First Synthesis of *C*-Phenyl *N-tert*-Butyl [^{15}N]nitrone (PBN-^{15}N) for the EPR Spin Trapping Methodology

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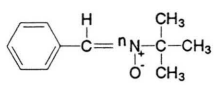
C-Phenyl *N-tert*-Butyl [^{15}N]nitrone, Nitrone, Spin Trap, EPR Spin Trapping, Aminoxyl (Nitroxide)

As compared to normal PBN, about fifty percent increase of electron paramagnetic resonance (EPR) spin trapping sensitivity has been gained by using a new 100% ^{15}N-enriched spin trap, *C*-phenyl *N-tert*-butyl[^{15}N]nitrone (PBN-^{15}N). PBN-^{15}N has been prepared by a convenient four-step route using ammonium-^{15}N chloride as the starting material. This synthetic method produces 2-methyl-2-[^{15}N]nitropropane which is useful for the synthesis of many other PBN-^{15}N type spin traps for the purpose of increasing spin trapping sensitivity. EPR spin trapping with PBN-^{15}N in benzene and in phosphate buffer has been investigated. The ^{15}N hyperfine splitting constant (^{15}N-hfsc) is larger than ^{14}N-hfsc by 40%. The larger ^{15}N-hfsc gives more opportunity to identify different radical addends within the same system.

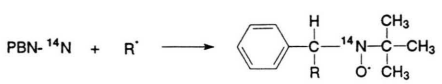
Several ^{15}N-enriched aminoxyls and nitrones have been synthesized for the purpose of increasing the sensitivity of the electron paramagnetic resonance (EPR) spin labelling and spin trapping methodologies [1]. A ^{15}N-enriched DMPO spin trap, 5,5-di([^{2}H$_3$]methyl) [^{15}N]-1-pyrroline *N*-oxide (DMPO-d_6-^{15}N), has been prepared, but no spin trapping results from this spin trap have been reported [1d]. Although the perdeuterated DMPO-^{15}N has also been synthesized [1d], the widespread use of this nitrone for enhancement of the spin trapping sensitivity is limited because the β-deuterium hyperfine splitting is so small that it is rarely useful for the diagnosis of free radical addends [1d]. For PBN (*C*-phenyl *N-tert*-butyl nitrone) [2] type spin traps, no ^{15}N-enriched compounds have been synthesized before and no spin trapping results are available. This communication describes the first synthesis of a new 100% ^{15}N-enriched spin trap, *C*-phenyl *N-tert*-butyl [^{15}N]nitrone (PBN-^{15}N), and spin trapping results with this spin trap are also presented. About 50% increase of spin trapping sensitivity has been gained as compared to normal PBN which consists of 99.63% of PBN-^{14}N and 0.37% of PBN-^{15}N [2].



n = 14, PBN-^{14}N

n = 15, PBN-^{15}N

As one of the most common spin traps, PBN-^{14}N has been used for about 30 years to detect reactive free radicals involved in chemical and biological processes [3]:



The nuclear spin (I) of ^{14}N is equal to 1 which produces the triplet hyperfine splitting for the spin adducts of PBN-^{14}N in addition to the doublet due to the β-H. Fig. 1(a) gives the EPR spectrum for the *n*-butoxyl radical adduct of PBN-^{14}N as an example. When ^{14}N is replaced with ^{15}N, ^{15}N (I = $^1/_2$) gives a doublet pattern as shown in the EPR spectrum of the *n*-butoxyl radical adduct of PBN-^{15}N in Fig. 1(b).

PBN-^{15}N has been synthesized by a convenient four-step route using 100% ^{15}N-enriched ammonium-^{15}N chloride as a starting material. As illustrated in Scheme 1, ammonium-^{15}N chloride

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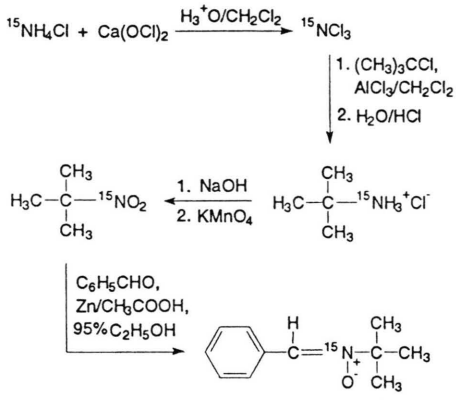


Scheme 1.

