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Bioactive Triterpenoids from the Twigs of *Chaenomeles sinensis*

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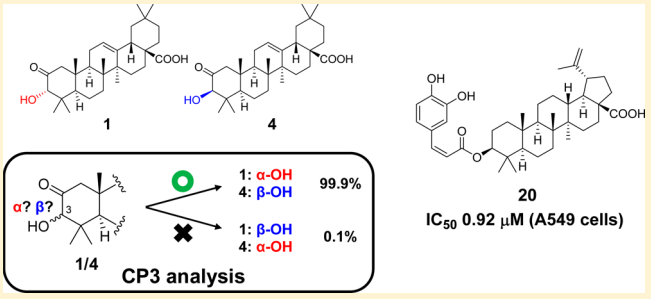
Supporting Information

ABSTRACT: *Chaenomeles sinensis* has been consumed traditionally for the treatment of throat diseases, diarrhea, inflammatory diseases, and dry beriberi. Repeated chromatography of the $CHCl_3$-soluble fraction from the 80% MeOH extract of *C. sinensis* twigs led to the isolation of three new triterpenoids, sinenic acid A (**1**), 3*β*-*O*-*cis*-feruloyl-2*α*,19*α*-dihydroxyurs-12-en-28-oic acid (**2**), and 3*β*-*O*-*cis*-caffeoylbetulin (**3**), together with 20 analogues. The chemical structures of **1**−**3** were determined using diverse NMR techniques and HRMS data analysis, chemical methods, and computational approaches supported by advanced statistics (CP3). All the purified compounds were evaluated not only for their cytotoxicity against four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15) but for their potential neuroprotective effects through induction of nerve growth factor in C6 glioma cells. Their anti-inflammatory effects were also assessed by measuring nitric oxide levels in lipopolysaccharide-insulted murine microglia BV2 cells.



Chaenomeles sinensis (Thouin) Koehne is a deciduous tree belonging to the family Rosaceae and is distributed widely in Korea, Japan, and mainland China. The ripened yellow fruit of *C. sinensis*, also called Chinese quince, has been used in Korean traditional medicine to treat throat diseases, diarrhea, inflammatory diseases, and dry beriberi.[1−3] Owing to this fruit being hard and possessing a strong acid taste, it is usually consumed in a candied form, as a tea, or in liquor in order to act as an effective cough medicine and diuretic.[4] The extract of *C. sinensis* fruit has been reported not only to possess antiviral, antidiabetic, antiacetylcholinesterase, antihyperglycemic, antihyperlipidemic, and antioxidant activities[5−8] but to produce triterpenoids, flavonoids, and phenolic compounds exhibiting tissue factor inhibitory and antipruritic activities.[1−3] In contrast to the relatively large number of studies on *C. sinensis* fruits due to its ethnopharmacological applications, there have been few investigations of the potentially bioactive constituents of *C. sinensis* twigs.[9,10]

In a continuing search for bioactive secondary metabolites from Korean medicinal plants, chemical investigations of *C. sinensis* twigs were carried out, leading to the isolation and characterization of three new (**1**−**3**) and 20 known triterpenoids (**4**−**23**). The structures of these purified compounds were established employing spectroscopic and spectrometric analysis, chemical methods, and computational analysis aided by statistics. All these phytochemicals (**1**−**23**) were also evaluated for their cytotoxic, neuroprotective, and anti-inflammatory activities.

RESULTS AND DISCUSSION

Compound **1** was isolated as a white powder, and its molecular formula was established as $C_{30}H_{46}O_4$ based on the deprotonated HRFABMS ion signal $[M - H]^-$ observed at *m*/*z* 469.3313 (calcd for $C_{30}H_{45}O_4$, *m*/*z* 469.3312). The ^1H NMR spectrum of **1** displayed resonances for seven singlet methyl protons [δ_H 1.24, 1.20, 0.99, 0.98, 0.95, and 0.92 (×2)], an olefinic proton [δ_H 5.45 (1H, brd, J = 3.6 Hz)], an oxygenated methine proton [δ_H 3.90 (1H, d, J = 3.7 Hz)], and two methylene protons [δ_H 3.01 (1H, d, J = 12.3 Hz) and 2.29 (1H, d, J = 12.3 Hz)]. The ^{13}C NMR spectrum of **1** revealed 30 resonances including a keto carbonyl (δ_C 213.1), a hydroxy carbonyl (δ_C 179.9), two olefinic carbons (δ_C 144.6 and 121.8), and an oxygenated methine carbon (δ_C 82.9). These spectroscopic data (Table 1) were comparable to those of 2*α*,3*α*-dihydroxyolean-12-en-28-oic acid (**7**) except for the presence of the keto carbonyl resonance (δ_C 213.1) and the

