# ROLE OF C-5 CHIRAL CENTER IN *R*-(+)-PULEGONE-MEDIATED HEPATOTOXICITY: METABOLIC DISPOSITION AND TOXICITY OF 5,5-DIMETHYL-2-(1-METHYLETHYLIDENE)-CYCLOHEXANONE IN RATS

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**ABSTRACT:**

Metabolic disposition of 5,5-dimethyl-2-(1-methylethylidene)-cyclo-hexanone (I) was examined in rats. Compound (I) was administered orally (250 mg/kg of body weight/day) to rats for 5 days. The following urinary metabolites were isolated and identified: 4,5,6,7-tetrahydro-3,6,6-trimethylbenzofuran (III), 3,3-dimethylcyclohexanone (VI), 5,5-di-methyl-3-hydroxy-2-(1-methylethylidene)-cyclohexanone (X), 5,5-di-methyl-2-(1-hydroxymethylethyl)-cyclohexanone (IX), 3-hydroxy-5-hydroxymethyl-5-methyl-2-(1-methylethylidene)-cyclohexanone (XI), 5,6-dihydro-3,6,6-trimethyl-2(4*H*)-benzofuranone (VIII), and 5,5-di-methyl-3-hydroxy-2-(1-carboxy ethylidene)-cyclohexanone (XIII). In-cubation of compound (I) with phenobarbital (PB)-induced rat liver microsomes in the presence of NADPH resulted in the formation of a metabolite, tentatively identified as a furanoterpene (III) based on proton magnetic resonance, gas chromatography, and gas chroma-tography-mass spectroscopy analyses. The formation of III was in-hibited to a significant extent by carbon monoxide, metyrapone, SKF 525-A, and cytochrome *c*, suggesting the participation of PB-induced microsomal cytochrome P-450 system in the conversion of I to III. Compound I gave type I spectral change in the PB-induced liver microsomes and the dissociation constant (*K*s) for I was 38.5 $\mu$M. Intraperitoneal administration of a single dose (250 mg/kg) of I to rats resulted in 26, 23, and 41% decreases in the levels of cytochrome P-450, glucose-6-phosphatase, and aminopyrine *N*-demethylase, re-spectively, at the end of 24 h. During this period, a 11-fold increase in serum glutamate pyruvate transaminase level was also observed. However, a decrease in the level of cytochrome P-450 and glucose-6-phosphatase, and an increase in serum glutamate pyruvate transaminase values were comparatively more pronounced when *R*-(+)-pulegone (250 mg/kg) or CCl$_4$ (0.6 ml/kg) was administered to rats. Pretreatment of rats with PB potentiated the hepatotoxicity caused by I, whereas pretreatment with 3-methylcholanthrene pro-tected from it. This suggests that PB-induced cytochrome P-450-catalyzed reactive metabolites may be responsible for the toxic ef-fects caused by I.

*R*-(+)-pulegone, a naturally occurring monoterpene ketone, is a potent hepatotoxin and the bioactivation of this compound to reactive metabolites is mostly responsible for the observed toxicity (Gordon et al., 1982, 1987; Thorup et al., 1983; Madyastha and Moorthy, 1989; McClanahan et al., 1989; Moorthy et al., 1989a,b, 1991; Madyastha and Raj, 1990, 1992). The hepatotoxin gets metabolized following two major pathways (Madyastha and Raj, 1993). One of the major pathways is initiated through the regiospecific hydroxylation of *R*-(+)-pulegone to 9-hydroxypulegone, which on intramolecular cy-clization followed by dehydration yields a furanoterpene, menthofu-ran (Gordon et al., 1987; McClanahan et al., 1989; Madyastha and Raj, 1990, 1993). The second major pathway is involved in the stereoselective hydroxylation of *R*-(+)-pulegone at C-5 position to form 5-hydroxypulegone which gets transformed to piperitenone (Madyastha and Raj, 1993). In fact, most of the metabolites of *R*-(+)-pulegone are derived from menthofuran and piperitenone

(Madyastha and Raj, 1992; Madyastha and Gaikwad, 1999). Mentho-furan, the proximate toxin, accounts for nearly half the toxicity mediated by *R*-(+)-pulegone (Thomassen et al., 1988, 1990), suggest-ing that the remaining 50% of toxicity is caused by metabolites derived independently of menthofuran. It appears that the metabolites derived from piperitenone could also contribute to *R*-(+)-pulegone-mediated toxicity because its biotransformation yields *p*-cresol and 6,7-dehydromenthofuran besides other metabolites (Madyastha and Gaikwad, 1999). *p*-Cresol is known to cause toxicity in both liver and lung (Deichmann and Keplinger, 1958; Thompson et al., 1994). The 6,7-dehydromenthofuran could also contribute to toxicity by virtue of its being a furanoterpene (Boyd, 1982). This suggests that the stereo-selective hydroxylation at the C-5 position is one of the key steps involved in the bioactivation of *R*-(+)-pulegone to reactive metabo-lites responsible for toxicity. The available evidences suggest that both the major pathways (5-hydroxylation and 9-hydroxylation path-ways) involved in the metabolism of *R*-(+)-pulegone yield reactive metabolites that can generate toxic effects. However, the contribution of the individual pathways toward *R*-(+)-pulegone-mediated toxicity has not been assessed (Scheme 1).

The characteristic structural features of *R*-(+)-pulegone are the presence of an $\alpha$-isopropylidene ketone unit and a chiral center at C-5. Both these structural features appear to be necessary for the com-pound to elicit maximum toxicity (Gordon et al., 1982). In fact, the

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R-(+)-pulegone

5,5-dimethyl-2-(1-methylethylidene)-
cyclohexanone(**I**)
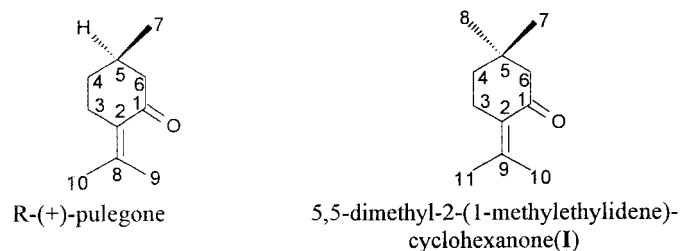
Scheme 1

C-5 methyl group should have a proper orientation as in *R*-(+)-pulegone. It is known that inversion of configuration of the C-5 methyl group in *R*-(+)-pulegone markedly affects the hepatotoxic potential. Thus, *S*-(−)-pulegone is approximately one-third as hepatotoxic as its enantiomer, *R*-(+)-pulegone (Gordon et al., 1982). It would be interesting to assess the hepatotoxic potential of a compound that is structurally similar to *R*-(+)-pulegone but without a chiral center at the C-5 position. One can envisage such a situation if the C-5 hydrogen in *R*-(+)-pulegone is replaced by another methyl group as in 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone (I), a compound structurally similar to *R*-(+)-pulegone. In this compound (I) the metabolic pathway initiated by C-5 hydroxylation is blocked due to an additional methyl substitution at the C-5 position. It would be interesting to find out whether compound I gets metabolized following a pathway initiated through regiospecific hydroxylation of the methyl group that is *syn* to the carbonyl group (C-10 methyl) or whether the compound I is not accepted by the hydroxylase system as a substrate. If C-10 hydroxylated product is formed from compound I, would such a compound undergo spontaneous intramolecular cyclization to a hemiketal, which upon dehydration yields a furanoterpene in a manner analogous to the formation of menthofuran from *R*-(+)-pulegone (Gordon et al., 1987; Moorthy et al., 1989a; Madyastha and Raj, 1990). To understand the role of chiral center at C-5 in *R*-(+)-pulegone-mediated toxicity, metabolic studies with 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone (compound I) were undertaken both in vivo and in vitro. This study describes the isolation and characterization of several novel metabolites from the urine of rats dosed with compound I. Some of the metabolites isolated and characterized are hitherto not known. The effects of compound I on hepatic microsomal enzymes in vivo are also reported.

## Materials and Methods

**Chemicals.** 3-Methylcholanthrene (3-MC)[1], glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP⁺, methylcellulose, and Tris-HCl were supplied by Sigma Chemical Co. (St. Louis, MO). Phenobarbital (PB) was a generous gift from IDPL (Hyderabad, India).

