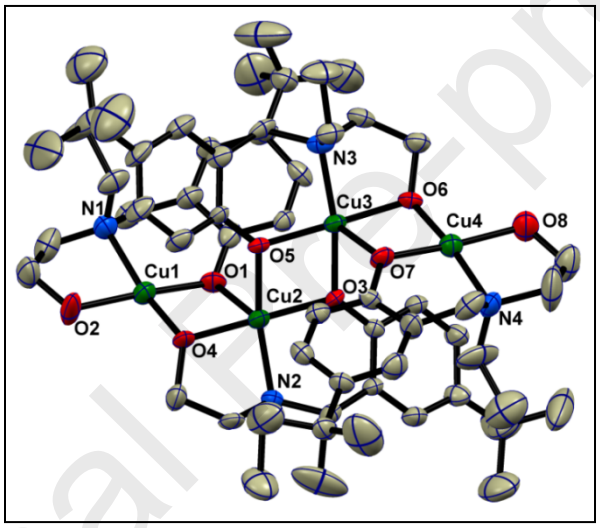
3.3. Structural description

The X-ray crystal structural analysis of complex **1** reveals that asymmetric unit contains one tetranuclear copper(II) di-cationic complex having a pseudo center of symmetry (Fig. 1a). The cationic unit is counterbalanced by two perchlorate anions and a lattice water molecule is present.

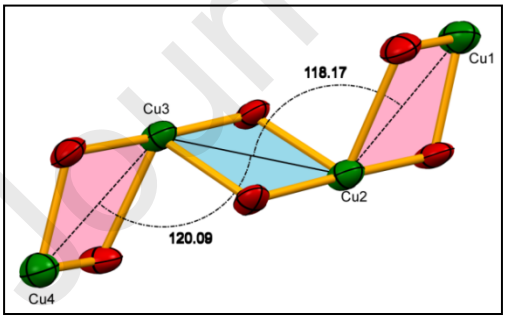
The complex shows a zig-zag chain of four copper atoms where Cu1/Cu2 and Cu3/Cu4 metal pairs are doubly bridged by an alkoxy and phenoxide oxygen atoms, while the central Cu2/Cu3 are doubly bridged by the participation of two phenoxide oxygen atoms to form three irregular Cu_2O_2 rhomboids (Fig. 1b). Of the four copper(II) atoms, Cu(1) and Cu(4) exhibit a square planar geometry, while Cu(2) and Cu(3) adopt a distorted square pyramidal geometry (Fig. 1c) as indicated by their trigonal index τ values[59] [τ for Cu(2) =0.29, Cu(3) = 0.22].

The coordination sphere of square planar copper centers is built by phenoxy oxygen, amine nitrogen, and the alcoholic oxygen atom of ligand **HL**, and the fourth site is occupied by deprotonated alkoxy oxygen atom of second **L**. On the other hand the square pyramidal positions in the base are occupied by the phenoxy oxygen, amine nitrogen, and the deprotonated alkoxy oxygen atom of **L**, and the alcoholic oxygen of nearby **HL**. A phenoxy oxygen is present at the apex.

The Cu-O bond distances in square planar and in square pyramidal base of all copper centers are close comparable as reported in Table S2, falling in a range 1.877(11)-1.996(10) Å. The Cu-N distances vary from 1.984(13) to 2.020(15) Å. The range of *cisoid* angles 77.9(4) - 101.2(4)° and those of *transoid* angles157.6(5) -176.1(5)° give indications of the distortion from ideal values. The axial Cu-phenoxide bond lengths are significantly longer, of 2.271(11)and 2.245(11)Å, as expected for Jahn-Teller effect. Finally the distance between pair of copper centers in the tetranuclear chain is 3.037(3) and 3.001(3), with an intermetallic angle averaging to 119°. All the coordination distances, although at lower accuracy, are comparable with those of analogous derivatives reported by others [22,60].



(a)



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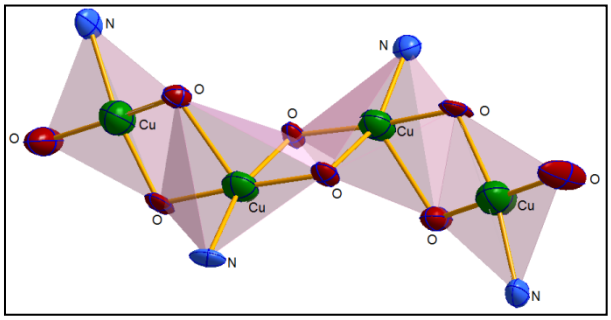
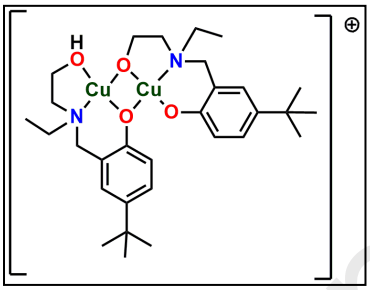


(c)

Fig. 1. (a) ORTEP view (30% probability) of complex **1** cation, H-atoms have been omitted (for clarity); (b) Zig-Zag chain of copper centers and (c) coordination geometry around two different Cu^{II} atoms

*3.4. ESI-MS study complex **1***

To get an idea of solution phase structure of complex **1** ESI-MS study is performed in positive mode using acetonitrile as solvent. The base peak observed at *m/z* = 625.2158 amu (Fig. S7) corroborates well with monocationic dinuclear species having molecular formula $C_{30}H_{47}Cu_2N_2O_4^+$ (calcd. *m/z* = 625.2128 amu). The dinuclear species (Fig. 2) is regarded as active species during all the catalytic activities.

