JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Biotransformation of α-Terpineol by the Larvae of Common Cutworm (*Spodoptera litura*)

MITSUO MIYAZAWA* AND MASASHI OHSAWA

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, 3-4-1 Kowakae, Higashiosaka-shi, Osaka 577-8502, Japan

Biotransformation of α-terpineol by the common cutworm (*Spodoptera litura*) larvae was investigated. α-Terpineol was mixed in an artificial diet, and the diet was fed to the larvae (fourth−fifth instar) of *S. litura*. Metabolites were isolated from the frass and analyzed spectroscopically. Main metabolites were 7-hydroxy-α-terpineol (*p*-menth-1-ene-7,8-diol) and oleuropeic acid (8-hydroxy-*p*-menth-1-en-7-oic acid). Intestinal bacteria from the frass of larvae did not participate in the metabolism of α-terpineol. α-Terpineol was preferentially oxidized at the C-7 position (allylic methyl group) by *S. litura* larvae.

**KEYWORDS: Common cutworm; *Spodoptera litura*; biotransformation; α-terpineol; 7-hydroxy-α-terpineol; oleuropeic acid; allylic methyl group; positional specific oxidation**

## INTRODUCTION

Terpenoids are known not only as raw materials for flavor and fragrance but also as biologically active substances. A great number of biologically active terpenoids are produced as plant secondary metabolites, and these have been shown to have biological activity against plants, microorganisms, and insects. Various attempts have been made to search for new biologically active terpenoids. Biotransformation is one way to produce biologically active terpenoids. In relation to this biotransformation of terpenoids using a biocatalyst for the purpose of producing new biologically active terpenoids, we have investigated the biotransformation of monoterpenoids by the larvae of the common cutworm (*Spodoptera litura*).

Previously we reported the biotransformation of monoterpenoids with a *p*-menthane skeleton [α-terpinene, (+)- and (−)-limonene, γ-terpinene, (−)-α-phellandrene, (+)- and (−)-menthol, and (*R*)- and (*S*)-terpinen-4-ol] by the common cutworm (*S. litura*) larvae (*1*−*5*). Consequently, we revealed that α-terpinene was preferentially oxidized at the C-7 position (allylic methyl group). The results indicated that intestinal bacteria probably participated in the metabolism of α-terpinene by *S. litura* larvae (*1*). (+)- and (−)-limonene were oxidized at the 8,9-double bond and the C-7 position (allylic methyl group) (*2*). γ-Terpinene and (−)-α-phellandrene were oxidized at the C-7 position (allylic methyl group) (*3*). (+)- and (−)-menthol were oxidized at the C-7 position (*4*). (*R*)- and (*S*)-terpinen-4-ol were oxidized at the C-7 position (allylic methyl group) (*5*).

In the present paper, the biotransformation of α-terpineol (**1**) by the larvae of *S. litura* was investigated for the purpose of estimating possible metabolic pathways in the larvae of insects belonging to Noctuidae. Compound **1**, a cyclic monoterpene alcohol, is known to be an important and widespread terpene, has a pine odor, and is used for the perfume of soap and its fruit flavor. It occurs in many essential oils. This paper describes the main metabolites and the metabolic pathway of **1** in the larvae of *S. litura*.

## MATERIALS AND METHODS

**Chemicals.** α-Terpineol [(±)-form, (**1**)] was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The reagent was crystallized and used as substrate for biotransformation. The specific rotation was as follows: $[\alpha]^{20}_{D}$ −40.4° ($CHCl_3$, *c* 3.0).

**Thin-Layer Chromatography (TLC).** TLC was performed on precoated plates [silica gel 60 $F_{254}$, 0.25 mm, Merck (Darmstadt, Germany)]. The solvent system was hexane/EtOAc (1:1 or 1:4, v/v). Compounds were visualized by spraying plates with 1% vanillin in 96% sulfuric acid followed by brief heating (120 °C, 1 min).

**Gas Chromatography (GC).** A Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (FID), a fused silica capillary column [DB-5 (liquid phase: 5% phenyl-methyl-polysiloxane), 30 m length, 0.25 mm i.d., film thickness = 1.0 μm], and a split injection of 25:1 were used. Helium at a flow rate of 1 mL/min was used as a carrier gas. The oven temperature was programmed from 80 to 240 °C at 4 °C/min. The injector and detector temperatures were 270 and 280 °C, respectively. The peak area was integrated with a Hewlett-Packard HP3396 series II integrator.

