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METABOLIC DISPOSITION OF A MONOTERPENE KETONE, PIPERITENONE, IN RATS: EVIDENCE FOR THE FORMATION OF A KNOWN TOXIN, *p*-CRESOL

K. MADHAVA MADYASTHA AND NILESH W. GAIKWAD

Bio-organic Section, Department of Organic Chemistry, Indian Institute of Science, Bangalore-560012 (K.M.M., N.W.G.), Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064, India (K.M.M.)

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ABSTRACT:

It was shown earlier that the monoterpene ketone, piperitenone (I) is one of the major metabolites of *R*-(+)-pulegone, a potent hepatotoxin. In the present studies, the metabolic disposition of piperitenone (I) was examined in rats. Piperitenone (I) was administered orally (400 mg/kg of the b. wt./day) to rats for 5 days. The following urinary metabolites were isolated and identified by various spectral analyses: *p*-cresol (VI), 6,7-dehydromenthofuran (III), *p*-mentha-1,3,5,8-tetraen-3-ol (IX), *p*-mentha-1, 3,5-triene-3, 8-diol (X), 5-hydroxypiperitenone (VIII), 7-hydroxypiperitenone (XI), 10-hydroxypiperitenone (XII), and 4-hydroxypiperitenone (VII). Incubation of piperitenone (I) with phenobarbital-induced rat liver microsomes in the presence of NADPH resulted in the formation of five metabolites which have been tentatively identified as metabolites III, VII, VIII, XI, XII, on the basis of gas chromatography retention time and gas chromatography-mass spectrometry analysis. Based on these results, a probable mechanism for the formation of *p*-cresol from piperitenone (I) via the intermediacy of metabolite III has been proposed.

The hepatotoxicity mediated by pennyroyal oil from *Mentha pulegium* has been attributed to its major constituent, *R*-(+)-pulegone (Gordon et al., 1982). This hepatotoxin gets extensively metabolized in the rat system, and the existence of two major pathways for its biotransformation has been demonstrated (Moorthy, et al., 1989; Madyastha and Raj, 1993). One of the major pathways is initiated through the regiospecific hydroxylation of *R*-(+)-pulegone to 9-hydroxypulegone, which is further transformed to menthofuran (Madyastha and Raj, 1990, Gordon et al., 1987). In the other major pathway, *R*-(+)-pulegone is subjected to stereoselective hydroxylation at C-5 position to form 5-hydroxypulegone, which, upon dehydration, yields piperitenone (**I**) (Madyastha and Raj, 1993). Most of the metabolites of *R*-(+)-pulegone are arising from these two common intermediates, viz. menthofuran and piperitenone (**I**). Using a rat model, it has been estimated that menthofuran accounts for nearly half the toxicity mediated by *R*-(+)-pulegone (Thomassen, et al., 1988). These observations suggest that pulegone elicits toxicity either directly or through metabolites formed independently of menthofuran. Because piperitenone (**I**) is one of the major metabolites of *R*-(+)-pulegone (Madyastha and Raj, 1993), it is quite possible that either piperitenone (**I**) or metabolites derived from it could also contribute to the toxicity mediated by *R*-(+)-pulegone.

It has been reported that *S*-(−)-pulegone is significantly less toxic than its *R*-(+)-enantiomer (Gordon et al., 1982). The only difference between these two enantiomers is the orientation of the methyl group at the C-5 position. Comparative metabolic studies carried out using *R*-(+) and *S*-(−)-pulegones have shown that all the major metabolites isolated and identified from both these enantiomers are the same (Madyastha and Gaikwad, 1998). However, there is a distinct difference between these two enantiomers with respect to the relative amounts of various metabolites formed. These studies have shown that the level of *p*-cresol (**VI**) and piperitenone (**I**) are significantly higher in the urine of rats treated with *R*-(+)-pulegone than of those treated with *S*-(−)-pulegone (Madyastha and Gaikwad, 1998). This suggests that piperitenone (**I**) could also independently contribute to *R*-(+)-pulegone-mediated toxicity. To better understand the mechanism and chemical basis of *R*-(+)-pulegone-mediated toxicity, metabolic studies with piperitenone (**I**) were undertaken both in vivo and in vitro. In fact, there has not been any report on the metabolic fate of piperitenone (**I**) in mammals. The present study describes the isolation and characterization of several novel metabolites from the urine of rats dosed with piperitenone (**I**), and some of these metabolites appear to be hitherto not known. The study also reports a new pathway for the formation of *p*-cresol, a known toxin from piperitenone (**I**).

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[1]Abbreviations used are: GC, gas chromatography; MS, mass spectrometry; TLC, thin-layer chromatography; IR, infrared; R_f, relative front; R_t, retention time.

Send reprint requests to: Prof. K. M. Madyastha, Bio-organic Section, Department of Organic Chemistry, Indian Institute of Science, Bangalore 560012, India.

Materials and Methods

Chemicals. Piperitenone (**I**) was synthesized as reported earlier (Nakanishi, et al., 1980) and purified by column chromatography over silica gel using ethyl acetate/hexane (5:95, v/v) as eluent. The purity of piperitenone used was more than 99% on the basis of gas chromatography (GC)[1] analysis. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $NADP^+$, methyl cellulose, and Tris-HCl were supplied by Sigma Chemical Co. (St. Louis, MO). Phenobarbital was a generous gift from IDPL (Hyderabad, India).

Animals and Dosing. Adult male rats (Wistar strain) weighing 180 to 200 g were used in these studies. Piperitenone (**I**) was administered to rats ($n = 30$) once daily for 5 days (400 mg/kg b. wt.) by gastric intubation as a suspension in 1% methylcellulose solution (1 ml). Control rats ($n = 5$) received only the vehicle. Control and experimental rats were housed separately in stainless steel metabolism cages with free access to food (laboratory animal food from Brook

Bond and Lipton, India) and water. Urine was collected in bottles maintained at 0–4°C.

