Transformation of a Monoterpene Ketone, (*R*)-(+)-Pulegone, a Potent Hepatotoxin, in *Mucor piriformis*

K. M. Madyastha*,†,‡ and H. V. Thulasiram†

Department of Organic Chemistry, Indian Institute of Science, Bangalore 560012, India, and Chemical Biology Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore 560064, India

Biotransformation of a monoterpene ketone, (*R*)-(+)-pulegone (**I**), a potent hepatotoxin, was studied using a fungal strain, *Mucor piriformis*. Eight metabolites, namely, 5-hydroxypulegone (**II**), piperitenone (**III**), 6-hydroxypulegone (**IV**), 3-hydroxypulegone (**V**), 5-methyl-2-(1-hydroxy-1-methylethyl)-2-cyclohexene-1-one (**VI**), 3-hydroxyisopulegone (**VII**), 7-hydroxypiperitenone (**VIII**), and 7-hydroxypulegone (**IX**), have been isolated from the fermentation medium and identified. GC analysis of the metabolites indicated that **II** was the major metabolite formed. The organism initiates transformation either by hydroxylation at the C-5 position or by hydroxylation of the ring methylenes, the former being the major activity. On the basis of the identification of the metabolites, pathways for the biotransformation of (*R*)-(+)-pulegone have been proposed. The mode of transformation of (*S*)-(−)-pulegone by this organism was shown to be similar to that of its (*R*)-(+)-enantiomer. When isopulegone (**X**) was used as the substrate, the organism isomerized it to pulegone (**I**), which was then transformed to metabolites **II**−**IX**.

Keywords: *(R)-(+)- and (S)-(−)-pulegone; monoterpene ketone; biotransformation; fungal system; hepatotoxin; metabolites*

INTRODUCTION

(*R*)-(+)-Pulegone (**I**), a monoterpene ketone, is the major constituent of pennyroyal oil from *Mentha pulegium*. The oil is used as a fragrance component and a flavoring agent. Studies carried out with animals have shown that (*R*)-(+)-pulegone is both hepatotoxic and pneumotoxic (Gordon et al., 1982; Thorup et al., 1983; Moorthy et al., 1991). Surprisingly, (*S*)-(−)-pulegone is far less toxic than its (*R*)-enantiomer (Gordon et al., 1982). The metabolic fate of (*R*)-(+)-pulegone (**I**) in rats has been studied in great detail, and these studies have indicated the existence of two major pathways for its biotransformation. One of the pathways is initiated through regiospecific hydroxylation of **I** to 9-hydroxypulegone, which is further converted to menthofuran (Gordon et al., 1987; Madyastha and Paul Raj, 1990). The other major pathway involves stereoselective hydroxylation of **I** to 5-hydroxypulegone, which upon dehydration yields piperitenone (Moorthy et al., 1989; Madyastha and Paul Raj, 1993).

Whereas considerable work has been carried out on the mammalian degradation of (*R*)-(+)-pulegone, very little is known regarding the microbial metabolism of this hepatotoxin. It is of interest to know whether microbes bring about transformation in (*R*)-(+)-pulegone in a manner similar to what has been observed in the mammalian system and, if so, then it would be easier to prepare some of the reactive metabolites in large quantities that can be used for their detailed study in the mammalian system. Earlier, *Mucor piriformis* was shown to carry out novel and preparatively useful transformations of steroids (Madyastha and Joseph, 1994, 1995) and alkaloids (Madyastha and Reddy, 1994). This fungal strain very efficiently transforms (*R*)-(+)- and (*S*)-(−)-pulegone to various metabolites. In fact, *M. piriformis* is more versatile in its ability to transform (*R*)-(+)-pulegone than some of the fungi tested earlier for carrying out the biotransformation of (*R*)-(+)-pulegone (Mustapha et al., 1992).

We report here the isolation and identification of eight metabolites, of which five of them have not been reported earlier. The mode of transformation of (*R*)-(+)-pulegone (**I**), its (*S*)-enantiomer, and isopulegone by *M. piriformis* are also compared.

