Notes

Lignans from *Dysosma versipellis* with Inhibitory Effects on Prostate Cancer Cell Lines[⊥]

Ren-Wang Jiang,[†] Jin-Rong Zhou,[‡] Po-Ming Hon,[†] Song-Lin Li,[†] Yan Zhou,[†] Ling-Lin Li,[‡] Wen-Cai Ye,[§] Hong-Xi Xu,[#] Pang-Chui Shaw,*[,†] and Paul Pui-Hay But*[,†]

Institute of Chinese Medicine, Department of Biochemistry and Department of Biology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, People's Republic of China, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, Boston, Massachusetts 02215, Institute of Chinese Medicine and Natural Products, Jinan University, Guangzhou, People's Republic of China, and Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong Science Park, Shatin, New Territories, Hong Kong, People's Republic of China

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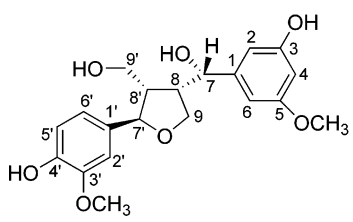
A new monoepoxylignan, dysosmarol (**1**), along with eight known compounds, podophyllotoxin (**2**), 4′-demethylpodophyllotoxin (**3**), deoxypodophyllotoxin (**4**), 4′-demethyldeoxypodophyllotoxin (**5**), diphyllin (**6**), kaempferol, quercetin, and β-sitosterol, were isolated from the roots of *Dysosma versipellis*. The structure of **1** was elucidated by spectroscopic methods. Aryltetralin lignans **2**−**4** showed the most potent inhibitory activities against the growth of androgen-sensitive (LNCaP) and androgen-independent (PC-3) human prostate cancer cell lines, with IC_{50} values in the ranges 0.030−0.056 and 0.032−0.082 μM, respectively. A quantitative HPLC analysis showed that compound **2** occurred at the highest concentration in the plant (37.21 mg/g) followed by compound **4** (5.01 mg/g) and compound **3** (2.75 mg/g). Furthermore, *D. versipellis* roots contain a similar content of compound **2** as compared with the rhizomes and roots of *Podophyllum hexandrum*, a commercial source of the lignan. Thus, cultivation of *D. versipellis* in suitable locations may serve as an alternative source for podophyllotoxin (**2**) production.

Lignans, with carbon skeletons composed of C_6-C_3 units linked in various modes, are present in different terrestrial and marine organisms.[1] Plants of the genus *Podophyllum* are rich sources of aryltetralin-type lignans. This type of compounds has stimulated considerable interest because of their antitumor,[2] antimitotic,[3] antiviral,[4] and insecticidal[5] activities. Furthermore, podophyllotoxin (**2**), a major bioactive lignan, has been used as a starting compound for the semisynthesis of etoposide, etopophos, and teniposide, which are used in the treatment of small-cell lung carcinoma and testicular cancer.[6−8]

Dysosma versipellis (Hance) M. Cheng, a herbaceous perennial species that grows in the understory of mixed evergreen and deciduous forests in China, belongs to the same family, Berberidaceae, as *Podophyllum*.[9] In some folk remedies, *D. versipellis* is used as a substitute for *Podophyllum hexandrum* Royle. However, only limited studies have been performed on the chemical components in *D. versipellis*.[10]

In the present investigation, a new compound, dysosmarol (**1**), was isolated and characterized from the roots of *D. versipellis* along with eight known compounds, podophyllotoxin (**2**), 4′-demethylpodophyllotoxin (**3**), deoxypodophyllotoxin (**4**), 4′-demethyldeoxypodophyllotoxin (**5**), diphyllin (**6**), kaempferol, quercetin, and β-sitosterol. The effects of these compounds were evaluated on growth inhibition of prostate cancer cells in vitro, and the amount of the most active components (**2**−**4**) was determined by HPLC analysis.



