Biotransformation of 1-Acetoxy-*p*-menth-4(8)-ene with a Suspension of Cultured Cells of *Nicotiana tabacum*

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The biotransformation of 1-acetoxy-*p*-menth-4(8)-ene with a suspension of cultured cells of *Nicotiana tabacum* was investigated. It was found that the cultured cells had the ability to hydroxylate stereoselectively the exocyclic double bond from the side opposite to the acetoxy group and regioselectively the allylic position of the exocyclic double bond.

Recently, increasing interest has been focused on the biochemical ability of plant cells to metabolize foreign substrates and/or convert them into more useful substances. Although the biotransformation of steroids, alkaloids, terpenoids, *etc.* with cultured plant cells has been studied,[1] little is known of how the transformation pattern depends on the structural variety of the foreign substrates. We recently found that a suspension of cultured cells of *Nicotiana tabacum* had the ability to hydroxylate the endocyclic and the terminal carbon–carbon double bonds and their allylic positions.[2,3] To clarify the transformation of the exocyclic carbon–carbon double bond, we investigated the biotransformation of 1-acetoxy-*p*-menth-4(8)-ene (1) with a suspension of cultured cells of *Nicotiana tabacum*.

Results and Discussion

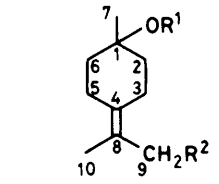
A suspension of cultured cells of *Nicotiana tabacum* ' Bright Yellow ' were used for this work. Incubation of the acetoxy-*p*-menthene (1) with the suspension of cultured cells in a similar manner to that described in our previous papers [4,5] yielded seven products, (2)—(8), as shown in Table 1. The products (2)—(5) are new compounds and their structures were elucidated as described below. The other products were characterized by direct comparison with authentic samples.

Structure Elucidation of the Products (2)—(5).—The major product (2), $C_{12}H_{22}O_4$, showed hydroxy stretching bands in the i.r. spectrum (Table 2) and in the n.m.r. spectrum the two methyl proton signals were at higher field than those of (1) (Table 3). These spectral data indicated that (2) may be 1-acetoxy-*p*-menthane-4,8-diol. The structure of this diol was finally determined by an *X*-ray crystallographic study, and an ORTEP drawing is given in Figure 1; the 1-acetoxy group was found to be *trans* to the 4-hydroxy group and (2) was thus established to be *r*-1-acetoxy-*p*-menthane-*t*-4,8-diol.

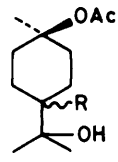
Complete separation of the product (3) from a mixture of compounds (2) and (3) failed, even on repeated preparative t.l.c. However, g.l.c.-mass spectrometric (-m.s.) analysis of the mixture suggested that (3) might be a geometrical isomer of (2). The identity of the product (3), *r*-1-acetoxy-*p*-menthane-*c*-4,8-diol, was established by comparison of the t.l.c., g.l.c., and mass fragmentation pattern with those of an authentic sample, which was prepared from compound (1) by osmium tetraoxide oxidation.[6]

The product (4), $C_{12}H_{20}O_3$, showed a ^1H n.m.r. signal at δ 4.12 assignable to a hydroxymethyl group (Table 3). Comparison of the ^{13}C n.m.r. signals of (4) with those of the *p*-menthane derivatives shown in Table 4 suggested that the product (4) is a 9-hydroxylated derivative of (1). The suggested structure was completely supported by the mass fragment peaks given in Table 2. Thus, the product (4) was established to be 1-acetoxy-*p*-menth-4(8)-en-9-ol.

