Bioactive Lignans from the Trunk of *Abies holophylla*

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ABSTRACT: Six new lignans (1–6) were isolated from the trunk of *Abies holophylla* MAXIM, together with 11 known lignans (7–17). The structures of 1–7 were elucidated by spectroscopic methods, acid hydrolysis, and use of the modified Mosher’s method. The effects of the isolates on nerve growth factor induction in a C6 rat glioma cell line were evaluated. Compounds 6, 7, and 13 showed significant induction of nerve growth factor secretion at concentrations of 10 μM. Compounds 1, 5, 6, and 16 showed moderate inhibitory effects on nitric oxide production in lipopolysaccharide-activated BV-2 cells (IC50 28.5–36.4 μM).

*Abies holophylla* MAXIM (Pinaceae), also known as Manchurian Fir or Needle Fir, is an evergreen and coniferous tree that is widely distributed in Korea, China, and Russia.1,2 Several *Abies* species have been used in Korean folk medicine for the treatment of colds, stomach aches, indigestion, rheumatic diseases, and vascular and pulmonary diseases.2 Previous phytochemical investigations on *A. holophylla* reported lignans, terpenoids, steroids, and phenolic compounds, and their cytotoxic activities against several tumor cell lines or inhibitory effect against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 macrophages.1,3

As part of our efforts to discover constituents of Korean medicinal plants with antineuroinflammatory activity, we found that an EtOAc phase obtained from a MeOH extract (see Experimental Section) of the trunk of *A. holophylla* exhibited inhibitory effects on NO production in murine microglia BV-2 cells. The EtOAc solubles were successively chromatographed through silica gel, Sephadex LH-20, and prep high-performance liquid chromatography (HPLC). The result was the isolation of six new lignans (1–6) and 11 known lignans (7–17). Their structures were determined by spectroscopic methods, including one-dimensional (1D) and two-dimensional (2D) NMR, HRMS, CD experiments, and acid hydrolysis, and the use of the modified Mosher’s method. Herein, we report the isolation and structural elucidation of compounds 1–7, along with NGE secretion and inhibitory effects on NO production of isolates (1–17). (see Figure S1 of the Supporting Information for structures of the known compounds).

Compound 1 had a molecular formula of C_{13}H_{20}O_{5}, as determined from the ion peak [M + H]^+ at m/z 391.1757 in positive ion high-resolution fast-atom bombardment mass spectrometry (HRFABMS). The 1H NMR spectrum showed the presence of two 1,3,4-trisubstituted aromatic rings (δ_H 6.77 (1H, d, J = 2.0 Hz, H-2), 6.71 (1H, d, J = 7.5 Hz, H-5), and 6.62 (1H, dd, J = 7.5, 2.0 Hz, H-6) and 6.87 (1H, d, J = 1.5 Hz, H-2′), 6.75 (1H, d, J = 8.0 Hz, H-5′), and 6.74 (1H, dd, J = 8.0, 1.5 Hz, H-6′)), one acetal methine [δ_H 4.67 (1H, d, J = 1.0 Hz, H-9)], one oxymethine [δ_H 4.52 (1H, d, J = 9.0 Hz, H-7′)], three OCH3 groups [δ_C 3.84 (3H, s, 3-OCH3)], 3.83 (3H, s, 3′-OCH3), and 3.19 (3H, s, 9-OCH3)], one oxymethylene [δ_H 3.71 (1H, t, J = 8.5 Hz, H-9′a) and 3.51 (1H, t, J = 8.5 Hz, H-9′b)], one methylene [δ_H 2.75 (1H, dd, J = 13.0, 4.5 Hz, H-7a) and 2.48 (1H, m, H-7b)], and two methine protons [δ_H 2.56 (1H, m, H-8) and 2.35 (1H, m, H-8′)]. The 13C NMR spectrum contained 21 signals, including 12 aromatic carbons for two aromatic rings, one acetal methine [δ_C 109.5 (C-9)], one oxymethine [δ_C 75.8 (C-7′)], one oxymethylene [δ_C 68.5 (C-9′)], three OCH3 groups [δ_C 55.2 (3-OCH3)], 55.1 (3′-OCH3), and 53.5 (9-OCH3)], two methine [δ_C 51.6 (C-8′) and 50.3 (C-8)], and one methylene [δ_C 39.2 (C-7′)] carbons. These spectroscopic data suggested that 1 was a 9-O-9′ subtype tetrahydrofuran lignan,4,5 and these data were similar to those of iso-α-intermedial except for the absence of signals of an OCH3 at C-7′ [δ_C 3.33 (3H, s); δ_C 56.3].5 1H–1H correlation spectroscopy (COSY), heteronuclear multiple quantum correlation spectroscopy (HMQC), and heteronuclear multiple bond correlation spectroscopy (HMBCC) spectra (Figure S2 of the Supporting Information) confirmed the planar structure of 1. The relative configuration of 1 was elucidated from the coupling constants and NOEY correlations. The small J value between H-8/H-9 (1.0 Hz) showed that H-8 and H-9 were in the trans form5,7 and the NOEY correlations of H-7/H-9 and H-8′ and H-7/H-8′ corroborated the relative configurations at C-8, C-9, C-7′, and C-8′ (Figure S3 of the Supporting Information). In the CD spectrum, negative Cotton effects at 224 and 284 nm indicated that 1 had 8R and 8′R absolute configuration.8,9 Through Mosher’s method with (R)- and (S)-
MPA, the absolute configuration at C-7’ was confirmed as S (Figure S4 of the Supporting Information).10,11 (-)-Koreanol12 was reported without determination of the configuration at C-7’, and its 1H and 13C NMR spectra were quite similar to those of 1. The J value of H-7’ in (-)-koreanol (d, J = 5.2 Hz) was reported to be different from that of 1 (d, J = 9.0 Hz), indicating that (-)-koreanol could be the C-7’ epimer of 1. Thus, compound 1 was determined to be (8R,9R,7’S,8’S’)-4,4’,7’-trihydroxy-3,3’-9-trimethoxy-9,9’-epoxy lignan, and it was named holophyllol A.

