Identification of Reactive Free Radicals with a New ^{31}P-Labeled DMPO Spin Trap[†]

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The ^{31}P atom ($I = 1/2$) can provide additional useful EPR information when it is introduced into the 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin trap. 2-(Diethylphosphono)-5,5-dimethyl-1-pyrroline *N*-oxide (DEP-DMPO) has been synthesized by addition of diethylphosphono anion to DMPO followed by oxidation with copper acetate/air in 95% ethanol. DEP-DMPO is a liquid at room temperature and soluble in both aqueous and organic solutions. A variety of spin trapping experiments have been explored. The main feature of the EPR spectra of DEP-DMPO spin adducts is the relatively large doublet of triplets. The ^{31}P hyperfine splitting constant (^{31}P-hfsc or a_p) in these examples is always larger than the N-hfsc. For oxygen-centered radical adducts, *e.g.*, from HOO•, HO•, *n*-BuO•, *i*-BuO•, and isoamylO•, a_P's are 32.3–34.5 G whereas a_N's are 13.4–14.1 G in water. For acyl adducts, such as $CH_3(CH_2)_4C(O)$•, $C_2H_5C(O)$•, $CH_3C(O)$•, and •CO_2, a_P's are 38.6–39.2 G whereas a_N's are 14.3–14.8 G in water; a_P's for alkyl and hydroxyalkyl radical adducts are between 48.1 and 54.6 G in water; for the phenyl adduct the value is exceptionally small, $a_p = 31.5$ G. Most of the adducts are very persistent in aqueous solutions, *e.g.*, $t_{1/2} = 3.0$ h for HO• adduct, 31.5 h for *i*-amylO• adduct, and 52.4 h for $CH_3C(O)$• adduct in water. However, the hydroperoxyl and *tert*-butylperoxyl adducts decay rapidly: $t_{1/2} = 3.6$ s in water and $t_{1/2} = 4.3$ s in isooctane, respectively.

Introduction

Since the use of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was proposed as a spin trap of general utility,[1] DMPO has become one of the most widely used spin traps.[2] DMPO rapidly scavenges free radicals, generating relatively persistent secondary radicals, so-called spin adducts:
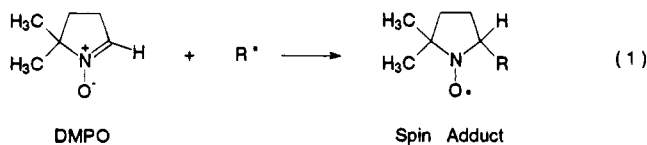
The β-H on the aminoxyl spin adduct provides remarkably suitable EPR information useful for the diagnosis of the structure of the free radical addend.[1] Unfortunately, however, the β-H is also assumed to be responsible for the degradation of the aminoxyl spin adduct:
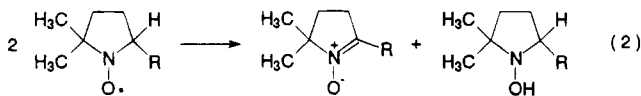
The stability of an aminoxyl spin adduct is a very important factor, within spin trapping methodology sometimes becoming the deciding factor in determining whether a spin trapping experiment is successful or not. In order to improve the stability of the spin adduct, replacement of the β-H on DMPO with an inert group is an initial consideration.[3–5] For example, the hydroxyl adduct of 2-methyl-DMPO (M_3PO)[3,4] is more stable than the hydroxyl adduct of DMPO in water.[6] 2-(Trifluoromethyl)-DMPO (TF-DMPO) can generate even much more stable aminoxyl spin adducts.[7] The phenyl, vinyl, and acyl adducts of 2-CF₃-DMPO can be heated to 110 °C in toluene and returned to room temperature with no loss in EPR signal. In order to recover the EPR information usually derived from the β-H hyperfine splitting (hfs) but now lost by substitution with a methyl and phenyl group, the ^{13}C-isotope has been introduced into some examples of DMPO-type spin traps.[8,9] Further improvements are under way to make the ^{13}C-hfsc (hyperfine splitting constant) more useful for the diagnosis of free radical structures.[10]