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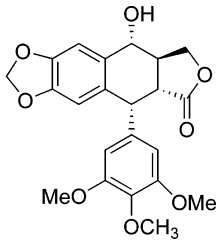

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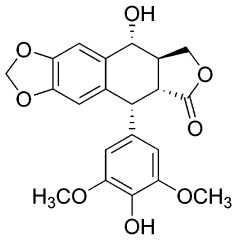

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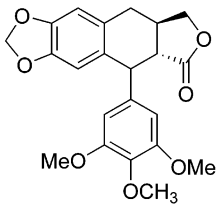

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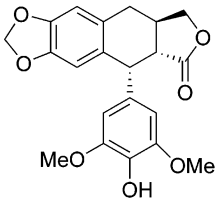

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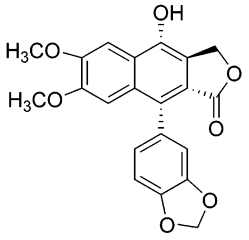

6

Compound **1** was isolated as a white, amorphous powder. HRESIMS analysis indicated a quasimolecular ion $[M + Na]^+$ at *m*/*z* 399.1419, corresponding to a molecular formula of $C_{20}H_{24}O_7$

[⊥] Dedicated to Professor Thomas C. W. Mak of the Chinese University of Hong Kong on the occasion of his 70th birthday.

* Corresponding authors. (P.-C.S) Tel: (852) 2609-6803. Fax: (852) 2603-5123. E-mail: pcshaw@cuhk.edu.hk. (P.P.-H.B.) Tel: (852) 2609-6299. Fax: (852) 2603-5646. E-mail: paulbut@cuhk.edu.hk.

[†] The Chinese University of Hong Kong.

[‡] Harvard Medical School.

[§] Jinan University.

[#] Hong Kong Jockey Club Institute of Chinese Medicine.

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Table 1. NMR Data for Dysosmarol (**1**)[a]

position	δ_C, mult.	δ_H (*J* in Hz)	HMBC (H to C)
1	136.16, qC		
2	111.63, CH	6.86, s	1, 3, 6, 7
3	147.15, qC		
4	115.97, CH	6.73, s	3, 6
5	148.97, qC		
6	120.79, CH	6.73, s	1, 2, 5, 7
7	76.61, CH	4.47, d (8.5)	8′, 1, 2, 6, 8, 9
8	50.81, CH	2.52, m	7′, 9′, 1, 7
9	71.48, CH_2	α: 3.92, dd (8.0, 8.6)	
		β: 4.24, dd (4.3, 8.6)	7, 8, 7′, 8′
1′	134.81, qC		
2′	111.18, CH	6.91, d (1.2)	1′, 3′, 4′, 5′, 6′, 7′
3′	149.01, qC		
4′	147.15, qC		
5′	115.97, CH	6,74, d (7.9)	2′, 3′, 4′
6′	120.27, CH	6.79, dd (1.2, 7.9)	1′, 2′, 4′, 7′
7′	85.03, CH	4.61, d (7.4)	1′, 2′, 6′, 8′, 9′, 8, 9
8′	53.51, CH	1.88, m	1′, 7′, 9′, 7, 8
9′	62.35, CH_2	α: 3.21, dd (5.5, 11.3)	7′, 8′, 8
		β: 3.29, m	
OCH_3 (3)	56.43, CH_3	3.81, s	3
OCH_3 (3′)	56,47, CH_3	3.84, s	3′

[a] Recorded in CD_3OD at 500 MHz (^1H NMR) and 125 MHz (^{13}C NMR). Carbon multiplicity was obtained from a DEPT experiment.

