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Biotransformation of (+)-(1*R*,2*S*)-fenchol by the larvae of common cutworm (*Spodoptera litura*)

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Abstract—Biotransformation of (+)-(1*R*,2*S*)-fenchol by the larvae of *Spodoptera litura* was carried out. Substrate was converted to three new terpenoids, (+)-(1*R*,2*S*)-10-hydroxyfenchol, (+)-(1*R*,2*R*,3*S*)-8-hydroxyfenchol and (−)-(1*S*,2*S*,6*S*)-6-*exo*-hydroxyfenchol, and one known terpenoid, (−)-(1*R*,2*R*,3*R*)-9-hydroxyfenchol. These structures were established by NMR, IR, specific rotation and mass spectral studies.

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. These points suggested that the biotransformation is easy method for produce the new organic compounds. We have reported the biotransformation of various compounds, including monoterpenoids, by the larvae of *Spodoptera litura* as biocatalyst.[1–5] The reasons for using the larvae of common cutworm (*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. In our previous paper, terpenoids have been used to regio-selective hydroxylation.[1–5] Fenchol is bicyclic monoterpenoid with a fenchane skeleton. The structure modification of fenchol by organic synthesis has been few reported.[6–10] However, biotransformation of (+)-fenchol has not been reported previously.

In the present paper, we report for the first time the biotransformation of (+)-(1*R*,2*S*)-fenchol by the larvae of *Spodoptera litura* and structural elucidation of the biotransformation products.

2. Results and discussion

Biotransformation by the larvae of *S. litura* was observed as follows: substrate was administered to the larvae through their diet (4 mg/g of diet); metabolite was then detected and isolated from the frass of larvae. The larvae were fed the artificial diet without substrate were used as control, and the extract of frass was analyzed by GC. Substrate and metabolites were observed in the frass of biotransformed extracts. Consume of substrate in the diet observed by the internal standard method in GC. The result was that consumption of (+)-(1*R*,2*S*)-fenchol (**1**) was 93.3%.

In the biotransformation of **1**, the four metabolites isolated from the frass were identified as (+)-(1*R*,2*S*)-10-hydroxyfenchol (**2**), (+)-(1*R*,2*R*,3*S*)-8-hydroxyfenchol (**3**), (−)-(1*S*,2*S*,6*S*)-6-*exo*-hydroxyfenchol (**4**) and (−)-(1*R*,2*R*,3*R*)-9-hydroxyfenchol (**5**) (**2**, **3** and **4** are new compounds). Percentage of recovered substrate and metabolites **2**, **3**, **4** and **5** in the frass extract were 6.7, 52.4, 18.6, 15.5 and 4.0%, respectively. Percentage was calculated from the peak area in the GC chromatogram of the extract of frass (Table 1).

Keywords: *Spodoptera litura*; Biotransformation; Regioselective; Hydroxylation; (+)-(1*R*,2*S*)-10-Hydroxyfenchol; (+)-(1*R*,2*R*,3*S*)-8-Hydroxyfenchol; (−)-(1*S*,2*S*,6*S*)-6-*exo*-Hydroxyfenchol; (−)-(1*R*,2*R*,3*R*)-9-Hydroxyfenchol.
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Table 1. The biotransformation of (+)-fenchol (**1**) by the *S. litura* larvae[a]

Substrate	**1**[b]	**2**	**3**	**4**	**5**
(+)-Fenchol (**1**)	6.7%	52.4%	18.6%	15.5%	4.0%

[a] Metabolites were obtained from the frass of *S. litura*. Percentage was calculated from the peak area in the gas chromatogram of the extract of frass.
[b] Recovered substrate.

Metabolite **2** showed a widely hydroxyl band (ν_{max} 3385 cm^{-1}) relatively compared with substrate in the IR spectrum. FABMS (neg.) had a fragmentation at *m/z* 151 which was assigned to $[M-H-H_2O]^+$, however parent ion has not indicate. As the conformation, metabolite **2** was acetylated at room temperature and subsequently examined by TLC. The formation of only one reaction product was confirmed (R_f 0.5) by TLC and obtained acetylated compound **2a**. Acetylated **2a**, the IR spectrum still had hydroxyl band (ν_{max} 3497 cm^{-1}) and the high resolution FABMS (pos.) had a peak at *m/z* 213.1509 which was assigned to $[M+H]^+$, characterized its monoacetate. These results indicate metabolite **2** had a primary alcohol. Therefore, metabolite **2** was contained two hydroxyl groups. The proton and carbon 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 (1.09 ppm) has characteristic coupling constant (J=10.3 Hz) with H_A-7 (1.48 ppm). This was confirmed by assignment of the NMR spectra using two-dimensional techniques (COSY, HMQC, HMBC and NOESY). COSY spectrum indicates correlation cross-peaks were observed between H-4 (1.77–1.74 ppm) and δ 1.48, 1.44 and 1.09 ppm (H_A-7, H-5$_{exo}$ and H_B-7, respectively), H-2$_{exo}$ (3.62 ppm) and δ 1.15 ppm (H-6$_{exo}$; a long distance *W*-coupling J=1.8 Hz). Then, the proton NMR spectrum showed two methyl groups located at δ 1.01 and 0.87 ppm and the two doublets located at δ 3.82 and 3.74 ppm (J=10.6 Hz), latter spectra are characteristic of the new methylene group. In the characteristic HMBC spectrum correlation were observed of two methyl groups (1.01 and 0.87 ppm) with one nonprotonated carbon (39.4 ppm; C-3), of new methylene group (3.82 and 3.74 ppm) with the other nonprotonated carbon (54.1 ppm; C-1). Therefore, metabolite **2** was produced by hydroxylation at the C-10 position of **1**. Furthermore, to determine the complete assignment of metabolite **2**, NOESY was measured. The spectrum indicates correlation cross-peaks were observed between H-5$_{endo}$,6$_{endo}$ (1.73 and 1.87 ppm, respectively) and one methyl signal at δ 0.87 ppm (H-9), H_A-7 (1.48 ppm) and other methyl signal at δ 1.01 ppm (H-8), so that was established. The specific rotation shows the (+)-form. From these data it was concluded that the structure of **2** is (+)-(1*R*,2*S*)-10-hydroxyfenchol, which is a new compound.

