5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-Oxide: A New Efficient Phosphorylated Nitrone for the *in Vitro* and *in Vivo* Spin Trapping of Oxygen-Centered Radicals

Claudine Frejaville, Hakim Karoui, Béatrice Tuccio, François Le Moigne, Marcel Culcasi, Sylvia Pietri, Robert Lauricella, and Paul Tordo*

Laboratoire Structure et Réactivité des Espèces Paramagnétiques, Case 521, Unité de Recherche Associée n° 1412 du Centre National de la Recherche Scientifique, Université de Provence, Centre de St Jérôme, 13397 Marseille Cedex 20, France

Received June 13, 1994[⊗]

5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO, **2**), a new spin trap, has been synthesized *via* a two-step synthetic route, and its ability to spin trap oxy radicals in biological milieu has been addressed. The *in vitro* spin trapping of hydroxyl and superoxide radicals was investigated in a phosphate buffer 0.1 M, and the hyperfine coupling constants of the spin adducts were determined. The rates of spin trapping of hydroxyl and superoxide radicals with **2** were found to be close to those reported for 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). However, the DEPMPO–superoxide spin adduct was shown to be significantly more persistent (15 times at pH 7) than the DMPO–superoxide spin adduct. Using **2** as a spin trap, the production of superoxide has been clearly characterized during the reperfusion of ischemic isolated rat hearts.

Introduction

Enormous efforts are still being devoted to addressing the role played by partially reduced oxygen species such as superoxide $O_2^{\bullet-}$ and hydroxyl HO$^\bullet$ radicals in mediating a variety of pathological conditions, including toxicity due to several chemicals and ionizing radiation, carcinogenesis, inflammation, and degenerative diseases such as immunodeficiencies, aging, and atherosclerosis.[2] In particular, these oxygen-derived free radicals have been implicated in the so-called ischemia/reperfusion injury,[3] the cytotoxicity occurring when tissues subjected to partial or total oxygen deprivation are reoxygenated.

Of the methods available for assessing free radical formation in biological systems, electron spin resonance (ESR) spin trapping appears one of the most appropriate and has been applied to investigate myocardial reperfusion injury in animal models.[4] Nitrones have emerged as the most popular spin traps for biological applications,[5] and out of several nitrone spin traps, the cyclic 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has received the most attention, since it yields distinct and characteristic spin adducts with $O_2^{\bullet-}$ and HO$^\bullet$ radicals.[5,6] Such results are worth considering on their own merits and have stimulated our research; however, the use of DMPO as a probe for oxyradical generation in biological milieu is not without its limitations.[5–7] Reaction of DMPO with superoxide is rather slow, having a second-order rate constant ranging from 10 M^{-1} s^{-1} at pH 7.8[8] to 1.2 M^{-1} s^{-1} at pH 7.4.[9] Its 1-octanol/water partition coefficient was found to be only 0.02–0.09,[10] indicating a preference for water over a lipid environment. On the other hand, the fate of DMPO spin adducts could also be a source of misinterpretation in biological ESR experiments. Firstly, 5,5-dimethyl-2-hydroperoxy-1-pyrrolidinyloxyl (DMPO–OOH), the superoxide spin adduct, undergoes a rapid chemical conversion to 5,5-dimethyl-2-hydroxy-1-pyrrolidinyloxyl (DMPO–OH), the hydroxyl radical spin adduct.[11] Secondly, several cellular components[5b,7,12] and even superoxide[13] itself are able to reduce DMPO–OOH and DMPO–OH into diamagnetic species. Finally, in aqueous solution, DMPO is susceptible to metal-ion-catalyzed addition of water, leading via a nonradical reaction to a variety of unwanted pyrrolidinoxyl radicals, including DMPO–OH.[5d,14]

All the above observations point out that new and more specific spin traps may help answer questions concerning how tissues and cells deal with oxygen-derived free radicals. Over the past 10 years, different synthetic approaches have allowed the improved synthesis and purification of DMPO for biological uses[15] as well as the design of more lipophilic[10b,15a,16] or isotopically substituted[17] five-membered ring pyrroline 1-oxides as more selective and sensitive spin traps for $O_2^{\bullet-}$ and HO$^\bullet$ radicals. However, most of these procedures are laborious, and the newly prepared pyrroline 1-oxides have similar limitations to or more severe than DMPO. Consequently, the commercially-available DMPO is still used as an universal spin trap for $O_2^{\bullet-}$ and HO$^\bullet$ radicals in biological studies.

In this paper, we report on the two-step synthesis of the first member of a new class of α-phosphorus-containing DMPO analogues, the 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline 1-oxide[18] (DEPMPO). The kinetics of the *in vitro* spin trapping reaction of DEPMPO with superoxide and hydroxyl radicals and the ESR characteristics and relative persistencies of the corresponding spin adducts were studied in detail. In addition, using the isolated perfused rat heart model, the enhanced potential for using DEPMPO as a probe for free radical formation during myocardial ischemia/reperfusion injury was addressed.

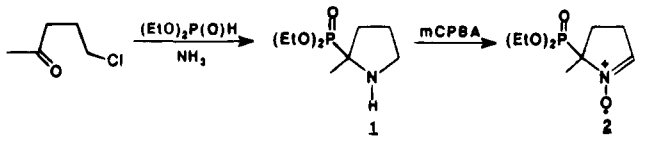
Results and Discussion

Synthesis. Diethyl (2-methyl-2-pyrrolidinyl)phosphonate, **1**, was obtained in a one-pot reaction by bubbling ammonia into an ethanolic solution of com-

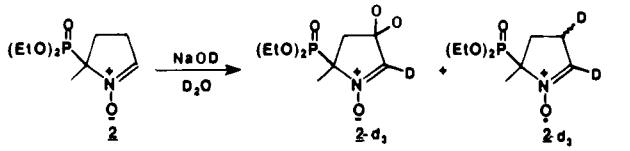
[⊗] Abstract published in *Advance ACS Abstracts*, December 15, 1994.

0022-2623/95/1838-0258$09.00/0

Scheme 1



Scheme 2



mercially-available 5-chloropentan-2-one and diethyl phosphite (Kabachnik–Fields' reaction)[19] (Scheme 1). Further, oxidation of a chloroform solution of **1** with *m*-chloroperbenzoic acid (mCPBA) led to the hygroscopic nitrone DEPMPO, **2**. Although the yield of the oxidation step was rather poor (30%), we found this method of synthesis the best overall, as **2** could be prepared in just two steps.

Stirring of DEPMPO with NaOD in D_2O for 24 h[17] led to a mixture of the 2,3,3-trideuterated DEPMPO **2**-d_3 and 2,3-dideuterated **2**-d_2 analogues (Scheme 2). The pure trideuterated molecule could not be obtained, due to the presence of water associated with DEPMPO.

Spin Trapping of the Hydroxyl Radical. The hydroxyl radical was generated in a phosphate buffer pH 7.0 in the presence of **2**, using two generators, *i.e.*, the Fenton (H_2O_2/$FeSO_4$) system or the superoxide-dependent Fenton system, consisting of a mixture of $FeNH_4(SO_4)_2$, hypoxanthine, and xanthine oxidase (HX/XO). Under these conditions, DMPO afforded a 1:2:2:1 ESR quartet characteristic of DMPO–OH, with the hyperfine splitting constants (hfsc's) $A_N = A_{H^\beta} = 14.9$ G.[6c] When DMPO was replaced by DEPMPO in the HO•-generating systems, the very intense and persistent ESR spectrum depicted in Figure 1 was observed in both cases. Since this signal was inhibited by the presence of either catalase or, in the case of the superoxide dependent Fenton system, superoxide dismutase (SOD), it was assigned to 2-(diethoxyphosphoryl)-2-methyl-5-hydroxy-1-pyrrolidinyloxyl (DEPMPO–OH), the DEPMPO/HO• spin adduct.

