Guaiane Sesquiterpenoids Isolated from the Fruits of *Torilis japonica* and Their Cytotoxic Activity

by **Dong Chun Kim**[a]), **Jeong Ah Kim**[b]), **Byung Sun Min**[c]), **Tae-Su Jang**[d]), **MinKyun Na***[a]), and **Seung Ho Lee***[a])

[a]) College of Pharmacy, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Korea (phone: +82-53-8102820; fax: +82-53-8104654; e-mail: mkna@ynu.ac.kr (*M. N.*); phone: +82-53-8102818; e-mail: seungho@ynu.ac.kr (*S. H. L.*))

[b]) College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea

[c]) College of Pharmacy, Catholic University of Daegu, Gyeongsan, Gyeongbuk 712-702, Korea

[d]) College of Medicine, CHA University, Seoul 135-080, Korea

Two new guaiane sesquiterpenoids, 11-(acetyloxy)-1,8-dihydroxyguai-4-en-3-one (**5**) and (1α,6β)-1,6-dihydroxytorilin (**6**), were isolated from the fruits of *Torilis japonica* (Umbelliferae), along with four known sesquiterpenes, torilin (**1**), torilolone (**2**), (1β)-1-hydroxytorilin (**3**), and (1α)-1-hydroxytorilin (**4**). During the phytochemical investigation, daucosterol, friedelin, and epifriedelanol were also isolated from the plant for the first time. The structures of the new sesquiterpenoids **5** and **6** were determined by comprehensive analyses of MS and NMR spectroscopic data. These isolates were evaluated against human breast cancer cells (MCF-7) and *Lewis* lung carcinoma (LLC) cells. Compounds **1**, **3**, and **4** exhibited cytotoxic activity against the LLC cells with IC_{50} values of 31.3, 32.5, and 34.0 μg/ml, respectively. However, no significant cytotoxicity was found against the MCF-7 cells for any of the compounds tested.

Introduction. – The fruits of *Torilis japonica* DC. (Umbelliferae) have been used as an anti-inflammatory traditional medicine to treat skin diseases and urogenital disorders. Torilin (=(3a*R*,4*S*,6*R*,7*S*)-7-[1-(acetyloxy)-1-methylethyl]-2,3,3a,4,5,6,7,8-octahydro-1,4-dimethyl-2-oxoazulen-6-yl (2*Z*)-2-methylbut-2-enoate; **1**), a guaiane sesquiterpenoid, is known as a major constituent of *T. japonica*, and its various bioactivities that include anti-inflammatory and anticancer activities have been demonstrated [1]. In addition, humulene-type, germacrane-type, oppositane-type, and eudesmane-type sesquiterpenoids were identified as constituents of this species [2]. Although there have been a number of studies on *T. japonica*, new metabolites are still discovered from its fruits [3]. In our phytochemical investigation on the species, we obtained two new guaiane sesquiterpenoids, (1β,8β)-11-(acetyloxy)-1,8-dihydroxyguai-4-en-3-one[1]) (**5**) and (1α,6β)-1,6-dihydroxytorilin[1]) (**6**) (*Fig. 1*), together with four known sesquiterpenes, torilin (**1**) [4], torilolone (**2**) [1], (1β)-1-hydroxytorilin (**3**) [5], and (1α)-1-hydroxytorilin (**4**) [6]. In this phytochemical study, daucosterol [7], friedelin [8], and epifriedelanol [8] were also identified as constituents of the plant for the first time. We now describe the isolation and structure determination of the new

[1]) Trivial atom numbering; for systematic names, see *Exper. Part.*

compounds **5** and **6**, and the evaluation of cytotoxic activities of the isolates against the MCF-7 human breast cancer cells and the *Lewis* lung carcinoma cells.

