The Hydroxylation of β-Terpineol and Its Acetate with the Cultured Cells of *Nicotiana tabacum*

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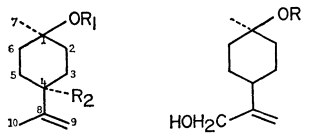
The biotransformation of *c*-4-*p*-menth-8(9)-en-*r*-1-ol (β-terpineol) and its acetate with the cultured suspension cells of *Nicotiana tabacum* were tested. It was found that the cultured cells have the ability to hydroxylate not only the allylic positions of the C–C double bond of these substrates, but also their terminal carbon-carbon double bond. The hydroxylation at the 4-position of their allylic positions was stereospecific, affording a *trans*-1,4-diol.

The biochemical capability of plant cells to metabolize foreign substrates and/or convert them into more useful substances is of considerable interest. In such a status, we recently investigated the transformation of monoterpenoid alcohols and ketones with the cultured cells of *Nicotiana tabacum* "Bright Yellow," and found that the tobacco cells have the ability not only to reduce stereoselectively the carbon–carbon double bond adjacent to the carbonyl group of carvone as well as the carbonyl group,[1] but also to hydroxylate regioselectively the allylic position of the carbon-carbon double bond of linalool.[2] Also, the cultured cells were found to have the ability to hydrolyze the acetoxyl group of linalyl acetate, dihydrolinalyl acetate, and α-terpinyl acetate, but the cells scarcely show such an ability to γ-terpinyl acetate.[3] To generalize how the transformation patterns depend on the type of the functional group of the substrates and the structure around the functional group, we now have investigated the biotransformation of *c*-4-*p*-menth-8(9)-en-*r*-1-ol (β-terpineol) (**1**) and its acetate (**2**). The result was in part outlined in the preliminary communication.[4] This paper will give a full detail of the result.

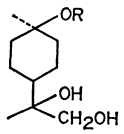
Results and Discussion

Callus tissues induced from the stem of *N. tabacum* "Bright Yellow" were used in this work. The callus tissues were cultured in Murashige and Skoog's medium[5] with continuous shaking for 3—4 weeks and then the monoterpenoids were administered to the cultures. The cultures were then incubated at 25 °C for 7—10 d with shaking in the dark.

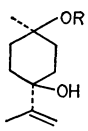


1: $R_1=R_2=H$
2: $R_1=Ac$, $R_2=H$
4: $R_1=H$, $R_2=OH$
8: $R_1=Ac$, $R_2=OH$

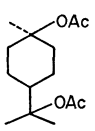
3: R=H
7: R=Ac



5: R=H
6: R=Ac



9: R=H
10: R=Ac



11

c-4-*p*-Menth-8(9)-en-*r*-1-ol (**1**) was transformed into three products, *c*-4-*p*-menth-8(9)-ene-*r*-1,10-diol (**3**), 4-*p*-menth-8(9)-ene-*r*-1,*t*-4-diol (**4**), and *c*-4-*p*-menthane-*r*-1,8,9-triol (**5**), which were characterized as follows. The major product (**3**) was indicated to be a 10-hydroxylated derivative of **1** on the basis of the fragment ion peaks at *m/z* 155.1042 (M^+-CH_3, $C_9H_{15}O_2$), 152.1185 (M^+-H_2O, $C_{10}H_6O$), and 134.1096 ($M^+-2\times H_2O$, $C_{10}H_{14}$) in the high resolution mass spectrum, the hydroxyl stretching bands at 3635 (primary) and 3618 cm^{-1} (tertiary) in the IR spectrum, and the ^1H NMR signal at δ 4.12 due to a hydroxymethyl group instead of the 10-methyl signal of **1**. Identity of the product **3** with *c*-4-*p*-menth-8(9)-ene-*r*-1,10-diol was established by comparisons of its spectral data with those of an authentic sample, which was prepared by oxidation of *r*-1-acetoxy-*c*-4-*p*-menth-8(9)-ene (**2**) with SeO_2 followed by hydrolysis of the oxidation product. On the other hand, the product **4** was established to be 4-*p*-menth-8(9)-ene-*r*-1,*t*-4-diol on the basis of comparisons of its spectral data with those of the literature[6-8] and those of an authentic sample. Identity of the product **5** with *c*-4-*p*-menthane-*r*-1,8,9-triol was established by comparisons of its TLC, IR and mass spectra with those of the authentic sample prepared by oxidation of **2** with OsO_4 followed by reduction with $LiAlH_4$.

