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Generation of Oxygen Radicals from Iron Complex of Orellanine, A Mushroom Nephrotoxin; Preliminary ESR and Spin-Trapping Studies

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Orellanine, [2,2'-bipyridine]-3,3',4,4'-tetrol-1,1'-dioxide, is the toxin responsible for the lethal nephrotoxicity of some *Cortinarius* mushrooms. Our present ESR and spin-trapping studies of the redox properties of the system of non-illuminated orellanine, ferrous iron and dioxygen contribute to understanding the molecular mechanism of its toxicity. UV-visible spectrophotometry, cyclic voltammetry and ESR in frozen medium showed the formation of a wine-red tris complex, $Fe(III)Or_3$. This ferric complex is easily reducible ($E_p = -565$ mV *vs* Ag/AgCl/3M KCl at pH 7), involving a one-electron reversible process. Spin-trapping using DMPO is employed to detect the generation of superoxide anion and hydroxyl radicals. The instantaneous one-electron oxidation of ferrous ions in the presence of the toxin under air is concomitant with dioxygen consumption as supported by dioxygen consumption. GSH involves the toxin and ferrous ions under air in a redox cycling process resulting in the production of glutathionyl and oxygen free radicals, observed for the first time with an iron complex of a mushroom toxin. In most cases, EDTA is not able to prevent the $Fe(III)Or_3$ and radical formation. The *ortho*-dihydroxylated groups borne by the di-*N*-oxidized bipyridine structure and not the bipyridine structure itself, are responsible for the formation of a stable ferric complex at pH 7, as they are for the generation of an apparently stable *ortho*-semiquinone anion radical. These one-electron mechanisms may play a major role in some of the known toxic effects of orellanine.

Keywords: Hydroxyl radical, superoxide radical, orellanine, iron complex, ESR, spin-trapping

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMSO, dimethylsulfoxide; EDTA, ethylenediamine tetraacetic acid; ESR, electron spin resonance; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; Or, orellanine ([2,2'-bipyridine]-3,3'-4,4'-tetrol-1,1'-dioxide); SOD, superoxide dismutase; Tris, *tris*(hydroxymethyl)-aminoethane.

INTRODUCTION

Orellanine, [2,2'-bipyridine]-3,3',4,4'-tetrol-1,1'-dioxide, is the toxin from several *Cortinarius* species from different geographic origins, including *Cortinarius orellanus*.[1] The lethal toxin induces acute renal failure after a few

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days delay.[2] Very severe or even fatal intoxications have occurred every autumn in the last few years in Europe and North America. No antidote and no specific therapy are known up to now. In the case of irreversible renal failure, only chronic hemodialysis or renal transplantation are efficient.

The purpose of our research is to elucidate the molecular mechanism of action of orellanine. We have recently reported[3] the existence of an *ortho*-semiquinone anion radical form of the toxin. It is generated after a one-electron oxidation, *e.g.* photochemically, at physiological pH under aerobic or anaerobic conditions. Bioreductive agents can involve *in vitro* the semiquinone radical and orellanine in a redox-cycling process. This process results in the continuous production of glutathionyl and oxygen free radicals, detected by spin-trapping. All these properties are due to the *ortho*-dihydroxylated groups borne by the di-*N*-oxidized bipyridine structure of the toxin and may at least partly explain its toxicity. No other ESR data are available in the literature for orellanine or closely related chelating molecules.

The aim of this paper is to examine another possible way of production of free radicals by orellanine. The bicyclic toxin, bearing several metal-binding sites (2,2'-bipyridine-di-*N*-oxide and two *ortho*-dihydroxylated groups), shows very strong complexation properties which we noticed by determining its pKs.[4] For a first approach, we chose iron among the transition metals because its orellanine-complex is soluble at physiological pH and iron plays an important role in the functioning of any cells and in free radicals generation. ESR spectroscopy in frozen medium was used to elucidate the oxidation state of metal in the iron compound formed when orellanine ligates ferrous iron in presence of dioxygen. ESR spectrometry with spin-trapping using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was employed to study the production of oxygen radicals from orellanine, ferrous iron and dioxygen. DMPO is also a commonly used spin trap for the detection of thiyl radicals generated from thiols which are candidates for cellular reducing agents for Fe(III)chelates. The experiments were carried out under aerobic conditions except where otherwise stated and at pH compatible with physiological functions. Other analytical methods such as UV-visible spectrophotometry, cyclic voltammetry and dioxygen consumption were also used to confirm the ESR results.

MATERIALS AND METHODS

Chemicals

Water was deionized and twice distilled. For a good reproducibility, all media employed should be as free as possible of transition metals other than the deliberately added iron. Catalase (EC 1.11.1.6) from beef liver (65,000 Units/mg) and superoxide dismutase (SOD, EC 1.15.1.1) from bovine erythrocytes (5000 Units/mg) were obtained from Boehringer Mannheim. GSH, Tris, EDTA, HEPES were supplied by Sigma. Ferrous sulfate was ACS reagent grade (Sigma), standardized manganimetrically and stored under nitrogen. Ferric nitrate (dissolved in H_3PO_4 pH 1), phosphoric acid, potassium hydroxide, potassium dihydrogen phosphate and disodium hydrogen phosphate were Suprapur reagent grade (Merck). DMSO was GR reagent grade (Merck).

The spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich and was purified by vacuum distillation to remove any ESR contaminating signal like the 1:2:2:1 quartet signal indicative of the DMPO-·OH adduct. Small aliquots were stored frozen under nitrogen. It was used at 300 mM concentration. All spin trap experiments were carried out in the dark to avoid photochemically induced reactions of DMPO and photooxidation of orellanine.

Orellanine was extracted from dry powdered carpophores of *C. orellanus* mushrooms collected locally and purified as previously described.[3] Stock solutions of orellanine (50 mM) were prepared in the dark by dissolving the toxin in 100 mM disodium hydrogen phosphate or 100 mM

Tris buffer pH 10. Then the pH was adjusted to 7 or 7.4 respectively. Stock solutions of the photosensitive and easily oxidizable toxin were stored in the dark at 4°C and used in the following two days.

ESR Experiments

For direct electron spin resonance measurements, the solutions were prepared by adding the iron solution (2 mM) at last in medium containing orellanine (6 mM) and 100 mM phosphate buffer pH 7 under air. After mixing, 0.2 ml of the wine-red solutions were transferred into cylindrical quartz ESR tubes and immediately frozen in liquid nitrogen. ESR spectra were recorded at –183°C on a Bruker ESP 300 E spectrophotometer at 9.41 GHz.