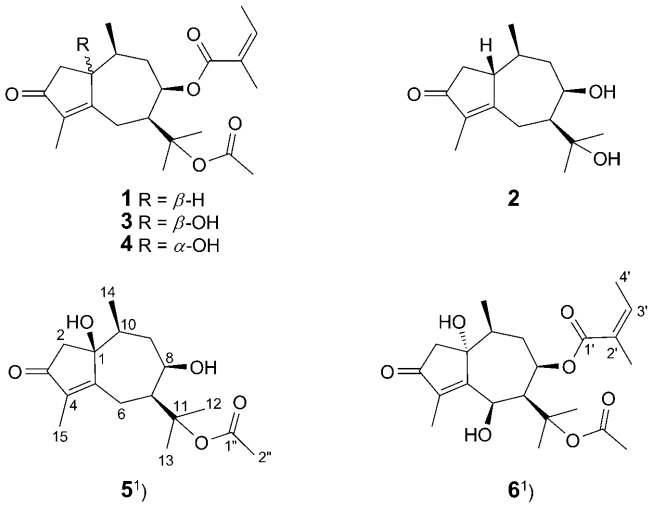
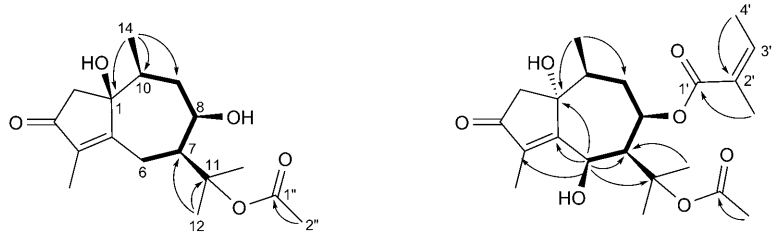


Fig. 1. *Compounds* **1**–**6**, *isolated from* T. japonica

Results and Discussion. – The MeOH extract of the fruits of *T. japonica* was partitioned between H_2O and AcOEt. The AcOEt-soluble fraction was separated by column chromatography (silica gel, gradient hexane/acetone 95:5 → 0:100), yielding 24 fractions. Purification of the 21st fraction by prep. HPLC afforded the two new sesquiterpenoids **5** and **6**.

Compound **5** was obtained as optically active colorless needles. A molecular formula $C_{17}H_{26}O_5$ was deduced from its HR-FAB-MS, ^{13}C-NMR, and DEPT data. The ^{1}H-NMR spectrum of **5** (*Table*) displayed characteristic signals for five Me groups at δ(H) 1.97, 1.63, 1.17, 1.14 (*s*, each), and 0.98 (*d*, $J = 6.8$ Hz), one O–CH group at δ(H) 5.09–5.17 (*m*), and a geminal spin system at δ(H) 2.45 and 2.30 (*d*, $J = 18.3$ Hz, each), as well as signals at δ(H) 2.77, 2.42–2.51 (each 1 H), and 1.55–1.77 (*m*, 4 H), which indicated that it would be a guaiane-type sesquiterpenoid similar to (1β)-1-hydroxytorilin (**3**). Comparison of the ^{1}H- and ^{13}C-NMR spectroscopic data of **5** with those of **3** clearly revealed the lack of an angeloyl moiety in **5** (angelic acid = (2*Z*)-2-methylbut-2-enoic acid). By means of ^{1}H,^{1}H-COSY, HMQC, and HMBC analyses (*Fig. 2*), all the NMR data for a partial structure C(14)–C(10)–C(9)–C(8)–C(7)–C(6) were assigned. The correlation of the Me signal at δ(H) 0.98 (*d*, $J = 6.8$ Hz, Me(14)) to C(1) (δ(C) 74.1) and C(9) (δ(C) 34.9) supported the attachment of an OH group at C(1). The relative configuration of **5** was determined by comparison of the ^{1}H-NMR data (chemical shifts and coupling constants) and analysis of NOESY data. The chemical-shift values of H–C(10) (δ(H) 1.58–1.61) and Me(14) (δ(H) 0.98) suggested the axial/equatorial disposition of OH–C(1) and Me(14) because the chemical shifts for a diaxial disposition of H–C(10) and Me(14) are δ(H) 2.33 and 0.80, respectively [9]. The NOESY data of **5** were similar to those of (1β)-1-hydroxytorilin (**3**) which

further supported the same relative configuration of **5** as that of **3**. The absolute configuration of **5** was deduced from the optical rotation value similar to those for torilin (**1**) and **3**. Consequently, the structure of **5** was determined to be (1β,8β)-11-(acetyloxy)-1,8-dihydroxyguai-4-en-3-one.



