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Preparation and use as spin trapping agents of new ester-nitrones

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The synthesis of two new nitrones, *N*-benzylidene-1,1-bis(ethoxycarbonyl)ethylamine *N*-oxide (DEEPN) and *N*-[(1-oxidopyridin-1-ium-4-yl)methylidene]-1-ethoxycarbonyl-1-methylethylamine *N*-oxide (EPPyON), is described. Measurement of their *n*-octanol–phosphate buffer partition coefficient permitted evaluation of their lipophilicity. Their capacity to act as spin trapping agents was investigated in aqueous media. Although these nitrones were unsuitable for detecting hydroxyl radical, they efficiently trapped various carbon- and oxygen-centred radicals, including superoxide, in aqueous media. The half-lives of their superoxide adducts were determined at pH 5.8 and 7.2. At neutral pH, the superoxide spin adduct of DEEPN was found to be as persistent as that of 5-diethoxyphosphoryl-5-methyl-3,4-dihydropyrrole *N*-oxide (DEPMPO). Consequently, DEEPN was believed to be an efficient trap for superoxide detection in aqueous media.

Introduction

Reactive oxygen species, and in particular oxygen-centred free radicals, have been implicated in a number of disease states arising from oxidative damage in cells, such as ischaemia/reperfusion injuries, diabetes, ageing, cancers, arthritis, and amyotrophic lateral sclerosis.[1–9] Consequently, the use of nitrones, in conjunction with EPR spectroscopy, is of growing importance in detecting free radicals in various biological media.[10–20] Among all the requirements for successful *in vivo* spin trapping of oxygen-centred radicals, the stability of the spin adducts has often been the key limitation. In this field, the synthesis of 5-diethoxyphosphoryl-5-methyl-3,4-dihydropyrrole *N*-oxide (DEPMPO), which was first published in 1994,[21] has been a major step forward. The various free radical adducts of DEPMPO are easily identified by EPR spectroscopy and its superoxide spin adduct was found to be *ca.* fifteen times more stable than that of 5,5-dimethyl-3,4-dihydropyrrole *N*-oxide (DMPO), its non-phosphorylated analogue, at pH 7.[22–25] However, DEPMPO also presents several drawbacks. It is rather expensive, highly hydrophilic, and not easily prepared at high purity. In addition, the EPR spectra of some of its adducts can be complicated by long range couplings, by the presence of two diastereoisomers or by conformational exchanges. A series of β-phosphorylated analogues of *N*-*tert*-butylbenzylideneamine *N*-oxide (PBN), the first members of which were *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PPN) and *N*-[(1-oxidopyridin-1-ium-4-yl)methylidene]-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PyOPN), has also been prepared.[26–29] These nitrones show various lipophilicities, and trap efficiently not only carbon-centred radicals but also superoxide, giving rise to rather persistent spin adducts. However, their superoxide adducts decompose more rapidly than that of DEPMPO. Thus, it appears clear that the spin trapping detection of free radicals in biological media requires the development of more nitrones with highly persistent spin adducts.

Recently, it has been shown that the presence of an electron withdrawing ester group in the β-position to the nitrone function could also stabilise the superoxide adducts. In the cyclic nitrone series, 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (EMPO)[30,31] and 2-*tert*-butoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (BocMPO)[32,33] were found to generate much more persistent superoxide spin adducts than DMPO, with EPR spectra simpler than those of DEPMPO. In the linear nitrone series, we found that *N*-benzylidene-1-ethoxycarbonyl-1-methylethylamine *N*-oxide (EPPN) allowed efficient detection of superoxide in an aqueous environment, leading to a superoxide adduct almost as persistent as that of PyOPN, and showing twice less lines.[34] These observations prompted us to prepare new ester-nitrones in order to increase the spin trapping capacities obtained with EPPN. We now report on the synthesis of *N*-benzylidene-1,1-bis(ethoxycarbonyl)ethylamine *N*-oxide (DEEPN) and of *N*-[(1-oxidopyridin-1-ium-4-yl)methylidene]-1-ethoxycarbonyl-1-methylethylamine *N*-oxide (EPPyON), and on their ability to trap free radicals, with particular attention to superoxide.