It assigned the structure of **2a**, which was confirmed by study of its 2D NMR spectra (COSY, HMQC, HMBC and NOESY).

The second metabolite **3**, the IR spectrum had a widely hydroxyl band (ν_{max} 3339 cm^{-1}) relatively compared with substrate. FABMS (neg.) had a fragmentation at *m/z* 151 which was assigned to $[M-H-H_2O]^+$, however parent ion has not indicate. As the conformation, metabolite **3** was acetylated at room temperature and subsequently examined by TLC. The formation of only one reaction product was confirmed (R_f 0.5) by TLC and obtained acetylated compound **3a**. Acetylated **3a**, the IR spectrum still had hydroxyl band (ν_{max} 3495 cm^{-1}) and the high resolution FABMS (pos.) had a peak at *m/z* 213.1483 which was assigned to $[M+H]^+$, characterized its monoacetate. These results indicate metabolite **3** had a primary alcohol. Therefore, metabolite **3** was contained two hydroxyl groups. The proton and carbon 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 (1.15 ppm) has characteristic coupling constant (J=10.3 Hz) with H_A-7 (1.40 ppm). This was confirmed by assignment of the NMR spectra using two-dimensional techniques (COSY, HMQC, HMBC and NOESY). COSY spectrum indicates correlation cross-peaks were observed between H-4 (1.80–1.77 ppm) and δ 1.40, 1.44 and 1.15 ppm (H_A-7, H-5$_{exo}$ and H_B-7, respectively), H-2$_{exo}$ (3.37 ppm) and δ 1.19 ppm (H-6$_{exo}$; a long distance *W*-coupling J=1.8 Hz). Then, the proton NMR spectrum showed two methyl groups located at δ 1.11 and 0.93 ppm and the two doublets located at δ 3.40 and 3.37 ppm (J=10.5 Hz), latter spectra are characteristic of the new methylene group. In the characteristic HMBC spectrum correlation were observed of C-7 (41.0 ppm) with one methyl group (1.11 ppm; H-10), of C-6 (25.7 ppm) with nonprotonated carbon (48.7 ppm; C-1), of new methylene group with the other nonprotonated carbon (44.6 ppm; C-3). NOESY spectrum indicates correlation cross-peaks were observed between H-5$_{endo}$,6$_{endo}$ (1.73–1.64 ppm, both of them) and one methyl signal at δ 0.93 ppm (H-9), H_A-7 (1.40 ppm) and new methylene group at δ 3.40 and 3.37 ppm (H-8), so that was established. Therefore, metabolite **3** was produced by hydroxylation at the C-8 position of **1**. The specific rotation shows the (+)-form. From the above data, it was concluded that the structure of **3** is (+)-(1*R*,2*R*,3*S*)-8-hydroxyfenchol, which is also a new compound.

It assigned the structure of **3a**, which was confirmed by study of its 2D NMR spectra (COSY, HMQC, HMBC and NOESY).

The third metabolite **4**, the IR spectrum had a widely hydroxyl band (ν_{max} 3343 cm^{-1}) relatively compared with substrate. FABMS (neg.) had a fragmentation at *m/z* 151 which was assigned to $[M-H-H_2O]^+$, however parent ion has not indicate. As the conformation, metabolite **4** was acetylated at room temperature and subsequently examined by TLC. However, reaction was not progress at all (no reaction). Therefore, metabolite **4** was acetylated in the usual manner and obtained acetylated compound **4a**. EIMS data for **4a** had a molecular formula of $C_{14}H_{22}O_4$ ($[M]^+$=254) and the high resolution FABMS (pos.) had a peak at *m/z* 255.1596 which was assigned to $[M+H]^+$, characterized its diacetate. The proton and carbon NMR spectra of **4** were similar to that of the substrate, except for the existence of new methine group and the disappearance of a methylene group. About the proton NMR, evidence for the presence of three methyl groups. As the conformation, assignment of the three methyl signals were achieved by

