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Journal of Molecular Catalysis B: Enzymatic 32 (2005) 123–130
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

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

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Received 16 January 2004; received in revised form 2 August 2004; accepted 9 August 2004
Available online 18 December 2004

Abstract

Biotransformation of (+)-(1*R*)- and (−)-(1*S*)-fenchone in *Spodoptera litura* larvae has been investigated. (+)- and (−)-Fenchone were regio- and stereo-selective hydroxylated. (+)-Fenchone was transformed to one new terpenoid, (+)-(1*S*,4*R*)-3-oxo-2,2,4-trimethyl-cyclopentylacetic acid and four known terpenoids, (+)-(1*S*,6*S*)-6-*exo*-hydroxyfenchone, (+)-(1*S*,6*R*)-6-*endo*-hydroxyfenchone, (+)-(1*R*)-10-hydroxyfenchone and (+)-(1*S*,5*R*)-5-*exo*-hydroxyfenchone. (−)-Fenchone was transformed to one new terpenoid, (−)-(1*S*)-10-hydroxyfenchone and four known terpenoids, (−)-(1*R*,6*R*)-6-*exo*-hydroxyfenchone, (−)-(1*R*,6*S*)-6-*endo*-hydroxyfenchone, (−)-(1*R*,5*S*)-5-*exo*-hydroxyfenchone and (−)-(1*R*,4*S*)-3-oxo-2,2,4-trimethyl-cyclopentylacetic acid. C-6 position of (+)- and (−)-fenchone was progressing to the carboxylic acid, which is characteristically metabolic pathway compared with any other biocatalysts. Intestinal bacteria from the frass of larvae did not participate in the metabolism of (+)- and (−)-fenchone.

Keywords: *Spodoptera litura*; Biotransformation; Regioselective; Stereoselective; Hydroxylation

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, using enzymes in the living body as biocatalysts. The characters of biotransformation are as follows: regio- and stereo-selective reaction under mild condition and producing optically active compounds. These points suggested that the biotransformation is easy method for production of the biologically active terpenoids.

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 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 that 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.

Fenchone (**1**) is one of the oldest known organic compounds [1]. Compound (+)-**1** is obtained from fennel oil (*Foeniculum vulgare*), and (−)-**1** from Thuja oil (*Thuja occidentalis*). They are used as a food flavour and in perfumes. There are several reports on the biotransformation of **1** by microorganisms and mammals, e.g. Baeyer–Villiger

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doi:10.1016/j.molcatb.2004.08.008

type oxidation of **1** by *Corynebacterium* sp. [2], 5-*exo* and 6-*exo* hydroxylation of **1** by *Absidia orchidis* [3], 5-*exo*, 6-*exo* and C-7 glucosylation of **1** by cultured plant cells of *Eucalyptus perriniana* [4], 5-*endo* and 6-*endo* hydroxylation of **1** by *Aspergillus nigar* [5] and hydroxylation of methyl groups of **1** by rabbits [6]. However, there is no report on the biotransformation of **1** by the insects.

We have reported that the larvae of common cutworm (*S. litura*) are able to biotransform the monoterpenes, e.g. α-terpinene [7], β-myrcene [8], α-terpineol [9] and (+)- and (−)-camphor [10], into their corresponding oxidized products. It was interesting whether regio-selectivity of the hydroxylation was controlled by allylic methyl group or oxygen functions.

In the present paper, the biotransformation of (+)- and (−)-fenchone (**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 new products in insects.

2. Experimental

2.1. Rearing of larvae

S. litura used in this study were obtained from Nissan Kagaku. The larvae of *S. litura* were reared in plastic cases (200 mm × 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 h light:8 h 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) [11].

2.2. Chemical compounds

The substrates used for the biotransformation experiments were (+)-(1*R*)-fenchone (Wako) and (−)-(1*S*)-fenchone (Wako).

2.3. General experimental procedures

Gas chromatography (GC) was performed on a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector (FID). The column was a fused silica capillary column (DB-5, 30 m length, 0.25 mm i.d.). Chromatographic conditions were as follows: oven temperature was programmed from 80 to 240 °C at 4 °C/min; injector and detector temperatures were 270 and 280 °C, respectively; split injection of 25:1; flow rate of helium gas is at 30.0 cm/s. Enantiomeric excess (e.e.%) of (−)-(1*R*,6*S*)-6-*endo*-hydroxyfenchone (**3**), (+)-(1*S*,5*R*)-5-*exo*-hydroxyfenchone (**5**) and (−)-(1*R*,4*S*)-methyl-3-oxo-2,2,4-trimethyl-cyclo pentylacetate (**6Me**) were detected by chiral column: CHROMPACK WCOAT fused silica CP-Cyclodextrin-β-236-M-19 (50 m length, 0.25 mm i.d.). Chromatographic conditions were as follows: oven temperature was held at 140 °C for 60 min; injector and detector temperatures were 270 and 280 °C, respectively; split injection of 40:1; flow rate of helium gas is at 36.0 cm/s. The peak area was integrated with a Hewlett-Packard HP3396 series II integrator. EI-MS measurements were obtained using gas chromatography–mass spectrometry (GC–MS). GC–MS was performed on a Hewlett-Packard 5972A mass selective detector interfaced with a Hewlett-Packard 5890A gas chromatograph fitted with a capillary column (HP-5MS, 30 m length, 0.25 mm i.d.). Chromatographic condition were the same as described above DB-5. The temperature of the ion source was 230 °C, and the electron energy was 70 eV. The IR spectra were obtained with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer. $CHCl_3$ was used as a solvent. The NMR spectra were obtained with a JEOL FX-500 (500.00 MHz, ^1H; 125.65 MHz, ^{13}C) spectrometer. Tetramethylsilane (TMS) was used as the internal standard in $CDCl_3$. Multiplicities were determined by the DEPT pulse sequence. The specific rotations were measured on a JASCO DIP-1000 digital polarimeter.

