Biotransformation of (*R*)- and (*S*)-Terpinen-4-ol by the Larvae of Common Cutworm (*Spodoptera litura*)

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(*R*)-Terpinen-4-ol 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. (*R*)-Terpinen-4-ol was transformed mainly to (*R*)-*p*-menth-1-en-4,7-diol. Similarly, (*S*)-terpinen-4-ol was transformed mainly to (*S*)-*p*-menth-1-en-4,7-diol. The C-7 position (allylic methyl group) of (*R*)- and (*S*)-terpinen-4-ol was preferentially oxidized.

Keywords: *Common cutworm; Spodoptera litura; biotransformation; terpinen-4-ol; (R)-p-menth-1-en-4,7-diol; (S)-p-menth-1-en-4,7-diol*

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 way to produce biologically active terpenoids.

Previously we reported the biotransformation of α-terpinene, (+)- and (−)-limonene, and (+)- and (−)-menthol by the larvae of common cutworm (*Spodoptera litura*) (*1*−*3*). 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. (+)- and (−)-limonene were oxidized at the 8,9-double bond and the C-7 position (allylic methyl group). (+)- and (−)-menthol were also oxidized at the C-7 position. In the present paper, the biotransformation of (*R*)- and (*S*)-terpinen-4-ol (**1**) by the larvae of *S. litura* was investigated for the purpose of estimating possible metabolic pathways in insects. Compound (*R*)-**1** has been isolated from *Eucalyptus dives*, *Xanthoxylum rhetsa*, and other essential oils. On the other hand, compound (*S*)-**1** occurs in many essential oils, for example, lavender, and *Cupressus macrocarpa*. It has a pleasant odor and taste and is widely used to flavor foods and oral pharmaceutical preparations. This paper describes the metabolites identified in frass.

MATERIALS AND METHODS

Chemicals. (*R*)-Terpinen-4-ol (**1**) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and (*S*)-terpinen-4-ol (**1**) was purchased from Nagaoka Perfumery Co., Ltd. (Nagaoka, Japan).

Thin-Layer Chromatography (TLC). TLC was performed on precoated plates [silica gel 60 F254, 0.25 mm, Merck (Darmstadt, Germany)]. The solvent system was hexane/EtOAc (1:1, v/v). Compounds were visualized by spraying plates with 1% vanillin in 96% sulfuric acid followed by brief heating.

Gas Chromatography (GC). A Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector, an HP-5MS capillary column (30 m length, 0.25 mm i.d.), and a split injection of 20:1 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 250 °C. The peak area was integrated with a Hewlett-Packard HP3396 series 2 integrator.

Gas Chromatography—Mass Spectrometry (GC-MS). A Hewlett-Packard 5890A gas chromatograph equipped with a split injector was combined by direct coupling to a Hewlett-Packard 5972A 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; 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.) 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; *4*).

Administration of (*R*)-Terpinen-4-ol (1). The artificial diet without the agar was mixed with a blender. Five hundred milligrams of (*R*)-**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 (*R*)-**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 (*R*)-**1** (actually 200−300 mg, 0.4−0.6 mg for a body) for 2 days, and then the artificial diet not containing (*R*)-**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). (*S*)-**1** was administered to 500

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larvae in the same manner. For diet and frass separation, the fresh frass was extracted as soon as the last instar larvae excreted.

Isolation and Identification of Metabolites from Frass. The extract solution was obtained by percolation three times with CH_2Cl_2 each time. The solvent was evaporated under reduced pressure, and 693 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 (292 mg) was obtained. The neutral fraction was analyzed by GC-MS; metabolite (*R*)-**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 (187 mg) was obtained. The acidic fraction was reacted with ethereal CH_2N_2 overnight and examined by GC-MS, but a metabolite from (*R*)-**1** did not occur. The neutral fraction was subjected to silica gel open-column chromatography (silica gel 60, 230−400 mesh, Merck) with a 9:1 *n*-hexane/EtOAc system (500 mL), and (*R*)-**2** (56 mg) was isolated. Metabolite (*R*)-**2** was identified by a comparison of established MS and NMR data.

(*R*)-*p*-Menth-1-en-4,7-diol (2) was obtained as a crystal: mp 87.5−89.5 °C; $[\alpha]_D$ −17.9° ($CHCl_3$, *c* 1.0); EIMS, *m*/*z* (rel intensity) 152 $[M - H_2O]^+$, 109, 81, 71 (base), 55, 43; ^{1}H NMR ($CDCl_3$) δ 0.93, 0.96 (each 3H, *d*, J = 7.0 Hz, Me-9 and Me-10), 4.01 (2H, *s*, H-7); ^{13}C NMR ($CDCl_3$) δ 16.7 (*t*, C-10), 16.8 (*t*, C-9), 22.6 (*q*, C-6), 30.4 (*q*, C-5), 34.0 (*q*, C-3), 37.1 (*d*, C-8), 66.8 (*q*, C-7), 72.2 (*s*, C-4), 120.2 (*d*, C-2), 137.1 (*s*, C-1).

Biotransformation of (*S*)-Terpinen-4-ol (1). The same procedure as described for (*R*)-**1** was used. Substrate (*S*)-**1** was transformed to metabolite (*S*)-**2** (64 mg).

