Synthesis and EPR Evaluation of the Nitrone PBN-[*tert*-^{13}C] for Spin Trapping Competition

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Electron Paramagnetic Resonance, Spin Trapping, Free Radicals

N-[*tert*-^{13}C]Butyl *C*-phenyl nitrone (PBN-[*tert*-^{13}C]) has been synthesized for an EPR spin trapping competition study. The newly synthesized PBN-[*tert*-^{13}C] shows different ^{13}C-hyperfine splitting constants ($a_{^{13}C}$) when it traps free radicals as compared to another ^{13}C-labeled PBN analogue, *N-tert*-butyl *C*-phenyl [*nitronyl*-^{13}C]nitrone (PBN-[*nitronyl*-^{13}C]). The PBN-[*tert*-^{13}C] hydroxyl adduct gives a larger $a_{^{13}C}$ value (5.14 G) as compared to the PBN-[*nitronyl*-^{13}C] hydroxyl adduct (4.36 G). This gain of the $a_{^{13}C}$ value decreases the chance of EPR signal overlap, thus providing a more resolved EPR spectrum when PBN-[*tert*-^{13}C] is used as an internal standard for EPR spin trapping competition studies of hydroxyl radical formation.

Introduction

Nitrone compounds are well known for their free radical-trapping ability and the wide-spread use as spin traps in the EPR spin trapping technique [1]. One of the most commonly used nitrone spin traps is *N-tert*-butyl *C*-phenyl nitrone (PBN) [2]. PBN is a good candidate for in vivo radical-trapping EPR studies because it exhibits reasonable pharmacokinetic properties. When PBN is administrated intraperitoneally to rats, its $t_{1/2}$ is 134 min and its concentration reaches a maximum at 15 min in plasma, and at 30 min in liver, kidney, brain, heart and lungs [3]. PBN also shows very interesting pharmacological effects. Chronic administration of PBN has been shown to reverse age-related parameters in Mongolian gerbils [4]. Pre-administration of PBN reduces the mortality associated with endotoxic shock in rats [5]. Treatment of mice with PBN before lipopolysaccharide administration significantly reduces the nitric oxide generation in liver [6]. PBN can prolong the life span of the senescence accelerated mouse [7]. PBN also can alleviate ischemia-reperfusion injury in animal brain and heart [8], and can mitigate liver edema in CCl_4 intoxicated rats [9]. Because of its interesting pharmacological properties of PBN and its radical-trapping ability, further improvement by modification of the PBN structure is desired for drug discovery. One of the examples is *N-tert*-butyl *C*-[2,4-di(sodiumsulfo)phenyl] nitrone (NXY-059), an analogue of PBN with higher solubility in water. NXY-059 has demonstrated its neuroprotective effects after transient focal cerebral ischemia in the rat [10] and is currently in human clinic trials for acute stroke.

In order to investigate the relationship between the radical-trapping capabilities of PBN-type analogues and their pharmacological effects, an internal spin trap standard is needed for the determination of relative radical-trapping rates with the EPR spin trapping methodology. Although PBN-^{15}N (*N-tert*-butyl *C*-phenyl [^{15}N]nitrone) [11] is a suitable spin trap for this purpose, the synthesis of PBN-^{15}N involves the preparation of $^{15}NCl_3$ which is highly toxic and may cause immediate headache and nausea [11–13] even if all of the procedures are kept in a hood. Another stable isotope-labeled PBN, *N-tert*-butyl *C*-phenyl [^{2}H]nitrone (PBN-D), is not a good candidate as an internal reference for spin trapping competition because the deuterium atom in spin adducts generally provides very small hyperfine splitting constants, splittings not large enough to differentiate the additional spectral lines from other PBN analogue adduct lines in the EPR spectrum. Very fortunately, spin adducts from a ^{13}C-labeled PBN, *i.e., N-tert*-butyl *C*-phenyl [*nitronyl*-^{13}C]nitrone (PBN-[*nitronyl*-^{13}C]), may provide ^{13}C-hyperfine splitting constants large enough for separating some of the reference EPR signals from the PBN analogue spin adduct EPR signals [14,15]. Since *N*-[*tert*-^{13}C]butyl *C*-phenyl ni-