The biotransformation of *r*-1-acetoxy-*c*-4-*p*-menth-8(9)-ene (**2**) with the cells yielded three products, **6**, **7**, and **8**. The ^1H NMR spectrum of **6** revealed a new signal at δ 3.47 due to a hydroxymethyl group with the disappearance of the signal of the terminal ethylenic linkage of **2**. Its mass spectrum exhibited the ion peaks at *m/z* 199 (M^+-CH_2OH), 139 ($M^+-CH_2OH-AcOH$), and 121 ($M^+-CH_2OH-AcOH-H_2O$). These data indicated the product **6** to be *r*-1-acetoxy-*c*-4-*p*-menthane-8,9-diol. Identity of the product with the diol was established by direct comparisons of its IR, ^1H NMR, and mass spectra with those of the authentic sample prepared by oxidation of **2** with OsO_4.[9] The formation of the diol (**6**) indicates the occurrence of hydroxylation of the terminal ethylenic linkage of **2**. On the other hand, the products **7** and **8** were identified as *r*-1-acetoxy-*c*-4-*p*-menth-8(9)-en-10-ol and *r*-1-acetoxy-4-*p*-menth-8(9)-en-*t*-4-ol, respectively, by direct comparisons of their physical and spectral data with those of the

Table 1. Biotransformation of *c*-4-*p*-menth-8(9)-en-*r*-1-ol (**1**) and its acetate (**2**) by the cultured cells of *N. tabacum*

Substrates	Products	Yield/%[a]
c-4-*p*-Menth-8(9)-en-*r*-1-ol (**1**)	*c*-4-*p*-Menth-8(9)-ene-*r*-1,10-diol (**3**)	13.1
	4-*p*-Menth-8(9)-ene-*r*-1,*t*-4-diol (**4**)	6.1
	c-4-*p*-Menthane-*r*-1,8,9-triol (**5**)	0.8
r-1-Acetoxy-*c*-4-*p*-menth-8(9)-ene (**2**)	*r*-1-Acetoxy-*c*-4-*p*-menthane-8,9-diol (**6**)	14.7
	r-1-Acetoxy-*c*-4-*p*-menth-8(9)-en-10-ol (**7**)	9.8
	r-1-Acetoxy-4-*p*-menth-8(9)-en-*t*-4-ol (**8**)	8.4

a) The weight percent of the products per the administered substrates.

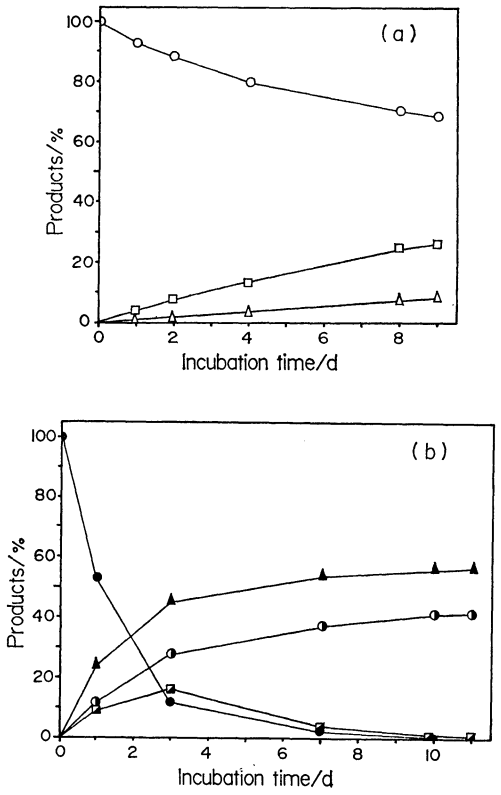


Fig. 1. The time-courses in the biotransformation of **1** (a) and **2** (b) by the cultured cells of *N. tabacum*. ○: **1**, ●: **2**, □: **3**, △: **4**, ▲: **6**, ◪: **7**, and ◑: **8**.

