JOURNAL OF NATURAL PRODUCTS

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Diterpenes from the Trunk of *Abies holophylla* and Their Potential Neuroprotective and Anti-inflammatory Activities

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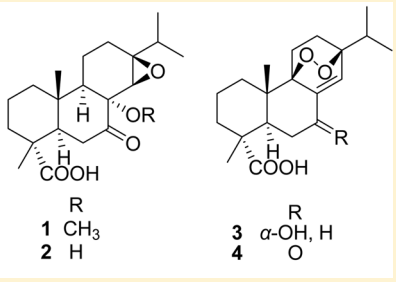
Supporting Information



ABSTRACT: Eleven new abietane-type diterpenes, holophyllins D–N (**1**–**11**), and 17 known analogues (**12**–**28**), were isolated from a MeOH extract of the trunk of *Abies holophylla*. The chemical structures of **1**–**11** were determined through spectroscopic data analysis, including NMR (^{1}H and ^{13}C NMR, DEPT, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) and HRFABMS methods. All isolated compounds (**1**–**28**) were evaluated for their cytotoxicity against four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-116), for their potential neuroprotective effects through induction of nerve growth factor in C6 glioma cells, and for their effects on nitric oxide levels in lipopolysaccharide-stimulated murine microglia BV2 cells.

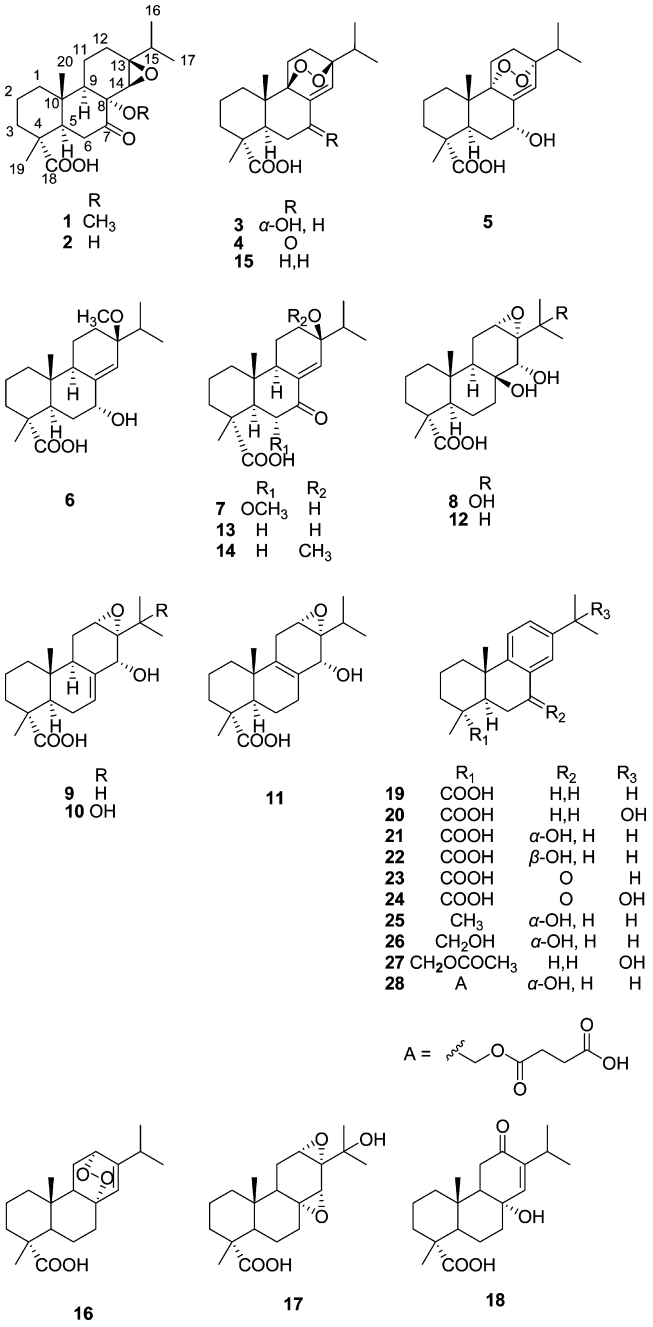
There are approximately 50 species in the genus *Abies* (Pinaceae), and some have been used in Korean traditional medicine for treating vascular and pulmonary diseases, indigestion, rheumatic diseases, and stomachache.[1,2] *A. holophylla* Maxim. is a coniferous tree distributed in the mountainous areas of Korea, mainland China, and Russia. Several bioactive monoterpenes, sesquiterpenes, triterpenes, and phenolic compounds have been isolated from this plant.[2,3] In a continuing search for bioactive constituents from Korean medicinal plants, it was found that the *n*-hexane and $CHCl_3$ layers of an *A. holophylla* MeOH extract were cytotoxic against A549, SK-OV-3, SK-MEL-2, and HCT-116 cells in a sulforhodamine B (SRB) bioassay (11.3–13.8 μg/mL for the *n*-hexane layer, 11.2–>20 μg/mL for the $CHCl_3$ layer) and reduced nitric oxide (NO) levels in lipopolysaccharide (LPS)-stimulated murine microglia BV2 cells. We have previously reported lignans and rearranged diterpenes from the *n*-hexane and EtOAc layers of an *A. holophylla* MeOH extract.[1,4] Herein, 11 further new diterpenes have been isolated, namely, holophyllins D–N (**1**–**11**), and 17 known diterpenes (**12**–**28**) from the active *n*-hexane and $CHCl_3$ layers. All isolated compounds (**1**–**28**) were evaluated for their cytotoxicity, potential neuroprotective activity, and effects on nitric oxide levels. In this paper, the isolation and structural elucidation of compounds **1**–**28** and their biological activities are presented.

RESULTS AND DISCUSSION

Holophyllin D (**1**) was isolated as a colorless gum. The molecular formula was $C_{21}H_{32}O_5$ from the $[M + Na]^+$ ion peak obtained by positive-ion high-resolution fast-atom bombardment mass spectrometry (HRFABMS). The ^{1}H and ^{13}C NMR spectra of **1** were similar to those of 7α,8α,13β,14β-diepoxyabietan-18-oic acid,[5] except for the presence of a carbonyl signal (δ_C 209.1), instead of an epoxide [δ_C 58.7; δ_H 3.19 (1H, brs)] signal at C-7 (Tables 1 and 2). Additionally, a methoxy group signal was observed [δ_C 52.3; δ_H 3.34 (3H, s)], for which the location was confirmed to be at C-8 based on the HMBC cross-peak of OCH_3-8 with C-8 (Figure 1). The planar structure of **1** was elucidated through two-dimensional (2D) NMR analysis, including the DEPT, COSY, HMQC, and HMBC spectra. A *trans*-fused A/B ring junction was corroborated from the NOESY cross-peaks of H-5/H-9 and H-19/H-20 (Figure 2). The relative configurations of the OCH_3-8 and 13-isopropyl groups were both α-oriented, based on the distinct NOESY correlations from OCH_3-8 to H-9 and H-16 (Figure 2). Thus, the structure of **1** was established as 13β,14β-epoxy-8α-methoxy-7-oxoabietan-18-oic acid.