For the spin-trapping method, X-Band ESR spectra were obtained using a quartz flat cell, a Bruker cavity and a Bruker ER 100D spectrometer (9.3 GHz). Cell temperature was 20°C or 37°C. Samples were mixed in the dark, pipetted into the cell with 100 mM phosphate buffer pH 7 or 100 mM Tris buffer pH 7.4. After being pipetted into the cell they were ready to scan within 30 s. The course of each reaction was monitored for at least 20 min after initiation.

Cyclic Voltammetry

Cyclic voltammetry was performed on a EG and G scanning potentiostat model 362. A three electrode assembly was used: a hanging mercury electrode as working electrode, a saturated aqueous Ag/AgCl/KCl electrode as reference electrode and a platinum wire as auxiliary electrode. Oxygen-free nitrogen was bubbled 5 min through the thermostated brown glass cell (25.0 ± 0.1°C).

UV-visible Spectrophotometry and Dioxygen Consumption

A UV-visible spectrophotometer Pye Unicam SP 7-500 was used to measure the changes in iron complex absorbance at 498 nm (quartz cell: 10 mm path length). Dioxygen consumption was measured polarographically in 100 mM phosphate buffer pH 7 at 28°C using a Clark-type electrode system purchased from Hansatech Ltd.

All the assays were carried out in triplicate or more and were assessed by varying the maximum number of parameters (different days, two different operators and daily-prepared reagents).

RESULTS

Studies of the System Non-illuminated Orellanine, Fe^{2+}, O_2

UV-visible Spectrophotometry

When ferrous iron is added to a colourless orellanine (Or) solution, (in presence or in absence of its semiquinone radical) in aerated phosphate buffer pH 7, a wine-red solution is instantaneously obtained. Ferrous iron reacts under air with ionized form of the toxin. Under rigorously anaerobic conditions, the solution remains colourless. The same wine-red colour is observed with ferric ions under aerobic or anaerobic conditions. In contrast to ferric iron, the ion of which is soluble at pH below 2, the wine-red compound formed is soluble above pH 5.

In order to elucidate the oxidation state of iron in the compound formed, UV-visible spectrometry was applied to solutions of Fe^{2+} and Or at pH 7 mixed under air or to mixtures of Fe^{3+} and Or. In both cases the spectra are identical, showing that the system Fe^{2+} and Or spontaneously (independently of light) autoxidizes under air, giving a soluble wine-red compound. The UV-visible spectrum of this compound at pH 7 presents two intense bands at 310 (ε = 11 700) and 282 nm (ε = 15 450) and a broad absorption band at 498 nm (ε = 2950). The two UV bands are similar to those of orellanine at this pH.[4]

In order to evaluate the metal to ligand ratio in the ferric orellanine complex at pH 7, varying amounts of ligand (0.1 to 0.6 mM) were added to a fixed amount of ferrous or ferric ions (0.1 mM). The

values of absorbance recorded at 498 nm increase with the amount of ligand added (Figure 1), and the maximal absorbance is obtained with a ratio 1:3 (metal to ligand). The absorbance does not change significantly above this ratio. Consequently, the wine-red compound is identified as a ternary complex, noted as Fe(III)Or_3.

Cyclic Voltammetry

Interestingly, the wine-red iron-orellanine compound is reducible on a stationary mercury electrode at pH 7. The electrochemical data were established with a ratio of three moles of Or (10^{-4} M in 100 mM phosphate buffer pH 7) for one of Fe^{3+}, on the basis of the spectrophotometric results. The voltammogram (Figure 2) of Fe(III)Or_3 shows a pseudoreversible mechanism with a cathodic potential E_p = –565 m V *vs* Ag/AgCl/3M KCl. The calculated values of the cyclic voltammetric criteria ΔE_p and *n* are found to be 55 mV and 1.018, respectively. The same voltammetric results are obtained with a solution prepared by mixing Or and Fe^{2+} under air. In addition, no cathodic voltammogram of ferric iron is observable. This shows the absence of free ferric iron in the medium, taking into account the limit of detection with this electrochemical technique. In contrast, as we previously established,[5] orellanine can be reduced on

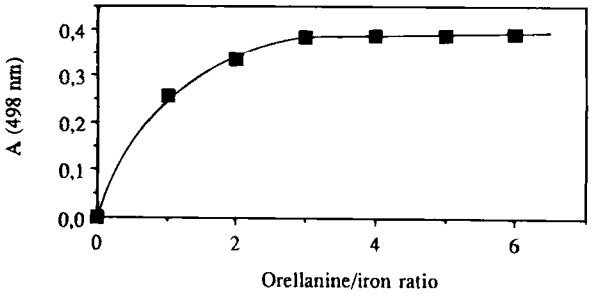

FIGURE 1 Absorbance at 498 nm of the ferric iron complexes formed at pH 7 by addition of various amounts of orellanine to a fixed amount of ferrous or ferric ions. Samples contained : Fe^{2+} or Fe^{3+} (0.1 mM) and Or (0.1 to 0.6 mM) in aerobic 100 mM phosphate buffer pH 7, mixed in the dark.

