Cytotoxic Constituents from the Roots of *Anthriscus sylvestris*

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Activity-guided fractionation of the roots of *Anthriscus sylvestris* resulted in the isolation and characterization of five cytotoxic compounds, deoxypodophyllotoxin (**1**), falcarindiol (**2**), and angeloyl podophyllotoxin (**5**) from the hexane soluble fraction and morelensin (**3**), bursehernin (**4**) from the chloroform soluble fraction. It is the first report of the occurrence of compound **5** in nature.

Key words : *Anthriscus sylvestris*, Anti-tumour agents, Cytotoxic lignans, Angeloyl podophyllotoxin

INTRODUCTION

Anthriscus sylvestris Hoffm. (Umbelliferae) is a perennial herb growing in Eurasia and eastern North America (Kozawa *et al.*, 1978a). The root of this plant has been used in Korean folk medicine as an antitussive and diuretic. Even now, it is employed as a hematinic or tonic under the name of "E shen" in Sichuan Sheng (Shisen Sho) in China. In addition, the young aerial part of this plant is sometimes used for food (Kozawa *et al.*, 1982). This plant has been demonstrated to afford a lignan, deoxypodophyllotoxin (Noguchi & Kawanami, 1940) which is known to have cytotoxicity (Ayres & Loike, 1990). Kozawa *et al.* (1978a, 1987b) have already made a study on the components of the dried root, isolating compounds such as lignans, phenylpropanoids and an acyloxycarboxylic acid.

In course of searching for anti-tumor agents from medicinal herbs, the MeOH extract of the roots of *A. sylvestris* was found to be active against human chronic myelogenous leukemia cell k562. Fractionation of the extract monitoring with bioassay led to the isolation of a novel cytotoxic lignan angeloyl podophyllotoxin (**5**), together with four known compounds deoxypodophyllotoxin (**1**), falcarindiol (**2**), morelensin (**3**) and bursehernin (**4**). Cytotoxic activity of the isolated compounds is also described.

MATERIALS AND METHODS

Instruments and reagents

Melting points were measured on a Perkin-Elmer DSC 7 and uncorrected. Optical rotations were taken in $CHCl_3$ on Polax Atago 11418 polarimeter at 20°C. IR spectra were recorded on a Unicam Mattson 1000 spectrophotometer in KBr disks. UV spectra were obtained with a Philips PU 8730 spectrophotometer. EI-MS were obtained on a HP-1100 LC-MSD. A JEOL Lambda-400 spectrometer was used to record NMR spectra 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR with TMS as an internal standard in $CDCl_3$. Open column chromatography was performed on Silica gel 60 (70~230 mesh, 7734, Merck) and Vacuum column chromatography (VCC) and MPLC on Silica gel (230~400 mesh, 9385, Merck). Precoated Kieselgel 60 F_{254} plates (thickness 0.25 mm, 5715, Merck) were used for TLC, with visualization conducted by spraying with 10% v/v ethanolic H_2SO_4 followed by heating at 110°C for 10 min. MPLC were performed using EYELA VSP-3050 pump and EYELA DC-1200 fraction collector. Preparative HPLC was performed on a Waters Delta Prep 4000 equipped with a J'sphere ODS-H80-prep column (YMC, 250×20 mm i.d.,ODS, 4 μm).

Plant materials

The dried roots of *A. sylvestris* were purchased from Kyung-Dong market, in Seoul, Korea and identified by Dr. H. J. Chi, Natural Products Research Institute, Seoul National University. The plant specimen is deposited in our laboratory.

