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EFFECT OF RING SIZE IN *R*-(+)-PULEGONE-MEDIATED HEPATOTOXICITY: STUDIES ON THE METABOLISM OF *R*-(+)-4-METHYL-2-(1-METHYLETHYLIDENE)-CYCLOPENTANONE AND DL-CAMPHORONE IN RATS

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ABSTRACT:

***R*-(+)-Pulegone, a monoterpene ketone, is a potent hepatotoxin. The present study was designed to evaluate whether the reduction of the ring size in *R*-(+)-pulegone would affect its mode of metabolism and its hepatotoxic potential. Metabolic fate of *R*-(+)-4-methyl-2-(1-methylethylidene)-cyclopentanone (I) and 5-methyl-2-(1-methylethylidene)-cyclopentanone (DL-camphorone; II) were examined in rats. Compounds I and II were administered orally (250 mg/kg of b.wt./day) to rats for 5 to 7 days. The following metabolites were isolated and identified from the urine of rats dosed with I: 3-methyl-5-(1-methylethylidene)-cyclopent-2-enone (Ie), Z-4-methyl-2-(1-hydroxymethylethylidene)-cyclopentanone (Ib), E-4-methyl-2-(1-hydroxymethylethylidene)-cyclopentanone (Ia), 3-hydroxy-4-methyl-2-(1-methylethylidene)-cyclopentanone (If), 4-hydroxy-4-methyl-2-(1-methylethylidene)-cyclopentanone (Ic), and E-4-methyl-2-(1-carboxyethylidene)-cyclopentanone (Id). Phenobarbital (PB)-induced rat liver microsomes in the presence of NADPH transformed compound I into metabolites, which were identified as Ia, Ib, Ic, Ie, and If. The following urinary metabolites were isolated and identified from compound II: 5-hydroxy-5-methyl-2-(1-methylethylidene)-cyclopentanone (IIc), 5-hydroxy-5-methyl-2-(1-methylethyl)-cyclopentanone (IIg), Z-5-methyl-2-(1-hydroxymethylethylidene)-cyclopentanone (IIb), 5-methyl-2-(1-hydroxymethylethyl)-cyclopentanone (IIf), E-5-methyl-2-(1-hydroxymethylethylidene)-cyclopentanone (IIa), E-5-methyl-2-(1-carboxyethylidene)-cyclopentanone (IId), and 5-methyl-2-(1-carboxyethyl)-cyclopentanone (IIe). PB-induced rat liver microsomes in the presence of NADPH were shown to transform compound II to IIa, IIb, and IIc. Studies carried out in vitro demonstrated that hydroxylation at the tertiary carbon atom or oxidation of the isopropylidene methyl groups in II can be specifically blocked through structural modifications as seen in compounds 2,2-dimethyl-5-(1-methylethylidene)-cyclopentanone (III) and 5-methyl-2-(1-ethyl-1-propylidene)-cyclopentanone (IV). Similar observation was also made when isopropylidene methyl groups in *R*-(+)-pulegone were replaced by ethyl groups. Intraperitoneal administration of a single dose (250 mg/kg) of I and II to rats did not elicit hepatotoxicity as judged by serum alanine aminotransaminase levels and liver microsomal drug metabolizing enzyme activities.**

R-(+)-Pulegone, a monoterpene ketone, a major constituent of pennyroyal oil from *Mentha pulegium* is a potent hepatotoxin (Gordon et al., 1982; Thorup et al., 1983; Moorthy et al., 1989a, 1991). This hepatotoxin is extensively metabolized in the rat system, and some of the reactive metabolites formed are mostly responsible for the observed toxicity (Gordon et al., 1987; Madyastha and Moorthy, 1989; McClanahan et al., 1989; Madyastha and Raj, 1990). Two major pathways are involved in the biotransformation of *R*-(+)-pulegone (Moorthy et al., 1989b; Madyastha and Raj, 1993). One of the major pathways is initiated through the regiospecific hydroxylation of *R*-(+)-pulegone to 9-hydroxypulegone, and this reaction is catalyzed by the liver microsomal cytochrome P450 system (Gordon et al., 1987; McClanahan et al., 1988; Madyastha and Moorthy, 1989; Madyastha and Raj, 1990). The 9-hydroxypulegone spontaneously cyclized intramolecularly to a hemiketal, followed by dehydration to menthofuran, a bicyclic furanoterpene (Gordon et al., 1987; Madyastha and Moorthy, 1989; McClanahan et al., 1989; Madyastha and Raj, 1990). In the other major pathway, *R*-(+)-pulegone is stereoselectively hydroxylated at the C-5 position, which upon dehydration yields piperitenone (Madyastha and Raj, 1991, 1993). Most of the metabolites of *R*-(+)-pulegone are derived from these two common intermediates, viz. menthofuran and piperitenone. Menthofuran upon further metabolism yields an α,β-unsaturated-γ-ketoaldehyde, a highly reactive metabolite capable of covalently interacting with liver proteins (McClanahan et al., 1989; Madyastha and Raj, 1992). It has been estimated that menthofuran is responsible for nearly half of the hepatotoxicity caused by *R*-(+)-pulegone (Thomassen et al., 1990). In fact, it is known that furano compounds upon metabolism yield strongly electrophilic metabolites that covalently interact with tissue macromolecules causing cell injury (Boyd, 1982). It has also been demonstrated earlier that piperitenone becomes biotransformed further to 6,7-dehydromenthofuran and *p*-cresol, besides other minor metabolites (Madyastha and Gaikwad, 1999). Both these metabolites could also contribute to *R*-(+)-pulegone-mediated toxicity (Deichman and Keplinger, 1958; Boyd, 1982; Thomson et al., 1994). This is

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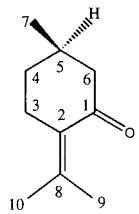
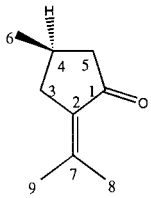
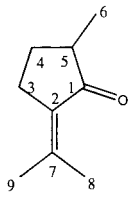


R-(+)-pulegone



R-(+)-4-methyl-2-(1-methylethylidene)-cyclopentanone (**I**)



5-methyl-2-(1-methylethylidene)-cyclopentanone (DL-camphorone, **II**)

SCHEME 1.

supported by the observation that the hepatotoxic potential of *R*-(+)-pulegone is reduced considerably when the C-5 hydroxylation pathway involved in the formation of piperitenone from pulegone is blocked through structural modification (Thulasiram et al., 2000).

Several compounds structurally similar or related to *R*-(+)-pulegone have been tested for their ability to elicit hepatotoxic effects, and these studies have indicated that the α-isopropylidene ketone group of pulegone is the necessary structural unit required for eliciting hepatotoxicity (Gordon et al., 1982). Any variation in this structural requirement such as reduction of either the ketone group or the isopropylidene double bond completely eliminates the hepatotoxic response (Gordon et al., 1982). Isomerization of the double bond to the alicyclic position, as in isopulegone, or combination of exocyclic and endocyclic double bonds, as in piperitenone, decreases the toxic potential (Gordon et al., 1982). It is also known that removal of the isopropylidene unit eliminates hepatotoxicity, whereas removal of the C-5 methyl group only decreases the toxic response (Gordon et al., 1982). In fact, inversion of configuration of the C-5 methyl group in *R*-(+)-pulegone markedly affects the hepatotoxic potential. Thus, *S*-(−)-pulegone is significantly less hepatotoxic than its enantiomer, *R*-(+)-pulegone (Gordon et al., 1982; Madyastha and Gaikwad, 1998). Recently, it has been demonstrated that the C-5 chiral center in *R*-(+)-pulegone contributes substantially to *R*-(+)-pulegone-mediated toxicity (Thulasiram et al., 2000). All these studies clearly indicate that some of the structural features of *R*-(+)-pulegone are the important determinants for its hepatotoxic response. However, it is not known whether reduction of the ring size in *R*-(+)-pulegone would affect its hepatotoxic potential. To explore this, metabolic studies with *R*-(+)-4-methyl-2-(1-methylethylidene)-cyclopentanone (**I**) and 5-methyl-2-(1-methylethylidene)-cyclopentanone (DL-camphorone, **II**) (Scheme 1) were undertaken both in vivo and in vitro. Compounds **I** and **II** (Scheme 1) are structurally very similar and have the same functional groups as in *R*-(+)-pulegone, except there is a reduction in the ring size. The purpose of the present investigation was to find out whether the rat system carries out the regiospecific oxidation of **I** and **II** to their allylic alcohols where the hydroxyl and keto groups are *syn* to each other and, if so, would they undergo intramolecular cyclization followed by dehydration to their corresponding furano compounds in a manner analogous to the formation of menthofuran (Gordon et al., 1987; McClanahan et al., 1989; Madyastha and Raj, 1990) and 6,7-dehydromenthofuran (Madyastha and Gaikwad, 1999) from *R*-(+)-pulegone and piperitenone, respectively. This information not only provides new insight into the toxic potential of compounds **I** and **II**, but also on their mode of metabolism. In fact, very little is known about the metabolism of five-membered cyclic ketones in the mammalian system. The present study describes the isolation and characterization of several novel metabolites from the urine of rats dosed with compounds **I** and **II**, and many of the metabolites characterized appear to be unknown. Preliminary studies pertaining to the hepatotoxic potential of compounds **I** and **II** are also presented.

