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BIOTRANSFORMATION OF 3-OXO-*p*-MENTHANE DERIVATIVES BY CULTURED CELLS OF *NICOTIANA TABACUM*

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Key Word Index—*Nicotiana tabacum*, Solanaceae, tissue culture, biotransformation, hydroxylation, reduction, stereoselectivity, 3-oxo-*p*-menthanes.

Abstract—The biotransformation of (1*R*)-(+)-*p*-menth-4(8)-en-3-one and (1*R*,4*S*)-(−)- and (1*R*,4*R*)-(+)-*p*-menth-3-ones with a suspension of cultured cells of *Nicotiana tabacum* was investigated. It was found that (i) the cultured cells regio- and stereoselectively hydroxylated the 4-position of (1*R*,4*S*)- and (1*R*,4*R*)-*p*-menth-3-ones to give (1*R*,4*R*)- and (1*R*,4*S*)-4-hydroxy-*p*-menth-3-ones, respectively and (ii) the cultured cells stereoselectively reduced the C–C double bond of the *p*-menth-4(8)-en-3-one from the *si*-face at C-4 and then the carbonyl group from the *re*-face.

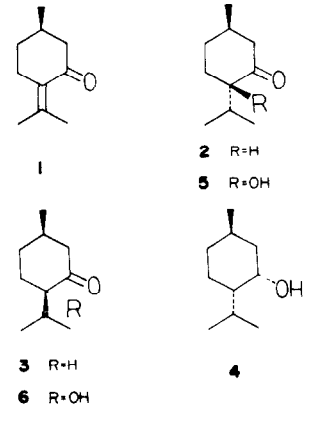
INTRODUCTION

Several studies have been focussed on the ability of plant cultured cells to metabolize foreign substrates and/or convert these substrates into more useful substances, because a specific transformation may be effected by such cells [1–7] A suspension of cultured cells of *Nicotiana tabacum* was recently found to have the ability to reduce stereoselectively the C–C double bond of an s-*trans*-α,β-unsaturated ketone and then the carbonyl group of the resultant ketone [6, 7] Furthermore, the conversion between cyclic ketones and their corresponding alcohols was reversible and the balance in the equilibrium of the interconversion can be predicted on the basis of the ^{13}C NMR chemical shift of the carbonyl carbon of the cyclic ketone [8, 9] The cultured cells of *Mentha* species exhibited the stereoselective preference in the reduction of pulegone and menthone [10, 11].

To generalize the stereospecificity in the reduction of the carbonyl group and the C–C double bond of α,β-unsaturated ketones with plant cultured cells, the metabolic behaviour of the cultured cells of *N tabacum* toward molecules having an s-*cis*-α,β-unsaturated and an isolated carbonyl groups has been examined with 3-oxo-*p*-methane derivatives as a foreign substrate. We found regio- and stereo-specific hydroxylation at the position adjacent to the carbonyl group as well as the stereospecific reduction of the C–C double bond and the carbonyl group with the cultured cells, and here describe the results

RESULTS AND DISCUSSION

The biotransformation of 3-oxo-*p*-menthane derivatives, such as (1*R*)-(+)-*p*-menth-4(8)-en-3-one (**1**) [(+)-pulegone], (1*R*,4*S*)-(−)-*p*-menth-3-one (**2**) [(−)-menthone] and (1*R*, 4*R*)-(+)-*p*-menth-3-one (**3**) [(+)-isomenthone], was performed with a suspension of cultured cells of *N tabacum* 'Bright Yellow' in a manner similar to that described in our previous papers [4, 6, 12]



Incubation of *p*-menth-4(8)-en-3-one (**1**) gave five products, **2**–**6**, as shown in Table 1 On the other hand, the *p*-menth-3-ones, **2** and **3**, were converted into three products, **4**–**6** (Table 1) Characterization of the products, such as (1*R*,4*S*)- and (1*R*,4*R*)-*p*-menth-3-ones (**2** and **3**) and (1*R*,3*R*,4*S*)-(+)-*p*-menth-3-ol (**4**) [(+)-neomenthol], was carried out by direct comparison of their physical and spectral data with those of authentic samples. Structures of the products, **5** and **6**, were elucidated to be (1*R*,4*R*)- and (1*R*,4*S*)-4-hydroxy-*p*-menth-3-ones, respectively, by interpretation of their spectral data and comparison of their ^{1}H NMR and CD spectral data with the reported data [13] Reliable evidence for the structures came from the H–H coupling pattern in the 500 MHz ^{1}H NMR spectra of **5** and **6**, as shown in Table 2 The coupling pattern was confirmed by a two-dimensional H–H shift correlation NMR spectra, all correlations reasonable for the structures were found in the spectra

