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SYNTHESIS AND EVALUATION OF DMPO-TYPE SPIN TRAPS

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The spin traps substituted with some groups at the 4-position of dimethyl-1-pyrroline N-oxide(DMPO) were compared with DMPO itself regarding their abilities as spin traps and their physical properties. 4,5,5-Trimethyl-1-pyrolline N-oxide (4MDMPO) and 5,5-dimethyl- 4-phenyl-1-pyrolline N-oxide (4PDMPO) were synthesized by the Bonnett method, and 5,5-dimethyl-4-hydroxymethyl-1-pyrolline N-oxide (4HMDMPO) was made by a unique method from 2(5H)-furanone. The melting points of 4MDMPO, 4PDMPO and 4HMDMPO were higher than that of DMPO. The magnitude of hydrophilicity was in the order of 4HMDMPO, DMPO, 4MDMPO, and 4PDMPO based on the partition coefficient experiments in a 1-octanol—water system. Several radicals, O_2^-, HO·, $\cdot CH_3$, $\cdot CH_2OH$, $\cdot CH(CH_3)OH$, $(CH_3)_3CO\cdot$ and H· radicals, were trapped with these DMPO derivatives for comparison with the trapping by DMPO itself. Spin adducts of O_2^- with the three DMPO derivatives showed ESR spectra similar to that of DMPO. In spite of the formation of diastereomers arising from spin trapping, the line-width enlargement was very small. The intensities and the decay rates of the spectra of 4MDMPO-O_2^-, 4PDMPO-O_2^-, 4HMDMPO-O_2^- and DMPO-O_2^- were almost equal. In the trapping of the ·OH radical by 4MDMPO, 4PDMPO and 4HMDMPO, the eight-line ESR spectra observed were different from the well-known four-line spectrum of DMPO-OH.

KEY WORDS: Spin trapping, DMPO, kinetics, diastereomer, partition coefficient.

INTRODUCTION

The use of the spin trapping technique has been expanding as a valuable tool for the studies of free radical processes in biology.[1,2] In spin trapping, nitroso compounds or nitrones have been widely used as spin traps. The former is superior to the latter in trapping carbon-centered radicals from the standpoint of ready discrimination of the trapped radicals and a faster trapping rate, while nitrones are more appropriate for trapping most important oxygen-centered radicals generated biologically.

Among several nitrones used as spin traps, DMPO has attracted the most attention, because the ESR spectra of the spin adducts with either superoxide or hydroxyl radicals are characteristic. However, this spin trap still has some problems from the following viewpoints: instability, troublesome purification because of the low melting point, a low rate constant for reaction with O_2^-, the rapid decay rate of the O_2^-

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adduct, its small partition coefficient, cellular toxicities because of the high concentration requirement for this trap and so on.

For these reasons, several studies of derivatives of DMPO, which are substituted at the 3- or 5-position of DMPO, were attempted to produce some improvements.[3-8] In this paper, spin traps with methyl (4MDMPO), phenyl (4PDMPO) and hydroxymethyl (4HMDMPO) groups substituted at the 4-position were synthesized and compared with DMPO itself in regard to their abilities as spin traps and their physical properties. The synthesis and some spin trapping chemistry of 4PDMPO has been reported before.[9]

EXPERIMENTAL

Materials

Chemicals for synthesis were obtained from Wako Pure Chemical Inc. (crotonaldehyde, 2-nitropropane and trans-cinnamaldehyde), Aldrich Chemical Co.(2(5H)-furanone and diisobutylaluminum hydride[DIBAL-H]), and Kanto Chemical Co. (all solvents). Chemicals for spin trapping were purchased from Dojin Chemical Co. (DMPO and diethylenetriamino-pentaacetic acid [DETAPAC]), Sigma Chemical Co.(hypoxanthine), Boehringer Mannheim GmbH.(xanthine oxidase from cow milk), Mitsubishi Gas Chemical (hydrogen peroxide), Nacalai Tesque (di-tert-butylperoxide) and Wako Pure Chemical Inc.(iron (II) sulfate heptahydrate).

Instruments

ESR spectra were recorded on an X-band JEOL JES-RE1X spectrometer equipped with 100 kHz modulation and an ES-UCX2 universal cavity with an ES-DM1 Mn^{2+} marker at room temperature. Hyperfine splitting constants(hfsc) and g-values were measured using this outside reference marker. An aqueous quartz flat cell (effective volume 160 μl) was used as a sample cell. In the irradiation experiments, USHIO MODEL U1-501C with a 500 watt Xenon bulb was used, and in the quantitative analysis of the partition coefficients, the Shimadzu LC 6AD HPLC system was employed.

