PPN-type nitrones: preparation and use of a new series of β-phosphorylated spin-trapping agents †

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The synthesis of six members of a new series of β-phosphorylated nitrones derived from α-phenyl-*N*-*tert*-butylnitrone (PBN) ‡ is described. A new method, based on HPLC, is used to evaluate their *n*-octanol–phosphate buffer partition coefficient. As is the case for other PBN-type traps, the lipophilicity of these nitrones is found to be greatly dependent on the structure of their aromatic moiety. The capacity of these compounds to act as spin trapping agents is surveyed by using them to trap various free radicals in organic or aqueous media, and the EPR parameters of the different spin adducts obtained are reported herein.

The technique of spin-trapping of short-lived radical intermediates by nitrones has become a valuable tool in the study of radical processes occurring in chemical or biochemical environments. In particular, the use of nitrones in the *in vivo* detection of transient radical species became increasingly important with the observation that these paramagnetic, highly reactive compounds could be involved in several biological responses and in many human pathologies.[1] Of all the commercially available nitrone spin traps, PBN (α-phenyl-*N*-*tert*-butylnitrone ‡) and its derivatives are certainly the most often used for *in vivo* experiments, since they have been shown to give persistent spin adducts with many carbon-centred radicals even in polar environments.[2] In addition, in the PBN series, the lipophilicity of the traps varies as a function of the nature of the aromatic moiety.[3] Thus, the α-(1-oxidopyridin-1-ium-4-yl)-*N*-*tert*-butylnitrone (4-PyOBN) was found to be quite hydrophilic, with an octanol–water partition coefficient (K_p) of 0.15, while α-(4-dodecyloxyphenyl)-*N*-*tert*-butylnitrone (4-DOPBN) appeared to be strongly hydrophobic. As for PBN itself ($K_p = 10$), this trap is quite lipophilic, although it can be solubilised in water at *ca.* 0.1 mol dm^{-3}. This large variety of lipophilicity in the PBN-type spin traps should be seen as a major advantage for their *in vivo* applications, since in biological cells free radicals can be generated in either water or lipid environments. But one of the most important drawbacks of these nitrones is that the EPR spectra of the various aminoxyl§ spin adducts are not very characteristic of the radical trapped. For example, the difference in the EPR signal total width of methyl and α-hydroxyethyl radical spin adducts of PBN [PBN–CH_3 and PBN–$CH(OH)CH_3$, respectively] was found to be only 0.04 mT in an aqueous environment.[4] The similarity of the shape of the various EPR spectra of PBN-type spin adducts, which has been at the origin of serious errors in identifying the radical trapped,[5] must be regarded as a severe restriction to the *in vivo* use of these spin trapping agents.

Recently, we described three new β-phosphorylated PBN-type traps:[6] PPN **2** (*N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide), 4-ClPPN **3** [*N*-(4-chlorobenzylidene)-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide] and 4-PyOPN **4** {1-diethoxyphosphoryl-1-methyl-*N*-[(1-oxidopyridin-1-ium-4-yl)methylidene]ethylamine *N*-oxide}. These new traps were found to trap efficiently not only carbon-centred radicals but also superoxide, giving rise to rather persistent spin adducts even in polar media.[7] Furthermore, the existence of an additional hyperfine splitting constant (hfsc) with the phosphorus nucleus allowed easy identification of the radical trapped.[8] These compounds were the first three members of a new series of PPN-type traps, *i.e.* of β-phosphorylated PBN-type nitrones. However, the method we initially used to prepare them was rather tedious and the last step of the synthesis gave the nitrones in poor yields, never exceeding 30%.[6] Another very simple synthetic route was found which allowed us to obtain these compounds easily with a high grade of purity and in yields exceeding 90%.[8] In this paper, we describe the preparation of several PPN-type nitrones, using this improved synthesis. The lipophilicity of these nitrones has been determined by a new method based on HPLC. The capacity of these compounds to act as spin-trapping agents has also been surveyed by using them to trap various carbon-centred radicals in aqueous or organic media.

Results and discussion

Diethyl [1-(hydroxyamino)-1-methylethyl]phosphonate **1** was first prepared following the method of Petrov *et al.*,[9] and purified by recrystallization in pentane. Then, PPN **2** itself and its derivatives were prepared in a one-step reaction by condensing the corresponding aldehyde with the β-phosphorylated hydroxylamine **1**, as shown in Scheme 1. Following this procedure, the various PPN-type spin traps indicated in Scheme 1 were obtained in a high grade of purity, and in yields often exceeding 90%.