Recently, attention was addressed to new phosphorus-bearing nitroxides (aminoxyls)[11] and spin-labeled sugar phosphonates.[12,13] In these cases the phosphorus hfs is sensitive enough so that the stereoisomers of a phospho-

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† Dedicated to Professor Glen A. Russell on the occasion of his 70th birthday.
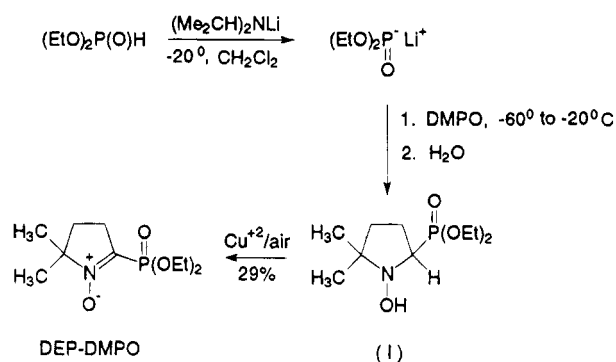‡ National Biomedical Center for Spin Trapping and Free Radicals.
§ Oklahoma Medical Research Foundation Spin Trap Source.
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Scheme 1

DEP-DMPO   (**I**)

rus-containing aminoxyl can be easily distinguished by EPR spectroscopy.[12] Therefore, it seemed that the phosphorus atom might be a potentially useful probe, sensitive to the nature and property of the free radical addend if the phosphorus atom could be introduced into the DMPO spin trap. Therefore, 2-(diethylphosphono)-5,5-dimethyl-1-pyrroline *N*-oxide (DEP-DMPO) was selected as the first member of a family of phosphorus-marked nitronyl spin traps to investigate:

DEP-DMPO
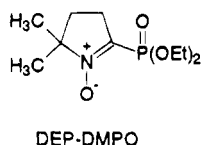
There might be several advantages for a phosphorus-labeled DMPO. First, as already mentioned, phosphorus hfs is normally large and could be very sensitive to the structure of the addend radical based on previous investigations.[11,12] Secondly, the phosphorus nuclear spin is 1/2, the same as that of the hydrogen nucleus; therefore, the EPR spectra of spin adducts derived from ^{31}P-labeled DMPO may have a simple pattern: namely a doublet of triplets. This results in convenient interpretations of EPR spectra for researchers. The third expectation is that the degradation of spin adducts of DMPO by disproportionation (see eq 2) is eliminated.

The synthetic route for the preparation of DEP-DMPO is illustrated in Scheme 1.

Experimental Section

Chemicals were purchased from Aldrich Chemical Co., Inc. DMPO was prepared in our laboratory.

Synthesis. The procedure in the literature[13] for the addition reaction of dimethylphosphono anion to nitrone compounds was adapted to our system. Under N_2, lithium diisopropylamide (LDA, 10 mL, 2.0 M solution, 20 mmol) was added dropwise to a solution of $(EtO)_2P(O)H$ (5.0 g, 36.2 mmol) in CH_2Cl_2 (40 mL) which had been precooled to −20 °C. The mixture was stirred for 15 min at −20 °C and then further cooled to −60 °C. A solution of absolutely dry DMPO (2.0 g, 17.7 mmol) in CH_2Cl_2 (4 mL) was added at this temperature. The reaction solution was allowed to warm slowly to −20 °C over a period of 3.5 h. Water (5 mL) was added to quench the reaction. The resulting solution was warmed to room temperature and then diluted with CH_2Cl_2 (100 mL). The solution was washed with NaCl-saturated aqueous solution (2 × 60 mL) and dried over Na_2SO_4. Filtration was followed by rotaevaporation. The residue was distilled to separate the excess $(EtO)_2P(O)H$ (<30 °C/1 Torr) and the desired hydroxylamine, 5-(diethylphosphono)-2,2-dimethyl-1-hydroxylpyrrolidine (**I**)

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Figure 1. EPR spectrum of the hydroxymethyl adduct of DEP-DMPO in water. EPR conditions: microwave power = 19.9 mW, receiver gain = 1 × 10⁴, modulation frequency = 100.0 kHz, modulation amplitude = 0.53 G, sweep time = 84 s, scale = 17, center field = 3473.0 G, and sweep width = 200.0 G; spin adduct from photolysis of the solution of DEP-DMPO (0.01M) and 12.5 vol % methanol in 1% hydrogen peroxide.

