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In Vivo Studies on the Metabolism of the Monoterpene Pulegone in Humans Using the Metabolism of Ingestion-Correlated Amounts (MICA) Approach: Explanation for the Toxicity Differences between (*S*)-(−)- and (*R*)-(+)-Pulegone

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The major in vivo metabolites of (*S*)-(−)-pulegone in humans using a metabolism of ingestion-correlated amounts (MICA) experiment were newly identified as 2-(2-hydroxy-1-methylethyl)-5-methylcyclohexanone (8-hydroxymenthone, **M1**), 3-hydroxy-3-methyl-6-(1-methylethyl)cyclohexanone (1-hydroxymenthone, **M2**), 3-methyl-6-(1-methylethyl)cyclohexanol (menthol), and *E*-2-(2-hydroxy-1-methylethylidene)-5-methylcyclohexanone (10-hydroxypulegone, **M4**) on the basis of mass spectrometric analysis in combination with syntheses and NMR experiments. Minor metabolites were be identified as 3-methyl-6-(1-methylethyl)-2-cyclohexenone (piperitone, **M5**) and α,α,4-trimethyl-1-cyclohexene-1-methanol (3-*p*-menthen-8-ol, **M6**). Menthofuran was not a major metabolite of pulegone and is most probably an artifact formed during workup from known (**M4**) and/or unknown precursors. The differences in toxicity between (*S*)-(−)- and (*R*)-(+)-pulegone can be explained by the strongly diminished ability for enzymatic reduction of the double bond in (*R*)-(+)-pulegone. This might lead to further oxidative metabolism of 10-hydroxypulegone (**M4**) and the formation of further currently undetected metabolites that might account for the observed hepatotoxic and pneumotoxic activity in humans.

KEYWORDS: Metabolism of ingestion correlated amounts (MICA); urinary metabolites; pulegone; menthol; piperitone; 2-(2-hydroxy-1-methylethyl)-5-methylcyclohexanone; 3-hydroxy-3-methyl-6-(1-methylethyl)-cyclohexanone; 3-methyl-6-(1-methylethyl)cyclohexanol; E-2-(2-hydroxy-1-methylethylidene)-5-methylcyclohexanone; 3-methyl-6-(1-methylethyl)-2-cyclohexenone; α,α,4-trimethyl-1-cyclohexene-1-methanol

INTRODUCTION

(*R*)-(+)-Pulegone (**Figure 1**) is a constituent of peppermint and pennyroyal oil. Additionally, it occurs in many other foods such as oregano, beans, and tea. It is widely used as a flavor constituent having Generally Recognized As Safe (GRA) status in the United States. In the European Union the following maximum levels for pulegone in foodstuffs have been set: 25 mg/kg in foodstuffs in general; 100 mg/kg in beverages; 250 mg/kg in peppermint- or mint-flavored beverages; and up to 350 mg/kg in mint confectionery. Despite its use in flavorings, there are severe toxic effects of pulegone at large doses (*1*, *2*), very recently discussed by the Scientific Committee of the European Commission (*3*). The other enantiomer, (*S*)-(−)-pulegone (**Figure 1**) is found rarely in essential oils.

There have been many investigations regarding the toxicity of pulegone, especially of the (*R*)-(+)-enantiomer, which seems to be much more toxic compared to the (*S*)-(−)-enantiomer.

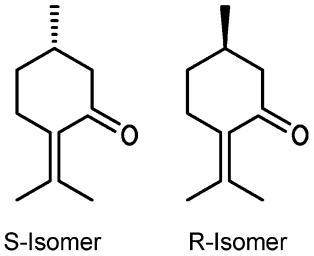

Figure 1. Structures of (*S*)-(−)-pulegone and (*R*)-(+)-pulegone.

Menthofuran was very early identified as a metabolite of pulegone formed after ingestion of large amounts of pennyroyal oil (*4*), and it is generally accepted that the hepatotoxicity of pulegone is due, at least in part, to this metabolite. There have been several studies of (*R*)-(+)- and (*S*)-(−)-pulegone metabolism using rats as test animals (*5*−*7*, *9*, *10*) and in vitro experiments with rat liver microsomes (*8*, *9*, *11*) and human cytochrome P450 enzymes (*12*). Additionally, very recently the focus was extended to the role of the enzyme glutathione

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S-transferase during phase I (*13*) and phase II (*10*) metabolism of pulegone.

From these data it seems that the metabolism of pulegone is very well established, even though the most recent publication (*10*) could not confirm some of the data published earlier, basically, that menthofuran is a major metabolite. However, to our knowledge there has never been a study focusing on the metabolism of pulegone in humans in general. Therefore, both enantiomers of pulegone were metabolized in MICA experiments in this study. The aims were, first, to clarify whether the metabolites found in animals can be also detected at ingestion levels of pulegone that are comparable to a diet, second, to clarify if there are other metabolites present that might have been overlooked because of the early focus on menthofuran, and, finally, to compare the data with the most recent publication (*10*), which has shed some doubt on the findings of the earlier publications.

MATERIALS AND METHODS

Chemicals. (*S*)-(−)-Pulegone, (*R*)-(+)-pulegone, and (+)-menthofuran were from Fluka (Buchs, Switzerland). Dess−Martin periodinane was from Lancaster (Newgate, U.K.). β-Glucuronidase H2 from *Helix pomatia* (100000 units/mL β-glucuronidase, 4500 units/mL sulfatase) was from Sigma (Steinheim, Germany). A racemic mixture of *p*-menthane-3,9-diol was a generous gift from Dr. Stefano Serra (CNR, Milano, Italy). Piperitone and mintlactone were generous gifts from Dr. Akira Fujita (T. Hasegawa, Tokyo, Japan).

MICA Experiment. Human experimentation was performed as previously described (*14*). The amount of (*R*)-(+)-pulegone applied was 35 mg (0.24 mmol; ∼500 μg/kg of body mass), whereas the amount of the less toxic (*S*)-(−)-pulegone was 70 mg (0.48 mmol; ∼1000 μg/kg of body mass).

Isolation of Metabolites from Urine and Derivatization Procedures. Isolation was performed as previously described (*14*). Briefly, after enzymatic hydrolysis at pH 5.0 with glucuronidase and sulfatase from *H. pomatia* and careful acidification to pH 2.0 with concentrated HCl, the metabolites were subsequently extracted with diethyl ether followed by a separation into neutral, phenolic, and acidic compounds. The metabolites in the acidic fraction were trimethylsilylated with BSTFA or ethylated with iodoethane (*14*).

Reference Compounds. Authentic standards were prepared according to the procedures described below. All syntheses started from either (*S*)-(−)-pulegone or (*R*)-(+)-pulegone, retaining the stereochemistry at C-1 of the menthane skeleton.

Synthesis of 2-(2-Hydroxy-1-methylethyl)-5-methylcyclohexanone (9-Hydroxy-*p*-menthan-3-one, M1). M1 was synthesized in a four-step sequence starting from isopulegol.

2-Hydroxy-β,4-dimethylcyclohexaneethanol (p-Menthane-3,9-diol). *p*-Menthane-3,9-diol was synthesized according to a published procedure (*15*) starting from isopulegol by hydroboration and oxidation with alkaline H_2O_2 solution. Independently, reference material was obtained from CNR, Milano, Italy.

Acetic Acid [2-(2-Hydroxy-4-methylcyclohexyl)propyl] Ester (9-Acetoxy-p-menthan-3-ol). To a solution of *p*-menthane-3,9-diol (1 mmol) in ethyl acetate (5 mL) were added acetic anhydride (1.0 mmol) and 4-(dimethylamino)pyridine (0.1 mmol). The mixture was stirred at room temperature until all of the diol was converted, as indicated by GC-MS of the reaction mixture. The solution was evaporated, diluted with CH_2Cl_2 (50 mL), and washed twice with water (5 mL), followed by freshly prepared saturated $NaHCO_3$ solution (5 mL). The solution (mixture of two diastereoisomers) was dried over Na_2SO_4 and used for the next step without further purification. MS (EI), *m*/*z* (relative intensity) 43 (58), 55 (40), 67 (35), 68 (37), 69 (44), 81 (82), 95 (53), 97 (70), 107 (38), 112 (100), 121 (40), 136 (34), 214 (0.5). The MS (EI) data were in excellent agreement with those previously published (*16*).

