Biotransformation of (±)-Lavandulol by the Plant Pathogenic Fungus *Glomerella cingulata*

Hirokazu Nankai, Mitsuo Miyazawa,* Shin-ichi Akazawa, and Hiromu Kameoka

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka, Osaka 577-8502, Japan

To clarify the structures of biotransformation products and the metabolic pathways, the microbial transformation of (±)-lavandulol has been investigated using the plant pathogenic fungus *Glomerella cingulata*. (±)-Lavandulol [(±)-5-methyl-2-(1-methylethenyl)-4-hexen-1-ol] was mainly oxidized at the C-4 double bond to give (−)-(2*S*,4*S*)-1,5-epoxy-5-methyl-2-(1-methylethenyl)-4-hexanol and *cis*- and *trans*-1,4-epoxy-5-methyl-2-(1-methylethenyl)-5-hexanol. 5-Methyl-2-(1-methylethenyl)-4-hexene-1,6-diol (6-hydroxylavandulol) was also produced through this biotransformation. These structures were confirmed by the mass, IR, ^{1}H NMR, and ^{13}C NMR spectral data.

Keywords: *Biotransformation; microbial transformation; Glomerella cingulata; plant pathogenic fungus; (±)-lavandulol; tetrahydropyran; tetrahydrofuran*

INTRODUCTION

In the course of our work related to investigation of the biotransformation of acyclic terpenoids by *Glomerella cingulata*, we have examined the conversion of some acyclic terpenoids, *cis*-nerolidol, nerylacetone, *trans*-nerolidol, geranylacetone, (2*E*,6*E*)-farnesol, citronellol, citronellal, tetrahydrogeraniol, tetrahydrolavandulol, and (2*Z*,6*Z*)-farnesol (Miyazawa et al., 1995a, 1996a−c; Nankai et al., 1996, 1997, 1998). In these conversions, it has been observed that the main reaction is the regioselective oxidation at the isopropyridene or isopropyl moiety located in the remote position from the oxygenated functional group. We have also reported the enantioselective formation of (−)-(2*S*,4*S*)-1,5-epoxy-5-methyl-2-(1-methylethenyl)-4-hexanol (**2**) from (±)-lavandulol [5-methyl-2-(1-methylethenyl)-4-hexen-1-ol] (**1**) by microbial transformation using *G. cingulata* (Miyazawa et al., 1997a). Compound **2** has a tetrahydropyran ring. We have taken an interest in the enantioselective formation of the tetrahydropyran ring, because the cyclic compounds have not been obtained in our previous research of biotransformations of acyclic terpenoids. In addition, compound **2** has been obtained as a novel compound. Compound **1**, an acyclic monoterpene alcohol, occurs in lavender oil (Ford et al., 1992). Artificial **1** (racemic form) is available inexpensively. Its acetate, which has a better odor than **1**, is widely used in fragrances (Opdyke, 1978). To investigate the biotransformation of compound **1**, we may produce new bioactive compounds. However, there is no report on the biotransformation of **1** using other microorganisms, plant cell cultures, or mammals. Accordingly, the biotransformation of **1** by *G. cingulata* was investigated to further clarify the products and their formation style.

In a 6-day incubation of **1** with *G. cingulata*, four major metabolites [including **2** (Miyazawa et al., 1997a)] were detected. We have now succeeded in elucidating the structure of the other three metabolites. This paper describes the time course and the structures of metabolites on the biotransformation of **1** by *G. cingulata*.

* Author to whom correspondence should be addressed (fax +81-6-727-4301; e-mail miyazawa@apch.kindai.ac.jp).

