Newer Aspects of the Synthesis and Chemistry of Nitroxide Spin Labels

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I. Introduction

Largely because nitroxide free radicals often exhibit a chemical inertness quite uncharacteristic of most other free radicals, nitroxides continue to be the subject of intensive investigation. Of particular interest from a theoretical viewpoint have been ESR studies of the magnetic interaction between two or more nitroxide groups within the same molecule.[1-10] On the other hand, NMR studies of nitroxides and other paramagnetic molecules[11] provide structural and spectroscopic information difficultly obtainable by ESR spectroscopy.[12-17] Perhaps the most conspicuous role payed by nitroxides today is that of a reporter group[18] in the study of biological systems by electron spin resonance (ESR) spectroscopy, i.e., the spin-labeling technique. Spin labeling was first introduced by McConnell[19,20] in 1965 and has rapidly evolved into one of the most powerful and versatile of the biophysists' tools.

Aspects of spin labeling have been the subject of numerous revews[21-41] as well as an entire book.[42] The practical side of spin labeling has been discussed by Jost and Griffith,[28] and new developments particularly in computerization continue.[43] Other advances include development of the saturation transfer ESR spectroscopic technique,[44-48] which has significantly extended the useful range of the rotational correlation time τ_2 in spin-labeling studies from the usual limit of 10^{-7} s or shorter to the range $10^{-7} < \tau_2 < 10^{-3}$ s.[49-51]

It is well to point out at the outset a distinction used by some investigators relating to the terms "spin label" and "spin probe".[42] When a nitroxide bearing molecule[52] is covalently attached to another molecule or macromolecule of interest, then the molecule or macromolecule is considered to be spin labeled. The intent, of course, is that the presence of the nitroxide grouping will not significantly perturb the behavior of the spin-labeled molecule in the system under study. The term spin probe, then, refers to a nitroxide containing molecule which is not covalently attached to molecules of the system under study. Spin probes may become immobilized through hydrophobic, ionic, or hydrogen-bonding interactions with proteins,[53] for example, or they may reflect different molecular environments within the system by a simple partitioning among these environments.[54] The distinction between the two terms is not always clear-cut, since spin-labeled molecules themselves often become spin probes when used to study complex systems. Thus, for purposes of this review, the distinction in terms will not be maintained.

The focus of this present review will be on the development of new stable nitroxide spin labels, new methods of attachment of the labels to molecules of interest, and new chemistry of the nitroxide grouping as it pertains to the spin-labeling method. The review is therefore not a compendium of molecules which have been spin labeled as these are summarized in the reviews cited above. The interesting branch of nitroxide chemistry relating to the "spin trapping" method[55-57] and the incidental generation of nitroxide radicals in studies unrelated to spin labeling also fall outside the scope of this review. Research appearing after 1970 will be emphasized, with literature coverage extending through about April 1977. Portions of this research have been reviewed by Gaffney,[58] wherein one also finds detailed procedures for the chemical synthesis of many nitroxide spin labels and representative procedures for the covalent attachment of these labels to biomolecules.

II. General Comments Regarding Stable Nitroxides

A. Stability of the Nitroxide Grouping

In order for a nitroxide free radical to be useful in conventional spin-labeling studies, it must be reasonably stable. Fortunately, a large number of nitroxide free radicals are chemically stable. The term "stable" is used here in the Ingold[59] sense; that is, the free radicals can be obtained in pure form, stored, and handled in the laboratory with no more precaution than that observed when working with conventional organic compounds. In order to place the chemistry of nitroxide spin labels into perspective, we now discuss some of the chemical properties of nitroxides pertinent to the design of new nitroxide spin labels.

The fundamental chemical and physical properties of stable nitroxides were established over the last 20 years largely through the pioneering research of E. G. Rozantsev in the USSR and A.

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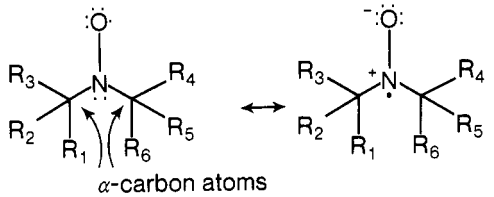
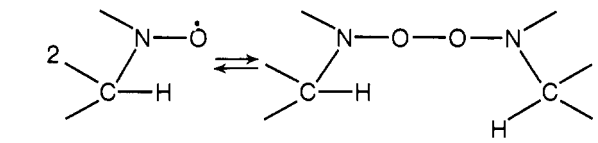
Rassat in France and their collaborators. Several books[60-62] and reviews[58,63-67] summarize the many contributions of these and other investigators in this area.

While a host of interesting nitroxides with various substituents on the nitroxyl nitrogen have been generated[68] and show a persistence[59] sufficient for a careful study of their ESR spectra or a study of the kinetics and mechanism of their decomposition, rather special structural features are required in order for the nitroxide to be stable. The vast majority of stable nitroxide free radicals are secondary amine *N*-oxides of the general structure **1** in which there are no hydrogens attached to the α-carbon atoms. This is obviously an important fact to keep in mind when contemplating the design of new nitroxide spin labels.



1a **1b**

When one or more hydrogen atoms are attached to the α-carbon atoms of the nitroxide group, the radicals typically undergo a disproportionation reaction,[69,70] producing a nitrone **3** and a *N*-hydroxyamine **4**, either or both of which may undergo further reaction. The rate of the disproportionation reaction strongly depends on the degree of substitution of the α-carbon atoms and on the solvent.[69] The following scheme for the decomposition has been proposed by Ingold.[69,71] The structure of the dimer **2** must be regarded as uncertain.[70,72] In fact, thermochemical considerations[69,72] suggest that formation of an O–O bond as in **2** may be endothermic by as much as 28 kcal/mol.



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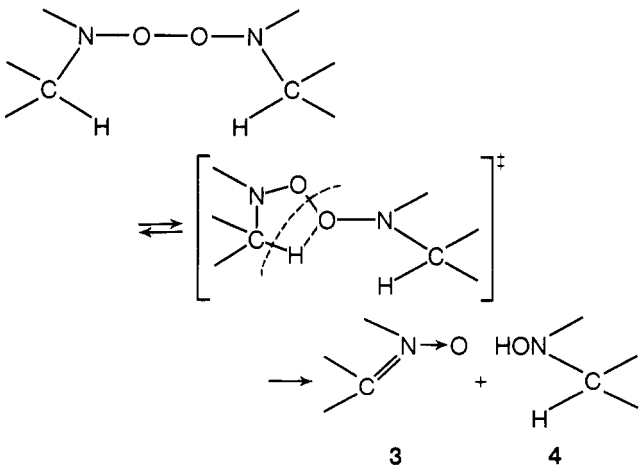
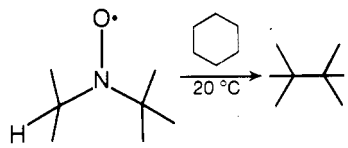


3 **4**

Briere and Rassat[73] have studied in some detail the decomposition of *tert*-butyl isopropyl nitroxide (**5**) in cyclohexane and have determined some of the products (eq 1). The reaction shows a very large primary kinetic isotope effect.

The presence of substituents on the α-carbon atoms not only prevents the above disproportionation reaction, but also the tendency toward dimerization is suppressed by the substituents, particularly in the solid state. In general, even relatively unhindered nitroxides show only a small tendency toward dimerization in solution. This was elegantly demonstrated by Rassat[74] with



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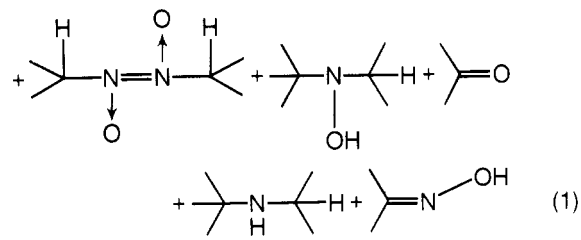
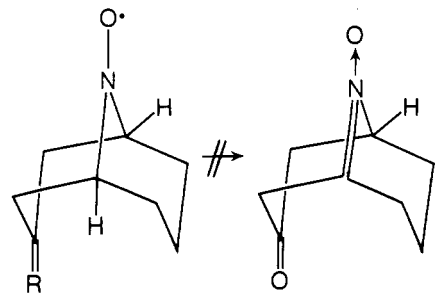


(1)

his preparation and study of the bicyclic nitroxide norpseudopelletierine-*N*-oxyl (**6**).

Nitroxide **6** could be obtained in pure crystalline form. Its ultraviolet, infrared, and ESR spectra in solution demonstrated the substance to be monomeric. The unusual stability of **6** toward

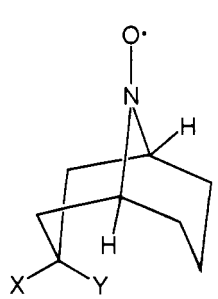


6, R = O
7, R = H_2

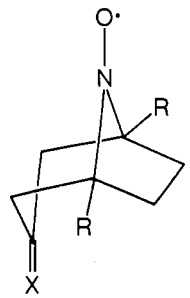
8

the disproportionation reaction is attributed to the observation that disproportionation would produce a nitrone **8** in which the carbon–nitrogen double bond would involve the bridgehead carbon atom, in violation of Bredt's rule.

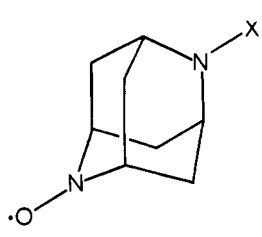
Several more quite stable bicyclic nitroxides have been prepared, including **7**,[72] **9–11**,[72] **12**,[75] **13**,[75] **14**,[76] **15**,[76] and **16**.[77] While single crystals of **11** are diamagnetic, those of **7** are



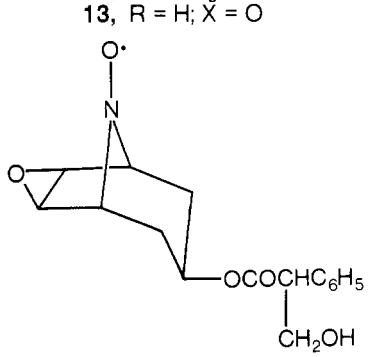
9, X = OH; Y = H
10, X = H; Y = OH



11, R = H; X = H_2
12, R = CH_3; X = O
13, R = H; X = O



14, X = CH_3
15, X = O·



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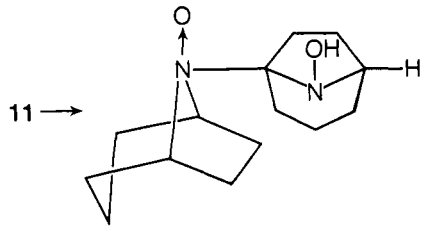
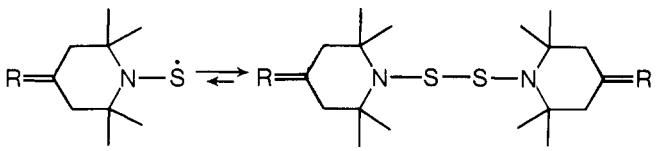


17

strongly paramagnetic. Solutions of **11** irreversibly afforded a dimeric product **17** upon standing.[78] Interestingly, dinitroxide **15** does exist as a dimer (or higher aggregate) in DMF solution (0.02 M) at room temperature. An ESR spectrum attributed to the monomeric species was observed by warming the DMF solution to 100 °C.[76]

Two interesting thionitroxides **18** and **19** were described by Bennett, et al.[79] in 1967. These molecules exist in the dimeric form **20** in solution at room temperature despite the presence of the bulky methyl groups on the α carbons. Apparently, both the greater size of the sulfur atom compared with oxygen and the greater strength of the S–S bond relative to the O–O bond contribute to the strong tendency of **18** and **19** to dimerize. The

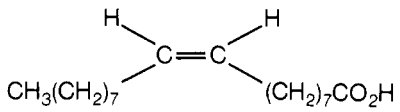


18, R = H_2
19, R = O

20

monomeric forms of both molecules were observed by ESR spectroscopy when either the solid form or a concentrated solution of the dimer in iodobenzene solution was heated. It is conceivable that valuable spin labels could be synthesized which capitalize on this tendency of thionitroxides to undergo reversible dimerization.

Relatively few nitroxides bearing one or more hydrogens on the α-carbon atoms have been actually used in spin-labeling studies. Keith[32] has claimed a procedure (**21** → **22**) for the attachment of an oxazolidine-*N*-oxyl ring at the site of a carbon–carbon double bond. The resulting nitroxide(s) showed a half-life



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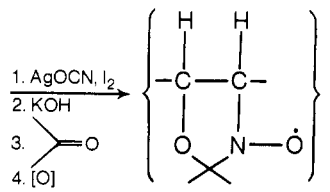
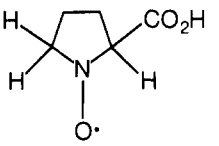


22

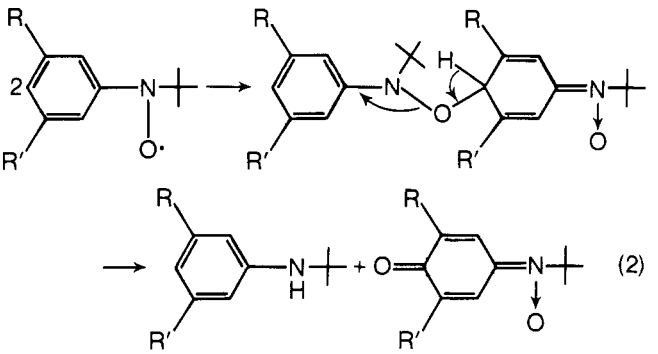
in ethyl oleate at 40 °C of about 2 h, the limited stability being attributed to the presence of a hydrogen on the α carbon. This potentially important method needs to be confirmed, however, since the intermediates and products were incompletely characterized and no details for the final oxidation step were provided.

Olcott[80] has prepared crystalline proline nitroxide (**23**) by oxidation of *N*-hydroxy-L-proline with *tert*-butyl hydroperoxide in aqueous ethanol. Nitroxide **23** was unexpectedly stable, showing a half-life in phosphate buffer at pH 7 of 16.5 h at 24 °C.



23

Conjugation of the nitroxide moiety with other groupings sometimes leads to nitroxides of considerable stability, and hence of potential applicability to the spin-labeling field. Chart I shows several representative conjugated nitroxides, all of which can be isolated in pure form. Certain of these do undergo slow decomposition. For example, Forrester[81] has shown that aryl *tert*-butyl nitroxides in general dimerize by coupling of the nitroxide oxygen atom to the para position of another molecule, giving after fragmentation, *p*-benzoquinone *tert*-butylimine *N*-oxides and *tert*-butylanilines (eq 2). Initial dimer formation is inhibited either by the presence of an ortho substituent which causes the aryl ring to be twisted out of conjugation with the nitroxide[82] or by the presence of certain substituents such as a *tert*-alkyl or methoxy group at the para position.[81,83]

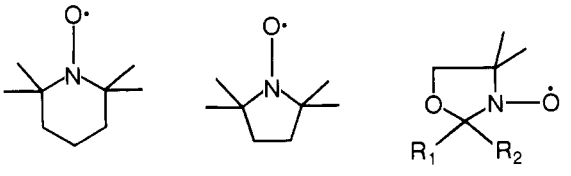


(2)

Chart II shows several representative conjugated nitroxides which, in general, have not been obtained in pure form owing to a limited stability. These molecules are included here, often with some indication of stability, for purposes of comparison with those which are stable.

B. Basic Building Block Nitroxides

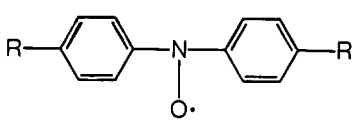
To date essentially all biological nitroxide spin-labeling studies[42] have utilized derivatives of three simple stable nitroxides. These are 2,2,6,6-tetramethylpiperidine-*N*-oxyl (**66**) (sometimes referred to as TEMPO), 2,2,5,5-tetramethylpyrroline-*N*-oxyl (**67**), and 4,4-dimethyloxazolidine-*N*-oxyl (**68**) (a doxyl nitroxide).



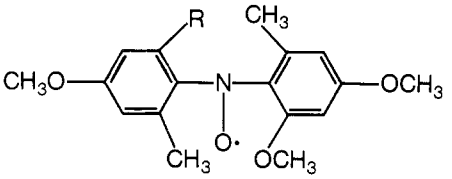
66 **67** **68**

Typically (see below, however), structures **66** and **67** bear a functional group at the 4 and 3 positions, respectively, capable of undergoing chemical reaction with some functional group of the biomolecule to be spin labeled. The doxyl group is normally attached to the molecule of interest at the site of a ketone group. Chemically reactive nitroxide spin labels have been divided by Morrisett[53] into four classes, relating to their use in the spin labeling of enzymes: alkylating agents, acylating agents, sulfonating agents, and phosphorylating agents. Representative members of each class are shown in Charts III–VI. Chart VII is a collection of miscellaneous chemically reactive nitroxide spin labels, which do not fall into the four other classifications. Ad-

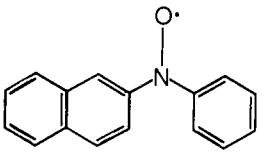
CHART I. Representative Isolable Nitroxides Stabilized by Conjugation



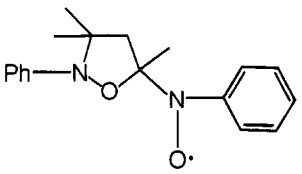
24, R = H;[84-87] CH_3, NO_2;[85,86] CH_3O[85,86,88-90]



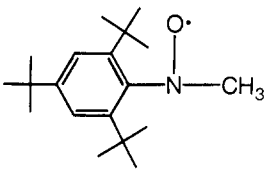
25, R = CH_3;[88,91] OCH_3[91]



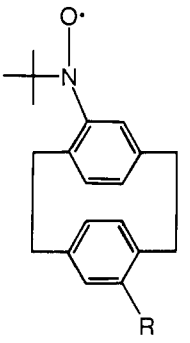
26[92]



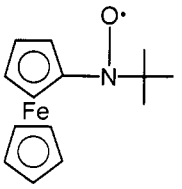
27,[93-95] Banfield and Kenyan nitroxide



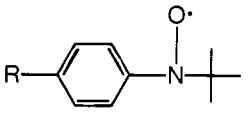
28[70]



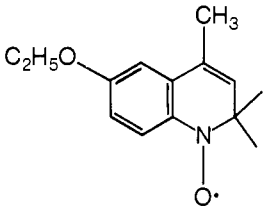
29, R = •ON-*t*-Bu;[96] H[97]



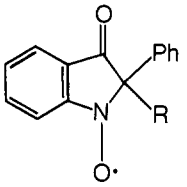
30[98]



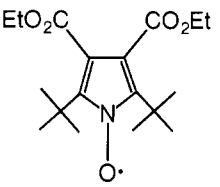
31,[99] R = C_2H_5, $CH(CH_3)_2$, *n*-C_4H_9, $C(CH_3)_3$, $Si(Me)_3$



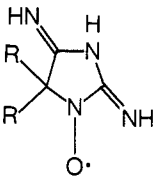
32[100]



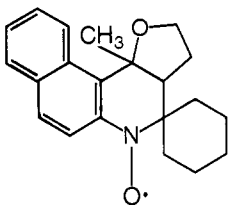
33,[101] R = Me, Et, Ph, $PhCH_2$



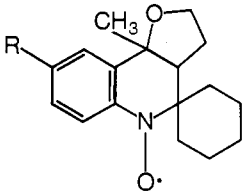
34[102]



