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ORIGINAL ARTICLE

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Development of novel copper(II) complexes of benzothiazole-*N*-sulfonamides as protective agents against superoxide anion. Crystal structures of [Cu(*N*–2-(4-methylbenzothiazole) benzenesulfonamidate)$_2$(py)$_2$] and [Cu(*N*–2-(6-nitrobenzothiazole) naphthalenesulfonamidate)$_2$(py)$_2$]

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Abstract Copper(II) ternary complexes based on the novel benzothiazole-*N*-sulfonamides, HL1 (*N*-2-(4-methylbenzothiazole)benzenesulfonamide) and HL2 (*N*-2-(6-nitrobenzothiazole)naphthalenesulfonamide) ligands, and pyridine have been synthesized and characterized. Complexes [Cu(L1)$_2$(py)$_2$] (**1**) and [Cu(L2)$_2$(py)$_2$] (**2**) were chemically characterized and their structures determined by means of single crystal X-ray analysis. In both compounds the Cu(II) ion is coordinated to four N atoms in a nearly square planar arrangement. The Cu-N bond distances are similar to those of Cu_2Zn_2SOD. The SOD mimetic activity of the complexes was determined both in vitro and in vivo. For determining the SOD-like activity of the complexes in vivo, we have developed a new method based on the complexes' protective effect on a Δ*sod1* mutant of *Saccharomyces cerevisiae* against free radicals generated by hydrogen peroxide and menadione as well as free radicals produced in the cellular respiration process. The results have shown that complex **1** presents a protective action against oxidative stress induced by menadione or H_2O_2 and that both complexes **1** and **2** protect against free radicals generated in cellular respiration.

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Introduction

The superoxide anion radical is a highly reactive toxic species found in many biological systems. It is involved in DNA damage, lipid peroxidation, radiation injury, and vascular diseases, among others [1, 2]. Superoxide dismutase (SOD) catalyzes O_2^- dismutation very efficiently [3], thus serving as an important means of defense against oxygen toxicity. In this reaction the copper(II) active site undergoes reduction to copper(I), followed by reoxidation to copper(II). The Cu(II) center has a distorted square pyramidal structure with a D_{2d} distortion toward a tetrahedral structure for the four His-N ligands in the equatorial plane [4]. During the catalytic process the metal center of SOD changes the square planar geometry [typical of Cu(II)] to assume a tetrahedral geometry [typical of Cu(I)]. Actually the 'plasticity' of copper(II) and the tetrahedral preference of copper(I) are the reasons for the uniqueness of SOD in protecting cells against an O_2^- attack.

Cu_2Zn_2SOD has been proposed for clinical uses [5, 6], but it has many shortcomings, including its short lifetime, high cost, tissue impermeability and immunogenicity. In contrast, the stable, non-toxic metal complexes which catalyze the dismutation of superoxide anion show considerable promise as SOD mimics for pharmaceutical application and have attracted much attention [7]. Although many SOD mimics, including copper(II) complexes of polypeptides [8, 9], polydentate Schiff bases [10, 11], mixed ligands [12, 13], and imidazolate-bridged heterobinuclear Cu-Zn complexes [14, 15] or binuclear copper complexes [16, 17] have been

reported, only a few are highly active in the range of physiological pH. Some factors have been proposed which may discriminate among the dismutation abilities of the copper complexes and these may include:

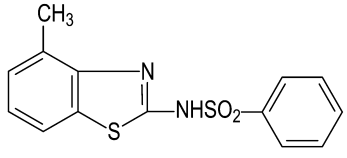
1. A limited steric hindrance to the approach of the superoxide anion is considered an essential requirement for the successful binding of the superoxide [18]
2. The flexibility of the copper(II) arrangement, which facilitates the interaction of the superoxide radical, followed by the rapid electron transfer reaction which results in reduction to copper(I) species [19]
3. The favorable response of π-electrons of the coordinated ligands in stabilizing the $Cu\text{-}O_2^-$ interaction so the presence of co-ordination sites belonging to nitrogen heteroaromatic rings such as imidazoles or pyridines is important for high SOD activity [20]

Accurate measurement of the ability of a given SOD mimic to catalyze the dismutation of superoxide is essential for establishing a correlation between pharmacological effect and SOD activity. Two types of methods – direct and indirect – are used for assaying the SOD-like activity of these metal complexes. Fridovich and McCord first discovered the activity of the SOD enzymes using an indirect method based on a cytochrome *c* assay. In this assay, ferricytochrome *c* is reduced by superoxide to its reduced form affording a spectrophotometric change or a fluorescence. Inhibition of this reduction of cytochrome *c* by scavenging or reducing the superoxide concentration is taken as a measure of SOD activity. In general, cytochrome *c* or nitro blue tetrazolium (NBT) are the most commonly used indicators in a system using xanthine/xanthine oxidase to generate steady state low levels of superoxide anion. Several problems have been noted concerning the use of indirect measurements to quantify SOD-like activity [21]. For example, the indirect assays rarely mirror natural conditions in that they are usually carried out with too little substrate compared to putative catalyst. In fact, there is often an excess of catalyst over the initial concentration of superoxide. This can lead to a false interpretation of catalytic activity in the case of a fast stoichiometric reaction with superoxide. Still, these methods can indicate whether a compound is an SOD mimic at lower levels of activity than direct methods can. Moreover, even with the aforementioned problems, the indirect method better approximates the in vivo conditions than do direct methods [22].

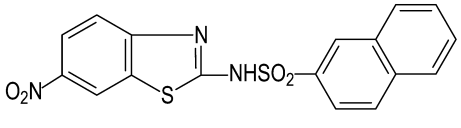
Direct methods, which are divided into the categories of stopped-flow kinetic analysis and pulse radiolysis, have been reported to be useful in properly assessing SOD activity, albeit with several disadvantages. For example, the stopped flow procedure is limited in that the rates can only be determined for compounds which possess catalytic activities greater than $K_{cat} > 10^{5.5}\ M^{-1}\ s^{-1}$ [21] that is due to a competition with background SOD degradation. Thus far, none of the assays currently employed can exactly replicate the environment encountered by these SOD mimics in the body. In fact, several SOD mimics which showed very efficient SOD-like activity in solution failed to do so in vivo [23]. Since a complex which is to be considered an efficient SOD mimic must be functional in vivo, having a well-defined assay for determining the SOD-like activity of low molecular weight metal complexes at conditions similar to the in vivo systems is of utmost importance. For these reasons we are interesting in developing a new method for determining the SOD-like activity of metal complexes in vivo.

