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Identification of a New Biflavonoid from *Selaginella doederleinii* Hieron

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A higher plant, *Selaginella doederleinii* Hieron (Selaginellaceae) has been used as a traditional herbal medicine to treat cancer and cardiovascular disease in Southeast Asia.[1,2] Previous phytochemical reports on this plant are including several biflavonoids, lignans, and alkaloids,[1-3,6,7] together with biological activities such as cytotoxic,[2,3] anti-viral,[6] and hypertensive effects.[7] In the present phytochemical study on *S. doederleinii*, a new biflavonoid, 2,3-dihydrorobustaflavone 7,7"-dimethyl ether (**1**) was isolated along with two known compounds, 2,3-dihydrorobustaflavone 7,4',7"-trimethyl ether (**2**) and 2",3"-dihydrorobustaflavone 7,4',7"-trimethyl ether (**3**), which were found in this plant for the first time (Figure 1). The structure of **1** was elucidated by physical and spectroscopic data analysis.

Compound **1** gave a molecular ion peak $[M+Na]^+$ at *m/z* 591.1286 in the HRESIMS, corresponding to an elemental formula of $C_{32}H_{24}O_{10}Na$. The absorption bands at 3376 and 1644 cm^{-1} in the IR spectrum of **1** suggested the presence of hydroxyl and conjugated carbonyl functionalities, respectively.[8] The UV spectrum exhibited absorption maxima at 333 and 285 nm, indicating the presence of aromatic ring system(s).[8] The ^{1}H-NMR spectrum of **1** showed a hydrogen-bonded hydroxyl(s) proton at δ_H 12.14 (2H, weak br s, OH).[3] The ^{1}H- and ^{13}C NMR spectra of **1** indicated the presence of a flavanone skelecton as in **1**, which showed characteristic signals at δ_H 5.51 (1H, dd, *J* = 12.8, 2.8 Hz, H-2)/δ_c 78.6 (C-2) and 3.33 (1H, H-3a) and 2.75 (1H, dd, *J* = 17.2, 2.8 Hz, H-3b)/41.9 (C-3),[9] together with signals for aromatic systems. In the ^{1}H-NMR spectrum of **1**, signals for A_2X_2 coupling system at δ_H 7.96 (2H, d, *J* = 8.6 Hz, H-2"' and 6"') and 6.90 (2H, d, *J* = 8.6 Hz, H-3"' and 5"'), signals for 1,3,4-trisubstituted aromatic ring system at δ_H 7.32 (1H, dd, *J* = 8.4, 2.2 Hz H-2'), 7.21 (1H, d, *J* = 2.2 Hz, H-6'), and 6.90 (1H, d, *J* = 8.4 Hz, H-3'), an aromatic singlet at δ_H 6.88 (1H, s, H-8"), and two *meta*-coupled aromatic protons at δ_H 6.12 (1H, d, *J* = 2.2 Hz, H-6) and 6.07 (1H, d, *J* = 2.2 Hz, H-8), were observed. The above data suggested that there were two sets of flavonoid, 5,7,3,4'-tetrasubstituted flavanone and 5",6",7",4"'-tetrasubstituted flavone, supported by the following HMBC correlations: H-2/C-4, C-6', H-3/C-2, C-2', H-6/C-10, H-8/C-6, C-9, H-3"/C-2", C-4", -10", C-1"', H-2"' and H-6"'/C-2", and H-3"' and H-5"'/C-1"' (Figure 2). They are connected by a C-3'–C-6" interflavonoid linkage, as evidenced by the HMBC cross-peaks of H-2'/C-6" and H-8"/C-6". Two methoxy protons at δ_H 3.78 (3H, s) and 3.81 (3H, s) were assigned at C-7 and C-7", respectively, according to the HMBC correlations with carbon signals at δ_C 167.4 (C-7) and 162.8 (C-7"), respectively. This was also supported by the NOESY correlations of OC<u>H</u>$_3$-7/H-6 and H-8 and OC<u>H</u>$_3$-7"/H-8". Further detailed analysis of ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, HSQC and HMBC NMR data allowed unambiguous assignments for all of the ^{1}H and ^{13}C NMR signals of **1**. Therefore, the structure of **1** corresponded to the robustaflavone series[10] and determined as a new compound, 2,3-dihydrorobustaflavone 7,7"-dimethyl ether. The circular dichroism (CD) experiment was performed to determine an absolute configuration at C-2 of **1**. However, the CD spectrum of **1** exhibited a flat CD band without a positive or negative cotton effect, indicating the presence of a racemic mixture, but not in a 1:1 mixture due to the slightly positive specific rotation observed.

