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# Metabolic stability of superoxide adducts derived from newly developed cyclic nitrone spin traps

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**Abstract**

Reactive oxygen species (ROS) are by-products of aerobic metabolism involved in the onset and evolution of various pathological conditions. Among them, superoxide radical is of special interest as the origin of several damaging species such as $H_2O_2$, hydroxyl radical, or peroxynitrite ($ONOO^-$). Spin trapping coupled with ESR is a method of choice to characterize these species in chemical and biological systems and the metabolic stability of the spin adducts derived from reaction of superoxide and hydroxyl radicals with nitrones is the main limit to the *in vivo* application of the method. Recently, new cyclic nitrones bearing a triphenylphosphonium or β–cyclodextrin moiety have been synthesized and their spin adducts demonstrated increased stability in buffer. In the present manuscript, we studied the stability of the superoxide adducts of four new cyclic nitrones in the presence of liver subcellular fractions and biologically relevant reductants using an original set up combining a stopped-flow device and an ESR spectrometer. The kinetics of disappearance of the spin adducts were analyzed using an appropriate simulation program. Our results highlight the interest of new spin trapping agents CD-DEPMPO and CD-DIPPMPO for specific detection of superoxide with high stability of the superoxide adducts in the presence of liver microsomes.

## Introduction

Reactive oxygen species (ROS) are important mediators in aging[1], cell signaling under physiological and pathological conditions such as inflammation, cancers, and neurodegenerative diseases[2,3]. Superoxide radical anion ($O_2^{\cdot -}$) is a major contributor to oxidative stress, as it is the origin of damaging species such as $H_2O_2$, $HO^{\cdot}$, and $ONOO^{-}$. Since its introduction in the 1970's[4], spin trapping coupled to electron spin resonance spectroscopy (ESR) has proved a valuable technique for free radical detection. The basic principle involves the reaction of a stable diamagnetic species (i.e. the spin trap) with a short-lived radical to form a persistent radical (i.e. the spin adduct) that accumulates and can be detected by ESR. Some drawbacks such as the poor spin trapping rate, the short lifetime of the adducts, and their instability in biological media limit the application of this method[5-7]. Many efforts have been dedicated to increasing the spin trapping rate and the intrinsic adduct stability by modifying the structure of the spin trap, sometimes using theoretical calculations for rational design[8-10]. The cyclic nitrone 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, Scheme 1)[11] has been commonly used to trap $O_2^{\cdot -}$ but its DMPO-OOH adduct spontaneously (non-enzymatically) decays with a short half-life close to 1 min at neutral pH[12]. Several DMPO analogues bearing an alkoxycarbonyl (e.g. EMPO[13], BMPO[14]) or dialkoxyphosphoryl substituent at position 5 (DEPMPO[15], or DIPPMPO[16]) are now available, and offer some advantages as their superoxide adducts exhibit longer half-lives in phosphate buffer and do not spontaneously decompose to the corresponding hydroxyl adducts. The stability of their superoxide adducts *in vivo* or in the presence of biological material remains low and these adducts are easily transformed to diamagnetic compounds. However, most publications dedicated to spin trapping in enzymatic systems, cells or organs make use of these spin traps[10, 17-24]. Recently, cyclic nitrones bearing a triphenylphosphonium group (Mito-DEPMPO and Mito-DIPPMPO, Scheme 1)[25,26], or conjugated to a β-cyclodextrin ring

(CDNMPO, CD-DEPMPO, CD-DIPPMPO)[27,28] (Scheme 1) have been synthesized. They display higher reaction rates with superoxide (three times that of DEPMPO for Mito-DEPMPO and CD-DEPMPO[10]) and their adducts show a highly increased stability in buffer in comparison with those of DEPMPO or BMPO. However the stability of the spin adducts of these new probes have not yet been evaluated in the presence of biological oxidoreductants and liver subcellular fractions that contain most of the enzymes involved in the metabolism of xenobiotics, such as cytochrome P450[29].

In a previous study[30], we investigated the stability of the superoxide and hydroxyl adducts of BMPO in the presence of rat liver microsomes (RLM) and rat liver cytosol (RLC) and identified the main pathways that result in the disappearance of the paramagnetic adducts. We highlighted the major roles of heme and hemeproteins, besides those of biological reductants such as ascorbic acid or glutathione, in the fast conversion of the BMPO-OOH and BMPO-OH spin adducts to diamagnetic species. In the present study, we performed a comparison of the stability of the superoxide and hydroxyl adducts of the last generation of cyclic nitrones (Mito-DEPMPO, Mito-DIPPMPO, CD-DEPMPO, and CD-DIPPMPO, Scheme 1) with those of BMPO, DEPMPO, and DIPPMPO in the presence of RLM, RLC, and some biological reductants. We identified structural characteristics that strongly increase the selectivity of trapping and the stability of the adducts. These results should be useful in the choice of the better spin trap for biological applications and the design of new probes.

## Experimental procedures

*Reagents*

DEPMPO was obtained from Radical Vision (Marseille, France). DIPPMPO, Mito-DEPMPO, and CD-DEPMPO were synthesized according to previously published protocols[16, 25, 26, 28]. The detailed synthesis and characterization of Mito-DIPPMPO and CD-DIPPMPO will be published elsewhere. TOMER was synthesized according to Le Moigne *et al*.[31] Purity was assayed by $^{1}$H NMR and HPLC coupled with mass spectrometry and was above 98%. NADPH, xanthine (X), xanthine oxidase (XO), superoxide dismutase (SOD), cytochrome c, catalase, methemoglobin (metHb), diethylene triamine pentaacetic acid (DTPA), ethylene diamine tetraacetic acid (EDTA), methyl orange, clotrimazole, and TEMPO were purchased from Sigma-Aldrich (St Quentin Fallavier, France). All cyclodextrins were obtained from Cyclolab Ltd, Hungary. All other chemicals and solvents were of the highest grade commercially available. Ultra pure water (ELGA, Antony, France, resistivity: 18.2 MΩ) was used for the preparation of 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM DTPA. $H_2O_2$ concentration was determined using $\varepsilon_{240} = 39.4\ M^{-1}cm^{-1}$[32].

*Preparation of rat liver microsomes (RLM) and cytosol (RLC)*

Male Sprague Dawley rats (200-250 g, Charles River, L'Arbresle, France) were provided laboratory chow and water *ad libitum*. After 7 days of adaptation, animals were treated with phenobarbital (PB) (20 mg $kg^{-1}$, in corn oil, i.p. for 4 days) or dexamethasone (Dexa) (50 mg $kg^{-1}$, in corn oil, i.p. for 4 days). RLC and RLM were prepared by differential centrifugation as previously reported[33] and stored at -80 °C until use. Protein concentrations were determined by the Bradford assay with bovine serum albumin (BSA) as a standard[34].

