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Reactivity of Cu_2(lonazolac)$_4$, a Lipophilic Copper Acetate Derivative

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An acetate-like copper complex of lonazolac (3-(p-chlorophenyl)-1-phenyl-pyrazole-4-acetate) was prepared and characterized. The copper of Cu_2-(lonazolac)$_4$ was spin-coupled and remained EPR-silent. Water and organic solvents did not affect this magnetic interaction. Superoxide dismutase activity of the Cu complex in micromolar concentrations was detectable in the presence of up to 900 μg per ml of serum albumin or whole serum protein. At 700 μM albumin concentration, a ternary complex between Cu_2(lonazolac)$_4$ and the protein was formed. The original acetate–copper coordination changed to a biuret-type copper bonding as seen from EPR and electron absorption spectrometry. Lonazolac did not induce a detectable conformational change of the protein near or at the copper binding site. Equilibrium dialysis and optical titration experiments revealed that essentially all copper of the Cu_2-(lonazolac)$_4$ complex was bound in the specific binding site of serum albumin. The copper complex proved to be an effective inhibitor of lipid peroxidation.

Introduction

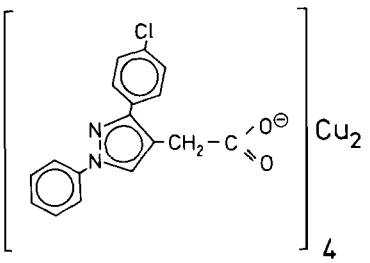
The superoxide dismutase activity of intracellular cuprein (Cu_2Zn_2-superoxide dismutase) and many low molecular weight copper chelates is an established phenomenon [for reviews see 1, 2]. They are thought to control superoxide concentration in biological systems. Among the superoxide dismutase mimics, copper complexes of the non-steroidal anti-inflammatory drugs are of special significance. These complexes are known to be lipophilic and are thus able to reach superoxide in a hydrophobic environment. A suitable lipophilic non-steroidal ligand proved to be 3-(p-chlorophenyl)-1-phenylpyrazole-4-acetate (lonazolac).

The copper complex was synthesized and chemically characterized. Superoxide dismutase activity and its possible suppression of lipid peroxidation were determined. Studies of the binding of Cu complexes to serum albumin by means of equilibrium dialysis were carried out to shed some light on the biological transport from the site of application to the possible site of action. These experiments should partially simulate the conditions in the extracellular fluid of inflamed regions. The reaction of Cu_2(lonazolac)$_4$ with serum albumin was controlled by circular dichroism measurement, electron absorption and electron paramagnetic resonance spectrometry.

Experimental

Reagents

All chemicals employed were of analytical grade quality. Bovine serum albumin was from Sigma, München, whole calf serum was purchased from Serva, Heidelberg. For the preparation of ultrafiltrate, whole serum was filtered at 2 °C under N_2 pressure through a nitrocellulose membrane, separation limit M_r = 10,000 (PM 10 Amicon, Oosterhout). Cu_2(salicylate)$_4$ and Cu_2(indomethacin)$_4$ were prepared according to refs. [3, 4]. Cu_2[3-(p-chlorophenyl)-1-phenylpyrazole-4-acetate]$_4$, Cu_2(lonazolac)$_4$, was prepared by dissolving 2 mmol of the ligand in 2.5 ml 0.1 *M* NaOH. After dilution with H_2O to 20 ml, 5 ml aqueous $CuSO_4$ (1 mmol) were slowly added. The greenish precipitate was repeatedly washed, centrifuged and dried over P_4O_{10}. Crystallization succeeded in dimethylsulphoxide heated to 70 °C. After 1 h dark green crystals were obtained. The elemental analysis was C 58.27%; H 3.88%; N 8.48%; Cu 10.4%; Cl 10.69% and was close to the theoretical values of a Cu(lonazolac)$_2$ complex: C 59.38%; H 3.49%; N 8.15%, Cu 9.24% and Cl 10.32%. Biogel P-2 chromatography revealed a M_r value near 800 suggesting the dimeric molecule Cu_2(lonazolac)$_4$ (M_r = 687.1).



