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Communications

Reactions of Nitric Oxide with Phenolic Antioxidants and Phenoxyl Radicals

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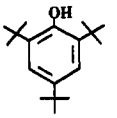
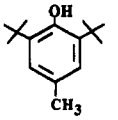
Summary: By EPR, NMR, and TLC methods it was possible to show that nitric oxide (•NO) reacts with five different methyl- or *tert*-butyl-substituted phenols including α-tocopherol to produce the phenoxyl radical which subsequently couples reversibly with excess •NO.

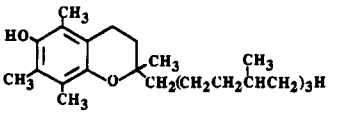
Although nitric oxide (•NO) has recently been recognized as an important biochemical free radical in a variety of animal and human tissues,[1] very little fundamental reaction chemistry is known about this molecule. In this report we describe preliminary results on the interaction of nitric oxide with hindered phenols commonly used as antioxidants. We have tried to answer the following questions: (1) Does •NO react with phenols to produce phenoxyl radicals? (2) Do the resulting phenoxyl radicals couple with •NO? (3) Is this reaction reversible? The phenols investigated were 2,4,6-tri-*tert*-butylphenol (I), 2,6-di-*tert*-butyl-4-methylphenol (or "butylated hydroxytoluene", BHT) (II), α-tocopherol (III), 4,4'-methylenebis-(2,6-di-*tert*-butylphenol) (IV), and phenyl-4,4'-methinebis-(2,6-di-*tert*-butylphenol) (V). The latter two examples are precursors to galvinoxyl[2] and phenylgalvinoxyl, respectively.



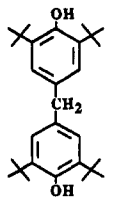
2,4,6-Tri-*t*-butylphenol
I



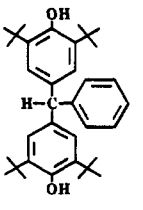
Butylated Hydroxytoluene (BHT)
(2,6-di-*t*-butyl-4-methylphenol)
II



α-Tocopherol
III



4-4'methylene-bis(2,6-di-*t*-butylphenol)
(Galvinoxyl precursor)
IV



Phenyl-4-4'methine-bis(2,6-di-*t*-butylphenol)
(Phenyl Galvinoxyl precursor)
V

The methods used involve producing •NO by reacting zinc metal with nitric acid (17% HNO_3)[3] in the absence of oxygen and passing the stream of •NO through 10%

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‡ Fleming Scholar, Summer 1992.

(1) (a) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109–142. (b) Lancaster, J. R. *Am. Sci.* **1992**, *80*, 248–259. (c) Stamler, J. L.; Singel, D. J.; Loscalzo, J. *Science* **1992**, *258*, 1898–1902.

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Table I. EPR Data for Selected Phenoxyl Radicals

phenol precursor	EPR pattern	obsd HFS (in G)	lit. value (in G)
2,4,6-tri-*tert*-butylphenol (I)	1:2:1 triplet	2.0	1.8[a]
2,6-di-*tert*-butyl-4-methylphenol (BHT, II)	1:3:3:1 quartet of 1:2:1 triplets	11.2, 1.8	10.7, 1.8[b]
α-tocopherol (III)	1:6:15:20:15:6:1 septet of multiplets	5.5	5.35[c]
galvinoxyl precursor (IV)	1:2:1 triplet of 1:2:1 triplets	9.3, 1.8	*d*
phenylgalvinoxyl precursor (V)	1:1 doublet of 1:2:1 triplets	7.8, 1.7	*e*
galvinoxyl (VI)	1:1 doublet of 1:4:6:4:1 quintets	6.0, 1.6	5.7, 1.3[f]

[a] Reference 4, Table 2, p 289. [b] Reference 4, p 313. [c] References 5 and 6. [d] Consistent with galvinoxyl precursor phenoxyl. [e] Consistent with phenylgalvinoxyl precursor phenoxyl. [f] Reference 4, p 282.