As mentioned above, one of the most important advantages of PBN-type nitrones is the great variety in the lipophilicity of these compounds. It was therefore of prime importance for us to verify that the same was true of the PPN series. Of the methods available to evaluate the lipophilicity of a spin trapping agent, one of the most often used is the determination of its *n*-octanol–water or *n*-octanol–phosphate buffer partition coefficient, *i.e.* K_p, by UV spectroscopy.[3,10] We have previously determined the value of K_p in this way for compounds **2** (PPN, $K_p = 10.1$), **3** (4-ClPPN, $K_p = 195$) and **4** (4-PyOPN, $K_p = 0.18$).[8] But the evaluation of the spin trap concentration in either the organic or aqueous phase by this method was not always very precise, particularly in the case of strongly hydrophobic or

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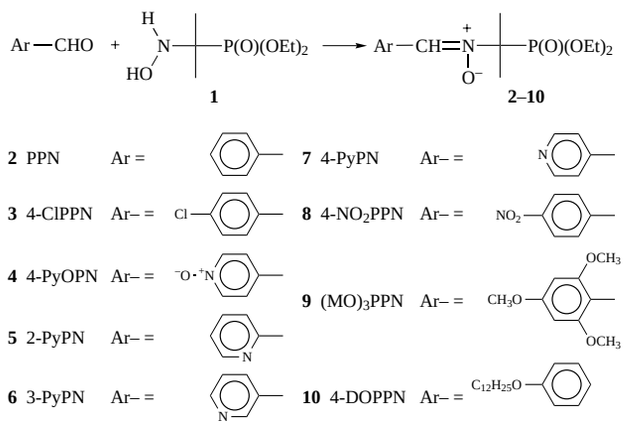
‡ IUPAC name: *N*-*tert*-butylbenzylideneamine *N*-oxide. The other nitrones can be named similarly.

§ Formerly known as nitroxide.

strongly hydrophilic compounds. We therefore felt that there was still a need for a convenient way to determine more precisely the spin-trap lipophilicity. We developed a new method to evaluate K_p by measuring the nitrone concentration, in either octanol or the aqueous phase, by HPLC and the results thus obtained for compounds **2**–**9** have been reported in Table 1. Note that differences can be observed in K_p values determined by either UV spectroscopy or HPLC. For example, in the case of the lipophilic 4-ClPPN, K_p was found to be 1.4 times higher by the HPLC method. Note that, generally, only the nitrone concentration in the aqueous phase is measured by UV spectroscopy, and this can be quite approximate in the case of lipophilic compounds. In fact, evaluation of the nitrone concentration in the octanol phase is almost impossible with this technique because of the presence of impurities in *n*-octanol which perturb UV measurements. However, the HPLC technique always permits the determination of the nitrone concentration in both phases. So we believe that the values indicated in Table 1 are more reliable than those previously determined by UV spectroscopy for nitrones **2**–**4**, and the new method described herein should be considered as a convenient way to evaluate K_p with reasonably good precision. However, it should be mentioned here that the HPLC technique did not permit evaluation of K_p for compound **10**, since the solvent mixture used was inappropriate to elute the strongly lipophilic 4-DOPPN.



Scheme 1 Synthesis of nitrones **2**–**10**

Table 1 Octanol–phosphate buffer partition coefficient of PPN-type nitrones determined by HPLC

Nitrone	K_p
PPN	10.2
4-PyOPN	0.21
4-ClPPN	273.3
2-PyPN	4.8
3-PyPN	2.6
4-PyPN	2.1
4-NO_2PPN	26.9
$(MO)_3$PPN	10.8

