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Holophyllin A, a rearranged abietane-type diterpenoid from the trunk of *Abies holophylla*

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ABSTRACT

Holophyllin A (**1**), a novel rearranged abietane-type diterpenoid was isolated, together with a new diterpene glycoside, holophyllin B (**2**), from the trunk of *Abies holophylla*. The structures of **1** and **2** were established by extensive spectroscopic analyses and their absolute configurations were determined by ECD calculation. All the isolates were tested for their inhibitory effects on NO production in LPS-activated murine microglial cells.

Abietane-type diterpenoids are rich in the genus *Abies* (Pinaceae) specifically[1] and associated with a variety of pharmacological effects that include anti-inflammatory, antiproliferative, antiviral, antiplasmodial, and antidepressant antivities.[2–6] Several species in this genus have been used in Korean traditional medicine for the treatment of stomach ache, rheumatic diseases, and vascular diseases.[7] *Abies holophylla* MAXIM (Pinaceae) is a evergreen tree that is widely distributed in Korea, China, and Russia. Our earlier phytochemical investigation on *A. holophylla* resulted in the isolation of anti-inflammatory lignans.[8] In our continuing search for bioactive constituents from Korean medicinal plants, we further isolated a novel rearranged abietane-type diterpene with spiro[4.5]decane structure, holophyllin A (**1**), together with a new diterpene glycoside, holophyllin B (**2**) (Fig. 1) from the *n*-hexane and EtOAc-soluble layers of the MeOH extract of *A. holophylla*, respectively. Shimagaki et al. reported two diterpenes as synthetic intermediates having the same skeleton as **2**.[9,10] However, this Letter described the first isolation of a diterpene possessing the 6/6/7 ring system from nature.

The trunk of *A. holophylla* (5.0 kg) was extracted with 80% aq MeOH (3 × 5 L) under reflux and 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. The *n*-hexane-soluble fraction and EtOAc-soluble fraction were subjected to repeated column chromatography on silica gel, Sephadex LH-20, and semi-preparative HPLC to give compounds **1** (3.2 mg) and **2** (17.2 mg), respectively.

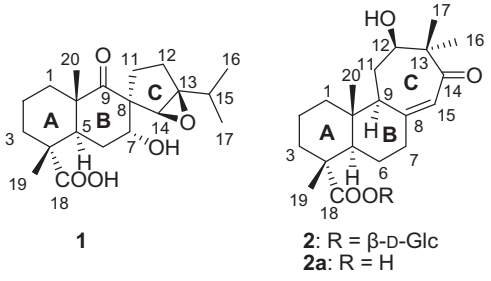

Figure 1. Structures of compounds **1**, **2**, and **2a**.

Holophyllin A (**1**)[11] was obtained as a colorless gum and had a molecular formula of $C_{20}H_{30}O_5$ by the positive ion HRFABMS (*m*/*z* 351.2171 $[M+H]^+$, calcd for $C_{20}H_{31}O_5$ 351.2171), requiring six degrees of unsaturation. The IR spectrum of **1** displayed absorption

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Table 1
^{1}H (700 MHz) and ^{13}C (175 MHz) NMR data for **1** in $CDCl_3$

Position	δ_H, mult (*J* in Hz)	δ_C
1ax	1.88, br d (13.3)	33.5 (CH_2)
1eq	1.50, overlap	
2	1.72, overlap	17.4 (CH_2)
3	1.74, overlap	36.7 (CH_2)
4		47.1 (C)
5	2.65, m	39.0 (CH)
6ax	2.24, br t (14.0)	29.7 (CH_2)
6eq	1.72, overlap	
7	3.96, br s	70.2 (CH)
8		61.1 (C)
9		210.6 (C)
10		47.8 (C)
11a	2.30, dt (13.3, 9.1)	23.1 (CH_2)
11b	1.82, overlap	
12a	1.95, dd (13.3, 9.1)	23.0 (CH_2)
12b	1.69, overlap	
13		72.5 (C)
14	3.35, s	66.4 (CH)
15	1.81, overlap	30.2 (CH)
16	1.02, d (7.0)	19.0 (CH_3)
17	1.02, d (7.0)	18.6 (CH_3)
18		181.4 (C)
19	1.35, br s	16.6 (CH_3)
20	1.31, s	18.6 (CH_3)

