Conversion of Menthyl Acetate or Neomenthyl Acetate into Menthol or Neomenthol by Cell Suspension Cultures of *Mentha canadensis* and *Mentha piperita*

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Suspension cultures of *Mentha canadensis* and *Mentha piperita* transformed added *l*-(−)-menthyl acetate and *d*-(+)-menthyl acetate such as *d*-(+)-neomenthyl acetate and *l*-(−)-neomenthyl acetate into their corresponding alcohols. Within 24 h most of the *l*-(−)-menthol was converted into *l*-(−)-menthyl glucoside. Small amounts of *d*-(+)-menthol and *d*-(+)-neomenthol were also transformed into their glucosides, while *l*-(−)-neomenthol decreased without glucosylation. In cultured *M. canadensis* the glucosylation was more enantioselective and stereospecific to *l*-menthol than in cultured *M. piperita*.

INTRODUCTION

The biochemical potential of *Mentha canadensis* and *Mentha piperita* suspension cultures to synthesize specific monoterpenes could become of considerable interest for their utilization in foods or drinks as well as for personal products or pharmaceutical applications. Until now the yield of essential oils produced by *M. piperita* suspension cultures has been very low (Cormier and Do, 1988; Kireeva et al., 1978; Rodov and Reznikova, 1982; Werrmann and Knorr, 1990). Consequently, attempts have been made to increase productivity.

Geraniol and limonene as added precursors were degraded by *M. piperita* callus cultures, and no oxygenated monoterpenes were identified (Godelmann, 1985; Werrmann and Knorr, 1989). However, it has been demonstrated that cultured *Mentha* cell lines reduced *d*-pulegone to *d*-isomenthone and *l*-menthone to *d*-neomenthol (Aviv and Galun, 1978; Aviv et al., 1981; Rodov et al., 1988). These results indicate stereospecificity with respect to precursors and products. Furthermore, callus cultures of *M. piperita* transformed added *l*-menthol into *l*-menthyl *β*-glucoside (Berger and Drawert, 1988). The known metabolic transformations of different precursors in *Mentha* cell cultures beginning with *d*-pulegone are shown in Figure 1.

According to Kjonaas and Croteau (1983), in intact *M. piperita* plants, *l*-limonene, the first cyclic intermediate, was converted into isopiperitenol, *l*-isopiperitone, *d*-pulegone, and *l*-menthone. *l*-Menthone was reduced by a dehydrogenase 1 to the enantiomeric alcohol *l*-menthol in the epidermal oil glands and by a dehydrogenase 2 to *d*-neomenthol in the mesophyll of the leaves (Kjonaas et al., 1982).

Croteau and Winters (1982) reported an acetyl transferase in the glands that converted *l*-menthol into *l*-menthyl acetate (10%) and small amounts of *d*-neomenthol to *d*-neomenthyl acetate. The bulk of *d*-neomenthol was transformed into *d*-neomenthyl *β*-glucoside and small amounts of *l*-menthol into 1-menthyl *β*-glucoside by a UDPglucose–monoterpenol glucosyl transferase present in the mesophyll (Croteau and Martinkus, 1979; Martinkus and Croteau, 1981). However, when menthol or neomenthol was added to leaf disks separately, they were converted nearly equally to menthyl or neomenthyl acetates and menthyl or neomenthyl glucosides (Croteau and Martinkus, 1979; Martinkus and Croteau, 1981). The metabolic transformation of *l*-menthone in *M. piperita* plants as reported in the literature is shown in Figure 2 (Croteau and Martinkus, 1979; Croteau and Winters, 1982; Martinkus and Croteau, 1981).

