A Novel Cytotoxic C-Methylated Biflavone, Taiwanhomoflavone-B from the Twigs of *Cephalotaxus wilsoniana*

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A novel C-methylated biflavone, taiwanhomoflavone-B (**1**), together with known compounds, 7,4',7"-tri-O-methylamentoflavone, 6-C-methylnaringenin and apigenin-7-O- β -glucoside were isolated from an ethanolic extract of *Cephalotaxus wilsoniana*. The structure of **1** was elucidated on the basis of spectroscopic analysis. Taiwanhomoflavone-B is cytotoxic with ED₅₀ values of 3.8 and 3.5 μ g/ml, against KB oral epidermoid carcinoma and Hepa-3B hepatoma cells, respectively.

Key words *Cephalotaxus wilsoniana*; cytotoxicity; taiwanhomoflavone-B; 7,4',7"-tri-O-methylamentoflavone; 6-C-methylnaringenin; kameferol; apigen-7-O- β -glucoside

Cephalotaxus wilsoniana HAYATA (Cephalotaxaceae) is an evergreen tree distributed over the middle mountains of Taiwan. Several antitumor components including hainanolide and the alkaloids homoharringtonine, harringtonine, *epi*-wilsonine, wilsonine and cephalotaxine have been reported from *Cephalotaxus* spp.^{1–7} Of these antitumor alkaloids, homoharringtonine has been selected as a candidate for phase II trial in the United States. Recently, we obtained a novel cytotoxic C₃₁ biflavone, taiwanhomoflavone-A in addition to a known biflavone, kayaflavone, and the known compounds, harringtonolide (=hainanolide), *epi*-wilsonine, and diterpenes as sugiol and isopimaric acid from the EtOH extract of the stem of *Cephalotaxus wilsoniana* HAYATA.⁸ Further investigation of the twigs of *C. wilsoniana* led to the isolation and characterization of a novel C-methylated biflavone, taiwanhomoflavone-B (**1**), along with known flavones, 7,4',7"-tri-O-methylamentoflavone (**2**), 6-C-methylnaringenin (**3**), and apigenin-7-O- β -glucoside (**4**). We report herein the structural elucidation of **1** determined by employing two dimensional (2D) NMR techniques including ¹H–¹H correlation spectroscopy (COSY), ¹H–¹³C heteronuclear multiple quantum coherence (HMQC), and ¹H–¹³C heteronuclear multiple bond coherence (HMBC) experiments.

Taiwanhomoflavone-B (**1**) was obtained as a pale yellow amorphous powder. Its molecular formula (C₃₂H₂₄O₁₀) was indicated by the molecular ion (*m/z* 568.1286 [M]⁺) in the high resolution electron impact (HR-EI)-MS spectra. The ¹H- and ¹³C-NMR spectra, together with IR absorption at 3400 (OH), 1660 (conjugated CO) and 1625 (aromatic) cm^{–1} revealed that **1** possessed two flavonoid units. The presence of signals at δ_H 7.56 (H-2', 6', d, *J*=8.5 Hz), δ_H 7.30 (H-3', 5', d, *J*=8.5 Hz), δ_H 7.98 (H-2'', 6'', d, *J*=8.5 Hz) and δ_H 7.27 (H-3'', 5'', d, *J*=8.5 Hz) in an A₂B₂ coupling system, together with the signals of δ_C 128.56 (C-2', 6'; C-2'', 6'') and 116.93 (C-3', C-5'; C-3'', C-5'') observed in the NMR spectra suggested two B-ring aromatic moieties in **1**. In the HMBC spectrum, two aromatic signals in a singlet were assigned at H-8 and 8'', respectively, due to the long-range coupling with C-7, 6 and C-7'', 6'', respectively. Then the methoxyl group at C-7'' was sequentially confirmed. Furthermore, in compari-

son with the chemical shifts of C-8 (δ_C 94.99) and C-8'' (δ_C 91.85), the higher chemical shift for C-8 by ca. δ_C 3–4 is consistent with that of similar flavonoids where a hydroxyl group was replaced by a methoxyl group at C-7''.⁹ The available evidence of C-6'' chemical shift (δ_C 126.56) suggests a linkage between two flavonoid units at C-4'–O–C-6''.¹⁰ This corroboration also excludes the possibility of linkage of the two flavonoid units by C-4'–O–C-8''. Otherwise, the chemical shift for C-6'' in **1** would be changed from general δ_C 98 to 95, due to a methoxyl group at C-7'' position.⁹

