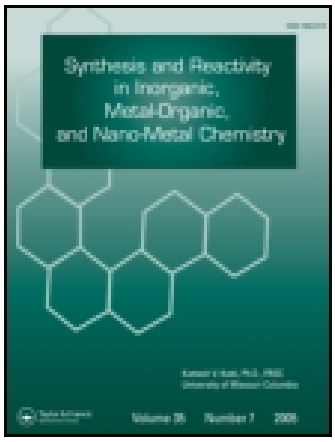

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# Synthesis, Characterization, and Measurement of Antioxidant Reactivity of Salicylidene-D,L-Tyrosine Ethyl Ester and Copper(II)(Salicylidene-D,L-Tyrosine Ethyl Ester)$_2$ in a Linoleic Acid Peroxidation Reaction System

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# Synthesis, Characterization, and Measurement of Antioxidant Reactivity of Salicylidene-D,L-Tyrosine Ethyl Ester and Copper(II)(Salicylidene-D,L-Tyrosine Ethyl Ester)$_2$ in a Linoleic Acid Peroxidation Reaction System

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**Salicylidene-D,L-tyrosine ethyl ester and Cu(II)(SalTyrOEt)$_2$ were synthesized, characterized, and chain-breaking antioxidant and prooxidant reactivities determined in a 2,2′-azoisobutyronitrile initiated linoleic acid peroxidation reaction. Kinetics of oxygen consumption were found to be comparable to the antioxidant reactivity of 2,6-ditertiary-butyl-4-hydroxytoluene, due to efficient removal of linoleic acid peroxyl radical with the formation of linoleic acid hydroperoxide, LOOH, and correlate with the presence of tyrosyl phenolic OH groups. Copper in Cu(II)(SalTyrOEt)$_2$ accounts for prooxidant reactivity, catalyzing removal of LOOH, except at low concentrations when its antioxidant properties dominate. It is concluded that Cu(II)(SalTyrOEt)$_2$ is a unique antioxidant with prooxidant reactivity.**

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**Keywords** Schiff base, salicylidene-tyrosine ethyl ester, copper(II) (salicylideneTyrOEt)$_2$, linoleic acid, peroxidation, antioxidant

## INTRODUCTION

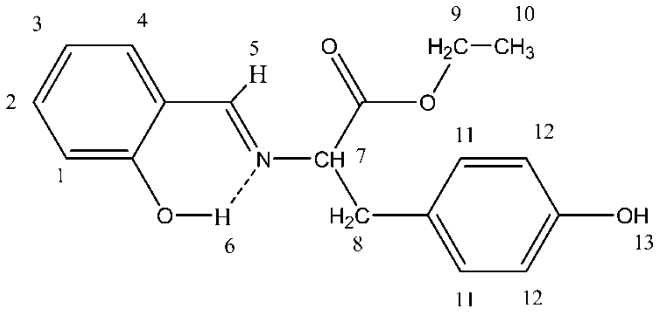
Copper(II) complexes are the principal forms of copper found in all tissues since the concentration of non-complexed free copper(II) is $\leq 10^{-18}$ M and not measurable with existing equipment.[1,2] These copper complexes are small molecular mass amino acid, carboxylic acid, and amino sugar complexes, a copper albumin complex, and all copper-dependent enzymes and storage proteins.[1,2] Thermodynamic stabilities and coordination competitions account for ligand exchange chemistry and the primary and ultimate existence of the variation in copper complexes found in all biochemically and physiologically relevant forms of copper in all biological systems.

Copper(II) complexes of amino acid Schiff bases have become important due to their varied pharmacological effects, which include anti-inflammatory, anti-ulcer, anti-convulsant, anti-diarrheal, antimicrobial, and anticancer

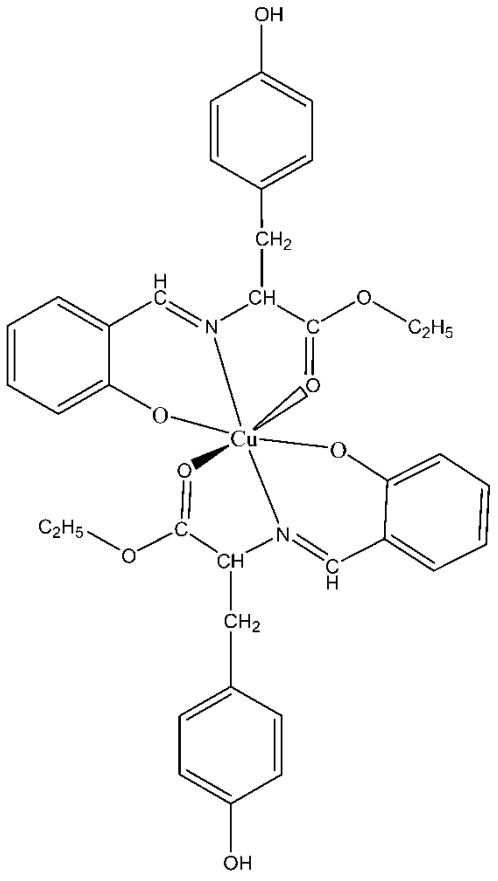
activities.[1–5] Schiff base Cu complexes are versatile models of metalloelement drugs and their structural and bonding modes provide a better understanding of protein-metalloelement bonding in ligand exchange processes.[6–11] These complexes can be used as catalysts for the decomposition of $H_2O_2$ and organic hydroperoxides as catalase-mimetic and peroxidase-mimetic agents respectively[1,2,12] and for the decomposition of superoxide as superoxide dismutase (SOD)-mimetic agents.[1–3,13]

The pharmacological effectiveness of copper complexes, consistent with their involvement in various biochemical process in normal tissues and in repair responses to overcome diseases states associated with free radical pathologies, are suggested to be due, in part, to their antiradical SOD-mimetic, catalase-mimetic, and peroxidase-mimetic reactivities.[1,2,13] In these roles the reaction center is the central metalloelement with redox reactivity having beneficial effects in overcoming reactions associated with many disease states. Since complexed forms of copper account for all measurable amounts of copper in tissues and ligands forming these complexes protect biochemical systems from the redox reactivity of free Cu(II), indiscriminate coordination reactivity, and chemical incompatibilities associated with physiological pH values,[14–18] these complexed forms of copper are most relevant for use as pharmacological agents.[1–5,11–13,19–23] These complexes may also serve as Cu delivery agents and facilitate *de novo* Cu-dependent enzyme syntheses need to overcome disease pathology.[1,2] Copper complexes do have prooxidant reactivity, which accounts for their peroxidase-mimetic reactivity in removing or metabolizing lipid hydroperoxides *in vivo*, which always requires an oxidizable co-substrate in biochemical systems.