Acetic Acid [2-(4-Methyl-2-oxocyclohexyl)propyl] Ester (9-Acetoxy-p-menthan-3-one). To a suspension of Dess−Martin periodinane (1.2 mmol) in CH_2Cl_2 (10 mL) was added a solution of 9-acetoxy-*p*-menthan-3-ol (∼1 mmol) in CH_2Cl_2 (50 mL). The mixture was stirred overnight at room temperature. The solution was concentrated to 5 mL using a rotary evaporator, and the white precipitate was removed by filtration. Column chromatography on a 100 mm × 30 mm, 25−40 μm, LiChroprep RP-18 column (water/methanol gradient) at 1.5 mL/min gave pure acetic acid [2-(4-methyl-2-oxocyclohexyl)propyl] ester as a colorless liquid: yield, 134 mg (63% based on *p*-menthane-3,9-diol); elemental composition (HRMS) $C_{12}H_{20}O_3$, found (212.1381), calcd (212.1412); MS (EI), *m*/*z* (relative intensity) 41 (27), 43 (63), 55 (27), 67 (17), 69 (29), 70 (18), 84 (15), 95 (14), 97 (20), 109 (15), 112 (100), 113 (19), 137 (16), 212 (2); ^{1}H NMR data (for the prevailing diastereoisomer) ($CDCl_3$) $\underline{CH_3}$−CH−CH_2O (0.99, d, J = 6.7 Hz, 3H), $\underline{CH_3}$−CH−CH_2−CO (1.02, d, J = 6.3 Hz, 3H), $\underline{CH_3}$−CO (2.05, s, 3H), $\underline{CH_2}$−O (4.05, m, 2H), C$\underline{H}$ and $\underline{CH_2}$ of cyclohexane ring (2.37, m, 1H; 2.27 m, 2H; 1.8−2.1, m, 4H; 1.3−1.5, m, 2H); ^{13}C NMR ($CDCl_3$) $\underline{CH_3}$−CH−CH_2O (15.25), $\underline{CH_3}$−CO (20.88), $\underline{CH_3}$−CH−CH_2−CO (22.21), CH_2−$\underline{CH_2}$−CH−CO (28.92), $\underline{CH_2}$−CH_2−CH−CO (33.96), $\underline{CH_2}$−CO (50.72), $\underline{CH_2}$−O (66.87), CH_3−$\underline{CH}$−CH_2CO (31.29), CH_3−$\underline{CH}$−CH_2O (35.30), $\underline{C}$H−CO (52.29), CH_3−$\underline{C}$OO (171.06), CH−$\underline{C}$O (211.00).

*2-(2-Hydroxy-1-methylethyl)-5-methylcyclohexanone (9-Hydroxy-p-menthan-3-one, **M1**).* The hydrolysis of pure 9-acetoxymenthone (10 mg) was performed using lithium methylate (5 μL of a 1.3 mmol/mL solution in methanol) in methanol (1 mL), HCl (5 μL) in aqueous tetrahydrofuran (1 mL; 50% w/w), Amberlyst (strongly acidic ion exchanger; 100 mg) in aqueous tetrahydrofuran (1 mL; 50% w/w), and potassium carbonate (50 mg) in aqueous methanol (1 mL; 50% w/w). In all cases GC-MS of the reaction mixture showed three compounds to be present in different proportions. It was impossible to purify any of the three compounds.

Synthesis of 3-Hydroxy-3-methyl-6-(1-methylethyl)cyclohexanone (1-Hydroxymenthone, M2). M2 was synthesized in a two-step sequence according to a published procedure (*17*) with piperitone as starting material. For NMR experiments 3-hydroxy-3-methyl-6-(1-methylethyl)cyclohexanone was purified by column chromatography on a 100 mm × 30 mm, 40 μm, Bakerbond Diol column (hexane/diethyl ether gradient) at 1.5 mL/min.

Synthesis of *E*-2-(2-Hydroxy-1-methylethylidene)-5-methylcyclohexanone [10-Hydroxy-*p*-menth-4(8)-en-3-one, M4]. 10-Hydroxy-*p*-menth-4(8)-en-3-one was synthesized in a four-step sequence starting from isopulegol.

2-[1-(Chloromethyl)ethenyl]-5-methylcyclohexanol (10-Chloroisopulegol). 10-Chloroisopulegol was synthesized according to a published procedure (*15*) starting from isopulegol by chlorination with NaOCl/CO_2. It was used without further purification for the next step. The yield was ∼90% on the basis of GC-MS. MS (EI), *m*/*z* (relative intensity) 41 (48), 53 (30), 55 (53), 67 (60), 71 (100), 79 (42), 81 (83), 93 (56), 102 (48), 108 (44), 109 (48), 123 (40), 170 (15), 188 (M^+; 8), 190 (M^+; 3).

2-[1-(Chloromethyl)ethenyl]-5-methylcyclohexanone (10-Chloroisopulegone). To a suspension of Dess−Martin periodinane (1.2 mmol) in CH_2Cl_2 (10 mL) was added a solution of 10-chloroisopulegol (∼1 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred for 1 h at room temperature. The suspension was diluted with pentane (40 mL), the precipitate removed by filtration, and the remaining solution concentrated to 5 mL using a rotary evaporator. The solvent was evaporated, and the crude 10-chloroisopulegone was used for the next step without further purification. MS (EI), *m*/*z* (relative intensity) 39 (63), 41 (58), 53 (45), 55 (27), 65 (29), 67 (65), 69 (39), 77 (26), 79 (43), 81 (37), 102 (30), 107 (68), 151 (100).

Acetic Acid [E-2-(2-Oxo-4-methylcyclohexylidene)propyl] Ester [10-Acetoxy-p-menth-4(8)-en-3-one]. The crude 2-[1-(chloromethyl)ethenyl]-5-methylcyclohexanone of the previous step (∼1 mmol) was dissolved in acetonitrile (4 mL). To this solution were added finely ground anhydrous sodium acetate (10 mmol, 620 mg) and hexadecyltributylphosphonium bromide (0.05 mmol, 25 mg). The suspension was heated for 2 h in a closed vessel at 80 °C. The resulting mixture consisted of approximately 30% menthofuran and 70% acetic acid [*E*-2-(2-oxo-4-methylcyclohexylidene)propyl] ester. Purification was performed by column chromatography on a 100 mm × 30 mm, 40 μm,

Bakerbond Diol column (40% *tert*-butyl methyl ether/60% hexane) at 1.5 mL/min. Yield, 90 mg (43% based on isopulegol); elemental composition (HRMS) $C_{12}H_{18}O_3$, found (210.1268), calcd (210.1256); MS (EI), *m/z* (relative intensity) 39 (39), 41 (39), 43 (100), 53 (35), 55 (39), 65(14), 67 (32), 69 (12), 77 (14), 79 (37), 81 (20), 93 (15), 107 (20), 108 (38), 139 (29), 149 (13), 150 (32), 151 (33), 168 (16); ^{1}H NMR ($CDCl_3$) $C\underline{H}_3-CH$ (1.02, d, $J = 6.7$ Hz, 3H), $C\underline{H}_3-C=$ (1.91, m, 3H), $C\underline{H}_3-CO$ (2.09, s, 3H), $C\underline{H}_2-CH_2-C=$ (1.38, m, 1H; 1.90, m, 1H), $C\underline{H}_2-CO$ (2.05, m, 1H; 2.54, m, 1H), $CH_2-C\underline{H}_2-C=$ (2.28, m, 1H; 2.82, m, 1H), $C\underline{H}_2-O$ (4.57, d, $J = 12.7$ Hz, 1H; 4,68, d, $J = 12.7$ Hz, 1H), $CH_3-C\underline{H}$ (2.05, m, 1H); ^{13}C NMR ($CDCl_3$) $\underline{C}H_3-C=$ (17.84), $\underline{C}H_3-CO$ (20.82), $\underline{C}H_3-CH$ (21.69), $CH_2-\underline{C}H_2-C=$ (28.72), $\underline{C}H_2-CH_2-C=$ (33.19), $\underline{C}H_2-CO$ (51.39), $\underline{C}H_2-O$ (64.36), $CH_3-\underline{C}H$ (32.32), $C=\underline{C}-CO$ (134.98), $\underline{C}=C-CO$ (136.72), $CH_3-\underline{C}OO$ (170.87), $CH_2-\underline{C}O$ (205.06).

