Synthesis of a New Spin Trap: 2-(Diethoxyphosphoryl)-2-phenyl-3,4-dihydro-2*H*-pyrrole 1-Oxide†

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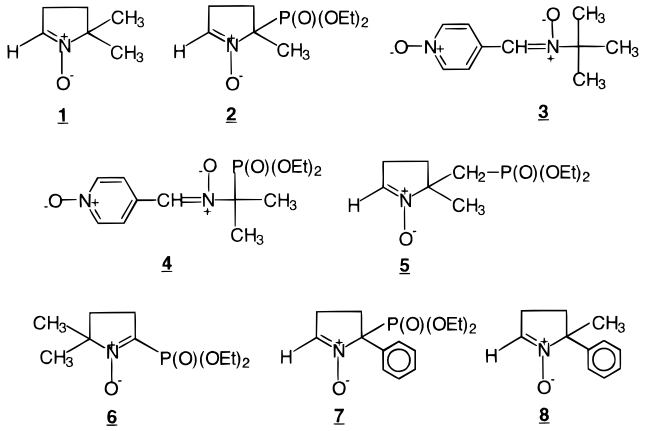
Recently, the synthesis of a new phosphorylated nitrone, 2-(diethoxyphosphoryl)-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DEPMPO) (**2**) was described. The presence of the phosphorylated group strongly stabilized the DEPMPO−superoxide spin adduct. To understand the role of the diethoxyphosphoryl group in this stabilization, a new phosphorylated nitrone, 2-(diethoxyphosphoryl)-2-phenyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DEPPPO) (**7**), was prepared through a four-step synthetic pathway, and its ability to trap free radicals was investigated. Data obtained from spin trapping experiments of a wide variety of free radicals generated in situ showed the formation of two diastereoisomers spin adducts with different phosphorus and hydrogen coupling constants. Superoxide trapping by DEPPPO gave a persistent nitroxide spin adduct, and its half-time life was measured and compared to that of the DEPMPO analogue.

Introduction

Superoxide anion radical ($O_2^{\bullet-}$) is the most important radical formed in aerobic organisms. During the mitochondrial respiratory chain, 1−4% of the metabolized oxygen escape the normal four-electron reduction process after accepting the first electron. Superoxide is also generated in vivo from various enzymatic processes and by the oxidation of a number of biologically significant compounds. Superoxide and reactive oxygen species derived therefrom ($HOO^{\bullet}$, $HO^{\bullet}$, and H_2O_2) are considered to be involved in a host of pathological processes including simple inflammation,[1] ischemia-reperfusion syndrome,[2] DNA damage,[3] and neuron death.[4]

ESR-spin trapping is a useful method to detect free radicals and has been widely used to trap oxygen-centered radicals in biological milieu.[5] 2,2-Dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DMPO) (**1**, Scheme 1) is the most popular spin trap, and ESR features of its resulting nitroxide spin adducts are well-known.[6] However, a major limitation with superoxide trapping is the low rate constant for the reaction between $O_2^{\bullet-}$ and nitrones and also the poor stability of the resulting nitroxide spin adduct.[7,8] DMPO−superoxide spin adduct has a half-life time estimated to be 1 min at physiological pH,[7] and a small percentage (3−5%) decomposes to DMPO−hydroxyl adduct,[8] thus, in some extent, limiting the use of DMPO to trap superoxide.

Scheme 1



Studies of β-phosphorylated five-membered ring nitroxides reported previously showed that their ESR phosphorus coupling was very sensitive to the ring conformation and represents a valuable structural probe.[9] We thus developed the synthesis of a new class of phosphorylated nitrones which would give β-phosphorylated five-membered ring nitroxide spin adducts by trapping free radicals (Scheme 2).

†**Abbreviations:** DEPPPO, 2-(diethoxyphosphoryl)-2-phenyl-3,4-dihydro-2*H*-pyrrole 1-oxide; DEPMPO, 2-(diethoxyphosphoryl)-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide; DEP-DMPO, 5-(diethoxyphosphoryl)-2,2-dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide; DMPO, 2,2-dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide; $O_2^{\bullet-}$, superoxide anion radical.

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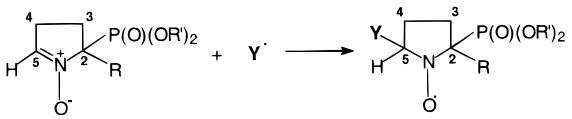
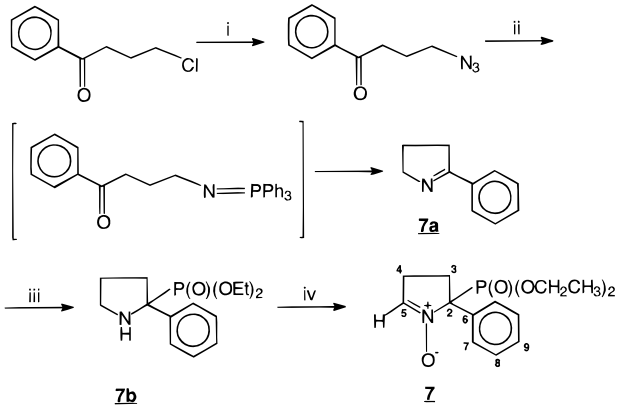
Scheme 2



