Daphnane Diterpene Esters with Anti-proliferative Activities against Human Lung Cancer Cells from *Daphne genkwa*

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Two new daphnane-type diterpene esters, yuanhuahine (1) and yuanhualine (2), were isolated from the flowers of *Daphne genkwa* (Thymelaeaceae) along with three known diterpene esters, yuanhuacine (3), yuanhuadine (4), and yuanhuagine (5). Their structures were determined by a combination of 1D and 2D NMR experiments, including correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and rotating frame Overhauser enhancement spectroscopy (ROESY) sequences, and mass spectrometry. All the isolated compounds were tested against A549 human lung cancer cells and MRC-5 human normal lung epithelial cells. Compounds 1—5 exhibited potent anti-proliferative effects against A549 lung cancer cells with IC_{50} values of 12—53 nM, whereas these compounds were relatively non-cytotoxic against MRC-5 normal lung epithelial cells.

Key words *Daphne genkwa*; Thymelaeaceae; daphnane diterpene; anti-proliferative activity; A549 human lung cancer cell

Daphne genkwa Sieb. *et* Zucc. (Thymelaeaceae) is a traditional oriental medicine that is widely distributed in Korea and China.[1)] The flower of this plant (Genkwa flos) has been traditionally used for abortifacient, diuretic, antitussive, expectorant, and anticancer effects.[1—3)] Previous phytochemical investigations of Genkwa flos led to the isolation of various compounds such as flavonoids, coumarins, amides, and diterpenes.[4—9)] Daphnane-type diterpenoids are typical constituents of plants from the families Thymelaeaceae and Euphorbiaceace. There have been reported several biological activities of daphnanes including antileukemia, piscicidal, toxicity, anticancer, abortion, and neurotrophy.[8,10,11)] As part of our ongoing study for discovery of plant-derived anticancer agents, two new daphnane diterpenoids, namely, yuanhuahine (**1**) and yuanhualine (**2**), were isolated from Genkwa flos. The present study describes the structural elucidation of the two new isolates **1** and **2**, along with their antiproliferative activities against human lung cancer cells and normal lung epithelial cells.

Results and Discussion

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined as $C_{33}H_{44}O_{10}$ by the pseudo molecular ion peak at *m*/*z* 601.3013 $[M+H]^+$ in positive HR-FAB-MS. The IR spectrum of **1** showed absorptions at 3456 cm^{-1} for the hydroxyl groups and at 1695 cm^{-1} for the conjugated carbonyl group. The ^{13}C- and distortionless enhancement by polarization transfer (DEPT) 135 spectra of **1** indicated the presence of five methyls, seven methylenes, twelve methines, and nine quaternary carbons. In particular, an unusual quaternary carbon (C-1′) at δ 117.2 was attributable to an orthoester group, which is a structural feature of daphnane-type diterpenoids. The quaternary carbon signals at δ 209.7 and 173.4 in the ^{13}C-NMR spectrum were assignable to an α,β-unsaturated cyclopentanone and an ester group, respectively. The resonances of δ_H 3.56 and δ_C 64.3 (C-7) for an oxygenated methine group and at δ_C 60.7 (C-6) for an oxygenated quaternary carbon were upfield shifted, suggesting the presence of an epoxy group between C-6 and C-7. The ^1H-NMR spectrum displayed two olefinic proton signals at δ_H 5.01 and δ_H 4.95 assignable to the exomethylene group (C-16) and two proton signals at δ_H 3.94 and 3.81 attributed to an oxygenated methylene (CH_2-20). All of these signals in the ^1H- and ^{13}C-NMR of **1** were similar to those of yuanhuadine (**4**), except for the ethyl signals (C-2″ and C-3″) of **1** instead of the methyl signal for the acetyl group in **4**.[8,12—14)] The methyl signal at δ_H 1.09 (3H, t, 7.5, H-3″) exhibited three-bond connectivity with the conjugated carbon at δ_C 173.4 (C-1″) in the heteronuclear multiple bond correlation (HMBC) spectrum of **1**, indicating that the ethyl group was attached to the carbon at C-1″ (Fig. 2).