Cytochrome P450 (P450) contents were determined by the method of Omura and Sato[35]. Determination of XO activity was performed using the initial rate of reduction of ferric cytochrome *c* to ferrous cytochrome *c* by superoxide. Quartz cuvettes (total volume 150 μL) contained 0.1 M phosphate buffer pH 7.4, 0.1 mM EDTA, 500 μM X, 100 U/mL catalase, 100 μM cytochrome *c*, and variable amounts of XO. Spectra were recorded on a Uvikon 941 spectrophotometer (Kontron Biotech) at 37°C and a $\Delta\varepsilon_{550}$ value of 21 000 $M^{-1}$ $cm^{-1}$ was used. Ascorbic acid[36] and glutathione[37] concentrations were determined following previously described methods.

*Spectral interactions between P450 $Fe^{III}$-heme and TRIMEB-$NH_2$*

Difference spectra were recorded at 21°C on a Uvikon 943 dual-beam scanning spectrophotometer (Kontron Biotech). Small volumes of $10^{-1}$ M stock solution of TRIMEB-$NH_2$ (Cyclolab Ltd, Hungary), a permethylated β-cyclodextrin with one amine group at position 6, were added to a 1-cm path length sample cuvette containing 150 μL of microsomal proteins (2 μM P450) from Dexa-treated rats in 0.1 M phosphate buffer pH 7.4. The same volumes of buffer were added to the reference cuvette containing the same amount of microsomal proteins and difference spectra were recorded between 380 and 500 nm. At the end of the experiment, the integrity of the P450 was checked after reduction of both cuvette contents with sodium dithionite and bubbling with carbon monoxide in the sample cuvette.

*Stopped-flow apparatus/ESR spectroscopy*

Experiments were performed with the help of a three-syringe Bio-Logic MPS-51 stopped-flow apparatus (Bio-Logic, Claix, France) connected to an AquaX quartz cell (Bruker, Wissembourg, France) fitted into an SHQ ESR-cavity (Fig. 1) following a previously described device[30]. All ESR spectra were recorded at 21°C with a Bruker Elexsys 500 ESR

spectrometer (Bruker, Wissembourg, France) operating at X-band (9.85 GHz). The following settings were used: modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; time constant, 40.96 ms; conversion time, 40.96 ms; center field, 349.0 mT; sweep width, 15 mT; sweep time, 41.94 s; microwave power, 10 mW. Data acquisition and processing were performed using Bruker Xepr software. Calibration of the spectrometer sensitivity was deduced from the accurate computer simulation of spectra of TEMPO or TOMER solutions of known concentrations. Computer simulations of the ESR spectra were performed using the program of Rockenbauer and Korecz[38]. Sets of parameters applied throughout in the determination of the proportions of different spin adducts were derived from the simulation of the best-resolved signals. Decay kinetics of ESR spectra were analyzed as previously described[30]. When first order decay rates were observed, the characteristic half-lives of the adducts ($t_{1/2}$, in min) are given. For reactions with ascorbic acid or glutathione, the initial kinetics were assumed to be second order and initial second order rate constants ($k_0$) were extracted:

$$-\left(\frac{d[\text{Adduct}]}{dt}\right)_0 = k_0[\text{Adduct}]_0[\text{Reductant}]_0$$

with $[\text{Reductant}]_0$, the initial concentration of ascorbic acid or glutathione; $[\text{Adduct}]_0$, the initial spin adduct concentration; and $-\left(\frac{d[\text{Adduct}]}{dt}\right)_0$, the initial decay rate of the adduct.

*Generation of the hydroxyl radical adducts and determination of their half-lives*

The hydroxyl radical adducts were generated by photolysis of a solution of 10 mM $H_2O_2$ in 0.1 M phosphate buffer, pH 7.4, containing the studied nitrones (0.5 - 1 mM)[39]. After 5 to 10 min of UV irradiation at 300 nm (Spectra Luminator, Oriel Instruments, Palaiseau, France), 100 U/mL catalase and 1 mM DTPA (final concentrations) were added, and the mixture was introduced into syringe 3 of the stopped-flow apparatus. Syringe 2 was filled with 0.1 M

phosphate buffer pH 7.4 containing 1 mM DTPA and the studied biological or chemical materials in 4-fold higher concentrations than those finally required. Mixing of 900 μL of syringe 3 (hydroxyl radical adduct) with 300 μL of syringe 2 (studied materials) was performed in 2 s and coordinated to ESR data acquisition.

*Generation of the superoxide adducts and determination of their half-lives*

The superoxide adducts of the studied nitrones were obtained from incubation of the spin traps (20 - 25 mM) in the presence of 250 μM X, 100 U/mL catalase, in 0.1 M phosphate buffer, pH 7.4, containing 1 mM DTPA, and the amount of XO adjusted to obtain a steady-state concentration of about 25 μM of the superoxide adduct after 15 min incubation in an open vial at 21°C. The solutions containing the superoxide adducts were then introduced into syringe 3 of the stopped-flow apparatus. Syringe 2 was filled with 0.1 M phosphate buffer, pH 7.4, containing 1 mM DTPA, 340 U/mL SOD, and the studied biological or chemical materials in 4-fold higher concentrations than those finally present in the AquaX cell. Typically the P450-concentration of RLM from PB-treated rats was 1 μM, NADPH was 1 mM, metHb or hemin were 1 μM, RLC from PB-treated rats protein content was 1 mg/mL, and methyl orange was 10 mM. The stopped-flow lines were primed with the solutions and 900 μL of syringe 3 (superoxide adduct) were mixed with 300 μL of syringe 2 (SOD + studied materials). Mixing was performed in 2 s and coordinated to ESR data acquisition.

## Results

*Hydroxyl radical adducts formation and decay*

The hydroxyl radical was generated by photolysis of a solution of $H_2O_2$ in phosphate buffer 0.1 M, pH 7.4 and spin trapping was performed in the presence of 0.5-1 mM of the studied cyclic nitrones. The spectra of DEPMPO-OH and DIPPMPO-OH adducts were simulated with the program of Rockenbauer and Korecz[38] using the hyperfine coupling constants ($A_P$, $A_N$, $A_{H\beta}$) given in Supplementary Table S1, and the observed ESR spectra displayed characteristic features in accordance with those previously described[16, 40]. In the case of DEPMPO and DIPPMPO, the hydroxyl radical adducts were the very predominant identified species. The half-lives of the DEPMPO-OH and DIPPMPO-OH adducts were greatly dependent upon their initial concentrations and their decays could be perfectly modeled using second order kinetics. In order to compare the stability of the spin adducts from the studied cyclic nitrones, all apparent half-lives were measured for an initial adduct concentration of 25 μM. Under these conditions, the spontaneous disappearance of the ESR signals of DEPMPO-OH, DIPPMPO-OH, and BMPO-OH in phosphate buffer occurred with very similar half-lives (38±3, 43±3, and 41±4 min, respectively).