**Synthetic Methods.** Piperitenone was synthesized as reported earlier (Nakanishi et al., 1980). 5,5-Dimethyl-2-(1-methylethylidene)-cyclohexanone (I) was synthesized as follows: Methyl magnesium iodide (in 60 ml of ether) prepared from dry magnesium turning (3.4 g) and methyl iodide (19.88 g) were taken in a round bottomed flask (250 ml) equipped with a sealed stirrer, a pressure equalizing funnel, and a reflux condenser. The contents of the flask were cooled to −27°C, 13.84 g of dried copper (I) chloride was added, and the mixture was stirred for 30 to 45 min at −27°C. Then with rapid stirring, a solution of piperitenone (15.0 g) in ether (75 ml) was added drop-wise. After the addition of piperitenone, stirring was continued for another period of 1 h at −27°C and overnight at room temperature. The reaction complex was

decomposed by adding 50 ml of cold saturated ammonium chloride solution with continued stirring and cooling, followed by 20 ml of 1 N hydrochloric acid to give a clear solution. The ethereal layer was separated and the aqueous phase was extracted with ether (100 ml × 2). The combined ether extract was dried over anhydrous sodium sulfate, and evaporated to a colorless liquid (90% yield). The compound was purified by column chromatography over neutral alumina using hexane as eluent. The purity of 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone used in this study was more than 99% on the basis of gas chromatography (GC) analysis. The compound has the following spectral characteristics. Relative front ($R_f$) 0.45 (system I), retention time ($R_t$) 13.1 min. Infrared (IR) spectrum (neat) showed absorptions at 1670 and 1610 cm⁻¹ (conjugated carbonyl group). Proton magnetic resonance (PMR) spectra (CDCl₃) were: δ 2.5 (t, 2H, J = 6.6 Hz, C-3 methylene protons), 2.28 (s, 2H, C-6 methylene protons), 1.99 (s, 3H, C-10 methyl protons), 1.72 (s, 3H, C-11 methyl protons), 1.52 (t, 2H, J = 6.8 Hz, C-4 protons), and 0.98 (s, 6H, C-7 and C-8 methyl protons). Mass spectra were: $m/z$ 166 (M⁺), 151 (M⁺-CH₃), 123 (M⁺-C₂H₃O), and 110 (M⁺-C₃H₄O). Compound I was converted to compound III following the procedure reported earlier (Bedoukian, 1948).

**Animals and Dosing.** Adult male rats (Wistar strain, 180–200 g) were used in these studies. For isolation of metabolites, 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone (I, 250 mg/kg of b.wt./day) was administered to rats (*n* = 20) once daily for 5 days by gastric intubation as a suspension in 1 ml of 1% methylcellulose solution. Control rats (*n* = 6) received only the vehicle. Control and experimental rats were housed separately in stainless steel metabolism cages with free access to food and water (laboratory animal food from Brooke Bond and Lipton, Bangalore, India). Urine was collected daily in bottles maintained at 0–4°C.

To evaluate the hepatotoxic potential of compound I and compare these values to *R*-(+)-pulegone or CCl₄-mediated toxicity, rats were treated with 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone (I, 250 mg/kg of b.wt.), *R*-(+)-pulegone (250 mg/kg of b.wt.), or CCl₄ (0.6 ml/kg of b.wt.) as a suspension in 0.3 ml of coconut oil. Control rats received only vehicle. Treatments were carried out i.p. Both control and experimental rats were housed separately in cages with free access to food and water. Animals were sacrificed by cervical dislocation 24 h after administration of test compounds.

In pretreatment experiments, rats were pretreated with PB (in 0.3 ml of 0.9% NaCl solution, 80 mg/kg of b.wt./day) or 3-MC (suspended in 0.3 ml of coconut oil, 40 mg/kg of b.wt./day) for 4 days before i.p. administration of compound I (250 mg/kg of b.wt.). However, at this dosage more than 50% of the PB-treated rats died within 24 h of treatment. PB and 3-MC were administered i.p. For serum glutamate pyruvate transaminase (SGPT) determinations, rats were sacrificed while under light anesthesia and blood was drawn from the heart by cardiac puncture.

PB-induced microsomes were prepared from livers of rats treated with PB (80 mg/kg of b.wt./day) for 4 days. The rats were sacrificed by cervical dislocation 24 h after the final dose and the livers were perfused with ice-cold 0.15 M KCl solution.

**Extraction of Urinary Metabolites.** The urine samples collected daily from control and experimental rats were adjusted to pH 4 to 5 with 1 N HCl and extracted separately with diethyl ether. Each day, ether extracts were pooled and stored at 0–4°C. The pooled ether extract was concentrated and extracted with 5% (w/v) aqueous NaHCO₃ to separate acidic and neutral metabolites. The bicarbonate phase acidified and extracted with ether to get the total acidic fraction. The ether extract, after removal of acidic metabolites, contained neutral metabolites.

**Preparation of Microsomes.** The livers were minced and homogenized in Tris-HCl buffer (0.05 M, pH 7.4) containing 0.25 M sucrose, and the microsomes were prepared as reported earlier (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 was estimated by the method of Lowry et al. (1951).

**Enzyme Assays.** Cytochrome P-450 (Omura and Sato, 1964), SGPT (Reitman and Frankel, 1957), glucose-6-phosphatase (Traiger and Plaa, 1971), and aminopyrine *N*-demethylase (Werringloer, 1978) were determined according to the published methods.

**Transformation In Vitro.** Microsomal protein (2 mg/ml) was incubated in the presence of 0.5 mM NADP⁺, 5.0 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 10 mM of MgCl₂, compound I (2 mM, in 50 μl

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TABLE 1

*Effect of various inhibitors on the transformation of I to III by PB-induced rat liver microsomes*

| Inhibitor | Concentration | Percentage of Inhibition |
|---|---|---|
| Control | ——— | 0.0 |
| CO | Bubbled for 2 min | 81* |
| Metyrapone | 1 mM | 74 |
| Piperinyl butoxide | 2 mM | 57 |
| SKF 525-A | 0.2 mM | 77 |
| Cyt. C | 5 $\mu$M | 86 |

* No inhibition was observed when the assay mixture was bubbled with $N_2$ for 1 to 2 min before the addition of compound I and NADPH.

100% activity (0.0% inhibition) represents 40 nmol of furan formed $mg^{-1}$ $h^{-1}$. The assays were carried out as described in the text.

of acetone), and 0.01 M Tris-HCl (pH 7.4) in a total volume of 5.0 ml. The reaction was initiated by the addition of NADPH-generating system, and incubated aerobically in a rotary shaker for 30 min at 37°C. At the end of the incubation period, the assay mixtures were cooled to 0–4°C, the protein was precipitated by adding 2.0 ml each of saturated $Ba(OH)_2$ and 0.25 M $ZnSO_4$ solution, and the mixture centrifuged (3000$g$). The supernatant was extracted with methylene chloride (20 ml × 3), concentrated, and an aliquot was subjected to GC and GC-mass spectroscopy (MS) analyses.

Effect of inhibitors on the transformation in vitro by PB-induced rat liver microsomes was determined as follows. Each inhibitor was preincubated with the microsomes at 37°C for 5 min, and then the assay was carried out as described above. Metyrapone, SKF 525-A, and piperonyl butoxide were added as a solution in 50 $\mu$l of acetone, and cytochrome $c$ in 100 $\mu$l of distilled water. The concentration of each inhibitor used is as mentioned in Table 1. In control assays only respective solvents were added and incubated at 37°C for 5 min before the assays were carried out. Carbon monoxide inhibition experiments were carried out by bubbling CO into the assay mixture for 1 to 2 min before the addition of compound I and NADPH-generating system. Similarly, $N_2$ was bubbled through the control assay mixture for 1 to 2 min.

**Binding Studies.** Binding studies were carried out in 50 mM potassium phosphate buffer (pH 7.4) with control and PB-induced rat liver microsomes (~3 mg/ml). Compound I (5–50 $\mu$M) dissolved in 1 to 10 $\mu$l of acetone was added to the sample cuvette and the same amount of acetone was added to the reference cuvette. The difference spectrum was recorded between 350 and 500 nm. A family of curves was obtained by adding increasing amounts of the compound I to the sample cuvette. Addition was continued until no additional increase in absorption occurred. The volume of acetone added was less than 1% of the final suspension. The extent of binding was measured by the difference in absorbance between the wavelengths of maximum and minimum absorption ($\Delta A_{max}$). The dissociation constant ($K_s$) was determined by plotting reciprocal $\Delta A_{max}$ versus reciprocal substrate concentration.

**Chromatographic Procedures.** Thin layer chromatography (TLC) was carried out on silica gel-G coated plates (0.25 mm for analytical, 0.75 mm for preparative TLC) developed with either hexane/ethyl acetate (system I, 90:10, v/v; system II, 75:25, v/v) or hexane/ethyl acetate/acetic acid (system III, 79:20:1, v/v) as the solvent systems. The compounds were visualized by spraying with 3% vanillin in 1% methanolic sulfuric acid followed by heating at 100°C for 5 to 10 min.
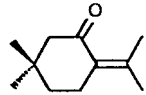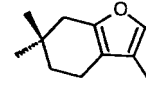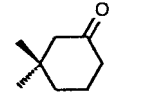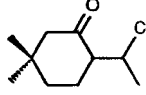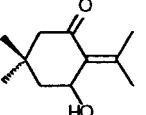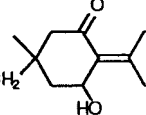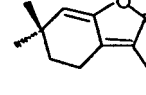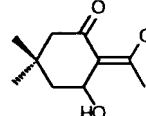
GC analyses were carried out on a Shimadzu model 14A instrument equipped with a hydrogen flame ionization detector and Shimadzu HR-1 wide bore capillary column (15-m × 0.5-mm diameter). Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. Initially, the column temperature was maintained at 60°C for 10 min, after which it was raised by 5°C/min to 150°C and maintained at 150°C for 5 min.

