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NEW BIFLAVONOIDS FROM *CEPHALOTAXUS HARRINGTONIA* VAR. *FASTIGIATA* (CEPHALOTAXACEAE)

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Abstract– Three new biflavonoids **1** (2,3-dihydro-6-methylginkgetin), **2** (2,3-dihydro-6-methylbilobetin) and **3** (2,3-dihydro-6-methylsequoiaflavone) have been isolated from the leaves of *Cephalotaxus harringtonia* K. Koch var. *fastigiata* Rehder (Cephalotaxaceae) and their structures were elucidated by spectral analysis.

INTRODUCTION

Biflavonoids are found in a limited number of Gymnosperm families such as Cycadaceae, Ginkgoaceae, Taxaceae, Podocarpaceae, Cephalotaxaceae, Taxodiaceae, Cupressaceae, and Araucariaceae.[1-3] Some amentoflavone-type biflavonoids, a dimer of flavones linked to each other at C3' and C8'' positions, were reported from the genus *Cephalotaxus* (Cephalotaxaceae). Amentoflavone, sequoiaflavone, ginkgetin, amentoflavone 7,4',7'',4'''-tetramethyl ether from *Cephalotaxus harringtonia* K. Koch var. *harringtonia*,[4] amentoflavone, bilobetin, sequoiaflavone, ginkgetin, amentoflavone 4',7''-dimethyl ether, amentoflavone 7,4',7'',4'''-tetramethyl ether from *C. koreana* Nakai,[5,6] amentoflavone, bilobetin, sciadopitysin from *C. griffithii* HK,[7] taiwanhomoflavone A-C, amentoflavone7,4',7"-trimethyl ether from *C. wilsoniana* Hayata,[8,9,10] oliveriflavone, sciadopitysin, amentoflavone 7,4',7'',4'''-tetramethyl ether from *C. oliveri* Mast,[11] sciadopitysin, amentoflavone 7,4',7'',4'''-tetramethyl ether from *C. fortunei* Hook. f. var. *alpina*[12] were reported. However an alkaloid, deoxyharringtonine, was reported only from the leaf of *Cephalotaxus harringtonia* K. Koch var. *fastigiata* Rehder (Cephalotaxaceae).[13] This is the first report of biflavonoids from *C. harringtonia* var. *fastigiata*.

RESULTS AND DISCUSSION

The structures of the three previously unknown compounds, **1** to **3,** were determined by spectroscopic analysis to be mixed dimers of flavones and flavanones (Figure 1).

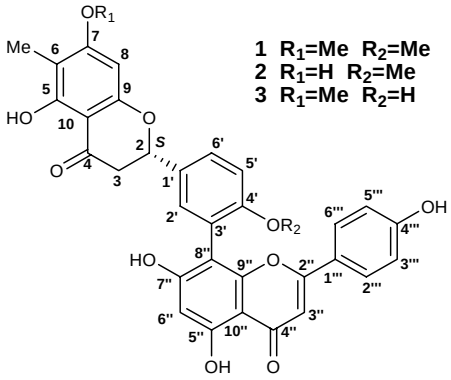

Figure 1. Structures of **1** - **3**

Compound **1**, which was obtained as a yellow powder, was assigned the molecular formula $C_{33}H_{26}O_{10}$ by the HREIMS spectra data *m/z* 582.1509 [M^+], (calcd 582.1526). The structure of **1** was determined by ^{1}H- and ^{13}C-NMR spectroscopy (Tables 1 and 2). ^{1}H-^{1}H COSY, HMQC and HMBC experiments were used to assign the observed resonances to individual atoms. The ^{1}H-^{1}H interactions and long-range ^{1}H-^{13}C interactions detectable in the ^{1}H-^{1}H COSY and HMBC experiments of **1** are summarized in Figure 2.