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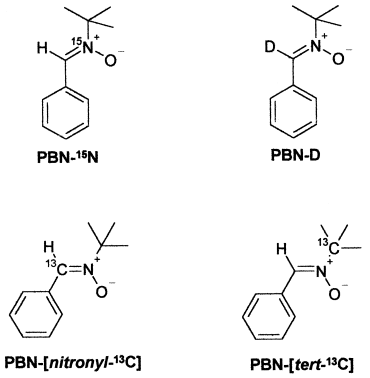
trone (PBN-[*tert*-^{13}C]) has not been previously reported and the ^{13}C-hyperfine splitting constants of its spin adducts remain unknown, it would be interesting to pursue this compound and investigate the EPR spectra of the spin adducts from this nitrone. This paper describes the synthesis of PBN-[*tert*-^{13}C] and the preliminary EPR study of this nitrone in comparison to PBN-[*nitronyl*-^{13}C]. The structures of the four stable isotope-labeled PBNs are illustrated in Scheme 1.



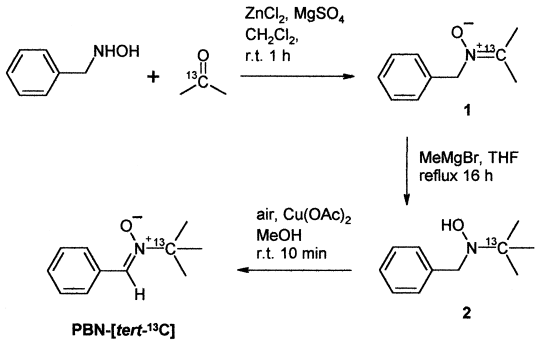
Scheme 1

PBN-[*tert*-^{13}C] has been synthesized by a convenient three-step route using acetone-[*carbonyl*-^{13}C] as the isotope-labeling starting material. As illustrated in Scheme 2, N-benzylhydroxylamine reacts with acetone-[*carbonyl*-^{13}C] in the presence of $ZnCl_2$ as a catalyst and $MgSO_4$ as a drying agent to generate the ^{13}C-labeled ketonitrone **1**. The ketonitrone **1** is treated with the Grignard reagent MeMgBr at refluxing temperature in dry tetrahydrofuran to provide the hydroxylamine intermediate **2**. This Grignard reaction does not occur when the reaction is performed at room temperature for 3 h. Oxidation of the hydroxylamine **2** with air catalyzed by $Cu(OAc)_2$ in methanol generates the desired nitrone product PBN-[*tert*-^{13}C]. An EPR grade pure sample is obtained after three sublimations.

Results and Discussion

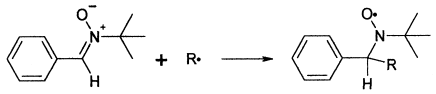
EPR spin trapping with PBN

Although the hyperfine splitting constants (hfsc's) for the spin adducts of PBN in Table 1 were previously described [15], they are collectively presented herein in order to compare easily



Scheme 2

Table 1. EPR hfsc's for spin adducts of PBN.

