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Enhanced Diagnostic EPR and ENDOR Spectroscopy of Radical Spin Adducts of Deuterated α-Phenyl *N-tert*-Butyl Nitrone†

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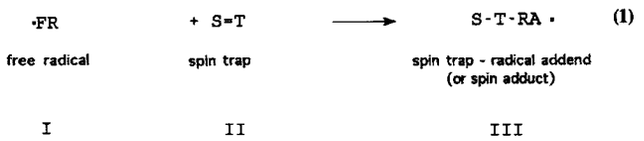
Deuterated analogues of *C*-phenyl *N-tert*-butyl nitrone (PBN) were synthesized to provide significant gains in spectral sensitivity and resolution in electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) applications. Three deuterated α-phenyl *N-tert*-butyl nitrones (PBNs) were prepared. EPR spectra of the corresponding radical spin adducts with the phenyl ring (PBN-d_5-R˙) or *tert*-butyl moiety (PBN-d_9-R˙) deuterated were found to enhance disclosure of the structure of the added radical. The most dramatic increases in EPR resolution, however, were not realized until both the phenyl and *tert*-butyl groups were deuterated (PBN-d_{14}-R˙). Here, baseline resolution of unique long-range (e.g. γ- and δ-) hyperfine splittings from the radical addend could be displayed. Representative radical spin adducts of PBN-d_{14} (methyl, hydroxyl, aminyl, cyanyl, carbamoyl, and vinyl) were prepared and compared with those of PBN to illustrate this point. It is also shown that when even higher spin adduct resolution is desired the combination of spin trap deuteration and ENDOR may be applied to advantage.

KEY WORDS EPR Free radicals ENDOR Deuterated spin traps High resolution

INTRODUCTION

The detection and identification of free radicals in complex (e.g. biological) systems along with the elucidation of their importance (*vis-á-vis* various physiological processes such as drug metabolism, ischemia reperfusion injury, radiation damage, lipid peroxidation and even carcinogenesis) continues to draw considerable attention.[1] One of the more promising approaches to the study of transient free radicals in complex milieu is a technique known as spin trapping[1–4] [Eqn (1)]. Here, the free radical (˙FR) (**I**) generally adds to an unsaturated spin trap molecule (S=T) (**II**) to provide a persistent molecule known as a spin adduct.



This addition product has the radical addend (RA) covalently bound to the spin trap (S–T–RA˙) (**III**). Detection of these species is achieved with the highly sensitive (10^{-6}–10^{-7} M) and selective electron paramagnetic resonance (EPR) spectroscopy. A modification to the EPR experiment called ENDOR (electron nuclear double resonance) is sometimes useful because it provides higher spectral resolution. It should be noted, however, that ENDOR is usually an order of magnitude or more less sensitive than EPR.

The main types of spin traps in current use are *C*-nitroso compounds (**IV**) and nitrones (**V**)[1–4] and in both cases the products are aminoxyl radicals (**VI** and **VII**) [Eqns (2) and (3), respectively].

In this account the focus is on deuterated nitrones, specifically deuterated α-phenyl *N-tert*-butyl nitrone (PBN).§ Deuterated *C*-nitroso spin traps (e.g. perdeuterio-2-methyl-2-nitrosopropane, MNP-d_9) may

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§ PBN is also known as *N-tert*-butyl *C*-phenyl nitrone or 2-methyl-*N*-phenylmethylene-2-propanamine-*N*-oxide.

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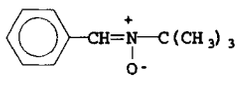
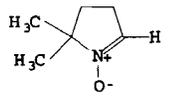
also provide enhanced EPR resolution, as has been shown by others.[5]

Ever since the early days of spin trapping[5] there has been a quest for modified and/or improved spin traps. Only lately, however, have some of these new nitrones been synthesized. Examples of new PBN-type spin traps have been published by Janzen and co-workers.[6,7] New spin traps of the DMPO type have also been recently reported by Zhang *et al.*,[8] Arya *et al.*,[9] Halpern *et al.*[10] and Pou *et al.*[11] These new nitrones with deuterium[7,9,10] and both[11] deuterium and nitrogen-15 substitution have been shown to be especially useful for EPR signal enhancement.



