A New Optical Biflavonoid, (2''R)-2'',3''-Dihydrorobustaflavone 7,4'-Dimethyl Ether, and Other Constituents from Selaginella trichoclada Alsto

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Abstract
A new optical biflavonoid, (2''R)-2'',3''-dihydrorobustaflavone 7,4'-dimethyl ether (1), and 6 known compounds (2-7) were isolated for the first time from the 70% ethanol extract of Selaginella trichoclada Alsto. The structures of the compounds were confirmed by extensive spectroscopic data analyses. Racemic compound 1 was separated by chiral-phase high-performance liquid chromatography, and the absolute configurations of (±)-1 were defined by circular dichroism spectroscopic data. Compound 1 exhibited moderate cytotoxicity against MCF-7, A549, and HepG2 human cancer cell lines.

Keywords
Selaginella trichoclada, robustaflavone, enantiomers

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The genus Selaginella (Selaginellaceae) is composed of approximately 70 species, many of which are distributed in China where many have been used for treating bleeding, jaundice, gonorrhea, and idiopathic thrombocytopenic purpura in traditional Chinese medicine.¹ The genus is known as a rich source of secondary metabolites including flavonoids,² selaginellin,³ alkaloids,⁴ lignans,⁵ and steroids,⁶ which have been reported to exhibit diverse pharmacological activities, such as antitumor,⁷,⁸ antibiosis,⁹,¹⁰ antiviral,¹¹ and antioxidant.¹² S. trichoclada Alsto, commonly known as “boyundan, yanbaicao and tubozi” in folk medicine, is mainly distributed in the southern area of China, where it is used widely as a herbal medicine for clearing heat and promoting diuresis and relieving cough.¹³ As a part of a systematic search for bioactive natural products from the 70% EtOH extracts of S. trichoclada, we report here the isolation and structural elucidation of (±)-2'',3''-dihydrorobustaflavone 7,4'-dimethyl ether (1) and 6 known compounds (2-7), as shown in Figure 1. The structures of these compounds were identified on the basis of extensive spectroscopic data, including UV-vis, infrared (IR), 1-dimensional (1D)-2D nuclear magnetic resonance (NMR), high-resolution electrospray ionization mass spectra (HRESIMS), and circular dichroism (CD). Compound 1 was a diastereoisomeric pair of enantiomers due to the presence of a chiral center at C-2'', but after being subjected to chiral-phase high-performance liquid chromatography (HPLC) analysis, finally yielded (±)-1. It is rare to obtain stereoisomers of a biflavonoid from the genus Selaginella. Compound 5 was isolated from the genus Selaginella for the first time. The cytotoxicity of compounds 1 to 7 was evaluated against 3 human cancer cell lines (MCF-7, A549, and HepG2).

