JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Lignans Isolated from *Campylotropis hirtella* (Franch.) Schindl. Decreased Prostate Specific Antigen and Androgen Receptor Expression in LNCaP Cells

HUI-YING HAN,[†] XIANG-HONG WANG,[†] NAI-LI WANG,[‡] MING-TAT LING,[†] YONG-CHUAN WONG,*[,†] AND XIN-SHENG YAO*[,§]

Cancer Biology Group, Department of Anatomy, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, SAR, China; Key Laboratory for Research and Development of Traditional Chinese Medicine and Natural Medicine, Shenzhen 518057, China; and China Institute of Traditional Chinese Medicine and Natural Products, Jinan University, 601 Huangpu Avenue West, Guangzhou 510632, China

Accumulating epidemiological data suggest that Asian men have lower incidences of prostate cancer and benign prostate hyperplasia (BPH) compared with American and European populations and may have benefited from their higher intake of phytoestrogens in their diet. However, how these phytochemicals affect prostatic diseases is still unclear. In this study, we isolated six lignans from a plant, *Campylotropis hirtella* (Franch.) Schindl., which has been used as a folk medicine for treatment of BPH in China, through bioassay guided fractionation. They were dehydrodiconiferyl alcohol (C**1**), 4-[(-6-hydroxy-2,3-dihydro-1-benzofuran-3-yl)methyl]-5-methoxybenzene-1,3-diol (C**2**), *erythro*-guaiacylglycerol-β-*O*-4′-coniferyl ether (C**3**), *threo*-guaiacylglycerol-β-*O*-4′-coniferyl ether (C**4**), secoisolariciresinol (C**5**), and prupaside (C**6**), where C**2** was identified as a new lignan analog. Their IC_{50} values for inhibition of prostate specific antigen (PSA) secretion were 19, 45, 110, 128, 137, and 186 μM, respectively, from C**1** to C**6** in LNCaP cells. Further study showed that C**1**−**5** down-regulated cellular PSA expression and C**1**−**4** also decreased androgen receptor (AR) expression in LNCaP cells. Furthermore, we investigated the proapoptotic effect of C**1** on LNCaP cells. The active forms of caspase 3 associated with the specific proteolysis of poly (ADP-ribose) polymerase (PARP) were detected, and the antiapoptotic protein Bcl-2 was down-regulated after the treatment with C**1**. These results collectively indicated that these lignans may have chemopreventive or therapeutic actions for prostate cancer through suppressing AR signaling pathway and inducing apoptosis.

KEYWORDS: Lignans; *Campylotropis hirtella* (Franch.) Schindl.; prostate specific antigen; androgen receptor; apoptosis

INTRODUCTION

Prostate cancer (PCa) is the most common malignancy in American men and second only to lung cancer in deaths (*1*). Despite the initial efficacy of androgen deprivation therapy, most patients eventually progress to hormone-refractory PCa, for which there is no curative therapy (*2*). Presently, more and more attentions begin to focus on the phytotherapeutic agents that may be more effective and safer (*3, 4*). Growing epidemiological data have reported that Asian men are less susceptible to BPH and PCa than Europeans and Americans, which may be due to their high dietary intake of phytoestrogens (*5, 6*). Phytoestrogens are widely distributed in plants and mainly fall into the three categories (i.e., isoflavones, lignans and flavornoids). The epidemiological evidence suggests that there may be some phytoestrogenic compounds in natural products that may be effective in chemoprevention or treatment of PCa and BPH. Our group previously has obtained a series of active flavonoids from *Brassica napus* L. pollen, which have a suppressive effect on prostate specific antigen (PSA) secretion in the androgen-dependent prostate cancer cell line LNCaP cells (*7*). In the present study, we further investigated the active constituents derived from a plant, *Campylotropis hirtella* (Franch.) Schindl, belonging to the family of leguminosaea, which has been used in traditional medicine for the treatment of BPH in China.

* To whom correspondence should be addressed: (X.-S. Y.) Phone: +86-20-8522-1352, +86-20-8522-5849. Fax: +86-20-8522-5849. E-mail: tyaoxs@jnu.edu.cn. (Y.-C. W.) Phone: 852 2819 9226. Fax: 852 2817 0857. E-mail: ycwong@hkucc.hku.hk.

[†] The University of Hong Kong.

[‡] Key Laboratory for Research and Development of Traditional Chinese Medicine and Natural Medicine.

[§] Jinan University.

10.1021/jf800476r CCC: $40.75
Published on Web 07/26/2008

Previous studies on this plant have reported that it contains tannins, steroids, and triterpenes compounds (*8, 9*). However, the nature of the compounds involved in regulation of PSA secretion remains unknown.

PSA is a well-accepted clinically marker for the diagnosis and prognosis of prostate diseases. It is also a functional biomarker of androgen receptor (AR) activation (*10*). AR functions as an androgen-inducible transcription factor. After its interaction with androgen, the AR is activated and translocated into the nucleus to regulate its target genes transcription. The AR/androgen signaling pathway plays critical roles in both normal prostate physiology and prostate derangement. Removal of androgen action on the prostate is the common therapy for both BPH and androgen-dependent PCa (*11*). More importantly, in many androgen-independent PCa cases, AR can be activated by low concentrations of androgens and other coactivators (*12*). This implies that AR itself could still mediate androgen-independent progression. Therefore, disrupting AR signaling is a particularly attractive approach to treat PCa. Recently, many approaches have been used to suppress AR expression or to block AR-mediated signaling, including antibodies (*13*), AR dominant negative factors (*14*), and small interfering RNAs (*15, 16*). These studies have further demonstrated that cell proliferation is inhibited by AR down-regulation. Thus, in this study, we further investigated the effects of these active compounds on cellular PSA and AR expression in LNCaP cells and their inhibition on the cell growth.

MATERIALS AND METHODS

Plant Material. Fresh roots of *Campylotropis hirtella* were collected from Kunming, Yunnan province, China, in October, 2004, and authenticated by Associate Professor Junkui Cai, Chinese Academy of Sciences in Kunming. A voucher specimen was kept in the Key Laboratory for Research and Development of New Drugs, Shenzhen 518057, China.