One of the longstanding goals of our group has been to gain insight into the coordination behavior of sulfonamides and *N*-substituted sulfonamides. To this end, we have synthesized a variety of mono- and dinuclear compounds and studied them extensively [24, 25, 26, 27, 28, 29, 30, 31, 32, 33]. Recently we have also become interested in ternary sulfonamide complexes with SOD-like activity. In this sense, a number of sulfathiazole (4-amino-*N*-(thiazol-2-yl)benzenesulfonamide) copper (II) complexes with imidazole, imidazole derivatives, or pyridine have been demonstrated to present high SOD-like activity in vitro [28, 29, 31]. Although a structural-SOD activity correlation for these copper(II) complexes was not clear enough, some structural features such as a distorted co-ordination polyhedron, that favors the reduction process from copper(II) to copper(I), and the different donor abilities of pyridine and imidazole could explain their different SOD-like activity. As a continuation of our study of sulfonamide derivatives and their metal complexes, we have developed a new class of aromatic sulfonamide ligands based on benzothiazole units. The presence of a coordination site belonging to nitrogen heteroaromatic and the possibility of accommodating the copper ion in different geometries are important features of these ligands for obtaining copper(II) complexes with high SOD-like activity.

In this paper, we describe the synthesis of two novel aromatic *N*-sulfonamide ligands [(*N*-2-(4-methylbenzothiazole)benzenesulfonamide (HL1) and *N*-2-(6-nitrobenzothiazole)naphthalenesulfonamide (HL2)] (Scheme 1) and their Cu(II) complexes with pyridine, $[Cu(L1)_2(py)_2]$ and $[Cu(L2)_2(py)_2]$. The crystal



N-2-(4-methylbenzothiazole)benzenesulfonamide (HL1)



N-2-(6-nitrobenzothiazole)naphtalenesulfonamide (HL2)

Scheme 1

structures and chemical characterization of these complexes and their SOD-mimetic activity are described. An indirect method has been used for assaying the SOD-like activity of our complexes, but due to the above mentioned limitations of the in vitro assays we propose a new in vivo method which is based on the protection of the complexes against oxidative stress over three different strains of *Saccharomyces cerevisiae*: a wild type, a strain defective in the mitochondrial manganese dependent SOD (Δ*sod2*), and a strain defective in the copper dependent SOD (Δ*sod1*).

Experimental

General

Reagents and solvents were commercially available and were used without further purification.

Elemental analyses were performed on a Carlo Erba AAS instrument. IR spectra were recorded as KBr pellets on a Mattson satellite FT-IR in the range 4000–400 cm^{-1}. FAB mass spectra were obtained on a VG Autospec spectrometer as a solution in 3-nitrobenzyl alcohol. Diffuse reflectance spectra of the Nujol mulls of the complexes were carried out on a Shimadzu UV-2101 PC spectrophotometer. EPR spectra were taken at room temperature with a Bruker ELEXSYS spectrometer operated at the X-band frequency.

Synthesis

Ligands. A solution containing 1 g of 2-amino-4-methylbenzothiazole or 1 g of 2-amino-6-nitrobenzothiazole and 2.5 g of the benzenesulfonylchoride or 2.5 g of 2-naphthalenesulfonylchloride in 6 ml of pyridine was heated at reflux for 1 h. The mixture was added to 10 ml of cold water and stirred for several minutes. The resulting solid was recrystallized from ethanol. Data for compound *N*-2-(4-methylbenzothiazole)benzenesulfonamide (**HL1**) (yield 68%). $C_{14}H_{12}N_2O_2S_2$ (304.4): calcd. C 55.2; H 4.0; N 9.2; S 21.1; found C 55.3; H 4.0; N 9.2; S 21.1; IR(KBr) (cm^{-1}): 1550 (thiazole); 1290, 1145 $\nu(SO_2)$, 948 ν(S-N). FAB: m/z 305 [M^+]; UV(λ_{max})(nm): 310 ($\pi\rightarrow\pi^*$). Data for compound *N*-2-(6-nitrobenzothiazole) naphthalenesulfonamide (**HL2**) (yield 62%). $C_{17}H_{11}N_3O_4S_2$ (385.4): calcd. C 53.0; H 2.9; N 10.9; S 16.6; found C 53.5; H 2.8; N 11.1; S 16.5; IR(KBr) (cm^{-1}): 1550 (thiazole); 1520, 1340 $\nu(NO_2)$; 1313, 1150, 1127 $\nu(SO_2)$; 960 ν(S-N). FAB: m/z 386 [M^+]; UV(λ_{max})(nm): 310, 370 ($\pi\rightarrow\pi^*$).

[Cu(L1)$_2$(py)$_2$] (*1*). A solution of 4 mmol of $CuSO_4.5H_2O$ in 20 ml of pyridine:H_2O [v:v = 1:1] was added dropwise under continuous stirring to a solution of the ligand (1 mmol) in 25 ml of pyridine:H_2O [v:v = 2:3]. The resulting solution was stirred for several hours and then left to stand at room temperature. Prismatic dark red crystals formed after a few days. Data for compound **1** (yield 89.5%). $C_{38}H_{32}N_6CuO_4S_4$ (828.5): calcd. C 55.1; H 3.9; N 10.1; S 15.5; found C 55.0; H 3.9; N 10.1; S 15.3; IR(KBr) (cm^{-1}): 1475d (thiazole); 1300, 1145 $\nu(SO_2)$, 980 ν(S-N). UV-vis (λ_{max})(nm): 470, 640sh.

[Cu(L2)$_2$(py)$_2$] (*2*). This compound was obtained by using a procedure similar to that described for complex **1**. A solution of 4 mmol of $CuSO_4.5H_2O$ in 20 ml of pyridine:H_2O [v:v = 1:1] was added dropwise under continuous stirring to a solution of the ligand (1 mmol) in 30 ml of pyridine:H_2O [v:v = 1:1]. The resulting solution was heated to 50°C during stirring and then left to stand at room temperature. Slow evaporation of this solution gave rise to prismatic brown crystals. Data for compound **2** (yield 68.5%). $C_{44}H_{30}N_8CuO_8S_4$ (990.6): calcd. C 53.3; H 3.0; N 11.3; S 12.9; found C 53.5; H 3.3; N 11.6; S 12.4; IR(KBr) (cm^{-1}): 1460 (thiazole); 1515, 1335 $\nu(NO_2)$; 1310, 1150, 1125 $\nu(SO_2)$, 980 ν(S-N). UV-vis (λ_{max})(nm): 490sh, 690sh.

