Highly Sensitive Colorimetric Detection and Facile Isolation of Diamagnetic Free Radical Adducts of Novel Chromotropic Nitrone Spin Trapping Agents Readily Derived from Guaiazulene

David A. Becker

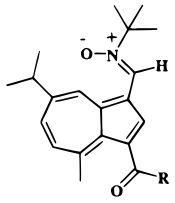
Department of Chemistry, Florida International University University Park, Miami, Florida 33199

Received August 22, 1995

The technique of spin trapping is an important method for garnering information on free radicals difficult or impossible to detect by direct spectroscopic observation due to their exceedingly short lifetimes and low concentrations.[1] To date, two classes of spin trapping agents have received the most attention, namely, nitroso compounds and nitrones. Of these, the latter have been more frequently used, especially in biological systems. The most commonly cited drawbacks to the application of spin trapping agents bearing nitroso functionality are instability and toxicity.[1a] On account of these undesirable characteristics, researchers often opt for nitrone spin traps despite the fact that their nitroxide spin adducts generally provide less structural information from ESR than do those from nitroso-based spin traps. Furthermore, the nitroxides obtained from the addition of certain carbon-centered radicals (tertiary alkyl and aryl) to the most widely used nitrone spin traps (PBN,[2] POBN,[3] and DMPO[4]) are, due primarily to disproportionation,[1a] less persistent than those obtained from addition of such radicals to nitroso compounds. Several groups have described the use of isotopically labeled spin traps[5] or the application of special equipment consisting of GC/MS or HPLC-interfaced ESR spectrometers[1a,6] designed to detect, isolate, and characterize free radical adducts of nitrone spin traps in biological systems with varied success. Herein is reported a new and simple colorimetric approach to the detection, isolation, and analysis of free radical adducts of nitrones employing the novel nitrone spin trapping agents **1** which are easily obtained from the abundant sesquiterpene guaiazulene.

Of particular importance regarding spin trapping with **1** is their capacity to tag free radicals by yielding characteristically colored and highly visible diamagnetic (and paramagnetic) spin adducts. *Thus, nitrones **1** provide the potential to implicate the intermediacy and establish the identity of free radicals in situations in which presently available ESR detection/isolation technology may fail.* Albeit considerably more persistent than most free radicals, nitroxides are nevertheless often subject to the usual free radical destruction processes of combination, disproportionation, and oxidation/reduction, yielding diamagnetic products. The rapid formation of diamagnetic spin adducts in traditional spin trapping experiments is an unwanted occurrence which can constitute a serious obstacle, since once such products are formed in biological systems employing conventional nitrone spin traps, they are lost among a vast number of diverse diamagnetic molecules.[1a,7] The ability to easily locate diamagnetic spin adducts in complex mixtures offers an appealing alternative should one be faced with technical difficulties often encountered while attempting to isolate nitroxides resulting from conventional nitrone spin traps before they decay into diamagnetic species.[1a,d] In spin trapping with **1**, the characteristic chromophore of the diamagnetic spin adducts arising from nitroxides via combination, disproportionation, or reduction, while crucially different from the chromophore of **1**, is in fact the same as that of the initially formed ESR-detectable nitroxide spin adducts. Therefore, this characteristic chromophore should also expedite the purification (and subsequent structure determination) of these paramagnetic species from reaction mixtures amenable to nitroxide longevity.[8]

Nitrone **1** (R = OEt) is a stable green solid (mp 43−45 °C) and is readily prepared in three steps from guaiazulene (Scheme 1). Exposure of guaiazulene to oxalyl bromide in ether at room temperature according to the method of Treibs[9] yields acyl bromide **2**, which is directly esterified with EtOH to provide the violet ethyl ester **3** in 80% yield. Oxidation of **3** with 2 equiv of DDQ in aqueous acetonitrile at room temperature in analogy to the method of Takase[10] affords a 74% yield of red aldehyde **4**. Condensation of **4** with *N-tert*-butylhydroxylamine hydrochloride in pyridine at 95 °C provides **1** (R = OEt) in nearly quantitative yield.[11]



