Synthesis, characterization and peroxidase activity of binuclear complexes of 2,6-bis(2-hydroxybenzylidene hydrazone)-4-methyl phenol

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The homo- and heterobinuclear complexes of Fe(II), Ni(II) and Cu(II) with 2,6-bis(2-hydroxybenzylidene hydrazone)-4-methyl phenol have been synthesized by template and non-template condensation methods. The probable structures of the complexes having general formula [MM'LCI(H₂O)₄]ₙH₂O (where M=M'=Fe, Ni or Cu; M = Fe, M' = Ni or Cu; H₃L = ligand; n = 0, 1, 2 or 4) have been proposed on the basis of elemental analyses, molecular weight determination, conductance measurements, magnetic moment, ESR and spectroscopic data. From spectroscopic studies, it has been concluded that coordination occurs through phenolic oxygen after deprotonation, nitrogen of azomethine and chlorine atom; the two metals being bridged through oxygen and chlorine atom. Of the three binucleating complexes of iron namely, (Fe, Fe), (Fe, Ni) and (Fe, Cu) tested for peroxidase activity, only (Fe, Cu) complex is found to be active.

A number of studies have been reported on the synthesis and characterization of macrocyclic and macroacyclic (I, II) homo- and heterobinuclear complexes of d and f metal ions with Schiff bases derived from the condensation of 2,6-diformyl-4-methyl phenol with ω,ω-diamines or other mono and bifunctional amines⁴⁻⁵. These complexes have been synthesized adopting non-template, direct template or transmetallation procedures⁶⁻⁷. A number of transition or inner transition metals have been incorporated onto the macrocycles/acycles (I, II) and there structural aspects have been well investigated, but little attention has been paid to the application of these compounds in different areas. To the best of our knowledge, no studies have been reported on synthetic homo- and hetero-binucleating models of peroxidase-like activity. Keeping this view in mind, the present paper is concerned with synthesis, characterization and peroxidase activity of a new series of novel binucleating complexes via condensation reaction of 2-hydroxybenzylidene hydrazone and 2,6-diformyl-4-methyl phenol in the presence of transition metal ions.

Materials and Methods

The ligand and all the complexes (homo- and heterobinuclear) were analysed for C, H, N on an automatic elemental analyzer, model 1600. ¹H NMR spectrum of the ligand was recorded on Varian EM 390 (90 MHz) spectrometer in CDCl₃ and two drops of DMSO-d₆ using TMS as an internal standard. Infrared spectra were recorded as KBr pellets on Biorad FTS-7 infrared spectrophotometer. Room temperature magnetic susceptibilities were determined using Magnetic Susceptibility Balance (Sherwood Scientific). The metals of the bimetallic complexes were determined with an Atomic Absorption Spectrophotometer, Varian Techtron, model AA-120. Electronic spectra of the metal complexes were recorded in DMSO or as Nujol mulls on Beckman (DU-64) spectrophotometer. Conductivity measurements were made on freshly prepared 10⁻³ M solutions in dry DMSO with a Digital
Conductivity Meter, model NDS 732. Molecular weights of the complexes were determined by cryoscopic method in dry nitrobenzene. Modified procedure of Shannon et. al was used for the assay of peroxidase activity. The assay mixture contained o-dianisidine (2.4 μmol), H₂O₂ (20 μmol), salt solution (0.05 mg/ml) and phosphate buffer (pH 6.0, 0.05 M) to make the volume 4 ml. Omission of H₂O₂ from the incubation mixture served as control. The assay mixture was preincubated at 37°C for 5 min prior to the addition of H₂O₂. The activity was measured at 470 nm in terms of OD measurements at an interval of 15 seconds.

Synthesis of ligand
The ligand was synthesized via two steps:

**Synthesis of 2-hydroxybenzylidene hydrazone**
2-Hydroxybenzylidene hydrazone was prepared by the condensation of 2-hydroxybenzaldehyde with hydrazine hydrate in ethanolic medium. The solid product obtained after usual workup was crystallized from ethanol. IR (νmax in cm⁻¹): 3330 (OH), 3240 (NH₂), 1625 (C=N) and 970 (N-N).

