5-(Diethoxyphosphorylmethyl)-5-methyl-4,5-dihydro-3*H*-pyrrole *N*-oxide: synthesis and evaluation of spin trapping properties

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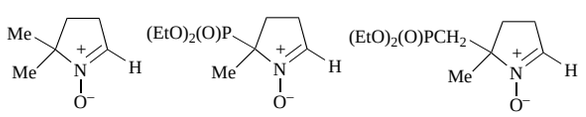
A new spin trap, the 5-(diethoxyphosphorylmethyl)-5-methyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (3), is prepared through a four-step synthetic route. The *in vitro* spin trapping properties of 3 have been investigated and the hydroxyl and superoxide spin adducts characterised. The persistence of the superoxide spin adduct obtained with 3 is compared with those of the analogues obtained with DEPMPO [5-(diethoxyphosphoryl)-5-methyl-4,5-dihydro-3*H*-pyrrole *N*-oxide] and DMPO in both phosphate buffer and pyridine. In 0.1 M phosphate buffer at pH 7.0, the HOO-3$^{\bullet}$ adduct is found to be 2.8 times more persistent than HOO-DMPO$^{\bullet}$, but 5.7 times less than HOO-DEPMPO$^{\bullet}$. In phosphate buffer, HOO-3$^{\bullet}$ decomposes to give HO-3$^{\bullet}$. The stereoselectivity of the addition of free radicals on 3 is generally poor, and mixtures of diastereomers have been observed by EPR. The overall spin trapping behaviour of nitrone 3 is more similar to that of DMPO than that of DEPMPO.

Introduction

Numerous spin trapping studies have been devoted to the role played by oxygen-centred radicals in biological processes.[1] DMPO (**1**) is a widely used spin trap but has been shown to have some limitations.[2–5] Its reaction with superoxide is rather slow (1.2 M^{-1} s^{-1} at pH 7.4)[6] and the HOO-DMPO$^{\bullet}$ spin adduct undergoes a rapid decomposition into HO-DMPO$^{\bullet}$, which may lead to misinterpretation in biological EPR experiments. In the course of our research on the design of new nitrone spin traps, we recently reported the synthesis of the 5-diethoxyphosphoryl-5-methyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DEPMPO, **2**).[7]



DMPO DEPMPO

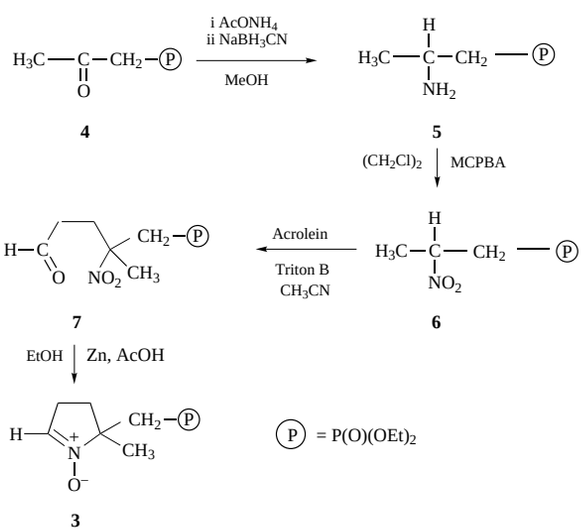
1 **2** **3**

DEPMPO was easily prepared in a two-step synthesis and was shown to trap efficiently hydroxyl and superoxide radicals. The HOO-DEPMPO$^{\bullet}$ spin adduct is much more persistent than HOO-DPMO$^{\bullet}$ (by about 15 times in phosphate buffer, pH 7), and we did not observe any detectable decay of HOO-DEPMPO$^{\bullet}$ to HO-DEPMPO$^{\bullet}$. In order to clarify the influence of the phosphonate group on the lifetime of the superoxide spin adduct, we prepared the 5-(diethoxyphosphorylmethyl)-5-methyl-4,5-dihydro-3*H*-pyrrole *N*-oxide **3**, where the phosphorus group is moved away from the nitrone moiety. We report hereafter the synthesis of **3** and an appraisal of its spin trapping properties.

