Isolation and Structure of Woodorien, a New Glucoside Having Antiviral Activity, from *Woodwardia orientalis*

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Hot aqueous and methanol extracts of the rhizomes of *Woodwardia orientalis* were tested for their *in vitro* antiviral activity against herpes simplex virus type 1 (HSV-1), poliovirus type 1, and measles virus by plaque reduction assay. The aqueous extract of *W. orientalis* reduced the plaque forming ability of HSV-1 and poliovirus more strongly than did the methanol extract. By bioassay-directed fractionation of the aqueous extract, a new glucoside, woodorien (1), along with five known compounds were isolated from an EtOAc-soluble fraction that had antiviral activity. The structures of these compounds were determined by the use of two dimensional (2D) NMR techniques (^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{13}C COSY and heteronuclear multiple-bond multiple-quantum coherence (HMBC)). Woodorien (1) was the most potent inhibitor against HSV-1 among the isolated compounds.

Keywords *Woodwardia orientalis*; herpes simplex virus type 1; antiviral activity; woodorien

Woodwardia orientalis is a small perennial fern which grows in shady, wet places. The rhizome of *W. orientalis* has been widely used in ancient Chinese folklore as an anti-inflammatory and anti-infective remedy.[1)]

In recent years, the antiviral activity of crude drugs has been widely studied,[2)] which drew our attention. In the course of our search for antiviral substances from natural sources, we screened several crude drugs for their antiviral activity using the plaque reduction assay. The aqueous extract of *W. orientalis* significantly reduced the plaque forming ability of herpes simplex virus type 1 (HSV-1) and poliovirus. Hence, antiviral activity guided fractionation was performed. The EtOAc-soluble fraction showed strong activity against HSV-1. Chemical analysis of the EtOAc-soluble fraction led us to isolate a new glucoside and five known compounds. The structures of these compounds were determined by spectroscopic methods.

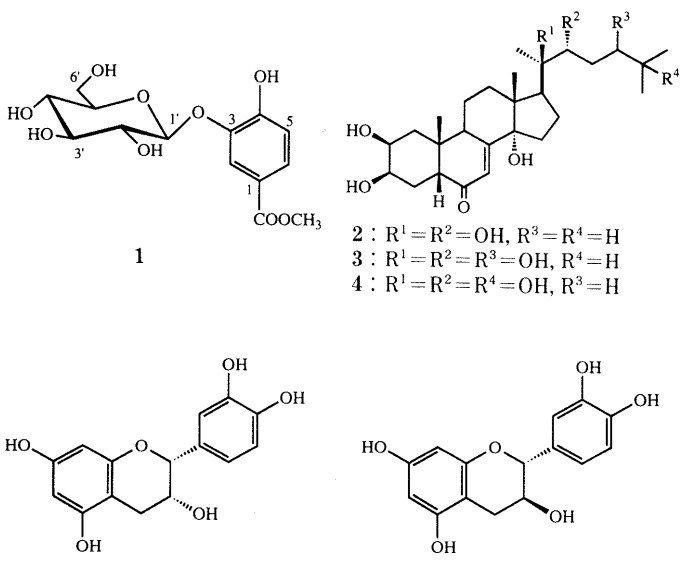

Chart 1

In the present paper, we report the isolation and characterization of the antiviral substance on HSV-1 from the active fraction of the aqueous extract of *W. orientalis*.

