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A New Noreudesmane-type Sesquiterpenoid from *Alpinia oxyphylla*

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Alpinia oxyphylla Miq. (Zingiberaceae) is widely cultivated and distributed in southern China. The fruits of this plant have been used as a traditional medicine in Korea and other oriental countries for the treatment of gastrointestinal disorders, urosis, diuresis, ulceration, and dementia. The plants in the genus of *Alpinia* are reported to be rich of diarylheptanoids, sesquiterpenoids, and flavonoids.[1] Previous pharmacological investigations have indicated that the fruits of *A. oxyphylla* possess various activities, including antioxidative,[2,3] anti-inflammatory,[4] anti-angiogenic,[5] anti-ulcer,[6] and neuroprotective activities.[7-9] Recently, diarylheptanoids and sesquiterpenes have been isolated from this plant, and some of which showed the inhibitory effects on nitric oxide production.[10-13] As part of our continuing search for inhibitors of nitric oxide (NO) production from medicinal plants, we investigated the active constituents of the fruits of *A. oxyphylla*. As a result, a new noreudesmane-type sesquiterpenoid, 4-methoxy-oxyphyllenone A (**1**), along with 15 known compounds was isolated and identified. The present paper describes the structure determination of the new compound **1** and the inhibitory effects of the isolated compounds on NO production.

The dried fruits of *A. oxyphylla* were extracted with methanol and fractionated successively with *n*-hexane, CH_2Cl_2, and water. The CH_2Cl_2-soluble fraction was subjected to various column chromatography and led to the isolation of the new noreudesmane-type sesquiterpenoid and 15 known compounds (Figure 1).

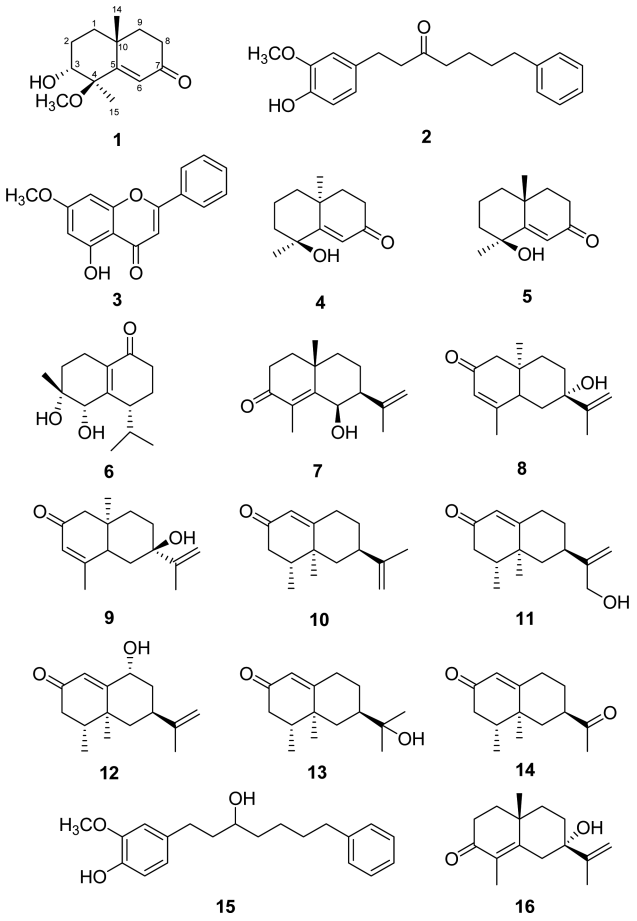

Figure 1. Structures of compounds **1-16**.