The first phosphorylated five-membered ring nitrone synthesized, (2-diethoxyphosphoryl)-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DEPMPO) (**2**, Scheme 1), exhibited interesting spin trapping properties and yielded long-lived superoxide spin adduct (half-life time estimated to 15 min at physiological pH).[10] Zeghdaoui et al. reported the synthesis of PBN-type phosphorylated nitrones,[11a] and Roubaud et al. showed that for these nitrones also the phosphorylated group significantly stabilized the superoxide spin adducts.[11b] In the case of diethyl (1-*N*-oxy-*N*-[4-(*N*′-oxypyridinyl)formylene]amino-1-methylethyl)phosphonate (4-PyOPN) (**4**), the phosphorylated analogue of commercially available α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-PyOBN) (**3**), the superoxide spin adduct is 22 times more persistent in 0.1 M phosphate buffer at pH 5.8.[11b] Recently, Roubaud et al. showed by synthesizing the 2-(diethoxyphosphorylmethyl)-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (**5**) that the lifetime of the superoxide spin adduct depended on the phosphorylated group position in the five-membered ring nitrone.[12] The same observations were reported by Janzen et al. who used 5-(diethoxyphosphoryl)-2,2-dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DEP-DMPO) (**6**).[13] The half-life time of DEP-DMPO−superoxide spin adduct was only 3.6 s.[13] Although the presence of a phosphorylated group on the double bond will facilitate a spectral pattern (6-line spectra), it decreased dramatically the superoxide adduct persistency.

For the DEPMPO−superoxide spin adduct, the average value of the phosphorus coupling is rather important ($a^P \approx 5.29$ mT at pH 7.4 in phosphate buffer). The large magnitude of the phosphorus coupling shows a significant hyperconjugative interaction between the carbon−phosphorus bond and the nitroxyl moiety. The existence of this interaction shows that in the DEPMPO−superoxide conformers the carbon−phosphorus bond adopts an axial or pseudoaxial orientation. To appreciate if stereoelectronic effects could influence the persistency of the DEPMPO−superoxide spin adduct, we sought to generate spin adducts in which the hyperconjugative interaction between the carbon−phosphorus bond and the nitroxyl moiety would be weakened. Rockenbauer has shown that for pyrrolidinoxyl radicals the phenyl group adopts an axial position.[14] We thus decided to generate spin adducts of the pyrrolidinoxyl series bearing a diethoxyphosphoryl and a phenyl group on the same carbon (Scheme 2, R = Ph) by trapping free radicals with a new nitrone, 2-(diethoxyphosphoryl)-2-phenyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DEPPPO) (**7**), and then investigating their ESR features.

Scheme 3[a]



[a](i) NaN_3, DME, 75 °C, 16 h (86%, crude product); (ii) PPh_3, Et_2O (70%); (iii) $HP(O)(OEt)_2$, cat. $BF_3(Et_2O)$ (68%); (iv) *m*-CPBA, $CHCl_3$, −5 °C (30%).

Results

Synthesis. According to a method described by Vaultier,[15] we obtained the γ-azidobutyrophenone (86%, crude product) by adding sodium azide to a solution of commercially available γ-chlorobutyrophenone in 1,2-dimethoxyethane. The reaction mixture was stirred at 75 °C during 16 h. The 2-phenyl-1-pyrroline **7a** (70%, after crystallization in pentane) was obtained in two steps from the γ-azidobutyrophenone via cyclization of the iminophosphorane (not isolated). Reaction of diethyl phosphite with **7a** in the presence of a catalytic amount of boron trifluoride diethyl etherate ($Et_2O{\cdot}BF_3$) at room temperature led to 68% of diethyl (2-phenylpyrrolidin-2-yl)phosphonate **7b** (Scheme 3) obtained as a colorless oil after column chromatography. Oxidation of **7b** was the most sensitive step of the synthetic pathway and was carried out with a solution of 70% *m*-chloroperbenzoic acid in chloroform at −5 °C to yield **7** as a yellow oil (30%) after column chromatography (CH_2Cl_2/EtOH 90/10).

ESR Studies. (a) Spin Trapping of Hydroxyl Radical. The hydroxyl radical was obtained by incubating DEPPPO (50 mM) with H_2O_2 (1 mM) and $FeSO_4$ (0.3 mM) in 0.1 M phosphate buffer at pH 7.0. We obtained an ESR signal simulated as a superimposition of spectra corresponding respectively to two diastereoisomers (a_N = 1.35 mT, a_H = 1.05 mT (1H), a_P = 3.06 mT (48%); a_N = 1.37 mT, a_H = 1.49 mT (1H), a_P = 3.18 mT, $a_{H\gamma}$ = 0.043 mT (1H) (52%)) (Figure 1a). This signal was inhibited by the presence of catalase (50 U mL^{-1}) in the incubation mixture. We observed the same signal by nucleophilic addition of water to **7** in the presence of Fe^{3+} (data not shown).[16] In this case, the percentage contribution for each diastereoisomer determined by computer simulation was 54/46% showing that we were getting a different ratio by radical addition. Due to the presence of two diastereoisomers, the DEPPPO/•OH signal is relatively

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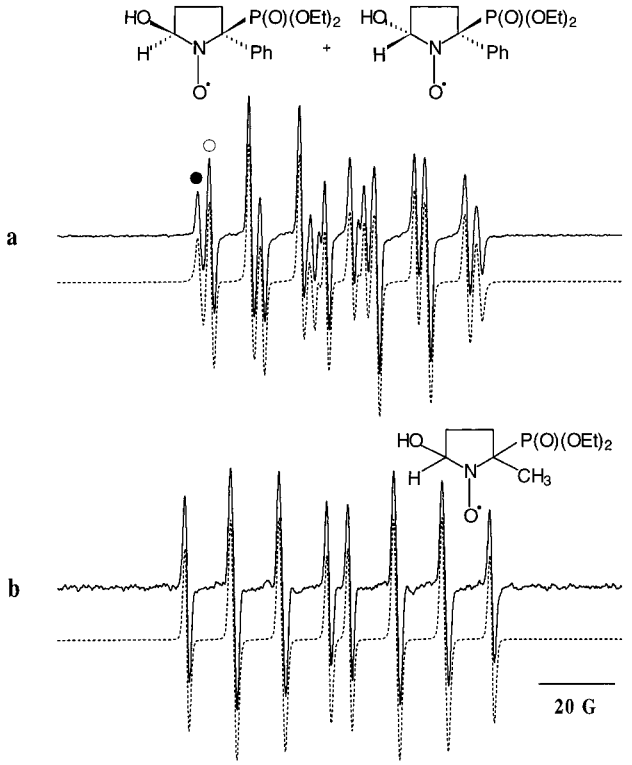

