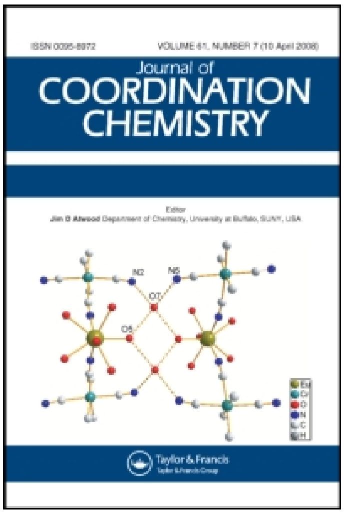

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Antioxidative properties of copper(II) complexes¶

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Eleven copper(II) complexes with amino acids, salicylate, 2,2′-phenanthroline, ethylenediamine, ethylenediaminetetraacetic acid, dithiocarbamates, and thiosemicarbazones were studied as scavengers of superoxide and hydroxyl radicals. These two radicals exist in biological systems and play an important role in oxidative stress. The complexes with the first three ligands are pro-oxidants. On the contrary, the *bis*(dithiocarbamato)copper(II) complexes are the most active against the studied radicals.

Keywords: Copper(II) complexes; Antioxidative properties; Superoxide radical

1. Introduction

When dioxygen accesses aerobic cells, Reactive Oxygen Species (ROS) can be produced. The most important ROS are superoxide radical ($O_2^{\cdot -}$), hydroxyl radical (OH.), hydrogen peroxide (H_2O_2), and singlet dioxygen ($^1O_2$). Different enzymes regulate these ROS *in vivo*, but sometimes an uncontrollable excess can arise provoking oxidative stress in the biological system [1]. Under such conditions the patient requires the administration of antioxidative drugs (antioxidants). Many diseases are related with the presence of considerable amounts of ROS, such as aging, inflammatory processes, etc. [2].

It is not easy to find compounds which are antioxidants against several types of ROS. Most commonly, copper(II) complexes can dismutate superoxide radical, but less efficiently than Cu, Zn-Superoxide Dismutase (SOD) does *in vivo* [3]. The reaction involved in this process corresponds to:

$$2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$$

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¶ This article is dedicated to Professor Alfredo Mederos who dedicate his life to develop Chemistry in Canary Islands, Spain.

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One of the products of the dismutation reaction is hydrogen peroxide, another ROS. In the presence of some transition metals H_2O_2 can be transformed into hydroxyl radical ($OH^{\cdot}$), the most dangerous ROS [4]:

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^{\cdot} + OH^-$$

called the Fenton reaction, or by the catalytic Haber–Weiss reaction:

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^-$$

Copper(I) can provoke the Fenton reaction, while copper(II) can catalyze the Haber–Weiss reaction. Therefore, copper(II) complexes able to mimic SOD are not only antioxidants but also potential pro-oxidants [5].

In the present article, we analyze the SOD-like activity of several copper(II) complexes by different methods and correlate this property with their thermodynamic stability and structure.

Together with Prof. Mederos, we studied two *bis*(dithiocarbamato)copper(II) complexes as a scavenger of nitric oxide [6].

2. Experimental

2.1. *Reagents and equipment*

All reagents (analytical grade) were purchased from Merck and used without purification. The enzymes were from Sigma. Freshly distilled water (CO_2-free) was used in all cases. The ligands commercially not available were synthesized as reported elsewhere: Glutamic Acid Dithiocarbamate (GluDTC) and glycine dithiocarbamate sodium salt trihydrate [7], sodium morpholyldithiocarbamate trihydrate [8], Pyruvic Acid Thiosemicarbazone (PTSC) [9], and Ribose *Bis*(thiosemicarbazone) (RibTSC) [10].

All copper(II) complexes were formed *in situ*, except for $Cu(RibTSC)_2$ which was prepared as reported elsewhere [11] and dissolved in Dimethyl Sulfoxide (DMSO).

Electron Paramagnetic Resonance (EPR) spectra were recorded at room temperature on a Bruker 200 D SRC X-band spectrometer. Microwave frequencies were measured with a XL Microwave (model 3120) counter. The modulation frequency and microwave frequency of the spectrophotometer were 100 kHz and 9.49 GHz, respectively. $CuSO_4$ and the copper(II) complexes were mixed in DMSO with Water H_2O_2 and 5,5-Dimethylpyrroline-*N*-oxide (DMPO) in order to achieve a final 10 mM concentration for the metal.

The spectrophotometric determinations were carried out on an Ultrospec 2100 *pro* (Amersham BioSci.) apparatus.