Compound **1** was isolated as a yellow gum. The HR-ESI-MS of compound **1** exhibited a protonated molecular ion peak at *m/z* 225.1485 $[M+H]^+$ (calcd. 225.1491) and established the molecular formula of $C_{13}H_{20}O_3$, implying four degrees of unsaturation. The IR spectrum showed absorptions at 3271 and 1647 cm^{-1}, which indicated the presence of a hydroxyl and α,β-unsaturated ketone. The ^1H NMR spectrum of compound **1** displayed the presence of two tertiary methyls at δ_H 1.37 (s, CH_3-15) and 1.41 (s, CH_3-14), an oxygenated methine at δ_H 3.70 (dd, J = 3.0, 3.0 Hz, H-3), a methoxyl group at δ_H 3.06 (s, OCH_3-4), and an olefinic proton at δ_H 5.99 (s, H-6). The ^{13}C NMR and HMQC spectra of compound **1** revealed 13 carbon signals, including a ketone carbon (δ_C 201.2), two olefinic carbons (δ_C 166.3 and 127.8), an oxygenated methine carbon (δ_C 74.5), an oxygenated quaternary carbon (δ_C 78.1), and a methoxy carbon (δ_C 48.6). All the above data indicated that compound **1** was a noreudesmane-type sesquiterpenoid.[10] The planar structure of compound **1** was further determined by 2D NMR experiments such as COSY, HMQC, and HMBC spectra (Figure 2). The ^1H-^1H COSY spectrum revealed the two spin systems corresponding to H-1/H-2/H-3 and H-8/H-9. In the HMBC spectrum, the correlations from CH_3-15 (δ_H 1.37) to C-3 (δ_C 74.5), C-4 (δ_C 78.1), and C-5 (δ_C 166.3), and from CH_3-14 (δ_H 1.41) to C-1 (δ_C 33.9), C-10 (δ_C 35.1), C-9 (δ_C 39.6), and C-5 (δ_C 166.3), clearly indicated that two methyl groups were located at C-4 and C-10, respectively. Further HMBC correlation from OCH_3-4 (δ_H 3.06) to C-4 (δ_C 78.1) revealed the location of a methoxy group at C-4.

The relative stereochemistry of compound **1** was determined from the coupling constant as well as from a NOESY

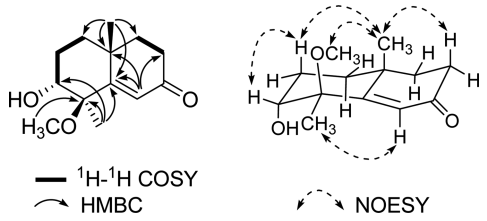

Figure 2. COSY, HMBC and NOESY correlations of compound **1**.

experiment (Figure 2). The small coupling constant of the oxygenated proton at C-3 (J = 3.0 and 3.0 Hz) and the observed NOE correlations between H-3β/H-2β and CH_3-15/H-6 confirmed the α-orientation of CH_3-15 and OH-3, and β-orientation of OCH_3-4, respectively. Further NOE correlations between CH_3-14/H-2β, CH_3-14/H-8β, and CH_3-14/OCH_3-4 clearly indicated the β-configuration of CH_3-14 and OCH_3-4, respectively. Therefore, compound **1** was identified as 4β-methoxy-11,12,13-trinor-5-eudesmene-7-one, and was named 4-methoxy-oxyphyllenone A. The previously known fifteen compounds were identified as yakuchinone A (**2**),[14] tectochrysin (**3**),[15] teuhetenone A (**4**),[16] (4*S**,5*E*,10*R**)-7-oxo-tri-nor-eudesm-5-en-4β-ol (**5**),[17] oxyphyllenodiol B (**6**),[10] (4a*S**,7*S**,8*R**)-8-hydroxy-1,4a-dimethyl-7-(prop-1-en-2-yl)-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one (**7**),[18] 7-*epi*-teucrenone (**8**),[16] teucrenone (**9**),[19] nootkatone (**10**),[20] 13-hydroxynootkatone (**11**),[20] oxyphyllol B (**12**),[21] 11-hydroxyvalenc-1(10)-en-2-one (**13**),[22] (4*R**,5*S**,7*R**)-7-acetyl-4,5-dimethyl-4,5,6,7,8,9-hexahydronaphthalen-2(3*H*)-one (**14**),[22] oxyphyllacinol (**15**)[2] and (4a*S**,7*S**)-7-hydroxy-1,4a-dimethyl-7-(prop-1-en-2-yl)-4,4a,5,6,7,8-hexahydronaphthalen-2(3*H*)-one (**16**)[12] by a comparison of their NMR data with those reported in the literature. Compound **5** was isolated for the first time from this plant.

