Mass Spectrometry and Electron Paramagnetic Resonance Study of Free Radicals Spontaneously Formed in Nitrone−Peracid Reactions

Hong Sang, Edward G. Janzen,* and Brian H. Lewis†

National Biomedical Center for Spin Trapping and Free Radicals, Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, Oklahoma 73104

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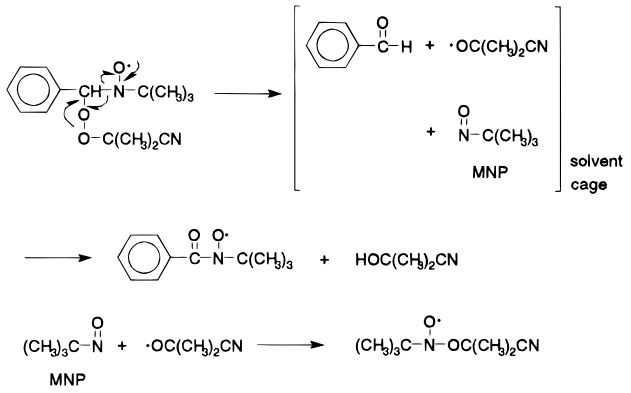
Reactions of spin traps (*C*-phenyl *N*-*tert*-butyl nitrone (PBN) and 5,5-dimethyl-2-phenyl-1-pyrroline *N*-oxide (2-Ph-DMPO)) with peracids have been investigated by both mass spectrometry (MS) and electron paramagnetic resonance (EPR). The peracids *m*-chloroperbenzoic acid, perbenzoic acid, and perpropionic acid, which can be considered models of biological peracids produced during lipid peroxidation, were found to react with spin traps to spontaneously produce significant amount of aminoxyl radicals. The radical products, as well as the nonradical products were detected and their structures identified by EPR and/or MS. Mechanisms for the formation of these products are proposed.

Introduction

C-Phenyl *N*-*tert*-butyl nitrone (PBN) is known to produce aminoxyl (nitroxide) spin adducts from the addition reaction with reactive free radicals.[1−3] Since almost all reactive radicals are trapped by PBN it has been assumed that this compound should be a good inhibitor for lipid peroxidation. The complete absence in the literature of reports on this topic must mean that this is not true. In fact in a homogeneous chemical lipid peroxidation system, PBN has no inhibitory effect whatsoever.[4] And yet we know that the peroxyl radical intermediates in this system are trapped by PBN.[5] How can inhibition be lacking if radical trapping occurs?

One explanation is that the peroxyl radical spin adduct (e.g. first molecule in Scheme 1) is itself unstable and decomposes to give more free reactive radicals. We have provided evidence for this intrinsic instability of peroxyl adducts by studying the products of 2-cyanoprop-2-yl radicals in the presence of oxygen.[6] Indeed the 2-cyanoprop-2-ylperoxyl radical spin adduct of PBN produces 2-cyanoprop-2-yloxyl radicals presumably by two-bond cleavage. Some of these radicals may be quenched in the solvent cage by reacting with the benzaldehyde formed in the decomposition presumably by cleavage of two bonds, since the combination of the benzoyl radical with 2-methyl-2-nitrosopropane (MNP) produces the well-known benzoyl *tert*-butyl aminoxyl or PBNOX. Also some 2-cyanoprop-2-yloxyl radicals are trapped by MNP and the resulting alkoxyl *tert*-butyl aminoxyl is detected by EPR (Scheme 1).

Scheme 1



In rat liver microsomal dispersions, lipid peroxidation is inhibited by PBN but not very efficiently. In a preliminary study we have reported that millimolar amounts of PBN are necessary for inhibition to be detected.[7] Clearly a very poor understanding exists for the inhibitory effect, or lack thereof, of PBN and other nitrones of this type. Further studies are underway in these laboratories on this topic.

In this connection we have considered the possibility that peracids themselves may react with nitrones spontaneously to produce reactive free radicals. Since peracids are not free radicals this reaction would be a case of nonradical molecular combination of peracid with a nitrone to produce radicals: a kind of molecule-induced free radical formation.[8] Since aldehydes produce peracids in the presence of oxygen or with some other oxidizing agents, any lipid peroxidation process producing aldehydes is also unavoidably producing peracids. The question we wanted to answer was, do these peracids in biological systems spontaneously produce reactive free radicals with PBN?

We have reported a preliminary study on this point using commercially available *m*-chloroperbenzoic acid (CPBA) as a model peracid.[9] Based on EPR results alone,

* To whom correspondence should be addressed. Tel. 405-271-7570; Fax 405-271-3980. Alternate address: Departments of Clinical Studies and Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, N1G2W1, Canada.

