temperature factors for non-hydrogen atoms. All hydrogen atoms were located from the difference Fourier map and refined with isotropic temperature factors. The final residual index (R factor) was 0.054. Calculations were carried out with the DIRECT-SEARCH program system.[13] Three tables consisting of atomic fractional coordinates, bond lengths, and bond angles have been deposited as supplementary material.

Crystal Structure of 25b. Recrystallization from Et_2O led to colorless prism crystals; the unit cell constants are a = 6.359 (1) Å, b = 10.145 (1) Å, c = 18.713 (1) Å, and β = 91.57 (1)° in space group $P2_1/a$ (Z = 4). Of the 2058 reflections measured with $2\theta \leq 130$ using Cu Kα, 1620 were independently observed at level $F \geq 3\sigma(F)$. Data reduction, least squares, electron-density synthesis, and related calculations were performed by using the methods and procedures described for **25a**. The final discrepancy factor was 0.076.

Crystal Structure of 25d. Crystals were formed in space group $P\bar{1}$ with a = 11.182 (3) Å, b = 11.733 (3) Å, c = 11.364 (3) Å, α = 110.95 (2)°, β = 70.92 (2)°, and γ = 118.95 (2)° for Z = 4. Of the 3203 reflections measured with $2\theta \leq 115$ using Cu Kα, 2190 were independently observed at level $F \geq 3\sigma(F)$. Refinement was carried out with block-diagonal least squares, and other calculations were performed by using the methods and procedures described for **25a**. The final R factor was 0.053.

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Acknowledgment. We thank Drs. Y. Tsujita and I. Kaneko for preparing the rabbit lung ACE used. Our appreciation is also extended to Dr. M. Yoshimoto for helpful discussions on the structure–activity relationship.

Registry No. **6a**, 111742-85-9; **6b**, 111820-50-9; **6c**, 111820-51-0; **6d**, 81938-91-2; **7**, 140-29-4; (±)-**8**, 111742-86-0; (±)-**10**, 111742-88-2; (±)-**11**, 111742-89-3; (±)-**12**, 111742-90-6; (±)-**13**·HCl, 111742-91-7; (±)-**14**, 111742-92-8; **15**, 4248-25-3; **16**, 111742-93-9; (±)-**19**, 111742-96-2; (±)-**20**, 111742-97-3; (±)-**21**·HCl, 111742-98-4; (±)-**22**, 111742-99-5; (±)-*cis*-**23**, 111743-00-1; (±)-***trans*-23**, 111742-87-1; (±)-*cis*-**24**, 111743-01-2; (γ)-*trans*-**24**, 111742-94-0; (±)-**25a**, 111743-02-3; (±)-**25b**, 111742-95-1; (±)-**25c**, 111743-07-8; (±)-**25d**, 111743-08-9; (±)-**26a**, 111743-03-4; (±)-**26b**, 111743-10-3; (±)-**26c**, 111743-10-3; (±)-**26d**, 111743-11-4; (±)-**27a**, 111743-04-5; (±)-**27b**, 111743-12-5; (±)-**27b**·maleate, 111743-15-8; (±)-**27c**, 111743-13-6; (±)-**27c**·maleate, 111743-16-9; (±)-**27d**, 111743-14-7; (±)-**27d**·maleate, 111743-17-0; **28a**, 111743-05-6; (3*R*,6*S*)-**28a**, 111820-57-6; **28b**, 111821-30-8; (3*R*,6*R*)-**28b**, 111820-58-7; **28c**, 111820-52-1; (3*R*,7*S*)-**28c**, 111820-59-8; **28d**, 111820-53-2; (3*R*,7*R*)-**28d**, 111820-60-1; **29a**·HCl, 111743-06-7; **29b**·HCl, 111820-54-3; **29c**·HCl, 111820-55-4; **29d**·HCl, 111820-56-5; (±)-**30**, 91417-30-0; ACE, 9015-82-1; $Br(CH_2)_3CO_2Et$, 2969-81-5; $BrCH_2CO_2Bu$-*t*, 5292-43-3; (*R*)-$Ph(CH_2)_2CH(OSO_2(F_3)CO_2Et$, 88767-98-0; *N*-hydroxysuccinimide, 6066-82-6.

Supplementary Material Available: Tables listing X-ray diffraction study data of **25a,b,d** (14 pages). Ordering information is given on any current masthead page.

