Cinnamylphenols from *Phyllodium pulchellum*

Chien-Chang Shen,[†] Shr-Ting Wang,[†] Show-Yinn Tsai,[‡] Hui-Chuan Yang,[†] Bor-Jinn Shieh,[‡] and Chien-Chih Chen*[,†,§]

National Research Institute of Chinese Medicine, No. 155-1, Sec. 2, Li Nung Street, Peitou, Taipei, Taiwan, Republic of China, Institute of Chemistry, Chung Yuan University, Chung-Li, Tao-Yuan Hsien, Taiwan, Republic of China, and Graduate Institute of Chinese Medical Science, China Medical University, No. 91 Hsueh-Shih Road, Taichung, Taiwan, Republic of China

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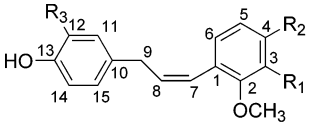
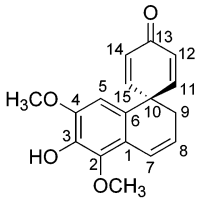
Four new cinnamylphenols, pulchelstyrenes A (**1**), B (**2**), C (**3**), and D (**4**), together with citrusinol, yukovanol, methyl piperitol, and 4-hydroxy-2,3-dimethoxybenzaldehyde were isolated from the aerial part of *Phyllodium pulchellum*. The structures of these compounds were determined by spectroscopic methods. Compounds **2, 3**, and citrusinol exhibited marginal cytotoxicity against KB cells, and citrusinol also showed mild cytotoxicity against the HepG2 cell line.

In Taiwan, *Phyllodium pulchellum* (L.) Desv. (Leguminosae) has been purported in folk medicine to treat fevers and liver fibrosis. The occurrence of some amines was reported previously in this plant.[1] In the course of our continuing study of bioactive natural products from folk medicinal plants used in Taiwan, we investigated the phenolic constituents of the H_2O-insoluble portion in a MeOH extract of *P. pulchellum*. A combination of several chromatographic techniques led to the isolation of eight phenols, which included four new cinnamylphenols, pulchelstyrenes A (**1**), B (**2**), C (**3**), and D (**4**), and four known compounds, citrusinol,[2] yukovanol,[3] methyl piperitol,[4] and 4-hydroxy-2,3-dimethoxybenzaldehyde.[5] Herein the structural characterization of four new compounds is described.



1 R_1=OCH_3, R_2=OH, R_3=H
2 R_1=OCH_3, R_2=OH, R_3=OCH_3
3 (*trans*) R_1=OH, R_2=OCH_3, R_3=OCH_3



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Compound **1** possessed the molecular formula $C_{17}H_{18}O_4$ as determined from EIMS and ^1H and ^{13}C NMR spectra. In its ^1H NMR spectrum, the chemical shifts of a double bond (δ 6.52 and 5.74) and a methylene group (δ 3.49) in the ABX_2 system were observed, which indicated the presence of an allyl functionality. The coupling constant (J_{AB} = 11.5 Hz) of the olefinic protons suggested a *cis* double bond. The ^1H NMR spectrum also showed two methoxy groups and six aromatic protons in two rings. One aromatic ring was 1,2,3,4-tetrasubstituted (AB system, J = 8.5 Hz), and the other ring was *para*-disubstituted (A_2B_2 system, J = 8.5 Hz). The positions of the substituents were further confirmed by the HMBC cross-peaks: H-5/C-1, C-3, C-4; H-6/C-2, C-4, C-7; H-7/C-2, C-6; H-8/C-1, C-9, C-10; 4-OH/C-3, C-4, C-5; 13-OH/C-13, C-12, C-14; H_2-9/C-7, C-8, C-10, C-11, C-15. The methoxy groups at δ_H 3.83 and 3.78 showed long-range correlations with carbons resonating at δ 141.1 and 152.0, respectively. The latter also correlated with H-6 and H-7, indicating a methoxy group at C-2. In addition, the carbon resonating at δ 141.1 correlated with H-5 and the carbon at δ 150.6 correlated with H-5 and H-6, which suggested that the other methoxy group was located at C-3 and a hydroxy group at C-4. The assignment of the upfield signal at δ_C 141.1 to C-3 was also supported by the report in the literature, where C-2 resonated at higher field than C-1 and C-3 in 1,2,3-trioxygenated aromatic compounds such as 2,3-dimethoxyphenol and 2,6-dimethoxyphenol.[6] The correlation of the hydroxyl group at δ_H 8.15 with C-12, C-13, and C-14 indicated that the other hydroxyl group was attached to C-13. Therefore, the structure of **1** was determined to be (*Z*)-1-(4-hydroxy-2,3-dimethoxyphenyl)-3-(4-hydroxyphenyl)propene, and this cinnamylphenol was given the trivial name pulchelstyrene A.

