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Regio- and Stereoselective Oxidation of (+)-Δ^3-Carene by the Larvae of Common Cutworm (*Spodoptera litura*)

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Biotransformation of (+)-Δ^3-carene (**1**) and (+)-(1*S*,3*S*,4*R*,6*R*)-3,4-epoxycarane (**1-1**) by larvae of *Spodoptera litura* was investigated. Compound **1** was transformed to (+)-(1*S*,3*S*,4*R*,6*R*,7*S*)-3,4-epoxycaran-9-ol (**1-2**) by *S. litura*. This structure was established by infrared, electron impact−mass spectrometry, one- and two-dimensional NMR spectral studies, and (+)-(1*S*,3*S*,4*R*,6*R*)-3,4-epoxycarane (**1-1**) was transformed for confirmation of a metabolic pathway. The results indicate that the metabolic reaction of compound **1** by the larvae of *S. litura* was regioselective hydroxylation at the methyl group of the geminal dimethyl (C-9 position) followed by stereoselective epoxidation at the carbon−carbon double bands (C-3 position). (+)-(1*S*,3*S*,4*R*,6*R*,7*S*)-3,4-Epoxycaran-9-ol (**1-2**) was a new compound.

KEYWORDS: Biotransformation; *Spodoptera litura*; (+)-Δ^3-carene; hydroxylation; epoxydation

INTRODUCTION

Terpenoids have been 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 secondary metabolites of plants, and these terpenoids have been shown to have biological activities against plants, microorganisms, and insects (*1*). However, there are few reports of the metabolic pathway of terpenoids. Recently, various attempts have been made to search for new biologically active terpenoids.

The investigation in the field of the biotransformation of monoterpenoids is gaining more interest: These reactions are performed by bacteria, fungi, yeasts, and even algae (*2*, *3*). However, there are few reports in the literature on the biotransformation of terpenoids by lepidoptera insects. The reasons for using the larvae of *Spodoptera litura* as a biological catalyst are as follows: Lepidoptera larvae always feed on plants containing terpenoids, so *S. litura* has a high level of enzymatic activity against terpenoids; the larvae consume a large amount of plants, making it possible to obtain more metabolites; and the larvae are easy to rear on a laboratory scale. Previously, we reported the biotransformation of [2.1.0]-bicyclic monoterpenoids, (+)-(1*R*)- and (−)-(1*S*)-camphor, (+)-(1*R*)- and (−)-(1*S*)-fenchone, and (+)-(1*R*,2*S*)-fenchol by *S. litura* (*4*−*7*). These studies revealed that substrates were regio- and stereoselectivity hydroxylated.

(+)-Δ^3-Carene (**1**) is a [4.1.0]-bicyclic monoterpenoid. Compound **1** is contained in the plant of the *Pinus* family as a main component (*8*). Biotransformations of **1** in microorganisms (*9*), cultured cells of plants (*10*, *11*), and mammals (*12*−*15*) have been published. However, there are no reports on the biotransformation by insects. In the present paper, the biotransformation of **1** and (+)-(1*S*,3*S*,4*R*,6*R*)-3,4-epoxycarane (**1-1**) by the larvae of *S. litura* is being reported for the first time, wherein we deal with the insects as biocatalysts and investigate for the purpose of estimating possible metabolic pathways and making products with high degrees in insects.

MATERIALS AND METHODS

Rearing of Larvae. 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 constant light. A commercial diet (Insecta LF; Nihon Nosan Kogyo Co., Ltd., Japan) was given to the larvae from the first instars. From the fourth instars, the diet was changed into an artificial diet composed of kidney beans (100 g), agar (12 g), and water (600 mL).

Materials. Compound **1** was purchased from Yasuhara Chemical Co. Ltd. (Hiroshima, Japan). Compound **1-1** (5.0 g) was synthesized from compound **1** by epoxydation according to the method of Crombie et al. (*16*). The overall yield was 89.4%. The spectral date was assigned by comparison with the previous paper.