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Chart 1

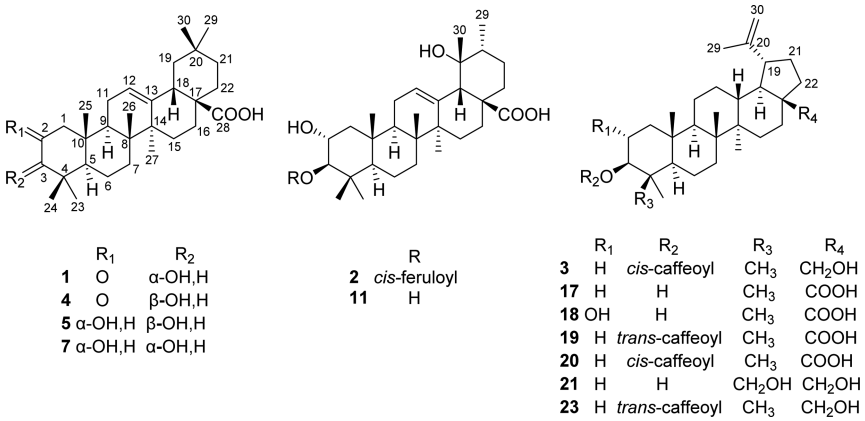

Table 1. ^{1}H [ppm, mult. (J in Hz)] and ^{13}C NMR Data of Compounds 1 and 4 in Pyridine-d_5

	1		4	
position	δ_C	δ_H	δ_C	δ_H
1ax	51.0	3.01, d (12.3)	53.4	2.20, d (12.0)
1eq		2.29, d (12.3)		2.48, d (12.0)
2	213.1		210.8	
3	82.9	3.90, d (3.7)	83.4	4.19, brs
4	41.9		45.5	
5	49.8	2.00, overlap	54.5	1.56, brd (13.6)
6ax	18.9	1.39, overlap	18.7	1.37, overlap
6eq		1.53, brd (14.0)		1.58, overlap
7ax	32.6	1.58, td (12.5, 3.3)	32.6	1.54, overlap
7ex		1.33, overlap		
8	40.0		39.8	
9	47.8	2.06, overlap	47.4	1.97, overlap
10	42.6		43.3	
11	23.4	1.96, overlap	23.5	1.83, overlap
12	121.8	5.45, brd (3.6)	121.7	5.46, t (3.6)
13	144.6		144.7	
14	42.1		42.0	
15ax	28.0	2.15, td (14.4, 4.6)	28.1	2.14, overlap
15eq		1.18, overlap		1.16, overlap
16a	23.5	2.08, overlap	23.5	2.09, overlap
16b		1.96, overlap		1.97, overlap
17	46.4		46.4	
18	41.7	3.29, dd (13.7, 4.0)	41.7	3.30, dd (14.1, 3.2)
19ax	46.2	1.77, t (13.7)	46.2	1.80, t (14.1)
19eq		1.28, overlap		1.29, overlap
20	30.7		30.7	
21ax	34.0	1.43, td (13.8, 4.0)	34.0	1.43, overlap
21eq		1.20, overlap		1.19, overlap
22ax	32.9	2.04, overlap	32.9	2.04, td (14.1, 4.4)
22eq		1.82, brd (13.4)		1.82, overlap
23	27.4	1.24, s	29.2	1.32, s
24	21.4	0.92, s	16.7	0.87, s
25	16.4	0.95, s	16.1	0.85, s
26	16.8	0.98, s	17.1	0.95, s
27	25.8	1.20, s	25.8	1.28, s
28	179.9		179.9	
29	33.0	0.92, s	33.0	0.95, s
30	23.5	0.99, s	23.5	1.00, s
OH-3		7.52, overlap		n/d

absence of the oxygenated carbon resonance (δ_C 66.1) observed in 2α,3α-dihydroxyolean-12-en-28-oic acid (**7**).[11] The keto carbonyl group was located at C-2 based on the HMBC cross-peak from H-1 [δ_H 3.01 (1H, d, J = 12.3 Hz) and 2.29 (1H, d, J = 12.3 Hz)] to C-2 (δ_C 213.1) (Figure 1). The 2D structure of **1** was established via thorough analysis of the COSY, HSQC, and HMBC NMR spectra (Figure 1). The α-orientation of OH-3 was confirmed given the strong and weak NOESY correlations from H-3 to H-24 and H-23, respectively. The configurations of the remaining stereogenic centers were identical to those of 2α,3α-dihydroxyolean-12-en-28-oic acid (**7**) based on the NOESY correlations of **1** (Figure 2) and their ^{13}C NMR chemical shift values similar to those of the reported oleanane derivatives (Table 1).[11,12] Therefore, the structure of **1** was established as 2-oxo-3α-hydroxyolean-12-en-28-oic acid, and it was assigned the trivial name sinenic acid A.