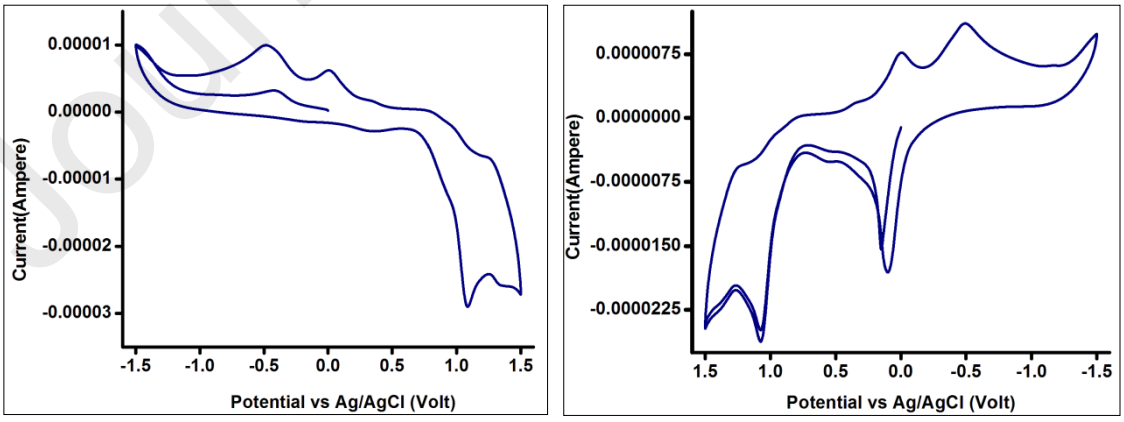


1b

Fig. 2. Probable composition of dinuclear species in solution as evidenced from ESI-MS study.

*3.5. Elecrochemical study of complex **1***

The cyclic voltammetry of complex **1** is carried out in DMF medium at a scan rate of 100 mV/sec. The Cu^{II}/Cu^{I} redox couple appeared at ~0.0 V (0.0078 volt) with respect to Ag/AgCl reference electrode and the cathodic peak at -0.48 V was attributed to Cu^{I}/Cu^{0} redox couple. The restricted scan from -0.6 volt to 0.6 volt resulted in a quasi-reversible redox couple as the observed E_{pc} = -0.422 volt with a ΔE_p value of 0.502 volt and i_{pc} / i_{pa} = 2.15 (Fig. 3).



(a) (b)

Fig. 3. Cyclic voltammogram of complex **1** in DMF medium during (a) cathodic scan and (b) anodic scan

3.6. EPR study

X-band EPR spectrum of complex **1** is recorded in DMF medium at room temperature. The spectrum consists of hyperfine lines which are due to ^{63}Cu and ^{65}Cu in the highfield (3/2) transition as is depicted in Fig. 4. It exhibited signals at $g_{\perp} = 2.07$ and the values of $g_{\parallel}$ are 2.12, 2.17, 2.21. The greater $g_{\parallel}$ value as compare to $g_{\perp}$ suggests that the unpaired electron is present at $d_{x^2-y^2}$ orbital. The spectral pattern reveals that metal center in solution phase adopts square planar geometry as is presented in Fig. 2 on the basis of ESI-MS study [61,62].

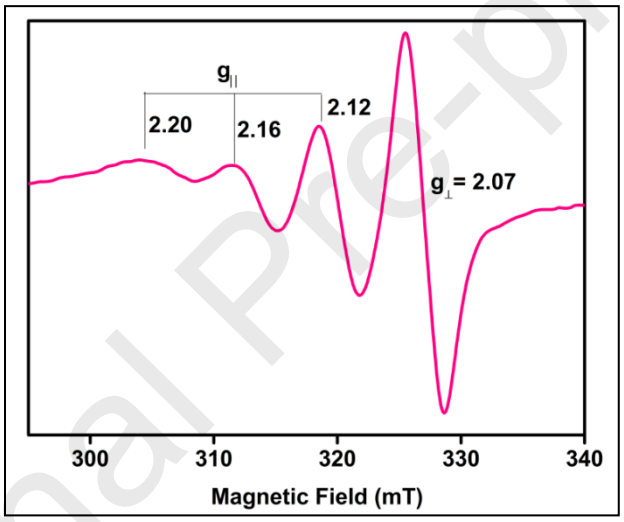
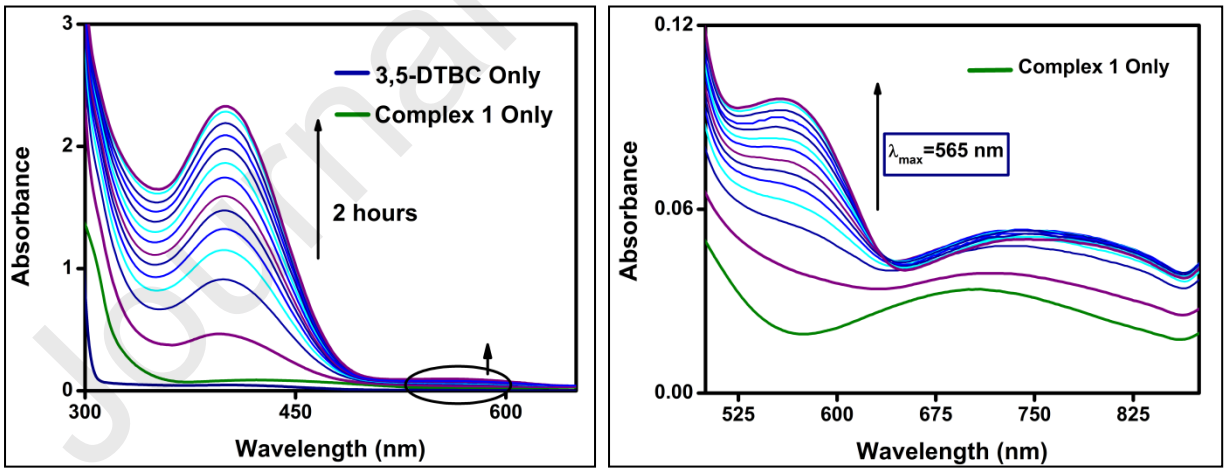


Fig. 4. X-band EPR spectrum of complex **1** at 298 K in DMF medium.

