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

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Biotransformation of sinesetin (1) by larvae of *Spodoptera litura* was investigated. Compound 1 was converted to a new flavonoid, (+)-5,7,3′,4′-tetramethoxyflavone-6-*O*-β-D-glucoside (4), and two known flavones, 4′-hydroxy-5,6,7,3′-tetramethoxyflavone (2) and 6-hydroxy-5,7,3′,4′-tetramethoxyflavone (3). These structures were established by IR, HR-EI-MS, HR-FAB-MS, 1D NMR, and 2D NMR spectral studies. The results indicate that the metabolic reaction of compound 1 by larvae of *S. litura* proceeded along two pathways; the main pathway is demethylation at the C-6 position followed by glucosylation, and the minor pathway is demethylation at the C-4′ position.

Key words biotransformation; *Spodoptera litura*; sinesetin; 6-hydroxy-5,7,8,3′,4′-pentamethoxyflavone; 5,7,8,3′,4′-pentamethoxyflavone-6-*O*-β-D-glucopyranoside; 4′-hydroxy-5,6,7,3′-pentamethoxyflavone

As part of our continuing program to investigate the biotransformation of natural products, we have been dealing with terpenoids and lignans with fungi and insects as biocatalysts.[1—3] Biotransformation has a number of advantages when compared to the corresponding chemical methods. The conditions for such processes are mild and in the majority of cases do not require the protection of other functional groups. Also, biotransformation precedes enantioselectively or regioselectively. Furthermore, some types of biotransformation can be cheaper and more direct than their chemical analogues and the conversions normally proceed under conditions that are regarded as ecologically acceptable. Thus biotransformation can be defined as the use of biological system to produce chemical changes in compounds witch are not their natural substrates.

Flavonoids are some of the most ubiquitous compounds found in fruits, vegetables, nuts, wine, and tea. Human dietary intake of these natural products is estimated to be about 1 g/d of mixed flavonoids. They display a wide range of biological and pharmacologic activity, including antioxidant, anticancer, and antimutagenic activity.[4—6] Previously, we reported the antimutagenic activity of flavonoids from Chinese traditional medicine.[7,8] The biotransformation of flavonoids has been investigated. Ibrahim and Abul-Hajj reported the C-4′ hydroxylation of three ring-A monohydroxy flavones by *Streptomyces fulvissimus*[9] and Ibrahim and Ibrahim *et al.* reported the sulfilation of flavanones by *Cunninghamella elegans*.[10,11] Murakami *et al.* also reported that nobiletin, 5,6,7,8,3′,4′-hexamethoxyflavone, was metabolized to 3′-demethylnobiletin by a rat liver S9 mixture.[12] However, there are no reports in the literature on the biotransformation of sinesetin (**1**) by larvae of *Spodoptera litura*. Sinesetin (**1**) is the polymethoxyflavanonid 5,6,7,8,3′,4′-hexamethoxyflavone occurring in *Citrus fruits*. Recently, we reported the antimutagenic activity of **1** against chemical mutagens.[7] In this study, the biotransformation of **1** by the larvae of *S. litura* was investigated for the purpose of estimating possible metabolic pathways in insects.

MATERIALS AND METHODS

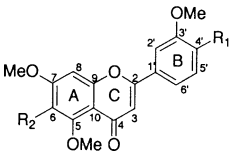
General The EI-MS and FAB-MS were obtained on a JEOL Tandem MStation JMS-700. The IR spectra were determined with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer. NMR spectra were obtained with a JEOL FX-500 (500.00 MHz, ^{1}H; 125.65 MHz, ^{13}C) spectrometer. The overpressured layer chromatography (OPLC) instrument was a Personal OPLC Basic System 50 (Bionisis-OPLC, France).

Chemical Sinesetin (**1**) was isolated from *Citrus aurantium* as antimutagenic compound.[7]

Overpressured Layer Chromatography Aluminum-backed silica gel TLC plates with perimeter seal (HTSorb, 5 μm, fluorescence 254 nm, 20 cm×20 cm, Bionisis-OPLC) was used for OPLC. The amount of sample (dissolved in acetone) applied to the plates was 50 mg. The mobile-phase composition was CH_2Cl_2/CH_3OH (9 : 1, v/v). The external pressure was 50 bars, the flow rate 800 μl/min, the volume of rapid delivery 300 μl, and the mobile phase volume 20000 μl. Fractions were collected at 1200 μl intervals and evaporated under reduced pressure. TLC analysis checked the purity of fractions.

