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A Dual Sensor Spin Trap for Use with EPR Spectroscopy

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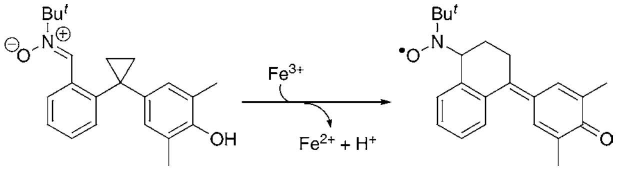
ABSTRACT



Redox active metal ions, carbon-centered radicals, and oxygen-centered radicals are important to oxidative stress. A radical detector combining a nitrone spin trap, a phenol, and a cyclopropane radical clocklike unit was prepared and used with EPR spectroscopy to detect and distinguish between hydroxyl radicals, methyl radicals, and iron(III) ions. Iron(III) reacts with the phenol unit inducing opening of the cyclopropane ring and cyclization to generate a stable nitroxyl radical.

There is convincing evidence that oxidative stress due to reactive oxygen species[1] (ROS) is a major contributor to the process of aging.[2] ROS are also involved in a wide range of pathologies including neurodegenerative diseases[3] such as Parkinson's and Alzheiemer's diseases and in ischemic events such as a stroke.[4]

The most damaging ROS are hydroxyl (HO•) and peroxyl radicals (ROO•).[1] These can also produce highly reactive carbon-centered radicals by hydrogen atom abstraction from biomolecules, particularly polyunsaturated fatty acids,[5] which may then reduce oxygen to regenerate peroxyl radicals. Redox active metal ions [e.g., copper(I) and iron(II)] are important to the generation of hydroxyl radicals by the Fenton reaction (Scheme 1a) and related processes, with their oxidation potential depending on ligands. The higher oxidation states of these metals can be reduced in situ by superoxide to give the superoxide-assisted Fenton reaction (Scheme 1a and b) and may also act as oxidizing agents themselves by single electron transfer. Thus, reaction between iron(III) and phenols, including α-tocopherol and flavonoids found in the diet, which are generally viewed as antioxidants,[6−8] would have a pro-oxidant effect.

Scheme 1. Superoxide-Assisted Fenton Reaction

$$\text{(a)}\quad Fe^{2\oplus} + H_2O_2 \longrightarrow Fe^{3\oplus} + HO^{\bullet} + HO^{\ominus}$$

$$\text{(b)}\quad Fe^{3\oplus} + O_2^{\bullet -} \longrightarrow Fe^{2\oplus} + O_2$$

Central to the study of oxidative stress is the ability to detect and distinguish between the various chemical contributors to it and to determine their locality in cells and organs. One approach is to use fluorescent probes, which are particularly good in vitro where confocal microscopy can

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[1] Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 3rd ed.; OUP: Oxford, 1999.

[2] Schriner, S. E.; Linford, N. J.; Martin, G. M.; Treuting, P.; Ogburn, C. E.; Emond, M.; Coskun, P. E.; Ladiges, W.; Wolf, N.; Van Remmen, H.; Wallace, D. C.; Rabinovitch, P. S. *Science* **2005**, *308*, 1909−1911.

[3] Lin, M. T.; Beal, M. F. *Nature* **2006**, *443*, 787−795.

[4] Adibhatla, R. M.; Hatcher, J. F. *Free Radical Biol. Med.* **2006**, *40*, 376−387.

[5] Yin, H.; Musiek, E. S.; Gao, L.; Porter, N. A.; Morrow, J. D. *J. Biol. Chem.* **2005**, *280*, 26600−26611.

[6] For a discussion of the importance and limitations of α-tocopherol as a biological antioxidant, see: Kim, H.-Y.; Pratt, D. A.; Seal, J. R.; Wijtmans, M.; Porter, N. A. *J. Med. Chem.* **2005**, *48*, 6787−6789 and refs 1−4 therein.