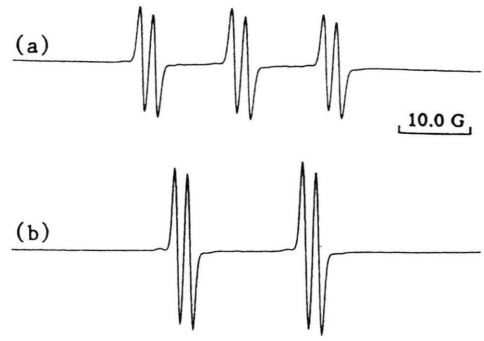
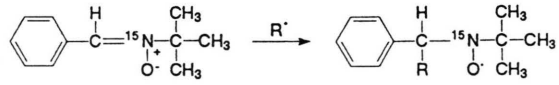


Fig. 1. (a) EPR spectrum for the *n*-butoxyl radical spin adduct of PBN-^{14}N (^{14}N-hfsc=13.70 G and H-hfsc=2.00 G), generated by photolysis of *n*-butyl nitrite (0.25 v.%) and PBN-^{14}N (10 mM) in benzene; (b) EPR spectrum of the *n*-butoxyl radical spin adduct of PBN-^{15}N (^{15}N-hfsc=19.21 G and H-hfsc=2.00 G), generated by photolysis of *n*-butyl nitrite (0.25 v.%) and PBN-^{15}N (10 mM) in benzene. EPR spectrometric conditions: modulation frequency = 100.0 kHz; modulation amplitude = 1.0 G; sweep speed=2.39 G/s; center field=3476.0 G; microwave frequency=9.65 GHz; microwave power=20 mW.

was reacted with calcium hypochlorite to generate trichloramine-^{15}N ($^{15}NCl_3$) which was treated with 2-chloro-2-methylpropane in the presence of aluminum chloride providing 2-[^{15}N]amino-2-methylpropane after hydrolysis [4,5]. Oxidation [6] of the [^{15}N]-labeled amine with potassium permanganate produced 2-methyl-2-[^{15}N]nitropropane which is expected to be widely useful for the synthesis of PBN-^{15}N type spin traps. *N-tert*-Butylhydroxyl-[^{15}N]amine generated *in situ* by reduction of the [^{15}N]nitro compound with zinc and acetic acid reacted further with benzaldehyde giving the PBN-^{15}N spin trap which was purified by the combination of column chromatography and repeated sublimations with 12% overall yield. Impurity EPR signals are not detected from a solution of the PBN-^{15}N product (50 mM) in benzene when the receiver gain of the EPR spectrometer is equal to 1.0×10^6 (for other spectrometric conditions, see Fig. 1).

Pure PBN-^{15}N was used to trap twelve different reactive free radicals in benzene (see Table I) and nine in phosphate buffer (see Table II). The hydrogen hyperfine splitting constants (H-hfsc) for the PBN-^{15}N radical adduct should be identical to the adduct of PBN-^{14}N because the difference in N-isotopes should have no EPR observable influence on the property of the β-hydrogen of the radical adduct [7]. Theoretically, as compared to PBN-^{14}N, the sensitivity of the adduct of PBN-^{15}N should be 50% greater because the triplet pattern due to the ^{14}N hfs is divided into the doublet due to ^{15}N hfs. This sensitivity improvement has been tested by using 50% PBN-^{15}N and 50% PBN-^{14}N contained in the same solution to trap the same free radical generated *in situ*. Fig. 2 shows the EPR spectrum of the *i*-amyloxyl adducts of PBN-^{15}N and PBN-^{14}N in the same benzene solution. The relative intensities of the peaks are clearly greater

Table I. EPR hyperfine splittings for radical spin adducts of PBN-^{15}N in benzene.[a]



