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A new bibenzyl and a new methylflavan from the tubers of *Bletilla striata*

Jin Woo Lee [a], Jun Gu Kim [a], Dongho Lee [b], Mi Kyeong Lee [a], Bang Yeon Hwang [a,*]

[a] College of Pharmacy, Chungbuk National University, Cheongju 28160, Republic of Korea
[b] Department of Plant Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea

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ABSTRACT

A new bibenzyl, (+)-(1*S*)-4′,5″-dihydroxy-1,3′,5′,3″-tetramethoxybibenzyl (**1**), and a new methylflavan, (2*S*)-7,4′-dihydroxy-5-methoxy-8-methylflavan (**2**), together with 10 known compounds were isolated from the methanolic extract of the tubers of *Bletilla striata*. Their chemical structures were elucidated based on spectrometric data interpretation, especially 1D and 2D NMR, HRESIMS, and electronic circular dichroism (ECD) data. All isolates (**1** - **12**) were evaluated for their inhibitory properties on nitric oxide production induced in RAW 264.7 cells, and dihydrophenantrenes **4** and **6** exhibited the best inhibitory effects with IC_{50} values of 29.5, and 6.5 μM, respectively. Additionally, significant activity possessed phenantrene **5** and methylflavane **2** with IC_{50} values of 38.9, and 44.9 μM, respectively.

1. Introduction

Bletilla striata (Thunb.) Reichb. f., (Orchidaceae) is a perennial herbaceous medicinal plant widely distributed in Korea, mainland China, and Japan (He et al., 2017). Plants in the genus *Bletilla* are reported as rich sources of phenanthrenes, biphenanthrenes, phenanthraquinones, lignans, bibenzyls, flavonoids, steroidal saponins, stilbenes, and polysaccharides (Bai et al., 1991, 1993; Yamaki et al., 1993a, 1993b; Feng et al., 2008; Park et al., 2014; Xu et al., 2019; Qian et al., 2015; Jiang et al., 2019; Zhou et al., 2019, 2020). Of these, phenanthrenes and 9, 10-dihydrophenanthrenes are a class of secondary metabolites with limited distribution in the Orchidaceae, Dioscoreaceae, and Combretaceae families (Xu et al., 2019; Sun et al., 2021; Ngan et al., 2020; Malan et al., 1993). Pharmacological investigations of the chemical constituents in the genus *Bletilla*, including phenanthrenes and dihydrophenanthrenes of *B. striata*, revealed a variety of biological activities, including hemostatic, wound-healing, cytotoxic, antibacterial, antiviral, and antioxidant activities (Park et al., 2014; Qian et al., 2015; Wang and Meng, 2015; He et al., 2017; Song et al., 2017; Jiang et al., 2019; Xu et al., 2019; Zhou et al., 2019, 2020, and Sun et al., 2021). To date, several types of bibenzyls and phenanthrene isolated from the genus *Bletilla* have been tested for their anti-inflammatory activities as well. For example, bleochrins A-J obtained from *Bletilla ochracea* Schltr have been tested, bleochrins A, B and F exhibited anti-inflammatory effects with IC_{50} values of 24.0, 18.1 and 16.8 μM, respectively (Li et al., 2018). Other 34 phenanthrene and 9,10-dihydrophenenthrene derivatives from the aqueous EtOH extract of *Bletilla striata* have been tested, and among them phochinenin K, bleformin F and 4,8,4’,8’-tetramethoxy-(1, 1’-biphenanthrene)-2,7,2’,7’-tetrol have been shown to possess potent anti-neuroinflammatory activities with IC_{50} values of 1.9, 5.0 and 1.0 μM, respectively (Zhou et al., 2019). An additional investigation is blestanonls A-M together with 12 known compounds, which were isolated from the tuber of *Bletilla striata*, and among them, 14 compounds exhibit inhibition of NO production with IC_{50} values of 1.4–8.3 μM (Sun et al., 2021). Also, recently, our group reported the isolation of two new C-methylated flavan-3-ols from the tubers of *B. striata* and their inhibitory effects on nitric oxide (NO) production (Bae et al., 2017).

