5-Carboxy-5-methyl-1-pyrroline *N*-oxide: a spin trap for the hydroxyl radical

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The *in vivo in situ* detection of hydroxyl radical (HO$^{\bullet}$) in real time has been one of the great challenges of free radicals in biology. While we have been able to identify this free radical as a secondary biomarker of HO$^{\bullet}$, the discovery that 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** can specifically spin trap HO$^{\bullet}$ at the expense of superoxide ($O_2^{\bullet -}$) opens new avenues of research. In particular, nitrone **2** will allow us to detect HO$^{\bullet}$ from low doses of radiation in animal tumors in real time.

Introduction

In 1895, Röntgen discovered that a Cookes tube was emitting a new penetrating form of electromagnetic radiation, which he called the X-ray.[1] Soon thereafter, Grubbé and associates used X-rays in the treatment of breast cancer.[2] Thus, began the field of radiation oncology. It would, nevertheless, be another forty-eight years before hydroxyl radical (HO$^{\bullet}$) generation was proposed to be the initiating event in radiation-induced tumor cell killing.[3]

Drawing upon these earlier findings,[3] we, in 1995, reported the *in vivo in situ* spin trapping of a free radical produced by radiation-induced HO$^{\bullet}$ in real time using low frequency EPR spectroscopy.[4] For these initial investigations, we used the spin trapping system consisting of 4-pyridyl(*N*-*tert*-butyl)methanimine *N*,*N*′-dioxide **1** in the presence of EtOH. Hydroxyl radical reacts with EtOH, yielding α-hydroxyethyl radical ($CH_3^{\bullet}CHOH$). The latter free radical was subsequently spin trapped by nitrone **1**, affording the corresponding aminoxyl.[5] Despite this success, we only detected the HO$^{\bullet}$ biomarker in the interstitial space of the tumor. While these studies[4] defined radiation-induced free radical reactions in this aqueous compartment, intracellular free radical events remain to be defined and await the development of spin traps that can compartmentalize within the tissue. One approach is to use readily hydrolyzable esters of, for instance, 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2**.[6] Once the ester of 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** has been taken-up by cells, hydrolysis by esterases therein would entrap nitrone **2**, as the pK_a of this compound is 2.95.[6a]

After the spin trapping specificity of 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** is defined, a reasonable preparative scheme for the synthesis of ^{15}N and deuterium-labeled esters of nitrone **2**,[6b] as was accomplished with 5,5-dimethyl-1-pyrroline *N*-oxide **3**, could be achieved.[7] First, however, it is important to define the sensitivity and selectivity of 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** toward HO$^{\bullet}$ and $O_2^{\bullet -}$. Herein, we report that 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** reacts with HO$^{\bullet}$ at near diffusion controlled rates, yielding the corresponding aminoxyl **6**. Using X-ray radiation, we were able to detect HO$^{\bullet}$ in a phosphate buffer with as little as 300 nM (1 Gy of radiation) of HO$^{\bullet}$. Furthermore, it was found that nitrone **2** does not react with $O_2^{\bullet -}$. Finally, 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** can easily be prepared from readily available chemicals, suggesting that isotope labeling is cost effective.

Results and discussion

The preparation of 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** is not unduly challenging, although attentiveness to experimental details will ensure a high yield of nitrone **2**.[6a] Once the synthesis was completed, we determined the specificity of 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** toward HO$^{\bullet}$ and $O_2^{\bullet -}$. One of the problems with 5,5-dimethyl-1-pyrroline *N*-oxide **3** is that aminoxyl **4** can be produced from either the spin trapping of HO$^{\bullet}$, from the decomposition of aminoxyl **5** or from metal ion-catalyzed aerobic oxidation (Scheme 1).[8] Thus, with nitrone **3**, it is frequently difficult to determine whether HO$^{\bullet}$ or $O_2^{\bullet -}$ has actually been spin trapped. This is a troubling problem to solve when attempts are made to identify either of these free radicals within cells.[9] Synthetic modifications to pyrroline *N*-oxides have afforded nitrones whose spin trapped adducts of $O_2^{\bullet -}$ do not decompose into aminoxyls analogous to **4**.[6b,10] Yet, we continue to seek a nitrone that can identify HO$^{\bullet}$ within a cellular compartment of a mammalian tissue in real time and at the site of its evolution.

