Neurite Outgrowth-Promoting Active Constituents of the Japanese Cypress (*Chamaecyparis obtusa*)

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Received August 30, 2007; accepted October 9, 2007

In the screening of biologically active constituents from woody plants, the methanol extract of leaves of *Chamaecyparis obtusa* showed potent neurite outgrowth-promoting activity in neuronal PC12 cells. The ethyl acetate-soluble fraction of the methanol extract showed potent activity and was separated by means of various chromatographic methods to give the two new compounds 1 and 2, as well as 11 known lignan and sesquiterpene derivatives. The structures of the new compounds were determined to be 9-*O*-acetyldihydrosesamin (1) and 9-*O*-(11-hydroxyeudesman-4-yl)dihydrosesamin (2), respectively, in NMR studies including 2D-NMR experiments. Of the 13 compounds, the known compound hinokinin (5) and the new compound 2 showed potent neurite outgrowth-promoting activity in PC 12 cells.

Key words *Chamaecyparis obtusa*; lignan; sesquiterpene–lignan conjugate; PC12 cell; neurite outgrowth-promoting activity

There are many severe social problems associated with aging and reduced brain function worldwide and especially in Japan due to lengthning of the average life span. There is currently no definitive treatment for such diseases. In the course of our biological test-guided screening of Japanese woody plant extracts, we condacted several tests of biological activity on isolated biologically active constituents from woody plants. We reported the isolation of antibacterial constituents,[1,2] antioxidative constituents,[3] and antitumor-promoting constituents[4] from woody plants. Neuronal PC12 cell[5] neurite outgrowth testing of methanol extracts of woody plants collected in the northern part of Hiroshima prefecture identified the methanol extract of leaves of the Japanese cypress (*Chamaecyparis obtusa*) as an active sample. Japanese cypress is a popular architectural wood in Japan and is cultivated all nationwide. We isolated neurite outgrowth-promoting active constituents from the leaves of the plant using biological tests to give two new compounds along with known lignan derivatives. The structures of the new compounds were determined using chemical and spectral methods including two-dimensional NMR spectrum. Some of these compounds showed potent neurite outgrowth activity. This report describes the isolation of the constituents of *C. obtusa* and determination of their structures. We also report their neurite outgrowth-promoting activity.

Results and Discussion

The methanol extract of the leaves of *C. obtusa* was fractionated to an ethyl acetate layer (AcOEt lay.), an *n*-butanol layer (*n*-BuOH lay.) and, aqueous lay. Of these, the AcOEt lay. showed the most potent neurite outgrowth-promoting activity in PC12 cells in the presence of an inactive amount of NGF, so the fraction was successively separated by means of silica gel column chromatography (SiO_2 c.c.), preparative thin layer chromatography (PLC), and HPLC using a reverse-phase column to give two new compounds **1** and **2** along with eleven known compounds **3**—**13**. Structures of the known compounds were determined by means of ^{1}H-NMR, ^{13}C-NMR, and $[\alpha]_D$ data (as shown in Fig. 1). Lignan derivatives (**3**—**10**) were identified as (−)-savinin (**3**),[6—8] (+)-sesamine (**4**),[9—11] (−)-hinokinin (**5**),[12,13] (+)-dihydrosesamin (**6**),[14,15] (−)-yatein (**7**),[16,17] (+)-piperitol (**8**),[14] (+)-xanthxylol (**9**),[18] and (+)-7-oxohinokinin (**10**),[19] respectively. Compounds **11** and **12** were identified as (−)-hinokiic acid (**11**)[20] and (+)-cryptomeridiol (**12**).[21,22] Compound **13** was identified as umbelliferone.[23]

