A new flavone C-glycoside and a new bibenzyl from *Bulbophyllum retusiusculum*

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**ABSTRACT**

A new flavone C-glycoside, apigenin 6-C-\(\alpha\)-arabinofuranosyl 8-C-\(\alpha\)-arabinopyranoside (1) and a new bibenzyl, bulbotetusine (2), were isolated from the tubers of *Bulbophyllum retusiusculum*. Their structures were established on the basis of extensive spectroscopic analyses. The absolute configuration of 2 was determined by the comparison of experimental and calculated electronic circular dichroism. Compounds 1 and 2 showed no obvious cytotoxic activity against any five human tumour cell lines with IC\(_{50}\) values >40 \(\mu\)M.

**1. Introduction**

Plants of the Orchidaceae are known to be a rich source of stilbenoids such as bibenzyls, phenanthrenes and 9,10-dihydrophenanthrenes (Majumder & Kar 1987; Leong et al. 1999; Zhang et al. 2007). The genus *Bulbophyllum*, belonging to the subtribe Bulbophyllinae of the Orchidaceae consists of over 1000 species found in Africa and Asia (Leong et al. 1997). Some *Bulbophyllum* plants were used as folk medicines to treat tuberculosis, chronic inflammation...
and fracture (Xu et al. 2009). The phytochemical research showed that phenanthrene derivatives and bibenzyls are the predominant constituents of this genus (Wang et al. 2014, Zhao et al. 2015). *Bulbophyllum retusiusculum*, a species from genus *Bulbophyllum*, is widely distributed in the Yunnan Province of China growing at an elevation of 500–2800 m. To the best of our knowledge, there has been no phytochemical research on this plant. To explore the new and bioactive constituents from genus *Bulbophyllum*, phytochemical investigation on the tubers of *B. retusiusculum* was carried out to afford a new flavone C-glycoside, which was rare in *Bulbophyllum* (Majumder & Sen 1991; Sun et al. 2014), and a new bibenzyl (Figure 1). Their cytotoxic activities were also investigated. This paper deals with the isolation and structural elucidation of these two new compounds from *B. retusiusculum*.

2. Results and discussion

Compound 1 was obtained as a yellowish powder that appears on TLC as a yellow spot after treatment with anisaldehyde/sulphuric acid. The negative HR-ESI-MS spectrum showed a quasimolecular peak [M – H]− at 533.1299 m/z, corresponding to the molecular formula C25H26O13, supported also by 13C nMR spectrum. The IR spectrum of 1 exhibited the characteristic absorption of hydroxyl group at 3416 cm−1 and carbonyl moiety at 1632 cm−1. Of the 25 carbons, 15 were assigned to the aglycon and 10 to the oligosaccharide moiety. In the 1H NMR spectrum obtained in DMSO-d6, a four-proton AB system was present (δH 6.88, 2H, d, J = 8.4 Hz; 8.30, 2H, d, J = 8.4 Hz), typical of a 1′,4′-disubstituted ring B of a flavonoid nucleus. Ring A and ring C showed only a resonance as one-proton singlet at δH 6.85, which was assigned to H-3 on the basis of HMBC correlations from H-3 to C-2 (δC 164.3 C), C-4 (δC 182.3 C), C-10 (δC 103.2 C), and C-1′ (δC 121.0 C). Thus, it can be concluded that ring A was a six-substituted benzene, which was consistent with an apigenin skeleton bearing two additional substituents on C-6 and C-8, respectively (Xie et al. 2003). Furthermore, the signals of two anomeric sugar protons were clearly visible as two doublets at δH 4.58 (J = 9.8 Hz) and δH 5.43 (J = 2.9 Hz). Analysis of the 13C NMR spectrum identified the two sugar units as α-arabinofuranose and α-arabinopyranose. The uncommon high field shift of two anomeric carbons (δC 74.4 CH, 80.0 CH) suggested the hypothesis of two C-glycosidic links (Pan et al. 2004; Vitalini et al. 2011), which was further supported by the larger coupling constants.
of two anomeric protons (Vitalini et al. 2011) comparing with normal O-glycosidic links of α-arabinofuranose (Hasan et al. 1996) and α-arabinopyranose (Mihci-Gaidi et al. 2010). NOE correlations (Figure S1) of H-1′′ (δ_H 5.43, d, J = 2.9 Hz) with H-4′′ (δ_H 3.89 m) and H-2′′ (δ_H 4.07, d, J = 2.1 Hz) with OH-3′′ (δ_H 3.93 brs) also suggested the α configuration of arabinofuranose. NOE correlations from H-1′′ to H-3′′, H-2′′ to OH-3′′ and H-2′ further supported that the configuration of arabinopyranose was α. Two glycosidic positions were established at C-6 and C-8 of apigenin on the basis of HMBC correlations (Figure S1) from H-1′′ (δ_H 5.43) to C-5 (δ_C 157.2), C-6 (δ_C 103.3), and C-7 (δ_C 162.8), H-1′′′ (δ_H 4.58) to C-7, C-8 (δ_C 104.6), and C-9 (δ_C 155.1). Therefore, 1 was identified as apigenin 6-C-α-arabinofuranosyl 8-C-α-arabinopyranoside.