Therefore, we investigated whether 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** might exhibit the necessary specificity. First, we determined if nitrone **2** (60 mM) would spin trap HO$^{\bullet}$. For these experiments we used three different sources of HO$^{\bullet}$: (a) photolysis of H_2O_2, (b) reduction of H_2O_2 by ferrous sulfate, and (c) X-ray radiation of H_2O. Direct H_2O radiolysis has been shown to be a convenient and quantitative source of HO$^{\bullet}$.[11] The

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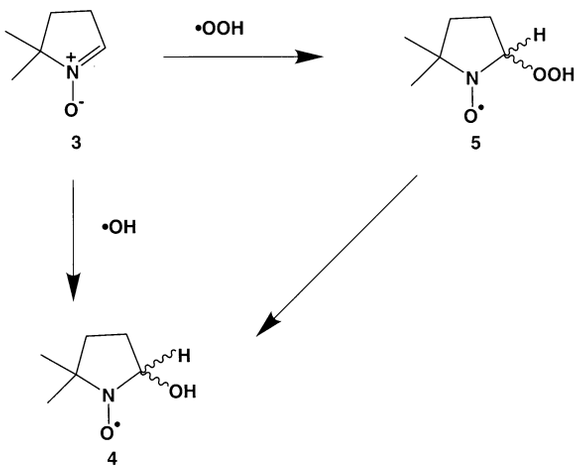
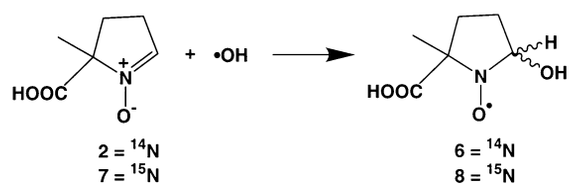


Scheme 1

relationship between radiation dose and HO• concentration is linear at conventional dose rates. Approximately, 300 nM of HO• is generated by each Gy of radiation. Although the proportionality constant depends on the source of ionizing radiation (γ-decay *versus* X-ray), the molar yield per Gy is a constant to within 5% over more than an order of magnitude in average photon energy, including ^{60}Co 150 kVp X-ray.[12]

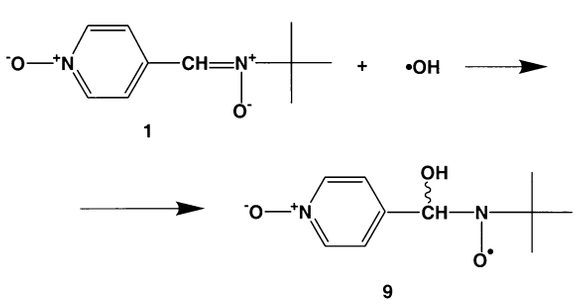
Independent of the experimental conditions, 5-carboxy-5-methyl-1-pyrroline ^{14}N-oxide **2** was able to spin trap HO•, leading to aminoxyl **6** (Scheme 2). The resultant EPR spectrum



Scheme 2

of aminoxyl **6** is shown in Fig. 1. It comprises two diastereomers, the dominant conformer (80%) has hyperfine splitting constants of a_N = 14.1 G and a_H = 15.8 G (Fig. 1). The lesser conformer (20%) displays hyperfine splitting constants of a_N = 14.1 G and a_H = 12.2 G (Fig. 1). The isomeric distribution was more clearly visible when HO• was spin trapped by 5-carboxy-5-methyl-1-pyrroline ^{15}N-oxide **7**, yielding aminoxyl **8** (Fig. 2). In this case, the major conformer (83%) exhibits hyperfine splitting constants of a_N = 20.1 G and a_H = 16.4 G (Fig. 2). The minor conformer (17%) shows hyperfine splitting constants of a_N = 20.1 G and a_H = 12.4 G (Fig. 2).

An apparent rate constant was obtained by estimating the relative rate of spin trapping HO• by 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** as compared to that for nitrone **1**.[13] Nitrone **1** (Scheme 3) was chosen as a competitive inhibitor, since the



Scheme 3

EPR spectrum for aminoxyl **9**, arising from the reaction of HO• with nitrone **1**, does not interfere with the EPR spectrum of

