New mono- and difunctionalized 2,2,5,5-tetramethylpyrrolidine- and Δ^3-pyrroline-1-oxyl nitroxide spin labels

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Several new nitroxide spin labels have been prepared. Nitroxide mesylate **5** and *p*-hydroxyacetophenone gave **6** which was selectively brominated with cupric bromide to give the alkylating agent **7**. The more water soluble phenacyl bromide analogue **17** was prepared either via the route **8** → **11** → **17** or else via the route **15** → **16** → **11** → **17**. Preliminary results indicate that toward aconitase, nitroxide alkylating agent **17** behaves similarly to phenacyl bromide. Several new difunctional nitroxides were prepared with an eye toward application as saturation transfer esr spin labels. Conjugate addition of HCN to **11** gave **18**, condensation of which with *p*-azidobenzaldehyde gave photolabile **19**. Azide **20** could similarly be prepared directly from **11**. Aldehyde **15** underwent condensation with *p*-azidoacetophenone to give azide **21**. This substance was allowed to react with hemoglobin. Upon photolysis the esr spectral mobile component was substantially reduced, suggesting covalent attachment at more than one site. Conjugate addition of HCN to **23** gave a mixture of *cis*, *trans* isomers **24** and **25**; the structure of **25** was established by X-ray crystallographic analysis to be the *trans* isomer. Conjugate addition of ethyl thioglycolate to **15** led to heterocycles **29–34**.

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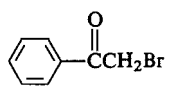
On a préparé plusieurs nouveaux marqueurs de spin de type nitroxyde. Le mésylate nitroxyde (**5**) et la *p*-hydroxyacétophénone donnent le composé **6** que l'on brome sélectivement avec le bromure cuivrique pour accéder à l'agent alkylant **7**. On prépare le bromure de phénacycle **17** analogue, plus soluble dans l'eau, soit selon la voie **8** → **11** → **17** ou selon la voie **15** → **16** → **11** → **17**. Des études préliminaires indiquent que l'agent alkylant nitroxyde **17** se comporte vis à vis de l'aconitase de façon analogue au bromure de phénacyle. On a préparé plusieurs nouveaux nitroxydes bifonctionnels dans le but de les utiliser comme transfert de saturation dans la rpe des marqueurs de spin. L'addition conjuguée du HCN sur le composé **11** conduit au composé **18** dont la condensation sur le *p*-azidobenzaldéhyde donne le composé photolabile **19**. On peut préparer de la même façon l'acide **20** en partant directement du composé **11**. L'aldéhyde **15**, par condensation sur le *p*-azidoacétophénone, conduit à l'azoture **21**. On a fait réagir ce dernier avec l'hémoglobine. Lors de la photolyse, le spectre rpe du composant mobile est réduit de façon significative, suggérant ainsi une liaison covalente en plusieurs endroits. L'addition conjuguée de HCN sur le composé **23** donne un mélange des isomères *cis* et *trans*, **24** et **25**; on a établi par cristallographie de rayons-X que le composé **25** est l'isomère *trans*. L'addition conjuguée du thioglycolate d'éthyle sur le composé **15** conduit aux hétérocycles **29** à **34**.

[Traduit par le journal]

The nitroxide spin labeling method constitutes a productive approach to the study of biological and other macromolecular systems by electron spin resonance (esr) spectroscopy (1). Central to this method is the availability of an array of functionalized stable nitroxide molecules which may be selectively attached to reactive sites on the molecule to be spin labeled (2). In connection with an ongoing collaborative investigation involving the non-heme iron–sulfur enzyme aconitase (EC 4.2.1.3), we required a series of nitroxide alkylating agents which were similar in reactivity to that of phenacyl bromide (**1**). Phenacyl bromide is known to react stoichiometrically with a single SH group near the active site of aconitase, leading to a loss of enzymatic activity (3). The first objective of this paper is to report the synthesis and some preliminary results with two new nitroxide alkylating agents modeled after phenacyl bromide.

The second objective of this paper is the development of several new hetero- and homodifunctionalized nitroxide spin labels. Difunctional spin



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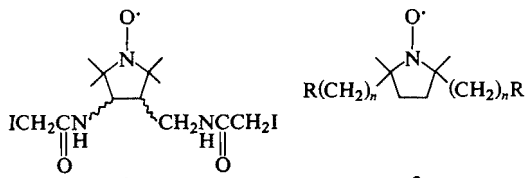
labels have the possibility of becoming rigidly attached to a macromolecule through simultaneous or sequential covalent bonding at more than one site. By essentially confining the motion experienced by such a label to that of the macromolecule itself, molecular motion of the macromolecule in the correlation time range $10^{-7} < \tau < 10^{-3}$ s may be conveniently studied using the relatively new saturation transfer electron paramagnetic resonance (STEPR) method (4, 5). Studies of protein motion in this time range are of considerable current interest owing to its possible functional significance (5, 6). Those relevant difunctional nitroxides already described are relatively few and include nitroxides **2** (7), **3** (8), and **4** (9).



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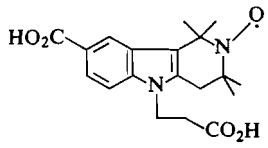

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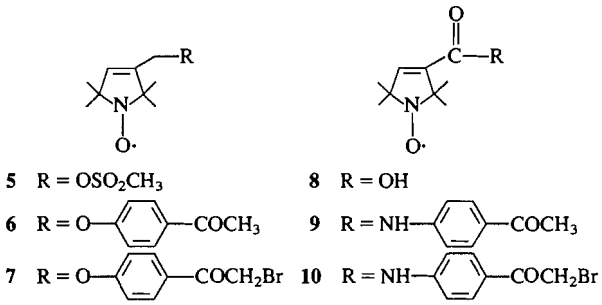
Results and discussion

Our first objective was to prepare several representative spin labeled phenacyl bromide analogues for subsequent studies with aconitase. Thus, nitroxide mesylate **5** (10) was allowed to react with *p*-hydroxyacetophenone in the presence of sodium hydroxide to give ketone **6** (52%), mp 101–102°C. Selective bromination of **6** was effected with cupric bromide (11), affording nitroxide phenacyl bromide **7** (14%), mp 73–74°C. The low yield of **7** resulted at least partially because of the difficulty in separating (by preparative tlc) the bromo derivative from the starting ketone.

Preliminary experiments with **7** and aconitase[2] were not promising owing to the low aqueous solubility of **7**, even in the presence of tolerable levels of organic cosolvents. For an alternative approach we prepared amide **9** (86%), mp 165–166°C, by reaction of the acid chloride of nitroxide acid **8** with *p*-aminoacetophenone. Unfortunately, initial attempts to prepare bromo derivative **10** led to intractable mixtures of many products.