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 inner diameter). Chromatographic conditions were as follows: the oven temperature was programmed from 80 to 270 at 4 °C/min; the intentional time and final time were kept at 5 min; the injector and detector temperatures were 270 and 280 °C, respectively; the split injection was 25:1; and the flow rate of helium gas was 30.0 cm/s. Electron impact−mass spectrometry (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 inner diameter). Chromatographic conditions were the same as described above for DB-5. The effluent of the GC column was introduced directly into the source of the via a transfer line (280 °C). The temperature of the ion source was 230 °C, and the electron energy was 70 eV. The acquisition mass range was 39−450 amu. High-resolution mass spectrometry

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Table 1. ^{1}H NMR Spectral Data for Compounds **1-1**, **1-2**, and **1-2Ac** (500.00 MHz, $CDCl_3$)[a]

	compounds		
carbon	**1-1**	**1-2**	**1-2Ac**
1	0.53 (1H, ddd, 9.2, 9.2, 2.0)	0.70 (1H, ddd, 9.2, 9.2, 2.3)	0.76 (1H, ddd, 9.2, 9.2, 2.0)
2	1.45 (1H, dd, 16.0, 2.0)	1.53 (1H, dd, 16.1, 2.3)	1.53 (1H, dd, 16.4, 2.0)
	2.14 (1H, dd, 16.0, 9.2)	2.23 (1H, dd, 16.1, 9.2)	2.23 (1H, dd, 16.4, 9.2)
3			
4	2.84 (1H,brs)	2.87 (1H, brs)	2.86 (1H, brs)
5	1.64 (1H, ddd, 16.4, 2.0, 2.0)	1.68 (1H, ddd, 16.5, 2.3, 2.3)	1.67 (1H, ddd, 16.8, 2.0, 2.0)
	2.30 (1H, ddd, 16.4, 9.2 2.0)	2.34 (1H, ddd, 16.5, 9.2 2.3)	2.34 (1H, ddd, 16.8, 9.2 2.0)
6	0.45 (1H, ddd, 9.2, 9.2, 2.0)	0.62 (1H, ddd, 9.2, 9.2, 2.3)	0.69 (1H, ddd, 9.2, 9.2, 2.0)
7			
8	0.73 (3H, s)	0.86 (3H, s)	0.82 (3H, s)
9	1.00 (3H, s)	3.32 (2H, s)	3.79 (2H, s)
10	1.26 (3H, s)	1.28 (3H, s)	1.28 (3H, s)
OAc			2.06 (3H, s)

[a] NMR spectra were recorded at 500 MHz (^{1}H) in $CDCl_3$ solution using TMS as an internal standard.

(HR-MS) was carried out with a JEOL JHX100 (with a JEOL JAMDA 5000 mass date system). The IR spectrum was obtained with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer. The NMR spectrum was obtained with a JEOL FX-500 (500.00 MHz, ^{1}H; 125.65 MHz, ^{13}C) spectrometer. The temperature of NMR measurements was 25 °C. Tetramethylsilane (TMS) was used as the internal standard in $CDCl_3$. Multiplicities were determined by the distortionless enhancement by polarization transfer (DEPT) pulse sequence. The specific rotations were measured on a JASCO DIP-1000 digital polarimeter.

Administration of the Substrate. The beans (100 g), water (600 mL), and compound **1** (1000 mg, 3 mg/g diet) were added into a blender and mixed for 5 min. Then, agar, which was dissolved in water, was added into a blender and boiled. The diet was then mixed and cooled in a stainless steel tray (220 mm × 310 mm wide, 30 mm high). The fourth and fifth instar larvae (average weight = 0.5 g) were moved into new cases (100 larvae/case), and the diet (1600 g) containing compound **1** was fed to the larvae for 2 days; then, the artificial diet not containing compound **1** was fed to the larvae for an additional 2 days. Compound **1-1** (1.0 g) was administered in the same manner.

