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BIOTRANSFORMATION OF GERANIOL, NEROL AND (+)- AND (−)-CARVONE BY SUSPENSION CULTURED CELLS OF *CATHARANTHUS ROSEUS*

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Key Word Index—*Catharanthus roseus*; hydroxylation; reduction; biotransformation; geraniol; nerol; (−)-carvone; (+)-carvone.

Abstract—Suspension cultured cells of *Catharanthus roseus* hydroxylate the allylic positions of geraniol, nerol and (+)- and (−)-carvone and reduce double bonds and ketone groups. After incubation for 7 days, the main products of (−)- and (+)-carvone were 5β-hydroxyneodihydrocarveol and 5α-hydroxycarvone, respectively.

INTRODUCTION

The ability of plant culture cells to convert foreign substrates into potentially useful substances is of great interest, as products may be formed which are difficult to prepare by synthetic chemical methods [1, 2]. We have studied the biotransformation of foreign substrates by plant suspension cell cultures [3–13] and found that many novel compounds can be produced, depending on the structure and functionality of the starting material.

We recently found that cultured suspension cells of *Catharanthus roseus* have the ability to hydroxylate regioselectively the allylic position of the cyclic monoterpenoid (−)-piperitone [14]. In order to better characterize the synthetic potential of this system with acyclic and cyclic monoterpenes, we have now investigated the biotransformation of geraniol (**1**), nerol (**2**), and (−)- and (+)-carvone (**3** and **4**) by these cultures.

RESULTS AND DISCUSSION

The callus tissues induced from the leaves of *C. roseus* that were used in our previous studies [14] were also used in this investigation. Just prior to use, callus tissue was transfered to freshly prepared Schenk and Hildebrandt medium [15] containing 1 ppm of 2,4-D and 3% sucrose, and then grown with continuous shaking for 2 weeks at 25° in the light. The metabolites formed from the substrates (**1–4**) were analysed by GC-mass spectrometry. The major components were isolated by preparative GC and their structures characterized by ^{1}H and ^{13}C NMR.

Biotransformation of geraniol (**1**) *and nerol* (**2**)

After 2 days of incubation, compounds **1** and **2** were converted to 10-hydroxygeraniol (**5**), 10-hydroxycitronellol (**6**) and 10-hydroxynerol (**7**) (Table 1). The structures of compounds **5**, **6** and **7** were determined by interpretation of their ^{1}H NMR and mass spectra and comparison with reported data [16–19]. The time course of the two experiments are shown in Figs 1 and 2. As shown in Fig. 1, compound **1** was converted to compounds **5**, **6** and **7** after 2 days incubation. At an early stage of the incubation, compound **1** was converted to compound **2** and citronellol (**8**). This observation shows that the cultured suspension cells isomerize compound **1** to compound **2** and reduce the C—C double bond at C-2 of **1** during the early stage of the incubation. The major product in the biotransformation of compound **1** after 2 days incubation was compound **5**. The formation of compound **5** indicates that compound **1** is regiospecifically hydroxylated at C-10. Furthermore, the formation of compounds **6** and **7** indicates that compounds **8** and **2** produced at the early stage of the incubation are also regiospecifically hydroxylated at C-10. These biotransformation products differ from those in the biotransformation of geraniol by *Nicotiana tabacum*, *Glycine max* and *Euphorbia characias* [20]. Figure 2 shows the time course for the biotransformation of