Compound **1** was obtained as colorless needles, mp 87—89 °C (MeOH). The HR-EI-MS of **1** showed a molecular ion *m*/*z* 398.1356 $[M]^+$ compatible with a molecular formula of $C_{22}H_{22}O_7$. The ^{1}H-NMR spectrum (in $CDCl_3$) of **1** showed the presence of an acetyl group [δ 2.04 (3H, s)], two methylenedioxy groups [δ 5.94 (2H, s), 5.95 (2H, s)] , two sets of 3,4-dioxyphenyl moieties [δ 6.73 (1H, d, J=8.0 Hz), 6.66 (1H, d, J=1.5 Hz), 6.62 (1H, dd, J=8.0, 1.5 Hz), and 6.76 (2H, overlap), 6.80 (1H, d, J=1.5 Hz)], three methylene groups [δ 4.31 (1H, dd, J=11.5, 7.0 Hz), 4.15 (1H, J=11.5, 7.5 Hz), 4.05 (1H, dd, J=9.0, 6.5 Hz), 3.71 (1H, dd, J=9.0, 7.0 Hz), 2.80 (1H, dd, J=13.5, 5.0 Hz), 2.50 (1H, overlap)], and three methine groups [δ 4.76 (1H, d, J=6.0 Hz), 2.70 (1H, m), 2.50 (1H, overlap)]. The ^{13}C-NMR spectrum of **1** showed the presence of an acetoxy group (δ_C 20.8, 170.9), 12 aromatic carbons (δ_C 147.8, 147.7, 146.9, 145.9, 136.4, 133.6, 121.4, 119.1, 108.8, 108.2, 108.0, 106.1), three carbinyl carbons (δ_C 83.0, 72.6, 62.5), three alkane carbons (δ_C 49.1, 42.3, 33.1), and methylenedioxy groups (δ_C 100.9). These data show that **1** is lignan monoacetate. The HMBC spectrum shown in Fig. 2 indicates that the structure of **1** is 9-acetoxy-3,4:3′,4′-bismethylenedioxy-7,9′-epoxylignan. Thus **1** should be an acetate of dihydrosesamin (**6**). Compound **1** ($[\alpha]_D$ +20.2°) was identified as an acetate of **6** in all respects including optical rotation ($[\alpha]_D$ +17.2°). These findings indicated that the configuration of **1** is identical to that

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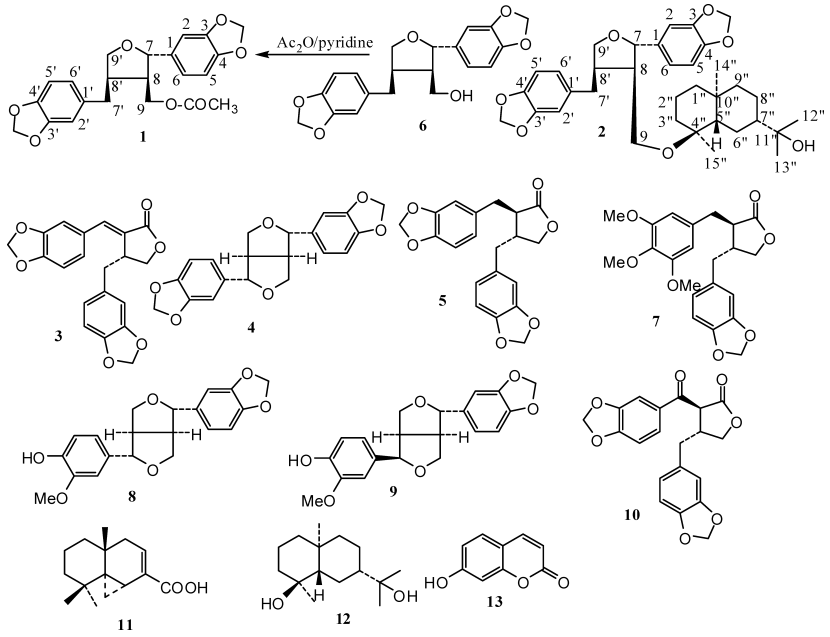


Fig. 1. Strucures of the Constituents of *C. obtuse* and a Chemical Transformation

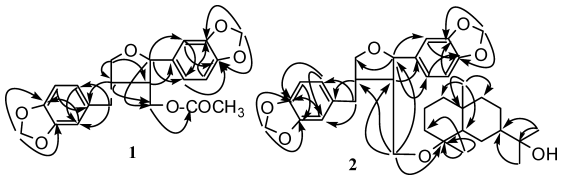


Fig. 2. Selected HMBC Correlations for **1** and **2**

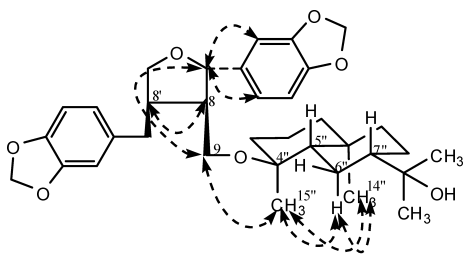
of **6**. Thus the structure of **1** was determined to be (7*S*,8*R*,8′*R*)-9-*O*-acetyldihydrosesamin.

