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BIOTRANSFORMATION OF PLANT SECONDARY METABOLITES BY SILKWORMS

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Abstract – Compounds resulting from the biotransformation of plant secondary metabolites by silkworms (*Bombyx mori*) were found in their feces. SilkMate 2S, an artificial diet, mixed with twelve powdered plant-derived crude drugs was fed to fifth instar silkworms. A glucoside was isolated from the MeOH extract of the feces ejected by the silkworms provided with Scutellariae Radix (Ogon). SilkMate 2S mixed with twelve flavonoids was fed to fifth instar silkworms. Two new compounds, 3-*O*-β-D-glucopyranosyloxy-6-methoxyflavone (**18**) and 3-*O*-β-D-glucopyranosyloxy-4'-hydroxyflavone (**19**), were obtained from the MeOH extracts of the feces ejected by the silkworms provided with 3-hydroxy-6-methoxyflavone (**10**) and 3,4'-dihydroxyflavone (**11**), respectively.

In recent years, it has become increasingly difficult to discover new compounds from natural resources because of limited access to genetic resources. Therefore, insect secondary metabolites have attracted attention in the search for new natural sources. For example, *N*-β-alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD) secreted from *Sarcophaga peregrina* and a derivative of cantharidin secreted from *Lytta vesicatoria* were reported to have cytotoxic activity against cancer cells.[1,2] We focused on silkworms (*Bombyx mori*) as a source of metabolites. Silkworms are used to evaluate drug toxicity and metabolism, and as animal models for disease states, because of the established method of their mass rearing for sericulture and the lack of ethical issues.[3–8] Bombycis Faeces (silkworm feces) is a traditional Chinese herbal medicine and is reported to contain terpenoids, steroids, flavonoids and plant growth hormones.[9–11] The numbers of glutathione S-transferase genes, ATP-binding cassette (ABC) transporter genes,

cytochrome P450 genes, and carboxylesterase (COE) genes in the silkworm genome are estimated to be 23, 52, 83, and 87, respectively.[12] In this study, plant-derived compounds were provided to silkworms, and compounds resulting from the biotransformation of plant secondary metabolites by the silkworms were isolated from their feces.

Silkworms feed on mulberry leaves and SilkMate 2S, an artificial diet containing mulberry leaves. Thus, SilkMate 2S mixed with twelve powdered plant-derived crude drugs at 10 or 20% was fed to fifth instar silkworms. Feces ejected by the silkworms were collected and sequentially extracted with $CHCl_3$ and MeOH, respectively. Similarly, powdered crude drugs and feces ejected by silkworms not provided with powdered plant-derived crude drugs were extracted with $CHCl_3$ and MeOH. Each extract (twelve $CHCl_3$ extracts and twelve MeOH extracts) from twelve powdered plant-derived crude drugs was compared to find compounds showing changes. In the MeOH extract of the feces ejected by the silkworms provided with Scutellariae Radix (Ogon), one of the powdered plant-derived crude drugs, a peak associated with compound showing a significant change was detected by liquid chromatography–mass spectrometry (LC-MS) regardless of the concentration of the powdered plant-derived crude drugs (Figure S1). To isolate this compound, a large amount of this MeOH extract was subjected to chromatography. Baicalein (**1**), baicalin (**2**), and wogonin (**3**) were contained in Scutellariae Radix as the main compounds (Figure 1, Figure SI).