Results and discussion

The nitrones DEEPN and EPPyON were obtained from *Z*-aldoximes **1** and **2**,[35,36] respectively, in a one-pot synthesis (Scheme 2). The aldoximate anions were first formed by adding metallic sodium to an aldoxime solution in absolute ethanol. Then, they were reacted with bromo-esters **3** and **4**, leading to DEEPN and EPPyON, respectively. EPPyON synthesis was achieved at room temperature, while DEEPN was prepared at −63 °C in order to avoid *O*-alkylation reaction. With this simple synthetic route, DEEPN and EPPyON were obtained as crystals in good yields and in high purity.

In biological systems, the success of spin trapping experiments greatly depends on the local concentration of the nitrone in the immediate vicinity of the free radical genesis. Thus, depending on the polarity of the medium where free radicals are produced, the use of a hydrophilic nitrone could in some cases give the best results, while in other cases a hydrophobic trap should be preferred. Therefore, it would be very useful to have at one's disposal a large variety of spin traps showing varying lipophilicity to perform a given spin trapping experiment. Of the methods available to evaluate the lipophilicity of a compound, one of the most often used is the determination of its *n*-octanol–water or *n*-octanol–phosphate buffer partition coefficient K_P. Following a technique based on HPLC,[29,37] partition coefficients (K_P) in *n*-octanol–phosphate buffer (0.1 mol dm^{-3}, pH 7) were evaluated for DEEPN and EPPyON. The results are reported in Table 1, along with K_P values of other nitrones.[38,39] As in the case of *N*-[(1-oxidopyridin-1-ium-4-yl)-methylidene]-1,1-isopropylamine *N*-oxide (PyOBN) and of PyOPN, the EPPyON partition coefficient K_P was found to be

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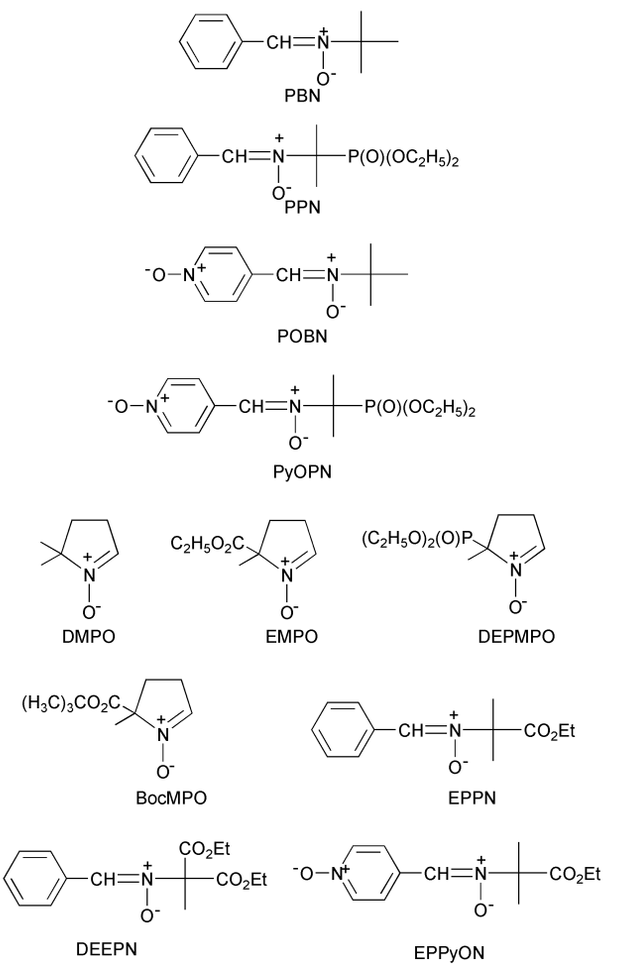


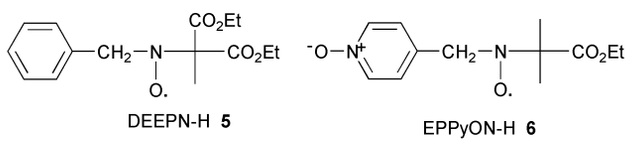
Scheme 1 Formulae of various nitrone spin traps.