$$\text{H}_2\text{CO}_3$$ $$\text{H}_2\text{CO}_3$$ $$\text{H}_2\text{CO}_3$$

Compound 2 was assigned the molecular formula of C25H34O10 by high-resolution electrospray ionization mass spectrometry (HRESIMS). The 1H and 13C NMR spectra of 2 closely resembled those of 1 but with an upfield shift of H-9’ (δH 1.418 and 3.999) and downfield shifts of H-7, 8’, 7’, and 8’ (δH 2.40 and 2.04, 1.95, 4.42, and 2.22, respectively), indicating that 2 was a stereoisomer of 1. The small J value between H-8/H-9 (1.0 Hz) and NOESY correlations of H-7/H-9 and H-7’ and H-8/H-8’ and H-9’-OCH3 confirmed the relative configuration of 2.5-7 The CD spectrum of 2, which showed negative Cotton effects at 228 and 286 nm, was quite similar to that of (8S,9R,7’S)-cubebin,5 thus, the absolute configurations at C-8 and C-8’ were determined as S and R, respectively. Mosher’s esterification with (R)- and (S)-MPA revealed that the absolute configuration at C-7’ of 2 was R. Therefore, the structure of 2, named holophyllol B, was unequivocally defined as (8S,9S,7’S,8’S’)-4,4’,7’-trihydroxy-3,3’-9-trimethoxy-9,9’-epoxy lignan.

Compound 3 also had a molecular formula of C26H34O12. Comparison of the NMR spectra of 3 with those of 2 showed they were very similar. However, upfield shifts of C-7 (δC 32.9) and C-9 (δC 105.4) were observed in 13C NMR spectrum of 3, suggesting that the relative configuration of 3 was identical with that of 2, except at C-9. The large J value H-8/H-9 (4.5 Hz) and NOESY correlations of H-7/H-7’ and H-8/H-8’ and H-9 confirmed the relative configuration of 3.5,6,13 The absolute configurations at C-8 and C-8’ were determined as S and R, respectively, from the CD spectrum which was very similar to that of 2, and the 7R configuration was determined by the modified Mosher’s method. Thus, the structure of 3 was established as (8S,9R,7’S,8’S’)-4,4’,7’-trihydroxy-3,3’-9-trimethoxy-9,9’-epoxy lignan, and it was named holophyllol C.

