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# SYNTHESIS OF DI-t-ALKYL NITROXIDES ENRICHED IN $^{2}H$ AND $^{12}C$

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## SUMMARY

The synthesis of a family of di-t-alkyl nitroxides is described, beginning with N-t-butyl-2-methyl-2-nitrosopropane $^{2}H_{18}$, $^{12}C_{8}$ ($^{12}C$-pd-DTBN) and including molecules with 9, 10, and 11 carbons. Eliminating the proton and, in some cases, the $^{13}C$ isotopes simplifies the resulting electron spin resonance (ESR) spectrum and improves the spectral resolution. Solvent dependent ESR parameters are given for this family of monofunctional spin probes. ESR evidence is seen for rotational isomerization within the most sterically hindered probe molecules. Some of these spin probes are especially useful for ESR spin probe studies in biomembranes where a range of probe hydrophobicity is desired.

Key words: perdeutero, nitroxide, spin probe, $^{12}C$, ESR.

## INTRODUCTION

N- t -butyl-2-methyl-2-nitrosopropane, or di- t -butyl nitroxide (DTBN), is the simplest air-stable free radical with the general structure $R_2NO$. The molecule is soluble in both aqueous and nonaqueous solvents, and is used as a monofunctional spin probe freely partitioning between biomembranes and the surrounding aqueous milieu (1). Probe electron spin resonance (ESR) signals are observed from at least two environments since the magnetic g value and hyperfine splitting parameters are medium dependent (figure 1). With good resolution, and/or with computer

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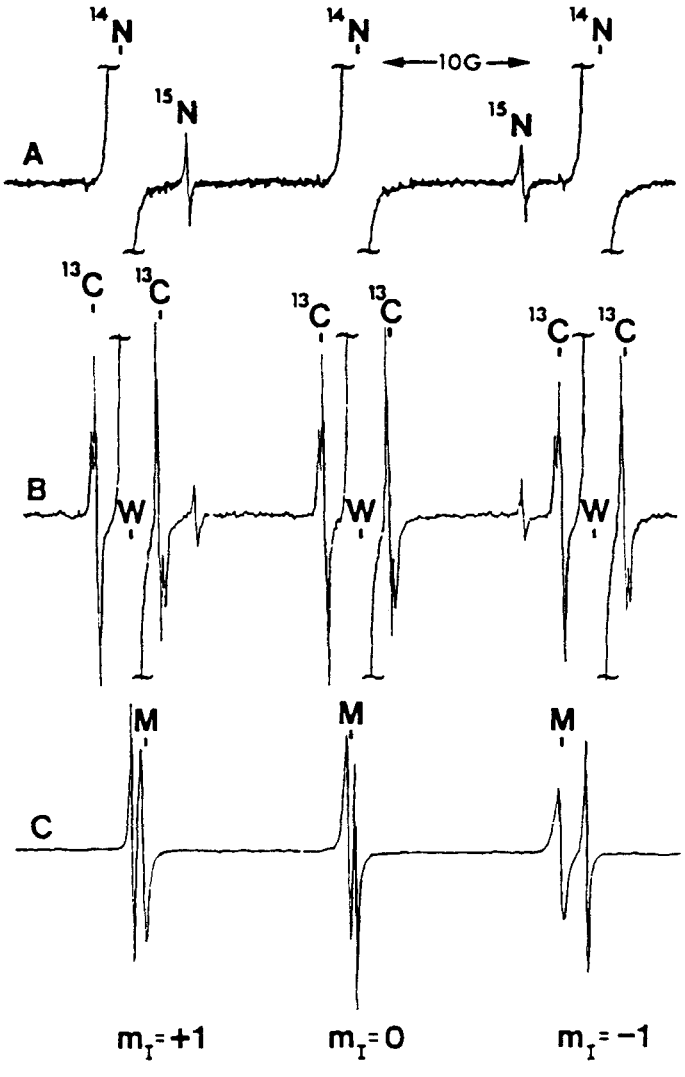


Figure 1:

A. $^{12}C$ pd-DTBN in water at high gain ($^{14}N$ lines are off scale) showing $^{15}N$ satellite peaks at natural abundance (0.37%). Note absence of $^{13}C$ satellite peaks.

B. pd-DTBN in water (gain about equal to A above). Note $^{13}C$ satellite peaks from both $\alpha$ and $\beta$ carbons due to $^{13}C$ at natural abundance (1.1%).