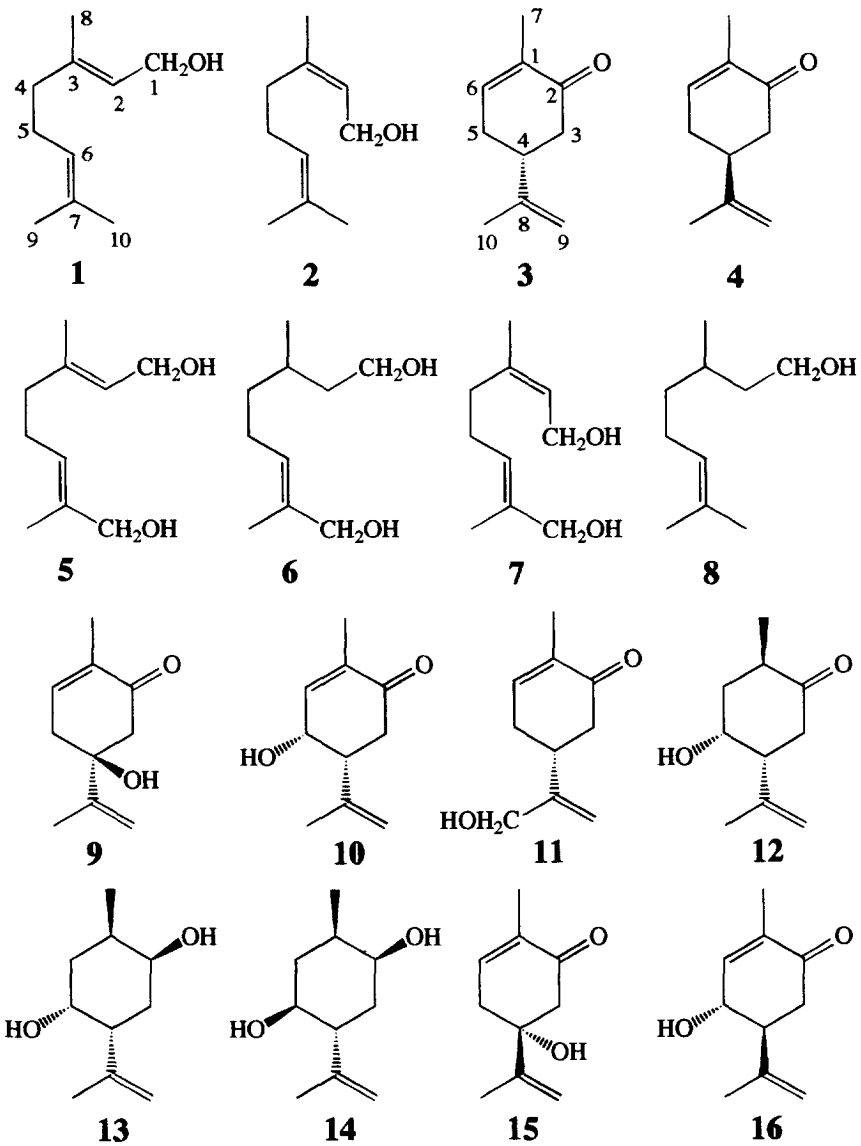

Table 1. Biotransformation of geraniol (**1**) and nerol (**2**) by suspension cultured cells of *Catharanthus roseus*.

Substrate	Product	Yield (%)*
Geraniol (**1**)	10-Hydroxygeraniol (**5**)	41.8
	10-Hydroxycitronellol (**6**)	32.9
	10-Hydroxynerol (**7**)	25.3
Nerol (**2**)	10-Hydroxygeraniol (**5**)	35.1
	10-Hydroxycitronellol (**6**)	25.7
	10-Hydroxynerol (**7**)	39.2

* Weight (%) of product relative to the substrate administered.

compound **2**. At the early stage of the incubation it was converted to compounds **1** and **8** in about 40% and 10% yield, respectively. Finally, the 10-hydroxylated compounds (**5**, **6** and **7**) were obtained as the main products after 2 days incubation. The formation of compound **7** indicates that compound **2** is regiospecifically hydroxylated at C-10 and the formation of compounds **5** and **6** indicates that compounds **1** and **8** produced at the early stage of the incubation are also regiospecifically hydroxylated at C-10. In both cases, significant isomerization took place, and the final products were the result of allylic oxidation and double bond reduction. Hydroxylation was at the C-10 position, indicating selectivity, as several allylic positions are available. Double-bond reduction occurred at the 2,3 position in the identified products, again indicating selectivity.

These results showed that cultured suspension cells of *C. roseus* reduce the C—C double bond at C-2 of **1** and regio- and stereo-selectively hydroxylate at the allylic C-10 position of the acyclic monoterpenoids **1**, **2** and **8**.