**Synthesis of 2,6-bis(2-hydroxybenzylidene hydrazone)-4-methyl phenol (H₃L)**
2,6-Diformyl-4-methyl phenol was prepared by the reported method. The title ligand was synthesized by heating 2-hydroxy benzylidene hydrazone with 2,6-diformyl-4-methyl phenol under reflux in ethanol (150 ml) for 2h. The light yellow microcrystalline product obtained on cooling was recrystallized from methanol. The elemental analysis is given in Table 1.

**Synthesis of complexes**
The homo- and heterobinuclear complexes of the ligand were synthesized by two different methods:

**Non-template synthesis of homobimetallic complexes**
All the complexes were synthesized by mixing metal chloride hydrate with the solution of the ligand (H₃L) in ethanol in 2:1 molar ratio. The solution was refluxed for 2h under dry nitrogen atmosphere. The solid thus obtained was filtered, washed with ethanol and dried in vacuo. The complexes obtained by non-template synthesis were of low yield (30-40%).

**Template synthesis of homobimetallic complexes**
A mixture of 2,6-diformyl-4-methyl phenol, 2-hydroxybenzylidene hydrazone and appropriate metal salt in 1:2:2 molar ratio suspended in ethanol was refluxed for 2 h under dry nitrogen atmosphere to yield homonuclear complexes.

**Template synthesis of heterobimetallic complexes**
Heteronuclear complexes were obtained by the similar procedure from 2,6-diformyl-4-methyl phenol, 2-hydroxybenzylidene hydrazone, iron(II) chloride tetrahydrate and MClₓ·nH₂O (M = Ni or Cu; n = 6 or 4) in 1:2:1:1 molar ratio. The final product was isolated in high yields (70-80%).

**Results and Discussion**
The absence of absorption bands at 1680 cm⁻¹ due to νC=O of aldehydic group of 2,6-diformyl-4-methyl phenol and a band at 3240 cm⁻¹ due to νNH₂ along with appearance of new band at 1630 cm⁻¹ (νC=N) in the IR spectrum of the ligand, H₃L confirmed that condensation had taken place. In the spectrum of the ligand a broad band centered at 3350 cm⁻¹ was due to ν(0-1-1). The broadness of this band suggested the presence of hydrogen bonds between azomethine nitrogen and the OH group. Further, a weak band at 2790 cm⁻¹ was attributed to the intramolecular hydrogen bonded OH group.

It was further confirmed by ¹H NMR spectrum of the ligand where singlet at 10.2 ppm due to HC=N proton was observed. A broad singlet at 11.4 ppm was due to phenolic hydroxyl group (exchangeable with D₂O), while a singlet at 2.4 ppm was attributed to methyl group attached to aromatic ring. A multiplet centered at 7.7 ppm was due to aromatic under dry nitrogen atmosphere. The solid compounds were filtered, washed with ethanol and dried in vacuo.
protons. The integrated proton ratio further corroborated the proposed structure of the ligand $H_2L$.

Two different synthetic procedures adopted for the formation of complexes (template and non-template) yielded the complexes with general formula $[MM'LCI(H_2O)_n]_2$ where $M=M'=Fe, Ni$ or $Cu$; $H_2L$ = ligand $n = 0, 1, 2$ or $4$ as evidenced by elemental (C, H, N), metal analyses, molecular weight determination. (Table 1), conductance measurements, magnetic moment and IR and electronic spectra. All these complexes were quite stable in solid state under atmospheric conditions. The complexes were insoluble in most of the organic solvents except in DMSO, DMF or nitrobenzene. Molar conductance values of these complexes (15-30 $\Omega^{-1}cm^2$mol$^{-1}$) in dry DMSO revealed non-electrolytic (triply deprotonated) nature of the complexes supporting thereby the presence of one chlorine atom in the coordination sphere.

