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A NOVEL DAPHNANE-TYPE DITERPENE FROM THE FLOWER BUD OF *Daphne genkwa*

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*A novel daphnane-type diterpene, genkwanin I (**1**), and a novel natural product, orthobenzoate 2 (**2**), were isolated from the flower bud of* Daphne genkwa*. The structures of the two compounds were elucidated by spectral techniques, viz.1D, 2D NMR spectra and HR-ESI-MS. Genkwanin I showed inhibitory activity against human promyelocytic HL-60 cells at an IC_{50} level of 11.74 μM.*

Keywords: *Daphne genkwa*, daphnane-type diterpene, structure elucidation, HL-60 cells.

Daphne genkwa Sieb. et Zucc. (Thymelaeaceae), which is widely distributed in most provinces along the Yangtze River and parts of provinces along the Yellow River, has a long history in medical practice and healthcare [1]. It was mainly used for diuretic, abortion, antitussive, expectorant, and antitumor purposes [2]. As the principal active components, daphnane-type diterpenes showed potent biological activities such as anti-leukemia [3, 4], antifertility [5], and pesticidal [6]. Our investigation on the daphnane-type diterpenes led to the isolation of a new compound, genkwanin I (**1**), and a novel natural product, orthobenzoate 2 (**2**). Here we described the isolation and structure elucidation of the compounds, and genkwanin I was tested for inhibitory activity against human promyelocytic HL-60 cells.

Compound **1** was obtained as a colorless needle crystal, $[\alpha]_D^{22}$ –14.0° (*c* 0.004, MeOH). The molecular formula was determined to be $C_{28}H_{32}O_9$ (*m/z* 535.1938 $[M + Na]^+$, calcd 535.1939) by HR-ESI-MS, indicating the presence of 13 degrees unsaturation. The proton signals of the ^1H NMR spectra and the carbon signals of the ^{13}C NMR spectra revealed the basic skeleton of daphnane-type diterpenes. In the ^1H NMR (600 MHz) spectra, 1-substituted phenyl group appeared as multiplets at δ 7.67 (m, H-2′, 6′) and 7.36 (m, H-3′, 4′, 5′), and three methyl groups appeared as one singlet at δ 1.84 (s, CH_3-17) and two doublets at δ 1.30 (d, J = 6.6, CH_3-18) and 1.13 (d, J = 6.0, CH_3-19); the only oxygenated methylene showed two doublets with the ^2J coupling constant resolved at δ 3.70 (1H, d, J = 12, H-20) and 3.93 (1H, d, J = 12, H-20). A typical feature of AB system olefin protons attached to a terminal double bond was observed at δ 5.09 (s, H-16) and 4.93 (s, H-16). The ^{13}C NMR (150 MHz) spectra of compound **1** further defined some of the above functionalities. Three methyl carbons (δ 12.8, C-19; δ 19.3, C-17; δ 21.4, C-18) and one terminal double bond (δ 147.9, C-15; δ 111.6, C-16) were distinguished. Two oxygenated carbons signals δ 62.7 (C-6) and 64.6 (C-7) were shifted upfield, suggesting the presence of 6,7-epoxy instead of 6,7-dihydroxyls. A quaternary carbon signal at δ 118.6 revealed the orthoester structure of daphnane-type diterpenes. Besides the characteristic signals above, the presence of an ester carbonyl was confirmed by a quaternary carbon signal at δ 156.6 (C-21). In the HMBC spectra, the carbonyl at δ_C 156.6 (C-21) correlated with the H-3 at δ_H 4.72, which was shifted downfield compared with that of compound **2** [7], suggesting that the carbonyl group was allocated to C-3. In the ^{13}C NMR, compared with compound **2**, the chemical shifts of C-3 and C-4 were significantly shifted downfield to δ 90.3 and 93.9, respectively, which was attributed to the structure of 3*β*,4*β*-dioxolan-21-one; this structure was further confirmed by the molecular formula $C_{28}H_{32}O_9$ obtained by HR-ESI-MS.