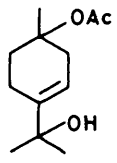
The product (5), $C_{12}H_{20}O_3$, showed a hydroxy stretching band in the i.r. spectrum (Table 2) and a ^1H n.m.r. signal at δ 5.57 due to >C=CH together with a sharp *gem*-dimethyl signal at higher field (δ 1.32) than that of compound (1) (Table 3); these indicated that the double bond had shifted from the 4(8)-position of (1) to the 3-position and that a hydroxy group had been introduced at C-8. The proposed structure possessed all the features necessary to explain the ^{13}C n.m.r. spectrum (Table 4) and the mass fragment peaks



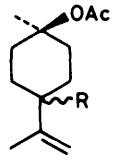
(1) R^1 = Ac, R^2 = H
(4) R^1 = Ac, R^2 = OH
(8) R^1 = R^2 = H



(2) R = ---OH
(3) R = ▬OH



(5)



(6) R = ---OH
(7) R = ▬OH

Table 1. The biotransformation of the acetoxy-*p*-menthene (1) with *Nicotiana tabacum* suspension cells

Products	Yield (%) [a] Run 1	Run 2
r-1-*Acetoxy*-p-*menthane*-t-4,8-*diol* (2)	11.3	9.8
r-1-*Acetoxy*-p-*menthane*-c-4,8-*diol* (3)	1.0	1.4
1-*Acetoxy*-p-*menth*-4(8)-*en*-9-*ol* (4)	4.6	4.4
1-*Acetoxy*-p-*menth*-3-*en*-8-*ol* (5)	5.0	8.6
r-1-Acetoxy-*p*-menth-8(9)-en-*t*-4-ol (6)	0.8	2.2
r-1-Acetoxy-*p*-menth-8(9)-en-*c*-4-ol (7)	0.7	2.4
p-Menth-4(8)-en-1-ol (8)	0.1	0.2

[a] Weight (%) of products relative to the substrate administered.

Table 2. Physical and selected spectral data of products (2)—(5)

Compd.	M.p. (°C)	Found (%) (Required) C	H	ν (cm^{-1}) OH	Ester C=O	*m/z* (fragment ion, %)
(2)	98.5—99.0	62.8 (62.6)	9.9 (9.6)	3 450s 3 530s	1 718s	171 (M^+ − C_3H_7O, 3), 137 (4), 111 (100), 93 (20), 69 (12), 59 (38), 43 (53); 171.1038 ($C_9H_{15}O_3$)
(3)						171 (M^+ − C_3H_7O, 1), 137 (4), 111 (100), 93 (17), 69 (16), 59 (38), 43 (80)
(4)	Viscous oil	68.0 (67.9)	9.9 (9.5)	3 410m	1 732s	152 (M^+ − AcOH, 5), 134 (100), 121 (46), 119 (55), 94 (51), 79 (30), 43 (51); 152.1187 ($C_{10}H_{16}O$)
(5)	Viscous oil	67.9 (67.9)	9.8 (9.5)	3 422s	1 726s	152 (M^+ − AcOH, 30), 137 (87), 134 (85), 119 (93); 109 (73), 91 (24), 79 (19), 59 (27), 43 (100); 152.1197 ($C_{10}H_{16}O$)

Table 3. ^{1}H N.m.r. chemical shifts (δ) ($CDCl_3$) of compounds (1), (2), (4), and (5)

Compound	7-Me	9-Me	10-Me	OAc	Others
(1)	1.48 (s)	1.66 (br s)	1.66 (br s)	1.98 (s)	
(2)	1.48 (s)	1.29 (s)	1.29 (s)	1.99 (s)	
(4)	1.48 (s)		1.74 (br s)	1.99 (s)	4.12 (s, CH_2OH)
(5)	1.48 (s)	1.32 (s)	1.32 (s)	1.94 (s)	5.57 (br s, =CH)

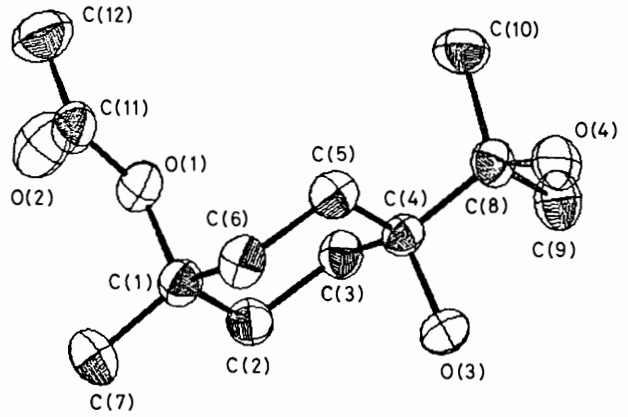


Figure 1. ORTEP drawing of the molecular structure of the *p*-menthane-4,8-diol (2), showing the crystallographic numbering scheme

