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Two New Isoflavone Glycosides from the Extracts of the Fungus *Monascus pilosus*-Fermented Black Soybean

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The fermented product of *Monascus* genus fungi has been used as a traditional food additive for improving the color of meat, fish, and soybean products, and is also known to have conserving properties in oriental countries for centuries.[1,2] *Monascus*-fermented red rice, called red mold rice (RMR), has useful secondary metabolites such as pigments, monacolin K, γ-aminobutyric acid (GABA), dimerumic acid, pyridine alkaloids and citrinin.[3-6] Several secondary metabolites from *M. pilosus* have recently been found to have some beneficial pharmacological effects such as decreasing the blood pressure, lowering the plasma cholesterol levels, antibacterial and anticancer activity.[7-9] Recently, we found that red mold black soybean (RMBS), a fermented product of *Monascus* using black soybean as the substrate has been shown to not only inhibit obesity and hyperlipidemia but also to decrease hepatic fat accumulation in high fat diet-induced obese mice.[10]

Obesity is one of the leading metabolic diseases worldwide and it is a serious health problem that is implicated in various pathological disorders including hypertension, type II diabetes, cardiovascular diseases, and certain types of cancers such as colorectal, breast and prostate cancers.[11,12] In obesity, adipocytes undergo abnormal growth characterized by hypertrophy (increase in cell size) and hyperplasia (increase in cell number) of fat-storing cells through excessive adipocyte differentiation.[13] Adipose tissue is a major energy reservoir in the body; it stores the excess energy in the form of lipids and releases it on demand. Furthermore, adipocytes constitute an endocrine system which secretes hormones such as adipokines.[14] Pathological adipocyte growth can be achieved by the processes of differentiation regulated by diverse factors.[15] Therefore, inhibition of fat accumulation by the disturbance of adipocyte differentiation is suggested to be an important therapeutic option in the treatment of obesity. Cultured 3T3-L1 adipocytes exhibit many properties that are similar to those of normal adipocytes.[16] Thus, this cell line has been well established to assess adipogenesis and adipocyte differentiation in an *in vitro* assay model. Herbal extracts from plants, such as *Gynostemma pentaphyllum*,[17] *Aegle marmelos*,[18] *Tecomella undulata*[19] and *Adenophora triphylla*[20] have been found to possess anti-obesity effects.

Moreover, natural products have also been a subject of an obesity study in 3T3-L1 cells. Previously, the effects of diarylheptanoids,[21] lanostane triterpenoids,[22] methyl cinnamate[23] and avicularin[24] on lipid accumulation in 3T3-L1 adipocytes were investigated. Hence crude drugs and natural products having similar properties would help to solve the obesity problem worldwide.

In continuation of our search for anti-adipogenic constituents of natural products, EtOAc-soluble fraction of RMBS showed inhibitory activity on adipocyte differentiation in 3T3-L1 cells as measured by fat accumulation using Oil Red O staining. Further fractionation and isolation of the EtOAc-soluble fraction of RMBS afforded four isoflavone glycoside derivatives including two new compounds. Herein, we describe the structure elucidation of these derivatives and evaluate their biological properties.

Purification of the EtOAc-soluble fraction of RMBS yielded two new isoflavone glycosides, 6"-*O*-crotonylgenistin (**1**) and genistein-7-*O*-α-D-ribofuranoside (**2**). The new isoflavone glycosides exhibited positive reactions to $FeCl_3$ (greenishbrown) reagents.

Compound **1**, a white powder, has the molecular formula $C_{25}H_{24}O_{11}$, as demonstrated by a negative HRESIMS at *m*/*z* 499.1233 $[M - H]^-$ (calcd for $C_{25}H_{23}O_{11}$, 499.1235), with 13

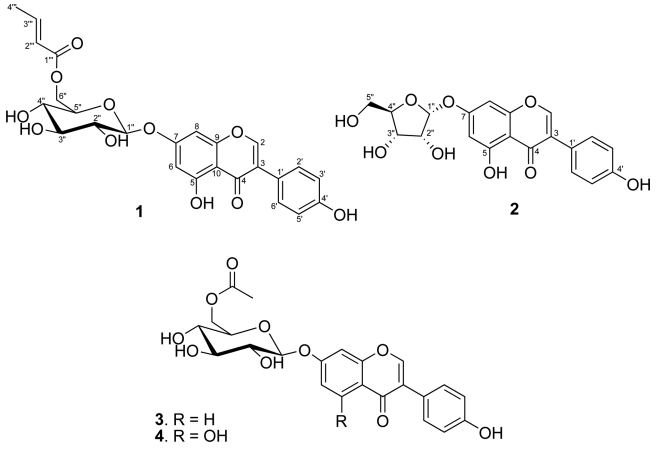

Figure 1. Chemical structures of compounds **1-4**.

