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BIOTRANSFORMATION OF (−)-α-BISABOLOL BY PLANT PATHOGENIC FUNGUS, *GLOMERELLA CINGULATA*

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Key Word Index—*Glomerella cingulata*; biotransformation; plant pathogenic fungus; (−)-α-bisabolol; (1*S*,3*R*,4*R*,7*S*)-3,4-dihydroxy-α-bisabolol; bisabolol oxide B; (1*S*,3*R*,4*R*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B; (1*S*,3*S*,4*S*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B.

Abstract—Microbial transformation of (−)-α-bisabolol by *Glomerella cingulata* has been investigated. (−)-α-Bisabolol was initially transformed to (1*S*,3*R*,4*R*,7*S*)-3,4-dihydroxy-α-bisabolol and bisabolol oxide B. (1*S*,3*R*,4*R*,7*S*)-3,4-Dihydroxy-α-bisabolol was further transformed to (1*S*,3*R*,4*R*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B. Bisabolol oxide B was also further transformed to (1*S*,3*R*,4*R*,7*S*,10*S*)- and (1*S*,3*S*,4*S*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B. The structures of the metabolic products were determined by spectroscopic methods. Metabolic pathways of the biotransformation of (−)-α-bisabolol by *G. cingulata* are also discussed.

INTRODUCTION

Many kind of terpenoids are known to have biological activity. We have been interested in the biotransformation of terpenoids using plant pathogenic fungi to produce new biologically active terpenoids. In our previous papers, we have reported the biotransformation of 1,8-cineole [1], (−)-α-bisabolol (**1**) [2, 3], (+)-cedrol [4] and (−)-globulol [5] to novel terpenoids by the plant pathogenic fungus, *Glomerella cingulata*.

(−)-α-Bisabolol (**1**), a major component of chamomile oil, is valued for its anti-inflammatory properties [6]. We have reported the biotransformation of **1** by *Aspergillus niger* to bisabolol oxide B (**3**) [7], (−)-α-tetrahydrobisabolene-2,5,6-triol [8] and 3,4-dihydroxy bisabolol oxide B [9]. On the biotransformation of **1** by *G. cingulata*, (1*S*,3*R*,4*R*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B (**4**) [2] and (1*S*,3*S*,4*S*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B (**5**) [3] are formed as the main products after seven days incubation. We have now succeeded in isolating two other minor metabolites, (1*S*,3*R*,4*R*,7*S*)-3,4-dihydroxy-α-bisabolol (**2**) and bisabolol oxide B (**3**). In the present paper we report the detailed structural determination of these metabolites, and show the time-course and metabolic pathways of the microbial transformation of **1** by *G. cingulata*.

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RESULTS AND DISCUSSION

A small amount of (−)-α-bisabolol (**1**) was incubated with *Glomerella cingulata* for seven days. One major compound (**4**) and some minor compounds were detected by TLC and GC analysis. These compounds were not detected on TLC analysis of the culture of *G. cingulata* to which no substrate was fed, or of a mixture of **1** with the medium being stirred for seven days. During this biotransformation, the time-course of the relative abundance of substrate **1** and its metabolites was observed by TLC, and measured quantitatively by GC (Fig. 1). As shown in Fig. 1, substrate **1** was transformed, and completely disappeared within four days; metabolites **2** and **3** decreased after three days, and **3** completely disappeared after five days; **2** and **3** were further transformed to **4** and **5**, and **4** was produced as a major metabolite, accounting for about 80%(GC) of **1** at six days.

In order to isolate these metabolites, large-scale incubation of **1** with *G. cingulata* was conducted for seven days. After the biotransformation, the culture was extracted as described in the Experimental. Metabolites **4** and **5** were isolated from the CH_2Cl_2 extract.