(2.45 g, bp 99−109 °C/1 Torr). The hydroxylamine **I** (2.45 g, 9.8 mmol) was dissolved in 95% EtOH and mixed with Cu-$(OAc)_2 \cdot H_2O$ (0.1 g) and NH_4OH (29% aqueous solution, 0.3 mL). The solution was stirred while being bubbled with air until a permanent blue remained (*ca.* 10 min). The residue after rotaevaporation was chromatographed on silica gel eluted with ethyl acetate. A liquid (1.3 g) was obtained: overall yield 29%; MS (rel int) *m/z* 250 (M^+ + 1, 5), 249 (M^+, 1), 234 (M^+ − CH_3, 6), 220 (M^+ − C_2H_5, 1), 206 (5), 204 (M^+ − OC_2H_5, 5), 168 (97), 138 ($C_4H_{10}O_3P^+$ + 1, 75), 123 (86), 112 ($C_6H_{10}NO^+$, 46), 111 (72), 108 (100), 95 (55), 82 (47), 70 (35), 68 (49), 57 (18); ^{1}H NMR (CDCl₃/TMS) δ 4.22 (quintet, $J_H = J_P = 7.7$ Hz, 4H, 2 OCH_2), 2.74 (dt, $J_H = 7.2$ Hz, $J_P = 3.1$ Hz, 2H, 3-CH_2), 2.06 (t, $J = 7.2$ Hz, 2H, 4-CH_2), 1.35 (s, 6H, 2CH_3), 1.29 (t, $J = 7.2$ Hz, 6H, 2 CH_3) ppm. Anal. Calcd for $C_{10}H_{20}NO_4P \cdot 0.75H_2O$: C, 45.71; H, 8.24; N, 5.33. Found: C, 45.74; H, 7.86; N, 5.76. GC/MS gave a single GC peak (impurities less than 1%) and a variety of fragments with largest *m/z* = 234 (M^+ − CH_3). The data are consistent with the structure of 2-(diethylphosphono)-5,5-dimethyl-1-pyrroline *N*-oxide (DEP-DMPO).