Extraction of Urinary Metabolites. The urine samples collected daily from control and experimental rats were adjusted to pH 5 to 6 with 1 N HCl and extracted three times with diethyl ether. The ether extracts for each day were pooled and stored at 0–4°C. The pooled ether extracts was concentrated and separated into acid and neutral fractions by extracting with 5% (w/v) aqueous $NaHCO_3$ solution. The bicarbonate phase was acidified and extracted with ether to obtain the total acid fraction. The ether extract, following removal of the acid fraction, contained the neutral/phenolic metabolites.

Preparation of Microsomes. Pretreatment of rats with phenobarbital (80 mg/kg b. wt.) was carried out as reported earlier (Madyastha and Srivatsan, 1987). The livers were minced and homogenized in Tris-HCl (0.05 M, pH 7.4) containing 0.25 M sucrose, and the microsomes were prepared following the method of Lu and Levin (1972). Microsomal pellets were suspended in Tris-HCl buffer (0.05 M, pH 7.8) containing 0.25 M sucrose and 20% glycerol (v/v) and were stored at −20°C. Protein determinations were conducted following the method of Lowry et al. (1951).

Studies In Vitro. Phenobarbital-induced rat liver microsomes (2 mg/ml) were incubated in the presence of glucose 6-phosphate (5 mM), $NADP^+$ (0.5 mM), glucose 6-phosphate dehydrogenase (2 unit), $MgCl_2$ (10 mM), piperitenone (2 mM, in 100 μl of acetone), and Tris-HCl (0.01 M, pH 7.4) in a total volume of 10 ml. The reaction was initiated by the addition of an NADPH-generating system, and the mixture was incubated aerobically in a rotary shaker for 1 h at 37°C. At the end of the incubation period, the assay tubes were immersed in ice, and the protein was precipitated by adding 4 ml each of saturated $Ba(OH)_2$ and 0.25 M $ZnSO_4$ solution. The precipitated protein was removed by centrifugation. The supernatant was extracted three times with 15 to 20 ml of methylene chloride, dried over anhydrous Na_2SO_4, concentrated, and an aliquot was subjected to GC and gas chromatography-mass spectrometry (GC-MS) analysis. This experiment was also repeated using uninduced (control) rat liver microsomes.

Chromatographic Procedures. Thin-layer chromatography (TLC) was carried out on Silica Gel-G coated plates (0.25 mm) developed with hexane/ethyl acetate as the solvent system (system 1, 70:30, v/v; system 2, 30:70, v/v; system 3, 90:10, v/v). Compounds were visualized on the chromatograms by spraying the TLC plates with 1% vanillin in 50% H_2SO_4 followed by heating at 100°C for 5 to 10 min.

GC. Analyses were carried out on a Shimadzu GC model 14A instrument equipped with a hydrogen flame ionization detector. The instrument was fitted with a Shimadzu HR-1 wide bore capillary column (15 m × 0.5 mm diameter). Nitrogen at a flow rate of 30 ml/min was used as the carrier gas. The temperature of the column was held at 80°C for 10 min, rising thereafter at 5°C/min to 120°C.

Spectra. Infrared (IR) spectra were recorded on Perkin-Elmer model 781. Proton NMR spectra were recorded on a JEOL FT-90 MHz spectrometer. Chemical shifts are reported in ppm, with respect to tetramethylsilane as the internal standard. Mass spectral analyses were performed on a JEOL-JMX-DX 303 instrument attached with a JMA-DA 5000 data system. A capillary column (50 m in length and 0.25 mm in diameter) containing either 3% SE-30 on chromosorb-W or 10% QF_1 on chromosorb-W was used for the effective separation of metabolites. The column temperature was programmed between 60° and 100°C at a rate of 2°C/min, and then to 220°C at 8°C/min. Helium was used as a carrier gas at a flow rate of 30 ml/min.

Results

Biotransformation of Piperitenone In Vivo. TLC analysis (system 1) of neutral/phenolic fraction (2.6 g) showed the presence of at least nine compounds which were absent in control urine extract. This fraction (2.6 g) was subjected to column chromatography over neutral alumina (45 g), and elution of the column with pentane yielded the least polar metabolite [relative front (R_f) 0.93, system 1, retention time (R_t) 3.9 min]. This compound had the following spectral characteristics: NMR spectrum ($CDCl_3$, Fig. 1) showed signals at δ 7.0 (1H, s, C-2 aromatic proton), 6.05 (1H, bs, C-7 olefinic proton), 2.7 to 2.1 (4H, m, C-4 & C-5 protons), 1.9 (3H, s, methyl protons on furan ring), and 1.85 (3H, s, C-8 methyl protons). Mass spectra (MS) (Fig. 1, inset) were: m/z 148(M^+), 133(M^+-CH_3), 105(M^+-C_2H_3O, base peak), and 91(M^+-C_3H_5O). Based on these spectral characteristics, the compound was tentatively assigned the structure 6,7-dehydromenthofuran (III, Fig. 2). This compound is unstable, and upon storage, it transformed into another compound with a mass spectral fragmentation pattern that corresponded well with that reported for 3,6-dimethyl benzofuran (Givens, et al., 1969) (Fig. 3). Formation of 3,6-dimethyl benzofuran from metabolite **III** further supports the structure assigned to the least polar metabolite (**III**) as 6,7-dehydromenthofuran.