Extraction and isolation

Dried and powdered roots (2 kg) were extracted exhaustively with hot MeOH for 3 h (×5) and concentrated under reduced pressure to give a dried MeOH

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extract. This extract (280 g) was suspended in H_2O and then partitioned successively with hexane, $CHCl_3$, EtOAc, and *n*-BuOH, leaving a residual H_2O soluble fraction. Hexane- and $CHCl_3$-soluble fractions showed cytotoxicity in k562 cell culture system. The hexane-soluble fraction was dissolved in a minimum amount of EtOAc and hexane was added until the solution was slightly turbid and left in a freezer overnight. The precipitate separated from the solution was removed by decapitation. Trituration of the precipitate with hexane gave a colorless residue, which was filtered. The residue (2.01 g) shows essentially a single spot on TLC (compound **1**). Solvent was removed under vacuum from the hexane-soluble fraction (16.9 g), which was then subjected to column chromatography on Silica gel 60 (600 g), eluting with hexane increasing concentration of EtOAc. Fractions showing similar TLC profiles were pooled to provide 37 combined frs. Fr. 22 was subjected to MPLC on silica gel, eluting with $CHCl_3$ and VCC on silica gel with hexane-EtOAc (20:1) to give compound **2** (18.4 mg). Fr. 25 was subjected to repeated VCC on silica gel, eluting with $CHCl_3$-MeOH (300:1) and hexane-EtOAc (12:1) to give compound **5** (10.8 mg). The $CHCl_3$-soluble extract (6.55 g) was chromatographed over silica gel (250 g) eluting with hexane increasing concentration of EtOAc. From this seperation, 28 combined frs. were provided. Fr. 20 was subjected to repeated VCC on silica gel, eluting with hexane-EtOAc (9:1) to give an active fraction which consisted of a mixture of closely related compounds, inseparable by further normal phase chromatography. Separation was effected readily, however, by reverse phase preparative HPLC with MeCN-H_2O (60:40) as a solvent, yielded two pure cytotoxic compounds **3** (12.4 mg) and **4** (8.5 mg). The spectral data for **1-4** were consistent with the published values (Boguchi & Charlton, 1995; Villegas *et al.*, 1988; Jolad *et al.*, 1977; Yamaguchi *et al.*, 1979).

Compound 1: Amorphous solid (Hexane:EtOAc), mp 166~168°C, $[\alpha]_D^{20}$ -113.0° ($CHCl_3$). Compound **1** was characterized as deoxypodophyllotoxin by comparison of physical and spectral data with the published values (Boguchi & Charlton, 1995).

Compound 2: Colorless oil, $[\alpha]_D^{20}$ +219.4° ($CHCl_3$). Compound **2** was characterized as falcarindiol by comparison of physical and spectral data with the published values (Villegas *et al.*, 1988).

Compound 3: Amorphous solid (Hexane:EtOAc), mp 181°C, $[\alpha]_D^{20}$ -125.0° ($CHCl_3$). Compound **3** was characterized as morelensin by comparison of physical and spectral data with the published values (Jolad *et al.*, 1977).

Compound 4: Colorless oil, $[\alpha]_D^{20}$ -151.0° ($CHCl_3$). Compound **4** was characterized as bursehernin by comparison of physical and spectral data with the published values (Yamaguchi *et al.*, 1979).

Compound 5: Amorphous solid (Hexane:EtOAc), $[\alpha]_D^{20}$ -109.1° ($CHCl_3$), IR (KBr) ν_{max} cm^{-1}: 2923 (C-H stretch), 1774 (C=O), 1712 (C=O), 1488 (C=C aromatic ring), 1234 (C-O), 1126 (C-O); EI-MS(70 eV) *m/z* (rel.int.) $[M]^+$ 496 (100.0), $[M-C_5H_7O_2$ (angelic acid)$]^+$ 397 (14.4), 263 (8.7), 229 (7.9), 185 (22.5), 168 (15.6), 135 (7.6), 83 (17.27), 55 (18.86); ^{1}H-(400 MHz) and ^{13}C-NMR (100 MHz) in $CDCl_3$:in Table I.

