DNA binding properties, Molecular Docking and Antioxidative Studies of Cu(II) complexes

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ABSTRACT

New three copper complexes of type [Cu(5-N02-3-OMe SALAAP)(L)H2O] (where L=2,2’ bipyridine(bpy), 1,10 phenonthroline(phen) and imidazole (Imz), (5-N02-3-OMe SALAAP = 2,3’-dimethyl-1-phenyl-4-(2-hydroxy-5-nitro-3-methoxybenzylidenamino)-pyrazole-5-one) have been synthesized. The structural features have obtained from their elemental analyses, magnetic susceptibility, molar conductance, $^1$H-NMR, Mass, IR and UV –Vis studies. Based on all these data, the complexes are assigned to be octahedral geometry. The interaction of metal complexes with Calf thymus DNA (CT-DNA) was carried out by absorption, fluorescence spectroscopy and viscosity measurement methods. A further molecular docking technique was employed to understand the binding of the complexes towards the molecular target DNA. Investigation of the antioxidative properties showed that the metal complexes have significant radical scavenging activity potency against DPPH radical.

Keywords: DNA Binding Properties, Molecular Docking, CU (II) Complexes, DPPH Radicals

INTRODUCTION

Schiff base metal complexes have been studied extensively [1] because of their attractive and physical properties and their wide range of applications such as catalysts, antimicrobials, corrosion inhibitors etc. Antipyrine and its derivatives exhibit a wide range of biological activities and applications[2-5]. DNA is a primary intracellular target of anti-cancer drugs because it regulates many biochemical processes that occur in the cellular system. Many small molecules exert their anticancer activities by binding with DNA, there by altering DNA replication, blocking the division of cancer cells and resulting in the cell death[6-8]. The Schiff bases obtained from different substituted salicylaldehydes can form intra and intermolecular hydrogen bonds which mostly determine the chemical and physicochemical properties of those compounds. Various studies have shown Schiff bases derived from salicylaldehyde [9-11] and its derivatives have considerable biological importance because of presence of many donor atoms (N and O) in molecules of these compounds and being to some extent analogous to biological systems[12].

Imines obtained from heteroaromatic compounds containing nitrogen atoms in the ring have efficient biological activities[13]. For this reason Schiff bases derived pyrazolones are now studying as a new kind of chemotherapeutics[14]. Further it has been revealed that free radicals can damage proteins, lipids and DNA of bio-tissues, leading to increased rates of cancer and fortunately antioxidants can prevent this damage due to their free radical scavenging activity[15]. Therefore much attention has been given to metal complexes that can bind to DNA and also with antioxidant properties.

The present investigation deals with the synthesis, characterization, DNA binding, molecular modeling and antioxidant activity of mixed ligand Cu(II) complexes of Schiff base (2,3’-dimethyl-1-phenyl-4-(2-hydroxy,5-nitro,3-methoxy benzylidenamino)-pyrazol-5-one) derived from 4-amino antipyrine and 5-Nitro 3-methoxy salicylaldehyde.

MATERIALS AND METHODS

All reagents, 4-Aminoantipyrine, Cu(NO$_3$)$_2$5H$_2$O, 5-Nitro 3-methoxy salicylaldehyde, Phenonthroline, 2,2 bipyridine, imidazole, Tris HCl, ethidium bromide (EB) and DPPH procured from Sigma Aldrich. CT DNA
was purchased from Banglore Genei (India). Commercial solvents were distilled and then used for the preparation of ligand and its complexes.

Micro analyses (C, H and N) were performed by using Perkin-Elmer 240c (USA) elemental analyser, molar conductivities in DMSO (10-3 M) at room temperature were measured using Digisun Digital conductivity meter. Magnetic susceptibility measurements of the complexes were carried out by Guoy balance using Hg[Co(NCS)2] as standard. IR spectra were recorded with Brucker FT-IR spectrometer in the 4000-400 cm\(^{-1}\) range using KBr pellets. The absorption spectra were recorded using Schimadzu model 1800 spectrophotometer at room temperature. NMR spectra were recorded on Brucker 400MHz Spectrometer in CDCl\(_3\). The mass spectra were recorded by ESI technique on LCQ ion trap Thermo Finnigan Sanjose CA (USA) mass spectrometer.