2.4. Administration of substrate

The artificial diet without the agar was mixed with a blender. Substrate **1** (3000 mg) 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 mm × 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 1.6 g, about 2.0 mg for a body) for 2 days, and then the artificial diet not containing (+)-**1** was fed to the larvae for additional 2 days. Frass was collected every 5 h (total 4 days) and stored in a solution of diethylether (300 mL). (−)-**1** was administered to 800 larvae in the same manner. The diet and frass was separated. The fresh frass was extracted as soon as the fourth to fifth instar larvae excreted.

2.5. Isolation and identification of metabolites from frass

The frass were extracted with diethylether (2× 300 mL) and then ethylacetate (2× 300 mL). Diethylether and ethylacetate extracts were mixed, the solvent was evaporated under reduced pressure, and 2970 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 (1644 mg) was obtained from the ethylacetate layer. The aqueous layer was separated, then acidified with 1N HCl (acidic fraction), and extracted with

ethylacetate. After shaking, acidic fraction (1061 mg) was obtained from the aqueous layer. Both of these two fractions were analyzed by GC–MS; metabolites (+)-**2**, (+)-**3**, (+)-**4** and (+)-**5** existed in neutral fraction and metabolite (+)-**6** existed in acidic fraction. The acidic fraction was reacted with ethereal CH_2N_2 overnight and subsequently analyzed by GC–MS, results indicates the existence of (+)-**6Me**. The neutral and acidic fraction were subjected to silica gel open-column chromatography (silica gel 60, 230–400 mesh, Merck) with a 9:1 hexane/diethylether solvent system, and three metabolites (+)-**2** (604 mg), (+)-**3** (155 mg) and (+)-**4** (53 mg) were isolated from neutral fraction and (+)-**6Me** (134 mg) was isolated from acidic fraction. Moreover, minor metabolite (+)-**5** was estimated by GC–MS and retention time in the GC chromatogram (used chiral column) from neutral fraction. On the other hand, substrate (−)-**1** was transformed to mainly (−)-**2** (230 mg), (−)-**4** (509 mg) and (−)-**5** (184 mg). Minor metabolites (−)-**3** and (−)-**6** were identified from GC–MS and retention time in the GC chromatogram (used chiral column). Amount of minor metabolites were calculated from the peak area in the GC chromatogram of the extract of frass (total extract; 3074 mg).

*2.5.1. (+)-(1S,6S)-6-exo-Hydroxyfenchone (**2**)*

White crystal, $[\alpha]_D^{20.0}$ + 28.1° ($CHCl_3$, *c* 1.00); HR-EI-MS, *m/z* 168.1155 $[M]^+$, calcd. for $C_{10}H_{16}O_2$, 168.1151; EI-MS, *m/z* (rel. intensity) 168 $[M]^+$ (18), 153 $[M–CH_3]^+$ (3), 140 (3), 124 (41), 109 (16), 107 (11), 97 (55), 96 (35), 88 (34), 81 (100), 70 (37), 69 (30), 55 (12), 53 (10), 43 (12), 41 (33); IR (KBr, ν_{max}, cm^{-1}) 3455, 2965, 1736, 1063; ^1H NMR ($CDCl_3$) δ 0.96 (3H, s, H-9), 1.06 (3H, s, H-8), 1.14 (3H, s, H-10), 1.55 (1H, ddd, *J* = 3.5, 4.5, 13.8 Hz, H-5$_{exo}$), 1.76 (1H, dddd, *J* = 1.2, 1.5, 2.9, 10.9 Hz, H_A-7; nearly C-2,3 position), 1.90 (1H, dd, *J* = 1.7, 10.9 Hz, H_B-7; nearly C-5,6 position), 2.18–2.20 (1H, m, H-4), 2.40 (1H, ddd, *J* = 2.9, 6.9, 13.8 Hz, H-5$_{endo}$), 3.62 (1H, ddd, *J* = 1.5, 3.5, 6.9 Hz, H-6$_{endo}$); ^{13}C NMR (see Table 2).

*2.5.2. (−)-(1R,6R)-6-exo-Hydroxyfenchone (**2**)*

White crystal, $[\alpha]_D^{20.0}$ −26.6° ($CHCl_3$, *c* 1.01); the spectral data of the enantiomer (−)-**2** were identical to those of (+)-**2**.

