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Iridoid Glycosides from the Twigs of *Sambucus williamsii* var. *coreana* and Their Biological Activities

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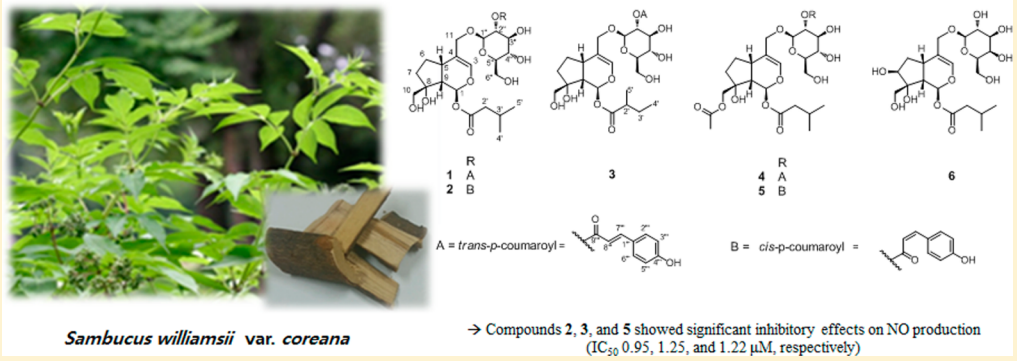
Supporting Information



ABSTRACT: Six new iridoid glycosides, sambucusides A–F (**1**–**6**), and two known derivatives (**7** and **8**) were isolated from a methanol extract of the twigs of *Sambucus williamsii* var. *coreana*. Their chemical structures were elucidated by spectroscopic methods, including NMR (^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY) and HRMS. All isolated compounds (**1**–**8**) were evaluated for their antiproliferative activities against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, and Bt549). Their effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV-2 cells and their neuroprotective effects through induction of nerve growth factor (NGF) in C6 glioma cells were also examined. Compounds **2**, **3**, and **5** showed cytotoxic effects (IC_{50} 1.3–8.7 μM) against the SK-MEL-2 and Bt549 cell lines and inhibitory effects on NO production (IC_{50} of 0.9, 1.3, and 1.2 μM, respectively). Compounds **2**, **4**, and **8** exhibited NGF-releasing effects (147.0 ± 5.8%, 158.7 ± 5.2%, and 152.6 ± 7.3%, respectively).

Sambucus williamsii var. *coreana* Nakai (Caprifoliaceae) is a deciduous shrub, widely distributed throughout Korea and mainland China. The stems and roots of this species have been used in Korean folk medicine for treating bone fractures, rheumatism, neuralgia, and osteoporosis.[1−3] Previous phytochemical investigations have led to the isolation of terpenes, fatty acids, phenolic compounds, iridoid glycosides, and lignans from this source.[3−8] Recently, it has been reported that an extract of *S. williamsii* was found to increase bone mass and bone strength in ovariectomized rats.[9] In addition, some lignans isolated from *S. williamsii* can inhibit the proliferation of osteoblast-like UMR106 cells.[3] However, few studies have been conducted to isolate potential anticancer, anti-inflammatory, and neuroprotective components from *S. williamsii* var. *coreana*. As part of a search for bioactive constituents from Korean medicinal plants,[10−12] the $CHCl_3$ and EtOAc layers of a *S. williamsii* var. *coreana* MeOH twig extract were studied based on the cytotoxic activity against A549, SK-OV-3, SK-MEL-2, and Bt549 cells in a sulforhodamine B (SRB) bioassay. They also decreased nitric oxide (NO) levels in lipopolysaccharide (LPS)-activated BV2 murine microglia cells. Some lignan derivatives have been isolated from this plant, and their biological activities have been reported previously.[8] Herein, the isolation of six new iridoid glycosides, sambucusides A–F (**1**–**6**), and two known derivatives (**7** and **8**) and their cytotoxic, anti-inflammatory, and neuroprotective activities are reported.

RESULTS AND DISCUSSION

The 80% MeOH extract from the twigs of *S. williamsii* var. *coreana* was fractionated to yield *n*-hexane-, $CHCl_3$-, EtOAc-, and *n*-BuOH-soluble fractions, and then each fraction was evaluated for inhibition of NO production in LPS-activated BV-2 cells and a nerve growth factor (NGF) release effect in C6 glioma cells. Among them, the EtOAc-soluble fraction showed moderate NO production inhibitory (IC_{50} values 72.7 μg/mL) and significant NGF release activities (196.1 ± 8.0%). Thus, the most active EtOAc-soluble fraction was investigated and

