SYNTHESIS OF *N-TERT*-BUTYL-α-(4-[^{18}F]FLUOROPHENYL)-NITRONE ([^{18}F]FPBN) FOR *IN VIVO* DETECTION OF FREE RADICALS.

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SUMMARY

We have synthesized the fluorine-18 labeled derivative of *N-tert*-butyl-α-phenylnitrone (PBN), a free radical spin trapping agent widely used with electron spin resonance (ESR). *N-tert*-Butyl-α-(4-[^{18}F]fluorophenyl)-nitrone ([^{18}F]FPBN) could be prepared with low radiochemical yield (3% decay corrected) by the direct aromatic nucleophilic substitution of *N-tert*-butyl-α-(4-nitrophenyl)nitrone with [^{18}F]fluoride. An alternate two step synthesis route consisted of the nucleophilic [^{18}F]fluoride substitution of 4-*N,N,N*-trimethylammoniumbenzaldehyde triflate to yield 4-[^{18}F]fluorobenzaldehyde, which was distilled into a vial containing *N-tert*-butylhydroxylamine in 2N NaOH. 4-[^{18}F]Fluorobenzaldehyde readily reacted with the hydroxylamine to form [^{18}F]FPBN . [^{18}F]FPBN was obtained in overall decay corrected yields of 24% in a total synthesis time <45 min. and was suitable for further applications in *in vivo* studies of free radicals.

Key-words: free radicals, PBN, fluorine-18, 4-[^{18}F]fluorobenzaldehyde

INTRODUCTION

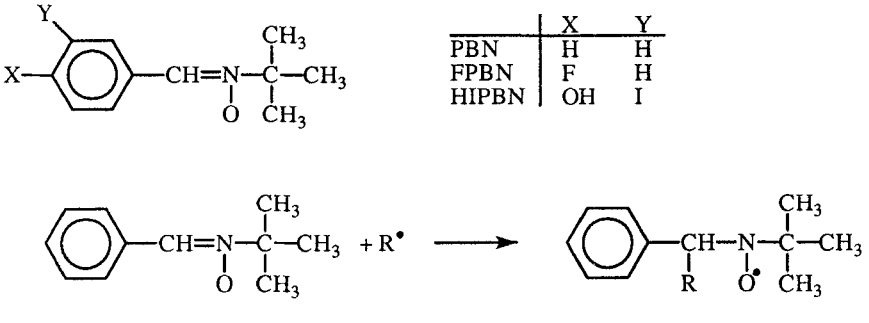
The role of free radicals related to the pathology of various diseases and aging has gained increasing attention during the last decade. Ischemia-reperfusion injuries, neurodegenerative diseases, cigarette smoke toxicity and inflammation are among the fast growing list of disorders that are linked to the presence of free radicals in cells and tissues. A recent issue of the British Medical Bulletin extensively reviewed the relationship of free radicals and disease (1).

CCC 0362–4803/95/020103–08

Received 10 April, 1994
Revised 22 August, 1994

The presence of free radicals can be detected by electron spin resonance spectroscopy (ESR) but direct detection in tissue is difficult due to the short half-life and the low concentrations of free radicals. Free radicals can however be reacted *in vivo* with spin-trapping agents to form a relatively stable adduct that can be extracted from the homogenized tissue to be finally assayed by ESR. Direct *in vivo* ESR measurements of free radicals adducts have been reported but provide only limited spatial information (2-4). Another approach for the *in vivo* detection of free radicals is the observation of brain chemiluminesence accompanying the production of free radicals (5). This method is however relatively invasive as it requires a cranial window and moreover does not provide any spatial information.

Positron emission tomography (PET) with an appropriate radiopharmaceutical that binds to free radicals or their secondary products would be a valuable tool for the *in vivo* visualization and quantification of free radicals and the evaluation of drugs designed to scavenge free radicals. *N-tert*-Butyl-α-phenylnitrone (PBN, Fig. 1) forms adducts with free radicals (6) that can be detected by ESR and is frequently used for spin trapping studies.



