Biotransformation of γ-Terpinene and (−)-α-Phellandrene by the Larvae of Common Cutworm (*Spodoptera litura*)

Mitsuo Miyazawa* and Takashi Wada

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

γ-Terpinene was mixed in artificial diet at a concentration of 1 mg/g of diet, and the diet was fed to the last instar larvae of common cutworm (*Spodoptera litura*). Metabolites were recovered from frass and analyzed spectroscopically. γ-Terpinene was transformed mainly to *p*-mentha-1,4-dien-7-oic acid and *p*-cymen-7-oic acid (cumic acid). Similarly, (−)-α-phellandrene was transformed mainly to (*4R*)-*p*-mentha-1,5-dien-7-oic acid and *p*-cymen-7-oic acid (cumic acid). The C-7 position (allylic methyl group) of γ-terpinene and (−)-α-phellandrene was preferentially oxidized.

Keywords: *Common cutworm; Spodoptera litura; biotransformation; γ-terpinene; α-phellandrene; p-mentha-1,5-dien-7-oic acid; p-mentha-1,5-dien-7-oic acid; cumic acid*

INTRODUCTION

Terpenoids are known as not only raw materials for flavor and fragrance but also biologically active substances. A great majority of biologically active terpenoids are produced as plant secondary metabolites, and these terpenoids have been shown to have biological activity against plants, microorganisms, and insects. Various ways are used to search for new biologically active terpenoids. Biotransformation is one way to produce biologically active terpenoids.

In a previous paper, the biotransformation of α-terpinene and (+)- and (−)-limonene by the larvae of the common cutworm (*Spodoptera litura*) was described (Miyazawa et al., 1996, 1998). Consequently, we revealed that the C-7 position (allylic methyl group) of α-terpinene was preferentially oxidized. The results indicated that the intestinal bacteria probably participated in the metabolism of α-terpinene. In the present paper, the biotransformation of γ-terpinene (**1**) and (−)-α-phellandrene (**4**) by the larvae of *S. litura* was investigated for the purpose of estimating possible metabolic pathways in insects. Compound **1** is a constituent of many essential oils (e.g., *Citrus*, *Eucalyptus*, *Juniperus*, *Mentha*, and *Pinus* species). On the other hand, compound **4** is a constituent of oils of pimento, bay, and *Eucalyptus phelandra* and other essential oils (e.g., *Citrus*, *Juniperus*, *Mentha*, and *Pinus* species). Compounds **1** and **4** are isomeric with one another on the difference of position of the endocyclic double bond. This paper deals with the metabolites from frass and the metabolic pathways.

MATERIALS AND METHODS

Chemicals. γ-Terpinene (**1**) and (−)-α-phellandrene (**4**) were purchased from Tokyo Kasei Kogyo Company, Ltd. (Tokyo, Japan).

Gas Chromatography (GC). A Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector, an OV-1 fused-silica capillary column (25 m length, 0.25 mm i.d.), and a split injection of 50:1 were used. Nitrogen 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 250 °C. The peak area was integrated with a Shimadzu C-R3A integrator.

Gas Chromatography−Mass Spectrometry (GC-MS). A Shimadzu GC-15A gas chromatograph equipped with a split injector was combined by direct coupling to a Shimadzu QP1000A mass spectrometer. The same type of column 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.

Nuclear Magnetic Resonance (NMR) Spectroscopy. The NMR spectra were obtained with a JEOL GSX-270 (270.05 MHz, ^{1}H) spectrometer.

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 °C, 70% relative humidity, and constant light. A commercial diet (Insecta LF; Nihon Nosan Kogyo Co., Ltd. Japan) was given to the larvae from the first instar. From the fourth instar, the diet was changed to an artificial diet composed of kidney beans (100 g), brewer's dried yeast (40 g), ascorbic acid (4 g), agar (12 g), and water (600 mL; Yushima et al., 1991).

Administration of γ-Terpinene (1). The artificial diet without the agar was mixed with a blender. Then, **1** was added directly into the blender at 1 mg/g of diet. 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 diet containing **1** was stored in a refrigerator until the time of administration. The last instar larvae (average weight = 0.5 g) were moved into new cases (100 larvae/case), and the diet was fed to the larvae in limited amounts. Groups of 500 larvae were fed for 2 days with 500 g of the diet containing **1** (483 mg), and then the artificial diet not containing **1** was fed to the larvae for an additional 2 days. The frass were collected daily (total of 4 days) and stored in a solution of CH_2Cl_2 (500 mL).