Results and discussion

Synthesis

Nitrone **3** was prepared according to a four-step synthesis (Scheme 1). In the first step, the diethyl (2-aminopropyl)-phosphonate **5** was obtained in 86% yield from the commercially available diethyl (2-oxopropyl)phosphonate **4** by a



4 **5** **6** **7** **3**

Scheme 1

reductive amination with sodium cyanoborohydride in the presence of ammonium acetate as previously reported by Varlet *et al.*[8] The oxidation of **5** with *M*CPBA in dichloroethane led to the diethyl (2-nitropropyl)phosphonate **6** (69%). The 1,4-addition of the carbanion of **6** (Triton B, acetonitrile) on acrolein, followed by hydrolysis, afforded the phoshorylated nitro ketone **7** (64% yield). Compounds **5** and **6** were pure enough to be used without further purification. Finally, zinc reduction of **7** in ethanol in the presence of acetic acid, led to **3** in 30% yield after purification.

EPR studies

The EPR characteristics of spin adducts of **3** are reported in Table 1.

Spin trapping of the hydroxyl radical. Hydroxyl radicals were generated by the Fenton system (H_2O_2–$FeSO_4$) in phosphate buffer pH 5.8. In the presence of **3**, a main quadruplet (1:2:2:1) of broad lines and a smaller signal were detected. Both signals were inhibited by catalase and were attributed to the two diastereomers of the hydroxyl adduct, HO-**3**$^{\bullet}$. When the

Table 1 EPR hyperfine splitting constants for spin adducts of nitrone **3** in phosphate buffer 0.1 M

Spin adduct	Source	%	A_N/G	$A_{H\beta}$/G	$A_{P\gamma}$/G [d]	$A_{H\gamma}$/G [d]	$A_{H\gamma}$/G [d]
HO-**3**$^{\bullet}$	H_2O–Fe^{2+} [a]	78	14.6	15.6			
		22	14.4	11.1			
HOO-**3**$^{\bullet}$ [e]	HX–XO [b]		13.7	11.8	1.3	0.9	0.8; 0.6
H_3C-**3**$^{\bullet}$	H_2O_2–Fe^{2+}–DMSO [b]	69	15.9	24.3			
		31	16.1	20.1			
HO_2C-**3**$^{\bullet}$	H_2O_2–Fe^{2+}–HCO_2Na [a]	60	15.4	16.7	2.1	0.7	0.5
		40	15.2	19.9	1.2	0.6	0.3
Bu^{t}OO-**3**$^{\bullet}$	Bu^{t}OOH 70%, *hν*	62	13.3	11.2	1.4	0.8	0.6
		38	13.3	8.0	1.4	0.9	0.7
GS-**3**$^{\bullet}$	GSSG, *hν* [c]	60	16.1	14.1	1.1	0.9	0.5
		40	14.4	15.0	2.4	0.9	0.6

[a] pH 5.8; [b] Ph 6.0; [c] pH 7.4; [d] The γ couplings are tentative assignments, the $A_{P\gamma}$ being attributed to the major ones.[17] [e] Ref. 16.