† OMRF Fleming Scholar, 1995 summer.

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(2) Perkins, M. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 1.

(3) Janzen, E. G. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press, Inc.: New York, 1980; pp 115−154.

(4) Unpublished results with Prof. Ross Barclay, Mt. Allison University.

(5) McCay, P. B.; Lia, E. K.; Poyer, J. L.; DuBose, C. M.; Janzen, E. G. *J. Biol. Chem.* **1984**, *259*, 2135−2143.

(6) Janzen, E. G.; Krygsman, P. H.; Lindsay, D. A.; Haire, H. D. *J. Am. Chem. Soc.* **1990**, *112*, 8279.

(7) Janzen, E. G.; West, M. S.; Poyer J. L. In *Frontiers of Reactive Oxygen Species in Biology and Medicine*; Asada, K., Yashikawa, T., Eds.; Amsterdam: Elservier Science Publishers, 1994; pp 431−434.

(8) Pryor, W. A. *Free Radicals*; McGraw-Hill: New York, 1966; pp 113−126.

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it was shown that free radicals are formed when CPBA is mixed with PBN in benzene or acetonitrile.

This paper describes a more detailed study of this system utilizing both mass spectrometry (MS) and EPR so that nonradical products as well as radical products can be detected and the structures identified. Also in addition to CPBA, perbenzoic and perpropionic acids produced *in situ* from their aldehyde precursors are investigated. Since perpropionic acid is an aliphatic peracid, the study of this system is relevant to biological peracids formed from lipid aldehydes produced in lipid peroxidation.

Experimental Section

Chemicals. *m*-Chloroperbenzoic acid (CPBA) (purity 57−86%, remainder *m*-chlorobenzoic acid and water), benzaldehyde, benzophenone (99%), and benzoyl peroxide were purchased from Aldrich. Propanal was obtained from Eastman Organic Chemicals. TEMPO was obtained from Sigma. All solvents used were HPLC grade. PBN, *p*-cyano-PBN, *p*-methoxy-PBN, PBN-d_{14}, 2-phenyl-DMPO, nitronyl-^{13}C labeled 2-Ph-DMPO, and MNP were obtained from OMRF Spin Trap Source (Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104).

Peracids. Three peracids were used in the nitrone−peracid reactions: *m*-chloroperbenzoic acid (ClC_6H_4COOOH), perbenzoic acid (C_6H_5COOOH), and perpropionic acid (C_2H_5COOOH). Perbenzoic acid was obtained but not isolated from a reaction between O_2 and benzaldehyde in the presence of UV light. After UV irradiation, the remaining benzaldehyde was washed away by hexane; then the residue was dissolved in benzene or acetonitrile. Because of the poor UV light absorption, perpropionic acid was obtained, but not isolated, by UV irradiation of propanal in the presence of benzophenone and O_2. Concentrations of the peracids were determined by NaS_2O_3/NaI titration,[10] using a 1.5 g of NaI, 50 mL of H_2O, 5 mL of glacial acetic acid, and 5 mL of chloroform mixture with a known volume of peracid solution. The mixture was then titrated with 0.1 M NaS_2O_3, where 1 mL = 0.0086 g of CPBA, 0.0069 g of perbenzoic acid, or 0.0044 g of perpropionic acid. By titration, the purity of CPBA used in this work was shown to be 80%. Calculated concentrations are listed below:

perbenzoic acid	perpropionic acid
a (mM) 6.16 ± 0.49	1.28 ± 0.03
b (mM) 17.50 ± 0.35	3.83 ± 0.77
c (mM)	21.60 ± 0.60
d (mM) 3.50 ± 0.07	4.32 ± 0.12

where *a* is the initial concentration present in the aldehyde, *b* is the concentration after 15 min of UV irradiation in the presence of O_2, *c* is the concentration after 15 min of UV irradiation in the presence of benzophenone and O_2, and *d* is the concentration used in PBN reaction.

Product Yields. Product yields were estimated from integrating EPR spectra which were calibrated by standard solutions of TEMPO ranging from 5×10^{-7} mM to 1×10^{-5} mM. Results presented throughout are an average of three independent experiments.

Electron Impact Ionization (EI) Measurements. EI mass spectra were obtained with a VG-Fisons Quattro triple stage quadrupole mass spectrometer. A direct insertion probe was used. Source temperature was 180 °C. The electron energy employed was 70 eV. The probe temperature was about 60 °C.