Isolation and Identification of Metabolite from Frass. Frass was collected every 5 h (total of 4 days) and extracted in a solution of diethyl ether (300 mL, two times). For diet and frass separation, the fresh frass was extracted as soon as the fourth and fifth instar larvae excreted. For the quantitative analysis of metabolites, GC analysis was used as an internal standard with the substrate. The extracts were evaporated under reduced pressure, and 1.8 g of extract was obtained. The extracts were dissolved in diethyl ether and then were added to the 5% $NaHCO_3$ solution. After the solution was shaken, the neutral fraction (960 mg) was obtained from the diethyl ether layer. The aqueous layer was separated, then acidified with 1 N HCl (acidic fraction), and extracted with diethyl ether. After the solution was shaken, the acidic fraction (842 mg) was obtained from the aqueous layer. The neutral fraction was analyzed by GC-MS; (+)-(1*S*,3*S*,4*R*,6*R*,7*S*)-3,4-epoxycaran-9-ol (**1-2**) occurred in this fraction. The acidic fraction was reacted with ethereal CH_2N_2 overnight and subsequently examined by GC-MS, but metabolites did not occur. The neutral fraction was analyzed by GC-MS; compound **1-2** occurred in this fraction. The neutral fraction was subjected to silica gel open-column chromatography (silica gel 300 mesh, Merck) with an 8:2 hexane/ethyl acetate solvent system, and compound **1-2** (220 mg) was isolated. In the case of compound **1-1**, the same procedure as described for **1** was used. Compound **1-1** was transformed to compound **1-2** (800 mg). The amount of metabolite was isolated from the extracts of frass (total extract of 1.98 g), and compound **1-1** was recovered at 200 mg. The metabolic compound from the frass was identified by a comparison of established GC-MS, IR, and NMR dates.

Structure of Metabolic Products. Compound **1-1** was a colorless oil. $[\alpha]_D^{25,0}$ +14.36° ($CHCl_3$, *c* 1.0). EI-MS, *m*/*z* (relative intensity): 152 $[M]^+$ (2), 137 (63), 119 (90), 109 (100), 91 (79), 77 (37), 67 (54), 55 (34), 43 (63). IR (film, ν_{max}, cm^{-1}): 2950, 1237, 1018. ^{1}H NMR: See **Table 1**. ^{13}C NMR: See **Table 2**.

Table 2. ^{13}C NMR Spectral Data for Compounds **1-1**, **1-2**, and **1-2Ac** (125.00 MHz, $CDCl_3$)[a]

	compounds		
carbon	**1-1**	**1-2**	**1-2Ac**
1	13.3 (d, CH)	13.3 (d, CH)	13.8 (d, CH)
2	23.3 (t, CH_2)	23.0 (t, CH_2)	23.0 (t, CH_2)
3	58.0 (s, C)	55.9 (s, C)	55.9 (s, C)
4	58.3 (d, CH)	58.0 (d, CH)	57.9 (d, CH)
5	19.9 (t, CH_2)	18.9 (t, CH_2)	18.9 (t, CH_2)
6	16.2 (d, CH)	11.2 (d,CH)	11.7 (d, CH)
7	23.0 (s, C)	23.1 (s,C)	22.1 (s, Q)
8	14.6 (q, CH_3)	10.2 (q, CH_3)	10.5 (q, CH_2)
9	27.5 (t, CH_2)	72.5 (t, CH_2)	73.6 (t, CH_2)
10	23.1 (q, $CH2_3$)	22.9 (q, CH2)	21.0 (q,CH_3)
OAc			171.3 (s, OAc)

[a] NMR spectra were recorded at 125 MHz (^{13}C) in $CDCl_3$ solution using TMS as an internal standard.

Compound **1-2** was a colorless oil. $[\alpha]_D^{28,0}$ +4.76° ($CHCl_3$, *c* 1.0). HREI-MS, *m*/*z*: 168.1156 $[M]^+$, calcd for $C_{10}H_{16}O_2$: 168.1151. EI-MS, *m*/*z* (relative intensity): 150 $[M - H_2O]^+$(4), 135 (18), 121 (37), 107 (37), 100 (45), 91 (52), 79 (38), 67 (26), 55 (35), 43 (100). IR (film, ν_{max}, cm^{-1}): 3425, 2920, 1202, 1017. ^{1}H NMR: See **Table 1**. ^{13}C NMR: See **Table 2**.