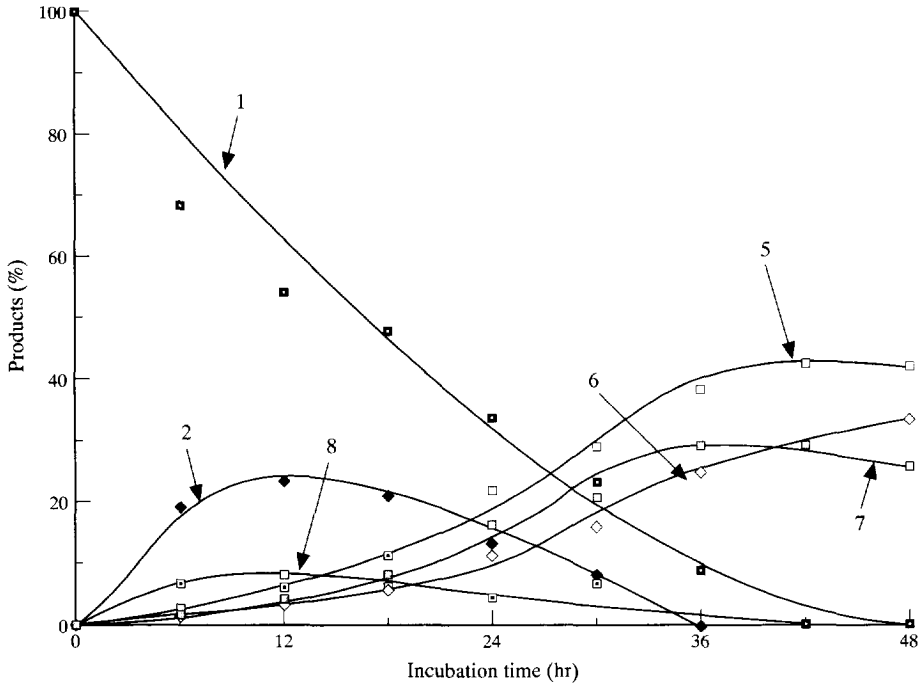

Fig. 1. Time course in the biotransformation of geraniol (**1**).

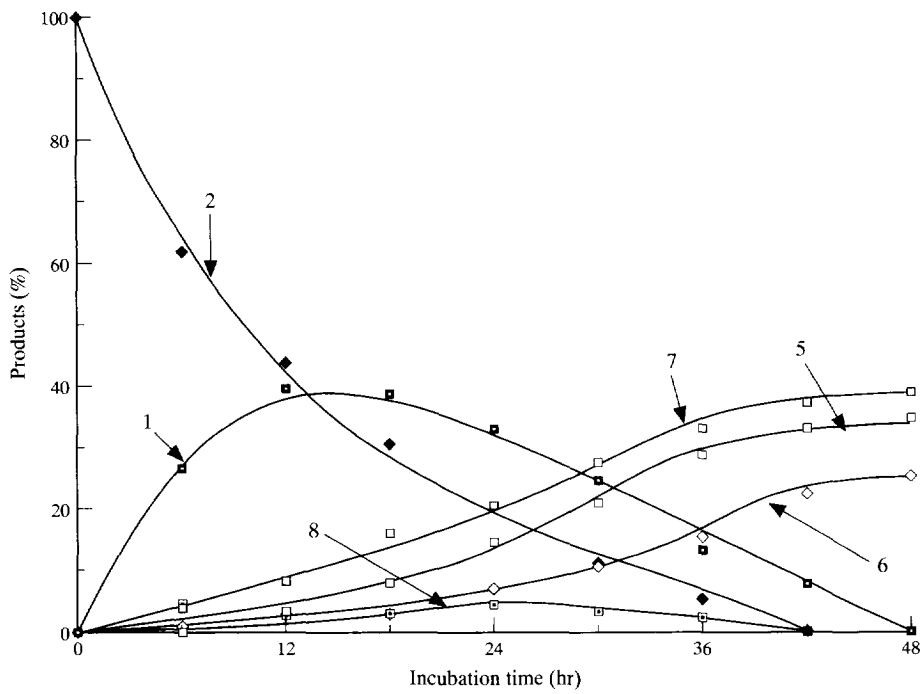

Fig. 2. Time course in the biotransformation of nerol (**2**).