HMBC and NOESY. In the characteristic HMBC spectrum correlation were observed of C-7 (36.8 ppm) with one methyl group (1.15 ppm; H-10), of C-6 (68.8 ppm) with nonprotonated carbon (53.5 ppm; C-1), of two methyl groups with the other nonprotonated carbon (39.3 ppm; C-3). NOESY spectrum indicates correlation cross-peaks were observed between H-5$_{endo}$,6$_{endo}$ (2.32 and 4.01 ppm, respectively) and one methyl signal at δ 0.85 ppm (H-9), H_A-7 (1.36–1.32 ppm) and the other methyl signal at δ 1.12 ppm (H-8), so that was established. Further, a coupling between H-2$_{exo}$ and H-6$_{exo}$ was disappearance (a long distance *W*-coupling). The above and 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 **4** is (−)-(1*S*,2*S*,6*S*)-6-*exo*-hydroxyfenchol, which is also a new compound.

It assigned the structure of **4a**, which was confirmed by study of its 2D NMR spectra (COSY, HMQC, HMBC and NOESY).

About metabolite **5**, the IR spectrum had a widely hydroxyl band (ν_{max} 3390 cm^{-1}) relatively compared with substrate. FABMS (neg.) had a fragmentation at *m/z* 151 which was assigned to $[M-H-H_2O]^+$, however parent ion has not indicate. As the conformation, metabolite **5** was acetylated at room temperature and subsequently examined by TLC. The formation of only one reaction product was confirmed (R_f 0.5) by TLC and obtained acetylated compound **5a**. Acetylated **5a**, the IR spectrum still had hydroxyl band (ν_{max} 3484 cm^{-1}) and the high resolution FABMS (pos.) had a peak at *m/z* 213.1479 which was assigned to $[M+H]^+$, characterized its monoacetate. These results indicate metabolite **5** had a primary alcohol. Therefore, metabolite **5** was contained two hydroxyl groups. The proton and carbon 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 (1.16 ppm) has characteristic coupling constant (J=10.3 Hz) with H_A-7 (1.50 ppm). This was confirmed by assignment of the NMR spectra using two-dimensional techniques (COSY, HMQC, HMBC and NOESY). COSY spectrum indicates correlation cross-peaks were observed between H-4 (1.74–1.72 ppm) and δ 1.50, 1.40 and 1.16 ppm (H_A-7, H-5$_{exo}$ and H_B-7, respectively), H-2$_{exo}$ (3.42 ppm) and δ 1.07 ppm (H-6$_{exo}$; a long distance *W*-coupling J=1.7 Hz). Then, the proton NMR spectrum showed two methyl groups located at δ 1.10 and 1.09 ppm and the two doublets located at δ 3.93 and 3.34 ppm (J=10.6 Hz), latter spectra are characteristic of the new methylene group. In the characteristic HMBC spectrum correlation were observed of C-7 (41.9 ppm) with one methyl group (1.09 ppm; H-10), of C-6 (25.2 ppm) with nonprotonated carbon (49.5 ppm; C-1), of new methylene group with the other nonprotonated carbon (43.4 ppm; C-3). NOESY spectrum indicates correlation cross-peaks were observed between H-5$_{endo}$, 6$_{endo}$ (1.62 and 1.80 ppm, respectively) and new methylene group at δ 3.93 and 3.34 ppm (H-9), H_A-7 (1.50 ppm) and one methyl signal at δ 1.10 ppm (H-8), so that was established. Therefore, metabolite **5** was produced by hydroxylation at the C-9 position of **1**. The specific rotation shows the (−)-form. From the above date, it was concluded that the structure of **5** is (−)-(1*R*,2*R*,3*R*)-9-hydroxyfenchol, which are reported in the literature,[6–8] however, they have not been published about completion assigned of structure and absolute stereochemistry.

It assigned the structure of **5a**, which was confirmed by study of its 2D NMR spectra (COSY, HMQC, HMBC and NOESY).

