(1 H, ddd, J = 14.5, 6.4, and 4.7 Hz), 1.77 (1 H, ddd, J = 7.4, 5.4, and 3.0 Hz), 1.82 (1 H, ddd, J = 7.4, 5.7, and 3.7 Hz), 1.91 (1 H, br t, J = 5.7 Hz), 2.17 (3 H, s COMe), 3.24 (3 H, s, OMe), 3.30 (3 H, s, OMe), 3.34 (3 H, s, OMe), 3.36 (3 H, s, OMe), 3.37 (1 H, m), 3.44 (2 H, m), 3.71 (1 H, t, J = 5.7 Hz), 5.08 (1 H, br d, J = 10.4 Hz), 5.10 (1 H, br d, J = 16.8 Hz), 5.81 (1 H, ddt, J = 16.8, 10.4, and 7.1 Hz); CIMS (isobutane) m/z 303 (MH^+), 271 (MH^+ - MeOH), 239 (MH^+ - 2MeOH), 207 (MH^+ - 3MeOH), 175 (MH^+- 4MeOH).

(3*R*,5*R*,7*S*,9*S*)-3,5,7,9-Tetramethoxy-10-oxoundecanal (15). To a stirred solution of **28** (17 mg, 0.056 mmol) in dioxane (1.2 mL) and water (0.4 mL) was added OsO_4 (1 mg). After stirring for 30 min at room temperature, $NaIO_4$ (50 mg, 0.27 mmol) was added and the reaction mixture was stirred for 1.5 h. The product was extracted from the mixture with ether and flash chromatographed (70% EtOAc–hexane) to give keto aldehyde **15** (12.4 mg, 73%): $[\alpha]_D$ -21.49° (c 1.0); IR ($CHCl_3$) 1720, 1460, 1355, 1010 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.62 (1 H, dt, J = 14.3 and 5.5 Hz), 1.66 (1 H, dt, J = 14.3 and 5.5 Hz), 1.78–1.97 (4 H), 2.16 (3 H, s, COMe), 2.63 (2 H, dd, J = 5.9 and 2.2 Hz), 3.24 (3 H, s, OMe), 3.28 (3 H, s, OMe), 3.34 (3 H, s, OMe), 3.35 (3 H, s, OMe), 3.42 (2 H, m), 3.70 (1 H, t, J = 6.2 Hz), 3.84 (1 H, quintet, J = 6.2 Hz), 9.80 (1 H, t, J = 2.2 Hz); ^{13}C (100 MHz, $CDCl_3$) δ 25.44, 35.94, 37.26, 37.78, 47.99, 56.07, 56.26, 56.67, 57.91, 73.51, 74.23, 74.69, 84.06, 201.21, 210.05; CIMS (isobutane) m/z 305 (MH^+), 273 (MH^+ - MeOH), 241 (MH^+ - 2MeOH), 209 (MH^+ - 3MeOH), 177 (MH^+ - 4MeOH).

Methyl (3*R*,5*R*,7*S*,9*S*)-3,5,7,9-Tetramethoxy-10-oxoundecanoate (16). To a solution of **28** (17.5 mg, 0.058 mmol) in dioxane (1.2 mL) and water (0.4 mL) were added $KMnO_4$ (2 mg) and $NaIO_4$ (62 mg), and the mixture was stirred for 2 h at room temperature. The reaction mixture was acidified with 1% HCl and the carboxylic acid extracted out with EtOAc. Treatment with ethereal diazomethane (2 mL) followed by flash chromatography (45% EtOAc–hexane) gave keto ester **16** (12.8 mg, 67%): $[\alpha]_D$ -6.76° (c 0.7); IR ($CHCl_3$) 1720, 1435, 1100 cm^{-1}; ^1H NMR (400 MHz, benzene-d_6) δ 1.64 (2 H, tt, J = 13.9 and 5.6 Hz), 1.84 (2 H, tt, J = 14.2 and 5.4 Hz), 1.93 (2 H, m), 1.94 (3 H, s, COMe), 2.41 (1 H, dd, J = 15.1 and 5.6 Hz), 2.54 (1 H, dd, J = 15.1 and 6.6 Hz), 3.05 (3 H, s, OMe), 3.11 (3 H, s, OMe), 3.12 (3 H, s, OMe), 3.16 (3 H, s, OMe), 3.35 (3 H, s, OMe), 3.46 (1 H, quintet, J = 5.6 Hz), 3.54 (1 H, quintet, J = 5.9 Hz), 3.60 (1 H, triplet, J = 5.9 Hz), 3.84 (1 H, quintet, J = 5.9 Hz); ^{13}C (100 MHz, $CDCl_3$) δ 25.48, 36.05, 37.44, 37.88, 39.25, 51.64, 56.16, 56.29, 56.86, 57.93, 74.32, 74.86, 74.96, 84.16, 171.98, 210.08; CIMS (isobutane) m/z 335 (MH^+), 303 (MH^+ - MeOH), 271 (MH^+ - 2MeOH), 239 (MH^+ - 3MeOH), 207 (MH^+ - 4MeOH).

Acknowledgment. This research was supported by Grant No. CA12623 from the National Cancer Institute, Department of Health and Human Services. A GN500-Omega NMR spectrometer at the University of Hawaii that was used in this study was purchased with a grant from the National Science Foundation. We thank Faith Caplan and Linda K. Larsen for determining the cytotoxicities and antifungal activities and Drs. Thomas Corbett and Frederick Valeriote (Division of Hematology and Oncology, Wayne State University School of Medicine) for evaluating the compounds for selective cytotoxicity.

Synthesis of Deuterium- and ^{15}N-Containing Pyrroline 1-Oxides: A Spin Trapping Study

Sovitj Pou and Gerald M. Rosen*

Department of Pharmacology & Toxicology, University of Maryland School of Pharmacy, Baltimore, Maryland 21201, and Veterans Administration Medical Center, Baltimore, Maryland 21218

Yexin Wu and John F. W. Keana*

Department of Chemistry, University of Oregon, Eugene, Oregon 97403

Received March 19, 1990

Our aim is to develop nitrone-based spin traps with improved sensitivity toward superoxide and hydroxyl radicals through isotopic substitution. Deuterated DMPO derivatives were prepared by either D_2O–NaOD or D_2O–DCl exchange reactions. The ^{15}N-substituted counterparts were synthesized starting with acetone-d_6 and (^{15}N)-hydroxylamine. These spin traps provide significantly enhanced sensitivity in the detection of superoxide and small carbon-centered free radicals.