**Gas Chromatography—Mass Spectrometry (GC-MS).** A Hewlett-Packard 5890A gas chromatograph equipped with a split injector and a fused silica capillary column [HP-5MS (liquid phase: 5% phenyl-methyl-polysiloxane), 30 m length, 0.25 mm i.d., film thickness = 0.25 μm] was combined by direct coupling to a Hewlett-Packard 5972A mass spectrometer, and the same temperature program as just described for GC were used. Helium at 1 mL/min was used as a carrier gas. The temperature of the ion source was 280 °C, and the electron energy was 70 eV. The electron impact (EI) mode was used.

**Infrared (IR) Spectroscopy.** The IR spectra were obtained with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer. $CHCl_3$ was used as a solvent.

* Author to whom correspondence should be addressed (telephone +81-6-6721-2332; fax +81-6-6727-4301; e-mail miyazawa@apch.kindai.ac.jp).

10.1021/jf020287e CCC: $22.00 
Published on Web 07/18/2002

**Nuclear Magnetic Resonance (NMR) Spectroscopy.** The NMR spectra were obtained with a JEOL FX-500 (500.00 MHz, $^1$H; 125.65 MHz, $^{13}$C) spectrometer. Tetramethylsilane (TMS) was used as the internal standard ($\delta$ 0.00).

**Specific Rotation.** The specific rotations were measured on a JASCO DIP-1000 digital polarimeter.

**Rearing of Larvae.** The larvae of *S. litura* were reared in plastic cases (200 × 300 mm wide, 100 mm high, 100 larvae/case) covered with a nylon mesh screen. The rearing conditions were as follows: 25−26 °C, 60−70% relative humidity, and 16 h light/8 h dark photocycle. A commercial diet (Insecta LF; Nihon Nosan Kogyo Co., Ltd.) was given to the larvae from the first to the sixth (last) instar.

**Oral Administration of α-Terpineol (1).** The diet of 800 larvae (fourth−fifth instar, body weight = 350−450 mg) was changed to an artificial diet composed of kidney beans (50 g), agar (6 g), and water (350 mL) (*6*). After 1 day, the artificial diet without the agar was mixed with a blender. Compound **1** (2.0 g) was then added directly into the blender. Agar was dissolved in water and boiled and then added into the blender. The diet was then mixed and cooled in a tray (220 × 310 mm wide, 30 mm high). The larvae were moved into new cases (100 larvae/case), and the diet was fed to the larvae in limited amounts. Groups of 800 larvae were fed the diet containing **1** (actually 1.4−1.5 g, 3.1−4.2 mg for a body) for 2 days, and then the artificial diet not containing **1** was fed to the larvae for an additional 1 day. Frass was collected daily (total of three days) and stored in a solution of $CH_2Cl_2$ (500 mL). For diet and frass separation, the fresh frass was extracted as soon as the larvae excreted.

**Isolation and Identification of Metabolites from Frass.** The frass were extracted three times with $CH_2Cl_2$ (500 mL) and two times with EtOAc (500 mL). $CH_2Cl_2$ and EtOAc extracts were mixed, the solvent was evaporated under reduced pressure, and 2.6 g of extract was obtained. The extract was dissolved in $CH_2Cl_2$ and then was added to the 5% $NaHCO_3$ solution. After shaking, the neutral fraction (870 mg) was obtained from the $CH_2Cl_2$ layer. The aqueous layer (acidic fraction) was separated, then acidified with 1 N HCl, and extracted with $CH_2Cl_2$. After shaking, the acidic fraction (1066 mg) was obtained from the aqueous layer.

The neutral fraction was analyzed with TLC, GC, and GC-MS. Compound **1** (recovered substrate) and metabolite **2** occurred in this fraction. The acidic fraction was reacted with ethereal $CH_2N_2$ and subsequently examined by TLC, GC, and GC-MS. The methyl ester of metabolite **3** occurred in this fraction. The neutral fraction was subjected to silica gel open-column chromatography (silica gel 60, 230−400 mesh, Merck) with a hexane/EtOAc gradient (9:1−1:1) system; compounds **1** (32 mg) and **2** (95 mg) were isolated. The methylated acidic fraction was subjected to silica gel open-column chromatography (silica gel 60, 230−400 mesh, Merck) with a hexane/EtOAc stepwise (19:1, 9:1, 6:1) system, and methylated metabolite **3** (282 mg) was isolated. Metabolite **2** and the methyl ester of **3** were identified by a comparison of established GC-MS, IR, and NMR data.