It is worth noting that flavonoids were not frequently isolated from genus Bulbophyllum. Compound 2, a white amorphous powder, gives a molecular formula of C_{15}H_{14}O_{5} by HR-ESI-MS (negative ion mode) at m/z 273.0767 [M − H] −. The IR spectrum revealed the presence of the hydroxyl group (3617 cm−1), as well as aromatic groups (1623, 1506, 1449, 1067 and 781 cm −1). The 1H nMR spectrum of 2 indicated the presence of one methylene group at δ_H 2.83 (1H, dd, J = 13.3, 7.2 Hz, Ha-7′) and 2.71 (1H, dd, J = 13.3, 6.4 Hz, Hb-7′), one dioxygenated methylene group at δ_H 5.77 (2H, s, −OCH2O−), one oxygenated methine proton at δ_H 4.55 (1H, t, J = 6.8 Hz, H-7), six aromatic protons appearing as a pair of m-coupled signals at δ_H 6.27 (1H, brs, H-2) and 6.25 (1H, brs, H-6), and four other protons at δ_H 6.93 (1H, t, J = 7.8 Hz, H-5′), 6.49 (1H, overlapped, H-6′), 6.48 (1H, overlapped, H-4′) and 6.47 (1H, brs, H-2′), which were consistent with a 1,3-disubstituted aromatic ring. In the 13C NMR spectrum of 2, 15 carbons were observed, including one dioxygenated methylene (δ_C 102.2 CH₂), one non-oxygenated methylene (δ_C 46.9 CH₂), one oxygenated methine (δ_C 76.5 CH), six aromatic methines and six aromatic quaternary carbons (including four oxygenated). The 1H and 13C NMR data of 2 were found to be similar to those of dendrosinen A (Chen et al. 2014), except that the two methoxyl signals were replaced by the signals of one dioxygenated methylene. Correlations between the dioxygenated methylene protons (δ_H 5.77) to C-3 (δ_C 150.2) and C-4 (δ_C 134.6) in HMBC experiment (Figure S2) indicated the dioxygenated methylene moiety was located at C-3 and C-4 (Figure S2). In addition, HMBC correlations from H-2 (δ_H 6.27) and H-6 (δ_H 6.25) to the oxygenated methane (δ_C 76.5) suggested the remaining hydroxyl group should be located at C-7.

The absolute configuration of C-7 was determined by the comparison of experimental and calculated electronic circular dichroism (ECD) in Figure S3. The results showed that the theoretical ECD data for 7R-isomer were in good agreement with the experimental spectrum. Thus, the absolute configuration of C-7 was determined as R, which was same as that of dendrocandin A (Li et al. 2008). Therefore, compound 2 was assigned as [R]-3,4-methylenedioxy-5,3′,7-trihydroxy bibenzyl and named bulbotetusine.