[a]These authors contributed equally to this work.

degrees of unsaturation in the molecule. Its IR spectrum revealed the presence of hydroxyl groups (3455 cm^{-1}) and carbonyl groups (1709 cm^{-1}). The UV spectrum of compound **1** showed absorption maxima (log ε) at 260 (4.12) nm, and in the ^{1}H-NMR, one proton signal at δ_H 8.16 and six aromatic protons were observed, indicating that compound **1** has an isoflavone skeleton.[25] Two *meta*-coupled doublets at δ_H 6.67 and 6.54 (each 1H, d, J = 1.4 Hz) could be assigned to H-6 and H-8, respectively and two sets of doublet signals for the A_2B_2 spin system at δ_H 7.41 and 6.87 (each 2H, d, J = 8.4 Hz) were assigned to a 1,4-disubstituted benzene, as found in the B-ring. This finding was supported by ^{13}C NMR and HSQC spectra, which showed a total of 25 carbons consisting of a methylene, 14 methines, one methyl, and nine quaternary carbons. The ^{1}H and ^{13}C NMR spectra revealed one anomeric signal [δ_H 5.05 (1H, d, J = 7.0 Hz) and δ_C 101.6] together with five carbon signals (δ_C 77.9, 75.8, 74.8, 71.9, 64.8) in the sugar region, which indicated that compound **1** was an isoflavone-*O*-glycoside. The β anomeric configuration for glucose was determined based on the coupling constant of the anomeric proton ($J_{1'',2''}$ > 7.0 Hz).[26] Identify of the sugar moiety was confirmed by acid hydrolysis and NMR data of compound **1** wherein glucose could be detected by TLC analysis. A cross peak in the HMBC spectrum between H-1" (δ_H 5.05) of the glucose unit and C-7 (δc 164.7) of the isoflavone moiety indicated that the glucose residue was attached to the 7-hydroxyl of the isoflavone moiety (Fig. 2). Based on the above finding, compound **1** was similar to genistin, except for an additional signal arising from the crotonyl group.[27] The presence of a crotonyl (*trans*-2-butenoic acid) group was deduced from the ^{1}H and ^{13}C NMR signals of a secondary methyl signal [δ_H 1.90 (1H, d, J = 7.0 Hz); δ_C 18.4], a *trans* olefinic group [δ_H 7.05 (1H, dq, J = 15.4, 7.0 Hz), 5.94 (1H, d, J = 15.4 Hz); δ_C 147.1, 123.3], and a carbonyl signal at δ_C 168.0. In the HMBC experiment, the glucose H-6" signals (δ_H 4.53 and 4.27) with C-1'" (δ_C 168.0) indicated that the crotonyl group was attached to C-6" of the glucose moiety. Therefore, compound **1** was identified as 5,4'-dihydroxy-isoflavone-7-*O*-β-D-(6"-crotonyl)glucoside and was named 6"-*O*-crotonylgenistin.

Table 1. NMR data for compounds **1** and **2** in CD_3OD[a]

No.	**1**		**2**	
	δ_H mult., (J Hz)	δ_C mult.	δ_H mult., (J Hz)	δ_C mult.
2	8.16 s	155.4 d	8.11 s	155.4 d
3		125.2 s		125.1 s
4		182.6 s		182.6 s
5		163.8 s		163.6 s
6	6.54 d (1.4)	101.1 d	6.57 d (2.1)	101.5 d
7		164.7 s		164.8 s
8	6.67 d (1.4)	96.2 d	6.73 d (2.1)	96.2 d
9		159.3 s		159.3 s
10		108.1 s		107.9 s
1'		123.2 s		123.3 s
2'	7.41 d (8.4)	131.5 d	7.38 d (8.4)	131.5 d
3'	6.87 d (8.4)	116.4 d	6.85 d (8.4)	116.4 d
4'		159.1 s		159.0 s
5'	6.87 d (8.4)	116.4 d	6.85 d (8.4)	116.4 d
6'	7.41 d (8.4)	131.5 d	7.38 d (8.4)	131.5 d
1"	5.05 d (7.0)	101.6 d	5.75 d (4.2)	102.1 d
2"	3.52[b]	74.8 d	4.24 dd (6.3, 4.9)	73.7 d
3"	3.52[b]	77.9 d	4.13 dd (6.3, 2.8)	71.2 d
4"	3.40 m	71.9 d		88.4 d
5"	3.79 t (7.0)	75.8 d	3.72 dd (11.9, 2.8) 3.66 dd (11.3, 3.5)	63.6 t
6"	4.53 d (11.2), 4.27 dd (11.2, 7.0)	64.8 t		
1'"		168.0 s		
2'"	5.94 d (15.4)	123.3 d		
3'"	7.05 dd (15.4, 7.0)	147.1 d		
4'"	1.90 d (7.0)	18.4 q		