Table 1 Partition coefficients K_P of nitrone spin traps in *n*-octanol–phosphate buffer

Nitrone	K_P	Reference
DEEPN	4.8	This work
EPPyON	0.33	This work
EPPN	29.8	34
PPN	10.2	29
PyOPN	0.21	29
PBN	10.4	38
	15	39
PyOBN	0.09	38
	0.15	39
DMPO	0.08	38
	0.1	39
DEPMPO	0.16	This work
EMPO	0.15	This work

rather low. This high hydrophilicity is the result of the replacement of a phenyl by a much more polar oxidopyridiniumyl group. The value determined for DEEPN ($K_P = 4.8$) indicates a preference for a lipid phase over a water environment, although this nitrone was found to be less lipophilic than PBN, PPN and EPPN. Consequently, DEEPN could eventually be able to penetrate biomembranes, while EPPyON should be expected to remain outside biological cells.

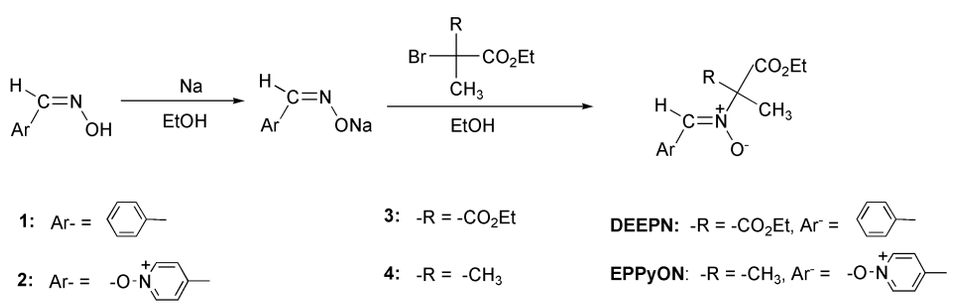
When in the presence of $NaBH_4$, nitrones undergo a chemical reduction resulting in the formation of the corresponding hydroxylamines.[29,34] These EPR silent compounds can be immediately autoxidised into the corresponding aminoxyl radicals. Incubating DEEPN and EPPyON in an oxygenated $NaBH_4$ solution in water resulted in the formation of aminoxyls **5** and **6**, respectively, denoted by DEEPN–H and EPPyON–H. Their EPR parameters are reported in Table 2. Note that the same aminoxyl radicals could be formed by trapping $^{\bullet}H$ in water, and both DEEPN–H and EPPyON–H could be considered as "pseudo-hydrogen radical spin adducts".



The capacities of our new compounds to detect short-lived radicals were then studied in buffered solutions (pH 7.2). Throughout this text, the aminoxyl formed by trapping the radical R by a nitrone N will be denoted by N–R. Whatever the spin adduct considered, the EPR spectrum recorded always consisted of a triplet of doublets, due to hyperfine couplings of the unpaired electron with nitrogen (a_N) and β-hydrogen (a_H) nuclei. They all have been simulated by the program elaborated by Rockenbauer and Korecz[40] and the hyperfine coupling constants (hfcc's) determined are reported in Table 2. The various spectra recorded were always very simple and easily analysed, although they were rather characteristic of the species trapped. For each nitrone, a_H was found to be lower for oxygen- than for carbon-centred radical adducts. Note however that much larger a_H variations are usually observed in the EPR spectra of the nitrone spin adducts in the DMPO series. Thus, the identification of the radical trapped could be less easy with our new compounds than with most cyclic nitrones.

Like many other nitrones, DEEPN and EPPyON were found to trap efficiently carbon-centred radicals ($^{\bullet}CH_3$, $^{\bullet}CH_2OH$, $^{\bullet}CO_2^-$), yielding spin adducts which lasted several hours and did not decompose into paramagnetic impurities. They also gave persistent spin adducts with $^{\bullet}OCH_3$ and $^{\bullet}SO_3H$ radicals.