PBN-Type
VIII



DMPO-Type
IX

Effect of deuteration on EPR and ENDOR lines

The main advantage of spin-trap deuteration for EPR is the reduction of broad spin adduct linewidths due to long-range unresolved hyperfine splittings. The magnetogyric ratio for deuterium to hydrogen (γ_{2H}/γ_{1H}) is approximately 1:7 and since the number of nuclear manifolds for one deuterium (spin 1) *vs.* one hydrogen (spin 1/2) is 3/2, at first glance the theoretical limit of linewidth reduction would appear to be $1/7 \times 3/2 \times 100$ or 21.4%. However, since increased deuteration is well known to produce narrower linewidths (e.g. by 50%),[12] the real reason why deuteration helps is because the intensities of the outer lines in deuterium multiplets $(a^2 + ab + b^2)^n$ taper off much more quickly than do those for hydrogen $(a + b)^n$ nuclei.[13]

Although ENDOR spectroscopy is intrinsically a more complex experiment than EPR, the effect of spin-trap deuteration is actually easier to assess. All ENDOR lines are simple doublets (separated by their hyperfine splittings) centered about their distinctive free nuclear (NMR) frequencies [Eqn (4)]. For hydrogen nuclei the ENDOR lines are centred around 14.48 MHz when the magnetic field is 3400 G. Deuterium lines are generally not seen owing to the low Larmor (NMR) frequency (2.22 MHz) and small (*vs.* H) hyperfine splittings.

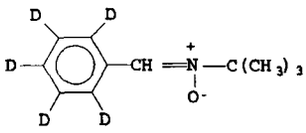
$$\nu_{ENDOR} = |\nu_\eta + A/2| \qquad (4)$$

where ν_η is the NMR free resonance frequency and A is the hyperfine splitting (when $|A/2| > \nu_\eta$, g_N (nuclear Zeeman splitting factor) > 0, $A > 0$).

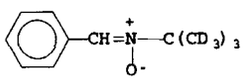
EXPERIMENTAL

Synthesis of deuterated PBNs

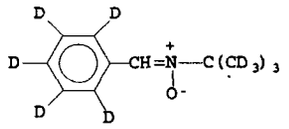
2-Methyl-2-nitropropane-d_9.[14] Deuterated *tert*-butylamine was available commercially; however, it may be synthesized via a modified Ritter reaction[15] with suitably deuterated precursors (e.g. *tert*-butanol-d_{10}). In the latter case the initial product is the deuterated *tert*-butylformamide, $(CD_3)_3CN(D)C(O)D$. The ^{13}C NMR spectrum shows the presence of two isomers owing to restricted rotation about the amide bond. ^{13}C NMR ($CDCl_3$), δ 28.46, 30.35 (methyls), 49.98, 50.69 (quaternary), 160.76, 163.08 ppm (carbonyl). The formamide hydrolysis products are the amine and amine hydrochloride. ^{13}C NMR for $(CD_3)_3CNH_2$ (in $CDCl_3$), δ 31.97 (methyls), 46.76 (quaternary); and for $(CD_3)_3CNH_3^+Cl^-$ (in D_2O), δ 29.54 (methyls), 54.94 ppm (quaternary).



PBN-d_5
X



PBN-d_9
XI



PBN-d_{14}
XII

To a magnetically stirred suspension of 28.9 g (188 mmol) of potassium permanganate in 135 ml of water at room temperature, 4.0 g (48.7 mmol) of *tert*-butylamine-d_9 (98 at.% D) is added dropwise in a three-necked flask fitted with an efficient reflux condenser and thermometer. After the addition is completed, the reaction mixture is heated 55 °C over a period of about 1 h and the mixture is kept at this temperature with stirring with a further 4 h. The dropping funnel and reflux condenser are replaced with stoppers and the flask is fitted with a short-path distillation unit. The reaction mixture is heated while stirring to steam distil the product. The condenser water is kept at around room temperature and not circulated because the nitroalkane may solidify. Approximately 45 ml of water are collected as distillate. The aqueous solution may then be placed in a refrigerator (*ca.* 4 °C). This causes the nitroalkane (which is denser than water) to solidify at the bottom of the container. The cooled aqueous layer containing the nitroalkane is extracted with diethyl ether (4 × 50 ml). The combined organic layers are washed with 2 × 5 ml of 2 M hydrochloric acid saturated with sodium chloride and 2 × 5 ml of saturated sodium chloride. The organic phase is dried with anhydrous sodium sulfate. The solvent is removed with a rotary evaporator (25 °C). It is important to stop the evaporation as soon as the last traces of ether have been removed because the volatile nitroalkane may also evaporate. The yield of white solid nitroalkane is 2.5 g (46% yield). ^{13}C NMR ($CDCl_3$), δ 27.73 (methyls), 85.00 ppm (quaternary).

