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Enantioselectivity in the Biotransformation of Mono- and Bicyclic Monoterpenoids with the Cultured Cells of *Nicotiana tabacum*

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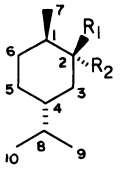
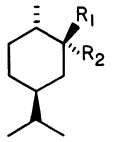
The biotransformation of the enantiomeric pairs of *p*-menthane and bicyclo[2.2.1] and [3.1.1]heptane derivatives with the cultured cells of *Nicotiana tabacum* was investigated. It was found that (i) the cultured cells discriminate the enantiomers of *p*-menthan-2-ol and bicyclo[2.2.1]heptan-2-ol and bicyclo[3.1.1]heptan-3-ol derivatives, and enantioselectively convert these alcohols to the corresponding ketones, (ii) the cells reduce the carbonyl group of *p*-menthan-2-one derivatives to a high extent, but not that of *p*-menthan-3-ones, and (iii) the cells discriminate the enantiomers of bicyclo[3.1.1]hept-2-en-4-one (verbenone) and enantioselectively reduce the C-C double bond of the (1*S*,5*S*)-enantiomer.

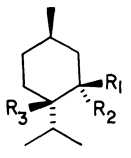
The ability of cultured plant cells to metabolize foreign substrates and/or to convert these substrates to more useful substances is of considerable interest because of the specificity of the transformation which may be effected by such cells.[1-4] In such a status, many investigations on the biotransformation of the monoterpenoids[5-13] with the cultured cells of *Nicotiana tabacum* "Bright Yellow" are performed, and the cultured cells were found to not only reduce stereoselectively the C-C double bond adjacent to the carbonyl group of carvone and then the carbonyl group,[8,9] but also discriminate the enantiomers of monoterpene acetates and enantioselectively hydrolize one of the enantiomers.[6,12] The author has now investigated the enantioselectivity in the oxidative and reductive transformations of the enantiomeric pairs of mono- and bicyclic monoterpenoids, such as *p*-menthane and bicyclo[2.2.1] and bicyclo[3.1.1]heptane derivatives, with the cultured cells of *N. tabacum*.[14]



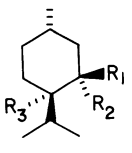
1a: R_1=H, R_2=OH
2a: R_1=OH, R_2=H
13a: R_1,R_2=O



1b: R_1=OH, R_2=H
2b: R_1=H, R_2=OH
13b: R_1,R_2=O

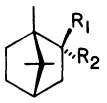


3a: R_1=OH, R_2,R_3=H
4a: R_1,R_3=H, R_2=OH
14a: R_1,R_2=O, R_3=H
23a: R_1,R_2=O, R_3=OH

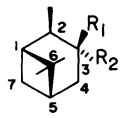


3b: R_1,R_3=H, R_2=OH
4b: R_1=OH, R_2,R_3=H
14b: R_1,R_2=O, R_3=H
23b: R_1,R_2=O, R_3=OH

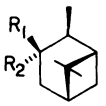
5a: R_1=H, R_2=OH
6a: R_1=OH, R_2=H
15a: R_1,R_2=O



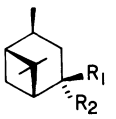
5b: R_1=H, R_2=OH
6b: R_1=OH, R_2=H
15b: R_1,R_2=O



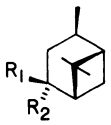
7a: R_1=H, R_2=OH
8a: R_1=OH, R_2=H
16a: R_1,R_2=O



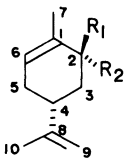
7b: R_1=H, R_2=OH
8b: R_1=OH, R_2=H
16b: R_1,R_2=O



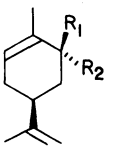
9a: R_1=OH, R_2=H
17a: R_1,R_2=O



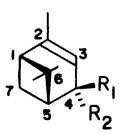
9b: R_1=OH, R_2=H
17b: R_1,R_2=O



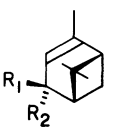
10a: R_1=H, R_2=OH
11a: R_1=OH, R_2=H
22a: R_1,R_2=O



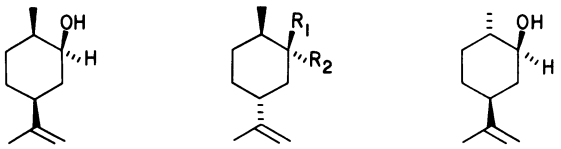
10b: R_1=OH, R_2=H
11b: R_1=H, R_2=OH
22b: R_1,R_2=O



12a: R_1=OH, R_2=H
18a: R_1,R_2=O



12b: R_1=OH, R_2=H
18b: R_1,R_2=O



19 20: R_1=OH, R_2=H 21: R_1,R_2=O 25

24a 24b

Results and Discussion

The same tobacco callus tissues induced from the stem of *N. tabacum* "Bright Yellow" as those used in our previous works were also used in this investigation. The callus tissues, just prior to use, were transplanted to the freshly prepared Murashige and Skoog's medium[18] containing 2 ppm of 2,4-dichlorophenoxyacetic acid as auxin and 3% of sucrose and then were grown with continuous shaking for 2—3 weeks at 25 °C in the dark. To these cultured cells, the filter-sterilized monoterpenoid was administered, and then the cultures were incubated at 25 °C for 7—10 d in a shaker. At a regular time interval, a part of the incubation mixture (suspension of cells and medium) was withdrawn with a pipette under sterile conditions and extracted with ether. Each ether extract, after removal of the solvent, was subjected to the GLC analysis to study the time-courses in the biotransformation of the enantiomeric pairs of the monoterpenoids.

Enantioselectivity in the Oxidation of the Secondary Alcohols. Enantioselectivity in the oxidation of monoterpenoids having a secondary hydroxyl group was examined with the enantiomeric pairs of 2- and 3-hydroxylated *p*-menthane derivatives, 2-hydroxylated bicyclo[2.2.1]heptane derivatives, and 3- and 4-hydroxylated bicyclo[3.1.1]heptane derivatives, such as (1*R*,2*R*,4*R*)-(−)- and (1*S*,2*S*,4*S*)-(+)-carvomenthols (**1a** and **1b**), (1*R*,2*S*,4*R*)-(+)- and (1*S*,2*R*,4*S*)-(−)-neocarvomenthols (**2a** and **2b**), (1*R*,3*R*,4*S*)-(−)- and (1*S*,3*S*,4*R*)-(+)-menthols (**3a** and **3b**), (1*R*,3*S*,4*S*)-(+)- and (1*S*,3*R*,4*R*)-(−)-neomenthols (**4a** and **4b**), (1*R*,2*S*,4*R*)-(+)- and (1*S*,2*R*,4*S*)-(−)-borneols (**5a** and **5b**), (1*R*,2*R*,4*R*)-(−)- and (1*S*,2*S*,4*S*)-(+)-isoborneols (**6a** and **6b**), (1*R*,2*R*,3*R*,5*S*)-(−)- and (1*S*,2*S*,3*S*,5*R*)-(+)-

