Identification of a New Cytotoxic Biflavanone from *Selaginella doederleinii*

Na-Youn Lee,[a] Hye-Young Min,[a] Jun Lee,[a,b] Joo-Won Nam,[a] Yoo-Jin Lee,[a] Ah-Reum Han,[a] Adam Wiryawan,[c] Wahyu Suprapto,[d] Sang Kook Lee,[a] and Eun-Kyoung Seo*[,a]

[a] *College of Pharmacy and The Center for Cell Signaling & Drug Discovery Research, Ewha Womans University; Seoul 120–750, Korea:* [b] *Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine; Daejeon 305–812, Korea:* [c] *Brawijaya University; Malang 65145, Indonesia: and* [d] *Batu Herba Medica Centre; East Java, Indonesia.* Received May 22, 2008; accepted June 23, 2008; published online June 26, 2008

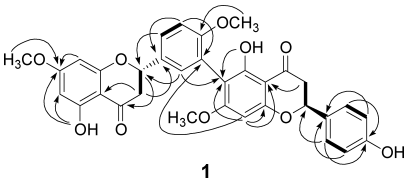
A new biflavanone, 2,2″,3,3″-tetrahydrorobustaflavone 7,4′,7″-trimethyl ether (1) was isolated from the whole plant of *Selaginella doederleinii* Hieron. (Selaginellaceae) together with the known biflavonoid, robustaflavone 7,4′,7″-trimethyl ether (2) as the cytotoxic constituents against the three human cancer cell lines, HCT, NCI-H358, and K562. The structure of the new compound 1 was elucidated by spectral analysis including various 1D- and 2D-NMR experiments.

Key words *Selaginella doederleinii*; Selaginellaceae; biflavanone; cytotoxicity

The genus *Selaginella* is composed of about 700 species and belongs to the family Selaginellaceae. In particular, *Selaginella doederleinii* Hieron. has been used as a traditional Chinese medicine[1] which is a well-known perennial Pteridophyte plant growing in South and Southwestern China at low altitude.[2] It has been used for the treatment of cancer and cardiovascular diseases.[1—3] Several biflavonoids, lignans, and alkaloids have been reported from this plant[4—7] together with some biological activities such as cytotoxicity,[4] anti-human immunodeficiency virus (HIV) activity, inhibition of human DNA polymerase, and anti-inflammatory effects.[4—7] In the present study, a new biflavanoid **1** was isolated from the whole plant of *S. doederleinii* with the known biflavonoid, robustaflavone 7,4′,7″-trimethyl ether (**2**).[8] Compound **2** was isolated from the family Sellaginellaceae for the first time. They were evaluated for their cytotoxic activity together with the known biflavonoid, robustaflavone 4′-methyl ether (**3**) which was previously reported from this plant.

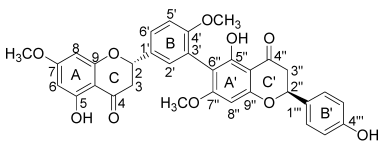
Compound **1** was obtained as pale yellow powder and showed a molecular ion peak at $[M+H]^+$ *m/z* 585.1755 (Calcd 585.1753) in the high-resolution ESI mass spectrometry, which was consistent with the molecular formula of $C_{33}H_{28}O_{10}$. The ^{1}H-NMR spectrum of **1** showed two downfield signals at δ_H 12.04 and 12.10, indicating the presence of hydrogen-bonded hydroxyls. Two sets of ABX signals at δ_H 2.79 (1H, dd, J=17.0, 2.6 Hz), 3.17 (1H, dd, J=17.0, 13.0 Hz), 5.43 (1H, dd, J=13.0, 2.6 Hz)/2.85 (1H, dd, J=17.0, 2.6 Hz), 3.12 (1H, dd, J=17.0, 2.6 Hz), 5.43 (1H, dd, J=13.0, 2.6 Hz) were corresponding to the protons, H-2, H-3/ H-2″, H-3″ of two flavanone moieties, respectively. Three methoxyl protons at δ_H 3.81 (3H, s), 3.80 (3H, s), and 3.75 (3H, s) were assigned at C-4′, C-7, and C-7″, respectively, according to the three-bond heteronuclear multiple bond connectivity (HMBC) connectivities with the carbon signals at δc 158.2, 170.0, and 165.4, respectively. Two *meta*-coupled aromatic proton signals at H-6 and H-8 appeared at δ_H 6.06 and 6.05 (J=2.6 Hz). The A_2X_2 coupling system was established from the signals at δ_H 6.90 (2H, d, J=8.2 Hz, H-3‴, 5‴) and 7.36 (2H, d, J=8.2 Hz, H-2‴, 6‴). The HMBC correlations of C-6″/OH-5″, H-2′, H-8″ and C-3′/H-5′, H-8″ indicated that **1** had an interflavonoid linkage between C-3′ and C-6″ corresponding to its biflavonoid skeleton of robustaflavone series.[1,4,8] The ^{1}H- and ^{13}C-NMR signals of **1** were assigned unambiguously by further detailed analysis of the ^{1}H–^{13}C HSQC, ^{1}H–^{13}C HMBC, and ^{1}H–^{1}H correlative spectroscopy (COSY) spectra as shown in Fig. 1.