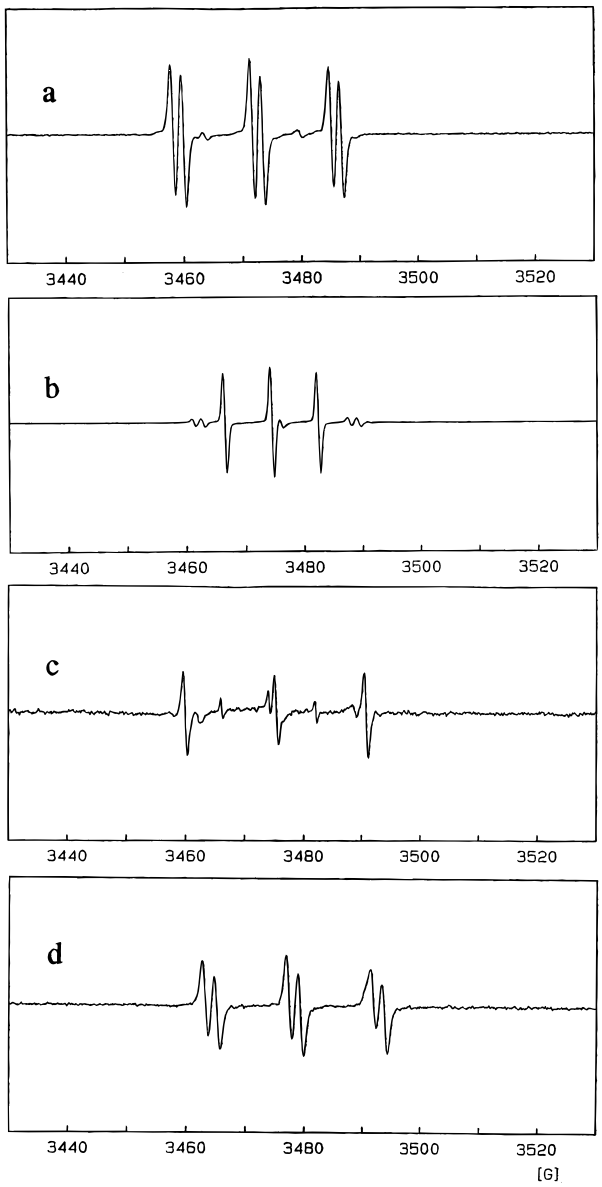

Figure 1. EPR spectrum obtained from 20 mM PBN and 1.74 mM CPBA in the absence of air at room temperature (a) in CH_3CN; (b) in benzene; (c) in benzene 3 days after the spectrum a was recorded; (d) in benzene 5 days after the spectrum a was recorded.

MS/MS Measurements. Argon gas was used for the collision-induced dissociation (CID). The collision energy was 100 eV.

EPR Measurement. EPR spectra were measured in a Bruker ER300 spectrometer with 100 KHz field modulation. Round quartz sample cells with *3.5 mm* i.d. were employed for benzene solutions, and flat quartz cells were used to record acetonitrile solutions.

Results

PBN-CPBA Systems. In an acetonitrile solution, EPR spectra of PBN−CPBA products consist of a triplet of doublets as the main component, as shown in Figure 1a. The N- and β-H hyperfine splitting constants (hfsc's) are indicative of *m*-chlorobenzoyloxyl adduct of PBN, **1,** as the major product; a_N = 13.43 G, $a_{\beta H}$ = 1.76 G.[11] A minor product in this solvent has only three lines due to

(9) Janzen, E. G.; Lin, C. R.; Hinton, R. D. *J. Org. Chem.* **1992**, *57*, 1633.

(10) Vogel, A. *Vogel's Textbook of Practical Organic Chemistry*, 4th ed.; Longman: London and New York, 1978; pp 307−308.

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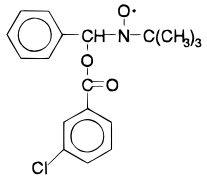
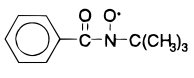
the well-known benzoyl *tert*-butyl aminoxyl radical (PBNOX), **2**, N-hfsc = 8.01 G.



1

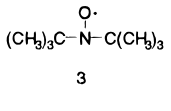
m.w. 332, 334



2

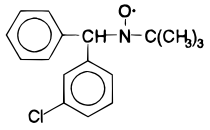
m.w. 192

In benzene the initial major product is **2** (Figure 1b). Evidence of further radical formation is found with time (Figure 1, parts c and d). As shown in Figure 1c, another set of three lines is clearly evident after 3 days in benzene with a N-hfsc of 15.44 G. This triplet is assigned to di-*tert*-butylaminoxyl, **3**, because this hyperfine splitting pattern is not changed by using the α-^{13}C-PBN instead of PBN. The spectrum recorded after 5 days (Figure 1d) consists solely of a triplet of doublets with larger values for the N- and β-H hfsc's (a_N = 14.24 G, $a_{\beta H}$ = 2.04 G) than for the comparable **1**. This spectrum is tentatively assigned to the *m*-chlorophenyl adduct of PBN, **4**, produced by decarboxylation of *m*-chlorobenzoyloxyl radicals.