E-2-(2-Hydroxy-1-methylethylidene)-5-methylcyclohexanone [10-hydroxy-p-menth-4(8)-en-3-one]. A solution of 10-acetoxy-*p*-menth-4(8)-en-3-one (1 mmol) in methanol (3.5 mL) was mixed with NaOH solution (3 mL; 0.1 mol/L). After exactly 5 min, hydrolysis was terminated by the addition of KH_2PO_4 (0.3 mmol) dissolved in water (10 mL). The mixture was immediately extracted with diethyl ether (2 × 30 mL). The combined organic phases were washed with water (1 × 1 mL) and brine (1 × 1 mL) and dried over Na_2SO_4. At this time a GC-MS run was performed indicating that the reaction mixture consisted of 20% menthofuran and 80% 10-hydroxy-*p*-menth-4(8)-en-3-one. The mixture was concentrated to ~1 mL, diluted with pentane (2 mL), and immediately purified by chromatography on a 100 mm × 30 mm, 40 μm, Bakerbond Diol column using a gradient starting with 40% diethyl ether/60% pentane at 10 °C and 3 mL/min. The fractions (2 × 20 mL) containing the target compound were combined, and $CDCl_3$ (0.75 mL) was added and concentrated by means of a rotary evaporator to ~0.5 mL. The solution was again diluted with $CDCl_3$ (0.75 mL) and concentrated to 0.6 mL. The solution contained ~99% 10-hydroxy-*p*-menth-4(8)-en-3-one and 1% menthofuran at this time besides a considerable amount of ether. The yield was in the range of 70−80% but was not determined exactly because the compound decomposed rapidly and seemed to decompose more quickly the more concentrated the solution. Elemental composition (HRMS) $C_{10}H_{16}O_2$, found (168.1133), calcd (168.1150); MS (EI), *m/z* (relative intensity) 41 (8), 55 (13), 67 (18), 69 (12), 77 (12), 79 (37), 80 (13), 81 (11), 91 (13), 93 (13), 107 (19), 108 (100), 109 (14), 135 (11), 139 (11), 149 (22), 150 (50), 168 (17); ^{1}H NMR ($CDCl_3$) $C\underline{H}_3-CH$ (1.01, d, $J = 6.2$ Hz, 3H), $C\underline{H}_3-C=$ (1.98, s, 3H), $C\underline{H}_2-CH_2-C=$ (1.37, m, 1H; 1.90, m, 1H), $C\underline{H}_2-CO$ (2.06, m, 1H; 2.52, m, 1H), $CH_2-C\underline{H}_2-C=$ (2.25, m, 1H; 2.81, m, 1H), $C\underline{H}_2-O$ (4.13, d, $J = 12.8$ Hz, 1H; 4.21, d, $J = 12.8$ Hz, 1H), $CH_3-C\underline{H}$ (2.03, m, 1H); ^{13}C NMR ($CDCl_3$) $\underline{C}H_3-C=$ (17.53), $\underline{C}H_3-CH$ (21.55), $CH_2-\underline{C}H_2-C=$ (28.03), $\underline{C}H_2-CH_2-C=$ (32.99), $\underline{C}H_2-CO$ (51.10), $\underline{C}H_2-O$ (62.62), $CH_3-\underline{C}H$ (31.96), $C=\underline{C}-CO$ (133.76), $\underline{C}=C-CO$ (141.07), $CH_2-\underline{C}O$ (205.10).

Synthesis of Z-2-(2-Hydroxy-1-methylethylidene)-5-methylcyclohexanone [9-Hydroxy-*p*-menth-4(8)-en-3-one]. 9-Hydroxy-*p*-menth-4(8)-en-3-one was synthesized in a four-step sequence starting from mintlactone.

Z-2-(2-Hydroxy-1-methylethylidene)-5-methylcyclohexanol [p-Menth-4(8)-ene-3,9-diol]. To a stirred suspension of $LiAlH_4$ (1 mmol, 38 mg) in dry diethyl ether (5 mL) was added dropwise a solution of mintlactone (1 mmol, 166 mg) in dry diethyl ether (3 mL). After 15 min of stirring, the excess of $LiAlH_4$ was destroyed by dropwise addition of methyl formate in diethyl ether. The reaction mixture was evaporated, and the remainder was treated with a solution of KH_2PO_4 (4 mmol, 544 mg) in water (10 mL), resulting in a pH of ~7. The diol was extracted with ethyl acetate (3 × 30 mL). The combined extracts were washed with saturated NaCl solution (1 × 3 mL), and the organic phase was dried over Na_2SO_4. The solution was used without further purification for the next step. No effort was made to purify the diol because of its poor chromatographic properties.

Acetic Acid [Z-2-(2-Hydroxy-4-methylcyclohexylidene)propyl] Ester [9-Acetoxy-p-menth-4(8)-en-3-ol]. To the crude solution of the *p*-menth-4(8)-ene-3,9-diol in ethyl acetate (~80 mL) were added acetic anhydride (1.2 mmol) and 4-(dimethylamino)pyridine (0.1 mmol). The reaction was monitored by GC-MS with the esterification being finished after 2 h at room temperature. The solution was washed with 5% citric acid (2 × 5 mL), with saturated $NaHCO_3$ solution (2 × 5 mL), and with saturated NaCl solution. The remaining organic phase was dried over Na_2SO_4 and concentrated to ~10 mL by means of a rotary evaporator. The product was sufficiently pure to be used for the next step without further purification. An analytical sample for NMR experiments was obtained by RP-HPLC on a 250 mm × 4.6 mm, 5 μm, Nucleosil C8 5-300 column at 1 mL/min with a water/methanol gradient. Elemental composition (HRMS) $C_{12}H_{20}O_3$, found (212.1429), calcd (212.1412); MS (EI), *m/z* (relative intensity) 41 (47), 43 (95), 53 (20), 55 (37), 67 (49), 68 (27), 69 (39), 79 (33), 81 (82), 82 (25), 91 (27), 93 (64), 94 (20), 95 (55), 109 (100), 108 (27), 110 (21), 119 (21), 123 (93), 137 (44), 139 (21), 152 ($M^+ - CH_3COOH$, 80), 170 (2), 194 ($M^+ - H_2O$, 5); ^{1}H NMR ($CDCl_3$) $C\underline{H}_3-CH$ (1.03, d, $J = 6.7$ Hz, 3H), $C\underline{H}_3-C=$ (1.73, s, 3H), $C\underline{H}_3-CO$ (2.05, s, 3H), $C\underline{H}_2-CH_2-C=$ (1.25, m, 1H; 1.65, m, 1H), $C\underline{H}_2-CO$ (1.44, m, 1H; 1.92, m, 1H), $CH_2-C\underline{H}_2-C=$ (1.96, m, 1H; 2.50, m, 1H), $C\underline{H}_2-O$ (4.82, d, $J = 11.8$ Hz, 1H; 4.95, d, $J = 11.8$ Hz, 1H), $CH_3-C\underline{H}$ (2.05, m, 1H); $C\underline{H}-OH$ (4.58, m, 1H); $CH-O\underline{H}$ (3.2, broad s, 1H); ^{13}C NMR ($CDCl_3$) $\underline{C}H_3-C=$ (17.22), $\underline{C}H_3-CO$ (21.15), $\underline{C}H_3-CH$ (21.49), $CH_2-\underline{C}H_2-C=$ (25.67), $\underline{C}H_2-CH_2-C=$ (33.21), $\underline{C}H_2-CHOH$ (42.06), $\underline{C}H_2-O$ (65.46), $CH_3-\underline{C}H$ (28.89), $CH_2-\underline{C}HOH$ (70.48), $C=\underline{C}-CHOH$ (141.07), $\underline{C}=C-CHOH$ (122.61), $CH_3-\underline{C}OO$ (171.98).