The presence of antiradical centers in ligands bonded to Cu, e.g., phenolic OH groups and the presence of redox active Cu(II) capable of removing singlet oxygen ($^1O_2$), superoxide, $O_2^{-}\bullet$, hydroxyl radical (HO•), and hydroperoxyl radical (HOO•), can play key roles in accounting for the antioxidant reactivity of these complexes and directly take part in preventing free radical processes associated with the oxidation of biological and model substrates.[19–23] copper(II) complexes of such ligands can produce beneficial antioxidant effects in addition to the antioxidant reactivity of the central metalloelement as an antiradical reaction center. According to Berthon et al.[19,20] ligands that have high antiradical reactivity decrease the manifestation of prooxidant reactivity of the central metalloelement and may be modified appropriately for utility in biological systems. It is then reasonable to search for potential pharmacologically effective Cu(II) complexes that possess a prooxidant to antioxidant converting mechanism of action or a combination of these reactivities in lipid media, taking into consideration the great importance of preventing abnormal non-metabolic lipid oxidation processes in biochemical systems. Accordingly, Cu(II) complexes formed with lipophilic ligands such as Schiff bases and in particular salicylidene-D,L-tyrosine ethyl ester [SalTyrOEt] (Figure 1a) were chosen for evaluation with regard to their antioxidant and prooxidant reactivities using a biochemically relevant linoleic acid peroxidation reaction system.[24]




(a)




(b)

FIG. 1. (a) The salicylidene-DL-tyrosine ethyl ester Schiff base ligand and (b) the Cu(II)$(SalTyrOEt)_2$ complex.

## MATERIALS AND METHODS

### Reagents

Chlorobenzene (Aldrich) was dried over anhydrous $CaCl_2$ and double distilled. Heptane, n-butanol, methanol, and acetonitrile, all from Aldrich, were purified by double distillation. Chlorobenzene and heptane were purified by passing them over a column of activated $Al_2O_3$; 2,2′-azoisobutyronitrile (AIBN) (Polyscience, Inc.) was purified by recrystallization from methanol; absolute ethanol was purified according to known procedures,[25] and 2,6-di-tert-butyl-4-methylphenol (BHT) (Aldrich) was repeatedly recrystallized from heptane to obtain a constant melting point, 69 to 70°C. Linoleic acid (LH) (Aldrich), tetrabutylammonium perchlorate (TBAP), D,L-tyrosine ethyl ester, salicylaldehyde, N,N′ bis(salicylidene-ethylenediamine), $Cu(II)_2(CH_3COO)_4(H_2O)_2$ (Fluka) and $Cu(II)_2$(stearate)$_4$ (Chemreactiv, Russia) were reagent grade and were used without further purification. All other chemicals used were of highest available purity. Synthesis of D,L-Tyrosine ethyl ester performed by a known method.[26] Synthesis of salicylidene-tyrosine ethyl ester [SalTyrOEt] (Figure 1a) and its Cu(II) complex (Figure 1b) were by reported literature methods[27–30] as detailed below.

### Synthesis of Salicylidene-D,L-Tyrosine Ethyl Ester

A 5°C chloroform solution of the ethyl ester of D,L-tyrosine, 10 mmol (2.09 g), was added drop wise to a stirred 50 ml chloroform solution of salicylaldehyde, 10 mmol (1.08 ml). The reaction mixture was stirred for 2 h at 20°C. Then 20 mmol (2.4 g) of anhydrous $MgSO_4$ was added with stirring to dry the chloroform solution. The mixture was stirred for 60 h, at 40°C under solvent-saturated high purity nitrogen gas. After the magnesium sulfate was removed by filtration the chloroform solution was extracted with 5 × 10 ml of twice distilled water, 5 × 10 ml of 0.5% solution of acetic acid, and again with 5 × 10 ml of water. This chloroform solution was dried over anhydrous sodium sulfate. The yellow solution was concentrated by evaporation in vacuo at 50°C to remove the chloroform and cooled to 30°C to obtain the yellow colored amorphous ethyl ester and dried in vacuo over $P_2O_5$. Yield: 1.85 g (59.3%). Melting point: 132 to 133°C (760 mm, Hg). Anal. Calc. For $C_{18}H_{19}NO_4$ (%): C, 69.07; H, 6.07; N, 4.47; Found: C, 69.10; H, 6.17; N, 4.55. A thin layer chromatogram gave a single spot having an $R_f$ of 0.79 using a propanol:water (7:3) solvent system.

### Synthesis of Copper(II) Salicylidene-D,L-Tyrosine Ethyl Ester

Copper(II)$_2$(acetate)$_4(H_2O)_2$, 1.5 mmol (0.544 g), was vigorously stirred in a mixture of 50 ml absolute ethanol and chloroform (1:1) to affect solution. Six mmol (1.88 g) of salicylidene-D,L-tyrosine ethyl ester dissolved in 30 ml of chloroform was added to the solution of copper acetate and gave a blue solution. This reaction mixture was stirred for 30 h at 30°C. After 20 h the solution was heated at 40°C for 10 h to obtain a light green solution, which when allowed to cool to room temperature gave a green precipitate. The green solid was filtered, washed with 5 × 20 ml diethyl ether and twice distilled water (5 × 20 ml), and after air-drying for 12 h gave a green powder, the Cu(II) complex of salicylidene-D,L-tyrosine ethyl ester. Yield: 1.31 g (63.7%). Melting point with decomposition: 265 to 270°C (760 mm, Hg). Anal. Calc. for $CuC_{36}H_{36}N_2O_8$ (%): C, 62.83; H, 5.27; N, 4.07; Cu 9.23. Found: C, 63.19; H, 5.43; N, 4.06; Cu 8.73.A thin layer chromatogram gave a single spot, $R_f = 0.79$, using a propanol:butanol:dimethylformamide:water (7:8:2:3) solvent system.