3

m.w. 144



4

m.w. 289, 291

MS presents further evidence for the formation of the above radical products. Because of the existence of the isotopes of ^{35}Cl (75.8%) and ^{37}Cl (24.2%), ions containing the chlorine atom can be recognized in the mass spectrum by observation of the isotope peaks. An ion containing one chlorine atom gives two peaks with relative intensity 100:32.5.[12] Therefore the presence of peaks at *m*/*z* 332 and 334 in Figure 2a indicates the formation of spin adduct **1.** The assignment of the peaks at *m*/*z* 331 and *m*/*z* 333 will be discussed later (see below). Peaks at *m*/*z* 276 and 278 in Figure 2a are the result of the loss of isobutylene from the molecular ions ($M^{\bullet+}$) by internal hydrogen transfer (McLafferty-like rearrangement).[13] Peaks at *m*/*z* 260 and 262 are the result of loss of both isobutylene and oxygen from $M^{\bullet+}$.[14]

Because PBN spin adducts usually produce *m*/*z* 57 as an abundant fragment, a precursor ion spectrum of *m*/*z* 57 (Figure 2b) is recorded during tandem mass spectrometry (MS/MS) analysis to find ions that are potentially due to PBN spin adducts from a mixture sample. Peaks at *m*/*z* 332 and 334 are due to **1**. The presence of the peak at *m*/*z* 193 is an indication of **2** plus a proton, **5**. Other peaks are probably due to fragments or peaks from nonradical species. The assignment of *m*/*z* 193 is further confirmed by observation of fragments at *m*/*z* 137 [M − C_4H_8]$^+$, *m*/*z* 105 [C_6H_5CO]$^+$ and *m*/*z* 57 [$C_4H_9^+$], which are produced by the collision-induced dissociation (CID) of ion at *m*/*z* 193 (Figure 2c).

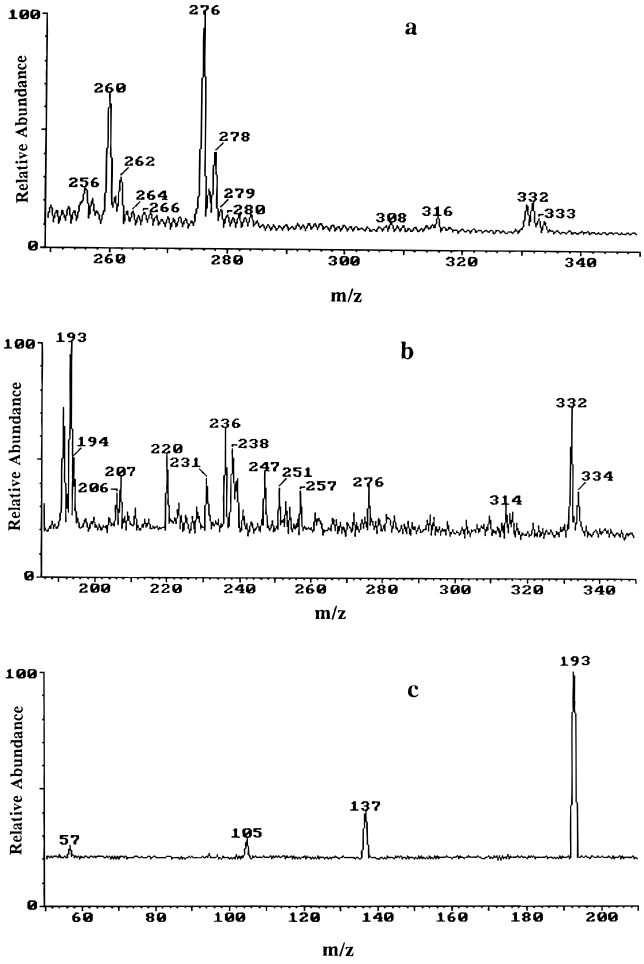
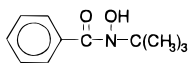


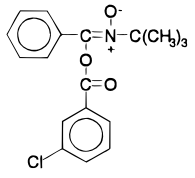
Figure 2. (a) The mass spectrum recorded from the reaction mixture of 20 mM PBN and 1.74 mM CPBA in CH_3CN in the absence of air at room temperature. (b) The precursor ion spectrum of *m*/*z* 57 recorded from the same system as in a. (c) The CID spectrum of *m*/*z* 193 recorded from the same system as in a.

