Isolation and Cytotoxicity of the Lignanoids from *Chamaecyparis formosensis*

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Key words
- *Chamaecyparis formosensis*
- Cupressaceae
- Taiwan red cypress
- cytotoxicity
- lignans
- 7,7′-(*S*)-dihydrotaiwanin C

Abstract

In this study, we assessed the antitumor activity of the methanol extract from wood chips of the heartwood of the Taiwan red cypress, *Chamaecyparis formosensis* Matsumura, which is a precious tree species endemic to Taiwan. A brine shrimp lethality test (BST) indicated that the ethyl acetate (EtOAc)-soluble extract from the MeOH extract was a suitable candidate (LC_{50} = 15.36 μg/mL) for further studies of the antitumor activity of its components. From this EtOAc fraction, we isolated six lignans and two norlignans and tested their cytotoxic activities *in vitro* against promyelocytic leukemia (HL-60) and hepatoma (Hepa-G2) cell lines. Among these compounds, 7,7′-(*S*)-dihydrotaiwanin C, isolated for the first time from nature, with its single crystal structure depicted in this study, exhibited significant cytotoxic activity against HL-60 cell lines *in vitro* (IC_{50} = 4.03 μg/mL) after 24 hours.

Abbreviations

BST:	brine shrimp lethality test
Hepa-G2:	human hepatoma
HL-60:	human leukemia
IC_{50}:	half-maximal inhibitory concentration
SARs:	structure-activity relationships
AS:	adenine sulfate

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Introduction

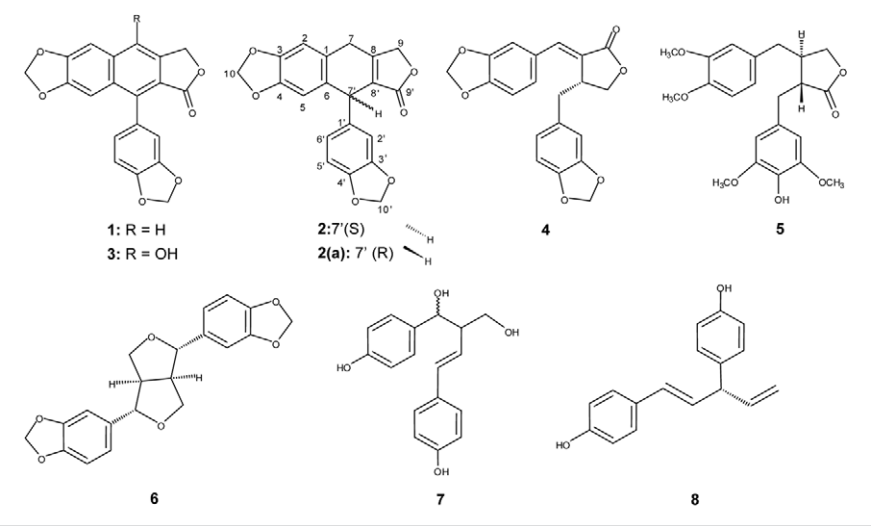
Many compounds isolated from woody plants have great potential for use as medicines for disease control or as raw materials for synthesizing useful analogues. Taxol and podophyllotoxin are well-known examples of antitumor compounds isolated from conifers [1]. When searching for such natural antitumor agents, cytotoxicity-based isolation and purification processes are used widely. In addition, it is well established that the natural durability of a tree is often dependent on the type and quantity of its chemical constituents.

Chamaecyparis formosensis Matsumura, known commonly as the Taiwan red cypress because its bark has a slightly reddish brown color, is indigenous to the high mountain area of Taiwan. Its wood is used frequently for housing construction and in expensive items of furniture. The Taiwan red cypress displays strong resistance against wood-decaying fungi [2], [3]. The chemical constituents of its roots, bark, wood, pericarps, and leaves have been investigated, resulting in the isolation of 18 sesquiterpenes, 40 diterpenes, 8 flavones, 7 lignans, and 11 miscellaneous compounds [4], [5], [6], [7]. In this paper, the isolation of a new lignan together with five known lignans plus two norlignans from the methanol extract of the heartwood of Taiwan red cypress are described. Furthermore, the chemical structure of the new lignan has been elucidated by X-ray crystallography and cytotoxic activities were evaluated for these eight lignans.