	R^1	R^2	R^3
1	OH	OH	H
2	OH	O-β-D-GlcA	H
3	H	OH	OMe
4	OMe	O-β-D-Glc	H

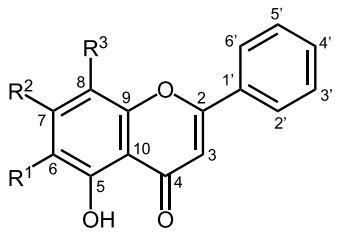

Figure 1. Structures of **1**–**4**

The compound showing a significant change was isolated as a compound **4**. From the ^1H and ^{13}C NMR deta, **4** has a flavonoid skeleton and glucose as sugar moiety. The structure of **4** was deduced from MS and 1D and 2D NMR (HMQC and HMBC) specra (Figure 2, Figures S14–17). The key HMBC correlations were from methoxy methyl protons at δ_H 3.79 to δ_C 132.6, and from δ_H 12.81 (hydroxy group at C-5) to δ_C 132.6, δ_C 152.3 (C-5), and δ_C 106.0 (C-10). Thus, the methoxy group was presented at C-6. And the hydrolyzed aglycon of **4** was identified as oroxylin (=baicalein 6-methyl ether).[13] The anomeric proton at δ_H 5.15 (d, J = 7.2 Hz) and carbon δ_C 100.1 were obtaind, and the coupling constants of the anomeric proton suggested that the sugar moiety of **4** was β-configuration. The absolute configuration of the sugar unit was determined as D-glucose by measuring the ODS HPLC retention time of sugar derivative.[14,15] The position of the glucopyranosyloxy group of **4** was determined to be C-7 from an observed HMBC correlation from

the anomeric proton at δ_H 5.15 to the C-7 carbon at δ_C 156.7. Thus, compound **4** was identified as oroxylin-7-*O*-β-D-glucoside.[16]

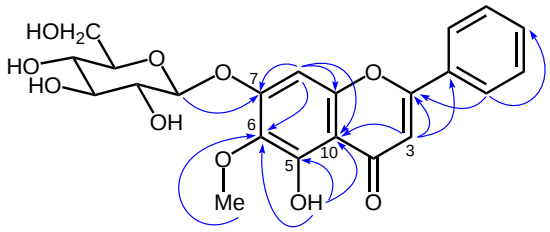

Figure 2. Key HMBC Correlations of **4** in DMSO-d_6

To investigate the relationship between the position and number of hydroxy group on flavonoids and the susceptibility to glycosylation, twelve compounds [3-hydroxyflavone (**5**), 5-hydroxyflavone (**6**), 6-hydroxyflavone (**7**), 7-hydroxyflavone (**8**), 3,6-dihydroxyflavone (**9**), 3-hydroxy-6-methoxyflavone (**10**), 3,4′-dihydroxyflavone (**11**), chrysin (**12**), 7,8-dihydroxyflavone (**13**), galangin (**14**), 5,7,2′-trihydroxyflavone (**15**), and apigenin (**16**)] were selected as starting materials from commercially available. Silkmate 2S mixed with each of twelve compounds was fed to fifth instar silkworms, respectively (Figure 3). In the MeOH extracts of the feces ejected by the silkworms provided with flavonoids **5**, **9**, **10**, **11**, and **14** with a hydroxy group at C-3, peaks associated with compounds derived from glycosides of the original compounds were detected by LC-MS (Figures S2–13). Feces ejected by the silkworms provided with **9**–**11** were enough needed to isolate the glycoside, we focused on their MeOH extracts and subjected to chromatography. Compounds **17**–**19** were isolated from the MeOH extracts of the feces ejected by the

	R^1	R^2	R^3	R^4	R^5	R^6	R^7
5	OH	H	H	H	H	H	H
6	H	OH	H	H	H	H	H
7	H	H	OH	H	H	H	H
8	H	H	H	OH	H	H	H
9	OH	H	OH	H	H	H	H
10	OH	H	OMe	H	H	H	H
11	OH	H	H	H	H	H	OH
12	H	OH	H	OH	H	H	H
13	H	H	H	OH	OH	H	H
14	OH	OH	H	OH	H	H	H
15	H	OH	H	OH	H	OH	H
16	H	OH	H	OH	H	H	OH
17	O-b-D-Glc	H	OH	H	H	H	H
18	O-b-D-Glc	H	OMe	H	H	H	H
19	O-b-D-Glc	H	H	H	H	H	OH