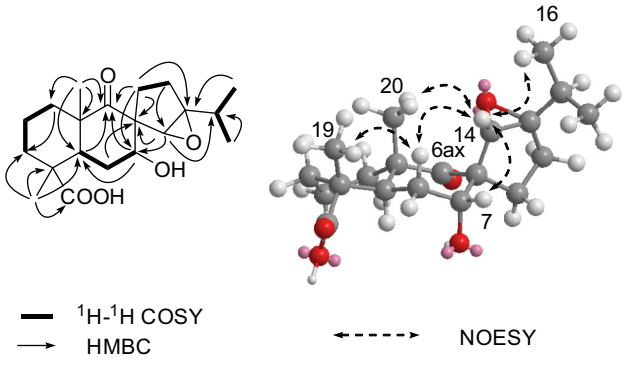


Figure 2. ^{1}H–^{1}H COSY, HMBC, and NOESY correlations of **1**.

characteristic of hydroxy (3384 cm^{-1}) and carbonyl groups (1697 cm^{-1}). The ^{13}C NMR spectrum revealed the presence of 20 carbon signals, which were assigned with the assistance of DEPT spectrum as two sp^2 quaternary carbonyl carbons [δ_C 210.6 and 181.4], four sp^3 methyls [δ_C 19.0, 18.6 (×2), and 16.6], six sp^3 methylenes [δ_C 36.7, 33.5, 29.7, 23.1, 23.0, and 17.4], four sp^3 methines [δ_C 70.2 (C—O), 66.4 (C—O), 39.0, and 30.2], and four sp^3 quaternary carbons [δ_C 72.5 (C—O), 61.1, 47.8, and 47.1]. From the ^{1}H NMR spectrum, the resonances of two oxymethine protons [δ_H 3.96, s; 3.35, s] and four methyls [δ_H 1.35, br s; 1.31, s; 1.02 (×2), d, *J* = 7.0 Hz] were observed. These spectroscopic data (Table 1) suggested an abietane-type origin diterpenoid for **1**.

Analysis of the ^{1}H–^{1}H COSY spectrum established four proton sequences from H_2-1 to H_2-3, H-5 to H-7, H_2-11 to H_2-12, and H_3-16 to H_3-17 (Fig. 2). HMBC correlations of H_3-19 with C-3 and C-5 provided the connection of C-3 and C-5 and formation of ring A was confirmed by HMBC cross-peaks of H_3-20 with C-1, C-5, and C-10. Ring B, which was adjacent to ring A could be deduced from the HMBC correlations of H-5, H-7 and H_3-20 with C-9, and H_2-6 with C-8. The HMBC cross-peaks of H_2-11 with C-7, C-8 and C-9, and H-14 with C-7 and C-8 provided the connection from C-11 to C-14 through the spiro carbon at C-8. The cyclopentane ring C was confirmed by HMBC correlations of H_2-11 with C-13 and H_2-12 with C-14, and the isopropyl group attached at C-13 was

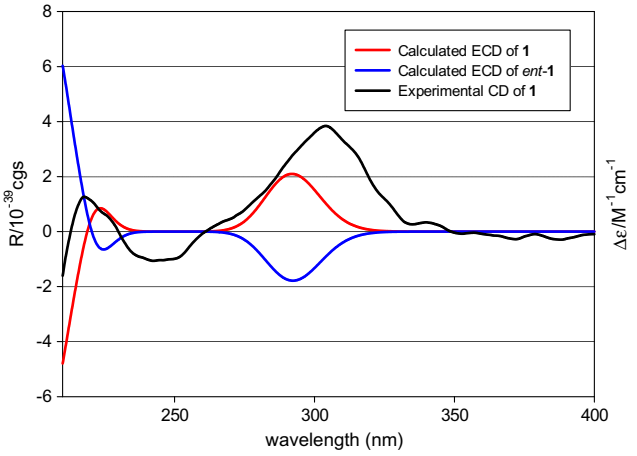


Figure 3. Experimental CD spectra of **1** (black), calculated ECD spectra of **1** (red), and *ent*-**1** (blue).

deduced from the HMBC correlations of H_3-16 and H_3-17 with C-13. The fact that the ^{13}C NMR signals of C-13 (δ_C 72.5) and C-14 (δ_C 66.4) were shifted upfield in comparison with those of normal oxyquaternary and oxymethine carbon groups,[12,13] respectively, implied an epoxide ring was to be located at C-13/C-14.