Materials and Methods

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

Synthetic Methods. *R*-(+)-4-Methyl-2-(1-methylethylidene)-cyclopentanone (**I**), 5-methyl-2-(1-methylethylidene)-cyclopentanone (DL-camphorone, **II**), and 2,2-dimethyl-5-(1-methylethylidene)-cyclopentanone (**III**) were synthesized as reported earlier (Gole, 1949), and were purified by column chromatography over neutral alumina using 1 to 5% ethyl acetate in hexane. 5-Methyl-2-(1-ethyl-1-propylidene)- cyclohexanone (**V**) was synthesized according to the method of Black et al. (1956). The compound was purified by column chromatography over neutral alumina using hexane as the eluting solvent. The compound (**V**) had the following spectral characteristics. Infrared (IR) spectrum (neat) indicated the presence of an enone (1685 and 1611 cm^{-1}). PMR spectra ($CDCl_3$) were: δ 2.65 and 2.60 (2t, 2H, J = 4.5 Hz, H-3), 1.7–2.4 (m, 9H, methylene protons) and 0.91–0.99 (m, 9H, methyl protons). ^{13}C NMR spectra (75 MHz, $CDCl_3$) were: δ 204.9 (C1), 151.05 (C2), 131.8 (C8), 51.4 (C6), 33.4 (C3), 32.2 (C5), 28.3 (C9), 26.7 (C11), 25.6 (C4), 21.8 (C7), 13.7 (C10), and 12.5 (C12). Mass spectra were: *m/z* 180 (M^+), 165 (M^+-CH_3), 151 (M^+-C_2H_5), and 109 (M^+-C_4H_7O).

5-Methyl-2-(1-ethyl-1-propylidene)-cyclopentanone (**IV**) was synthesized as follows: lithium diisopropylamide (17.9 mmol, in 40 ml anhydrous THF) was taken in a round-bottomed flask (150 ml) maintained under argon. The contents of the flask were cooled to −50°C and 2-methyl cyclopentanone (1.4 g, 15.6 mmol in 10 ml THF) was added at −50°C under argon. The resulting mixture was stirred at −50°C to −36°C for 30 min. Then, a solution of 3-pentanone (1.86 g, 21.6 mmol) in 10 ml of THF was added dropwise, and the mixture was stirred for 30 min at −36°C and overnight at room temperature. After completion of the reaction, the contents were cooled and a cold saturated ammonium chloride solution (~25 ml) was added, and the product was extracted with ether (50 ml × 3). The combined ether extracts were washed with water, brine, dried over anhydrous sodium sulfate, and evaporated to a colorless liquid (75% yield). The compound (**IV**) was purified by column chromatography over neutral alumina using 1% ethyl acetate in hexane as the eluent. The compound had the following spectral characteristics. IR spectrum (neat) showed absorptions at 1693 and 1610 cm^{-1} (conjugated carbonyl group). PMR spectra ($CDCl_3$) were: δ 2.67 (2 d, 2H, J = 1.96 Hz, C-3 methylene protons), 2.51 (m, 2H, C-4 methylene protons), 2.3 (m, 1H, C-5 methine proton), 2.12 (q, 4H, J = 5.8 Hz and 6.0 Hz, C-8 and C-10 methylene protons), 1.09 (d, 3H, J = 5.4 Hz, C-6 methyl protons), 1.03 (t, 3H, J = 6.2 Hz, C-9 methyl protons), and 1.0 (t, 3H, J = 6.12 Hz, C-11 methyl protons). ^{13}C NMR spectra (75 MHz, $CDCl_3$) were: δ 207.7 (C1), 157.9 (C2), 129.6 (C7), 45.0 (C5), 28.6 (C8), 28.5 (C3), 26.5 (C10), 24.0 (C4), 14.8 (C6), 13.1 (C9), and 11.6 (C11). Mass spectra were: *m/z* 166 (base peak, M^+), 151 (M^+-CH_3), 137 (M^+-C_2H_5), and 109 (M^+-C_3H_5O).

Hydrogenation of Compounds Ic, **Id**, and **If**. The compound (5.0 mg) was dissolved in dry methanol (0.5 ml), and a catalytic amount of palladium charcoal was added. The mixture was stirred for 2 h at room temperature under hydrogen atmosphere. The reaction mixture was then diluted with chloroform and passed through a celite bed, concentrated, and subjected to column chromatography over neutral alumina. The product was eluted using 1 to 5% ethyl acetate in hexane. The purified product was subjected to various spectral analyses.

Animals and Dosing. Adult male rats (Wistar strain, 180–200 g) were used in these studies. For isolation of metabolites, the test compound (**I** or **II**, 250 mg/kg of b.wt./day) was administered to rats (n = 20) once daily for 5 days in case of compound **I** and 7 days in case of compound **II** by gastric intubation as a suspension in 1 ml of 1% methylcellulose solution. Control rats (n = 6)

[1] Abbreviations used are: PB, phenobarbital; IR, infrared; PMR, proton magnetic resonance; THF, tetrahydrofuran; ALT, alanine aminotransferase; GC, gas chromatography; MS, mass spectroscopy; TLC, thin-layer chromatography; R_f, relative front; R_t, retention time; LRMS, low-resolution mass spectra; HRMS, high-resolution mass spectra.

TABLE 1

*Effect of i.p. administration of a single dose of DL-camphorone (**I**, 250 mg/kg), compound **II** (250 mg/kg), and R-(+)-pulegone (250 mg/kg) on rat liver microsomal enzymes and ALT 24 h after the treatment*

Enzyme Activity	Control*	Compound **I**	% Change Over Control	Compound **II**	% Change Over Control	*R*-(+)-Pulegone	% Change Over Control
Cytochrome P450 (nmol/mg protein)	0.86 ± 0.01	0.80 ± 0.03	7.0 (−)	0.76 ± 0.02	11.6 (−)	0.47 ± 0.04	45.30 (−)
Cytochrome b_5 (nmol/mg protein)	0.61 ± 0.02	0.69 ± 0.05	13.1 (+)	0.59 ± 0.03	3.3 (−)	0.55 ± 0.03	9.8 (−)
Glucose 6-phosphatase (nmol pi/min/mg protein)	193.13 ± 5.13	191.25 ± 3.75	0.95 (−)	178.25 ± 7.2	7.7 (−)	102.3 ± 4.23	47 (−)
Aminopyrine *N*-demethylase (nmol HCHO/min/mg protein)	7.5 ± 0.15	7.25 ± 0.36	3.33 (−)	6.6 ± 0.58	12.0 (−)	3.81 ± 0.25	49.2 (−)
ALT (units/ml)	29.34 ± 1.25	29.58 ± 1.4	0.008† (+)	38.66 ± 1.5	0.32† (+)	412.5 ± 17.5	13.06† (+)

* Animals treated with vehicle alone. Animals were killed 24 h after the administration of test compounds. Values represent means ± S.D. of three independent experiments consisting of tissues pooled from 6 rats. (−) indicates % decrease over control and (+) indicates % increase over control.