Spin Trapping Experiments

Superoxide Radical All reagents were dissolved in a 0.1 M phosphate buffer solution of pH 7.8 or in distilled water. To a mixture of 50 μl of a 2 mM hypoxanthine solution and 35μl of a 5.5 mM DETAPAC solution, 15μl of 0.2 M spin trap solutions and 50 μl of a 0.4 U/ml xanthine oxidase solution were added successively with vigorous stirring. Immediately the mixture was injected into a flat sample cell, and after about 40 sec, scanning of the magnetic field was started.

Hydroxyl Radical As the ·OH radical generation system, the so-called Fenton reaction was employed. Thus, to 75 μl of a 0.1 M phosphate buffer solution containing 1 mM of iron(II) sulfate heptahydrate and 1 mM of DETAPAC, 20 μl of 0.2 M aqueous spin traps and 75 μl of 1 mM aqueous hydrogen peroxide were added successively with vigorous stirring. Immediately the mixture was injected into a flat sample cell, and after about 40 sec, scanning of the magnetic field was started.

Other Radicals For the spin trapping experiments of the $\cdot CH_3$ radical, the $\cdot CH_2OH$ radical and the $\cdot CH(CH_3)OH$ radical, DMSO, methanol and ethanol of appropriate concentrations were added respectively to the above Fenton reaction system. For the spin trapping experiments of the $H\cdot$ atom and the $(CH_3)_3CO\cdot$ radical, methanolic solutions of 2% spin traps and 2% tributyltin hydride, and benzene solutions of 2% spin traps and 2% di-tert-butyl peroxide repectively were prepared. The solutions were then irradiated through the sample cell inserted into the cavity of the spectrometer.

Partition Coefficient Measurements

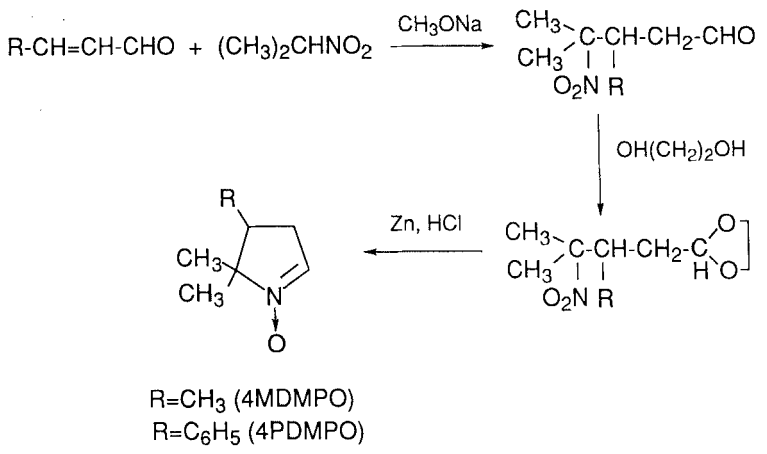
A spin trap was dissolved in 1 ml of purified water to make up concentrations of 0.2 mM, 0.4 mM, and 1 mM, and then 1 ml of 1-octanol was added to the above spin trap solutions. After vigorous stirring of the solutions by means of a table-mixer for 3 minutes at room temperature and allowing the solutions to stand for a while, the aqueous layer was analyzed by reverse phase HPLC. An arithmetic mean of three points was obtained as a concentration of the spin trap in the aqueous layer(A). By subtracting A from the parent concentration of the aqueous solution, the concentration of 1-octanol(B) was obtained. The partition coefficient(P) was afforded by dividing B by A.

Synthesis

4,5,5-Trimethyl-1-pyrroline N-oxide (4-MDMPO) 4MDMPO was prepared essentially by the Bonnett method.[10] Crotonaldehyde was allowed to react with 2-nitropropane in the presence of sodium methoxide in methanol at 60°C. After 3 hr, the reaction mixture was allowed to stand overnight at room temperature and was then acidified by adding acetic acid. After solvent evaporation, the residue was dissolved in water and extracted with ether. Upon vacuum distillation of the residue, which was obtained from the ethereal layer in the usual way, a main fraction of practically pure 3,4-dimethyl-4-nitropentanal (1) was obtained (58% yield, bp 63°C/0.25 mm Hg).