To resolve the absolute configuration at C-2 and C-2″ of **1**, the circular dichroism (CD) experiment was performed. The

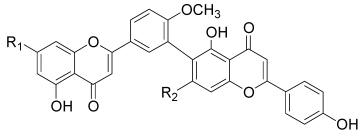


1

Fig. 1. Selected Correlations Observed in the HMBC (→) and COSY (—) NMR Spectra of **1**



1



2 R_1 = OCH_3, R_2 = OCH_3
3 R_1 = OH, R_2 = OH

Table 1. The Cytotoxicity of Isolates from *S. doederleinii*[a)]

Compounds	Cell lines[b)]		
	HCT116	NCI-H358	K562
1	19.1	23.5	28.8
2	15.6	20.1	22.5
3	>100	>100	>100
Ellipticin[c)]	1.0	1.6	1.2

a) Results are expressed as EC_{50} values in μM. *b*) Cell lines: HCT=colorectal carcinoma; NCI-H358=bronchioalveolar carcinoma (non-small cell lung cancer cells); K562=chronic myelogenous leukemia. *c*) Ellipticine was used as a positive control.

* To whom correspondence should be addressed. e-mail: yuny@ewha.ac.kr

CD spectrum of **1** exhibited a positive cotton effect at 328 nm and a negative cotton effect at 290 nm, respectively, indicating the typical "*S*" configuration at C-2 in flavanone skeleton.[9] Therefore, the structure of **1** was determined as a new biflavanone, 2,2″,3,3″-tetrahydrorobustaflavone 7,4′,7″-trimethyl ether. Compounds **2** and **3** were identified as the known biflavonoids, robustaflavone 7,4′,7″-trimethyl ether[8] and robustaflavone 4′-methyl ether,[1,4] respectively.

Compounds **1** (IC_{50} 19.1, 23.5, 28.8 μM) and **2** (IC_{50} 15.6, 20.1, 22.5 μM) exhibited cytotoxic activity against the three human cancer cell lines, HCT116, NCI-H358, and K562, whereas compound **3** did not show activity. Compounds **1** and **2** have methoxyls at C-7 and C-7″ in their flavonoid skeleton, whereas compound **3** has hydroxyls at C-7 and C-7″, indicating the methoxyls at C-7 and C-7″ seem to be important for their cytotoxic activity, although further studies are needed.

Experimental

General Optical rotations were measured with a P-1010 polarimeter (JASCO, Japan) at 25 °C. UV spectra (λ_{max}) were recorded on a U-3000 spectrophotometer (Hitachi, Japan). IR spectra (ν_{max}) were determined on a FTS 135 FT-IR spectrometer (Bio-Rad, CA, U.S.A.). Circular dichroism measurements were performed using JASCO J-715 CD/ORD spectropolarimeter. The 1D and 2D NMR experiments were conducted on a UNITY INOVA 400 MHz FT-NMR (varian, CA, U.S.A.), and TMS was used as an internal standard. ESI-MS, HR-ESI-MS and FAB-MS were obtained on a JMS 700 Mastation HR-MS spectrometer (JEOL, Japan). TLC analysis was performed on Kieselgel 60 F_{254} (Merck, Germany) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by dipping plates into 10% (v/v) H_2SO_4 reagent (Aldrich) followed by charring at 110 °C for 5—10 min. Silica gel (230—400 mesh, Merck, Germany), and RP-18 (YMCGEL ODS-A, 12 nm, S-150 μm) were used for column chromatography. All solvents used for chromatographic separations were distilled before use.

Plant Material The whole plants of *S. doedeleinii* Hieron. were collected at Batu Medica Centre, East Java, Indonesia, in May 2005 and were identified by Prof. Adam Wiryawan (Brawijaya University, Malang 65145, Indonesia). A voucher specimen has been deposited at the Batu Herba Medica Centre.

