Reaction of 5,5-Dimethyl-1-pyrroline 1-Oxide with Hypochlorite-Treated Ammonia: The Structure of a Novel *N*-Chloroimine Nitroxyl Radical

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A novel nitroxyl radical was observed by electron paramagnetic resonance spectroscopy (EPR) of reaction mixtures containing ammonium ion, hypochlorous acid (HOCl), and 5,5-dimethyl-1-pyrroline 1-oxide (DMPO). The structure of this radical was established as 2-(*N*-chloroimino)-5,5-dimethylpyrrolidine-1-oxyl on the basis of EPR studies of isotopically-labeled compounds, thin-layer chromatography (TLC), and chemical synthesis by an independent route. Incorporation of deuterium into DMPO and solvent stabilized the radical without affecting the multiplicity of the EPR signal. The use of [^{15}N]ammonium ion gave 2-(*N*-chloroimino)-5,5-dimethylpyrrolidine-1-oxyl radical. Detection of the radical upon TLC was based on its reaction with potassium iodide–starch to give a purple color, consistent with an *N*-chloroimine functionality. Synthesis of the new nitroxyl radical was accomplished by the Michael reaction of acrylonitrile and 2-nitropropane, catalytic hydrogenation of the Michael product, and reaction of the reduced compound with HOCl. The formation and EPR detection of 2-(*N*-chloroimino)-5,5-dimethylpyrrolidine-1-oxyl radical may be useful as a sensitive method for detecting hypochlorous acid and ammonium ion in biological systems.

Introduction

Neutrophils are phagocytic cells that circulate in the bloodstream and provide a first line of defense against invading microorganisms.[1] When activated, neutrophils undergo a burst of oxygen uptake (respiratory burst) that reflects the reduction of oxygen to superoxide ($O_2^{\bullet-}$).[2] The subsequent dismutation of $O_2^{\bullet-}$, catalyzed by the enzyme superoxide dismutase, results in the formation of H_2O_2.[3] By means of the iron-catalyzed Haber–Weiss reaction, $O_2^{\bullet-}$ and H_2O_2 can give rise to hydroxyl radical ($OH^{\bullet}$).[4] However, most of the H_2O_2 formed by neutrophils is utilized for the oxidation of chloride (Cl^-) to hypochlorous acid (HOCl), a reaction catalyzed by myeloperoxidase,[5] a major enzyme in the neutrophil that comprises 5% of its dry weight.[6] The killing of bacteria and the inflammatory effects of activated neutrophils on tissues[7] have been attributed, in large part, to the action of HOCl.[8]

The principal fate of HOCl produced by neutrophils is reaction with nitrogen compounds to form N–Cl derivatives that are relatively long-lived in cells.[9] One reactive nitrogen compound in cells is the ammonium ion (NH_4^+), which occurs as the product of various deamination reactions. NH_4^+ can react with HOCl to give a monochloramine (NH_2Cl) that is lipophilic and able to penetrate the cell membrane and cause oxidative damage.[9b,10] NH_2Cl exhibits properties that are microbicidal,[11] mutagenic,[12] and cytolytic,[10,13] and it has been implicated in pneumonitis[14] and lesions of the gastric mucosa.[15]

5,5-Dimethyl-1-pyrroline 1-oxide (DMPO) is frequently used as a spin trap[16] in the study of radical species produced during the respiratory burst, and spin adducts corresponding to $O_2^{\bullet-}$ and $OH^{\bullet}$ have been observed.[17] Recent studies have shown that the hydroxyl adduct of DMPO can also arise from the reaction of HOCl with DMPO,[18] suggesting that results from the spin trapping of activated neutrophils should be interpreted with caution. Clearly, it is important to characterize the reaction of DMPO with all cellular constituents that can lead to paramagnetic species. We have now examined by EPR spectroscopy the reaction of DMPO with HOCl-treated NH_4^+ (or NH_3) and have observed the formation of a novel *N*-chloroimine nitroxyl radical. This paper reports on the structure of this new radical product.

Experimental Section

General. $^{15}NH_4Cl$ (98 atom % ^{15}N), ND_4Cl (98+ atom % D), D_2O (99.8 atom % D or greater), 30% NaOD in D_2O (99+ atom % D), soluble starch, and DMPO were purchased from Aldrich or Sigma, and Cl_2 was purchased from Matheson. DMPO was vacuum distilled, stored in small amber vials under argon at −20 °C, and kept protected from light; a fresh vial was used for each

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set of experiments. Other chemicals were the best commercial grade available, and solutions were prepared with Pyrex-distilled water that was previously deionized. Three buffer systems were used: 0.2 M KH_2PO_4–H_3PO_4, pH 3.0 (buffer A); 0.2 M K_2HPO_4–KH_2PO_4, pH 7.0 (buffer B); 0.1 M sodium borate, pH 9.1 (buffer C).

