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Novel nitroxides for spin-labelling, -trapping, and magnetic resonance imaging applications

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Abstract--We describe the synthesis of the isotopically labelled nitrone spin traps **7**, **9**, and **10**, and spin labelled cytochalasin B derivatives **15** - **23**. We describe a new general route to proxyl nitroxide spin labelled fatty acids, for example, **28** and **29**, and the ^{15}N-nonadeutero 11-proxylpalmitic acid **P-OH**. New types of paramagnetic liposomes derived from bis nitroxide quaternary salts **31** and **32** have been prepared. Molecules designed as molecular amplifiers such as hexaamine **35a** are described. Paramagnetic centers such as a nitroxide epoxide or Gd complex **37** may be attached giving unique spin relaxers such as **35b** and **35c** to which an isothiocyanate reactive group may be added giving **36b** and **36c**.

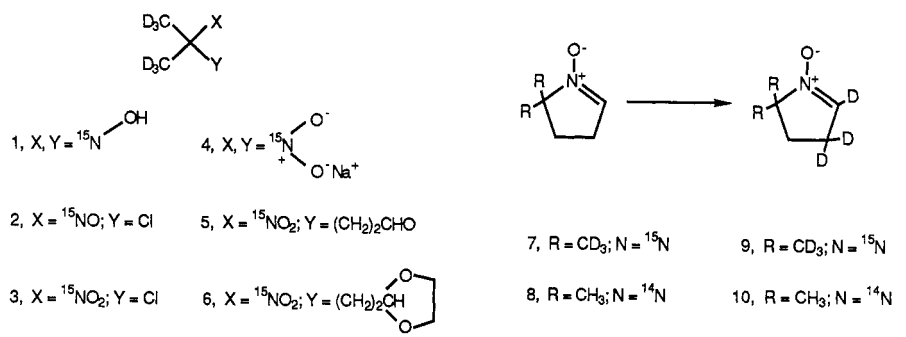
INTRODUCTION

Stable nitroxide free radicals continue to play a central role in a wide variety of investigations, especially involving spin labelling and spin trapping applications. More recently, novel applications have arisen in which nitroxides are being developed as contrast-enhancing agents for magnetic resonance imaging (MRI) and as paramagnetic agents for the relatively new field of imaging by electron spin resonance (ESR) spectroscopy. In this paper we summarize several recent developments in our laboratory in the nitroxide field.

I. Development of ^{2}H- and ^{15}N-isotopically substituted nitrone spin traps

Spin trapping[1,2] has proven to be a powerful tool for the identification of biologically generated transient organic free radicals. In this method an appropriate nitrone or nitroso compound reacts with the short lived radical intermediate producing a nitroxide with a lifetime considerably greater than that of the parent radical. Recent advances in spin trapping include the preparation of a family of pyrrolidine-1-oxide spin traps with enhanced lipophilicity and a decreased tendency toward oxidation[3] and the preparation of nitrones which react preferentially with hydroxyl radical over superoxide.[4]

One difficulty encountered in the spin trapping of certain free radical intermediates, for example superoxide, is the rather slow rate (~10 $M^{-1}sec^{-1}$) with which nitrones react with the intermediate. Thus potentially toxic concentrations of the nitrone are required for efficient trapping in biological systems. Our aim in this section is to develop nitrone-based spin traps with improved sensitivity owing to isotopic substitution. Our approach is based on the pioneering work of Beth et al.[5] and Janzen et al.[6] These groups showed that a significant increase in ESR sensitivity could be achieved by preparing nitroxides that contain ^{2}H and ^{15}N in place of ^{1}H and ^{14}N, respectively. Herein we report the synthesis of a family of such isotopically substituted nitrones and a study of their ability to spin trap superoxide and hydroxyl radicals generated from a model superoxide-generating system.



The original Bonnett et al.[7] synthesis was modified in order to maximize yields based on the key isotopically substituted starting materials acetone-d_6 and ^{15}N-hydroxylamine hydrochloride. Combination of the latter two reagents gave the volatile oxime **1**. Without purification of any intermediates,[8] oxime **1** was treated with Cl_2 to give **2** followed by ozone to give **3**. Catalytic hydrogenation of **3** in aqueous NaOH gave anion **4**. The pH was adjusted to 8 and the solution was evaporated to dryness. Then a Michael reaction between **4** and acrolein was effected at -60 °C in dry ethanol containing 0.1% 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxy gave aldehyde **5** (90%). Acetalization gave **6** which was subjected to the usual[7] reductive cyclization methodology to give hexadeutero ^{15}N- nitrone **7**. Upon treatment with D_2O-NaOD, **7** gave the nonadeutero ^{15}N-nitrone spin trap **9** (76%). Conditions for the three-proton exchange reaction were worked out using **8** → **10**.

