PERGAMON

Phytochemistry 50 (1999) 1347–1351

PHYTOCHEMISTRY

Biotransformation of (*S*)-(−)- and (*R*)-(+)-limonene using *Solanum aviculare* and *Dioscorea deltoidea* plant cells

Tomáš Vaněk*, Irena Valterová, Tomáš Vaisar†

Department of Plant Tissue Cultures, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10 Praha 6, Czech Republic

Received in revised form 21 July 1998

Abstract

(*S*)-(−)- and (*R*)-(+)-limonene was transformed to carvone via corresponding *cis*- and *trans*-carveol using *Solanum aviculare* and *Dioscorea deltoidea* plant cells. Both carveols and carvone formed were racemic in all biotransformations.

Keywords: *Solanum aviculare*; *Dioscorea deltoidea*; Monoterpenes; (*S*)-(−)-limonene; (*R*)-(+)-limonene; Carvone; *Cis*-carveol; *Trans*-carveol; Enantioselective separation

1. Introduction

Biotransformation has been extensively applied in the fermentation industry, for example in the production of L-aspartic acid from fumaric acid, L-malic acid from the same substrate and interconversion of a variety of steroids. Using the same approach, plant cell cultures have been used in studies on the formation of steroids and alkaloids on a laboratory scale.

Until now there has been described a large range of biotransformations, using plant cells established from many species and utilising many different substrates. According to conditions, one specific substrate may be converted to different products. Conversion of progesterone is one of the examples of the diversity of biotransformation. Nine different chemical reactions using 14 different plant cell cultures were described with this substrate (Stohs, 1980).

On the other hand, one cell culture may transform different substrates. In case of transformation of cyclic alcohols and monoterpenes using *Nicotiana tabacum* cells, the following reactions were described: regioselective hydroxylation of C=C double bond, enantioselective hydroxylation, stereoselective reduction of keto-group and reciprocal conversion of cyclic alcohols and ketones (Suga et al., 1982).

During our previous experiments, we described glycosidation of 2-(4-methoxybenzyl)-1-cyclohexanone using *Dioscorea deltoidea* (Vaněk, Urmanceva, Wimmer, & Macek, 1989) and 2-(4-methoxybenzyl)-1-cyclopentanone using *Rheum palmatum* cells (Vaněk, Wimmer, Macek, & Šaman, submitted), a stereoselective oxidation of (*S*)-*cis*-verbenol to (−)-verbenone (Vaněk, Macek, Stránský, & Ubik, 1989), and the effect of immobilisation on this reaction (Vaněk, Valterová, Pospíšilová, & Vaisar, 1994). In this paper, we describe a biotransformation of (*S*)-(−) and (*R*)-(+)-limonene using *Solanum aviculare* and *D. deltoidea* plant cells as biocatalysts.

2. Results and discussion

The biotransformation results are summarised in Table 1 and Scheme 1. In the reaction mixture after the biotransformation of (*S*)-(−)-limonene, we identified *cis*-

Table 1. Transformation of (*S*)-(−)-limonene by *Solanum aviculare* and *Dioscorea deltoidea* plant cells (after 10 days incubation)