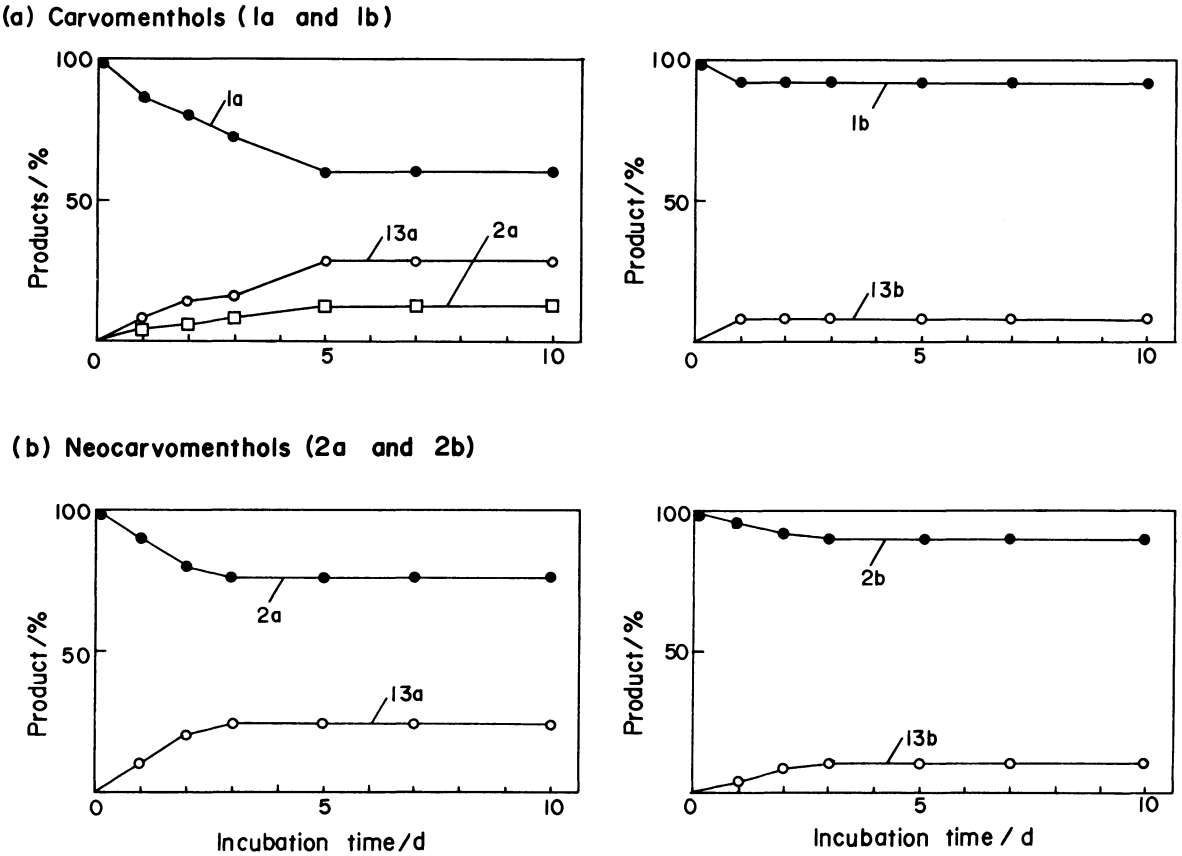


Fig. 1. The time-courses in the biotransformation of the enantiomeric pairs of (a) carvomenthols (**1a** and **1b**) and (b) neocarvomenthols (**2a** and **2b**).

isopinocampheols (**7a** and **7b**), (1*R*,2*R*,3*S*,5*S*)-(+)- and (1*S*,2*S*,3*R*,5*R*)-(−)-neoisopinocampheols (**8a** and **8b**) and (1*R*,2*S*,4*R*,5*R*)-(+)- and (1*S*,2*R*,4*S*,5*S*)-(−)-neoisoverbanols (**9a** and **9b**).

As shown in Fig. 1, the *p*-menthan-2-ols, **1a** and **2a**, were converted to (1*R*,4*R*)-(+)-carvomenthone (**13a**), whereas their enantiomers, **1b** and **2b**, were scarcely converted to the corresponding ketone **13b**. The biotransformation of **1a** gave, in addition to **13a**, (+)-neocarvomenthol (**2a**) as a minor product; the formation of **2a** may be caused by further reductive conversion of (+)-carvomenthone (**13a**). In the biotransformation of enantiomeric pairs of the *p*-menthan-3-ols, on the other hand, not only (1*R*,3*R*,4*S*)-(−)-menthol (**3a**) and (1*R*,3*S*,4*S*)-(+)-neomenthol (**4a**), but also their enantiomers, **3b** and **4b**, were scarcely converted to their corresponding ketones, **14a** and **14b**.

Figure 2 shows the time-courses in the biotransformation of the bicyclo[2.2.1]heptan-2-ols, such as borneols (**5a** and **5b**) and isoborneols (**6a** and **6b**). (1*R*,2*S*,4*R*)-(+)-Borneol (**5a**) and (1*R*,2*R*,4*R*)-(−)-isoborneol (**6a**) were quantitatively converted to (1*R*,4*R*)-(+)-camphor (**15a**), whereas the conversion of their enantiomers, **5b** and **6b**, to (1*S*,4*R*)-(−)-camphor (**15b**) occurred to a slight extent. Such an enantioselective transformation was further confirmed by feeding experiments of the racemic substrates of borneol (**5**) and isoborneol (**6**). After incubation of the racemic substrates with the cultured cells, the product and the unchanged substrate in the incubation mixture were separated from each other and then their optical

Table 1. Enantioselective Transformation of (±)-Borneol (**5**) and (±)-Isoborneol (**6**) into (+)-Camphor (**15a**) with the Cultured Cells of *N. tabacum*

Substrate used	**15a**			**5b** and **6b** recovered		
	$[\alpha]_D^{25}$/°	(*c*, solv.)	O.p.[a]	$[\alpha]_D^{25}$/°	(*c*, solv.)	O.p.[a]
(±)-Borneol (**5**)	+40.7	(1.4, EtOH)	92.1	−36.4	(0.6, EtOH)	95.5
(±)-Isoborneol (**6**)	+40.1	(1.0, EtOH)	90.7	+32.5	(2.0, EtOH)	94.8

a) O.p. denotes the optical purity (e. e. %), which was calculated on the basis of the specific rotation cited in the literature.[28]