EPR spectra were recorded at ambient temperature in a flat quartz cell or 3-mm bore quartz tube with an IBM Instruments, Model ER-200D, spectrometer equipped with a dual cavity and operated at a frequency of 9.79 GHz, with field modulation 100 kHz, microwave power 20 mW, receiver gain 2.52×10^4, and time constant 0.164 s. Computer simulations of EPR spectra were performed using software supplied with the spectrometer. NMR spectra with tetramethylsilane as internal standard were recorded on a Bruker, Model AC 200 or AM 500, instrument or a GE 500 PSG instrument. EIMS data were obtained with a VG Instruments, Model 70SQ, spectrometer, Kratos Concept 1-H mass spectrometer using a cooled direct insertion probe, or a Hewlett-Packard, Model 5988, GC–MS equipped with a 30-m × 0.32-mm i.d. DB-5 column (J & W Scientific). UV spectra were obtained at ambient temperature with a Hewlett-Packard, Model 8452A, diode array spectrophotometer and 10-mm light-path quartz cuvettes. pH values of EPR samples were determined with an Orion, Model 399A, pH meter equipped with a miniature glass electrode from Microelectrodes, Inc. Reported values are final pH readings of reaction mixtures unless stated otherwise. All additions to reaction mixtures and pH adjustments of buffer systems were conducted with vortex mixing. Analytical thin-layer chromatography (TLC) was performed on 100-μm silica gel plates (Kodak Chromagram) and preparative thin-layer chromatography (PTLC) on 1-mm silica gel plates (Whatman PLK5F). Column chromatography was performed with silica gel (40 μm, Baker) using 6-mL disposable filtration columns (Baker). The KI–starch solution used in TLC studies was freshly prepared by dissolving starch (0.5% w/v) and KI (1% w/v) in water. Melting points were determined with a Fisher-Johns apparatus and are uncorrected.

Preparation and Assay of HOCl. Hypochlorous anhydride, Cl_2O, was prepared by oxidation of Cl_2 in CCl_4 with mercuric oxide under anhydrous conditions as described by Cady.[19] The final preparation of Cl_2O in CCl_4 was stored in small amber bottles at −20 °C and kept protected from light.

Pure HOCl was freshly prepared by mixing stock Cl_2O with water in a vortex. The concentration of HOCl in the aqueous layer was determined from the absorbance (ϵ = 26 400 at 352 nm) of the complex formed from I_2 liberated in the presence of excess KI.[20] In the present study, 1 part of stock Cl_2O solution mixed with 5 parts of water yielded 40 mM HOCl.

Preparation of 5,5-Dimethyl[2,3,3-$^2H_3$]-1-pyrroline 1-Oxide (DMPO-d_3) (4). The trideuterated compound was prepared by heating DMPO with NaOD in D_2O (99.96 atom % D) as previously described.[21] The deuterated DMPO was obtained in 81% yield as a colorless liquid after two vacuum distillations: ^1H NMR ($CDCl_3$, 200 MHz) δ 1.42 (s, 6 H), 2.14 (s, 2 H); MS m/z 116 [M^+]. The distribution of deuterium calculated from the mass spectrum was as follows: DMPO-d_1, 1%, DMPO-d_2, 8%; DMPO-d_3, 88%; DMPO-d_4, 2%; DMPO-d_5, 1%.

EPR Examination of Reaction Mixtures. HOCl (10 μL, 0.4 μmol) was added to a solution of NH_4Cl (50 μL, 10 μmol) in buffer A (40 mM, final volume 250 μL, pH 3) in a 1.5-mL polypropylene centrifuge tube. The reaction mixture was stirred in a vortex and allowed to stand for 1 min. DMPO (10 μL, 90 μmol) was added to the solution and the mixture stirred briefly. The reaction was timed from the addition of DMPO. The reaction mixture was immediately transferred to the EPR cell and placed in the cavity of the spectrometer and the spectrum recorded. The g-value of EPR signals was determined by comparison with the g-value (2.0028) of a weak pitch standard placed in the dual cavity next to the flat cell.

Further studies were conducted by repeating the above reaction with the following variations:

(1) Replacement of NH_4Cl by $^{15}NH_4Cl$, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, HCO_2NH_4, and $CH_3CO_2NH_4$.

(2) Replacement of DMPO by DMPO-d_3.

(3) Replacement of DMPO by DMPO-d_3, NH_4Cl by ND_4Cl, HOCl by DOCl, KH_2PO_4–H_3PO_4 by KD_2PO_4–D_3PO_4, and H_2O by D_2O. DOCl was prepared by mixing 1 part of stock Cl_2O with 5 parts of D_2O. KD_2PO_4–D_3PO_4 was prepared from buffer A by repetitive (three times) lyophilization and dissolution in D_2O.

(4) Replacement of buffer A by buffers B and C.