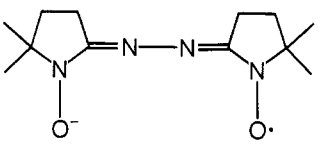
35, R = CH_3;[103-105] $-(CH_2)_5-$[106]



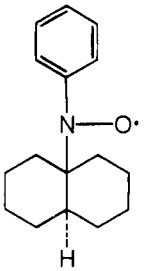
36[107]



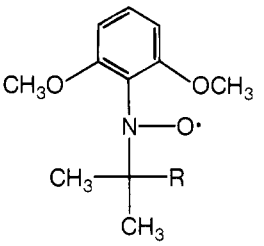
37,[108] R = H, CH_3O



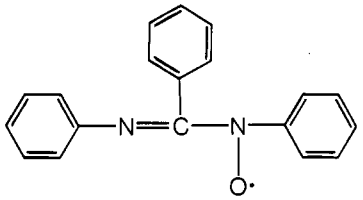
38[109]



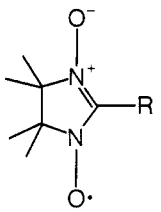
39[110,111]



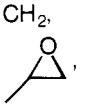
40,[112] R = CH_3, Ph

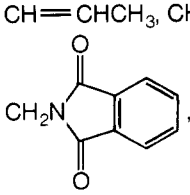


41[113]

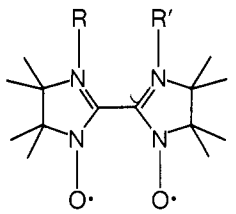


42, R = Ph;[114] H, CH_3, CH_3CH_2, $CH_3(CH_2)_{12}$, CH_2OH, $CH(OC_2H_5)_2$;[115] I, Br, CN, $C(=NH)OCH_3$;[116] *p*-$C_6H_4NO_2$, *p*-C_6H_4OH, *p*-$C_6H_4N(CH_3)_2$, $CH(CH_3)_2$, $C(CH_3)_3$, $CH_2CH(CH_3)_2$, $CH_2C_6H_5$;[117] $CH{=}CH_2$, $CH{=}CHCH_3$, $CH{=}CHC_6H_5$, $C{\equiv}C_6H_5$,

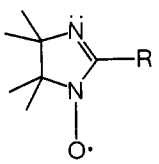




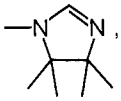
, CH_2NHTs, CH_2Cl, CH_2Br, CH_2I, CF_3[117]



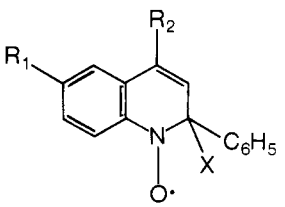
43,[118] R = R′ = an electron pair; R = O, R′ = an electron pair



44,[119] R = Ph, $CH_2CH(CH_3)_2$, $CH(CH_3)_2$, $C(CH_3)_3$, OCH_3, NH_2, $N(CH_3)_2$, $NHCH(CH_3)_2$,

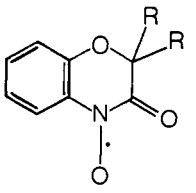


, I, Br, CN

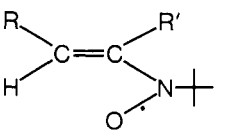


45,[120] R_1 = R_2 = H; R_1 = H; R_2 = OEt, $C(OH)(C_6H_5)_2$; R_1 = OMe; R_2 = H, Cl; X = Me, Et, C_6H_5

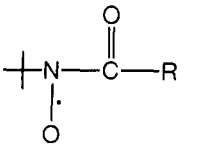
CHART II. Conjugated Nitroxides Which Are Unisolable



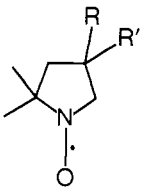
46,[121]
R = H, Me
($t_{1/2,\ dioxane}$ = 1 h)



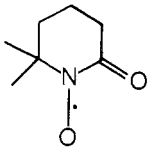
47[122]
R = $C_6H_5SO_2$; R′ = H
($t_{1/2,\ CHCl_3}$ = 2 h)
R = $C_6H_5SO_2$; R′ = C_6H_5
($t_{1/2,\ CHCl_3}$ = 10 min)
R = R′ = CO_2CH_3
($t_{1/2,\ Et_2O}$ = 2 h)



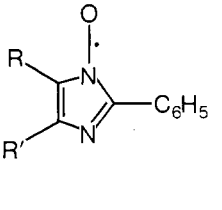
48[123]
R = H, CH_3, C_2H_5, *i*-C_3H_7, *t*-C_4H_9, C_6H_5
($t_{1/2,\ benzene}$ = several hours)



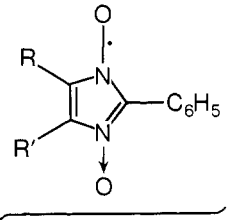
49[123]
R = R′ = H
R = H; R′ = CH_3
R = R′ = CH_3
($t_{1/2,\ benzene}$ = several hours)



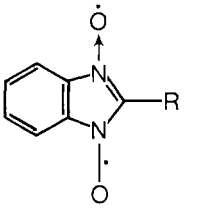
50[123]
($t_{1/2,\ benzene}$ = several hours)



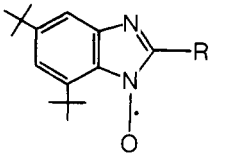
51[124]
R = R′ = C_6H_5



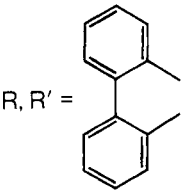
52[124]



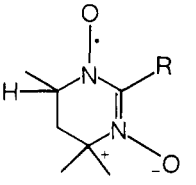
53
R = H[125]
R = C_2H_5, C_6H_5[126]



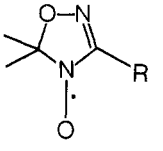
54[127]
R = *t*-C_4H_9; 2,4,6(MeO)$_3$–C_6H_2
(stable in benzene solution)



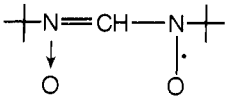
R, R′ =
(unstable in solution)



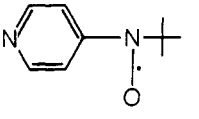
55[128]
R = H, Me
($t_{1/2}$ = "hours")



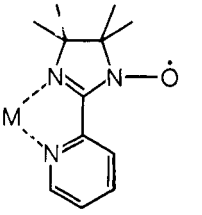
56[127]
R = C_6H_5, CH_3, $CH(CH_3)_2$, $C(CH_3)_3$
(stable for several hours in solution)



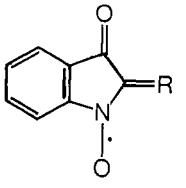
57[129]
(stable for 1 day in benzene solution)



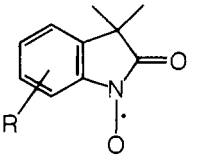
58[130]



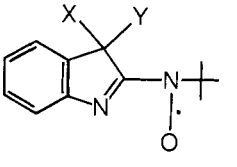
59[131, 132]
M = Zn, Cd, Hg



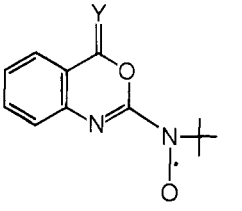
60[133]
R = C ($t_{1/2,\ benzene}$ = 4 h)
R = $NC(CH_3)_3$, $NCH(CH_3)_2$
($t_{1/2,\ benzene}$ = 30 min)



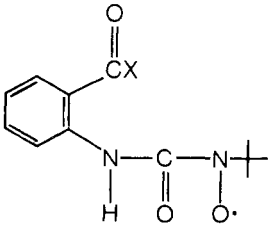
61[134]
R = H, 6-$C(CH_3)_3$, 6-C_6H_5, 6-NHAc, 6-Br, 6-NO_2, 5,7-$[C(CH_3)_3]_2$



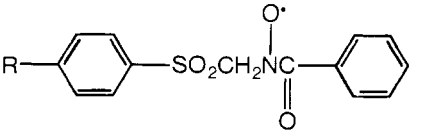
62[135]
X, Y = O
X = NH_2; Y = OH
X = CN; Y = OH
X = NEt_2; Y = OH or NEt_2



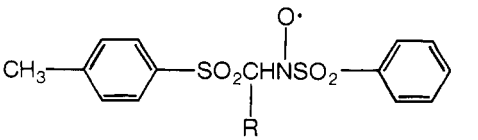
63a[135]
Y = O, $NC(CH_3)_3$



63b[135]
X = OH, NH_2

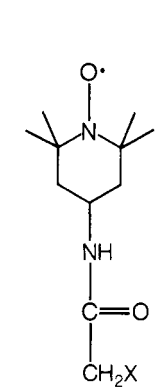


64[136]
R = H, CH_3, CH_3O, NO_2
(stable in CH_2Cl_2 solution > 5 h)

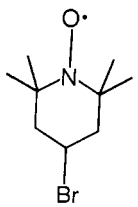


65[136]
R = H, Et
(stable in CH_2Cl_2 solution > 2 h)

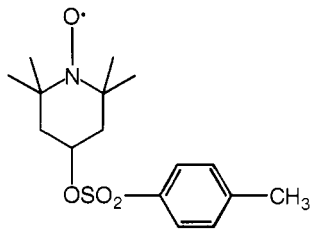
CHART III. Representative Building Block Nitroxide Alkylating Agents



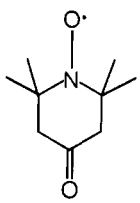
69,[137] X = Br, I



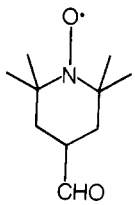
70[62]



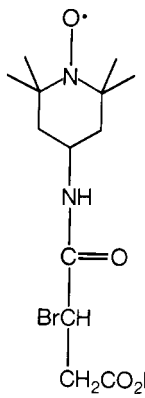
71[138]



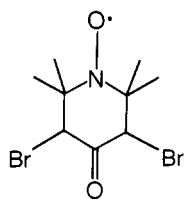
72[62]



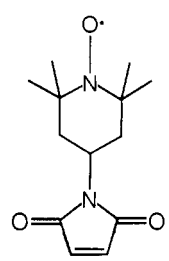
73[139]



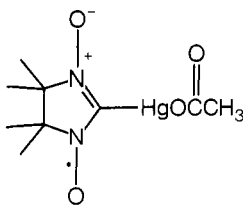
74[140]



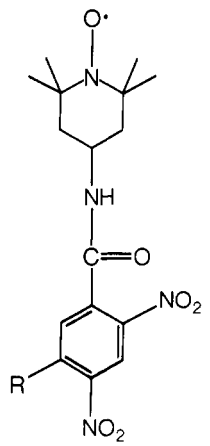
75[62, 141]



76[58, 140]

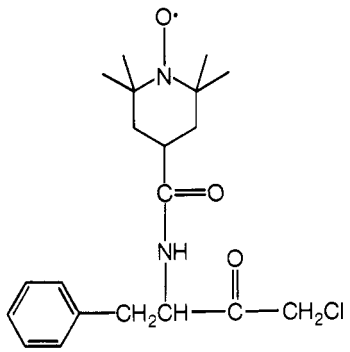


77[142]

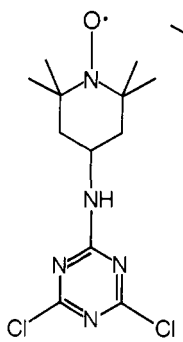


78[143]

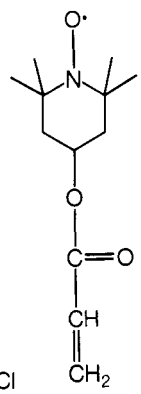
R = CH_2CH_2N—, Cl



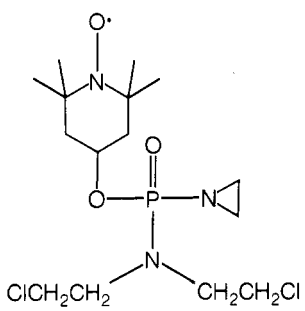
79[144]



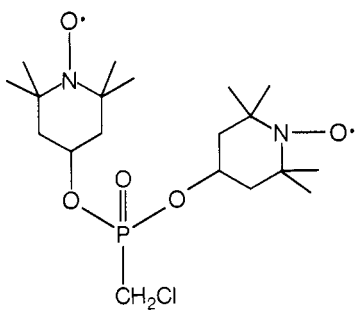
80[145]



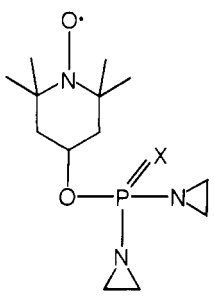
81[62]



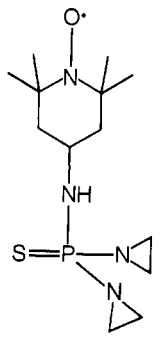
82[146]



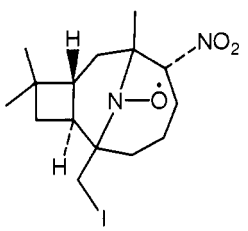
83[147]



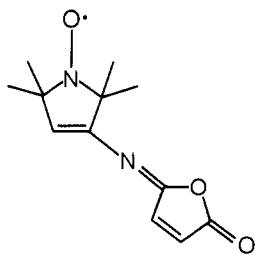
84,[148, 148a] X = O, S



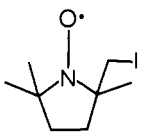
85[146, 148]



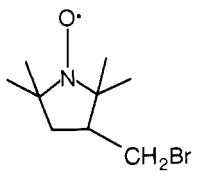
86[149]



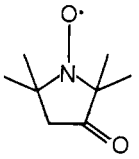
87[150]



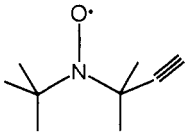
88[151]



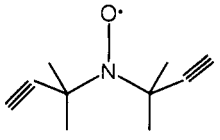
89[58]



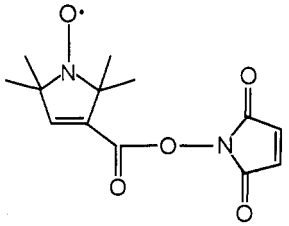
90[62]



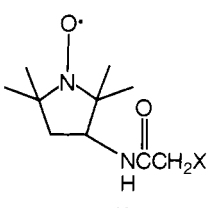
91[62]



92[62]

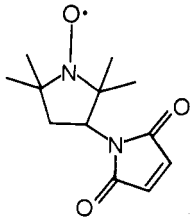


93[152]

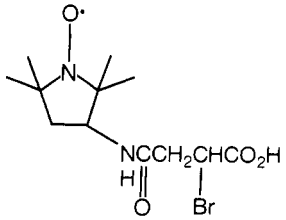


94[153]

X = Br, I

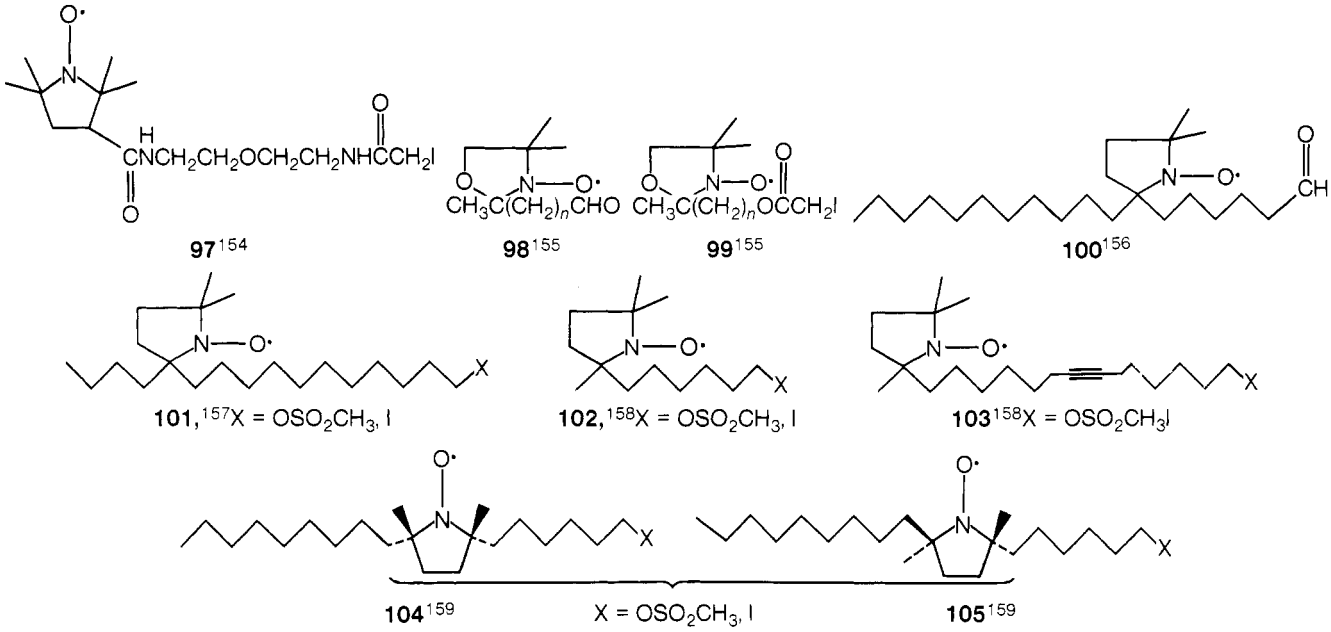


95[150]



96[140]

CHART III (Continued)

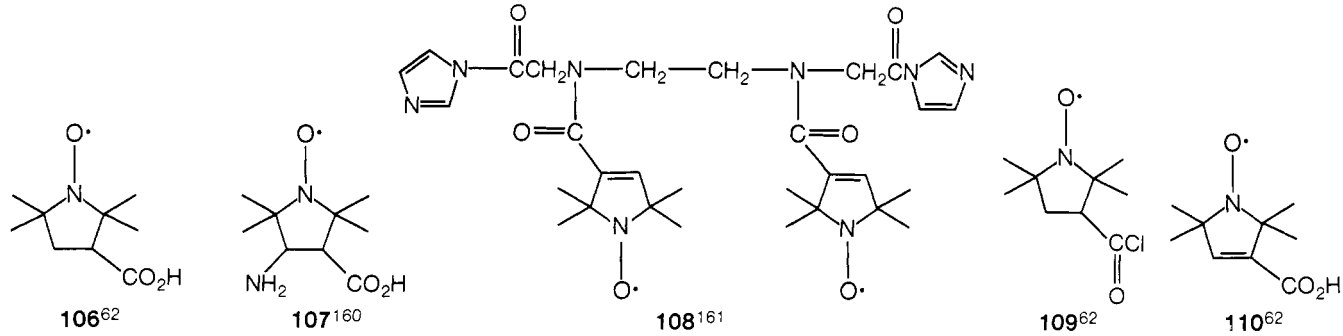


97[154] **98**[155] **99**[155] **100**[156]