Biotransformation of (−)- *and* (+)-*carvone* (**3** *and* **4**)

As shown in Table 2, after 7 days incubation **3** gave six hydroxylated compounds (**9**–**14**) and **4** gave two hydroxylated compounds (**15** and **16**). The characterization of the products, such as 4β-hydroxycarvone (**9**), 5α-hydroxycarvone (**10**), 10-hydroxycarvone (**11**), 5α-hydroxydihydrocarvone (**12**), 5α-hydroxyneodihydrocarveol (**13**), 5β-hydroxyneodihydrocarveol (**14**), 4α-hydroxycarvone (**15**) and 5α-hydroxycarvone (**16**), was carried out by interpretation of their ^{1}H and ^{13}C NMR spectral data (Table 3). The time courses of the biotransformation of compounds **3** and **4** are shown in Figs 3 and 4. As shown in Fig. 3, compound **3** was gradually converted to the hydroxylated compounds. At an early stage of the incubation, compound **11** was formed in high yield and after 2 days incubation compounds **9**, **10**, **12**, **13** and **14** were

Table 2. Biotransformation of (−)-carvone (**3**) and (+)-carvone (**4**) by the suspension cultured cells of *Catharanthus roseus*

Substrate	Product	Yield (%)*
(−)-Carvone (**3**)	4β-Hydroxycarvone (**9**)	6.3
	5α-Hydroxycarvone (**10**)	12.5
	10-Hydroxycarvone **11**)	1.4
	5α-Hydroxydihydrocarvone (**12**)	3.9
	5α-Hydroxyneodihydrocarveol (**13**)	18.4
	5β-Hydroxyneodihydrocarveol (**14**)	57.5
(+)-Carvone (**4**)	4α-Hydroxycarvone (**15**)	34.6
	5α-Hydroxycarvone (**16**)	65.4

* Weight (%) of product relative to the substrate administered.

formed and finally the formation of compound **14** reached a maximum after 7 days incubation. The yield of compounds **13** was one-third of that of compound **14**. The preferential formation of compound **14** indicates the occurrence of regio- and stereo-selective hydroxylation at C-5 of compound **3**. Figure 4 shows the time course for the biotransformation of compound **4**. After 7 days incubation compounds **15** and **16** were formed; the yield of **15** was one half of that of **16**. At this time compound **16** reached a maximum. The preferential formation of compound **16** indicates regio- and stereoselective hydroxylation at C-5 of compound **4**. In the biotransformation of compound **4** no product reduced at the C—C double bond or carbonyl group was observed during the 7-day incubation. This observation shows that cultured suspension cells of *C. roseus* discriminate between the enantiomers of carvone and preferentially hydroxylate C-4 and C-5 of compound **4**.

These results show that cultured suspension cells of *C. roseus* discriminate between the enantiomers of carvone and reduce the C—C double bond and the carbonyl group of compound **3** and then hydroxylate C-5 of the reduced alcohol, neodihydrocarveol; furthermore, the cells hydroxylate C-5 of compound **4** without reduction of the C—C double bond and/or the carbonyl group of compound **4**.

Isolation and structure elucidation

For spectroscopic investigation of the different metabolites, the crude product mixtures were purified by preparative GC, which resulted in pure fractions of compounds **15** and **16** from the *S*-(+)-carvone