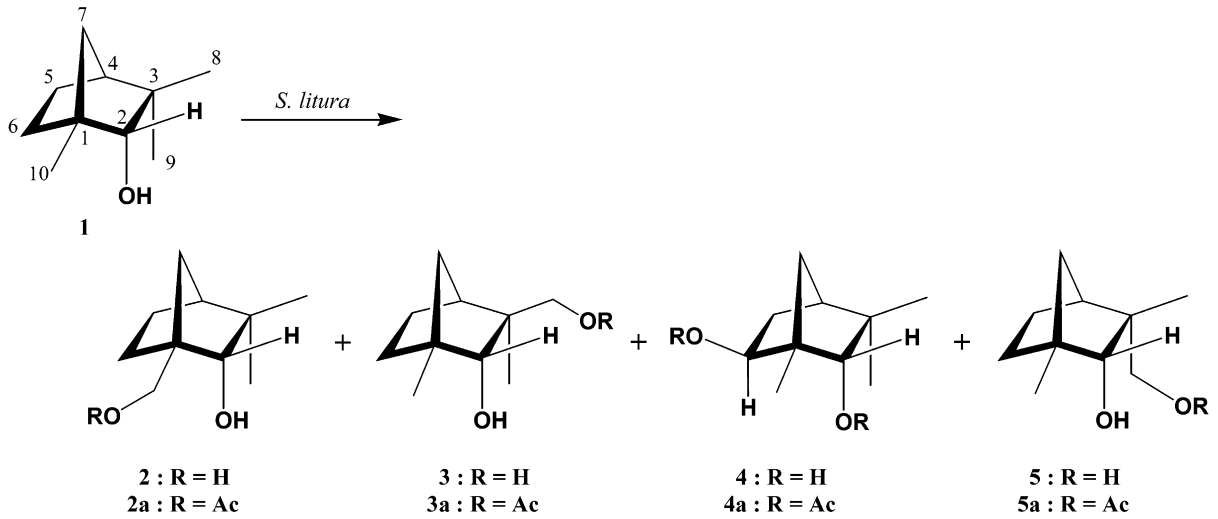
A previous paper described the participation of aerobically and anaerobically active intestinal bacteria in the metabolism of α-terpinene.[1] In the present study, the in vitro metabolism of **1** by intestinal bacteria was also examined in a manner similar to that of the previous paper.[1–3] However, substrate **1** was 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 **1**. The difference of reaction between **1** and α-terpinene was suggested to be due to the difference of substrate.

In the present study of biotransformation of **1**, the reaction was used the enzymatic activity which the larvae of *S. litura* possess. There are no reports on the biotransformation of **1** by biocatalysts, which is the new reaction. The larvae transformed **1** to mainly **2** (52.4%), **3** (18.6%), **4** (15.5%) and **5** (4.0%) (Scheme 1, Table 1). These results suggested that regioselective hydroxylation was progress in the metabolism of **1** by the larvae of *S. litura*. In this case, compound **1** is hydroxylated at C-6-*exo* position and methyl group of geminal dimethyl and C-10 position. These results indicate that the larvae of *S. litura* recognized the methyl groups and C-10 position of compound **1** being high degree of efficiency.

3. Experimental

3.1. General procedures

Thin-layer chromatography (TLC) was performed on precoated plates [silica gel 60 F_{254}, 0.25 mm (Merck)]. The solvent system was hexane–ethylacetate (1:1). Compounds were visualized by spraying plates with 1% vanillin in 96% sulfuric acid followed by brief heating. 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; carrier gas, Helium at a flow rate of 30.0 cm/s. The peak area was integrated with a Hewlett–Packard HP3396 series II integrator. EIMS 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. The temperature of the ion source was 230 °C, and the electron energy was 70 eV.



Scheme 1. The biotransformation of (+)-fenchol (**1**) by the larvae of *S. litura*.

FABMS was obtained on a JEOL the Tanden MStation JMS-700TKM. 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 (δ 0.00) for ^1H NMR spectra measured in $CDCl_3$. Residual $CHCl_3$ was used as internal reference (δ 77.00) for ^{13}C NMR spectra measured in $CDCl_3$. Multiplicities were determined by the DEPT pulse sequence. The specific rotations were measured on a JASCO DIP-1000 digital polarimeter.

3.2. Rearing of larvae

Spodoptera litura used in this study were obtained from Nissan Kagaku. It is getting to change the generation every biotransformation. The larvae of *S. litura* were reared in plastic cases (200×300 mm wide, 100 mm high, 200 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).[11]

3.3. Chemical compounds

(+)-(1*R*,2*S*)-Fenchol **1** was purchased from Fluka. It structure was characterized by ^1H NMR, ^{13}C NMR, specific rotation and MS spectra. The purity of this compound was judged to be >99% on analysis with GC–MS.

3.4. 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 and 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 1.4 g, about 1.75 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 five hours (total of 4 days) and stored in a solution of diethylether (300 mL). For diet and frass separation, the fresh frass was extracted as soon as the fourth to fifth instar larvae excreted.

3.5. 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 2992 mg of extract was obtained. The extract was dissolved in ethylacetate, and then was added to the 5% $NaHCO_3$ solution. After shaking, neutral fraction (1684 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 (939 mg) was obtained from the aqueous layer. The neutral fraction was analyzed by GC–MS; metabolites **2**, **3**, **4** and **5** occurred in this fraction. The acidic fraction was reacted with ethereal CH_2N_2 overnight and subsequently examined by GC–MS, but methylated metabolites did not exist. The neutral fraction was subjected to silica gel open-column chromatography (silica gel 60, 230–400 mesh, Merck) with a 9:1 hexane/ethylacetate solvent system, and four major metabolites **2** (502 mg), **3** (178 mg), **4** (148 mg) and **5** (38 mg) was isolated.