*2.5.3. (+)-(1S,6R)-6-endo-Hydroxyfenchone (**3**)*

White crystal, $[\alpha]_D^{19.5}$ + 60.5° ($CHCl_3$, *c* 0.38); HR-EI-MS, *m/z* 168.1144 $[M]^+$, calcd. for $C_{10}H_{16}O_2$, 168.1151; EI-MS, *m/z* (rel. intensity) 168 $[M]^+$ (2), 153 $[M–CH_3]^+$ (1), 140 (1), 124 (8), 109 (4), 107 (5), 97 (9), 88 (35), 81 (100), 69 (21), 55 (7), 53 (6), 43 (6), 41 (19); IR (KBr, ν_{max}, cm^{-1}) 3448, 2965, 1724, 1052; ^1H NMR ($CDCl_3$) δ 1.10 (3H, s, H-8), 1.12 (3H, s, H-9), 1.16 (3H, s, H-10), 1.62 (1H, dd, *J* = 1.7, 11.0 Hz, H_B-7; nearly C-5,6 position), 1.74 (1H, ddd, *J* = 2.6, 3.7, 13.8 Hz, H-5$_{endo}$), 1.85 (1H, ddd, *J* = 1.7, 2.6, 11.0 Hz, H_A-7; nearly C-2,3 position), 2.10–2.12 (1H, m, H-4), 2.15 (1H, ddd, *J* = 4.3, 9.6, 13.8 Hz, H-5$_{exo}$), 4.09 (1H, ddd, *J* = 1.7, 3.7, 9.6 Hz, H-6$_{exo}$); ^{13}C NMR (see Table 2).

*2.5.4. (−)-(1S)-10-Hydroxyfenchone (**4**)*

Colorless oil, $[\alpha]_D^{20.0}$ − 30.3° ($CHCl_3$, *c* 0.82); HR-EI-MS, *m/z* 168.1167 $[M]^+$, calcd. for $C_{10}H_{16}O_2$, 168.1151; EI-MS, *m/z* (rel. intensity) 168 $[M]^+$ (16), 153 $[M–CH_3]^+$ (2), 150 $[M–H_2O]^+$ (2), 135 $[153–H_2O]^+$ (0.5), 109 (22), 107 (23), 97 (71), 81 (71), 79 (61), 70 (58), 69 (100), 55 (24), 53 (18), 43 (29), 41 (86); IR (KBr, ν_{max}, cm^{-1}) 3447, 2968, 1736, 1040; ^1H NMR ($CDCl_3$) δ 1.05 (3H, s, H-9), 1.07 (3H, s, H-8), 1.47 (1H, dddd, *J* = 1.9, 5.5, 9.1, 12.5 Hz, H-6$_{endo}$), 1.49 (1H, dd, *J* = 1.7, 10.6 Hz, H_B-7; nearly C-5,6 position), 1.67 (1H, ddd, *J* = 3.2, 11.8, 12.5 Hz, H-6$_{exo}$), 1.74 (1H, dddd, *J* = 4.0, 5.5, 11.8, 12.2 Hz, H-5$_{exo}$), 1.84 (1H, dddd, *J* = 2.0, 3.2, 9.1, 12.2 Hz, H-5$_{endo}$), 2.01 (1H, dddd, *J* = 1.9, 1.9, 2.0, 10.6 Hz, H_A-7; nearly C-2,3 position), 2.20–2.23 (1H, m, H-4), 3.78 (1H, d, *J* = 11.8 Hz, H-10), 3.86 (1H, d, *J* = 11.8 Hz, H′-10); ^{13}C NMR (see Table 2).

*2.5.5. (+)-(1R)-10-Hydroxyfenchone (**4**)*

Colorless oil, $[\alpha]_D^{20.0}$ + 32.6° ($CHCl_3$, *c* 0.88); the spectral data of the enantiomer (+)-**4** were identical to those of (−)-**4**.

*2.5.6. (−)-(1R,5S)-5-exo-Hydroxyfenchone (**5**)*

White powder, $[\alpha]_D^{21.0}$ −15.2° ($CHCl_3$, *c* 0.39); HR-EI-MS, *m/z* 168.1155 $[M]^+$, calcd. for $C_{10}H_{16}O_2$, 168.1151; EI-MS, *m/z* (rel. intensity) 168 $[M]^+$ (11), 153 $[M–CH_3]^+$ (0.5), 150 $[M–H_2O]^+$ (4), 135 $[150–CH_3]^+$ (0.5), 122 (4), 121 (15), 109 (2), 107 (3), 97 (18), 85 (100), 81 (7), 79 (7), 72 (5), 69 (7), 55 (7), 53 (5), 43 (7), 41 (20); IR (KBr, ν_{max}, cm^{-1}) 3447, 2963, 1738, 1078; ^1H NMR ($CDCl_3$) δ 1.03 (3H, s, H-9), 1.06 (3H, s, H-8), 1.15 (3H, s, H-10), 1.46 (1H, ddd, *J* = 1.4, 1.8, 14.3 Hz, H-6$_{exo}$), 1.74 (1H, dddd, *J* = 1.5, 1.7, 2.3, 10.9 Hz, H_A-7; nearly C-2,3 position), 1.89 (1H, ddd, *J* = 2.3, 6.3, 14.3 Hz, H-6$_{endo}$), 2.07 (1H, dd, *J* = 2.0, 10.9 Hz, H_B-7; nearly C-5,6 position), 2.13–2.16 (1H, m, H-4), 4.38 (1H, ddd, *J* = 1.7, 1.8, 6.3 Hz, H-5$_{endo}$); ^{13}C NMR (see Table 2).