Spin trapping of superoxide utilized a superoxide generating system consisting of xanthine in the presence of xanthine oxidase at pH 7.8. Superoxide was produced at the rate of 10 μM/min (25 °C) as measured by the superoxide dismutase inhibitable reduction of cytochrome c. The addition of superoxide dismutase (60 μg/mL) to the reaction mixture confirmed that the observed ESR spectra resulted from the reaction of superoxide with the spin traps.[3] A factor of two increase in ESR peak height resulted from the trapping of superoxide by **10** compared to **8**. A further increase was seen with ^{15}N spin trap **9** (Figure 1). The advantage of using **9** in the spin trapping of hydroxyl radical by nitrones **7**, **8**, **9**, and **10** was likewise clear. Spin trapping of hydroxyl radical was conducted by the addition of $FeSO_4$ (0.1 mM) to the above superoxide generating system. The addition of catalase (300 U/mL) confirmed that the ESR spectra resulted from the reaction of hydroxyl radical with the spin traps.[3]

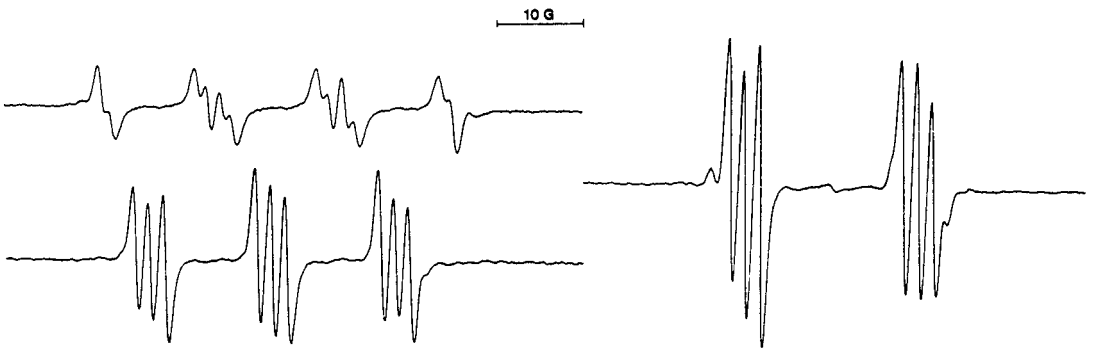


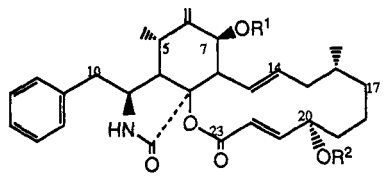
Fig. 1. Spin trapping of superoxide with **8**, **10** (lower), and **9** (right). Power, 20 mW; mod. freq., 100 kHz with ampl. of 0.63 G; sweep, 12.5 G/min; receiver gain, 2.3 x 10^3.

II. Spin labelling of the glucose transporter protein inhibitor Cytochalasin B

Essentially all cells depend on the intracellular availability of glucose. In most cells, glucose enters the cell driven by a concentration gradient (facilitated diffusion) and crosses the external membrane with the aid of a well studied transmembrane protein call the glucose transporter.[9,10] Cytochalasin-B (CB, **11**) is a potent competitive inhibitor of glucose efflux and a non-competitive inhibitor of glucose influx in erythrocytes and other cells and has therefore become an important tool for study of the transport process.[10,11]

We set out to study the motional characteristics of the CB binding site on the glucose transporter protein in erythrocytes using spin labelling. Herein, we describe the synthesis of a series of selectively spin labelled CB molecules each bearing a nitroxide ester on one or the other of the hydroxyl groups. Esterification on the C-20 hydroxy group was preferred based on the plausable model for CB binding developed by Griffin et al.[12] Their model proposed a three point H-bonding attachment involving N-2, O-7, and O-23 of CB, but not O-20. Earlier, Rothweiler and Tamm[13] reported a diesterification of CB and Masamune et al.[14] prepared the O-7 monoacetate by partial hydrolysis of the diacetate. Since we wished to use isotopically labeled nitroxide carboxylic acids in the synthesis, we chose to optimize yields based on direct partial esterification of CB, a process that has not been previously reported.