The HRFABMS of compound **2** (white powder), which exhibited a deprotonated ion at m/z 663.3892 [M − H]$^-$ (calcd for $C_{40}H_{55}O_8$, m/z 663.3891), led to its molecular formula being established as $C_{40}H_{56}O_8$. The comparison of its ^{1}H and ^{13}C NMR data (Table 2) with those of tormentic acid (**11**) indicated that **2** possesses an additional *cis*-feruloyl moiety, which was deduced from several characteristic NMR chemical shifts: δ_H 7.77 (1H, d, J = 2.0 Hz), 7.14 (1H, dd, J = 8.2, 2.0 Hz), 6.90 (1H, d, J = 12.9 Hz), 6.78 (1H, d, J = 8.2 Hz), 5.90 (1H, d, J = 12.9 Hz), and 3.88 (3H, s); δ_C 168.7, 149.5, 148.4, 145.2, 128.4, 126.7, 117.5, 115.7, 115.1, and 56.7.[13] Moreover an HMBC cross-peak from H-3 to C-9′ confirmed the *cis*-feruloyl motif as being substituted at C-3 (Figure 1). Alkaline hydrolysis of **2** afforded tormentic acid (**11**), which was substantiated by comparing the resultant ^{1}H NMR spectrum with previously reported data.[13] The full determination of the 1D and 2D NMR supported the assignment of structure of **2** as 3β-*O*-*cis*-feruloyl-2α,19α-dihydroxyurs-12-en-28-oic acid.

The molecular formula of compound **3** (white powder) was assigned as $C_{39}H_{56}O_5$ based on the deprotonated HRFABMS ion signal at m/z 603.4044 [M − H]$^-$ (calcd for $C_{39}H_{55}O_5$, m/z 603.4044). Inspection of the ^{1}H and ^{13}C NMR data of **3** (Table 2) suggested that this compound shares close structural similarities with betulin (**22**).[14] When compared to **22**, however, there was an additional *cis*-caffeoyl moiety in **3**, which was verified by the presence of the NMR resonances at δ_H 7.31 (1H, d, J = 2.1 Hz), 7.02 (1H, dd, J = 8.2, 2.1 Hz), 6.82 (1H, d, J = 12.7 Hz), 6.74 (1H, d, J = 8.2 Hz), and 5.76 (1H, d, J = 12.7 Hz) and δ_C 168.6, 148.2, 145.9, 144.8, 128.5, 124.7, 118.6, 117.6, and 115.8. The location of this additional *cis*-

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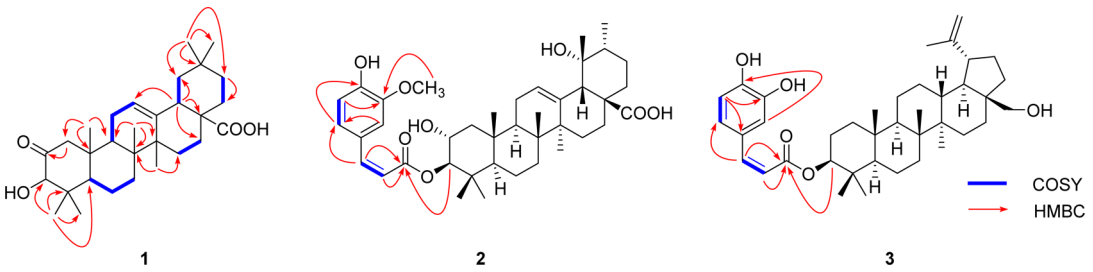

Figure 1. COSY (blue bold) and HMBC (red arrows) correlations of **1**−**3**.

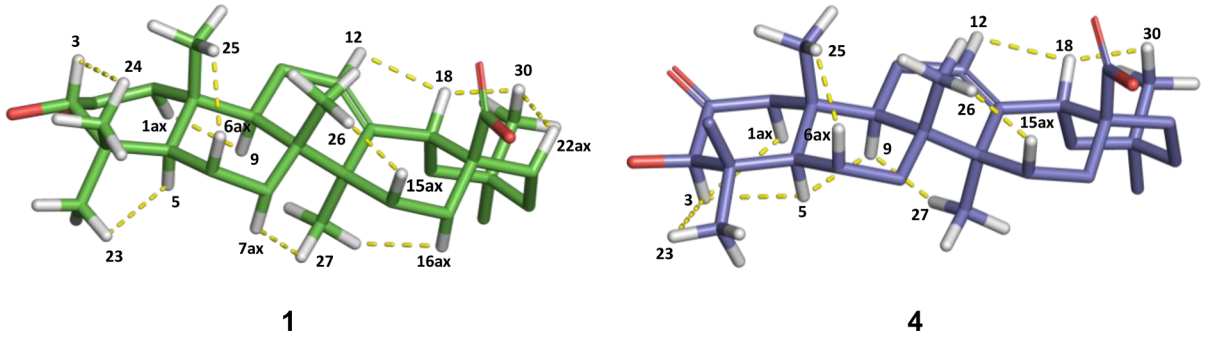

Figure 2. NOESY correlations (yellow dashed) of **1** and **4**. The geometry of the 3D structures was initially minimized at the MMFF force field and subsequently optimized at the B3LYP/6-31+G(d,p) level. Some protons were removed for a clearer presentation.

caffeoyl moiety was confirmed at C-3 via the HMBC cross-peak of H-3/C-9′ (Figure 1). The structure of **3** was verified when betulin (**22**) was produced from the alkaline hydrolysis of **3**, and this was confirmed by the comparison of the ^{1}H NMR spectrum of the hydrolysate with that of **22**.[14] The structure of **3** was consequently established as 3*β*-*O*-*cis*-caffeoylbetulin.