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Spectrometry

Copper was monitored on a Perkin Elmer absorption unit equipped with a HGA 76 B graphite cuvette. Electron absorption spectra were run on a Beckman Model 25 spectrophotometer. Circular dichroism was measured on a J 20 A spectropolarimeter (Jasco, Tokyo). Electron paramagnetic measurements were detected at 77 K on an E-109 spectrometer (Varian, Darmstadt).

Assays

Superoxide dismutase activity was measured using the nitro blue tetrazolium assay [5]. Both inorganic and enzymically generated superoxide was used. One ml of the assay mixture contained 195 μM nitro blue tetrazolium in 100 mM potassium phosphate buffer, pH 7.8 and different concentrations of the employed copper complex. The reaction was started with 1.5 μmol KO_2. Superoxide was produced enzymically by the xanthine/xanthine oxidase reaction with this procedure. One ml of the reaction volume contained 0.62 mM nitro blue tetrazolium, 50 mM HEPES buffer, pH 7.8; 0.2% (w/v) gelatine; 50 μM xanthine; 0.18 μM xanthine oxidase; copper and serum albumin or whole serum in variable concentrations. A_{540} was recorded in a 10 mm light path cell at 23 °C. The reproducibility of the data was better than ± 5%.

Lipid Peroxidation

Peroxidation of linolenic acid in the presence of chelated copper was quantified using a modification of the procedure given in ref. 6. Linolenic acid was oxidized using the xanthine oxidase/acetaldehyde system. One ml of the reaction medium contained 15 mM sodium phosphate buffer, pH 7.4; 500 μM linolenic acid; 10% (v/v) 1,2-dimethoxyethane; 0.18 μM xanthine oxidase; 48 mM acetaldehyde; start with 20 μl acetaldehyde (2.4 M). After 30 min A_{238} was recorded in a 10 mm light path quartz cell at 23 °C for another 30 min in the linear phase of the absorption increase. The standard error of the mean value was less than 2%.

Equilibrium Dialysis

The equilibrium dialysis unit consisted of two tightly fitting polyacrylic blocks (24 × 6 × 1.5 cm) each containing 5 cylindrical holes (15 mm × 5.7 mm) of 1000 μl volume. The two sets of holes were separated by a cellulose membrane, 1.5–2.0 nm pore size, Visking dialysis tubing (No. 27/32) (Serva, Heidelberg). Only 900 μl were injected perpendicularly into each cylindrical cell through channels 1.5 mm wide. A second channel ascertained the displacement of air. A remaining air bubble served as an internal stirrer. The apparatus was rotated at approx. 20 rpm. In the case of serum albumin dialysis was performed for 16 h at 23 °C. When whole calf serum was employed a dialysis time of 21 h at 2 °C became necessary.

Results

Molecular Properties

The relative molecular mass of the prepared copper complex below 1000, the chemical analyses, the relationship to coordinated acetate and the antiferromagnetic properties of the copper suggest a Cu_2-(acetate)$_4$-like metal binding centre. The copper core is surrounded by four lipophilic residues (Fig. 1).

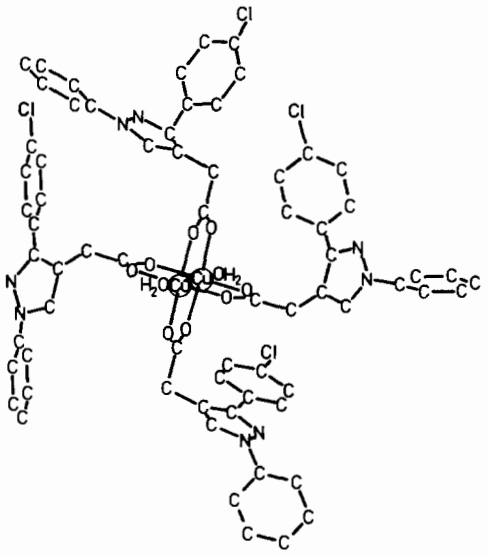


Fig. 1. Structure of Cu_2(lonazolac)$_4$. All hydrogen atoms are deleted.

The solubility in water was approximately 60 μM. The electron absorption of the aqueous complex was low ($\epsilon_{Cu,720}$, 45 M^{-1} cm^{-1}). In aprotic solvents the solubility increased substantially: dimethylsulphoxide, 500 μM; triolein, 2 mM and dioxane, 50 mM.