MS also provides evidence for another previously undetected product of the PBN−CPBA system, namely, *m*-chlorobenzoyloxyl PBN, **6**, which has only one mass dalton less than **1**. The nonradical product, **6**, is not identified by EPR. MS, however, confirms its structure in Figure 2a, where peaks at *m*/*z* 331 and 333 are appropriate molecular ions for **6**. The nitrone **6** must come from oxidation of the initially formed spin adduct **1**.



5

m.w. 193



6

m.w. 331, 333

To aid in the assignment of structure **6**, deuterated PBN (phenyl and *tert*-butyl perdeuterated—not the nitronyl hydrogen, PBN-d_{14}) was used. After using PBN-d_{14}, the molecular ions at *m*/*z* 332 and 334 of **1** in Figure 2a shift 14 daltons to *m*/*z* 346 and 348, respectively; molecular ions at *m*/*z* 331 and 333 of **6** also shift 14 daltons to *m*/*z* 345 and 347, respectively, which indicates

(12) MaLafferty, F. W. *Interpretation of Mass Spectra*; 3rd ed.; University Science Books: Mill Valley, CA, 1980; p 16.
(13) Janzen, E. G.; DuBose, C. M. *Anal. Lett.* **1993**, *26*, 2661.
(14) Janzen, E. G.; Sang, H.; Kotake, Y.; Arimura, M.; DuBose, C. M.; Poyer, J. L. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 847.

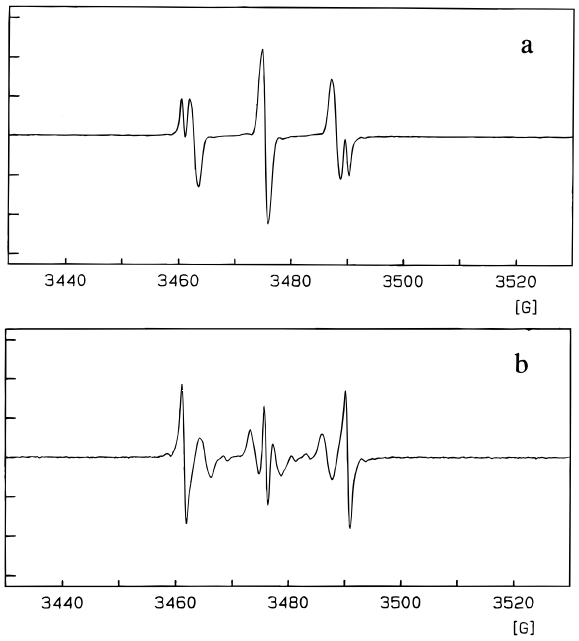


Figure 3. EPR spectrum obtained from 20 mM (a) 2-Ph-DMPO and (b) α-^{13}C-2-Ph-DMPO and 1.74 mM CPBA in the absence of air at room temperature.

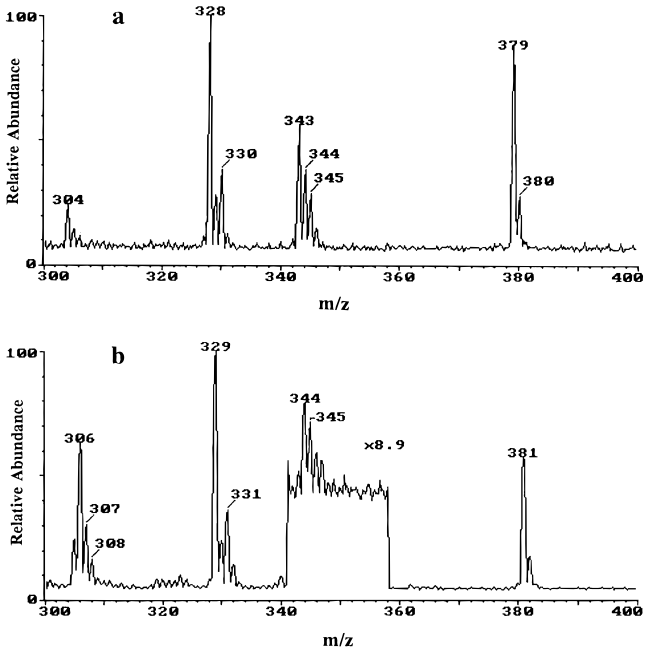
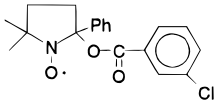


Figure 4. The mass spectrum recorded from the reaction mixture of 20 mM (a) 2-Ph-DMPO; (b) α-^{13}C-2-Ph-DMPO and 1.74 mM CPBA in benzene in the absence of air at room temperature.