Received: November 26, 2015
Published: January 26, 2016

DOI: 10.1021/acs.jnatprod.5b01053
J. Nat. Prod. 2016, 79, 387–394



Holophyllin E (**2**) was obtained as a colorless gum with the molecular formula $C_{20}H_{30}O_5$. The ^{1}H and ^{13}C NMR spectra of **2** resembled those of **1**, but no methoxy group signal was detected and an upfield-shifted carbon signal was detected at C-8 [**2**, δ_C 74.7; **1**, δ_C 78.9]. The occurrence of a hydroxy group at C-8 was corroborated through the HMBC correlations of H-9, H-11, and H-14 to C-8. The relative configuration of **2** was assumed to be the same as that of **1** based on the observed J values, which were confirmed by the NOESY cross-peaks between H-19/H-20 and H-14/H-16. The relative configuration of OH-8 was assigned as α through the quite similar pattern of the ^{1}H and ^{13}C NMR data to **1** (Tables 1 and 2) and the absence of NOESY correlations of H-9/H-12 and H-14. A full NMR analysis including 2D NMR data was used to confirm the structure of **2** as 13β,14β-epoxy-8α-hydroxy-7-oxoabietan-18-oic acid.

Holophyllin F (**3**) was obtained as a colorless gum with the same molecular formula of $C_{20}H_{30}O_5$ as **2**. Comparison of its ^{1}H and ^{13}C NMR data with those of **15** revealed the presence of hydroxy group signals in **3** [δ_C 65.5; δ_H 4.80 (1H, brt, J = 5.3 Hz, H-7)].[6] The location of the hydroxy group was established at C-7 based on the HMBC correlations from H-7 to C-5, C-6, C-8, C-9, and C-14 (Figure 1). The relative configuration of OH-7 was determined as α from the distinct NOESY cross-peak between H-7/H-20 (Figure 2). The β-configuration of an endoperoxide group was determined by comparing the ^{13}C NMR data of **3** with those reported for similar compounds. The downfield-shifted chemical shifts for C-11 and C-12 (δ_C 24.3 and 27.1, respectively) indicated that **3** possesses a β-oriented endoperoxide [δ_C 23.6−23.7 (C-11) and 25.3−26.6 (C-12)][7,8] rather than an α-oriented endoperoxide [δ_C 17.0−21.8 (C-11) and 24.2−24.7 (C-12)].[7,9,10] Through the modified Mosher ester method using (*R*)- and (*S*)-MTPA-Cl, the absolute configuration of C-7 was confirmed as *R* (Figure 3).[11,12] Thus, the structure of **3** was established as 7α-hydroxyabieta-8(14)-en-18-oic acid 9β,13β-endoperoxide.

Holophyllin G (**4**) was purified as a colorless gum. The molecular formula was found to be $C_{20}H_{28}O_5$ based on the HRFABMS. The presence of an α,β-unsaturated ketone was indicated from the UV maximum at 239 nm. Compounds **3** and **4** gave similar ^{1}H and ^{13}C NMR spectra, but the presence of a carbonyl signal (δ_C 194.7) and the absence of a hydroxy group signal were marked differences. The HMBC correlations from H-5, H-6, and H-14 to C-7 suggested the carbonyl carbon to be located at C-7. The planar structure of **4** was elucidated through 2D NMR analysis, including the ^{1}H−^{1}H COSY, HMQC, and HMBC spectra. The relative configuration of **4** was determined through the NOESY cross-peaks of H-1eq/H-20 and H-3eq/H-19. The β-configuration of an endoperoxide group present was assigned by comparing the carbon signals at C-11 and C-12 (δ_C 23.5 and 26.6, respectively), as described for **3**. Therefore, the structure of **4** was elucidated as 7-oxoabieta-8(14)-en-18-oic acid 9β,13β-endoperoxide.

The molecular formula of compound **5** (holophyllin H) was determined to be the same as that of **3** ($C_{20}H_{30}O_5$) from the positive-ion HRFABMS. Inspection of the ^{1}H and ^{13}C NMR data of **5** suggested that this compound is structurally very similar to **3**, with the major differences being upfield-shifted carbon signals at C-11 and C-12 (δ_C 21.7 and 24.6, **5**; δ_C 24.3 and 27.1, **3**, respectively). NMR analysis of the DEPT, ^{1}H−^{1}H COSY, HMQC, and HMBC data confirmed the planar structures of **5** and **3** to be identical. From the NOESY correlations between H-5/H-1ax and H-3ax, and H-20/H-7 and H-19, a *trans*-fused A/B ring and a OH-7α substituent were corroborated. The configuration of the endoperoxide group present was in the α-form based on the upfield-shifted ^{13}C NMR signals at C-11 and C-12 (δ_C 21.7 and 24.6, respectively).[7−10] Thus, the structure of **5** was assigned as 7α-hydroxyabieta-8(14)-en-18-oic acid 9α,13α-endoperoxide.