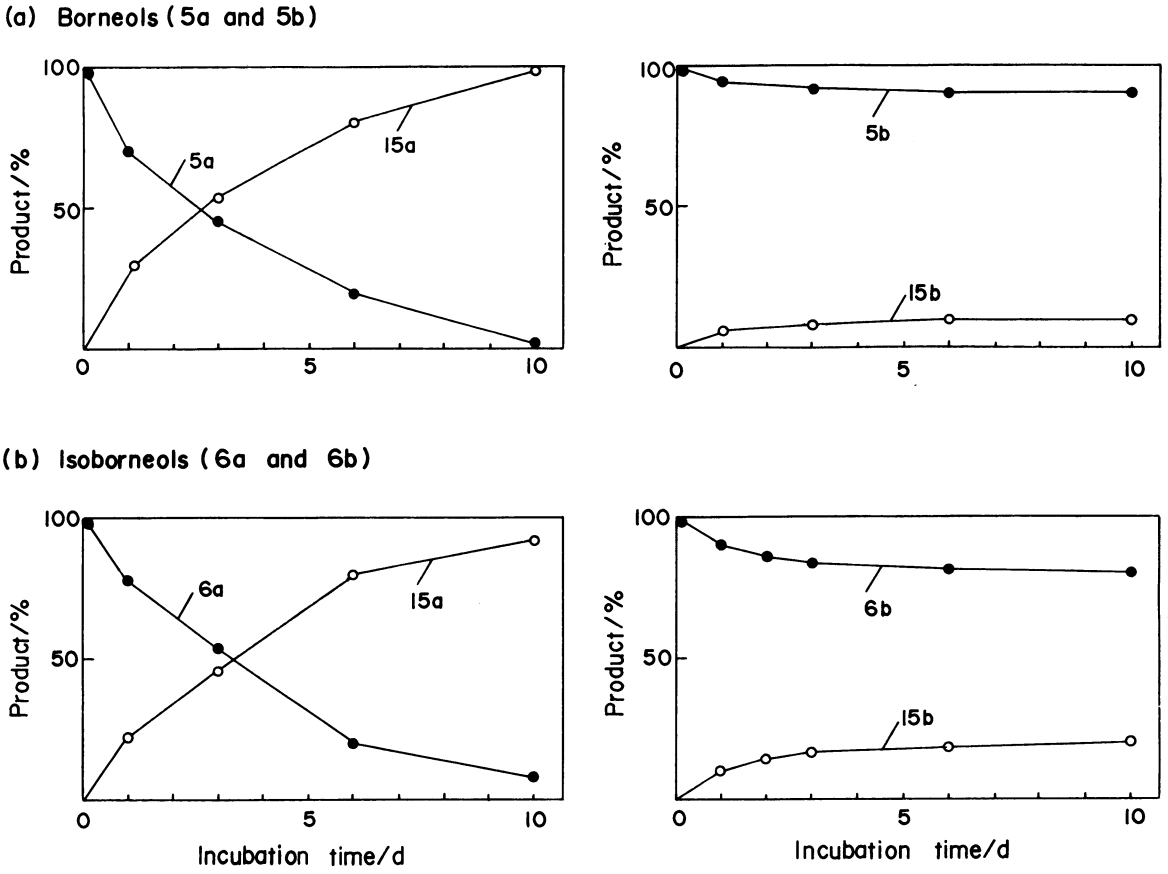


Fig. 2. The time-courses in the biotransformation of the enantiomeric pairs of (a) borneols (**5a** and **5b**) and (b) isoborneols (**6a** and **6b**).

purities were determined. The data given in Table 1 indicates that one enantiomer in the racemate was selectively susceptible to the oxidation with the cultured cells; (+)-borneol (**5a**) and (−)-isoborneol (**6a**) quantitatively transformed into (+)-camphor (**15a**), but their enantiomers remain unchanged.

The time-courses in the biotransformation of bicyclo[3.1.1]heptan-3-ols and -4-ols are shown in Figs. 3 and 4. (1*S*,2*S*,3*S*,5*R*)-(+)-isopinocampheol (**7b**) and (1*S*,2*S*,3*R*,5*R*)-(−)-neoisopinocampheol (**8b**) were quantitatively converted to (1*S*,2*S*,5*R*)-(−)-isopinocamphone (**16b**). However, their enantiomer, **7a** and **8a**, was hardly converted to their corresponding ketone. On the other hand, both enantiomers, **9a** and **9b**, of neoisoverbanol were quantitatively converted to their corresponding ketones **17a** and **17b**, respectively (Fig. 4).

Enantioselectivity in the Oxidation of the Allylic Alcohols. Enantioselectivity in the oxidation of monoterpenoids having an allylic hydroxyl group was