authentic samples, which had been prepared following to the reported procedure.[6,7] The yields of the transformation products are given in Table 1. The time-courses in the biotransformation of β-terpineol (**1**) and its acetate (**2**) with the cultured suspension cells were followed, and it was found that the transformation of **2** is more rapid than that of **1**. The products **3**, **4**, **6**, and **8** gradually increased in their amounts with the lapse of the incubation time, but, after the amounts of the product **7** became maximum at the 3-d incubation, it gradually decreased; this is probably due to the further conversion of **7** to another product. In spite of careful and repeated TLC and GLC analyses, no *cis*-1,4-glycols (**9** and **10**) were found in the reaction mixtures obtained at each regular interval in the time-course experiment. This fact indicates that the hydroxylation with the tobacco suspension cells is more stereoselective than that with SeO_2, which is known to convert **2** into *r*-1-acetoxy-4-*p*-menth-8(9)-en-*t*-4-ol (**8**) and its *cis*-isomer (**10**) in the ratio of 6:5.[6]

Thus, it has been clarified that β-terpineol (**1**) and its acetate (**2**) are hydroxylated not only at the allylic positions of the ethylenic linkage but also at the ethylenic linkage itself by the tobacco cultured suspension cells. However, in contrast to the predominant hydroxylation at the ethylenic linkage in the case of β-terpinyl acetate (**2**), only a small amount of β-terpineol (**1**) is hydroxylated at the ethylenic linkage. The hydroxylation of the 4-position of **1** and **2** is stereospecific and arises from the direction *trans* to the 1-hydroxyl or 1-acetoxyl group. Our recent studies on the biotransformation of carvone[1] showed that its carbon-carbon double bond adjacent to the carbonyl group was reduced, but its terminal carbon-carbon double bond remained unchanged. However, the terminal ethylenic linkage of β-terpinyl acetate (**2**) was hydroxylated to an appreciable extent. It is fascinating to note that the biotransformation pattern of the foreign substrates depends on the functional group of the substrate administered and the structure around the functional group.

Experimental

Analytical (0.25 mm thick) and preparative TLC (0.75 mm thick) were carried out on a silica-gel plate (Merck, Type 60, GF_{254}). GLC analyses were performed on an instrument equipped with an FID 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) by programming the column temperature at 100—200 °C with a rate of 3 °C/min for DEGS and at 90—250 °C, 2 °C/min for OV-17 and OV-101. The areas of the peaks on the gas-liquid chromatogram were calculated by use of a Shimadzu C-R1A Chromatopac recording data processor for chromatography. GC-MS were recorded on a mass spectrometer which was installed with a gas chromatograph equipped with an OV-101 capillary column (0.28 mm×50 m) at 80—210 °C with a rate of 3 °C/min using an EI ion source at 20 eV. ^1H NMR spectra were taken at 60 or 90 MHz for $CDCl_3$ solutions with TMS as an internal reference. FT-IR spectra were obtained on a JIR-40X FT-IR spectrophotometer.

Preparation of the Materials Used for Substrates. Following the method described in the literature,[10,11] *r*-1-acetoxy-*c*-4-*p*-menth-8(9)-ene (**2**) was prepared by pyrolysis of terpin diacetate (**11**) (n_D^{25} 1.4510, d_4^{25} 1.0217), which was derived from terpin hydrate[12] by acetylation, followed by purifications by column chromatography on 10% $AgNO_3$–silica gel with a hexane–EtOAc mixture with increasing EtOAc from 0 to 5% and then by distillation under a reduced pressure: bp 71 °C/3.5 mmHg, 1 mmHg≈133.322 Pa; n_D^{25} 1.4569; d_4^{25} 0.9414 (lit,[10] bp 81—82 °C/4 mmHg; n_D^{25} 1.4562; d_4^{25}

0.9411); >99.8% pure on GLC; IR (neat) 1737 (OAc), 3084, 1644, and 892 cm^{-1} (>C=CH_2); ^1H NMR ($CDCl_3$) δ=1.47 (3H, s, 7-CH_3), 1.69 (3H, bs, 10-CH_3), 1.99 (3H, s, OAc), and 4.69 (2H, bs, >C=CH_2). Found: C, 73.29; H, 10.24%. Calcd for $C_{12}H_{20}O_2$: C, 73.43; H, 10.27%.

c-4-*p*-Menth-8(9)-en-*r*-1-ol (**1**) was prepared from **2** by hydrolysis with 2.5% ethanolic KOH soln, followed by purification by preparative TLC on silica gel with hexane–EtOAc (7:3, v/v): mp 30.5—30.8 °C (lit,[7] 28—30 °C); >99.9% pure on GLC; IR (neat) 3400 (OH), 3095, 1648, and 890 cm^{-1} (>C=CH_2); ^1H NMR ($CDCl_3$) δ=1.23 (3H, s, 7-CH_3), 1.72 (3H, bs, 10-CH_3), and 4.70 (2H, bs, >C=CH_2). Found: C, 77.74; H, 11.73%. Calcd for $C_{10}H_{18}O$: C, 77.86; H, 11.76%.