Success was achieved through the synthesis of nitroxide bromoketone **17**. It was anticipated that the overall shape, solubility, polarity, and chemical reactivity of **17** might better approximate that of phenacyl bromide as compared with **7**. The precursor of **17**, nitroxide ketone **11**, was prepared in two ways. Firstly, nitroxide acid **8** was allowed to react with excess methyllithium (12), affording after air oxidation a readily separable mixture of ketone **11**, mp 71–72°C (18%) and *N*-methoxy derivative **13** (19%) as a colorless oil.

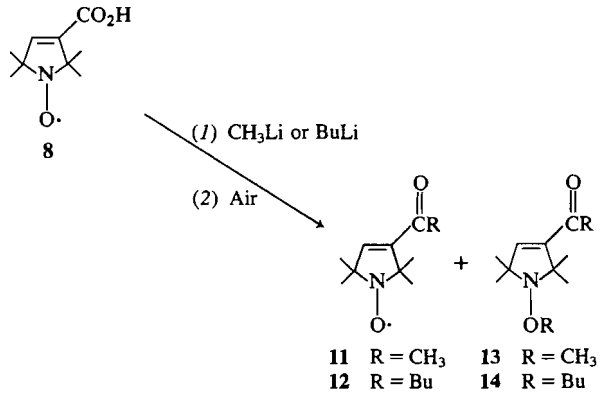


5 R = OSO_2CH_3 **8** R = OH

6 R = O—$COCH_3$ **9** R = NH—$COCH_3$

7 R = O—$COCH_2Br$ **10** R = NH—$COCH_2Br$

The successful reaction of a nitroxide carboxylic acid with methyllithium to produce (after reoxidation) a nitroxide methyl ketone is of special interest in view of the known rapid reaction of butyllithium with the nitroxide moiety of 2,2,6,6-tetramethylpiperidine-1-oxyl to give the corresponding N—H, N—OH, and N—OBu derivatives (13). Our results with **8** demonstrate that even though reaction at the nitroxide moiety of **8** does occur with methyllithium, nevertheless through use of excess reagent,



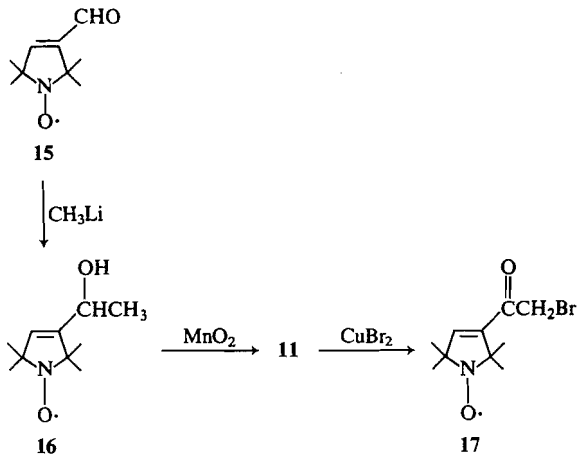
11 R = CH_3 **13** R = CH_3

12 R = Bu **14** R = Bu

the carboxyl group reacts in the expected manner to give the methyl ketone. In order to check the generality of this procedure, acid **8** was allowed to react with excess butyllithium. Useful amounts of the analogous product, nitroxide ketone **12** (oil, 24%), and *N*-butoxy derivative **14** (oil, 23%) were produced after air oxidation of the reaction mixture.

The versatile (see below) intermediate ketone **11** was also prepared by a second method. Reaction of

[2]Prof. H. Beinert, University of Wisconsin, Madison, WI, private communication.

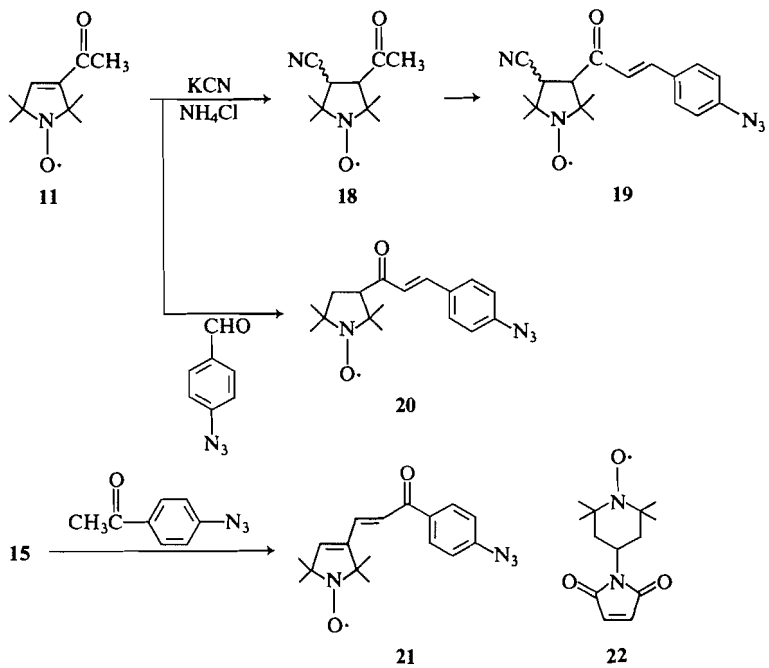


methyllithium with aldehyde **15** (14, 15) gave alcohol **16**, mp 80–81°C (45%). Oxidation of **16** with activated manganese dioxide afforded **11** in 83% yield.

Selective bromination of **11** with cupric bromide proceeded somewhat better than in the case of **6**, affording nitroxide bromoketone **17**, mp 67–69°C, in 25% yield (adjusted for recovered **11**). Preliminary experiments[2] demonstrate that treatment of aconitase with **17** under conditions similar to those employed with phenacyl bromide (3) likewise results in inactivation of the enzyme. These results and associated biophysical studies will be reported in detail later.

We next turned to the synthesis of several difunctional nitroxides while at the same time exploring some of the chemistry of ketone **11**, nitrile **23**, and aldehyde **15** (14, 15). The initial plan was to introduce one of the functions by way of a 1,4-conjugate addition reaction. The reaction of cyanide ion with **11** and **23** was first investigated with the ultimate aim of converting the cyano group into either an activated carbonyl group or else an imidate, for example. Thus, treatment of ketone **11** with potassium cyanide – ammonium chloride – DMF readily afforded the adduct **18** (29%), mp 104–107°C, likely as a mixture of stereoisomers. The adduct smoothly underwent a base-catalyzed condensation reaction with *p*-azidobenzaldehyde, giving phenylazidocyanonitroxide **19**, mp 110–114°C (41%). Alternatively, ketone **11** could be condensed directly with the azidoaldehyde, affording nitroxide **20**, mp 138–141°C (dec.) (47%). Also, aldehyde **15** underwent condensation with *p*-azidoacetophenone to give the α,β,γ,δ-unsaturated ketone **21**, mp 135–136°C (77%).