In the case of Mito-DEPMPO, Mito-DIPPMPO, CD-DEPMPO and CD-DIPPMPO, very weak ESR signals were observed in these experiments. Thus, the formation and stability of these adducts were not investigated further.

*Metabolic stability of hydroxyl radical adducts in the presence of RLM and of RLC*

Half-lives of the adducts were not straightforward to compare since the photolysis reaction did not allow to finely choose the initial adduct concentration for the study and since the second order kinetics cannot be neglected at higher adduct concentration. Rapid mixing of DEPMPO-OH and DIPPMPO-OH adducts with RLM or RLC resulted in high increases in

the decay rates of their ESR signals (20% increase with RLM containing 1 μM P450 and at least 100% increase with RLC containing 1 mg proteins/mL). The effect of RLM was further increased 2-fold by addition of NADPH, while the effect of RLC was mimicked by ascorbic acid. On the whole, it appears that the degradation mechanisms are not significantly different from those previously proposed for BMPO-OH[30].

*ESR study of superoxide radical adducts formation and decay*

The superoxide adducts of the studied cyclic nitrones (Scheme 1) were generated in open vials at 21°C by incubation in the presence of X and XO, that produced a constant and controlled flux of superoxide radical anion, and the formation of the spin adducts was monitored by ESR. In comparison with similar concentrations of BMPO-OOH, the presence of a dialkoxyphosphoryl moiety increased the number of lines in the ESR signal of the superoxide adducts and resulted in a lower signal/noise ratio. The initial adduct concentration had thus to be increased. Twenty - 25 mM of the studied nitrones and twice as much XO were used in the present work in comparison with our previous study (50 mM BMPO and XO producing 100 μM of $O_2^{\bullet-}$ in 15 min at 21°C)[30]. Under these conditions, the accumulation of superoxide spin adducts of these phosphorus-containing spin traps was more efficient than that of BMPO-OOH probably due to combined effects of a faster spin trapping reaction rate[10] and an increased stability of the spin adducts. A concentration of about 25 μM of the superoxide adducts was thus obtained in 15 min. Very minor formation of hydroxyl radical adducts was observed (Fig. 2). The signals of the superoxide adducts were simulated using the program of Rockenbauer and Korecz[38] using the hyperfine coupling constants ($A_P$, $A_N$, $A_{H\beta}$, $A_{H\gamma}$) given in Supplementary Table S1 and were in accordance with those described for DEPMPO-OOH and DIPPMPO-OOH in the literature[15, 16]. In phosphate buffer 0.1 M, pH 7.4, the ESR signals of all the superoxide adducts under study spontaneously decayed to

diamagnetic species with negligible formation of hydroxyl radical adduct (5-10%). Analysis of the kinetic decays yielded half-lives for Mito-DEPMPO, Mito-DIPPMPO, and CD-DEPMPO that were 1.5 – 2 fold higher than those of the parent nitrones DEPMPO and DIPPMPO and up to 3-fold in the case of CD-DIPPMPO (Fig. 2 and Supplementary Table S2). As previously observed with DEPMPO and DIPPMPO, the intrinsic stability of the superoxide adducts was increased when the pyrroline ring was substituted at position 5 with a diethoxyphosphoryl or a di-isopropyloxyphosphoryl group in comparison with BMPO[12]. For the DEPMPO and DIPPMPO analogues, *cis*-substitution at position $C_4$ by a β-cyclodextrin or a triphenylphosphonium moiety simplified the ESR spectral patterns due to slower conformational exchange and led to significant increase in half-lives, especially for β-cyclodextrin derivatives.

It should be noticed that half-lives of spin adducts are greatly dependent on experimental conditions (microwave power, temperature, buffer composition)[10] and some of our data appear significantly different than those previously reported in the literature[40]. However, our kinetics data were recorded under the same experimental conditions and could thus be safely compared.

*Stability of superoxide radical adducts in the presence of RLM*

Upon rapid mixing of the DEPMPO-OOH and DIPPMPO-OOH adducts with SOD and RLM (containing a final concentration of 1 μM P450), the corresponding ESR spectra quickly disappeared with minor (10%) formation of DEPMPO-OH or DIPPMPO-OH. The initial decays fitted well with first-order kinetics and the half-lives could be compared to that obtained previously for BMPO-OOH[30]. As previously observed in buffer alone, the stability of the superoxide adducts of DEPMPO and DIPPMPO in the presence of RLM were higher than that of BMPO ($t_{1/2}$ = 6.3 and 5.8 min versus 2.5 min, respectively) (Fig. 2 and

Supplementary Table S2). However the ratios of the $t_{1/2}$ values of these superoxide adducts in the presence of RLM to those in buffer alone were very similar to that of BMPO (0.17 and 0.15 versus 0.12, respectively), indicating that the phosphonate ester did not significantly protect against the disappearance of the ESR signals mediated by microsomal proteins. Mito-DEPMPO-OOH and Mito-DIPPMPO-OOH adducts also decayed rapidly in the presence of RLM ($t_{1/2}$ of 14.5 and 17 min, respectively), with a slightly higher conversion (20%) to Mito-DIPPMPO-OH in the latter case (Fig. 2 and Supplementary Table S2). Some protection against microsomal action linked to the triphenylphosphonium moiety was also observed since the ratios of the $t_{1/2}$ value in the presence of RLM to that in buffer alone represent 0.26 to 0.29 (almost twice the value obtained with the parent nitrones DEPMPO and DIPPMPO) (Fig. 2 and Supplementary Table S2). The addition of clotrimazole, a ligand of P450 heme iron[41], inhibited the effect of RLM on Mito-DEPMPO-OOH and Mito-DIPPMPO-OOH stability with significant increases in the $t_{1/2}$ values (35±2 and 36±4 min, respectively, i.e. 70 and 55% of the values in buffer alone). Finally, the implication of the heme in the degradation of the Mito-DEPMPO-OOH and Mito-DIPPMPO-OOH adducts was demonstrated in experiments performed in the presence of metHb or hemin where half-lives of the adducts were strongly reduced (Fig. 2 and Supplementary Table S2).