Materials and Methods

Melting points were measured with a Yanagimoto micro-melting point apparatus (Yanagimoto Co., Kyoto, Japan) and were uncorrected. IR spectra were taken on a Hitachi 260-10 IR spectrometer in KBr disc, and absorbance frequency is expressed in cm^{-1}. UV spectra were taken on a Shimadzu UV 2200 UV–visible spectrophotometer in MeOH and the λ_{max} was expressed in nanometers (nm). Optical rotation was measured on a JASCO DIP-4 automatic polarimeter at 26 °C. Mass spectra (MS) were measured in an electron impact (EI) mode at an ionization voltage of 70 eV with a JEOL DX-300 mass spectrometer. Fast atom bombardment (FAB) mass spectrum was carried out with glycerol as a matrix. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL GX-400 NMR spectrometer using tetramethylsilane (TMS) as an internal standard for ^{1}H-NMR, and chemical shifts were expressed in δ-value. ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{13}C COSY and ^{1}H-detected heteronuclear multiple-bond multiple-quantum coherence (HMBC) spectra were obtained with the usual pulse sequence, and data processing was performed with standard JEOL software. Column chromatography was done with Wako gel C-200 (Wako Pure Chemical Co., Osaka, Japan), and TLC and preparative TLC were carried out on pre-coated Merck Kieselgel F_{254} plates (0.25 or 0.5 mm).

Plant Material The rhizomes of *Woodwardia orientalis* Sw. were collected in Himi, Toyama prefecture of Japan in January, 1992. The sample was deposited in the Herbarium of Materia Medica of Toyama Medical and Pharmaceutical University, Toyama Japan.

Preparation of Aqueous and Methanol Extracts for Antiviral Assay The plant materials were chopped and dried in the shade. The dried rhizome (20 g) was refluxed with water (1.5 l) for 3 h. The solutions were filtered and the filtrate was concentrated under reduced pressure, then lyophilized. For methanol extracts: the dried rhizome (20 g) was extracted with methanol (1 l), and refluxed for 3 h. The extract was filtered and the filtrate was concentrated under reduced pressure to a small volume below 40 °C and then lyophilized. The lyophilized aqueous and methanol extracts were suspended in distilled water at various concentrations as indicated in the experiments. Each of the suspensions was boiled for 10 min and centrifuged at 3000 rpm for 15 min. The sterilized supernatant was used directly for antiviral assays.

Extraction and Isolation The dried rhizomes of *W. orientalis* (9 kg) were refluxed with water (32 l × 2) for 3 h. The combined water solution was concentrated under reduced pressure and lyophilized to give an extract (450 g). The aqueous extract (250 g) was suspended in distilled water (1 l) and successively extracted with EtOAc (3 l × 2) and *n*-BuOH (3 l × 2). The EtOAc-soluble fraction was concentrated under reduced pressure to afford an extract (5.2 g), and was chromatographed on silica gel with increasing

polarity ($CHCl_3$—30% MeOH : $CHCl_3$) to obtain seven fractions. Careful separation of these fractions with monitoring by *in vitro* bioassay led to the isolation of a new compound designated as woodorien (**1**) (6 mg), together with five known compounds, ponasterone A[3] (**2**) (37 mg), pterosterone[4] (**3**) (48 mg), ecdysterone[5] (**4**) (15 mg), (−)-epicatechin[6] (**5**) (45 mg), and (+)-catechin[7] (**6**) (25 mg).

Woodorien (1) A pale amorphous powder, $[\alpha]_D$ −2° (c=0.15, EtOH). Negative ion FAB-MS m/z: 329 $(M-H)^-$. IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 1710 (ester carbonyl), 1600 (phenyl). UV λ_{max}^{MeOH} nm (log ε): 250 (3.63). ^{1}H- and ^{13}C-NMR (pyridine-d_5): see Table I.

