Synthesis of Substituted, 2,2,5,5-Tetramethylpyrrolidin-1-oxyl Spin Labels--*p*H Sensitivity Studies

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The synthesis of derivatives of 2,2,5,5-tetramethylpyrrolidine nitroxide spin labels as *p*H sensitive probes is reported. The data on the *p*H sensitivity studies of these compounds indicate that the amino group increases the *p*H sensitivity of a nitroxide to a greater degree than a cyano, carboxyl or amido group.

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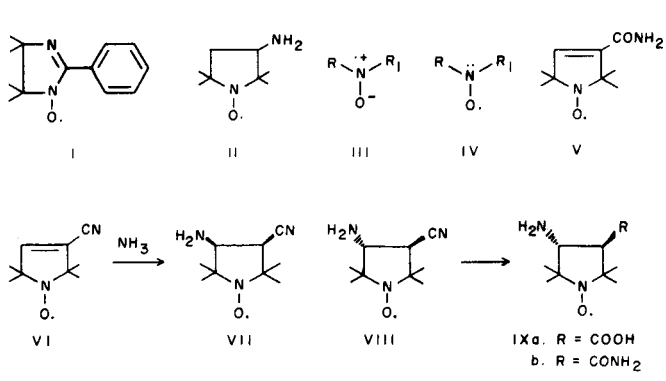
Spin labeling techniques have been used extensively in biological and biochemical investigations to probe structural features of biological systems and to study certain mechanistic aspects of biochemical reactions [2]. Nitroxide free radicals are most frequently used as reporter groups in spin labeling expriments because of their relatively high stability and simple esr spectral features. Nitroxide radicals typically exhibit esr spectra that are sensitive to their local environments. For example, the g factor (g) and hyperfine splitting constant (hfsc, a_N) of nitroxide spin labels vary slightly with the polarity of their environment [3,4]; consequently, nitroxide spin labels can be used to determine whether a given environment is hydrophilic or hydrophobic in nature.

Spin labels that could be used to measure *p*H would be very useful; such *p*H sensitive spin labels should be good probes for measuring the local acidities of biological systems [5]. Spin labels which are sensitive to *p*H, however, are very rare in the literature. Ullman reported an imidazole nitroxide [1] which showed a *p*H change of 3.9 gauss in acidic *p*H's in aqueous medium [6]. However, its esr spectral features are complex due to hyperfine interactions from nitrogen atoms, and therefore, these nitroxides have limited applications as *p*H probes. Reduced *p*H sensitivity was reported for structurally similar imidazolidine nitroxides [7]. Smith *et al.* found that the hyperfine splitting constant a_N of the 2,2,5,5-tetramethyl-3-aminopyrrolidin-1-oxyl nitroxide (II) changes in magnitude by about 0.5 gauss over the *p*H range 2 to 12 [8]. The thrust of the present work was to synthesize *p*H sensitive labels having simpler esr spectra with adequate *p*H sensitivity to monitor biological *p*H.

The presence of certain functional groups, such as amino group and/or a carboxyl group, near a nitroxide moiety in a molecule can be expected to enhance the sensitivity of the nitrogen hfsc of the nitroxide to *p*H changes. In general, the magnitude of the nitrogen hfsc in a nitroxide is dependent upon both the magnetic moment of the nitrogen nucleus and the unpaired electron density at the nitrogen nucleus [2].

In terms of resonance theory, the unpaired electron density at the nitrogen atom in a nitroxide depends upon the relative extent to which resonance structure (III) contributes to the resonance hybrid (III and IV) (Chart 1). On the basis of simple electrostatic considerations, an anionic moiety in the vicinity of the nitroxide nitrogen atom will tend to stabilize resonance structure III (relative to IV), resulting in it being a more important contributor. On the other hand, a cationic moiety placed near the nitroxide nitrogen atom will tend to destabilize structure III making it less important as a contributor. Thus, a nitroxide having a neighboring group that can either accept or lose a proton to become cationic or anionic is expected to exhibit greater variation in its nitrogen hfscs with *p*H than one not substituted in this manner. A nitroxide having two appropriate functional groups near the nitroxide moiety, perhaps amino and carboxyl, such that the nitroxide molecule can exist either in the cationic form or the anionic form (depending upon *p*H), might be particularly *p*H sensitive. That is to say, the magnitude of the nitrogen hfsc might vary appreciably with *p*H. Similarly, a nitroxide containing two basic groups, such as a diaminonitroxide, that can accept two protons at low *p*H to give a dicationic species may also be quite *p*H sensitive.