α-Phenyl *N-tert*-butyl nitrone-d_{14} (PBN-d_{14}) (XII). The method of choice (in terms of ease of synthesis, yield and purity) would appear to be the procedure described by Huie and Cherry.[16] Benzaldehyde-d_5 (99.7 at.% D) (0.991 g, 8.92 mmol), 2-methyl-2-nitropropane-d_9) (2.00 g, 17.83 mmol), and 1.75 g (26.8 mmol) of zinc dust were dissolved in 65 ml of 95% ethanol cooled to 10 °C. Acetic acid (3.21 g, 53.5 mmol) was added dropwise over about 30 min with brisk mechanical (overhead) stirring. The mixture was vigorously stirred for 2 h and stored in a refrigerator for 48 h. The solid zinc acetate was filtered off. The ethanol filtrate was rotoevaporated and the zinc acetate washed with 2 × 25 ml of diethyl ether. The organic portions were combined 1.5 g of crude nitrone was obtained although some zinc acetate may have remained. The crude product was sublimed at least onc (60 °C, 0.05 Torr) and recrystallized from 10% diethyl ether–light petroleum (b.p. 90–110 °C) followed by recrystallization from 20% diethyl ether–hexanes to give *ca.* 1 g (*ca.* 60% yield) of white crystalline PBN-d_{14} (m.p. 72–73 °C) suitable for spin trapping studies. ^{13}C NMR ($CDCl_3$), δ 28.30 (methyls), 70.77 (quaternary), 129.69 (nitronyl), 128–131 ppm (aryls). MS, *m/z* 191, 173, 157, 127, 110, 93, 82, 70, 66.

The nitrones PBN-d_5 (X) and PBN-d_9 (XI) wer prepared similarly from benzaldehyde-d_5 and 2-methyl-2-nitropropane and from benzaldehyde and 2-methyl-2-nitropropane-d_9, respectively. The ^{13}C NMR spectra were identical with that for PBN-d_{14}. The MS fragmentation patterns for PBN-d_5 (X) and PBN-d_9 (XI) were as follows: *m/z* 182, 167, 151, 126, 109, 94, 82, 70, 57 and *m/z* 186, 168, 152, 122, 105, 89, 77, 65, 66, respectively.

Spin adduct–radical addend generation and EPR spectra

Adduct synthesis was typically performed in toluene or aqueous solutions at room temperature. Approximately 1–2 ml of solutions containing 50 mM nitrone and 10 mM radical addend precursor were used. Alkyl spin adducts were formed by anion addition as described previously.[7] The aminyl, hydroxyl or carbamoyl adducts were formed in aqueous peroxodisulfate with the appropriate reagent (e.g. ammonia, formamide). The cyanyl adduct was generated by a 1,3-addition of trimethylsilyl cyanide to the nitrone followed by hydrolysis of the cyano-*O*-silyl ether[17] to the hydroxylamine and subsequently oxidized to the aminoxyl.

EPR and ENDOR spectra were recorded using a Bruker ER-200D EPR spectrometer (ST 4102 and EN 801 X-band cavities, respectively) and a Bruker ER-140 (Aspect 2000) data system. It should be noted that ordinary EPR spectrometer settings (e.g. modulation amplitude = 0.1 G and power = 10 mW) were sufficient to display the long-range (γ and δ) hyperfine splittings. EPR spectra were simulated (where necessary) with a BASIC program developed by Oehler and Janzen.[18]

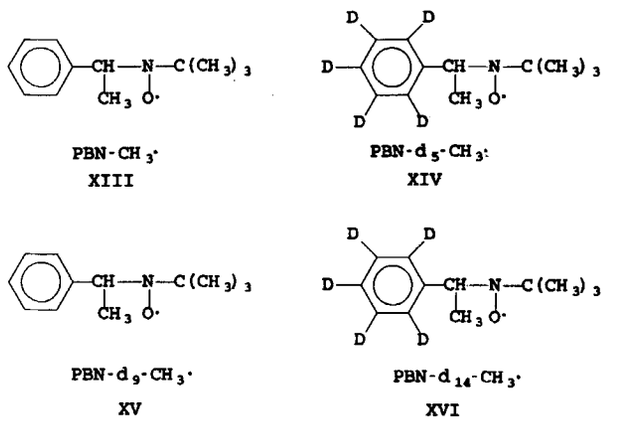
The ENDOR spectra were obtained with an E-N A-300 amplifier with 12.5 kHz radiofrequency modulation. The EPR center field was generally around 3400 G; therefore, the ^{1}H, ^{2}H, ^{14}N and ^{15}N NMR frequencies are 14.48, 2.22, 1.05 and 1.46 MHz, respectively. The sample temperature was controlled with a Bruker ER 4111 VT unit.

RESULTS AND DISCUSSION

The hyperfine splittings (hfs) of the deuterated spin adducts are given in Table 1.