By contrast, introduction of a β-cyclodextrin as substituent at position 4 of the spin traps resulted in a strong protection against degradation of the superoxide adducts mediated by the P450 heme. RLM alone did not affect the CD-DEPMPO-OOH and CD-DIPPMPO-OOH adducts half-lives in comparison with those observed in buffer alone ($t_{1/2}$ = 83 and 110 min, respectively, Fig. 2 and Supplementary Table S2). Addition of methyl orange (10 mM), a ligand of β-cyclodextrin that prevents interaction of the aminoxyl radical with the cyclodextrin[28, 42], did not significantly modify the stability of CD-DEPMPO-OOH and CD-DIPPMPO-OOH in buffer alone or in the presence of RLM (Fig. 2 and Supplementary Table

S2), suggesting that the protection was not due to inclusion of the superoxide adducts in the cyclodextrin ring. However, the addition of NADPH, the cofactor of P450 and P450 reductase, to RLM resulted in a rapid disappearance of the spin adduct ($t_{1/2}$ = 8.3 and 9.5 min, respectively, Fig. 2 and Supplementary Table S2). Finally, the increased stability of the CD-substituted superoxide adducts was not observed in the presence of hemin or metHb ($t_{1/2}$ = 4.0 and 7 min, respectively for CD-DEPMPO-OOH, $t_{1/2}$ = 4.4 min for CD-DIPPMPO-OOH with hemin) (Fig. 2 and Supplementary Table S2).

*Stability of superoxide radical adducts in the presence of RLC*

Rapid mixing of RLC with all the studied superoxide adducts generated by the X/XO system resulted in a fast decay of the corresponding ESR spectra with the appearance of the ESR signals of the hydroxyl radical adducts as intermediates on the way to diamagnetic products (Fig. 3), as previously observed with BMPO[30]. Since the reactions proceeded rapidly (half-lives for all adducts comprised between 1.5 and 6 min, Fig. 4 and Supplementary Table S3), small variations in the concentrations of each reactant yielded important variations in the half-lives and errors under these conditions were greater than in the previous study with RLM. Moreover, simulations of the spectra were complicated by the superimposition of superoxide and hydroxyl radical adducts. As a consequence, we could not make out any significant difference between the studied superoxide spin adducts. The effect of RLC on BMPO-OOH was previously attributed mainly to the reduction by ascorbic acid and glutathione contained in the cytosolic fraction[30]. Incubations of the superoxide adducts with ascorbic acid using the stopped-flow ESR device were thus performed. When one equivalent of ascorbic acid was used, the superoxide adducts decayed rapidly to ESR silent products within a few minutes (80% loss in 3 min for CD-DEPMPO-OOH, Fig. 5). When a concentration of ascorbic acid much lower to the concentration of the superoxide adduct was used, the kinetics showed two

phases: an initial rapid decay (< 1 min) until all ascorbic acid was consumed and a second phase with a half-life close to that observed in buffer alone. The reaction not being studied under pseudo first-order reaction conditions by using excess ascorbic acid, we extracted second-order rate constants from the initial decays (Table 1). The dead time due to mixing (≈ 2 s) led to underestimation of the true rate constants. However the results show that the initial rate constants of reaction of the various superoxide adducts with ascorbic acid were not significantly different, even though the β-cyclodextrin-substituted adducts appeared slightly less reactive (1.5 to 2-fold) (Table 1). The reaction rates were several order of magnitude higher than that of sterically shielded aminoxyl radicals such as 4-oxo-TEMPO or $CP^{\cdot}$, and were similar to the reaction rates of hydroxyl radical adducts. The published kinetic rate constant for DMPO-OH is one order of magnitude higher but compatible with our results since it was measured with a different experimental set-up characterized by a much shorter dead-time[45].

We performed the same study with glutathione. In this case, the superoxide adducts of the studied nitrones also decayed following second-order kinetics but were partially converted to the corresponding hydroxyl radical adduct, via a direct two-electron process, as previously described for BMPO[30] or DMPO[46]. The observed second order rate constants with glutathione were not significantly different for the various superoxide adducts ($k_0$ = 5-12 $M^{-1}$ $s^{-1}$), even though 2-fold slower values were recorded for β-cyclodextrin-substituted adducts ($k_0$ = 2.7-2.9 $M^{-1}$ $s^{-1}$) (Table 2).

**Discussion**

In the present study, we compared the behavior of the hydroxyl and superoxide adducts of seven cyclic nitrones (BMPO, DEPMPO, DIPPMPO, Mito-DEPMPO, Mito-DIPPMPO, CD-DEPMPO, and CD-DIPPMPO, Scheme 1) that result from the active research in the field of spin trap design in the last decades. All of these spin adducts displayed much higher half-lives in phosphate buffer, pH 7.4, than those of DMPO, a frequently used cyclic nitrone.

Application of the ESR/spin trapping technique to biological systems using cyclic nitrones is often impaired by the lack of stability of the spin adducts. The origins of the loss of ESR signal are partially understood since a limited number of studies have addressed this question[30, 39, 47, 48]. At the beginning of this study, we wondered whether the improvement in intrinsic half-lives of the spin adducts could correlate their metabolic stability when exposed to subcellular fractions, enzymes, or biological reductants and whether some lessons can be drawn regarding the design of new more metabolically stable spin traps.

An important data was the ability to use lower concentrations of the new spin traps bearing a Mito- or a CD-moiety to obtain EPR spectra of the superoxide adducts with as intense features as those obtained with DMPO or BMPO, despite the increased number of peaks due to the introduction of a P-atom (20-25 mM, compared to 50 mM). This result is of interest since the introduction of lower amounts of spin trap should reduce the potential toxicity of the molecules. This result could be due to 3-fold increased spin trapping rates[10] combined to lower rates of spontaneous decay of the spin-adducts.

Another important data concerns the selective trapping of superoxide radical by Mito- and CD-derived spin traps, these compounds leading to very low amounts of the hydroxyl radical adducts under our conditions (0.5-1 mM spin traps and photolysis of $H_2O_2$). When the spin trap concentration was increased (up to 20 mM), small amounts of Mito-DEPMPO-OH adduct were observed under Fenton reaction conditions[25]. This is likely due to competition

of several sites of the spin trap for the reaction with hydroxyl radical, the nitrone function playing a minor part. The rate of the reaction of hydroxyl radical with β-cyclodextrin, tetraphenylphosphonium ion or bromide ion have been measured as 4.2 $10^9$ $M^{-1}$ $s^{-1}$[49], 7.2 $10^9$ $M^{-1}$ $s^{-1}$[50], and 1.1 $10^{10}$ $M^{-1}$ $s^{-1}$[51], respectively, close to diffusion-controlled rates. This selectivity of Mito- and CD-derived spin traps towards superoxide could be of interest to discriminate between superoxide and hydroxyl formation in biological systems or cells since it makes a control incubation with SOD unnecessary.