1

The obvious chromotropism that accompanies conversion of nitrone spin traps **1** to diamagnetic free radical adducts arising

(1) (a) Janzen, E. G.; Haire, D. L. In *Advances in Free Radical Chemistry*; Tanner, D. D., Ed.; JAI Press: Greenwich, 1990; Vol. 1, p 253. (b) Knecht, K. T.; Mason, R. P. *Arch. Biochem. Biophys.* **1993**, *303*, 185. (c) Kalyanaraman, B. In *Reviews in Biochemical Toxicology*; Hodgson, E., Bend, J. R., Philpot, R. M., Eds.; Elsevier Biomedical: New York, 1980; Vol. 4, p 73. (d) Perkins, M. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 1. (e) Rehorek, D. *Chem. Soc. Rev.* **1991**, *20*, 341. (f) Mason, R. P.; Chignell, C. F. In *Free Radical Damage and Its Control*; Rice-Evans, C. A., Burdon, R. H., Eds.; Elsevier Science: New York, 1994; p 319.

(2) Janzen, E. G. *Acc. Chem. Res.* **1971**, *4*, 31.

(3) Janzen, E. G.; Wang, Y. Y.; Shetty, R. V. *J. Am. Chem. Soc.* **1978**, *100*, 2923.

(4) Iwamura, M.; Inamoto, N. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 703.

(5) (a) Barasch, D.; Krishna, M. C.; Russo, A. *J. Am. Chem. Soc.* **1994**, *116*, 7319. (b) Janzen, E. G.; Zhang, Y. K.; Haire, D. L. *J. Am. Chem. Soc.* **1994**, *116*, 3738. (c) Keana, J. F. W.; Lex, L.; Mann, J. S.; May, J. M.; Park, J. H.; Pou, S.; Prabhu, V. S.; Rosen, G. M.; Sweetman, B. J.; Wu, Y. *Pure Appl. Chem.* **1990**, *62*, 201. (d) Pou, S.; Rosen, G. M.; Wu, Y.; Keana, J. F. W. *J. Org. Chem.* **1990**, *55*, 4438. (e) Haire, D. L.; Oehler, U. M.; Krygsman, P. H.; Janzen, E. G. *J. Org. Chem.* **1988**, *53*, 4535. (f) Motten, A. G.; Levy, L. A.; London, R. E. *J. Magn. Reson.* **1988**, *80*, 112. (g) Janzen, E. G.; Oehler, U. M.; Haire, D. L.; Kotake, Y. *J. Am. Chem. Soc.* **1986**, *108*, 6858.

(6) (a) Kominami, S.; Rokushika, S.; Hatano, H. *Int. J. Radiat. Biol.* **1976**, *30*, 525. (b) Janzen, E. G.; Towner, R. A.; Krygsman, P. H. *Free Radical Res. Commun.* **1990**, *9*, 353. (c) Iwahashi, H.; Albro, P. W.; McGown, S. R.; Tomer, K. B.; Mason, R. P. *Arch. Biochem. Biophys.* **1991**, *285*, 172.

(7) (a) Iwamura, M.; Inamoto, N. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 702. (b) Janzen, E. G.; Krygsman, P. H.; Lindsay, D. A.; Haire, D. L. *J. Am. Chem. Soc.* **1990**, *112*, 8279. (c) Even mild biological reducing agents such as cysteine (in the presence of traces of ferric ion) and ascorbic acid will reduce nitroxides to the corresponding hydroxylamine. See: McConnell, H. M.; McFarland, B. G. *Q. Rev. Biophys.* **1970**, *3*, 91. See also: Sentjurc, M.; Mason, R. P. *Free Radical Biol. Med.* **1992**, *13*, 151.