### Spectroscopic Measurements

Fourier transform infrared (FTIR) spectra of the complex and ligand were measured from 4000 to 400 $cm^{-1}$ using KBr disks or nujol mulls respectively with a Nicolet FTIR Nexus Spectrometer, using 32 scans and a resolution of 4 $cm^{-1}$. UV-Vis spectra were obtained for methanol solutions from 205 to 900 nm with a Specord M50 (Germany) spectrophotometer. Proton NMR spectra were obtained in dimethyl sulphoxide-$d_6$ using a 300 MHz Mercury, Varian (U.S.A.) spectrometer. Copper analyses were performed with an AAS 1 atomic absorption spectrophotometer (Carl Zeiss, Jena, Germany).

### Measurements of the Relative Antioxidant Efficiency (RAE) using Oxygen Consumption Experiments

The method for determining antioxidant efficiencies was based upon the decrease in rate of oxygen consumption following the formation of linoleic acid radical ($L^\bullet$) in chlorobenzene, by thermal cleavage of 2,2′-azoisobutyronitrile (AIBN) and initiation of the oxidation of linoleic acid, reactions 1–5. Reaction 5, which is the rate limiting step, can be inhibited with an antioxidant capable of reacting with $LOO^\bullet$ and preventing subsequent oxidation of LH *via* chain propagating reactions, 4 and 5.

### Kinetic Analysis

The mechanism of the free radical chain reactions leading to the oxidation of linoleic acid in homogenous solution with a required concentration of oxygen $\geq 10^{-4}$ M needed to support the oxidation reaction (4) has been published.[31–36]

### Initiation

Oxidative chain reactions were initiated with the formation of ($R'^\bullet$) and ($R'OO^\bullet$) by reactions (1) and (2); and the conversion of these radicals to $L^\bullet$ by reaction (3):

$$R' - N = N - R' \xrightarrow{\Delta} [R'^\bullet N_2 R'^\bullet]_{\text{solvent cage}} \longrightarrow 2R'^\bullet + N_2 \qquad (1)$$

$$R'^\bullet + O_2 \overset{very\ fast}{\longrightarrow} R'OO^\bullet \qquad (2)$$

$$R'OO^\bullet + LH \longrightarrow R'OOH + L^\bullet \qquad (3)$$

where $R' - N = N - R'$ is the azo initiator, 2,2′-azoisobutyronitrile, $R'^{\bullet}$ is the cyano-iso-propyl radical, $(CH_3)_2C^{\bullet}C{\equiv}N$, and $R'OO^{\bullet}$ is the cyano-iso-propylperoxyl radical, $(CH_3)_2(N{\equiv}C)COO^{\bullet}$:

### Propagation

Propagation involves a cyclic process converting $L^{\bullet}$ to $LOO^{\bullet}$ in the presence of oxygen:

$$L^{\bullet} + O_2 \xrightarrow{v.fast} LOO^{\bullet} \quad (4)$$

$$LOO^{\bullet} + LH \xrightarrow{kp,slow} LOOH + L^{\bullet} \quad (5)$$

where $k_p$ is the rate constant for propagation.

### Termination

Chain termination reaction (6) limits the extent of lipid peroxidation in the absence of inhibitors, destroying two peroxyl radicals and yielding non-radical products.

$$LOO^{\bullet} + LOO^{\bullet} \xrightarrow{kt} \text{non-radical products} \quad (6)$$

where $k_t$ is the rate constant for termination. In the absence of a chain-breaking antioxidant and under a long-chain approximation, where the number of R• → ROO• cycles is large, the overall rate of uninhibited oxidation ($R_{uninh}$) is described by the kinetic equation:[32–34]

$$R_{uninh} = -\frac{d[O_2]_{uninh}}{dt} = \frac{k_p}{(2\,k_t)^{1/2}}[LH]R_i^{1/2} \quad (7)$$

where $R_i$ is the rate of initiation, and $-\mathrm{d}[O_2]_{uninh}/\mathrm{d}t$ is the rate of oxygen uptake for the uninhibited oxidation.

In the presence of a peroxyl radical trapping antioxidant, AH, the rate-controlling step for inhibition by the removal of $LOO^{\bullet}$ produced in reaction (4) is:

$$LOO^{\bullet} + AH \xrightarrow{k_{inh}} LOOH + A^{\bullet} \quad (8)$$

$$LOO^{\bullet} + A^{\bullet} \xrightarrow{fast} \text{non-radical products} \quad (9)$$

Under conditions of inhibition, the rate of oxidation is given by the kinetic equation;

$$R_{inh} = -\frac{d[O_2]_{inh}}{dt} = \frac{k_p}{nk_{inh}[AH]}[LH]R_i \quad (10)$$

where $-\mathrm{d}[O_2]_{inh}/\mathrm{d}t$ is the rate of oxygen uptake for the inhibited oxidation, $R_{inh}$ is the rate of inhibited oxidation of linoleic acid, $k_{inh}$ is the rate constant for the inhibition of the reaction of linoleic acid peroxyl radical with an antioxidant, and n is a stoichiometric factor for the removal of linoleic acid peroxyl radical by an antioxidant.[31–36]

### Oxygen Consumption Experiments

Stock solutions of 0.163 M LH and 0.1667 M AIBN were prepared in nitrogen-saturated chlorobenzene. Stock solutions of all tested compounds were prepared in butanol. The kinetics of oxygen consumption during the oxidation of linoleic acid was determined, at 760 torr $O_2$, using a highly sensitive calibrated 1 mm diameter capillary gas micro-volume meter to measure oxygen above the reaction mixture.[31,32] This oxidation reaction was performed in a 10 ml glass reaction vessel fitted with a water circulation jacket to facilitate temperature control and mounted on a magnetic stirrer. The reaction vessel was built to accommodate a membrane designed for the injection of reagent solutions without opening the cell. The vessel containing 4.6 ml 0.163 M LH in chlorobenzene was attached to the micro-volume meter. The stirred mixture was pre-equilibrated at the reaction temperature, 50°C, for 25 to 30 min to allow thermal equilibration and to stabilize the micro-volume meter reading prior to reaction initiation. Then the oxidation reaction was initiated as in the reaction sequence (1) to (3)[33–36] with the injection of 0.3 ml of a solution of AIBN in chlorobenzene through the membrane using a syringe. After 1 to 2 min, 0.1 ml of a butanol solution of the test antioxidant compound was introduced into the reaction mixture and, after 2 min, oxygen uptake was recorded as a function of time. The final volume of all reaction mixtures was 5 ml, the temperature was $50 \pm 0.1$°C, the final concentrations were 0.15 M LH, 0.01 M AIBN, and the concentration of the test compound varied in the range from $1.1 \times 10^{-6}$ to $5.5 \times 10^{-4}$ M. The reaction mixture was rapidly stirred at 600 rpm to ensure kinetic rather than diffusion control of the oxidation reaction rate.

