SYNTHESES OF BIFUNCTIONAL SPIN LABEL MOLECULES AND THEIR ORIENTATIONS IN MEMBRANES

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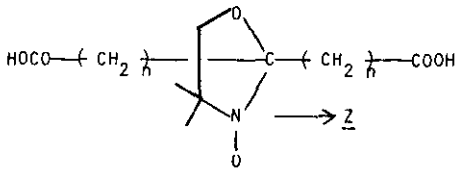
ABSTRACT: The synthesis of new derivatives of substituted pyrrolidine nitroxide free radicals is reported. The molecules are hydrophobic and bifunctional. A preliminary study of their orientation in lipid model membranes has been made by EPR. Molecules having chains of up to 17 atoms and polar end groups take up a conformation in membranes which is tentatively assigned to a bent configuration with both functional groups on one side of the membrane. These molecules are synthetic precursors of spin-labeled cross-linking reagents for membrane proteins.

INTRODUCTION. The lipid bilayer portions of biological membranes are essentially smectic liquid crystals and it is, therefore, possible to perferentially orient rod-shaped or planar molecules within membranes (1,2,3). An application of this property of lipid bilayers is that hydrophobic reagents may be directed to selected sites on membrane proteins (4). One example of this approach is the use of lipids which are photoreactive at points varying from the hydrophobic center of the bilayer to the lipid, water-interface region (5). The location of the reactive group within the bilayer can be estimated with reasonable certainty for the photoreactive reagents because they closely resemble the parent phospholipids in structure and because the arrangement of molecules in lipid bilayers is well defined from X-ray (6) and neutron diffraction studies (7). However, other useful membrane reagents have structures which do not resemble lipid molecules, and, therefore, it is not possible to deduce the orientation and location of the reagents within a membrane simply by analogy to those properties of the host lipids. Spin labeling (1-3), fluorescence (8) and photo-induced dichroism (7) techniques have been used to determine the orientation of chromophores in membranes. In this report, we describe the synthesis of a series of long-chain, bifunctional, spin-label molecules and the information obtained from their paramagnetic resonance (EPR) spectra about the conformation and orientation of the molecules in lipid membranes. These molecules are precursors of bifunctional protein-modification reagents which are currently under study in this laboratory.

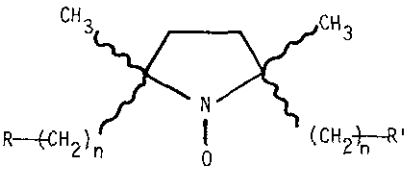
Seelig and co-workers showed (3) that spin-labeled dicarboxylic acids, of general structure I, may either extend across a smectic liquid crystal or take up a bent conformation with both carboxylic

acid groups on a single side of the bilayer structure. The preferred conformation is determined by relative lengths of the spin label molecule and of the amphiphiles of which the bilayers are comprised.



I

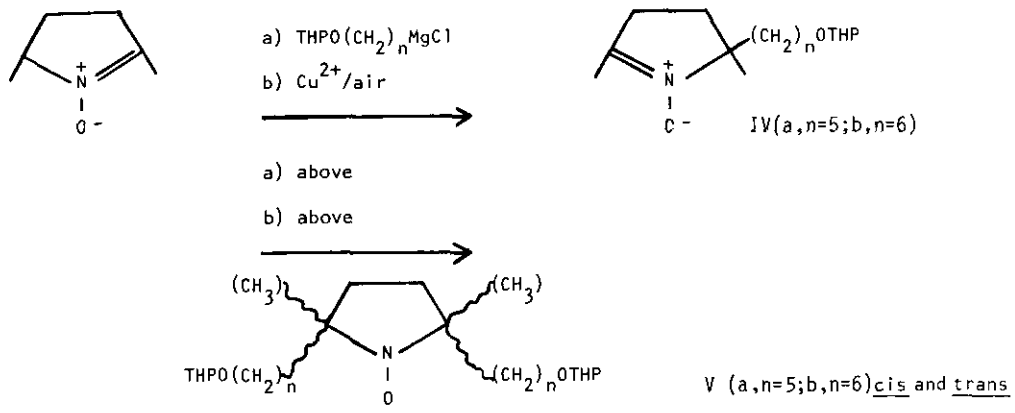
An important feature of the substituted oxazolidine nitroxides, I, for analysis of the EPR spectra of oriented bilayers is that the Z-principal axis of the nitroxide group is parallel to the extended chain. Recently, Keana and co-workers introduced synthetic methods (10,11) for preparing long chain nitroxides that effectively rotate the nitroxide coordinates by up to 90° from the orientation of these coordinates in the oxazolidine nitroxides (I). (The convention for the nitroxide principal axes is that X is along the N-O bond and Z is parallel to the pi-orbital.) The new nitroxide group is a 1-oxyl substituted pyrrolidine with the general structure II. Both cis- and trans- isomers result from the syntheses and these isomers can be separated for some R-groups. Because both R groups are the same in the molecules we will discuss here, the cis compounds are not



II (cis, trans)

chiral whereas the trans compounds are.