Fluorescence spectra were recorded with Schimadzu Spectrofluorimeter (RF 5301 PC). Viscosity experiments were carried out by means of an Ostawald Viscometer maintained at a constant temperature at 28±1°C in a thermostatic bath.

**Synthesis of 2-hydroxy-5-nitro,3-methoxy benzylidene -4-aminoantipyrine (5-NO\(_2\),3-OMe SALAAP)**

The ligand 2-hydroxy-5-nitro, 3-methoxy benzylidene-4-aminoantipyrine was prepared as reported earlier. An ethanolic solution of 4-aminoantipyrine (0.02 mol) was added to an ethanol solution of 5-Nitro 3-methoxy salicylaldehyde (0.02). The resultant mixture was refluxed for ca 3h. The solid product formed was filtered and recrystallized from ethanol.

**Data for Lignad (L): Yield: 88%, Mp: 148°C, Anal.Calcd for C\(_{19}\)H\(_{18}\)N\(_{4}\)O: C, 59.68%; H, 4.73%; N, 13.48%; Found: C, 59.65%; H, 4.73%; N, 13.46%. IR (KBr cm\(^{-1}\)): (O-H): 3724, (C=O): 1656, (C=N): 1595, (C-O): 1330, UV-Vis (MeOH) \(\lambda_{max}\): 250, 350. \(^1\)H-NMR (400MHz, CDCl\(_3\)): \(\delta\): 6-8 (m, Ar-H), \(\delta\): 2.4 (=C-CH\(_3\)), \(\delta\): 2.4 (=C-CH\(_3\)), \(\delta\): 13.4 (OH), [Fig.1]. ESI-MS (m/z): Calcd: 382, Found: 383 (M+1), [Fig.2].

![Fig.1 H-NMR Spectrum of Ligand](image)

**Synthesis of Complexes**

A solution of Cu(NO\(_3\))\(_2\)·5H\(_2\)O in ethanol (2mmol) was refluxed with an ethanolic solution of the Schiff base (2mmol) for 2 hours, then 2,2’ bipyridine / 1.10 phenanthroline (2mmol) / imidazole (2mmol) in ethanol was added, this mixture was refluxed for 3 hours. The resulting brown solution was evaporated to get the complexes.


**Data for [Cu(L)(Imd)(H\(_2\))]NO\(_3\) (Complex - 3): Yield: 78%, Mp: 248°C, Anal.Calcd for C\(_{42}\)H\(_{38}\)Cu N\(_{6}\): C, 48.17%; H, 4.56%; N, 15.32%; Found: C, 48.16%; H, 4.54%; N, 15.31%. IR (KBr/cm\(^{-1}\)): (O-H/H\(_2\)O): 3132,
(C=O): 1648,(C=N): 1591,(C-O): 1317,(M-O):590,(M-N):428, UV-Vis(MeOH) λ<sub>max </sub>250, 350,590,µ<sub>eff</sub>(BM)=1.73.ESI-MS(m/z): 513[M-2H₂O Fig.2].

Fig.2 Mass Spectra of ligand and metal complexes 1, 2 and 3

DNA Binding
Preparation of stock solution
Concentrated CT-DNA stock solution was prepared in 5mM Tris-HCl/50mM NaCl in water at pH 7.5 and the concentration of DNA solution was determined by UV absorbance at 260nm. The molar absorption coefficient was taken as 6600 M<sup>-1</sup>cm<sup>-1</sup>[16]. Solution of CT-DNA in 5 mM Tris–HCl/50mM NaCl (pH 7.5) gave a ratio of UV absorption at 260nm and 280 nm A<sub>260/A280</sub> of Ca.1.8-1.9, indicating that the DNA was sufficiently free of protein [17]. All stock solutions were stored at 4°C and were used within four days. The concentration of EB was determined spectrophotometrically at 480nm (ε = 5680 M<sup>-1</sup>cm<sup>-1</sup>) [18].

