New Guaiane Sesquiterpenes from the Fruits of *Torilis japonica*

In-Kyoung LEE,[a] Jeong-Hyung LEE,[b] Eui Il HWANG,[c] and Bong-Sik YUN*,[a]

[a] *Division of Biotechnology, College of Environmental and Bioresources Sciences, Chonbuk National University; Jeonju 561–756, Korea:* [b] *Department of Biochemistry, College of Natural Sciences, Kangwon National University; Chuncheon 200–701, Korea: and* [c] *KT&G Central Research Institute; Daejeon 305–805, Korea.*

Received May 23, 2008; accepted June 30, 2008; published online July 28, 2008

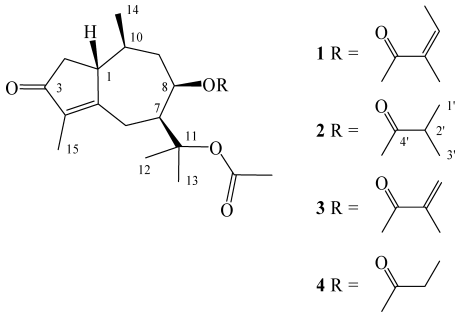
Three new guaiane type sesquiterpenes were isolated from the methanolic extract of the fruits of *Torilis japonica* together with a known compound, torilin (1). Their structures were established as 11-acetoxy-8-isobutyryl-4-guaien-3-one (2), 11-acetoxy-8-methacrylyl-4-guaien-3-one (3), and 11-acetoxy-8-propionyl-4-guaien-3-one (4) by spectroscopic methods. These compounds inhibited lipopolysaccharide (LPS)-induced nitric oxide production in murine macrophages RAW 264.7 cells.

Key words *Torilis japonica*; Umbelliferae; nitric oxide production inhibitor; guaiane sesquiterpenoid

Torilis japonica DECANDOLLE is a biennial plant that is found widely in East Asia. The fruits of this plant have been used as a traditional medicine for the treatment of skin disease, testitis, anthroneuralgia, impotence, and inflammation.[1)] In previous investigation on chemical constituents of *T. japonica*, torilin (**1**), a guaiane-type sesquiterpene angelate, was isolated as a major constituent. Torilin has been reported to have anti-inflammatory activity, anticancer activity including anti-angiogenetic effect, the ability to reverse multidrug-resistance in cancer cells, and anti-invasive activity in human fibrosarcoma cells.[2—5)] Besides guaiane-type sesquiterpene, humulene-type, germacrane-type, eudesmane-type, and oppositane-type sesquiterpenes were reported from this plant.[6)]

In our search for chemical constituents with anti-inflammatory activity, three new guaiane-type sesquiterpenes, 11-acetoxy-8-isobutyryl-4-guaien-3-one (**2**), 11-acetoxy-8-methacrylyl-4-guaien-3-one (**3**), and 11-acetoxy-8-propionyl-4-guaien-3-one (**4**) were isolated from the methanolic extract of the fruits of *Torilis japonica*. These compounds inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO) production in murine macrophages RAW 264.7 cells. Here, we describe the isolation and structure determination of compounds **1**—**4**, and their inhibitory effects on NO production in RAW 264.7 cells *in vitro*.

The dried fruits of *T. japonica* were extracted twice with a solution of $CHCl_3$–MeOH (50 : 50, v/v) at room temperature. After concentration of the extract under reduced pressure, the concentrate was chromatographed on a column of silica gel eluting with a gradient of increasing amount of ethyl acetate (1—50%) in hexane. The 2% ethyl acetate fraction was separated by consecutive Sephadex LH-20 and ODS column chromatographies and preparative HPLC to afford **1**—**4**.



1 R =

2 R =

3 R =

4 R =

Compounds **1**—**4** were similar in their physicochemical properties and NMR spectra. The IR spectra of **1**—**4** showed strong bands at 1730 cm^{-1} indicating the presence of acyl carbonyl carbon, and 1697 and 1639 cm^{-1} indicating the presence of a cyclopentenone moiety, which was supported by intensive absorption at 238 nm in their UV spectra. Compound **1** was identified as torilin (11-acetoxy-8-angeloyl-4-guaien-3-one), which was previously isolated from *T. japonica*,[7)] on the basis of EI-MS measurement and comparison of ^{1}H- and ^{13}C-NMR spectra. Its NMR spectra were consistent with those published in the literature on torilin.[8)]

