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Isolation and structural characterization of four diastereomeric lignan glycosides from *Abies holophylla* and their neuroprotective activity

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ABSTRACT

Diastereomers are a type of a stereoisomer and they are often isolated as a mixture due to their similar physical properties. Through HPLC technique, we isolated four stereoisomeric lignan glycosides, holophyllosides A–D (**1**–**4**), from the trunk of *Abies holophylla* Maxim. which were first isolated as a mixture 25 years ago. The planar structures of **1**–**4** were characterized by conventional 1D and 2D NMR data analysis and their stereochemistry was determined via empirical comparison of their ^{13}C NMR chemical shifts and $^{3}J_{H\text{-}H}$ coupling constants with the reported values, enzymatic hydrolysis followed by LC-MS analysis, and ECD experiment. Of the four stereoisomers isolated, only compounds **1**, **3**, and **4** showed moderate neuroprotective activity by inducing NGF secretion in C6 cells whereas **2** did not. This study highlights again the stereochemical importance of molecules in their biological and pharmaceutical application.

1. Introduction

In organic chemistry, isomers are molecules with the same molecular formulas but different spatial arrangements. They often share similar chemical and/or physical properties including behaviors in column chromatography for isolation of natural products. This is evidenced by many cases in which two or more isomers (constitutional [1–10] and stereoisomers [11–18]) have been isolated as an inseparable mixture. The major issue of isolating mixtures, rather than pure compounds, is that it is not possible to evaluate biological activity of each compound and to further develop it for new drug candidates without total synthesis of all the individual compound.

A lignan glycoside mixture, *erythro*- and *threo*-1-(4'-hydroxy-3'-methoxy-phenyl)-2-[4"-(3-hydroxypropyl)-2"-methoxyphenoxy]-1,3-propandiol 4'-*O*-β-D-xylopyranoside, was previously isolated from *Picea abies* (L.) Karst. (Pinaceae) in 1995 [19] and *Pseudolarix kaempferi* Gord. (Pinaceae) in 2011 [20] without purification of each compound as well as assignment of its absolute configuration. During ongoing search for bioactive secondary metabolites from Korean medicinal plants, we isolated and identified all four possible diastereomers of 1-(4'-hydroxy-3'-methoxy-phenyl)-2-[4"-(3-hydroxypropyl)-2"-methoxyphenoxy]-1,3-propandiol 4'-*O*-β-D-xylopyranoside, holophyllosides A–D (**1**–**4**, Fig. 1), from *Abies holophylla* Maxim., which belongs to Pinaceae family. Herein, described are structural characterization of **1**–**4** including their relative and absolute configuration and their biological activity.

2. Results and discussion

Holophylloside A (**1**) was isolated as a colorless gum. The molecular formula of this compound was determined as $C_{25}H_{34}O_{11}$ based on the sodiated molecular ion peak at *m/z* 533.1994 (calcd for $C_{25}H_{34}O_{11}Na^{+}$, 533.1993, error = 0.2 ppm). The ^{1}H NMR and HSQC data of **1** indicated the presence of two 1,3,4-trisubstituted benzene rings [δ_H 7.09 (1H, d, J = 2.0 Hz), 7.02 (1H, d, J = 8.3 Hz), and 6.93 (1H, dd, J = 8.3, 2.0 Hz); δ_H 6.80 (1H, d, J = 8.0 Hz), 6.78 (1H, d,

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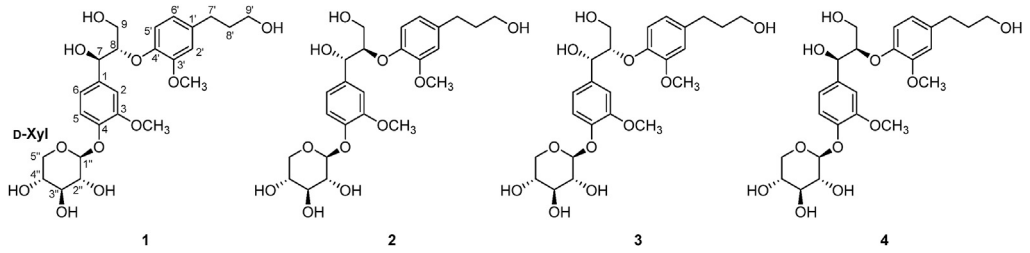

Fig. 1. Chemical structures of compounds **1**–**4**.