Cell culture assay for cytotoxic activity

Each of the human tumor cells was cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin and periodically subcultured once or twice per week. 10,000 tumor cells were plated on 96-well microplate 24 h before the test of samples. Each fraction of *A. sylvestris* was dissolved in DMSO, sterilized with a

Table I. ^{1}H-(400 MHz) and ^{13}C-NMR (100 MHz) data and COSY correlations for compound **5** ($CDCl_3$)

	δ C[a]	δ H[b]	^{1}H-^{1}H COSY
1	132.29s		
2	128.51s		
3	109.63d	6.48 (s)	H-7'
4	148.05s		
5	147.57s		
6	107.06d	6.74 (s)	-
7	73.12d	5.89 (d,9.0)	H-8
8	38.88d	2.82 (m)	H-7,H-9a, H-9b,H-8'
9a	71.60t	4.20 (t,9.8)	H-8,H-9b
9b		4.35 (dd,9.3,6.8)	H-8,H-9a
1'	134.87s		
2', 6'	107.80d	6.33 (s)	H-7', 3'-,5'-OCH_3
3'	152.57s		
4'	136.89s		
5'	152.57s		
7'	43.71d	4.55 (d,4.2)	H-3,H-2',6',H-8'
8'	45.60d	2.90 (dd,4.4,14.6)	H-7,H-7'
9'	173.75s		
-OCH_2O-	101.54t	5.92 (s)	-
3',5'-OCH_3	55.93q	3.68 (s)	H-2',6'
4'-OCH_3	60.74q	3.73 (s)	-
1"	167.99s		
2"	126.78s		
3"	140.28d	6.14 (qq,1.5,7.3)	H-4",2"-CH_3
4"	16.01q	1.96 (qd,1.5,7.3)	H-3"
2"-CH_3	20.57q	1.86 (q[c]1.5)	H-3"

[a]Multiplicities were obtained from DEPT experiment.
[b]Multiplicities and coupling constants in Hz are given.
[c]δ1.86 is splited to quintet.

0.22 μm PVDF filter, and serially 5-fold diluted with RPMI 1640 from 40 μg/ml to 0.00256 μg/ml. It was then added into a 96-well microplate containing the human tumor cells. The final concentration of each fraction was from 20 μg/ml to 0.00128 μg/ml and that of DMSO was below 0.5%. After the cells were cultured for 48 h in a 37°C, 5% CO_2 incubator, 25 μl of 2 mg/ml MTT(3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide) was added to cultures of each well and then the cells were cultured for additional 4 h. Microplates were centrifuged at 1000 rpm for 10 min, and the supernatants were removed by flicking. The formazan formed by mitochondrial succinate dehydrogenase from MTT was dissolved in 100 μl of DMSO. Absorbance of each well was measured at 540 nm with a ELISA reader (Bio-Tek, Winooski, VT). The IC_{50} (50% inhibition concentration) was calculated by Linear Regression method (Carmichael *et al.*, 1987).

RESULTS AND DISCUSSION

Hexane-soluble and $CHCl_3$-soluble fractions were separated by silica gel and octadecylsilanized (ODS) silica gel column chromatographies to give compounds **1-5**. The structures of **1-4** were identified as deoxypodophyllotoxin (**1**), falcarindiol (**2**), morelensin (**3**) and bursehernin (**4**), respectively (Fig. 1), on the basis of their spectroscopic properties and by comparison of their physical and spectral data with published values (Boguchi & Charlton, 1995; Villegas *et al.*, 1988; Jolad *et al.*, 1977; Yamaguchi *et al.*, 1979).

Compound **5** was isolated as an amorphous solid, $[\alpha]_D^{20}$ -109.1° ($CHCl_3$). Compound **5** gave a violet coloration with sulfuric acid and showed IR bands at 1774, 1712 (C=O), 1488 (C=C), 1234, 1126 (C-O). The EI mass spectrum of compound **5** displayed a molecular ion peak at *m/z* 496. The ion at *m/z* 397 $[M\text{-}C_5H_7O_2$ (angelic acid)$]^+$ represented the fragment $C_{22}H_{21}O_7$.