Compound **2** was isolated as a colorless oil with rotation values of −30.0 and its molecular formula was determined to be $C_{21}H_{32}O_5$ by HR-EI-MS (*m/z* 364.2252 $[M]^+$, +0.2 mmu). The ^{1}H- and ^{13}C-NMR spectra of **2** were very similar to those of **1**, except for the angeloyl group at the C-8 position. The ^{1}H-NMR spectrum of **2** showed characteristic signals of an isobutyric acid moiety at δ 2.42 (1H, m, H-2′), 1.10 (3H, d, J=8.4, H-1′), and 1.08 (3H, d, J=8.4, H-3′) and in the ^{13}C-NMR spectrum, at δ 175.9 (C-4′), 34.3 (C-2′), 19.1 (C-1′), and 18.6 (C-3′). These results indicated that compound **2** had an isobutyryl moiety instead of the angeloyl in **1**. The structure of **2** was confirmed by interpretation of the HMBC spectrum, which exhibited a long-range correlation from a methine proton at δ 5.22 (H-8) to the carbonyl carbon at δ 175.9 (C-4′) and from methyl protons at δ 1.10 (H-1′) and δ 1.08 (H-3′) to the carbons at δ 34.3 (C-2′) and 175.9 (C-4′). Other HMBC correlations were consistent with the structure of compound **2**. Therefore, the structure of **2** was unambiguously assigned as 11-acetoxy-8-isobutyryl-4-guaien-3-one.

The molecular formula of compound **3**, which was isolated as a colorless oil with a specific rotation value of −31.5, was established as $C_{21}H_{30}O_5$ by HR-EI-MS (*m/z* 362.2083 $[M]^+$, −1.0 mmu). Comparison of the ^{1}H- and ^{13}C-NMR spectra of **3** with those of **1** suggested that **3** was the same structure as **1**, except for the presence of a substituent of methacrylyl moiety at C-8 position instead of an angeloyl group. The ^{1}H-NMR spectrum of **3** exhibited the signals due to a methacrylic acid at δ 6.10 (1H, br s, H-1′), 5.62 (1H, br s, H-1′), and 2.01 (3H, s, H-3′). Methacrylyl moiety was supported by the HMBC correlations from H-1′ at δ 6.10 to C-4′, C-2′, and C-3′ and from H-3′ at δ 2.01 to C-1′, C-2′, and C-4′. Substitution position of methacrylic acid was determined to be C-8 by the HMBC correlations from H-8 to

* To whom correspondence should be addressed. e-mail: ybs@kribb.re.kr

the carbonyl carbon at δ 166.3. Therefore, the structure of **3** was determined to be 11-acetoxy-8-methacrylyl-4-guaien-3-one.

Compound **4** was isolated as a colorless oil and its molecular formula was determined to be $C_{21}H_{32}O_5$ by HR-EI-MS (*m/z* 350.2094 $[M]^+$, +0.1 mmu). The ^{1}H- and ^{13}C-NMR spectra of **4** were very similar to those of **1**, except for the presence of propionyl moiety instead of angeloyl group at the C-8 position. The ^{1}H- and ^{13}C-NMR spectra of **4** showed the characteristic signals of a propionic acid at δ 1.15 (3H, t, J=8.0, H-1′) and 2.31 (2H, m, H-2′) in the ^{1}H-NMR and at δ 173.5 (C-3′), 28.3 (C-2′), and 9.3 (C-1′) in the ^{13}C-NMR. HMBC correlation of H-8 at δ 5.32 to the carbonyl carbon of C-3′ connected the propionyl group to C-8. Other HMBC correlations were consistent with the structure of **3**. Therefore, the structure of **3** was determined to be 11-acetoxy-8-propionyl-4-guaien-3-one.

The stereochemistry of new torilin derivatives **2**—**4** was determined by comparison of the proton coupling constants, NOESY data, and specific rotation values with the known torilin. The ^{1}H-NMR coupling constants of **2**—**4** were almost same as those of torilin, suggesting that these compounds had the same relative stereochemistry as torilin. This suggestion was supported by the NOESY experiment, which showed no difference between new derivatives and torilin in correlation. Absolute stereochemistry was deduced from similar rotation values between torilin and **2**—**4**.

The cellular events in macrophage-like cells (*e.g.*, RAW264.7 cell) induced by LPS are regarded as useful *in vitro* models for evaluating the potency of anti-inflammatory compounds.[9] We estimated the inhibitory activity of **1**—**4** against LPS-stimulated NO production in RAW 264.7 cells using Griess reaction. The IC_{50} values of **1**—**4** were 35.5, 95.0, 22.5, and >100 μM, respectively. Although **2** and **4** showed marginal activity, **1** and **3** displayed significant activity. These results suggested that substituents at C-8 position would be important for NO production inhibitory activity. These compounds showed no cytotoxic effect at concentrations necessary to inhibit NO production. Although specific activity of the isolated compounds was less than other anti-inflammatory agents, high contents of **1** and no cytotoxic effect reveal that these compounds might be useful as an anti-inflammatory agent.