**Metabolite 2** was obtained as an oil: $[\alpha]^{17}_D$ −22.4° ($CHCl_3$, *c* 2.0); EI-MS, *m*/*z* (rel intensity) 152 $[M - H_2O]^+$ (17), 137 (5), 134 $[M - 2H_2O]^+$ (2), 121 $[152 - CH_2OH]^+$ (14), 109 (29), 79 (100), 59 (98) 43 (59); IR (liquid film), $\upsilon_{max}$ cm$^{-1}$ 3250, 1670, 1144, 1018; $^1$H NMR ($CDCl_3$) $\delta$ 1.19 (3H, s, H-10), 1.20 (3H, s, H-9), 1.54 (1H, m, H-4), 4.00 (2H, br dd, $J$ = 13.0, 17.5 Hz, H-7), 5.69 (1H, br s, H-2); $^{13}$C NMR (**Table 1**).

**Methyl ester of metabolite 3** was obtained as an oil: $[\alpha]^{17}_D$ −39.8° ($CHCl_3$, *c* 1.24); EI-MS, *m*/*z* (rel intensity) 180 $[M - H_2O]^+$ (15), 167 $[M - OCH_3]^+$ (2), 151 (8), 149 (5), 140 (36), 125 (11), 108 (15), 105 (10), 93 (8), 79 (35), 59 (100), 43 (42); IR (liquid film), $\upsilon_{max}$ cm$^{-1}$ 3405, 1735, 1670, 1262, 1100, 1050; $^1$H NMR ($CDCl_3$) $\delta$ 1.21 (3H, s, H-10), 1.22 (3H, s, H-9), 1.55 (1H, m, H-4), 3.73 (3H, s, −$OCH_3$), 6.99 (1H, m, H-2); $^{13}$C NMR (**Table 1**).

**Incubation of Intestinal Bacteria with α-Terpineol (1).** This experiment was carried out under sterile conditions. Petri dishes, pipets, and solutions were autoclaved. GAM broth (Nissui Pharmaceutical Co., Ltd.; 5.9 g) and water (100 mL) were mixed, adjusted to pH 8.6 with $KH_2PO_4$, and placed in Petri dishes at 10 mL/Petri dish. The fresh frass (3 g) of the fourth−fifth instar larvae were suspended in physiological saline (100 mL), and the suspension (1 mL) was pipetted in the medium.

**Table 1.** $^{13}$C NMR Spectral Data for Compounds **1** and **2** and the Methyl Ester of **3** (Recorded at 125.65 MHz, Residual $CHCl_3$ Used as Internal Reference, $\delta$ = 77.00)

| C | 1 | 2 | 3 |
|---|---|---|---|
| 1 | 134.0 (s) | 137.4 (s) | 130.0 (s) |
| 2 | 120.5 (d) | 122.3 (d) | 139.4 (d) |
| 3 | 23.9 (t) | 23.6 (t) | 23.2 (t) |
| 4 | 45.0 (d) | 45.0 (d) | 44.1 (d) |
| 5 | 26.8 (t) | 26.3 (t) | 25.1 (t) |
| 6 | 31.0 (t) | 26.5 (t) | 27.3 (t) |
| 7 | 23.3 (q) | 66.9 (t) | 167.7 (s) |
| 8 | 72.7 (s) | 72.6 (s) | 72.1 (s) |
| 9 | 26.2 (q) | 27.3 (q) | 26.5 (q) |
| 10 | 27.4 (q) | 26.5 (q) | 27.1 (q) |
| $COOCH_3$ | | | 51.4 (q) |

[a] Chemical shifts are in ppm; multiplicities were determined by the DEPT pulse sequence.

**Table 2.** Recovery and Yield of Metabolites of Compounds **1**–**3** by the *S. litura* Larvae[a]

| substrate | **1** administered in the artificial diet (g) | compd **1**–**3** in metabolites[c] (g) | recovery (%) | yield[b] (%) | | |
|---|---|---|---|---|---|---|
| | | | | **1** | **2** | **3** |
| α-terpineol (**1**) | 1.5 | 1.2 | 80 | 8.6[d] | 26.7 | 57.6 |

[a] Metabolites were obtained from the frass of *S. litura* larvae, which were orally administered **1**–**3**. [b] Percentage estimated by GC. [c] Calculated from the peak area in the gas chromatogram used an internal standard (α-terpineol). [d] Recovered substrate.