X-ray crystal structure determination

Data collection of [Cu(L1)$_2$(py)$_2$] (*1*). Data collection of **1** covered almost the whole sphere of reciprocal space with a completeness of 78.1% in the maximum resolution shell (0.92–0.85 Å) and 87.5% for all data. Crystal to detector distance was 2.9 cm. Crystal decay was checked by observing the scale factors for all the frames collected; no obvious decay was observed. The title compound crystallized in the monoclinic system, space group $P2_1/n$, as indicated by the systematic absences. A total of 140 images were collected, with an oscillation of 50 and an exposure time of 75 s per image. Final mosaicity calculated was 0.884°. χ^2 for all reflections between 20 and 0.85 Å was 1.087; the ratio I:σ was 3:1 for higher resolution shell reflections (0.92–0.85 Å) and 10:1 for all data combined. Internal symmetry was observed in the molecules, with an inversion center at the Cu position; therefore, only half of the molecule was refined as the asymmetric unit.

Data collection: Nonius KCCD Diffractometer Control Software [34]. Cell refinement: Scalepack [35]. Data reduction: Denzo [35]. Program(s) used to solve structure: Patterson methods using the program PATTY [36]. Program(s) used to refine structure: SHELXL-97 [37]. Atomic Scattering Factors were taken from International Tables for Crystallography [38]. Molecular Graphics: EUCLID [39]. All calculations were made at the University of Oviedo on the SGI-computers of the Scientific Computer Center and X-Ray group. Experimental details are given in Table 1.

Data collection for compound [Cu(L2)$_2$(py)$_2$] (*2*). A monoclinic orange crystal measuring 0.20×0.13×0.13 mm, space group $P2_1/c$ (as determined from the systematic absences) was used for the data collection. Data collection was performed at 200(2) K on a Nonius Kappa CCD single crystal diffractometer, using Cu-Kα radiation (λ = 1.5418 Å). Crystal to detector distance was fixed at 29 mm, and a total of 734 images were collected using the oscillation method (both f and w oscillations were necessary to fill the Ewald Sphere) with 2° oscillation and 40 s exposure time per image. Data collection strategy was calculated with the program Collect [40]. Data reduction and cell refinement were performed with the programs DENZO and SCALEPACK [35]. A total of 13599 reflections were collected between θ 2° and 70°. Unit cell dimensions were determined from 8379 reflections. Multiple measured reflections were averaged, R_{merge} = 0.057, resulting in 7368 unique reflections (hkl range $0 < h < 20$, $0 < k < 20$, $-17 < l < 17$), of which 4464 were observed with $I > 2\sigma(I)$. Final mosaicity was 0.38°. Data completeness was 89.9%. The intensity to error ratio for all reflections was 38.3:3.4.

The crystal structure was solved with the program DIRDIF-96 using Patterson methods [41]. Anisotropic least-squares refinement was carried out with SHELXL-97 [37]. All non-hydrogen atoms were anisotropically refined. All hydrogen atoms were treated with a mixture of independent and constrained refinement. The final cycle of full-matrix least-squares refinement based on 7368

Table 1 Crystal data and structure refinement for $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**)

	1	**2**
Empirical formula	$C_{19}H_{16}Cu_{0.5}N_3O_2S_2$	$C_{44}H_{30}CuN_8O_8S_4$
fw	414.24	990.54
Crystal system	Monoclinic	Monoclinic
Space group	$P2_1/n$	$P2_1/c$
a, Å	13.6480(10)	16.6934(13)
b, Å	9.8820(10)	17.8194(14)
c, Å	14.3190(10)	14.7844(12)
β (deg)	106.058(10)	98.793(5)
V, Å^3	1855.8(3)	4346.2(6)
Z	4	4
λ, Å	1.54184	1.54184
μ, mm^{-1}	3.345	3.050
ρ_{calcd} g/cm^3	1.483	1.514
T, K	200(2)	200(2)
R1	0.0643	0.0450
wR2	0.1359	0.1033

reflections and 586 parameters converged to a final value of R1 ($F^2 > 2\sigma(F^2)$) = 0.0450, wR2 ($F^2 > 2\sigma(F^2)$) = 0.1033, R1 (F^2) = 0.0810, wR2(F^2) = 0.1156. Final difference Fourier maps showed no peaks higher than 0.330 e $Å^{-3}$ or deeper than –0.407 e $Å^{-3}$. Atomic scattering factors were taken from the International Tables for Crystallography [38].

Geometrical calculations were made with PARST [42]. The crystallographic plots were made with PLATON [43]. All calculations were made at the University of Oviedo on the Scientific Computer Center and X-Ray group computers. Experimental details are given in Table 1.

Crystallographic data without structure factors for the two structures reported in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publications no. CCDC 163318 and CCDC 163319. Copies of the data can be obtained free of charge from the CDCC, 12 Union Road, Cambridge CB2 1EZ, UK; e-mail deposit@cdcc.cam.a.uk; www: http://ccdc.cam.ac.uk; tel +44–1223–336408; fax: + 44–1223–336003.

In vitro evaluation of SOD-like activity

The in vitro SOD activities of the compounds $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**) were evaluated with the Oberley and Spitz method with minor modifications [44]. Xanthine (1.5×10^{-4} mol/l) and xanthine oxidase in 50 mmol/l potassium phosphate buffer, pH = 7.8, were used to generate a reproducible and constant flux of superoxide anions. Superoxide radical anions generated by this enzymatic system were detected by the reduction of nitro blue tetrazolium (NBT) (5.6×10^{-5} mol/l) to blue formazane which was followed spectrophotometrically at 560 nm. The appropriate amount of xanthine oxidase to give in the control assays an ΔA_{560} rate of 0.05/min was used. This ΔA_{560} rate of 0.05/min corresponds to a rate of production of superoxide radical of 1.2 μmol/l·min. Solutions of the copper complexes **1** and **2** at different concentrations were prepared in 50 mmol/l Tris-HCl buffer, pH = 7.8, and added to the assay mixture in a volume representing one-tenth of the total. In a typical experiment 0.1 ml of the solution of the complex to be assayed and 0.1 ml of xanthine oxidase were added to 0.8 ml of a solution containing 0.69 ml of potassium phosphate buffer (pH = 7.8), 0.025 ml of NBT, and 0.085 ml of xanthine. The percentage inhibition of NBT reduction was used as a measure of SOD activity of the compounds. The enzymatic system was also tested against a possible inactivation caused by the copper(II) complexes. The formation of uric acid from xanthine was followed at 310 nm to evaluate if the assayed complexes affect the generation of superoxide anions by directly interacting with the enzymatic system. The inhibition percentage of enzyme activity was subtracted from that of NBT. The concentration of complex required to yield 50% inhibition of NBT reduction (named IC_{50}) was determined from a plot of percentage inhibition vs complex concentration. Assays were carried out both with and without bovine serum albumin (BSA). Xanthine, xanthine oxidase, NBT, and superoxide dismutase (bovine erytrocyte) and BSA were from Sigma Chemical Co.