*2.5.7. (+)-(1S,4R)-Methyl-3-oxo-2,2,4-trimethyl-cyclopentylacetate (**6Me**)*

Colorless oil, $[\alpha]_D^{20.2}$ + 35.6° ($CHCl_3$, *c* 0.43); HR-EI-MS, *m/z* 198.1242 $[M]^+$, calcd. for $C_{11}H_{18}O_3$, 198.1256; EI-MS, *m/z* (rel. intensity) 198 $[M]^+$ (65), 183 $[M–CH_3]^+$ (41), 167 $[M–OCH_3]^+$ (21), 128 (100), 124 (36), 109 (15), 96 (63), 69 (95), 68 (51), 55 (21), 41 (39); IR (KBr, ν_{max}, cm^{-1}) 2965, 1738, 1458, 1383, 1219, 1167; ^1H NMR ($CDCl_3$) δ 0.82, 1.06 (each 3H, s, H-6, 7), 1.15 (1H, d, *J* = 7.2 Hz, H-8), 1.20 (1H, dd, *J* = 10.9, 12.6 Hz, H-5), 2.17–2.24 (2H, m, H-1, 4), 2.25 (1H, dd, *J* = 9.5, 13.4 Hz, H-9), 2.34 (1H, ddd, *J* = 5.2, 8.6, 12.6 Hz, H-5), 2.48 (1H, dd, *J* = 3.7, 13.4 Hz, H-9); ^{13}C NMR (see Table 3).

Methyl ester **6Me** (12 mg) was dissolved in 1N NaOH (10 mL), and the solution was stirred for 3 h at room temp

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

Substrate	Yield[b] (%)						
	1[c]	2	3	4	5	6	Unidentified metabolites[d]
(+)-Fenchone (1)	0.5 ± 0.3	61.5 ± 0.9	15.6 ± 2.9	4.5 ± 1.4	2.5 ± 2.2	11.9 ± 1.8	3.5
(−)-Fenchone (1)	0.3 ± 0.3	19.5 ± 2.1	13.5 ± 1.4	42.1 ± 0.8	15.6 ± 3.8	2.9 ± 2.7	6.1

[a] Metabolites were obtained from the frass of *S. litura*. One group contains 15 larvae. The data 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.

Table 2
^{13}C NMR spectral data for metabolites (**2–5**) (125.00 MHz, $CDCl_3$)

Carbon	Compounds			
	2	**3**	**4**	**5**
1	60.8 (s)	60.1 (s)	60.0 (s)	53.6 (s)
2	222.7 (s)	220.8 (s)	224.0 (s)	222.1 (s)
3	46.9 (s)	48.0 (s)	48.0 (s)	45.3 (s)
4	44.0 (d)	43.9 (d)	45.2 (d)	53.2 (d)
5	37.3 (t)	34.6 (t)	24.1 (t)	70.7 (d)
6	72.0 (d)	77.4 (d)	27.1 (t)	43.2 (t)
7	37.2 (t)	41.0 (t)	37.4 (t)	37.4 (t)
8	23.6 (q)	23.9 (q)	23.1 (q)	23.6 (q)
9	21.2 (q)	20.8 (q)	21.4 (q)	21.3 (q)
10	10.5 (q)	12.6 (q)	62.6 (t)	14.2 (q)

(25 °C). The product was isolated in the usual manner and obtained metabolite **6** (10 mg).

*2.5.8. (+)-(1S,4R)-3-oxo-2,2,4-Trimethyl-cyclopentylacetic acid (**6**)*

Colorless oil, $[\alpha]_D^{17.4}$ + 36.7° ($CHCl_3$, *c* 0.50); HR-EI-MS, *m/z* 184.1095 $[M]^+$, calcd. for $C_{10}H_{16}O_3$, 184.1100; EI-MS, *m/z* (rel. intensity) 184 $[M]^+$ (37), 169 $[M–CH_3]^+$ (22), 156 (3), 151 $[M–H_2O]^+$ (2), 137 (3), 124 (11), 114 (77), 96 (15), 81 (14), 69 (100), 55 (29), 41 (51); IR (KBr, ν_{max}, cm^{-1}) 3300-2500, 2965, 1738, 1459, 1303, 1240, 1170, 900; ^{1}H NMR ($CDCl_3$) δ 0.83, 1.09 (each 3H, s, H-6, 7), 1.14 (1H, d, *J* = 7.2 Hz, H-8), 1.22 (1H, dd, *J* = 9.9, 11.8 Hz, H-5), 2.19–2.29 (2H, m, H-1, 4), 2.29 (1H, dd, *J* = 10.1, 14.7 Hz, H-9), 2.41 (1H, ddd, *J* = 5.9, 8.6, 11.8 Hz, H-5), 2.54 (1H, dd, *J* = 4.3, 14.7 Hz, H-9); ^{13}C NMR (see Table 3).