(+)-(1*S*,3*S*,4*R*,6*R*,7*S*)-9-Acetyl-3,4-epoxycaran (compound **1-2Ac**) was obtained as a colorless oil by reaction with Ac_2O and pyridine. $[\alpha]_D^{25,0}$ +4.98° ($CHCl_3$, *c* 1.0). HREI-MS, *m*/*z*: 210.1261 $[M]^+$, calcd for $C_{12}H_{18}O_3$: 210.1256. EI-MS, *m*/*z* (relative intensity): 150 $[M - OAc]^+$ (4), 135 (18), 121 (37), 107 (37), 93 (45), 91 (52), 67 (38), 79 (26), 43 (100). IR (film, ν_{max}, cm^{-1}): 1739, 1380, 1242, 1029. ^{1}H NMR: See **Table 1**. ^{13}C NMR: See **Table 2**.

RESULTS AND DISCUSSION

Biotransformation of 1 by the Larvae of *S. litura*. In the biotransformation of compound **1**, **1** was converted to **1-2**. Alcohol was detected by GC analysis, and more oxidation compounds (carboxylic acid and aldehyde) were not detected in this experimental. Frass of the larvae, which were fed a diet without substrate, were used as control, and the extracts from the frass were analyzed by GC-MS. The result was that compounds **1** and **1-2** were not observed in the frass of control. For the quantitative analysis of metabolite, we varied the quantity of substrate in the extract by the internal standard method in GC. The percentage was calculated from the peak area in the GC chromatogram of the frass extract. The frass extract was 78.0% recovered substrate **1** and 22.0% converted metabolite **1-2** by peak area in GC (see **Table 3**). Metabolic reaction by the larvae of *S. litura* was observed as follows: Compound **1** was administered

to the larvae through their diet; the metabolite was then confirmed and isolated from the frass of larvae. The larvae were fed with artificial diet, and the extract of frass was analyzed by GC-MS. The result was that **1** was not observed in the frass. Compound **1** was transformed to **1-2** completely.

The IR spectrum of compound **1-2** had a hydroxyl band at 3425 cm^{-1} and epoxide band at 1017 cm^{-1}. The EI-MS spectrum showed a significant mass fragment $[M - H_2O]^+$ at m/z 150. The ^1H NMR spectrum of **1-2Ac** (acetylated compound derived from **1-2**) displayed one signal at δ 2.06 due to monoacetate: Other signals are summarized in **Table 1**. To confirm the structure of compound **1-2**, H−H correlation spectroscopy, heteronuclear multiple quantum coherence, heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect (NOE) experiments were conducted. The hydroxyl group at δ 3.32 was placed at C-9 based on the ^3J correlation of three carbons with C-1 (13.3 ppm), C-6 (11.2 ppm), and C-8 (10.2 ppm) in its HMBC spectrum and NOE correlation with H-1 (0.70 ppm) and H-6 (0.62 ppm) (see **Figure 1**). The epoxide proton at δ 3.32 was placed at C-4 based on the ^3J correlation of three carbons with C-5 (18.9 ppm), C-6 (11.2 ppm), and C-10 (22.9 ppm) in its HMBC spectrum and NOE correlation with H-2 (1.53 ppm) (see **Figure 1**). The specific rotation shows the (+)-form. From these dates, it was concluded that the structure of **1-2** was (+)-(1*S*,3*S*,4*R*,6*R*,7*S*)-3,4-epoxycaran-9-ol (see **Scheme 1**). Compound **1-2** was a new compound.

Table 3. Recovery and Yield of Metabolite of Compound **1-2** by *S. litura*

				yield[b] (%)	
substrate	administered in the artificial diet (g)	frass extract[a] (g)	recovery (%)	**1**	**1-2**
1	1.0	1.80	100	78.0[c]	22.0
1-1	1.0	1.98	100	20.0[c]	80.0

[a] Calculated from the peak area in the gas chromatogram using an internal standard (**1**). [b] Percentage estimated by GC. [c] Recovered substrate.