† Values represent number of fold change over control. Other details of the experiment are as described under *Materials and Methods.*

received only the vehicle. In our earlier studies (Madyastha and Raj, 1993), *R*-(+)-pulegone was administered orally to rats (250 mg/kg) to investigate the nature of urinary metabolites. LD_{50} in rats for *R*-(+)-pulegone is 245 to 250 mg/kg (Moorthy et al., 1989a). Since we wanted to compare the toxic potential, as well as mode of metabolism of compounds **I** and **II** with that of *R*-(+)-pulegone, we used the same amount of test compounds (250 mg/kg) in all our experiments. 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° to 4°C.

To evaluate the hepatotoxic potential of compounds **I** and **II**, and compare these values to *R*-(+)-pulegone-mediated toxicity, rats were treated with *R*-(+)-4-methyl-2-(1-methylethylidene)-cyclopentanone (**I**, 250 mg/kg of b.wt.) or 5-methyl-2-(1-methylethylidene)-cyclopentanone (**II**, 250 mg/kg of b.wt.) or *R*-(+)-pulegone (250 mg/kg of b.wt.) as a suspension in coconut oil (0.3 ml). Control rats received only vehicle. Treatments were carried out intraperitoneally (i.p.). It has been noted earlier that i.p. administration of a single dose of *R*-(+)-pulegone caused a significant change in the levels of liver microsomal glucose-6-phosphatase, aminopyrine *N*-demethylase, and serum alanine aminotransferase (ALT). However, when the same dosage was administered orally, the changes observed in these activities were comparatively less pronounced (Moorthy et al., 1989a). Both control and experimental rats were housed separately in cages with free access to food and water. Animals were killed by cervical dislocation 24 h after administration of test compounds.

In pretreatment experiments, rats were pretreated with phenobarbital (in 0.3 ml of 0.9% NaCl solution, 80 mg/kg of b.wt./day) for 4 days prior to i.p. administration of test compounds (**I** or **II**, 250 mg/kg of b.wt.). For ALT determinations, rats were killed while under light ether 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. Rats were killed 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. Urine samples collected daily from control and experimental rats were adjusted to pH 4 to 5 with 1 N HCl and extracted three times with diethyl ether. The ether extracts from each day were pooled, concentrated, and separated into acidic and neutral fractions, as reported earlier (Madyastha and Raj, 1993).

Preparation of Microsomes. PB-induced rat liver microsomes were prepared as reported earlier (Madyastha and Raj, 1992). 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 P450 (Omura and Sato, 1964), serum ALT (earlier referred as glutamate pyruvate transaminase) (Reitman and Frankel, 1957), glucose-6-phosphatase (Traiger and Plaa, 1971), and aminopyrine *N*-demethylase (Werringloer, 1978) were determined according to the reported methods.

Studies in Vitro. Microsomal protein (2 mg/ml) was incubated in the presence of $NADP^+$ (0.5 mM), glucose 6-phosphate (5.0 mM), glucose-6-phosphate dehydrogenase (1 unit), $MgCl_2$ (10 mM), test compounds (**I–V**, 2 mM, in 50 μl acetone), and Tris-HCl (0.01 M, pH 7.4) in a total volume of 5.0 ml. The reaction was initiated by the addition of an 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° to 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 was centrifuged (3000*g*). The supernatant was extracted with methylene chloride (20 ml × 3), concentrated, and an aliquot was subjected to GC and GC-MS analyses. For isolating the metabolites, organic extracts of several assays were pooled and processed.

Binding studies were conducted in potassium phosphate buffer (50 mM, pH 7.4) with control and phenobarbital-induced rat liver microsomes (~3 mg/ml) as reported earlier (Madyastha and Gaikwad, 1998).

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) developed with either hexane-ethyl acetate (8:2, v/v, system I) or hexane-ethyl acetate (7.5:2.5, v/v, system II) or chloroform-methanol (9.6:0.4, v/v, system III) or ethyl acetate-methanol-acetic acid (96.5:3:0.5, v/v, system IV). 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 conducted 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 Perkin-Elmer model 781 spectrophotometer. Proton and ^{13}C NMR spectra were recorded on 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 with a JMA-DA-5000 data system.

Results

Effect of Compounds I, II, and *R*-(+)-pulegone on Hepatic Microsomal Enzymes. A number of liver microsomal enzymes and serum ALT levels were determined 24 h after the i.p. administration of compounds **I** and **II** (250 mg/kg of b.wt.) to rats. The effects of compounds **I** and **II** on the hepatic microsomal enzymes were compared with the effects observed after a single dose of i.p. administration of *R*-(+)-pulegone (250 mg/kg of b.wt.), a known hepatotoxin. These preliminary results are summarized in Table 1. Consistent with the earlier reports (Gordon et al., 1982; Moorthy et al., 1989b, 1991), it was noted that i.p. administration of a single dose of *R*-(+)-pulegone to rats resulted in a marked decrease in microsomal cytochrome P450, aminopyrine *N*-demethylase, and glucose-6-phosphatase activities (Table 1). However, these activities were not significantly affected after the administration of a single dose of compounds **I** and **II**. Even the increase in ALT levels was only marginal. It was also noted that pretreatment of rats with PB for 4 days prior to the administration of **I** and **II** (as described under *Materials and Methods*) did not alter significantly the levels of hepatic microsomal enzymes, as well as ALT levels (data not shown).

TABLE 2

Spectral data for metabolites derived from compound **I**

Compound	IR (neat) γ_{max}	^{1}H NMR (δ) $CDCl_3$	MS (LRMS and HRMS)
Ia	3400 cm^{-1} (—OH), 1703 and 1627 cm^{-1} (conjugated carbonyl)	4.2 (s, 2H, H—9), 2.8 (d, 2H, J = 10.8 Hz, H—3), 2.5 (2d, 2H, J = 7.5 Hz, H—5), 2.27 (s, 4H, H—8 and H—4), 1.1 (d, 3H, J = 7.5 Hz, H—6)	*m/z*: 154 (M^+, bp), 139 (M^+—CH_3), 136 (M^+—H_2O), 125 (M^+—C_2H_5), 121 (M^+—CH_3—H_2O), 111 (M^+—CO—H_2O) HRMS: $C_9H_{14}O_2$ requires 154.0994, found 154.0997
Ib	3400 cm^{-1} (—OH), 1680 and 1610 cm^{-1} (conjugated carbonyl)	4.3 (s, 2H, H—8), 2.2–2.6 (m, 5H, ring protons), 1.9 (s, 3H, H—9), 1.05 (d, 3H, J = 6.0 Hz, H—6)	*m/z*: 154 (M^+), 139 (M^+—CH_3), 136 (M^+—H_2O), 121 (M^+—CH_3—H_2O) HRMS: $C_9H_{14}O_2$ requires 154.0994, found 154.0996
Ic	3440 cm^{-1} (—OH), 1690 and 1629 cm^{-1} (conjugated carbonyl)	2.6 (bs, 2H, H—3), 2.4 (s, 2H, H—5), 2.1 (s, 3H, H—8), 1.77 (s, 3H, H—9) 1.38 (s, 3H, H—6)	*m/z*: 154 (M^+), 139 (M^+—CH_3), 136 (M^+—H_2O), 121 (M^+—CH_3—H_2O), 111 (M^+—CO—CH_3), 96 (M^+—C_3H_6O) HRMS: $C_9H_{14}O_2$ requires 154.0994, found 154.1001
Id	3100 cm^{-1} (carboxyl), 1706 and 1690 cm^{-1} (carbonyl groups)	2.35 (s, 3H, H—8), 2.06–2.6 (m, 5H, ring protons), 1.11 (d, 3H, J = 6.44 Hz, H—6)	*m/z*: 168 (M^+), 150 (M^+—H_2O), 140 (M^+—CO), 122 (M^+—CO—H_2O)
Ie	1695 and 1620 cm^{-1} (conjugated carbonyl)	6.05 (s, 1H, H—2), 3.1 (s, 2H, H—4), 2.3 (s, 3H, H—8), 2.1 (s, 3H, H—6), 1.86 (s, 3H, H—9)	*m/z*: 136 (M^+, bp), 121 (M^+—CH_3), 93 (M^+—CO—CH_3) HRMS: $C_9H_{12}O$ requires 136.0888, found 136.0886
If	3390 cm^{-1} (—OH), 1690 and 1620 cm^{-1} (conjugated carbonyl)	4.6 (s, 1H, H—3), 2.7 (2d, 2H, J = 8.1 Hz, H—5), 2.3 (bs, 4H, H—8, and H—4), 2.04 (s, 3H, H—9), 1.0 (d, 3H, J = 6.9 Hz, H—6)	*m/z*: 154 (M^+), 139 (M^+—CH_3), 136 (M^+—H_2O), 121 (M^+—CH_3—H_2O), 111 (M^+—CO—CH_3), 83 (M^+—C_4H_7O, bp) HRMS: $C_9H_{14}O_2$ requires 154.0994, found 154.0993