The ESR parameters of DEPMPO–OH and 2-(diethoxyphosphoryl)-2-methyl-5-hydroxy-4,4,5-trideuterio-1-pyrrolidinyloxyl DEPMPO-d_3–OH, obtained by trapping HO• with **2**-d_3, are listed in Table 1. As with DMPO–OH, the A_N (14.0 G) and A_{H^β} (13.0 G) hfsc's for DEPMPO–OH are close, giving rise to a quartet pattern which is split by a large phosphorus coupling (A_P = 47.4 G). When methanol or ethanol was added to the hydroxyl radical generating systems in the presence of **2**, the corresponding hydroxymethyl or (methylhydroxy)-methyl radical spin adduct (Table 1) was observed, instead of DEPMPO–OH.

Many examples of stereospecific and stereoselective radical additions on pyrroline 1-oxides have already been mentioned in the literature.[16b-d,20] In the HO• trapping experiments with **2**, we detected only one isomer of the expected spin adduct. The same result was observed for the trapping with **2** of different radicals ($CO_2^{\bullet-}$; $SO_3^{\bullet-}$; methyl; α-hydroxyalkyl; $(RO)_2P(O)^\bullet$) either in aqueous solutions or in organic solvents.

The formation of the DMPO–OH radical has been reported as occurring through nucleophilic addition of water in the presence of aqueous ferric chloride,[14a] followed by air oxidation of the generated hydroxylamine. However, when the reaction is carried out in various buffers or in the presence of metal ion chelators, addition of water on DMPO is suppressed.[14b] Due to the serious implications of these results with respect to many spin-trapping studies using nitrones, we investigated the reactivity of DEPMPO toward water in the presence of ferric ions. A strong ESR signal from the DEPMPO–OH spin adduct was observed within 1 min from a sample containing 1 mM $FeNH_4(SO_4)_2$ and 25 mM DEPMPO in water. We observed that the intensity of this DEPMPO–OH signal did not change significantly when the concentration of $FeNH_4(SO_4)_2$ was increased from 0.5 to 1 mM. When the concentration was lower than 0.25 mM, however, the formation of DEPMPO–OH was not observed (with DMPO this lower concentration was 0.1 mM). On the other hand, no formation of DEPMPO–OH was observed when the reaction was carried out in a phosphate buffer at pH 7.0 with a concentration 1 mM in $FeNH_4(SO_4)_2$.

The method of kinetic competition,[8] with DMPO as a competitive scavenger, was used to determine the rate constant for the trapping of HO• on DEPMPO. The HO• radical was generated by a Fenton reaction in a phosphate buffer at pH 7.4. The spin-trapping rate was monitored by measuring the intensity (as the peak height or the peak area) of the first or the second low-field peak of the DEPMPO–OH ESR signal. Assuming a steady-state concentration of HO•, the kinetic model can be described by eqs 1–5 (Scheme 3), in which

Scheme 3

$$\mathrm{HO^\bullet + DEPMPO} \xrightarrow{k_{\mathrm{DEPMPO}}} \mathrm{DEPMPO{-}OH} \quad (1)$$

$$\mathrm{HO^\bullet + DMPO} \xrightarrow{k_{\mathrm{DMPO}}} \mathrm{DMPO{-}OH} \quad (2)$$

$$\frac{-\mathrm{d[HO^\bullet]}}{\mathrm{d}t} = k_{\mathrm{DEPMPO}}[\mathrm{DEPMPO}][\mathrm{HO^\bullet}] + k_{\mathrm{DMPO}}[\mathrm{DMPO}][\mathrm{HO^\bullet}] \quad (3)$$

$$\frac{\mathrm{d[DEPMPO{-}OH]}}{\mathrm{d}t} = k_{\mathrm{DEPMPO}}[\mathrm{DEPMPO}][\mathrm{HO^\bullet}] \quad (4)$$

$$V/v = 1 + (k_{\mathrm{DMPO}}/k_{\mathrm{DEPMPO}})([\mathrm{DMPO}]/[\mathrm{DEPMPO}]) \quad (5)$$

k_{DEPMPO} and k_{DMPO} represent the rate constants for the trapping of HO• with DEPMPO and DMPO, respectively. At a high concentration of DEPMPO and in the absence of DMPO, the rate of spin trapping is equal to the rate of HO• generation. If V and v represent the rate of spin trapping in the respective absence and presence of DMPO, then eq 5 is obtained by dividing eq 3 by eq 4. By plotting the V/v ratio as a function of the [DMPO]/[DEPMPO] ratio (kept in between 0 and 1.0) a straight line is obtained with the slope equal to 0.43 and the correlation coefficient equal to 0.99 (measuring V/v from the peak height) or 0.47 and 0.97 (measuring V/v from the peak area). Thus, using[8] $k_{\mathrm{DMPO}} = 3.4 \times 10^9$ M^{-1} s^{-1} led to $k_{\mathrm{DEPMPO}} = 7.14$ (7.82) $\times 10^9$ M^{-1} s^{-1}.

Spin Trapping of Superoxide. Identification of the Spin Adduct. The spin trapping of superoxide with DEPMPO was conducted in phosphate buffers at

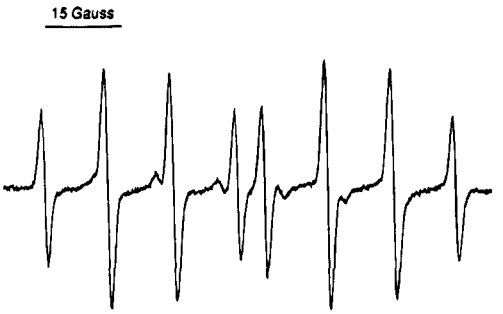


Figure 1. ESR spectrum of DEPMPO–OH obtained by carrying a Fenton reaction in the presence of DEPMPO.

Table 1. ESR hyperfine Splitting Constants for Spin Adducts of DEPMPO

spin adduct (source, solvent)	A_N (G)	A_{H^β} (G)	A_{H^γ} (G)	A_P (G)
DEPMPO–OH[a,b] (H_2O_2, Fe^{2+}, H_2O)	14.0	13.0	0.27 (3H)	47.4
DEPMPO-d_3–OH[b,e] (H_2O_2, Fe^{2+}, H_2O)	14.0	2.0(1D)	0.27 (3H)	47.5
DEPMPO-CH_2OH (H_2O, CH_3OH, Fe^{2+}, H_2O)	14.5	20.7		49.95
DEPMPO–OOH[a–c]	A[d] 13.4	11.9	0.8 (1H), 0.43 (6H)	52.5
(HX, XO, DTPA, buffer pH 7)	B[d] 13.2	10.3	0.9 (1H), 0.43 (6H)	48.5

[a] $g = 2.0059$. [b] From calculated spectrum. [c] $K_{A\rightarrow B} = 7 \times 10^7\ s^{-1}$. [d] 50%, line width 0.22 G. [e] Using a mixture **2-d_3/2-d_2**: 80/20.

