

Substituent Effect on the Rate of the Hydroxyl and Phenyl Radical Spin Trapping with Nitrones

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(Received March 18, 2002)

Hydroxyl and phenyl radical spin trapping rates by α-phenyl-*N*-*t*-butylnitrone (PBN, *N*-benzylidene-*t*-butylamine *N*-oxide) and its analogs were determined using a competitive trapping method. Hydroxyl radical was generated from hydrogen peroxide in water using UV photolysis, in the presence of the selected spin trap plus 5,5-dimethyl-pyrroline *N*-oxide (DMPO). Phenyl radical was produced with UV photolysis of tetraphenyllead, and spin trapping was performed in benzene. Spin trapping rate constants were calculated using EPR signal intensity ratios for the DMPO spin adducts vs PBN-type spin adducts. The rate constants were strongly dependent on the kind of substituent in the spin trap; the magnitude of the substituent effect was also dependent on the kind of free radicals trapped, i.e., hydroxyl or phenyl radicals. For example, in phenyl radical trapping, the spin trapping rate in hydroxy-substituted PBNs followed Hammett's equation, while there is no such correlation in hydroxyl radical trapping. Hydroxy-substituted PBNs such as 2-, 3-, and 4-hydroxy-PBNs showed much lower apparent spin trapping rates than that of PBN. The reaction between hydroxyl radical and phenoxyl group is a likely cause.

The spin trapping technique has been proved to be a useful tool in free radical chemistry for many years.[1,2] Recently, spin trapping has also been shown to be useful in biological systems, where attention is focused on trapping of superoxide and hydroxyl radicals.[3,4] The hydroxyl radical is considered to be a most damaging reactive oxygen species in biological systems, and it is detectable in some cases using electron paramagnetic resonance (EPR) spin trapping.[5] However, at present only a few spin trapping compounds (spin traps) are in practical use for hydroxyl radical detection, because the stability of the hydroxyl spin adduct is usually low.[6] The spin trap 5,5-dimethyl-pyrroline *N*-oxide (DMPO) has been most often used for hydroxyl radical detection due to the unique EPR spectrum and the stability of the spin adduct. α-Phenyl-*N*-*t*-butylnitrone (PBN, *N*-benzylidene-*t*-butylamine *N*-oxide) and its substituted-phenyl analogs are not as advantageous as DMPO for the use as hydroxyl radical spin traps, however, these compounds have shown a wide variety of therapeutic activities in animal disease models.[7] Such activities were at least in part attributed to the spin traps' hydroxyl radical trapping capabilities.

It is speculated that in biological systems nitrones that have higher hydroxyl radical trapping rates may show more potent pharmacologic activity. However, this hypothesis has not been proven yet. The objective of this study was to collect basic data for free radical trapping rate of various nitrone spin traps, which would aid in correlating the trap's biological activity to trapping rate. We determined hydroxyl and phenyl radical spin trapping rates for several substituted PBN-type spin traps, using a competitive trapping method. We utilized a photolytic method to generate free radicals, which should exert minimum influence on the rate constants to be determined. The effects of various substituents in PBN analogs on the hydroxyl radical spin trapping rates are compared with those of phenyl radical, and their mechanistic implications are discussed.

Experimental

Materials. Spin traps illustrated in Scheme 1 were used in the present study. PBN, 4-POBN, 2-SO_3-PBN, and DMPO were purchased from Aldrich Chemical Company, Inc. Other PBN-type spin traps and 2-Ph-DMPO were the gift from Dr. Edward G. Janzen.[8] Hydrogen peroxide (30%) and tetraphenyllead were obtained from Wako Pure Chemicals, and were used as sources of hydroxyl and phenyl radicals, respectively. Benzene and water were purified by distillation and used as solvents.

Free Radical Generation and EPR Measurements. Hydroxyl and phenyl radicals were generated with UV irradiation (2 second irradiation with a 75 W medium-pressure Mercury arc). For instance, hydrogen peroxide (0.2 mol dm^{-3}) and spin traps (5×10^{-3} mol dm^{-3}) were mixed in phosphate buffer (pH = 6.4) and loaded in the EPR flat cell.[4] After UV light was irradiated to the sample cell outside the cavity, EPR signals were immediately recorded with a JEOL JES-FE3XG EPR spectrometer. Benzene solution of tetraphenyllead was bubbled with nitrogen gas to deoxy-

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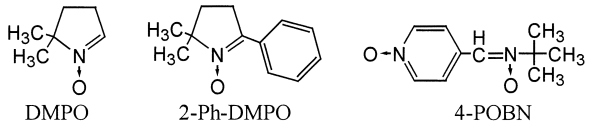
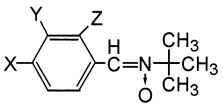