Materials and Methods

Reagents

GR grade solvents (> 99.5 %) used in column chromatography including hexane, ethyl acetate, chloroform, methanol, and HPLC grade (> 99.9 %) solvents, hexane, ethyl acetate, dimethyl sulfoxide (DMSO) and methanol were purchased from



Merck. Standards of curcumin (≥ 94%), plumbagin (practical grade) and MTT (4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich Co.

Plant material

The wood was collected from an 80-year-old *Chamaecyparis formosensis* in forestland at the Experimental Forest of National Taiwan University (NTU) in 2005, and was identified by Assistant Prof. Yen-Hsueh Tseng, Department of Forestry, National Chung Hsing University (NCHU), Taiwan. The voucher specimen [CF001-CF004] was deposited in the herbarium of NCHU.

General experimental procedures

High-performance liquid chromatography (HPLC) was performed using an Agilent 1100 series system (Agilent Technologies). ^{1}H- and ^{13}C-NMR spectra were recorded using Varian Plus

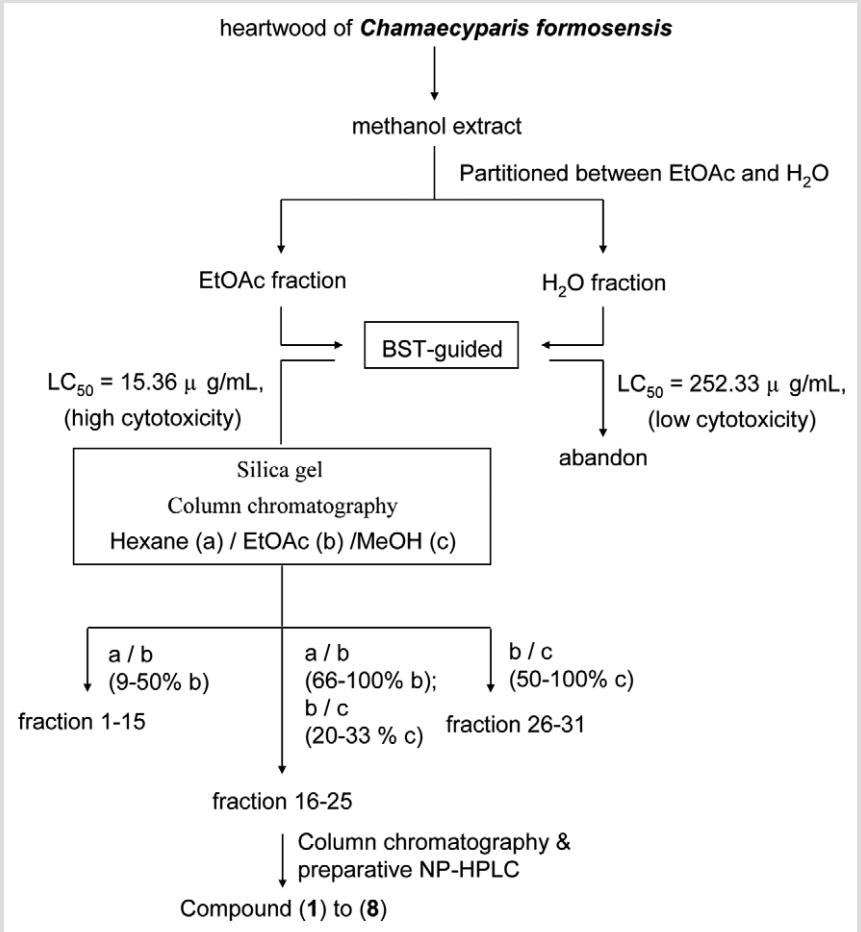


Fig. 1 BST-guided isolation of lignans from heartwood of *Chamaecyparis formosensis*.