MATERIALS AND METHODS

General Procedure. Compound **1** was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Thin-layer chromatography (TLC) was performed on precoated plates [silica gel 60 F_{254}, 0.25 mm, Merck (Darmstadt, Germany)]. Solvent systems were hexane/diethyl ether [1:1, v/v; solvent system 1 (S1)] and hexane/ethyl acetate [1:1, v/v; solvent system 2 (S2)]. Compounds were visualized by spraying plates with 1% vanillin in 96% sulfuric acid followed by brief heating. Gas chromatography (GC) was performed on an HP 5890 Series II Plus gas chromatograph equipped with a flame ionization detector (FID). The column was a fused silica capillary column [DB-5, 30 m × 0.25 mm i.d., film thickness 1.0 μm (J&W Scientific, Folsom, CA)]. Chromatographic conditions were as follows: column temperature, raised from 80 to 260 °C at 4 °C min^{-1}; injector temperature, 270 °C; detector temperature, 280 °C; carrier gas, He at 1.8 mL min^{-1}. Yields of individual constituents were determined by peak areas as measured by an HP 3396 Series II integrator. FAB-MS was obtained on a JEOL JMS-HX 100 mass spectrometer, and the matrix was glycerin. EI-MS measurements were obtained using gas chromatography/mass spectrometry (GC/MS). GC/MS was performed on an HP 5972A mass selective detector interfaced with an HP 5890 Series II Plus gas chromatograph fitted with a column [HP-5MS, 30 m × 0.25 mm i.d., film thickness 0.25 μm]. Chromatographic conditions were the same as described above. Infrared spectra (IR) were determined with a Perkin-Elmer 1760-x IR Fourier transform spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GSX 270 NMR spectrometer (^{1}H NMR, 270.05 MHz; ^{13}C NMR: 67.80 MHz). Tetramethylsilane (TMS) was used as the internal standard (δ 0.00) for ^{1}H NMR spectra measured in $CDCl_3$. Residual $CHCl_3$ was used as internal reference (δ 77.00) for ^{13}C NMR spectra measured in $CDCl_3$. Multiplicities were determined by DEPT pulse sequence.

Preculture of *G. cingulata*. *G. cingulata* was precultured according to a procedure described in previous papers (Miyazawa et al., 1996a; Nankai et al., 1997).

Time Course Experiment. Precultured *G. cingulata* (1 mL) was transferred into a 200 mL Erlenmeyer flask contain-

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Table 1. ^{1}H NMR Spectral Data[a] of Compounds 1−5

H	1	2	3 and 4	5
1	3.50 dd (8, 10.5)	3.37 t (11.5)	3.63 m	3.52 dd (8, 11)
1′	3.57 dd (5.5, 10.5)	3.65 ddd (2, 4.5, 11.5)	3.81 m	3.58 dd (5.5, 11)
2	1.96−2.34 m	2.33 m	2.75−2.30 m	2.04−2.37 m
3	1.96−2.34 m	1.56 ddd (11.5, 11.5, 12.5)	1.81 m	2.04−2.37 m
3′	1.96−2.34 m	1.91 dddd (2, 3.5, 4.5, 12.5)	1.95 m	2.04−2.37 m
4	5.08 m	3.50 dd (4.5,11.5)	4.02 m	5.38 t (7)
6	1.61 s	1.17 s	1.14 s	3.99 s
7	1.70 m	1.29 s	1.24 s	1.67 s
9	4.82 m	4.73 m	4.77 m	4.82 m
9′	4.93 m	4.81 m	4.77 m	4.93 m
10	5.09 m	1.73 s	1.76/1.74 s	1.71 s

[a] Chemical shifts in ppm; coupling constants in Hz.

Table 2. ^{13}C NMR Spectral Data[a] of Compounds 1−5

C	1	2	3 and 4	5
1	63.6 (CH_2)	64.5 (CH_2)	72.4/72.1 (CH_2)	63.6 (CH_2)
2	49.9 (CH)	43.7 (CH)	46.2/46.9 (CH)	49.6 (CH)
3	28.4 (CH_2)	33.3 (CH_2)	31.2/31.7 (CH_2)	27.8 (CH_2)
4	122.0 (CH)	74.1 (CH)	85.5/86.5 (CH)	123.5 (CH)
5	132.8 (C)	74.8 (C)	71.6/71.3 (C)	136.0 (C)
6	25.7 (Me)	27.8 (Me)	21.3/20.8 (Me)	68.7 (CH_2)
7	17.8 (Me)	15.8 (Me)	24.4/24.3 (Me)	13.8 (Me)
8	145.4 (C)	144.7 (C)	145.0/144.1 (C)	145.0 (C)
9	113.1 (CH_2)	110.5 (CH_2)	110.1/110.5 (CH_2)	111.3 (CH_2)
10	19.5 (Me)	21.4 (Me)	27.0/27.3 (Me)	19.5 (Me)

[a] Chemical shifts in ppm.

ing 100 mL of medium (same medium used in preculture) and stirred (∼100 rpm) for 3 days. After the growth of *G. cingulata*, compound **1** (50 mg) was added to the medium and cultivated for 7 more days. Every day 5 mL of the culture medium was removed, saturated with NaCl, and extracted with EtOAc, and the solvent was then evaporated. The crude extract was analyzed by TLC, GC, and GC/MS.