In the present study, further investigation of potential bioactive compounds from *B. striata* was carried out, and a new bibenzyl, (+)-(1*S*)-4′,5″-dihydroxy-1,3′,5′,3″-tetramethoxybibenzyl (**1**), and a new methylflavan, (2*S*)-7,4′-dihydroxy-5-methoxy-8-methylflavan (**2**), together with 10 known metabolites were isolated and determined (Fig. 1). Moreover, the inhibitory effects on LPS-induced NO production in RAW 264.7 cells of all isolates were evaluated.

2. Results and discussion

Compound **1** was obtained as a colorless oil. The molecular formula was established as $C_{18}H_{22}O_6$ by HRESIMS (*m/z* 357.1306 $[M + Na]^+$, calcd for $C_{18}H_{22}NaO_6$, 357.1309) and ^{13}C NMR data (Table 1), indicating eight indices of hydrogen deficiency. The ^1H NMR data of **1** displayed signals for the five aromatic protons at δ_H 6.51 (2H, s, H-2′,6′),

* Corresponding author.
E-mail address: byhwang@chungbuk.ac.kr (B.Y. Hwang).

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6.17 (2H, br s, H-2″,6″), and 6.13 (1H, t, J =2.4 Hz, H-4″), one oxymethine proton at δ_H 4.24 (1H, dd, J = 7.6, 5.6 Hz, H-1), four methoxy groups at δ_H 3.72 (6H, s, OMe-3′,5′), 3.63 (3H, s, OMe-3″), and 3.06 (3H, s, OMe-1), two methylene protons at δ_H 2.86 (1H, dd, J = 14.0, 7.6 Hz, Ha-2) and 2.69 (1H, dd, J = 14.0, 5.6 Hz, Hb-2) (Table 1). The ^{13}C NMR and HSQC spectra of **1** revealed 18 carbon resonances, which were assignable to 12 aromatic carbons, one oxymethine carbon, four methoxy carbons, and one methylene carbon. In the ^{1}H and ^{13}C NMR spectra, the proton signals at δ_H 6.51 (2H, s, H-2′,6′) and δ_H 3.72 (6H, s, OMe-3′,5′), and the carbon signals at δ_C 148.2 (C-3′,5′), 135.2 (C-4′), 104.6 (C-2′,6′), and 56.4 (OMe-3′,5′) indicated the existence of a symmetrically 1′,3′,4′,5′-tetrasubstituted benzene ring with 3′,5′-dimethoxy and 4′-hydroxy groups, which was further corroborated by the HMBC correlation from two methoxy protons at δ_H 3.72 to C-3′ and C-5′ at δ_C 148.2 (Fig. 2). Additionally, a symmetrically 1″,3″,5″-trisubstituted benzene ring with 3″-methoxy and 5″-hydroxy groups was evident from the proton signals at δ_H 6.17 (2H, br s, H-2″,6″) and 6.13 (1H, t, J =2.4 Hz, H-4″), and the carbon signals at δ_C 160.4 (C-3″), 158.4 (C-5″), 109.4 (C-6″), 106.3 (C-2″), and 99.3 (C-4″). This structure was further confirmed by the observed HMBC correlations from H-4″ to C-2″, C-3″, C-5″, and C-6″, and from OH-5″ to C-4″, C-5″, and C-6″ (Fig. 2). The remaining oxymethine proton at δ_H 4.24 (1H, dd, J = 7.6, 5.6 Hz, H-1) coupled with two geminal methylene protons at δ_H 2.86 (1H, dd, J = 14.0, 7.6 Hz, Ha-2) and 2.69 (1H, dd, J = 14.0, 5.6 Hz, Hb-2) suggested that one methylene of the bibenzyl moiety was connected with an oxygenated carbon. The HMBC correlation from methoxy proton at δ_H 3.06 to the oxymethine carbon at δ_C 84.4 supported the position of a methoxy group at C-1 (Fig. 2). This proposed structure was also supported by the NOESY correlations of H-2',6'/1,3',5'-OMe and 3''-OMe/H-2'',4''. The absolute configuration at C-1 in **1** was established as *S* according to its optical rotation ($[\alpha]_D^{25}$ +4.5) and the positive Cotton effect at 230 nm ($\Delta\varepsilon$ +0.36) (Fig. S7, Supplementary data), which were similar to those of dendrocandin C and nobilin B, and opposite to those of dendrocandin A and loddigesiinol C obtained from other Orchidaceae species (Zhang et al., 2006; Li et al., 2008, 2009; Ito et al., 2010). Therefore, the structure of **1** was determined as (+)-(1*S*)-4′,5″-dihydroxy-1,3′,5′,3″-tetramethoxybibenzyl.