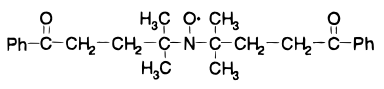
that the β-H must be lost from structure **1** to form structure **6**. If this was not the case, a 13 daltons shift should be observed after using PBN-d_{14} because the loss of deuterium atom causes 2 mass dalton change.

2-Ph-DMPO−CPBA System. 2-Ph-DMPO reacts with CPBA in a similar way to that of PBN. The EPR spectrum recorded from this system shows two triplets in benzene (Figure 3a) with hfsc's a_N = 14.35 G and a_N = 12.80 G, respectively. The signal with hfsc a_N = 12.80 G is assigned to the *m*-chlorobenzoyloxyl adduct of 2-Ph-DMPO, **7**, based on its hfsc value.[15] The assignment of the second triplet was made based on the use of α-^{13}C-2-Ph-DMPO[15] instead of 2-Ph-DMPO. The α-^{13}C-2-Ph-DMPO gives more hyperfine splitting information due to the existence of the α-^{13}C.[16] When α-^{13}C-2-Ph-DMPO was used, the EPR spectrum (Figure 3b) consists of one triplet of doublets with hfsc's a_N = 12.80 G and $a_{\beta^{13}C}$ = 3.76 G and one triplet with a_N = 14.35 G. Because no ^{13}C hyperfine splitting was observed for the second nitroxide, this radical is assigned to the structure **8.** The mechanism of formation of this radical is probably similar to that of **3** in the PBN−CPBA system (see Discussion).



7

m.w. 344, 346

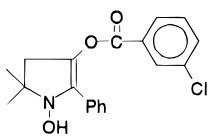


8

m.w. 380

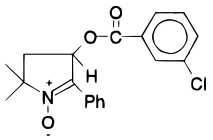
The assignments of structure **7** and **8** are also confirmed by MS experiments. Figure 4a is the mass spectrum recorded from the system of 2-Ph-DMPO and CPBA in benzene. The isotope peaks at *m*/*z* 344 and 346 are due to **7**. The ion at m/z 379 is considered to be the product **8** minus a proton, which is further confirmed by the observation of 2 mass daltons shift after using of α-^{13}C-2-Ph-DMPO instead of 2-Ph-DMPO (Figure 4b).

In addition to the identification of the above free radical adducts, another nonradical product is also identified by MS, much like the PBN−CPBA system. A significant relative abundance of *m*/*z* 343 and *m*/*z* 345 ions which have 1 mass dalton less than **7** was observed in Figure 3a. Unlike PBN, here no β-H exists in spin adducts of 2-Ph-DMPO which can be lost. This product might be due to structure **9a** or **9b**.



9a

m.w. 343, 345



9b

m.w. 343, 345

The peaks at *m*/*z* 328 and *m*/*z* 330 are considered to be fragments of *m*/*z* 343 and *m*/*z* 345 probably produced from **7** by loss of oxygen. All these assignments are supported by the experiment using α-^{13}C-2-Ph-DMPO (see Figure 4b).

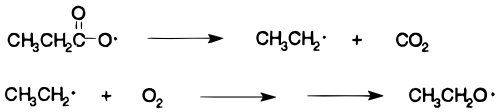
PBN-Perbenzoic Acid System (in Situ). Perbenzoic acid behaves in much the same way as CPBA when mixed with PBN. Analysis of the EPR spectrum indicates the formation of the benzoyloxyl adduct and PBNOX. The hfcs's of the benzoloxyl adduct are a_N = 13.39 G, $a_{\beta H}$ = 1.50 G in benzene;[11] the hfsc of PBNOX is a_N = 8.01 G.

PBN-Perpropionic Acid System (in Situ). Perpropionic acid produces the EPR spectrum shown in Figure 5, thus confirming that aliphatic peracids also react with PBN to form free radical products. When benzene or CH_3CN is used as a solvent, the EPR spectrum shows two triplets of doublets. The main spin adduct formed in this system has hfcs's as follows: a_N = 13.95 G, $a_{\beta H}$ = 2.08 G in benzene. These values are consistent with an

(15) Janzen, E. G.; Zhang, Y.-K.; Haire, D. L. *J. Am. Chem. Soc.* **1994**, *116*, 3738.

(16) Haire, D. L.; Oehler, U. M.; Krygsman, P. H.; Janzen, E. G. *J. Org. Chem.* **1988**, *53*, 4535.

alkoxyl adduct of PBN.[17] The minor component has hfcs's typical of alkyl radical adducts: $a_N = 14.60$ G and $a_{\beta H} = 3.25$ G.[18] We suggest that these two species are the ethoxyl and ethyl spin adducts, respectively, produced from the decarboxylation of the propionoyloxyl radicals and production of alkoxyl radicals from alkylperoxyl radical intermediates. These results are preliminary, and further work is planned to understand systems involving aliphatic peracid and PBN.