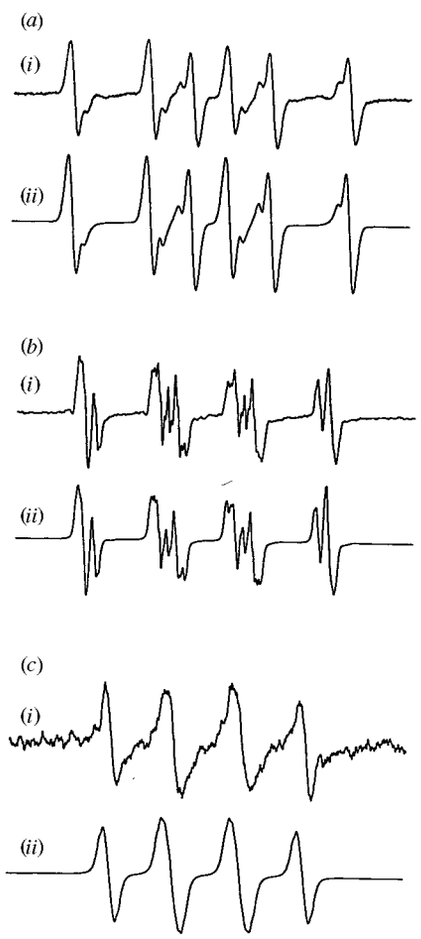


Fig. 1 EPR spectra of spin adducts (*a*) H_3C-**3**$^{\bullet}$ (pH 5.8); (*b*) HO_2C-**3**$^{\bullet}$ (pH 5.8); (*c*) HOO-**3**$^{\bullet}$ (pH 6.0) in phosphate buffer 0.1 M. (*i*) Experimental spectra. (*ii*) Calculated spectra.

Fenton system was used in the presence of DMSO or sodium formate, the corresponding methyl [Fig. 1(*a*)] or carboxyl [Fig. 1(*b*)] spin adducts (two diasteromers) were observed in place of HO-**3**$^{\bullet}$ (Table 1).

Spin trapping of superoxide. Spin trapping of superoxide was performed in phosphate buffer (pH 5.8 and 7.0) using hypoxanthine–xanthine oxidase (HX–XO) or riboflavin–light–diethylenetriamine pentaacetic acid (DTPA) as superoxide generating systems. The overall aspect of the EPR spectra [Fig. 1(*c*)] was a four line signal. The assignment of this signal to the superoxide adduct HOO-**3**$^{\bullet}$ was supported by its inhibition with superoxide dismutase (85 units ml^{-1}) and by its reduction to HO-**3**$^{\bullet}$ by glutathione (GSH)–glutathione peroxidase (GSH-Px) (10 units ml^{-1}). The HOO-**3**$^{\bullet}$ decay was shown to generate the HO-**3**$^{\bullet}$ spin adduct even at pH 5.8 and in chelexed buffer. This behaviour is similar to that of DMPO but was not observed with DEPMPO under the same conditions. An important alternate linewidth was observed in the HOO-DEPMPO$^{\bullet}$ EPR spectrum and was attributed to the occurrence of a chemical exchange.[7b] For nitrone **3**, the EPR spectrum of HOO-**3**$^{\bullet}$ was easy to differentiate from that of HO-**3**$^{\bullet}$. However, owing to the lack of a large phosphorus coupling involved in the chemical exchange,[9] the HOO-**3**$^{\bullet}$ EPR spectrum did not exhibit any significant alternate linewidth. The superoxide adduct HOO-**3**$^{\bullet}$ was also produced in pyridine by the lumiflavin–light–DTPA system and the spectrum was identical to the one observed by nucleophilic addition of H_2O_2 in the same solvent (Table 2).

Table 2 EPR hyperfine splitting constants of HOO-**3**$^{\bullet}$ in pyridine

Spin adduct	Source	A_N/G	$A_{H\beta}$/G	$A_{P\gamma}$/G	$A_{H\gamma}$/G
HOO-**3**$^{\bullet}$	H_2O_2, pyridine	12.9	10.6	1.7	1.4; 1.2; 0.7

Kinetics of decay of the superoxide adduct. In phosphate buffer (pH 5.8 and 7.0), the superoxide was generated with the riboflavin–light–DTPA system. After the end of illumination, the decay of HOO-**3**$^{\bullet}$ was monitored by measuring the decrease of the low field line in its EPR spectrum. The reaction followed pure first order kinetics, and the rate constant at pH 7.0 was 5.0×10^{-3} s^{-1}, corresponding to a half-life of 138 s. The decay rate constants of the superoxide spin adducts obtained with DEPMPO, DMPO and **3**, in the phosphate buffer and in pyridine, are reported in Table 3. For HOO-**3**$^{\bullet}$ in pyridine, the modelling of the decay curve showed a mixture of first and second order processes (which can be accounted for by a disproportionation reaction[2,4,10]), and the rate of decay of the spin adduct (SA) is given by eqn. (1).