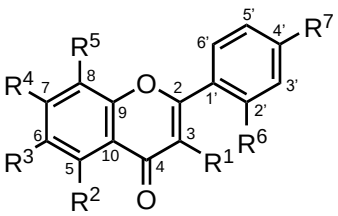

Figure 3. Structures of **5**–**16** provided to silkworms and **17**–**19** produced by the transformation

silkworms provided with **9**–**11**, respectively. From the ^{1}H and ^{13}C NMR deta, **17**–**19** show a flavonoid skeleton and glucose. The structures of **17**–**19** were deduced from MS and 1D and 2D (HMQC and HMBC) NMR specra (Figure 4, Table 1, Figures S18–27). The coupling constants of the anomeric protons of **17** (J = 7.7 Hz), **18** (J = 7.4 Hz) and **19** (J = 7.4 Hz) suggested that the sugar moiety of **17**–**19** were β-configuration. The absolute configuration of each sugar unit (D-glucose) of **17**–**19** was determined measuring the ODS HPLC retention time of sugar derivatives according to the same method of compound **4**.[14,15] The position

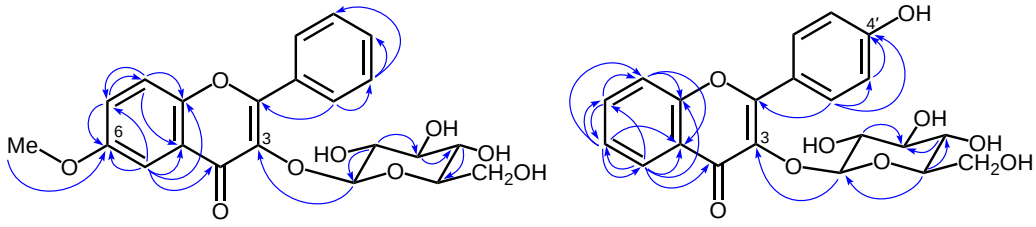

Figure 4. HMBC Correlations of **18** and **19** in DMSO-d_6

Table 1. ^{1}H and ^{13}C NMR spectroscopic data for **18** and **19** in DMSO-d_6

	18		**19**	
Position	δ_C	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)
2	155.7		156.0	
3	136.1		135.5	
4	173.5		173.3	
5	104.5	7.56 (d, 3.0)	125.0	8.06 (m)
6	156.6		125.0	7.49 (dd, 7.6, 7.6)
7	124.2	7.43 (dd, 9.2, 3.0)	133.9	7.80 (m)
8	120.3	7.71 (d, 9.1)	118.3	7.72 (d, 8.4)
9	149.8		154.5	
10	123.9		121.2	
1′	131.0		123.3	
2′	128.4	8.15 (dd, 6.7, 3.0)	131.0	8.09 (d, 8.4)
3′	129.2	7.53 (m)	115.8	6.90 (d, 8.7)
4′	130.8	7.53 (m)	159.9	
5′	129.2	7.53 (m)	115.8	6.90 (d, 8.7)
6′	128.4	8.15 (dd, 6.7, 3.0)	131.0	8.09 (d, 8.4)
1″	100.7	5.30 (d, 7.4)	100.1	5.54 (d, 7.4)
2″	74.3	3.15 (m)	74.2	3.18 (m)
3″	76.7	3.18 (m)	76.5	3.18 (m)
4″	70.0	3.06 (overlap)	69.9	3.05 (m)
5″	77.7	3.06 (overlap)	77.4	3.05 (m)
6″	61.0	3.54 (dd, 11.4, 5.3) 3.26 (overlap)	60.8	3.53 (dd, 11.0, 3.7) 3.30 (overlap)
6-OMe	56.0	3.88 (s)		
4′-OH				10.16 (s)

of the glucopyranosyloxy group of **17**–**19** was determined to be C-3 from an observed HMBC correlation from the anomeric proton (H-1") to the C-3 carbon. Thus, compound **17** was identified as 3-*O*-β-D-glucopyranosyloxy-6-hydroxyflavone, a glycoside of **9**.[17] Compounds **18** and **19** were determined to 3-*O*-β-D-glucopyranosyloxy-6-methoxyflavone and 3-*O*-β-D-glucopyranosyloxy-4′-hydroxyflavone as new compounds, respectively. It has been reported that glycosylation of coumarin and other compounds by cytochrome P450 occurs in silkworms.[18–20] This paper demonstrates biotransformation by silkworms, which may be applied to the search for new medicinal resources.