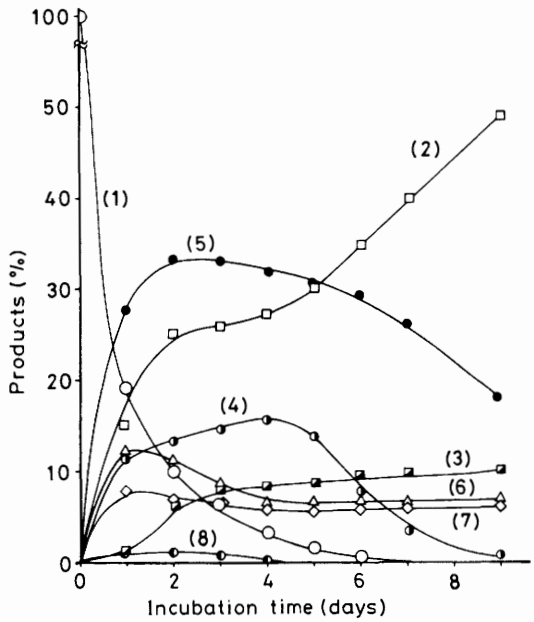


Figure 2. Time-course in the biotransformation of the acetoxy-*p*-menthene (1) with *Nicotiana tabacum* suspension cells

(Table 2). Thus, the product (5) was established to be 1-acetoxy-*p*-menth-3-en-8-ol.

The Transformation Patterns.—The time-course in the biotransformation of the acetoxy-*p*-menthene (1) with the suspension of cultured cells was followed and is shown in Figure 2. Products (2) and (5) were formed in high yields in the early part of the incubation, but the yield of (5) gradually decreased after 5 days. A similar decrease was also observed in the production of (4). These effects are probably due to the further conversion of (4) and (5) into other products, which unfortunately could not be identified.

The formation of *r*-1-acetoxy-*p*-menthane-*t*-4,8-diol (2) predominated over that of *r*-1-acetoxy-*p*-menthane-*c*-4,8-diol (3); this indicates the occurrence of stereoselective hydroxylation at the exocyclic double bond of (1). On the other hand, in spite of careful analyses by a combination of t.l.c., g.l.c., and g.l.c.-m.s., not even a trace of the 3- or 5-hydroxylated product of (1) was found. This fact indicates the occurrence of regioselective hydroxylation of the methyl group at the allylic position of the exocyclic double bond of (1).

The formation of the products (5)—(7) might be due to dehydration of the 4- or 8-hydroxy group of the glycol (2), or to migration of the carbon–carbon double bond at the 4(8)-position of (1) to the 3- or 8(9)-position followed by hydroxylation at the position allylic to the migrated double bond. However, none of the products (5)—(7) were formed when the product (2) was incubated with the suspension of cultured cells under the same conditions as above. Also, no product having the double bond at the 3- or 8-position of the *p*-menthane skeleton was formed when the acetoxy-*p*-menthene (1) was treated with Murashige and Skoog's medium. These facts may indicate the direct conversion of compound (1) into the products (5)—(7) by the cultured tobacco cells, but the mechanism is still ambiguous.

Thus, it was shown that the suspension of cultured cells of *Nicotiana tabacum* are able to hydroxylate stereoselectively

Table 4. ^{13}C Chemical shifts (δ_C) ($CDCl_3$) of compounds (1), (2), and (4)—(8)

