Biotransformation of Geraniol by the Larvae of Common Cutworm (*Spodoptera litura*)

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Abstract: Biotransformation of geraniol by the common cutworm (*Spodoptera litura*) larvae was investigated. Geraniol was mixed in an artificial diet, and the diet was fed to the larvae (fourth-fifth instar) of *S. litura*. Geraniol was transformed to 8-hydroxygeraniol, 9-hydroxygeraniol, (2*E*,6*E*)-8-hydroxy-3,7-dimethyl-2,6-octadienoic acid, (2*E*,6*E*)-3,7-dimethyl-2,6-octadiene-1,8-dioic acid and (2*E*,6*Z*)-3,7-dimethyl-2,6-octadiene-1,9-dioic acid. Geraniol was preferentially oxidized at the allylic methyl group and primary alcohol by *S. litura* larvae.

Key words: common cutworm, *Spodoptera litura*, biotransformation, hydroxylation, geraniol

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 one of the way to produce biologically active terpenoids. The characters of biotransformation are as follows: regio- and stereo-selective reaction under mild conditions and produced optical active compounds easily.

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 references for biotransformation of monoterpenoids by lepidoptera insects. The reasons for using the larvae of *Spodoptera litura* as a biological catalyst are as followed: lepi-

dopteran 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.

Geraniol (**1**) is one of the oldest known organic compounds. Compound **1** is the general terpenoids and are contained in various plants (e.g., *Cymbopogon nardus*, *Pelargonium roseum*, *Rosa damascaeana*). The biotransformation of **1** in plants, microorganisms and mammals have been published (1-28). Previously we reported biotransformation of α -terpinene (29), β -myrcene (30), (–)-dihydromyrcenol and (–)-dihydromyrcenyl acetate (31) by the larvae of common cutworm (*S. litura*). Consequently, we revealed that α -terpinene was preferentially oxidized at the C-7 position (allylic methyl group). β -myrcene was oxidized at the 3,10- and 1,2- double bonds. (–)-dihydromyrcenol and (–)-dihydromyrcenyl acetate were oxidized at the 1,2- double bond and C-3 position.

This paper describes the main metabolites and the metabolic pathway of **1** in the larvae of *S. litura*.

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2 Material and Methods

2.1 Chemical Compound

The substrate used for the biotransformation experiments was geraniol (Fluka).

2.2 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 follow: oven temperature was programmed from 80 to 280°C at 4°C/min; injector and detector temperatures were 270°C and 280°C, respectively; split injection of 25:1; flow rate of helium gas is at 30.0 cm/s. 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. The NMR spectra were obtained with a JEOL FX-500 (500.00 MHz, ¹H; 125.65 MHz, ¹³C) spectrometer. Tetramethylsilane (TMS) was used as the internal standard in CDCl₃. Multiplicities were determined by the DEPT pulse sequence.

2.3 Rearing of Larvae

The larvae of *S. litura* were reared in plastic cases (200 × 300 mm wide, 100 mm high, 100 larvae/case) covered with a nylon mesh screen. The rearing conditions were as follows: 25°C, 70% relative humidity, and constant light. A commercial diet (Insecta LF; Nihon Nosan Kogyo Co., Ltd. Japan) was given to the larvae from the first instar. From the fourth instar, the diet was changed to an artificial diet composed of kidney beans (100 g), agar (15 g), and water (600 mL) (32).

2.4 Administration of Substrate

The artificial diet without the agar was mixed with a blender. Substrate **1** (2200 mg) was then added directly into the blender at 3 mg/g of diet. After agar was dissolved in water, it 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 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 1.8 g, about 2.2 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).

