

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

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Biotransformation of the 5,7,4'-trimethoxyisoflavone (1), 6,7,4'-trimethoxyisoflavone (2), and 7,4'-dimethoxyisoflavone (3) by insects, *Spodoptera litura* was investigated. Compound 1 was transformed to 5-hydroxy-7,4'-dimethoxyisoflavone (4), 7-hydroxy-5,4'-dimethoxyisoflavone (5) and 4'-hydroxy-5,7-dimethoxyisoflavone (6) by *S. litura*. Compounds 2 and 3 were hardly metabolized by *S. litura*. This suggested that compound 1 was converted to compounds 4, 5 and 6 by demethylation at the C-5, C-7 and C-4' position, respectively.

Key words biotransformation; isoflavone; *Spodoptera litura*; biocatalyst; demethylation

The naturally occurring isoflavones are reported to possess diverse biological activities, including antioxidative,¹⁾ antifungal²⁾ and estrogenic^{3,4)} activity. Recently, an osteoporosis preventive effect⁵⁾ and mitigation of menopausal disorders⁶⁾ has attracted attention and various physiological activities of isoflavone are expected. It has been reported that these activities were enhanced in the biotransformation.^{7,8)} Biotransformation possesses the advantage of proceeding under mild conditions and with high regio- and enantioselectivity. Esaki *et al.* reported the biotransformation of soybean isoflavones to 8-hydroxydaidzein and 8-hydroxygenistein by *Aspergillus satoii* and these isoflavones exhibited significantly stronger antioxidative activity than daidzein and genistein.⁷⁾ Therefore, it is important to produce the bioactive isoflavone derivatives and to elucidate their metabolic pathways in microorganisms and insects.

In the course of our work, we have studied the biotransformation of isoflavonoids and terpenoids by fungi and insects as a biocatalyst.^{9–14)} In a previous paper, we reported the biotransformation of the isoflavones, 7,4'-dimethoxyisoflavone and 7,4'-diacetoxyisoflavone, by *Aspergillus niger*. 7,4'-Dimethoxyisoflavone was transformed to 6-hydroxy-7,4'-dimethoxyisoflavone and daidzein, while 7,4'-diacetoxyisoflavone was also transformed to daidzein by hydrolysis.¹⁰⁾ In this paper, we report the synthesis of isoflavones, 5,7,4'-trimethoxyisoflavone (1), 6,7,4'-trimethoxyisoflavone (2) and 7,4'-dimethoxyisoflavone (3) and the biotransformation of compounds 1, 2 and 3 by insect, *Spodoptera litura*.

Results and Discussion

Compound 1 was metabolized by *S. litura*. Compounds 2 and 3 were hardly metabolized by *S. litura*.

The transformation of 5,7,4'-trimethoxyisoflavone (1) by *S. litura* was examined. Extracts were chromatographed on silica gel column chromatography to give metabolites. Metabolites 4 (6 mg), 5 (5 mg), 6 (3 mg) and substrate 1 (130 mg) were isolated. In order to determination of structure of the metabolites, metabolites 5 and 6 were acetylated.

Metabolites 4, 5 and 6 showed the molecular formula of C₁₇H₁₄O₅ in their HR-EI-MS, respectively. The ¹H-NMR spectrum of compounds 4, 5 and 6 showed the presence of two methoxyl groups, which indicated that one methoxy sig-

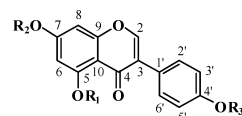
nal has disappeared compared with compound 1. Furthermore, compound 4 had a signal of hydrogen bond at δ 12.85. Compounds 5 and 6 were converted monoacetyl compounds, 5Ac and 6Ac by reaction with acetylation. It results that compounds 4, 5 and 6 showed the presence of two methoxyl groups and one hydroxy group, respectively (see Table 1).

Compounds 4 and 5 also showed a significant mass fragment at m/z 166 and 132, and compound 6 also showed a significant mass fragment at m/z 180 and 118, respectively. In a previous article, Talukdar *et al.* reported the structure of 5-hydroxy-7,4'-dimethoxyisoflavone by the *retro* Diels–Alder reaction indicating the presence of one methoxyl and one hydroxy groups in ring A, and one methoxy group should be present in ring B.¹⁵⁾ In the identification of the isoflavones, the *retro* Diels–Alder fragmentation was found to be important.^{16,17)}

In our previous paper, we reported the biotransformation of sinesetin by larvae of *S. litura*. The main pathway was demethylation at C-6 position followed by glucosylation, and the minor pathway was demethylation at the C-4' position.¹³⁾

These results showed that compound 4 had the hydroxy group at C-5 position and compound 5 had the hydroxy group at C-7 position. Compound 6 also showed a significant mass fragment at m/z 180 and 118 formed after the *retro* Diels–Alder reaction indicating the presence of two methoxyl groups in ring A, and one hydroxy group should be present in ring B. Thus from compound 6 had the hydroxy group at C-4' position.