[a]Spectra were recorded at 700 MHz for ^{1}H NMR and 175 MHz for ^{13}C NMR using TMS as internal standard. The assignments were based on ^{1}H-^{1}H COSY, HSQC, and HMBC experiments. [b]Overlapped with other signals.

Compound **2**, a white amorphous powder, had the molecular formula $C_{20}H_{18}O_9$, as determined by negative HRESIMS (*m/z* 401.0868 $[M - H]^-$; calcd 401.0867). The ^{1}H NMR spectrum exhibited a flavonoid pattern and showed a signal at δ_H 8.11 (1H, s), typical of the proton at C-2 of an isoflavonoid skeleton. The ^{1}H and ^{13}C NMR data of compound **2** suggested that its structure was similar to that of genistin.[28] The only difference between these two compounds was the sugar moiety. Analysis of aromatic region of the ^{1}H NMR spectrum of compound **2** confirmed that the compound was a genistein derivative, with characteristic resonances for an A_2B_2-spin coupling system of two *ortho* doublets at δ_H 7.38 and 6.85 (each 2H, d, J = 8.4 Hz), characteristic for H-2'/6' and H-3'/5' of a 1,4-disubstituted B-ring. Also, two *meta*-coupled doublets at δ_H 6.73 and 6.57 (each 1H, d, J = 2.1 Hz) represented H-8 and H-6, respectively. The remaining resonances in the ^{1}H NMR spectrum at δ_H 4.3 to 3.6 indicated the presence of a sugar moiety. A loss of 132 mass unit from the molecular ion in the ESIMS suggested the presence of furanoside.[29] The connection between the sugar and isoflavone moieties through the *O* bond was established by the key HMBC correlation from H-1" (δ_H 5.75) to C-7 (δ_C 164.8). These correlations indicated that a sugar moiety was attached at C-7 of genistein (Figure 2). The ribose residue was confirmed by comparing the ^{13}C NMR data of compound **2** with those of several furanosides, such as naphthyl ribo-

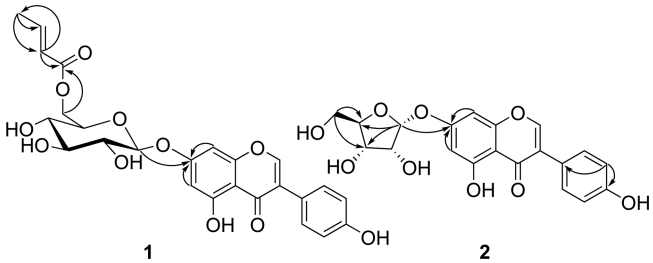

Figure 2. Key HMBC correlations of compounds **1** and **2**.

Table 2. Effect of isolated compounds **1-4** adipocyte differentiation in 3T3-L1

Relative fat accumulation (% of control)			
Differentiated control		100 ± 5.6	
Compounds	50 μM	100 μM	200 μM
1	69.3 ± 9.0[a]	22.8 ± 8.7	21.2 ± 6.8
2	91.9 ± 15.5	54.7 ± 11.5	23.1 ± 6.6
3	83.9 ± 7.2	81.3 ± 16.5	80.5 ± 18.3
4	89.1 ± 6.0	80.1 ± 4.5	62.9 ± 6.3
GL[b]	24.8 ± 4.6	10.3 ± 1.4	-

Relative lipid contents (%) were calculated as 100 × [(absorbance of sample-treated – absorbance of undifferentiated control)/[(absorbance of differentiated control – absorbance of undifferentiated control)]. [a]The experiments were repeated in triplicate, and the values were expressed as mean ± standard deviation. [b]Galanolactone (GL) was used as a positive control.