[7] Radical scavenging properties of flavonoids are well established: (a) McPhail, D. B.; Hartley, R. C.; Gardner, P. T.; Duthie, G. G. *J. Agric. Food Chem.* **2003**, *51*, 1684−1690. (b) Bors, W.; Michel, C.; Stettmaier, K. *Methods Enzymol.* **2001**, *335*, 166−180 and refs therein.

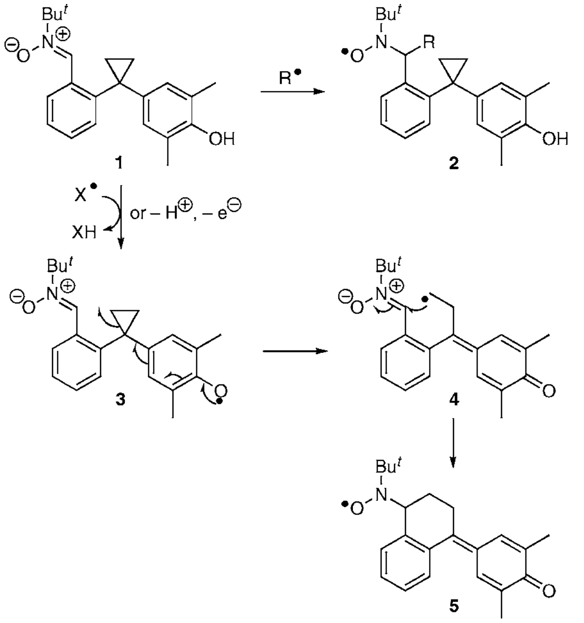
[8] The role of flavonoids as dietary antioxidants in vivo is disputed: Lotito, S. B.; Frei, B. *Free Radical Biol. Med.* **2006**, *41*, 1727−1746.

10.1021/ol071285o CCC: $37.00

be employed, and recently great advances have been made in developing probes that detect different ROS selectively.[9] Although simple to use, sometimes results from fluorescent probes can be misleading; e.g., probes that were at first believed to be responding to ROS have since been found to be measuring free cytochrome C instead.[10] Electroparamagnetic resonance (EPR) spectroscopy has the advantage that it only detects radicals and can be used both in vitro and in vivo (albeit at different operating frequencies). Although many of the radicals found in biological systems are too short-lived for direct detection by EPR spectroscopy, spin traps have been developed that convert highly reactive radicals into more stable radicals. Nitrones are generally employed as these trap highly reactive radicals to give stable nitroxyl radicals,[11] which can be detected, and the adducts of specific ROS and carbon-centered radicals can have characteristic spectra.[12] Thus, the nitrone can be regarded as a radical sensor and the resulting nitroxyl as the actuator, for display by EPR spectroscopy.

