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5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-Oxide (DEPMPO): a New Phosphorylated Nitrone for the efficient *In Vitro* and *In Vivo* Spin Trapping of Oxygen-centred Radicals

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5-Diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), a newly synthesized spin trap, is used for the *in vitro* spin trapping of hydroxyl and superoxide radicals, and though the spin trapping rates are close to those reported for 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), the DEPMPO–superoxide spin adduct is significantly more persistent than its DMPO analogue, a difference which allows the detection of superoxide during the reperfusion of ischaemic isolated rat hearts.

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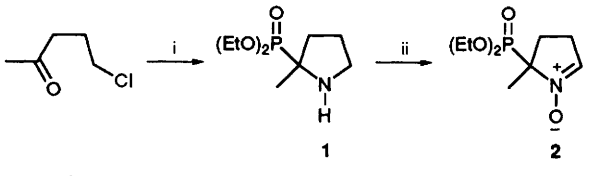
Enormous efforts are still being devoted to addressing the role played by partially reduced oxygen species such as superoxide $O_2^{-\cdot}$ and hydroxyl $HO^{\cdot}$ radicals in mediating a variety of pathological conditions. Of the methods available for assessing free radical formation in biological systems, EPR spin trapping appears one of the most appropriate and has been extensively applied to investigate myocardial reperfusion injury in animal models. Out of several nitrone spin traps, the cyclic 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has received the most attention,[1] since it yields distinct and characteristic spin adducts with $O_2^{-\cdot}$ and $HO^{\cdot}$ radicals.[1,2] However, reaction of DMPO with superoxide is rather slow, having a second order rate constant ranging from 10 $dm^3\ mol^{-1}\ s^{-1}$ at pH 7.8[3] to 1.2 $dm^3\ mol^{-1}\ s^{-1}$ at pH 7.4.[4] On the other hand, 5,5-dimethyl-2-hydroperoxy-1-pyrrolidinyloxyl (DMPO–OOH), the superoxide spin adduct, is short lived and can undergo a rapid chemical conversion to 5,5-dimethyl-2-hydroxy-1-pyrrolidinyloxyl (DMPO–OH), the hydroxyl radical spin adduct.[5] Many other limitations exist to the use of DMPO as a probe for oxyradical generation in biological milieu and we now 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 (DEPMPO),[6] and on its ability to spin trap oxygen-derived radicals.

Diethyl (2-methyl-2-pyrrolidinyl) phosphonate **1** was obtained (62% yield) in a one-pot reaction by bubbling ammonia into an ethanolic solution of commercially available 5-chloro-2-pentanone and diethylphosphite (Scheme 1). Next, using MCPBA, oxidation of **1** in $CHCl_3$ led to the hygroscopic nitrone DEPMPO **2** (30% yield).

At room temp., solutions of **2** in pure water or phosphate buffers were stable, and no formation of artifactual nitroxides was detected by EPR.

When 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, a very intense and persistent EPR spectrum was observed in both cases. 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 also observed during the photolysis of H_2O_2 and was assigned to one diastereoisomer of 2-diethoxyphosphoryl-2-methyl-5-hydroxy-1-pyrrolidinyloxyl (DEPMPO-OH), the DEPMPO/$HO^{\cdot}$ spin adduct. The a_N (14.1 G) and a_H^{β} (13.2 G) hfscs for DEPMPO-OH are close, giving rise to a quartet pattern which is split by a large phosphorus coupling (a_P = 47.3 G). When methanol was added to the hydroxyl radical generating systems in the presence of **2**, the corresponding $\cdot CH_2OH$ radical spin adduct was observed, instead of DEPMPO–OH.



Scheme 1 Synthesis of **2**: i, $(EtO)_2P(O)H$, NH_3; ii, MCPBA, $CHCl_3$

The spin trapping of superoxide with DEPMPO was conducted in phosphate buffers at different pH values, by using superoxide-generating systems consisting either of hypoxanthine in the presence of xanthine oxidase or of a light-riboflavin-electron donor combination.[3] A typical EPR spectrum of the spin adduct obtained during these experiments is shown in Fig. 1. The formation of this spin adduct was completely inhibited by SOD, and when the trapping of superoxide was conducted in the presence of glutathione peroxidase system, only the DEPMPO–OH spin adduct was observed. On the other hand, the same spectrum, exhibiting slightly different hfscs, was observed by reacting KO_2 with **2**, either in DMF or pyridine. These results unambiguously support the spectrum shown in Fig. 1 corresponding with one diastereoisomer of the DEPMPO–OOH spin adduct.