MATERIALS AND METHODS

Materials. (*R*)-(+)-Pulegone (**I**) and isopulegol were obtained from Aldrich Chemical Co. (Milwaukee, WI). (*S*)-(−)-Pulegone was synthesized as reported earlier (Corey et al., 1976). Isopulegone was prepared by the pyridinium chlorochromate oxidation of isopulegol (Corey and Suggs, 1975). Isopulegone obtained was purified by column chromatography over silica gel using 5% ethyl acetate in hexane. ^{1}H NMR ($CDCl_3$) spectrum showed signals at δ 5.14 (s, 2H, olefinic protons), 2.18−1.8 (m, 8H, ring protons), 1.78 (s, 3H, allylic methyl), and 0.98 (d, 3H, J = 7.2 Hz, ring methyl). Mass spectrum gave the following: m/z 152 (M^+), 137 ($M^+ - CH_3$) 109 ($M^+ - C_3H_7$), 93 ($M^+ - C_3H_7O$), 81 ($M^+ - C_4H_7O$), and 67 ($M^+ - C_5H_9O$).

Microorganism. The organism used in the present study was isolated from soil and identified as *Mucor piriformis* (Madyastha and Srivatsan, 1987). It was maintained and propagated as reported earlier (Madyastha and Srivatsan 1987).

Conditions for Fermentation. Fermentations were carried out in modified Czapek Dox medium (Prema and Bhat-

* Author to whom correspondence should be addressed (telephone 091-080-3092541; fax 091-080-3341683; e-mail kmm@orgchem.iisc.ernet.in).

† Indian Institute of Science.

‡ Jawaharlal Nehru Centre for Advanced Scientific Research.

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tacharyya, 1962). The pH of the medium was adjusted to 6.8−7.0 with 1 N NaOH. Flasks (500 mL) containing 100 mL of sterile medium were inoculated with 1 mL of a spore suspension from a 5-day-old culture grown on potato dextrose agar (PDA) slants and were incubated at 29−30 °C on a rotary shaker (220 rpm) for 36 h. After this growth period, the pH of the medium was again adjusted to 7.0 by the addition of sterile 1 N NaOH, 60 mg of (*R*)-(+)-pulegone in acetone (0.2 mL) was added to each flask, and the incubation was continued for an additional period of 48 h (increase in the substrate concentration considerably decreased the yields of the metabolites). A control experiment was also run with the substrate but without the organism. In a similar way fermentation was carried out using (*S*)-(−)-pulegone and isopulegone as substrates.

In time course experiments, incubations were carried out for 24, 48, 72, and 96 h and the metabolites formed at the end of each incubation period were monitored by GC analysis. Quantification was made by measuring the area under the peak. The levels of different metabolites formed were determined on the basis of the area under the respective peaks and compared with the standard graphs obtained for each metabolite by injecting known amounts of these metabolites. All analyses were carried out under identical conditions.

Extraction of Metabolites. At the end of the incubation period, the contents from all of the flasks were pooled, the pH was adjusted to 5.5−6.0, and the mixture was filtered. The filtrate (broth) and mycelia were separately extracted with $CHCl_3$. The two $CHCl_3$ extracts were combined, concentrated, and separated into acidic and neutral fractions by treatment with 5% sodium bicarbonate solution. The bicarbonate phase was acidified and re-extracted with $CHCl_3$. The acidic fraction contained compounds derived from the organism and hence was not processed further. The neutral fraction was subjected to column chromatography and preparative TLC to isolate the metabolites in the pure form.

Chromatography. 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 hexanes/ethyl acetate (8:2, v/v, system I) or chloroform/methanol (9.7:0.3, v/v, system II). Compounds were visualized by spraying with 3% vanillin in 1% methanolic H_2SO_4, followed by heating at 100 °C for 5−10 min. GC analyses were carried out on a Shimadzu model 14A instrument equipped with a hydrogen flame ionization detector and a Shimadzu HR-1 wide bore capillary column (15 cm × 0.5 mm). N_2 was used as the carrier gas at a flow rate of 30 mL/min. Initially the column temperature was maintained at 80 °C for 10 min, after which time the temperature was raised at 5 °C/min to 150 °C and maintained at 150 °C for 5 min.

Hydrogenation of Compounds VIII and IX. The compound (5.0 mg, **VIII**/**IX**) 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, passed through a Celite bed, concentrated, and subjected to column chromatography over silica gel, and the compound was eluted using 5% ethyl acetate in hexane.