Figure 1. Spin trapping of hydroxyl radical. (a) Spectrum obtained upon incubating H_2O_2 (1 mM), $FeSO_4$ (0.3 mM), and DEPPPO (50 mM) at room temperature in phosphate buffer (0.1 M, pH 7.4). (b) As in a but in the presence of DEPMPO (50 mM). The dotted lines in a and b represent computer simulation of the respective spectrum. Open and closed circles indicate the low-field line of DEPPPO/•OH diastereoisomers. Spectrometer settings: microwave power, 10 mW; modulation amplitude, 0.05 mT; time constant, 0.128 s; gain, 2.5×10^4; scan range, 20 mT; and scan time, 120 s.

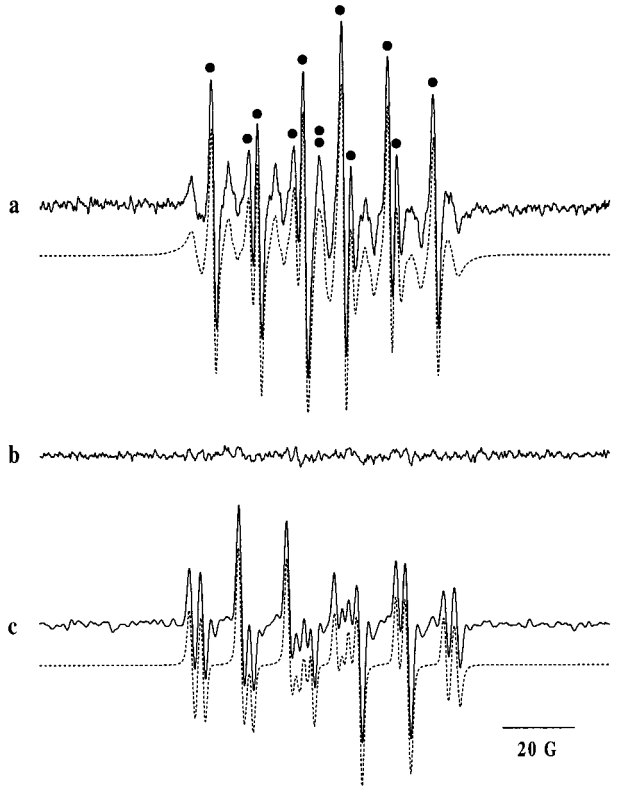

Figure 2. Spin trapping of superoxide radical anion. (a) Incubation mixture contained hypoxanthine (0.4 mM), xanthine oxidase (0.4 U mL^{-1}), DTPA (1 mM), and DEPPPO (100 mM) in phosphate buffer (0.1 M, pH 7.4). (b) As in a but in the presence of SOD (0.5 $mg.mL^{-1}$). (c) As in a but in the presence of Gpx (10 U mL^{-1}) and GSH (0.3 mM). The dotted lines in a and c represents computer simulation of the respective spectrum and the closed circles (●) indicate the lines corresponding to the diastereoisomer A. Spectrometer settings: microwave power, 10 mW; modulation amplitude, 0.1 mT; time constant, 0.128 s; gain, 5×10^4; scan range, 20 mT; and scan time, 120 s.

complicated compared to its DEPMPO analogue (Figure 1b). Monitoring of the ESR signal showed that the two diastereoisomers decayed with a different rate (data not shown).

(b) Spin Trapping of Superoxide Anion Radical. Spin trapping of superoxide with DEPPPO was performed at pH 7.4 by using two different superoxide-generating systems. The same signal was observed by using either hypoxanthine (0.4 mM) in the presence of xanthine oxidase (0.4 U mL^{-1}) or a light−riboflavin−DTPA combination (Figure 2a). With both superoxide generating systems, the ESR signal was inhibited by addition of SOD (0.5 mg mL^{-1}) proving that the spectra were due to the trapping of $O_2^{\bullet -}$ (Figure 2b). In the presence of a glutathione (0.3 mM)−glutathione peroxidase (10 U mL^{-1}) system with both superoxide-generating systems, we only observed the hydroxyl radical spin adduct with a percentage contribution for each diastereoisomer of 33/67% determined by computer simulation of the experimental spectrum (Figure 2c). **7**−superoxide spin adduct was detected as a complicated signal which was computer-simulated considering two diastereoisomers **A** and **B**, both involved in a chemical exchange (Table 1). The existence of a chemical exchange between two conformers inducing an important alternate line width in the ESR spectrum has been reported for the DEPMPO−superoxide spin adduct.[10b] Trapping of *tert*-butylperoxyl radical (*t*-BuOO•) gave also a complicated signal pattern, computer simulated as two diastereoisomers with only one diastereoisomer involved in a chemical exchange (Figure 3, Table 1).