In order to appreciate the potential of our new nitrones in the detection of short-lived radicals, a series of free radicals was trapped by each one of these compounds, in organic or aqueous media. Since our main purpose here was to rapidly assess the capacity of the various nitrones to act as spin traps, only the trapping of a few oxygen- and carbon-centred radicals has been surveyed. In order to simplify the notation, the aminoxyl obtained by trapping a free radical $R^{\bullet}$ by a nitrone N will be noted N–R. For example, 2-PyPN–CH_3 represents the methyl-radical spin adduct of the nitrone 2-PyPN. All the EPR spectra thus recorded have been fully analysed and simulated using a computer program elaborated by Duling.[11] The EPR parameters thus obtained for the various spin adducts are reported in Tables 2–6. All the spectra recorded are easily analysed and consist of a triplet of doublets, due to hyperfine splitting constants (hfscs) with the nitrogen and the β-hydrogen, split by a large phosphorus coupling. As an example, the EPR spectrum of the spin adduct 4-NO_2PPN–CH_3 recorded in water is shown in Fig. 1. In the case of nitrones **2**–**4**, many spin trapping experiments have already been described in a previous paper[8] and are not discussed again here. For all the nitrones studied, the EPR parameters of the $H^{\bullet}$ radical adduct are reported in Tables 2–6. These aminoxyls were prepared by reduction by $NaBH_4$ of the corresponding nitrone followed by an autoxidation in water.

Except in the case of the strongly lipophilic 4-DOPPN, which will be discussed later, all the nitrones tested were found to trap every kind of carbon-centred radical. Whatever the

Table 2 EPR parameters of various spin adducts of 2-PyPN

Aminoxyl	Solvent	a_N/mT	a_H/mT	a_P/mT
2-PyPN–CH_3	Water	1.462	0.364	4.651
	DMSO–water (90:10)	1.398	0.288	4.658
2-PyPN–CH_2OH	Water	1.423	0.320	4.320
	Methanol–water (90:10)	1.399	0.298	4.027
2-PyPN–$CH(CH_3)OH$	Water	1.438	0.324	4.248
	Ethanol–water (90:10)	1.412	0.356	4.036
2-PyPN–$CO_2^-(H^+)$	Water	1.430	0.300	4.558
2-PyPN–C_6H_5	Benzene	1.371	0.249	4.107
2-PyPN–H	Water	1.510	1.025 (2H)	4.800
2-PyPN–OH	Water	1.405	0.228	4.160

Table 3 EPR parameters of various spin adducts of 3-PyPN

Aminoxyl	Solvent	a_N/mT	a_H/mT	a_P/mT
3-PyPN–CH_3	Water	1.469	0.243	4.673
	DMSO–water (90:10)	1.407	0.212	4.561
3-PyPN–CH_2OH	Water	1.429	0.216	4.480
	Methanol–water (90:10)	1.387	0.192	4.153
3-PyPN–$CH(CH_3)OH$	Water	1.441	0.116	4.510
	Ethanol–water (90:10)	1.401	0.235	4.041
3-PyPN–$CO_2^-(H^+)$	Water	1.428	0.324	4.985
3-PyPN–C_6H_5	Benzene	1.362	0.265	4.582
3-PyPN–H	Water	1.516	1.071 (2H)	4.829
3-PyPN–OH	Water	1.392	0.175	4.376

Table 4 EPR parameters of various spin adducts of 4-PyPN

Aminoxyl	Solvent	a_N/mT	a_H/mT	a_P/mT
4-PyPN–CH_3	Water	1.435	0.266	4.640
	DMSO–water (90:10)	1.395	0.242	4.588
4-PyPN–CH_2OH	Water	1.420	0.247	4.347
	Methanol–water (90:10)	1.401	0.235	4.141
4-PyPN–$CH(CH_3)OH$	Water	1.427	0.244	4.246
	Ethanol–water (90:10)	1.412	0.228	3.993
4-PyPN–$CO_2^-(H^+)$	Water	1.432	0.243	4.310
4-PyPN–C_6H_5	Benzene	1.352	0.244	4.477
4-PyPN–H	Water	1.492	0.991 (2H)	4.800
4-PyPN–OH	Water	1.418	0.217	4.328

Table 5 EPR parameters of various spin adducts of 4-NO_2PPN

Aminoxyl	Solvent	a_N/mT	a_H/mT	a_P/mT
4-NO_2PPN–CH_3	Water	1.455	0.307	4.633
	DMSO–water (90:10)	1.370	0.274	4.562
4-NO_2PPN–CH_2OH	Water	1.435	0.287	4.305
	Methanol–water (90:10)	1.403	0.260	4.049
4-NO_2PPN–$CH(CH_3)OH$	Water	1.449	0.264	4.279
	Ethanol–water (90:10)	1.402	0.261	3.914
4-NO_2PPN–$CO_2^-(H^+)$	Water	1.414	0.350	4.919
4-NO_2PPN–C_6H_5	Benzene	1.352	0.273	4.546
4-NO_2PPN–H	Water	1.507	1.039 (2H)	4.794
4-NO_2PPN–OH	Water	1.440	0.228	4.742

