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DETECTION OF FREE RADICALS GENERATED FROM THE *IN VITRO* METABOLISM OF CARBON TETRACHLORIDE USING IMPROVED ESR SPIN TRAPPING TECHNIQUES

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The spin trapping chemistry of carbon tetrachloride has been previously investigated in rat liver, both *in vitro* and *in vivo*. In addition to the trichloromethyl radical, both a 'carbon-centred' and an 'oxygen-centred' radical have been detected *in vitro*. These spin adducts have been assigned to 'lipid' and 'lipid oxyl' radicals. However, no specific structural characterization has been provided to date. The spin trapping chemistry of this system was reinvestigated with the use of deuterated α-phenyl N-*tert*-butyl nitrones to obtain better spectral resolution. Results indicate that the PBN trapped carbon-centred lipid radical is of a primary alkyl type.

KEY WORDS: spin trapping, carbon tetrachloride, lipid peroxidation, trichloromethyl radical, lipid and lipid oxyl radicals, deuterated spin traps, g-values, freeze-pump-thaw techniques, aminoxyl (nitroxide) spin adducts.

ABBREVIATIONS: CCl_4 – Carbon tetrachloride; $\cdot CCl_3$ – Trichloromethyl radical; PBN – α-phenyl N-*tert*-butyl nitrone; PBN(d_9) – α-phenyl N-*tert*-butyl(d_9) nitrone; PBN(d_{14}) – α-phenyl(d_5) N-*tert*-butyl(d_9) nitrone; PBN-L(R) – PBN-lipid spin adduct; PBN-CH_2-L – PBN-CH_2-lipid adduct; PBN-OL(OR) – PBN-lipid oxyl adduct.

INTRODUCTION

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin in many mammalian systems, including humans.[1–4] It is generally believed that CCl_4 is reductively cleaved via the NADPH-cytochrome P-450 electron transport chain, to the trichloromethyl radical ($\cdot CCl_3$) and a chloride anion (Cl^-).[1–4] In the presence of oxygen, it is expected that most of the $\cdot CCl_3$ radicals react to form trichloromethyl peroxyl radicals ($\cdot OOCCl_3$). The reaction of membrane lipid components with reactive free radicals results in the formation of various lipid radicals such as alkyl (L·), alkoxyl (LO·) and peroxyl (LOO·) radicals. These lipid radicals propagate peroxidative reactions which eventually lead to cell necrosis.[2,5–9]

Spin trapping techniques, in general, are useful for the detection of reactive short-lived free radicals since the addition reaction to chemical traps, such as nitrones leads to more persistent radical adducts that may be detected by electron spin resonance (ESR) spectroscopy.[8] Spin trapping studies on the metabolism of CCl_4 in rat liver have been reported.[9,10] The positive identification of the $\cdot CCl_3$ radical is now widely accepted based on the detection of the trichloromethyl spin adduct of PBN in both

in vitro (microsomal fractions[10,11] and isolated hepatocytes)[12] and *in vivo* in the rat.[7,9,11] From *in vitro* studies, with varying amounts of oxygen, other PBN spin adducts have also been detected. These spin adducts are proposed to be trapped lipid radicals of L· and LO· type. The assignments of these adducts have only been made in general terms, i.e. a 'carbon-centred' radical, other than $\cdot CCl_3$, and an 'oxygen-centred' radical.[7,11]

It is this aspect of the previously published results that we have undertaken to investigate further in this study. Deuteration of PBN, at either the *tert*-butyl group PBN(d_9), the phenyl group PBN(d_5)[13] or both PBN(d_{14}) reduces the spin adduct spectral linewidths so that long-range hyperfine splittings of magnetic nuclei present in the group which was the radical may be resolved.[14] We have used PBN-d_{14} to resolve additional splittings (e.g. γ-splittings) in the PBN-lipid adducts, as well as to determine more accurate g-values.

