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MASS SPECTROSCOPY AND CHROMATOGRAPHY OF THE TRICHLOROMETHYL RADICAL ADDUCT OF PHENYL *TERT*-BUTYL NITRONE

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Positive structural identification of the PBN-trichloromethyl spin adduct *in vitro* was accomplished with the use of high pressure liquid chromatography and/or gas chromatography coupled with mass spectrometry. Both thin layer and liquid chromatography were used to separate a complex mixture of compounds from rat liver extracts treated with CCl_4 *in vitro* and *in vivo*. Deuterated PBN's (PBN-d_9; *tert*-butyl deuteration, or PBN-d_{14}; both phenyl and *tert*-butyl deuteration) were also used to aid in the mass spectral analysis of spin adducts from liver extracts of CCl_4 exposed rat livers, since the *tert*-butyl group fragment ion, $C_4D_9^+$ (m/z = 66) is always present for PBN and PBN spin adducts. In addition, the masses of the ion peaks increase by the amount of deuteration, i.e. an increase of 9 for PBN-d_9 or PBN-d_{14} in comparison to normally synthesized PBN.

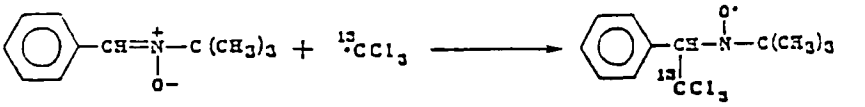
KEY WORDS: α-Phenyl *tert*-butyl nitrone (PBN), carbon tetrachloride (CCl_4), PBN-trichloromethyl adduct (PBN-CCl_3), thin layer chromatography (TLC), high pressure chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS), electron spin resonance (ESR).

INTRODUCTION

Numerous spin trapping studies have confirmed that trichloromethyl radicals ($\cdot CCl_3$) are produced in rat liver when the animal is subjected to carbon tetrachloride (CCl_4)[1-5] or when rat liver microsomes are treated with NADPH and CCl_4.[6-10] α-Phenyl *tert*-butyl nitrone (PBN) is most commonly used as a spin trap and the isotopically enriched $^{13}CCl_4$ is the most preferred form of the halocarbon.

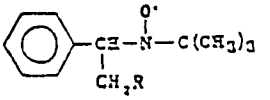
The $^{\cdot 13}CCl_3$ spin adduct of PBN gives a 12-line ESR spectrum which is composed of a triplet of double doublets which is distinctly different from the spectrum of any other carbon- or oxygen-centered spin adducts:

*deceased

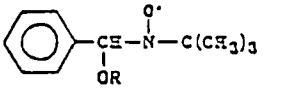


$a_N = 14.1, a_\beta^H = 1.8, a_\beta^{13C} = 9.6\,G$

Carbon- and oxygen-centered spin adducts are also detected in these systems and the values of the hyperfine splitting constants (hfsc's) for the PBN spin adducts have been published before:



$a_N = 14.49, a_\beta^H = 3.35, a_\gamma^H = 0.53\,G$



$a_N = 13.70, a_\beta^H = 1.88\,G$

In previous publications we have shown that high pressure liquid chromatography (HPLC) can be used to separate PBN spin adducts and the hydroxylamines of corresponding structure using electrochemical oxidation as the detection method.[11,12] For carbon-centered adducts, gas chromatography and mass spectroscopy (GC/MS) provides determinations of the molecular weights of both the nitroxides as well as the hydroxylamines of corresponding structure provided the group which was the radical is relatively small (methyl to n-butyl). In this communication we report on the use of TLC and HPLC separation in conjunction with GC/MS analysis of the CCl_4 rat liver system exposed to PBN and deuterated PBN's.

MATERIALS AND METHODS

(a) Materials

PBN was obtained from Aldrich and further purified by vacuum sublimation. The synthesis of PBN-d_9 and PBN-d_{14} was done in this laboratory.[13] All solvents used were HPLC grade and the water was purified on a Millipore filtration unit.