Nitroxides **19**, **20**, and **21** constitute a potentially versatile series of difunctional spin labels. Each is capable of reacting with a protein SH or NH_2 group in a 1,4-conjugate addition reaction analogous to the reaction of maleimide spin label **22** (16) with proteins. While only a single point of covalent attachment to the protein is possible with label **22**, a second point of attachment may be introduced by photolysis of protein adducts of **19**, **20**, and **21**. The reactive phenylnitrene intermediate so generated



should be capable of forming a covalent bond with an adjacent protein residue (or the solvent (17)).

In order to test this approach nitroxide **21** and maleimide label **22** were attached covalently to hemoglobin under identical conditions. The top spectrum in Fig. 1 is a conventional esr spectrum of **21** bound to hemoglobin. The spectrum is dominated by a large bound component, but a small amount of mobile component (indicated by the arrows) is also evident. After photolysis (bottom spectrum of Fig. 1) the mobile component was reduced substantially. The amount of mobile component in the maleimide nitroxide **22**-labeled preparation was comparable to that of **21**-labeled hemoglobin before photolysis. After photolysis, however, the esr spectrum of maleimide **22**-labeled hemoglobin was unchanged.

It is useful to compare the splitting between the low field maximum and the high field minimum ($2A_{max}$) for hemoglobin labeled with the maleimide label **22** and difunctional nitroxide **21** (after photolysis). At 25°C $2A_{max}$ for maleimide-labeled hemoglobin is 66.3 gauss while for hemoglobin labeled with nitroxide **21**, $2A_{max}$ is 69.9 gauss. This difference could result from either polarity differences (a more polar environment would cause $2A_{max}$ to be larger) or from the maleimide label undergoing a small amount of molecular motion independent of that of the protein. By obtaining esr spectra at −196°C where molecular motion is negligible, any differences in $2A_{max}$ should be due to polarity effects alone. The esr spectra recorded at −196°C of hemoglobin labeled with the two spin labels are essentially the same, indicating that differences in polarity are not significant. We conclude, therefore, that differences in $2A_{max}$ in the room temperature esr spectra of these labels covalently bound to hemoglobin are due to nitroxide **21** being more motion-restricted than the maleimide label.

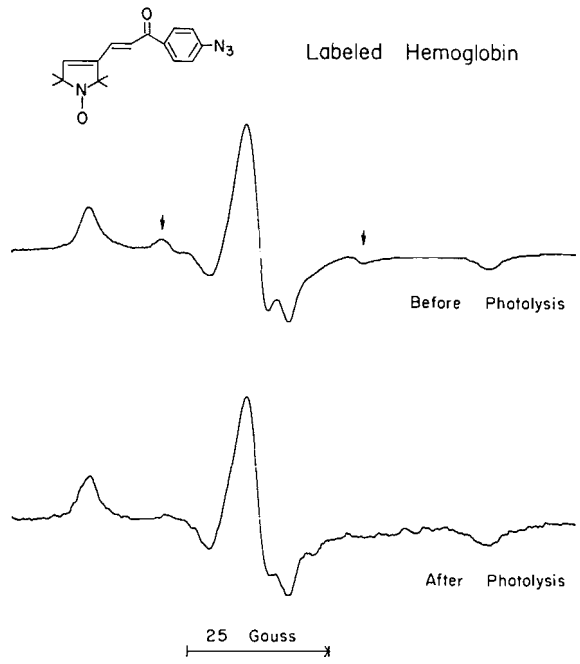
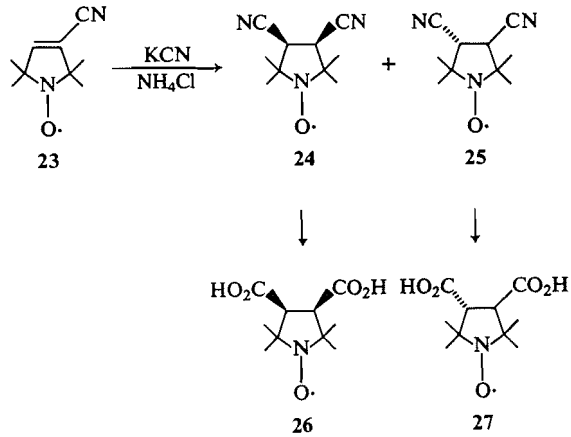


FIG. 1. The 25°C esr spectra of hemoglobin covalently labeled with nitroxide **21** before and after photolysis. The arrows in the top spectrum indicate the mobile component, the relative amount of which is reduced when the preparation is exposed to visible light.

With the 1,4-addition of potassium cyanide to ketone **11** in hand, we next investigated the corresponding reaction with unsaturated nitrile **23**. A separable mixture of *cis* dinitrile **24**, mp 85°C (21%) and *trans* dinitrile **25**, mp 146–147°C (18%) was produced. Vigorous hydrolysis of **24** and **25** with aqueous sodium hydroxide gave acids **26**, mp >250°C, and **27**, mp 220°C, respectively.



Initially, we had planned to establish the stereochemistry of **24** and **25** through formation of the cyclic anhydride from the *cis* acid **26**. Preliminary attempts to form a cyclic anhydride from either isomer using for example, heat, hot acetic anhydride, or dicyclohexylcarbodiimide were not promising. Eventually, we turned to an X-ray crystallographic analysis of the higher melting dinitrile isomer **25** which unambiguously establishes that it has the *trans* configuration (Fig. 2). The crystal structure (Fig. 3) contains discrete molecules separated by normal van der Waals distances. Molecular dimensions are in Table 1. The N—O distance, 1.269(4) Å, is within the usual range for unconjugated radicals (18) and other distances (N—Csp^3 1.476 and 1.484(4), Csp^3—Csp^3 1.503–1.555(4), Csp^3—C(N) 1.468 and 1.488(5), C≡N 1.104 and 1.109(4) Å) are unexceptional. The five membered ring adopts a slightly distorted envelope conforma-

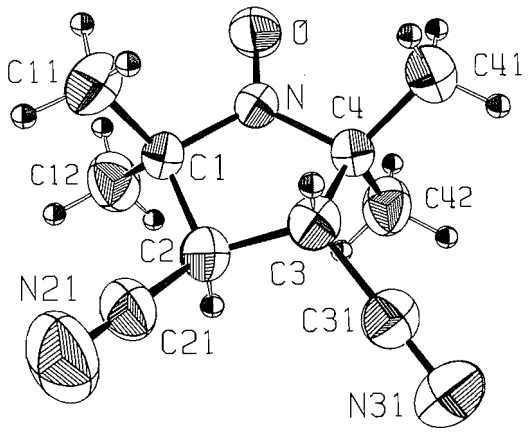


FIG. 2. View of molecule **25** showing the *trans*-configuration of the nitrile groups.