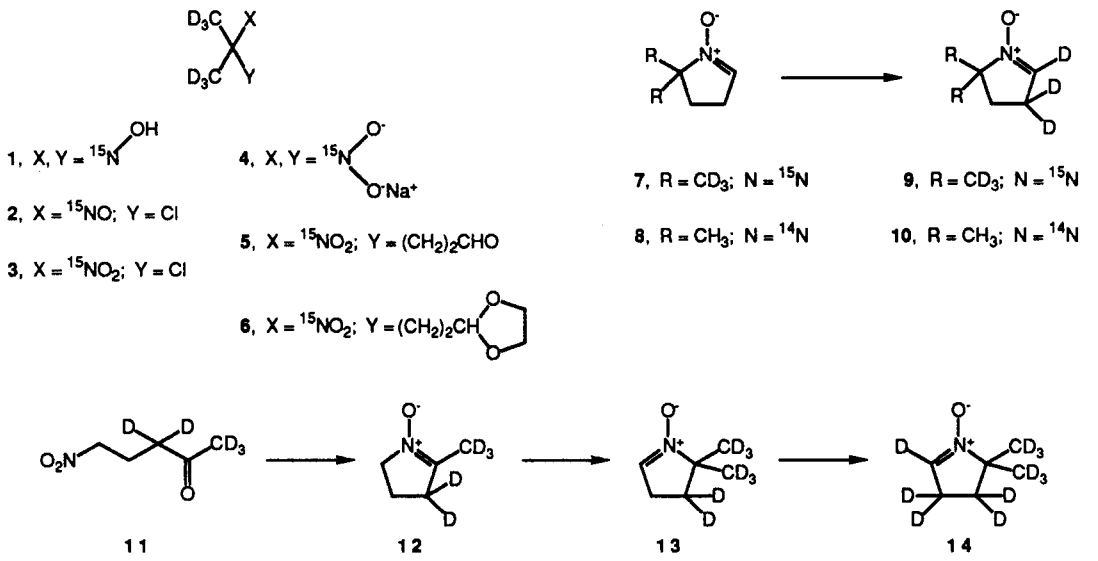
In recent years reduced oxygen species, including superoxide and hydroxyl radical, have been studied intensively as these reactive intermediates appear to play an important role in mediating a variety of pathologic conditions. For example, it has been proposed that during ischemia/reperfusion injury, free radicals initiate events leading to cellular necrosis.[1,2] Yet, data in support of this hypothesis is largely indirect, coming from the observation that in vivo, free radical scavengers significantly ameliorate the injury.

Of the available methods for the detection of free radicals, only spin trapping offers the opportunity to simultaneously measure and distinguish among a variety of important biologically generated free radicals.[3-5] In this technique, a nitrone or nitroso compound reacts with a short-lived free radical to produce a nitroxide whose lifetime is considerably greater than that of the parent free radical.[6] The spin trap 5,5-dimethyl-1-pyrroline 1-oxide (DMPO, **8**) is most frequently used; however, this nitrone has several limitations. Its reaction with superoxide is rather slow, having a second-order rate constant of only 10 M^{-1} s^{-1}.[7] Its partition coefficient was found to be only

(1) Granger, D. N.; Rutili, G.; McCord, J. M. *Gastroenterology* **1981**, *81*, 22.
(2) Bulkley, G. B. *Brit. J. Cancer* **1987**, 55, Supp. VIII, 66.
(3) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. *Arch. Biochem. Biophys.* **1980**, *200*, 1.
(4) Janzen, E. G. In *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1980; Vol. 4, p 116.
(5) Rosen, G. M.; Finkelstein, E. *Adv. Free Rad. Biol. Med.* **1985**, *1*, 345.
(6) Janzen, E. G. *Acc. Chem. Res.* **1971**, *4*, 31.
(7) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. *J. Am. Chem. Soc.* **1980**, *102*, 4994.

0022-3263/90/1955-4438$02.50/0

Scheme I



0.08, indicating a preference for water over a lipid environment.[8] Finally, its susceptibility toward metal ion catalyzed air oxidation yielding 2-hydroxy-5,5-dimethyl-1-pyrrolidinyloxy (DMPO-OH) as well as a nitroxide exhibiting a three-line ESR spectrum[9] suggests the need for new spin traps. We have recently prepared a family of pyrroline 1-oxide nitrones with enhanced lipophilicity and decreased susceptibility toward air oxidation[10] as well as nitrones that react preferentially with hydroxyl radical at the expense of superoxide.[11] Despite these accomplishments, we have been unable to synthesize nitrones that react with superoxide at rates greater than 10 M^{-1} s^{-1}. Consequently, the spin trapping of free radicals in biological systems has tended to require high concentrations of the nitrone, which can lead to cellular toxicity. One approach around this problem is to increase the sensitivity of the spin trapping procedure. On the basis of the pioneering work of Beth et al.[12] and Janzen et al.,[13] a significant increase in sensitivity may be realized by preparing spin traps containing deuterium and ^{15}N in place of hydrogen and ^{14}N. Herein, we report the synthesis of a family of deuterium- and ^{15}N-containing pyrroline 1-oxides and compare their ability to spin trap superoxide, hydroxyl, and small carbon-centered free radicals generated from the model superoxide-generating system, xanthine/xanthine oxidase.

Results and Discussion

New conditions for each step in the Bonnett et al.[14] synthesis of DMPO were required for the efficient preparation of the (^{15}N)nitrone spin traps in order to optimize yields based on the respective ^{15}N starting materials. In the event, (^{15}N)hydroxylamine hydrochloride was allowed to react with acetone-d_6, forming the volatile ketoxime 1 (Scheme I). Attempts to prepare (^{15}N)-2-nitropropane from 1 using Iffland's method (NBS bromination, nitric acid oxidation, sodium borohydride reduction)[15] or direct oxidation with trifluoroperacetic acid[16] were unsuccessful.

