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Identification of Antitumor Lignans from the Seeds of Morning Glory (*Pharbitis nil*)

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Supporting Information

ABSTRACT: In the search for antitumor compounds from Korean natural resources, activity-guided fractionation and purification processes were used on seeds of morning glory (*Pharbitis nil*). Air-dried *P. nil* seeds were extracted with ethanol and separated into *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. Four new lignans, pharbilignans A–D (**1**–**4**) were isolated from the most active ethyl acetate fraction of the ethanol extract. Their structures were characterized on the basis of spectroscopic methods, including one- and two-dimensional nuclear magnetic resonance (NMR) techniques, high resolution mass spectrometry (HRMS), and circular dichroism (CD) spectroscopy. The cytotoxic activities of the isolates (**1**–**4**) were evaluated by determining their inhibitory effects on four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15) using a sulforhodamine B (SRB) bioassay. Pharbilignan C (**3**) showed potent cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines with IC_{50} values of 1.42, 0.16, 0.20, and 0.14 μM, respectively. On the basis of the expanded understanding that inflammation is a crucial cause in tumor progress, we also evaluated anti-inflammatory activity of the isolates (**1**–**4**). Pharbilignan C (**3**) strongly inhibited nitric oxide (NO) production in the lipopolysaccharide (LPS)-activated BV-2 microglia cell line with an IC_{50} value of 12.8 μM.

KEYWORDS: *Pharbitis nil, Convolvulaceae, lignans, structural elucidation, cytotoxicity, anti-inflammation*

INTRODUCTION

Lignans collectively refer to a diverse class of phenylpropanoid dimers and oligomers that occur naturally in many plants. Sources that are particularly rich in lignan phytochemcials are flax seeds, sesame, sunflower, pumpkin seeds, and whole grain breads. Lignans are valuable antioxidants and phytoestrogens with both estrogenic and antiestrogenic activities.[1,2] A positive association between high levels of lignans in the body and reduced risks of prostate and breast cancer exists.[3] In recent decades, renewed interest in investigating natural products has led to the discovery of several important anticancer drugs including vinblastine, vincristine, paclitaxel, and the semi-synthetic substances, etoposide, etopophos, and teniposide. The latter three compounds are chemical derivatives of podophyllotoxin, a natural product belonging to the lignan group. Thus, the natural products approach to discovery and development of new lignan derivatives for anticancer drugs is an attractive and promising avenue.

We have identified lignan analogues with diverse bioactivities from Korean natural resources. These include compounds with antimelanogenic, antitumor, and anti-inflammatory activities.[4−8] Previously, we found that an ethanol extract of the seeds of morning glory (*Pharbitis nil*) exhibited considerable cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 human tumor cell lines in our screening test and reported several cytotoxic phenolic constituents including lignans from ethanol (EtOH) extracts.[8]

Morning glory (Convolvulaceae) is a famous flower that is popular as an ornamental plant. The seeds of *P. nil* (Pharbitidis Semen) is both a food and a medicinal agent that has traditionally been used as a purgative in Korea, China, and Japan.[9] The seeds have also been used in traditional Chinese medicine food for their analgesic effects against abdominal pain and for the treatment of a variety of digestive problems.[10] The seeds are an enriched source of diverse phytochemicals, particularly resin glycosides and gibberellins.[11−13] However, to our knowledge, only our previous study has investigated the lignan phytochemicals of Pharbitidis Semen.[8] Presently, we used activity-guided fractionation combined with chromatography to focus on identifying antitumor lignan deriviatives from the seeds of morning glory. In this article, we describe the isolation and structural determination of four new lignans named pharbilignans A-D (**1**–**4**) (Figure 1) as well as their biological activity with respect to cytotoxic and anti-inflammatory activities.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA).

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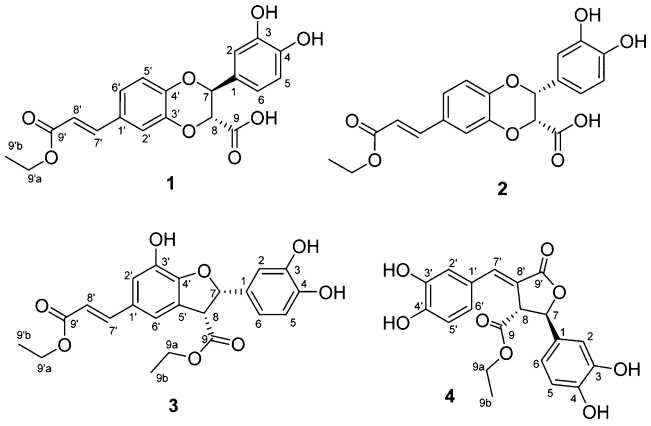

Figure 1. Structures of compounds **1**−**4**.

IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). Circular dichroism (CD) spectra were measured on a Jasco J-715 spectropolarimeter (Jasco, Easton, MD, USA). Ultraviolet (UV) spectra were recorded with a Shimadzu UV-1601 UV−visible spectrophotometer (Shimadzu, Tokyo, Japan). High-resolution (HR)-electrospray ionization (ESI) mass spectra were recorded on a SI-2/LCQ DecaXP liquid chromatography (LC)-mass spectrometer (Thermo Scientific, West Palm Beach, FL, USA). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative high performance liquid chromatography (HPLC) used a Gilson 306 pump (Gilson, Middleton, WI, USA) with a Shodex refractive index detector (Shodex, New York, NY, USA). Column chromatography was performed with a silica gel 60 (Merck, 70−230 mesh and 230−400 mesh) and RP-C_{18} silica gel (Merck, 230−400 mesh). The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Merck precoated silica gel F_{254} plates and reversed-phase (RP)-18 F_{254s} plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

Plant Material. The seeds of *P. nil* were purchased at Kyungdong herbal market, Seoul, Korea, in July 2006, and were identified by one of the authors (K. R. Lee). A voucher specimen (SKKU 2006-7) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. Air-dried *P. nil* seeds (10 kg) were extracted with 50% EtOH (3 × 4 L, on each of 3 days) at room temperature and filtered. The filtrate was evaporated under reduced pressure using a rotavapor to obtain the EtOH extract (1.4 kg), which was suspended in distilled H_2O (7.2 L) in a separatory funnel to be partitioned in sequence with *n*-hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), and butanol (*n*-BuOH), which yielded 10, 7, 10, and 550 g of dried organic fractions, respectively. A bioactivity-guided fractionation method was used for isolation work. Each fraction was tested for cytotoxic activity against A549, SK-OV-3, SK-MEL-2, and HCT-15 human tumor cell lines using a sulforhodamine B (SRB) bioassay, which proved that the EtOAc-soluble fraction showed the strongest activity (IC_{50}: 28.74, 23.45, 10.32, and 17.81 μM, respectively against A549, SK-OV-3, SK-MEL-2, and HCT-15 cells). The most active EtOAc-soluble fraction (10 g) was chromatographed on the open-column of silica gel (230−400 mesh, 300 g) eluting with $CHCl_3$/methanol (MeOH) (10:1 → 1:1, gradient system) to give six fractions (A−F). Each fraction obtained from the EtOAc-soluble fraction was further tested for cytotoxic activity against the four aforementioned cell lines to identify the active fraction. Fractions B, D, and F displayed activity: fraction B (IC_{50}: 5.31, 3.10, 8.39, and 6.11 μM, respectively, against A549, SK-OV-3, SK-MEL-2, and HCT-15), fraction D (IC_{50}: 40.24, 33.97, 12.60, and 20.37 μM, respectively), and fraction F (IC_{50}: 48.92, 27.09, 15.74, and 19.40 μM, respectively). Fraction B (2.5 g) was chromatographed further on the open-column of RP-C_{18} silica gel (230−400 mesh, 150 g) eluting with MeOH/H_2O (3:2 → 4:1, gradient system) to give seven subfractions (B1−B7). Fraction B7 (50 mg) was purified by semipreparative normal-phase HPLC (*n*-hexane/EtOAc, 1:1), using an Apollo Silica column (250 mm × 10 mm i.d., 5 μm, Alltech, Nicholasville, KY, USA) to give **3** (24 mg, t_R = 16.0 min). Fraction D (2.5 g) was applied to an RP-C_{18} silica gel open-column chromatograph (60% MeOH) to yield six subfractions (D1−D6). Fraction D2 (340 mg) was passed through a Sephadex LH-20 column eluted with 100% MeOH and further purified by semipreparative reversed-phase HPLC (30% MeCN), using an Econosil RP-18 column (250 mm × 10 mm i.d., 10 μm, Alltech, Nicholasville, KY, USA) to give **4** (16 mg, t_R = 15.0 min). Fraction F (3.6 g) was passed through a Sephadex LH-20 column and eluted with CH_2Cl_2/MeOH (1:1) to obtain four subfractions (F1−F4). Finally, fraction F3 (700 mg) was purified by semipreparative reversed-phase HPLC (18% MeCN) to yield **1** (20 mg, t_R = 18.0 min) and **2** (13 mg, t_R = 18.5 min).