Assuming that no resolved long-range couplings with the ring hydrogens would be resolved, the ESR spectrum of DEPMPO–OOH should be composed of twelve identical lines. Twelve lines were indeed observed (Fig. 1), but they clearly exhibited an alternate linewidth, which resulted in the unusual shape of the whole EPR spectrum. 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

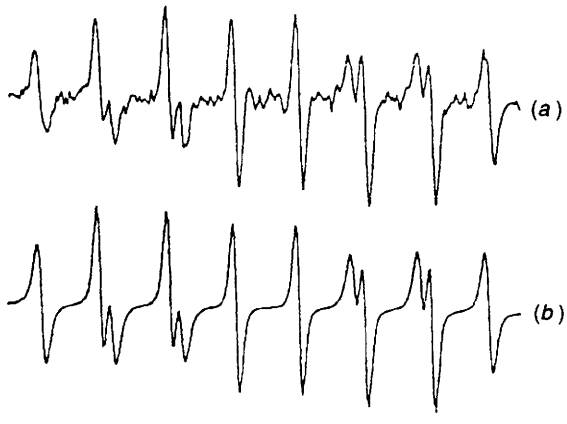


Fig. 1 Spectrum of DEPMPO–OOH, obtained as a consequence of the generation of $O_2^{-\cdot}$ in the presence of DEPMPO: (*a*) experimental; (*b*) calculated, assuming an exchange between two conformers *A* and *B* of DEPMPO–OOH and the following parameters: *A* (50%): a_N = 13.2 G, a_H^{β} = 11.9 G, a_H^{γ} (1H) = 0.7 G, a_H^{γ} (6H) = 0.43 G, a_p = 52.5 G, LW = 0.45 G; *B* (50%): a_N = 13.4 G, a_H^{β} = 10.3 G, a_H^{γ} (1H) = 0.8 G, a_H^{γ} (6H) = 0.43 G, a_P = 48.5 G, LW = 0.45 G; $k_{exchange}$ = 4 $10^7\ s^{-1}$

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constants should have different values. Actually, satisfactory simulation of the experimental spectrum was obtained (Fig. 1) assuming a fast exchange between two equally populated conformers of DEPMPO–OOH.

The method of kinetic competition, with DMPO as a competitive scavenger, was used to determine, in phosphate buffer at pH 7, the rate constants, k_{DEPMPO} and k'_{DEPMPO}, for the trapping of HO· and O_2^{-}· on **2**. Using[3] $k_{DMPO} = 3.4 \times 10^9$ $dm^3\ mol^{-1}\ s^{-1}$ and $k'_{DMPO} = 60\ dm^3\ mol^{-1}\ s^{-1}$ led to $k_{DEPMPO} = 7.8 \times 10^9\ dm^3\ mol^{-1}\ s^{-1}$ and $k'_{DEPMPO} = 90\ dm^3\ mol^{-1}\ s^{-1}$. The decay kinetic of the DEPMPO–OOH spin adduct, generated in phosphate buffer at pH 7, was monitored by EPR. The initial decay had both first- and second-order components but after 10 min, the decay was pure first order with a rate constant of $7.8 \times 10^{-4}\ s^{-1}$, ($t_{1/2} = 890$ s). Under the same conditions, the decay of the DMPO–OOH spin adduct was pure first order and had a rate constant of $1.4 \times 10^{-2}\ s^{-1}$ ($t_{1/2} = 60$ s).

Using the Langendorff method, reperfusion experiments were performed on three ischaemic isolated rat hearts,[7] DEPMPO (2.12 mmol dm^{-3}) being continuously infused for the entire reperfusion period. The DEPMPO–OOH spin adduct was easily detected in the early stage of the reoxygenation and its formation was suppressed when SOD was added to the perfusate.

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