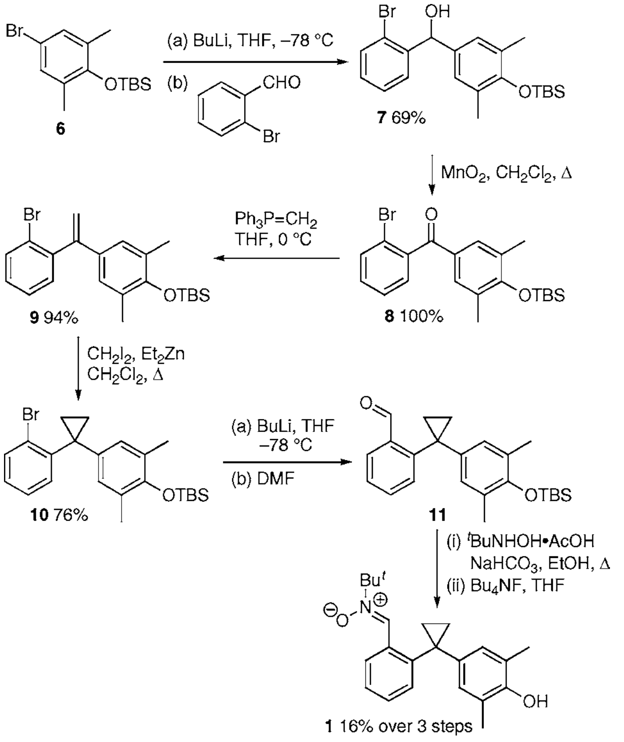
We designed probe **1**, which has two different sensor moieties and the same actuator, to allow the detection and identification of a wide range of species involved in oxidative stress. The probe **1** consists of two noncommunicating aromatic rings, linked by a cyclopropane ring, with the nitrone and phenol groups acting as sensors for different types of radicals by different mechanisms (Scheme 2). Sterically unhindered electron-rich carbon-centered radicals and very reactive, and hydroxyl radicals, which are very reactive, would be expected to form adducts with the nitrone moiety rapidly, generating the sterically protected nitroxyl radicals **2**.[12] On the other hand, electron-poor species might be expected to react with the phenolic moiety by hydrogen atom abstraction or by sequential proton loss−electron transfer to give phenoxyl radical **3**, with the rate and mechanism depending on the solvent and the species involved.[13] The cyclopropane is related to known radical clocks[14] and should open rapidly to give an unstable primary radical **4**, which will cyclize onto the nitrone to generate a nitroxyl actuator **5**. The different nitroxyl radicals should have different EPR spectra allowing the processes to be distinguished. Only radical processes would be detected.

Scheme 2. Design of Dual Sensor Probe



Our strategy for the construction of the double detector probe involved aldehyde **11** as a key intermediate (Scheme 3). Although benzophenone derivatives bearing masked aldehydes are easily accessible,[15] the choice of protecting group is problematic as unmasking must tolerate the cyclo-

Scheme 3. Preparation of Dual Sensor Probe **1**



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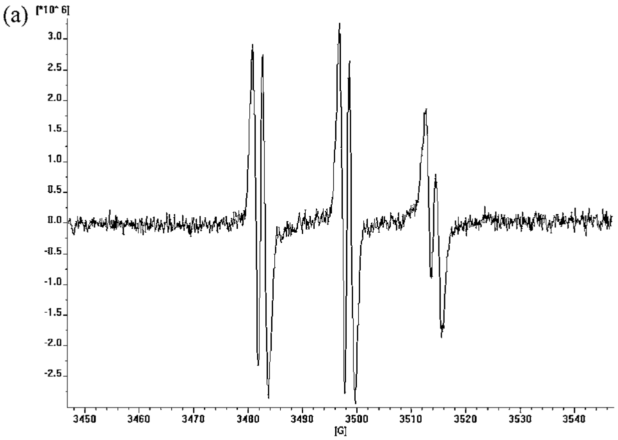
[9] Soh, N. *Anal. Bioanal. Chem.* **2006**, *386*, 532−543.

[10] Burkitt, M.; Jones, C.; Lawrence, A.; Wardman, P. *Biochem. Soc. Symp.* **2004**, *71*, 97−106 and refs 3−5 therein.