*Pharbilignanol A (**1**).* White amorphous powder. $[\alpha]_D^{25}$ −9.1 (*c* 0.80, MeOH). UV (MeOH) λ_{max} (log ε): 259 (4.05), 306 (3.62) nm. CD (MeOH): $[\theta]_{220}$ −2810, $[\theta]_{234}$ +3140, $[\theta]_{282}$ +1530. IR (KBr) ν_{max}: 3361, 2946, 2833, 1726, 1630, 1610, 1511, 1451, 1370, 1033, 670 cm^{-1}. For ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data, see Table 1. Electrospray ionization (ESI)-MS (positive-ion mode) *m*/*z*: 409 $[M + Na]^+$. High-resolution (HR)-ESIMS (positive-ion mode) *m*/*z*: 409.0902 $[M + Na]^+$ (calcd for $C_{20}H_{18}NaO_8$, 409.0899).

Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds 1−2 in CD_3OD[a]

	1		2	
position	δ_C	δ_H	δ_C	δ_H
1	130.0		129.9	
2	115.7	6.88 d (2.0)	116.1	6.84 d (2.0)
3	145.2		144.7	
4	146.2		146.1	
5	116.3	6.74 d (8.5)	116.2	6.63 d (8.5)
6	120.3	6.76 dd (8.5, 2.0)	120.8	6.73 dd (8.5, 2.0)
7	78.2	5.16 d (6.0)	77.4	5.59 d (3.5)
8	81.0	4.53 d (6.0)	78.2	4.68 d (3.5)
9	174.5		173.4	
1′	129.1		129.7	
2′	117.5	7.15 d (2.0)	117.5	7.15 d (2.0)
3′	146.7		146.9	
4′	147.1		147.2	
5′	118.6	6.96 d (8.0)	118.9	7.02 d (8.0)
6′	123.2	7.11 dd (8.0, 2.0)	122.9	7.11 dd (8.0, 2.0)
7′	146.2	7.58 d (16.0)	146.4	7.58 d (16.0)
8′	116.7	6.34 d (16.0)	117.0	6.35 d (16.0)
9′	169.2	169.2		
9′a	61.6	4.22 q (7.0)	61.6	4.22 q (7.0)
9′b	14.7	1.32 t (7.0)	14.7	1.32 t (7.0)

[a] δ is in ppm, and *J* values are in parentheses. The assignments were based on ^{1}H,^{1}H−COSY, HMQC, and HMBC experiments.

*Pharbilignanol B (**2**).* White amorphous powder. $[\alpha]_D^{25}$ −15.6 (*c* 0.35, MeOH). UV (MeOH) λ_{max} (log ε): 259 (4.05), 306 (3.63) nm. CD (MeOH): $[\theta]_{234}$ −2610, $[\theta]_{250}$ +2530, $[\theta]_{288}$ −890. IR (KBr) ν_{max}: 3361, 2947, 2833, 1727, 1630, 1608, 1507, 1450, 1355, 1033, 670 cm^{-1}. For ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data, see Table 2. ESIMS (positive-ion mode) *m*/*z*: 409 $[M + Na]^+$. HR-ESIMS (positive-ion mode) *m*/*z*: 409.0906 $[M + Na]^+$ (calcd for $C_{20}H_{18}NaO_8$, 409.0899).

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Table 2. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds 3−4[a]

	3[b]		4[c]	
position	δ_C	δ_H	δ_C	δ_H
1	132.0		132.5	
2	113.0	6.84 d (2.0)	113.4	6.74 d (2.5)
3	144.0		147.0	
4	144.3		147.3	
5	115.6	6.77 d (8.0)	116.7	6.78 d (8.0)
6	118.7	6.70 dd (8.0, 2.0)	118.4	6.67 dd (8.0, 2.5)
7	87.1	5.97 d (8.0)	82.5	5.59 d (3.0)
8	55.6	4.21 d (8.0)	54.6	4.24 dd (3.0, 2.0)
9	170.8		172.0	
9a	62.1	4.25 q (7.0)	63.2	4.19 q (7.0)
9b	14.1	1.27 t (7.0)	14.4	1.20 t (7.0)
1′	128.7		126.8	
2′	116.4	6.97 br s	118.2	7.06 d (2.0)
3′	140.3		150.1	
4′	148.5		146.9	
5′	125.6		116.7	6.83 d (8.0)
6′	117.5	7.03 br s	125.5	6.99 dd (8.0, 2.5)
7′	144.9	7.52 d (16.0)	142.4	7.59 d (2.5)
8′	115.6	6.21 d (16.0)	119.8	
9′	168.1		173.8	
9′a	60.8	4.25 q (7.0)		
9′b	14.2	1.32 t (7.0)		

