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BIOTRANSFORMATION OF Δ^2-CARENE BY CALLUS TISSUES

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Key Word Index—*Myrtillocactus geometrizans*; Cactaceae; *Nicotiana tabacum*; Solanaceae; plant tissue culture; biotransformation; Δ^2-carene; carenol; carenone; allylic hydroxylation.

Abstract—Dissociated callus tissues of *Myrtillocactus geometrizans* and *Nicotiana tabacum* convert Δ^2-carene into carenols and carenones in good yield via allylic hydroxylation. The time-course of the biotransformation was monitored in *M. geometrizans*.

INTRODUCTION

Biological transformations using plant cell suspensions allow structural modifications to provide useful substances [1–4]. Several studies have reported on the ability of plant cell suspensions to transform exogenous substrates. We previously reported the biotransformation of geraniol, a monoterpenic alcohol, and the production of paraffinic hydrocarbons and fatty acids by phototrophic, photomixotrophic and heterotrophic plant cell suspensions [5–7]. We found only one report on the biotransformation by plant cell cultures of a terpenic hydrocarbon, a sesquiterpenic one, valencene [8]. The present paper reports on the biotransformation of the monoterpene hydrocarbon, Δ^2-carene, performed by dissociated callus tissues, of *Myrtillocactus geometrizans* and *Nicotiana tabacum*.

RESULTS AND DISCUSSION

The biotransformation of exogenous Δ^2carene, produced by *Pinus sylvestris*, has been performed with two different dissociated callus tissues from *Myrtillocactus geometrizans* (Martius) Console (T.) and *Nicotiana tabacum* L. var. Wisconsin 38. Preliminary experiments were performed using 10 g fr. wt of 28-day-old callus tissues culture transferred into a 250 ml Erlenmeyer flask containing 100 ml of liquid medium. After 48 hr, the substrate (100 mg) was added to the shaken culture flask. After three days, we observed only 10% of substrate transformation whether added neat or in 20% ethanol and no transformation with 50% hexadecane as co-solvent. When the terpene was added neat in the culture medium containing Tween 80 (1 g l^{-1}), complete transformation was observed after 3 days incubation. Under these conditions, no transformation occurred when the culture was inactivated by autoclaving for 20 min at 120° just prior to inoculation with carene.

For each callus tissues suspension, the addition of 100–500 mg l^{-1} of terpene maintained cell viability higher than 80% of control experiment three days after addition. At higher concentrations, the viability decreased rapidly and, at 1 g l^{-1}, complete cell death was observed after 2 days of incubation. Similar substrate toxicity on the growth and viability of cell cultures was also reported for other monoterpenes [9]. The final amount of Tween 80 alone in the medium was not toxic to the cells.

In order to identify the biotransformation products, carene (2 g) was added to dissociated callus suspensions corresponding to a fresh weight of 400 g (see Experimental). After 6 days of incubation, extraction provided 1.9 and 1.8 g of crude extract, respectively, from *M. geometrizans* and *N. tabacum* experiments.

Preparative chromatography of *M. geometrizans* extracts afforded **2** and **3** and a mixture (**4**) of four products besides minor substances (Table 1).

Compounds **2** and **3**, the major compounds in *M. geometrizans* biotransformation, were identified as 3-care-2-one **2** and 2-care-4-one **3**, respectively, by comparing their ^{1}H (Table 2) and ^{13}C NMR spectra and mass spectra with those previously described [10–14].