3.7. Catecholase activity

From the above discussion it is clear that complex **1** is the catalyst precursor and the dinuclear obtained on dissolution of **1**, most probably a square planar dinuclear Cu^{II} complex is the active catalyst. The catalytic activity of **1** towards the oxidation of 3,5-DTBC, a model substrate generally used to investigate the catecholase activity has been monitored by time dependent UV-Vis spectral scan in DMF medium. The change in spectral pattern of complex **1** in presence of 3,5-DTBC has been depicted in Figure 5. The peak at $\lambda_{max} \sim 400$ nm characteristics of 3,5-DTBQ ($\varepsilon = 1900$ L mol^{-1} cm^{-1}) increases with time which proves that complex **1** is a potential catalyst

for the oxidation of 3,5-DTBC. It is generally demonstrated by nearly all the investigators working on modeling of catechol oxidase that during catalysis by dinuclear copper(II) complexes the substrate catechol interact with the copper center(s). Then two electron reduction of the two copper centers occurred to generate copper(I) species with concomitant oxidation of catechol to quinone [63]. The copper(II) species is re-generated by reaction of oxygen with copper(I) species. Now two situations may be visualized in UV-Vis spectral study: (i) disappearance of d-d band indicating the formation of Cu^{I} with fast electron transfer and the re-oxidation by oxygen of the formed copper(I) complex which is often the rate-determining step; (ii) the d-d band remains intact or a blue or red shifting, indicating intramolecular electron transfer should be the rate-determining step[64]. In case of complex **1** d-d band undergoes red shift to λ_{max} ~ 752 nm upon addition of 3,5-DTBC. Therefore it may be presumed that intramolecular electron transfer is likely to be the rate-determining step. A striking feature has been noticed along with the above spectral changes is the gradual increase of a new band at λ_{max} ~ 565 nm. The previous reports suggest that the increment of peak at λ_{max} ~ 565 nm is the signature of phenoxyl radical formation [32, 65-67]. Therefore, this particular band makes us curious to find out the origin of the catalysis: is there any possibility of radical pathway in copper based model system in catechol oxidation?



(a) (b)

Fig. 5. (a) Oxidation of 3, 5-DTBC by species **1b** (substrate: catalyst = 100:1) observed in UV-Vis spectroscopy with time in DMF medium at 25 °C, (b) change in d-d band of species **1b** with oxidation of 3, 5-DTBC.

Before to address this intriguing issue, it is essential to know the fate of dioxygen in the catalytic cycle. During the re-generation of catalyst dioxygen may be converted to either water (4 electrons are involved) or hydrogen peroxide (2 electrons are involved). Spectroscopic study in presence of the catalyst, KI and catechol (3,5-DTBC) detects I_3^-, which is a clear indication of H_2O_2 formation. The band at λ_{max} = 356 nm is observed due to generation of I_3^- and the plot of absorption maxima at 356 nm at different time interval is presented in Fig. S8. Therefore a radical pathway is most probably involved in this catalysis.

A controlled experiment has been carried out without using catalyst *i.e.* species **1b** and the absorbance of the characteristics band of 3,5-DTBQ around 400 nm is not observed to increase with time (Fig. S9).

3.8. ESI-MS study

In order to identify the intermediates formed during the course of catecholase activity ESI-MS study was carried out in positive mode. A small amount of reaction mixture was taken and diluted with acetonitrile to record the mass spectrum. The base peak at *m/z* = 866.7215 amu corresponds to 1:1 adduct of dinuclear species and 3,5- DTBC which is consistent with molecular formula $C_{44}H_{69}Cu_2N_2O_6^+ + H_2O$ (Calcd. *m/z* = 867.1357 amu). The peak at *m/z* = 624.0012 amu corresponds to dinuclear entity of complex **1**. The peak at *m/z* = 243.1362 amu represents the sodium adduct of 3,5-DTBQ (Calcd. *m/z* = 243.1361 amu) (Fig. S10).

3.9. Cyclic voltammetry Study

Cyclic voltammetry study was performed to monitor the redox reaction occurring during the oxidation of catechol catalyzed by complex **1**. Extensive analysis of cyclic voltammogram of species **1b** in presence of 3,5-DTBC showed that a new cathodic peak was generated at -0.06 V and a new anodic peak at 0.5 V. Cathodic peak at -0.06 V can be tentatively assigned to the reduction of free 3,5-DTBQ to copper(II) bound deprotonated 3,5-DTBC and the anodic peak at 0.5 V is due to oxidation of 3,5-DTBC to free 3,5-DTBQ (Fig. 6) [18].

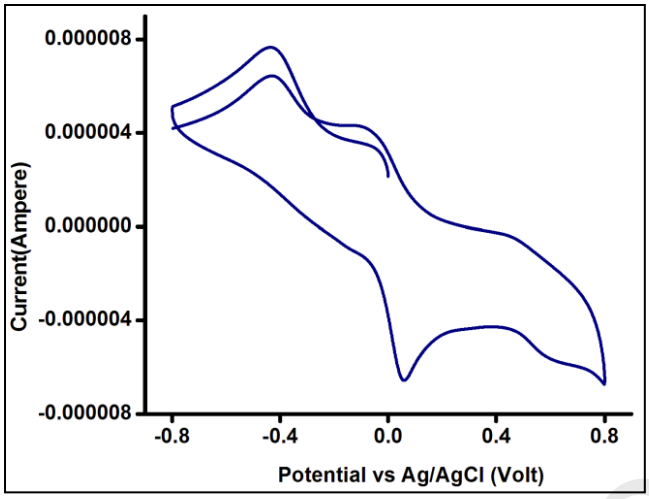


Fig. 6. Cyclic voltammogram of reaction mixture of complex **1** and 3,5-DTBC in DMF medium.

3.10. EPR study

Finally to confirm the formation of organic radical generated during catalytic cycle X-band EPR study has been carried out at 298 K. When 3,5-DTBC is added to the solution of complex **1** a new sharp peak is observed to originate at g = 2.05 as a signature of organic radical formation with the retention of the peaks observed for complex **1** only (Fig. 7). This observation becomes a guiding factor to establish a new finding in catecholase activity study catalyzed by dicopper(II) model system *i.e.* feasibility of radical pathway. Now as far as the nature of the radical is concerned there is two possibilities, either arene anion radical or phenoxyl radical [31,32]. On the basis of the EPR spectral pattern and the electronic spectral band at ~565 nm (*vide supra*) it may be stated that the radical is phenoxy radical and that very proposition has been authenticated by DFT calculations (*vide infra*).

