Isoflavone Tetraglycosides from *Sophora japonica* Leaves[§]

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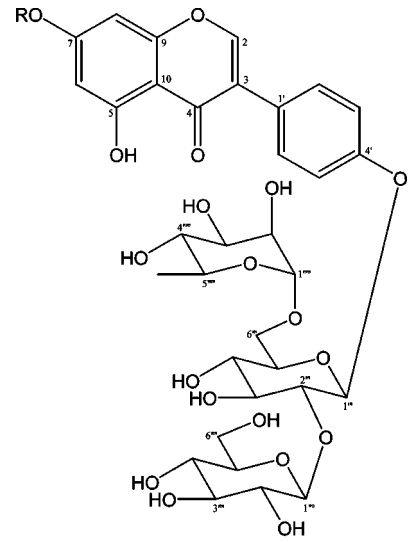
Received August 3, 2007

Two new isoflavone tetraglycosides (**1** and **2**) and six known compounds were isolated from the leaves of *Sophora japonica*. The new glycosides are genistein 7-*O*-*β*-D-glucopyranoside-4′-*O*-(6‴-*O*-α-L-rhamnopyranosyl)-*β*-sophoroside (**1**) and genistein 7-*O*-α-L-rhamnopyranoside-4′-*O*-(6‴-*O*-α-L-rhamnopyranosyl)-*β*-sophoroside (**2**). The structures of compounds **1** and **2** were established primarily by NMR experiments and chemical methods, and they are the first reported naturally occurring isoflavone glycosides with four attached sugar residues.

The genus *Sophora*, family Leguminosae, is a rich source of flavonoids,[1] alkaloids,[2] and saponins.[3] *Sophora japonica* L. (Leguminosae) is widely distributed throughout China. Its buds and fruits have been used as a hemostatic agent in traditional Chinese medicine, and flavonoids were discovered as hemostatic constituents from the buds of *S. japonica*.[1b] Triterpenes, phospholipids, alkaloids, amino acids, polysaccharides, and fatty acids have also been reported from its seeds.[4,5] Recently we systematically investigated the chemical constituents in the pericarps[1c] and seeds[1d] of *S. japonica* and isolated and identified many compounds including flavonols, isoflavones, and their glycosides. To our knowledge, no phytochemical investigation on the leaves of this species has been reported. The current report describes the isolation and structure elucidation of two new isoflavone glycosides, genistein 7-*O*-*β*-D-glucopyranoside-4′-*O*-(6‴-*O*-α-L-rhamnopyranosyl)-*β*-sophoroside (**1**) and genistein 7-*O*-α-L-rhamnopyranoside-4′-*O*-(6‴-*O*-α-L-rhamnopyranosyl)-*β*-sophoroside (**2**), from the leaves of *S. japonica*, together with six known compounds. The structures of **1** and **2** have been determined on the basis of the spectroscopic data including 2D NMR spectra and chemical evidence. The known compounds were identified as genistein 7-*O*-*β*-D-glucopyranoside-4′-*O*-*β*-D-glucopyranoside, sophorabioside, genistin, rutin, quercetin 3-*O*-*β*-D-glucopyranoside, and kaemgferol 3-*O*-*β*-D-glucopyranoside by comparison of their spectroscopic data with those reported[6,7] and by comparison with authentic samples. Compounds **1** and **2** are the first reported naturally occurring isoflavone glycosides having four sugar residues.

Compound **1** was isolated as a colorless, amorphous powder. The molecular formula was established as $C_{39}H_{49}O_{24}$ on the basis of the HRFABMS data. The exact mass of the $[M - H]^-$ ion at *m/z* 901.2582 matched well with the expected molecular formula of $C_{39}H_{49}O_{24}$ (calcd 901.2598). The IR spectrum of compound **1** showed strong absorption bands at 3415 (OH), 1652 (α,*β*-unsaturated C═O), 1612, 1582, 1495 (C═O, aromatic), and a broad band at 1160−1000 cm^{-1}, indicating its glycosidic nature. The UV spectrum (λ_{max} 260 nm) of compound **1** was typical of compounds having an isoflavone skeleton.[6] A characteristic resonance for H-2 of an isoflavone was observed at δ_H 8.48 (1H, s, δ_C 155.2) in the ^{1}H NMR spectrum.[6,8] This assignment was confirmed by long-range correlation with δ_C 180.4 (C-4), 157.2 (C-9), and 122.2 (C-1′) in the corresponding HMBC spectrum. Upon acid hydrolysis of **1**, genistein, glucose, and rhamnose were identified by TLC. Genistein was also identified by UV and ^{1}H NMR spectroscopy.[7]