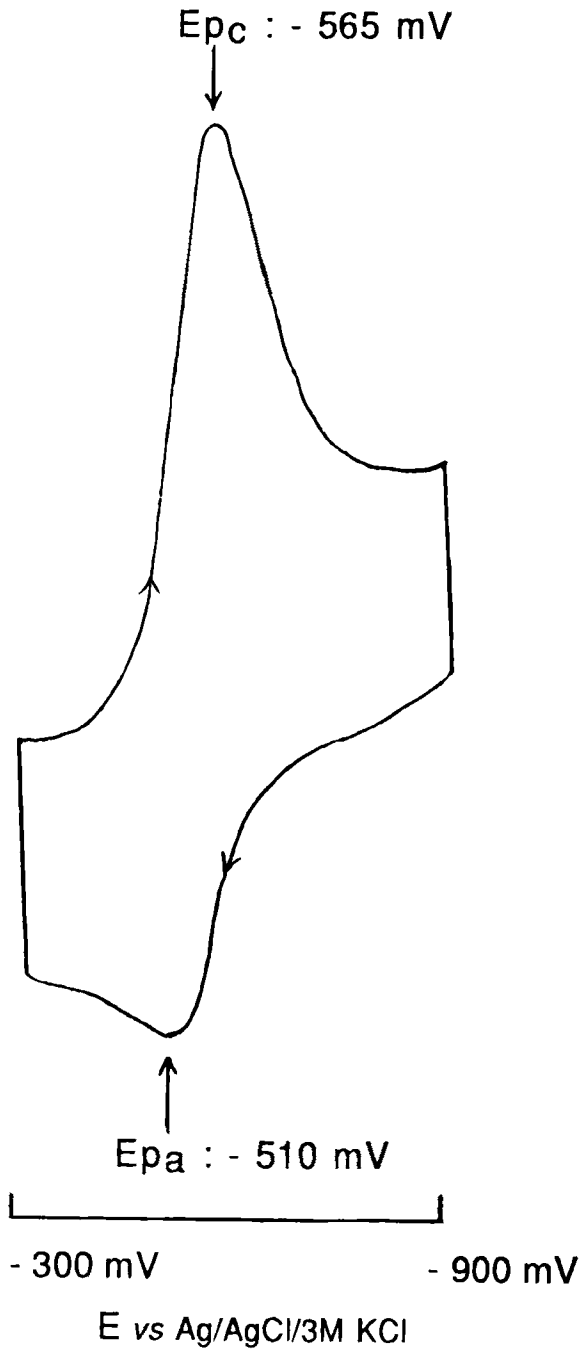

FIGURE 2 Cyclic voltammogram of the Fe(III)Or_3 complex at a hanging mercury electrode. Fe^{3+} (0.033 mM) and Or (0.1 mM) in 100 mM phosphate buffer as supporting electrolyte (final pH: 7.0). Scan rate: 100 mV s^{-1}; working electrode: hanging mercury electrode; drop size: large; reference electrode: Ag/AgCl/3M KCl; cell temperature : 25.0 ± 0.1°C.

mercury only under limited conditions of pH (0.0 to 4.3) and of concentration (very diluted solutions 10^{-6} to 10^{-7} M). Only one cathodic peak is observed on the voltammogram (E_p = –1076 mV *vs* Ag/AgCl/3M KCl at pH 2.3) without anodic peak. So the highly irreversible four-electon reduction of the free toxin is due to a catalytic process connected with the reduction of the protonated *N*-oxide functions.

ESR Spectroscopy

Many Fe(III)complexes are paramagnetic and give, as a rule, a g = 4.3 ESR signal; thus we recorded ESR spectra of the system Or (0.6 mM), Fe^{2+} (0.2 mM) and O_2 at –183°C in 100 mM phosphate buffer pH 7. The spectrum (Figure 3) exhibits a broad absorption derivative feature centred near a g = 4.26. No ESR signal is obtained with the corresponding ferrous blanks. The spectra recorded with a mixture of Fe^{3+} and Or show a g = 4.23 broad band. In addition, a broad signal of uncertain identity is observed at g = 2.22 for this last ferric chelate and for the ferric iron blanks too. Similar ESR results have been reported[6] for monocyclic catechol-ferric iron complex at different pH (4.5 to 11). Note that, under our experimental conditions, no free-radical semiquinone of orellanine is seen at pH 7.

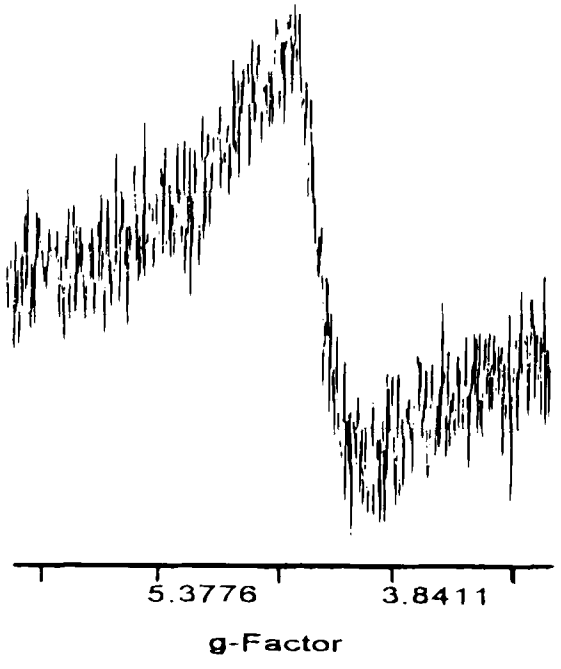

FIGURE 3 ESR spectrum of Fe(III)Or_3 complex in frozen medium. Final concentrations: Fe^{2+} (0.2 mM), Or (0.6 mM) in 100 mM phosphate buffer pH 7. Spectrometer settings : cell temperature, –180°C; microwave power, 10 mW; modulation amplitude, 0.312 mT; modulation frequency, 100 kHz; receiver gain, 1.25×10^5; time constant, 1.28 ms; sweep time, 167.8 s; sweep width, 400.2 mT.

Spin-trapping Experiments

On the basis of the preceding experiments, a ratio of 3 moles of orellanine for 1 iron atom was chosen for our spin-trapping study. When non-illuminated Or (1 mM) is mixed with DMPO (300 mM) in phosphate buffer (100 mM pH 7) and then Fe^{2+} (0.33 mM) is added to the medium, the wine-red characteristic colour of the complex Fe(III)Or_3 appears. In the presence of the diamagnetic and water-soluble spin trap DMPO, an intense signal of DMPO-hydroxyl radical spin adduct (DMPO-$^{\cdot}$OH) is immediately recorded ($a_N = a_H = 1.49$ mT). This ESR signal increases during 10 min at 20°C or during 3 min at 37°C and then slowly decreases with $t_{1/2}$ = 120 min at 20°C. If mixing is made within 30 s, a spectrum of DMPO-superoxide anion radical spin adduct (DMPO-$^{\cdot}$OOH) can be seen on the first scan (a_N = 1.43, a_H = 1.17, a_H = 0.13 mT), superimposed to the signal of the spin-trap adduct of $^{\cdot}$OH (Figure 4b), as confirmed by simulation (Figure 4c). The characteristic signal of the superoxide radical rapidly turns into the one of DMPO-$^{\cdot}$OH (Figure 4d).