In vivo evaluation of SOD-like activity

The in vivo SOD-like activities of the complexes $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**) were evaluated over three different strains of *Saccharomyces cerevisiae*: W303–1A, wild type (*MATa ade2–1 ura3–1 his3–11 trp1 leu2–3 leu2–112 can1–100)*; ATCC96687, strain defective in the copper dependent SOD (*MATa ura3–52 trp1–289 his3-Δ1 leu2–3 leu2–112 sod1::URA3)*, and ATCC96688, strain defective in the mitochondrial manganese dependent SOD (*MATa ura3–52 trp1–289 his3-Δ1 leu2–3 leu2–112 sod2::TRP1*). The strains were purchased from American Type Culture Collection (ATCC). The protective action of the complexes against free radicals produced in the respiratory process as well as those generated by menadione or H_2O_2 was determined. The parameters used as a measure of the protective effect of the compounds in both types of experiments are indicated below.

In vivo evaluation of the protective effect of the complexes against free radicals produced in respiration. Solutions of compounds $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**) in DMSO:EtOH (1:4) at increasing concentrations were added to 15 ml of melted YPgly medium (1% yeast extract, 2% peptone, 2% glycerol, 1.5% agar, and 2% ethanol) kept at 45°C. Media were poured in Petri dishes and allowed to solidify at room temperature. Different dilutions of cell cultures were grown overnight at 28°C in stirred liquid YPD reach medium (1% yeast extract, 2% peptone, and 2% glucose). Then 5-μl drops of each of the three strains at decreasing concentrations (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were added to the Petri dishes, which were then incubated at 28°C for four days. Untreated cultures (without the copper(II) complexes **1** and **2**) were incubated in parallel. The increase of growth of cells found in presence of the copper(II) compounds compared with cell growth in absence of the complexes was taken as a qualitative estimate of the protective effect based on serial dilution drops assays.

In vivo evaluation of the protective effect of the complexes against free radicals produced by oxidative agents. Yeast cells were grown in YPD reach medium (1% yeast extract, 2% peptone, and 2% glucose). Solid media contained 1.5% agar. Cell density from cultures grown overnight was determined by cell counting in a "Nebauer" *hematimetre*. Then 10^6 cells were resuspended in 15 ml of melted solid YPD media kept at 45°C. Solutions of the complexes $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**) in a mixture of DMSO: EtOH (1:4) at increasing concentrations were added to the growth medium. Cell suspensions were poured into Petri dishes and allowed to solidify at room temperature. Paper disks measuring 6 mm in diameter (Antibiotica test Blättchen) and containing different solutions for the different strains were placed over the media. For the wild (W303–1A) and Δ*sod2* (ATCC96688) strains, 15 μl of a 40 mmol/l menadione solution in ethanol or 5 μl of 35% H_2O_2 was used, whereas 5 μl of a 5 mmol/l menadione solution in ethanol or 5 μl of 17.5% H_2O_2 was employed for the Δ*sod1* (ATCC96687) strain. Differences in the amounts of oxidants used depended on the differences in sensibility of the different strains [45]. The diameters of the clear zones around the disks, measured after three days of incubation at 28°C, were taken as a quantitative estimate of the protective action.

Toxicity assays

The putative toxicity of the complexes $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**) on the wild, the Δ*sod1*, and the Δ*sod2 Saccharomyces cerevisiae* strains was determined using the following procedure: solutions of complexes in DMSO:EtOH (1:4) were added to 15 ml of melted YPD medium (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar) kept at 45°C. Media were poured into Petri dishes and allowed to solidify at room temperature. Different dilutions of cell cultures were grown overnight at 28°C in stirred liquid YPD reach medium (1% yeast extract, 2% peptone, and 2% glucose). Then 5-μl drops of the three strains at decreasing concentrations (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were poured into the Petri dishes which were then incubated at 28°C for four days. Untreated cultures (without the copper(II) complexes **1** and **2**) were incubated in parallel. Cells viability in the presence of complexes **1** and **2** compared with that in absence of these compounds was taken as a qualitative measure of the putative toxicity of the copper(II) complexes assayed.

Results and discussion

Crystal structures of compounds $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**)

The crystal structures of compounds **1** and **2** are shown in Figs. 1 and 2, respectively. Significant bond lengths and angles are listed in Table 2.

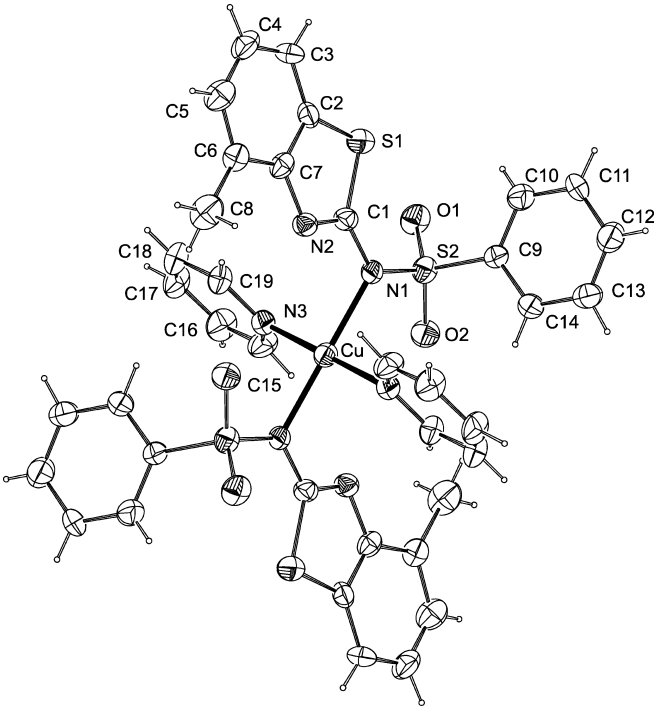


Fig. 1 ORTEP drawing of the $[Cu(L1)_2(py)_2]$ (**1**) complex

The crystal structures of the compounds consist of a metal center surrounded by four N atoms which correspond to two pyridine molecules and two deprotonated