Chromatography. An aqueous solution (250 μL) of the ammonium salt, HOCl, and DMPO was prepared as in the EPR studies and mixed in a vortex with $CHCl_3$ (100 μL). The $CHCl_3$ extract was separated by centrifugation, spotted (3 μL) on a TLC plate, and the plate developed with 10% EtOAc in $CHCl_3$. The plate was air-dried and lightly sprayed with an aqueous solution of KI–starch.

A reaction mixture containing $NH_4H_2PO_4$ (40 μL, 8 μmol), NaOCl (50 μL, 23 μmol), and DMPO (20 μL, 180 μmol) in buffer B (80 mM, final volume 2.5 mL) was mixed in a 4-mL reactivial (Pierce) and the reaction mixture extracted with $CHCl_3$ (1 mL). The organic layer was briefly dried (Na_2SO_4) and placed on a column of silica gel (1.5 g) and the column eluted with $CHCl_3$ at a rate of 1 mL min^{-1}. The fractions (ca. 1 mL) were examined by EPR: fractions 15 and 16 displayed a weak but unidentified EPR signal.

A second reaction mixture containing $NH_4H_2PO_4$ (100 μL, 20 μmol), NaOCl (100 μL, 46 μmol), and DMPO (50 μL, 450 μmol) in water (final volume 2.5 mL) was prepared and worked up as above. The $CHCl_3$ extract was applied to a PTLC plate and the plate developed with 10% EtOAc in $CHCl_3$. One edge of the air-dried plate was sprayed with an aqueous solution of KI–starch, and bands (R_f 0.48 and 0.74) that immediately turned purple were scraped off and examined by EPR. Part of the solid from each band was also eluted with $CHCl_3$ and the eluents examined by EPR before and after treatment with PbO_2. Results are discussed below.

Synthesis of 2-(*N*-Chloroimino)-5,5-dimethylpyrrolidine-1-oxyl Radical (8). Acrylonitrile was heated with 2-nitropropane in alcoholic KOH under reflux as previously described[22] to obtain 4-methyl-4-nitropentylnitrile (5): ^1H NMR ($CDCl_3$, 200 MHz) δ 2.42–2.32 (4 H, m, $-CH_2CH_2-$), 1.65 (6 H, s, 2 CH_3); MS (rel intensity) m/z 96 (95, M − NO_2), 79 (11), 69 (81), 55 (100), 41 (93). Catalytic hydrogenation of **5** over Raney Ni gave 2-amino-5,5-dimethylpyrroline 1-oxide (6):[23] mp 225–228 °C dec, 238 °C,[23] 247–248 °C (sealed evacuated capillary);[24] UV λ_{max} (H_2O) 226 nm (ϵ 8000); ^1H NMR (CD_3OD, 500 MHz) δ 5.386 (2 H, s, NH_2), 2.661 (2 H, t, J = 7.6 Hz, H-3), 1.981 (2 H, t, J = 7.6 Hz, H-4), 1.309 (6 H, s, 2 CH_3); ^{13}C NMR (CD_3OD, 125.76 MHz) δ 150.58 (C-2), 67.43 (C-3), 31.55 (C-4), 24.25 (2 CH_3), the peak for C-5 was probably buried in the solvent signal at δ 48.5–47.5; MS (rel intensity) m/z 128 (100, M), 113 (63), 111 (43), 97 (52), 96 (61). HRMS calcd for $C_6H_{12}N_2O$ (M^+) 128.0590, found 128.0949. A solution of the amino compound **6** and HOCl (1 equiv) in buffers A–C or water gave an EPR spectrum that was identical to that obtained from the mixture of ammonium salt, HOCl, and DMPO.

A solution of **6** (200 μL, 40 μmol) in water (final volume 2.5 mL) was treated with NaOCl (100 μL, 46 μmol). The resulting yellow solution was stirred briefly and extracted with $CHCl_3$ (1 mL) and the yellow organic layer dried (Na_2SO_4) and subjected to PTLC using 5% EtOAc in $CHCl_3$. One edge of the air-dried plate was sprayed with KI–starch. A band at R_f 0.55 that was originally yellow displayed a strong purple coloration with KI–starch; additional bands at R_f 0.74, 0.69, and 0.33 showed much less purple coloration. These bands were scraped off and the solids examined by EPR; an arbitrarily-chosen area of the plate at R_f 0.18 that appeared to be free of any UV- or KI–starch-visible compound was examined by EPR as a control. The bands at R_f