2.5 Isolation and Identification of Metabolites from Frass

The frass were extracted with diethylether (300 mL × 2) and then ethylacetate (300 mL × 2). The extract solution was evaporated under reduced pressure, and 4066 mg of extract was obtained. The extract was dissolved in ethylacetate, and then was added to the 5% NaHCO₃ solution. After shaking, neutral fraction (1226 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 (2122 mg) was obtained from the aqueous layer. The acidic fraction was reacted with ethereal CH₂N₂ overnight and subsequently examined by GC/MS, **4Me**, **5Me** and **6Me** were existed. The neutral fraction was subjected to silica gel open-column chromatography (silica gel 60, 230-400 mesh, Merck) with a hexane/ethylacetate gradient (9:1-1:1) system; two metabolites **2** (195 mg) and **3** (78 mg) were isolated. The methylated acidic fraction was subjected to silica gel open-column chromatography (silica gel 60, 230-400 mesh, Merck) with a hexane/diethylether gradient (9:1-1:1) system; two methylated metabolites **4Me** (649 mg) and **5Me** (725 mg) were isolated. These metabolites were identified by the comparison of established MS, IR, and NMR data. Compound **6Me** was identified by comparison with literature data of GC-MS (33).

2.5.1 8-hydroxygeraniol (**2**)

Colorless oil; EI-MS, *m/z* (rel. intensity) 152 [M-H₂O]⁺ (1), 137 [152-CH₃]⁺ (6), 134 [152-H₂O]⁺ (3), 121 (11), 109 (8), 94 (12), 84 (32), 68 (63), 55 (25), 43 (100); IR (KBr, *v*_{max}, cm⁻¹) 3340, 2922, 2856, 1669, 1444, 1383, 1237, 1005; ¹H-NMR (CDCl₃) δ 1.66 (3H, *brs*, H-9), 1.68 (3H, *brs*, H-10), 2.06-2.09 (2H, *brt*, *J* = 8.0 Hz, H-4), 2.15-2.19 (2H, *m*, H-5), 3.99 (2H, *brs*, H-

8), 4.16 (2H, *brd*, $J = 7.1$ Hz, H-1), 5.38 (1H, *brd*, $J = 1.2$, 7.0 Hz, H-6), 5.41 (1H, *qt*, $J = 1.2$, 7.1 Hz, H-2); ^{13}C -NMR (**Table 1**)

2·5·2 9-hydroxygeraniol (**3**)

Colorless oil; EI-MS, m/z (rel. intensity) 152 [$\text{M}-\text{H}_2\text{O}$] $^+$ (1), 137 [$152-\text{CH}_3$] $^+$ (7), 135 [$152-\text{H}_2\text{O}$] $^+$ (5), 119 (17), 95 (9), 84 (44), 68 (58), 55 (27), 43 (100); IR (KBr, ν_{max} , cm^{-1}) 3354, 2927, 1730, 1669, 1446, 1375, 1246, 1003; ^1H -NMR (CDCl_3) δ 1.68 (3H, *brs*, H-10), 1.79 (3H, *brs*, H-8), 2.02-2.07 (2H, *brt*, $J = 7.5$ Hz, H-4), 2.13-2.20 (2H, *m*, H-5), 4.09 (2H, *brs*, H-9), 4.14 (2H, *brd*, $J = 7.0$ Hz, H-1), 5.25 (1H, *brt*, $J = 7.2$ Hz, H-6), 5.39 (1H, *qt*, $J = 1.5$, 7.0 Hz, H-2); ^{13}C -NMR (**Table 1**)

Methyl ester **4Me** was dissolved in 1N NaOH, and the soln was stirred for 3 hr at room temp (25°C). The product was isolated in the usual manner and obtained metabolite **4**.

2·5·3 (2*E*,6*E*)-8-hydroxy-3,7-dimethyl-2,6-octadienoic acid (**4**)

Colorless oil; EI-MS, m/z (rel. intensity) 166 [$\text{M}-\text{H}_2\text{O}$] $^+$ (4), 151, 148 [$166-\text{H}_2\text{O}$] $^+$ (2), 138 (5), 121 (10), 100 (55), 82 (41), 55 (15), 43 (100); IR (KBr, ν_{max} , cm^{-1}) 3500-2400 (COOH), 1699, 1644; ^1H -NMR (CDCl_3) δ 1.65 (3H, *bs*, H-10), 2.10 (3H, *d*, $J = 1.3$ Hz, H-9), 2.20 (4H, *m*, H-4, 5), 3.91 (2H, *s*, H-8), 5.38 (1H, *bt*, $J = 5.9$ Hz, H-6), 5.66 (1H, *s*, H-2); ^{13}C -NMR (**Table 1**)