(8) Even though nitroxides possess a visible chromophore of their own, their characteristic red color is due to an absorption with a very low extinction coefficient centered around 460 nm. For example, the visible absorption spectrum in hexane for di-*tert*-butylnitroxide shows a maximum at 465 nm with log $\epsilon = 0.95$. The extinction coefficient for the absorption giving rise to the color of the diamagnetic azulene-containing spin adducts described herein is between 1 and 2 orders of magnitude greater. See: Smith, P. A. S. *Open-Chain Nitrogen Compounds*; W. A. Benjamin, Inc.: New York, 1965; Vol. 2, p 105 and references cited therein.

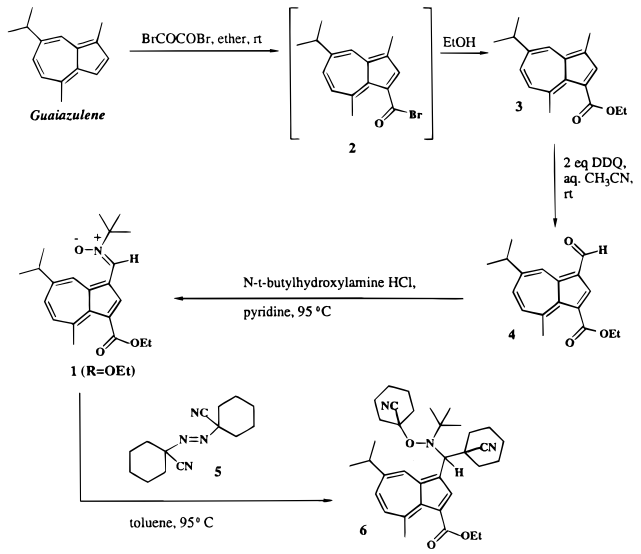
(9) Treibs, W.; Orttmann, H.; Schlimper, R.; Lindig, C. *Chem. Ber.* **1959**, *92*, 2152.

(10) Amemiya, T.; Yasunami, M.; Takase, K. *Chem. Lett.* **1977**, 587.

(11) Spectral data for **1** (R = OEt): ^{1}H NMR (300 MHz, $CDCl_3$) 9.74 (s, 1H), 8.36 (s, 1H), 8.17 (s, 1H), 7.54 (d, J = 11 Hz, 1H), 7.43 (d, J = 11 Hz, 1H), 4.37 (q, J = 7 Hz, 2H), 3.17 (m, 1H), 2.97 (s, 3H), 1.71 (s, 9H), and 1.38−1.43 (m, 9H); ^{13}C NMR (75.4 MHz, $CDCl_3$) 167.2, 148.7, 145.8, 141.3, 141.0, 137.7, 136.8, 132.7, 132.5, 123.0, 120.7, 117.1, 69.6, 60.8, 38.3, 28.4, 27.9, 24.4, and 14.4; IR (neat) 3135, 2965, 2930, 2905, 2870, 1715, 1580, 1560, 1460, 1335, 1300, 1245, 1195, 1150, 1105, 1040, 920 cm^{-1}; UV−vis λ_{max} (hexane) 313 nm (ϵ = 26 071), 358 (15 526), 417 (8390), and 588 (532); exact mass (FABMS, NBA) calcd for $C_{22}H_{30}NO_3$ ($M^+ + 1$) 356.2226, found 356.2230.

0002-7863/96/1518-0905$12.00/0

Scheme 1



via either combination, disproportionation, or reduction of intermediate nitroxides is unprecedented and may render them useful in tracking free radical residues in frequently encountered cases involving fast annihilation of nitroxide spin adducts via any of the aforementioned processes. Thus, when an emerald green solution of nitrone **1** (R = OEt) in toluene (60 mM) is heated to 95 °C in the presence of azo compound **5** under argon, TLC analysis of the progress of the reaction reveals the formation of a violet product of lower polarity than **1** (R = OEt). When the reaction mixture is poured onto a flash chromatography column containing chloroform-saturated silica gel and eluted with chloroform, a violet band descends and is easily collected. Further purification by preparative TLC (chloroform) affords the violet double spin adduct **6**.[12] An inspection of the ^{1}H NMR spectrum of the reaction mixture formed in a competitive spin trapping experiment entailing thermolysis (95 °C, 6 h) of a toluene solution containing 100 mM concentrations of **1** (R = OEt), PBN, and **5** indicates that, relative to PBN, nitrone **1** (R = OEt) produces a roughly equal amount of the corresponding double spin adduct.[13] Early results concerning the use of **1** in trapping other types of carbon-centered radicals (such as aryl radicals) have been encouraging.[14]