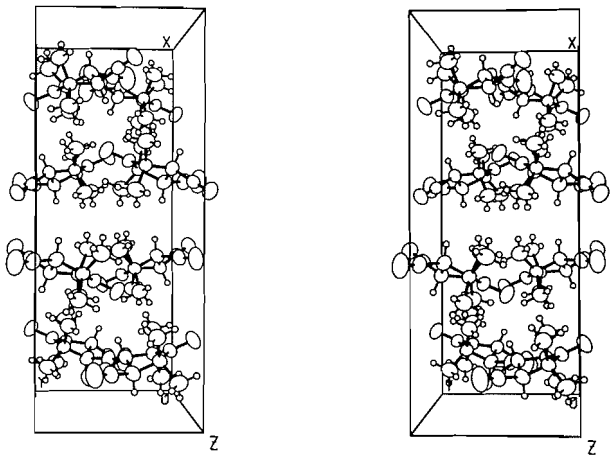
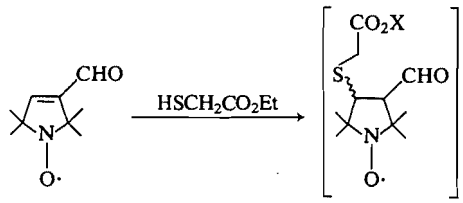


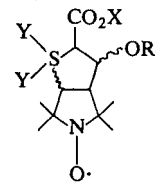
FIG. 3. Stereoview of the crystal structure of **25**.

tion with C(3) (see Fig. 2 for crystallographic numbering scheme) at the flap.

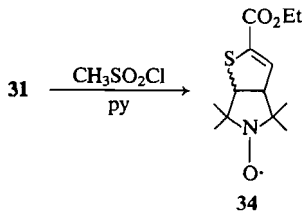
The 1,4-conjugate addition of sulfur reagents to nitroxide acceptors was particularly interesting owing to the generally excellent nucleophilicity of sulfur and also the presence of free reactive sulfhydryl groups on many proteins. Preliminary model experiments involving the addition of sodium phenylsulfinate to aldehyde **15** were not encouraging. Reaction of **15** with ethyl thioglycolate, however, led to the sulfur heterocycle **29**, mp 86–88°C (49%) (mixture of stereoisomers), likely through the intermediacy of adduct **28**. Preparative tlc of **29** followed by two recrystallizations of one of the bands gave a single pure isomer, mp 138–139°C.

Several other heterocycles were prepared from the stereoisomeric mixture **29**. Acetylation of **29** gave acetate **31**, mp 126–127°C (82%), while hydrogen peroxide oxidation of **29** gave sulfone **32**, mp





29 R = H; X = Et; Y = :
30 R = H; X = Me; Y = :
31 R = Ac; X = Et; Y = :
32 R = H; X = Et; Y = O
33 R = X = H; Y = :



156–157°C (63%). Reaction of **29** with methanesulfonyl chloride in pyridine gave olefin **34**, mp 113–116°C, (74%) as the major product, confirming the β-hydroxyester formulation for **29**. Reaction of **15** with methyl thioglycolate gave ester **30**, mp 136–137°C (44%), hydrolysis of which led to the acid **33**, mp 212–213°C (81%).

Experimental

Infrared spectra were recorded with either a Beckman IR-5 or IR-7 spectrometer. The nmr spectra were recorded on a Varian XL-100 high resolution spectrometer in $CDCl_3$ and only the characteristic peaks are reported. Chemical shifts are reported in parts per million (δ) downfield from internal Me_4Si; mass spectra (70 eV) (*m/e*) were determined on a CEC 110-2B double-focusing mass spectrometer equipped with a direct inlet. Elemental analyses were performed at the University of Oregon by Dr. R. Wielesek. Ultraviolet spectra were determined on a Cary 15 uv spectrometer. Silica gel column chromatography was done with Baker 60-200 mesh silica gel. Preparative thin layer chromatography (tlc) was done on either Analtech 1000 μ silica gel GF 254 or Whatman PKF6 plates. Melting points were recorded on a Thomas–Hoover apparatus. Solvents were routinely distilled.

1-Oxyl-2,2,5,5-tetramethyl-3-(4-acetylphenoxymethyl)pyrroline (6)

To a stirred solution of 4-hydroxyacetophenone (150 mg, 1.10 mmol) and nitroxide mesylate **5** (10) (248 mg, 1.00 mmol) in MeOH (5 mL) was added a solution of Na_2CO_3 (100 mg) in water

TABLE 1. Interatomic distances and angles

Bond	Distance (Å)	Bond	Angle (°)
O—N	1.269(4)	O—N—C(1)	121.7(3)
N—C(1)	1.476(4)	O—N—C(4)	121.2(3)
N—C(4)	1.484(4)	C(1)—N—C(4)	117.0(2)
C(1)—C(2)	1.538(4)	N—C(1)—C(2)	99.8(2)
C(1)—C(11)	1.514(4)	N—C(1)—C(11)	109.7(3)
C(1)—C(12)	1.503(4)	N—C(1)—C(12)	110.0(3)
C(2)—C(3)	1.514(5)	C(2)—C(1)—C(11)	115.5(3)
C(2)—C(21)	1.468(5)	C(2)—C(1)—C(12)	110.3(3)
C(3)—C(4)	1.555(5)	C(11)—C(1)—C(12)	110.9(3)
C(3)—C(31)	1.488(5)	C(1)—C(2)—C(3)	105.0(3)
C(4)—C(41)	1.504(4)	C(1)—C(2)—C(21)	113.5(3)
C(4)—C(42)	1.512(4)	C(3)—C(2)—C(21)	114.1(3)
N(21)—C(21)	1.104(4)	C(2)—C(3)—C(4)	104.1(3)
N(31)—C(31)	1.109(4)	C(2)—C(3)—C(31)	114.6(3)
		C(4)—C(3)—C(31)	113.3(3)
Ring torsion angles (°)		N—C(4)—C(3)	97.4(2)
		N—C(4)—C(41)	111.1(3)
C(4)—N—C(1)—C(2)	−3.2	N—C(4)—C(42)	109.7(3)
N—C(1)—C(2)—O—C(3)	27.0	C(3)—C(4)—C(41)	111.2(3)
C(1)—C(2)—C(3)—C(4)	−41.4	C(3)—C(4)—C(42)	115.7(3)
C(2)—C(3)—C(4)—N	36.3	C(41)—C(4)—C(42)	111.0(3)
C(3)—C(4)—N—C(1)	−20.7	N(21)—C(21)—C(2)	179.3(4)
		N(31)—C(31)—C(3)	177.6 (4)

(3 mL). After a 3 h stir at 25°C, the precipitated product was collected and washed with water, affording 150 mg (52%) of nitroxide **6** as long yellow needles. Recrystallization from MeOH/water afforded the analytical specimen, mp 101–102°C; esr ($CHCl_3$) 3 lines (a_n = 15.0 G); ir (Nujol): 1690 and 1610 cm^{-1}. *Anal.* calcd. for $C_{17}H_{22}NO_3$: C 70.80, H 7.69, N 4.86; found: C 70.91, H 7.76, N 4.84.

