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A New Tigliane-Type Diterpenoid from *Daphne genkwa*

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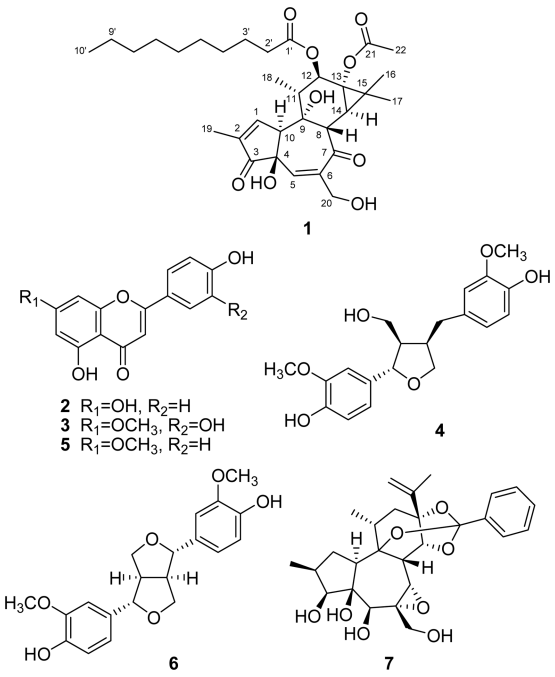
Daphne genkwa Sieb. et Zucc., a member of the family Thymelaeaceae, has been used as well known traditional medicine for diuretic, antitussive, expectorant, anticancer, and, anti-inflammatory purposes in Korea and China.[1,2] A number of compounds including daphnane-type diterpene esters, lignans, flavonoids, and coumarins were reported previously in this plant.[3] The daphnane-type diterpenoids from the genus *Daphne* have been showed various biological activities, such as cytotoxic, antifertility, topoisomerase I inhibitory, anti-melanogenesis, and anti-HIV activities.[4-9]

In the course of searching for anti-inflammatory compounds from medicinal plants, the MeOH extract of the flower buds of *D. genkwa* showed the inhibitory effect on the nitric oxide (NO) production in RAW 264.7 cells. NO plays a key role in neurotransmission, control of blood pressure, and cellular defense mechanisms. However, an enormous and uncontrollable amount of NO, is synthesized by inducible NO synthase, is responsible for inflammation.[10] Therefore, the methanol extract of the flower buds of *D. genkwa* was partitioned by successive extraction with *n*-hexane, CH_2Cl_2, and water. The CH_2Cl_2-soluble extract was subjected to column chromatography on a silica gel, RP-18, Sephadex LH-20, and preparative HPLC to afford a new tigliane-type diterpenoid compound (**1**) along with six known compounds (**2-7**). The structure of **1** was determined by spectroscopic data interpretation, particularly by extensive 1D and 2D NMR experiments. This paper deals with the isolation and structure determination of a new tigliane-type diterpenoid compound (**1**) as well as the inhibitory effects of all isolates on the NO production in LPS-stimulated RAW 264.7 cells.

