Biotransformation of (+)- and (−)-Limonene by the Larvae of Common Cutworm (*Spodoptera litura*)

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(+)-Limonene was mixed in an 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. (+)-Limonene was transformed mainly to (+)-*p*-menth-1-ene-8,9-diol (uroterpenol) and (+)-*p*-mentha-1,8-dien-7-oic acid (perillic acid). Similarly, (−)-limonene was transformed mainly to (−)-*p*-menth-1-ene-8,9-diol (uroterpenol) and (−)-*p*-mentha-1,8-dien-7-oic acid (perillic acid). The results indicate that there is little difference in metabolic pathway between the (+)- and (−)-forms.

Keywords: *Common cutworm; Spodoptera litura; biotransformation; limonene; uroterpenol; perillic 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 attempts have been made to search for new biologically active terpenoids. Biotransformation is one of way to produce biologically active terpenoids.

In a previous paper, the biotransformation of α-terpinene (**7**) by the larvae of common cutworm (*Spodoptera litura*) was described (Miyazawa et al., 1996). Consequently, we revealed that the C-7 position (allylic methyl group) of **7** was preferentially oxidized. The results indicated that the intestinal bacteria probably participated in the metabolism of **7**. In the present paper, the biotransformation of (+)- and (−)-limonene (**1**) by the larvae of *S. litura* was investigated for the purpose of estimating possible metabolic pathways in insects. Compound **1** is the most important and widespread terpene known and is extensively used in the perfumery and flavor industries. Compound (+)-**1** is a major constituent of the oil of orange rind, dill oil, oil of cumin, neroli, bergamot, caraway, and lemon (*Citrus*, *Anethum*, *Juniperus*, and *Peucedanum* species). On the other hand, compound (−)-**1** is a constituent of pine needle oil. This paper deals with the difference in metabolism between the (+)- and (−)-forms.

MATERIALS AND METHODS

Chemicals. The (+)- and (−)-forms of limonene (**1**) were purchased from Tokyo Kasei Kogyo Co., 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.

Infrared (IR) Spectroscopy. The IR spectra were obtained with a Perkin-Elmer 1760X spectrometer. $CHCl_3$ was used as a solvent.

Nuclear Magnetic Resonance (NMR) Spectroscopy. The NMR spectra were obtained with a JEOL GSX-270 (270.05 MHz, ^1H; 67.80 MHz, ^{13}C) 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 (+)-Limonene (1). The artificial diet without the agar was mixed with a blender. Five hundred milligrams of (+)-**1** was then 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 the diet containing (+)-**1** (actually 200−300 mg, 0.4−0.6 mg for a body) for 2 days, and then the artificial diet not containing (+)-**1** was fed to the larvae for an additional 2 days. Frass was collected daily (total of 4 days) and stored in a solution of CH_2Cl_2 (500 mL). (−)-**1** was administered to the last instar larvae as well as (+)-**1**. For diet and frass separation, the fresh frass was extracted as soon as the last instar larvae excreted.

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Isolation and Identification of Metabolites from Frass. The frasses were extracted three times with CH_2Cl_2 each time. The extract solution was evaporated under reduced pressure, and 1439 mg of extract was obtained. The extract was distributed between 5% $NaHCO_3$(aq) and CH_2Cl_2, the CH_2Cl_2 phase was evaporated, and the neutral fraction (850 mg) was obtained. The neutral fraction was analyzed by GC/MS; metabolite (+)-**2** occurred in this fraction. 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 (314 mg) was obtained. The acidic fraction was dissolved in CH_2Cl_2 (20 mL), and CH_2N_2 (5 mL, for acidic metabolites isolate simple) was added to the solution. The solution was evaporated, and the methylated fraction (358 mg) was obtained. The methylated fraction was analyzed by GC/MS; metabolites (+)-**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*-hexane/$CHCl_3$ solvent system, and (+)-**2** (66 mg) and methylated (+)-**3** (47 mg) were isolated. Metabolites (+)-**2** and methylated (+)-**3** were identified by a comparison of established MS, IR, and NMR data. Methylated (+)-**3** was dissolved in 5% NaOH/water/MeOH (10 mL), and the solution was refluxed for 1 h at 120 °C. The solution was acidified with 1 N HCl and distributed between Et_2O and water. The Et_2O phase was evaporated, yielding 41 mg of (+)-**3**.