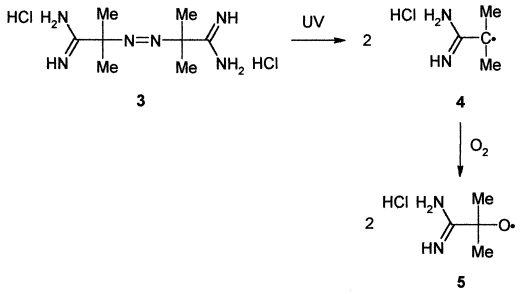


Radical R• Trapped	Radical Source	Hfsc's of Spin Adduct [a] a_N (G)	a_H (G)	a_{13C} (G)
[a]				
	+ air [b] + UV	15.64	4.08	--
•OH	H_2O_2 + UV	15.64	2.76	--
•CH_2OH	$CH_3OH + H_2O_2$ + UV	16.07	3.87	--
•CD_2OH	$CD_3OH + H_2O_2$ + UV	16.08	3.89	--
•$^{13}CH_2OH$	$^{13}CH_3OH + H_2O_2$ + UV	16.08	3.89	3.89
•$CH(CH_3)OH$	$C_2H_5OH + H_2O_2$ + UV	16.16	3.36	--
•$^{13}CH(CH_3)OH$	$CH_3{}^{13}CH_2OH + H_2O_2$ + UV	16.17	3.48	3.48
•$C(CH_3)_2OH$	$(CH_3)_2CHOH + H_2O_2$ + UV	16.15	3.57	--
•CH_3	DMSO+ H_2O_2 + UV [c]	16.51	3.56	--
•OCH_3 [d]	DMSO+ H_2O_2 + air + UV	15.15	3.30	--
•CD_3	DMSO-d_6 + H_2O_2 + UV [c]	16.48	3.62	--
•OCD_3 [e]	DMSO-d_6 + H_2O_2 + air + UV	15.15	3.33	--
	THF + H_2O_2 + UV	16.07	3.28	--
	1,4-dioxane + H_2O_2 + UV	15.96	3.01	--

[a] Hfsc's stand for the hyperfine splitting constants. Phosphate buffer (100 mM, pH=7.4) was used as a solvent. The concentration of hydrogen peroxide was 1% and the substrate such as methanol is 20%. Radical **4** could not be trapped with the nitrone, see text; data a_N and a_H refer to **5**; [b] air was needed to generate the oxygen-centered radical **5**. EPR signal was not observed if argon gas was bubbled through the solution before UV irradiation; [c] argon gas was bubbled through the solution before UV irradiation; [d] methyl radical adducts were also identified as minor components; [e] deuterated methyl radical adducts were also identified as minor components.

with the hfsc's for the corresponding spin adducts of PBN-[*nitronyl*-^{13}C] and PBN-[*tert*-^{13}C]. When the water-soluble azo-compound **3** was irradiated with UV light under an inert atmosphere such as argon gas, the generated carbon-centered radical **4** was so bulky and sterically hindered that it could not be trapped by PBN, resulting in a silent EPR spectrum. In contrast, when air was present, the bulky radical **4**, as illustrated in Scheme 3, reacted with molecular oxygen to generate the oxygen-

centered radical **5** that was trapped by PBN, giving a typical six-line EPR spectrum.



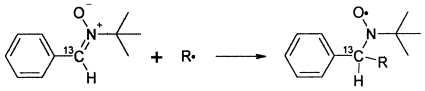
Scheme 3

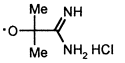
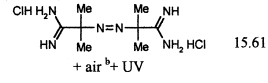
The free radicals in Table 1 were chosen mainly based on four reasons: (1) These radicals could easily be generated in an aqueous phosphate-buffered solution. (2) Deuterated carbon-centered radicals provide better resolution of the spin adduct EPR spectra due to smaller EPR line-width as compared to the corresponding non-deuterated radical adducts. (3) ^{13}C-labeled carbon-centered radicals confirm the corresponding non-labeled carbon radicals. For example, hydroxyl radicals from hydrogen peroxide can be trapped with PBN. By adding an alcohol, such as methanol or ethanol, one expects a reaction between a hydroxyl radical and the alcohol molecule giving a new alcohol carbon radical that is then trapped by PBN resulting in a different EPR spectrum. ^{13}C-labeled alcohols such as $^{13}CH_3OH$ and $CH_3{}^{13}CH_2OH$ may confirm the corresponding alcohol carbon radical adduct by providing additional ^{13}C-splitting. (4) These radicals basically represent water-soluble carbon-centered radicals and oxygen-centered radicals.

EPR spin trapping with PBN-[nitronyl-^{13}C]

As shown in Table 2, the spin adducts of PBN-[*nitronyl*-^{13}C] provide additional ^{13}C-hfsc's as compared to the corresponding PBN adducts [15]. The ^{13}C-hfsc is derived from the interaction between the nitroxyl odd-electron and the ^{13}C-atom. The ^{13}C-hfsc is a sensitive probe to the free radical addend because the free radical is covalently linked to the ^{13}C-atom in the spin adduct [14–16]. For oxygen-centered radical adducts, because the oxygen is a strong electron-withdrawing atom, ^{13}C-hfsc's with the range of 4.36–5.61 G are

Table 2. EPR hfsc's for spin adducts of PBN-[*nitronyl*-^{13}C].