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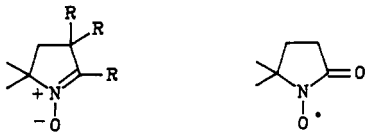
0.74, 0.69, 0.33, and 0.18 were found to be EPR silent and were not studied further. The band at R_f 0.55 showed a strong EPR signal; it was eluted with $CHCl_3$, and EPR and mass spectra of the yellow eluent were recorded. To obtain sufficient material for NMR spectra, the reaction was performed in duplicate on a larger scale (6, 120 μmol; NaOCl, 138 μmol; final volume 2.9 mL). The PTLC band at R_f 0.55 after eluting with $CHCl_3$ and concentrating in vacuo afforded 8 as a yellow viscous oil (18 mg, 46%): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.428 (2 H, m, H-3), 2.337 (2 H, m, H-4), 1.667 (6 H, s, 2 CH_3); MS (rel intensity) m/z 163 [M (^{37}Cl), 10], 161 [M (^{35}Cl), 29], 148 [M (^{37}Cl) - CH_3, 3], 146 [M (^{35}Cl) - CH_3, 10], 131 (6), 127 (8), 97 (29), 96 (100). The ^{1}H NMR sample appeared to contain a small amount of hydrocarbon as an impurity as well as a few minor peaks that may be attributable to decomposition products arising from 8: HRMS calcd for $C_6H_{10}N_2OCl$ (M^+) 161.0482, found 161.0486.

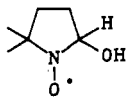
Results and Discussion

The addition of either DMPO (1) to an aqueous solution of NH_4Cl and HOCl, or HOCl to an aqueous solution of 1 and NH_4Cl, produced an EPR signal at g = 2.0068, which



1 R=H DMPO 2 DMPOX

4 R=^{2}H DMPO-d_3



3 DMPO-OH

was composed of a triplet of triplets with further hyperfine splitting (Figure 1A). The aqueous solution of NH_4Cl and DMPO[25] or NH_4Cl and HOCl was EPR silent while the aqueous solution of HOCl and DMPO gave 5,5-dimethyl-2-pyrrolidone-1-oxyl radical (DMPOX) (2) through the intermediacy of 5,5-dimethyl-2-hydroxypyrrolidine-1-oxyl radical (DMPO-OH) (3).[18b] These observations indicated that the signal shown in Figure 1A corresponded to a radical species produced from a reaction involving all three species: NH_4Cl, HOCl, and DMPO.

Isotope Labeling and Deuterium Effects. To assign the structure of the new radical, EPR spectra of reaction mixtures containing specifically-labeled components were recorded. When NH_4Cl in the reaction mixture was replaced by $^{15}NH_4Cl$, the signal shown in Figure 1A changed to a triplet of doublets at g = 2.0068, as depicted in Figure 2A. This clearly indicated that the N of NH_4Cl was incorporated into the radical species and that the corresponding hyperfine splitting caused by this N was 3.71 G (^{14}N, Figure 1A) or 5.25 G (^{15}N, Figure 2A). It also follows that the triplet splitting that is common to the signals in Figures 1A and 2A and which has a hyperfine splitting constant of 8.9 G may be assigned to the nitroxyl N initially present in DMPO. The additional fine splitting in the signals (Figures 1A and 2A) probably arises from either two nonequivalent hydrogen atoms or a chlorine atom.

The β-H and sometimes the two γ-Hs of DMPO-derived radicals can contribute to the hyperfine splitting of their EPR spectra.[26] To determine if DMPO hydrogens were

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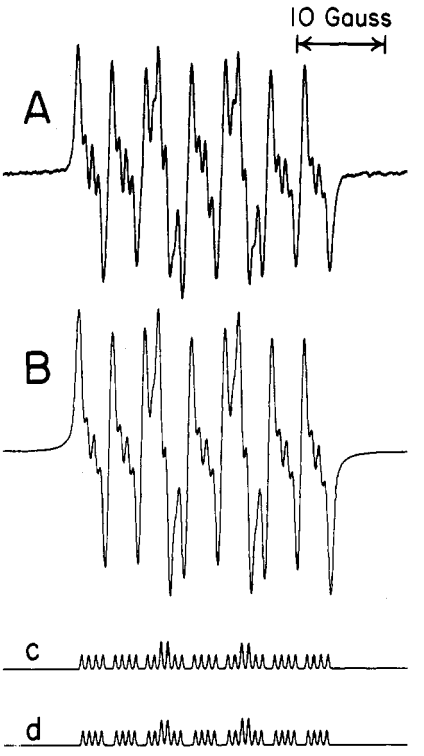

Figure 1. (A) EPR spectrum of a solution containing 40 mM NH_4Cl, 1.6 mM HOCl, and 359 mM DMPO in 40 mM KH_2PO_4–H_3PO_4 (pH 3). Recording of the spectrum was begun 0.65 min after mixing the reagents. See Experimental Section for instrumental parameters. (B) Computer simulation of the spectrum shown in Figure 1A. Stick diagrams c and d represent lines due to the ^{35}Cl- and ^{37}Cl-isomers of 8. Parameters: A_{14N} = 8.9 G, $A_{14N}\beta$ = 3.71 G (spin = 1), A_{35Cl} = 0.78 (75%), A_{37Cl} = 0.7 G (25%), line width = 1.1 G, line shape = 0.67 Gaussian and 0.33 Lorentzian.