Effect of Buffer

The first assays were made in 0.1 M phosphate buffer pH 7 because phosphate ions are contained in biological fluids, especially in intracellular fluids and serve as part of the buffering capacity of the cells. It is well known that phosphate ions

have relatively high binding effect on iron, thus we tested Tris buffer, the iron chelating ability of which is weaker. The $^{\cdot}OH$ production by Or, Fe^{2+} and O_2 system was duplicated in 0.1 M Tris buffer pH 7.4 to compare the two sets of results, and a significant decrease of the DMPO-$^{\cdot}OH$ signal (–25%) was observed in Tris. It is in good agreement with its ability to scavenge $^{\cdot}OH$. A more important decrease (–74%) is obtained in 0.1 M HEPES buffer pH 7. HEPES competes with DMPO for $^{\cdot}OH$ according to results previously published for the system Fe^{2+}, bleomycin, O_2.[7] Therefore, the study of the strong chelating agent orellanine was carried out in phosphate buffer to be close to biological conditions.

Controls

Unchelated Fe^{2+} does not readily autoxidize in saline solution at neutral pH and ambient O_2 tension, under time and concentration conditions used in our experiments as shown by the absence of oxygen radicals adducts on the spectra of controls (Figure 4a). These results are supported by the absence of dioxygen consumption by Fe^{2+} controls in phosphate medium as described below. In contrast, 0.18 mM Fe^{3+} at pH below 2 (to make ferric iron soluble) induces a weak DMPO-$^{\cdot}OH$ signal as reported by other authors.[8,9] Its intensity is six times as low as the one induced by the studied system. This adduct is observed within three minutes and is persistent for at least 60 min after Fe^{3+} addition to DMPO solution. The adduct formation is due to the Fe^{3+}-induced nucleophilic attack of H_2O at the 2-position of the DMPO ring.[8,9] Note that, in these controls, the free Fe^{3+} concentration is widely higher than in the samples containing Or at pH 7, samples in which Fe^{3+} is quite predominantly complexed. Thus, the observation of $^{\cdot}OH$-spin adduct in aerated mixtures of Fe^{2+} and Or is not artifactual.

Effect of Metal-ligand Ratio

When the iron-to-orellanine ratio is varied (from 1 to 6) with fixed concentration of Fe^{2+} (0.33 mM), the intensity of the DMPO-$^{\cdot}OH$ signal is maximal for a 1:3 metal to ligand ratio (Figure 5). Above this ratio, the ESR signal significantly decreases.

Effect of SOD, Catalase and DMSO

In the presence of SOD (250 U/ml), no DMPO-$^{\cdot}OOH$ signal is observed on the first scan. Moreover, the DMPO-$^{\cdot}OH$ signal is lowered (–15%). Addition of 650 U/ml of catalase decreases the DMPO-$^{\cdot}OH$ signal more efficiently (–25%). In the presence of both SOD and catalase, a strong decrease is observed (–70%). All the shifts were measured 5 min after mixing the solutions.

After addition of DMSO (1% v/v) in the aqueous system, a DMPO-$^{\cdot}CH_3$ signal (a_N = 1.53 mT and a_H = 2.2 mT) is observed, superimposed to a DMPO-$^{\cdot}OH$ signal (Figure 4e). This ESR spectrum is comparable to the simulated one (Figure 4f). Furthermore, the line height of the DMPO-$^{\cdot}CH_3$ signal increases at the expense of that of the DMPO-$^{\cdot}OH$ signal. These results indicate that the DMPO-$^{\cdot}OH$ spectrum recorded is not an artifact.

Effect of Dioxygen Content and pH

When Fe^{2+} is added to a mixture of Or and DMPO in phosphate buffer pH 7 in which the dissolved dioxygen has been partly removed by bubbling nitrogen, the DMPO-$^{\cdot}OH$ signal is substantially lowered, and the solution is only slightly coloured. Not surprisingly, under rigorously anaerobic conditions (under argon), no ESR signal is recorded. However, if the anaerobic ESR-silent sample is exposed to air, DMPO-$^{\cdot}OH$ is instantaneously formed. All these results indicate the role of dioxygen in the formation of the superoxide anion radical.

The influence of pH is examined in a chosen range (pH 5 to 9) in which the complex Fe(III)Or_3 remains soluble and stable in phosphate buffer. The intensity of the DMPO-$^{\cdot}OH$ signals recorded after the same incubation time (10 min) remains constant in this pH range.

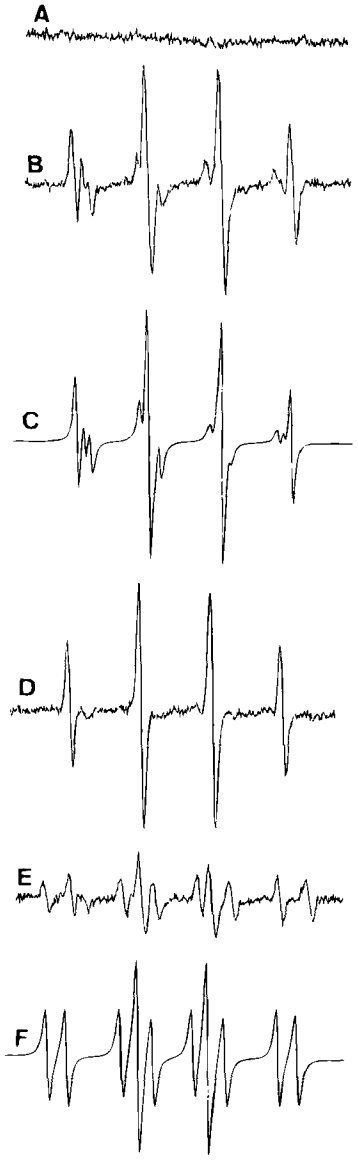

FIGURE 4 ESR spectra of radical adducts of DMPO produced at physiological pH by the system non-illuminated orellanine, Fe^{2+} and O_2.
Samples contained : (**A**) Control, Fe^{2+} (0.33 mM), DMPO (300 mM) in aerobic 100 mM phosphate buffer pH 7. (**B**) Spectrum (first scan) of the wine-red solution containing DMPO (300 mM) in 100 mM phosphate buffer pH 7, Or (1 mM) and Fe^{2+} (0.33 mM) added at last, mixed under air and ready for scan within 30 s. (**C**) Computer simulation of spectrum B : 33% of signal with splitting constants of DMPO-$^{\cdot}$OOH adduct (a_N = 1.43, a_H = 1.17, a_H = 0.13 mT), and 67% of signal with splitting constants of DMPO-$^{\cdot}$OH adduct ($a_N = a_H$ = 1.49 mT) were mixed in the first half of the spectrum, 17% and 85% were mixed in the second half. (**D**) Spectrum recorded 200 s after the spectrum shown in B. (**E**) Spectrum of DMPO (300 mM) in 100 mM phosphate buffer pH 7, Or (1 mM), Fe^{2+} (0.33 mM) and DMSO 5% (v/v). (**F**) Computer simulation of spectrum E : 50% of signal with splitting constants of DMPO-$^{\cdot}$OH adduct and 50% of signal with splitting constants of DMPO-$^{\cdot}CH_3$ adduct (a_N = 1.53, a_H = 2.2 mT). Spectrometer settings : cell temperature, 20°C; microwave power, 20 mW; modulation amplitude, 0.08 mT; modulation frequency, 100 kHz; receiver gain, 4×10^5; time constant, 200 ms; scan time, 200 s; scan range, 10 mT.