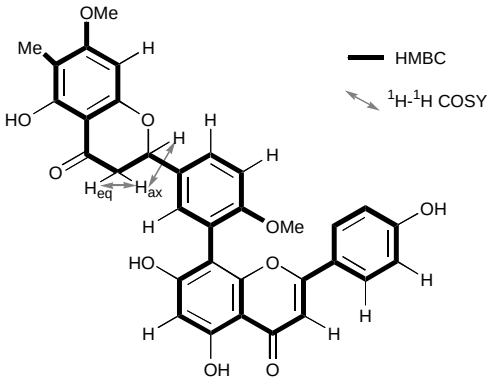

Figure 2. HMBC, ^{1}H-^{1}H COSY of **1**

The NMR spectra of compound **1** exhibited a number of doublets. The presence of a flavanone moiety was revealed by the proton signals at δ 2.75 (2.80) and 3.40 (3.43) attributed to C-3 and at δ 5.60 (5.61) assigned to the proton at C-2. The large coupling constant ($J_{H2,H3ax}$ = 13.2 Hz) is characteristic of diaxial protons and indicates flavanone B-ring is in an equatorial position on C-2.[3] All carbon signals excepting one (C-9") were identified by the combination of ^{1}H-^{1}H COSY and HMBC experiments. The presence of methoxyl and methyl groups in position C-7, C-4' and C-6 of **1** was confirmed by the data of EIMS and HMBC spectra, respectively. The proton signal at δ7.51 and δ7.52 (2' position) showed HMBC correlations at δ 104.19 and δ104.21 (C-8''). This HMBC correlation data suggested that C-3' was linked

to C-8'' position which located of the biphenyl bond. The bond between C-3' and C-8" were also determined through the EIMS spectrum.[2]

It was already reported that 2,3-dihydroxysciadopitysin (Figure 3) exists as a dynamic equilibrium of two diastereomers due to be the presence of a chiral center on C-2 and atropisomerism induced by restricted rotation along the C3'-C8'' bond.[14] This is the same case for **1**. Therefore the resulting doubling of proton and carbon resonances were observed in the spectra. The absolute configuration at C-2 of **1** was determined by the circular dichroism (CD) data. (2*S*)-naringenin contains almost the same chromophore system as the part flavanone.[15]

Table 1. ^{1}H-NMR Spectrum Data (DMSO-d_6) of Compounds **1**-**3**.

position	**1**	**2**	**3**
2	5.60, 5.61 (each 1H, dd, *J* = 13.2, 2.9 Hz)	5.53, 5.54 (each 1H, dd, *J* = 13.2, 2.7 Hz)	5.52 (1H, dd, *J* = 13.4, 2.4 Hz)
3_{eq}	2.75, 2.80 (each 1H, dd, *J* = 17.1, 2.9 Hz)	2.71, 2.74 (each 1H, dd, *J* = 17.1, 2.7 Hz)	2.72 (1H, brd, *J* = 17.1 Hz)
3_{ax}	3.40, 3.43 (each 1H, dd, *J* = 17.1, 13.2 Hz)	3.40 (2H, m)	3.39 (1H, dd, *J* = 13.4, 17.1 Hz)
8	6.18, 6.22 (each 1H, s)	5.99, 6.00 (each 1H, s)	6.18, (1H, brs)
2'	7.51, 7.52 (each 1H, d, *J* = 2.4 Hz)	7.45, 7.49 (each 1H, d, *J* = 2.4 Hz)	7.42 (1H, brs)
5'	7.22 (2H, d, *J* = 8.8 Hz)	7.20, 7.21 (each 1H, d, *J* = 8.8 Hz)	7.00 (1H, d, *J* = 8.9 Hz)
6'	7.60, 7.61 (each 1H, dd, *J* = 8.8, 2.4 Hz)	7.57, 7.60 (each 1H, dd, *J* = 8.8, 2.4 Hz)	7.42 (1H, brd, *J* = 8.9 Hz)
3''	6.80 (2H, s)	6.79 (2H, s)	6.77 (1H, s)
6''	6.39 (2H, s)	6.38 (2H, s)	6.33 (1H, s)
2''', 5'''	7.53, 7.54 (each 2H, d, *J* = 8.8 Hz)	7.50, 7.51 (each 2H, d, *J* = 8.9 Hz)	7.60 (2H, d, *J* = 8.9 Hz)
3''', 6'''	6.79, 6.81 (each 2H, d, *J* = 8.8 Hz)	6.77, 6.78 (each 2H, d, *J* = 8.9 Hz)	6.79 (2H, d, *J* = 8.9 Hz)
6-CH_3	1.90 (6H, s)	1.87, 1.88 (each 3H, s)	1.90 (3H, s)
7-OCH_3	3.79 (6H, s)	-	3.79 (3H, s)
7-OH	-	10.34 (2H, brs)[*1]	-
4'-OCH_3	3.72, 3.73 (each 3H, s)	3.70 (6H, s)	-
4'-OH	-	-	10.30 (1H, brs)[*2]
7''-OH	10.71 (2H, s)	10.71 (2H, brs)[*1]	10.30 (1H, brs)[*2]
4'''-OH	10.34 (2H, s)	10.34 (2H, brs)[*1]	10.30 (1H, brs)[*2]
5-OH	12.21, 12.22 (each 1H, s)	12.40 (2H, s)	12.22 (1H, s)
5''-OH	13.07, 13.08 (each 1H, s)	13.05, 13.06 (each 1H, s)	13.06 (1H, s)

[*1, *2] may be exchanged within the same column.