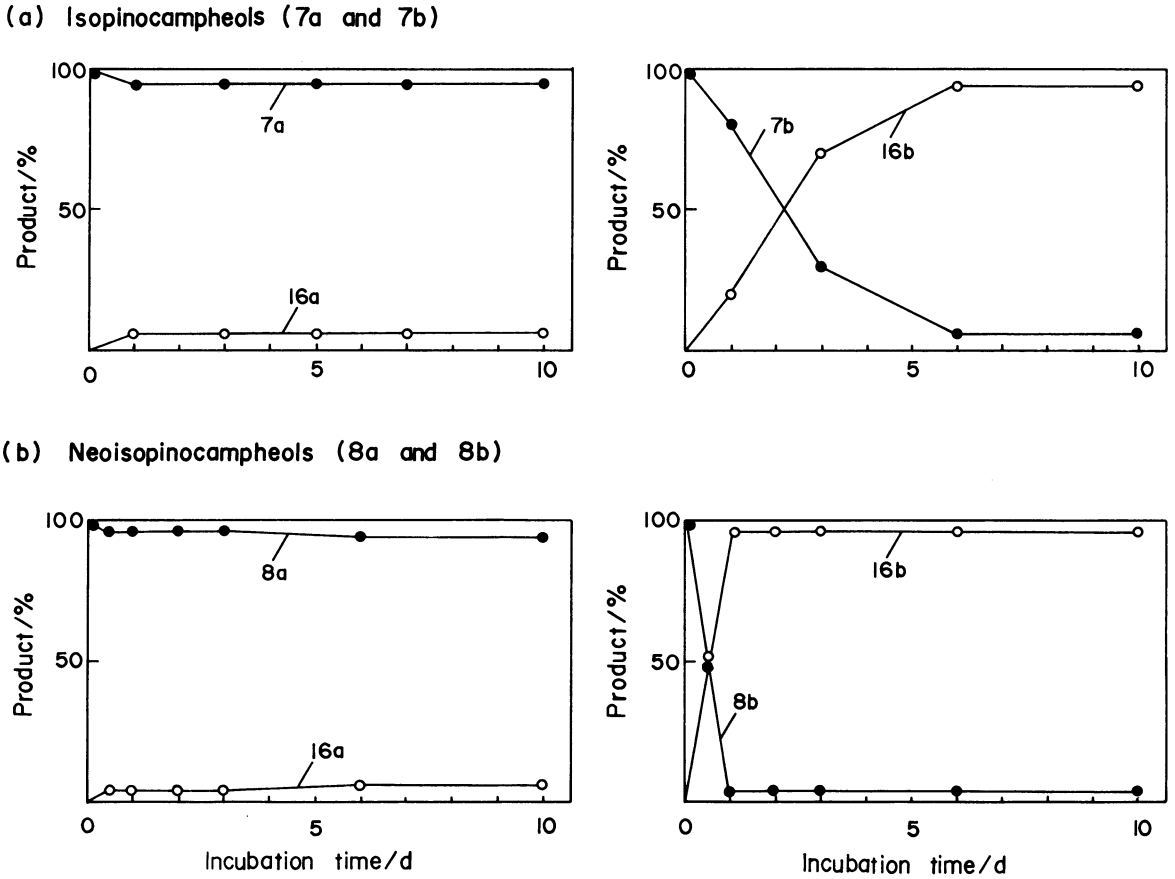


Fig. 3. The time-courses in the biotransformation of the enantiomeric pairs of (a) isopinocampheols (**7a** and **7b**) and (b) neoisopinocampheols (**8a** and **8b**).

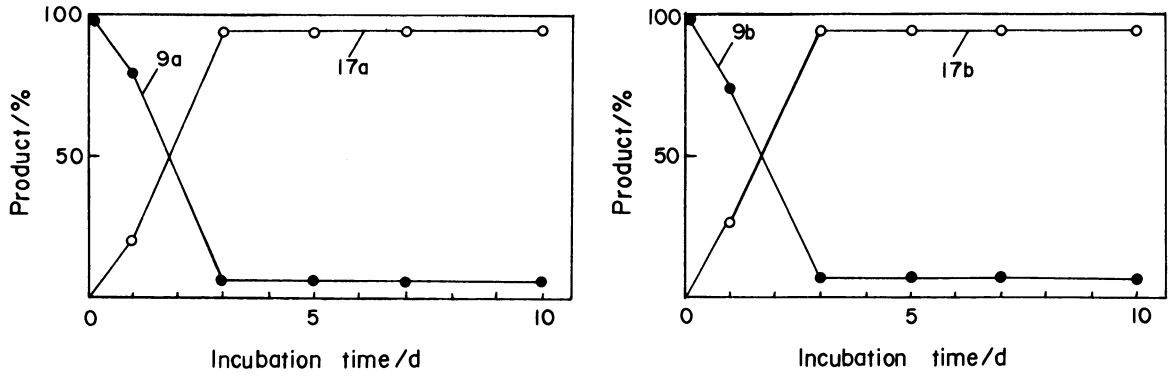


Fig. 4. The time-courses in the biotransformation of the enantiomeric pairs of neoisoverbanols (**9a** and **9b**).

examined with the enantiomeric pairs of 2-hydroxylated *p*-menthene derivatives and 4-hydroxylated bicyclo[3.1.1]heptene derivatives, such as (2*R*,4*R*)-(−)- and (2*S*,4*S*)-(+)-*cis*-carveols (**10a** and **10b**), (2*S*,4*R*)-(−)- and (2*R*,4*S*)-(+)-*trans*-carveols (**11a** and **11b**), and (1*R*,4*R*,5*R*)-(+)- and (1*S*,4*S*,5*S*)-(−)-*cis*-verbenols (**12a** and **12b**). Figure 5 shows the time-courses in the biotransformation of enantiomeric pairs of *cis*-carveol (**10a** and **10b**) and *trans*-carveol (**11a** and **11b**). The (2*S*,4*S*)-*cis*- and (2*S*,4*R*)-*trans*-carveols (**10b** and **11a**) were converted to the corresponding ketones **22b** and **22a** to a large extent, respectively, whereas the conversion of their enantiomers **10a** and **11b** to their corresponding ketones **22a** and **22b** occurred to a slight extent. These facts indicate that the cultured cells preferentially oxidize the allylic alcohols having the chirality of *S* at C-2 and this oxidation is independent of the chirality at C-4. In the biotransformation of *cis*-carveol (**10b**) a remarkable increase in the formation of neoisodihydrocarveol (**19**) after 3 d incubation was observed. However, it is not clear whether this formation was caused by the