Acetic Acid [Z-2-(2-Oxo-4-methylcyclohexylidene)propyl] Ester [9-Acetoxy-p-menth-4(8)-en-3-one]. To Dess−Martin periodinane (1 mmol) suspended in CH_2Cl_2 (50 mL) was added a solution of 9-acetoxy-*p*-menth-4(8)-en-3-ol (~0.9 mmol) in ethyl acetate (9 mL). The mixture was stirred overnight at room temperature. It was taken to dryness, and the white precipitate was extracted with pentane/diethyl ether [50:50 (v/v); 4 × 40 mL]. The organic phase was concentrated and purified by RP-HPLC on a 250 mm × 4.6 mm, 5 μm, Nucleosil C8 5-300 column at 1 mL/min with 25% methanol/75% water. Yield, 48% (101 mg) based on starting material mintlactone; elemental composition (HRMS) $C_{12}H_{18}O_3$, found (210.1265), calcd (210.1256); MS (EI), *m/z* (relative intensity) 39 (11), 41 (17), 43 (50), 53 (12), 55 (16), 67 (13), 69 (12), 79 (30), 81 (14), 91 (13), 93 (15), 95 (12), 97 (14), 107 (18), 108 (100), 109 (19), 111 (12), 124 (10), 139 (90), 140 (10), 150 ($M^+ - CH_3COOH$; 65), 151 (29), 168 (38), 210 (M^+; 1); ^{1}H NMR ($CDCl_3$) $C\underline{H}_3-CH$ (1.02, d, $J = 6.7$ Hz, 3H), $C\underline{H}_3-C=$ (1.77, s, 3H), $C\underline{H}_3-CO$ (2.06, s, 3H), $C\underline{H}_2-CH_2-C=$ (1.38, m, 1H; 1.90, m, 1H), $C\underline{H}_2-CO$ (2.06, m, 1H; 2.54, m, 1H), $CH_2-C\underline{H}_2-C=$ (2.30, m, 1H; 2.74, m, 1H), $C\underline{H}_2-O$ (4.76, d, $J = 11.8$ Hz, 1H; 4.93, d, $J = 11.8$ Hz, 1H), $CH_3-C\underline{H}$ (2.00, m, 1H); ^{13}C NMR ($CDCl_3$) $\underline{C}H_3-C=$ (15.86), $\underline{C}H_3-CO$ (20.77), $\underline{C}H_3-CH$ (21.53), $CH_2-\underline{C}H_2-C=$ (28.70), $\underline{C}H_2-CH_2-C=$ (33.52), $\underline{C}H_2-CO$ (50.56), $\underline{C}H_2-O$ (65.55), $CH_3-\underline{C}H$ (31.63), $C=\underline{C}-CO$ (136.05), $\underline{C}=C-CO$ (138.10), $CH_3-\underline{C}OO$ (170.66), $CH_2-\underline{C}O$ (203.59).

Z-2-(2-hydroxy-1-methylethylidene)-5-methyl-cyclohexanone; (9-hydroxy-p-menth-4(8)-en-3-one). Hydrolysis of 9-acetoxy-*p*-menth-4(8)-en-3-one with either acid or weak base always led to menthofuran as the single product. No trace of 9-hydroxy-*p*-menth-4(8)-en-3-one was detectable by either HPLC or GC-MS.

Synthesis of α,α,4-Trimethyl-1-cyclohexenemethanol (3-*p*-Menthen-8-ol, M6). M6 was synthesized in a two-step sequence according to a published procedure (*18*) starting from pulegone. For NMR experiments, 4-trimethyl-1-cyclohexenemethanol was purified by chromatography on a 100 mm × 30 mm, 40 μm, Bakerbond Diol column at 1.5 mL/min using a hexane/diethyl ether gradient. Yield, 78% based on starting material pulegone; elemental composition (HRMS) $C_{10}H_{18}O$, found (154.1341), calcd (154.1358); MS (EI), *m/z* (relative intensity) 43 (60), 59 (26), 79 (30), 81 (29), 93 (27), 95 (23), 107 (18), 121 (43), 136 (17), 139 (100), 154 (15); ^{1}H NMR ($CDCl_3$) $C\underline{H}_3-CH$ (0.95, d, 3H), $CH_3-C\underline{H}$ (1.20, m, 1H), $(C\underline{H}_3)2-C-OH$ (1.30, s, 3H and 1.31, s, 3H), $O\underline{H}$, $C\underline{H}_2$,$C\underline{H}_2$,$C\underline{H}_2$ (1.5−1.8 and 2.0−2.2, m, 7H), $-C\underline{H}=C$ (5.70, m, 1H); ^{13}C NMR ($CDCl_3$) $\underline{C}H_3-CH$ (21.67), $CH_3-\underline{C}H$ (28.22), $(\underline{C}H_3)_2-C-OH$ (28.89), $\underline{C}H_2$, $\underline{C}H_2$, $\underline{C}H_2$ (24.44; 31.34; 33.70), $C-OH$ (72.85), $-\underline{C}=CH$ (143.48), $-\underline{C}H=C$ (118.43).

NMR Spectroscopy. NMR spectra (^{1}H, ^{13}C, 135° DEPT) were recorded on a Bruker AM 360 instrument in $CDCl_3$ (unless otherwise stated) with TMS as internal standard ($\delta = 0$ ppm). Carbon multiplicities of all compounds were determined by DEPT. Two-dimensional

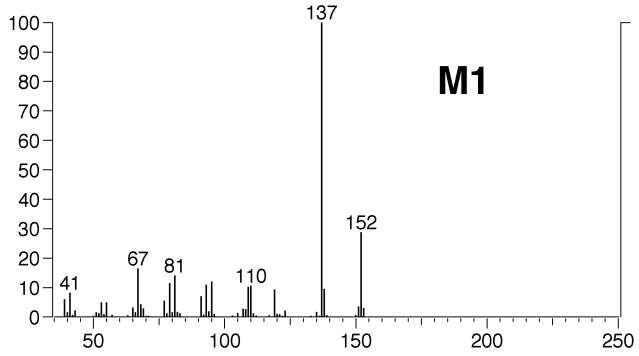

Figure 2. Mass spectrum of metabolite **M1**.

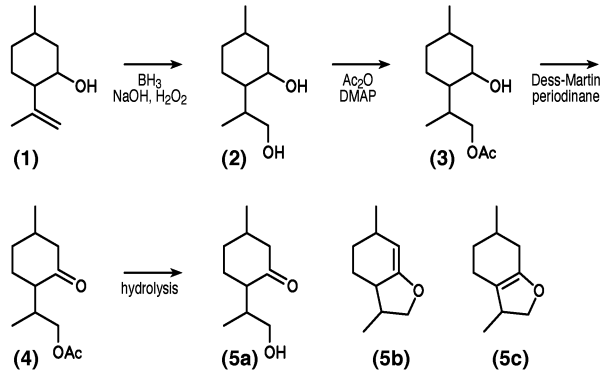

Figure 3. Synthesis of 9-acetoxymenthone (**4**), a precursor of **M1**.

experiments (COSY, HMBC, and HMQC) were recorded on a Bruker AMX 400 instrument in $CDCl_3$ (unless otherwise stated).

High-Resolution Gas Chromatography (HRGC)—Mass Spectrometry (MS). HRGC was performed using a 30 m × 0.32 mm i.d. DB-5 capillary column (J&W Scientific, Folsom, CA) in a gas chromatograph type 8000 (ThermoQuest, Egelsbach, Germany). Sample injection was performed using the split technique at a temperature of 230 °C and a split ratio of 1:25. The volumes injected ranged from 1 to 20 μL. Temperature during HRGC was held at 35 °C for 1 min and raised to a final temperature of 230 °C at a rate of 6 °C/min. Mass spectra were recorded on an MD 800 quadrupole mass spectrometer (ThermoQuest) in the electron ionization (EI) mode at 70 eV.

HRGC—High-Resolution Mass Spectrometry (HR-MS). HRGC was performed on a 30 m × 0.25 mm i.d. DB-5 capillary column (J&W Scientific) in a gas chromatograph type 5890 (Hewlett-Packard, Heilbronn, Germany). Sample injection was performed using the cold on-column technique. The volumes injected ranged from 0.2 to 0.5 μL. Temperature during HRGC was held at 35 °C for 1 min and raised to 230 °C at a rate of 6 °C/min. Mass spectra were recorded with a Finnigan MAT 95S (Finnigan, Bremen, Germany) mass spectrometer in the EI mode at 70 eV, in the chemical ionization (CI) mode at 115 eV with isobutane as reagent gas, and in high-resolution (HR) mode using perfluorokerosene as internal standard.