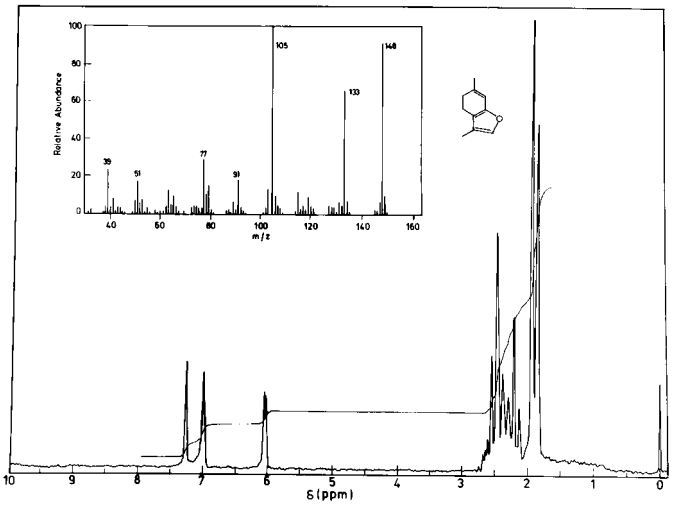

FIG. 1. *^{1}HNMR spectra and electron impact mass spectra (inset) of 6,7-dehydromenthofuran (**III**).*

Additional elution of the column with hexane yielded a fraction that upon GC (R_t 4.0 and 8.1 min) and TLC (system 1, R_f 0.66 and 0.57) analyses showed the presence of two major compounds. These two compounds were separated by preparative TLC (system 3). The compound with R_f 0.66 and R_t 4.0 min showed the following spectral characteristics: NMR spectra ($CDCl_3$, Fig.4) δ 7.1 to 6.7 (2H, two doublets, C-5 & C-6 aromatic protons), 6.75 (1H, s, C-2 aromatic proton), 5.7 (1H, bs, hydroxyl proton), 5.4 (1H, bs, C-9a olefinic proton), 5.15 (1H, bs, C-9b olefinic proton), 2.3 (3H, s, methyl protons on aromatic ring), and 2.1 (3H, s, allylic methyl proton). IR (neat) spectrum showed absorptions around 3500 cm^{-1} (hydroxyl group) and 1620 cm^{-1} (aromatic). Mass Spectra (Fig. 4, inset) were: m/z 148(M^+), 133(M^+-CH_3), 119(M^+-C_2H_5), 105(M^+-C_3H_7), and 43(M^+-C_7H_5O, base peak). Based on the spectral data the compound was identified as *p*-mentha-1, 3,5,8-tetraen-3-ol (**IX**, Fig. 2). The structure assigned was also confirmed by comparing the spectral characteristics (NMR and IR) with that reported for this compound (Divakar, et al., 1977).

The compound with R_f 0.57 was identified as unmetabolized piperitenone (**I**) by comparing its GC retention time (R_t 8.1 min) and NMR, IR and mass spectra with that of authentic compound.

The metabolite with R_f 0.56 (system 1) was eluted from the neutral alumina column with 19:1 hexane/ethyl acetate. The metabolite was identified as *p*-cresol (**VI**) by comparing its spectral data [NMR, IR, and mass spectrometry (MS)] to that reported earlier (Madyastha and Raj, 1992). Further elution of the column with the same solvent system yielded a compound with R_f 0.5 (system 1) and R_t 12.3 min. This compound had the following spectral characteristics. IR spectrum (neat) showed absorptions at 3300 cm^{-1} (hydroxyl group), 1570 cm^{-1} and 1620 cm^{-1} (aromatic ring). NMR spectra ($CDCl_3$, Fig. 5) were: δ 8.9 (1H, bs, phenolic hydroxyl), 6.97 to 6.61 (2H, two