1 R = glc
2 R = rha

Its ^{1}H and ^{13}C NMR spectra showed the presence of a genistein moiety and four sugar residues.[6,7] A ^{13}C NMR signal at δ 163.1 was assigned to C-7 on the basis of its long-range ^{13}C−^{1}H correlations to both H-6 (δ 6.48) and H-8 (δ 6.74), whereas C-7 showed a three-bond correlation with an anomeric proton at δ 5.07 in the HMBC spectrum. The 2D NMR spectra allowed the assignment of all ^{1}H and ^{13}C NMR signals of the 7-glycosyl residue (Table 1), which was identified as a glucopyranoside unit.[10] A large coupling constant (J = 7.2 Hz) for the anomeric proton of the glucose in the ^{1}H NMR spectrum suggested a *β*-orientation.[11] A methyl doublet, which appeared at δ 1.20 in the ^{1}H NMR spectrum of **1**, was assigned to the C-6 methyl protons of a rhamnose residue. All ^{1}H and ^{13}C NMR signals of the rhamnosyl moiety were assigned on the basis of 2D NMR spectral data (Table 1). A TOCSY experiment showed a correlation between the C-6 methyl protons of the rhamnose residue and the anomeric proton at δ 4.34, demonstrating that they belong to the same spin system. The anomeric proton of the rhamnosyl residue showed a long-range correlation with a ^{13}C NMR signal at δ 66.0, which was assigned to C-6‴ of the second glucosyl moiety. Therefore, glycosylation of the second glucose at the C-6‴ position became evident. A TOCSY experiment showed a correlation between the two C-6 protons (δ 3.19, 3.34) of the second glucosyl moiety and the anomeric proton at δ 5.54, demonstrating that they belong to the same spin system. A ^{13}C NMR signal at δ 157.1 was assigned to C-4′, on the basis of its long-range ^{13}C−^{1}H correlations observed in the HMBC experiment with the two ^{1}H NMR signals at δ 7.04

[§] Dedicated to Dr. G. Robert Pettit of Arizona State University for his pioneering work on bioactive natural products.

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10.1021/np070396t CCC: $40.75
Published on Web 11/30/2007

Table 1. ^{1}H and ^{13}C NMR Assignments (δ/ppm) for Compounds **1** and **2** in DMSO-d_6[a]

	1		2	
position	^{13}C	^{1}H	^{13}C	^{1}H
2	155.2 d	8.48 (s)	155.1 d	8.49 (s)
3	124.1 s		124.0 s	
4	180.4 s		180.5 s	
5	161.5 s		161.6 s	
6	99.7 d	6.48 (d, 1.8)	99.8 d	6.46 (d, 1.8)
7	163.1 s		163.2 s	
8	94.7 d	6.74 (d, 1.8)	94.7 d	6.71 (d, 1.8)
9	157.2 s		157.3 s	
10	106.2 s		106.2 s	
1′	122.2 s		122.4 s	
2′,6′	130.2 d	7.51 (d, 8.6)	130.3 d	7.50 (d, 8.6)
3′,5′	115.6 d	7.04 (d, 8.6)	115.7 d	7.04 (d, 8.6)
4′	157.1 s		157.1 s	
5-OH		12.93 (s)		12.91 (s)
1″	99.8 d	5.07 (d, 7.2)	98.5 d	5.60 (brs)
2″	73.2 d	3.72 (m)	70.2 d	3.71 (m)
3″	76.4 d	3.33 (m)	69.9 d	3.91 (m)
4″	69.5 d	3.20 (m)	71.8 d	3.39 (m)
5″	77.2 d	3.45 (m)	70.1 d	3.53 (m)
6″	60.6 t	3.69 (m), 3.70 (m)	18.1 q	1.19 (d, 6.2)
1‴	98.2 d	5.54 (d, 7.0)	98.3 d	5.54 (d, 7.0)
2‴	82.2 d	3.53 (m)	82.2 d	3.52 (m)
3‴	76.4 d	3.56 (m)	76.5 d	3.56 (m)
4‴	69.7 d	3.17 (m)	69.7 d	3.16 (m)
5‴	75.6 d	3.19 (m)	75.5 d	3.19 (m)
6‴	66.0 t	3.33 (m), 3.34 (m)	66.1 t	3.33 (m), 3.34 (m)
1⁗	104.0 d	4.65 (d, 7.6)	104.1 d	4.65 (d, 7.6)
2⁗	74.4 d	3.13 (m)	74.4 d	3.14 (m)
3⁗	76.6 d	3.24 (m)	76.6 d	3.25 (m)
4⁗	70.1 d	3.20 (m)	70.1 d	3.21 (m)
5⁗	77.0 d	3.17 (m)	77.1 d	3.17 (m)
6⁗	60.8 t	3.55 (m), 3.64 (m)	60.8 t	3.54 (m), 3.65 (m)
1⁗′	100.3 d	4.34 (brs)	100.3 d	4.34 (brs)
2⁗′	70.2 d	3.37 (m)	70.2 d	3.38 (m)
3⁗′	69.9 d	3.69 (m)	69.9 d	3.69 (m)
4⁗′	71.8 d	3.20 (m)	71.8 d	3.20 (m)
5⁗′	68.1 d	3.81 (m)	68.2 d	3.80 (m)
6⁗′	17.7 q	1.20 (d, 6.2)	17.8 q	1.20 (d, 6.2)