Radical R• Trapped	Radical Source	Hfsc's of Spin Adduct [a] a_N (G)	a_H (G)	a_{13C} (G)
	a  + air [b] + UV	15.61	4.11	5.61
•OH	H_2O_2 + UV	15.62	2.71	4.36
•CH_2OH	$CH_3OH + H_2O_2$ + UV	16.06	3.87	6.17
•CD_2OH	$CD_3OH + H_2O_2$ + UV	16.08	3.89	6.17
•$CH(CH_3)OH$	$C_2H_5OH + H_2O_2$ + UV	16.16	3.37	6.01
•$C(CH_3)_2OH$	$(CH_3)_2CHOH + H_2O_2$ + UV	16.12	3.61	6.04
•CH_3	DMSO+ H_2O_2 + UV [c]	16.47	3.57	5.97
•OCH_3 [d]	DMSO+ H_2O_2 + air + UV	15.13	3.33	5.30
•CD_3	DMSO-d_6 + H_2O_2 + UV [c]	16.48	3.60	6.01
•OCD_3 [c]	DMSO-d_6 + H_2O_2 + air + UV	15.15	3.32	5.32
	THF + H_2O_2 + UV	16.05	3.34	5.94
	1,4-dioxane + H_2O_2 + UV	15.98	3.12	5.97

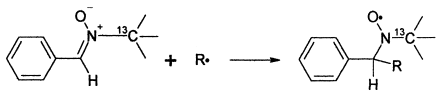
[a] Hfsc's stand for the hyperfine splitting constants. Phosphate buffer (100 mM, pH=7.4) was used as a solvent. The concentration of hydrogen peroxide was 1% and the substrate such as methanol is 20%. Radical **4** could not be trapped with the nitrone, see text; data a_N and a_H refer to **5**; [b] air was needed to generate the oxygen-centered radical **5**. EPR signal was not observed if argon gas was bubbled through the solution before UV irradiation; [c] argon gas was bubbled through the solution before UV irradiation; [d] methyl radical adducts were also identified as minor components; [e] deuterated methyl radical adducts were also identified as minor components.

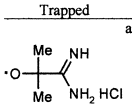
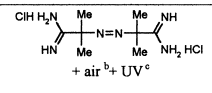
smaller than those for carbon-centered radical adducts with the range of 5.94–6.17 G, as shown in Table 2. With regard to the N-hfsc's and H-hfsc's, the data for PBN-[nitronyl-^{13}C] adducts are consistent with those for PBN, which indicates the reproducibility of these radical-generating systems.

EPR spin trapping with PBN-[tert-^{13}C]

The hfsc's of spin adducts from PBN-[*tert*-^{13}C] are shown in Table 3. Since the ^{13}C-atom is the tertiary carbon that is three single bonds away from the free radical addend, the ^{13}C-hfsc values are not very sensitive to the variety of these free radical addend. The ^{13}C-hfsc values for PBN-[*tert*-^{13}C] adducts are in the range of 5.06–5.57 G which are much narrower than the range of 4.36–6.17 G for PBN-[*nitronyl*-^{13}C] adducts. Although most of the ^{13}C-hfsc values for PBN-[*tert*-^{13}C] adducts are smaller than those for the corresponding PBN-[*nitronyl*-^{13}C] adducts, the PBN-[*tert*-^{13}C] hydroxyl adduct does give larger a_{13C} (5.14 G) as compared to 4.36 G of the PBN-[*nitronyl*-^{13}C] hydroxyl adduct (Scheme 4). This gain of the a_{13C} value may decrease the chance of EPR signal overlap, thus

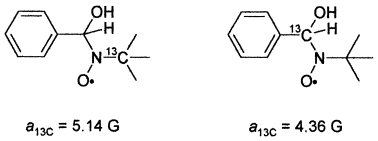
Table 3. EPR hfsc's for spin adducts of PBN-[*tert*-^{13}C].