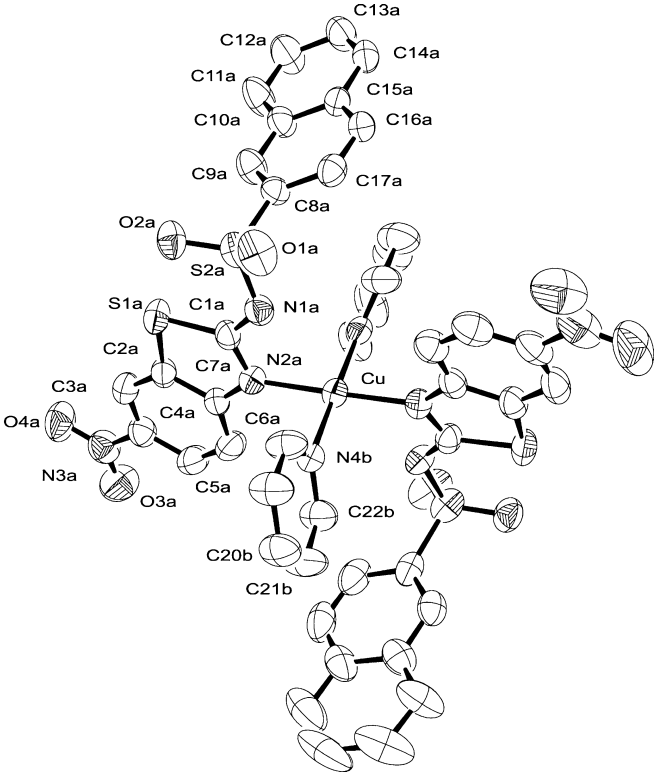


Fig. 2 ORTEP drawing of the $[Cu(L2)_2(py)_2]$ (**2**) complex

Table 2 Selected bond lengths (Å) and angles (°) for $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**)

1		**2**	
Cu1-N1	2.028(4)	Cu1-N2B	1.952(2)
Cu1-N3	1.989(4)	Cu1-N2A	1.963(3)
		Cu1-N4B	1.993(3)
		Cu1-N4A	1.999(3)
N1-Cu1-N3	89.46(2)	N2B-Cu1-N2A	178.57(11)
		N2B-Cu1-N4B	89.68(10)
		N2A-Cu1-N4B	91.13(10)
		N2B-Cu1-N4A	89.87(10)
		N2A-Cu1-N4A	89.31(10)
		N4B-Cu1-N4A	179.55(10)

N-sulfonamide derivative ligands. The metal ion in complex **1** is located at an inversion symmetry center; hence, its coordination polyhedron is strictly square planar. In complex **2** the tetrahedrality value, τ, of 1.18° indicates that the copper ion adopts a nearly square planar geometry [46]. The Cu-N bond distances lie in the range of 1.952(2)–2.028(4) Å, which are normal lengths for Cu-N_{py}, Cu-$N_{sulfonamido}$, and Cu-$N_{thiazole}$ coordination bonds [28, 29, 30]. The Cu-$N_{thiazole}$ [$1.96(2)_{av}$ Å] in **2** is shorter than the Cu-$N_{sulfonamido}$ in **1** [2.028(4) Å]. The complexes present some similarities with the copper(II) center of the Cu_2Zn_2SOD; for example, the Cu-N distances are close to those of the enzyme (2.06 Å) [47]. However, the angles N-Cu-N are somewhat different from those of the Cu_2Zn_2SOD [N_{His60}-Cu-N_{His120} 157°, N_{His46}-Cu-N_{His48} 140°]. Although compounds **1** and **2** cannot be considered perfect structural models of an SOD enzyme, they do fulfill two important requirements for superoxide dismutase activity; namely, they both contain a flexible coordination polyhedron as well as at least one accessible site to allow the reaction with the superoxide anion to occur.

It is worth noting that in spite of the similar structure and monodentate behavior of the two ligands, they coordinate differently. While $L1^-$ interacts through the sulfonamido N atom, $L2^-$ links the copper(II) ion via the benzothiazole N atom.

Spectroscopic properties

The IR spectra of both complexes present a similar pattern. The most remarkable difference occurs in the band corresponding to the stretching vibration of the thiazole ring, which is shifted from 1550 cm^{-1} in the free ligands to 1470 cm^{-1} in the complexes. The $\nu(SO_2)_{as}$ and $\nu(SO_2)_s$ bands do not show significant differences with respect to those of the ligands. The characteristic band corresponding to the ν(S-N) appears near 980 cm^{-1}, shifted ca. 30–20 cm^{-1} to higher frequencies with respect to those of the uncoordinated ligands. In general, the patterns of the IR spectra are similar to those observed for other copper *N*-sulfonamide derivatives [29, 30, 31].

The solid electronic spectrum of complex **1** presents a band at 470 nm and a shoulder at 640 nm, which correspond to ligand field transitions. In compound **2** these d-d transitions are observed as shoulders at 490 and 690 nm. Both spectra are characteristic of square planar complexes with CuN_4 chromophores [48]. Cu_2Zn_2SOD has its absorption maximum at 680 nm [11], close to the shoulders found in the spectra of compounds $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**).

The polycrystalline X-band EPR spectra of the complexes at room temperature are axial in nature. The EPR parameters, calculated by simulation [49], are $g_{\|} = 2.26$, $g_{\perp} = 2.05$, and $A_{\|} = 160 \times 10^{-4}$ for complex **1** and $g_{\|} = 2.22$, $g_{\perp} = 2.04$, and $A_{\|} = 180 \times 10^{-4}cm^{-1}$ for complex **2**. The quotient $g_{\|}/A_{\|}$ is a measure for the degree of tetrahedral distortion. This quotient ranges from ca. 105 to 135 cm for square-planar structures and increases markedly on the introduction of tetrahedral distortion [50]. The $g_{\|}/A_{\|}$ values for the compounds **1** and **2** are 141 and 123 cm, respectively. These values suggest that complex **2** is square planar while complex **1** presents slight tetrahedral distortion. The EPR parameters of Cu_2Zn_2SOD are $g_{\|} = 2.268$, $g_{\perp} = 2.087$, and $A_{\|} = 142 \times 10^{-4}cm^{-1}$ [51]. The value of $g_{\|}/A_{\|}$ equal to 160 cm indicates some tetrahedral distortion of the copper(II) arrangement at the active site of the native enzyme. In terms of the empirical factor $g_{\|}/A_{\|}$ the $[Cu(L1)_2(py)_2]$ (**1**) complex has more structural similarity to Cu_2Zn_2SOD than the $[Cu(L2)_2(py)_2]$ (**2**) compound.