Pulchelstyrene B (**2**) had the molecular formula $C_{18}H_{20}O_5$, 30 mass units greater than that of **1**. The ^1H NMR spectrum suggested that compound **2** also possessed a *Z*-propene unit of cinnamylphenol in addition to five aromatic protons and three methoxy groups. Signals at δ_H 6.59 (1H, d, J = 8.0 Hz, H-5) and 6.88 (1H, d, J = 8.0 Hz, H-6) were assigned to protons on the 1,2,3,4-tetrasubstituted benzene ring, and an ABX [δ 6.71 (1H, d, J = 8.0 Hz, H-14), 6.62 (1H, dd, J = 1.5, 8.0 Hz, H-15), and 6.73 (1H, d, J = 1.5 Hz, H-11)] system was observed in the other benzene ring. The positions of substituents were further confirmed by the HMBC cross-peaks: H-5/C-1, C-3, C-4; H-6/C-2, C-4, C-7; H-7/C-2, C-6; H-8/C-1, C-10; H-11/C-9, C-10, C-12, C-13, C-15; H-14/C-10, C-12, C-13; H-15/C-9, C-11, C-13. Similar to **1**, two of three methoxy groups in compound **2** were located at C-2 and C-3, which was deduced from the HMBC correlations of the tetrasubstituted ring. In the other ring, H-11 correlated with two oxygenated carbons, which were attached to a hydroxy group and a methoxy group, respectively, and H-15 correlated only with the oxygenated carbon with a hydroxy

* Corresponding author. Phone: 886-2-28201999, ext. 6701. Fax: 886-2-28264276. E-mail: ccchen@cma23.nricm.edu.tw.
[†] National Research Institute of Chinese Medicine.
[‡] Institute of Chemistry, Chung Yuan University.
[§] China Medical University.

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group attached. Thus, the location of the third methoxy group was confirmed to be at C-12, and a hydroxyl group was attached to C-13. Consequently, the structure of pulchelstyrene B (**2**) was concluded to be (*Z*)-1-(4-hydroxy-2,3-dimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)propene.

Pulchelstyrene C (**3**) had the same molecular formula, $C_{18}H_{20}O_5$, as **2**. In its ^{1}H NMR spectrum, an ABX_2 (δ 6.57, 6.23, and 3.42) system with a large coupling constant (J_{AB} = 15.5 Hz) was attributed to the *E*-propene unit of a cinnamylphenol structure[7] with five aromatic protons and three methoxy groups. For these five aromatic protons, an AB [δ 6.66 (1H, d, J = 8.5 Hz, H-5) and 6.89 (1H, d, J = 8.5 Hz, H-6)] system in one benzene ring and an ABX [δ 6.71 (1H, d, J = 8.5 Hz, H-14), 6.65 (1H, dd, J = 2.0, 8.5 Hz, H-15), and 6.79 (1H, d, J = 2.0 Hz, H-11)] system in the other benzene ring were observed. The location of three methoxy groups on the benzene rings was deduced by HMBC and NOE difference experiments. The HMBC spectrum showed correlations of H_2-9/C-10, C-11, C-15, H-11/C-9, C-12, C-13, C-15, H-14/C-10, C-12, C-13, and H-15/C-11, C-13, which were similar to those of **2** and suggested the presence of a 3-methoxy-4-hydroxyphenyl group. Furthermore, cross-peaks of H-7/C-6, C-9 and H-6/C-2, C-4, C-7 were observed, and two methoxy groups at δ_H 3.74 and 3.83 showed long-range heteronuclear correlations with C-2 and C-4, which indicated that they were attached to C-2 and C-4, respectively. In addition, nuclear Overhauser effects were observed between 2-OCH_3/H-7, 4-OCH_3/H-5, and 12-OCH_3/H-11 in NOE experiments. Hence, these three methoxy groups were determined to be located at C-2, C-4, and C-12, respectively. Thus, the structure of pulchelstyrene C (**3**) was established as (*E*)-1-(3-hydroxy-2,4-dimethoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)propene.