[a] δ is in ppm, and J values are in parentheses. The assignments were based on ^{1}H,^{1}H−COSY, HMQC, and HMBC experiments. [b] ^{1}H and ^{13}C NMR data were recorded in $CDCl_3$. [c] ^{1}H and ^{13}C NMR data were recorded in CD_3OD.

*Pharbilignanol C (**3**).* White amorphous powder. $[\alpha]_D^{25}$ −26.3 (*c* 0.65, MeOH). UV (MeOH) λ_{max} (log ε): 229 (4.23), 256 (3.98), 322 (3.75) nm. CD (MeOH): $[\theta]_{212}$ +3630, $[\theta]_{234}$ +4570, $[\theta]_{254}$ +8870, $[\theta]_{279}$ −1210, $[\theta]_{335}$ +4820. IR (KBr) ν_{max}: 3356, 2947, 2832, 1723, 1690, 1603, 1512, 1451, 1350, 1275, 1033, 670 cm^{-1}. For ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data, see Table 2. ESIMS (positive-ion mode) *m*/*z*: 415 $[M + H]^+$. HR-ESIMS (positive-ion mode) *m*/*z*: 415.1388 $[M + H]^+$ (calcd for $C_{22}H_{23}O_8$, 415.1393).

*Pharbilignanol D (**4**).* White amorphous powder. $[\alpha]_D^{25}$ +27.7 (*c* 0.40, EtOH). UV (MeOH) λ_{max} (log ε): 258 (4.03), 310 (3.74) nm. CD (MeOH): $[\theta]_{211}$ +2160, $[\theta]_{231}$ +4550, $[\theta]_{245}$ +4710, $[\theta]_{263}$ −5020, $[\theta]_{276}$ +6780. IR (KBr) ν_{max}: 3361, 2947, 2833, 1734, 1642, 1604, 1526, 1450, 1294, 1202, 1032, 670 cm^{-1}. For ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data, see Table 2. ESIMS (positive-ion mode) *m*/*z*: 387 $[M + H]^+$. HR-ESIMS (positive-ion mode) *m*/*z*: 387.1087 $[M + H]^+$ (Calcd for $C_{20}H_{19}O_8$, 387.1080).

Cell Cultures. All tumor cell cultures were maintained using RPMI1640 cell growth medium (Gibco, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin, and 100 mg/mL streptomycin. The human tumor cell lines, A549 (nonsmall cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) were provided by the National Cancer Institute (NCI). BV2 murine microglia cells were generously provided by Dr. E. Choi from Korea University (Seoul, Korea) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. All cells were incubated at 37 °C in a humidified incubator with 5% CO_2.

Cytotoxicity Assessment. The cytotoxicity of the isolates (**1**−**4**) against cultured human tumor cell lines was evaluated by the SRB method. The assays were performed at the Korea Research Institute of Chemical Technology. Each tumor cell line was inoculated over standard 96-well flat-bottom microplates and then incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The attached cells were then incubated with the serially diluted samples. After continuous exposure to the compounds for 48 h, the culture medium was removed from each well, and the cells were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. After washing with tap water, the cells were stained with 0.4% SRB dye and incubated for 30 min at room temperature. The cells were washed again and then solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 nm with a microtiter plate reader. Doxorubicin (Sigma-Aldrich, St. Luuis, Mo, USA; ≥98% purity) was used as the positive control. Doxorubicin had IC_{50} values against A549, SK-OV-3, SK-MEL-2, and HCT15 of 0.02, 0.02, 0.03, and 0.08 μM, respectively.

Measurement of Nitric Oxide (NO) Production and Cell Viability in Lipopolysaccharide (LPS)-Activated BV-2 Cells. Murine microglia BV-2 cells were plated in wells of a 96-well plate (3×10^4 cells/well). After 24 h, cells were pretreated with samples for 30 min and then stimulated with 100 ng/mL LPS for another 24 h. Nitrite in the culture media, a soluble oxidation product of NO, was measured by the Griess reaction. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader (Emax, Molecular Device, Sunnyvale, CA, USA). Graded sodium nitrite solutions were used as standards to calculate nitrite concentration. Cell viability was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA, Sigma-Aldrich), served as the positive control.