The methyl adduct ($^{\cdot}CH_3$)

The degree of deuteration of the spin-trap moiety has a striking effect on the resolution of the spectrum due to the radical addend hfs. The EPR spectra of the methyl spin adducts (**XIII–XVI**) from the more deuterated spin traps exquisitely illustrate the greater view of the radical addend hfs [Fig. 1(A)–(D)]. The quartet from the methyl moiety exhibits baseline resolution only when the highly deuterated analogue, PBN-d_{14} (**XII**), is utilized.



The hydroxyl adduct ($^{\cdot}OH$)

The EPR spectrum of the hydroxyl adduct of PBN (**XVII**) yields an undistinguished triplet of doublets [Fig. 2(A)]. Introduction of PBN-d_{14} (**XVIII**), however, provides a uniquely discriminating resolution of γ-H hfs (doublet) of the radical addend [Fig. 2(B)]. Proof of this assignment was shown by the disappearance of this

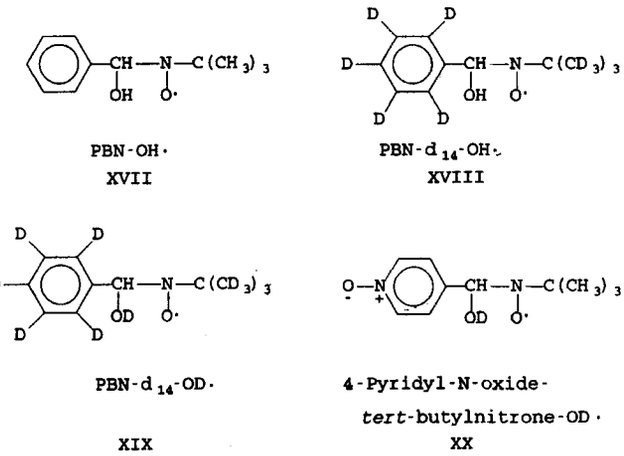


Table 1. EPR hyperfine splittings constants of representative spin adducts of PBN-d_{14} [a,b]

Radical addend	a^N	a_β^H	$a_\beta^{Nucleus}$	$a_\gamma^{Nucleus}$	$a_\delta^{Nucleus}$	Medium
CH_3	14.81	3.58	$^{13}C = 3.11$ [c]	$^{1}H = 0.45$ [d]	—	Toluene
OH	15.46	2.70	$^{17}O = 3.36$ [e]	$^{1}H = 0.21$ [d]	—	Water
NH_2	16.06	3.54	$^{14}N = 0.82$	$^{1}H = 0.39$ [d]	—	Water
CN [f]	14.84	1.76	—	$^{14}N = 0.21$	—	Toluene
$C(O)NH_2$	15.73	3.27	$^{13}C = 10.55$	$^{14}N = 0.54$	$^{1}H = 0.54$ [d]	Water
$CH{=}CH_2$	14.78	2.62	—	$^{1}H = 0.85$	$^{1}H = 0.40$, $^{1}H = 0.14$ [g]	Toluene

[a] All hfs are given in gauss and were measured at room temperature unless noted otherwise.
[b] Apart from the usual a^N and a_β^H hyperfine splittings for the spin adduct, all other hfs are attributed to the radical addend.
[c] See Ref. 7.
[d] These assignments were confirmed by isotopic labeling with deuterium.
[e] See Ref. 19.
[f] In CH_3CN $a^N = 15.02$; $a_\beta^H = 2.03$; $a_\beta^{13C} = 9.85$ G.[20]
[g] Hfs detectable only by ENDOR at 200 K.