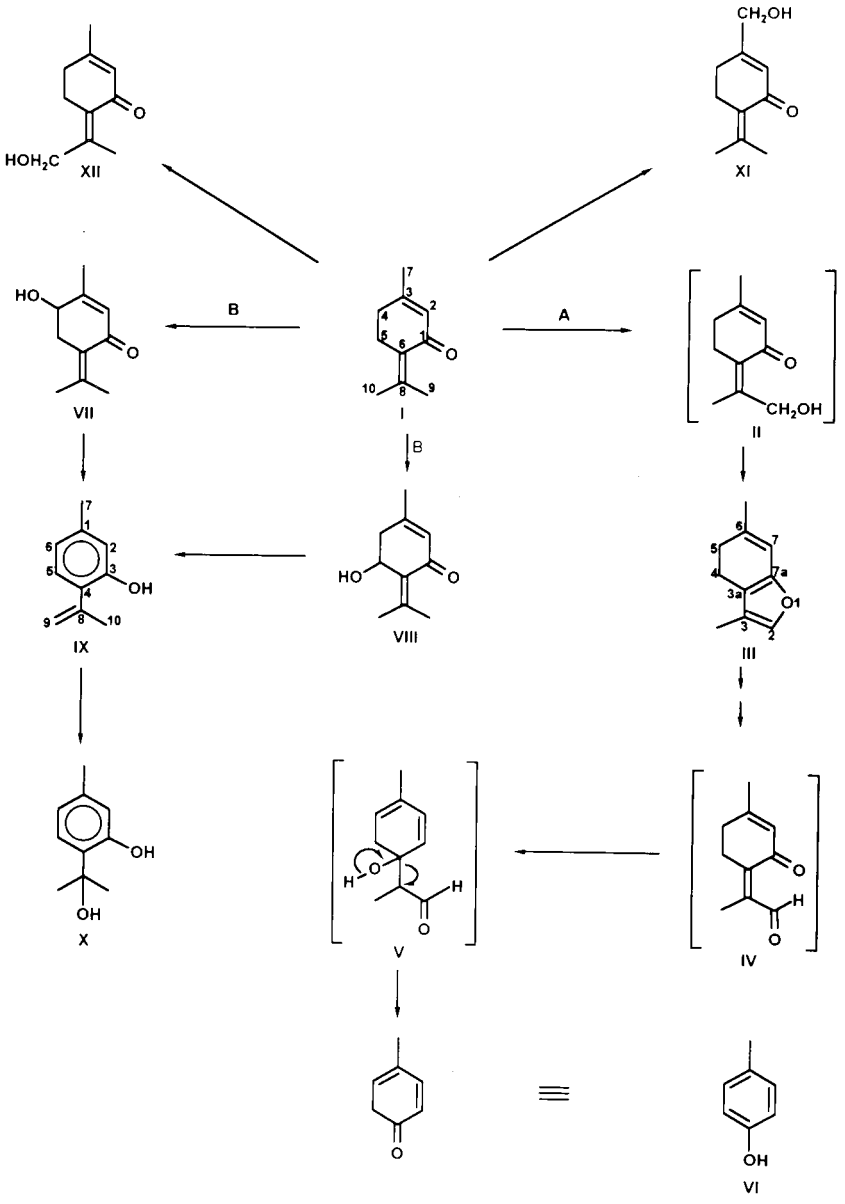

FIG. 2. *Probable metabolic pathways of piperitenone* ***(I).***

doublets, C-5 and C-6 aromatic protons), 6.68 (1H, s, C-2 aromatic proton), 2.24 (3H, s, methyl on aromatic ring), and 1.65 (6H, s, dimethyl group on C-8). MS analysis showed molecular ion peak at m/z 166 and fragmentation pattern (Fig. 5, Inset) m/z 166(M^+), 148(M^+-H_2O, base peak), 133(M^+-CH_5O) and 105 (M^+-C_3H_9O). Based on the spectral characteristics, the metabolite was identified as *p*-mentha-1, 3,5-triene-3, 8-diol (**X**, Fig. 2) The spectral characteristics of metabolite **X** agreed with the earlier report on this compound (Desai, et al., 1984).

Elution of the column with hexane/ethyl acetate (9:1, v/v) yielded a compound having R_f 0.6 (system 2) and R_t 15.9 min. The compound had the following spectral characteristics. NMR spectra ($CDCl_3$, Fig. 6) were: δ 5.9 (1H, s, olefinic proton), 4,27 (1H, m, C-5 proton), 2.87 (2H, m, C-4 methylene protons), 2.15 (3H, s, C-9 methyl protons), 2.04 (3H, s, C-7 methyl protons) and 1.9 (3H, s, C-10 methyl protons). IR spectrum (neat) showed absorptions at 3380 cm^{-1} (hydroxyl group), 1650 cm^{-1} (conjugated ketone), and 1600 cm^{-1} (double bond). Mass spectra (Fig. 6, inset) were: m/z 166(M^+, base peak), 151(M^+-CH_3), 148(M^+-H_2O), 123(M^+-C_2H_3O), and 108(M^+-C_3H_6O). Based on these spectral characteristics, the compound was assigned the structure 5-hydroxypiperitenone (**VIII,** Fig. 2).

The metabolites with R_f 0.5 (system 2) and R_t 20.5 min was eluted from the neutral alumina column with 8:2 hexane/ethyl acetate. The compound had the following spectral characteristics: IR spectrum (neat) showed absorptions at 3380 cm^{-1} (hydroxyl group), 1650 cm^{-1} (conjugated ketone) and 1600 cm^{-1} (double bond). NMR spectra ($CDCl_3$, Fig. 7) δ 6.1 (1H, s, olefinic proton), 4.2 (2H, bs, C-7 protons), 2.7 (2H, t, C-4 protons), 2.25 (2H, t, C-5 protons), 2.1 (3H, s, C-9 methyl protons) and 1.85 (3H, s, C-10 methyl protons). Mass spectra (Fig. 7, inset) were: m/z 166(M^+, base peak), 137(M^+-C_2H_5), 135(M^+-CH_2OH), 123(M^+-C_2H_3O), and 109(M^+-C_3H_5O). From the spectral data, the metabolite was identified as 7-hydroxypiperitenone (**XI**, Fig. 2).

Additional elution of column with 8:2 hexane/ethyl acetate gave a fraction that upon GC analysis showed the presence of two compounds (R_t 19.2 and 15.8 min). The TLC analysis (system 2) of this

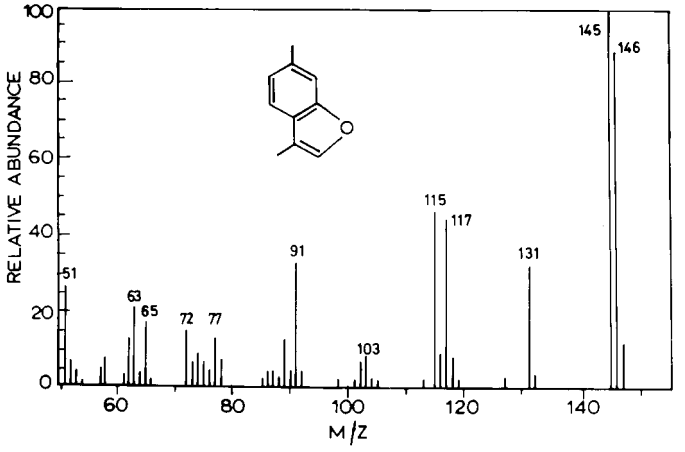

FIG. 3. *Electron impact mass spectra of 3,6-dimethylbenzofuran.*

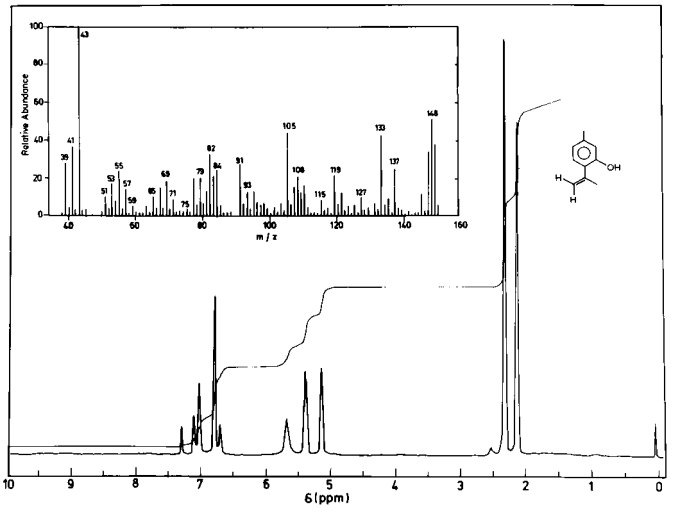

FIG. 4. *^{1}HNMR spectra and electron impact mass spectra (inset) of p-mentha-1,3,5,8-tetraen-3-ol (**IX**).*