CHART I



Rassat has reported the synthesis of 2,2,5,5-tetramethyl-3-cyano-4-aminopyrrolidin-1-oxyl (VIII) and 2,2,5,5-tetramethyl-3-carboxyl-4-aminopyrrolidin-1-oxyl (IXa) [11]. The amination of 2,2,5,5-tetramethyl-3-cyanopyrrolidin-1-oxyl (VI) with either aqueous or gaseous ammonia was reported to afford very small amounts of nitroxide VIII. The

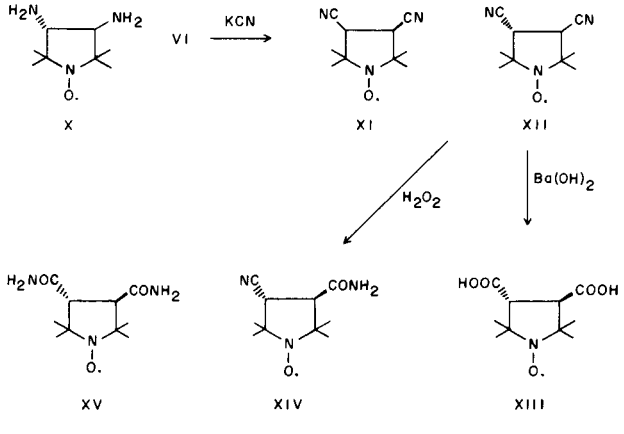
main product in this case was 2,2,5,5-tetramethyl-3-carbamoylpyrrolidin-1-oxyl (V) which is the usual starting material for the synthesis of nitroxide (VI).

We have carried out the amination reaction of 2,2,5,5-tetramethyl-3-cyanopyrrolidin-1-oxyl (VI) using liquid ammonia and isolated a mixture of *trans* and *cis*-2,2,5,5-tetramethyl-3-cyano-4-aminopyrrolidin-1-oxyl (VIII and VII, respectively) in approximately 28 percent yield. The major *trans* isomer mp 84-85° is identical (ir and mp, 85°) to that reported [11]. A larger scale run at atmospheric pressure, however, afforded a poorer yield of these products. The amination of VI with liquid ammonia under high pressure proceeded well and afforded a mixture of nitroxide VIII (65%) and nitroxide VII (15%). The isomers VII and VIII were separated by preparative tlc on silica gel plates and were further purified by crystallization.

The nmr of the major isomer VIII (mp 84-85°) contained a pair of doublets at δ 2.45 and 3.15 due to the methine protons. The nmr of the minor isomer VII (mp 83°) contained an AB quartet (δ 3.05, J_{AB} = 7 Hz) from the methine protons. The *trans*-isomer VIII was subjected to hydrolysis with barium hydroxide and afforded a 54% yield of 2,2,5,5-tetramethyl-3,-carboxyl-4-aminopyrrolidin-1-oxyl (IXa).

Nitroxide VIII was selected for use in the (attempted) synthesis of 2,2,5,5-tetramethyl-3,4-diaminopyrrolidin-1-oxyl (X) (Chart 2), a nitroxide that might be particularly sensitive to *p*H changes. Our initial plan was to hydrolyze the cyano group of VIII to the corresponding amido group, and then to subject the amido-substituted nitroxide to Hofmann degradation to afford the desired nitroxide X. Hydrogen peroxide could not, however, be used for the conversion of the cyano group to the amino group in case of VIII because of the sensitivity of the amino group in VIII toward oxidation by this reagent [12].

CHART 2



The above conversion was thus attempted using methods not involving the use of hydrogen peroxide. Several attempts to effect the above conversion by hydrolysis with hydrochloric acid of varying concentration at several different temperatures did not afford the desired amido nitroxide IXb. At higher temperatures (~95°) and at higher acid concentrations (2*N*), the nitroxide was destroyed.