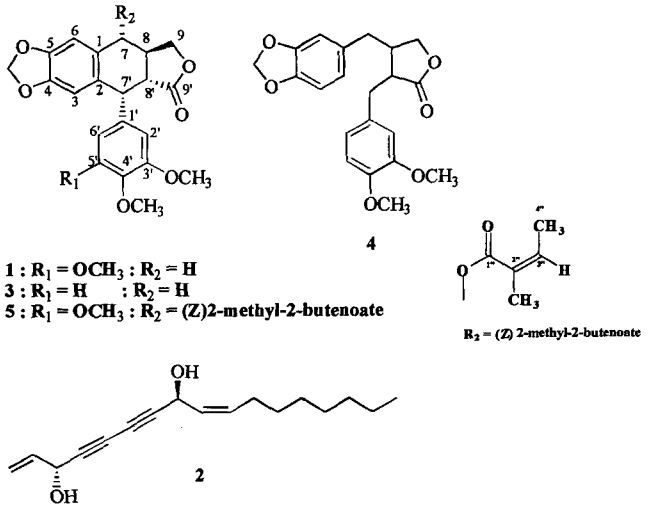

Fig. 1. Structures of the isolated compounds from *Anthriscus sylvestris*

The ^{1}H-NMR spectrum of compound **5** showed signals (δ ppm, $CDCl_3$) at 1.86 (3H, quintet), 1.96 (3H, qd) and 6.14 (1H, qq) assignable to angeloyl group (Kozawa *et al.*, 1978a). Two 6H and 3H singlets at δ 3.68 and 3.73, which could be assigned to three methoxy groups, two of which have identical magnetic environments. A downfield methylene singlet at δ 5.91 was characteristic of a methylenedioxy group. A set of geminal-coupled protons resonating at δ 4.20 and 4.35 represented a methylene, i.e. C-9 protons sandwiched between an oxygen and methine. A COSY spectrum displayed cross-peaks between signals at δ 4.19 and 4.35 due to their geminal disposition, while the cross-peaks between δ 4.19 and 2.87 represented vicinal coupling between one of the methylenic protons with the neighbouring methine proton (C-8H). A 2H singlet in the aromatic region of the spectrum at δ 6.33 was assigned to the two aromatic protons in an identical magnetic environment, i.e. C-2' and C-6' protons. Two more downfield signals appearing as broad singlet at δ 6.74 and 6.48 were ascribed to the two remaining aromatic protons of the skeleton. The absence of coupling interactions between these aromatic protons indicated their para-disposition and they were therefore assigned to the C-6 and C-3 protons, respectively (Table I).

The ^{13}C-NMR spectrum of **5** showed 24 signals representing 27 carbons. An examination of the structure shows that the aromatic ring substituted at C-7' contains three pairs of identical carbons (the two -OCH_3 carbons appearing at δ 55.93, the two carbons at which the -OCH_3 groups are attached resonating at δ 152.57 and the two carbons ortho to a methoxy group resonating at δ 107.80). An ester carbonyl carbon (C-9') resonated at δ 173.75, a methylene-dioxy carbon appeared at δ 101.54 and a downfield methylene carbon resonated at δ 71.60. The methine carbons resonated at δ 43.71, 45.60, 38.88 and 73.12 representing the carbon atoms of ring B. Aromatic carbons appeared in two groups. The signals between δ 107 and 110 were due to nonoxygenated aromatic carbons, while the signals resonating between δ 128 and 153 represented either oxygen-bearing or quaternary aromatic carbons.

In the heteronuclear multiple quantum coherence (HMQC) experiment, C-6 and C-3 carbons (δ 107.06 and 109.63) in the aromatic moiety displayed one-bond interaction with the protons resonating at δ 6.74 and 6.48, respectively, while another set of carbons resonating at δ 107.80 (C-2' and C-6') in ring C showed correlation with C-2' and C-6' protons (δ 6.33). C-9 methylenic protons exhibited heteronuclear coupling with C-9 carbon (δ 71.60).