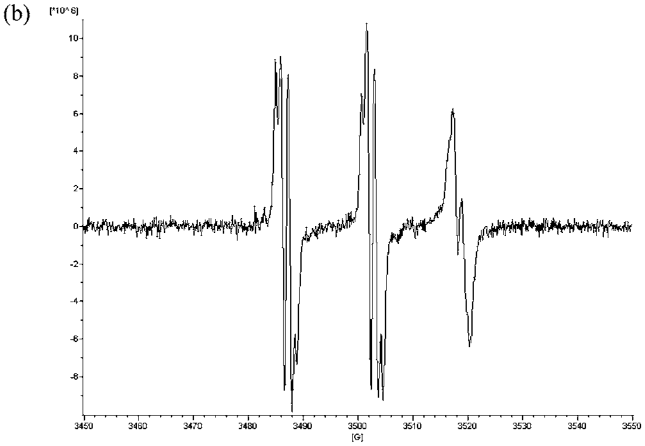
[11] Recent examples of nitrones as probes and antioxidants include: (a) Xu, Y. K.; Kalyanaraman, B. *Free Radical Res.* **2007**, *41*, 1−7. (b) Sklavounou, E.; Hay, A.; Ashraf, N.; Lamb, K.; Brown, E.; MacIntyre, A.; George, W. D.; Hartley, R. C.; Shiels, P. G. *Biochem. Biophys. Res. Commun.* **2006**, *347*, 420−427. (c) Kamibayashi, M.; Oowada, S.; Kameda, H.; Okada, T.; Inanami, O.; Ohta, S.; Ozawa, T.; Makino, K.; Kotake, Y. *Free Radical Res.* **2006**, *40*, 1166−1172. (d) Ionita, P. *Free Radical Res.* **2006**, *40*, 59−65. (e) Ortial, S.; Durand, G.; Poeggeler, B.; Polidori, A.; Pappolla, M. A.; Böker, J.; Hardeland, R.; Pucci, B. *J. Med. Chem.* **2006**, *49*, 2812−2820. (f) Hardy, M.; Ouari, O.; Charles, L.; Finet, J.-P.; Iacazio, G.; Monnier, V.; Rockenbauer, A.; Tordo, P. *J. Org. Chem.* **2005**, *70*, 10426−10433. (g) Hay, A.; Burkitt, M. J.; Jones, C. M.; Hartley, R. C. *Arch. Biochem. Biophys.* **2005**, *435*, 336−346. (h) Allouch, A.; Roubaud, V.; Lauricella, R.; Bouteiller, J.-C.; Tuccio, B. *Org. Biomol. Chem.* **2005**, *3*, 2458−2462. (i) Liu, Y. P.; Ji, Y. Q.; Song, Y. G.; Liu, K. J.; Liu, B.; Tian, Q.; Liu, Y. *Chem. Commun.* **2005**, 4943−4945.

[12] Rosen, G. M.; Britigan, B. E.; Halpern, H. J.; Pou, S. *Free Radicals: Biology and Detection by Spin Trapping*; OUP: Oxford, 1999.

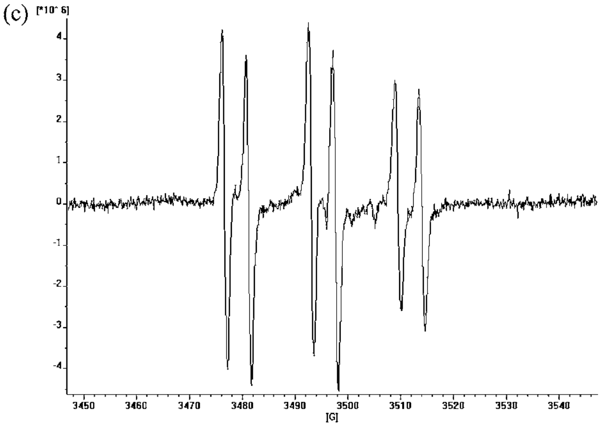
[13] Musialik, M.; Litwinienko, G. *Org. Lett.* **2005**, *7*, 4951−4954.



g = 2.0059, A_N = 15.93 G (t), A_H^{β} = 1.85 G (d)



g = 2.0055, A_N = 15.70 G (t), A_H^{β} = 1.42 G (d), $A_H^{\gamma/\delta}$ = 1.05 and 0.99 G (dd).



g = 2.0056, A_N = 16.32 G (t), A_H^{β} = 4.57 G (d).