Compound **4** was obtained as a white powder with the identical molecular formula with that of **1** ($C_{30}H_{46}O_4$), from its HRFABMS showing a deprotonated ion at *m*/*z* 469.3311 [M − H]$^-$ (calcd for $C_{30}H_{45}O_4$, *m*/*z* 469.3312). The ^{1}H and ^{13}C NMR data of **4** were found to be closely comparable to those of **1**, but there were slight shifts in the resonances of an oxygenated methine [δ_H 4.19 (1H, brs), δ_C 83.4, for **4**; δ_H 3.90 (1H, d, *J* = 3.7 Hz), δ_C 82.9, for **1**] and a keto carbonyl (δ_C 210.8, for **4**; δ_C 213.1, for **1**) group, suggesting that **4** is the C-3 epimer of **1**. Indeed, the planar structure of **4** was determined to be identical with that of **1** via NMR data analysis. The *β*-orientation of the C-3 hydroxy group was determined based upon the NOESY correlations from H-3 to H-1$_{ax}$, H-5, and H-23 (Figure 2), confirming that **4** was the C-3 epimer of **1**. The structure of **4** was consequently established as 2-oxo-3*β*-hydroxyolean-12-en-28-oic acid and named sinenic acid B. Compound **4** was purified from a natural source for the first time, although it has been reported as a synthetic product.[15]

The epimeric relationship of **1** with **4** was reaffirmed using the CP3 protocol featuring quantum mechanics (QM)-based calculations of NMR shielding tensors coupled with advanced statistics. This strategy has been extensively applied in structural validation and revision when the NMR spectra of a pair of diastereomers are available.[16] Major conformers of **1** and **4** were identified upon conformational searches utilizing Macromodel (Schrodinger LLC), and their GIAO shielding constant calculations were computed at the B3LYP/6-31G(d,p) level employing the Gaussian 09 package (Gaussian Inc.) (see the Experimental Section). These resultant chemical shift values were averaged given their Boltzmann populations calculated based upon the relative MMFF94 potential energies (Table S1, Supporting Information), and these averaged values were subjected to the calculation of CP3 analysis (see the Experimental Section; Tables S2 and S3, Supporting Information). The CP3 analysis supported the NMR-based assignments produced for each epimer (i.e., **1** and **4**) with 99.9% probability when both the ^{1}H and ^{13}C NMR chemical shift values were considered (Figure 3; Figure S29, Supporting Information).

The other 19 reported phytochemicals were identified via comparison of their observed physical and spectroscopic data as maslinic acid (**5**),[12] arjunic acid (**6**),[17] 2*α*,3*α*-dihydroxyolean-12-en-28-oic acid (**7**),[11] (2*α*)-hydroxy-3-oxoolean-12-en-28-oic acid (**8**),[18] euscaphic acid (**9**),[19] 2*α*-hydroxypomolic acid (**10**),[19] tormentic acid (**11**),[13] 2*α*-hydroxyursolic acid (**12**),[19] 2*α*,19*α*-dihydroxy-3-oxours-12-en-28-oic acid (**13**),[20] 3-epi-2-oxopomolic acid (**14**),[21] pomolic acid 3-acetate (**15**),[22] 3*β*-*O*-*trans*-feruloyl-2*α*,19*α*-dihydroxyurs-12-en-28-oic acid (**16**),[23] betulinic acid (**17**),[14] alphitolic acid (**18**),[24] 3-*O*-*trans*-caffeoylbetulinic acid (**19**),[25] 3-*O*-*cis*-caffeoylbetulinic acid (**20**),[25] ilekudinol C (**21**),[26] betulin (**22**),[14] and 3*β*-*O*-*trans*-caffeoylbetulin (**23**).[27]

Using a sulforhodamine B (SRB) assay, the cytotoxicities of the purified compounds **1**−**23** were evaluated against four human cancer cell lines, namely, A549 (non-small-cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT15 (colon adenocarcinoma). According to Table 3, the lupane-type triterpenoids **3**, **17**, **19**−**21**, and **23** showed cytotoxic potencies with IC_{50} values ranging from 0.9 to 9.9 *μ*M. Interestingly, betulin (**22**) displayed no activity (IC_{50} > 10.0 *μ*M), whereas its 3-*O*-caffeoyl derivatives **3** and **23** showed cytotoxicity toward SK-OV-3 and SK-MEL-2 cancer cell lines, with the former possessing a *cis*-3-*O*-caffeoyl functionality and being slightly more potent than its geometric isomer (**3**, 8.4 and 2.0 *μ*M; **23**, 9.1 and 2.9 *μ*M). In line with this finding, compound **20**, the

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Table 2. ^{1}H [ppm, mult. (J in Hz)] and ^{13}C NMR Data of Compounds 2 and 3 in Methanol-d_4