5[1]) **6**[1])

Fig. 2. *Key HMBCs* (H → C) *and COSYs* (—) *of compounds* **5** *and* **6**

Table. *^{1}H- and ^{13}C-NMR Data* ($CDCl_3$, 250 and 63 MHz, resp.) *of Compounds* **5** *and* **6**. δ in ppm, J in Hz.

	5		**6**	
	δ(C)	δ(H)	δ(C)	δ(H)
C(1)	74.1		82.8	
CH_2(2)	50.4	2.45 (*d*, *J* = 18.3), 2.30 (*d*, *J* = 18.3)	49.8	2.29 (*d*, *J* = 17.5), 2.53 (*d*, *J* = 17.5)
C(3)	209.6		208.7	
C(4)	135.4		141.2	
C(5)	177.1		172.1	
CH_2(6) or CH(6)	23.3	2.77 (*d*, *J* = 13.0), 2.42–2.51 (*m*)	70.1	6.07 (*d*, *J* = 7.5)
CH(7)	51.8	1.55–1.57 (*m*)	54.6	2.69 (*d*, *J* = 7.5)
CH(8)	73.2	5.09–5.17 (*m*)	71.7	5.64–5.67 (*m*)
CH_2(9)	34.9	2.03–2.12 (*m*), 1.71–1.79 (*m*)	38.6	2.22–2.25 (*m*), 1.55–1.69 (*m*)
CH(10)	38.3	1.58–1.61 (*m*)	39.0	2.42–2.48 (*m*)
C(11)	79.7		73.2	
Me(12)	28.6	1.17 (*s*)	28.8	1.20 (*s*)
Me(13)	26.7	1.14 (*s*)	27.7	1.20 (*s*)
Me(14)	18.6	0.98 (*d*, *J* = 6.8)	16.5	0.89 (*d*, *J* = 6.9)
Me(15)	7.8	1.63 (*s*)	8.9	1.88 (*s*)
C(1′)			166.6	
C(2′)			129.4	
CH(3′)			137.1	5.90 (*dq*, *J* = 1.3, 7.2)
Me(4′)			15.6	1.81 (*dd*, *J* = 1.3, 7.2)
Me–C(2′)			20.5	1.69 (*t*-like, *J* = 1.4)
C(1″)	172.0		169.1	
Me(2″)	21.5	1.97 (*s*)	21.3	2.02 (*s*)

Compound **6** was obtained as an optically active white powder. A molecular formula $C_{22}H_{32}O_7$ was determined for this compound on the basis of its HR-FAB-MS and ^{13}C-NMR data. The overall feature of the ^{1}H- and ^{13}C-NMR data (*Table*) of **6** was similar to those of (1α)-1-hydroxytorilin (**4**). The most striking difference in the ^{1}H-NMR spectrum was a signal at δ(H) 6.07 (*d*, *J* = 7.5 Hz, 1 H) implying the presence

of an O–CH group in **6**. In the ^{13}C-NMR spectrum of **6**, one more oxygenated C-atom signal was observed at δ(C) 70.1, which suggested that this compound should be a derivative of **4** bearing an additional OH group. A difference of 16 mass units compared to **4** further supported the inference. The position of this OH group was determined to be at C(6) by means of ^{1}H,^{1}H-COSY and HMBC analyses. The sequential H-atom spin system Me(14)–H–C(10)–CH_2(9)–H–C(8)–H–C(7)–H–C(6) was identified by the ^{1}H,^{1}H-COSY data, and the partial structure C(14)–C(10)–C(9)–C(8)–C(7)–C(6) was further confirmed by the HMBCs (*Fig. 2*). The relative configuration of this new compound was determined by NOESY data in which H–C(10), H–C(8), H–C(7), and H–C(6) showed a correlation to each other (*Fig. 3*). The diaxial disposition of OH–C(1) and Me(14) was determined from the chemical shift values of H–C(10) (δ(H) 2.42–2.48) and Me(14) (δ(H) 0.89) similar to those of **4** [9]. Since this new sesquiterpene **6** displayed a positive optical-rotation value similar to **4**, the absolute configuration of **6** was deduced to be the same as that of **4**. Therefore, the structure of **6** was determined to be (1α,6β)-1,6-dihydroxytorilin (=(6β,8β)-8-(angeloyloxy)-11-(acetyloxy)-1,6-dihydroxyguai-4-en-3-one).