MATERIALS AND METHODS

1. Preparation of Rat Liver Microsomes

One-month old male Wistar rats (90–100 g) were allowed free access to Purina Certified Rat Chow No. 5002 and water. Rats were killed by CO_2 asphyxiation and the livers were subsequently removed. The livers were perfused via the hepatic portal vein with ice-cold isotonic saline (0.85% NaCl) supplemented with EDTA (0.1 mM). The perfused livers were homogenized in a 0.15 M potassium phosphate buffer (pH 7.4) supplemented with sucrose (0.25 M) and EDTA (0.1 mM). The liver homogenates were initially centrifuged at 10,000 × g to obtain a supernatant, which was further centrifuged at 105,000 × g to obtain the microsomal fraction from the pellet. The concentration of the protein was determined by the method of Lowry *et al.*[15] using bovine serum albumin as the standard. The final concentration of the microsomal protein was diluted to 40 mg/ml. The microsomal preparation was kept frozen at −70 °C until ready for use.

2. ESR Analysis of the PBN-Spin Adducts

The incubation systems were all carried out in an air atmosphere for 15 min at 25 °C. The reaction mixtures consisted of rat liver microsomes (40 mg/ml of protein in a 0.15 M potassium phosphate buffer (pH 7.4) with 0.25 M sucrose and 0.1 mM EDTA), 0.1 M PBN, PBN(d_9) or PBN(d_{14}), 0.2 M CCl_4 or $^{13}CCl_4$ (99 atom % ^{13}C) and 0.3 mM each of NADPH and NADH. The synthesis of PBN(d_9) and PBN(d_{14}) will be reported elsewhere.[13]

(a) Whole microsomal system For the whole microsomal systems, the reaction mixture was transferred to a flat cell and flushed with nitrogen gas for 10–15 min to remove oxygen. ESR spectra of the reaction mixture were then determined with the use of a Bruker AQ TM cavity.

(b) Rat liver lipid extracts For the lipid extract experiments, the lipids were extracted from the whole microsomal reaction mixture with hexane (50 ml). The hexane extracts were collected and roto evaporated to dryness, and subsequently resuspended in benzene (0.5–1.0 ml). The benzene solution was transferred to a round cell and

subsequently subjected to 3 freeze-pump-thaw cycles to remove oxygen. ESR spectra of the lipid extracts, in benzene, were then determined with the use of an ESR ST cavity at room temperature.

ESR spectra were recorded using a Bruker EPR ER-200D spectrometer, and spectral accumulation was done by using a Bruker ER-140 (ASPECT 2000) data system. For long-term accumulations, a field frequency lock was used (with DPPH as the internal standard). The ESR spectral analysis was performed with the use of a computer simulation program.[16]

3. *Determination of g-Values for PBN Spin Adducts*

(a) PBN-CCl_3 The PBN-CCl_3 spin adduct was obtained by photolysis (15 min irradiation) of a reaction mixture consisting of 0.05 M PBN and 0.2 M CCl_4 in benzene. Prior to photolysis the reaction mixture was subjected to 3 freeze-pump-thaw cycles. The PBN-CCl_3 g-value was determined by using a solution of Fremy's salt as a reference (g-value = 2.00550 ± 0.00005; a_N = 13.091 ± 0.004).[17,18]

(b) PBN-L and PBN-OL The g-values for both the PBN-L and the PBN-OL spin adducts were determined by using the g-value for the PBN-CCl_3 as an internal standard in combination with computer simulation techniques.

RESULTS AND DISCUSSION

The presence of oxygen in the solutions during ESR investigations tends to hinder high resolution analysis of spin-adduct spectra. Thus, all spectra were obtained in the complete absence of air either by extensive degassing with nitrogen gas (whole microsomal preparations) or via a freeze-pump-thaw cycle technique, i.e. vacuum