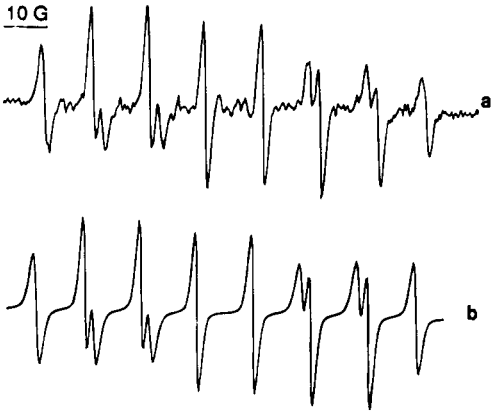


Figure 2. (A) ESR spectrum of DEPMPO–OOH obtained as a consequence of the reaction of hypoxanthine with xanthine oxidase. Spectrometer settings were microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G; receiver gain 6.3×10^3; response time 0.128 s; sweep time 52.5 G/min. (b) Computer simulation of (a) obtained with the parameters listed in Table 1.

different pH values, by using superoxide-generating systems consisting either of hypoxanthine in the presence of xanthine oxidase (HX/XO) or of a light riboflavine electron donor (LRED) combination.[8] A typical ESR spectrum of the spin adduct obtained during these experiments is shown in Figure 2. The formation of this spin adduct was completely inhibited by SOD (85 units/mL), and when the trapping of superoxide was conducted in the presence of glutathione peroxidase system (10 units/mL), only the DEPMPO–OH spin adduct was observed. Moreover, nucleophilic addition of H_2O_2[21] to DEPMPO in pyridine led to an ESR spectrum identical to that shown in Figure 2, and all the above results unambiguously support this spectrum corresponding with the DEPMPO–superoxide spin adduct.

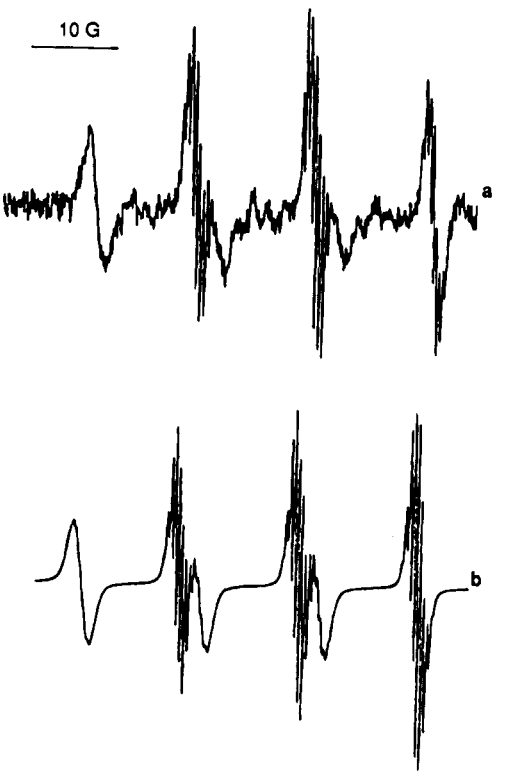


Figure 3. (a) Half ESR spectrum of DEPMPO–OOH recorded at low modulation amplitude (0.6 G), all the other spectrometer settings being identical to those used to record the ESR spectrum shown in Figure 2a. (b) Computer simulation of (a).

When trace metal impurities were carefully removed from the medium by chelex treatment, no sign of decomposition of the DEPMPO–superoxide adduct into the DEPMPO–OH adduct was observed. When superoxide was generated by the HX/XO system, the decay of the DEPMPO–superoxide adduct did not give any new detectable nitroxide. However, when superoxide was generated by the LRED system, the concomitant formation of a carbon-centered radical spin adduct (A_N = 15.0 G, A_{H^β} = 21.0 G, A_P = 47.6 G) was observed.

Assuming that no long-range couplings with the ring hydrogens would be resolved, the ESR spectrum of the DEPMPO–superoxide spin adduct should exhibit 12 lines of equal intensity. Twelve lines were indeed observed (Figure 2a), but the shape of some of them was unusual, and the analysis or the simulation of the spectrum was not straightforward. When the modulation frequency of the recording was 100 kHz and the modulation amplitude lower than 0.8 G, the ESR spectrum of the DEPMPO–superoxide spin adduct clearly exhibited a dramatic alternate line width (Figure 3a). This effect could be accounted for by the existence of an exchange between different forms of the DEPMPO–superoxide spin adduct, for which the conformationally-dependent hyperfine coupling constants should have different values. Actually, quite satisfactory simulations of the experimental spectra were obtained (Figures 2b, 3b) assuming a fast exchange between two forms of the DEPMPO–superoxide spin adduct and using the parameters given in Table 1.

Either an exchange between the protonated and deprotonated forms of the DEPMPO–superoxide spin adducts or an exchange between two conformers could account for the observed alternate linewidth. In order to address this problem, we first generated DEPMPO–

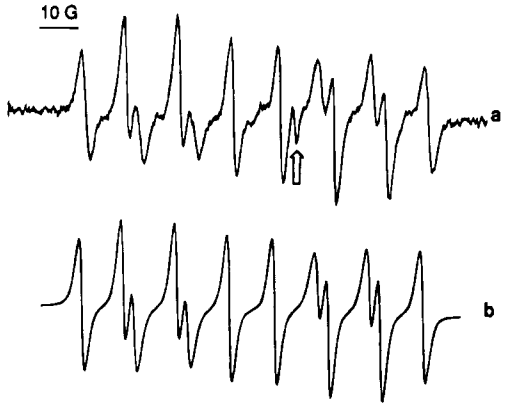


Figure 4. (a) Spectrum of DEPMPO–$OOCH_3$ obtained as a consequence of the generation[23] of $CH_3OO^{\bullet}$ in the presence of DEPMPO. (b) Computer simulation of (a) obtained assuming an exchange between two conformers A and B of DEPMPO–$OOCH_3$ and the following parameters. A (40%): A_N = 13.5 G, A_{H^β} = 9.5 G, A_{H^γ} (1H) = 1.2 G, A_{H^γ} (6H) = 0.43 G, A_P = 45.8 G, LW = 0.9 G. B (60%): A_N = 13.1 G, A_{H^β} = 10.9 G, A_{H^γ} (1H) = 0.9 G, A_{H^γ} (6H) = 0.43 G, A_P = 49.8 G, LW = 0.9 G. k (exchange) = 5×10^7 s^{-1}. The peak marked with an arrow is a background signal from the spectrometer cavity.

OOH at different pHs and observed that its ESR spectrum was not significantly changed when the pH increased from 5.6 to 8.2. On the other hand, we also observed a similar line width effect on the ESR spectrum of the DEPMPO–$OOCH_3$ (Figure 4), and DEPMPO–OOt-Bu spin adducts. These results suggest that the alternate line width observed on the ESR spectra of DEPMPO–OOH and DEPMPO–alkylperoxy spin adducts originates from conformational equilibria. It is also worth mentioning that in phosphate buffers, the well-known DMPO–OOH ESR spectrum exhibits an asymmetry which cannot be reproduced using traditional simulation softwares.[22] However, the asymmetrical DMPO–OOH spectrum can be effectively reproduced[23] by assuming an exchange between two forms of equal populations and using the following parameters: A_{N1} = 14.4 G, A_{H^β} = 12.8 G, A_{H^γ} = 1.1 G, LW = 0.3 G; A_{N2} = 14.2 G, A_{H^β} = 10.6 G, A_{H^γ} = 1.4 G, LW = 0.3 G, $k_e = 1.5 \times 10^7$. Further experiments are in progress in order to determine the nature of these conformational equilibria.