$J = 2.1$ Hz), and 6.66 (1H, dd, $J = 8.0$, 2.1 Hz)], six oxygenated methine protons [δ_H 4.85 (1H, overlap), 4.82 (1H, d, $J = 7.3$ Hz), 4.29 (1H, td, $J = 5.7$, 3.6 Hz), 3.56 (1H, overlap), 3.47 (1H, dd, $J = 9.0$, 7.3 Hz), and 3.41 (1H, t, $J = 9.0$ Hz)], six oxygenated methylene protons [δ_H 3.90 (1H, dd, $J = 11.4$, 5.3 Hz) and 3.30 (1H, overlap); δ_H 3.85 (1H, dd, $J = 11.9$, 5.7 Hz), and 3.75 (1H, dd, $J = 11.9$, 3.6 Hz); δ_H 3.54 (2H, t, $J = 6.5$ Hz)], two methoxy groups [δ_H 3.81 (3H, s) and 3.77 (3H, s)], and four methylene protons [δ_H 2.60 (2H, m) and 1.80 (2H, m)]. The ^{13}C NMR spectrum of **1** showed total 25 peaks supporting the above structural suggestion; 12 signals for the two 1,3,4-trisubstituted benzene rings [δ_C 152.0, 151.0, 147.3, 147.0, 138.2 ($\times$2), 122.0, 121.0, 119.7, 118.1, 114.1, and 112.8], 11 signals for oxygenated carbons [δ_C 103.7 (dioxygenated), 86.6, 77.5, 74.8, 74.0, 71.2, 67.0, 62.4, 62.3, 56.7 (methoxy), and 56.6 (methoxy)], and two signals for methylene groups (δ_C 35.7 and 32.8). In addition to these spectroscopic data (Table 1), COSY and HMBC data analysis of **1** (Fig. 2A) confirmed that the planar structure of **1** is identical to that of 1-(4'-hydroxy-3'-methoxy-phenyl)-2-[4"-(3-hydroxypropyl)-2"-methoxyphenoxy]-1,3-propandiol 4'-*O*-β-D-xylopyranoside, which was isolated from *P. abies* and *P. kaempferi* (both Pinaceae) in 1995 and 2011, respectively [19,20]. However, this compound was reported as a 7:1 mixture of its *erythro*- and *threo*-isomers without assignment of their absolute configuration which led us to further charaterization of full stereochemistry of **1** as well as **2**–**4**. Holophyllosides B–D (**2**–**4**) were obtained from the same subfraction B2-4 where **1** was isolated (see Materials and Methods section). Inspection of 1D and 2D NMR and HRMS data of **2**–**4** revealed that these three compounds possess the same planar structure as that of **1** (Fig. 1). The pyranose form of xylose in **1**–**4** was confirmed by HMBC correlation of H-5"/C-1" (Fig. 1A). Assuming the configuration of xylopyranose in **1**–**4** as D-form based on its higher natural abundance than L-form, we tentatively concluded that **1**–**4** are diastereomers with different configuration at C-7 and C-8.