(c) Kinetic Decay of the Superoxide Adduct. The use of the light−riboflavin−DTPA superoxide generating system was not possible in this study because a residual carbon-centered radical coming from the electron donor arises.[8c] Carbon-centered radical spin adducts are very persistent and overlapping of lines can occur, leading to an overestimation of the measured half-life times. So we used the xanthine−xanthine oxidase system to generate superoxide in 0.1 M phosphate buffer, pH 7.4 and 8.2. After incubating the spin trap (17 mM) with hypoxanthine (0.4 mM), xanthine oxidase (0.04 U mL^{-1}) in the presence of DTPA (300 μM) for 10 min to reach the steady-state concentration, we added superoxide dismutase (0.5 mg mL^{-1}) to dismutate superoxide and thus to stop the formation of the DEPPPO−superoxide spin adduct. Decay of the superoxide spin adduct was then monitored by measuring the decrease of the ESR spectrum low-field line of the diastereoisomer with the most intense signal considered as the major diastereoisomer. The kinetic was simulated as a pseudo-first-order process. Calculated half-life times for DEPPPO−superoxide adduct were (13.1 ± 0.1) min at pH 7.4 and (8.5 ± 0.5) min at pH 8.2. Under the same conditions, the DEPMPO superoxide spin adduct had a similar persistency: (14.8

Table 1: ESR Hyperfine Splitting Constants for Peroxyl Adducts of Nitrone 7

spin adduct	source	solvent	%		A_N/mT	A_P/mT	$A_{H\beta}$/mT	$A_{H\gamma}$/mT	$k^a/s^{-1} \times 10^{-6}$
$O_2^{\bullet-}$/HOO$^\bullet$	HY/XO	water[b]	(A): 51[c]	40[d]	1.17	3.72	0.83		23
				60[d]	1.35	1.88	1.19		
			(B): 49[c]	28[d]	1.25	4.64	1.16		2
				72[d]	1.31	3.32	1.02		
t-BuOO$^\bullet$	*t*-BuOOH/*hν*	benzene	83[c]	47[d]	1.25	4.20	0.95	0.16	2.5
				53[d]	1.24	3.99	0.71	0.18	
			17[c]	17[c]	1.30	2.50	0.95	0.10	

[a] Exchange rate. [b] 0.1 M phosphate buffer, pH 7.4. [c] Percentage contribution of diastereoisomer. [d] Percentage contribution of conformer.

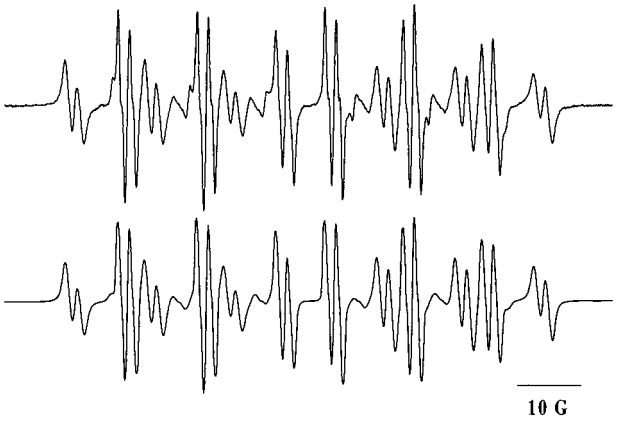

Figure 3. *tert*-Butyl peroxyl spin adduct of DEPPPO and its computer simulation. Spectrum obtained upon UV photolysis of a solution containing *tert*-butyl hydroperoxide (1.5 M) and DEPPPO (50 mM) at room temperature in benzene. Spectrometer settings: microwave power, 10 mW; modulation amplitude, 0.032 mT; time constant, 0.128 s; gain, 2.5×10^4; scan range, 20 mT; and scan time, 480 s.

± 1.4) min at pH 7.4 and (9.9 ± 0.2) min at pH 8.2. A comparison with the commonly used spin trap, DMPO, was impossible to perform under the same conditions because of the short half-life time of the corresponding superoxide spin adduct (~50 s at pH 7.0). However, it is important to recall that DEPMPO−superoxide spin adduct is 15 times more persistent than DMPO−superoxide spin adduct.

(d) Spin Trapping of Other Radicals. *tert*-Butoxyl radical (*t*-BuO$^\bullet$) was produced by UV photolysis of a solution of di-*tert*-butyl peroxide in benzene. In the presence of DEPPPO, we observed a spectrum, computer-simulated as a superimposition of two diastereoisomers (Table 2). Spectra obtained by trapping $^\bullet CO_2^-$, Ph$^\bullet$, and thiyl radicals showed the presence of two species, probably diastereoisomers, with slightly different hyperfine coupling constants (Table 2). For most of the radicals generated and trapped by DEPPPO, we obtained a similar percentage contribution for the two diastereoisomers (approximately 70% of the diastereoisomer with the largest phosphorus coupling value and 30% of the other). Trapping of the glutathionyl radical (GS$^\bullet$) generated by UV photolysis of GSNO in water gave a broad 12-line spectrum with alternating line width (Table 2). We simulated this signal by assuming the existence of two diastereoisomers (data not shown). In the case of the DEPPPO/$^\bullet$SG spin adduct, the major diastereoisomer was the one with the lowest phosphorus coupling value. Spin adducts obtained by trapping of thiyl radicals were not persistent, and continuous photolysis was needed to observe them.

Different results were obtained during the generation of the DEPPPO−OBu^t spin adduct depending on the nature of the generating system. By UV photolysis of a solution containing DEPPPO and di-*tert*-butyl peroxide in benzene, two diastereoisomers were observed (Table 2). Surprisingly, only one species (24-line spectra) was observed when *t*-BuO$^\bullet$ was tentatively generated by addition of a small amount of $Pb(OAc)_4$ to a solution of the *tert*-butyl alcohol in benzene. The same results were obtained with CH_3OH, CH_3CH_2OH, and *i*-PrOH. On the other hand, in the presence of $Pb(OAc)_4$, we always observed the same paramagnetic byproducts. Our results suggest that a nucleophilic addition of the alcohol to the nitrone assisted by $Pb(OAc)_4$ could occur followed by a rapid oxidation of the hydroxylamine to the corresponding aminoxyl.