Isolation and Identification of Metabolites from Frass. The frass were extracted three times with CH_2Cl_2 each time. The extract solution was evaporated under reduced pressure, and 1681 mg of the extract was obtained. The extract was distributed between 5% aqueous $NaHCO_3$ and CH_2Cl_2, the CH_2Cl_2 phase was evaporated, and the neutral fraction (864

* 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/jf9900581 CCC: $19.00
Published on Web 06/07/2000

mg) was obtained. The alkali phase was acidified with 1 N HCl and distributed between water and CH_2Cl_2. The CH_2Cl_2 phase was evaporated, and the acidic fraction (450 mg) was obtained. The acidic fraction was dissolved in CH_2Cl_2 (20 mL), and CH_2N_2 (5 mL) was added to the solution. The solution was evaporated, and the methylated fraction (478 mg) was obtained. The methylated fraction was analyzed by GC-MS, and methylated **2** and methylated **3** occurred in this fraction. The methylated fraction was subjected to silica gel open-column chromatography (silica gel 60, 230−400 mesh, Merck) with a 9:1 *n*-hexanee/$CHCl_3$ solvent system, and methylated **2** (28 mg) and methylated **3** (34 mg) were isolated. Methylated **2** and methylated **3** were identified by a comparison of established MS and ^{1}H NMR data.

***p*-Mentha-1,4-dien-7-oic Acid (2) as methyl ester** was obtained as an oil: EIMS *m*/*z* (rel int) 180 $[M]^+$ (12), 137 (72), 105 (64), 91 (54), 77 (66), 59 (69), 43 (100); ^{1}H NMR ($CDCl_3$) δ 1.03 (6H, d, J = 7.0 Hz, Me-9 and Me-10), 2.22 (1H, septet, J = 7.0 Hz, H-8), 2.81 (2H, m, H-3), 2.92 (2H, m, H-6), 3.74 (3H, s, OMe), 5.49 (1H, m, H-5), 6.99 (1H, m, H-2).

***p*-Cymen-7-oic acid (cumic acid) (3) as methyl ester** was obtained as an oil: EIMS *m*/*z* (rel int) 178 $[M]^+$ (34), 163 (100), 119 (69), 91 (63), 77 (41), 59 (44), 51 (39); ^{1}H NMR ($CDCl_3$) δ 1.27 (6H, d, J = 7.6 Hz, Me-9 and Me-10), 2.96 (1H, septet, J = 7.6 Hz, H-8), 3.90 (3H, s, OMe), 7.29 (2H, d, J = 8.1 Hz, H-3 and H-5), 7.96 (2H, d, J = 8.1 Hz, H-2 and H-6).

Biotransformation of (—)-α-Phellandrene (4). The same procedure as described for **1** was used. Substrate **4** (507 mg) was transformed to metabolites **5** (42 mg, as methyl ester) and **3** (35 mg, as methyl ester).

***(4R)*-*p*-Mentha-1,5-dien-7-oic acid (5) as methyl ester** was obtained as an oil: EIMS *m*/*z* (rel int) 180 $[M]^+$ (13), 137 (32), 105 (74), 77 (100), 59 (60); ^{1}H NMR ($CDCl_3$) δ 0.91 (6H, dd, J = 3.2 and 6.8 Hz, Me-9 and Me-10), 1.57 (2H, m, H-3), 3.76 (3H, s, OMe), 5.81 (1H, m, H-5), 6.37 (1H, m, H-6), 6.93 (1H, m, H-1).

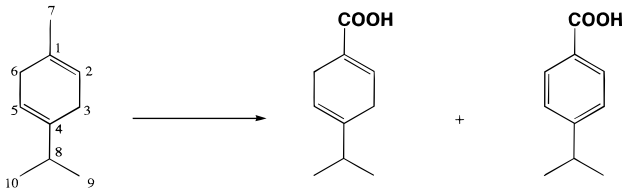
RESULTS AND DISCUSSION

Metabolites from Frass. Metabolic reaction by the larvae of *S. litura* was observed as follows: substrate was administered to the larvae as their diet; the metabolite was then detected and isolated from the frass of larvae. In the previous paper, α-terpinene was mixed in the diet of larvae at a high concentration (10 mg/g of diet) to increase the production of potential metabolites (Miyazawa et al., 1996). However, intermediary metabolites (alcohols and aldehydes) were not isolated, although alcohols were detected by GC analysis. These results suggested that intermediary metabolites were insignificantly excreted into the frass. In the present study, a concentration of 1 mg/g of diet was therefore chosen as optimum for administration (i.e., the concentration consumes a substrate completely).

In the biotransformation of γ-terpinene (**1**), the two metabolites isolated from the frass were identified as *p*-mentha-1,4-dien-7-oic acid (**2**) and *p*-cymen-7-oic acid (**3**). The majority of metabolites were **2** (46%) and **3** (48%). Percentage was calculated from the peak area in the GC spectra of the extract of frass; 100% was defined as total metabolites of **1**. Substrate **1** and intermediary metabolites (alcohols and aldehydes) were not detected in the frass by GC analysis. Metabolites **2** and **3** were produced by oxidation at the C-7 position of **1**, and allylic oxidation was the only metabolic pathway.