Compound **2** was obtained as a colorless amorphous solid. The HR-FAB-MS of **2** showed a pseudo-molecular ion *m*/*z* 601.3137 $[M+Na]^+$ compatible with the molecular formula $C_{35}H_{46}O_7Na$. The ^{1}H-NMR spectrum of **2** showed the presence of four singlet methyl groups [δ 1.18 (3H, s), 1.16 (3H, s), 1.10 (3H, s), 0.90 (3H, s)], two sets of 3,4-methylenedioxyphenyl groups [δ 5.86 (4H, s), 6.68 (1H, d, J=1.5 Hz), 6.72 (1H, d, J=8.0 Hz), 6.62 (1H, dd, J=8.0, 1.5 Hz), 6.85 (1H, J=1.5 Hz), 6.74 (1H, d, J=8.5 Hz), 6.78 (1H, dd, J=8.5, 1.5 Hz)], as well as methine and methylene groups like those of dihydrosesamine (**6**) and criptomeridiol (**12**) (Table 1). The ^{13}C-NMR spectral data (Table 1) of **2** showed a summation of carbon chemical shifts belonging to **6** and **12**. These indicate that **2** is a condensation product of **6** and **12**. The HMBC spectrum of **2** showed the presence of units of **6** and **12**, as shown in Fig. 1. The H-9 methylene protons of the dihydrosesamin portion showed an HMBC correlation with C-4″ of the criptomeridiol portion. One of the H-9 protons showed a correlation with Me-15″ protons in the differential NOE spectrum (as shown in Fig. 3). These finding indicate that **2** is linked between C-9 and C-4″ through an ether linkage. The ^{13}C-NMR signals of the dihydrosesamine portion showed almost the same signal pattern as those of **6**, except for the carbons near the connection point. Differential NOE for **2** showed correlations between Me-15″ and Me-14″, Me-14″ and H-6″α as well as Me-15″ and H-6″α as shown in Fig. 3. H-6″α gave a quartet signal at δ 0.99 with a large coupling constant with H-5″ (J=10.0 Hz) and the H-7″ (J=10.0 Hz). This indicates that Me-14″, Me-15″ and H-6″α have 1,3-diaxial correlations with each other and the isopropyl group at C-7 is equatorial. Thus the relative configuration of the sesquiterpene portion of **2** was identified as that of criptomeridiol (**12**). The optical rotation of criptomeridiol (**12**) isolated here is $[\alpha]_D$ +17.0°, which shows the opposite sign to the reported $[\alpha]_D$ −17.9° for (4*R*,5*R*,7*R*,10*R*)-criptomeridiol.[21] The absolute stereochemistry of the criptomeridiol portion of **2** should be the same as that of the isolated **12**. Thus the structure of **2** was determined to be (7*S*,8*R*,8′*R*,4″*S*,5″*S*,7″*S*,10″*S*)-9-*O*-(11-hydroxyeudesman-4-yl)-dihydrosesamin.

Compounds isolated from *C. obtusa* were examined for neurite outgrowth-promoting activity in neuronal PC12 cells in the presence or absence of NGF (2 ng/ml). The percentage of neurite-bearing cells to the total number of cells counted was examined in each culture well. The concentration of NGF added weakly induced neurite outgrowth, showing activity of 7.1±2.6%. Of the known lignan derivatives, hinokinin (**5**) and sesamin (**4**) showed potent neurite outgrowth-promoting activities: 76.0±6.0% and 46.8±3.9% at 10 μg/ml, and 50.9±2.6% and 25.2±4.1% at 5 μg/ml, respectively, when cultured with NGF. The new compound **1** and dihydrosesamin (**6**) showed weak activity: 19.9±0.9% and 16.0±0.1% at 5 μg/ml with NGF. Hinokinin (**5**) also showed neurite outgrowth-promoting activity: 33.2±5.4% at 10 μg/ml and 16.5±2.6% at 5 μg/ml without NGF. The new compound **2** showed cytotoxicity (20% cell survival) for PC12 cells at the concentration of 5 μg/ml. However, **2** showed more potent neurite outgrowth-promoting activity (42.6±4.8%) at a lower concentration of 1.25 μg/ml (80% cell survival) in the presence of NGF. Compound **2** also showed activity (32.1±1.7%) at the same concentration (1.25 μg/ml) without NGF. Some lignan derivatives showed neurite outgrowth activity in PC12 cells. New compound **2**, a

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for **1** and **2** in $CDCl_3$ (500 MHz and 125 MHz)

