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Biotransformation of (1*R*)-(+)- and (1*S*)-(−)-camphor by the larvae of common cutworm (*Spodoptera litura*)

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Abstract

Biotransformation of (1*R*)-(+)- and (1*S*)-(−)-camphor in *Spodoptera litura* larvae has been investigated. (+)- and (−)-Camphor was hydroxylated, regio-selective transformation, (+)-camphor to (1*R*,5*S*)-(+)-5-*endo*-hydroxycamphor, (1*R*,5*R*)-(+)-5-*exo*-hydroxycamphor and (1*R*,7*R*)-(+)-8-hydroxycamphor. Similarly, (−)-camphor was transformed to (1*S*,5*R*)-(−)-5-*endo*-hydroxycamphor, (1*S*,5*S*)-(−)-5-*exo*-hydroxycamphor and (1*S*,7*S*)-(−)-8-hydroxycamphor. C-8 position of (+)- and (−)-camphor being hydroxylated to a high degree of efficiency compared with any other biocatalysts. Intestinal bacteria from the frass of larvae did not participate in the metabolism of (+)- and (−)-camphor.

Keywords: *Spodoptera litura*; Biotransformation; Regio-selective; Hydroxylation; Geminal dimethyl

1. 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. However, it is difficult that these active compounds were produced by organic synthesis. Biotransformation is the biologically synthetic process that using enzymes in the living body as biocatalysts. The characters of biotransformation are as follows: regio- and stereo-selective reaction under mild condition and produced optical active compounds easily. These points suggested that the biotransformation is one of the ways to produce the biologically active terpenoids. Previously, it biotransformed using mainly the mammals and microorganisms.

The investigation in the field of biotransformation of monoterpenoids is gaining more interest: these reactions are performed by bacteria, fungi, yeasts and even algae. However, there are few reports in the literature on the biotransformation of terpenoids by lepidoptera in insects. In the present study, the biotransformation of terpenoids was attempted by the larvae of common cutworm (*Spodoptera litura*). The reasons for using the larvae of *S. litura* as a biological catalyst are as followed: lepidopteran larvae feed on plants contained terpenoids as their diet and, therefore possess a high level of enzymatic activity against terpenoids; the worm consumes a large amount of plants, making it possible to obtain more metabolites; and the worm is easy to rear on a laboratory scale.

Camphor (**1**) is one of the oldest known organic compounds. Many reports on the metabolism of **1** in mammals and microorganisms have been published, e.g. dogs [1,2], rabbits [2–4], *Pseudomonas putida* [4–11], *Rhodococcus rhodocrous* [12,13] and *Streptomyces griseus* [14]. Also there are reports on the biotransformation of **1** by cultured plant cells of Sage (*Salvia officinalis*) [15] and *Eucalyptus perriniana* [16], however, there is no literature on the biotransformation by the insects.

Previously we reported biotransformation of α-terpinene, (+)- and (−)-limonene (*p*-menthane skeleton), and β-myrcene (acyclic monoterpenoid) by the larvae of common cutworm (*S. litura*) [17–19]. These studies revealed that the C-7 position (allylic methyl group) of α-terpinene was preferentially oxidized. Then (+)- and (−)-limonene were oxidized at 8,9-double bond and the C-7 position (allylic methyl group). β-Myrcene was oxidized at the 3,10- and

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1,2-double bonds. There have been no studies on the biotransformation of bicyclic monoterpenoid by the larvae of *S. litura*.

Compound **1** is isolated from the camphor tree (*Cinnamomum camphora*) and one of the major constituents of the essential oil of common sage (*S. officinalis*) [20,21]. It has a camphoraceous oder and is used commercially as a moth repellent, and as a preservative in pharmaceuticals and cosmetics [22].

In the present paper, the biotransformation of (+)- and (−)-camphor (**1**) by the larvae of *S. litura* is being first reported wherein we deal with the insects as biocatalysts, and investigate for the purpose of estimating possible metabolic pathways and make products with high degree in insects.