The medium without frass was also prepared for a blank experiment. These media were incubated (20 °C, darkness, 3 days) under aerobic and anaerobic conditions. After growth of bacteria, **1** (2 mg/Petri dish) was added to the medium and the incubation was continued an additional 2 days. The medium was acidified with 1 N HCl and distributed between $Et_2O$ and a saturated solution of salt. The $Et_2O$ phase was evaporated, and the extract was obtained. For the quantitative analysis of metabolites, GC analysis was used as an internal standard with **1**.

**Biotransformation of Metabolite 2.** A small amount of metabolite **2** (80 mg) was dissolved in acetone (20 mL), and the solution was painted on the surface of the artificial diet (20 mg). The diet was fed to 15 larvae (fourth−fifth inster). The frass was collected and extracted with $CH_2Cl_2$ (100 mL) and EtOAc (100 mL). Metabolite **3** was identified from the retention time in the GC chromatogram of the extract from the frass.

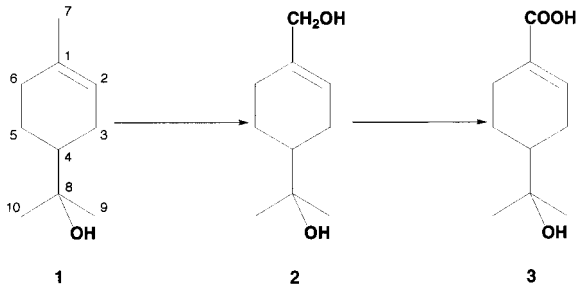
## RESULTS AND DISCUSSION

**Metabolites from Frass.** Although alcohol and carboxylic acid were detected by GC analysis, intermediary metabolites (aldehyde) were not isolated. This suggested that intermediary metabolites were hardly excreted into the frass. The larvae that were fed the diet without substrate were used as control, and the extract from the frass was analyzed by GC. The result was that compounds **1**−**3** were not observed in the frass.

For the quantitative analysis of metabolites, we varied the quantity of substrate in the extract by the internal standard method in GC. Percentage was calculated from the peak area in the GC chromatogram of the extract of frass. One hundred percent was defined as total metabolites of **1** and recovered **1**. The result was that recovered **1** in the frass extract was 8.6% and recovered metabolites **2** and **3** were 26.7 and 57.6%, respectively (**Table 2**).

Metabolite **2** had a molecular formula of $C_{10}H_{18}O_2$ based on its EI-MS spectral. Other spectral data indicated the presence of a secondary hydroxyl group ($\delta_H$ 4.00; $\delta_C$ 66.9; $\upsilon_{max}$ 3250,

**Scheme 1.** Possible Metabolic Pathway of α-Terpineol (**1**) by the Larvae of *S. litura*




**1** **2** **3**

1018 $cm^{-1}$). The $^1H$ and $^{13}C$ NMR spectra were assigned by comparison with the spectral data for **1** and the previous paper (*7*). These spectral data suggested that metabolite **2** was 7-hydroxy-α-terpineol (*p*-menth-1-ene-7,8-diol).

The methyl ester of metabolite **3** had a molecular formula of $C_{11}H_{18}O_3$ based on its EI-MS spectral. Other spectral data indicated the presence of a carbonyl group and the methyl group of the ester ($\delta_H$ 3.73; $\delta_C$ 51.4, 167.7; $\upsilon_{max}$ 3405, 1723, 1262 $cm^{-1}$). $^1H$ and $^{13}C$ NMR spectra were assigned by comparison with the spectral data for **1** and **2** and the previous paper (*8*). These spectral data suggested that metabolite **3** was oleuropeic acid (8-hydroxy-*p*-menth-1-en-7-oic acid).

**Intestinal Bacteria.** A previous paper described the participation of *S. litura* intestinal bacteria in the metabolism of α-terpinene (*1*). The aerobically active intestinal bacteria transformed α-terpinene to *p*-mentha-1,3-dien-7-ol, and the anaerobically active intestinal bacteria transformed α-terpinene to *p*-cymene. In the present study, the in vitro metabolism of **1** by intestinal bacteria was also examined in a manner similar to that of the previous paper. However, **1** was not metabolized at all. These results suggested that the intestinal bacteria did not participate in the metabolism of **1**. The difference of reaction between **1** and α-terpinene was suggested to be due to the difference of substrate.