Recognizing that the anion 4 was the species that was needed for the Michael reaction with acrolein, we adapted the procedure of Brane and Patterson[17] for the one-pot generation of 4. Thus, ketoxime 1 was treated with chlorine gas to give nitroso derivative 2. Without isolation, 2 was treated with ozone, giving nitro derivative 3. Without purification, 3 was catalytically hydrogenated over Pd/C in aqueous NaOH[17,18] to give anion 4. In a trial run, acidification of 4 gave mostly acetone (Nef reaction) rather than 2-nitropropane. Therefore, 4 was used directly in a low-temperature Michael reaction with acrolein in the presence of the polymerization inhibitor 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxy to give the nitro aldehyde 5. It is important to note that the NaOH hydrogenation solution must first be neutralized to pH 8 in order to minimize anionic polymerization of the acrolein. Utilization of acrolein at this stage instead of the expensive acrolein-d_4 was based on our finding that gentle warming of DMPO with NaOD in D_2O led to complete exchange of the hydrogens at positions 2 and 3, giving the trideuterated analogue 10 in good yield.

Aldehyde 5 was converted into ethylene acetal 6. The nitro group was then reduced with zinc in aqueous ammonium chloride to the *N*-hydroxy stage. Acidification led to hydrolysis of the acetal group and effected cyclization to hexadeutero (^{15}N)nitrone 7. Finally, the D_2O–NaOD exchange reaction on 7 gave nonadeutero (^{15}N)-nitrone spin trap 9.

Our next objective was to synthesize the perdeuterated nitrone 14. The deuterated nitropentanone 11 was prepared by proton exchange in D_2O and DCl. Reductive cyclization of 11 with zinc dust in aqueous ammonium chloride gave nitrone 12. Treatment of 12 with (methyl-d_3)magnesium bromide followed by Cu^{2+}-catalyzed air oxidation afforded 13 as a colorless oil. Under the same conditions as described above, D_2O/NaOD exchange of 13 gave the perdeuterated nitrone 14.

The spin trapping of superoxide with nitrones 8, 9, 10, and 14 was conducted by using a superoxide-generating

(8) Rosen, G. M.; Finkelstein, E.; Rauckman, E. J. *Arch. Biochem. Biophys.* **1982**, *215*, 367.
(9) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J.; Paxton, J. *Mol. Pharmacol.* **1979**, *16*, 676.
(10) Turner, M. J., III; Rosen, G. M. *J. Med. Chem.* **1986**, *29*, 2439.
(11) Rosen, G. M.; Turner, M. J., III. *J. Med. Chem.* **1988**, *31*, 428.
(12) Beth, A. H.; Venkataramu, S. D.; Balasubramanian, K.; Dalton, L. K.; Robinson, B. H.; Pearson, D. E.; Park, C. R.; Park, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 967.
(13) Janzen, E. G.; Oehler, U. M.; Haire, D. L.; Kotake, Y. *J. Am. Chem. Soc.* **1986**, *108*, 6858.
(14) Bonnett, R.; Brown, R. F. C.; Clark, V. M.; Sutherland, I. O.; Todd, A. *J. Chem. Soc.* **1959**, 2094.
(15) Iffland, D.; Yen, T. F. *J. Am. Chem. Soc.* **1954**, *76*, 4083.
(16) Emmons, W. D.; Pagano, A. S. *J. Am. Chem. Soc.* **1955**, *77*, 4557.
(17) Brane, M. W.; Patterson, J. M. *J. Org. Chem.* **1976**, *41*, 733.
(18) Robertson, J. A. *J. Org. Chem.* **1948**, *13*, 3958.

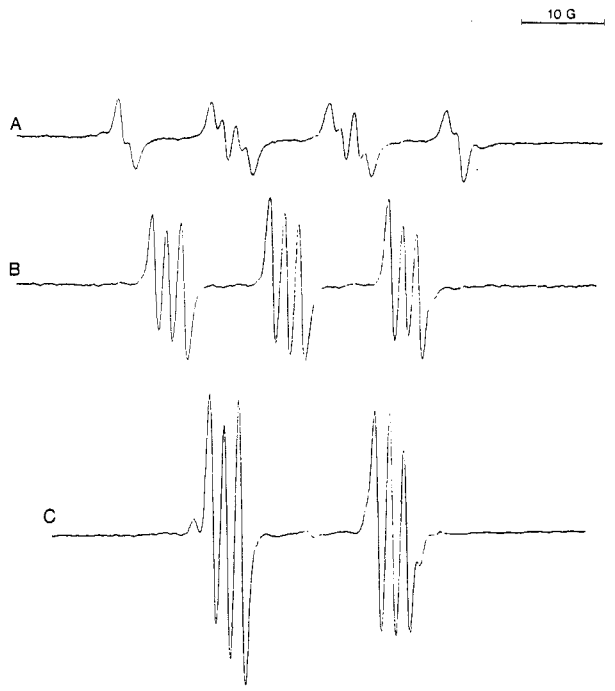


Figure 1. ESR spectra of superoxide spin adducts obtained as a consequence of the reaction of xanthine with xanthine oxidase in the presence of (A) **8**, (B) **10**, and (C) **9**. Microwave power was 20 mW; modulation frequency was 100 KHz, with a modulation amplitude of 1 G; sweep time was 12.5 G/min; and the receiver gain was 2.3×10^3, with a response time of 1 s. Hyperfine splitting constants are presented in Table I.

Table I. Hyperfine Splitting Constants (G) for Spin Trapped Adducts

nitrones	8	9	10	14
A_N ($O_2^{\bullet-}$)	14.1	20.0	14.0	14.0
A_{H^β} ($O_2^{\bullet-}$)	11.2	1.7	1.7	1.0
A_{H^α} ($O_2^{\bullet-}$)	1.2			
A_N ($HO^\bullet$)	14.8	21.0	15.0	15.0
A_H ($HO^\bullet$)	14.8	2.2	2.2	2.2
A_N ($HOCHCH_3$)	15.5	22.5	16.0	16.0
A_H ($HOCHCH_3$)	22.5	3.5	3.5	3.5

system consisting of xanthine in the presence of 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 production of nitrone–superoxide adduct was completely inhibited by superoxide dismutase (60 μg/mL), confirming that the observed ESR spectra resulted from the reaction of superoxide with each nitrone (Figures 1 and 2). Hyperfine splitting constants for superoxide spin trapped adducts are summarized in Table I.