RESULTS AND DISCUSSION

Isolation and Structural Elucidation of Compounds. Air-dried seeds of *P. nil* were extracted with 50% aqueous EtOH. The hydroethanolic extract showed considerable cytotoxicity against some human tumor cell lines using the SRB bioassay. The hydroethanolic extract was subjected to solvent-partition into *n*-hexane, chloroform, ethyl acetate, and *n*-butanol fractions. The ethyl acetate fraction showed the strongest activity and was subjected to separation and purification by successive column chromatography over silica gel and Sephadex LH-20, and semipreparative HPLC, which resulted in the isolation and identification of four new lignans, named pharbilignans A−D (**1**−**4**) (Figure 1).

Pharbilignan A (**1**) was isolated as a white, amorphous powder. Its HR-ESIMS at *m*/*z* 409.0902 $[M + Na]^+$ (calcd for $C_{20}H_{18}NaO_8$, 409.0899) and NMR spectroscopic data (Table 1) established the molecular formula as $C_{20}H_{18}O_8$, implying the existence of 12 of unsaturation. The UV absorption maxima at 259 and 306 nm were indicative of a lignan skeleton.[14] The IR absorption bands of **1** were characteristic of hydroxy (3361 cm^{-1}), carbonyl (1726 cm^{-1}), and aromatic ring (1610 and 1451 cm^{-1}) functional groups. The ^{1}H NMR spectrum of **1** showed signals attributable to a deshielded benzylic oxymethine proton at δ_H 5.16 (H-7) and an oxygenated proton at δ_H 4.53 (H-8), implying the linkage of two phenylpropanoid units via a 1,4-dioxane bridge.[15,16] Detailed inspection of the ^{1}H and ^{13}C NMR spectra (Table 1) of **1** showed that the signals were similar to those of americanol A[15] and were almost identical to those of 7-[(1*E*)-2-carboxyethenyl]-3-(3,4-dihydroxyphenyl)-2,3-dihydro-1,4-benzodioxin-2-carboxylic acid, except for the presence of an ethoxy group in **1**.[16,17] The skeleton was confirmed by heteronuclear multiple bond correlation (HMBC), showing correlations from H-7 (δ_H 5.16) to C-2, C-6, C-9, and C-4′ and correlations from H-8 (δ_H 4.53) to C-1, and C-3′. The HMBC correlation between H-9′a (δ_H 4.22) and

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C-9′ (δ_C 169.2) indicated the presence of an ethoxy group at C-9′ (Figure 2).

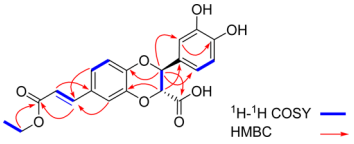

Figure 2. Key ^{1}H−^{1}H COSY (blue line) and HMBC (red arrow) correlations of **1**.

It is difficult to determine whether benzodioxane-type neolignans belong to the americanol or isoamericanol type. Recently, it was reported that the chemical shift differences between H-2′ and H-5′ in the ^{1}H NMR spectra measured in CD_3OD allow for an unambiguous identification of the isomeric structures.[15,16] The ^{1}H NMR spectrum of **1** showed a large difference of 0.19 ppm between H-2′ (δ_H 7.15) and H-5′ (δ_H 6.96), consistent with that of the americanol type.[15,16] Difference nuclear Overhauser effect (NOE) experiments were used to assign the structure, where significant long-range NOE enhancements could be observed between H-2′ and H-8 and between H-5′ and H-7, confirming the americanol type. Additionally, the americanol type of **1** was supported by a small difference of 0.4 ppm between C-3′ (δ_C 146.7) and C-4′ (δ_H 147.1) in the ^{13}C NMR data of **1**.[15,16] The coupling constant ($J_{7,8}$ = 6.0 Hz) between H-7 and H-8, and nuclear Overhauser effect spectroscopy (NOESY) correlation (H-6/H-8) indicated a *trans*-relationship of H-7/H-8 on the 1,4-benzodioxane ring.[17,18] The absolute configuration of the C-7 and C-8 was designated as *S* and *R*, respectively, on the basis of CD data ($[\theta]_{220}$ −2810, $[\theta]_{234}$ +3140, $[\theta]_{282}$ +1530), which was in good agreement with the configuration reported in the literature.[19] Therefore, the structure of **1** was established as depicted in Figure 1.