Using either DEEPN or EPPyON, and as observed previously with EPPN,[34] attempts to trap hydroxyl radical in aqueous environments were never successful. Regardless of the $^{\bullet}OH$ generator, we generally obtained weak signals, which most probably corresponded to carbon-centred radical adducts, denoted by DEEPN–C and EPPyON–C. We made the assumption that $^{\bullet}OH$ could abstract $^{\bullet}H$ from the ethoxy function of the nitrones, as previously observed with EPPN,[34] thereby yielding a carbon-centred radical which was consequently trapped by the nitrones. In some cases, an additional EPR signal was also

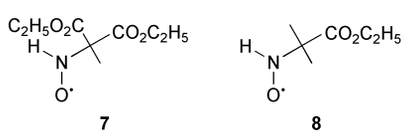


Scheme 2 Synthesis of DEEPN and of EPPyON.

Table 2 EPR hyperfine coupling constants for spin adducts of nitrones DEEPN and EPPyON in aqueous media (tridistilled water or 0.1 mol dm^{-3} phosphate buffer, pH 7.2)

Nitrone	Spin adduct	Source	a_N/mT	a_H/mT
DEEPN	DEEPN–H	$NaBH_4$ reduction, autoxidation	1.58	1.05 (2H)
	DEEPN–CH_3	Fenton system + DMSO	1.49	0.40
	DEEPN–CH_2OH	Fenton system + methanol	1.34	0.36
	DEEPN–CO_2^-	Fenton system + HCO_2Na	1.46	0.36
	DEEPN–SO_3^-	Fenton system + Na_2SO_3	1.38	0.19
	DEEPN–OCH_3	Methanol, K_2SO_8, heating	1.38	0.27
	DEEPN–O_2H	Xanthine, xanthine oxidase	1.38	0.24
	DEEPN–C	Fenton system or H_2O_2 *hv*	1.47	0.32
EPPyON	EPPyON–H	$NaBH_4$ reduction, autoxidation	1.57	1.01 (2H)
	EPPyON–CH_3	Fenton system + DMSO	1.52	0.27
	EPPyON–CH_2OH	Fenton system + methanol	1.48	0.26
	EPPyON–CO_2^-	Fenton system + HCO_2Na	1.49	0.30
	EPPyON–SO_3^-	Fenton system + Na_2SO_3	1.40	0.14
	EPPyON–OCH_3	Methanol, K_2SO_8, heating	1.33	0.17
	EPPyON–O_2H	Xanthine, xanthine oxidase	1.35	0.15
	EPPyON–C	Fenton system or H_2O_2 *hv*	1.48	0.28

observed in these experiments. According to previous results on the decay mechanisms of $^{\bullet}OH$ adducts of PBN- and PPN-type nitrones,[37,41–44] these spectra have been attributed to aminoxyls **7** (a_N = 1.26 mT, a_H = 1.33 mT) and **8** (a_N = 1.35 mT, a_H = 1.34 mT), formed after the decomposition of DEEPN–OH and EPPyON–OH, respectively. Attempts to obtain DEEPN–OH and EPPyON–OH by nucleophilic addition of water to the nitrones, followed by an autoxidation, also failed. As previously observed with various PBN-type nitrones, we concluded that both DEEPN–OH and EPPyON–OH decomposed much too rapidly in water to be detectable by conventional EPR spectroscopy.



7 **8**

More interesting results were obtained when superoxide was produced by a xanthine–xanthine oxidase (X–XO) system in the presence of either DEEPN or EPPyON. In each case, a rather intense EPR spectrum of the superoxide spin adduct was recorded, as shown in Fig. 1. We verified that the formation of