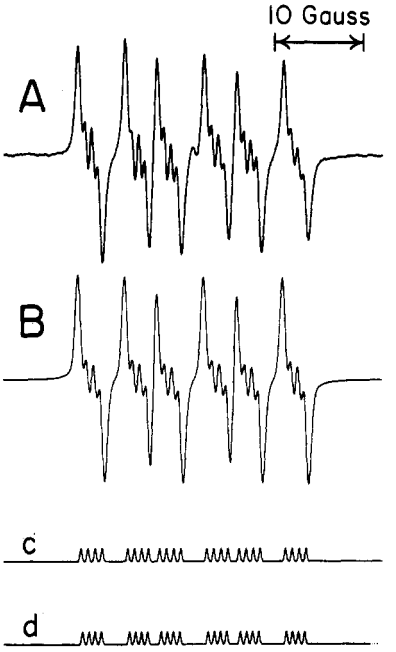

Figure 2. (A) EPR spectrum of a solution containing 40 mM $^{15}NH_4Cl$, 1.6 mM HOCl, and 359 mM DMPO in 40 mM KH_2PO_4–H_3PO_4 (pH 3). Recording of the spectrum was begun 0.9 min after mixing the reagents. (B) Computer simulation of the spectrum shown in Figure 2A. Stick diagrams c and d represent lines due to the ^{35}Cl- and ^{37}Cl-isomers of 9. Parameters: A_{14N} = 8.9 G, $A_{15N}\beta$ = 5.25 G (spin = $^1/_2$), A_{35Cl} = 0.78 (75%), A_{37Cl} = 0.7 G (25%), line width = 1.1 G, line shape = 0.67 Gaussian and 0.33 Lorentzian.

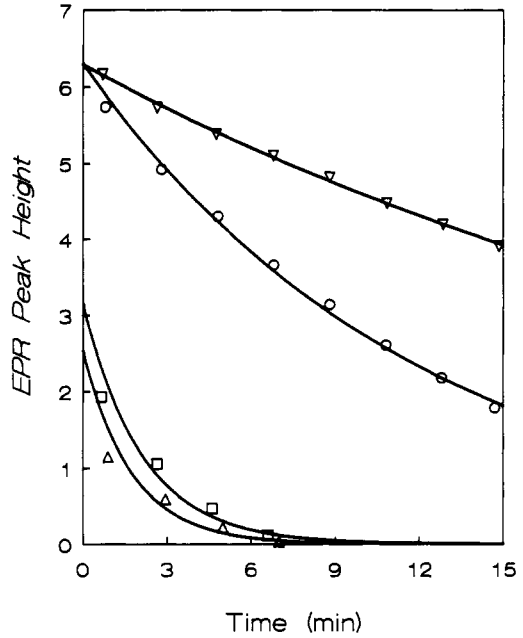

Figure 3. Effect of deuteration and ^{15}N-substitution on the amplitude and the decay of the EPR signals of *N*-chloroimine nitroxyl radicals **8–10**. Triangles (△) refer to a solution containing 40 mM NH_4Cl, 1.6 mM HOCl, and 359 mM DMPO in 40 mM KH_2PO_4–H_3PO_4 (pH 3) (**8**); *R* value for the exponential fit = 0.971. Circles (○) refer to a solution containing 40 mM NH_4Cl, 1.6 mM HOCl, and 359 mM DMPO-d_3 in 40 mM KH_2PO_4–H_3PO_4 (pH 3) (**10**); *R* value for the exponential fit = 0.998. Inverted triangles (▽) refer to a solution containing 40 mM ND_4Cl, 1.6 mM DOCl, and 359 mM DMPO-d_3 in 40 mM KD_2PO_4–D_3PO_4 ("pH" 3) (**10**); *R* value for the exponential fit = 0.999. Squares (□) refer to a solution containing 40 mM $^{15}NH_4Cl$, 1.6 mM HOCl, and 359 mM DMPO in 40 mM KH_2PO_4–H_3PO_4 (pH 3) (**9**): *R* value for the exponential fit = 0.981. Peak heights were measured for the outermost low field line of the EPR spectra (e.g., Figure 1A) of **8–10** and are given in arbitrary units.

involved in the hyperfine splitting (Figure 1A), DMPO-d_3 (**4**) in which the β-H and both γ-Hs were substituted by deuterium was prepared.[21] It was found that the reaction mixture containing NH_4Cl, HOCl, and DMPO-d_3 gave the same EPR signal as that shown in Figure 1A, indicating that the β- and γ-Hs of DMPO did not contribute to the hyperfine splitting. Further, the radical species responsible for the Figure 1A signal appears to lack a β-H because all known DMPO adducts having a β-H exhibit hyperfine splitting from the β-H.[26] Treatment of DMPO-d_3 with ND_4Cl and DOCl in deuterated buffer also produced the same signal, thus eliminating the possibility that hydrogens from NH_4Cl, H_2O, or HOCl contributed to the multiplicity of the signal shown in Figure 1A.