DMPO 2-Ph-DMPO 4-POBN



Spin Trap	Substituent		
	X	Y	Z
PBN	H	H	H
4-NO_2-PBN	NO_2	H	H
4-CH_3O-PBN	CH_3O	H	H
4-HO-PBN	OH	H	H
3-HO-PBN	H	OH	H
2-HO-PBN	H	H	OH
2-SO_3-PBN	H	H	SO_3Na
SALBN	COONa	OH	H

Scheme 1.

genate before UV irradiation. Spin adduct concentration was calculated using double integration of first-derivative EPR signal with the aid of a computer program (WIN-RAD system, Radical Research Inc.). The g-values were calculated using a frequency counter (Advantest TR5214) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a standard.

Results and Discussion

Spin Trapping of Hydroxyl Radical. Figure 1(1) shows the typical EPR spectrum obtained in an irradiated solution of hydrogen peroxide plus DMPO plus PBN. Two spin adduct species are visible in this spectrum: A_N = 1.53 mT and A_H = 1.53 mT assigned to •DMPO-OH (•OH spin adduct of DMPO); and A_N = 1.58 mT and A_H = 0.281 mT assigned to •PBN-OH (•OH spin adduct of PBN).[3,4] Because the lowest field lines in the EPR spectra of •DMPO-OH and •PBN-OH (arrows in Fig. 1(1)) were well separated, the integrated intensity of each line was used to determine the concentration ratio.

Spin trapping rates were determined using a competitive trapping method.[9] The reaction scheme for spin trapping of hydroxyl radical in the presence of two spin trapping compounds (for example, PBN and DMPO) is:

$$H_2O_2 \xrightarrow{h\nu} 2^{\bullet}OH$$
$$DMPO + {}^{\bullet}OH \xrightarrow{k_1} {}^{\bullet}DMPO\text{-}OH$$
$$PBN + {}^{\bullet}OH \xrightarrow{k_2} {}^{\bullet}PBN\text{-}OH$$

Then, the ratio of the first order rates of formation for •PBN-OH and •DMPO-OH adducts can be expressed as follows:

$$\frac{d[{}^{\bullet}PBN\text{-}OH]/dt}{d[{}^{\bullet}DMPO\text{-}OH]/dt} = \frac{k_2}{k_1}\frac{[PBN]_0}{[DMPO]_0}, \quad (1)$$

where $[PBN]_0$ and $[DMPO]_0$ denote the initial concentrations of spin traps. Thus, k_2/k_1 can be calculated from the concentration ratio of •DMPO-OH and •PBN-OH, i.e., the plot of the concentration ratio of spin adducts against initial concentrations of spin traps ($[PBN]_0/[DMPO]_0$) gives a line with the slope k_2/k_1. A typical plot for the PBN/DMPO system is shown in Fig. 2 with a straight line passing through the origin (correlation coefficient R = 0.99), suggesting that the calculation of relative spin trapping rate constants (k_2/k_1) using Eq. 1 is justifiable.

We determined ratios of spin-trapping rate constant (k_2/k_1) of hydroxyl radical for 10 PBN-type spin traps (Table 1). Previously, DMPO spin trapping rate of hydroxyl radical was determined using a time-resolved spectrophotometric technique, and was reported to be $k_1 = 3.6 \times 10^9$ dm^3 mol^{-1} s^{-1}.[10] Therefore, this value was utilized to calculate hydroxyl radical trapping rate constants (k_2) for the present spin traps (Table 1).

The inspection of Table 1 indicated that 2-Ph-DMPO exhibits a low rate in trapping hydroxyl radical as compared with that of DMPO. This may be attributed to steric hindrance caused by the phenyl group at 2-position, at which free radical trapping occurs. It should be noted that •OH trapping rate constants by hydroxy-substituted PBNs, such as 2-, 3-, and 4-HO-PBNs and SALBN, are much lower than others.