Table 3
^{13}C NMR spectral data for metabolites (**6** and **6Me)** (125.00 MHz, $CDCl_3$)

Carbon	Compounds	
	6	**6Me**
1	41.8 (d)	42.1 (d)
2	47.4 (s)	47.3 (s)
3	223.7 (s)	223.8 (s)
4	43.0 (d)	43.0 (d)
5	34.5 (t)	34.6 (t)
6 or 7	18.6 (q), 23.3 (q)	18.5 (q), 23.2 (q)
8	15.0 (q)	15.0 (q)
9	34.5 (t)	34.6 (t)
10	178.6 (s)	173.2 (s)
COOMe		51.7 (q)

*2.5.9. (−)-(1R,6S)-6-endo-Hydroxyfenchone (**3**), (+)-(1S,5R)-5-exo-hydroxyfenchone (**5**), (−)-(1R,4S)-3-oxo-2,2,4-trimethyl-pentylacetic acid (**6**)*

These samples were estimated by GC–MS and gas chromatography (used chiral column). The fragmentation pattern was consistent with each enantiomer. The retention time was different from each enantiomer.

2.6. Incubation of Intestinal Bacteria

This experiment was intentionally carried out under sterile conditions. Petri dishes, pipettes, and solutions were autoclaved. A GAM Broth (Nissui Pharmaceutical) was adjusted to pH 8.9 and placed in Petri dishes at 10 mL per 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 NaCl. 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**.

3. Results and discussion

3.1. Biotransformation of (+)-(1R)- and (−)-(1S)-fenchone 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. The larvae fed with artificial diet without substrate were used as control, and the extract of frass was analyzed by GC. Compounds **1**–**6** and unidentified metabolites were not observed in the frass of controls.

In the biotransformation of (+)-**1**, the four major metabolites isolated from the frass were identified as (+)-(1*S*,6*S*)-6-*exo*-hydroxyfenchone (**2**), (+)-(1*S*,6*R*)-6-*endo*-hydroxyfenchone (**3**), (+)-(1*R*)-10-hydroxyfenchone (**4**) and (+)-(1*S*,4*R*)-3-oxo-2,2,4-trimethyl-cyclopentylacetic acid (**6**) ((+)-**6** is a new compound), and minor metabolites exists. One of the minor metabolite had led to its estimate as (+)-(1*S*,5*R*)-5-*exo*-hydroxyfenchone (**5**), with supporting structural evidence provided by the mass spectral fragmentation pattern and retention time in the GC chromatogram (used chiral column). In the biotransformation of (−)-**1**, the three major metabolites isolated from the frass were identified as (−)-(1*R*,6*R*)-6-*exo*-hydroxyfenchone (**2**), (−)-(1*S*)-10-hydroxyfenchone (**4**) and (−)-(1*R*,5*S*)-5-*exo*-hydroxyfenchone (**5**) ((−)-**4** is a new compound), and minor metabolites exists. One of the minor metabolite had led to its estimate as (−)-(1*R*,6*S*)-6-*endo*-hydroxyfenchone (**3**), and other one as (−)-(1*R*,4*S*)-methyl-3-oxo-2,2,4-trimethyl-cyclopentylacetate (**6Me**), with supporting structural evidence provided by the mass spectral fragmentation pattern and retention time in the GC chromatogram (used chiral column). Furthermore, about all metabolites, enantiomeric excess (e.e.%) was identified by GC (used chiral column). The result indicates that these are enantiomerically pure compounds (e.e.100%).

The structures of following metabolites were confirmed by assignment of the NMR spectra using two-dimensional techniques (COSY, HMQC, HMBC and NOESY).

The metabolite **2** had a molecular formula of $C_{10}H_{16}O_2$ that was estimated by its HR-EI-MS spectral. The IR spectrum contained a new hydroxyl band at 3455 cm^{-1}. In the ^1H- and ^{13}C NMR spectra were assigned by comparison with the substrate **1** and the previous paper [12,13]. In the characteristic HMBC spectrum, some correlation cross-peaks were observed of C-7 (37.2 ppm) with one methyl group (1.14 ppm; H-10), C-10 (10.5 ppm) with new methine proton (3.62 ppm; H-6), two methyl groups with the other quaternary carbon (46.9 ppm; C-3). NOESY spectrum indicates the structure established with some correlation cross-peaks that were observed between H-5_{endo}, H-6_{endo} (2.40 and 3.62 ppm, respectively) and one methyl signal at 0.96 ppm (H-9), H_A-7 (1.76 ppm) and the other methyl signal at 1.06 ppm (H-8). Moreover, it confirmed by COSY and HMQC, so that configuration of the hydroxyl group at C-6 was *exo*. The specific rotation shows the (+)-form. From these data, it was concluded that the structure of (+)-**2** is (+)-(1*S*,6*S*)-6-*exo*-hydroxyfenchone. About (−)-(1*R*,6*R*)-6-*exo*-hydroxyfenchone, these spectral data were identified comparison with enantiomer (+)-**2**.