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Table 1 Biotransformation of (1*R*)-*p*-menth-4(8)-en-3-one (**1**), (1*R*,4*S*)-*p*-menth-3-one (**2**) and (1*R*,4*R*)-*p*-menth-3-one (**3**) with a suspension of cultured cells of *N tabacum*

	Products (%)*				
Substrates	**2**	**3**	**4**	**5**	**6**
1	29	145	10	02	02
2	–	58	69	121	06
3	22	—	001	17	53

*Yield (%) of the products for the substrate administered

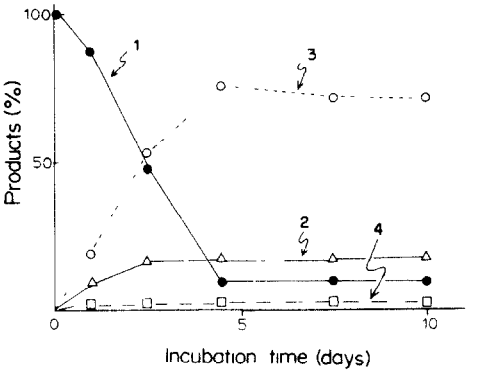

Fig 1 The time-course in the biotransformation of (1*R*)-(+)-*p*-menth-4(8)-en-3-one (**1**) with a suspension of the cultured cells of *N tabacum*

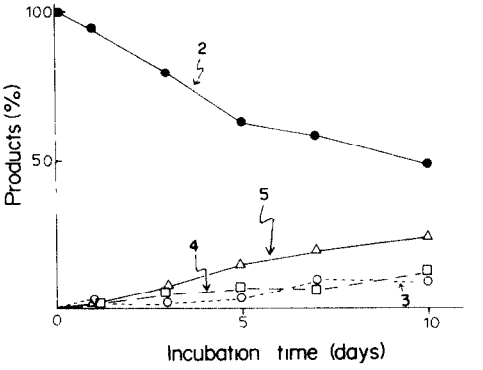

Fig 2 The time-course in the biotransformation of (1*R*,4*S*)-(−)-*p*-menth-3-one (**2**) with a suspension of the cultured cells of *N tabacum*

The time-courses in the biotransformation of (1*R*)-(+)-*p*-menth-4(8)-en-3-one (**1**) with a suspension of the cultured cells was followed, and is shown in Fig 1 The formation of (1*R*,4*R*)-*p*-menth-3-one (**3**) became a maximum after four days incubation However, the yield of (1*R*,4*S*)-*p*-menth-3-one (**2**) was one-quarter of that of the *p*-menth-3-one (**3**). The preferential formation of the *p*-menth-3-one (**3**) indicates the occurrence of the stereoselective reduction of the C–C double bond adjacent to the carbonyl group from the *si*-face

The time-courses in the biotransformations of (1*R*,4*S*)- and (1*R*,4*R*)-*p*-menth-3-ones, **2** and **3**, are shown in Figs 2 and 3, respectively The major products in the biotransformation of **2** and **3** were (1*R*,4*R*)- and (1*R*,4*S*)-4-hydroxy-*p*-menth-3-ones (**5** and **6**), respectively The formation of **5** and **6** indicates that the 3-oxo-*p*-menthanes are regio- and stereospecifically hydroxylated to the compounds with the hydroxyl group at C-4 The biotransformation of **2** gave (1*R*,3*R*,4*S*)-*p*-menth-3-ol (**4**) as a reduction product, but the reduction of the carbonyl group hardly occurred in the case of **3** The formation of **4** from **2** indicates that the carbonyl group of **2** is stereospecifically reduced from the *re*-face The low reductive transformation of **3** may be caused by the steric hindrance owing to the axial methylethyl group [14] adjacent to the carbonyl group of **3**

