CHEMICAL AND METABOLIC STUDIES ON N-BENZYL-*tert*-BUTYLAMINE AND ITS POTENTIAL METABOLITES

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SUMMARY

The metabolism of N-benzyl-*tert*-butylamine was studied *in vitro* using male hamster hepatic microsomal preparations. This substrate produced the corresponding nitrone, benzaldehyde and an uncharacterised metabolite. No metabolites were detected which corresponded to either authentic amide or oxaziridine. The results indicate that the nitrone observed as a metabolite in this experiment is not an intermediate leading to the formation of an oxaziridine and hence an amide, under careful experimental conditions excluding light.

KEY WORDS

N-benzyl-*tert*-butylamine, nitrones, microsomes, hamster, metabolism, oxaziridines

INTRODUCTION

Previous studies have revealed that amides and nitrones are metabolites arising from certain secondary aromatic amines and metabolic pathways have been proposed for the formation of these amides /1-6/. It was later established that nitrones are not metabolic intermediates in the formation of amides /7/. However, they may be involved in the chemical production of amides via oxaziridines during analysis /7/. In subsequent studies, it was also demonstrated that

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certain imines were metabonates (chemical artifacts) arising from secondary aromatic amine metabolism /8/ and that these imines could be substrates for amide formation /9/.

We have recently shown that no metabolites were observed which corresponded to authentic amide or oxaziridine following the *in vitro* metabolism of N-benzylidene-*tert*-butylamine (NBDTBA; Fig. 1c) and other aryl-alkylimines /10/. It was proposed that this could be due to the high polarity of NBDTBA and the other imines because of the aliphatic moiety in their structure /10/. Although these aryl-alkylimines were stable under our incubation conditions and it was possible to prepare the corresponding oxaziridines, they were poor substrates for oxidative microsomal metabolism /10/ in contrast to the diarylimines studied previously /9/.

The instability of diaryl oxaziridines has previously been reported /11-14/; however, the corresponding oxaziridine (TBPO; Fig. 1d) of N-benzylidene-*tert*-butylamine was known to be stable /12,13/. Since there is no report on the metabolic formation of amides and oxaziridines from N-benzylic secondary aliphatic amines, we studied the *in vitro* microsomal metabolism of N-benzylidene-*tert*-butylamine (NBTBA; Fig. 1a). A study of the chemical reactions of proposed metabolic intermediates, i.e., nitrone and imine, was also carried out to see whether they are involved in the production of the corresponding amide via an oxaziridine (Fig. 1). The substrate and proposed metabolites were synthesised and characterised using spectroscopic and spectrometric techniques and separated using HPLC. The results of a study of the *in vitro* oxidative microsomal metabolism of NBTBA with hepatic washed hamster microsomes fortified with $NADPH_2$ are presented.

MATERIALS AND METHODS

Synthesis and characterisation of NBTBA and its potential metabolites

Chemicals:

Benzyl and benzoyl chlorides, m-chlorperbenzoic acid (m-CPBA) and benzaldehyde were purchased from Aldrich Chemical Company, U.K. *tert*-Butylamine and the corresponding nitrone, α-phenyl-N-*tert*-butylnitrone (TBNP; Fig. 1b) were purchased from Fluka, Chemica-Biochemica, Buchs, Switzerland. All the chromatographic solvents