EXPERIMENTAL

General experimental procedures. ^{1}H and ^{13}C NMR spectra were measured with a JEOL JNM-ECS400 (^{1}H 400 and ^{13}C 100 MHz) spectrometer, using a residual solvent signal as an internal standard. HR-MS spectra were obtained using a JEOL JMS-T1000LC spectrometer. Column chromatography was conducted on 63-210 μm Silica Gel 60N (Kanto Chemical). Compounds **5**–**13** from Tokyo Chemical Industry, **14** and **16** from Extrasynthese, and **15** from ICC Chemical Co. were used as commercial reagents.

Liquid chromatography-mass spectrometry conditions. LC-MS spectra were measured with a Shimadzu LCMS-8040 (Triple Quadrupole Mass Spectrometer). The analytical column was a 2.1×100 mm JASCO J-Pak Symphonia C18 column with a particle size of 3 μm. The plant-derived crude drugs extracts and the flavonoid extracts were dissolved in MeCN at the concentration of 10.0 mg/mL and 1.0 mg/mL, respectively, and filtered with a 0.22 μm micropore syringe filter. The mobile phase consisted of (A) distilled water and (B) MeCN. The separations were conducted at 25 °C and a flow-rate of 0.2 mL/min. A gradient program was adopted as follows: held at 20% B (0–10 min), linear from 20 to 100% B (10–50 min), and held at 100% B (10–50 min). The injection amount was 10 μL. UV spectra were recorded at 220 nm. Electrospray ionization MS (ESI-MS) spectra were acquired in positive and negative ion modes using an electrospray interface. The ionization parameters for scan mode were as follows: ESI temperature, 250 °C; heat-block temperature, 400 °C; nebulizing gas flow, 3.0 L/min.

Silkworms were fed an artificial feed mixed with powdered plant-derived crude drugs and flavonoid reagents.

Feeding silkworms on artificial diet mixed with powdered plant-derived crude drugs and flavonoid reagents. Silkworm infection experiments were performed according to a previous report.[21] Eggs of silkworms (Hu·Yo × Tukuba·Ne) were purchased from Ehime-Sanshu Co, Ltd. (Ehime, Japan). The silkworms were fed an artificial diet, Silkmate 2S, containing antibiotics purchased from Ehime-Sanshu

Co., Ltd. Silkworm fifth instar larvae were fed the artificial diet (Silkmate 2S; Ehime-Sanshu Co., Ltd.) overnight.

Mixing of compounds in Silkmate 2S was performed according to a previous report.[22] The powder of plant-derived crude drugs or flavonoid reagents was added to the artificial diet and mixed thoroughly. The diet containing the test sample was fed to the fifth instar larvae for 4 days.

Extraction and separation of feces ejected by silkworms provided with Scutellariae Radix. The dried feces (246.2 g) from silkworms provided with Scutellariae Radix were sequentially extracted with $CHCl_3$ (500 mL, 3 times) and MeOH to yield a concentrated $CHCl_3$ extract (2.11 g) and MeOH extract (5.11 g), respectively. The concentrated MeOH extract partitioned between H_2O and *n*-BuOH to yield a concentrated *n*-BuOH extract (3.48 g). The *n*-BuOH extract was subjected to silica gel column chromatography (Si. C. C.) and eluted with $CHCl_3$-MeOH ($CHCl_3$, 50:1, 10:1, 2:1, 1:1, MeOH) to afford seven fractions (A1–A7). Fraction A3 (540 mg) was subjected to Si. C. C. and eluted with $CHCl_3$-MeOH (50:1, 30:1, 20:1, 10:1, 5:1, MeOH) to afford eight fractions (A3A–A3H). Fraction A3F (205.5 mg) was filtered with MeOH to yield **4** (102.1 mg), isolated as a solid. **4** was hydrolyzed with 2M HCl at 110 °C for 2 h. Acid hydrolysis of **4** was subjected to Si. C. C. to give aglycon. The aglycon was identified as oroxylin (=baicalein 6-methyl ether).[13]