Biotransformation of (±)-Lavandulol (1) for 6 Days. Precultured *G. cingulata* (∼10 mL) was transplanted into a stirred fermentor (3.5 L) containing 3 L of medium. Cultivation was carried out at 27 °C and stirring for 3 days under aeration (∼200 mL min^{-1}). After the growth of *G. cingulata*, **1** (1.5 g) was added into the medium and cultivated for 6 more days.

Isolation of Metabolites. After the fermentation, culture medium and mycelia were separated by filtration. The medium was saturated with NaCl and extracted with CH_2Cl_2. The mycelia were also extracted with CH_2Cl_2. Both CH_2Cl_2 extracts were mixed, and then the solvent was evaporated to give crude extract (1.2 g). The extract was suspended in CH_2Cl_2 and partitioned between CH_2Cl_2 and 5% aqueous $NaHCO_3$. The solvent of the CH_2Cl_2 layer was evaporated to give the neutral part (860 mg). The water layer was acidified to pH 2 with HCl, saturated with NaCl, and extracted with CH_2Cl_2. The solvent (CH_2Cl_2) of the 5% aqueous $NaHCO_3$ soluble part was evaporated to give the acidic part (90 mg). The neutral part was chromatographed on Si-60 columns with a hexanes/EtOAc gradient (19:1 to 1:1); substrate and four metabolites were isolated and purified [**1** (151 mg) (Rf = 0.4, S1; Rf = 0.54, S2); **2** (100 mg) (Rf = 0.26, S1); mixture of **3** and **4** (300 mg) (Rf = 0.28, S1); **5** (95 mg) (Rf = 0.16, S2)].

Compound 2: colorless oil; FAB-MS (positive) *m/z* 171 (MH^+); EI-MS see Figure 2a; IR ν_{max} 3461, 2978, 1646, 1455, 1375, 1153, 1095, 1054, 895, 831 cm^{-1}; ^{1}H NMR see Table 1; ^{13}C NMR see Table 2.

Mixture of 3 and 4: colorless oil; FAB-MS (positive) *m/z* 171 (MH^+); EI-MS see Figure 2b,c; IR ν_{max} 3431, 3083, 2976, 1647, 1458, 1377, 1237, 1163, 1073, 1019, 961, 942, 891, 856 cm^{-1}; ^{1}H NMR see Table 1; ^{13}C NMR see Table 2.

Compound 5: colorless oil; $[\alpha]_D$ −8.7° ($CHCl_3$; *c* 0.95); FAB-MS (positive) *m/z* 171 (MH^+); EI-MS see Figure 2d; IR ν_{max} 3350, 2926, 1646, 1441, 1013, 891 cm^{-1}; ^{1}H NMR see Table 1; ^{13}C NMR see Table 2.

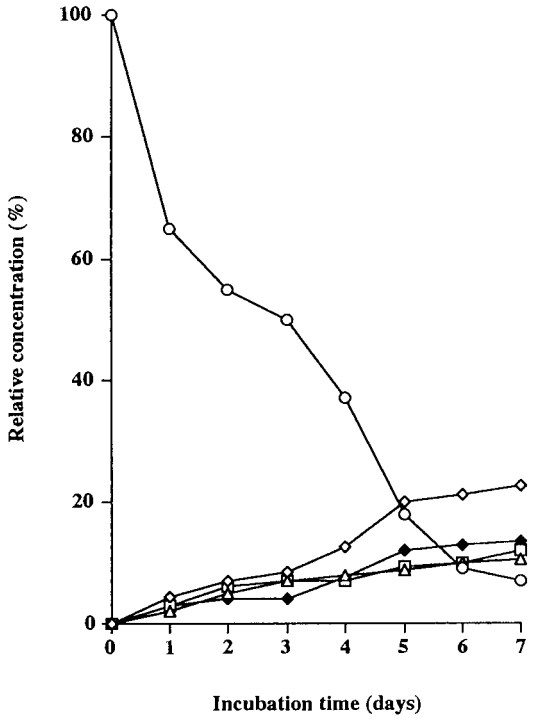


Figure 1. Time course of the biotransformation of **1** by *G. cingulata*: (○) (±)-lavandulol (**1**); (□) (−)-(2*S*,4*S*)-1,5-epoxy-5-methyl-2-(1-methylethenyl)-4-hexanol (**2**); (◇, ◆) *cis*- and *trans*-1,4-epoxy-5-methyl-2-(1-methylethenyl)-5-hexanol (**3** and **4**); (△) 5-methyl-2-(1-methylethenyl)-4-hexene-1,6-diol (**5**).