Absorption spectra
Absorption spectra for the DNA binding studies were recorded on Schimadzu model 1800 UV-Vis spectrophotometer using 1-cm quartz micro cuvettes. Absorption titrations were performed by keeping the concentration of the complexes constant and by varying the [CT-DNA] from 0-100µM. For the complexes, the binding constants (K<sub>b</sub>) have been determined from the spectroscopic titration data using the following equation:

\[
\frac{[DNA]}{(\varepsilon_f - \varepsilon_b)} = \frac{[DNA]}{(\varepsilon_f - \varepsilon_b)} + 1/K_b \frac{(\varepsilon_f - \varepsilon_b)}{(\varepsilon_f - \varepsilon_b)}
\]

The 'apparent' extinction coefficient (\(\varepsilon_f\)) was obtained by calculating \([\text{Absorbance}]/[\text{Cu}]\). The terms \(\varepsilon_f\) and \(\varepsilon_b\) correspond to the extinction coefficients of free (unbound) and fully bound complexes respectively. A plot of \([\text{DNA}] / (\varepsilon_f - \varepsilon_b)\) Vs [DNA], will give a slope of \(1/(\varepsilon_f - \varepsilon_b)\) and an intercept \(1/K_b \frac{(\varepsilon_f - \varepsilon_b)}{(\varepsilon_f - \varepsilon_b)}\). K<sub>b</sub> is the ratio of the slope to the intercept.

Viscosity measurements
Viscometric titrations were performed with an Ostwald viscometer at room temperature. The concentration of DNA was 200µM, complex concentration varied from 0-120 µM and the flow times were measured with a digital timer and each sample was measured three times for accuracy and an average flow time was calculated, data was presented as \(1/\eta_0^{1/3}\) versus [complex]/[DNA], where \(\eta\) is the
viscosity of DNA in the presence of complex and ηυ that of alone. Viscosity values were calculated from the observed flow time of DNA containing solutions (τ) corrected for that buffer alone (5mM tris –HCl /50mM NaCl) (τo), ηυ = (t−t0) [19].

**Fluorescence Spectroscopy**

Fluorescence spectra were recorded with Schimadzu Spectrofluorimeter (RF 5301 PC) equipped with Xenon lamp. All fluorescence titrations were carried out in 5mM Tris –HCl/50 mM NaCl (PH 7.5). Solution containing DNA and EB was titrated with varying concentrations of 1, 2 and 3. The solutions were excited at 540 nm and fluorescence emission ,which corresponds to 595-600 nm were recorded. The samples were shaken and kept for 2-3 min for equilibrium and then spectra were recorded. The DNA concentration was always 10µM. The concentration of 1, 2 and 3 were in the range of 0-160µM and EB concentration was 5µM.

The spectra were analyzed according to the classical Stern-Volmer equation [20].

\[ I_o / I = 1+K_{sv} r \] .......................... (2)

Where I_o and I are the fluorescence intensities at 595 - 600 nm in the absence and presence of the quencher, respectively, Ksv is the linear Stern-Volmer constant, r is the concentration of the quencher.

**DNA Docking Studies**

**Methodology:**

Metal complexes were constructed and minimized in ChemDraw and a thorough search for putative binding sites of metal complex on the DNA was performed by using previously reported blind docking approach [21] with the AutoDock 4.2 program package[22]. Crystal structure of DNA was downloaded from protein data bank (www.rcsb.org pdb id: 5JU4), [23] it was prepared by protein preparation wizard applying OPLS 2005 force field in Schrodinger suite. Grid maps were generated with 0.375 Å spacing by the AutoGrid program for the whole DNA target. For each metal complex 50 docking runs were evaluated by Lamarckian genetic algorithm (LGA). Representative groups were collected and ordered based on the calculated binding free energy of the complexes. The low binding energy conformer was used for binding interaction analysis, Molecular interaction diagrams are obtained from PMV[24].

**Antioxidant Assays**

The 2, 2-diphenyl-2-picryl –hydrayzl (DPPH) radical scavenging activity of the complexes was measured according to the literature [25], the DPPH radical is a stable free radical having a λmax at 517nm. A fixed concentration of the complex (100µg/ml to 500µg/ml) was added to a solution of DPPH in methanol (0.004%, 4ml). DPPH solution with methanol was used as blank. The solution was incubated at 37° C for 30 min in dark. The decrease in absorbance of DPPH was measured at 517 nm on UV- Vis Spectrophotometer [26]

The Percentage of inhibition of radical DPPH(%) was calculated according to the equation

\[ I% = \frac{A_o - A_i}{A_i} \times 100 \]

Where, A_o is the absorbance of the control and A_i is the absorbance of the sample.