RESULTS AND DISCUSSION. The syntheses of the 2,2,5,5-tetra-substituted pyrrolidines II are achieved by two cycles of (a) Grignard addition to a substituted nitrone and (b) air oxidation as shown on the following page. In the course of investigating conditions for preparing the nitrone IIIa, we have also prepared the 3,3-dimethyl derivative IIIb, in somewhat higher yield than IIIa was prepared. The nitrones III and IV are very hygroscopic and care must be taken to dry and distill them before the Grignard additions. The Grignard reagents from the α-chloro-ω-tetrahydropyranylalkanes formed well in refluxing tetrahydrofuran, but not in diethylether. Our syntheses differ from those of Lee and Keana (11) in that two Grignard additions of tetrahydropyranyl ethers are employed. Our yields, however, were similar to theirs in which one alkyl Grignard and one tetrahydropyranyl ether Grignard were used.



We report the synthesis of a number of derivatives of the general structure II with the R-groups shown below for V-X.

R=	n=	
OTHP	5,6	Va,b
OH	5,6	VIa,b
I	5,6	VIIa,b
CN	5,6	VIIIa,b
COOH	5,6	IXa,b
CH_3	7	X

In the syntheses carried out by Lee and Keana (11), reaction sequences in which the fourth substituent added to the pryrrolidine ring was a long chain one gave primarily the trans substituted derivative. When methyl was the final substituent added, the cis geometry predominated. Based on these results, it is reasonable to expect that the di-tetrahydropyranyl ether compounds Va and Vb are mixtures of cis and trans isomers with trans predominant. The earlier work (11) also showed that some of the cis and trans derivatives could be separated by tlc. In our syntheses, there were several products moving with similar, but not identical, R_f's for a number of derivatives. Since, in each case, the major component was isolated, the trans compounds may have been selectively purified in the multi-step synthetic sequences. Indeed, ^{1}HNMR of the chemically reduced (and thus diamagnetic) nitroxides VIb and VIIIa,b tends to confirm this.

The ^{1}HNMR patterns ($CDCl_3$) for VIb and VIIIb, after chemical reduction of the nitroxides to the hydroxylamines, were quite similar in the region δ1.0-1.9 except for a peak at δ1.82 which was present in the spectrum of VIb (less than 2H) but apparently absent from that of VIIIb (a very small peak in this region cannot be ruled out). Lee and Keana found that all cis acetate derivatives of nitroxides ($N\text{-}O\text{-}COCH_3$) gave peaks at δ1.6-1.75 which were not found for the trans analogs. However, rigorous proof of the trans geometry for VIIIb requires further isolation and characteri-

zation of some of the minor (presumably cis) components of VII or VIII.

In associated work, we have prepared bifunctional derivatives of 1-oxyloxazolidines, I (12). We had two goals in preparing the pyrrolidine derivatives, II. We wished to know if the conformation of the molecules in membranes was significantly different for the substituted pyrrolidines, compared to the oxazolidine derivatives. In addition, we wished to prepare two sets of protein modification reagents in which the nitroxide principal axes were at differing angles relative to the molecular coordinates in order to facilitate future EPR studies of anisotropic rotation of membrane proteins. The orientation, and thus the conformation, of nitroxides in membranes can be investigated by preparing multilayer membranes oriented on a flat surface. We have made membranes of this type from L-α-dilauroylphosphatidylcholine (DLL). When a dry film of DLL is exposed to water vapor for several hours, a limited amount of water (25% by weight) is taken up and lipid bilayers, separated by water layers, are formed. Gentle pressure of this hydrated material between two glass plates produces well-oriented bilayer stacks. We chose DLL for our studies for several reasons. It does not contain unsaturated fatty acid chains and is thus not subject to air oxidation; it has a phase transition only below room temperature so that fluid bilayers can readily be prepared; it has relatively short fatty acid chains (12 carbon atoms) so that it might be possible for the longest of the bifunctional reagents to take up an extended conformation, spanning the bilayer. Figure 1 shows three possible orientations of the trans molecules II in a membrane bilayer. Each of the molecular orientations shown corresponds to a unique set of EPR spectra for oriented bilayer samples. It is assumed that each orientation drawn in Figure 1 exists in an array generated by rotation of the orientation depicted about the bilayer normal, N. Table 1 summarizes the number and position of gauche rotations in the flexible polymethylene chains that can provide some of the bent (Fig. 1a) and extended (Fig. 1b,c) conformations of II.