In order to facilitate NMR, TLC, and HPLC analysis of the products, the acylation of CB[15] was first investigated using cyclopentenone carboxylic acid **X-OH**, DCC and a trace of 4-pyrrolidinopyridine (PPY) in CH_2Cl_2. We introduced **X-OH** earlier as a diamagnetic isostere for a pyrroline nitroxide.[16] In the event, only diester **12** could be isolated, undoubtedly owing to the low solubility of CB in CH_2Cl_2. The H-20 and H-7 protons in **12** were assigned with the aid of double resonance experiments aided by the original CB NMR assignments of Garden and Lynn.[17] Optimal partial esterification was achieved by using 2:1 CH_2Cl_2-$CHCl_3$ as the solvent, giving 7-monoester **13** (7%), 20-monoester **14** (25%), and diester **12** (30%). Next, nitroxide acid **Y-OH** (1.2 equiv) was used as the acylating agent, giving a mixture of 7,20-diester **15**, 7-monoester **16**, and 20-monoester **17**. Esters **15** and **16** were obtained pure by tlc, however, ester **17** co-eluted with starting CB. Eventually, all three esters were obtained in analytically pure form by reverse phase HPLC. Similarly prepared were the corresponding ^{15}N-perdeuterated analogs **18**, **19**, and **20** and the corresponding 11-proxylpalmitate (see below) esters **21**, **22**, and **23**.

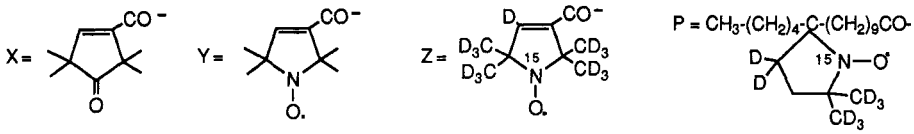


11, $R^1 = R^2 = H$

12, $R^1 = R^2 = X$ 15, $R^1 = R^2 = Y$ 18, $R^1 = R^2 = Z$ 21, $R^1 = R^2 = P$

13, $R^1 = X$; $R^2 = H$ 16, $R^1 = Y$; $R^2 = H$ 19, $R^1 = Z$; $R^2 = H$ 22, $R^1 = P$; $R^2 = H$

14, $R^1 = H$; $R^2 = X$ 17, $R^1 = H$; $R^2 = Y$ 20, $R^1 = H$; $R^2 = Z$ 23, $R^1 = H$; $R^2 = P$



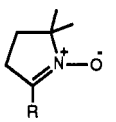
With regard to glucose transport inhibition, CB was 10-15 fold more potent than the most potent of the CB derivatives which was the diamagnetic 20-monoester **14**. Ester **14** inhibited transport half maximally at about 10 mM and also inhibited CB binding half maximally at about 8 mM. The other two diamagnetic CB esters, **12** and **13** were on the average about 10-fold less potent in both binding inhibition and transport inhibition than was ester **14**. A comparison of diamagnetic CB 20-ester **14** with paramagnetic CB 20-ester **17** showed **17** to be ~10-fold less potent in transport inhibition and CB binding experiments than **14**. An ESR spectrum of **17** (30 μM) in the presence of red blood cells (PBS, pH 7.4) clearly showed spectral line broadening indicative of motion restriction of the spin label. Further ESR studies of the interaction of these new CB spin labels with red cells is underway.

III. Novel MRI contrast enhancing agents

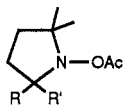
Magnetic resonance imaging (MRI) is a powerful noninvasive medical diagnostic tool that is currently undergoing rapid development. Agents that can selectively enhance the contrast among various tissues, organs and fluids or of lesions within the body can add significantly to the versatility of MRI. Owing to their paramagnetic nature and thus their ability to affect the relaxation times T_1 and T_2 of nearby nuclei, nitroxides are under active investigation as contrast enhancing agents for MRI. In order to achieve significant enhancement in a given target area, it is necessary to introduce many paramagnetic centers at the site, especially if each center contains only one unpaired electron. Below we describe two methods for achieving this objective.

1. An Improved Synthesis of Proxyl Nitroxide-Labelled Fatty Acids; Synthesis of Novel Paramagnetic Liposomes. Liposomes[18] provide the possibility for targeting various agents to certain sites within the body, serving as vessels to contain encapsulated paramagnetic material.[19] We have recently described a new type of liposome containing long chain nitroxide esters in the bilayer and an oxidant trapped inside whose function is to regenerate the nitroxide group by a flip-flop process once it has become reduced to the N-hydroxy stage by the outside reductants.[20] Another way to solve the "nitroxide reduction problem" in liposomes is to prepare liposomes made up entirely of paramagnetic amphipathic molecules. In this section we describe an improved synthetic route to proxyl nitroxide labelled fatty acids. 3-Proxylpalmitic acid (**28**) was chosen as one target since this residue, when incorporated into a liposome, would have its nitroxide group near the polar headgroup and thus easily accessible to the bulk water.