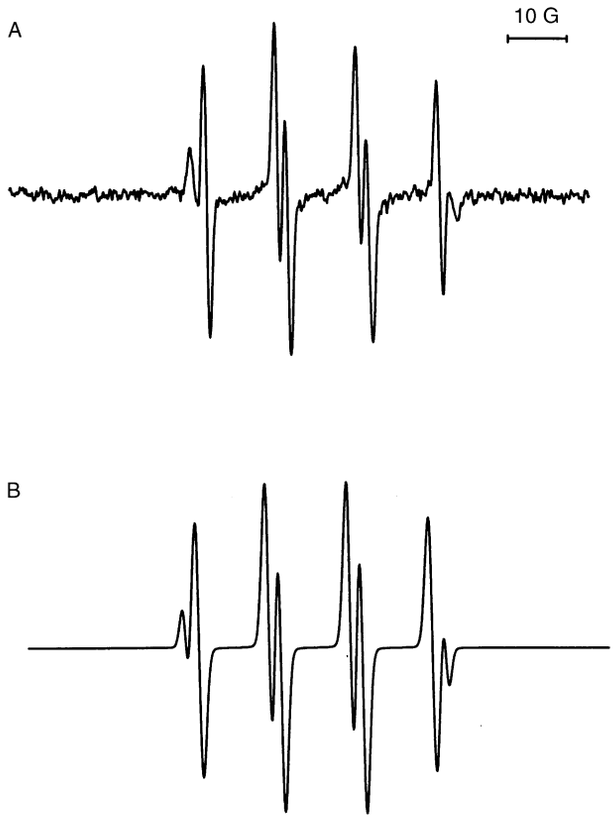


Fig. 1 **A**. EPR spectrum of aminoxyl **6**, generated by the reaction of nitrone **2** (60 mM in 50 mM phosphate buffer, containing 1 mM DTPA at pH 7.4) with H_2O_2 (17.6 mM). The EPR spectrum was recorded 1 min after photolysis, which lasted for 1 min. Receiver gain was 1.6×10^4. **B**. Computer simulation of the two isomers of aminoxyl **6**. The larger conformer, which comprises 80% of the composite EPR spectrum, has hyperfine splitting constants of a_N = 14.1 G and a_H = 12.2 G. The smaller conformer, which encompasses 20% of the composite EPR spectrum, has hyperfine splitting constants of a_N = 14.1 G and a_H = 15.8 G.

aminoxyl **6**. Reactions of nitrone **2** and nitrone **1** with HO• can be expressed as shown in eqn. (1) and (2).

$$\text{HO}^\bullet + \text{nitrone } \mathbf{2} \longrightarrow \text{aminoxyl } \mathbf{6} \quad (1)$$

$$\text{HO}^\bullet + \text{nitrone } \mathbf{1} \longrightarrow \text{aminoxyl } \mathbf{9} \quad (2)$$

From eqn. (1) and (2), the rate of HO• elimination can be represented as shown in eqn. (3).

$$V = -\text{d[HO}^\bullet\text{]}/\text{d}t = k_{app}[\text{nitrone } \mathbf{2}][\text{HO}^\bullet] + k_{\text{aminoxyl 9}}[\text{nitrone } \mathbf{1}][\text{HO}^\bullet] \quad (3)$$

In the absence of nitrone **1**, eqn. (3) can be described as shown in eqn. (4).

$$v = -\text{d[HO}^\bullet\text{]}/\text{d}t = k_{app}[\text{nitrone } \mathbf{2}][\text{HO}^\bullet] \quad (4)$$

By dividing eqn. (4) into eqn. (3) and rearranging the terms, the competing reactions can be represented as shown in eqn. (5).

$$V/v = 1 + k_{\text{aminoxyl 9}}[\text{nitrone } \mathbf{1}]/k_{app}[\text{nitrone } \mathbf{2}] \quad (5)$$

Determining the rates V and v are cumbersome, resulting too often in estimations that are prone to error. However, the relative concentration of the spin trapped adduct, as determined by EPR spectral peak height, is directly related to the k_{app} and k_{aminoxyl} **9**, the rate constants for the spin trapping of HO• by nitrone **2** and nitrone **1**, respectively, at given concen-

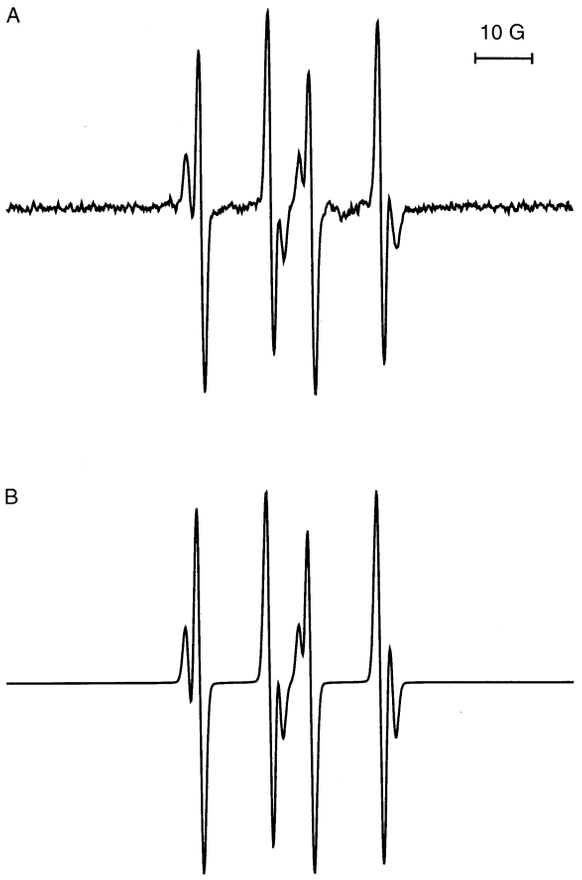
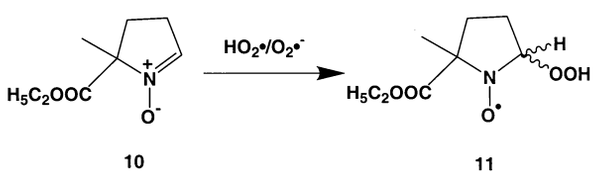