Table 2. ^{13}C-NMR Spectrum Data (DMSO-d_6) of Compounds **1**-**3**.

position	**1**	**2**	**3**
2	78.39, 78.49	78.19, 78.23	78.8
3	41.99, 42.45	42.15, 42.60	42.4
4	196.45, 196.49	196.17, 196.21	197.1
5	159.09	160.71	159.4
6	103.91, 103.92	103.25, 103.27	104.0
7	164.79	164.47, 164.50	165.1
8	90.89, 90.96	94.23	91.1
9	160.87	160.32, 160.35[*3]	161.3
10	102.00, 102.02	101.47	102.0
1’	129.69, 129.90	130.19, 130.41	128.4[*4]
2’	130.77, 131.24	130.99, 131.04	127.5[*4]
3’	120.57, 120.60	120.64, 120.81	119.3
4’	157.27, 157.31	157.46, 157.52	156.2
5’	110.83, 110.88	110.99, 110.20	115.9
6’	127.63, 127.74	127.64, 127.97	128.4[*4]
2’’	163.06	163.46, 163.48	163.4
3’’	102.17	102.35, 102.41	102.3
4’’	181.64	182.02	182.0
5’’	160.01	160.32[*3]	160.2
6’’	98.30, 98.33	98.46, 98.49	98.8
7’’	161.36, 161.40	161.63, 161.66	161.4
8’’	104.19, 104.21	104.37	105.0
9’’	153.84, 153.87	154.17, 154.18	154.4
10’’	103.33	103.5	103.3
1’’’	120.94, 120.96	121.2	121.0
2’’’, 5’’’	127.82, 127.89	128.04, 128.06	128.2
3’’’, 6’’’	115.59	115.77, 115.78	115.7
4’’’	160.74	161.05	161.0
6-CH_3	6.93	6.89	6.9
7-OCH_3	55.95, 55.97	-	56.0
4’-OCH_3	55.45, 55.46	55.49	-

[*3, *4] may be exchanged within the same column.

If the same configurational assignment for naringenin were applied to the flavanone moiety of **1**, the C-2 stereogenic center could be determined. The CD spectrum of **1** exhibits a weak positive Cotton effect around 330 nm and a strong negative Cotton effect due to both the *p*-hydroxybenzoyl and *p*-hydroxyphenyl moieties around 290 nm. The spectrum of **1** almost overlaps that of (2*S*)-naringenin.[15]

This means that **1** and (2*S*)-naringenin are homochiral at C-2. This would imply that C-2 of **1** has a 2*S* absolute configuration. On the basis of the above results, the structure of this compound was first determined and named (2*S*)-2,3-dihydro-6-methylginkgetin (**1**).

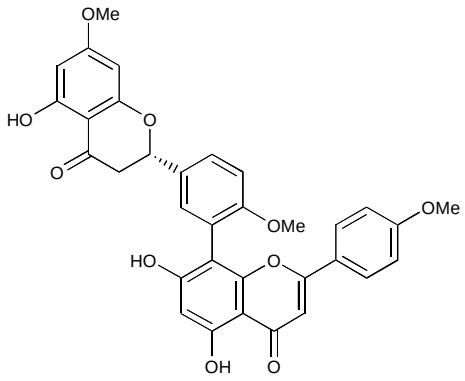

Figure 3. Structure of 2,3-dihydroxysciadopitysin

Compound **2** was obtained as a yellow powder, and the molecular formula of **2** was established to be $C_{32}H_{24}O_{10}$ using the HREIMS spectra data *m/z* 568.1375 [M^+] (calcd 568.1369). The structure of **2** was elucidated using similar methods of spectroscopic analysis as those used for **1**. ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of **1**. The significant differences were the absence of a second methoxyl group and presence of an additional hydroxyl compared to **1**. NMR correlation (Figure 4) and EIMS fragmentations of **2** were similar to **1**. NMR data of compound **2** also exhibited doubling of peaks in the spectra (Table 1 and 2). It was revealed that **2** also existed as a diastereomeric mixture due to an atropiomerism. Its CD spectrum was almost identical to that of (2*S*)-naringenin. The CD spectrum of **2** also overlaps that of (2*S*)-naringenin.[15] On the basis of the above results, this compound was structurally determined and named (2*S*)-2,3-dihydro-6-methylbilobetin (**2**).