Thus from the foregoing spectral studies the structure of compounds 4, 5 and 6 were identified as 5-hydroxy-7,4'-dimethoxyisoflavone, 7-hydroxy-5,4'-dimethoxyisoflavone and 4'-hydroxy-5,7-dimethoxyisoflavone, respectively. Spectral data of compound 4 were assigned by comparison with the previous paper.¹⁵⁾ Compound 5 was obtained by synthesis



- 1 $R_1=R_2=R_3=Me$
- 4 $R_1=H, R_2=R_3=Me$
- 5 $R_1=R_3=Me, R_2=H$
- 6 $R_1=R_2=Me, R_3=H$

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from the chalcone.¹⁸⁾ In our previous paper, compound **6** was obtained by microbial transformation.¹⁷⁾ The ¹H-NMR spectrum analysis of compounds **5Ac** and **6Ac** were not reported, and thus this is the first report of the ¹H-NMR spectrum analysis of compounds **5Ac** and **6Ac**.

Compound **1** was transformed to compounds **4**, **5** and **6**. Metabolites **4**, **5** and **6** were compounds in the demethylation of compound **1** at the C-5 and C-7 and C-4' position, respectively. On the other hand, compounds **2** and **3** were not converted. These results indicate that compound **1** showed substrate specificity for this biotransformation. This is the first report of biotransformation of 5,7,4'-trimethoxyisoflavone (**1**) by *S. litura*. It is interesting to note that demethylation of one position are easily obtained by insects.

Experimental

General Procedure Nuclear magnetic resonance (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 (δ 0.00). IR spectra were determined with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer. EI-MS spectra were obtained on a JEOL the Tandem MS station JMS-700 TKM. Gas chromatograph (GC) was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID). GC-MS was performed on a Hewlett-Packard 5972 series mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a column (HP-5MS, 30 m length, 0.25 mm i.d.).

Preparation of Isoflavones Genistein, glycitein and daidzein were isolated soyaflabon HG (powder of Glycine max, purchased from FUJI Oil Co., Ltd. (Osaka, Japan).

Synthesis of Substrates Methylation of isoflavones were carried out according to the synthetic method of Matsuda *et al.*¹⁹⁾ A solution of genistein (200 mg) in *N,N*-dimethylformamide (DMF, 12 ml) was treated with methyl iodide (CH₃I, 0.6 ml) in the presence of sodium hydride (NaH, 60 mg) and the mixture was stirred at room temperature for 12 h. 5,7,4'-Trimethoxyisoflavone (**1**) (180 mg) was obtained. Through a similar procedure of methylation, 6,7,4'-trimethoxyisoflavone (**2**) (188 mg) was obtained from

glycitein and 7,4'-dimethoxyisoflavone (**3**) (190 mg) was obtained from daidzein. These spectral data were assigned by comparison with the previous paper.^{10,17)}

Rearing of Larvae The larvae of *S. litura* were reared in plasticcases (200×300 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.) was given to the larvae from the first to sixth (last) instar.

Administration of 5,7,4'-Trimethoxyisoflavone (1) The diet of 100 larvae (fourth–fifth instar) was changed to an artificial diet composed of kidney beans (100 g), agar (12 g), and water (600 ml). After 1 d, the artificial diet without the agar was mixed with a blender. Compound **1** (150 mg) was then added to the blender at 1 mg/g of diet. Agar was dissolved in water and boiled and then added to the blender. The diet was then mixed and cooled in a tray. The larvae were moved into new cases, and the diet containing compound **1** (3 mg for a body) were fed for 2 d, and then the artificial diet not containing compound **1** was fed to the larvae for an additional 2 d. Frass was collected daily and stored in a solution of CHCl₃ (100 ml).

The frass was extracted with CHCl₃ (100 ml×3) and EtOAc (100 ml×2). These extracts were mixed, the solvent was evaporated under reduced pressure, and 211.9 mg of extract was obtained. The extract was analyzed by TLC and GC-MS; metabolites **4**, **5** and **6** occurred in this extract. The extract was fractionated by SiO₂ column chromatography with CH₂Cl₂/acetone as eluents. Recovery substrate **1** (130 mg) was isolated. Three metabolites **4** (6 mg), **5** (5 mg) and **6** (3 mg) were isolated. The acetylated derivatives of compounds **5** (**5Ac**) and **6** (**6Ac**) were obtained by reaction with Ac₂O and pyridine.