101,[157] X = OSO_2CH_3, I **102**,[158] X = OSO_2CH_3, I **103**[158] X = OSO_2CH_3, I

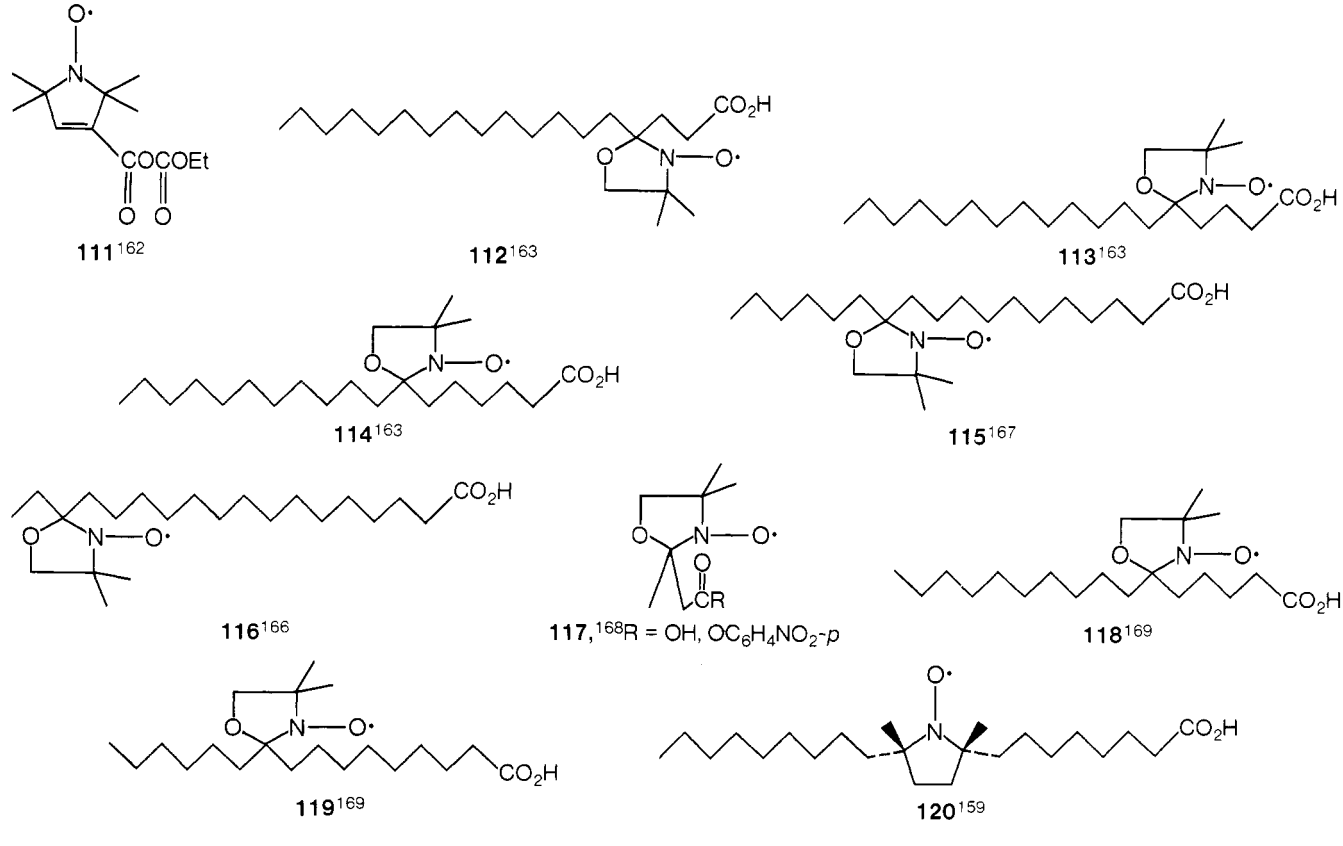
104[159] X = OSO_2CH_3, I **105**[159]

CHART IV. Representative Building Block Nitroxide Acylating Agents



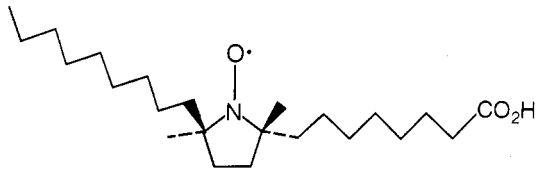
106[62] **107**[160] **108**[161] **109**[62] **110**[62]

R-(+) enantiomer[164, 165]

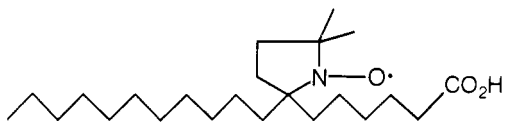


111[162] **112**[163] **113**[163] **114**[163] **115**[167] **116**[166] **117**,[168] R = OH, $OC_6H_4NO_2$-*p* **118**[169] **119**[169] **120**[159]

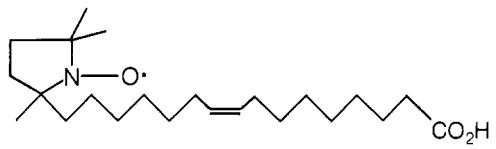
CHART IV (Continued)



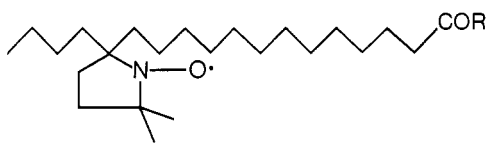
121[159]



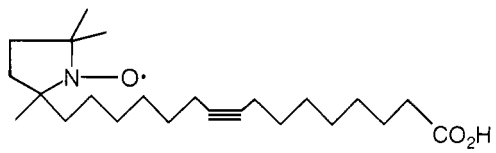
122[156]



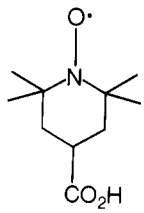
123[158]



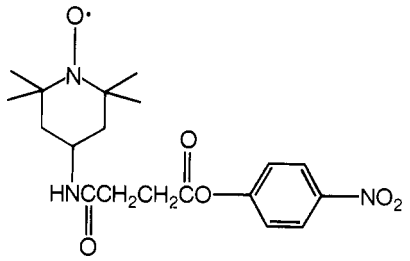
124,[157] R = OH, Cl



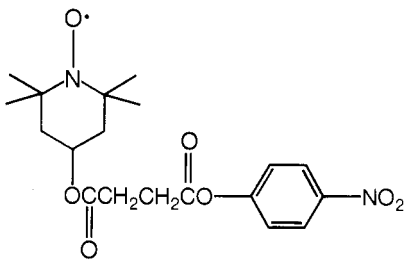
125[158]



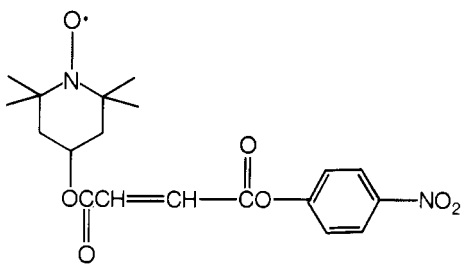
126[170]



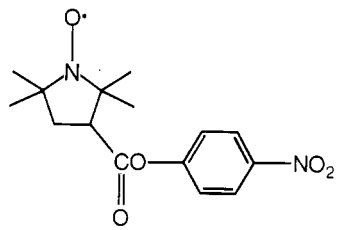
127[171]



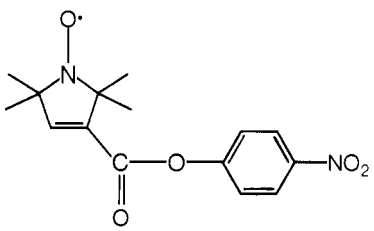
128[171]



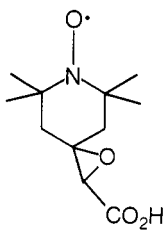
129[171]



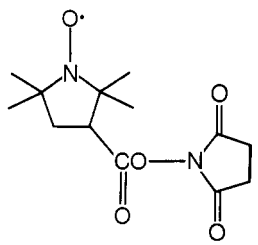
130[172]



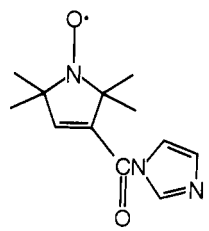
131[173]



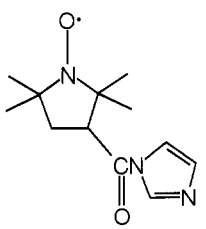
132[62]



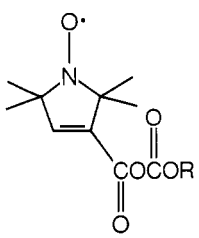
133[152]



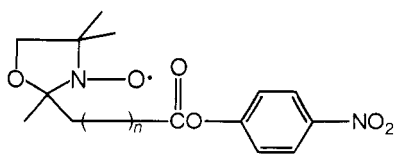
134[150]



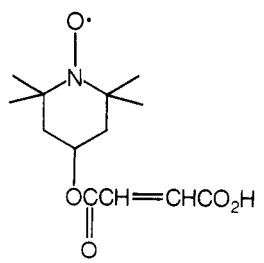
135[174]



136,[162] R = Et, C_6H_5



137[155]



138[171]

ditional examples of each class will be found in the discussion of newer methods of synthesis below.

C. Spectra of Nitroxides

a. NMR Spectra. Conventional NMR spectra of nitroxide-containing molecules are quite broad and are therefore of limited use for monitoring the changes in structure taking place during a chemical synthesis. The line broadening comes about because the unpaired electron in the nitroxide group interacts with nuclear spins and thereby causes rapid intramolecular and intermolecular relaxation of nearby protons. Valuable information, nevertheless, such as the sign and magnitude of the proton hyperfine interaction can be obtained from chemical shifts in the NMR spectra of nitroxide free radicals.[12-17] Typically NMR spectra are determined at concentrations as high as 3 M in order that the electron spin relaxation time becomes shorter than the inverse of the proton hyperfine coupling constant.[193] Alternatively though

CHART V. Representative Building Block Nitroxide Sulfonating Agents[175,176]

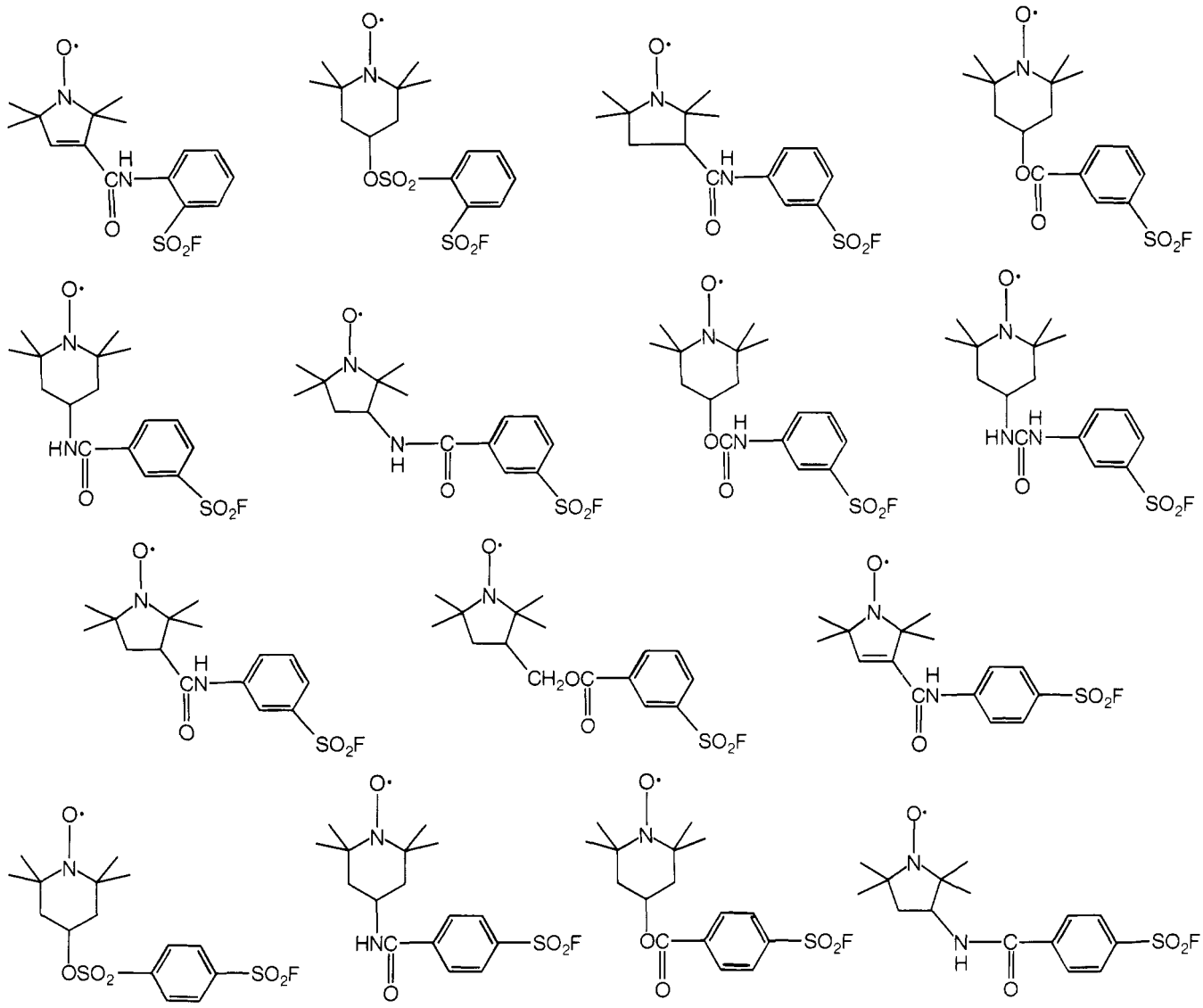
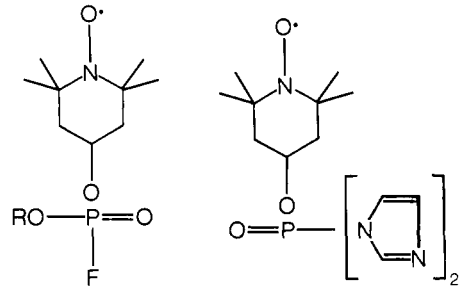
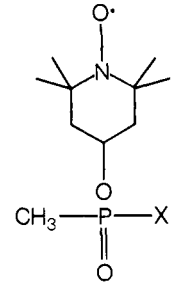


CHART VI. Representative Building Block Nitroxide Phosphor(n)ylating Agents

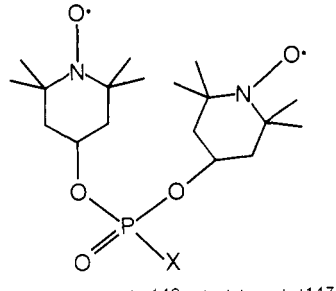


139, R = Me, *i*-Pr, *c*-C_6H_{11};[177] Et[178]

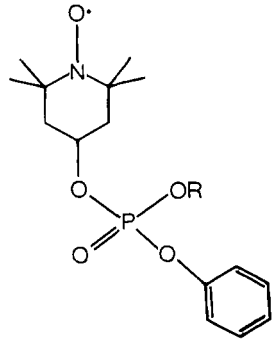
140[147, 179, 180]



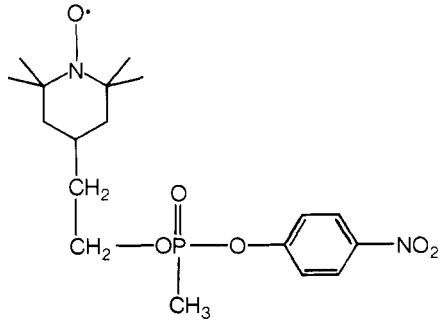
141, X = F;[181] $OC_6H_4NO_2$-*p*;[182] 3-doxylandrostan-17β-yl[181]



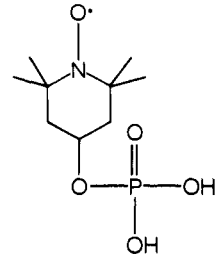
142, X = F, Cl;[146] 1-imidazolyl[147]



143, R = $C_6H_4NO_2$-*p*;[182] 1-imidazolyl;[183, 184] H[183]

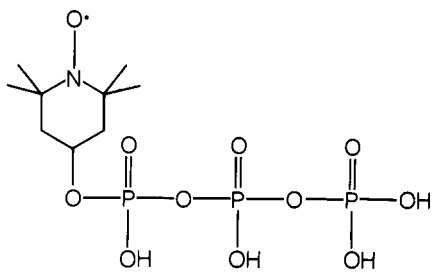


144[182]

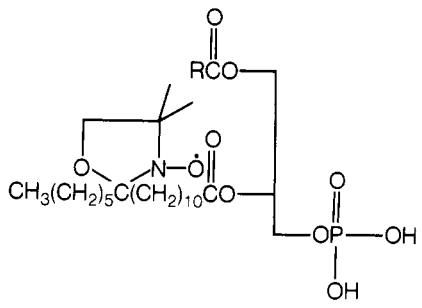


145[185]

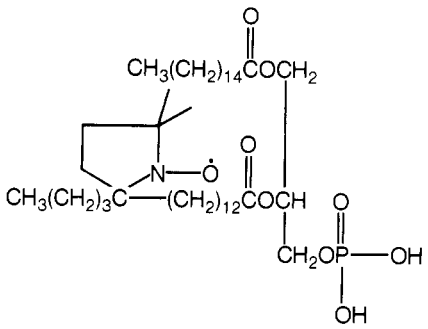
CHART VI (Continued)



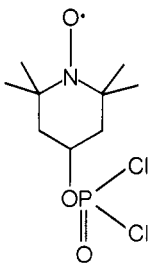
146[26]



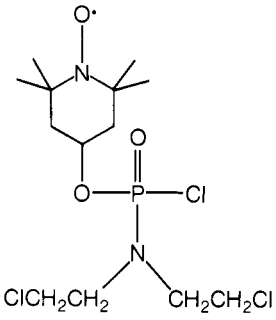
147[186]



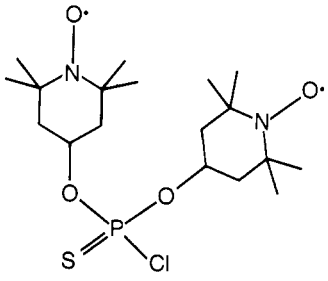
148[157]



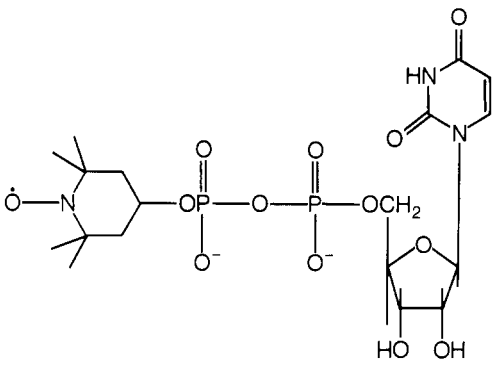
149[147]



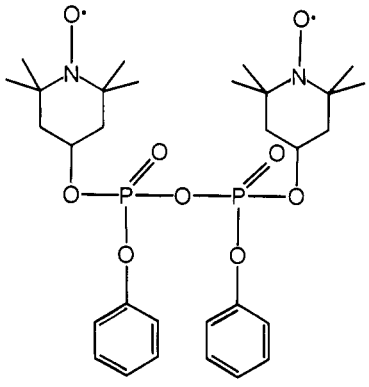
150[146]



151[146]