**RESULTS AND DISCUSSION**

**Synthesis and Characterization**

The syntheses of ligand and complexes of 1, 2 and 3 are schematically presented in schemes 1 and 2. The complexes are soluble in DMSO, ethanol and methanol. They were non-hygrosopic and stable in both solid and solution phase. The molar conductance data of the complexes were measured in DMSO solution for the 0.001M solutions. The complexes 1, 2 and 3 showed the molar conductivity in the range of 105-136Ω -1cm²mol⁻¹ indicating electrolytic nature of complexes.

**Infrared Spectra**

The important IR spectral data of the ligand and its complexes[ Fig.3] are summarized in Table 1. The νC≡O and νC=N (azomethine) observed at 1656 cm⁻¹ and 1595 cm⁻¹ respectively in the spectra of the ligand show downward shift/upward shift in all the complexes. These are suggestive of the participation of the pyrazol-carbonyl and azomethine groups in coordination [27-31]. The IR broad bands of metal complexes in the range 3049–3122 cm⁻¹ indicate the presence of lattice water molecules[32]. The νC=O(phenolic) modes of the ligand appear at 1342 cm⁻¹, shifted in the complexes indicate the complex formation via deprotonation of phenolic OH group [33]. Then non ligand bands 563-590 cm⁻¹ and 416-428 cm⁻¹ are assigned to νM=O and νM=N respectively [34]. The peaks corresponding to the ring stretching frequencies νC=C and νC=N at 1503, 1421 cm⁻¹ of free phenanthroline and at 1527,1443 cm⁻¹ of free bipy were shifted to higher frequencies upon complexation, indicating the coordination of the heterocyclic nitrogen atoms.
to the metal ion. The characteristic out of plane hydrogen bending modes of free phenanthroline observed at 853 and 738 cm\(^{-1}\) (for bipyridine at 851, 691 cm\(^{-1}\)) were shifted upon metal complexation [35].

**Electronic Absorption Spectra and Magnetic Moment Studies**

UV-VIS Spectra of Cu(II) complexes 1, 2 and 3 in MeOH show a broad band [Fig.4] in 570nm- 590nm region corresponding to d-d transitions which is in consistent with that of distorted octahedral geometry [36,37]. It is also supported by magnetic moments of the complexes. The broadness and position of the band favour distorted octahedral geometry. In the UV-Vis band at 225nm for complex 1 and 250nm for complex 2 and 250nm for complex 3 are due to the \(\pi-\pi^*\) transitions of azomethine (C=N) function of Schiff base and remaining bands in the UV region at 400nm for complex 1 and 400nm for complex 2 and 350 nm for complex 3 are due to the \(\pi-\pi^*\) transitions of coordinated phen and bipy ligands respectively [38]. The important uv spectral data and magnetic moment data of the ligand and its complexes are summarized in Table 2.

**ESR Spectral Studies**

ESR spectral studies of paramagnetic transition metal (II) complexes yield information about the distribution of the unpaired electrons and hence about the nature of the bonding between the metal ion and its ligands. The Cu(II) complexes exhibited well resolved anisotropic signals in the parallel and perpendicular regions as shown in Fig.5. The observed data showed that \(g_\perp = 2.19\) and \(g_\parallel = 2.12\). The \(g_\perp\) values are greater than \(g_\parallel\), suggesting major distortion from octahedral symmetry in Cu(II) complex[39]. The \(g_\perp\) is a moderately sensitive function for indicating covalency. The \(g_\perp >2.3\) is characteristic of anionic environment and \(g_\perp <2.3\) is of covalent environment in M-L bonding[40]. The observed \(g_\perp\) value for the Cu(II) complex is less than 2.3, in agreement with the covalent character of the M-L bond. Thus a distorted geometry is proposed for the complexes [41]. Data summarized in Table 3.

**DNA binding studies**

The mode and propensity of binding of Cu(II) complexes to CT-DNA were studied by different techniques.