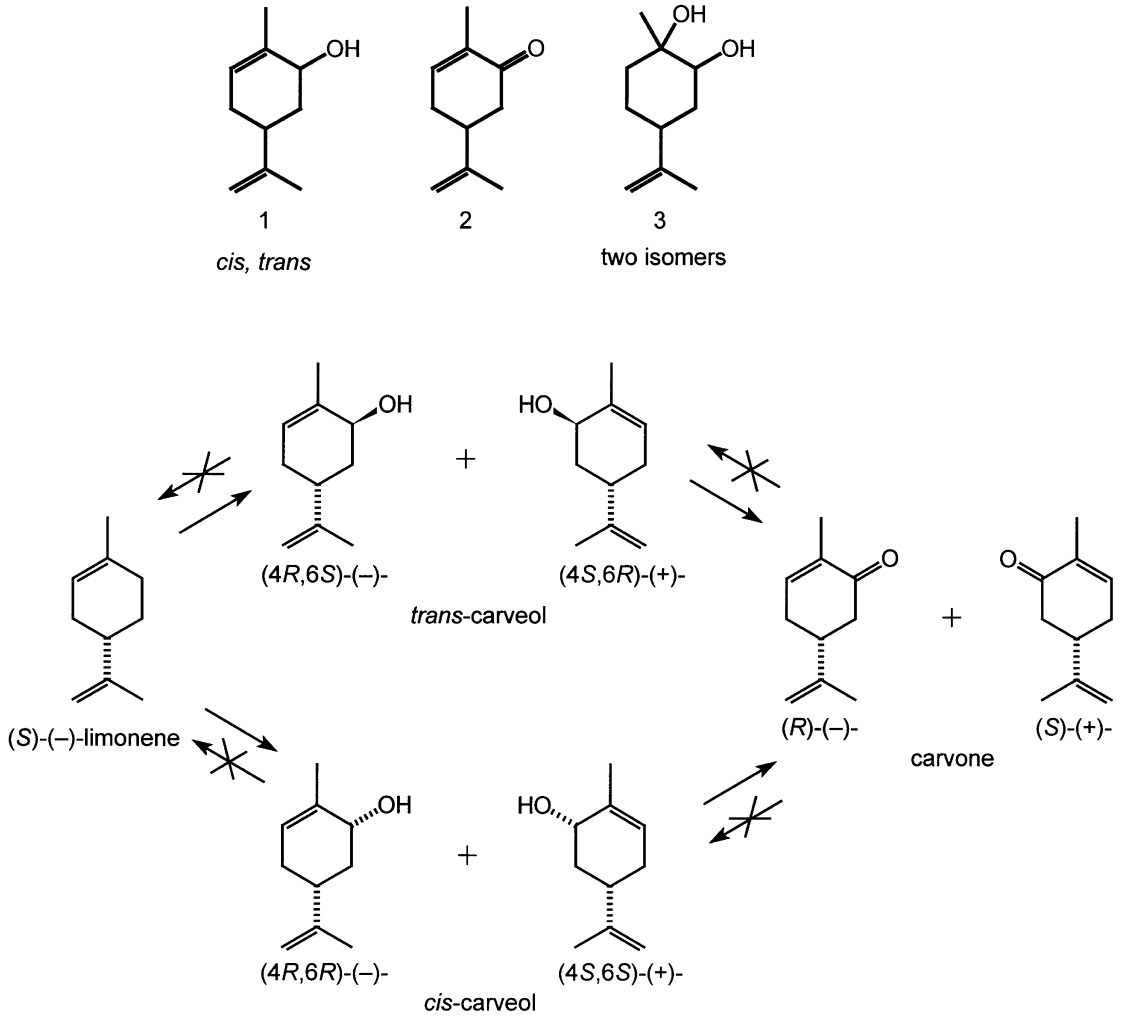
Plant cells	Products (relative %)[a]		
	cis-carveol	*trans*-carveol	carvone
Solanum aviculare	19	6	34
Dioscorea deltoidea	20	19	13

[a] Beside the starting compound.

* Corresponding author.

† Present address: Molecumetics, 2023 120th Ave NE, Bellevue, WA 98005-2928, USA.

0031-9422/99/$ - see front matter
PII: S0031-9422(98)00659-1



Scheme 1. The course of biotransformation of (*S*)-(−)-limonene by *Solanum aviculare* and *Dioscorea deltoidea* plant cells. Main products (1 and 2); minor product (3).

and *trans*-carveol (**1**) and carvone (**2**) beside the starting compound. Two minor products were found, mass spectra of which were identical with that of *p*-menth-8-ene-1,2-diol (**3**, two isomers in ratio 9:1). Low content of these compounds did not allow us to determine which isomers were formed. Mass spectra of these two minor products were very similar (Garg & Agarwal, 1988). The biocatalytic oxidation of a monoterpenic hydrocarbon to an alcohol and ketone is in agreement with the knowledge of enzymatic systems in higher plants (Croteau, 1987).

The resulting mixtures extracted from plant cells and the nutrient medium were identical. The time course of the transformations is illustrated in Figs 1 and 2. The highest yield of carvone (34%) was found using *S. aviculare* plant cells as a biocatalyst (Fig. 1). In the case of the biocatalysis by *D. deltoidea*, the reaction seemed to be somewhat slower (Fig. 2).