As a general observation from our study, the half-lives of the studied spin adducts in phosphate buffer were very significantly increased in comparison with those of BMPO adducts. However the mechanisms of their decay in the presence of RLM and RLC seem to remain very similar to those we previously described in the case of BMPO (Scheme 2)[30]. We highlighted the role of the ferric heme proteins in the degradation of both adducts involving oxidation of the aminoxyl moiety of BMPO-OH to an unstable oxoammonium cation that further evolved to yield a β-hydroxynitrone and its cyclic hydroxamic acid tautomers[52-54] (Scheme 2, c), while the alkylhydroperoxide moiety of BMPO-OOH would be cleaved either homolytically into ring-opened diamagnetic products or heterolytically into BMPO-OH[29] (Scheme 2, a). Besides, biological reductants such as ascorbic acid and glutathione, and flavin dependent reductases, including P450 reductase, were involved in the rapid loss of the ESR signals of both BMPO-OOH and BMPO-OH in the presence of RLC. Thiols and glutathione were able to reduce BMPO-OOH to BMPO-OH (Scheme 2, a)., while ascorbic acid alone reduced both adducts probably to an unstable hydroxylamine that would yield back BMPO after dehydration or elimination of $H_2O_2$[48] (Scheme 2, b).

In the present study, clotrimazole, a ligand of $Fe^{III}$ heme[41], inhibited the disappearance of Mito-DEPMPO-OOH and Mito-DIPPMPO-OOH in the presence of RLM, suggesting that this effect was also dependent upon $Fe^{III}$ P450 (Supplementary Table S2). Interestingly,

distinct behaviors of the spin adducts were identified with CD-derived nitrones where RLM alone had no effect on the decay rates of the superoxide adducts. However addition of NADPH led to a rapid reduction of the CD-spin adducts. This could be explained by the hypothesis that steric constraints would prevent the entrance to the P450 active site and inhibit the reaction of the spin adducts (hydroperoxides) catalyzed by the $Fe^{III}$ heme. The approximate dimensions of the β-cyclodextrin moiety are 0.78 nm in height and 1.53 nm in its larger diameter[55]. Supporting this hypothesis, our spectral interaction study did not allow any variation in the spin state of the P450 heme to be detected in the presence of up to 2.6 mM TRIMEB-$NH_2$ and it is thus likely that the β-cyclodextrin does not significantly enter the active site of P450. It is noteworthy that randomly substituted methyl-β-cyclodextrin can diversely affect (inhibit or enhance) P450 activity, these effects being attributed to the disruption of the microsomal membrane[56]. In our case, such an effect cannot be ruled out. By contrast, the fast disappearance of CD-DEPMPO-OOH and CD-DIPPMPO-OOH signals observed with RLM in the presence of NADPH could involve a direct reaction of the adducts with the reductases, including P450 reductases.

All the presently studied spin adducts seem to behave roughly the same towards biological reductants such as ascorbic acid and glutathione, the main reducing agents contained in the cytosol (Fig. 4). However, the second order rate constants of the reaction of the spin adducts with either ascorbic acid or glutathione showed some differences (Tables 1 and 2) with smaller reaction rates observed with CD-derived spin adducts. This could be consistent with the fact that the aminoxyl radical is involved in a dynamic exchange between the bulk of the solution and the primary methoxy crown of the β-cyclodextrin, thus affording a partial protection against reduction[28].

## Conclusion

Regarding the detection of intracellular superoxide production, the spin adducts from cyclic nitrones display weaknesses that reside in the hydroperoxide function that is the target of peroxidase activities (P450[30], glutathione peroxidase[47], thioredoxine reductase[57] ...) and in the nitroxide (aminoxyl radical) moiety that supports the ESR signature and that cannot be shielded from reduction by bulky substituents (a strategy proposed for "stable" nitroxides) without lowering the rate of the spin trapping reaction[10]. However our last results highlight the fact that the spin adducts have to get access to the sites of transformations, the $Fe^{III}$ heme of hemeproteins for oxidative pathways, or the cellular compartments that contain biological reductants or flavin-dependent reductases, in order to be converted to diamagnetic compounds. In this respect, CD-derived spin traps are not likely to enter the cells, since the β-cyclodextrin moiety is highly hydrophilic and was shown not to significantly enter membranes or cells[58, 59]. Therefore their adducts are potentially less subject to degradation by heme proteins, reductases, and biological reducing agents, which are mainly located within the cell. CD-DEPMPO and CD-DIPPMPO could then prove superior spin traps to study extracellular superoxide production, with high specificity on superoxide detection over other ROS, provided that they are used at low concentrations, so as not to disturb the cell membrane[58]. Assays of these spin traps with cells are currently underway to test this hypothesis.

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## Abbreviations

BMPO, 5-tert-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; CD-DEPMPO, 6-monodeoxy-6-mono-4-[(5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxyde)-ethylenecarbamoyl-(2,3-di-*O*-methyl) hexakis (2,3,6-tri-*O*-methyl)]-β-cyclodextrin; CD-DIPPMPO, 6-monodeoxy-6-mono-4-[(5-diisopropoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxyde)-ethylenecarbamoyl-(2,3-di-*O*-methyl) hexakis (2,3,6-tri-*O*-methyl)]-β-cyclodextrin; CDNMPO, 5-*N*-*β*-cyclodextrin-5-carboxamide-5-methyl-1-pyrroline *N*-oxide; $CP^{\cdot}$, 3-carboxy-2,2,5,5-tetramethyl-pyrrolidin-1-oxyl; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; Dexa, dexamethasone; DIPPMPO, 5-diisopropoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylene triamine pentaacetic acid; EDTA, ethylene diamine tetraacetic acid; ESR, electron spin resonance (equivalent to EPR, electron paramagnetic resonance); metHb, methemoglobin; Mito-DEPMPO, (4*R**, 5*R**)-5-(diethoxyphosphoryl)-5-methyl-4-[({[2-(triphenylphosphonio)ethyl]carbamoyl}oxy)methyl]pyrroline *N*-oxide bromide; Mito-DIPPMPO, (4*R**, 5*R**)-5-(diisopropyloxyphosphoryl)-5-methyl-4-[({[2-(triphenylphosphonio)ethyl]carbamoyl}oxy)methyl]pyrroline *N*-oxide bromide; P450, cytochrome P450; PB, phenobarbital; RLC, rat liver cytosol; RLM, rat liver microsomes; ROS, reactive oxygen species; SOD, superoxide dismutase; $t_{1/2}$, half lifetime; TEMPO, (2,2,6,6-tetramethyl)piperidine-1-oxyl; TOMER, 2-diethoxyphosphoryl-2,5,5-trimethyl-pyrrolidin-1-oxyl

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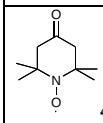
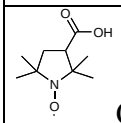
**Tables**