The experimental EPR spectra of molecules V, VI and VIII-X in DLL membranes (both dispersions and oriented bilayers) have been obtained. Four characteristic line shapes were observed. Some samples gave the superimposed spectra of two or three of the characteristic line shapes. The molecules VIII and X, with non-polar R groups, gave nearly isotropic spectra in oriented bilayers at room temperature. Two other characteristic line shapes are overlapping in the spectrum shown in Figure 2a. Line shapes very similar to this were obtained for molecules V and IX with R groups -OH and -COOH. We are able to simulate the spectra in Fig. 2a, with DPPH (α,α'-diphenyl-β-picryl hydrazyl) as a marker, by superimposing spectra with g = 2.0035 (minor component) and 2.0074 (major component) and, for the major component, with Z-molecular axis tilted by 75° from the normal to the membrane plane. The g-value 2.0074, together with the considerable motional averaging, does not allow an unambiguous choice between preferential alignment of the molecular X or Y axis with the membrane normal. However, a number of considerations, including the predominance of the trans geometrical

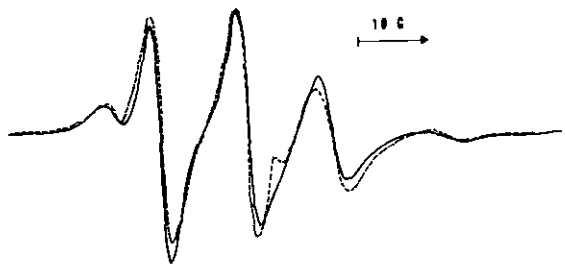


Fig. 2a

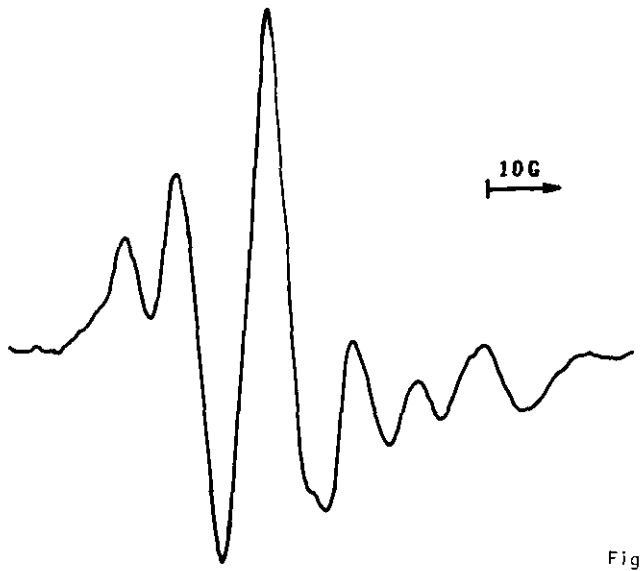


Fig. 2b

Figure 2a: Experimental and calculated spectra for VIb in oriented bilayer; magnetic field parallel to bilayer normal; parameters for major component: tilt angle = 75°, $T_{\parallel}'$ = 21.6, $T_{\perp}'$ = 11.2 gauss, Δ g = .0026, line widths (low to high field) = 3.5, 3.5, 5.0 gauss; minor component: tilt angle = 0°, $T_{\parallel}'$ = 24.4, $T_{\perp}'$ = 9.5 gauss, Δ g = .0026, line widths = 3.5, 3.0, 6.0 gauss. Ratio of major/minor component is 8/1.

Figure 2b: Same as experimental spectrum in Fig. 2a except that label is IXb; note additional component apparent particularly in high-field region.