Preparation of (±)-2. Compound **1** (4.2 g) was dissolved in chloroform (210 mL) and cooled on ice. *m*-Chloroperoxobenzoic acid (4.3 g) was added and stirred at 0 °C. After 3 h, saturated $Na_2S_2O_3$ (aq, 150 mL) and saturated $NaHCO_3$ (aq, 150 mL) were added. Extraction was performed with dichloromethane (200 mL). The dichloromethane layer was washed with saturated NaCl (aq), dried with Na_2SO_4, and evaporated in vacuo. To the extract was added a little 5% HCl (aq). Five percent $NaHCO_3$ (aq) was added to adjust the pH value of the solution to ∼7. Extraction with dichloromethane gave a crude extract (2.8 g). The crude extract was chromatographed on Si-60 columns with a hexanes/EtOAc gradient (19:1 to 1:1) to give compound (±)-**2** (350 mg).

Biotransformation of (±)-2. This experiment was carried out in the same manner described under Time Course Experiment.

RESULTS

To investigate the capacity of *G. cingulata* to metabolize **1**, the time course experiment was carried out as described under Materials and Methods. Four major metabolites and many minor metabolites were detected by TLC, GC, and GC/MS analyses. These metabolites were not detected by TLC or GC analyses of the culture of *G. cingulata* to which no substrate (**1**) was added, nor were they produced in a mixture of **1** and the medium that was stirred for 7 days. It was demonstrated, therefore, that *G. cingulata* transformed **1** into various metabolites. The time course of relative concentration changes of **1** and metabolites was monitored by TLC and quantitatively measured by GC analysis (Figure 1). The starting substrate **1** was ∼90% consumed after 7 days. Metabolites **2**−**5** increased to ∼10−25% after 7 days. In addition, many minor metabolites were detected.

To isolate metabolites **2**−**5**, incubation of **1** (1.5 g) using *G. cingulata* was carried out for 6 days. After the biotransformation, the culture was extracted as de-