Figure 1. EPR experiments with nitrone **1**.[19]

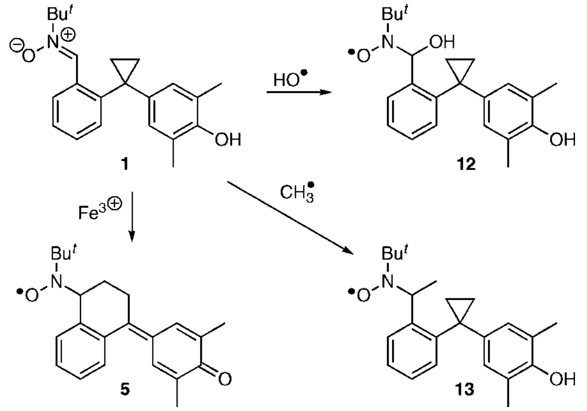
propyl unit which is in close proximity.[16] Consequently, we decided to introduce the aldehyde group late in the synthesis using a cyclopropanation, lithiation, formylation sequence demonstrated by Jason.[17] Thus, bromination and silyl protection of 2,6-dimethylphenol gave bromide **6**. Lithiation and reaction with 2-bromobenzaldehyde gave alcohol **7**, which was oxidized to the benzophenone **8**. Wittig methylenation gave alkene **9**, and modified Simmons−Smith cyclopropanation[18] proceeded in good yield to give 1,1-diarylcyclopropane **10**. Lithiation and reaction with DMF gave the key intermediate **11**, which was converted into a nitrone and deprotected to give the desired target **1** in modest yield over three steps.

Nitrone **1** was reacted with hydroxyl radicals generated from hydrogen peroxide and iron(II) sulfate[19] (Scheme 1a), and the EPR spectrum was recorded (Figure 1a).

This signal slowly declined, and a more complex stable signal began to appear. Because iron(III) is a byproduct of the Fenton reaction, we reasoned that this new signal might arise from the metal ion behaving as an oxidant. Sure enough, when nitrone **1** was treated with iron(III) chloride, a strong long-lasting signal was recorded (Figure 1b). Finally, methyl radicals were generated from hydroxyl radicals by reaction with DMSO[19] and trapped to give a product that had a different spectrum (Figure 1c).

The hyperfine splittings in spectra a and c shown in Figure 1 were easily derived by simulation. We reasoned that reaction with the hydroxyl and methyl radicals occurred at the nitrone to give adducts **12** and **13** (Scheme 4)[20] and that iron(III) ions caused oxidation of the phenol moiety of nitrone **1** and production of tricyclic nitroxide **5**, where hyperfine coupling to the γ and/or δ hydrogen atoms is observed.

Scheme 4. Reactions of Dual Sensor Probe **1**



To confirm the assignment of tricycle **5** and to assist in the interpretation of spectrum b (Figure 1), tetradeuterated

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(14) (a) Lemieux, R. P.; Beak, P. *J. Org. Chem.* **1990**, *55*, 5454−5460. (b) Newcomb, M. *Tetrahedron* **1993**, *49*, 1151−1176.

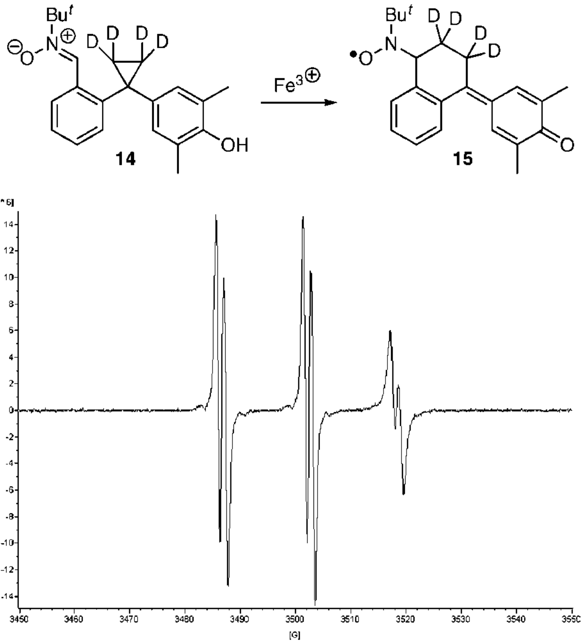
(15) (a) Flanagan, S. R.; Harrowven, D. C.; Bradley, M. *Tetrahedron* **2002**, *58*, 5989−6001. (b) Ukita, T.; Nakamura, Y.; Kubo, A.; Yamamoto, Y.; Moritani, Y.; Saruta, K.; Higashijima, T.; Kotera, J.; Takagi, M.; Kikkawa, K.; Omori, K. *J. Med. Chem.* **2001**, *44*, 2204−2218. (c) Couture, A.; Deniau, E.; Lebrun, S.; Grandclaudon, P. *J. Chem. Soc., Perkin 1* **1999**, 789−794. (d) Patil, P. A.; Joshi, R. R.; Narasimhan, N. S. *Indian J. Chem. Sect. B* **1987**, *26*, 1025−1029.