400 and Varian Inova 600 spectrometers; chemical shifts are presented in ppm (δ) downfield from internal TMS. Silica gel 60 (Merck 70 – 230 mesh, 230 – 400 mesh; ASTM) was used for column chromatography and silica gel 60 F_{254} (Merck) for thin-layer chromatography.

Extraction and isolation

The air-dried heartwood chips (10.0 kg) were exhaustively extracted with MeOH (50 L) at ambient temperature. The extracts were concentrated under vacuum to yield the MeOH extract (420.5 g) and partitioned between ethyl acetate (EtOAc) and H_2O (1 : 1, each 3 L) to separate the EtOAc- (2.5 L) and H_2O-soluble fractions. The EtOAc fraction was concentrated under vacuum to obtain the extract (70.8 g) and further subjected to column chromatography over silica gel (9 × 35 cm; 70 – 230 mesh) through elution with a gradient of hexane, EtOAc, and MeOH (hexane:EtOAc = 10 : 1; 8 : 1; 6 : 1; 4 : 1; 2 : 1; 1 : 1; 1 : 2; 1 : 4; 1 : 8; 0 : 10 and EtOAc:MeOH = 4 : 1; 2 : 1; 1 : 1; 1 : 2; 0 : 1; each 0.5 L); in total, 31 fractions were collected. This separation of chemical components was monitored through TLC analysis. The compounds collected from fractions 16 – 25 were further purified through column chromatography (silica gel, 230 – 400 mesh) and HPLC (5SL-II, 250 × 10 mm; Luna silica column; Phenomenex Co.). The gradient of HPLC was isocratic elution with EtOAc/hexane (20 : 80) at a flow rate of 3.2 mL/min to obtain compounds **1** to **8**. The amount and purity (generally based upon peak area in HPLC) of each compound obtained is as follows: compound **1**, 18 mg, 98.6 %; compound **2**, 6 mg, 99.5 %; compound **3**, 11 mg, 98.4 %; compound **4**, 10 mg, 99.1 %; compound **5**, 7 mg, 98.4 %; compound **6**, 8 mg, 98.5 %; compound **7**, 22 mg, 99.2 % and compound **8**, 20 mg, 99.3 %.

7,7′-(S)-Dihydrotaiwanin C (**2**): White powder; m. p. 245 – 246 °C; $[\alpha]_D^{25}$: –100 (*c* 0.4, $CHCl_3$); UV (MeOH): λ_{max} nm (log ε) = 292 nm (3.47); IR (KBr): ν = 2920, 1750, 1484 cm^{-1}; EI-MS (probe) 70eV: *m/z* (rel. int.) = 350 $[M]^+$ (100), 305 $[M-45]^+$ (29), 275 $[M-75]^+$ (21), 185 $[M-165]^+$ (20), 122 $[M-128]^+$ (26); HR-EI-MS: *m/z* = 350.0781 $[M]^+$ (calcd. for $C_{20}H_{14}O_6$: 350.0790); ^{1}H-NMR (600 MHz, $CDCl_3$): δ = 3.66 (1H, dd, *J* = 4.2, 22.2 Hz, H-7), 3.87 (1H, dd, *J* = 4.2, 22.2 Hz, H-7), 4.77 (1H, br. s, H-7′), 4.82 (1H, d, *J* = 16.2 Hz, H-9), 4.88 (1H, d, *J* = 16.2 Hz, H-9), 5.88 (1H, d, *J* = 1.2 Hz, H-10), 5.89 (1H, d, *J* = 1.2 Hz, H-10′), 5.90 (1H, d, *J* = 1.2 Hz, H-10′), 5.94 (1H, d, *J* = 1.2 Hz, H-10), 6.52 (1H, d, *J* = 2.4 Hz, H-2′), 6.59 (1H, s, H-5), 6.70 (1H, s, H-2), 6.71 (1H, d, *J* = 7.8 Hz, H-5′), 6.74 (1H, dd, *J* = 2.4, 7.8 Hz, H-6′); ^{13}C NMR (600 MHz, $CDCl_3$): δ = 29.1 (C-7), 42.2 (C-7′), 71.0 (C-9), 101.1 (C-10 or C-10′), 101.2 (C-10 or C-10′), 107.7 (C-2), 108.3 (C-5′), 108.5 (C-2′), 109.5 (C-5), 121.7 (C-6′), 123.3 (C-6), 127.9 (C-8′), 130.0 (C-1), 136.6 (C-1′), 146.5 (C-4′), 146.9 (C-3), 147.2 (C-4), 147.8 (C-3′), 157.1 (C-8), 172.1 (C-9′).