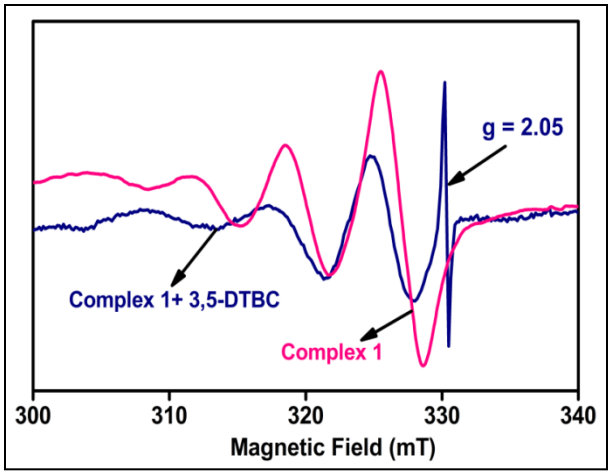
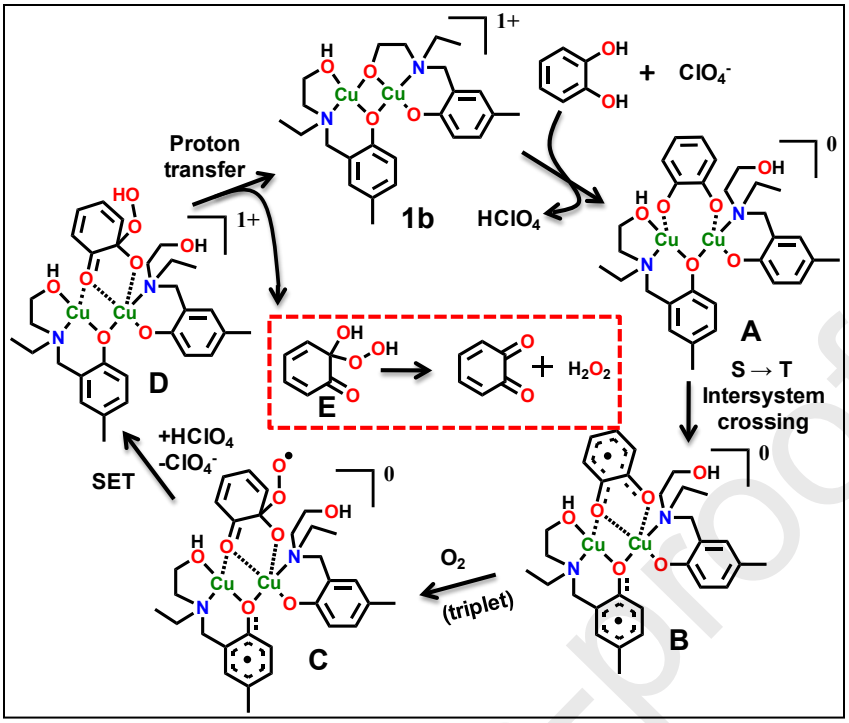


Fig. 7. X-band EPR spectrum of reaction mixture of complex **1** and 3,5-DTBC in DMF medium at 298 K.

3.11. Mechanistic pathway of catecholase activity and DFT study

In case of catecholase activity, metal centre redox participation likely to be occurred when redox active metal centre(s) such as copper(II), manganese(III) is present in the active site of bio-mimicking models as observed from earlier reports. Based on the experimental findings such as; i) the UV-Vis band corresponding to the d-d transition of Cu^{II} (d^9) at 752 nm remained intact, indicating that Cu^{II} (d^9) → Cu^{I} (d^{10}) reduction do not occur during the course of catalytic cycle; ii) observation of phenoxy radical in the UV-Vis (565 nm) and EPR spectral studies during catalysis and iii) production of hydrogen peroxide in the reaction. Hence it is evident that phenoxyl radical is formed during oxidation of 3,5-DTBC and thus ruling out the probability of redox participation. These unusual experimental findings intrigued us to perform DFT calculations. We have envisioned the catalytic cycle, as represented in Scheme 2, based on the foregoing experimental observations for our DFT calculations.



Scheme 2. Plausible catalytic cycle of catecholase activity catalyzed by active species **1b**.

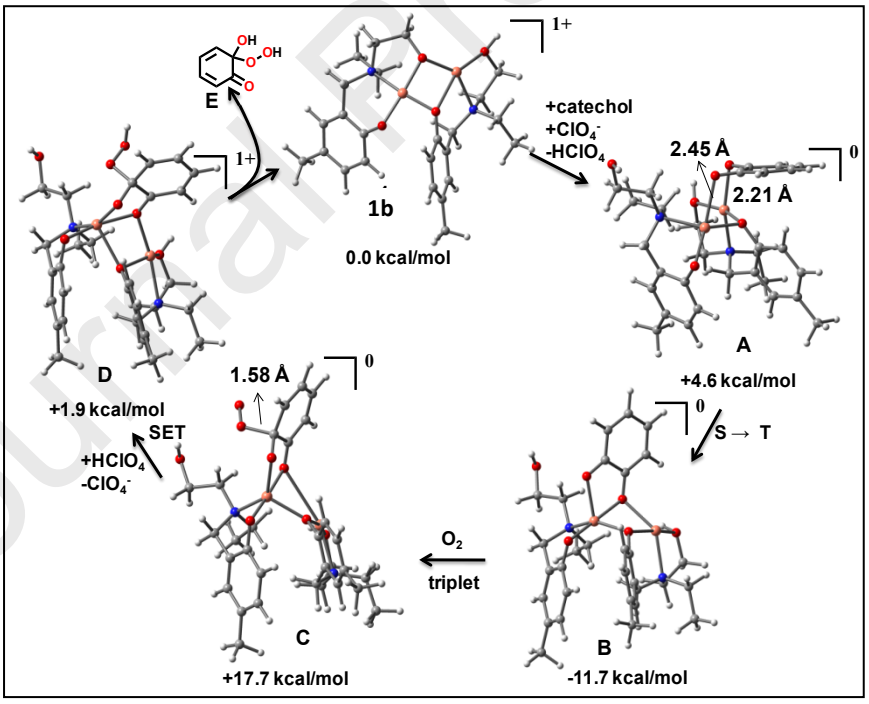


Fig. 8. M06-2X optimized geometries for the intermediates of catalytic catechol oxidation by the dimeric species **1b**.

