Antiproliferative Constituents in Umbelliferae Plants II.¹⁾ Screening for Polyacetylenes in Some Umbelliferae Plants, and Isolation of Panaxynol and Falcarindiol from the Root of *Heracleum moellendorffii*

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Methanol extracts of 36 samples of 21 Umbelliferae plants were screened for polyacetylenic compounds using the ELISA for panaxytriol, and their antiproliferative activity was checked by MTT assay using the tumor cell lines MK-1, HeLa and B16F10. The presence of antiproliferative polyacetylenes was suggested in *Angelica acutiloba* (fruit), *Anethum graveolens* (root), *Bupleurum rotundifolium* (fruit), *Carum carvi* (fruit and root), *Coriandrum sativum* (fruit), *Cryptotaenia japonica* (leaf), *Glehnia littoralis* (fruit), *Heracleum moellendorffii* (root) and *Torilis japonica* (fruit).

Panaxynol and falcarindiol were successfully isolated from the root of *Heracleum moellendorffii* as antiproliferative polyacetylenes.

Key words *Heracleum moellendorffii*; Umbelliferae; antiproliferative activity; polyacetylene; panaxynol; falcarindiol

The authors have identified the antiproliferative constituents in "red ginseng" (the steamed roots of *Panax ginseng* C. A. MEYER) as panaxytriol, panaxynol and panaxydol,²⁾ and found that panaxytriol enhances the cytotoxicity of mitomycin C on a human gastric adenocarcinoma cell line, MK-1³⁾; it also inhibits cellular respiration and disrupts the cellular energy balance in a human breast carcinoma cell line, Breast M25-SF.⁴⁾ These findings suggest the possibility of polyacetylenes becoming new anticancer drugs or materials which can be expected to have a synergistic effect on anticancer drugs. This possibility has prompted us to investigate the polyacetylenes in the plants, and in the preceding paper we reported the establishment of a highly sensitive enzyme-linked immunosorbent assay (ELISA) for panaxytriol⁵⁾ as well as the results of screening polyacetylenes in crude drugs using the ELISA.¹⁾ We found that the methanol extracts of all 9 crude drugs examined originating from the Umbelliferae plants showed a positive reaction in the ELISA, and we isolated panaxynol and heptadeca-1,8-diene-3,10-diol from *Saposhnikovia* Radix, falcarindiol from *Peucedani* Radix, and 9,10-epoxy-3-hydroxy-octadeca-1-ene-4,6-diyne-18-al from *Foeniculi* Fructus. All these polyacetylenes showed inhibitory effects on the colony growth of MK-1 cells.

In a continuation of the search for antiproliferative polyacetylenes, we have extended the range of investigation to other Umbelliferae plants. This paper deals with the results of the screening of 36 samples of 21 Umbelliferae plants, and the identification of two polyacetylenes in the root of *Heracleum moellendorffii*.

MATERIALS AND METHODS

Equipment Proton nuclear magnetic resonance (¹H-NMR) (500 MHz) and carbon-13 magnetic resonance (¹³C-NMR) (125 MHz) spectra were recorded with a JEOL JNM-A500 spectrometer in CDCl₃ solution using tetramethylsilane as an internal standard. FAB-MS were measured with a

JEOL JMS-HX 110 spectrometer using *m*-nitrobenzyl alcohol containing NaI as the matrix. Nunc F Immunoplates (Nunc, Roskilde, Denmark) and a Molecular Devices UV_{max} ELISA analyzer (Menlo Park, California) were used in the ELISA experiment. Column chromatography was carried out on Silica gel 60 (230–400 mesh, Merck).

Plant Materials Plants used for the screening test were collected in a suburb of Fukuoka City, or cultivated in the medicinal plant garden of the Faculty of Pharmaceutical Sciences, Fukuoka University. All plant materials were dried at room temperature. The roots of *Heracleum moellendorffii* were collected in Fukuoka City.

Preparation of the Test Samples for ELISA The dried and powdered plant material (10 g) was extracted with MeOH (100 ml) under reflux for 1 h. The MeOH solution was evaporated under reduced pressure and the residue was dried *in vacuo*. A 1/10 portion of the MeOH extract (1 g equivalent of the dried plant material) was suspended in 10 ml of 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1% bovine serum albumin (BSA) (buffer A). The solution was filtered and diluted 50, 250 and 1250 times with the same buffer (1 ml each solution contains the extract in quantifies of 2, 0.4 and 0.08 mg equivalent of the dried plant material, respectively), and 50 μl of each solution was used for the ELISA.