Oroxylin-7-*O*-β-D-glucoside (4): Pale yellow powder; positive HRESI-MS *m/z* 469.1106 $[M+Na]^+$ (calcd. for $C_{22}H_{22}O_{10}Na$, 469.1111 $[M+Na]^+$) ; ^{1}H NMR (DMSO-d_6) δ ppm: 3.21 (1H, m, C-4”), 3.32 (1H, m, C-2”), 3.33 (1H, m, H-3”), 3.48 (1H, m, H-6”), 3.50 (1H, H-5”), 3.70 (1H, m, H-6”), 3.79 (3H, s, O<u>C</u>H$_3$), 5.15 (1H, d, *J* = 7.2 Hz, H-1”), 7.07 (1H, s, H-3), 7.08 (1H, s, H-8), 7.54-7.65 (2H, m, H-3’, 5’), 7.61 (2H, m, H-4’), 8.10 (2H, dd, *J* = 8.0, 1.6 Hz, H-2’, 6’), 12.81 (1H, s, 5-OH) and ^{13}C NMR (DMSO-d_6) δ ppm: 60.3 (O<u>C</u>H$_3$), 60.6 (C-6”), 69.5 (C-4”), 73.2 (C-2”), 76.7 (C-3”), 77.3 (C-5”), 94.5 (C-8), 100.1 (C-1”), 105.0 (C-3), 106.0 (C-10), 126.5 (C-2’, 6’), 129.2 (C-3’, 5’), 130.6 (C-1’), 132.2 (C-4’), 132.6 (C-6), 152.3 (C-5 or C-9), 152.4 (C-9 or C-5), 156.7 (C-7), 163.7 (C-2), 182.5 (C-4).

Oroxylin (=baicalein 6-methyl ether)[13]: ^{1}H NMR (DMSO-d_6) δ ppm: 3.75 (3H, s, O<u>C</u>H$_3$), 6.66 (1H, s, H-3 or H-8), 6.98 (1H, s, H-8 or H-3), 7.52−7.30 (3H, m, H-3’, 4’, 5’), 8.07 (2H, d, *J* = 6.4 Hz, H-2’, 6’); 12.93 (1H, s, 5-OH); ^{13}C NMR (DMSO-d_6) δ ppm: 60.0 (O<u>C</u>H$_3$), 94.4 (C-8), 104.3 (C-10), 104.7 (C-3), 126.4 (C-2’, 6’), 129.2 (C-3’, 5’), 130.7 (C-1’), 131.5 (C-6), 132.0 (C-4’), 152.6 (C-5 or C-9), 152.7 (C-9 or C-5), 157.6 (C-7), 163.2 (C-2), 182.3 (C-4).

Extraction and separation of feces ejected by silkworms provided with 9. Silkmate 2S mixed with **9** (2.50 g) was fed to 250 fifth instar silkworms. The dried feces (47.5 g) from silkworms provided with **9**

were extracted with $CHCl_3$ (500 mL, 3 times) and MeOH to yield a concentrated $CHCl_3$ extract (0.72 g) and MeOH extract (3.16 g), respectively. The MeOH extract was subjected to Si. C. C. and eluted with $CHCl_3$-MeOH (100:1, 50:1, 30:1, 10:1, 5:1, 2:1, 1:1, MeOH) to afford six fractions (B1–B6). Fraction B4 was **17** (1.15 g), isolated as a solid.

Extraction and separation of feces ejected by silkworms provided with 10. Silkmate 2S mixed with **10** (2.56 g) was fed to 256 fifth instar silkworms. The dried feces (56.5 g) from silkworms provided with **10** were extracted with $CHCl_3$ (500 mL, 3 times) to yield a concentrated $CHCl_3$ extract (2.23 g). The residues of the dried feces were extracted with MeOH (500 mL, 3 times) to yield a concentrated MeOH extract (1.01 g). The MeOH extract was subjected to silica gel column chromatography (Si. C. C.) and eluted with $CHCl_3$-MeOH (100:1, 50:1, 30:1, 10:1, 5:1, 2:1, 1:1, MeOH), to afford six fractions (C1–C6). Fraction C3 (67.0 mg) was subjected to Si. C. C. and eluted with $CHCl_3$-MeOH (15:1, 10:1, 7:1, 5:1, 1:1) to afford four fractions (C3A–C3D). Fraction C3A–C3C (48.5 mg) was subjected to Si. C. C. and eluted with $CHCl_3$-MeOH (30:1, 10:1, 1:1, MeOH) to afford four fractions (C3A1–C3A4). Fraction C3A3 was **18** (16.5 mg), isolated as a solid.