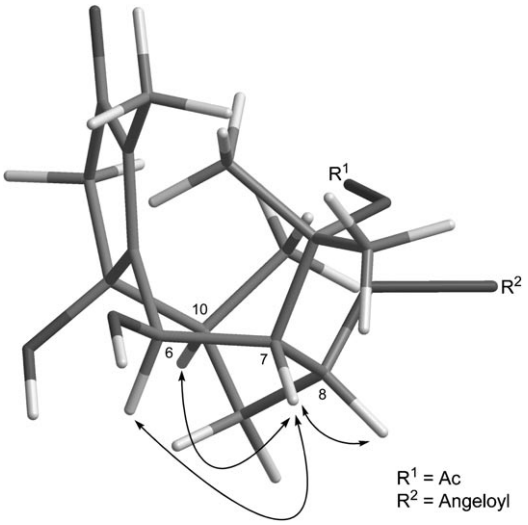


Fig. 3. *Key NOE correlations* (H↔H) *of compound* **6**[1])

The isolated compounds were evaluated for their cytotoxic activity against the cancer cell lines MCF-7 and LLC performed *via* the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay [10]. Of these, compounds **1**, **3**, and **4** were found to be cytotoxic toward the LLC cancer cells with IC_{50} values of 31.3, 32.5, and 34.0 μg/ml, respectively. However, no significant cytotoxicity was found against the MCF-7 cancer cells for any of the compounds tested.

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Experimental Part

General. All chemicals and solvents were of anal. grade and used without further purification. TLC: aluminium plates precoated with silica gel *60 F_{254}* (*Merck*). Column chromatography (CC): silica gel (SiO_2; 70–230 mesh) and *Lichroprep-RP-18* gel (40–63 μm; *Merck*). Prep. HPLC: *Shim-Pack-Prep-ODS* (20 × 250 mm) column, *LC-10AD* pump (*Shimadzu*), and *SPD-10A* detector (*Shimadzu*). Optical rotations: *Jasco-DIP-1000* automatic digital polarimeter (*Jasco Corporation*, Tokyo, Japan). UV Spectra: *Jasco-V-550* spectrophotometer; λ_{max} (log ε) in nm. ^{1}H- and ^{13}C-NMR Spectra: *Bruker-ARX* spectrometer (250 MHz); δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-FAB-MS: *JMS700* (*Jeol*); in *m*/*z* (rel. %).

Plant Material. The dried fruits of *T. japonica* DC. were purchased from a folk-medicine market 'Yak-ryoung-si' in Daegu, Korea, in May 2007. A voucher specimen was deposited with the herbarium of the College of Pharmacy, Yeungnam University.