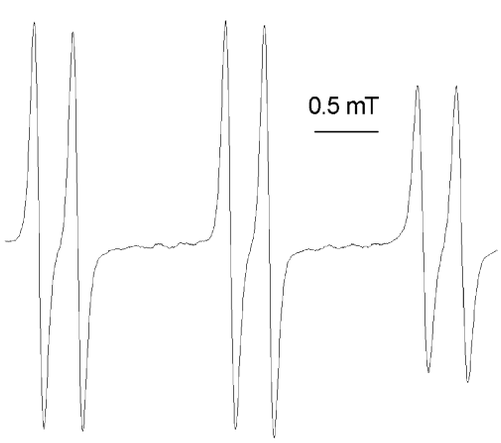


Fig. 1 EPR spectrum of aminoxyl EPPyON–O_2H obtained in a pH 7.2 buffer by generating superoxide with a xanthine–xanthine oxidase system in the presence of EPPyON (a_N = 1.35 mT and a_H = 0.15 mT).

DEEPN–O_2H and EPPyON–O_2H signal was completely inhibited by superoxide dismutase (SOD, 600–1200 units cm^{-3}) and unaffected by catalase (600 units cm^{-3}).

As mentioned in the introduction, the short life-time of the adduct at physiological pH has certainly been a key limitation to trapping superoxide with nitrones in biological media. Actually, nitrones that could yield more persistent superoxide adducts are still needed. It was then of prime importance to evaluate the half-lives of both DEEPN–O_2H and EPPyON–O_2H, and to compare their persistence to that of other nitrone–superoxide adducts. Experiments were performed at pH 5.8 and 7.2, using an X–XO superoxide generator. Superoxide was first produced in the presence of DEEPN or EPPyON (10 mmol dm^{-3}), and the adduct formation was stopped by adding SOD 3 min after the reaction had begun. The adduct EPR spectrum was then recorded over 1200–2400 s, in order to observe the adduct decrease in a single spectrum. A typical EPR signal thus recorded is shown in Fig. 2. The various spectra obtained were

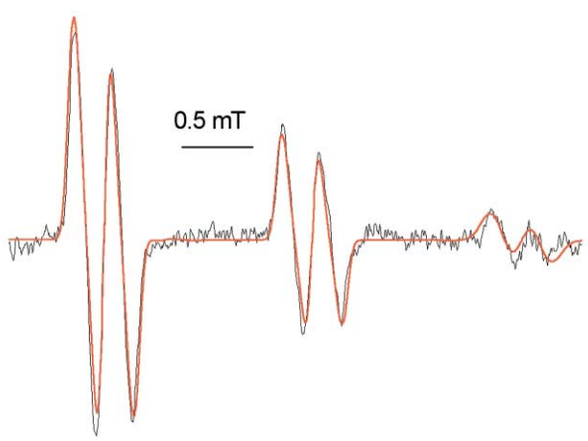


Fig. 2 Experimental EPR spectrum of the aminoxyl DEEPN–O_2H (pH 5.8) recorded with a 20 min scan time after addition of SOD, and its superimposed simulation. The experimental signal was obtained by generating superoxide with a xanthine–xanthine oxidase system in the presence of DEEPN. The simulation led to the following parameters: hfcc's, a_N = 1.38 mT and a_H = 0.24 mT; first order decay rate constant, $k_D = 0.68 \times 10^{-3}$ s^{-1}.

simulated with the computer program of Rockenbauer and Korecz,[40] which allowed us to determine the first-order rate constant k_D for the superoxide adduct decay (see Fig. 2). The values obtained for k_D are listed in Table 3, along with the half-lives $t_{½}$ of the superoxide spin adducts. In order to facilitate comparisons, k_D and $t_{½}$ previously published for other nitrone–superoxide adducts are also given in this table.[23,28,30,33,34]

Despite differences in the superoxide generator used, we noticed that both DEEPN–O_2H and EPPyON–O_2H decayed less rapidly than most adducts listed in Table 3. In particular,

Table 3 Rate constant (k_D) and half-life ($t_{½}$) determined for the first-order decay of the superoxide spin adduct for various nitrones in aqueous media