The absorption band due to $v$(O-H) was found to be completely absent in the complexes suggesting thereby the involvement of all the three phenolic hydroxyl groups, after deprotonation, in coordination. Moreover, the phenolic C-O stretching and bending vibrations, at 1530 and 1280 cm$^{-1}$, respectively, as expected for an uncoordinated phenol, were found to be shifted to higher frequency by 20-30 cm$^{-1}$. This upward displacement of the bands suggested the involvement of phenoxy bridging$^{10}$.$^1$. It was further confirmed by the appearance of new bands at 710-740 cm$^{-1}$ due to $M-O-M$ vibrations$^{11}$. The shift of these bands may be speculated to be due to the increase in C-O bond strength for extended delocalization of the $\pi$-system of azine moiety. The complexes showed negative spectral shift in $v$(C-N) mode from 1630 in the free ligand to 1580-75 cm$^{-1}$ indicating the participation of nitrogen atom of azomethine group in coordination. It was further confirmed by the shifting of $v$(N-N) mode from 1070 in the ligand to 1045-40 cm$^{-1}$ in the complexes. The water molecules present in the complexes, indicated by the broad adsorption band at 3450 cm$^{-1}$, have probably lattice and/or coordination water. In the far infrared region, the bands at 430-445, 360-380 and 310-355 cm$^{-1}$ were assigned to $v$-M-O, $v$-M-N and $v$-M-Cl, respectively.$^9$

The magnetic susceptibility determined for homobinuclear complex of Fe(II) obtained by both the methods (template and non-template) revealed high spin nature of the complex. Iron (II) complex had a magnetic moment of 5.13 B.M. slightly lower than spin-only value which is due to very weak spin-spin coupling. The nickel(II) complexes obtained by template and non-template synthesis exhibited effective magnetic moment values of 3.1 and 3.2 B.M., respectively at room temperature indicating spin-exchange interactions between two $S = 1$ states. The effective magnetic moment of copper (II) complexes was found to be $\sim 1.83$ B.M., which corresponded to a single unpaired electron with significant orbital contribution.

The heterobinuclear complexes viz. (Fe, Ni) and (Fe, Cu) exhibited $\mu_{eff}$ equal to 2.7 and 2.9 B.M., respectively. A definite inference could not be deduced for lack of magnetic moment values at variable temperatures.

In the electronic spectra of diiron(II) complex, two absorptions observed at 18382 and 15698 cm$^{-1}$ were assigned to $5E_1 - 5E_2$ and $5E_1 - 5A_1$ transitions, respectively. The electronic spectrum of dinickel(II) complex exhibited four $d-d$ transitions at ~8800, 11450, 15900 and 27100 cm$^{-1}$. The first two bands appeared to be the split of the $3B_{1g}$ and $3B_{2g}$ transitions and may be assigned as $3B_{1g} - 3T_{1g}$ and $3B_{1g} - 3B_{2g}$ while rest of the two transitions were due to $3A_{2g} - 3T_{1g}$ and $3A_{2g} - 3T_{1g}$ respectively with probably octahedral geometry. The electronic spectrum of the dicopper(II) complex exhibited a broad $d-d$ transition

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mol. form.</th>
<th>m.p./ decomp. temp (°C)</th>
<th>Mol. Wt. Obs.(Calc.)</th>
<th>Found (Calcd.), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_2L$</td>
<td>$C_3H_5N_2O_3$</td>
<td>125</td>
<td>400 (405)</td>
<td>69.0 (68.2)</td>
</tr>
<tr>
<td>[Fe$_2$LCI(H$_2$O)$_4$.2H$_2$O]</td>
<td>$C_3H_5N_2O_3ClFe_2$</td>
<td>198</td>
<td>652.2 (644)</td>
<td>42.3 (43.1)</td>
</tr>
<tr>
<td>[Ni$_2$LCI(H$_2$O)$_4$.4H$_2$O]</td>
<td>$C_3H_5N_2O_3ClNi_2$</td>
<td>210</td>
<td>693.9 (701)</td>
<td>39.8 (38.9)</td>
</tr>
<tr>
<td>[Cu$_2$LCI(H$_2$O)$_4$.4H$_2$O]</td>
<td>$C_3H_5N_2O_3ClCu_2$</td>
<td>165</td>
<td>649.6 (656)</td>
<td>42.5 (42.0)</td>
</tr>
<tr>
<td>[FeNiLCI(H$_2$O)$_4$]</td>
<td>$C_3H_5N_2O_3ClFeNi$</td>
<td>290</td>
<td>619.4 (624)</td>
<td>44.6 (43.8)</td>
</tr>
<tr>
<td>[FeCuLCI(H$_2$O)$_4$]</td>
<td>$C_3H_5N_2O_3ClFeCu$</td>
<td>230</td>
<td>623.9 (615)</td>
<td>44.2 (44.8)</td>
</tr>
</tbody>
</table>