ELISA for the Polyacetylenic Compounds The ELISA for panaxytriol was used to detect polyacetylenic compounds in the plant materials. Details of the method are described in the preceding paper.⁵⁾

(1) Coating of the anti-panaxytriol IgG to the microtiter wells: The wells in microtiter plates were coated by loading an anti-panaxytriol IgG solution (150 μl) (4 μg/ml in buffer B) and allowing it to stand overnight at 4 °C. After the plates were washed twice with buffer C, they were incubated with 2% BSA in phosphate-buffered saline (PBS) (200 μl) for 20 min at 37 °C to prevent nonspecific adsorption.

(2) Immunoreaction of the polyacetylenes and the panaxytriol-horseradish peroxidase (HRP) conjugate against

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the anti-panaxytriol IgG: The anti-panaxytriol IgG-coated wells, thus prepared, were then filled with the test sample solution (50 μ l), or PBS (50 μ l) as a control, immediately followed by the addition of the panaxytriol-HRP conjugate suspension (50 μ l) in buffer A. The wells were then incubated overnight at 4 °C, and washed briskly with buffer C.

(3) Peroxidase-catalyzed oxidation of 3,3',5,5'-tetramethylbenzidine (TMB): 0.42 mM of TMB solution (150 μ l) in buffer D containing 3% dimethylsulfoxide (DMSO) and 0.01% hydrogen peroxide was added to the above wells, and the wells were incubated at 37 °C for a period suitable to allow the enzyme-catalyzed oxidation of TMB. The oxidation reaction was quenched by adding 2.0 M H_2SO_4 (50 μ l) to each well. The absorbance (B) of the oxidation product in the test sample well and that (B_0) of the control well were measured spectrophotometrically at 450 nm using the ELISA analyzer.

The amount of panaxytriol-HRP conjugate bound to the anti-panaxytriol IgG in the test sample well was inversely proportional to the amount of polyacetylenes in the test sample. The amount of panaxytriol-HRP conjugate bound to the antibody was measured in terms of peroxidase activity. The absorbance was proportional to the amount of oxidation product, which was proportional to the amount of panaxytriol-peroxidase conjugate bound to the antibody and inversely proportional to the amount of polyacetylenes in the test sample; therefore, the higher B/B_0 value suggests a lower content, and the lower B/B_0 value means a higher content of free panaxytriol and/or the related polyacetylenic compounds in the test sample.

The buffers used in this experiment are as follows: A, 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1% BSA; B, 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM Na_2S_3 ; C, 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.05% (v/v) Tween 20; D, 0.05 M acetate-citric acid buffer (pH 5.5).

Measurement of Antiproliferative Activity against Tumor Cell Lines Nude mouse-transplantable human gastric adenocarcinoma cells (MK-1), human uterus carcinoma cells (HeLa) and murine malignant melanoma cells (B16F10) were used as tumor cell lines. Cellular growth was determined using the MTT-microculture tetrazolium assay.⁶⁾

Thus, the tumor cells were maintained in RPMI 1640 containing 10% fetal calf serum. Fifty μ l of the tumor cell suspension (2×10^5 cells/ml) and 50 μ l of the test sample suspension were plated in flat-bottomed microtiter wells and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After cultivation, 10 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/ml) was added to the microtiter wells. After 2 h incubation at 37 °C, 100 μ l was taken out from each well, to which 100 μ l of DMSO was added to solubilize the MTT-formazan product. The absorbance at 590 nm was measured with an ELISA analyzer. The percent cell growth inhibition (ED) = $(1 - A/B) \times 100$ (A: OD_{590} of the tumor cells in RPMI 1640 with the test sample, B: OD_{590} of tumor cells in RPMI 1640 without the test sample).

Isolation of Polyacetylenic Compounds from the Roots of *Heracleum moellendorffii* The powdered dried root (150 g) was packed in a glass tube and percolated with $CHCl_3$ (1 l). The $CHCl_3$ eluate was evaporated and concen-