Table 3 The IC_{50} values taken from reports on SOD-like activities of copper(II) complexes

Complex	IC_{50} (μmol/l)	Ref
$[Cu(L1)_2(py)_2]$ (**1**)	0.146	This work
$[Cu(L2)_2(py)_2]$ (**2**)	0.172	This work
$[Cu(stz)_2(Him)_2]MeOH$	0.664	26
$[Cu(stz)_2(mHim)_2]H_2O$	0.429	26
$[Cu(stz)_2(4,4\text{-dmHim})_2]$	0.742	28
$[Cu(stz)_2(1,2\text{-dmHim})_2]$	1.03	28
$[Cu(stz)_2(4\text{-mHim})_2]$	0.586	28
$CuL_{1,4py}(ClO_4)_2.0.5\ H_2O$	1.4	52
$CuL_{1,4im}(ClO_4)_2.0.5\ H_2O$	4.0	52
$Cu(acetylsalicylate)_2$	23.0	52
$Cu(lysine)_2$	86.0	52
[Cu(Mc)]	0.13	30
[Cu(TAAB)]	0.14	30
$[Cu(Pu\text{-}6\text{-}MePy)(H_2O)](ClO_4)_2$	2.25	11
Cu_2Zn_2SOD	0.006	This work
Cu_2Zn_2SOD	0.0081	11

Abbreviations: stz = sulfathiazolate, Him = imidazole, mHim = *N*-methylimidazole; 4,4-dmHim = 4,4-dimethylimidazoline; 1,2-dm Him = 1,2-dimethylimidazole; 4-mHim = 4-methylimidazole; $L_{1,4py}$ = Schiff base formed from 1,4-diaminobutane and pyridine-2-aldehyde; $L_{1,4im}$ = Schiff base formed from 1,4-diaminobutane and imidazole-2-aldehyde Mc = macrocyclic ligand formed by 1,3-bis (5-methylpyrazol-1-yl)propane and 1,3-bis(diethylaminopropane); TAAB = tetraanhydroaminobenzaldehyde; Pu-6-MePy = Schiff base formed from 1,4-diaminobutane and 6-methylpyridine-2-aldehyde

In vitro superoxide-dismutase activity

The SOD-like activities of the two complexes were tested with indirect methods in which the xanthine/xanthine-oxidase system served as the source for superoxide radicals. By way of comparison, the activity of the Cu_2Zn_2SOD and the SOD-like activity of $Cu(NO_3)_2.6H_2O$ were assayed under the same conditions. Both complexes presented SOD-like activity at biological pH. The IC_{50} values were 0.146 μmol/l and 0.172 μmol/l for compounds **1** and **2**, respectively. These values are lower than that for Cu(II) ($IC_{50} = 0.450$ μmol/l), indicating that their activity is not due to a possible partial dissociation of the complexes. The IC_{50} determined for both complexes is higher than the value found for native Cu_2Zn_2SOD (0.006 μmol/l); in fact the activities exhibited by the two compounds are 4 and 3% the activity of the enzyme. On the other hand, it must be emphasized that compounds **1** and **2** present lower IC_{50} values than those of copper(II) complexes previously described in the literature as good SOD mimics [29, 31, 52, 53] (Table 3). In our work, we found that the activity of the complexes was influenced by the physiological chelator albumin. In the presence of a tenfold excess of bovine serum albumin (BSA), complex **1** was observed to be less active, $IC_{50} = 0.280$ μmol/l, which indicates that, to some extent, albumin mobilizes the copper(II) ion of the complex. Complex **2** exhibited similar behavior.

In vivo SOD-like activity

Before evaluating the in vivo superoxide-dismutase mimetic activity of the complexes, their putative toxicity was determined using YPD medium. In this medium, fermentation is the initial pathway of sugar metabolism in actively growing cells of *S. cerevisiae* due to the Crabtree effect. This phenomenon relates glucose concentration with the particular catabolic route adopted by *S. cerevisiae* in the presence of oxygen and states that, even under aerobic conditions, fermentation predominates over respiration. At all concentrations assayed (30, 50, 70, and 90 μmol/l), the presence of the complexes did not modify the growth of the three strains, which suggests that complexes **1** and **2** do not present significant toxicity (data not shown).

To quantify the in vivo SOD-like activity of the Cu(II) complexes we have developed a new method based on the protection against free radicals provided by the compounds to the yeast *Saccharomyces cerevisiae* (a wild type strain, the copper defective Δ*sod1* mutant, and the manganese defective Δ*sod2* mutant). We have evaluated the protective effect of the complexes against free radicals produced in the respiratory process and also against free radicals generated by oxidative agents, such as hydrogen peroxide or menadione.

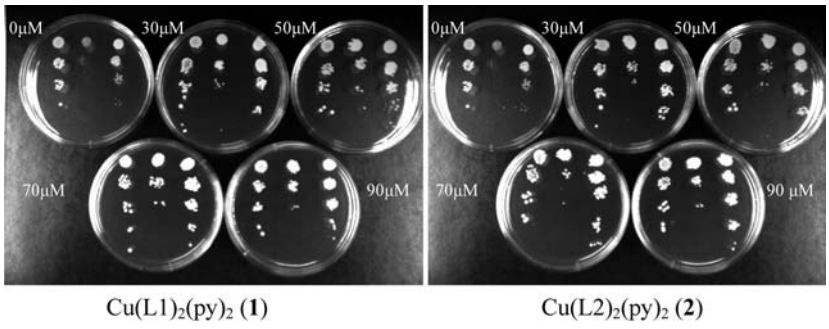


Fig. 3 Effect of complex **1** and **2** on the growth of the *Saccharomyces cerevisiae* against free radicals produced by respiration. In each Petri dish: first column: wild strain; second column: Δ*sod1* mutant; third column: Δ*sod2* mutant. In each column: strains at decreasing concentrations: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}

Complex protection against free radicals produced in respiration

Reactive Oxygen Species (ROS) can be generated as a consequence of normal cellular metabolic processes such as respiration or β-oxidation of fatty acids. The toxicity of these free radicals is actually due to their ability to damage a large number of cellular constituents. To evaluate the protective effect of complexes **1** and **2** against free radicals generated in respiration, three strains of *Saccharomyces cerevisiae* – wild, Δ*sod1*, and Δ*sod2* – were grown in a culture containing glycerol, which is a non-fermentable carbon source for *S. cerevisiae* [54]. Figure 3 shows the growth of several dilutions of these three strains treated with increasing concentrations of complexes $[Cu(L1)_2(py)_2]$ (**1**) and $[Cu(L2)_2(py)_2]$ (**2**). As expected, the growth of the wild strain was not significantly modified by the complexes; however, the protective effect of the complexes on the Δ*sod1* strain was found to be significant. These results provide strong evidence that both **1** and **2** are able to penetrate the cells to compensate for the Cu_2Zn_2SOD deficiency in the Δ*sod1*, thus protecting the cells against free radicals produced in respiration. Moreover, in the presence of both compounds, a growth increase was observed for the Δ*sod2*. This indicates that the complexes can, to some extent, perform the function of the mitochondrially located MnSOD, the role of which appears to be restricted to protecting cells against superoxide anions generated in respiration [45].