The structures of the metabolites were determined by spectral data. The major product, **4**, has a molecular formula $C_{15}H_{28}O_4$ based on FAB-mass spectrometry (m/z 273, $[MH]^+$). Its ^1H and ^{13}C NMR spectra indicated the presence of a secondary hydroxyl group (δ_H 3.61; δ_C 73.8) and four methyl groups (δ_H 1.12, 1.13, 1.22,

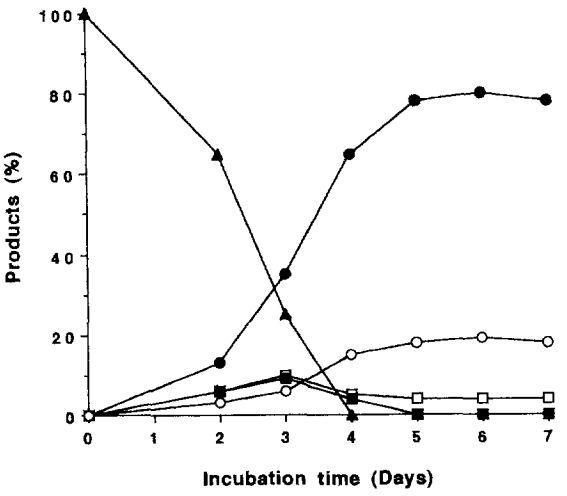

Fig. 1. Time-course in the biotransformation of **1** by *Glomerella cingulata*. ▲ = (−)-α-bisabolol (**1**); □ = (1*S*,3*R*,4*R*,7*S*)-3,4-dihydroxy-α-bisabolol (**2**); ■ = bisabolol oxide B (**3**); ● = (1*S*,3*R*,4*R*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B (**4**); ○ = (1*S*,3*S*,4*S*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B (**5**).

1.28; δ_C 22.4, 24.2, 27.3, 27.6) and no double bond. Comparison of spectral data between **4** and the metabolites of **1** by *A. niger* [7–9] indicate that **4** is 3,4-dihydroxy-bisabolol oxide B. The absolute configuration at C-3 was revealed by Horeau's method [10]. In the case of **4**, C-3 is in the *R*-configuration, because dextrorotatory acid ($[\alpha]_D^{20}$ + 9.41°) was obtained. The orientation of H-3 was shown to be equatorial from the $W_{h/2}$ of the H-3 signal of the ^{1}H NMR. Comparison with the known ^{13}C NMR data (*p*-menthane-1,2,8-triols [11] and 4-*tert*-butyl-1-methylcyclohexan-1,2-diols [12]) reveal that 4-Me exists in an equatorial position, and 3,4-diol in a 1,2-diaxial configuration. So, C-4 is in the *R*-configuration. C-10 was revealed to be *S*-configuration by comparison with the ^{13}C NMR data of bisabolol oxide B and epi-bisabolol oxide B [13]. Thus, **4** was determined to be (1*S*,3*R*,4*R*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B.

Product **5** has a molecular formula $C_{15}H_{28}O_4$ based on FAB-mass spectrometry (*m/z* 273, $[MH]^+$). Its ^{1}H and ^{13}C NMR spectra indicated the presence of a secondary hydroxyl group (δ_H 3.53; δ_C 76.9) and four methyl groups (δ_H 1.12, 1.12, 1.17, 1.20; δ_C 22.3, 24.3, 27.0, 18.8) and no double bond. The comparison of spectral data between **5** and **4** indicate that **5** is a stereoisomer of **4**. The absolute configuration at C-3 was revealed by Horeau's method [10]. In the case of **5**, C-3 is in the *S*-configuration, because a laevorotatory acid ($[\alpha]_D^{20}$ − 14.18°) was obtained. The orientation of H-3 was determined to be axial from the $W_{h/2}$ of the H-3 signal of the ^{1}H NMR. The ^{13}C NMR chemical shift of the 4-Me group of **5** is in a higher magnetic field than that of **4**. This is dependent on a *γ-gauche* effect of the equatorial γ-OH [11, 12]. So, 4-Me exists in the axial position. Consequently, the 3,4-diol is in the 1,2-diequatorial configuration, i.e. C-4 is in the *S*-configuration. C-10 was revealed to be in the *S*-configuration by comparison with the ^{13}C NMR data of bisabolol oxide B and epi-bisabolol oxide B [13]. Thus, **5** was determined to be (1*S*,3*S*,4*S*,7*S*,10*S*)-3,4-dihydroxy-bisabolol oxide B.