$$-\mathrm{d[SA]}/\mathrm{d}t = k_a[\mathrm{SA}] + k_b[\mathrm{SA}]^2 \quad (1)$$

However, after 2 min, the decay occurred by pure first order kinetics ($k_a = 0.85 \times 10^{-3}$ s^{-1}) corresponding to a half-life of 815 s. In all cases, the HOO-**3**$^{\bullet}$ adduct is slightly more persistent than HOO-DMPO$^{\bullet}$ but considerably less than HOO-DEPMPO$^{\bullet}$ (Table 3). Furthermore, the decomposition of HOO-**3**$^{\bullet}$ is accompanied by the formation of the hydroxyl adduct, as reported for DMPO[2,4,11] but not for DEPMPO.

Spin trapping of GS$^{\bullet}$ and Bu^{t}OO$^{\bullet}$. The glutathionyl radical GS$^{\bullet}$ was produced by UV photolysis of glutathione disulfide in phosphate buffer at pH 5.8 and 7.4 (Table 1). The spectrum of GS-**3**$^{\bullet}$ corresponds to a 60:40 mixture of two diastereomers. Bu^{t}OO$^{\bullet}$ formed by UV photolysis of 70% Bu^{t}OOH, and two diastereomers were observed in a 62:38 ratio.

Conclusion

Nitrone **3** has been shown to trap efficiently oxygen-, sulfur- and carbon-centred radicals, giving rise, in many cases, to mixtures of diastereomeric spin adducts, as recently reported for other nitrones.[12] However, the behaviour of **3** toward the trap-

Table 3 Kinetic parameters for the decay of HOO-DPMO•, HOO-DEPMPO• and HOO-**3**• in 0.1 M phosphate buffer and in pyridine. The half-lives $t_{\frac{1}{2}}$ were calculated from the first order rate constants k_a

Spin adduct	Solvent	$k_a/10^{-3}$ s^{-1}	$k_b[SA]_0/10^{-4}$ s^{-1}	$t_{\frac{1}{2}}$/s
HOO-DMPO•	Pyridine	1.36	—	510
HOO-DMPO•	Buffer, pH 5.8	7.73	—	89
HOO-DMPO•[a]	Buffer, pH 7.0	14	—	50
HOO-DEPMPO•	Pyridine	0.25	1.3	2772
HOO-DEPMPO•[a]	Buffer, pH 5.6	0.38	8.1	1824
HOO-DEPMPO•[a]	Buffer, pH 7.0	0.90	6.1	780
HOO-**3**•	Pyridine	0.85	10.2	815
HOO-**3**•	Buffer, pH 5.8	2.28	—	304
HOO-**3**•	Buffer, pH 7.0	5.01	—	138

[a] Ref. 7(*c*).

ping of superoxide is very similar to that of DMPO. The half-life of HOO-**3**• at pH 7.0 in phosphate buffer is only approximately three times higher than the half-life of HOO-DMPO•, and its decay gives rise to the hydroxyl spin adduct HO-**3**•. These results show that for DEPMPO the phosphorus group in the 5-position plays an important role in the stabilisation of the superoxide spin adduct. It is likely that the strong electron-withdrawing effect of the diethoxyphosphoryl group[13] is involved in this strong stabilisation. Studies are in progress in our laboratory to explain the influence of electronic and steric effects of β substituents.