Pulchelstyrene D (**4**) was isolated as a brown amorphous powder and gave a molecular formula of $C_{17}H_{16}O_4$ according to HREIMS data. The ^{1}H NMR spectrum displayed the characteristic ABX_2-type signals for the *cis*-CH_A=$CH_B$$CH_2$ moiety, in which the *cis* configuration was deduced by the coupling constant of the olefinic protons (J_{AB} = 10.0 Hz). In contrast to pulchelstyrenes A−C, the methylene group resonating at δ_H 2.47 indicated that it was linked to a sp^3-carbon (C-10) instead of an aromatic ring. In the ^{13}C NMR spectrum, the signal at δ 186.0 was derived from an $\alpha,\beta,\alpha',\beta'$-unsaturated carbonyl system, which also exhibited an absorption band at 1656 cm^{-1} in the IR spectrum. The attachment of two double bonds, resonating at δ_H 7.08 (2H, d, J = 10.0 Hz, H-11 and H-15) and 6.27 (2H, d, J = 10.0 Hz, H-12 and H-14), to the carbonyl group was confirmed by the HMBC experiment and demonstrated the correlation between H-11, H-15 and C-13. The ^{1}H HMR spectrum also exhibited two methoxy groups (δ 3.88 and 3.79) and one aromatic proton [δ 6.32 (1H, s)] from a pentasubstituted aromatic ring. In the HMBC spectrum, cross-peaks of H-8/C-1, C-10, H-9/C-6, C-10, and H-5/C-10 suggested a 1,2-dihydronaphthalene skeleton. This was also connected to the $\alpha,\beta,\alpha',\beta'$-unsaturated carbonyl system through the C-10 spiro carbon, as evidenced by the cross-peaks of H-11, H-15/C-6, C-9, C-10 and H-12, H-14/C-10. The location of two methoxy groups was determined by NOE difference experiments. The H-5 signal was enhanced by irradiation of the methoxy group at δ_H 3.79, and the irradiation of the methoxy group at δ_H 3.88 resulted in an enhancement of the H-7 signal, which revealed that these two methoxy groups were located at C-4 and C-2, respectively, and a hydroxyl group was at C-3. Thus the structure of pulchelstyrene D (**4**) was established as 6′-hydroxy-5′,7′-dimethoxy-2′*H*,4*H*-spiro[cyclohexa-2,5-diene-1,1′-naphthalen]-4-one.

The in vitro cytotoxicity was evaluated against both KB and HepG2 cell lines for the eight isolates obtained in the present study. Compounds **2** and **3** showed selective cytotoxicity against KB cells with IC_{50} values of 55.6 and 39.8 μM, respectively. Citrusinol exhibited cytotoxicity against both KB and HepG2 cell lines with IC_{50} values of 42.3 and 43.3 μM, respectively, while the other compounds were inactive (IC_{50} > 100 μM).

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Inova 500 spectrometer. UV spectra were measured on a Hitachi U-3200 spectrophotometer. IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrophotometer. EIMS and HREIMS spectra were obtained on Finnigan GCQ and Finnigan/Thermo Quest MAT 95XL spectrometers, respectively.

Plant Material. The aerial part of *Phyllodium pulchellum* was collected in August 2003 in Taipei. A voucher specimen (NRICM-03-021) is deposited at the herbarium of National Research Institute of Chinese Medicine.