Carbon no.	Compound						
	(1)	(2)	(4)	(5)	(6)	(7)	(8)
C-1	81.3	81.0	81.2	79.9	80.8	81.3	69.5
C-2	37.4	31.7	37.1	32.9	31.7 [a]	32.8 [a]	40.2
C-3	25.3	26.7	25.0	116.0	31.3 [a]	32.4 [a]	25.7
C-4	121.3	74.4 [a]	109.8	143.1	72.5	72.4	120.5
C-5	25.3	26.7	25.0	29.0	31.3 [b]	32.4 [b]	25.7
C-6	37.4	31.7	37.1	37.1	31.7 [b]	32.8 [b]	40.2
C-7	25.3	25.9	25.7	29.0	25.8	22.4	29.5
C-8	126.6	75.3 [a]	135.3	72.6	151.9	149.4	130.6
C-9	20.0	24.8	63.3	22.1	109.3	110.8	20.0
C-10	20.0	24.8	16.3	22.1	18.9	18.7	20.0
COMe	22.2	22.2	22.3	24.0	22.2	22.4	
COMe	170.0	170.6	169.9	170.5	170.5	170.4	

[a,b] The values in each vertical column may be reversed.

the exocyclic double bond of the acetoxy-*p*-menthene (1) from the side opposite to that of the acetoxy group, and to hydroxylate regioselectively the methyl group at the allylic position of the double bond. Recently, reduction of the ethylenic linkage adjacent to the carbonyl group was found to occur in the biotransformation of pulegone [7] with the cultured tobacco cells. However, in the case of the acetoxy-*p*-menthene (1) having an isolated exocyclic double bond, the cultured tobacco cells effected the hydroxylation rather than the reduction.

Thus, the transformation patterns of foreign substrates with the cultured cells of *Nicotiana tabacum* are determined by the structure of the foreign substrates administered, in particular, by their functional group and the structural features around the functional group.

Experimental

Analytical and preparative t.l.c. were carried out on 0.25-mm and 0.75-mm thick silica-gel plates (Merck silica 60, GF_{254}), respectively. G.l.c. analyses were performed on an instrument equipped with an f.i.d. and a glass column (3 mm × 2 m) packed with 15% DEGS, 2% OV-17, or 2% OV-101 on Chromosorb W (AW-DMCS; 80—100 mesh), varying the column temperature over the range 100—200 °C at a rate of 3 °C min^{-1} for DEGS and over the range 90—250 °C at a rate of 2 °C min^{-1} for OV-17 and OV-101. G.l.c.-m.s. were recorded on a mass spectrometer which was equipped with an e.i. ion source operating at 20 eV, with a gas chromatograph equipped with an OV-101 capillary column (0.28 mm × 50 m) operating in the range 80—210 °C at a rate of 3 °C min^{-1}. ^1H and ^{13}C N.m.r. spectra were obtained at 60 and 22.6 MHz, respectively, with tetramethylsilane as internal reference.

Substrate.—Following the method described in the literature,[8] 1-acetoxy-*p*-menth-4(8)-ene (1) was synthesized from 1,4,8-tribromo-*p*-menthane (m.p. 108—109 °C) which was prepared by bromination of limonene.[9] The purification of (1) was carried out by column chromatography on silica gel with hexane–ethyl acetate (97 : 3, v/v), followed by distillation: b.p. 105.5—106.0 °C at 9 mmHg (1 199.7 Pa); n_D^{25} 1.4665; d_D^{25} 0.9556; >99.9% pure on g.l.c.; $\nu_{max.}$ (film) 1 739s cm^{-1} (ester C=O); ^1H and ^{13}C n.m.r. (Tables 3 and 4); *m/z* 136 (M^+ − AcOH, 85%), 121 (100), and 93 (63).

Incubation of 1-*Acetoxy*-p-*menth*-4(8)-*ene* (1) *with the Suspension of Tobacco Cells.*—The suspension of cells of *Nicotiana tabacum* 'Bright Yellow' were prepared as described in refs. 4 and 5; the cells were cultured in a 300-ml conical flask containing 100 ml of Murashige and Skoog's medium [10] in each case. To the flask containing the suspension of cells, the acetoxy-*p*-menthene (1) (10 mg) was added; 600 and 540 mg of substrate were used in Runs 1 and 2, respectively. The transformation of the acetoxy-*p*-menthene (1) was brought about by incubating the cultured mixture at 25 °C for 10 days on a rotary shaker (70 r.p.m.) in the dark.