Spin trapping of hydroxyl radical was conducted by the addition of ferrous sulfate (0.1 mM) to the above superoxide-generating system. The addition of catalase (300 U/mL) confirmed that the ESR spectrum resulted from the reaction of hydroxyl radical with each spin trap (Figures 3 and 4). In addition, when ethanol (0.28 M, final concentration) was added to the hydroxyl radical generating system in the presence of nitrones **8**, **9**, **10**, and **14**, α-hydroxyethyl spin trapped adducts were observed (Figures 5 and 6). The hyperfine splitting constants are presented in Table I.

A literature search revealed that most spin trapping experiments using nitrone **8** were conducted with a modulation amplitude setting of 1.0 G. For purposes of comparison, some of our spin trapping experiments were performed using 1.0 G (Figures 1, 3, and 5). However, a 0.2 G setting (Figures 2, 4, and 6) proved to be more

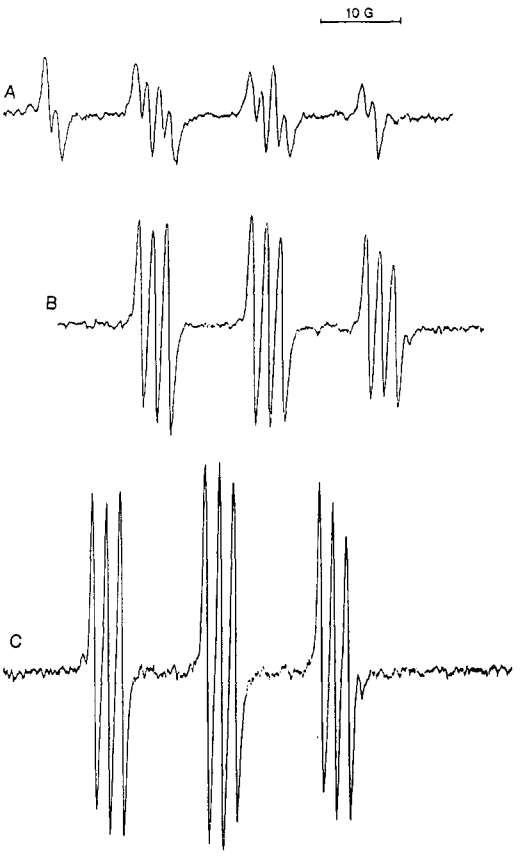


Figure 2. ESR spectra of superoxide spin adducts obtained as a consequence of the reaction of xanthine with xanthine oxidase in the presence of (A) **8**, (B) **10**, and (C) **14**. Instrumentation settings were the same as described in Figure 1, except that the receiver gain was 10×10^3 and modulation amplitude of 0.2 G.

suitable (cf. Figure 1B versus 2B) for spin adducts derived from deuterium- and ^{15}N-containing nitrones.

ESR spectra shown in Figure 1 (A and B) demonstrate the utility of placing deuterium in positions 2 and 3 of nitrone **8**, independent of the presence of ^{15}N. In the case of **10**, the spin trapping of superoxide led to a doubling in sensitivity (as compared to **8**), which significantly increased either by replacing ^{14}N with ^{15}N (spin trap **9**) or by using the perdeuterated spin trap **14** (Figure 2C).

ESR spectra derived from the spin trapping of hydroxyl radical with nitrones **9**, **10**, and **14** showed no significant enhancement when compared to studies using **8** (Figures 3 and 4). Apparently, the increase in individual ESR peak heights resulting from overlap of the hyperfine splittings in the $HO^\bullet$–**8** spin adduct ($A_N = A_H = 14.8$ G) approximately offsets the expected sensitivity gains by isotope labeling. However, in the spin trapping of α-hydroxyethyl radical by nitrones **9**, **10**, and **14**, markedly enhanced ESR spectra were observed when compared to spin trapping experiments conducted using **8**. The enhancement due to deuterium substitution is seen by comparing Figure 5A to 5B and 6A to 6C. Further enhancement due to ^{15}N substitution is seen by comparing Figure 5B to 5C.

In conclusion, we have shown that combinations of deuterium and ^{15}N substitutions in DMPO can lead to a significant increase in ESR signal sensitivity when spin trapping superoxide radical, hydroxyl radical, or α-hydroxyethyl radical. In our model superoxide-generating system, where high steady state levels of this free radical are easily produced, increased sensitivity is clearly unnecessary. However, in cell systems, where rates of superoxide (as well as hydroxyl radical) production are very small, a significant increase in sensitivity may make it