Feeding of the Monoterpenoids to the Tobacco Suspension Cells. The callus tissues used in this study were induced from the stem of *Nicotiana tabacum* "Bright Yellow" and have been maintained for about 8 years. Just prior to use for this work, the callus tissue was transplanted to freshly prepared Murashige and Skoog's medium[5] (100 ml per 300 ml-conical flask) containing 2 ppm 2,4-dichlorophenoxyacetic acid and 3% sucrose, and it was grown with continuous shaking for 3—4 weeks at 25 °C in the dark. After the substrate (10 mg per one flask; total 250—400 mg) had been added to the suspension cultures (about 50—70 g cells per one flask), the cultures was incubated at 25 °C for 7—10 d on a rotary shaker (70 min^{-1}) in the dark.

Isolation and Identification of the Products. The suspension cells were filtered off and triturated with MeOH. Removal of the solvent from the methanol solution gave an extract, which was again extracted with $CHCl_3$. On the other hand, the culture medium separated from the mass of the cells was extracted with $CHCl_3$. The two $CHCl_3$ extracts were put together, since they exhibited the same behavior on TLC and GLC. Transformation products **3**, **4**, and **6**—**8** were isolated from the combined $CHCl_3$ extract by preparative TLC on silica gel with hexane–EtOAc (1:2 or 7:3, v/v), and identified by direct comparisons of their physical constants, TLC, GLC, and spectral data with those of synthetic specimens prepared as described below.

In the case of β-terpineol (**1**), the aqueous layers separated from the $CHCl_3$ solution were put together, and the combined aqueous solution (850 ml) was exactly neutralized and concentrated into 50 ml by lyophilization. The concentrated aqueous solution was extracted continuously with EtOAc for 17 h by use of a liquid-liquid extractor. Triol **5** was isolated from the EtOAc extract by preparative TLC (0.5 mm thick) on silica gel with EtOAc–MeOH (97:3, v/v), and identified by direct comparisons of its TLC, IR and mass spectra with those of a synthetic sample. To confirm the complete extraction of the triol **5** from the aqueous solution, the residue (6.20 g) obtained from the residual aqueous solution by lyophilization was extracted with MeOH (50 ml×2), and then the sirup (3.75 g) obtained from the MeOH solution on evaporation was subjected to the analytical TLC. However, no spot of the triol (**5**) was observed.

The yields of the products were as shown in Table 1, and the physical constants and spectral data of the products were as follows.

c-*4*-p-*Menth-8(9)-ene*-r-*1,10-diol (3)*: Mp 75.0—76.2 °C (from hexane); IR (KBr) 3327 (OH) and 3100, 1652, and 901 cm^{-1} (>C=CH_2); IR (1.8×10^{-3} M, CCl_4) 3635 (primary OH), 3618 (tertiary OH); ^1H NMR ($CDCl_3$) δ=1.24 (3H, s, 7-CH_3), 4.12 (2H, bs, -$C\underline{H}_2OH$), 4.92 and 5.01 (2H, each d, >C=CH_2); MS (70 eV), *m/z* (rel intensity) 155 (6), 152 (33), 137 (12), 134 (20), 119 (17), 109 (34), 94 (33), 84 (34), 71 (66), 55 (30), and 43 (100); High-resolution MS *m/z* 155.1042 (M^+-CH_3, $C_9H_{15}O_2$), 152.1185 (M^+-H_2O, $C_{10}H_6O$), and 134.1096 ($M^+-2\times H_2O$, $C_{10}H_{14}$).

