Biosynthesis of Menthofuran in *Mentha* × *piperita*: Stereoselective and Mechanistic Studies

Sabine Fuchs, Silvia Zinn, Thomas Beck, and Armin Mosandl*

Institut für Lebensmittelchemie, Johann Wolfgang Goethe-Universität Frankfurt, Marie-Curie-Strasse 9, D-60439 Frankfurt (Main), Germany

Mentha × *piperita* shoot tips and first leaf pair were fed with aqueous solutions of [$^2H_2$]- and [$^2H_2$]/[^{18}O]-labeled pulegone. The essential oil was analyzed by solid phase microextraction and enantioselective multidimensional gas chromatography/mass spectrometry. After feeding experiments with labeled pulegone racemate, both labeled (*S*)-menthofuran and (*R*)-menthofuran were detectable simultaneously together with genuine (*R*)-menthofuran. It could be shown that both labeled pulegone enantiomers are converted by *Mentha* × *piperita* to the corresponding labeled menthofuran enantiomers, favoring the labeled analogue of the nongenuine (*S*)-pulegone. The oxygen in menthofuran is introduced by enzymatic oxidation of pulegone, as concluded from feeding experiments with mixed labeled [$^2H_2$]/[^{18}O]pulegone.

Keywords: *Menthofuran; stable isotope labeling; Mentha* × *piperita biosynthesis; solid-phase microextraction (SPME); enantioselective multidimensional gas chromatography/mass spectrometry (enantio-MDGC/MS)*

INTRODUCTION

Menthofuran is a characteristic monoterpene in *Mentha* with an undesirable influence on mint essential oil quality. The oxidative bioconversion of pulegone leading to menthofuran was assumed by Reitsema (1958) and confirmed by Battaile and Loomis (1961), using incubation of very young leaf slices with labeled pulegone. Although there have been several in vivo and in vitro investigations on the conversion of pulegone into menthofuran in mammals (McClanahan et al., 1988; Moorthy et al., 1989; Nelson et al., 1992a,b; Madyastha and Gaikwad, 1998), little recent research has been carried out on the biosynthetic origin of menthofuran in *Mentha* species. As in mammals, a cytochrome P-450 oxidase is assumed to be involved in the biosynthesis of menthofuran. The *cis*-methyl group of the pulegone isopropylidene moiety may be hydroxylated, followed by an intramolecular cyclization and dehydration (Croteau and Gershenzon, 1994; McCaskill and Croteau, 1997). Genetic aspects of the menthofuran biosynthesis as well as environmental effects on the menthofuran content have been studied (Murray and Hefendehl, 1972; Burbott and Loomis, 1967; Clark and Menary, 1980).

Recently, enantioseparation by gas chromatography was reported by Werkhoff et al. (1995). As expected, they found enantiopure (*R*)-menthofuran in *Mentha piperita* oil.

This paper reports on in vivo feeding experiments with deuterium as well as deuterium and ^{18}O-labeled pulegone precursors using intact plant material of *Mentha* × *piperita*. The essential oil of each single shoot tip and first leaf pair was analyzed by solid-phase microextraction (SPME) and enantioselective multidimensional gas chromatography/mass spectrometry (enantio-MDGC/MS).

MATERIALS AND METHODS

Enantioselective Gas Chromatography (Enantio-GC). Enantioseparation of the synthesized labeled pulegone was performed on an HP 5890 Series II gas chromatograph, equipped with a duran glass capillary (30 m × 0.23 mm i.d.) coated with 15% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin in SE 52 (film thickness = 0.1 μm). Conditions were as follows: carrier gas, hydrogen, 130 kPa; split, 30 mL/min; injector temperature, 250 °C; detector, FID, 260 °C; oven temperature 80 °C, raised at 1 °C/min to 200 °C (30 min isothermal).

GC/MS. A Fisons Instruments GC 8065, coupled to a Fisons Instruments MD 800 mass spectrometer, equipped with a BPX-5 column (50 m × 0.32 mm i.d., film thickness = 0.25 μm; SGE-Analytik, Weiterstadt, Germany) was employed for the GC/MS analysis of the synthesized monoterpenes. Conditions were as follows: carrier gas, helium, 109 kPa; split, 30 mL/min; injector temperature, 230 °C; oven temperature, 40 °C (5 min isothermal), raised at 2.5 °C/min to 320 °C (30 min isothermal); ion source temperature, 200 °C; interface temperature, 250 °C; mass range, 40−250 amu; EI, 70 eV. The molecular ion (M^+) and the fragmentation ions were given as *m*/*z* with relative peak intensities to the base peak (percent).