The relative configuration between C-7 and C-8 in **1**–**4** were determined by the well-established empirical rules in three different ways. First, a systemic analysis on the chemical shift difference between H-9a and H-9b in diverse 8–*O*–4'-oxyneolignan derivatives revealed that the $\delta_{H9a\text{-}H9b}$ value of *threo*-isomers are larger than that of *erythro*-isomers when measured in methanol-d_4, DMSO-d_6, D_2O, and acetic acid-d_4 [21]. As shown in Fig. 2B and Table 1, we observed the smaller $\delta_{H9a\text{-}H9b}$ value in **1** and **2** (both 0.10 ppm) than that in **3** and **4** (both 0.28 ppm). Second, the chemical shift differences between C-7 and C-8 showed a contrast between *erythro*- and *threo*-isomers; $\Delta\delta_{C8-C7}$ of *erythro*-isomer (~12.1 ppm in methanol-d_4) was smaller than that of *threo*-isomer (~13.1 ppm in methanol-d_4) [22]. Similar pattern was observed from **1**–**4**; the $\Delta\delta_{C8-C7}$ value of **1** and **2** were both 12.6 ppm while that of **3** and **4** were 13.5 ppm (Table 2). Finally, the conventional method comparing coupling constants between H-7 and H-8 was applied for confirmation of their relative configuration. A clear difference of $^3J_{H\text{-}7,H\text{-}8}$ value in *erythro* and *threo*-isomers were observed only in the NMR solvent of chloroform-*d* and acetone-

Table 1
^{1}H [ppm, mult., (*J* in Hz)] NMR data of compounds **1**–**4** in methanol-d_4.

position	δ_H			
	1	**2**	**3**	**4**
2	7.09, d (2.0)	7.08, d (2.0)	7.12, d (2.0)	7.12, d (2.0)
5	7.02, d (8.3)	7.02, d (8.3)	7.04, d (8.3)	7.04, d (8.3)
6	6.93, dd (8.3, 2.0)	6.93, dd (8.3, 2.0)	6.97, dd (8.3, 2.0)	6.96, dd (8.3, 2.0)
7	4.85, overlap[a]	4.86, overlap[a]	4.93, d (5.4)	4.93, d (5.4)
8	4.29, td (5.7, 3.6)	4.29, td (5.7, 3.6)	4.24, td (5.4, 4.3)	4.24, td (5.4, 4.3)
9a	**3.85**, dd (11.9, 5.7)	**3.85**, dd (11.9, 5.7)	**3.75**, dd (11.9, 5.4)	**3.75**, dd (11.9, 5.4)
9 b	**3.75**, dd (11.9, 3.6)	**3.75**, dd (11.9, 3.6)	**3.47**, dd (11.9, 4.3)	**3.47**, dd (11.9, 4.3)
(ΔH9a-H9b)	(**0.10**)	(**0.10**)	(**0.28**)	(**0.28**)
2′	6.78, d (2.1)	6.78, d (2.0)	6.84, d (2.0)	6.84, d (2.0)
5′	6.80, d (8.0)	6.81, d (8.2)	6.93, d (8.2)	6.92, d (8.2)
6′	6.66, dd (8.0, 2.1)	6.66, dd (8.2, 2.0)	6.70, dd (8.2, 2.0)	6.69, dd (8.2, 2.0)
7′	2.60, m	2.60, m	2.62, m	2.62, m
8′	1.80, m	1.79, m	1.81, m	1.80, m
9′	3.54, t (6.5)	3.54, t (6.5)	3.55, t (6.5)	3.55, t (6.5)
1′′(Xyl)	4.82, overlap[a]	4.83, d (7.3)	4.85, d (7.4)	4.84, d (7.4)
2″	3.47, dd (9.0, 7.3)	3.47, dd (8.9, 7.3)	3.47, dd (8.9, 7.4)	3.47, dd (8.9, 7.4)
3″	3.41, t (9.0)	3.41, t (9.0)	3.42, t (8.9)	3.42, t (8.9)
4″	3.56, overlap[a]	3.56, overlap[a]	3.57, overlap[a]	3.57, overlap[a]
5″a	3.90, dd (11.4, 5.3)	3.90, dd (11.5, 5.3)	3.90, dd (11.4, 5.3)	3.90, dd (11.4, 5.3)
5″b	3.30, overlap[a]	3.30, overlap[a]	3.30, overlap[a]	3.30, overlap[a]
OCH_3-3	3.81, s	3.81, s	3.83, s	3.83, s
OCH_3-3′	3.77, s	3.77, s	3.84, s	3.84, s

[a] Overlapped with the peak from the other atom(s) in the molecule or solvent.