2. Experimental

2.1. Rearing of larvae

S. litura used in this study were obtained from Nissan Kagaku. It is getting to change the generation of every biotransformation. 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 16:8 L:D (light:dark) photoperiod. A commercial diet (Insecta LFS; Nihon Nosan Kogyo) 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), agar (12 g), and water (600 ml) [23].

2.2. Chemical compounds

The substrates used for the biotransformation experiments were (1*R*)-(+)-camphor (Nacalai Tesque) and (1*S*)-(−)-camphor (Fluka).

2.3. Administration of substrate

The artificial diet without the agar was mixed with a blender. Three thousands milligrams of (+)-**1** was then added directly into the blender. Agar was dissolved in water, boiled and then added into the blender. The diet was then mixed and cooled in a stainless steel tray (220 × 310 mm wide, 30 mm high). The diet containing (+)-**1** was stored in a refrigerator until the time of administration. The fourth to fifth 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 800 larvae were fed the diet containing (+)-**1** (actually 2.1 g, about 2.6 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 every 5 h (total of 4 days) and stored in a solution of diethylether (300 ml). (−)-**1** was administered to 800 larvae in the same manner. For diet and frass separation, the fresh frass was extracted as soon as the fourth to fifth instar larvae excreted.

2.4. Isolation and identification of metabolites from frass

The frass were extracted by diethylether (300 ml × 2) and then ethylacetate (300 ml × 2). Diethylether and ethylacetate extracts were mixed, the solvent was evaporated under reduced pressure, and 3089 mg of extract was obtained (from about 300 g of frass, a larva excreted about 300–400 mg of frass). The extract was dissolved in ethylacetate, and then was added to the 5% $NaHCO_3$ solution. After shaking, neutral fraction (1815 mg) was obtained from the ethylacetate layer. The aqueous layer (acidic fraction) was separated, then acidified with 1 N HCl, and extracted with ethylacetate. After shaking, acidic fraction (1005 mg) was obtained from the aqueous layer. The neutral fraction was analyzed by GC–MS; metabolites (+)-**2**, (+)-**3** and (+)-**4** occurred in this fraction. The acidic fraction was reacted with ethereal CH_2N_2 overnight and subsequently examined by GC–MS, but metabolites from (+)-**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 hexane/diethylether solvent system, and three major metabolites (+)-**2** (498 mg), (+)-**3** (271 mg) and (+)-**4** (348 mg) was isolated. On the other hand, substrate (−)-**1** was transformed to two major metabolites (−)-**2** (469 mg) and (−)-**3** (234 mg) and a minor metabolite (−)-**4** (43 mg). Amount of (−)-**4** was calculated from the peak area in the GC chromatogram of the extract of frass and total extract (2916 mg).

2.4.1. (1R,5S)-(+)-5-endo-hydroxycamphor (**2**)

Colorless crystal, $[\alpha]_D^{24.1}$ +41.5° ($CHCl_3$, *c* 1.09); EI-MS, *m*/*z* (rel. intensity) 168 $[M]^+$ (21), 153 $[M - CH_3]^+$ (100), 150 $[M - H_2O]^+$ (2), 135 $[153 - H_2O]^+$ (15), 125 (15), 111 (68), 109 (42), 108 (87), 107 (83), 93 (62), 83 (30), 81 (29), 69 (47), 57 (19), 55 (63), 41 (85); IR (KBr, υ_{max}, cm^{-1}) 3434 (OH), 1734 (C=O), 1091 (CH_2OH); ^1H NMR ($CDCl_3$) δ 0.86 (3H, s, H-9), 0.87 (3H, s, H-10), 1.01 (3H, s, H-8), 1.24 (1H, dd, J = 4.0, 14.5 Hz, H-6_{endo}), 2.17 (1H, ddd, J = 1.5, 9.5, 14.5 Hz, H-6_{exo}), 2.20 (1H, ddd, J = 2.0, 4.5, 19.5 Hz, H-3_{exo}), 2.18–2.22 (1H, m, H-4), 2.70 (1H, d, J = 19.5 Hz, H-3_{endo}), 4.64 (1H, dddd, J = 2.0, 4.0, 4.5, 9.5 Hz, H-5_{exo}); ^{13}C NMR ($CDCl_3$) δ 9.3 (q, C-10), 19.3 (q, C-8), 20.3 (q, C-9), 34.6 (t, C-3), 40.9 (t, C-6), 47.6 (s, C-7), 48.8 (d, C-4), 59.0 (s, C-1), 69.5 (d, C-5), 218.6 (s, C-2).