Extraction and Isolation. Dried roots of the plant (15 kg) were ground and extracted with 60% EtOH three times. After evaporation of the solvent in vacuo, the residues (900 g) were resuspended in water and partitioned with EtOAc and *n*-BuOH, successively. Three fractions were obtained as EtOAc-soluble, *n*-BuOH-soluble, and water-soluble fractions. The EtOAc-soluble fraction showed strong activity in decreasing the secretion of PSA in LNCaP cells compared to the two other extracts (data not shown). Thus, the EtOAc-soluble fraction was further subjected to open column chromatography on silica gel with elution by a $CHCl_3$−MeOH gradient (100:0→0:100), which resulted in 11 fractions by TLC analysis. Among these fractions, fractions 2∼7 showed various degrees of inhibitory ability on PSA secretion (data not shown). The most active fractions 4 and 6 were passed through an open Sephadex LH-20 column with $CHCl_3$−MeOH (1:1) as an eluent and then chromatographied on an open ODS column with MeOH−H_2O as an eluent (3:7→9:1). The eluents of 50% MeOH were further purified by ODS preparative HPLC (45% MeOH) after being subjected to analytical ODS HPLC with solvent MeOH−H_2O (3:7→9:1), which yielded compound **6** (C**6**) (6 mg) from fraction 4 and compounds **1** (C**1**) (23 mg), **2** (C**2**) (5 mg), **3** (C**3**) (30 mg), **4** (C**4**) (20 mg), and **5** (C**5**) (10 mg) from fraction 6.

Open column chromatography was carried out on silica gel H60 (Qingdao Haiyang Chemical Group Corp., Qingdao, China), Sephadex LH-20 (Amersham Biosciences AB) and ODS (60−80 μm, Merck), which were used as packing materials, and operated under the common pressure and room temperature conditions. Silica gel G254 was used for analytical TLC with developing solution acetone−cyclohexane (3:7→1:1), and the color reaction was carried out using 10% H_2SO_4 in ethanol under heating condition. The analytical and preparative HPLC were performed on a Shimadzu Pak with a UV detector using a Shim-pack VP-ODS column (4.6 mm × 250 mm) and a Shim-pack PREP-ODS column (10 mm × 250 mm), respectively. The flow rate was 1 mL/min for analysis and 10 mL/min for preparation; the temperature of both columns was 28 °C.

Identification of Isolated Lignans. All isolated lignans were elucidated by spectroscopic methods including optical rotation, UV spectra, ESI-MS, NMR, and HR-ESIMS. The known compounds were further confirmed by comparison with previously reported data. Optical rotations were measured using a Jasco P-1020 polarimeter. UV spectra were obtained with a Shimadzu UV2301PC UV−vis recording spectrophotometer in MeOH. NMR spectra were determined on a Bruker AVANCE 400 NMR spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C) with TMS as the internal standard. ESI-MS spectra were performed on a Bruker Esquire 2000 mass spectrometer, and HR-TOF-MS spectra were obtained on a Micromass Q-TOF mass spectrometer.

Dehydrodiconiferyl Alcohol (C1). $C_{20}H_{22}O_6$; pale yellow needle crystal. $[\alpha]D^{26}$ −2.4° (c = 0.1 MeOH). ESI-MS: m/z 381 $[M + Na]^+$. UV (CH_3OH) λ_{max} nm: 277 nm (log ε 4.54). ^{1}H NMR (400 MHz, in CD_3OD): δ 6.94 (3H, m, H-2, 2′, 6), 6.82 (1H, dd, J = 2.0, 8.2 Hz, H-6′), 6.76 (1H, d, J = 8.2 Hz, H-5′), 6.53 (1H, d, J = 15.8 Hz, H-7), 6.22 (1H, td, J = 5.8, 16.0 Hz, H-8), 5.52 (1H, d, J = 6.3 Hz, H-7′), 3.48 (1H, dd, J = 6.3, 12.4 Hz, H-8′), 4.18 (2H, dd, J = 1.4, 5.8 Hz, H-9), 3.80 (2H, m, H-9′), 3.80 (3H, s, H-2-OCH_3), 3.86 (3H, s, H-3′-OCH_3).

***erythro*-Guaiacylglycerol-*β*-*O*-4′-coniferyl Ether (C3).** $C_{20}H_{24}O_7$; buff oil. $[\alpha]D^{25}$ −1.1° (c = 0.1, MeOH). ESI-MS: m/z 399 $[M + Na]^+$, m/z 375 $[M - H]^-$. UV (CH_3OH) λ_{max} nm: 265 nm (log ε 4.45). ^{1}H NMR (400 MHz, in CD_3OD): δ 7.01 (1H, d, J = 2.0 Hz, H-2), 6.98 (1H, brs, H-2′), 6.86 (2H, brs, H-5′), 6.83 (1H, dd, J = 8.0, 2.0 Hz, H-6), 6.72 (1H, d, J = 8.0 Hz, H-5), 6.50 (1H, dt, J = 15.9, 1.4 Hz, H-7′), 6.22 (1H, dt, J = 15.9, 5.8 Hz, H-8′), 4.82 (1H, overlapped, H-7), 4.35 (1H, m, H-8), 4.18 (2H, dd, J = 5.8, 1.4 Hz, H-9′), 3.80 (2H, m, H-9), 3.80 (6H, s, H-3,3′-OCH_3).

***threo*-Guaiacylglycerol-*β*-*O*-4′-coniferyl Ether (C4).** $C_{20}H_{24}O_7$; buff oil. $[\alpha]D^{25}$ +1.0° (c = 0.1, MeOH). ESI-MS: m/z 399 $[M + Na]^+$, m/z 375 $[M - H]^-$. UV (CH_3OH) λ_{max} nm: 264 nm (log ε 4.19). ^{1}H NMR (400 MHz, in CD_3OD): δ 7.04 (1H, d, J = 1.8 Hz, H-2′), 7.02 (1H, d, J = 1.7 Hz, H-2), 7.00 (1H, d, J = 8.3 Hz, H-5′), 6.90 (1H, dd, J = 8.3, 1.8 Hz, H-6′), 6.85 (1H, dd, J = 8.1, 1.7 Hz, H-6), 6.75 (1H, d, J = 8.1 Hz, H-5), 6.52 (1H, brd, J = 15.9 Hz, H-7′), 6.25 (1H, td, J = 15.9, 5.9 Hz, H-8′), 4.87 (1H, d, J = 5.8 Hz, H-7), 4.29 (1H, m, H-8), 4.20 (2H, br d, J = 5.9 Hz, H-9′), 3.72 (1H, dd, J = 11.9, 4.0 Hz, H-9), 3.46 (1H, dd, J = 11.9, 5.4 Hz, H-9), 3.86 (3H, s, H-3-OCH_3), 3.81(3H, s, H-3′-OCH_3).