	2		3	
position	δ_C	δ_H	δ_C	δ_H
1ax	48.9	2.05, overlap	39.8	1.76, overlap
1eq		1.08, overlap		1.05, overlap
2	67.7	3.84, overlap	24.8	1.72, overlap
3	85.5	4.64, d (9.9)	82.4	4.52, dd (9.1, 7.3)
4	40.6		39.1	
5	56.6	1.03, brd (12.0)	57.0	0.87, overlap
6a	19.7	1.61, overlap	19.5	1.56, overlap
6b		1.52, overlap		1.50, overlap
7a	34.2	1.64, overlap	35.5	1.50, overlap
7b		1.38, overlap		1.46, overlap
8	42.8		42.3	
9	48.7	1.83, overlap	51.9	1.42, overlap
10	39.4		38.4	
11a	24.9	2.08, overlap	22.2	1.48, overlap
11b				1.30, overlap
12a	129.2	5.33, brt (3.4)	26.7	1.73, overlap
12b				1.13, overlap
13	140.4		38.8	1.74, overlap
14	41.3		44.0	
15a	29.8	1.86, overlap	28.3	1.82, td (13.3, 4.2)
15b		1.04, overlap		1.05, overlap
16ax	26.8	2.59, td (12.7, 5.0)	30.5	1.22, td (13.3, 4.0)
16eq		1.56		2.00, overlap
17	48.7		49.1	
18	55.3	2.55, s	50.2	1.64, t (11.7)
19	73.8		49.7	2.45, td (11.1, 5.7)
20	43.3	1.40, overlap	152.0	
21a	27.5	1.79, overlap	31.0	2.00, overlap
21b		1.25, overlap		1.40, overlap
22a	39.4	1.77, overlap	35.2	1.93, dd (12.0, 8.9)
22b		1.68, overlap		
23	29.4	0.93, s	28.7	0.88, s
24	18.3	0.89, s	17.1	0.81, s
25	17.2	1.07, s	16.9	0.92, s
26	17.7	0.85, s	16.7	1.11, s
27	25.0	1.39, s	15.4	1.06, s
28a	182.7		60.5	3.77, brd (10.6)
28b				3.31, overlap
29	16.8	0.96, d (6.7)	19.4	1.72, s
30a	27.2	1.23, s	110.4	4.71, m
30b				4.60, m
1′	128.4		128.5	
2′	115.1	7.77, d (2.0)	118.6	7.31, d (2.1)
3′	148.4		145.9	
4′	149.5		148.2	
5′	115.7	6.78, d (8.2)	115.8	6.74, d (8.2)
6′	126.7	7.14, dd (8.2, 2.0)	124.7	7.02, dd (8.2, 2.1)
7′	145.2	6.90, d (12.9)	144.8	6.82, d (12.7)
8′	117.5	5.90, d (12.9)	117.6	5.76, d (12.7)
9′	168.7		168.6	
OCH_3-3′	56.7	3.88, s		

cis-3-*O*-caffeoyl derivative of betulinic acid (**17**), exhibited more potent activity than **17** and its geometric isomer (**19**) (Table 3).[28]

The neuroprotective activities of the bioactive entities (**1**–**23**) were also evaluated in terms of their inducing potential on nerve growth factor (NGF) secretion in C6 cells (Table 4).

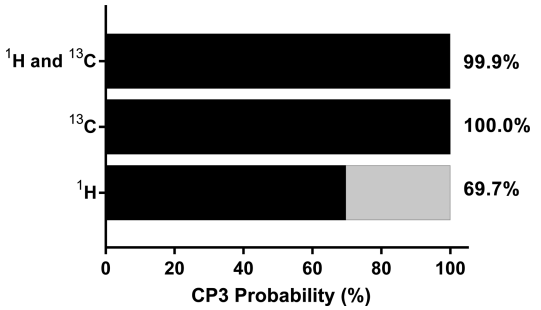

Figure 3. CP3 analysis with experimental and calculated NMR chemical shifts of **1** and **4**. Percentiles indicate the probabilities where the experimental ^{1}H, ^{13}C, or both NMR chemical shift values of **1** and **4** matched their respective computed values.

Table 3. Cytotoxicity of Selected Compounds against Four Cultured Human Cancer Cell Lines in the SRB Bioassay

	IC_{50} (μM)[a]			
compound	A549	SK-OV-3	SK-MEL-2	HCT15
3	>10.0	8.4	2.0	>10.0
17	3.1	>10.0	>10.0	8.7
19	9.9	>10.0	>10.0	>10.0
20	0.9	7.5	6.7	3.8
21	5.1	>10.0	>10.0	>10.0
23	>10.0	9.0	2.9	>10.0
cisplatin[b]	3.3	3.8	2.0	4.3

[a]50% inhibitory concentration; the concentration of compound that caused a 50% inhibition in cell growth. [b]Cisplatin was used as a positive control.

