β-Phosphorylated α-phenyl-*N*-*tert*-butylnitrone (PBN) analogues: a new series of spin traps for oxyl radicals

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The two β-phosphorylated nitrones, *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide **3** (PPN) and 1-diethoxyphosphoryl-1-methyl-*N*-[(1-oxidopyridin-1-ium-4-yl)methylidene]ethylamine *N*-oxide **4** (4-PyOPN), which are the phosphorylated analogues of α-phenyl-*N*-*tert*-butylnitrone (PBN) and α-(1-oxidopyridin-1-ium-4-yl)-*N*-*tert*-butylnitrone (4-PyOBN), respectively, have been prepared and their ability to trap oxygen-centred radicals has been investigated.

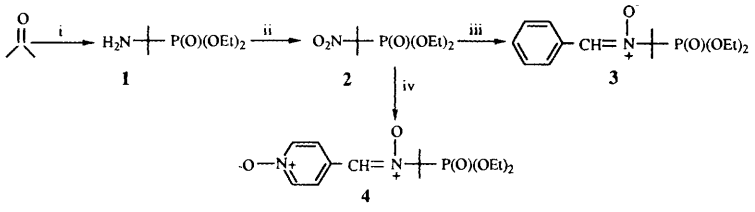
Oxygen-centred radicals are involved in many processes of chemical and biological interest[1–4] and their spin trapping by nitrones in organic, aqueous and biological media has been widely investigated. Nowadays, special attention is devoted to the spin trapping of superoxide–hydroperoxyl ($O_2^{\bullet-}$–HOO$^\bullet$) and hydroxyl (HO$^\bullet$) radicals in biological milieu.[5–8] For this purpose, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and α-phenyl-*N*-*tert*-butylnitrone (PBN) are the traps most widely employed, but their use is not without its limitations. The DMPO–superoxide spin adducts (DMPO–O_2H) decompose quickly in a polar environment and in most cases its EPR characterization in biological milieu is uncertain and tricky.[9,10] On the other hand, the strong hydrophilicity of DMPO limits its biological uses to extracellular spin trapping. PBN is much more lipophilic than DMPO,[11,12] but the hydroxyl radical spin adduct of PBN (PBN–OH) disappears in aqueous solution in a matter of seconds, while the superoxide spin adduct (PBN–O_2H) decays even more rapidly.[6,13–17] Furthermore, the EPR spectra of PBN–OH and PBN–O_2H are very similar, exhibiting very close hyperfine splitting constants (hfsc) and this may be a source of misinterpretation in spin trapping experiments. Various analogues of PBN bearing substituents on the phenyl ring have been proposed, but they all present more or less the same limitations as PBN.[15–20] Among these nitrones, the α-(1-oxidopyridin-1-ium-4-yl)-*N*-*tert*-butylnitrone (4-PyOBN) seems the most interesting with which to trap superoxide radicals generated by a xanthine–xanthine oxidase system.[7,15–17] However, the intensity of the corresponding EPR spectrum decreased during its recording, showing that even this spin adduct (4-PyOBN–O_2H) is not very persistent in aqueous media. Note that PBN and its analogues have been shown to be particularly efficient at trapping free radicals derived from lipids and resulting from oxidative stress.[21–23] On the other hand, PBN has been successfully used to protect different kinds of tissues against ischemia-reperfusion injury.[24,25]

We recently reported that the superoxide spin adduct obtained with 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) is much more persistent than DMPO–O_2H (*ca.* 15 times at pH 7 in phosphate buffer).[26–28] Moreover, for many spin adducts obtained with DEPMPO, additional information is obtained from the large phosphorus coupling observed. These observations prompted us to prepare PBN analogues bearing a β-dialkoxyphosphoryl substituent.[29] We now report preliminary results on the synthesis of *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide **3** (PPN) and 1-diethoxyphosphoryl-1-methyl-*N*-[(1-oxidopyridin-1-ium-4-yl)methylidene]ethylamine *N*-oxide **4** (4-PyOPN) and on their ability to trap oxygen-centred radicals in an aqueous environment.

