Lignans and Coumarins from the Roots of *Anthriscus sylvestris* and Their Increase of Caspase-3 Activity in HL-60 Cells

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A new lignan, sylvestrin (1), was isolated from the MeOH-soluble fraction of the roots of *Anthriscus sylvestris* HOFFM. (Umbelliferae), along with six lignans (2—7), three coumarins (8—10), and a polyacetylene (11). The structure of sylvestrin was determined to be 2-(3′,4′,5′-trimethoxybenzylidene)-3-(3″,4″-methylendioxybenzyl)-γ-butyrolactone (1) by spectroscopic means, including 2D-NMR. The eleven compounds were assessed for their abilities to activate a caspase-3 in human promyeloid leukemic HL-60 cells. The intracellular caspase-3 activity of (−)-deoxypodophyllotoxin (3), angeloyl podophyllotoxin (5), deoxypicropodophyllin (6), picropodophyllotoxin (7), and falcarindiol (11) increased approximately 4.6, 3.6, 3.7, 3.9, and 3.9-fold, at 0.001, 1, 1, 1, and 20 μM, respectively, over that of the untreated control. In addition, compounds 3, 5, 6, and 7 showed apoptosis-inducing activities that were measured by DNA fragmentation in HL-60 cells.

Key words *Anthriscus sylvestris*; Umbelliferae; apoptosis; sylvestrin; caspase-3; DNA fragmentation

Anthriscus sylvestris HOFFM. (Umbelliferae) is a perennial herb that grows in Europe, Caucasus, Siberia, Kamchatka, Kuriles, Sakhalin, China, Manchuria, and Korea. The root of this plant has been used in Korean traditional medicine as an antitussive, an antipyretic, an analgesic, a diuretic, and a cough remedy. This plant has been demonstrated to possess a lignan, deoxypodophyllotoxin,[1] which is known to have antitumor activity,[2,3] anti-platelet aggregation activity, antiviral activity, antiproliferative activity, broad insecticidal activity, inhibition of passive cutaneous anaphylaxis reactions,[4] liver-protective action,[5] and anti-inflammatory activity.[6] Kozawa *et al.* have reported compounds such as lignans, phenylpropanoids, and an acyloxycarboxylic acid in this plant.[7,8] In addition, several reports have indicated the isolation of monoterpenes, anthricinol, deoxypodophyllotoxin, angeloylbutenoic acid, and anthriscusin from the hexane-soluble fraction; anthricin, isoanthricin, and crocactone were obtained from the EtOAc-soluble fraction.[7—9] During the course of our search for new caspase-3 activators from natural sources,[10] we found that the MeOH extract of the roots of *A. sylvestris* showed activity. In this study, we describe the characterization of a new lignan as well as the increase of caspase-3 activity and internucleosomal DNA fragmentation of compounds from this source.

MATERIALS AND METHODS

General Experimental Procedures Melting points were measured on a Yanagimoto micro hot-stage melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter in $CHCl_3$. UV spectra were recorded on a Shimadzu UV-2450 spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AMX 600 spectrometer. EI-MS spectrum was recorded on a JMX-AX 505 HAD mass spectrometer (JEOL Co.). Column chromatography was carried out using silica gel (Kieselgel 60, 63—200 μm, Merck Co.) and RP-18 silica gel (YMC AA12S75). Thin layer chromatography (TLC) was carried out using plates coated with silica gel 60 F_{254} (Merck Co.) and spots were visualized under UV light and by 10% sulfuric acid (in H_2O) following by heating.

Plant Material The roots of *A. sylvestris* HOFFM. (Umbelliferae) were collected at Jeombong Mountain, Gangwon province, Korea in June, 2003. The voucher specimen is deposited at the PLANT Extract Bank (No. 01880), Korea Research Institute of Bioscience and Biotechnology (KRIBB), Korea.