The optimized structures of the intermediates A, B, C and D have been presented in Fig. 8. Density functional theoretical computations have been performed at M06-2X/6-31G* level to compute Gibbs free energy of the intermediates for the evaluation of reaction feasibility at room temperature. In the first step (**1b→A**) of the catalytic cycle, one molecule of catechol binds to the dinuclear complex (S_0) through bridging fashion with concomitant deprotonation of catechol. The process is energetically facile as the catechol addition raises free energy of the system by 4.6 kcal/mol only. In the intermediate **A**, Cu^{II}-O (catechol) bond distances are 2.21 and 2.45 Å. In the next step, **A** undergoes spin flipping from singlet to triplet state **B** and this intersystem crossing is energetically favorable by 16.3 kcal/mol. The frontier molecular orbital analysis (FMO) of the triplet state displayed two singly occupied molecular orbitals (SOMOs), one situated on the ligand (-6.37 eV) and the other one on catechol moiety (-6.67 eV) (Fig. 9). At this juncture, the catechol moiety can covalently combine with dioxygen in its triplet state to produce intermediate **C** and the process raises the system's energy to 17.7 kcal/mol. The C-O (O_2) bond distance is 1.58 Å. The FMO analysis of **C** revealed that the two SOMOs are situated on the ligand with -6.68 eV and -6.36 eV of energy whereas the LUMO is located over catechol and dioxygen units (Fig. 9). Therefore, a single electron transfer (SET) process can occur from the ligand to the dioxygen unit accompanied by a proton transfer process yielding intermediate **D**. The transformation is thermodynamically favourable as it brings down the system's energy to 1.9 kcal/mol. In the last step, catechol fragment (**E**) release occurs easily to complete the catalytic cycle. The intermediate **E** eventually produces benzoquinone and hydrogen peroxide.

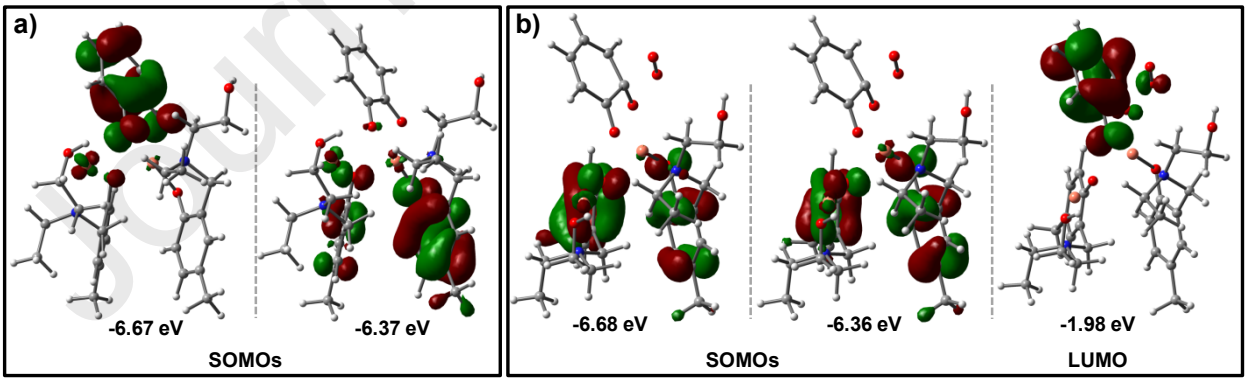


Fig. 9. Frontier molecular orbital plots for intermediate a) **B** and b) **C**.

3.12. Phenoxazinone Synthase Activity

Phenoxazinone synthase activity of complex **1** has been monitored using ortho-Aminophenol (OAP) as model substrate in DMF medium under aerobic condition. The course of the reaction is monitored by time dependent UV-Vis spectroscopy at 25 °C. The broad peak at λ_{max} = 422 nm, signature of phenoxazinone (APX) increases with time (Fig. 10), indicating that complex **1** is a potential catalyst for the oxidation of ortho-aminophenol. The changes noticed in the d-d bands which have been shown in Fig. 10 during the course of the reaction are noteworthy.

During oxidation of ortho-aminophenol the d-d band of complex **1** is disappeared immediately after the start of oxidation. A plateau like nature of d-d band is observed in the region of 664 nm but the enhancement of absorbance value is not appreciable. This phenomenon indicates that the copper(II) centre is like to underwent reduction to copper(I).

In this case a controlled experiment is also carried out without using catalyst and the characteristics band of APX around λ_{max} ~ 422 nm was not found to increase with time (Fig. S11).