Extraction and Isolation The dried and milled plant material (335 g) was extracted with MeOH (3×8 l) by maceration. The MeOH extracts (25 g) were separated by liquid column chromatograph [(glass column (6.4×22 cm); silica gel (230—400 mesh, 360 g)] using gradient mixtures of *n*-hexane–EtOAc–MeOH gradients (from *n*-hexane–EtOAc=49 : 1 to 1 : 1, *n*-hexane–EtOAc–MeOH=1 : 1 : 0 to 0 : 0 : 1) as mobile phases to give 13 fractions (FI—FXIII). Fraction FXII (7.5 g, *n*-hexane–EtOAc–MeOH=1 : 1 : 5) was subjected to Sephadex LH-20 column chromatography using 100% MeOH as a solvent system (F01—F05). The F01 fraction (15 mg) was chromatographed over reverse-phase column (ϕ 2.2 cm; ODS-A, 12 nm, S-150 μm, 40 g) with MeOH–H_2O gradient (from 6 : 4 to 8 : 2 v/v), giving **1** (2.1 mg, 0.00039% w/w). F02 (90.1 mg) was eluted with MeOH–H_2O (ϕ 2.2 cm; ODS-A, 12 nm, S-150 μm, 40 g) by reverse-phase column chromatography, providing 5 fractions (F0201—F0205). Subfraction, F0205 (30.5 mg) was further separated using a preparative TLC ($CHCl_3$–MeOH=49 : 1) to afford compound **2** (3 mg, 0.00056%, *Rf* 0.25). F05 fraction (1.1 g) was further fractionated by flash silica gel column chromatography (ϕ 2.8 cm; 230—400 mesh, 70 g) using $CHCl_3$–MeOH (gradient from 49 : 1 to MeOH 100% v/v) as eluents, affording **3** (14.4 mg, 0.0026% w/w).

2,2″,3,3″-Tetrahydrorobustaflavone 7,4′,7″-Trimethyl Ether (**1**): A pale yellow powder; $[\alpha]_D^{25}$ −16.4° (c=0.17, $CHCl_3$); CD (c=2.0×10^{-3} mM, $CHCl_3$) $\Delta\varepsilon_{289}$ −6.70, $\Delta\varepsilon_{327}$ +2.40; IR (KBr) cm^{-1}: 2930, 1735, 1639, 1574, 1455, 1204, 1110, 1028, 816, 758; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 12.10 (OH-5″), 12.04 (OH-5), 7.44 (1H, dd, J=8.6, 2.6 Hz, H-6′), 7.36 (2H, d, J=8.2 Hz, H-2‴, 6‴), 7.28 (1H, d, J=2.6 Hz, H-2′), 7.04 (1H, d, J=8.6 Hz, H-5′), 6.90 (2H, d, J=8.2 Hz, H-3‴, 5‴), 6.18 (1H, s, H-8″), 6.06 (1H, d, J=2.6 Hz, H-6), 6.05 (1H, d, J=2.6 Hz, H-8), 5.43 (1H, dd, J=13.0, 2.6 Hz, H-2β), 5.41 (1H, dd, J=13.0, 2.6 Hz, H-2″β), 3.81 (3H, s, OC$\underline{H}_3$-4′), 3.80 (3H, s, OC$\underline{H}_3$-7), 3.75 (3H, s, OC$\underline{H}_3$-7″), 3.17 (1H, dd, J=17.0, 13.0 Hz, H-3α), 3.12 (1H, dd, J=17.0, 2.6 Hz, H-3″α), 2.85 (1H, dd, J=17.0, 2.6 Hz, H-3″β), 2.79 (1H, dd, J=17.0, 2.6 Hz, H-3β); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 196.3 (C-4″), 196.1 (C-4), 170.0 (C-7), 165.4 (C-7″), 164.2 (C-5), 163.1 (C-9), 163.0 (C-9″), 160.5 (C-5″), 158.2 (C-4′), 156.3 (C-4‴), 130.8 (C-2′), 130.6 (C-1‴), 130.0 (C-1′), 128.0 (C-2‴, 6‴), 127.2 (C-6′), 121.9 (C-3′), 115.8 (C-3‴, 5‴), 111.4 (C-5′), 107.5 (C-6″), 103.2 (C-10), 103.0 (C-10″), 95.1 (C-6), 94.3 (C-8), 91.4 (C-8″), 79.3 (C-2″), 79.1 (C-2), 56.1 (O$\underline{C}H_3$-4′), 56.0 (O$\underline{C}H_3$-7), 55.7 (O$\underline{C}H_3$-7″), 43.4 (C-2, 3), 43.2 (C-2″, 3″); HMBC correlation: OH-5/C-5, C-6, C-7, C-10; OH-5″/C-5″, C-6″, C-10″; H-2β/C-4, C-3, C-1′, C-6′; H-3/C-4, C-10, C-1′; H-6/C-5, C-7, C8, C-10; H-8/C-6, C-8, C-9, C-10; H-2/C-2, C-4′, C-2′; H-5′/C-1′, C-4′, C-3′; H-6′/C-1′, C-6′, C-4′, C-6″; H-2″/C-4″; H-8″/C-6″, C-7″, C-9″, C-10″; H-2‴ and 6‴/C-3‴, C-4‴, C-5‴; H-3‴ and 5‴/C-1‴, C-4‴; OC$\underline{H}_3$-7/C-7; OC$\underline{H}_3$-4′/C-4′; OC$\underline{H}_3$-7″/C-7″; NOESY correlation: OC$\underline{H}_3$-7/H-6; OC$\underline{H}_3$-4′/H-5′; OC$\underline{H}_3$-7″/H-8″; ESI-MS *m/z* (% rel. int.) 585 $[M+H]^+$ (100), 580 (44), 546 (90), 544 (65), 522 (40), 507 (83), 503 (90); HR-ESI-MS *m/z* 585.1753 $[M+H]^+$ (Calcd for $C_{33}H_{28}O_{10}$: 585.1755).