It is interesting to note that the intensity and lifetime of the EPR signal were significantly enhanced when DMPO-d_3 was substituted for DMPO in the above experiments. The change in amplitude of the EPR signal with time is shown in Figure 3. The amplitude, which is a measure of signal intensity, followed exponential decay in the examples shown in Figure 3. The rate of radical formation was too fast to be measured under our experimental conditions and is not reflected in the Figure 3 curves. The first-order rate constant of the decay with respect to nondeuterated (k_H), DMPO-deuterated (k_D), and completely-deuterated (k_{D_3}) reaction mixtures was found to be 9.5×10^{-3}, 1.4×10^{-3}, and 5.3×10^{-4} s^{-1}, respectively; the corresponding value for the reaction mixture containing $^{15}NH_4Cl$ was 7.8×10^{-3} s^{-1}. The data in Figure 3 suggest

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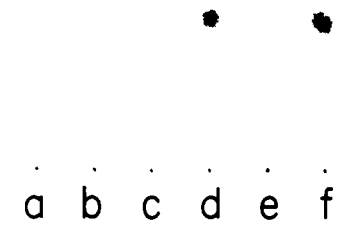

Figure 4. Thin-layer chromatography. The $CHCl_3$ extracts of the following aqueous solutions (a–d) were spotted on the TLC plate. Color development occurred only under conditions that also yielded EPR signals. See Experimental Section for further details. (a) 80 mM $NH_4H_2PO_4$ and 8 mM NaOCl in 80 mM KH_2PO_4–K_2HPO_4 (pH 7). (b) 3.2 mM $NH_4H_2PO_4$ and 8 mM NaOCl in 80 mM KH_2PO_4–K_2HPO_4 (pH 7). (c) 80 mM $NH_4H_2PO_4$, 8 mM NaOCl, and 72 mM DMPO in 80 mM KH_2PO_4–K_2HPO_4 (pH 7). (d) 3.2 mM $NH_4H_2PO_4$, 8 mM NaOCl, and 72 mM DMPO in 80 mM KH_2PO_4–K_2HPO_4 (pH 7). (e) Spotted 0.2 μmol of DMPO in $CHCl_3$ followed by 3 μL of a. (f) Spotted 0.2 μmol of DMPO in $CHCl_3$ followed by 3 μL of b.

that the initial signal intensity for both mixtures containing DMPO-d_3 were the same, and this value was about 2.5-fold higher than that of the nondeuterated mixture. These results are consistent with previous observations that deuteration of DMPO[21] and various spin labels[27] resulted in increased EPR intensity. The incorporation of ^{15}N (from $^{15}NH_4Cl$) only slightly enhanced the stability of the radical.

Further analysis of Figure 3 reveals substantial deuterium isotope effects. The signals from reaction mixtures containing DMPO-d_3 survived longer, the lowest rate of decay being observed for the mixture containing all deuterated components including the medium. The radical **10** formed from DMPO-d_3, which should contain two deuterium atoms at the γ-position, showed an isotope effect (k_H/k_D) of 6.9 in its decay. This clearly suggests that the γ-hydrogens make a significant contribution to the activation energy of the transition state associated with the decay of the new radical **8**, perhaps in the form of hyperconjugation.[28] The other isotope effect, k_D/k_{D_3} = 2.6, was due to deuteration of the solvent because no hydrogens from NH_4^+ or HOCl were incorporated into radical **8**. The latter isotope effect suggests[29] that, in the decay process, the solvent (water) might function as a reactant or that there is hydrogen bonding[30] or a rapid exchange of hydrogens between radical **8** and water.

Other Ammonium Salts. The fine splitting in the Figure 1A signal appears to be due to a chlorine atom present in the radical species because experiments with the deuterated compounds clearly demonstrated that hydrogen atoms were not involved in the splitting. The Cl atom could be derived from NH_4Cl or HOCl. To distinguish between these possibilities, EPR spectra were obtained after replacing NH_4Cl in the reaction mixture with several ammonium salts: $(NH_4)_2SO_4$, $NH_4H_2PO_4$, HCO_2NH_4, and $CH_3CO_2NH_4$. All reaction mixtures exhibited the same Figure 1A signal, thus indicating that Cl in the radical species was derived from HOCl.