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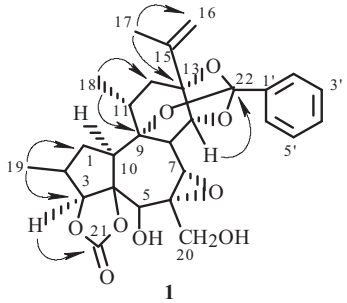
TABLE 1. ^{1}H (600 MHz) and ^{13}C NMR Data (150 MHz) of Compound **1** (CD_3OD, δ, ppm, J/Hz)

C atom	δ_H	δ_C	HMBC	C atom	δ_H	δ_C	HMBC
1β	2.02 (1H, m)	36.1	H-2, 3	15		147.9	H-14, 16β, 17
1α	1.57 (1H, m)			16	5.09 (1H, s)	111.6	H-14
2	1.94 (1H, m)	37.7	H-1α, 1β, 10, 19		4.93 (1H, s)		
3	4.72 (1H, d, J = 5.2)	90.3	H-1β, 5, 19	17	1.84 (3H, s)	19.3	H-16α
4		93.9	H-1β, 10	18	1.30 (3H, d, J = 6.6)	21.4	H-12β, 12α
5	4.10 (1H, s)	71.0	H-3, 10	19	1.13 (3H, d, J = 6.0)	12.8	H-1α, 2
6		62.7	H-5, 7, 8, 20	20	3.70 (1H, d, J = 12)	64.9	H-7, 8
7	3.44 (1H, s)	64.6	H-5, 14, 20		3.93 (1H, d, J = 12)		
8	2.78 (1H, d, J = 2.7)	37.0	H-7	21		156.6	H-3
9		81.3	H-1α, 7, 8, 10, 12α, 14, 18	22		118.6	H-14, 2′, 6′
10	2.92 (1H, dd, J = 13.6, 6.6)	50.6	H-1α, 1β, 3	1′		137.6	H-3′, 5′
11	2.11 (1H, m)	37.2	H-12α, 18	2′	7.67 (1H, m)	127.2	H-6′, 3′,4′
12β	2.35 (1H, dd, J = 14.1, 7.8)	37.1	H-11	3′	7.36 (1H, m)	128.8	H-4′, 5′
12α	1.79 (1H, d, J = 14.1)			4′	7.36 (1H, m)	130.3	H-3′, 5′
13		85.6	H-11, 16β, 17	5′	7.36 (1H, m)	128.8	H-3′, 4′
14	4.64 (1H, d, J = 2.7)	84.0	H-7, 8, 12β	6′	7.67 (1H, m)	127.2	H-2′, 4′, 5′



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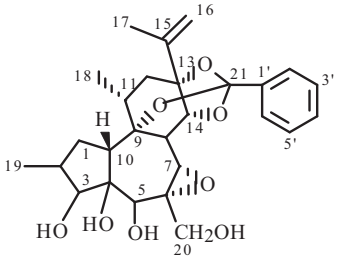

2

Based on the analysis above, compound **1** was elucidated as 1,2α-dehydro-3β,4β-dioxolan-daphnetoxin-21-one, a novel structure named genkwanin I. The ^{1}H NMR and ^{13}C NMR assignments interpreted by HMBC spectra are shown in Table 1.

Compound **2** was obtained as a white amorphous powder. An ion peak at *m/z* 487.2326 $[M + H]^+$ was revealed by HR-ESI-MS, and the molecular formula was determined to be $C_{27}H_{34}O_8$ (*m/z* 487.2326 $[M + H]^+$, calcd 487.2326). The ^{1}H NMR (300 MHz) spectrum was in correspondence with the reported data [7]. Herein, the data of ^{13}C NMR (75 MHz) spectra are reported for the first time. Furthermore, compound **2** was obtained by transesterification of the mixture of M_1 and M_2 from the roots of *Wikstroemia mekongenia* W. W. Sm. in the literature [7]. In our investigation, compound **2** was identified as orthobenzoate 2, which was isolated from the natural product for the first time.

The inhibitory activity against human promyelocytic HL-60 cells of genkwanin I was evaluated by MTT assay. In order to verify the activity of genkwanin I, the known HL-60 cell inhibitor yuanhuacin was chosen for comparison. Genkwanin I exhibited cell growth inhibition against HL-60 cells at the IC_{50} level of 11.74 μM, which value can be compared to that displayed by yuanhuacin (IC_{50} = 12.90 μM). These result indicate that genkwanin I is a potent cell growth inhibitor constituent of *Daphne genkwa*, and might be potent as an antitumor agent.

EXPERIMENTAL

Plant Material. The flower bud of *Daphne genkwa* was collected from the Sichuan Mianyang area, People′s Republic of China in August 2006. It was authenticated by Prof. Sun-Qi Shi, Department of Pharmacognosy, Shenyang Pharmaceutical University. The voucher specimens are kept in the Natural Products Laboratory of Shenyang Pharmaceutical University, Shenyang, China.