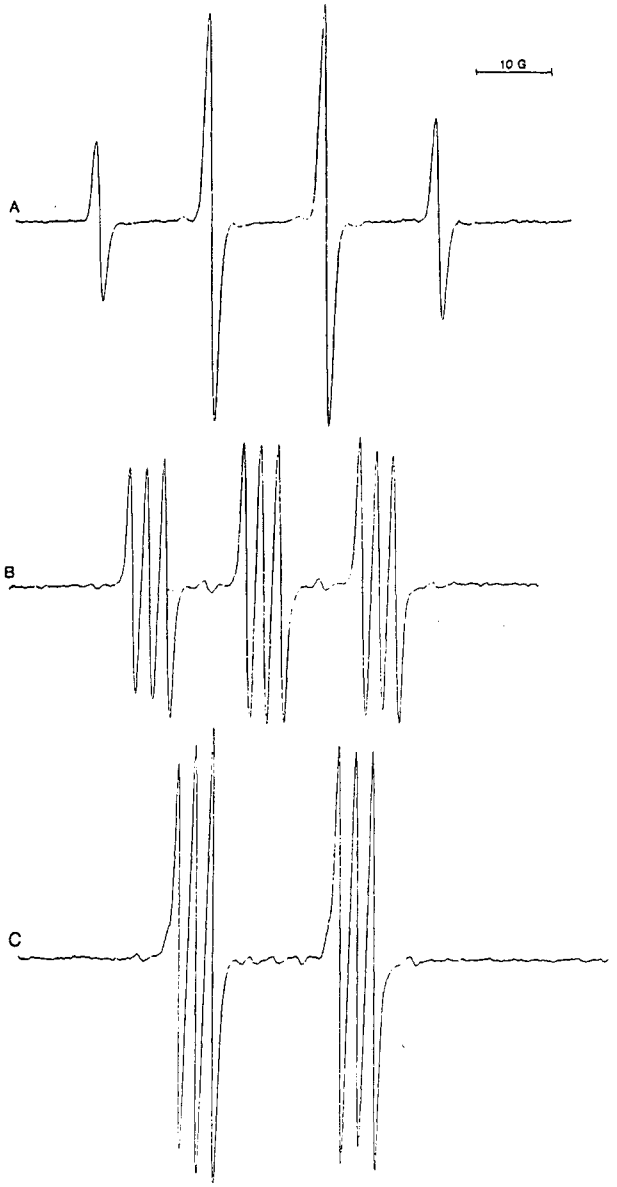


Figure 3. ESR spectra of hydroxyl radical spin adducts obtained as a consequence of the reaction of xanthine with xanthine oxidase in the presence of ferrous sulfate (0.1 mM) and (A) **8**, (B) **10**, and (C) **9**. Instrumentation settings were the same as described in Figure 1, except that the receiver gain was 4×10^3.

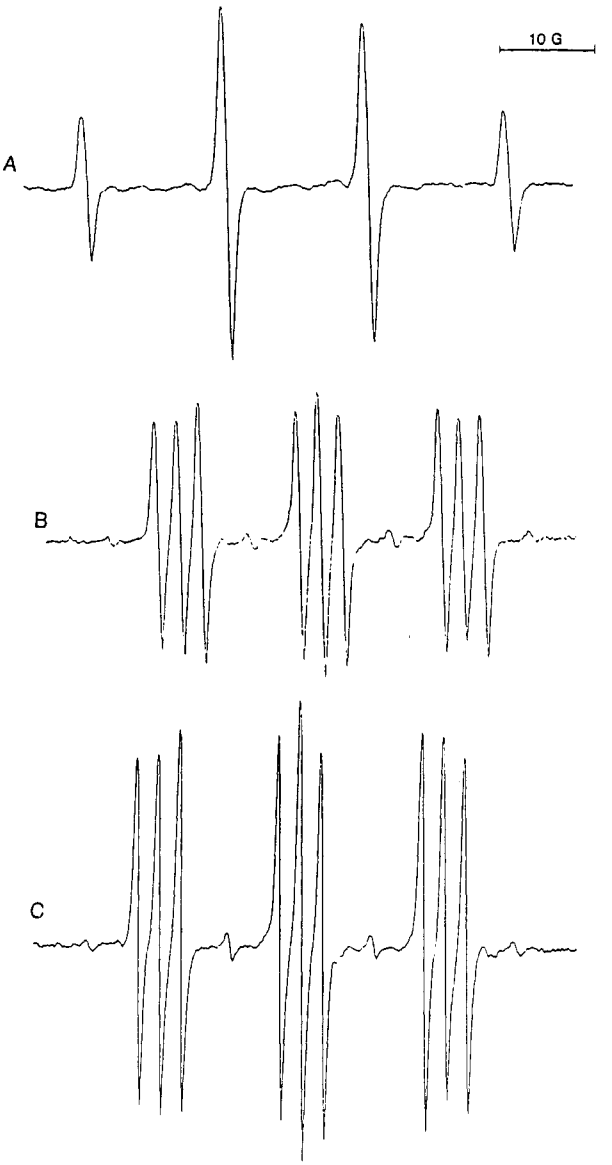


Figure 4. ESR spectra of hydroxyl radical spin adducts obtained as a result of the reaction of xanthine with xanthine oxidase in the presence of ferrous sulfate (0.1 mM) and (A) **8**, (B) **10**, and (C) **14**. Instrumentation settings were the same as described in Figure 2, except that the receiver gain was 5×10^3.

possible to unequivocally identify these reactive species, which might be impossible in the absence of deuterium- and ^{15}N-containing DMPO. Thus, we have recently been able to use **10** and **14** but not **8** to spin trap superoxide generated by phorbol myristate acetate (PMA)-stimulated human neutrophils. The experiments were conducted in the cavity of a low-frequency ESR spectrometer, which allows the study of free radical processes in an isolated perfused organ.[22,23]

Experimental Section

General Comments. Diethylenetriaminepentaacetic acid (DTPA), xanthine, and superoxide dismutase were obtained from Sigma Chemical Company (St. Louis, MO). Catalase was purchased from Calbiochem, Behring Diagnostics (San Diego, CA). Xanthine oxidase was obtained from Dr. Irwin Fridovich (Department of Biochemistry, Duke University Medical Center, Durham, NC). Chelex-100 was purchased from Bio-Rad (Richmond, CA). All buffers were passed through a Chelex-100 ion exchange column before use, according to the method of Poyer and McCay,[19] to remove trace metal ion impurities. All spin trapping experiments were conducted in an air-saturated 50 mM sodium phosphate buffer, pH 7.8, containing 1 mM DTPA.

Acetone-d_6 was purchased from Aldrich Chemical Company (Milwaukee, WI) and [^{15}N]hydroxylamine hydrochloride was obtained from MSD Isotopes, Merck Chemical Division (St. Louis, MO). ^1H NMR spectra were recorded in $CDCl_3$. GC-MS were obtained with an HP-1 (cross-linked-methyl silicone gum, 12 m × 0.2 mm × 0.33 mm film thickness) column.

4-([$^2H_3$]Methyl)-4-([^{15}N]nitro)[5,5,5-$^2H_3$]pentanal (5). To a D_2O solution (5 mL) of (^{15}N)hydroxylamine hydrochloride (1.5 g, 15.6 mmol) was added NaOD (960 mg, 23.4 mmol) in D_2O (2 mL). The reaction mixture was stirred for 15 min at 25 °C. To this was added acetone-d_6 (99.6%, 2 mL, 27.3 mmol) dropwise. After 2 h the solution was saturated with NaCl (2.5 g) and extracted with ether (5 × 10 mL). The combined ether extracts were dried over anhydrous K_2CO_3, filtered, and evaporated to near dryness, giving oxime **1** as a white solid. Chlorine was bubbled

(19) Poyer, J. L.; McCay, P. B. *J. Biol. Chem.* **1971**, *246*, 263.
(20) Warner, D. T.; Moe, O. A. *J. Am. Chem. Soc.* **1952**, *74*, 1064.
(21) Kloetzel, M. C. *J. Am. Chem. Soc.* **1947**, *69*, 2271.
(22) Pou, S.; Peric, M.; Halpern, H. J.; Rosen, G. M. *Free Rad. Res. Commun.* In press.
(23) Halpern, H. J.; Peric, M.; Pou, S.; Rosen, G. M. Manuscript in preparation.