**Spectra.** IR spectra were recorded on a Perkin-Elmer model 781 spectrophotometer. Proton and $^{13}C$ NMR spectra were recorded on a JEOL FT-300 MHz spectrometer. Chemical shifts are reported in ppm, with respect to tetramethylsilane as the internal standard. MS analyses were performed on a JEOL-JMX-DX 303 instrument attached to a JMA-DA-5000 data system. GC-MS analyses were performed on a Shimadzu QP-2000 instrument. A capillary column ULBON HR-1 (50 m × 0.25 mm in diameter) was used for separation of metabolites. The column temperature was programmed from 100–250°C. The initial temperature was 100°C for 6 min and then heated at

TABLE 2

*GC analysis of the total urine extract*

Analysis was carried out as described in the text. Roman numbers represent the different metabolites. Metabolites **VIII** and **XIII** do not separate under the GC conditions used. The structure of the metabolites, their retention time, and percentage of composition are presented. Purified metabolites **VIII** and **XIII** have retention times of 28.1 and 28.0 min, respectively.

| Compound | Rt | Percentage of Composition of Total Urine Extract |
|---|---|---|
| | *min* | |
| I | 13.1 | 20.2 |
| III | 7.7 | 5.3 |
| VI | 1.7 | 2.4 |
| IX | 22.2 | 5.7 |
| X | 21.5 | 6.4 |
| XI | 23.8 | 5.4 |
| VIII / XIII | 28.0 | 47.1 |
| Benzoic acid (Endogenous compound) | | 2 |
| Unidentified (minor) metabolites | | 5.5 |

the rate of 10°C/min to 250°C. Helium was used as a carrier gas at a flow rate of 2 ml/min. High resolution mass spectral analyses (HRMS) were carried out using a VG Auto spec M mass spectrometer. Perfluorokerosene was used as internal reference and the data was acquired at 5000 resolution.

## Results

**Biotransformation of 5,5-Dimethyl-2-(1-methylethylidene)-cyclohexanone(I) In Vivo.** The composition of the total urine extract
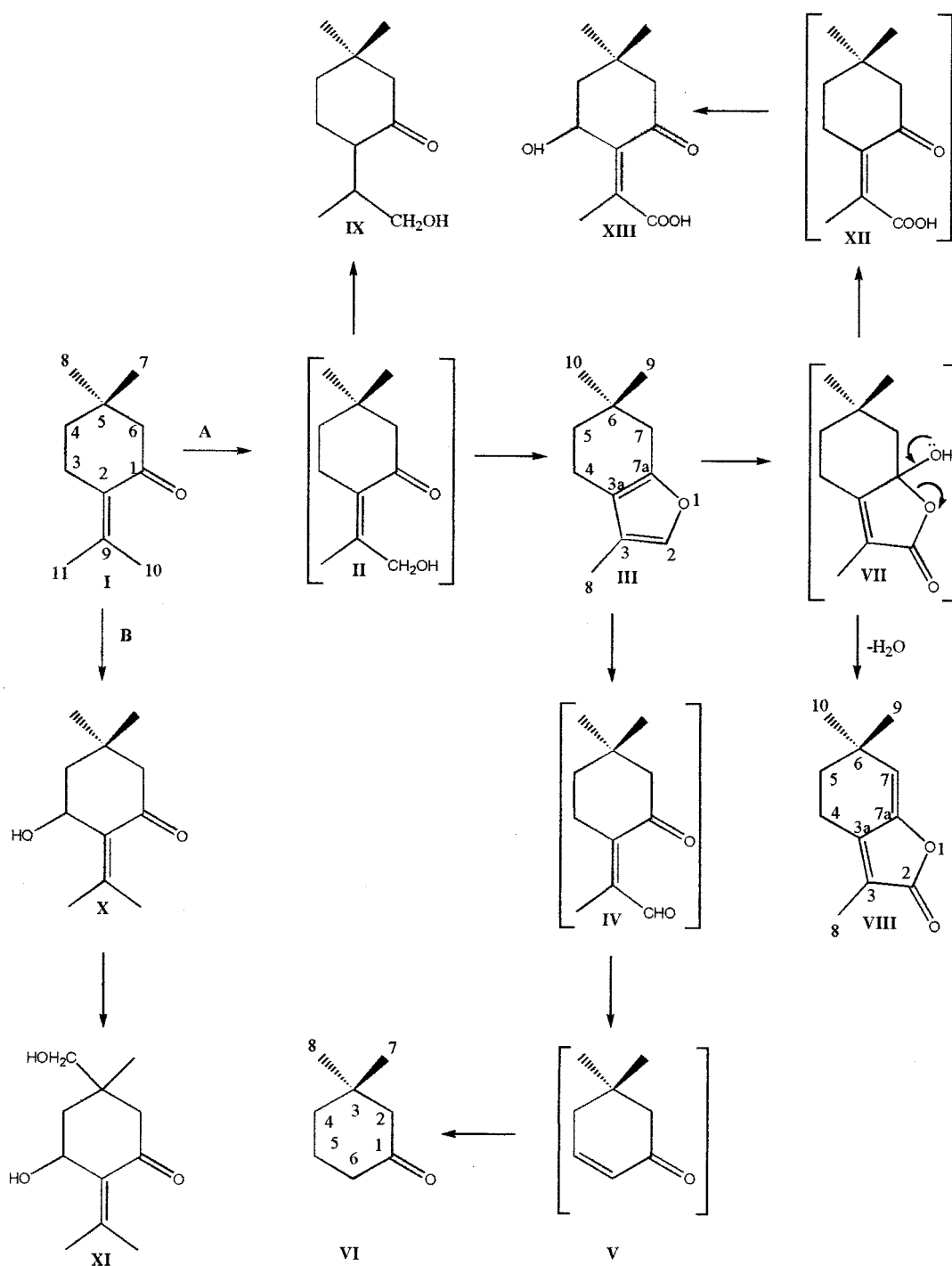
FIG. 1. *Probable metabolic pathways of 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone (I).*

A and B represent a major and a minor pathway, respectively.

(Table 2) was determined by GC analyses. The analyses showed the presence of eight peaks corresponding to metabolites III, VI, VIII, IX, X, XI, XIII, and unmetabolized compound I (Fig. 1). The peaks corresponding to these metabolites were enhanced when mixed with the purified metabolites isolated by column chromatography. GC profile also showed a couple of very minor peaks, which accounted for 5.5% of the total metabolites formed. Nearly 45% of the administered dose was excreted in the urine as metabolites and unmetabolized substrate. However, it should be noted here that both substrate

and its metabolites are volatile and a considerable amount must have been lost during extraction. Unmetabolized compound I was nearly 20% of the total urine extract. Metabolites VIII and XIII could not be separated under the GC conditions used.

TLC analysis (system I) of neutral fraction (0.9 g) showed the presence of at least seven compounds that were absent in control urine extract. The metabolites present in this fraction were separated by column chromatography over neutral alumina (50 g), and the column was initially eluted with pentane followed by hexane/ethyl acetate
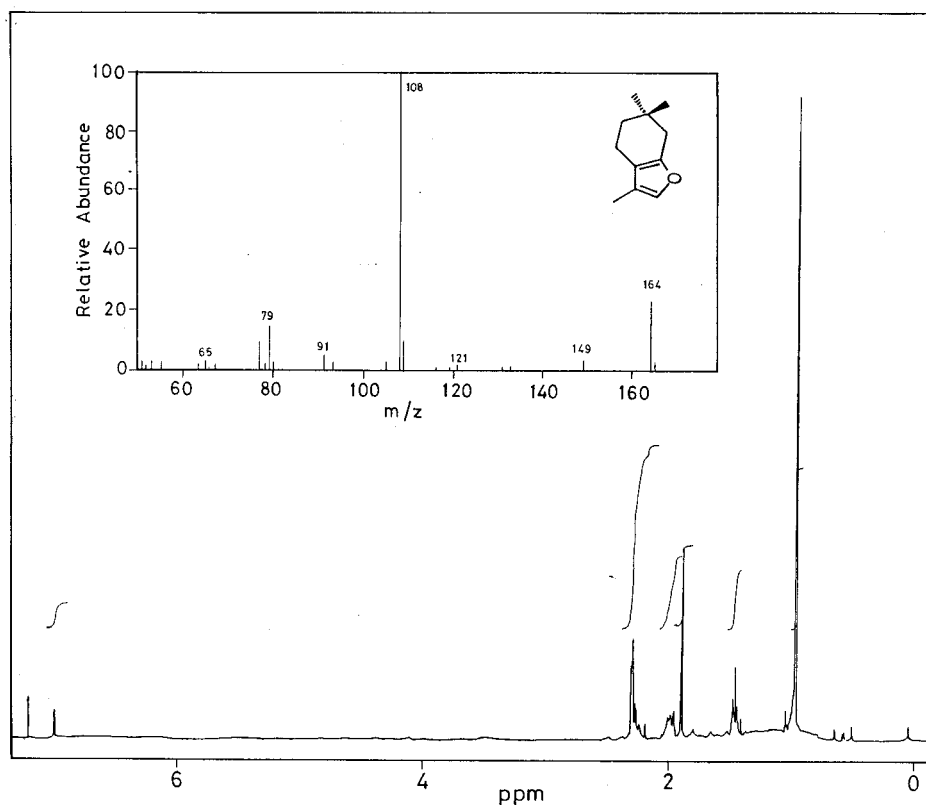
Fig. 2. *PMR spectra and electron impact mass spectra (inset) of 4,5,6,7-tetrahydro-3,6,6-trimethyl benzofuran (III).*