The relative stereochemistry was determined by analysis of the rotating frame Overhauser enhancement spectroscopy (ROESY) data of **1** as shown in Fig. 3. The ROE correlation between H-12 and CH_3-18 indicated the *trans* configuration between the functional groups at C-11 and C-12. Additional correlations between H-7 and H_2-20, and between H-8 and H-11 and H-14 confirmed their *cis* positions to each other. Continuous correlations of H-14 with H-16 and CH_3-17 pro-

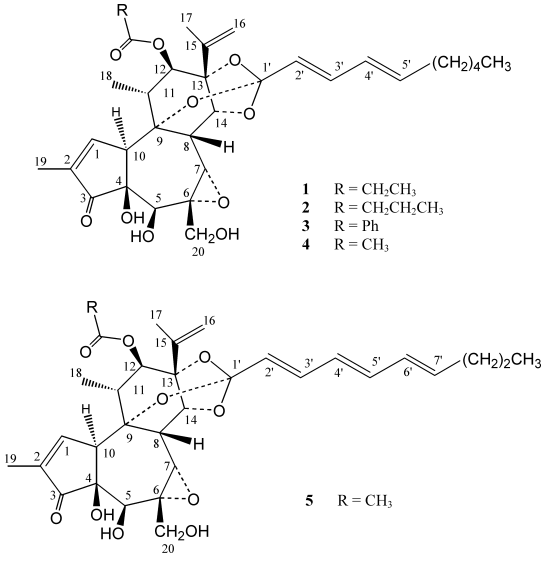

Fig. 1. Structures of **1**—**5**

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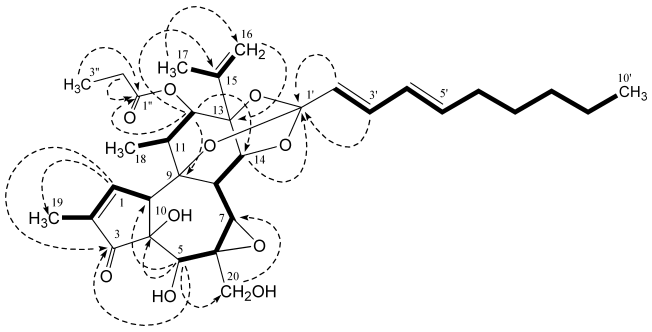

Fig. 2. ^{1}H–^{1}H COSY (———) and Selected ^{1}H–^{13}C HMBC (·····➤) Correlations of Compound **1**

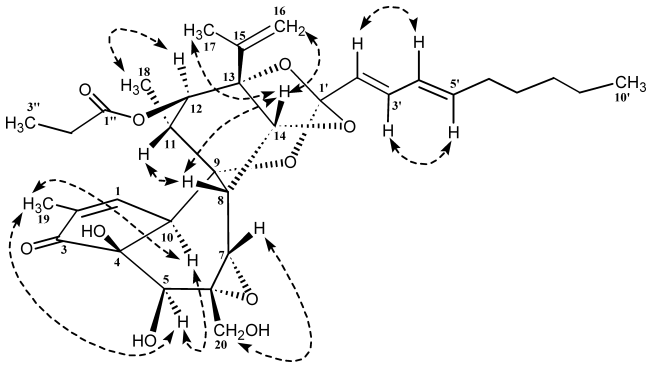

Fig. 3. Selected ^{1}H–^{1}H ROESY (◀·····➤) Correlations of Compound **1**

vided strong evidence for the β-orientations for these protons. On the other hand, the ROE correlation between H-5 and H-10 demonstrated their α orientation. Although its absolute configuration could not be resolved, the relative configuration was determined unambiguously. As a result, compound **1** was elucidated as the new daphnane-type diterpenoid, 6α,7α-epoxy-5β-hydroxy-9,13,14-*ortho*-[(2*E*,4*E*)-deca-2,4-dien-1-yl]-resiniferonol-12β-yl propionate, namely, yuanhuahine.