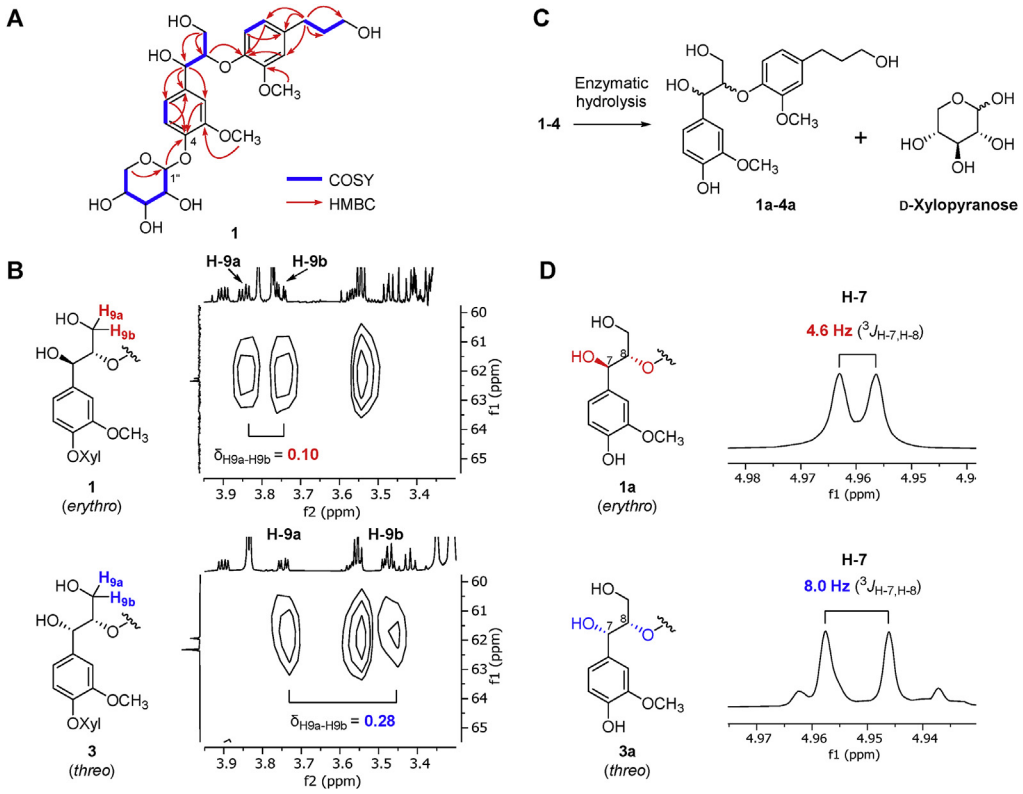

Fig. 2. Determination of the planar structure and relative configuration of **1**–**4**. (A) Key COSY (blue bold) and HMBC (red arrows) correlations of **1**. (B) Structures of **1** (*erythro*) and **3** (*threo*), and their zoomed-in HSQC spectra for H-9a and H-9b. (C) Enzymatic hydrolysis of **1**–**4** that yielded their aglycones **1a**–**4a** and D-xylopyranose. (D) Structures of **1a** (*erythro*) and **3a** (*threo*) and their ^{1}H NMR peak of H-7 with coupling constant.

Table 2
^{13}C NMR data of compounds **1**–**4** in methanol-d_4.