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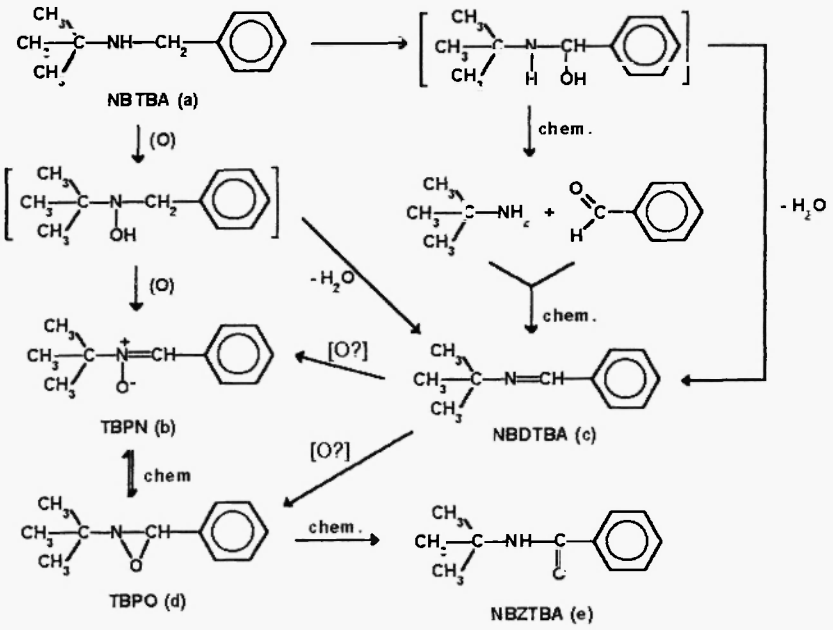

Fig. 1: Possible intermediates involved in the metabolism of N-benzyl-*tert*-butylamine (a).

were obtained from British Drug Houses, Poole, Dorset (BDH) as S.L.R. grade. Plastic-backed TLC plates precoated with silica-gel $60F_{254}$ were obtained from E. Merck, Darmstadt, Germany. Silica-gel 60, particle size 0.063-0.200 mm (70-230 mesh ASTM) (E. Merck, Darmstadt, Germany) was used as column chromatography stationary phase.

Instrumentation:

UV spectra were recorded either on a KONTRON UVIKON860 UV spectrophotometer or a Rapiscan HPLC-UV detector. EI-mass spectra were determined by direct insertion of samples on a mass spectrometer with 70 eV ionisation potentials.

The substrate, NBTBA (Fig. 1a) was prepared by the method of Bortnick *et al.* /14/. *tert*-Butylamine (1 mole) and 33% aqueous sodium hydroxide solution (1 mole) were added to benzylchloride (1 mole) in benzene (12 ml) while stirring and heating under reflux.

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Heating was continued for 12 h. The unreacted amine and benzyl chloride were removed from the reaction mixture by the use of column chromatography with petroleum ether (b.p. 40-60°C):acetone (50:50, v/v) as solvent system. Mass spectra showed molecular ion peaks and correct fragmentation pattern (Table 1). The NMR spectrum (not shown) also confirmed the structure. The corresponding imine, NBDTBA (Fig. 1c) was prepared by condensation of equimolar amounts of *tert*-butylamine and benzaldehyde as described by Pews /15/. Mass spectra showed molecular ion peaks and correct fragmentation patterns for imines (Table 1). The amide, NBZTBA (Fig. 1e), was rapidly prepared in a pure state using the Schotten-Baumann reaction and recrystallized from ethanol /16/. The amide had the correct mass fragmentation pattern and its melting point was as recorded in the literature (Table 1) /17/.

Attempted preparation of α-phenyl-N-tert-butyloxaziridine (TBPO; Fig. 1d)

i) The first attempt to prepare TBPO utilised the method of Pews /15/ in which mCPBA in dichloromethane (DCM) was added to the imine (NBDTBA; Fig. 1c) dropwise over thirty minutes. The solution was filtered to remove the m-chlorbenzoic acid, washed with dilute sodium sulphite solution followed by dilute sodium carbonate solution and dried over sodium carbonate. The DCM was evaporated at room temperature using a rotary film evaporator. The reaction mixture was examined by TLC using a variety of chromatographic solvent systems. Following spraying with Ehrlich reagent, it was shown that a new substance was present which slowly gave a yellow colour, in contrast to the authentic imine which did not respond to this reagent. The mixture was analysed by mass spectrometry which showed a M^++16 molecular ion peak. This peak was not attributable to the isomeric nitrone or amide since authentic standards for these structures, which can be separated from the new compound by TLC, were available (Table 1). This mixture was also examined by HPLC (details given below) when a new peak was observed which was separated from starting imine, amide and nitrone (Fig. 2a). The UV spectra showed that the maximum absorbance of this peak was different from the corresponding nitrone and amide (Fig. 3). These findings strongly suggest that oxaziridine was present in the synthetic reaction mixture. No further attempts were made to isolate the oxaziridine.