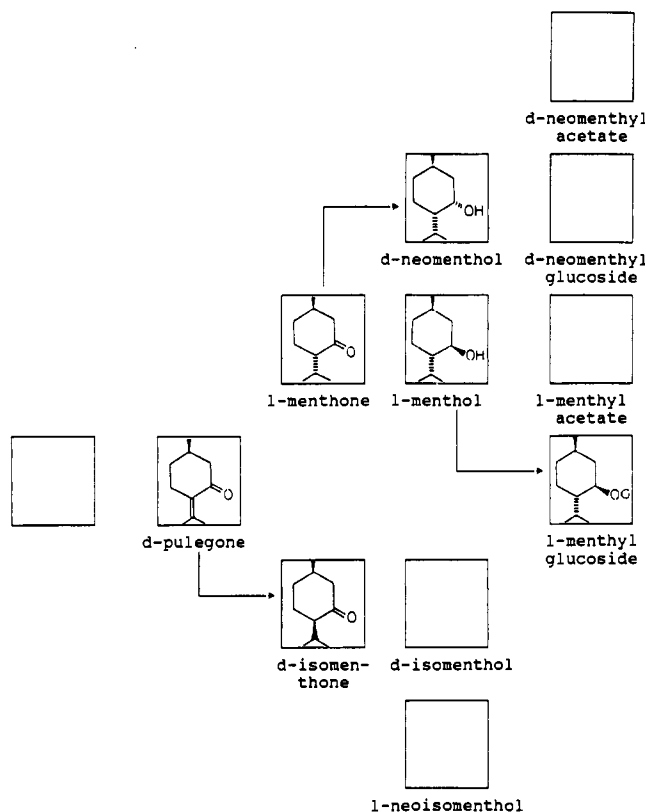

Figure 1. Precursor addition and conversion in different *Mentha* suspension cultures (Aviv and Galun, 1978; Aviv et al., 1981; Rodov et al., 1988).

To our knowledge no studies exist about the conversion of menthyl acetate or neomenthyl acetate in *Mentha* cell cultures. In this paper the occurrence of a deacetylating activity of *M. canadensis* and *M. piperita* suspension cultures as well as the stereospecificity and enantioselectivity of the reaction will be discussed. Cell cultures might have a potential to produce natural *l*-menthol by

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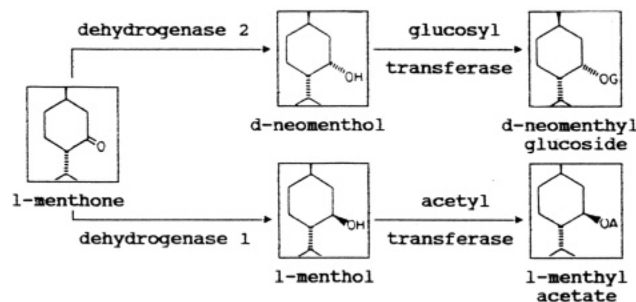


Figure 2. Pathway of *l*-menthol beginning with *l*-menthone in *M. piperita* leaves (Croteau and Martinkus, 1979; Croteau and Winters, 1982; Kjonaas et al., 1982; Martinkus and Croteau, 1981).

deacetylation of *l*-menthyl acetate as natural compound in the essential oil of different *Mentha* species.

EXPERIMENTAL PROCEDURES

Material. Sterile plants of *M. canadensis* 174 were obtained from the Germplasm Center, Corvallis, OR, and *M. piperita* plants were received from the Botanical Garden of Berlin. Shoots were surface sterilized; 1-cm pieces of *M. piperita* were plated on Petri dishes with 10 mL of B5 agar medium (Gamborg et al., 1968) and pieces of *M. canadensis* on MSk agar medium (Murashige and Skoog, 1962). Cell suspensions were initiated from rapid growing calli by transfer into the analogous liquid medium and maintained for 14 months by transfer into fresh medium every 2 weeks.

l-(−)-Menthyl acetate, *d*-(+)-menthyl acetate, *d*-(+)-neomenthyl acetate, *l*-(−)-neomenthyl acetate, *l*-(−)-menthol, *d*-(+)-menthol, *d*-(+)-neomenthol, and *l*-(−)-neomenthol were purchased from Fluka (Neu-Ulm, FRG). Acetate, 2-octanol, *n*-pentane, and ether were from Merck (Darmstadt, FRG). Acetate enzyme test from Boehringer (Mannheim, FRG) and β-glucosidase from Sigma (Karlsruhe, FRG) were used.

Chemical Analyses. Using a micro steam distillation/extraction apparatus (Chrompack, Mainz-Kastel, FRG), 25 mL of homogenized suspensions was distilled at 95 °C. Distillates were extracted with 15 mL of pentane/ether (1/1) at 65 ± 2 °C for 1 h. The solvent mixture was dried with anhydrous Na_2SO_4 and concentrated to 100–200 μL. 2-Octanol (10 μg/25 mL of medium) was added as an internal standard just before extraction.

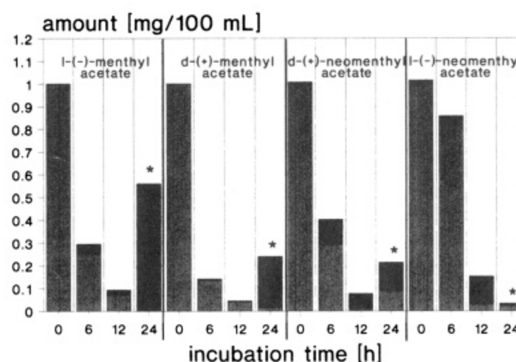
Acetate and monoterpenes could be separated by a GLC (Carlo Erba, Model HRGC 5160 Mega Series, Frankfurt, FRG) with a flame ionization detector and a glass column (DB-Wax, 50 m × 32 mm, 0.5-μm film). The flow rate of the carrier gas was 2 mL/min (He) and that of the burning gas 25 mL/min (H_2) and 350 mL/min (compressed air). Split was 20 mL/min. Balance of split and circulation of septum was 20:1. Column temperature started at 80 °C and was raised at a rate of 2 °C/min to 230 °C.