Reaction of Cu_2(lonazolac)$_4$ with Bovine Serum Albumin

The reaction of Cu_2(lonazolac)$_4$ with serum albumin was examined by electron absorption, circular dichroism and electron paramagnetic resonance measurements.

Titration of serum albumin with both copper sulphate and Cu_2(lonazolac)$_4$ showed the blue shift of the 720 nm band to 530 nm ($\epsilon_{Cu,530} = 100$ M^{-1} cm^{-1}). Plotting of either copper compound *versus* A_{530} made no difference (Fig. 2). In the presence of substoichiometric copper to albumin concentrations, especially at concentrations similar to those found under physiological conditions (700 μM albumin, 15 μM copper), essentially all copper was bound in the specific copper-binding site of serum albumin characterized by its absorption at 530 nm. This specific copper coordination has been assigned to the N-terminal tripeptide NH_2·asp·thr·his– [7].

When copper and albumin concentrations are identical, some 10% of the copper is extraneously bound by serum albumin which parallels a broad absorption increase around 670 nm. Equilibrium dialysis showed that essentially no low molecular weight copper species were detected in the dialysate.

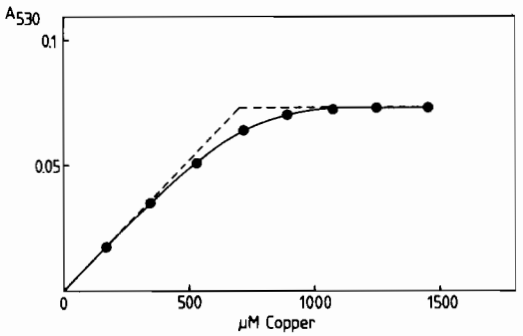


Fig. 2. Titration of serum albumin with Cu_2(lonazolac)$_4$ and $CuSO_4$. One ml contained 700 μM bovine serum albumin in 20 mM HEPES buffer pH 7.4; 150 mM NaCl and different concentrations of either Cu_2(lonazolac)$_4$ or $CuSO_4$. A_{530} was measured at 23 °C.

More important, the coordination sphere had changed from a homogeneous oxygen–copper bonding of the Cu_2(lonazolac)$_4$ into a biuret type compound indicative for the 530 nm absorption. This conclusion was supported by EPR data (Fig. 3).

In aqueous Cu_2(lonazolac)$_4$ all copper proved to be fully antiferromagnetically coupled. Only the baseline was seen. The same baseline was recorded using the solid compound or after dissolving in aprotic solvents.

The addition of bovine serum albumin gave rise to the appearance of a classical biuret type EPR signal, indicative that the low relative molecular mass copper complex has been disrupted by the large biopolymer. Nevertheless, according to equilibrium dialysis experiments the lonazolac ligand remained tenaciously bound to serum albumin. However, direct coordination to the copper cannot be deduced as the addition of $CuSO_4$ alone resulted in exactly the same EPR spectrum both in shape and magnitude. The conclusion that copper alone is coordinated to the specific copper binding site of serum albumin is supported.

Circular dichroism measurements were carried out to examine whether or not lonazolac is able to induce a conformational change of the specific copper–albumin complex. In the absence and presence of 700 μM lonazolac no detectable difference in the Cotton extrema at 490 nm (θ = 1780 degree X cm^2 X decimol^{-1}) and 575 nm (θ = −4340 degree X cm^2 X decimol^{-1}) was measured. Bovine serum albumin and Cu^{2+} were 700 μM and 350 μM, respectively.

Superoxide Dismutase Activity

The nitro blue tetrazolium reduction with KO_2 in the absence of gelatine was inhibited 50% at 1.5 μM