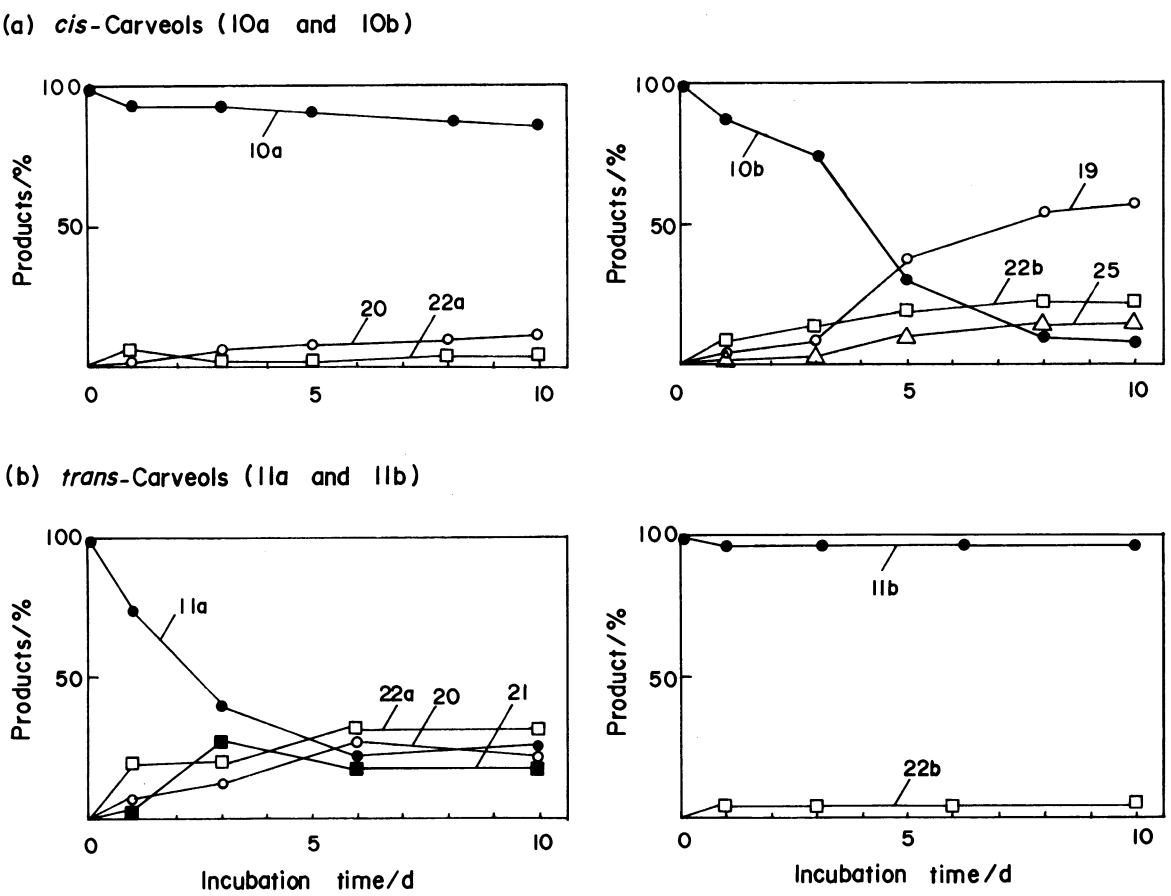


Fig. 5. The time-courses in the biotransformation of the enantiomeric pairs of (a) *cis*-carveols (**10a** and **10b**) and (b) *trans*-carveols (**11a** and **11b**).

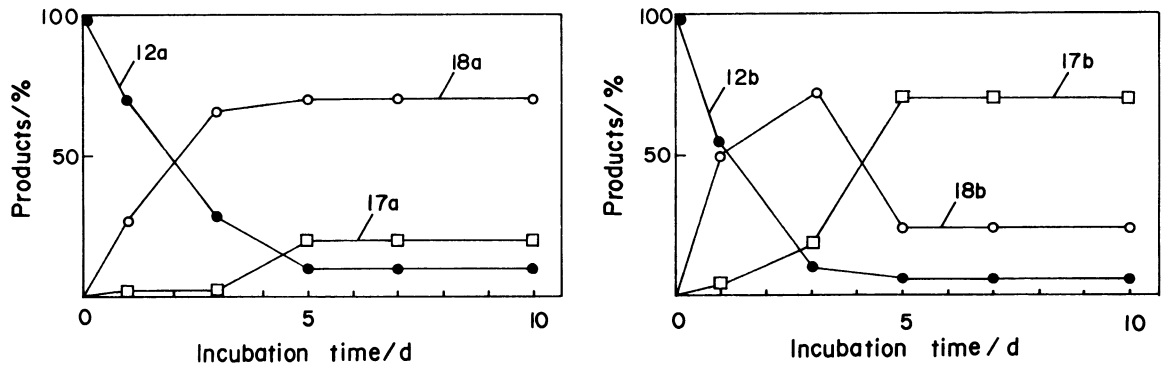


Fig. 6. The time-courses in the biotransformation of the enantiomeric pairs of *cis*-verbenols (**12a** and **12b**).

reduction of carvone (**22b**) produced or by the direct reduction of the C–C double bond of *cis*-carveol (**10b**). The time-courses in the biotransformation of enantiomeric pairs of *cis*-verbenol (**12a** and **12b**) are shown in Fig. 6. Both enantiomers were quantitatively transformed to their corresponding ketones **18a** and **18b** respectively, after 3 day's incubation. In the biotransformation of **12b**, the product **18b** was formed in high yield in the early stage of the incubation, but **18b** decreased after 3 d with a gradual increase in the formation of (1*S*,2*R*,5*S*)-*cis*-verbanone (**17b**). Such further transformation occurred to a small extent in the case of **12a**. Accordingly, in the biotransformation of the monoterpenoids having an allylic hydroxyl group, this hydroxyl group is enantioselectively oxidized to the carbonyl group and then the C–C double bond adjacent to the carbonyl group of α,β-unsaturated carbonyl compounds produced is stereoselectively hydrogenated to a saturated carbonyl compounds. However, the C–C double bond in the 1-methylethenyl group is not at all hydrogenated.

Enantioselectivity in the Reduction of the Carbonyl Compounds. Enantioselectivity in the reduction of monoterpenoids having a carbonyl group was examined with the enantiomeric pairs of *p*-menthan-2-one and -3-one derivatives and bicyclo[2.2.1]heptan-2-one and bicyclo[3.1.1]heptan-3-one and -4-one derivatives, such as (1*R*,4*R*)-(+)- and (1*S*,4*S*)-(−)-carvomenthones (**13a** and **13b**), (1*R*,4*S*)-(−)- and (1*S*,4*R*)-(+)-menthones (**14a** and **14b**), (1*R*,4*R*)-(+)- and (1*S*,4*S*)-(−)-camphors (**15a** and **15b**), (1*R*,2*R*,5*S*)-(+)- and (1*S*,2*S*,5*R*)-isopinocamphones (**16a** and **16b**), and (1*R*,2*S*,5*R*)-(+)- and (1*S*,2*R*,5*S*)-(−)-*cis*-verbanones (**17a** and **17b**). The time-courses in the biotransformation of carvomenthones (**13a** and **13b**) and menthones (**14a** and **14b**) are shown in Figs. 7 and 8. (1*R*,4*R*)-(+)-Carvomenthone (**13a**) was quantitatively converted to (1*R*,2*S*,4*R*)-(+)-neocarvomenthol (**2a**), whereas its (1*S*,4*S*)-(−)-enantiomer **13b** was converted to (1*S*,2*S*,4*S*)-(+)-carvomenthol (**1b**) and (1*S*,2*R*,4*S*)-(−)-neocarvomenthol (**2b**) in a ratio of 2:1. The preferential formation of (+)-neocarvomenthol (**2a**) and (+)-carvomenthol (**1b**) indicates that the carvomenthones (**13a** and **13b**) are stereospecifically reduced to the hydroxy compounds with the chirarity of *S* at the C-2. The extent of the stereoselectivity in the reduction of the enantiomers was different each other; the selectivity was very high for the reduction of (1*R*,4*R*)-carvomenthone (**13a**), but low for that of the enantiomer **13b**. On the other hand, the biotransformation of (1*R*,4*S*)-(−)- and (1*S*,4*R*)-(+)-menthones (**14a** and **14b**) gave (1*R*,4*R*)- and (1*S*,4*S*)-4-hydroxy-*p*-menthan-3-ones (**23a** and **23b**), respectively,[19] in addition to isomenthones (**24a** and **24b**) and menthols (**3b**, **4a**, and **4b**) (Fig. 8). The time-course experiments show that the reduction of the carbonyl group of