	X	Y
PBN	H	H
FPBN	F	H
HIPBN	OH	I

Fig. 1 Structure of *N-tert*-butyl-α-phenylnitrone (PBN), *N-tert*-butyl-α-(4-fluorophenyl)nitrone (FPBN), *N-tert*-butyl-α-(4-hydroxy-3-iodophenyl)nitrone (HIPBN) and the formation of a PBN radical adduct

In animal experiments, PBN moreover appears to have neuroprotective (7), cardioprotective (8) and anti-aging (9) characteristics that are attributed to its free-radical scavenging abilities. Biodistribution studies in mice with [^{14}C]PBN (10) indicate that this molecule is evenly distributed in various tissues including heart and brain. Wada et al. (11) have recently synthesized a radioiodinated derivative of PBN

(^{125}I-HIPBN, Fig. 1) to pursue the detection of free radicals with single photon emission tomography (SPECT).

In view of the appealing characteristics of PBN we have synthesized the fluorine-18 labeled derivative *N-tert*-butyl-α-(4-[^{18}F]fluorophenyl)nitrone ([^{18}F]FPBN, Fig. 1) in order to investigate the possibility of detecting free radicals *in vivo* with PET.

METHODS AND MATERIALS

N-tert-Butyl-α-(4-nitrophenyl)nitrone, *N-tert*-butyl-α-phenylnitrone, *N-tert*-butylhydroxylamine hydrochloride, 4-fluorobenzaldehyde and Kryptofix 2.2.2. were commercially obtained from Aldrich Chem. Co (Milwaukee, MI). 4-*N,N,N*-Trimethylammoniumbenzaldehyde trifluoromethanesulfonate was synthesized according to the procedure published by Haka et al. (12). NMR spectra were acquired on a Bruker 300 NMR spectrometer. High resolution mass spectra were recorded on a VG 70-250S spectrometer. [^{18}F]Fluoride was produced by proton irradiation of 10% enrichment [^{18}O]water (Swann Chem. Inc., Port Chester NY) using a TCC CS30 cyclotron. The contents of the target were passed over an ion exchange column (Toray TIN-200 quartenary ammonium, carbonate form, 1.6 mm x 20 mm) to trap the [^{18}F]fluoride and to recover the [^{18}O]water. [^{18}F]Fluoride was eluted from the column with 2.3 mg K_2CO_3 dissolved in 500 µl H_2O. The aqueous solution was used after a waiting period of 1 hour to allow the decay of ^{13}N formed by the (p,α) reaction on natural water.

HPLC analyses were performed on a 250x4.6mm Ultremex 5µ C18 (Phenomenex, Torrance CA) column eluted with CH_3CN:H_2O (50:50) at a flowrate of 1.5 ml/min. (10). UV absorption was detected at 289 nm. The retention time in this system of the different compounds is presented in Table 1. Recovery of the radioactivity of a [^{18}F]FPBN preparation from the HPLC column was 103±4 % (mean±sd, n=3), as determined by measurement of radioactivity in the total HPLC

Table 1. HPLC retention time of different benzaldehydes and nitrones

Compound	Retention time (min.)
4-fluorobenzaldehyde	4.74
4-NO_2-benzaldehyde	4.78
PBN	3.90
FPBN	4.24
NO_2PBN	5.45

eluent after injection of an equal volume of [^{18}F]FPBN solution, with and without the HPLC column connected. A series of concentrations of authentic FPBN was used to construct a calibration curve for the determination of the specific activity.

N-tert-Butyl-α-(4-fluorophenyl)nitrone (FPBN).