(b) Preparation of in vitro samples for TLC and normal phase HPLC (Oklahoma Medical Research Foundation)

Rat liver microsomal preparations were obtained as reported previously.[3] The reaction mixture consisted of rat liver microsomes (2.0 mg/ml of protein) in a 0.05 M phosphate buffer at pH 7.4, 0.14 M, PBN, 0.20 M ^{13}C-carbon tetrachloride and a NADPH/$NADP^+$ generating system.[3] A Folch extract ($CHCl_3$:CH_3OH/2:1) of the rat liver microsomal incubation system was evaporated to almost dryness, taken up in $CHCl_3$ and spotted onto a TLC plate. The TLC plates were chromatographed with a petroleum ether and diethyl ether (70:30) mixture for 60 min, and arbitrarily divided into 5 zones, zone 1 beginning at the origin and zone 5 ending at the solvent front. The silica gel bands in the 5 zones were scraped from the plates individually, loaded into separate columns, and eluted with heptane. HPLC separation was done on an Ultrasphere Si 5 μm 4.6 × 250 mm column using heptane-isopropyl alcohol (97:3) as the mobile phase at a flow rate of 1.0 ml/min. Sample detection was achieved using a variable wavelength UV detector at 291 nm.

(c) Preparation of in vitro and in vivo samples for reverse phase HPLC (Chemistry and Biochemistry Department, University of Guelph)

Microsomal preparations were obtained as reported from a previous study.[9] The reaction mixture consisted of rat liver microsomes (30 mg/ml of protein) in a 0.15 M phosphate buffer at pH 7.4, 0.14 M PBN-d_9 or PBN-d_{14}, 0.20 M $^{13}CCl_4$ and 0.3 mM each of NADPH and NADH.

For *in vivo* studies, rats were administered $^{13}CCl_4$ (120 μl/200 g rat in saline and 5% Emulphor solution) and PBN (0.14 M, 1 ml; either non-deuterated PBN, PBN-d_9 or PBN-d_{14}) via an i.p. injection. Animals were killed 30 min after administration of the above mixture. Livers were subsequently removed and homogenized.

Spin adducts, obtained from either rat liver microsomal mixtures or homogenized livers from rats administered $^{13}CCl_4$ and PBN *in vivo*, were extracted by the method of Burton *et al.* with some modification.[14] Sodium dodecyl sulfate (3 ml of a 0.40 M aqueous solution) was added to 1 ml of liver homogenate mixture, followed by vigorous agitation on a vortex mixer. Absolute ethanol (4 ml) was added subsequently to precipitate the microsomal proteins. The spin adducts were extracted via the addition of 2 ml of hexane, agitated by vortexing, centrifuged (bench-top) and the hexane layer finally removed.

HPLC purification of PBN adduct mixtures was performed with a Spherisorb 5 ODS 210 mm id. × 25 cm column. The mobile phase consisted of 70% acetonitrile and 30% 0.02 M KH_2PO_4 buffer, pH 5.8. It was pumped isocratically at flow rates of 2.0 to 2.5 ml/min. The solvent delivery system and the detectors were used as previously described.[15]

(d) ESR analysis of PBN spin adducts

Rat liver extracts obtained from either *in vitro* or *in vivo* experiments were transferred to a round quartz ESR cell and degassed under N_2 for 15 min before being placed in an ST-ESR cavity. ESR spectra were recorded with a Bruker EPR ER-200D X-band spectrometer. Spectral accumulation and averaging was done using a Bruker ER-140 (Aspect 2000) data system.

(e) GC/MS analysis

Samples were concentrated prior to GC/MS analysis. The samples were analyzed with a Hewlett-Packard series 5790 GC interfaced with a VG model 12000 quadrupole mass spectrometer equipped with a VG model 11/250 data system. The mode of operation was either electron ionization (EI) at 70 eV or chemical ionization (CI) with isobutane. Chromatographic separation was carried out on a J&W 0.25 mm i.d. × 15 m DB-5 (Durabond 5% phenyl and 95% methyl siloxane) fused silica capillary column with a temperature program from 100°C to 320°C at 10°C/min.