fraction revealed the presence of two compounds (R_f 0.47 and 0.42) which were separated by repeated preparative TLC (system 2). The compound with R_f 0.47 showed the following spectral characteristics: IR spectrum (neat) showed absorption at 3375 cm^{-1} (hydroxyl group), 1645 cm^{-1} (conjugated ketone), and 1600 cm^{-1} (double bond). NMR spectra ($CDCl_3$, Fig. 8) were: δ 5.92 (1H, s, olefinic proton), 4.25 (2H, s, C-10 protons), 2.82 (2H, t, C-4 protons), 2.35 (2H, t, C-5protons), 2.11 (3H, s, C-9 methyl protons), and 1.93 (3H, s, C-7 methyl protons). Mass spectra (Fig. 8, inset) were: m/z 166(M^+, base peak), 148(M^+-H_2O), 147(M^+-H_3O), 133(M^+-CH_5O) and 105(M^+-C_3H_9O). Based on the spectral data, the compound was identified as 10-hydroxypiperitenone (**XII**, Fig. 2). The compound with R_f 0.42 (system 2) and R_t 15.8 min showed the following spectral properties: IR spectrum (liquid film) showed absorption at 3385 cm^{-1} (hydroxyl group), 1650 cm^{-1} (conjugated ketone), and 1590 cm^{-1} (double bond). NMR spectrum ($CDCl_3$, Fig. 9) δ 5.98 (1H, s, olefinic proton), 5.1 (1H, m, C-4 proton), 2.58 (2H, bs, C-5 proton), 2.14 (3H, s, C-9 methyl protons), and 1.98 (6H, s, C-10 and C-7 methyl protons). Mass spectra (Fig. 9, inset) were: m/z 166(M^+, base peak), 149(M^+-OH), 151(M^+-CH_3), 137(M^+-C_2H_5), 133(M^+-CH_5O), 123(M^+-C_2H_3O), and 109(M^+-C_3H_5O). From the spectral data, the metabolite was identified as 4-hydroxypiperitenone (**VII**, Fig. 2).

The composition of the total neutral/phenolic fraction (Fig. 10) was

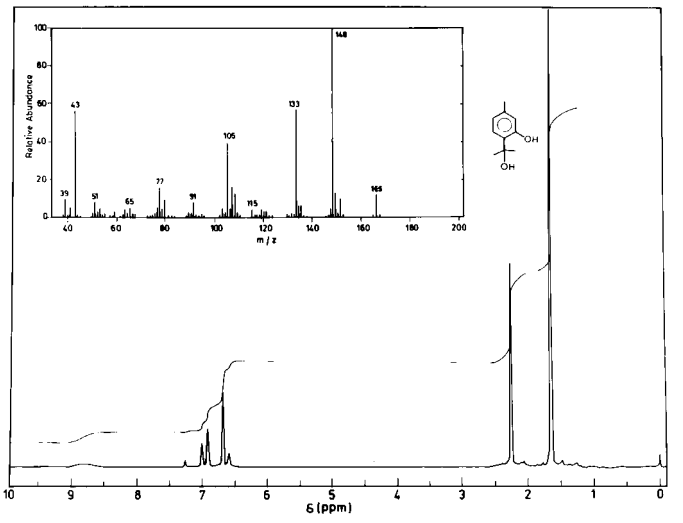

FIG. 5. *^{1}HNMR spectra and electron impact mass spectra (inset) of p-mentha-1,3,5-triene-3, 8-diol (**X**).*

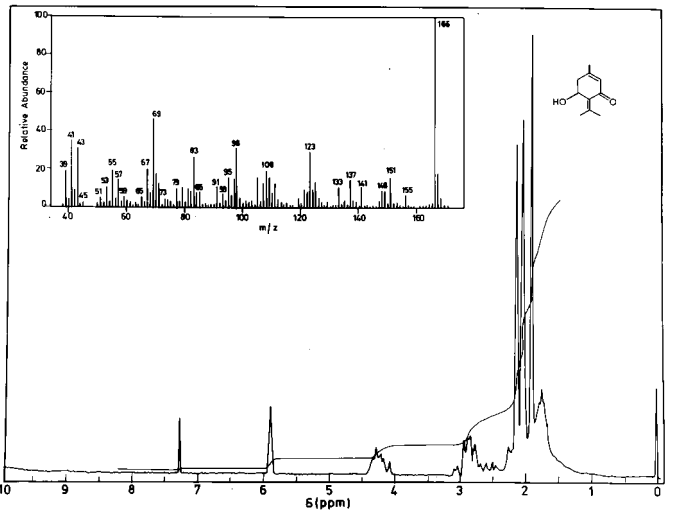

FIG. 6. *^{1}HNMR spectra and electron impact mass spectra (inset) of 5-hydroxypiperitenone (**VIII**).*