Moreover, based on the HMBC spectrum, two carbonyl carbons at C-4 and C-4'' (δ_C 182.98, 196.21) were unambiguously assigned due to long-range coupling with an olefinic H-3 and the saturated H-2'' and H-3'', respectively. Together with the above evidence, **1** was deduced as a 2'',3''-dihydroisocryptomerin analogue except for an additional methyl signal (δ_H 2.35) was observed in **1**. These findings revealed that **1** had a new C₃₁ skeleton with two flavones linked by C-4'–O–C-6''. After detailed examination of the HMBC spectrum of **1**, the methyl group was assigned at C-6 due to the correlation between the methyl group and C-5 and C-6, and between H-8 and C-6, respectively. Thus, the structure of 6-C-methyl-2'',3''-dihydroisocryptomerin (**1**) was confirmed completely and tentatively named taiwanhomoflavone-B.

Compounds **1** and **4**, hainanolide (**5**), *epi*-wilsonine (**6**), sugiol (**7**) and isopimaric acid (**8**) were tested against four cancer cell lines: KB, COLO-205, Hepa-3B and Hela. The bioassay data exhibited that only diterpenes **7** and **8** were inactive, whereas the other entries showed the cytotoxicity (Table 1). Not surprisingly, hainanolide (**5**) possessed the

Taiwanhomoflavone B (**1**)

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most active effect against the above four tumor cells in this experiment; it was also report to exhibit the potent cytotoxicity against several other tumor cell lines.¹¹⁾ The other active

principles isolated from the titled plant as well as their structure–activity relationships are still under investigation.

Table 1. Cytotoxicity of Compounds **1**, **4**, **5**–**8** against Tumor Cell Lines

Entries	Cell line (ED ₅₀ , $\mu\text{g/ml}$)			
	KB ^{a)}	COLO-205 ^{a)}	Hepa-3B ^{a)}	Hela ^{a)}
1	3.8	^{b)}	3.5	^{b)}
4	3.5	^{b)}	8.7	^{b)}
5	0.11	^{b)}	0.05	0.37
6	1.94	^{b)}	^{b)}	^{b)}
7	— ^{c)}	—	—	—
8	—	—	—	—

a) Hepa-3B: hepatoma, KB: oral epidermoid carcinoma, COLO-205: colon carcinoma, Hela: cervix carcinoma. b) No test. c) Inactive, ED₅₀>10 $\mu\text{g/ml}$.

Table 2. ¹H- and ¹³C-NMR Data^{a)} (Pyridine-*d*₅) for Compound **1**

Carbon	¹³ C (ppm)	¹ H (ppm)	¹³ C– ¹ H connectivities ^{b)}
2	165.08 s	—	H-3
3	103.79 d	6.98 (s)	—
4	182.98 s	—	H-3
4a	102.46 s	—	H-8, 5-OH
5	162.18 s	—	6-Me, 5-OH
6	104.99 s	—	H-8, 6-Me, 5-OH
7	166.21 s	—	H-8, 6-Me
8	94.99 d	6.41 (s)	7-OH
8a	161.32 s	—	H-8
1'	121.93 s	—	H-3, H-3', H-5'
2'	128.56 d	7.56 (d, 8.5)	H-6'
3'	116.93 d	7.30 (d, 8.5)	H-5'
4'	162.97 s	—	H-3', H-5'
5'	116.93 d	7.30 (d, 8.5)	H-3'
6'	128.56 d	7.56 (d, 8.5)	H-2'
2''	79.23 d	5.44 (dd, 13, 3)	H-3''
3''	43.35 t	2.80 (dd, 17.0, 3)	H-2''
		3.20 (dd, 17.0, 13.0)	
4''	196.21 s	—	H-3''
4''a	106.27 s	—	H-8''
5''	153.92 s	—	—
6''	126.56 s	—	H-8''
7''	159.13 s	—	7''-OMe, H-8''
8''	91.85 d	6.91 (s)	—
8''a	154.65 s	—	H-8''
1'''	132.90 s	—	H-3''', 5''', 2'', 3''
2'''	129.01 d	7.98 (d, 8.5)	H-6''', H-2''
3'''	115.46 d	7.27 (d, 8.5)	H-5'''
4'''	159.05 s	—	H-3''', 5'''
5'''	115.46 d	7.27 (d, 8.5)	H-3'''
6'''	129.01 d	7.98 (d, 8.5)	H-2''', H-2''
6-Me	7.55 q	2.35 (s)	— ^{c)}
7''-O-Me	56.60 s	3.80 (s)	— ^{c)}

a) All assignments (¹³C; 75.5 MHz, multiplicity; ¹H; 300 MHz) are based on one dimensional (1D) and two dimensional (2D) NMR experiments, including COSY 90, HETCOR, and HMBC spectra. b) ¹H–¹³C long-range correlation (HMBC) corresponded to two or three bonds connectivities. c) These assignments were explained in the text.