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signals ($\delta_{C} = 196.8$ and $182.4$). The $^1$H NMR and $^1$H-$^1$H correlation spectroscopy (COSY) data (supplemental material Figures S5 and S7) showed the characteristic resonances at $\delta_{C} \approx 8.11$ (1H, dd, $J = 1.9$, 8.7 Hz, H-6'), $\delta_{C} \approx 7.82$ (1H, d, $J = 1.9$ Hz, H-2'), and $\delta_{C} \approx 7.22$ (1H, d, $J = 8.7$ Hz, H-5') for an ABX coupling system. The flavanone II B-rings units of compound 1 showed A$_2$X$_2$ coupling system signals at $\delta_{C} \approx 7.35$ (2H, dd, $J = 2.9$, 8.1 Hz, H-2''/H-6'') and $\delta_{C} \approx 6.82$ (2H, dd, $J = 2.9$, 8.1 Hz, H-3''/H-5''). Moreover, 2 meta-coupled proton resonances appeared at $\delta_{C} \approx 6.37$ (1H, m, H-6) and $\delta_{C} \approx 6.87$ (1H, m, H-8), and 1 aromatic proton signal of the flavanone II A-ring units at $\delta_{C} \approx 6.02$ (1H, s, H-8''). The key correlations of H-8'' to C-6'', C-7'', and C-9'', of H-2' to C-3' and C-6'', and of H-5' to C-3' in the heteronuclear multiple bond correlation (HMBC) spectrum (Figure 2) indicated that the interlinkage position between the flavanone unit I B-rings and the flavanone unit II A-ring of compound 1 was linked between C-3' and C-6'', corresponding to the robustaflavone series. Two methoxyl groups were located at C-7 and C-4', respectively, based on distortions enhancement by polarization transfer (DEPT), heteronuclear single quantum correlation (HSQC), and HMBCs (supplemental material Figures S8–S10). In the 1D-NMR spectrum, it is remarkable that a hindered rotation isomerism phenomenon of some signals was observed, such as H-3 ($\delta_{C} \approx 6.96$, 6.97), H-6 ($\delta_{C} \approx 6.36$, 6.37, 6.38), H-8 ($\delta_{C} \approx 6.86$, 6.87, 6.88), H-2'' ($\delta_{C} \approx 5.44$, 5.51), OCH$_3$-7 ($\delta_{C} \approx 3.86$, 3.87), OCH$_3$-4' ($\delta_{C} \approx 3.80$, 3.81), C-2'' ($\delta_{C} \approx 79.2$, 79.4, 79.6), C-6'' ($\delta_{C} \approx 105.2$, 105.3), C-8'' ($\delta_{C} \approx 93.1$, 93.2), C-2''/6'' ($\delta_{C} \approx 128.7$, 128.8), and C-3''/5'' ($\delta_{C} \approx 115.5$, 115.7), due to the chirality of C-2''. The lack of optical activity and Cotton effect in the CD spectrum indicated that 1 was a racemic mixture. Compound 1 was successfully resolved by HPLC using a CHIRALPAK AD-H column to afford 2 enantiomers of (+)-1 ($\left[\alpha\right]_{25}^{D} = +25.6$, $t_{R} = 10.18$ minutes) and (−)-1 ($\left[\alpha\right]_{25}^{D} = +23.5$, $t_{R} = 12.07$ minutes). Compound (−)-1 displayed a positive Cotton effect at 289 nm and a negative Cotton effect at 327 nm in the experimental CD data (supplemental material Figure S3), suggesting that the absolute configuration of (−)-1 was assigned as 2''R. The absolute configuration of (+)-1 was defined as 2''S by comparing the CD data of (+)-1 and (−)-1 (supplemental material Figure S3). Thus, the structure of 1 was identified as 2'',3''-dihydrorobustaflavone 7,4'-dimethyl ether; (−)-1 was defined as a new optical compound, (2''R)-2'',3''-dihydrorobustaflavone 7,4'-dimethyl ether.

By analysis of spectroscopic data (UV-vis, IR, 1D/2D NMR, and HRESIMS) and comparison with literature values, the structures of 6 known compounds (3-7) were elucidated as (2''S)-2'',3''-dihydrorobustaflavone 4'-methyl ether, (2''S)-2'',3''-dihydrorobustaflavone, 1H-indole-3-carboxaldehyde, phenazime-1-carboxamide, honokid, and magnolol, respectively.

Additionally, compounds 1 to 7 were tested for their cytotoxic activity against 3 human cancer cell lines, MCF-7, A549, and HepG2 by MTT assay (Table 1). Oxaliplatin was used as the positive control. Compound 1 had a moderate cytotoxic activity against MCF-7, A549, and HepG2 cell lines with half-maximal inhibitory concentration values of 41.6, 35.2, and 37.8 µM, respectively.