Note: $^9$ The water molecules present in the complexes, indicated by the broad adsorption band at 3450 cm$^{-1}$, have probably lattice and/or coordination water. In the far infrared region, the bands at 430-445, 360-380 and 310-355 cm$^{-1}$ were assigned to $v$-M-O, $v$-M-N and $v$-M-Cl, respectively.

$^{10}$ The magnetic susceptibility determined for homobinuclear complex of Fe(II) obtained by both the methods (template and non-template) revealed high spin nature of the complex. Iron (II) complex had a magnetic moment of 5.13 B.M. slightly lower than spin-only value which is due to very weak spin-spin coupling. The nickel(II) complexes obtained by template and non-template synthesis exhibited effective magnetic moment values of 3.1 and 3.2 B.M., respectively at room temperature indicating spin-exchange interactions between two $S = 1$ states. The effective magnetic moment of copper (II) complexes was found to be $\sim 1.83$ B.M., which corresponded to a single unpaired electron with significant orbital contribution.

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band at 1724 cm\(^{-1}\) suggesting an octahedral configuration around each copper ion.

The X-band ESR spectrum of the powdered sample of dicopper (II) complex at room temperature exhibited an axial type signal with two \(g\) values, \(g_0\) (2.20) and \(g_\perp\) (2.05) which were in well agreement with an essentially \(d_{x^2-y^2}\) Cu (II) ground state\(^\text{12}\). The \(G\) factor [defined as \(G = (g_\perp-1)/g_0-2\)] indicative of exchange interactions between the Cu(II) sites in polycrystalline solids close to 4, suggested the negligible exchange interaction. The order \(g > g_\perp > 2.0027\) showed that the ground state of Cu(II) was predominantly \(d_{x^2-y^2}\). Lack of resolution of \(g\) components suggested a possible \(D_{4h}\) symmetry.

**Peroxidase Activity**

Peroxidase (donor: \(H_2O_2\)-oxidoreductase, EC 1.11.17) is a group of non-specific enzymes from different sources and is mainly involved in detoxification of \(H_2O_2\). Peroxidase catalyses the dehydrogenation of a large number of aromatic compounds including \(o\)-dianisidine. In the present investigation, the three synthesized binucleating complexes (Fe, Fe), (Fe, Ni) and (Fe, Cu) were tested for peroxidase activity by comparison with crude enzyme extract prepared from water stressed rice leaves. \(o\)-Dianisidine was taken as hydrogen donor. It was observed that out of three complexes, only (Fe, Cu) complex showed peroxidase activity (100 ml of 1.5 mg/ml solution of complex in DMF, caused 0.66 units change in optical density/min), thus catalysing the following reaction:

\[
H_2O_2 + o\text{-Dianisidine} \rightarrow H_2O + \text{oxidised o-dianisidine}
\]

(green colour, measured at 470 nm). Since (Fe, Fe) and (Fe, Ni) complexes did not exhibit any peroxidase activity, it is speculated that the activity of (Fe, Cu) complex may be due to Cu component of (Fe, Cu), a reaction analogous to dismutation of superoxide anion by superoxide dismutase (having Cu-Zn binucleating centre).

**References**