In phosphate buffers at pH 5.6 and 7.0, the addition of superoxide on DEPMPO is almost stereoselective and only one diastereoisomer of the spin adduct was clearly detected. However, in organic solvents like DMF or DMSO, the minor diastereoisomer (10–15%) was also clearly identified.

Owing to the quick decay of superoxide spin adducts, reliable delayed ESR analysis requires the possibility of freezing and storing such nitroxides for the spin-trapping technique to be applied to the investigation of cellular responses to oxidative stress. After thawing frozen aqueous samples which contained high concentrations of enzymatically-generated DMPO–OOH, we observed that only small DMPO–superoxide ESR signals and intense DMPO–OH ESR signals could be detected (data not shown). On the other hand, Pou and Rosen[24] reported that the freezing and thawing of DMPO–OOH samples formed by stimulation of neutrophils with a phorbol diester dramatically decreased the amount of residual DMPO–OOH. Furthermore, we observed that freezing and storing in liquid N_2 a sample

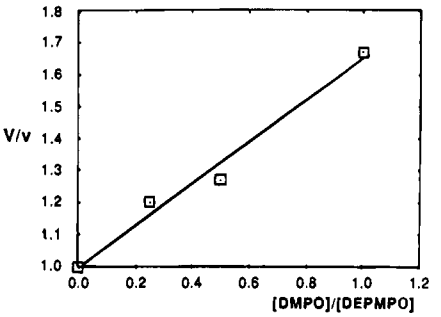


Figure 5. Inhibition by DMPO of superoxide trapping with DEPMPO. $O_2^{\bullet-}$ was produced by the light/riboflavin/DTPA system at pH 7 in 0.1 M phosphate buffer. The data were plotted according to equation $V/v = 1 - k'_{DMPO}[I]k'_{DEPMPO}[D]$, where V and v are the rates of superoxide trapping in the absence and presence of DMPO, k'_{DMPO} and k'_{DEPMPO} are second-order rate constants for the reaction of superoxide with the inhibitor DMPO and with DEPMPO, respectively, and [I] and [D] refer to the inhibitor DMPO and to the DEPMPO concentrations. The correlation was 0.98.

of DEPMPO–superoxide spin adduct in a phosphate buffer at pH 7.0 did not induce any significant change in its ESR spectrum, recorded at room temperature after thawing.

Spin Trapping of Superoxide: Kinetics of Spin Trapping. The light/riboflavine/DTPA system was used as a source of superoxide in phosphate buffer at pH 7.0 in the presence of DEPMPO. The method of kinetic competition was used to determine the apparent spin-trapping rate constant with DMPO as a competitive scavenger for superoxide. The spin-trapping rate was estimated by following the intensity of the ESR signal of the DEPMPO–superoxide spin adduct.

Ignoring the dismutation of superoxide and assuming a steady state for its concentration, the kinetic model is the same as that described in Scheme 3 (replacing $HO^{\bullet}$ by $O_2^{\bullet-}$, and k_{DEPMPO} and k_{DMPO} by k'_{DEPMPO} and k'_{DMPO}, respectively). In the absence of DMPO, the rate of spin trapping is equal to the rate of superoxide generation. Consequently, if V and v represent the rate of spin trapping in the respective absence and presence of DMPO, and if the V/v ratio is plotted as a function of the [DMPO]/[DEPMPO] ratio (kept in between 0 and 1), a straight line is obtained (Figure 5) with the slope equal to 0.65, and thus $k'_{DEPMPO} = 1.5k'_{DMPO}$.

Spin Trapping of Superoxide: Kinetics of the Decay of the Spin Adduct. The small half-life of DMPO–OOH at physiological pH is one of the most important drawbacks of using DMPO to study oxidative stress. In order to appreciate the perspective which DEPMPO could open in this field, it was of prime importance to compare the half-lives of DEPMPO–superoxide and DMPO–superoxide generated under the same conditions.

The superoxide was generated using the light/riboflavine/DTPA (diethylenetriaminepentaacetic acid) system in a phosphate buffer at pH 7.0. The decay kinetics of the spin adducts were monitored by measuring the decrease in appropriate ESR peak heights after termination of illumination. The decay of DMPO–superoxide produced under these conditions was pure first-order and had a rate constant of 1.4×10^{-2} s^{-1}, which corresponds to an half-life of 50 s. The decay of DMPO–superoxide was accompanied by the formation of DMPO–OH. However, the decay of DEPMPO–superoxide

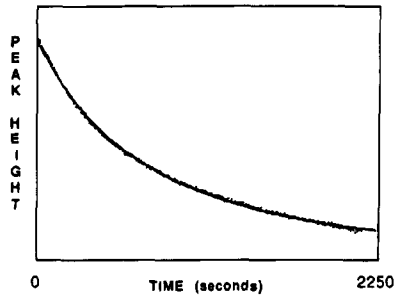


Figure 6. Kinetic of decay of DEPMPO–OOH. The experimental curve is represented by the dotted line. The superoxide spin adduct was generated at pH 7 by a light/riboflavin/DTPA system in 0.1 M phosphate buffer containing 0.1 M DEPMPO. After the light was shut off, the signal decay was monitored at a fixed magnetic field corresponding to a pure line of the DEPMPO–OOH ESR spectrum. The calculated curve is represented in continuous line and was obtained from computer integration of equation $-d[S]/dt = k_d[S] + k_d'[S]^2$, in which k_d and k_d' represent, respectively, the first-order and the second-order kinetic constants of the decay, and [S] the DEPMPO–OOH concentration. A good match between experimental and calculated curves was obtained when $k_d = 9 \times 10^{-4}$ s^{-1} and $k_d'[S]_0 = 6 \times 10^{-4}$ s^{-1}.

produced under the same conditions was slower and had both first- and second-order components (Figure 6). After 10 min, the decay was pure first-order with a rate constant of 7.8×10^{-4} s^{-1}, which corresponds to an half-life of 890 s. The concentration reached by the DEPMPO–superoxide spin adduct was significantly higher than that reached by the DMPO–superoxide, and the second-order component in the decay of the former might be due to a disproportionation process. In organic solvents, the concentration reached by DMPO–OOH spin adduct was higher, and a second-order reaction was shown to contribute to DMPO–OOH decay[25] in DMF and DMSO.

Spin Trapping of Superoxide with DEPMPO during Reperfusion of Ischemic Isolated Rat Heart. Using the Langendorff method, perfusion experiments were performed on three isolated perfused hearts from anesthetized (5 mg/100 g of body weight of intraperitoneally injected sodium pentobarbital) male Wistar-strain rats (300–350 g body weight). The hearts were allowed to equilibrate for 30 min at the normal coronary flow of 14 mL/min (normoxic control period) and were then submitted to a sequence including 30 min of zero-flow ischemia at 37 °C and 10 min reperfusion at the control flow rate. The spin trap DEPMPO was continuously infused for the last 5 min of the control and for the entire reperfusion periods. To minimize decomposition of the nitrone, a cold (4 °C) nonoxygenated DEPMPO solution in chelexed Krebs medium (40 mM) was mixed to the perfusion medium at 5% of the total perfusion rate, just before the aortic cannula, to yield a final concentration of 2.12 mM. At this point, the DEPMPO solutions were diamagnetic. In one experiment, SOD (100 μg/mL) was added to the perfusion medium during the 15 last min of the control period and throughout reperfusion.