RESULTS AND DISCUSSION

The urine samples of six volunteers (three males and three females) were collected for 24 h before (control) and after (test) ingestion of 1000 μg of (*S*)-(−)- or 500 μg of (*R*)-(+)-pulegone/kg of body weight. Because it can be assumed that most of the metabolites of pulegone have been condensed with either sulfuric acid or glucuronic acid during phase II reactions, the samples were treated with glucuronidase and sulfatase to liberate the apolar metabolites presumably formed in phase I reactions. After extraction with diethyl ether and separation into acidic, phenolic, and neutral compounds, each fraction was analyzed by HRGC-MS either before or after trimethylsilylation or ethylation. For analysis of unconjugated metabolites the urines were extracted directly with diethyl ether after the pH had been adjusted to either 2 or 7. All metabolites where detected in the neutral fraction. Concentrated samples of test and control urine were also tested by HPLC. However, no metabolites could be detected in terms of new peaks present in the test sample (data not shown). It would be desirable to analyze the conjugated metabolites directly; however, only an HPLC method in combination with radioactive ^{14}C labeling would be reasonable, but also inconsistent with experimentation on humans.

Metabolite M1. Surprisingly, the mass spectrum (**Figure 2**) of the major metabolite of (*S*)-(−)-pulegone (based on GC-MS area) was not identical with menthofuran as expected from the results of most of the available published literature. It is noteworthy that the molecular weight of the major metabolite appeared to be the same as that of pulegone itself. Usually, metabolites have a higher molecular weight because in most cases oxygen is attached to the molecule during phase I reaction. **M1** was also present in untreated urine; however, its amount differed considerably and ranged from extremely high to barely detectable. Most probably **M1** is bound to glucuronic acid or sulfuric acid via a very labile bond, which might be hydrolyzed partly even under the pH conditions of urine itself.

From HRGC-HRMS the exact mass was consistent with the elemental composition $C_{10}H_{16}O$. The molecular weight was also confirmed by HRGC-MS-CI with isobutane as reagent gas. Therefore, the very intense base peak with *m*/*z* 137 is formed by removal of a methyl radical from the molecular ion with *m*/*z* 152. On the basis of those facts it is most probable that the methyl group is in an allylic position to a double bond present in the molecule. In addition to the cyclic C6 ring system of pulegone, it is most likely that there is a second ring structure present in the molecule, therefore forming a partly hydrogenated benzofuran skeleton. From a combination of all assumptions the structure of **M1** was proposed to be either 2,3,4,5,6,7-hexahydro-3,6-dimethylbenzofuran (8,9-dihydromenthofuran, **5c** in **Figure 3**) or 2,3,3a,4,5,6-hexahydro-3,6-dimethylbenzofuran (**5b** in **Figure 3**). Both compounds have the benzofuran ring system and from both the removal of only one methyl group during HRGC-MS, either from the cyclohexene ring or from the dihydrofuran ring, is very probable.

To confirm the proposed structure for **M1**, the major metabolite of (*S*)-(−)-pulegone, its precursor, 9-acetoxymenthone, was synthesized by the sequence shown in **Figure 3**. The synthesis started from isopulegol (**1**), which was hydroborinated and oxidized to the diol (**2**). The unhindered hydroxy group was then esterified with acetic acid anhydride, forming **3**, and the remaining hydroxy group converted to a carbonyl function using Dess−Martin reagent, furnishing 9-acetoxymenthone (**4**). The hydrolysis of pure 9-acetoxymenthone was performed using either lithium methylate in methanol, hydrochloric acid in aqueous tetrahydrofuran, a strongly acidic ion exchanger in aqueous tetrahydrofuran, or potassium carbonate in aqueous methanol. In all cases GC-MS of the reaction mixture showed all three compounds (**5a**, **5b**, and **5c**) to be present in different proportions. As an example, the GC-MS of the reaction mixture dissolved in chloroform and methanol after hydrolysis with lithium methylate is shown with mass spectra of the corresponding peaks (**Figure 4**).

5a elutes last from the GC column and must therefore be the alcohol 6-(2-hydroxy-1-methylethyl)-3-methylcyclohexanone (9-hydroxymenthone). Because of the intense McLafferty rearrangement ion at *m*/*z* 112 the hemiketal tautomer can probably be excluded to be present during gas chromatographic conditions. 9-Hydroxymenthone is the major detectable product, in

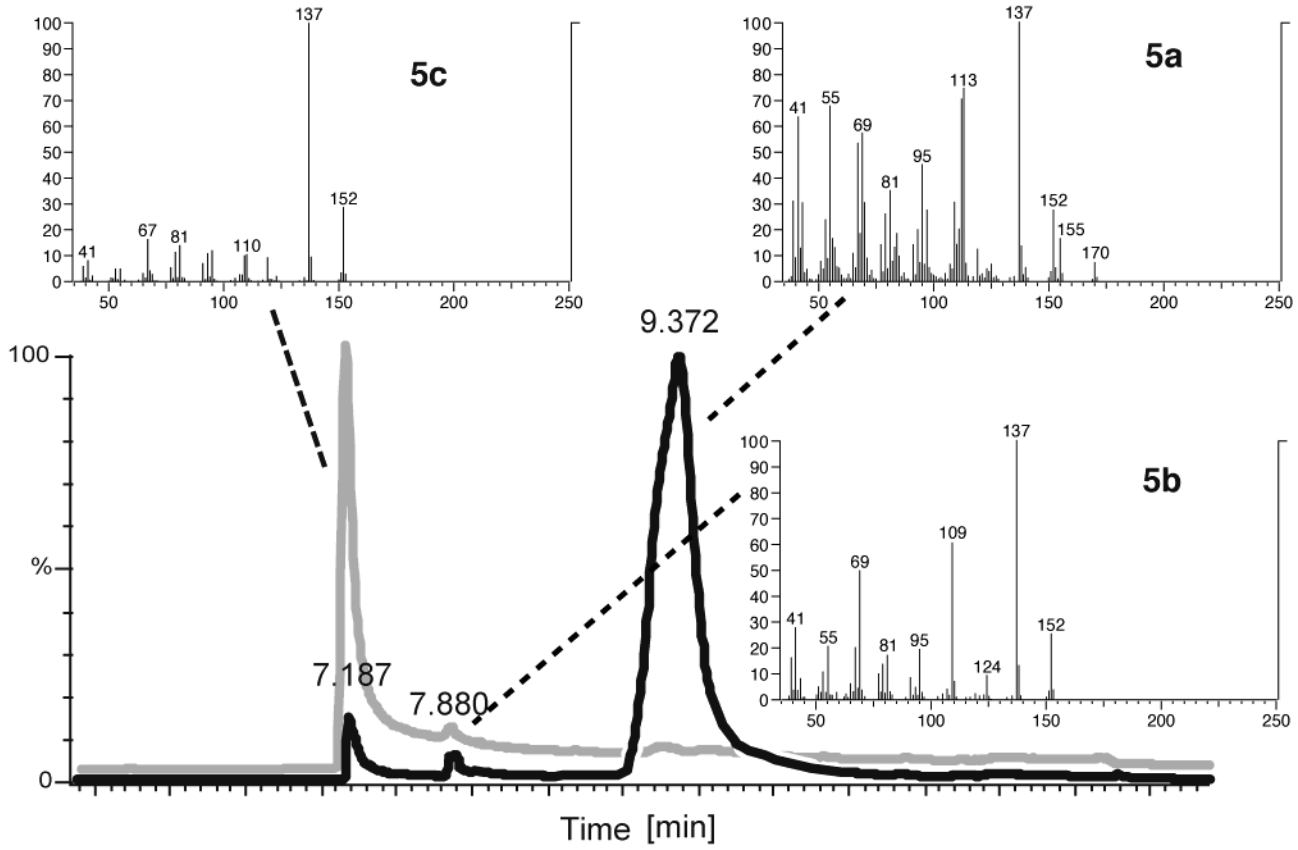

Figure 4. GC-MS of the hydrolysate of **IV** dissolved in chloroform (black trace) and methanol (gray trace).

the case when the methanol initially used is removed under vacuum and replaced with chloroform. On the basis of GC-MS analysis the methanolic solution consists mainly of peak **5c**. Discrimination between **5c** and **5b** is easily possible by interpretation of the MS data. By retro-Diels−Alder rearrangement from **5c** an ion at *m*/*z* 110 is formed by loss of propene, whereas from **5b** the ion at *m*/*z* 124 is formed by loss of ethene followed by loss of the furan methyl group, forming the ion at *m*/*z* 109.