2.4.2. (1R,5R)-(+)-5-exo-hydroxycamphor (**3**)

Colorless amorphous crystal, $[\alpha]_D^{24.4}$ +42.2° ($CHCl_3$, *c* 0.32); EI-MS, *m*/*z* (rel. intensity) 168 $[M]^+$ (36), 153 $[M - CH_3]^+$ (15), 150 $[M - H_2O]^+$ (2), 135 $[153 - H_2O]^+$ (3), 125 (38), 111 (100), 109 (22), 107 (23), 93 (11), 83 (37), 69 (48), 55 (53), 41 (66); IR (KBr, υ_{max}, cm^{-1}) 3450 (OH), 1740 (C=O), 1100 (CH_2OH); ^1H NMR ($CDCl_3$) δ 0.85 (3H, s, H-9), 0.93 (3H, s, H-10), 1.25 (3H, s, H-8), 1.70 (1H, d, J = 18.5 Hz, H-3_{endo}), 1.79 (1H, ddd, J = 1.0, 3.5, 14.0 Hz,

H-6$_{exo}$), 1.85 (1H, dd, J = 7.5, 14.0 Hz, H-6$_{endo}$), 2.16 (1H, dd, J = 1.0, 5.0 Hz, H-4), 2.33 (1H, dd, J = 5.0, 18.5 Hz, H-3$_{exo}$), 4.02 (1H, dd, J = 3.5, 7.5 Hz, H-5$_{endo}$); ^{13}C NMR ($CDCl_3$) δ 8.9 (q, C-10), 20.1 (q, C-8), 21.0 (q, C-9), 40.0 (t, C-3), 40.4 (t, C-6), 46.5 (s, C-7), 50.8 (d, C-4), 58.7 (s, C-1), 74.6 (d, C-5), 218.4 (s, C-2).

*2.4.3. (1R,7R)-(+)-8-hydroxycamphor (**4**)*

Colorless crystal, $[\alpha]_D^{24.8}$ +26.9° ($CHCl_3$, *c* 0.70); EI-MS, *m*/*z* (rel. intensity) 168 $[M]^+$ (9), 153 $[M - CH_3]^+$ (1), 137 $[M - CH_2OH]^+$ (12), 109 (18), 108 (37), 95 (100), 91 (10), 79 (15), 67 (22), 55 (16), 43 (27), 41 (27); IR (KBr, υ_{max}, cm^{-1}) 3438 (OH), 1741 (C=O), 1083 (CHOH); ^{1}H NMR ($CDCl_3$) δ 0.97 (6H, s, H-9, 10), 1.42 (1 H, ddd, J = 4.0, 9.0, 12.0 Hz, H-5$_{endo}$), 1.46 (1 H, ddd, J = 4.5, 9.0, 13.5 Hz, H-6$_{endo}$), 1.74 (1 H, ddd, J = 4.0, 12.0, 13.5 Hz, H-6$_{exo}$), 1.90 (1 H, d, J = 18.0 Hz, H-3$_{endo}$), 1.93–2.03 (1 H, m, H-5$_{exo}$), 2.36 (1H, ddd, J = 3.5, 4.5, 18.0 Hz, H-3$_{exo}$), 2.39 (1H, dd, J = 4.0, 4.5 Hz, H-4), 3.52 (1H, d, J = 11.0 Hz, H-8), 3.74 (1H, d, J = 11.0 Hz, H-8′); ^{13}C NMR ($CDCl_3$) δ 10.1 (q, C-10), 14.9 (q, C-9), 26.6 (t, C-5), 29.7 (t, C-6), 39.4 (d, C-4), 42.9 (t, C-3), 51.3 (s, C-7), 57.4 (s, C-1), 64.7 (t, C-8), 218.4 (s, C-2).