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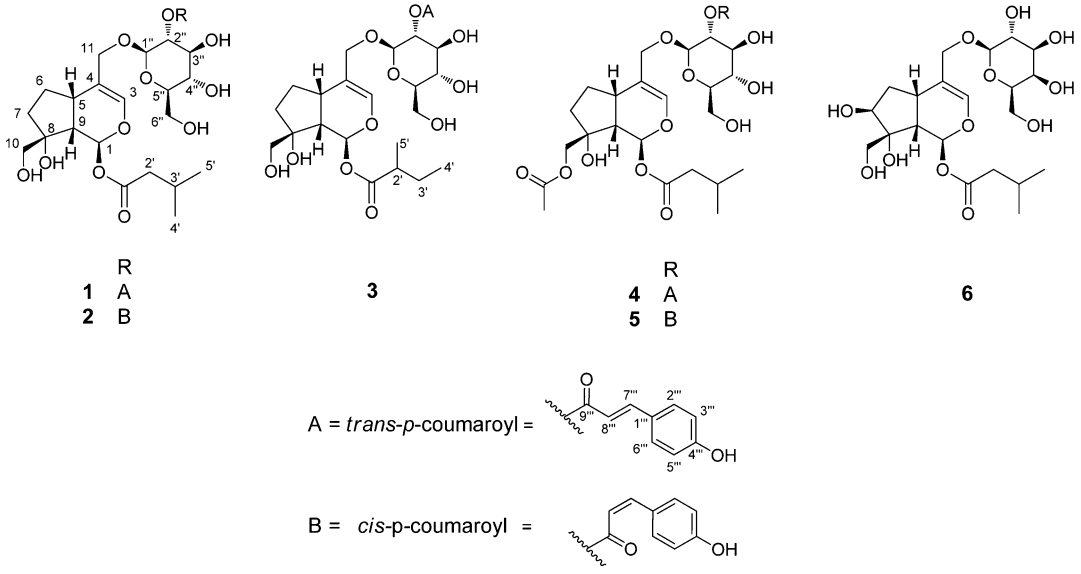
Chart 1



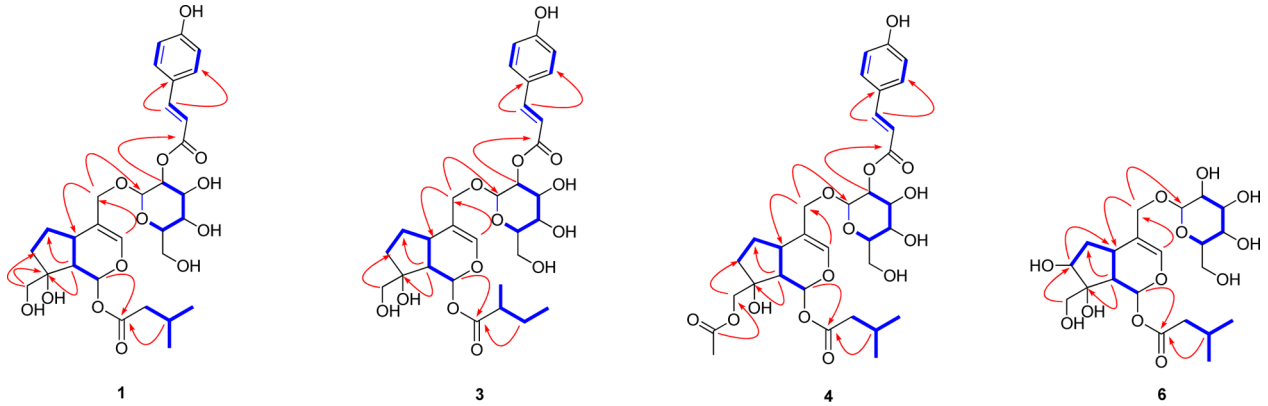


Figure 1. $^{1}H-^{1}H$ COSY (bold) and HMBC (arrow) correlations of **1**, **3**, **4**, and **6**.

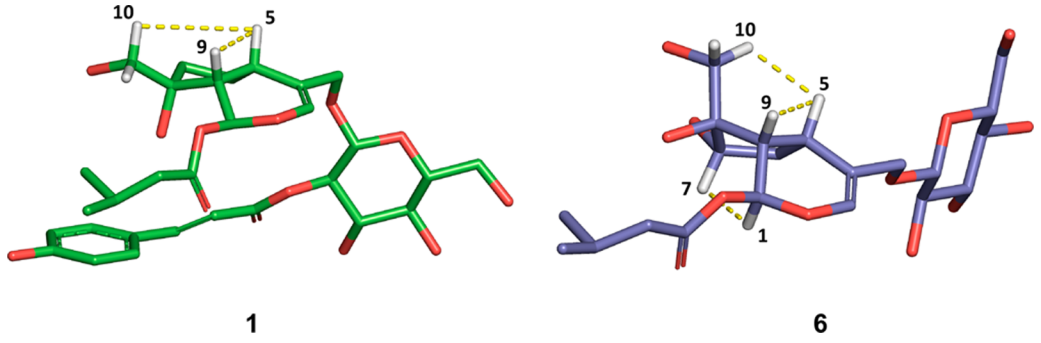


Figure 2. Key NOESY correlations (yellow dashed) of **1** and **6**. Some protons were removed for a clearer presentation.

resulted in the isolation of six new iridoid glycosides (**1**–**6**), together with two known iridoid derivatives (**7** and **8**).

Compound **1** was obtained as a yellowish gum with a negative optical rotation ($[\alpha]_D^{25}$ −121.5). Its molecular formula was determined to be $C_{30}H_{40}O_{13}$ based on its molecular ion peak $[M + Na]^+$ at m/z 631.2360 (calcd for $C_{30}H_{40}O_{13}Na$, 631.2367) in the positive-ion HRESIMS. The IR spectrum of **1** showed the presence of hydroxy group (3450 cm^{-1}) and ester carbonyl group (1725 cm^{-1}) absorption. The ^{1}H NMR spectrum of **1** showed signals for a 1,4-disubstituted aromatic ring [δ_H 7.48 (2H, d, J = 8.6 Hz, H-2‴ and 6‴) and 6.84 (2H, d, J = 8.6 Hz, H-3‴ and 5‴)], a *trans*-olefinic moiety [δ_H 7.67 (1H, d, J = 16.0 Hz, H-7‴) and 6.38 (1H, d, J = 16.0 Hz, H-8‴)], an anomeric proton of glucose [δ_H 4.60 (1H, d, J = 8.0 Hz, H-1″)], an isobutyl group [δ_H 2.21 (2H, m, H-2′), 2.06 (1H, m, H-3′), and 0.96 (6H, d, J = 6.7 Hz, H-4′, and 5′)], and two oxymethylenes [δ_H 4.12 and 4.26 (each 1H, d, J = 11.5 Hz, H-11) and 3.38 and 3.36 (each 1H, m, H-10)]. The ^{13}C NMR spectrum exhibited 30 carbon signals, indicating an iridoid moiety (δ_C 140.9, 115.4, 91.7, 82.6, 69.9, 69.6, 46.0, 37.3, 36.3,