C. $^{12}C$ pd-DTBN in a DMPC dispersion in water at normal gain. Note good resolution of membrane (M) and water (W) signals. The absence of $^{13}C$ satellites from the aqueous probe allows for undistorted membrane probe signals.

aided deconvolution, the probe ESR spectrum can yield motional and polarity information about the probe sites as well as the partition coefficient of the probe between the diverse environments.

The ESR spectrum of dilute DTBN with normal isotopic composition in a homogeneous solvent appears to have a hyperfine splitting from only the $^{14}N$ nucleus, resulting in a three line spectrum with a solvent dependent line separation (hyperfine splitting $a_N$). The hyperfine splitting is about 15.2 Gauss in hydrocarbon solvents and 17.2 Gauss in water (1). Inhomogeneous peak broadening due to proton hyperfine splitting is observed (2,3). Satellite lines due to natural abundance $^{13}C$ in both the alpha and the beta positions have an intensity of about 4% of the parent lines. The increased ESR resolution, improved lineshape, and sensitivity enhancement observed with deuterium substitution in spin probes increases the information content of the spectra (4,5). The synthesis of pd-DTBN has been published (5). DTBN has also been synthesized with partial $^{13}C$ enrichment at the alpha carbon (6).

The low field $^{13}C$ satellites of the high field aqueous (W) line ($m_I$=-1) are especially undesirable for X-band ESR membrane partitioning studies since they occur at a magnetic field value essentially coincident with the probe signal from the membrane (M) ($m_I$=-1) line (figure 1b,c). This spectral coincidence distorts the area, shape, and intensity of this, the best resolved, membrane signal. The problem is especially severe for those studies where the membrane signal may be relatively small. Our attempts to computer deconvolute membrane probe signals in the presence of these satellites have not been fully satisfactory, and we have therefore synthesized the pd-DTBN probe from $^{13}C$-depleted starting materials. This pd-$^{12}C$-DTBN probe has been recently used for a model biomembrane study (7).

Reactions 1-5 resulted in the $^{12}C$-enriched pd-DTBN. The symbol *C denotes $^{12}C$. The isotope $^{15}N$ can be inserted in step 2 for increased sensitivity or for dual probe experiments.

1) $*CD_3*CO*CD_3 + *CD_3MgI \longrightarrow (*CD_3)_3*COD$ (I)

2) $I + CH_3CN \longrightarrow (*CD_3)_3*CNHCOCH_3$ (II)

3) $II + OH^- \longrightarrow (*CD_3)_3*CNH_2$ (III)

4) $III + KMnO_4 \longrightarrow (*CD_3)_3*CNO_2$ (IV)

5) $IV + Na \longrightarrow ((*CD_3)_3*C)_2NO$ (V)

Substitution of one or both of the t -butyl R groups with a more hydrophobic R group, such as 2-methyl-2-butyl or 3-methyl-3-pentyl, results in related spin probes that have an increased lipid bilayer-to-water partition coefficient, $K_{par}$. $K_{par}$ is defined as the probe concentration in the bilayer membrane (site M) divided by the probe concentration in the surrounding water (site W). We have made the N- t -butyl-2-methyl-2-nitrosobutane, or (4,5)NO, the N-methyl-2-butyl-2-methyl-2-nitrosobutane-$d_{22}$, or pd-(5,5)NO, the N-t-butyl-3-methyl-3-nitrosopentane, or (5,6)NO, and the N- t -butyl-3-ethyl-3-nitrosopentane-$d_{24}$, or pd-(4,7)NO. Each added methylene group was found to increase $K_{par}$ by about a factor of two to three.

Those bulky and more hydrophobic spin probes containing nine or more carbons could not be made by reactions analogous to those in sequence 1-5. (4,5)NO was made using a Grignard reagent (8). The remaining probes were made by oxidation of the corresponding secondary amines. The condensation reactions yielding these bulky secondary amines require that at least one of the R groups exist as the corresponding alkyne (9). We produced the saturated di-pd- t -alkyl amines by base catalyzed exchange of the alkyne proton followed by catalytic deuteration. All the saturated spin probes were stable free radicals. Small amounts of alkyne spin probes (5,5≡)NO, (5≡,5≡)NO, and (4,7≡)NO were obtained by oxidation of a

fraction of the corresponding acetylenic amine. These alkyne probes proved to be unstable in membrane preparations above $50^{\circ}C$. The intensity of the $^{13}C$ satellite peaks for nitroxides with more than nine carbons was found to be anomalously small (see Discussion) so that $^{12}C$ enrichment was not attempted in these cases.