*2.4.4. (1S,5R)-(−)-5-endo-hydroxycamphor (**2**)*

Colorless crystal, $[\alpha]_D^{22.9}$ −40.5° ($CHCl_3$, *c* 0.55); the spectral data of the enantiomer (−)-**2** were identical to those of (+)-**2**.

*2.4.5. (1S,5S)-(−)-5-exo-hydroxycamphor (**3**)*

Colorless amorphous crystal, $[\alpha]_D^{25.1}$ −43.9° ($CHCl_3$, *c* 0.38); the spectral data of the enantiomer (−)-**3** were identical to those of (+)-**3**.

*2.4.6. (1S,7S)-(−)-8-hydroxycamphor (**4**)*

This sample was identified by GC–MS and gas chromatography (used chiral column): EI-MS, *m*/*z* (rel. intensity) 168 $[M]^+$ (9), 153 $[M - CH_3]^+$ (2), 137 $[M - CH_2OH]^+$ (12), 109 (19), 108 (37), 95 (100), 91 (10), 79 (15), 67 (22), 55 (16), 43 (25), 41 (24).

2.5. Incubation of intestinal bacteria

This experiment was intentionally carried out under sterile conditions. Petri dishes, pipets, and solutions were autoclaved. A GAM Broth (Nissui Pharmaceutical) was adjusted to pH 8.9 and placed in Petri dishes at 10 ml/Petri dish. The fresh frass (5 g) of the fourth to fifth 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 (20 °C, darkness, 2 days) under aerobic and anaerobic conditions. After growth of bacteria, (+)-**1** (0.3 mg/ml) was added to the medium and the incubation was continued. The medium was distributed between ethylacetate and saturated solution of salt. The ethylacetate layer was evaporated under reduced pressure, 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**.

2.6. General experimental procedures

2.6.1. Gas chromatography (GC)

A Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector, fused silica DB-5 capillary column (30 m length, 0.25 mm i.d.), and a split injection of 25:1 were used. Helium at a flow rate of 30.0 cm/s 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. Enantiomeric excess (e.e. %) of (1*S*,7*S*)-(−)-8-hydroxycamphor (**4**) was detected by chiral column: CHROMPACK WCOAT fused silica CP-Cyclodextrin-β-236-M-19 (50 m length, 0.25 mm i.d.), and a split injection of 40:1 were used. Helium at a flow rate of 36.0 cm/s was used as a carrier gas. The oven temperature was held at 140 °C for 60 min. The injector and detector temperatures were 270 and 280 °C. The peak area was integrated with a Hewlett-Packard HP3396 series II integrator.

2.6.2. Gas chromatography/mass spectrometry (GC/MS)

A Hewlett-Packard 5890A gas chromatograph equipped with a split injector, an HP-5MS capillary column (30 m length, 0.25 mm i.d.) 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 30.0 cm/s was used as a carrier gas. The temperature of the ion source was 230 °C, and the electron energy was 70 eV. The electron ionization (EI) mode was used.

2.6.3. 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.

2.6.4. 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 (δ 0.00).

2.6.5. Specific rotation

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

3. Results and discussion

3.1. Biotransformation of (1R)-(+)-camphor by the larvae of Spodoptera litura

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 [17]. Although alcohols were detected by GC analysis, intermediary metabolites (epoxides and aldehydes) and acids were not isolated. This suggested that they were hardly excreted into the frass. In the present study, a concentration of 4 mg/g of diet was, therefore chosen as optimum for administration (i.e., the concentration consumes a substrate completely). The larvae that were fed the diet without substrate were used as control, and the extract of frass was analyzed by GC. Compounds **1**–**4** and unidentified metabolites were not observed in the frass of controls. For the consumption of substrate in the diet observed, we varied the quantity of substrate in the diet by the internal standard method in GC. The result was that consumption of (+)-camphor (**1**) was 93.1%. The result for (−)-**1** was 95.7%.