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and 29.8), an isovaleryl ester moiety [δ_C 173.3, 44.3, 26.8, and 22.8 (×2)], a glucopyranosyl unit (δ_C 101.5, 78.1, 76.2, 75.3, 71.8, and 62.8), and a *trans*-coumaroyl group [δ_C 168.4, 161.4, 146.9, 131.3 (×2), 127.2, 117.0 (×2), 115.0]. The ^{1}H and ^{13}C NMR spectra of **1** were similar to those of viburtinoside II,[13] except for the presence of a methylene [δ_H 1.63 (1H, m) and 1.52 (1H, m)] at C-7 with the absence of an oxymethine carbon (δ_C 78.9) and an acetyl group (δ_H 2.01). This was supported by the ^{1}H−^{1}H COSY cross-peak between CH_2-7/CH_2-6. The 3-methylbutyroyl group was located at C-1 based on the HMBC correlation from H-1 [δ_H 6.10 (1H, d, J = 5.0 Hz)] to C-1′ (δ_C 173.3). The location of the glucose unit was determined to be at C-11 by analysis of the HMBC data showing a correlation from H-11 to C-1″. The HMBC cross-peak from H-2″ [δ_H 4.87 (1H, overlap)] to C-1‴ (δ_C 168.4) also indicated a coumaroyl group to be present at C-2″ of the glucose unit. The planar structure of **1** was confirmed from the ^{1}H−^{1}H COSY, HMQC, and HMBC spectra (Figure 1). The relative configuration of **1** was established by analyzing the NOESY and ^{13}C NMR data. In the NOESY spectrum (Figure 2), correlations of H-5/H-9 and H-10 and of H-9/H-10 indicated that H-5, H-9, and CH_2-10 are all β-oriented. Through the chemical shift of C-9 (δ_C 46.0) and the hydroxymethyl group located at C-8, it was determined to have a β-configuration.[14] Acid hydrolysis of **1** afforded glucose and *trans*-*p*-coumaric acid. The ^{1}H NMR data of a *trans*-*p*-coumaric acid unit corresponded to previously reported data.[15] D-Glucose was identified by TLC comparison and GC analysis after derivatization with an authentic sample.[16] The anomeric configuration of D-glucose was determined to be β based on the J value (8.0 Hz) of the anomeric proton in D-glucose.[17] Thus, the structure of **1** was established as 8-hydroxy-2″-*O*-*trans*-*p*-coumaroyldihydropenstemide, and it was named sambucuside A.

Compound **2** was obtained as a yellowish gum. The molecular formula of **2** was established as being $C_{30}H_{40}O_{13}$ from the HRFABMS. It showed a negative ion $[M - H]^-$ at m/z 607.2385 (calcd for $C_{30}H_{39}O_{13}$ 607.2385). The ^{1}H and ^{13}C NMR data of **2** were very similar to those of **1**, except for the chemical shifts of H-7‴ and H-8‴ (δ_H 6.89 and 5.82 for **2**; δ_H 7.67 and 6.38 for **1**). Their relatively small J values (12.9 Hz for **2**; 16.0 Hz for **1**) indicated that the *p*-coumaroyl moiety is in the *cis*-form.[18] The structure of **2** was confirmed using the ^{1}H−^{1}H COSY, HMQC, and HMBC data (Figure 1). D-Glucopyranose was identified in a similar manner to that described for **1**. The structure of **2** (sambucuside B) was established as 8-hydroxy-2″-*O*-*cis*-*p*-coumaroyldihydropenstemide.

Compound **3** was obtained as a yellowish gum. Its molecular formula was determined as $C_{30}H_{40}O_{13}$ from the molecular ion peak $[M + Na]^+$ at m/z 631.2368 (calcd for $C_{30}H_{40}O_{13}Na$, 631.2367) in the positive-ion HRESIMS. The ^{1}H and ^{13}C NMR data of **3** were quite similar to those of **1**, except for the presence of the 2-methylbutyroyl group, rather than a 3-methylbutyroyl group at C-1 [δ_H 2.38 (1H, m, H-2′), 1.64 (1H, overlap, H-3′a), 1.49 (1H, overlap, H-3′b), 0.92 (3H, t, J = 7.5 Hz, H-4′), and 1.13 (3H, d, J = 7.0 Hz), δ_C 176.9 (C-1′), 42.3 (C-2′), 27.9 (C-3′), 11.9 (C-4′), and 16.8 (C-5′) for **3**; δ_H 2.21 (1H, dd, J = 7.1, 1.9 Hz, H-2′), 2.06 (1H, m, H-3), and 0.96 (6H, t, J = 6.7 Hz, H-4′, 5′), δ_C 173.3 (C-1′), 44.3 (C-2′), 26.8 (C-3′), and 22.8 (C-4′,5′) for **1**].[19] This was supported by the HMBC cross-peaks of H-5′/C-1′, C-2′, and C-3′. The structure of **3** (sambucuside C) was confirmed using ^{1}H−^{1}H COSY, HMQC, and HMBC experiments (Figure 1) and was assigned as 1-(2-methylbutyroyl)-8-hydroxy-2″-*O*-*trans*-*p*-coumaroyldihydropenstemide.