Compound 4 was obtained as a colorless gum. The molecular formula was determined to be C25H32O9 from the [M + Na]+ ion in the positive ion HRESIMS. The 1H and 13C NMR spectra of 4 suggested that 4 was a dihydrobenzofuran neolignan glycoside and was similar to those of 11.14 Except for the absence of signals of an OH group at C-5 and NOESY correlations of H-7/H-7’ and H-8/H-8’ and H-9, the 1H and 13C NMR spectra were very similar. However, upon comparison of its 1H NMR and MS data,24 and 3-trimethoxy-9,9’-epoxy lignan. The CD spectrum of 4 showed an HRESIMS ion peak, [M + Na]+ at m/z 529.2052, consistent with the molecular formula of C25H32O10. The 1H and 13C NMR spectra were very similar to those of 13,23 except for absence of signals of an OCH3 (δH 3.86 (3H, s); δC 55.6). The location of the two OCH3 groups in 5 was established by the HMBC correlations from 3-OCH3 (δH 3.85) to C-3 (δC 152.2) and from 3″-OMe (δH 3.52) to C-3′″ (δC 82.0). Additionally, the HMBC correlation of H-1′ to C-7 showed that the sugar unit was located at C-4, and the J value of anomeric proton (J = 1.5 Hz) confirmed it as α-rhamnose.15 Acid hydrolysis of 4 afforded the aglycone, cedrusinin, and L-rhamnose ([(α)]23D +9.0), which was identified by co-TLC confirmation and gas chromatography (GC) analysis.16,17 The identification of the aglycone was by comparison of its 1H NMR and MS data.16,17 The trans-configuration between H-7 and H-8 was confirmed through the J value (6.0 Hz),20 and its CD spectrum showed the negative Cotton effect at 236 nm confirming the absolute configurations as 7R and 8S.21,22 Cedrusinin-4-O-α-L-rhamnopyranoside was isolated without determination of the absolute configuration at C-7 and C-8,15 and its 1H and 13C NMR spectra were quite similar to those of 4. From the opposite sign of optical rotation values between cedrusinin-4-O-α-L-rhamnopyranoside ([(α)]23D +50.0) and 4 ([(α)]23D +16.0), cedrusinin-4-O-α-L-rhamnopyranoside should be 7S and 8R forms. Thus, the structure of 4 was determined to be (7R,8S)-cedrusinin 4-O-α-L-rhamnose.

Compound 5 displayed an HRESIMS ion peak, [M + Na]+ at m/z 529.2052, consistent with the molecular formula C25H32O10. The 1H and 13C NMR spectra were very similar to those of 13,23 except for absence of signals of an OCH3 (δH 3.86 (3H, s); δC 55.6). The location of the two OCH3 groups in 5 was established by the HMBC correlations from 3-OCH3 (δH 3.85) to C-3 (δC 152.2) and from 3″-OMe (δH 3.52) to C-3′″ (δC 82.0). Additionally, the HMBC correlation of H-1′ to C-7 showed that the sugar unit was located at C-4. Acid hydrolysis of 5 gave the aglycone, 8, which was identified as cedrusin by comparison of its 1H NMR and MS data,24 and 3-O-methyl-L-rhamnose, which was identified by co-TLC confirmation, optical rotation ([(α)]23D +10.3),19 1H NMR and MS data,5,26 and GC analysis. The relative configuration between H-7 and H-8 of 5 was determined by the J value (6.0 Hz) as trans,20 and the negative Cotton effect at 245 nm in the CD spectrum confirmed the 7R and 8S configuration.21,22 Thus, 5 was elucidated as (7R,8S)-cedrusin 4-O-(3-O-methyl-α-L-rhamnopyranoside).
Compound 6 had a molecular formula of C_{25}H_{34}O_{10} as determined by the ion peak [M + Na]^+ at m/z 517.2048. The 1^H and 13C NMR spectra were similar to those of ligaranin E,\(^\text{27}\) except for the presence of signals for a xylose unit (δH 4.80 (1H, d, J = 7.0, H-1)), 3.89 and 3.29 (each 1H, m, H-5′), 3.55 (1H, m, H-4′), 3.46 (1H, m, H-2′), and 3.42 (1H, m, H-3′); δC 106.2) in the 13C NMR spectrum and a small change of chemical shift of methine and methylene protons at H-7, 8, 9, δC 102.2 (C-5) in the 13C NMR spectrum and a small change of δC 106.2 (C-4′) in the 13C NMR spectrum. This was supported by 1H-1H COSY, HMBC, and HMBC spectra. The xylose unit was placed at C-4 by the observation of an HMBC correlation from H-1 and HMBC spectra. The xylose unit was placed at C-4 by the

Antineuroinflammatory activities of the isolates (1–17) were also tested via measurement of NO levels using bacterial endotoxin, LPS, in the murine microglia BV-2 cell line. Compounds 1, 5, 6, and 16 moderately inhibited NO production with IC_{50} values of 36.4, 32.9, 31.0, and 28.5 μM, respectively, in LPS-stimulated BV-2 cells without cell toxicity (Table S2 of the Supporting Information).