RESULTS AND DISCUSSION

After TLC fractionation of a Folch extract of rat liver microsomes, incubated with $^{13}CCl_4$ and PBN, the plates were divided into five zones. The zone nearest the solvent front (# 5) contained the trichloromethyl adduct of PBN. These results were obtained consistently with a number of different samples. Zone 2 gave a triplet of doublets with

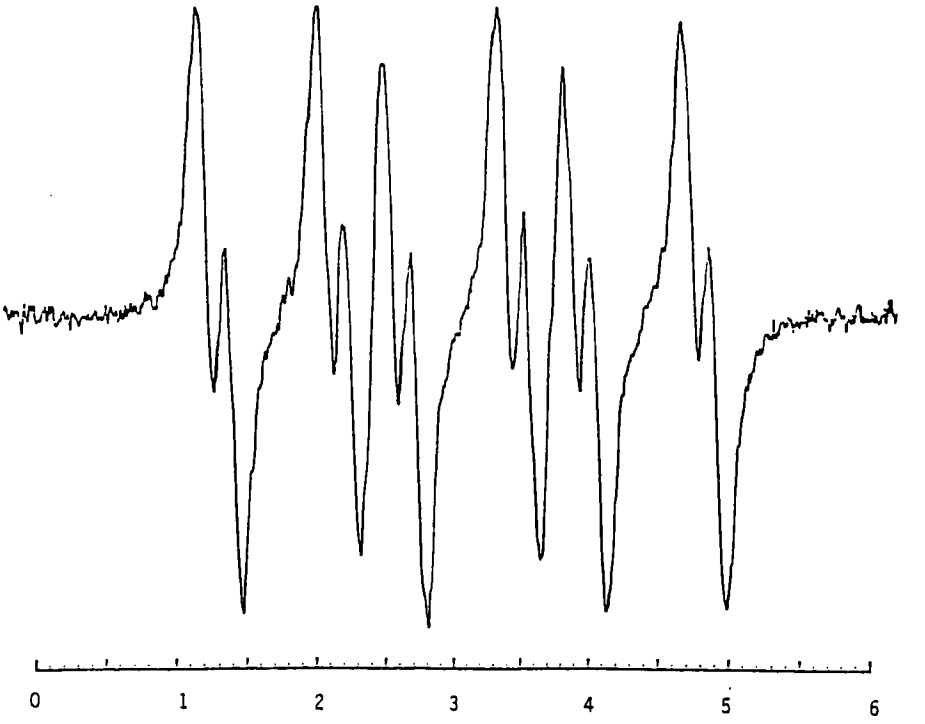
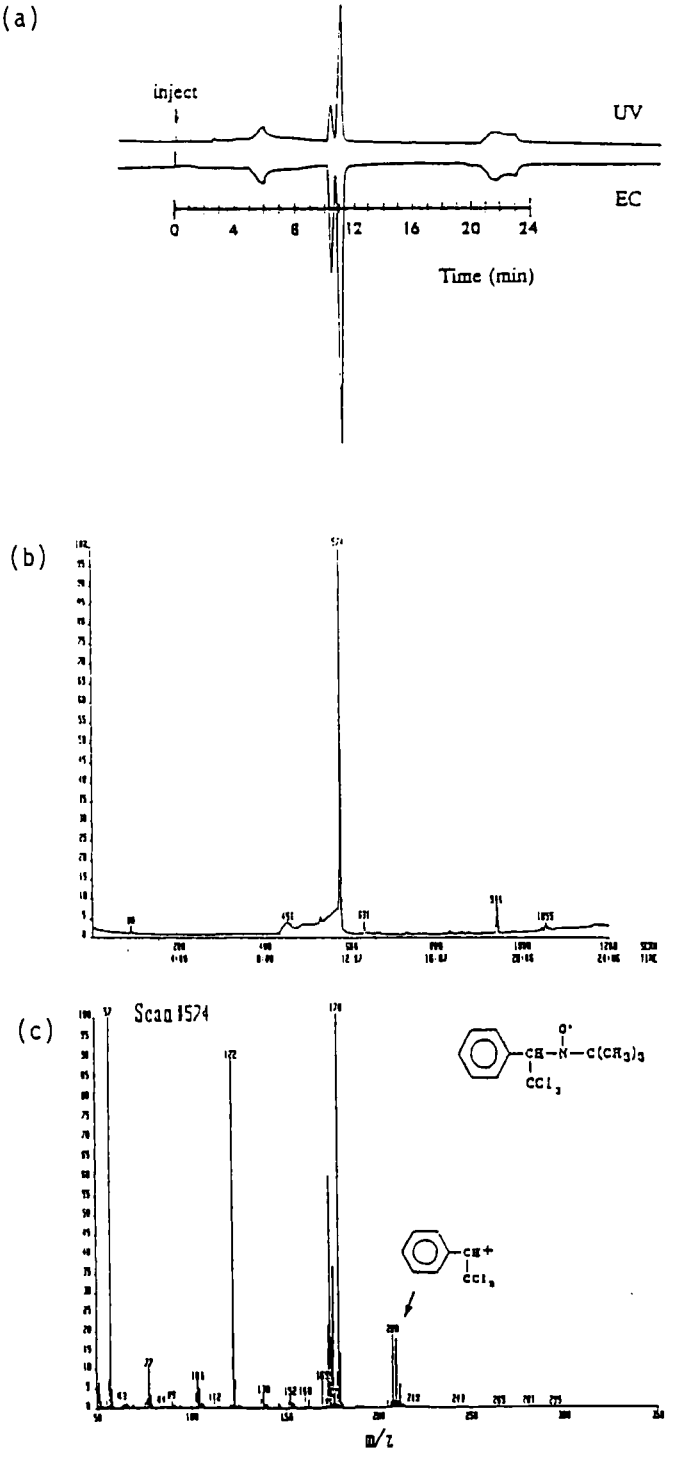