Robustaflavone 7,4′,7″-Trimethyl Ether (**2**): Yellow powder; UV (MeOH) λ_{max} (log ε) 341.0 nm (4.34), 269.0 nm (4.28); IR (KBr) cm^{-1} 2894, 1649, 1503, 1349, 1117, 1037, 804; ESI-MS *m/z* (%) 581 $[M+H]^+$ (70), 575 (60), 573 (55); ^{1}H-NMR data was in agreement with reported values.[8]

Robustaflavone 4′-Methyl Ether (**3**): Yellow powder; UV (MeOH) λ_{max} (log ε) 340.0 nm (4.38), 270.0 nm (4.47); IR (KBr) cm^{-1} 2873, 1649, 1557, 1539, 1359, 1168, 1022; FAB-MS *m/z* (% rel. int.) 575 $[M+Na]^+$ (2), 553 (3), 530 (1), 521 (1), 491 (1); ^{1}H- and ^{13}C-NMR data were in agreement with reported values.[1,4]

***In Vitro* Cytotoxicity Assay** Cytotoxic potential was determined as described previously.[11] Briefly, cells (in log growth phase) were counted, diluted to 5×10^4 cells/ml with fresh medium, and added to 96-well microtiter plates (190 μl/well) containing test materials (10 μl in 10% aqueous DMSO). Test plates were incubated for 3 d at 37 °C in CO_2 incubator. All treatments were performed in triplicate. After the incubation periods, cells were fixed by the addition of 50 μM of cold 50% aqueous trichloroacetic acid (4 °C for 30 min), washed 4—5 times with tap water, and air-dried. The fixed cells were stained with sulforhodamine B (SRB) (0.4% w/w SRB in 1% aqueous acetic acid) for 30 min. Free SRB solution was then air-dried, the bound dye was solubilized with 200 μl of 10 mM tris-base (pH 10.0), and absorbance was determined at 515 nm using an ELISA plate reader. Finally, the absorbance values obtained with each of the treatment procedures were averaged, and the averaged value obtained with the zero day control was subtracted. These results were expressed as a percentage, relative to solvent-treated control incubations, and EC_{50} values were calculated using non-linear regression analyses (percent survival *versus* concentration). Ellipticine was used as a positive control.

Acknowledgments This work was supported by a grant from the Brain Korea 21 Project and by grant No. R15-2006-020 from the National Core Research Center (NCRC) program of the Ministry of Education, Science and Technology (MEST) and the Korea Science and Engineering Foundation (KOSEF) through the Center for Cell Signalling & Drug Discovery Research at Ewha Womans University.

References

1) Lu Y., Chen Y., Wen J., *Yunnan Zhiwu Yanjiu*, **26**, 226—228 (2004).
2) Lin R. C., Skaltsounis A.-L., Seguin E., Tillequin F., Koch M., *Planta Med.*, **60**, 168—170 (1994).
3) Chao L. R., Seguin E., Tillequin F., *J. Nat. Prod.*, **50**, 422—426 (1987).
4) Lin L.-C., Kuo Y.-C, Chou C.-J., *J. Nat. Prod.*, **63**, 627—630 (2000).
5) Ma S.-C., But P. P.-H., Ooi V. E.-C., *Biol. Pharm. Bull.*, **24**, 311—312 (2001).
6) Liu R. C., Peyroux J., Seguin E., Koch M., *Phytother. Res.*, **5**, 188—190 (1991).
7) Pan K. Y., Lin J. L., Chen J. S., *J. Toxicol. Clin. Toxicol.*, **39**, 637—639 (2001).
8) Gu Y., Xu Y., Fang S., He Q., *Zhiwu Xuebao*, **32**, 631—636 (1990).
9) Gaffield W., *Tetrahedron*, **26**, 4093—4108 (1970).
10) Lee S. K., Cui B., Mehta R. R., Kinghorn A. D., Pezzuto J. M., *Chem. Biol. Interact.*, **115**, 215—228 (1998).