**Experimental**

**General Experimental Procedures**

HRESIMS were recorded on an Agilent 1290 UPLC linked with a Q-TOF mass spectrometer. The UV spectra were measured on a UV-2450 spectrometer (SHIMADZU, Kyoto, Japan). NMR spectra, including $^1$H- and $^{13}$C-NMR, $^1$H-$^1$H
COSY, DEPT, HSQC, and HMBC, were recorded on an Avance III 400 MHz Bruker spectrometer (Bruker BioSpin, Rheinstetten, Germany). Silica gel GF-254 (Qingdao Marine Chemical Factory, Qingdao, China) was used for thin-layer chromatography (TLC), and Sephadex LH-20 (TOYOPEARL TOSOH, Tokyo, Japan) for column chromatography (CC). Semipreparative HPLC was accomplished using an Agilent 1200 HPLC system carried out on a preparative YMC Pack ODS-A (10 μm, 250 × 10 mm, YMC Co., Ltd., Kyoto, Japan).

Optical rotations were measured with a Rudolph AUTOPOL IV polarimeter. CD spectra were measured on an Applied Photophysics spectrometer.

**Plant Material**

Whole herbs of *S. trichoclada* were collected from Shaoyang (Hunan province, China) in July 2016, and authenticated by the authors. A voucher specimen was deposited in School of Pharmaceutical Sciences, Hunan University of Medicine.

**Extraction and Isolation**

The air-dried whole herbs of *S. trichoclada* (8.0 kg) were cut into pieces and refluxed in 70% EtOH-H₂O (80 L × 2, 2 hours/each). The extract was concentrated under reduced pressure, and the concentrate (358.6 g) partitioned into H₂O, light petroleum (22.5 g), EtOAc (44.6 g), and n-BuOH (68.2 g) fractions, successively. The EtOAc fraction (44.6 g) was subjected to silica gel CC, eluting with a gradient of Cl₂H₂/MeOH (from 100:0 to 0:100), to obtain 8 fractions A-H (Fr. A-H). Then, Fr. C was separated on Sephadex LH-20 with MeOH/H₂O (60:40, v/v, isocratic elution) to yield 6 subfractions (Fr. C₁-6), based on TLC analyses (Cl₂H₂/MeOH, 15:1, with 2 drops of acetic acid). Fr. C₃ was further purified by semipreparative HPLC, eluting with ACN/H₂O (45:55, v/v, 3.0 mL/min) to yield compounds **1** (1.3 mg), **2** (3.9 mg), and **3** (2.8 mg). By using chiral-phase HPLC (CHIRALPAK AD-H, 5 μm, 4.6 mm × 250 mm, cyclohexane/isopropanol [60:40, v/v], 1.0 mL/min), racemic compound **1** was further resolved into (+)-**1** and (−)-**1**. Fr. C₄ was repeatedly purified by semipreparative HPLC eluting with ACN/H₂O (45:55, v/v, 3.0 mL/min) to obtain compounds **4** (3.1 mg), **5** (3.4 mg), **6** (4.8 mg), and **7** (5.3 mg).

**Table 1. Cytotoxicity of Compounds 1 to 7 Against Human Cancer Cell Lines.**

| Compound | MCF-7  | A549    | HepG2  |
|----------|--------|---------|--------|
| 1        | 41.6 ± 0.1 | 35.2 ± 0.2 | 37.8 ± 0.1 |
| 2        | 52.2 ± 0.7 | 63.5 ± 1.2 | 48.5 ± 0.9 |
| 3        | 58.7 ± 0.3 | 67.1 ± 0.4 | 64.6 ± 1.3 |
| 4        | 68.8 ± 0.3 | 72.1 ± 0.5 | 72.5 ± 0.9 |
| 5        | 69.6 ± 0.5 | 43.5 ± 0.2 | 39.6 ± 0.3 |
| 6        | >100    | >100    | >100   |
| 7        | >100    | >100    | >100   |
| Oxaliplatin | 5.2 ± 0.2 | 6.5 ± 0.1 | 4.3 ± 0.1 |

*Results expressed as the mean half-maximal inhibitory concentration values in μM from triplicate measurements.