Spin adduct	Source[a]	$k_D/10^{-3}$ s^{-1}	$t_{½}$/s	Reference
DEEPN–O_2H	X–XO, pH 7.2	0.73	950	This work
DEEPN–O_2H	X–XO, pH 5.8	0.68	1019	This work
EPPyON–O_2H	X–XO, pH 7.2	1.88	369	This work
EPPyON–O_2H	X–XO, pH 5.8	1.2	578	This work
EPPN–O_2H	LRED, pH 7	2.1	315	34
EPPN–O_2H	LRED, pH 5.8	2.0	408	34
PPN–O_2H	LRED, pH 5.8	2.26	307	28
PyOPN–O_2H	LRED, pH 5.8	1.63	425	28
PyOBN–O_2H	LRED, pH 5.8	37	19	28
DMPO–O_2H	LRED, pH 7	14	50	23
DMPO–O_2H	LRED, pH 5.8	10.3	67	28
DEPMPO–O_2H	LRED, pH 7	0.9	770	23
DEPMPO–O_2H	LRED, pH 5.6	0.38	1824	23
EMPO–O_2H	HX–XO, pH 7	2.4	289	30
BocMPO–O_2H	LRED, pH 7	1.35	513	33
BocMPO–O_2H	LRED, pH 5.6	0.77	900	33

[a] Superoxide was generated by a light–riboflavin–electron donor system (LRED), a xanthine–xanthine oxidase system (X–XO), or a hypoxanthine–xanthine oxidase system (HX–XO).

they were found to be more persistent than EPPN–O_2H, which shows that the replacement of a phenyl by an oxidopyridinium group, and above all the presence of a second electron-withdrawing ethoxycarbonyl group, enhanced the stability of the superoxide spin adduct in aqueous media. Note also that the half lives of DEEPN–O_2H and of DEPMPO–O_2H were found to be in the same range at pH 7–7.2. This is an important result since DEPMPO–O_2H is to date the most persistent nitrone–superoxide spin adduct in aqueous medium. Thus, DEEPN seems to be an efficient spin trap for superoxide detection in neutral buffered media.

Conclusion

The two spin traps DEEPN and EPPyON can be easily obtained as pure crystals and trap efficiently various kinds of free radicals in aqueous media, although they cannot be used for $^{\bullet}$OH detection. In particular, DEEPN presents interesting superoxide spin trapping possibilities, and its results should be compared to those obtained with DEPMPO. DEEPN synthesis is simpler and its preparation does not require distillation or chromatographic purification. It is more lipophilic than DEPMPO, which could make it suitable for various biological experiments. The superoxide spin adducts of these two nitrones decay approximately at the same rate at neutral pH, but the DEEPN–O_2H EPR spectrum is simpler, as it contains fewer lines and corresponds to only one diastereoisomer with no conformational exchange and no long range couplings. All these results show that DEEPN is an efficacious spin trap for superoxide detection. However, DEEPN also presents several drawbacks, inherent to the structure of PBN-type traps. The EPR spectra of its various spin adducts are less characteristic than in the case of cyclic nitrones, essentially because of the small a_H coupling. In addition, it cannot be used to detect $^{\bullet}$OH in aqueous media. Lastly, the spin trapping rates are usually higher for DMPO-type nitrones than for PBN- or PyOBN-type traps. Therefore, DEEPN should be expected to trap superoxide less rapidly than DEPMPO. It is likely that DEPMPO is still superior for detecting superoxide, but DEEPN appears the most efficient trap described to date in the PBN series. Further experimental work, such as the determination of the superoxide spin trapping rate and the study of the toxicity and of the biodistribution of this trap, would permit confirmation that DEEPN could present interesting biological applications. On the other hand, the results obtained with EPPyON show that the replacement of the phenyl by an oxidopyridinium group also results in a stabilisation of the superoxide spin adduct. Thus, a nitrone that bears an oxidopyridinium and two ethoxycarbonyl groups should be even more efficacious than DEEPN for superoxide detection, and we are now trying to prepare such a trap.