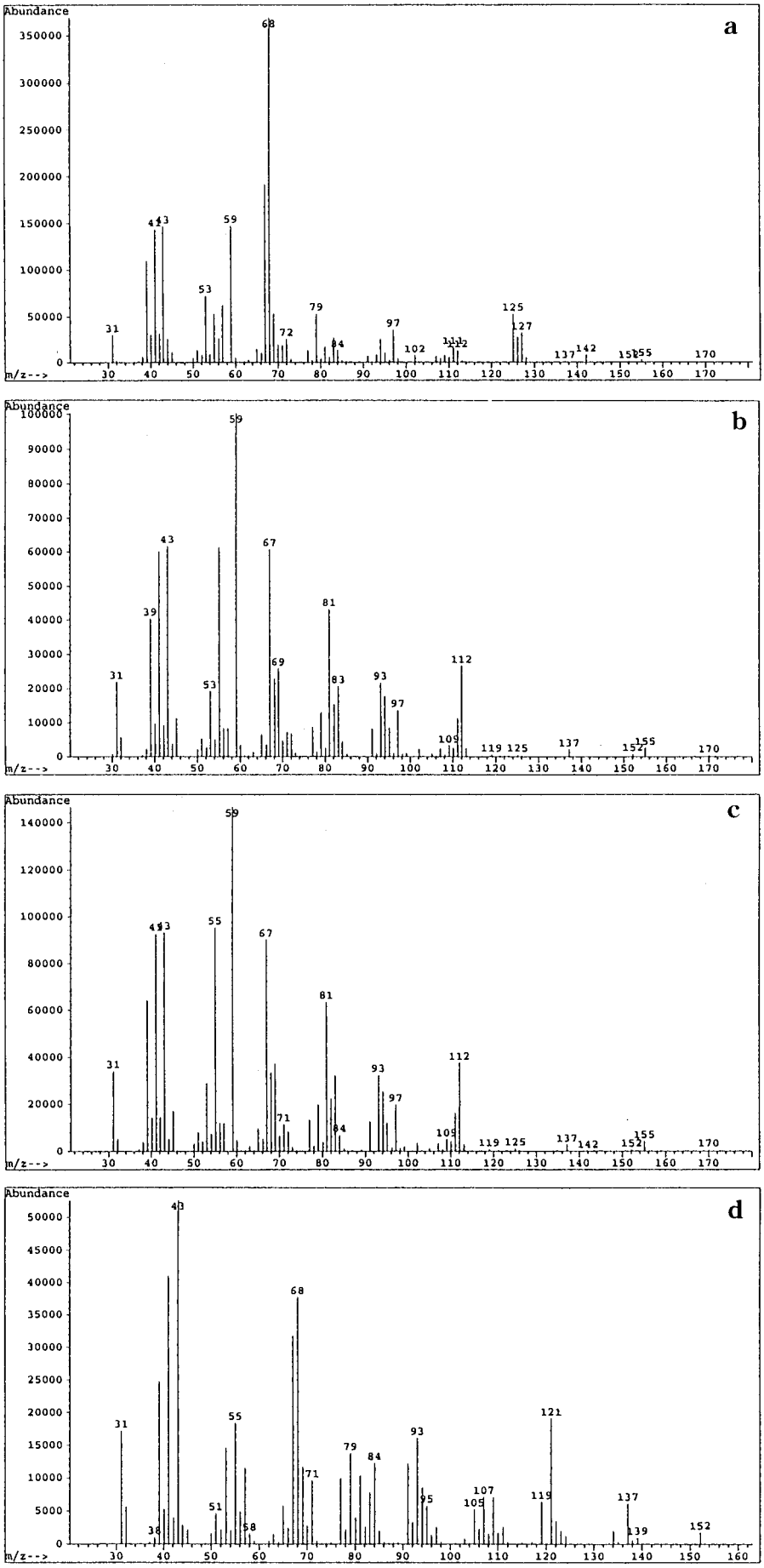
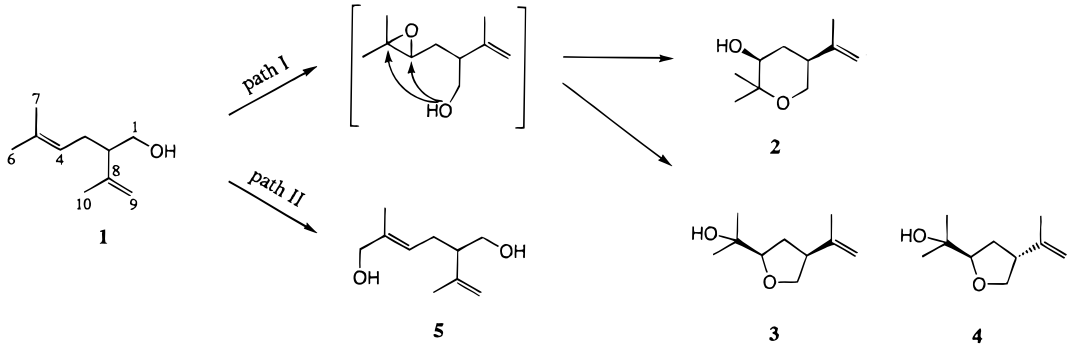


Figure 2. EI-MS of compounds **2**−**5**: (a) compound **2**; (b) forward peak on GC of mixture of **3** and **4**; (c) backward peak on GC of mixture of **3** and **4**; (d) compound **5**.

Scheme 1. Possible Metabolic Pathways of 1 by *G. cingulata*



scribed under Materials and Methods and metabolites **2**, **5**, and a mixture of **3** and **4** were obtained from the dichloromethane extract using a column chromatography. The structures of **2**−**5** were determined by spectral data.

The structure of compound **2** has been reported as (−)-(2*S*,4*S*)-1,5-epoxy-5-methyl-2-(1-methylethenyl)-4-hexanol.