Table 4. Effects of Compounds 11, 17, and 19 on NGF Secretion in C6 Cells

compound	NGF secretion[a] (%)	cell viability[b] (%)
11	167.4 ± 9.57	100.5 ± 4.07
17	177.6 ± 2.74	77.3 ± 4.18
19	355.7 ± 2.94	95.0 ± 0.18
6-shogaol[c]	168.6 ± 7.16	125.8 ± 0.93

[a]C6 cells were treated with 20 μM of each compound. After 24 h, the content of NGF secreted in the C6-conditioned medium was measured by ELISA. The level of secreted NGF is expressed as the percentage of the untreated control (set as 100%). [b]Cell viability after treatment with 20 μM of each compound was determined by an MTT assay and is expressed as a percentage (%). Results are the means of three independent experiments, and the data are expressed as mean ± SD. [c]Positive control substance.

Compounds **17** and **19** strongly induced NGF release (177.6 ± 2.74% and 355.7 ± 2.94%, respectively), when compared with the positive control, 6-shogaol, 168.6 ± 7.16%, but with no significant cell toxicity toward the C6 cells at the tested dose (20 μM). These data suggest that the *trans*-oriented caffeoyl moiety substituted at C-3 of betulinic acid (**17**) might be a key functionality in augmenting NGF secretion. Contrary to this observation, 3-*O*-*cis*-caffeoylbetulinic acid (**20**) showed poor NGF secretion (52.49 ± 9.53%) presumably because of its potent cytotoxicity (23.56 ± 1.21%). Compound **11** also exhibited strong NGF secretion in C6 cells (167.37 ± 9.57%), implying that some functionalized triterpenoids can serve as prototypes for the development of noncytotoxic neuroprotective agents.

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The potential anti-inflammatory effects on the purified molecules (**1**–**23**) were also tested via the measurement of produced nitric oxide (NO) levels in lipopolysaccharide (LPS)-stressed murine microglia BV-2 cells (Table 5). Compounds **7**

Table 5. Inhibitory Effect of Selected Compounds on NO Production in LPS-Activated BV-2 Cells

compound	IC_{50} (μM)[a]	cell viability(%)[b]
2	37.1	110.9 ± 7.63
5	17.8	30.4 ± 5.22
7	21.6	87.0 ± 4.30
12	47.1	100.3 ± 3.53
17	4.5	59.6 ± 3.18
18	14.5	71.0 ± 4.11
20	13.4	35.3 ± 2.16
21	25.5	88.0 ± 4.94
L-NMMA[c]	21.8	114.1 ± 2.96

[a]IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells. [b]Cell viability following treatment with 20 μM of each compound was determined using the MTT assay and is expressed as a percentage (%). Data are expressed as the mean ± SD of three independent experiments. [c]Positive control substance.

and **18** exhibited potent inhibition of NO production, with IC_{50} values of 21.6 and 14.5 μM, which were comparably potent to the positive control N^G-monomethyl-L-arginine (L-NMMA) (21.8 μM), without significant cell toxicity. Although compounds **5**, **17**, and **20** also exhibited strong activity (17.8, 4.5, and 13.4 μM), they diminished the cell viability of BV-2 cells with 30.4 ± 5.22%, 59.6 ± 3.18%, and 35.3 ± 2.16% at the tested concentration (20 μM) as well. This implies that the observed inhibitory activity of **5**, **17**, and **20** on NO production was influenced by their cytotoxicity.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were obtained on a JASCO P-1020 polarimeter (JASCO, Easton, MD, USA). UV spectra were acquired with a Shimadzu UV-1601 UV–visible spectrophotometer (Shimadzu, Tokyo, Japan). IR spectra were measured on a Bruker IFS-66/S Fourier-transform IR spectrometer (Bruker, Karlsruhe, Germany). NMR spectra were generated using a Bruker AVANCE III 700 NMR spectrometer (Bruker, Karlsruhe, Germany). The HRFABMS chromatograms were recorded on a Waters SYNAPT G2 (Waters, Milford, MA, USA) mass spectrometer. Semipreparative HPLC was conducted utilizing a Gilson 306 pump (Gilson, Middleton, WI, USA) and a Shodex refractive index detector (Shodex, New York, NY, USA) with a Phenomenex Luna C_{18} 10 μm column (10 × 250 mm, Phenomenex, Torrance, CA, USA) and an Apollo silica 5 μm column (10 × 250 mm, Apollo, Manchester, UK) at a flow rate 2 mL/min. LPLC was accomplished employing a LiChroprep Lobar-A Si 60 column (240 × 10 mm, 40–63 μm, Merck, Darmstadt, Germany) equipped with an FMI QSY-0 pump (Teledyne Isco, Lincoln, NE, USA). Open column chromatography was implemented with silica gel 60 (70–230 and 230–400 mesh; Merck, Darmstadt, Germany) and RP-C_{18} silica gel (230–400 mesh, Merck, Darmstadt, Germany). TLC was carried out with precoated silica gel F_{254} plates and RP-18 F_{254s} plates (Merck, Darmstadt, Germany).