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TABLE 1

Analytical and spectral data of NBTBA and its potential metabolites

Compound (Abbreviation)	M w	Yield (%)	Description	Major mass spectral fragments m/e (% relative abundance)	TLC Rfx100 values	HPLC retention time (min)	UV (maximum absorbance) (nm)
N-Benzyl-*tert*-butylamine (NBTBA)	163	40	pale yellow liquid b.p. 91°C* (11 Mm)	57(11),71(6),91(100), 148(94),149(18),163(6) 164(2)	23.4	2 2	200
N-Benzylidene-*tert*-butylamine (NBDTBA)	161	55	liquid b.p. 90°C** (11Mm)	57(55),66(73),77(41), 78(42),79(47),90(67), 106(100),116(18), 130(37),147(75), 161(54)	67.7	5 2	242,210
N-(*tert*-butyl)-α-phenyl oxaziridine (TBPO)	177	N C.	liquid	57(20),63(11),77(93), 105(100), 121(70), 134(9), 162(55), 177(58), 178(10)	71.3	10.3	244
N-(*tert*-buty)-α-phenyl nitrone (TBPN)	177	C.A.	solid m.p. 74°C	N.D.	56.3	5 5	292
N-benzoyl-*tert*-butyl amine (NBZTBA)	177	85	white crystalls mp 134 °C***	77(42),105(100),134(5) 122(39),162(33), 177(54),178(10)	40.1	6 1	200

For HPCL conditions see text.
TLC solvent system: (Petroleum |b.p.: 40-60°|: acetone) (50:50,v/v)
*Bortnick *et al.* /14/; **Emmons /11/; ***Clarke /17/.
N.C. = not calculated as it had an impurity from the imine (NBDTBA)

C.A. = commercially available
N.D. = not determined

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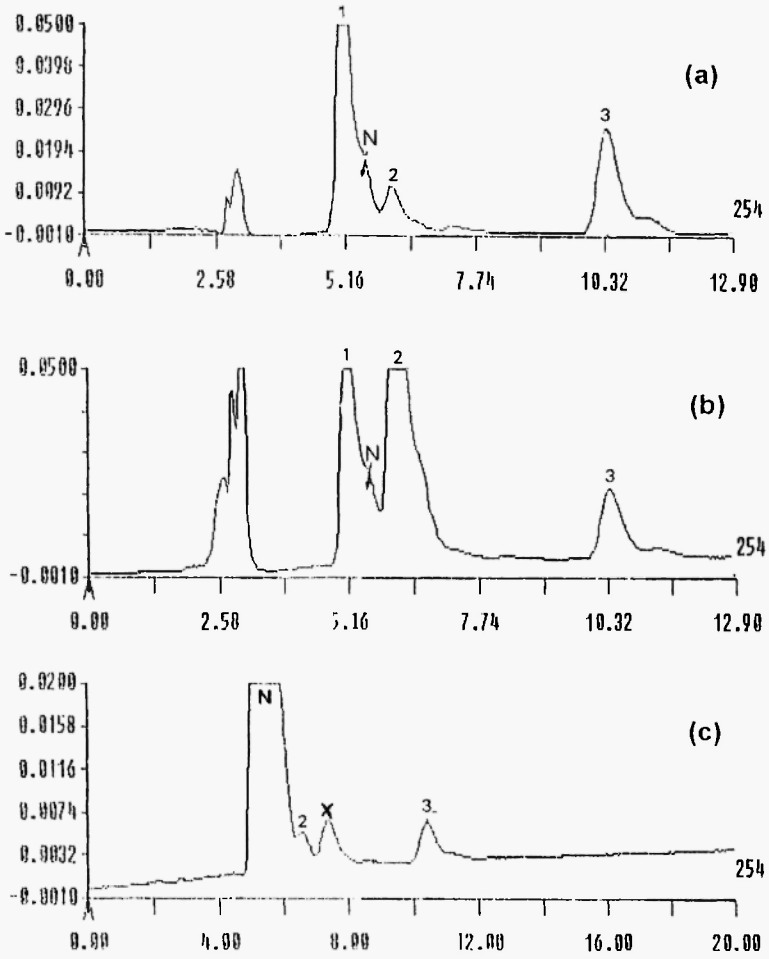