To examine deacetylation of monoterpenyl acetates, *l*-menthyl, *d*-menthyl, *d*-neomenthyl, and *l*-neomenthyl acetate (1 mg/100 mL) were applied to each culture (45 ± 5 g of fresh weight) at the 13th day of cultivation. The cultures were analyzed after incubation periods of 0, 6, 12, and 24 h.

For determination of acetate the enzymatic test of Boehringer (Mannheim, FRG) was used. The absorbance of the samples was measured at 340 nm. To examine glucosylation of monoterpenols in cell cultures, menthol or neomenthol (2 mg/100 mL) was applied to each culture at the 13th day of cultivation. After an incubation time of 23 h, 10 units of β-glucosidase/100 mL was added. After 1 h, glucosidically bound alcohols converted into free alcohols, which were measured.

RESULTS AND DISCUSSION

Initially, neither menthol nor any other native or acetylated alcohols were detected in significant amounts in suspension cultures of *M. canadensis* and *M. piperita*. The addition of *d*/*l*-menthyl acetate and *d*-neomenthyl acetate resulted in corresponding alcohols.



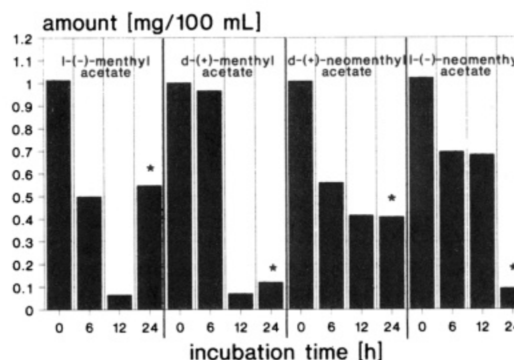


Figure 3. Degradation of added monoterpene acetates and concurrent synthesis of corresponding monoterpene alcohols in *Mentha* suspension cultures. (Light shading) monoterpene acetate; (dark shading) monoterpene alcohol; (dark shading with asterisk) 1 h after β-glucosidase addition.

More than 60% of the *l*-(−)-menthyl acetate disappeared, approaching total disappearance within 6 h after 24 h in both *Mentha* suspension cultures (Figure 3). *l*-Menthol was observed to appear within 6 h. Further incubation resulted in a complete degradation of synthesized *l*-menthol. Furthermore, both *Mentha* cultures quite efficiently transformed *d*-(+)-menthyl acetate, *d*-(+)-neomenthyl acetate, and *l*-(−)-neomenthyl acetate into corresponding alcohols. Within 6 h *M. canadensis* degraded approximately 80% of *d*-menthyl acetate, 60% of *d*-neomenthyl acetate, and almost 30% of *l*-neomenthyl acetate. During the same time *d*-menthyl acetate decreased by 20%, *d*-neomenthyl acetate by 50%, and *d*-neomenthyl acetate by 30% in *M. piperita* cultures. During the conversion of menthyl acetate or neomenthyl acetate no acetate was identified by GLC or by an acetate enzyme test (data not shown). It is not clear what happened to the remaining portions of the substrate. However, since the main emphasis of this work was on menthol production, the fate of each individual degradation step was not followed.

The results demonstrated that monoterpenyl acetates added to *Mentha* suspension cultures were degraded within 6 or 12 h while the corresponding alcohols appeared. After further incubation, all synthesized alcohols and acetates were completely degraded. Both *Mentha* species exhibited the same conversion of *l*-menthyl acetate to *l*-menthol. Similar results were obtained from deacetylation of *d*-menthyl acetate, *d*-neomenthyl acetate, and *l*-neomenthyl acetate. Such effects were also found for the conversion of *d*-pulegone to *d*-isomenthone (Aviv and Galun, 1978) and *l*-menthone to *d*-neomenthol (Aviv et al., 1981; Rodov et al., 1988).