(+)-(4*R*)-*p*-Menth-1-ene-8,9-diol (Uroterpenol) (2): 6:4 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-forms; oil; $[\alpha]_D$ +83.3° ($CHCl_3$, *c* 1.4); EIMS, *m*/*z* (rel intensity) 121 (32), 93 (19), 71 (21), 43 (100); IR (ν_{max} cm^{-1}) 3366, 2924, 1439, 1051; ^1H NMR ($CDCl_3$) δ 1.12 (*R*), 1.09 (*S*) (3H, *s*, Me-10), 1.64 (3H, *s*, Me-7), 1.98 (2H, *m*, 2 × OH), 3.43, 3.58 (*R*), 3.39, 3.54 (*S*) (2H, AB*q*, J = 10.8 Hz, H-9), 5.40 (*R*), 5.35 (*S*) (1H, *m*, H-2); ^{13}C NMR ($CDCl_3$) δ 20.4 (*t*, C-10), 23.3 (*t*, C-7), 24.2 (*q*, C-5), 25.8 (*q*, C-3), 30.8 (*q*, C-6), 40.6 (*d*, C-4), 68.2 (*q*, C-9), 74.7 (*s*, C-8), 120.5 (*d*, C-2), 133.8 (*s*, C-1).

(+)-(4*R*)-*p*-Mentha-1,8-dien-7-oic Acid (Perillic Acid) (3) as Methyl Ester: oil; EIMS, *m*/*z* (rel intensity) 180 $[M]^+$ (4), 137 (11), 121 (23), 93 (27), 68 (100), 39 (48); IR (ν_{max} cm^{-1}) 1718, 1436, 1253, 1085; ^1H NMR ($CDCl_3$) δ 1.75 (3H, *s*, Me-10), 3.73 (3H, *s*, OMe), 4.74 (2H, *m*, H-9), 7.00 (1H, *m*, H-2); ^{13}C NMR ($CDCl_3$) δ 20.7 (*t*, C-10), 24.5 (*q*, C-5), 27.0 (*q*, C-6), 31.0 (*q*, C-3), 40.0 (*d*, C-4), 51.5 (*t*, OMe), 109.2 (*q*, C-9), 129.9 (*s*, C-8), 139.1 (*d*, C-2), 148.7 (*s*, C-1), 167.8 (*s*, C-7).

(+)-(4*R*)-*p*-Mentha-1,8-dien-7-oic Acid (Perillic Acid) (3): white crystal; mp 124 °C; $[\alpha]_D$ +90.6° ($CHCl_3$, *c* 1.6); ^1H NMR ($CDCl_3$) δ 1.75 (3H, *s*, Me-10), 4.75 (2H, *m*, H-9), 7.14 (1H, *m*, H-2); ^{13}C NMR ($CDCl_3$) δ 20.7 (*t*, C-10), 24.2 (*q*, C-5), 27.0 (*q*, C-6), 31.3 (*q*, C-3), 40.0 (*d*, C-4), 109.3 (*q*, C-9), 129.5 (*s*, C-8), 141.9 (*d*, C-2), 148.6 (*s*, C-1), 172.7 (*s*, C-7).

Biotransformation of (−)-Limonene (1). Same procedure as described for (+)-**1**. Substrate (−)-**1** was transformed to metabolites (−)-**2** (57 mg) and (−)-**3** (33 mg).

(−)-(4*S*)-*p*-Menth-1-ene-8,9-diol (Uroterpenol) (2): 6:4 mixture of (4*S*,8*R*)- and (4*S*,8*S*)-forms; oil; $[\alpha]_D$ −59.0° ($CHCl_3$, *c* 1.85); EIMS, *m*/*z* (rel intensity) 139 (14), 121 (46), 95 (26), 75 (24), 43 (100); ^1H NMR ($CDCl_3$) δ 1.10 (*R*), 1.13 (*S*) (3H, *s*, Me-10), 1.65 (3H, *s*, Me-7), 1.98 (2H, *m*, 2 × OH), 3.40, 3.54 (*R*), 3.44, 3.58 (*S*) (2H, AB*q*, J = 10.8 Hz, H-9), 5.35 (*R*), 5.41 (*S*) (1H, *m*, H-2); ^{13}C NMR ($CDCl_3$) δ 20.5 (*t*, C-10), 23.0 (*t*, C-7), 24.3 (*q*, C-5), 25.8 (*q*, C-3), 30.8 (*q*, C-6), 40.8 (*d*, C-4), 68.6 (*q*, C-9), 74.7 (*s*, C-8), 120.0 (*d*, C-2), 134.3 (*s*, C-1).