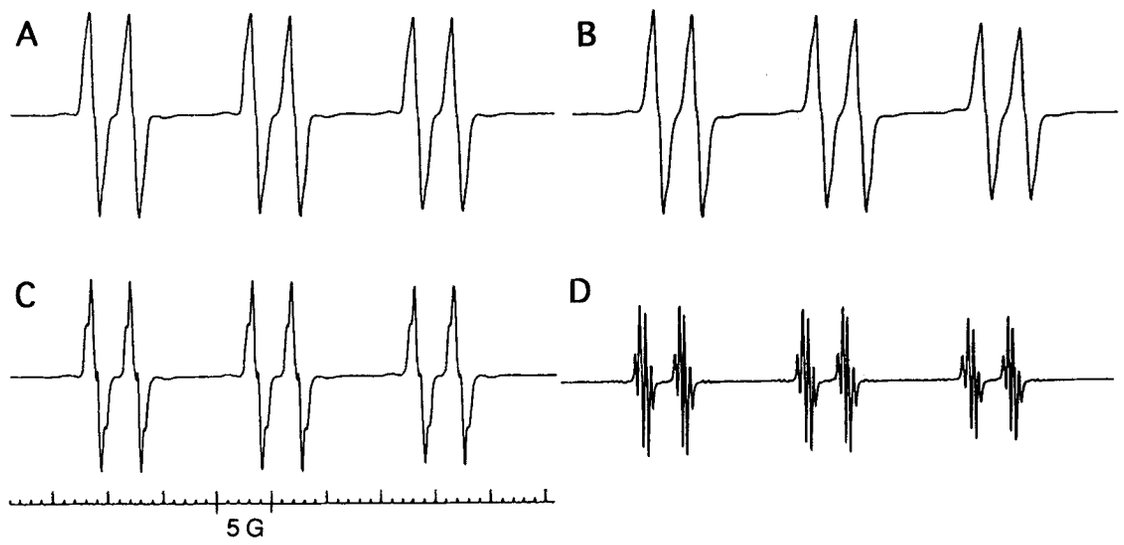


Figure 1. EPR spectra of (A) PBN-$CH_3^{\bullet}$, (B) PBN-d_5-$CH_3^{\bullet}$, (C) PBN-d_9-$CH_3^{\bullet}$ and (D) PBN-d_{14}-$CH_3^{\bullet}$ in toluene.

doublet on treatment with D_2O (**XIX**) [Fig. 2(C)]. This feature has been noted previously with the PBN-type spin trap α-(4-pyridyl-*N*-oxide) *N*-*tert*-butyl nitrone (PyOBN) (**XX**).[21]

The aminyl adduct ($^{\bullet}NH_2$)

A broad, complicated EPR spectrum results from PBN-NH_2 (**XXI**) [Fig. 3(A)]. With PBN-d_{14} (**XXII**), the spectrum becomes easier to interpret as the radical addend hfs become clearly resolved [Fig. 3(B)]. A computer simulation [Fig. 3(C)] reveals that the β-N hfs is almost exactly twice the amino δ-H hfs. This leads to a 1:2:2:2:2:2:1 septet. Confirmation of this assignment was verified by D_2O exchange (**XXIII**) [Fig. 3(D)].

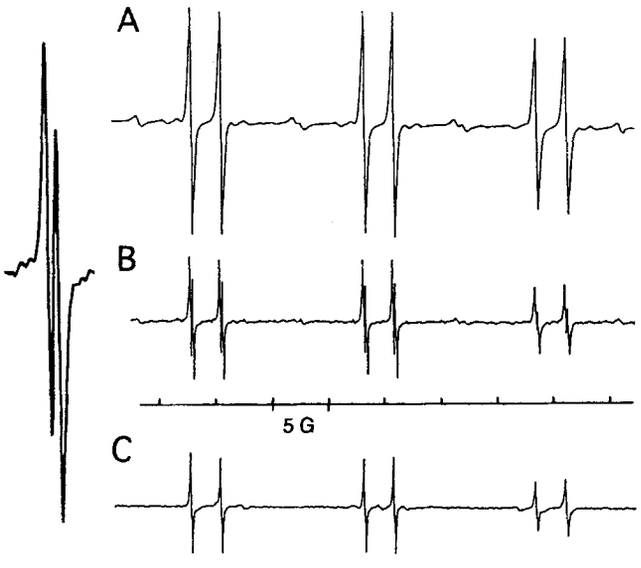
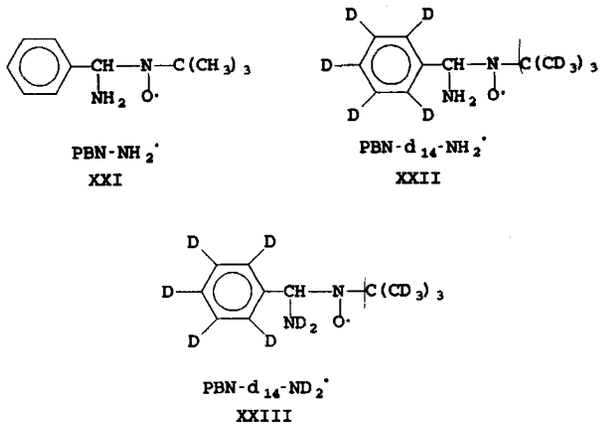
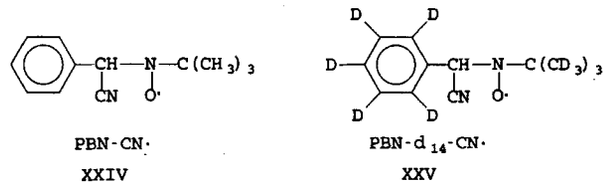


Figure 2. EPR spectra of (A) PBN-OH$^{\bullet}$ and (B) PBN-d_{14}-OH$^{\bullet}$ in water and (C) PBN-d_{14}-OD$^{\bullet}$ in deuterium oxide.