Experimental

Synthesis and characterisations

General. NMR spectra were recorded on a Bruker AC 100 (^{1}H, 100 MHz; ^{31}P, 40.53 MHz) or a Bruker AC 200 (^{1}H, 200 MHz; ^{13}C, 50.32 MHz) spectrometer. δ Values are given in ppm and *J* values in Hz. Elemental analyses were determined in the University of Aix-Marseille III. Mass spectra and HRMS were recorded at the University of Rennes. The diethyl(2-aminopropyl)phosphonate was prepared and characterised according to the results published by Varlet *et al.*[8] The diethyl (2-oxopropyl)phosphonate was purchased from Aldrich.

Diethyl(2-aminopropyl)phosphonate (5). A solution of diethyl(2-oxopropyl)phosphonate (1 g, 5.2 mmol), ammonium acetate (3.96 g, 51.5 mmol) and sodium cyanoborohydride (0.32 g, 5.2 mmol) in 15 ml of methanol was stirred for 72 h at room temperature, then concentrated HCl was added until the solution reached pH 2 and the methanol was then removed. The residue was taken up in 10 ml of water and washed three times with 10 ml of diethyl ether. The aqueous solution was brought to pH 12 with potassium hydroxide, saturated with NaCl and extracted with three 12 ml portions of CH_2Cl_2. The organic layer was dried over magnesium sulfate. Filtration and removal of the solvent afforded 0.86 g (4.4 mmol, 86%) of **5**.

Diethyl (2-nitropropyl)phosphonate (6). Aminophosphonate **5** (1 g, 5.15 mmol) in 1,2-dichloroethane (2.5 ml) was added dropwise to a refluxing solution of *m*-chloroperbenzoic acid (75%, 4.7 g, 27.45 mmol) in 15 ml of 1,2-dichloroethane. The mixture was stirred under reflux for 16 h, then cooled, washed with saturated aqueous Na_2CO_3 (3 × 10 ml) and dried over magnesium sulfate. Removal of the solvent gave 0.8 g (3 mmol, 69%) of nitro compound **6**. δ_P($CDCl_3$) 23.90; δ_H(100.13 MHz, $CDCl_3$) 1.34 (6 H, t, *J* 7.0, 2C*H*$_3$CH$_2$O), 1.68 (3 H, d, *J* 6.7, C*H*$_3$CHNO$_2$), 1.7–2.7 (2 H, m, C*H*$_2$P), 4.12 (4 H, qt, *J* 7.0, 2 OC*H*$_2$CH$_3$), 4.85 (1 H, m, *H*CNO$_2$); δ_C(50.32 MHz, $CDCl_3$) 16.21 (d, *J* 5.5, OCH$_2$*C*H$_3$), 16.32 (d, *J* 5.3, OCH$_2$*C*H$_3$), 20.57 (d, *J* 7.3, *C*H$_3$CHNO$_2$), 31.08 (d, *J* 143.5, *C*H$_2$P), 62.20 (d, *J* 2.7, 2 O*C*H$_2$CH$_3$), 78.22 (H*C*NO$_2$). (Calc. for $C_7H_{16}NO_5P$: C, 37.33; H, 7.16; N, 6.22. Found: C, 37.42; H, 7.08; N, 6.20%).