Spectral Studies. Infrared (IR) spectra were recorded either on a Perkin-Elmer model 781 or on a Hitachi 270-50 spectrophotometer. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a JEOL FT-90 MHz spectrometer. Chemical shifts are reported in parts per million, with respect to tetramethylsilane as the internal standard. Mass spectra (MS) were recorded on a JEOL-JMS-DX 303 instrument with JMA-DA 5000 data system.

RESULTS

A batch of 50 flasks was inoculated with *M. piriformis*, and at the end of 2 days after the addition of substrate, the contents of all the flasks were pooled and processed as described under Materials and Methods. The neutral fraction (2.6 g) upon TLC analysis (system I) showed

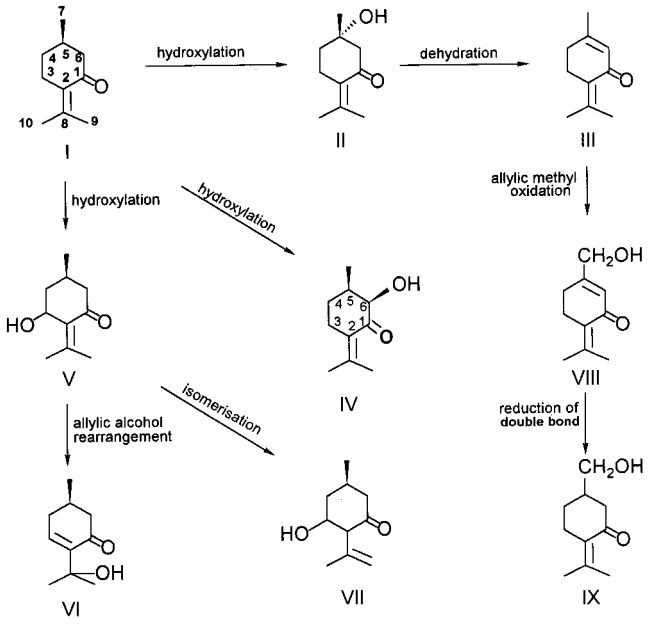

Figure 1. Transformation of (*R*)-(+)-pulegone by *M. piriformis*.

the presence of at least eight compounds that were absent in the control experiment. This fraction was subjected to column chromatography on silica gel (80 g), and the metabolites were eluted with hexane/ethyl acetate mixtures. Elution of the column with hexane yielded a fraction containing the least polar compound (R_f 0.79, system I). The GC analysis of this fraction showed the presence of one major peak (t_R = 3.7 min) and one minor peak (t_R = 2.4 min). The major and minor peaks were enhanced when mixed with pulegone (**I**) and isopulegone (**X**), respectively. The mass spectral analysis of these two peaks (t_R = 3.7 and 2.4 min) corresponded well with those of authentic pulegone (**I**) and isopulegone (**X**).

The fraction eluted with ethyl acetate (0.5%) in hexane contained a mixture of two compounds with R_f values of 0.62 (**IV**) and 0.59 (**III**) (system I), which were separated and purified by repeated preparative TLC (system I). Compound **IV** showed an optical rotation of +66 (*c* 1.0, $CHCl_3$) and UV absorption maximum at 254 nm. The IR, ^{1}H NMR, and MS data for this compound are given in Table 1. From the spectral characteristics, the compound was identified as 6-hydroxypulegone (**IV**, Figure 1). The spectral data agreed with an earlier report for this compound (Imamura et al., 1975). Earlier studies also suggested that the C-5 methyl group and the C-6 hydroxyl group in **IV** are cis to each other (Imamura et al., 1975). Compound **III** (R_f 0.59, system I; t_R = 6.8 min) showed UV absorption maxima at 276 and 244 nm. From the spectral characteristics (Table 1), the compound was identified as piperitenone (**III**, Figure 1). The identity of the metabolite was further confirmed by comparing the NMR and MS data with those of an authentic compound prepared as reported earlier (Nakanishi et al., 1980).