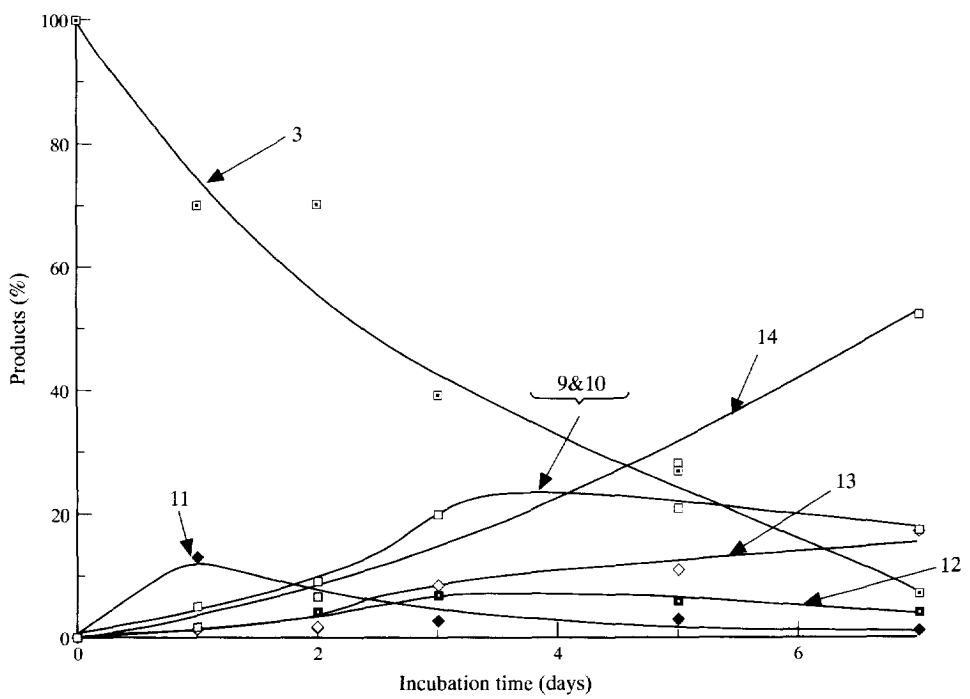

Fig. 3. Time course in the biotransformation of (−)-carvone (**3**).

Table 3. ^{1}H NMR data of carvone biotransformation products.

	9, 15*	10*	11*	12*	13*	14*	16*
1-H				2.86 *m*†	1.97 *m*‡	1.65 *m br*, covered	
2-H					3.90 *m*, ⩽ 3	3.79 *m*, ⩽ 3.5	
3-H_{ax}	2.81 *d*, 16.1	2.79 *dd*, 15.4, 12	2.39 *dd*, 16.0, 13.2	2.80 *dd*, 13.6, 13.6	1.92 *ddd*, 13.4, 13.4, 2.4	1.50 *dddd*, 13.8, 13.8, 3.4	2.38 *dd*, 16.3, 13.9
3-H_{eq}	2.62 *dd*, 16.0, ⩽ 3	2.39 *ddd*, 15.4, 2.5, ⩽ 1	2.60 *ddd*, 16.0, 3.7, 1.4	2.19 *dd*, 13.0, 3.7	1.61 *m*	1.83 *ddd*, 13.8, 3.4, 3.4	2.51 *dd*, 16.3, 3.9
4-H		2.75 *m*, covered	2.81 *m*	2.45 *m br*, covered	2.45 *m br*, covered	2.35 *ddd*, 13.8, 10.5, 3.4	2.69 *ddd*, 13.9, 9.8, 3.9
5-H_{ax}	2.70 *ddd*, 18.4, ⩽ 3		2.31 *dddd*, 18.2, 10.7, 2.5, 2.5			3.48 *ddd*, 11.0, 10.5, 4.1	4.43 *ddd*, 9.8, ⩽ 3
5-H_{eq}	2.49 *dddd*, 18.4, 5.0, ⩽ 2	4.38 *m*, ⩽ 2	2.49 *dddd*, 18.2, 4.5, 4.5, 1.4	4.08 *m*, ⩽ 3	3.95 *m*, ⩽ 3		
6-H/6-H_{ax}	6.60 *m*	6.77 *m*, ⩽ 5	6.73 *m*, ⩽ 2	2.34 *m*	1.65–1.78 *m*	1.43 *ddd*, 13.6, 12.4, 11.0	6.70 *m*
6-H_{eq}				2.34 *m*	1.65–1.78 *m*	1.76 *ddd*, 12.4, 4.1, 4.1	
7-H_3	1.8 *s br*	1.8 *s br*	1.77 *s*	1.01 *d*, 6.8	0.94 *d*, 7.2	1.0 *d* 6.9	1.79 *t*, ⩽ 2
9-H_2	4.91 *t*, ⩽ 2	4.83 *s*	4.94 *s*	4.69 *s*	4.79 *s*	4.89 *s br*	4.95 *s br*
	5.02 *s br*	5.10 *s*	5.14 *s*	5.05 *s*	4.75 *s*	4.92 *t*, ⩽ 2	4.99 *t*, ⩽ 2
10-H_3/10-H_2	1.8 *s br*	1.8 *s*	4.14 *s*	1.80 *s br*	1.78 *s*	1.69 *s*	1.75 *s br*

* $CDCl_3$, 500 MHz.
† After decoupling of 10-H_3: *dd*, 13.2, 5.6.
‡ After decoupling of 10-H_3: *ddd*, 12.9, 4.4, 2.8.