mixtures. Elution of the column with pentane yielded a fraction containing mainly one compound ($R_f$ 0.76, system I; $R_t$ 7.7 min). This compound had the following spectral characteristics. IR spectrum (neat) showed absorptions around 1630 and 1460 cm$^{-1}$ (characteristic of furan ring). PMR spectra (CDCl$_3$, Fig. 2) were: $\delta$ 7.0 (s, 1H, C-2 aromatic proton), 2.3 to 2.25 (m, 4H, C-4 and C-7 methylene protons), 1.9 (s, 3H, C-10 methyl protons), 1.5 (t, 2H, J = 3Hz, C-5 methylene protons), 0.99 (s, 6H, C-8 and C-9 methyl protons). MS (Fig. 2, inset) were: $m/z$ 164 (M$^+$), 149 (M$^+$-CH$_3$), 121 [M$^+$-(CO+CH$_3$)], and 108 (M$^+$-C$_4$H$_8$, base peak). The base peak at $m/z$ 108 is characteristic of the furanoid fragment (Pinder, 1977). Based on the spectral characteristics, the metabolite was identified as 4,5,6,7-tetrahydro-3,6,6-trimethyl benzofuran (III, Fig. 1). The structure assigned was also confirmed by synthesizing this compound following the reported procedure (Bedoukian, 1948) and comparing the PMR and mass spectrum of the synthetic compound with that of the isolated metabolite (III, Fig. 2).

Elution of the column with hexane gave a fraction containing unmetabolized compound I ($R_f$ 0.45, system I; $R_t$ 13.1 min). This was also confirmed by comparing its mass spectra with that of authentic compound I.

Additional elution of the column with hexane/ethyl acetate (9.5:0.5, v/v) yielded fractions containing mainly one compound ($R_f$ 0.35, system I; $R_t$ 1.77 min) that was purified by preparative TLC (system I). This compound had the following spectral characteristics. IR spectrum (neat) showed absorptions at 1720 cm$^{-1}$ (carbonyl group). PMR spectra (CDCl$_3$) were: $\delta$ 2.24 (t, 2H, J = 6.6Hz, C-6 protons), 2.12 (s, 2H, C-2 protons), 1.85 (q, 2H, C-5 protons), 1.55 (t, 2H, J = 5.7Hz, C-4 protons), and 0.95 (s, 6H, C-7 and C-8 methyl protons). MS spectra were: $m/z$ 126 (M$^+$), 111 (M$^+$-CH$_3$), 83 (M$^+$-C$_2$H$_3$O, base peak), and 69 (M$^+$-C$_3$H$_5$O). From the spectral data, the metabolite was identified as 3,3-dimethyl cyclohexanone (VI, Fig. 1). The

structure assigned was also confirmed by synthesizing this metabolite (VI) following the reported procedure (House and Wilkins, 1976) and then comparing its spectral data with those of the metabolite isolated (VI).

The metabolite with $R_f$ 0.52 (system II) and $R_t$ 21.5 min was eluted from the column with hexane/ethyl acetate (24:1, v/v). It was also purified by preparative TLC (system II), and the purified metabolite had the following spectral characteristics. IR spectrum (neat) showed absorptions around 3445 cm$^{-1}$ (hydroxyl group), 1677, and 1620 cm$^{-1}$ (conjugated carbonyl group). PMR spectra (CDCl$_3$, Fig. 3) were: $\delta$ 3.6 (2 d, 1H, J = 4.8Hz, C-3 proton), 2.35 (d, 2H, J = 15.6Hz, C-6 protons), 2.15 (d, 2H, J = 19.2Hz, C-4 protons), 1.95 (s, 3H, C-10 methyl protons), 1.75 (s, 3H, C-11 methyl protons), and 0.95 and 0.92 (2s, 6H, C-7 and C-8 methyl protons). Mass spectra (Fig. 3, inset) were: $m/z$ 182 (M$^+$), 164 (M$^+$-H$_2$O), 149 (M$^+$-H$_2$O-CH$_3$), 121 (M$^+$-CO-H$_2$O- CH$_3$), and 83 (M$^+$-C$_6$H$_{11}$O). HRMS found M$^+$, 182.1304; C$_{11}$H$_{18}$O$_2$ requires 182.1306. From the spectral data, the metabolite was identified as 5,5-dimethyl-3-hydroxy-2-(1-methylethylidene)-cyclohexanone (X, Fig. 1).

Elution of the column with hexane/ethyl acetate (47:3, v/v) gave a fraction containing mostly one compound ($R_f$ 0.48, system II; $R_t$ 22.2 min), which was additionally purified by preparative TLC (system II). This compound had the following spectral characteristics. IR spectrum (neat) indicated the presence of a hydroxyl (3450 cm$^{-1}$) and carbonyl (1706 cm$^{-1}$) groups. PMR spectrum (CDCl$_3$, Fig. 4) were: $\delta$ 3.3 (d, 2H, J = 3.3 Hz, C-10 hydroxy methyl protons), 2.3 to 1.5 (m, 8H, ring methylene protons), 0.85 (d, 3H, J = 7.8 Hz, C-11 methyl protons), and 0.81 (s, 6H, C-7 and C-8 methyl protons). Mass spectra (Fig. 4, inset) were: $m/z$ 184 (M$^+$), 169 (M$^+$-CH$_3$), 153 (M$^+$-CH$_3$-H$_2$O), 142 (M$^+$-C$_3$H$_6$), 111 (M$^+$-C$_5$H$_{13}$), and 69 (base peak). HRMS: C$_{11}$H$_{20}$O$_2$ requires 184.1463, found M$^+$, 184.1470. Based on these
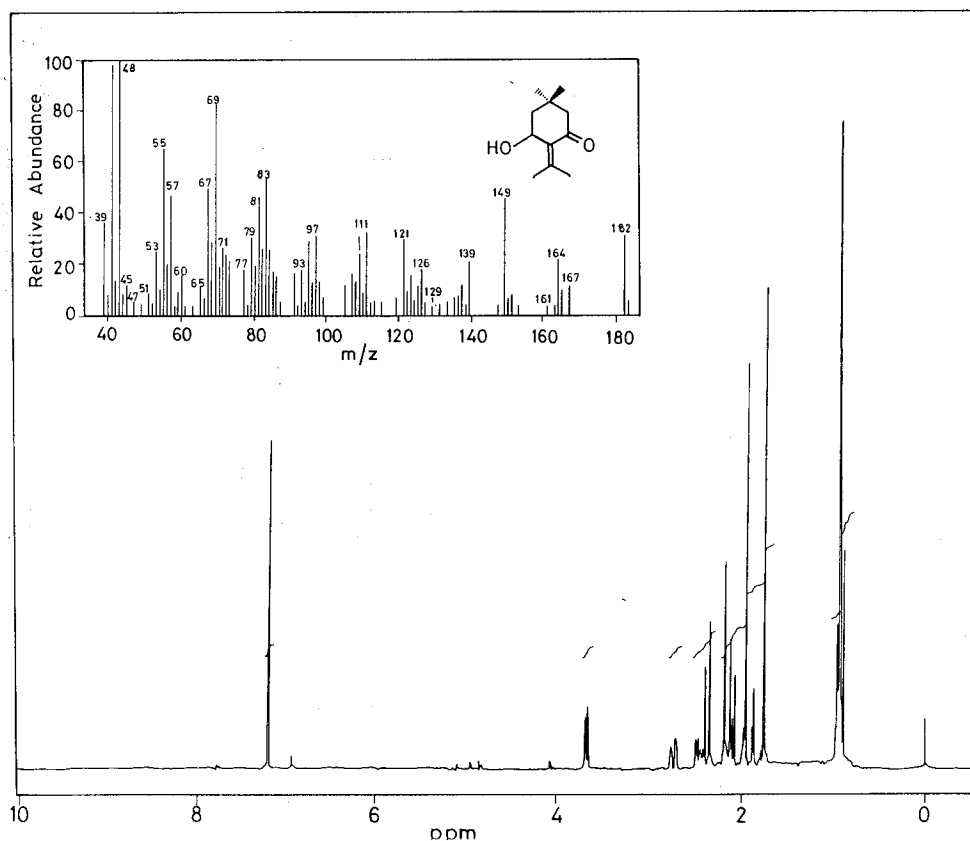
FIG. 3. *PMR spectra and electron impact mass spectra (inset) of 5,5-dimethyl-3-hydroxy-2-(1-methylethylidene)-cyclohexanone (X).*

spectral characteristics the metabolite was identified as 5,5-dimethyl-2-(1-hydroxymethylethyl)-cyclohexanone (IX, Fig. 1).