X-ray crystallographic analysis of 7,7′-(*S*)-dihydrotaiwanin C (2)

Crystal data: $C_{20}H_{14}O_6$; MW 350.31; space group *P*-1; *a* = 7.9503(11) Å; *b* = 9.5256(13) Å; *c* = 11.0575(15) Å; *V* = 773.74(18) $Å^3$; *Z* = 2; D_{calcd} = 1.504 Mg/m^3; *F*(000) = 364. Intensity data were collected on a Siemens R3 m/V diffractometer using monochromatized Mo Kα radiation (λ = 0.71073 Å) via the $\Theta - 2\Theta$ scan technique; those 2990 data with $I > 2.0\sigma(I)$ were considered to be observed. The crystal structure was solved using direct methods. At convergence: *Rf* = 5.95 %; goodness-of-fit on F^2 = 1.098. Neutral atom scattering factors used in the structure factor calculations were taken from International Tables for X-ray Crystallography. Crystallographic calculations were performed using the Bruker Analytical X-Ray Systems (AXS). Final non-hydrogen atom coordinates, bond lengths, and bond angles can be obtained from the authors. The above crystal structure has been deposited at the Cambridge Crystallographic Data Center and allocated the deposition number CCDC 687 872.

Brine shrimp lethality bioassay

The brine shrimp lethality tests (BSTs) were performed according to the procedures described previously [8], [9], [10]. Brine shrimp (*Artemia salina* Leach) eggs were obtained from a local aquarium shop; artificial saline was prepared by dissolving sea salt (3.8 g) in H_2O (1 L) followed by filtering. A small tank incorporating a perforated dividing dam was prepared from a plastic soap container. A sample of salt water (ca. 10 mL) was placed in the tank and a pinch of the eggs was placed on one side of the tank. An opaque cover was placed on top of this side of the tank. The other side of the tank was irradiated with light from a normal lamp. The eggs were left for 48 h to hatch, at which point the brine shrimps had swum to the lighted side of the tank. Four sets of BSTs were conducted at concentrations of 1000, 100, 10,

Table 1 NMR spectral data of 7,7′-(*S*)-dihydrotaiwanin C (**2**)[a]

Position	7,7′-(S)-Dihydrotaiwanin C (2)		
	^{1}H-NMR δ, (600 MHz)[b]	^{13}C-NMR δ, (150 MHz)[b]	HMBC
1		130.0	
2	6.70 (1H, s)	107.7	C-1, C-3, C-4,
3		146.9	
4		147.2	
5	6.59 (1H, s)	109.5	C-3, C-4, C-6
6		123.3	
7	3.66 (1H, dd, *J* = 4.2, 22.2 Hz), 3.87 (1H, dd, *J* = 4.2, 22.2 Hz)	29.1	C-1, C-6, C-8, C-8′
8		157.1	
9	4.82 (1H, d, *J* = 16.2 Hz), 4.88 (1H, d, *J* = 16.2 Hz)	71.0	C-8, C-8′
10	5.88 (1H, d, *J* = 1.2 Hz), 5.94 (1H, d, *J* = 1.2 Hz)	101.1[c]	C-3, C-4
1′		136.6	
2′	6.52 (1H, d, *J* = 2.4 Hz)	108.5	C-3′, C-4′, C-6′
3′		147.8	
4′		146.5	
5′	6.71 (1H, d, *J* = 7.8 Hz)	108.3	C-1′, C-3′, C-4′
6′	6.74 (1H, dd, *J* = 2.4, 7.8 Hz)	121.7	C-2′, C-5′, C-4′
7′	4.77 (1H, br. s)	42.2	C-8, C-1′, C-6′, C-8′
8′		127.9	
9′		172.1	
10′	5.89 (1H, d, *J* = 1.2 Hz), 5.90 (1H, d, *J* = 1.2 Hz)	101.2[c]	C-3′, C-4′