Diethyl (4-formyl-2-methyl-2-nitrobutyl)phosphonate (7). A solution of acrolein (0.28 g, 5 mmol) in 2.5 ml of CH_3CN was added dropwise to compound **6** (1 g, 4.4 mmol) at 0 °C. The mixture was cooled to −5 °C and 18 µl of Triton B (40%) were added dropwise over 40 min. The mixture was stirred at 0 °C over 2 h, then at 10 °C over 20 h, then cooled to 0 °C and acrolein (0.14 g, 2.4 mmol) added dropwise. The mixture was stirred at 0 °C for 2 h, then at 10 °C for 20 h. The mixture was finally cooled to 0 °C and water (2 ml) and HCl solution (4%, until pH 3) were added. The organic layer was dried over $MgSO_4$. After removal of the solvent, 0.8 g (2.8 mmol, 64%) of the crude product **7** was obtained, which was pure enough for further use. δ_P($CDCl_3$) 22.17; δ_H(200.13 MHz, $CDCl_3$) 1.31 and 1.32 (6 H, 2t, *J* 7.2, 2C*H*$_3$CH$_2$O), 1.76 (3 H, s, C*H*$_3$CNO$_2$), 2.2–2.8 (6 H, m, 3C*H*$_2$), 4.10 (4 H, qt, *J* 7.2, 2 OC*H*$_2$CH$_3$), 9.74 (1 H, s, C*H*O); δ_C(50.32 MHz, $CDCl_3$) 16.19 (d, *J* 7.1, 2 OCH$_2$*C*H$_3$), 23.94 (d, *J* 3.3, *C*H$_3$CCH$_2$P), 31.97 (d, *J* 8.4, *C*H$_2$CCH$_2$P), 34.83 (d, *J* 142.4, *C*H$_2$P), 38.12 (*C*H$_2$CHO), 62.23 (d, *J* 6.9, 2 O*C*H$_2$CH$_3$), 87.72 (*C*NO$_2$), 199.80 (*C*HO).

5-(Diethoxyphosphorylmethyl)-5-methyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (3). At 2 °C, to a solution of **7** (1 g, 3.6 mmol) in 8 ml of ethanol (95%) were added, firstly, zinc dust (0.6 g, 9.2 mmol) (slowly, with mechanical stirring), then acetic acid (0.85 g, 14.2 mmol) diluted in 3 ml of ethanol (dropwise, keeping the temperature below 8 °C). The mixture was stirred at 2 °C for 15 h. The sample was filtered to remove the zinc acetate; the residue was washed with ethanol. Then, the ethanol was removed from the filtrate and the crude product dissolved in 50 ml of water. The impurities were removed by continuous workup with diethyl ether, then the nitrone extracted from the aqueous layer by continuous workup with CH_2Cl_2. The organic layer was dried with Na_2SO_4 and the solvent removed to give 300 mg of nitrone **3** (1.2 mmol, 30%). δ_P($CDCl_3$) 24.94; δ_H(200.13 MHz, $CDCl_3$) 1.34 (6 H, t, *J* 7.2, 2C*H*$_3$CH$_2$O), 1.57 (3 H, s, C*H*$_3$-CCH$_2$O), 1.7–2.8 (6 H, m, 3C*H*$_2$), 4.12 (4 H, qt, *J* 7.2, 2OC*H*$_2$CH$_3$), 6.90 (1 H, s, =C*H*); δ_C(50.32 MHz, $CDCl_3$) 16.38 (d, *J* 6.1, 2OCH$_2$*C*H$_3$), 24.67 (d, *J* 4.7, *C*H$_3$C*CH$_2$P), 24.94 (*C*H$_2$), 31.85 (*C*H$_2$), 34.06 (d, *J* 142.1, *C*H$_2$P), 61.91 (d, *J* 5.5, O*C*H$_2$CH$_3$), 62.01 (d, *J* 5.0, O*C*H$_2$CH$_3$), 74.28 (*C**CH$_3$CH$_2$P), 133.42 (*C*H); (Calc. for $C_{10}H_{20}NO_4P$: 249.1130. Found: 249.1137.) *m*/*z* 232 (13.40%), 204 (*M* − Et, 3.50), 138 [HP(O)(OEt)$_2$, 1.38], 125 (13.13), 94 (100), 80 (7.01), 28 (9.7).

Spin-trapping studies

General. Xanthine oxidase (XO) and bovine erythrocyte superoxide dismutase (SOD) were purchased from Boerhringer Mannheim Biochemica Co.; catalase, glutathione peroxidase, diethylenetriaminepentaacetic acid (DTPA) and other chemicals were obtained from Sigma Chemical Co.