Plant Material. Twigs of two-year-old *C. sinensis* were collected in Seoul, Republic of Korea, in January 2012. A voucher specimen for the plant (SKKU-NPL 1206) was authenticated by one of the authors (K.R.L.) and stored at the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea.

Extraction and Isolation. Twigs of *C. sinensis* (7.0 kg) were extracted three times using 80% aqueous MeOH for 1 day under reflux, and the combined extract was filtered. The filtrate was concentrated in vacuo to obtain a crude MeOH extract (320 g). The slurry was suspended in H_2O and sequentially extracted with hexanes, $CHCl_3$, EtOAc, and *n*-BuOH to produce 3, 15, 6, and 30 g of residues, respectively. The $CHCl_3$-soluble phase (15 g) was subjected to passage over a silica gel open column ($CHCl_3$–MeOH, 50:1 → 1:1) to furnish nine pooled fractions (C1–C9). Fraction C1 (1.8 g) was chromatographed on an RP-C_{18} silica gel column eluting with 60% aqueous MeOH to give 10 subfractions (C1-1–C1-10). Fraction C1-3 (50 mg) was purified using semipreparative HPLC, eluting with an isocratic mixture of 55% aqueous CH_3CN, to garner compounds **13** (t_R 15.5 min, 2 mg) and **14** (t_R 17.3 min, 3 mg). Fraction C1-5 (50 mg) was separated by semipreparative HPLC (80% aqueous MeCN) to obtain compounds **1** (t_R 16.6 min, 2 mg), **4** (t_R 12.2 min, 2 mg), **8** (t_R 15.8 min, 2 mg), and **15** (t_R 11.1 min, 3 mg). Compound **17** (t_R 20.3 min, 6 mg) was acquired upon the purification of fraction C1-6 (50 mg) with semipreparative HPLC (90% aqueous MeCN). Fraction C3 (1.6 g) was subjected to separation on an RP-C_{18} silica gel open column, eluting with 60% aqueous MeOH, to give 10 subfractions (C3-1–C3-10). Among those subfractions, compounds **2** (t_R 16.2 min, 2 mg), **7** (t_R 12.9 min, 11 mg), and **16** (t_R 19.2 min, 2 mg) were purified from fraction C3-7 (50 mg) by a semipreparative HPLC (90% aqueous MeOH). Fraction C3-8 (40 mg) was purified employing semipreparative HPLC, with an isocratic solvent system (90% aqueous CH_3CN), to furnish compounds **21** (t_R 19.9 min, 11 mg) and **22** (t_R 21.2 min, 2 mg). Fraction C3-9 (60 mg) was purified with semipreparative HPLC, eluting with 80% aqueous CH_3CN, to afford compound **20** (t_R 13.3 min, 5 mg). Compounds **3** (t_R 20.3 min, 10 mg) and **23** (t_R 22.9 min, 11 mg) were isolated upon purification of fraction C3-10 (100 mg) by an isolation strategy using semipreparative HPLC (85% aqueous CH_3CN). Fraction C4 (1.5 g) was fractionated into 12 subfractions (C4-1–C4-12) using an RP-C_{18} silica gel open column eluting with 70% aqueous MeOH. Fraction C4-6 (20 mg) was purified by semipreparative HPLC (75% aqueous MeOH) to acquire compound **10** (t_R 21.2 min, 2 mg). Fraction C4-7 (120 mg) was separated using semipreparative HPLC ($CHCl_3$–MeOH, 30:1) to yield compounds **6** (t_R 15.6 min, 4 mg) and **9** (t_R 11.3 min, 7 mg). Fraction C4-9 (50 mg) was purified by semipreparative HPLC ($CHCl_3$–MeOH, 30:1) to yield compounds **11** (t_R 17.7 min, 10 mg) and **18** (t_R 18.8 min, 20 mg). Compound **5** (t_R 10.3 min, 20 mg) was obtained from fraction C4-10 (110 mg) employing semipreparative HPLC with a solvent mixture of $CHCl_3$–MeOH (30:1). Compounds **12** (t_R 19.3 min, 3 mg) and **19** (t_R 17.6 min, 2 mg) were afforded from fraction C4-11 (200 mg) using a LiChroprep Lobar-A Si 60 column followed by semipreparative HPLC (70% aqueous CH_3CN).

*Sinenic Acid A (**1**):* white powder; $[\alpha]_D^{25}$ −11.0 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3395, 2944, 2870, 1640 cm^{-1}; ^1H (700 MHz) and ^{13}C (175 MHz) NMR data in pyridine-d_5, see Table 1; HRFABMS (negative-ion mode) *m/z* 469.3313 $[M - H]^-$ (calcd for $C_{30}H_{45}O_4$, *m/z* 469.3312).

*3β-O-cis-Feruloyl-2α,19α-dihydroxyurs-12-en-28-oic acid (**2**):* white powder; $[\alpha]_D^{25}$ +35.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 285 (2.80), 229 (2.75) nm; IR (KBr) ν_{max} 3430, 2950, 2880, 1716, 1700, 1625, 1588, 1512 cm^{-1}; ^1H (700 MHz) and ^{13}C (175 MHz) NMR data in methanol-d_4, see Table 2; HRFABMS (negative-ion mode) *m/z* 663.3892 $[M - H]^-$ (calcd for $C_{40}H_{55}O_8$, *m/z* 663.3891).