A mixed benzene solution of (1) and ethylene glycol containing a small amount of *p*-toluene sulfonic acid was refluxed in a round-bottomed flask equipped with a Dean-Stark condenser for 4 hr. Upon vacuum distillation of the residue, which was obtained from the benzene solution by a conventional method, a main fraction of practically pure 2-(2,3-dimethyl-3-nitrobutyl)-1,3-dioxolane (2) was obtained (75% yield, bp 75°C/0.08 mm Hg).

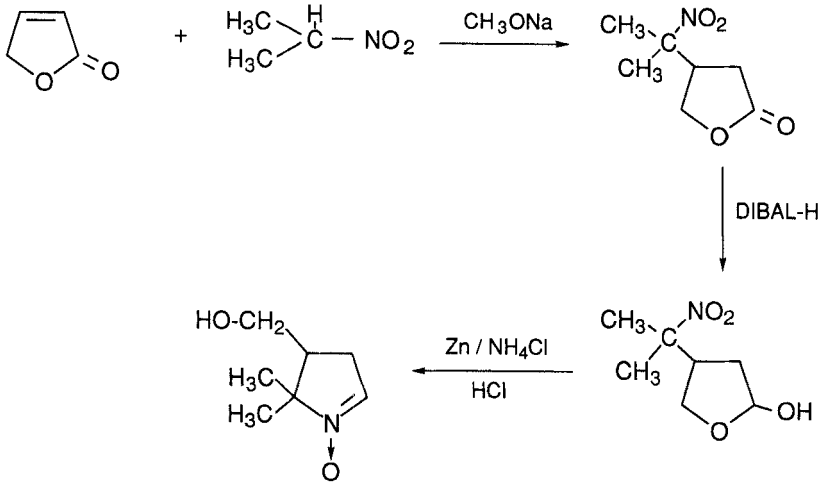
Activated zinc dust was gradually added to an aqueous solution of (2) and NH_4Cl at 15°C with brisk mechanical stirring for 30 min. The resulting precipitate was filtered off, washed with a small amount of warm water, and then the mixture of filtrate and washings was acidified with HCl and allowed to stand in a refrigerator overnight. After the solution was heated at 70°C for 40 min, it was cooled to room temperature and made alkaline with an aqueous Na_2CO_3 solution. The rough product afforded from the aqueous solution by concentrating under reduced pressure was extracted with chloroform. After conventional treatment, the residue was distilled in vacuo to give a viscous oil at 70°C/1.1 mm Hg as a main fraction which solidified immediately. After recrystallization from ethyl acetate, 4,5,5-trimethyl-1-pyrroline N-oxide (4MDMPO) was obtained (32% yield, mp 40°C).



SCHEME 1 Synthetic route of 4,5,5-trimethyl-1-pyrroline N-oxide (4MDMPO) and 5,5-dimethyl-4-phenyl-1-pyrroline N-oxide (4PDMPO).

5,5-Dimethyl-4-phenyl-1-pyrroline N-oxide (4PDMPO) 4PDMPO was also prepared by the Bonnett method.[10] *Trans*-cinnamaldehyde was allowed to react with 2-nitropropane in the presence of sodium methoxide in methanol at 65°C with stirring for 3 hr. The reaction mixture was handled in a way similar to the preparation of 3,4-dimethyl-4-nitropentanal to yield a main fraction of practically pure 4-methyl-4-nitro-3-phenylpentanal (3) (49% yield, bp 133°C/0.2 mm Hg).

The compound (3) was allowed to react with ethylene glycol in a way similar to the preparation of (2). The resulting viscous oil, which solidified immediately, was obtained at 138°C/0.17 mm Hg as a main fraction. After recrystallization from a mixed solvent of methanol-water, pure crystals of 2-(3-methyl-3-nitro-2-phenylbutyl)-1,3-dioxolane (4) were obtained (80.7% yield, mp 90°C).



SCHEME 2 Synthetic route of 5,5-dimethyl-4-hydroxymethyl-1-pyrroline N-oxide (4HMDMPO).

