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 15N-nonadeuerto 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 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-I-oxide spin traps with enhanced lipophilicity and a decreased tendency toward oxidation 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 ($\sim$10 M$^{-1}$sec$^{-1}$) with which nitrones react with the intermediate. Thus potentially toxic concentrations of the nitrene are required for efficient trapping in biological systems. Our aim in this section is to develop nitroxide-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 nitroxides and a study of their ability to spin trap superoxide and hydroxyl radicals generated from a model superoxide-generating system.

\[ \text{D}_\text{O}_\text{H} \]

1, $X, Y = {^1}N$

2, $X = {^{15}}NO_2; Y = Cl$

3, $X = {^{15}}NO_2; Y = Cl$

4, $X, Y = {^{15}}N$

5, $X = {^{15}}NO_2; Y = (CH_3)_2OH$

6, $X = {^{15}}NO_2; Y = (CH_3)_2OH$

7, $R = CD_3; N = {^1}N$

8, $R = CH_3; N = {^1}N$

9, $R = CD_3; N = {^{15}}N$

10, $R = CH_3; N = {^{15}}N$
The original Bonnett et al.\textsuperscript{7} synthesis was modified in order to maximize yields based on the key isotopically substituted starting materials acetone-\textsuperscript{14}\textsuperscript{N} and 15\textsuperscript{N}-hydroxylamine hydrochloride. Combination of the latter two reagents gave the volatile oxime 1. Without purification of any intermediates,\textsuperscript{8} oxime 1 was treated with Cl\textsubscript{2} to give 2 followed by ozone to give 3. Catalytic hydrogenation of 3 in aqueous NaOH gave aldehyde 5 (90\%). Acetilation gave 6 which was subjected to the usual\textsuperscript{7} reductive cyclization methodology to give hexadeutero 15\textsuperscript{N}-nitrone 7. Upon treatment with D\textsubscript{2}O-NaOD, 7 gave the nonadeutero 15\textsuperscript{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 \textmu M/min (25 °C) as measured by the superoxide dismutase inhibitable reduction of cytochrome c. The addition of superoxide dismutase (60 \textmu g/mL) to the reaction mixture confirmed the observed ESR spectra resulted from the reaction of superoxide with the spin traps.\textsuperscript{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\textsuperscript{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\textsubscript{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.\textsuperscript{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 called the glucose transporter.\textsuperscript{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.\textsuperscript{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 plausible model for CB binding developed by Griffin et al.\textsuperscript{12} Their model proposed a three point H-bonding attachment involving N-2, O-7, and 0-23 of CB, but not O-20. Earlier, Rothweiler and Tamml\textsuperscript{13} reported a diesterification of CB and Masamune et al.\textsuperscript{14} prepared the 0-7 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\textsuperscript{15} was first investigated using cyclopentenone carboxylic acid X-OH, DCC and a trace of 4-pyrrolidinopyridine (PPY) in CH\textsubscript{2}Cl\textsubscript{2}. We introduced X-OH earlier as a diamagnetic isostere for a pyrroline nitroxide.\textsuperscript{16} In the event, only diester 12 could be isolated, undoubtedly owing to the low solubility of CB in CH\textsubscript{2}Cl\textsubscript{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.\textsuperscript{17} Optimal partial esterification was achieved by using 2:1 CH\textsubscript{2}Cl\textsubscript{2}-CHCl\textsubscript{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\textsuperscript{N}-perdeuterated analogs 18, 19, and 20 and the corresponding 11-proxylpalmitate (see below) esters 21, 22, and 23.
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 provide the possibility for targeting various agents to certain sites within the body, serving as vessels to contain encapsulated paramagnetic material. 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. 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 proxy 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. and related to that used in other systems by Hiedeg and Lex. 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 1-proxylpalmitic acid (29) using pentylmagnesium bromide and the Grignard reagent derived from 11-bromoundecene as the source of the alkyl chains. Finally, starting with 15N-nonadeutero nitrone 9, the method was used to prepare the 15N-nonadeutero-11-proxylpalmitic acid (P-OH) which was then used to form the CB esters described above.
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-tetramethylpyrrolidine-N-oxide gave trinitroxide salt 32. Electron microscopy 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 T1 measurements at 360 MHz. Whereas the T1 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, BuN4+HSO4-) (N3CH2)3CCH2OH, giving hexaazide 34. Latent functionality in the form of a 1,3-diene was introduced at the carbonyl group by hydrolysis followed by CDI mediated condensation with 1-amino-3,5-hexadiene to give the corresponding amide.
Next, the amide was converted into hexaamine 35a by treatment with Ph3P and aqueous THF. This hexaamine 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 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 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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REFERENCES
15. Commercial CB was found by mass spectrometry to be contaminated by a small amount of m/e M+ + 16 material which was likely the corresponding epoxide.