Synthesis of Spin Traps Specific for Hydroxyl Radical

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Two nitrones, 3,3-diethyl-5,5-dimethylpyrroline 1-oxide (DEDMPO) and 3,3,5,5-tetramethylpyrroline 1-oxide (M_4PO), were synthesized by the zinc/ammonium chloride reduction of appropriately substituted γ-nitrocarbonyl compounds, followed by addition of methylmagnesium bromide to the resulting intermediate nitrones. The lipophilicities of these nitrones were estimated by determining their partition coefficients in an 1-octanol/water system. They were found to be considerably more lipophilic than 5,5-dimethylpyrroline 1-oxide (DMPO). The spin trapping of hydroxyl and superoxide radicals with these nitrones was investigated, and the hyperfine coupling constants were determined. M_4PO was found to spin trap both free radicals, while DEDMPO spin trapped only hydroxyl radical. DEDMPO was used to determine if hydroxyl radical was produced during the metabolism of menadione or nitrazepam by porcine thoracic aorta endothelial cells. Our results indicate, in conjunction with spin-trapping studies utilizing DMPO, that only superoxide is generated during cellular metabolism of quinones and aromatic nitro-containing compounds by endothelial cells.

Free radicals have been proposed to initiate a variety of pathological conditions, including ischemia/reperfusion injury.[1] Yet, verification of the role that these reactive intermediates play in mediating cellular injury is limited, in part, to our ability to be able to monitor free-radical reactions in vivo or at least in an in vitro cell model. With the application of spin-trapping techniques to this problem, great advances have been made in understanding the mechanism by which free radicals induce cellular injury.[2] From these studies, superoxide and hydroxyl radicals appear to be the most important biologically generated free radicals. Because of this, nitrones have emerged as the principal spin traps due to the rapid decomposition of the corresponding nitroxides derived from the reaction of nitroso compounds (e.g., 2-methyl-2-nitrosopropane) with these free radicals.[3] Among the several nitrones used as spin traps,[2] 5,5-dimethylpyrroline 1-oxide (DMPO) has received the most attention. Reaction of this spin trap with either superoxide or hydroxyl radical produces spin-trapped adducts with characteristic ESR spectra.[4] When both free radicals are generated in the presence of DMPO, the resulting ESR signal is a composite of the individual spectrum of the two spin-trapped adducts. In theory, differences in the hyperfine splitting pattern of each spin-trapped adduct should provide a means to distinguish among these free radicals. However, the kinetics of superoxide spin trapping and ongoing side reactions involving the spin-trapped adduct, 2-hydroperoxy-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OOH), make data interpretation more complex. For example, DMPO-OOH is unstable and

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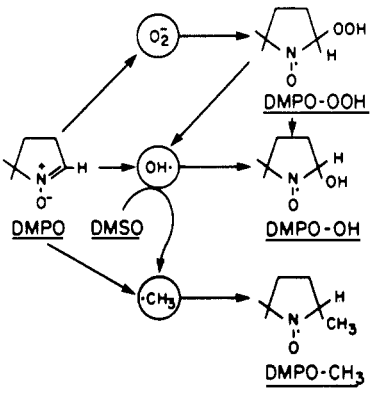


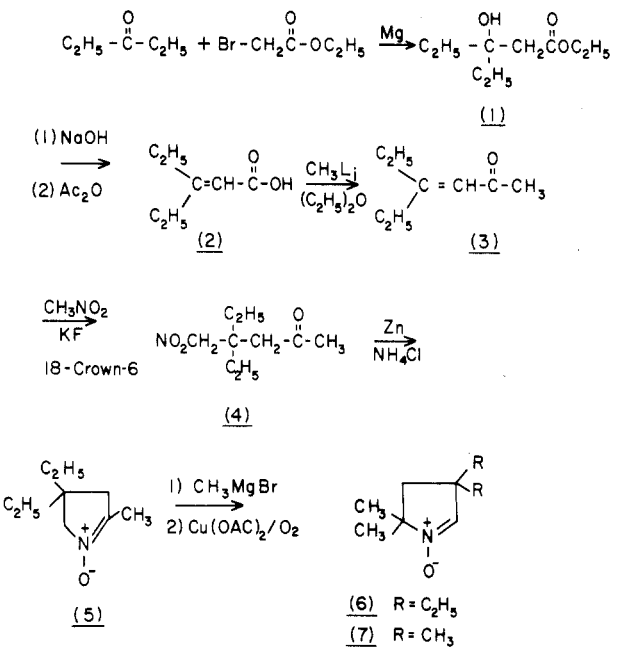
Figure 1. Spin trapping of free radicals by DMPO. The diagram depicts alternate mechanisms by which DMPO-OH can be generated.