A β orientation of H_3-19 and H_3-20 was confirmed from the NOE correlations of H-6ax with H_3-19 and H_3-20. The distinct NOE cross-peaks of H-14 with H-6ax, H-7, H_3-16, and H_3-20 indicated a β orientation of H-7 and an α orientation of H-14 and the isopropyl group. Therefore, the relative configuration of **1** was assigned as 4*R**,5*R**,7*R**,8*R**,10*S**,13*S**, and 14*S**. The absolute configuration of **1** was determined by comparing the CD spectrum of **1** and the ECD spectra of the two possible enantiomers of **1**. These spectra were calculated using time-dependent density-functional theory (TD-DFT) at the B3LYP/def2-TZVPP//B3LYP/def-SV(P) level for all

Table 2
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for **2** in CD_3OD

Position	δ_H, mult (*J* in Hz)	δ_C
1ax	1.27, m	38.9 (CH_2)
1eq	1.67, overlap	
2	1.62, overlap	19.3 (CH_2)
3ax	1.89, m	37.7 (CH_2)
3eq	1.62, overlap	
4		49.0 (C)
5	2.10, t (8.0)	50.2 (CH)
6	1.54, m	27.0 (CH_2)
7ax	2.30, overlap	39.5 (CH_2)
8		157.6 (C)
9	2.36, dd (11.0, 5.0)	58.4 (CH)
10		40.8 (C)
11a	1.79, dd (14.3, 5.0)	33.0 (CH_2)
11b	1.64, overlap	
12	3.75, d (8.8)	74.3 (CH)
13		55.9 (C)
14		211.0 (C)
15	5.81, br s	125.8 (CH)
16	1.08, s	27.3 (CH_3)
17	1.12, s	16.7 (CH_3)
18		178.8 (C)
19	1.20, s	17.3 (CH_3)
20	0.71, s	15.2 (CH_3)
1′	5.46, d (8.1)	96.2 (CH)
2′	3.35, overlap	74.1 (CH)
3′	3.41, overlap	78.4 (CH)
4′	3.37, overlap	71.2 (CH)
5′	3.37, overlap	79.0 (CH)
6′a	3.83, d (11.5)	62.5 (CH_2)
6′b	3.68, dd (11.5, 2.2)	

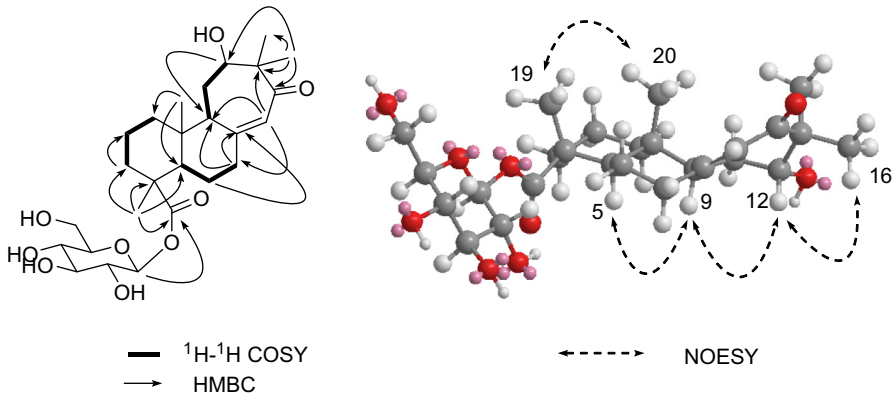


Figure 4. ^{1}H–^{1}H COSY, HMBC, and NOESY correlations of **2**.