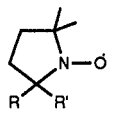
Acid **28** was synthesized as follows. Nitrone **24** was allowed to react with tridecylmagnesium bromide and the resulting intermediate was oxidized with Cu^{2+}/O_2 to give nitrone **25**. Next, **25** was allowed to react with allylmagnesium bromide and then the reaction was quenched with acetyl chloride to give N-acetoxy pyrrolidine **26**. Selective oxidation of the terminal double bond of **26** to give acid **27** was accomplished using the $RuO_4/NaIO_4/CCl_4/MeCN$ reagent combination described by Carlsen et al.[21] and related to that used in other systems by Hideg and Lex.[22] Basic hydrolysis of **27** under air followed by acidification gave acid **28** in good overall yield. Alternatively, nitrone **24** was converted by the above method into 11-proxylpalmitic acid (**29**) using pentylmagnesium bromide and the Grignard reagent derived from 11-bromoundecene as the source of the alkyl chains. Finally, starting with ^{15}N-nonadeutero nitrone **9**, the method was used to prepare the ^{15}N-nonadeutero-11-proxylpalmitic acid (**P-OH**) which was then used to form the CB esters described above.



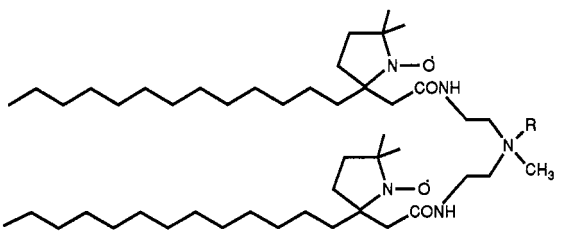
24, R = H
25, R = $(CH_2)_{12}CH_3$



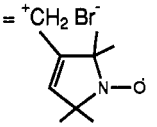
26, R = $(CH_2)_{12}CH_3$; R' = $CH_2CH{=}CH_2$
27, R = $(CH_2)_{12}CH_3$; R' = CH_2CO_2H