rapidly decomposes into three species: (a) 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxyl (DMPO-OH), (b) a nonparamagnetic species, and (c) a small but detectable level of hydroxyl radical, which may then react with DMPO to generate DMPO-OH[5,6] (Figure 1). In addition, DMPO-OOH can be rapidly reduced to DMPO-OH by the enzyme glutathione peroxidase.[7] Thus, DMPO-OH may arise through pathways that are independent of biologically generated hydroxyl radical, making the characteristic spectrum of DMPO-OH insufficient proof for the generation of this free radical.

Verification that an ESR spectrum was derived from the spin trapping of superoxide, even though the hyperfine splitting pattern is characteristic of DMPO-OH, can be accomplished by noting the effect of superoxide dismutase (SOD) and catalase on the magnitude of the signal. Since SOD accelerates the rate of superoxide dismutation to hydrogen peroxide, a dose-dependent diminution in the ESR spectrum should be observed. Again, when catalase, which reduces hydrogen peroxide to water, is added to the reaction, no decrease in signal intensity would be noted if the ESR spectrum characteristic of DMPO-OH resulted from the spin trapping of superoxide. Because the molecular weights of SOD and catalase are large, inhibitory actions of these enzymes are limited to free-radical reactions taking place either in homogeneous solutions or in extracellular milieu.

Several years ago, we reported the spin trapping of free radicals as the result of endothelial cell metabolism of the quinone menadione and the aromatic nitro-containing compound nitrazepam.[7] When menadione was incubated with cultured endothelial cells in the presence of DMPO, we obtained two ESR spectra characteristic of DMPO-OOH and DMPO-OH. With the addition of SOD to the above reaction, we observe only the ESR signal corresponding to DMPO-OH. For nitrazepam, only the ESR spectrum for DMPO-OH was recorded, which was not inhibited by the addition of SOD. Finally, in the absence of either menadione or nitrazepam, no ESR spectrum was noted. Although we undertook a number of experiments to determine the source of DMPO-OH, we were unable to unequivocally prove whether superoxide or hydroxyl radical was spin trapped. On the basis of circumstantial evidence, we suggested[7] that this nitroxide arose as the consequence of the initial spin trapping of superoxide followed by the rapid bioreduction of DMPO-OOH to DMPO-OH, presumably by the enzyme glutathione peroxidase.

Scheme I



Because of the uncertainty as to whether superoxide or hydroxyl radical is spin trapped during the metabolism of either menadione or nitrazepam by endothelial cells, we decided to synthesize several 3,3-dialkyl-5,5-dimethylpyrroline 1-oxides, which would spin trap hydroxyl radical at the expense of superoxide.

Results and Discussion

On the basis of our earlier kinetic studies on the spin trapping of superoxide and hydroxyl radicals with DMPO,[8] we felt that it might be possible to synthesize a series of nitrones that would react selectively with hydroxyl radical and not with superoxide. We envisioned that by increasing the steric hindrance around the third position of the pyrroline ring, the rate of superoxide spin trapping might approach zero, while the rate of spin trapping of hydroxyl radical would not be so dramatically affected. For these initial investigations, we synthesizeds two nitrones, 3,3-diethyl-5,5-dimethylpyrroline 1-oxide (**6**, DEDMPO) and 3,3,5,5-tetramethylpyrroline 1-oxide (**7**, M_4PO), and studied their ability to spin trap both superoxide and hydroxyl radicals.

The initial step in the synthesis of DEDMPO involved a Reformansky reaction in which ethyl bromoacetate, 3-pentanone, and magnesium powder are stirred under closely monitored conditions (this reaction is very exothermic once it commences) to give ethyl 3-ethyl-3-hydroxypentanoate (**1**; Scheme I). Hydrolysis followed by dehydration with acetic anhydride yielded 3-ethyl-2-pentenoic acid (**2**). Reaction of (**2**) with methyllithium in ether gave the corresponding ketone, 4-ethyl-3-hexen-2-one (**3**). Refluxing (**3**) with acetonitrile and nitromethane in a phase-transfer reaction involving potassium fluoride and 18-crown-6 ether led to the corresponding nitro ketone, 4,4-diethyl-5-nitro-2-pentanone (**4**). Zinc/ammonium chloride reduction of (**4**) yielded 4,4-diethyl-2-methylpyrroline 1-oxide (**5**). Addition of methylmagnesium (**5**)