In the biotransformation of (+)-**1**, the three major metabolites isolated from the frass were identified as (1*R*,5*S*)-(+)-5-*endo*-hydroxycamphor (**2**), (1*R*,5*R*)-(+)-5-*exo*-hydroxycamphor (**3**) and (1*R*,7*R*)-(+)-8-hydroxycamphor (**4**). Percentage of substrate **1** and metabolites **2**, **3** and **4** in frass extract were 6.9±1.4, 34.4±2.6, 18.7±3.9 and 24.1±1.8%, respectively (Table 1).

The metabolite **2** had a molecular formula of $C_{10}H_{16}O_2$ that was estimated by its EI-MS spectral. The IR spectrum contained a new hydroxyl band at 3434 cm^{-1}. ^1H and ^{13}C NMR spectra were assigned by comparison with the spectral date for **1** and the previous paper [4,24–26]. ^1H NMR evidence for the presence of three methyl groups. A coupling of 2.0 Hz between H-3$_{exo}$ (2.20 ppm) and H-5$_{exo}$ (4.64 ppm) indicates the long-range coupling. In addition, a coupling of 1.5 Hz between H-4 (2.18–2.22 ppm) and H-6$_{exo}$ (2.17 ppm) indicates the *W*-relationship of these protons. The hydroxylated methane proton observed at H-5$_{exo}$ (4.64 ppm) was coupled with H-3$_{exo}$ (J = 2.0 Hz), H-4 (J = 4.5 Hz), H-6$_{endo}$ (J = 4.0 Hz) and H-6$_{exo}$ (J = 9.5 Hz), so that configuration of the hydroxyl group at C-5 was *endo*. As the conformation, assignment of the three methyl signals was achieved by NOE. Irradiation of the signal at 2.20 ppm (H-3$_{exo}$) caused the enhancement of the signal due to the H-9, while irradiation of the signal at 4.64 ppm (H-5$_{exo}$) enhanced the H-8. Thus, assignment of the signals at 0.86, 1.01 and 0.87 ppm to be made to C-9, C-8 and C-10, respectively. To determine the structure of C-3 and C-6 two methine parts and C-8 and C-9 two methyl parts of **2**, HMQC was measured. Correlation cross-peaks were observed between 2.70 and 2.20 ppm (H-3$_{endo}$, 3$_{exo}$, respectively) and C-3 (34.6 ppm), 2.17 and 1.24 ppm (H-6$_{exo}$, 6$_{endo}$, respectively) and C-6 (40.9 ppm), 1.01 ppm (H-8) and C-8 (19.3 ppm), and 0.86 ppm (H-9) and C-9 (20.3 ppm), so that was established. The specific rotation shows the (+)-form. From these date it was concluded that the structure of **2** is (1*R*,5*S*)-(+)-5-*endo*-hydroxycamphor.

Metabolite **3** had a molecular formula of $C_{10}H_{16}O_2$ based on its EI-MS spectral. The IR spectrum contained a new hydroxyl band at 3450 cm^{-1}. ^1H and ^{13}C NMR spectra were compared with those of the closely related compound **1**, which has been reported in the literature [4,24–28]. ^1H NMR evidence for the presence of three methyl groups. A coupling of 1.0 Hz between H-4 (2.16 ppm) and H-6$_{exo}$ (1.79 ppm) indicates the long-range coupling. The hydroxylated methane proton observed at H-5$_{endo}$ (4.02 ppm) was coupled with H-6$_{exo}$ (J = 3.5 Hz) and H-6$_{endo}$ (J = 7.5 Hz), so that configuration of the hydroxyl group at C-5 was *exo*. As the conformation, assignment of the three methyl signals was achieved by NOE. Irradiation of the signal at 2.33 ppm (H-3$_{exo}$) caused the enhancement of the signal due to the H-9, while irradiation of the signal at 1.79 ppm (H-6$_{exo}$) enhanced the H-8. Thus, assignment of the signals at 0.85, 1.25 and 0.93 ppm to be made to C-9, C-8 and C-10, respectively. To determine the structure of C-3 and C-6 two methine parts and C-8 and C-9 two methyl parts of **3**, HMQC was measured. Correlation cross-peaks were observed between 2.33 and 1.70 ppm (H-3$_{exo}$, 3$_{endo}$, respectively) and C-3 (40.0 ppm), 1.79 and 1.85 ppm (H-6$_{exo}$, 6$_{endo}$, respectively) and C-6 (40.4 ppm), 1.25 ppm (H-8) and C-8 (20.1 ppm), and 0.85 ppm (H-9) and C-9 (21.0 ppm), so that was established. The specific rotation shows the (+)-form. From the above date, it was concluded that the structure of **3** is (1*R*,5*R*)-(+)-5-*exo*-hydroxycamphor.