Compound **2** gave a pseudo molecular ion peak at *m*/*z* 615.3169 $[M+H]^+$ in positive HR-FAB-MS, corresponding to the molecular formula $C_{34}H_{46}O_{10}$. The UV and IR of **2** displayed identical absorptions with those of **1**. The ^{1}H- and ^{13}C-NMR spectra of **2** were also similar with those of compound **1** except for a propyl group instead of the ethyl group in **1**. In the ^{1}H-NMR spectrum of **2**, the propyl signals appeared at δ_H 2.06 (2H, t, 7.5, H-2″), 1.42 (2H, m), and 0.75 (3H, t, 7.5), which were correlated with the ^{13}C-NMR signals at δ_C 36.2, 18.0, and 13.3, respectively, in the HMQC experiment. The HMBC correlations of C-1″ with CH_2-2″ and CH_2-3″ afforded strong evidence for the presence of one 1-butanoxyl ($CH_3CH_2CH_2CO_2$) system, which was assigned to C-12 (δ 172.9) due to the HMBC correlation between H-12 and C-1″. The ROESY spectrum of **2** showed almost identical correlations with those of **1**, indicating their identical stereochemistry. The structure of **2** was thereby elucidated as the new compound, 6α,7α-epoxy-5β-hydroxy-9,13,14-*ortho*-[(2*E*,4*E*)-deca-2,4-dien-1-yl]-resiniferonol-12β-yl butanoate, namely, yuanhualine.

Isolates **1** and **2** were tested for their inhibitory activity against cell proliferation in A549 human lung cancer cells along with the known compounds **3**—**5**, yuanhuacine,[5,8] yuanhuadine,[8,15] and yuanhuagine,[8] which were previously isolated from this plant. As shown in Table 2, all compounds exhibited potent anti-proliferative activity against A549 cells with 50% growth inhibition (IC_{50}) values ranging at 11.7—53 nM, while the positive control, ellipticine, showed the IC_{50} value of 1.1 μM. Among these compounds, yuahuadine (**4**), which has an acetyl group at C-12, exhibited the most potent activity. The only differences between the two new compounds and **4** were the side chains attached to the carbonyl at C-1″, indicating that the methyl group at C-1″ in **4** is probably better than the ethyl or the propyl side chains in **1** and **2**, respectively, for exerting anti-proliferative activity. On the other hand, although compound **5** has a methyl group at C-1″, it did not show as potent activity as **4**, probably due to the different side chain at C-1′. Therefore, the side chain at C-1″ seems to affect the anti-proliferative activity. Furthermore, using nonneoplastic human lung epithelial MRC-5 cells, it was assessed whether compounds **1**—**5** have any differential sensitivity to normal *versus* cancer cells. As shown in Table 2, the IC_{50} values of compounds **1**—**5** were >1000 times more potent against MRC-5 human normal lung cells compared with A549 human lung cancer cells. These results suggest that compounds **1**—**5** are relatively non-cytotoxic against normal lung epithelial cells compared with lung cancer cells. To the best of our knowledge, the antiproliferative activity against A549 human lung cancer cells and structure elucidation for compounds **1** and **2** are reported in the present study for the first time.

Experimental

General Experimental Procedures Optical rotations were measured on a P-1010 automatic digital polarimeter (Jasco, Japan) at 25 °C. UV spectra were obtained by U-3000 spectrophotometer (Hitachi, Japan). IR spectra were run on a FTS-135 FT-IR spectrometer (Bio-Rad, CA, U.S.A.). NMR spectroscopic data were recorded at room temperature on Bruker DMX 500 spectrometer. Tetramethylsilane (TMS) was used as internal standard. Mass spectra were measured with a JMS 700 Mstation HRMS spectrometer (JEOL, Japan) and high resolution FAB mass spectra measured by dual target inlet probe. Silica gel (230—400 mesh; Merck) and octadecyl silica (ODS) YMC gel (20 μm; YMC Europe GMBH) were also used for column chromatography and silica gel 60 GF_{254} nm for TLC. HPLC (Younglin SP930D pump; column: GROMSIL120 ODS-5 ST, 10 μm, 40×250 mm) attached with UV absorbance detector 730D (Younglin) was used for final purification.

Plant Material The flowers of *D. genkwa* were purchased from Hanyakyutong Co. at Kyeongdong medicinal market in Seoul, Korea, in 2003. The sample was identified by Prof. Wan-Kyunn Whang at the College of Pharmacy of Chung-Ang University, Seoul, Korea. A voucher specimen (EWHA-2003-10) was deposited at the herbarium in the College of Pharmacy, Ewha Womans University.