Ponasterone A (2) White needles, mp 256—257 °C (lit.[3] 259—260 °C), $[\alpha]_D$ +90° (c=1.0, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 0.83, 0.84 (each 3H, d, J=7.0 Hz, 26-, 27-H_3), 1.08 (3H, s, 19-H_3), 1.23 (3H, s, 18-H_3), 1.42 (1H, m, 24-H), 1.49 (1H, m, 25-H), 1.52 (1H, m, 23-H), 1.57 (3H, s, 21-H_3), 1.65—1.80 (2H, m, 23-, 24-H), 1.76 (1H, m, 11-H), 1.80 (1H, m, 4-H), 1.85—1.95 (3H, m, 1-, 11-, 15-H), 2.00—2.10 (3H, m, 4-, 12-, 16-H), 2.14 (1H, dd, J=13.0, 4.0 Hz, 1-H), 2.21 (1H, ddd, J=12.0, 6.5, 2.0 Hz, 15-H), 2.46 (1H, ddd, J=20.5, 10.0, 2.0 Hz, 16-H), 2.61 (1H, td, J=13.5, 4.5 Hz, 12-H), 2.93 (1H, t, J=9.0 Hz, 17-H), 3.00 (1H, dd, J=13.0, 4.0 Hz, 5-H), 3.60 (1H, br s, 9-H), 3.80 (1H, br d, J=11.0 Hz, 22-H), 4.17 (1H, td, J=12.0, 4.0 Hz, 2-H), 4.23 (1H, br s, 3-H), 6.26 (1H, d, J=2.0 Hz, 7-H). The assignment of ^{1}H-NMR signals was accomplished by means of ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY. Compound **2** was identified by comparing the MS, ^{1}H- and ^{13}C-NMR data of previous literature.[3]

Pterosterone (3) White needles, mp 227—228 °C (lit.[4] 229—230 °C), $[\alpha]_D$ +7.4° (c=1.0, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 1.02, 1.04 (each 3H, J=7.0 Hz, 26-, 27-H_3), 1.23 (3H, s, 18-H_3), 1.60 (3H, s, 21-H_3), 1.70—1.80 (3H, m, 4-, 11-, 25-H), 1.83 (1H, m, 23-H), 1.84—2.00 (3H, m, 1-, 11-, 15-H), 2.00—2.10 (3H, m, 4-, 12-, 23-H), 2.10—2.25 (3H, m, 1-, 15-, 16-H), 2.48 (1H, ddd, J=20.0, 11.0, 2.0 Hz, 16-H), 2.61 (1H, td, J=13.5, 5.5 Hz, 12-H), 2.93 (1H, t, J=9.0 Hz, 17-H), 3.00 (1H, dd, J=13.0, 4.0 Hz, 5-H), 3.60 (1H, m, 9-H), 3.95 (1H, dd, J=8.5, 4.0 Hz, 22-H), 4.13 (1H, m, 2-H), 4.18 (1H, m, 24-H), 4.23 (1H, br s, 3-H), 6.26 (1H, d, J=2.5 Hz, 7-H). Assignment of ^{1}H-NMR signals was accomplished by means of ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY. Compound **3** was identified by comparing the MS, ^{1}H- and ^{13}C-NMR data of previous literature.[4]

Ecdysterone (4) White needles, mp 242 °C (lit.[5] 235—239 °C), $[\alpha]_D$ +63° (c=1.0, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 1.08 (3H, s, 19-H_3), 1.23 (3H, s, 18-H_3), 1.38 (6H, br s, 26-, 27-H_3), 1.60 (3H, s, 21-H_3), 1.76 (1H, m, 11-H), 1.80 (2H, m, 4-, 15-H), 1.82 (1H, m, 24-H), 1.83 (1H, m, 25-H), 1.84 (1H, m, 23-H), 1.90 (1H, m, 11-H), 2.03 (1H, m, 4-H), 2.04 (1H, m, 12-H), 2.07 (2H, m, 15-, 16-H), 2.29 (1H, m, 24-H), 2.45 (1H, dd, J=20.0, 11.0 Hz, 16-H), 2.58 (1H, ddd, J=14.5, 13.0, 4.5 Hz, 12-H), 3.01 (1H, br d, J=9.0 Hz, 17-H), 3.03 (1H, br s, 5-H), 3.61 (1H, br s, 9-H), 3.88 (1H, br d, J=8.4 Hz, 22-H), 4.24 (2H, m, 2-, 3-H), 6.26 (1H, d, J=2.5 Hz, 7-H). Assignment of ^{1}H-NMR signals was accomplished by means of ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY. Compound **4** was identified by comparing the MS, ^{1}H- and ^{13}C-NMR data of previous literature.[5]

TABLE I. ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR Data for Woodorien (**1**) in Pyridine-d_5