28, R = $(CH_2)_{12}CH_3$; R' = CH_2CO_2H
29, R = $(CH_2)_4CH_3$; R' = $(CH_2)_9CO_2H$

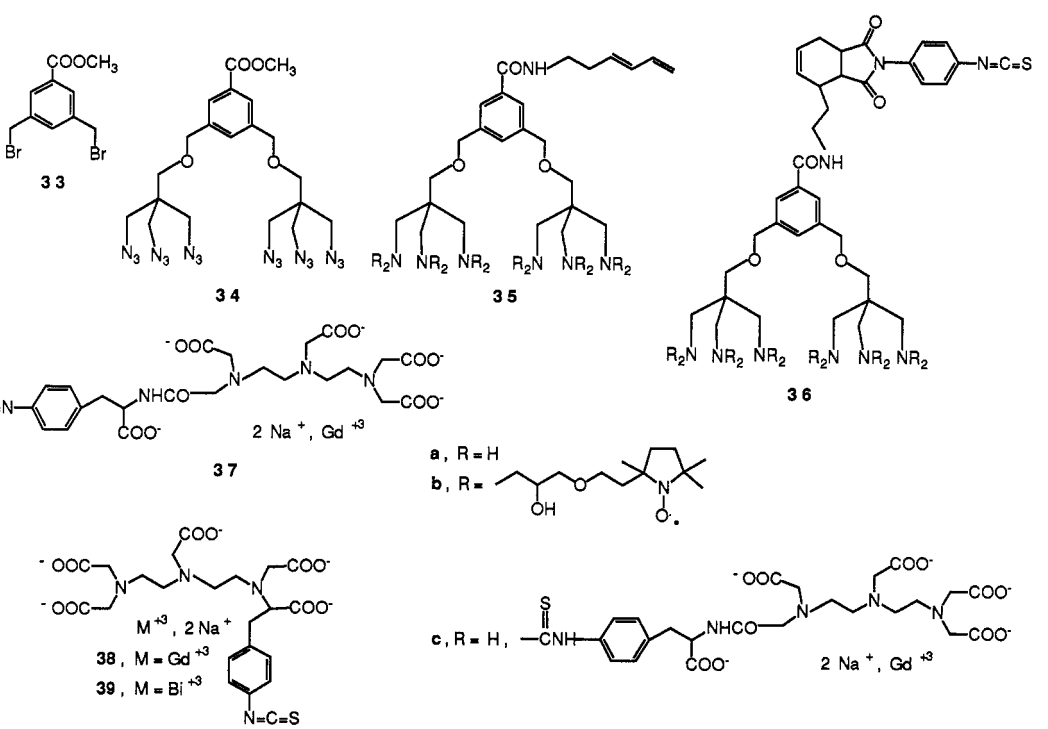


30, R = :
31, R = $CH_3{}^+I^-$
32, R = $^+CH_2\,Br^-$



The synthesis of the new amphipathic nitroxide bearing molecules proceeded as follows. The ethoxycarbonyl mixed anhydride of acid **28** was used to diacylate the two primary amino groups of N,N-bis(2-aminoethyl)methyl amine, giving diamide **30**. Quaternization with MeI gave the dinitroxide salt **31** while quaternization with 5-bromomethyl-2,2,5,5-tetramethylpyrroline-N-oxy[23] gave trinitroxide salt **32**. Electron microscopy[24] was used to demonstrate that salts **31** and **32** indeed formed liposomes upon sonication of their aqueous dispersions. That the nitroxide groups were in a position to effectively relax bulk water was demonstrated by T_1 measurements at 360 mHz. Whereas the T_1 value for pure water was 3.3 s (control), a value of 0.7 s was measured for a 5 x 10^{-3} M "solution" of **31** in water.

2. Synthesis of a New Series of Paramagnetic Molecular Amplifiers. We have developed a new series of multifunctional molecules that permit the attachment of many paramagnetic centers to a targeting device such as a monoclonal antibody through a single, uniquely functionalized reactive arm. Methyl 3,5-bis-(bromomethyl)benzoate (**33**) was used to alkylate (50% NaOH, $BuN_4{}^+HSO_4{}^-$) $(N_3CH_2)_3CCH_2OH$, giving hexaazide **34**. Latent functionality in the form of a 1,3-diene[25] was introduced at the carbonyl group by hydrolysis followed by CDI mediated condensation with 1-amino-3,5-hexadiene to give the corresponding amide.



33

34

35

36

37

a, R = H
b, R =

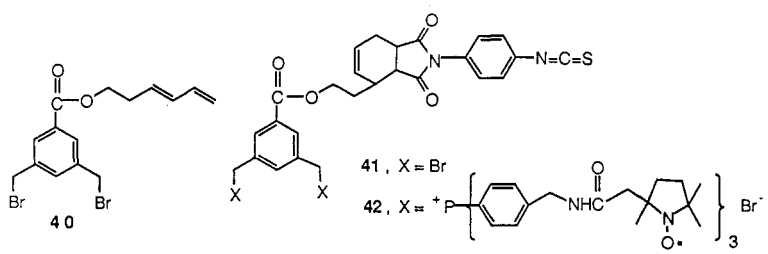
38, M = Gd^{+3}
39, M = Bi^{+3}

c, R = H,

Next, the amide was converted into hexaamine **35a** by treatment with Ph_3P and aqueous THF. This hexamine was then alkylated by treatment with an excess of a nitroxide epoxide to give dodecanitroxide **35b**.

The crucial reactive group was then introduced by subjecting **35b** to a Diels-Alder reaction[25] with p-aminophenylmaleimide followed by a selective reaction with thiophosgene to give the analytically pure isothiocyanate **36b**. Alternatively, hexaamine **35a** may be condensed with a variety of other reagents including functionalized chelates **37** - **39**, ultimately giving rise to hexachelates such as **36c** which also bear a reactive isothiocyanate group for attachment of the assembly to some target molecule.

Amplification may also be based on a triphenylphosphonium group. Thus, dibromide **40** underwent the Diels Alder reaction with p-isothiocyanatophenylmaleimide to give **41**. Reaction of **41** with the appropriate nitroxide-substituted triphenylphosphine gave the bis salt **42** containing six nitroxide groups per molecule. The isothiocyanate grouping in these new amplifier molecules is well positioned to react with amino groups present in target molecules such as antibodies or liposomes. In principle, higher levels of amplification might be achievable by reaction of one or the other of **37**, **39**, or **42** with hexaamine **35a**.



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