Table 6 EPR parameters of various spin adducts of $(MO)_3$PPN

Aminoxyl	Solvent	a_N/mT	a_H/mT	a_P/mT
$(MO)_3$PPN–CH_3	Water	1.569	1.337	4.687
	DMSO–water (90:10)	1.485	1.246	4.832
$(MO)_3$PPN–CH_2OH	Methanol–water (90:10)	1.458	0.487	4.748
$(MO)_3$PPN–$CH(CH_3)OH$	Water	1.535	0.924	4.124
	Ethanol–water (90:10)	1.490	0.350	4.170
$(MO)_3$PPN–$CO_2^-(H^+)$	Water	1.494	0.529	4.124
$(MO)_3$PPN–H	Water	1.505	1.621 (2H)	4.921

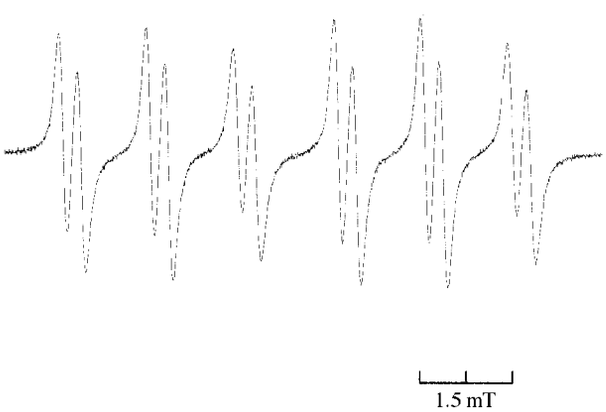


Fig. 1 EPR spectrum of the methyl adduct of 4-NO_2PPN nitrone (4-NO_2PPN–CH_3 $a_N = 1.455$, $a_H = 0.307$ and $a_P = 4.633$ mT)

nature of the medium, we always observed rather intense and persistent spin adducts, which could be measured over a few minutes in the case of $^{\bullet}OH$ spin adducts in water and a few hours in the other cases. In addition, when compared to their non-phosphorylated analogues, the various spin adducts showed EPR spectra very characteristic of the radical trapped. This is directly due to the presence of a strong extra coupling of the unpaired electron with the phosphorus nucleus and should be regarded as an important advantage of our new traps over other PBN-type nitrones. Such an important hyperfine coupling with a phosphorus in the β-position with respect to the nitrogen is not surprising and the same kind of phenomenon has already been observed, for example, in the case of cyclic[12] or linear[13] β-phosphorylated stable aminoxyls. It also appeared that slight modifications in the aromatic moiety of the trap could induce strong modifications in the EPR parameters of the spin adducts. Thus, the EPR spectrum total width of the adducts 3-PyPN–CO_2^- and 4-PyPN–CO_2^- differed by *ca.* 0.75 mT.

The lipophilic compounds 4-NO_2PPN **8** and $(MO)_3$PPN **9** were poorly soluble in water, and saturated solutions of these nitrones have been used to trap free radicals in aqueous media. Even under these unfavourable conditions, persistent spin adducts have been observed by trapping carbon-centred radicals with **8**, but the nitrone **9** seems to be a particular case, and should be discussed. Whatever the structure of the radical trapped, note that in the case of $(MO)_3$PPN **9**, the hfsc with the hydrogen was always found to be much higher than those with other PPN-type nitrones. Thus, for $(MO)_3$PPN–CH_3 in water, for example, a_H can reach 1.337 mT, although this coupling constant was always found to be lower than 0.4 mT for the methyl radical spin adducts of the other nitrones in the same environment. The presence of two methoxy groups in the 2- and 6-positions on the aromatic rings certainly induces steric hindrance which results in a particular conformation of the various spin adducts of $(MO)_3$PPN. The same kind of phenomenon has previously been observed by Janzen *et al.*[14] for the various spin adducts of $(MO)_3$PBN, the non-phosphorylated analogue of compound **9**; in this case, the β-H hfsc values are in general larger than in the equivalent structure derived from PBN. Nitrone **9** did not trap the phenyl radical in benzene, and this may be because of the presence of the methoxy groups which hindered the radical approach. More surprisingly, when a Fen-