Enantio-MDGC/MS. A Siemens SiChromat 2, equipped with two independent column oven programs and a live-T-switching device, was used for the enantio-MDGC/MS analysis of the SPME headspace extracts. The main column was coupled to the transfer line of a Finnigan MAT ITD 800, using an open split interface. Precolumn conditions were as follows: duran glass capillary (30 m × 0.23 mm i.d.) coated with a 0.23 μm film of SE 52; carrier gas, hydrogen, 120 kPa; split, 8 mL/min, injector temperature, 220 °C; detector, FID, 250 °C; oven temperature, 50 °C (5 min isothermal), raised at 5 °C/min to 250 °C (49 min isothermal); cut times 24.5−28.5 min. Main column conditions were as follows: duran glass capillary (30 m × 0.23 mm i.d.) coated with a 0.23 μm film of 50% octakis-(2,3-di-*O*-butyryl-6-*O*-*tert*-butyldimethylsilyl)-γ-cyclodextrin in OV-1701vi (Schmarr, 1992); carrier gas, hydrogen, 100 kPa; oven temperature, 40 °C (30 min isothermal), raised at 0.5 °C/

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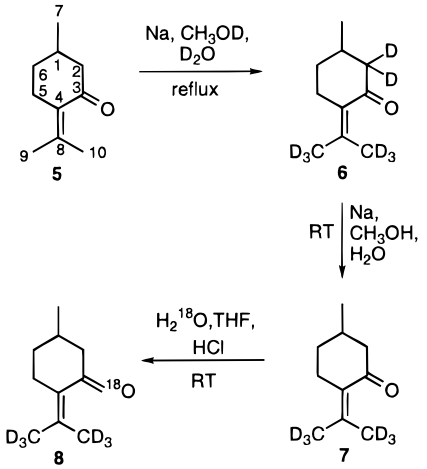
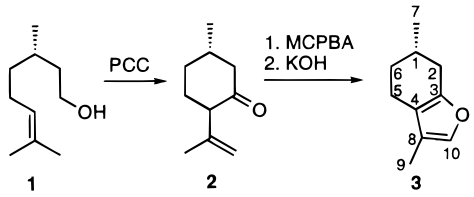
Scheme 1. Synthesis of Different Deuterium- and ^{18}O-Labeled Racemic Pulegones (6−8)



Scheme 2. Synthesis of (*S*)-Menthofuran (3) Starting from (*S*)-Citronellol (1)



min to 60 °C (0 min isothermal), raised at 2.5 °C/min to 120 °C; detector, ITD 800, transfer line 250 °C; open split interface, 250 °C; helium sweeping flow, 1 mL/min; ion trap manifold, 195 °C; EI, 70 eV.

^{1}H Nuclear Magnetic Resonance (NMR). A Bruker ARX 300, at 300 MHz, was employed for recording the ^{1}H NMR spectra. $CDCl_3$ was used as solvent. Abbrevations are as follows: s, singlet; d, doublet; t, triplet; m, multiplet; *J*, spin−spin coupling constant (Hz); a, axial; e, equatorial. The terpene nomenclature, given in Schemes 1 and 2, was used for assignment.

Synthesis of 5-(*R*/*S*)-2-[[1-^{2}H$_3$]Methyl[2,2,2-^{2}H$_3$]ethylidene]-5-methyl-[6,6-^{2}H$_2$]cyclohexanone (d_8-Pulegone) (6). Pulegone was synthesized according to the method of Gibson (1983). Three millimoles (470 mg) of (*R*)-pulegone and 3 mmol (470 mg) of (*S*)-pulegone (both from Fluka, Deisenhofen, Germany) were added to a solution containing 3 mL of D_2O, 6 mL of CH_3OD, and 4 mmol (92 mg) of sodium metal. The mixture was heated under reflux for 1.5 h, subsequently cooled, diluted with diethyl ether, and extracted twice with water and once with brine. After drying over sodium sulfate, the diethyl ether was removed in a vacuum. The crude product was used for two further H/D-exchange reactions under identical conditions. After three reaction steps, 280 mg of crude d_8-pulegone was purified by flash chromatography. Chromatographic conditions were as follows: column diameter, 20 mm; silica gel, 30−60 μm (Baker 7024-01); eluent pentane/diethyl ether 10:1 (v/v). After removal of the eluent, 0.07 mmol (11 mg) of pure **6** was obtained: MS 160 (M^+, 66), 142 (16), 117 (28), 114 (24), 82 (89), 70 (76); ^{1}H NMR δ 1.00 (d, 3H, 7-H, J = 6.5 Hz), 1.32 (m, 1H, 6a-H), 1.86 (m, 1H, 6e-H), 1.95 (m, 1H, 1-H), 2.25 (m, 1H, 5a-H), 2.70 (dt, 1H, 5e-H, J = 15.5 Hz, J = 4.5 Hz). No traces of 2e-H, 2a−H, 9-H, and 10-H were found at δ 2.50, 1.8−2.0, 1.79, and 2.00, respectively (Tori et al., 1975; Bambridge et al., 1995). Enantiomeric distribution was R/S = 1/1 (enantio-GC). Deuterium contents were [$^2H_7$] 23% and [$^2H_8$] 77%, calculated by MS data. Enantiopure deuterium-labeled [$^2H_8$]pulegone was synthesized starting from enantiopure pulegone in one reaction step.