product mixture and of compounds **11** and **14**, and inseparable binary mixtures of compounds **9** and **10**, and compounds **12** and **13** for the *R*-(−)-metabolites. In the high-resolution ^{1}H NMR spectra the proton signals of the highly substituted small-ring systems were well separated and characteristic signals for different types of methyl groups, allylic protons or those close to hydroxylated positions served as good indicators of the oxidation states of the compounds. To ensure unequivocal assignments, homonuclear decoupling experiments were performed in order to clarify coupling patterns and confirm assignments to individual compounds in mixed spectra. Absolute configurations were determined by careful interpretation of coupling patterns of vicinal protons and their relation to the stereocenter at C-4. The ^{1}H data of all carvone metabolites are summarized in Table 3. Owing to the small amounts of samples, ^{13}C data could only be acquired for compound **11**. Comparison with reference data provided further structural proof for compounds **11** [21] and **16** [22]. The enantiomer of the latter is known as a constituent of spearmint oil and *Boltania* oil [23, 24]. Compound **9** is described in the literature as a plant metabolite [25] and also a synthetic intermediate [26], but no complete NMR spectroscopic data could be found.

EXPERIMENTAL

Analytical. TLC: Merck 60 GF_{254} silica gel (0.25 and 0.75 mm); GC; silica capillary column (30 m × 0.25 mm) coated with polyethylene glycol 20 M at an operating temp. of 180° and 130–230° at 8° min^{-1}; EI-MS 70 eV; NMR: 500 MHz (^{1}H) and 125 MHz (^{13}C) with samples in $CDCl_3$.

Substrates. Geraniol, nerol, and (−)- and (+)-carvone (Aldrich) were purified by CC on silica gel with hexane–EtOAc (4:1; 99% pure on GC, $[\alpha]_D^{25}$ −60.5° (neat) and $[\alpha]_D^{25}$ +59.8° (neat)).

Feeding of substrates to suspension cells of C. roseus *and time-course experiments.* The callus tissues were induced from the leaves of *C. roseus* and have been maintained for approx. 4 years. Just prior to use in this study, the tissues were transferred to freshly prepared SH medium [15] containing 2 ppm of 2,4-D and 3% sucrose, and were then grown with continuous shaking (120 rpm) for 1 week at 25°. The substrate (20 mg) was added to the culture suspension cells and the cultures incubated at 25° for 2–7 days in a shaker. To study the time course of the biotransformation, a portion of the incubation mixt. was pipetted out at regular intervals under sterile conditions for subsequent extraction with EtOAc.

The details of the time-course experiments are described below in the case of **1** as an example. A part of the callus tissues was transferred to 200 ml SH medium [15] in a 500 ml conical flask and grown with continuous shaking for 1 week at 25° in the light (about 2000 lx). The substrate **1** (20 mg) was administered to the precultured cells (60–70 g) in a 500 ml