trated under reduced pressure and dried *in vacuo* to give a reddish brown oily residue (HMR-I; 7 g). The residual root powder after percolation with $CHCl_3$ was percolated with MeOH (1 l). The MeOH eluate was concentrated *in vacuo*, the residue was suspended in water (200 ml), and the turbid solution was passed through a column of a polystyrene resin Daion HP-20 (50 ml). After washing the column with water (200 ml), the materials absorbed in the resin were eluted with MeOH (200 ml). The MeOH eluate was concentrated and dried *in vacuo* to give a brown powder (HMR-II, 1.5 g). The antiproliferative activity (ED_{50}) of HMR-I and HMR-II against MK-1 were *ca.* 2.5 μ g/ml and 20 μ g/ml, respectively, suggesting that antiproliferative polyacetylenes are contained in HMR-I. HMR-I (immunoreactivity B/B_0 : 33.3% at 40 μ g/ml; antiproliferative activity: ED_{50} , *ca.* 2.5 μ g/ml) (7 g) was suspended in a hexane-AcOEt (3 : 2, v/v) mixture, and the insoluble material (HMR-Ia, 5.24 g, B/B_0 : 73.7% at 40 μ g/ml, ED_{50} : *ca.* 12 μ g/ml) was removed by centrifugation. The supernatant was concentrated and dried *in vacuo* to give a reddish oil (HMR-Ib, 1.65 g, B/B_0 : 22.8% at 40 μ g/ml; ED_{50} : *ca.* 0.8 μ g/ml). HMR-Ib was repeatedly chromatographed on silica gel using hexane-AcOEt (3 : 1, v/v) as an eluting solvent, then fractionated into 9 fractions monitored by TLC. Among these fractions, the 3rd and the 8th fractions showed high antiproliferative activity (ED_{50} : both less than 0.2 μ g/ml) and low B/B_0 values (the 3rd fraction, 29.6%; the 8th fraction, 30.6% both at 40 μ g/ml). Other fractions showed low antiproliferative activity and higher B/B_0 values. Two fractions were purified by silica gel column chromatography to give compound **1** (55 mg) from the 3rd fraction, and compound **2** (626 mg) from the 8th fraction, both as colorless oils.

RESULTS

Screening for Polyacetylenic Compounds Using the ELISA for Panaxytriol and Examination of Their Antiproliferative Activity The 36 MeOH extracts of 21 Umbelliferae plants were screened for polyacetylenic compounds using the ELISA for panaxytriol, and their antiproliferative activity was examined against three tumor cell lines, MK-1, HeLa and B16F10. The results are shown in Table 1.

Identification of the Polyacetylenic Compounds Compound **1** was obtained as a colorless oil; however, it gradually turned a reddish yellow color on standing at room temperature. The molecular formula, $C_{17}H_{24}O$, was provided by high-resolution FAB-MS. The 1H -NMR spectrum showed signals of a normal C_7H_{15} group linked to an olefin carbon, a methylene group linked to an olefin carbon and a quaternary carbon, one $>CH-OH$ group, a *cis* disubstituted olefin group, and a monosubstituted olefin (a terminal olefin) group (Table 2). The ^{13}C -NMR spectrum showed four acetylenic carbon signals in addition to the signals correlative to the proton signals described above (Table 3). These data suggest that compound **1** is a panaxynol. The identity was confirmed by comparison of the NMR data with those reported.⁷⁾

Compound **2**, a colorless oil, $C_{17}H_{24}O_2$, showed the 1H -NMR signals of a normal C_7H_{15} group, two $>CH-OH$ groups linked to olefin groups, a *cis* disubstituted olefin group, and a terminal olefin group (Table 2), and the ^{13}C -NMR spectrum showed signals of four acetylenic carbons in

Table 1. Immunoreactivity with Anti Panaxytriol Antibody and Antiproliferative Activity of Extracts of Umbelliferae Plants