Position	δ_H	δ_C
1	—	121.9 s
2	8.35 d (2.0)	120.6 d
3	—	146.3 s
4	—	154.4 s
5	7.25 d (8.5)	117.1 d
6	7.93 dd (8.5, 2.0)	126.7 d
1′	5.59 d (7.5)	104.5 d
2′	4.19 t (7.5)	74.9 d
3′	4.28 t (7.5)	78.2 d
4′	4.34 t (7.5)	70.8 d
5′	3.39 ddd (7.5, 4.5, 2.5)	79.0 d
6′	4.40 dd (11.5, 4.5)	62.0 t
	4.47 dd (11.5, 2.5)	
$COOCH_3$	3.65 s	—
$COOCH_3$	—	51.6 q
$COOCH_3$	—	166.6 s

δ values are in ppm and the figures in parentheses are coupling constants in Hz. Multiplicities of carbon signals were determined by means of the DEPT method and are indicated as s, d, t, and q, and the ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY and HMBC were measured.

(−)-Epicatechin (5) A pale brown amorphous powder, $[\alpha]_D$ −54.2° (c=1.5, EtOH). EI-MS m/z: 290 (M^+), 151, 139, 123, 91. Compound **5** was identified by comparison with the MS, ^{1}H- and ^{13}C-NMR spectral data of the authentic sample of (−)-epicatechin.

(+)-Catechin (6) A pale brown amorphous powder, $[\alpha]_D$ +56° (c=0.9, EtOH). EI-MS m/z: 290 (M^+), 151, 139, 123, 91. Compound **6** was identified by comparison with the MS, ^{1}H- and ^{13}C-NMR spectral data of the authentic sample of (+)-catechin.

Cells and Viruses Vero cells were grown at 37 °C in Eagle's minimal essential medium (MEM) supplemented with 5% and 2% calf serum, respectively. HSV-1 (Seibert strain[8] or 7401H strain[9]) provided from Mori, Kyushu University, Japan), poliovirus type 1 (Sabin strain), and measles virus (Tanabe strain) were propagated in Vero E6 cells. The infected cultures were frozen and thawed three times, and centrifuged at 3000 rpm for 15 min. Their supernatants were stored at −80 °C until use.[10]

Antiviral Assay Duplicate cultures of Vero cells in 60 mm plastic dishes were infected with 100 plaque-forming units (PFU)/0.2 ml of HSV-1 (Seibert strain), poliovirus, or measles virus for 1 h. Then the cells were overlaid with 5 ml of a nutrient methylcellulose (0.8%) medium containing 100 μg/ml and 300 μg/ml of aqueous and methanol extracts, and incubated for 2, 3 and 5 d at 37 °C for HSV-1, poliovirus, and measles virus, respectively. The infected cells were fixed and stained, and the number of plaques was counted. The cytotoxicity of the samples was evaluated by the extent of omission of uninfected cells from the surface of stained dishes in the plaque reduction assay. Strong (+) and weak (±) cytotoxicity were scored as the omission of more than 50% and less than 50%, respectively, of uninfected cells as compared with untreated dishes (controls) as shown in Table III.

Results and Discussion

The aqueous and methanol extracts of the rhizomes of *W. orientalis* were tested for their *in vitro* antiviral activity against HSV-1, poliovirus type 1, and measles virus by the plaque reduction assay at a concentration of 100 and 300 μg/ml. As shown in Table II, the aqueous extracts of *W. orientalis* showed a significant antiviral effect on HSV-1 and poliovirus type 1, while no effect was observed on the measles virus.