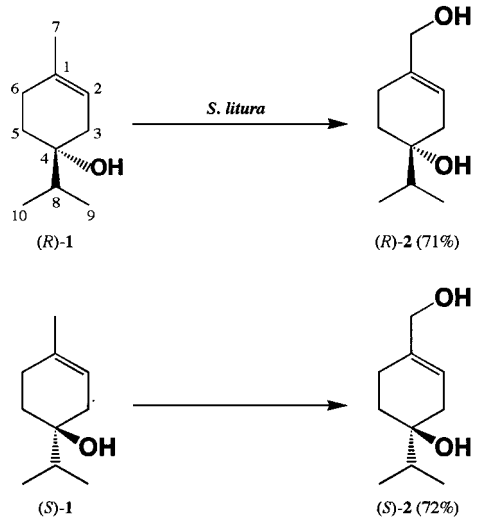
(*S*)-*p*-Menth-1-en-4,7-diol (2) was obtained as a crystal: $[\alpha]_D$ +17.8° ($CHCl_3$, *c* 1.4); the spectral data of the enantiomer (*S*)-**1** were identical to those of (*R*)-**1**.

Incubation of Intestinal Bacteria with (*R*)-Terpinen-4-ol (1). This experiment was intentionally carried out under sterile conditions. Petri dishes, pipets, and solutions were autoclaved. A GAM broth (Nissui Pharmaceutical Co., Ltd.) was adjusted to pH 9.0 and placed in Petri dishes at 10 mL/Petri dish. The fresh frass (5 g) of the last instar larvae was 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, (*R*)-**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 the addition of (*R*)-**1**. 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, the GC analysis was used as an internal standard with (*R*)-**1**. (*S*)-**1** was tested as well as (*R*)-**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 was mixed in the diet of larvae at a high concentration (10 mg/g of diet) to increase the production of potential metabolites (*1*). Although alcohols were detected by GC analysis, intermediary metabolites (for example, aldehyde) were not isolated. This suggested that intermediary metabolites (for example, aldehyde) 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 (*R*)-terpinen-4-ol (**1**) was 11%. The result for (*S*)-**1** was 13%.

Scheme 1. Metabolites of (*R*)- and (*S*)-Terpinen-4-ol (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 each **1**.

In the biotransformation of (*R*)-**1**, the one metabolite isolated from the frass was identified as (*R*)-*p*-menth-1-en-4,7-diol (**2**) (see Scheme 1). The percentage conversion of metabolite (*R*)-**2** was 71%. This 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 (*R*)-**1**. Substrate (*R*)-**1** and some minor products were detected in the frass by GC analysis. These products were not detected by TLC and GC analysis of the frass of *S. litura* to which substrate was not fed but the artificial diet not containing (*R*)-**1** was. From the above result, it is demonstrated that *S. litura* transformed (*R*)-**1** into (*R*)-**2** and some minor products. Metabolite (*R*)-**2** was produced by oxidation at the C-7 position (allylic methyl group) of (*R*)-**1**.

In the biotransformation of (*S*)-**1**, similarly, the one metabolite isolated from the frass was identified as (*S*)-*p*-menth-1-en-4,7-diol (**2**). The percentage conversion of metabolite (*S*)-**2** was 72%. Substrate (*S*)-**1** and some minor products were detected in the frass by GC analysis. These products were not detected by TLC and GC analysis of the frass of *S. litura* to which substrate was not fed but the artificial diet not containing (*S*)-**1** was. From the above result, it is demonstrated that *S. litura* transformed (*S*)-**1** into (*S*)-**2** and some minor products. These results were similar to those for (*R*)-**1**.

Intestinal Bacteria. A previous paper described the participation of 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 (*R*)- and (*S*)-**1** by intestinal bacteria was also examined in a manner similar to that of the previous paper. However, (*R*)- and (*S*)-**1** were not metabolized at all (no reaction). These results suggested that the intestinal bacteria did not

participate in the metabolism of (*R*)- and (*S*)-**1**. The difference of reaction between (*R*)- and (*S*)-**1** and α-terpinene was suggested to be due to the difference of substrate.

Metabolic Pathways. In the present study of biotransformation of (*R*)- and (*S*)-**1**, the larvae transformed (*R*)-**1** to (*R*)-**2**; similarly, the larvae transformed (*S*)-**1** to (*S*)-**2** (Scheme 1). The C-7 positions (allylic methyl group) of (*R*)- and (*S*)-**1** were preferentially oxidized like the biotransformation of α-terpinene. These results indicate C-7 is the preferred position for oxidation. The larvae did not recognize the difference between (*R*)- and (*S*)-forms. In brief, the influence of the asymmetric C-4 carbon atom between (*R*)- and (*S*)-forms in the bodies of larvae was not observed.

This is only one report on the biotransformation of **1** by other organisms. It seems natural to obtain different metabolites with different species of organisms. The hydroxylation at C-1 and C-2 positions is the main metabolic pathway in the biotransformation of **1** by *Gibberella cyanea* DSM 62719 (*5*). The present study is the first report of the C-7 position (allylic methyl group) of compound **1** being hydroxylated to a high degree of efficiency.

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