4-p-*Menth-8(9)-ene*-r-*1*,t-*4-diol (4)*: Mp 139—140 °C (from hexane) (lit,[8] 140—141 °C); IR (KBr) 3357 (OH), 3098, 1641, and 895 cm^{-1} (>C=CH_2); IR (4.1×10^{-3} M, CCl_4) 3617 cm^{-1} (tertiary OH); ^1H NMR ($CDCl_3$) δ=1.27 (3H, s, 7-CH_3), 1.83 (3H, bs, 10-CH_3), 4.82 and 5.05 (2H, m, >C=CH_2); MS (70 eV), *m/z* (rel intensity) 170 (M^+, 10), 155 (13), 152 (23), 137 (24), 123 (38), 109 (31), 98 (100), 84 (38), 73 (53), 69 (59), and 43 (74); High-resolution MS *m/z* 155.1086 (M^+-CH_3, $C_9H_{15}O_2$), 152.1207 (M^+-H_2O, $C_{10}H_{16}O$), and 134.1109 ($M^+-2\times H_2O$, $C_{10}H_{14}$).

c-*4*-p-*Menthane*-r-*1,8,9-triol (5)*: FT-IR (neat) 3355 (OH), 1453, 1380, 1168 1048 (C–O), 900 cm^{-1}; MS (70 eV), *m/z* (rel. intensity) 157 (1), 155 (1.5), 152 (1), 149 (1), 139 (35), 121 (3), 109 (3), 95 (18), 81 (20), 75 (9), 71 (20), 55 (13), and 43 (100); High-resolution MS *m/z* 157.1238 (M^+-CH_2OH, $C_9H_{17}O_2$), 139.1124 ($M^+-H_2O-CH_2OH$, $C_9H_{15}O$), and 121.1005 ($M^+-2\times H_2O-CH_2OH$, C_9H_{13}).

r-*1*-*Acetoxy*-c-*4*-p-*menthane-8,9-diol (6)*: n_D^{25} 1.4749; IR (neat) 3421 (OH) and 1727 cm^{-1} (OAc); ^1H NMR ($CDCl_3$) δ=1.09 (3H, s, 10-CH_3), 1.46 (3H, s, 7-CH_3), 1.99 (3H, s, OAc), and 3.47 (2H, dd, J=16 and 11 Hz, -$C\underline{H}_2OH$); MS (70 eV), *m/z* (rel intensity) 199 (0.5), 155 (2), 152 (4), 139 (49), 135 (5), 121 (11), 95 (26), 82 (26), 75 (17), and 43 (100); High-resolution MS *m/z* 152.1172 ($M^+-AcOH-H_2O$, $C_{10}H_{16}O$), 139.1119 ($M^+-AcOH-CH_2OH$, $C_9H_{15}O$), 135.1173 ($M^+-AcOH-H_2O-OH$, $C_{10}H_{15}$), and 121.1027 ($M^+-AcOH-CH_2OH-H_2O$, C_9H_{13}).

r-*1*-*Acetoxy*-c-*4*-p-*menth-8(9)-en-10-ol (7)*: n_D^{25} 1.4764 (lit,[6] n_D^{25} 1.4757); IR (neat) 3427 (OH), 1728 (OAc), and 3100, 1651, and 898 cm^{-1} (>C=CH_2); ^1H NMR ($CDCl_3$) δ=1.48 (3H, s, 7-CH_3), 2.00 (3H, s, OAc), 4.19 (2H, bs, -$C\underline{H}_2OH$), 4.75 and 5.01 (2H, m, >C=CH_2); MS (70 eV), *m/z* (rel intensity) 194 (M^+-H_2O, 1), 152 (20), 137 (20), 134 (27), 121 (14), 119 (26), 109 (27), 106 (27), 94 (30), 84 (28), 79 (26), and 43 (100). The structure of **7** was further confirmed by its conversion to *c*-4-*p*-menth-8(9)-ene-*r*-1,10-diol (**3**) (mp 74.0—76.0 °C) on reduction with $LiAlH_4$ in dry ether. The diol (**3**) was identified by direct comparisons of the IR and ^1H NMR spectra with those of an authentic specimen.

r-*1*-*Acetoxy*-*4*-p-*menth-8(9)-en*-t-*4-ol (8)*: Mp 88.5—89.5 °C (from hexane) (lit,[6] 88—89 °C); IR (KBr) 3480 (OH), 1708 (OAc), 3100, 1642, and 905 cm^{-1} (>C=CH_2); ^1H NMR ($CDCl_3$) δ=1.49 (3H, s, 7-CH_3), 1.78 (3H, m, 10-CH_3), 1.99 (3H, s, OAc), 4.79 and 5.02 (2H, m, >C=CH_2); MS (70 eV), *m/z* (rel intensity) 171 (2), 152 (50), 137 (19), 124 (30), 123 (55), 119 (7), 111 (23), 109 (28), 97 (40), 84 (45), 69 (59), and 43 (100). The structure of **8** was further confirmed by its conversion to 4-*p*-menth-8(9)-ene-*r*-1,*t*-4-diol (**4**) (mp 135—139 °C; lit,[8] 140—141 °C) on reduction with $LiAlH_4$ in dry ether. Identification of **4** was performed by direct comparisons of its spectra (IR and ^1H NMR) with those of an authentic sample.