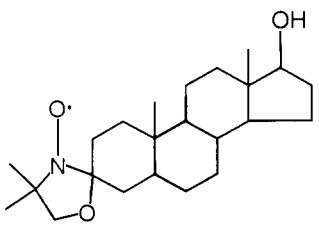


152[187, 187a]

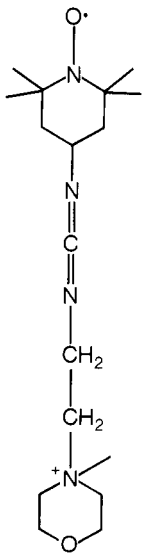


153[183]

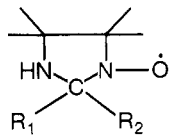
CHART VII. Miscellaneous Building Block Nitroxide Spin Labels



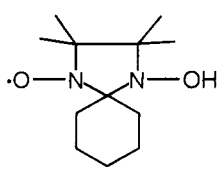
154[188]



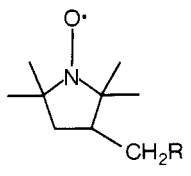
155[189]



156,[190] $R_1, R_2 = -(CH_2)_5-$; $R_1 = R_2 = CH_3, CH_3CH_2CH_2$

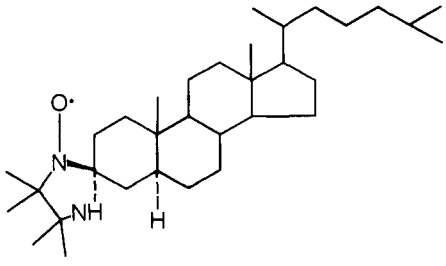


157[190]

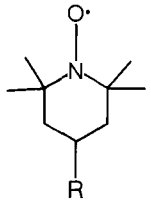


158, R = NH_2;[192] OH[58]

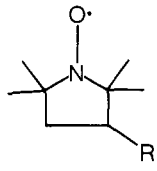
CHART VII (Continued)



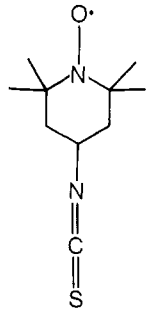
159[190]



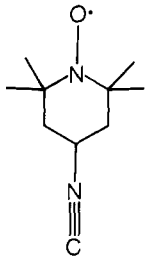
160,[62] R = NH_2, OH



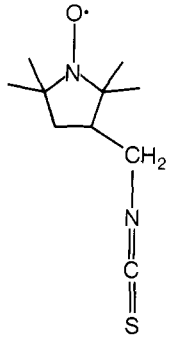
161,[62] R = NH_2, OH



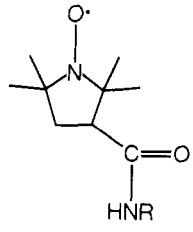
162[58]



163[191]

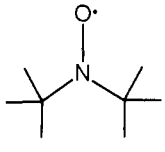


164[142]



165,[142] R = $(CH_2)_2N{=}C{=}S$, $(CH_2)_3N{=}C{=}S$, $(CH_2)_2O(CH_2)_2N{=}C{=}S$

less conveniently, one may employ a paramagnetic solvent such as di-*tert*-butyl nitroxide (**166**), 2-doxylpropane (**167**), or the corresponding completely deuterated analogues[194] as spin-relaxer[195] solvents for NMR studies of nitroxide free radicals.



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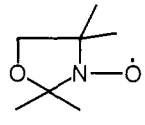
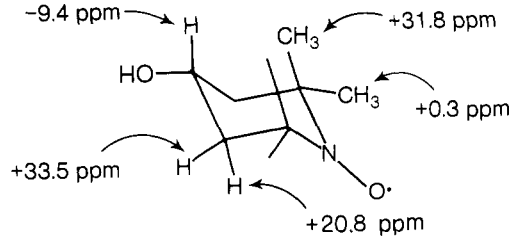


167

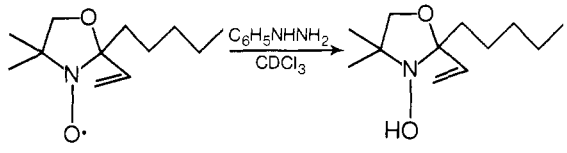
Spin density on a given proton gives rise to relatively large chemical shifts (compared with a diamagnetic molecule of similar structure) as can be seen in the NMR spectrum of nitroxide **168.**[16,196]



168

Lee and Keana[197] recently described a simple method for gaining structural information on a nitroxide by NMR spectroscopy. The method involves the addition of ~1.5 equiv of phenylhydrazine to the NMR tube containing the nitroxide dissolved in $CDCl_3$. The nitroxide is reduced in situ to the diamagnetic *N*-hydroxy derivative (for example, **169** → **170**). The nitroxide may be recovered by air oxidation in MeOH containing a trace of cupric ion. Rassat and Rey[198] have independently used the in situ phenylhydrazine reduction technique for structural studies on a series of tetrahydrooxazine nitroxides (see below).

Kreilich[199,200] has measured the ^{13}C NMR spectra of several nitroxides.



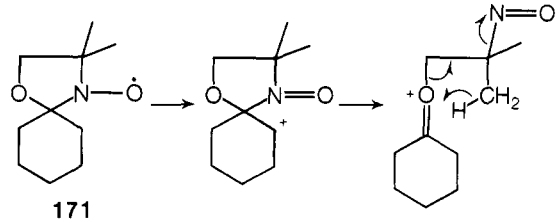
169 170

b. Infrared Spectra. Rassat[201] has reported the infrared stretching frequency of several piperidine nitroxides to be ν_{NO}[14] = 1373 ± 7 cm^{-1}. He used ^{15}N-labeled nitroxides in order to confirm the band positions. Other nitroxides show absorption in the range 1310–1370 cm^{-1}.[61]

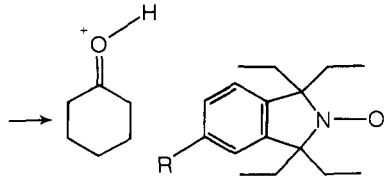
c. UV-Visible Spectra. Nitroxides are yellow in color owing to an absorption band in the region 410–450 nm (ϵ <20) in the visible region. A much more intense maximum is observed at about 230 nm (ϵ ~3000).[61]

d. Circular Dichroism. Rassat[202] has measured the circular dichroism spectra of a number of optically active nitroxides. Results are interpreted in terms of an octant rule similar to the one for ketones.

e. Mass Spectra. The mass spectra of a series of piperidine,[203] pyrrolidine,[204] and doxyl[205] nitroxides have been measured and interpreted. The base peak in the mass spectra



171



172 173, R = H, NO_2, NH_2, $NHC_6H_2(NO_2)_3$

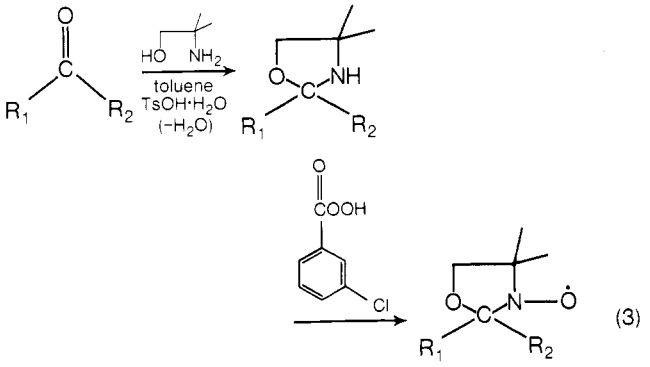
of simple doxyl nitroxides is that of the corresponding protonated ketone (for example, **171** → **172**). The mass spectra of nitroxides **173** have also been interpreted.[206]

Recently, a gas chromatography–mass spectroscopy procedure for the identification of isomeric doxyl stearic acids has been described.[207]

III. Newer Methods of Nitroxide Spin Label Synthesis

A. Doxyl Nitroxides

A general method for the rigid attachment of a stable nitroxide grouping at the site of a ketone group in a molecule was introduced by Keana et al.[208] in 1967. Thus, condensation (eq 3) of



(3)

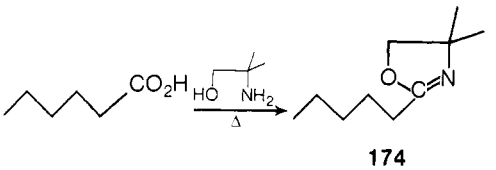
a doxyl nitroxide

the ketone with 2-amino-2-methylpropanol in benzene, toluene, or xylene using toluenesulfonic acid monohydrate as catalyst affords the corresponding oxazolidine.[209] The yield of oxazolidine derived from sluggishly reacting ketones is generally significantly improved and reaction time shortened by the use of xylene as solvent and a Dean–Stark water separator containing anhydrous K_2CO_3.[210] With certain hindered ketones, the amino alcohol has been used as solvent.[211] Oxidation of the oxazolidine with *m*-chloroperbenzoic acid (MCPA) then affords the corresponding stable nitroxide, typically in about 30% yield (based on starting oxazolidine). Such nitroxides are generally referred to as doxyl nitroxides, which is an acronym for 4,4-dimethyloxazolidine-*N*-oxyl.

Charts IV, VI, and VII include several representative doxyl nitroxide spin labels which have been prepared by this method. The doxyl group has also been attached to ketone groups of a polymer.[212]

Recently, an alternative synthetic route to doxyl nitroxides which bipasses the usual ketone precursors was described.[213,214] Advantage is taken of the availability of a variety of 2-substituted 4,4-dimethyloxazolines from the elegant work of Meyers.[215] Oxidation[214,216] of the representative oxazoline **174** with MCPA led to the corresponding oxaziridine **175** which underwent isomerization to the nitrone **176** upon chromatography over silica gel. Reaction of **176** with several organometallic reagents led to the *N*-hydroxy intermediate **177** which could be easily oxidized to the doxyl nitroxide **178** with air in the presence of Cu^{2+} ion. Analogous reactions of nitrone **179** with methyllithium and butyllithium afforded tetrahydrooxazine nitroxides **180** and **181.**

It is well to point out that the peracid oxidation step occurs before the addition of the organometallic reagent; therefore, the organometallic fragment may well contain peracid-sensitive groups such as a carbon–carbon double bond. Thus, **178** (R = vinyl) could be prepared by this method in 29% yield.[214] Equation 11, however (see below), demonstrates that in certain cases, at least, it is feasible to form a nitroxide by oxidation with MCPA in the presence of a carbon–carbon double bond.



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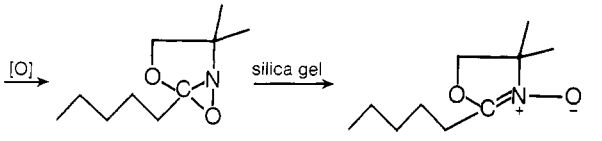
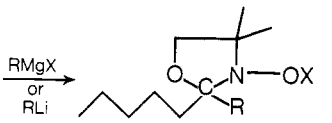
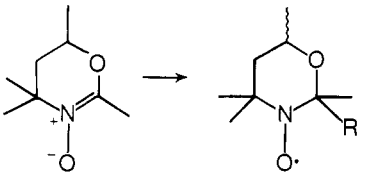


175 **176** (moisture sensitive)



177, X = H
178, X = ·

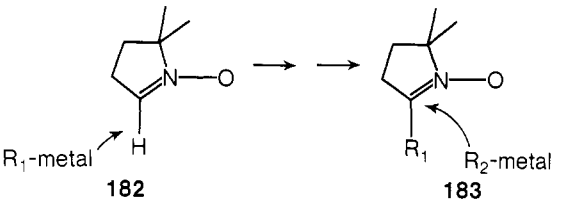


179 **180**, R = CH_3
181, R = $CH_3(CH_2)_3$

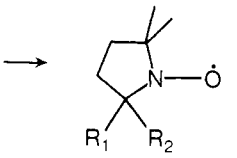
B. Proxyl Nitroxides

When compared with the pyrrolidine-*N*-oxyl nitroxide ring system, doxyl nitroxides at times show two shortcomings owing to the presence of the ring oxygen atom of the doxyl nitroxides, namely, a diminished chemical stability, and an increased polarity of the spin label.

Synthetic routes have now been developed[156] leading to a series of side-chain-substituted 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (proxyl) nitroxide lipid spin labels **184.** Thus, commercially available (Aldrich Co.) nitrone **182** is treated with an

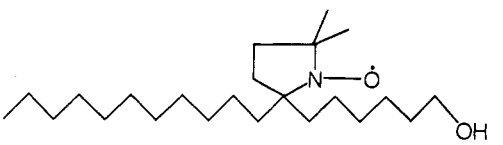


182 **183**



184

a proxyl nitroxide



185

186 **187**

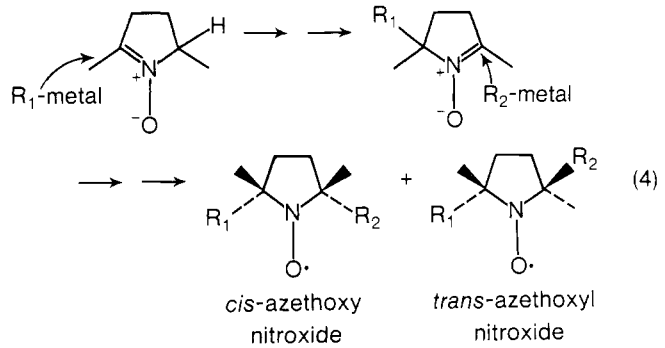
organometallic reagent, affording after Cu^{2+}–air oxidation of the intermediate, a new nitrone **183.** Reaction of **183** with a second organometallic reagent which also bears a protected alcohol group at the end of the chain followed by Cu^{2+}–air oxidation of the product gives the proxyl nitroxide **184.** Representative proxyl nitroxides prepared in this way are included in Charts III, IV, and VI.

The chemical inertness of the proxyl nitroxide grouping in these new lipid spin labels was demonstrated[156] by the several oxidation and substitution reactions performed on proxyl alcohol **185,** leading to the synthesis of, for example, proxyl nitroxides **100** and **122.** Experiments involving the partitioning of proxyl nitroxide **186** and doxyl nitroxide **187** between dodecane and water indicated the significantly lower polarity of the proxyl group. Moreover, the ESR spectrum of proxyl nitroxide **186** was more sensitive to changes in polarity of the medium than that of doxyl nitroxide **187.**

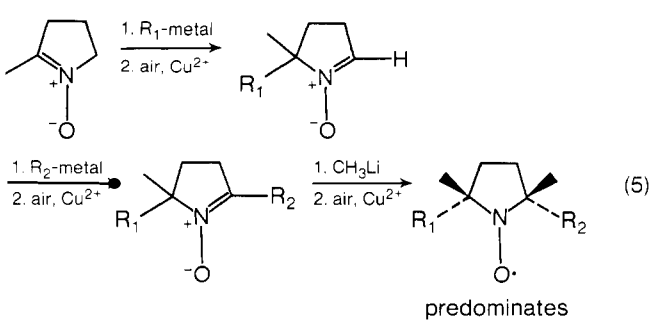
C. Azethoxyl Nitroxides (Minimum Steric Perturbation Spin Labels)

One criticism of the spin-labeling method has been that the steric bulk of the spin label may perturb the system in the vicinity of the spin label; i.e., "one must ensure that the reporter group is *reporting* the news, not *making* the news".[217] A new series of nitroxide lipid spin labels which minimizes the steric perturbation of the nitroxide group on the spin-labeled molecule has recently been described.[159] This class of nitroxides is called azethoxyl[218] (2,5-dialkyl-2,5-dimethylpyrrolidine-*N*-oxyl) nitroxides in order to distinguish them from the chemically similar but structurally different proxyl nitroxides (see above). In the azethoxyl series the nitroxide nitrogen atom is actually embedded in the hydrocarbon chain. Cis–trans isomerism is possible. Molecular models suggest that the trans isomer should resemble a saturated lipid, whereas the cis isomer introduces a bend in the chain which approximates that observed with a cis carbon–carbon double bond.

The general synthetic route to the azethoxyl nitroxide spin labels is similar to that of the proxyl nitroxides, except that a different nitrone is used in the beginning (eq 4). With the route shown in eq 4, normally the trans isomer predominates, probably because for steric reasons the second organometallic reagent prefers to add to the nitrone intermediate from the less hindered side.



(4)

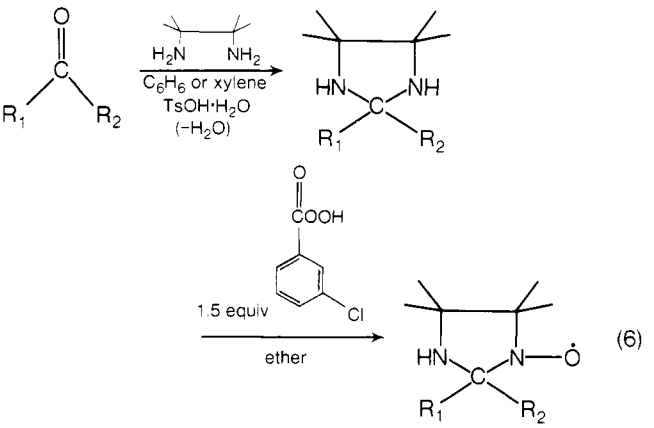


(5)

The cis isomer may be made to predominate by choosing the synthetic route outlined in eq 5. The smaller size of azethoxyl nitroxides as compared with proxyl or doxyl nitroxides was demonstrated by trapping several *cis*- and *trans*-azethoxyl nitroxides in the tubular cavities of thiourea crystals.[159] Doxyl and proxyl nitroxides, in contrast, are apparently too large to put into the cavities.

D. Imidazolidine-Derived Nitroxides

The first imidazolidine-derived nitroxide spin labels have been recently described by Keana et al.[190] The nitroxides were prepared (eq 6) by condensation of 2,3-diamino-2,3-dimethylbutane

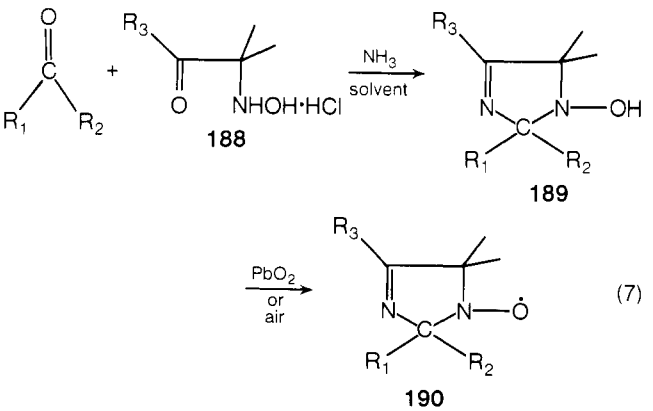


(6)

with a ketone followed by oxidation of the resulting imidazolidine with MCPA (see Chart VII for examples). Because of the rigid attachment of the nitroxide grouping to the parent molecule, this new series of labels should show many of the advantages of doxyl nitroxides in ESR spin-labeling studies. The remaining unreacted amino function additionally offers a site for the attachment of a second grouping to the nitroxide moiety via an alkylation or acylation reaction. Chart VII includes several imidazolidine nitroxides prepared by this method.