Extraction and Isolation. The dried fruits of *T. japonica* DC. (10 kg) were extracted three times with MeOH at r.t. The MeOH soln. was concentrated to give a residue (672 g) which was partitioned between H_2O and AcOEt. The AcOEt-soluble fraction (300 g) was subjected to CC (SiO_2, hexane/acetone 95 : 5, 90 : 10, 85 : 15, 80 : 20, 70 : 30, 60 : 40, 40 : 60, 20 : 80, and 0 : 100) *Fractions 1–24*. Torilin (**1**; 1470 mg) was obtained as a major constituent from *Fr. 9* by recrystallization in MeOH. From *Fr. 19*, torilolone (**2**; 170 mg) was also isolated by recrystallization in MeOH. *Fr. 15* (5 g) was fractionated by CC (SiO_2, CH_2Cl_2/acetone 99 : 1, 97 : 3, 95 : 5, 93 : 7, 90 : 10, 80 : 20, 50 : 50, and 0 : 100): *Frs. 15.1–15.9*. The (1β)-1-hydroxytorilin (**3**; 1810 mg) was obtained as another major constituent from *Fr. 15.6*. *Fr. 17* (14.5 g) was subjected to CC (SiO_2, CH_2Cl_2/acetone 95 : 5 → 0 : 100): *Frs. 17.1–17.5*. *Fr. 17.2* (1.2 g) was subjected to CC (SiO_2, CH_2Cl_2/acetone 95 : 5 → 0 : 100): (1α)-1-hydroxytorilin (**4**; 250 mg). *Fr. 21* (6.5 g) was subjected to CC (SiO_2, hexane/AcOEt 90 : 10 → 0 : 100): *Frs. 21.1–21.9*. Compound **5** (50 mg) was isolated from *Fr. 21.3* (1070 mg) by recrystallization in CH_2Cl_2. *Fr. 21.5* (1690 mg) was purified by prep. HPLC (*Sim Pack Prep ODS* (25 × 350 mm), MeOH/H_2O 40 : 60 → 75 : 25, flow rate 6 ml/min): **6** (10 mg).

(1β,8β)-*11-(Acetyloxy)-1,8-dihydroxyguai-4-en-3-one* (= *(5*S*,6*R*,8*S*,8a*S*)-5-[1-(Acetyloxy)-1-methylethyl]-4,5,6,7,8,8a-hexahydro-6,8a-dihydroxy-3,8-dimethylazulen-2(1*H*)-one*; **5**): Colorless needles. $[\alpha]_D^{25} = -22.8$ ($c = 0.1$, MeOH). UV: 235 (4.01). ^{1}H- and ^{13}C-NMR (250 and 63 MHz, CD_3OD): *Table*. HR-FAB-MS (pos.): 311.1861 ($[M+H]^+$, $C_{17}H_{27}O_5^+$; calc. 311.1858).

(1α,6β)-1,6-Dihydroxytorilin (= *(3a*R*,4*S*,6*R*,7*S*,8*R*)-7-[1-(Acetyloxy)-1-methylethyl]-2,3,3a,4,5,6,7,8-octahydro-3a,8-dihydroxy-1,4-dimethyl-2-oxoazulen-6-yl (2*Z*)-2-Methylbut-2-enoate*; **6**): White powder. $[\alpha]_D^{25} = +15$ ($c = 0.1$, MeOH). UV: 225 (4.10). ^{1}H- and ^{13}C-NMR (250 and 63 MHz, CD_3OD): *Table*. HR-FAB-MS (pos.): 409.2229 ($[M+H]^+$, $C_{22}H_{33}O_7^+$; calc. 409.2226).

Cytotoxicity Assay [10]. The cancer cell lines (MCF-7 and LLC) were maintained in RPMI 1640, which included L-glutamine with 10% fetal bovine serum (FBS) and 2% penicillin–streptomycin. Cells were cultured at 37° in a 5% CO_2 incubator. Cytotoxicity was measured by means of a modified MTT assay. Viable cells were seeded in the growth medium (100 μl) into 96-well microtiter plates ($1 \cdot 10^4$ cells/well) and incubated at 37° in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 5.0 to 150 μM by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to $< 0.1\%$. After standing for 24 h, 10 μl of the test sample was added to each well. The same volume of DMSO was added to the control wells. On removing medium after 48 h of the test-sample treatment, MTT (10 μl) was also added to each well (final concentration, 5 mg/ml). After 4 h incubation, the plates were removed, and the resulting formazan crystals were dissolved in DMSO (150 ml). The *OD* was measured at 570 nm. The IC_{50} value was defined as the concentration of sample that reduced absorbance by 50% relative to the vehicle-treated control.

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