EPR Spin Trapping. The concentration of DEP-DMPO used in spin trapping experiments is 1 × 10⁻² M. When ultraviolet (UV) light was utilized to generate free radicals, the UV light beam from a 75-W high-pressure mercury UV lamp was directly focused into an EPR cavity. Generally, the sample solution was irradiated with UV light just for a few seconds. Hydroperoxyl (HOO•) radicals were generated by ultraviolet (UV) light irradiation of 30% H_2O_2. *tert*-Butylperoxyl (*t*-BuOO•) radicals were produced by UV light irradiation of concentrated *t*-BuOOH solution in isooctane (5.5 M). Hydroxyl (HO•) radicals were generated by UV light irradiation of 1% H_2O_2. *n*-Butoxyl (*n*-BuO•), isobutoxyl (*i*-BuO•), and isoamyloxyl ($Me_2CHCH_2CH_2O$•) radicals were produced by UV light irradiation of the corresponding nitrite in C_6H_6. The three spin adducts were transferred into H_2O by removing C_6H_6 and dissolving the residue into H_2O. Hexanoyl [CH_3-$(CH_2)_4C•(O)$], propionyl [$EtC•(O)$], and acetyl [$MeC•(O)$] radicals were produced by UV light irradiation of a mixture solution of the corresponding aldehyde and *t*-BuOOH in C_6H_6. After the EPR spectrum of the C_6H_6 solution had been measured, removal of C_6H_6 by vigorous bubbling with nitrogen left a residue which was dissolved in H_2O and then measured on the EPR spectrometer. UV light irradiation of $Na_2C_2O_4$ in 1% H_2O_2 generated •CO_2^- radicals. Hydroxyalkyl and 2-tetrahydrofuryl radicals were produced by UV light irradiation of the corresponding alcohol or THF in 1% H_2O_2. Methyl radicals were generated by UV light irradiation of DMSO in 1% H_2O_2, thermolysis of (*t*-BuOOCO)₂ in C_6H_6, and also decomposition of $PhCO_2OBu^t$. Decomposition of $PhCO_2OBu^t$ and (PhCOO)₂ under UV light irradiation produced also C_6H_5• radicals. *m*-ClC₆H₅• radicals were generated by decomposition of *m*-ClC₆H₄CO₃H under UV light irradiation. Additional peaks of low intensity in Figures 1 and 2 are due to an EPR active
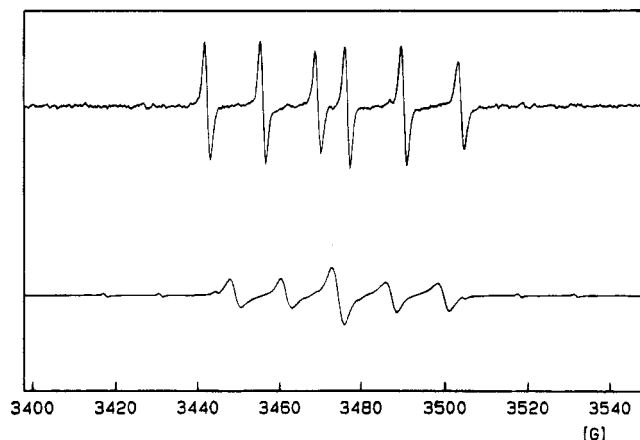
Figure 2. EPR spectrum of the n-butoxyl adduct of DEP-DMPO in water (top) and in benzene. EPR conditions: microwave power = 19.9 mW, modulation frequency = 100.0 kHz, modulation amplitude = 0.1 G, sweep time = 84 s, scale = 16, sweep width = 150.0 G, and receiver gain = 5 × 10⁵ (top) and 2 × 10⁴ (bottom); spin adduct from photolysis of the solution of DEP-DMPO (0.01 M) and 1.0 vol % n-BuONO in benzene.

Table 1. EPR Hyperfine Splitting Constants of DEP-DMPO Spin adducts in H_2O[a]

radical	a_N	a_P
HOO•	13.41	32.57
HO•	14.11	32.26
n-BuO•	13.53	34.46
i-BuO•	13.54	34.41
isoamylO•	13.48	34.15
$CH_3(CH_2)_4C•(O)$	14.35	38.64
$C_2H_5C•(O)$	14.30	39.07
$CH_3C•(O)$	14.33	39.18
•CO_2^-	14.82	38.75
HOCH₂•	14.83	50.26
HOCH•Me	14.77	52.08
HOCH•Et	14.89	52.16
HOC•Me₂	14.54	54.57
2-THFyl	14.97	50.71 A[b]
	14.60	49.68 B[b]
CH₃•	15.25	48.10[c]
	15.38	48.02[d]
	15.34	48.03[e]
C_6H_5•	15.04	31.53[f]
	14.94	31.66[g]

[a] EPR hfsc's are presented in gauss. [b] The ratio of isomer A to isomer B is 1.4:1. [c] Methyl radical source is 1% H_2O_2 + Me_2SO + UV. [d] Methyl radical resulted from (t-BuOOCO)₂. [e] Methyl radical is from decomposition of t-BuO• radical which is generated from PhCO₂OBu^t. [f] Phenyl radical is from decomposition of PhCO₂OBu^t. [g] Phenyl radical is also from decomposition of (PhCOO)₂.

impurity in DEP-DMPO itself (a_P = 87.17 G and a_N = 13.65 G in C_6H_6).