**Table 2. ¹H NMR (400 MHz) and ¹³C NMR (100 MHz) Spectroscopic Data of Compound 1 in DMSO-d₆.**

| Position | δH (J in Hz) | δC | Position | δH (J in Hz) | δC |
|----------|--------------|-----|----------|--------------|-----|
| 2        | 164.3        | 2"  | [5.44, 5.51] (1H, dd, 2.7, 12.3) | 79.2, 79.4, 79.6 |
| 3        | [6.96, 6.97 (1H, m)] | 104.1 | 3" | 3.25 (1H, m, H-3") | 42.5 |
| 4        | 182.4        | 4"  | 2.64 (1H, m, H-3") | 196.8 |
| 5        | 13.00 (1H, brs) | 162.3 | 5" | 12.43 (1H, brs) | 161.6 |
| 6        | [6.36, 6.37, 6.38 (1H, m)] | 98.7 | 6" | 9.64 (1H, brs) | (105.2, 105.3) |
| 7        | 165.7        | 7"  | 6.02 (1H, s) | 164.8 |
| 8        | [6.86, 6.87, 6.88 (1H, m)] | 93.4 | 8" | 6.02 (1H, s) | (93.1, 93.2) |
| 9        | 158.2        | 9"  | 101.6 |
| 10       | 104.7        | 10" | 101.6 |
| 1'       | 130.8        | 1"" | 128.5 |
| 2'       | 7.82 (1H, d, 1.9) | 130.9 | 2"/6" | 7.35 (2H, dd, 2.9, 8.1) | (128.7, 128.8) |
| 3'       | 122.6        | 3"/5" | 6.82 (2H, dd, 2.9, 8.1) | (115.5, 115.7) |
| 4'       | 161.3        | 4"  | 157.8 |
| 5'       | 7.22 (1H, d, 8.7) | 112.2 | OCH3-7 | 3.86, 3.87 (3H, m) | 56.6 |
| 6'       | 8.11         | 128.3 | OCH3-4" | 3.80, 3.81 (3H, m) | 56.3 |

DMSO, dimethyl sulfoxide.

*Multiple signals by rotation isomerism due to the presence of chirality at C-2".*
2",3"-Dihydrorobustaflavone 7,4'-Dimethyl Ether (1)

Yellow amorphous powder. (+)-I: [α]D25° +25.6 (c 0.1, MeOH); CD (MeOH) λmax (Δ ε): 289 (-2.6), 327 (+1.0). (-)-I: [α]D25° −23.5 (c 0.1, MeOH); CD (MeOH) λmax (Δ ε): 289 (+3.9), 327 (-1.1).

UV (MeOH) λmax (nm) (log ε): 224 (1.52), 292 (0.78), 331 (0.23). IR νmax (KBr): 3150, 1656, 1499, 1449, 1358, 1295, 1235, 1170, 1125, 836 cm⁻¹. 1H NMR and 13C NMR data in dimethyl sulfoxide (DMSO)-d6. HRESIMS: m/z 569.1441 [M + H]⁺ (calculated for C32H25O10 569.1442).

Cell Cytotoxicity Assay

Compounds 1 to 7 were tested for cytotoxicity against MCF-7 (breast cancer), A549 (lung cancer), and HepG2 (liver cancer) cell lines using the MTT method. The cells were treated with compounds 1 to 7 dissolved in DMSO at various concentrations (1, 4, 16, 64, and 100 µg/mL). Oxaliplatin (5 µg/mL), dissolved in DMSO, was used as the positive control. These cell lines were then cultured in 96-well plates at a cell density of 4000 cells/well. After incubation of the cells with compounds 1 to 7 for 24 hours, 10 µL of MTT (0.5 mg/mL) solution was added to each well and incubated for 4 hours at 37°C. DMSO (100 µL) was then added to each well and incubated for another 10 minutes. The optical density value of each well was measured at 490 nm using an ELISA reader.

Declaration of Conflicting Interests

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