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 ED_{50} values of 3.8 and 3.5 μg/ml, against KB oral epidermoid carcinoma and Hepa-3B hepatoma cells, respectively.

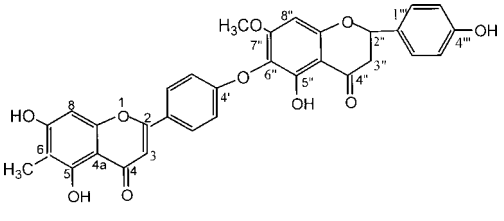
Key words *Cephalotaxus wilsoniana*; cytotoxicity; taiwanhomoflavone-B; 7,4′,7″-tri-*O*-methylamentoflavone; 6-*C*-methylnaringenin; kamepferol; apigen-7-*O*-β-glucoside

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 C_{31} 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–^{13}C heteronuclear multiple quantum coherence (HMQC), and ^1H–^{13}C heteronuclear multiple bond coherence (HMBC) experiments.

Taiwanhomoflavone-B (**1**) was obtained as a pale yellow amorphous powder. Its molecular formula ($C_{32}H_{24}O_{10}$) was indicated by the molecular ion (*m/z* 568.1286 $[M]^+$) in the high resolution electron impact (HR-EI)-MS spectra. The ^1H- and ^{13}C-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 A_2B_2 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 comparison 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″-dihydroisocrytomerin analogue except for an additional methyl signal (δ_H 2.35) was observed in **1**. These findings revealed that **1** had a new C_{31} 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″-dihydroisocrytomerin (**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 bioassy 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}, μg/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, $ED_{50}>10\,\mu$g/ml.

Table 2. ^{1}H- and ^{13}C-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) 3.20 (dd, 17.0, 13.0)	H-2″
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 (^{13}C; 75.5 MHz, multiplicity; ^{1}H; 300 MHz) are based on one dimensional (1D) and two dimensional (2D) NMR experiments, including COSY 90, HETCOR, and HMBC spectra. *b*) ^{1}H–^{13}C long-range correlation (HMBC) corresponded to two or three bonds connectivities. *c*) These assignments were explained in the text.

Experimental

General Experimental Procedures ^{1}H- and ^{13}C-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 C_{18} 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 $CHCl_3$. The $CHCl_3$ 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 ($5C_{18}$, 250×10 mm) with MeOH–H_2O (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}; ^{1}H- and ^{13}C-NMR, see Table 2; FAB-MS *m/z* 567 $[M-H]^+$; HR-EI-MS *m/z* 568.1286 $[M]^+$ (Calcd for $C_{32}H_{24}O_{10}$, 568.1369).

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

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