ton reaction was conducted in the presence of 20% MeOH and of $(MO)_3PPN$, the hydroxymethyl radical spin adduct of the nitrone was never detected, but we observed the appearance of an aminoxyl EPR signal consisting of 18 lines of equal intensities. More precisely, its coupling pattern corresponded to a triplet of triplets, split by a large phosphorus coupling. A computer simulation of this signal led to $a_N = 1.40$, $a_H = 0.62$ (1H), $a_H = 0.58$ (1H), $a_P = 4.621$ mT. This aminoxyl has not been clearly identified yet, and further experiments are now in progress in our laboratory in order to determine its structure and to fully elucidate the mechanism of its formation.

It should be mentioned here that the different nitrones tested did not very efficiently trap oxygen-centred radicals in an aqueous environment. Thus, when we tried to trap superoxide, generated by either a standard hypoxanthine–xanthine oxidase system[15] or a standard light–riboflavin–electron donor system,[16] in phosphate buffer with each one of the nitrones studied, the EPR signals observed were generally much too weak to be correctly analysed. The performance of the nitrones **5**–**9** in the spin trapping of superoxide appeared less interesting than those of both the hydrophilic 4-PyOPN **4** and the lipophilic PPN **2**, which have been shown to trap superoxide efficiently even in a polar environment.[6–8] On the other hand, by generating hydroxyl radicals in phosphate buffer by a standard system in the presence of 4-NO_2PPN, only a weak signal, corresponding to the adduct 4-NO_2PPN–OH, was observed. Using nitrones **5**–**7** under the same conditions, the EPR spectra of the corresponding hydroxyl adducts were slightly more intense. But whatever the nitrone was, the same second paramagnetic species ($a_N = 1.33$, $a_H = 1.31$ and $a_P = 5.20$ mT) was always detected in the aqueous media. As previously mentioned,[6] this aminoxyl has been identified as compound **11** and has been shown to result from the decomposition of the various hydroxyl spin adducts in the water environment. It should also be mentioned here that when $(MO)_3PPN$ was employed to trap hydroxyl radical in water media, only the EPR signal of the decomposition product **11** was detected.

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Lastly, we would like to discuss the case of the nitrone 4-DOPPN. This nitrone was first synthesised because we thought that it could be interesting to study a strongly lipophilic trap. But 4-DOPPN was too poorly water soluble and not useful to trap free radicals in an aqueous environment. In addition, when 4-DOPPN was used to trap oxygen- or carbon-centred free radicals in organic media, the EPR signals observed always showed very broad lines. This strong line broadening, which remained unchanged when oxygen was carefully removed from the medium by argon bubbling, could be due to the existence of a great number of conformations of the aminoxyls considered, corresponding to different positions of the long alkoxyl chain on the phenyl ring. The line width was always larger than the β-H hfsc, and the various spin adducts' spectra were too difficult to analyse to permit a correct determination of the various EPR parameters. In fact, rather precise hfsc values have been determined only in the case of the phenyl radical spin adduct of 4-DOPPN recorded in benzene (4-DOPPN–C_6H_5, $a_N = 1.375$, $a_H = 0.295$ and $a_P = 4.595$ mT). For all the other spin adducts of 4-DOPPN, the error in the determination of these hfscs was found to be very high so they are not included here. In conclusion, it appeared that the nitrone 4-DOPPN offered only little in the way of advantages for spin trapping in any medium investigated.

Conclusion

The synthesis pathway described in this paper permitted β-phosphorylated PBN-type nitrones to be prepared in a one-step reaction by condensing an aromatic aldehyde with the hydroxylamine **1**, the preparation of which has been previously described by Petrov *et al.*[9] This method has been employed successfully to prepare PPN itself and eight other compounds of the same series, but many other phosphorylated nitrones could be synthesised by the same technique, providing that the corresponding aldehyde is available.

The *n*-octanol–phosphate buffer partition coefficient of nitrones **2**–**9** has been evaluated by a new method, based on HPLC, with good precision, even in the case of strongly hydrophobic compounds. In the PPN series, the lipophilicity of the various traps tested was found to be greatly dependent on the structure of the aromatic moiety. Thus, lipophilic nitrones such as 4-NO_2PPN could be useful tools in organic solvents or in a lipid environment, while 4-PyOPN is preferred for studies in aqueous media.