### EXPERIMENTAL SECTION

#### General Experimental Procedures.
Optical rotations were measured on a JASCO P-1020 polarimeter. Infrared spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. CD spectra were measured on a JASCO J-810 spectropolarimeter. UV spectra were recorded using an Agilent 8453 UV-visible spectrophotometer. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (1H) and 125 MHz (13C), respectively. HRFABMS spectra were obtained on a JEOL JMS700 mass spectrometer and HREIMS spectra were recorded on a Micromass QTOF2-MS. Preparative HPLC was performed using a Gilson 306 pump with a Shodex refractive index detector. Silica gel 60 (Merck, 230–400 mesh) and RP-C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography (CC). TLC was performed using Merck precoated silica gel F_{254} plates and RP-18 F_{254}c plates. The packing material for molecular sieve CC was Sephadex LH-20 (Pharmacia Company). Low-pressure liquid chromatography was performed on Merck Lichroprep Lobar-A (240 × 10 mm) column with an FMI QSY-0 pump (ISCO).

#### Plant Material.
_A. holophylla_ trunk material was collected in Seoul, Korea, in January 2012, and the plant was identified by one of the authors (K.R.L.). A voucher specimen (SKKU-NPL 1205) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

#### Extraction and Isolation.
The trunk material of _A. holophylla_ (5.0 kg) was extracted with 80% aq MeOH under reflux and was filtered. The filtrate was evaporated under reduced pressure to obtain a 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 of residues, respectively. The EtOAc-soluble fraction (5 mg) was separated on a RP-C_{18} silica gel column (20% aq CH_3CN) and then by preparative HPLC (30% aq CH_3CN), to yield ten subfractions (E31, E32, E33, E34, E35, E36, E37, E38, E39, E40) (6 mg). Fraction E35 (50 mg) was separated by preparative HPLC (30% aq CH_3CN) to yield compounds 11 (7 mg) and 12 (6 mg). Fraction E33 (50 mg) was separated by preparative HPLC (30% aq CH_3CN) to yield compounds 8 (2 mg), 9 (2 mg), and 10 (4 mg). Fraction E32 (10 mg) was separated on a RP-C_{18} silica gel column (20% aq CH_3CN) and further by preparative HPLC (25% aq CH_3CN), to yield compounds 6 (4 mg), 7 (2 mg), and 13 (2 mg). Fraction E31 (20 mg) was separated on a SEP-PAK Vac C_{18} (CHCl_3–MeOH, 8:1) and further by preparative HPLC (20% aq CH_3CN), to give compounds 5 (4 mg), 14 (1 mg), and 15 (2 mg). Fraction E55 (300 mg) was separated on a silica gel Waters Sep-Pak Vac C_{18} (CHCl_3–MeOH, 30:1) and then by preparative HPLC (40% aq CH_3CN) to yield compounds 1 (15 mg), 2 (8 mg), 3 (2 mg), and 4 (2 mg). Fraction E61 (1.2 mg) was separated by silica gel CC (CHCl_3–MeOH, 7:1) to give eight subfractions (E61–E68). Fraction E61 (79 mg) was purified by preparative HPLC (30% aq CH_3CN) to yield compounds 2 (5 mg), 3 (3 mg), and 16 (4 mg). Fraction E68 (100 mg) was separated on a Sephadex LH-20 column (CHCl_3–H_2O, 20:1) to yield compounds 4, 5, and 6. Fraction E65 (10 mg) was separated on a silica gel CC (CHCl_3–MeOH, 7:1) to give four subfractions (E65–E69). Fraction E65 (20 mg) was separated on a silica gel Waters Sep-Pak Vac C_{18} (CHCl_3–MeOH, 30:1) and then by preparative HPLC (40% aq CH_3CN) to yield compounds 1 (15 mg), 2 (8 mg), 3 (2 mg), and 4 (2 mg). Fraction E61 (1.2 mg) was separated by silica gel CC (CHCl_3–MeOH, 7:1) to give eight subfractions (E61–E68). Fraction E61 (79 mg) was purified by preparative HPLC (30% aq CH_3CN) to yield compounds 2 (5 mg), 3 (3 mg), and 16 (4 mg). Fraction E68 (100 mg) was separated on a Sephadex LH-20 column (CHCl_3–
MeOH, 1:1) and further by preparative HPLC (30% ac CH3CN for to give compound 4 (mg).