Secoisolariciresinol. (C**5**). $C_{20}H_{26}O_6$; buff oil. ESI-MS: m/z 385 $[M + Na]^+$, m/z 361 $[M - H]^-$. UV (CH_3OH) λ_{max} nm: 282nm (log ε 3.72). ^{1}H NMR (400 MHz, in CD_3OD), δ 6.65 (2H, d, J = 8.0 Hz, H-5, 5′), 6.58 (2H, d, J = 1.8 Hz, H-2, 2′), 6.54 (2H, dd, J = 1.8, 8.0 Hz, H-6, 6′), 3.58 (4H, t, J = 4.4 Hz, H-9, 9′), 3.73 (6H, s, H-3,3′-OCH_3), 1.90 (2H, m, H-8, 8′), 2.65 (2H, dd, J = 7.0, 13.8 Hz, H-7, 7′), 2.55 (2H, dd, J = 7.7, 13.8 Hz, H-7, 7′).

5, 5′-Dimethoxylariciresino (C6). $C_{22}H_{28}O_8$; colorless needle crystal. $[\alpha]D^{23}$ +4.2° (c = 0.03,MeOH). ESI-MS: m/z 443 $[M + Na]^+$, m/z 419 $[M - H]^-$. UV (CH_3OH) λ_{max} nm: 271 (log ε 3.38). ^{1}H NMR (400 MHz, in CD_3OD): δ 6.62 (2H, s, H-2, 6), 6.50 (2H, s, H-2′, 6′), 4.76 (1H, d, J = 6.8 Hz, H-7), 4.00(1H, dd, J = 6.4, 8.4 Hz, H-9′), 3.85 (1H, overlapped, H-9), 3.81 (12H, overlapped, H-3, 5, 3′, 5′-OCH_3), 3.74 (1H, dd, J = 6.0, 8.4 Hz, H-9′), 3.65 (1H, dd, J = 6.6, 8.4 Hz, H-9), 2.92 (1H, dd, J = 4.8, 13.4 Hz, H-7′), 2.72 (1H, m, H-8′), 2.48 (1H, dd, J = 11.3, 13.4 Hz, H-7′), 2.38 (1H, m, H-8).

Bioassays. *Cell Culture and Test Compounds Preparation.* LNCaP cell line was a gift from Dr. Xianpin LU (Shenzhen Chipscreen Biosciences Ltd. and Research Center for Small Molecule Drug, China). The cells were maintained at 37 °C in RPMI 1640 (Gibco, CA) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin−streptomycin (Hyclone, UT). The isolated compounds(C**1**−**6**) were dissolved in DMSO to obtain the desired concentrations.

MTS Assay. Cell viability was measured using the MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) proliferation assay kit (Promega) as described by the manufacturer. After the cells were treated with the test compounds for the designated time, the culture medium was completely discarded, and

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Data of C**2** (in CD_3OD)

position	δ_H	δ_C	position	δ_H	δ_C
1					
2	4.16(1H,ddd,*J*=10.2,3.4,2.0);3.87(1H,t,*J*=10.2)	71.0	10	2.76(1H,ddd,*J*=16.3,5.6,2.0);2.59(1H,dd,*J*=16.3,10.8)	26.1
3	3.34(1H, m)	30.6	11		104.1
4		120.5	12		160.1
5		157.0	13	6.22(1H, d,*J*=2.4)	92.3
6	6.31(1H, d,*J*=2.3)	103.6	14		157.2
7		157.9	15	5.88(1H, d,*J*=2.4)	96.5
8	6.24(1H, dd,*J*=2.3,8.3)	107.6	16		157.7
9	6.86(1H, d,*J*=8.3)	128.8	12−OCH_3	3.74(3H, s)	55.8

60 μL/well of solution was added, which consisted of 1.1 mL of 2 mg/mL MTS (Promega, WI) solution, 55 μL of 0.92 mg/mL PMS (Sigma Chemical Co, Germany) solution, and 5.5 mL of 5% FBS/RPMI 1640. They were incubated for another 1.5 h at 37 °C in the dark, and the ODs were measured at a wavelength of 490 nm.

Detection of Secreted PSA by ELISA. 10 000 cells were seeded in 96-well plates and cultured for 2 days. The cells were subsequently incubated with 4−5 different concentrations of each test compound for another 48 h. The secreted PSA in the medium was detected by ELISA, as previously described (*7*). In brief, ELISA 96-well plates (Costar) were first coated with rabbit antibody to human PSA (Biodesign, NY) overnight at 4 °C. The plates were then blocked with 1% BSA for 1 h at 37 °C. After washing with PBST, the medium and various concentrations of standard PSA were added and incubated at 37 °C for 2 h. Then, the plates were added with monoclonal antibody to human PSA (Biodesign, NY) and incubated for 1 h at 37 °C, followed by secondary antibody Goat-Anti-Mouse Ig(H+L)-HRP (Southern Biotechnology Associates, AL) incubation at 37 °C for 30 min. Finally, the plates were developed with substrate solution (1 mL of 1% OPD, 9 mL of substrate buffer, and 15 μL of H_2O_2) for 10 min. The reaction was stopped by adding 1 M H_2SO_4, and the optical densities (ODs) were measured at a wavelength of 490 nm on a microplate spectrophotometer (Molecular Devices Corp.). The mean absorbance value (OD_{490}) for each set of reference standards, controls, and samples was calculated. A standard curve was constructed by plotting the mean absorbance of each reference standard against its concentration in nanograms per milliliter. The absorbance values were placed on the *y* axis and concentrations on the *x* axis. The mean absorbance values for each specimen were used to determine the corresponding concentration of PSA in nanograms per milliliter from the standard curve. The calculated concentrations were further normalized to MTS OD values, which offset the possible influence on PSA resulted from cytotoxicity of the tested compounds. The final results were represented as the ratio of PSA released from the compound-treated cells over that of the vehicle-treated cells. Each data point represented the mean and standard deviation of triplicate experiments.

Western Blotting Assay. Experiments were carried out as previously described (*17*). Briefly, 250 000 cells were seeded in 6-well plates. After 48 h, different concentrations of test compounds were added, and the cells were incubated for the designated time. The medium was removed completely, and the cells were collected. The collected cells were then added with 30∼50 μL of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride), resuspended, and then centrifuged to afford cell lysates. Protein concentrations were measured using the DC protein assay kit (Bio-Rad, CA). Equal amounts of proteins (20 μg) were loaded into a 10% SDS polyacrylamide gel for electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Amersham, NJ). The membrane was blocked with milk for 1 h and then incubated with primary antibodies for 1 h at room temperature against AR (Santa Cruz Biotechnology, CA), PSA (DakoCytomation, Denmark), Bcl-2, Bcl-xl, Bax, Caspase-3, PARP (Cell Signaling Technology, MA), and Actin (Santa Cruz Biotechnology), which was used as an internal control. After incubating with appropriate secondary antibodies, signals were visualized by enhanced chemiluminescence (Amersham).