Compound **4** (sambucuside D) was isolated as a yellowish gum, and its molecular formula was established as $C_{32}H_{42}O_{14}$ on the basis of its ^{13}C NMR data and the molecular ion peak $[M - H]^-$ at m/z 649.2491 (calcd for $C_{32}H_{41}O_{14}$, 649.2491) in the HRFABMS. The ^{1}H and ^{13}C NMR data of **4** were quite similar to those of **1**, except for the presence of an additional acetyl group [δ_C 172.9, 20.9; δ_H 2.01 (3H, s)]. In the HMBC spectrum, a correlation from H-10 [δ_H 3.97 (1H, d, J = 11.0 Hz) and 3.85 (1H, d, J = 11.0 Hz)] to an acetyl carbonyl carbon (δ_C 172.9) was used to assign the additional acetyl group at C-10 (Figure 1). The relative configuration of **4** was established as being the same as that of **1** based on the coupling constant between H-1 and H-9 (5.7 Hz for **4**; 5.0 Hz for **1**) and the NOESY cross-peaks of H-5/H-9 and H-10, H-9/H-10, and H-10/H-3′ of **4** (Figure 2). Thus, the structure of **4** was established as 1-(2-methylbutyroyl)-8-hydroxy-10-acetyl-2″-*O*-*trans*-*p*-coumaroyldihydropenstemide.

Compound **5** (sambucuside E) was obtained as a yellowish gum, and its molecular formula was established as $C_{32}H_{42}O_{14}$ using HRESIMS, which showed a positive-ion peak $[M + Na]^+$ at m/z 673.2470 (calcd for $C_{32}H_{42}O_{14}Na$ 673.2472). The ^{1}H and ^{13}C NMR data of **5** were similar to those of **4**, except that a *trans*-coumaroyl moiety of **4** was replaced with a *cis*-coumaroyl moiety in **5**. This was supported by the *cis*-coupling constant (12.9 Hz) for H-7‴ and H-8‴ of **5**. The structure of **5** was confirmed from the ^{1}H−^{1}H COSY, HMQC, and HMBC spectra obtained (Figure 1), as 1-(2-methylbutyroyl)-8-hydroxy-10-acetyl-2″-*O*-*cis*-*p*-coumaroyldihydropenstemide.

Compound **6** was obtained as a colorless gum, and its molecular formula was determined to be $C_{21}H_{34}O_{12}$ based on the negative-ion HRFABMS and ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectra of **6** were similar to those of supensolide F,[20] except for signals assigned to the sugar unit [δ_C 101.5 (C-1″), 72.5 (C-2″), 73.1 (C-3″), 75.5 (C-4″), 69.1 (C-5″), and 63.4 (C-6″) for **6**; δ_C 103.6 (C-1″), 75.3. (C-2″), 78.2 (C-3″), 71.8 (C-4″), 78.0 (C-5″), and 62.9 (C-6″) for supensolide F]. This indicated that **6** has a galactopyranose moiety instead of a glucopyranose moiety as in supensolide F. The HMBC correlation from H-1″ [δ_H 4.68 (1H, d, J = 8.0 Hz)] to C-11 (δ_C 69.9) showed that the galactopyranose unit was located at C-11. Acid hydrolysis of **6** afforded D-galactose, which was confirmed by co-TLC with an authentic sample and GC analysis.[16] The relative configuration of **6** was confirmed by the NOESY spectrum, in which correlations between CH_2-10/H-5 and H-9 and H-9/H-5 suggested that H-10, H-9, and H-5 are all β-oriented. The orientation of H-7 could be expected to be α-oriented, based on the correlation between H-7/H-1. Thus, the structure of **6** (sambucuside F) was established as 7β,8β-dihydroxydihydropenstemide.

The two known compounds were identified as 8-methoxy-10-methylene-2,9-dioxatricyclo(4,3,1,$0^{3,7}$)decane (**7**)[21] and 4-hydroxy-8-methoxy-10-methylene-2,9-dioxatricyclo(4,3,1,$0^{3,7}$)-decane (**8**)[22] by comparison with NMR data in the literature.

The cytotoxic activities of compounds **1**−**8** against the A549 (non-small-cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and Bt549 (invasive ductal carcinoma) cell lines were evaluated using an SRB bioassay. Compounds **2**, **3**, and **5** showed cytotoxic activities against SK-MEL-2 and Bt549 of the human cancer cell lines tested, with IC_{50} values ranging from 1.3 to 8.7 μM (Table 3).