Only a weak signal was observed with other carbon-centered radicals such as $^\bullet CH_3$ and $^\bullet CH(CH_3)OH$ generated via a Fenton reaction (H_2O_2, Fe^{2+}) in the presence of dimethyl sulfoxide (DMSO) and ethanol (EtOH), respectively (data not shown). UV photolysis of CH_3I gave no detectable signals.

(e) Partition Coefficient (K_p). In a previous paper, we reported the value of the DEPMPO partition coefficient to be between those of 1-octanol and water (K_p = 0.06).[10b] Using a method described by Konorev et al.,[17] we evaluated the DEPPPO partition coefficient (K_p = 2.4). It is interesting to note that the presence of a phenyl group greatly enhanced lipophilicity of the spin trap; 5-phenyl-2,2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (**8**),[18] a phenylated analogue of DMPO, exhibited a partition coefficient 100 times greater than that of DMPO (K_p(DMPO) = 0.1).[19]

Discussion

Conformation of the Diethoxyphosphoryl Group. We have already shown in a previous paper that for DEPMPO the position of the diethoxyphosphoryl group is very important in the stabilization of its superoxide spin adduct.[12] Thus if the diethoxyphosphoryl group is moved away from the C2 position as in the 2-(diethoxyphosphorylmethyl)-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (**5**), the corresponding superoxide spin adduct has a half-life time comparable to that of its DMPO analogue.[12] Removing the diethoxyphosphoryl group from the C2 position can modify both the steric and electronic influences on the stabilization of the resulting superoxide spin adduct.

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Table 2: ESR Hyperfine Splitting Constants for Spin Adducts of Nitrone 7

adduct	source	solvent	%[a]	A_N/mT	A_P/mT	$A_{H\beta}$/mT	$A_{H\gamma}$/mT	other
HO•	H_2O_2/Fe^{2+}	water[b]	52	1.37	3.18	1.49	0.043	
			48	1.35	3.06	1.05		
	H_2O/Fe^{3+}	water[c]	46	1.37	3.18	1.49	0.043	
			54	1.35	3.06	1.05		
CH_3O[d]	$CH_3OH/Pb(OAc)_4$	benzene	100	1.28	3.81	0.71	0.18	
EtO[d]	$EtOH/Pb(OAc)_4$	benzene	100	1.28	3.84	0.72	0.18	
i-PrO[d]	iPrOH/$Pb(OAc)_4$	benzene	100	1.29	3.80	0.73	0.18	
t-BuO[d]	tBuOH/$Pb(OAc)_4$	benzene	100	1.31	3.70	0.94	0.17	0.02 0.07
tBuO•	(t-BuO)$_2$/$h\nu$	benzene	67.5	1.29	3.82	0.80	0.19	
			32.5	1.24	3.02	0.92	0.11	
Ph•	(PhCOO)$_2$/$h\nu$	benzene	63	1.37	3.41	2.0		
			37	1.33	3.26	1.92		
$•CO_2^-$	H_2O_2/Fe^{2+}	water[a]	67.5	1.44	3.45	1.94		
	/HCO_2Na		32.5	1.42	2.60	1.80		
CH_3S•	(CH_3S)$_2$/$h\nu$	benzene	68	1.31	3.62	1.36		
			32	1.28	3.01	1.36		
t-BuS•	(tBuS)$_2$/$h\nu$	benzene	67	1.34	3.49	1.37		
			33	1.29	3.18	1.42		
PhS•	(PhS)$_2$/$h\nu$	benzene	68	1.29	3.50	1.43		
			32	1.25	3.10	1.42		
GS•	GSNO/$h\nu$	water[a]	64	1.39	3.09	1.62		
			36	1.41	3.25	1.54		
H•	**2**/DMD[e]	water[a]	100	1.40	3.42	1.63		$A_{H\beta 2}$ 2.02

[a] Percentage contribution of diastereoisomer. [b] 0.1 M phosphate buffer, pH 7.4. [c] Doubly distilled water, pH 7.0. [d] The spin adducts are formed via nucleophilic attack. [e] DMD = dimethyldioxirane.

If we could maintain the diethoxyphosphoryl group in the C2 position and change the resulting spin adducts conformation, we should observe the influence of the conformation on the spin adduct persistency. Rockenbauer et al. have reported in pyrrolidinoxyl radicals that a phenyl group will adopt preferentially a pseudoaxial position.[14] To study the influence of the conformation of the phosphorylated group, we prepared DEPPPO, a phenylated analogue of DEPMPO bearing a phenyl and a diethoxyphosphoryl group on the C2 position (see Scheme 2).

For DEPMPO, ^{13}C NMR data showed a 7.35 Hz $^3J_{P-C4}$ coupling; however, for DEPPPO this coupling was not resolved. $^3J_{P-C4}$ couplings have an angular dependence, and by using the Karplus relation proposed by Thiem and Meyer we found dihedral angle ($PC_2C_3C_4$) values equal to 131° and 98° for DEPPPO and DEPMPO, respectively.[20] Thus, the preferred geometry of the diethoxyphosphoryl group is pseudoaxial for DEPMPO and pseudoequatorial for DEPPPO.