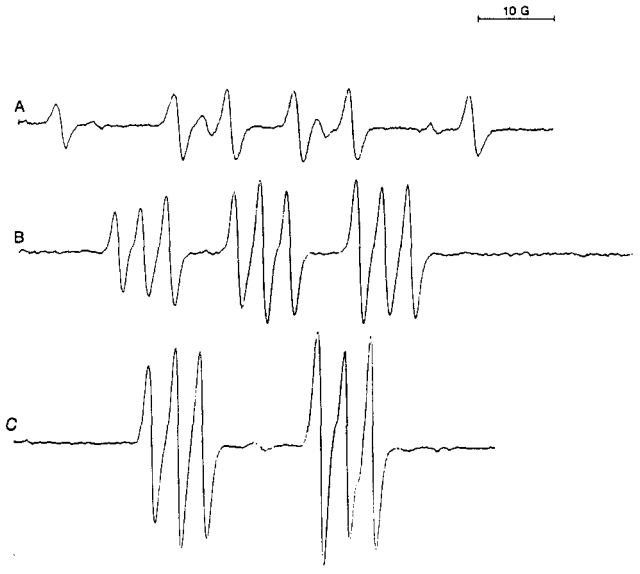


Figure 5. ESR spectra of ($HO\dot{C}HCH_3$) spin adducts obtained as a result of the reaction of xanthine with xanthine oxidase in the presence of ferrous sulfate (0.1 mM), ethanol (0.28 M), and in the presence of (A) **8**, (B) **10**, and (C) **9**. Instrumentation settings were the same as described in Figure 1. In scan A, one observes a small amount of hydroxyl spin trapped by DMPO, giving DMPO-OH. As the concentration of ethanol increased, this ESR spectrum disappears. In scans B and C, ESR spectra corresponding to hydroxyl radical spin trapped adducts are not observed because of the overlap with the more dominant α-hydroxyethyl spin trapped products.

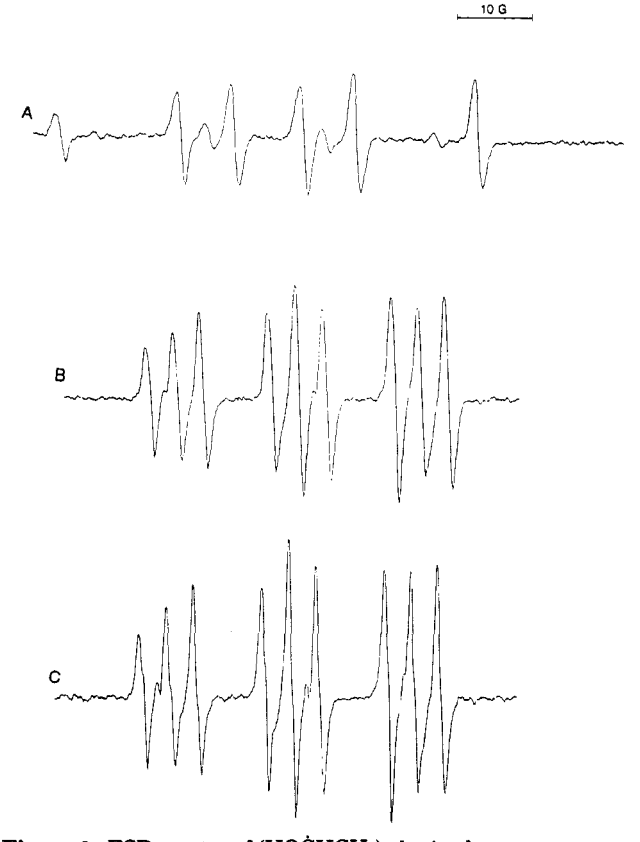


Figure 6. ESR spectra of ($HO\dot{C}HCH_3$) obtained as a consequence of the reaction of xanthine with xanthine oxidase in the presence of ferrous sulfate (0.1 mM), ethanol (0.28 M), and in the presence of (A) **8**, (B) **10**, and (C) **14**. Instrumentation settings were as described in Figure 2, except the receiver gain was 6.3×10^3.

into a solution of **1** in CH_2Cl_2 (20 mL) at −10 °C until a blue-green color persisted. This was followed by bubbling ozone into the solution until the blue color was completely discharged. The solvent was removed in vacuo and the residual oil was taken up in NaOH (1.80 g, 45 mmol in 20 mL) and hydrogenated using Pd/C (10%, 150 mg) and H_2 under 60 psi for 10 h. The Pd/C was filtered and the pH of the remaining solution was adjusted to 8 with 2 N HCl. The solution was then evaporated to dryness, giving a white powder, which is a mixture of **4** and NaCl. This white solid was further dried by azeotropic distillation with ethanol (99.5%) followed by subjection to high vacuum. To a suspension of the white powder in ethanol (99.5%, 30 mL), cooled below −60 °C, was added freshly distilled acrolein (2.60 g, 46.4 mmol, containing 0.1% 4-hydroxyl-2,2,6,6-tetramethylpiperidinoxyl) dropwise at such a rate that the temperature did not exceed −60 °C. After addition, the temperature was allowed to slowly rise to −15 °C over 4 h. To the resulting yellow suspension was added glacial acetic acid (1.50 g, 25.1 mmol), and the mixture was stirred for 15 min, giving a white suspension. The ethanol was evaporated to give a sticky light yellow residue, which was dissolved in CH_2Cl_2 (20 mL), filtered, evaporated, and distilled, giving 2.91 g (90%) of **5** as a colorless liquid: bp 59–65 °C (0.1 mm) (lit.[20] bp 58–60 °C at 0.1 mm); ^1H NMR 2.24 (t, 2), 2.51 (t, 2), 9.78 (s, 1).