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Table 1. ^{1}H NMR [ppm, mult, (J in Hz)] Data of Compounds 1−6 in Methanol-d_4 (700 MHz)

position	1	2	3	4	5	6
1	6.10, d (5.0)	6.09, d (4.9)	6.09, d (5.1)	6.07, d (5.7)	6.11, d (5.1)	6.17, d (4.9)
3	6.36, brs	6.35, brs	6.36, brs	6.36, brs	6.36, brs	6.38 brs
5	2.64, m	2.62, m	2.65, m	2.63, m	2.62, m	3.06, m
6a	1.74, m	1.73, m	1.74, m	1.80, m	1.74, m	2.02, dd (7.8, 4.1)
6b	1.69, m	1.70, m	1.70, m	1.68, m	1.68, m	
7α	1.52, m	1.56, m	1.51, overlap	1.53, overlap	1.57, m	4.00, brt (3.9)
7β	1.63, m	1.64, m	1.63, overlap	1.58, overlap	1.62, m	
9	2.14, dd (9.0, 5.0)	2.03, overlap	2.14, dd (9.1, 5.0)	2.09, overlap	1.99, dd (9.3, 5.1)	2.36, dd (9.8, 4.9)
10a	3.37, overlap	3.38, overlap	3.38, overlap	3.97, d (11.0)	3.94, d (11.0)	3.79, d (11.2)
10b	3.34, overlap	3.34, overlap	3.34, overlap	3.85, d (11.0)	3.88, d (11.0)	3.68, d (11.2)
11a	4.26, d (11.5)	4.26, d (11.4)	4.26, d (11.5)	4.29, d (11.4)	4.28, d (11.3)	4.28, d (11.5)
11b	4.12, d (11.5)	4.09, d (11.4)	4.13, d (11.5)	4.09, d (11.4)	4.07, d (11.3)	4.10, d (11.5)
2′	2.21, dd (7.1, 1.9)	2.20, dd (7.2, 1.8)	2.38, m	2.20, brd (7.1)	2.20, brd (7.2)	2.23, brd (7.3)
3′	2.06, m	2.07, m	1.64, overlap	2.06, overlap	2.07, overlap	2.10, m
			1.49, overlap			
4′	0.96, d (6.7)	0.97, d (6.7)	0.92, t (7.5)	0.96, d (6.7)	0.96, d (6.7)	0.99, d (6.7)
5′	0.96, d (6.7)	0.97, d (6.7)	1.13, d (7.0)	0.96, d (6.7)	0.96, d (6.7)	0.99, d (6.7)
1″	4.60, d (8.0)	4.53, d (8.0)	4.59, d (8.0)	4.58, d (8.0)	4.52, d (8.0)	4.68, d (8.0)
2″	4.85, overlap	4.83, overlap	4.86, overlap	4.86, overlap	4.83, overlap	3.33, overlap
3″	3.65, m	3.58, m	3.64, m	3.64, m	3.59, m	4.06, m
4″	3.42, m	3.42, m	3.42, m	3.42, m	3.40, m	3.49, m
5″	3.38, overlap	3.34, overlap	3.37, overlap	3.37, m	3.35, overlap	3.70, overlap
6″a	3.93, dd (12.0, 2.0)	3.91, dd (11.9, 2.1)	3.93, dd (11.9, 1.9)	3.93, dd (11.9, 2.2)	3.91, dd (12.0, 2.2)	3.86, overlap
6″b	3.72, dd (12.0, 5.8)	3.72, dd (11.9, 5.8)	3.72, dd (12.0, 5.8)	3.73, dd (12.0, 5.8)	3.72, dd (12.0, 5.9)	3.67, overlap
2‴,6‴	7.48, d (8.6)	7.75, d (8.7)	7.47, d (8.6)	7.48, d (8.6)	7.77, d (8.7)	
3‴,5‴	6.84, d (8.6)	6.77, d (8.7)	6.81, d (8.6)	6.83, d (8.6)	6.75, d (8.7)	
7‴	7.67, d (16.0)	6.89, d (12.9)	7.67, d (15.9)	7.67, d (15.9)	6.89, d (12.9)	
8‴	6.38, d (16.0)	5.82, d (12.9)	6.37, d (15.9)	6.38, d (15.9)	5.82, d (12.9)	
CH_3CO				2.01, s	2.05, s	

In particular, compound **3** displayed quite potent cytotoxic activity against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, and Bt549 cells), with IC_{50} values of 3.8, 1.9, 1.4, and 1.3 μM, respectively. Interestingly, although compounds **1** and **3** have similar structures except for the functional group at C-1, only compound **3** was active, suggesting that the presence of the 2-methylbutyroyl group at C-1 plays an important role in mediating the cytotoxicity against the cancer cell lines tested. Compounds **1** and **4**, possessing a *trans*-coumaroyl substituent in the glucosyl unit, exhibited no cytotoxic activities. However, compounds **2** and **5**, having a *cis*-coumaroyl group, showed strong cytotoxic activities to the SK-MEL-2 and Bt549 cancer cell lines tested. These data suggest that the geometry of the double bond in the coumaroyl group is of relevance for their cytotoxic effects in SK-MEL-2 and Bt549 cancer cell lines.

Anti-neuroinflammatory effects of all the isolated compounds (**1**−**8**) were evaluated by measuring NO levels in LPS-stimulated murine microglia BV2 cells (Table 4). Among them, compounds **2**, **3**, and **5** inhibited the NO level with IC_{50} values of 0.9, 1.3, and 1.2 μM, respectively. They showed more potent NO-inhibitory activity than N^G-monomethyl-L-arginine (L-NMMA), the positive control (IC_{50} 13.2 μM), without demonstrating any cell toxicity at a concentration of 20 μM.

Neuroprotective activities of the isolated compounds (**1**−**8**) were examined by measuring NGF secretion in C6 cells (Table 5). The level of NGF released into the medium was measured, and cell viability was determined using an MTT assay. Among the compounds isolated, compounds **2**−**4** and **8** were stimulants of NGF release, with levels of NGF stimulated at 147.0 ± 5.8%, 145.4 ± 5.8%, 158.7 ± 5.2%, and 152.6 ± 7.3%, respectively (146.3 ± 7.2% for 6-shogaol, a positive control) without producing cytotoxic effects at a concentration of 20 μM.