Compound **1** was obtained as a white amorphous powder. The molecular formula was established as $C_{32}H_{46}O_9$ from the HRESIMS data at *m/z* 573.3054 $[M-H]^-$ ($C_{32}H_{45}O_9$, calcd. for 573.3064). The ^{1}H-NMR spectrum (Table 1) of **1** showed four methyl protons [δ_H 1.21 (3H, s, CH_3-16), 1.18 (3H, s, CH_3-17), 0.94 (3H, d, J = 7.5 Hz, CH_3-18), and 1.80 (3H, br s, CH_3-19)], four methine protons [δ_H 3.81 (1H, d, J = 5.5 Hz, H-8), 3.10 (1H, m, H-10), 2.23 (1H, m, H-11), 1.81 (1H, d, J = 5.5 Hz, H-14)], an oxygenated methine proton [δ_H 5.45 (1H, d, J = 11.5 Hz, H-12)], an oxygenated methylene proton [δ_H 4.25 (2H, m), CH_2-20)], and two olefinic protons [δ_H 7.60 (1H, br s, H-1) and 6.95 (1H, br s, H-5)], which suggested that **1** had a tigliane-type skeleton.[11,12] The remaining ^{1}H-NMR data indicated the presence of an acetyl group [δ_H 2.06 (3H, s, CH_3-22)] and a decanoyl group [0.90 (3H, br t, J = 7.0 Hz, CH_3-10'), 1.28-1.31 (12H, m, CH_2-4' to CH_2-9'), 1.58 (2H, m, CH_2-3'), and 2.30 (2H, br t, J = 7.4 Hz, CH_2-2')]. The ^{13}C-NMR spectrum (Table 1) of **1** showed 32 carbon resonances, including two ketone carbonyl signals [δ_C 205.7 (C-3) and 202.1 (C-7)] and two ester carbonyl signals [δ_C 175.3 (C-21) and 175.5 (C-1')]. The ^{1}H-NMR and ^{13}C-NMR spectra of compound **1** were very similar to those of trigowiin A, which had been recently isolated from *Trigonostemon howii* (Euphorbiaceae). The only noticeable structural difference between compound **1** and trigowiin A is the substituent at C-12; compound **1** has a decanoyl instead of dodecanoyl group as in trigowiin A.[11]



In the HMBC spectrum (Figure 1), the correlation between H-12 (δ_H 5.45) and C-1' (δ_C 175.5), C-11 (δ_C 45.7), C-13 (δ_C 67.2), and C-15 (δ_C 26.8) indicated that the decanoyl moiety was attached at C-12. Further HMBC correlations from H-12 (δ_H 5.45) and CH_3-22 (δ_H 2.06) to C-21 (δ_C 175.3) allowed the acetyl group to be located at C-13. The location of the carbonyl group at C-3 were confirmed by the HMBC correlations between H-1 (δ_H 7.60) and C-3 (δ_C 205.7) and C-4 (δ_C 74.3), and between H-19 (δ_H 1.80) and C-3 (δ_C 205.7). The remaining carbonyl group was placed at C-7 based on further HMBC correlations between H-5 (δ_H 6.95) and C-7 (δ_C 202.1), C-10 (δ_C 60.3), and C-20 (δ_C 62.4).

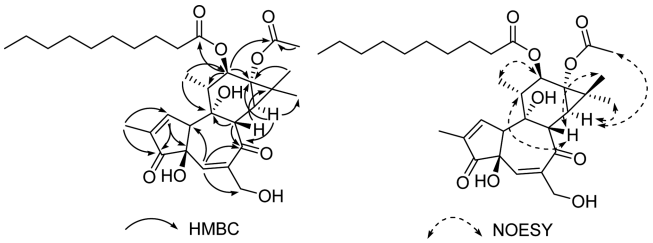


Figure 1. Key HMBC and NOESY correlations of **1**.

The relative configuration of compound **1** was confirmed by the NOESY data and comparison with literature.[11,12] The NOE correlation (Figure 1) between H-12 (δ_H 5.45) and CH_3-18 (δ_H 0.94), suggested that the decanoyl moiety is β-orientation. The proton signal of H-8 (δ_H 3.81) displayed cross peaks with H-11 (δ_H 2.23) and H-16 (δ_H 1.21), in NOESY spectrum, which indicated they were in the same β-orientation. Further NOE correlations with H-14 (δ_H 1.81) and H-17 (δ_H 1.18) and CH_3-22 (δ_H 2.06), were indicated they were in the α-orientation. Therefore, the structure of **1** was identified as 12-*O*-decanoyl-7-oxo-5-ene-phorbol-13-acetate, and given the trivial name daphwanin.

The six known compounds were identified as apigenin (**2**),[13] luteolin-7-methyl ether (**3**),[14] (+)-lariciresinol (**4**),[15] genkwanin (**5**),[16] (+)-pinoresinol (**6**),[15] 3-deoxo-1,2-dihydro-3-hydroxy-(2β,3β)-daphnetoxin (orthobenzoate 2) (**7**)[5] by comparing their physicochemical and spectroscopic data with those reported in the literature.