Although systematic tests to determine the correlation between the spin trapping rate and pharmacologic activity have

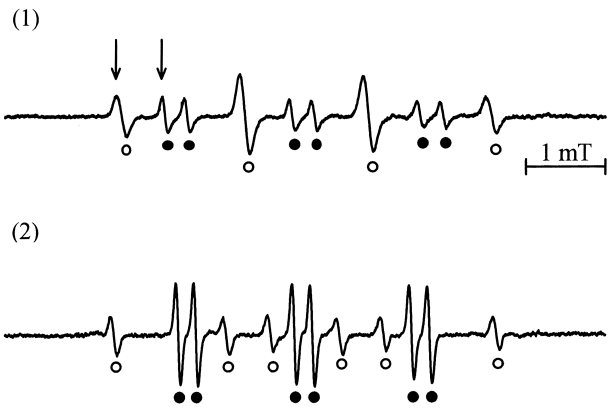


Fig. 1. (1) EPR spectrum obtained after UV-photolysis in the aqueous solution of hydrogen peroxide, DMPO, and PBN: (○) •DMPO-OH (g = 2.0059) and (●) •PBN-OH (g = 2.0057). (2) EPR spectrum obtained after UV-photolysis in the benzene solution of tetraphenyllead, DMPO, and PBN: (○) •DMPO-Ph (g = 2.0045) and (●) •PBN-Ph (g = 2.0054). Peaks marked with arrows were used to determine the concentration of spin adducts.

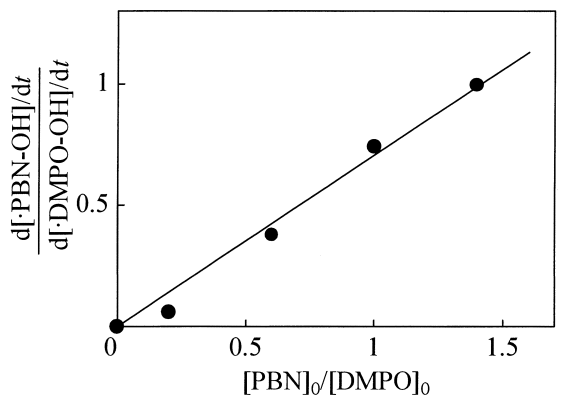


Fig. 2. The ratio of hydroxyl radical spin trapping rates for PBN and DMPO plotted as a function of the ratio of initial concentration of spin traps ($[PBN]_0/[DMPO]_0$): correlation coefficient R = 0.99.

Table 1. Hyperfine Coupling Constants (hfcc) and Rate Constants for Hydroxyl Radical Spin Trapping at 298 K

Spin Trap	hfsc/mT		k_2/k_1	$10^{-9}\ k_2$/dm^3 mol^{-1} s^{-1}	log P
	A_N	A_H			
4-POBN	1.51	0.178	1.48 ± 0.02	5.33	−0.8
2-SO_3-PBN	1.62	0.536	1.34 ± 0.02	4.82	< −1
DMPO	1.53	1.53	1	($k_1 = 3.6 \times 10^9$ dm^3 mol^{-1} s^{-1})	−1.0
4-NO_2-PBN	1.56	0.230	0.85 ± 0.01	3.06	1.4
PBN	1.58	0.281	0.71 ± 0.01	2.56	1.2
2-Ph-DMPO	1.52		0.44 ± 0.01	1.58	1.0
4-CH_3O-PBN	1.58	0.296	0.31 ± 0.01	1.12	1.2
3-HO-PBN	1.56	0.288	0.29 ± 0.01	1.04	1.0
4-HO-PBN	1.60	0.293	0.25 ± 0.01	0.90	0.6
2-HO-PBN	1.58	0.331	0.06 ± 0.01	0.22	1.3
SALBN	1.56	0.277	0.04 ± 0.01	0.14	< −1

not been performed, Hamburger and McCay compared the potencies of PBN, DMPO, and 4-POBN in the protection of rats from the lethality caused by endotoxin (lipopolysccharide) administration.[11] At optimized doses, PBN showed the highest protective action (approximately 80% protection), followed by 4-POBN (40% protection), and DMPO (25% protection), i.e., PBN > 4-POBN > DMPO. The apparent trapping rates for hydroxyl radical were: 4-POBN > DMPO > PBN (Table 1), which does not agree with the results of the animal model experiments. Although a very limited comparison is possible at present, we speculate that hydroxyl radical trapping capability is not necessarily responsible for the pharmacologic activity in this specific disease model. Moreover, hydrophobicity of the spin trap may greatly modulate its tissue concentration in vivo. The log P (where P is the 1-octanol/water partition coefficient) values are widely used as a measure of hydrophobicity.[12] In the present system, however, the trapping rates (k_2) show no appreciable correlation to log P values (Table 1).