3.5.1. (1*R*,2*S*)-10-Hydroxyfenchol (2). (+)-(1*R*,2*S*)-1-Hydroxymethyl-3,3-dimethylbicyclo[2.2.1]heptan-2-ol; colorless viscous oil; $[\alpha]_D^{24.4}$ +15.5° ($CHCl_3$, *c* 0.66); FABMS (neg.), *m/z* 151 $[M-H-H_2O]^+$; EI-MS, *m/z* (rel. intensity) 137 $[M-H_2O-CH_3]^+$ (2), 134 $[M-2H_2O]^+$ (0.5), 127 (0.5), 121 (6), 115 (0.5), 109 (11), 105 (2), 103 (0.5), 95 (7), 93 (7), 87 (9), 81 (100), 80 (36), 79 (20), 77 (7), 72 (33), 69 (26), 67 (20), 57 (15), 55 (18), 43 (22), 41 (34); IR (KBr, ν_{max}, cm^{-1}) 3385, 2956, 1073; ^1H NMR ($CDCl_3$) δ

0.87 (3H, s, H-9), 1.01 (3H, s, H-8), 1.09 (1H, dd, J=1.8, 10.3 Hz, H_B-7; nearly C-5,6 position), 1.15 (1H, dddd, J=1.8, 3.3, 12.3, 12.5 Hz, H-6$_{exo}$), 1.44 (1H, dddd, J=4.0, 6.0, 12.3, 12.5 Hz, H-5$_{exo}$), 1.48 (1H, dddd, J=2.0, 2.2, 2.8, 10.3 Hz, H_A-7; nearly C-2,3 position), 1.73 (1H, dddd, J=2.8, 3.3, 9.1, 12.3 Hz, H-5$_{endo}$), 1.74–1.77 (1H, m, H-4), 1.87 (1H, dddd, J=2.2, 6.0, 9.1, 12.3 Hz, H-6$_{endo}$), 3.62 (1H, d, J=1.8 Hz, H-2$_{exo}$), 3.74 (1H, d, J=10.6 Hz, H-10), 3.82 (1H, d, J=10.6 Hz, H-10′); ^{13}C NMR see Table 2.

Acetylation of **2**. Pyridine (1.0 mL) was added to a solution of **2** (8 mg) in acetic anhydride (18 mL), and the solution was stirred for 4 h at room temp (25 °C). The products were isolated in the usual manner and separated by silica gel column chromatography with a hexane–ethylacetate solvent system. The monoacetate **2a** (7 mg) was obtained.

(1*R*,2*S*)-10-Acetoxyfenchol (**2a**). (−)-(1*R*,2*S*)-1-Acetoxymethyl-3,3-dimethylbicyclo [2.2.1]heptan-2-ol; colorless viscous oil; $[\alpha]_D^{25.3}$ −4.1° ($CHCl_3$, *c* 1.54); HRFAB-MS (pos.), *m*/*z* 213.1509 $[M+H]^+$, calcd for $C_{12}H_{21}O_3$, 213.1491; EI-MS, *m*/*z* (rel. intensity) 212 $[M]^+$ (0.5), 194 $[M-H_2O]^+$ (0.5), 170 $[M-CH_2CO]^+$ (2), 169 (17), 152 $[M-CH_3COOH]^+$ (20), 137 (7), 134 (18), 129 (4), 123 (14), 121 (16), 109 (38), 95 (16), 91 (17), 81 (100), 80 (43), 79 (38) 77 (15), 72 (58), 69 (27), 67 (32), 55 (20); IR (KBr, ν_{max}, cm^{-1}) 3497, 2959, 1722, 1462, 1364, 1259, 1034; ^{1}H NMR ($CDCl_3$) δ 0.90 (3H, s, H-9), 1.01 (3H, s, H-8), 1.09–1.15 (1H, m, H-6$_{exo}$), 1.14 (1H, dd, J=2.0, 10.3 Hz, H_B-7; nearly C-5,6 position), 1.39–1.48 (1H, m, H-5$_{exo}$), 1.58 (1H, dddd, J=1.9, 2.0, 2.5, 10.3 Hz, H_A-7; nearly C-2,3 position), 1.70–1.79 (2H, m, H-5$_{endo}$, 6$_{endo}$), 1.78–1.80 (1H, m, H-4), 2.08 (3H, s, 10-OCO*Me*), 3.46 (1H, dd, J=1.6, 3.8 Hz, H-2$_{exo}$), 4.04 (1H, d, J=11.4 Hz, H-10), 4.29 (1H, d, J=11.4 Hz, H-10′); ^{13}C NMR see Table 2.