Radical R• Trapped	Radical Source	Hfsc's of Spin Adduct [a] a_N (G)	a_H (G)	a_{13C} (G)
	 + air [b] + UV [c]	15.60	4.19	5.06
•OH	H_2O_2 + UV [c]	15.61	2.74	5.14
•CH_2OH	$CH_3OH + H_2O_2$ + UV [c]	16.06	3.91	5.43
•CD_2OH	$CD_3OH + H_2O_2$ + UV [c]	16.08	3.90	5.47
•$CH(CH_3)OH$	$C_2H_5OH + H_2O_2$ + UV [c]	16.14	3.37	5.46
•$C(CH_3)_2OH$	$(CH_3)_2CHOH + H_2O_2$ + UV [c]	16.13	3.63	5.57
•CH_3	DMSO+ H_2O_2 + UV [c, d]	16.45	3.62	5.45
•OCH_3 [e]	DMSO+ H_2O_2 + air + UV [c]	15.11	3.31	5.35
•CD_3	DMSO-d_6 + H_2O_2 + UV [c, d]	16.48	3.62	5.46
•OCD_3 [f]	DMSO-d_6 + H_2O_2 + air + UV [c]	15.12	3.31	5.33
	THF + H_2O_2 + UV [c]	16.05	3.36	5.37
	1,4-dioxane + H_2O_2 + UV [c]	15.93	3.07	5.36

[a] Hfsc's stand for the hyperfine splitting constants. Phosphate buffer (100 mM, pH=7.4) was used as a solvent. The concentration of hydrogen peroxide was 1% and the substrate such as methanol is 20%. Radical **4** could not be trapped with the nitrone, see text; data aN and aH refer to **5**; [b] air was needed to generate the oxygen-centered radical **5**. EPR signal was not observed if argon gas was bubbled through the solution before UV irradiation; [c] argon gas was bubbled through the solution before UV irradiation; [d] methyl radical adducts were also identified as minor components; [e] deuterated methyl radical adducts were also identified as minor components.

providing more resolved EPR spectrum when PBN-[*tert*-^{13}C] is used as an internal standard for EPR spin trapping competition study of hydroxyl radical formation.



a_{13C} = 5.14 G a_{13C} = 4.36 G

Scheme 4

Experimental Section

Melting points were measured on a Perkin Elmer DSC-6 differential scanning calorimeter. Elemental analysis was performed by Galbraith Laboratories, Inc. (Knoxville, TN, USA). ^{13}C-Labeled chemical reagents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Other chemical reagents were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Organic solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA).

EPR spin trapping

PBN-[*nitronyl*-^{13}C] and PBN were synthesized in house by reacting benzaldehyde-[*carbonyl*-^{13}C] or benzaldehyde, respectively, with N-*tert*-butylhydroxylamine, and purified by sublimation until no EPR-active impurity was detectable at 50 mM concentration of the nitrone and 1×10^6 of the EPR receiver gain. EPR spectra were recorded at room temperature on a Bruker ESP-300 electron paramagnetic resonance spectrometer. The EPR instrument settings were as following: microwave frequency at 9.76 GHz, microwave power at 10 dB, field modulation intensity at 0.2 G, receiver gain between 1×10^5 to 1×10^6, time constant at 0.64 s, phase at 280°, scan range 100 G, scan time from 100 s to 240 s. The concentration of PBN-[*tert*-^{13}C] and PBN-[*nitronyl*-^{13}C] used in spin trapping experiments was 50 mM. When ultraviolet (UV)-light was used to generate free radicals, the UV-light beam from a 100-W mercury lamp (Oriel model #6281) was directly focused into an EPR cavity within which an EPR flat cell containing the interested solution had been placed. The mercury lamp was assembled with an igniter (Oriel model #66001) which was power-supplied with a universal Oriel unit (Oriel model #68805). The electric current for the mercury lamp was 5A. The mixed solution in an EPR flat cell in the EPR cavity was irradiated with UV for a few to 20 seconds depending on the radical system.