Compounds 1 and 2 were examined in a panel of cancer cell lines, including HL-60, SMMC-7721, A-549, MCF-7 and SW-480 human tumour cell lines in vitro with cisplatin and paclitaxel as the positive controls. The cytotoxic activity was estimated by a method described previously (Wu et al. 2014). The result showed that both compounds 1 and 2 exhibited no obvious cytotoxic activities against any tested cell lines (IC_{50} > 40 μM). Many bibenzyl compounds were reported to exhibit cytotoxic activities (Yang et al. 2014). The absence of cytotoxic activity for 2 indicated that the presence of OH-7 had negative effect on cytotoxic activity, which was consistent with literatures (Chen et al. 2014).
3. Experimental procedures

3.1. General procedure

Melting point was determined on a XRC-1 melting point apparatus and uncorrected. Optical rotation was measured with a Jasco P-1020 digital polarimeter. A Nicolet Magna-IR 550 spectrometer was used for scanning IR spectroscopy with KBr pellets. NMR spectra were acquired with either a Bruker AM-400 spectrometer (400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR) or a Bruker DRX-600 (600 MHz for $^1$H NMR and 150 MHz for $^{13}$C NMR) spectrometer. HR-ESI-MS analyses were recorded with Agilent G3250AA and Auto Spec Premier P776 spectrometer. Silica gel (200–300 mesh or 300–400 mesh) and Sephadex LH-20 were used for column chromatography.

3.2. Plant material

The tubers of *B. retusiusculum* were collected in Yangbi County, Dali City, in Yunnan Province of China, in June 2014, and identified by associate researcher Jiang-Miao Hu from the Kunming Institute of Botany, The Chinese Academy of Sciences, PR China. The voucher specimen (2014-JBL-1) has been deposited in the school of Chemical Science and Technology of Yunnan University.

3.3. Extraction and isolation

Dried powdered tubers of *B. retusiusculum* (3.8 kg) were extracted three times with MeOH at 60 °C. The extracts were combined, and evaporated under reduced pressure. The crude residue (200 g) was subjected to a macroporous resin column eluted with water and pure MeOH successively. The MeOH fraction (80 g) was passed through a silica gel column eluted with CHCl$_3$/MeOH (10:1), CHCl$_3$/MeOH (3:1) and pure MeOH successively. The CHCl$_3$/MeOH (10:1) fraction (25 g) was chromatographed over silica gel eluting with CHCl$_3$/MeOH (200:1–1:1) gradient systems to give six fractions (FrA1–FrA6). Further, silica gel column chromatography purification of FrA2 (0.8 g) was accomplished by elution with PE/acetone (5:1) to afford 2 (7 mg). The CHCl$_3$/MeOH (3:1) fraction (45 g) was subjected to a silica gel column using an EtOAc/MeOH gradient (50:1–1:1) to yield eight fractions (FrB1–FrB8). FrB4 (387 mg) was separated by repeated silica gel and Sephadex LH-20 column chromatography to yield compound 1 (43 mg).

