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INFLUENCE OF CHEMICAL STRUCTURE OF NITROXYL SPIN LABELS ON THEIR REDUCTION BY ASCORBIC ACID

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Abstract - The influence of structure on the reduction of nitroxyl spin labels by ascorbic acid was examined using both piperidine and pyrrolidine nitroxyls. A five-fold molar excess of ascorbic acid and pH of 7.4 were used. The nitroxyl concentration was measured by electron spin resonance spectrometry. The five-membered (pyrrolidine) nitroxyls were more stable than the six-membered derivatives. Ring substituents also influenced the reaction. The anionic derivatives were more stable than the unionized compounds which, in turn, were more stable than the amines (cations at pH 7.4).

INTRODUCTION

Because of the presence of an unpaired electron, nitroxyl spin labels can be used to enhance contrast in proton magnetic resonance imaging (MRI) and thereby increase the diagnostic usefulness of this new technique (1). Potential applications of contrast-enhancing nitroxyls include: differentiation of isomagnetic tissues, direct evaluation of organ function and pathology, and evaluation of tissue perfusion (2-4).

Two water soluble nitroxyls, succinic acid N-(2,2,6,6-tetramethyl-1-oxyl-4-piperidinyl) monoamide [6C] and 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl [5B] have been administered intravenously to experimental animals prior to proton imaging by magnetic resonance techniques. These nitroxyls aided in the definition of renal functional abnormalities, defects in the blood-brain-barrier, and perfusion abnormalities in infarcted myocardium. Both nitroxyls were extensively reduced *in vivo*, presumably to their corresponding hydroxylamine derivatives (5). This reduction process is undesirable, because the hydroxylamine is not paramagnetic and produces no contrast enhancing effect in MRI. Nitroxyl derivatives with high resistance to *in vivo* reduction would, therefore, be advantageous for contrast enhancement.

The rates of reduction of 6C and 5B by ascorbic acid have been shown to differ significantly when measured under identical conditions (5), indicating that chemical structure influences the rate of biologic reduction and that certain structures may be particularly resistant to reduction *in vivo*.

The objective of this study was to use a representative spectrum or nitroxyl compounds to assess chemical structure as a factor in the rate of their reduction by ascorbic acid. Many of the compounds were newly synthesized to permit meaningful comparisons. The rate of nitroxyl reduction for each compound was assessed by incubation with a five-fold molar excess of ascorbic acid; the amount of nitroxyl remaining as a function of time was measured by electron paramagnetic resonance.

The nitroxyls used in these studies are listed in Table 1. They were assigned a 2-character code. The number in the code indicates a pyrrolinyl or pyrrolidinyl, 5, or a piperidinyl, 6, compound. The letters, A-M, indicate a particular substituent.

Table 1.
Nitroxide structures and reduction by ascorbic acid

		Percent remaining	
		At 1 minute	At 2 minutes
SIX-MEMBERED RINGS[a]			
H	**6A**	50.6±4.0[b]	26.7±5.1
COOH	**6B**	32.5±3.2	11.4±2.0
$NHCO-(CH_2)_2-COOH$	**6C**	13.1±2.5	2.5±0.8
OH	**6D**	14.6±0.8	3.1±0.4
$CONH_2$	**6E**	13.0±1.9	2.7±0.1
= O	**6F**	13.1±3.8	2.6±1.0
NH_2	**6G**	1.0±0.3	0.4±0.1
OPO_3H	**6H**	31.7±6.1	8.9±2.0
$NHCO-(CH_2)_2-CH_2OH$	**6I**	8.6±0.1	1.0±0.6
$NH-(CH_2)_2-CH_2OH$	**6J**	0.5±0.3	0.3±0.2
FIVE MEMBERED RINGS			
a) Pyrrolidine Derivatives			
H	**5A**	97.9±0.2	95.2±0.7
COOH	**5B**	96.0±1.4	92.7±1.7
$NHCO-(CH_2)_2-COOH$	**5C**	90.8±0.3	83.6±0.5
OH	**5D**	93.5±0.7	87.8±1.0
$CONH_2$	**5E**	94.2±0.5	89.6±0.6
= O	**5F**	16.4±3.6	6.3±2.0
NH_2	**5G**	72.4±4.0	58.4±4.8
$NHCOCOOCH_3$	**5K**	89.2±1.0	80.8±1.0
NHCOCOOH	**5L**	94.1±1.2	89.2±1.4
b) Pyrroline Derivative			
COOH	**5M**	91.3±1.2	83.6±3.0