Biotransformation of *R*-(+)-4-Methyl-2-(1-methylethylidene)-cyclopentanone (I). *Neutral metabolites.* Examination of the neutral fraction (0.76 g) by TLC (system I) showed the presence of five metabolites (R_f 0.43, 0.2, 0.18, 0.15, and 0.12) that were absent in the control urine extract. This fraction was subjected to column chromatography over neutral alumina (20 g), and the metabolites were eluted with hexane-ethyl acetate mixtures. Elution of the column with hexane yielded a compound (R_f 0.59 system I, R_t 5.4 min) identified as unmetabolized substrate (**I**) by comparing its GC retention time, PMR, and mass spectra with that of authentic compound.

The metabolite corresponding to R_f 0.43 (system I; R_t 11.8 min) was eluted from the column with hexane-ethyl acetate (9.8: 0.2, v/v). From the spectral characteristics (Table 2), it was identified as 3-methyl-5-(1-methylethylidene)-cyclopent-2-enone (**Ie**, Fig. 1A). Elution of the column with hexane-ethyl acetate (9:1, v/v) yielded two fractions, one containing a compound with R_f 0.2 (system I; R_t 15.0 min) and the other with R_f 0.18 (system I; R_t 15.9 min). These two metabolites were further purified by preparative TLC (system I). Based on the spectral characteristics (Table 2), the metabolites with R_f 0.2 and 0.18 were identified as Z-4-methyl-2-(1-hydroxymethylethylidene)-cyclopentanone (**Ib**, Fig. 1A) and E-4-methyl-2-(1-hydroxymethylethylidene)-cyclopentanone (**Ia**, Fig. 1A), respectively.

Further elution of the column with hexane-ethyl acetate (8.8:1.2, v/v) gave two compounds with R_f 0.15 and 0.12 (system I). The spectral data of these compounds are presented in Table 2. From the spectral characteristics, the compounds with R_f 0.15 (R_t 15.9 min) and 0.12 (R_t 15.0 min) were identified as 3-hydroxy-4-methyl-2-(1-methylethylidene)-cyclopentanone (**If**, Fig. 1A) and 4-hydroxy-4-methyl-2-(1-methylethylidene)-cyclopentanone (**Ic**, Fig. 1A), respectively. It was observed that the GC analysis of **Ic** (R_t 15.0 min) upon storage indicated the appearance of a new peak (R_t 11.8 min), which was enhanced when mixed with 3-methyl-5-(1-methylethylidene)-cyclopent-2-enone (**Ie**, Fig. 1A).

Acidic metabolites. TLC examination (system IV) of the acidic fraction showed the presence of one compound (R_f 0.51, R_t 19.9 min), which was absent in control urine extract. This fraction (0.8 g) was chromatographed on a silica gel column (30 g), and the compound corresponding to R_f 0.51 was eluted with hexane-ethyl acetate (19:1, v/v). From the spectral characteristics (Table 2), the compound was tentatively identified as E-4-methyl-2-(1-carboxyethylidene)-cyclopentanone (**Id**, Fig. 1A).

Composition of the total urine extract was determined by GC analyses, which showed the presence of seven peaks corresponding to metabolites and unmetabolized compound **I**. These peaks were enhanced when mixed with the purified metabolites (**Ia**–**If**) isolated by column chromatography. GC profile also showed a couple of very minor peaks, which accounted for 5% of the total metabolites formed, and these minor metabolites could not be characterized. Nearly 35% of the administered dose was excreted in the urine as metabolites and

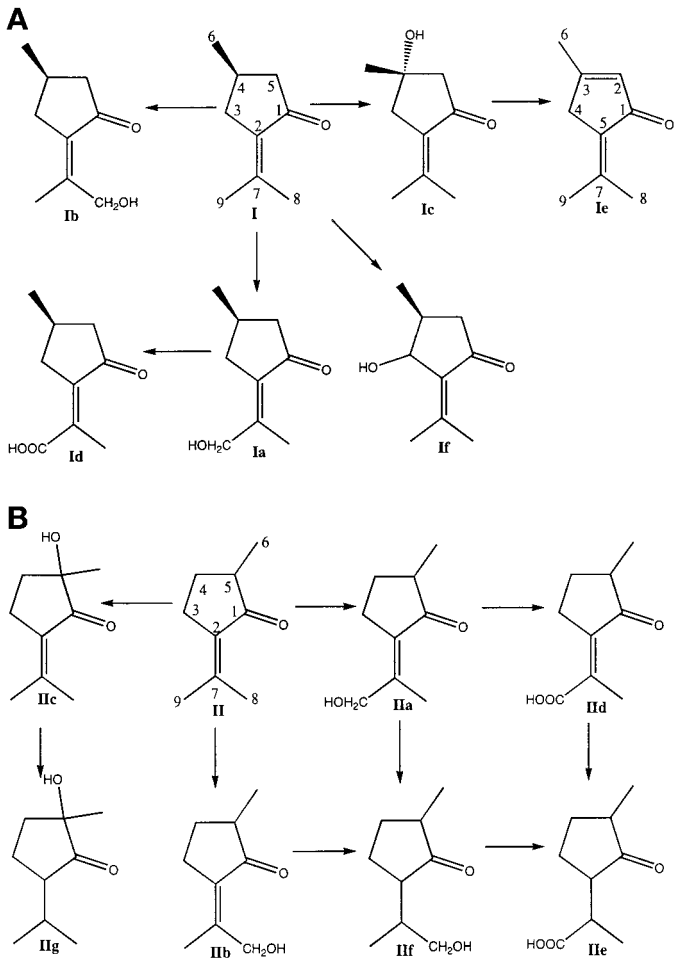


FIG. 1. *A, probable metabolic pathways of R-(+)-4-methyl-2-(1-methylethylidene)-cyclopentanone (**I**); B, probable metabolic pathways of 5-methyl-2-(1-methylethylidene)-cyclopentanone (**II**).*