Experimental

General Experimental Procedures ¹H- and ¹³C-NMR spectra were recorded at 300.13 and 75.46 MHz, respectively, on a Bruker 300 AC spectrometer. The spectra of heteronuclear correlation, HMBC was established by the coupling of 8 Hz. Electron impact (EI)-MS and FAB-MS were performed on a JEOL SX-102A instrument. Si gel (Merck 70–230 mesh) was used for column chromatography, and precoated Si gel (Merck 60F-254) plates were used for TLC. HPLC was accomplished on an SPD-6AV liquid chromatograph using a preparative C₁₈ column. Melting points were determined on a Fisher-Johns apparatus and are uncorrected.

Plant Material Twigs of *Cephalotaxus wilsoniana* were collected in July 1998 in Taipei, Taiwan, R.O.C. A voucher specimen is deposited at the National Research Institute of Chinese Medicine, Shih-Pai, Taipei.

Extraction and Isolation The dried twigs of *C. wilsoniana* (5.7 kg) were extracted exhaustively with ethanol. An EtOH extract (160 g) was extracted successively with *n*-hexane and CHCl₃. The CHCl₃ extract was chromatographed by column chromatography over Si gel and eluted with *n*-hexane–EtOAc and EtOAc to give 7 fractions. The bioactive fr. 4 (*n*-hexane:EtOAc=3:1) was further separated by HPLC (5C₁₈, 250×10 mm) with MeOH–H₂O (8.5:1) to furnish taiwanhomoflavone-B (**1**) (9 mg).

Taiwanhomoflavone-B (**1**): Pale yellow powder; IR ν_{max} (KBr) 3400 (OH), 1660 (conjugated CO) and 1625 (aromatic) cm^{−1}; ¹H- and ¹³C-NMR, see Table 2; FAB-MS *m/z* 567 [M–H]⁺; HR-EI-MS *m/z* 568.1286 [M]⁺ (Calcd for C₃₂H₂₄O₁₀, 568.1369).

Cytotoxicity Assay An *in vitro* cytotoxicity assay was performed as previously described.¹²⁾

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References and Notes

- Powell R. G., Weisleder D., Smith C. R., Jr., Wolff I. A., *Tetrahedron Lett.*, **1969**, 4081–4084 (1969).
- Powell R. G., Weisleder D., Smith C. R., Jr., Rohwedder W. K., *Tetrahedron Lett.*, **1970**, 815–818 (1970).
- Khan N. U., Ilyas M., Rahman W., Okigawa M., Kawano N., *Phytochemistry*, **10**, 2541–2545 (1971).
- Powell R. G., Mikolajczak K. L., Weisleder D., Smith C. R., Jr., *Phytochemistry*, **11**, 3317–3320 (1972).
- Sun N., Xue Z., Liang S., Huang L., *Acta Pharmaceut. Sinica*, **1**, 39–43 (1979).
- Qiu M., Lu B. M. X., Nie R., *Acta Botanica Yunnanica*, **1**, 97–99 (1997).
- Furukawa H., Itoigawa M., Haruna M., Jinno Y., Ito K., Lu S. T., *Yakugaku Zasshi*, **96**, 1373–1377 (1976).
- Kuo Y. H., Lin C. H., Hwang S. Y., Shen Y. C., Lee Y. L., Li S. Y., *Chem. Pharm. Bull.*, **48**, 440–441 (2000).
- Markham K. R., Sheppard C., Geiger H., *Phytochemistry*, **26**, 3335–3337 (1987).
- Silva G. L., Chai H., Gupta M. P., Farnsworth N. R., Cordell G. A., Pezzuto J. M., Beecher C. W., Kinghorn A. D., *Phytochemistry*, **40**, 129–134 (1995).
- Sun N., Zhao Z., Chen R., Lin W., Zhou Y., *Acta Pharmaceut. Sinica*, **16**, 233–234 (1981).
- Kuo Y. H., Huang H. C., Kuo Yang L. M., Chen C. F., *J. Org. Chem.*, **64**, 7023–7027 (1999).