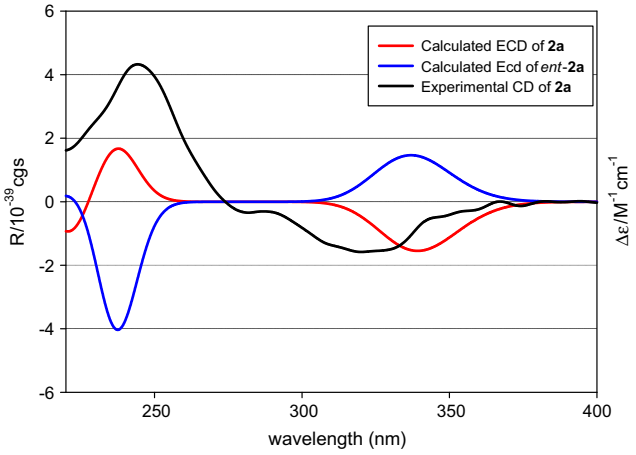
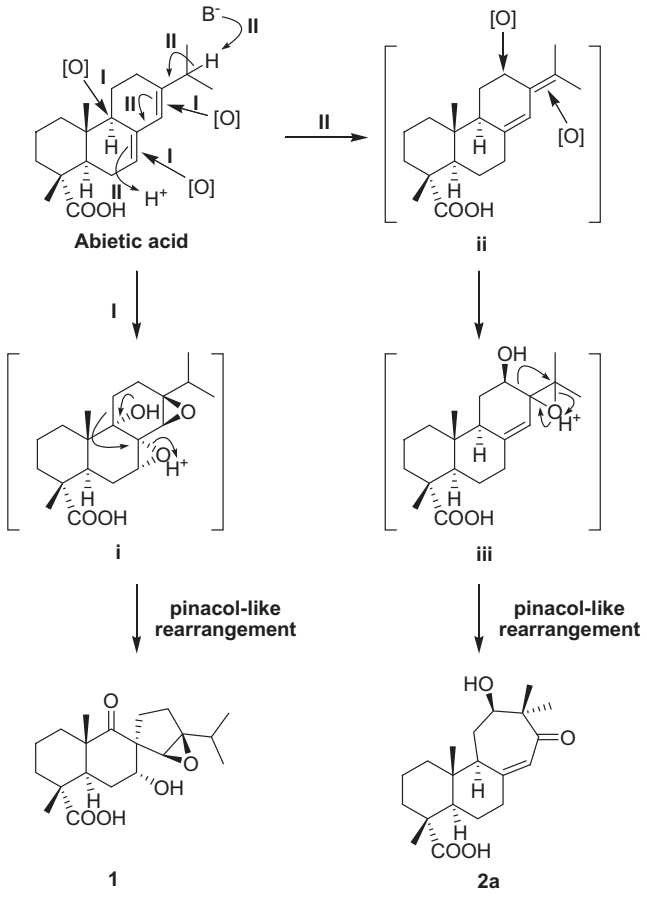


Figure 5. Experimental CD spectra of **2a** (black), calculated ECD spectra of **2a** (red), and *ent*-**2a** (blue).

atoms.[14] The CD spectrum of **1** displayed a positive n–π* Cotton effect at 304 nm ($\Delta\varepsilon = 3.8$), matching well with the calculated ECD spectrum for **1** (Fig. 3). Thus, the absolute configuration of **1** was assigned as 4*R*,5*R*,7*R*,8*R*,10*S*,13*S*, and 14*S*.

Holophyllin B (**2**)[15] was purified as a colorless gum. The molecular formula was determined to be $C_{26}H_{40}O_9$ from the $[M+Na]^+$ ion in the positive ion HRFABMS (*m*/*z* 519.2569; calcd for $C_{26}H_{40}O_9Na$ 519.2570), indicating seven degrees of unsaturation. The presence of α,β-unsaturated ketone was deduced from the UV maximum at 239 nm. The analysis of ^{13}C NMR spectrum revealed that **2** possessed 26 carbons, of which six carbons were characteristic of a glucopyronosyl unit [δ_C 96.2, 79.0, 78.4, 74.1, 71.2, and 62.5]. The remaining 20 carbons were assigned as four sp^2 carbons [δ_C 211.0 (C=O), 178.8 (C=O), 157.6 (C=C), and 125.8 (C=C)], four sp^3 methyls [δ_C 27.3, 17.3, 16.7, and 15.2], six sp^3 methylenes [δ_C 39.5, 38.9, 37.7, 33.0, 27.0, and 19.3], three sp^3 methines [δ_C 74.3 (C–O), 58.4, and 50.2], and three sp^3 quaternary carbons [δ_C 55.9, 49.0, and 40.8] with the assistance of DEPT spectrum. The ^{1}H NMR spectrum showed one olefinic proton [δ_H 5.81, br s], one oxymethine proton [δ_H 3.75, d, J = 8.8 Hz], and four tertiary methyls [δ_H 1.20, 1.12, 1.08, and 0.71 (each s)], as well as one anomeric proton [δ_H 5.46, d, J = 8.1 Hz]. The above spectral data (Table 2) suggested that **2** could be a glycoside of abietane-type origin diterpenoid.

The ^{1}H and ^{13}C NMR data assigned to ring A and B, with the aid of ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY, were similar to those of 8(14)-podocarpen-7,13-dion-18-oic acid.[16] The ^{1}H–^{1}H COSY correlations of H_2-11 with H-9 and H-12 and the HMBC cross-peaks of H_3-16 with C-12, C-13, and C-14 provided the connections from



Scheme 1. Hypothetical biogenetic pathway of **1** and **2a**.

C-9 to C-14 (Fig. 4). The seven-membered ring C was confirmed by HMBC correlations of H-15 with C-7, C-9, and C-13. The large J value of H-1′ (8.1 Hz) suggested that the sugar was a β-glucopyronosyl unit and the HMBC cross-peak of H-1′ with C-18 unambiguously located this sugar on the aglycon.