The effluent perfusate from the normoxic and reperfused hearts was sampled from the pulmonary artery in 5-s aliquots at the end of the control period and sequentially during reperfusion. Samples were immediately frozen in liquid N_2 and successively thawed prior to ESR analysis. Control samples of perfusate

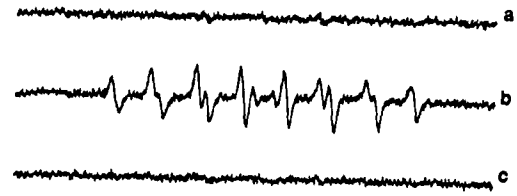


Figure 7. Trapping of superoxide with DEPMPO during reperfusion of ischemic isolated rat heart. (a) Control (b) ESR signal recorded after freezing and thawing a sample collected (during 5 s) 50 s after the begining of reperfusion. The reperfusion followed a 30 min period of zero-flow ischemia. (c) Same as (b) in the presence of SOD (100 μg/mL) added to the perfusion medium during the last 15 min of the control period and throughout reperfusion.

from all hearts showed no appreciable ESR signals, and therefore little or no free radicals were spin trapped on DEPMPO (Figure 7a). Whereas $O_2^{\bullet -}$ is thought to be produced as a primary species during myocardial reperfusion injury, most of the previously published experiments on isolated hearts subjected to ischemia have reported the detection of DMPO–OH spin adducts upon reperfusion, when DMPO was used as a spin trap in the concentration range 10–100 mM.[4] On the basis of the short life-span of DMPO–OOH and on SOD-dependent inhibition of DMPO–OH in these experiments, it was postulated that DMPO–OH could, in part, originate from intramolecular cleavage of DMPO-OOH.[4] Only two studies have reported on the transient detection of weak DMPO–OOH ESR signals in coronary effluents from rat hearts during initial postischemic reflow.[4a,f] In coronary effluents from the two hearts reperfused with 2.12 mM DEPMPO-supplemented normal medium, intense ESR spectra were recorded, mainly consisting of DEPMPO–superoxide spin adducts with little contamination with DEPMPO–OH. The signals were easily detected from 20 s to 6 min following restoration of control flow (Figure 7b). Involvement of $O_2^{\bullet -}$ was confirmed since ESR spectra were totally inhibited when ischemia and reperfusion was performed in the presence of 100 μg/mL SOD (Figure 7c).

Conclusion

5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide, DEPMPO, was prepared in a reasonable yield by a two-step synthesis from the commercially available 5-chloro-2-pentanone. Solutions of DEPMPO in chelexed phosphate buffers 0.1 M stored for 1 day at 0–6 °C did not generate any ESR signal which could hamper the interpretation of a spin trapping experiment. To estimate the lipophilicity of DEPMPO, we determined its partition coefficient in a 1-octanol/water system. DEPMPO exhibited a partition coefficient of only 0.06, very similar to that of DMPO.[10]

Reaction of $HO^{\bullet}$ with DEPMPO produced the very persistent DEPMPO–OH spin adduct. The reaction was shown to be stereospecific and roughly twice as rapid as the reaction of $HO^{\bullet}$ with DMPO.

Reaction of $O_2^{\bullet -}$/$HOO^{\bullet}$ with DEPMPO produced the DEPMPO–superoxide spin adduct, the ESR spectrum of which exhibited a dramatic alternate line width effect. The reaction was shown to be highly stereoselective and slightly (1.5 times) more rapid than the reaction of $O_2^{\bullet -}$/$HOO^{\bullet}$ with DMPO. The decay of DEPMPO–superoxide was shown to involve both first- and second-order reactions and to be much slower (15 times less in

phosphate buffer at pH 7) than the decay of DMPO–superoxide.

The ESR signal of DEPMPO–superoxide in phosphate buffer did not exhibit any significant change after thawing of a sample stored in liquid nitrogen for two days.

Finally, using a low concentration of DEPMPO, we easily detected the formation of superoxide during the reperfusion of ischemic isolated rat hearts.

These preliminary data suggest that DEPMPO offers a very promising addition to DMPO as a useful tool in biological spin-trapping experiments. In our laboratory, the synthesis of a series of cyclic and acyclic nitrones bearing different kinds of phosphorus groups in different positions relative to the nitrone function is now in progress. The relevance of these new nitrones in trapping free radicals in biological milieu will then be fully investigated.

Experimental Section

1. Synthesis and Characterization of Starting Materials and Products. Instrumentation. IR spectra were recorded on a Unicam Mattson 1000 instrument. Mass spectra were obtained using a Varian Mat 311 instrument. ^{31}P NMR spectra were recorded at 40.53 MHz (Bruker AC 100 instrument), with 85% H_3PO_4 as external reference. ^1H and ^{13}C NMR spectra were recorded on Bruker AC 200 or AM 400X instruments respectively at 200 or 400 MHz and 50.32 or 100.61 MHz.

Diethyl (2-Methyl-2-pyrrolidinyl)phosphonate (1). Gaseous ammonia was bubbled through a solution of 5-chloropentan-2-one (Aldrich) (11.8 g, 0.1 mol) and diethyl phosphite (14 g, 0.1 mol) in ethanol (50 mL) for 4 h at 50 °C. After filtration and removal of the solvent under vacuum, the residue was poured into a 2 N HCl solution (40 mL) which was then extracted with dichloromethane (2 × 50 mL). Sodium bicarbonate was added to the aqueous layer up to pH 10. After extraction with chloroform (4 × 50 mL), the organic layer was dried over sodium sulfate, filtered, and evaporated to dryness, resulting in a colorless oil **1** (13.7 g, 62%). Anal. Found: C, 48.5; H, 9.2; N, 6.15. Calcd for $C_9H_{20}NO_3P$: C, 48.8; H, 9.1; N, 6.3. IR (film, cm^{-1}): 3315, 1235, 1055, 1031, 958. ^1H NMR (C_6D_6): δ 1.10 (6H, t, J = 7.0 Hz), 1.28 (3H, d, J = 15.0 Hz), 1.2–1.8 (3H, m), 2.0–2.5 (1H, m), 2.7–3.0 (2H, m), 3.7–4.5 (4H, m). ^{13}C NMR (C_6D_6): δ 16.6, 16.7, 26.1 (d, J = 4.5 Hz), 35.2 (d, J = 2.5 Hz), 47.3 (d, J = 7.1 Hz), 50.4 (d, J = 150 Hz), 62.1 (d, J = 7.2 Hz), 62.3 (d, J = 7.2 Hz). ^{31}P NMR (C_6D_6): δ 29.8.