Since the efficiency for hydroxyl radical spin trapping of 2-SO_3-PBN is high, the very low efficiency for trapping of 2-HO-PBN can not be accounted for by the steric hindrance due to 2-substituent group. As can be seen in Table 1, 3-HO- and 4-HO-PBNs as well as 2-HO-PBN show low trapping efficiency, in addition, the spin trapping rates in hydroxy-substituted PBNs did not follow Hammett's equation. Therefore, Fig. 3(1) shows a Hammett type relationship for •OH spin trapping of 4 monosubstituted PBN-type spin traps by excluding HO- and 2-substituted PBNs. The reaction constant ρ for hydroxyl radical spin trapping was 0.45.

There is a tendency that an electron-withdrawing group such as nitro group tends to increase •OH trapping rate, and an electron-donating group such as methoxy group tends to decrease it. Previously, in a spin trapping study of *t*-butoxy radical, Janzen and Evans have shown the same trend in the substituent effect.[2] Hirota et al.[13] studied the substituted PBN spin trapping reaction using molecular-orbital and molecular-mechanics calculations. They suggested that the electron densities in the lowest unoccupied molecular orbital (LUMO) of the spin trap and the highest occupied molecular orbital (HOMO) of the hydroxyl radical could be determinants for the trapping reaction rate. The positive reaction constant ($\rho = 0.45$) for hydroxyl radical spin trapping reaction obtained in this study

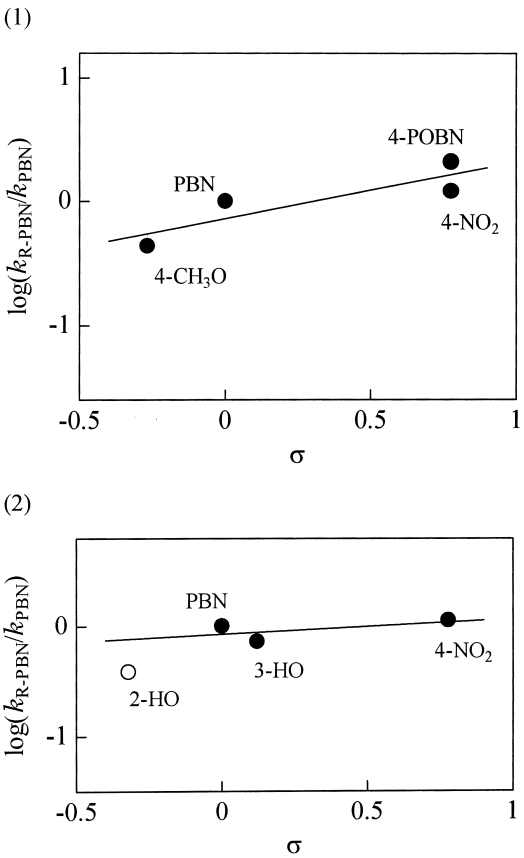


Fig. 3. Plots of logarithms of the relative rates ($k_{R\text{-}PBN}/k_{PBN}$) vs Hammett σ-constants on monosubstituted PBNs (R-PBN): (1) hydroxyl radical spin trapping and (2) phenyl radical spin trapping.

supports the notion that the nucleophilic attack of hydroxyl radical occurs at the double bond in the nitrone group.

Spin Trapping of Phenyl Radical. Phenyl radical spin trapping rates by monosubstituted PBNs were determined using a competitive trapping method. Figure 1(2) shows the EPR spectrum obtained for phenyl radical spin trapping. Hfsc's of the two spin adducts are: $A_N = 1.39$ mT and $A_H = 1.93$ mT assigned to •DMPO-Ph (phenyl radical adduct of DMPO); and

Table 2. Hyperfine Coupling Constants (hfcc) and Rate Constants for Phenyl Radical Spin Trapping at 298 K

Spin Trap	hfsc/mT		k_2/k_1	$10^{-7}\ k_2$/dm^3 mol^{-1} s^{-1}
	A_N	A_H		
4-NO_2-PBN	1.41	0.210	1.75 ± 0.09	1.35
PBN	1.43	0.220	1.56 ± 0.01	1.2
3-HO-PBN	1.44	0.231	1.13 ± 0.04	0.87
DMPO	1.39	1.93	1	0.77
2-HO-PBN	1.54	0.179	0.60 ± 0.03	0.46

$A_N = 1.43$ mT and $A_H = 0.220$ mT assigned to •PBN-Ph (phenyl radical adduct of PBN).[14,15] The relative spin trapping rate constants of phenyl radical for 4 monosubstituted PBN spin traps are listed in Table 2. Because 2-SO_3- and 4-HO-PBNs are insoluble in benzene, the rates were not measured. The absolute trapping rate constant of phenyl radical by PBN was reported as 1.2×10^7 dm^3 mol^{-1} s^{-1} in methanol,[16] and this value was used to calculate the rate constants (k_2) for PBN-type spin traps (Table 2).