1-Oxyl-2,2,5,5-tetramethyl-3-(4-bromoacetylphenoxymethyl)-pyrroline (7)

To a well stirred solution of ketone **6** (86 mg, 0.30 mmol) in ethyl acetate/$CHCl_3$ (dry) (1:1) (20 mL) was added $CuBr_2$ (134 mg, 0.600 mmol). The mixture was refluxed for 5 h and then filtered. The filtrate was treated briefly with activated charcoal, filtered, and evaporated to dryness. Preparative tlc (Whatman) (ethyl acetate/hexanes (1:1)) gave two major bands which were eluted with ethyl acetate: R_f 0.56 amounted to 45 mg (52%) of unreacted ketone **2**: R_f 0.65 amounted to 15 mg (14%) of desired crystalline **7**. Recrystallization from ether/hexanes gave the analytical specimen, mp 73–74°C; esr ($CHCl_3$) 3 lines (a_n = 15.0 G); ms *m/e*: 368 (8), 366.070 (10) (calcd. for $C_{17}H_{21}NO_3Br$, 366.071), 288 (14), 168 (32), 149 (55), 138 (90), 123 (100), 122 (40), 107 (40).

1-Oxyl-2,2,5,5-tetramethylpyrroline-3-(N′-p-acetylphenyl)-carboxamide (9)

To a stirred solution of 1-oxyl-2,2,5,5-tetramethyl-3-carboxypyrroline (**8**) (3.68 g, 20.0 mmol) in benzene (20 mL) and dry pyridine (5 mL) at 0°C was added dropwise a solution of thionyl chloride 3.0 g (0.025 mmol) in benzene (10 mL). After 30 min a solution of 4-aminoacetophenone (2.7 g, 20 mmol) in benzene (50 mL) was added dropwise and the resulting solution was refluxed for 1 h. The cooled reaction mixture was extracted with 1 *N* HCl followed by 10% aqueous K_2CO_3 and water. Concentration of the dried (Na_2SO_4) benzene layer gave a yellow oil which was triturated in ether. The resulting solid was filtered, affording 5.2 g (86%) of crude **9** suitable for further reactions. Recrystallization of a portion from $CHCl_3$/hexanes afforded the analytical specimen, mp 165–166°C; ir (Nujol): 3125, 1670, and 1590 cm^{-1}. *Anal.* calcd. for $C_{17}H_{21}N_2O_3$: C 67.75, H 7.02, N 9.30; found: C 67.54, H 7.27, N 9.28.

1-Oxyl-2,2,5,5-tetramethyl-3-acetylpyrroline (11) and 1-methoxy-2,2,5,5-tetramethyl-3-acetylpyrroline (13)

To a stirred suspension (N_2) of acid **8** (552 mg, 3.00 mmol) in ether (10 mL) was added dropwise 5.0 mL (7.5 mmol) of a 1.5 *M* solution of MeLi in ether at a rate such that a gentle reflux was maintained. After addition was complete, the mixture was quenched by dropwise addition of saturated aqueous NH_4Cl. The mixture was diluted with brine and extracted with ether. The almost colorless ether phase was dried (Na_2SO_4) and concentrated, affording a pale yellow oil. In order to facilitate the oxidation of the *N*-hydroxy intermediate, the oil was dissolved in $CHCl_3$ (15 mL) and vigorously stirred under air for 12 h. Evaporation of the solvent gave 310 mg of a viscous yellow oil which was subjected to preparative tlc (Analtech) (ethyl acetate/hexanes (1:1)). The band at R_f 0.93 was *N*-methoxy ketone **13**, obtained as a colorless oil; 110 mg (19%); ir (film): 1675 and 1622 cm^{-1}; nmr δ: 1.30 (s, 6), 1.38 (s, 6), 2.30 (s, 3), 3.72 (s, 3), 6.42 (s, 1); ms *m/e*: 197.142 (11) (calcd. for $C_{11}H_{19}NO_2$, 197.142), 196 (19), 182 (72), 136 (100), 105 (20).

The band at R_f 0.75 afforded nitroxide **11** as yellow needles (98 mg, 18%) from hexanes, mp 71–72°C; esr ($CHCl_3$) 3 lines (a_n = 14.8 G); ir (Nujol): 1675 and 1620 cm^{-1}; ms *m/e*: 183 (6), 182.118 (28) (calcd. for $C_{10}H_{16}NO_2$, 182.118), 152 (53), 138 (11), 137 (100), 126 (16), 110 (16), 109 (53). *Anal.* calcd. for $C_{10}H_{16}NO_2$: C 65.90, H 8.85, N 7.69; found: C 65.82, H 8.90, N 7.73.

1-Oxyl-2,2,5,5-tetramethyl-3-butanoylpyrroline (12) and 1-butoxy-2,2,5,5-tetramethyl-3-butanoylpyrroline (14)

Preparation of **12** and **14** was similar to that of **11** and **13**. From acid **8** (184 mg, 1.00 mmol) and 3.0 mL (4.8 mmol) of 1.6 *M* butyllithium in hexane, there was obtained after preparative tlc 65 mg (23%) of oily *N*-butoxy ketone **14** (R_f 0.95; ir (film): 1675 and 1623 cm^{-1}; nmr δ: 2.63 (t, 2), 3.86 (t, 2), 6.20 (s, 1); ms *m/e*: 282 (10), 281.237 (36) (calcd. for $C_{17}H_{31}NO_2$, 281.235), 267 (28), 266 (100), 210 (30), 182 (57), 126 (15), 109 (12)) and 53 mg (24%) of yellow oily nitroxide **12** (R_f 0.73; esr ($CHCl_3$) 3 lines (a_n = 14.8

G); ir (film): 1675 and 1620 cm^{-1}; ms *m/e*: 226 (25), 225 (29), 224.165 (59) (calcd. for $C_{13}H_{22}NO_2$, 224.165), 210 (61), 194 (59), 179 (25), 137 (100), 126 (32), 110 (33), 109 (79)). The pale yellow aqueous phase from the original work-up of this reaction was acidified (pH 3) and extracted with $CHCl_3$. From the extract there was obtained 25 mg (14%) of the starting acid **8**.

1-Oxyl-2,2,5,5-tetramethyl-3-(1-hydroxyethyl)pyrroline (16)

To a stirred solution of nitroxide aldehyde **15** (14, 15) (505 mg, 3.00 mmol) in dry ether (15 mL) at 25°C was added dropwise 4.0 mL (3.9 mmol) of a 1.3 *M* solution of MeLi in ether. After 30 min the reaction was quenched by addition of saturated aqueous NH_4Cl (1 mL) followed by brine (20 mL). The mixture was extracted with ether. The extract was dried (Na_2SO_4) and concentrated. The pale yellow residue was dissolved in $CHCl_3$ (20 mL) and stirred under air for 12 h (solution now deep yellow). The solvent was evaporated and the residue was crystallized from ether–hexane (0°C), affording 250 mg (45%) of crystalline **16**, suitable for further reactions. Recrystallization from $CHCl_3$/hexanes afforded the analytical specimen, mp 80–81°C; ir (Nujol): 3400 cm^{-1}. *Anal.* calcd. for $C_{10}H_{18}NO_2$: C 65.18, H 9.85, N 7.60; found: C 64.70, H 9.80, N 7.68.

