Biflavone Glucosides from *Ginkgo biloba* Yellow Leaves

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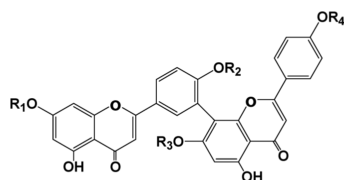
Phytochemical investigation of *Ginkgo biloba* (Ginkgoaceae) has resulted in the isolation of two new biflavone glucosides, ginkgetin 7″-*O*-β-D-glucopyranoside (1) and isoginkgetin 7-*O*-β-D-glucopyranoside (2). The structures were determined on the basis of chemical and spectroscopic evidences.

Key words *Ginkgo biloba*; Ginkgoaceae; biflavone glucoside; ginkgetin 7″-*O*-β-D-glucopyranoside; isoginkgetin 7-*O*-β-D-glucopyranoside

The dried leaves of *Ginkgo biloba* (Ginkgoaceae) have been used therapeutically for centuries, in the treatment of asthma, bronchitis, ischemia, arteriosclerosis, and rheumatism.[1,2] Earlier phytochemical investigation into this species resulted in the isolation of flavonoids,[2—12] diterpenes,[13—15] sesquiterpenes,[15,16] polyphenols, and others.[9,17] In this paper, we report the isolation and structural identification of new biflavone glucosides (**1**, **2**) from the leaves of *G. biloba* collected in Korea.

The combination of silica gel and RP-18 column chromatography with the EtOAc soluble portion of the methanolic extract from the leaves of *G. biloba* yielded compounds **1** and **2**. Both compounds were isolated as yellow amorphous powders, which exhibited characteristic flavonol glycoside color reactions, *i.e.*, reddish-pink on Mg–HCl tests, and positive on Molisch tests.

The high-resolution (HR)-FAB-MS spectrum of 729.1819 $[M+H]^+$ for compound **1** yielded a molecular formula of $C_{38}H_{32}O_{15}$, which is consistent with biflavonoid glycoside. Acid hydrolysis of compound **1** resulted in the isolation of ginkgetin (**1a**) and glucose, both of which were identified *via* direct comparison with TLC analysis, using authentic samples.[18] The glucose was assumed to be in the common D-configuration, as this is the configuration most often encountered among the flavonoid glycosides. The anomeric proton signal at δ 5.16 (J=7.8 Hz) in the ^{1}H-NMR spectrum indicated the β-configuration for the glucopyranosyl moiety. The UV spectrum of compound **1** in methanol exhibited absorption maxima similar to those of ginkgetin.[19] The addition of sodium methoxide induced a shift in band I to 391 nm, thereby indicating the presence of a free 4′ or 4‴-hydroxyl group. The failure to detect any bathochromic shifts in band II after NaOAc treatment suggested that the glucose was attached to the gingetin at C-7″.[20] The glycosidic linkage site of β-D-glucose at C-7″ was further verified by the HMBC experiments, in which the anomeric proton at δ 5.16 was correlated with the C-7″ at δ 160.1. Detailed analyses of the HMQC and HMBC spectra allowed for the assignment of all of the ^{1}H- and ^{13}C-NMR signals of both the glucosyl and the aglycone moieties. From the above data and the ROESY cor-



1: R1=R2= CH3, R3=Glu, R4= H
2: R1= Glu, R2= R4= CH3, R3= H

Fig. 1. Structures of **1** and **2**

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Compounds **1** and **2** in DMSO-d_6