Fig. 2 **A**. EPR spectrum of aminoxyl **8**, generated by the reaction of nitrone **7** (60 mM in 50 mM phosphate buffer, containing 1 mM DTPA at pH 7.4) with H_2O_2 (17.6 mM). The EPR spectrum was recorded 1 min after photolysis, which lasted for 1 min. Receiver gain was 1.6×10^4. **B**. Computer simulation of the two isomers of aminoxyl **8**. The larger conformer, which comprises 77% of the composite EPR spectrum, has hyperfine splitting constants of $a_N = 19.7$ G and $a_H = 12.4$ G. The smaller, which encompasses 23% of the composite EPR spectrum, has hyperfine splitting constants of $a_N = 16.4$ G and $a_H = 20.1$ G.

tration of nitrone **2**. As such, we can assign the term A_o to be the peak height of aminoxyl **6** in the absence of nitrone **1** and the term A to be the peak height of aminoxyl **6** in the presence of nitrone **1** at various concentrations. If the concentration of the nitrone **2** is fixed, then a plot of A_o/A *versus* [nitrone **1**]/[nitrone **2**] generates a straight line, the slope is $k_{\text{aminoxyl 9}}$ / k_{app}[nitrone **2**].[13] By using the known rate constant[8a] for the formation of aminoxyl **9**, $k = 1.9 \times 10^9$ $M^{-1}s^{-1}$, the k_{app} value for nitrone **2** can be calculated. A representative experiment is shown in Fig. 3. Here photolysis of H_2O_2, as a source of $HO^{\bullet}$, led to $k_{app} = 7.7 \times 10^8$ $M^{-1}s^{-1}$. Repeated experiments under identical conditions resulted in an average $k_{app} = 6.8 \pm 1.5 \times 10^8$ $M^{-1}s^{-1}$.

Next, we investigated whether 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** would spin trap $O_2^{\bullet -}$. For these studies, we used NADPH–FMN as the source of $O_2^{\bullet -}$. Under these conditions, we found that 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **10** (60 mM) spin trapped $O_2^{\bullet -}$ at pH 7.4[6b] and $HO_2^{\bullet}$ at pH 1.95 (Scheme 4, Fig. 4**A** and Fig. 4**C**). In contrast, 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** did not react with $HO_2^{\bullet}/O_2^{\bullet -}$ at either pH (Fig. 4**B** and Fig. 4**D**). The pK_a of $HO_2^{\bullet}/O_2^{\bullet -}$ is 4.8.[14] From these experiments, several observations are worth noting. First, as in the case of 5,5-dimethyl-1-pyrroline *N*-oxide **3**,[8a] the efficiency of spin trapping $O_2^{\bullet -}$ by 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide is far greater at low pH, where $HO_2^{\bullet}$ dominates, than it is at high pH where $O_2^{\bullet -}$ is most prevalent.



10 11

Scheme 4

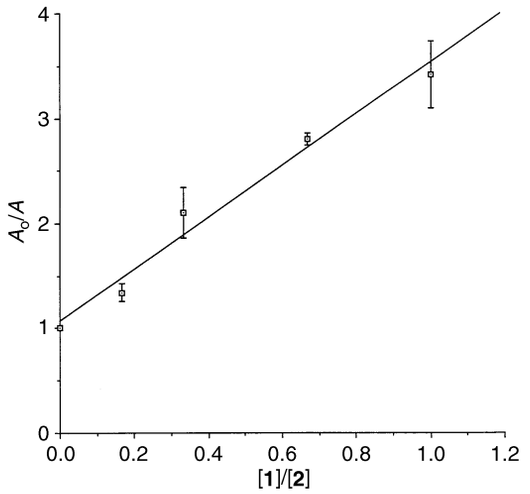


Fig. 3 A representative plot of the inhibition by nitrone **1** of the spin trapping of $HO^{\bullet}$ by nitrone **2**. From these data, the apparent rate constant was found to be 7.7×10^8 $M^{-1}s^{-1}$. Details are presented in the Results and discussion section.