Isolation and Characterization of the Products.—The cultured mixture was worked up as described in refs. 4 and 5. An incubation product was obtained and was subjected to repeated preparative t.l.c. on silica gel with hexane–ethyl acetate (7 : 3 and 1 : 1, v/v). As described in the Discussion, the structures of the new compounds isolated, (2), (4), and (5), were elucidated on the basis of the physical constants and spectral data given in Tables 2—4. The product (3) could not be isolated in a pure state, and it was identified by comparing its t.l.c., g.l.c., and g.l.c.-m.s. with those of a synthetic specimen prepared as described below. The products (6)—(8) were identified by direct comparison of their physical and spectral data with those of authentic samples.[11,12]

Time-course in the Biotransformation of the Acetoxy-p-*menthene* (1).—The acetate (1) was administered to the precultured suspension of cells in each flask, as described above. One of the cultured mixtures was extracted with chloroform at regular time intervals. Each chloroform extract (5—9 mg) was dissolved in ethyl acetate (0.4 ml) and then subjected to g.l.c. The products were identified by co-t.l.c. and co-g.l.c. with authentic samples. The yields of the products were determined on the basis of the peak areas from g.l.c. and are expressed relative (%) to the total amount of the whole reaction product extracted.

Incubation of the Product (2) *with the Suspension of Tobacco Cells.*—*r*-1-Acetoxy-*p*-menthane-*t*-4,8-diol (2) (4 mg) was incubated with the cultured cells under the same conditions as above. No compound other than the starting substrate (2) was found in the chloroform extract (5 mg) even on carefully repeated t.l.c. and g.l.c. analyses.

Treatment of the Acetoxy-p-*menthene* (1) *with Murashige and Skoog's Medium.*—After the acetate (1) (44 mg) had been shaken with Murashige and Skoog's medium (100 ml) at 25 °C for 7 days in the dark, the reaction mixture was extracted with chloroform to give an oily substance (18 mg). Careful t.l.c. and g.l.c. analyses indicated that the oil was composed of only the unchanged acetate (1).

Table 5. Final atomic co-ordinates (× 10^4) and thermal parameters of the non-hydrogen atoms of the *p*-menthane-4,8-diol (2), with e.s.d.s in parentheses

Atoms	*x*	*y*	*z*	B_{eq} [a] (Å^2)
C(1)	2 755(5)	1 233(8)	5 379(4)	2.9
C(2)	1 377(6)	1 267(9)	5 132(4)	3.0
C(3)	943(5)	2 908(9)	4 602(4)	2.9
C(4)	1 625(5)	3 114(8)	3 813(4)	2.5
C(5)	2 999(5)	3 107(9)	4 063(4)	3.1
C(6)	3 446(6)	1 456(9)	4 595(4)	3.2
C(7)	3 117(7)	−500(10)	5 875(5)	4.3
C(8)	1 193(6)	4 840(9)	3 297(4)	3.0
C(9)	1 681(7)	6 641(9)	3 706(5)	4.1
C(10)	−211(6)	4 905(10)	3 111(4)	3.9
C(11)	4 066(6)	3 316(9)	6 292(4)	3.5
C(12)	3 982(7)	4 965(10)	6 851(5)	5.0
O(1)	2 949(4)	2 850(6)	5 937(3)	3.2
O(2)	5 010(4)	2 502(8)	6 199(3)	4.8
O(3)	1 288(4)	1 553(6)	3 296(3)	3.2
O(4)	1 741(4)	4 748(6)	2 497(3)	3.9

[a] $B_{eq} = 8\pi^2(U_1 + U_2 + U_3)/3$, where U_1, U_2, and U_3 are the principal components of the *U* matrix.