Elution of the column with 3% ethyl acetate in hexane gave fractions containing mainly one compound (R_f 0.33, system I; t_R = 4.9 min), which was further purified by preparative TLC (system I). The IR, ^{1}H NMR, and MS data for this compound {**VI**, $[\alpha]_D^{26} = -32°$ (*c* 1.0, in $CHCl_3$)} are shown in Table 1. On the basis of the spectral characteristics, the compound was assigned the structure 5-methyl-2-(1-hydroxy-1-methylethyl)-2-cyclo-

Table 1. Spectral Data for Metabolites II−IX[a]

compd	IR (neat) γ_{max}	^{1}H NMR (δ) $CDCl_3$	MS (LR and HRMS)
II	3400 cm^{-1} (−OH) 1665 and 1600 cm^{-1} (conjugated carbonyl)	2.48 (4H, m, H-3 and H-6), 1.95 (3H, s, H-9), 1.8−2.0 (2H, m, H-4), 1.8 (3H, s, H-10), 1.25 (3H, s, H-7)	*m/z*: 168 (M^+, 31%), 150 ($M^+ - H_2O$, 20%), 135 ($M^+ - CH_3 - H_2O$, 30%), 107 ($M^+ - C_2H_5O$, 39%), 67 ($M^+ - C_5H_9O_2$, 48%) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1154
III	1640 and 1595 cm^{-1} (conjugated carbonyl)	5.8 (1H, br s, H-6), 2.6 (2H, t, H-4), 2.2 (2H, t, H-3), 2.0 (3H, s, H-7), 1.85 (3H, s, H-9), 1.8 (3H, s, H-10)	*m/z*: 150 (M^+, 100%), 135 ($M^+ - CH_3$, 50%), 122 ($M^+ - CO$, 12%), 91 ($M^+ - C_3H_7O$, 17%)
IV	3460 cm^{-1} (−OH) 1675 and 1610 cm^{-1} (conjugated carbonyl)	4.2 (1H, d, J = 6.4 Hz, H-6), 3.98 (1H, br s, 6-OH), 2.2−2.6 (4H, m, H-3 and H-4), 1.98 (3H, s, H-9), 1.83 (3H, s, H-10), 0.88 (3H, d, J = 7.7 Hz, H-7)	*m/z*: 168 (M^+, 50%), 150 ($M^+ - H_2O$, 13%), 125 ($M^+ - C_3H_7$, 100%) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1140
V	3400 cm^{-1} (−OH) 1670 and 1600 cm^{-1} (conjugated carbonyl)	4.95 (1H, t, H-3), 1.92−2.6 (5H, m, H-4, H-5, and H-6), 1.9 (3H, s, H-9), 1.85 (3H, s, H-10), 0.95 (3H, d, J = 6.4 Hz, H-7)	*m/z*: 168 (M^+, 9%), 150 ($M^+ - H_2O$, 100%), 135 ($M^+ - CH_3 - H_2O$, 49%) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1144
VI	3420 cm^{-1} (−OH) 655 cm^{-1} (conjugated carbonyl)	6.88 (1H, dd, J = 5.6 and 2.4 Hz, H-3), 4.38 (1H, br s 8-OH), 2.1−2.5 (5H, m, H-4, H-5, and H-6), 1.4 (6H, s, H-9 and H-10), 1.07 (3H, d, J = 5.98 Hz, H-7)	*m/z*: 168 (M^+, 4%), 153 ($M^+ - CH_3$, 100%), 150 ($M^+ - H_2O$, 26%), 135 ($M^+ - CH_3 - H_2O$, 29%), 109 ($M^+ - C_3H_7O$, 19%) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1157
VII	3400 cm^{-1} (−OH) 700 cm^{-1} (carbonyl)	5.28 and 4.98 (2H, 2s, H-9), 3.8 (1H, m, H-3), 2.9 (1H, d, J = 11.6 Hz, H-2), 2.48−1.7 (5H, m, H-4, H-5 and H-6), 1.88 (3H, s, H-10), 1.1 (3H, d, J = 7.7 Hz, H-7)	*m/z*: 168 (M^+, 20%), 153 ($M^+ - CH_3$, 30%), 150 ($M^+ - H_2O$, 43%), 135 ($M^+ - CH_3 - H_2O$, 30%), 109 ($M^+ - C_3H_7O$, 58%) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1152
VIII	3400 cm^{-1} (−OH) 1650 and 1590 cm^{-1} (conjugated carbonyl)	6.2 (1H, s, H-6), 4.3 (2H, s, H-7), 2.68 (2H, t, H-4), 2.28 (2H, t, H-3), 2.15 (3H, s, H-9), 1.85 (3H, s, H-10)	*m/z*: 166 (M^+, 100%), 135 ($M^+ - CH_2OH$, 31%), 123 ($M^+ - C_3H_7$, 29%) HRMS: $C_{10}H_{14}O_2$ requires 166.0994, found 166.0998
IX	3400 cm^{-1} (−OH) 1660 and 1590 cm[1] (conjugated carbonyl)	3.55 (2H, d, J = 5.1 Hz, H-7), 2.6−2.05 (7H, m, ring protons), 2.0 (3H, s, H-9), 1.8 (3H, s, H-10), 1.68 (1H, s, 7-OH)	*m/z*: 168 (M^+, 29%), 137 ($M^+ - CH_2OH$, 12%), 84 ($M^+ - C_5H_8O$, 100%) HRMS: $C_{10}H_{16}O_2$ requires 168.1150, found 168.1142