Experiments to determine prooxidant reactivity of the test compound in the presence of LOOH were performed after allowing LOOH to accumulate in the absence of the test compound. Only after the accumulation of ~0.001 M LOOH, based upon the amount of consumed oxygen as determined earlier under the same reaction conditions, was a solution of the studied compound injected into the reaction mixture.

### Calculation of Oxygen Consumption Rates

The kinetics of oxygen consumption measured in the presence of various concentrations of the studied compounds based upon a kinetic plot of oxygen concentration change with time are shown in Figure 2. Initial rates of oxygen consumption were determined from these kinetic plots. The initial slope for the plot of $t_o$ to $t$ for these kinetic plots was calculated to obtain the initial rate of LH oxidation, $\mathrm{d}[O_2]_{inh}/\mathrm{d}t(t_o)$.

### Measurements of the Antioxidant Efficiency (AE), Relative Antioxidant Efficiency (RAE), and Antiperoxyl Radical Capacity (ARC)

Calculations of AE and RAE have been described in detail.[33] The plot of the initial rate of oxygen consumption, $\mathrm{d}[O_2]_{inh}/\mathrm{d}t\,(t_o)$, is dependent upon the inverse concentration of antioxidant, $[AH]_o^{-1}$, added to the oxidation reaction system. The concentration range was chosen to obtain a

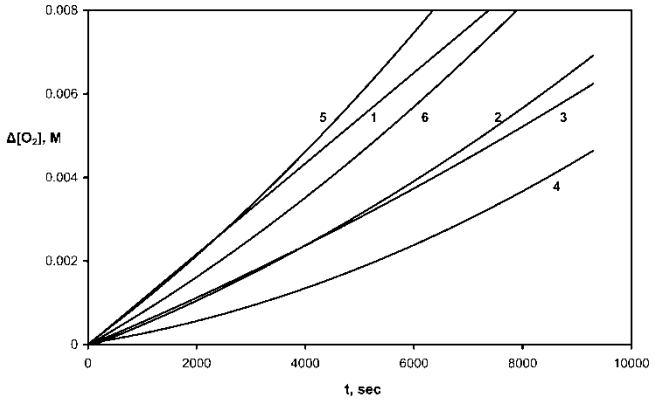



FIG 2. Kinetic plots of oxygen consumption during thermally initiated oxidation of 0.15 M linoleic acid by $10^{-2}$ M AIBN in chlorobenzene at 50°C ($R_i = 4 \times 10^{-8}$ $Ms^{-1}$): without antioxidant (1); $1.1 \times 10^{-4}$ M SalTyrOEt (2); $0.55 \times 10^{-4}$ M Cu(II)(SalTyrOEt)$_2$ (3); $1.1 \times 10^{-4}$ M BHT (4); in the presence of $0.275 \times 10^{-4}$ M Cu(II)(stearate)$_2$ (5); or $1.1 \times 10^{-4}$ M SalTyrOEt and $0.275 \times 10^{-4}$ M Cu(II)(stearate)$_2$ mixture (6).

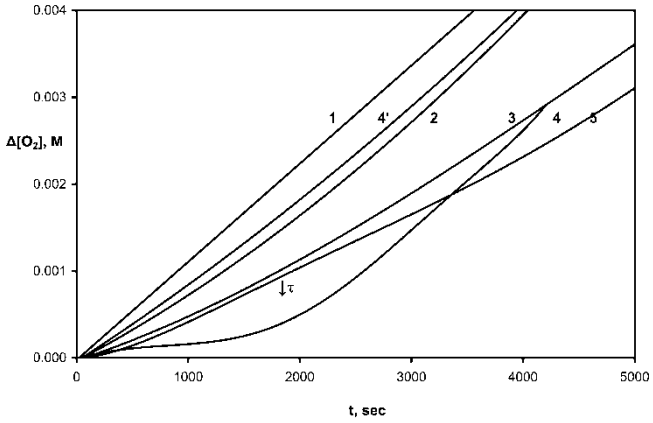



FIG. 3. The effect of various concentrations of Cu(II)(SalTyrOEt)$_2$ on oxygen consumption during thermally initiated oxidation of 0.15 M linoleic acid by $10^{-2}$ M AIBN in chlorobenzene at 50°C. Without antioxidant (1), or in the presence of $5.5 \times 10^{-6}$ M (2), $11 \times 10^{-6}$ M (3), $22 \times 10^{-6}$ M (4), or $55 \times 10^{-6}$ M (5) Cu(II)(SalTyrOEt)$_2$, or in the presence of both $22 \times 10^{-6}$ M Cu(II)(SalTyrOEt)$_2$ and $1 \times 10^{-3}$ M LOOH (4′) ($R_i = 4 \times 10^{-8}$ $Ms^{-1}$). The induction period, $\tau$, is indicated by an arrow.

rectilinear plot. From equation (10), a plot was calculated with the slope of S where S was defined in equation (11):

$$S = \frac{k_p[LH]R_i}{nk_{inh}} \tag{11}$$

The ratio of $k_{\text{ind}}$ to $k_{\text{p}}$ shown in equation (12) is defined as AE,

$$AE = \frac{k_{inhib}}{k_p} = \frac{[LH]R_i}{nS} \tag{12}$$

These values can be calculated from S, $R_i$, and n, which usually equals 2 for a monophenolic antioxidant.[31–36] The initiation rate, $R_i$, was calculated by measuring the time of the induction period, $\tau$ (see Figure 3), in the presence of a reference antioxidant, BHT, according to equation (13):

$$R_i = 2[BHT]_o\tau \tag{13}$$

where $[\text{BHT}]_\text{o}$ is the initial concentration of BHT.