The cyanyl adduct ($^{\bullet}CN$)

Resolution of the γ-N hfs of PBN-CN$^{\bullet}$ (**XXIV**) is not possible [Fig. 4(A)], but with PBN-d_{14}-CN$^{\bullet}$ (**XXV**) this characteristic triplet becomes readily apparent [Fig. 4(B)].



The carbamoyl adduct ($^{\bullet}C(O)NH_2$)

The '^{13}C-labelled carbamoyl adduct' of PBN (**XXVI**) was chosen to avoid possible spectral interferences from the ^{12}C analogue and the hydroxyl adduct (**XVII**) [Fig. 5(A), black dots]. This general approach (β-^{13}C labelling) has been successfully utilized previously.[22] The spectrum from PBN-d_{14}-C(O)$NH_2^{\bullet}$ (**XXVII**) yields two additional radical addend hfs [Fig. 5(B)]. These are

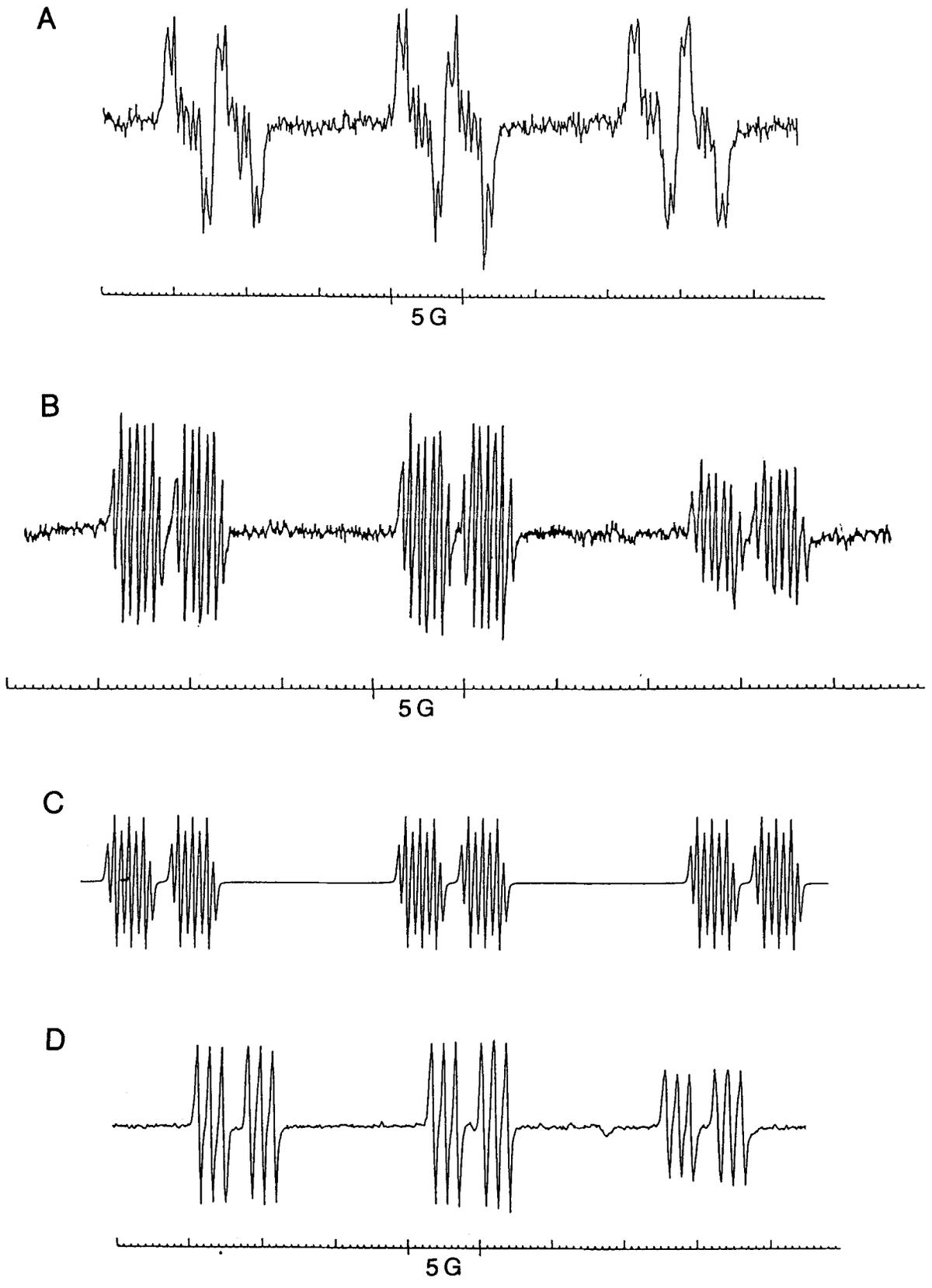


Figure 3. EPR spectra of (A) PBN-$NH_2^{\cdot}$ and (B) PBN-d_{14}-$NH_2^{\cdot}$ in water (with computer simulation (C)) and (D) PBN-d_{14}-$ND_2^{\cdot}$ in deuterium oxide.