**Table 1:** Second order rate constants ($k_0$) for the reaction of various aminoxyl radicals with ascorbic acid extracted from initial reaction rates or from the literature.

| **Aminoxyl radical** | $k_0$ **($M^{-1}$ $s^{-1}$)** | **Reference** |
|---|---|---|
| BMPO-OOH | >300±10[a] | [30], this work |
| DEPMPO-OOH | >240±20[a] | this work |
| Mito-DEPMPO-OOH | >350±40[a] | this work |
| CD-DEPMPO-OOH | >180±40[a] | this work |
| CD-DIPPMPO-OOH | >160±30[a] | this work |
|  4-oxo-TEMPO | 7.2±0.8[b]<br>6.9±0.7[a] | [44]<br>this work |
|  $CP^{\cdot}$ | 0.11±0.01[b] | [44] |
| DMPO-OH | >5200±200[c] | [45] |
| BMPO-OH | >210±10[a] | [30], this work |
| DEPMPO-OH | >350±110[a] | this work |

[a] Solutions of superoxide or hydroxyl radical adducts were prepared as described under Experimental procedures, and loaded into syringe 3 of the stopped-flow apparatus. Alternatively a solution of 4-oxo-TEMPO was used. They were rapidly mixed with the SOD + ascorbic acid or ascorbic acid solution in buffer contained in syringe 2. The ESR signals were recorded (with a ≈ 2 s dead-time) and analyzed as described under Experimental procedures.

[b] Rates were measured at 20°C in sodium phosphate buffer (0.3 M, pH 7.4).

[c] DMPO-OH was prepared by sonication of an argon-saturated solution of DMPO (2.0 mM) in sodium phosphate buffer (0.1 M, pH 7.4). Rapid mixing with ascorbic acid was performed in 50 ms using a stopped-flow module. ESR measurements were performed at 25°C.

**Table 2:** Second order rate constants ($k_0$) for the reaction of various superoxide spin adducts with glutathione extracted from initial reaction rates.[a]

| **Aminoxyl radical** | $k_0$ **($M^{-1}$ $s^{-1}$)** |
|---|---|
| BMPO-OOH | 7.5±0.8 |
| DEPMPO-OOH | 5.6±0.6 |
| Mito-DEPMPO-OOH | 11±2 |
| Mito-DIPPMPO-OOH | 9±3 |
| CD-DEPMPO-OOH | 2.9±0.4 |
| CD-DIPPMPO-OOH | 2.7±0.3 |

[a] Solutions of superoxide adducts were prepared as described under Experimental procedures, and loaded into syringe 3 of the stopped-flow apparatus. They were rapidly mixed with the SOD + glutathione solution in buffer contained in syringe 2. The ESR signals were recorded (with a ≈ 2 s dead-time) and analyzed as described under Experimental procedures.

**Legends for Figures and Schemes**

**Figure 1:** Schematic description of the stopped-flow apparatus connected to the AquaX cell fitted in the SHQ ESR cavity of a X-band ESR spectrometer.

**Figure 2:** Half-lives ($t_{1/2}$ in min) of the superoxide adducts in the presence of buffer alone, RLM with or without NADPH cofactor, or hemin. A solution of about 25 μM superoxide adduct prepared by incubation of 20-25 mM cyclic nitrone in the presence of a X/XO generating system in 0.1 M phosphate buffer pH 7.4 with 1 mM DTPA, was rapidly mixed with a solution containing SOD alone, SOD + RLM (from PB-treated rats, 1 μM P450) with or without NADPH (1 mM), or SOD + hemin (1 μM). The ESR signals were recorded and analyzed as described under Experimental procedures. Half-lives are the means ± S.D. from 2-3 experiments. Values marked with an asterisk have not been determined. See Supplementary Table S2 for more details.

**Figure 3: A.** Evolution with time of the ESR spectra of CD-DIPPMPO-OOH in the presence of RLC (1.0 mg protein/mL) showing decay and partial conversion to CD-DIPPMPO-OH. Noise in the spectra was filtered using the SVD procedure described by Lauricella *et al.* [43]. **B.** The kinetic curves were obtained from simulation of the above spectra as described under Experimental procedures. Simulated spectra for CD-DIPPMPO-OOH and CD-DIPPMPO-OH are given in the inset.

**Figure 4:** Half-lives ($t_{1/2}$ in min) of the superoxide spin-adducts in the presence of buffer alone or RLC. A solution of about 25 μM superoxide adduct prepared by incubation of 20-25 mM cyclic nitrone in the presence of a X/XO generating system in 0.1 M phosphate buffer pH 7.4 with 1 mM DTPA, was rapidly mixed with a solution containing SOD alone or SOD + RLC (from PB-treated rats, 1 mg protein/mL). The ESR signals were recorded and analyzed as described under Experimental procedures. Half-lives are the means ± S.D. from 2-3 experiments. See Supplementary Table S3 for more details.

**Figure 5:** Decay of CD-DEPMPO-OOH in the absence or presence of ascorbic acid. In this typical experiment, CD-DEPMPO-OOH was generated at 21°C by the reaction of X with XO in 0.1 M phosphate buffer, pH 7.4, containing 1 mM DTPA and 20 mM CD-DEPMPO. Spin adduct was stored in syringe 3 of the stopped-flow apparatus and rapidly mixed with the content of syringe 2 containing SOD in buffer alone (open circles), or SOD and ascorbic acid, either 25 μM final (open triangles) or 50 μM final (open squares). Mixing of the solutions was coordinated to ESR data acquisition. Further analysis and simulation of the ESR spectra was performed as described in Experimental procedures.

**Scheme 1:** Structures of the spin traps under study and general structures for superoxide and

hydroxyl adducts.

**Scheme 2:** Possible mechanisms for the disappearance of superoxide or hydroxyl radical adducts of the studied cyclic nitrones, involving the oxidation or reduction of the hydroperoxide (a) or the aminoxyl radical (nitroxide) functions (b and c). (See Bézière *et al.*[30], and references therein for further details).