Fig. 2: a. HPLC chromatogram from oxaziridine (TBPO) synthetic mixture from m-CPBA oxidation of imine (NBDTBA). b. HPLC chromatogram from the reaction of corresponding imine (NBDTBA) with benzoyl peroxide. c. HPLC chromotogram from a methanolic solution of authentic nitrone (TBPN) following one day irradiation with direct sunlight. 1: Imine (NBDTBA); 2: amide (NBZTBA); 3: oxaziridine (TBPO); N: nitrone (TBPN); X: an unknown substance. Note: the shoulder on peak 1 corresponded to nitrone (NBPN).

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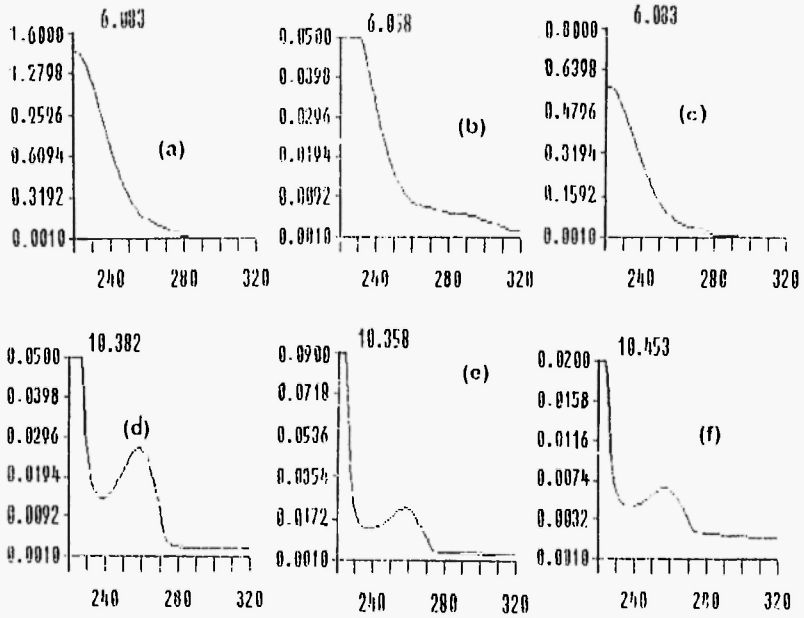

Fig. 3: UV spectra of amide (NBZTBA): (a) authentic; (b) from oxaziridine synthetic mixture; (c) from benzoyl peroxide experiment, and oxaziridine (TBPO): (d) from oxaziridine synthetic mixture, (e) from benzoyl peroxide experiment; (f) from nitrone irradiation experiment.

ii) An alternative route to the oxaziridine was attempted which involved the oxidation of the imine in DCM with benzoylperoxide at room temperature for two days with continuous stirring. The mixture was filtered and analysed by HPLC (details given below) (Fig. 2b) which showed the presence of the nitrone and amide together with a compound having spectral and chromatographic properties identical to the new compound detected above (Figs. 2a and 3).

iii) In order to further confirm the oxaziridine structure the corresponding nitrone (TBPN; Fig. 1b) was dissolved in acetonitrile /13/ and exposed to direct sunlight for one day in a glass vessel and examined by HPLC (details given below). The formation of two new compounds which had chromatographic properties identical to those of the corresponding amide and the proposed oxaziridine were observed (Fig. 2c). A novel compound eluting between the amide and the putative oxaziridine on HPLC was detected (Fig. 2c), but was not further characterised.

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In all cases, the UV spectrum of the proposed oxaziridine was quite different from the corresponding nitrone or amide and its absorption maximum was shifted to a shorter wavelength (Fig. 3). The UV spectrum of the new compound was similar to that of the oxaziridines detected in an earlier study /13/. This combined evidence supports the proposal that the new compound detected in each experiment was the required oxaziridine.