The RAE was obtained directly from the ratio of the plot slopes by dividing the value obtained for the test antioxidant, AH, by the value obtained by the reference antioxidant, BHT, as shown in equation (14):

$$RAE = -\frac{AEAH}{AEBHT} = \frac{k_{inh}}{k_{inh,BHT}} = \frac{SBHT}{SAH} \tag{14}$$

The RAE can also be determined by dividing the value of AE for the antioxidant by the value of AE for BHT. The AE and RAE values are the quantitative kinetic parameters for antioxidant reactivity. Using experimental values for AE and RAE as well as literature data for BHT $k_{inh}$, $3 \times 10^4\,M^{-1}s^{-1}$,[32] $k_{inh}$ was determined for a test antioxidant from equation (14).

Antiperoxyl radical capacity (ARC), which is the measure of the number of radicals that react per antioxidant molecule.[33] For SalTyrOEt and Cu(II)(SalTyrOEt)$_2$ these values were calculated by measuring the induction period in the presence of the reference BHT antioxidant, $\tau_{\text{BHT}}$, and the test compound, $\tau_{\text{AH}}$, according to equation (15):

$$\text{ARC}_{\text{AH}} = (2[\text{BHT}]_\text{o}\tau_{\text{AH}})/([\text{AH}]_\text{o}\tau_{\text{BHT}}) \tag{15}$$

where $[\text{AH}]_\text{o}$ is the initial concentration of AH.

## RESULTS AND DISCUSSION

### Spectral Characterization of SalTyrOEt and Cu(II)(SalTyrOEt)$_2$ Complex

The SalTyrOEt ligand was characterized by $^1$H NMR, FTIR, and UV-Visible spectra. Proton NMR spectra were obtained in DMSO-d$_6$ and contained absorbances ($\delta$, ppm) at 1.26 t (3H, J = 7.1 Hz, $C^{10}H_3$); 2.97 dd (1H, J = 13.6, 8.3 Hz, $C^8H_a$); 3.18 dd (1H, J = 13.6, 5.4 Hz, $C^8H_b$); 4.16 dd (1H, J = 8.3, 5.4 Hz, $NC^7H$), 4.16 q (2H, J = 7.1, Hz, $OC^9H_2$); 6.61 m (2H, $C^{12}H$); 6.81 td (1H, J = 7.4, 1.3 Hz, $C^3H$); 6.84 br.d (1H, J = 8.5, Hz, $C^1H$); 6.91 m (2H, $C^{11}H$); 7.22 dd (1H, J = 7.6, 1.8 Hz, $C^4H$), 7.27 ddd (1H, J = 8.5, 7.2, 1.8 Hz, $C^2H$); 8.15 s (1H, N = $C^5H$); 8.83 br.(1H, salicyl-$O^6H$); 12.84 br. (1H, tyrosyl-$O^{13}H$). The salicyl OH evidences the greatest shift due to intramolecular hydrogen bonding shown in Figure 1a. The tyrosine phenolic group is also shifted due to intermolecular hydrogen bonding with DMSO.

The UV-Visible spectrum of SalTyrOEt contained three maxima, $\lambda_{\text{max}}$ (nm) and $\varepsilon$ ($M^{-1}cm^{-1}$), at 214.6 (25640) for C═O, 258.8 (11340) for the salicyl ring, and 317.2 (4060) for the tyrosyl ring.

The FTIR spectrum of the Schiff base contains absorptions at 1735 and 1643 $cm^{-1}$ assignable to the ester carbonyl group and the azomethyne, CH═N, group vibrations, respectively. The salicyl phenolic OH group exhibits a very broad absorption through the range of 2200 to 3200 $cm^{-1}$ due to overlapping aromatic absorptions centered at 2520, 2600, 2683, and 2806 $cm^{-1}$. A broad absorption through the range of 2800 to 3200 $cm^{-1}$ is assigned to aromatic and aliphatic CH absorbances, which overlap with the 2806 $cm^{-1}$ absorption. The tyrosyl-phenolic OH group gave a broad absorption through the range of 3200 to 3600 $cm^{-1}$, centered at 3454 $cm^{-1}$ and overlapping with absorptions for the CH and salicyl-phenolic OH absorptions. The broad absorption for the salicyl OH group was attributed to intramolecular hydrogen bonding between the phenolic OH hydrogen atom and the nitrogen of the azomethyne group as shown in Figure 1a. The absorption observed at 1242 $cm^{-1}$ is attributed to salicyl phenolic C–O stretching and the absorption observed at 1258 $cm^{-1}$, is attributed to tyrosyl phenolic C–O stretching

The Cu(II) complex is soluble in DMF, DMSO, methanol, butanol, and other polar solvents and is quite stable in air under dry and dark conditions, giving reproducible kinetics as an antioxidant over a 6 month period. Elemental analysis data support the formation of a 1:2 complex, Cu(II)(SalTyrOEt)$_2$. The UV-Visible spectrum obtained in methanol had absorptions at $\lambda_{max}$ nm with $\varepsilon$ ($M^{-1}cm^{-1}$) = 223.4 (55160), 242 (34950) shoulder, 269.4 (23000), 370 (8260), and 632 (120). The broad absorption centered at 632 nm was assigned to a d-d transition and indicates a distorted octahedral geometry around the Cu(II) atom with two nitrogen ligand atoms. The band at 370 nm is due to a phenolate → Cu(II) charge transfer transition, which indicates bonding of the salicyl-phenolate oxygen atom to Cu(II).[37,38] The FTIR spectrum of the Cu(II) complex contained absorptions at 3419, 1714, and 1619 $cm^{-1}$ assignable to the tyrosyl-phenolic OH group, the ester carbonyl group, and the azomethyne group vibrations, respectively. The 21 $cm^{-1}$ shift to lower frequency for the carbonyl group and a 24 $cm^{-1}$ shift for the azomethyne group as well as the decrease in absorption intensity for the carbonyl oxygen suggest bonding of the carbonyl oxygen and azomethyne N atoms to copper, consistent with published literature for complexes with similar ligands[37]-[40]. The salicyl phenolic C–O absorbance at 1242 $cm^{-1}$ is lost and new broad absorptions occur at 1258 $cm^{-1}$ and 756 $cm^{-1}$ upon complex formation, indicating bonding of the salicyl phenolic oxygen atom and the Cu(II) atom, which is also consistent with the published literature.[37–40] The very broad 2200 to 3200 $cm^{-1}$ absorption of the free salicyl phenolic OH group of the ligand is not present in the copper complex, which supports deprotonation of the salicyl-phenolic OH group with Cu(II) coordination. This is also supported by the appearance of a new band at 460 $cm^{-1}$ which is attributed to Cu(II)–O salicyl-phenolate vibrations.[37–40]

Results of elemental analyses, FTIR, and UV-Vis spectra show that SalTyrOEt coordinates with the Cu(II) atom in a tridentate manner *via* the carbonyl oxygen, azomethine nitrogen, and the salicyl-phenolate $O^-$ groups to form a mononuclear complex with proton loss from the salicyl phenolic OH group consistent with the structure shown in Figure 1b.