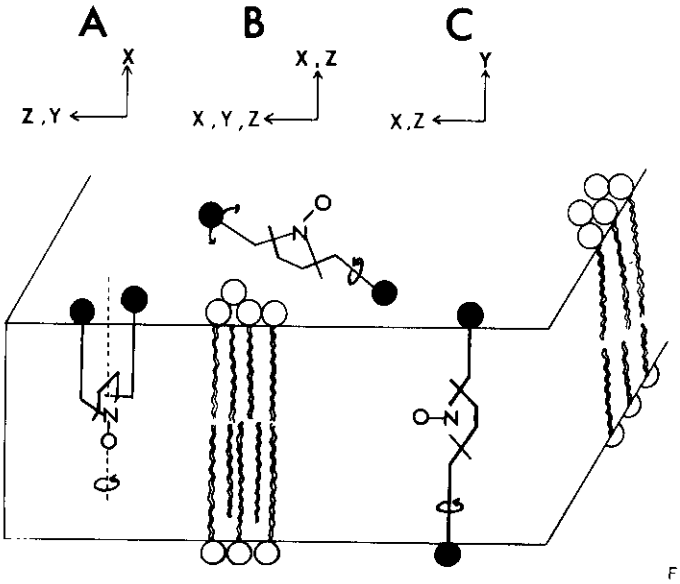


Fig. 1

Figure 1: Three possible conformations of the molecule II in a membrane bilayer

TABLE 1.

Conformations of II Derivatives for Various Gauche Bond Arrangements*

(*Based on CPK molecular models)

#Gauche Bonds	Gauche Bond Location	Conformation	Nitroxide Axis Parallel Bilayer Normal (b)
Trans Isomer			
0	---	extended	Z minus 30°
2g	2-3	extended	Z minus ∿10°
2g	3-4	extended	Y
2g	1-2	bent	X
4g	1-2, 4-5	bent	X
Cis Isomer			
0	---	wide bend	Z
2g	2-3	bent	∿Z
2g	1-2	bent	X
2g	1-2	extended	Y
2g	3-4	extended	Y

a) The chains are numbered so that C-1 is next to a carbon atom on the pyrrolidine ring.

b) Only the alignment for the arrangement of the extended conformation shown in Fig. 1c is tabulated. The orientation 1b is expected to give nearly isotropic spectra in oriented bilayers.

isomer, the length of the molecules involved, the high mobility of molecules contributing the major spectral component ($T_{11}' = 22$ gauss) and analogy to the more easily discerned conformations of the oxazolidine nitroxides, I, (3) suggests that the bent conformation shown in Fig. 1a is the predominant one for molecules VI and IX in membranes. The minor component of the spectra could arise from the extended trans isomer or from the bent cis one. A fourth characteristic line shape was obtained as the major component in the spectrum of Vb and as a minor component in the sepctrum of IXb (Figure 2b). We are not able to unambiguously assign this spectrum to a conformation at present. However, since it is only found for the longest molecules prepared, a tentative conclusion is that it corresponds to molecules in some trans-membrane conformation.

EXPERIMENTAL. Computer simulation of EPR line shapes were carried out using an approach described earlier (2).

2,5-Dimethyl-1-pyrroline-1-oxide (IIIa). The condensation of methyl vinyl ketone and nitroethane was carried out as described by Shecter, et al. (13) to give 49.3% yield of 5-nitro-2-hexanone after distillation (b.p. 115-119°/10mm); IR (CCl_4) 1720 (C=O).
Cyclization (14) of 5-nitro-2-hexanone with zinc dust and ammonium chloride gave 2,5-dimethyl-1-pyrroline-1-oxide in 50% yield (b.p. 72°/0.7mm). IR (CCl_4) 1605 cm^{-1} (C=N); NMR (CCl_4/TMS) δ(ppm) 1.35 (3H, d, CH_3), 1.92 (3H, s, C=C-CH_3), 1.9-2.7 (4H, m, -CH_2-), 3.9 (1H, m, $HC\overset{+}{N}(O^-)C=C$).

2,4,4,5-Tetramethyl-1-pyrroline-1-oxide (IIIb). Condensation of mesityl oxide (4-methyl-3-penten-2-one) with nitroethane gave 4,4-dimethyl-5-nitro-2-hexanone (b.p. 82°/1.0mm); IR (CCl_4) 1720 cm^{-1} (C=O); NMR (CCl_4/TMS) δ(ppm) 1.0 (3H, s, C-CH_3), 1.1 (3H, s, C-CH_3), 1.45 (3H, d, $CH(NO_2)CH_3$), 2.08 (3H, s, (C=O)CH_3), 2.5 (2H, CH_2-C), 5.0 (1H, q, C<u>H</u>-CH_3).
Cyclization as above gave IIIb in 70% yield (b.p. 72°/1.0mm); IR (CCl_4) 1605 cm^{-1} (C=N); NMR (CCl_4/TMS) δ(ppm) 1.07 (3H, s, CH_3), 1.18 (6H, CH_3), 1.88 (3H, s, C=CCH_3), 2.56 (2H, s, C-CH_2), 3.58 (1H, m, $HC\overset{+}{N}(O^-)C=C$).