position	δ_C			
	1	**2**	**3**	**4**
1	138.2	138.1	137.8	137.8
2	112.8	112.9	112.7	112.7
3	151.0	150.9	151.0	151.0
4	147.0	147.1	147.1	147.1
5	118.1	118.1	118.2	118.2
6	121.0	121.0	120.6	120.6
7	**74.0**	**74.0**	**73.8**	**73.9**
8	**86.6**	**86.6**	**87.3**	**87.4**
(ΔC8-C7)	**(12.6)**	**(12.6)**	**(13.5)**	**(13.5)**
9	62.3	62.3	62.0	62.0
1′	138.2	138.2	138.3	138.3
2′	114.1	114.1	114.1	114.1
3′	152.0	152.0	151.8	151.8
4′	147.3	147.3	147.6	147.6
5′	119.7	119.7	119.5	119.5
6′	122.0	121.9	122.1	122.1
7′	32.8	32.9	32.8	32.9
8′	35.7	35.7	35.7	35.7
9′	62.4	62.4	62.3	62.3
1″(Xyl)	103.7	103.7	103.6	103.6
2″	74.8	74.8	74.8	74.8
3″	77.5	77.5	77.5	77.6
4″	71.2	71.2	71.1	71.2
5″	67.0	67.0	67.0	67.0
OCH_3-3	56.6	56.6	56.6	56.6
OCH_3-3′	56.7	56.7	56.7	56.7

d_6 + D_2O, not in methanol-d_4, DMSO-d_6, pyridine-d_5, and D_2O [22,23]; $^{3}J_{H\text{-}7,H\text{-}8}$ of *erythro*-isomer (~3.0–5.4 Hz in acetone-d_6 + D_2O; ~3.6–4.2 Hz in chlroroform-*d*) was smaller than that of *threo*-isomer (~6.0–6.6 Hz in acetone-d_6 + D_2O; ~8.4 Hz in chlroroform-*d*). We found that the $^{3}J_{H\text{-}7,H\text{-}8}$ values of **1a**–**4a**, aglycones of **1**–**4,** respectively, measured in chloroform-*d* were 4.6 Hz for **1a** and **2a** and 8.0 Hz for **3a** and **4a** (Fig. 2C and D, Table 3). Collectively, based on these three different empirical rules the relative configuration between C-7 and C-8 in **1**–**4** were assigned as *erythro* for **1** and **2** and as *threo* for **3** and **4**.

The absolute configuration of **1**–**4** were assigned by ECD data analysis; the positive and negative Cotton effect at ~230–240 nm

Table 3
^{1}H [ppm, mult., (*J* in Hz)] NMR data of compounds **1a** and **3a** in chloroform-*d*.[a]

position	δ_H	
	1a	**3a**
2	6.97, d (1.7)	6.97, d (1.8)
5	6.88, d (8.1)	7.02, d (8.1)
6	6.82, dd (8.1, 1.4)	6.92, dd (8.2, 1.8)
7	4.96, d (**4.6**)	4.95, d (**8.0**)
8	4.11, ddd (6.0, 4.6, 3.4)	3.89, overlap[b]
9a	3.89, overlap[b]	3.62, dd (12.5, 3.2)
9 b	3.64, overlap[b]	3.47, dd (12.5, 3.9)
2′	6.77, d (1.7)	6.78, d (1.9)
5′	6.88, d (8.1)	6.89, d (8.1)
6′	6.74, dd (8.1, 1.9)	6.76, dd (8.1, 2.0)
7′	2.68, m	2.68, m
8′	1.89, m	1.88, m
9′	3.68, t (6.4)	3.68, t (6.4)
OCH_3-3	3.89, s	3.91, s
OCH_3-3′	3.88, s	3.89, s

[a] The ^{1}H NMR data of **2a** and **4a** were identical with their enantiomeric isomers, **1a** and **3a**, respectively.
[b] overlapped with the peak from the other atom(s) in the molecule or solvent.