Synthetic procedure for the preparation of PBN-[tert-^{13}C]

A mixture of acetone-[*carbonyl*-^{13}C] (1.00 g, 16.93 mmol) and zinc chloride (2.31 g, 16.93 mmol) in methylene chloride was stirred at 20 °C for 15 min. To this mixture were added *N*-benzylhydroxylamine (2.2 g, 17.86 mmol) and $MgSO_4$ (2.1 g, 16.93 mmol) at 20 °C. The reaction mixture was stirred for 1 h, filtered and evaporated to dryness. The residue was chromatographed on silica gel with ethyl acetate as elution to remove zinc cloride providing 3.50 g (yield 100%) of the ketonitrone (**1**). ^{1}H NMR (270.17 MHz, $CDCl_3$): δ = 7.40–7.25 (m, 5H, C_6H_5), 5.16 (d, J_{13C} = 1.5 Hz, 2H, CH_2), 2.33 (d, J_{13C} = 13.6 Hz, 3H, CH_3), 2.28 (d, J_{13C} = 13.6 Hz, 3H, CH_3). ^{13}C NMR (67.94 MHz, $CDCl_3$): δ = 163.46 (^{13}C isotope, nitronyl carbon), 132.11 (C_1 of phenyl), 129.11 (C_3 and C_5 of phenyl), 128.86 (C_4 of phenyl), 128.44 (C_2 and C_6 of phenyl), 63.10 (d, J_{13C} = 2.1 Hz, CH_2), 22.45 (d, J_{13C} = 13.0 Hz, CH_3), 21.79 (d, J_{13C} = 11.9 Hz, CH_3).

To a solution of the ketonitrone **1** (2.60 g, 15.83 mmol) in anhydrous THF (150 ml) was added MeMgBr (3.0 M, 10 ml, 30 mmol) at 20 °C under argon atmosphere. The mixture was refluxed for 16 h and then cooled to 0 °C before NH_4Cl-saturated water (8 ml) was slowly added. The solvent was rotary evaporated and the residue was dissolved in methylene chloride. Removal of the solvent gave a yellowish solid (1.90 g) which contained at least three components without the presence of the ketonitrone **1** according to ^{1}H NMR. The ^{1}H NMR spectrum also suggested the presence of the desired hydroxylamine intermediate **2** in this mixture. To a solution of this mixture in methanol (100 ml) was added a mixed solution of $Cu(OAc)_2$ monohydrate (0.05 g) and 38% NH_4OH (1 ml) in methanol (10 ml). The mixture was stirred under open air until a blue color appeared. The solvent was rotary evaporated and the obtained residue was chromatographed on silica gel eluted with a mixed solvent of hexanes and ethyl acetate (3:1, v/v). The target nitrone PBN-[*tert*-^{13}C] was obtained in 14.2% yield (0.4 g). Further purification by three sublimations at 65 °C/1 torr provided EPR grade sample for the spin trapping study. In comparison, the TLC profile of this product was the same as that for unlabeled PBN; R_f = 0.12 (silica gel plate, $CHCl_3$); m.p. 71.4 °C (DSC method). ^{1}H NMR (270.17 MHz, $CDCl_3$): δ = 8.28 – 8.25 (m, 2H, *o*-H of C_6H_5), 7.52 (d, J_{13C} = 1.5 Hz, 1H, C**H**=N(O)), 7.40 – 7.36 (m, 3H, *p*-H, *m*-H of C_6H_5), 1.58 (d, J_{13C} = 4.0 Hz, 9H, 3 CH_3) ppm. ^{13}C NMR (67.94 MHz, $CDCl_3$): δ = 131.10 (C_1 of phenyl), 130.16 (C_4 of phenyl), 129.88 (broad, *C*H=N(O)), 128.83 (C_2 and C_6 of phenyl), 128.48 (C_3 and C_5 of phenyl), 70.86 (^{13}C isotope, *tert*-C of butyl), 28.40 (d, J_{13C} = 38.4 Hz, *C*H_3). The assignment of ^{1}H and ^{13}C NMR data was made with the help of the corresponding CH correlation NMR spectrum of this nitrone product. $^{13}CC_{10}H_{15}NO$ (178.24): calcd. C 74.68, H 8.48, N 7.86; found C 74.28, H 8.71, N 7.85.

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