Extraction and Isolation The dried powder of the flower of *D. genkwa* (10 kg) was extracted with $CHCl_3$/acetone/MeOH (1 : 1 : 1, 80 l) at room temperature to give a greenish-black crude extract (1st extract; 386 g). $CHCl_3$/acetone/MeOH extracts were dissolved in MeOH/$CHCl_3$ (1 : 4, 800 ml) then filtered. The filtrate was concentrated in vacuum to afford a residue (2nd extract; 216 g). MeOH/$CHCl_3$ extracts (216 g) were subjected to silica flash chromatography eluted with $CHCl_3$, CH_2Cl_2/acetone (1 : 1), and $CHCl_3$/MeOH (1 : 1) solvent system to give three fractions. During the execution of isolation, the anti-proliferation activity test against A549 human lung cancer cells was used to guide the fractionation. The most active fraction (10% MeOH/$CHCl_3$, 64 g) was further fractionated by ODS flash column chromatography [50%, 75%, 90%, 100% MeOH in H_2O]. The 90% fraction (39 g) was subjected again to silica open chromatography with acetone/CH_2Cl_2 (5%, 10%, 15%, 20%, and 30%). Final purification of 15% fractions (2.1 g) was performed by preparation ODS HPLC with elution of 93% MeOH in H_2O, providing **3** (202 mg; 0.00202%). In addition, final purification of 20% fraction (2.6 g) was performed by preparation ODS HPLC with 93% MeOH in H_2O, providing **1** (40 mg; 0.0004%), **2** (40 mg;

Table 1. NMR Spectroscopic Data of Compounds **1** and **2**

Position	**1**			**2**		
	δ_C	δ_H (J)	$^1H\rightarrow^{13}C$ HMBC	δ_C	δ_H (J)	$^1H\rightarrow^{13}C$ HMBC
1	160.7	7.57 (1H, br s)	2, 3, 4, 10, 19	159.4	7.36 (1H, br s)	2, 3, 4, 9, 10, 19
2	137.1	—	—	136.8	—	—
3	209.7	—	—	208.9	—	—
4	72.7	—	—	72.7	—	—
5	72.4	4.26 (1H, s)	3, 4, 6, 20	70.2	4.00 (1H, d, 2.1)	3, 4, 6, 7, 10, 20
6	60.7	—	—	61.5	—	—
7	64.3	3.56 (1H, s)	5, 6, 9, 14, 20	63.8	3.33 (1H, s)	5, 6, 9, 20
8	35.6	3.51 (1H, d, 2.7)	6, 9, 11, 12, 14, 20	35.2	3.32 (1H, 3.9)	6, 9, 11, 14, 20
9	78.3	—	—	78.3	—	—
10	47.7	3.92 (H, br s)	2, 9, 11	47.3	3.70 (1H, br s)	1,2, 4, 5, 9, 11
11	44.3	2.36 (1H, q, 7.2)	10, 12, 13, 18	43.8	2.24 (1H, m)	10, 12, 13, 18
12	78.3	4.99 (1H, s)	9, 11, 13, 14, 15, 1″	78.1	4.08 (1H, s)	9, 13, 14, 15, 1″
13	83.9	—	—	83.6	—	—
14	80.7	4.76 (1H, d, 2.7)	7, 8, 9, 12, 1′	80.3	4.61 (1H, d, 2.7)	7, 8, 9, 10, 1′
15	143.3	—	—	143.0	—	—
16	113.6	5.01 and 4.95 (2H, br s)	13, 17	113.2	4.85 and 4.79 (2H, br s)	13, 17
17	18.9	1.84 (3H, s)	13, 15, 16	18.4	1.67 (3H, s)	13, 15, 16
18	18.5	1.31 (3H, d, 7.2)	9, 11, 12	18.0	1.12 (3H, d, 7.2)	8, 9, 11, 12
19	10.1	1.80 (3H, dd, 2.7, 1.2)	1, 2, 3	9.6	1.61 (3H, br s)	1, 2
20	65.2	3.94 and 3.81 (2H, d, 11.7)	6, 7	64.7	3.56 and 3.76 (2H, br d, 12.3)	5, 6, 7
1′	117.2	—	—	116.9	—	—
2′	122.5	5.65 (1H, d, 16.5)	1′, 4′	122.2	5.46 (1H, d, 15.3)	1′, 3′, 4′, 5′
3′	135.3	6.67 (1H, dd, 16.5, 10.5)	1′, 5′	135.0	6.49 (1H, dd, 15.3 10.5)	1′, 2′, 4′, 5′
4′	128.8	6.05 (1H, 15.0, 10.5)	2′, 6′	128.4	5.88 (1H, m)	2′, 3′, 5, 6′, 7′
5′	139.6	5.87 (1H, m)	3′, 6′	139.3	5.70 (1H, m)	3′, 4′, 6′, 7′
6′	32.9	2.10 (2H, q, 7.2)	4′, 5′, 7′, 8′	32.5	1.94 (2H, q, 6.9)	4′, 5′, 7′, 8′
7′	28.9	1.39 (2H, m)	5′, 6′, 8′, 9′	28.5	1.21 (2H, m)	5′, 8′, 9′
8′	31.5	1.26 (2H, m)	7′, 9′	31.2	1.14 (2H, m)	7′, 9′, 10′
9′	22.7	1.31 (2H, overlapped)	6′	22.3	1.10 (2H, m)	10′
10′	14.2	0.89 (3H, t, 6.9)	7′, 8′, 9′	13.7	0.72 (3H, t, 6.6)	8′, 9′
1″	173.4	—	—	172.9	—	—
2″	28.0	2.25 (2H, q, 7.5)	1″, 3″	36.2	2.06 (2H, t, 7.5)	1″, 3″, 4″
3″	9.2	1.09 (3H, t, 7.5)	1″, 2″	18.0	1.42 (2H, m)	1″, 2″, 4″
4″				13.3	0.75 (3H, t, 7.5)	2″, 3″