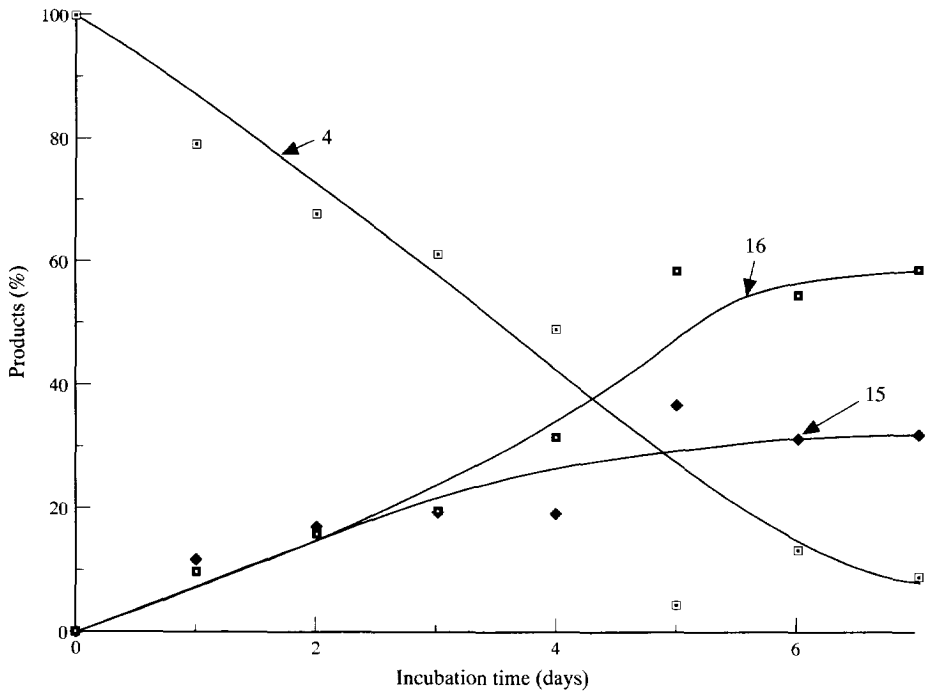

Fig. 4. Time course in the biotransformation of (+)-carvone (**4**).

conical flask and the cultures were incubated at 25° in a rotary shaker (120 rpm) in the light. At regular intervals, some incubation mixture (10 ml) was pipetted out under the sterile conditions and extracted with EtOAc. The EtOAc extract was made up to 0.2 ml and 2 μl of the soln was subjected to GC. The yields of the products were determined on the basis of the peak area from GC and are expressed as a relative percentage to the total amount of the total reaction products extracted. Identification of the products were performed by comparison (co-GC and GC-MS) with authentic samples.

Isolation and identification of products. The products extracted with EtOAc were purified by prep. GC (1.83 m × 4 mm i.d. glass column packed with 3% OV-101 on chromosorb 750, programmed 80–230° at 10 min^{-1}) equipped with a liquid N_2 cooled thermal gradient detector, and collected in glass tubes. Products were then identified by GC-MS and ^1H NMR.

10-*Hydroxygeraniol* (**5**). Oil; MS *m/z* (rel. int.): 152 $[M-H_2O]^+$ (5), 137 $[M-H_2O-CH_3]^+$ (13), 134 (15), 121 (27), 109 (15), 94 (30), 84 (49), 68 (94), 43 (100).

10-*Hydroxycitronellol* (**6**). Oil; MS *m/z* (rel. int.): 154 $[M-H_2O]^+$ (22), 139 $[M-H_2O-CH_3]^+$ (30), 121 (50), 109 (37), 95 (33), 81 (76), 55 (90), 43 (100).

10-*Hydroxynerol* (**7**). Oil; MS *m/z* (rel. int.): 152 $[M-H_2O]^+$ (7), 137 $[M-H_2O-CH_3]^+$ (21), 134 (18), 121 (26), 109 (25), 94 (56), 84 (75), 68 (93), 43 (100).

Citronellol (**8**). Oil; MS *m/z* (rel. int.): 156 $[M]^+$ (20), 138 $[M-H_2O]^+$ (22), 123 $[M-H_2O-CH_3]^+$ (38), 109 (23), 95 (57), 82 (66), 69 (100).

4β-*Hydroxycarvone* (**9**). Oil; MS *m/z* (rel. int.): 166 $[M]^+$ (5), 148 $[M-H_2O]^+$ (36), 133 (15), 108 (26), 98 (100), 91 (27), 70 (60).

5α-*Hydroxycarvone* (**10**). Oil; MS *m/z* (rel. int.): 166 $[M]^+$ (12), 148 $[M-H_2O]^+$ (82), 133 $[M-H_2O-CH_3]^+$ (28), 108 (23), 82 (100), 77 (21), 69 (25).

10-*Hydroxycarvone* (**11**). Oil; MS *m/z* (rel. int.): 167 $[M+1]^+$ (3), 166 $[M]^+$ (15), 148 $[M-H_2O]^+$ (3), 112 (17), 95 $[M+1-CH_3-C(CH_2OH)=CH_2]^+$ (100), 67 (24).

5α-*Hydroxydihydrocarvone* (**12**). Oil; MS *m/z* (rel. int.): 168 $[M]^+$ (36), 150 $[M-H_2O]^+$ (4), 135 $[M-H_2O-CH_3]^+$ (5), 99 (100), 71 (62).

5α-*Hydroxyneodihydrocarveol* (**13**). Oil; MS *m/z* (rel. int.): 170 $[M]^+$ (3), 152 $[M-H_2O]^+$ (17), 134 $[M-H_2O-H_2O]^+$ (29), 109 (26), 101 (100), 93 (52), 81 (42), 70 (52).