## Antioxidant Efficiencies

As shown in Figure 2, plot 1, there was a rapid linear consumption of oxygen with time in the absence of antioxidant for the uninhibited reaction: $LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ (Reaction 5) leading to the regeneration of $LOO^{\bullet}$ in the subsequent reaction: $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ (Reaction 4). Addition of $1.1 \times 10^{-4}$ M SalTyrOEt, $5.5 \times 10^{-5}$ M Cu(II)(SalTyrOEt)$_2$ or $1.1 \times 10^{-4}$ M BHT, representing equal concentrations of the phenolic OH group, caused a marked decrease in rate of oxygen uptake (plots 2, 3, and 4, respectively) compared to the uninhibited reaction rate (plot 1). This shows that the studied compounds have chain-breaking antioxidant reactivity, which may be due to the antioxidant reactivity of SalTyrOEt and the removal of $LOO^{\bullet}$ (Reactions 16–18 in the case of SalTyrOEt). The decrease in the oxygen consumption rate is also consistent with antioxidant reactivity in the case of TyrOEt. When TyrOEt, SalTyrOEt or BHT were introduced into the oxidation system at concentrations ranging from $5.5 \times 10^{-6}$ to $5.5 \times 10^{-4}$ M, the initial rate of oxygen consumption decreased linearly with increasing concentration. As shown in Figure 3, addition of increasing concentrations of Cu(II)(SalTyrOEt)$_2$ ranging from $5.5 \times 10^{-6}$ to $2.2 \times 10^{-5}$ M (plots 2 to 4) also decreased the initial rate of oxygen consumption in a concentration related manner when compared with the uninhibited reaction (plot 1), with a nearly constant decrease in the initial rate of oxygen uptake over the first 2000 sec for plots 2, 3 and 4 when compared to plot 1.

Quantitative kinetic antioxidant parameters obtained using experimental curves for oxygen consumption in the presence of various concentrations of the studied compounds and equations (12–15) for the addition of TyrOEt, SalTyrOEt, Cu(II)(SalTyrOEt)$_2$, or BHT to the linoleic acid oxidation reaction mixture are presented in Table 1.

Based upon the RAE, AE, $k_{inh}$, and ARC data shown in Table 1, Cu(II)(SalTyrOEt)$_2$ and SalTyrOEt have antioxidant, antiperoxyl radical reactivity, similar to that of BHT in inhibiting oxidations caused by linoleic acid peroxyl radical in a lipid reaction medium. Dividing the RAE and ARC values obtained for Cu(II)(SalTyrOEt)$_2$ by 2 to obtain an equivalent number of tyrosyl-phenolic OH groups (Table 1) shows that the apparent greater antioxidant reactivity of this complex is due to its greater number of tyrosyl-phenolic OH groups. Thus, these results indicate a BHT-like chain-breaking antioxidant mechanism[32] implying that mechanisms of antiperoxyl radical and antioxidant reactivity of Cu(II)(SalTyrOEt)$_2$, SalTyrOEt, and TyrOEt involve abstraction of the hydrogen atom from the tyrosyl-phenolic OH group by linoleic acid peroxyl

TABLE 1
Values of RAE, AE, $k_{inh}$ and ARC

| Antioxidant | RAE | AE = $k_{inh}/k_p$ | $k_{inh}$ | ARC |
|---|---|---|---|---|
| BHT | 1 | [a]300 | [b]$3 \times 10^4$ | 2 |
| TyrOEt | 0.189 | 57 | $5.66 \times 10^3$ | [c] |
| SalTyrOEt | 0.316 | 94 | $9.35 \times 10^3$ | $2.55 \pm 0.20$ |
| Cu(II)(SalTyrOEt)$_2$ | 1.92 | 578 | $5.78 \times 10^4$ | $3.58 \pm 0.20$ |
| Cu(II)(SalTyrOEt)$_2 \div 2$ | 0.96 | 289 | $2.89 \times 10^4$ | $1.79 \pm 0.10$ |

[a]The $k_{inh}/k_p$ value for BHT agrees with the literature,[32] [b]$k_p = 100$ $M^{-1}s^{-1}$,[41] [c]not determined.
Obtained for test compounds in the thermally initiated oxidation of 0.15 M linoleic acid with $10^{-2}$ M AIBN ($R_i = 4 \times 10^{-8}$ $Ms^{-1}$) in chlorobenzene at 50°C, equations (12) to (14).



radical to form a tyrosyl-phenoxyl radical, TyrO$^\bullet$, as shown in reaction (16), which rapidly reacts to form non-radical products, as in reactions (17) and (18):

$$LOO^\bullet + TyrOH \xrightarrow{k_{inh}} LOOH + TyrO^\bullet \quad (16)$$

$$LOO^\bullet + TyrO^\bullet \xrightarrow{fast} \text{non-radical products} \quad (17)$$

$$TyrO^\bullet + TyrO^\bullet \xrightarrow{fast} \text{non-radical products} \quad (18)$$

Additional evidence that the antioxidant or antiperoxyl radical reactivities of SalTyrOEt and Cu(II)(SalTyrOEt)$_2$ are due to the presence of the tyrosyl-phenolic OH group in these molecules comes from the fact that Schiff bases that do not have an equivalent tyrosyl-phenolic OH group such as N,N′-bis (salicylidene)ethylenediamine and salicylidene Schiff bases of ethyl esters of γ-aminobutyric acid or tryptophan do not possess measurable antiperoxyl radical reactivity (our unpublished observation). The phenolic OH of the salicyl group present in N,N′-bis(salicylidene)ethylenediamine, in salicylidene Schiff bases of γ-aminobutyric acid ethyl ester and tryptophan, as well as in SalTyrOEt, does not contribute to antiperoxyradical reactivity because of intramolecular hydrogen bonding between the hydrogen atom of the salicyl-phenolic OH group and the nitrogen of azomethyne group as shown in Figure 1a for SalTyrOEt. This intramolecular hydrogen bonding prevents phenoxyl radical formation and markedly reduces the antioxidant or antiperoxyl radical reactivity of these phenolic hydroxyl groups, consistent with literature reports.[23,42–44]