Pharbilignan B (**2**) was obtained as a white, amorphous powder. Its molecular formula $C_{20}H_{18}O_8$ (12 of unsaturation) was derived by HR-ESIMS *m*/*z* 409.0906 $[M + Na]^+$ (calcd for $C_{20}H_{18}NaO_8$, 409.0899). The similarity of UV and IR absorption bands in **1** and **2** suggested that **2** had the same lignan skeleton as **1**. The ^{1}H and ^{13}C NMR spectra (Table 1) of **2** also showed a similarity with those of **1**, except for the chemical shifts of C-7 and C-8 in **2** (δ_H 5.59, H-7; δ_C 77.4, C-7 and δ_H 4.68, H-8; δ_C 78.2, C-8). Thus, compound **2** is an isomer of **1** with a 1,4-dioxane bridge. A large difference of 0.13 ppm between H-2′ (δ_H 7.15) and H-5′ (δ_H 7.02) and a small difference of 0.3 ppm between C-3′ (δ_C 146.9) and C-4′ (δ_H 147.2) in the NMR data of **2** indicated that compound **2** should be americanol type **1**.[15,16] A relatively small coupling constant ($J_{7,8}$ = 3.5 Hz) between H-7 and H-8 suggested a *cis*-configuration of H-7 and H-8 in the molecule.[17,18] The absolute configuration of the C-7 and C-8 was assigned on the basis of CD data ($[\theta]_{234}$ −2610, $[\theta]_{250}$ +2530, $[\theta]_{288}$ −890), allowing the assignment of 7*R* and 8*R* configurations, respectively.[20] Accordingly, the structure of **2** was elucidated as depicted in Figure 1.

Pharbilignan C (**3**) was isolated as a white, amorphous powder. It had the molecular formula $C_{22}H_{22}O_8$ with 12 of unsaturation, as deduced by HR-ESIMS *m*/*z* 415.1388 $[M + H]^+$ (calcd for $C_{22}H_{23}O_8$, 415.1393). The UV absorption maxima at 256 and 322 nm indicated a lignan skeleton for **3**.[14] The IR absorption bands of **3** exhibited the presence of hydroxy (3356 cm^{-1}), carbonyl (1723 cm^{-1}), and aromatic ring (1603 and 1451 cm^{-1}) moieties. The ^{1}H and ^{13}C NMR data for C-7 and C-8 in **3** (δ_H 5.97, H-7; δ_C 87.1, C-7; and δ_H 4.21, H-8; δ_C 55.6, C-8) supported that compound **3** was a dihydro[*b*]-benzofuran-type neolignan, and the ^{1}H and ^{13}C NMR spectra (Table 2) of **3** were very similar to those of caffeicin F, except for the presence of two ethoxy groups, indicating that compound **3** was an analogue of caffeicin F with two ethoxy groups as a side chain.[18] The structure was verified by an HMBC experiment showing correlations from H-7 (δ_H 5.97) to C-2, C-6, C-9, and C-4′ and from H-8 (δ_H 4.53) to C-1, C-4′, and C-6′. The HMBC correlations H-9a/C-9 (δ_C 170.8) and H-9′a/C-9′ (δ_C 168.1) allowed the assignment of an ethoxy group at C-9 and the other ethoxy group at C-9′ (Figure 3).

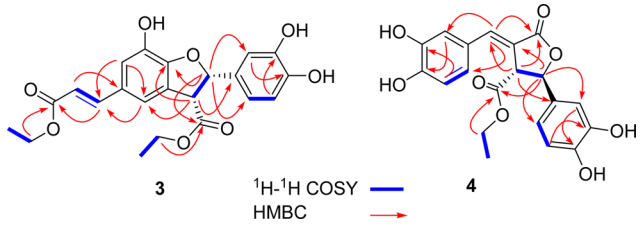

Figure 3. Key ^{1}H−^{1}H COSY (blue line) and HMBC (red arrow) correlations of **3** and **4**.