Compound **2** was obtained as a white amorphous powder, and the molecular formula was determined to be $C_{17}H_{18}O_4$ from the HRESIMS (m/z 287.1268 $[M+H]^+$, calcd for $C_{17}H_{19}O_4$, 287.1278) and ^{13}C NMR data (Table 2). The ^{1}H NMR data of **2** showed signals for a 1,4-disubstituted benzene group at δ_H 7.21 (2H, d, J =8.4 Hz, H-2′,6′) and 6.75 (2H, d, J =8.4 Hz, H-3′,5′), an isolated aromatic singlet at δ_H 6.05 (1H, s, H-6), an oxymethine proton at δ_H 4.87 (1H, d, J =10.0 Hz, H-2), four methylene protons at δ_H 1.80 (1H, m, H-3a), 2.06 (1H, m, H-3b), and 2.52 (2H, m, H-4), a methyl group at δ_H 1.87 (3H, s, Me-8), and a methoxy group at δ_H 3.67 (3H, s, OMe-5). The ^{13}C NMR and HSQC spectra of **2** revealed the presence of 17 carbon signals, corresponding to 12 aromatic carbons, an oxygenated methine carbon, two methylene carbons, a methyl carbon, and a methoxy carbon. The position of the methyl group at C-8 was confirmed by the HMBC correlation from Me-8 to C-7, C-8, and C-8a (Fig. 3). The HMBC correlation from OMe-5 to C-5 suggested that the methoxy group was located at C-5 position (Fig. 3). The absolute configuration at C-2 was assigned as *S* by its optical rotation, $[\alpha]_D^{25}$ -20.6, opposite that of (2*R*)-7,4′-dihydroxy-5-methoxy-8-methylflavan (Awale et al., 2009). Although the structure of **2** having a negative $[\alpha]_D^{25}$ value was reported by Xu et al. in 2016, details of its structure elucidation had not described because it was misinterpreted as a known structure (2*R*) in the paper (Xu et al., 2016). The ECD data of **2**, which showed a negative Cotton effect at 280 ($\Delta\varepsilon$ -0.62) nm (Fig. S15, Supplementary data), was further confirmed the 2*S* absolute configuration. Therefore, compound **2** was identified as (2*S*)-7,4′-dihydroxy-5-methoxy-8-methylflavan.

In addition, the 10 known compounds were identified as 7,4′-

Table 1
^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for **1** in DMSO-d_6[a].

Position	δ_C	δ_H (J in Hz)
1	84.4	4.24, dd (7.6, 5.6)
2	44.3	2.86, dd (14.0, 7.6) 2.69, dd (14.0, 5.6)
1'	132.1	–
2', 6'	104.6	6.51, s
3', 5'	148.2	–
4'	135.2	–
1"	141.3	–
2"	106.3	6.17, br s
3"	160.4	–
4"	99.3	6.13, t (2.4)
5"	158.4	–
6"	109.4	6.17, br s
OMe-1	55.2	3.06, s
OMe-3',5'	56.4	3.72, s
OMe-3"	56.3	3.63, s
OH-4'	–	8.24
OH-5"	–	9.21

[a] Assignments are supported with COSY, HSQC, and HMBC experiments.