Compound **6** (holophyllin I) gave the molecular formula $C_{21}H_{34}O_4$, and the ^{1}H and ^{13}C NMR data showed that its structure is related closely to that of methyl 13β-ethoxy-7α-hydroxyabiet-8(14)-enoate.[13] The major differences were the presence of a methoxy group at C-13 [δ_H 3.18 (3H, s); δ_C 49.4] and a free carboxylic acid at C-18 in **6** instead of a ethoxy group at C-13 [δ_H 3.35 (1H, dq, J = 8.4, 6.9 Hz), 3.28 (1H, dq, J = 8.4, 6.9 Hz), 1.08 (3H, t, J = 6.9 Hz); δ_C 56.5, 16.2] as well as the carboxylic acid methyl ester group at C-18 in methyl 13β-ethoxy-7α-hydroxyabiet-8(14)-enoate. The HMBC cross-peak

DOI: 10.1021/acs.jnatprod.5b01053
J. Nat. Prod. 2016, 79, 387−394

Table 1. ^{1}H NMR [ppm, mult., (J in Hz)] Data of Compounds 1−11 in $CDCl_3$

position	1[a]	2[b]	3[a]	4[b]	5[b]	6[b]	7[a]	8[b]	9[b]	10[b]	11[b]
1ax	1.14, td (12.8, 3.5)	1.15, m	1.67, m	1.77, overlap	1.90, td (12.6, 3.5)	1.21, overlap	1.28, overlap	1.03, td (13.3, 4.2)	1.17, td (13.3, 4.2)	1.18 td (13.3, 4.2)	1.24, overlap
1eq	1.83, brd (12.8)	1.89, overlap		1.82, brd (12.0)	1.46, overlap	1.75, brd (12.7)	1.89, brd (13.1)	1.70, overlap	1.79, brd (13.3)	1.83, overlap	1.78, brd (12.6)
2a	1.58, overlap	1.60, overlap	1.63, m	1.68, m	1.61, m	1.61, overlap	1.67, m	1.58, overlap	1.59, overlap	1.60, overlap	1.67, overlap
2b							1.58, m				
3ax	1.79, overlap	1.80, overlap	1.75, m	1.75, overlap	1.79, m	1.86, td (12.6, 4.2)	1.86, overlap	1.81, td (13.3, 4.2)	1.86, td (14.0, 4.2)	1.82, overlap	1.84, overlap
3eq				1.86, brd (12.9)	1.66, brd (11.6)	1.70, overlap	1.75, overlap	1.67, overlap	1.68, brd (14.0)	1.71, brd (12.6)	1.66, overlap
5	3.02, dd (12.1, 7.2)	2.71, dd (11.2, 7.0)	2.34, overlap	2.55, dd (13.6, 2.4)	2.65, brt (9.1)	2.46, dd (14.0, 2.1)	2.57, d (12.7)	1.72, overlap	1.99, overlap	2.01, overlap	1.99, d (12.6)
6a	2.53, dd (18.3, 7.2)	2.52, overlap	2.18, m	2.57, dd (17.0, 13.6)	ax 2.25, m	1.81, td (14.0, 2.8)	3.58, d (12.7)	1.68, overlap	2.03, overlap	2.05, overlap	1.60, overlap
6b	2.39, dd (18.3, 12.1)	2.51, overlap	1.48, overlap	2.34, dd (17.0, 2.4)	eq 1.47, overlap	1.56, overlap		1.22, overlap	1.83, overlap	1.87, overlap	1.46, dd (12.6, 7.7)
7a			4.80, brt (5.3)		4.73, brt (7.0)	4.34, brt (2.8)		2.21, td (14.0, 4.9)	5.92, brs	5.92, brs	2.48, m
7b								1.41, dt (14.0, 2.8)			2.01, brd (4.9)
9	1.73, overlap	1.69, dd (9.1, 6.3)				2.25, brt (7.7)	2.14, brd (8.6)	1.22, overlap	2.24, m	2.26, m	
11a	1.49, dd (13.2, 3.6)	1.53, m	2.36, overlap	2.43, ddd (13.3, 9.1, 4.2)	2.12, ddd (12.6, 9.1, 3.5)	1.62, overlap	1.77, overlap	eq 2.04, ddd (14.7, 4.9, 2.1)	eq 2.13, overlap	2.17, ddd (15.4, 6.3, 3.5)	eq 2.65, brd (18.2)
11b	1.44, dd (13.2, 3.6)		1.66, overlap	1.72, overlap	1.48, td (12.6, 3.5)		1.52, overlap	ax 1.95, ddd (14.7, 11.9, 2.1)	ax 1.58, overlap	1.60, overlap	ax 2.29, brd (18.2)
12a	1.98, brd (14.2)	1.99, m	2.10, overlap	2.20, ddd (12.6, 9.1, 2.8)	2.02, ddd (12.6, 9.1, 3.5)	eq 1.64, overlap	eq 1.75, overlap	3.82, t (2.1)	3.37, d (2.1)	3.62, s	3.40, d (2.8)
12b	1.76, overlap	1.84, m	1.46, overalp	1.48, td (12.6, 4.2)	1.53, td (12.6, 3.5)	ax 1.33, td (14.0, 4.2)	ax 1.50, overlap				
14	3.21, s	2.90, s	6.61, s	7.56, s	6.57, brs	5.67, brs	6.71, brs	3.43, brs	4.39, s	4.55, s	4.19, s
15	1.69, overlap	1.62, overlap	1.96, sept (7.0)	2.00, sept (7.0)	1.98, sept (7.0)	2.04, sept (7.0)	1.82, overlap		2.11, sept (7.0)		2.15, sept (7.0)
16	1.04, d (6.8)	1.03, d (7.0)	1.03, d (7.0)	1.05, d (7.0)	1.02, d (7.0)	0.92, d (7.0)	0.98, d (7.0)	1.35, s	1.08, d (7.0)	1.43, s	1.11, d (7.0)
17	1.01, d (7.1)	1.00, d (7.0)	1.03, d (7.0)	1.05, d (7.0)	1.02, d (7.0)	0.86, d (7.0)	0.89, d (6.8)	1.30, s	0.91, d (7.0)	1.24, s	0.94, d (7.0)
19	1.32, s	1.31, s	1.36, s	1.39, s	1.33, s	1.24, s	1.35, s	1.25, s	1.27, s	1.29, s	1.22, s
20	0.81, s	0.96, s	1.11, s	1.17, s	1.05, s	0.82, s	0.99, s	1.09, s	0.83, s	0.85, s	1.06, s
OCH_3	3.34, s					3.18, s	3.61, s				

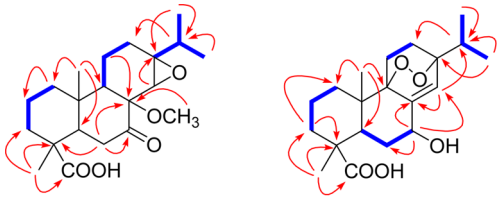
[a]Recorded at 500 MHz. [b]Recorded at 700 MHz.