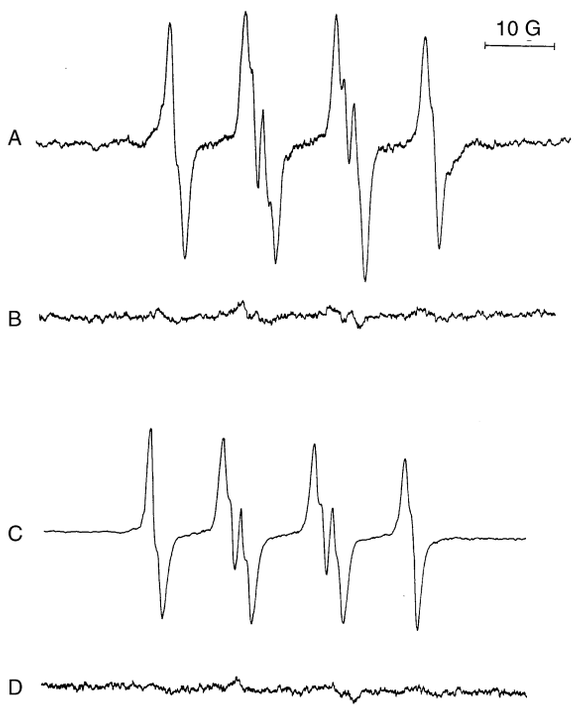


Fig. 4 **A**. EPR spectrum of the aminoxyl **11**, produced by the spin trapping of $O_2^{\bullet -}$ by 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide **10** (60 mM in 50 mM phosphate buffer, containing 1 mM DTPA at pH 7.4). The EPR spectrum is a composite of two conformers with hyperfine splitting constants of $a_N = 12.8$ G, $a_H^{\beta} = 12.1$ G and $a_H^{\gamma} = 0.2$ G for one isomer and $a_N = 12.8$ G and $a_H^{\beta} = 8.6$ G for the other isomer.[6b] The source of $O_2^{\bullet -}$ was NADPH (1 mM)–FMN (1 mM). The EPR spectrum was recorded 3 min after mixing the reactants. Receiver gain was 1.0×10^4. **B**. Same as **A**, except that nitrone **2** (60 mM) was used in place of 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide. Receiver gain was 1.0×10^4. **C**. EPR spectrum of the aminoxyl **11**, produced by the spin trapping of $O_2^{\bullet -}$ by nitrone **10** (60 mM in 50 mM KCl and HCl solution, containing 1 mM DTPA at pH 1.95). The EPR spectrum was recorded 3 min after mixing the reactants. Receiver gain was 2.5×10^3. **D**. Same as **C**, except that nitrone **2** (60 mM) was used in place of nitrone **10**. Receiver gain was 1.0×10^4.

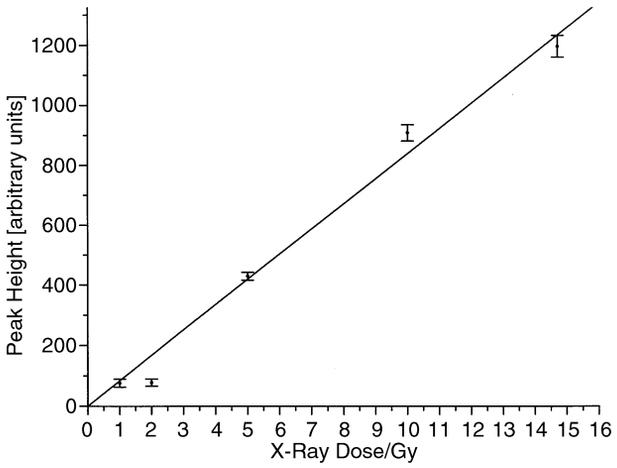


Fig. 5 The height of the highest feature from the EPR spectrum of aminoxyl **6**, generated by the reaction of nitrone **2** (40 mM in 50 mM phosphate buffer, containing 1 mM DTPA at pH 7.4) with $HO^{\bullet}$, produced by water radiolysis as a function of radiation dose in Gray (Gy). The third spectral feature was the line chosen as the highest in the spectrum, the high-field doublet. The measured heights were extrapolated to the time at the end of the dose delivery. Radiation was delivered using 150 kVp X-ray from a PANTAK pmc100 X-ray source as described above. EPR spectrometer settings are described for the radiation measurements in the text.

Second, we originally speculated that the reason 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** did not react with $O_2^{\bullet -}$ at pH 7.4 was due to the repulsive effects of the two negative charges, namely that on nitrone **2** and $O_2^{\bullet -}$, which prevented the spin trapping of this free radical. However, even at a pH where the equilibrium for 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** is shifted in the direction of the free acid, nitrone **2** still did not spin trap $HO_2^{\bullet}$. We are currently exploring alternative mechanisms to account for this finding.