The absolute configuration of carvone formed from (−)-limonene would be expected to be *R*, without change at the chiral centre (Scheme 1). Kjonaas and Croteau (1983) have shown that (−)-limonene was a progenitor of (−)-carvone in *Mentha spicata*. However, according to the CD spectra of our obtained product of biotransformation, the resulting carvone was racemic. We did not obtain the CD curve of (*R*)-carvone as expected ($\Delta\varepsilon_{350}$ 0.06, $\Delta\varepsilon_{313}$-0.09, $\Delta\varepsilon_{250}$-1.59, $\Delta\varepsilon_{220}$ 2.63). This surprising fact led us to the assumption that the racemisation must have taken place already in the first reaction step of oxidation of limonene to carveols. This hypothesis was confirmed by analysing the reaction mixture on a chiral gas chromatographic column. Fig. 3 shows that both *cis*- and *trans*-carveols formed from enantiopure limonene were racemic. An identical racemic mixture of carveols was obtained from the biotransformation of (*S*)-(−)- and (*R*)-(+)-limonene, respectively. The mechanism of this racemisation is most probably via a symmetric intermediate. The shift of double bond in position 1 of the substituted cyclohexene ring leads to the series of products with opposite absolute configuration (Scheme 1).

For the elucidation of the reaction course of

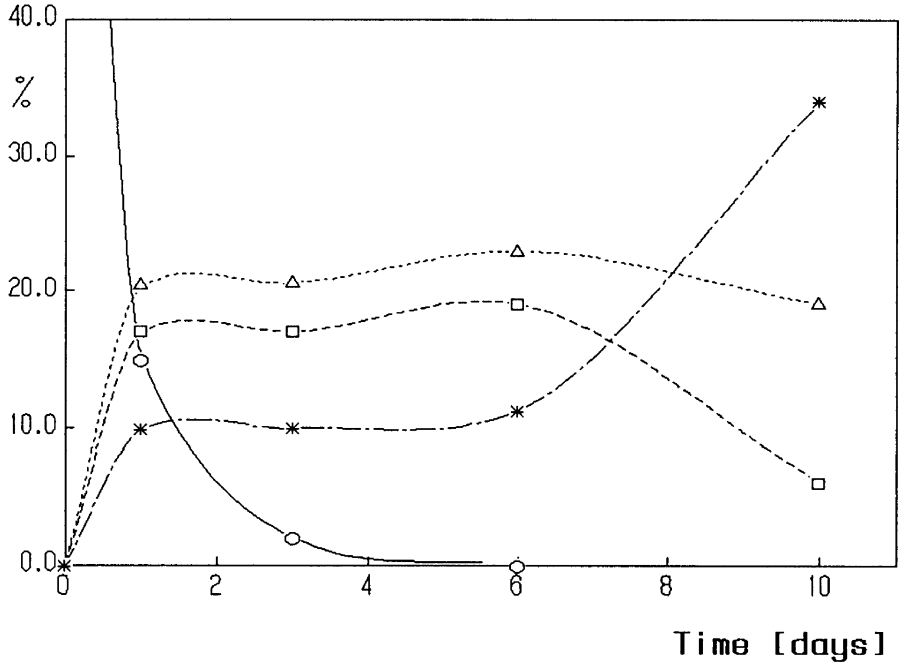


Fig. 1. Time course of the biotransformation of (*S*)-(−)-limonene by *Solanum aviculare* plant cells; (○) limonene; (□) *cis*-carveol; (△) trans-carveol; (★) carvone.