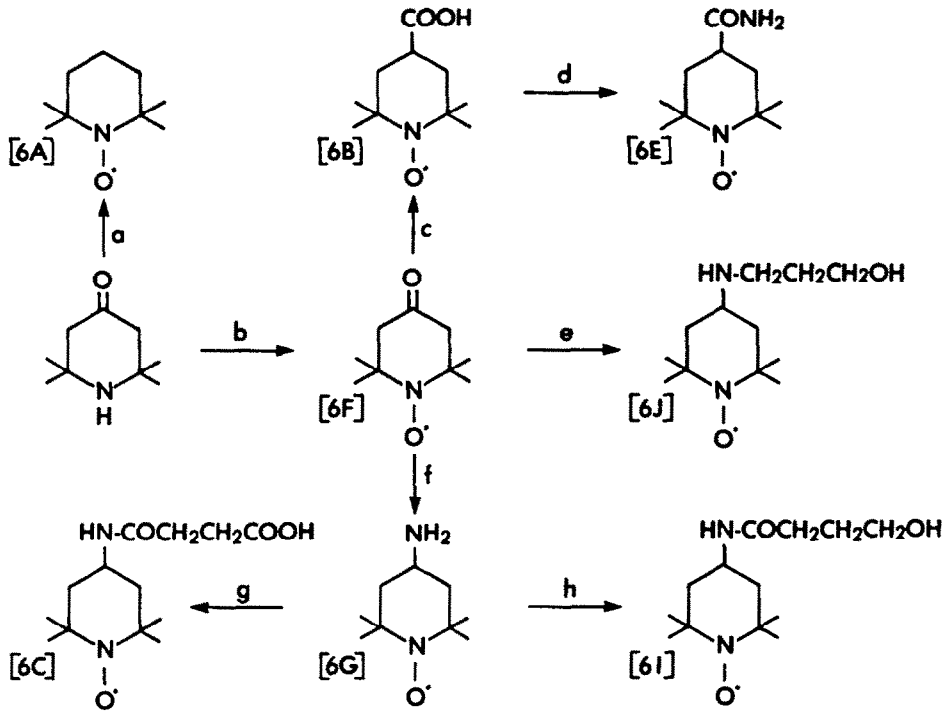
[a]Ring structures are shown in schemes 1 and 2.
[b]Mean (S.D.) of at least 3 experiments.

A brief comment on nomenclature is in order. "Nitroxide" is often applied to free radicals with the >N-O•functionality. In this paper, the more appropriate "nitroxyl" term is used.

RESULTS AND DISCUSSION

Synthesis of Six-Membered Nitroxyls: The synthetic strategies for the preparation of piperidine-1-oxyl derivatives are shown in Scheme I.

Scheme I: Synthesis of Six-Membered Nitroxyls.