5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (2). A solution of *m*-chloroperbenzoic acid 70% (4.4 g, 18 mmol) in chloroform (40 mL) was added over a period of 1 h to a stirred solution of **1** (2 g, 9 mmol) in chloroform (30 mL) at −10 °C. The reaction mixture was then washed with saturated aqueous $NaHCO_3$ solution (2 × 15 mL) and brine (15 mL). The organic layer was dried over sodium sulfate. The solvent was removed under vacuum. Column chromatography of the residue (silica, 15% ethanol in dichloromethane) afforded **2** as a very hygroscopic oil (0.6 g, 30%). Anal. Found: C, 44.32; H, 7.99; N, 5.81; P, 12.90. Calcd for $C_9H_{18}NO_4P$, 2.5% H_2O: C, 44.81; H, 7.74; N, 5.81; P, 12.86. IR (film, cm^{-1}): 1574, 1248, 1051, 1023, 970. UV (methanol, nm): 240 (ϵ 6900). ^1H NMR ($CDCl_3$): δ 1.35 (3H, t, J = 7.1 Hz), 1.36 (3H, t, J = 7.1 Hz), 1.70 (3H, d, J = 14.9 Hz), 2.0–2.2 (1H, m), 2.5–2.7 (1H, m), 2.7–2.9 (2H, m), 4.1–4.4 (4H, m), 6.9–7.0 (1H, m); (C_6D_6) δ 1.02 (3H, t, J = 6.9 Hz), 1.12 (3H, dt, J = 0.6 and 6.9 Hz), 1.4–1.6 (1H, m), 1.58 (3H, d, J = 14.6 Hz), 1.7–1.8 (1H, m), 2.3–2.4 (1H, m), 2.5–2.6 (1H, m), 3.8–4.0 (2H, m), 4.2–4.4 (2H, m), 6.5 (1H, q, J = 2.9 Hz). ^{13}C NMR ($CDCl_3$): δ 16.3, 16.4, 20.8, 25.8, 30.9, 62.8 (d, J = 6.0 Hz), 63.9 (d, J = 6.0 Hz), 75.0 (d, J = 155 Hz), 134.9 (d, J = 8.0 Hz); (C_6D_6) δ 16.3 (d, J = 4.4 Hz), 16.4 (d, J = 5.2 Hz), 21.0, 25.7, 30.9, 61.9 (d, J = 6.6 Hz), 64.0 (d, J = 5.7 Hz), 75.1 (d, J = 155 Hz) 133.1 (d, J = 8.5 Hz). ^{31}P NMR (C_6D_6): δ 22.52 ($CDCl_3$) δ 21.60; (D_2O) δ 24.92. MS: *m/z* 235 (M^+, 10), 218 (2.5), 162 (3.5), 139 (4.6), 111 (11), 98 (100), 84 (65), 82 (32). ^{31}P NMR (C_6D_6): δ 22.52; ($CDCl_3$) δ 21.60; (D_2O) δ 24.92. MS: *m/z* 235 (M^+, 10), 218 (2.5), 162 (3.5), 139 (4.6), 111 (11), 98 (100), 84 (65), 82 (32).

5-(Diethoxyphosphoryl)-5-methyl[2,3,3-$^2H_3$]-1-pyrroline *N*-oxide (2-*d*$_3$). NaOD (0.18 N, 10 mL) was added slowly under a N_2 atmosphere to a stirred solution of nitrone **2** (0.5 g) in D_2O (3 mL) at 5 °C. The reaction was monitored by ^1H NMR (disappearance of the vinylic proton). The mixture was neutralized with D_2SO_4 and extracted with dry DMF. The solvent was removed under vacuum, and molecular distillation (10^{-6} mmHg) of the residual brown oil (0.3 g) gave a colorless oil (80 mg, 16%, **2**-*d*$_3$/**2**-*d*$_2$: 80/20). ^1H NMR (C_6D_6): δ 1.02 (3H, t, J = 7.1 Hz), 1.12 (3H, dt, J = 7.1 and 0.5 Hz), 1.5–1.7 (1H, m), 1.58 (3H, d, J = 14.6 Hz), 2.4–2.8 (1.2H, m, 0.2 H excess due to **2**-*d*$_2$), 3.9–4.0 (2H, m), 4.2–4.5 (2H, m). ^{13}C NMR (C_6D_6): δ 16.4 (d, J = 5.6 Hz), 16.5 (d, J = 5.8 Hz), 21.1, 30.9, 61.9 (d, J = 6.9 Hz), 64.2 (d, J = 5.7 Hz), 75.2 (d, J = 154 Hz); the small triplet observed at δ 25.22 (J = 20 Hz) was attributed to the dideuterated compound **2**-*d*$_2$. ^{31}P NMR (C_6D_6): δ 22.52.

2. Spin-Trapping Studies. General. Xanthine oxidase and bovine erythrocyte superoxide dismutase (SOD) were purchased from Boehringer Mannheim Biochemica Co.; catalase, glutathione peroxidase and all chemicals were obtained from Sigma Chemical Co. All buffers were stirred for 6 h in the presence of a chelating iminodiacetic acid resin (4 g/100 mL) to remove trace metal impurities. The use of DTPA as chelating agent further minimizes artifacts resulting from such metal impurities. ESR assays were carried out in a flat cell by using a computer-controlled Varian E-9 ESR spectrometer at pH values ranging from 5.5 to 8.6. The ESR spectra were also recorded on a Bruker ESP 300 spectrometer equipped with an NMR gaussmeter for magnetic field calibration. An HP 5350B microwave frequency counter was used for the determination of g factors.

HO· Trapping. Fenton Reaction System. A standard Fenton reaction system was used to generate HO·. $FeSO_4$ (2 mM) were added to a solution containing 0.1 M phosphate buffer, 2 mM DTPA, 2 mM H_2O_2, and 0.1 M DEPMPO. The ESR spectrum of the spin adduct was recorded 1 min after the addition of ferrous sulfate. No ESR signal was observed when catalase (600 units/mL) was added before $FeSO_4$.

HO· Trapping. Superoxide Dependent Fenton Reaction. HO· was also produced by adding ferric ammonium sulfate to a classical superoxide-generating system. The medium contained 0.1 M phosphate buffer, 0.4 mM hypoxanthine, 1 mM DTPA, 2 mM $FeNH_4(SO_4)_2$, 0.1 DEPMPO, and 0.4 units/mL xanthine oxidase. In these conditions the ESR spectrum of DEPMPO–OH was easily observed, while no signal was produced when catalase (600 units/mL) or SOD (85 units/mL) were added before xanthine oxidase.

Nucleophilic Addition of Water. When a solution containing 25 mM of DEPMPO and 1 mM of $FeNH_4(SO_4)_2$ in oxygenated pure distilled water was prepared, the ESR signal of DEPMPO–OH was observed. When the same experiment was realized in 0.1 M phosphate buffer at pH 7, no ESR signal was observed.

·CH_2OH and ·$CH(CH_3)OH$ Trapping. The Fenton reaction system in methanolic (20%) or ethanolic (10%) solution was used to generate ·CH_2OH or ·$CH(CH_3)OH$.

Superoxide Trapping. Hypoxanthine/Xanthine Oxidase System. This superoxide-generating system contained 0.1 M phosphate buffer, 0.4 mM hypoxanthine, 0.4 unit/mL xanthine oxidase, 1 mM DTPA, and 1 mM DEPMPO as spin trap. ESR spectra were recorded 40 s after the addition of xanthine oxidase. No signal occurred when SOD (85 units/mL) was added before xanthine oxidase.

Superoxide Trapping. Light/Riboflavin/DTPA System. The light/riboflavin/DTPA system used in our experiments contained 0.1 M phosphate buffer, 4.5 mM DTPA, 0.1 mM riboflavin, and 0.1 M DEPMPO. The superoxide generation was initiated by irradiating the ESR cell directly in the cavity, using a tungsten filament 100 W lamp. (The main

advantage of such a system is that it permits the recording of spectra right from the start of the spin trapping). No signal was produced in the presence of SOD (85 units/mL).