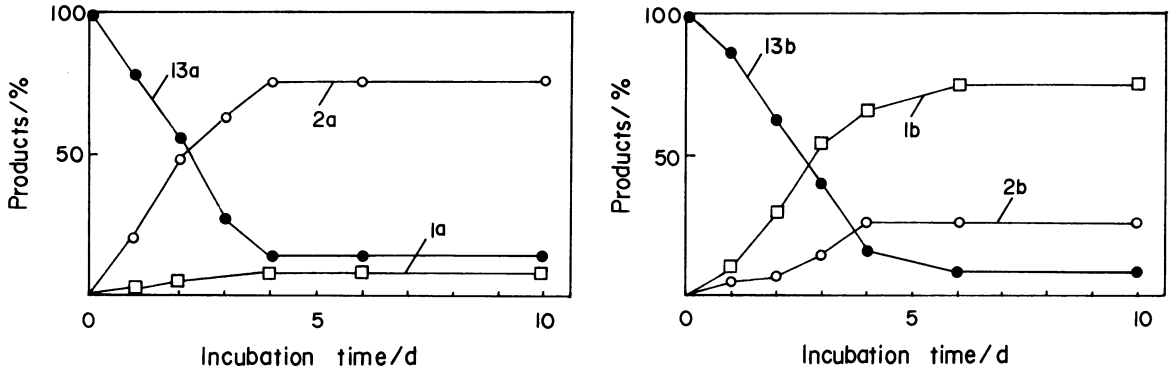


Fig. 7. The time-courses in the biotransformation of the enantiomeric pairs of carvomenthones (**13a** and **13b**).

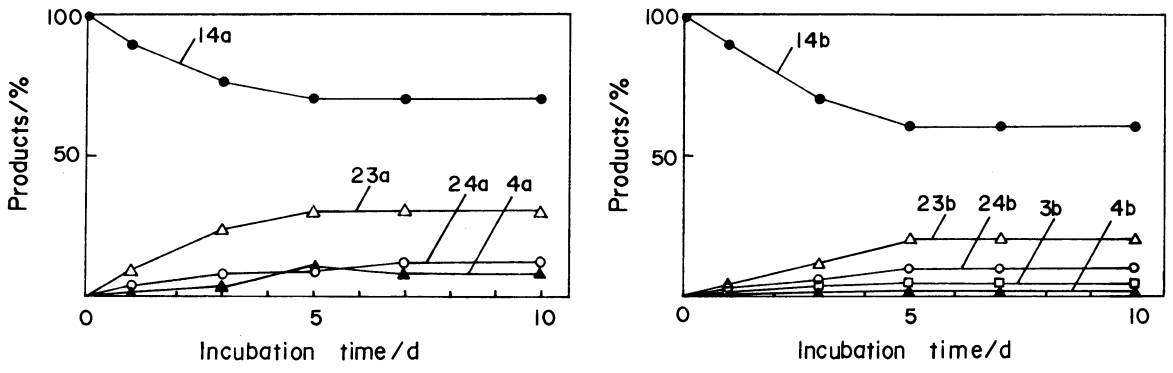


Fig. 8. The time-courses in the biotransformation of the enantiomeric pairs of menthones (**14a** and **14b**).

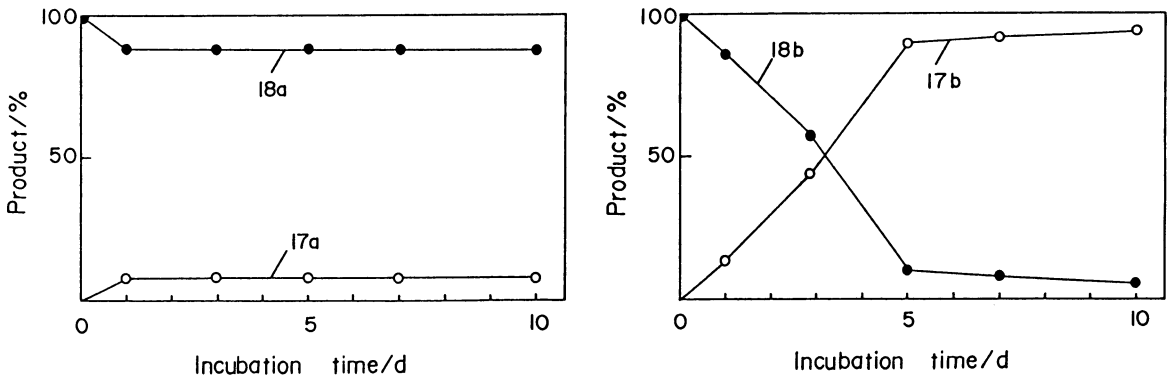


Fig. 9. The time-courses in the biotransformation of the enantiomeric pairs of verbenones (**18a** and **18b**).

menthones (**14a** and **14b**) occurred to a slight extent. This low conversion, as compared with the case of *p*-menthan-2-ones, **1a** and **1b**, may be caused by the steric hindrance owing to the 1-methyl ethyl group adjacent to the carbonyl group. On the other hand, (1*R*,4*R*)-(+)- and (1*S*,4*S*)-(−)-camphors (**15a** and **15b**), (1*R*,2*R*,5*S*)-(+)- and (1*S*,2*S*,5*R*)-(−)-isopinocamphones (**16a** and **16b**), and (1*R*,2*S*,5*R*)-(+)- and (1*S*,2*R*,5*S*)-(−)-*cis*-verbanones (**17a** and **17b**) were scarcely converted to their corresponding alcohols. These results may be explained by assuming that the balance of the equilibrium between the ketones and the corresponding alcohols in the oxidoreduction in the cultured cells is predicted to lie toward the side of the ketones.[10]

Enantioselectivity in the Reduction of the C–C Double Bond of α,β-Unsaturated Carbonyl Compounds. Enantioselectivity in the reduction of the C–C double bond of monoterpenoids having an α,β-unsaturated carbonyl group was examined with the enantiomeric pairs of bicyclo[3.1.1]hept-2-en-4-ones, such as (1*R*,5*R*)-(+)- and (1*S*,5*S*)-(−)-verbenones (**18a** and **18b**). Figure 9 shows the time-courses in the biotransformation of (1*R*,5*R*)-(+)-verbenone (**18a**) and its enantiomer **18b**. The (1*S*,5*S*)-enantiomer **18b** was quantitatively converted to (1*S*,2*R*,5*S*)-(−)-*cis*-verbanone (**17b**) after 10 d incubation, whereas the conversion of (1*R*,5*R*)-enantiomer **18a** to the corresponding ketone **17a** scarecely occurred. These facts indicate that the cultured cells discriminate the enantiomers and reduce the C–C double bond of the (1*S*,5*S*)-enantiomer **18b**. In addition, the hydrogenation of **18b** with the cultured cells gave only *cis*-verbanone (**17b**), but not its trans-isomer. This indicates that the hydrogen attack to the C–C double bond of **18b** stereospecifically occurs from the *re*-face at C-2 of **18b**. No further conversion of the *cis*-verbanone (**17b**) into neoisoverbanol (**9b**) was observed. This may be explained by assuming that the balance of the equilibrium between **17b** and its corresponding alcohol **9b** in the oxidoreduction in the cultured cells is predicted to lie toward the side of the *cis*-verbanone (**17b**), because the equilibrium constant in the oxidoreduction of **9b**⇄**17b** is estimated to be about 1.4 on the basis of the ^{13}C NMR chemical shift (δ 214.1) of the carbonyl carbon of **17b**.[10]