2·5·4 (2*E*,6*E*)-8-hydroxy-3,7-dimethyl-2,6-octadienoic acid (**4**) as methyl ester

Colorless oil; EI-MS, m/z (rel. intensity) 198 [M] $^+$

(1), 180 (3), 166 (4), 165 (4), 148 (5), 138 (6), 137 (6), 121 (25), 114 (66), 83 (76), 82 (42), 43 (100); IR (KBr, ν_{max} , cm^{-1}) 3340, 2925, 1719, 1649, 1149; ^1H -NMR (CDCl_3) δ 1.66 (3H, *brs*, H-9), 2.17 (3H, *d*, $J = 1.1$ Hz, H-10), 2.20-2.23 (4H, *m*, H-4, 5), 3.68 (3H, *s*, 1-COOMe), 4.00 (2H, *brs*, H-8), 5.37 (1H, *brt*, $J = 6.0$ Hz, H-6), 5.67 (1H, *q*, $J = 1.1$ Hz, H-2); ^{13}C -NMR (**Table 1**)

Methyl ester **5Me** was dissolved in 1N NaOH, and the soln was stirred for 4 hr at room temp (25°C). The product was isolated in the usual manner and obtained metabolite **5**.

2·5·5 (2*E*,6*E*)-3,7-dimethyl-2,6-octadiene-1,8-dioic acid (**5**)

Colorless oil; EI-MS, m/z (rel. intensity) 180 [$\text{M}-\text{H}_2\text{O}$] $^+$, 162, 134; IR (KBr, ν_{max} , cm^{-1}) 3300-2400 (COOH), 1691, 1645, 1154; ^1H -NMR (CDCl_3) δ 1.76 (3H, *s*, H-9), 1.91 (3H, *s*, H-10), 2.18 (2H, *t*, $J = 7.0$ Hz, H-5), 2.28 (2H, *q*, $J = 7.0$ Hz, H-5), 5.67 (1H, *s*, H-2), 6.34 (1H, *t*, $J = 7.0$ Hz, H-6); ^{13}C -NMR (**Table 1**)

2·5·6 (2*E*,6*E*)-3,7-dimethyl-2,6-octadiene-1,8-dioic acid (**5**) as dimethyl ester

Colorless oil; EI-MS, m/z (rel. intensity) 226 [M] $^+$ (1), 194 (42), 179 (7), 166 (88), 162 (41), 134 (82), 107 (100), 91 (38), 82 (52); IR (KBr, ν_{max} , cm^{-1}) 2925, 1718, 1649, 1150; ^1H -NMR (CDCl_3) δ 1.84 (3H, *brs*, $J = 1.5$ Hz, H-9), 2.17 (3H, *d*, $J = 1.5$ Hz, H-10), 2.27 (2H, *brt*, $J = 7.5$ Hz, H-4), 2.33-2.38 (2H, *m*, H-5), 3.37 (3H, *s*, 1-COOMe), 3.69 (3H, *s*, 8-COOMe), 5.68 (1H, *q*, $J = 1.5$ Hz, H-2), 6.69 (1H, *qt*, $J = 1.5$, 7.5 Hz, H-6); ^{13}C -

Table 1 ^{13}C NMR Spectral Data for Metabolites **2-5** (125.00 MHz, CDCl_3) .

Carbon	Compounds					
	2	3	4	4Me	5	5Me
1	59.3 (t)	59.1 (t)	166.4 (s)	167.1 (s)	166.6 (s)	167.0 (s)
2	123.7 (d)	124.0 (d)	116.2 (d)	115.4 (d)	115.6 (d)	115.7 (d)
3	139.9 (s)	139.1 (s)	163.6 (s)	159.5 (s)	158.0 (s)	158.4 (s)
4	39.0 (t)	39.2 (t)	41.3 (t)	40.4 (t)	39.0 (t)	39.3 (t)
5	25.7 (t)	25.8 (t)	26.7 (t)	25.5 (t)	26.1 (t)	26.5 (t)
6	125.5 (d)	127.3 (d)	124.9 (d)	124.3 (d)	140.0 (d)	140.3 (d)
7	135.1 (s)	134.9 (s)	137.0 (s)	135.8 (s)	128.2 (s)	128.5 (s)
8	68.9 (t)	21.1 (q)	68.6 (t)	68.7 (t)	167.9 (q)	168.3 (q)
9	13.7 (q)	61.3 (t)	13.7 (q)	13.6 (q)	12.2 (q)	12.4 (q)
10	16.2 (q)	16.3 (q)	19.0 (q)	18.7 (q)	18.3 (q)	18.7 (q)
1-COOMe				50.8 (q)		50.8 (q)
8-COOMe						51.7 (q)