**Figure 1:**

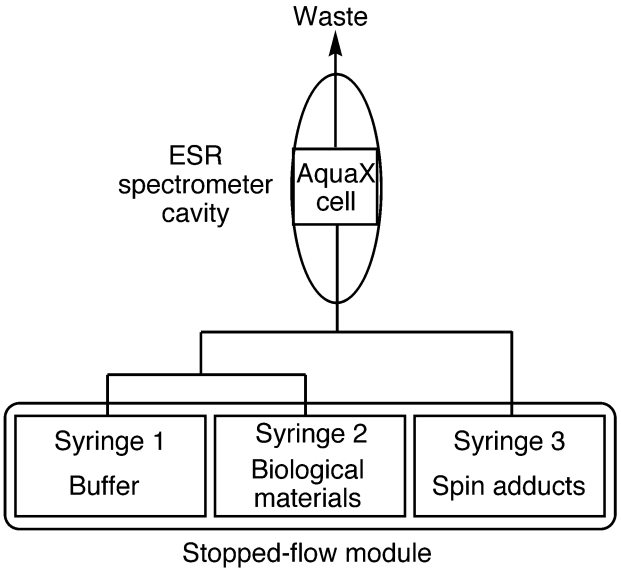



**Figure 2:**

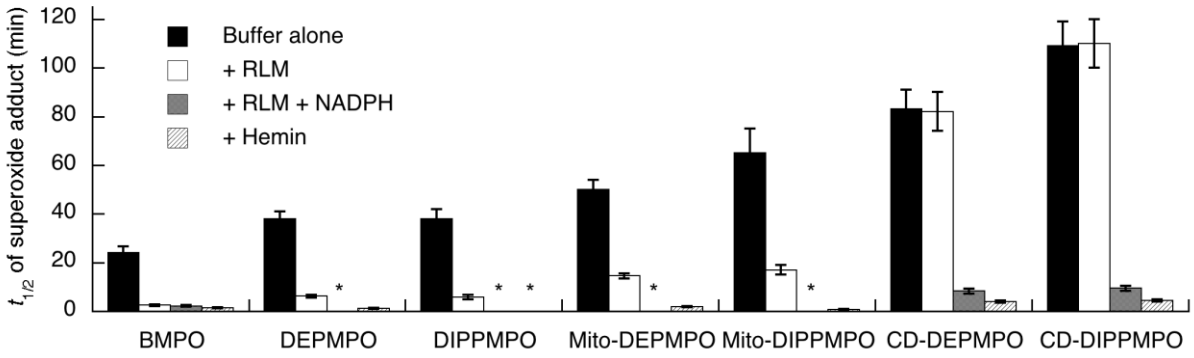


**Figure 3:**

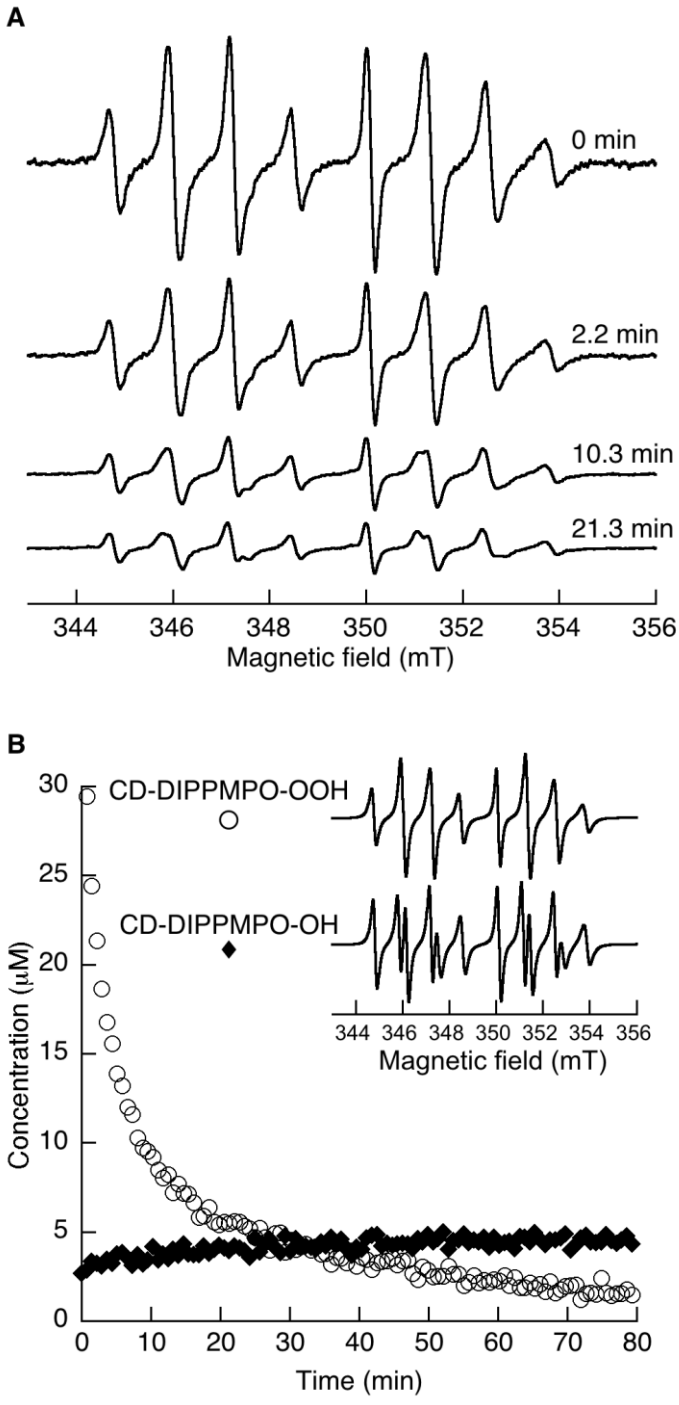

A
0 min
2.2 min
10.3 min
21.3 min
344 346 348 350 352 354 356
Magnetic field (mT)
B
CD-DIPPMPO-OOH
CD-DIPPMPO-OH
344 346 348 350 352 354 356
Magnetic field (mT)
Concentration (μM)
0 5 10 15 20 25 30
0 10 20 30 40 50 60 70 80
Time (min)