Radical additions on DEPMPO are stereoselective. The bulkiness of the diethoxyphosphoryl group in the pseudoaxial position hinders one nitronyl face and favors radical addition on the opposite face. As a consequence, in most cases, only one diastereoisomer was observed (RS•, HO•, R_3C•, ...).[21] Although two diastereoisomers were obtained in some cases, the second diastereoisomer had a very low percentage contribution.[21] For DEPMPO spin adducts, the average phosphorus coupling value is 5 mT, indicating that in the most populated conformers the diethoxyphosphoryl group adopts a pseudoaxial position. Concerning the DEPPPO spin adducts, the phosphorus coupling values are smaller than 4 mT, which is in a good agreement with a phosphorus mainly in the pseudoequatorial position. Furthermore, the selectivity observed for DEPPPO is poor, indicating that both faces of the nitronyl moiety are hindered. We can reasonably assume that the steric hindrance of the phenyl group in the pseudoaxial position is more important than that of the pseudoequatorial diethoxyphosphoryl group. By trapping free radicals with DEPPPO, we obtained two diastereoisomers with different percentage contributions, and the major diastereoisomer should be the one resulting from the radical addition on the opposite face of the phenyl group. Half-life times for the diastereoisomer A of the DEPPPO−superoxide spin adduct exhibiting the smaller average phosphorus coupling (Table 1, Figure 2) were measured at two different pHs and compared to those obtained under the same conditions for the DEPMPO analogue. Our results suggest that the diethoxyphosphoryl group conformation does not have a major influence on superoxide spin adduct persistency.

Trapping of the hydroxyl radical with DEPPPO yielded an intense and persistent ESR signal corresponding to two diastereoisomer−spin adducts that decayed at different rates. It is interesting to mention that, keeping all the EPR parameters (coupling constants, line widths) equal, the calculated diastereoisomer ratio was different when the DEPPPO−OH spin adducts were generated via nucleophilic addition of water (Table 2). This observation could be used to differentiate the two modes of formation of DEPPPO−OH. However, it is necessary to mention that a good fit between experimental and calculated spectra could also be obtained using the same diastereoisomer ratio but with a different set of parameters.

Conclusion

2-(Diethoxyphosphoryl)-2-phenyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DEPPPO) was prepared according to an original four-step synthesis, and the ability of DEPPPO to trap free radicals, especially hydroxyl and superoxide, was investigated. For DEPPPO and its spin adducts the preferred geometry of the diethoxyphosphoryl group is pseudoequatorial while a pseudoaxial geometry is favored for DEPMPO and its spin adducts. We found that the DEPMPO− and DEPPPO−superoxide spin adducts have almost the same half-life time, thus indicating that the

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conformational geometry of the diethoxyphosphoryl group does not influence the decay of these phosphorylated spin adducts. The two faces of DEPPPO are hindered, and the free radical additions on the nitronyl moiety were not stereoselective. Although persistent spin adducts were observed by trapping $^{\bullet}CO_2^-$ and Ph$^{\bullet}$, the observation of an unstable signal during methyl and hydroxyethyl radical trapping remains unexplainable. Concerning the trapping of $O_2^{\bullet-}$ and HO$^{\bullet}$, DEPPPO appears as a promising lipophilic analogue of DEPMPO.

Experimental Section

Synthesis and Characterizations. (a) General. ^{1}H NMR spectra were recorded at 100 and 400 MHz in $CDCl_3$ and C_6D_6 using TMS as internal references. ^{31}P NMR (40.53 MHz) was taken in $CDCl_3$ and C_6D_6 using 85% H_3PO_4 as an internal standard with broad-band ^{1}H decoupling. ^{13}C NMR spectral measurements were performed at 100.6 MHz using $CDCl_3$ and C_6D_6 as internal standards. δ values are given in ppm and J values in hertz. Elemental analyses were determined at the University of Aix-Marseille III.

(b) 2-Phenyl-1-pyrroline (7a). Sodium azide (7.6 g, 117 mmol) was added to a solution of γ-chlorobutyrophenone (14 g, 78 mmol) and tetrabutylammonium chloride (0.5 g, 1.8 mmol) in 1,2-dimethoxyethane (60 mL). After stirring for 16 h at 75 °C, the solution was filtered on Celite 545 and washed with ether, and the solvent was evaporated under reduced pressure to yield a brown oil. To this oil diluted in dry ether (200 mL) was added triphenylphosphine (20.4 g, 777 mmol). When the nitrogen release was over, we added 100 mL of pentane, and the mixture was stirred at room temperature for 12 h. The solution was then filtered and the solvent evaporated under reduced pressure to yield a yellow oil which was purified by crystallization in pentane (7.7 g, 68%). NMR ^{1}H (100 MHz, $CDCl_3$, TMS): 1.9−2.1 (m, 2H), 2.8−2.9 (tt, 2H), 4−4.1 (tt, 2H), 7.3−7.4 (m, 3H), 7.8−7.9 (m, 2H) (Calcd for $C_{10}H_{11}N$ (145.20): C, 82.72; H, 7.64; N, 9.65. Found: C, 82.81; H, 7.62; N, 9.58).