The mother liquors were combined, evaporated, and the residue was dissolved in $CHCl_3$ (30 mL). Active MnO_2 (3 g) was added to the stirred solution and the mixture was refluxed for 3 h and then filtered. The filtrate was concentrated and then subjected to preparative tlc (Analtech) (ethyl acetate/hexanes, 1:1). There was obtained 120 mg (20%) of *N*-methoxy ketone **13** and 30 mg (6%) of nitroxide ketone **11**.

Alternatively, pure nitroxide alcohol **16** (46 mg, 0.25 mmol) was dissolved in $CHCl_3$ (10 mL) and treated with active MnO_2 (0.5 g). The mixture was stirred under reflux for 3 h (monitored by tlc) and filtered. The filtrate was subjected to preparative tlc, affording 38 mg (83%) of ketone **11**, mp 71–72°C.

1-Oxyl-2,2,5,5-tetramethyl-3-(bromoacetyl)pyrroline (18)

To a stirred solution of ketone **11** (91 mg, 0.50 mmol) in ethyl acetate/$CHCl_3$ (20 mL) (1:1) was added powdered $CuBr_2$ (223 mg, 1.00 mmol). The mixture was refluxed for 7 h after which period the black $CuBr_2$ was nearly completely replaced by a white precipitate of CuBr. The green supernatant was treated with activated charcoal and filtered. The filtrate was evaporated to dryness and the residue was subjected to preparative tlc (Whatman), giving two major bands which were eluted with ethyl acetate: R_f 0.43 amounted to 35 mg (38%) of unreacted ketone **11**. R_f 0.55 amounted to 20 mg (15%) of desired bromo ketone **17**, mp 67–69°C; esr ($CHCl_3$) 3 lines, a_n = 14.7 G; ms *m/e*: 262 (32), 260.029 (32) (calcd. for $C_{10}H_{15}BrNO_2$, 260.029), 152 (24), 151 (100), 137 (38), 110 (24), 109 (67).

1-Oxyl-2,2,5,5-tetramethyl-3-acetyl-4-cyanopyrroline (18)

A mixture of ketone **11** (91 mg, 0.50 mmol) KCN (65 mg, 1.0 mmol), and NH_4Cl (59 mg, 1.1 mmol) was dissolved in water–ethanol (10 mL, 8:2) and refluxed for 2 days. Brine (20 mL) was added and the mixture was extracted with ether. The extract was dried (Na_2SO_4), evaporated, and the residue was subjected to preparative tlc (Analtech) ($CHCl_3$/ether, 1:1). Starting ketone **11** (15 mg, 16%) was recovered from the upper band. The lower band afforded 30 mg (29%) of adduct **18**, likely as a mixture of stereoisomers, mp 104–107°C; ir ($CHCl_3$): 2230 and 1720 cm^{-1}; ms *m/e*: 210 (7), 209.128 (26) (calcd. for $C_{11}H_{17}N_2O_2$, 209.129), 195 (16), 179 (45), 135 (22), 110 (100). *Anal.* calcd. for $C_{11}H_{17}N_2O_2$: C 63.13, H 8.19, N 13.39; found: C 62.51, H 8.64, N 13.15.

1-Oxyl-2,2,5,5-tetramethyl-3-(4-azidocinnamoyl)pyrroline (20)

To a stirred solution of ketone **11** (109 mg, 0.600 mmol) in MeOH (3 mL) (protected from light) was added aqueous NaOH (0.3 mL, 1 *N*), followed by a solution of 4-azidobenzaldehyde (97 mg, 0.66 mmol) in MeOH (1 mL). During the addition the color became deep orange. Afte 2 h the precipitated yellow crystals were collected, washed with MeOH–water, and dried in the dark, affording 87 mg (47%) of azide **20**, mp 138–141°C (dec.); ir (Nujol): 2100 and 1655 cm^{-1}; ms *m/e*: 312 (46), 311.150 (100) (calcd. for $C_{17}H_{19}N_4O_2$, 311.151), 297 (62), 253 (56), 238 (50), 210 (27), 146 (62), 144 (81), 125 (56), 116 (100), 109 (42), 108 (30), 106 (42).

1-Oxyl-2,2,5,5-tetramethyl-3-(4-azidocinnamoyl)-4-cyanopyrroline (19)

The above procedure was followed. From cyano ketone **18** (52 mg, 0.25 mmol) and 4-azidobenzaldehyde (37 mg, 0.25 mmol) there was obtained 35 mg (41%) of yellow crystalline **19**, mp 110–114°C (dec.); ir (Nujol): 2245, 2210, 1685, 1655, and 1595 cm^{-1}; ms *m/e*: 339 (11), 338.161 (19) (calcd. for $C_{18}H_{20}N_5O_2$, 338.162), 172 (26), 146 (35), 145 (21), 144 (100), 116 (58), 110 (22).

1-Oxyl-2,2,5,5-tetramethyl-3-(2-(4-azidobenzoyl)ethenyl)-pyrroline (21)

To a stirred solution of aldehyde **15** (16.8 mg, 0.100 mmol) in MeOH (1.0 mL) (protected from light) was added a solution of 4-azidoacetophenone (16.1 mg, 0.100 mmol) in MeOH (3.0 mL) followed by 1.0 *N* NaOH (0.1 mL). After a 3 h stirring period at 25°C the precipitated yellow crystalline plates were collected and washed with aqueous MeOH, affording 24 mg (77%) of crystalline ketone **21**, mp 125–129°C. Recrystallization from aqueous MeOH afforded the analytical specimen: mp 135–136°C; esr ($CHCl_3$) 3 lines (a_n = 14.7 G); ir (Nujol): 2120, 1665, and 1610 cm^{-1}; ms *m/e*: 312 (36), 311.151 (100) (calcd. for $C_{17}H_{19}N_4O_2$, 311.151), 297 (23), 283 (14), 269 (22), 253 (26), 238 (43), 225 (48), 212 (40), 210 (28), 182 (14), 135 (20), 120 (41), 105 (18). *Anal.* calcd. for $C_{17}H_{18}N_4O_2$: C 65.57, H 6.15, N 18.00; found: C 65.62, H 6.23, N 17.98.