About metabolite **3,** the ^1H- and ^{13}C NMR spectra were similar to that of the substrate, except for the existence of new methine group and the disappearance of a methylene group, which has been reported in the literature [14]. It had a molecular formula of $C_{10}H_{16}O_2$ that was estimated by its HR-EI-MS spectral and the IR spectrum contained a new hydroxyl band at 3448 cm^{-1}. Assignment of the three methyl signals was achieved by HMBC and NOESY. In the characteristic HMBC spectrum, some correlation cross-peaks were observed of C-7 (41.0 ppm) with one methyl group (1.16 ppm; H-10), C-10 (12.6 ppm) with new methine proton (4.09 ppm; H-6), two methyl groups with the other quaternary carbon (48.0 ppm; C-3). NOESY spectrum indicates the structure established with some correlation cross-peaks that were observed between H_B-7 (1.62 ppm) and H-6_{exo} and H-5_{exo} (4.09 and 2.15 ppm, respectively), H-5_{endo} (1.74 ppm) and one methyl signal at 1.12 ppm (H-9). Moreover, it was confirmed by COSY and HMQC, so that configuration of the hydroxyl group at C-6 was *endo*. The specific rotation shows the (+)-form. From these data, it was concluded that the structure of **3** is (+)-(1*S*,6*R*)-6-*endo*-hydroxyfenchone. About (−)-(1*R*,6*S*)-6-*endo*-hydroxyfenchone, it was estimated from mass spectral fragmentation pattern and retention time in the GC chromatogram (used chiral column). The fragmentation pattern was consistent with enantiomer (+)-**3**. Moreover, retention time was different between (+)- and (−)-**3**.

The metabolite **4** had a molecular formula of $C_{10}H_{16}O_2$ that was estimated by its HR-EI-MS spectral. The IR spectrum contained a new hydroxyl band at 3447 cm^{-1}. The ^1H- and ^{13}C NMR spectra were similar to that of the substrate, except for the existence of new methylene group and the disappearance of a methyl group. About the proton NMR, H_B-7 (1.49 ppm) has characteristic coupling constant (J = 10.6 Hz) with H_A-7 (2.01 ppm). COSY spectrum indicates the structure established with some correlation cross-peaks that were observed between H-4 (2.23–2.20 ppm), and H_A-7, H-5_{exo} and H_B-7 (2.01, 1.74 and 1.49 ppm, respectively). Then, the ^1H NMR spectrum showed two methyl groups located at 1.07 and 1.05 ppm and the two doublets located at 3.86 and 3.78 ppm (J = 11.8 Hz), latter spectra are characteristic of a new methylene group. In the characteristic HMBC spectrum, some correlation cross-peaks were observed of two methyl groups (1.07 and 1.05 ppm) with one quaternary carbon (48.0 ppm; C-3), new methylene group (3.86 and 3.78 ppm) with the other quaternary carbon (60.0 ppm; C-1). Therefore, metabolite **4** was produced by hydroxylation at the C-10 position of **1**. Furthermore, to determine the complete assignment

of metabolite **4**, NOESY was measured. The spectrum indicates the structure established with some correlation cross-peaks that were observed between H-5$_{endo}$, H-6$_{endo}$ (1.84 and 1.47 ppm, respectively) and one methyl signal at 1.05 ppm (H-9), H_A-7 (2.01 ppm) and other methyl signal at 1.07 ppm (H-8). The specific rotation shows the (−)-form. From these data, it was concluded that the structure of **4** is (−)-(1*S*)-10-hydroxyfenchone, which is a new compound. About (+)-(1*R*)-10-hydroxyfenchone, these spectral data were identified comparison with the previous paper [6,15–18].

The metabolite **5** had a molecular formula of $C_{10}H_{16}O_2$ that was estimated by its HR-EI-MS spectral. The IR spectrum contained a new hydroxyl band at 3447 cm^{-1}. The ^{1}H- and ^{13}C NMR spectra were similar to that of the substrate, except for the existence of new methine group and the disappearance of a methylene group, which has been reported in the literature [13]. About the ^{1}H NMR, H_B-7 (2.07 ppm) has characteristic coupling constant (J = 10.9 Hz) with H_A-7 (2.01 ppm). COSY spectrum indicates correlation cross-peaks that were observed between H-4 (2.16–2.13 ppm), and H_A-7 and H_B-7 (1.74 and 2.07 ppm, respectively), H_A-7 (1.74 ppm), and H-5$_{endo}$ and H-6$_{endo}$ (4.38 and 1.89 ppm, respectively; a long distance *W*-coupling). In the characteristic HMBC spectrum, some correlation cross-peaks were observed of C-7 (37.4 ppm) with one methyl group (1.15 ppm; H-10), C-6 (43.2 ppm) with quaternary carbon (53.6 ppm; C-1), two methyl groups with the other quaternary carbon (45.3 ppm; C-3). NOESY spectrum indicates the structure established with some correlation cross-peaks that were observed between H-5$_{endo}$, H-6$_{endo}$ (4.38 and 1.89 ppm, respectively) and one methyl signal at 1.03 ppm (H-9), H_A-7 (1.74 ppm) and the other methyl signal at 1.06 ppm (H-8). Moreover, it confirmed by HMQC, so that configuration of the hydroxyl group at C-5 was *exo*. The specific rotation shows the (−)-form. From these data, it was concluded that the structure of **5** is (−)-(1*R*,5*S*)-5-*exo*-hydroxyfenchone. About (+)-(1*S*,5*R*)-5-*exo*-hydroxyfenchone, it was estimated from mass spectral fragmentation pattern and retention time in the GC chromatogram (used chiral column). The fragmentation pattern was consistent with enantiomer (+)-**5**. Moreover, retention time was different between (+)- and (−)-**5**.