Preparation of tetrahydropyranyl ethers. Equimolar quantities of α-chloro-ω-hydroxyalkanes and dihydropyran, with a trace of hydrochloric acid, were shaken and allowed to stand for 3-5 hours. The reactions are exothermic. A few pellets of sodium hydroxide were added to destroy the acid and the solution was purified by distillation. The pentyl derivative was obtained in 58% yield (b.p. 78°/.55mm) and the hexyl derivative in 46% yield (b.p. 94°/0.5mm).

2,5-Dimethyl-2,5-di(6'-tetrahydropyranyloxyhexyl)-1-pyrrolidine-1-oxyl (Vb). A solution of 4.6 g (39.7 mmole) of nitrone IIIa in 30 ml of tetrahydrofuran was added at 21° to a stirred 100 ml solution of the Grignard reagent derived from the tetrahydropyranyl ether of 6-chlorohexanol (13.14 g, 59.6 mmole) and 1.45 g of magnesium turnings (59.6 mmole). [Addition of the nitrone to a refluxing solution of the Grignard reagent in tetrahydrofuran gave lower yields of the desired product.] The mixture was stirred for 30 min at room temperature, after the addition was complete.

Saturated ammonium chloride solution was added and the organic layer was separated, dried over sodium sulfate and evaporated to yield a yellow oil which was immediately taken up in 50 ml of methanol and 5 ml of concentrated ammonium hydroxide. Copper acetate [$Cu(OAc)_2 \cdot H_2O$, 1g] was added to the solution and the mixture was stirred in air until a deep blue color developed (~30 min). The mixture was diluted with water and ether. The ether layer was collected, dried and evaporated to give a brown oil from which a low boiling fraction was removed by distillation (40°/1mm). The residue was used directly for the second Grignard addition without further purification.

The second addition to the trisubstituted nitrone was performed in the same manner as above using a 20 ml solution of the crude nitrone in tetrahydrofuran and the Grignard reagent prepared from 0.92 g magnesium (38.0 mmole) and 8.58 g (39.0 mmole) of the tetrahydropyranyl ether of 6-chlorohexanol. After work-up, the product was purified by column chromatography on silica gel (elution with 1% methanol in chloroform) followed by preparative thin layer chromatography (chloroform, R_f 0.1) to give IVb in 20% yield (based on the original 4.5 g of nitrone); IR (neat) 1135 cm^{-1} (C-O-C). (Calculated for $C_{28}H_{52}NO_5$, 482.73: C, 69.66; H, 10.85; N, 2.90. Found: C, 67.97; H, 10.72; N, 1.97.

2,5-Dimethyl-2,5-di(6'-tetrahydropyranyloxypentyl)pyrrolidine-1-oxyl Va. The nitroxide substituted with two tetrahydropyranyloxypentyl chains (IVa) was prepared by the procedure described above for IVb. (Calculated for $C_{26}H_{48}NO_5$, 454.68: C, 68.86; H, 10.64;N, 3.08. Found: C, 68.75; H, 10.70; N, 3.00).

2,5-Dimethyl-2,5,-di(6'-hydroxyhexyl)pyrrolidine-1-oxyl (VIb) and the corresponding di(5'-hydroxypentyl) analog (VIa). The di-tetrahydropyranyl ether Vb (200 mg, 0.41 mmol) was cleaved in 50 ml methanol containing 13.2 mg of p-toluenesulfonic acid after standing for 4 hr at room temperature. The solution was diluted with water and ether and the ether layer was washed with water and saturated sodium chloride, dried and evaporated. The residue was dissolved in chloroform and purified on a dry silica gel column. The column was eluted with chloroform, followed by ether. The ether fractions gave 91 mg (70.6%) of the diol (tlc, 5% methanol-chloroform R_f .405). (Calculated for $C_{18}H_{38}NO_3$, 314.49: C, 68.74; H, 11.53; N, 4.45. Found: C, 68.59; H, 11.62; N, 4.42).