Reactions 6-14 gave the pd-(4,7)NO spin probe. (5,5)NO, (4,6)NO, and (5,6)NO may be obtained by obvious substitution:

6) $CD_3CD_2OD + HBr \longrightarrow CD_3CD_2Br$ (VI)

VI + Mg $\longrightarrow CD_3CD_2MgBr$

7) $CD_3CD_2MgBr + HCO_2Et \longrightarrow (CD_3CD_2)_2CHOH$ (VII)

8) VII + PCC $\longrightarrow (CD_3CD_2)_2CO$ (VIII)

9) VIII + LiC≡CH $\longrightarrow (CD_3CD_2)_2C(OH)(C{\equiv}CH)$ (IX)

10) IX + DCl $\longrightarrow (CD_3CD_2)_2C(Cl)(C{\equiv}CH)$ (X)

11) X + III $\longrightarrow (CD_3)_3CN(H)C(C_2D_5)_2(C{\equiv}CH)$ (XI)

12) XI + $OD^-$ $\longrightarrow (CD_3)_3CN(H)C(C_2D_5)_2(C{\equiv}CD)$ (XII)

13) XII + $2D_2 \longrightarrow (CD_3)_3CN(D)C(C_2D_5)_3$ (XIII)

14) XIII + $H_2O_2 \longrightarrow (CD_3)_3CN(O)C(C_2D_5)_3$ (XIV)

Molecule III in step 11 of this sequence was not $^{13}C$ depleted.

## MATERIALS AND METHODS

The $^{12}C$-pd-DTBN synthesis in reactions 1-5 used methyl iodide $^2H_3,^{12}C$ (Merck, St. Louis, MO) and acetone $^2H_6,^{12}C_3$ (Wilmad, Buena, NJ) as starting materials. All other perdeutero starting materials were purchased from the Aldrich Chemical Company (Milwaukee, WI). The ESR spectrum of dilute solutions of the $^{12}C$-pd-DTBN probe (figure 1a) demonstrates that product V is at least 99.95 mol% $^{12}C$. The final $^2H$ level in all pd-free radical products is approximately 98 mol% ($^1H$ NMR and IR). The phospholipid, 2,3-ditetradecanoyl sn-glycero-3-phosphocholine (DMPC), was obtained from the Sigma Chemical Co. (St. Louis, MO).

ESR spectra were obtained on a Varian E-12 ESR spectrometer with an interfaced Varian E-900 data system. NMR spectra were obtained on a General Electric GE-300 NMR spectrometer. Infra-red spectra were obtained on a Nicolet DX-20 FTIR spectrometer.

## EXPERIMENTAL

t-butanol $-^{2}H_{9},^{12}C_{4}$ (I) Approximately 68 mmol of $^{12}CD_{3}MgI$ in 150 mL of ether was prepared. The acetone was dissolved in 10 mL of ether and then added to the Grignard at $0^{o}C$.

t-butylamine $-^{2}H_{9},^{12}C_{4}$ (III) Acetonitrile (35 mmol) was added to I at $0^{o}C$ in a mixed $DOAc/D_{2}SO_{4}$ solvent. Work up (10) yielded 11.2 mmol of labeled N-t-butylacetamide (II). Basic hydrolysis (ethylene glycol) at $200^{o}C$ in a closed system at reduced pressure yielded 10.7 mmol of the amine (III) in a $-78^{o}C$ trap after several hours.

t-nitrobutane $-^{2}H_{9},^{12}C_{4}$ (IV) III (10.7 mmol) was reacted with excess $KMnO_{4}$ (11). After steam distillation and acid washing 6.6 mmol of IV was obtained.

N-t-butyl-2-methyl-2-nitrosopropane $-^{2}H_{18},^{12}C_{8}$ (V) The procedure of Hoffman, et al., (12) was modified by using sodium sand in place of the chunks of sodium metal. IV was added to the Na sand in dry 1,2-dimethoxyethane in an ice bath with stirring. The sodium surface turned gold, and the solution a light purple, within a few minutes. The next day the solvent was removed, cold methanol was added to destroy excess sodium, and water was added to hydrolyze the salt to nitroxide V. After washing and drying the product was purified on a 40 cm column of alumina using a 50/50 (v/v) pentane/ether solvent. Following solvent stripping the residue was found to be 90% pure (gas chromatography) with only one paramagnetic product (ESR). Yield: 0.12 g or 0.74 mmol of V (22% based on the nitrobutane).