Position	**1**		**2**	
	δ_C	δ_H (multi. Hz)	δ_C	δ_H (multi. Hz)
C-1	136.4		137.7	
2	106.1	6.80 (1H, d, 1.5)	106.4	6.85 (1H, d, 1.5)
3	147.8		147.5	
4	146.9		146.5	
5	108.0	6.76 (1H, overlap)	107.8	6.74 (1H, d, 8.5)
6	119.1	6.76 (1H, overlap)	118.9	6.78 (1H, dd, 8.5, 1.5)
7	83.0	4.76 (1H, d, 6.0)	83.1	4.77 (1H, d, 6.0)
8	49.1	2.50 (1H, overlap)	50.9	2.31 (1H, qui, 6.5)
9	62.5	4.15 (1H, dd, 11.5, 7.5)	57.2	3.49 (1H, dd, 8.5, 6.5)
		4.31 (1H, dd, 11.5, 7.0)		3.37 (1H, dd, 8.5, 7.0)
1′	133.6		134.6	
2′	108.8	6.66 (1H, d, 1.5)	109.0	6.68 (1H, d, 1.5)
3′	147.7		147.6	
4′	145.9		145.7	
5′	108.2	6.73 (1H, d, 8.0)	108.1	6.72 (1H, d, 8.0)
6′	121.4	6.62 (1H, dd, 8.0, 1.5)	121.4	6.62 (1H, dd, 8.0, 1.5)
7′	33.1	2.80 (1H, dd, 13.5, 5.0)	33.0	2.85 (1H, dd, 13.5, 4.5)
		2.50 (1H, overlap)		2.49 (1H, dd, 13.5, 11.0)
8′	42.3	2.70 (1H, m)	42.7	2.61 (1H, m)
9′	72.6	4.05(1H, dd, 9.0, 6.5)	72.7	3.97 (1H, dd, 8.5, 6.5)
		3.71 (1H, dd, 9.0, 7.0)		3.68 (1H, dd, 8.5, 7.0)
O–CH_2O	100.9	5.94 (2H, s), 5.95 (2H, s)	100.8	5.86 (4H, s)
CO	170.9			
CH_3	20.8	2.04 (3H, s)		
1″			40.8	1.59 (1H, overlap)
				1.04 (1H, dt, 3.0, 11.0)
2″			19.7	1.58 (1H, overlap)
				1.50 (1H, tq, 2.5, 11.5)
3″			37.6	1.82 (1H, dt, 10.0, 2.5)
				1.38 (1H, overlap)
4″			76.1	
5″			52.3	1.33 (1H, overlap)
6″			21.6	1.97 (1H, br d, 11.5)
				0.99 (1H, q, 10.0)
7″			50.1	1.31 (1H, overlap)
8″			22.5	1.59 (1H, overlap)
				1.29 (1H, overlap)
9″			45.0	1.45 (1H, dt, 10. 5,2.5)
				1.18 (1H, overlap)
10″			34.3	
11″			72.8	
12″			26.8	1.16 (3H, s)
13″			27.3	1.18 (3H, s)
14″			18.4	1.10 (3H, s)
15″			19.1	0.90 (3H, s)



6"α-axial H; δ 0.99 (q, J = 10.0 Hz)
$J_{5",6"\alpha}$ = 10.0 Hz
$J_{6"\alpha,7"}$ = 10.0 Hz
$J_{6"\alpha,6"\beta}$ = 10.0 Hz

Fig. 3. Selected NOEs of **2**

lignan–sesquiterpene conjugate, showed especially potent activity with and without NGF. Compound **2** is thus an interesting compound in terms of its chemical structure and biological activity.

Experimental

Melting points were recorded on a Yanagimoto micro melting point apparatus andares uncorrected. The UV spectra were recorded on a Hitachi U-2001 spectrophotometer. The IR spectra were recorded on a JASCO FT/IR-6300 spectrometer with ATR. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL α-500 NMR spectrometer (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR). Chemical shifts were shown as δ-values (ppm) with tetramethyl silane (TMS) as an internal standard. HR-EI-MS and HR-FAB-MS data were measured on a JEOL HX110 mass spectrometer. $[\alpha]_D$ was measured on a JASCO P-1010 polarimeter at 25°. Analytical and preparative HPLC was carried out using a reverse-phase column (Mightysil RP-18, Kantho Chemical Co., Ltd.) with a CH_3CN–H_2O solvent system. TLC was performed using precoated silica gel $60F_{254}$ (Merck, 200×200×0.25 mm for analysis

and 200×200×0.5 mm for the preparation of constituents).

Plant Materials Leaves of the Japanese cypress (*C. obtusa*) were collected in October 2003 in the mountainous northern part of Hiroshima prefecture, Japan. The plants were identified by Mr. T. Sano of the Hiroshima Prefectural Forestry Research Center. Voucher specimens have been deposited in the laboratory of the Prefectural University of Hiroshima.