It is noted that, in phenyl radical spin trapping, the low trapping efficiency that was seen in hydroxyl radical spin trapping by hydroxylated PBNs was absent. Hammett-type plot for phenyl radical trapping in benzene is shown in Fig. 3(2). The Hammett σ-constant for 2-HO-group is not available, and thus the σ-constant at 4-position is tentatively used. By this reason, we excluded 2-HO-PBN from the calculation of the reaction constant ρ, and so the slope of the Hammett plot is slightly positive, i.e., $\rho = 0.14$. It is noted that the HO-trapping rates are about 100 times faster than the Ph-trapping rates. In a kinetic study of superoxide and hydroxyl radicals, Finkelstein et al.[3] estimated the rate ratio for DMPO hydroxyl radical spin trapping (k_{DMPO}) and •OH radical hydrogen abstraction from ethanol (k_{EtOH}) as $k_{DMPO}/k_{EtOH} = 1.91$. Using the k_{DMPO} value,[10] we calculate the k_{EtOH} value as 1.89×10^9 dm^3 mol^{-1} s^{-1}, which is comparable to the •OH radical spin trapping rate of PBN (k_{PBN}) shown in Table 1. The ratio is calculated to be $k_{PBN}/k_{EtOH} = 1.35$.

•OH Radical Reaction with Hydroxylated PBN. There are many reports on hydrogen abstraction by free radicals from OH group in phenol compounds. Hogg et al.[17] studied the hydrogen abstraction by DPPH from phenols, and have shown that •OH radicals cause 1) hydrogen abstraction reaction from the OH group in hydroxy-substituted PBNs, and 2) •OH radical addition to the traps. Thus, after UV irradiation, the possible •OH radical reactions may be shown as follows:

$$HO^\bullet + DMPO \xrightarrow{k_1} {}^\bullet DMPO\text{-}OH$$

$$HO^\bullet + HO\text{-}PBN \xrightarrow{k_2} HO\text{-}PBN(^\bullet)\text{-}OH$$

$$HO^\bullet + HO\text{-}PBN \xrightarrow{k_3} {}^\bullet O\text{-}PBN$$

$${}^\bullet O\text{-}PBN + HO\text{-}PBN \xrightarrow{k_4} HO\text{-}PBN(^\bullet)\text{-}O\text{-}PBN \xrightarrow{fast} Decomposition$$

$${}^\bullet O\text{-}PBN + HO\text{-}PBN(^\bullet)\text{-}OH \xrightarrow{k_5} {}^\bullet O\text{-}PBN(^\bullet)\text{-}OH \xrightarrow{fast} Decomposition$$

$${}^\bullet O\text{-}PBN + HO\text{-}PBN \xrightarrow{k_6} HO\text{-}PBN + {}^\bullet O\text{-}PBN,$$

where •O-PBN and •O-PBN(•)-OH denote phenoxyl radicals formed by hydrogen abstraction from spin trap (HO-PBN) and spin adduct (HO-PBN(•)-OH), respectively. The reactions shown above consume originally added spin trap as well as •OH radicals, and could lead to diminishing apparent trapping efficiency by hydroxy-substituted PBN.

In a rat model, Reinke et al.[18] investigated the metabolic fate of PBN in vivo and showed that the phenyl group in PBN is hydroxylated to form 2-, 3-, and 4-hydroxy-PBNs. A majority (80%) of hydroxyl radical attack occurs on the aromatic ring of PBN rather than on the nitrone group trapping. This result was surprising, because it was thought that the hydroxyl radical attack occurs primarily at the double bond in the nitrone group. Nevertheless, hydroxy-substituted PBN should still retain free radical-trapping capabilities and phenolic group should have scavenging capability against hydroxyl radical. Since hydroxyl radicals show high reactivity with spin traps at the sites other than nitrone group sites, it is possible to speculate that free radical trapping capability may not be a unique determinant for the pharmacologic activity.

In conclusion, we determined spin trapping rates in various substituted PBNs for hydroxyl radical (in water) and phenyl radical (in benzene) using a competitive trapping method with DMPO. We show a reason why hydroxy-substituted PBN exhibits apparent low spin trapping rates. Although no correlation was found between the pharmacologic activities and trapping rate constants, this study may provide helpful models for the interpretation of biological data in the future.

We thank Dr. Edward G. Janzen (Belwood, Ontario Canada) for the gift of spin traps used in this study. Claudio Olea-Azar is grateful to the research fellowship provided by the American Chemical Society to visit the University of Oklahoma Health Sciences Center.

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