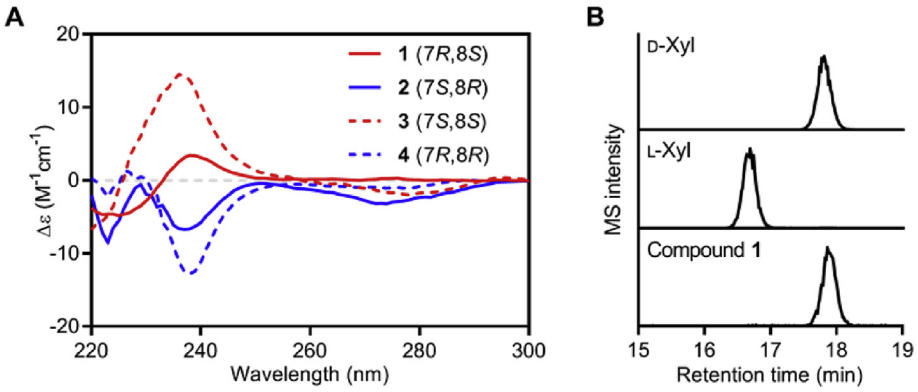

Fig. 3. Absolute configuration assignment for **1**–**4**. (A) ECD spectra of **1**–**4**. (B) Extracted ion chromatograms (EICs, *m/z* 417.1149, 20 ppm window) of D- and L-xylopyranose and **1** derivatized with chiral agents.

Table 4
Effect of compounds **1**–**4** on NGF secretion in C6 cells.

compound	NGF secreation[a]	Cell viability (%)[b]
1	133.76 ± 10.69	102.99 ± 0.19
2	104.64 ± 12.36	109.50 ± 1.90
3	144.93 ± 10.27	112.46 ± 7.33
4	136.79 ± 10.81	113.99 ± 4.95
6-shogaol[c]	168.58 ± 7.16	125.80 ± 0.93

[a] C6 cells were treated with 20 μM of each compound. The level of secreted NGF cell is expressed as percentage of the untreated control.
[b] The 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.

indicate 8*S* and 8*R* configuration, respectively, in 8-*O*-4' neolignan derivatives [23]. Observing a positive Cotton effect at 238 nm from **1** and 236 nm from **3** and a negative Cotton effect at 237 nm from **2** and 238 nm from **4**, we assigned the absolute configuration of **1** and **3** as 8*S*, and **2** and **4** as 8*R* (Fig. 3A). Finally, D-configuration of xylopyranose unit in **1**–**4** were confirmed by chrial derivatization followed by LC-MS analysis which is a well-established method [24,25]. Xylopyranose obtained from enzymatic hydrolysis of **1**–**4** was chiral derivatized with L-cysteine methyl ester hydrochloride and *o*-tolyl isothiocyanate and further analyzed by LC-MS. The retention time of xylopyranose from the **1**–**4** (17.8 min) were identical to that of authentic D-xylopyranose (17.8 min), rather than L-xylopyranose (16.7 min) (Fig. 3B). Collectively, the structures of **1**–**4** were elucidated as (7*R*,8*S*)-, (7*S*,8*R*)-, (7*S*,8*S*)-, and (7*R*,8*R*)-7,9,9'-trihydroxy-3,3'-dimethoxy-8-*O*-4'-neolignan-4-*O*-β-D-xylopyranose, respectively.

In our continuing research for neuroprotective, anti-inflammatory, and cytotoxic secondary metabolites from medicinal plants [11,26–32], we evaluated **1**–**4** for these biological activities. The potential neuroprotective activity of compounds **1**–**4** were tested by assessing nerve growth factor (NGF) secretion level in C6 cells (Table 4). Compounds **1**, **3**, and **4** displayed mild effects on NGF release with stimulation levels of 133.76 ± 10.69, 144.93 ± 10.27, and 136.79 ± 10.81%, respectively, without significant cell toxicity, whereas compound **2** did not (104.64 ± 12.36%). It is quite interesting that among four diastereomers **1**–**4** only compound **2** showed no activity. Although further studies are needed the stereochemistry at C-7 and C-8 in 8-*O*-4' neolignan derivatives may play an important role in inducing NGF from C6 cells. All the isolates did not show anti-inflammatory and cytotoxic activity.

3. Conclusion

In summary, we successfully isolated four diastereomeric lignan glycosides, holophyllosides A–D (**1**–**4**), from *A. holophylla*, characterized their structures including absolute configuration by spectroscopic and chemical methods, and evaluated biological activity of the isolates. Through this study we solved the 25-year-old problem by purifying each diastereomer of **1**–**4** and expect that further biological study on these compounds would be investigated.

