Selective Cytotoxicity of Ginkgetin from *Selaginella moellendorffii*

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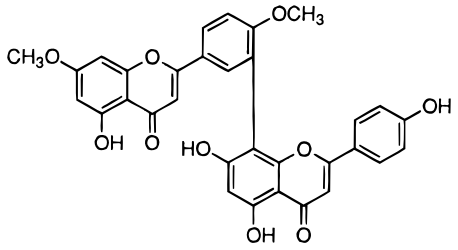
Bioassay-directed fractionation of an ethanolic extract of *Selaginella moellendorffii* has led to the isolation of a known biflavone, ginkgetin (**1**). A dose-dependent inhibition was observed with **1** on the growth of OVCAR-3 (human ovarian adenocarcinoma) cells with 50% inhibition occurring at 1.8 μg/mL. Nonbioactive fractions yielded four additional known biflavones, amentoflavone 7,4′,7″,4‴-tetramethyl ether, kayaflavone, podocarpusflavone A, and amentoflavone.

Selaginella moellendorffii Hieron (Selaginellaceae) has been used to treat jaundice, gonorrhea, bleeding, and acute hepatitis.[1] Some other *Selaginella* species have been reported to exhibit various biological activities. For example, a water extract of *S. tamariscina* has shown antineoplastic activity in a Sarcoma-180A model,[2] and a methanolic extract of *S. doederleinii* demonstrated inhibitory activity against the growth of HeLa cells *in vitro*.[3] In addition, a water extract of *S. doederleinii* was shown to have an antimutagenic effect agaist benzo[α]pyrene-induced mutations in a *Salmonella typhimurium* microsomal system.[4] Moreover, *S. doederleinii* has also manifested antitumor activity using syngeneic Meth A tumors in BALB/c mice;[5] the mechanism of this may be the enhancement of T cell-mediated tumor immunity, particularly the tumor-specific DTH (delayed-type hypersensitivity) response.[6] Furthermore, the crude drug from *S. doederleinii* acted as a priming agent for tumor necrosis factor (TNF) production in mice without accompanying liver and spleen hyperplasia.[7]

In a continuing search for antitumor agents, we have found that a crude ethanolic extract of *S. moellendorffii* possessed an *in vitro* inhibitory effect on the growth of the cancer cell line, OVCAR-3. In this paper, we report that the bioassay-directed fractionation of the ethanolic extract of *S. moellendorffii* has led to the isolation of one cytotoxic and four noncytotoxic biflavones. Only four chemical constituents, isopimpinellin, palmitic acid, stearic acid, and β-sitosterol, have been previously reported from *S. moellendorffii*.[8]

The crude ethanolic extract of *S. moellendorffii* was subjected to chromatography, and fractions were assayed for cytotoxicity against OVCAR-3 cells. The bioactive fractions were rechromatographed to result in the isolation of an active compound identified as ginkgetin (**1**). Purification of the other fractions yielded the compounds amentoflavone 7,4′,7″,4‴-tetramethyl ether, kayaflavone, podocarpusflavone A, and amentoflavone. The structures of the biflavones were elucidated from their ^{1}H- and ^{13}C-NMR spectra with the aid of NOE, ^{1}H-^{13}C COSY, and ^{1}H-^{13}C COLOC experiments and by comparison with existing data from the literature.[9−14] The positions of the methoxy groups of these biflavones were established by NOE experiments. The ^{13}C-NMR chemical shifts at C-2′ and C-6′ of these compounds were determined by ^{1}H-^{13}C COSY NMR experiments, and the interflavonoid linkages were determined by ^{1}H-^{13}C COLOC NMR spectra, indicating that they all have a C-3′,C-8″ linkage.



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Although *Selaginella* species are known to produce biflavones,[10,15−22] the five biflavones reported herein were isolated from *S. moellendorffii* for the first time, while **1**, kayaflavone, and podocarpusflavone A have not yet been previously reported from the genus *Selaginella*. Compound **1** has also been found in several other plant genera.[23−27]

Recently, some biflavones from *Selaginella* species have been reported to show cytotoxicity against a panel of human cancer cell lines.[22] Here, the biflavones from *S. moellendorffii* were evaluated for growth inhibition of OVCAR-3 (ovarian adenocarcinoma) cells. Amentoflavone 7,4′,7″,4‴-tetramethyl ether, kayaflavone, podocarpusflavone A, and amentoflavone gave no apparent inhibition at concentrations up to 10 μg/mL. Moreover, a dose-dependent inhibition of the growth of OVCAR-3 cells was observed with ginkgetin (**1**) with 50% inhibition being observed at a concentration of 1.8 μg/mL (Figure 1). Such OVCAR-3 cytotoxicity was cell-type specific; two other human tumor cell lines (HeLa, cervical epitheloid carcinoma cells; HepG2, hepatocellular carcinoma cells) and one normal human cell line (HEK, embryonic kidney cells) were also included in the present investigation. However, no evidence was found that their growth was adversely affected by **1**. This selective cytotoxic effect of **1** could be extended to other cell lines, including human embryonic lung (HEL) cells and monkey kidney (Vero) cells (data not shown).[28]