DOI: 10.1021/acs.jnatprod.5b01053
J. Nat. Prod. 2016, 79, 387−394

Table 2. ^{13}C NMR (ppm, mult) Data of Compounds 1−11 in $CDCl_3$

carbon	1[a]	2[b]	3[a]	4[b]	5[b]	6[b]	7[a]	8[b]	9[b]	10[b]	11[b]
1	40.3, t	40.2, t	33.5, t	32.7, t	30.8, t	37.8, t	38.7, t	38.6, t	38.2, t	38.5, t	36.2, t
2	17.9, t	17.7, t	17.4, t	17.2, t	17.4, t	18.0, t	17.7, t	17.4, t	17.9, t	17.8, t	18.3, t
3	37.0, t	36.5, t	36.9, t	37.1, t	36.3, t	36.9, t	37.5, t	36.3, t	36.5, t	36.6, t	36.0, t
4	45.5, s	45.9, s	46.7, s	45.8, s	46.3, s	46.6, s	44.3, s	47.0, s	46.0, s	45.9, s	47.1, s
5	40.8, d	42.9, d	37.8, d	37.2, d	38.4, d	41.9, d	50.2, d	49.4, d	44.7, d	44.5, d	45.5, d
6	37.7, t	37.1, t	31.5, t	37.4, t	32.3, t	31.7, t	82.0, d	20.1, t	25.3, t	25.2, t	20.9, t
7	209.1, s	214.0, s	65.5, d	194.7, s	65.0, d	72.9, d	200.3, s	35.4, t	126.8, d	127.7, d	28.0, t
8	78.9, s	74.7, s	147.4, s	141.5, s	147.5, s	145.4, s	139.1, s	72.5, s	137.1, s	136.6, s	126.5, s
9	57.0, d	57.7, d	81.6, s	81.8, s	80.8, s	47.0, d	51.4, d	41.4, d	43.6, d	42.7, d	135.3, s
10	36.4, s	37.3, s	38.8, s	38.4, s	39.1, s	38.1, s	35.7, s	35.7, s	34.2, s	34.2, s	36.9, s
11	16.3, t	16.2, t	24.3, t	23.5, t	21.7, t	16.7, t	18.2, t	20.5, t	22.2, t	22.3, t	23.4, t
12	22.6, t	21.8, t	27.1, t	26.6, t	24.6, t	26.5, t	29.4, t	60.4, d	59.0, d	58.7, d	58.0, d
13	62.1, s	62.3, s	79.9, s	80.2, s	79.4, s	75.4, s	71.8, s	66.3, s	65.3, s	65.4, s	65.5, s
14	56.4, d	59.9, d	130.3, d	141.1, d	126.2, d	129.9, d	140.3, d	71.2, d	69.3, d	69.0, d	69.2, d
15	33.8, d	34.0, d	32.2, d	32.1, d	32.2, d	32.2, d	37.9, d	70.0, s	30.5, d	71.0, s	30.6, d
16	17.8, q	17.4, q	17.2, q	17.3, q	17.1, q	16.1, q	16.2, q	25.6, q	18.9, q	27.1, q	19.3, q
17	18.2, q	18.1, q	17.5, q	17.1, q	17.1, q	17.7, q	17.3, q	24.7, q	17.0, q	25.0, q	17.1, q
18	182.6, s	181.1, s	182.2, s	181.4, s	182.2, s	182.2, s	181.8, s	181.2, s	182.4, s	182.2, s	182.5, s
19	17.4, q	17.0, q	17.7, q	18.0, q	17.4, q	16.8, q	16.6, q	16.8, q	16.9, q	17.2, q	16.5, q
20	16.3, q	16.5, q	18.0, q	17.1, q	19.6, q	13.9, q	15.9, q	15.7, q	15.0, q	15.3, q	20.5, q
OCH_3	52.3, q					49.4, q	60.9, q				

[a]Recorded at 125 MHz. [b]Recorded at 175 MHz.

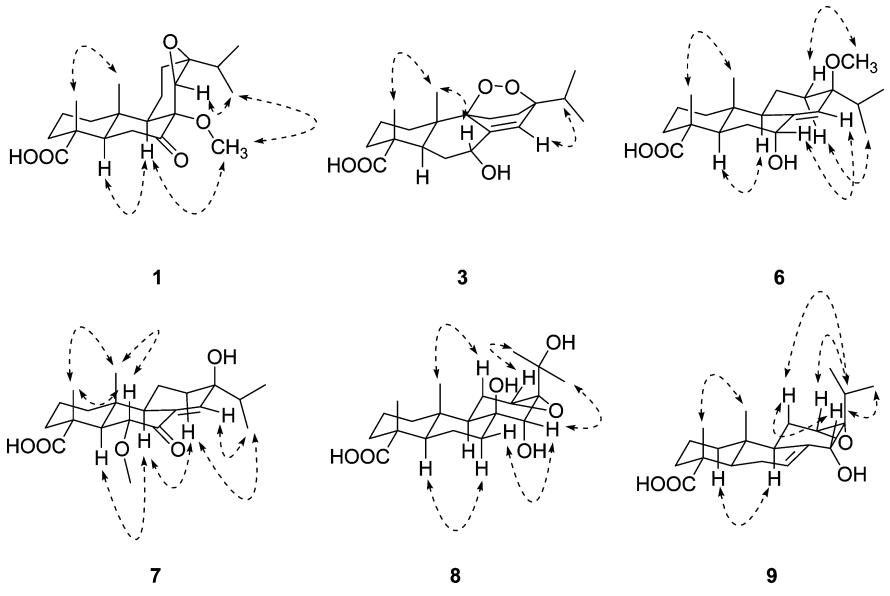


1 3

Figure 1. ^{1}H−^{1}H COSY (bold) and HMBC (arrows) correlations of 1 and 3.

between OCH_3-13 and C-13 corroborated the location of the methoxy group at C-13. The relative configuration of 6 was confirmed through NOESY correlations of H-5/H-9, H-7/H-14, H-12ax/H-17, H-12eq/OCH_3, and H-19/H-20 (Figure 2). The absolute configuration of C-7 was determined as *R* by the modified Mosher ester method. Thus, 6 was established as 7*α*-hydroxy-13*β*-methoxyabiet-8(14)-en-18-oic acid.

Holophyllin J (7) was obtained as a colorless gum with the molecular formula $C_{21}H_{32}O_5$. Its ^{1}H and ^{13}C NMR spectra resembled those of 7-oxo-13*β*-hydroxyabiet-8(14)-en-18-oic acid,[14] but there were signals for an additional methoxy group [δ_H 3.61 (3H, s); δ_C 60.9] at C-6 based on the HMBC correlation of OCH_3-6/C-6. The NOESY cross-peaks between H-6/H-19 and H-20, H-12ax/H-9 and H-17, and H-14/H-17 suggested that the OCH_3-6 and OH-13 substituents are in the *α*- and *β*-forms, respectively. Thus, the structure of 7 was



1 3 6

7 8 9

Figure 2. Key NOESY correlations of 1, 3, and 6−9.