Thin-Layer Chromatography TLC was performed on precoated plates (silica gel 60 F_{254}, 0.25 mm, Merk, Darmstadt, Germany). The solvent system was CH_2Cl_2/acetone (9 : 1, v/v, for compounds **1**—**3**), CH_2Cl_2/MeOH (8 : 2, v/v, for compound **4**), and butanol/acetic acid/water (3 : 2 : 2, v/v/v, for glucose). Compounds **1**—**4** were visualized by spraying the plates with 1% vanillin in concentrated sulfuric acid followed by brief heating (120 °C, 1 min). Glucose was visualized by heating the plates at 110 °C for 10 min after spraying with 10% (v/v) sulfuric acid in EtOH.

Rearing of Larvae The larvae of *S. litura* were reared



	R1	R2
1	OMe	OMe
2	OMe	OH
3	OH	OMe
4	OMe	OGlu

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in plastic cages (200×300 width, 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 Sinesetin (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 agar was mixed with a blender. Compound **1** (150 mg) was then added directly 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. Fifty larvae were moved into new cages. The larvae were fed the diet containing **1** (3 mg/body) for 2 d, and then the artificial diet not containing compound **1** was fed to the larvae for an additional 2 d. Feces were collected daily (total of 4 d), and stored in a solution of $CHCl_3$ (100 ml).

Isolation of Metabolic Compounds from Feces The feces from 50 larvae were extracted with $CHCl_3$ (100 ml×3) and AcOEt (100 ml×2). These extracts were gathered, the solvent was evaporated under reduced pressure, and 602.4 mg of extract was obtained. The extract was fractionated by SiO_2 column chromatography with CH_2Cl_2/acetone (97 : 3 and 1 : 1) and CH_2Cl_2/CH_3OH (1 : 1) as eluents. Recovery substrate **1** (110 mg), and metabolites **2** (4 mg) and **3** (13 mg) were isolated from the CH_2Cl_2/acetone fraction. Metabolite **4** (20 mg) was isolated from CH_2Cl_2/CH_3OH fraction by OPLC.

6-Hydroxy-5,7,3′,4′-tetramethoxyflavone (**3**): White crystals, mp 140.2—141.9 °C. IR ν_{max}^{KBr} cm^{-1}: 3124, 1630, 1597, 1510, 1486, 1323, 1264, 1119. ^{1}H- and ^{13}C-NMR: see Tables 1 and 2. EI-MS *m/z* (%): 358 $[M]^+$ (100), 343 (40), 340 (55), 195 (4), 167 (6), 165 (6), 162 (21). HR-EI-MS *m/z*: 358.1026 $[M]^+$ (Calcd for $C_{19}H_{18}O_7$: 358.1053).

5,7,3′,4′-Tetramethoxyflavone-6-*O*-*β*-D-glucoside (**4**): White crystals, mp 153—154 °C (dec.), $[\alpha]_D^{18}$ +10.6° (*c*=0.4, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3419, 2932, 1648, 1598, 1521, 1489, 1263, 1119. ^{1}H- and ^{13}C-NMR see Tables 1 and 2. FAB-MS (positive mode) *m/z*: 551 $[M+H]^+$, 389 $[M+H-162]$. HR-FAB-MS (positive mode) *m/z*: 521.1672 $[M+H]^+$ (Calcd for $C_{25}H_{29}O_{12}$: 551.1764).

RESULTS AND DISCUSSION

Sinesetin (**1**) was incorporated into the artificial diet using cellulose powder as an inert carrier. Artificial diet containing 1 mg/g diet of **1** was fed to the 50 larvae for 2 d and then artificial diet without **1** was fed for an additional 2 d. Feces were collected and extracted with dichloromethane and ethyl acetate. These extracts were subjected to silica gel column chromatography and OPLC to give metabolites. Metabolites **2** (4 mg), **3** (13 mg), and **4** (20 mg) and substrate **1** (110 mg) were isolated. Other metabolites were not detected in TLC analysis. The structures of metabolites **2**, **3**, and **4** were determined based on spectral data.