High performance liquid chromatography

The substrate and its potential metabolites were analysed using a reverse-phase isocratic HPLC system which comprised an ACB pump, a model 7125 syringe loading sample injector valve fitted with a 20 µl sample loop, a Milton Roy variable wavelength UV detector and a computer based detection system (Dell3168X, Epson LX-800, Roseate Software). The analytical column (Phase Separations Ltd, Deeside, U.K.) contained Spherisorb 5 µm ODS (250x4.6 mm i.d.) and the pre-column material was co-pellicular ODS (Whatman International Ltd., Maidstone, Kent). HPLC analyses were also carried out using the above system in conjunction with a high speed, multiple wavelength UV/VIS detector (Rapiscan SA6508, Severn Analytical, U.K.), scanning from 190 to 364 nm. The detector was coupled to a PC (Tandon AT) allowing data capture and manipulation via the Rapiscan programme version 2.0. The mobile-phase composition was acetonitrile:water (50:50, v/v) at a flow rate of 1 ml/min. HPLC retention times of the substrate and its potential metabolites are shown in Table 1. The Rapiscan UV detector connected to the standard HPLC system was used to obtain UV spectra of both authentic synthetic compounds and products of NBTBA metabolism.

Incubation and extraction procedures

Dichloromethane (SLR grade) was obtained from BDH. β-Nicotinamide dinucleotide phosphate (disodium salt, NADP) and glucose-6-phosphate (disodium salt, G-6-P) were purchased from BDH. Glucose-6-phosphate dehydrogenase suspension (Reinheit grade II, 10 mg per 2 ml, G-6-PD) was obtained from Boehringer Mannheim Corporation (London). All other laboratory chemicals were obtained from BDH as the purest grade available.

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Adult male hamsters (Syrian, 80-110 g) were used in this study. The animals were deprived of food overnight prior to sacrifice, but were allowed water *ad libitum*. They were previously fed on a balanced diet. Hepatic washed microsomes were prepared as described by Schenkman and Cinti /19/.

Incubations were carried out in a shaking water-bath at 37°C using a standard co-factor solution consisting of NADP (2 μmole), G-6-P (10 μmole), G-6-PD suspension (1 unit) and aqueous $MgCl_2$ (50% w/w) (20 μmole) in phosphate buffer (0.2 M, pH 7.4, 2 ml) at pH 7.4. Co-factors were pre-incubated for 5 min to generate $NADPH_2$, before the addition of microsomes (1 ml equivalent to 0.5 g original liver) and substrate (5 μmol) in methanol (5 μl). The incubation was continued for 30 min, terminated and extracted with dichloromethane (2x5 ml). The organic extracts were evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 200 μl of methanol for HPLC. Because of the known chemical lability of nitrone and oxaziridine functional groups /20/, incubations were carried out in the dark and light was rigorously avoided during subsequent extraction and concentration procedures. These conditions were used to prevent the chemical conversion of any oxaziridine present to the isomeric amide during analysis. The reconstituted extracts were analysed using the reverse-phase isocratic HPLC system described above.

RESULTS AND DISCUSSION

Following the *in vitro* metabolism of NBTBA, no metabolites were observed which corresponded to authentic amide or oxaziridine. However, the corresponding nitrone (TBPN), benzaldehyde and an uncharacterised metabolite (possibly an N-hydroxy product) were detected. This could not be the corresponding imine as the imine had an identical retention time to that of benzaldehyde. Figure 4a shows a HPLC chromatogram following NBTBA metabolism by hamster microsomes. No metabolites were observed in control experiments either in the presence of denatured microsomes or in the absence of co-factors (data not shown). The UV spectra of the nitrone and benzaldehyde metabolites corresponded to those of authentic compounds and are presented in Figure 5.