Chemicals RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco Laboratories. Ac-ASP-Glu-Val-Asp 7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) was obtained from Enzyme Systems Products (Livermore, CA, U.S.A.). Camptothecin, penicillin, streptomycin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), propidium iodide (PI), and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Annexin V-FITC was provided by BD Biosciences (U.S.A.). Apoptotic DNA-ladder kit was obtained from Roche (Germany).

Extraction and Isolation The dried roots (2.6 kg) of *A. sylvestris* were extracted with MeOH (20 l×3) at room temperature for 4 d. The combined MeOH extracts were concentrated under reduced pressure and obtained the MeOH extract (471.0 g). The extract was suspended in H_2O (1 l), and the resulting H_2O layer was partitioned with hexane (5 l×3), $CHCl_3$ (5 l×3) and EtOAc (5 l×3), successively, to give the hexane (41 g), $CHCl_3$ (37 g) and EtOAc-soluble fractions (4 g), respectively. The hexane-soluble fraction (41 g) was chromatographed on a silica gel column using hexane : acetone (12 : 1→4 : 1, gradient) to obtain 6 fractions (Fr. ASH-1—6). Fr. ASH-3 (1.8 g) was subjected to CC on a silica gel

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(hexane : acetone, 10 : 1) and RP-18 silica gel column (MeOH, 40%→80%; gradient) to give **8** (3 mg). Fr. ASH-4 (6.52 g) was subjected to chromatographed on a silica gel column (hexane : acetone, 4 : 1→2 : 1; gradient) and RP C-18 silica gel column (MeOH, 0%→80%; gradient) to yield **1** (9 mg), **2** (8 mg), **3** (150 mg), **4** (32 mg), **5** (7 mg), and **11** (830 mg).

The $CHCl_3$-soluble fraction (37 g) was chromatographed on a silica gel column using hexane : EtOAc (8 : 1→1 : 1, gradient) to obtain 8 fractions (Fr. ASC-1—8). Compound **3** (6.7 g) was obtained by crystallization with MeOH from fraction ASC-6 kept overnight at room temperature. The mother liquid of Fr. ASC-6 (8.4 g) was subjected to column chromatography on a silica gel (hexane : EtOAc, 2 : 1) and on MPLC RP-18 (MeOH, 50%→100% in H_2O) to obtain **6** (28 mg), **7** (4.8 mg), **9** (6.3 mg), and **10** (5.4 mg).

Sylvestrin (**1**): Amorphous solid; UV ($CHCl_3$) λ_{max} (log ε): 240 (4.27), 316 (4.25) nm; $[\alpha]_D^{25}$ +21.9° (c=0.1, $CHCl_3$); IR ν_{max} cm^{-1}: 1720 (C=O), 1630 (C=C), 1600, 1480 (aromatic C=C), 910; EI-MS *m/z*: 398 $[M]^+$; HR-EI-MS *m/z*: 398.1364 (Calcd for $C_{22}H_{18}O_7$ $[M]^+$ 398.1365).

Nemerosin (**2**): Amorphous solid; UV ($CHCl_3$) λ_{max} (log ε): 241 (4.27), 318 (4.25) nm; $[\alpha]_D^{25}$ −52.0° (c=0.15, $CHCl_3$); EI-MS *m/z*: 398 $[M]^+$.

(−)-Deoxypodophyllotoxin (**3**): Colorless needles; mp 168—170 °C; UV ($CHCl_3$) λ_{max} (log ε): 241 (3.82), 293 (3.46) nm; $[\alpha]_D^{25}$ −122.4° (c=0.25, $CHCl_3$); EI-MS *m/z*: 398 $[M]^+$.

(−)-Deoxypodorhizone (**4**): Colorless oil; UV ($CHCl_3$) λ_{max} (log ε): 240 sh (4.11), 287 (3.83) nm; $[\alpha]_D^{25}$ −27.7° (c=0.3, $CHCl_3$); EI-MS *m/z*: 400 $[M]^+$.