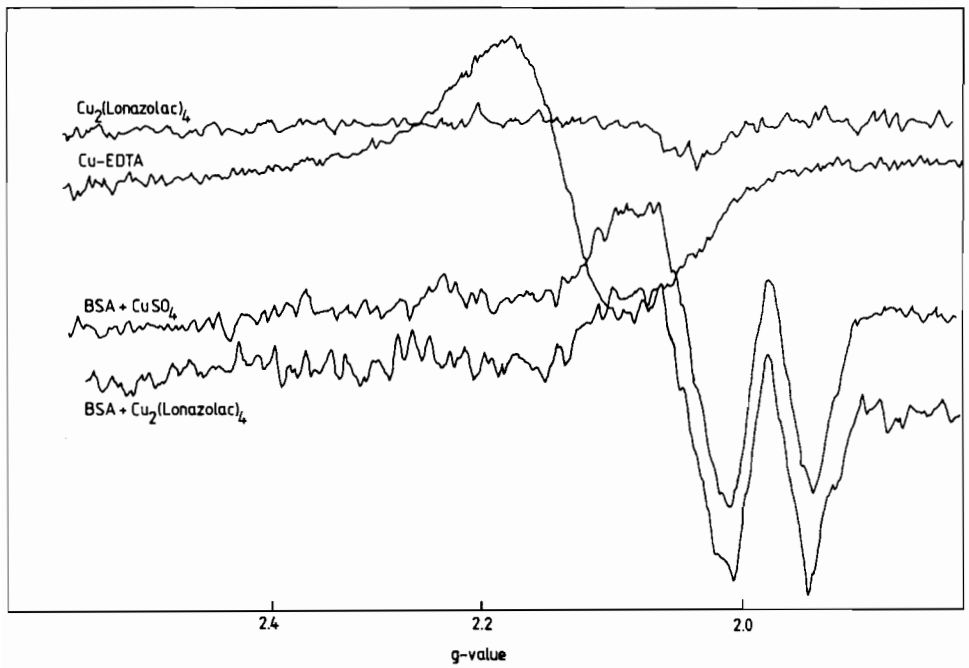


Fig. 3. Electron paramagnetic resonance of Cu_2(lonazolac)$_4$ in the presence of serum albumin. The copper concentration of all employed complexes was identical at 61 μM, albumin 82 μM. Cu–EDTA served as the standard. Recording conditions: Modulation amplitude 10 G; modulation frequency 100 kHz; microwave power 20 mW; microwave frequency 9.24 GHz; the temperature was 77 K.

TABLE I. Superoxide Dismutase Activity of Copper Complexes in the Presence of Serum Albumin. One ml of the reaction volume contained 0.62 m*M* nitro blue tetrazolium; 50 m*M* HEPES buffer, pH 7.8; 0.2% (w/v) gelatine; 50 μM xanthine; 0.18 μM xanthine oxidase; 3 μM copper and serum albumin in variable concentrations. A_{540} was recorded at 23 °C. It should be noticed that the addition of $CuSO_4$ may result in the chelation of Cu by xanthine oxidase, xanthine, gelatine or nitro blue tetrazolium.

Cu Complex [3 μM Cu(II) throughout]	Inhibition of NBT-Reduction [%]					
$CuSO_4$	59	60	45	29	9	7
Cu_2(Salicylate)$_4$	62	60	55	28	10	4
Cu_2(Indomethacin)$_4$	60	56	44	30	11	5
Cu_2(Lonazolac)$_4$	58	55	46	33	15	11
μM bovine serum albumin added	0	0.17	0.83	1.7	8.3	17.0

concentrations of Cu complexes. Twice the concentration of copper chelate became necessary when xanthine/xanthine oxidase was the $\cdot O_2^-$ source. It should be emphasized that there are essentially no aqueous cupric ions. The dissociated Cu^{2+} of $CuSO_4$ will have reacted with the buffer, xanthine, nitro blue tetrazolium or even with the enzyme protein. Upon titrating with increasing concentrations of serum albumin the superoxide dismutase activity is progressively diminished. In the case of Cu_2(lonazolac)$_4$ there is still a detectable activity when a 6 fold excess of serum albumin is used (Table I).

A similar reactivity is seen when whole serum is used. The enzymic reduction of nitro blue tetrazolium was inhibited by 50% usually at 2.4 μM concentrations of added copper. In the presence of increasing amounts of whole serum a reduction in the superoxide dismutase activity was noted. Even at 900 $\mu g \times ml^{-1}$ of protein Cu_2(lonazolac)$_4$ displayed some detectable activity. In the presence of physiological protein concentrations the superoxide dismutase activity was below the detection limit.