(c) Diethyl (2-Phenylpyrrolidin-2-yl)phosphonate (7b). To a solution of **7a** (12.5 g, 86 mmol) was added diethyl phosphite (14.2 g, 100 mmol) in the presence of boron trifluoride ethyl etherate (1 mL, 8 mmol). The mixture was stirred at room temperature under nitrogen for 24 h. A 2 N HCl solution (40 mL) was added in the mixture up to pH 2. After having been extracted with dichloromethane (2 × 30 mL), the aqueous layer was treated first with sodium hydroxide and then with sodium bicarbonate to reach pH 10. After extraction with dichloromethane (4 × 30 mL), the organic layer was dried over magnesium sulfate, filtered, and evaporated under reduced pressure to yield a yellow oil which was chromatographed on a silica column (acetone/pentane 1:3) and evaporated as a colorless oil (6.6 g, 60%). NMR ^{31}P (40.53 MHz): δ 26.9 ppm (C_6D_6), 26.4 ppm ($CDCl_3$). ^{1}H (400 MHz, C_6D_6): 0.92 (3H, t, OCH_2CH_3, $^3J_{HH}$ = 7.07 Hz), 0.97 (3H, t, OCH_2CH_3, $^3J_{HH}$ = 7.07 Hz), 1.34−1.45 (1H, m, CH), 1.58−1.69 (1H, m, CH), 2,15−2,24 (1H, m, CH), 2.49 (1H, s large, NH), 2.51−2.63 (1H, m, CH), 2.79 (1H, dd, CH), 2.94−3.0 (1H, m, CH), 3.67−3.85 (2H, m, OCH_2CH_3), 3.87−3.94 (2H, m, OCH_2CH_3), 7.06−7.11 (1H, m, H_{ar}), 7.20−7.24 (2H, m, H_{ar}), 7.79−7.83 (2H, m, H_{ar}). ^{13}C (100.6 MHz, C_6D_6): 16.78−16.83 (OCH_2CH_3), 25.94 (CH_2-(4), J = 9.05 Hz), 37.33 (CH_2(3)); 47.22 (CH_2(5), J = 9.05 Hz), 62.66 et 63.04 (OCH_2CH_3, J = 7.0 Hz), 67.7 (Ph-C_2−P, J = 149.9 Hz), 127.41 (CH(9), J = 3.0 Hz), 128.36 (CH(7), J = 3.0 Hz), 128.49 (CH(8), J = 4.01 Hz), 142,7 (C(6)) (Calcd for $C_{14}H_{22}NO_3P$: 283.31 C, 59.35; H, 7.83; N, 4.94; O, 16.94; P, 10.93. Found: C, 59.37; H, 7.82; N, 4.94).

(d) 2-(Diethoxyphosphoryl)-2-phenyl-3,4-dihydro-2*H*-pyrrole 1-Oxide (DEPPPO) (7). A solution of 70% *m*-chloroperbenzoic acid (3.4 g, 14 mmol) in chloroform (30 mL) was added over a period of 1 h to a stirred solution of **7b** (2 g, 7 mmol) in choloroform (20 mL) at −5 °C. The reaction mixture was then washed with saturated aqueous sodium bicarbonate (20 mL) and sodium chloride (20 mL) solutions. The organic layer was dried over magnesium sulfate. The solvent was dried under vacuum. Column chromatography of the residue (silica, THF/EtOH 9:1 and CH_2Cl_2/EtOH 9:1) afforded **7** as a hygroscopic yellow oil (0.5 g, 25%) which solidified at low temperature. NMR ^{31}P (40.53 MHz): δ 18.22 ppm ($CDCl_3$), 18.87 ppm (C_6D_6). ^{1}H (400 MHz, C_6D_6): 0.96 (3H, t, OCH_2CH_3, $^3J_{HH}$ = 7.1 Hz), 1.02 (3H, t, OCH_2CH_3, $^3J_{HH}$ = 7.1 Hz, J_{PH} = 0.3 Hz), 1.7−1.8 (1H, m, CH), 1.91−2.0 (1H, m, CH), 2.20−2.28 (1H, m, CH), 2.94−3.06 (1H, m, CH), 3.87−3.95 (2H, m, OCH_2CH_3), 4.31−4.4 (2H, m, OCH_2CH_3), 6.57 (1H, dt, =CH, $^3J_{HH}$ = 2.8 Hz, $^5J_{PH}$ = 1.5 Hz), 7.1 (1H, m, CH_{ar}), 7.2 (2H, m, CH_{ar}), and 8.0 (2H, m, CH_{ar}). ^{13}C (100.06 MHz, C_6D_6): 16.70 and 16.76 (OCH_2CH_3, J = 11.1 and 11.0 Hz, respectively), 26.33 (CH_2-(3), J = 7.2 Hz), 33.77 (CH_2(4)), 63.10 (OCH_2CH_3, J = 7.0 Hz), 64.78 (OCH_2CH_3, J = 6.0 Hz), 82.97 (Ph−C_2−P, J = 158 Hz), 127.9 (CH(9), J = 4 Hz), 128.45 (CH(7), J = 3 Hz), 128.77 (CH-(8), J = 2 Hz), 134.41 (CH(5), J = 8 Hz), 137.75 (C(6)) (Calcd for $C_{14}H_{20}NO_4P$ (297.29): C, 56.56; H, 6.78; N, 4.71 O, 21.53; P, 10.42. Found: C, 56.36; H, 6.83; N, 4.34).

Spin Trapping Studies. (a) General. Xanthine oxidase (XOD), bovine erythrocyte superoxide dismutase (SOD), and catalase were purchased from Boehringer Mannheim Biochemical Co.; glutathione (GSH), glutathione peroxidase (Gpx), diethylenetriaminepentaacetic acid (DTPA), di-*tert*-butyl peroxide, dibenzoyl peroxide, *tert*-butyl hydroperoxide, riboflavin, and other chemicals were from Sigma Chemical Co. *S*-Nitrosoglutathione (GSNO) was kindly provided by Pr. B. Kalyanaraman from the Biophysics Research Institute in Milwaukee, USA.