Table 1. Biotransformation of Δ^2-carene (0.5 g l^{-1}) by dissociated callus suspensions of *M. geometrizans* and *N. tabacum* after 6 days of incubation

Compounds	*M. geometrizans* Yield%	*N. tabacum* Yield%
1	2	8
2	20	4
3	64	6
4	10	75
Unidentified	2	4

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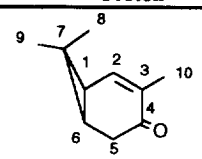
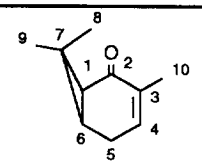
Table 2. ^{1}H NMR spectral data of compounds **2** and **3** (in $CDCl_3$ in ppm from TMS)

Proton	1	2	3	4	5	6	7	8	9	10
 **2** δ(^{1}H)	1.48	6.68	—	—	2.50 2.60	1.32	—	0.80	1.20	1.75
 **3** δ(^{1}H)	1.62	—	—	6.41	2.46 2.71	1.44	—	1.08	1.18	1.75

The structure of the compounds in the mixture **4** was determined to be the diastereomeric alcohols shown in Scheme 1 on the following basis. The IR spectrum showed an OH band at 3350 cm^{-1}, and a proton geminal to a hydroxyl group appears in the ^{1}H NMR spectrum as a complex signal at δ4–5. Finally, mild chemical oxidation of **4** by pyridinium chlorochromate [15] led to a mixture (6:19) of the carenones, **2** and **3**.

In *N. tabacum* experiments, the major compounds were the diastereomeric alcohols **4** (Table 1). These alcohols gave a mixture (2:3) of **2** and **3** upon oxidation.

The time-course of conversion was studied only with *M. geometrizans* (Fig. 1). The maximum concentration of hydroxy compounds **4** was reached after three days, followed by a slow decrease. After 5 days, the ketone concentration reached its maximum; the ratio between **2** and **3** was similar throughout the experiment. Within the same period, the Δ^2-carene concentration decreased to a very low level.

The activity of the culture was examined during the biotransformation pathway (Fig. 1). Control experiments were performed in the absence of terpene and with or without Tween 80. There was a steady glucose consumption during the experiment. The addition of surfactant led to a faster glucose consumption with a stabilization around the third day. In the biotransformation medium, glucose consumption stopped on the second day after terpene addition. The pH value was lower for the experiment with Tween and Tween + terpene than that for the glucose alone. Cell viability decreased to 80% and was only affected by terpene addition (0.5 gl^{-1}).

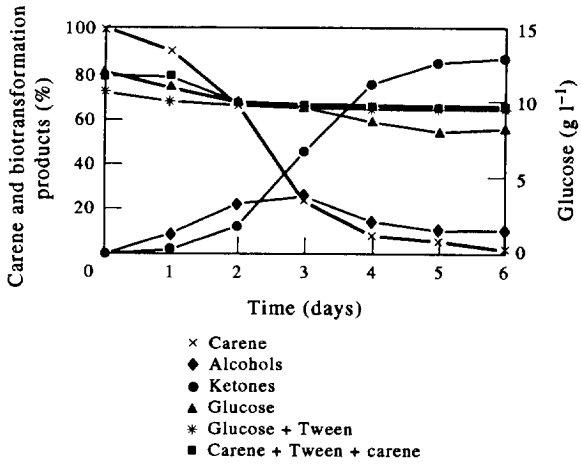
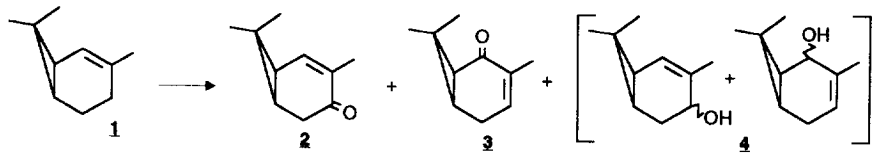


Fig. 1. Biotransformation activity of *M. geometrizans* callus suspensions with 0.5 gl^{-1} carene.

In this investigation, we have shown that Δ^2-carene was transformed by allylic hydroxylation and oxidation reactions. The two plant species gave a high level of biotransformation (up to 90%) of carene into carenols **4**, which were the main products in *N. tabacum* experiments. With *M. geometrizans* callus suspensions, these alcohols were further oxidized into carenones which were obtained in yields comparable to chemical oxidation [10, 14, 16]. Both ketones would be potentially useful intermediates for the synthesis of sesqui- and diterpenoids.



Scheme 1. Biotransformation products.