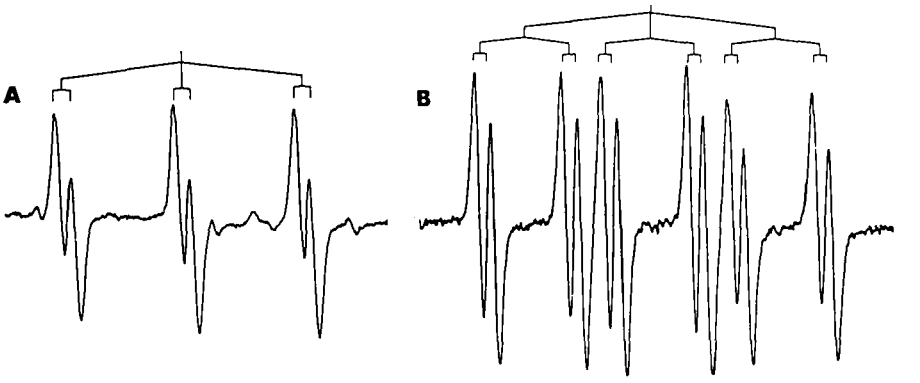

FIGURE 1 ESR spectra of the PBN-CCl_3 spin adduct. These signals were observed after incubation (15 min) of rat liver microsomes (25 °C) with $^{12}CCl_4$ or $^{13}CCl_4$, NADPH, NADH and PBN. The samples were flushed with nitrogen (10–15 min) to remove oxygen prior to ESR analysis. (A) Spectrum of the PBN-$^{12}CCl_3$ spin adduct; a_N = 13.9, a_β^H = 1.75 G; (B) Spectrum of the PBN-$^{13}CCl_3$ spin adduct; a_N = 14.1, a_β^H = 1.8, a_β^{13C} = 9.6 G.

TABLE I

Comparison of hyperfine coupling constants of PBN-spin adducts derived from *in vitro* CCl_4 metabolism to those of previously published PBN-spin adducts

PBN-adduct	Solvent	a_N	a_β^H	Ref.
PBN-CCl_3	microsomal preparation	13.9	1.75	exp.[a]
	water	14.1	1.8	19
	benzene	13.88	1.62	exp.[b]
	benzene	14.00	1.68	20
PBN-R				
R = L	benzene[c]	14.49	3.32	exp.[b]
-CH_3	benzene	14.79	3.73	21
-CH_2CH_3	benzene	14.68	3.25	21
-$CH_2CH_2CH_2CH_3$	benzene	14.64	3.21	21
-$CH_2(CH_2)_{10}CH_3$	toluene	14.66	3.33	22
-$CH(CH_3)_2$	benzene	14.67	2.59	24
-$C(CH_3)_3$	benzene	14.66	2.32	24
PBN-OR				
OR = OL	benzene[c]	13.70	1.88	exp.[b]
OCH_3	benzene	13.77	2.05	25
OCH_2CH_3	benzene	13.88	2.09	23
$OCH_2CH_2CH_2CH_3$	benzene	13.65	1.97	21
$OC(CH_3)_3$	benzene	14.11	1.83	26

[a] Experimental values from Fig. 1 (using PBN).
[b] Experimental values from Fig. 4 (using PBN(d_{14})).
[c] The benzene solution contains variable amounts of microsomal lipids from the lipid extraction procedure, which may modify the splitting constants slightly (±0.2 G).

evacuation, 0.05 torr (microsomal lipid extraction studies) following incubation in an air atmosphere.

Figure 1 illustrates results from studies involving the trapping of the $\cdot CCl_3$ radical *in vitro* (rat liver microsomes) under aerobic (air atmosphere) conditions. When rat liver microsomes, NADPH, NADH, $^{12}CCl_4$ and PBN are incubated under an aerobic atmosphere, a 6-line hyperfine splitting pattern due to the trichloromethyl radical adduct of PBN was observed (Fig. 1(a)). When the same experiments were repeated, by replacing $^{13}CCl_4$ for $^{12}CCl_4$, the spectrum shown in Fig. 1(b) was obtained. Spin adducts with ^{13}C-labeling have a characteristic doubling of each spectral line (which is not observed for $^{12}CCl_4$) due to the nuclear spin of 1/2 for ^{13}C, thus establishing that $\cdot CCl_3$ is derived from CCl_4. Comparison of the hyperfine splitting constants for PBN-CCl_3 in a microsomal system to those in water (Table I) indicates that they are quite similar, irrespective of the presence of protein and lipid components in the microsomal preparation.

Figure 2 is a spectrum of a microsomal lipid extract in benzene (subjected to 3 freeze-pump-thaw cycles) obtained from a reaction mixture consisting of rat liver microsomes, NADPH, NADH, $^{13}CCl_4$ and PBN (incubated under aerobic conditions). At least three PBN-spin adducts are observed simultaneously. In addition to the characteristic 12-line pattern (A) of the PBN-$^{13}CCl_3$ adduct, there are two additional sets of overlapping triplet of doublets attributed to a 'carbon-centred' PBN-L adduct (pattern B) and 'oxygen-centred' PBN-OL adduct (pattern C).