The aqueous extract of *W. orientalis* was therefore extracted successively with EtOAc and *n*-BuOH. Table III shows that the EtOAc-soluble fraction from the aqueous extract significantly and dose-dependently reduced the plaque forming ability of HSV-1, while the water-soluble fractions did not. The *n*-BuOH-soluble fraction showed very strong cytotoxicity at concentrations of 100 and 200 μg/ml so that plaque formation (%) could not be measured, but showed only weak activity at the concentration of 50 μg/ml. From these results, the EtOAc-soluble fraction which showed dose-dependent activity and low cytotoxicity was chosen and further chromatographed on silica gel to afford seven fractions (frs. 1—7), which were examined for antiviral activity on HSV-1 (Table III). The results showed that frs. 2—5 exhibited antiviral activity, while frs. 1, 6 and 7 did

TABLE II. Antiviral Activity of Aqueous and Methanol Extracts of *Woodwardia orientalis*

Sample	Concentration (μg/ml)	Plaque formation (%)		
		HSV-1	Polio	Measles
Aqueous ext.	300	0.0	0.0	0(+)[a]
	100	51.6	30.5	105.4
Methanol ext.	300	0.0	0.0	0(+)
	100	70.6	89.0	85.3

a) Due to strong cytotoxicity, plaque formation (%) could not be measured. Strong (+) cytotoxicity was scored as the omission of more than 50% of uninfected cells as compared with untreated dishes (controls).

TABLE III. Antiviral Activity of Different Fractions from the Aqueous Extract of *Woodwardia orientalis* against HSV-1

Fraction	Plaque formation (%)			
	Concentration (μg/ml)			
	200	100	50	25
EtOAc-soluble	0.0	28.9	76.8	80.7
BuOH-soluble	0(+)[a]	0(+)	66.4	58.6
H_2O-soluble	0(+)	79.6	83.2	71.4
Fr.-1	61.1	68.2	78.6	71.4
Fr.-2	29.6	54.3	76.8	82.5
Fr.-3	0(±)	76.1	79.6	88.2
Fr.-4	0(±)	60.7	74.6	80.7
Fr.-5	0.0	27.1	57.9	75.4
Fr.-6	0(+)	0(+)	76.4	83.2
Fr.-7	0(+)	80.0	83.5	86.2

a) Due to strong cytotoxicity, plaque formation (%) could not be measured. Strong (+) and weak (±) cytotoxicity was scored as the omission of more than 50% and less than 50%, respectively, of uninfected cells as compared with untreated dishes (controls).

TABLE IV. Antiviral Activity of Compounds **1**—**6** against HSV-1

Compound	Plaque formation (%)[a]
Woodorien (**1**)	16.1
Ponasterone A (**2**)	57.9
Pterosterone (**3**)	64.8
Ecdysterone (**4**)	70.4
(−)-Epicatechin (**5**)	50.4
(+)-Catechin (**6**)	32.7

a) Concentration 100 μg/ml.

not show activity. After re-chromatography and/or preparative TLC of frs. 2—5, compounds **1**, **2** and **3**, **4**, **5** and **6** were isolated from frs. 5, 2, 3 and 4, respectively.

Compounds **2**—**6** were known compounds, ponasterone A (**2**), pterosterone (**3**), ecdysterone (**4**), (−)-epicatechin (**5**) and (+)-catechin (**6**). The structures for these compounds were determined by comparing the MS, ^{1}H-NMR and ^{13}C-NMR data of previous literature. They were further confirmed by ^{1}H–^{1}H COSY, distortionless enhancement by polarization transfer (DEPT) and ^{1}H–^{13}C COSY spectra. Compounds **3**—**6** were first isolated from *W. orientalis*.

Compound **1** was obtained as a pale amorphous powder and showed $[\alpha]_D$ −2° (c=0.15, MeOH). The molecular formula for **1** was determined to be $C_{14}H_{18}O_9$ on the basis of the negative ion FAB-MS, which exhibited a $[M-H]^-$ peak at m/z 329. In the UV spectrum, **1** showed an absorption band at 250 nm (log ε 3.63) and in the IR spectrum strong absorptions appeared at 3340 (OH), 1710 (ester carbonyl), and 1600 cm^{-1} (aromatic). The ^{1}H-NMR spectrum of **1** showed signals due to an ester methyl group (δ_H 3.65, s) and 1,3,4-trisubstituted benzene protons [δ_H 7.25 (d, J=8.5 Hz), 7.93 (dd, J=8.5, 2.0 Hz), and 8.35 (d, J=2.0 Hz)] along with sugar protons (δ_H 3.39, 4.19, 4.28, 4.34, 4.40, 4.47, 5.59). The ^{13}C-NMR of **1** showed a carbonyl carbon (δ_C 166.6), three aromatic methine carbons (δ_C 117.1, 120.6, 126.7), six sugar carbons (δ_C 62.0, 70.8, 74.9, 78.2, 79.0, 104.5), and an ester methyl carbon (δ_C 51.6) along with three quaternary carbons (δ_C 121.9, 146.3, 154.4) (Table I).