DOI: 10.1021/acs.jnatprod.5b01053
J. Nat. Prod. 2016, 79, 387−394

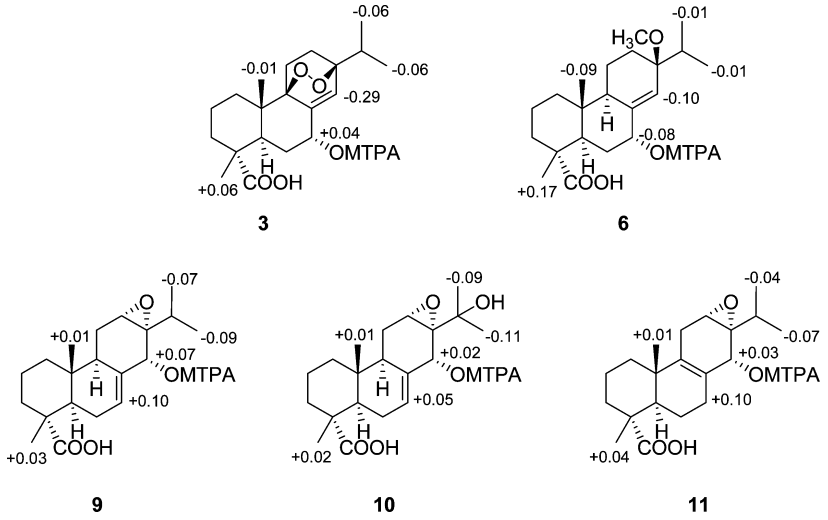


Figure 3. $\Delta\delta$ values ($\delta_S - \delta_R$) in ppm of the two MTPA esters derived from **3**, **6**, **9**, **10**, and **11**.

Table 3. Effects of Selected Compounds on NGF Secretion in C6 Cells

compound	NGF secretion (%)[a]	cell viability (%)[b]	compound	NGF secretion (%)[a]	cell viability(%)[b]
2	155.0 ± 0.6	91.4 ± 2.9	**19**	170.2 ± 0.8	92.3 ± 3.6
5	154.3 ± 0.8	126.7 ± 2.8	**20**	152.9 ± 7.0	94.3 ± 4.8
6	169.4 ± 2.4	78.1 ± 6.5	**21**	204.3 ± 2.2	101.4 ± 9.2
7	170.2 ± 4.9	96.2 ± 2.1	**22**	165.1 ± 2.3	96.2 ± 7.2
9	193.9 ± 3.0	73.1 ± 0.6	**23**	201.8 ± 1.3	103.8 ± 3.7
10	158.6 ± 2.3	92.5 ± 1.1	**24**	161.7 ± 1.2	94.6 ± 5.3
13	175.6 ± 0.7	94.5 ± 8.7	**26**	177.1 ± 0.9	89.3 ± 6.0
15	175.9 ± 1.3	109.3 ± 0.6	**27**	171.3 ± 5.8	85.5 ± 10.1
16	153.0 ± 0.1	101.5 ± 3.0	**28**	142.2 ± 3.3	98.2 ± 8.8
18	166.1 ± 2.3	91.2 ± 5.8	6-shogaol[c]	141.8 ± 9.4	118.6 ± 3.1

[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%). Data are means ± SD of three independent experiments performed in triplicate. [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 means of three independent experiments, and the data are expressed as means ± SD. [c]Positive control substance.

elucidated as 13β-hydroxy-6α-methoxy-7-oxoabiet-8(14)-en-18-oic acid.

The HRFABMS data {*m*/*z* 391.2097 [M + Na]$^+$ (calcd for $C_{20}H_{32}O_6Na$, 391.2097)} of **8** (holophyllin K) indicated that this molecule possesses the molecular formula $C_{20}H_{32}O_6$. The ^{1}H and ^{13}C NMR spectra of **8** were similar to those of **12**,[6] except for the presence of an oxygenated carbon signal (δ_C 70.0), instead of a methine signal [δ_C 34.0; δ_H 1.49 (1H, m)]. The oxygenation location was deduced as C-15 based on the two singlet peaks observed for H-16 and H-17 and the HMBC correlations from H-16 and H-17 to C-15. The OH-8 and OH-14 configurations were established as β- and α-oriented, respectively, based on the NOESY cross-peak of H-7eq/H-14 (Figure 2). In addition, distinct NOESY cross-peaks between H-12/H-16 and H-14/H-17 suggested that the 12,13-epoxy group configuration is in the α-form (Figure 2). Therefore, the structure of **8** was established as 12α,13α-epoxy-8β,14α,15-trihydroxyabieta-18-oic acid.

Holophyllin L (**9**) was isolated as a colorless gum, and the molecular formula was $C_{20}H_{30}O_4$ based on HRFABMS. The ^{1}H and ^{13}C NMR data of **9** indicated that the structure of this compound is similar to that of **12**. A difference found was that double-bond signals [δ_C 137.1, 126.8; δ_H 5.92 (1H, brs)] were observed in **9**, instead of an oxygenated quaternary carbon signal [δ_C 71.8] in **12**. The HMBC correlations from H-7 to C-5, C-6, C-9, and C-14 indicated that this double bond could be located at C-7 and C-8. The α-configurations of the 12,13-epoxy and OH-14 groups were assigned through the NOESY correlations of H-11ax/H-14 and H-15, H-12/H-15, and H-14/H-16. The absolute configuration of C-14 was elucidated as *R* using the same method as described for **3**. Thus, compound **9** was elucidated as 12α,13α-epoxy-14α-hydroxyabiet-7-en-18-oic acid.

The ^{1}H and ^{13}C NMR data of **10** (holophyllin M) were very similar to those of **9**, except for the presence of an additional hydroxy group (δ_C 71.0). The hydroxy group location was C-15 based on the HMBC cross-peaks from H-12, H-14, H-16, and H-17 to C-15. Full assignments of the ^{1}H and ^{13}C NMR spectra were carried out by analyzing the DEPT, ^{1}H−^{1}H COSY, HMQC, and HMBC data. The relative configuration of **10** was assumed to be the same as that of **9** based on the observed *J* values and by the NOESY cross-peaks of H-14/H-7, H-16, and H-20. The *R* configuration of C-14 was determined by the modified Mosher ester method. Therefore, **10** was established as 12α,13α-epoxy-14α,15-dihydroxyabiet-7-en-18-oic acid.