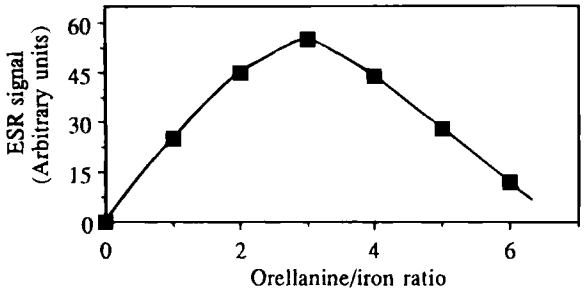

FIGURE 5 Variation in the intensity of one of the line for DMPO-·OH adduct generated by the system non-illuminated orellanine, Fe^{2+} and O_2 for varied iron to orellanine ratio. Samples contained : fixed concentration of Fe^{2+} (0.33 mM), DMPO (300 mM) and various concentrations of Or (0–3.3 mM) in aerobic 100 mM phosphate buffer pH 7. Spectrometer settings : cell temperature, 20°C; microwave power, 20 mW; modulation amplitude, 0.08 mT; modulation frequency, 100 kHz; time constant, 1 s; scan time, 200 s; scan range, 10 mT.

Effect of Oxidation State of Iron

In order to confirm the role of autoxidation of Fe^{2+} in presence of Or and O_2 in the generation of oxygen radicals, Fe^{3+} ions (0.33 mM) are added at last to a medium containing Or and DMPO in phosphate buffer pH 7. In these conditions, a wine-red colour instantaneously appears and ferric iron is solubilized in presence of the toxin giving the Fe(III)Or_3 complex. However, only a weak DMPO-·OH signal is recorded three minutes after mixing the solutions. The signal peak height is much weaker than that which is observed with the controls without Or (Fe^{3+} 0.33 mM, DMPO 300 mM at pH below 2). Note that the presence of both chelators, orellanine and phosphate ions, decreases the DMPO-·OH signal caused by the nucleophilic attack of the spin trap by ferric iron in water at pH 2. Ferric iron is not able to generate an intense and significant oxygen radicals production in presence of Or and O_2 like that obtained with ferrous iron.

Oxygen Consumption Experiments

The role of molecular dioxygen in the formation of the DMPO-·OH adducts was further supported by the dioxygen consumption experiment as shown in Figure 6. The oxygen consumption by controls containing various concentrations of non illuminated Or (0.5 to 1 mM) or Fe^{2+} (0.15 to 0.33 mM) in 100 mM phosphate buffer pH 7 is null. Addition of 0.33 mM Fe^{2+} in solutions containing 0.99 mM Or

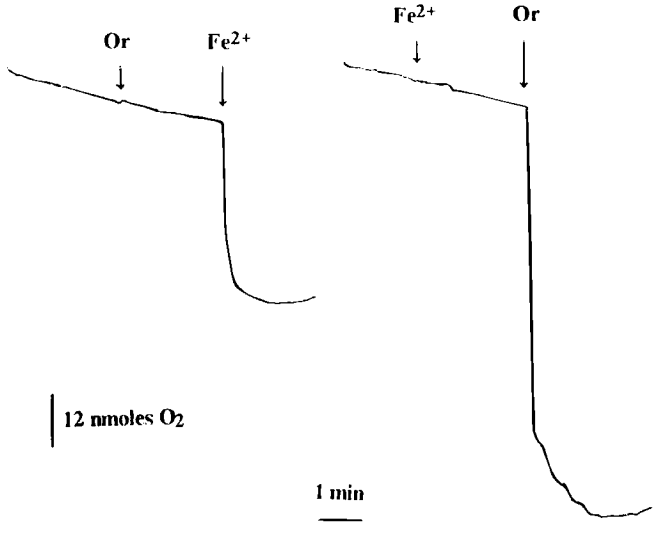

FIGURE 6 Dioxygen consumption by the system non-illuminated orellanine, Fe^{2+} and O_2 under physiological conditions. Reaction medium : phosphate buffer 100 mM pH 7 equilibrated with the dioxygen electrode at 28°C. (**A**) Addition of 0.31 mM Or followed by an addition of 0.15 mM Fe^{2+}. (**B**) Addition of 0.33 mM Fe^{2+} followed by an addition of 0.99 mM Or.

induces an instantaneous oxygen consumption. This consumption is proportional to the Fe^{2+} concentration for the ratio 1 : 3 metal to ligand.

Study of the System, Non-Illuminated Orellanine, Fe^{2+}, O_2 and Thiol by Spin-Trapping

As shown in our recent report[3], bioreductive agents like GSH and cysteine can involve the semiquinone radical of orellanine and the toxin itself in a redox cycling process resulting in the production of oxygen free radicals. Moreover, the reduction of metal-chelates can be accomplished by superoxide, but also by other biological reductants. Therefore, we have checked the influence of GSH (30 mM) on the system Or (1mM), Fe^{2+} (0.33 mM) and O_2 in the presence of DMPO (300 mM) in phosphate buffer pH 7. The results are different according to the order of addition of the reagents.