**Metabolic Pathways.** In the present study of the biotransformation of **1**, the larvae transformed **1** to **2** and **3** (**Scheme 1**). The C-7 position of **1** was preferentially oxidized as in the previous paper (*1*−*5*). The results indicate that oxidation at the C-7 position (allylic methyl group) is the main metabolic pathway of monoterpene alcohol with *p*-menthane skeleton in *S. litura* larvae. In the biotransformation of **2**, the metabolite was **3**. Metabolite **2** was oxidized at the C-7 position by the larvae of *S. litura*. The results of the biotransformations of **1** and **2** revealed that in the biotransformation of **1** by the larvae of *S. litura*, the main metabolite **3** was formed by one metabolic pathway (**1** → **2** → **3**) in the larvae of *S. litura* (**Scheme 1**).

There have been some reports of the biotransformation of **1** by other organisms. In the biotransformation of **1** by the suspension cells of *Nicotiana tabacum*, compound **2**, *trans*-6-hydroxy-α-terpineol, and *cis*-6-hydroxy-α-terpineol are produced. Oxidation at the C-6 and C-7 positions (allylic methyl groups) is the main metabolic pathway (*7*). In the biotransformation of **1** in the rat, compound **1** is transformed to compound **3**, *p*-menthane-1,2,8-triol, and dihydrooleuropeic acid (8-hydroxy-*p*-menthan-7-oic acid). Oxidation of the allylic methyl group and reduction of the endocyclic double bond are the metabolic pathways in rat (*8*). In the biotransformation of **1** by *Armillariella mellea*, oxidation of the C-6 position and the 1,2-double bond is the metabolic pathway (*9*).

In this study, compound **1** was oxidized at only the C-7 position of the allylyic methyl group in the *S. litura* larvae; that is, positional specific oxidation was progress in the metabolism of **1**. There is only one metabolic pathway for metabolism of **1** in the *S. litura* larvae.

## LITERATURE CITED

(1) Miyazawa, M.; Wada, T.; Kameoka, H. Biotransformation of α-terpinene by the larvae of common cutworm (*Spodoptera litura*). *J. Agric. Food Chem.* **1996**, *44*, 2889−2893.

(2) Miyazawa, M.; Wada, T.; Kameoka, H. Biotransformation of (+)- and (−)-limonene by the larvae of common cutworm (*Spodoptera litura*). *J. Agric. Food Chem.* **1998**, *46*, 300−303.

(3) Miyazawa, M.; Takashi, W. Biotransformation of γ-terpinene and (−)-α-phellandrene by the larvae of common cutworm (*Spodoptera litura*). *J. Agric. Food Chem.* **2000**, *48*, 2893−2895.

(4) Miyazawa, M.; Kumagae, S.; Kameoka, H. Biotransformation of (+)- and (−)-menthol by the larvae of common cutworm (*Spodoptera litura*). *J. Agric. Food Chem.* **1999**, *47*, 3938−3940.

(5) Miyazawa, M.; Kumagae, S.; Kameoka, H. Biotransformation of (*R*)- and (*S*)-terpinene-4-ol by the larvae of common cutworm (*Spodoptera litura*). *J. Agric. Food Chem.* **2001**, *49*, 4312−4314.

(6) Yushima, K.; Kamano, S.; Tamaki, Y. *Rearing Methods of Insects*; Japan Plant Protection Association: Tokyo, Japan, 1991; pp 214−218.

(7) Suga, T.; Aoki, T.; Hirata, T.; Ym Sook Lee; Nishimura, O.; Utsumi, M. Biotransformation of foreign substrates with callus tissues. Transformation of terpineols with tobacco suspension cells. *Chem. Lett.* **1980**, 229−230.

(8) Madyastha, K. M.; Sriratsar, V. Biotransformation of α-terpineol in rat: Its effects on the liver microsomal cytochrome P-450 system. *Bull. Environ. Contam. Toxicol.* **1985**, *15* (2), 165−170.

(9) Draczynska-Lusiak, B.; Siewinski, A. Enantioselectivity of the metabolism of some monoterpenic components of coniferous tree resin by *Armillariella mellea* (honey fungus). *J. Basic Microbiol.* **1989**, *29* (5), 269−275.

**Received for review March 6, 2002. Revised manuscript received June 6, 2002. Accepted June 6, 2002.**

JF020287E