Alternate routes for the synthesis of nitroxide X as well as 2,2,5,5-tetramethyl-3,4-dicarboxylpyrrolidin-1-oxyl (XIII) were investigated. Refluxing VI with ethanolic potassium cyanide gave a mixture of *cis*- and *trans*-2,2,5,5-tetramethyl-3,4-dicyanopyrrolidin-1-oxyl (XI and XII, respectively), [13]. The major *trans* compound XII (rf 0.65, ethyl acetate-chloroform 1:1) was isolated in 51% yield. The ir of XII contained a strong absorption at 2215 cm^{-1} attributable to the cyano moiety and did not contain C=C absorption. The nmr of the hydroxylamine derivative of XII (the reduced nitroxide) exhibited methine proton absorption as a singlet at δ 3.00 and methyl proton absorption as a pair of overlapping singlets at δ 1.25 and 1.28. The mass spectrum exhibited a molecular ion peak at m/e 192. The minor compound XI (the *cis*-isomer) upon reduction to its corresponding hydroxylamine exhibited nmr absorption at δ 3.15 as a singlet for the methine protons and at δ 1.22 and δ 1.4 as a singlet for the methyl protons. The observation that two separate methyl proton resonances occur in the case of XI provides support for the *cis*-stereochemistry assigned to this compound. *trans*-2,2,5,5-Tetramethyl-3,4-dicarboxylpyrrolidin-1-oxyl (XIII) was obtained upon mild hydrolysis of nitroxide XII with barium hydroxide (mp 222-223°) [13].

The *trans*-isomer XII upon reaction with hydrogen peroxide in the presence of sodium tungstate yielded 2,2,5,5-tetramethyl-3-amido-4-cyanopyrrolidin-1-oxyl (XIV, mp 212-213°) and 2,2,5,5-tetramethyl-3,4-diamidopyrrolidin-1-oxyl (XV, mp 258-260°). Nitroxide XIV exhibited characteristic ir absorptions due to the cyano and amido moieties present and showed the expected parent ion at m/e = 210 in its mass spectrum. The nmr of the hydroxylamine derivative of XIV exhibited two singlets at δ 1.4 and δ 1.57 for the methyl protons and a pair of doublets at δ 3.4 and δ 3.9 (J = 11 Hz) for the methine protons. Nitroxide XV exhibited strong ir absorption at 1660 cm^{-1} because of the amido moieties and lack of any C=N absorption. The mass spectrum of XV showed the expected parent ion at m/e = 228.

Several attempts were made to convert the nitroxide XV into nitroxide X *via* a Hofmann degradation using sodium hypobromite. In all cases, however, no detectable X was formed under the usual conditions of Hofmann degradation and only the starting nitroxide XV was recovered.

All of the above nitroxides that were prepared in pure form were examined for sensitivity of their nitrogen hfsc toward changes in *p*H. Dilute solutions (5×10^{-5} *M*) of

each nitroxide were prepared at different *p*Hs over the *p*H range of 2 to 12, and the magnitude of the nitrogen hfsc was determined for each sample by esr measurements. The results are summarized in Table 1. None of the nitroxides studied exhibited an appreciably greater *p*H sensitivity than does the amino-substituted nitroxide II. The data does indicate that the amino group increases the *p*H sensitivity of a nitroxide to a greater degree than does a cyano, a carboxyl, or an amido group.

Our results, furthermore, indicate that placement of a group such as amino at a position *beta* to the nitroxide moiety is probably too far removed from the nitroxide to have a significant effect upon the spin density at the nitroxide to have a significant effect upon the spin density at the nitroxide nitrogen atom with changing *p*H. Placement of such a group in the *alpha*-position would probably be an improvement in this regard, although the resulting nitroxide without the four methyl groups in the *alpha*- positions may not be very stable. Although the new nitroxides are not promising for *p*H sensitivity studies, they are valuable as functionalized nitroxides for a wide variety of other possible spin label studies.

Table 1

Sensitivity of the Nitrogen hfsc of Some Bifunctionalized Nitroxides Toward *p*H Changes

Nitroxide	a_N [a] (Gauss)
IXa	0.52
XII	0.30
XIII	0.50
XV	0.45

[a] a_N = Change in the magnitude of the nitroxide nitrogen hfsc over the *p*H range 2 to 12.

EXPERIMENTAL

General.

The starting nitroxides were purchased from Eastman Chemical Company. All melting points are uncorrected. The nmr spectra were recorded on a Varian EM-360 or Jeol 100-MHz spectrometer using pyridine-d_5 as solvent and tetramethylsilane as the internal standard. The nmr spectrum of the hydroxylamine derivative of each nitroxide studied was obtained after *in situ* reduction of the nitroxide in an nmr tube using 0.5 equivalent of phenylhydrazine [14]. The ir spectra were recorded on a Perkin-Elmer Model 257-grating infrared spectrophotometer. The esr spectra were recorded on a Varian E-104 spectrometer. The uv spectra were recorded on a Cary 14 uv-vis spectrometer. Preparative tlc was done on a Silicar 7GF (Mallinkrodt).