Concluding Remarks. The enantioselectivity in the oxidative and reductive transformation of the enantiomeric pairs of *p*-menthane and bicyclo[2.2.1] and bicyclo[3.1.1]heptane derivatives with the cultured cells of *N. tabacum* was established as follows. (i) The cultured cells discriminated the enantiomers of *p*-menthan-2-ols, bicyclo[2.2.1]heptan-2-ols, and bicyclo[3.1.1]heptan-3-ols, and enantioselectively oxidized these secondary alcohols, but this was not the case for the *p*-menthan-3-ols and bicyclo[3.1.1]heptan-4-ols. (ii) In the case of allylic alcohols, also, the cultured cells discriminated the enantiomers of 6-*p*-menthen-2-ols, and enantioselectively oxidized these allylic alcohols, but this was not the case for the bicyclo[3.1.1]hept-2-en-4-ol. (iii) The cultured cells discriminated the enantiomers of *p*-menthan-2-ones in their reductive conversion to the corresponding hydroxy compounds, but this was not the case for the *p*-menthan-3-ones. The hydrogen attack in the reduction took place preferentially from the *re*-face of the carbonyl group to give the alcohols with the chirality of *S* at the position bearing the hydroxyl group. (iv) The cultured cells discriminated the enantiomers of bicyclo[3.1.1]hept-2-en-4-one, and enantioselectively reduced its C–C double bond. The hydrogen attack in the hydrogenation took place stereospecifically from the *re*-face of the C–C double bond.

Thus, the main finding in the oxidative and reductive transformation is that the enantioselective transformation takes place for the only substrate having the methyl group on the vicinal position of the functional group.

Experimental

Analytical and preparative TLC were carried out on 0.25-mm and 0.5-mm thick silica-gel plates (Merck silica gel 60,

GF_{254}), respectively. GLC analyses were performed on an instrument equipped with FID and a glass column (3 mm×2 m) packed with 15% DEGS, 5% PEG-20M, and 2% OV-17 on Chromosorb W (AW-DMCS; 80—100 mesh) at 100, 120, and 90—200 °C (3 °C min^{-1}), respectively. ^1H NMR spectra were obtained at 60 and 90 MHz in $CDCl_3$ with TMS as an internal standared. GC-MS spectra were recorded on a mass spectrometer equipped with a gas chromatograph with 15% DEGS column (3 mm×2 m) by EI mode at 70 ev. The areas of the peaks on the gas liquid chromatogram were determined by using a Shimadzu C-R1B Chromatopac recording data processor for chromatography.

Substrates. (i) Monoterpene Alcohols. (1*R*,2*R*,4*R*)-(−)-Carvomenthol (**1a**) was prepared from (−)-dihydrocarveols[20] by hydrogenation in the presence of 10% Pd-C. The hydrogenation of (+)-neodihydrocarveol[20] in the presence of 10% Pd-C gave (1*R*,2*S*,4*R*)-(+)-neocarvomenthol (**2a**). (1*R*,3*R*,4*S*)-(−)-Menthol (**3a**) and its enantiomer **3b** were commercial materials of Aldrich Chem. Company. (1*R*,3*S*,4*S*)-(+)-Neomenthol (**4a**) was prepared from (−)-menthone[33] by reduction with $NaBH_4$. The reduction of (+)-camphor[28] with $LiAlH_4$ gave (1*R*,2*S*,4*R*)-(+)-borneol (**5a**) and (1*R*,2*R*,4*R*)-(−)-isoborneol (**6a**). (1*R*,2*R*,3*R*,5*S*)-(−)-Isopinocampheol (**7a**) was prepared by the hydroboration-oxidation of (+)-α-pinene.[21] The isopinocampheol (**7a**) was oxidized by pyridinium dichromate[23] to yield (+)-isopinocamphone,[21] which was reduced by $NaBH_4$ to give (1*R*,2*R*,3*S*,5*S*)-(+)-neoisopinocampheol (**8a**). (1*R*,2*S*,4*R*,5*R*)-(+)-Neoisoverbanol (**9a**) was prepared from (+)-*cis*-verbanone[34] by reduction with $LiAlH_4$. (2*R*,4*R*)-(−)-*cis*-Carveol (**10a**) and (2*S*,4*R*)-(−)-*trans*-carveol (**11a**) were prepared by the Meerwein-Pondorf reduction of (−)-carvone.[22] The reduction of (−)-verbenone[31] with $NaBH_4$ in MeOH at 0 °C gave (1*R*,4*R*,5*R*)-(−)-*cis*-verbenol (**12a**). On the other hand, the enantiomers of the alcohols described above were prepared from the corresponding compounds in the same manner as above.

(ii) Monoterpene Ketones. (1*R*,4*R*)-(+)-Carvomenthone (**13a**) was prepared from (+)-dihydrocarvone[20] by hydrogenation in the presence of 10% Pd-C. (1*R*,4*S*)-(−)-Menthone (**14a**) was prepared from (−)-menthol[26] by oxidation with pyridinium dichromate.[23] (1*R*,4*R*)-(+)-Camphor (**15a**) was prepared from (+)-borneol[28] by pyridinium dichromate oxidation.[23] Oxidation of (+)-neoisopinocampheol[21] with $Na_2Cr_2O_7$ gave (1*R*,2*R*,5*S*)-(+)-isopinocamphone (**16a**). (1*R*,2*S*,5*R*)-(+)-*cis*-Verbanone (**17a**) was prepared from (+)-verbenone[31] by hydrogenation in the presence of 10% Pd-C. (1*R*,5*R*)-(+)-Verbenone (**18a**) was prepared from (−)-α-pinene by oxidation with *t*-butyl chromate.[24] The enantiomers of the ketones described