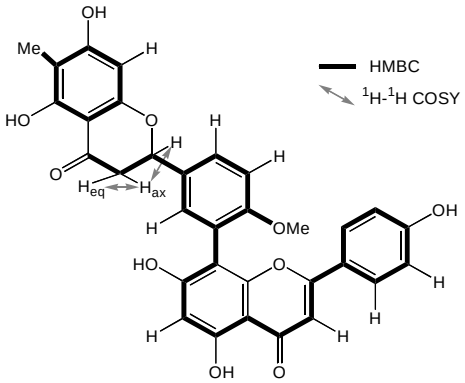

Figure 4. HMBC and ^{1}H-^{1}H COSY of **2**

Compound **3** was obtained as a yellow powder and assigned the molecular formula $C_{32}H_{24}O_{10}$ by the HREIMS spectra data *m/z* 568.1365 [M^+], (calcd 582.1369). The structure of **3** was determined by spectroscopic analysis. NMR spectra of **3** were similar to those of **1**. The significant differences were the absence of a C-4' methoxyl group and presence of an additional hydroxyl compared to **1**. The substitution of the phenyl ring (A-ring) is identical to that of **1**, as proved by the ^{1}H-NMR spectrum. These signals of

3, were different from those of **1** and **2** as they were not doubled unlike for **1** and **2** (Table 1 and 2). ^{1}H-^{1}H COSY and HMBC are summarized in Figure 5. The CD spectrum was almost identical to that of (2*S*)-naringenin. The CD spectrum of **3** also overlaps that of (2*S*)-naringenin.[15] The results of the observed NMR signals and the CD spectrum indicates that there was no atropic isomerism caused by limited rotation along the C-3' and C-8" bond. On the basis of these results **3** was determined as (2*S*)-2,3-dihydro-6-methylsequoiaflavone (**3**).

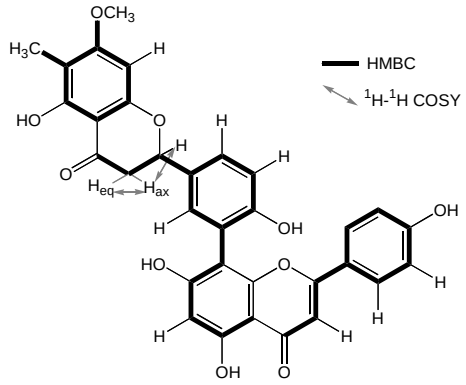

Figure 5. HMBC and ^{1}H-^{1}H COSY of **3**

This is the first report of the isolation of biflavonoids from *C. harringtonia* var. *fastigiata* and the structural determination of new compounds **1** to **3**.

EXPERIMENTAL

General Procedure

Melting points were measured with a Yanagimoto MP micro melting point apparatus. Optical rotation ($[\alpha]_D$) was measured with a HORIBA SEPA-300. IR spectra were measured with a JASCO IR Report-100 infrared spectrometer. UV spectra were measured with a Shimadzu UV-240. MS spectra were measured with a JEOL JMS-700. NMR spectra were measured with a JEOL AL-400 (^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz), or a JEOL JNM-LA500 (^{1}H-NMR: 500 MHz, ^{13}C-NMR: 125 MHz) spectrometer. Circular dichroism (CD) measurement was carried out under dry N_2 on a JASCO-820 spectropolarimeter at 25℃.

Plant material

Leaves of *Cephalotaxus* K. Koch var. *harringtonia* var. *fastigiata* Rehder were collected in April 2005 in Saitama prefecture, Japan. A voucher specimen (2005AP-1) was deposited at The Department of Pharmacognosy and Phytochemistry, Meiji Pharmaceutical University. Species identification was confirmed by Dr. Kiyotaka Koyama.