Extraction and Isolation. The aerial part of *P. pulchellum* (6.0 kg) was cut into pieces and extracted with MeOH (3 × 40 L) three times under reflux. The combined MeOH extract was concentrated, suspended in distilled H_2O, and then centrifuged to provide H_2O-soluble and H_2O-insoluble portions. The H_2O-insoluble portion was separated by extensive column chromatography over silica gel using an *n*-hexane−EtOAc gradient system to give seven fractions. The fourth fraction, eluted with *n*-hexane−EtOAc (3:1), was further chromatographed on a Sephadex LH-20 column with MeOH followed by purification on preparative TLC plates with *n*-hexane−EtOAc (3:1) to afford four compounds, citrusinol (57.7 mg), **1** (12.5 mg), 4-hydroxy-2,3-dimethoxybenzaldehyde (3.6 mg), and **2** (10.6 mg). The fifth fraction, eluted with *n*-hexane−EtOAc (1:1), was further repeatedly chromatographed on Sephadex LH-20 (MeOH) and silica gel columns to obtain four compounds, **3** (13.6 mg), yukovanol (17.5 mg), **4** (6.3 mg), and methyl piperitol (6.0 mg).

Pulchelstyrene A (1): brown amorphous powder; IR (KBr) 3475, 2927, 1592, 1508, 1461, 1225, 1062 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 204 (4.69), 230 (sh), 277 (4.03) nm; ^{1}H NMR (acetone-d_6) δ 3.49 (2H, dd, J = 1.5, 6.0 Hz, H_2-9), 3.78 (3H, s, 2-OCH_3), 3.83 (3H, s, 3-OCH_3), 5.74 (1H, m, H-8), 6.52 (1H, d, J = 11.5 Hz, H-7), 6.62 (1H, d, J = 8.5 Hz, H-5), 6.75 (2H, d, J = 8.5 Hz, H-12 and H-14), 6.92 (1H, d, J = 8.5 Hz, H-6), 7.02 (2H, d, J = 8.5 Hz, H-11 and H-15), 8.04 (1H, s, 4-OH), 8.15 (1H, s, 13-OH); ^{13}C NMR (acetone-d_6) δ 33.9 (C-9), 60.2 (2-OCH_3), 60.3 (3-OCH_3), 111.1 (C-5), 115.5 (C-12 and C-14), 122.8 (C-1), 124.7 (C-6 and C-7), 129.3 (C-11 and C-15), 130.5 (C-8), 131.9 (C-10), 141.1 (C-3), 150.6 (C-4), 152.0 (C-2), 156.0 (C-13); EIMS *m/z* $[M]^+$ 286 (100), 271 (20), 255 (32), 199 (20), 183 (78).

Pulchelstyrene B (2): brown amorphous powder; IR (KBr) 3480, 2933, 1603, 1514, 1493, 1461, 1267, 1235, 1067 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 230 (sh), 254 (4.68) nm; ^{1}H NMR (CD_3-OD) δ 3.45 (2H, br d, J = 7.0 Hz, H_2-9), 3.78 (3H, s, 2-OCH_3), 3.79 (3H, s, 12-OCH_3), 3.83 (3H, s, 3-OCH_3), 5.75 (1H, m, H-8), 6.50 (1H, d, J = 11.5 Hz, H-7), 6.59 (1H, d, J = 8.0 Hz, H-5), 6.62 (1H, dd, J = 1.5, 8.0 Hz, H-15), 6.71 (1H, d, J = 8.0 Hz, H-14), 6.73 (1H, d, J = 1.5, H-11), 6.88 (1H, d, J = 8.0 Hz, H-6); ^{13}C NMR (CD_3OD) δ 35.3 (C-9), 56.3 (12-OCH_3), 61.1 (2-OCH_3), 61.2 (3-OCH_3), 112.3 (C-5), 113.0 (C-11), 116.2 (C-14), 121.6 (C-15), 123.8 (C-1), 125.8 (C-6 and C-7), 131.5 (C-8), 133.9 (C-10), 142.3 (C-3), 145.7 (C-13), 148.9 (C-12), 151.7 (C-4), 153.0 (C-2); EIMS *m/z* $[M]^+$ 316 (100), 301 (15), 285 (30), 162 (10); HREIMS *m/z* 316.1311, calcd for $C_{18}H_{20}O_5$ 316.1305.

