Abietane diterpenoids with neuroprotective activities from Phlegmariurus carinatus

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ABSTRACT
Two new abietane diterpenoids, phlecarinatone A (1) and phlecarinatone B (2), along with two known analogues (3 and 4), were isolated from Phlegmariurus carinatus. The structures of 1–4 were unambiguously elucidated by comprehensive spectroscopic analyses. Abietane diterpenoids were isolated from the plant for the first time. All isolates were tested for their neuroprotective activities against H2O2-induced SH-SY5Y cells injury, and compound 2 showed moderate effect at the concentrations ranging from 5 to 20 μM in vitro assay.

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1. Introduction
Phlegmariurus carinatus (Desv.) Ching, belonging to the family Huperziaceae, is an epiphytic club moss mainly distributed in tropical and subtropical regions (Ching 1982). For thousands of years, the plant has been used as folk medicine for the treatment of...
rheumatism, pain and swelling (Wu and Song 1998; Zhang and Zhang 2004). To date, more than 600 Lycopodium alkaloids have been reported. Especially, the well-known compound huperzine A, a high selectivity acetylcholinesterase (AChE) inhibitor obtained from Huperzia serrata, has been used for the treatment of myasthenia gravis (MG) and Alzheimer’s disease (AD) in China (Liu et al. 1986; Kozikowski and Tückmantel 1999; Bai et al. 2000; Cheng et al. 1996). Hence, phytochemical investigations of Huperziaceae family have generally focused on Lycopodium alkaloids. However, the AChE inhibitory activity of these alkaloids was lower than that of huperzine A (Ying et al. 2014; Xiong et al. 2019; Vallejo et al. 2020; Zhu et al. 2020).

Abietane diterpenoids, a large family of naturally occurring diterpenoids, were found to exhibit potent cytotoxic, anti-microbial and anti-inflammatory activities (Sun and Li 2012; Wu et al. 2014; Ngo et al. 2021; Ulubelen et al. 2001).

The first investigation on the phytochemical constituents of P. carinatus was reported in 2014. In order to discover structurally interesting and bioactive substances, we studied the entire P. carinatus plant. In this report, two new abietane diterpenoids (1 and 2), together with two known analogues (3 and 4) (Figure 1), were isolated. All compounds were screened for their neuroprotective activities. Herein, the isolation, structural elucidation and biological properties of these compounds are described.

Figure 1. Structures of compounds 1–4.
2. Results and discussion

2.1. Chemistry

Compound 1 was obtained as a yellow amorphous powder. The molecular formula was determined to be C$_{21}$H$_{28}$O$_{5}$ (m/z 361.2019 [M + H]$^+$, calcd for C$_{21}$H$_{29}$O$_{5}$+$^+$, 361.2015) by HRESIMS data, with eight degrees of unsaturation. The IR spectrum showed absorption bands for hydroxy group (3393 cm$^{-1}$) and carbonyl group (1620 cm$^{-1}$) functionalities. The $^1$H NMR spectrum (Table S1) showed the signals of an oxygen-bearing methine proton at $\delta$H 4.59 (1H, d, $J = 13.5$ Hz, H-6), a methoxy proton at $\delta$H 3.89 (3H, s, OCH$_3$-21), and five methyl protons at $\delta$H 1.21 (3H, d, $J = 6.4$ Hz, CH$_3$-16), 1.19 (3H, d, $J = 6.4$ Hz, CH$_3$-17), 1.17 (3H, s, CH$_3$-18), 1.23 (3H, s, CH$_3$-19), and 1.66 (3H, s, CH$_3$-20). The $^{13}$C NMR and DEPT data (Table S1) exhibited 21 carbon atoms, including five methyls ($\delta$C 20.0, 20.3, 20.4, 22.4 and 35.5), a methoxy ($\delta$C 60.3), three methylenes ($\delta$C 18.5, 35.9 and 41.6), two methines ($\delta$C 24.8 and 55.9), a oxygenated methine ($\delta$C 74.0), three keto carbonyl ($\delta$C 184.4, 184.8 and 199.2), and six quarternary carbons ($\delta$C 34.1, 41.5, 127.4, 136.3, 156.7 and 159.2).