Holophyllol A (1). Colorless needles; mp 175–178 °C; [α]D25 +50.6 (c 0.75, MeOH); IR (KBr) νmax 3379, 2941, 2834, 1517, 1453, 1274, 1032 cm−1; 1H (CD3OD, 500 MHz) δ 7.01 (1H, d, J = 8.5 Hz, H-2), 6.68 (1H, br d, J = 8.5 Hz, H-4), 6.70 (1H, s, H-6), 6.57 (1H, s, H-2′), 5.48 (1H, d, J = 6.5 Hz, H-7), 3.85 (1H, m, H-9a), 3.82 (3H, s, 3-OCH3), 3.74 (1H, d, J = 11.0, 7.5 Hz, H-9b), 3.55 (2H, t, J = 6.5 Hz, H-9′), 3.45 (1H, dd, J = 11.0, 6.5 Hz, H-8), 2.56 (2H, t, J = 8.0 Hz, H-7′), 1.79 (2H, m, H-8′); positive ESIMS m/z 369 [M + Na]+.

Lignaril E. Colorless gum; CD (MeOH) [Δ]25 +33.0 (2.0) nm; UV (MeOH) λmax (log e) 279 (2.0), 231 (4.8) nm; 1H NMR (CD3OD, 500 MHz) δ 6.82 (1H, d, J = 8.5 Hz, H-5′), 6.81 (2H, br s, H-2 and 2′), 6.70 (1H, br d, J = 8.5 Hz, H-6′), 6.68 (1H, br d, J = 8.5 Hz, H-6), 6.67 (1H, d, J = 8.5 Hz, H-5), 4.35 (1H, m, H-8), 3.81 (3H, s, 3-OCH3), 3.79 (3H, s, 3-OCH3), 3.63 (2H, m, H-9), 3.55 (2H, t, J = 6.5 Hz, H-9′), 2.89 (2H, dd, J = 6.0, 2.0 Hz, H-7′), 2.61 (2H, t, J = 8.0 Hz, H-7′), 1.79 (2H, m, H-8′); positive ESIMS m/z 385 [M + Na]+.

NGF and Cell Viability Assay. C6 Glioma cells were used to measure NGF release into the medium.35 C6 cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) in a humidified incubator with 5% CO2. To measure NGF content in medium and cell viability, C6 cells were seeded into 24-well plates (1 × 105 cells/well). After 24 h, the cells were treated with DMEM containing 2% FBS and 1% PS with 20 μM of each sample for one day. Media supernatant was used for the NGF assay using an ELISA development kit (R&D System, Minneapolis, MN). Cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

Measurement of NO Production and Cell Viability. BV-2 cells were maintained in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin. To measure NO production, BV-2 cells were plated onto a 96-well plate (3.3 × 104 cells/well) and further by preparative HPLC (30% ac CH3CN) to yield the organic layers evaporated under reduced pressure to yield a colorless gum; CD (MeOH) [Δ]25 +20.0 (2.0) nm; UV (MeOH) λmax (log e) 279 (2.0), 231 (4.8) nm; 1H NMR (CD3OD, 500 MHz) δ 6.82 (1H, d, J = 8.5 Hz, H-5′), 6.81 (2H, br s, H-2 and 2′), 6.70 (1H, br d, J = 8.5 Hz, H-6′), 6.68 (1H, br d, J = 8.5 Hz, H-6), 6.67 (1H, d, J = 8.5 Hz, H-5), 4.35 (1H, m, H-8), 3.81 (3H, s, 3-OCH3), 3.79 (3H, s, 3-OCH3), 3.63 (2H, m, H-9), 3.55 (2H, t, J = 6.5 Hz, H-9′), 2.89 (2H, dd, J = 6.0, 2.0 Hz, H-7′), 2.61 (2H, t, J = 8.0 Hz, H-7′), 1.79 (2H, m, H-8′); positive ESIMS m/z 385 [M + Na]+.

Notes

**ASSOCIATED CONTENT**

Supporting Information

Bioactivities of 1–17, 1H and 13C NMR data of 1–7, structures of known compounds 8–17, HRMS and 1D and 2D NMR data of 1–7, CD spectra and ΔΔ95 values for the (R)- and (S)-MPA esters of 1–3 and 7, 1H NMR data of 1r–3r and 7r, 1s–3s and 7s, 4a–6a, and 3-O-methyl-l-ribose. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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