The relative configuration at H-7 and H-8 was established on the basis of their coupling constant ($J_{7,8}$ = 8.0 Hz), which was consistent with a *cis*-configuration of H-7 and H-8 in **3**.[21] This arrangement was also supported by a NOESY correlation between H-7 and H-8 but the lack of NOESY correlations between H-7 and H-9a or between H-8 and H-2/H-6. Finally, the positive Cotton effect at 254 nm in the CD spectrum of **3** suggested the absolute configuration at C-7 as 7*S*.[22,23] This analysis also determined an 8*R* configuration based on the relative stereochemistry previously determined as *cis*-configuration between H-7 and H-8. Therefore, the structure of **3** was assigned the structure depicted in Figure 1. A survey of the literature revealed that an epimer of **3** with the 7*S*,8*S*-form was synthesized previously.[24]

Pharbilignan D (**4**) had the molecular formula $C_{20}H_{18}O_8$, with 12 of unsaturation, deduced by HR-ESIMS *m*/*z* 387.1087 $[M + H]^+$ (calcd for $C_{20}H_{19}O_8$, 387.1080). The ^{1}H and ^{13}C NMR spectra (Table 2) of **4** were similar to those of phellinsin A, except for the presence of an ethoxy group, which suggested that compound **4** was the same lignan-type as phellinsin A.[25] The lignan skeleton was verified by HMBC correlations of H-7/C-2, C-6, C-9, C-8′, and C-9′; H-8/C-1, C-7′, and C-9′; and H-7′/C-8, C-2′, C-6′, and C-9′ (Figure 3). The presence of an ethoxy group at C-9 in **4** was confirmed by the HMBC correlation of H-9a/C-9. The geometry of the double bond at C-7′ was established to be *E* on the basis of the chemical shift of H-7′ (δ_H 7.59) and the long-range coupling constant ($J_{7',8}$ = 2.5 Hz).[26,27] This was confirmed by the NOESY experiment where the correlations between H-8 (δ_H 4.24) and H-2′ (δ_H 7.06)/ H-6′ (δ_H 6.99) are shown. Furthermore, a *trans*-configuration of H-7 and H-8 was concluded by the relatively small coupling constant ($J_{7,8}$ = 2.5 Hz), combined with a NOESY correlation between H-8 and H-2/H-6.[26,27] The configurations of C-7 and C-8 were assigned as 7*R** and 8*R** on the basis of the optical rotation value of **4** ($[\alpha]_D^{25}$ +27.7).[27] Moreover, its CD spectrum showed Cotton effects at 231 and 263 nm ($[\theta]_{231}$ +4550, $[\theta]_{263}$ −5020). According to the above

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Table 3. Cytotoxic Activities of Compounds 1−4 against Four Cultured Human Tumor Cell Lines Using the SRB Assay

	IC_{50} (μM)[a]			
compd	A549	SK-OV-3	SK-MEL-2	HCT-15
1	>30.0	27.45 ± 0.75	15.23 ± 1.30	19.84 ± 0.13
2	>30.0	22.67 ± 2.15	17.64 ± 0.52	21.72 ± 1.18
3	1.42 ± 0.38	0.16 ± 0.03	0.20 ± 0.11	0.14 ± 0.07
4	>30.0	21.59 ± 0.83	13.77 ± 0.28	16.52 ± 1.20
doxorubicin[b]	0.02 ± 0.003	0.02 ± 0.002	0.03 ± 0.007	0.08 ± 0.008

[a]IC_{50} value of compounds against each tumor cell line. The IC_{50} value was defined as the concentration (μM) that caused 50% inhibition in cell growth in vitro. Data are expressed as the mean ± SD of three distinct experiments [b]Doxorubicin as a positive control.

analysis, the structure of **4** was elucidated as depicted in Figure 1.

It was likely that compounds **1**−**4** were artifacts of the isolation procedure because 50% EtOH was used as the solvent for extraction. In order to verify that compounds **1**−**4** are authentic natural products, another supply of the plant was obtained and extracted with MeOH and EtOAc, respectively. The resulting extracts were tested using LC-MS analyses, alone and coinjected with the pure compounds **1**−**4**. Compounds **1**−**4** were all identified on the HPLC chromatogram of the crude extracts. The increase in peak areas corresponding to **1**−**4** supported that these compounds are indeed naturally occurring metabolites.

Evaluation of Antitumor Activity. All isolated compounds (**1**−**4**) were evaluated for antitumor activity by determining their inhibitory effects on four human tumor cell lines including A549 (nonsmall cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) using a SRB bioassay.[28] The results (Table 3) showed that all tested lignans had consistent cytotoxicity against the SK-OV-3, SK-MEL-2, and HCT-15 cell lines but not A549, with IC_{50} values of 0.14 to 27.45 μM. In particular, compound **3** exhibited the most potent cytotoxicity against the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines with IC_{50} values of 1.42 ± 0.38, 0.16 ± 0.03, 0.20 ± 0.11, and 0.14 ± 0.07 μM, respectively, which was slightly less active but comparable to that of the positive control, doxorubicin.