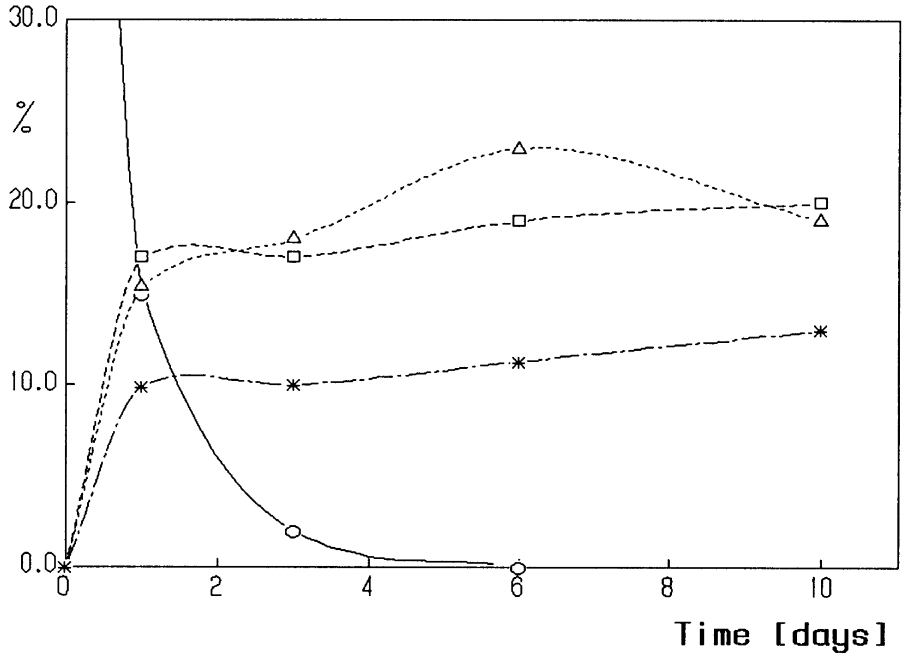


Fig. 2. Time course of the biotransformation of (*S*)-(−)-limonene by *Dioscorea deltoidea* plant cells; (○) limonene; (□) *cis*-carveol; (△) trans-carveol; (★) carvone.

biotransformation of (*S*)-(−)-limonene using *S. aviculare* and *D. deltoidea* plant cells, we studied separately the effect of both types of plant cells on all products found in the reaction mixture after transformation. In the case of a synthetic mixture of carveols (*cis*:*trans* 88:12, prepared from commercial carvone by reduction with $NaBH_4$), we found carvone as product of biotransformation beside the starting alcohols. The ratio of the enantiomers in the starting mixture of carveols did not change during the transformation. The enantiomeric purity of carvone formed from carveols was not checked in this case because the enantiomeric pair did not separate on the chiral chromatographic column. When we used carvone as a substrate for the biotransformation, no

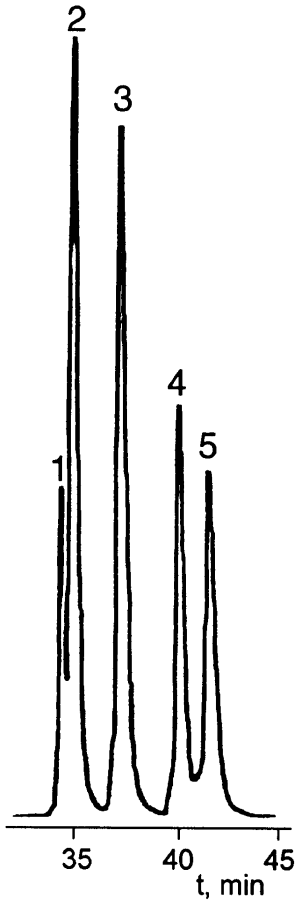


Fig. 3. Part of the gas chromatogram of the biotransformation products showing a separation of carveols on a cydex B column; (1) carvone (enantiomers not separated); (2) (4*R*,6*S*)-(−)-*trans*-carveol); (3) (4*S*,6*R*)-(+)-*trans*-carveol; (4) (4*S*,6*S*)-(+)-*cis*-carveol; (5) (4*R*,6*R*)-(−)-*cis*-carveol.

products were found. These results confirm the proposed reaction course as shown in Scheme 1. The same scheme can be drawn for (*R*)-(+)-limonene, too.