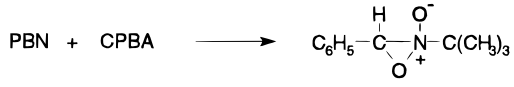
Product Yields. Acetonitrile and benzene produced significant differences in the initial yields of product **1** and **2**, as shown in Table 1. Product **2** is the major initial product formed in the benzene system. The concentration of **1** is increased with the use of acetonitrile, while oxidized PBN yield is drastically lowered.

Para-substituted PBN's display different effects in the reaction with CPBA. With electron-withdrawing substituents, e.g. CN-PBN, very small amounts of either radical are produced. When an electron-donating substituent is used, e.g. CH_3O-PBN, the highest total yield of **1** and **2** is found compared to CN-PBN or PBN.

With an increase of CPBA concentration in the region of 0.5 mM to 3 mM, the product yields also increase. The presence of air changes product amounts. In the presence of air, a larger amount of PBNOX is formed in benzene. Thus in benzene, 2-Ph-DMPO yields more *m*-chlorobenzoyloxyl adduct than PBN (results shown in Table 1).

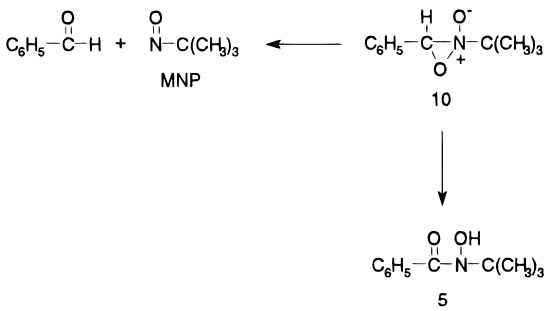
Discussion

The mechanism of reaction between a peracid and a nitronyl function should first of all include an "epoxidation". Since imines react with peracids to produce oxaziranes, nitrones might be expected to produce oxazirane N-oxides:



10

The chemistry of **10** is unknown, but hydride rearrangement or cleavage could account for some of the products observed:

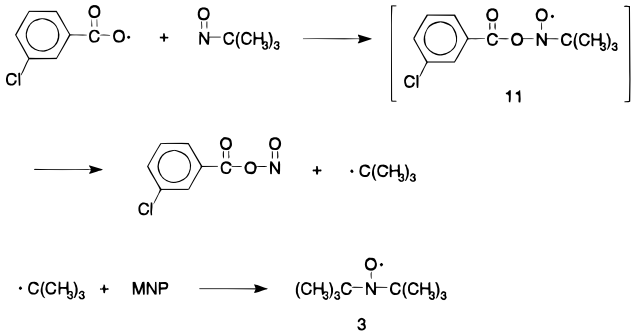


MNP

10

5

The production of large amounts of di-*tert*-butylaminoxyl, **3**, can come from reactions of MNP with acyloxyl radicals since the spin adduct is not stable, producing *tert*-butyl radical as one of the cleavage products:

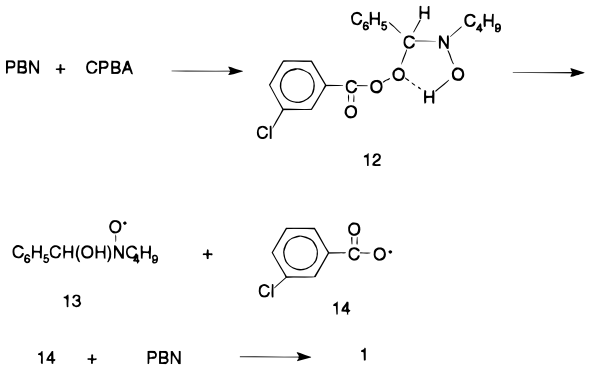


11

3

Indeed when benzoyl peroxide in benzene was mixed with MNP at near room temperature where benzoyloxyl radicals are known to be formed,[19] strong EPR signals due to **3** were detected without any trace of spectra attributed to the benzoyloxyl radical adduct of MNP.

However, the detection of **1** and **4** indicates that free *m*-chlorobenzoyloxyl radicals are also formed when CPBA is used. Perhaps a precursor molecular addition product, **12**, is formed. This product has the structure of a hydroxylamine of the peroxyl adduct of PBN, which might be expected to produce radicals spontaneously by an internal redox reaction:



12

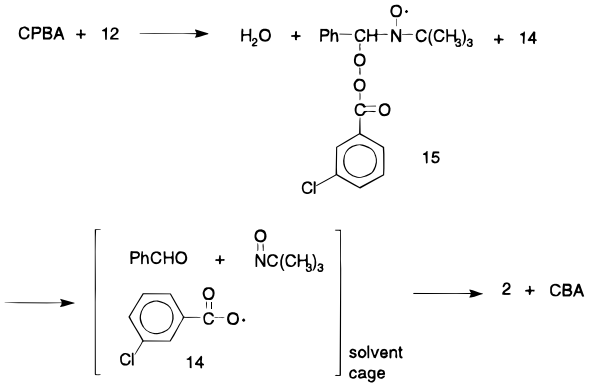
13

14

1

However, the hydroxyl adduct of PBN has not been detected in any systems reported in this investigation.