3-*O*-β-D-Glucopyranosyloxy-6-methoxyflavone (18): Pale yellow powder; mp 193–195 °C; $[\alpha]_D^{18}$ −31.4 (c 0.50, MeOH); IR ν_{max} (ATR) cm^{-1}: 3294, 2922, 2851, 1614, 1552, 1491, 1470, 1378, 1276, 1252, 1218, 1081, 1059, 1028; UV λ_{max} (MeOH) nm (log ε): 206 (4.40), 228 (4.35), 260 (4.34), 310 (4.26); HR-ESI-MS (positive) *m/z*: 453.1167 $[M+Na]^+$ (calcd for 453.1162, $C_{22}H_{22}O_9Na$); ^1H and ^{13}C NMR (DMSO-d_6) see Table 1.

Extraction and separation of feces ejected by silkworms provided with 11. Silkmate 2S mixed with **11** (2.15 g) was fed to 215 fifth instar silkworms. The dried feces (39.8 g) from silkworms provided with **11** were extracted with $CHCl_3$ (500 mL, 3 times) to yield a concentrated $CHCl_3$ extract (0.49 g). The residues of the dried feces were extracted with MeOH (500 mL, 3 times) to yield a concentrated MeOH extract (1.96 g). The MeOH extract was subjected to silica gel column chromatography and eluted with $CHCl_3$-MeOH (100:1, 50:1, 30:1, 10:1, 5:1, 2:1, 1:1, MeOH), to afford six fractions (D1–D6). Fraction D4 was **19** (450.6 mg), isolated as a solid.

3-*O*-β-D-Glucopyranosyloxy-4'-hydroxyflavone (19): Pale yellow powder; mp 149–151 °C; $[\alpha]_D^{17}$ −6.3 (c 1.0, MeOH); IR ν_{max} (ATR) cm^{-1}: 3124, 1599, 1544, 1480, 1470, 1400, 1290, 1183, 1076, 1062, 1004; UV λ_{max} (MeOH) nm (log ε): 206 (4.07), 230 (4.35), 336 (4.10); HR-ESI-MS (positive) *m/z*: 439.1002 $[M+Na]^+$ (calcd for 439.1005, $C_{21}H_{20}O_9Na$); ^1H and ^{13}C NMR (DMSO-d_6) see Table 1.

Hydrolysis and determination of absolute configuration of sugars[14,15] Compounds **4**, and **17**–**19** (1.0 mg, respectively) in 2M HCl (4.0 mL) was heated at 110 °C for 2 h. The reaction mixture was extracted with EtOAc (2 × 4 mL), and the aqueous phase was evaporated. The residues of each aqueous phase and authentic sugar samples (D/L-glucose) was dissolved in pyridine (1.0 mL), respectively, containing L-cysteine methyl ester (1.0 mg) and heated at 60 °C for 1 h, and then *o*-toly isothiocyanate (1.0 mL) was added to the mixture and heated further for 1 h. Then each reaction mixture was analyzed using the Shimadzu Prominence HPLC system using SPD-M20A PDA detector (at 250 nm). Analytical HPLC was performed on the YMC- Pack-ODS-A column (250 × 4.6 mm, 5 μm) eluting with A (0.1% formic acid): B (MeCN) = 80:20 (v/v) at 1.0 mL/min. The absolute configuration of sugars in each compound was established by a comparison of the retention times with the derivatives of sugar standards (D/L-glucose). Retention time of D- and L-glucose derivatives were 16.9 min and 15.8 min, respectively. Retention time for the derivatives of compounds **4**, and **17**–**19** were consistented with that of D-glucose derivative.

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