4. Experimental section

4.1. General experimental procedures

Optical rotation data were recorded utilizing a JASCO P-1020 polarimeter (Jasco, Easton, MD, USA). ECD spectra were recorded with a JASCO J-810 spectropolarimeter (Jasco, Tokyo, Japan). The NMR studies were accomplished employing a Bruker AVANCE III 700 NMR spectrometer (Bruker, Karlsruhe, Germany) and resultant spectra were processed using MestReNova (Mnova) (version 14.1.2–25024) with default weighting functions. HRFABMS data were acquired on a Waters SYNAPT G2 (Milford, MA, USA). HRESIMS and LC-MS data were measured using an Agilent 1290 Infinity II HPLC instrument (Foster City, CA, USA) coupled to a G6545B quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies) with a Kinetex C_{18} 5 μm column (250 mm length × 4.6 mm i.d.; Phenomenex, Torrance, CA, USA). Purification was achieved using a semi-preparative HPLC system equipped with a Gilson 306 pump (Middleton, WI, USA), a Shodex refractive index detector (New York, NY, USA), a Luna C_{18} 10 μm column (250 mm length × 10 mm i.d.; Phenomenex, Torrance, CA, USA) and a J'sphere ODS H80 column (250 mm length × 10 mm i.d., S-4 μm, 8 nm; YMC, Kyoto, Japan). Open columns packed with silica gel 60 (70–230 and 230–400 mesh; Merck) or RP-C_{18} silica gel (230–400 mesh; Merck, Darmstadt, Germany) were implemented for crude fractionation and separation. Precoated silica gel F_{254} plates and RP-18 F_{254s} plates (Merck) were utilized for thin-layer chromatography (TLC).

4.2. Plant material

The trunk of *A. holophylla* was collected in Seoul, Korea in January 2012. A voucher specimen (SKKU-NPL 1205) was authenticated by Dr. Kang Ro Lee (Sungkyunkwan University, Korea) and has been deposited in the herbarium of the School of Pharmacy,

Sungkyunkwan University, Suwon, Korea.

4.3. Extraction and isolation

The trunk of *A. holophylla* (5.0 kg) were extracted with 80% aqueous MeOH under reflux and filtered to yield 280 g of crude extract, 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, respectively. The *n*-butanol-soluble fraction (30 g) separated over a silica gel column ($CHCl_3$–MeOH–H_2O, 3:1:0.1 → 1:1:0.2) to yield nine fractions (B1–B9). The fraction B2 (0.6 g) was chromatographed on a RP-C_{18} silica gel column with 40% aqueous MeOH to give 11 subfractions (B2-1–B2-11). By separation of subfraction B2-4 (100 mg) using a C_{18} semi-preparative HPLC [Phenomenex Luna C_{18} column (10 × 250 mm), 2 mL/min, 18% aqueous CH_3CN], mixtures of **1** and **2** (35 mg), and **3** and **4** (20 mg) were obtained. The compounds **1**–**4** were purified (4, 5, 7, and 5 mg, respectively) from each mixture by an additional semi-preparative HPLC step [YMC J'sphere ODS H80 column (10 × 250 mm), 2 mL/min, 15% aqueous CH_3CN].

4.3.1. Holophylloside A (*1*)

Colorless gum; $[\alpha]_D^{25}$ +24 (c 0.05, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$): 238 (+3.4), 276 (+0.3) nm; ^1H (700 MHz) and ^{13}C NMR (175 MHz) data, see Tables 1 and 2, respectively; HRFABMS (positive-ion mode) *m/z* 533.1994 $[M + Na]^+$ (calcd for $C_{25}H_{34}O_{11}Na^+$, 533.1993, error = 0.2 ppm).

4.3.2. Holophylloside B (*2*)

Colorless gum; $[\alpha]_D^{25}$ −3 (c 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$): 237 (− 6.7), 274 (− 3.2) nm; ^1H (700 MHz) and ^{13}C NMR (175 MHz) data, see Tables 1 and 2, respectively; HRFABMS (positive-ion mode) *m/z* 533.1993 $[M + Na]^+$ (calcd for $C_{25}H_{34}O_{11}Na^+$, 533.1993, error = 0 ppm).