Bromoethane $-^2H_5$ (VI) Commercial perdeuterated ethanol (0.91 mol, used without purification) was reacted with 48% HBr in the presence of sulfuric acid (reaction 6) (13). The product was removed as it was formed by slow distillation from the reaction vessel into a trap immersed in ice water. The product, washed twice with cold water and stored over $CaCl_2$, was shown to be 99+% free of protons (IR). The yield was 0.14 mol (75%).

3-Pentanone $-^2H_{10}$ (VIII) Deuterated 3-pentanol (VII) was readily synthesized as previously described for di- n -butyl carbinol (14). The ethyl formate used for the reaction was purified by shaking with potassium carbonate followed by distillation from phosphorus pentoxide. The addition of the ethyl formate to the prepared ethyl-$d_5$ Grignard reagent was quite vigorous (reaction 7). Following workup the product was purified by fractional distillation to yield 52 mmol of alcohol (75%, b.p.= 115°C). The alcohol was then reacted with a fivefold excess of pyridinium chlorochromate, PCC, in methylene chloride solution (reaction 8)(15). The supernatant was decanted off and the residue washed with ether. The combined solutions were eluted through a short bed of silica and the product was isolated by fractional distillation. The yield was 30 mmol, 60%.

3-(ethyl- $^2H_5$) 1-pentyne-3-ol-4,4,5,5,5- $^2H_5$ (IX) and

3-(ethyl- $^2H_5$) 3-chloro-1-pentyne-4,4,5,5,5- $^2H_5$ (X)

Reactions 9 and 10 were run as previously described (16,17). After addition of VIII (30 mmol) to a solution of LiC≡CH, the reaction mixture was heated gently to 35°C and stirred for 1 hour. After workup, 27 mmol of alcohol was obtained (90%, b.p.= 71°C at 150 mmHg). The alcohol was added to an ice cold solution of $CaCl_2$ (60 mmol), CuCl (50 mmol) and Cu powder (25 mg) in 25 mL con. $DCl/D_2O$. The mixture was stirred at 0°C for 30 min. The resulting chloride was washed with cold $DCl/D_2O$ and dried over $CaCl_2$. The product (22 mmol, 80%) was used without further purification.

(1',1'-dimethylethyl),(1,1-diethyl-2-propynyl)amine $-^2H_{20}$ (XII)
The t -butylamine-$^2H_9$ used in reaction 11 was produced from commercial t -butyl alcohol-$^2H_{10}$ ($^{13}C$ natural abundance) as described for compound III above. The amine was alkylated with the chloride X in the presence of KOH by adding the chloride and KOH in 10 aliquots at 4 hour intervals (10). The product was isolated the next day by steam distillation to yield 11 mmol of the secondary amine XI (50% yield). The acetylenic proton was exchanged for deuterium by stirring the amine with 0.5 molar NaOD in $D_2O$ for 16 hours (18). The acetylenic function was completely deuterated after two treatments (IR). The amine functionality, less accessible to solvent (17,19), remained substantially protonated.

(1',1'-dimethylethyl)(1,1-diethylpropyl)amine $-^2H_{24}$ (XIII)
The acetylenic amine XII (15 mmol) was dissolved in 12 mL of ethanol-O-$^2H$ along with 0.5 g of Raney nickel. The mixture was charged with 60 psi $D_2$ in a Parr micro-hydrogenation apparatus and shaken 18 hours. The ethanol was evaporated under reduced pressure and the residue was purified by steam distillation. The yield of saturated amine was 67% (10 mmol).

N-t-butyl-3-ethyl-3-nitrosopentane $-^2H_{24}$ (XIV) The amine XIII was oxidized with $H_2O_2$ by a procedure previously used for cyclic amines (reaction 14) (20). To 2 mmol of XIII were added in order 0.5 mL of acetonitrile, 250 mg of $NaHCO_3$, 36 mg of $Na_2WO_4 \cdot 2H_2O$ and 1.4 mL of 30% $H_2O_2$. The reaction was stoppered and stirred for 40 hours at room temperature. Following workup the yellow orange product was purified on a silica gel column (0.5 x 15 cm) with 20% methanol in hexane to yield 1 mmol of nitroxide XIV (50% yield).

## DISCUSSION

Freely partitioning, monofunctional, and approximately

spherical spin probes continue to be useful in biomembrane research (7,21). By increasing the hydrophobicity of such probes in measured amounts we can extend their application to difficult spin labeling problems, e.g. the red blood cell membrane (22), which do not yield to less hydrophobic spin probes without resorting to covalent binding or to polyfunctional probes. Our measure of the hydrophobicity of the spin probes is $K_{par}$ for the probe in aqueous dispersions of the phospholipid DMPC at 37°C. $K_{par}$ was determined from the integrated area of the two probe signals, M and W in Figure 1, in a dispersion of known composition. $K_{par}$ is tabulated for the spin probes in Table 1. The $K_{par}$ values increase rapidly as the carbon number of the probe is increased. The unsaturated spin probes have smaller values of $K_{par}$ than their saturated counterparts.