We found that the strongly lipophilic nitrone 4-DOPPN was not a useful spin trapping agent, since the EPR signal of its various spin adducts, when observed, always showed very broad lines and were almost impossible to analyse. But, except for 4-DOPPN, all the nitrones studied were found to trap very efficiently carbon-centred free radicals in every kind of medium, yielding persistent spin adducts showing intense EPR spectra. However, the lipophilic 4-NO_2PPN and $(MO)_3PPN$ were found to be too poorly water-soluble to trap hydroxyl radicals in aqueous milieu. In addition, it should be mentioned that nitrones **5**–**9** are not usable to detect superoxide in water environment. In contrast, in a previous study, we found that the rather lipophilic PPN **2** and the hydrophilic 4-PyOPN **4** trapped very efficiently superoxide even in polar media, and it appeared then that these two nitrones are still the best spin trapping agents in the PPN series for the detection of superoxide in aqueous environment. One of the most important advantages of the new series of spin traps described herein, when compared to their non-phosphorylated analogues, is that their various spin adducts are easily identified by their EPR spectra, because of the existence of a strong hyperfine coupling with the phosphorus nucleus. Thus, we have given in this paper the EPR parameters of a few spin adducts of these nitrones, hoping that nitrones **2**–**9** will be useful tools for chemists and biologists involved in spin trapping experiments. Other spin trapping experiments of a wide range of carbon-, oxygen-, sulfur-, nitrogen- and phosphorus-centred radicals are now in progress with the nitrones showing the most interesting performances, *i.e.* compounds **2**–**7**.

Experimental

All chemicals and solvents used were purchased from either Sigma or Aldrich Chemical Companies. The solvents were of the highest grade of purity and twice-distilled before use.

Synthesis of compounds 2–10

The hydroxylamine **1** was synthesised using the method of Petrov *et al.*,[9] and recrystallized in pentane. Its identification was achieved by ^1H, ^{13}C and ^{31}P NMR spectroscopy, as described previously.[7] Nitrones **2**–**10** were prepared by heating an ethanolic solution of the corresponding aldehyde (10 mmol dm^{-3}) at 55 °C for 3 h in the presence of the hydroxylamine **1** (10 mmol dm^{-3}), as shown in Scheme 1. The various nitrones were then usually obtained in a high grade of purity. However, in the case of compounds **5**–**7**, purification was achieved by washing an aqueous solution of the nitrones with diethyl ether; the nitrones were thus extracted from the remaining aqueous layer with dichloromethane, and were obtained as viscous oils.

For compounds **8**–**10**, purification was achieved by recrystallization in either diethyl ether or pentane.

All the nitrones obtained have been identified on the basis of their ^{1}H, ^{13}C and ^{31}P NMR spectra, recorded on Bruker AC 100, 200 and Bruker AM 400X spectrometers. The chemical shifts (δ) in ppm were referred to internal TMS for ^{1}H and ^{13}C NMR, and to external 85% H_3PO_4 for ^{31}P NMR. *J* values are given in Hz. The characteristics of the NMR spectra of compounds **2**–**4** have been previously described[7] and are not given here. In the case of compounds **5**–**10**, the following NMR parameters have been obtained.

***N*-[(Pyridinium-2-yl)methylidene]-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide 2-PyPN 5.** δ_H($CDCl_3$, 200 MHz) 9.17 (1H, d, $J_{2,3}$ 8.1), 8.65 (1H, dd, $J_{5,4}$ 4.7, $J_{5,3}$ 1.5), 8.00 (1H, d, J_P 2.5), 7.78 (1H, dt, $J_{3,2}$ 8.1, $J_{3,4}$ 8.0, $J_{3,5}$ 1.5), 7.30 (1H, dd, $J_{4,3}$ 8.0, $J_{4,5}$ 4.7), 4.27 (4H, dq, J_H 7.0, J_P 7.3), 1.85 (6H, d, J_P 14.9), 1.31 (6H, t, J_H 7.0); δ_C($CDCl_3$, 50.32 MHz) 149.4 (s), 149.2 (s), 136.8 (s), 133.9 (d, J_P 5.0), 124.2 (s), 123.8 (s), 73.8 (d, J_P 155.5), 63.2 (d, J_P 7.1), 23.1 (s), 16.2 (d, J_P 5.7); δ_P($CDCl_3$, 40.5 MHz) 21.40.