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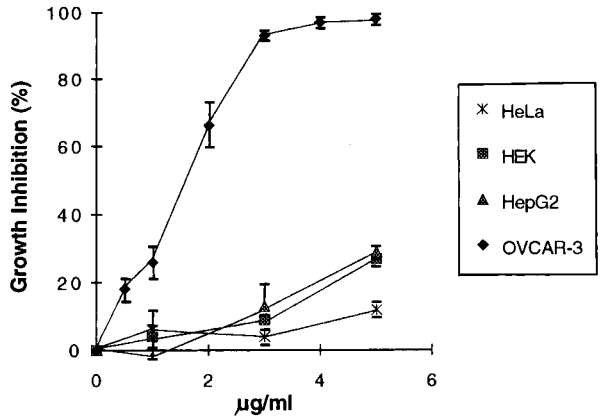

Figure 1. Effects of ginkgetin (**1**) on the growth of different cell lines after 48 h incubation. Increasing concentrations of **1** dose-dependently inhibited the growth of OVCAR-3 cells. In comparison, the growth of three other human cell lines was relatively unaffected.

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-, COSY, and COLOC NMR spectra were determined on a Varian Gemini-200 spectrometer, and FAB-MS spectra were measured on a JEOL JMS-HX110 mass spectrometer. E. Merck Si gel 60 was used for column chromatography, and analytical TLC was carried out on E. Merck aluminum sheets precoated with Si gel 60 F_{254} (0.2 mm thickness). The chromatograms were visualized under UV light (254 or 365 nm) or by spraying with 5% phosphomolybdic acid containing a trace of ceric sulfate in 5% H_2SO_4, followed by heating on a hot plate (120 °C).

Plant Material. The whole plant of *S. moellendorffii*, imported from the People's Republic of China, was purchased from a Chinese medicine shop in Taipei in October, 1994. The plant was identified by Dr. Chen-Meng Kuo, Department of Botany, National Taiwan University. A voucher specimen (NRICM 83001) was deposited in the National Research Institute of Chinese Medicine, Taipei.

Extraction and Isolation. Whole specimens of the air-dried plant (3 kg) were extracted with 95% ethanol (20 L) three times at 60 °C for 24 h. The ethanolic extracts were combined and concentrated *in vacuo* to 1 L. The concentrated extract was then mixed with 235 g of Si gel (230−400 mesh). The air-dried mixture was subjected to chromatography on a Si gel column (10 × 60 cm, 70−230 mesh) and then eluted with $CHCl_3$ (8 L) followed by 1%, 2%, 3%, 5%, 7%, and 10% MeOH/$CHCl_3$ (6 L each). Fractions (1 L each) were collected, and like fractions were combined to afford a total of 15 pooled fractions. The 13−15th fractions showed cytotoxicity to OVCAR-3 cells and were rechromatographed over Si gel (5 × 90 cm, 230−400 mesh) using gradient elution. The solvent gradient used was 1%, 2%, 3%, 5%, 7%, and 10% MeOH/$CHCl_3$ (4 L each). Fractions (500 mL per flask) were collected, and like fractions were combined to yield a total of 10 subfractions. Cytotoxic activity was observed in subfraction 5 (flasks 23−28), which was further purified by MPLC on Si gel (1 × 50 cm, 230−400 mesh) using gradient solvents of 1−10% MeOH/$CHCl_3$ to give **1** (35 mg). Purification of subfractions 2 (flasks 10−14), 4 (flasks 19−22), 8 (flasks 36−41), and 9 (flasks 42−47) in a similar manner produced amentoflavone 7,4′,7″,4‴-tetramethyl ether (15 mg), kayaflavone (8 mg), podocarpusflavone A (40 mg), and amentoflavone (38 mg), respectively.

Ginkgetin (1): pale yellow solid; ^{1}H-NMR data (DMSO-d_6), in good agreement with published data; ^{13}C-NMR data, in good agreement with published data[11] except C-2′ (δ 130.98) and C-6′ (δ 128.33); negative FAB-MS m/z 565 $[M - H]^-$; R_f 0.31 (5% MeOH/$CHCl_3$, Si gel).

Amentoflavone 7,4′,7″,4‴-tetramethyl ether: pale yellow solid; ^{1}H-NMR data ($CDCl_3$), in good agreement with published data;[10] ^{13}C-NMR data, in good agreement with published data[11] except C-2′ (δ 131.12) and C-6′ (δ 127.92); positive FAB-MS m/z 595 $[M + H]^+$; R_f 0.84 (5% MeOH/$CHCl_3$, Si gel).