(16) Preliminary investigation of such routes showed that cyclizations competed at various stages.

(17) Jason, M. E. *Tetrahedron Lett.* **1982**, *23*, 1635−1638.

(18) Charette, A. B.; Beauchemin, A. *Org. React.* **2001**, *58*, 1−415.

cyclopropane **14** was prepared by the same route, but using $Ph_3P{=}CD_2$ in the alkenation step and CD_2I_2 for the cyclopropanation step. Reaction between nitrone **14** and iron(III) chloride gave rise to a simplified EPR spectrum corresponding to nitroxide **15** (Figure 2), confirming the original assignment of nitroxide **5**. The spectrum b of nitroxide **5** was simulated using the values for A_N and A_H^{β} determined for nitroxide **15**. Coupling to two of the γ and/or δ hydrogen atoms in nitroxyl radical **5** is observed, and the A_H^{γ} or A_H^{δ} values of about 1 G derived from simulation are consistent with those in the literature for *N*-*tert*-butyl-*N*-cyclohexyl nitroxide.[21]



g = 2.0055, A_N = 15.70 G (t), A_H^{β} = 1.42 G (d).

Figure 2. Reaction of deuterated nitrone **14** with $FeCl_3$ and the EPR spectrum of its adduct **15**.[22]

In conclusion, we have demonstrated a proof of the principle that combination of two different radical sensors, a radical clocklike relay system, and a nitrone allows oxygen-centered radicals, carbon-centered radicals, and an oxidizing metal ion to be detected and distinguished by EPR spectroscopy.

Acknowledgment. We thank EPSRC and SPARC for funding, EPSRC National Service for EPR Spectroscopy, and in particular, Dr. Radoslaw Kowalczyk and Prof. Eric McInnes for advice and training.

Supporting Information Available: Full experimental procedures, spectroscopic data, and scanned NMR spectra for compounds **1**, **6**−**11**, and **14** are included. Details of EPR experiments and simulations are also included. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) Conditions for generation of hydroxyl and methyl radicals were adapted from: Rosen, G. M.; Tsai, P.; Barth, E. D.; Dorey, G.; Casara, P.; Spedding, M.; Halpern, H. J. *J. Org. Chem.* **2000**, *65*, 4460−4463. Polyakov, N. E.; Kruppa, A. I.; Leshina, T. V.; Konovalova, T. A., Kispert, L. D. *Free Radical Biol. Med.* **2001**, *31*, 43−52. (a) 3.3 mM **1**, 0.33 mM $FeSO_4$, 0.33 mM H_2O_2, DMF−H_2O (1:2). (b) 3.3 mM **1**, 0.33 mM $FeCl_3$, DMF−H_2O (1:2). (c) 3.3 mM **1**, 0.33 mM $FeSO_4$, 0.33 mM H_2O_2, DMSO−H_2O (1:2). *g* values referenced to strong pitch.

(20) Hydroxyl radicals are very highly reactive, and some reaction at the phenolic position may have occurred; however, any signal for the tricyclic nitroxide **5** is initially obscured by the signal from the more abundant nitroxyl radical **12**.

(21) Wajer, Th. A. J. W.; Mackor, A.; de Boer, Th. J. *Recl. Trav. Chim. Pays-Bas* **1971**, *90*, 568−576.

(22) Conditions: 3.3 mM **14**, 0.33 mM $FeCl_3$, DMF−H_2O (1:2).