[a] Assignments are confirmed by ^{1}H-^{1}H COSY, DEPT, HSQC and HMBC.

[b] Solvent: $CDCl_3$.

[c] Interchangeable.

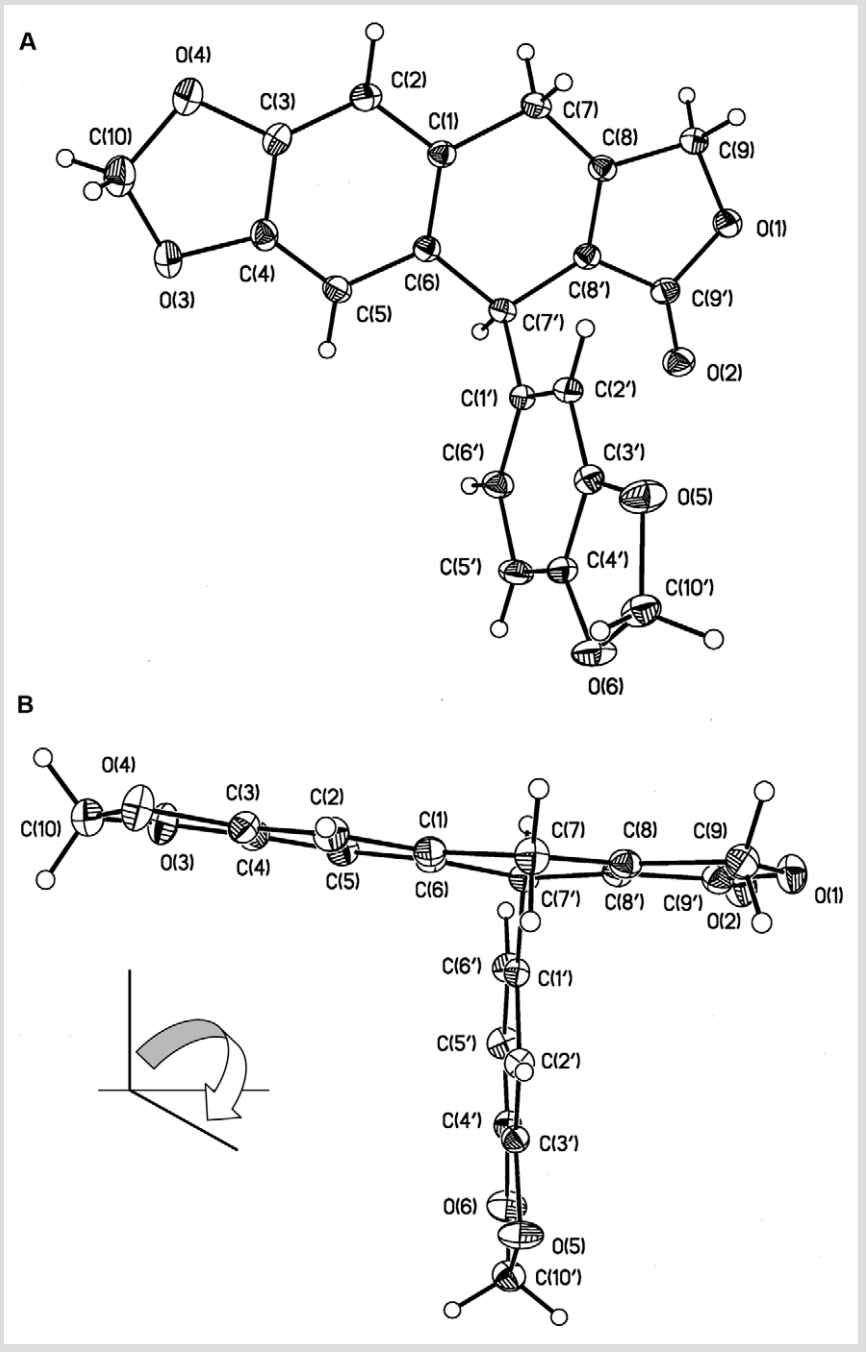


Fig. 2 Molecular structure of 7,7′-(*S*)-dihydrotaiwanin C (**2**); (**B**) is the rotated diagram of (**A**).

and 1 μg/mL; each set was performed in triplicate. Saline was used as the control (excluding the test sample and solvent). The samples were prepared by dissolving the extracted compounds (20 mg) in DMSO (2 mL). Volumes of 500, 50, 5 and 0.5 μL were transferred from this solution into the glass vials, and to each vial, brine (ca. 5 mL) was added to achieve the concentration of 1000, 100, 10 and 1 μg/mL, respectively, followed by 10 brine shrimps. Survivors were counted 24 h later, and the percentage of deaths at each dose was recorded.

Tumor cell growth inhibition assay

The cytotoxicity was examined using an MTT (4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay [11], [12]. Briefly, exponentially growing cells (human tumor cells, including HL-60 promyelocytic leukemia cells and Hepa-G2 hepatoma cells) were seeded (1×10^5 cells/mL) into a 96-well plate in triplicate and then they were pre-incubated for 4 h for cell attachment. The medium was then aspirated off and fresh medium (100 μL) containing various concentrations (20, 10, 5, 1 μg/mL) of the test compound was added to the cultures. The cells were incubated in the presence of each compound at 37 °C for 24 h under humidified air containing 5 % CO_2. Cell survival was evaluated after adding tetrazolium salt solution (1 mg MTT/mL PBS; 10 mL). After 4 h of incubation at 37 °C, DMSO (100 μL) was added to dissolve the precipitated MTT. The microplates were shaken for 15 min and then the absorbance was determined at 570 nm using a multi-well scanning spectrophotometer.