Radical (R•)	Source	^{15}N-hfsc	H-hfsc
C_6H_5COO•	$(C_6H_5COO)_2$	18.55	1.50
C_6H_5COO•	C_6H_5COOOH[b]	18.62	1.52
t-BuOO•	*t*-BuOOH+UV	19.16	1.36
t-BuO•	$(t\text{-BuO})_2$+UV	19.55	2.02
n-BuO•	*n*-BuONO+UV	19.21	2.00
i-BuO•	*i*-BuONO+UV	19.21	1.91
i-AmylO•	*i*-AmylONO+UV	19.21	1.96
$Me_2C(CN)O$•	AIBN+O_2+UV	19.45	2.05
$Me(CH_2)_4C(O)$•	$Me(CH_2)_4CHO+(t\text{-BuO})_2$+UV	20.01	3.13
$EtC(O)$•	$EtCHO+(t\text{-BuO})_2$+UV	20.09	3.18
c-$C_6H_{11}C(O)$•	*c*-$C_6H_{11}CHO+(t\text{-BuO})_2$+UV	19.95	3.81
$C_6H_5C(O)$•	$C_6H_5CHO+(t\text{-BuO})_2$+UV	20.04	4.50
C_6H_5•	$(C_6H_5COO)_2$+UV	20.19	2.20

[a] The spectra were recorded at room temperature. The hyperfine splittings are given in Gauss (1 G = 0.1 mT). The error is estimated to be ± 0.05 G. Photolysis with UV was usually for a number of seconds; [b] Benzaldehyde was used; C_6H_5COOOH is present in benzaldehyde as an impurity from air oxidation.

Table II. EPR hyperfine splittings for radical spin adducts of PBN-^{15}N in phosphate buffer.[a]

Radical (R•)	Source	^{15}N-hfsc	H-hfsc
•OH	$1\%H_2O_2$+UV	21.70	2.74
•CH_2OH	CH_3OH+$1\%H_2O_2$+UV	22.39	3.81
•$CH(CH_3)OH$	C_2H_5OH+$1\%H_2O_2$+UV	22.43	3.27
•$CH(C_2H_5)OH$	n-C_3H_7OH+$1\%H_2O_2$+UV	22.58	3.13
•$C(CH_3)_2OH$	$(CH_3)_2CHOH$+$1\%H_2O_2$+UV	22.48	3.62
•CH_3	$CH_3S(O)CH_3$+$1\%H_2O_2$+UV	22.92	3.67
•CD_3	$CD_3S(O)CD_3$+$1\%H_2O_2$+UV	22.97	3.62
•C_6H_5	$(C_6H_5COO)_2$[b]	22.39	4.20
•N_3[c]	NaN_3+$1\%H_2O_2$	21.24	2.34

[a] The spectra were recorded at room temperature. The hyperfine splittings are given in Gauss (1 G = 0.1 mT). The error is estimated to be ± 0.05 G. Photolysis with UV was usually for a number of seconds. The concentration of the phosphate buffer is 0.1 M; [b] the phenyl adduct was generated in benzene and the residue after removal of benzene was dissolved in phosphate buffer; [c] the splitting from one of the nitrogens in $^{14}N_3$ addend is equal to 1.98 G consistent with the value in ref. 7.

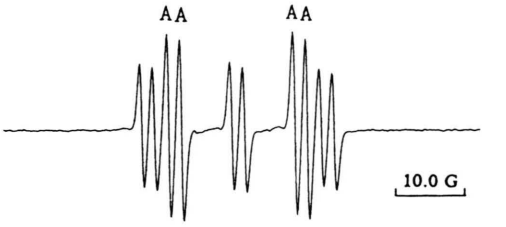


Fig. 2. EPR spectrum of the *i*-amyloxyl radical spin adducts of PBN-^{15}N (labelled with "A", ^{15}N-hfsc=19.16 G and H-hfsc=1.96 G) and PBN-^{14}N (^{14}N-hfsc=13.69 G and H-hfsc=1.96 G), generated by photolysis of *i*-amyl nitrite (0.25 v.%), PBN-^{15}N (5 mM) and PBN-^{14}N (5 mM) in benzene. EPR spectrometric conditions are identical to in Fig. 1.

for PBN-^{15}N as compared to PBN-^{14}N spin adduct.

Six free radicals have been investigated for comparison and the results are organized in Table III. This Table shows 42–55% sensitivity increase of the PBN-^{15}N adduct over PBN-^{14}N. The ^{15}N-hfsc is also larger than ^{14}N-hfsc by 40% which is expected from the difference of their nuclear magnetic moments (μ, −0.283 for ^{15}N and +0.404 for ^{14}N) [8]. When more than one radical adduct is present in the same solution, the larger ^{15}N-hfsc (*i.e.* more spacing) might give more opportunity to identify different radical addends.