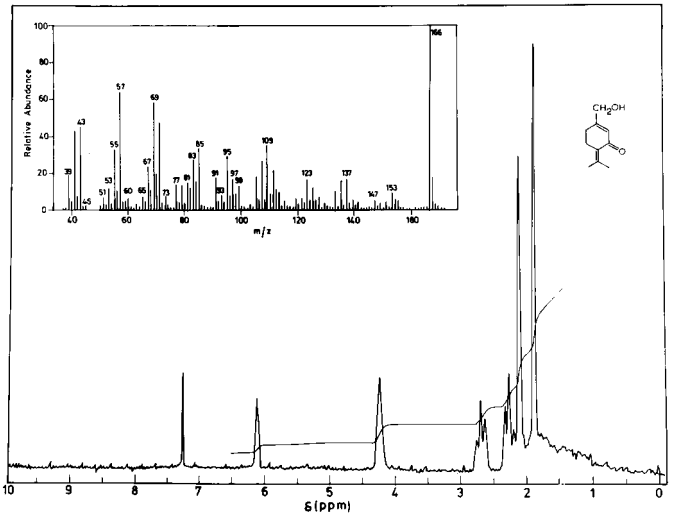

FIG. 7. *^{1}HNMR spectra and electron impact mass spectra (inset) of 7-hydroxypiperitenone (**XI**).*

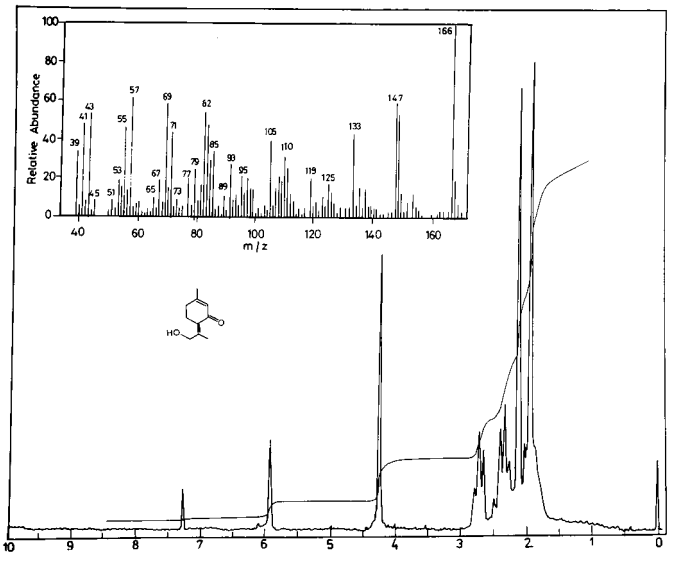

FIG. 8. *[1]HNMR spectra and electron impact mass spectra (inset) of 10-hydroxypiperitenone (**XII**).*

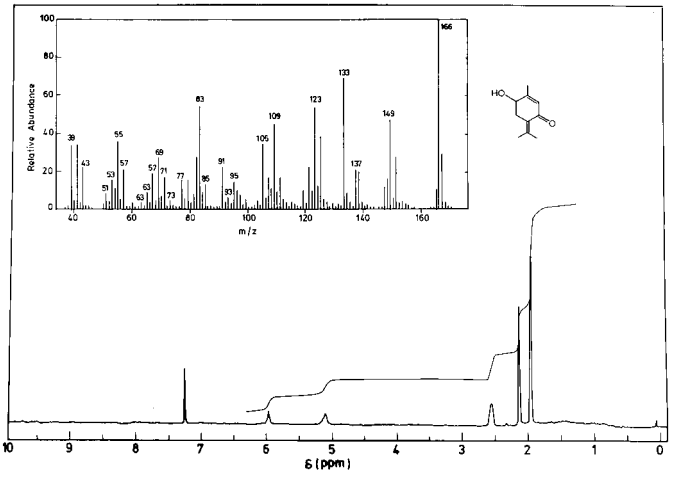

FIG. 9. *[1]HNMR spectra and electron impact mass spectra (inset) of 4-hydroxypiperitenone (**VII**).*

determined by GC analyses. The analyses showed the presence of seven major peaks corresponding to metabolites **III**, **VI**, **VII**, **VIII**, **IX**, **X**, **XI**, and **XII** and unmetabolized piperitenone (**I**) (Fig. 2). GC profile also showed few minor peaks, which could not be identified. The peaks corresponding to these metabolites (**III**, **VI**, **VII**, **VIII**, **IX**, **X**, **XI**, and **XII**) were enhanced when mixed with the purified metabolites isolated by column chromatography. On the basis of GC analysis, nearly 85% of the total neutral/phenolic fraction was identified. Two pairs of metabolites (**III** and **IX**; **VII** and **VIII**) could not be separated well under the GC conditions used.

Acidic Metabolites. Very little acidic fraction was obtained, and it did not contain any metabolites derived from piperitenone. Hence this fraction was not processed further.

Biotransformation of Piperitenone (I) by Liver Microsomes. Phenobarbital-induced rat liver microsomes were incubated with piperitenone (**I**) in the presence of NADPH and O_2 as described in *Materials and Methods*. The methylene chloride extract of the assay mixture upon GC analysis indicated the presence of metabolites **VII**, **VIII**, **XI**, **XII** and **III**. All of the peaks corresponding to these metabolites were enhanced when mixed with samples isolated by