Simulation of EPR Spectra and Analysis of Hyperfine Splitting Constants. The signals shown in Figures 1A and 2A were simulated by assuming an interaction of the electron spin with only two nonequivalent

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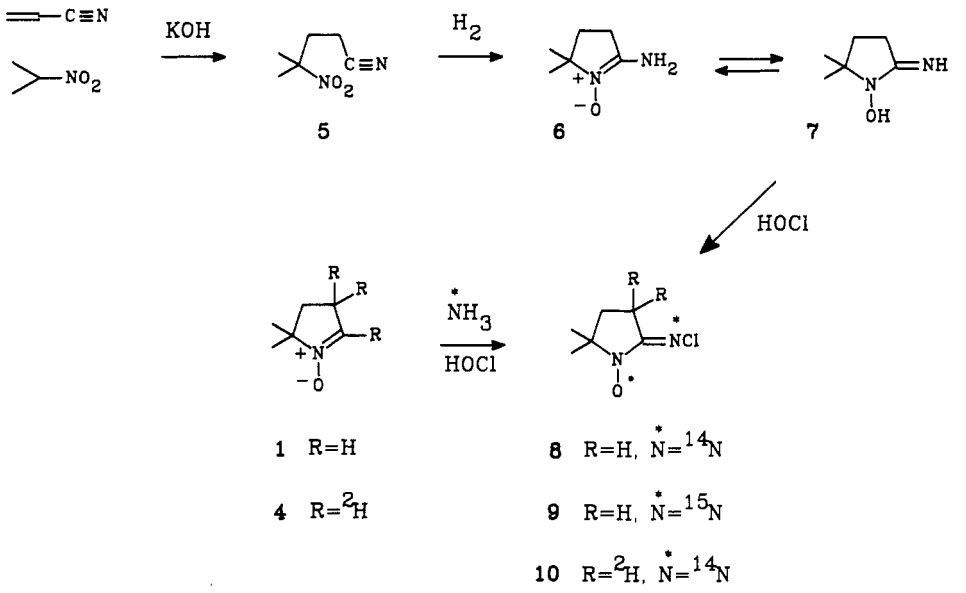


Figure 5. Outline of independent synthesis of 8 and the formation of 8, 9, and 10 from hypochlorite-treated ammonium ion (or ammonia) and DMPO.

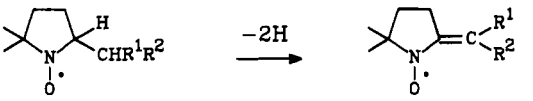
N atoms and a Cl atom in the radical. The simulated spectra are shown in Figures 1B and 2B, respectively. The good agreement between the observed and the computed spectra confirms that no other atoms contributed to the hyperfine splitting. The results presented above demonstrate that the two N atoms were derived from the ammonium salt and DMPO and that the Cl atom arose from HOCl. The new radical 8 is thus a DMPO-derived nitroxyl containing a Cl atom and one additional N atom.

An important clue to the structural assembly of the exogenous N and Cl atoms in the radical 8 is provided by the hyperfine splitting constant (A_N, 8.9 G) of the nitroxyl N. The A_N is known to be related approximately linearly to the spin density on the nitroxyl N.[31] The A_N values of DMPO adducts in which the β-carbon is tetrahedral often fall in the range of 14–16.7 G.[26] When the β-carbon is trigonal, permitting delocalization, the spin-density of the nitroxyl N is reduced, and hence lower values for A_N are expected. Known DMPO derivatives with a trigonal β-carbon have significantly lower A_N values as shown by the examples in Table I. Acyclic nitroxyl radicals also show similar trends in A_N values which correspond to structural changes at the β-carbon.[26,32] From these considerations, it appears that the new radical whose A_N is 8.9 G has a trigonal carbon at the β-position with provision for delocalizing the spin density. As noted above, the radical also lacks a β-H. Therefore, the N atom derived from NH_4^+ must be attached to the β-carbon to constitute an imine group. The chlorine atom derived from HOCl should then reside on the imine N. The radical thus appears to be an *N*-chloroimine nitroxyl; to the best of our knowledge, this is the first example of a nitroxyl radical having an N–Cl group.

Chromatography. A TLC method was used to gain further support for the presence of an N–Cl group in radical 8. It is known that N–Cl compounds oxidize iodide to iodine,[20b] which forms a purple-blue complex with starch.[33] $CHCl_3$ extracts of reaction mixtures containing an ammonium salt, HOCl, and DMPO were chromatographed on silica gel plates using 10% EtOAc in $CHCl_3$ and the plates sprayed with a solution of KI and starch. Those mixtures that gave EPR signals always produced a purple spot at R_f 0.43, pointing to a possible correlation between the radical and a constituent N–Cl group. A representative chromatogram is shown in Figure 4. EPR studies showed that, under neutral (pH 7) and alkaline (pH 9) conditions, the EPR signal (Figure 1A) was detected only at low ratios (<1) of ammonium salt to hypochlorite, and parallel observations were made in the TLC studies (Figure 4). However, under acidic conditions (pH 3), the EPR signal was detected at both high (20) and low (0.5) ratios of ammonium salt to HOCl. The latter finding will be addressed in more detail in a subsequent paper.