The finding of a nitrone as a metabolite suggests that N-hydroxylation of NBTBA occurred, followed by further oxidation /21/, although other routes to this compound as suggested earlier /9/ cannot

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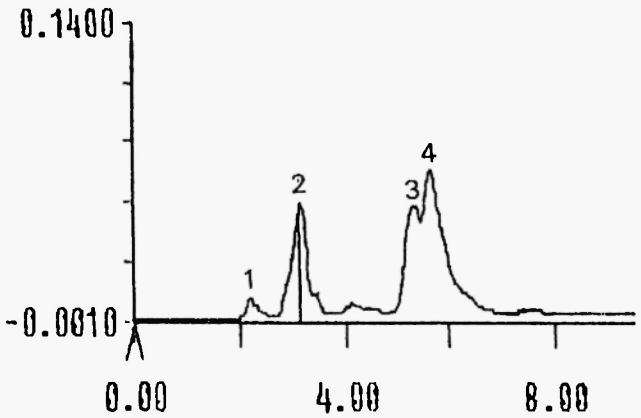

Fig. 4: HPLC chromatogram obtained following extraction of male hamster microsomal incubation mixture with NBTBA as substrate. 1=Substrate (NBTBA); 2=uncharacterised metabolite; 3–benzaldehyde; 4=nitrone (TBPN).

be ruled out at this stage. The failure to find the corresponding amide under our carefully controlled incubation and analytical conditions clearly shows that the nitrone is not an intermediate giving rise to amide from NBTBA.

Further work is required to elucidate the structure of the uncharacterised metabolite and establish its position in the total metabolic picture of this arylalkylamine.

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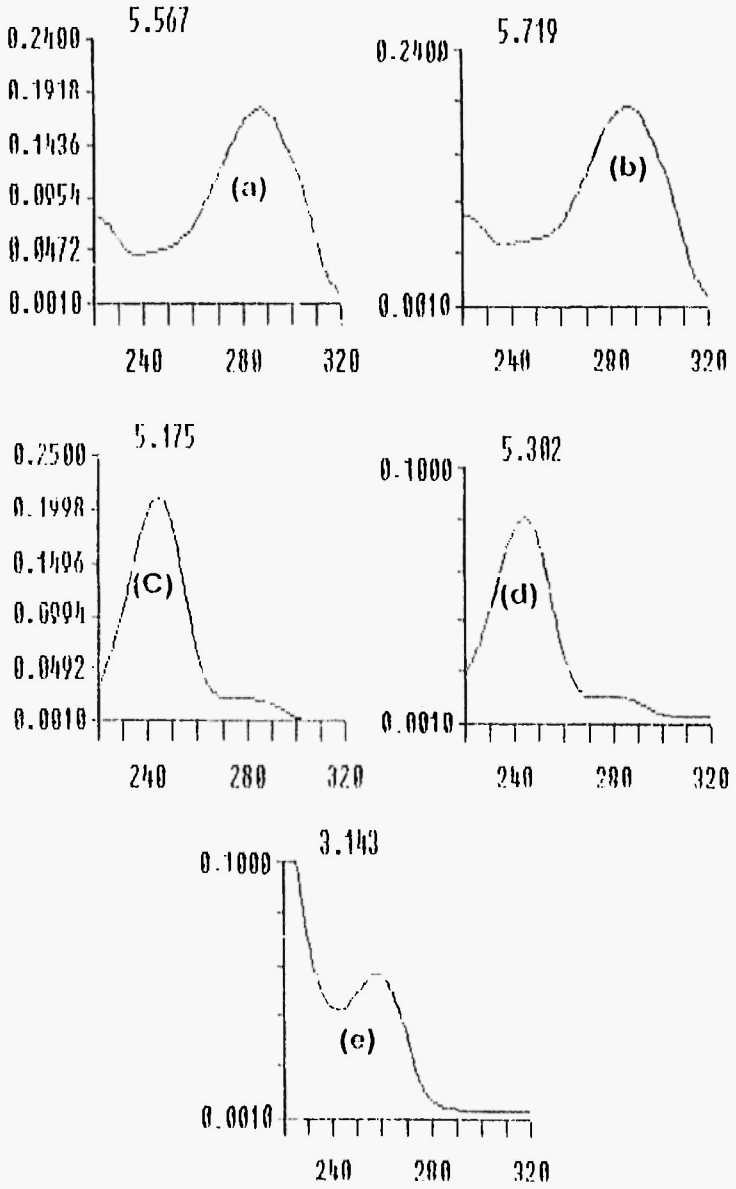

Fig. 5: UV spectra of: (a) authentic nitrone; (b) metabolic nitrone (TBPN); (c) authentic benzaldehyde; (d) metabolic benzaldehyde, and (e) uncharacterised metabolite.

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