In conclusion, a synthetic route has been developed to produce 2-methyl-2-[^{15}N]nitropropane which is useful for the preparation of PBN-^{15}N type of spin traps to increase spin trapping sensitivity. PBN-^{15}N is the first example of this type of spin trap which has been synthesized and used to trap reactive free radicals. It has been shown that about 50% sensitivity improvement is realized and 40% more spacing between lines is found as compared to normal PBN. Other PBN-^{15}N type spin traps are being developed for further improvement in spin trapping sensitivity.

Experimental

EPR spectra were measured on a Bruker ESP 300E or 300 spectrometer. MS spectra were determined on a FISONS/VG QUATTRO MS/MS triple quadrupole spectrometer with the condition of 70 eV (EI). Ammonium-^{15}N chloride was purchased from ISOTEC, Inc. Other chemicals were purchased from Aldrich Chemical Company, Inc.

Table III. Comparison of EPR spin trapping of PBN-^{15}N with PBN-^{14}N in the same solution.[a]

Radical (R•)	Solvent	PBN-^{15}N		PBN-^{14}N		EPR intensity increase[b]
		^{15}N-hfsc	H-hfsc	^{14}N-hfsc	H-hfsc	$[(^{15}N-^{14}N)/^{14}N]\times100\%$
n-BuO•	benzene	19.26	1.96	13.73	1.96	42%
i-AmylO•	benzene	19.16	1.96	13.69	1.96	48%
C_6H_5•	benzene	20.19	2.20	14.42	2.22	42%
•CH_2OH	buffer[c]	22.39	3.81	16.03	3.81	55%
•CHMeOH	buffer[c]	22.53	3.27	16.03	3.30	45%
•CD_3	buffer[c]	22.94	3.62	16.37	3.62	43%

[a] The spectra were recorded at room temperature. The hyperfine splittings are given in Gauss (1 G = 0.1 mT). The error of the splitting is estimated to be ± 0.05 G; [b] the average intensity of PBN-^{15}N adduct subtracts the average intensity of PBN-^{14}N adduct, then divided by the average intensity of PBN-^{14}N adduct. The obtaining result times 100% to give the percentage of the EPR intensity increase; [c] the concentration of phosphate buffer is 0.1 M.

EPR spin trapping

The concentration of PBN-^{15}N and PBN-^{14}N used in spin trapping experiments was 1×10^{-2} M. When ultraviolet(UV)-light was utilized to generate free radicals, the UV-light beam from a 75 W high-pressure mercury UV lamp was directly focused into an EPR cavity. Within the cavity, an EPR sample tube containing the interested solution had been placed. Generally, the sample solution was irradiated with UV-light just for a few seconds.

The preparation of C-phenyl N-tert-butyl [^{15}N]nitrone (PBN-^{15}N)

The literature procedure for the preparation of trichloramine (NCl_3) [3,4] was adapted for the synthesis of trichloramine-^{15}N ($^{15}NCl_3$) in our system. A 250-ml three-necked flask, equipped with a mechanical stirrer, a thermometer and an addition funnel, was cooled to 1–2 °C. Calcium hypochlorite (70%, 12.35 g, 58.7 mmol), water (27 ml) and methylene chloride (41 ml) were added successively. With vigorous stirring, a solution of ammonium-^{15}N chloride (2.0 g, 36.7 mmol) in a mixed solvent of 37% hydrochloric acid (7 ml) and water (21 ml) was added dropwise for 20 min at ≤ 5 °C. The solution was stirred for additional 25 min with cooling. The two layers were separated and methylene chloride (20 ml) was used to wash the flask. The combination of the methylene chloride was washed with cold water (2 × 40 ml) and dried over anhydrous sodium sulfate. The obtained solution contained the desired intermediate of trichloramine-^{15}N ($^{15}NCl_3$) and was kept below -50 °C for 2 h before use.