The methyl ester **6Me** had a molecular formula of $C_{11}H_{18}O_3$ that was estimated by its HR-EI-MS spectral. The specific rotation shows the (+)-form. It had four methyl (including a esterified methyl observed at 51.7 ppm), two methylene, two methine and three quaternary carbons (including a ketone carbon and a carboxyl carbon observed at 223.8 and 173.2 ppm, respectively), one methyl and one methine carbon more and one methylene less than **1**. The ^{1}H- and ^{13}C NMR spectra were assigned by comparison with the substrate **1** and the previous paper [19–22]. However, they have not been isolated and published about completion assigned of structure and absolute configuration. Assignment of the three quaternary carbon signals was achieved by HMBC. In the characteristic HMBC spectrum, some correlation cross-peaks were observed of two methyl groups with quaternary carbon (47.3 ppm; C-2). One methyl singlet changed into doublet compared with **1** and the coupling between methine proton observed at 2.17–2.24 ppm (H-4) were made clear by the analysis of COSY spectrum, so that the metabolite **6Me** was cleavage of carbocyclic ring. The configuration of metabolite **6** was produced by oxidation at the C-6 position of **1**. The carboxylic acid **6** had a molecular formula of $C_{10}H_{16}O_3$ that was estimated by its HR-EI-MS spectral. The IR spectrum contained a widely band at 3300–2500 cm^{-1}. From these data, it was concluded that the structure of **6** is (+)-(1*S*,4*R*)-3-oxo-2,2,4-trimethyl-cyclopentylacetic acid, which is also a new compound. About (−)-(1*R*,4*S*)-3-oxo-2,2,4-trimethyl-cyclopentylacetic acid, it was estimated from mass spectral fragmentation pattern and retention time in the GC chromatogram (used chiral column). The fragmentation pattern was consistent with enantiomer (+)-**6**. Moreover, retention time was different between (+)- and (−)-**6**.

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 HR-EI-MS spectrum. Therefore, hydroxyl group was supposed to have been introduced in substrate.

3.2. Biotransformation of metabolites **2** and **3**

A small amount of metabolite **2** (40 mg) was dissolved in ethanol (10 mL), and the solution was painted on the surface of the artificial diet. The diet was fed to larvae (fourth to fifth instar). The frass was collected and extracted with diethylether and then ethylacetate. Metabolite **6** was identified from the retention time in the GC chromatogram of the extract from the frass. On the other hand, metabolite **3** was administered to larvae in the same manner. The result indicated metabolite **6** was identified.

3.3. Study of the difference among the individuals of larvae

The diet which was prepared in the 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. This experiment was done to five groups. However, there were few differences of each group (Table 1). These results suggested that the biotransformation of (+)- and (−)-fenchone by the larvae of *S. litura* has reappearance.

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

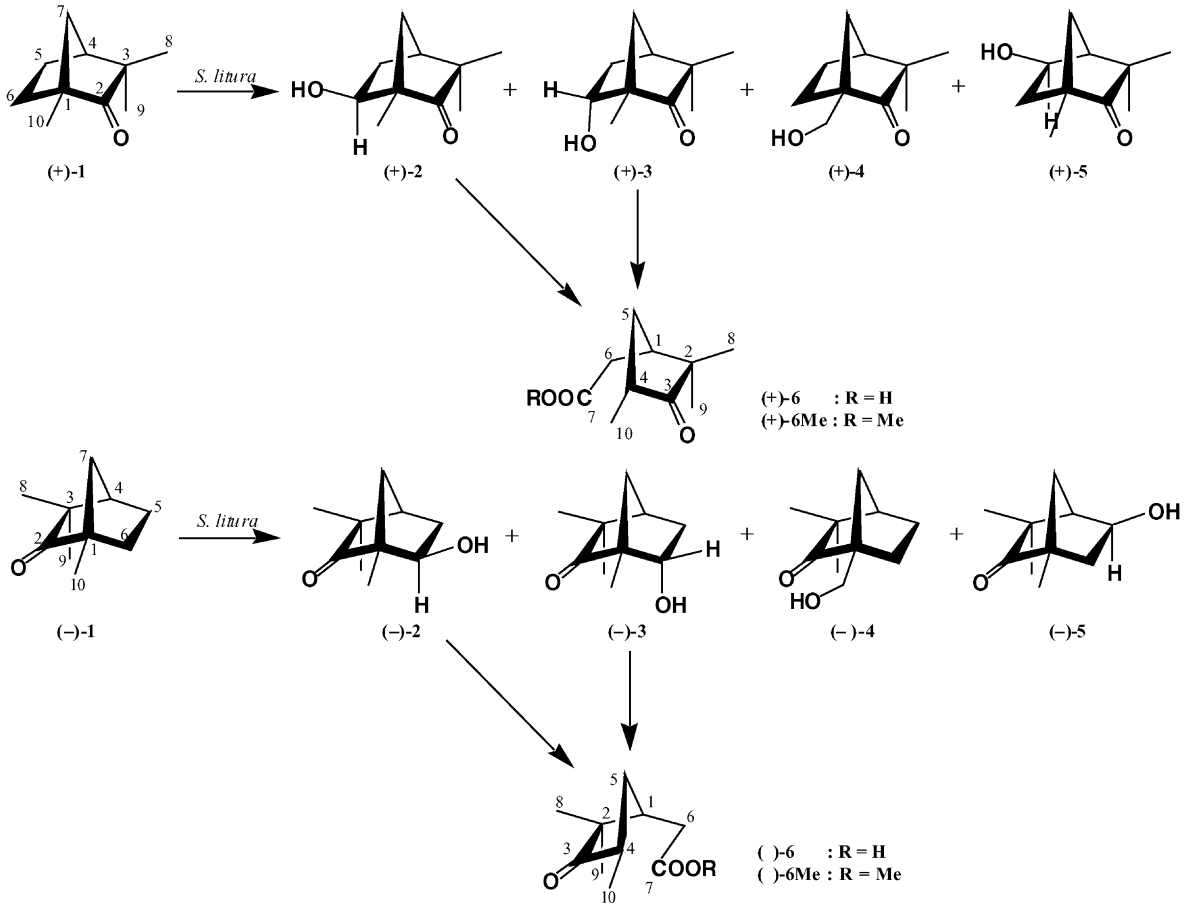
A previous paper described the participation of aerobically and anaerobically active intestinal bacteria in the metabolism of α-terpinene [7]. In the present study, the in