N-tert-Butylhydroxylamine.HCl (225 mg, 1.8 mmol) was dissolved in 5 ml NaOH 0.5N and heated in a water bath to 60°C. 4-Fluorobenzaldehyde (210 mg, 1.7 mmol) dissolved in 5 ml ethanol was added drop by drop to this solution over a period of 5 min. The reaction mixture was stirred for 3 hours at 60°C to yield a yellow solution that was evaporated. Diethylether (15 ml) was added to the remaining oil, yielding a precipitate that was removed by filtration. The filtrate was evaporated to yield white waxy crystals that were purified by sublimation under vacuum to yield 120 mg (36% yield) of the pure product: mp 80-81°C; ^{1}H NMR ($CDCl_3$) δ 3.77 (s, 9H); δ 9.25 (m, 2H);δ 9.69 (s, 1H), δ 10.49 (m, 2H); high resolution mass spec (exact mass) : found 195.1060, calc 195.1059.

N-tert-Butyl-α-(4-[^{18}F]fluorophenyl)nitrone ([^{18}F]FPBN)

Method A. From *N-tert*-butyl-α-(4-nitrophenyl)nitrone (Fig. 2 (A)). The [^{18}F]fluoride (typically 50 mCi)/K_2CO_3 solution was added to a solution of 26 mg 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8] hexacosane (Kryptofix 2.2.2) in 500 µl CH_3CN. The solution was evaporated at a temperature of 105°C under a continuous nitrogen stream (about 20 ml/min.). Residual water was removed by two additions of 1 ml anhydrous CH_3CN and subsequent heating at 105°C under a flow of nitrogen. To the residue was added *N-tert*-butyl-α-(4-nitrophenyl)nitrone (8 mg) dissolved in 500 µl anhydrous DMSO and the vessel was sealed and heated at 130°C for 15 min, during which the color of the reaction mixture changed from yellow to dark green. The mixture was diluted with 7 ml H_2O and passed through an activated (2x5 ml ethanol followed by 10 ml H_2O) C18 Sep-Pak. The Sep-Pak was rinsed twice with 5 ml H_2O and [^{18}F]FPBN was eluted with 5 ml ethanol. Radiochemical yields of 3.0±0.3% (mean±sd, n=3, decay corrected) were obtained in a total synthesis time of 35 min. HPLC analysis exhibited a single radioactive peak coeluting with authentic *N-tert*-butyl-α-(4-fluorophenyl)nitrone, and two chemical mass peaks corresponding to *N-tert*-butyl-α-(4-nitrophenyl)nitrone and *N-tert*-butyl-α-(4-fluorophenyl)nitrone. No attempts were made to separate these compounds.

Method B. From 4-[^{18}F]fluorobenzaldehyde (Fig. 2 (B)). The [^{18}F]fluoride /K_2CO_3/ Kryptofix solution was dried by the procedure described above. After the drying step the nitrogen flow from the reactor was directed to a second reaction vial containing 10 mg *N-tert*-butyl hydroxylamine.HCl in 1 ml 2N NaOH. 4-*N,N,N*-Trimethylammoniumbenzaldehyde triflate (8 mg) in 500 µl anhydrous DMSO was added to the dried [^{18}F]fluoride/K+/Kryptofix mixture and was heated to 130°C for 15 min. under a continuous nitrogen stream. The 4-[^{18}F]fluorobenzaldehyde produced was continuously distilled into the *N-tert*-butylhydroxylamine solution. After 15 min, the nitrogen flow was stopped, and the [^{18}F]fluorobenzaldehyde allowed to react with the hydroxylamine for an additional 10 min. at room temperature.