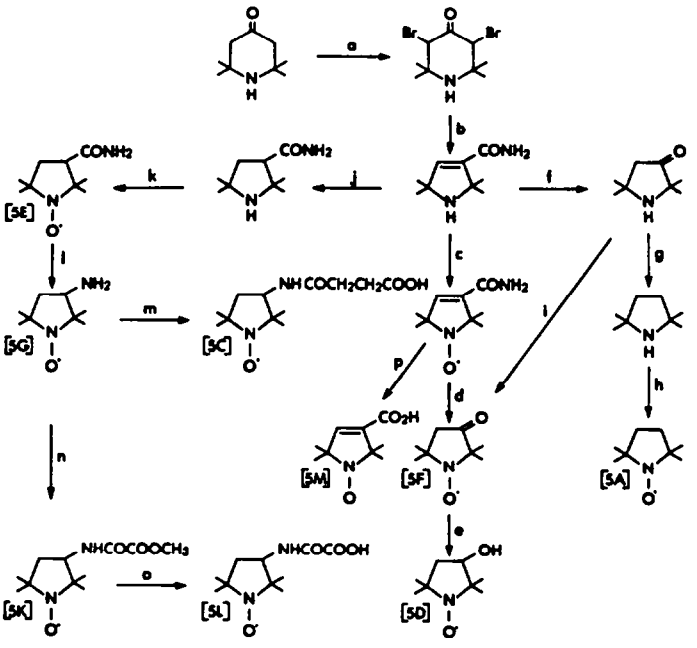


[a] N_2H_4/diethylene glycol/KOH, 30% $H_2O_2/Na_2WO_4/H_2O$; [b] 30% $H_2O_2/Na_2WO_4/H_2O$; [c] $TsCH_2NC$/t-BuOK, $Ba(OH)_2/H_2O$/100°C; [d] CH_2N_2, NH_3/CH_3OH; [e] $HO(CH_2)_3NH_2/NaBH_3CN/CH_3OH$; [f] $NH_4OAc/NaBH_3CN/CH_3OH$; [g] Succinic anhydride/$(C_2H_5)_2O$/125-130°C; [h] γ-Butyrolactone.

The 4-substituted piperidinoxyl derivative, **6J**, was prepared in a 41% yield by an adaptation of the general procedure reported earlier (6,7). Since the original work (6) contained neither yield nor physical data, the details for the preparation of **6J** are given in the experimental section. The reaction of γ-butyrolactone with **6G** at 125-130° gave a 59% yield of **6I**. Carboxylic acid, **6B**, was obtained by the reaction of the ketone, **6F**, with tosylmethyl isocyanate, followed by hydrolysis of the intermediate cyanoderivative (8). The reaction of **6B** with diazomethane gave the corresponding ester (9) which was converted by aminolysis to the amide **6E**. The reaction of succinic anhydride with **6G** in ethyl ether yielded compound **6C**. The most abundant peak in the mass spectrum of **6C** was observed at m/e 257 which is attributed to the loss of a methyl group from the molecular ion.

Synthesis of Five-Membered Nitroxyls: The syntheses of the pyrrolidine-oxyl derivatives are shown in Scheme II.

Scheme II: Synthesis of Five-Membered Nitroxyls.



[a] Br_2/AcOH; [b] NH_4OH; [c] 30% $H_2O_2/Na_2WO_4/H_2O$; [d] NaOBr/H_2O; [e] $NaBH_4/C_2H_5OH$; [f] NaOBr/H_2O; [g] N_2H_4/diethylene glycol monomethyl ether; [h] 30% $H_2O_2/Na_2WO_4/H_2O$; [i] 30% $H_2O_2/Na_2WO_4/H_2O$; [j] H_2/Ni_R; [k] 30% $H_2O_2/Na_2WO_4/H_2O$; [l] NaOBr/H_2O; [m] Succinic anhydride/$(C_2H_5)_2O$; [n] $(COOCH_3)_2/CH_3OH$/64 °C; [o] 10% NaOH/CH_3OH; [p] 10% NaOH/H_2O/100 °C.

The keto radical, **5F**, was synthesized from 3-carboxamide-2,2,5,5-tetramethyl-3-pyrroline, either by oxidation with hydrogen peroxide in the presence of sodium tungstate, followed by the Hofman degradation, or by the reversed sequence of these two reactions. The reduction of **5F** using aluminum iso-propoxide was reported earlier (10) to give a 80% yield of **5D**. The yield was now improved to 90% using sodium borohydride as the reducing agent. The Wolff-Kishner reduction of the 2,2,5,5-tetramethylpyrrolidine-3-one to 2,2,5,5-tetramethylpyrrolidine was reported (11) to give a 28% yield. In the present work, extensive modifications of the experimental procedure resulted in a 73% yield of 2,2,5,5-tetramethylpyrrolidine. The oxidation of 2,2,5,5-tetramethylpyrrolidine to **5A** was reported earlier (12) without a yield. Now compound **5A** was obtained in a 54% yield. The reaction of **5G** with dimethyl oxalate in methanol resulted in **5K** which was then converted to **5L** by hydrolysis. The most abundant peak in the mass spectrum of **5L** was observed at m/e 215 which is attributed to a loss of methyl group from the molecular ion. The reaction of succinic anhydride with [5G] in ethyl ether resulted in [5C]. The most abundant peak in the mass spectrum of [5C] was observed at m/e 243 which is attributed to the loss of a methyl group from the molecular ion. Another peak at m/e 214 may arise by the loss of the carbon dioxide from the molecular ion.