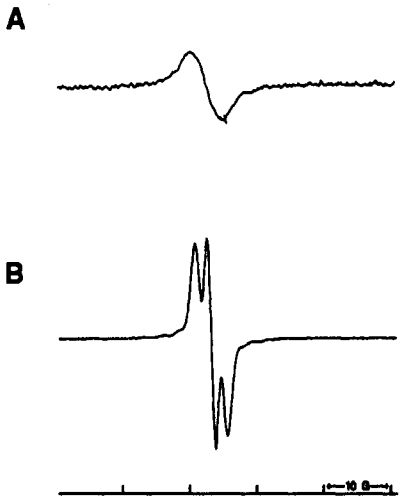
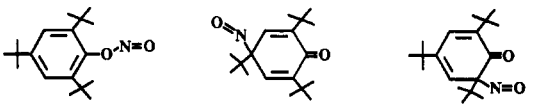


Figure 1. EPR spectrum obtained from 2,4,6-tri-*tert*-butylphenol (A) in cyclohexane saturated with nitric oxide and (B) in cyclohexane bubbled with nitrogen after exposure to nitric oxide.

NaOH to remove higher oxides of nitrogen. This •NO is bubbled into cyclohexane containing the phenol. The presence of phenoxyl radicals was determined by EPR spectroscopy using a Bruker ER 300 spectrometer. Authentic EPR spectra of phenoxyl radicals were obtained by lead dioxide oxidation in the same solvent. The splitting patterns and hyperfine coupling constants were consistent with the literature as shown in Table I. NMR spectra were recorded on a Varian XL300 spectrometer at 300 MHz.

The reaction between •NO and I in cyclohexane produces an EPR signal consisting of one broad line of low intensity (Figure 1A). This signal resolves into a triplet spectrum characteristic of 2,4,6-tri-*tert*-butylphenoxyl radical when nitrogen is bubbled into the cyclohexane solution previously saturated with nitric oxide (Figure 1B). The intensity of this signal is much greater than that found in the presence of •NO (approximately 20×) and continues to increase with time. The single line in Figure 1A can be computer generated by increasing the line width in Figure 1B. This analysis is consistent with the interpretation that the single line in Figure 1A is due to an •NO broadened triplet as shown in Figure 1B.

Thus, we conclude that •NO oxidized I to produce the phenoxyl radical which subsequently couples with excess nitric oxide to leave only a small amount of EPR-detectable phenoxyl radical in cyclohexane solution in the presence of •NO. When excess •NO is removed the nitric oxide adduct slowly dissociates back to phenoxyl radical and •NO. The structure of the coupled product could be the nitrite or either the 2- or 4-nitrosocyclohexadienone:



VI VII VIII

Analysis of the products by TLC (hexane on silica gel) shows three new substances, one of which tests positive for C-nitroso compounds by the "Griese test".[7] Proton NMR spectra could be assigned to C-nitroso compounds as the major product. Thus

	aromatic	vinyl	*tert*-butyl	OH
I	7.374		1.414, 1.363	4.82
VII		7.196 (s)	1.226, 0.816	
VIII		6.48 (s)	1.316, 0.905	
		*	*	

where an * indicates an additional peak is expected but covered by overlap. The ratio of VII:VIII is approximately 4:1. It appears that the para isomer is favored over the ortho isomer by 4×. Further work is necessary to ascertain whether these unstable NO-adducts survive TLC separation unchanged.

Similar results are obtained with the precursors to galvinoxyl and phenylgalvinoxyl. Although no EPR spectra were obtained from reacting •NO with the phenols, subsequent bubbling with nitrogen after exposure to •NO gave the characteristic patterns due to the phenoxyl radicals after 24 h. We conclude that the phenoxyl radicals are formed initially and couple with excess •NO.

BHT and α-tocopherol differ from I, IV, and V in that methyl groups are present either ortho or para to the hydroxyl group in the phenol. If phenoxyl radicals are formed from the reaction of nitric oxide with the phenolic function, subsequent reactions may not be confined to reversible coupling. Irreversible hydrogen abstraction reactions may occur with •NO to produce quinone methide compounds.[8]

Thus, when α-tocopherol is exposed to •NO in cyclohexane, only a very weak broad EPR signal is obtained (Figure 2A). However, when this solution is bubbled with nitrogen a better resolved spectrum of greater intensity could be detected (approximately 8× stronger) (Figure 2B). This spectrum decreases in intensity with time. The Griese test showed no C-nitroso compounds in the cyclohexane reaction mixture; however, many new products can be detected by TLC (hexane/ethyl acetate (80:20)).

(6) Lambelet, P.; and Löliger, J. *Chem. Phys. Lipids* **1984**, *35*, 185–198.