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Table I. Summary of Experimental Data for Nitrones

	DEDMPO (6)	M_4PO (7)	DMPO
A_N ($O_2^{\bullet-}$)	*a*	13.7 (15.67)[b] G	14.3[c] G
A_H ($O_2^{\bullet-}$)	*a*	7.5 (20.02)[b] G	11.7[c] G
A_H ($O_2^{\bullet-}$)	*a*		1.25[c] G
A_N ($HO^{\bullet}$)	13 G	15.3 (15.3)[b] G	14.9[c] G
A_H ($HO^{\bullet}$)	13 G	16.5 (16.8)[b] G	14.9[c] G
k (M^{-1} s^{-1})[e]	0	1.0	10[d]
P(octanol/H_2O)	1.22	0.14	0.02[f]

[a] Spin trapping of superoxide was not observed. [b] Reference 16. [c] Reference 4. [d] Reference 8. [e] Second-order rate constant for the reaction of superoxide with each nitrone. [f] Reference 10.

resulted in the corresponding hydroxylamine, which was aerial oxidized in the presence of copper acetate to nitrone (**6**). Nitrone **7** was prepared according to the procedures outlined by Bonnett et al.[9]

Nitrones synthesized by the methods described above could then be purified to the degree necessary for spin-trapping experiments. This purification was twofold. First, fractional elution was performed by using silica gel (230–400 mesh) and a dichloromethane/methanol solvent system. Second, the purified fraction containing the nitrone was Kugelrohr distilled under low pressures. Nitrones at this point were essentially devoid of any paramagnetic impurities and were subsequently stored under argon at −70 °C until use.

The spin trapping of superoxide with nitrones **6**, **7**, and DMPO was conducted with a superoxide-generating system consisting of hypoxanthine in the presence of the enzyme xanthine oxidase at pH 7.8. The rate of superoxide production, as measured by superoxide dismutase-inhibitable reduction of cytochrome *c*, was 10 μM/min. The addition of SOD (10 μg/mL) to the above reaction mixture confirmed that the ESR spectrum resulted from the reaction of superoxide with the nitrone. Hyperfine splitting constants are summarized in Table I.

Rate constants for the reaction of **7** and DMPO with superoxide in phosphate buffer (0.1 M at pH 7.8) are given in Table I. We chose hypoxanthine/xanthine oxidase as a source of superoxide because the rate of its production by this enzyme system was found to be independent of nitrone concentration. In earlier articles,[8,10] we developed equations to determine the rate constants for the reaction of superoxide with various nitrones and discussed experimental conditions necessary to achieve accurate determinations. For this reason, we will not repeat that discussion here.

As shown in Table I, the rate constant for the reaction of **7** with superoxide is only 10% of that calculated when DMPO is used in the above reaction. This finding suggests that if we further increase the bulkiness at the third position of the pyrroline ring, the rate of superoxide spin trapping should ideally continue to decrease. Thus, it was not surprising to observe that when **6** was employed in place of **7** in our model superoxide-generating system, we were unable to spin trap superoxide (Table I). These data point to the fact that it is now possible to synthesize a family of nitrones that are unable to spin trap superoxide.

We then turned our attention to hydroxyl radical. Spin trapping of this free radical was conducted by the addition of ferric ammonium sulfate (0.1 mM) in the presence of DTPA (0.1 mM) to our model superoxide-generating system. The addition of catalase (300 units/mL) to our reaction mixture confirmed that the ESR spectrum resulted from the reaction of hydroxyl radical with each nitrone. Hyperfine splitting constants are presented in Table I.