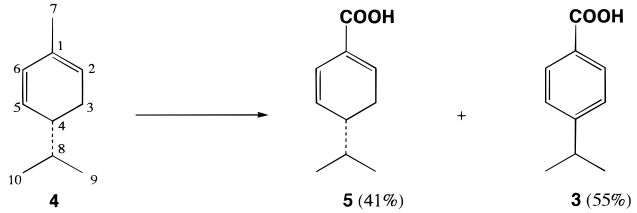
In the biotransformation of (−)-α-phellandrene (**4**), similarly, the two metabolites isolated from the frass were identified as (*4R*)-*p*-mentha-1,5-dien-7-oic acid (**5**) and *p*-cymen-7-oic acid (**3**). The majority of metabolites were **5** (41%) and **3** (55%). Percentage was calculated from the peak area in the GC spectra of the extract of frass; 100% was defined as total metabolites of **4**. Substrate **4** and intermediary metabolites (alcohols and aldehydes) were not detected in the frass by GC analysis. Metabolites **5** and **3** were produced by oxidation at the C-7 position of **4**, and allylic oxidation was the only metabolic pathway.

Scheme 1. Metabolites of γ-Terpinene (1) by the Larvae of *S. litura*[a]



[a] Percentage was calculated from the peak area in the GC spectra of the extract of frass. One hundred percent was defined as total metabolites of **1**.

Scheme 2. Metabolites of α-Phellandrene (4) by the Larvae of *S. litura*[a]



[a] Percentage was calculated from the peak area in the GC spectra of the extract of frass. One hundred percent was defined as total metabolites of **4**.

Intestinal Bacteria. A previous paper described the participation of intestinal bacteria in the metabolism of α-terpinene (Miyazawa et al., 1996). 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** and **4** by intestinal bacteria was also examined in a similar manner in the previous paper. However, **1** and **4** were not metabolized at all (no reaction). These results suggested that the intestinal bacteria did not participate in the metabolism of **1** and **4**.

Metabolic Pathways. In the present study of the biotransformation of **1** and **4**, the larvae transformed **1** to **2** and **3** (Scheme 1); similarly, the larvae transformed **4** to **5** and **3** (Scheme 2). In addition, the previous paper described that the larvae mainly transformed α-terpinene to *p*-mentha-1,3-dien-7-oic acid and **3** (Miyazawa et al., 1996). The three substrates, **1**, **4**, and α-terpinene, are isomeric with one another on the difference of position of the endocyclic double bond (1,3-, 1,4-, and 1,5-diene, respectively). These results indicate that there is little difference in the metabolic pathways (the oxidation at the C-7 position and the dehydrogenation) among **1**, **4**, and α-terpinene. However, the biotransformation of α-terpinene differs from that of **1** and **4** in terms of the enzymatic activity (the concentration of substrate in diet), the proportion of products, and the participation of intestinal bacteria. α-Terpinene did not remain in the frass of larvae fed α-terpinene; on the other hand, a large quantity of substrates **1** and **4** remained in the frass of larvae fed **1** and **4**, respectively, at a concentration of 10 mg/g of diet. The proportion of two metabolites of **1** was about 5:5 of 7-oic acid (**2**) and

dehydrogenated 7-oic acid (**3**), and the proportion of two metabolites of **4** was about 5:5 of 7-oic acid (**5**) and dehydrogenated 7-oic acid (**3**); on the other hand, the proportion of two metabolites of α-terpinene was about 9:1 of *p*-mentha-1,3-dien-7-oic acid and dehydrogenated 7-oic acid (**3**). Furthermore, only α-terpinene was metabolized by intestinal bacteria.

Compounds **1** and **4** are reported in the literature as constituents of many essential oils. However, to date there are only two reports on the metabolism of **1**; namely, fungi, *Corynespora cassiicola* and *Diplodia gossyina*, converted **1** to (*1R*,*2R*)-*p*-menth-4-ene-1,2-diol and cuminyl alcohol (Abraham et al., 1984, 1986); and beetles, *Dendroctonus ponderosae*, converted **1** to *p*-mentha-1,4-dien-7-ol and cuminyl alcohol (Pierce et al., 1987). There are only two reports on the metabolism of **4**; namely, fungi, *Corynespora cassiicola* and *Diplodia gossypina*, converted **4** to (*1S*,*2S*,*4R*)-*p*-menth-5-ene-1,2-diol, yabunikkeol, and *p*-menth-5-en-2-one (Abraham et al., 1984, 1986); and beetles, *Dendroctonus ponderosae*, converted **4** to *p*-mentha-1,5-dien-7-ol and cuminyl alcohol (Pieece et al., 1987). This fact indicates that the larvae of *S. litura* exhibit a metabolic pathway similar to that of beetles.

LITERATURE CITED

Abraham, W. R.; Kieslich, K.; Reng, H.; Stumpf, B. Formation and production of 1,2-*trans*-glycols from various monoterpenes with 1-menthene skeleton by microbial transformation with *Diplodia gossypina*. *Eur. Congr. Biotechnol., 3rd* **1984**, *1*, 245−248.

Abraham, W. R.; Stumpf, B.; Kieslich, K. Microbial transformation of terpenoids with 1-*p*-menthene skeleton. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 24−30.

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

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.

Pierce, H. D., Jr.; Conn, J. E.; Oehlschlager, A. C.; Borden, J. H. Monoterpene metabolism in female mountain pine beetles, *Dendroctonus ponderosae* Hopkins, attacking ponderosa pine. *J. Chem. Ecol.* **1987**, *13*, 1455−1480.

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

Received for review January 22, 1999. Revised manuscript received February 28, 2000. Accepted March 13, 2000.

JF9900581