2.2. *Methods used in the determination of the antioxidative properties*

The inhibition of pyrogallol auto-oxidation was performed spectrophotometrically (440 nm) based on a method reported elsewhere [8]. Only freshly prepared pyrogallol aqueous solutions (2 mM) were used. To 3 mL of pyrogallol solution, containing phosphate buffer at pH 7.4 and H_2O_2 (3%, 40 μL), the corresponding amount of copper(II) complex solution was added. The degree of inhibition of pyrogallol auto-oxidation was expressed as IC_{50} value, which corresponds to the concentration of copper(II) complex for which the auto-oxidation of pyragollol (in the presence of H_2O_2) is inhibited to 50%.

The SOD-like activity was determined spectrophotometrically (560 nm) using superoxide radicals generated by the xanthine–xanthine oxidase system with NBT (nitroblue tetrazolium chloride) as indicator [12–14]. The degree of the inhibition of the concentration of superoxide radical was expressed as IC_{50}, corresponding to the concentration of copper(II) complex for which the concentration of superoxide radical was reduced by 50% (determined indirectly by the indicator).

The kinetic determinations (pyrogallol auto-oxidation and SOD-like activity) were carried out spectrophotometrically using the *Kinetic Enzyme* software. The assay time was generally fixed to not less than 10 min. In each experiment, four cuvettes (10 mm) were used. The first cuvette was always the reference system: pyrogallol (auto-oxidation) and xanthine oxidase (SOD-like); the other three cuvettes contained solutions of the studied compound at different concentrations. The percent of inhibition of these reactions was calculated through slopes (ΔAbs/min) obtained from the cuvettes containing the assay at different concentrations (assay slope) referred to the slope of the reference sample (ref slope) according to the following equation:

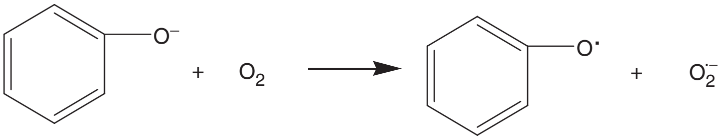
$$\%\ \text{inhibition} = (\text{assay slope} - \text{ref slope}) \cdot 100/\text{ref slope}$$

The IC_{50} value was obtained by regression analysis and interpolation of the % inhibition *versus* assay concentration curve for no less than five experimental points. Only a linearity greater than 0.96 was considered acceptable.

3. Results and discussion

3.1. *Pyrogallol model*

Phenols oxidize through formation of phenoxyl radical according to reaction 1 [15].



(1)

In the case of pyrogallol (1, 2, 3-trihydroxybenzene) this process is spontaneous in basic medium. The oxidation of pyrogallol by dioxygen involves formation of superoxide radical [16]. Therefore, as we confirmed, SOD inhibits pyrogallol auto-oxidation. On the contrary, the xanthine–xanthine oxidase system and hydrogen peroxide accelerate it. Neither catalase nor peroxidase affect the auto-oxidation of pyrogallol. These observations confirm that:

- Superoxide radical, $O_2^{\cdot -}$, is involved in the auto-oxidation reaction.
- Hydroxyl radical, and not hydrogen peroxide, is formed with formation of a phenoxyl radical.

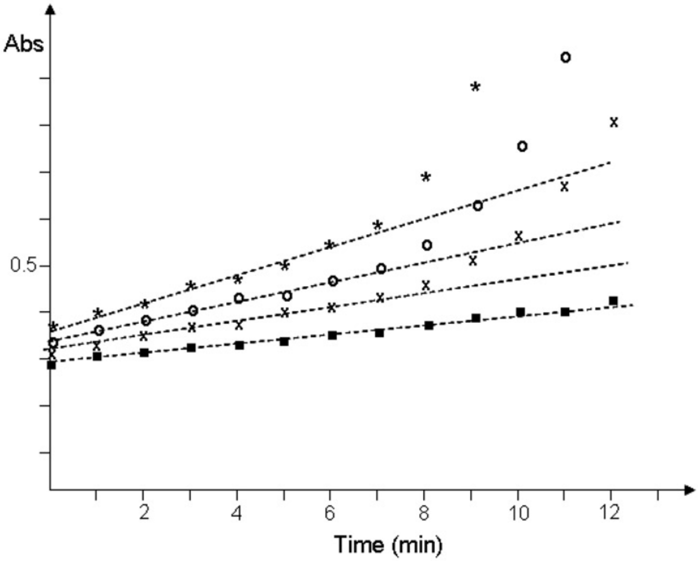
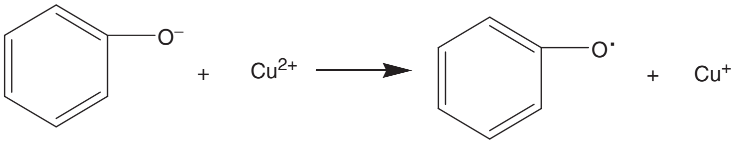


Figure 1. Variation of absorbance of pyrogallol auto-oxidation in the presence of different concentrations of $CuSO_4$.