Reduction of Nitroxyls by Ascorbic Acid: We observed that the reduction of both the five- and six-membered nitroxyls does not always show first-order kinetics (5), as previously reported (13,14). Hydroxylamines which are formed during the reduction of nitroxyls by ascorbic acid (15,16), can spontaneously oxidize to their corresponding nitroxyls in the presence of air oxygen (5). The oxidation is known to be catalyzed by copper ions (17,18). To ascertain if impurities catalyzed the reaction in our studies, EDTA was added to complex any metal ions present. No difference was observed between experiments with and without EDTA, indicating that spontaneous metal ion-catalyzed oxidation of the hydroxylamine did not occur under our experimental conditions.

Results of the present ascorbic acid reduction study are presented in Table I and are expressed as the percentage of nitroxyl remaining 1 and 2 minutes after the beginning of the reaction. At these times, differences in the stabilities among the 20 nitroxyls were readily apparent. For example, after 1 minute, the percentage of 5A remaining was approximately 200 times more than that of the structurally-different nitroxyl, 6J.

With one exception, [5F], the five-membered ring nitroxyls [5A-5M] were reduced more slowly than the six-membered derivatives [6A-6J]. This trend was observed for both the substituted and unsubstituted heterocycles. Therefore, we conclude that the reduction rate of nitroxyls by ascorbic acid is strongly influenced by the nature of the ring system. It appears that the planar five-membered ring system possesses a greater resistance to ascorbate reduction than the puckered six-membered ring derivatives. The presence of a double bond in the five-membered structure [5M] seems to enhance reducibility.

Substituents on the heterocyclic ring further influence, although to a lesser extent, the stability of the nitroxyls. Substitution has an apparent destabilizing effect, since substituted nitroxides were always more rapidly reduced by ascorbic acid than their corresponding unsubstituted derivatives. The carboxylic derivatives (anions at pH 7.4) were more stable than the nonionized compounds which, in turn, were more stable than the amines (cations at pH 7.4).

We observed that nitroxyls carrying substituents with a positive charge were more rapidly reduced than those with a negative charge. It was recently proposed (14) that the mono-anion is the form of ascorbic acid responsible for the reduction of nitroxyls. This theory suggests that anionic nitroxyls would be repulsed by the reducing agent and thereby more slowly reduced than cationic derivatives which would be attracted. Our observations were generally consistent with this theory. Comparison of 6G and 6J indicates that the secondary amine, [6J], which is presumably a stronger base than 6G and therefore more protonated at pH 7.4, is also more rapidly reduced by ascorbic acid than the primary amine [6G]. In comparison, compound 6I containing an amide function instead of the secondary amine function in 6J, is not protonated at pH 7.4 and therefore is less rapidly reduced than 6J. In contrast, compounds 6B and 6H which possess a negatively-charged substituent close to the piperidine ring are the most stable among all the substituted six-membered ring nitroxyls. If the mono-anion is the active form of ascorbic acid, the rate of reduction of the unsubstituted nitroxyls should be intermediate between the anionic and cationic derivatives. However, the unsubstituted compounds 5A and 6A were more stable than their corresponding negatively-charged five- and six-membered substituted nitroxyls. The ranking of the stability by the nature of the substituents was the same in both ring systems, except for 5E which was more rapidly reduced than expected.

Nitroxyls have been frequently used as spin labels (19,20) and their chemical properties have been studied extensively over the past 25 years, including their reducibility by chemical agents in biological environments (19,20). However, a systematic study of the influence of structure of nitroxyls on their susceptibility to reduction has not been reported. In this study, we show marked differences that relate to basic structural characteristics. These results have practical implications, for example in biochemical experiments involving the disappearance rates of different nitroxyls within cells. In past reports nitroxyls, differing by their charge (21) or by their lipid-water partition coefficient (22), were compared assuming that the differences in the observed reduction rates were related to these properties alone. However, in these experiments, the spin labels also differed in their ring system. Thus, the conclusions drawn are open to question.