(−)-(4*S*)-*p*-Mentha-1,8-dien-7-oic Acid (Perillic Acid) (3) as Methyl Ester: oil; EIMS, *m*/*z* (rel intensity) 180 $[M]^+$ (5), 165 (12), 137 (22), 121 (44), 105 (34), 68 (100); IR (ν_{max} cm^{-1}) 1718, 1436, 1253, 1085; ^1H NMR ($CDCl_3$) δ 1.75 (3H, *s*, Me-10), 3.73 (3H, *s*, OMe), 4.74 (2H, *m*, H-9), 7.00 (1H, *m*, H-2); ^{13}C NMR ($CDCl_3$) δ 20.7 (*t*, C-10), 24.5 (*q*, C-5), 27.0 (*q*, C-6), 31.0 (*q*, C-3), 40.0 (*d*, C-4), 51.5 (*t*, OMe), 109.2 (*q*, C-9), 129.9 (*s*, C-8), 139.1 (*d*, C-2), 148.7 (*s*, C-1) 167.8 (*s*, C-7).

(−)-(4*S*)-*p*-Mentha-1,8-dien-7-oic Acid (Perillic Acid) (3): white crystal; mp 125 °C; $[\alpha]_D$ −81.5° ($CHCl_3$, *c* 1.7); ^1H NMR ($CDCl_3$) δ 1.75 (3H, *s*, Me-10), 4.75 (2H, *m*, H-9), 7.14 (1H, *m*, H-2); ^{13}C NMR ($CDCl_3$) δ 20.7 (*t*, C-10), 24.2 (*q*, C-5), 27.0 (*q*, C-6), 31.3 (*q*, C-3), 40.0 (*d*, C-4), 109.3 (*q*, C-9), 129.4 (*s*, C-8), 141.9 (*d*, C-2), 148.6 (*s*, C-1), 172.4 (*s*, C-7).

Incubation of Intestinal Bacteria with (+)-Limonene (1). This experiment was intentionally carried out under sterile condition. Petri dishes, pipets, and solutions were autoclaved. A GAM Broth (Nissui Pharmaceutical Co., Ltd., Japan) was adjusted to pH 9.0 and placed in Petri dishes at 10 mL/Petri dish. The fresh frasses (5 g) of the last instar larvae were suspended in physiological saline (100 mL), and the suspension (1 mL) was pipetted in the medium. The medium without frass was also prepared for a blank experiment. These media were incubated (18 °C, darkness, 2 days) under aerobic and anaerobic conditions. After growth of bacteria, **1** (10 mg/Petri dish) was added to the medium and the incubation was continued. The percentage of metabolites in the medium was determined 12, 24, and 48 h after addition of **1**. The medium was acidified with 1 N HCl and distributed between Et_2O and saturated solution of salt. The Et_2O phase was evaporated, and the extract was obtained. For the quantitative analysis of metabolites, the GC analysis was used as an internal standard with **1**. (−)-**1** was tested as well as (+)-**1**.

RESULTS AND DISCUSSION

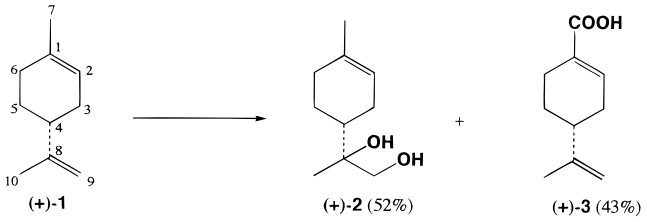
Metabolites from Frass. Biotransformation by the larvae of *S. litura* was observed as follows: substrate was administered to the larvae through their diet; metabolite was then detected and isolated from the frass of larvae. In a previous paper, α-terpinene (**7**) was mixed in the diet of larvae at a high concentration (10 mg/g of diet) to increase the production of potential metabolites (Miyazawa et al., 1996). Although alcohols were detected by GC analysis, intermediary metabolites (alcohols and aldehydes) were not isolated. This suggested that intermediary metabolites were hardly excreted into the frass. In the present study, a concentration of 1 mg/g of diet was therefore chosen as optimum for administration. "Optimum" means the concentration results in complete consumption of substrate. The larvae that were fed the diet without substrate were used as control, and the extract of frass was analyzed by GC. The result was that terpenoids in the frass were not observed. For the consumption of substrate in the diet observed, we varied the quantity of substrate in the diet by the internal standard method. The result was that consumption of (+)-limonene (**1**) was 5%. The result for (−)-**1** was similar.