Table 1. ^{1}H- and ^{13}C-NMR data of compound **1** (CD_3OD)[a]

Carbon No.	**1**	
	δ_H	δ_C
1	7.60 (1H, br s)	158.9 d[b]
2	-	136.8 s
3	-	205.7 s
4	-	74.3 s
5	6.95 (1H, br s)	138.3 d
6	-	149.7 s
7	-	202.1 s
8	3.81 (1H, d, *J* = 5.5 Hz)	55.7 d
9	-	77.2 s
10	3.10 (1H, m)	60.3 d
11	2.23 (1H, m)	45.7 d
12	5.45 (1H, d, *J* = 11.5 Hz)	77.7 d
13	-	67.2 s
14	1.81 (1H, d, *J* = 5.5 Hz)	30.8 d
15	-	26.8 s
16	1.21 (3H, s)	17.2 q
17	1.18 (3H, s)	23.8 q
18	0.94 (3H, d, *J* = 7.5 Hz)	14.8 q
19	1.80 (3H, br s)	10.4 q
20	4.25 (2H, m)	62.4 t
21	-	175.3 s
22	2.06 (3H, s)	21.0 q
1'	-	175.5 s
2'	2.30 (2H, br t, *J* = 7.4 Hz)	35.3 t
3'	1.58 (2H, m)	26.3 t
4'-9'	1.28-1.31 (12H, m)	30.0-33.1 t
10'	0.90 (3H, br t, *J* = 7.0 Hz)	14.4 q

[a]Assignments aided by a combination of DEPT, HMQC, and HMBC experiments. [b]Carbon multiplicity.

All isolated compounds were examined for their inhibitory effects on LPS-induced NO productions in RAW 264.7 cells. The new tigliane-type diterpenoid, daphwanin (**1**) (IC_{50} = 7.2 μM), and daphnane-type diterpenoid, 3-deoxo-1,2-dihydro-3-hydroxy-(2β,3β)-daphnetoxin (**7**) (IC_{50} = 5.4 μM), showed strong inhibitory activities than positive control aminoguanidine (IC_{50} = 17.4 μM). Apigenin also showed moderate inhibitory activity, with IC_{50} value of 37.5 μM. However, the other compounds were inactive in this assay, with IC_{50} values > 50 μM. The cell viability assay results indicated that the inhibitory effects of NO production were not caused by cytotoxic effects of these compounds (data not shown). Recently, diterpenoids with phorbol- and daphnane-type skeleton from the stem bark of *Daphne aurantiaca* showed potent inhibitory activities against NO production.[17] Biflavonoids, coumarins, and lignans from the genus *Daphne* also showed moderate NO inhibitory and anti-inflammatory activity.[18-21]

Taken together, our results indicate that the diterpenoid compounds from the flower buds of *D. genkwa* might be valuable candidates for the treatment of various inflammatory disease caused by excessive production of NO.

Experimental

General Procedures. Optical rotations were determined with a JASCO DIP-1000 polarimeter. UV spectra were obtained on JASCO UV-550 spectrometer. NMR spectra were recorded on a Bruker DRX 500 MHz NMR spectrometer using CD_3OD as a solvent. High resolution electrospray ionization mass (HRESIMS) spectra were measured on a Waters QTOF micromass spectrometer. Semipreparative HPLC was performed using a Waters HPLC system equipped with two Waters 515 pumps and a 2996 photodiode array detector using a YMC J'sphere ODS-H80 column (4 μm, 150 × 20 mm, i.d.). Open column chromatography was performed using a silica gel (70-230 mesh, Merck) and Lichroprep RP-18 (40-63 μm, Merck). Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F_{254} (0.24 mm, Merck) plates.