Previous studies[10] have indicated that L· can only be detected when oxygen is absent

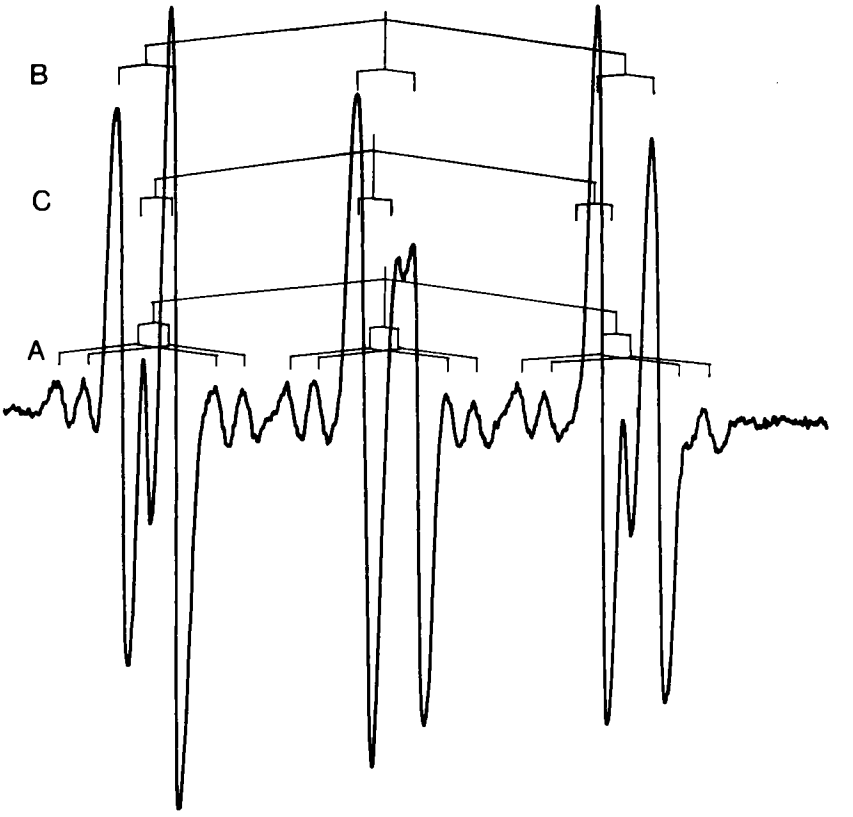

FIGURE 2 ESR spectrum of a microsomal lipid extract obtained after incubation (15 min) of rat liver microsomes (25 °C) with $^{13}CCl_4$, NADPH, NADH and PBN. The microsomal lipids were extracted with hexane, evaporated to dryness and resuspended in benzene. Prior to ESR analysis, the lipid extract in benzene was subjected to freeze-pump-thaw (15–20 min) to remove oxygen. (A) The PBN-$^{13}CCl_3$ adduct; a_N = 13.9, a_β^H = 1.7, a_β^{13C} = 9.6G; (B) The PBN-L adduct; a_N = 14.6, a_β^H = 3.5G; (C) The PBN-OL adduct; a_N = 13.3, a_β^H = 1.9G.

or completely consumed in the presence of the spin trap, since L· reacts more rapidly with oxygen than with PBN. Thus, as the oxygen tension decreases, L· becomes a principal radical, which is spin trapped by PBN, producing the mixed signal seen in Fig. 2.

The assignment of the PBN-OL adduct is based on a comparison of similar a_N and a_β^H values to previously published primary alkoxyl adducts of PBN (Table I), as well as a characteristic g-value (Table II). A g-value of 2.00631 for the PBN-OL adduct is characteristic for 'oxygen-centred' adducts of PBN. The g-values for the two 'carbon-centred' PBN adducts, PBN-CCl_3 and PBN-L were found to be 2.00628 and 2.00612, respectively. The higher g-value for PBN-CCl_3 is probably due to the electronegative chlorine groups adjacent to the carbon.

Substitution of PBN with the deuterated analogue, PBN(d_9), and subsequent lipid extraction of the microsomal reaction mixture (Fig. 3) resulted in the detection of

TABLE II

Comparison of g-values for the different PBN(d_{14}) spin adducts obtained from the *in vitro* mmetabolism of CCl_4.