Partial structures a (C-1–C-2–C-3), b (C-5–C-6), and c (C-16–C-15–C-17) were determined by a detailed analysis of $^1$H–$^1$H COSY spectrum (Figure S1). The key HMBC correlations of H$_3$-19 ($\delta$H 1.23) with C-3/C-4/C-5/C-18, H$_2$-15 ($\delta$H 3.16) with C-12/C-13/C-14, H$_3$-17 ($\delta$H 1.66) with C-1/C-5/C-9/C-10, and 21-OCH$_3$ ($\delta$H 3.89) with C-12 confirmed that 1 had the similar planar structure with 7-oxoroyleanone-12-methyl ether (Kabouche et al. 2007) (Figure S1). The major difference between them was that the methylene at $\delta$C 35.5 (C-6) in 7-oxoroyleanone-12-methyl ether was replaced by an oxygenated methine carbon ($\delta$C 74.0) in 1. The NOESY of H-6 ($\delta$H 4.59)/H$_3$-19 ($\delta$H 1.23) and H$_3$-20 ($\delta$H 1.66), H$_2$-2 ($\delta$H 1.54)/H$_3$-19 and H$_3$-20 indicated that 6-OH of 1 was $\alpha$-oriented (Figure S2). Moreover, by comparing the optical rotation of 1 ($[\alpha]_D^{20} – 92$) with that of 6$\alpha$-hydroxy-7-oxoroyleanone ($[\alpha]_D^{25} 11$) (Zheng et al. 2020), the hydroxyl group at C-6 in 1 was deduced to be $\alpha$-oriented. Thus, compound 1 was deduced as 6$\alpha$-hydroxy-7-oxoroyleanone-12-methyl ether and named phlecarinatone A.

Compound 2 was isolated as a white powder. It was assigned the molecular formula C$_{21}$H$_{28}$O$_{5}$ based on HRESIMS (m/z 361.2019 [M + H]$^+$, calcd for C$_{21}$H$_{29}$O$_{5}$+$^+$, 361.2015) data analyses. A detailed comparison of the 1D NMR data of 2 and 3 revealed that they are similar compounds. The 1D NMR data of 2 closely resembled those of 3, except that a hydroxy group at C-11 ($\delta$C 138.6) and a methoxy group at C-12 ($\delta$C 152.2) in 3 was replaced by a methylenedioxy group ($\delta$C 100.5) in 2, which was confirmed by the HMBC correlations from H$_2$-21 ($\delta$H 5.83) to C-11 ($\delta$C 135.8) and C-12 ($\delta$C 153.5). In addition, the methylene at $\delta$C 35.9 (C-6) in 3 was replaced by an oxygenated methine carbon ($\delta$C 72.6) in 2 which was confirmed by HMBC correlations of H-6 ($\delta$H 4.49) with C-5 ($\delta$C 54.7), C-7 ($\delta$C 203.1) and C-10 ($\delta$C 39.4). The NOESY correlations of H-6 ($\delta$H 4.49)/H$_3$-19 ($\delta$H 1.11) and H-6/H$_3$-20 ($\delta$H 1.32) indicated a $\alpha$-orientation of 6-OH. Hence, compound 2 was determined as 6,14-dihydroxy-11,12-methylenedioxy-abieta-8,11,13-triene-7-dione and named phlecarinatone B.

Additionally, two known compounds, inuroyleanol (3) (Frontana et al. 1994) and 7-dehydroabietanone (4) (Su et al. 1994), were identified by comparison of their reported 1D NMR data.
2.2. Neuroprotective activity assay

The neuroprotective effects of all the isolates on H₂O₂-induced decrease in cell viability were evaluated in human SH-SY5Y neuroblastoma cells. Compound 2 showed moderate neuroprotective activity (Figure S3). The rest of the compounds exerted weak protective effects (Table S2).

3. Experimental

3.1. General experimental procedures

UV spectra were acquired on a SPECPRD PLUS-50 spectrometer (Analytik Jena, Jena, Germany). IR spectra were measured on a Nicolet iS50 FTIR spectrometer (Thermo Scientific, Waltham, USA). Optical rotations were recorded on a INESA SGW-533 polarimeter (INESA, Shanghai, China). HRESIMS data was performed on an Waters Xevo G2-XS Q-TOF mass spectrometer (Waters Corp., Ltd., Massachusetts, USA). 1D and 2D NMR spectra were obtained on a Bruker AV-400 MHz spectrometers (Bruker, Karlsruhe, Germany). Analytical HPLC was performed on a Agilent Technologies 1200 connected to a DAD detector (Agilent, G1315B) and equipped with a Silgreen ODS C₁₈ column (250 mm × 4.6 mm, 5 μm; Peking, China). Semi-preparative HPLC was performed on an Agilent Technologies 1260 instrument with a VWD detector (Agilent, G1314A), using a Silgreen ODS C₁₈ column (250 mm × 10.0 mm, 5 μm). Silica gel (200–300 mesh, Qingdao Haiyang Chemical Corp, Ltd., Qingdao, China) and Sephadex LH-20 (GE Healthcare, Sweden) were used for open column chromatography (CC). All solvents were analytical pure.