Synthesis of 5-(*R*/*S*)-2-[[1-^{2}H$_3$]Methyl[2,2,2-^{2}H$_3$]ethylidene]-5-methylcyclohexanone (d_6-Pulegone) (7). Three millimoles (470 mg) of crude **6** was added to a solution containing 6 mL of CH_3OH, 5 mL of H_2O, and 4 mmol (92 mg) of sodium metal. The solution was allowed to stand at room temperature for 63 h. Crude **7** (1.4 mmol, 220 mg) was purified by flash chromatography, yielding 0.3 mmol (48 mg) of pure **7**: for conditions see above; MS 158 (M^+, 44), 140 (14), 115 (20), 112 (16), 81 (100), 70 (24); ^{1}H NMR δ 1.00 (d, 3H, 7-H, J = 6.5 Hz), 1.33 (m, 1H, 6a-H), 1.86 (m, 1H, 6e-H), 1.95−2.25 (m, 2H, 1-H, 2a-H), 2.24 (m, 1H, 5a-H), 2.49 (dd, 1H, 2e-H, J = 11.0 Hz, J = 2 Hz), 2,70 (dt, 1H, 5e-H, J = 15.5 Hz, J = 4.5 Hz). Traces of 9-H and 10-H were found at δ 1.75 and 2.00, respectively. Deuterium contents were [$^2H_5$] 25% and [$^2H_6$] 75%.

Synthesis of 5-(*R*/*S*)-2-[[1-^{2}H$_3$]Methyl[2,2,2-^{2}H$_3$]ethylidene]-5-methyl-[^{18}O]cyclohexanone (d_6-[^{18}O]Pulegone) (8). Compound **8** was synthesized according to the method of Vederas (1980) with modifications. Six millimoles (93 mg) of crude **7** was added to a solution containing 1 mL of 0.3 N HCl/dry THF and 80 μL of $H_2{}^{18}O$ (95.7% atom ^{18}O, 0.6% atom ^{17}O, 3.7% atom ^{16}O; Euriso-top, Saint Aubin Cedex, France). After standing at room temperature for 70 h, the solutions was diluted with diethyl ether. The organic layer was extracted with brine and dried over sodium sulfate. A second step was performed. The resulting crude **8** was purified by preparative thin-layer chromatography (TLC). Chromatographic conditions were as follows: silica gel, 60 F 254 (Merck); mobile phase pentane/diethyl ether 10:1 (v:v); detection, UV 254 nm; R_f 0.4; yield, 19 mg (0.1 mmol); MS 160 (M^+, 35), 142 (14), 115 (19), 112 (17), 81 (100), 72 (36); ^{1}H NMR δ 1.00 (d, 3H, 7-H, J = 6.5 Hz), 1,13 (m, 1H, 6a-H), 1.86 (m, 1H, 6e-H), 1.95−2.05 (m, 2H, 1-H, 2a-H), 2.24 (m, 1H, 5a-H), 2.49 (dd, 1H, 2e-H, J = 11 Hz, J = 2 Hz), 2.71 (dt, 1H, 5e-H, J = 15.5 Hz, J = 4.5 Hz). Deuterium and ^{18}O contents were [$^2H_5$,^{16}O] 5%, [$^2H_6$,^{16}O] 12%, [$^2H_5$,^{18}O] 35%, and [$^2H_6$,^{18}O] 48%.

