A Novel Cytotoxic *C*-Methylated Biflavone from the Stem of *Cephalotaxus wilsoniana*

Yao-Haur KUO,*[,a,b] Chin-Hou LIN,[a,b] Shy-Yuan HWANG,[c] Ya-Ching SHEN,[d] Yi-Ling LEE,[a,b] and Shyh-Yuan LI*[,a,b]

National Research Institute of Chinese Medicine,[a] Shih-Pai, Taipei, 112, Taiwan, R.O.C., Department of Applied Chemistry, Chinese Culture University,[b] Taipei,113, Taiwan, R.O.C., Department of Agriculture and Forestry, Taiwan Endemic Species Research Institute,[c] Nantou County, 552, Taiwan, R.O.C., and Institute of Marine Resources, National Sun Yat-Sen University,[d] Kaohsiung, 804, Taiwan, R.O.C. Received October 12, 1999; accepted November 12, 1999

Bioassay-directed fractionation of an ethanolic extract of *Cephalotaxus wilsoniana* has resulted in the isolation of a novel *C*-methylated biflavone, taiwanhomoflavone-A (1). Its structure was elucidated on the basis of spectroscopic analysis. Taiwanhomoflavone-A is cytotoxic with ED_{50} values of 3.4, 1.0, 2.0 and 2.5 μg/ml, respectively, against KB epidermoid carcinoma of nasopharynx, COLO-205 colon carcinoma, Hepa-3B hepatoma, and Hela cervix tumor cells.

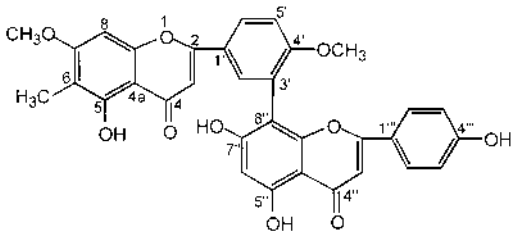
Key words *Cephalotaxus wilsoniana*; cytotoxicity; taiwanhomoflavone-A; 6-*C*-methyl-7, 4′-*O*-dimethylamentoflavone

Several antitumor alkaloids[1—6] have been reported from *Cephalotaxus* spp. *Cephalotaxus wilsoniana* HAYATA (Cephalotaxaceae) is an evergreen tree distributed over the middle mountain of Taiwan. During a search for antitumor agents from the terrestrial plants of Taiwan,[8—11] we found a crude extract from *C. wilsoniana* which possesses inhibitory effect against KB, Hepa-3B, and Hela cancer cell lines. Bioassay-guided fractionation of an EtOH extract led to the isolation of a novel *C*-methylated biflavone (**1**) in addition to the known compounds, harringtonolide (=hainanolide), *epi*-wilsonine, sugiol, isopimaric acid and kayaflavone. In this paper we describe the structural elucidation of **1** using H–H correlation spectroscope (COSY), ^{13}C–^{1}H heteronuclear multiple quantum coherence (HMQC) and ^{13}C–^{1}H heteronuclear multiple bonds coherence (HMBC) experiments.

The molecular formula of taiwanhomoflavone (**1**) ($C_{33}H_{24}O_{10}$) was indicated by a molecular ion (*m/z* 579 $[M-H]^+$) in the FAB-MS spectra. The IR spectrum suggested that **1** contained hydroxyl, conjugated carbonyl and aromatic functions.

Observing the ^{1}H-NMR spectrum of **1**, an AMX coupling system with signals at δ 8.49 (H-2′), 8.08 (H-6′), and 7.29 (H-5′) revealed a 1,3, 4-trisubstituted benzene ring. The signals at δ_H 7.76 (H-2‴, 6‴, d, J=8.5 Hz) and δ_H 7.15 (H-3‴, 5‴, d, J=8.5 Hz), together with the signals of δ_C 128.56 (C-2‴, 6‴), and 116.81 (3‴, 5‴) in the ^{13}C-NMR spectrum indicated another aromatic moiety in an A_2B_2 coupling system. In addition, four aromatic protons in each singlet, two aromatic methoxyls in singlet, and a unique methyl group in singlet were observed in the ^{1}H-NMR spectrum. This evidence revealed that **1** has a biflavone skeleton with a *C*-methyl group and excluded the possibility of linkage between the two flavone moieties at C-2‴, -6‴, -3‴ and -5‴ in the B-ring.