**Figure 4:**

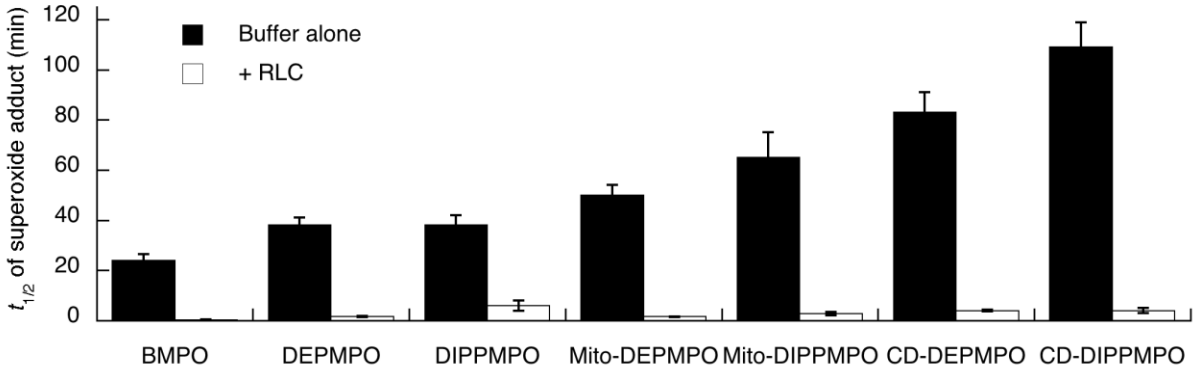
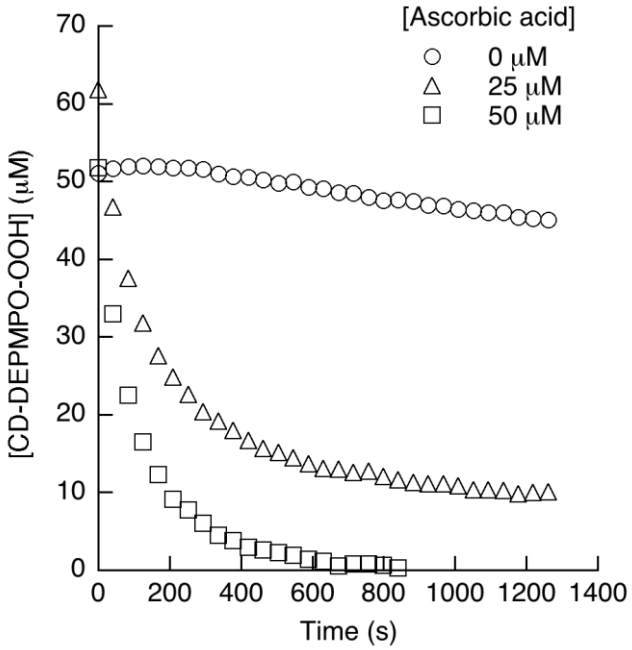
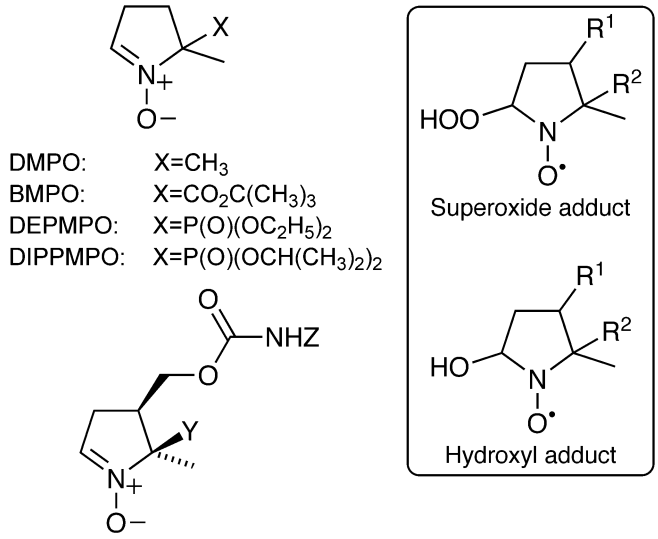



**Figure 5:**



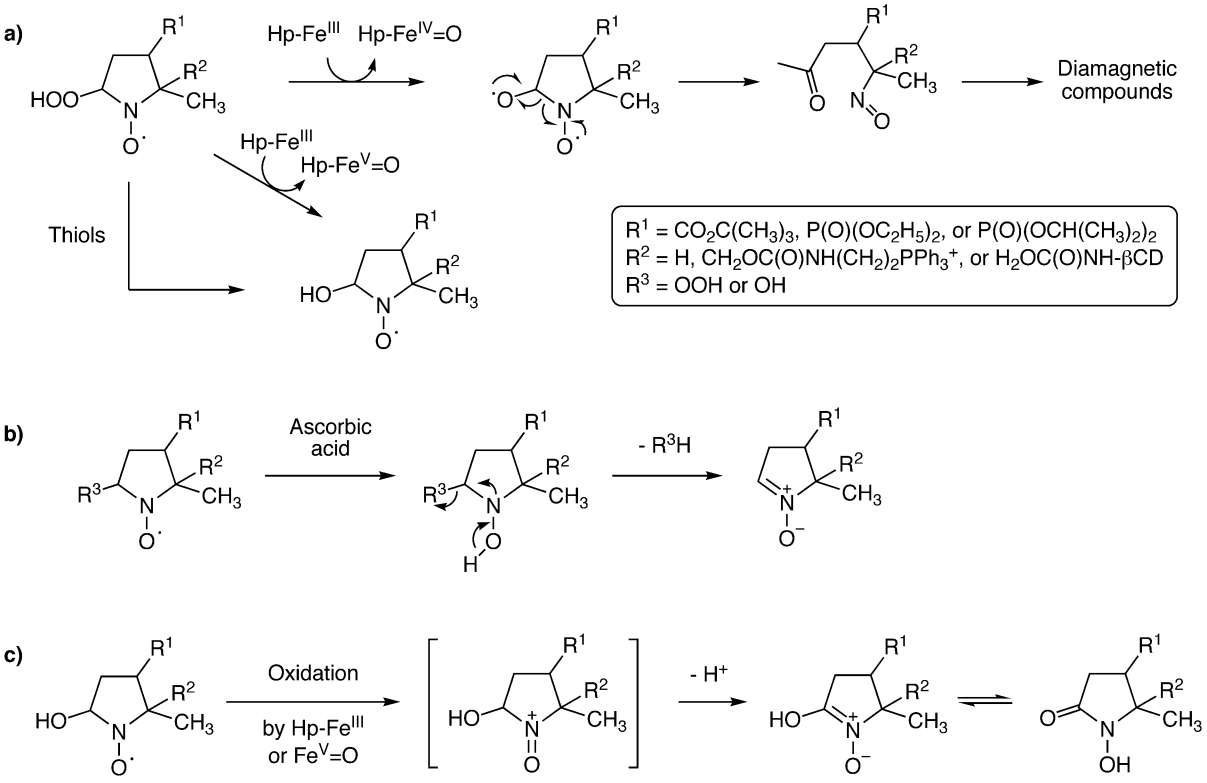
## Schemes

### Scheme 1:




Mito-DEPMPO: Y=P(O)($OC_2H_5$)$_2$; Z=$CH_2CH_2PPh_3^+Br^-$
Mito-DIPPMPO: Y=P(O)($OCH(CH_3)_2$)$_2$; Z=$CH_2CH_2PPh_3^+Br^-$
CD-DEPMPO: Y=P(O)($OC_2H_5$)$_2$; Z=methyl-β-cyclodextrin
CD-DIPPMPO: Y=P(O)($OCH(CH_3)_2$)$_2$; Z=methyl-β-cyclodextrin

### Scheme 2:




## Appendix A: Supplementary material

**Supplementary Material**

Click here to download Supplementary Material: Supplementary material.pdf