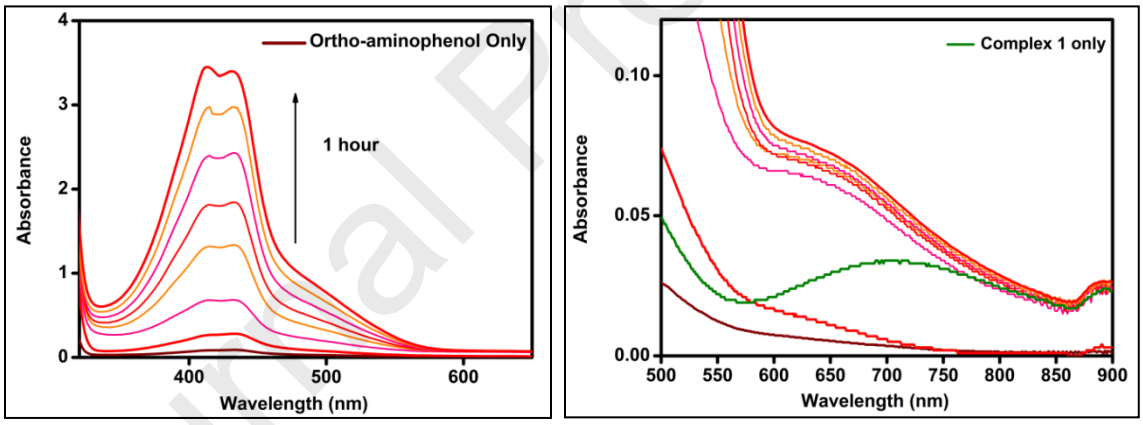


Fig. 10. Oxidation of ortho-aminophenol catalysed by complex **1** (substrate: catalyst = 100:1) observed in UV-Vis spectroscopy with time in DMF medium at 25 °C. (b) Change in d-d band of complex **1** with oxidation of ortho-aminophenol.

3.13. ESI-MS Study

In order to detect the composition of the intermediate forming during phenoxazinone synthase activity ESI-MS study has been performed in positive mode. A very small amount of reaction mixture has been taken and diluted with acetonitrile. The peak present at *m/z* = 625.2156 amu is

signature of a monocationic dinuclear species as discussed earlier. The base peak is present at *m/z* = 843.1345 amu which corroborated with the molecular formula [$C_{42}H_{60}Cu_2N_4O_6$ + H^+] and is consistent with 1:2 adduct between dinuclear species and ortho-aminophenol. The peak at *m/z* = 213.2041amu is observed due to formation of phenoxazinone, the peak being indicative of H adduct to phenoxazinone (Calcd. *m/z* = 213.2121amu)(Fig. S12).

3.14. EPR study

X-band EPR study has been performed to detect the intermediate formed during catalytic cycle. Complex **1** contains four line EPR signal. When ortho-aminophenol was added to complex **1** in DMF medium an isotropic signal at g = 2.12 (Fig. 9) appears which is attributed to formation of organic radical during catalytic cycle. The characteristics peak of complex **1** disappears upon addition of OAP thus suggesting that metal center may undergo reduction. Previous reports suggest that when redox active metal centre is present in the active site metal centered reduction took place with generation of radical although ligand centered radical formation is another alternative of metal centered redox participation during catalysis[68,69].

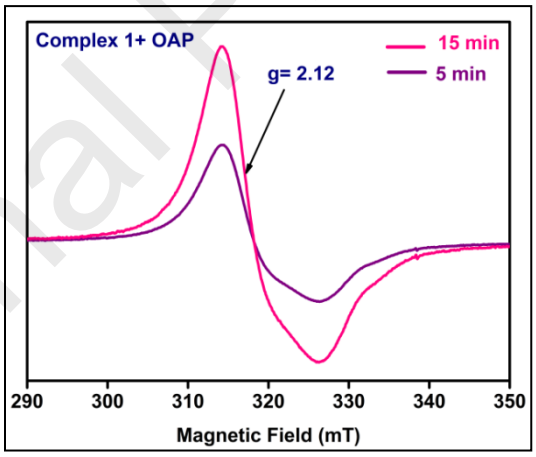


Fig. 11. X-band EPR spectrum of reaction mixture of complex **1** and ortho-aminophenol in DMF medium at 298 K.

3.15. Cyclic voltammetry study

For complete assessment of the redox reaction occurring during catalysis, cyclic voltammetry of the dinuclear copper species **1b** in presence of ortho-amino phenol has been performed in DMF medium at 100 mV/sec scan rate. The cyclic voltammogram of the reaction mixture consists of

two adjacent cathodic peaks at E_{pc} = 0.11 V and E_{pc} = -0.18 V, which may be attributed to reduction of Cu^{II} to Cu^{I} during the catalytic cycle (Fig. 10).

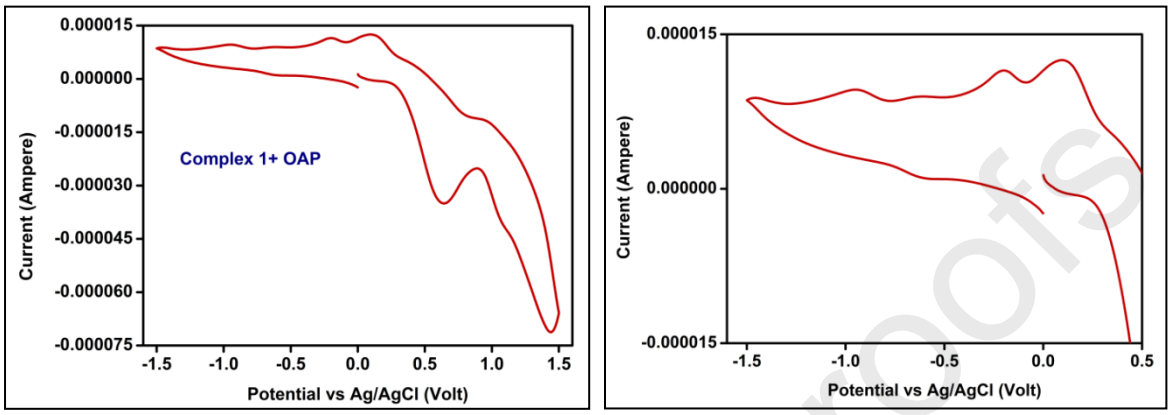
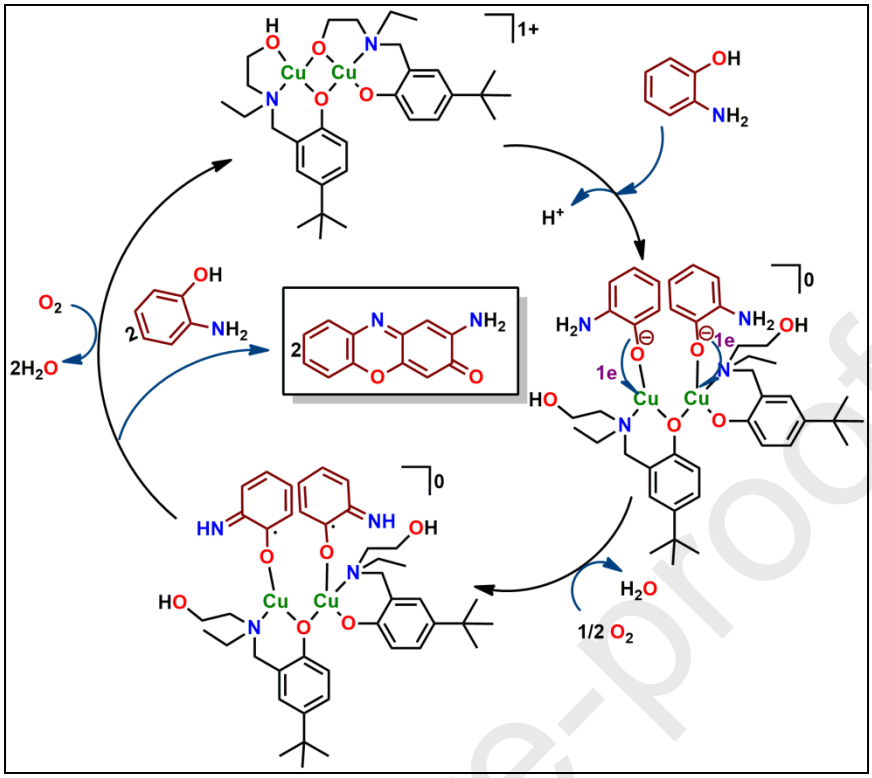