Synthesis of (6*S*)-3,6-Dimethyl-4,5,6,7-tetrahydrobenzo[*b*]furan [(*S*)-Menthofuran] (3). Isopulegon (**2**) was synthesized according to the method of Corey et al. (1976) with a modified cleanup; menthofuran (**3**) was synthesized according to the method of Friedrich and Bohlmann (1988). Pyridinium chlorochromate (PCC; 8.9 mmol, 1.93 mg) was dissolved in 11 mL of dry methylene chloride. (*S*)-Citronellol (**1**; 430 mg, 2.7 mmol) (Fluka) was added. It was stirred for 36 h, pentane/diethyl ether 1:1 (v:v) was added, and the suspension was filtered (Celite). The solution was washed with 10% HCl, 10% $NaHCO_3$, and H_2O and dried over sodium sulfate. The crude product isopulegone (**2**) (1.5 mmol, 230 mg) was dissolved in 10 mL of dry benzene; 2.6 mmol (450 mg) of 85% *m*-chloroperbenzoic acid was added, and the solution was stirred for 24 h. The benzene was evaporated, and the residue was taken up in petrol and filtered. After evaporation of the solvent, 5.9 mL of methanol and 3.2 mL of 40% KOH solution were added, and the solution was stirred for 4 h at room temperature. Then diethyl ether was added to the solution. After extraction with water (twice) and brine (once), the solution was dried over sodium sulfate. Purification by flash chromatography (conditions see above) yielded 0.07 mmol (11 mg) of pure **3**: MS 150 (M+, 38), 108 (100), 79 (29); ^{1}H NMR δ 1.07 (d, 3H, 7-H, J = 6.5 Hz), 1.2−1.4 (m, 1H, 6-H), 1.8−1.9 (m, 2H, 1-H, 6-H) 1.92 (s, 3H, 9-H), 2.1−2.4 (m, 3H, 2-H, 5-H), 2.64 (dd, 1H, 2-H, J = 11.0 Hz, J = 5.0 Hz), 7.03 (s, 1H, 10-H) (Friedrich and Bohlmann, 1988).

Synthesis of (5*S*)-2-Isopropyliden-5-[^{2}H$_3$]methyl-[4,4-^{2}H$_2$]cyclohexanone [d_5-(*S*)-Pulegone] (9). 9 was prepared according to the method of Fuchs et al. (1999). Enantiomeric distribution was S/R = 92:8.

Plant Material. Cuttings from *Mentha* × *piperita* were kindly provided by the Botanical Garden of Frankfurt University. The plants selected for the experiments were 5−7 cm high with 4−6 leaf pairs.

Feeding Experiments. *Precursor Solution.* A solution of the labeled pulegones (**6**−**9**) with a concentration of 0.1 mg/mL was prepared by dissolving the same amounts of **6**−**9** and Tween 20 in distilled water.