1-Oxyl-2,2,5,5-tetramethyl-3,4-dicyanopyrrolidine cis, trans *isomers (24 and 25)*

A mixture of nitrile **23** (165 mg, 1.00 mmol), KCN (130 mg, 2.00 mmol), and NH_4Cl (107 mg, 2.00 mmol) was dissolved in DMF (3 mL)/water (30 mL), heated at 70°C for 3 h, and then set aside at 25°C for 48 h. Brine (15 mL) was added and the mixture was extracted with ether. The extract was dried (Na_2SO_4) and evaporated to dryness. Preparative tlc (Analtech) of the residue gave three major bands ($CHCl_3$/ether, 1:1): R_f 0.80 amounted to 43 mg (26%) of starting **23**. R_f 0.63 amounted to 35 mg (18%) of yellow crystalline *trans* dinitrile isomer **25**, mp 146–147°C ($CHCl_3$/hexane); ir (Nujol): 2220 cm^{-1}; ms *m/e*: 193 (11), 192.113 (59) (calcd. for $C_{10}H_{14}N_3O$, 192.114), 179 (4), 178 (35), 177 (31), 163 (6), 162 (46), 83 (11), 82 (100), 81 (73). *Anal.* calcd. for $C_{10}H_{14}N_3O$: C 62.48, H 7.34, N 21.86; found: C 61.82, H 7.37, N 21.36. R_f 0.37 amounted to 40 mg (21%) of the *cis* isomer **24** which slowly crystallized, mp 85°C; ir (film): 2220 cm^{-1}; ms *m/e*: 193 (3), 192.113 (15) (calcd. for $C_{10}H_{14}N_3O$, 192.114), 178 (18), 162 (16), 85 (28), 83 (100), 82 (19). *Anal.* found: C 62.1, H 6.9, N 21.0.

X-ray analysis of 25

Crystal data:

$C_{10}H_{14}N_3O$ f.w. = 192.1

Orthorhombic, a = 19.155(2), b = 7.634(1), c = 15.059(3) Å, U = 2202.1 $Å^3$, Z = 8, D_c = 1.16, $F(000)$ = 824, $MoK_\alpha(\lambda = 0.71069$ Å), μ = 0.5 cm^{-1}, space group $Pbcn$ (D_{2h}^{14}) from the systematic absences $hk0$, $k = 2n + 1$; $h0l$, $h + l = 2n + 1$.

A small crystal of dimensions 0.40 × 0.33 × 0.13 mm was used in the analysis. Unit cell contstants and intensity data were determined using an Enraf–Nonius computer-controlled CAD-4 diffractometer. For the cell dimension determination, the setting angles of 25 reflexions with θ near 15° were used in a

least-squares refinement. During data collection three well separated reflections were monitored at regular intervals and showed no reduction in intensity. The intensities of 1930 reflections with $2 < \theta < 25°$ were measured by the $\theta/2\theta$ scan technique. The 1068 reflections with $I > 3\sigma(I)$ were labelled "observed" and used, after correction for Lorentz and polarization factors, in the determination and refinement of the structure.

The structure was determined by direct methods using the SHELX program (19). The first E-map calculated with E's > 1.2 revealed all 14 non-hydrogen atoms. Four cycles of full-matrix isotropic refinement reduced R to 0.14 and a subsequent difference map revealed positions for all 14 protons. These were then allowed for in idealized positions (C—H 0.95 Å) and only an overall isotropic temperature factor was refined for protons in subsequent refinement. After six further rounds of full-matrix calculations, with the non-hydrogen atoms allowed anisotropic motion, the refinement was completely converged with $R = 0.058$ and $R' = \Sigma w\Delta^2/\Sigma wF_o^2 = 0.074$. In the refinement cycles, weights were derived from the counting statistics, $w = 1/(\sigma^2 F + 0.005F^2)$, and scattering factors were taken from refs. 20 and 21. A final difference map was free of any significant features. Final atomic coordinates are given in Table 2. The measured and calculated structure factors and thermal parameters have been placed in the Depository of Unpublished Data.[3]

trans-*1-Oxyl-2,2,5,5-tetramethyl-3,4-dicarboxypyrrolidine (27)*

A solution of **25** (10 mg, 0.52 mmol) and 2 *N* aqueous NaOH (0.62 mL, 1.2 mmol) was heated at 90°C until evolution of NH_3 (22) ceased (34 h). The solution was cooled, carefully neutralized to pH 4 by addition of 6 *N* HCl, and extracted with ether. Evaporation of the extract afforded 2.8 mg of crude partially hydrolyzed nitroxide which still contained cyano absorption in the ir spectrum. The original aqueous phase was further acidified to pH 2 and again extracted with ether. The extract was dried (Na_2SO_4) and evaporated. The residue was recrystallized from ether–hexane, affording 7.3 mg (61%) of **27**, mp 220°C; ms *m/e*: 230.102 (70) (calcd. for $C_{10}H_{16}NO_5$, 230.103), 216 (23), 197 (20), 170 (18), 169 (30), 154 (25), 110 (22), 109 (25), 101 (95), 100 (65), 83 (100), 82 (60).

cis-1-Oxyl-2,2,5,5-tetramethyl-3,4-dicarboxypyrrolidine (26)

The *cis* dinitrile isomer **24** (27 mg) was hydrolyzed as in the previous experiment, affording 18 mg (56%) of diacid **26** as a yellow powder, mp >250°C; ms *m/e*: 231 (15), 230.104 (50) (calcd. for $C_{10}H_{16}NO_5$, 230.103), 216 (45), 197 (14), 170 (30), 169 (23), 154 (21), 110 (24), 109 (21), 101 (79), 100 (49), 83 (100), 82 (57).

2-Carboethoxy-3-hydroxy-4,4,6,6-tetramethyl-2,3,3a,4,6,6a-hexahydrothieno[2,3-c]pyrrole-5-oxyl (29)

Ethyl thioglycolate (120 mg, 1.00 mmol) was added to a solution of aldehyde **15** (168 mg, 1.00 mmol) in MeOH (5 mL) containing diethylamine (14.6 mg, 0.200 mmol). After a 24 h reflux period, the solution was concentrated and subjected to preparative tlc (Analtech) (ethyl acetate/hexanes, 1:1). The R_f 0.64 band amounted to 35 mg (21%) of starting aldehyde **15**. Two very close lower bands, R_f 0.54, were collected together, affording 140 mg (49%) of the title compound, likely as a mixture of stereoisomers, mp 86–88°C; ir (Nujol): 3550–3250 and 1740 cm^{-1}; ms *m/e*: 290 (2), 289 (8), 288.128 (31) (calcd. for $C_{13}H_{22}NO_4S$, 288.127), 215 (16), 174 (59), 143 (4), 142 (13), 141 (100), 110 (75), 101 (52). *Anal.* calcd. for $C_{13}H_{22}NO_4S$: C 54.14, H 7.69, N 4.85; found: C 53.94, H 7.56, N 5.35. Repeated preparative tlc followed by two crystallizations from ether–hexane gave one pure isomer of **29**, mp 138–139°C.[3]

2-Carbomethoxy-3-hydroxy-4,4,6,6-tetramethyl-2,3,3a,4,6,6a-hexahydrothieno[2,3-c]pyrrole-5-oxyl (30)

The above procedure was followed. From methyl thioglycolate (106 mg, 1.00 mmol) and aldehyde **15** (168 mg, 1.00 mmol) there was obtained 40 mg (24%) of recovered aldehyde and 120 mg (44%) of ester **30**, mp 136–137°C (ether/hexane); ms *m/e*: 275 (36), 274.111 (91) (calcd. for $C_{12}H_{20}NO_4S$, 274.111), 260 (56), 244 (31), 160 (100), 127 (87), 110 (69), 101 (44), 99 (80).