The $^{14}N$ hyperfine splitting, $a_N$, of each probe in water and in hydrocarbon is given in Table 1. In a given solvent the magnitude of $a_N$ decreases as the probe size increases. The hyperfine splitting of all the probes remains very solvent dependent so that all the probes give good resolution of the M and W sites for the $m_I$=-1 hyperfine lines in membrane studies.

The ESR line width, $\Delta H_{pp}$ (Gauss), of the $m_I$=0 hyperfine line at 25°C in water is also given in Table 1. This line width is much less than one Gauss for all the perdeuterated probes. The sensitivity and resolution enhancement gained from narrow probe line widths is largely due to the replacement of all hydrogen species by deuterium (significant long range coupling (23) was observed for these free radicals from protons on gamma carbons). The temperature dependence of the probe line width is complex as discussed below.

The ESR spectrum of nondeuterated (4,7)NO at -10°C gives evidence for a weak multiplet (species A) overlapping each $m_I$ line of the high temperature species (species B). At -92°C

(hexane) species A dominates and each $m_I$ signal has the appearance of a 1:2:1 triplet with a splitting of about 1.4 Gauss, suggesting very strong and approximately equivalent coupling of the unpaired electron to 2 of the 24 protons in the molecule. This temperature effect is completely reversible. We postulate that significant barriers to rotation exist about some of the single bonds of the (4,7)NO radical and we assign the A and B forms to rotational isomers. Less dramatic ESR evidence for the two isomers is also seen for the perdeuterated (4,7)NO radical. ESR evidence for rotational isomers and inequivalent beta -methylene protons in other free radicals has been reported (23,24). For the (4,7)NO probe at 37°C there is very little evidence for the low temperature A form, but a rapid equilibrium between the two forms may account for some of the additional temperature dependent ESR line width found for this probe.

Further evidence for restricted single bond rotation in our most sterically hindered spin probes is found in the anomalous intensities of the $^{13}C$ hyperfine satellites. In contrast to the DTBN probe and the (4,5)NO probe, where natural abundance $^{13}C$ satellites have an intensity of almost 4% of the parent line, the intensities of the $^{13}C$ satellites in the bulky spin probes are often less than 1% of the parent line and the temperature dependence of both the magnitudes and the intensities of these satellites is complex. $^{13}C$ satellites are routinely observed only from the two carbons alpha to the nitrogen as these splittings are independent of the internal rotations. A dynamic exchange mechanism involving ring inversion has been proposed to explain $^{13}C$ satellites from beta -carbons in cyclic nitroxide radicals (25,26). With our sterically hindered spin probes a similar mechanism may be operating, but the exchange process with our probes appears to be a temperature dependent interconversion between rotational isomers.

## CONCLUSION

Di-t-alkyl nitroxide radicals are superior spin probes following $^{2}H$ and $^{12}C$ isotopic substitution due to spectral simplification and reduced line width. Probe hydrophobicity increases with increasing carbon number. ESR evidence for restricted bond rotations may limit the usefulness of those spin probes with more than nine carbons.

TABLE 1

| spin probe | $a_N$ pentane | $a_N$ water | $\Delta H_{pp}$ water | $K_{par}$ |
|---|---|---|---|---|
| pd-DTBN | 15.10 | 17.15 | 0.26 | 13 |
| (4,5)NO | 15.0 | 16.99 | 0.55 | 25 |
| pd-(5,5)NO | 14.78 | 16.96 | 0.29 | 180 |
| (4,6)NO | 14.61 | 16.8 | 0.9 | n.a. |
| pd-(4,7)NO | 14.36 | 16.61 | 0.39 | 300 |
| (5,6)NO | 14.24 | 15.62 | 0.87 | n.a. |
| pd-(5≡,5≡)NO | 15.0 | 16.55 | 0.22 | 122 |
| pd-(5,5≡)NO | 14.86 | 16.6 | n.a. | 160 |
| pd-(4,7≡)NO | 14.45 | 16.39 | 0.45 | 290 |

All values, except $K_{par}$, are in Gauss, measured at 25°C.
$K_{par}$ is at 37°C in an aqueous DMPC dispersion.
The line width, $\Delta H_{pp}$, is for the $m_I$=0 line.

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