$CH_3OO^\bullet$ Trapping. $CH_3OO^\bullet$ was produced[26] by UV photolysis of an oxygenated aqueous solution containing 5 mM H_2O_2, 100 mM DMSO, and 5 mM DEPMPO as the spin trap. The ESR spectrum obtained under these conditions corresponded exactly and exclusively to the DEPMPO–$OOCH_3$ spin adduct.

Kinetic Studies of $HO^\bullet$ Trapping. The Fenton reaction system described previously was employed to generate $HO^\bullet$, and the second-order rate constant for $HO^\bullet$ trapping with DEPMPO was determined using the method of kinetic competition, with DMPO as competitive inhibitor. On every assay, a DEPMPO concentration of 0.05M was fixed, while the concentration of DMPO was varied from 0 to 0.075 M. The ESR signal of the DEPMPO–OH spin adduct was recorded exactly 1 min after the addition of ferrous sulfate, and the spin-trapping rate was determined by monitoring the intensity of a pure line of the ESR spectrum.

Kinetic Studies of Superoxide Trapping. The method of kinetic competition was used to determine the rate constant for superoxide trapping by DEPMPO employing DMPO as competitive inhibitor. The superoxide was generated in 0.1 M phosphate buffer at pH 7 using the light/riboflavin/DTPA system described previously. For kinetic experiments, a fixed DEPMPO concentration of 0.1 M was chosen, and the DMPO concentration was varied from 0 to 0.1 M. The ESR spectrum of the superoxide adduct was recorded exactly 10 s after the beginning of irradiation, and the spin-trapping rate was determined by monitoring the intensity of a pure line of the DEPMPO–OOH ESR signal.

Kinetic of Decay of Superoxide Spin Adducts. The light/riboflavin/DTPA system described previously was used to produce superoxide in 0.1 M phosphate buffer at pH 7, with a concentration of nitrone (DEPMPO or DMPO) fixed to 0.1 M. The superoxide adduct formation occurred solely during irradiation and was suppressed immediately the light was shut off. The decay of the spin adducts was then followed by monitoring the decrease of an appropriate line of DEPMPO–OOH or DMPO–OOH ESR spectrum.

Taking for granted the contribution of both first- and second-order processes to the decay of DEPMPO–OOH or DMPO–OOH, the rate of decay is given by eq 1.

$$-\mathrm{d}[\text{spin adduct}]/\mathrm{d}t = k_\mathrm{d}[\text{spin adduct}] + k_{\mathrm{d}'}[\text{spin adduct}]^2 \quad (1)$$

The value of k_d was calculated for DEPMPO–OOH and for DMPO–OOH with a least-squares multifunction computer program which fitted the curves calculated from eq 1 to the experimental curves.

3. Perfused Isolated Rat Heart: Perfusion Techniques and Perfusion Medium. Perfusion experiments were performed by the Langendorff method on three isolated perfused hearts from anesthetized (5 mg/100 g of body weight of intraperitoneally injected sodium pentobarbital) male Wistar-Strain rats (300–350 g body weight). Briefly, the hearts were excised and rapidly retrogradely perfused at 37 °C via the aorta, at a constant coronary flow of 14 mL/min, yielding an aortic pressure of 75–80 mmHg. The nonrecirculating perfusion medium was a Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 120 mM NaCl, 24 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 11 mM glucose. The perfusion medium was treated with chelex 100 resin (1% w/w) according to the batch method of Buettner[27] and gassed with a mixture of 95% O_2 and 5% CO_2 prior to use.

Perfusion Protocol. Hearts were allowed to equilibrate for 30 min at the normal coronary flow of 14 mL/min (normoxic control period) and were then submitted to a sequence including 30 min of zero-flow ischemia at 37 °C and 10 min reperfusion at the control flow rate. The spin trap DEPMPO was infused continuously for the last 5 min of the control and for the entire reperfusion periods. To minimize decomposition of the nitrone, a cold (4 °C) nonoxygenated DEPMPO solution in chelexed Krebs medium (40 mM) was mixed to the perfusion medium at 5% of the total perfusion rate just before the aortic cannula to yield a final concentration of 2.12 mM. At this point, the DEPMPO solutions were diamagnetic. In one experiment, SOD (100 μg/mL) was added to the perfusion medium during the 15 last min of the control period and throughout reperfusion.

The effluent perfusate from the normoxic and reperfused hearts were sampled from the pulmonary artery in 5-s aliquots at the end of the control period and sequentially during reperfusion. Samples were immediately frozen in liquid N_2 and successively thawed prior to ESR analysis.

ESR Measurements. ESR spectra were recorded at room temperature in the same standard quartz flat cell using a Bruker ESP 300 spectrometer operating at X-band (9.79 GHz) with a 100-kHz modulation frequency. The instruments settings were as follows: nonsaturating microwave power, 10 mW; modulation amplitude, 0.787 G; receiver gain, 8×10^5; time constant, 81.92 ms; sweep rate, 50 G/min.

References

(1) Initial presentation of results at the 4th International Symposium on Spin Trapping and Organic EPR Spectroscopy with Applications in Chemistry, Biology and Medicine; Oklahoma City, October 24, 1993.

(2) (a) Fridovich, I. *Annu. Rev. Pharmacol. Toxicol.* **1983**, *23*, 239–257. (b) Pryor, W. A. *Annu. Rev. Physiol.* **1986**, *48*, 657–667. (c) Sies, H. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1058–1071. (d) Sies, H. *Am. J. Med.* **1991**, *91* (Suppl. 3C), 31S–38S. (e) Halliwell, B.; Gutteridge, J. M. C.; Cross, C. E. *J. Lab. Clin. Med.* **1992**, *119*, 598–620. (f) Bast, A.; Haenen, G. R. M. M.; Doelman, C. J. A. *Am. J. Med.* **1991**, *91* (Suppl. 3C), 2S–13S. (g) Sies, H.; De Groot, H. *Toxicol. Lett.* **1992**, *64/65*, 547–551. (h) Buettner, G. R.; Jurkiewicz, B. A. *Free Rad. Biol. Med.* **1993**, *14*, 49–55.

(3) (a) McCord, J. M. *N. Engl. J. Med.* **1985**, *312*, 159–163. (b) Bolli, R. *J. Am. Coll. Cardiol.* **1988**, *12*, 239–249. (c) Kloner, R. A.; Przyklenk, K.; Whittaker, P. *Circulation* **1989**, *80*, 1115–1127. (d) Sekili, S.; McCay, P. B.; Li, X. Y.; Zughaib, M.; Sun, J. Z.; Tang, L.; Thornby, J. I.; Bolli, R. *Circ. Res.* **1993**, *73*, 705–723.

(4) (a) Arroyo, C. M.; Kramer, J. H.; Dickens, B. F.; Weglicki, W. B. *FEBS Lett.* **1987**, *221*, 101–104. (b) Garlick, P. B.; Davies, M. J.; Hearse, D. J.; Slater, T. F. *Circ. Res.* **1987**, *61*, 757–760. (c) Bolli, R.; Patel, B. S.; Jeroudi, M. O.; Lai, E. K.; McCay, P. B. *J. Clin. Invest.* **1988**, *82*, 476–485. (d) Zweier, J. L. *J. Biol. Chem.* **1988**, *263*, 1353–1357. (e) Pietri, S.; Culcasi, M.; Cozzone, P. J. *Eur. J. Biochem.* **1989**, *186*, 163–173. (f) Nakazawa, H.; Arroyo, C. M.; Ichimori, K.; Saigusa, Y.; Minezaki, K. K.; Pronai, L. *Free Rad. Res. Commun.* **1991**, *14*, 297–302. (g) Mergner, G. W.; Weglicki, W. B.; Kramer, J. H. *Circulation* **1991**, *84*, 2079–2090. (h) Boucher, F.; Pucheu, S.; Coudray, C.; Favier, A.; de Leiris, J. *FEBS Lett.* **1992**, *302*, 261–264.