with nine units of unsaturation. The ^1H NMR spectrum (Table 1) indicated the presence of two aromatic methoxy groups at δ_H 3.81 (3H, s) and 3.84 (3H, s), four geminal protons attached to carbon atoms bearing an oxygen functionality at δ_H 3.92 (1H, dd, $J = 8.0$, 8.6 Hz, H-9α), 4.24 (1H, dd, $J = 4.3$, 8.6 Hz, H-9*β*), 3.21 (1H, dd, $J = 5.5$, 11.3 Hz, H-9′α), and 3.29 (1H, m, H-9′*β*), and two methines and two oxymethines at δ_H 2.52 (1H, m, H-8), 1.88 (1H, m, H-8′), 4.47 (1H, d, $J = 8.5$ Hz, H-7), and 4.61 (1H, d, $J = 7.4$ Hz, H-7′). In the low-field region, six aromatic protons could be divided into two groups, with the first showing an ABX coupling system assignable to a 1,3,4-trisubstituted benzene ring that appeared at δ 6.91 (1H, d, $J = 1.2$ Hz, H-2′), 6.79 (1H, dd, $J = 1.2$, 7.9 Hz, H-6′), and 6.74 (1H, d, $J = 7.9$ Hz, H-5′) and the second including two broad singlets at δ 6.73 (2H, s, H-4 and H-6) and 6.86 (1H, s, H-2), indicating a 1,3,5-trisubstituted benzene ring. Analysis of the ^{13}C NMR spectrum of **1** revealed 12 aromatic carbon atoms, two methoxy groups, two oxymethylenes, and four methines, with two of these attached to oxygen atoms. These spectroscopic data were consistent with **1** being a monoepoxy-type lignan.[11]

The full NMR assignments and connectivities of **1** were determined by $^1H-^1H$ COSY, HSQC, and HMBC spectroscopic data analysis. The ^1H-H COSY spectrum showed three spin systems: (i) H-7 → H-8 → H_2-9; (ii) H-7′ → H-8′ → H_2-9′, and (iii) H-8 → H-8′, which connected (i) and (ii). The HMQC spectrum revealed that the proton at δ_H 4.61 (H-7′) is attached to the carbon at δ 85.0 (C-7′), and the HMBC spectrum showed that H-7′ was correlated to C-8, C-8′, and C-9, suggesting that a tetrahydrofuran unit was formed by ring closure involving an oxygen atom bridged to C-7′ and C-9. Furthermore, the HMBC correlations of H-7′ to C-1′, C-2′, and C-6′, in addition to the ABX coupling system (H-2′, H-5′, and H-6′), established that the tetrahydrofuran unit was attached to the 1,3,4-trisubstituted benzene ring at C-1. Similarly, the HMQC spectrum revealed that the proton at δ_H 4.47 (H-7) was correlated to the C-7 signal at δ 76.6, and the HMBC spectrum showed that H-7 was correlated to C-8, C-8′, C-9, C-1, C-2, and C-6, suggesting that C-7 is connected to the tetrahydrofuran ring and the 1,3,5-trisubstituted benzene ring through a C-1 → C-7 → C-8 linkage.

The relative configuration of **1** was established from the ROESY spectrum. The key ROESY correlations of **1** are shown in Figure 1. The three-dimensional structure was constructed by Chem3D Pro 9.0. Only hydrogen atoms binding to the chiral carbons are shown for clarity. The correlations between H-7 → H-8, H-7 → H-8′, and H-7 → H-9*β* indicated that H-7, H-8, H-8′, and H-9*β* were all oriented on one side. The correlation of protons between H-7′ → H-9α indicated H-7′ and H-9α were mutually oriented on the other side. Accordingly, the relative configuration of **1** was established as 7*S*, 8*S*, 8′*S*, 7′*R* (**1a**) and 7*R*, 8*R*, 8′*R*, 7′*S* (**1b**), respectively. It is interesting to note that the steric energy of **1a** (12.117 kcal/mol) was lower than that of **1b** (20.794 kcal/mol), as calculated by Chem3D MM2 software,[12] which indicates that **1a** is more stable than **1b**, and thus the configuration **1a** for **1** is shown.