Experimental

All chemicals were purchased from ACROS or Sigma-Aldrich Chemical Companies. The enzymes were obtained from Boehringer Mannheim Biochemica Company. Solvents were of the highest grade of purity commercially available and were used without further purification. Aqueous media were prepared from tridistilled water and buffers were stirred for 6 h in the presence of a chelating iminodiacetic acid resin (4 g dm^{-3}), in order to remove trace metal impurities. All the compounds synthesised were identified by microanalysis and on the basis of their ^{1}H and ^{13}C NMR spectra, recorded on a Bruker AC 200 instrument. The chemical shifts (δ) in ppm are reported with respect to internal TMS, and J values are given in Hz. The purity of DEEPN and of EPPyON was verified by HPLC on a Waters Model 600E multisolvent delivery system, equipped with a Waters Photodiode Array Detector (PAD) 996, a Waters Millenium Chromatography Manager, a Waters 717 autosampler, and a Kromasil 5 µ C18 column (25 cm length, 4.6 mm id). We also verified by EPR spectroscopy that these nitrones did not contain any paramagnetic impurities.

Synthesis of DEEPN

The *Z*-benzaldoxime was prepared beforehand from the *E*-isomer, according to the procedure described by Polonski and Chimiak.[35] Then, a solution containing Na (2 mmol) and *Z*-benzaldoxime (2 mmol) was prepared in absolute ethanol (5 cm^3) and cooled down in a liquid nitrogen–chloroform bath (−63 °C). A diethyl bromomethylmalonate solution (2 mmol) in absolute ethanol (10 cm^3) was added to this mixture, the medium was kept 3 h at −63 °C, and then slowly brought back to room temperature. Ethanol was evaporated under reduced pressure, and the residue was dissolved in 5 cm^3 water. After extraction with $CHCl_3$, the organic layer was dried over $MgSO_4$, and filtered. Evaporation of $CHCl_3$ and recrystallisation from methyl *tert*-butyl ether–cyclohexane yielded crystals (450 mg, 76%); mp 75.1 °C. Elemental analysis calculated for $C_{15}H_{19}NO_5 \cdot 0.33\ H_2O$ (299.32): C, 60.19; H, 6.62; N, 4.68; found: C, 60.16; H, 6.40; N, 4.74%. δ_H ($CDCl_3$, 200.13 MHz) 1.31 (6H, t, J 7.1, OCH_2-CH_3), 2.06 (3H, s, CH_3), 4.34 (4H,

q, *J* 7.1, OCH_2), 7.40–7.45 (3H, m, *aromatic H*), 7.58 (1H, s, N=C*H*), 8.20–8.30 (2H, m, *aromatic H*); δ_C ($CDCl_3$, 50.32 MHz) 13.81 (2C, OCH_2-CH_3), 21.02 (1C, CH_3), 62.97 (2C, OCH_2), 83.10 (1C, *C*-CH_3), 128.46 (2C, *aromatic C*), 129.27 (2C, *aromatic C*), 129.50 (1C, *aromatic C*),130.90 (1C, *aromatic C*), 135.09 (1C, N=*C*H), 166.22 (2C, *C*=O).

Synthesis of EPPyON

The *Z*-(1-oxidopyridin-1-ium-4-yl)-4-carbaldoxime was prepared beforehand from the 4-methylpyridine *N*-oxide, after the method of Schnekenburger.[36] Then, EPPyON was synthesised following the same procedure as that described above, by using *Z*-(1-oxidopyridin-1-ium-4-yl)-4-carbaldoxime and ethyl bromodimethylacetate instead of *Z*-benzaldoxime and diethyl bromomethylmalonate, respectively. Only one procedural modification was made, since all reactions were performed at room temperature. EPPyON, obtained in 72% yield, was recrystallised from diethyl ether–pentane; mp 119.5 °C. Elemental analysis calculated for $C_{12}H_{16}N_2O_4$ (252.27): C, 57.13; H, 6.39; N, 11.10; found: C, 56.90; H, 6.27; N, 11.04%. δ_H ($CDCl_3$, 200.13 MHz) 1.27 (3H, t, *J* 7.2, OCH_2-CH_3), 1.81 (6H, s, CH_3), 4.25 (2H, q, *J* 7.2, OCH_2), 7.47 (1H, s, N=C*H*), 8.15 (4H, s, *aromatic H*); δ_C ($CDCl_3$, 50.32 MHz) 13.92 (1C, OCH_2-CH_3), 24.38 (2C, CH_3), 62.37 (1C, OCH_2), 77.93 (1C, *C*-$(CH_3)_2$), 124.63 (2C, *aromatic C*H), 127.25 (1C, *aromatic C*), 127.46 (1C, N=*C*H), 139.02 (2C, *aromatic C*H), 169.93 (1C, *C*=O).