RESULTS

Isolation and Identification of Main Active Compounds Which Down-Regulated PSA Secretion in LNCaP Cells from *C. hirtella.* In the present study, using repeated chromatography, we analyzed the active components from *C. hirtella* by bioassay guided isolation. The root of *C. hirtella* was extracted with 60% EtOH. After evaporation of the solvent, the extract was resuspended in H_2O and partitioned with EtOAc and *n*-BuOH, successively. The EtOAc-soluble fraction, which showed significant inhibition on PSA secretion in LNCaP cells (data not shown), was subject to bioassay-guided isolation with repeated column chromatography on silica gel, Sephadex LH-20, and ODS and further purified by reversed-phase HPLC to obtain compounds C**1**−**6**. C**2** turned out to be a new compound.

C**2** was obtained as buff oil, $[\alpha]D^{25}$ −11.1° (*c* 1.0, MeOH). The HR-TOF-MS (*m*/*z* 311.0895, $[M + Na]^+$) and NMR (in CD_3OD, **Table 1**) analyses revealed the molecular formula as $C_{16}H_{16}O_5$. The UV spectrum showed its absorption λ_{max} (MeOH) is 280 nm (log ε 3.90). In combination with the HSQC spectrum, the ^{1}H NMR spectrum of C**2** exhibited signals of one methoxyl group at 3.74 (3H, s), one methane proton at δ 3.34 (1H, m), four methylene protons at δ 4.16 (1H, ddd, J = 10.2, 3.4, 2.0 Hz), 3.87 (1H, t, J = 10.2 Hz), 2.76 (1H, ddd, J = 16.3, 5.6, 2.0 Hz), and 2.59 (1H, dd, J = 16.3, 10.8 Hz), as well as five aromatic protons. These five aromatic protons appeared as an ABX coupling system at δ 6.31 (1H, d, J = 2.3 Hz), 6.24 (1H, dd, J = 2.3, 8.3 Hz), and 6.86 (1H, d, J = 8.3 Hz) and a pair of *meta*-coupled aromatic protons at δ 6.22 (1H, d, J = 2.4 Hz) and 5.88 (1H, d, J = 2.4 Hz). In the ^{13}C NMR and DEPT spectra of C**2**, 16 carbon signals were observed, which were due to one methyl (oxygenated), one methine, two methylene (one oxygenated), five aromatic methines, and seven aromatic quaternary carbons (five oxygenated). According to the ^{1}H-^{1}H COSY spectrum, the correlations between protons for one methylene at δ 4.16 (1H, ddd, J = 10.2, 3.4, 2.0 Hz), 3.87 (1H, t, J = 10.2 Hz) and the methane proton at δ 3.34 (1H, m) and between the methane proton and the protons for the other methylene at 2.76 (1H, ddd, J = 16.3, 5.6, 2.0 Hz), 2.59 (1H, dd, J = 16.3, 10.8 Hz) indicated the presence of a group

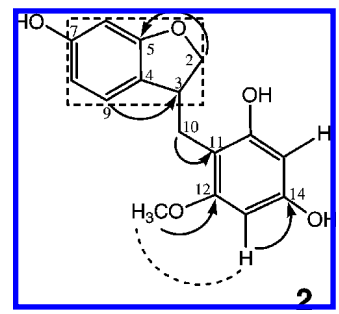

Figure 1. The HMBC and NOE correlations of C**2**.

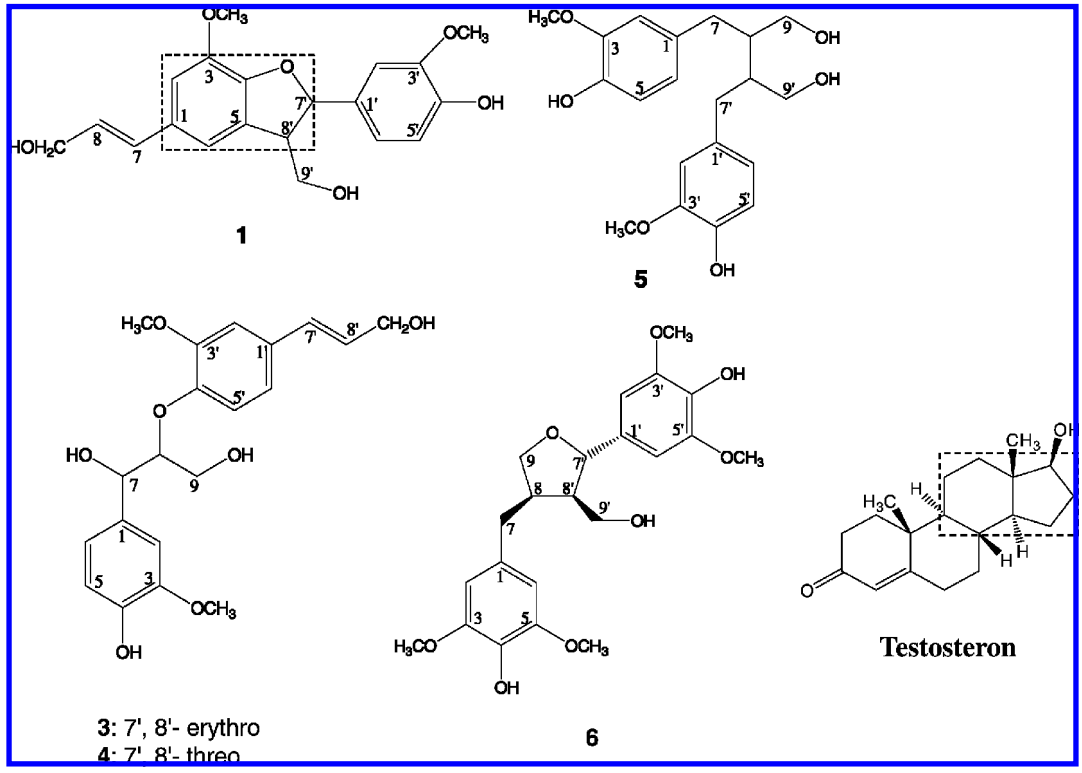

Figure 2. Five known lignans isolated from *Campylotropis hirtella* (Franch.) Schind. and testosterone.