Plant Materials. The flower buds of *D. genkwa* were purchased from Kyung-dong market, Seoul, Korea, in March 2012. The origin of the herbal material was identified by on of the authors (B. Y. Hwang) and a voucher specimen was deposited at the Herbarium of College of Pharmacy, Chungbuk National University, Korea.

Extraction and Isolation. The dried and powdered flower buds of *D. genkwa* (2 kg) were extracted with MeOH three times at room temperature. The combined MeOH extracts were concentrated *in vaccuo* at 40 °C to yield 120 g of residue. The residue was suspended in H_2O (1.5 L) and then successively partitioned with *n*-hexane (3 × 1.5 L), CH_2Cl_2

(3 × 1.5 L), EtOAc (3 × 1.5 L) to give *n*-hexane (24.6 g), CH_2Cl_2 (21.5 g), EtOAc (27.5 g), and water-soluble extract (46.4 g), respectively. The CH_2Cl_2-soluble extract (21.5 g) was then subjected to column chromatography on silica gel eluted with CH_2Cl_2-methanol gradient system (100:0 to 0:100) to yield five subfractions (C1-C5). Fraction C3 (6.1 g) was further subjected to column chromatography over silica gel eluted with *n*-hexane-EtOAc (100:0 to 0:100) to yield five subfractions (C31-C35). Fraction C33 (0.8 g) was chromatographed over silica gel chromatography by using CH_2Cl_2-EtOAc gradient system (100:0 to 0:100), and further purified by semipreparative HPLC (MeCN-H_2O gradient from 30:70 to 100:0, flow rate 6.0 mL/min) to afford compound **1** (3.5 mg). Fraction C32 (0.7 g) was chromatographed on a RP-18 column chromatography eluted with MeOH-H_2O gradient (from 20:80 to 100:0) to obtain four fractions (C321-C324). Fraction C323 (0.3 g) was further purified by HPLC (MeCN-H_2O gradient from 40:60 to 100:0, flow rate 6.0 mL/min) to yield compounds **2** (4 mg), **3** (34 mg), **4** (5 mg). Fraction C31 (0.8 g) was chromatographed over silica gel column chromatography with CH_2Cl_2-MeOH gradient system (100:0 to 0:100) to afford compound **5** (500 mg). Compound **6** (17 mg) was obtained from fraction C34 by silica gel column chromatography with CH_2Cl_2-MeOH gradient system (100:0 to 0:100). Fraction C35 (2.3 g) was further chromatographed over silica gel column chromatography with CH_2Cl_2-EtOAc gradient system (100:0 to 0:100) to give compound **7** (38 mg).

Daphwanin (1): White amorphous powder; $[\alpha]_D^{25}$ +102 (*c* 0.01, MeOH), UV_{max} (MeOH) λ_{max} (log ε) 213 (3.38) nm; IR (KBr) ν_{max} 3350, 1725, 1680, 1620, 1465 cm^{-1}; ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD), see Table 1; HRESIMS *m/z* 573.3054 $[M-H]^-$ (calcd for $C_{32}H_{45}O_9$: 573.3064).

Measurement of NO Production and Cell Viability Assay. Nitric oxide production was determined by measuring the amount of nitrite from cell culture supernatant as previously described.[22] Briefly, RAW 264.7 cells (1×10^5 cells/well) were cultured in flat bottom 96 well microtitre plate in quadruplicate for 12 h. Thereafter, 100 μL of media were replaced with fresh medium containing either compound, 1 μg/mL of LPS (Sigma Chemical Co., St.Louis, MO, USA) and further cultured for 24 h. The culture supernatant was collected at the end of culture for nitrite assay, which was used as a measurement of NO production. The culture supernatant (100 μL) was mixed with equal volume of Griess reagent at room temperature for 10 min. The absorbance was measured at 540 nm by a microplate reader. The remaining cells after Griess assay were used for cell viability with the MTT-based colorimetric assay.

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Supporting Information. ^{1}H-, ^{13}C-NMR, DEPT, HMQC, HMBC, NOESY, and HRESIMS spectra of **1** are available as Supporting Information.

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