Angeloyl podophyllotoxin (**5**): Amorphous solid; UV ($CHCl_3$) λ_{max} (log ε): 240 (4.16), 270 (3.68), 286 (3.76) nm; $[\alpha]_D^{25}$ −102.7° (c=0.11, $CHCl_3$); EI-MS *m/z*: 496 $[M]^+$.

Deoxypicropodophyllotoxin (**6**): White needles; mp 168—170 °C; UV ($CHCl_3$) λ_{max} (log ε): 240 (4.23), 294 (3.88) nm; $[\alpha]_D^{25}$ +8.7° (c=0.15, $CHCl_3$); EI-MS *m/z*: 398 $[M]^+$.

Picropodophyllotoxin (**7**): Colorless needles; mp 171—173 °C; UV ($CHCl_3$) λ_{max} (log ε): 240 (4.14), 291 (3.73) nm; $[\alpha]_D^{25}$ −7.3° (c=0.15, $CHCl_3$); EI-MS *m/z*: 414 $[M]^+$.

5-Methoxypsoralen (**8**): Colorless needles; mp 185—187 °C; UV ($CHCl_3$) λ_{max} (log ε): 251 (4.23), 260 (4.19), 269 (4.20), 310 (4.13) nm; $[\alpha]_D^{25}$ +10.0° (c=0.1, $CHCl_3$); EI-MS *m/z*: 216 $[M]^+$.

Isoscopoletin (**9**): Yellow needles; mp 182—184 °C; UV ($CHCl_3$) λ_{max} (log ε): 240 (3.79), 251 (3.74), 259 (3.71), 295 (3.77), 341 (4.06) nm; $[\alpha]_D^{25}$ −4.7° (c=0.19, $CHCl_3$); EI-MS *m/z*: 192 $[M]^+$.

Scopoletin (**10**): Yellow needles; mp 181—183 °C; UV ($CHCl_3$) λ_{max} (log ε): 240 (4.14), 251 (3.84), 259 (3.80), 295 (3.86), 341 (4.15) nm; $[\alpha]_D^{25}$ +8.3° (c=0.12, $CHCl_3$); EI-MS *m/z*: 192 $[M]^+$.

Falcarindiol (**11**): Colorless oil; $[\alpha]_D^{25}$ +150.2° (c=0.1, $CHCl_3$); EI-MS *m/z*: 260 $[M]^+$.

Cell Culture Human promyelocytic leukemia HL-60 cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured in IMDM medium supplemented with 20% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell culture was maintained at 37 °C in a 5% CO_2 humidified incubator.

Cell Viability Cell viability was assessed by the standard MTT viability. Cells were seeded to a concentration of 5×10^5 cells/ml, then compounds **1**—**11** were added to the suspension. After 24 h incubation (37 °C), 10 μl MTT (5 mg/ml) was added to each well. After 4 h incubation (37 °C) and 5 min centrifugation (3000 rpm), the resulting formazan precipitate was dissolved with 100 μl DMSO and the absorption was measured at 570 nm on a microplate reader (BIO-RAD, U.S.A.).[11] The growth inhibition was determined using;

growth inhibitor=(control's OD−sample's OD)/control's OD

Assay of Caspase-3 Activity Caspase-3 enzyme activity was measured by proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC by counting on a microplate fluorometer (Perkin-Elmer, LS50B, U.S.A.). After incubation with compounds **1**—**11** for 24 h, cells were harvested and washed once with cold PBS. The pellets were lysed using 15 μl of lysis buffer containing 10 mM EDTA, 0.5% Triton X-100, and 10 mM Tris–HCl (pH 8.0) at room temperature for 10 min, and then 100 μl of assay buffer (100 mM HEPES; pH 7.5, 10 mM dithiothreitol, 10% sucrose, 0.1% CHAPS, 0.1% BSA) and 10 μl of substrate solution (10 μl of 20 mM substrate+1000 μl assay buffer) were added on ice. Fluoroscene at 400 nm (excitation) and 505 nm (emission) was measured after incubation at 37 °C for 1 h.[12] Control is 0.1% DMSO-treated cells.