[a] Details are as mentioned under methods.

hexene-1-one (**VI**, Figure 1). The spectral data agreed with those given in an earlier paper on this compound (Nagasawa et al., 1975).

Elution of the column with 7% ethyl acetate in hexane yielded fractions containing a compound (R_f 0.22, system I; t_R = 5.2 min) that was further purified by preparative TLC (system I). The spectral data of this optically active compound {$[\alpha]_D^{26}$ = +20° (*c* 1.0, in $CHCl_3$)} are presented in Table 1. From the spectral characteristics, the compound was identified as 3-hydroxyisopulegone (**VII**, Figure 1).

Further elution of the column with 9% ethyl acetate in hexane gave a fraction which upon TLC analysis revealed the presence of one major compound and one minor compound (R_f 0.36 and 0.43, system II). In GC analysis, these two compounds did not separate (conditions as mentioned under Materials and Methods) and gave a single peak (t_R = 10.7min). It was also observed from GC analysis of this mixture that the peak corresponding to t_R = 10.7 min decreased upon storage at room temperature, with the appearance of a new peak (t_R = 6.8 min) that was enhanced when mixed with piperitenone (**III**, Figure 1). These two compounds were separated by preparative TLC (system II). The minor compound {R_f 0.43, system II; $[\alpha]_D^{26}$ = +12° (*c* 1.0, in $CHCl_3$)} was identified as 3-hydroxypulegone (**V**, Figure 1) on the basis of various spectral characteristics (Table 1). The major compound {R_f 0.36, system II; $[\alpha]_D^{26}$ −31° (*c* 1.0, in $CHCl_3$)} was identified as 5-hydroxypulegone (**II**, Figure 1) by comparing the spectral characteristics with the earlier study on this compound (Mustapha et al., 1992; Madyastha and Paul Raj, 1993).

The fraction eluted with 12% ethyl acetate in hexane contained a mixture of two compounds with R_f values of 0.30 and 0.26 (system II). These two compounds were separated and purified by preparative TLC (system II). The IR, ^{1}H NMR, and mass spectral data for these compounds (**VIII**, **IX**) are given in Table 1. From the spectral characteristics, the compounds with R_f 0.30 and 0.26 (system II) were identified as 7-hydroxypulegone (**VIII**, Figure 1) and 7-hydroxypiperitenone (**IX**, Figure 1), respectively. The structures assigned (**VIII** and **IX**) were further confirmed by hydrogenation of **VIII** and **IX** using palladium charcoal as a catalyst. Both **VIII** and **IX** upon hydrogenation yielded the same product, which had the following spectral characteristics: IR spectrum (neat) γ_{max}, 3400 cm^{-1} (hydroxyl) and 1695 cm^{-1} (carbonyl); ^{1}H NMR ($CDCl_3$) δ 3.49 (2H, d, J = 5.42 Hz, H-7), 2.3−1.62 (9H, m, ring protons), and 0.86, 0.79 (6H, 2d, J = 6.58, 6.54 Hz, H-9 and H-10); MS, *m/z* 170 (M^+, 52%), 155 ($M^+ - CH_3$, 40%), 152 ($M^+ - H_2O$, 10%), 139 ($M^+ - CH_2OH$, 33%), 128 ($M^+ - C_3H_6$, 65%), and 69 (100%); HRMS, $C_{10}H_{18}O_2$ requires 170.1307, found 170.1302. The hydrogenated product was identified as 2-isopropyl-5-(hydroxymethyl)cyclohexanone.