Chromotropism has also been observed in preliminary experiments employing nitrone spin trap **1** (R = OEt) in lipid peroxidation studies. Dissolution of 10 mg of **1** (R = OEt) in 50 mL of corn oil and bubbling of air through the resulting green oil at 90 °C for 9.5 h yields a bright red oil from which, after partitioning between hexane and acetonitrile, one can isolate from the acetonitrile layer 300 mg of crude reddish material, which, when subjected to TLC analysis, shows the presence of a major red product.[15] When a control experiment is conducted with argon bubbling in the presence or absence of water, this product is not detected and the recovered green oil contains no azulenic products other than the starting nitrone. Similarly, no observable chromotropism and complete recovery of the unreacted nitrone is the outcome of an aerobic control experiment substituting chlorobenzene, a solvent devoid of easily abstractable hydrogen atoms, in place of corn oil. This data strongly suggests that the observed change in color from green to red is instigated by addition to nitrone **1** (R = OEt) of free radicals formed by autoxidation, presumably involving linoleic acid subunits of the corn oil glycerides. The application of these nitrones as indicators of oxidative stress in lipids is a focus of ongoing research.

By virtue of the presence of acyl bromide intermediate **2** in the synthesis of these nitrones, one can envision making a wide range of easily prepared derivatives whose physical properties can be modulated by judicious choice of a nucleophile (alcohol, amine, etc.) to employ in acylation reactions with **2**. Lipophilic or hydrophilic side chains at this position should drastically influence solubility properties, and the exploitation of this electrophilic site for the preparation of bioconjugates may afford interesting spin traps with efficient targeting capacities.[16]

The free radical scavenging and antioxidant properties of nitrones have been recent topics fostering intense activity in the biological/pharmacological arena.[17−20] Much evidence points to the role of free radical damage in the etiology of a number of pathological conditions such as atherosclerosis,[17] Alzheimer's disease,[18] cancer,[21] ischemia−reperfusion injury,[19] and senescence.[20] Research into the efficacy of these new nitrones in the implication and elucidation of pathways of free radical processes and in providing possible therapeutics for free radical mediated diseases is in progress and will be reported in due course.

Acknowledgment. The financial support of this work by Florida International University is gratefully acknowledged. Mass spectra were generously provided by the Hudlicky group and measured at the UF Department of Chemistry facilities in Gainesville, FL. Discussions with Professor William R. Dolbier, Jr., and Professor Steven A. Benner (Department of Chemistry, UF) were of great assistance in the preparation of this manuscript.

JA952895Z

(12) Spectral data for **6**: ^{1}H NMR (300 MHz, $CDCl_3$) 8.74 (s, 1H), 8.20 (s, 1H), 7.57 (d, J = 11 Hz, 1H), 7.36 (d, J = 11 Hz, 1H), 4.57 (s, 1H), 4.39 (q, J = 7 Hz, 2H), 3.13, (m, 1H), 3.02 (s, 3H), 1.53−2.78 (m, 20H), 1.35−1.49 (m, 9H), and 1.14 (s, 9H); IR (neat) 2960, 2940, 2860, 2220, 2200, 1705, 1415, 1260, 1195, 1095, 1040, and 800 cm^{-1}; UV−vis λ_{max} (hexane) 301 nm (ϵ = 10 209), 351 (2097), 370 (2558), and 548 (198); exact mass (FABMS, NBA) calcd for $C_{36}H_{50}N_3O_3$ (M^+ + 1) 572.3852, found 572.3853.