Fig. 12. Cyclic voltammogram of reaction mixture of complex **1** and ortho-aminophenol in DMF medium

3.16. Mechanistic pathway of Phenoxazinone Synthase activity

On combining the experimental results obtained from ESI-MS spectrum, EPR spectrum and cyclic voltammogram of reaction mixture of complex **1** and OAP a mechanistic pathway has been proposed. The catalytic cycle is initiated by the formation of 1:2 adduct between complex **1** and ortho-aminophenol. In the next step there occurs the formation of an organic radical located on the ortho-aminophenol moiety with simultaneous reduction of copper(II) centre. In the next step ortho-aminophenol is converted to phenoxazinone (APX) in presence of aerial oxygen. The plausible mechanistic pathway of phenoxazinone synthase activity catalyzed by complex **1** is presented in Scheme 3.



Scheme 3. Plausible catalytic cycle of phenoxazinone synthase activity catalyzed by species **1b**.

3.17. Phosphatase Like Activity

To explore the hydrolytic property of complex **1**, phosphatase like activity is studied in 97.5 % (v/v) DMF-H_2O mixture using disodium salt of (4-nitrophenyl)phosphate(4-NPP)hexahydrate (4-NPP) as the model substrate and progress of the reaction has been monitored by time dependent UV-Vis spectral scan at 25 °C. The complex **1** is capable to catalyze the hydrolysis of the phosphate-ester bond of the substrate as is evident from the time dependent UV-Vis spectral study. The spectrum of reaction mixture shows an increment of peak at λ_{max} ~ 424 nm with time, characteristics of 4-nitrophenolate ion (Fig. 11).

A controlled experiment has been carried out with copper(II) perchlorate hexahydrate and ligand **H_2L** and in both the cases hydrolysis of phosphate ester bond did not occur (Fig. S11).

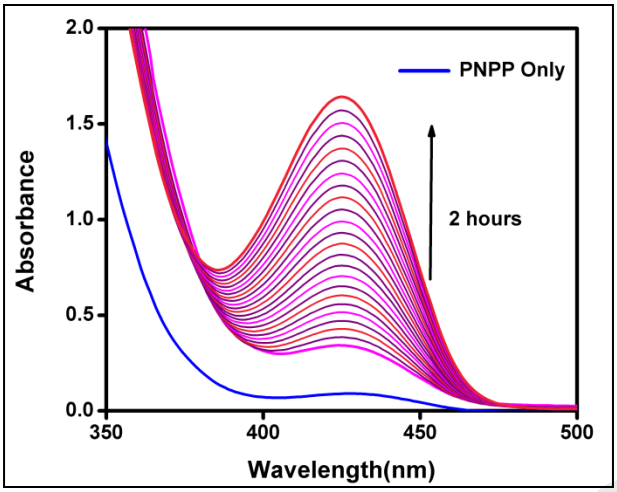


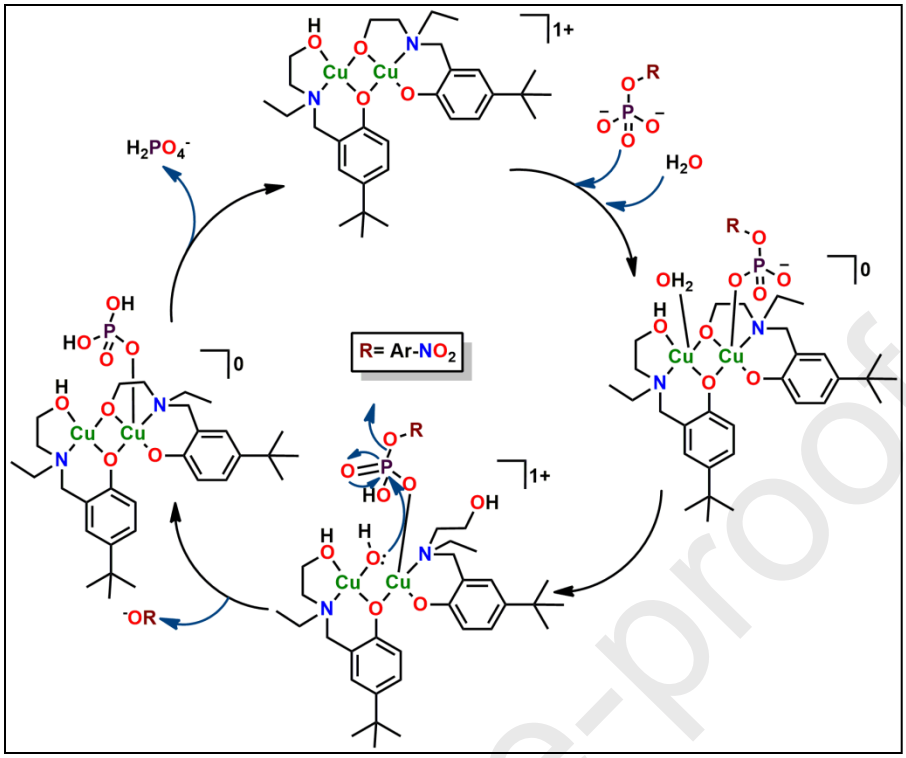
Fig. 13. Hydrolysis of 4-NPP catalyzed by complex **1** (substrate: catalyst = 20:1) in 97.5% (v/v) DMF-H_2O observed in UV-Vis spectroscopy with time at 25 °C at intervals of 5 min.