*3β-O-cis-Caffeoylbetulin (**3**):* white powder; $[\alpha]_D^{25}$ +44.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 288 (3.05), 231 (2.90) nm; IR (KBr) ν_{max} 3500, 2930, 2888, 1700, 1632, 1600, 1515 cm^{-1}; ^1H (700 MHz) and ^{13}C (175 MHz) NMR data in methanol-d_4, see Table 2; HRFABMS (negative-ion mode) *m/z* 603.4044 $[M - H]^-$ (calcd for $C_{39}H_{55}O_5$, *m/z* 603.4044).

*Sinenic acid B (**4**):* white powder; $[\alpha]_D^{25}$ +76.0 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3401, 2940, 2866, 1639 cm^{-1}; ^1H (700 MHz) and ^{13}C (175 MHz) NMR data in pyridine-d_5, see Table 1; HRFABMS (negative-ion mode) *m/z* 469.3311 $[M - H]^-$ (calcd for $C_{30}H_{45}O_4$, *m/z* 469.3312).

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Alkaline Hydrolysis of 2 and 3. Compounds **2** and **3** (each 1 mg) were individually hydrolyzed using 0.1 N KOH (2 mL) and stirred at room temperature for 12 h. The reaction mixture was neutralized with Dowex HCR W2 (H^+ form) resin, and the resin was removed by filtration. A portion of the reaction product was partitioned between $CHCl_3$–H_2O (1 mL each). The $CHCl_3$-soluble phase was purified by semipreparative HPLC (90% aqueous MeCN) to afford the aglycones **2a** (=**11**) and **3a** (=**22**), which were identified utilizing ^1H NMR and MS data as tormentic acid and betulin, respectively.[13,14]

Cytotoxicity Assessment. The cytotoxicity of the compounds addressed in this study against the cultured human tumor cell lines A549, SK-OV-3, SK-MEL-2, and HCT15 was evaluated using the SRB assay.[29] Cells addressed in the current study were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained at the Korea Research Institute of Chemical Technology.

NGF and Cell Viability Assays. The C6 glioma cells (the Korean Cell Line Bank, Seoul, Republic of Korea) were used to assess the release of NGF into the culture medium. The test cells were seeded onto 24-well plates at a density of 1×10^5 cells/well. After 24 h, the cells were treated with serum-free DMEM and incubated with various concentrations of compounds for an additional 24 h. The medium supernatant was collected from the culture plates, and NGF levels were evaluated using an ELISA development kit. Cell viability was also assessed with a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, in which the results were expressed as a percentage of the control group (untreated cells).

NO Production and Viability in LPS-Stressed BV-2 Cells. The inhibitory effect of the test compounds on LPS-stimulated NO production was scrutinized using BV2 cells, as developed by Dr. V. Bocchini at the University of Perugia (Perugia, Italy).[30,31] The target cells were seeded on a 96-well plate at 4×10^4 cells/well and treated with/without different concentrations of the purified molecules. LPS (100 ng/mL) was added to BV2 cells, and they were incubated for 24 h. Nitrite (NO_2, soluble oxidation product of NO) concentrations present in the culture medium were measured using the Gries reagent (0.1% *N*-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). The supernatant (50 μL) was mixed with an identical volume of the Gries reagent. After 10 min, absorbance at 570 nm was measured utilizing a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA). Generated nitrite concentrations were gauged using graded sodium nitrite solution as a standard. Cell viability was evaluated using the MTT assay.

Computational Analysis.[16,32] All conformers addressed in the study were found using the Macromodel (version 2015-2, Schrodinger LLC) module with "mixed torsional/low-mode sampling" in the MMFF94 force field. The searches were initially implemented in the gas phase with a 50 kJ/mol energy window limit and 10 000 maximum number of steps to exhaustively explore all potential conformers. The Polak–Ribiere conjugate gradient protocol was utilized to minimize conformers with 10 000 maximum iterations and a 0.001 kJ $(mol\ Å)^{-1}$ convergence threshold on the rms gradient. Conformers within 10 kJ/mol of each global minimum were not subjected to geometry optimization steps and proceeded to gauge-invariant atomic orbital (GIAO) shielding constant calculations using the Gaussian 09 package (Gaussian Inc.) at the B3LYP/6-31G(d,p) level in the gas phase. The calculated NMR properties of compounds **1** and **4** were Boltzmann-averaged given their respective Boltzmann populations (Tables S1–S3, Supporting Information) and used for calculations of CP3 probability analysis facilitated by the applets available at http://www-jmg.ch.cam.ac.uk/tools/nmr/. The stereo images in Figure 2 were rendered using Pymol 1.6.x (Schrödinger LLC).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00111.

Additional information (PDF)

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Notes

The authors declare no competing financial interest.

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