When GSH is immediately added to a wine-red solution of $Fe(III)Or_3$ complex preliminary formed by mixing Or and Fe^{2+} under air, no DMPO-$^{\cdot}$OOH signal is observed on the first scan. A DMPO-$^{\cdot}$OH signal is nevertheless recorded, its peak height is slightly lowered and its rate of decay ($t_{1/2}$ = 80 min at 20°C) is enhanced in comparison with the one obtained in controls without GSH ($t_{1/2}$ = 120 min at 20°C). The decrease of the DMPO-$^{\cdot}$OH signal intensity may be partly explained by the ability of the thiols, like cysteine, captopril and GSH to scavenge $^{\cdot}$OH radicals.[10] The rate constant of $^{\cdot}$OH scavenging by GSH (1.4×10^{10} M^{-1} s^{-1}) is to be compared with the rate constant of the reaction of DMPO with $^{\cdot}$OH radicals (3.4×10^{9} M^{-1} s^{-1}). It is noticeable that a minor production of a new DMPO adduct identified as a DMPO-GS$^{\cdot}$ (see below) is recorded within the first 200 s at 37°C only. No visible discolouration of the solution occurs even 85 min after GSH addition.

When GSH is immediately mixed with DMPO and Or prior to addition of Fe^{2+}, the wine-red complex appears. No DMPO-$^{\cdot}$OOH signal is present on the first scan but a new DMPO adduct is observed (Figure 7b). Taking into account that the DMPO adduct of the glutathione radical has ESR splitting constants (a_N = 1.52 mT and a_H = 1.61 mT) close to those of $^{\cdot}$OH (a_N = 1.52 mT and a_H = 1.52 mT) the question has arisen whether thiyl and hydroxyl radicals are simultaneously produced in the reaction of iron orellanine with sulfhydryl compounds. The recorded spectrum could be simulated using the splitting constants of DMPO-GS$^{\cdot}$ and DMPO-$^{\cdot}$OH. The simulated spectrum is given in Figure 7c. So, we have unambiguously identified the adduct as a DMPO-GS$^{\cdot}$ one. Thus we can conclude that a significant DMPO-GS$^{\cdot}$ signal appears superimposed to a DMPO-$^{\cdot}$OH one. The decreasing DMPO-GS$^{\cdot}$ signal can nevertheless be recorded at 37°C during 15 min after mixing the solutions. The intensity of one of the lines for the DMPO-$^{\cdot}$OH spin adduct, measured after the DMPO-GS$^{\cdot}$ signal has disappeared, is about 12% inferior to that obtained with the controls without GSH. In addition, the DMPO-GS$^{\cdot}$ generation is GSH concentration dependent. Consistently, the production of thiyl radicals decreases with decreasing concentration of the reducing thiol in the studied range 30 to 1 mM.

When GSH is immediately added to a mixture of DMPO and Fe^{2+} prior to addition of Or, no colour appears immediately. During approximately the first 3 min, only an intense DMPO-GS$^{\cdot}$ adduct with a distinctive ESR spectrum consisting of four relatively broad lines, is recorded at 37°C (Figure 7d). In addition, hydroxyl radicals are trapped 3 min after reaction initiation, giving an ESR signal superimposed to the latter one (Figure 7e). The DMPO-GS$^{\cdot}$ adduct signal vanishes 20 min after mixing the solutions. The $t_{1/2}$ of the DMPO-$^{\cdot}$OH adduct is about 75 min at 37°C and the intensity of this signal is much lower (10%) than in the previous case. The colourless medium turns very slightly wine-red with time. By reducing dioxygen with catalysis by ferrous iron and orellanine, GSH gives the thiyl species. With the large concentration of thiol utilized, the

solution will rapidly become anaerobic, preventing the oxidation of the iron-orellanine system and the formation of hydroxyl radicals. By redissolution of dioxygen from air with time into the medium, oxidation of a part of iron and the concomitant formation of ferric-orellanine complex can occur, visualized by the appearance of a slight wine-red colour and hydroxyl radicals trapped by DMPO.

It is noticeable that in controls with DMPO, 0.33 mM Fe^{2+} and 30 mM GSH, in aerobic phosphate, a slight signal is observed after about five minutes after reaction initiation at 37°C (Figure 7a). The ESR signal intensity is much lower than that recorded in presence of the toxin and is in good agreement with results reported by other authors.[7]

Study of the System Non-Illuminated Orellanine, Fe^{2+}, O_2 and EDTA by Spin-Trapping

In this preliminary report, we have studied the effect of the chelating agent EDTA (1mM) on the iron-orellanine system (1 mM Or and 0.33 mM Fe^{2+}) in presence of DMPO (300 mM) in phosphate buffer pH 7, under air. Two cases will be depicted according to the order of addition of the reagents.

When EDTA is added at last in the medium containing the wine-red complex formed by mixing Fe^{2+} and Or, the intensity of DMPO-$^{\cdot}$OH signal is not significantly different from that of controls without EDTA and ferric iron is not withdrawn from its complex. No visible discolouration of the solution occurs after a 15 min incubation time. It is known[11] that EDTA itself scavenges some $^{\cdot}$OH radicals (2.8×10^9 M^{-1} s^{-1}). Taking into account the rate constant value for hydroxyl radical trapping with DMPO (3.4×10^9 M^{-1} s^{-1}) which is closed to the previous one and our results, it seems that no noticeable EDTA scavenging effect can be observed in these experiments.

On the other hand, when Or is added to a mixture containing EDTA and Fe^{2+}, no colouration appears after a 15 min incubation time. Moreover, no DMPO-$^{\cdot}$OH signal is recorded, only a weak DMPO carbon centred radical signal which is in form and intensity identical to the signal noticed in controls without Or (data not shown). Thus fer-