Complex protection against exogenous free radicals

We have also tested the effect of the complexes on cell growth with a Δ*sod1* mutant treated with menadione or H_2O_2. The results are given in Fig. 4, which shows the growth inhibition area (black region) of the Δ*sod1* strain in the presence of $[Cu(L1)_2(py)_2]$ (**1**), $[Cu(L2)_2(py)_2]$ (**2**), and $CuCl_2$. In this assay, disks at the top of each Petri dish contained the oxidative stress inducer menadione while the bottom disks contained hydrogen peroxide. By way of comparison, both the wild and the Δ*sod2* mutant strains, which share the copper dependent SOD, were also studied.

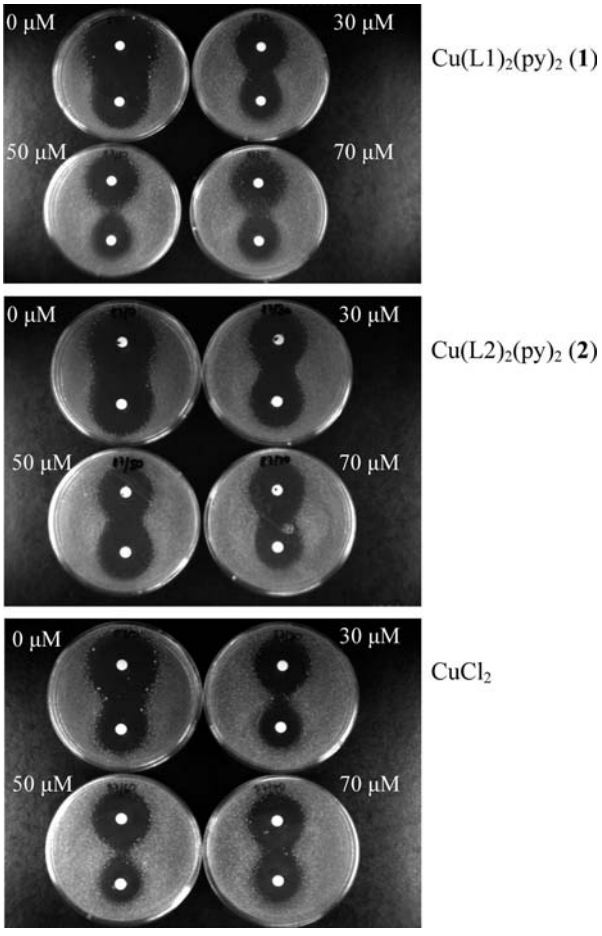


Fig. 4 Effect of complex **1**, complex **2** and $CuCl_2$ on the growth of the Δ*sod1* mutant against free radicals produced by menadione (disk at the top of each Petri dish) and H_2O_2 (disk at the bottom of each Petri dish)

In the presence of complex **1**, a growth increase was observed for the Δ*sod1* strain (smaller diameter of the inhibition area at complex concentrations 30, 50, and 70 μmol/l). This clearly suggests that complex **1** provides protection against the oxidative stress generated both by menadione and by H_2O_2. This protective action does not seem to depend significantly on complex concentration; however, at the maximum concentration assayed, a fraction of the cells seemed to enjoy higher levels of protection.

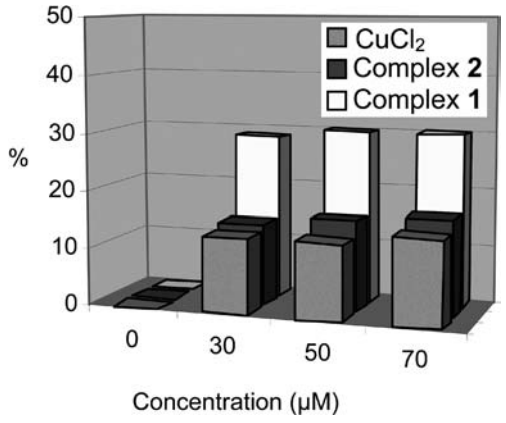


Fig. 5 Percentage reduction of the diameter of the growth inhibition area vs concentration of complex **1,** complex **2**, and $CuCl_2$ when oxidative stress was produced by menadione

Complex **2** slightly improved the growth of the Δ*sod1* strain (Fig. 4). The reduction of the diameter of the inhibition area for complex **2** is smaller than for complex **1** which indicates that complex **2** has less protective action than compound **1**. Moreover, the protective effect of compound **2** is similar to that observed for the Cu(II) salt. These results are summarized in Fig. 5 that represents the reduction (%) of the diameter of the growth inhibition area vs concentration of complex **1**/complex **2**/$CuCl_2$ when oxidative stress was produced by menadione.

As was expected, no protection effect for complexes **1** and **2** or $CuCl_2$ was observed in the wild type or the Δ*sod2* strains (data not shown) since both strains have the copper dependent SOD.

The results obtained from these assays clearly show that, at least in the range of the concentrations assayed, complex **1** demonstrates SOD-like activity in vivo. This activity derives from the nature of the complex itself since it presents an even higher activity than Cu(II). According to our results, then, complex **1** can be considered a promising protective agent against toxicity of superoxide anion.

Although the new method presented here is more time-consuming than the more commonly used indirect in vitro method, the fact that it better approximates the conditions in biological systems is highly advantageous. This method permits one to differentiate between the SOD-like activities of the complexes. In fact, taking into account the IC_{50} values of the complexes **1** and **2** determined by the indirect method, both present similar SOD-like activities; however they show protective effects against free radicals clearly different in vivo. Moreover, the results obtained with the indirect method indicate that complex **2** is more active than Cu(II) but both complex **2** and Cu(II) exhibit similar activities in vivo.