FIGURE 1 ESR spectrum of the PBN-$^{13}CCl_3$ spin adduct obtained from a TLC separation (zone 5) of a rat liver microsomal Folch extract previously incubated with $^{13}CCl_4$ PBN and NADPH.

the following ESR parameters a_N = 13.63, a_H^β = 2.10 G in benzene (the solvent was evaporated from the material eluted from zone 2 and the sample was taken up in benzene to permit comparison of coupling constants with literature values). This spectrum is assigned to an oxyl radical adduct of PBN on the basis of the hfsc's and g-value. Occasionally zone 2 also contains a small amount of another triplet of doublets which may be the carbon-centered radical adduct.

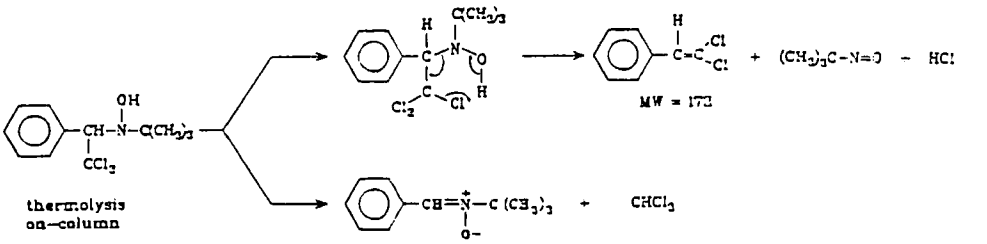
Normal phase HPLC separation of a sample from zone 5 using 97% heptane: 3% isopropyl alcohol at room temperature gave three closely spaced peaks (A, B, C) where peak B contained a strong signal due to the trichloromethyl adduct and peaks A and C also gave a weak signal due to the same species. An ESR spectrum of peak B is shown in Figure 1. Reverse phase HPLC on the sample obtained from peak B (named 5-B) using 70% CH_3CN: 30% H_2O containing phosphate buffer gave two closely spaced peaks using electrochemical detection (Figure 2a) with retention times of 9.56 and 10.10 minutes respectively (named 5-Ba and 5-Bb). Peak A from zone 5 gave essentially the same two peaks (named 5-Aa and 5-Ab) except that the overall

FIGURE 2 (a) Reverse phase HPLC (C-18 column) chromatograms of TLC zone 5, normal phase HPLC peak B, obtained from a rat liver microsomal Folch extraction previously incubated with $^{13}CCl_4$, PBN and NADPH. The solvent used was 30:70 H_2O:CH_3CN in 0.05 M phosphate buffer. The flow rate was 1 ml/min with a pressure of 800 psi. The chart speed was 0.5 cm/min. The top chromatogram was detected on a UV detector at 214 nm; 0.5 AUFS. The bottom chromatogram was detected on an electrochemical detector set at + 0.7 V and 50 nA/V. The retention times of the two major peaks are 9.56 and 10.10 min, respectively. (b) Gas chromarogram of TLC zone 5, normal phase HPLC peak B, as described in (a). (c) Mass spectrum (EI, 70 eV) for peak # 574 of the above gas chromatogram. PBN-$^{13}CCl_3$, MW = 295.