One of the neutral metabolites with $R_f$ 0.4 (system II) and $R_t$ 23.83 min was eluted with 9:1 hexane/ethyl acetate and had the following spectral characteristics. IR spectrum (neat) indicated the presence of hydroxyl group (3440 cm$^{-1}$) and conjugated carbonyl group (1668 and 1620 cm$^{-1}$). PMR spectra (CDCl$_3$, Fig. 5) were: δ 3.33 (m, 3H, hydroxy methyl attached to C-5 and C-3 methylene protons), 2.34 (2s, 2H, C-6 methylene protons), 2.17δ and 2.07 (2 d, 2H, J = 1.5 Hz, C-4 protons), 1.96 (s, 3H, C-10 methyl protons), 1.73 (s, 3H, C-11 methyl protons), and 0.93 (s, 3H, methyl protons at C-5). Mass spectra (Fig. 5, inset) were: $m/z$ 198 (M$^+$), 182 (M$^+$-O), 162 (M$^+$-2H$_2$O), 151 (M$^+$-CH$_3$O$_2$), 135 (M$^+$-C$_2$H$_7$O$_2$), 121 (M$^+$-C$_3$H$_9$O$_2$), 109 (M$^+$-C$_4$H$_9$O$_2$), and 69 (base peak). HRMS: found (M$^+$-16), 182.1301; C$_{11}$H$_{18}$O$_2$ requires 182.1306. The mass spectra of metabolite XI (Fig. 5, inset) showed that the abundance of molecular ion (M$^+$) was extremely low hence (M$^+$-O) ion was considered for HRMS analysis. Based on these spectral characteristics, the compound was assigned the structure 3-hydroxy-5-hydroxymethyl-5-methyl-2-(1-methylethylidene)-cyclohexanone (XI, Fig. 1).

Additional elution of the column with 17:3 hexane/ethyl acetate gave a fraction, which upon TLC analysis (system II) showed the presence of a compound ($R_f$ 0.38, system II; $R_t$ 28.0 min) along with some minor impurities. The compound ($R_f$ 0.38) was additionally purified by preparative TLC (system II) and it had the following spectral characteristics. IR spectrum (neat) indicated the presence of a conjugated carbonyl group (1682 and 1620 cm$^{-1}$). PMR spectrum (CDCl$_3$, Fig. 6) were: δ 5.34 (s, 1H, C-7 olefinic proton), 2.5 (t, 2H, J = 6.6 Hz, C-4 protons), 1.78 (s, 3H, C-10 methyl protons), 1.64 (t, 2H, J = 7.2 Hz, C-5 protons), and 1.09 (s, 6H, C-8 and C-9 methyl

protons). $^{13}$C NMR spectrum (75 MHz, CDCl$_3$, Fig. 7) were: δ 188.5 (C$_2$), 140.9 (C$_{7a}$), 135.4 (C$_{3a}$), 124.3 (C$_3$), 119.3 (C$_7$), 37.2 (C$_4$), 32.6 (C$_6$), 28.9 (C$_9$ and C$_{10}$), 19.6 (C$_5$), and 8.2 (C$_8$). Mass spectra (Fig. 6, inset) were: $m/z$ 178 (M$^+$), 162 (M$^+$-O), 147 (M$^+$-CH$_3$O), 134 (M$^+$-CO$_2$), and 107 (M$^+$-C$_3$H$_3$O$_2$). Based on all these spectral characteristics, the metabolite was identified as 5,6-dihydro-3,6,6-trimethyl-2(4H)-benzofuranone (VIII, Fig. 1).

**Acidic Metabolites.** Examination of the acidic fraction by TLC (system III) showed one compound ($R_f$ 0.49 system III) that is not present in control urine extract. The fraction (0.8 g) was chromatographed on a silica gel column (40 g), and the metabolite corresponding to compound $R_f$ 0.49 (system III) was eluted with 19:1 hexane/ethyl acetate. The metabolite had the following spectral characteristics. IR spectrum (neat) showed broad absorption around 3464 cm$^{-1}$ (acid hydroxyl group) and at 1706 and 1630 cm$^{-1}$ (conjugated carbonyl group). PMR spectrum (CDCl$_3$ Fig. 8) were: δ 6.95 (s, 1H, acidic proton), 3.45 (t, H, J = 5.7 Hz, C-5 proton), 2.19 (s, 3H, C-11 methyl protons), 1.6 to 2.7 (m, 4H. ring methylene protons), and 1.14 and 1.08 (2s, 6H, C-7 and C-8 methyl protons). Mass spectra (Fig. 8, inset) were: $m/z$ 212 (M$^+$), 196 (M$^+$-O), 178 (M$^+$-H$_2$O$_2$), 151 (M$^+$-CO$_3$H), and 136 (M$^+$-C$_2$H$_4$O$_3$). Based on the spectral characteristics, the compound was tentatively identified as 5,5-dimethyl-3-hydroxy-2-(1-carboxyethylidene)-cyclohexanone (XIII, Fig. 1).

**Transformation of Compound I by PB-Induced Rat Liver Microsomes.** PB-induced rat liver microsomes were incubated aerobically with compound I in the presence of NADPH as described under *Materials and Methods*. The methylene chloride extract of the assay mixture on TLC (system I) and GC analyses indicated the presence of only one metabolite ($R_f$ 0.76, system I; $R_t$ 7.7 min), which corresponded to that of 4,5,6,7-tetrahydro-3,6,6-trimethyl benzofuran (III,
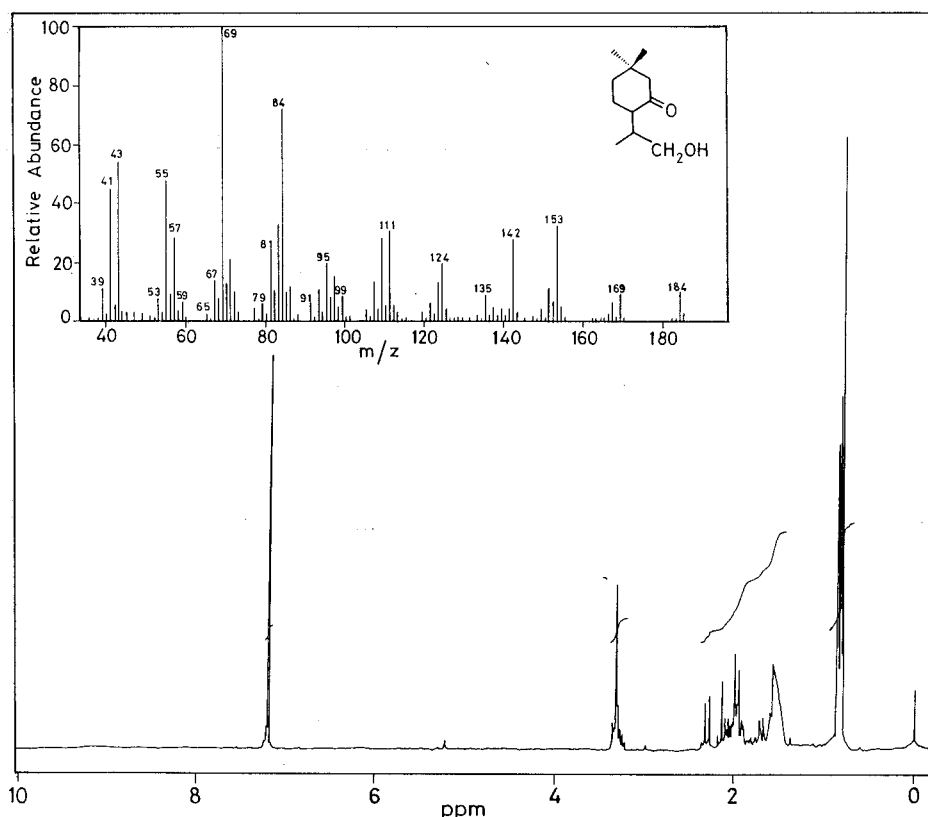
FIG. 4. *PMR spectra and electron impact mass spectra (inset) of 5,5-dimethyl-2-(1-hydroxy methylethyl)-cyclohexanone (IX).*

Fig. 1). The identity of the metabolite formed (III) was also confirmed by comparing its mass spectral analysis with that of compound III isolated from the urine extract and compound III prepared following the reported procedure (Bedoukian, 1948) [$m/z$ 164 (M$^+$), 149 (M$^+$-CH$_3$), 121 (M$^+$-CO-CH$_3$), and 108 (M$^+$-C$_4$H$_8$, base peak, characteristic of a furan ring)]. The rate of compound III formed was shown to be 40 nmol mg$^{-1}$ h$^{-1}$. NADH could not support the enzymatic conversion of compound I to III. The transformation of compound I to compound III was significantly greater (40 nmol mg$^{-1}$h$^{-1}$) in microsomes obtained from PB-pretreated rats than in control microsomes (20.2 nmol mg$^{-1}$h$^{-1}$). However, the formation of compound III was not observed when 3-MC-treated microsomes were used. The transformation of I to III is initiated by the regiospecific hydroxylation (C-10 hydroxylation) of I to its 10-hydroxy compound, which is highly unstable and so spontaneously undergoes cyclization followed by dehydration to a furanoterpene (III, Fig. 1).

**Inhibitor Studies.** Table 1 shows the effect of various inhibitors on the transformation of I to III (C-10 hydroxylation) by PB-pretreated rat liver microsomes. Carbon monoxide bubbled for 2 min resulted in the loss of 81% of the original activity. SKF 525-A seems to be a better inhibitor of the 10-hydroxylase than is metyrapone. SKF 525-A at a concentration of 0.2 mM inhibited the reaction to the extent of 77% whereas metyrapone inhibited to nearly the same extent (74%, Table 1) at a concentration of 1.0 mM. Both cytochrome *c* and piperinyl butoxide inhibited the hydroxylase activity 86 and 57%, respectively (Table 1).