DNA Fragmentation After incubation with compounds **3**, **5**, **6**, **7**, **11** (at 0.01, 1, 1, 1, 10 μl) for 24 h, HL-60 cells were harvested and washed with PBS. Apoptotic DNA was purified using an Apoptotic DNA Ladder Kit (Roche, Mannleim, Germany). Then cell pellets were lysed in 200 μl of lysis buffer (10 mM Tris–HCl; pH 8.0, 10 mM urea, 6 M guanidine–HCl, and 20% Triton X-100; pH 4.4) for 10 min. The lysate flowed to the filter and the filter was washed with 4 mM NaCl, 0.4 mM Tris–HCl (pH 7.5) and 80% EtOH. The DNA bound at the filter was eluted with 200 μl elution buffer (10 nM Tris–HCl, pH 8.5). The isolated DNA fragments were separated by 1% agarose gel containing 0.5 μg/ml of ethidium bromide and analyzed under an ultraviolet illuminator.[13] Negative control is 0.1% DMSO-treated cells and positive control treated with 0.5 μM campthothecin.

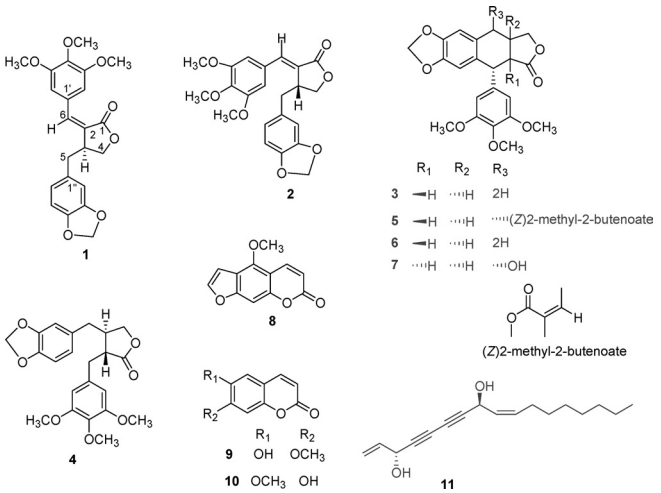

Fig. 1. Chemical Structures of Compounds **1**—**11** Isolated from *A. sylvestris*

RESULTS AND DISCUSSION

As part of the ongoing program of research in our laboratory to detect natural products through the increased activation of caspase-3, the activated properties of compounds from the roots of *Anthriscus sylvestris* HOFFM. (Umbelliferae) were investigated. Eleven compounds were isolated from the roots of *A. sylvestris* by using normal and reversed-phase silica gel column chromatography. The structures of isolated compounds were identified by comparison of their melting point, UV, NMR, and MS spectral data with those data in the literature for nemerosin (**2**), (−)-deoxypodophyllotoxin (**3**), (−)-deoxypodorhizone (**4**), angeloyl podophyllotoxin (**5**), deoxypicropodophyllin (**6**), picropodophyllotoxin (**7**), 5-methoxypsoralen (**8**), isoscopoletin (**9**), scopoletin (**10**), and falcarindiol (**11**).