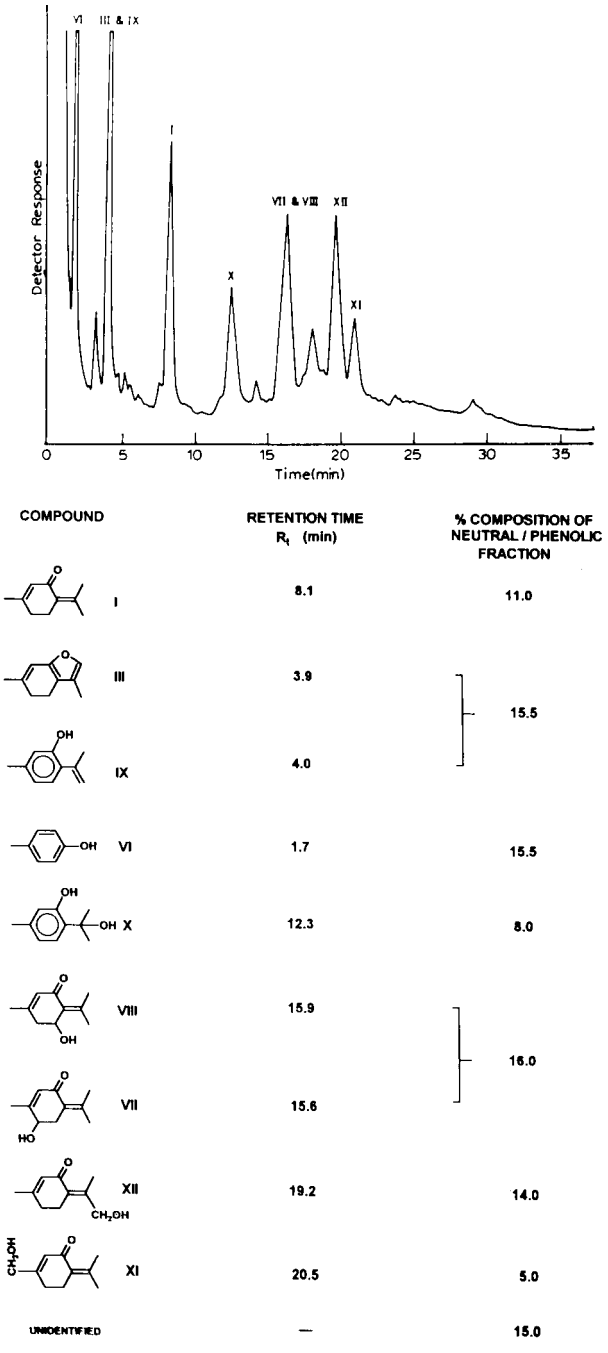

FIG. 10. *The GC analysis of the total neutral/phenolic fraction.*

Analysis was carried out as described in *Materials and Methods*. Roman numbers represent the different metabolites. Two pairs of metabolites (**III** and **IX** and **VII** and **VIII**) do not separate under the GC conditions employed. The structure of the metabolites, their retention time and percentage composition are presented. Purified metabolites **III**, **IX**, **VIII** and **VII** have the retention time as 3.9, 4.0, 15.9and 15.8 min respectively. However, when the total neutral/phenolic fraction was injected, metabolite pairs **III** and **IX** and **VIII** and **VII** have retention time (R_t) as 3.98 and 15.9 min, respectively.

column chromatography. The GC-MS analysis of the assay mixture indicated the presence of metabolites **VII**, **VIII**, **XI**, **XII** and **III**. Nearly 10% of the substrate (**I**) added was converted into these five metabolites (**VII**, **VIII**, **XI**, **XII** and **III**). Among the metabolites formed, compound **III** was present in higher levels. Incubation of piperitenone (**I**) with uninduced (control) rat liver microsomes in the presence of NADPH and O_2 as described in Materials and Methods,

and GC analysis of the methylene chloride extract of the reaction mixture indicated that metabolites were present in very low amounts.

Discussion

The present study has demonstrated the ability of the rat's system to carry out various transformations in piperitenone (**I**). Most of the metabolites present in the urine of rats dosed with piperitenone (**I**) have been isolated and characterized based on various spectral analyses. It is interesting to note that all of the five positions in piperitenone (**I**) which are allylic to double bonds are hydroxylated, unlike in *R*-(+)-pulegone (Madyastha and Raj, 1993). Cursory examination of different metabolites isolated from the urine extract indicated the existence of two major pathways (A and B, Fig. 2) for the biotransformation of **I**. However, it should be noted here that the pathways proposed are speculative and certainly need more experimental evidence to substantiate the sequence of reactions. Suitable experimental precautions were taken to minimize the loss and nonenzymatic transformations of the metabolites during their isolation.

Pathway A (Fig. 2) appears to be the most interesting and significant, as it is involved in the formation of *p*-cresol (**VI**, Fig. 2). It has been reported earlier that *p*-cresol (**VI**) causes severe toxicity in both liver and lung (Deichmann and Keplinger, 1958; US Department of Health, 1992; Thompson et al., 1994). It (**VI**) is also one of the major metabolites of *R*-(+)-pulegone and menthofuran in vivo (Madyastha and Raj, 1992, 1993). It appears that the first step in pathway A in the biotransformation sequence (Fig. 2) is the oxygenation of the C-9 methyl group, resulting in the formation of 9-hydroxypiperitenone (**II**), which upon cyclization followed by dehydration yields 6,7-dehydromenthofuran (**III**). The fact that this metabolite (**III**) is unstable and gets converted to 3,6-dimethylbenzofuran (Fig. 3) lends further support to the structure assigned to this metabolite as 6,7-dehydromenthofuran (**III**). Studies carried out in vitro using phenobarbital-induced rat liver microsomes gave corroborative experimental evidence for the above sequence of reactions. In fact, the sequence of reactions involved in the formation of 6,7-dehydromenthofuran (**III**) appears to be very similar to the earlier reports on the formation of menthofuran from *R*-(+)-pulegone (Gordon et al., 1987; Madyastha and Raj, 1990). One can envisage the formation of *p*-cresol (**VI**) from 6,7-dehydromenthofuran (**III**) as shown in Fig. 2. The liver microsomal cytochrome P-450 system can convert compound **III** to its 2,3-epoxide, and opening of the epoxide can give rise to an unsaturated ketoaldehyde (**IV**, Fig. 2). Reduction of the keto group in **IV** to a secondary alcohol and its elimination would generate 1,2-double bond with a simultaneous migration of the 2,3-double bond to 3,4-position. This sequence of reaction is followed by hydration of the exocyclic double bond (**V**, Fig. 2) which facilitates a spontaneous nonezymatic retroaldol type reaction to yield *p*-cresol (**VI**, Fig. 2). The proposed catabolic sequence (Fig. 2) appears to be analogous to the pathway established earlier for the conversion of menthofuran to *p*-cresol via the intermediacy of an α,β-unsaturated-γ-ketoaldehyde (McClanahan et al., 1989; Madyastha and Raj, 1990, 1992). Earlier studies have shown that rat liver microsomes in the presence of NADPH and O_2 convert menthofuran to an highly reactive α,β-unsaturated-γ-ketoaldehyde which interacts with tissue macromolecules to elicit toxicity (Madyastha and Moorthy, 1989; McClanahan et al., 1989; Madyastha and Raj, 1990). Alternatively, this reactive metabolite is further transformed to 4-methyl-2-cyclohexenone, which yields *p*-cresol upon hydroxylation (Madyastha and Raj, 1991, 1992). In fact, mechanistically, the conversion of 6,7-dehydromenthofuran (**III**) to *p*-cresol (**VI**) appears to be comparatively simpler than the transformation of menthofuran to *p*-cresol (**VI**) due to the presence of 6,7-double bond in compound III (Fig. 2). The sequence of reactions postulated in the conversion of **III** to *p*-cresol (**VI**) could not be tested in vitro using 6,7-dehydromenthofuran (**III**), as this compound appears to be unstable and all attempts to synthesize it were not successful.