About metabolite **4**, the molecular formula was estimated as $C_{10}H_{16}O_2$, based on its EI-MS spectrum. The IR spectrum contained a new hydroxyl band at 3438 cm^{-1}. ^1H and ^{13}C NMR spectra were assigned by comparison with the

Table 1
Metabolites of (+)- and (−)-camphor (**1**) by the S. *litura* larvae[a]

Substrate	Yield[b] (%)				
	1[c]	**2**	**3**	**4**	Unidentified metabolites[d]
(+)-Camphor (**1**)	6.9 ± 1.4	34.4 ± 2.6	18.7 ± 3.9	24.1 ± 1.8	16.1
(−)-Camphor (**1**)	4.3 ± 2.4	46.3 ± 3.3	23.1 ± 4.0	4.5 ± 1.2	21.8

[a] Metabolites were obtained from the frass of *S. litura*. One group is contained 15 larvae. The date represent means ± S.E. of five determinations (n = 5).
[b] Percentage was calculated from the peak area in the gas chromatogram of the extract of frass.
[c] Recovered substrate.
[d] Analyzed by GC–MS.

previous paper [25,26,28–32]. The NMR spectra of **4** were similar to those of **1** except for the existence of hydroxyl group and the disappearance of a methyl group. The ^{1}H NMR spectrum showed two methyl groups located at δ 0.97 ppm. The two doublets located at δ 3.52 and 3.74 ppm ($J = 11.0$ Hz), these spectrum is characteristic of a CH_2OH group. For the purpose of determine the structure of geminal dimethyl of C-8 and C-9 position, NOESY was measured. The presence of the CH_2OH group attached to different side (C-8) as the C=O group, correlation H-3$_{exo}$ and H-9, and H-5$_{exo}$ and H-8. All other couplings are as expected for metabolite **4** was produced by hydroxylation at the C-8 position of geminal dimethyl of **1**. Further, assignment of the three ddd signals (δ_H 1.42, 1.46 and 1.74 ppm) were achieved by HMQC. Correlation cross-peaks were observed between 26.6 ppm (C-5) and H-5$_{exo}$, 5$_{endo}$ (1.93–2.03 and 1.42 ppm, respectively), and 29.7 ppm (C-6) and H-6$_{exo}$, 6$_{endo}$ (1.74 and 1.46 ppm, respectively), so that was established. The specific rotation showed the (+)-form, these date indicates **4** is (1*R*,7*R*)-(+)-8-hydroxycamphor.

On the other hand, there are unidentified metabolites. One of the metabolites has molecular formula was estimated as $C_{10}H_{16}O_2$, based on its EI-MS spectrum. Therefore, hydroxyl was supposed to have been introduced in substrate. Other metabolites were not inferences.

3.2. Biotransformation of (1S)-(−)-camphor by the larvae of Spodoptera litura

In the biotransformation of (−)-**1**, the two major metabolites isolated from the frass were identified as (1*S*,5*R*)-(−)-5-*endo*-hydroxycamphor (**2**) and (1*S*,5*S*)-(−)-5-*exo*-hydroxycamphor (**3**). On the other hand, a minor metabolite exists. One of the minor metabolite has led to its assignment as 8-hydroxycamphor, with supporting structural evidence provided by the mass spectral fragmentation pattern [28,30]. The fragmentation pattern are consistent with (+)-8-hydroxycamphor. As the confirmation, enantiomeric excess (e.e. %) was identified GC (used chiral column). The result indicate that **4** is an enantiomerically pure compound (e.e. 100%). The presence of substance **1** and metabolites **2**, **3** and **4** in frass extract were 4.3 ± 2.4, 46.3 ± 3.3, 23.1 ± 4.0 and 4.5 ± 1.2%, respectively (Table 1).