Following the procedure of Petrov *et al.*,[30] amine **1** was prepared by reaction of acetone and diethyl phosphite under a flow of ammonia and was then oxidised with $KMnO_4$ to afford diethyl 1-methyl-1-nitroethyl phosphonate **2** (Scheme 1). Nitrone **3** was obtained in 30% yield by addition of acetic acid to a suspension of benzaldehyde, the phosphonate **2** and zinc in ethanol. Nitrone **4** was obtained in 27% yield by reduction of the phosphonate **2** with zinc and ammonium chloride in ethanol, filtration of the mixture and then addition of 4-formylpyridine *N*-oxide to the filtrate.

The hydroxyl radical spin adduct of **3** (PPN–OH) was obtained in 0.1 mol dm^{-3} phosphate buffer at pH 5.6, 7 and 8.2, either by a standard Fenton system (H_2O_2–$FeSO_4$) or by a superoxide dependent Fenton reaction consisting of a mixture of $FeNH_4(SO_4)_2$, hypoxanthine and xanthine oxidase.[27] In both cases, the resultant 12 line EPR spectrum showed the following hfscs determined by simulation of the experimental signal:[31] a_H = 2.7, a_N = 14.6 and a_P = 43.2 G, the total spectrum width being 75.1 G. This signal was inhibited by the presence of either catalase or, in the case of the superoxide dependent Fenton system, superoxide dismutase (SOD). When 10% methanol was added to the hydroxyl radical generating system in the presence of **3**, the corresponding $^\bullet CH_2OH$ radical spin adduct was observed instead of PPN–OH and the following hfscs were then determined: a_H = 3.4, a_N = 14.7 and a_P = 42.4 G.

Using the same hydroxyl radical generating systems in the



Scheme 1 Synthesis of **3** and **4**: i, NH_3, $HP(O)(OEt)_2$; ii, $KMnO_4$; iii, Zn, EtOH, AcOH, C_6H_5CHO; iv, Zn, EtOH, NH_4Cl, 4-formylpyridine *N*-oxide

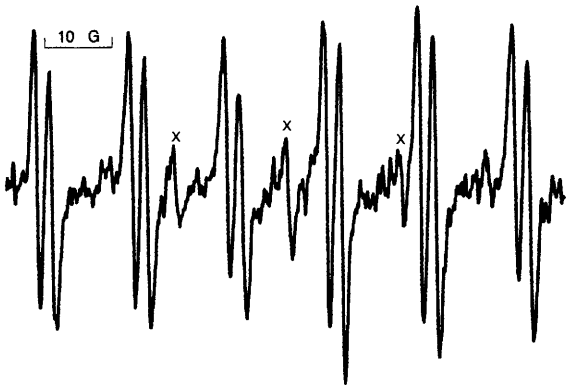


Fig. 1 EPR spectrum of the hydroperoxyl spin adduct of **3** (PPN–O_2H) recorded in a pH 7 phosphate buffer; the hfscs are a_H = 2.1, a_N = 13.5 and a_P = 41.32 G. The minor three line signal (×) corresponds to decomposition product of **3** (a_N = 16.1 G).

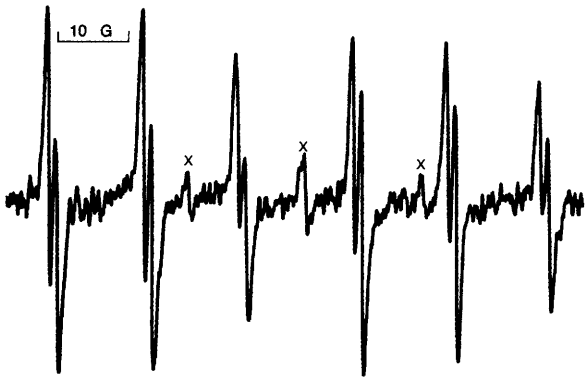
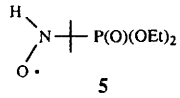


Fig. 2 EPR spectrum of the hydroperoxyl spin adduct of **4** (4-PyOPN–O_2H) recorded in phosphate buffer at pH 7; the hfscs are a_H = 1.2, a_N = 13.0 and a_P = 42.1 G. The minor three line signal (×) corresponds to a decomposition product of **3** (a_N = 16.32 G).