(5) (a) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. *Arch. Biochem. Biophys.* **1980**, *200*, 1–16. (b) Rosen, G. M.; Finkelstein, E. *Adv. Free Rad. Biol. Med.* **1985**, *1*, 345–375. (c) Janzen, E. G. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1980; Vol. 4, pp 115–154. (d) Thornalley, P. J. *Life Chem. Rep.* **1986**, *4*, 57–112. (e) Mottley, C.; Mason, R. P. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum Publishing Corp.: New York, 1989; Vol. 8, pp 489–546.

(6) (a) Harbour, J. R.; Chow, V.; Bolton, J. R. *Can. J. Chem.* **1974**, *52*, 3549–3553. (b) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. *Mol. Pharmacol.* **1979**, *16*, 676–685. (c) Buettner, G. R. *Free Rad. Biol. Med.* **1987**, *3*, 259–303.

(7) Pou, S.; Hassett, D. J.; Britigan, E. B.; Cohen, M. S.; Rosen, G. M. *Anal. Biochem.* **1989**, *177*, 1–6.

(8) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. *J. Am. Chem. Soc.* **1980**, *102*, 4994–4999.

(9) Yamazaki, I.; Piette, L. H.; Grover, T. A. *J. Biol. Chem.* **1990**, *265*, 652–659.

(10) (a) Rosen, G. M.; Finkelstein, E.; Rauckman, E. J. *Arch. Biochem. Biophys.* **1982**, *215*, 367–378. (b) Turner, M. J. III; G. M. Rosen, *J. Med. Chem.* **1986**, *29*, 2439–2444.

(11) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. *Mol. Pharmacol.* **1982**, *21*, 262–265.

(12) (a) Chen, K.; Glockner, J. F.; Morse, P. D.; Swartz, H. M. *Biochemistry* **1989**, *28*, 2496–2501. (b) Iannone, A.; Tomasi, A.; Vannini, V.; Swartz, H. M. *Biochim. Biophys. Acta* **1990**, *1034*, 285–289. (c) Iannone, A.; Tomasi, A.; Vannini, V.; Swartz, H. M. *Biochim. Biophys. Acta* **1990**, *1034*, 290–293. (d) Sentjurc, M.; Pecar, S.; Chen, K.; Wu, M.; Swartz, H. M. *Biochim. Biophys. Acta* **1991**, *1073*, 329–335. (e) Samuni, A.; Carmichael, A. J.; Russo, A.; Mitchell, J. B.; Riesz, P. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7593–7597.

(13) (a) Samuni, A.; Murali-Krishna, C.; Riesz, P.; Finkelstein, E.; Russo, A. *Free Rad. Biol. Med.* **1989**, *6*, 141–148. (b) Samuni, A.; Samuni, U.; Swartz, H. M. *Free Rad. Biol. Med.* **1989**, *6*, 179–183. (c) Buettner, G. R. *Free Rad. Res. Commun.* **1990**, *10*, 11–15.

(14) (a) Makino, K.; Hagiwara, T.; Hagi, A.; Nishi, M.; Murakami, A. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 1073–1080. (b) Hanna, P. M.; Chamulitrat, W.; Mason, R. P. *Arch. Biochem. Biophys.* **1992**, *296*, 640–644.

(15) (a) Haire, D. L.; Hilborn, J. W.; Janzen, E. G. *J. Org. Chem.* **1986**, *51*, 4298–4300. (b) Janzen, E. G.; Jandrisits, L. T.; Shetty, R. V.; Haire, D. L.; Hilborn, J. W. *Chem.-Biol. Interact.* **1989**, *70*, 167–172.

(16) (a) Haire, D. L.; Janzen, E. G. *Can. J. Chem.* **1982**, *60*, 1514–1522. (b) Dehnel, A.; Griller, D.; Kanabus-Kaminska, J. M. *J. Org. Chem.* **1988**, *53*, 1566–1567. (c) Zhang, Y.-K.; Lu, D.-H.; Xu, G.-Z. *Z. Naturforsch.* **1990**, *45B*, 1075–1083. (d) Zhang, Y.-K.; Lu, D.-H.; Xu, G.-Z. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1855–1860.

(17) (a) Pou, S.; Rosen, G. M.; Wu, Y.; Keana, J. F. W. *J. Org. Chem.* **1990**, *55*, 4438–4443. (b) Halpern, H. J.; Pou, S.; Peric, M.; Yu, C.; Barth, E.; Rosen, G. M. *J. Am. Chem. Soc.* **1993**, *115*, 218–223.

(18) (a) Frejaville, C.; Karoui, H.; Le Moigne, F.; Culcasi, M.; Pietri, S.; Tordo, P. *French Patent* N° PV 9308906, 20 July, **1993**. (b) Frejaville, C.; Karoui, H.; Tuccio, B.; Le Moigne, F.; Culcasi, M.; Pietri, S.; Lauricella, R.; Tordo, P. *J. Chem. Soc., Chem. Commun.* **1994**, 1793–1794.

(19) (a) Fields, E. K. *J. Am. Chem. Soc.* **1952**, *74*, 1528–1531. (b) Kabachnik, M. I.; Medved, T. Ya. *Dokl. Akad. Nauk. SSSR.* **1952**, *83*, 689–692.

(20) (a) Zhang, Y.; Xu, G. *J. Chem. Soc., Chem. Commun.* **1988**, 1629. (b) Zhang, Y.; Xu, G. *Magn. Reson. Chem.* **1989**, *27*, 846–851. (c) Arya, P.; Stephens, J. C.; Griller, D.; Pou, S.; Ramos, C. L.; Pou, W. S.; Rosen, G. M. *J. Org. Chem.* **1992**, *57*, 2297–2301. (d) Dembkowski, L.; Finet, J. P.; Frejaville, C.; Le Moigne, F.; Maurin, R.; Mercier, A.; Pages, P.; Stipa, P.; Tordo, P. *Free Rad. Res. Commun.* **1993**, *19*, S23–S32.

(21) Reszka, K.; Bilski, P.; Chignell, C. F. *Free Rad. Res. Commun.* **1992**, *17*, 377–385.

(22) Buettner was the first to pay attention to the asymmetry of the DMPO–OOH ESR spectrum. He was able to obtain a reasonably good simulation of the spectrum assuming the existence of two species at the same concentration but exhibiting different ESR parameters. Buettner, G. R. *Free Rad. Res. Commun.* **1990**, *10*, 11–15.

(23) Lauricella, R.; Tordo, P. To be published.

(24) Pou, S.; Rosen, G. M. *Anal. Biochem.* **1990**, *190*, 321–325.

(25) Tuccio, B.; Lauricella, R.; Frejaville, C.; Bouteiller, J. C.; Tordo, P. *J. Chem. Soc., Perkin Trans. 2*, **1994**, in press.

(26) Bors, W.; Michel, C.; Stettmaier, K. *J. Chem. Soc., Perkin Trans. 2*, **1992**, 1513–1517.

(27) Buettner, G. R. *J. Biochem. Biophys. Meth.* **1988**, *16*, 27–40.

JM940386M