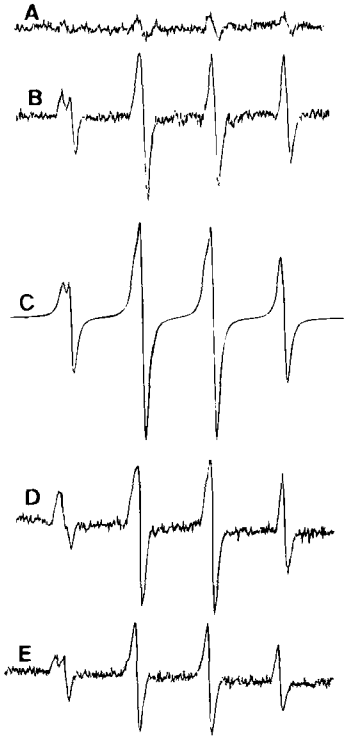

FIGURE 7 ESR spectra of radical adducts of DMPO produced at physiological pH by the system non-illuminated orellanine, Fe^{2+}, O_2 and GSH according to the order of reagents addition. Samples contained : (**A**) Control, GSH (30 mM) added to a mixture containing DMPO (300 mM) in aerobic 100 mM phosphate buffer pH 7 and Fe^{2+} (0.33 mM). (**B**) Spectrum (first scan) of DMPO (300 mM) in 100 mM phosphate buffer pH 7, Or (1 mM), GSH (30 mM) and Fe^{2+} (0.33 mM) added at last to the aerated medium and ready for scan within 30 s. (**C**) Computer simulation of spectrum B : 75% of signal with splitting constants of DMPO-GS$^{\cdot}$ adduct (a_N = 1.53, a_H = 1.62mT), and 30% of signal with splitting constants of DMPO-$^{\cdot}$OH adduct (a_N = a_H = 1.49 mT). The DMPO-GS$^{\cdot}$ adduct is simulated with the g factor 0.00065 units higher than the DMPO-$^{\cdot}$OH one. (**D**) Spectrum (first scan) of DMPO (300 mM) in 100 mM phosphate buffer pH 7, Fe^{2+} (0.33 mM), GSH (30 mM) and Or (1 mM) added at last to the aerated medium and ready for scan within 30 s. (**E**) Spectrum recorded 13 min after the spectrum shown in D. Spectrometer settings : cell temperature, 37°C; microwave power, 10 mW; modulation amplitude, 0.08 mT; modulation frequency, 100 kHz; receiver gain, 4×10^5; time constant, 1 s; scan time, 200 s; scan range, 10 mT.

rous ions in presence of EDTA give only the colourless Fe(II)EDTA complex, without oxygen radicals production.

DISCUSSION

The present study demonstrates the formation of a $Fe(III)Or_3$ complex and the involvement of $O_2^{\cdot-}$ and $^{\cdot}OH$ generation during the oxidation of Fe^{2+} in presence of Or and O_2 at physiological pH.

For this study of iron dependent oxygen radicals producing processes the three common buffers, phosphate, Tris and HEPES have been tested despite they are not "good buffers".[12] Phosphate and Tris buffers are known to ligate metals like iron and, in the presence of a weak iron chelate, both buffers will effectively compete with the studied chelator of iron. Moreover, phosphate catalyzes rapid Fe^{2+} oxidation because of its preference for Fe^{3+} and it is likely that significant superoxide anion radical is produced. On the other hand, Tris is also an effective $^{\cdot}OH$ scavenger like HEPES buffer. The latter buffer, which is frequently used, can affect iron redox chemistry.[12] Finally, for our present study, it seems to be impossible to avoid buffer problems taking into account the literature data. However, our spin trapping and dioxygen consumption experiments are in favour of the use of phosphate buffer in which hydroxyl radical production is the most intense and controls the most satisfactory, because their ESR signals are quite negligible.

The ionized species of orellanine react with ferrous iron under aerobic conditions or with ferric iron to give a stable ferric complex which is water-soluble at pH > 5. Between pH 5.8 and 11, which are the values of the two higher apparent pKs of orellanine, the *ortho*-dihydroxylated groups are dianionic in the predominant zwitterionic-like species.[4] So the ionized *ortho*-hydroquinone functionalities of orellanine appear well suited for metal chelation. The tris ferric complexes, formed either by direct association with Fe^{3+} or by autoxidation of the Fe^{2+}, orellanine system, exhibit indistinguishable visible spectra. In addition, the visible band is in agreement with the one classically observed with Fe(III)-catechol complexes. Moreover, it is recognized that ferric ions can form very stables complexes with catechol ligands.[6] With 1,2-benzenediol, a benzenic monocyclic compound, the ferric iron is bound as the bis complex between pH 6 and 7. But at pH > 9.5 essentially all of the ferric iron exists as the tris (catecholato) complex.[6] Other *ortho*-diphenolic-monocyclic compounds like tiron are known to give at pH 7 very stable violet tris complexes with iron at its ferric state. On the contrary, 2,2′-bipyridine, the basic bicyclic skeleton of the toxin, gives a red tris complex with ferrous iron only (λ_{max} = 520 nm). Our UV-visible data are in favour of an *ortho*-hydroxyl-complex for the iron-orellanine one and the spectrophotometric titration agrees with a tris-complex.

The cyclic voltammetric study of the readily reducible $Fe(III)Or_3$ complex allows us to estimate the number of electrons (n) involved in the overall electrode process which is in close agreement with a one-electron reduction. Using the relation established for a reversible process, an $E^{o\prime}$ value of 280 mV *vs* N.H.E. (pH 7) is calculated for the $Fe(III)Or_3/Fe(II)Or_3$ redox couple. Thus, the reduction potential of the Fe^{3+}/Fe^{2+} redox couple is decreased by approximately 500 mV by chelation to orellanine. It is known that chelators in which oxygen atoms ligate the metal tend to prefer the oxidized forms of iron, thus decreasing the redox potential of these metals.[12] On the other hand, chelators which bind Fe^{3+} with greater affinity than Fe^{2+}, increase the rate of Fe^{2+} autoxidation. Orellanine acts as a low molecular weight chelating agent to selectively bind, solubilize, and probably mobilize ferric iron.

The ESR observations in frozen medium seem to suggest that the ferric state is completely stabilized by the toxin. When orellanine is mixed either with Fe^{3+} or with Fe^{2+}, ESR signals attributable to high spin iron occurred in both cases, which testified to the presence of $Fe(III)Or_3$ complex in both samples. These data confirm the

hypothesis of a fast Fe^{2+} oxidation in presence of orellanine and dioxygen. For example, Fe^{3+} chelator desferal (desferrioxamine B) possesses such a property. In an dioxygen-containing system, ferrous iron binds with desferal and then oxidizes very quickly giving Fe(III)desferal complex which exhibits a g = 4.3 ESR signal too.[13]

Thus the wine-red compound obtained with the system Or, Fe^{2+}, O_2, at pH 7 was identified with a $Fe(III)Or_3$ complex.

The present spin trapping experiments are in favour of the above proposed structure for the iron complex of orellanine as stated by visible spectroscopy, voltammetry and ESR. They also evidence an oxygen radical production. Interestingly, the reaction of Fe^{2+} and orellanine (1 : 1 metal to ligand ratio) in the presence of dioxygen produces a detectable concentration of hydroxyl radical. In contrast, when orellanine is in excess with regard to the proposed stoichiometry (1 : 3 metal to ligand ratio), the intensity of the DMPO-$^{\cdot}$OH signal decreases and is dependent for orellanine concentration. This fall in adduct detected is probably due to the ability of orellanine to scavenge superoxide radicals like what is known of catechol(amine)s.[14] In addition, the proposed "closed" structure of the $Fe(III)Or_3$ complex shown in Figure 8, would be expected to scavenge some $^{\cdot}$OH radicals generated at the iron centre of the complex. Ferric-bipyridyl complex which also exhibits a "closed" structure is known[15] to release in presence of H_2O_2 small numbers of $^{\cdot}$OH radicals into free solution in comparison to ferric-EDTA complex which have an "open structure".