We observed that addition of a copper(II) salt to a solution of pyrogallol (pH 7.4) accelerated exponentially its auto-oxidation (figure 1) according to reaction (2):



(2)

We considered that ligands able to form a stable bond with copper(II) would restrict the Cu(II)/Cu(I) reduction process and, therefore, not accelerate the auto-oxidation of pyrogallol. For that reason, $Cu(Gly)_2$, $Cu(Glu)_2$, and $Cu(salicylate)_2$, which are complexes that can easily dissociate, accelerated the auto-oxidation of pyrogallol. On the other hand, inhibition of the auto-oxidation of pyrogallol can be considered as an indirect way to measure inhibition of the Fenton reaction.

$Cu(phen)_2^{2+}$ presented a particular behavior against pyrogallol. This complex accelerated pyrogallol auto-oxidation in a logarithmic way. It is well known that in this complex Cu(II) is easily reduced to Cu(I) and also that $Cu(phen)_2^{+}$ produces hydroxyl radicals [17]. These two facts explain the strong effect of $Cu(phen)_2^{2+}$ on the auto-oxidation of pyrogallol.

SOD inhibited the auto-oxidation of pyrogallol at $IC_{50} = 0.16\,\mu M$ of the enzyme indicating that superoxide radical is involved in this process. Seven other copper(II) complexes studied also inhibited this reaction (table 1) but less efficiently.

The copper(II) complexes that expressed the highest inhibition of the auto-oxidation of pyrogallol corresponded to the three *bis*(dithiocarbamato) complexes studied. This ligand is known to form highly stable copper(II) complexes with strong resistance to reduction to Cu(I), a limiting factor [8].

Table 1. Concentrations at which each compound inhibited by 50% (IC_{50} in μM) the concentration of the reaction products.

Compound	Structure	Log β_N	IC_{50} (μM) (pyrogallol auto-oxid.)	IC_{50} (μM) (SOD-like activity)
SOD	Tetragonal	–	0.16	0.002
$Cu(GluDTC)_2$	Square-planar	19.35 [22]	11	1.2
$Cu(MorDTC)_2$	Square-planar	26.05 [23]	15	3.2
$Cu(GlyDTC)_2$	Square-planar	–	43	20
Cu(EDTA)	Distorted octahedral	18.9 [24]	280	52
$Cu(PTSC)_2$	Square-planar	–	370	26
Cu(RibTSC)	Square-planar	–	830	100
$Cu(en)_2^{2+}$	Square-planar	19.9 [24]	1100	5.4

3.2. *SOD-like activity*

The SOD-like activity was determined spectrophotometrically using NBT as indicator to detect the amount of superoxide radical present (table 1). All the studied copper(II) complexes presented a relatively low SOD-like activity, higher than 1 μM.

The *bis*(dithiocarbamato)copper(II) complexes are characterized by two interrelated factors that affect SOD-like activity:

- The ligand stabilizes Cu^{2+} oxidation state
- The complexes are highly square planar with low tendency to distort to tetrahedral (Cu^{+} structure).

Cu(RibTSC) is also square planar where the ligand is tetradentate κ^4-N,S,S,N. According to EPR determinations $Cu(PTSC)_2$, the other thiosemicarbazonato complex studied has a more distorted square-planar structure [18]. Cu(EDTA) (Ethylenediaminetetraacetic Acid, EDTA) presented the second highest IC_{50} value (low SOD-like activity), an understandable result considering its octahedral structure.

In table 1 the complexes were organized for an increase in the IC_{50} values (pyrogallol auto-oxidation) proceeding down. Similar behavior can be observed in the second column (SOD-like activity), with the exception of Cu(EDTA) and $Cu(en)_2^{2+}$. The fact that superoxide radical is involved in the auto-oxidation of pyrogallol explains the observed correlation between this property and the SOD-like activity.

The IC_{50} (SOD-like) of $Cu(en)_2^{2+}$ does not correlate with the effect on the auto-oxidation of pyrogallol, probably due to the high flexibility of the square-planar structure of this complex. More important is the fact that this complex is the only one carrying positive charge, enhancing electrostatic attraction of the substrate, an anionic radical ($O_2^{\cdot-}$).