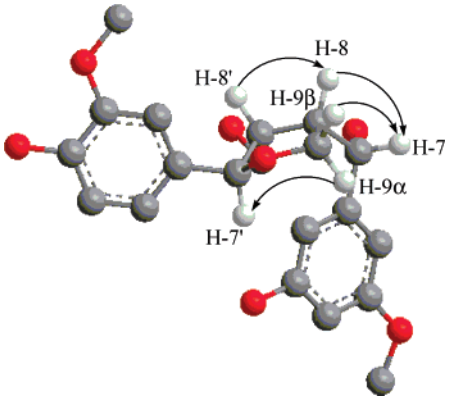

Figure 1. Key ROESY NMR correlations of compound **1**.

Table 2. Inhibitory Effects of Lignans **1**−**6** on Prostate Cancer Cell Lines

compound	IC_{50} (μM) LNCaP	IC_{50} (μM) PC-3
dysosmarol (**1**)	16	16
podophyllotoxin (**2**)	0.031	0.034
4′-demethylpodophyllotoxin (**3**)	0.056	0.082
deoxypodophyllotoxin (**4**)	0.03	0.032
4′-demethyldeoxypodophyllotoxin (**5**)	0.15	0.14
diphyllin (**6**)	11.5	7.2

Monoepoxy-type lignans occur in a variety of plants and include tanegool from *Helianthus annuus* L.,[11] (−)-tanegool from *Brassica fruticulosa* Cirillo,[13] and 7′-hydroxylariciresinol from *Taxus yunnanensis* W.C. Cheng & L.K. Fu[14] and *Araucaria angustifolia* (Bertol.) Kuntze.[15] All these compounds share the same skeleton as that of **1** but possess a 1,3,4-trisubstitution pattern in both phenyl rings and different configurations at the chiral centers. Compound **1** represents the first monoepoxy-type lignan bearing a 1,3,5-trisubstituted phenyl ring. Accordingly, **1** was proposed to be a new lignan and has been accorded the trivial name dysosmarol.

The structures of eight other known compounds were identified by comparing their spectroscopic data (UV, ESIMS, ^1H and ^{13}C NMR) with those of reported values and found to be podophyllotoxin (**2**),[16] 4′-demethylpodophyllotoxin (**3**),[16] deoxypodophyllotoxin (**4**),[16] 4′-demethyldeoxypodophyllotoxin (**5**),[16] diphyllin (**6**),[17] kaempferol,[18] quercetin,[18] and *β*-sitosterol.[19]

Aryltetralin lignans **2**−**4** showed potent inhibitory activity against the prostate cell lines LNCaP and PC-3, with IC_{50} values in the ranges 0.030−0.056 and 0.032−0.082 μM, respectively. The monoepoxylignan **1** and compounds **5** and **6** exhibited less potent activity (Table 2). Compound **6** has been reported to show potent cytotoxicity against KB cells,[20] but it exhibited only weak activity against the prostate cell lines.

Compounds **2**−**4** exhibited the most potent activity. Thus, a HPLC-UV method was established for the quantification of their contents in the roots of *D. versipellis* from three locations. Linear calibration curves were obtained for **2** ($t_R = 30.5$ min, $y = 2281.7x - 16.25$, $r^2 = 0.9998$), **3** ($t_R = 22.8$ min, $y = 2010.9x - 66.81$, $r^2 = 1.0000$), and **4** ($t_R = 42.2$ min, $y = 2816.5x + 61.99$, $r^2 = 0.9999$). Compound **2** showed the highest content (average 37.21 from 61.73, 39.22, and 10.69 mg/g in the samples of Zhejiang,

Yunnan and Anhui Provinces of China, respectively) followed by **4** (average 5.01 from 6.13, 6.03, and 2.86 mg/g) and **3** (average 2.75 from 3.16, 1.93, and 3.17 mg/g).