NMR (Table 1)

2·5·7 (2*E*,6*Z*)-3,7-dimethyl-2,6-octadiene-1,8-dioic acid (**6**) as dimethyl ester

EI-MS, m/z (rel. intensity) 226 [M]⁺ (1), 195 (4), 194 (18), 166 (13), 162 (83), 135 (45), 134 (100), 107 (44), 91 (32)

3 Results and Discussion

3.1 Biotransformation of Geraniol (1) by the Larvae of *S. 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 that were fed the diet without substrate were used as control, and the extract of frass was analyzed by GC. Compounds **1-6** metabolites were not observed in the frass of controls.

In the biotransformation of **1**, the five metabolites isolated from the frass were identified as 8-hydroxygeraniol (**2**), 9-hydroxygeraniol (**3**), (2*E*,6*E*)-8-hydroxy-3,7-dimethyl-octadienoic acid (**4**), (2*E*,6*E*)-3,7-dimethyl-octadiene-1,8-dioic acid (**5**) and (2*E*,6*Z*)-3,7-dimethyl-octadiene-1,9-dioic acid (**6**) (Scheme 1). Intermediary metabolites (aldehyde and epoxide) were not detected in the frass by GC-MS analysis.

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 $C_{10}H_{18}O_2$ based on its EI-MS spectral. The IR spectrum

observed increase of hydroxy band. In 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, which has been reported in the literature (34). NOESY spectrum indicates the structure established with correlation cross-peak that were observed between H-6 (5.38 ppm) and H-8 (3.99 ppm). From these data, it was concluded that the structure of **2** is 8-hydroxygeraniol.

The metabolite **3** had a molecular formula of $C_{10}H_{18}O_2$ that was estimated by its EI-MS spectral and the IR spectrum observed increase of hydroxy band. In 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, which has been reported in the literature (34). Assignment of new methylene group was achieved by HMBC and NOESY. In the characteristic HMBC spectrum, some correlation cross-peaks were observed of C-6 (139.1 ppm) with one methyl group (1.79 ppm; H-8) and new methylene group (4.09 ppm; H-9), three methylene groups with the quaternary carbon (139.1 ppm; C-3). NOESY spectrum indicate the structure established with correlation cross-peak that were observed between H-5 (2.13-2.20 ppm) and H-9 (4.09 ppm). From these data, it was concluded that the structure of **3** is 9-hydroxygeraniol.

The methyl ester **4Me** had a molecular formula of $C_{11}H_{18}O_3$ that was estimated by its EI-MS spectral. It had three methyl (including a esterified methyl observed at 3.68 ppm), three methylene, two methine and three quaternary carbons (including a carboxy carbon observed at 167.1 ppm), one quaternary carbon

Scheme 1 Possible Metabolic Pathway of Geraniol (**1**) by the Larvae of *S. litura*.

more than **1**. The ^1H - and ^{13}C -NMR spectra were assigned by comparison with the substrate **1** and the previous paper (35). Assignment of the carboxy carbon and methylene of hydroxy group signals was achieved by HMBC and NOESY. In the characteristic HMBC spectrum, some correlation cross-peak were observed of methine group (5.67 ppm; H-2) with carboxy carbon (167.1 ppm; C-1), methyl group (18.7 ppm; C-10) and methylene group (40.4 ppm; C-4). On the other hands, some correlation cross-peak were observed of methine group (5.37 ppm; H-6) with methyl group (13.6 ppm; C-9) methylene group (68.7 ppm; C-8) and quaternary carbon (135.8 ppm; C-7). NOESY spectrum indicates the structure established with correlation cross-peak that observed between H-6 (5.37 ppm) and H-8 (4.00 ppm). From these data, it was concluded that the structure of **4** is (2*E*,6*E*)-8-hydroxy-3,7-dimethyl-octadienoic acid.