3.18. ESI-MS Study

A very small amount of reaction mixture has been taken and diluted with acetonitrile and ESI-MS spectrum was recorded in positive mode. The base peak is observed at *m/z* = 667.1119 amu which corroborates exactly with the molecular formula $C_{30}H_{47}Cu_2N_2O_4$+ CH_3CN (Calcd. *m/z* = 666.8491 amu) *i.e.* it represents the acetonilrile adduct of dinuclear entity. The peak at *m/z* = 862.1069 amu (Calcd. *m/z* = 862.2171 amu) corresponds to hydroxo bridged dinuclear species having molecular formula $[C_{36}H_{54}Cu_2N_3O_{11}P](2H^+)$ (Fig. S14) and this is supposed to be the key intermediate in P-O bond hydrolysis reaction[43].

3.19. Mechanistic pathway of Phosphatase activity

The catalytic cycle is likely to be initiated by attack of water and 4-nitrophenylphosphate (4-NPP) followed by the conversion of water molecule to hydroxo species. In the next step nucleophilic attack of hydroxo to the phosphorus centre takes place and this step is believed to be the rate determining step.[70] Actually, this step involves the formation of dihydrogen phosphate species with the concomitant liberation of 4-nitrophenolate ion. In the last step dihydrogenphosphate is released from dinuclear copper center with regeneration of the catalyst, **1b**.



Scheme 4. Plausible catalytic cycle of phosphatase activity catalyzed by dinuclear species.

3.20. Kinetics Study of complex 1 for all three catalytic activity

The kinetic studies of complex **1** for catecholase activity, phenoxazinone synthase activity and phosphatase have been performed to quantify the catalytic efficacy of the newly synthesized complex. Michaelis-Menten approach is found to be the best because the complex showed saturation kinetics and the kinetic parameters were calculated from Lineweaver Burk plot (Fig. S15-S17). The k_{cat}/ K_M value dictates the catalytic efficiency of the enzyme and it is observed that in case phosphatase activity k_{cat}/K_M value is the highest among the all three activity. The kinetic parameters of complex **1** are presented in Table 1.

Table 1

Kinetic parameters of complex **1** for various catalytic activity

	V_{max} (M s^{-1})	K_M (M)	k_{cat}	k_{cat}/K_M (M^{-1} s^{-1})	S.D	R^2
Catecholase activity	6.44×10^{-7}	2.38×10^{-3}	23 h^{-1}	2.70	0.153	0.999
Phenoxazinone synthase activity	1.07×10^{-6}	3.55×10^{-3}	38.52 h^{-1}	3.01	0.988	0.981
Phosphatase activity	2.66×10^{-4}	8.58×10^{-4}	5.22 s^{-1}	6.08×10^{3}	1.040	0.995

The rate constant values of some catalytic promiscuous homometallic and heterometallic complexes are presented in Table S3. On considering the k_{cat} value of catecholase activity with the previously reported complexes it may be stated that complex **1** is moderately active catalyst but the mechanistic pathway of **1** makes it unique from the reported catalysts. It is also capable in catalyzing the oxidation of ortho-aminophenol with moderate efficiency. The species show appreciable hydrolyzing property, only fewer zinc(II) based models showed higher activity than this newly synthesized species. Hence, it may be concluded that the newly synthesized complex **1** is a unique species having the capability to catalyze oxidation of catechol, oxidation of ortho amino phenol and hydrolysis of phosphate monoester dianion.

4. Conclusions

Flexible ligand design by utilizing Mannich base ligand synthesis becomes fruitful in making polynuclear complex of our interest. An exciting catalytic promiscuity has been demonstrated in the present report where our synthesized tetranuclear Cu^{II} complex is observed to act as catalyst precursor. The dinuclear species, which has been originated from the catalyst precursor in solution, is actually the active catalyst. **1** has shown its potential as hydrolytic catalyst with high efficiency to catalyze P-O bond hydrolysis and thereby acts as a functional model of phosphatase enzyme. Activity of **1** as redox catalyst to catalyze phenoxazinone synthases is moderate. On the other hand, **1** becomes a unique member in the model study of catechol oxidase. A completely new finding of radical pathway in copper based models, rather than copper centered redox participation has been explored by combined experimental and theoretical investigation.

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Highlights

Catalytic promiscuity of a copper(II)-Mannich base complex having unprecedented radical pathway in catecholase activity

- A flexible single-side Mannich base ligand (**H_2L**) has been synthesized with the view to prepare a tetranuclear copper (II) complex, **$[Cu_4(L)_2(HL)_2]\cdot(ClO_4)_2\cdot H_2O$** (**1**)
- Complex **1** exhibits excellent catalytic activities like catecholase, phenoxazinone synthase and phosphatase activities.
- Complex **1** catalyses the aerobic oxidation of 3,5-DTBC not by well established metal centered redox participation, rather via a ligand centered radical pathway.
- It is a completely new finding in copper(II) based catechol oxidase modeling.

[73]