3.4. Apigenin 6-C-α-arabinofuranosyl 8-C-α-arabinopyranoside (1)

Yellowish powder. m.p. 227–228 °C. [$\alpha$]$^{D}_{19}$ -19.63 (c 1.00, MeOH). IR (KBr) $\nu_{max}$: 3416, 1632, 1576, 1442, 1364, 1220, 1082, 832, 653 cm$^{-1}$. HR-ESI-MS $m/z$: [M+H]$^+$ Calcd for C$_{25}$H$_{29}$O$_{13}$ 533.1300, Found: $m/z$ 533.1299. $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$, ppm: 6.85 (1H, s, H-3), 8.30 (1H, d, $J = 8.4$ Hz, H-2'), 6.88 (1H, d, $J = 8.4$ Hz, H-3'), 8.30 (1H, d, $J = 8.4$ Hz, H-6'), 8.43 (1H, d, $J = 2.9$ Hz, H-1'), 4.07 (1H, d, $J = 2.1$ Hz, H-2''), 3.93 (1H, brs, H-3''), 3.89 (1H, m, H-4''), 3.65 (2H, m, H-5''), 4.58 (1H, d, $J = 9.8$ Hz, H-1'''), 4.29 (1H, m, H-2'''), 3.41 (1H, m, H-3'''), 3.84 (1H, m, H-4'''), 3.55 (1H, d, $J = 12$ Hz, H-5'''), 3.87 (1H, b, H-5''''), 10.29 (1H, s, OH-6'), 13.92 (1H, s, OH-5) and 5.48 (1H, d, $J = 3.7$ Hz, OH-3''). $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$, ppm: 164.3 (C-2), 101.8 (C-3), 182.3 (C-4), 157.2 (C-5), 103.3 (C-6), 162.8 (C-7), 104.6 (C-8), 155.1 (C-9), 103.2 (C-10), 121.0 (C-1'), 129.8 (C-2'), 116.1 (C-3'), 161.2 (C-4'), 116.1
(C-5′), 129.8 (C-6′), 80.0 (C-1′′), 77.5 (C-2′′), 77.6 (C-3′′), 87.2 (C-4′′), 61.4 (C-5′′), 74.4 (C-1′′′), 68.1 (C-2′′′), 75.1 (C-3′′′), 69.2 (C-4′′′) and 71.3 (C-5′′′).

3.5. Bulbotetusine (2)

White amorphous powder m.p. 69–70 °C. \([\alpha]_D^{20} = -24.70\) (c 1.00, MeOH). IR (KBr) \(\nu_{\text{max}}\): 3417, 1623, 1506, 1449, 1373, 1067, 781 cm\(^{-1}\). HR-EI-MS \(m/z\): [M – H]\(^-\) Calcd for C\(_{15}\)H\(_{13}\)O\(_5\) 273.0768, Found: \(m/z\) 273.0767. \(^1\)H NMR (400 MHz, CD\(_3\)OD) \(\delta\), ppm: 6.27 (1H, brs, H-2), 6.26 (1H, brs, H-6), 4.55 (1H, t, J = 6.8 Hz, H-7), 6.47 (1H, brs, H-2′), 6.48 (1H, overlapped, H-4′), 6.93 (1H, t, J = 7.8 Hz, H-5′), 6.49 (1H, overlapped, H-6′), 2.83 (1H, dd, J = 13.3, 7.2 Hz, Ha-7′), 2.71 (1H, dd, J = 13.3, 6.4 Hz, Hb-7′) and 5.77 (2H, s, –OCH\(_2\)O–). \(^{13}\)C NMR (100 MHz, CD\(_3\)OD) \(\delta\), ppm: 140.5 (C-1), 110.3 (C-2), 150.2 (C-3), 134.6 (C-4), 141.6 (C-5), 99.8 (C-6), 76.5 (C-7), 141.3 (C-1′), 117.4 (C-2′), 158.1 (C-3′), 114.1 (C-4′), 130.0 (C-5′), 121.9 (C-6′), 46.9 (C-7′) and 102.2 (–OCH\(_2\)O–).

3.6. Calculation

The theoretical calculations of compound 2 were performed using Gaussian Program by Yunnan Electronic Computing Center. Two possible \(R\) and \(S\) geometries of compound 2 were previously optimised by Density Functional Theory methods at the B3LYP/6-31G(d) level (Ding et al. 2009) and excitation energies and rotational strengths were calculated using time-dependent Density Functional Theory at the B3LYP/6-31G(d,p) level in acetonitrile with PCM model (Liao et al. 2015). The ECD spectrum is simulated from electronic excitation energies and velocity rotational strengths.

4. Conclusion

The chemical investigation of the tubers of \(B. \text{retusiusculum}\) resulted in the isolation of a new flavone C-glycoside, apigenin 6-C-\(\alpha\)-arabinofuranosyl 8-C-\(\alpha\)-arabinopyranoside (1) and a new bibenzyl, bulbotetusine (2). The absolute configuration of 2 was determined by the comparison of experimental and calculated ECD. Compounds 1 and 2 showed no obvious cytotoxic activity against any five human tumour cell lines.

Disclosure statement

No potential conflict of interest was reported by the authors.

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