The methyl ester **5Me** had a molecular of $\text{C}_{12}\text{H}_{18}\text{O}_4$ that was estimated by its EI-MS spectral. It had four methyl (including a esterified methyl observed at 3.37 and 3.69 ppm), two methylene and four quaternary carbons (including two carboxy carbon observed at 50.8 and 51.7 ppm, respectively), one methyl and two quaternary carbons more than **1**. The ^1H - and ^{13}C -NMR spectra were assigned by comparison with the substrate **1** and the previous paper (33). Assignment of two carboxy carbons was achieved by HMBC spectrum, some correlation cross-peak was observed of methine group (5.68 ppm; H-2) with carboxy carbon (167.0 ppm; C-1), methyl group (18.7 ppm; C-10) and methylene group (39.3 ppm; C-4). On the other hands, some correlation cross-peaks were observed of methine group (140.3 ppm; H-6) with methyl group (C-9), carboxy carbon (168.3 ppm; C-8) and quaternary carbon (128.5 ppm; C-7). NOESY spectrum indicates the structure established with correlation cross-peak that observed between H-6 (6.69 ppm) and methoxy group (3.37 ppm; C-8). From these data, it was concluded that the structure of **5** is (2*E*,6*E*)-3,7-dimethyl-octadiene-1,8-dioic acid.

3.2 Metabolic Pathways

In the present study of biotransformation of **1**, the larvae transformed **1** to **2** (16.1%), **3** (6.4%), **4** (30.6%), **5** (34.2%) and **6** (4.0%) (Table 2). There was a tendency to oxidize at the C-1, C-8 and C-9 positions of **1**. Also, there were two metabolic pathways with biotrans-

formation of **1**. The result of the biotransformation of **1**, revealed that oxidation at the main metabolic pathway (**1** → **2** → **4** → **5**) (Scheme 1). On the other hand, oxidation at the C-1 and C-9 positions is the minor metabolic pathway (**1** → **3** → **6**) (Scheme 1). Previously we reported biotransformation of acyclic monoterpenes by the larvae of *S. litura* (30, 31). But the reaction wasn't progressing to the carboxylic acid. The present study is first report on obtaining the carboxylic acid from acyclic monoterpenes in biotransformation by *S. litura*.

There have been some reports of the biotransformation of **1** by other organisms. In the biotransformation of **1** by the suspension cells of *C. roseus*, compound **2**, 8-hydroxycitronellol and 8-hydroxyneryl are produced. Oxidation at the C-8 position (allylic methyl groups) is the main metabolic pathway (1). On the other hands, biotransformation in suspension cultured cells of plants (e.g. *A. millefolium*, *R. centifolia*, *A. graveolens* and *V. vinifera*), failed to oxidize the C-8 methyl group of **1**, and instead preferred to oxidize the primary alcoholic group to citral and interconversion of **1** into nerol (2-11). But the biotransformation of **1** by the suspension cell aren't progressing to the carboxylic acid. In the biotransformation of **1** by microorganisms observed oxidation of the primary alcohol group to the carboxy group (23-27). But these biotransformation also aren't progressing to the dicarboxylic acid. In the biotransformation of **1** in the rat, compound **1** is transformed to compound **2**, compound **5**, 8-carboxygeraniol, geranic acid and 3-hydroxycitronellic acid are produced. Oxidation at the C-1 and C-8 positions (primary alcohol and allylic methyl group) are the metabolic pathways in rat (28).

In the present study, the larvae of *S. litura* employ a metabolic pathway similar to that used by the rat. But the minor metabolic pathway isn't obtained previous

Table 2 Metabolites of Geraniol (**1**) by the *S. litura* Larvae^a.

Substrate	Yield ^b (%)					
	1 ^c	2	3	4	5	6
Geraniol	8.7	16.1	6.4	30.6	34.2	4.0

^a Metabolites were obtained from the frass of *S. litura*.

^b Percentage was calculated from the peak area in the gas chromatogram of the extract of frass. Analyzed by GC-MS.

^c Recovered substrate.

paper. Therefore, the minor metabolic pathway is only one metabolic pathway for metabolism of **1** in the *S. litura* larvae.

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