2,2,5,5-Tetramethyl-3-amino-4-cyanopyrrolidin-1-oxyl (VII and VIII).

a. Synthesis at Atmospheric Pressure.

Nitroxide VI (0.2 g) was added to liquid ammonia (80 ml) and water (20 ml). The mixture was stirred at ambient temperature for 3 days. The reaction mixture was then diluted with brine and extracted with ether. The ether extract was washed with water once and then dried over sodium sulfate. Removal of the solvent afforded a crude material which was chromatographed over alumina (10 g). The unreacted starting material was eluted from the column with benzene. The crude product was then eluted from the column with benzene-ether (5:1). The product was found to be a mixture of *cis*- and *trans*-isomers which were separated by preparative tlc on silica gel plates [solvent: chloroform-ethyl acetate (1:1)].

b. Synthesis at High Pressure.

A mixture of nitroxide VI (2.5 g) in liquid ammonia (20 ml) was placed in a thick-walled glass tube; the glass tube was then immersed in liquid nitrogen and sealed *in vacuo*. The sealed tube was transferred to a high-pressure bomb and then allowed to warm to ambient temperature and then remain at ambient temperature for 60 hours. The tube was carefully opened, and the ammonia was allowed to evaporate. The residue was taken up in water, and the resulting aqueous solution was extracted several times with ether. The ether extract was dried over anhydrous sodium sulfate, and the ether solvent was then removed. The crude material (2.8 g), which remained, was chromatographed over alumina (40 g, 20 cm). Elution with benzene removed a small amount of starting nitroxide. Elution with benzene-ether (5:1) gave a crude product which was crystallized from ether-hexane (1:2). The first crop of crystals contained mainly the *trans*-isomer VIII (1.8 g).

An analytical sample of VIII was prepared by preaparative tlc over silica gel plates using chloroform-ethyl acetate (1:1) followed by crystallization from ether-hexane (1:2) mp 83-84°; ir (chloroform): ν max 2240 cm^{-1}; nmr (deuteriochloroform containing 5 mg of phenylhydrazine): δ 1.0, 1.2, 1.32 (s, 12H, CH_3), 2.45 (d, J = 10 Hz, 1H, C*H*), 3.15 (d, J = 10 Hz, 1H, C*H*), and 2.3 to 3.4 (broad s, 2H, NH_2).

The mother liquor from the crystallization of the major isomer contained mainly the *cis*-isomer VII (which was slower moving by tlc than VIII); nitroxide VII was purified by preparative tlc [chloroform-ethyl acetate (1:1)] followed by crystallization from ether-hexane (1:2) to give 0.51 g of pure VII, mp 82-83°; ir (chloroform): 2215 cm^{-1}; nmr (deuteriochloroform): δ 1.15, 1.2, 1.4 (s, 12H, CH_3), 3.05 (q, J = 7 Hz, 2H, C*H*), and 2.5 to 3.5 (broad s, 2H, NH_2).

Anal. Calcd. for $C_9H_{16}N_3O$: C, 59.31; H, 8.85; N, 23.05. Found: C, 59.00; H, 9.05; N, 23.00.

2,2,5,5-Tetramethyl-3,4-dicyanopyrrolidin-1-oxyl (XI and XII).

A mixture of nitroxide VI (3.2 g), potassium cyanide (3.05 g), ammonium chloride (2.6 g), ethanol (100 ml), and water (100 ml) was heated at 70° for 5 hours. The reaction mixture was cooled, saturated with brine, and then extracted with ether. The ether extact was washed once with brine and then dried over anhydrous sodium sulfate. The ether solvent was removed and the resulting crude reaction product was chromatographed using an alumina column. Elution with benzene-hexane (1:1) removed the unreacted starting material. Further elution with benzene removed the faster-moving *trans*-isomer XI (rf 0.65, ethyl acetate-chloroform 1:1). Subsequent elution with benzene-chloroform (2:1) removed the minor *cis*-isomer XI.

The major isomer XII was purified by crystallization from ether-hexane (1:2) affording 1.91 g (52%) of light yellow crystals, mp 142-144°; ir (chloroform): ν max 2215 cm^{-1}; nmr (deuteriochloroform): δ 1.25, 1.28 (s, 12H, CH_3), 3.0 (s, 2H, C*H*).