Extraction and Isolation Powdered leaves (2.3 kg) of *C. obtusa* were extracted with methanol (MeOH) under reflux to give an MeOH extract, which showed strong neurite outgrowth activity. The MeOH extract was poured into water and extracted with AcOEt to give an AcOEt extract. The residual aqueous solution was extracted with *n*-BuOH to give an *n*-BuOH extract and an aqueous layer. These three fractions were examined for neurite outgrowth activity, and the AcOEt fraction showed the most potent activity. The AcOEt fraction (127 g) was therefore chromatographed on a silica gel column using a chloroform ($CHCl_3$)–MeOH gradient-solvent system to give many fractions, which were compiled into 12 fractions (COMA-1—COMA-12) according to TLC profiles. COMA-2 (50 g) was chromatographed on a silica gel column using a gradient *n*-hexane–AcOEt solvent system to give 10 fractions. These fractions were further successively purified by column chromatography, preparative layer chromatography (PLC), and HPLC using a reverse-phase column (ODS) to give compounds **1** (8 mg), **3** (254 mg), **4** (1.34 g), **5** (190 mg), **7** (48 mg), **10** (154 mg), and **11** (4.0 g). COMA-3 (27.0 g) was chromatographed on a silica gel column using a gradient-*n*-hexane–AcOEt solvent system to give 10 fractions. These fractions were further purified by HPLC using an ODS column and CH_3CN–H_2O solvent system to give compounds **1** (17 mg), **2** (28 mg), **4** (1.2 g), **6** (250 mg), **7** (590 mg), **8** (70 mg), **9** (200 mg), **10** (295 mg), and **11** (270 mg). COMA-4 (2.2 g) was chromatographed on a silica gel column using a gradient *n*-hexae–AcOEt solvent system to give 8 fractions. These fractions were further successively purified by PLC and HPLC to give **4** (21 mg), **6** (50 mg), **12** (27 mg), and **13** (26 mg).

Compound **1**: Colorless needles (from MeOH). mp 87—89 °C. HR-EI-MS: *m/z* 398.1356 $[M]^+$ (Calcd for $C_{22}H_{22}O_7$: 398.1356), $[\alpha]_D^{25}$ +20.2°. (*c*=0.034, MeOH), UV (MeOH) λ_{max} (ε) 236 (5062), 287 (6049). IR (ATR) cm^{-1}: 2894, 1741, 1240, 1032, 938. ^1H- and ^{13}C-NMR spectral data are shown in Table 1.

Compound **2**: Colorless amorphous solid. HR-FAB-MS; *m/z* 601.3137 $[M+Na]^+$ (Calcd for $C_{35}H_{46}O_7Na$: 601.3144). $[\alpha]_D^{25}$ −8.3° (*c*=0.024, MeOH). UV (MeOH) λ_{max} (ε) 237 (5439), 287 (6746). IR (ATR) cm^{-1}: 3453, 2928, 1243, 1036, 926. ^1H- and ^{13}C-NMR spectral data of **2** are shown in Table 1.

Acetylation of Compound 6 Compound **6** (50 mg) was acetylated with acetic anhydride (0.5 ml) and pyridine (0.5 ml) at room temperature overnight. The reaction mixture was poured into ice water. The precipitates were filtered and washed with water to give an acetate, which was identified as compound **1** in all respects including optical rotation, $[\alpha]_D^{25}$ +17.2° (*c*=0.02, MeOH).

Neurite Outgrowth Activity Test Rat PC12 cells were obtained from Riken Cell Bank (Saitama, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, NY, U.S.A.) supplemented with 10% fetal bovine serum (Filtron, Brooklyn, Australia) and 10% horse serum (Gibco BRL) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells (3.5×10^4 cells/well) were seeded in a 12-well collagen-coated plate (Iwaki, Chiba, Japan) and cultured overnight. After NGF (10 ng/ml) and various concentrations of each sample had been added to the medium, the cells were cultured for a further 48 h. All samples were dissolved in DMSO and added at a final concentration of less than 0.1% DMSO. In another experiment for determining the activity of authentic compounds, PC12 cells were differentiated in serum-free medium. Precultured cells were washed once with DMEM and then cultured in fresh serum-free DMEM containing an N-2 supplement (Gibco BRL). After 2 h, NGF (10 ng/ml) and each test compound were added to the medium. The neurite formation of PC12 cells was examined under a phase-contrast microscope, and processes with a length greater than the cell diameter were scored as neurites. The ratio of neurite-bearing cells to the total number of cells counted was examined for each culture well. Each value is expressed as the mean±S.D. from triplicate wells for each condition. Cytotoxicity was evaluated from the cell survival ratio counted in the field of view in the neurite outgrowth test.

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