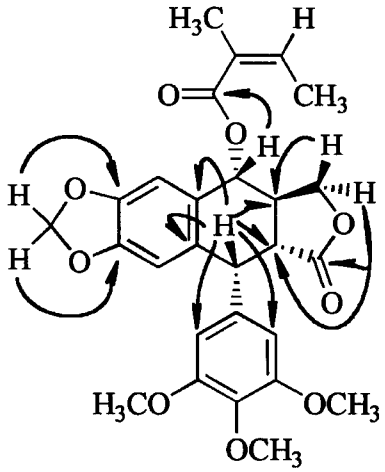

Fig. 2. HMBC correlations of compound **5** in $CDCl_3$

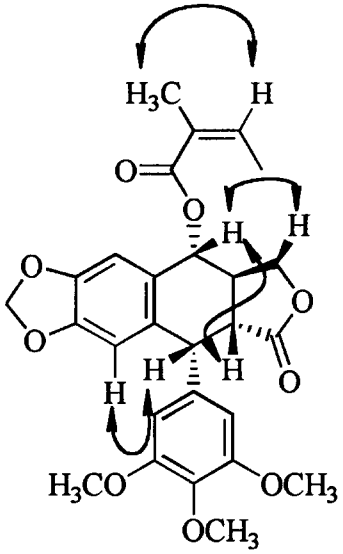

Fig. 3. NOE correlations of compound **5** in $CDCl_3$

In the heteronuclear multiple bond connectivity (HMBC) experiment, the long-range interactions between the protons at δ 6.33 (H-2' and H-6') with the carbons resonating at δ 152.57 (C-5') and δ 136.89 (C-4') suggested that they are part of ring C. Carbon-9 methylene protons displayed HMBC interaction with C-8 (δ 38.88) and C-9b proton exhibited long-range interactions with the carbons at δ 45.60 (C-8') and 173.75 (C-9'). The proton resonating at δ 4.55 (C-7'H) had long-range interaction with C-2, C-1, C-8, C-8' and also with C-2' and C-6' carbons in ring C. Similarly C-6 proton (δ 6.74) in the aromatic moiety exhibited interactions with C-5, C-4, C-7, and C-1, while the aromatic proton resonating at δ 6.48 (C-3H) showed interaction with C-2, C-7', C-5, and C-4. C-7 proton (δ 5.89) exhibited HMBC interaction with C-1" (δ 167.99) which suggested that angeloyl moiety attached at C-7 (Fig. 2).

A nuclear overhauser effect spectroscopy (NOESY) experiment shows that 2"-methyl protons correlates with H-3" but not with H-4". Thus it is unambiguous that the moiety is angelic acid (Fig. 3).

Complete analysis of ^{1}H and ^{13}C NMR spectra with the aid of COSY, HMQC and HMBC experiments led to the identification of compound **5** as shown in Fig. 1. The chemical shift of the lignan part of compound **5** was comparable to those of podophyllotoxin (Brewer *et al.*, 1979). Thus the structure of compound **5** was determined as angeloyl podophyllotoxin.

In the ^{1}H-NMR spectrum, coupling constants (4.4 Hz) of H-7', H-8' show that they are configured with eclipsed *cis*. Each coupling constants(14.6Hz) of H-8, H-8' and coupling constants(9.0Hz) of H-7, H-8 show that they are configured with anti *trans*. So stereo configuration of compound **5** must be *R-R-R-R* (C7-C8-C8'-C7') or *S-S-S-S*. In the natural compound configuration of podophyllotoxin is *R-R-R-R* and the ^{1}H-NMR spectrum of compound **5** is similar to that of podophyllotoxin (Brewer *et al.*, 1979). Thus, we determined that the stereo configuration of compound 5 is *R-R-R-R* (C7-C8-C8'-C7')

Table II. Cytotoxic activity of the isolated compounds (IC_{50}, μg/ml)

Compounds	Colo205 (Human Colon)	k562 (Human Leukemia)
1	0.0958	0.0184
2	0.819	0.577
3	>20	16.445
4	16.445	0.426
5	0.0958	0.0184
Etoposide	>20	1.477
Doxorubicin	0.682	

The isolated compounds were evaluated for their cytotoxic activity on human chronic myelogenous leukemia cell k562 and human colon tumor cell Colo 205. The results are shown in Table II. Compound **1** and **5** showed exceptionally strong cytotoxic activity against the leukemia cells, with IC_{50} value of 0.0184 μg/ml, which was approximately 5 and 100 times greater than doxorubicin and etoposide, positive control anticancer agents, respectively. *In vivo* evaluation of compound **5** on anti-tumor activity remains to be carried out.

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