(7) The Griese test indicates production of the nitrosoniumion or compounds that produce nitrosonium ion by acid hydrolysis. The Griese reagent is an aqueous HCl (8.3%) solution of 4-sulfanilamide (15 mM) and *N*-1-naphthylethylenediamine (2 mM). A positive test gives a *pink* color.

(8) For 2,6-di-*tert*-butylmethylphenol see: Loy, B. R. *J. Org. Chem.* **1966**, *31*, 2386–2388. Also see: ref 3, p 311–315.

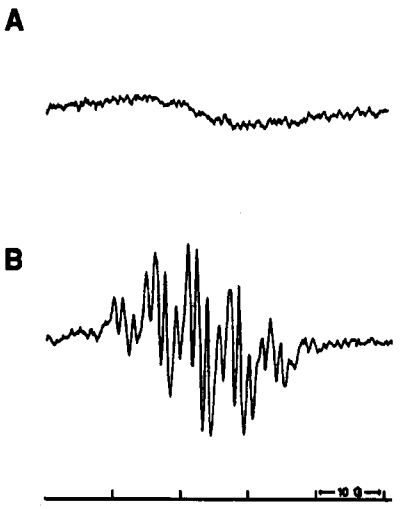


Figure 2. EPR spectrum obtained from α-tocopherol (A) in cyclohexane saturated with nitric oxide and (B) in cyclohexane bubbled with nitrogen after exposure to nitric oxide.

Experiments with BHT gave no EPR signals, either in the presence of •NO or after bubbling the cyclohexane solution with nitrogen. However, both TLC and NMR show that a new product is produced. Thus:

	aromatic	vinyl	methyl	*tert*-butyl	OH
II	7.06		2.245	1.385	4.78
para adduct		6.56 (s)	1.225	1.189	

Assignments were made with deuterated BHT, deuterated in all positions except the methyl group.[9]

Thus, the •NO adduct of BHT does not dissociate to EPR-detectable amounts of phenoxyl radical and •NO in the absence of excess nitric oxide. This may be due to decomposition of the BHT/NO adduct to quinone methide and/or production of dimeric compounds which involves hydrogen atom abstraction from the *p*-methyl group. However, α-tocopheroxyl *is* produced from reaction with nitric oxide, but further reaction with •NO either produces adducts which do not decompose to α-tocopheroxyl or new products which do not contain the •NO group.

We conclude from these experiments that sterically hindered phenolic antioxidants react with nitric oxide first to produce the phenoxyl radical and subsequently to form •NO adducts. These •NO adducts dissociate slowly in the absence of excess nitric oxide to produce the phenoxyl radicals they originated from. The rate of the latter dissociation depends on the structure of the phenoxyl radical.[4] One could expect more "stable" phenoxyl radicals would form a weaker •NO bond to nitric oxide and dissociate more rapidly and more completely in an equilibrium situation. The equilibrium constant and the rates of dissociation and association should depend on temperature and on solvent polarity.

The reversibility between stable phenoxyl radicals and nitric oxide is reminiscent of the reversibility of the triplet ground-state dioxygen molecule and stable radicals. It has been demonstrated that the triphenylmethyl radical reacts reversibly with oxygen to produce the peroxyl radical when locked in a crystal powder of triphenylacetic acid.[10,11] However, the reaction between *tert*-butyl radicals and dioxygen is apparently not reversible.[12] At biological temperatures bisallylic radicals from unsaturated lipids in bilayer membrane models are believed to react reversibly with oxygen[13] and radicals derived from β-carotene are proposed to add to oxygen reversibly.[14] Oxygen may also react reversibly with phenoxyl radicals but subsequent fast reactions of the peroxyl radical may mask this reaction.

Nitric oxide and dioxygen thus may have similar reactivities. However, •NO appears to be slightly more reactive than dioxygen. While •NO reacts directly with phenols at observable rates to produce phenoxyl radicals, dioxygen does not or the reaction is much slower. Phenoxyl radicals react both with •NO and with oxygen. The extent of reversibility of this reaction may depend on the structure and "stability" of the phenoxyl radical. We propose that phenoxyl radicals such as those derived from α-tocopherol could function as nitric oxide carriers in biological systems. The NO groups appears to be quite labile and may respond differently depending on the local environment and structure of the phenol. Further studies are underway to investigate these possibilities.

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(9) A sample of deuterated BHT, deuterated in all positions except for the methyl group, was kindly provided by Prof. Ken Jeffrey of the Physics Department of the University of Guelph.

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