Our results also influence the design and selection of nitroxyls for contrast enhancement for MRI. We previously observed an extensive reduction of two nitroxyls *in vivo* (5). A good correlation existed between the rates of reduction by ascorbic acid and *in vivo* stability for these two nitroxyls (5). We now conclude that pyrrolidine nitroxyls can be of greater potential value for MRI contrast enhancement than piperidine derivatives because of their greater resistance to reduction.

EXPERIMENTAL

Materials: All reagents were of the finest quality available commercially. All solvents were distilled prior to use. Ethyl ether was distilled and stored over lithium aluminum hydride. The pyridine was distilled and stored over solid potassium hydroxide. The triethylamine was stored over potassium hydroxide. Ascorbic acid was purchased from Sigma Chemical Company (St. Louis, MO).

4-Phosphonoxy-2,2,6,6-tetramethylpiperidine-1-oxyl [**6H**] was obtained from Aldrich Chemical Company (Milwaukee, WI). The following nitroxides were obtained from Eastman Kodak Company (Rochester, NY) and, in some cases, were also synthesized in our laboratory: 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl [**6D**], 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl [**6F**], 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl bonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl [**5E**], 3-carboxy-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl [**5H**].

The following nitroxides and precursors were prepared by literature procedures: 2,2,6,6-tetramethylpiperidine-1-oxyl (10), 2,2,5,5-tetramethyl-3-pyrrolidone (23), 3-carboxy-2,2,5,5-tetramethylpyrroline-1-oxyl (10), 3-aminocarbonyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl (24), 2,2,5,5-tetramethylpyrrolidine (24), 3-aminocarbonyl-2,2,5,5-tetramethylpyrrolidine (24), 2,2,5,5-tetramethyl-3-oxopyrrolidine-1-oxyl (24), and 3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl (25).

Analytical procedures: Melting points were determined on a Thomas Hoover melting point apparatus, model 6406-K, with a calibrated thermometer. The IR analyses were performed on a Perkin-Elmer Spectrophotometer, model 735 B. Mass spectra were recorded on a Hewlett-Packard Mass Spectrometer, model 5985 GS, using a direct insertion probe, a source pressure of 2×10^{-7} torr, and methane as the reactant gas. Therefore, $M^+ + 1$ values are reported. Microanalyses were performed either on a F & M Scientific Corporation Carbon, Hydrogen, Nitrogen Analyzer, model 185, or by Atlantic Microlab, Inc., Atlanta, Georgia. Column Chromatography was performed either using the Flash Chromatography Technique (26) on Silica gel 60 (Fluka) finer than 230 mesh, or by conventional column chromatography on alumina (MCB, Type F-20, 80-200 mesh). Thin-layer chromatographic (T.L.C.) analyses were performed on Silica gel 60 F_{254} or aluminum oxide 60 F_{254} precoated sheets (Merck), layer thickness 0.2 mm with visualization using UV light and/or iodine chamber. The electron paramagnetic resonance spectra of $\sim 10^{-4}$M benzene or chloroform solutions were obtained using a Varian E-115 spectrometer. All spectra consisted of 3 lines with a_N= 14.5-16G.

Preparation of 4-[N(3-hydroxypropyl)amino]-2,2,6,6-tetramethylpiperidine-1-oxyl [**6J**]. Sodium cyanoborohydride (0.63 g, 0.010 mol) was added to a solution of 3-amino-1-propanol (2.25 g, 0.030 mol) and 2,2,6,6-tetramethyl-4-oxopiperidine-1-oxyl (1.70 g, 0.010 mol) in methanol (100 ml) and the resultant solution was kept for 48h at 25°C. After removing the solvent on a rotating evaporator at 40°C/20 torr, the oily residue was dissolved in water (20 ml) and acidified with 2 N hydrochloric acid to pH 2. The excess of the unreacted ketone was removed from the acidic aqueous solution by extraction with chloroform (3x30 ml) and the extracts were discarded. The aqueous layer was then adjusted to pH 10 with a 6N solution of sodium hydroxide followed by chloroform extractions (3x30 ml). The combined chloroform extracts were dried with magnesium sulfate, filtered and the filtrate concentrated on a rotating evaporator at 40°C/20 torr. The remaining, red, oily material was purified by column chromatography on alumina using a mixture of t-butyl methyl ether and methanol (v/v 7:1) as eluant to give 1.05 g of a red oil which crystallized on cooling to 0°C. Recrystallization from a mixture of benzene and cyclohexane gave 0.95 g (41%) of pure **6J**, m.p. 103-104°C. Purity control by the T.L.C. (Silica gel, methanol) indicated one compound.