(b) ESR Measurements. ESR spectra were recorded at room temperature using a computer-controlled Varian E-3 or a Bruker ESP 300 ESR spectrometer at 9.5 GHz (X-band) employing 100 kHz field modulation. Reaction mixtures were prepared in a Chelex-treated phosphate buffer (0.1 M, pH 7.4 and 8.2). Standard ESR spectra were simulated using ESR software developed by D. Duling from the Laboratory of Molecular Biophysics, NIEHS[22] (this software is available via the World Wide Web at http://www.epr.niehs.nih.gov/PEST/) and with the ESR software developed by A. Rockenbauer from the Central Research Institute of Chemistry, Hungary.[23] The UV photolysis was performed by a 1000 W xenon−mercury Oriel lamp.

(c) Superoxide Trapping. Hypoxanthine−Xanthine Oxidase System. Xanthine oxidase (0.4 U mL^{-1}) was added to a solution of DEPPPO (0.1 M), DTPA (1 mM), and hypoxanthine (0.4 mM) in phosphate buffer (0.1 M, pH 7.4). The ESR spectrum was recorded 60 s after the addition of XOD.

(d) Superoxide Trapping. Riboflavin−DTPA−Light System. This superoxide generating system contained DEPPPO (0.1 M), DTPA (4.5 mM), and riboflavin (0.1 mM) in phosphate buffer (0.1 M, pH 7.4). The ESR spectrum was recorded 2 min after irradiating the ESR cell inside the spectrometer cavity (blue light λ = 430 nm, 600 mCd).

With both superoxide generating systems, the ESR signal was inhibited by addition of SOD (0.5 mg mL^{-1}). In the presence of GSH (0.3 mM) and Gpx (10 U mL^{-1}), only the hydroxyl adduct was observed.

(e) HO$^{\bullet}$ Trapping. Fenton System. The hydroxyl radical was generated by adding $FeSO_4$ (0.5 mM) to a solution of DEPPPO (50 mM) and H_2O_2 (1 mM) in phosphate buffer (0.1 M, pH 7.4). The ESR spectrum of the hydroxyl adduct was recorded 60 s after addition of ferrous sulfate. No ESR signal was observed in the presence of catalase (50 U mL^{-1}) in the incubation mixture.

(f) Nucleophilic Addition of Water. The hydroxyl spin adduct was obtained by adding $FeCl_3$ (1 mM) to a solution of DEPPPO (50 mM) in doubly distilled water.

(g) $^{\bullet}CO_2^-$ ($^{\bullet}CO_2H$) Trapping. A Fenton system in the presence of $NaHCO_2$ was used to generate $^{\bullet}CO_2^-$ ($^{\bullet}CO_2H$). $FeSO_4$ (0.5 mM) was added to a solution of DEPPPO (50 mM), H_2O_2 (1 mM), and $NaHCO_2$ (0.1 M) in phosphate buffer (0.1

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M, pH 7.4). An ESR spectrum of the hydroxyl adduct was recorded 60 s after addition of ferrous sulfate.

(h) *t*-BuOO• Trapping. *t*-BuOO• was produced by UV photolysis of a solution of *tert*-butyl hydroperoxide (1.5 M) and DEPPPO (50 mM) in benzene.

(i) Ph• Trapping. Ph• was generated by UV photolysis of a solution of dibenzoyl peroxide (1 M) in the presence of DEPPPO (50 mM) in benzene.

(j) Thiyl Radicals Trapping. CH_3S•, PhS•, and *t*-BuS• were produced by UV photolysis of the respective dialkyl sulfide (1.5 M) and DEPPPO (50 mM) in benzene. Glutathion thiyl radical (GS•) was produced by UV photolysis of GSNO (2 mM) and DEPPPO (50 mM) in phosphate buffer (0.1 M, pH 7.4).

(k) Alkoxyl Radicals Trapping. DEPPPO/•OCH_3, DEPPPO/•OCH_2CH_3, and DEPPPO/ •O-*i*-Pr were produced by addition of a small amount of $Pb(OAc)_4$ in a benzenic solution containing DEPPPO (50 mM) and 1.5 M of the desired alcohol. *t*-BuO• was generated by UV photolysis of a solution of di-*tert*-butyl peroxide (0.15 M) and DEPPPO (50 mM) in benzene.

(l) Kinetics of Decay of Superoxide Spin Adducts. We used the hypoxanthine−xanthine oxidase system described previously to generate superoxide in phosphate buffer (0.1 M, pH 7.4 and 8.2). The spin trap concentration was 0.017 M for DEPPPO and DEPMPO. The superoxide generation was initiated by incubating xanthine oxidase in the reaction mixture for 10 min and suppressed by adding SOD (0.5 mg mL^{-1}). The spin adduct decay was followed by monitoring the decrease of an appropriate line of the spin adduct. Computer simulations were performed using the DAPHNIS program developed by R. Lauricella at Laboratoire SREP: 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-squares method was then applied to fit the calculated curves with the experimental ones. In these calculations, the monitored ESR peak intensity is related to the actual radical concentration [SA] by a scale factor. The first-order constant k_a and the product $k_b[SA]_0$ are independent of this scale factor. Half-life times are given as means ± SD ($n \geq 3$).

(m) Partition Coefficient (K_p) Determination. The lipophilicity of DEPPPO was evaluated from its 1-octanol−water partition coefficient as it was previously described by Konorev et al.[17] A solution of DEPPPO was prepared in 1-octanol at a concentration of 0.25 mM. The concentration of spin trap was measured at its optical absorption maximum ($\lambda_{max} = 245$ nm in 1-octanol and $\lambda_{max} = 238$ nm in water) using a computer-controlled UNICAM UV/visible UV4 spectrometer. Equal volumes (5 mL) of freshly prepared 1-octanol solution of DEPPPO and of doubly distilled water were vigorously mixed at 37 °C during 1 h, and the two phases were separated by brief centrifugation (3500*g*, 2 min). K_p was measured as the ratio between the absorbency of the spin trap in 1-octanol to that in water.

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