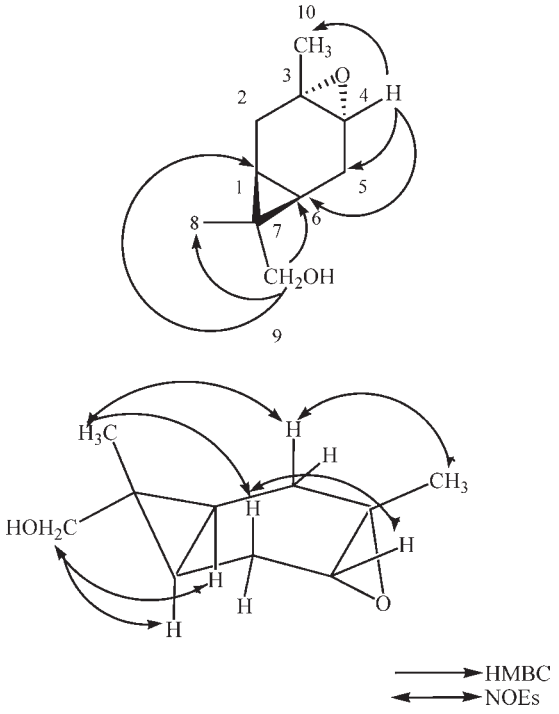
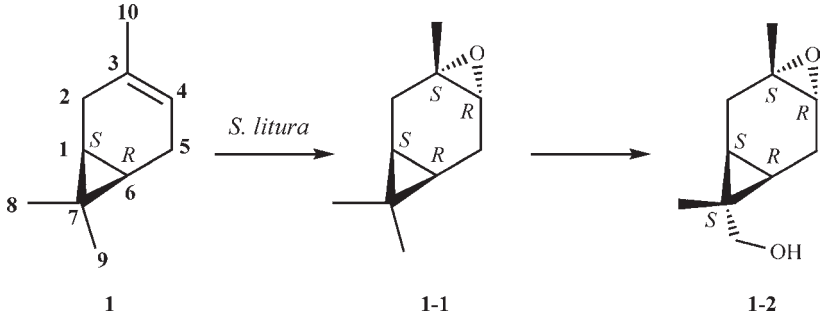


Figure 1. Cross-peaks observed in compound **1-2**.

Biotransformation of (+)-(1*S*,3*S*,4*R*,6*R*)-3,4-Epoxycaran (1-1) by the Larvae of *S. litura*. Compound **1-1** was transformed for confirmation of the metabolic pathway. In the biotransformation of compound **1-1** , **1-1** was converted to **1-2**, too. The frass extract was 20.0% recovered substrate **1-1** and 80.0% converted metabolite **1-2** by peak area in GC (see **Table 3**). The conversion rate of **1-1** was higher than **1**.

There have been some reports of biotransformation of compound **1** by other biocatalysts. In the biotransformation of **1** by *Acetobacter aceti*, caren-3,4-diol-5-one and caren-3,4-diol-carboxylic acid are produced. The reaction of microorganism included hydroxylation C=C double bonds (C-3) and allylic oxidation. In the biotransformation of **1** by *Acetobacter aceti*, caren-3,4-diol-5-one and caren-3,4-diol-carboxylic acid are produced. The reaction of microorganism included hydroxylation C=C double bonds (C-3) and allylic oxidation. In the biotransformation of **1** by cultured cells of *Rosa centifolia*, *p*-cymen-7-ol, *p*-isopropenyltoluene, and *trans*-epoxycarane are produced. In the biotransformation of **1** by cultured cells of *N. tabacum* and *C. rose*, 3,4-epoxycarane, 3,4-caranediol, 3-carn-5-one, 3,3,6-trimethylcyclohepta-2,4-dien-1-one, and 8-hydroxy-*m*-cymene are produced. The reaction of plants cultured cells included hydroxylation C=C double bonds (C-3), allylic oxidation, and cleavage of cyclopropane ling. In the biotransformation of **1** by rabbits, 3-caren-9-ol, 3-caren-9-carboxylic acid, caren-9,10-dicarboxylic acid, and 3-caren-10-carboxylic acid are produced. In the biotransformation of **1** by human liver microsomes, *trans*-epoxycarane and caren-10-ol are produced. The reactions of mammals (rabbits and human liver microsomes) included allylic oxidation, epoxydation, and geminal dimethyl hydroxylation. As compared with other biocatalysts, the characteristics of the larvae of *S. litura* were regioselective geminal dimethyl hydroxylation (C-9) and stereoselective epoxidation at C=C double bonds (C-3). These reactions are not usually found in other catalysts. This study revealed that **1** was transformed to **1-2** via **1-1**.

Scheme 1. Biotransformation of Δ^3-Carene (**1**) by *S. litura*



1 **1-1** **1-2**

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