(13) That double spin adduct **6** is not an artifact produced via a mechanism involving the intermediacy of carbanionic species is supported by the absence of (1-cyanocyclohexyl)diphenylmethanol in the reaction mixture (as determined by ^{1}H NMR and TLC comparison with an authentic sample) when the thermolysis is conducted in the presence of an equimolar concentration of benzophenone. In toluene solution, benzophenone is preferentially attacked by carbanions (organolithium derivatives) in the presence of equimolar concentrations of nitrone **1** (R = OEt).

(14) For example, on the basis of analysis of the ^{1}H NMR spectra of the violet and green products formed at room temperature when nitrone **1** (R = OEt, 10 mg, 100 mM in benzene) is subjected to conditions for the generation of the 4-bromophenyl radical (according to the Gokel modification of the Gomberg−Bachmann reaction), their structures have been assigned as the corresponding hydroxylamine (violet) and nitrone (green) resulting from disproportionation of the expected intermediate nitroxide radical. That these products are also formed in 9:1 benzene−*t*-BuOH argues strongly against their being artifacts formed via the involvement of aryl carbanion intermediates. See: Beadle, J. R.; Korzeniowski, S. H.; Rosenberg, D. E.; Garcia-Slanga, B. J.; Gokel, G. W. *J. Org. Chem.* **1984**, *49*, 1594.

(15) This red product has been identified as aldehyde **4** and is postulated to arise from decomposition of a spin adduct between nitrone **1** (R = OEt) and an oxygen-centered radical. Nitrone **1** (R = OEt, 5.65 × 10^{-4} M) is unchanged in 98:1:1 EtOAc−HOAc−water after 10 h at 90 °C in a sealed tube. Spin trapping studies of **1** with active oxygen radicals have not yet been conducted. See also ref 18b.

(16) Laayoun, A.; Coulombeau, J. F.; Constant, J. F.; Berger, M.; Cadet, J.; Lhomme, J. *Int. J. Radiat. Biol.* **1994**, *66*, 259.

(17) (a) Thomas, C. E.; Ohlweiler, D. F.; Kalyanaraman, B. *J. Biol. Chem.* **1994**, *269*, 28055. (b) Lee, D. M. In *Methods in Enzymology*; Packer, L., Ed.; Academic Press: San Diego, 1994; Vol. 234, p 513. See also: Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. *N. Engl. J. Med.* **1989**, *320*, 915.

(18) (a) Hensley, K.; Carney, J. M.; Mattson, M. P.; Aksenova, M.; Harris, M.; Wu, J. F.; Floyd, R. A.; Butterfield, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3270. (b) Hensley, K.; Aksenova, M.; Carney, J. M.; Harris, M.; Butterfield, D. A. *NeuroReport*, **1995**, *6*, 493.

(19) Floyd, R. A.; Carney, J. M. *Ann. Neurol.* **1992**, *32 Suppl.*, S22.

(20) (a) Edamatsu, R.; Mori, A.; Packer, L. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 847. (b) Chen, Q.; Fischer, A.; Reagan, J. D.; Yan, L.-Y.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4337. See also: Harman, D. *J. Gerontol.* **1956**, *11*, 298.

(21) (a) Hix, S.; Da Silva Morais, M.; Augusto, O. *Free Radical Biol. Med.* **1995**, *19*, 293. (b) Jurkiewicz, B. A.; Buettner, G. R. *Photochem. Photobiol.* **1994**, *59*, 1. (c) Buettner, G. R.; Kelley, E. E.; Burns, C. P. *Cancer Res.* **1993**, *53*, 3670. (d) Hiramoto, K.; Kaku, M.; Kato, T.; Kikugawa, K. *Chem. Biol. Interact.* **1995**, *94*, 21. See also: Ames, B. N. *Science* **1983**, *221*, 1256. Pryor, W. A. In *Anticarcinogenesis and Radiation Protection*; Cerutti, P. A., Nygaard, O. F., Simic, M. G., Eds.; Plenum Press: New York, 1987; p 1.