No.	1		2	
	^{13}C	^{1}H	^{13}C	^{1}H
2	163.9		162.7	
3	103.9	6.97 (s)	103.6	6.79 (s)
4	182.0		181.2	
5	161.1		161.3	
6	98.0	6.37 (d, 1.9)	99.7	6.02 (s)
7	165.2		160.2	
8	92.7	6.79 (d, 1.9)	94.7	6.31 (s)
9	157.4		157.7	
10	105.0		102.4	
1′	122.5		123.0	
2′	131.0	8.11 (d, 2.6)	131.3	8.04 (d, 2.2)
3′	122.1		120.6	
4′	160.9		159.8	
5′	111.8	7.35 (d, 8.9)	111.4	7.36 (d, 8.9)
6′	128.4	8.22 (dd, 2.6, 8.9)	127.9	8.14 (dd, 2.2, 8.9)
2″	164.2		163.6	
3″	103.8	6.84 (s)	103.3	6.98 (s)
4″	182.2		182.3	
5″	161.0		160.9	
6″	98.2	6.77 (s)	98.0	6.76 (s)
7″	160.1		160.8	
8″	105.9		105.2	
9″	153.6		153.6	
10″	104.8		104.9	
1‴	121.0		122.6	
2‴, 6‴	128.2	7.49 (d, 8.9)	127.9	7.64 (d, 8.6)
3‴, 5‴	116.1	6.66 (d, 8.9)	114.6	6.98 (d, 8.6)
4‴	160.6		162.4	
1⁗	99.7	5.16 (d, 7.8)	99.7	5.16 (d, 7.8)
2⁗	73.0	3.03 (m)	73.3	3.02 (m)
3⁗	76.7	3.28 (t, 8.6)	76.7	3.29 (t, 8.6)
4⁗	69.7	3.11 (t, 8.6)	69.5	3.09 (t, 8.6)
5⁗	77.2	3.40 (m)	77.1	3.46 (m)
6⁗	60.7	3.44 (d, 12.0), 3.76 (dd, 2.0, 12.0)	60.6	3.43 (d, 13.2), 3.76 (dd, 2.1, 13.2)
OCH_3	56.1	3.84	55.8	3.78
OCH_3	55.9	3.76	55.5	3.76
OH		13.22, 12.97		13.14, 12.91

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relations (H-2′/H-3, H-6′/H-5′, H-2″″/H-3″″, H-8/H-7), therefore, the structure of compound **1** was ginkgetin 7″-*O*-β-D-glucopyranoside.

The HR-FAB-MS spectrum of compound **2** exhibited a *quasi*-molecular ion peak at *m/z* 729.1811 $[M+H]^+$ (Calcd 729.1819) , which is consistent with a molecular formula of $C_{38}H_{32}O_{15}$, corresponding to the biflavonoid glycoside. The IR and UV spectrum of compound **2** were determined to be almost identical to those of compound **1**, thereby pointing to a similarity in their natures. Acid hydrolysis of compound **2** resulted in the isolation of isoginkgetin (**2a**) and glucose, both of which were identified *via* direct comparison in TLC analysis using authentic samples.[18] The glycosidic linkage site of D-glucose was determined to be C-7, based on the HMBC spectrum. In the HMBC spectrum, the anomeric proton peak located at δ 5.16 was correlated with the C-7 at δ 160.2. Compound **2** was, therefore, determined to be isoginkgetin 7-*O*-β-D-glucopyranoside. To the best of our knowledge, this study is the first report of the natural occurrence of these compounds.

Experimental

General ^{1}H- and ^{13}C-NMR spectra were measured using a JEOL JNM-ECP 400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) spectrometer. ROESY, HMQC, and HMBC spectra were recorded using pulsed field gradients. The chemical shifts were referenced to the respective residual solvent peaks, and the values recorded in δ. FAB-MS data were collected on a JEOL JMS-HX110/110A spectrometer. Column chromatography was carried out using silica (Si) gel 60 (70—230 mesh, Merck, Germany) and RP-18 Lichroprep (Merck, Germany). TLC was carried out on precoated Merck Kieselgel 60 F_{254s} plates (0.25 mm) and RP-18 F_{254s} plates (Merck) and spots were detected under UV light using 50% H_2SO_4 reagent. All solvents for column chromatography were of reagent grade, and were acquired from commercial sources.

Plant Materials The leaves were collected directly from a *G. biloba* tree, which had been grown at Andong National University, Andong, Korea. The collection took place in November, 2001.