E. Imidazoline-Derived Nitroxides

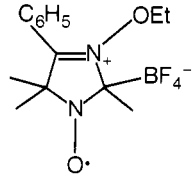
Within the past several years several classes of stable nitroxides which possess the imidazoline structure and which have some potential as spin labels have been prepared. A series of Δ^3-imidazoline nitroxides have been described by Sevastyanova and Volodarskii[219] in the Russian literature. Thus, condensation (eq 7) of 1 equiv of 2-hydroxyamino-2-methylbutanone (**188**) with a ketone in the presence of ammonia or ammonium acetate afforded the *N*-hydroxyimidazoline **189,** oxidation of which with PbO_2 afforded the nitroxide **190.**



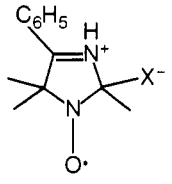
(7)

$R_1 = R_2 = CH_3$; $R_3 = C_6H_5$
$R_1, R_2 = (CH_2)_4$; $R_3 = C_6H_5$
$R_1 = R_2 = R_3 = CH_3$

The method may prove especially valuable in the synthesis of spin labels since the condensation takes place under mildly alkaline conditions as opposed to the acidic conditions normally employed for the synthesis of doxyl nitroxides. The ease with which the *N*-hydroxy intermediate may be oxidized to the nitroxide is also an advantage. Further chemical transformations with these substances and the corresponding nitrones **193** include formation of several stable salts, for example, **191** and **192.**

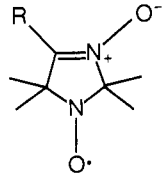


191[220]

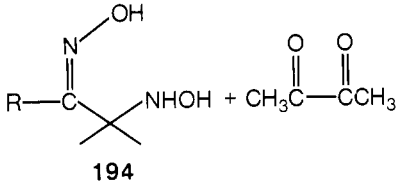


192, [220] X = ClO_4, BF_4, $SbCl_6$, $SnCl_6$, $FeCl_4$, Cl

Several interesting and versatile nitrone derivatives **193** of the Δ^3-imidazoline nitroxides have been prepared, including **195** and **196** derived by reaction of biacetyl with *N*-hydroxy oxime **194.**[228]



193, R = $CONH_2$;[221] CH_3;[222] CH_2Br;[222,223] CH_3CHBr;[224] $CHBr_2$;[225] CH_3CO;[224] *t*-BuNHCO, *i*-PrNHCO,[225] $C_6H_{11}NHCO$;[225] CO_2H;[226] $C_6H_5CH{=}CH{-}$;[226] $CH{=}NOH$[227]



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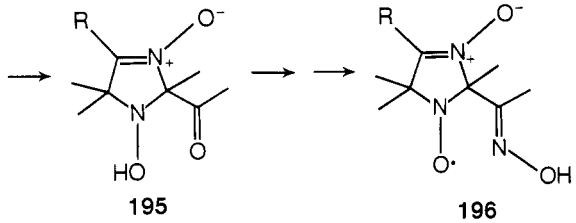
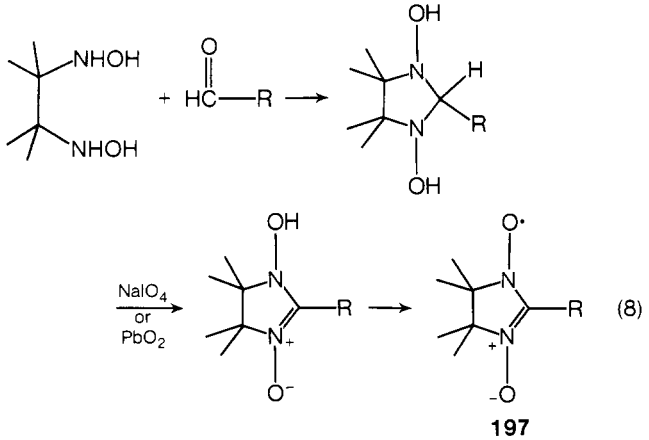


195 **196**

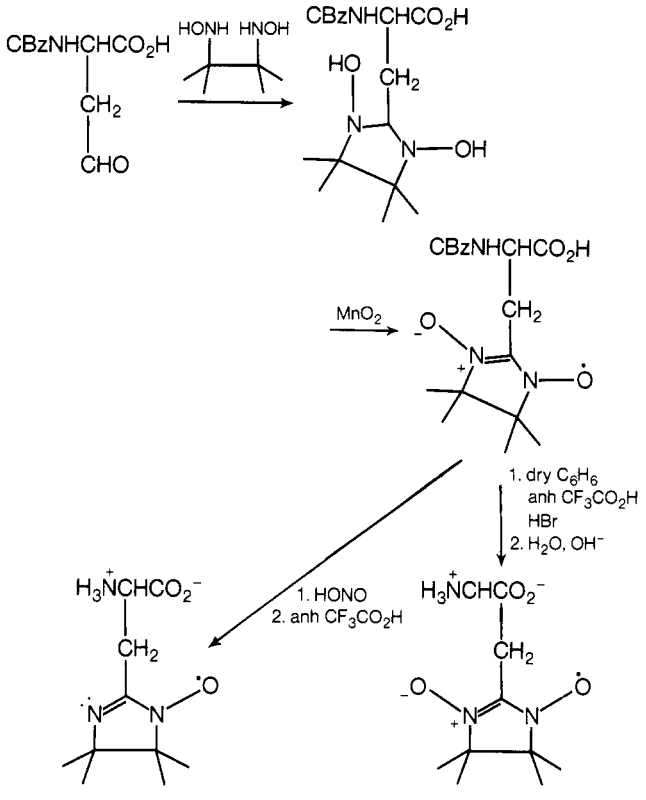
A versatile series of stable nitroxide free radicals possessing a Δ^2-imidazoline-1-oxyl 3-oxide grouping **197** has been intro-



197

(8)

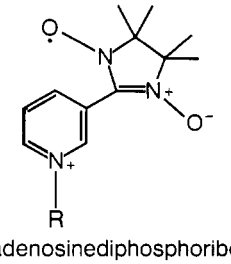
SCHEME I



duced by Ullman and co-workers.[114-117] Referred to as nitronyl nitroxides, these molecules are readily prepared by condensation of 2,3-dihydroxyamino-2,3-dimethylbutane with an aliphatic or aromatic aldehyde followed by oxidation of the resulting di-*N*-hydroxy intermediate with PbO_2 or $NaIO_4$ (eq 8). Chart I includes several examples of nitronyl nitroxides prepared by the Ullman group. One disadvantage of these molecules in spin-labeling studies is that, depending on the structure of the aldehyde, the ESR spectra are complicated by hyperfine splitting due to the α hydrogens. Thus, nitronyl nitroxides derived from α-monosubstituted aldehydes give five 1:2:1 triplets. Five sets are observed because the unpaired electron is coupled to two identical nitrogens of the imidazoline ring.

Weinkam and Jorgensen[229] have taken advantage of the structural similarity between imidazole and the nitronyl nitroxide grouping in order to prepare spin-labeled analogues of the amino acid histidine. Their route is outlined in Scheme I. These analogues were used to prepare nitroxide free radical containing peptides in the angiotensin series.[230]

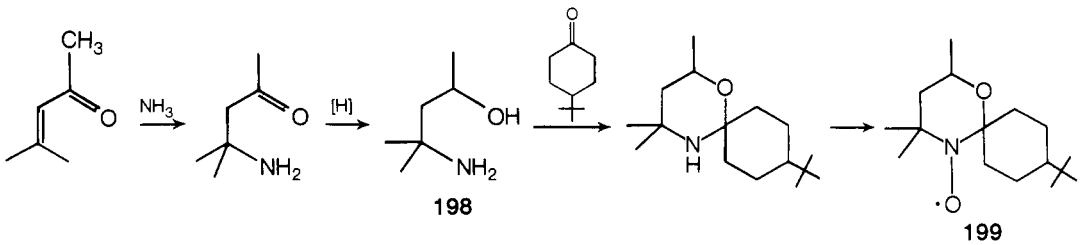
Very recently, a new nitronyl nitroxide spin-labeled analogue of nicotinamide adenine dinucleotide was described by Abdallah et al.[230a] Novel metal chelated imidazoline nitroxides **59**[131,132] have also been reported.



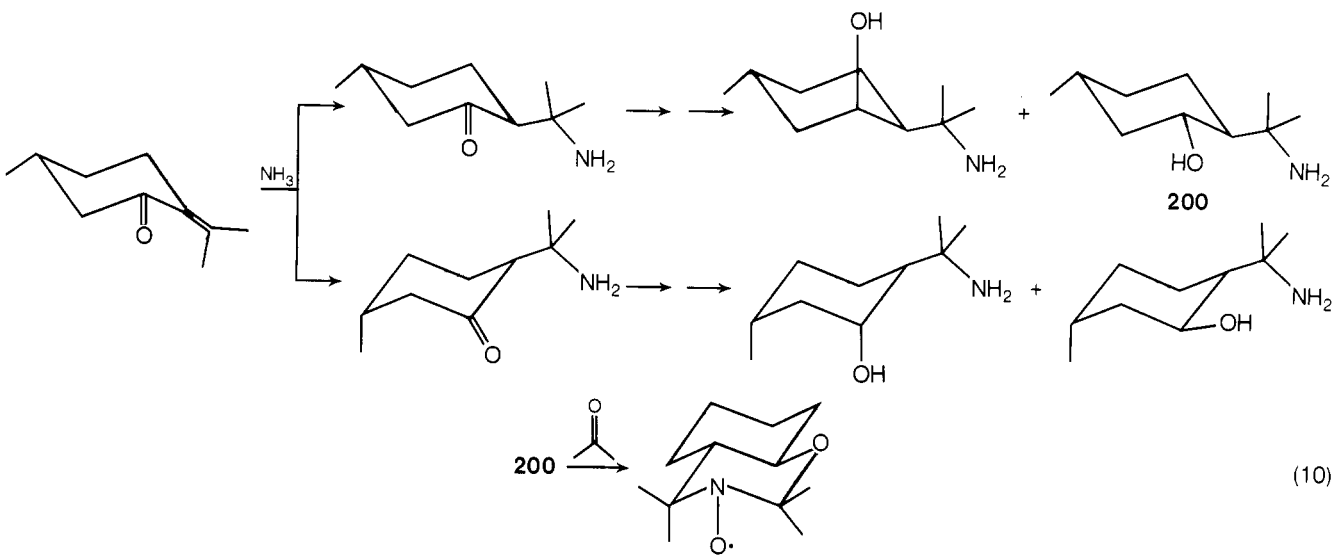
R = adenosinediphosphoribosyl

F. Tetrahydrooxazine-Derived Nitroxides

Rassat and Rey[198] have synthesized 2-amino-2-methylpentan-4-ol (**198**) and have used the material to prepare a series of tetrahydrooxazine nitroxides, for example, **199** (eq 9), in a



(9)



(10)

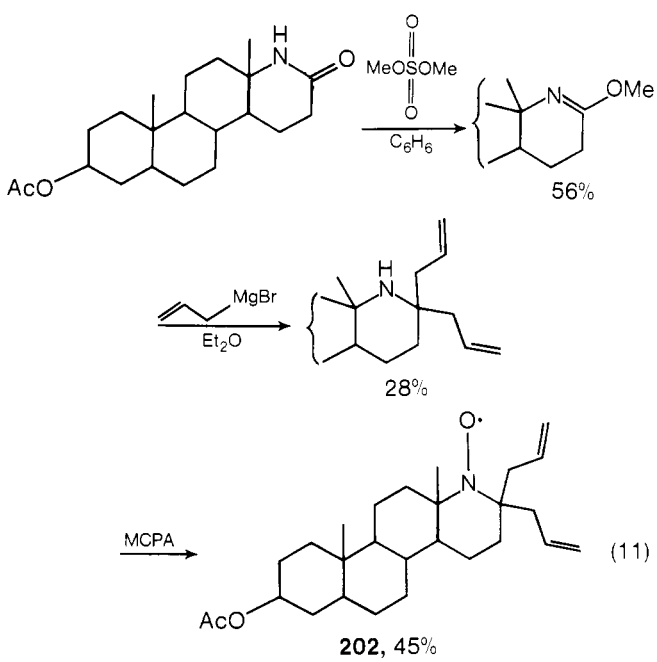
manner analogous to the synthesis of doxyl nitroxides.

An alternative synthesis of tetrahydrooxazine nitroxides has been described by Lee and Kearra[214] (see **179** → **180** and **181** above). The additional methyl substituent attached to C-6 leads to pesky isomer possibilities when unsymmetrical ketones are used.

Starting with (+)-pulegone, Rassat and Rey[198] prepared another interesting series of tetrahydrooxazine nitroxides (eq 10), one example of which is **201.** Stereochemistry in this series was established by NMR spectroscopy on the corresponding *N*-hydroxy intermediates.

G. A New Aza Steroid Nitroxide

Ramasseul and Rassat[231] have reported the synthesis of steroid nitroxide **202** in which the nitroxide group is part of the steroid skeleton. Their synthesis is outlined in eq 11 and would appear to be generally applicable for the synthesis of other unique nitroxides, starting with appropriately substituted six-membered ring lactams. A spin-labeling study of **202** with bovine serum albumin has also been described.[232]

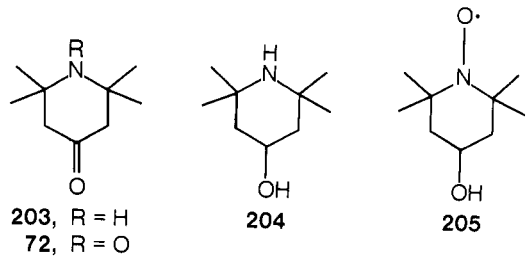


(11)

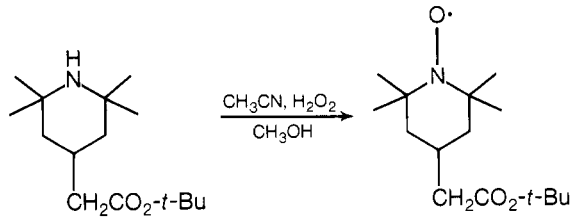
H. 2,2,6,6-Tetramethylpiperidine-*N*-oxyl and 2,2,5,5-Tetramethylpyrrolidine-*N*-oxyl Nitroxides

4-Oxo-2,2,6,6-tetramethylpiperidine (**72**) has served as the starting material for the synthesis of a wide variety of piperidine and pyrrolidine nitroxide spin labels.[58,61,62] Several of the more recent syntheses are described below. While **72** is available commercially from several sources,[42] Sosnovsky[233] has recently published an improved procedure for the synthesis of **203** by condensation of acetone with ammonia in the presence of calcium chloride. Conditions have also been worked out for the oxidation of 4-hydroxy-2,2,6,6-tetramethylpiperidine (**204**) to the corresponding nitroxide **205** in near-quantitative yield.[234]

The final oxidation of the secondary amine to the nitroxide has been investigated by Rosen et al.[235] While MCPA is an effective

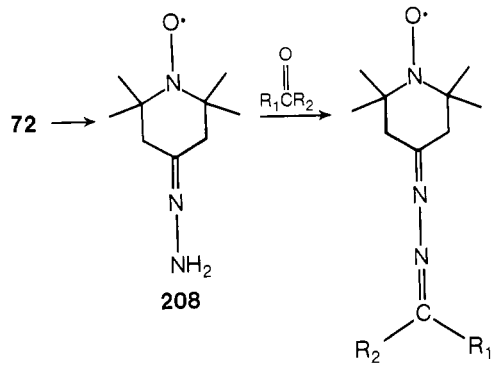


oxidizing agent for amines of limited water solubility, Rosen suggests the use of the acetonitrile–hydrogen peroxide method of Payne.[236] With this method, ester amine **206** was oxidized to nitroxide **207** in 88% yield.



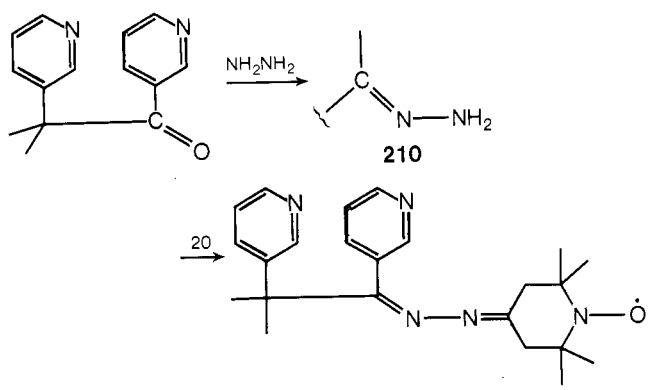
206 207

A new reagent **208** for the spin labeling of aldehydes and ketones has been reported by Schlude.[237] Thus, nitroxide ketone **72** was converted into the hydrazone **208,** a substance which readily afforded azines **209** upon reaction with aldehydes or ketones.



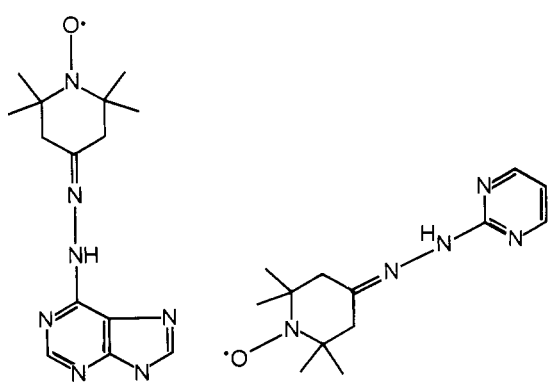
208 209

One example of the alternative order of covalently binding **72** to a substrate via an azine linkage is the method whereby Ruf and Nastainczyk[238] synthesized the spin-labeled metyrapone (**211**) for spin-labeling studies with cytochrome P-450. They first prepared metyrapone hydrazone (**210**) and then condensed this material with nitroxide **72** to obtain the spin-labeled derivative **211.**



210 211

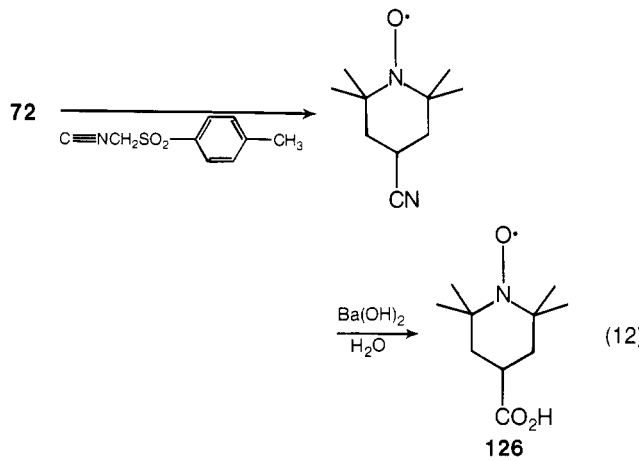
A second example is the synthesis[239] of several spin-labeled purine and pyrimidine derivatives (e.g., **212** and **213**) by reaction



212 213

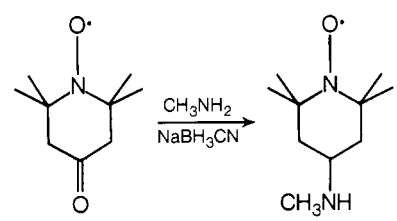
of 6-hydrazinopurine and 2-hydrazinopyrimidine with nitroxide **72.**

A convenient synthesis (eq 12) of the nitroxide carboxylic acid spin label **126** from nitroxide **72** has been recently described by Rosen.[240] This route circumvents difficulties in repeating an earlier synthesis[241] which likely yielded the α,β-unsaturated analogue of **126** rather than **126** itself.[240]



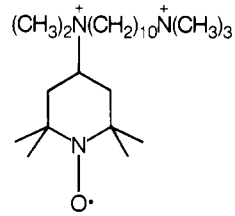
126 (12)

Sodium cyanoborohydride[242] ($NaBH_3CN$) has been shown by Rosen[243,244] to be an effective agent for the reductive amination of **72** with primary and secondary amines (eq 13). Reaction takes place in the pH range of 5–9, optimally at pH 7–8, and the nitroxide group is not reduced.