4.3.3. Holophylloside C (*3*)

Colorless gum; $[\alpha]_D^{25}$ +9 (c 0.15, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$): 236 (+14.5), 278 (− 1.9) nm; ^1H (700 MHz) and ^{13}C NMR (175 MHz) data, see Tables 1 and 2, respectively; HRFABMS (positive-ion mode) *m/z* 533.1993 $[M + Na]^+$ (calcd for $C_{25}H_{34}O_{11}Na^+$, 533.1993, error = 0 ppm).

4.3.4. Holophylloside D (*4*)

Colorless gum; $[\alpha]_D^{25}$ −9 (c 0.15, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$): 238 (− 12.8), 277 (− 1.07) nm; ^1H (700 MHz) and ^{13}C NMR (175 MHz) data, see Tables 1 and 2, respectively; HRFABMS (positive-ion mode) *m/z* 533.1993 $[M + Na]^+$ (calcd for $C_{25}H_{34}O_{11}Na^+$, 533.1993, error = 0 ppm).

4.4. Enzymatic hydrolysis of *1*–*4* and identification of their aglycone and sugar unit

Compounds **1**–**4** (1 mg) were individually hydrolyzed with β-glucosidase (10 mg) (almonds, Sigma) in 1 mL of H_2O at 37 °C for 48 h $CHCl_3$ was used to extract organic layers from each reaction mixture. The aglycones **1a**–**4a** were obtained from $CHCl_3$ fractions. The monosaccharides acquired from H_2O-soluble phases were added to pyridine (0.5 mL) containing L-cysteine methyl ester hydrochloride (0.5 mg) and the respect reaction mixtures were stirred at 60 °C for 1 h. Then *o*-tolyl isothiocyanate (0.1 mL) was added and stirred at 60 °C for another 1 h. Each reaction mixture was analyzed without purification by LC-MS analysis (0.7 mL/min; 25% aqueous CH_3CN with 0.1% formic acid for 30 min). The authentic samples of D-xylopyranose and L-xylopyranose were derivatized and analyzed by the same method as described above. The hydrolysate derivatives of **1**–**4** were detected at 17.8 min for D-xylopyranose in the LC-MS analysis, which corresponded with those of D-forms of authentic sugar (17.8 min for D-xylopyranose, 16.7 min for L-xylopyranose).

4.4.1. (7R,8S)-, (7S,8R)-, (7S,8S)-, and (7R,8R)-7,9,9'-trihydroxy-3,3'-dimethoxy-8-O-4'-neolignan (*1a*–*4a*, respectively)

Colorless gum; ^1H NMR (700 MHz) data, see Table 3. HRESIMS (positive-ion mode) *m/z* 401.1580 $[M + Na]^+$ (calcd for $C_{20}H_{26}O_7Na^+$, 401.1571, error = 2.2 ppm) for **1a**, *m/z* 401.1575 $[M + Na]^+$ (calcd for $C_{20}H_{26}O_7Na^+$, 401.1571, error = 1.0 ppm) for **3a**.

4.5. Assays for NGF release from C6 cells

C6 glioma cell lines were used to measure the nerve growth factor (NGF) of the culture medium, which were fixed with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) in an incubator filled with 5% CO_2. The cells were seeded in a 24-well culture plate (1×10^5 cells/well) and incubated for 24 h. The cells were treated with or without 20 μM of the compounds (**1**–**4**), together with serum-free Dulbecco's modified Eagle's medium (DMEM) for another 24 h. Released NGF levels from the supernatants from each cell were measured using an ELISA development kit (R&D System, Minneapolis, MN, USA). Besides, the cell viability was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay by comparison with 6-shogaol as a positive control and the results are expressed as percentage of the control group.

4.6. NO generation from BV-2 cells, and cytotoxicity against four human cell lines

As described in our previous communications [11,26–29,31,32].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tet.2020.131735.

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