TABLE 3

Spectral data for metabolites derived from compound **II**

Compound	IR (neat) γ_{max}	^{1}H NMR (δ) $CDCl_3$	MS (LRMS and HRMS)
IIa	3380 cm^{-1} (—OH), 1690 and 1615 cm^{-1} (conjugated carbonyl)	4.2 (s, 2H, H—9), 2.27 (s, 3H, H—8), 2.15 (m, 1H, exchanges with D_2O), 1.95–2.7 (m, 5H, ring protons), 1.1 (d, 3H, J = 7.7 Hz, H—6)	*m/z*: 154 (M^+), 136 (M^+—H_2O), 125 (M^+—C_2H_5), 121 (M^+—CH_3—H_2O), 111 (M^+—CO—CH_3), 83 (M^+—C_4H_7O) HRMS: $C_9H_{14}O_2$ requires 154.0994, found 154.0994
IIb	3400 cm^{-1} (—OH), 1680 and 1610 cm^{-1} (conjugated carbonyl)	4.3 (s, 2H, H—8), 2.2–2.6 (m, 5H, ring protons), 1.9 (s, 3H, H—9), 1.05 (d, 3H, J = 6.4 Hz, H—6)	*m/z*: 154 (M^+, bp), 139 (M^+—CH_3), 136 (M^+—H_2O), 125 (M^+—C_2H_5), 121 (M^+—CH_3—H_2O), 111 (M^+—CO—CH_3), 84 (M^+—C_4H_6O) HRMS: $C_9H_{14}O_2$ requires 154.0994, found 154.0997
IIc	3440 cm^{-1} (—OH), 1700 and 1610 cm^{-1} (conjugated carbonyl)	2.8 (s, 1H, exchanges with D_2O), 2.25 (s, 3H, H—8), 1.87 (s, 3H, H—9), 1.9–2.5 (m, 4H, H—3 & H—4), 1.23 (s, 3H, H—6)	*m/z*: 154 (M^+), 139 (M^+—CH_3), 136 (M^+—H_2O), 126 (M^+—CO), 121 (M^+—CH_3—H_2O), 111 (M^+—CO—CH_3), 93 (M^+—CO—H_2O—CH_3) HRMS: $C_9H_{14}O_2$ requires 154.0994, found 154.1007
IId	3000 cm^{-1} (carboxyl), 1700 and 1670 cm^{-1} (carbonyl groups)	8.5 (bs, 1H, acidic proton), 2.32 (s, 3H, H—8), 2.2–3.1 (m, 5H, ring protons), 1.15 (d, 3H, J = 6.6 Hz, H—6)	*m/z*: 168 (M^+), 150 (M^+—H_2O), 140 (M^+—CO), 123 (M^+—COOH), 98 (M^+—C_4H_6O) HRMS: $C_9H_{12}O_3$ requires 168.0786, found 168.0771
IIe	3120 cm^{-1} (carboxyl), 1720 and 1690 cm^{-1} (carbonyl groups)	6.7 (bs, 1H, acidic proton), 1.7–3.2 (m, 7H, H—2, H—3, H—4, H—5, and H—7), 1.1 (d, 6H, J = 6.4 Hz, H—6 and H—8)	*m/z*: 170 (M^+), 152 (M^+—H_2O), 142 (M^+—CO), 125 (M^+—COOH), 98 (M^+—$C_3H_4O_2$) HRMS: $C_9H_{14}O_3$ requires 170.0943, found 170.0954
IIf	3390 cm^{-1} (—OH), 1720 cm^{-1} (carbonyl)	3.6 (m, 2H, H—8), 1.8–2.6 (m, 7H, H—2, H—3, H—4, H—5, H—7), 1.1 (d, 3H, J = 6.4 Hz, H—6), 0.95 (d, 3H, J = 7.7 Hz, H—9)	*m/z*: 156 (M^+), 141 (M^+—CH_3), 125 (M^+—CH_3O), 113 (M^+—C_2H_3O), 98 (M^+—C_3H_6O, bp) HRMS: $C_9H_{16}O_2$ requires 156.1150, found 156.1148
IIg	3440 cm^{-1} (—OH), 1740 cm^{-1} (carbonyl)	1.6–2.6 (m, 6H, H—2, H—3, H—4, and H—7), 1.2 (s, 3H, H—6), 0.95 (2d, 6H, J = 7.7 Hz, H—8 and H—9)	*m/z*: 156 (M^+), 139 (M^+—OH), 128 (M^+—CO), 58 (M^+—$C_6H_{10}O$) HRMS: $C_9H_{16}O_2$ requires 156.1150, found 156.1136

unmetabolized substrate. However, these values are approximate, since both substrate and metabolites are highly volatile and a considerable amount must have been lost during extraction and storage.

*Biotransformation of 5-methyl-2-(1-methylethylidene)-cyclopentanone (**II**, DL-camphorone) in vivo*. The neutral fraction (1.4 g) upon TLC analysis (system II) revealed the presence of six compounds, which were absent in the control urine extract. This fraction was subjected to column chromatography over silica gel (30 g), and elution of the column with hexane yielded a compound (R_f 0.76, system II) identified as unmetabolized camphorone (**II**) by comparing its PMR, IR, and mass spectra with that of authentic compound.

Elution of the column with hexane-ethyl acetate (9.8:0.2, v/v) yielded two fractions, each containing mainly one compound and they (R_f 0.35, 0.34, system II) were further purified by preparative TLC (system II). The compound with R_f 0.35 showed a UV absorption maximum at 255 nm. Based on the spectral characteristics (Table 3), these two metabolites were identified as 5-hydroxy-5-methyl-2-(1-methylethylidene)-cyclopentanone (R_f 0.35, R_t 9.1 min, **IIc**, Fig. 1B) and 5-hydroxy-5-methyl-2-(1-methylethyl)-cyclopentanone (R_f 0.34, R_t 5.3 min, **IIg**, Fig. 1B). Hydrogenation of **IIc** in the presence of palladium charcoal resulted in the formation of **IIg**, as judged by various spectral analyses.

The compounds with R_f 0.27 and 0.21 (system II) were eluted from the column with hexane-ethyl acetate (9.6:0.4, v/v). The compound with R_f 0.27 showed a UV absorption maximum at 257 nm. From the spectral data (Table 3), these two metabolites were identified as Z-5-methyl-2-(1-hydroxymethylethylidene)-cyclopentanone (R_f 0.27, R_t 15.7 min, **IIb**, Fig. 1B) and 5-methyl-2-(1-hydroxymethylethyl)-cyclopentanone (R_f 0.21, R_t 12 min **IIf**, Fig. 1B).

Additional elution of the column with hexane-ethyl acetate (9.6:0.4, v/v) yielded a compound with R_f 0.2 (system II) and R_t 18.6 min. This compound showed an UV absorption maximum at 250 nm. From the spectral data (Table 3), the metabolite was identified as E-5-methyl-2-(1-hydroxymethylethylidene)-cyclopentanone (**IIa**, Fig. 1B). Hydrogenation of **IIa** and **IIb** using palladium charcoal as catalyst yielded the same product whose spectral characteristics (PMR and MS) matched well with that of metabolite **IIf** (Fig. 1B). This observation further supports the structures assigned for **IIa** and **IIb** (Fig. 1B).

Acidic metabolites. Examination of the acidic fraction by TLC showed two compounds (R_f 0.6 and 0.62, system IV), which were absent in the control urine extract. These two compounds with R_f 0.6 (R_t 20.7 min) and R_f 0.61 (R_t 19.3 min) were eluted from the column with 9.7:0.3 and 9.6:0.4 hexane-ethyl acetate (v/v), respectively. The compounds were further purified by preparative TLC (system IV), and their spectral data are presented in Table 3. Based on the spectral characteristics, they were identified as E-5-methyl-2-(1-carboxyethylidene)-cyclopentanone (R_f 0.6, **IId**, Fig. 1B) and 5-methyl-2-(1-carboxyethyl)-cyclopentanone (R_f 0.61, **IIe**, Fig. 1B). Hydrogenation of **IId** in the presence of palladium charcoal as catalyst yielded a product with spectral characteristics (PMR and MS), the same as that of metabolite **IIe**.

GC analyses of the total urine extract showed the presence of seven peaks corresponding to the metabolites (**IIa**–**IIg**). The peaks corresponding to these metabolites were enhanced when mixed with the purified metabolites isolated by column chromatography. GC profile also showed couple of very minor peaks, which accounted for 9.4% of the total metabolites formed. Nearly 37% of the administered dose was excreted in the urine as metabolites and unmetabolized substrate. These values may not be accurate, since both substrate and its metabolites are highly volatile and a considerable amount must have been lost during extraction.

Binding studies. Both compounds **I** and **II** elicit type I binding spectrum (λ_{max} 385–388 nm, λ_{min} 420–423 nm), with control and PB-induced microsomes. The value of spectral dissociation constant (K_s) calculated from the double-reciprocal plot (as described under *Materials and Methods*) for compound **I** with control and PB-induced microsomes was 90.9 μM and 35.7 μM, respectively, and for compound **II** was 55.5 μM and 29 μM, respectively. Binding affinity of both compounds **I** and **II** toward PB-induced microsomes is comparatively higher than with control microsomes.