Table 6. Bond lengths (Å) of the *p*-menthane-4,8-diol (2), with e.s.d.s in parentheses

C(1)–C(2)	1.515(7)	C(4)–O(3)	1.435(6)
C(1)–C(6)	1.521(7)	C(5)–C(6)	1.526(7)
C(1)–C(7)	1.523(7)	C(8)–C(9)	1.536(7)
C(1)–O(1)	1.481(6)	C(8)–C(10)	1.540(7)
C(2)–C(3)	1.518(7)	C(8)–O(4)	1.450(6)
C(3)–C(4)	1.523(7)	C(11)–C(12)	1.505(8)
C(4)–C(5)	1.515(7)	C(11)–O(1)	1.340(6)
C(4)–C(8)	1.554(7)	C(11)–O(2)	1.206(6)

Table 7. Bond angles (°) of the *p*-menthane-4,8-diol (2), with e.s.d.s in parentheses

C(2)–C(1)–C(6)	109.5(4)	C(8)–C(4)–O(3)	107.1(4)
C(2)–C(1)–C(7)	110.7(4)	C(4)–C(5)–C(6)	113.9(4)
C(2)–C(1)–O(1)	103.1(4)	C(1)–C(6)–C(5)	112.7(4)
C(6)–C(1)–C(7)	113.1(4)	C(4)–C(8)–C(9)	113.4(4)
C(6)–C(1)–O(1)	110.5(4)	C(4)–C(8)–C(10)	112.0(4)
C(7)–C(1)–O(1)	109.6(4)	C(9)–C(8)–C(10)	110.5(4)
C(1)–C(2)–C(3)	114.3(4)	C(4)–C(8)–O(4)	107.4(4)
C(2)–C(3)–C(4)	112.5(4)	C(9)–C(8)–O(4)	105.2(4)
C(3)–C(4)–C(5)	109.1(4)	C(10)–C(8)–O(4)	108.0(4)
C(3)–C(4)–C(8)	111.7(4)	C(12)–C(11)–O(1)	110.7(4)
C(5)–C(4)–C(8)	112.5(4)	C(12)–C(11)–O(2)	124.3(5)
C(3)–C(4)–O(3)	106.0(4)	O(1)–C(11)–O(2)	125.1(5)
C(5)–C(4)–O(3)	110.1(4)	C(1)–O(1)–C(11)	121.9(4)

X-Ray Crystallographic Analysis of the Product (2).—The *X*-ray crystallographic analysis was performed on a Syntex R3 diffractometer using graphite-monochromated Mo-K_α radiation. The crystal analysed was 0.47 mm × 0.45 mm × 0.35 mm in size.

Crystal data. $C_{12}H_{22}O_4$, $M = 230.3$, monoclinic, $a = 10.886(13)$, $b = 7.309(7)$, $c = 15.906(22)$ Å, $\beta = 95.1(1)°$, $U = 1260.6$ Å^3, $Z = 4$, $D_c = 1.21$ g cm^{-3}, $D_m = 1.20$ g cm^{-3}, μ(Mo-K_α) = 1.0 cm^{-1}, space group $P2_1/n$. A total of 1 603 reflections were collected by ω-scan ($\theta_{max.} = 50.0$), of which 1 460 reflections with intensities >1.96 times that of the standard deviations were used in the structure determination. The phases of 168 reflections with $|E| > 1.50$ were determined by MULTAN.[13] An *E* map for the best solution yielded positions for all non-hydrogen atoms. Anisotropic refinement for carbon and oxygen atoms and isotropic refinement for hydrogen atoms by full-matrix least-squares calculation reduced the *R* index finally to 0.071. Final atomic co-ordinates are given in Table 5, and bond lengths and bond angles in Tables 6 and 7. Structure factor tables are available as a Supplementary Publication * (SUP No. 23686, 16 pages).

Preparation of Authentic Samples.—Following the procedure described in the literature,[6] the acetate (1) was oxidized with osmium tetraoxide. G.l.c. and g.l.c.-m.s. analyses indicated that the resultant product comprised compound (2) [g.l.c.-m.s. *m/z* 171 ($M^+ - C_3H_7O$, 3%), 111 (100), 93 (20), and 59 (38)] and compound (3) [g.l.c.-m.s., *m/z* 171 ($M^+ - C_3H_7O$, 2%), 111 (100), 93 (16), and 59 (34)] in the ratio 5 : 2.

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* For details of the Supplementary Publications Scheme see Instructions to Authors (1983), *J. Chem. Soc., Perkin Trans. 1*, 1983, Issue 1.

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