Plant name	Part	Immunoreactivity (B/B ₀ , %) ^{a)}			Antiproliferative activity (ED ₅₀ , µg/ml) ^{b)}		
		Dilution (fold)			Tumor cell lines		
		×50	×250	×1250	MK-1	HeLa	B16F10
<i>Angelica acutiloba</i>	leaf	71.3	89.1	94.0	>100	>100	>100
	fruit	27.9	40.0	50.4	70	>100	>100
<i>Angelica dahurica</i>	fruit	39.9	62.2	69.3	40	>100	50
<i>Angelica decursiva</i>	leaf	58.8	79.1	98.9	50	>100	>100
	root	43.7	70.0	84.8	30	>100	>100
<i>Angelica japonica</i>	leaf	58.0	78.2	94.0	>100	>100	>100
	root	43.2	60.4	69.6	25	>100	>100
	fruit	45.7	58.5	68.6	40	>100	30
<i>Angelica keiskei</i>	leaf	72.9	88.8	98.8	>100	>100	>100
	root	53.5	77.3	82.7	90	>100	50
<i>Anethum graveolens</i>	leaf	62.2	87.5	101.0	>100	>100	50
	fruit	35.2	57.2	59.3	>100	>100	>100
	root	11.9	22.2	30.7	25	>100	>100
<i>Anthriscus cerefolium</i>	fruit	52.0	73.6	76.2	50	>100	100
<i>Anthriscus sylvestris</i>	leaf	61.4	84.6	92.4	<1.56	1.56	1.56
	fruit	82.7	82.3	84.0	<1.56	1.56	1.56
	root	38.4	66.4	74.4	<1.56	1.56	1.56
<i>Bupleurum falcatum</i>	leaf	59.6	78.0	82.7	>100	>100	>100
<i>Bupleurum rotundifolium</i>	fruit	38.3	47.7	71.1	6.25	50	25
<i>Carum carvi</i>	leaf	56.3	81.8	97.1	75	>100	>100
	fruit	13.3	21.8	26.8	50	>100	70
	root	19.3	37.2	40.5	12	>100	80
<i>Centella asiatica</i>	leaf	31.4	58.2	65.8	30	>100	50
<i>Cnidium officinale</i>	leaf	81.7	92.0	96.9	>100	>100	>100
<i>Coriandrum sativum</i>	leaf	73.6	89.2	99.9	>100	>100	>100
	fruit	9.0	16.6	28.2	40	>100	50
<i>Cryptotaenia japonica</i>	leaf	29.6	49.5	62.8	40	>100	70
<i>Daucus carota ssp sativus</i>	root	35.4	58.3	70.0	>100	>100	>100
<i>Foeniculum vulgare</i>	leaf	76.4	86.8	96.0	75	>100	100
<i>Glehnia littoralis</i>	fruit	14.1	21.9	29.2	5	>100	25
<i>Heracleum moellendorffii</i>	leaf	44.4	67.6	81.7	80	>100	>100
	root	39.4	64.3	71.4	25	>100	>100
<i>Peucedanum japonicum</i>	root	54.9	76.2	85.9	>100	>100	>100
<i>Torilis japonica</i>	leaf	47.4	71.3	83.7	>100	>100	>100
	fruit	22.3	31.4	46.6	12.5	>100	12.5
	leaf	29.8	50.9	69.4	25	>100	75

a) The value shows the amount (percentage) of bound enzyme activity for test samples (B) as a ratio of that bound using panaxytriol-HRP alone (B₀). Decreased value of B/B₀ reflects an increase in the amount of polyacetylenic compounds in test samples. b) ED₅₀ is the concentration of each test sample required to obtain 50% growth inhibition.

Table 2. ¹H-NMR Assignments for Compounds 1 and 2 (500 MHz, ppm, CDCl₃)

	Compound 1	Compound 2
H-1	5.23 (ddd, J=1.0, 1.5, 10.5 Hz)	5.25 (ddd, J=1.0, 1.5, 10.5 Hz)
	5.46 (ddd, J=1.0, 1.5, 17.0 Hz)	5.47 (ddd, J=1.0, 1.5, 17.0 Hz)
H-2	5.94 (ddd, J=5.5, 10.5, 17.0 Hz)	5.94 (ddd, J=5.5, 10.5, 17.0 Hz)
H-3	4.90 (br d, J=5.5 Hz)	4.94 (br d, J=5.5 Hz)
H-8	3.03 (br d, J=7.0 Hz)	5.20 (br d, J=8.0 Hz)
H-9	5.37 (dddd, J=1.5, 1.5, 7.0, 7.0, 11.0 Hz)	5.51 (dddd, J=1.5, 1.5, 8.0, 10.5 Hz)
H-10	5.51 (dddd, J=1.5, 1.5, 7.0, 7.0, 11.0 Hz)	5.61 (dddd, J=1.0, 7.5, 7.5, 10.5 Hz)
H-11	2.02 (br ddd, J=7.0, 7.0, 7.0 Hz)	2.11 (dddd, J=1.5, 7.5, 7.5, 7.5 Hz)
H-12	1.36 (t-like, J=7.0 Hz)	1.38 (t-like, J=7.5 Hz)
H-13	1.28 (m)	1.28 (m)
H-14	1.28 (m)	1.28 (m)
H-15	1.28 (m)	1.28 (m)
H-16	1.28 (m)	1.28 (m)
H-17	0.88 (t-like, J=7.5 Hz)	0.88 (t-like, J=7.0 Hz)

addition to the signals corresponding to the above-mentioned functional groups (Table 3). Compound 2 was identified as falcariindiol by comparison of the NMR data with those reported.⁸⁾

Antiproliferative Activity of Panaxynol and Falcariindiol The antiproliferative activity of these polyacetylenic compounds, panaxynol and falcariindiol, was determined by MTT assay using B16F10 cells as a target.