The data obtained in this biological test suggested that the dihydrobenzofuran-type lignan, the skeleton of compound **3**, could be a promising class for antitumor agents toward human tumor cell lines. A series of such synthetic dihydrobenzofuran lignans and related derivatives have been explored for their potential antitumor and antiproliferative activities.[24,29] In our previous search for bioactive compounds from Korean natural resources, 3-(β-D-glucopyranosyloxymethyl)-2-(4-hydroxy-3-methoxyphenyl)-5-(3-hydroxypropyl)-7-methoxydihydrobenzofuran, (7*S*,8*R*)-dihydrodehydrodiconiferyl alcohol, and (7*S*,8*R*)-dehydrodiconiferyl alcohol 4-*O*-β-D-glucopyranoside, belonging to the dihydrobenzofuran-type lignan, were identified as minor constituents in Korean traditional medicines.[30−32] However, none exhibited a promising cytotoxic effect in the previously used human tumor cell lines (IC_{50} > 30.0 μM). This result was in agreement with the structure−activity relationship (SAR) analysis of dihydrobenzofuran lignans for antitumor activity in human tumor cell lines, where it was reported that (i) apparently, methylation of the hydroxyl group in two aromatic moieties reduces the activity, (ii) reduction of the double bond in the C_3 side chain causes a decrease in the activity, and (iii) reduction of the ester functionality to a primary alcohol leads to loss in the activity.[29] The SAR analysis suggests that the dihydrobenzofuran-type lignan, compound **3**, which contains the ethyl ester functionality and the double bond in the side chain, but no methoxyl group, yields the greatest cytotoxicity against all tumor cell lines presently examined. However, the dihydrobenzofuran-type lignan has been reported to have a pharmacophore for inhibitors of tubulin polymerization binding at the colchicine site, which can lead to the display of antimitotic and potential antitumor properties.[29] Particularly, methyl(*E*)-3-{2-(3,4-dihydroxyphenyl)-7-hydroxy-3-methoxycarbonyl-2,3-dihydro-1-benzofuran-5yl}prop-2-enoate, which is very similar to compound **3** with the only difference being the replacement of methyl ester functionality, was recently determined to be a potential antitumor agent by causing G2/M arrest and apoptosis involving the mitochondrial controlled pathway.[33]

Progression of cancer is associated with inflammatory responses.[34] Inflammatory gene expression is often negatively correlated with cancer stage and prognosis.[35−37] Nonsteroidal anti-inflammatory drugs are effective for cancer prevention, further supporting a role for inflammation in tumor progression.[38] On the basis of the expanded understanding that an anti-inflammatory agent could be a potential antitumor molecule, we evaluated the anti-inflammatory activities of the isolates (**1**−**4**) through the measurement of nitrite, a soluble oxidation product of NO, in the culture medium using the Griess reaction in LPS-activated BV-2 microglial cells.[39] BV-2 has both the phenotypic and functional properties of reactive microglia cells and is activated following stimulation by various agents including LPS.[40,41] Activated BV-2 cells exhibit various inflammatory responses including the production of the proinflammatory factor NO.[42] Among the tested compounds, compound **3** showed significant inhibitory effects on LPS-stimulated NO production with an IC_{50} of 12.8 μM in BV-2 microglial cells at 20 μM without cytotoxicity, which actually is more potent than N^G-monomethyl-L-arginine (L-NMMA), an inducible NO synthase (iNOS) inhibitor (IC_{50} value of 17.7 μM).[39] The rest of the compounds did not show any significant inhibitory effects on NO production in the range of 1 to 20 μM. Toxicity induced by overproduction of NO, when it becomes chronic, can lead to enhanced cell replication that is a risk factor for many cancers,[43] and oxidative damage to DNA by NO has been associated with the development of cancer.[44] Compound **3** thus could be a potential antitumor agent via the inhibition of NO formation.

In conclusion, it appears that lignans **1**−**4** isolated from the most active EtOAc fraction are at least partially responsible for the antitumor effect of the crude EtOH extract of the morning glory seeds. Among them, pharbilignan C (**3**) is the most promising by virtue of the most potent cytotoxicity in various human tumor cell lines and anti-inflammatory activity involving the inhibited production of NO.

■ ASSOCIATED CONTENT

Supporting Information

NMR data for compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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