Iridoid glycosides with a methylbutyroyl group at C-1 and a sugar at C-11 have been reported in species of *Viburnum*, *Valeriana*, and *Sambucus*.[23−25] These iridoid derivatives were evaluated for inhibitory activity against HeLa S3 cancer cells and induced a significant hepatoprotection as a reduction in serum ALT and AST. The iridoid glycosides with a methylbutytoyl group at position C-1 and with a sugar at position C-11, as isolated from this plant source in the present investigation, showed cytotoxic activities to the SK-MEL-2 and Bt549 cancer cell lines, inhibition of NO levels in LPS-stimulated murin microglia BV2 cells, and NGF secretion-inducing activities.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 polarimeter (Easton, MD, USA). Infrared (IR) spectra were recorded using 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 with a Bruker AVANCE III 700 NMR spectrometer at 700 MHz (^{1}H) and 175 MHz (^{13}C). HRESIMS data were obtained with a Waters SYNAPT G2 mass spectrometer. HRFABMS was performed with a JEOL JMS700 mass spectrometer. A Hewlett-Packard (HP) GC 6890 Series system equipped with a 5973 mass selective detector system was controlled by the Enhanced ChemStation version B.01.00 software. The capillary column used for GC was an Agilent J&W HP-5MS UI (30.0 m × 0.25 mm i.d., 0.25 μm film thickness coated 5% diphenyl, 95% dimethylpolysiloxane). The preparative high-perform-

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Table 2. ^{13}C NMR (ppm, mult) Data of Compounds 1−6 in methanol-d_4 (175 MHz)

carbon	1	2	3	4	5	6
1	91.7	91.7	91.8	91.7	91.6	92.0
3	140.9	140.8	140.9	140.8	140.7	140.1
4	115.4	115.2	115.1	115.3	115.2	116.9
5	36.3	36.1	36.3	36.5	36.0	33.1
6	29.8	29.7	29.8	29.9	29.7	38.4
7	37.3	37.4	37.4	38.1	38.0	79.7
8	82.6	82.7	82.7	80.9	80.8	84.1
9	46.0	46.1	46.1	46.7	46.7	45.0
10	69.6	69.8	69.7	71.7	71.8	66.5
11	69.9	70.0	69.9	70.1	70.0	69.9
1′	173.3	173.4	176.9	173.2	173.3	173.3
2′	44.3	44.4	42.3	44.4	44.4	44.4
3′	26.8	26.9	27.9	27.0	26.9	26.9
4′	22.8	22.8	11.9	22.8	22.8	22.8
5′	22.8	22.8	16.8	22.8	22.8	22.8
1″	101.5	101.7	101.6	102.0	102.0	101.3
2″	75.3	75.4	75.4	75.4	75.0	72.5
3″	76.2	76.2	76.3	76.2	76.2	73.1
4″	71.8	71.9	71.9	71.9	71.9	69.1
5″	78.1	78.2	78.2	78.2	78.2	75.5
6″	62.8	62.8	62.8	62.8	62.8	63.4
1‴	127.2	127.4	126.9	127.2	127.5	
2‴,6‴	131.3	134.3	131.4	131.4	134.4	
3‴,5‴	117.0	116.1	117.3	117.1	116.2	
4‴	161.4	160.8	162.1	161.6	160.8	
7‴	146.9	145.8	147.1	146.9	145.7	
8‴	115.0	116.2	115.0	115.3	116.3	
9‴	168.4	167.2	168.5	168.4	167.2	
CH_3CO				172.9	173.0	
CH_3CO				20.9	20.9	

Table 3. Cytotoxicity of Compounds 1−8 against Four Cultured Human Cancer Cell Lines

	IC_{50} (μM)[a]			
compound	A549	SK-OV-3	SK-MEL-2	Bt549
1	>10	>10	>10	>10
2	>10	>10	8.4	8.7
3	3.8	1.9	1.4	1.3
4	>10	>10	>10	>10
5	>10	7.5	5.2	4.0
6	>10	>10	>10	>10
7	>10	>10	>10	>10
8	>10	>10	>10	>10
etoposide[b]	1.1	2.9	0.2	1.0

[a]50% inhibitory concentration; the concentration of compound that caused a 50% inhibition in cell growth. The cells tested were treated with serial dilutions (30, 10, 3, 1, 0.3, 0.1 μM) of each compounds. After 48 h, the cell survival rates were measured using an SRB assay. Results are means of three independent experiments, and the data are expressed as means ± SD. [b]Positive control.

ance liquid chromatography (HPLC) system used was equipped with a Gilson 306 pump (Middleton, WI, USA) and a Shodex refractive index detector (New York, NY, USA). Column chromatography was performed using 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 reversed-phase (RP)-18 F_{254s} plates were used for thin-layer chromatography (TLC). Spots were

Table 4. Inhibitory Effect of Compounds 1−8 on NO Production in LPS-Activated BV-2 Cells after 24 h of Treatment

compound	IC_{50} (μM)[a]	cell viability (%)[b]
1	45.5	102.3 ± 9.9
2	0.9	103.0 ± 3.1
3	1.3	136.4 ± 2.1
4	29.8	142.7 ± 0.6
5	1.2	130.4 ± 8.3
6	112.5	103.1 ± 3.7
7	94.2	103.7 ± 9.9
8	110.1	36.0 ± 9.9
L-NMMA[c]	13.2	120.3 ± 6.3

[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]The cell viability after treatment with 20 μM of each compound was determined by the 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.