Reduction of (4) with zinc dust and the ring closure reaction to form the pyrroline compound were performed in a manner similar to the preparation of 4MDMPO. The resulting rough crystals were recrystallized from ethyl acetate to give pure crystals of 5,5-dimethyl-4-phenyl-1-pyrroline N-oxide (4PDMPO) (40% yield, mp 110°C).

5,5-Dimethyl-4-hydroxymethyl-1-pyrroline N-oxide (4HMDMPO) A mixture of 5 g (0.06 mol) of 2(5H)-furanone and 10 g (0.11 mol) of 2-nitropropane was added dropwise to a methanol solution of 40.5 g (0.45 mol) of 2-nitropropane containing 3.3 g (0.06 mol) of sodium methoxide for 1 hr on cooling at −20°C and was then refluxed for 3 hr. Upon cooling, the solution was acidified with acetic acid, and the solvent was evaporated. The residue was dissolved in water and extracted with chloroform. After the usual treatment of the solution, the residue was distilled in vacuo to give brown crystals. On recrystallization from hexane-ether, colorless needles of 4-(1-methyl-1-nitroethyl)tetrahydrofuran-2-one(5) were obtained (64% yield, mp 67–69°C).

A toluene solution (23 ml) of 1.5 M of diisobutylaluminum hydride was added dropwise with a syringe to a toluene solution (1 l) of 2.6 g (0.015 mol) of (5) at −70°C by means of methanol-liquid N_2 with stirring and under flowing nitrogen. After 30 min, the reaction mixture was poured into ice-water, acidified with acetic acid, and extracted with chloroform. After the usual treatment of the organic layer, the residue obtained by evaporation of the solution at reduced pressure was recrystallized from *n*-hexane–methylene chloride to give colorless needles of 2-hydroxy-4-(1-methyl-1-nitroethyl)-tetrahydro-furan(6) (1.1 g, 42% yield, mp 88–89°C).

Activated zinc dust (1.1 g, 0.017 mol) was gradually added to a mixed solution of methanol (5 ml) and water (10 ml) containing 0.8 g (0.0046 mol) of (6) and 0.25 g (0.0046 mol) of NH_4Cl with brisk mechanical stirring at an ice-water point, and the reaction was continued at this temperature. After 30 min, the precipitate was filtered off and washed with a small amount of warm water and methanol several times, and then the mixed solution of filtrate and washings was acidified with HCl and stored in a refrigerator overnight. After heating at 70°C for 40 min with stirring, the solution was cooled to room temperature and made alkaline with an aqueous solution of Na_2CO_3. After concentration of the mother liquor under reduced pressure, ethyl acetate was added to the residue to form a precipitate. This precipitate was filtered off, and on recrystallization from ethyl acetate, 5,5-dimethyl-4-hydroxy-1-pyrroline N-oxide (4HMDMPO) was obtained (0.36 g, 55% yield, mp 87°C, colorless needles).

RESULTS AND DISCUSSION

Synthesis and Physical Properties of 4MDMPO, 4PDMPO and 4HMDMPO

According to the most popular method devised by Bonnett *et al.*,[10] 4MDMPO and 4PDMPO were synthesized from crotonaldehyde and *trans*-cinnamaldehyde respectively as shown in Scheme 1. On the other hand, 4HMDMPO was synthesized by a unique method from 2(5H)-furanone as outlined in Scheme 2. This method will be available for synthesis of analogous nitrone compounds in the future. The melting points of 4MDMPO, 4PDMPO and 4HMDMPO were 40°C, 110°C and 87°C, respectively. These values are superior to that of DMPO, whose melting point is below 40°C, from the standpoints of purification, handling and stability.

In the application of the spin trapping technique, it is important to use a spin trap consistent with the environment, whether lipophilic or hydrophilic, especially in

biological studies. The value of the partition coefficient is an appropriate guide in this respect. Values of the partition coefficients of the three DMPO derivatives are shown in Table 1, compared with those of DMPO itself and phenyl N-*tert*-butylnitrone (PBN) as references. These spin traps should always be used according to the purpose of the study.

Spin Trapping of Superoxide Radical

It was expected that the three DMPO derivatives substituted at the 4-position remote from the 2-position might afford ESR spectra similar to that of DMPO on trapping the superoxide radical. In fact, values of ESR parameters similar to that of DMPO except that a little smaller values of β-hydrogen hfsc's of the 4MDMPO,4PDMPO and 4HMDMPO adducts with O_2^- were obtained as shown in Table 2. The ESR spectrum of 4MDMPO-O_2^- is shown in Figure 1 as an example. The effects of diastereomers in those spin adducts on the spectra were so small that no problems were observed.