M.S.: m/e = 230(100), 213(12). IR (KBr): "ν" = 3160, 2900, 2850, 2770, 1435, 1350, 1330, 1220, 1150, 1090, 890 cm^{-1}. $C_{12}H_{25}N_2O_2$ (229.34). Calcd: C 63.49% H 11.10% N 12.34%
Found: C 63.17% H 11.13% N 12.02%

Preparation of 4-(4-hydroxybutanoylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl [**6I**]. A mixture of **6G** (0.80 g; 0.0046 mol) and γ-butyrolactone (1.20 g, 0.0140 mol) was heated at 125-130°C for 2.5 h. After removal of the excess of the γ-butyrolactone on a rotating evaporator at 100°C/1 torr, the crude product was purified by chromatography on alumina using a 2% solution of methanol in t-butyl methyl ether as eluant. Removal of the solvent gave a red, oily product, which solidified on cooling to 0°C. Crystallization from ethyl acetate afforded 0.70 g (59%) of pure **6I**, m.p. 100-101°C. Purity control by T.L.C. (Silica gel, t-butyl methyl ether: methanol = 5:1) indicated one compound.

M.S.: m/e = 257(27), 171(100), 154(20), 124(28). IR (KBr): "ν" = 3270, 3220, 2920, 2870, 1640, 1520, 1320, 1220, 1025 cm^{-1}. $C_{13}H_{25}N_2O_3$ (257.35). Calcd: C 60.67% H 9.79% N 10.89%
Found: C 61.09% H 9.92% N 10.74%

Preparation of succinic acid N-(2,2,6,6-tetramethyl-1-oxyl-4-piperidinyl)-monoamide [**6C**]. A solution of succinic anhydride (0.10 g, 1.0 mmol) in ethyl ether (5.0 ml) was added dropwise with vigorous stirring to a solution of **6G** (0.17 g, 1.0 mmol) in ethyl ether (5.0 ml). After the addition, the solution was boiled with reflux for 1 h and stirred for an additional 15 h at 25°C. The pink precipitate was filtered, washed with ethyl ether and dried in a vacuum dessicator at 25°C/1 torr. Recrystallization from a mixture of chloroform and hexane (v/v, 3:1) gave 0.23 g (85%) of pure **6C**, m.p. 152-154°C. Purity control by T.L.C. (Silica gel, chloroform:methanol = 9:1) indicated one product.

M.S.: m/e = 272(36), 257(100). $C_{13}H_{23}N_2O_4$ (271.33). Calcd: C 57.56% H 8.49% N 10.33%
Found: C 57.72% H 8.35% N 10.36%

Preparation of 4-methoxycarbonyl-2,2,6,6-tetramethylpiperidine-1-oxyl. To a solution of the carboxylic acid **6B** (8) (0.15 g, 0.75 mmol) in methanol (5 ml) was introduced at 0°C a freshly prepared ether solution of diazomethane (10 mmol). The reaction was monitored by T.L.C. using silica gel and methylene chloride. After 30 minutes the solvents were removed on a rotating evaporator at 25°/20 torr. The remaining gummy orange solid, 0.158 g (98%), was purified by flash chromatography on Silica gel using methylene chloride as eluant. Removal of the solvent on a rotating evaporator gave a deep orange solid, m.p. 77-80°C (lit. (10) m.p. 80-82°C).