Results

Table 1 presents EPR hfsc's of 16 spin adducts of DEP-DMPO in H_2O. Table 2 collects EPR hfsc's of 10 radical adducts of DEP-DMPO in C_6H_6. The ^{31}P-hfsc in these examples is always larger than the N-hfsc. The range of ^{31}P and ^{14}N-hfsc's may be summarized as shown in Scheme 2 (SA stands for spin adduct).

Table 3 presents half-life times ($t_{1/2}$) of several spin adducts. The half-life time is the time which is taken for EPR signals to decay to half of the starting EPR signal height.

Table 2. EPR Hyperfine Splitting Constants of DEP-DMPO Spin Adducts in C_6H_6[a]

radical	a_N	a_P
t-BuOO•	12.60	31.91[b]
n-BuO•	12.47	25.79
i-BuO•	12.40	25.98
isoAmylO•	12.44	26.15
$CH_3(CH_2)_4C•(O)$	13.38	37.76
$C_2H_5C•(O)$	13.42	37.96
$CH_3C•(O)$	13.38	38.14
CH₃•	13.86	49.85[c]
	13.86	49.97[d]
C_6H_5•	13.62	34.50[e]
	13.67	34.59[f]
m-ClC₆H₅•	13.63	34.60

[a] EPR hfsc's are presented in gauss. [b] The solvent is isooctane. [c] Methyl radical resulted from (t-BuOOCO)₂. [d] Methyl radical is also generated from decomposition of t-BuO• radical which resulted from PhCO₂OBu^t. [e] Phenyl radical is from decomposition of PhCO₂OBu^t. [f] Phenyl radical is also from decomposition of (PhCOO)₂.

Discussion

(1) EPR Spectra and Hyperfine Splitting Constants for Spin Adducts of DEP-DMPO. Figure 1 shows the EPR spectrum of the hydroxymethyl adduct of DEP-DMPO as a typical example to illustrate the pattern of the EPR spectra of DEP-DMPO spin adducts. When a_P is larger than $2a_N$, the EPR spectrum of the spin adduct shows two separated sets of triplets. Most of the spin adduct EPR spectra listed in Table 1 and 2 fit this case. If a_P is very close to $2a_N$, i.e., ($a_P - 2a_N$) ≈ 1 G, and the signals are broadened, the EPR spectrum may show the pattern 1:1:2:1:1. The EPR spectra of n-butoxyl, isobutoxyl and isoamyloxyl adducts belong to this case as illustrated in the bottom spectrum of Figure 2 with the n-butoxyl adduct of DEP-DMPO as an example. One difference in comparing DEP-DMPO radical adducts with DMPO radical adducts is that no γ-H hfs is found in the former case. Alkoxyl, alkylperoxyl, and superoxide adducts of DMPO exhibit γ-H hfsc's.[1,14-16] Superoxide, n-butylperoxyl, n-butoxyl, isobutoxyl, and isoamyloxyl adducts of DEP-DMPO in water do not exhibit distinct γ-H hfs with a line width of about 1.0 G.

Radical adducts of DEP-DMPO described here have large phosphorus hyperfine splittings varying from 25 to 55 G, i.e., a 30 G range, whereas spin adducts of DMPO show β-H hyperfine splittings from 6 to 25 G, i.e., a 19 G range.[1,17] In EPR spectroscopy, the pertinent dihedral angle is directly related to the hyperfine splitting constant, e.g., a_P for DEP-DMPO adducts and a_H for DMPO adducts. The angular dependence of a_H for simple aminoxyls can be expressed as $a_H = 25-26\ \cos^2 y$[12-14] where y is the dihedral angle between the p orbital of the aminoxyl nitrogen atom and the C_2–H bond. A similar relationship for 2-phosphorus-substituted aminoxyls has been proposed, namely, $a_P = 148\ \cos^2 y$,[12] where y stands for the dihedral angle between the p orbital of the aminoxyl nitrogen atom and the C_2–P bond. From these equations, it can be concluded that a_P is five
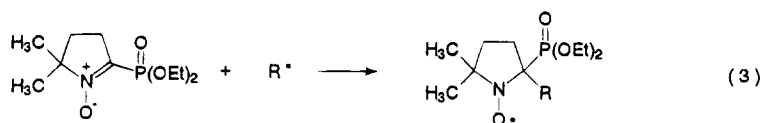
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Scheme 2