Since our long-term goal is *in vivo in situ* detection of $HO^{\bullet}$ after exposing a tumor to radiation, experiments were designed to estimate the efficiency of nitrone **5** in capturing low concentrations of $HO^{\bullet}$ after X-ray radiation of an aqueous sodium phosphate buffer (50 mM, pH 7.4). Fig. 5 shows a linear relationship between EPR spectral peak height of the second line of aminoxyl **6** and the radiation dose. Interestingly, we were able to detect aminoxyl **6** with as little as 1 Gy (300 nM of $HO^{\bullet}$) of radiation, increasing linearly up to 15 Gy. The half-life of aminoxyl **6** under these experimental conditions was 30 ± 2 min, sufficiently long to allow the identification of $HO^{\bullet}$ generated at the site of evolution in real time. We anticipate that this approach will, in the future, allow for the direct comparison of $HO^{\bullet}$ spin trapping effectiveness as new spin traps are developed for the detection of this free radical.

Experimental

Reagents

Diethylenetriaminepentaacetic acid (DTPA), NADPH (nicotinamide adenine dinucleotide phosphate) and FMN (flavin mononucleotide) were purchased from Sigma Chemical Company (St. Louis, MO). Superoxide dismutase (SOD) was obtained from Boehringer Mannheim (Indianapolis, IN). Sodium [^{15}N]nitrite was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA). All other reagents were of reagent grade unless indicated otherwise. IR spectra were recorded on a FT-IR spectrometer (Perkin-Elmer, Norwalk, CT) in $CHCl_3$. ^{1}H NMR spectra were obtained using a GE QE-300/Tecmake NMR spectrometer. The melting point of 5-carboxy-5-methyl-1-pyrroline ^{14}*N*-oxide **2** and 5-carboxy-5-methyl-1-pyrroline ^{15}*N*-oxide **7** was measured on a Thomas Hoover capillary melting point apparatus and was corrected. Mass spectra were run on a Finnigan LCQ/Quadrupole ion trap Mass Spectrometer (Metrics, Greenville, NC), housed at the University of Maryland School of Pharmacy Mass Spectrometry Center.

Syntheses

Ethyl 2-nitropropionate. Synthesis of the nitro-ester was performed by following literature procedures with some modifications.[15] Ethyl 2-bromopropionate (20 g, 14.3 mL, 110 mmol, Aldrich) was added to a solution of dimethyl sulfoxide (90 mL) containing sodium [^{14}N]nitrite (13.2 g, 192 mmol) and phloroglucinol dihydrate (1,3,5-trihydroxybenzene·2H_2O, 19.1 g, 118 mmol, Aldrich). This reaction was run in the dark for 2 h at room temperature during which time it turned brown. This reaction was then poured over ice–water (200 mL) and extracted extensively with ether. The remaining etheral solution was washed with an aqueous saturated solution of NaCl (30 mL) and dried over anhydrous $MgSO_4$. After filtration, the solution was rotary evaporated to dryness to give an oil. Distillation afforded ethyl 2-nitropropionate, as a colorless liquid, bp, 43–45 °C at 0.05 mmHg, 8 g (49%).[15] IR ($CHCl_3$) *v*: 1752 (C=O), 1564 (N–O), 1475 (N–O) cm^{-1}.

2-Ethoxycarbonyl-2-nitropentanal. To a solution of sodium ethoxide (0.2 g sodium in 200 mL absolute ethanol) was added ethyl 2-nitropropionate (8 g, 54.4 mmol). The reaction was cooled in an ice bath. Acrolein (3 g, 3,67 mL, 54.4 mmol, Aldrich, freshly distilled over drierite) was then added slowly to the mixture, maintaining the temperature at 10 °C. The reaction was, at this point, stirred at 45 °C for 3 h, whereupon the mixture was cooled in an ice bath and acetic acid (1 mL) was added thereto. Evaporation, *in vacuo*, left a residual oil that was taken up in $CHCl_3$ and washed with a saturated solution of NaCl (20 mL). The organic layer was dried over anhydrous $MgSO_4$ and evaporated to dryness. Fractional distillation gave 2-ethoxycarbonyl-2-nitropentanal, as a colorless liquid, bp 78–80 °C at 0.05 mmHg, 6 g (55%).[6a] IR ($CHCl_3$) *v*: 1754 (C=O), 1546 (N–O), 1450 (N–O) cm^{-1}. This aldehyde-ester formed a 2,4-dinitrophenylhydrazone.