Table 3. ^{13}C -NMR Signal Assignments for Compounds 1 and 2 (125 MHz, ppm, CDCl_3)

	Compound 1	Compound 2
C-1	117.0	117.2
C-2	136.2	135.9
C-3	63.6	63.4
C-4	74.3	78.3
C-5	71.3	70.3
C-6	64.1	68.7
C-7	80.3	79.9
C-8	17.7	58.6
C-9	121.9	127.7 ^{a)}
C-10	133.1	134.6 ^{a)}
C-11	27.2	27.7 ^{a)}
C-12	29.1	29.2 ^{a)}
C-13	29.2	29.1
C-14	29.2	29.1
C-15	31.8	31.8
C-16	22.6	22.6
C-17	14.1	14.0

a) Signal assignments by Kern *et al.*⁸⁾ were revised on the basis of the HMBC experiment.

Fig. 1. Dose-Effect Relationships of Panaxynol and Falcariindiol on Growth of B16F10 Cells

B16F10 cells were incubated with various concentration of panaxynol (●) or falcariindiol (○) for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Percent growth inhibition = $(1 - \text{OD}_{590} \text{ of B16F10 cells in RPMI 1640 with the test sample} / \text{OD}_{590} \text{ of B16F10 cells in RPMI 1640 without the test sample}) \times 100$. Results are the mean $\pm$ S.D. of three experiments.

Panaxynol and falcariindiol suppressed their proliferation in a dose-dependent fashion, as shown in Fig. 1. The ED_{50} of panaxynol and falcariindiol was 3.7 ± 1.4 and 23.2 ± 0.5 $\mu\text{g/ml}$, respectively.

DISCUSSION

The ELISA for panaxytriol was originally established to detect panaxytriol in the serum. We examined the structure requirement of this assay using panaxytriol (3) and a limited number of polyacetylenes having similar partial structures, $\text{CH}_2=\text{CH}-\text{CH}(\text{OH})\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{R}$. The cross-reactivity (panaxytriol; 100%) of panaxydol (4) is 2.7%, panaxynol (1): 0.04%; heptadeca-1,8-diene-4,6-diyne-3,10-diol (5): 0.32%; and falcariindiol (2): 0.042%.⁵⁾ These data show that the specificity of this method largely depends on partial structures, $\text{CH}_2=\text{CH}-\text{CH}(\text{OH})\text{C}\equiv\text{C}-\text{C}\equiv\text{C}-\text{CH}_2-\text{CH}(\text{OH})-$

$\text{CH}(\text{OH})-$, and the cross-reactivity varies depending on variations of the glycol moiety of this partial structure. Therefore, the B/B_0 value of the extract of the plant material does not necessarily reflect the total amount of polyacetylenic compounds; however, judging from the results shown in the preceding paper,¹⁾ it may safely be said that the ELISA for panaxytriol is a powerful tool for detecting this type of polyacetylenic compound in plant materials.

Among plant materials tested in this study, *Angelica acutiloba* (fruit), *Anethum graveolens* (root), *Bupleurum rotundifolium* (fruit), *Carum carvi* (fruit and root), *Coriandrum sativum* (fruit), *Cryptotaenia japonica* (leaf), *Glehnia littoralis* (fruit) and *Torilis japonica* (fruit) showed B/B_0 values of less than 50% at 250 times dilution. These materials, except for *Angelica acutiloba* (fruit) and *Carum carvi* (fruit), showed high antiproliferative activity against tumor cells and they are highly likely to contain antiproliferative polyacetylenic compounds.

The extracts of the leaf, fruit and root of *Anthriscus sylvestris* showed extremely high antiproliferative activity, while the B/B_0 values were much higher than those of the above-mentioned plant materials. Its root might contain polyacetylenic compounds; however, it is difficult to consider that the activity is due only to the polyacetylenic compounds. The high activity strongly suggests the presence of other antiproliferative compounds in this plant.

The extract of the root of *Heracleum moellendorfii* did not show as low a B/B_0 value as those of the above-mentioned plant materials; however, the value suggested the presence of polyacetylenic compounds related to panaxytriol. So far, there has been no report of the isolation of polyacetylenes from this plant. Therefore, we checked the extract using a combination of the ELISA and the MTT assay for the antiproliferative polyacetylenes. The CHCl_3 extract of the root was fractionated monitored by the ELISA, MTT assay and TLC, and two polyacetylenic compounds, panaxynol and falcariindiol, were isolated. In the course of fractionation, a mixture of crystalline compounds was obtained, and it showed properties suggesting it to be coumarins. It showed no antiproliferative activity; therefore, further investigation on this mixture was not carried out.

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