The ARC value for SalTyrOEt and Cu(II)(SalTyrOEt)$_2$, calculated per tyrosine ligand, is close to 2, see Table 1, which indicates that the ligand has one phenolic antiperoxyl radical reaction center. The somewhat larger ARC value for SalTyrOEt than for BHT or Cu(II)(SalTyrOEt)$_2$, suggests that the salicylic phenolic OH group may play some role in reactions with peroxyl radicals, although this role is probably small since the difference in ARC values is not large. Conversely, the value of $k_{inh}$ is larger for Cu(II)(SalTyrOEt)$_2$ than for compounds having salicylic phenolic OH groups.

Since the ARC value for Cu(II)(SalTyrOEt)$_2$ is close to 2, the Cu(II) atom cannot be involved in antiperoxyl radical reactions of Cu(II)(SalTyrOEt)$_2$. Direct participation of Cu(II) in the removal of peroxyl radicals would result in a larger ARC value for the complex, close to 2.5. The high oxidation potential of Cu(II), $E^\circ_{Cu(II)/Cu(III)} = -2.6$ V at pH ≈ 7,[45] indicates that the reaction Cu(II) + LOO$^\bullet$ → Cu(III) is much less likely to occur than oxidation reactions involving peroxyl radicals such as tert-butylOO$^\bullet$, $E^{\circ -}_{\text{tert-butylOO}\bullet/\text{tert-butylOO}} = -0.7$ V.[46] The lack of a measurable reaction between Cu(II) and *tert*-butyl peroxyl radical has been previously reported.[23]

## Prooxidant Reactivities

TyrOEt, SalTyrOEt, and BHT have no prooxidant reactivity in our experimental conditions. Copper(II) complexes possess prooxidant reactivities including peroxidase-mimetic reactivity, which is due to the ability of Cu(II) complexes to catalyze the removal of accumulated LOOH formed in the chain oxidation of linoleic acid as well as in the antioxidation reaction of LOO$^\bullet$ with tyrosyl phenolic OH groups. Products of prooxidation, LOO$^\bullet$ and LO$^\bullet$, formed in these reaction processes then co-initiate the chain peroxidation of linoleic acid.[14–21] The prooxidant reactivity of Cu(II) complexes, which involves the oxidation of LOOH (19), the homolysis of LOOH (20) by Cu(I) formed in reaction (19) and the peroxidase-mimetic reactivity (21), was evaluated for Cu(II)(SalTyrOEt)$_2$ at times long after the peroxidation reaction induction period, τ, to allow for accumulation of LOOH. The increased oxidation rate in the presence of Cu(II)(SalTyrOEt)$_2$, in comparison with the uninhibited oxidation reaction rate, indicates that this prooxidant reactivity, reactions (19) and (20), is observable once linoleic acid hydroperoxide accumulates as a reaction product.

$$Cu(II) + LOOH \longrightarrow Cu(I) + LOO^\bullet + H^+ \quad (19)$$

$$Cu(I) + LOOH \longrightarrow Cu(II) + LO^\bullet + OH^- \quad (20)$$

$$Cu(I) + LOOH \longrightarrow LO - Cu(III) - OH \quad (21)$$

An alternative explanation for the ability of Cu(II) to induce lipid peroxidation in the presence of a lipid hydroperoxide but

not a reducing agent, involves the oxidation of Cu(II) by the hydroperoxide, forming Cu(III) and the lipoxyl radical, reaction 22:[18]

$$Cu(II) + LOOH \longrightarrow Cu(III) + LO^{\bullet} + OH \qquad (22)$$

Both the lipoxyl radical $LO^{\bullet}$ and Cu(III) generated according to reaction (22) are capable of initiating lipid peroxidation, reaction (23), leading to the regeneration of $LOO^{\bullet}$, reaction (4):

$$Cu(III) + LH \longrightarrow Cu(II) + L^{\bullet} + H^{+} \qquad (23)$$

$$L^{\bullet} + O_2 \xrightarrow{v.fast} LOO^{\bullet}$$

It should be noted that as the oxidation reaction proceeds, the antioxidant copper complex becomes depleted and other copper complexes accumulate in the system, the ligands of which are the products of peroxyl radical reactions with tyrosine OH groups. These ligands, which do not have antiperoxyl radical centers, can influence the Cu(I)/Cu(II), Cu(II)/Cu(III), Cu(I)/Cu(III) redox potentials and strengthen the prooxidant reactivity of the metabolites of Cu(II)(SalTyrOEt)$_2$. This could explain the observed increase in rate of oxygen consumption at the end of uninhibited oxidations in the presence of Cu(II)(SalTyrOEt)$_2$ (Figure 3, plots 2 and/or 4).

Copper chelation by the SalTyrOEt ligand suppresses the prooxidant reactivity of the central metalloelement. This is evidenced by the fact that Cu(II)(SalTyrOEt)$_2$ more effectively suppresses the rate of oxygen consumption during the oxidation of linoleic acid than does an equimolar mixture of Cu(II)$_2$(stearate)$_4$ and SalTyrOEt (see Figure 2, plots 3 and 5). Additional evidence for this comes from the observation that Cu(II)$_2$(stearate)$_4$ does not show antioxidant reactivity, but instead has prooxidant reactivity as shown in Figure 2, plot 6. Addition of $2.75 \times 10^{-5}$ M Cu(II)$_2$(stearate)$_4$ to the reaction mixture caused a rapid consumption of oxygen at a level which was slightly greater than that observed for the uninhibited reaction.

One possible mechanism explaining the prooxidant reactivity of Cu(II) complexes could be oxygen activation. However, when the oxidation reaction of linoleic acid is performed in air (at an oxygen partial pressure of 150 mm Hg, no change in kinetics or initial rate was observed for the oxidation reaction. This result suggests that oxygen activation involving coordination to Cu(II) does not occur under our experimental conditions.