However, after enzymatic hydrolysis of the urine samples only the structure **5c** was found and never **5a** or **5b**. Because **M1** was not always found in the urines without treatment with glucuronidase and sulfatase, it has to be concluded that the original metabolite is most probably **5a** or its cyclic hemiacetal. Only these have a hydroxy group that can be linked to glucuronic acid. The identified **M1** might be formed spontaneously during enzymatic hydrolysis or during workup of the enzymatically hydrolyzed urine. Complete loss of **5a** and formation of **M1** during GC can be excluded, because chromatography of the synthetic mixture has proven that the equilibrium is much more dependent on the solvent than on temperature or injection technique.

It is noteworthy that it was not possible to purify any of the three compounds and, therefore, NMR data for only acetylated **M1** were obtained. This is most probably because traces of water present in any chromatographic system give rise to an equilibrium between all possible structures (**Figure 5**). Additionally, in more concentrated solutions as used for NMR the compound will obviously form dimers to some extent by self-addition to the enolether function, producing an even more complex spectrum of compounds. The equilibrium might also explain why this major metabolite has never been described in earlier literature in cases when enzymatic hydrolysis was used to free the primary metabolites from their conjugates. **M1** is quite polar,

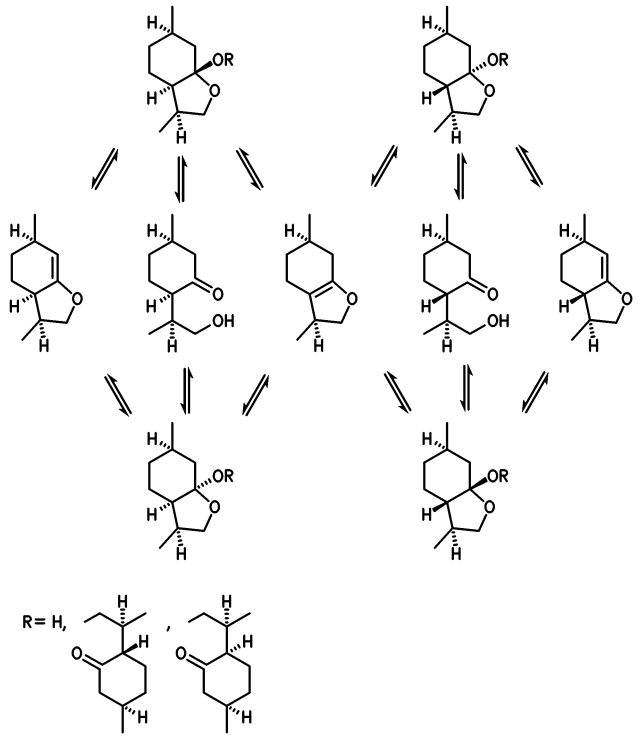

Figure 5. Polymorphism of **M1** [only the 8-(*S*)-isomer is shown; numbering refers to menthane skeleton].

showing a behavior comparable to that of uroterpenolone (*14*), a metabolite of carvone in humans, not being very well extracted from aqueous solutions.

However, **M1** was reported as being detected in a very recent publication (*10*) and found to be conjugated to glucuronic acid.

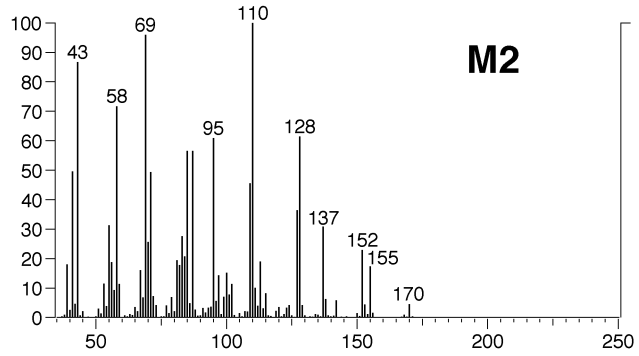

Figure 6. Mass spectrum of metabolite **M2**.

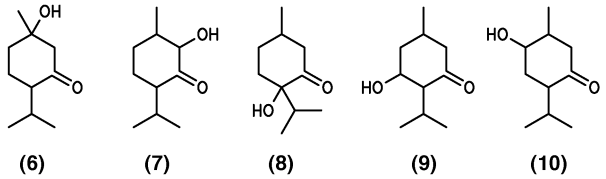

Figure 7. Possible structures of **M2**.

In conjugated form **M1** cannot take part in the equilibrium (**Figure 5**). It is noteworthy that only ^{1}H NMR data are available for the **M1**−glucuronic acid conjugate (*10*) and, therefore, it is still not definite whether the open-chain form or the hemiketal form is the one to be conjugated during phase II metabolism.

Metabolite **M1** was also found as metabolite of the more toxic (*R*)-(+)-pulegone; however, its amount was apparently much lower compared to the amount found after ingestion of (*S*)-(−)-pulegone. Because the hepatotoxicity of pulegone is linked, at least in part, to menthofuran formation during metabolism, the formation of 9-hydroxymenthone can be considered as a true detoxification. Menthofuran formation should not be possible from this metabolite. Therefore, the increased ability of 9-hydroxymenthone formation from (*S*)-(−)-pulegone in the body might very well explain the lower toxicity of the latter.

Metabolite M2. For metabolite 2 (**M2**) a mass spectrum was obtained from the neutral fraction after enzymatic hydrolysis (**Figure 6**). From HRGC-HRMS the exact mass was consistent with the elemental composition $C_{10}H_{18}O_2$. The molecular weight was also confirmed by HRGC-MS in the CI mode. The formation of *m*/*z* 152, that is, the loss of water, indicated that there is at least one hydroxy group present in the molecule. From the intense fragmentation pattern a hydrogenated benzofuran ring structure as in **M1** could be excluded, and therefore only a cyclohexanone ring system should be present. From the quite intense fragment at *m*/*z* 128, which is most probably formed from *m*/*z* 170 via McLafferty rearrangement by removal of C_3H_6 from the isopropyl side chain, it was concluded that the hydroxy group has to be attached to the ring system. The following structures (**Figure 7**) were therefore considered as theoretically possible structures of metabolite M2.

Structures **9** and **10** were considered to be unlikely as the position of the oxygen is unusual because tertiary carbon atoms are normally strongly favored over secondary carbon atoms by phase I oxidizing enzymes. Structures **7** and **8**, where the hydroxy group is in an α-position to the carbonyl group, would not explain the intense fragment at *m*/*z* 110, because the removal of water is not favored from this position. Structure **6** seemed ideal to be to explain the observed mass spectrum. It is the only structure easily generating the fragment at *m*/*z* 58 after retroaldol reaction followed by McLafferty rearrangement under mass spectrometric conditions. The intense formation of the ion

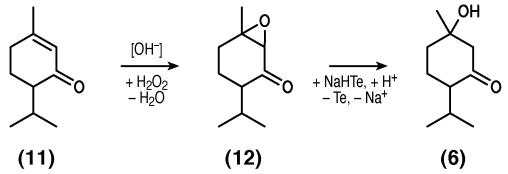

Figure 8. Synthesis of 3-hydroxy-3-methyl-6-(1-methylethyl)cyclohexanone.

at *m*/*z* 43 can be also explained by the open-chain form, which contains two acetyl groups. Additionally, the hydroxy group is attached in a β-position to the carbonyl group, from which removal of water can be explained best. Finally, the position of the hydroxy group at a tertiary carbon atom is well in line with the expected behavior of phase I enzymes metabolizing a saturated hydrocarbon.

To confirm the structural proposal, **M2** was synthesized according to a published (*17*) procedure (**Figure 8**) starting from piperitone (**11**) via piperitone epoxide (**12**). The synthesized sample showed identical retention times and mass spectrometric decomposition and, therefore, **M2** was successfully identified as 3-hydroxy-3-methyl-6-(1-methylethyl)cyclohexanone (1-hydroxymenthan-3-one). This is the first identification of **M2** as a metabolite of pulegone in humans.

Metabolite M3. Metabolite **M3** was identified as the well-known compound menthol [3-methyl-6-(1-methylethyl)cyclohexanol] by its mass spectrum and cochromatography with an authentic standard. A total of three diastereoisomers of menthol were found in the urine of test persons metabolizing (*S*)-(−)-pulegone, but only one was found in those metabolizing (*R*)-(+)-pulegone. The amount of menthol was apparently much higher in the case of (*S*)-(−)-pulegone compared to (*R*)-(+)-pulegone, again indicating that differences in toxicity are most probably closely related to the ability of to reduce pulegone rather than to metabolize it by oxidation. To our knowledge this is the first identification of menthol as a metabolite of pulegone.