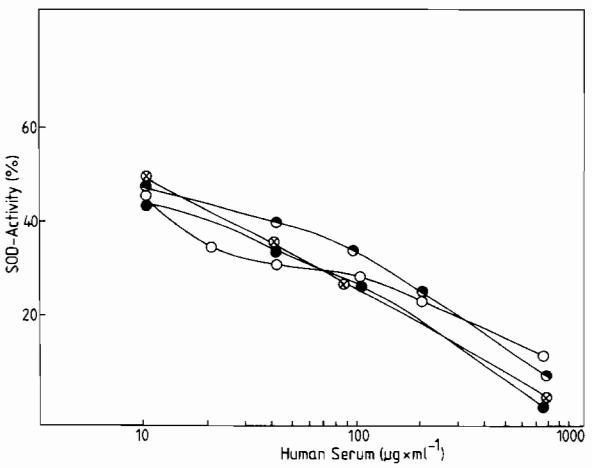


Fig. 4. Superoxide dismutase activity of copper chelates in the presence of whole human serum. ○Cu_2(lonazolac)$_4$, ⊗ Cu_2(salicylate)$_4$, ◑Cu_2(indomethacin)$_4$, ●$CuSO_4$. The xanthine/xanthine oxidase mediated reduction of nitro blue tetrazolium was measured. Further details as in the legend to Table I.

Equilibrium Dialysis

It was of interest to examine whether or not the observed ternary complex between copper, lonazolac and serum albumin would dissociate copper and/or some Cu_2(lonazolac)$_4$. Equilibrium dialysis of 700 μM bovine serum albumin containing 15 μM Cu_2-(lonazolac)$_4$ against an isotonic HEPES buffer solution for more than 16 h yielded only 0.4 μM Cu and less than 1% of the original lonazolac in the dialysate. Titration with either $CuSO_4$ or Cu_2(lonazolac)$_4$ on the albumin side up to 400 μM neither increased copper nor lonazolac release into the dialysate significantly. These results support the spectrometric data.

The same experiment was repeated using whole bovine serum which was dialysed against ultrafiltrate of the same serum. Unlike the case of albumin some 10 μM Cu were detected in the dialysate when the copper concentration was raised to 400 μM. Again the addition of 2 m*M* lonazolac did not change the dialysis pattern. Thus, the slight increase of copper in the dialysate must be attributed to copper complexes other than Cu_2(lonazolac)$_4$ (Fig. 5). In the light of these observations the appearance of Cu_2-(indomethacin)$_4$ in serum described earlier will have to be reconsidered [4]. The gel chromatographic separation following the application of the ligand *in vitro* and *in vivo* will have to stand up to rigorous questioning.

Lipid Peroxidation

As the solubility of Cu_2(lonazolac)$_4$ in aprotic solvents is increased by several orders of magnitude, it was expected that this copper complex would reach many a lipophilic section in the cell. Inhibition of superoxide-dependent lipid peroxidation could be the consequence. A suitable method to shed some light on this question was considered to be the

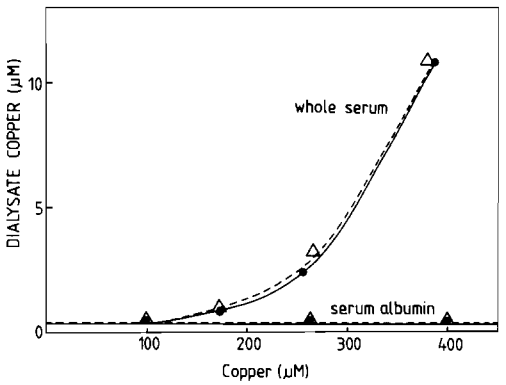


Fig. 5. Equilibrium dialysis of bovine serum and serum albumin in the presence of Cu_2(lonazolac)$_4$ or lonazolac and $CuSO_4$ (– – –), $CuSO_4$ alone (——). The concentration of serum albumin was 700 μM which was dialysed against HEPES-buffered isotonic saline, pH 7.4. Two one ml chambers were separated by a cellulose membrane of 1.5–2.0 nm pore size. Dialysis of serum albumin was performed at 23 °C for 16 h. Whole serum was dialysed against serum ultrafiltrate at 2 °C for 21 h. Lonazolac was determined spectrophotometrically at 280 nm in the dialysate.