Time-courses in the Biotransformation of 1 and 2. The substrate (10 mg) was administered to each of five flasks containing 100 ml of the precultured suspension. The cultured mixtures were worked up at a regular time interval in the same manner as described above. The oily product (5—10 mg) obtained from the $CHCl_3$ extract was dissolved in 0.4 ml of ethyl acetate to subject to GLC. Components of the product were identified by co-TLC and co-GLC with authentic samples and then by GC-MS measurements. The yields of the products were determined on the basis of the peak areas on GLC, and expressed as relative percent to the whole products extracted, as shown in Fig. 1.

Preparation of the Authentic Samples. *Preparation of* ***7***, ***8***, *and* ***10***: Following the method described in the literature,[7] *r*-1-acetoxy-*c*-4-*p*-menth-8(9)-ene (**2**) (2.46 g) was oxidized with SeO_2 (0.75 g) dissolved in a mixture of *t*-BuOH (6.0 g) and benzene (8 ml) at 40—50 °C for 9 h under a nitrogen atmosphere. The reaction mixture was subjected to preparative TLC with hexane–EtOAc (7:3, v/v) to give *r*-1-acetoxy-*c*-4-*p*-menth-8(9)-en-10-ol (**7**) (0.11 g; n_D^{25} 1.4769), *r*-1-acetoxy-4-*p*-menth-8(9)-en-*t*-4-ol (**8**) (0.31 g; mp 88.5—89.5 °C), and *r*-1-acetoxy-4-*p*-menth-8(9)-en-*c*-4-ol (**10**) (0.25 g; n_D^{25} 1.4778).

Preparation of ***3*** *and* ***4***: *c*-4-*p*-Menth-8(9)-ene-*r*-1,10-diol (**3**) (22 mg; mp 75.0—76.0 °C) and 4-*p*-menth-8(9)-ene-*r*-1,*t*-4-diol (**4**) (31 mg; mp 139—140 °C) were obtained from **7** (30 mg) and **8** (45 mg) by reduction with $LiAlH_4$ in dry ether for 3 h at 0 °C, respectively.

Preparation of **6**: Following the procedure described in the literature,[9] β-terpinyl acetate (**2**) (0.196 g) was oxidized with osmium tetraoxide (0.275 g) in a mixture of ethyl ether (20 ml) and pyridine (0.4 ml) at room temp for 2 d to give brownish precipitates. The precipitates were filtered off and hydrolyzed with a sodium hydroxide solution (1%) in the presence of mannitol. *r*-1-Acetoxy-*c*-4-*p*-menthane-8,9-diol (**6**) (0.190 g, n_D^{25} 1.4736) was isolated from the reaction mixture by preparative TLC with hexane–EtOAc (3:7, v/v).

Preparation of ***5***: *c*-4-*p*-Menthane-*r*-1,8,9-triol (**5**) (48 mg) was obtained from **6** (68 mg) by reduction with $LiAlH_4$ in dry ether for 8 h at 0 °C: mp 118.0—118.5 °C (from EtOAc) (lit,[13] 118—118.5 °C); FT-IR (neat) 3355 (OH), 1453, 1380, 1168, 1048 (C–O), 900 cm^{-1}; ^1H NMR ($CDCl_3$) δ=1.10 (3H, s, 10-CH_3), 1.16 (3H, s, 7-CH_3), and 3.36 (2H, d, J=3.7 Hz, -$C\underline{H}_2OH$); ^{13}C NMR ($CDCl_3$) δ_c= 74.9(s), 69.1(s), 68.3(t), 44.1(d), 38.8(t, 2×C), 31.4(q), 22.8(t), 21.8(t), and 20.7(q); MS (70 eV), *m/z* (rel intensity) 157 (1), 155 (1.5), 152 (1), 149 (2), 139 (39), 121 (3), 109 (3), 95 (19), 81 (17), 75 (9), 71 (19), 55 (10), and 43 (100).

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