In the decay experiments using the same concentrations of the spin traps, the ESR spectra of spin adducts of 4MDMPO, 4PDMPO and 4HMDMPO in the O_2^- generation system with hypoxanthine-xanthine oxidase, disappeared after about 8 minutes

TABLE 1
Partition coefficients of 4MDMPO, 4PDMPO, 4HMDMPO, DMPO and PBN at room temperature
Partition Coefficient in 1-Octanol–Water System

$$P = \frac{[\text{Conc. in 1-Octanol}]}{[\text{Conc. in water}]}$$

	PBN	4PDMPO	4MDMPO	DMPO	4HMDMPO
P (r.temp.)	21	9	0.3	0.1	0.01

Concentration range: 1–0.2 mM, Quantitative analysis: HPLC.

TABLE 2
Hyperfine splitting constants[a] for 4-substituted DMPO spin adducts

Trapped radical	4PDMPO			4MDMPO			4HMDMPO			DMPO		
	a_N	$a_H\beta$	$a_H\gamma$	a_N	$a_H\beta$	$a_H\gamma$	a_N	$a_H\beta$	$a_H\gamma$	a_N	$a_H\beta$	$a_H\gamma$
O_2^-	14.11	9.6	1.7	13.97	10.06	1.68	14.30	8.25	1.9	14.1	11.4	1.3
HO·	14.40	10.03	1.1	14.72	10.79	1.1	15.35	11.23	1.2	14.72	14.72	
CH_3·	15.97	17.64		16.11	18.75		16.28	18.49		16.39	23.19	
CH_2OH·	15.69	18.67		15.83	19.36		15.73	19.18		15.83	22.36	
$CH(CH_3)OH$·	15.83	18.89		15.97	19.86		15.87	19.32		15.83	22.92	
$(CH_3)_3CO$·*	13.06	6.67	2.08	13.19	7.22	2.08	13.24	6.76	2.1	13.19	8.06	1.95
H·#		22.50			21.94			24.01			18.89	
	14.17			14.44			15.59			14.44		
		15.83			16.39			17.66			18.89	

(a) In gauss at room temperature.
Solvent: H_2O, *Benzene, #Methanol.
g-Value: 2.0056–2.0062.

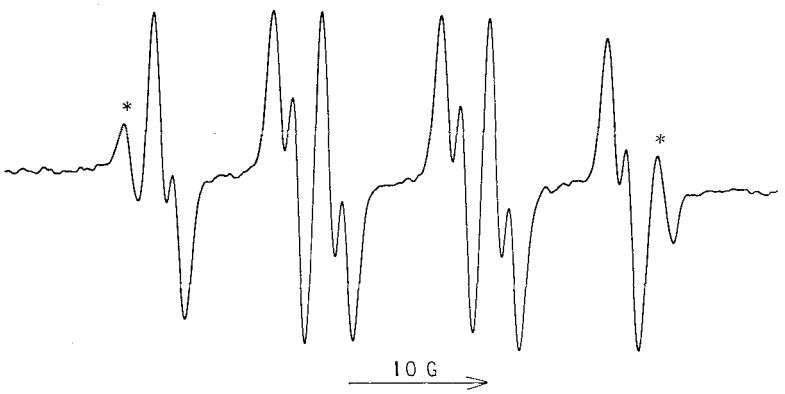


FIGURE 1 ESR spectrum of spin adduct of 4MDMPO with superoxide radical. Hfsc's are shown in Table 2. (*: unknown peaks).

in analogy with the case of DMPO. The time course of the spectrum of the 4PDMPO spin adduct of O_2^- is shown along with that of DMPO itself in Figure 2. Usually in the case of decay of the DMPO adduct, the four-line spectrum of the hydroxyl adduct gradually appears. After disappearance of the spectrum of the O_2^- adduct, only the four-line spectrum is observed. However, it is interesting that in the cases of the three DMPO derivatives, after disappearance of the spectra of their spin adducts with O_2^-, no other spectrum was observed.