It is possible that **12** could also be oxidized by another CPBA molecule to form **15** before internal disproportionation occurs:



15

14

solvent cage

(17) Bluhm, A. L.; Weinstein, J.; Sousa, J. A. *Nature* **1971**, *229*, 500.
(18) Bluhm, A. L.; Weinstein, J. *J. Org. Chem.* **1972**, *37*, 1748.
(19) Janzen, E. G.; Blackburn, B. J. *J. Am. Chem. Soc.* **1968**, *90*, 5909−5910.

Table 1. Yields of Spin Adducts Produced from the CPBA System in the Absence of Air

	benzene yield (M) × 10^7			CH_3CN yield (M) × 10^7	
	1[a]	**2**	**3**	**1**	**2**
CN-PBN	<1.0	<1.0	0	3.7 ± 1.1	0
PBN	4.9 ± 1.6	22.5 ± 9.5	0	44.5 ± 5.5	2.9 ± 0.9
CH_3O-PBN	3.2 ± 3.2	152.0 ± 46.2	24.5 ± 0.5	113.5 ± 16.5	2.6 ± 1.0
	7	**8**		**7**	**8**
2-Ph-DMPO	19.9 ± 5.6	9.7 ± 2.4		36.8 ± 0.4	<1.0

[a] **1**, **2**, **3**, **7**, **8** are product **1, 2, 3, 7, 8** respectively, which are described in text.

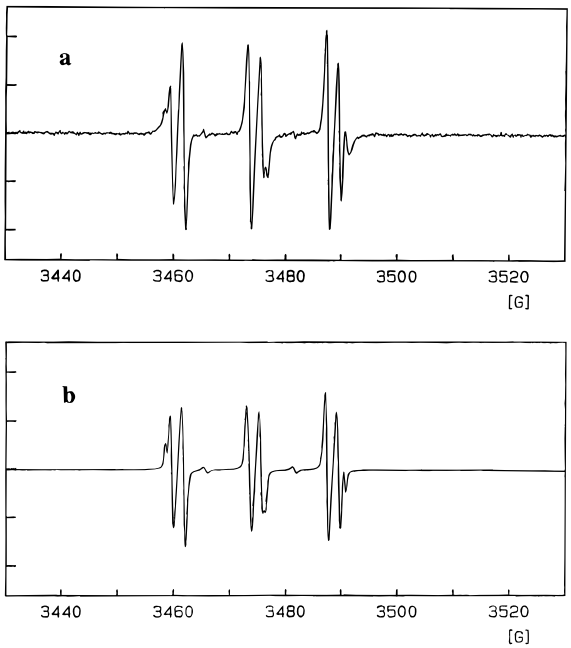


Figure 5. (a) EPR spectrum obtained from 20 mM PBN and 4.32 mM perpropionic acid (*in situ*) in benzene in the absence of air at room temperature. (b) Computer simulation of spectrum a.

If these reactions described above occur faster than the disproportionation reaction indicated earlier, spin adduct **2** will be detected as a main product. Since the peroxyl adduct of PBN, **15**, is not expected to be stable,[6] decomposition will spontaneously produce benzaldehyde, *m*-chlorobenzoyloxyl **14**, and MNP. Some of **14** will be quenched in the solvent cage by reacting with PhCHO; the produced benzoyl radical will then react with MNP to yield **2**.

As shown from yield analysis, **2** is the major product in benzene; however, **1** becomes the predominant product in CH_3CN. A possible explanation for this might be that the molecular addition product, **12**, is more likely to be produced in polar media.

Conclusions

Spin traps PBN and 2-Ph-DMPO react with peracid to spontaneously produce free radicals. PBN and CPBA initially produce the *m*-chlorobenzoyloxyl adduct and PBNOX. The product yields depend on the solvent polarity, peracid concentration, and substituent group on PBN. The radical products and nonradical products can be identified by EPR and/or MS. Perbenzoic acid prepared *in situ* behaves in much the same way as CPBA. The aliphatic acid, perpropionic acid, also prepared *in situ* reacts with PBN to produce C_2H_5OPBN as a main radical spin adduct.

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