In order to confirm the structures of **2** and **3**, a large-scale incubation of **1** with *G. cingulata* was conducted for three days. After biotransformation, the culture was extracted as described in the experimental. Compounds **1**–**4** were isolated from the CH_2Cl_2 extract.

Product **2** has a molecular formula $C_{15}H_{28}O_3$ based on FAB-mass spectrometry (*m/z* 255, $[M - H]^-$). Its ^{1}H and ^{13}C NMR signals indicate the presence of a secondary hydroxyl group (δ_H 3.62, *br s*; δ_C 73.9) and only one trisubstituted double bond [δ_H 5.12, *m*; δ_C 124.5 (CH) and 131.8 (C)] bearing two methyl groups (δ_H 1.62 and 1.68, *s*; δ_C 17.7 and 25.7). Comparison of the NMR data of **2** with those of **1**, **4** and **5** suggested that the double bond in the C-10(11) position remained; on the other hand, the double bond in the C-3(4) position was oxidized to *trans vic*-diol by *G. cingulata*. The ^{13}C NMR data of **2**, on comparison with those of **4** and **5**, indicate that the stereochemistry at C-3 and C-4 were 3*R* and 4*R*. From the above data it was concluded that the structure of **2** is (1*S*,3*R*,4*R*,7*S*)-3,4-dihydroxy-α-bisabolol. The metabolite **3** was identified to be bisabolol oxide B by comparison with spectral data of bisabolol oxide B [7, 13].

The metabolic pathways in the biotransformation of **1** by *G. cingulata* (Fig. 2) were derived from the time-course (Fig. 1) and the structure of the metabolites. The major metabolite **4** was formed via two pathways (**1** → **2** → **4** and **1** → **3** → **4**), and **5** was also formed via two pathways [**1** → **3** → **5** and **1** → **6** → **5** (**6** was not detected in this experiment)]. The first step (**1** → **2**, **3** or **6**) involves oxidation of one double bond of **1**, and the second step (**2** → **4**, **3** → **4** or **5**, and **6** → **5**) involves oxidation of the remaining double bond. In order to clarify the second step, small amounts of **2** and **3** respectively, were incubated with *G. cingulata*, for two days. In the biotransformation of **2**, compound **4** was detected by TLC and GC-mass spectrometry. In the biotransformation of **3**, compounds **4** and **5** were detected by TLC and GC-mass spectrometry. So, the second step was proved, as described above. The oxidation of double bonds in this system probably proceeded by way of the epoxidation of the double bond, although the intermediate epoxide could not be isolated. That is to say, 3,4-diols of **2**, **4**, **5** and **6** could be formed by hydrolysis of the 3,4-epoxide. In this hydrolysis, the 3*R*,4*R*-form (**2** and **4**) was formed more than the 3*S*,4*S*-form (**5** and **6**) by *G. cingulata*. The 7,10-epoxide of **3**–**5** was formed by nucleophilic attack of 7-OH on the 10,11-epoxide, and the 10*S* form was only produced by *G. cingulata*. In this bioconversion, oxidation of the endocyclic double bond proceeded more rapidly than that of the exocyclic double bond, because more **2** was obtained than **3**, and **3** disappeared after five days. From the above result, the major metabolic pathway is **1** → **2** → **4** shown by bold arrows in Fig. 2.