Attempts were made to isolate radical 8 by column and preparative thin-layer chromatography (PTLC) of $CHCl_3$ extracts of reaction mixtures containing an ammonium salt, hypochlorite, and DMPO. A component eluted from the column with $CHCl_3$ gave a weak but unidentified EPR signal (data not shown). PTLC was also unsuccessful but provided the same unidentified EPR signal when $CHCl_3$ eluents of appropriate EPR-silent bands were treated with PbO_2, suggesting that the unidentified radical in the column fraction may have resulted from air oxidation of a diamagnetic reaction product. Failure to isolate 8 by chromatography from reaction mixtures containing DMPO can be attributed to further reaction of 8 with DMPO (see

Table I. Hyperfine Splitting Constant (A_N) of the Nitroxyl Nitrogen of DMPO Derivatives Containing Tetrahedral and Trigonal β-Carbon Atoms



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radical	A_N (G) (tetrahedral β-C)	A_N (G) (trigonal β-C)	ref
R^1 = CN, R^2 = Ph (I, II)	14.0 (I)	7.1 (II)	32
R^1 = CN, R^2 = CN (I, II)	13.5 (I)	5.6 (II)	32
R^1 = COPh, R^2 = Ph (I, II)	14.6 (I)	8.5 (II)	32
DMPO-OH (3), DMPOX (2)	14–15 (3)	7 (2)	26

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below). The purple spot at R_f 0.43 in the earlier TLC studies corresponded, as discussed below, to a reaction product of 8 and DMPO, which retained the active Cl atom.

Chemical Synthesis. Confirmatory evidence for the structure of radical 8 was obtained by independent chemical synthesis as outlined in Figure 5. Briefly, the Michael reaction of acrylonitrile and 2-nitropropane in the presence of ethanolic KOH gave 5[22] which, on catalytic hydrogenation, provided 6.[23] The products from the acetylation, benzoylation, and methylation of the amino compound 6 have been found to be mainly the derivatives of the tautomeric imino structure 7.[34] The reaction of 6 with HOCl furnished an EPR spectrum that was identical to the spectrum produced from NH_4^+, HOCl, and DMPO (Figure 1A).

Although radical 8 could be produced from 6 and HOCl or from NH_4^+, HOCl, and DMPO, chromatographic behavior and decay of 8 were significantly influenced by the presence of DMPO. The $CHCl_3$ extract of 6 and HOCl displayed a purple TLC spot (KI–starch) at R_f 0.55 while the corresponding spot from the $CHCl_3$ extract of NH_4^+, HOCl, and DMPO appeared at R_f 0.43 (10% EtOAc in $CHCl_3$). Co-TLC of these two $CHCl_3$ extracts showed only a single purple spot at R_f 0.43. The EPR signal of 8 produced from NH_4^+, HOCl, and DMPO decayed completely within a short period of time (<10 min, Figure 3) whereas that from 6 and HOCl, despite its gradual decomposition, was detected by EPR even after 24 h (data not shown). Moreover, the addition of DMPO to a solution of 6 and HOCl greatly reduced the lifetime of the radical (<10 min), and the resulting solution produced a single purple TLC spot (KI–starch) at R_f 0.43. Thus, the apparent lower mobility on TLC and the reduced stability of 8 in reaction mixtures of NH_4^+, HOCl, and DMPO may be rationalized in terms of a rapid reaction of 8 (R_f 0.55) and DMPO to form a diamagnetic species (R_f 0.43).[35]

For further studies by MS and ^1H NMR, radical 8 was purified by PTLC of the $CHCl_3$ extract obtained from the reaction mixture of 6 and HOCl. The intensity ratio of the peaks at m/z 161 [M (^{35}Cl)] and 163 [M (^{37}Cl)] and those at m/z 146 [M (^{35}Cl) – CH_3] and 148 [M (^{37}Cl) – CH_3] in the mass spectrum was found to be 3:1, providing further support for the presence of a Cl atom in 8. High-resolution mass spectrometry established the molecular formula of 8 as $C_6H_{10}N_2OCl$, and the ^1H NMR spectrum was consistent with this structure.

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Registry No. 1, 3317-61-1; 4, 128217-05-0; 5, 16507-00-9; 6, 34211-69-3; 8, 139871-44-6; 9, 139871-47-9; 10, 139871-48-0; DMPO-d_5, 139871-45-7; DMPO-d_4, 139871-46-8; NH_4^+, 14798-03-9; HOCl, 7790-92-3; NH_4Cl, 12125-02-9; $(NH_4)_2SO_4$, 7783-20-2; $NH_4PO_4H_2$, 7722-76-1; HCO_2NH_4, 540-69-2; CH_3CONH_4, 631-61-8; $^{15}NH_4Cl$, 39466-62-1; ND_4Cl, 12015-14-4; DOCl, 13770-22-4; KH_2PO_4, 7778-77-0; H_3PO_4, 7664-38-2; KD_2PO_4, 13761-79-0; D_3PO_4, 14335-33-2; D_2O, 7789-20-0; D_2, 7782-39-0; NaClO, 7681-52-9; acrylonitrile, 107-13-1; 2-nitropropane, 79-46-9.

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