Thus, it was found that a suspension of the cultured cells of *N tabacum* regio- and stereo-selectively hydroxylates the 4-position of the 3-oxo-*p*-menthanes, the hydroxyl group occupies the same spatial arrangement as that of the leaving methine proton Such a hydroxylation had not been observed yet in the biotransformation of the menthones with the cultured cells of *Mentha* species [10] No hydroxylation took place in the biotransform-

Table 2 ^{1}H NMR spectral data* of the products **5** and **6**

	5		**6**	
H	δ	*J*(Hz)	δ	*J*(Hz)
1	2 56 *qdddd*	6 5, 6 4, 4 4, 2 7, 2 4	1 89 *ddqt*	13 1, 12 1, 6 7, 4 3
2α	2 70 *dd*	13 2, 6 4	2 22 *t*	13 1
2β	2 23 *dt*	13 2, 2 4	2 46 *ddd*	13 1, 4 3, 2 4
5α	2 17 *ddd*	14 2, 4 5, 4 4	1 43 *dt*	12 2, 2 9
5β	1 65 *td*	14 2, 4 4	1 30 *ddd*	12 2, 11 1, 2 6
6α	1 94 *tt*	14 2, 4 4	2 33 *dtd*	12 1, 11 1, 2 9
6β	1 49 *ddddd*	14 2, 4 5, 4 4, 2 7, 2 4	1 73 *ddddd*	11 0, 4 2, 2 9, 2 6, 2 4
8	2 21 *m*†	6 5	2 19 *m*†	6 7
Me-1	0 94 *d*	6 5	1 06 *d*	6 7
Me-8	0 68 *d*	6 5	0 70 *d*	6 7
Me-8	0 99 *d*	6 5	1 00 *d*	6 7

*Measured at 500 MHz in $CDCl_3$

†*m* denotes sevenfold lines

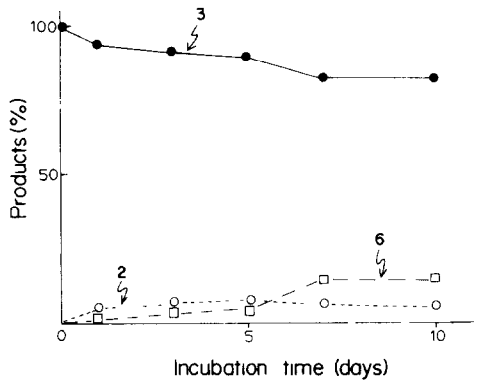

Fig 3 The time-course in the biotransformation of (1*R*,4*R*)-(+)-*p*-menth-3-one (**3**) with a suspension of the cultured cells of *N tabacum*

ation of 2-oxo-*p*-menthane derivatives with the cultured cells of *N. tabacum* [6] This fact indicates that the enzyme participating in the hydroxylation seems to have a strict substrate specificity. In the biotransformation of s-*cis*-α,β-unsaturated ketone, on the other hand, the cultured cells stereospecifically reduced the C–C double bond adjacent to the carbonyl group and then the carbonyl group of the resultant ketone The stereospecificity in these reductions was also observed in the biotransformation of *p*-menth-6,8-dien-2-one (carvone) with the cultured cells of *N tabacum*

EXPERIMENTAL

Analytical and prep TLC were carried out on Merck 60 GF_{254} Si-gel plates with 0 25 and 0 75 mm layers of the adsorbent, respectively GC was performed on an instrument equipped with an FID and a glass column (3 mm × 2 m) packed with 15% DEGS and 2% OV-17 on Chromosorb W (AW-DMCS, 80–100 mesh) at 120° and 90–150° (2°/min), respectively GC-MS were taken on a mass spectrometer which was installed with an EI ion source operating at 20 eV and a gas chromatograph equipped with a PEG-20 M capillary column (0 28 mm × 30 m) operating in the range 100–200° (3°/min) High-resolution mass spectra were recorded on a Hitachi M-80 mass spectrometer at 70 eV ^{1}H NMR spectra were obtained on a Hitachi R-600 and/or a JEOL GX-500 FT-NMR spectrometers at 60 and 500 MHz, respectively, with $SiMe_4$ as internal standard