*Biotransformation of compounds **I** and **II** by rat liver microsomes.* Phenobarbital-induced rat liver microsomes were incubated aerobically with *R*-(+)-4-methyl-2-(1-methylethylidene)-cyclopentanone

TABLE 4

Spectral data for metabolites derived from compounds **III, IV,** and **V**

Compound	IR (neat) γ_{max}	^{1}H NMR (δ) $CDCl_3$	MS (LRMS and HRMS)
IIIa	3400 cm^{-1} (—OH), 1690 and 1610 cm^{-1} (conjugated carbonyl)	4.92 (d, 1H, J = 6.0 Hz, H—4), 2.27 (s, 3H, H—9), 2.03 (s, 3H, H—10), 1.79 (2d, 2H, J = 2.1 Hz, H—3), 1.19 and 1.02 (2s, 6H, H—6 and H—7)	*m/z*: 168 (M^+), 153 (M^+—CH_3), 150 (M^+—H_2O), 135 (M^+—CH_3—H_2O, bp), 107 (M^+—CO—CH_3—H_2O) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1147
IIIb	3400 cm^{-1} (—OH), 1690 and 1610 cm^{-1} (conjugated carbonyl)	4.26 (s, 2H, H—9), 2.4 (t, 2H, J = 7.2 Hz, H—4), 1.87 (s, 3H, H—10), 1.7 (t, 2H, J = 7.5 Hz, H—3), 1.02 (s, 6H, H—6 and H—7)	*m/z*: 168 (M^+), 153 (M^+—CH_3), 150 (M^+—H_2O), 135 (M^+—CH_3—H_2O, bp), 107 (M^+—CO—CH_3—H_2O) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1157
IIIc	3400 cm^{-1} (—OH), 1690 and 1613 cm^{-1} (conjugated carbonyl)	4.15 (s, 2H, H—10), 2.46 (m, 2H, H—4), 2.22 (bs, 3H, H—9), 1.68 (t, 2H, J = 7.8 Hz, H—3), 0.97 (s, 6H, H—6 and H—7)	*m/z*: 168 (M^+), 153 (M^+—CH_3), 150 (M^+—H_2O), 135 (M^+—CH_3—H_2O, bp) 125 (M^+—CO—CH_3), 107 (M^+—CO—H_2O—CH_3) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1153
IVa	3400 cm^{-1} (—OH), 1690 and 1605 cm^{-1} (conjugated carbonyl)	2.71 (m, 2H, H—8), 2.49 (m, 2H, H—3), 2.18 (q, 2H, J = 6.1 Hz, H—10), 1.95 (m, 2H, H—4), 1.23 (s, 3H, H—6), 1.07 (t, 3H, J = 6.2 Hz, H—9), 1.04 (t, 3H, J = 6.04 Hz, H—11)	*m/z*: 182 (M^+), 164 (M^+—H_2O), 149 (M^+—CH_3—H_2O, bp), 121 (M^+—CO—CH_3—H_2O) HRMS: $C_{11}H_{18}O_2$ requires 182.1307, found 182.1302
Va	3412 cm^{-1} (—OH), 1678 and 1610 cm^{-1} (conjugated carbonyl)	2.04–2.57 (m, 10H, methylene protons), 1.33 (s, 3H, H—7), 1.03 (q, 6H, J = 7.5 Hz, H—10 and H—12)	*m/z*: 192 (M^+), 178 (M^+—H_2O), 163 (M^+—CH_3—H_2O), 135 (M^+—CO—CH_3—H_2O) HRMS: $C_{12}H_{20}O_2$ requires 196.1463, found 196.11462
Vb	1655 and 1610 cm^{-1} (conjugated carbonyl)	5.88 (bs, 1H, H—2), 2.63 (t, 2H, J = 6.3 Hz, H—4), 2.42 (q, 2H, J = 7.5 Hz, H—9), 2.29 (t, 2H, J = 6.6 Hz, H—5), 2.17 (q, 2H, J = 7.5 Hz, H—11), 1.93 (s, 3H, H—7), 1.07 (t, 3H, J = 7.5 Hz, H—10), 1.03 (t, 3H, J = 7.5 Hz, H—12)	*m/z*: 178 (M^+), 163 (M^+—CH_3), 149 (M^+—C_2H_5), 96 (M^+—C_5H_6O), 82 (M^+—C_7H_{12}) HRMS: $C_{12}H_{18}O$ requires 178.1357, found 178.1343

(**I**) and 5-methyl-2-(1-methylethylidene)-cyclopentanone (DL-camphorone, **II**) in the presence of NADPH as described under *Materials and Methods*.

*Transformation of **I**.* TLC (system I) and GC analyses of the methylene chloride extract of the assay mixture indicated the presence of five metabolites (**Ia**, **Ib**, **Ic**, **Id**, and **Ie**). The peaks corresponding to these metabolites were enhanced when admixed with samples isolated from the urine of rats treated with compound **I**. GC-MS analysis of the methylene chloride extract also revealed the presence of five metabolites whose fragmentation pattern matched well with that of **Ia**, **Ib**, **Ic**, **Id**, and **Ie**, isolated and characterized from the urine extract. It was observed that PB-induced liver microsomes convert nearly 21% of the added substrate (**I**) into these five metabolites (**Ia**, **Ib**, **Ic**, **Id**, and **Ie**), whereas control microsomes were shown to transform only 5% of added substrate (**I**) into these metabolites.

*Transformation of **II***. The methylene chloride extract of the assay mixture upon TLC (system I) and GC analyses indicated the presence of three metabolites that corresponded well with **IIa**, **IIb**, and **IIc**. All of the peaks corresponding to these metabolites were enhanced when mixed with samples isolated from the urine extract. GC-MS analysis of the methylene chloride extract of the reaction mixture indicated the presence of three compounds whose fragmentation pattern matched well with that of **IIa**, **IIb**, and **IIc** isolated from the urine of rats dosed with **II**. It was demonstrated that PB-induced rat liver microsomes convert nearly 16% of the substrate (**II**) to various metabolites (**IIa**, **IIb**, and **IIc**) under the assay condition used, whereas uninduced microsomes (control) transform only 4% of the substrate (**II**) into **IIa**, **IIb**, and **IIc**.

*Biotransformation of 2,2-dimethyl-5-(1-methylethylidene)-cyclopentanone (**III**).* PB-induced rat liver microsomes were incubated aerobically with **III** in the presence of NADPH as described under *Materials and Methods*. The methylene chloride extract of the assay mixture upon TLC (system II) and GC analyses indicated the presence of three metabolites (R_f 0.38, 0.35 and 0.34, system II) which were absent in control experiment. The organic extract (60 mg) was subjected to column chromatography on neutral alumina (6.0 g) and the metabolites were eluted with hexane-ethyl acetate mixtures. Elution of the column with hexane yielded a fraction containing unmetabolized compound **III** (R_f 0.93 system II; R_t 5.7 min).

The metabolite with R_f 0.38 (R_t 16.8 min) was eluted from the column with hexane-ethyl acetate (9.5:0.5, v/v) and from the spectral data (Table 4), the compound was identified as 4-hydroxy-2,2-dimethyl-5-(1-methylethylidene)-cyclopentanone (**IIIa**, Fig. 2A). Further elution of the column with hexane-ethyl acetate (9.4:0.6, v/v) yielded two fractions, each containing mainly one compound. These two metabolites (R_f 0.35, 0.34) were further purified by preparative TLC (system II). From their spectral data (Table 4), the metabolites with R_f 0.35 (R_t 18.4 min) and R_f 0.34 (R_t 20.7 min) were identified as Z-2,2-dimethyl-5-(1-hydroxymethylethylidene)-cyclopentanone (**IIIb**, Fig. 2A) and E-2,2-dimethyl-5-(1-hydroxymethylethylidene)-cyclopentanone (**IIIc**, Fig. 2A), respectively. It was noted that PB-induced liver microsomes convert nearly 35% of the substrate (**III**) into these three metabolites (**IIIa**, **IIIb**, and **IIIc**). However, the percentage of conversion was significantly lower (~6%) when uninduced (control) rat liver microsomes were used.