The spectral data of the enantiomer (−)-**2** and (−)-**3** were identical to those of (+)-**2** and (+)-**3**, respectively and comparison with previous paper [4,24–28]. The specific rotation showed that (−)-**2** ($[\alpha]_D^{22.9}$ −40.5° ($CHCl_3$, *c* 0.55)) and (−)-**3** ($[\alpha]_D^{25.1}$ −43.9° ($CHCl_3$, *c* 0.38)) were the (−)-form. These spectral data suggested that metabolite (−)-**2** and (−)-**3** were (1*S*,5*R*)-(−)-5-*endo*-hydroxycamphor and (1*S*,5*S*)-(−)-5-*exo*-hydroxycamphor, respectively.

On the other hand, there are unidentified metabolites. One of the metabolites has a molecular formula which was estimated as $C_{10}H_{16}O_2$, based on its EI-MS spectrum. Therefore, hydroxyl was supposed to have been introduced in substrate. Other metabolites were not inferences.

3.3. Study of the difference among the individuals of larva

The diet which was prepared same manner was fed to 15 larvae (fourth to fifth instar). The frass was collected and extracted with diethylether and then ethylacetate. Metabolites were identified from the retention time and calculated from the peak area in the GC chromatogram of the extract of frass. It did this experiment to five groups. However, there were few differences of each group (Table 1). These results suggested that the biotransformation of (+)- and (−)-camphor by the larvae of *S. litura* has reappeared.

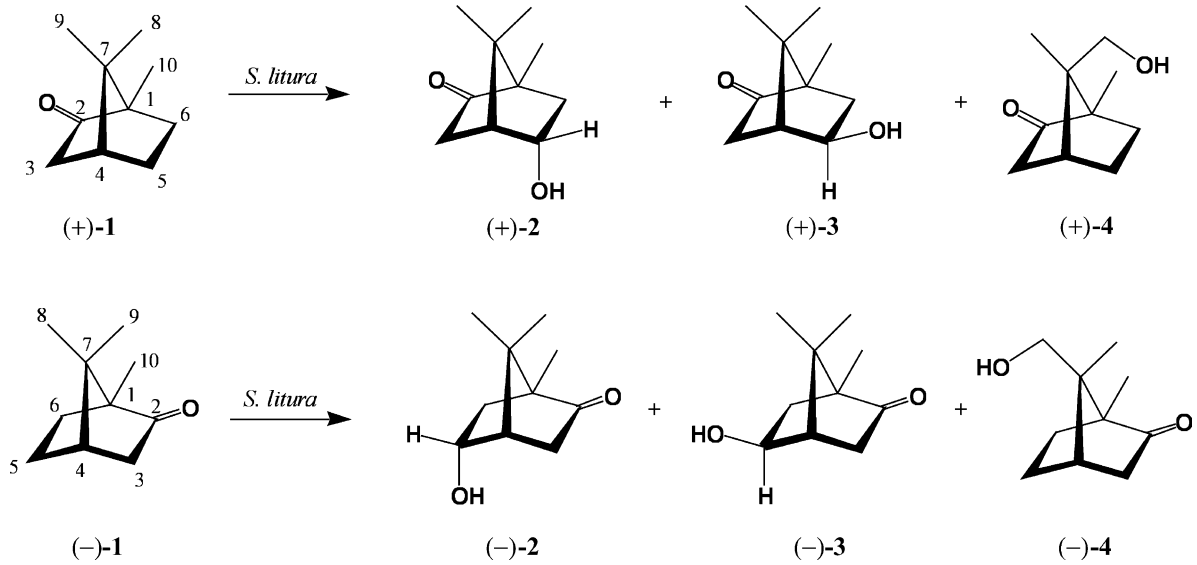
3.4. Biotransformation of (+)- and (−)-camphor by the intestinal bacteria of Spodoptera litura

A previous paper described the participation of aerobically and anaerobically active intestinal bacteria in the metabolism of α-terpinene [17]. 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) both aerobic and anaerobic condition. These results suggested that the intestinal bacteria did not participate in the metabolism of (+)- and (−)-**1**. The difference of reaction between (+)- and (−)-**1** and α-terpinene was suggested to be due to the difference of substrate.