The biotransformation of (+)- and (−)-limonene by microorganisms *Aspergillus cellulosae* was described by Noma, Yamasaki, and Asakawa (1992). They found slightly different products formed from the respective enantiomers. While (+)-limonene gave perillyl alcohol, neodihydrocarveol, *p*-menth-8-en-1,2-diol, and isopiperitenone, the transformation of (−)-limonene led to a mixture with a different proportion of products, neodihydrocarveol being replaced by *cis*-carveol. *Trans*-carveol and carvone were not found among the products. Although the authors suppose the unchanged configuration at $C_{(4)}$ in the transformation products, no evidence about their enantiomeric purity is given (Noma et al., 1992).

In the transformation of limonene, we found a similar pattern of the reaction course as in the case of transformation of *cis*-verbenol to verbenone by *S. aviculare* free and immobilised plant cells (Vaněk et al., 1994). In both cases, the plant cells were able to oxidise alcohols to ketones, but no reduction of keto-group was observed. However, certain differences in the reaction course of the two above-mentioned substrates can be found. In the case of verbenols, a partial isomerisation of *cis*-verbenol to the *trans*-isomer was observed (Vaněk et al., 1994). No isomerisation was observed in the case of carveols. Furthermore, no reduction of the endocyclic (1,2-) double bond in carvone was observed while biotransformation of verbenone provided a detectable amount of the reduced product (Vaněk et al., 1994).

3. Experimental

3.1. Plant cell cultures

Suspension culture of the *S. aviculare* Forst was obtained in 1980 from callus (strain KK1N) which was derived from the leaf of a plant cultivated aseptically from seed supplied by the Botanical Garden in Kew (Macek, 1989). This culture was subcultivated in 5-days intervals in the nutrient medium according to Murashige and Skoog in the modification of Linsmayer and Skoog, containing 1×10^{-6} mol l^{-1} 2,4-dichlorophenoxyacetic acid and 1×10^{-6} mol l^{-1} of kinetine, at the temperature 27°C in the dark on a roller (5 rpm).

Suspension culture of the *D. deltoidea* Wall. was obtained from the Institute of Plant Physiology, Moscow. This culture was subcultivated in 14-days intervals in the nutrient medium according to Murashige and Skoog, containing 1×10^{-6} mol l^{-1} 2,4-dichlorophenoxyacetic acid and 5×10^{-6} mol l^{-1} of kinetine, at the temperature 27°C in the dark on a roller (5 rpm).

3.2. Biotransformation

During the biotransformation experiments, 100 ml of 5 days old suspension containing 15 g of cell fresh weight was incubated at standard conditions in 500 ml flasks with 15 mg of substrate. The degree of conversion was measured using gas chromatography.

After incubation time, the cells were separated from the nutrient medium by filtration. Then the cells were homogenised in acetone (50 ml) and after 24 h, they were mixed with water (200 ml) and extracted with light petroleum (3×50 ml). The organic extracts were combined and dried over sodium sulphate, filtered and evaporated in vacuo. The nutrient medium was extracted in the same manner. The residues obtained were dissolved in 2 ml hexane and used for GC analysis.

Kinetic studies and the biotransformation on a preparative scale was done with (*S*)-(−)-limonene (120 mg, 92% purity) as a substrate. After a preparative TLC (plate 20×20 cm, elution mixture light petroleum–ether 8:2), 24 mg of carvone were obtained. The biotransfor-

mation of (*R*)-(+)-limonene (85% purity) was done only in analytical scale for comparison of the chirality of carveols using a chiral GC analysis. Both starting compounds (*S*)-(−)- and (*R*)-(+)-limonene were enantiomerically pure as checked by chiral GC analyses.

3.3. Analysis

The resulting compounds were separated by GC and HPLC. They were identified by comparison of their retention times with standards and using GC–MS, GC–FTIR, and UV spectra. In case of the minor products 3, the structures were suggested on the basis of their mass spectra only (Garg & Agarwal, 1988). Carvone as the main product was isolated by preparative TLC and characterised by MS, IR and UV spectra.

3.4. GC

An HP 5890A gas chromatograph with FID was used with a fused silica capillary column DB-1 (methyl silicone), 30 m × 0.25 mm, film thickness 0.25 μm, carrier gas H_2, linear velocity 63 cm s^{-1} at 40°C. The temperature programme was 40°C to 100°C at 2°C min^{-1}, then to 300°C at 20°C min^{-1}; injector temperature 200°C, split ratio 50:1, detector temperature 250°C.