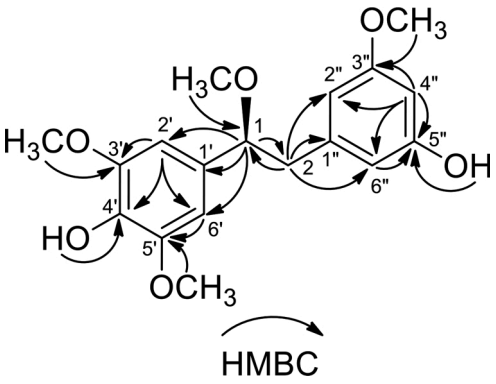

Fig. 2. Key HMBC correlations of compound **1**.

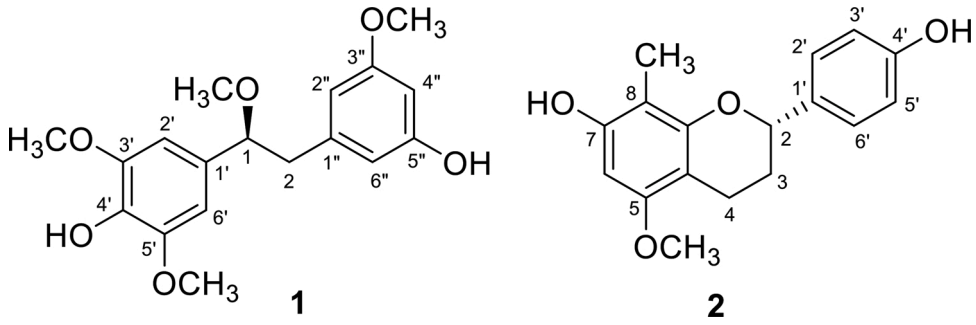

Fig. 1. Structures of compounds **1** and **2**.

Table 2
^{1}H (900 MHz) and ^{13}C NMR (225 MHz) spectroscopic data for **2** in DMSO-d_6[a].

Position	δ_C	δ_H (*J* in Hz)
1	–	–
2	76.2	4.87, d (10.0)
3	28.6	1.80, m
		2.06, m
4	18.9	2.52, m
4a	100.8	–
5	155.0	–
6	90.9	6.05, s
7	153.8	–
8	102.6	–
8a	153.6	–
1'	132.1	–
2', 6'	127.0	7.21, d, (8.4)
3', 5'	114.9	6.75, d, (8.4)
4'	156.7	–
OMe-5	54.9	3.67, s
Me-8	7.9	1.87, s
OH-7	–	8.97, s
OH-4'	–	9.38, s

[a] Assignments are supported with COSY, HSQC, and HMBC experiments.

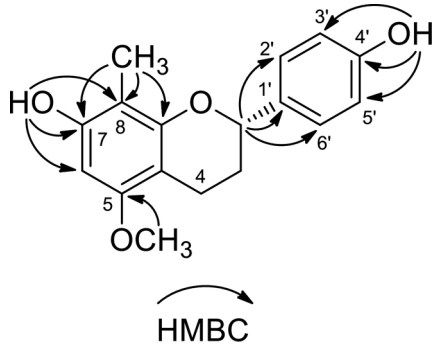

Fig. 3. Key HMBC correlations of compound **2**.