4-([$^2H_3$]Methyl)-4-([^{15}N]nitro)[5,5,5-$^2H_3$]pentanal Ethylene Acetal (6). A benzene solution (20 mL) of **5** (2.91 g, 19.3 mmol), ethylene glycol (1.8 g, 29 mmol), and *p*-toluenesulfonic acid monohydrate (4 mg) was refluxed for 3 h while the water was azeotropically removed in a Dean–Stark trap containing $CaSO_4$ (2 g). Upon cooling, the solution was washed with a saturated $NaHCO_3$ solution (2 mL) and brine (2 mL), dried over anhydrous K_2CO_3, and evaporated to dryness, and the remaining oil was distilled to give 2.64 g (70%) of **6** as a colorless liquid: bp 85–86 °C (0.05 mm) (lit.[14] 105 °C at 0.5 mm); ^1H NMR 1.60–1.67 (m, 2), 1.99–2.03 (m, 2), 3.85–3.98 (m, 4), 4.86 (t, 1).

5,5-Di([$^2H_3$]methyl)[^{15}N]-1-pyrroline 1-Oxide (7). To an aqueous solution (10 mL) of **6** (2.64 g, 13.5 mmol) and NH_4Cl (850 mg, 16 mmol) at 0 °C was added Zn dust (2.20 g, 33.6 mmol) portionwise over 2 h at such a rate as to maintain the temperature at ~0 °C. After being stirred at this temperature for an additional 4 h, the mixture was filtered and washed with boiling water. The combined filtrate was acidified with 12 N HCl (1.8 mL) and warmed to 70 °C for 1 h. The volume was then reduced on a rotary evaporator until a yellow-white precipitate appeared. This was neutralized with NaOH (1 g in 3 mL of H_2O) at 0 °C. The resultant solution was then saturated with sodium borate (2 g). The mixture was extracted with $CHCl_3$ (5 × 5 mL), dried over K_2CO_3, filtered, evaporated, and distilled, giving 543 mg (34%) of **7** as a colorless liquid: bp 45–47 °C (0.1 mm); ^1H NMR 2.09 (t, 2), 2.14 (m, 2), 6.78 (t, 1); high resolution MS (70 eV, *m/z*) calcd for $C_6H_5D_6{}^{15}NO$ 120.1187, observed 120.1190.

5,5-Di([$^2H_3$]methyl)[^{15}N,2,3,3-$^2H_3$]-1-pyrroline 1-Oxide (9). A D_2O solution (7 mL) of **7** (443 mg, 3.69 mmol) and NaOD (133 mg, 3.24 mmol) was heated at 70 °C for 20 h. The solvent was evaporated to near dryness. The brown residue was extracted with $CHCl_3$ (5 × 5 mL), dried over K_2CO_3, evaporated to dryness, and distilled, giving 346 mg (76%) of **9** as a colorless liquid, which became a solid below 0 °C: R_f 0.45 ($CHCl_3$/MeOH, 10:1); IR ($CDCl_3$) 1572 cm^{-1}; ^1H NMR 2.03 (s, 2); high resolution MS (70 eV, *m/z*) calcd for $C_6H_2D_9{}^{15}NO$ 123.1376, observed 123.1376.

5,5-Dimethyl[2,3,3-$^2H_3$]-1-pyrroline 1-Oxide (10). 5,5-Dimethyl-1-pyrroline 1-oxide (1.00 g, 8.62 mmol) and NaOD (310 mg, 7.56 mmol) in D_2O (10 mL) were heated at 70 °C for 12 h. The solution was evaporated to near dryness, and the brown residue was extracted with $CHCl_3$ (5 × 5 mL), dried over anhydrous K_2CO_3, evaporated to dryness, and distilled, giving 910 mg (90%) of **10** as colorless liquid, which became solid at temperatures below 0 °C: R_f 0.44 ($CHCl_3$/MeOH, 10:1); ^1H NMR 1.42 (s, 6), 2.10 (s, 2); IR (neat) 1530 cm^{-1}; MS $C_6H_2D_3NO$, *m/e* 116 (M^+). Anal. Calcd for $C_6H_2D_3NO$: C, 62.05; H, 9.55; N, 12.06. Found: C, 61.76; H, 9.59; N, 11.89.

5-Nitro(1,1,1,3,3-$^2H_5$)pentanone (11). A two-phase emulsion consisting of D_2O (45 mL), concentrated DCl (9 mL), and 5-nitropentanone[21] (45.0 g, 0.34 mmol) was stirred at 55 °C for 48 h. The organic layer was separated and the aqueous solution was extracted with ether (3 × 50 mL). The combined organic solutions were dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness, giving partially deuterated (~80% incorporation) **11**, as determined by NMR. The exchange procedure was repeated two more times until incorporation of deuterium was complete,

affording 32.4 g (70%) of colorless liquid, bp 46–50 °C (0.3 mmHg). This material was of sufficient purity as shown by NMR for use in the next step. ^{1}H NMR: 2.21 (t, 2), 4.42 (t, 2).

1-(($^2H_3$)-Methyl)(2,2-$^2H_3$)-1-pyrroline 1-Oxide (12). Nitro ketone 11 (15.0 g, 0.11 mol) and a solution of ammonium chloride (6.36 g) in D_2O (45 mL) were combined in a 250-mL three-necked flask fitted with a mechanical stirrer and cooled in a bath maintained at −20 °C. Zinc dust (36 g) was added in small portions over a period of 3 h such that the temperature of the reaction did not exceed 5 °C. After the addition was completed, the reaction was stirred for an additional 0.5 h at 25 °C. The mixture was filtered and the filtered cake was washed with methanol (5 × 40 mL). The combined filtrates were concentrated, and the oil was extracted with $CHCl_3$ (5 × 20 mL), dried over anhydrous $MgSO_4$, filtered, evaporated, and distilled to give 7.05 g (62%) of 12 as a white oil, bp 65–67 °C (0.3 mmHg). This material was of sufficient purity as shown by NMR for use in the next step. ^{1}H NMR: 2.08 (t, 2), 3.99 (t, 2).