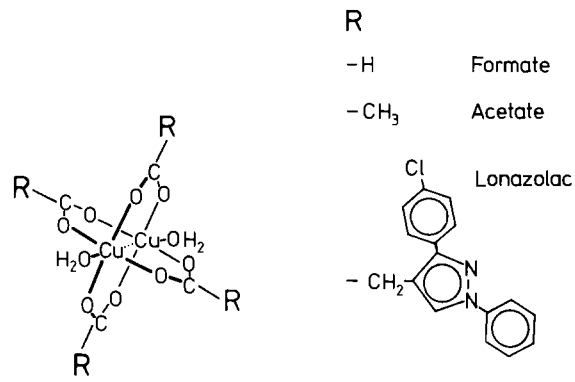
TABLE II. Inhibition of Linolenic Acid Peroxidation in the Presence of Cu_2(lonazolac)$_4$. Reaction conditions: One ml contained 15 mM sodium phosphate, pH 7.4; 500 μM linolenic acid; 10% (v/v) 1,2-dimethoxyethane; 2% (v/v) dioxane; 0.18 μM xanthine oxidase, 48 mM acetaldehyde; start with 20 μl acetaldehyde (2.4 M). After 30 min of reaction A_{238} was recorded at 23 °C for another 30 min in the linear phase of absorption increase.

Cu_2(lonazolac)$_4$ [μM Cu(II)]	$\Delta A_{238} \times h^{-1} \times 10^3$	Inhibition [%]
0	18.2	0
1	14.8	19
5	11.6	40
10	2.0	89

acetaldehyde/xanthine oxidase-mediated peroxidation of linolenic acid (Table II). Indeed, there was a marked and clear inhibition of lipid peroxidation in the presence of micromolar concentrations of Cu_2-(lonazolac)$_4$.

Discussion

Replacement of one methyl hydrogen on the acetate moiety by 3-(p-chlorophenyl)-1-phenyl-pyrazole (lonazolac) increases the lipophilicity of the corresponding copper complex very sharply. It has been shown that the dimeric Cu_2(lonazolac)$_4$ is stable in both water and aprotic solvents. The magnetic coupling of the two copper ions is close to a metallic type interaction. This complex does not survive treatment with higher concentrations of serum albumin. A ternary complex is formed in which lonazolac does not contribute to the first coordination shell of the copper. It is intriguing to consider



the strong antiinflammatory action of many copper acetate-like compounds *in vivo* [8]. Throughout they are perfect mimics of superoxide dismutase activity [2, 9]. However, the competitive action of many macromolecular ligands in aqueous systems must be dealt with. Nevertheless, not all stages of inflammation are fully understood including both the generation and scavenging of superoxide radicals.

The second order rate constants for the reaction between $\cdot O_2^-$ and the acetate type copper complexes are near $5 \times 10^9\ M^{-1}\ s^{-1}$ and twice as high as those obtained with the native Cu_2Zn_2–superoxide dismutase [4]. In spite of this remarkable observation only 10 nM of Cu_2Zn_2–superoxide dismutase are required to inhibit the reaction of superoxide in indirect assays. By way of contrast a hundredfold excess of Cu complexes is needed to show the same reactivity.

According to the present results obtained from the reaction of serum albumin with copper chelates, not very much can be said about the actual concentration of superoxide dismutase mimetic complexes in all these indirect assays. They contain many different metal chelators. For example, copper binding to xanthine oxidase protein similar to that observed for serum albumin is possible. Numerous other chelators including nitro blue tetrazolium, gelatine and xanthine may react with the added copper complex. Thus, the actual concentration of the superoxide dismutase mimicking complex is probably much lower. It may come close to that of the 10 nM Cu_2Zn_2 superoxide dismutase. The assignment of the superoxide dismutase activity to 'free Cu^{2+} ions can be discarded. It has been shown that the free divalent copper ion concentration in complexes with amino acids or peptides lies between 10^{-12}–$10^{-18}\ M$ [9, 10].

At present the following facts can be summarized. The acetate-like copper complexes are perfect mimics of superoxide dismutase activity in the absence of high concentrations of competitive chelating agents.

In the presence of these ligands the concentration of intact complexes is very low. However, the absence of any superoxide dismutase activity at all cannot be ascertained. Some residual activity exerted by traces of undissociated copper complexes beyond the detection limit has to be considered. These traces may be sufficient to explain the antiinflammatory action of many copper complexes observed *in vivo* [8]. The power of the acetate-like superoxide dismutase mimics could be attributed to the lipophilicity of these complexes. Both the aromatic residues and the charge compensation following the reaction with divalent copper facilitate transportation of the complex through lipophilic systems.

Acknowledgements

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