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References

1. Barton JK (1994) In: Bertini I, Gray HB, Lippard SJ, Valentine JS (eds) Bioinorganic chemistry. University Science Press, Mill Valley CA
2. Baudoin O, Teulade-Fichou MP, Vigneron JP, Lehn JM (1998) Chem. Commun 2349
3. McCord JM, Fridovich I (1969) J Biol Chem 244:6049
4. Holm RH, Kennepohl P, Solomon EI (1996) Chem Rev 26:2239–2314
5. Michelson AM, Jadot G, Puget K (1988) Free Radical Res Commun 4:209
6. Emerit J, Pelletier D, Tosoni-Verlignue D, Mollet M (1989) Free Radical Biol Med 7:1457
7. Sorenson JRJ (1984) Chem Br 20:1110–1113
8. Kubota S, Yang J-T (1984) Proc Natl Acad Sci USA 81: 3283
9. Patel RN, Pandeya KB (1998) J Inorg Biochem 72:109
10. Whang J, Luo Q-H, Zhang J-J, Shen MC, Liu AD, Gu HC, Li F-M, Di S-J (1997) Radiat Phys Chem 49:527
11. Muller J, Schubl D, Maichle-Mosser C, Strahle J, Wesser U (1999) J Inorg Biochem 75:63
12. Bhirud RG, Srivastava TS (1991) Inorg Chim Acta 179:125
13. Cao R, Travieso N, Fragoso A, Villalonga R, Diaz A, Martinez M E, Alpizar JD, West X (1997) J Inorg Biochem 66:213
14. Lu Q, Luo Q-H, Dai AB, Zhou ZY, Hu GZ (1990) J Chem Soc Chem Commmun 1429
15. Pierre JL, Chautemps P, Refaif S, Beguin C, Marzouki AE, Serratrice G, Saint-Aman E, Rey P (1995) J Am Chem Soc 117:1965
16. Tabbi G, Driessen WL, Reedijk J, Bonomo RP, Veldman N, Spek AL (1997) Inorg Chem 36:1168
17. Zhang J-J, Luo Q-H, Long D-L, Chen JT, Li FM, Liu AD (2000) J Chem Soc Dalton Trans 1893
18. Boden N, Holmes MC, Knowles PF (1975) Biochem Biophys Res Commun 4:225
19. Bonomo RP, Conte E, Impellizzeri G, Pappalardo G, Purrello R, Rizzarelli E (1996) J Chem Soc Dalton Trans 3093
20. Bienvenue E, Chona S, Lobo-Recio MA, Marzin C, Pacheco P, Seta P, Tarrago G (1995) J Inorg Biochem 55:157
21. Riley DP (1999) Chem Rev 99:2573–2587
22. Goldstein S, Czaspski G (1991) Free Radical Res Commun 12/ 13:5
23. Aranovitch J, Samuni A, Godinger D, Czapski G (1986) In: Rotilio G (ed) Superoxide and superoxide dismutase in chemistry, biology and medicine. Elsevier Science Publishers
24. Alzuet G, Ferrer S, Borrás J, Solans X, Font-Bardía M (1993) Inorg Chim Acta 203:257–261
25. Casanova J, Alzuet G, Borrás J, Timoneda J, García-Granda S, Cándano-Gonzalez I (1994) J Inorg Biochem 56:65–67
26. Alzuet G, Casella L, Perotti A, Borrás J (1994) J Chem Soc Dalton Trans 2347–2351
27. Alzuet G, Casanova J, Ramirez JA, Borrás J, Carugo O (1995) J Inorg Biochem 57:219–234
28. Casanova J, Alzuet G, Borrás J, Latorre J, Sanau M, García-Granda S (1995) J Inorg Biochem 60:219–230
29. Casanova J, Alzuet G, Borrás J, Carugo O (1996) J Chem Soc Dalton Trans 2239–2244
30. Casanova J, Alzuet G, Latorre J, Borrás J (1997) Inorg Chem 36:2052–2058
31. Casanova J, Alzuet G, Ferrer S, Latorre J, Ramírez JA, Borrás J (2000) Inorg Chim Acta 304(2):170–179
32. Gutierrez L, Alzuet G, Real JA, Cano J, Borrás J, Castiñeiras A (2000) Inorg Chem 39:3608–3614
33. Gutierrez L, Alzuet G, Borrás J, Castiñeiras A, Rodríguez-Fortea A, Ruiz E (2001) Inorg Chem 40:3089–3096
34. Kappa-CCD Control Software (1997) Nonius BV Delf, The Netherlands
35. Otwinowski Z, Minor W (1997) Processing of X-ray diffraction. Data collected in oscillation mode in methods in

enzymology, vol 276. Macromolecular crystallography, part A. Carter and Sweet, Academic Press, New York, pp 307–326
36. Beurskens PT, Admiraal G, Beurskens G, Bosman WP, García-Granda S, Gould RO, Smits JMM, Smykalla C (1992). The DIRDIF program system. Technical Report of the Crystallographic Laboratory, University of Nijmegen, The Netherlands
37. Sheldrick GM (1997) SHELXL-97: a program for the refinement of crystal structures. University of Gottingen, Germany
38. International tables for X-ray crystallography (1974) Birmingham: Kynoch Press (Present distributor: Kluwer Academic Publishers: Dordrecht, The Netherlands)
39. Spek AL (1982) The EUCLID package. In: Computational crystallography. Clarendon Press, Oxford, p 528
40. Collect Nonius BV (1997–2000)
41. Beurskens PT, Beurskens G, Bosman WP, de Gelder R, García-Granda S, Gould RO, Israël R, Smits JMM (1996).The DIRDIF-96 program system. Crystallography Laboratory, University of Nijmegen, The Netherlands
42. Nardelli M (1983) Comput Chem 7:95–98
43. Spek AL (2000) PLATON, a multipurpose crystallographic tool. Utrecht University, Utrecht, The Netherlands
44. Oberley LW, Spitz DR (1986) In: Greewald A (ed) Handbook of methods for oxygen radicals research. CRC Press, Boca Raton
45. Jamienson DJ, Rivers SL, Stephen WS (1994) Microbiology 140:3277–3283
46. Addison W, Rao TN, Reedijk J, van Rijn J, Verschoor GC (1984) J Chem Soc Dalton Trans 1349
47. Bertini I, Banci L, Piccioli M (1990) Coord Chem Rev 100:67
48. Gutierrez L, Alzuet G, Borrás J, Liu-González M, Sanz F, Castiñeiras A (2001) Polyhedron 20:703–709
49. WINEPR-Simfonia.1.25 (1994–1996) Bruker Analytik GmbH, Karlsruhe, FRG
50. Sakaguchi U, Addison AW (1979) J Chem Soc Dalton 600
51. Wesser U, Miessel R, Linss M (1990) In Antioxidants in therapy and preventive medicine. Plenum Press, New York
52. Linss M, Wesser U (1987) Inorg Chim Acta 138:163
53. Bonomo RP, Impellizzeri G, Pappalardo G, Purrello R, Rizzarelli E, Tabbi G (1998) J Chem Soc Dalton Trans 3851
54. Walker GM (1998) In: Yeast physiology and biotechnology. Wiley, Chichester