In the HMBC spectrum, the correlations between C-8″ and H-6″ and H-2′ revealed the connective positions to be at C-3′



1

Table 1. ^{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	164.14 s	—	H-3
3	104.76 d	7.12 (s)	—
4	182.73 s	—	H-3
4a	105.54 s	—	H-3
5	158.83 s	—	6-Me
6	108.67 s	—	H-8, 6-Me
7	163.64 s	—	H-8, OMe
8	90.31 d	6.71 (s)	—
8a	156.31 s	—	H-8
1′	123.25 s	—	H-5′
2′	131.87 d	8.49 (d, 2.0)	H-6′
3′	104.93 s	—	H-5′
4′	161.44 s	—	H-2′, 6′, OMe
5′	111.82 d	7.29 (d, 8.5)	—
6′	128.40 d	8.08 (dd, 2.0,8.5)	H-2′
2″	164.36 s	—	H-3″
3″	103.44 d	6.95 (s)	—
4″	183.02 s	—	H-3″
4″a	104.85 s	—	H-3″, 6″
5″	162.40 s	—	H-3″, 6″
6″	99.31 d	6.93 (s)	—
7″	163.64 s	—	—
8″	104.76 s	—	H-2′, 6″
8″a	155.53 s	—	—
1‴	122.18 s	—	H-3‴, 5‴
2‴	128.56 d	7.76 (d, 8.5)	H-6‴
3‴	116.81 d	7.15 (d, 8.5)	H-5‴
4‴	162.67 s	—	H-3‴, 5‴, 2‴, 6‴
5‴	116.81 d	7.15 (d, 8.5)	H-3‴
6‴	128.56 d	7.76 (d, 8.5)	H-2‴
6-Me	7.60 q	2.22 (s)	—[c]
7-*O*-Me	56.02 q	3.74 (s)	—[c]
4′-*O*-Me	55.98 q	3.77 (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*) HMBC corresponded to two or three bonds connectivities. *c*) These assignments were explained in the text.

* To whom correspondence should be addressed.

and C-8″ between the flavones. Generally, the carbonyl carbon signal (C-4 or -4″) observed near δ_C 183 for biflavones such as ginkgetin[12] would permit the assignment of a peri-OH at C-5 or -5″. The signal for C-8 at δ_C 90.3 in **1** was consistent with the carbon bearing a methoxyl group at the C-7 position; otherwise, a chemical shift for C-8 would appear around δ_C 94.0 suggesting a hydroxyl group at C-7.[12] After detailed examination of the HMBC spectrum of **1**, a methyl group was assigned at C-6 due to the correlation between the methyl group and C-5 and C-6, respectively. Together with the above evidence, the structure of **1** was deduced to be an analogue of 6-*C*-methyl-7-*O*-methylamentoflavone (**2**)[13] except for a methoxyl group in **1** and a hydroxyl group in **2**. The remaining methoxyl group in **1** was assigned at C-4′ due to the HMBC spectrum. Then, the structure of **1** was confirmed unambiguously, and was tentatively named taiwanhomoflavone-A.

Furthermore, bioassays showed that taiwanhomoflavone-A (**1**) exhibited cytotoxic effects against four cancer cell lines: KB, COLO-205, Hepa-3B and Hela with ED_{50} of 3.45, 1.06, 2.03 and 2.53 μg/ml, respectively. To our knowledge, this is the first report that 6-*C*-methyl biflavone as compound **1** has cytotoxic activity.