EXPERIMENTAL

General. Δ^2-Carene, commercially available (Fluka), was sealed in a tube and sterilized by autoclaving for 15 min at 12°. GC of biotransformation products was performed on a 25 m Carbowax 20 M capillary column with FID. Injector and det. temps were 210° and 240°, respectively. After sample injection, and a delay time of 30 sec at 70°, a linear temp. prog. ranging from 70° to 200° (3° min^{-1}) was used. Carrier gas was H_2 at 30 ml min^{-1}. MS were obtained by GC-MS. ^1H and ^{13}C NMR spectra were recorded on a Varian XL 200 spectrometer in $CDCl_3$ solns with TMS as int. standard.

Cell lines. The two plant tissue culture strains used for these studies had been established several years previously. Calli were cultivated in 250 ml conical flasks each containing 100 ml Lindsmaier and Shoog medium supplemented with sucrose (15 g l^{-1}), agar (8 g l^{-1}), auxin (2 mg l^{-1} AII), kinetin (200 μg l^{-1} for *M. geometrizans*; 600 μg l^{-1} for *N. tabacum*), vitamins (inositol: 100 mg l^{-1}; thiamine: 400 mg l^{-1}) at pH 5.6. Culture conditions were 23°, under 100 μE m^{-2} sec^{-1} of PAR for 18 hr per day. For the prepn of dissociated callus tissues, 250 ml flasks containing 100 ml of modified Lindsmaier and Shoog liquid medium were each inoculated with 100 g l^{-1} fr. wt of 28-day-old callus tissues, closed with an additional plastic cap and maintained at 110 rpm on an orbital shaker. The modified liquid medium was supplemented with sucrose (15 g l^{-1}), agar (8 g l^{-1}), auxin (2 mg l^{-1} AII), kinetin (200 mg l^{-1} for *M. geometrizans*; 600 μg l^{-1} for *N. tabacum*), vitamins (inositol: 100 μg l^{-1}; thiamin: 400 mg l^{-1}) at pH 5.6. In some expts, Tween 80 (1 g l^{-1}) was also added to the liquid medium (see Results and Discussion). Cell viability was determined after staining in erythrosin soln [17]. Enzymatic sucrose determination was conducted according to standard methods.

Biotransformation experiments. Carene (0.1 to 1 g l^{-1}) was inoculated aseptically to 24 hr-old dissociated callus tissues in 20% MeOH, 50% hexadecane or neat (experiment with Tween 80). Control flasks with plant cells but without carene and vice versa were included in each expt. Flasks were shaken at 110 rpm in the light. Time-course expts (250 ml flasks) were started with the addition of 50 mg of carene (0.5 g l^{-1}). For prep. biotransformation, 4 experiments for each strain were performed with 100 g (fr. wt) of callus tissues dissociated in 1 l of culture medium in 4 l flasks containing 1 g l^{-1} of Tween 80. Carene (0.5 g l^{-1}) was added to the shaken flask after 24 hr. After 6 days of incubation, the contents of the 4 flasks were combined for analysis.

Analysis. Cells and medium were extracted with distilled Et_2O for 48 hr using a liquid–liquid extractor. Hexadecane (1 mg 100 ml^{-1}) was added prior to extraction as an int. standard for quantitative analysis. The organic phase was dried (Na_2SO_4), the solvent evapd at 45° and the residue analysed by GC. In prep. expts, the residue (1.9 g and 1.8 g of crude extract, respectively, for *M. geometrizans* and *N. tabacum*) was sepd and purified by CC on Merck 70–230 mesh silica gel with pentane–Et_2O mixts (4:1–7:3–1:1–Et_2O) to give 5 frs. Each fr. was submitted to GC analysis. NMR and MS of the major frs (2–4) were recorded.

Oxidation of compounds **4** (100 mg) was performed with pyridinium chlorochromate [15]. By using a 24 hr, reaction time, the mixture was transformed in 93.5% overall yield into 3-care-2-one (**2**) and 2-care-4-one (**3**).

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