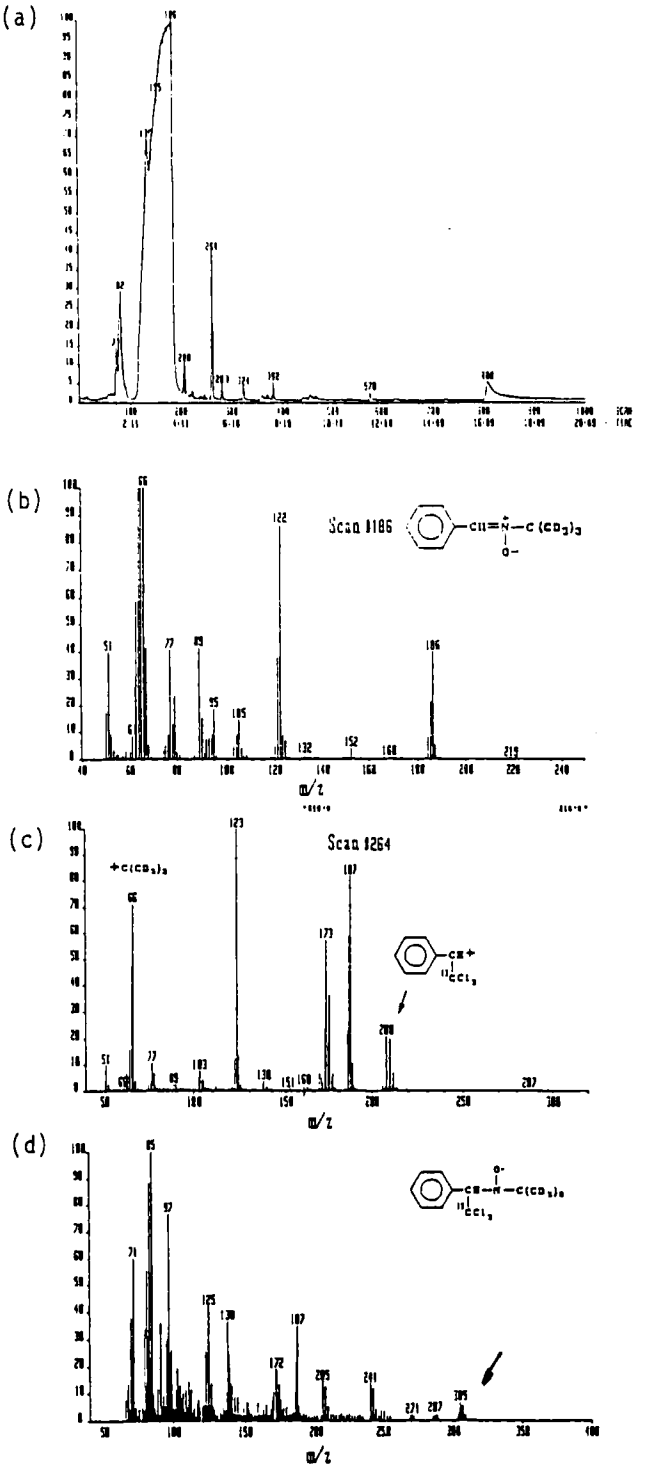
intensity was less. By analogy to previous experience with alkyl spin adducts of PBN, the second HPLC peak with slightly longer retention time in the latter separation could be assigned to the hydroxylamine of the trichloromethyl radical spin adduct but this conclusion has not been absolutely proved at this time. If the relative positions found in the reverse phase column are simply the opposite of that in the normal phase column then peak 5A is the hydroxylamine of the spin adduct nitroxide radical in 5B. The fact that both components appear in the HPLC reverse phase analysis of both 5B and 5A simply means that an incomplete cut was made for sample collection. An assignment of peak 5C has not been made at this time.