3.5.2. (1*R*,2*R*,3*S*)-8-Hydroxyfenchol (3). (+)-(1*R*,2*R*,3*S*)-3-Hydroxymethyl-1,3-dimethylbicyclo[2.2.1]heptan-2-ol; colorless amorphous crystals; $[\alpha]_D^{24.2}$ +9.7° ($CHCl_3$, *c* 1.21); FABMS (neg.), *m*/*z* 151 $[M-H-H_2O]^+$; EI-MS, *m*/*z* (rel. intensity) 152 $[M-H_2O]^+$ (2), 139 (2), 137 [152-CH_3]$^+$ (5), 123 (4), 121 [139-H_2O]$^+$ (7), 109 (7), 108 (4), 95 (9), 93 (6), 81 (100), 80 (21), 79 (13), 71 (6), 69 (17), 55 (16), 43 (20), 41 (21); IR (KBr, ν_{max}, cm^{-1}) 3339, 2950, 1025; ^{1}H NMR ($CDCl_3$) δ 0.93 (3H, s, H-9), 1.08 (1H, dddd, J=1.8, 5.1, 7.6, 12.0 Hz, H-6$_{exo}$), 1.11 (3H, s, H-10), 1.15 (1H, dd, J=1.7, 10.3 Hz, H_B-7; nearly C-5,6 position), 1.39 (1H, dddd, J=2.1, 2.2, 2.7, 10.3 Hz, H_A-7; nearly C-2,3 position), 1.41–1.50 (1H, m, H-5$_{exo}$), 1.64–1.73 (2H, m, H-5$_{endo}$, 6$_{endo}$), 1.77–1.80 (1H, m, H-4), 3.37 (1H, d, J=1.8 Hz, H-2$_{exo}$), 3.37 (1H, d, J=10.5 Hz, H-8), 3.40 (1H, d, J=10.5 Hz, H-8′); ^{13}C NMR see Table 2.

Acetylation of **3**. Pyridine (1.0 mL) was added to a solution of **3** (10 mg) in acetic anhydride (18 mL), and the solution was stirred for 4 h at room temp (25 °C). The products were isolated in the usual manner and separated by silica gel column chromatography with a hexane–ethylacetate solvent system. The monoacetate **3a** (9 mg) was obtained.

(1*R*,2*R*,3*S*)-8-Acetoxyfenchol (**3a**). (−)-(1*R*,2*R*,3*S*)-3-Acetoxymethyl-1,3-dimethylbicyclo [2.2.1]heptan-2-ol; colorless viscous oil; $[\alpha]_D^{25.8}$ −4.3° ($CHCl_3$, *c* 0.52); HRFAB-MS (pos.), *m*/*z* 213.1483 $[M+H]^+$, calcd for $C_{12}H_{21}O_3$, 213.1491; EI-MS, *m*/*z* (rel. intensity) 152 $[M-CH_3COOH]^+$ (4), 137 [152-CH_3]$^+$ (15), 123 (6), 121 (6), 109 (9), 95 (7), 93 (6), 81 (100), 80 (9), 79 (9), 69 (13), 67 (13), 55 (10); IR (KBr, ν_{max}, cm^{-1}) 3495, 2952, 1740, 1465, 1375, 1246, 1033; ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, s, H-9), 1.04–1.12 (1H, m, H-6$_{exo}$), 1.11 (3H, s, H-10), 1.19 (1H, dd, J=1.6, 10.3 Hz, H_B-7; nearly C-5,6 position), 1.43 (1H, dddd, J=1.4, 2.2, 2.4, 10.3 Hz, H_A-7; nearly C-2,3 position), 1.43–1.52 (1H, m, H-5$_{exo}$), 1.63–1.72 (2H, m, H-5$_{endo}$, 6$_{endo}$), 1.96–1.99 (1H, m, H-4), 2.08 (3H, s, 8-OCO*Me*), 3.33 (1H, d, J=1.8 Hz, H-2$_{exo}$), 3.83 (1H, d, J=10.9 Hz, H-8), 3.86 (1H, d, J=10.9 Hz, H-8′); ^{13}C NMR see Table 2.

3.5.3. (1*S*,2*S*,6*S*)-6-*exo*-Hydroxyfenchol (4). (+)-(1*S*,2*S*,6*S*)-1,3,3-Trimethylbicyclo[2.2.1]heptan-2,6-diol; white amorphous crystals; $[\alpha]_D^{25.0}$ −4.4° ($CHCl_3$, *c* 0.42); FABMS (neg.), *m*/*z* 151 $[M-H-H_2O]^+$; EI-MS, *m*/*z* (rel. intensity) 155 $[M-CH_3]^+$ (18), 152 $[M-H_2O]^+$ (15), 137 (20), 126

Table 2. ^{13}C NMR spectral data for (+)-fenchol (**1**) and their metabolites (**2**-**5**) and derivatives (**2a**-**5a**) (125.65 MHz, $CDCl_3$)