3.5. GC–MS

An integrated system consisting of mass spectrometer ZAB-EQ and an HP-5890A gas chromatograph was used with a fused silica capillary column OV-1 (25 m × 0.25 mm, film thickness 0.25 μm), carrier gas helium, linear velocity 50 cm s^{-1} at 40°C. The temperature programme was similar to that mentioned above; injector temperature 150°C, split ratio 50:1; EI ionisation, electron energy 70 eV, temperature of ion source from 80 to 180°C.

3.6. Enantioselective separations

A Cydex B column (permethylated β-cyclodextrin, 50 m × 0.22 mm, film thickness 0.25 μm) was used for the separation of the enantiomeric pairs of *cis*- and *trans*-carveols. The compounds were identified by their mass spectra (Fisons MD 800 instrument). The separation was performed isothermally at 120°C (helium linear velocity 25.6 cm/s). The separation is shown on Fig. 3. Retention times of products: 34.69 min (carvone, enantiomers not separated); 35.14 min ((4*R*,6*S*)-(−)-*trans*-carveol); 37.38 min ((4*S*,6*R*)-(+)-*trans*-carveol); 40.18 min ((4*S*,6*S*)-(+)-*cis*-carveol); 41.54 min ((4*R*,6*R*)-(−)-*cis*-carveol). Products of the biotransformation of both (*S*)-(−)- and (*R*)-(+)-limonene were analysed and gave an identical mixture of products. The absolute configuration of the two isomers of diol 3 could not be determined due to their low content in the reaction mixture.

3.7. HPLC

A gradient pump SP8700, an injection valve Rheodyne 7125, and a diode-array detector LKB 2140 were used. Compounds were separated on a stainless steel column 250 × 4.6 mm filled with reverse phase Vydac 5 μm; mobile phase methanol–water, linear gradient 0 min 25% methanol, 10 min 50% methanol, 20 min 75% methanol; flow 1 mol/min.

3.8. GC–FTIR

Products were characterised by their infrared spectra recorded on a Bruker IFS 85 instrument. GC conditions were identical with those used for GC–MS analyses.

3.9. CD

The optical purity of the resulting carvone was determined by circular dichroism after the isolation by prep. TLC. The CD curve was recorded on a Jobin–Yvon Mark V dichrograph in methanol. The observed values Δε were close to zero line within the instrument error. The CD curve did not exhibit the pattern of enantiopure carvone.

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic (grant No. 203/94/0644) and COST No. 66.20. The authors also wish to thank Dr S. Vašíčková for measuring the CD and GC–FTIR data.

References

Croteau, R. (1987). *Chemical Reviews, 87*, 929.
Garg, S. N., & Agarwal, S. K. (1988). *Phytochemistry, 27*, 936.
Kjonaas, R., & Croteau, R. (1983). *Archives of Biochemistry and Biophysics, 220*, 79.
Macek, T. (1989). In Y. P. S. Bajaj (Ed.), *Biotechnology in Agriculture and Forestry*, (p. 443). Berlin: Springer Verlag.
Noma, Y., Yamasaki, S., Asakawa, Y. (1992). *Phytochemistry, 31*, 2725.
Stohs, S. J. (1980). In A. Fiechter (Ed.), *Plant Cell Cultures I*., (p. 85). Berlin: Akademie-Verlag.
Suga, T., Hirata, T., Aoki, T., Lee, Y. S., Hamada, H., & Futatsugi, M. (1982). In A. Fujivara (Ed), *Proc. 5th International Congress on Plant Tissue and Cell Culture* (p. 381). Tokyo: Maruzen Co.
Vaněk, T., Macek, T., Stránský, K., & Ubik, K. (1989). *Biotechnology Techniques, 6*, 411.
Vaněk, T., Urmanceva, V., Wimmer, Z., & Macek, T. (1989). *Biotechnology Letters, 11*, 243.
Vaněk, T., Valterová, I., Pospíšilová, R., Vaisar, T. (1994). *Biotechnology Techniques, 5*, 289.
Vaněk, T., Wimmer, Z., Macek, T., & Šaman, D. (submitted). *Biocatalysis and Biotransformations.*