M.S.: m/e = 215(21), 214(66). IR (Nujol) V = 1740 cm^{-1}. $C_{11}H_{20}NO_3$ (214.28)

Preparation of 4-aminocarbonyl-2,2,6,6-tetramethylpiperidine-1-oxyl [**6E**]. A solution of 4-methoxycarbonyl-2,2,6,6-tetramethylpiperidine-1-oxyl (0.150 g, 0.7 mmol) in methanol (5 ml) was saturated with ammonia gas at 0°C. The reaction mixture was stored for 22 h in a refrigerator, saturated once again with ammonia gas and boiled with reflux for 1 h. The reaction was monitored by T.L.C. using silica gel and methylene chloride. The reaction was stopped when additional components to 4-methoxycarbonyl-2,6,6-tetramethylpiperidine-1-oxyl and **6E** appeared. The solvent was removed on a rotating evaporator at 25°/20 torr, and the remaining orange gum was flash chromatographed on silica gel using methylene chloride (100 ml) and methylene chloride: methanol = 9:1 (150 ml). Removal of the solvent from fraction 2-3 (30 ml) gave an orange solid, 0.061 g (40%) m.p. 77-80°C, of the recovered methyl ester 4-methoxycarbonyl-2,6,6-tetramethylpiperidine-1-oxyl. Removal of the solvent from the fractions 9-10 gave light orange crystals, 0.082 g (97%, based on reacted 4-methoxycarbonyl-2,2,6,6-tetramethylpiperidine-1-oxyl) of compound **6E**, m.p. 115-119°C.

M.S.: m/e = 201(15), 200(20). $C_{10}H_{19}N_2O_2$ (199.27). Calcd: C 60.27% H 9.61% N 14.06%
Found: C 59.75% H 10.51% N 14.35%

Preparation of 2,2,5,5-tetramethylpyrrolidine. A mixture of 2,2,5,5-tetramethyl-3-oxopyrrolidine (1.97 g, 0.014 mol), hydrazine hydrate (2.1 ml, 0.042 mol), potassium hydroxide (2.8 g, 0.050 mol) and diethylene glycol monoethyl ether (10 ml) was heated at 135°C until the evolution of nitrogen ceased (14 h). The reflux condenser was then replaced with a distillation condenser and the bath temperature was gradually raised to 195°C. The distillate was saturated with anhydrous potassium carbonate, and the organic layer separated and distilled at atmospheric pressure, collecting a fraction boiling at 105-125°C. This material was redistilled to give 1.3 g (73%) of pure 2,2,5,5-tetramethylpyrrolidine, b.p. 110-115°C (lit. (12): b.p. 108-111°C). Purity control by T.L.C. (Silica gel, ethyl ether: methanol = 9:1) indicated one product.

Preparation of 2,2,5,5-tetramethylpyrrolidine-1-oxyl [**5A**]. Aqueous hydrogen peroxide (30%, 3.0 ml) was added dropwise with stirring at 0°C to a solution of 2,2,5,5-tetramethylpyrrolidine (1.00 g, 7.9 mmol) and sodium tungstate (0.01 g, 0.3 mmol) in aqueous methanol (45%, 8.0 ml). Following the addition, the solution was stirred at 25°C for 24 h, then diluted with water (20 ml), saturated with anhydrous potassium carbonate, and extracted with ethyl ether (4x10 ml). The combined ether extracts were dried with anhydrous magnesium sulfate, filtered and the solvent was removed on a rotating evaporator at 25°C/20 torr. The remaining oily product was purified by flash chromatography on silica gel, using a mixture of chloroform and ethyl acetate (v/v, 9:1) as eluant. Removal of the solvents gave 0.60 g (54%) of pure **5A** as the orange oil with strong camphor-like odor, (lit. (13): oil, m.p. -4°C). Purity control by T.L.C. (Silica gel, chloroform: ethyl acetate = 9:1) indicated one product.

M.S.: m/e = 143(100). $C_8H_{16}NO$ (142.22).

Preparation of 3-methoxalylamino-2,2,5,5-tetramethylpyrrolidine-1-oxyl [**5K**]. A solution of **5G** (0.157 g, 1.0 mmol) and dimethyl oxalate (0.118 g, 1.0 mmol) in methanol (10 ml) was boiled with reflux for 4 h. The solution was then cooled and the solvent was removed on a rotating evaporator at 25°C/20 torr. Recrystallization of the remaining solid from a mixture of chloroform and hexane (v/v, 3:1) gave 0.196 g (81%) of **5K**, m.p. 185-7°C. Purity control by T.L.C. (Silica gel, ethyl ether) indicated one product.