[a] All assignments are based on extensive 1D and 2D NMR measurements (DEPT, DQF-COSY, TOCSY, NOESY, HMQC, and HMBC); 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR. Carbon multiplicities were determined by DEPT experiments. s = quaternary, d = methine, t = methylene, q = methyl carbons.

(H-3′,5′) and δ 7.51 (H-2′,6′), and the C-4′ signal showed a three-bond correlation with the anomeric proton of a second glucosyl unit at δ 5.54. The anomeric proton of the third glucosyl residue at δ 4.65 showed a long-range correlation with a ^{13}C NMR signal at δ 82.2, corresponding to a proton at δ 3.53 in the HMQC spectrum. The latter signal showed a ^{1}H−^{1}H correlation, observed in the DQF-COSY experiment, with the anomeric proton of the second glucosyl moiety at δ 5.54. Therefore, glycosylation of the second glucosyl moiety at the C-2‴ position also became evident. 2D NMR allowed the assignment of all ^{1}H and ^{13}C NMR signals of the second and third glucosyl moieties (Table 1). The β-orientation of two anomeric protons was evident from the coupling constant of H-1‴ (J = 7.2 Hz) and H-1⁗ (J = 7.6 Hz) observed in the ^{1}H NMR spectrum.[10] These showed the presence of a 4′-*O*-(6‴-*O*-α-L- rhamnopyranosyl)-β-sophorosyl residue in compound **1**. The NMR signals of the 4′-*O*-(6‴-*O*-α-L-rhamnopyranosyl)-β-sophorosyl residue were the same as those of a flavonoid 4′-*O*-(6‴-*O*-α-L-rhamnopyranosyl)-β-sophoroside described in the literature.[12] All of these considerations were used to characterize compound **1** as genistein 7-*O*-β-D-glucopyranoside-4′-*O*-(6‴-*O*-α-L- rhamnopyranosyl)-β-sophoroside.

The molecular formula of **2**, determined to be $C_{39}H_{49}O_{23}$ by HRFABMS, suggested the presence of a rhamnose instead of glucose in comparison with compound **1**. The ^{1}H and ^{13}C NMR spectra of **2** showed the presence of a genistein moiety and four sugar residues.[6,7] Its ^{1}H and ^{13}C NMR and 2D NMR spectra were indicative of a genistein 4′-*O*-(6‴-*O*-α-L-rhamnopyranosyl)-β-sophoroside unit, as found for **1** (Table 1). A ^{13}C NMR signal at δ 161.3 was assigned to C-7 on the basis of its long-range ^{13}C−^{1}H correlations observed in a HMBC experiment with the ^{1}H NMR signals at δ 6.45 (H-6) and 6.70 (H-8). The C-7 signal showed a three-bond correlation with an anomeric proton at δ 5.60 in the HMBC spectrum. Another methyl doublet, observed at δ 1.19 in the ^{1}H NMR spectrum of **2**, was assigned to the C-6 methyl group of a rhamnose residue. All ^{1}H and ^{13}C NMR signals of the rhamnosyl moiety were assigned on the basis of the 2D NMR spectroscopic data (Table 1). A TOCSY experiment showed a correlation between the rhamnose methyl protons at C-6 and the anomeric proton at δ 5.60, demonstrating that they belonged to the same spin system, consistent with a 7-*O*-rhamnosyl residue. The NMR signals of the 7-*O*-rhamnosyl residue were comparable with literature values for flavonoid 7-*O*-rhamnoside.[4,5,8] Therefore, compound **2** was identified as genistein 7-*O*-α-L-rhamnopyranoside-4′-*O*-(6‴-*O*-α-L- rhamnopyranosyl)-β-sophoroside.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer model 241 polarimeter. UV spectra were obtained on a Shimadzu UV-1601 spectrophotometer. IR (KBr) spectra were recorded using a Perkin-Elmer 983 spectrometer. ^{1}H, ^{13}C, and 2D NMR spectra were run on a Bruker DRX-500 instrument. Chemical shifts were reported with reference to the respective residual solvent or deuterated solvent peaks (δ_H 2.5 and δ_C 39.5 from DMSO-d_6). FABMS data were obtained on a JEOL JMS DX-303HF mass spectrometer. HPLC separations were performed on a HP 1100 apparatus equipped with a diode array UV detector and XTERRA C_{18} (Waters, 10 μm, 15 × 200 mm, flow rate 12 mL/min) column. GC was carried out on a GC-14C gas chromatograph (Shimadzu, Japan) with an AC-1 fused silica capillary column (30 m × 0.25 mm × 0.25 μm) (SGE); detector, 230 °C; temperature gradient system for the oven, 150 °C for 1 min and then raised to 230 °C at rate of 5 °C/min. Silica gel plates for TLC and silica gel for column chromatography were produced by Qingdao Marine Chemical Campany, Qingdao, China.