Sylvestrin (**1**) was obtained as an amorphous solid. The EI-MS spectrum showed a molecular ion peak at *m/z* 398. Its UV spectrum exhibited absorbance at 316 and 240 nm. The ^{1}H-NMR spectrum showed signals for a methine proton at δ 3.42 (m), an oxygenated methylene at δ 4.36 (dd, J=9.0, 7.2 Hz) and 4.13 (dd, J=9.0, 3.6 Hz), three methoxyl protons at δ 3.93 and δ 3.91 (6H), a methylenedioxy group at δ 5.98 and 5.97 (each d, J=1.2 Hz), two aromatic protons at δ 6.62 (s), an ABX system of phenyl protons at δ 6.71 (d, J=1.8 Hz), 6.78 (d, J=7.8 Hz), and 6.66 (dd, J=7.8, 1.8 Hz), two benzylic protons at δ 2.97 (dd, J=13.8, 6.6 Hz) and 2.82 (dd, J=13.8, 9.0 Hz), and an olefinic proton at δ 6.64 (d, J=1.8 Hz). The ^{13}C-NMR spectrum, in combination with the DEPT and HMQC spectra, showed signals for a carbonyl carbon (δ 169.7), two sp^3 methylenes (δ 70.3, 41.1), an sp^3 methine (δ 44.8), eight sp^2 quaternary carbons (δ 153.1; ×2, 148.4, 146.9, 140.2, 131.7, 129.2, 126.8), and six sp^2 methines (δ 141.0, 122.7, 109.7, 109.2; ×2, 108.8). This indicated a dibenzylbutyrolactone lignan with a structure similar to nemerosin (**2**) from *A. sylvestris*.[14] Careful examination of the spectroscopic data revealed several differences between compound **1** and compound **2**. The chemical shift of the olefin proton at H-6 of compound **1** was observed at δ 6.64 (d, J=1.8 Hz), while the corresponding resonance of the *E*-isomer of compound **2** appeared at δ 7.53 due to the deshielding effect of the neighboring carbonyl group.[15,16] Furthermore, the chemical shift of H-3 of compound **1** (δ 3.42) was shifted to a higher field than that of compound **2** (δ 3.86). From these data, the stereochemistry at H-6 of compound **1** was determined as *Z*-configuration, and this finding was supported by the comparison of its physical and spectral properties to those of gadain.[14] The optical rotation of compound **1** (+21°, $CHCl_3$, c=0.1) is opposite that of isochaihulactone (−29°, $CHCl_3$, c=0.5) isolated from *Bupleurum scorzonerifolium*.[17] Therefore, the absolute configuration of C-3 of compound **1** opposite that of isochaihulactone and should be 3*S*. Consequently, the structure of compound **1** was elucidated as (3*S*,6*Z*)-2-(3′,4′,5′-trimethoxybenzylidene)-3-(3″,4″-methylenedioxybenzyl)-γ-butyrolactone. This is the first time that sylvestrin was reported to be obtained from a natural source.

Compounds **1**—**11** were tested for their increasing activity of caspase-3 enzyme in human promyelocytic leukemia HL-60 cells (doubling time about 24 h) for 24 h. Activation of the caspase cascade appears to be a crucial event during the apoptotic process. In particular, caspase-3 activity is the common effector of most apoptotic pathways. Figure 2 shows the effects of compounds on caspase-3 activity. In this experiment, camptothecin (0.5 μM), a potent apoptosis-inducing agent, was used as a positive control. Compounds **3**, **5**, **6**, **7**, and **11** were found to increase caspase-3 activity at the concentrations of 0.001, 1, 1, 1, and 20 μM. Among the compounds tested, compound **3** increased caspase-3 activity the most potently, and was able to induce apoptosis at very low concentrations (1×10^{-3}, 1×10^{-4}, 1×10^{-5}). Compounds **3**, **5**, **6**, and **7** indicated that a cyclohexane ring of dibenzyl-γ-butyrolactone lignan seems to significantly enhance caspase-3 activity.

Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1** and **2** (in $CDCl_3$)