Statistical analysis

The values of cytotoxicity against HL-60 and Hepa-G2 are presented in terms of mean ± standard deviation. In the brine shrimp lethality bioassay, the data obtained were analyzed using a Finney computer program provided by Professor Jerry McLaughlin, Purdue University, Indiana, USA, for determination of LC_{50} values with 95 % confidence intervals. The raw X-ray data were processed to obtain the crystal information and the calculation of displacements for the atoms to the defined plane were using the software SHELXTL-NT V5.1 (Bruker, AXS).

Supporting information

Original spectral data for compound **2** are available as Supporting Information.

Results and Discussion

To study the cytotoxicity of the components of the Taiwan red cypress, we first assessed the effects of its MeOH extract with a BST. The BST indicated that the EtOAc-soluble portion of the MeOH extract of the Taiwan red cypress was a suitable candidate (LC_{50} = 15.36 μg/mL) for further studies of the antitumor activities of its components. **Fig. 1** is a flow diagram illustrating the BST-guided isolation.

The EtOAc-soluble portion was separated by column chromatography and HPLC, and six lignans were isolated from fractions 16 – 25, including three arylnaphthalene-type lignans [taiwanin C (**1**), 7,7′-(*S*)-dihydrotaiwanin C (**2**), and taiwanin E (**3**)], two dibenzyl-γ-butyrolactone-type lignans [savinin (**4**) and 4-(3,4-dimethoxybenzyl)-3-(4-hydroxy-3,5-dimethoxybenzyl)dihydrofuran-2-one (**5**)], and one tetrahydrofuran-type lignan [sesamin (**6**)]. In addition, two norlignans – yatersinol (**7**) and hinokiresinol (**8**) – were also isolated and identified through comparison of their UV, IR, and ^{1}H- and ^{13}C-NMR spectroscopic data with those of authentic materials [13], [14], [15], [16], [17].