Spin Trapping of Hydroxyl Radical

As is well known, the spectrum of the hydroxyl radical adduct of DMPO in water shows the characteristic four lines indicating that the hfsc of nitrogen is the same as that of β-hydrogen. The three DMPO derivatives displayed different spectra depending on the group in the 4-position. Even when photolysis of hydrogen peroxide was applied instead of the Fenton system, the same spectra were obtained. Figure 3 shows the spectra of the hydroxyl radical adduct of 4MDMPO along with the second differential spectrum. A tentative analysis, which afforded ESR parameters shown in Table 2, was performed as follows. The spectrum in Figure 3 is composed of two different spectra. One consists of a triplet of smaller β-H doublets and the additional smaller γ-H doublets observed on the second differential spectrum ($a_N = 14.72$G, $a_H^\beta = 10.79^G$, $a_H^\gamma = 1.1$G). This spectrum is perhaps due to the hydroxyl adduct. Another spectrum is composed of a triplet of larger β-H doublets ($a_N = 14.90$ G, $a_H\beta = 19.32$ G) probably due to a carbon-centered adduct of 4MDMPO. The generation of this carbon-centered radical is possibly due to the easy abstraction of the 4-position tertiary hydrogen with the hyroxyl radical. Both spectra partially overlap each other. The spectra in trapping of the hydroxyl radical with 4PDMPO and 4HMDMPO can be analyzed in the same way as 4MDMPO, and are assigned to the hydroxyl adducts as well as adducts due to carbon-centered radicals ($a_N = 15.12$ G, $a_H\beta = 18.98$ G in 4PDMPO; $a_N = 15.18$ G, $a_H\beta = 18.77$ G in 4HMDMPO). Further explorations should be performed in the future to confirm the above analysis.