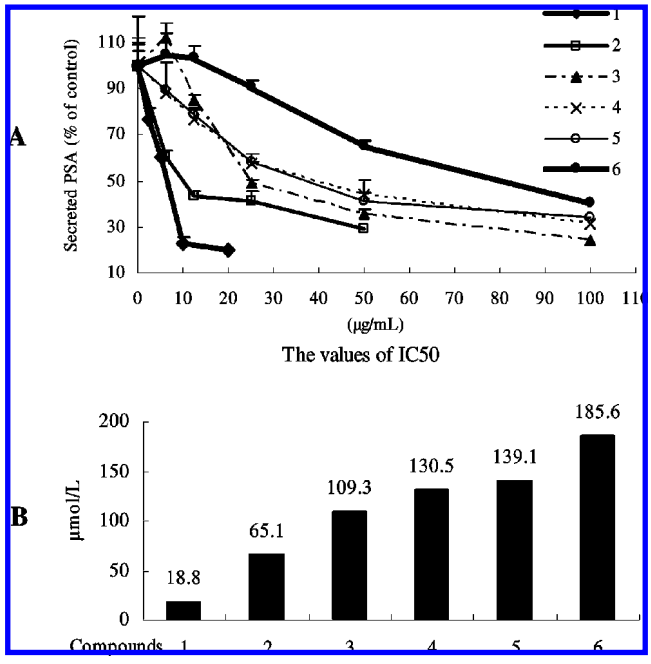

Figure 3. Six lignans inhibited the secretion of PSA in LNCaP cells. (**A**) The results were shown as a percentage of solvent control and represented as mean ± sem of the tested compounds from triplicate of experiments. (**B**) The values of IC_{50} of inhibition in PSA secretion, which were derived from the dose-dependent action trend of the correspondent compounds in **A**.

−O−CH2−CH−CH2−. The above evidence suggested that C**2** had a phenylpropane monomer. On the basis of the two-dimensional NMR spectra of C**2** (HMBC, HSQC, ^{1}H-^{1}H COSY) and the splitting patterns of aromatic protons, most proton and carbon signals in the structure were assigned. In the HMBC spectrum of C**2**, ^{13}C-^{1}H long-range correlation signals (**Figure 1A**) for δ 6.31(H-9)/δ 30.6 (C-3) and δ 4.16, 3.87 (H-2a, b)/ δ 157.0 (C-5) were observed, in combination with the unsaturation degree, which suggested the group −O−CH2−CH−CH2− was looped with the ABX coupling system phenyl and formed a benzodihydrofuran group. Furthermore, the ^{13}C-^{1}H long-range correlation between δ 2.76, 2.59 (H-10a, b) and δ 104.1(C-11) supported that the benzoterahydrofuran unit was linked to the *meta*-coupled pattern phenyl at δ 104.1(C-11) through the exocyclic methylene. In the NOESY spectrum of C**2**, the methoxyl group at 3.74 (3H, s) correlated with δ 6.22 (H-13) but not δ 5.88 (H-15) (**Figure 1**). According to HMBC correlation, the methoxyl group was attached to δ 160.1. Therefore, the carbon signal δ 160.1 should be assigned at C-12 but not at C-14. A strong ^{13}C-^{1}H long-range correlation signal between H-13 and δ 157.2 was observed, whereas no correlation between H-13 and δ 157.7 was found. Thus, δ 157.2 was assigned at C-14, and δ 157.7 was assigned at C-16. In combination with the molecular weight, the other four oxygenated aromatic quaternary carbons were hydroxyl-substituted. So, all proton and carbon signals in the structure were assigned, and C**2** was deduced as 4-[(-6-hydroxy-2,3-dihydro-1-benzofuran-3-yl)methyl]-5-methoxybenzene-1,3-diol.

The other five lignans have been also isolated from other plants (*18–23*). They were identified by means of similar analysis described above and comparison with their previously reported data (*18–23*). Their structures were dehydrodiconiferyl alcohol (C**1**) (*18*), *erythro*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether (C**3**) (*19–21*), *threo*-guaiacylglycerol-*β*-*O*-4′-coniferyl ether(C**4**) *(19–21)*, secoisolariciresinol (C**5**) (*22*), and prupaside (C**6**) (*23*) (**Figure 2**). All these known compounds were reported to be present in this plant for the first time.

Effect of the Isolated Compounds on PSA Secretion in LNCap Cells. The effects on PSA secretion in LNCaP cells have been suggested to be correlated with the response of treatment of prostate diseases (*7*). In this study, we resorted to PSA secretion as a parameter for selecting active compounds