Experimental

General Experimental Procedures Specific rotation was determined using a JASCO P-1020 polarimeter. HR-EI-MS was taken on a JMS-700 JEOL mass spectrometer. UV and IR spectra were recorded on a Shimadzu UV-300 and a FT-IR Equinox 55 spectrometer, respectively. NMR spectra were obtained on a Varian UNITY Inova NMR spectrometer with ^{1}H-NMR at 400 MHz and ^{13}C-NMR at 100 MHz in $CDCl_3$. Chemical shifts are given in ppm (δ) using TMS as internal standard.

Plant Material The dried fruits of *Torilis japonics* were purchased at an herbal drug store at Keumsan, Korea. A voucher specimen was deposited in the herbarium of the Bioactive Metabolite Research Center, KRIBB.

Extraction and Isolation The dried fruits of *T. japonica* (10 kg) were extracted twice with a solution of $CHCl_3$–MeOH (50 : 50, v/v) at room temperature. After removal of solvent under reduced pressure, the concentrate was chromatographed on a column of silica gel eluting with a gradient of an increasing amount of ethyl acetate (1—50%) in hexane. The 2% ethyl acetate fraction was subjected to a column of Sephadex LH-20 eluted with $CHCl_3$/MeOH (1 : 1, v/v), followed by octadecyl silane (ODS) column chromatography eluting with a gradient of an increasing amount of MeOH (50—90%) in H_2O. Finally, an 80% aqueous MeOH fraction was purified by preparative HPLC (Cosmosil C_{18}, 20×150 mm; flow rate 8 ml/min; MeOH/H_2O, 70 : 30; UV detection at 210 nm) to afford **1** (rt 28.9 min, 3 g), **2** (rt 20.8 min, 150 mg), **3** (rt 17.9 min, 4 mg), and **4** (rt 15.1 min, 210 mg) as colorless oils.

11-Acetoxy-8-isobutyryl-4-guaien-3-one (**2**): Colorless oil; $[\alpha]_D$ −30.0 (c=14.96, MeOH); UV λ_{max} (MeOH) (log ε): 238 (4.24) nm; IR (KBr) cm^{-1}: 3444, 2981, 2877, 2360, 1729, 1697, 1637, 1458; ^{1}H- and ^{13}C-NMR:

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for Compounds **2**—**4** in $CDCl_3$

No.	**2**		**3**		**4**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	51.0	2.33 (m)[a)]	51.3	2.46 (m)	51.4	2.38 (m)
2	41.2	2.48 (dd, J=18.4, 6.0)	41.5	2.61 (dd, J=18.4, 6.0)	41.4	2.58 (dd, J=18.4, 6.4)
		1.96 (dd, J=18.4, 3.2)		2.08 (dd, J=18.4, 3.2)		2.04 (dd, J=18.4, 3.6)
3	208.0		208.4		208.3	
4	135.1		135.4		135.3	
5	173.9		174.0		174.1	
6	25.5	2.74 (d, J=13.6)	25.8	2.88 (d, J=13.6)	25.7	2.81 (d, J=13.2)
		2.43 (br dd, J=13.6)		2.53 (br d, J=13.6)		2.49 (br d, J=13.2)
7	46.2	2.29 (dd, J=10.4, 4.0)	46.7	2.42 (dd, J=10.4, 3.6)	46.4	2.36 (dd, J=10.4, 4.0)
8	71.0	5.22 (ddd, J=8.0, 7.6, 4.0)	71.7	5.44 (ddd, J=7.6, 7.6, 3.6)	71.4	5.32 (ddd, J=8.0, 7.6, 4.0)
9	40.1	2.09 (br dd, J=14.0, 7.6)	40.4	2.25 (br dd, J=14.4, 8.4)	40.3	2.21 (br dd, J=14.0, 7.6)
		1.47 (ddd, J=14.0, 10.0, 8.4)		1.62 (m)		1.59 (ddd, J=14.0, 10.0, 8.4)
10	33.3	1.37 (m)	33.6	1.48 (m)	33.5	1.44 (m)
11	84.3		84.6		84.6	
12	24.7	1.46 (s)	24.8	1.54 (s)	24.9	1.53 (s)
13	24.0	1.39 (s)	24.2	1.52 (s)	24.2	1.47 (s)
14	22.7	0.92 (d, J=6.4)	22.9	1.04 (d, J=6.4)	22.9	1.01 (d, J=6.4)
15	8.0	1.62 (d, J=2.0)	8.3	1.73 (d, J=2.0)	8.2	1.71 (d, J=2.0)
1′	19.1	1.10 (d, J=8.4)	126.1	6.10 (br s)	9.3	1.15 (t, J=8.0)
				5.62 (br s)		
2′	34.3	2.42 (m)	136.8		28.3	2.31 (m)
3′	18.6	1.08 (d, J=8.4)	18.6	2.01 (s)	173.5	
4′	175.9		166.3			
$COCH_3$	22.6	1.88 (s)	22.9	1.98 (s)	22.8	1.88 (s)
	170.2		170.6		170.5	