Table 1. ^{1}H and ^{13}C NMR data for compound **1** (CD_3OD)[a]

Carbon No.	Compound **1**	
	δ_C	δ_H
1	33.9	1.41 (1H, m)
	–	1.85 (1H, m)
2	23.8	2.41 (1H, m)
	–	1.57 (1H, m)
3	74.5	3.70 (1H, dd, J = 3.0, 3.0 Hz)
4	78.1	–
5	166.3	–
6	127.8	5.99 (1H, s)
7	201.2	–
8	33.5	2.67 (1H, m)
	–	2.35 (1H, m)
9	39.6	1.94 (1H, m)
	–	1.75 (1H, m)
10	35.1	–
14	22.7	1.41 (3H, s)
15	17.7	1.37 (3H, s)
OCH_3-4	48.6	3.06 s

[a]Measured at 500 and 125 MHz. The assignments were based on the COSY, HMQC and HMBC experiments.

All the isolates were tested for their inhibitory effects on NO production in LPS-induced RAW 264.7 cells. The results showed that compounds **2**, **11** and **15** exhibited significant inhibitory activity with IC_{50} values of 27.4, 38.2 and 38.1 μM, respectively. Compounds **3**, **10** and **16** showed moderate inhibitory activity on NO production with IC_{50} values of 69.4, 56.9 and 78.3 μM, respectively. Aminoguanidine was used as a positive control (IC_{50} value of 18.5 μM). However, the other compounds showed no inhibitory activity in this assay (IC_{50} values > 100 μM). Cell viability assay indicated that none of the compounds exhibited significant cytotoxicity at their effective concentration for the inhibition of NO production (data not shown). It has been reported that several diaryheptanoid and sesquiterpenoid from the fruits of *A. oxyphylla* were found to have inhibitory activity on NO production.[10-13,22] Taken together, our results suggested that *A. oxyphylla* may be a possible candidate for the treatment of inflammatory disease.

Experimental

General Procedures. IR spectra were recorded on a JASCO FT-IR 4100 spectrophotometer (Jasco, Japan). Optical rotations were measured using a JASCO DIP-1000 polarimeter (Jasco, Japan). The NMR spectra were recorded on Bruker ADVANCE III 400 MHz and ADVANCE 500 MHz (Bruker, Germany) spectrometers. The HR-ESI-MS spectra were performed on maXis 4G mass spectrometer (Bruker, Germany). Preparative HPLC was performed using a Waters 515 HPLC Pump with a Waters 2996 Photodiode-array detector and YMC J'sphere ODS-H80 column (4 μm, 150 × 20 mm, USA). Column chromatography was performed using a silica gel (70-230 mesh, Merck) and Lichroprep RP-18 (40-63 μm, Merck). TLC was performed using aluminum plates precoated with Kieselgel 60 F254 (Merck).

Plant Materials. The dried fruits of *A. oxyphylla* were purchased from Kyungdong herbal market, Seoul, Korea, in March 2011. The plant material was identified by Kyong Soon Lee, Emeritus Professor, Chungbuk National University. A voucher specimen (CBNU-11AO) was deposited at the Herbarium of the College of Pharmacy, Chungbuk National University, Korea.

Extraction and Isolation. Dried fruits of *A. oxyphylla* (3 Kg) were extracted with three times MeOH at room temperature. After filtration and evaporation of the solvent under reduced pressure, the combined methanol extract (470 g) was suspended in water (1.5 L), and partitioned with *n*-hexane, CH_2Cl_2 and EtOAc to afford extracts of *n*-hexane (66.3 g), CH_2Cl_2 (156.1 g), EtOAc (24.6 g) and water layer, respectively. The CH_2Cl_2-soluble extract (156.1 g) was subjected to a silica gel column chromatography with a solvent system (*n*-hexane:CH_2Cl_2 = 2:1 to CH_2Cl_2:MeOH = 1:1) to give six fractions (AOC1 - AOC6). Fraction 1 (35.5 g) was separated over a silica gel column with a solvent system (*n*-hexane:CH_2Cl_2 = 5:1 to CH_2Cl_2:MeOH = 1:1) to give