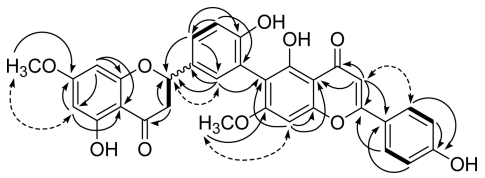
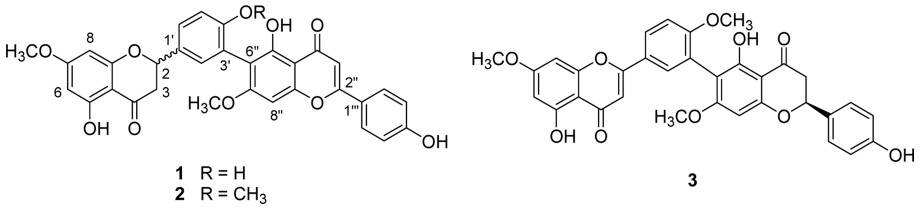


Figure 2. Selected ^{1}H-^{1}H COSY (▬), ^{1}H-^{1}H NOESY (◂⋯▸), and HMBC (→) correlations of compound **1**.



1 R = H
2 R = CH_3

3

Figure 1. Structures of the isolates **1-3** from the whole plants of *S. doedeleinii*.

Compounds **2** and **3** were identified as 2,3-dihydrorobustaflavone 7,4',7''-trimethyl ether (**2**)[11] and 2'',3''-dihydrorobustaflavone 7,4',7''-trimethyl ether (**3**),[12] respectively, by analyses of their physical and spectroscopic data as well as by comparison of their data with the published values. Compound **2** was optically active ($[\alpha]_D^{25}$: –10.60), however, the absolute configuration at C-2 could not be determined due to the flat CD band shown in the CD spectrum of **2**. This observation indicated that **2** present as a racemic mixture. The configuration at C-2'' of **3** was determined as *S*, due to its CD spectrum (a positive cotton effect at 333 nm and a negative cotton effect at 287 nm).[13] To the best of our knowledge, **2** and **3** were isolated from *S. doederleinii* for the first time.

Experimental Section

General Experimental Procedures. 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). 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, USA), and TMS was used as an internal standard. ESIMS and HRESIMS were obtained on a JMS 700 Mastation HRMS spectrometer (JEOL, Japan). Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} and RP-18 F_{254s} silica gel plates (Merck, Germany) with visualization under UV light (254 and 365 nm) and 10% (v/v) sulfuric acid spray followed by heating (120 °C, 5 min). Silica gel (230-400 mesh, Merck, Germany), YMC*GEL ODS-A (S-150 μm, YMC Co., Ltd., Japan), and Sephadex LH-20 (Amersham Pharmacia Biotech, U.K.) were used for column chromatography.