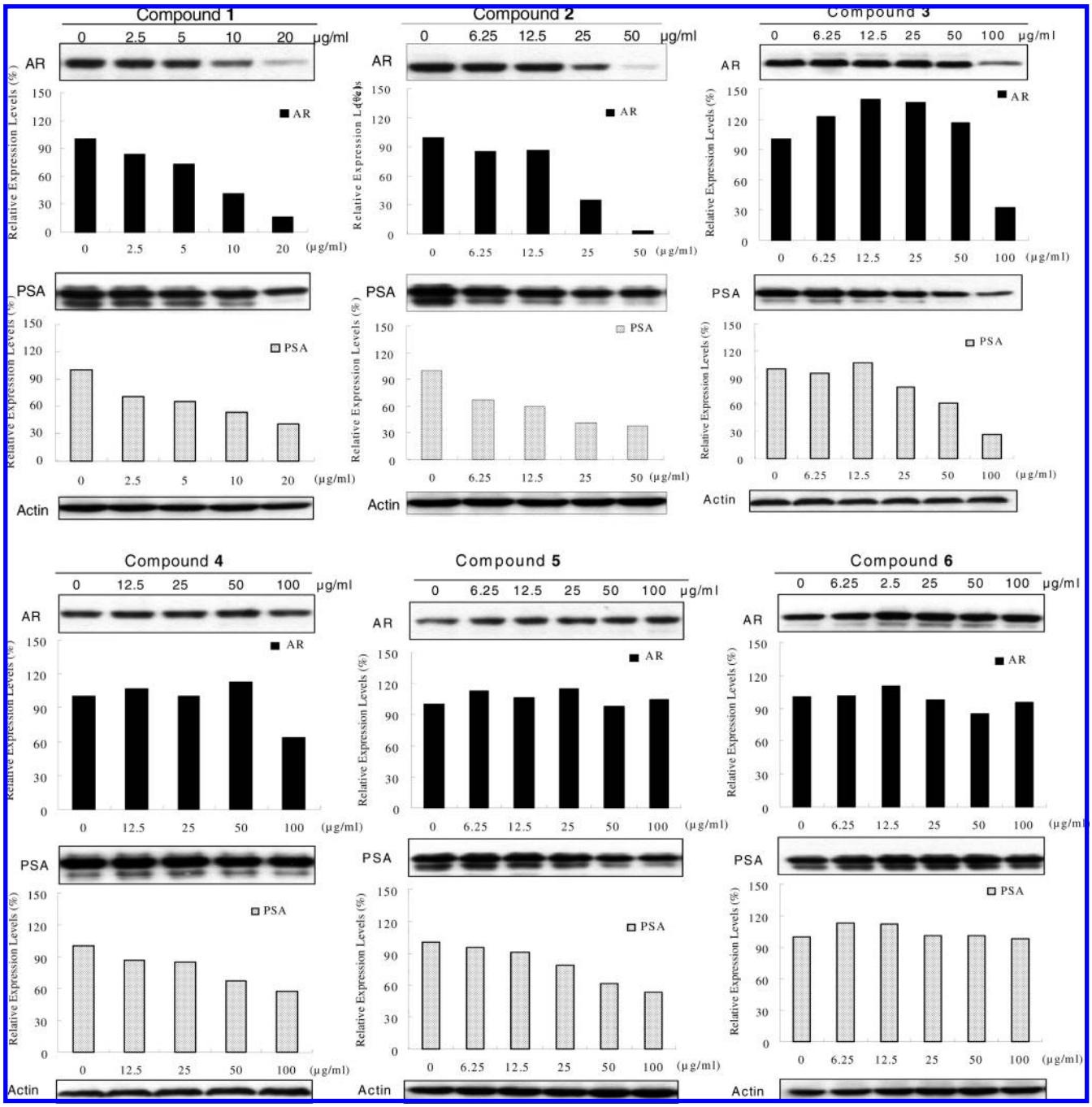

Figure 4. Western blot analysis of PSA and AR expression levels in LNCaP cells treated with the six lignans. The Actin protein band was used as a loading control and for normalization. The graphs show the normalized densitometric results in percentage of control (no treatment).

from *C. hirtella*. Considering that high level of cell cytotoxicity will complicate the interpretation of the results, we used various concentrations of the test compounds to ensure no high cytotoxicity is induced. In addition, the detected PSA concentration data were normalized to cell viability in order to eliminate the influence caused by different cell numbers, which may result from the subtle cytotoxicity of tested compounds. The six lignans (**C1**−**6**) all inhibited PSA secretion in a dose dependent manner, as shown in **Figure 3A**. Their IC_{50} values ranged from about 19 to 190 μM (**Figure 3B**). Their relative activities appeared to be **C1** > **C2** >**C3** >**C4** >**C5** > **C6** ranging from stronger to weaker, as indicated. With respect to their structures, **C1** and **C2** both have a benzoterahydrofuran unit, **C3** and **C4** are stereomers, and all of them contain phenylpropane groups. Though these compounds exhibited common characteristics structurally, modifications in other structures may contribute to their different effects on PSA secretion.

Effect of the Compounds on Cellular PSA and AR Expression in LNCap cCells. PSA is well-known to be regulated by AR. When androgen binds to AR, it will be activated and will interact with androgen response elements in the promoters of target genes such as PSA, regulating the transcription of target genes (*10*). In LNCaP cells, AR is mutated but remains functional (*24*). Since these lignan compounds decreased PSA secretion, we hypothesized that they may also decrease cellular PSA expression, which may indicate the suppression of AR transcriptional activity. Therefore, we further investigated cellular PSA and AR

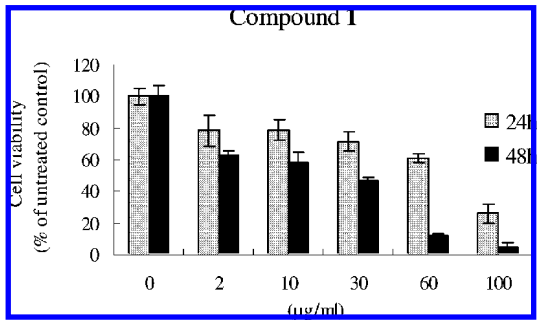

Figure 5. Cell viability of C**1** examined by the MTS assay. Results represented the OD ratio between the treated and untreated cells. Each data point is represented by mean ± standard deviations.

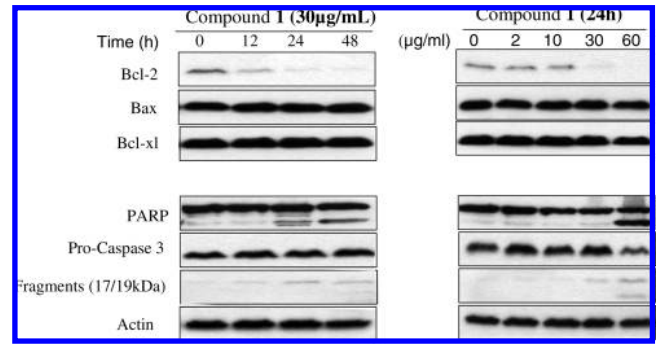

Figure 6. Effect of C**1** on the expression of apoptosis-related proteins. Apoptosis-related protein expression in LNCaP cells was examined by Western blotting after treatment with 30 μg/mL C**1** for four time points and four concentrations of C**1** for 24 h.

proteins in LNCaP cells in response to these compounds by Western blotting assay. The concentrations of the compounds used were mostly similar with those used in ELISA. **Figure 4** showed that C**1**−**5** down-regulated PSA expression. In addition, C**1**−**4** further suppressed AR expression. However, C**6** has no perceivable effect on either cellular PSA or AR expression. These data suggested that the compounds with stronger activity on inhibition of PSA secretion also significantly affected cellular PSA and AR expression.

C1 Inhibited LNCap Cell Proliferation and Induced Apoptosis. Several previous studies have indicated that AR downregulation results in significant cell growth inhibition and increase in apoptotic cells (*13–16*). To investigate whether these lignans also affect cell proliferation, we focused on C**1**, which was most effective in the above assays, and studied its antiproliferation and proapoptosis on LNCaP cells. As shown in **Figure 5**, C**1** significantly inhibited cell proliferation in a dose- and time-dependent manner by the MTS assay. The value of IC_{50} for 48 h treatment was 18.3 μg/mL (51 μM). To further investigate whether the antiproliferation effects of C**1** were caused by its activation of the apoptosis pathway, we examined the cleavages of caspase 3 and poly (ADP-ribose) polymerase (PARP). As shown in **Figure 6**, cleaved caspase 3 and PARP were detected in a dose- and time-dependent manner. Furthermore, several key apoptosis related proteins were also evaluated after the treatment with C**1**, and we found that (**Figure 6**) the antiapoptotic protein Bcl-2 was down-regulated, while there was no obvious effect on the levels of protein Bcl-xl and Bax, which indicated that C**1** could decrease the ratio between Bcl-2 and Bax. These results suggested that C**1** induced apoptosis and finally resulted in cell death.