Metabolite **2** showed an $[M]^+$ peak at *m/z* 358.1042, corresponding to the molecular formula $C_{19}H_{18}O$ in its HR-EI-MS. This was supported by the appearance of 19 carbon signals in its ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum of **2** showed four methoxy proton signals δ 3.92, 3.98, 3.99, and 4.00. It also showed a significant mass fragment at *m/z* 195 formed after the *retro* Diels–Alder reaction indicated the presence of three methoxyl groups in ring A, and the remaining one methoxyl and one hydroxyl group should be present in ring B.[13] To confirm the location of the hydroxyl group in the metabolite, H–H COSY, NOESY, HMQC, and HMBC experiments were conducted. Three aromatic proton signals at δ 7.31 (1H, d, *J*=2.0), 7.03 (1H, d, *J*=8.3), and 7.49 (1H, dd, *J*=2.0, 8.3) were assigned to H-2′, H-5′, and H-6′, respectively, of ring B. The methoxy group at δ 4.00 was placed at C-3′ based on the ^3J correlation of these protons with C-3′ at 146.8 ppm in its HMBC spectrum and NOE correlation with H-2′ (δ 7.31) in its NOESY spectrum (Fig. 1). The aromatic proton signals at H-2′ and H-6′ showed the ^3J correlation, and H-5′ also showed the ^2J correlation to the carbon signal (C-4′) at 148.7 ppm. In addition, the presence of three methoxy groups in ring A was confirmed by NOESY and HMBC spectra (Fig. 1). Thus from the foregoing spectral studies the structure of **2** was identified as 4′-hydroxy-5,6,7,3′-tetramethoxyflavone. These spectral data were assigned by comparison with the spectral data for **2** in previous papers.[14,15]

Metabolite **3** showed an $[M]^+$ peak at *m/z* 358.1026, corresponding to the molecular formula $C_{19}H_{18}O_7$ in its HR-EI-MS. This was supported by the appearance of 19 carbon signals in its ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum of **3** showed the presence of four methoxy groups at δ 3.97, 3.99, 4.03, and 4.04, which indicated that one methoxy signal has disappeared compared with **1**. Two methoxy signals at δ 3.97 and 3.99 were placed at C-4′ and C-3′ in ring B as they showed NOE correlations with H-5′ (δ 6.98) and H-2′ (δ 7.34), respectively, in its NOESY spectrum (Fig. 1). The methoxy group at δ 4.04 was placed at C-7 based on NOE

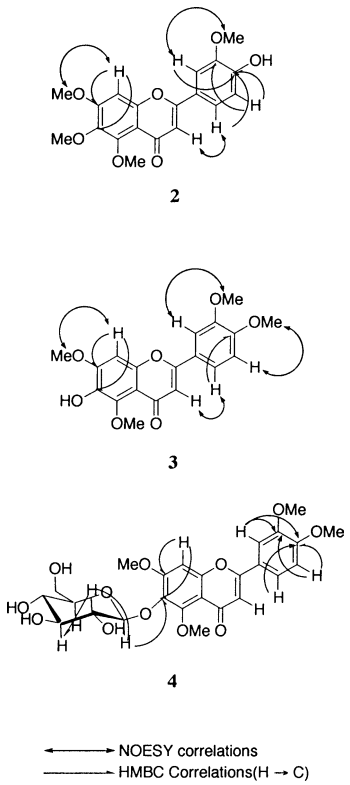

Fig. 1. Cross Peaks Observed in Compounds **2**, **3**, and **4**

Table 1. ^{1}H-NMR Spectral Data of Sinesetin (**1**) and Metabolic Compounds

H	**1**	**2**	**3**	**4**
3	6.60 s	6.59 s	6.62 s	6.61 s
8	6.80 s	6.80 s	6.83 s	7.10 s
2′	7.33 d (1.9)	7.31 d (2.0)	7.34 d (2.3)	7.46 d (2.0)
5′	6.97 d (8.4)	7.03 d (8.3)	6.98 d (8.3)	7.07d (8.6)
6′	7.51 dd (1.9, 8.4)	7.49 dd (2.0, 8.3)	7.52 dd (2.3, 8.3)	7.584 dd (2.0, 8.6)
OMe				
5	3.99 s	3.99 s	4.03 s	3.95 s
6	3.92 s	3.92 s		
7	3.98 s	3.98 s	4.04 s	3.99 s
3′	3.99 s	4.00 s	3.99 s	3.92 s
4′	3.96 s		3.97 s	3.89 s
OH				
6			5.88 br s	
Glu				
1				5.03 d (7.5)
2				3.50 dd (7.5, 8.90)
3				3.44 dd (8.9, 8.9)
4				3.39 dd (8.9, 9.5)
5				3.21 ddd (2.5, 5.5, 9.5)
6				3.64 dd (2.5, 12.0)
				3.77 dd (5.5, 12.0)

The ^{1}H-NMR spectrum recorded at 500.0 MHz in $CDCl_3$ (**1**—**3**) and CD_3OD (**4**), with TMS as an internal standard.