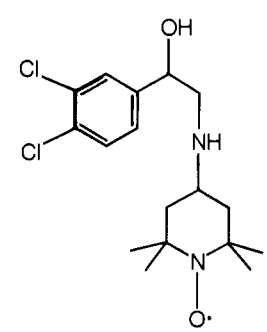


72 75% (13)

Sinha and Chignell[245] have used $NaBH_3CN$ as well as other reagents to prepare a series of spin-labeled analogues **214, 215,** and **216** of decamethonium, dichloroisoproterenol, and propanolol, respectively, and of certain other drug molecules[246] including 9-aminoacridines.[247] Yields in the reduction step were 35–40%. Rosen[248] has indicated that with high molecular weight



214



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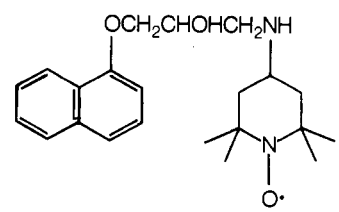

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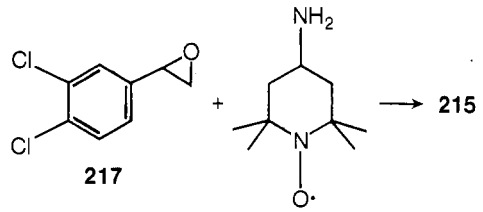
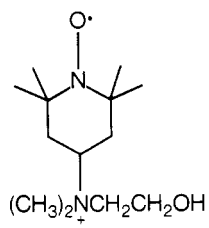


217 218 215

or hindered amines alternative routes not involving a reductive amination with $NaBH_3CN$ may result in better yields. Thus, reaction of 3,4-dichlorophenyloxirane (**217**) with nitroxide amine **218** gave **215** in 83% yield.

Since the pioneering work of McConnell[249] leading to the synthesis of spin-labeled choline derivatives **219** and spin-labeled phosphatidylcholine derivative **220,** amino nitroxide **218** has enjoyed wide use in the spin-labeling field. For example, using conventional chemistry and beginning with **218,** Gargiulo[250] prepared several spin-labeled analogues of local anesthetics (eq 14).



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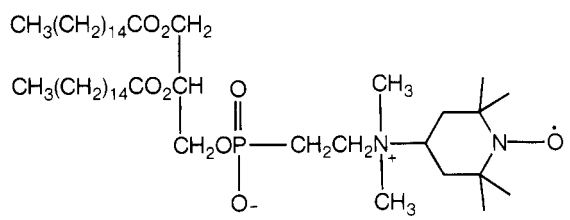

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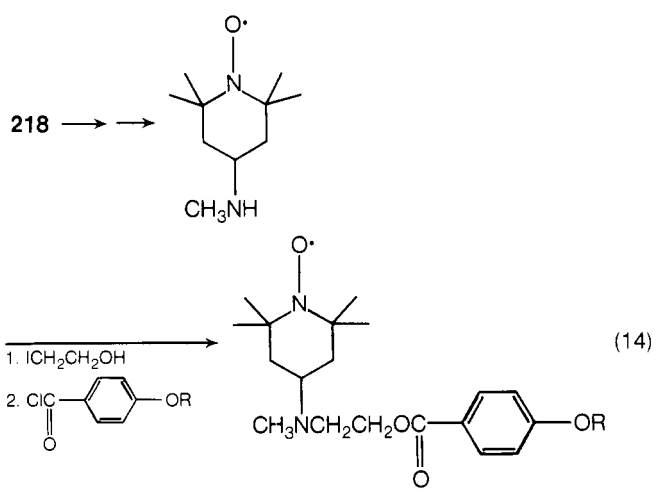
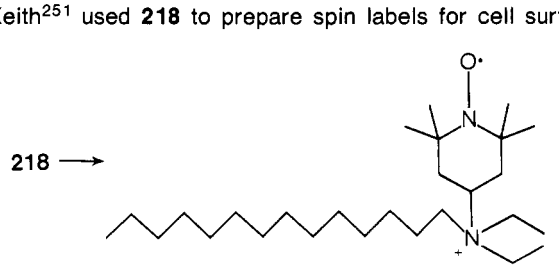


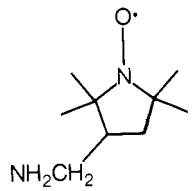
(14)

Keith[251] used **218** to prepare spin labels for cell surface studies. Reaction of **218** with tetradecyl bromide followed by excess ethyl iodide led to spin label **221.**

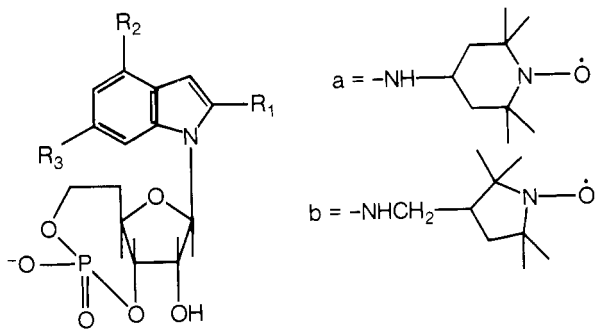


221

Hoppe and Wagner[252] allowed **218** and pyrrolidine nitroxide amine **222** to react with several halogen-substituted cyclic AMP compounds in order to prepare the series of spin-labeled cyclic AMPs **223.**

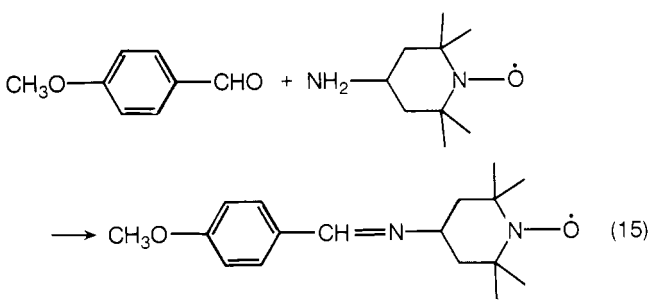


222



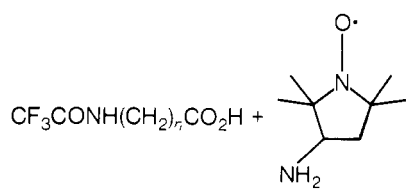
223, R_2 = a; R_1 = R_3 = H
R_2 = b; R_1 = R_3 = H
R_1 = a; R_2 = NH_2; R_3 = H
R_1 = b; R_2 = NH_2; R_3 = H
R_3 = a; R_2 = NH_2; R_1 = H

Amine **218** also readily condenses with aromatic aldehydes to form Schiff's bases useful for the study of nematic liquid crystals (eq 15).[253]

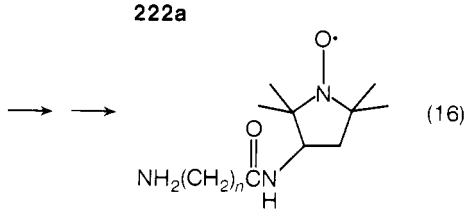


(15)

Pyrrolidine nitroxide amine **222a** was recently employed[254] in the synthesis of several spin-labeled ω-amino acid amides. The key step was condensation of the corresponding ω-trifluoroacetylamide with **222** followed by selective cleavage of the ω-trifluoroacetylamide linkage (eq 16).

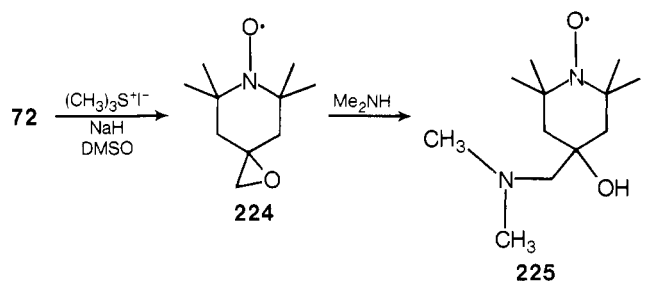


222a



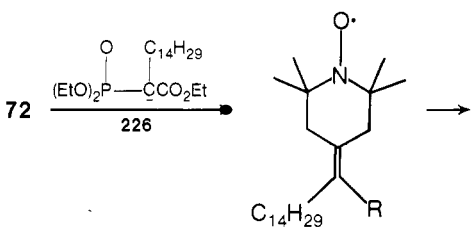
(16)

Nitroxide **72** has recently been used to prepare epoxide **224**[255] (71% yield) by reaction with trimethylsulfonium iodide–sodium hydride–$(CH_3)_2SO$. Subsequent reaction of **224** with dimethylamine produced the choline analogue **225.**

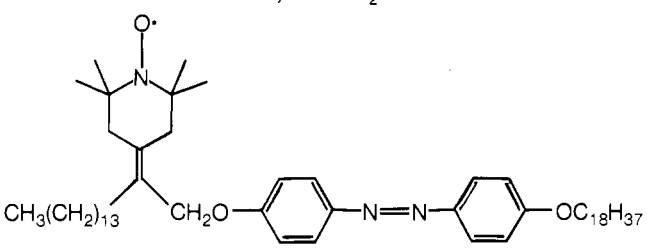


224 **225**

Dvolaitzky et al.[256] synthesized from **72** an interesting spin-labeled molecule **230** which forms liquid crystals. Thus, reaction of **72** with the modified Wittig reagent **226** gave olefin **227.** Reduction of **227** with $LiAlH_4$ gave alcohol **228** which was converted into the mesylate **229** and then allowed to react with the anion of the appropriate azo derivative, forming the desired **230.**

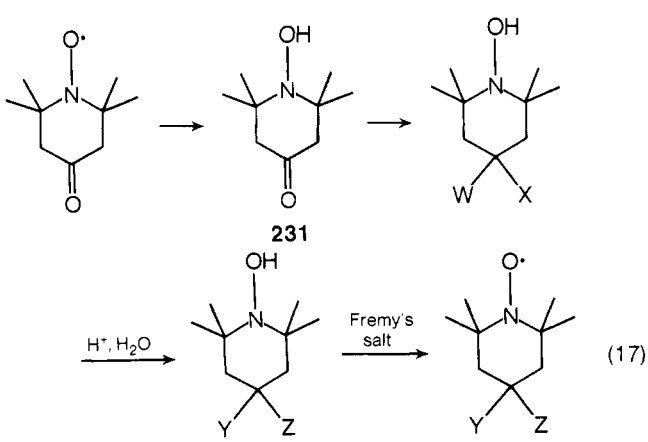


227, R = CO_2Et
228, R = CH_2OH
229, R = CH_2OMs



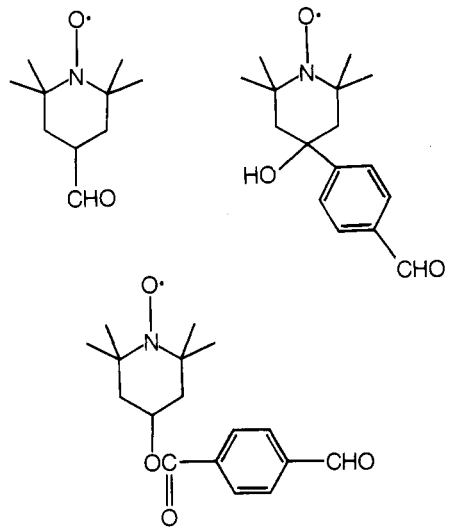
230

For reactions with **72** which require a subsequent acid hydrolysis step, Schlude[139,257] has recommended prior protection of the nitroxide moiety by catalytic reduction to the *N*-hydroxyl amine **231** (eq 17). This circumvents possible problems associated with the tendency of nitroxide groups to undergo a disproportionation reaction under acidic conditions. Then, after the reaction and hydrolysis steps, the nitroxide is regenerated by oxidation with Fremy's salt ($(KOSO_2)_2NO$).

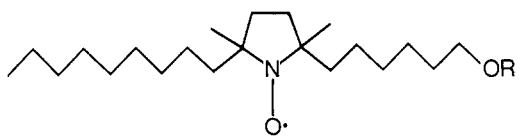


231 (17)

The following representative nitroxide aldehydes were prepared from **72** by this sequence.[139]

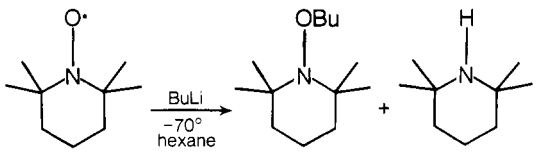


Certainly, it is not necessary to protect the nitroxide group during an acid-catalyzed reaction in every case. For example, Lee[258] has shown that routine acid-catalyzed hydrolysis of a tetrahydropyranyl ether (**232** → **233**) protecting group proceeds well (76% yield) without affecting the nitroxide linkage in substituted pyrrolidine nitroxides.

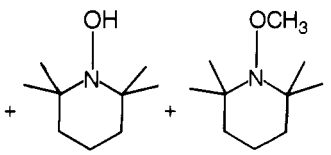


232, R = THP
233, R = H

Aside from the well-known instability of the nitroxide grouping toward disproportionation in strongly acidic media,[62,259] two other side reactions have been investigated in some detail. The first involves the reaction of *n*-butyllithium and 2,2,6,6-tetramethylpiperidine-*N*-oxyl (**234**). Whitesides and Newirth[260] showed that nitroxide **234** is rapidly destroyed in hexane solution

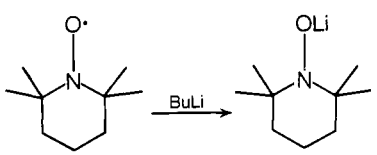


234 46% 11%



11% 33% (18)

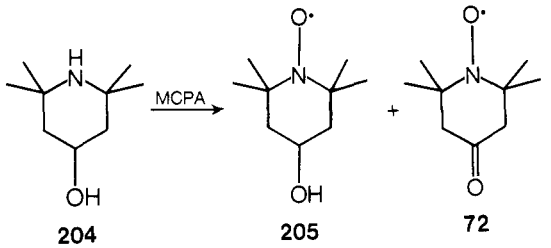
at −70 °C when treated with BuLi. Products after a quench with dimethyl sulfate are as shown in eq 18. The authors suggest that the first step may be a one-electron oxidation of the organolithium reagent by the nitroxyl radical (eq 19). This reaction is a potential side reaction whenever a highly reactive carbanion (an organolithium or Grignard reagent,[261] for example) is allowed



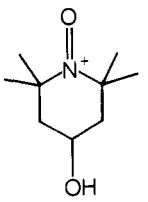
(19)

to react with a nitroxide bearing a functional group elsewhere in the molecule. For example, Harcus et al.[239] observed reduction of the nitroxide group of **72** during an attempted reaction with a 6-lithiomethylpyrimidine.[262] It should be possible to avoid the side reaction, however, by first reducing the nitroxide group to the *N*-hydroxy derivative.

The second side reaction involves a nitroxide-catalyzed oxidation of alcohols in the presence of an oxidizing agent such as MCPA. The reaction was first observed during the oxidation of alcohol amine **204,** producing both nitroxide **205** and nitroxide **72,**[263] and has since been developed as a general procedure for the oxidation of primary and secondary alcohols to the corresponding carbonyl compounds.[264,265] The active intermediate is thought[264,265] to be the corresponding oxoammonium derivative **205a** of the nitroxide.

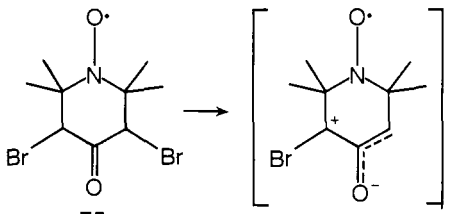


204 **205** **72**



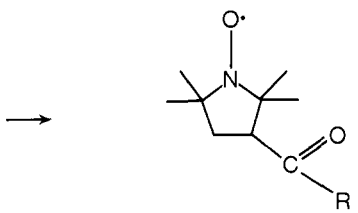
205a

Nitroxide **72** can be brominated twice to give **75.** This substance is reported[141] to react readily with primary and secondary amines, converting them directly into the pyrrolidine amide. Examples of amides **236** which have been synthesized from **75** are shown in eq 20. Zwitterion **235** is proposed as an intermediate.



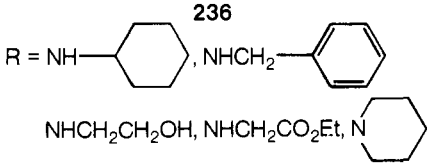
75

235

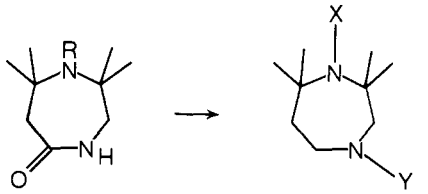


(20)

236



Rassat[266] has recently synthesized aminonitroxide **243** from lactam **237,** itself obtainable from amine **203** by the method of Rosantsev.[267] Reduction of **237** afforded diamine **239** which was converted to the β,β,β-trichloroethoxy carbonyl derivative **240** and then oxidized to the nitroxide **241** with MCPA. Cleavage of the protecting group with Zn dust in acetic acid following the

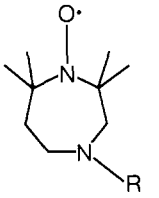


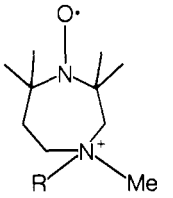
237, R = H
238, R = O·

239, X = Y = H
240, X = H, Y = $COOCH_2CCl_3$
241, X = O·; Y = $COOCH_2CCl_3$
242, X = OH, Y = H
243, X = O·; Y = H

procedure of Windholz and Johnston[268] afforded the *N*-hydroxyamine **242** which was oxidized to nitroxide **243** with Ag_2O.

Amino nitroxide **243** could also be prepared by reduction of lactam nitroxide **238** with $LiAlH_4$.[269] Reaction of **243** with formic acid–formaldehyde gave tertiary amine **244,** a substance which could be quaternized with either methyl iodide or ethyl bromoacetate, affording **245** and **246.**[269]





243, R = H
244, R = Me

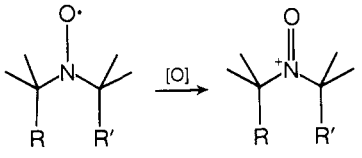
245, R = Me
246, R = CH_2CO_2Et

IV. Chemistry of Nitroxide Spin Labels

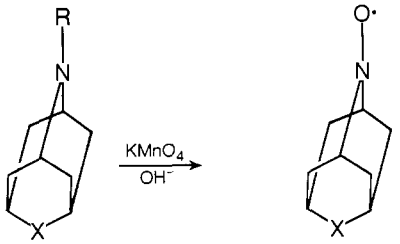
Frequently, it is important to chemically modify a molecule already bearing a nitroxide grouping without affecting the nitroxide moiety. This section focuses on newer examples of what can be done.