vitro metabolism of (+)- and (−)-**1** by intestinal bacteria was also examined in a manner similar to that of the previous paper [7–10]. 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**.

3.5. Study of the metabolic pathways

In the present study of biotransformation of (+)- and (−)-**1**, the larvae transformed (+)-**1** to mainly (+)-**2** (61.5 ± 0.9%), (+)-**3** (15.6 ± 2.9%), (+)-**4** (4.5 ± 1.4%), (+)-**6** (11.9 ± 1.8%) and a minor compound (+)-**5** (2.5 ± 2.2%); on the other hand, the larvae transformed (−)-**1** to mainly (−)-**4** (42.1 ± 0.8%), (−)-**2** (19.5 ± 2.1%), (−)-**5** (15.6 ± 3.8%) and minor compounds (−)-**3** (13.5 ± 1.4%), (−)-**6** (2.9 ± 2.7%) (Scheme 1, Table 1). Compounds **2** and **4** are remarkably different percentage between (+)- and (−)-**1**. In the biotransformation of (+)-**1**, it was predominantly hydroxylated at C-6$_{exo}$, on the other hand, in the biotransformation of (−)-**1**, predominantly hydroxylated at C-10. These results indicate that (i) each enantiomer was preferentially hydroxylation at different position and (ii) stereoselective oxidation at C-6 position was progressed in the metabolism of **1** by the larvae of *S. litura*. The results of the biotransformation of **1**, **2** and **3** revealed that in the biotransformation of **1** by the larvae of *S. litura*, the metabolite **6** was formed by two metabolic pathways (**1** → **2** → **6** and **1** → **3** → **6**) (Scheme 1).

Compound **1** is the most important and widespread terpene known; metabolism of **1** in mammals and microorganisms has been published. It seems natural to obtain different metabolites with different species of organisms [2–6]. In *Corynebacterium* sp., Baeyer–Villiger type oxidation was progressed and obtained 1,2- and 2,3-fencholides [2]. The mycelium of *A. orchidis*, produced 5-*exo*- and 6-*exo*-hydroxyfenchone both enantiomer [3]. The cultured cells of *E. perriniana* produced hydroxyfenchone glycosides at C-5$_{exo}$, C-6$_{exo}$ and C-7 position, the first step of the biotransformation was supposed to be hydroxylation of fenchone [4]. Literature identified the hydroxyfenchones obtained from the bioconversion of (+)-fenchone in *A. nigar* as 5-*endo*- and 6-*endo*-hydroxyfenchone [5]. Literature identified the hydroxyfenchones obtained from the hydrolyzed urine of rabbits that had been fed (+)-fenchone as 8-, 9- and 10-hydroxyfenchone [6]. In the present study, the larvae of *S. litura* employ a metabolic pathway similar to that used by the rabbit and mycelium of *A. orchidis*. Compound **1** is hydroxylated at C-6, C-10 and C-5$_{exo}$ position. In particular, about C-6 position, the reaction was cleavage of



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

carbocyclic ling and progressing to the carboxylic acid. There are two metabolic pathways for metabolism of **1**. Therefore, characteristic reaction is C-6 position of **1** by the larvae of *S. litura* compared with other organisms. Moreover, the regio- and stereo-selective hydroxylation of fenchone is different main metabolite. It is the first reaction compared with previous paper [7–10]. The present study is first report on obtaining a carboxylic acid from (+)-**1** as the biotransformation product with the C-10 position of (−)-**1** being hydroxylated with high degree of efficiency.

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