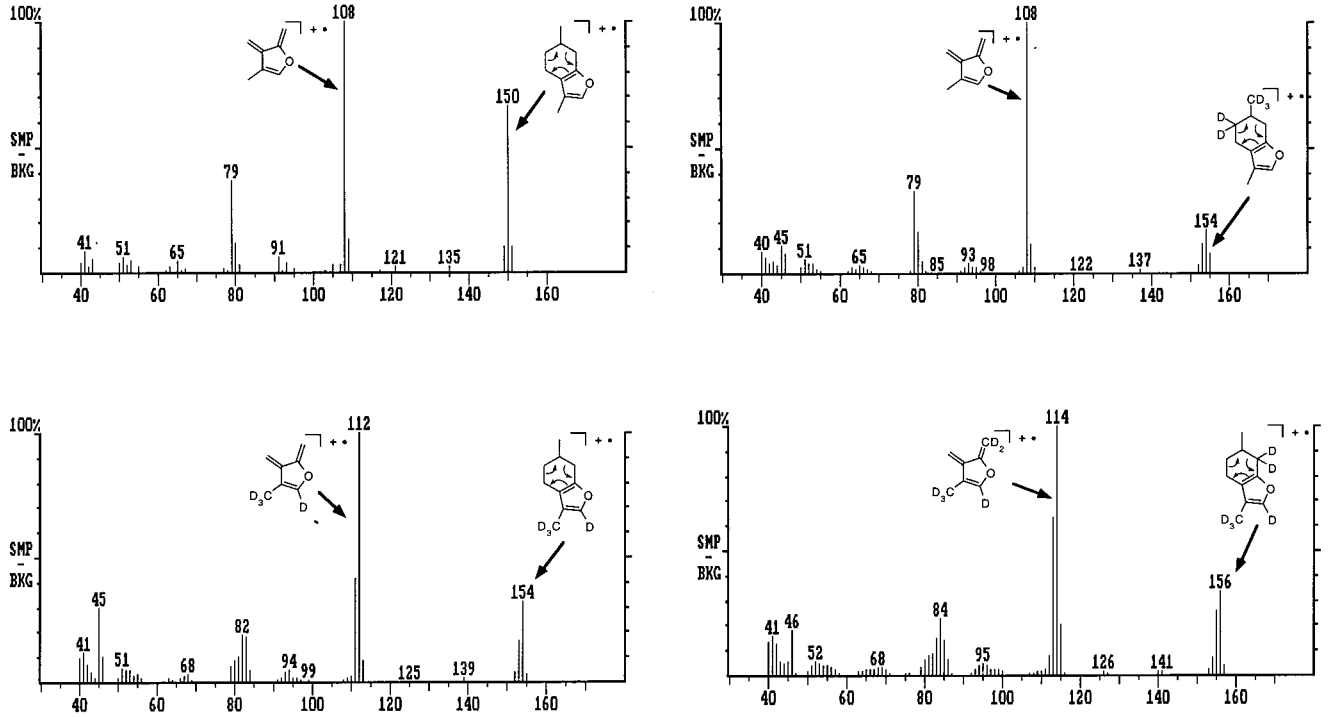


Figure 1. Mass spectra of deuterium-labeled menthofurans obtained from feeding experiments with different deuterium-labeled pulegones. Retro-Diels−Alder fragmentation is proposed.

*(S)-Menthofuran (**3**) (Internal Standard, IST) Solution.* The solution contained 0.1 mg/mL Tween 20 and 0.01 mg/mL (*S*)-menthofuran. For feeding experiments the respective precursor solution and the internal standard solution were mixed (2:1, v/v). In feeding experiments with d_5-(*S*)-pulegone (**9**), only the precursor solution was used. The shoot tip and first leaf pair and 400 μL of the feeding solution were incubated in a sealed 2 mL vial at room temperature for 17−21 h in the dark. Blank cultures were maintained with distilled water containing 0.1 mg/mL Tween 20. The plant material was then placed in a new vial, sealed, and incubated for 1 h in the dark. Afterward, the essential oil evaporating from the glandular trichomes as well as the feeding solution was analyzed using headspace SPME.

SPME. An SPME fiber, coated with a 100 μm film of poly(dimethylsiloxane), installed in an SPME fiber holder for manual use (both Supelco, Munich, Germany) was used for headspace sampling. For thermal desorption, the SPME fiber remained in the injector for 3 min, with the split valve being opened after 2 min.

RESULTS AND DISCUSSION

Feeding experiments with deuterium-labeled monoterpene precursors are powerful tools for in vivo studies of monoterpene biosynthesis in plants. The essential oil of the plants was analyzed by SPME and enantio-MDGC/MS. This is a highly efficient and noninvasive micro method for the evaluation of stereochemical pathways during bioconversion reactions (Wüst et al., 1996, 1998).

For the determination of the stereoselectivity in menthofuran biogenesis, deuterium-labeled pulegone precursors (**6** and **7**) were used. They are readily available using hydrogen−deuterium-exchange reactions and different reaction conditions. Deuterium and ^{18}O-labeled pulegone (**8**) was synthesized using a special exchange reaction (Scheme 1).

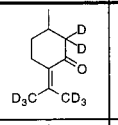
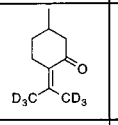
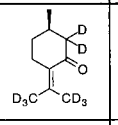
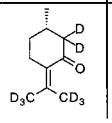
The procedure of mixed labeling proves whether the oxygen-18 is lost during bioconversion.

Labeled menthofurans were identified by their retention times on the precolumn and main column as well as by their specific mass spectra. The mass spectra showed a characteristic base peak, most probably due to a retro-Diels−Alder reaction, as proved by the mass spectra of different deuterium-labeled menthofurans (Figure 1).