Compound **11** (holophyllin N) showed ^{1}H and ^{13}C NMR data similar to those of **9**, except for the absence of an olefinic proton [δ_H 5.92 (1H, brs)] in **9**, indicating a shift of the double bond to C-8/C-9 in **11**. The cross-peaks from H-5, H-7, H-11, H-14, and H-20 to C-9 in the HMBC spectrum corroborated

DOI: 10.1021/acs.jnatprod.5b01053
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that the double bond is located between C-8 and C-9. The α-configurations of the OH-14 and 12,13-epoxy groups were confirmed through distinct NOESY correlations of H-7eq/H-14 and H-15/H-12 and H-14, respectively. The absolute configuration of C-14 was assigned as *R* by the modified Mosher ester method. A full NMR analysis was used to elucidate the structure of **11** as 12α,13α-epoxy-14α-hydroxyabiet-8(9)-en-18-oic acid.

The 17 known compounds isolated were identified as abiesadine C (**12**),[6] 7-oxo-13β-hydroxyabiet-8(14)-en-18-oic acid (**13**), 7-oxo-13β-methoxyabiet-8(14)-en-18-oic acid (**14**),[15] 9,13β-epidioxy-8(14)-abieten-18-oic acid (**15**),[6] levopimaric acid endoperoxide (**16**),[16] 15-hydroxy-8α,14α,12α,13α-diepoxyabietan-18-oic acid (**17**),[5] 12-oxo-8-hydroxyabiet-13(14)-enoic acid (**18**),[17] dehydroabietic acid (**19**),[18] 15-hydroxydehydroabietic acid (**20**),[6] 7α-hydroxydehydroabietic acid (**21**), 7β-hydroxydehydroabietic acid (**22**),[18] 7-oxo-dehydroabietic acid (**23**),[16] 15-hydroxy-7-oxo-8,11,13-abietatrien-18-oic acid (**24**),[19] (−)-8,11,13-abietatrien-7α-ol (**25**),[20] 7α,18-dihydroxydehydroabietanol (**26**),[21] 8,11,13-abietatriene-15,18-diol 18-acetate (**27**),[18] and abiesadine F (**28**),[6] by comparison with NMR and MS data in the literature.

Many diterpenes are associated with their neuroprotective activity measured by secretion of nerve growth factor (NGF) from C6 glioma cells.[22−24] Therefore, compounds **1**−**28** were tested for their neuroprotective effects using an enzyme-linked immunosorbent assay (ELISA) development kit that measured NGF release from C6 glioma cells into the medium. Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Table 3, compounds **9**, **21**, and **23** induced NGF secretion most significantly by 193.9 ± 3.0%, 204.3 ± 2.2%, and 201.8 ± 1.3%, respectively, without producing a cytotoxic effect at 20 μM, and displayed more potent effects than a positive control, 6-shogaol (141.8 ± 9.4%).[25,26]

The effects of the isolated compounds (**1**−**28**) on NO levels in LPS-stimulated murine microglia BV2 cells were evaluated. Compounds **4**, **15**, and **18** exhibited moderate activities, with IC_{50} values of 30.1 ± 6.5, 57.3 ± 0.2, and 56.1 ± 3.3 μM, respectively, without significant cell toxicity at 20 μM. The other compounds showed weak or no activity. L-NMMA was used as the positive control (IC_{50} 24.8 ± 4.6 μM).

The cytotoxic activities of compounds **1**−**28** were evaluated against the A549, SK-OV-3, SK-MEL-2, and HCT-116 cell lines using the SRB bioassay,[27] but they were inactive (IC_{50} > 10 μM).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter (JASCO, Easton, MD, USA). Infrared (IR) spectra were recorded on a Bruker IFS-66/S Fourier-transform IR spectrometer (Bruker, Karlsruhe, Germany). Ultraviolet (UV) spectra were recorded with a Shimadzu UV-1601 UV−visible spectrophotometer (Shimadzu, Tokyo, Japan). NMR spectra were recorded on a Varian Unity INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C) and a Bruker AVANCE III 700 NMR spectrometer at 700 MHz (^{1}H) and 175 MHz (^{13}C). HRFABMS were measured on either a Waters SYNAPT G2 (Milford, MA, USA) or a JEOL JMS700 mass spectrometer (Tokyo, Japan). The semi-preparative high-performance liquid chromatography (HPLC) system used had a Gilson 306 pump (Middleton, WI, USA) with a Shodex refractive index detector (New York, NY, USA). Column chromatography was performed with silica gel 60 (70−230 and 230−400 mesh; Merck, Darmstadt, Germany) and RP-C_{18} silica gel (Merck, 230−400 mesh). Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates (Merck) were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying the samples with anisaldehyde−sulfuric acid.

Plant Material. The trunk of *Abies holophylla* was collected in Seoul, Korea, in January 2012. A voucher specimen (SKKU-NPL 1205) was authenticated by K.R.L. and deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. The *A. holophylla* (5.0 kg) trunk was extracted with 80% aqueous MeOH under reflux and filtered. The filtrate was evaporated under reduced pressure to obtain the MeOH extract (280 g), which was suspended in distilled H_2O and successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-butanol, yielding 23, 43, 17, and 35 g residues, respectively. The *n*-hexane-soluble fraction (23 g) was separated over a silica gel column ($CHCl_3$−MeOH, 70:1 → 1:1) to yield nine fractions (H1−H9). Fraction H7 (0.8 g) was chromatographed on an RP-C_{18} silica gel column with 85% aqueous MeOH to give 10 subfractions (H71−H710). Fractions H72 (50 mg) and H74 (70 mg) were purified by semipreparative HPLC (2 mL/min, 40% aqueous CH_3CN) to yield compounds **10** (2 mg) and **9** (5 mg), respectively. Compounds **8** (6 mg), **11** (6 mg), **20** (4 mg), and **28** (3 mg) were obtained by purifying fractions H76 (20 mg), H77 (50 mg), H78 (25 mg), and H710 (100 mg) using preparative HPLC (55−60% aqueous CH_3CN). Fraction H8 (1.0 g) was separated over an RP-C_{18} silica gel column with 85% aqueous MeOH to give nine subfractions (H81−H89). Fractions H82 (90 mg), H83 (80 mg), H84 (50 mg), H85 (70 mg), H86 (40 mg), and H87 (100 mg) were purified by semipreparative HPLC (2 mL/min, 45−65% aqueous CH_3CN) to yield compounds **2** (3 mg), **4** (4 mg), **5** (8 mg), **6** (4 mg), **17** (3 mg), **18** (5 mg), **21** (13 mg), **24** (5 mg), and **27** (3 mg). The $CHCl_3$-soluble fraction (20 g) was chromatographed on a silica gel column ($CHCl_3$−MeOH, 50:1 → 1:1). Ten crude fractions (C1−C10) were collected after a TLC analysis. Fraction C2 (8.2 g) was separated on RP-C_{18} silica gel, eluted with a gradient solvent system of 90 → 100% aqueous MeOH, to give 10 subfractions (C21−C210). Fractions C21 (50 mg), C22 (80 mg) C23 (320 mg), C24 (600 mg), C25 (400 mg), and C27 (800 mg) were subjected to repeated column chromatography (including a silica gel column and a Lobar-A Si 60 column) and further purified by semipreparative HPLC (50−90% aqueous CH_3CN) to yield compounds **1** (4 mg), **3** (3 mg), **7** (5 mg), **14** (4 mg), **15** (3 mg), **16** (7 mg), **19** (24 mg), **23** (12 mg), **25** (8 mg), and **26** (2 mg). Fraction C3 (3.2 g) was separated on an RP-C_{18} silica gel column (65 → 100% aqueous MeOH) and further purified by semipreparative HPLC (50−55% aqueous CH_3CN) to yield compounds **12** (3 mg) and **13** (7 mg).