*Transformation of 5-methyl-2-(1-ethyl-1-propylidene)-cyclopentanone (**IV**).* Incubation of **IV** with PB-induced rat liver microsomes in the presence of NADPH resulted in the formation of a metabolite (R_f 0.44, system I, R_t 17.0 min), which was absent in the control experiment. This metabolite was separated by column chromatography on neutral alumina using hexane-ethyl acetate (9.4:0.6, v/v) as the eluent. From the spectral data (Table 4), this metabolite was identified as 5-hydroxy-5-methyl-2-(1-ethyl-1-propylidene)-cyclopentanone (**IVa**, Fig. 2B). Nearly 16% of the substrate (**IV**) was converted into **IVa**. However, the conversion was significantly less (4%) when uninduced (control) rat liver microsomes were used.

*Transformation of 2-(1-ethyl-1-propylidene)-5-methyl-cyclohexanone (**V**).* PB-induced rat liver microsomes were incubated aerobically with compound (**V**) in the presence of NADPH as described in *Materials and Methods.* The methylene chloride extract of the assay mixture upon TLC (system I) and GC analyses showed the presence of two metabolites (R_f 0.6 and 0.25 system I; R_t 12.3 min and 15.2 min, respectively) that were absent in the control experiment. This fraction was subjected to column chromatography over neutral alu-

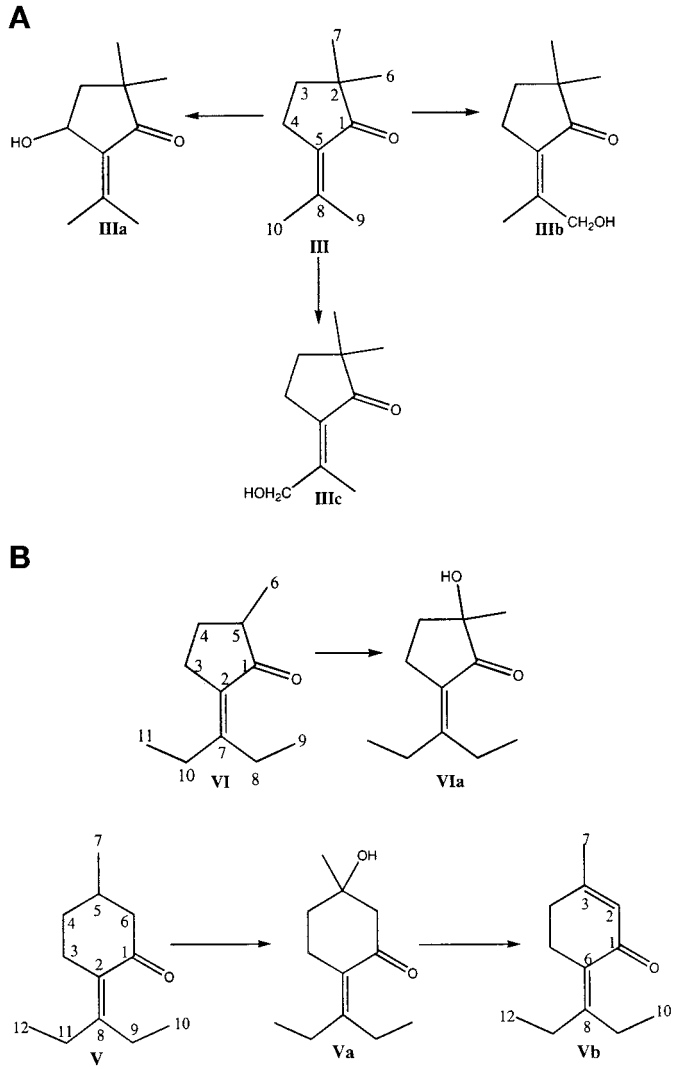


FIG. 2. *Transformation of (A) 2,2-dimethyl-5-(1-methylethylidene)-cyclopentanone (**III**), (B) 4-methyl-2-(1-ethyl-1-propylidene)-cyclopentanone (**IV**), and (C) 5-methyl-2-(1-ethyl-1-propylidene)-cyclohexanone (**V**) by PB-induced rat liver microsomes.*

mina, and the metabolites were eluted with hexane-ethyl acetate mixtures. Elution of the column with hexane yielded a fraction containing the unmetabolized substrate (**V**, R_f 0.74, system I; R_t 7.2 min). The metabolites with R_f 0.6 and 0.25 (system I) were eluted with 9.9:0.1, v/v and 9.4:0.6, v/v hexane-ethyl acetate, respectively. From the spectral characteristics (Table 4), these metabolites were identified as 6-(1-ethyl-1-propylidene)-3-methyl-2-cyclohexen-1-one (R_f 0.6, R_t 12.3 min; **Vb**, Fig. 2C) and 6-(1-ethyl-1-propylidene)-5-hydroxy-5-methyl-cyclohexanone (R_f 0.25, R_t 15.2 min; **Va**, Fig. 2C). Nearly 8.5% of **V** was converted into these two metabolites.

Discussion

The objective of the present investigation was to find out whether reduction in the ring size in *R*-(+)-pulegone would affect its hepatotoxic potential and mode of metabolism. This aspect was evaluated using *R*-(+)-4-methyl-2-(1-methylethylidene)-cyclopentanone (**I**) as the test compound, which is structurally very similar to the potent hepatotoxin, *R*-(+)-pulegone. Both the compounds (pulegone and compound **I**) possess an α-isopropylidene ketone unit with a *R*-configuration methyl group positioned β to the carbonyl group, a characteristic structural feature of *R*-(+)-pulegone required for eliciting hepatotoxicity (Gordon et al., 1982). The only striking difference between them is the size of the ring; pulegone is a cyclohexanone derivative, whereas compound **I** is a cyclopentanone derivative. Compound **II** is the positional isomer of **I**. The present study has demonstrated that structural modification of *R*-(+)-pulegone by reducing the ring size, as in **I** and **II**, eliminates its toxic potential. Studies carried out with **I** and **II**, both in vivo and in vitro, clearly indicate the existence of significant difference in the mode of biotransformation between *R*-(+)-pulegone and compounds **I** and **II**. This difference in the mode of biotransformation appears to be responsible for their (**I** and **II**) inability to elicit hepatotoxicity.

The present study represents characterization of various metabolites isolated from the urine of the rats dosed with **I** and **II**. We have assigned chemical structures mainly by comparing the ^{1}H-NMR spectra of the metabolites with that of the substrates used. The structures assigned were further confirmed by mass spectral analyses (LRMS and HRMS). Most of the metabolites isolated and characterized appear to be hitherto unknown. It was not possible to accurately quantify the metabolites formed due to their high volatility, although suitable precautions were taken to minimize the loss and nonenzymatic transformations of the metabolites during extraction and isolation. Speculative pathways have been proposed for the biotransformation of **I** and **II** (Fig. 1, A and B) on the basis of various metabolites isolated and characterized. In fact, these hypothetical pathways are based more on chemical logic than on direct experimental proof. However, studies carried out in vitro using PB-induced rat liver microsomes supported the in vivo findings and the proposed sequence of reactions.