In first prescreening experiments with labeled racemic pulegone, small amounts of labeled racemic menthofuran were detected in the pure feeding solution by SPME/enantio-MDGC/MS, despite exclusion of light and without incubation of any plant material. Consequently, photochemical reactions cannot be the cause for this finding. However, the detection of small amounts of labeled menthofuran racemate suggested a nonselective autoxidation process. Furthermore, when using mixed labeled pulegone, the ^{18}O/^{16}O ratio in pulegone and corresponding menthofuran remained unchanged; however, when plant material was fed with mixed labeled pulegone under identical experimental conditions, significant differences were detectable. Enantiodiscrimination as well as a change in ^{18}O/^{16}O ratio in menthofuran took place, indicating an enzymatic process in the plant. Therefore, the labeled menthofuran in the essential oil analyzed consists mainly of biotransformed pulegone, but a small amount of menthofuran from the feeding solution also has to be taken into account. For exact calculations, (*S*)-menthofuran (**3**) was chosen as an ideal internal standard (IST), because this enantiomer (**3**) is not detectable in blank cultures of *Mentha* × *piperita*. IST (**3**) was synthesized using oxidation and cyclization of (*S*)-citronellol (**1**) to isopulegone (**2**). Epoxidation and cyclization of an epoxyketone intermediate led to (*S*)-menthofuran (**3**) (Scheme 2).

In feeding experiments with regioselectively deuterated precursors (**6** and **7**) the corresponding menthofuran enantiomers were calculated versus the internal standard (*S*)-menthofuran (**3**). Thus, it was clearly

Table 1. Ratios of Deuterium-Labeled Menthofuran Enantiomers versus Internal Standard (*S*)-Menthofuran (3) in Feeding Solutions and Corresponding Plants[a]

labeled terpene administered	analysis of	ratio labeled (S)-menthofuran/IST	ratio labeled (R)-menthofuran/IST
6 	feeding solution	1,6	1,6
	plant ess. oil	38,3	17,4
7 	feeding solution	0,2	0,2
	plant ess. oil	8,2	5,9
(R)-6 	feeding solution	-	0,01
	plant ess. oil	-	2894
(S)-6 	feeding solution	0,01	-
	plant ess. oil	5121	-

[a] Ess. oil is essential oil.

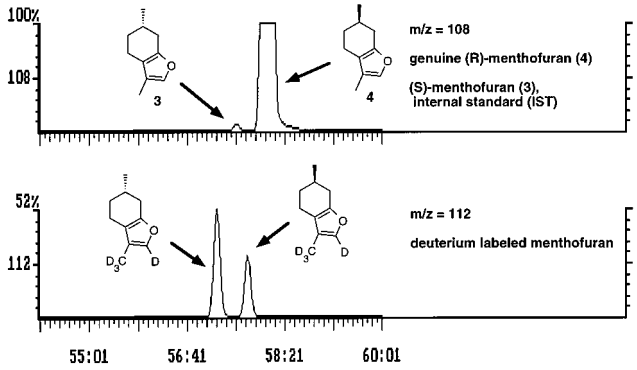


Figure 2. Enantio-MDGC/MS analysis of the headspace extract of *M.* × *piperita* fed with racemic d_6-pulegone (**7**); (*S*)-menthofuran (**3**) is used as internal standard (main column chromatogram).

shown that the labeled menthofuran species could be traced back almost exclusively to the bioconversion of the corresponding pulegone precursors (Table 1).

The main column chromatogram (enantio-MDGC/MS) of a feeding experiment using racemic d_6-pulegone (**7**) is shown in Figure 2.

At *m*/*z* 108 the genuine (*R*)-menthofuran (**4**) and the IST (*S*)-menthofuran (**3**) are detected. The deuterium-labeled menthofuran was detected at *m*/*z* 112. During the bioconversion of labeled pulegone, an enantiodiscrimination was observed. When a racemic mixture of labeled pulegone was fed, labeled (*S*)-menthofuran, the analogue of the nongenuine menthofuran enantiomer, was preferably biosynthesized in *Mentha* × *piperita*. The main result of all feeding experiments, using labeled racemic pulegone, was 58% (*S*)-menthofuran to 42% (*R*)-menthofuran (Table 2).

Feeding experiments with enantiopure d_8-pulegone [(*R*)-**6**, (*S*)-**6**] proved labeled (*S*)-menthofuran and (*R*)-menthofuran, respectively, to be exclusively biosynthesized from the corresponding pulegone precursor.