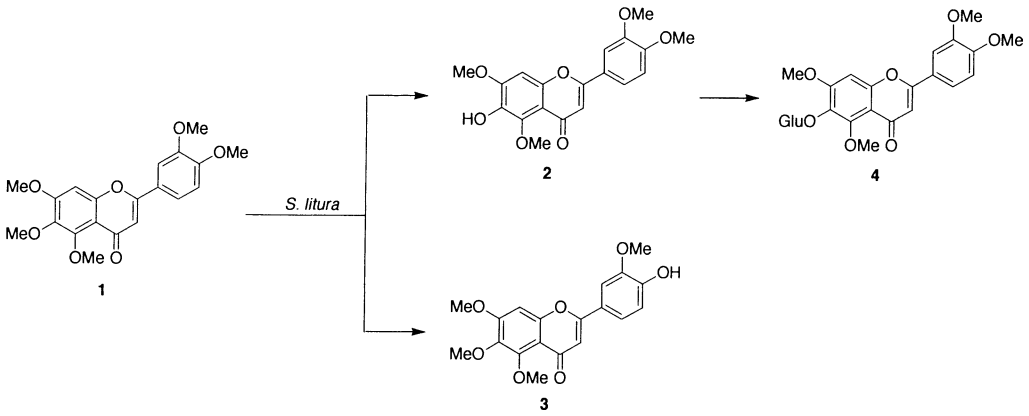

Chart 1. Possible Metabolic Pathway of Sinesetin (**1**) by *S. litura*

correlation with H-8 (δ 6.83) in its NOESY spectrum (Fig. 1). The methoxy group at δ 4.03 showed cross-peak correlation to the carbon signal at δ 144.0 in its HMBC spectrum. The H-8 proton signal showed the ^3J correlation with the carbon signal at δ 136.7, suggesting that the hydroxyl group was connected to the C-6 position. Thus from the foregoing spectral studies, the structure of **3** was identified as 6-hydroxy-5,7,3,4′-tetramethoxyflavone. Previously, this compound was reported to be the intermediate for synthesizing 5,6,7-trihydroxyflavonoids.[16,17] However, the NMR spectrum analysis of compound **3** was not reported, and thus this is the first report of the NMR spectrum analysis of compound **3**.

Metabolite **4** showed on an $[M+H]^+$ peak at *m/z* 521.1672, corresponding to the molecular formula $C_{25}H_{28}O_{12}$ in its HR-FAB-MS. A peak at *m/z* 359 $[M+H-162]^+$ was due to the loss of a hexose moiety. Acidic hydrolysis of **4** gave glucose, which was identified by TLC and FAB-MS, and aglycone, which was identified as compound **3** by TLC, EI-MS, and ^{1}H-NMR spectrum. As shown in Table 1, the anomeric proton signal of **4** at δ 5.03 (1H, d, J=7.5) indicated the α-configuration and hence of a β-glucoside linkage. The glucose protons were conclusively confirmed by HMBC (Fig. 1). The specific optical rotation shows the (+)-form; therefore **4** was identified as (+)-5,7,3′,4′-tetramethoxyflavone-6-*O*-β-D-glucoside. β-Glucosylation appears to be a more important pathway for detoxication of phenolics by many insects, rather than the glucuronidation that predominates in mammals.[18,19] In our previous paper, β-glucoside was the main metabolite in the biotransformation of lignans by larvae of *S. litura*.[1,2]

The possible metabolic pathway of **1** is shown in Chart 1. Compound **1** was metabolized to **2** and **3**; furthermore **3** was glucoside conjugated and excreted in the feces. Metabolite **3** was the major compound in the demethylation of **1**, and the glucoside of **2** was not detected. From these results, the main metabolic pathway is demethylation at the C-6 position on ring A followed by glucosylation. This is the first report of biotransformation of sinesetin (**1**) in insects. Furthermore, metabolite **3** is a new compound.

Table 2. ^{13}C-NMR Spectral Data of Sinensetin (**1**) and Metabolites

No.	**1**	**2**	**3**	**4**
2	161.2	161.2	161.4	163.7
3	107.5	107.2	107.2	107.1
4	177.3	177.3	177.0	179.5
5	152.7	152.6	144.0	153.3
6	140.4	140.3	136.7	137.9
7	157.7	157.7	152.2	159.6
8	96.3	96.2	96.3	98.1
9	154.6	154.5	151.8	156.5
10	112.9	114.8	112.3	113.0
1′	124.2	123.6	124.2	124.8
2′	108.7	108.1	108.7	110.4
3′	149.3	146.8	149.3	150.9
4′	151.9	148.7	151.9	153.8
5′	111.2	114.9	111.2	112.7
6′	119.6	120.2	119.6	121.2
OMe				
5	62.3	62.2	62.6	57.2
6	61.6	61.5		
7	56.2	56.3	56.5	63.0
3′	56.1	56.1	56.1	56.7
4′	56.4		56.0	56.5
Glu				
1				104.6
2				75.7
3				77.9
4				71.4
5				78.5
6				62.5

The ^{13}C-NMR spectrum was recorded at 125.65 MHz in $CDCl_3$ (**1**—**3**) and CD_3OD (**4**).

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