a) Proton resonance multiplicity and coupling constant (J=Hz) in parenthesis. All spectra were recorded at 400 MHz for proton and at 100 MHz for carbon.

see Table 1; EI-MS *m/z*: 365.5 $[M]^+$; HR-EI-MS *m/z*: 364.2252 $[M]^+$ (Calcd for $C_{21}H_{32}O_5$, 364.2250).

11-Acetoxy-8-methacrylyl-4-guaien-3-one (**3**): Colorless oil; $[\alpha]_D$ −31.5 (c=0.37, MeOH); UV λ_{max} (MeOH) (log ε): 238 (4.29) nm; IR (KBr) cm^{-1}: 3440, 2982, 2877, 2360, 1730, 1697, 1638, 1458; ^{1}H- and ^{13}C-NMR: see Table 1; EI-MS *m/z*: 362 $[M]^+$; HR-EI-MS *m/z*: 362.2083 $[M]^+$ (Calcd for $C_{21}H_{30}O_5$, 362.2093).

11-Acetoxy-8-propionyl-4-guaien-3-one (**4**): Colorless oil; $[\alpha]_D$ −34.8 (c=2.22, MeOH); UV λ_{max} (MeOH) (log ε): 238 (4.28) nm; IR (KBr) cm^{-1}: 3444, 2981, 2877, 2360, 1732, 1697, 1639; ^{1}H- and ^{13}C-NMR: see Table 1; EI-MS *m/z*: 350$[M]^+$; HR-EI-MS *m/z*: 350.2094 $[M]^+$ (Calcd for $C_{20}H_{30}O_5$, 350.2093).

Measurement of NO Production and Cell Viability Assay RAW264.7 cells grown on a 100 mm culture dish were harvested and seeded in 96-well plates at 2×10^5 cells/well. The plates were pretreated with various concentrations of the compounds for 30 min and then incubated for another 24 h with or without 1 μg/ml of LPS. Nitrite concentration in the culture supernatant was measured by the Griess reaction. Cell viability was measured with a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based colorimetric assay.

Acknowledgment This work was supported by the grants from the KRIBB Research Initiative Program, Korea Research Institute of Bioscience and Biotechnology and from the Biogreen 21 Program (20070301034026) of Rural Development Administration, Republic of Korea.

References

1) Lee M. C., Ryu K. S., *Bull. KH Pharma. Sci.*, **6**, 61—65 (1978).
2) Lee E. B., Cho S. I., Kang S. S., Kim K. R., Kim T. H., *Korean J. Pharmacogn.*, **30**, 137—144 (1999).
3) Kim M. S., Lee Y. M., Moon E. J., Kim S. E., Lee J. J., Kim K. W., *Int. J. Cancer*, **87**, 269—275 (2000).
4) Kim S. E., Kim Y. H., Kim Y. C., Lee J. J., *Planta Med.*, **64**, 332—334 (1998).
5) Kim M. S., Baek J. H., Park M. T., Sohn T. K., Kim S. E., Lee J. J., Kim K. W., *Oncol. Rep.*, **8**, 359—364 (2001).
6) Kitajima J., Suzuki N., Satoh M., Watanabe M., *Phytochemistry*, **59**, 811—815 (2002).
7) Chikamatsu H., Maeda M., Nakazaki M., *Tetrahedron*, **25**, 4751—4765 (1969).
8) Kang S. S., Lee E. B., Kim T. H., Kim K. R., Jung J. H., *Arch. Pharm. Res.*, **17**, 284—286 (1994).
9) Kobori M., Yoshida M., Ohnishi-Kameyama M., Shinmoto H., *Br. J. Pharmacol.*, **150**, 209—219 (2007).