General Procedures. HR-ESI-MS were carried out on a MicroTOF spectrometer (Bruker Daltonics). 1D and 2D NMR spectra were run on Bruker AM-300 MHz and ARX-600 MHz spectrometers using TMS as an internal standard. Silica gel (200–300 mesh, Marine Chemical Factory, Qingdao, China), MCI gel (CHP20P, 75–150 μ, Mitsubishi Chemical Corporation, Japan), and C_{18} silica gel (60–80 μm, Merck, Germany) were used for column chromatography. Spots were visualized on TLC by heating the chromatoplates at 100°C in an oven after spraying with 10% H_2SO_4.

Extraction and Isolation. Air-dried flower bud of *Daphne genkwa* (6.0 kg) was extracted with 95% ethanol (25 L × 6) extensively at room temperature for 20 days to give a dark crude extract (450 g), and the extract was dissolved in water (10 L) to form a suspension and partitioned with $CHCl_3$ to afford a $CHCl_3$-soluble fraction A (250 g). Fraction A was subjected to column chromatography on silica gel (200–300 mesh) eluted with gradient petroleum–acetone (100:1–2:1) to yield 6 fractions (Fr. 1–6). Fraction 4 (16 g) was purified by column chromatography on MCI gel eluted with MeOH–H_2O (3:7–10:0) to afford compound **1** (8 mg). Fraction 6 was further chromatographed over a C_{18} silica gel column eluted with MeOH–H_2O (6:4–8:2) to give compound **2** (30 mg).

Genkwanin I (1). $C_{28}H_{32}O_9$, $[\alpha]_D^{22}$ −14.0° (*c* 0.004, MeOH), HR-ESI-MS (*m/z*: 535.1938 $[M + Na]^+$, calcd 535.1939). The NMR data are listed in Table 1.

Orthobenzoate 2 (2). $C_{27}H_{34}O_8$; HR-ESI-MS (*m/z* 487.2326 $[M + H]^+$, calcd 487.2326); ^1H NMR (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.0 (1H, m, H-1*β*), 1.88 (1H, m, H-1*α*), 1.80 (1H, m, H-2), 3.77 (1H, d, J = 3, H-3), 3.76 (1H, s, H-5), 3.40 (1H, s, H-7), 2.94 (1H, d, J = 2.7, H-8), 2.65 (1H, dd, J = 12.6, 5.7, H-10), 2.34 (1H, m, H-11), 2.20 (1H, d, J = 13.8, H-12*β*), 1.78 (1H, dd, J = 13.8, 6, H-12*α*), 4.49 (1H, d, J = 2.7, H-14), 5.02 (1H, s, H-16), 4.92 (1H, s, H-16), 1.83 (3H, s, CH_3-17), 1.28 (3H, d, J = 6.6, CH_3-18), 1.01 (3H, d, J = 6.0, CH_3-19), 3.93 (1H, d, J = 12.3, H-20), 3.70 (1H, d, J = 12.3, H-20), 7.73 (2H, m, H-2′, 6′), 7.35 (3H, m, H-3′, 4′, 5′); ^{13}C NMR (75 MHz, $CDCl_3$, δ): 34.6 (C-1), 36.8 (C-2), 77.9 (C-3), 79.9 (C-4), 72.7 (C-5), 61.5 (C-6), 64.1 (C-7), 36.5 (C-8), 80.4 (C-9), 48.8 (C-10), 35.4 (C-11), 36.0 (C-12), 84.4 (C-13), 82.7 (C-14), 146.5 (C-15), 111.3 (C-16), 20.9 (C-17), 19.3 (C-18), 13.1 (C-19), 65.8 (C-20), 117.4 (C-21), 136.2 (C-1′), 126.2 (C-2′, 6′), 129.3 (C-4′), 128.1 (C-3′, 5′).

Cell Growth Inhibition Assay. Cell growth inhibition was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All compounds were dissolved in DMSO, and the final concentration of DMSO in the culture medium was controlled at less than 0.1% (v/v). Cultivate HL-60 (human promyelocytic leukemia) cells were seeded in each well of the 96-well plates with 1 × 105 cells/well. After incubation with the compounds for 48 h, MTT solution (2.5 mg/mL in PBS) was added (10 μL/well), and the plates were incubated for an additional 4 h at 37°C. The produced formazan crystals were dissolved in 100 μL of DMSO, and the optical density of the solution was measured at 492 nm using a microplate reader (TECAN, Austria). Growth inhibition was estimated from the optical density of the solution.

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