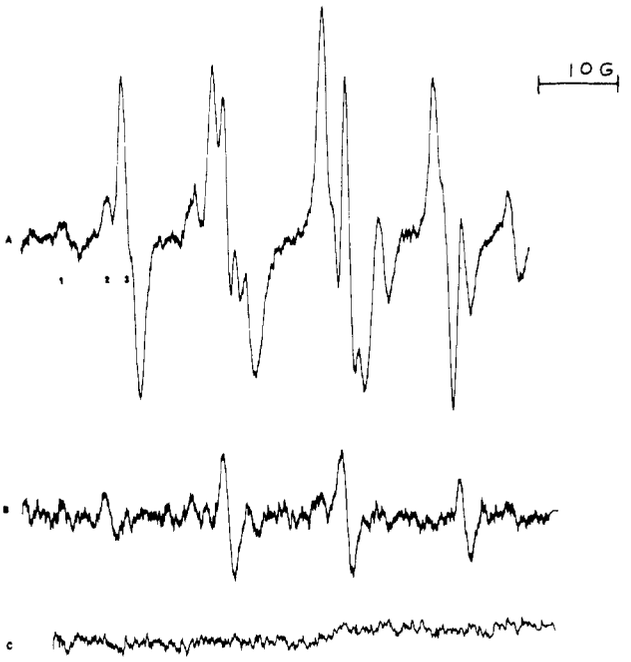

Figure 2. ESR spectra obtained when endothelial cells (1 000 000 cells/mL) were incubated with menadione (0.1 mM in 0.14 M DMSO) in the presence of 0.1 M DMPO (for A and B) or 0.1 M DEDMPO (for C). (A) The spectrum is a composite of three different spin-trapped adducts: (1) 2,2,5-trimethyl-1-pyrrolidinyloxyl, (2) 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxyl, and (3) 2-hydroperoxy-5,5-dimethyl-1-pyrrolidinyloxyl. Microwave power was 20 mW, and the modulation frequency was 100 kHz with an amplitude of 1.0 G. Sweep time was 6.25 G/min, and the receiver gain was 3.2×10^4 with a response time of 3.0 s. (B) the same as (A) except superoxide dismutase (10 μg/mL) was added to the reaction mixture. Microwave power was 20 mW, and the modulation frequency was 100 kHz with an amplitude of 1.0 G. Sweep time was 6.25 G/min, and the receiver gain was 4.0×10^4 with a resonse time of 3.0 s; $A_N = A_H = 14.9$ G. (C) The same as (A) except DEDMPO was substituted in place of DMPO.

There are several observations worth noting. First, all nitrones spin trap hydroxyl radical, even though **6** is less efficient than either **7** or DMPO. Second, the relative lipophilicity (as determined by an octanol/water biphasic environment) of these nitrones increased as the total number of carbon atoms at the third position of the pyrroline ring increased. Finally, none of the nitrones exhibits overt cellular toxicity as cell viability, estimated by Trypan blue exclusion, was unchanged in the presence of these spin traps.

Our initial series of experiments were aimed at determining what free radical was spin trapped intracellularly as a consequence of cellular drug metabolism. When cultured endothelial cells were incubated with menadione (0.1 mM in 0.14 M DMSO) in the presence of DMPO (0.1 M) and DTPA (1 mM), the ESR spectrum shown in Figure 2A rapidly arose, which is a composite of DMPO-CH_3, DMPO-OOH, and DMPO-OH. For a detailed discussion of the spin trapping of free radicals during endothelial cell metabolism, see our earlier studies on this topic.[7] With the addition of SOD (10 μg/mL), the ESR spectrum changed to that characteristic of DMPO-OH (Figure 2B. When nitrazepam (1 mM in 0.14 M DMSO) was substituted for menadione in the above reaction mixture, the ESR spectrum shown in Figure 3A was recorded. The question must now be asked: Is the ESR spectrum characteristic of DMPO-OH an artifact arising from the

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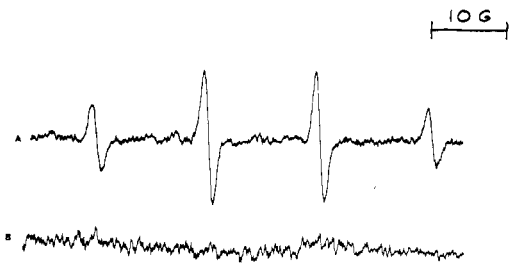

Figure 3. ESR spectra obtained when endothelial cells (1 000 000 cells/mL) were incubated with nitrazepam (1 mM in 0.14 M DMSO) and DTPA (1 mM) in the presence of (A) DMPO (0.1 M), $A_N = A_H = 14.9$ G; (B) DEDMPO (0.1 M). Scanning conditions were the same as described in Figure 2.