	a_N/G	a_P/G	
hydroxyl SA	14.1	32.3	in water
hydroperoxyl SA	13.4	32.6	in water
alkoxyl SA	13.5	34.1-34.5	in water
	12.4-12.5	25.8-26.2	in benzene
acyl SA (including $^\bullet CO_2^-$)	14.3-14.8	38.6-39.2	in water
	13.4	37.8-38.1	in benzene
alkyl SA	14.5-15.4	48.0-54.6	in water
	13.9	49.9	in benzene

Table 3. Half-Life Times of DEP-DMPO and DMPO Spin Adducts

spin adduct	solvent	lifetime ($t_{1/2}$)
DEP-DMPO-**OOH**	water/30%H_2O_2	3.6 s
DEP-DMPO-**OOBu**-*t*	*i*-octane/*t*-BuOOH	4.3 s
DEP-DMPO-**OH**	water/1%H_2O_2	3.0 h
DEP-DMPO-**Oamyl**-*i*	water[a]	31.5 h
DEP-DMPO-**C(O)CH₃**	water[a]	52.4 h
DEP-DMPO-**C₆H₅**	water[a]	$\gg$ 16.5 h[b]
DMPO-**OOH**[c]	water	58 s
DMPO-**OOBu**-*t*	*i*-octane/*t*-BuOOH	28 s
DMPO-**OH**	water	1.0 h
DMPO-**OBu**-*t*[d]	benzene	2.1 h
DMPO-**C₆H₅**[c]	water	hours

[a] These spin adducts were generated in C_6H_6. Removal of C_6H_6 by vigorous bubbling with N_2 left a residue which was dissolved in H_2O and then measured on the EPR spectrometer. [b] EPR spectra show no decay of the $C_6H_5^\bullet$ adduct within 16.5 h. [c] See: Janzen, E. G.; Haire, D. L. Two Decades of Spin Trapping In *Advances in Free Radical Chemistry*; JAI Press, Inc.: Greenwich, 1990; Vol 1, pp 253–295. [d] Detection of this radical adduct in H_2O was not successful due to its rapid decomposition in H_2O over the workup period.

to six times more sensitive to a variation in the dihedral angle than a_H in the β-position.

(2) Solvent Effect on EPR Spectra of DEP-DMPO Spin Adducts. For spin adducts of DEP-DMPO studied, a polar solvent such as H_2O always increases the a_N value but does not necessarily increase the a_P value although most of the adducts have larger a_P values in H_2O than in C_6H_6. However, for the $C_6H_5^\bullet$ adduct of DEP-DMPO, a_P in H_2O (31.66 G) is smaller than in C_6H_6 (a_P = 34.59 G). This result indicates that the conformation of the $C_6H_5^\bullet$ adduct in H_2O is different from that in C_6H_6.

Solvents can also dramatically change the line width of the EPR signal. As shown in Figure 2, the line width of the n-BuO$^\bullet$ adduct signal is significantly larger in C_6H_6 than in H_2O (2.6 G vs 1.2 G). This phenomenon is also observed for the i-BuO$^\bullet$ adduct and the isoamyloxyl adduct. The large line width in C_6H_6 might be partly due to some γ-H hfs from of the preferential conformation of the adduct in this solvent. The possibility of the broadening influence coming from dissolved O_2 has been

eliminated by careful bubbling of the C_6H_6 solution with N_2.