**Absorption Spectra**

The Absorption spectra of complexes 1, 2 and 3 [Fig.6] show both hypochromic and bathochromic shifts on the addition of increasing amounts of CT-DNA. The hypochromic and bathochromic shifts are indicative of an intercalative mode of binding where the aromatic chromophore of the complex stacks between the base pairs of DNA [42-44]. The binding constant (\(K_b\)) for the association of 1, 2 and 3 with CT –DNA (insets of respective figures) were determined as \(1.74 \times 10^5\), \(2.32 \times 10^5\) and \(1.35 \times 10^5\) respectively tabulated in Table 4 (Eq (1)[45,46]). The binding of 2 is higher than 1 & 3 due to the presence of an extended aromatic phenyl ring.

**Fluorescence spectroscopy**

The fluorescence quenching experiments were performed to get an estimate on the relative binding affinity of the complexes to CT-DNA with respect to EB. It is well known[47] that free EB displays a decrease in emission intensity in Tris –HCl buffer medium because of quenching by solvent molecules. However EB strongly fluoresces in the presence of DNA due to the complete intercalation between the adjacent DNA pairs, a process that can be reversed by the addition of a competing agent[48,49]. The quenching curves of EB bound to DNA in the absence and presence of 1, 2 and 3 are given in Fig.7. The addition of 1 to DNA pretreated with EB caused appreciable reduction in emission intensity, indicating that the complex binds to DNA at the site occupied by EB. Similar trends were observed for 2 and 3. The above data were analysed by means of the Stern-Volmer equation [49]. The quenching plots (insets of respective figure) illustrates that the fluorescence quenching of EB bound to DNA by 1, 2 and 3 is in linear agreement with the Stern –Volmer relationship, which corroborates that the two complexes bind to DNA. In the plot of \(I/I_0\) versus [complex]/[DNA], \(K_{sv}\) is given by the ratio of slope to intercept. The \(K_{sv}\) values for 1, 2 and 3 are 1.618, 2.05 and 0.512 respectively tabulated in Table 4.

**Viscosity Measurements**

To authenticate the intercalative binding, viscosity experiments were performed. It is well known that the relative viscosity of CT-DNA solution on interaction with complex will increase for intercalative binding mode, remain same for classical groove binding and decrease for partial intercalation[50-52]. The effect of 1, 2 and 3 on the viscosity of DNA depicted in Fig.8 shows steady increase in the viscosity of the DNA with the addition of increasing amounts of the complexes. Such behavior is consistent with the addition of other intercalators (i.e. EB), which increase the relative specific viscosity for the lengthening of the DNA double helix resulting from intercalation. The results clearly indicate that both the complexes intercalate between adjacent DNA base pairs, causing an extention in the helix there by increasing the viscosity of DNA[53,54].
DNA Docking Results
Molecular docking of Copper metal complex was carried out in Autodock 4.2 by applying blind docking protocol. The process provides the putative binding site of the complex in DNA. The most high binding affinity conformer of the complex was selected from the process to analysis the mode of interaction. The binding energy values of the complex are provided in Table 5. The dock scores absolutely correlate to the experimental DNA binding values $K_d$. Complex 2 having highest binding values showed highest dock score followed by complex 1 and complex 2. Dock pose analysis of these complex illustrate the possible mode of binding, complexes 1 and 2 were docked in the minor groove of the DNA and Complex 3 was docked at the major groove (Fig 9). Complex 1 formed a hydrogen bond interaction with phosphate group of T7 while complex 2 interacted with C21 (Fig.10). In case of complex 3, no hydrogen bond interactions were observed, that is reflected in its lower binding to DNA compared to other two complexes.