spin trapping of superoxide followed by rapid bioreduction to DMPO-OH or is hydroxyl radical produced during the metabolism of either menadione or nitrazepam by endothelial cells? When **6** (0.1 M) was incubated with menadione (0.1 mM in 0.14 M DMSO), endothelial cells, and DTPA (1 mM) at pH 7.4, no ESR spectrum was observed (Figure 2C). Similar results were obtained when nitrazepam (1 mM in 0.14 M DMSO) was substituted in place of menadione (Figure 3Be. These findings, when viewed in light of our earlier observations,[7] point to the fact that during cellular metabolism of quinones and aromatic nitro-containing compounds by endothelial cells only superoxide is spin trapped. There is no evidence for the formation of hydroxyl radical, even though the ESR spectrum characteristic of DMPO-OH is recorded. Our data suggest that DMPO-OH arose as a consequence of the spin trapping of superoxide by DMPO, giving DMPO-OOH. Subsequent degradation, both chemical and enzymatic, led to the formation of DMPO-OH. Finally, it is important to note that our conclusions may be limited to this cell type and to these experimental conditions. For this reason, we are currently exploring the generality of these findings.

Experimental Section

General Comments. Xanthine oxidase, bovine erythrocyte superoxide dismutase (SOD), *N,N*-bis[2-[bis(carboxymethyl)-amino]ethyl]glycine (DTPA), and hypoxanthine were purchased from Sigma Chemical Co. (St. Louis, MO). Chelex-100 was purchased from Bio-Rad (Richmond, CA). All buffers were passed through a Chelex-100 ion-exchange column according to the method of Poyer and McCay[11] to remove trace metal ion impurities. All other reagents were used as obtained from commercial suppliers unless otherwise noted.

Infrared spectra were recorded on a Perkin-Elmer Model 727 infrared spectrophotometer. Proton NMR spectra were obtained on a Varian VXR 200-MHz spectrometer with deuteriochloroform as the solvent. ESR spectra were recorded on a Varian Associates Model E-9 spectrometer. Ultraviolet absorption measurements were obtained on a Perkin-Elmer 559 spectrophotometer. Elemental analyses were performed by Atlantic Microlab, Inc. (Atlanta, GA). Where analyses are indicated only by the symbols of the elements, the analytical results were within the acceptable range of ±0.4% of the theoretical values for those elements.

Column chromatographic separations were conducted with silica gel (230–400 mesh). Elution of the components was monitored by analytical thin-layer chromatography utilizing Eastman Chromagram silica gel sheets with fluorescent indicator. Visualization was performed by using a UV light and an iodine chamber. All solvents were used as obtained commercially.

5,5-Dimethylpyrroline 1-oxide (DMPO) and 3,3,5,5-tetramethylpyrroline 1-oxide (M_4PO) were synthesized by the methods of Bonnett et al.[9]

Procine thoracic aorta endothelial cells were grown according to published procedures.[7,12] For ESR studies, cells were harvested by trypsinization, washed, and resuspended in 50 mM sodium phosphate/0.15 M NaCl/5 mM glucose at pH 7.4, which had previously been passed through a Chelex-100 ion-exchange column. Cells were kept on ice after harvest, and studies involving free-radical production as the result of cell metabolism were conducted at ambient temperature.

Syntheses. 3-Ethyl-2-pentenoic Acid (2). The synthesis of **2** was adopted from the earlier work of Kon and Linstead[13] and more recently by Solas.[14]

To a 2-L, three-necked, round-bottom flask fitted with two condensers in series was added 20 g (0.23 mol) of 3-pentanone, 6 g (0.25 mol) of magnesium powder, 22 mL (0.20 mol) of ethyl bromoacetate, and 100 mL of benzene. The reaction was stirred at room temperature under careful scrutiny since this *reaction commences without warning and may be quite violent*, requiring ice to control. After the exothermic reaction had subsided, this mixture was warmed to 70 °C for 1 h, followed by decomposition with cold sulfuric acid. An additional 200 mL of benzene was then added, and the mixture was washed extensively with water and 10% sodium hydroxide. The benzene solution was dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo, giving 21 g (60%) of ethyl 3-ethyl-3-hydroxypentanoate (**1**): IR (neat film) 3500–3300 (br, O—H), 1730 (C=O) cm^{-1}.