Extensive analysis of the ^{1}H- and ^{13}C-NMR spectra of

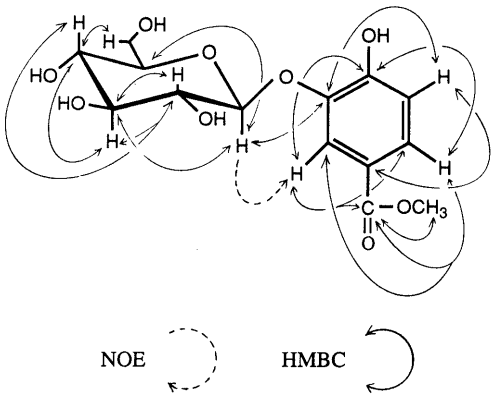

Chart 2

1, with the aid of ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY, allowed us to deduce that compound **1** is a β-D-*O*-glucoside of methyl protocatechuate. Then, we measured the HMBC[11] spectrum to establish the connectivities of three substituents in the benzene ring. Both the *meta*- and *meta*-, *ortho*-coupling protons [δ_H 8.35 (d, J=2.0 Hz), 7.93 (dd, J=8.5, 2.0 Hz)] showed long-range correlation with the carbonyl carbon (δ_C 166.6), suggesting that the ester group is located between two aromatic protons. Hence, two other substituents, glucose and hydroxyl groups, must be in the 3 or 4 positions with respect to the ester group, rather than other isomers. Next, we measured the nuclear Overhauser effect (NOE) spectrum. On irradiation of the anomeric proton signal at δ_H 5.59, the NOE effect was observed on the *meta*-coupling proton at δ_H 8.35 which clearly suggested that a glucose group lies adjacent to the *meta*-coupling proton. Also, the quaternary carbon at δ_C 146.3 (C-3) showed long-range correlations with the proton δ_H 7.25 (5-H) and 5.59 (1′-H), indicating a connection of the glucose moiety with the benzene ring at the C-3 position. On the other hand, the quaternary carbon at δ_C 154.4 (C-4) was correlated with the protons at δ_H 8.35 (2-H) and 7.93 (6-H). In turn, the carbon at δ_C 74.9 (C-2′) showed correlations with δ_H 4.28 (3′-H) and 4.34 (4′-H). Furthermore, the carbon at δ_C 79.0 (C-5) showed a correlation with the proton δ_H 5.59 (1′-H). Other significant long-range correlations observed are shown by arrows in Chart 2.

On the basis of the above findings, the structure of **1** was fully established and it was named woodorien.

Compounds **1**—**6** were tested for their inhibitory effect against HSV-1 infection on Vero cells. The results showed that all the compounds tested reduced the plaque-forming ability of HSV-1 at a concentration of 100 μg/ml, although to a different extent. Of these, woodorien (**1**) was the most potent inhibitor, and no cytotoxicity was found (Table IV). The structure–function relationship that underlies the antiviral activity of these compounds remains to be elucidated.

In our experiment, the *n*-BuOH-soluble fraction also showed weak activity at a lower concentration, although it was found to be strongly cytotoxic at higher concentrations. The chemical analysis of the *n*-BuOH soluble fraction is in progress.

Various crude drugs have been used to treat virus infections in traditional systems of medicine, but most of them have not been fully evaluated using modern scientific

techniques. An *in vitro* bioassay system using the plaque reduction assay is quite convenient, and it enables us to evaluate many crude drug extracts and a great number of compounds isolated from the active drugs. The isolation and characterization of antiviral substances from other crude drugs is now in progress.

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