DISCUSSION

In this study, we first found that a new lignan analog (C**2**) and five known lignans (C**1**, C**3**−**6**) from *C. hirtella* inhibited PSA secretion in LNCaP cells. Furthermore, most of these compounds down-regulated cellular PSA and AR expression. These results provided evidence for the first time that lignans, as a major subtype of phytoestrogens, can suppress AR function signaling. In addition, C**1** significantly inhibited LNCaP growth and possibly through induction of apoptosis. These results are consistent with the recently established notion that AR downregulation results in prostate cancer cell growth inhibition (*25*).

PSA is a marker widely used clinically for detecting PCa. A post-therapeutic decline in PSA also supports the prognostic outcome of the treatment for PCa (*26*). PSA itself exhibits biological functions. Previous studies indicated that, in the tumor microenvironment, PSA could potentially cleave a number of proteins that are involved in inhibiting PCa genesis and progression (*27–30*). Therefore, the decrease of circulating PSA level in PCa patients indicates suppression of PCa growth. In this study, all six tested lignans inhibited PSA secretion from LNCaP cells. These results indicate that these compounds may also decrease PSA level in vivo and may have positive effects on PCa.

It is critical to understand the mechanisms by which these compounds inhibit PSA secretion. Whether cellular PSA is also down-regulated is a conceivable reason. Given that PSA expression in cells is controlled by AR transcription activity, down-regulation of PSA may suggest that these compounds may also have effects on AR function. More importantly, AR signaling exerts crucial roles in the etiology of PCa and is an important target for the treatment of BPH and PCa (*12*). For BPH and early PCa, which are androgen-dependent, reducing the levels of circulation androgens to suppress AR/androgen signaling is an effective treatment choice. For example, finasteride, a 5α-reductase inhibitor, which prevents conversion of testosterone to its more active form 5α-dihydrotestosterone (DHT) in the prostate, is an effective drug for a significant proportion of BPH patients. Androgen deprivation is also an effective therapeutic strategy for early PCa. However, most PCa patients eventually develop androgen-independent tumors that are resistant to this form of therapy. A significant body of evidence has suggested that in many androgen-independent PCa cases, AR is highly expressed and hypersensitive to low, castrated-level of androgens or even can be activated by nonandrogens to induce tumor cell growth. Therefore, the strategy targeting the inactivation of AR function is particularly attractive to treat hormone refractory prostate tumors. On the basis of these observations, we examined the cellular PSA and AR expression levels in the LNCaP cells and found that the expression of both were down-regulated by most of the compounds tested, which suggested that the AR signaling pathway was suppressed by these compounds. It is interesting that the most potent C**1** and C**2** both have a benzoterahydrofuran unit, which is similar to testosterone as indicated in **Figures 1** and **2**. We propose that this similarity may contribute to their potential effects on AR function. In addition, C**3** and C**4** were different in their configurations, which may explain their different activities. C**6** had no obvious effect on cellular PSA and AR expression, which may lead to its weak inhibition of PSA secretion. Alternatively, this may be resulted from other changes on cell membrane kinetics or cell situations.

Despite these results, whether these compounds inhibit cell proliferation is a key question. We focused on C**1**, which had the highest activity on AR inhibition, and studied its effects on LNCaP cells proliferation. As expected, C**1** significantly inhibited cell growth. Moreover, it also induced apoptosis. These results provided further evidence that AR down-regulation could inhibit prostate cancer cell growth, as indicated by several previous publications (*13–16, 25*).

Actually, lignans are rich in a variety of dietary sources such as in most cereals, fruits, and vegetables in traditional Asian diets (*31*). Increasing epidemiological evidence suggested that different contents of phytoestrogens in the diet between Eastern and Western cultures may be one of the reasons for the dramatic difference in the incidence of PCa and possibly BPH (*32, 33*). Previous studies have suggested that lignans play a role in the prevention and treatment of PCa, including antiproliferative activity, inhibition of human aromatase, 17-hydroxysteroid dehydrogenase, and 5α-reductase, antioxidant activity, and modulatin of cellular growth factors (*34*). However, there was no definite evidence showing that lignans had effects on AR function. Our present results provided new insight that lignans could suppress the AR signaling pathway. Though limited to only several lignans, the data provided some molecular evidence to support that lignans may exert their affects on PCa and possibly also on BPH through suppression of AR pathway.

ACKNOWLEDGMENT

We thank Shanghai Institute of Materia Medica of Chinese Academy of Science for measuring the HR-ESI-MS. We also thank Huang Jinghui and Jin Sanlin for NMR spectra and Zhang Jiayong for UV spectroscopy measurements.

LITERATURE CITED

(1) Stewart, A. B.; Lwaleed, B. A.; Douglas, D. A.; Birch, B. R. Current drug therapy for prostate cancer: an overview. *Curr. Med. Chem.: Anti−Cancer Agents* **2005**, *5*, 603–612.

(2) Bracarda, S.; de Cobelli, O.; Greco, C.; Prayer-Galetti, T.; Valdagni, R.; Gatta, G.; de Braud, F.; Bartsch, G. Cancer of the prostate. *Crit. Rev. Oncol. Hematol.* **2005**, *56*, 379–396.

(3) Capodice, J. L.; Katz, A. E. What patients take without telling you: holistic approach for BPH. *World J. Urol.* **2006**, *24*, 378–382.

(4) Katz, A. E. Flavonoid and botanical approaches to prostate health. *J. Altern. Complement Med.* **2002**, *8*, 813–821.

(5) Denis, L.; Morton, M. S.; Griffiths, K. Diet and its preventive role in prostatic disease. *Eur. Urol.* **1999**, *35*, 377–387.

(6) McCann, M. J.; Gill, C. I.; McGlynn, H.; Rowland, I. R. Role of mammalian lignans in the prevention and treatment of prostate cancer. *Nutr. Cancer* **2005**, *52*, 1–14.

(7) Han, H. Y.; Shan, S.; Zhang, X.; Wang, N. L.; Lu, X. P.; Yao, X. S. Down-regulationof prostate specific antigen in LNCaP cells by flavonoids from the pollen of *Brassica napus* L. *Phytomedicine* **2007**, *14*, 338–343.

(8) Lu, X. Z.; Xu, W. B.; Shen, J. X. Chemical studies on *Campylotropis hirtella* (Franch. Schindl.). *Zhongguo Zhongyao Zazhi* **1997**, *22* (11), 680–682.

(9) Qin, L.; Chen, X. M.; Chen, W. X.; Yang, L.; Zhang, C. K. Study on tannins from *Campylotropis hirtella*. *Tianran Chanwu Yanjiu Yu Kaifa* **1991**, *3*, 31–34.

(10) Kim, J.; Coetzee, G. A. Prostate specific antigen gene regulation by androgen receptor. *J Cell. Biochem.* **2004**, *93*, 233–241.