No.	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1		169.7		172.7
2		126.8		127.5
3	3.42, m	44.8	3.86, m	39.9
4	4.13, dd, (9.0, 3.6)	70.3	4.27, dd, (9.0, 2.1)	70.1
	4.36, dd, (9.0, 7.2)		4.33, dd, (9.0, 6.3)	
5	2.82, dd, (13.8, 9.0)	41.1	2.66, dd, (15.0, 10.0)	38.2
	2.97, dd, (13.8, 6.6)		3.07, dd, (15.0, 5.0)	
6	6.64, d, (1.8)	141.0	7.53, d, (1.5)	138.1
1′		131.7		129.8
2′	6.62, s	109.2	6.79, s	107.9
3′		153.1		153.8
4′		140.2		140.4
5′		153.1		153.8
6′	6.62, s	109.2	6.79, s	107.9
1″		129.2		131.7
2″	6.71, d, (1.8)	108.8	6.64, d, (1.5)	109.4
3″		148.4		147.0
4″		146.9		148.4
5″	6.78, d, (7.8)	109.7	6.74, d, (7.8)	108.8
6″	6.66, dd, (7.8, 1.8)	122.7	6.60, dd, (7.8, 1.5)	122.3
$OCH_3(C_3')$	3.91, s	56.7	3.88, s	56.7
$OCH_3(C_4')$	3.93, s	61.3	3.92, s	61.4
$OCH_3(C_5')$	3.91, s	56.7	3.88, s	56.7
OCH_2O	5.97, d, (1.2)	101.4	5.95, d, (1.2)	101.5
	5.98, d, (1.2)		5.96, d, (1.2)	

δ values in ppm and coupling constants in Hz.

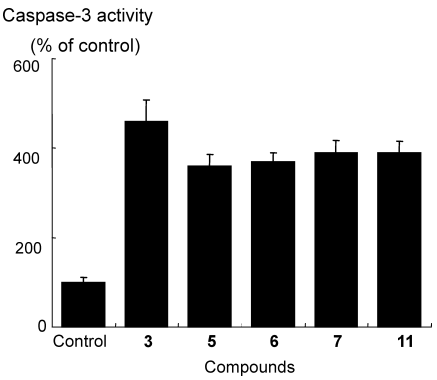

Fig. 2. Effect of (−)-Deoxypodophyllotoxin (**3**), Angeloyl Podophyllotoxin (**5**), Deoxypicropodophyllin (**6**), Picropodophyllotoxin (**7**), and Falcarindiol (**11**) on Caspase-3 Activation

HL-60 cells were treated with compounds **3**, **5**, **6**, **7**, and **11** at 0.001, 1, 1, 1, and 20 μM for 24 h, then collected and washed with PBS. Cell lysate was incubated at 37 °C with Ac-DEVD-AFC for 1 h, after which the fluroscence intensity was measured.

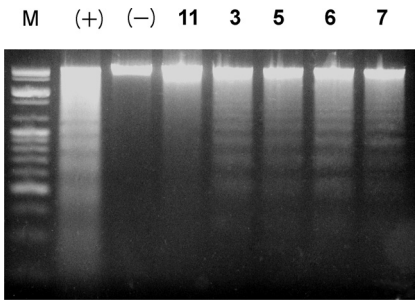

Fig. 3. DNA Fragmentation Induced by (−)-Deoxypodophyllotoxin (**3**), Angeloyl Podophyllotoxin (**5**), Deoxypicropodophyllin (**6**), Picropodophyllotoxin (**7**), and Falcarindiol (**11**)

HL-60 cells were treated with compounds **3**, **5**, **6**, **7**, and **11** at 0.01, 1, 1, 1, and 10 μM for 24 h and then collected. DNA fragmentation of treated cells was detected by 1% agarose gel electrophoresis. M is a DNA size marker.

A DNA ladder pattern can be observed on agarose gel using electrophoresis. When apoptosis occurs, 180—200 bp of repeated DNA fragmentation can be observed due to endonuclease activity. Compounds **3**, **5**, **6**, **7**, and **11** were chosen for the DNA fragmentation experiment after reviewing the results of caspase-3 activity. As shown in Fig. 3, typical ladders of DNA fragmentation were shown in columns treated with compound **5**, **7**, and **8** at 1 μM and compound **3** at 0.01 μM. In contrast, compound **11** did not reveal a DNA ladder pattern at 10 μM.

In summary, the results of the present study demonstrated that falcarindiol (**11**), (−)-deoxypodophyllotoxin (**3**), angeloyl podophyllotoxin (**5**), deoxypicropodophyllin (**6**), and picropodophyllotoxin (**7**) have an apoptosis-inducing effect in HL-60 cells as determined by caspase-3 activation and DNA fragmentation.

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