(3) Steric Hindrance on the Spin Trapping Reaction. When (t-BuOOCO)$_2$ is used as a radical source, an EPR spectrum of t-BuO$^\bullet$ adduct of DEP-DMPO might be expected because t-BuO$^\bullet$ radicals are produced in the following decomposition reaction (eq 4):

$$t\text{-BuOOCOCOOOBu-}t \rightarrow 2\ t\text{-BuO}^\bullet + 2CO_2 \quad (4)$$
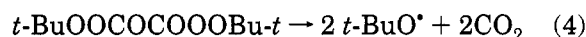
However, the spectrum was not consistent with the t-BuO$^\bullet$ radical adducts of DEP-DMPO. Instead, the EPR hfsc's of the adduct are the same as those obtained from $CH_3^\bullet$ radicals from 1% H_2O_2 + DMSO in H_2O. It is concluded that $CH_3^\bullet$ radicals are trapped by DEP-DMPO instead of t-BuO$^\bullet$ radicals. Methyl radicals are generated from β-cleavage of t-BuO$^\bullet$ radicals (eq 5):

$$t\text{-BuO}^\bullet \rightarrow CH_3^\bullet + CH_3COCH_3 \quad (5)$$
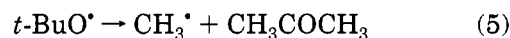
(t-BuO)$_2$ and t-BuONO are also utilized as a radical source, but no EPR signal corresponding to t-BuO$^\bullet$ adducts of DEP-DMPO was observed. The $Cl_3C^\bullet$ radical adduct of DEP-DMPO is also not found in the EPR spectrum of the solution when photolysis of $BrCCl_3$ is used as the radical source. We conclude that bulky t-BuO$^\bullet$ and $Cl_3C^\bullet$ radicals are not trapped by DEP-DMPO.

(4) Stabilities of Several Kinds of DEP-DMPO Spin Adducts. Half-life times of HOO$^\bullet$, t-BuOO$^\bullet$, HO$^\bullet$, isoamyloxyl, $CH_3C(O)^\bullet$, and $C_6H_5^\bullet$ adducts of DEP-DMPO are collected in Table 3. As shown in Table 3, hydroperoxyl and *tert*-butylperoxyl adducts of DEP-DMPO decay very rapidly. Two possibilities exist for the disappearance of hydroperoxyl and *tert*-butylperoxyl adducts. The first is that active free radicals, *i.e.*, HOO$^\bullet$($O_2^{\bullet-}$) or t-BuOO$^\bullet$ may react with aminoxyl adducts resulting in diamagnetic products. The second is that the spin adducts decompose rapidly. Hydroxyl adducts of DEP-DMPO are quite persistent. This adduct is stable enough for normal spin trapping experiments. In aqueous solution, the half-life time of the isoamyloxyl adduct (31.5 h) and the acetyl adduct (52.4 h) of DEP-DMPO is so long that DEP-DMPO might be useful for a biological spin

trapping study of a peroxidation system. For phenyl adducts, the intensity of the EPR signal does not decay in water within 16.5 h. It is reasonable to propose that other alkyl and aryl adducts of DEP-DMPO may also be very persistent.

Summary

A new ^{31}P-marked DMPO, 2-(diethylphosphono)-5,5-dimethyl-1-pyrroline *N*-oxide (DEP-DMPO), has been conveniently prepared. The spin trap can be used to study free radicals presented in both aqueous and organic solutions. The EPR spin trapping results demonstrate that the phosphorus atom is a probe very sensitive to the nature of radical addend and much more sensitive than hydrogen atom. The persistent property of its hydroxyl, alkoxyl, acyl, and C-centered radical adducts makes spin trapping experiments easily handled. Spin adducts derived from DEP-DMPO do not contain β-H. Therefore, the primary decay routes such as β-H abstraction and disproportionation do not occur in the case of DEP-DMPO spin adducts. Further challenges are still left for stabilizing superoxide and alkylperoxyl radical adducts. A research program is being conducted to discover superoxide-adduct-persistent spin traps.

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