When a sample from Peak 5B was submitted to GC/MS analysis the GC trace gave only one major peak (#574 in Figure 2b) although the broad ion current trace at 430–574 seconds indicated on-column decomposition of some component of the injected sample. Inspection of the mass spectrum of peak 574 (Figure 2c) clearly points to the trichloromethyl radical adduct of PBN (see later). Mass analysis of the peak at 451 gives a spectrum clearly due to PBN, but PBN has a much shorter retention time (250–300 seconds) and should have come through much earlier if present as an impurity in sample 5B. Peak 527 has a major peak in the mass spectrum due to a dichlorinated species with a mass of 173. This information indicates that a component of sample 5B decomposes on the column to give PBN and/or a dichlorinated molecule of mass 173. We suggest that this component is the hydroxylamine of the trichloromethyl adduct of PBN and that the nitroxide of corresponding structure is stable enough for GC separation and MS detection. A mechanism which would account for this avenue of decomposition is as follows:



Extracts from rat liver microsomal samples treated with $^{13}CCl_4$ in the presence of PBN-d_9 were found to be complex mixtures, as illustrated by GC/MS analysis (Figure 3). The gas chromatogram (Figure 3a) indicates the presence of numerous compounds. To aid in the analysis of PBN adducts from the other compounds present in the mixture PBN-d_9 was used. The advantage of using PBN-d_9 or PBN-d_{14} is that a major fragment ion present in the mass spectra of PBN or PBN spin adducts is $C_4D_9^+$ (m/z = 66) as a result of fragmentation of the deuterated *tert*-butyl group. With the use of undeuterated PBN (as shown in Figure 2c) the *tert*-butyl group fragment ion, $C_4H_9^+$, has an ion mass of m/z = 57 which is a common fragment ion found in the mass spectra of numerous organic compounds. In contrast, m/z = 66 is not as abundant.

FIGURE 3 (a) GC chromatogram of a rat liver microsomal hexane extract previously incubated with PBN-d_9, $^{13}CCl_4$ and NADPH/NADH. (b) Mass spectrum (EI, 70 eV) of GC peak #186, PBN-d_9, MW = 186. (c) Mass spectrum (EI, 70.eV) of GC peak #264, PBN-d_9-$^{13}CCl_3$ spin adduct, MW = 305 (parent ion not visible by EI). (d) Mass spectrum (CI, isobutane reagent gas) of the PBN-d_9-CCl_3 spin adduct, MW + H^+ = 305, obtained from a rat liver microsomal hexane extract of an incubation mixture containing microsomes, CCl_4, PBN-d_9 and NADPH/NADH.



Of the GC peaks shown in Figure 3a only peak #186 and #264 contained a significant fragment ion at $m/z = 66$. GC peak #186 was unreacted PBN-d_9(MW = 186) (Figure 3b). GC peak #264 is assigned to the ^{13}C-labelled trichloromethyl spin adduct of PBN-d_9(MW = 304) (Figure 3c). The structure of this compound was assigned by analysis of the mass spectrum which did not have an observable parent ion peak, although the presence of a trichlorinated fragment ion of mass 208 with a characteristic three chlorine isotopic pattern is observed. A similar fragmentation pattern was seen for a rat liver microsomal extract previously incubated with $^{13}CCl_4$ and PBN-d_{14} which has been published elsewhere.[14] This assignment has also been confirmed by chemical ionization GC/MS of a rat liver microsomal extract previously exposed to $^{13}CCl_4$ and PBN-d_9. A parent ion peak (MW + 1) of $m/z = 305$ was observed (Figure 3d). The *in vivo* liver homogenate extract GC/MS spectrum of the PBN-CCl_3 adduct was similar to that shown in Figure 3c for the *in vitro* sample.

Even though the presence of lipid-type 'carbon-centered' (PBN-CH_2R) and 'oxygen-centered' (PBN-OL) PBN spin adducts have been detected by ESR previously,[9] the use of GC/MS analysis has not to date given any additional structural information for these compounds. This may be due to the instability and heat-sensitive nature of these spin adducts.

The positive structural identification of spin adducts in a biological mixture requires not only the use of ESR but also the use of various chromatographic techniques (including TLC, HPLC and GC) in conjunction with mass spectrometric analysis. In addition, the mass spectral marker of deuterated PBN's, $m/z = 66$, allows rapid identification of PBN and PBN spin adducts in a sample containing a complex mixture of compounds.

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