furanoside and asperflavin ribofuranoside.[30,31] The absolute configuration of D-ribose in compound **2** was established by measurement of its optical rotation following acid hydrolysis ($[\alpha]_D^{28}$ –25.3°, c 0.03, H_2O).[30] The sugar moiety was further determined to be α-anomeric glycoside by chemical shifts and coupling constant at C-1 [δ_H 5.75 (d, J = 4.2 Hz), δ_C 102.1 (d)] and by the comparison of 1D NMR data of ribofuranose moiety with the reported value.[32,33] Thus, compound **2** was identified as genistein-7-*O*-α-D-ribofuranoside. In addition to the above mentioned two new isoflavone glycosides, two known isoflavone derivatives were identified, by the analysis of spectroscopic data and comparison with the literature values, as 6"-*O*-acetyldaidzin (**3**)[34] and 6"-*O*-acetylgenistin (**4**)[35] (Figure 1).

Anti-adipogenic activity of the isolated compounds **1-4**, together with galanolactone (GL) as a positive control, was evaluated by assessing fat accumulation in 3T3-L1 cells.[36] To determine the non-toxic concentrations of compounds, 3T3-L1 cells were treated with various concentrations (50, 100, 200 μM) of compounds **1-4**, and the cell viability was measured by MTT assay. None of the compounds were cytotoxic at concentrations up to 200 μM. Hence, we treated the cells with compounds at this concentration. During differentiation (days 0-8), preadipocytes differentiated into mature adipocytes with cytoplasmic lipid vesicles. On day 8, the differentiated adipocytes were stained with Oil Red O solution, and the lipid contents were quantified spectrophotometrically at 510 nm. Among the isolated compounds, 6"-*O*-crotonylgenistin (**1**) and genistein-7-*O*-α-D-ribofuranoside (**2**) significantly inhibited fat accumulation in differentiated adipocytes (Table 2). 6"-*O*-crotonylgenistin (**1**), which exerted the most potent activity, inhibited fat accumulation up to 21.2% of fully differentiated cells (Figure 3).

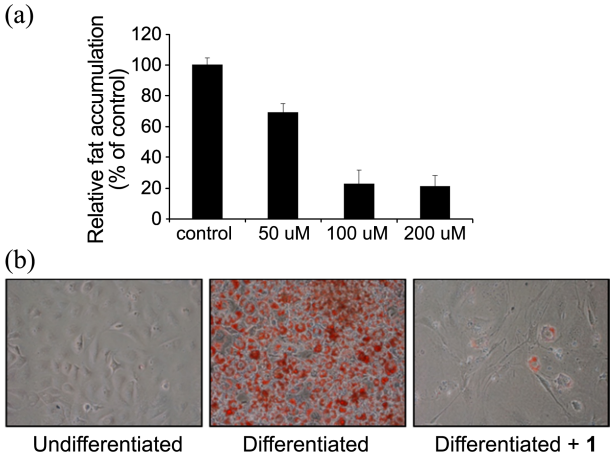

Figure 3. Effects of compound **1** on adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells were induced to differentiate in the absence or in the presence of compound **1** (50, 100, 200 μM) for 8 day and then the stained lipid contents were quantified by measuring absorbance (a). Cultured were treated with 200 μM compound **1** for whole period of differentiation (b).

In summary, the present study demonstrates that the EtOAc-soluble fraction and four isoflavone glycosides including two new isoflavone glycosides isolated from the RMBS inhibit adipocyte differentiation in 3T3-L1 cells by decreasing fat accumulation. These results suggest that RMBS and its isoflavone glycoside components may be effective agents for preventing obesity or obesity-related diseases.

Experimental Section

General Procedures. Optical rotations were recorded on a Jasco P-200 digital polarimeter. UV and IR spectra were obtained using a Shimadzu UV-1650PC and a Jasco FT/IR-4100 spectrometer, respectively. 1D and 2D NMR spectra were measured on a Bruker Ascend 700 MHz NMR spectrometer with tetramethylsilane as an internal standard, and chemical shifts expressed in terms of δ values. Electrospray ionization (ESI) mass spectra were obtained on a LTQ Orbitrap XL (Thermo Scientific) mass spectrometer. Preparative HPLC was performed using a Shimadzu system (LC-8A pump and SPD-20A UV/VIS detector) and a YMC-Pack ODS A column (250 × 20 mm i.d.), using a mixed solvent system of methanol-water at a flow rate of 8 mL/min. Medium pressure liquid chromatography (MPLC, Combi Flash RF, Teledyne ISCO) separations were performed using a RediSep Rf silica column (40 g) with a flow rate of 40 mL/min. Open column chromatography was performed using a silica gel (Kieselgel 60, 70-230 mesh, Merck) and Diaion HP-20 (Mitsubishi Kasei, Tokyo); and thin layer chromatography (TLC) was performed using a pre-coated silica gel 60 F_{254} (0.25 mm, Merck) and RP-18 $F_{254}S$ (0.25 mm, Merck Co., Darmstadt). All other chemicals and reagents were analytical grade.