Currently, a commercial source of podophyllotoxin (**2**) is the rhizomes and roots of *Podophyllum hexandrum*. However, excessive collection of this plant has made it an endangered species.[21] Total synthesis of compound **2** has been achieved,[22,23] but is not economical. The results of the present study not only have shown that the roots of *D. versipellis* contain a similar concentration level of **2** (average 3.72%) as compared to the roots and rhizomes of *P. hexandrum* (4.25−5.22%)[24] but also have revealed that the concentration level of **2** varied in plants from different sources. A recent study by capillary electrophoresis showed that the distribution of **2** also varied in different parts of this plant.[25] Since some localities may produce *D. versipellis* with a high content of **2**, through cultivation, this plant may be used as an alternative source of podophyllotoxin. We do not recommend the collection of the wild plant, as *D. versipellis* has also become an endangered species in recent years,[26] and the chemical contents in wild plants may vary extensively.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 341 polarimeter in MeOH solution. The UV spectra were obtained on an online Beckman 168 DAD spectrophotometer. NMR spectra were obtained (^{1}H, ^{13}C, DEPT, ROESY, ^{1}H−^{1}H COSY, HSQC, and HMBC) on a Bruker spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. Chemical shifts are reported in ppm with reference to the solvent signals CD_3OD (δ_H 4.87 and δ_H 49.2), and coupling constants are in Hz. ESIMS were recorded on a Finnigan MAT TSQ 7000 instrument in a negative mode. HRESIMS measurements were made on an API QSTAR PULSAR*i* system Q-TOF mass spectrometer. Column chromatography was performed with silica gel (Merck, Germany). TLC was performed on precoated silica gel 60 F_{254} plates (0.2 mm thick, Merck), and spots were detected by UV illumination or by spraying with vanillin−H_2SO_4 (1%) and 1% $FeCl_3$ reagents.

Plant Material. The plants were collected in Panan, Zhejiang Province (ICM2006-2985), Haozhou, Anhui Province (ICM2006-2987), and Dongshan, Yunnan Province (ICM2006-2990), People's Republic of China, in May 2004 by Dr. Hui Cao. The samples were identified carefully by morphological characteristics. Voucher specimens were deposited in the Museum of Chinese Medicine, Institute of Chinese Medicine, The Chinese University of Hong Kong.

Extraction and Isolation. The dried roots (1 kg) of *D. versipellis* from Zhejiang Province were chopped into small pieces (2−4 mm) and refluxed with 95% EtOH (3 L × 2) for 2 h. After removing the solvent, the residue was partitioned between 10% aqueous MeOH and hexane. The MeOH layer was concentrated and suspended in distilled water (500 mL) and then extracted with CH_2Cl_2 (400 mL × 2), EtOAc (400 mL × 2), and *n*-BuOH (400 mL × 2), successively, to afford CH_2Cl_2 (21.1 g), EtOAc (23.5 g), and *n*-BuOH extracts (6.2 g), respectively.

The CH_2Cl_2 extract (20 g) was subject to silica gel column chromatography and eluted with CH_2Cl_2−EtOAc (7:3). The eluates were monitored by TLC, combined, and then recrystallized from CH_2Cl_2−MeOH to give the white crystalline **6** (300 mg).

The EtOAc extract (20 g) was fractionated by silica gel column chromatography, using elution with a CH_2Cl_2−EtOAc (7:3 → 4:6) gradient. The eluates were combined into 10 fractions (F1−F10) based on TLC profiles. Compound **4** (790 mg) was purified from F3 by crystallization in a mixture of MeOH and EtOAc. The remaining solution of F3 was evaporated to dryness, and then the residue was recrystallized in MeOH to yield β-sitosterol (94 mg). Similarly, the residue of F4 was recrystallized in EtOAc−hexane to afford crystalline **5** (50 mg), and the residue of F5 was recrystallized in EtOAc−hexane to give the light yellow powder kaempferol (600 mg). The residue of F8 was recrystallized in CH_2Cl_2 to give the light-yellow powder quercetin (190 mg), and the remaining solution was concentrated and separated by column chromatography on silica gel with CH_2Cl_2−EtOAc (7:3) as the eluent to give **2** (4.6 g). Compound **3** (1.3 g) was isolated by recrystallization of the residue of F10 in a mixture of hexane and EtOAc.