Metabolite M4. Metabolite **M4** was only a minor metabolite of (*S*)-(−)-pulegone, but it was one of the major metabolites of (*R*)-(+)-pulegone. For **M4** a mass spectrum was obtained from the neutral fraction showing a high degree of similarity compared to the mass spectrum of menthofuran (**Figure 9**). However, **M4** was detected after enzymatic hydrolysis only, again indicating conjugation to either glucuronic acid or sulfuric acid.

From HRGC-HRMS the exact mass of the molecular ion at *m*/*z* 168 was determined to be consistent with the elemental composition $C_{10}H_{16}O_2$. The molecular weight was also confirmed by HRGC-MS in the CI mode. The formation of the ion at *m*/*z* 150, that is, the loss of water, indicated that there is one hydroxy group present in the molecule. Because of the similar mass spectra of menthofuran and **M4**, the latter was considered to be a potential precursor for menthofuran earlier identified as a metabolite of pulegone. Consequently, the only useful possible structures for **M4** were *E*- or *Z*-2-(2-hydroxy-1-methylethylidene)-5-methylcyclohexanone. However, a review of the literature revealed that neither compound has apparently ever been synthesized or isolated and fully characterized. Apparently, only a mass spectrum of *Z*-2-(2-hydroxy-1-methylethylidene)-5-methylcyclohexanone was published [available as Supporting Information (*19*)] and without verification of the proposed *Z*-configuration. Therefore, for both compounds different synthetic methods were elaborated, which should allow the unequivocal discrimination between both forms.

Z-2-(2-Hydroxy-1-methylethylidene)-5-methylcyclohexanone (9-hydroxypulegone) was synthesized from mintlactone

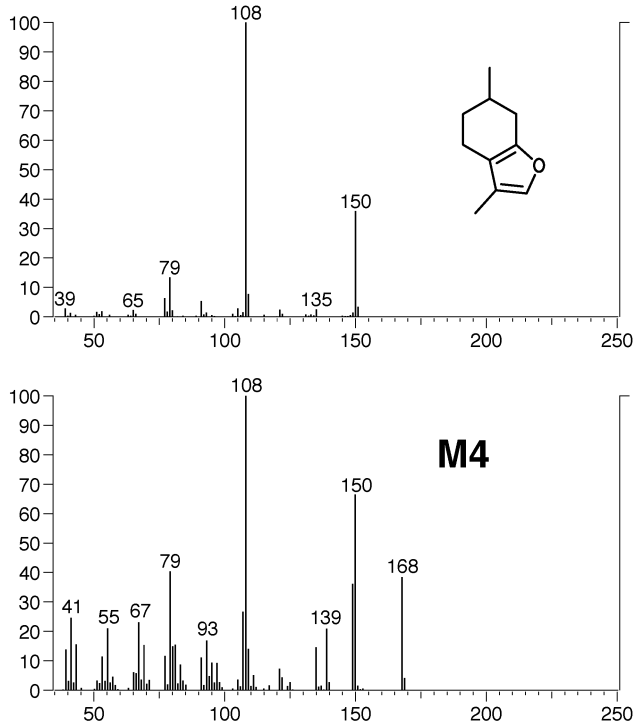

Figure 9. Comparison of mass spectra of menthofuran and metabolite **M4**.

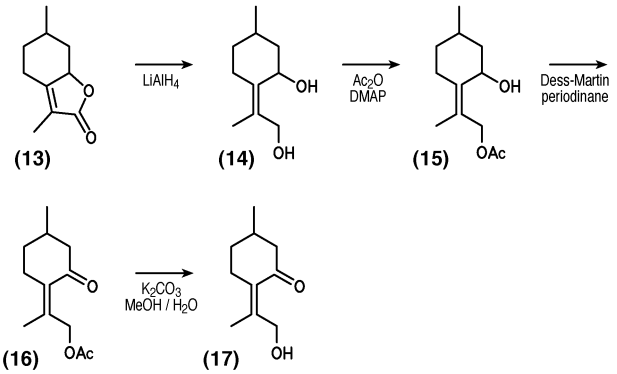

Figure 10. Synthesis of *Z*-2-(2-hydroxy-1-methylethylidene)-5-methylcyclohexanone (9-hydroxypulegone, **17**).

(**13**) in a four-step sequence (**Figure 10**). In the first step mintlactone (**13**) was reduced, forming a diol (**14**) with the required *Z*-configuration of the double bond. In the next step the primary hydroxy group was protected by acetylation and the resulting acetoxy alcohol (**15**) was oxidized to a direct precursor (**16**) of the desired 9-hydroxypulegone (**17**). However, during any kind of hydrolysis with different bases or under acidic conditions no trace of 9-hydroxypulegone was ever detected by either GC-MS or HPLC (chromatograms not shown), and only menthofuran was formed from the acetylated precursor (**16**) in quantitative yield within minutes. It has to be concluded that 9-hydroxypulegone cannot exist and, therefore, must be excluded as the structure for **M4**.

E-2-(2-Hydroxy-1-methylethylidene)-5-methylcyclohexanone (10-hydroxypulegone) was synthesized from isopulegol (**1**) in a four-step procedure (**Figure 11**). In the first step isopulegol was chlorinated and the resulting 10-chloroisopulegol (**18**) oxidized using Dess−Martin periodinane, furnishing 10-chloroisopulegone (**19**). Treatment of the latter with a suspension of sodium acetate in acetonitrile in the presence of a phase transfer catalyst gave a mixture of *E*-2-(2-acetoxy-1-methyl-

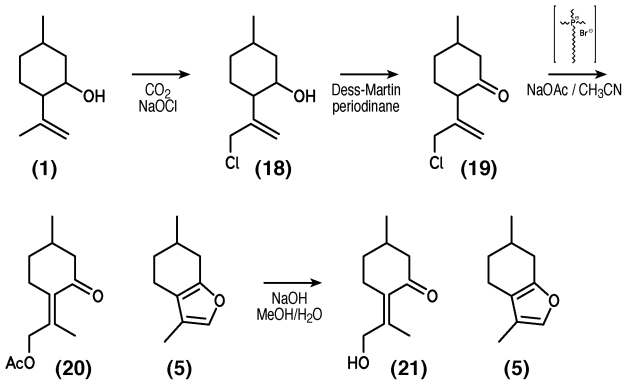

Figure 11. Synthesis of *E*-2-(2-hydroxy-1-methylethylidene)-5-methylcyclohexanone (10-hydroxypulegone, **21**).

ethylidene)-5-methylcyclohexanone (10-acetoxypulegone, **21**) and menthofuran (**5**). This reaction consists of two steps, the first being the isomerization of the double bond and intermediate formation of 10-chloropulegone and 9-chloropulegone, depending on the conformation of the isopropenyl side chain. However, 9-chloropulegone is not stable under these conditions and immediately cyclizes, forming menthofuran. In 10-chloropulegone cyclization is impossible because of the *Z*-configuration of the double bond, and the chlorine atom is displaced in an S_N2 reaction by the unsolvated acetate anion. In the last step the ester bond of **21** is hydrolyzed with dilute sodium hydroxide solution in aqueous methanol. Menthofuran is stable under the chosen hydrolysis conditions and can be removed during purification of the product.

Surprisingly, in the first experiment after hydrolysis with dilute K_2CO_3 solution in aqueous methanol for 1 h the yield of 10-hydroxypulegone was very low compared to that of the byproduct, menthofuran, and it was not possible to obtain any NMR data. Nevertheless, the synthesized sample showed identical retention times and mass spectrometric fragmentation during GC-MS and, therefore, **M4** was successfully identified as *E*-2-(2-hydroxy-1-methylethylidene)-5-methylcyclohexanone (10-hydroxypulegone). This is the first identification of **M4** as a metabolite of pulegone in humans in vivo.

To find out more about the reactivity of 10-hydroxypulegone, its stable precursor 10-acetoxypulegone was purified by column chromatography and subjected to hydrolysis alone without menthofuran being present. Surprisingly, depending on the hydrolysis conditions, again ~30% to as much as 100% menthofuran was formed from 10-acetoxypulegone. The optimal hydrolysis conditions to obtain 10-hydroxypulegone were treatment of the precursor with a mixture of methanol/0.1 mol/L NaOH (50:50, v/v) for a few minutes at room temperature. After subsequent termination of hydrolysis and immediate extraction of the target compound, column chromatography yielded pure 10-hydroxypulegone in sufficient amounts for NMR experiments. It is noteworthy that even at −20 °C in the NMR solvent $CDCl_3$ under aprotic conditions, 10-hydroxypulegone decays first very quickly and then slowly (**Table 1**), forming menthofuran as the sole product. Therefore, we cannot confirm the stability observed previously (*19*).