PBN-Adduct	g-value (in benzene)
PBN-CCl_3	2.00628 ± 0.00001
PBN-L	2.00612 ± 0.00002
PBN-OL	2.00631 ± 0.00002

Fremy's salt (reference aminoxyl radical)[18]
g-value = 2.00550 ± 0.00005,
a_N = 13.091 ± 0.004[17]

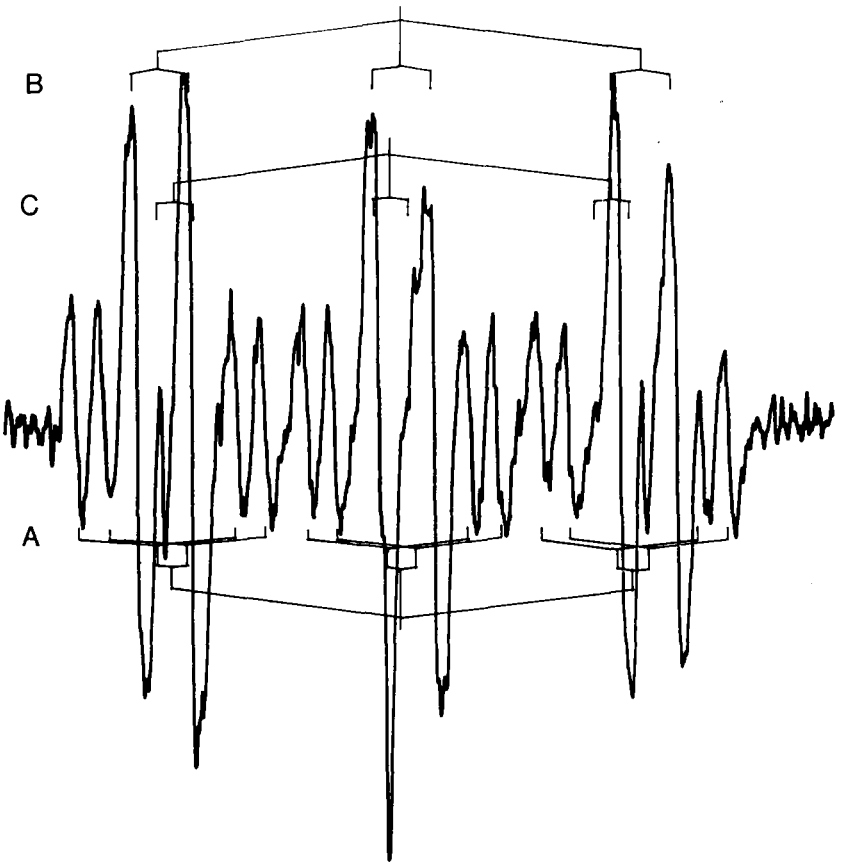

FIGURE 3 ESR spectrum of a microsomal lipid extract obtained after incubation (15 min) of rat liver microsomes (25 °C) with $^{13}CCl_4$, NADPH, NADH and PBN(d_9). Treatment of the lipid extract was similar to that described in Fig. 2. (A) The PBN(d_9)-$^{13}CCl_3$ adduct; a_N = 14.00, a_β^H = 1.60, a_β^{13C} = 9.64 G; (B) The PBN(d_9)-L adduct; a_N = 14.65, a_β^H = 3.33 G; (C) The PBN(d_9)-OL adduct; a_N = 13.71, a_β^H = 1.98 G.

PBN-adducts similar to those observed in Fig. 2 with a moderate increase in resolution. Long-range hyperfine splitting of the PBN-L adduct is observed to a minor extent, but the resolution is still not sufficient to permit additional spectral assignments to be made. $PBN(d_5)$ has been shown to give similar spectra as $PBN(d_9)$ (results not shown).[13] Due to the degree of overlap between the spectral signals of the PBN-L and PBN-OL adducts, it is difficult, without the use of computer simulation, to determine the a_N and a_β^H values for the PBN-OL adduct.