(11) Chatterjee, B. The role of the androgen receptor in the development of prostatic hyperplasia and prostate cancer. *Mol. Cell. Biochem.* **2003**, *253*, 89–101.

(12) Culig, Z.; Hobisch, A.; Hittmair, A.; Peterziel, H.; Cato, A. C.; Bartsch, G.; Klocker, H. Expression, structure and function of androgen receptor in advanced prostatetic carcinoma. *Prostate* **1998**, *35*, 63–70.

(13) Zegarra-Moro, O. L.; Schmidt, L. J.; Huang, H.; Tindall, D. J. Disruption of androgen receptor function inhibits proliferation of androgenrefractory prostate cancer cells. *Cancer Res.* **2002**, *62*, 1008–1013.

(14) Butler, L. M.; Centenera, M. M.; Neufing, P. J.; Buchanan, G.; Choong, C. S.; Ricciardelli, C.; Saint, K.; Lee, M.; Ochnik, A.; Yang, M.; Brown, M. P.; Tilley, W. D. Suppression of androgen receptor signaling in prostate cancer cells by an inhibitory receptor variant. *Mol. Endocrinol.* **2006**, *20*, 1009–1024.

(15) Liao, X.; Tang, S.; Thrasher, J. B.; Griebling, T. L.; Li, B. Small-interfering RNA induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. *Mol. Cancer Ther.* **2005**, *4*, 505–515.

(16) Haag, P.; Bektic, J.; Bartsch, G.; Klocker, H.; Eder, I. E. Androgen receptor down regulation by small interference RNA induces cell growth inhibition in androgen sensitive as well as in androgen independent prostate cancer cells. *J. Steroid Biochem. Mol. Biol.* **2005**, *96*, 251–258.

(17) Cheung, H. W.; Jin, D. Y.; Ling, M. T.; Wong, Y. C.; Wang, Q.; Tsao, S. W.; Wang, X. Mitotic arrest deficient 2 expression induces chemosensitization to a DNA-damaging agent, cisplatin, in nasopharyngeal carcinoma cells. *Cancer Res.* **2005**, *65*, 1450–1458.

(18) Yuen, M. S. M.; Xue, F.; Mak, T. C. W.; Wong, H. N. C. On the absolute structure of optically active neolignans containing a dihydrobenzo[*b*]furan skeleton. *Tetrahedron* **1998**, *54*, 12429–12444.

(19) Li, S.; Lundquist, K.; Wallis, A. F. A. Revised structure for a neolignan from *Brucea jaVanica*. *Phytochemistry* **1998**, *49*, 2125–2128.

(20) Miki, K.; Takehara, T.; Sasaya, T.; Sakakibara, A. Lignans of *Larix leptolepis*. *Phytochemistry* **1980**, *19*, 449–453.

(21) Sawabe, A.; Mathubara, Y.; Kumamoto, H.; Iizuka, Y.; Okamoto, K. Structure and physiological activity of phenyl propanoid glycosides of hassaku (*Citrus hassaku* Hort.) and orange (*Citrus sinensis* Osbeck.) peels. *Nippon Nogei Kagaku Kaishi* **1986**, *60*, 593–599.

(22) Fonseca, S. B.; De Paiva Campello, J.; Barata, L. E. S.; Ruveda, E. A. 13C-NMR analysis of lignans from *Araucaria augustifolia*. *Phytochemistry* **1978**, *17*, 499–502.

(23) Yoshinari, K.; Sashida, Y.; Shimomura, H. Two new lignan xylosides from the barks of *Prunus ssiori* and *Prunus padus*. *Chem. Pharm. Bull.* **1989**, *37*, 3301–3303.

(24) Suzuki, H.; Ueda, T.; Ichikawa, T.; Ito, H. Androgen receptor involvement in the progression of prostate cancer. *Endocr.−Relat. Cancer* **2003**, *10*, 209–216.

(25) Chiu, F. L.; Lin, J. K. Downregulation of androgen receptor expression by luteolin causes inhibition of cell proliferation and induction of apoptosis in human prostate cancer cells and xenografts. *Prostate* **2008**, *68*, 61–71.

(26) Kelly, W. K.; Scher, H. I.; Mazumdar, M.; Vlamis, V.; Schwartz, M.; Fossa, S. D. Prostate specific antigen as a measure of disease outcome in hormone-refractory prostatic cancer. *J. Clin. Oncol.* **1993**, *11*, 607–615.

(27) Cohen, P.; Graves, H. C.; Peehl, D. M.; Kamarei, M.; Giudice, L. C.; Rosenfeld, R. G. Prostate-specific antigen (PSA) is an insulin-like growth factor binding protein-3 protease found in seminal plasma. *J. Clin. Endocrinol. Metab.* **1992**, *75*, 1046–1053.

(28) Mantzoros, C. S.; Tzonou, A.; Signorello, L. B.; Stampfer, M.; Trichopoulos, D.; Adami, H. O. Insulin-like growth factor-1 in relation to prostate cancer and benign prostatic hyperplasia. *Br. J. Cancer* **1997**, *76*, 1115–1118.

(29) Chan, J. M.; Stampfer, M. J.; Giovannucci, E.; Gann, P. H.; Ma, J.; Wilkinson, P.; Hennekens, C. H.; Pollak, M. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* **1998**, *279*, 563–566.

(30) Cohen, P.; Peehl, D. M.; Graves, H. C.; Rosenfeld, R. G. Biological effects of prostate specific antigen as an insulin-like

growth factor binding protein-3 protease. *J. Endocrinol.* **1994**, *142*, 407–415.
(31) Potter, J. D. Patterns of diet. In *Food, Nutrition, and the Prevention of Cancer: A Global Perspective*; American Institute for Cancer Research: Washington, DC, 1997; pp 22−34.
(32) Adlercreutz, H.; Mazur, W. Phyto-oestrogens and Western diseases. *Ann. Med.* **1997**, *29*, 95–120.
(33) Dhom, G. Epidemiology of hormone-depending tumours. In *Endocrine Dependent Tumours*; Voigt, K., Knabbe, C., eds.; Raven Press: New York, 1991, pp 1−42.
(34) Lin, X.; Swtizer, B. R.; Demark-Wahnefried, W. Effect of mammalian lignans on the growth of prostate cancer cell lines. *Anticancer Res.* **2001**, *21*, 3995–4000.

Received for review February 15, 2008. Revised manuscript received April 21, 2008. Accepted May 27, 2008. This work was supported by grants from Traditional Chinese Medicine and Nature Drug Research Institute in the Zhejiang province of China.

JF800476R