All values given in ppm downfield from TMS were determined in $CDCl_3$ (**1**) or $CDCl_3$+MeOH-d_4 (**2**) at 500 MHz.

Table 2. Inhibitory Effects of the Isolates from the Genkwa Flos on the Proliferation of A549 Human Lung Cancer Cells and MRC-5 Human Lung Epithelial Cells

Compound	IC_{50},[a] (μM)	
	A549 cells	MRC-5 cells
1	0.053	>20
2	0.023	>20
3	0.019	>20
4	0.012	11.1
5	0.027	>20
Ellipticine[b]	1.1	2.0

a) A half maximal (50%) inhibitory concentration, *b*) positive control.

0.0004%), **4** (340 mg; 0.0034%), and **5** (48 mg; 0.00048%).

Yuanhuahine (**1**): White amorphous, $[\alpha]_D^{20}$ +20.3 (c=0.2, $CHCl_3$). UV ($CHCl_3$) λ_{max} nm (log ε): 241 (4.2). IR (KBr) ν_{max}: 3456, 1695 and 936 cm^{+1}. HR-FAB-MS *m/z*: 601.3013 $[M+H]^+$ (Calcd for $C_{33}H_{45}O_{10}$, 601.3007). ^1H- and ^{13}C-NMR data, see Table 1.

Yuanhualine (**2**): White amorphous, $[\alpha]_D^{20}$ +61.9 (c=0.62, $CHCl_3$). UV ($CHCl_3$) λ_{max} nm (log ε): 242 (4.2). IR (KBr) ν_{max}: 3459, 1696 and 935 cm^{+1}. HR-FAB-MS *m/z*: 615.3169 $[M+H]^+$ (Calcd for $C_{34}H_{47}O_{10}$, 615.3162). ^1H- and ^{13}C-NMR data, see Table 1.

Yuanhuadine (**4**): White amorphous, $[\alpha]_D^{20}$ +9.4 (c=0.09, $CHCl_3$). ESI-MS *m/z*: 587 ($C_{32}H_{42}O_{10}$). All physical and spectral data were comparable to the published values.[8,15]

***In Vitro* Cytotoxicity Assay** The potential of compounds isolated from *D. genkwa* to inhibit cell proliferation in A549 cells was evaluated according to the SRB assay. A549 cells (5×10^4 cells/ml) were treated with various concentrations of tested compounds for 3 d. After treatment, cells were fixed with 10% TCA solution, and cell viability was determined by the sulforhodamine B (SRB) protein staining method.[16] The result was expressed as a percentage relative to solvent-treated control incubations, and the IC_{50} values were calculated by nonlinear regression analysis (percent survival *versus* concentration).

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