Table I. Glucosylation of *l*-Menthol, *d*-Menthol, *d*-Neomenthol, and *l*-Neomenthol by *Mentha* Suspension Cultures after Addition of 2 mg of Monoterpenol/100 mL at the 13th Day and Incubation for 24 h

	M. canadensis, mg of alcohol/100 mL	*M. piperita*, mg of alcohol/100 mL
l-(−)-menthol		
free	0.12 ± 0.00	0.04 ± 0.01
glucosidically bound	1.18 ± 0.07	1.09 ± 0.03
d-(+)-menthol		
free	0.01 ± 0.01	0.01 ± 0.00
glucosidically bound	0.45 ± 0.22	0.22 ± 0.01
d-(+)-neomenthol		
free	0.02 ± 0.00	0.02 ± 0.01
glucosidically bound	0.35 ± 0.03	0.79 ± 0.12
l-(−)-neomenthol		
free	0.01 ± 0.01	0.00 ± 0.00
glucosidically bound	0.01 ± 0.00	0.04 ± 0.02

The deacetylation in *Mentha* suspension cultures was reverse to the monoterpene pathway in *M. piperita* leaves, where separated acetyl transferase of the epidermal oil glands acetylated menthol and neomenthol (Croteau and Winters, 1982; Karp and Croteau, 1988; Martinkus and Croteau, 1981). The reverse behavior of acetylation/deacetylation in plant leaves and cell cultures of *Mentha* could be the result of enzyme types. Since no acetate was released during the deacetylation of tested monoterpenyl acetates, occurrence of an acetyltransferase in suspension cultures of *M. canadensis* and *M. piperita* is more probable than occurrence of pure acetate deacetylase.

The conversion of enantiomeric alcohols to the corresponding β-*d*-glucoside occurred in both *Mentha* suspension cultures (Table I). In *M. piperita* the conversion rates of *d*/*l*-menthol and *d*/*l*-neomenthol of their β-glucosides were approximately 55/10% and 3/40%, respectively. Similar results occurred in *M. canadensis* cultures. The glucosylation rate of *l*-menthol was nearly 60%, of *d*-menthol 22%, of *d*-neomenthol 17%, and of *l*-neomenthol 0%. The glucosylation of epimeric menthol was more selective for *l*-menthol than for diastereomeric *d*-menthol as well as for *d*-neomenthol and diastereomeric *l*-neomenthol in both *Mentha* suspension cultures. According to Berger and Drawert (1988), glucosylation of *l*/*d*-menthol could also be found. These authors reported that in *M. piperita* suspension cultures added menthol was converted by approximately 70% into menthyl glucoside within 12 h.

In comparison to intact plants of *M. piperita* (Croteau and Martinkus, 1979; Croteau and Winters, 1982; Martinkus and Croteau, 1981) the occurrence of a glucosyltransferase in suspension cultures will be discussed. The conversion of monoterpenols to their β-*d*-glucosides seems to be analogous to the reactions observed in the mesophyll of *M. piperita* leaves. The enzyme systems of both *Mentha* cell cultures converted *l*-menthol to menthyl β-glucoside more efficiently than *d*-menthol, *d*-neomenthol, and *l*-neomenthol to *d*-menthyl-, *d*-neomenthyl-, and *l*-neomenthyl β-glucoside, similar to isolated UDPglucose–monoterpenol glucosyl transferase (Croteau and Martinkus, 1979; Martinkus and Croteau, 1981).

With respect to deacetylation of *l*-menthyl acetate more than half of the synthesized *l*-menthol was glucosidically bound in both *Mentha* suspension cultures within 24 h, and the remainder was completely degraded (Table I). The highest amount of free *l*-menthol (25%) was synthesized in *M. piperita* cultures within 6 h (Figure 3). *M. canadensis* cultures synthesized basically no free *l*-menthol.

Similar to the deacetylation of *l*-menthyl acetate, the highest amount of unbound *d*-menthol and *d*-neomenthol (Figure 3) was found in *M. piperita* cultures (15%) within 6 h, while *M. canadensis* cultures synthesized no free *d*-menthol and 10% *d*-neomenthol. With respect to glucosylation (Table I), no more than 20% of deacetylated *d*-menthyl acetate and *d*-neomenthyl acetate was converted into their glucosides by *M. canadensis* cultures within 24 h. In contrast, only 10% of deacetylated *d*-menthyl acetate but up to 40% of deacetylated *d*-neomenthyl acetate was glucosidically bound in *M. piperita* cultures. After 24 h, in both *Mentha* cultures all free alcohols and corresponding acetates were completely degraded.

In conclusion, the transformation of menthyl acetate and neomenthyl acetate to enantiomeric alcohols was not equally selective in both *Mentha* suspension cultures. The glucosyltransferase showed more enantioselectivity and stereospecificity to *l*-menthol in *M. canadensis* than in *M. piperita*. In contrast to these results, no significant differences in glucosylation activity between glucosylation of *l*-menthol and *d*-neomenthol in *M. piperita* were found, while *l*-neomenthyl glucoside could not be identified.

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