Table 2. Physical Properties of the Substrates

Compound	Mp θ_m/°C	n_D^{25}	$[\alpha]_D^{25}$/°
1a		1.4628	−21.0 (*c* 1.0, MeOH) (lit,[25] −22.0)
1b		1.4624	+21.7 (*c* 1.8, MeOH)
2a		1.4647	+40.3 (*c* 1.5, MeOH) (lit,[25] +43.7)
2b		1.4643	−40.6 (*c* 2.0, MeOH)
3a			−49.3 (*c* 2.0, EtOH) (lit.,[26] −49.6)
3b			+48.7 (*c* 1.5, EtOH)
4a		1.4601	+20.1 (*c* 1.0, EtOH) (lit,[27] +19.6)
4b		1.4600	−20.7 (*c* 2.3, EtOH)
5a	201—202		+37.6 (*c* 1.0, EtOH) (lit,[28] +37.9)
5b			−37.1 (*c* 1.0, EtOH)
6a	210—211		−33.6 (*c* 1.0, EtOH) (lit,[28] −34.3)
6b			+34.1 (*c* 2.0, EtOH)
7a	55—57		−31.7 (*c* 0.8, C_6H_6) (lit,[21] −32.8)
7b			+30.1 (*c* 1.6, C_6H_6)
8a	45—47		+34.5 (*c* 1.3, C_6H_6) (lit,[21] +36.0)
8b			−33.8 (*c* 2.0, C_6H_6)
9a			+5.2 (*c* 1.3, C_6H_6) (lit,[29] +5.3)
9b			−5.0 (*c* 1.5, C_6H_6)
10a		1.4925	−21.5 (*c* 0.75, $CHCl_3$)
10b		1.4932	+22.1 (*c* 1.9, $CHCl_3$) (lit,[30] +23.9)
11a		1.4930	−207.0 (*c* 1.0, $CHCl_3$)
11b		1.4938	+210.2 (*c* 2.0, $CHCl_3$) (lit,[30] +213.1)
12a			+8.7 (*c* 1.0, $CHCl_3$) (lit,[31] +9.3)
12b			−8.3 (*c* 2.1, $CHCl_3$)
13a		1.4546	+5.9 (*c* 1.0, EtOH)
13b		1.4542	−5.3 (*c* 2.3, EtOH) (lit,[32] −6.0)
14a		1.4503	−27.3 (*c* 1.0, EtOH) (lit,[33] −29.9)
14b		1.4505	+28.0 (*c* 1.5, EtOH)
15a	174—175		+42.9 (*c* 1.1, EtOH) (lit,[28] +44.2)
15b			−42.6 (*c* 1.5, EtOH)
16a		1.4741	+11.2 (*c* 1.5, EtOH) (lit,[21] +10.5)
16b		1.4747	−10.0 (*c* 2.0, EtOH)
17a		1.4773	+55.3 (*c* 1.7, $CHCl_3$) (lit,[34] +52.5)
17b		1.4775	−53.3 (*c* 2.0, $CHCl_3$)
18a		1.4966	+210.5 (*c* 1.5, $CHCl_3$)
18b		1.4968	−209.3 (*c* 2.3, $CHCl_3$) (lit,[31] −208)

Table 3. Physical and Spectral Data of the Products

Compound	$[\alpha]_D^{25}$/°	^{1}H NMR in $CDCl_3$	m/z (rel intensity)
13	+6.4 (*c* 0.5, EtOH)	0.87 (3H, d, *J*=6.0 Hz, 1-Me) 0.97 (6H, 9,10-Me)	154 (M^+, 21), 111 ($M^+-CH(Me)_2$, 100), 83 (27), 69 (24)
15	+44.4 (*c* 1.1, EtOH)	0.86 (s, 8-Me), 0.92 (s, 9-Me) 0.98 (s, 10-Me)	152 (M^+, 40), 137 (M^+-Me, 7), 109 (30), 95 (100)
16	−9.9 (*c* 2.0, EtOH)	0.88 (s, 9-Me), 1.20 (d, *J*=6.0 Hz, 10-Me), 1.31 (s, 8-Me)	152 (M^+, 15), 110 ($M^+-C(Me)_2$, 13), 95 (30), 83 (78), 69 (90)
17	+53.2 (*c* 0.8, $CHCl_3$)	1.07 (3H, d, *J*=6.0 Hz, 7-Me), 1.23 (3H, s, 8-Me) 1.35 (3H, s, 9-Me)	152 (M^+, 10), 137 (M^+-Me, 10), 109 (27), 95 (48), 83 (100)

above were prepared from the corresponding alcohols in the same manner as above. All the samples were >99% pure by GLC, and these physical data are given in Table 2.

Feeding of the Monoterpenes to the Tobacco Cultured Cells. The callus tissues used in this study were induced from the stem of *N. tabacum* "Bright Yellow" and have been maintained for about 15 years.[5] Just prior to use for this work, a part of the callus tissuse as transplanted to freshly prepared Murashige and Skoog's medium[18] (100 cm^3 in a 300 cm^3-conical flask) containing 2 ppm of 2,4-dichlorophenoxyacetic acid and 3% sucrose and grown with continuous shaking for 2—3 weeks at 25 °C in the dark. The substrate (10 mg/flask) was added to the suspension cultures (about 50—70 g cells/flask) and the cultures were incubated at 25 °C for 7—10 d on a rotary shaker (70 rpm) in the dark.

Isolation and Identification of the Products. The incubation mixture was filtered and the mass obtained was triturated with MeOH. The MeOH extract was concentrated and extracted with ether. The culture medium filtered was extracted with ether. The two ether extracts were bulked, since they exhibited the same behavior on TLC and GLC. Transformation products were isolated from the extract by prep. TLC on silica gel (EtOAc–hexane, 1:4) and identified by direct comparison of physical constants, TLC, GLC, and spectral data with those of authentic samples. The physical constants and spectral data of the products are given in Table 3. The minor products were identified by direct comparison of TLC, GLC and/or GC-MS with those of authentic samples.

Time-Course Experiments in the Biotransformation of the Substrates. The substrate (10 mg) was incubated at 25 °C for 10 d with shaking (70 rpm) in the dark. At a regular time interval, a part (10 cm^3) of the incubation mixture was pipetted out under sterile conditions and then extracted with ether. The yields of the products were determined on the basis of the peak area from GLC and are expressed as a relative percentage to the total amount of the whole reaction products extracted. These results are shown in Figs. 1—9 in the text.

The details of the time-course experiments are described below in the case of **1a** as an example. A part of the callus tissues was transplanted to 100 cm^3 of Murashige and Skoog's medium[18] in a 300 cm^3-conical flask and grown with continuous shaking for 2—3 weeks at 25 °C in the dark. The substrate **1a** (10 mg) was administered to the precultured cells (about 70 g) in a 300 cm^3-conical flask and the cultures were incubated at 25 °C in a rotary shaker (70 rpm) in the dark. At a regular time interval, a part of the incubation mixture (10 cm^3) was pipetted out under sterile conditions and extracted with ether. The ether extract was made up to 0.2 cm^3 and 0.002 cm^3 of the ether solution was subjected to GLC with a 15% DEGS column at 120 °C by use of a microsyringe. The yields of the products were determined on the basis of the peak area from the GLC and are expressed as a relative percentage to the total amount of the whole reaction products extracted. Identification of the products were performed by comparison (co-GLC and GC-MS) with authentic samples.[25,32] Thus, the time-course in the biotransformation of **1a** as shown in Fig. 1 was obtained.

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