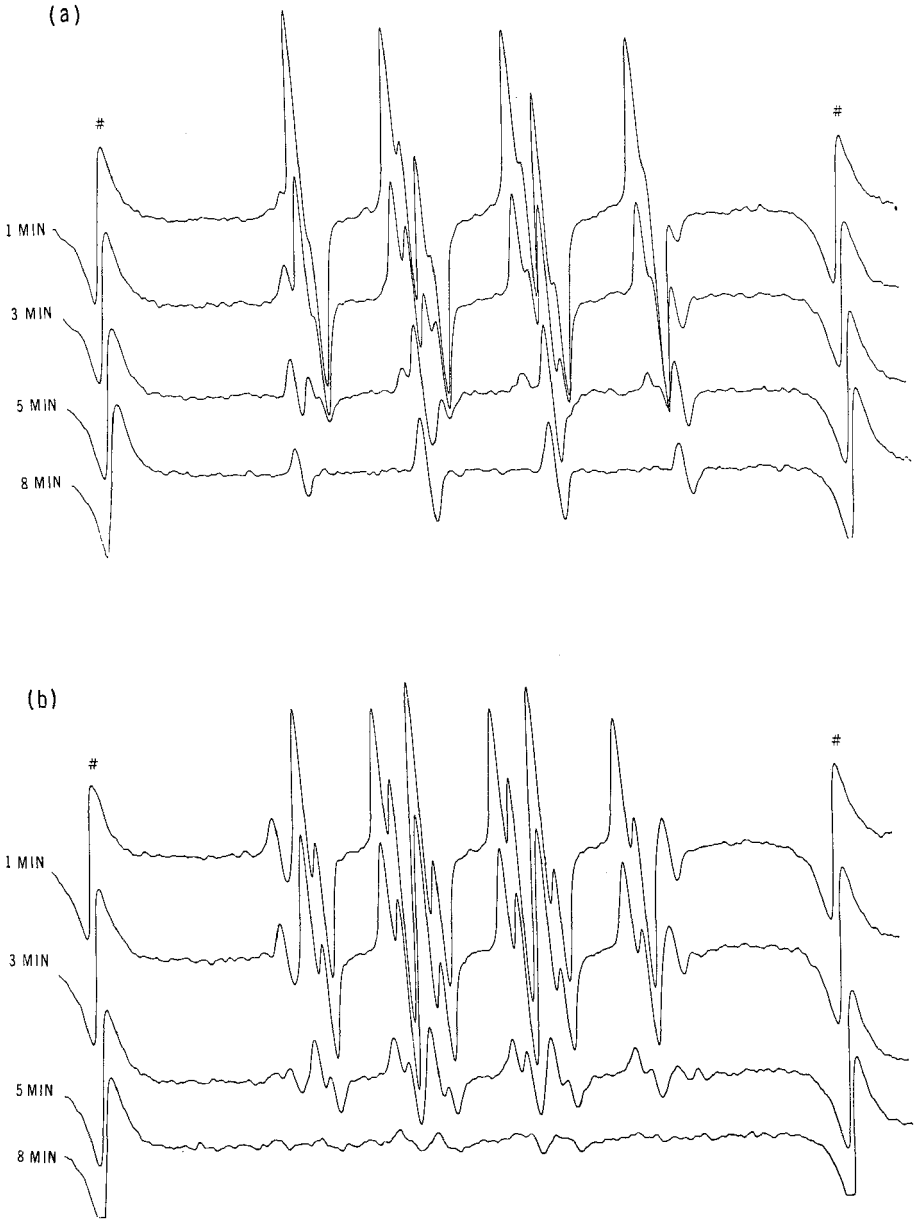


FIGURE 2 ESR spectra showing decay of spin adducts of DMPO and 4PDMPO with superoxide radical. (a) DMPO adduct (b) 4PDMPO adduct. (#: Mn^{2+} peaks).

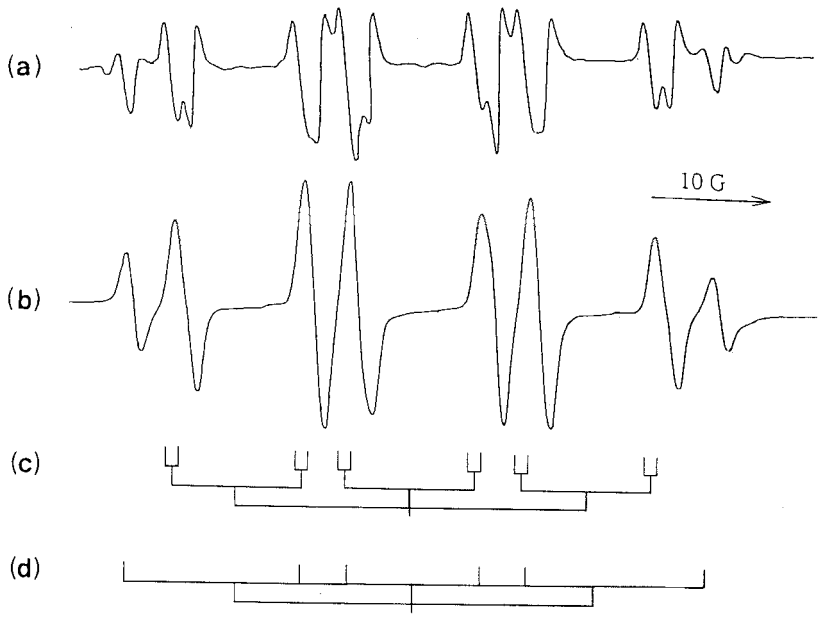


FIGURE 3 ESR spectrum of hydroxyl adduct of 4MDMPO. (a) second differential spectrum (b) first differential spectrum (c) splitting pattern of hydroxyl adduct (d) splitting pattern of carbon-centered radical adduct. Hfsc's of hydoxyl adduct and carbon-centered radical adduct are shown in Table 2 and text respectively.

Spin Trapping of Other Radicals

Various kinds of radicals, $\cdot CH_3$, $\cdot CH_2OH$, $\cdot CH(CH_3)OH$, H$\cdot$ and $(CH_3)_3CO\cdot$ radicals, were trapped with 4MDMPO, 4PDMPO and 4HMDMPO in each case in order to compare to the trapping by DMPO itself. As the ESR parameters in Table 2 indicate, the spectra of the spin adducts were almost similar to that of DMPO itself, although in trapping of the carbon-centered radicals the β-hydrogen hfsc's of the spin adducts of the three derivatives are a little smaller than those of the DMPO adducts. Some typical spectra of the spin adducts, i.e., 4MDMPO-$CH(CH_3)OH$, 4HMDMPO-$OC(CH_3)_3$ and 4HMDMPO-H, are shown in Figure 4. It was predicted that mixture spectra might be observed, because the spin adducts of the substituted DMPOs at the 4-position are diastereomers. However, in fact the deformations of the spectra were so small that practically no problems were encountered. As shown in Figure 4-(c), the ESR spectrum of the H$\cdot$ radical adduct of 4HMDMPO indicates that the two β-hydrogens at the 2-position are magnetically non-equivalent and thus show hfsc's different from that of the DMPO-H adduct (Table 2).