The solution was then neutralized by addition of 1.2 ml 1M H_3PO_4, diluted to 10 ml with water and passed through an activated (2x5ml ethanol followed by 10 ml H_2O) C18L Sep-Pak (Waters) to separate [^{18}F]FPBN from excess *N-tert*-butyl-hydroxylamine. The Sep-Pak was rinsed twice with 5 ml 0.5M NaH_2PO_4 and once with 5 ml H_2O to remove all hydroxylamine. [^{18}F]FPBN was finally eluted from the Sep-Pak with 1.5 ml ethanol followed by 0.5 ml H_20. A sample (10 µl) of this solution was injected on the HPLC to determine the specific activity. The concentration of *N-tert*-butylhydroxylamine in the ethanol eluate was below the detection limit (0.1 µmol/ml) of TLC (MeOH HOAc 0.5%) with iodine vapor detection . The ethanolic solution was diluted to a total volume of 15 ml with saline and sterilized by filtration (Anotop 25, 0.2 µm Whatman). Radiochemical yields of 24±10% (mean±sd, n=3, decay corrected) were obtained in a total synthesis time of 45 min. HPLC analysis showed a single radioactive peak coeluting with authentic *N-tert*-butyl-α-(4-fluorophenyl)nitrone. The specific activity was 402±16 mCi/µmol (mean±sd, n=3) at end of synthesis.

RESULTS AND DISCUSSION

A sample of authentic *N-tert*-butyl-α-(4-fluorophenyl)nitrone (FPBN) was synthesized by the procedure described by Angelini et al. (10) for the synthesis of [^{14}C]PBN. Although the synthesis is a one-pot reaction, it involves two steps, the nucleophilic attack of the hydroxylamine nitrogen on the benzaldehyde carbonyl group

followed by proton migration and elimination of water. Fluorine-18 labeled FPBN can be prepared by the aromatic nucleophilic substitution by [^{18}F]fluoride ion on the commercially available *N-tert*-butyl-α-(4-nitro-phenyl)nitrone (Fig. 2 (A)). The nitrone group is only moderately activating resulting in a low radiochemical yield (3%), and this synthesis method would require a final HPLC purification to separate [^{18}F]FPBN from the corresponding nitro-substituted nitrone. Better overall radiochemical yields of [^{18}F]FPBN are obtained by the two-step synthesis, involving the intermediate preparation of 4-[^{18}F]fluorobenzaldehyde followed by reaction with *N-tert*-butylhydroxylamine (Fig. 2 (B)).

4-[^{18}F]Fluorobenzaldehyde is a commonly used intermediate and is prepared by the nucleophilic substitution by [^{18}F]fluoride ion on 4-NO_2-benzaldehyde or 4-*N,N,N*-trimethylammoniumbenzaldehyde triflate.