5-Ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (10). The 1,3-dioxolan was initially prepared followed by Zn reduction as described in the literature with modifications.[6a] 2-Ethoxycarbonyl-2-nitropentanal (9.05 g, 44.58 mmol) in benzene (100 mL, freshly distilled to remove water) containing ethylene glycol (3.23 g, 52.14 mmol) and toluene-*p*-sulfonic acid (100 mg) were refluxed, the generated water was collected in a Dean–Stark trap until the theoretical amount was achieved. The reaction was then cooled, washed with a saturated solution of $NaHCO_3$ (50 mL) and dried over anhydrous $MgSO_4$. Evaporation to dryness left a residual oil that was distilled to yield the dioxolan, as a colorless oil, bp 88–92 °C at 0.05 mmHg, 8.2 g (75%).[6a] IR ($CHCl_3$) *v*: 1748 (C=O), 1551 (N–O), 1454 (N–O) cm^{-1}.

The dioxolan (8.2 g, 33.20 mmol) was dissolved in 50% aqueous ethanol (70 mL), containing NH_4Cl (1.82 g, 34 mmol) and cooled to 10–15 °C. Zinc dust (11.9 g, 182 mmol) was slowly added, maintaining the temperature ≤15 °C. After stirring for 3 h at this temperature, the reaction mixture was filtered, the filter cake was washed with 95% ethanol (50 mL) and evaporated to dryness. The remaining oil was taken up in $CHCl_3$, dried over anhydrous $MgSO_4$ and evaporated to dryness. The hydroxylamine was dissolved in dilute hydrochloric acid (0.3 M, 100 mL) and left to stand for 24 h at ambient temperature. Upon neutralization with ammonia (30%), the solution was evaporated *in vacuo*. The residue was dissolved in $CHCl_3$, dried over anhydrous Na_2SO_4 and evaporated to dryness. Distillation yielded 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (**10**), as a viscous oil, bp 104–106 °C at

0.05 mmHg, 2.9 g (48%).[6a] IR ($CHCl_3$) 1740 (C=O), 1585 (C=N–O) cm^{-1}.

5-Carboxy-5-methyl-1-pyrroline ^{14}N-oxide (2) and 5-carboxy-5-methyl-1-pyrroline ^{15}N-oxide (7). 5-Ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (2 g) was hydrolyzed by treatment with an aqueous solution of sodium hydroxide (2.5%, 20 mL) at 100 °C for 1 h. Upon cooling, this solution was passed through a column containing Dowex 50 (H^+ form, Biorad, Richmond, CA). Fractions were collected and rotary evaporated under vacuum to dryness, giving 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2**. Crude nitrone **2** was purified by flash chromatography using silica gel (mesh 230–400). By eluting the column with chloroform–methanol (11 : 1), 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** (1.2 g, 70%) was obtained as a white solid, a portion of which was recrystallized from $CHCl_3$, mp 135–136 °C.[6a] IR ($CHCl_3$) *v*: 3500–3300 (O–H), 1715 (C=O), 1585 (C=N–O) cm^{-1}; NMR (D_2O) δ: 1.60 (s, 3H), 2.10–2.30 (m, 1H), 2.40–2.65 (m, 1H), 2.65–2.90 (m, 2H), 7.35 (s, 1H); *m*/*z* 143.

5-Carboxy-5-methyl-1-pyrroline ^{15}N-oxide **7** was prepared as described above, except sodium [^{15}N]nitrite was used in place of sodium [^{14}N]nitrite in the first step. Nitrone **7** was isolated as a white solid, mp 135–136 °C (from $CHCl_3$).[6a] IR ($CHCl_3$) *v*: 3500–3300 (O–H), 1715 (C=O), 1585 (C=N–O) cm^{-1}; NMR (D_2O) δ: 1.60 (s, 3H), 2.10–2.30 (m, 1H), 2.40–2.65 (m, 1H), 2.65–2.90 (m, 2H), 7.35 (s, 1H); *m*/*z* 143.9.

Generation of superoxide

Superoxide was generated using NADPH and FMN at pH 7.4 and 1.95.[16] Specifically, $O_2^{\bullet -}$ production was achieved by mixing NADPH (1 mM) and FMN (1 mM) in sodium phosphate buffer (50 mM) containing DTPA (1 mM), at pH 7.4 and KCl (50 mM and HCl solution titrated to pH 1.95 with a NaOH solution) containing DTPA (1 mM).