**Binding Studies.** Compound I elicited type I binding spectrum ($\lambda_{max}$ 385–388 nm, $\lambda_{min}$ 420–423 nm) with control and PB-induced microsomes whereas with 3-MC-induced microsomes it produced a reverse type I difference spectrum ($\lambda_{max}$ 420–425 nm, $\lambda_{min}$ 385–390 nm). The value of spectral dissociation constant ($K_s$) calculated from the double reciprocal plot (as mentioned under *Materials and Meth-*

*ods*) for this compound with control and PB-induced microsomes was 62.5 and 38.5 $\mu$M, respectively. The binding affinity of compound I toward PB-induced microsomes is comparatively higher than with control microsomes.

**Effect of Compound I, *R*-(+)-Pulegone, and Carbon Tetrachloride on Hepatic Microsomal Enzymes.** A number of liver microsomal enzymes and SGPT levels were studied 24 h after the i.p. administration of 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone (I) to rats. The effects of compound I on the hepatic microsomal enzymes were compared with the effects observed after a single dose of i.p. administration of known hepatotoxins viz. *R*-(+)-pulegone (250 mg/kg of b.wt.) and CCl$_4$ (0.6 ml/kg of b.wt.) to rats. The results are summarized in Table 3. These studies were carried out 24 h after the treatment with test compounds.

Consistent with the earlier reports (Boyd et al., 1980; Mannering et al., 1981; Plaa, 1981; Gordon et al., 1982; Moorthy et al., 1989b, 1991), it was observed that i.p. administration of a single dose of *R*-(+)-pulegone or CCl$_4$ to rats caused marked decrease in microsomal cytochrome P-450, aminopyrine *N*-demethylase, and glucose-6-phosphatase activities (Table 3). During this period, a significant increase in SGPT level was also observed (Table 3). The effects of i.p. administration of a single dose of compound I resulted in 26, 23, and 41% decrease in cytochrome P-450, glucose-6-phosphatase, and aminopyrine *N*-demethylase activities, respectively, after 24 h of administration (Table 3). An 11-fold increase in SGPT level was also observed (Table 3). However, the decrease in the level of cytochrome P-450 and glucose-6-phosphatase and increase in SGPT value after the administration of test compounds were considerably more in the case of *R*-(+)-pulegone and CCl$_4$ than with compound I (Table 3), whereas aminopyrine *N*-demethylase was affected to the same extent with all the compounds tested during this period (Table 3). There was
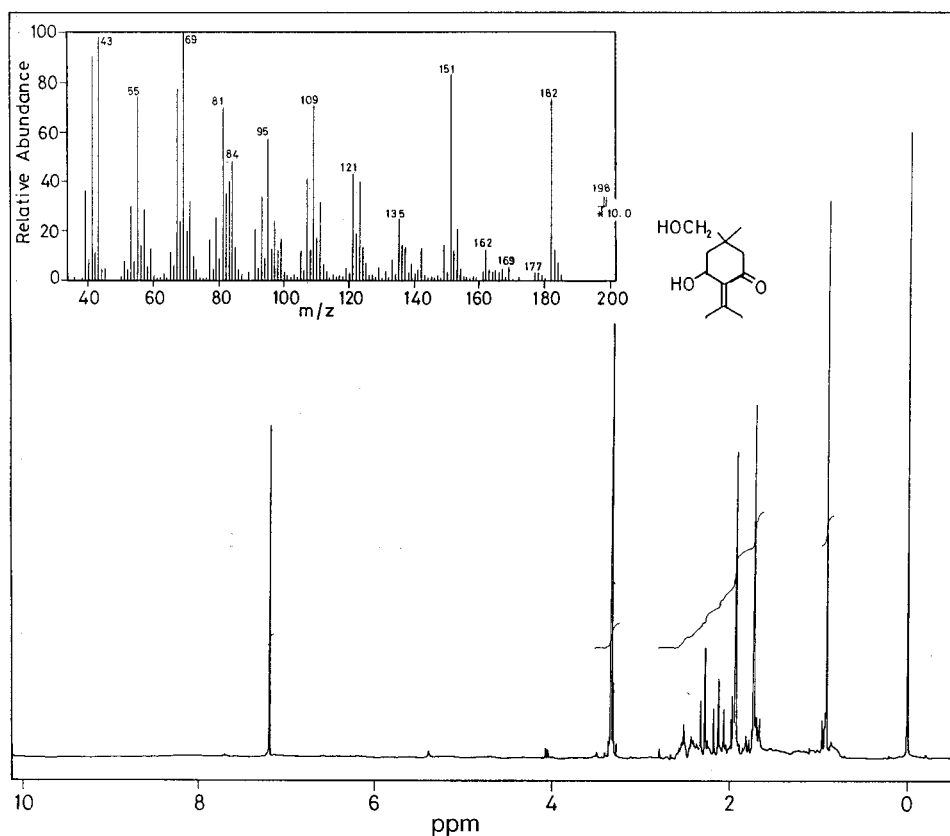
FIG. 5. *PMR spectra and electron impact mass spectra (inset) of 3-hydroxy-5-hydroxymethyl-5-methyl-2-(1-methylethylidene)-cyclohexanone (XI).*

no significant change in the level of cytochrome $b_5$ after the administration of compound I (data not shown).

**Pretreatment Studies.** Pretreatment of rats with PB for 4 days before i.p. administration (250 mg/kg of b.wt.) of compound I potentiated the hepatotoxicity caused by I (Table 4). Compound I caused 58, 49, and 48% decrease in the levels of cytochrome P-450, glucose-6-phosphatase, and aminopyrine *N*-demethylase in PB-treated rats as against 26, 23, and 40% in untreated rats, respectively (Table 4). It was also noticed that SGPT was 23-fold higher than the control when compound I was administered to PB-treated rats. Contrary to these observations, pretreatments of rats with 3-MC resulted in complete protection from the toxicity mediated by compound I. Administration of compound I to 3-MC pretreated rats did not alter significantly cytochrome P-450, glucose-6-phosphatase, and aminopyrine *N*-demethylase (Table 4). There was not any noticeable change in the level of SGPT as compared with that of rats treated with 3-MC alone (Table 4).

### Discussion

The purpose of this study was to probe further the significance of a chiral center at C-5 in *R*-(+)-pulegone-mediated toxicity. To evaluate this aspect, 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone (I) was chosen as the test compound because it is structurally similar to the potent hepatotoxin, *R*-(+)-pulegone except that the C-5 hydrogen in pulegone is substituted by a methyl group with the result that it has lost its chirality. This structural modification has blocked one of the major metabolic pathways of *R*-(+)-pulegone initiated through hydroxylation at the C-5 position (Madyastha and Raj, 1993).

This study represents the characterization of various metabolites isolated from the urine of the rats dosed with I. The identification of these metabolites has provided not only a basis for understanding the