Extraction and Isolation The dried *G. biloba* leaves (1.18 kg) were refluxed for 3 h with MeOH. The total filtrate was then concentrated to dryness, *in vacuo*, at 40 °C, in order to make the MeOH extract (524 g). This extract was suspended in H_2O, and then successively partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH, in order to yield CH_2Cl_2 (111.7 g), EtOAc (48.3 g), and *n*-BuOH fractions (175.6 g), as well as H_2O residue (146.0 g). The EtOAc fraction (48.3 g) was then chromatographed on a Si gel column, using CH_2Cl_2–MeOH (stepwise) and generating 20 subfractions (Fr. 1 to 20). Fraction 11 (6.03 g) was chromatographed over Si gel, using CH_2Cl_2–MeOH (40 : 1 to pure MeOH) in order to obtain 13 fractions (Fr. 11-1 to 11-13). Fraction 8 (Fr. 11-8, 0.77 g) was subjected to column chromatography over a Si gel column with CH_2Cl_2–MeOH–H_2O (5 : 1 : 1 to MeOH), yielding 8 fractions (Fr. 11-8-1 to 11-8-8). Compounds **1** (12.8 mg) and **2** (12.9 mg) were obtained *via* the RP-18 column chromatography (70% MeOH) performed on Fraction 7 (Fr. 11-8-7, 0.43 g).

Ginkgetin 7″-*O*-β-D-glucopyranoside (**1**): Yellow amorphous powder. $[\alpha]_D^{20}$ +5.5° (c=0.004, MeOH). IR ν_{max} cm^{-1}: 3436, 2359, 1657, 1607, 1575, 1506, 1370, 1259, 1186, 1169, 1026, 828. UV λ_{max} (MeOH) 270 (log ε 4.53), 330 (4.49) nm; +NaOMe 272 (4.52), 391 (4.34); +NaOAc 270 (4.53), 331 (4.45); +NaOAc+H_3BO_3 270 (4.53), 331 (4.49) ; +$AlCl_3$ 279 (4.50), 302 (4.38), 344 (4.48), 389 (4.31); +$AlCl_3$+HCl 279 (4.50), 302 (4.39), 343 (4.48), 389 (4.28). HR-FAB-MS *m/z*: 729.1819 $[M+H]^+$ (Calcd for $C_{38}H_{33}O_{15}$: 729.1819). ^{1}H- and ^{13}C-NMR: see Table 1.

Isoginkgetin 7-*O*-β-D-glucopyranoside (**2**): Yellow amorphous powder. $[\alpha]_D^{20}$ +0.77° (c=0.003, MeOH). IR ν_{max} cm^{-1}: 3445, 2360, 1634, 1558, 1362, 1260, 1180, 1068, 828. UV λ_{max} (MeOH) 271 (log ε 4.68), 328 (4.58) nm; +NaOMe 277 (4.75), 298 sh (4.66), 369 (4.36); +NaOAc 271 (4.67), 329 (4.52); +NaOAc+H_3BO_3 272 (4.63), 328 (4.54); +$AlCl_3$ 281 (4.65), 341 (4.56), 391 (4.33); +$AlCl_3$+HCl 280 (4.66), 341 (4.56), 391 (4.31). HR-FAB-MS *m/z*: 729.1811 $[M+H]^+$ (Calcd for $C_{38}H_{33}O_{15}$: 729.1819), 567 $[M-162+H]^+$. ^{1}H- and ^{13}C-NMR: see Table 1.

Acid Hydrolysis of 1 and 2 Compounds **1** and **2** (each 5 mg) were dissolved in 5% aqueous HCl (5 ml), and separately refluxed for 3 h each. The reaction mixture was extracted with EtOAc. The EtOAc fraction (aglycone) and aqueous fraction (sugar) were concentrated to dryness for the purposes of identification. The aglycone was crystallized from MeOH, in order to yield **1a** (mp 230—233°) from compound **1**, and **2a** (mp 209—210°) from compound **2**. The success of this step was confirmed by direct comparison with TLC analysis, using authentic samples.[18] The sugar component was identified as glucose by TLC on Si gel (developing solvent EtOAc/MeOH/H_2O/AcOH=13 : 3 : 3 : 4, *Rf* 0.30), *via* direct comparison with authentic samples.

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