To determine whether the oxygen of the labeled pulegone is the same as that in menthofuran, feeding

Table 2. Enantiomeric Distribution of Menthofuran Obtained from Feeding Experiments with Labeled Racemic Pulegones[a]

	labeled (*S*)-menthofuran (%)	labeled (*R*)-menthofuran (%)
labeled racemic pulegone (**6**−**8**) ($n = 26$, $\sigma_{n-1} = 5.38$)	58	42
(*R*)-d_8-pulegone [(*R*)-**6**]	nd	>98
(*S*)-d_8-pulegone [(*S*)-**6**]	>98	nd
blank cultures	nd	nd

[a] *n*, numbers of analysis; σ_{n-1}, standard deviation; nd, not detectable.

experiments with deuterium and ^{18}O-labeled pulegone were carried out. The obtained mass spectra of menthofuran from feeding experiments with d_6-pulegone (**7**) and d_6-[^{18}O]pulegone (**8**) were identical, indicating that the oxygen of the pulegone was lost during bioconversion. The loss of oxygen-18 of compound **8** by hydrolysis was excluded, as indicated by GC/MS analysis of **8** in the blank feeding solution (deuterium and ^{18}O contents: [$^2H_5$,^{16}O] 6%, [$^2H_6$,^{16}O] 11%, [$^2H_5$,^{18}O] 39%, [$^2H_6$,^{18}O] 43%). In the plant, no hydrolytic loss of ^{18}O labeling took place, as proved by the mass spectra of menthone and isomenthone, also generated from d_6-[^{18}O]pulegone (**8**). Their mass spectra showed the same ^{18}O/^{16}O ratio as the labeled pulegone precursor (Figure 3).

Obviously, during bioconversion of labeled pulegone a loss of deuterium occurs, proved by the mass spectra of the generated menthofuran. After feeding labeled pulegones (**6**−**8**), the mass spectra of corresponding menthofurans showed high-intensity peaks at *m*/*z* 113 and 111, respectively, sometimes being the base peak. The deuterium content of pulegones (**6**−**8**) in the feeding solution remained almost unaffected [e.g., deuterium content of feeding solution (**6**): [$^2H_6$] 2%, [$^2H_7$] 23%, [$^2H_8$] 75%], so the loss of the deuterium in the corresponding labeled menthofurans cannot be explained as yet.

Our feeding experiments show that an enzymatic process is responsible for the bioconversion of labeled pulegone to labeled menthofuran. Photooxygenation can be excluded because the feeding experiments were carried out in the dark. Furthermore, autoxidative processes are negligible, because no ^{18}O-labeled menthofuran was detectable.

The enzymatic conversion of labeled pulegone into labeled menthofuran in *Mentha* × *piperita* is not specific, as both pulegone enantiomers are converted into menthofuran with corresponding stereochemistry at C-1. Furthermore, a discrimination in favor of labeled (*S*)-pulegone, the analogue of the nongenuine (*S*)-pulegone, was observed (Scheme 3).

From the literature, differences between (*S*)-pulegone and (*R*)-pulegone in the metabolism in mammals are known (Madyastha and Gaikwad, 1998). Both pulegone enantiomers are metabolized to menthofuran, although higher levels of menthofuran were found in the case of (*R*)-pulegone. However, in this context, it must be taken into account that both pulegone enantiomers do not occur as genuine compounds in mammals.

On the basis of the results obtained from our feeding experiments, obviously an enzymatic oxidation of labeled pulegone (probably by a cytochrome oxidase) is involved in the bioconversion of labeled pulegone into