significance of two major pathways, viz. C-5 and C-9 hydroxylation pathways involved in the metabolism of pulegone, but also indicated the possible mode of metabolism if the C-5 hydroxylation pathway is blocked through structural modification. It can be inferred from this study that compound I is essentially metabolized following a major pathway A (Fig. 1). However, the proposed pathway is speculative, but it is supported by in vitro study, precedence from the early work on metabolism of *R*-(+)-pulegone, and chemical logic. As expected, the major pathway is initiated through the regiospecific oxidation of the methyl group *syn* to the carbonyl group resulting in the formation of ω-hydroxylated derivative (II, Fig. 1). It is interesting to note that most of the metabolites (75–80% of the total metabolites formed) isolated appear to have been derived from a common intermediate, viz. 10-hydroxy compound (II, Fig. 1). The metabolite II readily undergoes intramolecular cyclization followed by dehydration to yield a furanoterpene (III, Fig. 1) in a manner analogous to the formation of *R*-(+)-menthofuran (Gordon et al., 1987; Madyastha and Raj, 1990), *S*-(−)-menthofuran (Madyastha and Gaikwad, 1998) and 6,7-dehydromenthofuran (Madyastha and Gaikwad, 1999) from *R*-(+)-pulegone, *S*-(−)-pulegone, and piperitenone, respectively. In support of this pathway, it was demonstrated that PB-induced rat liver microsomes convert I to a furanoterpene (III, Fig. 1) in the presence of NADPH and air, presumably through the intermediacy of II (Fig. 1). It was also noticed that the transformation of I to III was significantly higher with PB-induced than control microsomes. Formation of furanoterpene (III) from I was inhibited to a significant extent by reagents, which are recognized inhibitors of the reactions mediated by the cytochrome P-450 system (Table 1). All these observations clearly suggest that the major pathway A (Fig. 1) initiated through regiospecific hydroxylation of I is catalyzed by the liver microsomal cytochrome P-450 system. In support of this assumption, it was demon-
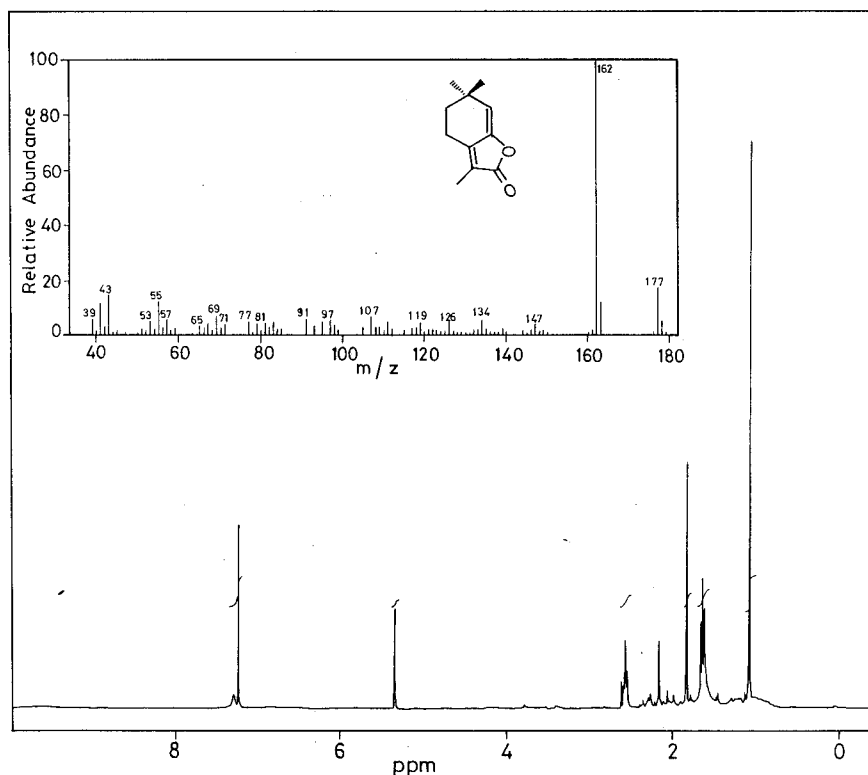
FIG. 6. *PMR spectra and electron impact mass spectra (inset) of 5,6-dihydro-3,6,6-trimethyl-2(4H)-benzofuranone (VIII).*
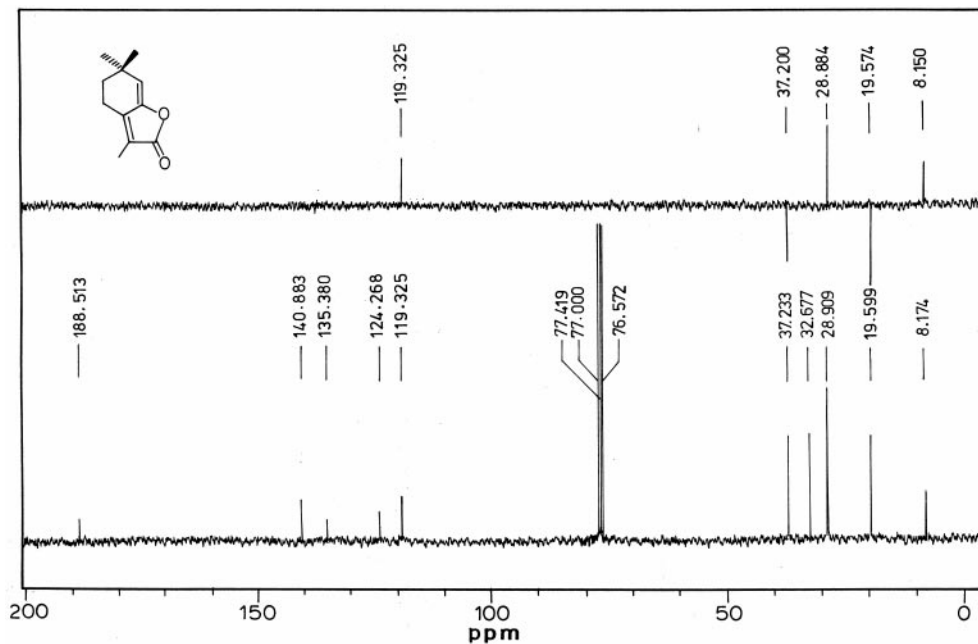


FIG. 7. *$^{13}C$ NMR spectra of 5,6-dihydro-3,6,6-trimethyl-2(4H)-benzofuranone (VIII).*

strated earlier that the microsomal cytochrome P-450 system carries out the regiospecific oxidation of $R$-(+)-pulegone to its allylic alcohol (9-hydroxypulegone) where the hydroxyl and the keto groups are *syn* to each other. The allylic alcohol readily undergoes intramolecular cyclization followed by dehydration to menthofuran, a furanoterpene (Gordon et al., 1987; McClanahan et al., 1989; Madyastha and Raj, 1990). A similar sequence of reactions has also been shown to be involved in the transformation of piperitenone to 6,7-dehydromentho-

furan (Madyastha and Gaikwad, 1999). In this study it is reasonable to assume that the allylic alcohol (II, Fig. 1) formed from I gets converted to a furanoterpene (III, Fig. 1) in a manner analogous to the formation of menthofuran and 6,7-dehydromenthofuran from $R$-(+)-pulegone and piperitenone, respectively. However, II could not be isolated from both in vivo and in vitro experiments because it readily undergoes intramolecular cyclization to a hemiketal. A similar observation was made earlier during the enzymatic conversion of $R$-(+)-
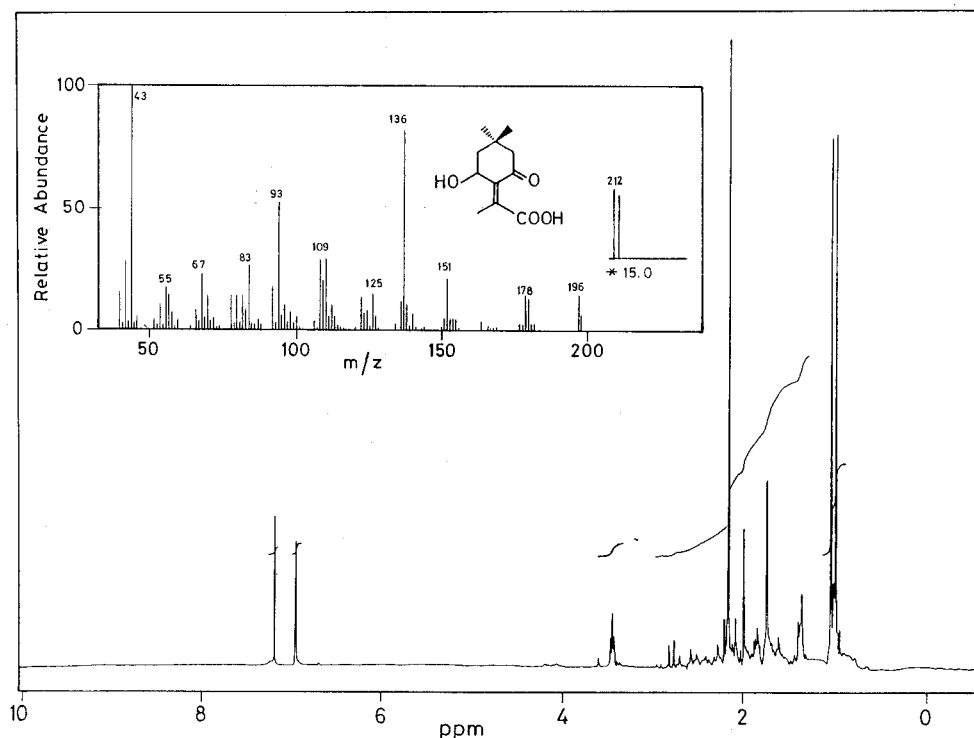
FIG. 8. *PMR spectra and electron impact mass spectra (inset) of 5,5-dimethyl-3-hydroxy-2-(1-carboxyethylidene)-cyclohexanone (XIII).*

TABLE 3

*Effect of i.p. administration of a single dose of 5,5-dimethyl-2-(1-methylethylidene)-cyclohexanone (I, 250 mg/kg), R-(+)-pulegone (250 mg/kg), and CCl$_4$ (0.6 ml/kg) on rat liver microsomal enzymes and SGPT 24 h after the treatment*

| Enzyme Activity | Control[a] | Test Compounds | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | Compound I | Percentage of change over control | R-(+)-pulegone | Percentage of change over control | CCl$_4$ | Percentage of change over control |
| Cytochrome P-450 (nmol/ mg of protein) | 0.50 ± 0.01 | 0.37 ± 0.02 | 25.80 (−) | 0.28 ± 0.06 | 44.00 (−) | 0.205 ± 0.05 | 59.00 (−) |
| Glucose 6-phosphatase (nmol pi/min/ mg of protein) | 226.90 ± 20.6 | 174.00 ± 11.25 | 23.30 (−) | 131.25 ± 22.5 | 42.20 (−) | 133.10 ± 1.9 | 41.00 (−) |
| Aminopyrine N-demethylase (nmol HCHO/ min/mg of protein) | 6.40 ± 0.9 | 3.80 ± 0.2 | 40.60 (−) | 3.75 ± 0.5 | 41.00 (−) | 3.90 ± 0.3 | 39.00 (−) |
| SGPT (U/ml) | 31.15 ± 3.15 | 378.70 ± 1.4 | 11.20[†] (+) | 639.50 ± 4.5 | 19.50[†] (+) | 488.00 ± 7 | 14.70[†](+) |

[a] Animals treated with vehicle alone. Animals were sacrificed 24 h after the administration of test compounds. Values represent mean ± S.D. of three independent experiments consisting of tissues pooled from six rats. (−) indicates percent decrease over control and (+) indicates percent increase over control.
[†], Values represent number of fold change over control. Other details of the experiment are as mentioned in *Materials and Methods.*

pulegone to menthofuran via the intermediacy of 9-hydroxy pulegone (Gordon et al., 1987; Madyastha and Raj, 1990). When the exocyclic double bond in II is reduced as in IX (Fig. 1), the compound does not get cyclized, hence we could isolate IX from the urine extract as a minor metabolite possibly derived from II.