Extract and Isolation

The dried leaves (147 g) were extracted with acetone at rt (2L×5). Removal of the solvent in vacuo yielded an acetone extract (1.92 g). The acetone extract was chromatographed by silica gel column chromatography (Kanto Kagaku Silica Gel 60 N 63-210 mesh) with a solvent gradient system of $CHCl_3$-MeOH (100:1, 50:1, 30:1, 20:1, 10:1-MeOH) to yield Fr. 1-4. Fr. 1-4 were subjected to Silica gel HPLC (Senshu Pak PEGASIL Silica 60-5, 10ϕ X 250 mm) which column was eluting with $CHCl_3$-MeOH (50:1) to yield compound **1** (30.0 mg) from Fr. 1. Compounds **2** (3.3 mg) and **3** (9.7 mg) were isolated from Fr. 2 in the same manner. Six known biflavonoids were isolated and identified by comparing to the reported spectral data, amentoflavone 7,4',7'',4'''-tetramethyl ether[5] (45.6 mg), amentoflavone 7,4',7''-trimethyl ether[5] (15.2 mg) and sciadopitysin[5] (48.4 mg) from Fr. 1, ginkgetin[5] (271 mg) from Fr. 2, bilobetin[5] (66.3 mg) from Fr. 3 and amentoflavone[16] (2.2 mg) from Fr. 4, respectively.

2,3-Dihydro-6-methylginkgetin (1)

Yellow powder, mp 140-144°C. $[\alpha]_D^{25}$ −35.9 (*c* 0.40, MeOH). IR ν_{max} (KBr) cm^{-1}: 3425 (sh), 1650, 1605, 1580, 1505, 1450, 1365, 1290, 1245, 1200, 1180, 1160, 1130, 1010, 1000, 840. UV (MeOH) λ_{max} nm (log ε): 207 (4.71), 217 (4.68), 285sh (4.49), 331 (4.30). CD (MeOH) λ_{max} nm ($\Delta\varepsilon$): 333 (+5.0), 291 (-11.4), 248 (+1.9), 243 (+1.2). HREIMS *m/z*: 582.1509 (M^+, Calcd for $C_{33}H_{26}O_{10}$, 582.1526). EIMS *m/z* (rel. int. %): 582 (M^+, 100), 551 (20), 403 (32), 402 (24), 396 (9), 389 (9), 376 (13), 377 (5), 371 (5), 181 (6), 152 (7), 121 (10). ^{1}H- and ^{13}C-NMR: see Table 1 and 2.

2,3-Dihydro-6-methylbilobetin (2)

Yellow powder, mp 180-188°C. $[\alpha]_D^{25}$ −34.0 (*c* 0.20, MeOH). IR ν_{max} (KBr) cm^{-1}: 3400 (sh), 1655, 1605, 1560, 1505, 1440, 1360, 1260, 1180, 1160, 1110, 1030, 800. UV (MeOH) λ_{max} nm (log ε): 203 (4.69), 213sh (4.65), 296 (4.37), 329 (4.21). CD (MeOH) λ_{max} nm ($\Delta\varepsilon$): 329 (+2.9), 291 (-10.3), 253 (+1.3), 236 (-0.2). HREIMS *m/z*: 568.1375 (M^+, Calcd for $C_{32}H_{24}O_{10}$, 568.1369). EIMS *m/z* (rel. int. %): 568 (M^+, 100), 537 (35), 403 (24), 402 (24). 389 (9), 376 (18), 371 (7), 284 (6), 251 (5), 167 (6), 138 (6), 121 (12). ^{1}H- and ^{13}C-NMR: see Table 1 and 2.

2,3-Dihydro-6-methylsequoiaflavone (3)

Yellow powder, mp 220-222°C. $[\alpha]_D^{25}$ −34.7 (*c* 0.10, MeOH). IR ν_{max} (KBr) cm^{-1}: 3425 (sh), 1650, 1605, 1580, 1560, 1450, 1360, 1290, 1240, 1180, 1160, 1130, 840. UV (MeOH) λ_{max} nm (log ε): 206 (4.50), 215 (4.49), 289 (4.32), 332sh (4.10). CD (MeOH) λ_{max} nm ($\Delta\varepsilon$): 332 (+3.6), 291 (-10.9), 250 (+0.1), 242 (-0.3). HREIMS *m/z*: 568.1365 (M^+, Calcd for $C_{32}H_{24}O_{10}$, 568.1369). EIMS *m/z* (rel. int. %):

568 (M^+, 100), 551 (20), 550 (50), 390 (11), 389 (44), 388 (19), 375 (20), 371 (35), 370 (41), 362 (14), 357 (6), 252 (16), 226 (5), 207 (7), 181 (18), 180 (8), 152 (13), 121 (20), 109 (7). ^{1}H- and ^{13}C-NMR: see Table 1 and 2.

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