5,5-Di(($^2H_3$)-methyl)(4,4-$^2H_2$)-1-pyrroline 1-Oxide (13). To a stirred solution of deuterated methylmagnesium bromide (1 M, 100 mL) in ether was added a solution of nitrone 12 (5.2 g, 50 mmol) in dry ether (50 mL) at a rate sufficient to mtaintain gentle reflux. After being stirred at 25 °C for 0.5 h, the solution was treated at 0 °C with saturated aqueous ammonium chloride (20 mL). The ether layer was decanted and combined with ether extracts (5 × 50 mL) from the aqueous layer. The solvent was evaporated to yield a yellow oil (2.89 g). To this oil were added methanol (50 mL), concentrated aqueous NH_4OH (5 mL), and copper acetate (500 mg) in the presence of a stream of air. Within 10–15 min a deep blue color developed, at which point the solution was evaporated. The blue oil was then taken up in ether (50 mL), washed with saturated aqueous sodium bicarbonate (10 mL) and brine (10 mL), dried over anhydrous $MgSO_4$, filtered, evaporated to dryness, and distilled to give 1.00 g (17%) of 13 as a white oil, bp 35–37 °C (0.03 mmHg). This material was pure by NMR. ^{1}H NMR: 2.55 (br s, 2), 6.79 (t, 1).

5,5-Di(($^2H_3$)-methyl)(2,3,3,4,4-$^2H_5$)-1-pyrroline 1-Oxide (14). A D_2O solution (10 mL) of 13 (1.00 g, 8.26 mmol) and NaOD (310 mg, 7.56 mmol) was heated at 70 °C for 12 h. The solution was evaporated to near dryness. The brown residue was extracted with $CHCl_3$ (5 × 5 mL), dried over anhydrous $MgSO_4$, filtered, evaporated, and distilled to give 900 mg (88%) of 14 as a colorless oil, which became a solid below 0 °C. This material shows only one peak by GC. Analysis by GC-MS indicated that 14 consisted of 62% of the $^2H_{11}$ species, 29% of the $^2H_{10}$, $^1H_1$ species, 6% of the $^2H_9$, $^1H_2$ species, and 1% of the $^2H_8$, $^1H_3$ species. MS: $C_6D_{11}NO$, m/e 124 (M^+).

Purification of Spin Traps. The following procedures were used to purify spin traps 9 and 14. Immediately prior to use, the spin traps (100–200 mg) were passed through a short silica gel column (1.0 g) using $CHCl_3/CH_3OH$ (100:1). The solvent was evaporated and the white oil was bulb-to-bulb distilled using a Kugelrohr apparatus (38–40 °C/0.03 mmHg) to give the spin trap as colorless oil, which became solid below 0 °C. All spin traps were stored under argon at −70 °C.

Spin Trapping of Superoxide. The superoxide-generating system consisted of xanthine (400 μM) and xanthine oxidase such that the rate of superoxide production was 10 μM/min at 25 °C. Measurement of superoxide generation was determined optically by following the reduction of cytochrome c at 550 nm, using a molar absorptivity of 20 mM^{-1} cm^{-1}. The reaction was initiated by the addition of xanthine oxidase to xanthine (400 μM) and the various spin traps (0.1 M) to a final volume of 0.25 mL. No free radical could be spin trapped if any of the components of the above reaction were not present.

Spin Trapping of Hydroxyl Radical. The spin trapping of hydroxyl radical was undertaken by the addition of ferrous sulfate (0.1 mM) to the superoxide-generating system described above.

Spin Trapping of α-Hydroxyethyl Radical. The spin trapping of α-hydroxyethyl radical was undertaken by the addition of absolute ethanol (0.28 M) to the hydroxyl radical generating system described above.

Acknowledgment. We thank Dr. Howard Halpern, University of Chicago, for his many valuable suggestions. This work was supported in part by grants from the NIH, HL-33550 (G.M.R.) and GM 27137 (J.F.W.K.); The Council for Tobacco Research-USA, Inc. (G.M.R.); and the National Science Foundation, DCB-8616115 (G.M.R.).

The Product of Reserpine Autoxidation

D. V. C. Awang,* B. A. Dawson, M. Girard, and A. Vincent

Bureau of Drug Research, Health and Welfare Canada, Tunney's Pasture, Ottawa, Canada, K1A 0L2

I. Ekiel

Division of Biological Sciences, National Research Council, Ottawa, Canada, K1A 0R6

Received November 6, 1989

Oxidation of reserpine (1) usually leads to the anhydronium bases, 3,4-dehydroreserpine (2) and 3,4,5,6-tetradehydroreserpine (3), products of overall dehydrogenation. Indoles characteristically undergo autoxidation to produce primarily allylic indolenine hydroperoxides, which are susceptible to subsequent reactions by which other oxidation products are formed. The autoxidation of indole alkaloids has not been thoroughly examined. This work describes the characterization of the hitherto unreported major product of reserpine autoxidation, 6, formed in association with a minor proportion of the expected indolenine hydroperoxide, 5. Some evidence is advanced in support of a proposed mechanism of formation of these products.

Reserpine (1) reacts with a variety of reagents, undergoing oxidative dehydrogenation to produce the fluorescent anhydronium bases, 3,4-dehydroreserpine (3-dehydroreserpine) (2) and 3,4,5,6-tetradehydroreserpine ("lumireserpine") (3). Lead tetraacetate has been employed in their synthesis,[1] and vanadium pentoxide–phosphoric acid[2] and nitrous acid[3] reactions (which produce mainly 2) have been applied, with fluorescence detection, to the determination of reserpine in pharmaceutical formulations.

The nitrous acid reaction forms the basis of the official USP reserpine assay procedure[4] and has been investigated in some detail. Rescinnamine similarly produces the

(1) Wright, G. E.; Tang, T. Y. *J. Pharm. Sci.* **1972**, *61*, 299.
(2) Urbanyl, T.; Stober, H. *J. Pharm. Sci.* **1970**, *59*, 1824.
(3) Szalkowski, C. R.; Mader, W. J. *J. Am. Pharm. Assoc. (Sci. Ed.)* **1956**, *45*, 613. Banes, D.; Wolff, J.; Fallscheer, H. O.; Carol, J. *Ibid.* **45**, 710, **1956**.
(4) *The United States Pharmacopeia 21th rev*; U.S. Pharmacopeial Convention, Inc.: Rockville, MD, 1985; pp 935–940.

0022-3263/90/1955-4443$02.50/0 Published 1990 by the American Chemical Society