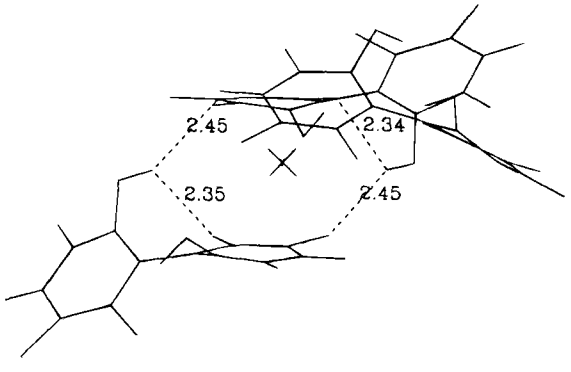

FIGURE 8 Proposed structure of $Fe(III)Or_3$ complex.

The inhibition of DMPO-$^{\cdot}$OH formation by SOD and catalase indicates that this formation involves $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}$OH produced in the studied system. If hydroxyl radicals are really generated, DMSO (1 % v/v), added in the aqueous medium, would react with these free radicals to produce methyl radicals as we have observed. These radicals have been spin-trapped, giving DMPO-$^{\cdot}CH_3$ at the expense of DMPO-$^{\cdot}$OH.

The ESR spin-trapping experiments unambiguously demonstrate that the system of non-illuminated orellanine, Fe^{2+} and O_2 at physiological pH generates instantaneously after mixing on the one hand a wine-red complex $Fe(III)Or_3$ and on the other hand superoxide anion radicals and the subsequent hydroxyl radicals. Moreover, the rate of oxygen consumption indicates the high level of autoxidation of this system. These results are consistent with superoxide anion radicals being formed at the expense of dioxygen. (Eq. 1):

$$Fe^{2+} + 3Or + O_2 \rightarrow Fe(III)Or_3 + O_2^{\cdot-} \quad (1)$$

Then, superoxide anion radical turns into hydrogen peroxide by disproportionation, and formation of hydroxyl radical occurs from hydrogen peroxide by the iron-catalyzed Haber-Weiss reaction. Superoxide anion radical and hydroxyl radical are spin-trapped by DMPO; no oxy radical is trapped under rigorously anaerobic conditions, showing the role of the molecular oxygen in the formation of the superoxide anion radical.

Recently, we observed that non-illuminated orellanine in combination with Fe^{2+} and dioxygen also cleaves DNA *in vitro* under physiological conditions (unpublished work), in good agreement with the production of oxygen radicals shown in the present spin-trapping study.

GSH, which is present in animal cells at mM levels, may participate in the mechanism of cytotoxicity of orellanine. Addition of this reducing thiol, in

a medium containing at the same time Fe(III)Or_3 and oxygen radicals generated by oxidation of Fe^{2+} in presence of Or and O_2, is not able to induce noticeable thiyl radical formation. Thus GSH is a poor reducing agent for Fe(III)Or_3. Similar results were reported for Fe(III)bleomycin complex.[7] In addition, oxygen radicals are not able to react with GSH to give thiyl radicals. On the other hand, a small amount of $GS^{\cdot}$ is revealed with hydroxyl radicals immediately after mixing Fe^{2+}, Or and GSH under aerobic conditions at 37°C. It is more clearly seen when GSH competes with free orellanine in the reaction mixture. Under particular experimental conditions no hydroxyl radical is trapped during the first 3 min, but an intense production of thiyl radicals is observed. Thus direct one-electron oxidation is likely to occur in the reaction of thiol, and in particular its anionic form GS^-, with an electron acceptor such as Fe(III)Or_3 (Eq 2):

$$GS^- + Fe(III)Or_3 \rightleftarrows GS^{\bullet} + Fe(II)Or_3 \quad (2)$$

To qualitatively estimate the relative ability of EDTA and Or to bind iron, interaction of EDTA with our system was studied. No detectable loss of iron from its previously formed complex with one of the ligands by the other one could be observed during the course of our experiments (15 min) at pH 7. So if a displacement of iron would take place, it should likely be very slow. However, one could surmise that the toxin is a very strong chelating agent for ferric iron. Moreover, solutions of the Fe(III)Or_3 complex seem to be indefinitely stable in the dark as is known for Fe(III)EDTA solutions. When $^{\cdot}OH$ radicals are instantaneously formed by the system Or, Fe^{2+} and O_2, an excess of EDTA is not able to suppress the concomitant oxygen radical generation.

The results related in this present paper confirm a fast Fe^{2+} oxidation in presence of orellanine and dissolved dioxygen as well as a concomitant generation of $O_2^{\cdot-}$ and $^{\cdot}OH$. Therefore, our data suggest that when ferrous iron is the main free catalytic metal, prooxidant action of orellanine may dominate, via the formation of $^{\cdot}OH$ or $GS^{\cdot}$ radicals.

There are two possible monoelectronic redox mechanisms of toxicity of the nephrotoxin orellanine. As we previously reported, under aerobic conditions, the oxidation of orellanine to an apparently stable *ortho*-semiquinone radical is coupled to the generation of $O_2^{\cdot-}$ and $^{\cdot}OH$. This mechanism can be cyclized by bioreductive agents such as GSH or enzymatic bioreductants.[3] Formation of semiquinone and highly reactive hydroxyl radicals under physiological conditions may partly explain the toxic properties of this fungal toxin. The second mechanism, described in this paper, involves the spontaneous aerobic oxidation of the system Fe^{2+}, orellanine resulting in $O_2^{\cdot-}$ and $^{\cdot}OH$ production. This mechanism might be also cyclised by bioreductive agents. By these two pathways, orellanine could act as a catalyst of the formation of $O_2^{\cdot-}$ and $^{\cdot}OH$ at its renal site of toxicity. The two proposed mechanisms may lead to a large dioxygen consumption, creating hypoxic conditions, as well as the dramatic depletion of renal glutathione levels observed in rats.[16] This radical production may cause cell death and tubular necrosis as we have previously described.[17]

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