All buffers were stirred for 4 h in the presence of a chelating iminodiacetic acid resin (4 g per 100 ml) to remove traces of metal impurities. EPR spectra were recorded on a computer controlled Varian E-3 ESR spectrometer and on a Bruker ESP 300 spectrometer equipped with an NMR gaussmeter for field calibration. An HP 5350B microwave was used for determination of *g* factors. The UV photolysis was produced by a 1000 W xenon–mercury Oriel lamp. The EPR spectra were simulated with the EPR software developed by D. Dulling from the Laboratory of Molecular Biophysics NIEASCN.[14]

HO$^{\bullet}$ trapping—Fenton reaction system. A standard Fenton reaction system was used to generate HO$^{\bullet}$. $FeSO_4$ (3.3 mM) was added to a solution containing 0.1 M phosphate buffer (pH 5.8), 0.1 M nitrone, 2 mM H_2O_2. The EPR spectrum of the spin adduct was recorded 40 s after the addition of ferrous sulfate. No EPR signal was observed when catalase (400 unit ml^{-1}) was added before $FeSO_4$.

$^{\bullet}CH_3$ and $CO_2^{\bullet-}$–$^{\bullet}CO_2H$ trapping. A Fenton reaction system in presence of DMSO (or HCO_2Na) was used to generate $^{\bullet}CH_3$ (or $CO_2^{\bullet-}$–$^{\bullet}CO_2H$). $FeSO_4$ (3.3 mM) was added to a solution containing: 0.1 M nitrone **3**, 2 mM H_2O_2, 100 mM DMSO (or 0.5 mM HCO_2Na) in 0.1 M phosphate buffer (pH 5.8–6.0). The EPR spectrum of the spin adduct was recorded 40 s after the addition of ferrous sulfate.

Superoxide trapping—hypoxanthine–xanthine oxidase system (HX–XO). This superoxide-generating system contained 0.4 mM hypoxanthine, 0.4 unit ml^{-1} xanthine oxidase and 0.2 M nitrone as spin trap in 0.1 M phosphate buffer. Oxygen was bubbled into the reaction mixture for 30 s and the EPR spectrum was recorded 40 s after the addition of xanthine oxidase.

Superoxide trapping—light–riboflavin–DTPA system. The light–riboflavin–DTPA system used in our experiments contained 0.1 M nitrone, 4 mM DTPA, 0.1 mM riboflavin in 0.1 M phosphate buffer. In pyridine, the riboflavin was replaced by lumiflavin. Oxygen was bubbled into the reaction mixture for 60 s. The superoxide generation was initiated by irradiating the EPR cell using a tungsten filament 100 W lamp.

For the two superoxide generating systems, in buffer solutions, the EPR signal was completely inhibited when the experiment was driven in the presence of SOD (85 units ml^{-1}). When glutathione (0.1 M) and glutathione peroxidase (12 units ml^{-1}) were added, only the hydroxyl adduct was observed.

GS$^{\bullet}$ and Bu^{t}OO$^{\bullet}$ trapping. GS$^{\bullet}$ was produced by UV photolysis of a solution containing 5×10^{-2} M glutathione disulfide and 1.7×10^{-2} M nitrone as spin trap in 0.1 M phosphate buffer. Bu^{t}OO$^{\bullet}$ was produced by UV photolysis of a Bu^{t}OOH solution (70%) containing 0.05 M nitrone as spin-trap.

Kinetics 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 5.8 and 7 (light–lumiflavin–DTPA in pyridine). The nitrone concentration was 0.1 M. The superoxide adduct formation occurred during irradiation and was immediately suppressed when the light was shut off. The decay of the spin adduct was followed by monitoring the decrease of an appropriate line of the spin adduct spectrum. Computer simulations of the kinetic EPR data were performed using the home-made DAPHNIS program:[15] the signal amplitude at time t_n was calculated from the signal amplitude at time t_{n-1} using the chosen rate equation. The standard least square method was then applied to fit the calculated curves with the experimental ones. In these calculations, the intensity of the monitored EPR peak is related to the actual radical concentration [SA] by a scale factor. The first order rate constant k_a and the product $k_b[SA]_0$ reported in Table 3 are independent of this scale factor.

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