presence of **4**, another 12 line EPR spectrum was observed with a_H = 1.7, a_N = 13.8 and a_P = 42.8 G (total width: 72.1 G). Since this signal was inhibited by either catalase or, in the case of the superoxide dependent Fenton reaction, by SOD, it was assigned to the hydroxyl radical adduct of **4** (4-PyOPN–OH). When 10% methanol was added to the HO$^\bullet$ generating system in the presence of **4**, only the $^\bullet CH_2OH$ radical spin adduct of **4** was observed with a_H = 2.1, a_N = 14.1 and a_P = 44.1 G. In some cases, the adducts PPN–OH and 4-PyOPN–OH partially decomposed into the same paramagnetic species, exhibiting an EPR spectrum composed of a quartet (a_N = 13.3 and a_H = 13.1 G) split by a large phosphorus coupling (a_P = 52 G). This species was identified as the aminoxyl radical **5** formed either



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from PPN–OH or 4-PyOPN–OH after elimination of benzaldehyde or 4-formylpyridine *N*-oxide, respectively. The same behaviour for the PBN–OH has been already observed by Kotake and Janzen.[15]

By using a standard hypoxanthine–xanthine oxidase superoxide generating system[27] in phosphate buffer at acid or neutral pH in the presence of **3**, a rather intense 12 line EPR spectrum was observed with a_H = 2.1, a_N = 13.5 and a_P = 41.3 G (total width: 70.4 G). Since this signal was inhibited by SOD and transformed into the PPN–OH spectrum by the glutathione–glutathione peroxidase system,[27] it could be reasonably assigned to the hydroperoxyl radical spin adduct of **3** (PPN–O_2H, Fig. 1). When the same experiments were carried out with **4**, the persistent 12 line EPR signal of the hydroperoxyl radical spin adduct of **4** (4-PyOPPN–O_2H) was clearly identified (a_H = 1.2, a_N = 13 and a_P = 42.1 G, the total width being 69.3 G) (Fig. 2). We also noticed that at room temperature, **3** and **4** in pure water or in phosphate buffer can partially decompose, giving rise to a 3 line EPR spectrum of an unidentified aminoxyl radical (a_N = 16.1 G with **3** and a_N = 16.3 G with **4**). However, this paramagnetic species did not really limit the use of **3** or **4** in spin trapping experiments, since it did not interfere with the EPR signals of the spin adducts. When we tried the hypoxanthine–xanthine oxidase system in the presence of PBN, we always failed to record the EPR spectrum of PBN–O_2H, as this adduct decomposed too quickly in a polar environment even at acid pH. Under the same conditions, after several unsuccessful attempts, we were able, using 4-PyOBN as the spin trap, to detect a weak spectrum of 4-PyOBN–O_2H whose intensity decreased significantly during its recording. These results clearly show that for **3** and **4**, as it was reported for DEPMPO, the replacement of a methyl group by a diethoxyphosphoryl group increases significantly the half-lives of oxyl radical spin adducts compared with the half-lives of the same spin adducts formed with PBN, 4-PyOBN or DMPO.

With both **3** and **4**, the hfscs of hydroxyl or hydroperoxyl radical spin adducts were unaffected in the pH range 5.6–8.2, although the persistence of all these spin adducts was greater at acid pH. In contrast with PBN and 4-PyOBN, these two phosphorylated nitrones can be considered to be efficient spin traps for the hydroperoxyl radical in aqueous media. Furthermore, the existence of an additional hfsc with the phosphorus allows an easy distinction between hydroxyl and hydroperoxyl radical adducts: the spectrum of PPN–OH is more than 4 G larger than the PPN–O_2H one and the total width of 4-PyOPN–OH and of 4-PyOPN–O_2H differ by almost 3 G.

Compounds **3** and **4**, the first members of a new series of phosphorylated acyclic nitrones, seem then to be promising spin traps for oxyl radicals in a polar environment. Further experiments are now in progress to study the kinetics of the hydroxyl and hydroperoxyl radical spin adduct decays in various media and the results will be reported in due course.

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