Kayaflavone: pale yellow solid; ^{1}H NMR (DMSO-d_6) δ 13.21 (1H, s, OH-5), 12.91 (1H, s, OH-5″), 10.82 (1H, s, OH-7), 8.18 (1H, dd, J = 2 Hz, 8.8 Hz, H-6′), 8.06 (1H, d, J = 2 Hz, H-2′), 7.61 (2H, d, J = 8.4 Hz, H-2‴), 7.35 (1H, d, J = 8.8 Hz, H-5′), 6.93 (1H, s, H-3), 6.91 (2H, d, J = 8.4 Hz, H-3‴), 6.90 (1H, s, H-3‴), 6.67 (1H, s, H-6″), 6.47 (1H, d, J = 1.2 Hz, H-8), 6.18 (1H, d, J = 1.2 Hz, H-6), 3.82 (3H, s, OMe-7″), 3.77 (3H, s, OMe-4′), 3.74 (3H, s, OMe-4‴); ^{13}C NMR δ 182.33 (C-4″), 181.77 (C-4), 164.19 (C-7), 163.47 (C-2), 163.24 (C-2″), 162.62 (C-7″), 162.31 (C-4‴), 161.46, 161.42 (C-5, C-5″), 160.32 (C-4′), 157.39 (C-8a), 153.46 (C-8″a), 130.82 (C-2′), 128.35 (C-6′), 127.91 (C-2‴), 122.71, 122.63 (C-1′, C-1‴), 121.19 (C-3′), 114.54 (C-3‴), 111.78 (C-5′), 104.61 (C-8″), 104.11 (C-4a), 103.77 (C-4″a), 103.77, 103.21 (C-3, C-3″), 98.88 (C-6), 95.57 (C-6″), 94.11 (C-8), 56.50, 55.96, 55.50 (3 × −OMe); positive FAB-MS m/z 581 $[M + H]^+$; R_f 0.41 (5% MeOH/$CHCl_3$, Si gel).

Podocarpusflavone A: pale yellow solid; ^{1}H NMR (acetone-d_6) δ 13.14 (1H, s, OH-5), 13.01 (1H, s, OH-5″), 8.13 (1H, d, J = 2.4 Hz, H-2′), 8.03 (1H, dd, J = 2.4 Hz, 8.6 Hz, H-6′), 7.72 (2H, d, J = 9.1 Hz, H-2‴), 7.24 (1H, d, J = 8.6 Hz, H-5′), 6.92 (2H, d, J = 9.1 Hz, H-3‴), 6.72 (1H, s, H-3), 6.70 (1H, s, H-3″), 6.50 (1H, d, J = 2.1 Hz, H-8), 6.44 (1H, s, H-6″), 6.23 (1H, d, J = 2.1 Hz, H-6), 3.78 (3H, s, OMe-4‴); ^{13}C-NMR data, in good agreement with published data[11] except C-2′ (δ 132.57) and C-6′ (δ 128.77); negative FAB-MS m/z 551 $[M - H]^-$; R_f 0.15 (5% MeOH/$CHCl_3$, Si gel).

Amentoflavone: pale yellow solid; ^{1}H- and ^{13}C-NMR data (acetone-d_6), in good agreement with published data;[12,14] negative FAB-MS m/z 537 $[M - H]^-$; R_f 0.25 (10% MeOH/$CHCl_3$, Si gel).

Biological Assays. OVCAR-3 cells were obtained from the American Type Culture Collection (ATCC: HTB-161). HEK, HeLa, and HepG2 cells were kindly provided by the Cell Bank, Veterans General Hospital, Taipei, Taiwan. Cells were grown in RPMI 1640 medium supplemented with glutamine and 10% fetal calf serum. To perform the growth inhibition assays, cells were seeded into 12-well tissue culture plates at a density of 0.2−1 × 10^5 cells in 1 mL medium per well. Prior to each assay, the seeded cells were preincubated overnight at 37 °C in a humidified incubator containing 5% CO_2. Each experiment was carried out in triplicate. At time zero, the spent medium was replaced with 1 mL of fresh medium containing 0.5−10 μg of test compound dissolved in DMSO. Cells were harvested for viability counting subsequent to incubation at timed intervals. The medium was removed, and the wells were rinsed with 1 mL of phosphate-buffered saline. The

cells were harvested with trypsin-EDTA, suspended in culture media, and microscopically counted after the trypan blue exclusion staining. Cytotoxicity of the compounds at various concentrations was calculated as the net growth inhibition (%) of the cells relative to that of controls containing an equivalent amount of DMSO only.

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