The solution after immediate filtration was added in one-portion to a precooled dry flask containing aluminum chloride (7.33 g, 55 mmol) and methylene chloride (15 ml) under an atmosphere of pure nitrogen at -10 °C. With magnetic stirring, a solution of 2-chloro-2-methylpropane (10.18 g, 110 mmol) in methylene chloride (10 ml) was added dropwise over 20 min to the reaction flask while the temperature was controlled between -10 °C and -5 °C. After the reaction mixture was stirred for an additional hour at this temperature, it was poured over a mixture of 37% hydrochloric acid (5 ml) and ice (50 g). The mixture was stirred for several minutes, then left to stand overnight. The two layers were separated and the organic layer was washed with water (2 × 40 ml). The combined aqueous solution was extracted with diethyl ether (2 × 50 ml) and bubbled with nitrogen gas for 10 minutes. This solution contained the hydrochloric salt of 2-[^{15}N]amino-2-methylpropane. The solution was cooled to 0–5 °C and another solution of sodium hydroxide in water (50%, 30 g) was added. The resulting basic solution was steam-distilled immediately. The distillate (*ca.* 100 ml) was carefully collected in a precooled flask containing potassium permanganate (10 g, 63.3 mmol). Another portion of potassium permanganate (10 g) was added and the solution was stirred for 2 h at 0–5 °C and overnight at 20 °C. Again, potassium permanganate (10 g) was added and the mixture was stirred for 3 h at 20 °C. Steam-distillation was conducted and the distillate (*ca.* 100 ml) was collected in a flask cooled to 0–5 °C. The distillate was extracted with diethyl ether (2 × 40 ml). The ether solution was dried over anhydrous magnesium sulfate. After filtration, ether was removed by slow fractional distillation to give 1.6 g of clear liquid. TLC (thin layer chromatography) analysis showed that the R_f value of the liquid was the same as a known sample of 2-methyl-2-nitropropane (R_f=0.5 with hexane as eluent). This liquid product is the desired intermediate of 2-methyl-2-[^{15}N]nitropropane which was used directly for further preparation.

This crude liquid was mixed with benzaldehyde (3.26 g, 30.76 mmol) in 95% ethanol (25 ml). After cooling to 0–5 °C, zinc dust (2.0 g, 30.76 mmol) was added with magnetic stirring. Acetic acid (3.67 g, 61.52 mmol) was added dropwise for 20 min at < 9 °C. The reaction mixture was stirred for additional 7.5 h at < 3 °C, then stored in a refrigerator overnight. The mixture was filtered and the cake was washed with ethanol (2 × 30 ml). The residue after rotaevaporation was dissolved in chloroform (75 ml) and washed with water (2 × 30 ml). The organic solution was dried over anhydrous magnesium sulfate, filtered and rotaevaporated. The residue was chromatographed on silica gel eluted successively with methylene chloride and chloroform to give 1.18 g of sticky solid. One sublimation at 60–65 °C/0.1 torr gave 0.79 g of white crystals with 12% overall yield. This product was further purified by repeated sublimations (5-times more) at 50–55 °C/0.1 torr affording 0.65 g of nice white crystals of *C*-phenyl *N*-*tert*-butyl [^{15}N]nitrone (PBN-^{15}N). TLC analysis with UV detection method showed only one spot. The R_f value of the product is the same as a known sample of normal PBN. R_f=0.12 (silica gel plate, $CHCl_3$); m.p. 70–71 °C.

^{1}H NMR (300 MHz, $CDCl_3$/TMS, 298 K) δ 8.31–8.28 (m, 2H, Ar-H), 7.56 (d, J=2.4 Hz, 1H, CH=NO), 7.43–7.40 (m, 3H, Ar-H), 1.62 (d, J=2.4 Hz, 9H, $C(CH_3)_3$) ppm.

MS (rel. int.%) *m/z* 179 (M^++1, 1), 178 (M^+, 8), 123 (2), 122 (11), 121 (4), 105 (4), 89 (14), 77 (11), 66 (9), 65 (14), 63 (7), 57 (*t*-Bu^+, 100), 41 (40), 39 (17).

$C_{11}H_{15}{}^{15}NO$ (178.2)

Calcd	C 74.12	H 8.48	N 8.42%,
Found	C 73.99	H 8.57	N 8.36%.

These data are consistent with the desired structure of the novel compound *C*-phenyl *N-tert*-butyl[^{15}N]nitrone (PBN-^{15}N). The spin trapping experiments described in this paper also support the correct structure of this compound.

Acknowledgment

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[2] At least six names for PBN are currently being used in the literature: α-phenyl *N-tert*-butyl nitrone, *C*-phenyl *N-tert*-butyl nitrone, *N-tert*-butyl α-phenyl nitrone, *N-tert*-butyl *C*-phenyl nitrone, *N*-benzylidene *tert*-butylamine *N*-oxide, and 2-methyl-*N*-phenylmethylene-2-propanamine *N*-oxide. Because PBN-^{14}N is 269-times more naturally abundant than PBN-^{15}N in normal PBN, PBN-^{14}N will be used to refer to normal PBN in the description of this work.

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