dihydroxy-8-methylflavan (**3**) (Ioset et al., 2001), flavanthridin (**4**) (Majumder and Banerjee, 1990), 2,8-dihydroxy-3,4,7-trimethoxyphenanthrene (**5**) (Yang et al., 2007), coelonin (**6**) (Majumder et al., 1982), farrerol (**7**) (Youssef et al., 1998), 1-(4-hydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-one (**8**) (Suksamrarn et al., 2008), 1-(4-hydroxyphenyl)-7-phenyl-(6*E*)-6-hepten-3-ol (**9**) (Suksamrarn et al., 1994), eucomic acid dimethyl ester (**10**) (Heller et al., 1974), 3, 4-dihydroxyallylbenzene (**11**) (Rathee et al., 2006), and *p*-hydroxy-benzaldehyde (**12**) (Kim et al., 2003) by comparison of their observed and reported spectroscopic data. Among these known metabolites, compounds **3**, **4** and **7–9** were found in the genus *Bletilla* for the first time.

Excessive production of nitric oxide (NO), generated by inducible NO synthase in macrophages, may cause a variety of inflammatory diseases. Therefore, inhibition of excessive NO production has been considered as therapeutic strategy for anti-inflammatory agents (Minhas et al., 2020). The MTT-based cell viability assay indicated that inhibition of NO production was not caused by cytotoxic effects of all tested compounds (data not shown). Compounds **1-12** were tested for their ability to inhibit NO production in LPS-stimulated RAW 264.7 macrophages. Aminoguanidine was used as positive control (IC_{50} = 18.6 μM) (Table 3). In order to detect clear structure-activity trends, we used other bibenzyls, phenanthrenes and dihydrophenanthrenes reported in our previous investigation to extent the number of the comparable structures. In general, the bibenzyl compounds (IC_{50} values of >50) were less potent than the phenanthrenes (average IC_{50} = 23.7 μM) and dihydrophenanthrenes (average IC_{50} = 51.2 μM) (Bae et al., 2017). In the phenanthrene skeletons, compounds **4** and **6** showed relatively

Table 3
Inhibitory effects of **1-12** on LPS-induced NO production in RAW 264.7 cells[a].

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	>50	**7**	>50
2	44.9	**8**	>50
3	>50	**9**	>50
4	29.5	**10**	>50
5	38.9	**11**	>50
6	6.5	**12**	>50

Aminoguanidine was used as the positive control (IC_{50} = 18.6 μM).
[a] Results are expressed as the mean IC_{50} values in μM from triplicate experiments.

stronger inhibition on NO production with IC_{50} values of 29.5 and 6.5 μM, respectively, as compared with compound **5** with IC_{50} values of 38.9 μM. These results indicated that the presence of $\Delta^{9(10)}$ double bond in the phenanthrene skeleton seems responsible for the loss of activity, which is supported by the fact that 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol showed better activity than 3,7-dihydroxy-2,4-dimethoxyphenanthrene and 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene (Bae et al., 2017). However, particular features of the presence or absence of hydroxyl or methoxy group in the aromatic rings didn't seem to impact biological activity in those structures. Therefore, dihydrophenantrenes **4** and **6** from *B. striata* have potential for further development as anti-inflammatory agents.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-1000 polarimeter. ECD spectra were obtained on a JASCO J-715 spectrometer. 1D and 2D NMR spectra were recorded on a Bruker AVANCE 400 and 900 MHz spectrometers using DMSO-d_6 as a solvent. HRESIMS were recorded on a Bruker maXis 4 G or Waters Q-Tof Premier mass spectrometer. Silica gel (70-230 mesh; Merck) was used for open column chromatography. MPLC was performed using a Biotage Isolera system with Lichroprep RP-18 (40−63 μm, Merck). Semi-preparative HPLC was performed on a Waters system (two 515 pumps and 2996 photodiode-array detector) with a YMC J'sphere ODS-H80 column (150 × 20 mm, i.d., 4 μm), using a mixed solvent system of CH_3CN-H_2O at a flow rate of 6 mL/min. TLC was performed with pre-coated silica gel 60 F_{254} aluminum sheets (0.25 mm, Merck, Darmstadt, Germany), and the spots were visualized under UV light and by spraying with 10 % vanillin-H_2SO_4 in water reagent followed by heating.