Pulchelstyrene C (3): brown amorphous powder; IR (KBr) 3480, 2922, 1614, 1514, 1461, 1261, 1088 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ϵ) 228 (4.03), 272 (3.65) nm; ^{1}H NMR (CD_3OD) δ 3.42 (2H, d, J = 7.0 Hz, H_2-9), 3.74 (3H, s, 2-OCH_3), 3.82 (3H, s,

12-OCH_3), 3.83 (3H, s, 4-OCH_3), 6.23 (1H, m, H-8), 6.57 (1H, d, J = 15.5 Hz, H-7), 6.65 (1H, dd, J = 2.0, 8.5 Hz, H-15), 6.66 (1H, d, J = 8.5 Hz, H-5), 6.71 (1H, d, J = 8.5 Hz, H-14), 6.79 (1H, d, J = 2.0 Hz, H-11), 6.89 (1H, d, J = 8.5 Hz, H-6); ^{13}C NMR (CD_3OD) δ 40.2 (C-9), 56.4 (12-OCH_3), 56.7 (4-OCH_3), 61.0 (2-OCH_3), 108.7 (C-5), 113.3 (C-11), 116.2 (C-14), 117.2 (C-6), 122.0 (C-15), 125.8 (C-1), 126.1 (C-7), 130.4 (C-8), 133.4 (C-10), 140.6 (C-3), 145.8 (C-13), 146.7 (C-2), 148.9 (C-12), 149.3 (C-4); EIMS m/z $[M]^+$ 316 (100), 301 (20), 285 (40), 161 (15); HREIMS m/z 316.1302, calcd for $C_{18}H_{20}O_5$ 316.1305.

Pulchelstyrene D (4): brown amorphous powder; IR (KBr) 3520, 2927, 1656, 1614, 1493, 1461, 1309, 1120, 1088 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ϵ) 237 (4.44), 278 (3.86) nm; ^1H NMR ($CDCl_3$) δ 2.47 (2H, dd, J = 1.5, 4.5 Hz, H_2-9), 3.79 (3H, s, 4-OCH_3), 3.88 (3H, s, 2-OCH_3), 5.93 (1H, dt, J = 4.5, 10.0 Hz, H-8), 6.27 (2H, d, J = 10.0 Hz, H-12 and H-14), 6.32 (1H, s, H-5), 6.85 (1H, d, J = 10.0 Hz, H-7), 7.08 (2H, d, J = 10.0 Hz, H-11 and H-15); ^{13}C NMR ($CDCl_3$) δ 33.9 (C-9), 44.1 (C-10), 56.3 (4-OCH_3), 61.3 (2-OCH_3), 104.7 (C-5), 120.1 (C-1), 121.8 (C-7), 122.7 (C-8), 124.2 (C-6), 128.1 (C-12, C-14), 138.4 (C-3), 143.8 (C-2), 147.1 (C-4), 153.3 (C-11 and C-15), 186.0 (C-13); EIMS m/z $[M]^+$ 284 (100), 252 (20); HREIMS m/z 284.1044, calcd for $C_{17}H_{16}O_4$ 316.1305.

Cytotoxic Activity against KB and HepG2 Cells. The HepG2 cells (hepatoma cell line, HA22T) were provided by the Cell Bank of Veterans General Hospital, Taipei, Taiwan. The KB cells (epidermoid carcinoma cell line, CCRC 60017) were purchased from Food Industry Research and Development Institute, Hsinchu, Taiwan.

An MTT colorimetric assay was performed in 96-well plates. The assay was based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product, which could be measured spectrophotometrically. Tumor cells ($(1-1.5) \times 10^4$ /mL) were inoculated in each well, and the plates were incubated overnight at 37 °C in 5% CO_2. Twenty-four hours after seeding, 200 μL treated or nontreated in triplicate with various concentrations of compounds was added, and the plates were incubated for 2 days. At day 3, 20 μL of MTT solution (5 mg/mL) per well was added to each cultured medium. After 4 h incubation, the medium was discarded and formazan blue formed in the cells was resolved by adding 100 μL of DMSO. The plates were read on a Dynatech MR5000 Microelisa reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. Cytotoxicity was expressed as 50% inhibitory concentration (IC_{50}) of cell growth.

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