Table 5. Effects of Compounds 1−8 on NGF Secretion in C6 Cells after 24 h of Cell Treatment

compound	NGF secretion (%)[a]	cell viability (%)[b]
1	130.4 ± 5.0	115.0 ± 2.1
2	147.0 ± 5.8	101.7 ± 3.1
3	145.4 ± 5.8	101.3 ± 3.4
4	158.7 ± 5.2	119.7 ± 2.7
5	134.1 ± 4.4	104.0 ± 4.2
6	107.4 ± 12.0	115.7 ± 11.3
7	133.4 ± 11.4	105.6 ± 0.9
8	152.6 ± 7.3	110.7 ± 0.5
6-shogaol[c]	146.3 ± 7.2	98.8 ± 5.2

[a]C6 cells were treated with 20 μM of each compounds. 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 a percentage of the untreated control (set as 100%). Data are means ± SD of three independent experiments performed in triplicate. [b]The cell viability after treatment with 20 μM of each compound was determined by an MTT assay and is expressed as percentages (%). Results are means of three independent experiments, and the data are expressed as means ± SD. [c]Positive control.

detected on TLC under UV light or by heating after spraying samples with anisaldehyde−sulfuric acid.

Plant Material. Twigs of *S. williamsii* var. *coreana* were collected from Chungbuk Goesan, Korea, in August 2012. The plant materials were identified by one of the authors of this study (K.R.L.). A voucher specimen (SKKU-NPL-1214) has been deposited at the Herbarium of School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. Twigs of *S. williamsii* var. *coreana* (10.0 kg) were extracted with 80% aqueous MeOH under reflux and filtered. The filtrate was evaporated under reduced pressure to obtain a MeOH extract (280 g), which was then suspended in distilled H_2O and successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-butanol, yielding 16, 30, 17, and 40 g of residue, respectively. The EtOAc-soluble fraction (20 g) was separated on an RP-C_{18} silica gel column using gradient elution with 30% aqueous MeOH → 80% aqueous MeOH, which yielded seven pooled fractions (E1−E7). Fraction E2 (5.1 g) was chromatographed on a silica gel column using $CHCl_3$−MeOH−H_2O (10:1:0.1 → 3:1:0.1), which resulted in eight fractions (E2a−E2h). Fractions E2a (60 mg) and E22 (69 mg) were purified by semipreparative HPLC (2 mL/min, 40% aqueous MeOH), which yielded compounds **7** (8 mg) and **8** (5 mg), respectively. Fraction E2g (1.2 g) was separated over an RP-C_{18} silica gel column using 35% aqueous MeOH, which gave seven additional fractions (E2ga−E2gg).

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Fraction E2a (169 mg) was purified by semipreparative HPLC (2 mL/min, 55% aqueous MeOH), which yielded compound **6** (4 mg). Fraction E3 (2.1 g) was separated over a silica gel column using $CHCl_3$−MeOH−H_2O (8:1:0.1 → 3:1:0.1), which gave seven subfractions (E3a−E3g). Compounds **1** (11 mg), **2** (3 mg), and **3** (4 mg) were obtained by purifying fraction E3f (196 mg) using semipreparative HPLC (55% aqueous MeOH). Fraction E4 (2.0 g) was separated over a silica gel column using $CHCl_3$−MeOH−H_2O (8:1:0.1 → 3:1:0.1), which gave five subfractions (E4a−E4e). Compounds **4** (9 mg) and **5** (5 mg) were obtained by purifying fraction E4b (275 mg) through semipreparative HPLC using 55% aqueous MeOH.

*Sambucuside A (**1**):* yellowish gum; $[\alpha]_D^{25}$ −122 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 312 (1.11), 226 (0.75), 211 (0.75) nm; IR (KBr) ν_{max} 3450, 2967, 2845, 1725, 1605, 1168, 1003 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRESIMS (positive-ion mode) *m*/*z* 631.2360 $[M + Na]^+$ (calcd for $C_{30}H_{40}O_{13}Na$, *m*/*z* 631.2367).

*Sambucuside B (**2**):* yellowish gum; $[\alpha]_D^{25}$ −76 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 315 (2.40), 226 (1.80), 211 (1.81) nm; IR (KBr) ν_{max} 3702, 3410, 2970, 2824, 1600, 1032 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (negative-ion mode) *m*/*z* 607.2385 $[M - H]^-$ (calcd for $C_{30}H_{39}O_{13}$, *m*/*z* 607.2385).

*Sambucuside C (**3**):* yellowish gum; $[\alpha]_D^{25}$ −68 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 315 (3.98), 227 (3.04), 211 (3.08) nm; IR (KBr) ν_{max} 3434, 2964, 2853, 1715, 1602, 1169, 1035, 999 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRESIMS (positive-ion mode) *m*/*z* 631.2368 $[M + Na]^+$ (calcd for $C_{30}H_{40}O_{13}Na$, *m*/*z* 631.2367).

*Sambucuside D (**4**):* yellowish gum; $[\alpha]_D^{25}$ −95 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 313 (3.04), 227 (1.95), 210 (1.99) nm; IR (KBr) ν_{max} 3701, 3442, 2969, 2866, 1725, 1605, 1168, 1032, 756 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (negative-ion mode) *m*/*z* 649.2491 $[M - H]^-$ (calcd for $C_{32}H_{41}O_{14}$, *m*/*z* 649.2491).