Plant Material. The whole plants of *S. doedeleinii* were collected in 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 whole plants of *S. doedeleinii* (335 g) was extracted with 95% MeOH (3 × 8 L) overnight at room temperature. The solvent was evaporated *in vacuo* to afford a MeOH extract (25 g), which was subjected to silica gel column chromatography (CC) using gradient mixtures of hexanes-EtOAc (49:1 → 0:1) to give 13 fractions (FI-FXIII). Fraction FXII (7.4 g) was chromatographed on Sephadex LH-20 (100% MeOH), providing five sub-fractions (FXII-1 – FXII-5). The sub-fraction FXII-1 (15 mg) was subjected to reversed-phase CC eluted with gradient mixtures of MeOH-H_2O (3:2 → 4:1), yielding **3** (1.8 mg). The sub-fraction FXII-2 (90 mg) was separated by reversed-phase CC eluted with gradient mixtures of MeOH-H_2O (4:1 → 2:1), affording five sub-fractions (FXII-2-1 – FXII-2-5). The sub-fraction FXII-2-5 (30 mg) was carried out preparative TLC ($CHCl_3$-MeOH = 49:1) to afford **2** (2.0 mg). The combined fractions of FXII-3 and FXII-4 (300 mg) were chromatographed over silica gel eluted with gradient mixtures of $CHCl_3$-MeOH (99:1 → 1:1), yielding eight sub-fractions (FXII-3-1 – FXII-3-8). FXII-3-3 (24 mg) was purified by preparative TLC using a solvent system of $CHCl_3$-MeOH (49:1) to provide **1** (1.5 mg).

2,3-Dihydrorobustaflavone 7,7''-dimethyl ether (1): Yellow powder. $[\alpha]_D^{25}$: +13.00 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 333 (4.6), 285 (4.7) nm; IR (KBr) ν_{max} cm^{-1}: 3376, 1644, 1604, 1450, 1349, 1202, 1156; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 12.14 (2H, s, OH), 7.96 (2H, d, *J* = 8.6 Hz, H-2''' and 6'''), 7.32 (1H, dd, *J* = 8.4, 2.2 Hz H-6'), 7.21 (1H, d, *J* = 2.2 Hz, H-2'), 6.90 (1H, d, *J* = 8.4 Hz, H-5'), 6.90 (2H, d, *J* = 8.6 Hz, H-3''' and 5'''), 6.88 (1H, s, H-8''), 6.80 (1H, s, H-3''), 6.12 (1H, d, *J* = 2.2 Hz, H-6), 6.07 (1H, d, *J* = 2.2 Hz, H-8), 5.51 (1H, dd, *J* = 12.8, 2.8 Hz, H-2), 3.81 (3H, s, OC<u>H</u>$_3$-7''), 3.78 (3H, s, OC<u>H</u>$_3$-7), 3.33(1H, H-3), 2.75 (1H, dd, *J* = 17.2, 2.8 Hz, H-3); ^{13}C-NMR (DMSO-d_6, 100 MHz) δ 197.0 (C-4), 181.6 (C-4''), 167.4 (C-7), 163.8 (C-2''), 163.2 (C-9), 162.9 (C-5), 162.8 (C-7''), 162.5 (C-4'''), 156.8 (C-9''), 156.2 (C-4'), 131.1 (C-6'), 128.4 (C-2''' and 6'''), 128.0 (C-2'), 127.2 (C-1'), 120.1 (C-5' and 1'''), 116.2 (C-3', 5''', and 3'''), 110.3 (C-6''), 104.9 (C-10''), 102.8 (C-10), 102.6 (C-3''), 94.6 (C-8), 93.7 (C-6), 90.1 (C-8''), 78.6 (C-2), 56.1 (O<u>C</u>H$_3$-7''), 55.8 (O<u>C</u>H$_3$-7), 41.9 (C-3); HRESIMS *m/z* = 591.1286 $[M+Na]^+$ (calcd for $C_{32}H_{24}O_{10}Na$: 591.1261).

2,3-Dihydrorobustaflavone 7,4',7''-Trimethyl Ether (2): $[\alpha]_D^{25}$: –10.60 (*c* 0.15, MeOH; literature values: –13.3, *c* 0.75, dioxane[11]).

2'',3''-Dihydrorobustaflavone 7,4',7''-Trimethyl Ether (3): $[\alpha]_D^{25}$: –5.55 (*c* 0.18, MeOH; literature values: –2.3, *c* 0.44, dioxane[12]); CD (*c* 2.0 × 10^{-3} mM, MeOH) $\Delta\varepsilon_{287}$ –0.48, $\Delta\varepsilon_{333}$ +0.22 nm.

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Supporting Information. The spectral data of compound **1** are available on request from the correspondence author.

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