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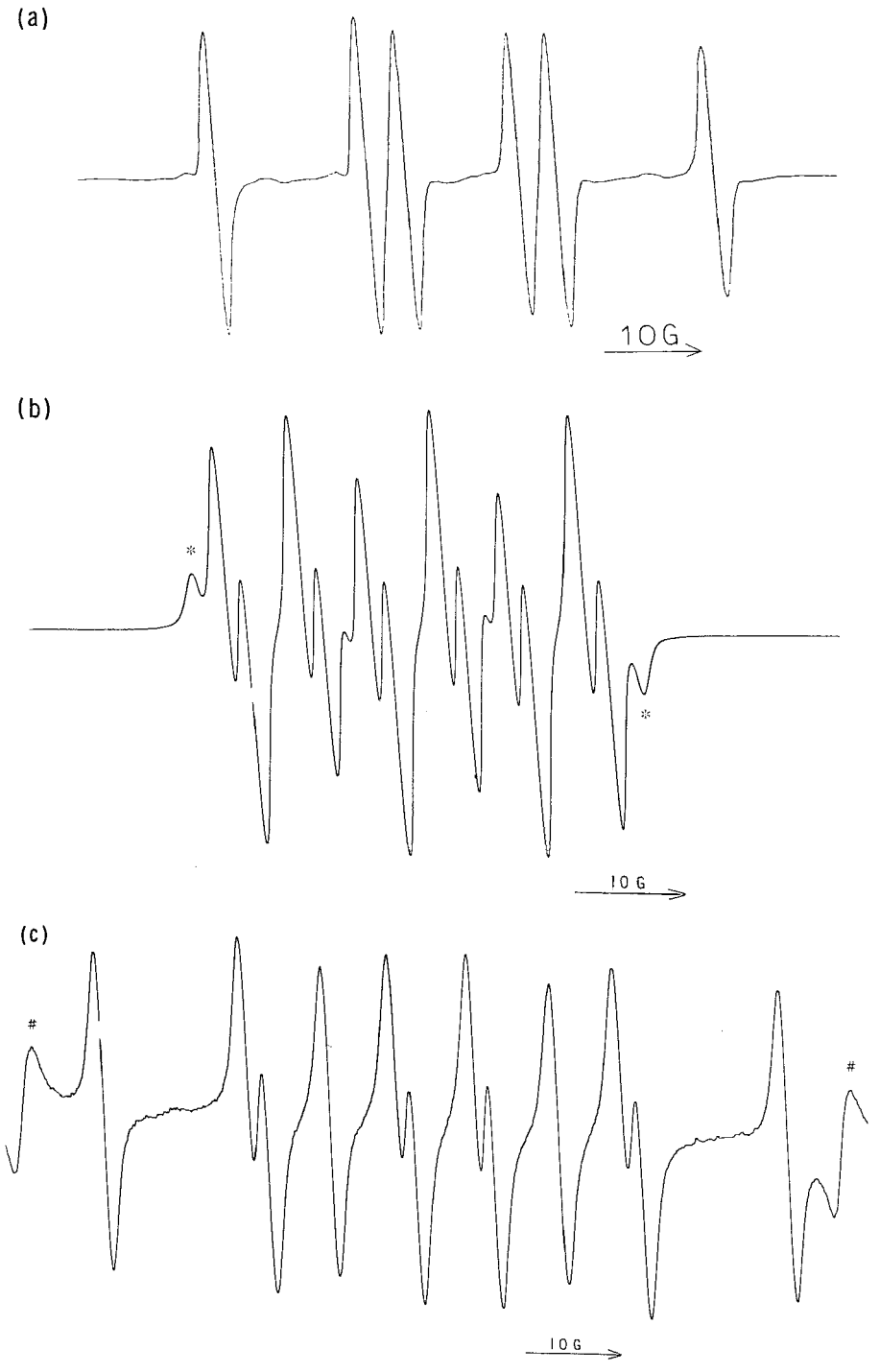

FIGURE 4 ESR spectra of some spin adducts of 4MDMPO and 4HMDMPO. (a) 4MDMPO-$CH(CH_3)OH$ (b) 4HMDMPO-$OC(CH_3)_3$ (c) 4HMDMPO-H. Hfsc's are shown in Table 2. (*: unknown peaks, #: Mn^{2+} peaks).

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