A. Oxidation in the Presence of a Nitroxide Group

The nitroxide group is reasonably easily oxidized by agents such as Cl_2, Br_2,[270-273] and moist Ag_2O[324] likely leading to oxoammonium salt **247** which then may decompose via several pathways. However, Rassat[275] has reported the synthesis of unsubstituted bicyclic nitroxides **249–251** in moderate yield by oxidation of the corresponding tertiary amine **248** with alkaline



247

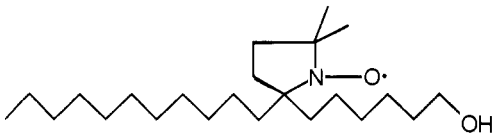


248 50–60%

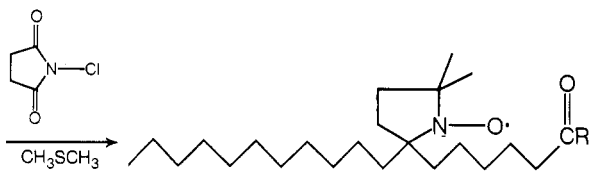
249, X = H, H
250, X = CH_2
251, X = a single bond

$KMnO_4$, a rather mild oxidizing agent. The electrochemical oxidation of several stable aliphatic nitroxides has been examined.[275a]

Whereas attempted oxidation of proxyl nitroxide alcohol **185**[156] with Jones reagent gave a mixture of nonparamagnetic products,[274] oxidation to the aldehyde **100** was readily accomplished in 78% yield with the *N*-chlorosuccinimide–dimethyl sulfide reagent of Corey and Kim.[276] Aldehyde **100** was then oxidized to carboxylic acid **122** with Ag^+ ion (Tollen's reagent, two-phase system).

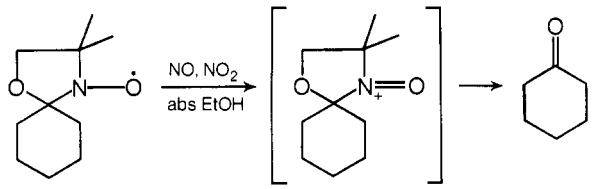


185



100, R = H
122, R = OH

A novel oxidative method of cleaving a doxyl group back to the parent ketone has been described by Spencer et al.[205,277] Thus, treatment of the doxyl derivative **252** of cyclohexanone, for example, with commercial nitric oxide in absolute ethanol leads to cyclohexanone in near-quantitative yield. The actual oxidizing agent was shown to be small amounts of NO_2 in the commercial NO. The oxoammonium ion **253** was suggested as a likely intermediate.



252 **253**

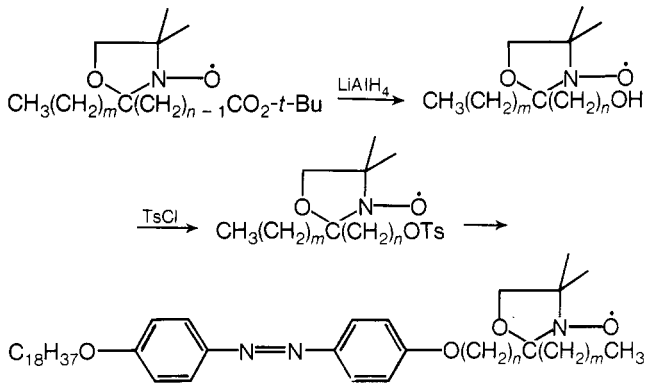
MCPA is also able to "over-oxidize" a nitroxide group to the corresponding oxoammonium ion if used in excess. This reaction is described above (**204** → **72**)[264,265] and is likely the reason why the MCPA oxidation step used in the synthesis[208] of doxyl nitroxides typically affords doxyl nitroxides in yields of about 30%.[277a]

B. Substitution Reactions on Molecules Bearing a Nitroxide Group

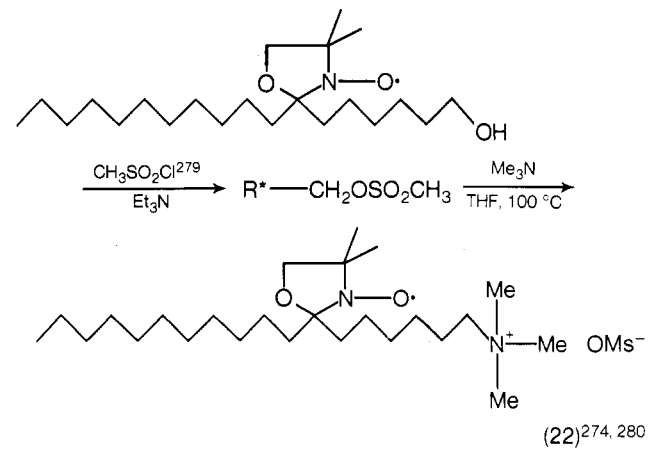
The most common method of covalent attachment of a nitroxide spin label to another molecule is, of course, by way of a substitution reaction on the nitroxide molecule (e.g., nitroxide alkylating agents or acylating agents). Typically, no further chemical transformations are performed after this stage. In this section we focus on the continued alteration of the functional group(s) on nitroxide bearing molecules via substitution reactions.

Several doxyl nitroxide alcohols have been synthesized. These have served as starting material for a variety of interesting and useful doxyl spin labels as shown by the following examples (eq 21–24).

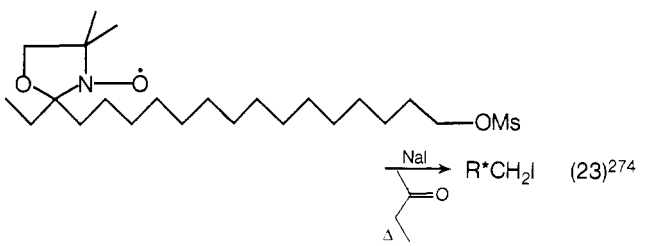
Proxyl and azethoxyl nitroxides are suitable for further chemical elaboration on remote portions of the molecule, as shown by the following examples (eq 25–28).



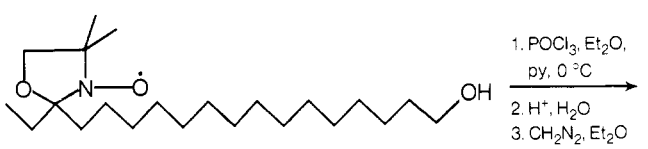
(21)[278]

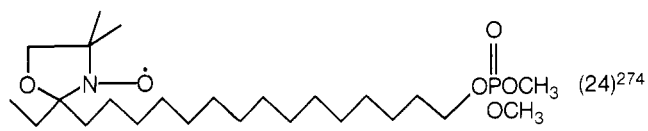


(22)[274, 280]

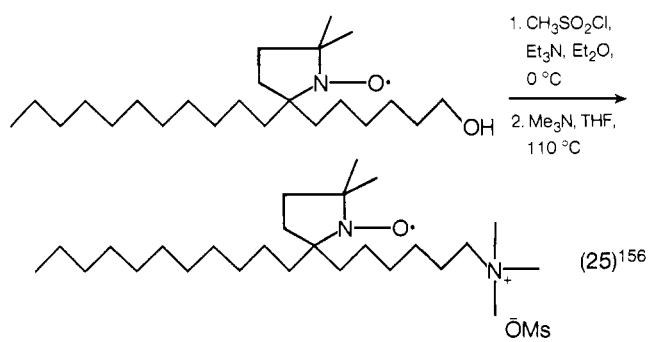


(23)[274]

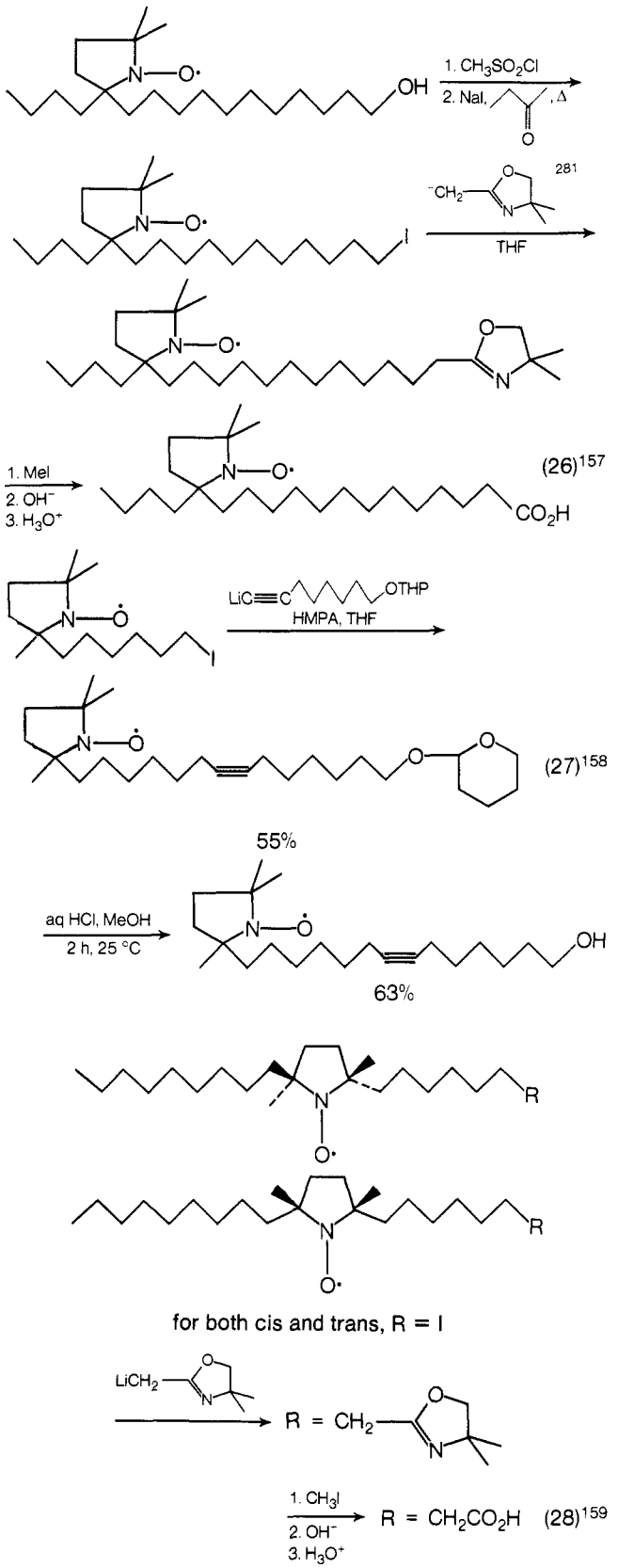


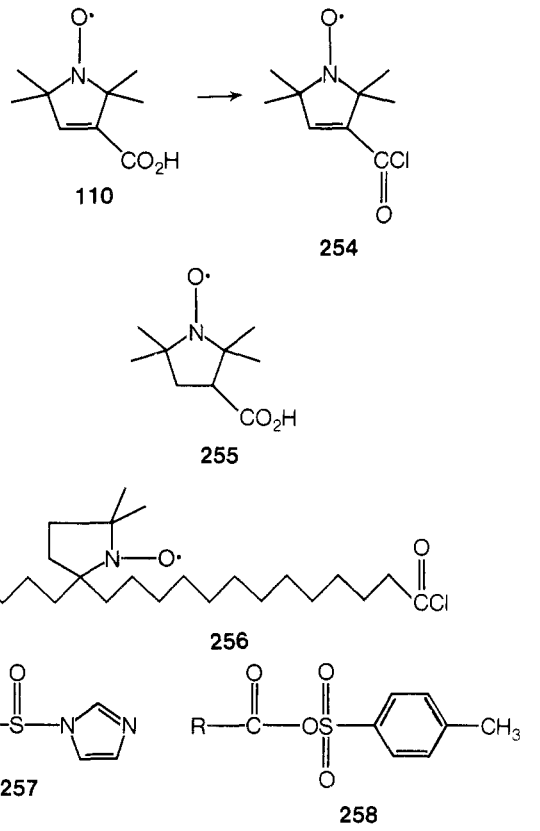


(24)[274]



(25)[156]

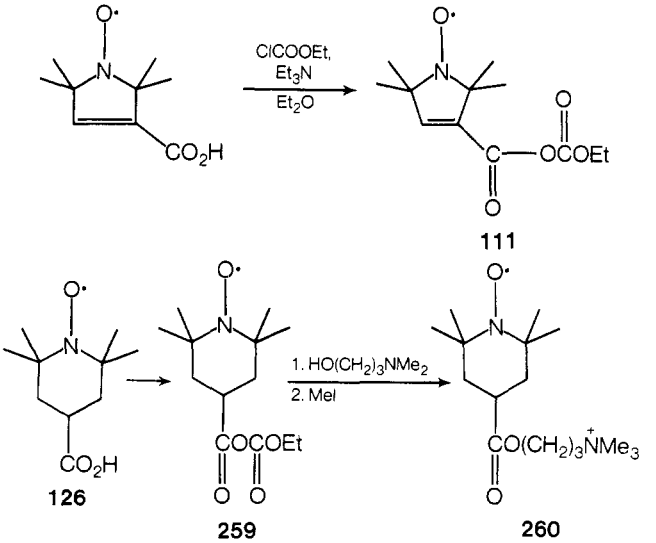




and Lewis[285] were not able to prepare the acid chloride of nitroxide acid **110** without destruction of the nitroxide group. Instead, *N,N'*-thionyldiimidazole (**257**) was used[285] as the condensation agent with **110** in order to prepare, in low yield, several steroid esters from **110.** More recently, Dodd[286] has found that dicyclohexylcarbodiimide in dry pyridine gives higher yields of spin-labeled steroid esters from **110** than does **257.**

Proxyl nitroxide acid chloride **256** could be readily prepared from the corresponding acid by treatment with oxalyl chloride in pyridine–chloroform.[157] The ability to form acid chlorides of nitroxide acids is important for use of a new powerful but mild lipid acylation procedure recently developed, which employs carboxylic–sulfonic acid mixed anhydrides **258** as the active acylating agent.[287]

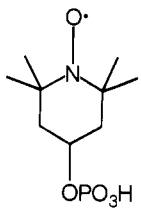
Nitroxide carboxylic–carbonic acid anhydrides (for example, **111**) were first introduced by Griffith et al.[162] as a new class of versatile spin-label acylating agents. Recently,[288] the method has been used to prepare mixed anhydride **259** which was in turn used to prepare **260.**



C. Acylation Reactions of Nitroxide Spin Labels

Representative nitroxide acylating agents are shown in Chart IV. A variety of reagents including thionyl chloride–pyridine–benzene,[282] phosphorus pentachloride–toluene–hexane,[283] phosphorus pentachloride–pyridine–chloroform,[284] and triphenylphosphine–carbon tetrachloride[170] have been used to convert nitroxide acid **110** into acid chloride **254.** However, Dodd

A ganglioside has recently been spin labeled[289] with nitroxide phosphate **145** using the triisopropylbenzenesulfonyl chloride–pyridine method employed by McConnell[249] and others.[290] Presumably, the reaction proceeds through the mixed sulfonic–phosphoric acid anhydride **261.** Sosnovsky[183,184] has recommended the use of activated phosphate **262** for the synthesis of spin-labeled phosphates. This and other phosphorylating agents are collected in Chart VI.



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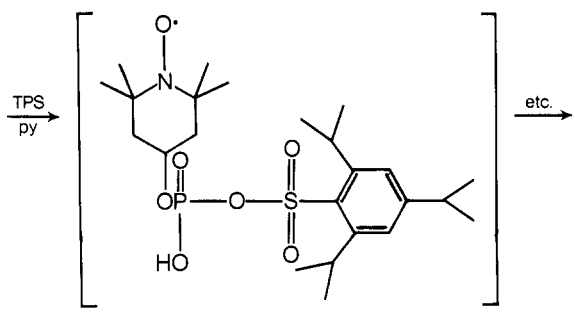

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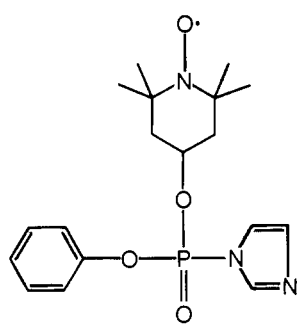
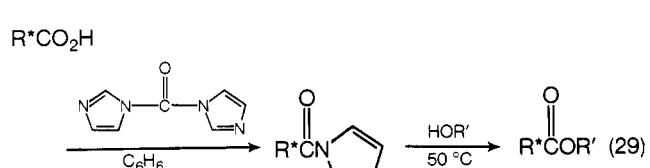


262

Symmetrical nitroxide carboxylic acid anhydrides have been widely used as active acylating agents in spin-labeling chemistry. Hubbell and McConnell[291] were the first to synthesize phosphatidyl cholines bearing a spin label in the side chain. They prepared the symmetrical anhydrides derived from a series of doxyl stearic acids by reaction of the latter with dicyclohexylcarbodiimide in CCl_4. Subsequent reaction of the anhydride with egg lysolecithin in the presence of Na_2O led to the spin-labeled phospholipid in low yield. The symmetrical anhydride method has since been used to prepare a variety of spin-labeled biomolecules, for example, spin-labeled acyl-coenzyme A,[292] spin-labeled triglycerides and sterols,[292a] spin-labeled long-chain acyl cholines,[293] and spin-labeled phosphatidic acid, the latter via phospholipase D hydrolysis of the spin-labeled lecithin.[294]

Recently, an acyl imidazole procedure (eq 29) has been shown by Boss[295] to lead to spin-labeled phospholipids in improved yield. Among the spin-labeled phospholipids prepared by the Boss[295] procedure are **263–266.**



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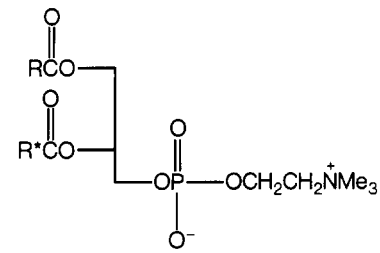


263, R*C(=O)– = 5-doxylstearoyl (14%)[295]

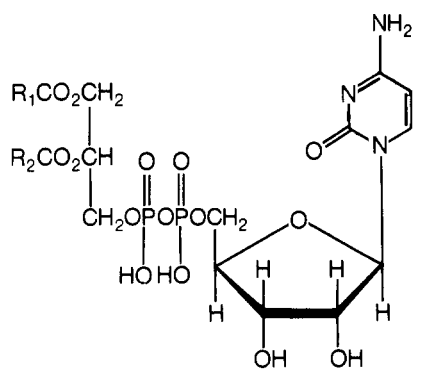
264, R*C(=O)– = 12-doxylstearoyl[295]

265, R*C(=O)– = 14-proxylstearoyl (60%)[157]

266, R*C(=O)– = 9-proxyldecanoyl (52%)[158]

Very recently, the nitroxide acyl imidazole **135** has been described by Adackaparayil and Smith.[174] They observed that whereas the unsaturated analogue **134**[150] is unstable, **135** is a nicely crystalline, stable acylating agent, capable of, for example, acylating testosterone at the 17 position in 19% yield. Substance **135** is somewhat more reactive toward water than is acetylimidazole.