seven subfractions (AOC1-1 - AOC1-7). AOC1-4 (8 g) was chromatographed on a silica gel column using a gradient of CH_2Cl_2:MeOH (1:0 to 0:1) to obtain ten subfractions. AOC1-4-7 (3 g) was purified by a silica gel column chromatography with a gradient of CH_2Cl_2:MeOH (1:0 to 0:1) to afford compounds **10** (251.3 mg) and **2** (1 g). Compound **3** (103 mg) was obtained from AOC1-4-7 by recrystallization in MeOH. AOC1-5 (5 g) was subjected to a MCI gel MPLC with a gradient of H_2O:acetone (1:0 to 0:1) to afford five subfractions (AOC1-5-1 - AOC1-5-5). AOC1-5-3 (106 mg) was separated by preparative HPLC with a gradient of MeCN:H_2O (1:5 to 1:0) to afford compounds **1** (7 mg) and **14** (3 mg). Fraction 2 (5 g) was subjected to a RP gel MPLC using a gradient of MeOH:H_2O (3:7 to 1:0) to yield seven subfractions (AOC2-1 - AOC2-7). AOC2-3 (300 mg) was separated by using preparative HPLC with a gradient of MeCN:H_2O (1:5 to 1:0), resulting in the isolation of compounds **4** (2.1 mg), **5** (5.2 mg), **8** (10 mg), and **13** (3.7 mg). AOC2-4 (350 mg) was purified by using preparative HPLC with a gradient of MeCN:H_2O (1:5 to 1:0) to yield compounds **9** (3 mg), **11** (1.1 mg) and **16** (3 mg). AOC2-5 (300 mg) was also purified by using preparative HPLC with gradient of MeCN:H_2O (2:5 to 1:0) to yield compound **7** (4 mg). AOC2-6 (215 mg) was separated by using preparative HPLC with gradient of MeCN:H_2O (2:5 to 1:0) to yield compounds **12** (5 mg) and **15** (4 mg). Fraction 4 (26.1 g) was chromatographed on a silica gel column eluted with a solvent system (*n*-hexane:CH_2Cl_2 = 2:1 to CH_2Cl_2:MeOH = 1:1) to give four fractions (AOC4-1 - AOC4-4). AOC4-1 (3 g) was subjected to a RP gel MPLC using a gradient of H_2O: MeCN (1:0 to 4:6) to yield ten subfractions (AOC4-1-1 - AOC4-1-10). AOC4-1-5 (100 mg) was purified by using preparative HPLC with gradient of MeCN:H_2O (2:5 to 5:5) to yield compound **6** (6.5 mg).

4-Methoxy-oxyphyllenone A (1): Yellow gum; $[\alpha]_{20}^{D}$ +59.0 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3271, 1647 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD), see Table 1; HR-ESI-MS *m/z*: 225.1485 $[M+H]^+$ (calcd. for $C_{13}H_{21}O_3$, 225.1491).

Measurement of NO Production and Cell Viability Assay. The nitrite concentration in the medium was determined as an indicator of NO production according to the Griess method previously described.[23] Briefly, RAW 264.7 cells were seeded into 96-well tissue culture plates at a density of 2×10^5 cells/mL, and stimulated with 1 μg/mL of LPS in the presence or absence of compounds. After incubation at 37 °C and 5% CO_2 atmosphere for 24 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess reagent containing equal volumes 0.2% (w/v) sulfanilamide in 5% (w/v) phosphoric acid and 0.2% (w/v) of *N*-(1-naphthyl)ethylenediamine solution to determine nitrite production. The absorbance was measured at 540 nm by a microplate reader. The remaining cells after Griess assay were used for viability with the CCK (Dojindo, Tokyo, Japan)-based colorimetric assay.

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Supporting Information. ^1H-, ^{13}C-NMR, COSY, HMQC, HMBC, NOESY, and HR-ESI-MS spectra of **1** are available as Supporting Information.

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