3.3. *Thermodynamic stability*

Thermodynamic stability is such an important property when studying biological applications of copper complexes that even for Cu(I) they can present SOD-like activity [19].

As mentioned above, Cu(II) accelerates the auto-oxidation of pyrogallol and can be involved in the Fenton and Haber–Weiss reactions with an undesirable pro-oxidant effect. Therefore, it is important to use highly stable copper(II) complexes.

Table 2. Concentrations at which some copper(II) complexes increased by 50% the concentration of oxidized pyrogallol (AC_{50}) compared with their stability constants.

Compound	AC_{50} (μM)	Log β_2 [24]
$CuSO_4$	610	–
$Cu(Glu)_2$	300	13.95
$Cu(Gly)_2$	180	15.19
$Cu(Sal)_2$	21	17.60

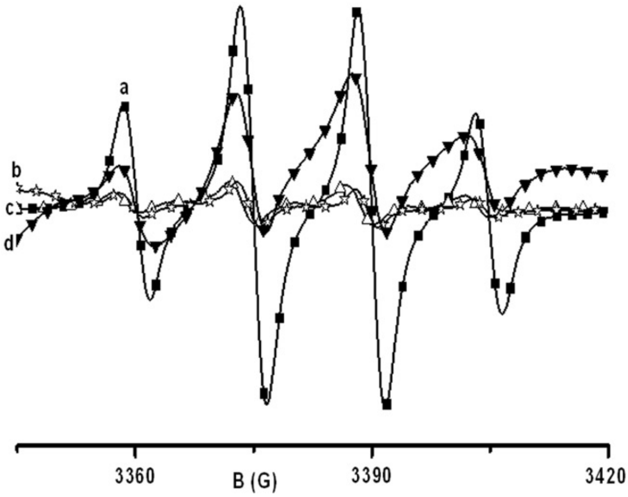


Figure 2. EPR spectra of the systems containing DMPO (20 mM) + H_2O_2 (40 μL, 3%) and 10 mM concentrations of: (a) $CuSO_4$, (b) $Cu(PTSC–H)_2$, (c) Cu(EDTA), (d) $Cu(en)_2^{2+}$.

This condition is not met by $Cu(Gly)_2$, $Cu(Glu)_2$, and $Cu(salicylate)_2$, which accelerate the auto-oxidation of pyrogallol as $CuSO_4$ did. Evidently, this is due to the relatively high degree of dissociation of the complexes. In table 2, the concentrations at which these complexes increased by 50% the concentration of oxidized pyrogallol (AC_{50}) are compared with their stability constants. Evidently, the relatively low stabilities of $Cu(Gly)_2$, $Cu(Glu)_2$, and $Cu(salicylate)_2$ permit significant dissociation of Cu^{2+} which induces the auto-oxidation of pyrogallol.

The other seven complexes studied (reported in table 1) are characterized by a high thermodynamic stability and should not be considered as pro-oxidants but, on the contrary, antioxidants. For those cases for which it was technically possible, the radical scavenging properties were also studied by EPR.

3.4. *Copper(II) scavenging properties studied by EPR*

Cu(EDTA), $Cu(PTSC)_2$ and $Cu(en)_2^{2+}$ were studied by EPR spectroscopy using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), a known spin trap of superoxide and hydroxyl radicals [20, 21]. The three complexes inhibited the EPR signals of DMPO (figure 2) when compared to $CuSO_4$. In all cases, H_2O_2 was added (40 μL, 3%). The percent of inhibition of radicals achieved was: Cu(EDTA), 86%, $Cu(PTSC)_2$, 86%, and $Cu(en)_2^{2+}$, only 43%. The low inhibition expressed by $Cu(en)_2^{2+}$ should be related to the observed low inhibition of pyrogallol auto-oxidation (table 1). The other complexes could not be studied due to interference with the signals of the corresponding complexes.

4. Conclusions

The 11 copper(II) complexes selected have two different characteristics when studied in the presence of the auto-oxidation of pyrogallol:

- $Cu(Gly)_2$, $Cu(Glu)_2$, $Cu(salicylate)_2$ and $Cu(phen)_2^{2+}$ accelerated the reaction and were defined as pro-oxidants.
- The three dithiocarbamato copper(II) complexes, Cu(EDTA), the two thiosemicarbazonato complexes and $Cu(en)_2^{2+}$ inhibited pyrogallol auto-oxidation.

The seven copper(II) complexes that inhibited the auto-oxidation of pyrogallol were also studied as SOD mimetics, but the activity was relatively low. Nevertheless, three of these complexes inhibited the EPR signals of DMPO, a spin trap, indicating a significant antioxidative activity.

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