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 chromatograph, and precoated Si gel (Merck 60F-254) plates were used for TLC. HPLC was accomplished on an SPD-6AV liquid chromatography using a preparative C_{18} column. Melting points were determined on a Fisher-Johns apparatus and are uncorrected.

Plant Material The stems of *Cephalotaxus wilsoniana* were collected in Septemper 1995 on Taipei, Taiwan. A voucher specimen is deposited at the National Research Institute of Chinese Medicine, Shih-Pai, Taipei, Taiwan, R.O.C.

Extraction and Isolation The dried stems of *C. wilsoniana* (6.3 kg) were extracted exhaustively with ethanol . An EtOH extract (200 g) of dried stems of *Cephalotaxus wilsoniana* 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 8 fractions. The bioactive fr. 5 (*n*-hexane : EtOAc = 2 : 1) was further separated by HPLC ($5C_{18}$, 250×10 mm) with MeOH–H_2O (9 : 1) to furnish taiwanhomoflavone-A (**1**) (11 mg).

Taiwanhomoflavone-A (**1**): Pale yellow crystal, mp 245—248 °C; IR ν_{max} (KBr) 3400 (OH), 1660 (conjugated CO), 1620 (aromatic rings) cm^{-1}; ^{1}H- and ^{13}C-NMR, see Table 1; FAB-MS *m/z* 579 $[M-H]^+$; HR-EI-MS *m/z* 580.1379 $[M]^+$ (Calcd for $C_{33}H_{24}O_{10}$, 580.1369).

Cytotoxicity Assay An *in vitro* cytotoxicity assay was performed as previously described.[11]

Acknowledgments The authors thank the National Science Council, R.O.C. (NSC 88-2314-B-077-007) for financial support to Y. H. Kuo. We also thank Mr. Shih-Jen Wang, NSC Regional Instrument Center of HSINCHU, for measuring the FABMS data.

References

1) Powell R. G., Weisleder D., Smith C. R., Jr., Wolff I. A., *Tetradron Lett*., **1969**, 4081—4084.
2) Powell R. G., Weisleder D., Smith C. R., Jr., Rohwedder W. K., *Tetradron Lett*., **1970**, 815—818.
3) Khan N. U., Ilyas M., Rahman W., Okigawa M., Kawano N., *Phytochemistry*, **10**, 2541—2545 (1971).
4) Powell R. G., Mikolajczak K. L., Weisleder D., Smith C. R., Jr., P*hytochemistry*, **11**, 3317—3320 (1972).
5) Sun N., Xue Z., Liang S., Huang L., *Acta Pharmaceut. Sinica*, **1**, 39—43 (1979).
6) Qiu M., Lu B. M. X., Nie R., *Acta Botanica Yunnanica*, **1**, 97—99 (1997).
7) Furukawa H., Itoigawa M., Haruna M., Jinno Y., Ito K., Lu S. T., *Yakugaku Zasshi*, **96**, 1373—1377 (1976).
8) Kuo Y. H., Kuo Yang L. M., Chen C. F., *J. Org. Chem*., **62**, 3242—3245 (1997).
9) Kuo Y. H., Chang Chi. I., Li S. Y., Chou C. J., Chen C. F., Kuo Y. H., Lee K. H., *Planta Med*., **63**, 363—365 (1997).
10) Kuo Y. H., Kuo Yang L. M., *Phytochemistry*, **44**, 1275—1281 (1997).
11) Kuo Y. H., Huang H. C., Kuo Yang L. M., Chen C. F., *J. Org. Chem*., **64**, 7023—7027 (1999).
12) Markham K. R., Sheppard C., Geiger H., *Phytochemistry*, **26**, 3335—3337 (1987).
13) Aqil M., Rahman W., Hasaka N., Okigawa M., Kawano N., *J. Chem. Soc*., *Perkin Trans. 1*, **1981**, 1389—1392.