However, Cu(II)(SalTyrOEt)$_2$ has prooxidant reactivity. The initial reaction kinetics for the reactivity of Cu(II)(SalTyrOEt)$_2$ prooxidation were studied in the presence of 0.001M hydroperoxide, LOOH. As shown in Figure 3, plot 4′, the rate of oxygen consumption markedly increased, demonstrating prooxidant reactivity for Cu(II)(SalTyrOEt)$_2$, with an ARC value for Cu(II)(SalTyrOEt)$_2$ being smaller than 4 (Table 1). These experimental data directly show that reactions (19), (20), and/or (22) occur in the oxidation system. This prooxidant reactivity is also consistent with the observation the oxygen consumption rate decreased as the complex concentration increased up to $2.2 \times 10^{-5}$ M Cu(II)(SalTyrOEt)$_2$ (Figure 3, plots 2, 3 and 4). Upon a further increase in concentration to $5.5 \times 10^{-5}$ M, the antioxidant reactivity begins to exceed the prooxidant reactivity (Figure 3, plot 5), as expected when compared with the decrease shown for $2.2 \times 10^{-5}$ M Cu(II)(SalTyrOEt)$_2$. In addition, when the concentration of Cu(II)(SalTyrOEt)$_2$ is increased, the decrease in the rate of oxygen consumption persists longer. These results indicate that Cu(II)(SalTyrOEt)$_2$ possesses low prooxidant reactivity at concentrations $\leq 5.5 \times 10^{-5}$ M.

### Comparison of Antioxidant and Prooxidant Reactivities

The marked chain-breaking antioxidant reactivities of Cu(II)(SalTyrOEt)$_2$ are kinetically dominant and Cu(II)(SalTyrOEt)$_2$ acts as a phenolic antioxidant as does the free ligand, SalTyrOEt, which does not possess any measurable prooxidant properties under conditions of these experiments.

The antiradical tyrosyl-phenol OH reaction center of Cu(II)(SalTyrOEt)$_2$ removes $LOO^{\bullet}$ formed in reaction (5), as well as $LOO^{\bullet}$ and $LO^{\bullet}$ formed in the removal of LOOH by reactions (19), (20), and (22), and thus suppresses the free radical oxidative reactions initiated by these species as well as the chain propagation reactions (4), and (5). During the induction period, when the concentration of LOOH is still low and the concentration of the tyrosine centers is still appreciable, the formation of non-radical products occurs as in reaction (21). Therefore this ligand converts potential prooxidant reactivity of Cu(II) to a lipid hydroperoxide detoxifying and utilizing antioxidant compound in a lipid medium. Under these conditions the resultant complex manifests properties of a sterically non-hindered phenolic antioxidant without direct involvement of the central metalloelement in antiperoxyl radical reactions, without demonstrating prooxidant reactivity.

## SUMMARY AND CONCLUSIONS

We report here the synthesis, characterization, and evaluation of a new Schiff base, salicylidene-D,L-tyrosine ethyl ester ligand, SalTyrOEt (Figure 1a), and the Cu(II) complex, Cu(II)(SalTyrOEt)$_2$ (Figure 1b) for antioxidant and prooxidant reactivities. Kinetic parameters for chain-breaking antioxidant efficiencies were determined in an in vitro linoleic acid initiated peroxidation reaction system selected as a biochemically relevant lipid model system.

Results of this research show that SalTyrOEt coordinates with Cu(II) in a monobasic tridentate manner via a carbonyl oxygen, the azomethine nitrogen, and the salicyl-phenolate oxygen produced by loss of the proton from the salicyl-phenolic OH group. Cu(II)(SalTyrOEt)$_2$ and its ligand were found to possess antiperoxyl radical antioxidant reactivity as a phenolic antioxidant in a lipid medium. This complex and its ligands react as sterically unhindered phenols to remove peroxyl radicals, which are responsible for the chain

reaction process of linoleic acid peroxidation. It has also been established that this complex does not manifest any prooxidant reactivity due to the presence of Cu(II) at low concentrations $\leq 5.5 \times 10^{-5}$ M. The decreased prooxidant efficiency is due to two factors: (a) Bonding of the SalTyrOEt ligand with the central metalloelement suppresses redox reactivity due to reactions (19) to (21); and (b) the existence of an antioxidant reactivity center on the ligand that is capable of removing both $LOO^{\bullet}$ and $LO^{\bullet}$. Thus, Cu(II)(SalTyrOEt)$_2$ combines very low prooxidant and high chain-breaking antioxidant reactivities. As a result of this combination, this copper complex as well as copper complexes of other ligands manifesting high chain-breaking antioxidant reactivity, can assist in nonradical mediated removal of elevated concentrations of lipohydroperoxides and lipoperoxyl radicals that may accumulate in lipids and can produce a positive therapeutic effect in disease states caused by oxidative stress. These observations provide valuable information for the design and synthesis of new transition metalloelement complexes with potential utility as pharmacologically effective bioactive complexes.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| LH | Linoleic acid |
| $L^{\bullet}$ | Linoleic acid alkyl radical |
| $LOO^{\bullet}$ | Linoleic acid alkyl peroxyl radical |
| $R'^{\bullet}$ | Cyanoisopropyl radical |
| $R'OO^{\bullet}$ | Cyanoisopropylperoxyl radical |
| $R_o$ | Rate of peroxidation in the absence of anti-oxidant |
| $R_i$ | Rate of initiation |
| BHT | 2,6-di-tert-butyl-4-methylphenol |
| $k_{inh}$ | Rate constant of inhibition |
| N | Stoichiometric factor |
| R | Rate of peroxidation |
| $R^2$ | Squared correlation factor |
| LH | Linoleic acid |
| LOOH | Linoleic acid hydroperoxide |
| $LO^{\bullet}$ | Linoleic acid oxyl radical |
| ARC | Antiperoxyl radical capacity |
| $ArO^{\bullet}$ | Phenoxyl radical |
| FTIR | Fourier Transform Infrared |
| T | Temperature |
| AIBN | 2,2′-Azobis(isobutyronitrile) |
| AH | Antioxidant |
| NMR | Nuclear Magnetic Resonance |
| UV | Ultraviolet |

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