EXPERIMENTAL

Pre-culture of Glomerella cingulata. Spores of *Glomerella cingulata* which had been preserved at low temp., were inoculated into sterilized culture medium

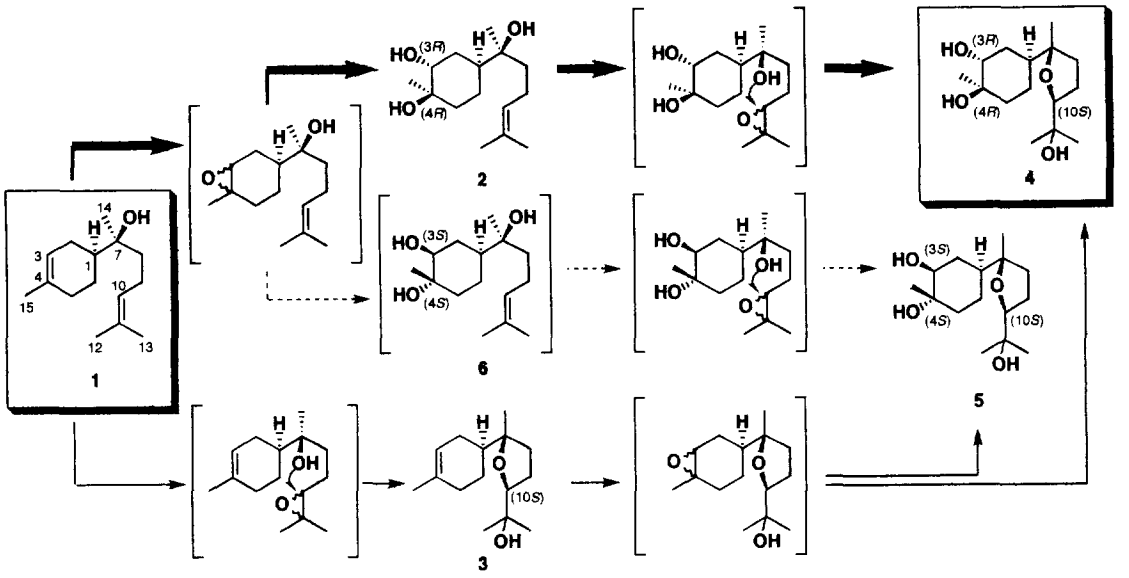

Fig. 2. Metabolic pathway of **1** by *G. cingulata*.

(1.5% saccharose, 1.5% glucose, 0.5% polypeptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.1% K_2HPO_4 and 0.001% $FeSO_4 \cdot 7H_2O$ in distilled water, pH 7.2), and the flask shaken at 27° for 3 days.

Time-course experiment. Pre-cultured *G. cingulata* were transplanted into a 100 ml conical flask containing 50 ml medium, and were stirred for 3 days. After growth of *G. cingulata*, **1** (100 mg) was added to the medium and incubated for 7 more days. Every day, 5 ml of the culture medium was removed and acidified to pH 2 with 1M HCl and satd with NaCl. Then, the culture medium was extracted with Et_2O. The extract was analysed by GC and TLC. The ratio between the substrate (**1**) and metabolic products was determined on the basis of the peak area of GC and is shown in Fig. 1.

Biotransformation of (−)-α-bisabolol (**1**) *for 7 days.* Pre-cultured *G. cingulata* was transplanted into a 1 l stirred fermentor containing 750 ml medium, and this was incubated at 27° with stirring for 3 days under aeration. Compound **1** (1.44 g) was then added to the medium and incubation continued for 7 more days.

Isolation of metabolites. After the fermentation, culture medium and mycelia were sepd by filtration. The medium was acidified to pH 2 with 1M HCl and satd with NaCl, and extracted with CH_2Cl_2. The mycelium was also extracted with CH_2Cl_2. Both CH_2Cl_2 extracts were combined, the solvent evapd and crude extract (1.38 g) was obtained. The extract was sepd into the neutral part (1.08 g) and the acidic part (0.18 g) in the usual manner. The neutral part was chromatographed on Si-60 columns with a hexane–EtOAc gradient (from 19:1 to 1:1), and two metabolites isolated and purified (**4**:406 mg, **5**:95 mg).

Biotransformation of (−)-α-bisabolol (**1**) *for 3 days.* Pre-cultured *G. cingulata* was transplanted into a 1 l stirred fermentor containing 500 ml medium. Cultivation was conducted at 27° with stirring for 3 days under aeration. Compound **1** (1.01 g) was then added to the medium and incubation continued for 3 more days.