Spin trapping of hydroxyl radical

Photolysis of H_2O_2 was used as a source of $HO^{\bullet}$. A solution of either 5-carboxy-5-methyl-1-pyrroline ^{14}N-oxide **2** (60 mM) or 5-carboxy-5-methyl-1-pyrroline ^{15}N-oxide **7** (60 mM), containing H_2O_2 (17.6 mM) was irradiated by exposing this reaction to UV light (Ultra-Violet Products, Inc, San Gabriel, CA, SCTI model) for 1 min in sodium phosphate buffer (50 mM) at pH 7.4 containing DTPA (1 mM). EPR Spectra were recorded 1 min after termination of photolysis. The Fenton reaction was used as an alternative source of $HO^{\bullet}$ by mixing H_2O_2 (17.6 mM) and Fe^{2+} (80 μM) in phosphate buffer at pH 7.4. All spectra were recorded at room temperature using an EPR spectrometer (Varian Associates E-9 or E-109). Reaction mixtures were transferred to a flat quartz cell and fitted into the cavity of the EPR spectrometer. Instrumentation settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; response time, 1 s; and sweep, 12.5 G min^{-1}.

X-Ray irradiation of H_2O was used as an alternative source of $HO^{\bullet}$. 5-Carboxy-5-methyl-1-pyrroline *N*-oxide **2** (1 mL, 40 mM) in sodium phosphate buffer (50 mM) at pH 7.4 containing DTPA (1 mM) was irradiated with 1, 2, 5, 10 or 14.7 Gy at a dose rate of 2.12 Gy min^{-1} (PANTAK pmc1000, 150kVp, 25 mA, 1.6 mm Al filter, half value layer (HVL) = 1.9 mm Cu). Approximately 2 min after irradiation was completed, the sample was transferred to a quartz TM flat cell and fitted into the cavity of a Varian E-12 EPR spectrometer equipped with a century series bridge (E-102) and with a TM011 cavity mounted horizontally in the magnet. EPR spectra were recorded at X-band (9.45 GHz), using 30 mW of power, with time constant of 0.3 s, a dwell time of 0.3 s, 512 points per spectrum, (~2.5 min per spectrum), receiver gain of 2×10^5 and modulation amplitude of 0.5 G. Data were logged on a PC interfaced with the Varian console *via* an interface built by R. Quine, University of Denver, a set of 12 bit A/D and D/A channels on a Data Translation (Marlboro, MA) DT2721 computer interface board and locally written acquisition software. Data for signal height *versus* dose were obtained from multiple measurements on samples exposed to given radiation doses as a function of time after radiation. These were extrapolated to the signal height at the end of radiation using a linear exponential regression. Error estimates on the signal height *versus* time scans derived from two sources. The first was the standard signal-to-noise (S/N). A portion of the baseline representing approximately 1/10 the spectral scan was selected as typical. Peak-to-peak deviation was measured. The ratio of this signal to the interval was multiplied by 3 to represent the signal to RMS noise. A second source of measurement uncertainty was the insertion error. This is estimated to be 6% based on the reinsertion of stable sample into the cavity. The final errors reflect the larger of the two sources.

Spin trapping superoxide

Spin trapping of $O_2^{\bullet -}$ by nitrone **2** (60 mM) and nitrone **10** (60 mM) was achieved by mixing the appropriate reactants as described above. All spectra were recorded at room temperature using an EPR spectrometer (Varian Associates E-9). Reaction mixtures were transferred to a flat quartz cell and fitted into the cavity of the spectrometer. Instrumentation settings were: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; response time, 1 s; and sweep, 12.5 G min^{-1}.

Rate of the spin trapping of $HO^{\bullet}$

To determine the apparent rate constant for the spin trapping of $HO^{\bullet}$ by 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2**, the mixture of nitrone **2** (60 mM), H_2O_2 (17.6 mM) and nitrone **1** (0 – 60 mM) were photolyzed for 1 min, as previously described.[13] Reaction mixtures were immediately transferred to an EPR flat quartz cell and introduced into the cavity of the EPR spectrometer. EPR spectra were recorded 1 min after the termination of the photolysis.

Stability of the spin trapped adduct of $HO^{\bullet}$

The stability of the spin trapped adduct of $HO^{\bullet}$ was estimated using methods detailed earlier. In a typical experiment, 1 mL of 5-carboxy-5-methyl-1-pyrroline *N*-oxide **2** (40 mM) in sodium phosphate buffer (50 mM) at pH 7.4 containing DTPA (1 mM) was irradiated with 10 Gy at a dose rate of 2.12 Gy min^{-1} (PANTAK pmc1000, 150kVp, 25 mA, 1.6 mm Al filter, HVL = 1.9 mm Cu). Approximately 2 min after irradiation was completed, the sample was transferred to a quartz TM flat cell and fitted into the cavity of a Varian E-12 EPR spectrometer. EPR spectra were recorded at room temperature at various time intervals until the peak height decreased to several half-lives.

Modeling of EPR spectra

EPR spectra were modeled using the Bruker Symphonia and Bruker WinEPR programs.

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