3.2. Plant material

The dried tubers of *B. striata* were obtained from Kyung-dong herbal market, Seoul, Korea, in April 2016 and identified by B. Y. Hwang. A voucher specimen (CBNU 2016-04BS) has been deposited with the Herbarium of College of Pharmacy, Chungbuk National University, Korea.

3.3. Extraction and isolation

The dried and powdered tubers of *B. striata* (5.4 kg) were extracted with MeOH (3 × 18 L) at room temperature for 3 days. The extract was evaporated under reduced pressure, and the residue (1080 g) was suspended in water and partitioned successively with hexane (2 × 2 L), CH_2Cl_2 (2 × 2 L), and EtOAc (2 × 2 L). The ethylacetate-soluble fraction (20.1 g) was chromatographed on a silica gel column and eluted with CH_2Cl_2-MeOH gradient system (100:0 to 0:100) to obtain nine fractions, BSE1 – BSE9. BSE2 fraction (1.2 g) was separated on MPLC (RP-18) and eluted with MeOH-H_2O (30:70 to 100:0) to obtain nine fractions, BSE2-1 – BSE2-9. BSE2-4 fraction (30 mg) was further purified by semi-

preparative HPLC (MeCN-H_2O, 20:80 to 40:60) to yield compound **11** (1 mg). BSE2-7 fraction (80 mg) was further separated by semi-preparative HPLC (MeCN-H_2O, 40:60 to 60:40) to yield compounds **4** (10 mg), **5** (2 mg), and **3** (1 mg). BSE2-8 fraction (100 mg) was further separated by semi-preparative HPLC (MeCN-H_2O, 50:50 to 70:30) to yield compounds **2** (8 mg), **7** (9 mg), **8** (3 mg), and **9** (2 mg). BSE3 fraction (1.1 g) was chromatographed on MPLC (RP-18) and eluted with MeOH-H_2O (20:80 to 100:0) to yield ten fractions, BSE3-1 – BSE3-10. BSE3-3 fraction (40 mg) was purified by semi-preparative HPLC (MeCN-H_2O, 30:70 to 50:50) to obtain compound **12** (5 mg). BSE3-5 fraction (50 mg) was purified by semi-preparative HPLC (MeCN-H_2O, 40:60 to 60:40) to obtain compound **10** (5 mg). BSE3-7 fraction (100 mg) was further purified by semi-preparative HPLC (MeCN-H_2O, 40:60 to 60:40) to afford compounds **1** (3 mg) and **6** (8 mg).

3.3.1. (+)-(1S)-4′,5″-Dihydroxy-1,3′,5′,3″-tetramethoxybibenzyl (1)

Colorless oil; $[\alpha]_D^{25}$ +4.5 (c = 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 230 (+0.36), 300 (−0.11), 320 (+0.12), 360 (−0.16); ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6), see Table 1; HRESIMS: m/z 357.1306 $[M + Na]^+$, calcd 357.1309.

3.3.2. (2S)-7,4′-dihydroxy-5-methoxy-8-methylflavan (2)

White amorphous powder; $[\alpha]_D^{25}$ −20.6 (c = 0.1, MeOH); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 230 (−2.35), 280.0 (−0.62); ^{1}H NMR (900 MHz, DMSO-d_6) and ^{13}C NMR (225 MHz, DMSO-d_6), see Table 2; HRESIMS: m/z 287.1268 $[M+H]^+$, calcd 287.1278.

3.4. Measurement of LPS-induced NO production and cell viability

RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL. Louis, MO, USA) with 10 % heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL) at 37 °C humidified air containing 5% CO_2. Cell viability and NO production were tested using MTT and Griess assays, respectively, as described in our previous study (Le et al., 2021).

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2021.06.007.

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