Enzymes also can be effective reagents for the introduction of a spin label into a molecule. For example, Ohnishi[296] has synthesized a new phosphatidyl serine spin label by reaction of spin-labeled cytidine diphosphate diglyceride with L-serine catalyzed by the rare enzyme phosphatidylserine synthetase. Stuhne-Sekalec and Stanecev[297,297a] have synthesized spin-labeled radioactive cytidine diphospho-*sn*-1,2-diacylglycerol enzymatically. Incorporated spin labels were 5-,[298] 12-,[297] and 16-doxylstearic acid.[298]



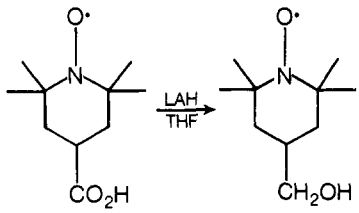
cytidine diphospho-*sn*-1,2-diacylglycerol

The action of phospholipase D on spin-labeled phosphatidyl choline has led to the synthesis of spin-labeled phosphatidic acid.[294,296] The enzyme also catalyzes the exchange of polar headgroups. Thus, when the phospholipase D reaction is run in the presence of excess ethanolamine or glycerol then spin-labeled phosphatidylethanolamine and spin-labeled phosphatidylglycerol may be isolated.[299]

D. Reduction of Nitroxide Spin Labels

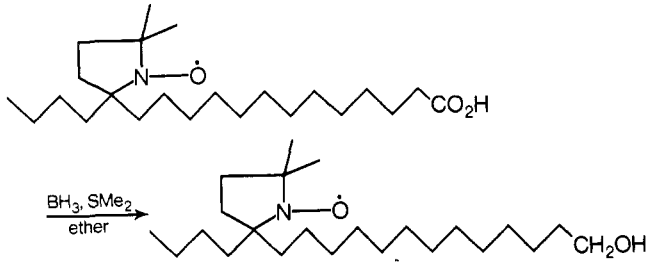
Chemical reduction of nitroxide groups has been summarized by Gaffney.[58] Other pertinent observations are the following. Rosen[288] has recently confirmed that $LiAlH_4$, even in excess,

can be used with a nitroxide bearing molecule without reduction of the nitroxide (eq 30). Sodium sulfide, on the other hand, reduces nitroxides to the corresponding amines.[299a]



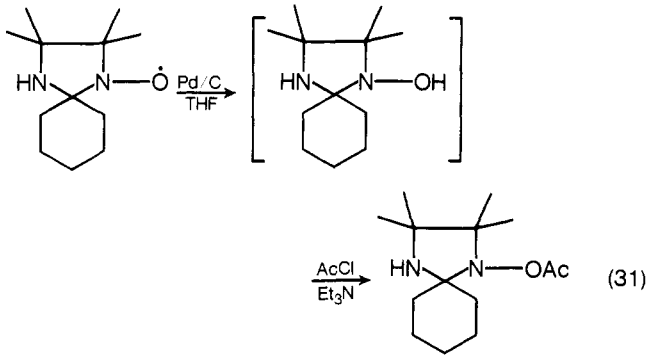
(30)

As indicated above, the nitroxide group is also stable toward $NaBH_3CN$[243,244] (eq 13). Keana et al.[157] have shown that 14-proxylstearic acid can be smoothly reduced to 14-proxylstearyl alcohol **267** in 90% yield with borane–methyl sulfide. Unlike $LiAlH_4$, which reduces most other functional groups more rapidly than it reduces free carboxyl groups, borane–methyl sulfide reduces carboxyl groups most rapidly.[300]



267

Although Rozantsev[62] has indicated that catalytic reduction of α-tetramethyl-substituted piperidine and pyrrolidine nitroxides with H_2 over a palladium catalyst leads to the secondary amine, Keana et al. have shown that with H_2–10% palladium on carbon in THF solution, proxyl[157] azethoxyl,[159] and imidazolidine-*N*-oxyl nitroxides[190] are near quantitatively converted to the corresponding *N*-hydroxy compounds. These substances may be conveniently "quenched" in situ with acetyl chloride and Et_3N, affording the corresponding *N*-acetoxy compound (eq 31).[190]



(31)

In ESR spin-labeling studies advantage is often taken of the ability of nitroxide groups to undergo chemical (or photochemical) reduction to the diamagnetic *N*-hydroxy derivative, a method exploited by McConnell.[188,249] Ascorbic acid in excess is a widely used reducing agent for spin labels.[188,301-302b]

A useful alternative to ascorbic acid is phenylhydrazine.[197] Daniel and Cohn[303] used this reagent in order to reduce nitroxide spin labels in NMR studies of t-RNA derivatives since, unlike ascorbic acid, phenylhydrazine does not obscure the methyl region in the NMR spectrum.

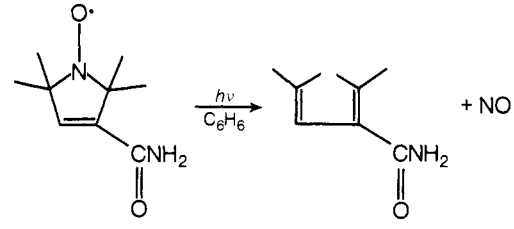
Reductase activity may also be present in the biological system, leading to loss of ESR signal.[304,305] For example, Ho,[306] in a spin-label study of energy coupling of active transport in *E. coli* membrane vesicles, observed that ESR signal decrease obeyed pseudo-first-order kinetics and the rate of decrease was dependent on the added donor, D-lactate or succinate. A series of experiments with various sulfhydryl reagents led to the suggestion that nitroxide reduction was due to certain sulfhydryl groups which are coupled to the respiratory chain between the flavin-linked dehydrogenase and cytochrome b_1. Sulfhydryl groups were previously known to effect reduction of nitroxide radicals.[307,308]

In a very recent comparison study[159] doxyl nitroxide **115** was shown to be much more rapidly reduced by sodium ascorbate or dithiothreitol than either proxyl nitroxide **124** (R = H) or *trans*-azethoxyl nitroxide (**121**). The nitroxides (1.1×10^{-4} M) were dissolved in 0.1 M phosphate buffer, pH 7.5, containing sucrose (0.25 M), EDTA (10^{-3} M), and either sodium ascorbate (0.011 M) or dithiothreitol (0.11 M). After 20 min in the ascorbate solution only 3% of the original signal intensity remained for doxyl nitroxide **115,** whereas 90% remained for proxyl nitroxide **124** (R = H) and 94% remained for azethoxyl nitroxide **121.** After 20 min in the dithiothreitol solution 75% of the original signal intensity remained in the case of **115,** while 95% remained in the case of both **124** (R = H) and **121.**

It is well here to point out that Trommer et al.[309] has mentioned the inadvertent reduction of certain nitroxide spin labels by stainless steel syringe needles. They recommend use of platinum needles and Teflon tipped plungers for handling certain nitroxides.

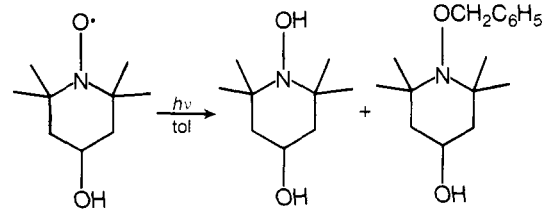
E. Nitroxide Photolysis and Thermolysis

The stable nitroxide **268** upon irradiation in benzene with a high-pressure mercury lamp through Pyrex results in an elimination of NO and formation of the diene **269** in 95% yield.[310]

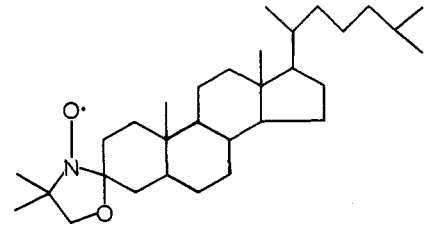


268 **269**

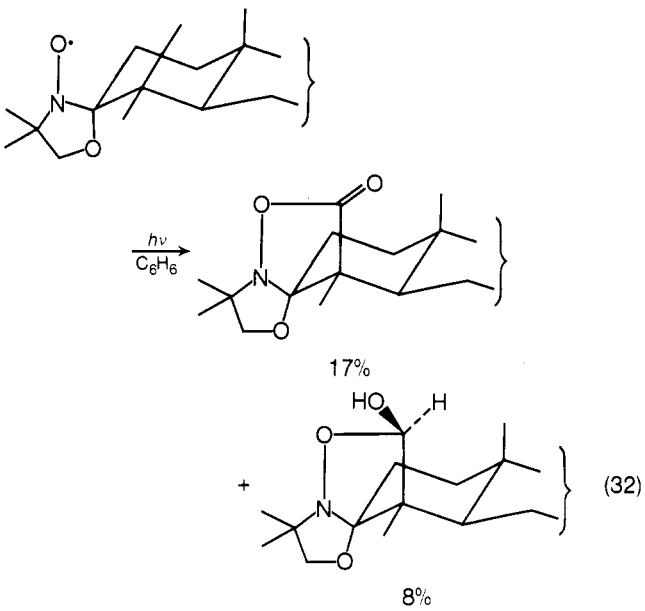
The elimination of NO from a nitroxide upon photolysis, however, is not a general reaction. For example, irradiation of nitroxide alcohol **205** in toluene gives about equal amounts of *N*-hydroxy derivative **270** and *O*-benzyl derivative **271.**[311] Comparable products are observed upon irradiation of 3-doxylcholestane **272.**[311] The nature of the photoproducts suggests that the excited nitroxide is an effective hydrogen atom abstractor.



205 **270** **271**



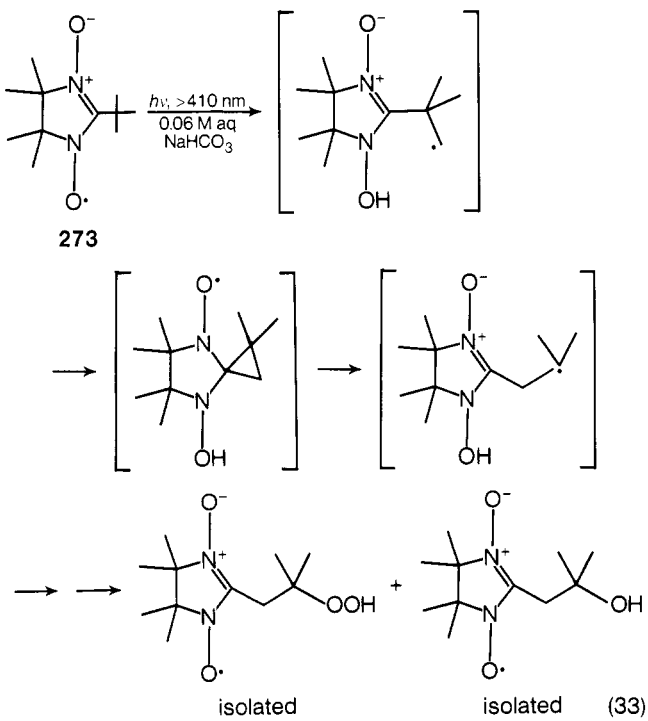
272



(32)

Spencer[211] has capitalized on the photochemistry of doxyl nitroxides in developing a new method for oxidative demethylation at C-4 of a steroid (eq 32).

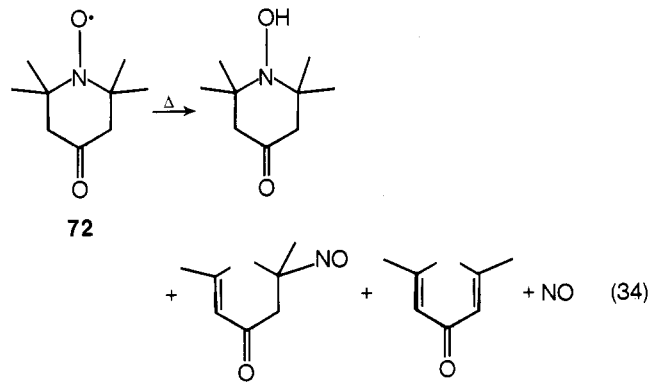
Despite the (albeit quite low) photochemical reactivity of the nitroxide group itself, useful information may be obtained by irradiation of other components of biological samples in the presence of nitroxide spin labels. For example, the localization of phenothiazine derivatives within biological membranes has been studied utilizing the ability of phenothiazine derivatives to reduce fatty acid spin labels upon irradiation (~310 nm), presumably by capture of phenothiazine radicals by the nitroxide.[312] In an unrelated study probing the mechanism of light-induced phenothiazine-sensitized liposomal membrane breakdown, Copeland[313] irradiated (near uv light) liposomes which contained a spin label trapped in the inside aqueous phase. Loss of membrane integrity was monitored by observing release of the spin label in the presence of a series of phenothiazines. The method takes advantage of the appearance of sharp ESR lines as the spin labels are released and diffuse apart.



(33)

Call and Ullman[314] studied the photolysis of nitronyl nitroxide **273** (eq 33). The proposed mechanism further illustrates the hydrogen abstraction ability of photoexcited nitroxides.

Thermolysis of nitroxide **72** has been shown to proceed according to eq 34.[315] More recently Rassat and Rey[316] have studied the thermolysis of several isoquinuclidine nitroxides. For example, upon heating nitroxide **274** at 110 °C for 1 h, a mixture of **275** and **276** was obtained in good yield.



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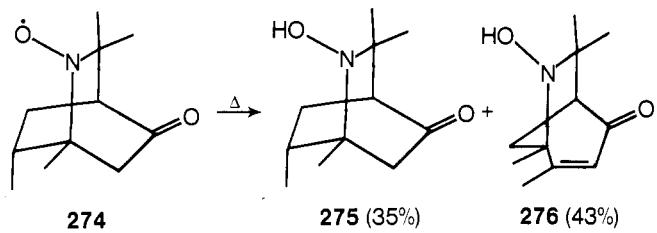
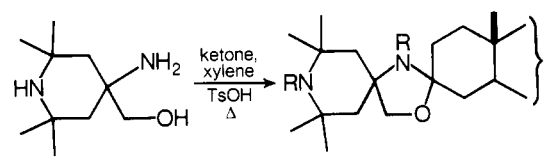


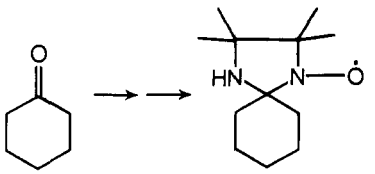
F. Dinitroxide Spin Labels

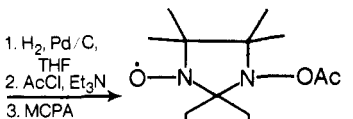
A host of di- and polynitroxides with interesting spectroscopic properties have been synthesized by chemical attachment of two or more stable nitroxide groups to bi- or polyfunctional molecules.[3,62,117,161,317,318] Relatively few methods are available for the rigid attachment of a dinitroxide framework to an existing molecule. The first such dinitroxide spin label was reported by Keana and Dinerstein.[319] Thus, precursor **277** was synthesized and then condensed with cholestan-3-one, affording diamine **278.** Oxidation with MCPA afforded the rather rigidly attached dinitroxide **279.** As expected, the ESR spectra of **279**

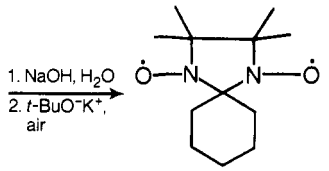


incorporated into a multilayer on a glass slide was highly dependent on the orientation of the multilayer with respect to the laboratory magnetic field. The distance between the two outermost lines varied from ~450 to about ~250 G. Rassat[4] has synthesized other derivatives bearing this dinitroxide label. Such biradicals have been used, for example, in the study of cyclodextrin inclusion compounds by ESR spectroscopy.[320]

Very recently, Keana et al.[190] described the synthesis of a new dinitroxide ketone spin label in which the nitroxide groups are separated from each other by only one carbon atom. The synthesis of dinitroxide **280** is shown in eq 35. The dipolar splitting parameter of this substance, 2*D*, is 1606 G. Steroid dinitroxide **159** was similarly prepared. The rigid attachment of the dinitroxide grouping to the molecule of interest together with the relatively small steric size of the dinitroxide grouping and the large dipolar splitting make these molecules of considerable

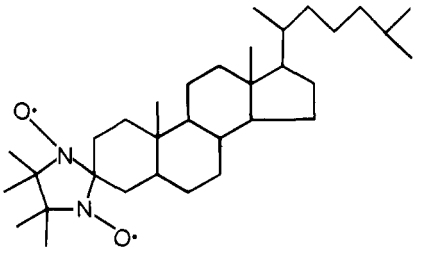






(35)

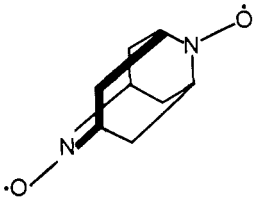
280 (structure by x-ray)



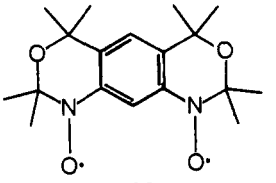
159

interest in both theoretical studies and in spin-labeling studies where extreme sensitivity to orientation is important.

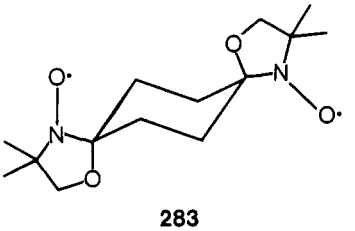
Other stable, quite rigid dinitroxides, of theoretical interest,[6,321] have been prepared but have not as yet been used as spin labels, largely because it is not easy to attach them to another molecule. These include dinitroxides **281**,[76] **282**,[322] **283**,[1,9] **284**,[8] and **285**.[6] Such molecules certainly lend themselves to use as spin probes, however.



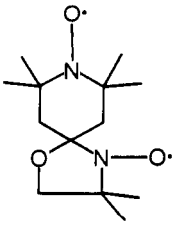
281



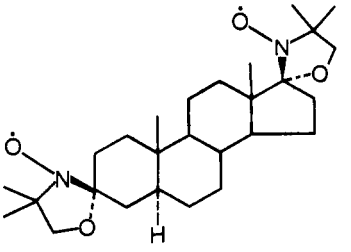
282



283



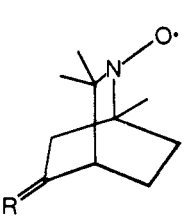
284



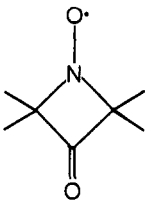
285

G. Unexploited Nitroxides of Some Potential as Spin Labels or Spin Probes

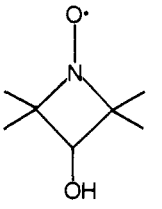
This review concludes with a listing of several interesting stable nitroxides which have been prepared but which have not yet enjoyed significant use in the spin-labeling field.



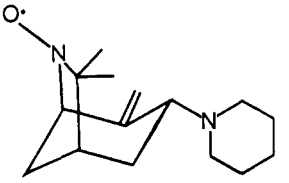
286
R = O[323, 324]
R = H, H[325]



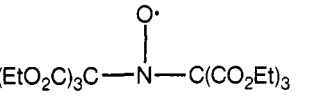
287[326]



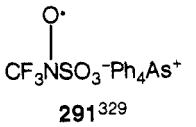
288[326]



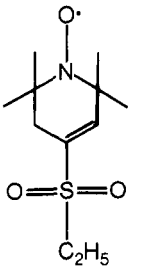
289[327]



290[328] (stable in solution)



291[329]



292[330]

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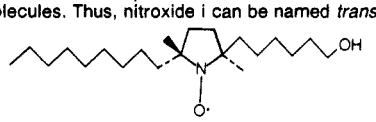

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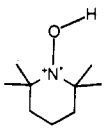

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