5β-*Hydroxyneodihydrocarveol* (**14**). Oil; MS *m/z* (rel. int.): 170 $[M]^+$ (30), 152 $[M-H_2O]^+$ (20), 134 $[M-H_2O-H_2O]^+$ (12), 108 (22), 101 (100), 93 (62), 81 (36), 68 (51).

4α-*Hydroxycarvone* (**15**). Oil; MS *m/z* (rel. int.): 166 $[M]^+$ (5), 148 $[M-H_2O]^+$ (36), 133 (15), 108 (26), 98 (100), 91 (27), 70 (60).

5α-*Hydroxycarvone* (**16**). Oil; MS *m/z* (rel. int.): 166 $[M]^+$ (10), 148 $[M-H_2O]^+$ (80), 133 $[M-H_2O-CH_3]^+$ (28), 108 (21), 82 (100), 77 (21), 69 (25).

REFERENCES

1. Pras, N., *Journal of Biotechnology*, 1992, **26**, 29.
2. Suga, T. and Hirata, T., *Phytochemistry*, 1990, **29**, 2393.
3. Hamada, H., *Bulletin of the Chemistry Society of Japan*, 1988, **61**, 869.

4. Hamada, H., Umeda, N., Otsuka, N. and Kawabe, S., *Plant Cell Reports*, 1988, **7**, 493.
5. Hamada, H., Nakamura, N., Ito, S., Kawabe, S. and Funamoto, T., *Phytochemistry*, 1988, **27**, 3807.
6. Suga, T., Hirata, T., Hamada, H. and Murakami, S., *Phytochemistry*, 1988, **27**, 1041.
7. Hamada, H., Jacobson, A. R., Williams, H. J. and Scott, A. I., *Biotechnology Letters*, 1990, **12**, 897.
8. Hamada, H. and Nakazawa, K., *Biotechnology Letters*, **12**,1991, 805.
9. Hamada, H. and Kawabe, S., *Life Sciences*, 1991, **48**, 613.
10. Hamada, H., Konishi, H., Williams, H. J. and Scott, A. I., *Phytochemistry*, 1991, **30**, 2269.
11. Hamada, H. and Nakata, S., *Plant Tissue Culture Letters*, 1992, **9**, 32.
12. Hamada, H., Fuchikami, Y., Jansing, R. L. and Kaminsky, L. S., *Phytochemistry*, 1993, **33**, 599.
13. Nakamura, K., Miyoshi, H., Sugiyama, Y. and Hamada, H., *Phytochemistry*, 1995, **40**, 1419.
14. Hamada, H., Fuchikami, Y., Ikematsu, Y., Hirata, T., Williams, H. J. and Scott, A. I., *Phytochemistry*, 1994, **37**, 1037.
15. Schenk, R. U. and Hildebrandt, A. C., *Canadian Journal of Botany*, 1972, **50**, 199.
16. Umbreit, M. A. and Sharpless, K. B., *Journal of the American Chemical Society*, 1977, **99**, 5526.
17. Madyastha, K. M., Meehan, T. D. and Coscia, C. J., *Canadian Journal of Biochemistry*, 1976, **15**, 1097.
18. Balsevich, J., *Planta Medica*, 1985, 128.
19. Madyastha, K. M. and Murthy N. S. R. K., *Tetrahedron Letters*, 1988, **29**, 579.
20. Carriere, F., Gil, G., Tapie, P. and Chagvardieff, P., *Phytochemistry*, 1989, **28**, 1087.
21. Weinges, K. and Schwarz, G., *Liebigs Annals of Chemistry*, 1993, 811.
22. Miyashita, M., Suzuki, T. and Yoshikoshi, A., *Journal of Organic Chemistry*, 1985, **50**, 3377.
23. Canova, L., *Anais Academia Brasileira de Ciencias*, 1972, **44**, 273.
24. Diaz, J. G., Barba, B. and Herz, W., *Phytochemistry*, 1994, **36**, 703.
25. Castro, V., Jakupovic, J. and Dominguez, X. A., *Phytochemistry*, 1989, **28**, 2727.
26. Buchi, G. and Wuest, H., *Journal of Organic Chemistry*, 1969, **34**, 857.