Substrates (1*R*)-(+)-*p*-menth-4(8)-en-3-one (**1**) (pulegone), donated from Takasago Perfumery Co Ltd, was purified by distillation under a red pres, bp 94–95°/1068 Pa (10 mmHg), $[\alpha]_D^{25}$ +22.3° (EtOH, *c* 4 6), n_D^{25} 1 4831, d_4^{25} 0 9370 (lit [15] $[\alpha]_D$ +22 4°, n_D^{20} 1 4864, d_4^{20} 0 9374), >99% pure on GC

(1*R*,4*S*)-(−)-*p*-Menth-3-one (**2**) was prepared from (−)-menthol (Aldrich) by oxidation with chromium trioxide [16] followed by CC on Si gel using a hexane–AcOEt mixt with increasing AcOEt from 0 to 3% $[\alpha]_D^{25}$ −25 7° (EtOH, *c* 1.1), n_D^{25} 1 4472, d_4^{25} 0 8908 (lit [15] $[\alpha]_D^{20}$ −29 4°, n_D^{25} 1 4481, d_4^{25} 0 8895); >99% pure on GC

(1*R*,4*R*)-(+)-*p*-Menth-3-one (**3**) was prepared from the *p*-menth-3-one (**2**) by isomerization with NaOEt A soln of **2** (4 0 g) and NaOEt (0 5 g) in EtOH (50 ml) was stirred for 40 hr at 45° The reaction mixt was extracted with Et_2O to give a mixture (3 7 g) of **2** and **3** (7 13 on GC peak) The desired compound **3** was isolated from the mixture by prep TLC with hexane–AcOEt (4 1). $[\alpha]_D^{25}$ +94 8° (EtOH, *c* 1 0), n_D^{25} 1 4558, d_4^{25} 0.8889 (lit [15] $[\alpha]_D^{20}$ +95 0°, n_D^{20} 1 4511, d_4^{25} 0 8952), >99% pure by GC

Incubation of the substrates with a suspension of cultured cells of N tabacum A suspension of the cultured cells was prepared as described in refs [4] and [6], the cells were cultured in a 300 ml conical flask containing 100 ml of Murashige and Skoog's medium [17] in each case To the flask containing the suspension of the cells, the substrate (10 mg) was added, 250–300 mg of the substrate were used in each experiment The transformation was performed by incubating the mixture at 25° for 7–10 days on a rotary shaker (70 rpm) in the dark

Isolation and identification of the products The cultured mixture was worked up as described in refs [6] and [12] The incubation product obtained was subjected to GC analysis and then prep. TLC on Si gel with hexane–EtOAc (8 2) to separate the products The products **2–4** were identified by comparison of their TLC, GC, and spectral data with those of authentic samples The structures of the products **5** and **6** were elucidated by interpretation of their spectral data and comparison of their ^{1}H NMR and CD spectra with the reported data [13] Yields of the products were as shown in Table 1, and the physical constant and the spectral data of the products were described below

(1R,4S)-p-*Menth*-3-*one* (**2**) $[\alpha]_D^{25}$ −26 5° (EtOH, *c* 0 2), ν_{max}^{liq} cm^{-1} 1708 (C=O), ^{1}H NMR ($CDCl_3$) δ0 86 (3H, *d*, *J* = 6 Hz, 7-Me), 0 94 (3H, *d*, *J* = 6 Hz, 9-Me) and 1 02 (3H, *d*, *J* = 6 Hz, 10-Me), MS *m/z* (rel int) 154 [M$^+$] (66), 139 (85), 112 (100) and 69 (55)

(1R,4R)-p-*Menth*-3-*one* (**3**) $[\alpha]_D^{25}$ +94 4° (EtOH, *c* 0 4), ν_{max}^{liq} cm^{-1} 1711 (C=O), ^{1}H NMR ($CDCl_3$) δ0 83 (3H, *d*, *J* = 6 Hz, 7-Me), 0.95 (3H, *d*, *J* = 6 Hz, 9-Me) and 0 98 (3H, *d*, *J* = 6 Hz, 10-Me), MS *m/z* (rel int) 154 [M$^+$] (64), 139 (76), 112 (100), and 69 (50)