*Holophyllin D (**1**):* colorless gum; $[\alpha]_D^{25}$ −46.0 (*c* 0.20, MeOH); IR (KBr) ν_{max} 3384, 2950, 2843, 1698, 1650, 1457, 1032 cm^{-1}; ^{1}H NMR (500 MHz) data, see Table 1; ^{13}C NMR (125 MHz) data, see Table 2; HRFABMS (positive-ion mode) *m*/*z* 387.2145 $[M + Na]^+$ (calcd for $C_{21}H_{32}O_5Na$, 387.2147).

*Holophyllin E (**2**):* colorless gum; $[\alpha]_D^{25}$ −38.0 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3383, 2951, 2844, 1699, 1650, 1457, 1033 cm^{-1}; ^{1}H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (positive-ion mode) *m*/*z* 351.2171 $[M + H]^+$ (calcd for $C_{20}H_{31}O_5$, 351.2171).

*Holophyllin F (**3**):* colorless gum; $[\alpha]_D^{25}$ +2.0 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3383, 2961, 2779, 1721, 1615, 1408, 1029 cm^{-1}; ^{1}H NMR (500 MHz) data, see Table 1; ^{13}C NMR (125 MHz) data, see Table 2; HRFABMS (positive-ion mode) *m*/*z* 351.2170 $[M + H]^+$ (calcd for $C_{20}H_{31}O_5$, 351.2171).

*Holophyllin G (**4**):* colorless gum; $[\alpha]_D^{25}$ −33.5 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 239 (4.34) nm; IR (KBr) ν_{max} 3443, 2942, 2853, 1701, 1613, 1405, 1079 cm^{-1}; ^{1}H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (positive-ion mode) *m*/*z* 349.2015 $[M + H]^+$ (calcd for $C_{20}H_{29}O_5$, 349.2015).

*Holophyllin H (**5**):* colorless gum; $[\alpha]_D^{25}$ −23.5 (*c* 0.08, MeOH); IR (KBr) ν_{max} 3384, 2960, 2780, 1723, 1605, 1412, 1030 cm^{-1}; ^{1}H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2;

DOI: 10.1021/acs.jnatprod.5b01053
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HRFABMS (positive-ion mode) m/z 373.1991 $[M + Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1991).

Holophyllin I (6): colorless gum; $[\alpha]_D^{25}$ −36.0 (c 0.20, MeOH); IR (KBr) ν_{max} 3379, 2958, 2788, 1721, 1611, 1410, 1033 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (positive-ion mode) m/z 373.2355 $[M + Na]^+$ (calcd for $C_{21}H_{34}O_4Na$, 373.2355).

Holophyllin J (7): colorless gum; $[\alpha]_D^{25}$ +16.0 (c 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 240 (4.81) nm; IR (KBr) ν_{max} 3455, 2943, 2879, 1700, 1611, 1411, 1088 cm^{-1}; ^1H NMR (500 MHz) data, see Table 1; ^{13}C NMR (125 MHz) data, see Table 2; HRFABMS (positive-ion mode) m/z 387.2149 $[M + Na]^+$ (calcd for $C_{21}H_{32}O_5Na$, 387.2147).

Holophyllin K (8): colorless gum; $[\alpha]_D^{25}$ +3.6 (c 0.30, MeOH); IR (KBr) ν_{max} 3485, 2964, 2921, 1703, 1470, 1047 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (positive-ion mode) m/z 391.2097 $[M + Na]^+$ (calcd for $C_{20}H_{32}O_6Na$, 391.2097).

Holophyllin L (9): colorless gum; $[\alpha]_D^{25}$ −17.2 (c 0.25, MeOH); IR (KBr) ν_{max} 3380, 2966, 2777, 1719, 1615, 1405, 1032 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (positive-ion mode) m/z 357.2042 $[M + Na]^+$ (calcd for $C_{20}H_{30}O_4Na$, 357.2042).

Holophyllin M (10): colorless gum; $[\alpha]_D^{25}$ +1.9 (c 0.20, MeOH); IR (KBr) ν_{max} 3379, 2957, 2785, 1725, 1611, 1408, 1031 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (positive-ion mode) m/z 373.1991 $[M + Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1991).

Holophyllin N (11): colorless gum; $[\alpha]_D^{25}$ +4.7 (c 0.08, MeOH); IR (KBr) ν_{max} 3381, 2962, 2788, 1720, 1620, 1401, 1036 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (positive-ion mode) m/z 335.2222 $[M + H]^+$ (calcd for $C_{20}H_{31}O_4$, 335.2222).