M.S.: M/e = 244(100). $C_{11}H_{19}N_2O_4$ (243.28). Calcd: C 54.32% H 7.82% N 11.52%
Found: C 53.85% H 8.23% N 11.43%

Preparation of oxalic acid N-(2,2,5,5-tetramethyl-1-oxyl-4-pyrrolidinyl) monoamide [**5L**]. A solution of **5K** (0.150 g, 0.66 mmol) and a 10% sodium hydroxide solution (2 ml) in methanol (10 ml) was stirred at 25°C for 24 hr. The reaction mixture was subsequently diluted with water (20 ml) and acidified with 0.1 N hydrochloric acid. The acidified solution was extracted with ethyl ether (3x10 ml). The ether extracts were dried with anhydrous magnesium sulfate, filtered, and the solvent removed on a rotating evaporator at 20°C/20 torr. Purification of the crude **5L** by flash chromatography on silica gel, using ethyl ether as the eluant gave 0.120 g (85%) of pure **5L**, m.p. 230-32°C. Purity control by T.L.C. (Silica gel, ethyl ether) indicated one product.

M.S.: m/e = 230(46), 215(100). $C_{10}H_{17}N_2O_4$ (229.24). Calcd: C 52.40% H 7.42% N 12.23%
Found: C 51.93% H 7.42% N 11.73%

Preparation of succinic acid N-(2,2,5,5-tetramethyl-1-oxyl-3-pyrrolidinyl)-monoamide [**5C**]. To a solution of **5G** (0.157 g, 1.0 mmol) in anhydrous ethyl ether (5.0 ml), was added dropwise a solution of succinic anhydride (0.100 g, 1.0 mmol) in anhydrous ethyl ether (5.0 ml) at 25°C. After the addition, the solution was boiled with reflux for 1 h, followed by stirring for 15 h at 25°C. The solvent was removed on a rotating evaporator at 20°C/20 torr. Purification of the crude, yellow product by flash chromatography on silica gel, using a mixture of ethyl ether and chloroform (v/v, 9:1) as eluant, gave 0.180 g (70%) of pure **5C**, m.p. 170-172°C. Purity control by T.L.C. (Silica

gel, methanol: chloroform = 1:9) indicated one product.

M.S.: m/e = 258(56), 243(100). $C_{12}H_{21}N_2O_4$ (257.29). Calcd: C 56.03% H 8.17% N 10.89%
Found: C 55.82% H 8.10% N 10.67%

Preparation of 3-hydroxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl [5D]. A solution of 5F (0.624 g, 4.0 mmol) and sodium borohydride (0.076 g, 2.0 mmol) in 95% ethanol (15.0 ml) was stirred at 25°C for 8 h, while maintaining the reaction temperature below 25°C. The solvent was then removed on a rotating evaporator at 40°C/20 torr, and the remaining residue was treated with water (10 ml). The water was removed on a rotating evaporator at 65°C/20 torr and the remaining residue was extracted with ethyl ether (3x15.0 ml). The combined ether extracts were washed with water (2.0 ml) and dried with anhydrous magnesium sulfate. Concentration of the ether extracts on a rotating evaporator at 25°C/20 torr gave a yellow solid. Recrystallization from hexane gave 0.600 g (95%) of a yellow, crystalline 5D, m.p. 125-126°C (lit. (10):m.p. 125.5-126°C. Purity control by T.L.C. (Silica gel, ethyl ether) indicated one product.

Reduction of Nitroxyls by Ascorbic Acid: Fresh solutions of 2 mM nitroxyl (in phosphate buffer 0.067 M, pH 7.4) and of 10 mM ascorbic acid (in phosphate buffer 0.067 M, pH 7.4, with and without EDTA 2 mM) were used for these experiments. For each compound, equal volumes of the nitroxyl and ascorbic acid solutions were mixed at time zero and immediately introduced into a 0.04 ml flat cell (Varian Instrument Division) placed within the electron paramagnetic resonance spectrometer cavity. Measurements were made at room temperature (23°C - 26°C) with a Varian E 104A electron paramagnetic resonance spectrometer. The rate of nitroxyl reduction was evaluated by the decrease of height of the low field peak of the first derivative spectrum. This peak was chosen because the ascorbate radical anion interferes with measurements near the middle nitroxyl peak (26).

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