The reaction of 10-hydroxypulegone forming menthofuran is considerably faster in aqueous solution at room temperature at any pH, leading to complete transformation of 10-hydroxypulegone to menthofuran, usually within hours. Therefore, this reaction has to be considered as a major source of menthofuran. As a consequence, the amount of menthofuran detected in metabolism experiments will be strongly dependent on the

Table 1. Decomposition of 10-Hydroxypulegone in $CDCl_3$ at −20 °C

time (h)	10-hydroxypulegone (%)	methofuran (%)
0	1.2	98.9
4	4.9	95.1
24	7.7	92.3
48	8.4	91.6
120	8.7	91.3

workup method. Our experiments with highly purified 10-hydroxypulegone (**M4**) support the opinion proposed very recently (*10*) that menthofuran is most probably not a metabolite of pulegone but an artifact resulting from workup. The precursor, 10-acetoxypulegone, is also not indefinitely stable and slowly isomerizes, forming 9-acetoxypulegone. The driving force might be explained with the orientation of the dipoles found in the molecule. However, this conversion seemed to be apparently slower (data not shown) compared to the decay of 10-hydroxypulegone.

Minor Metabolites. There were some other metabolites present in the test urines; however, all of their amounts were very low compared to **M1**, **M2**, **M3**, and **M4**. Some of them have been identified previously in rats. The following minor metabolites were successfully identified by comparison with authentic samples: piperitone (**M5**) and 4-trimethyl-1-cyclohexene-1-methanol (**M6**, 3-*p*-menthen-8-ol). Piperitone might also be formed as an artifact from **M2** during enzymatic hydrolysis. There were traces of other compounds present only in test urines; however, due to their low amount it was impossible to identify them by interpreting the mass spectrometric data. Because their amount was much lower compared to the metabolites identified, they might also stem from impurities in pulegone and therefore might not be metabolites of the latter.

Menthofuran. After enzymatic hydrolysis, a mass spectrum was obtained from test urines consistent with menthofuran. An authentic sample of menthofuran showed identical retention times and mass spectrometric fragmentation and, therefore, the compound was identified as the latter. However, only trace amounts of menthofuran were found in urines that were extracted directly. As menthofuran itself has no hydroxy group by which it could have been attached to either glucuronic acid or sulfuric acid, it should have been present in the glucuronidase/sulfatase-treated and in the untreated samples in equal amounts, which was not the case.

The only explanation for this behavior is that menthofuran is not formed in relevant amounts during metabolism but is generated from one or more precursors during enzymatic hydrolysis or workup as it was already shown for **M4**. However, even after enzymatic hydrolysis, the amount of menthofuran is much lower compared to **M1** and also lower compared to **M2**. Therefore, if menthofuran is really a major metabolite in humans, it must have been further metabolized via epoxymenthofuran and hydroxymenthofuran, forming mintlactone (*12*). Mintlactone could be only tentatively detected in the test urines in this study, and only a noisy mass spectrum was obtained, further excluding menthofuran as a major metabolite of pulegone in humans. In recent literature the role of menthofuran as major metabolite was questioned (*1*) because the amounts of menthofuran compared to pulegone found in blood were extremely low. Additionally, menthofuran was also not found among the metabolites of (*R*)-(+)-pulegone in F334 rats (*10*).

Metabolism of Pulegone in Humans. On the basis of the metabolites identified in this study, the following pathways

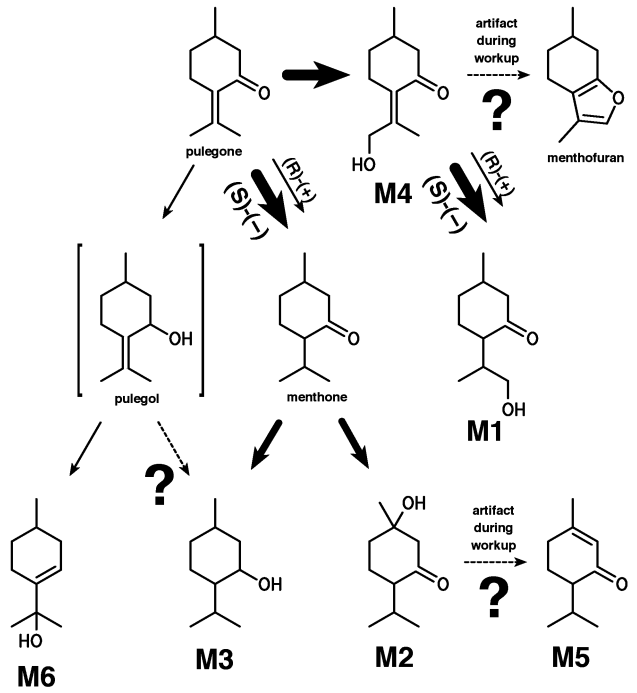

Figure 12. Proposed metabolism scheme for pulegone in humans.

(**Figure 12**) are proposed during metabolism of pulegone in humans at ingestion-correlated nontoxic concentrations. In the first step pulegone seems to be oxidized selectively in the 10-position, forming 10-hydroxypulegone (**M4**). Alternatively, it may be reduced to menthone, which was detected at trace levels in all test urines (data not shown). It might be possible that pulegone is also reduced at the carbonyl group first; however, no trace of pulegol was found in the urine samples. Consequently, pulegol is either reduced very efficiently to menthol (**M3**) or rearranged under the conditions in the human body forming α,α,4-trimethyl-1-cyclohexene-1-methanol (**M6**, 3-*p*-menthen-8-ol), which was found as a minor metabolite. However, a more reasonable pathway leading to **M3** is the reduction of the carbonyl group in menthone. Metabolite **M2** is a typical menthone metabolite and therefore should be formed by oxidation of the latter at C5.

In the literature allylomerization of pulegol has been reported (*18*) to take place at a nearly physiologic pH of 6.5 under certain conditions, leading to α,α,4-trimethyl-1-cyclohexene-1-methanol (**M6**). However, the formation of **M6** from pulegol as an artifact during enzymatic hydrolysis with sulfatase and glucuronidase also cannot be totally excluded. Some authors recently claimed identification of pulegol as a metabolite of (*S*)-(−)-pulegone (*6*). However, their published mass spectrum of pulegol was totally different from the one we have obtained for synthetic pulegol prepared according to a known method (*18*). In fact, the mass spectra of pulegol and of **M6** are very similar and, therefore, likely to be confused, especially in cases when only GC-MS has been used as an identification method without the backup of synthesis.

The major metabolite **M1** is formed from the primary oxidation product **M4** by reduction of the exocyclic double bond. The polymorphism of **M1** has already been discussed. It remains unclear whether or to what extent **M4** can be a precursor for menthofuran in humans in vivo or if menthofuran has to be considered solely as an artifact during workup. In a recent study a nonvolatile metabolite in F344 rats was characterized (*10*), which would also be a suitable precursor explaining the observed formation of menthofuran during workup. However, at least at

low doses of pulegone, menthofuran is not a relevant metabolite in humans in either (*S*)-(−)- or (*R*)-(+)-pulegone.

It seems that for (*R*)-(+)-pulegone both reduction steps to menthone and from **M4** to **M1** are highly stereoselective and much less favored by the conformation of the terpene compared to (*S*)-(−)-pulegone, where **M4** is accumulated. In case **M4** cannot be removed quickly enough by reduction, further oxidation at the hydroxy group might take place, forming the obviously very reactive pulegone-10-aldehyde. This compound might also be responsible for the toxic effects caused preferentially by (*R*)-(+)-pulegone. Therefore, the differences in toxicity between (*R*)-(+)- and (*S*)-(−)-pulegone can be very well explained by the stereospecifity of the unknown enzyme responsible for reducing the exocyclic double bond.

We were not able to clarify the observations published earlier (*20*) that *p*-cresol is a major metabolite of menthofuran and, therefore, also responsible for the toxic effects associated with pulegone ingestion. *p*-Cresol was always present in the urines of the test persons in amounts >100 times more compared to the major metabolite **M1** in both test and control urines. Further studies with ^{13}C-labeled pulegone are underway to clarify this issue.

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