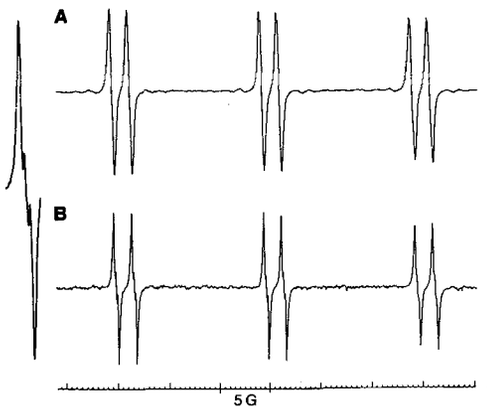
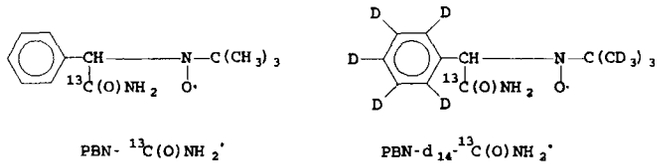


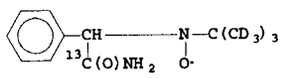
Figure 4. EPR spectra of (A) PBN-CN$^{\bullet}$ and (B) PBN-d_{14}-CN$^{\bullet}$ in toluene.

the δ-N and one δ-H hfs which just happen to be approximately equal in value (0.54 G, Table 1). Since only one of the δ-H hfs is detected it can be concluded that hindered rotation about the amide–spin adduct bond is prevalent. This feature should not be surprising because NMR[23] (which is intrinsically slower than EPR) displays slow rotation with amide compounds. Deuteration of the carbamoyl moiety (**XXVIII**) [Fig. 5(C)] causes the spectrum to lose the carbamoyl δ-H hfs. A computer simulation [Fig. 5(D)] for **XXVIII** confirms this assignment.



PBN-^{13}C(O)NH$_2^{\bullet}$
XXVI

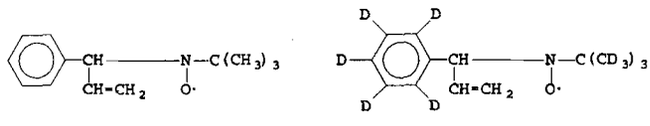
PBN-d$_{14}$-^{13}C(O)NH$_2^{\bullet}$
XXVII



PBN-d$_{14}$-^{13}C(O)ND$_2^{\bullet}$
XXVIII

The vinyl adduct ($^{\bullet}$CH=CH$_2$)

Only one radical addend hfs (δ-H) is visible with PBN-CH=CH$_2^{\bullet}$ (**XXIX**) by EPR spectroscopy [Fig. 6(A)]. With PBN-d_{14}-CH=CH$_2^{\bullet}$ (**XXX**) one more radical addend hfs is seen [Fig. 6(B)]. However, to exhibit all three radical addend proton hfs one must utilize ENDOR spectroscopy along with spin-trap deuteration [Fig. 6(C)]. The largest doublet centered about ν_{1H} is the β-H hfs whereas the three smaller doublets are attributed to the radical addend [Fig. 6(C)]. Since the two δ-H hfs are of different magnitudes (0.40 and 0.14 G), hindered rotation between the vinyl and spin-trap moieties is evident.