Isolation of metabolite. After fermentation, the culture was extracted as described above. From the crude extract (0.95 g), substrate **1** (210 mg) and three metabolites (**2**:82 mg, **3**:75 mg, **4**:220 mg) were isolated.

Biotransformation of **2** *and* **3**. Pre-cultered *G. cingulata* was transplanted into Petri dishes (ϕ 90 × 15 mm) containing 15 ml medium. This was cultured at 27° under static conditions for 3 days. After this time, **2** and **3** (*ca* 10 mg) were added to the Petri dishes, respectively, and these were incubated for 2 more days. After the fermentation, culture medium and mycelia were sepd by filtration. The medium was acidified to pH 2 with 1M HCl and satd with NaCl, and extracted with CH_2Cl_2. The mycelium was also extracted with CH_2Cl_2. Both CH_2Cl_2 extracts were combined, the solvent evapd and crude extract was obtained. The extract was analysed by TLC and GC-MS.

Compound **2**. Crystal; mp 105.5–107.1°; $[\alpha]_D^{20}$ − 23.59° ($CHCl_3$; *c* 1.0); FAB-MS (neg.) *m/z*: 255 $[M - H]^-$; EIMS *m/z* (rel. int.): 238 $[M - H_2O]^+$ (7), 220 (6), 193 (15), 127 (12), 109 (100), 93 (20), 82 (36), 69 (55), 42 (90); IR ν_{max} cm^{-1}: 3400, 2964, 2939, 1376, 1178, 1038, 912; ^1H NMR (500 MHz, $CDCl_3$, TMS as int. standard): δ1.14, 1.25 (each, 3H, *s*, H-14, H-15), 1.62, 1.68 (each 3H, *s*, H-12, H-13), 3.62 (1H, *br s*, $W_{h/2}$ = 6.5 Hz, H-3), 5.12 (1H, *m*, H-10); ^{13}C NMR: see Table 1.

Compound **3**. Oil; $[\alpha]_D^{20}$ − 30.26° ($CHCl_3$; *c* 0.5); EIMS *m/z* (rel. int.): 238 $[M]^+$ (2), 223 (4), 220 (5), 205 (4), 202 (3), 180 (20), 162 (45), 143 (100), 134 (30), 121 (30), 105 (60), 93 (40), 85 (70), 71 (45), 59 (55), 42 (75); IR ν_{max} cm^{-1}: 3450, 2990, 2960, 1470, 1375, 1325, 1320, 1230, 1175, 1156, 1092, 1080, 1055, 1040, 951, 895, 810; ^1H NMR (500 MHz, $CDCl_3$, TMS as int. standard): δ1.12, 1.13 (each, 3H, *s*, H-12, H-13), 1.21 (3H, *s*, H-14), 1.64 (3H, *s*, H-15), 3.78 (1H, *t*, *J* = 6.8 Hz, H-10), 5.37 (1H, *m*, H-3); ^{13}C NMR: see Table 1.

Table 1. ^{13}C NMR spectral data for compounds **1–5** (125.65 MHz, $CDCl_3$, residual $CHCl_3$ used as int. ref., $\delta = 77.00$)