When PBN is replaced with $PBN(d_{14})$, the spectrum shown in Fig. 4 is obtained. The dominant PBN-spin adducts observed in both Figs. 2 and 3 are present, but there is a considerable increase in the spectral resolution for all three adducts. Determination of the a_N and a_β^H values for both the PBN-CCl_3 and the PBN-L adducts can be made with much better accuracy. Of greater importance, the spectral resolution has increased to the extent where γ-hydrogen hyperfine splitting for the PBN-L adduct is observable with a characteristic 1:2:1 relative intensity pattern (pattern B). The 1:2:1

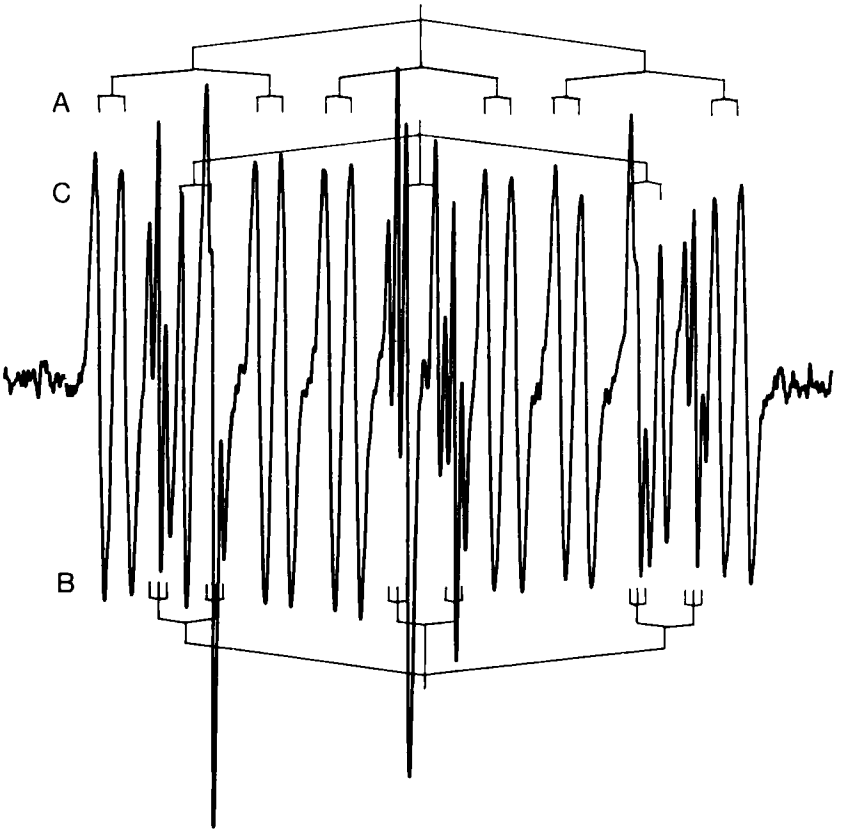

FIGURE 4 ESR spectrum of a microsomal lipid extract obtained after incubation (15 min) of rat liver microsomes (25 °C) with $^{13}CCl_4$, NADPH, NADH and $PBN(d_{14})$. Treatment of the lipid extract was similar to that described in Fig. 2. (A) The $PBN(d_{14})$-$^{13}CCl_3$ adduct; a_N = 13.88, a_β^H = 1.61, a_β^{13C} = 9.67 G; (B) The $PBN(d_{14})$-CH_2-L adduct; a_N = 14.49, a_β^H = 3.35 G, a_γ^H = 0.53 G (2H); (C) The $PBN(d_{14})$-OL adduct; a_N = 13.70, a_β^H = 1.88 G.

spectral pattern is proof that a methylene alkyl group is coupled to the α-carbon of the spin adduct. This finding suggests that the lipid radical trapped is of a primary alkyl type ($\cdot CH_2$-R) and thus quite different from the structure of the radical usually proposed resulting from hydrogen abstraction from the bis-allylic position of the unsaturated hydrocarbon chain.[6] Other studies in our laboratory indicate that PBN may be partially associated with a polar region of the membrane structure in a microsomal preparation.[27] Therefore, it is possible that PBN may be trapping lipid radicals that are formed as a result of hydrogen abstraction near the phospholipid polar head group.

In conclusion, it is apparent that substituting PBN(d_{14}) for PBN in spin trapping experiments greatly enhances the spectral resolution, thus making the assignments of a mixture of PBN-spin adducts in a biological sample more dependable.

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