The time course experiment carried out with (*R*)-(+)-pulegone (**I**) revealed that during the early stages of incubation (24 h) nearly 40% of **I** was transformed into various metabolites (**II**−**IX**, Figure 1). By prolonging the incubation period to 48 and 72 h, the transformation was increased to 62% and 70%, respectively. However, incubation carried out beyond 72 h did not result in any appreciable improvement in the yields of the metabolites. GC profiles of the total metabolites formed at different time intervals (24, 48, and 72 h) clearly indicated that 5-hydroxypulegone (**II**) was the major metabolite formed (Figure 2).

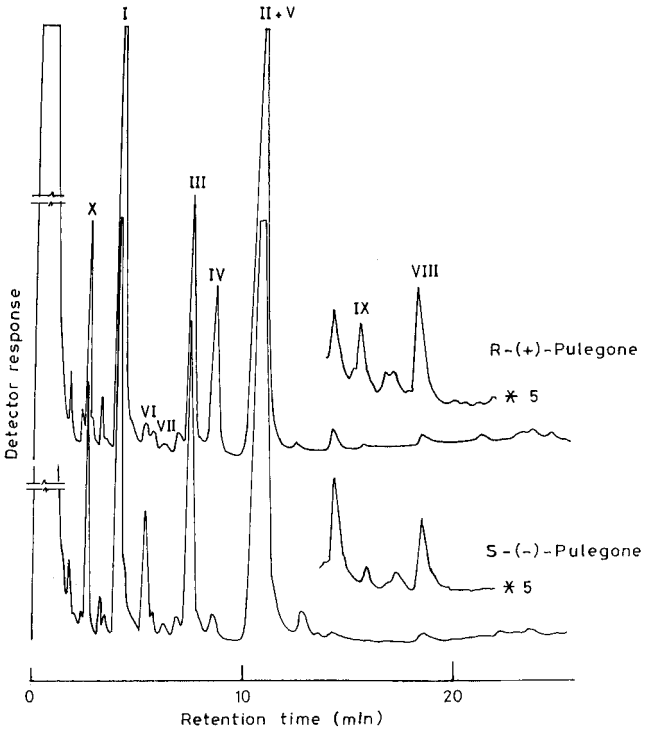

Figure 2. GC separation of the neutral metabolites obtained by incubating (*R*)-(+)- and (*S*)-(−)-pulegone with *M. piriformis* for 48 h. Experimental conditions are as reported under Materials and Methods. **I**, pulegone; **II**, 5-hydroxypulegone; **III**, piperitenone; **IV**, 6-hydroxypulegone; **V**, 3-hydroxypulegone; **VI**, 5-methyl-2-(1-hydroxy-1-mthylethyl)-2-cyclohexene-1-one; **VII**, 3-hydroxyisopulegone; **VIII**, 7-hydroxypiperitenone; **IX**, 7-hydroxypulegone; **X**, isopulegone. *5 indicates that particular area is magnified 5 times.

Biotransformation of (*S*)-(−)-Pulegone. The organism *M. piriformis* also accepts (*S*)-(−)-pulegone as substrate. The fermentation of (*S*)-(−)-pulegone was carried out under the same experimental conditions as those used for its (*R*)-enantiomer (**I**). Nearly 60% of (*S*)-(−)-pulegone was biotransformed at the end of 48 h of incubation. The GC analysis of the total neutral fraction from (*S*)-(−)-pulegone clearly indicated that all of the metabolites isolated and identified (**II**−**IX**) from (*R*)-(+)-pulegone (**I**) were also shown to be formed from its (*S*)-enantiomer (Figure 2) as judged by their retention time and peak enhancement, which occurred when mixed with the metabolites (**II**−**IX**) isolated and identified from (*R*)-(+)-pulegone. There was no significant difference in the levels of various metabolites formed from both the enantiomers except that the level of 6-hydroxypulegone (**IV**) formed was appreciably higher (70−80% more) in the case of (*R*)-(+)-pulegone than (*S*)-(−)-pulegone (Figure 2). When isopulegone was used as the substrate, it was observed that the organism initiated the biotransformation by converting it to pulegone, which was further transformed into various metabolites (**II**−**IX**, Figure 1).