The relative configuration was deduced from the distinct NOESY correlations of H_3-19 with H_3-20 and H-9 with H-5 and H-12 (Fig. 4). The absolute configuration of **2** was determined through the same method as **1**. The CD spectrum of **2a**,[17] the product of acid hydrolysis of **2**, showed a negative n→π* Cotton effect at 320 nm ($\Delta\varepsilon = -1.6$) and a positive π→π* Cotton effect at 244 nm ($\Delta\varepsilon = 4.3$), corresponding well with the calculated ECD spectrum

for **2a** (Fig. 5). Therefore, the absolute configuration of **2** was assigned as 4*R*,5*R*,9*S*,10*R*, and 12*R*. The D-configuration of sugar was determined by GC/MS analysis.[8]

The biogenetic origin of **1** and **2a** can plausibly be tracked back to the most common compound, abietic acid (Scheme 1). Allylic oxidation at C-9 and epoxidation at C-7/C-8 and C-13/C-14 of abietic acid generates intermediate **i**. After opening of the epoxide ring at C-7/C-8 and a pinacol-like rearrangement, compound **1** might be formed having a spiro[4,5]decane structure. Compound **2a** could be derived through biosynthetic intermediates **ii** and **iii**. Abietic acid underwent isomerization to yield **ii** with the formation of $\Delta^{8(14)}$ and $\Delta^{13(15)}$, and further C-12 and C-14/C-15 may be oxidized and epoxidized, respectively, to give intermediate **iii**. The 6/6/7 ring system of **2a** could be formed through a pinacol-like rearrangement.

Antineuroinflammatory activities of the **1**, **2**, and **2a** were evaluated for their inhibitory effects on NO production in lipopolysaccharide (LPS)-activated murine microglial cells because the *n*-hexane and EtOAc-soluble layers of the MeOH extract of *A. holophylla* showed inhibitory activities on NO production (IC_{50} 8.21 and 38.33 μg/mL, respectively). Compounds **1**, **2**, and **2a** moderately inhibited NO production with IC_{50} values of 18.59, 29.78, and 24.80 μM, respectively, in LPS-stimulated BV-2 cells without cell toxicity (L-NMMA, positive control, IC_{50} 17.24 μM). We also tested the cytotoxicity of the compounds **1**, **2**, and **2a** against A549, SK-OV-3, SK-MEL-2, and HCT-15 human tumor cell lines using the sulforhodamine B (SRB) assays in vitro, but they were inactive (IC_{50} > 10.0 μM).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2014.10.030.

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17. *Holophyllin C* (***2a***)*:* Colorless gum; $[\alpha]_D^{25}$ +43.3 (*c* 0.02, MeOH); IR (KBr) ν_{max} 3381, 2948, 2842, 1648, 1454, 1032 cm^{-1}; UV (MeOH) λ_{max} (log ε): 240 (10.0) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$): 320 (−1.6), 244 (4.4) nm; ^1H NMR (CD_3OD, 500 MHz) δ_H 5.81 (1H, br s, H-15), 3.75 (1H, d, *J* = 8.8 Hz), 2.35 (1H, overlap, H-9), 2.30 (1H, overlap, H-7ax), 2.28 (1H, td, *J* = 14.2, 5.5 Hz, H-7eq), 2.10 (1H, dd, *J* = 12.0, 2.4 Hz, H-5), 1.83 (1H, overlap, H-3ax), 1.79 (1H, dd, *J* = 14.2, 5.5 Hz, H-11a), 1.66 (1H, overlap, H-1eq), 1.64 (1H, overlap, H-11b), 1.59 (2H, overlap, H-2), 1.58 (1H, overlap, H-3eq), 1.55 (2H, overlap, H-6), 1.25 (1H, td, *J* = 12.3, 4.4 Hz, H-1ax), 1.13 (3H, s, H-19), 1.11 (3H, s, H-17), 1.08 (3H, s, H-16), 0.69 (3H, s, H-20); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 211.1 (C-14), 183.9 (C-18), 158.0 (C-8), 125.7 (C-15), 74.3 (C-12), 58.7 (C-9), 55.9 (C-13), 50.6 (C-5), 48.9 (C-4), 40.9 (C-10), 39.7 (C-7), 39.3 (C-1), 38.5 (C-3), 33.0 (C-11), 27.4 (C-6), 27.3 (C-16), 19.6 (C-2), 17.8 (C-19), 16.7 (C-17), 15.1 (C-20); positive HRFABMS *m*/*z* 335.2222 $[M+H]^+$ (calcd for $C_{20}H_{31}O_4$ 335.2222).