Similarly, Va was converted to VIa. (Calculated for $C_{16}H_{32}NO_3$, 286.43: C, 67.09; H, 11.26; N, 4.89 Found: C, 64.97; H, 10.71; N, 4.54).

2,5-Dimethyl-2,5-di(6'-iodohexyl)pyrrolidine-1-oxyl VIIb). Methanesulfonyl chloride (114 mg, 0.1 mmole) was added to a stirred solution of the diol VIb (91 mg, 0.2 mmole) and 122 mg (1.2 mmole) of triethylamine in 10 ml of dry dichloromethane at -20°C (Dry Ice - CCl_4 bath). After addition, the mixture was allowed to warm to 20°C and 80 ml of cyclohexane was added. Solids were removed by filtration and the filtrate was evaporated. The crude dimethanesulfonate was dissolved

in 10 ml of methyl ethyl ketone containing 540 mg of sodium iodide. The solution was heated under reflux for 30 min, diluted with cyclohexane and filtered. Evaporation gave the crude diiodide (tlc, chloroform, R_f .88, .95).

The preparation of VIIa was similar to that of VIIb.

2,5-Dimethyl-2,5-di(6'-cyanatohexyl)pyrrolidine-1-oxyl (VIIIb). The crude diiodide VIIb was mixed with 10 ml absolute ethanol and 0.2 g (4.0 mmole) sodium cyanide and the mixture was heated under reflux for 20 hrs. The reaction mixture was partitioned between water and chloroform and the chloroform layer was dried over sodium sulfate. Preparative tlc (chloroform) of the residue gave the dinitrile (R_f .27) IR (CCl_4) 2240 (C≡N) cm^{-1}. Calculated for $C_{20}H_{36}N_3O$, 332.5: C, 72.24; H, 10.30; N, 12.38. Found: C, 71.98; H, 10.22; N, 12.62).

The corresponding di(5'-cyanatopentyl) derivative, VIIa, was prepared similarly. (Calculated for $C_{18}H_{32}N_3O$, 304.46: C, 71.00; H, 9.93; N, 13.79. Found: C, 70.93; H, 9.87; N, 13.69).

2,5-Dimethyl-2,5-di(6'-carboxyhexyl)pyrrolidine-1-oxyl (IXb). Hydrolysis of the dinitrile (72 mg, 0.22 mmole) was achieved by heating a methanol (10 ml) solution with 2 ml, 4N sodium hydroxide at reflux for 16 hrs. The solution was acidified with cold, 1N hydrochloric acid and the diacid was extracted into ethylacetate. The organic layer was washed with water and saturated sodium chloride, dried over sodium sulfate and evaporated to give a yellow oil. Preparative thin layer chromatography (5% methanol/chloroform) gave pure diacid. (Calculated for $C_{20}H_{38}NO_5$, 370.51: C, 64.83; H, 9.79; N, 3.78. Found: C, 64.63; H, 9.62; N, 3.58).

The corresponding di(5'-carboxypentyl) compound was prepared in a small amount and used for oriented bilayers studies, but was not analysed.

2,5-Dimethyl-2,5-dioctylpyrrolidine-1-oxyl (X). A solution of 2,5-dimethylpyrroline-1-oxide (3 g) in 30 ml of ether was added to a stirred solution of octylmagnesium bromide (prepared from 5.68 g 1-bromooctane and 0.716 g magnesium in 100 ml ether) at a rate sufficient to maintain gentle reflux. The solution was stirred for an additional 30 min at room temperature. Saturated ammonium chloride was added and the ether layer which separated was dried and evaporated to give a yellow oil which was immediately taken up in 50 ml of methanol and 5 ml of concentrated ammonium hydroxide and stirred with 1 g of cupric acetate until a blue color developed. The solution was diluted with 50 ml water and extracted with ether. The ether extract was dried and evaporated to give a residue which was purified on a silica gel column (5% methanol in chloroform). A slightly impure (tlc) product was obtained in 50% yield and used directly for the next step.

The nitrone (2.6 g, 23.0 mmole) in 10 ml ether was added at 20°C to 50 ml of the Grignard reagent prepared from 0.42 g magnesium and 3.3 g 1-bromooctane. After 30 minutes of stirring, the mixture was worked up in the usual way and oxidized with copper/air. Column chromatography on silica gel (chloroform) gave X. (tlc ($CHCl_3$) R_f 0.79) (Calculated for $C_{22}H_{44}NO$,

338.59: C, 78.04; H, 13.10; N, 4.13. Found: C, 78.25; H, 13.22; N, 4.24).

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