DISCUSSION

Earlier studies have clearly demonstrated that the hepatotoxin (*R*)-(+)-pulegone is extensively metabolized in the rat system (Madyastha and Paul Raj, 1993). One of the major pathways and perhaps the most significant pathway involved in the biotransformation is the regiospecific oxidation of (*R*)-(+)-pulegone (**I**) to 9-hydroxypulegone, in which the hydroxy and keto groups are syn to each other. This allylic alcohol readily undergoes intramolecular cyclization followed by dehydration to menthofuran, which is considered as the proximate toxin responsible for at least 50% of the toxicity elicited by (*R*)-(+)-pulegone (Thomassen et al., 1988). Menthofuran is further metabolized to an α,β-unsaturated-γ-keto aldehyde and *p*-cresol (McClanahan et al., 1989; Madyastha and Paul Raj, 1992), and these two metabolites substantially contribute toward (*R*)-(+)-pulegone-mediated toxicity. In contrast, the present studies have clearly demonstrated that *M. piriformis* does not carry out 9-hydroxylation of both (*R*)-(+) and (*S*)-(−)-pulegone, suggesting that these enantiomers are not transformed to menthofuran. However, the organism very efficiently transformed both (*R*)-(+) and (*S*)-(−)-pulegone to various metabolites, and the mode of metabolism seems to be operating either through hydroxylation at the C-5 position or through ring methylene hydroxylation. The 5-hydroxypulegone (**II**) upon dehydration yields piperitenone (**III**). Piperitenone (**III**) has also been shown to be formed in the mammalian system as one of the metabolites of both (*R*)-(+)- and (*S*)-(−)-pulegone, although its formation is significantly greater from (*R*)-(+)-pulegone than from its (*S*)-enantiomer (Madyastha and Paul Raj, 1993; Madyastha and Gaikwad, 1998), suggesting hydroxylation at C-5 position is stereoselective. On the contrary, *M. piriformis* transformed both of the enantiomers of pulegone to **III** to the same extent, indicating that hydroxylation at the C-5 position is not stereoselective. It is interesting to note that mammals convert piperitenone (**III**) to 9-hydroxypiperitenone, which upon cyclization yields 6,7-dehydromenthofuran (unpublished observation), a furan compound that has the potential to generate toxicity. However, such a transformation is not possible in *M. piriformis* because the organism is not capable of carrying out 9-hydroxylation of piperitenone (**III**). In the mammalian system, further metabolism of **III** leads to the formation of *p*-cresol, a known toxin (Sax, 1951). It is believed that part of the toxicity mediated by (*R*)-(+)-pulegone is due to the formation of *p*-cresol (Madyastha and Paul Raj, 1991; Thompson et al., 1994). Present studies have also noted that *M. piriformis* does not transform **III** to *p*-cresol.

Although C-5 hydroxylation seems to be the major activity, the organism also carries out hydroxylation at C-3 and C-6 positions to form 3-hydroxy- (**V**) and 6-hydroxypulegone (**IV**), respectively. It is possible to envisage the formation of **VI** and **VII** from **V** through an allylic alcohol rearrangement and double-bond isomerization. 7-Hydroxypiperitenone (**VIII**) could have been formed through oxidation of the allylic methyl group in **III**. In fact, hydroxylation of the allylic methyl group in some of the monoterpenes has been observed earlier in microbial (Ranganathan and Madyastha, 1983; Madyastha and Murthy, 1988) and mammalian systems (Madyastha and Chadha, 1982). In the present studies, it was observed that significantly higher levels of 6-hydroxypulegone (**IV**) were formed from (*R*)-(+)-pulegone than from its (*S*)-enantiomer (Figure 2). It is quite possible that inversion of configuration of the C-5 methyl group would affect the hydroxylation reaction at the C-6 position. From the present and earlier studies (Mustapha et al., 1992) it can be inferred that fungal species tested so far do not produce reactive metabolites which have been shown to be formed during

the biotransformation of (*R*)-(+)- pulegone in the mammalian system (Madyastha and Paul Raj, 1993).

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