***N*-[(Pyridinium-3-yl)methylidene]-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide 3-PyPN 6.** δ_H($CDCl_3$, 200 MHz) 9.04 (1H, s), 9.02 (1H, d, $J_{2,3}$ 8.8), 8.59 (1H, d, $J_{4,3}$ 4.4), 7.81 (1H, d, J_P 2.7), 7.37 (1H, dd, $J_{3,2}$ 8.8, $J_{3,4}$ 4.4), 4.21 (4H, dq, J_H J_P 7.0), 1.85 (6H, d, J_P 14.8), 1.34 (6H, t, J_H 7.0); δ_C($CDCl_3$, 50.32 MHz) 150.0 (s), 149.8 (s), 134.9 (s), 130.1 (d, J_P 5.6), 127.1 (s), 123.3 (s), 73.2 (d, J_P 155.5), 63.2 (d, J_P 6.7), 23.0 (s), 16.2 (d, J_P 5.8); δ_P($CDCl_3$, 40.5 MHz) 22.00.

***N*-[(Pyridinium-4-yl)methylidene]-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide 4-PyPN 7.** δ_H($CDCl_3$, 200 MHz) 8.64 (2H, d, J_H 6.0), 8.02 (2H, d, J_H 6.0), 7.77 (1H, d, J_P 2.6), 4.16 (4H, dq, J_H J_P 7.2), 1.80 (6H, d, J_P 14.7), 1.30 (6H, t, J_H 7.2); δ_C($CDCl_3$, 50.32 MHz) 137.1 (s), 131.4 (d, J_P 5.8), 121.8 (s), 74.1 (d, J_P 153.2), 63.6 (d, J_P 7.3), 23.2 (s), 16.4 (d, J_P 6.0); δ_P($CDCl_3$, 40.5 MHz) 21.00.

***N*-(4-Nitrobenzylidene)-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide 4-NO_2PPN 8.** δ_H($CDCl_3$, 400 MHz) 8.39 (2H, d, J_H 9.0), 8.21 (2H, d, J_H 9.0), 7.88 (1H, d, J_P 2.7), 4.15 (4H, dq, J_H 7.0, J_P 7.6), 1.82 (6H, d, J_P 14.7), 1.30 (6H, dt, J_H 7.0, J_P 3.3); δ_C($CDCl_3$, 100.6 MHz) 147.8 (s), 136.3 (s), 131.5 (d, J_P 4.0), 129.3 (s), 123.8 (s), 74.1 (d, J_P 152.9), 63.7 (d, J_P 6.4), 23.3 (s), 16.5 (d, J_P 5.3); δ_P($CDCl_3$, 40.5 MHz) 21.50.

***N*-(2,4,6-Trimethoxybenzylidene)-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide $(MO)_3$PPN 9.** δ_H($CDCl_3$, 100 MHz) 7.68 (1H, d, J_P 3.0), 6.11 (2H), 4.25 (4H, dq, J_H 7.0, J_P 6.9), 3.80 (9H), 1.83 (H, t, J_P 14.8), 1.34 (6H, t, J_H 7.0); δ_C($CDCl_3$, 50.32 MHz) 187.6 (s), 162.7 (s), 159.6 (s), 128.2 (d, J_P 5.3), 101.7 (s), 90.8 (s), 90.3 (s), 72.0 (d, J_P 154.0), 63.1 (d, J_P 7.1), 55.8 (s), 55.4 (s), 23.6 (s), 16.4 (d, J_P 5.8); δ_P(40.5 MHz, $CDCl_3$) 22.11.