(1R,3R,4S)-p-*Menth*-3-*ol* (**4**) $[\alpha]_D^{25}$ +16 8° (EtOH, *c* 0 4), ν_{max}^{liq} cm^{-1} 3435 (OH), ^{1}H NMR ($CDCl_3$) δ0 76 (3H, *d*, *J* = 6 Hz, 7-Me), 0 92 (3H, *d*, *J* = 6 Hz, 9-Me) and 0 98 (3H, *d*, *J* = 6 Hz, 10-Me), MS *m/z* (rel int) 156 [M$^+$] (3), 138 (40), 123 (42), 95 (60), 81 (80), 71 (95), 67 (98) and 64 (100)

(1R,4R)-(−)-4-*Hydroxy*-p-*menth*-3-*one* (**5**) $[\alpha]_D^{25}$ −30 9° (EtOH, *c* 0 2), $\lambda_{max}^{CCl_4}$ nm (log ε) 275 (1 72), $\nu_{max}^{CCl_4}$ cm^{-1} (0 0028 M) 3488, ^{1}H NMR (Table 2), ^{13}C NMR ($CDCl_3$) δ_c 15 5 (C-9), 16 2 (C-10), 18 8 (C-7), 27 9 (C-6), 30 3 (C-8), 32 3 (C-5), 33 1 (C-1), 44 4 (C-2), 80.7 (C-4) and 214 9 (C-3), MS *m/z* (rel int) 170 [M$^+$] (16), 126 (22), 99 (100), 86 (29), 81 (32), 71 (22) and 43 (89), high-resolution MS *m/z* (rel int) 170 1332 [M$^+$, $C_{10}H_{18}O_2$] (100), 152 1190 [$C_{10}H_{16}O$, M$^+ - H_2O$] (4) and 137 0927 [$C_9H_{13}O$, M$^+ - H_2O$ − Me] (2), CD (MeOH, *c* 0 032 M), $[\theta]_{330}$ 0, $[\theta]_{321}$ +198, $[\theta]_{286}$ −2181, $[\theta]_{240}$ 0, (CCl_4, *c* 0 022 M) $[\theta]_{320}$ 0, $[\theta]_{284}$ −5180, $[\theta]_{243}$ 0

(1R,4S)-(+)-4-*Hydroxy*-p-*menth*-3-*one* (**6**). $[\alpha]_D^{25}$ +72 0° (EtOH, *c* 0 1), $\lambda_{max}^{CCl_4}$ nm (log ε) 271 (1 96), $\nu_{max}^{CCl_4}$ cm^{-1} (0 0025 M) 3491, ^{1}H NMR (Table 2), MS *m/z* (rel int) 170 [M$^+$] (18), 126 (28), 99 (100), 86 (32), 81 (36), 71 (26) and 43 (76), high-resolution MS *m/z* (rel int.) 170 1319 [M$^+$, $C_{10}H_{18}O_2$] (45), 152 1244 [$C_{10}H_{16}O$, M$^+ - H_2O$] (2) and 137.0997 [$C_9H_{13}O$, M$^+ - H_2O$–Me] (2), CD (MeOH, *c* 0 021) $[\theta]_{328}$ 0, $[\theta]_{284}$ +11500, $[\theta]_{240}$ 0, (CCl_4, *c* 0 030) $[\theta]_{324}$ 0, $[\theta]_{283}$ +9810, $[\theta]_{248}$ 0.

Time-course in the biotransformation of the substrates Each substrate was incubated at 25° for 10 days with shaking (70 rpm) in the dark At a regular time interval, a part (10 ml) of the incubation mixture was pipetted out under sterile conditions and then extracted with Et_2O. Each Et_2O extract was subjected to GC with a 15% DEGS column at 120° The yields of the products were determined on the basis of the peak area on GC and are expressed as a relative percentage to the total amount of the whole reaction product extracted

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