It is quite possible that the furanoterpene (III) formed could be transformed into an hydroxylactone (VII), which can either dehydrate to produce an unsaturated lactone (VIII, Fig. 1) or opening of the hydroxylactone (VII) as shown in Fig. 1 followed by allylic hydroxylation could yield the metabolite XIII (Fig. 1). The first step in this sequence of reactions (III → VII, Fig. 1) could be nonenzymatic. In support of this proposed catabolic sequence (Fig. 1), it has been reported earlier that lower furanoterpenes (Kubota, 1969) and furanoeremophilanes (Pinder, 1977) easily get transformed into their

corresponding hydroxylactone or unsaturated lactones either in the presence of a mild oxidizing agent or by auto-oxidation. This facile transformation is normally used in structure determination of furanoterpenes (Novotny and Sörm, 1965). It appears that the furanoterpene III is the major metabolite of I although a significant portion of it gets further transformed to metabolites VIII and XIII (Fig. 1). In fact, metabolites III, VIII, and XIII together constitute nearly 67% of the total metabolites formed (Table 2).

Studies carried out earlier both in vivo and in vitro have demonstrated that R-(+)-pulegone, S-(−)-pulegone, and menthofuran get converted to an α, β-unsaturated-γ-ketoaldehyde (McClanahan et al., 1989; Madyastha and Raj, 1990, 1992; Madyastha and Gaikwad, 1998). These studies have indicated the possible involvement of liver microsomal cytochrome P-450 in this transformation and the conver-

TABLE 4

*Effect of a single dose of 5,5-dimethy1-2-(1-methyl ethylidene)-cyclohexanone (I) administered i.p. to rats pretreated with PB or 3-MC*

| Enzyme Activity Tested | PB-Control | PB + Compound I | Percentage of Change over Control | 3-MC-Control | 3-MC + Compound I | Percentage of Change over Control |
|---|---|---|---|---|---|---|
| Cytochrome P-450 (nmol/mg of protein) | $1.34 \pm 0.02$ | $0.57 \pm 0.01$ | $57.6\ (-)$ | $1.33 \pm 0.02$ | $1.19 \pm 0.08$ | $10.5\ (-)$ |
| Glucose 6-phosphatase (nmol pi/min/mg of protein) | $202.75 \pm 15$ | $105 \pm 3.75$ | $48.5(-)$ | $129.37 \pm 5.62$ | $111.25 \pm 1.25$ | $14.2\ (-)$ |
| Aminopyrine $N$-demethylase (nmol HCHO/ min/ mg of protein) | $7.88 \pm 0.07$ | $4.08 \pm 0.08$ | $48.2\ (-)$ | $5.38 \pm 0.3$ | $4.75 \pm 0.25$ | $11.7\ (-)$ |
| SGPT(U/ml) | $37.95 \pm 1.05$ | $914.5 \pm 18.5$ | $23.1^{\dagger}(+)$ | $25.9 \pm 2.1$ | $30.8 \pm 0.7$ | $0.2^{\dagger}(+)$ |

[†], Represents number of fold change over control.

Methods of pretreatment are described under *Materials and Methods*. Compound I (250 mg/Kg bw) was administered i.p. to PB- and 3-MC-pretreated rats. More than 50% of the PB-treated rats died within 24 h whereas in the case of 3-MC-treated rats, the survival rate was 100% after the administration of the compound I. Animals were sacrificed 24 h after the administration of compound I. Values represent mean $\pm$ S.D. of three independent experiments consisting of tissues pooled from six rats. $(-)$ indicates percent decrease over control and $(+)$ indicates percent increase over control.

sion is possibly through the intermediacy of an epoxide. So it is quite possible that the transformation of III to IV (Fig. 1) may take place in a manner analogous to the formation of $\alpha$, $\beta$-unsaturated-$\gamma$-ketoaldehyde from $R$-(+)-pulegone and menthofuran. Additional metabolism of this unsaturated ketoaldehyde results in the formation of 4-methyl-2-cyclohexenone, which also gets transformed to $p$-cresol, one of the major toxic metabolites of pulegone and menthofuran (Madyastha and Raj, 1991, 1992, 1993; Madyastha and Gaikwad, 1998). The sequence of reactions involved in this transformation appears to be reductive elimination of the keto group in $\alpha$, $\beta$-unsaturated-$\gamma$-ketoaldehyde and hydration of the exocyclic double bond followed by a retro-aldol type reaction, which could easily yield 4-methyl-2-cyclohexanone. In this investigation, it has been noticed that the metabolism of compound I does not yield $p$-cresol as one of the metabolites, suggesting that the $\alpha$, $\beta$-unsaturated-$\gamma$-ketoaldehyde (IV, Fig. 1) is not subjected to the above sequence of reactions. However, this study has demonstrated that 3,3-dimethylcyclohexanone (VI, Fig. 1) is one of the minor metabolites of I. This minor metabolite (VI) could have been formed as a consequence of nonenzymatic cleavage of the exocyclic double bond in IV followed by reduction as shown in the Fig. 1. Similar nonenzymatic cleavage has been proposed earlier for the formation of 5-methyl-2-cyclohexenone and 3-methylcyclohexanone from an $\alpha$, $\beta$-unsaturated-$\gamma$-ketoaldehyde derived from menthofuran (Madyastha and Raj, 1992).

The minor pathway B (Fig. 1) is involved in the formation of metabolites X and XI. These minor metabolites could have been formed through allylic hydroxylation of I followed by methyl oxidation. It is quite possible that metabolites formed via the major pathway (A, Fig. 1) may be mostly responsible for the observed toxicity mediated by I. The major metabolites formed along this pathway are furanoterpene (III) and a lactone (VIII). In fact, menthofuran, which is structurally very similar to metabolite III, is a potent hepatotoxin (Madyastha and Raj, 1994). Many sesquiterpene lactones with an $\alpha$, $\beta$-unsaturated-$\gamma$-lactone structural unit as seen in metabolite VIII, have been shown to possess significant antitumor and cytotoxic activities (Rodriguez et al., 1976).

In this study we have compared the hepatotoxic potential of compound I with known hepatotoxins such as $R$-(+)-pulegone and $CCl_4$. It is to be noted that one cannot draw any real conclusion from the relative toxicity studies without obtaining kinetic data for the metabolism of $R$-(+)-pulegone and its analog (I). However, these are preliminary studies that point out that compound I is significantly less hepatotoxic than $R$-(+)-pulegone and $CCl_4$. This study has demonstrated that compound I causes considerable hepatotoxicity in rats as evidenced by a 11-fold increase in SGPT level and a 23% loss of glucose-6-phosphatase 24 h after a single dose of I (Table 3). In addition, decrease in cytochrome P-450 as well as aminopyrine $N$-demethylase was also observed (Table 3), indicating that compound I interferes with cytochrome P-450-mediated metabolism possibly through its destruction. Increased levels of SGPT as well as glucose-6-phosphatase indicate possible hepatic injury caused by compound I. Earlier it was reported that rats treated with hepatotoxic agents such as $CCl_4$ showed elevated SGPT levels as well as extensive centrilobular necrosis (Balazs et al., 1961). However, the increased levels of SGPT as well as decreased levels of glucose-6-phosphatase observed in the case of compound I are significantly lower than these values obtained from rats treated with hepatotoxic agents such as $R$-(+)-pulegone or $CCl_4$ (Table 3). This suggests that compound I is not as potent a hepatotoxin as $R$-(+)-pulegone or $CCl_4$. It appears that substitution of C-5 hydrogen in $R$-(+)-pulegone with a methyl group considerably decreases its hepatotoxic potential. This could possibly be due to the fact that in such a compound, C-5 hydroxylation is not possible. As a consequence of this, compound I is predominantly metabolized following a pathway initiated through regiospecific hydroxylation of a methyl *syn* to the carbonyl, resulting in the formation of a furanoterpene (III) as shown in Fig. 1. In fact, most of the metabolites isolated and characterized in this study appear to have been derived from this metabolite (III). Our studies point out that metabolites derived through the C-5 hydroxylation pathway involved in the biotransformation of $R$-(+)-pulegone play a considerable role in eliciting $R$-(+)-pulegone-mediated toxicity.

Pretreatment of rats with PB, a known inducer of cytochrome P-450, before administration of I, resulted in potentiation of hepatotoxicity as evidenced by a significant increase in SGPT levels, whereas pretreatment with 3-MC protected from it (Table 4). It appears that PB-induced cytochrome P-450-catalyzed reactive metabolite(s) may be responsible for the toxicity caused by compound I. Our results also suggest that the hepatotoxic potential of $R$-(+)-pulegone can be reduced considerably if the C-5 hydroxylation pathway involved in its metabolism is blocked through structural modification. It appears that the C-5 chiral center contributes substantially to $R$-(+)-pulegone-mediated toxicity.

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