5-Hydroxy-7,4'-dimethoxyisoflavone (**4**): White crystals, HR-EI-MS, *m/z* 298.0833 [M]⁺, Calcd for C₁₇H₁₄O₅, 298.0842; EI-MS, *m/z* (%) (rel. int.) 298 [M]⁺ (100), 297 (13), 283 (10), 255 (4), 166 (12), 149 (7), 132 (17), 117 (5); IR (KBr) ν_{\max} cm⁻¹ 1614, 1576, 1515, 1441; ¹H-NMR (CDCl₃) see Table 1.

7-Hydroxy-5,4'-dimethoxyisoflavone (**5**): White crystals, HR-EI-MS, *m/z* 298.0860 [M]⁺, Calcd for C₁₇H₁₄O₅, 298.0842; EI-MS, *m/z* (%) (rel. int.) 298 [M]⁺ (100), 297 (17), 283 (10), 269 (3), 166 (22), 132 (25), 117 (10).

4'-Hydroxy-7,4'-dimethoxyisoflavone (**6**): White crystals, HR-EI-MS, *m/z* 298.0845 [M]⁺, Calcd for C₁₇H₁₄O₅, 298.0842; EI-MS, *m/z* (%) (rel. int.) 298 [M]⁺ (100), 297 (25), 283 (10), 269 (3), 180 (5), 118 (20).

7-Acetoxy-5,4'-dimethoxyisoflavone (**5Ac**): White crystals, EI-MS, *m/z* (%) (rel. int.) 340 [M]⁺ (100), ¹H-NMR (CDCl₃) see Table 1.

4'-Acetoxy-7,4'-dimethoxyisoflavone (**6Ac**): White crystals, EI-MS, *m/z* (%) (rel. int.) 340 [M]⁺ (100), ¹H-NMR (CDCl₃) see Table 1.

Administration of 6,7,4'-Trimethoxyisoflavone (2) and 7,4'-Dimethoxyisoflavone (3) Compounds **2** (150 mg) and **3** (150 mg) were administered in the same way. Extracts were mixed, the solvent was evaporated under reduced pressure, and 215.0 mg and 210.0 mg of extract was obtained. Recovery substrates **2** (148 mg) and **3** (148 mg) were isolated.

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Table 1. ¹H-NMR Spectral Data of Compounds **1**, **4**, **5Ac** and **6Ac**

No.	1	4	5Ac	6Ac
2	7.76 s	7.86 s	7.82 s	7.79 s
6	6.36 d (2.3)	6.40 d (2.4)	6.58 d (2.5)	6.38 d (2.3)
8	6.44 d (2.3)	6.38 d (2.4)	6.85 d (2.5)	6.46 d (2.3)
2',6'	7.47 dt (8.9, 2.0)	7.45 dt (8.9, 2.4)	7.48 dt (8.4, 1.9)	7.56 dt (8.4, 2.0)
3',5'	6.93 dt (8.9, 2.0)	6.98 dt (8.9, 2.4)	6.94 dt (8.4, 1.9)	7.13 dt (8.4, 2.0)
OMe	3.93 s	3.87 s	3.96 s	3.94 s
	3.88 s	3.84 s	3.84 s	3.90 s
	3.82 s			
OAc			2.35 s	2.31 s
OH		12.85 s		

¹H-NMR recorded at 500.0 MHz in CDCl₃, TMS as internal standard.

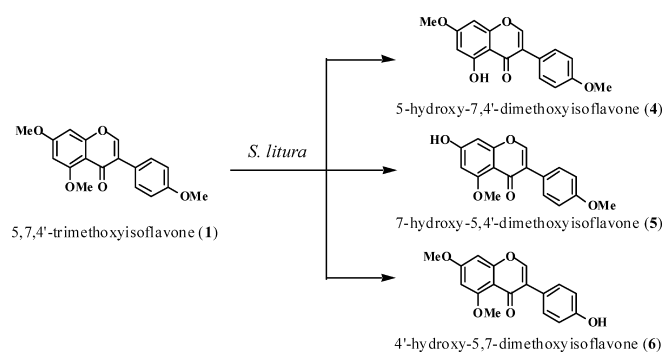


Chart 1. Biotransformation of compound **1** by *S. litura*

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