Compound **2**, which is an enantiomer of the known compound 1,4-dihydrotaiwanin C (**2a**), had not been isolated from nature previously [18]. The molecular formula was $C_{20}H_{14}O_6$ based on the molecular ion at m/z = 350.0781 in its high-resolution mass spectrum. The IR spectrum of **2** revealed the presence of a lactone carbonyl group at 1750 cm^{-1}. The ^{1}H-NMR spectrum revealed five aromatic protons constituted by two singlets (each 1 H) at δ = 6.59 and 6.70 and an ABX system characteristic of a 1,2,4-trisubstituted phenyl unit at δ = 6.52 (d, J = 2.4 Hz, 1 H), 6.71 (d, J = 7.8 Hz, 1 H), and 6.74 (dd J = 2.4, 7.8 Hz, 1 H); in addition, the spectrum contained signals for a downfield methylene [δ = 3.66 (dd, J = 4.2, 22.2 Hz, 1 H); δ = 3.87 (dd, J = 4.2, 22.2 Hz, 1 H)]. Furthermore, a lactone methylene [δ = 4.82 (d, J = 16.2 Hz, 1 H); δ = 4.88 (d, J = 16.2 Hz, 1 H)] and two methylenedioxy groups [(δ = 5.88 – 5.94 (4 H)] were also observed. A comparison of these data with those of congeneric lignans [15], [16], [17], [18] revealed that compound **2** was an arylnaphthalene-type lignan, but with ring B partially hydrogenated. It is interesting to note that the germinal coupling for the two methylene protons on C-7 is fairly large (J = 22.2 Hz) and there is an unusual ^5J long range coupling constant (^5J = 4.2 Hz) between H_2-7 and H-7′(this proton displayed as a broad triplet-like a singlet when amplified), and this can be further confirmed by their ^{1}H-^{1}H COSY correlations obtained and as reported for compound 1,4-dihydrotaiwanin C (**2a**) [18]. By investigation of the X-ray structure of **2**, the two double bonds (C1 = C6 and C8 = C8′) adjacent to the H_2-7 and H-7′on the same six-membered ring can transfer the spin information between the protons via their *p*-orbitals, which may explain the long-distance coupling. **Table 1** shows the NMR spectral data and HMBC correlations of **2**. Based on the similarity of its spectral data with those of 1,4-dihydrotaiwanin C (**2a**), this new compound was assigned as **2**. The configuration at C-7′was assigned tentatively as *S*, based on its specific rotation being opposite to that of compound **2a** ([α] = + 107.4). From this analysis, compound **2** was determined to be 7,7′-(*S*)-dihydrotaiwanin C, isolated for the first time from nature.

The structure of **2** has also been confirmed using X-ray crystallography (**Fig. 2A**). In the X-ray structure of **2**, the linear four rings constructed virtually a plane (**Fig. 2B**). Furthermore, from the calculation of the analysis, **Fig. 3** exhibited the displacement of each atom to the ideal plane, built by atoms includ-

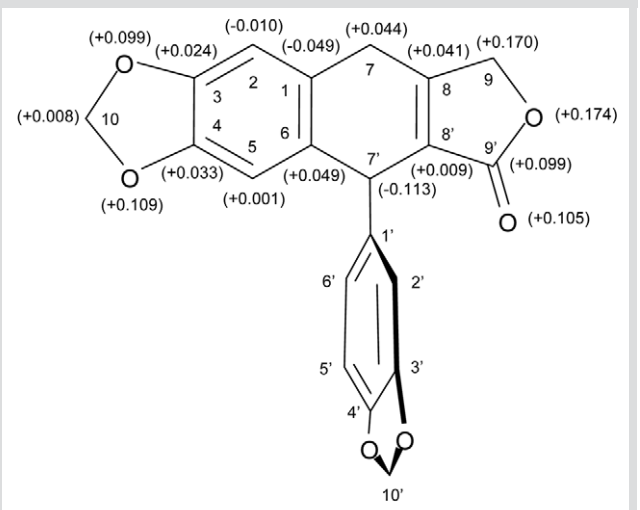


Fig. 3 Diagram of 7,7′-(*S*)-dihydrotaiwanin C (**2**). The values in parenthesis represent the displacements in angstroms (Å) of the atoms to the flat plane, built by C(1) to C(6), C(8) and C(8′).