Carbon	Compounds							
	2	**2a**	**3**	**3a**	**4**	**4a**	**5**	**5a**
1	54.1 (s)	52.7 (s)	48.7 (s)	48.8 (s)	53.5 (s)	51.6 (s)	49.5 (s)	49.1 (s)
2	83.4 (d)	80.5 (d)	80.3 (d)	80.3 (d)	84.9 (d)	85.1 (d)	85.8 (d)	85.0 (d)
3	39.4 (s)	38.9 (s)	44.6 (s)	42.9 (s)	39.3 (s)	39.4 (s)	43.4 (s)	42.1 (s)
4	48.1 (d)	47.8 (d)	43.4 (d)	43.5 (d)	46.9 (d)	47.2 (d)	46.1 (d)	45.8 (d)
5	25.6 (t)	25.3 (t)	26.0 (t)	25.8 (t)	38.6 (t)	36.5 (t)	25.4 (t)	25.6 (t)
6	21.7 (t)	21.3 (t)	25.7 (t)	25.5 (t)	68.8 (d)	72.8 (d)	25.2 (t)[a]	25.1 (t)
7	36.6 (t)	36.9 (t)	41.0 (t)	40.8 (t)	36.8 (t)	38.0 (t)	41.9 (t)	41.3 (t)
8	30.6 (q)	30.6 (q)	71.8 (t)	72.1 (t)	30.6 (q)	29.5 (q)	25.2 (q)[b]	25.2 (q)
9	20.0 (q)	20.0 (q)	15.1 (q)	15.6 (q)	20.0 (q)	19.8 (q)	66.7 (t)	67.8 (t)
10	68.2 (t)	67.0 (t)	19.3 (q)	19.2 (q)	14.8 (q)	14.7 (q)	19.1 (q)	19.0 (q)
CO*Me*		20.9 (q)		21.0 (q)		20.8 (q)[c]		21.0 (q)
*C*OMe		171.5 (s)		171.6 (s)		171.3 (s)[c]		171.4 (s)
CO*Me*						21.2[d]		
*C*OMe						170.8 (s)[d]		

[a] 25.20 ppm.
[b] 25.23 ppm.
[c] C-2 position.
[d] C-6 position.

(14), 121 (52), 119 (6), 111 (29), 109 (35), 97 (41), 95 (23), 81 (93), 80 (76), 72 (51), 69 (40), 65 (9), 57 (41), 55 (53), 43 (100), 41 (86); IR (KBr, ν_{max}, cm^{-1}) 3343, 2948, 1054; ^1H NMR ($CDCl_3$) δ 0.85 (3H, s, H-9), 1.02 (3H, s, H-8), 1.15 (3H, s, H-10), 1.21 (1H, ddd, J=3.8, 4.0, 13.5 Hz, H-5_{exo}), 1.32–1.36 (1H, m, H_A-7; nearly C-2,3 position), 1.38 (1H, dd, J=1.5, 10.3 Hz, H_B-7; nearly C-5,6 position), 1.68–1.73 (1H, m, H-4), 2.32 (1H, ddd, J=2.3, 7.2, 13.5 Hz, H-5_{endo}), 3.37 (1H, s, H-2_{exo}), 4.01 (1H, ddd, J=0.9, 3.8, 7.2 Hz, H-6_{endo}); ^{13}C NMR see Table 2.

Acetylation of **4**. Pyridine (2.0 mL) was added to a solution of **4** (5 mg) in acetic anhydride (30 mL), and the solution was refluxed for 5 h. The products were isolated in the usual manner and separated by silica gel column chromatography with a hexane–ethylacetate solvent system. The diacetate **4a** (4 mg) was obtained.

(1*S*,2*S*,6*S*)-2,6-Diacetoxyfenchol (**4a**). (−)-(1*S*,2*S*,6*S*)-1,3,3-Trimethylbicyclo[2.2.1]heptan-2,6-diacetate; colorless viscous oil; $[\alpha]_D^{25.3}$ −15.6° ($CHCl_3$, *c* 0.18); HRFAB-MS (pos.), *m/z* 255.1596 $[M+H]^+$, calcd for $C_{14}H_{23}O_4$, 255.1597; EI-MS, *m/z* (rel. intensity) 254 $[M]^+$ (0.5), 212 $[M-CH_2CO]^+$ (7), 194 $[M-CH_3COOH]^+$ (4), 179 (0.5), 170 $[212-CH_2CO]^+$ (0.5), 152 (18), 137 (10), 123 (18), 114 (31), 109 (24), 108 (26), 97 (13), 93 (13), 81 (93), 80 (100), 72 (26), 69 (27), 55 (13); IR (KBr, ν_{max}, cm^{-1}) 2961, 1740, 1463, 1375, 1238; ^1H NMR ($CDCl_3$) δ 0.78 (3H, s, H-9), 1.05 (3H, s, H-10), 1.14 (3H, s, H-8), 1.30 (1H, ddd, J=3.8, 4.1, 13.8 Hz, H-5_{exo}), 1.50 (1H, ddd, J=1.5, 10.6 Hz, H_B-7; nearly C-5,6 position), 1.56 (1H, dddd, J=1.2, 1.5, 2.9, 10.6 Hz, H_A-7; nearly C-2,3 position), 1.76–1.78 (1H, m, H-4), 2.04 (3H, s, 6-OCO*Me*), 2.08 (3H, s, 2-OCO*Me*), 2.41 (1H, ddd, J=2.9, 7.2, 13.8 Hz, H-5_{endo}), 4.46 (1H, s, H-2_{exo}), 5.02 (1H, ddd, J=1.2, 3.8, 7.2 Hz, H-6_{endo}); ^{13}C NMR see Table 2.