***N*-[(4-Dodecyloxyphenyl)methylidene]-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide 4-DOPPN 10.** δ_H($CDCl_3$, 200 MHz) 8.26 (2H, d, *J* 8.8), 7.66 (1H, d, J_P 2.5), 6.91 (2H, d, *J* 8.9), 4.19 (4H, dq, J_H 7.3, J_P 7.1), 3.98 (2H, t, *J* 6.4), 1.81 (6H, d, J_P 14.9), 1.75 (2H, m), 1.34 (24H, m), 0.87 (3H, t, *J* 6.8); δ_C($CDCl_3$, 50.32 MHz) 160.7 (s), 132.9 (d, J_P 4.4), 130.9 (s), 123.5 (s), 114.2 (s), 70.8 (d, J_P 279), 63.3 (d, J_P 7.2), 31.9 (s, CH_3), 29.4 (m), 26.0 (s), 23.3 (s), 22.7 (s), 16.4 (d, J_P 5.8), 14.1 (s); δ_P($CDCl_3$, 40.5 MHz) 22.55.

K_p Determination

The lipophilicity of nitrones **2**–**10** was evaluated from their *n*-octanol–phosphate buffer (0.1 mol dm^{-3}, pH 7) phosphate buffer partition coefficient (K_p) as follows. Solutions of nitrones were prepared in *n*-octanol at a concentration of 0.25 mmol dm^{-3}. Equal volumes of freshly prepared octanolic solution of nitrone and of 10 mmol dm^{-3} phosphate buffer at pH 7.4 were vigorously mixed at 37 °C for 1 h and the two phases were separated by a brief centrifugation (1000*g* for 20 s). The nitrone concentration in either octanolic or aqueous solution was determined by HPLC, by using a Waters model 600E multisolvent delivery system, equipped with a Waters UV detector, a Spectra Physics SP4600 integrator and a Kromasil 5 μm C18 column (25 cm length, 4.6 mm id). HPLC column conditions were as follows: flow rate, 1 cm^3 min^{-1}; injection volume, 20 μl; isocratic elution solvent (68% methanol, 0.5% triethylamine, 31.5% water). A 3×10^{-2} mmol dm^{-3} acetophenone solution was used as internal reference. For each one of the nitrones tested, K_p was evaluated as the ratio of the nitrone concentration in *n*-octanol to that in phosphate buffer.

Reduction of nitrones by $NaBH_4$

An aqueous solution containing 0.05 mol dm^{-3} nitrone and saturated with $NaBH_4$ was prepared. Autoxidation of the hydroxylamine obtained led to the corresponding aminoxyl.

Spin trapping

In all spin trapping experiments, the concentration of the nitrones was 0.05 mol dm^{-3}, except for the strongly hydrophobic traps used in aqueous media, for which we prepared saturated solutions. In aqueous media, $^{\bullet}CH_3$, $^{\bullet}CH_2OH$, $^{\bullet}CH(CH_3)OH$ and $^{\bullet}CO_2^-$ radicals were generated in the presence of the nitrone studied by a standard Fenton system [0.2% H_2O_2, 2 mmol dm^{-3} ethylenediaminetetraacetic acid (EDTA) and 1 mmol dm^{-3} $FeSO_4$] in the presence of dimethyl sulfoxide (DMSO, 10%), methanol (20%), ethanol (10%) or sodium formate (0.2 mol dm^{-3}), respectively, to yield the corresponding spin adducts. The same aminoxyls have also been obtained in organic media using the same Fenton system in DMSO, methanol or ethanol. The phenyl radical spin adducts have been generated in benzene by photolysis of a solution of C_6H_4I (1.5 mol dm^{-3}) in the presence of the nitrone studied. The $HO^{\bullet}$ spin adducts of the various nitrones have also been obtained in water by using either a Fenton system or by photolysis of 3% aqueous H_2O_2. Following the method described by Rosen *et al.*,[17] the same nitroxides have also been obtained by adding acetic acid (7%) to an aqueous solution containing 15% H_2O_2 and the appropriate nitrone. Since the same EPR signal was recorded with the two methods, it has been attributed to the nitrone/$HO^{\bullet}$ spin adduct.

EPR measurement

EPR assays were carried out at 20 °C in EPR tubes by using a computer-controlled Bruker EMX spectrometer operating at X-band with 100 kHz modulation frequency, and equipped with an NMR gaussmeter for magnetic field calibration. The instrument settings were as follows: non-saturating microwave power, 10 mW; modulation amplitude ranging from 0.06 to 0.1 mT; scan time, 180 s; time constant, 0.128 s; receiver gain ranging from 1.2×10^4 to 6.3×10^4. For the various spin adducts, the hfsc values were determined by EPR signal simulations using the computer program elaborated by Duling.[11]

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