Anal. Calcd. for $C_{10}H_{14}N_3O$: C, 62.46; H, 7.34; N, 21.85. Found: C, 62.38; H, 7.35; N, 21.78.

The more polar isomer was crystallized from ether-hexane to afford 0.84 g (22%) of crystals, mp 84°; ir (chloroform): ν max 2220 cm^{-1}; nmr (deuteriochloroform): δ 1.22, 1.42 (s, 12H, CH_3), and 3.15 (s, 2H, C*H*).

Anal. Calcd. for $C_{10}H_{14}N_3O$: C, 62.46; H, 7.34; N, 21.85. Found: C, 62.52; H, 7.46; N, 21.72.

2,2,5,5-Tetramethyl-3,4-dicarboxylpyrrolidin-1-oxyl (XIII).

A mixture of nitroxide XII (0.2 g) and a solution of barium hydroxide (1.5 g) in water (25 ml) was sealed in a thick-walled pyrex tube. The resulting mixture was heated to 120° for 1.5 hours. The tube, upon cooling, was broken open, and excess solid carbon dioxide was then immediately added to the tube. The resulting precipitate was filtered off, and the aqueous filtrate was evaporated. The solid residue was dissolved in eth-

anol and filtered. Removal of solvent *in vacuo* from the filtrate afforded a gummy substance which was crystallized from acetone-benzene to yield 0.13 g of light yellow crystals, mp 222-223°.

Anal. Calcd. for $C_{10}H_{16}NO_5 \cdot \frac{1}{2}H_2O$: C, 46.68; H, 7.44. Found: C, 46.43; H, 7.21.

2,2,5,5-Tetramethyl-3,4-dicarbamoylpyrrolidin-1-oxyl (XV) and 2,2,5,5-Tetramethyl-3-carbamoyl-4-cyanopyrrolidin-1-oxyl (XIV).

To a solution of nitroxide XII (0.3 g) in water (10 ml) and ethanol (10 ml) was added 30% hydrogen peroxide (5 ml) and sodium tungstate (50 mg). The resulting mixture was stirred at room temperature for 2 days. The reaction mixture was then extracted with a mixture of chloroform and ethyl acetate. The combined extract was dried, and the solvent was removed. The aqueous phase was evaporated *in vacuo*, and the residue was extracted with ethanol. The solvent was removed, and the resulting residue was combined with that from above. The combined crude product was subjected to preparative tlc on silica gel plates [solvent chloroform-ethanol (80:20)]. A fast-moving component was eluted with chloroform and then further purified by crystallization from ethanol to afford 123 mg of pure XIV, mp 212-213°; ir (chloroform): ν max 2320 cm^{-1} (C=N), 1675 cm^{-1} ($CONH_2$); nmr (pyridine-d_5): δ 1.4, 1.57 (s, 12H, CH_3), 3.4 (d, J = 11 Hz, 1H, C*H*), 3.9 δ (d, J = 11 Hz, 1H, C*H*); ms: M^+ at m/e = 210.

Anal. Calcd. for $C_{10}H_{16}N_3O_2$: C, 57.11; H, 7.62; N, 19.99. Found: C, 57.23; H, 7.75; N, 19.90.

The slower-moving component was eluted with ethanol containing 1% water. The solvent was removed, and the crude product was crystallized from ethanol-water to afford 65 mg of pure XV, mp 258-260°; ir (potassium bromide): ν max 1680 cm^{-1}; ms: M^+ at m/e = 228.

Anal. Calcd. for $C_{10}H_{18}N_3O_3$: C, 52.61; H, 7.94; N, 18.40. Found: C, 52.80; H, 8.34; N, 18.40.

ESR Measurements of HFSCs.

The esr measurements of the magnitudes of the nitroxide nitrogen hfscs for each nitroxide at different *p*Hs were made at ambient temperature using dilute solutions ($5 \times 10^{-5} M$) of a given nitroxide in different phosphate buffers. A 1×10^{-3} solution of a nitroxide in water was diluted to $5 \times 10^{-5} M$ with a phosphate buffer of appropriate *p*H. The *p*H of the resulting nitroxide solution in each case was checked immediately before the esr spectrometer had earlier been calibrated using galvinoxyl free radical to determine the gauss per cm of esr chart paper at the appropriate scan ranges.

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REFERENCES AND NOTES

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