3.5.4. (1*R*,2*R*,3*R*)-9-Hydroxyfenchol (5). (−)-(1*R*,2*R*,3*R*)-3-Hydroxymethyl-1,3-dimethylbicyclo[2.2.1]heptan-2-ol; colorless amorphous crystals; $[\alpha]_D^{26.1}$ −19.0° ($CHCl_3$, *c* 0.40), [$[\alpha]_D^{28.5}$ −21.2° $CHCl_3$, *c* 2.8[6,7]; $[\alpha]_D^{20}$ −23° $CHCl_3$, *c* 3.0[8]]; FABMS (neg.), *m/z* 151 $[M-H-H_2O]^+$; EI-MS, *m/z* (rel. intensity) 152 $[M-H_2O]^+$ (0.5), 137 $[152-CH_3]^+$ (5), 123 (4), 121 (5), 109 (6), 108 (5), 95 (6), 93 (6), 81 (100), 80 (18), 79 (11), 71 (6), 69 (16), 67 (15), 55 (13), 53 (6), 43 (16), 41 (18); IR (KBr, ν_{max}, cm^{-1}) 3390, 2926, 1067; ^1H NMR ($CDCl_3$) δ 1.07 (1H, dddd, J=1.7, 3.2, 12.6, 12.6 Hz, H-6_{exo}), 1.09 (3H, s, H-10), 1.10 (3H, s, H-8), 1.16 (1H, dd, J=1.6, 10.3 Hz, H_B-7; nearly C-5,6 position), 1.40 (1H, dddd, J=4.1, 6.3, 12.6, 12.6 Hz, H-5_{exo}), 1.50 (1H, dddd, J=2.0, 2.2, 2.9, 10.3 Hz, H_A-7; nearly C-2,3 position), 1.62 (1H, dddd, J=2.9, 3.2, 8.6, 12.6 Hz, H-5_{endo}), 1.72–1.74 (1H, m, H-4), 1.80 (1H, dddd, J=2.0, 6.3, 8.6, 12.6 Hz, H-6_{endo}), 3.34 (1H, d, J=10.6 Hz, H-9), 3.42 (1H, d, J=1.7 Hz, H-2_{exo}), 3.93 (1H, d, J=10.6 Hz, H-9′); ^{13}C NMR see Table 2.

Acetylation of **5**. Pyridine (1.0 mL) was added to a solution of **5** (5 mg) in acetic anhydride (18 mL), and the solution was stirred for 4 h at room temp (25 °C). The products were isolated in the usual manner and separated by silica gel column chromatography with a hexane–ethylacetate solvent system. The monoacetate **5a** (4 mg) was obtained.

(1*R*,2*R*,3*R*)-9-Acetoxyfenchol (**5a**). (+)-(1*R*,2*R*,3*R*)-3-Acetoxymethyl-1,3-dimethylbicyclo [2.2.1]heptan-2-ol; colorless amorphous crystals; $[\alpha]_D^{26.1}$ +6.4° ($CHCl_3$, *c* 0.19); HRFAB-MS (pos.), *m/z* 213.1479 $[M+H]^+$, calcd for $C_{12}H_{21}O_3$, 213.1491; EI-MS, *m/z* (rel. intensity) 152 $[M-CH_3COOH]^+$ (4), 137 $[152-CH_3]^+$ (6), 123 (6), 121 (5), 109 (6), 95 (6), 93 (6), 81 (100), 80 (17), 79 (8), 69 (13), 67 (13), 55 (9); IR (KBr, ν_{max}, cm^{-1}) 3484, 2954, 1720, 1460, 1373, 1248, 1034; ^1H NMR ($CDCl_3$) δ 1.04 (3H, s, H-8), 1.07 (1H, dddd, J=1.7, 3.4, 12.5, 12.6 Hz, H-6_{exo}), 1.10 (3H, s, H-10), 1.19 (1H, dd, J=1.7, 10.3 Hz, H_B-7; nearly C-5,6 position), 1.46 (1H, dddd, J=4.0, 6.1, 12.5, 12.6 Hz, H-5_{exo}), 1.47–1.51 (1H, m, H_A-7; nearly C-2,3 position), 1.57 (1H, dddd, J=2.9, 3.4, 8.9, 12.6 Hz, H-5_{endo}), 1.77 (1H, dddd, J=2.3, 6.1, 8.9, 12.6 Hz, H-6_{endo}), 1.83–1.85 (1H, m, H-4), 2.06 (3H, s, 9-OCO*Me*), 3.39–3.41 (1H, m, H-2_{exo}), 4.05 (1H, d, J=11.3 Hz, H-9), 4.10 (1H, d, J=11.3 Hz, H-9′); ^{13}C NMR see Table 2.

3.6. 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, substrate **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**.

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