Preparation of Mosher Ester Derivatives 3r, 6r, 9r−11r, 3s, 6s, and 9s−11s. Compound **3** (0.5 mg) in deuterated pyridine (0.6 mL) was transferred into a clean NMR tube. (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (10 μL) (Sigma, St. Louis, MO, USA) was added immediately into the NMR tube under a N_2 gas stream, and then the NMR tube was shaken carefully to mix the sample and MTPA chloride evenly. The NMR reaction tube was left at room temperature overnight. The reaction was then completed to afford the (*R*)-MTPA ester derivative (**3r**) of **3**. The (*S*)-MTPA ester derivative of **3** (**3s**) was obtained as described for **3r**. The ^1H NMR spectra of **3r** and **3s** were measured directly in the NMR reaction tubes. Similarly, treatment of **6** and **9**−**11** with (*S*)- and (*R*)-MTPA-Cl afforded the respective Mosher esters **6r**, **9r**−**11r**, **6s**, and **9s**−**11s**.

***3*-(*R*)-MTPA ester (3r):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.87 (1H, s, H-14), 6.39 (1H, brs, H-7), 1.27 (3H, s, H-19), 1.13 (3H, s, H-20), 1.01 (3H, s, H-16), 0.96 (3H, s, H-17); positive FABMS m/z 566.2 $[M + Na]^+$.

***3*-(*S*)-MTPA ester (3s):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.58 (1H, s, H-14), 6.43 (1H, brs, H-7), 1.33 (3H, s, H-19), 1.12 (3H, s, H-20), 0.95 (3H, s, H-16), 0.90 (3H, s, H-17); positive FABMS m/z 566.2 $[M + Na]^+$.

***6*-(*R*)-MTPA ester (6r):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.59 (1H, brs, H-7), 6.55 (1H, s, H-14), 1.33 (3H, s, H-19), 1.32 (3H, s, H-20), 1.18 (6H, d, J = 6.9 Hz, H-16 and H-17); positive FABMS m/z 566.3 $[M + Na]^+$.

***6*-(*S*)-MTPA ester (6s):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.51 (1H, brs, H-7), 6.45 (1H, s, H-14), 1.50 (3H, s, H-19), 1.23 (3H, s, H-20), 1.17 (6H, d, J = 6.9 Hz, H-16 and H-17); positive FABMS m/z 566.3 $[M + Na]^+$.

***9*-(*R*)-MTPA ester (9r):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.42 (1H, s, H-14), 5.98 (1H, brs, H-7), 1.20 (3H, s, H-19), 1.08 (3H, s, H-16), 0.89 (3H, s, H-17), 0.66 (3H, s, H-20); positive FABMS m/z 550.3 $[M + Na]^+$.

***9*-(*S*)-MTPA ester (9s):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.49 (1H, s, H-14), 6.08 (1H, brs, H-7), 1.23 (3H, s, H-19), 1.01 (3H, s, H-16), 0.80 (3H, s, H-17), 0.67 (3H, s, H-20); positive FABMS m/z 550.3 $[M + Na]^+$.

***10*-(*R*)-MTPA ester (10r):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.80 (1H, s, H-14), 6.25 (1H, brs, H-7), 1.19 (3H, s, H-19), 1.52 (3H, s, H-17), 1.50 (3H, s, H-16), 0.67 (3H, s, H-20); positive FABMS m/z 566.2 $[M + Na]^+$.

***10*-(*S*)-MTPA ester (10s):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.82 (1H, s, H-14), 6.30 (1H, brs, H-7), 1.21 (3H, s, H-19), 1.41 (6H, s, H-16 and H-17), 0.68 (3H, s, H-20); positive FABMS m/z 566.2 $[M + Na]^+$.

***11*-(*R*)-MTPA ester (11r):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.37 (1H, s, H-14), 1.13 (3H, s, H-19), 1.10 (3H, s, H-16), 0.85 (3H, s, H-17), 0.90 (3H, s, H-20); positive FABMS m/z 550.3 $[M + Na]^+$.

***11*-(*S*)-MTPA ester (11s):** ^1H NMR (pyridine-d_5, 700 MHz) δ_H 6.40 (1H, s, H-14), 1.17 (3H, s, H-19), 1.06 (3H, s, H-16), 0.78 (3H, s, H-17), 0.91 (3H, s, H-20); positive FABMS m/z 550.3 $[M + Na]^+$.

Cytotoxicity Assessment. The cytotoxicity of the compounds against cultured human tumor cell lines such as A549 (non-small-cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-116 (colon adenocarcinoma) was evaluated by the SRB method as described in our previous paper.[4] Etoposide (≥98%; Sigma Chemical Co., St. Louis, MO, USA) was used as the positive control. This compound exhibited IC_{50} values of 1.4 ± 0.22, 2.6 ± 0.31, 1.5 ± 0.15, and 1.0 ± 0.09 μM against the A549, SK-OV-3, SK-MEL-2, and HCT-116 cell lines, respectively.

NGF and Cell Viability Assays. In this study, C6 glioma cells were used to measure the release of NGF into the culture medium. C6 cells were seeded onto 24-well plates at a density of 1×10^5 cells/well, and, after 24 h, the cells were treated with serum-free DMEM and different concentrations of compound for an additional 24 h. The medium supernatant was collected from cultured plates, and the NGF level was measured using an ELISA development kit. Cell viability was measured using an MTT assay, as described previously.[28] The results are expressed as a percentage of the control group (untreated cells). 6-Shogaol was used as the positive control.[29]

Measurement of NO Production and Cell Viability in LPS-Activated BV-2 Cells. The inhibitory effect of the test compounds on LPS-stimulated NO production was studied using BV2 cells. BV2 cells were seeded on a 96-well plate (4×10^4 cells/well) and treated with or without different concentrations of the compounds. These cells were stimulated with LPS (100 ng/mL) and incubated for 24 h. The concentration of nitrite (NO_2), a soluble oxidation product of NO, in the culture medium was measured using Gries reagent (0.1% *N*-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). Fifty microliters of supernatant was mixed with an equal volume of the Gries reagent. Absorbance was measured after 10 min using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA) at 570 nm wavelength. L-NMMA, a well-known nitric oxide synthase (NOS) inhibitor,[28] was used as a positive control. Graded sodium nitrite solution was used as a standard to calculate nitrite concentrations. Cell viability was evaluated by observing the ability of viable cells to reduce the yellow-colored MTT to a purple-colored formazan, using an MTT assay.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b01053.

HRMS and NMR data of **1**−**11** (PDF)

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Notes

The authors declare no competing financial interest.

DOI: 10.1021/acs.jnatprod.5b01053
J. Nat. Prod. 2016, 79, 387−394

■ ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2013R1A1A2A10005315). We are thankful to the Korea Basic Science Institute (KBSI) for the measurements on the mass spectra.

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DOI: 10.1021/acs.jnatprod.5b01053
J. Nat. Prod. 2016, 79, 387−394