2-Carboethoxy-3-acetoxy-4,4,6,6-trimethyl-2,3,3a,4,6,6a-hexahydrothieno[2,3-c]pyrrole-5-oxyl (31)

Acetic anhydride (20.4 mg, 0.200 mmol) was added to alcohol **29** (28.8 mg, 0.100 mmol) in pyridine (1.0 mL) and the solution was allowed to stand at 25°C for 3 h. The usual work-up afforded a yellow solid which crystallized from ether/hexane to give 27 mg (82%) of acetate **31**, mp 126–127°C; ms *m/e*: 332 (6), 331 (25), 330.136 (69) (calcd. for $C_{15}H_{24}NO_5S$, 330.137), 242 (11), 241 (16), 240 (82), 216 (34), 174 (100), 167 (40), 141 (41), 99 (72).

2-Carboethoxy-3-hydroxy-4,4,6,6-tetramethyl-2,3,3a,4,6,6a-hexahydrothieno[2,3-c]pyrrole-5-oxyl-1,1-dioxide (32)

Hydrogen peroxide (30%) (1.0 mL) was added to a solution of alcohol **29** (28.8 mg) in MeOH (2.0 mL). Sodium tungstate (3 mg) was then added and the solution was allowed to stand for 3 h at 25°C. The solution was diluted with brine (10 mL) and extracted with $CHCl_3$. The extract was dried (Na_2SO_4) and evaporated to dryness, affording a solid residue which was triturated with ether. Filtration afforded 20 mg (63%) of crystalline sulfone **32**, mp 156–157°C; ms *m/e*: 320.118 (10) (calcd. for $C_{13}H_{22}NO_6S$, 320.117), 164 (20), 139 (3), 111 (35), 110 (100), 109 (13), 95 (29).

TABLE 2. Final fractional coordinates (× 10^4) with standard deviations in parentheses

Atom	*x*	*y*	*z*
O	1861(1)	313(3)	577(2)
N	1591(1)	−1203(4)	610(2)
N(21)	1084(3)	−3742(5)	−2784(3)
N(31)	761(2)	−6374(5)	−749(2)
C(1)	1384(1)	−2013(4)	1460(2)
C(2)	1035(2)	−6308(5)	−3889(2)
C(3)	1385(2)	−4052(4)	229(2)
C(4)	1447(1)	−2216(4)	−211(2)
C(11)	2023(2)	−2290(5)	2035(2)
C(12)	857(2)	−879(5)	1924(3)
C(21)	1066(2)	−4840(5)	−3260(2)
C(31)	1019(2)	−5358(5)	−337(3)
C(41)	2057(2)	−2141(6)	−838(2)
C(42)	791(2)	−1532(6)	−649(2)
H(21)	546	−6452	−3965
H(31)	1830	−4576	314
H(111)	1897	−2809	2586
H(112)	2238	−1188	2141
H(113)	2340	−3035	1732
H(121)	719	−1409	2467
H(122)	460	−738	1553
H(123)	1058	234	2041
H(411)	1958	−2794	−1360
H(412)	2453	−2629	−550
H(413)	2152	−959	−994
H(421)	694	−2186	−1171
H(422)	851	−333	−799
H(423)	413	−1645	−245

[3]The tables are available, at nominal charge, from the Depository of Unpublished Data, CISTI, National Research Council of Canada, Ottawa, Ont., Canada, K1A 0S2.

*2-Carboxy-3-hydroxy-4,4,6,6-tetramethyl-2,3,3a,4,6,6a-hexahydro[2,3-c]pyrrole-5-oxyl (**33**)*

A solution of methyl ester **30** (144 mg, 0.500 mmol) and 1.0 mL (1.0 mmol) of 1 *N* NaOH in MeOH (3.0 mL) was allowed to stand at 25°C for 3 h. The solution was diluted with brine, acidified, and extracted with ether. Evaporation of the dried (Na_2SO_4) extract gave 105 mg (81%) of crystalline acid **33**. Recrystallization from $CHCl_3$/ether/hexenes gave the analytical specimen, mp 212–213°C; ms *m/e*: 260.096 (5) (calcd. for $C_{11}H_{18}NO_4S$, 260.096), 169 (29), 154 (95), 138 (67), 126 (100), 123 (38), 110 (86), 109 (33), 108 (29), 95 (52), 92 (71).

*2-Carboethoxy-4,4,6,6-tetramethyl-3a,4,6,6a-tetrahydrothieno[2,3-c]pyrrole-5-oxyl (**34**)*

Methanesulfonyl chloride (22.9 mg, 0.200 mmol) was added to a solution of alcohol **29** (28.9 mg, 0.100 mmol) in pyridine (1.0 mL) and the solution was allowed to stand at 25°C for 6 h. The mixture was diluted with brine, acidified to pH 4 with 6% hydrochloric acid, and extracted with ether. The dried (Na_2SO_4) extract was evaporated and the residue was subjected to preparative tlc (Analtech) (ethyl acetate/hexanes, 1:1). The major band, R_f 0.74, afforded 20 mg (74%) of crystalline **34**. Recrystallization from ether/hexanes gave the analytical specimen, mp 113–116°C; ir (Nujol): 1720 and 1560 cm^{-1}; ms *m/e*: 272 (5), 271 (14), 270.116 (54) (calcd. for $C_{13}H_{20}NO_3S$, 270.116), 241 (11), 240 (63), 226 (16), 225 (100), 198 (16), 197 (87), 184 (14), 183 (89), 181 (16), 169 (53), 167 (84), 155 (37), 153 (42), 151 (47), 133 (33), 125 (73), 113 (94), 111 (65).

Spin labeled hemoglobin

Stock solutions (5 mg/mL) of 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (**22**) (Syva Co.) and nitroxide **21** were prepared in ethanol. Hemoglobin (20 mg, Sigma, Type I, bovine) was dissolved in 30 m*M* Tris pH 8.0 buffer (0.5 mL) in subdued light. The spin label stock solution (6 μL) was added and the resulting solution was stirred 2 h at 25°C. After removal of unreacted spin label by dialysis, the sample was concentrated to 50 μL using a collodion bag apparatus (Schleicher & Schuell, MW cutoff 25 000) before esr spectra were recorded. Samples to be photolyzed were diluted to 0.5 mL with the Tris buffer and then illuminated 5 min at 0°C with filtered (1 *M* KNO_2) radiation from a 1000 W quartz–halogen lamp. After the photolysis, the samples were concentrated to 50 μL and esr spectra (Varian E-Line spectrometer) were again recorded.

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