A mixture of crude **1** (21 g, 0.12 mol), 10 g of KOH, 100 mL of methanol, and 20 mL of water was refluxed overnight. Then, the majority of the methanol was removed in vacuo, 200 mL of water was added, and the resultant solution was extracted with ether. Following acidification with HCl, the mixture was extracted with ether. The ether solution was dried over anhydrous $MgSO_4$ and evaporated in vacuo to give 11 g (63%) of 3-ethyl-3-hydroxypentanoic acid: IR (neat film) 3500–3300 (br, O—H), 1720 (C=O) cm^{-1}.

To 10 g (68.5 mmol) of 3-ethyl-3-hydroxypentanoic acid was added 20.96 g (205.5 mmol) of acetic anhydride. This solution was gently refluxed overnight. The crude reaction mixture was then steam distilled, collecting approximately 1 L of distillate. The distillate was saturated with NaCl and extracted with ether. The ether extracts were combined, dried over anhydrous MgSO, and evaporated to give an oil, which was fractionally distilled to yield 4.4 g (50%) of **2** as a colorless liquid: bp 120–123 °C (921 mm) [lit.[13] 129 °C (23 mm)]; IR (neat film) 3500–3100 (br, O—H), 1690 (C=O), 1650 (C=C) cm^{-1}.

4-Ethyl-3-hexen-2-one (3). To 5 g (39.1 mmol) of **2** in 200 mL of dry ether at 0 °C was added 56 mL (78 mmol) of methyllithium in ether (1.4 M; Aldrich) over a 2-h period. After the addition was complete, the reaction mixture was stirred at this temperature for an additional 2 h, refluxed for 2 h, and then stirred at room temperature overnight. The reaction mixture was then added to a crushed ice/10% HCl solution. The ether layer was removed, and the aqueous solution was extracted with ether. The ether extracts were combined and dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The residue was fractionally distilled to give 2 g (41%) of **3** as a colorless liquid: bp 65–68 °C (20 mm) [lit.[15] 59 °C (14 mm)]; IR (neat film) 1690 (C=O), 1620 (C=C) cm^{-1}. Two grams of **2** was reclaimed.

4,4-Diethyl-5-nitro-2-pentanone (4). A solution of 4-ethyl-3-hexen-2-one (31.6 g, 0.25 mol), nitromethane (135 mL, 2.5 mol), anhydrous potassium fluoride (2.91 g, 0.05 mol), and 18-crown-6 ether (3.30 g, 12.5 mmol) in 150 mL of dry acetonitrile was allowed to reflux for 1 week under a protected atmosphere. The solution was then cooled, and the solvent was removed. The residue was dissolved in 200 mL dichloromethane, and the resulting solution was washed with 100 mL of 1 N HCl and then dried over anhydrous $MgSO_4$. Removal of the solvent and fractional distillation afforded 13 g (28%) of **4** as a pale yellow oil: bp 89–91 °C (0.025 mm); IR (neat film) 1710 (C=O), 1547

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(N—O), 1380 (N—O) cm^{-1}; ^1H NMR δ 4.61 (s, 2 H, CH_2NO_2), 2.52 (s, 2 H, CH_2CO), 2.14 (s, 3 H, CH_3CO), 1.45 (m, 4 H, CH_2CH_3), 0.82 (t, 6 H, CH_2CH_3). Anal. ($C_9H_{17}NO_3$) C, H, N.

4,4-Diethyl-2-methylpyrroline 1-Oxide (5). Zinc dust (17.7 g) was added in small portions to a solution of 4,4-diethyl-5-nitro-2-pentanone (12.5 g, 66.7 mmol) and ammonium chloride (3.8 g) in 200 mL of 60% aqueous ethanol at 10 °C. The resulting suspension was stirred vigorously for 5 h at 10–15 °C, the suspension was filtered, and the filter cake was washed with four 50-mL portions of warm water. The combined filtrate and washings were concentrated to about 20 mL in vacuo and the residue was extracted with four 50-mL portions of dichloromethane. The combined extracts were dried over anhydrous sodium sulfate, the solvent was removed, and the residue was distilled to afford 4.68 g (45%) of **5** as a colorless oil: bp 128–131 °C (0.5 mm). IR (neat film) 1630 (C=N), 1225 (N—O) cm^{-1}; ^1H NMR δ 3.73 (m, 2 H, CH_2NO), 2 (s, 3 H, CH_3C=N), 1.47 (br m, 6 H, CH_2CH_3 + $CH2_2C(CH_3)$=N), 0.85 (m, 6 H, CH_2CH_3). Anal. ($C_9H_{17}NO$) C, H, N.