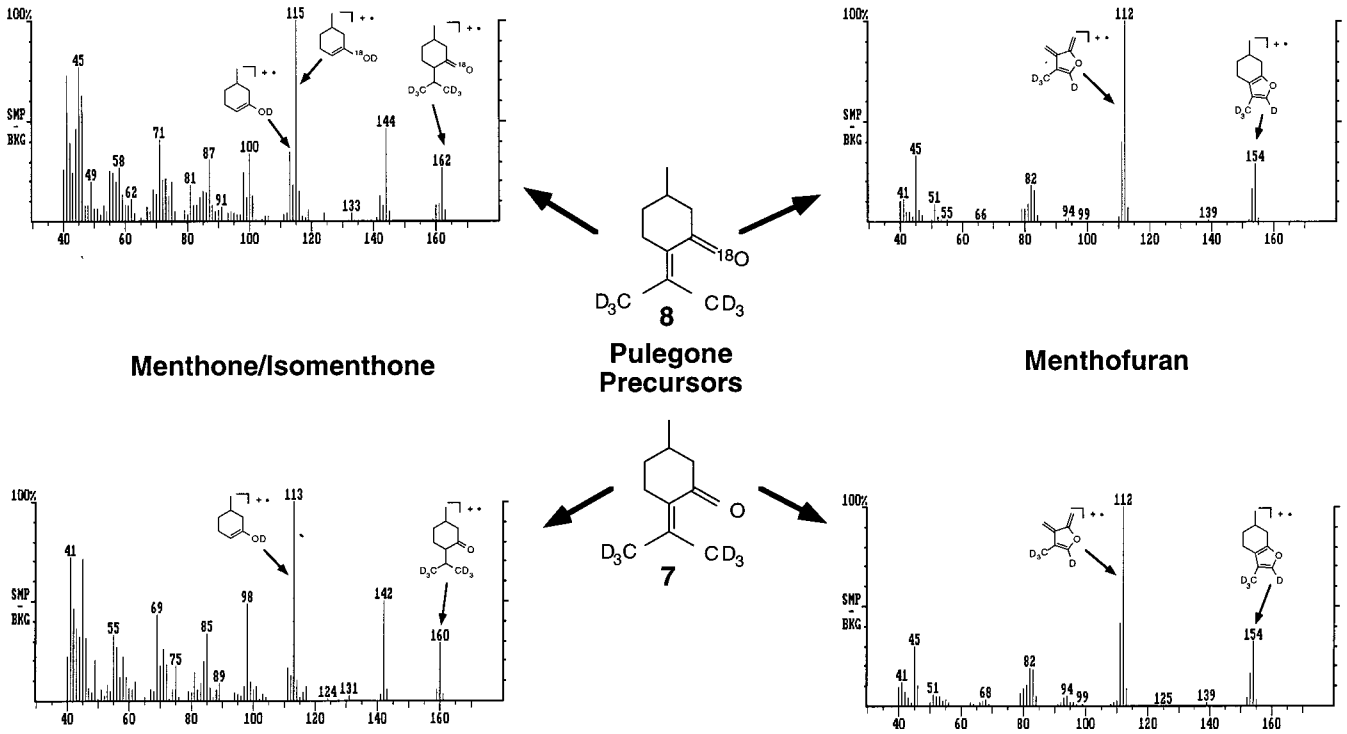
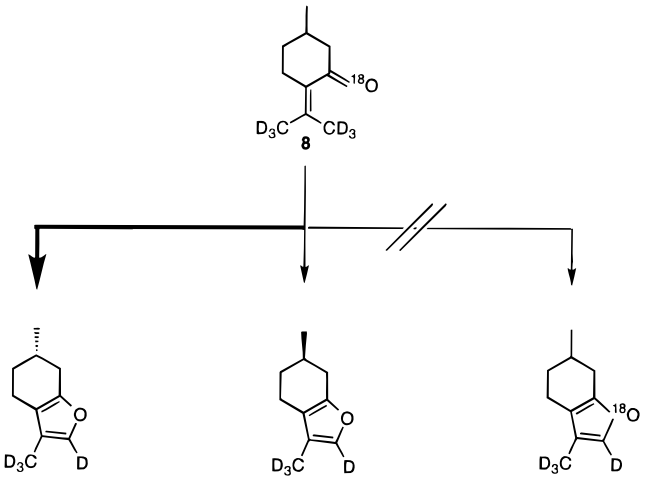


Figure 3. Mass spectra of labeled menthofuran and labeled menthone/isomenthone obtained after feeding experiments with d_6-pulegone (**7**) and d_6-[^{18}O]-pulegone (**8**).

Scheme 3. Proposed Pathway for the Bioconversion of Pulegone to Menthofuran in *M.* × *piperita*



menthofuran. The oxygen introduced by oxidation of pulegone remains in the menthofuran, whereas the original oxygen of the pulegone carbonyl group is lost during bioconversion. This finding is in agreement with investigations in mammals using experiments carried out under an $^{18}O_2$ atmosphere, where complete incorporation of $^{18}O_2$ into menthofuran was observed (Nelson et al., 1992b).

The labeling pattern of the menthofuran biosynthesized in *Mentha* × *piperita* does not contradict the mechanism proposed for the bioconversion of pulegone to menthofurane in mammals. Nelson et al. (1992b) suggested a cytochrome P-450-catalyzed oxidation of the *cis*-methyl group of the pulegone isopropylidene moiety, subsequently cyclized and dehydrated. Therefore, further investigations must be carried out to obtain more details of menthofuran biosynthesis in *Mentha* × *piperita*.

CONCLUSIONS

Feeding experiments with stable isotope labeled pulegone enantiomers proved that *Mentha* × *piperita* is able to convert both pulegone enantiomers into the corresponding (*R*)- and (*S*)-configured menthofurans. Furthermore, when racemic pulegone precursors were administered, a discrimination was observed, favoring the labeled analogue of the nongenuine (*S*)-pulegone. An enzymatic oxidation is involved in menthofuran biosynthesis, as shown by incubation in the dark and by loss of the pulegone oxygen.

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