K_P determination

The lipophilicity of DEEPN and EPPyON was evaluated from their *n*-octanol–phosphate buffer (0.1 mol dm^{-3}, pH 7) partition coefficient, K_P, as follows. Equal volumes of freshly prepared octanolic solutions of nitrone (0.25 mol dm^{-3}) and of buffer were mixed and vigorously stirred at 37 °C for 1 h, and the two phases were separated by brief centrifugation (1000 *g* for 20 s). The nitrone concentration in either the octanolic or the aqueous phase was determined by HPLC, by using a Waters 996 PAD, a Waters 717 plus autosampler, and a Waters 600 pump. Data handling was accomplished using the Millenium 32 software. A 30 μmol dm^{-3} acetophenone solution was used as internal standard. In each case, K_P was evaluated as the ratio of the nitrone concentration in *n*-octanol to that in buffer.

Reduction of the nitrones by $NaBH_4$

Reduction of DEEPN and of EPPyON (30 mmol dm^{-3}) by $NaBH_4$ (30 mmol dm^{-3}) was performed in water. Autoxidation of the hydroxylamines thus formed led to the corresponding aminoxyl radicals.

Spin trapping studies

Hydroxyl radical was produced by a standard Fenton system (0.2% H_2O_2, 2 mmol dm^{-3} ethylenediaminetetraacetic acid, and 1 mmol dm^{-3} $FeSO_4$), or by UV photolysis of a 3% H_2O_2 solution in water. Alternatively, aminoxyl radicals DEEPN–OH and EPPyON–OH were generated by nucleophilic addition of water in the presence of $FeCl_3$ (1 mmol dm^{-3}). The free radicals $^{\bullet}CH_3$, $^{\bullet}CH_2OH$, $^{\bullet}CO_2^-$, and $^{\bullet}SO_3^-$ were produced by performing a Fenton reaction in the presence of dimethyl sulfoxide (DMSO, 10%), methanol (10%), sodium formate (0.2 mol dm^{-3}), or Na_2SO_3 (10 mmol dm^{-3}), respectively. Methoxyl radical $CH_3O^{\bullet}$ was generated by heating (60 °C, 2 min) a 0.1 mol dm^{-3} $K_2S_2O_8$ solution in phosphate buffer–methanol (80 : 20, vol./vol.). The superoxide generating system used contained 0.4 mmol dm^{-3} xanthine, 1 mmol dm^{-3} diethylenetriaminepentaacetic acid (DTPA), and 0.4 unit cm^{-3} xanthine oxidase (X–XO system). Spin trapping experiments were performed in 0.1 mol dm^{-3} phosphate buffer (pH 5.8 and 7.2) in the presence of 10 mmol dm^{-3} nitrone, unless otherwise stated. EPR essays were carried out at room temperature in capillary tubes by using a computer-controlled Bruker EMX spectrometer operating at X-band with 100 kHz modulation frequency. For the various spin adducts, hyperfine coupling constant values were determined by EPR signal simulation using the computer program elaborated by Rockenbauer and Korecz.[40]

Decay kinetics of superoxide adducts

The X–XO system described above was used to generate superoxide in the presence of DEEPN or EPPyON (10 mmol dm^{-3}). Superoxide dismutase (SOD, 300 units cm^{-3}) was added to the medium 3 min after the reaction had begun. The medium was then transferred into EPR tubes and the signal was recorded over 20–40 min in order to observe the superoxide adduct decay in a single spectrum. The spectra obtained were simulated by the computer program of Rockenbauer and Korecz[40] which permitted calculation of the rate constants k_D for the first-order decay of the superoxide adducts.

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