Extraction and Isolation. The dried RMBS (10 Kg) were extracted with 80% EtOH (3 × 18 L) at room temperature. After filtration and evaporation of the solvent *in vacuo*, the EtOH extract was suspended in distilled water and then partitioned, in turn, with *n*-Hexane, CH_2Cl_2, EtOAc and *n*-BuOH. The EtOAc-soluble fraction (23 g), which showed the most potent activity, was subjected to further chromatographic separation. The EtOAc-soluble fraction was subjected to Diaion HP-20 column chromatography with the mixture of methanol/water (0%, 20%, 40%, 60%, 80%, 100% MeOH in water) to give six fractions (G44-56-31 ~

36). G44-56-35 was subjected to MPLC on silica gel eluting with the mixture of $CHCl_3$/MeOH (1:0 → 0:1, RediSep Rf silica column, 40 g, flow rate 40 mL/min) to give 11 subfractions (G44-92-1 ~ 11). Subfraction G44-92-7 was further purified by means of semi-preparative HPLC eluting with acetonitrile/water (30 to 50% acetonitrile) at a flow speed of 8 mL/min to yield compounds **1** (8 mg), **2** (12 mg), **3** (25 mg) and **4** (120 mg).

Acid Hydrolysis of Compound 2 and Determination of Stereochemistry of Ribofuranose. A solution of compound **2** (5 mg) in 9% aq. HCl (1 mL) was reacted for 2 h at 80 °C. The reaction mixture was extracted with EtOAc repeatedly to remove the aglycone fraction, which was identical to genistein. The H_2O layer was then concentrated to furnish the sugar residue (1.1 mg). The rotation recorded for the ribose isolated in this study was $[\alpha]_D^{28}$ –25.3° (*c* 0.03, H_2O), which closely matched that for the D-ribose (ref. –18.5°).

6''-*O*-Crotonylgenistin (1). White amorphous powder; $[\alpha]_D^{28}$ –76.9° (*c* 0.1, MeOH); IR λ_{max} 3516, 3455, 2918, 2853, 1709, 1656, 1511, 1442, 1370, 1239, 1182, 1073 cm^{-1}; UV (MeOH) λ_{max} nm (log ε): 260 (4.05), 329 (3.25); ^1H (700 MHz) and ^{13}C (175 MHz) NMR spectroscopic data, see Table 1; ESI-MS (negative mode) *m/z* 499 $[M–H]^-$, 545 $[M+COOH]^-$, 999 $[2M–H]^-$, 1045 $[2M+COOH]^-$; HRESIMS (negative mode) *m/z* 499.1233 (calcd for $C_{25}H_{23}O_{11}$, 499.1235).

Genistein-7-*O*-α-D-ribofuranoside (2). White amorphous powder; $[\alpha]_D^{28}$ +78.8° (*c* 0.2, MeOH); IR λ_{max} 3366, 2931, 2353, 1651, 1612, 1445, 1368, 1244, 1039 cm^{-1}; UV (MeOH) λ_{max} nm (log ε): 260 (4.11), 331 (3.52); ^1H (700 MHz, CD_3OD) and ^{13}C (175 MHz, CD_3OD) NMR spectroscopic data, see Table 1; ^1H NMR (700 MHz, DMSO-d_6) and ^{13}C (175 MHz, DMSO-d_6) NMR spectroscopic data, see Supporting Information; ESI-MS (negative mode) *m/z* 269 $[M–ribose]^-$, 401 $[M–H]^-$, 447 $[M+COOH]^-$; HRESIMS (negative mode) *m/z* 401.0868 (calcd for $C_{20}H_{17}O_9$, 401.0867).

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Supporting Information. Experimental details (fermentation, assay procedures, and acid hydrolysis of **1**), NMR and HRESI-MS spectra of **1** and **2**, and NMR data for **2-4** are available as Supporting Information.

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