3.5. Study of the metabolic pathways

In the present study of biotransformation of (+)- and (−)-**1**, the larvae transformed (+)-**1** to mainly (+)-**2** (34.4 ± 2.6%), (+)-**3** (18.7 ± 3.9%) and (+)-**4** (24.1 ± 1.8%); on the other hand, the larvae transformed (−)-**1** to mainly (−)-**2** (46.3 ± 3.3%), (−)-**3** (23.1 ± 4.0%) and a minor compound (−)-**4** (4.5 ± 1.2%) (Scheme 1, Table 1). Compounds **2** and **3** account for the rate with the equal degree to the total metabolites of (+)- and (−)-**1**. However, compound **4** is remarkable in having different percentages between (+)- and (−)-**1**. These results suggested that (i) both compounds were preferentially hydroxylated at C-5$_{endo}$ position and (ii) stereoselective *gem*-dimethyl hydroxylation was in progress in the metabolism of **1** by the larvae of *S. litura*. Previously, we reported the biotransformation of *p*-menthan and acyclic monoterpenoids, C-7 position (allylic methyl group), 8,9-double bond and the double bond of the side chain (8,9-double bond) were preferentially hydroxylated, respectively [17–19]. In this case, (+)- and (−)-**1** are hydroxylated at C-5 position and methyl group of geminal dimethyl (C-8 position). These results indicate C-5 position and C-8 position are rather the preferred position for hydroxylation. These are far away from the C=O group.

Compound **1** is the most important and widespread terpene known; many reports on the metabolism of **1** in mammals and microorganisms have been published. The hydroxylation step of this biotransformation is probably



Scheme 1. Possible metabolic pathway of (+)- and (−)-camphor (**1**) by the larvae of *S. litura*.

catalyzed by a cytochrome P450-dependent monooxygenase as in the case of mammals and microorganisms. The regio- and stereo-selectivity of hydroxylation of camphor is different main metabolite. It seems natural to obtain different metabolites with different species of organisms [1–16]. In dogs, after feeding camphor, from the urine, a glycosidic substance was obtained which was shown to consist of a mixture of glucuronides of hydroxycamphor. Primary metabolite was 5-*exo*-hydroxycamphor [1,2]. Literature identified the hydroxycamphors obtained from the hydrolyzed urine of rabbits that had been fed (+)- and (−)-camphor as 3- and 5-*endo*-hydroxycamphor, they also demonstrated the reduction of (+)-camphor to (+)-borneol in vivo in rabbits [2–4]. In *P. putida*, which can employ (+)-camphor as the sole carbon source, cleavage of the bicyclic skeleton of the monoterpene is initiated by hydroxylation at C-5$_{exo}$ position with the subsequent formation of 5-oxocamphor followed by lactonization reactions; in some instances lactonization may precede C-5-hydroxylation. *P. putida* produced 8-hydroxycamphor as a minor metabolite, however others did not [4–11]. In the present study, the main product converted by the larvae of *S. litura* is the same as in rabbit; therefore, the larvae of *S. litura* employ a metabolic pathway similar to that used by the rabbit [2–4]. Compound **1** is hydroxylated at C-5$_{endo}$, C-5$_{exo}$ and C-8 position (methyl group of *gem*-dimethyl). In particular, characteristic hydroxylation is C-8 position of **1** by the larvae of *S. litura* comparision with other organisms. The present study is the first report of C-8 position of (+)-**1** being hydroxylated to a high degree of efficiency.

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