C	1	2	3	4	5
1	42.9 (CH)	39.4 (CH)	43.8 (CH)	39.9 (CH)	46.2 (CH)
2	26.9 (CH_2)	29.7 (CH_2)	26.3 (CH_2)	30.7 (CH_2)	32.8 (CH_2)
3	120.5 (CH)	73.9 (CH)	120.5 (CH)	73.8 (CH)	76.9 (CH)
4	134.0 (C)	70.9 (C)	134.1 (C)	71.0 (C)	74.0 (C)
5	31.0 (CH_2)	33.5 (CH_2)	30.9 (CH_2)	33.4 (CH_2)	38.2 (CH_2)
6	22.0 (CH_2)	21.4 (CH_2)	24.3 (CH_2)	22.3 (CH_2)	24.5 (CH_2)
7	74.2 (C)	74.3 (C)	85.2 (C)	85.0 (C)	84.5 (C)
8	40.1 (CH_2)	39.2 (CH_2)	35.2 (CH_2)	35.1 (CH_2)	35.1 (CH_2)
9	23.3 (CH_2)	22.2 (CH_2)	27.3 (CH_2)	26.3 (CH_2)	26.2 (CH_2)
10	124.6 (CH)	124.5 (CH)	83.9 (CH)	83.9 (CH)	83.9 (CH)
11	131.5 (C)	131.8 (C)	71.5 (C)	71.6 (C)	71.6 (C)
12	17.6 (Me)	17.7 (Me)	22.0 (Me)	22.4 (Me)	22.3 (Me)
13	25.7 (Me)	25.7 (Me)	24.2 (Me)	24.2 (Me)	24.3 (Me)
14	23.3 (Me)	24.3 (Me)	27.4 (Me)	27.3 (Me)	27.0 (Me)
15	23.2 (Me)	27.4 (Me)	23.4 (Me)	27.6 (Me)	18.8 (Me)

Chemical shifts in ppm; multiplicities were determined by the DEPT pulse sequence.

Compound **4**. Crystal; mp 49.2–51.7°; $[\alpha]_D^{20} - 17.53°$ ($CHCl_3$; *c* 1.0); FAB-MS (pos.) *m/z*: 273 $[MH]^+$; EIMS *m/z* (rel. int.): 254 $[M - H_2O]^+$ (0.3), 239 (3), 213 (5), 195 (10), 177 (40), 159 (22), 143 (100), 125 (42), 107 (20), 85 (40), 71 (35), 60 (18), 45 (52); IR ν_{max} cm^{-1}: 3430, 2973, 2942, 2879, 1461, 1375, 1175, 1082, 1034, 908; ^1H NMR (500 MHz, $CDCl_3$, TMS as int. standard): δ1.12, 1.13 (each, 3H, *s*, H-12, H-13), 1.22, 1.28 (each 3H, *s*, H-14, H-15), 3.61 (1H, *br s*, $W_{h/2} = 6.5$ Hz, H-3), 3.77 (1H, *dd*, $J = 7.0$, 7.0 Hz, H-10); ^{13}C NMR: see Table 1.

Compound **5**. Crystal; mp 62.3–63.6°; $[\alpha]_D^{20} - 3.60°$ ($CHCl_3$; *c* 1.0); FAB-MS (pos.) *m/z*: 273 $[MH]^+$; EIMS *m/z* (rel. int.): 239 $[M - H_2O - CH_3]^+$ (3), 221 (3), 213 (5), 195 (8), 177 (25), 159 (18), 143 (100), 125 (28), 107 (16), 85 (42), 71 (52), 60 (42), 45 (90); IR ν_{max} cm^{-1}: 3406, 2971, 2940, 2873, 1467, 1377, 1141, 1065, 946; ^1H NMR (500 MHz, $CDCl_3$, TMS as int. standard): δ1.12 (6H, *s*, H-12, H-13), 1.17, 1.20 (each 3H, *s*, H-14, H-15), 3.53 (1H, *dd*, $J = 4.5$, 11.5 Hz, $W_{h/2} = 16$ Hz, H-3), 3.77 (1H, *dd*, $J = 7.0$, 7.0 Hz, H-10); ^{13}C NMR: see Table 1.

Horeau's method [10]. Treatment of a pyridine solution of **4** (20 mg) with excess of racemic 2-phenylbutanoic anhydride (48 mg), followed by hydrolysis of the anhydride at the end of the reaction, extraction of the resulting acid with 5% aq. $NaHCO_3$ and acidification of the basic extract, gives a dextrorotatory acid (16 mg) ($[\alpha]_D^{20} + 9.41°$). Compound **5** (10 mg) was treated in the same manner, and a laevorotatory acid (5 mg) ($[\alpha]_D^{20} - 14.18°$) was obtained.

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