The *n*-BuOH extract (6.2 g) was subjected to silica gel column chromatography with CH_2Cl_2−EtOAc−MeOH (6:3:1) as the eluting solvent to give crude **1**, which was further purified by silica gel column chromatography using hexane−CH_2Cl_2−acetone (3:3:4) as an eluent to give pure **1** (15 mg).

Dysosmarol (1): whitish, amorphous powder; $[\alpha]_D^{20}$ +32.0 (*c* 0.1, MeOH); ^{1}H NMR (500 MHz, MeOH-d_4) and ^{13}C NMR (125 MHz, MeOH-d_4), shown in Table 1; EIMS *m*/*z* 376 $[M]^+$ (75), 358 $[M - H_2O]^+$ (30), 327 $[M - H_2O - OCH_3]$ (10); ESIMS *m*/*z* 375 $[M - H]^-$, 345 $[M - H - CH_2O]^-$, 327 $[M - H - CH_2O - H_2O]^-$, 297 $[M - H - CH_2O - H_2O - CH_2O]^-$; HRESIMS *m*/*z* 399.1419 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_7Na$, 399.1419).

HPLC Analysis. The powdered roots (0.5 g) of *D. versipellis* were extracted with methanol (10 mL) under ultrasonic conditions (20 °C, 60 Hz) for 1 h. The extracted solutions were diluted four times and filtered through a 0.22 μm PTFE syringe filter. An aliquot of the filtrate (2 μL) was injected in the HPLC instrument for analysis. Analytical HPLC was performed on a HP 1100 instrument system equipped with a G1322 degasser, a G1311A pump, a G1328 VWD detector, and a G1313A autosampler. Chromatographic separation was carried out on an Alltima C_{18} reversed-phase column (150 × 4.6 mm, 3 μm; Alltech), using a gradient solvent system comprised of water containing 0.25% acetic acid (A) and CH_3CN (B). Gradient profile: 0−15 min, 10−30% B; 15−45 min, 30−45% B; 45−60 min, 45−70% B; and 60−65 min, 70−10% B, with a flow rate of 0.8 mL/min and UV detection at 236 nm.

Calibration of Compounds 2−4. Pure compounds **2**−**4** were dissolved in methanol and diluted into appropriate concentration ranges for the construction of calibration curves. Duplicate injections were made at five concentration levels. The calibration curve of each standard was constructed by plotting the peak area versus injection amount. The amounts of **2**−**4** in the samples were calculated from the corresponding curves.

Cytotoxicity Assays. Androgen-sensitive LNCaP and androgen-independent PC-3 human prostate cancer cell lines were maintained as monolayer cultures in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U penicillin/mL, and 0.1 mg streptomycin/mL in a 95% air, 5% CO_2, and a water-saturated atmosphere. The effects of purified lignans **1**−**6** on the growth inhibition of prostate cancer cells were determined by using Cell Titer 96 Aqueous One solution reagent, MTS (Promega, Madison, WI), following a published method.[27] Briefly, cells were cultured in 96-well plates at a concentration of 3000 (PC-3) or 5000 (LNCaP) cells/well and allowed to attach overnight. Cells were then treated with compounds at the desired concentrations or the vehicle (DMSO) and incubated for 48 h. MTS (20 μL/well) was added and incubated for 2−4 h at 37 °C in 5% CO_2, and the absorbance of formazan was measured at 490 nm in a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). The experiments were performed at least three times, each in triplicate. Because podophyllotoxin (**2**) was found to show pronounced inhibitory effects against a variety of tumor cell lines including prostate cancer,[28] no additional positive control was used in this bioassay.

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