It is interesting to note that the early sequence of reactions involved in the biotransformation of **I** and **II** (Fig. 1, A and B) is similar to that of *R*-(+)- and *S*-(−)-pulegone. The metabolic fate of *R*-(+)-pulegone (Moorthy et al., 1989a; Madyastha and Raj, 1993) and its enantiomer *S*-(−)-pulegone (Madyastha and Gaikwad, 1998) has been studied in great detail. These studies have established that the methyl group *syn* to the carbonyl in pulegone gets hydroxylated to 9-hydroxypulegone, which on intramolecular cyclization followed by dehydration yields a furanoterpene, menthofuran (Gordon et al., 1987; McClanahan et al., 1988; Madyastha and Raj, 1990; Madyastha and Gaikwad, 1998). In contrast, it was observed that both the isopropylidene methyl groups (C-8 and C-9 methyls) in compounds **I** and **II** are hydroxylated yielding **Ia**, **Ib**, **IIa**, and **IIb** (Fig. 1, A and B). In fact, in these compounds (**I** and **II**), the methyl group *syn* to the carbonyl is poorly hydroxylated. This observation is based on the fact that **Ib** and **IIb** are the minor metabolites, whereas **Ia** and **IIa** are the major metabolites (Fig. 1, A and B) isolated from the urine of rats treated with **I** and **II**, respectively. Interestingly, unlike in the case of *R*-(+)- and *S*-(−)-pulegone (Gordon et al., 1987; McClanahan et al., 1988; Madyastha and Raj, 1990; Madyastha and Gaikwad, 1998), the metabolites **Ib** and **IIb** do not undergo intramolecular cyclization to yield the corresponding furano compounds. On the other hand, the major metabolites **Ia** and **IIa** get further oxidized to the corresponding 9-carboxy derivatives (**Id** and **IId**; Fig. 1, A and B). Earlier studies have established that menthofuran, a furanoterpene, was one of the metabolites responsible for at least half of the hepatocellular necrosis caused by *R*-(+)-pulegone (Thomassen et al., 1988). Further metabolism of menthofuran results in the formation of an α,β-unsaturated-γ-ketoaldehyde (McClanahan et al., 1988; Moorthy et al., 1989b; Madyastha and Raj, 1990) and *p*-cresol (Madyastha and Raj, 1991, 1993) as major metabolites, the former binding irreversibly to the hepatic microsomal fraction in vitro (McClanahan et al., 1989) and the latter a known toxin, as well as a glutathione depletor (Thomassen et al., 1990). So, it is quite possible that the inability of the rat system to produce

furanoterpene from **I** and **II** could be one of the reasons for their failure to elicit hepatotoxicity.

Earlier studies have also shown the hydroxylation of *R*-(+)- and *S*-(−)-pulegone at the C-5 position (hydroxylation at the tertiary carbon atom) to form 5-hydroxypulegone, which upon dehydration yields piperitenone (Madyastha and Raj, 1993; Madyastha and Gaikwad, 1998). It is interesting to note that compounds **I** and **II** are also hydroxylated at the tertiary carbon atom yielding **Ic** and **IIc** (4-hydroxy and 5-hydroxy compounds; Fig. 1, A and B), respectively. The 4-hydroxy compound (**Ic**) undergoes dehydration to form **Ie**, in a manner analogous to the formation of piperitenone from 5-hydroxypulegone. It appears that the keto group allylic to the C-5 methylene protons in **Ic** facilitates the dehydration and formation of the C4-C5 double bond in **Ie** (Fig. 1A). Such a situation does not exist in **IIc** and that may be the reason for its resistance to dehydration. On the other hand, **IIc** gets converted to **IIg** (Fig. 1B) through reduction of the isopropylidene double bond. In a similar way, reduction of the exocyclic double bond in **IIb** and **IId** results in the formation of **IIf** and **IIe**, respectively (Fig. 1B). Earlier, it was shown that reduction of the isopropylidene double bond in pulegone eliminates its hepatotoxic response (Gordon et al., 1982). Hence, it is reasonable to assume that metabolites (Fig. 1B) **IIe**, **IIf**, and **IIg** may not elicit toxicity.

It can be inferred from the present study that the rat system initiates the biotransformation of **I** and **II** either through hydroxylation at the tertiary carbon atom or oxidation of the isopropylidene methyl groups (C-8 and C-9 methyl groups). Studies carried out in vitro using phenobarbital-induced rat liver microsomes in the presence of NADPH and O_2 support this observation and suggest direct hydroxylation of **I** at the C-8, C-9, C-4, and C-3 positions (Fig. 1A). Compound **II** gets hydroxylated at the C-8, C-9, and C-5 positions (Fig. 1B). GC analysis of the assays carried out in vitro indicated that the levels of different metabolites formed from **I** and **II** were significantly higher with phenobarbital-induced rat liver microsomes than with uninduced liver microsomes, suggesting the involvement of the liver microsomal cytochrome P450 system in the formation of these metabolites. In fact, earlier it was demonstrated that the microsomal cytochrome P450 system carries out the regiospecific oxidation of *R*-(+)-pulegone to its allylic alcohol (Gordon et al., 1987; McClanahan et al., 1988; Madyastha and Raj, 1990). It is interesting to note that the hydroxylation at the tertiary carbon atom or oxidation of the isopropylidene methyl groups in compounds **I** and **II** can be specifically blocked through structural modification. This is evident from our studies carried out in vitro using compounds **III** and **IV**. When C-5 hydrogen in **II** is replaced by another methyl group as in 2,2-dimethyl-5-(1-methylethylidene)-cyclopentanone (**III**), the phenobarbital-induced rat liver microsomes transform **III** into metabolites, where the isopropylidene methyl groups are oxidized (Fig. 2A). On the other hand, when the allylic methyl groups in **II** are replaced by ethyl groups as in 5-methyl-2-(1-ethyl-1-propylidene)-cyclopentanone (**IV**), the microsomes carry out hydroxylation only at the tertiary carbon atom to yield **IVa**. Earlier studies have demonstrated that structural modification of *R*-(+)-pulegone by replacing the C-5 hydrogen with a methyl group has blocked one of the major metabolic pathways of *R*-(+)-pulegone initiated through hydroxylation at the C-5 position, although the rat system has retained its ability to carry out allylic methyl hydroxylation (Thulasiram et al., 2000). Opposite of this, phenobarbital-induced rat liver microsomes exclusively carry out hydroxylation at the C-5 position (hydroxylation at the tertiary carbon atom) when the isopropylidene methyl groups in *R*-(+)-pulegone are replaced by ethyl groups as in 2-(1-ethyl-1-propylidene)-5-methylcyclohexanone (**V**, Fig. 2C). The present and the earlier findings (Thulasiram et al., 2000) indicate that it is possible to block the C-5 and C-9 hydroxylation pathways involved in the metabolism of *R*-(+)-pulegone through structural modification. Both these pathways generate toxic metabolites and hence blocking one of the pathways would significantly reduce the hepatotoxic potential of the structurally modified *R*-(+)-pulegone (Thulasiram et al., 2000).

In the present study, we have compared the hepatotoxic potential of compounds **I** and **II** with *R*-(+)-pulegone, a potent hepatotoxin. These preliminary studies point out that **I** and **II** are not hepatotoxic. This observation is based on the fact that i.p. administration (250 mg/kg of b.wt.) of **I**/**II** to rats did not significantly alter the levels of hepatic microsomal cytochrome P450, aminopyrine *N*-demethylase, glucose-6-phosphatase, and serum ALT (Table 1). Pretreatment of rats with phenobarbital, prior to the administration of compounds **I** and **II**, did not significantly change these parameters suggesting that these compounds do not elicit toxicity (results are not presented). In fact, studies carried out in vitro clearly indicate that phenobarbital-induced rat liver microsomes transform **I** and **II** to various metabolites more efficiently than uninduced microsomes. So, if any of these metabolites have the ability to elicit toxicity, then one would expect to see significant changes in the levels of glucose-6-phosphatase, aminopyrine *N*-demethylase, and ALT after the administration of **I** or **II** to rats pretreated with phenobarbital. Since the levels of microsomal enzymes and ALT have not changed, it appears that the metabolism of **I** and **II** does not result in the formation of reactive metabolites responsible for eliciting toxicity. However, consistent with the earlier report (Moorthy et al., 1989a, 1991), *R*-(+)-pulegone caused severe hepatotoxicity in rats, as evidenced by a significant increase in ALT levels (a 13-fold increase, Table 1) and potentiation of hepatotoxicity when it was administered to PB-treated rats (results not shown). Our results suggest that the hepatotoxic potential of *R*-(+)-pulegone can be eliminated by reducing the ring size as in **I** and **II**. This could be possibly due to the differences in the mode of metabolism between *R*-(+)-pulegone and compounds **I** and **II**.

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