Table 2 Cytotoxicity of lignans (**1 – 6**) and norlignans (**7, 8**) isolated from *Chamaecyparis formosensis* towards two human cancer lines HL-60 and Hepa-G2[a]

Compounds	IC_{50} (μg/mL)[b]	
	HL-60	Hepa-G2
taiwanin C (**1**)	ca. 20	> 20
7,7′-(*S*)-dihydrotaiwanin C (**2**)	4.03 ± 0.66	> 20
taiwanin E (**3**)	18.56 ± 0.40	16.68 ± 0.95
savinin (**4**)	> 20	> 20
4-(3,4-dimethoxybenzyl)-3-(4-hydroxy-3,5-dimethoxybenzyl)-dihydrofuran-2-one (**5**)	> 20	> 20
sesamin (**6**)	> 20	> 20
yatersinol (**7**)	> 20	> 20
hinokiresinol (**8**)	15.55 ± 0.51	> 20
curcumin[c]	5.21 ± 0.19	–
plumbagin[d]	–	4.54 ± 0.23

[a] HL-60 is the cell line of leukemia; Hepa-G2 is the cell line of hepatoma.

[b] IC_{50} is half-maximal inhibitory concentration represented as mean ± standard deviation; three replicates.

[c] Used as positive control for HL-60.

[d] Used as positive control for Hepa-G2.

ing C(1) to C(6), C(8) and C(8′) of **2**. The symbol of plus (+) or minus (–) represents the atom to be above or below the plane as defined. Based on the results of these calculations, the eight atoms, C(1) to C(6), C(8) and C(8′), lie within 0.049 Å of a common plane, whereas the other atoms of the four rings lie within 0.170 Å. In particular, the hexadiene ring in **2** has a fairly flat envelope form with C(7′) as the slightly out-of-plane atom (Δ = –0.113 Å), and the dihedral angle between plane C(7')–C(8′)–C(8) and plane C(7)–C(8)–C(8′) or between plane C(7′)–C(6)–C(1) and plane C(7)–C(1)–C(6) is only 6.2 °. In addition, the methylenedioxyphenyl ring, attached to C(7′), is almost perpendicular to the four rings plane with an angle of 84.0 °.

Next, we tested the six lignans **1**–**6** and the two norlignans **7** and **8** for their cytotoxicities against HL-60 and Hepa-G2 cancer cells. **Table 2** provides structure-activity relationships (SARs) for the lignans. The phenyltetralin-type (or arylnaphthalene-type) of lignans (i.e., compounds **1**, **2**, and **3**) were more potent than the dibenzylbutane- and tetrahydrofuran-type lignans (**4**, **5**, and **6**) towards HL-60 cells. The dihydrophenyltetralin lignan (i.e., compound **2** with its partially hydrogenated B-ring) displayed higher potency relative to that of the aromatic phenyltetralin-type lignans (compounds **1** and **3**). The hydroxy group at the C7 position appears to enhance the cytotoxicity of taiwanin E (**3**) relative to that of taiwanin C (**1**).

In summary, one novel lignan (**2**), 7,7′-(S)-dihydrotaiwanin C, together with 5 known lignans and 2 known norlignans, was isolated from the heartwood of *Chamaecyparis formosensis*. The structure of **2** has been further confirmed using X-ray crystallography. In addition, compound **2** exhibited significant cytotoxic activity against HL-60 cell lines *in vitro* (IC_{50} = 4.03 μg/mL) after 24 hours.

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