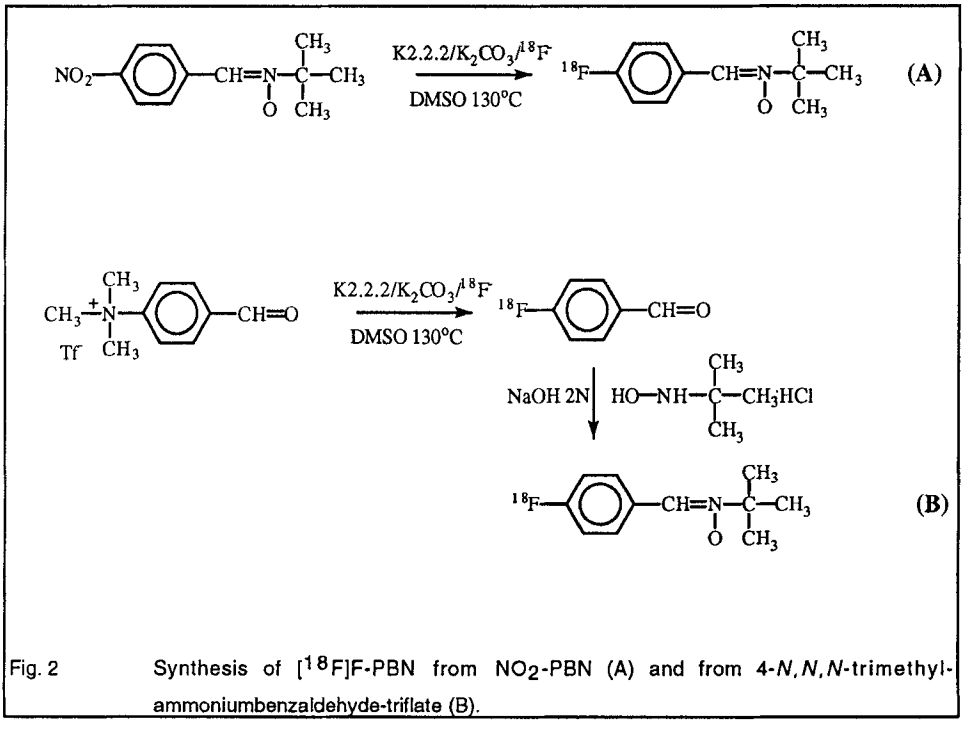


Fig. 2 Synthesis of [^{18}F]F-PBN from NO_2-PBN (A) and from 4-*N,N,N*-trimethyl-ammoniumbenzaldehyde-triflate (B).

4-[^{18}F]Fluorobenzaldehyde is then usually isolated from the labeling reaction mixture by solid phase extraction. We found that 4-[^{18}F]fluorobenzaldehyde can be easily removed from the labeling reaction mixture by maintaining a nitrogen flow through the

reaction solution. In this way 4-[^{18}F]fluorobenzaldehyde distills upon its formation together with some DMSO, the amount of which depends on the labeling temperature and the nitrogen flow. Analysis after heating at 130°C for 10 min. under a continuous stream of nitrogen showed that more than 98% of total 4-[^{18}F]fluorobenzaldehyde was distilled from the labeling reaction mixture together with about 300 µl of DMSO. Both the 4-*N,N,N*-trimethylammoniumbenzaldehyde triflate and the nitrobenzaldehyde are not volatile under these conditions and remain in the initial reaction vial obviating the necessity of an eventual HPLC purification to separate nitro- and fluorine-18 labeled nitrones.

For the second step in formation of [^{18}F]FPBN, the 4-[^{18}F]fluorobenzaldehyde is distilled into a vial where it is efficiently trapped (>95%) in a basic aqueous solution containing *N-tert*-butylhydroxylamine. The rate of conversion of the aldehyde to the nitrone is largely dependent on the concentration of NaOH. The conversion is virtually quantitative after 10 min. in 2N NaOH at room temperature.

After completion of the condensation reaction, the reaction mixture is neutralized and excess *N-tert*-butylhydroxylamine, inorganic salts and DMSO are removed by Sep-Pak purification yielding pure [^{18}F]FPBN exhibiting an identical retention time on HPLC as the authentic cold FPBN. The radiochemical purity was better than 98% and [^{18}F]FPBN remains stable for more than 6 hours in a 10% ethanol solution in saline, as determined by HPLC.

The overall yield of [^{18}F]FPBN is mainly dependent on the initial [^{18}F]fluorobenzaldehyde formation, for which similar yields (12) have been reported in the literature (70% from resolubilized [^{18}F]fluoride and 30-40% from total fluoride). The lower yields obtained here can probably be attributed to variability in reactivity of the [^{18}F]fluoride ion used in this study, which is also reflected in the range of yields obtained. The specific activity of the [^{18}F]FPBN appears to be low (about 400 mCi/µmol) but is reasonable in view of the low amount of starting activity (50 mCi [^{18}F]fluoride).

In conclusion, we have synthesized [^{18}F]FPBN by reacting *N-tert*-butylhydroxylamine with 4-[^{18}F]fluorobenzaldehyde, which was in turn obtained by distillation from a [^{18}F]fluoride-4-*N,N,N*-trimethylammoniumbenzaldehyde triflate reaction mixture. The final product was obtained in yields, chemical and

radiochemical purities, and specific activities sufficient for utilization in *in vivo* animal experiments. The results of these studies will be presented elsewhere.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health NS15655, the Department of Energy DE-FGO2-88ER60639 and the North Atlantic Treaty Organization.

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