The most interesting aspect of pathway A (Fig. 2) is that it represents a new route for the formation of *p*-cresol (**VI**) from *R*-(+)-pulegone via the intermediacy of piperitenone (**I**) and 6,7-dehydromenthofuran (**III**). Earlier studies have clearly established that piperitenone is one of the metabolites of *R*-(+)-pulegone in vivo (Madyastha and Raj, 1993). It is quite possible that the metabolites such as *p*-cresol (**VI**), 6,7-dehydromenthofuran (**III**) formed via pathway A may also contribute toward the pulegone-mediated toxicity in mammals. In fact, it has been shown that *p*-cresol rapidly depletes intracellular glutathione levels (Thompson et al., 1994). Because *p*-cresol (**VI**) is one of the major metabolites formed from both *R*-(+)-pulegone and menthofuran in vivo, it is reasonable to assume that it (**VI**) could contribute to the overall toxicity mediated by *R*-(+)-pulegone by decreasing the availability of glutathione for conjugation with reactive metabolites derived from *R*-(+)-pulegone. In support of this argument, Thomassen and his coworkers have shown that *R*-(+)-pulegone depletes glutathione in vivo and oxidative metabolites of pulegone are responsible for the glutathione depletion (Thomassen, et al., 1990). Alternatively, *p*-cresol can be further transformed either to an epoxide or to a quinone methide as reactive intermediates which can generate toxic effects by interacting with tissue macromolecules (Thompson et al., 1994).

The present study has demonstrated that in piperitenone (**I**) both of the isopropylidene methyl groups (C-9 and C-10) are hydroxylated as evidenced by the isolation of 10-hydroxypiperitenone (**XII**) and 6,7-dehydromenthofuran (**III**). The 9-hydroxypiperitenone (**II**) formed readily undergoes intramolecular cyclization followed by dehydration to yield compound **III**. However, such a cyclization is not possible in the case of 10-hydroxypiperitenone (**XII**). These results are supported by the in vitro studies carried out using phenobarbital-induced rat liver microsomes. Contrary to these observations, it was noted that in the case of *R*-(+)-pulegone only the methyl group *syn* to the carbonyl group is hydroxylated by the liver microsomal cytochrome P-450 system (Gordon et al., 1987; Madyastha and Raj, 1990).

Pathway B for the biodegradation of piperitenone (**I**) is initiated through ring hydroxylation resulting in the formation of 4-hydroxy (**VII**) and 5-hydroxy (**VIII**) piperitenones (Fig. 2). Both of these compounds (**VII** and **VIII**), upon dehydration, could yield compound **IX** (Fig. 2). Hydration of the exocyclic double bond in compound **IX** could yield metabolite **X** (Fig. 2). Metabolite **XI** (Fig. 2) might have been formed through allylic methyl (C-7 methyl) hydroxylation. Direct hydroxylation of piperitenone at the C_4, C_5, C_7, and C_{10} positions is amply supported by the in vitro studies conducted using phenobarbital-induced rat liver microsomes in the presence of NADPH and O_2. In fact, studies carried out in vitro also suggested the possible involvement of phenobarbital-induced cytochrome P-450 system in the hydroxylation reaction because uninduced (control) liver microsomes very poorly transformed piperitenone to various metabolites. However, this observation needs additional experimental support.

In summary, we have demonstrated that *p*-cresol (**VI**) is one of the major metabolites of piperitenone in vivo. Earlier studies have clearly shown that both piperitenone (**I**) and menthofuran are the major metabolites of *R*-(+)-pulegone in vivo (Madyastha and Raj, 1993). Hence, it can be inferred that both of these metabolites of *R*-(+)-pulegone are independently further biotransformed to *p*-cresol (**VI**). This is a significant observation from the toxicological point of view. We have proposed the sequence of reactions that can lead to the formation of *p*-cresol (**VI**) from piperitenone (**I**) via the intermediacy

of 6,7-dehydromenthofuran (**III**) and an unsaturated ketoaldehyde (**IV**, Fig. 2). This hypothetical scheme is based on chemical logic and the earlier report on the conversion of menthofuran to an α,β-unsaturated-γ-ketoaldehyde, by studies carried out in vivo and in vitro (McClanahan et al., 1989, Madyastha and Raj, 1990, 1992).

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