3,3-Diethyl-5,5-dimethylpyrroline 1-Oxide (6). A solution of 4,4-diethyl-2-methyl-1-pyrroline 1-oxide (3 g, 19.3 mmol) was added dropwise to a cold solution (~0 °C) of methylmagnesium bromide (3.2 M, 12 mL, 38.6 mmol; Aldrich) under nitrogen. After completion, the mixture was allowed to warm to reflux for 1 h. The mixture was then cooled and treated with 10 mL of a saturated ammonium chloride solution, and the ether layer was removed. The aqueous solution was extracted thrice with ether and the ether solutions were combined, dried over anhydrous $MgSO_4$, and evaporated to dryness. The remaining oil was dissolved in methanol and cupric acetate (50 mg), and ammonium hydroxide (1 mL) was added. The solution was continually bubbled with oxygen until a blue color persisted. The mixture was evaporated to dryness, and the remaining mixture was taken up in ether, dried over anhydrous sodium sulfate, and evaporated to dryness to give an oil. This oil was purified by chromatographic separation on a silica gel column. Elution of the column with 5% methanol/dichloromethane afforded the product (R_f 0.76) as a colorless oil. This product was then Kugelrohr distilled at 45–48 °C (0.1 mm) to give 1.91 g (58%) of **6** as a colorless oil: IR (neat film) 1580 (C=N), 1245 (N—O) cm^{-1}, ^1H NMR δ 6.63 (s, 1 H, N=CH), 1.90 (s, 2 H), 1.48 (s = q, 10 H), 0.84 (t, 6 H, CH_2CH_3). Anal. ($C_{10}H_{19}NO$) C, H, N.

Biochemical Studies. Spin Trapping of Superoxide. The superoxide-generating system contained 400 μM hypoxanthine, 1 mM DTPA, 0.1 M sodium phosphate buffer (pH 7.8), and xanthine oxidase such that the rate of superoxide production was 10 μM/min at 25 °C. Measurement of superoxide was determined optically by the reduction of cytochrome *c* at 550 nm with an extinction coefficient of 20 mM^{-1} cm^{-1}. The concentration of the various spin traps ranged from 10 mM to 0.1 M. The reaction was initiated by the addition of xanthine oxidase. By monitoring the conversion of hypoxanthine to uric acid at 292 nm, it was determined that the various nitrones did not inhibit the enzyme. The formation of superoxide spin-trapped adducts was monitored by ESR as the low-field peak and was observed to increase linearly for several minutes. The production of this nitroxide was completely inhibited by SOD (10 μg/mL). No free radical could be spin trapped if any component of the above reaction was absent.

Spin Trapping of Hydroxyl Radical. The spin trapping of hydroxyl radical was undertaken by the addition of ferric ammonium sulfate (0.1 mM) to the above superoxide-generating system. No free radical was spin trapped in the presence of catalase (300 units/mL).

Partition Coefficient Studies. A stock solution (0.1 mM) of each nitrone in deionized water was prepared. The stock solution was diluted to prepare solutions of varying concentrations, and a calibration curve was obtained from the absorbance measurements at 230 nm. Then, 5 mL of each stock solution was combined with an equal volume of 1-octanol (Aldrich, gold label), and the biphasic mixture was shaken for 30 min. After separation, the lower aqueous layer was drained, and the concentration of the nitrone was determined from the calibration curve. The concentration of each nitrone in the 1-octanol layer was obtained by difference, and the partition coefficient was calculated.

Spin Trapping of Free Radicals Generated by Endothelial Cell Metabolism. Spin-trapping experiments were designed to detect free radicals generated as a consequence of cellular metabolism of either menadione or nitrazepam by endothelial cells. A typical reaction mixture contained 1 000 000 cells, menadione (0.1 mM in 0.14 M DMSO) or nitrazepam (1 mM in 0.14 M DMSO), DTPA (1 mM), the spin trap (either DMPO, or DEDMPO, 0.1 M), and sufficient buffer (50 mM sodium phosphate/0.15 M NaCl/5 mM glucose, pH 7.4, which had previously been passed through a Chelex-100 ion-exchange column) to bring the final volume to 0.5 mL. Reaction mixtures were then transferred to a flat quartz cell and fitted into the cavity of the ESR spectrometer at room temperature. The ESR spectrum was obtained within 1 min after initiation of the reaction.

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