*Sambucuside E (**5**):* yellowish gum; $[\alpha]_D^{25}$ −117 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 311 (0.47), 227 (0.45), 204 (0.60) nm; IR (KBr) ν_{max} 3450, 2972, 2853, 1727, 1606, 1170, 1031, 755 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRESIMS (positive-ion mode) *m*/*z* 673.2470 $[M + Na]^+$ (calcd for $C_{32}H_{42}O_{14}Na$, *m*/*z* 673.2472).

*Sambucuside F (**6**):* colorless gum; $[\alpha]_D^{25}$ −90 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 285 (0.34), 204 (1.42) nm; IR (KBr) ν_{max} 3435, 3187, 1718, 1667, 1014, 756 cm^{-1}; ^1H NMR (700 MHz) data, see Table 1; ^{13}C NMR (175 MHz) data, see Table 2; HRFABMS (negative-ion mode) *m*/*z* 477.1966 $[M - H]^-$ (calcd for $C_{21}H_{33}O_{12}$, *m*/*z* 477.1967).

Acid Hydrolysis of Compounds 1−6 and Sugar Analysis. Each compound (2.0 mg each) was dissolved in 1 mL of 1 N HCl. The solution was then heated at 80 °C for 2 h. This reaction mixture was diluted with H_2O and extracted with CH_2Cl_2. The CH_2Cl_2 extract was evaporated in vacuo to yield aglycone. The H_2O layer was dried in vacuo and mixed with pyridine (0.1 mL) and L-cysteine methyl ester hydrochloride (2.0 mg). The mixture was stirred at 60 °C for 1.5 h. After the reaction mixture was dried in vacuo, the residue was trimethylsilylated with 1-trimethylsilylimidazole (0.1 mL) for 2 h. The mixture was then partitioned between *n*-hexane and H_2O (0.3 mL). The organic layer (1 μL) was analyzed by GC-MS under the following conditions: capillary column, HP-5MS UI (30 m × 0.25 mm × 0.25 μm, Agilent); temperatures of the injector and detector, both at 200 °C. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing to 180 °C at a rate of 5 °C/min. D-Glucose and D-galactose were treated in the same manner. D-Glucose (**1**: 15.37 min, **2**: 15.33 min, **3**: 15.36 min, **4**: 15.34 min, **5**: 15.36 min) and D-galactose (**6**: 14.86 min) were detected for compounds **1**−**6**. Identification of D-glucose (15.35 min) and D-galactose (14.85 min) detected in each case was performed by co-injection of the hydrolysate with standard silylated sugars.

Cytotoxicity Assessment. The cytotoxicity of the isolated compounds against the cultured human tumor cell lines A549, SK-OV-3, SK-MEL-2, and Bt549 was evaluated by an SRB method.[26] These assays were performed at the Korea Research Institute of Chemical Technology. Cells used 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. Each tumor cell line was inoculated into standard 96-well flat-bottom microplates and incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO_2. Attached cells were incubated with serially diluted compounds (30, 10, 3, 1, 0.3, and 0.1 μM). After 48 h of continuous exposure to these compounds, the culture medium was removed and cells were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. After washing with tap water, cells were stained with 0.4% SRB dye and incubated at room temperature for 30 min. These cells were washed again and then solubilized with 10 mM unbuffered Tris base solution (pH 10.5). Absorbance was measured spectrophotometrically at 520 nm with a microtiter plate reader. Etoposide (≥98%; Sigma Chemical Co., St. Louis, MO, USA) was used as a positive control. The 50% inhibitory concentration of cell growth (IC_{50}) was expressed as the mean of three independent experiments ± standard deviation (SD).

Measurement of NO Production and Cell Viability in LPS-Activated BV-2 Cells. BV-2 cells, developed by Dr. V. Bocchini at the University of Perugia (Perugia, Italy), were used for this study. The BV-2 microglia cells were first treated with the test compounds and incubated for 30 min. After 30 min of incubation, the treated cells were stimulated by adding LPS (100 ng/mL) and incubated for 24 h for treatment. Nitrite (a soluble oxidation product of NO) was measured in the culture medium using the Griess reaction after 24 h of treatment. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min of incubation, absorbance was measured at a wavelength of 570 nm using a microplate reader (Emax, Molecular Device, Sunnyvale, CA, USA). Graded sodium nitrite solution was used as standard to calculate nitrite concentration. Cell viability was measured using the MTT assay. N^G-Monomethyl-L-arginine (Sigma), a NO synthase inhibitor, was used as a positive control.

NGF and Cell Viability Assays. C6 glioma cells were used to measure NGF release. C6 cells were purchased from the Korean Cell Line Bank and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin in a humidified incubator with 5% CO_2. The C6 cells were seeded into 24-well cell culture plates at a density of 1×10^5 cells/well to measure NGF content in the medium and cell viability. After 24 h, the cells were treated with DMEM containing 2% FBS, 1% penicillin−streptomycin, and 20 μM of each compound for 1 day. The medium supernatant was used for NGF ELISA (R&D Systems, Minneapolis, MN, USA). Cell viability was measured using an MTT assay. After 24 h of treatments, cells were incubated with MTT reagent for 1 h in a humidified incubator with 5% CO_2 at 37 °C. Blue-stained viable cells were then converted into purple-colored solution in the presence of DMSO. Absorbance value was then measured at a wavelength of 570 nm on a microplate reader. The positive control was 6-shogaol.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00410.

HRMS and NMR data of **1**−**6** (PDF)

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Notes

The authors declare no competing financial interest.

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