In the biotransformation of (+)-**1**, the two metabolites isolated from the frass were identified as (+)-(4*R*)-*p*-menth-1-ene-8,9-diol (**2**) and (+)-(4*R*)-*p*-mentha-1,8-dien-7-oic acid (**3**). The majority of metabolites were (+)-**2** (52%) and (+)-**3** (43%). 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 (alcohol, aldehyde, and epoxide) were not detected in the frass by GC analysis. Metabolite (+)-**2** was produced by oxidation at the 8,9-double bond of (+)-**1**, and metabolite (+)-**3** was produced by oxidation at the C-7 position of (+)-**1**.

In the biotransformation of (−)-**1**, similarly, the two metabolites isolated from the frass were identified as (−)-(4*S*)-*p*-menth-1-ene-8,9-diol (**2**) and (−)-(4*S*)-*p*-mentha-1,8-dien-7-oic acid (**3**). The majority of metabolites were (−)-**2** (50%) and (−)-**3** (44%). These results were similar to those for (+)-**1**.

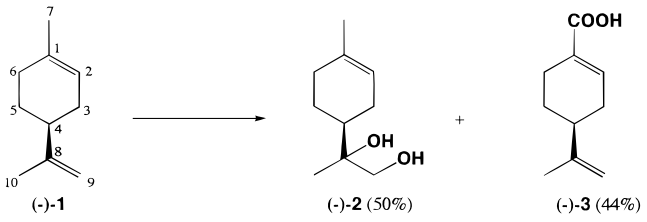
Intestinal Bacteria. A previous paper described the participation of intestinal bacteria in the metabolism of **7** (Miyazawa et al., 1996). The aerobically active

Scheme 1. Metabolites of (+)-Limonene (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. 100% was defined as total metabolites of (+)-**1**.

Scheme 2. Metabolites of (−)-Limonene (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. 100% was defined as total metabolites of (−)-**1**.

intestinal bacteria transformed **7** to *p*-mentha-1,3-dien-7-ol, and the anaerobically active intestinal bacteria transformed **7** to *p*-cymene. In the present study, the in vitro metabolism of (+)- and (−)-**1** by intestinal bacteria was also examined in a manner similar to that of the previous paper. However, (+)- and (−)-**1** were not metabolized at all (no reaction). These results suggested that the intestinal bacteria did not participate in the metabolism of (+)- and (−)-**1**. The difference of reaction between (+)- and (−)-**1** and **7** was suggested to be due to the difference of substrate.

Metabolic Pathways. In the present study of biotransformation of (+)- and (−)-**1**, the larvae transformed (+)-**1** to (+)-**2** and (+)-**3** (Scheme 1); similarly, the larvae transformed (−)-**1** to (−)-**2** and (−)-**3** (Scheme 2). These results indicate that there is little difference in the products and the proportion of products between (+)- and (−)-forms. Furthermore, we revealed metabolites (+)- and (−)-**2** as a mixture of diastereoisomers, respectively, by a comparison of ^{1}H-NMR data in the literature (Carman et al., 1986). Consequently, (+)-**2** was a 6:4 mixture of (4*R*,8*R*)- and (4*R*,8*S*)-forms; on the other hand, (−)-**2** was a 6:4 mixture of (4*S*,8*R*)- and (4*S*,8*S*)-forms. This shows that the larvae did not recognize the difference between (+)- and (−)-forms. In brief, the influence of the asymmetric C-4 carbon atom between (+)- and (−)-forms in the bodies of larvae was not observed.

Compound **1** is the most important and widespread terpene known; therefore, there are many reports on the biotransformation of **1** by other organisms. It seems natural to obtain different metabolites with different species of organisms. However, species of microorganisms or mammals have similar metabolic pathways (oxidative positions). In general, oxidations at the 1,2-double bond, C-7 position, and C-6 position are the main metabolic pathways in the biotransformation of 1 by microorganisms (Dhavalikar and Bhattacharyya, 1966; Dhavalikar et al., 1966; Dhere and Dhavalikar, 1970; Mukherjee et al., 1973; Bowen, 1976; Devi and Bhattacharyya, 1977; Abraham et al., 1984, 1986; Cadwallader et al., 1989; Noma et al., 1992). On the other hand, oxidations at the 8,9-double bond and C-7 position are the main metabolic pathways in the biotransformation of **1** by mammals (Igimi et al., 1974; Kodama et al., 1974, 1976; Regan and Bjeldanes, 1976; Watabe et al., 1980, 1981). This indicates that the larvae of *S. litura* employ metabolic pathways similar to those of mammals.

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