PBN-CH=CH$_2$
XXIX

PBN-d$_{14}$-CH=CH$_2$
XXX

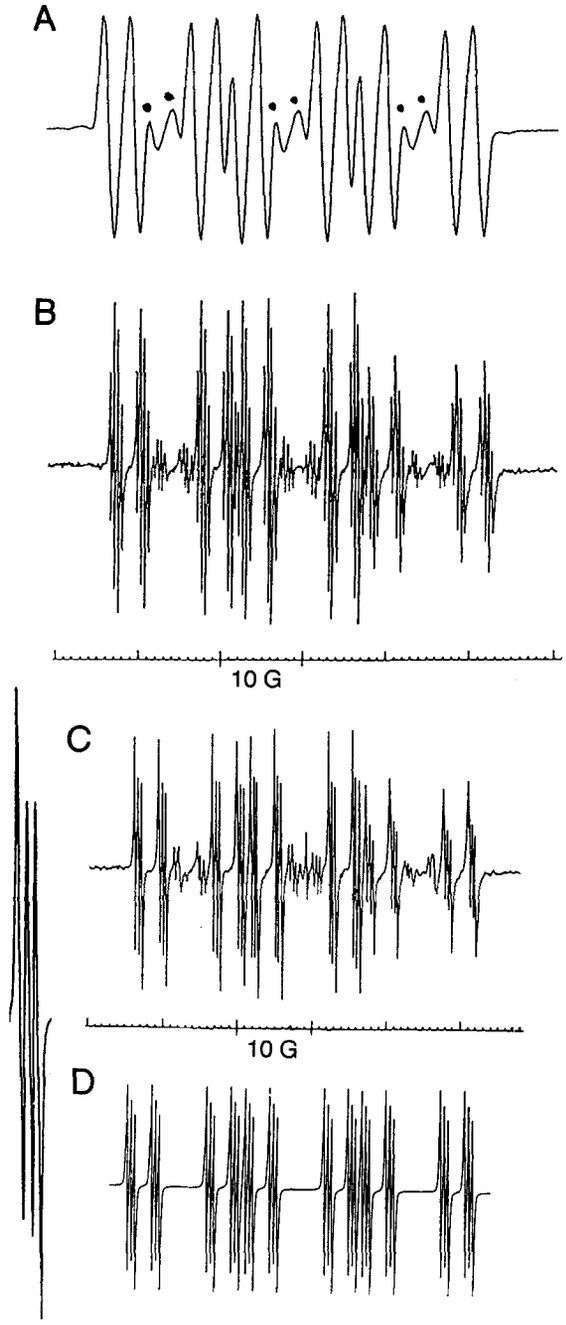


Figure 5. EPR spectra of (A) PBN-C(O)NH$_2^{\bullet}$ and (B) PBN-d_{14}-C(O)NH$_2^{\bullet}$ in water and (C) PBN-d_{14}-^{13}C(O)ND$_2^{\bullet}$ in deuterium oxide (with computer simulation (D)).

CONCLUSION

We have described the synthetic routes to three new deuterated spin traps: α-phenyl (d_5) *N-tert*-butyl nitrone, α-phenyl *N-tert*-butyl (d_9) nitrone and α-phenyl (d_5) *N-tert*-butyl (d_9) nitrone. The latter, which is fully deuterated (except for the nitronyl H) was shown to clearly be the superior spin trap for EPR and ENDOR owing to increased resolution and sensitivity. The signal enhancing ability of PBN-d_{14} was recently demonstrated in a study of 1-hydroxyethyl radicals from the *in*

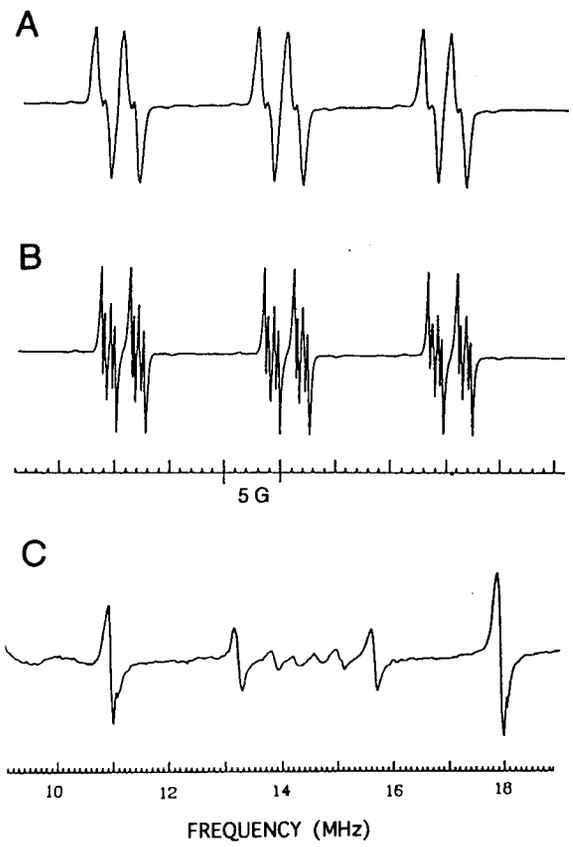


Figure 6. EPR spectra of (A) PBN-CH=CH_2· and (B) PBN-d_{14}-CH=CH_2· in toluene and the ENDOR spectrum of PBN-d_4-CH=CH_2· in toluene at 200 K.

vitro metabolism of ethanol.[24] Here, a sharp characteristic EPR triplet revealed the substructure of the added radical to be a methylene-type group (i.e. ·CH_2R). An additional side benefit of deuteration is that spin adducts of PBN-d_{14} (and PBN-d_9) in complex chemical[25] or *in vitro* biochemical systems[26] (analysed by mass spectrometry) are much easier to identify owing to the highly characteristic *m/z* 66 deuterated *tert*-butyl fragment.

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