Compounds **3** and **4** had a molecular formula of $C_{10}H_{18}O_2$ based on their GC-EIMS. ^{1}H and ^{13}C NMR signals and the IR spectrum indicated compounds **3** and **4** had a tertiary hydroxyl group [δ_C 71.6/71.3 (C); ν_{max} 3431, 1163 cm^{-1}], an ether linkage [δ_H 3.63, 3.81 and 4.02; δ_C 85.5/86.5 (CH), 72.4/72.1 (CH_2)], and an isopropenyl group but no trisubstituted double bond. These spectral data indicated that the structures of compounds **3** and **4** were elucidated to be *cis*- and *trans*-1,4-epoxy-5-methyl-2-(1-methylethenyl)-5-hexanol.

GC-EIMS indicated compound **5** had a molecular formula of $C_{10}H_{18}O_2$. ^{1}H and ^{13}C NMR signals and the IR spectrum showed **5** had two primary hydroxyl groups [δ_H 3.99; δ_C 68.7 (CH_2) and δ_H 3.52 and 3.58; δ_C 63.6 (CH_2)], an isopropenyl group, and a trisubstituted double bond. From the spectral data, compound **5** was elucidated to be 5-methyl-2-(1-methylethenyl)-4-hexene-1,6-diol (6-hydroxylavandulol).

DISCUSSION

On this biotransformation, two main metabolic pathways were observed (Scheme 1): (I) regioselective epoxidation at the double bond distant from the hydroxyl group (the remote double bond) and subsequent attack of the lone pair of the hydroxyl group to the C-4 or C-5 position, although the intermediate epoxide could not be detected; (II) regioselective hydroxylation of the methyl group (C-6 position).

Similarly to our previous results (Miyazawa et al., 1995a, 1996a−c; Nankai et al., 1996, 1997, 1998), oxidation at the remote double bond (path I) is the main metabolic pathway. Such cyclization compound was obtained on the microbial transformation of linalool (Abraham et al., 1990; Bock et al., 1986; Madyastha et al., 1977; Rapp and Mandery, 1988). It was reported that linalool was transformed to linalool oxide pyranoid and furanoid (cyclized product) via 7,8-epoxylinalool (Abraham et al., 1990; Bock et al., 1986).

Regarding the ether ring formation, furanoids (**3** and **4**) were obtained as mixtures of the cis- and trans-forms, while pyranoid (**2**) was obtained only as the cis-form. In addition, only the (−)-(2*S*,4*S*)-form of *cis*-pyranoid (**2**) was produced on this biotransformation. We recently reported the enantioselective esterification of four compounds having a tetrahydropyran ring, (±)-*cis*-(**6**) and (±)-*trans*-linalool-oxide pyranoid (**7**) (Miyazawa et al., 1995b), (±)-2,6,6-trimethyl-7-oxabicyclo[3.1.1]octan-2-ol (**8**) (Miyazawa et al., 1997b), and 1,6,6-trimethyl-7-oxabicyclo[3.1.1]octan-2-ol (**9**) (Miyazawa et al., 1997b). (*R*)-Alcohols of **6**−**9** were esterified with malonic acid enantioselectively by *G. cingulata*. Therefore, two pathways were considered on the formation of **2**; (A) after formation of (±)-**2**, (+)-**2** was esterified with malonic acid enantioselectively and (−)-**2** remained; (B) stereospecific epoxidation at the remote double bond and subsequent attack of the lone pair of the hydroxyl group to the C-5 position. If malonate of (+)-**2** was produced, it could be detected by GC/MS. Malonate of **2**, however, could not be detected in this study. To clarify this pathway, we also examined the biotransformation of (±)-**2** by *G. cingulata*. (±)-**2** was prepared from (±)-**1** by oxidation using *m*-chloroperoxobenzoic acid. (±)-**2**, however, was little transformed. Thus, the formation of (−)-**2** would be considered by way of pathway B.

On the biotransformation of **1** by *G. cingulata*, hydroxylation of the methyl group (C-6) also proceeded (metabolic pathway II). This hydroxylation of the methyl group located in the remote position from the oxygenated functional group had not been observed on the other biotransformations of acyclic terpenoids by *G. cingulata* (Miyazawa et al., 1995a, 1996a−c; Nankai et al., 1996, 1997, 1998). This difference may be explained by the influence of the distance between the methyl group and the oxygenated functional group.

Work is now in progress to clarify the metabolic pathway of **1** to **2** and to produce pyranoids or furanoids selectively.

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