Plant Material. Leaves of *S. japonica* L. were collected from mature trees, growing in Nanjing, China, in 2000. The plant material was identified by Prof. Luoshan Xu (China Pharmaceutical University), and a voucher specimen (No. CPUT-001128) was deposited in the herbarium of China Pharmaceutical University.

Extraction and Isolation. The air-dried leaves (10.0 kg) were finely pulverized and extracted by percolation with methanol for two weeks at room temperature. The combined extracts were filtered and concentrated under vacuum to obtain a crude extract (370 g). The extract was suspended in water and successively partitioned with cyclohexane, EtOAc, and *n*-BuOH. The *n*-BuOH-soluble fraction was concentrated and subjected to silica gel column chromatography (CC) eluting with CH_2Cl_2−CH_3OH (20:1) followed by stepwise addition of CH_3OH to yield 13 fractions. Fraction 9 (31.6 g) was subjected to silica gel CC (200–300 mesh, 1000 g) eluted with a CH_2Cl_2−CH_3OH system (10:3) and Sephadex LH-20 CC (300 g) eluted with CH_3OH and purified by RP-18 preparative HPLC (CH_3OH−0.5% acetic acid, 17:83; **1**, t_R = 10.87 min; **2**, t_R = 11.89 min) to give compounds **1** (12 mg) and **2** (10 mg), respectively. Fraction 7 (58.2 g) was subjected to silica gel CC (200–300 mesh, 1300 g) eluted with CH_2Cl_2−CH_3OH (10:2) to give sophorabioside (5.5 g), rutin (10.2 g), and genistein 7-*O*-β-D-glucopyranoside-4′-*O*-β-D-glucopyranoside (38 mg). Fraction 5 (20.2 g) was subjected to silica gel CC (200–300 mesh, 800 g) eluted with CH_2Cl_2−CH_3OH (10:2) to give genistin (89 mg), quercetin 3-*O*-β-D-glucopyranoside (18 mg), and kaemgferol 3-*O*-β-D-glucopyranoside (20 mg).

Genistein 7-*O*-β-D-glucopyranoside-4′-O-(6‴-O-α-L-rhamnopyranosyl)-β-sophoroside (1): colorless, amorphous powder; $[\alpha]^{25}_D$ −68 (*c* 0.002, DMSO); UV (CH_3OH) λ_{max} 260 nm (log ε 4.20); IR ν_{max}^{KBr} 3415, 2975, 2930, 1652, 1612, 1582, 1512, 1495, 1442, 1384, 1367, 1304, 1241, 1183, 1160−1000 cm^{-1}; ^{1}H and ^{13}C NMR (DMSO-d_6), see Table 1; negative HRFABMS *m/z* found 901.2582 [M − H]$^-$ (calcd for $C_{39}H_{49}O_{24}$, 901.2598).

Genistein 7-*O*-α-L-rhamnopyranoside-4′-*O*-(6‴-O-α-L-rhamnopyranosyl)-β-sophoroside (2): colorless, amorphous powder; $[\alpha]^{25}_D$ −87 (*c* 0.002, DMSO); UV (CH_3OH) λ_{max} 260 nm (log ε 4.26); IR

ν_{max}^{KBr} 3413, 2975, 2929, 1651, 1612, 1583, 1511, 1496, 1442, 1385, 1366, 1306, 1240, 1182, 1160∼1000 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) see Table 1; negative HRFABMS *m*/*z* found 885.2667 $[M - H]^-$ (calcd for $C_{39}H_{49}O_{24}$, 885.2653).