3.2. Plant material

Whole herbs of *P. carinatus* were collected in the county of Jingzhou, Hunan Province, People’s Republic of China, in the winter of 2021. The plant was authenticated by Prof. Gui-Shan Tan (Central South University, Changsha, China). A voucher specimen (No. 20210108) was deposited in the Key Laboratory of Prevention and Treatment of Cardiovascular and Cerebrovascular Diseases, Ministry of Education, Gannan Medical University.

3.3. Extraction and isolation

The whole plant of *P. carinatus* (8.1 kg) were crushed into powder and then extracted with 75% EtOH (80 L) under reflux for three times (2 h each time) to afford a crude extract (0.9 kg). After suspended in water (7.0 L), the water-soluble residue was successively partitioned with petroleum ether (PE), ethyl acetate, and n-butanol, successively. The PE layer (275.0 g) was chromatographed over silica gel CC with a gradient elution of PE–EtOAc (100:1 → 0:1) to yield eight fractions (A–H). Separation of fraction A (32.6 g) was separated by CC on silica gel, eluting with PE–EtOAc (100:1 → 50:1), yielding five sub-fractions (A₁–A₅). Fraction A₁ (5.3 g) was separated on silica gel CC using PE–EtOAc (100:1 → 70:1) to yield three sub-fractions (A₁-1 – A₃-3). Fraction A₁-2
(1.5 g) was separated by Sephadex LH-20 column (CH₂Cl₂–MeOH, 2:1) and then purified by semi-preparative HPLC, using CH₃CN–H₂O (90:10 → 95:5, 15 min) as the mobile phase, to obtained compound 2 (2.5 mg, t₁R=12.3 min). The fraction A2 (3.1 g) was separated by Sephadex LH-20 column using a gradient elution of CH₂Cl₂–MeOH (2:1), then further purified by semi-preparative HPLC (CH₃CN–H₂O, 85:15 → 95:5, 20 min) to give compounds 1 (10.8 mg, t₁R=13.6 min), 3 (48.5 mg, t₁R=18.3 min), and 4 (54.7 mg, t₁R=19.4 min).

3.3.1. Phlecarinatone A (1)
Yellow amorphous powder; [α]ᵦ⁰° −92 (c 0.10, MeOH); UV (MeOH) λmax: (log ε) 202 (3.96), 240 (3.58), 279 (3.50), 373 (3.11) nm; IR (KBr) νmax: 3393, 2922, 2850, 1707, 1620, 1456, 1414, 1370, 1296 and 1024 cm⁻¹; for 1H NMR (CDCl₃, 400 MHz) and 13C NMR (CDCl₃, 100 MHz) data see Tables S1; positive HRESIMS at m/z 361.2019 [M+H]+ (calcd for C₂₁H₂₉O₅, 361.2015).

3.3.2. Phlecarinatone B (2)
Yellow amorphous powder; [α]ᵦ⁰° +27 (c 0.10, MeOH); UV (MeOH) λmax: (log ε) 202 (4.42), 244 (3.86), 292 (3.84), 363 (3.70) nm; IR (KBr) νmax: 3391, 2927, 2852, 1610, 1421, 1370, 1290 1200 and 1103 cm⁻¹; for 1H NMR (CDCl₃, 400 MHz) and 13C NMR (CDCl₃, 100 MHz) data see Tables S1; positive HRESIMS at m/z 361.2019 [M+H]+ (calcd for C₂₁H₂₉O₅, 361.2015).

3.4. Bioassay
SH-SY5Y cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and with 100 units/mL penicillin and 100 μg/mL streptomycin at at 37 °C in a 5% CO₂ humidified incubator. Cells were plated at a density of 5000 cells per well in 96-well plates and cultured for 24 h. All compounds tested were dissolved in dimethylsulfoxide (DMSO) and then diluted to corresponding concentrations with cell culture medium. The antioxidant N-acetylcysteine (NAC, 100 μM) was used as a positive control for H₂O₂-induced cell damage models. Cells were pretreated with the test compounds 2 h then incubated with 100 μM H₂O₂ for another 24 h without changing the culture medium. Then cells were incubated with the 10 μL of the MTT solution (5 mg/mL) for 2 h, and then 100 μL of DMSO to solubilize the formed formazan. The optical density (OD) levels were measured at 490 nm using a microplate reader.

4. Conclusions
In conclusion, four compounds, including two new abietane diterpenoids (1 and 2) and two known compounds (3 and 4), were isolated from the whole plant of P. carinatus. All compounds were tested for their neuroprotective activities toward damaged SH-SY5Y cells induced by H₂O₂. The bioassays suggested that only compound 2 significantly attenuated H₂O₂-induced cell damage at concentrations of 10 and 20 μM.
Disclosure statement
There is no potential conflict of interest for authors.

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