Antioxidant Activity
The antioxidant activities of the metal complexes and ascorbic acid (standard) as found by scavenging DPPH radical, are shown in Fig.11. When the concentration of compounds was increased from 100µg/ml to 500 µg/ml. DPPH radical scavenging activities were increased. The DPPH radical scavenging activities were found to be 42.9% for complex 1, 43.5% for complex 2 and 41.2% for complex 3 at the concentration of 500 µg/ml. The radical scavenging activity of the compound depends on the structural factors such as the phenolic hydroxyl, carboxylic and other structural features [55]. The order of antioxidant activity of the complexes is ascorbic acid > 2 > 1 > 3.
Fig. 4 Electronic Spectra of ligand and metal complexes 1, 2 and 3

Fig. 5 ESR Spectra of complexes 1, 2 and 3
Fig. 6 Absorption spectra of complexes 1, 2 and 3 in the presence of increasing amounts of CT-DNA. Conditions: [Complex] = 10 μM, [CT-DNA] = 0-100 μM. 1-3 The arrow indicates the change in absorbance upon increasing the DNA concentration. Inset: Linear plots for the calculation of intrinsic binding constant $K_b$. 
Fig. 7. Emission spectra of EB bound to DNA in the absence and presence of complexes (10 μM) 1-3. The arrow indicates the change in absorbance upon increasing the DNA concentration, \( r = [\text{complex}]/[\text{DNA}] \). Inset: Stern–Volmer quenching curves

Fig. 8. Effects of increasing amount of Ethidium Bromide (EB), complexes 1, 2 and 3 on the relative viscosity of CT-DNA at 25°C ± 0.1, \( r = [\text{complex}]/[\text{DNA}] \).

Fig. 9. Dockpose of copper complex on the DNA showing groove binding (a) Complex 2 (b) Complex 1 and (c) Complex 3 DNA is shown in molecular surface form.
Fig. 10 Dock pose of Copper complex showing hydrogen bond interaction (a) Complex 2 with T7 (b) Complex 1 with C21 and (c) Complex 3 lacks hydrogen bond interaction.

Fig. 11. Antioxidant Activity of the complexes

Scheme 1

4-Amino antipyrine

5-Nitro 3-methoxy salicylaldehyde

Schiff Base

Refluxed for 2 hours in ethanol
**Scheme-2**

**Table 1. Infrared (IR) Spectra of Ligand and Metal complexes**

<table>
<thead>
<tr>
<th>Ligand/Complex</th>
<th>$\nu$ O-H/H$_2$O</th>
<th>$\nu$ C=N</th>
<th>$\nu$ C=O</th>
<th>$\nu$ C-OH</th>
<th>$\nu$ M-O</th>
<th>$\nu$ M-N</th>
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<tr>
<td>Ligand (L)</td>
<td>3724</td>
<td>1595</td>
<td>1656</td>
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<td>416</td>
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<td>1571</td>
<td>1636</td>
<td>1324</td>
<td>563</td>
<td>426</td>
</tr>
<tr>
<td>Complex-3</td>
<td>3132</td>
<td>1591</td>
<td>1648</td>
<td>1317</td>
<td>590</td>
<td>428</td>
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</table>

**Table 2. Electronic Absorption Spectra of Ligand and Metal complexes**

<table>
<thead>
<tr>
<th>Ligand/Complex</th>
<th>Absorption Region (nm)</th>
<th>Band Assignment</th>
<th>$\mu_{eff}$</th>
<th>Geometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligand (L)</td>
<td>250, 350</td>
<td>$\pi$-$\pi^*$</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Complex-1</td>
<td>225, 400, 570</td>
<td>$\pi$-$\pi^*$</td>
<td>1.69</td>
<td>octahedral</td>
</tr>
<tr>
<td>Complex-2</td>
<td>225, 400, 590</td>
<td>$\pi$-$\pi^*$</td>
<td>1.71</td>
<td>octahedral</td>
</tr>
<tr>
<td>Complex-3</td>
<td>250, 350, 590</td>
<td>$\pi$-$\pi^*$</td>
<td>1.73</td>
<td>octahedral</td>
</tr>
</tbody>
</table>
CONCLUSIONS
The Schiff base ligand and its Cu(II) complexes have been synthesized and structurally characterized. Based on analytical, molar conductance, magnetic and spectral data, all these complexes are assigned to be octahedral geometry. DNA binding studies of the complexes was investigated by UV-VIS and Fluorescence spectroscopy and Viscometry methods. The order of antioxidant activity of the complexes is 2>1>3. Docking of copper complexes to DNA has shown that they bind to minor and major grove of DNA and the dock score profoundly correlate with the experimental binding constant.

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