Acid Hydrolysis of 1 and 2. A solution of each compound (3 mg) in 10% HCl was stirred at 90 °C for 5 h. After cooling, the reaction mixture was filtered. The filtrate was examined by TLC together with authentic sugar samples (EtOAc−CH_3OH−H_2O−HOAc, 6:1:1:1, glucose, R_f = 0.31; rhamnose, R_f = 0.55). The remaining filtrate was concentrated to dryness to give a residue, which was dissolved in dry pyridine (0.1 mL). After addition of L-cysteine methyl ester hydrochloride (1.0 mg), the mixture was stirred and warmed at 60 °C for 1 h. Trimethylsilylimidazole (150 μL) was added, and the mixture was stirred at 60 °C for 30 min After centrifugation, the supernatant was directly subjected to GC analysis.[13] Standard monosaccharides were subjected to the same reaction and GC analysis, and peaks were observed with t_R (min) of 14.5 (L-rhamnose) and 22.3 (D-glucose). L-Rhamnose and D-glucose were obtained in the ratio 1:3 and 2:2, respectively.

Acknowledgment. This research was supported by the Returned Student Initial Funding from Nanjing University of Chinese Medicine. We are grateful to Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, for the NMR and FABMS experiments. We are also pleased to thank Prof. Luoshan Xu (Division of Pharmacognosy, China Pharmaceutical University) for the plant identification.

References and Notes

(1) (a) Deng, Y. H.; Xu, K. P.; Zhou, Y. J.; Li, F. S.; Zeng, G. Y.; Tan, G. S. *J. Asian Nat. Prod. Res.* **2007**, *9*, 45–48. (b) Ishida, H.; Umino, T.; Tsuji, K.; Kosuge, T. *Chem. Pharm. Bull.* **1989**, *37*, 1616–1618. (c) Tang, Y.; Lou, F.; Wang, J.; Zhuang, S. *J. Nat. Prod.* **2001**, *64*, 1107–1110. (d) Wang, J.; Lou, F.; Wang, Y.; Tang, Y. *Phytochemistry* **2003**, *63*, 463–465.

(2) (a) Ding, P. L.; Huang, H.; Zhou, P.; Chen, D. F. *Planta Med.* **2006**, *72*, 854–856. (b) Wu, Y.; Shao, Q.; Zhen, Z.; Cheng, Y. *Biomed. Chromatogr.* **2006**, *20*, 446–450. (c) Ding, P. L.; Yu, Y. Q.; Chen, D. F. *Phytochem. Anal.* **2005**, *16*, 257–263.

(3) Byun, J. H.; Kim, J. S.; Kang, S. S.; Son, K. H.; Chang, H. W.; Kim, H. P.; Bae, K. *Chem. Pharm. Bull.* **2004**, *52*, 870–873.

(4) Mukhamedova, K. H. S.; Glushenkova, A. I. *Chem. Nat. Compd.* **1997**, *33*, 445–448.

(5) Grishkovets, V. I.; Gorbacheva, L. A. *Chem. Nat. Compd.* **1995**, *31*, 596–599.

(6) Watanabe, K.; Kinjo, J.; Nohara, T. *Chem. Pharm. Bull.* **1993**, *41*, 394–396.

(7) Markham, K. R.; Terlai, B.; Stanley, R.; Geiger, H.; Mabry, T. L. *Tetrahedron* **1978**, *34*, 1389–1397.

(8) Moon, H. I.; Lee, J.; Zee, O. P.; Chung, J. H. *Biol. Pharm. Bull.* **2005**, *28*, 1123–1125.

(9) Huang, W.-Z.; Duan, J.-A.; Li, Z.-L. *Zhongguo Yaoke Daxue Xuebao* **2000**, *31*, 8–10.

(10) Markham, K. R.; Chari, V. M.; Mabry, T. J. In *The Flavonoids: Advances in Research*; Harborne, J. B., Mabry, T. J., Eds.; Chapman & Hall: London, 1982; pp 19–134..

(11) Markham, K. R.; Geiger, H. In *The Flavonoids: Advances in Research since 1986*; Harborne, J. B., Ed.; Chapman & Hall: London, 1994; pp 441–497..

(12) Budzianowski, J. *Phytochemistry* **1990**, *29*, 3643–3647.

(13) Hara, S.; Okabe, H.; Mihashi, K. *Chem. Pharm. Bull.* **1987**, *35*, 501–506.

NP070396T