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Chemical constituents from fruiting bodies of Basidiomycete *Perenniporia subacida*

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ABSTRACT

Four new aromatic abietane diterpenoids and two new benzene derivatives, namely perenacidins A–F (**1**–**6**), have been isolated from the fruiting bodies of Basidiomycete *Perenniporia subacida*. The structures were elucidated by means of extensive spectroscopic methods and computational ECD method. The antifungal activities against *Canidia albicans* and the cytotoxic activities against four cancer cell lines (including K-562, A-549, SMMC-7721, MCF-7) were evaluated *in vitro*.

1. Introduction

Perenniporia Murrill is a cosmopolitan genus of bracket-forming or encrusting polypores containing about 60 currently recognized species, 29 of which have been reported in China [1,2]. Some species in the genus could attack living or dead hardwood and conifers, playing a key role in the substance circulation of forest ecosystem. *Perenniporia subacida* have been used as a medicine in treatment for tumor and pruritus in China. Previous phytochemical investigations on this genus revealed the presence of triterpenoids [3,4], naphthalenones [5], and sesquiterpenoids [6]. However, secondary metabolites produced by the fungus *P. subacida* have not been reported. As part of our efforts to search for bioactive secondary metabolites from higher fungi [7–9], we have carried out a chemical investigation on the EtOH extract of the fruiting bodies of *P. subacida*, which led to the isolation of four new aromatic abietane diterpenoids and two new benzene derivatives, namely perenacidins A–F (**1**–**6**). Herein, we report the isolation, structural elucidation and biological activities of these compounds.

2. Experimental section

2.1. General experimental procedures

Optical rotations were measured with a P-1020 polarimeter (Jasco, Japan). UV spectra were recorded using a UV-2401A spectrophotometer (Shimadzu, Japan) equipped with a DAD and a 1-cm path-length cell. Samples in methanol solution were scanned from 190 to 400 nm in 1 nm steps. IR spectra were obtained on a Bruker FT-IR Tensor 27 spectrometer using KBr pellets. 1D and 2D NMR spectra were run on a Bruker Avance III-600 MHz spectrometer (Karlsruhe, Germany). Chemical shifts (δ) were expressed in ppm with reference to solvent signals. HR-MS were recorded on a Waters Auto Premier P776 spectrometer (Waters, USA) or an Agilent G6230AA Accurate Mass TOF LC/MS instrument (Agilent, USA). An Agilent 1200 series instrument equipped with Zorbax SB-C18 column (5 μm, 4.6 mm × 150 mm, Agilent, USA; detector: DAD) was used for high performance liquid chromatography (HPLC) analysis with a flow rate of 1.0 mL/min, and an Agilent 1100 series instrument with a reverse-phase preparative Zorbax SB-C18 column (5 μm, 9.4 mm × 150 mm, Agilent, USA) was used for the sample preparation with a flow rate of 10 mL/min. Column chromatography (CC) was performed on silica gel (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, China), RP-18 (5 μm, Fuji Silyisa Chemical Ltd., Japan), and Sephadex LH-20 (Amersham Biosciences, Sweden). Fractions were monitored by TLC (GF_{254}, Qingdao Haiyang Chemical

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Co. Ltd., Qingdao, China), and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol.

2.2. Fungal material

Fruiting bodies of *P. subacida* were collected in a suburb of Helsinki, Finland in September, 2014 and identified by Prof. Yu-Cheng Dai (Beijing Forestry University). A specimen (No. KIB2014-7#) was deposited at Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The dried and powdered fruiting bodies (860 g) of *P. subacida* were extracted four times (48 h each time) with MeOH at room temperature. The organic layer was concentrated under reduced pressure to afford a crude extract (71 g), and the residue was subjected to silica gel column chromatography separated on a silica gel column (8 × 40 cm) eluted with step-gradient of $CHCl_3$–MeOH (from 1:0 to 0:1) to yield ten fractions (Fr. 1–10). Fr. 3 (13 g) was separated on a reverse-phase (RP) C-18 column (5 μm, 4 × 18 cm) using a step-gradient of MeOH–H_2O (*v*/*v*: 8:2, 4:6, 6:4, 8:2, 0:10) to yield seven sub-fractions (3a–3 g). Fraction 3e (570 mg) was separated by preparative-HPLC (MeCN–H_2O, from 20:80 to 40:60 in 20 min) to give compounds **3** (3.3 mg, retention time (t_R) = 11.5 min), and **4** (1.5 mg, t_R = 9.8 min). Fraction 5 (6.3 g) was subjected on a RP-C18 silica gel with MeOH–H_2O (v/v, 0:100, 30:70, 60:40, 100:0) to get five sub-fractions (5a–5e). After preparative-HPLC (MeCN–H_2O, from 15:85 to 30:70 in 15 min) and Sephadex LH-20 (acetone) gel column, **1** (35 mg, t_R = 7.9 min) and **2** (12 mg, t_R = 10.1 min) were obtained from fraction 5b (815 mg). Fraction 6 (1.4 g) was also applied to a RP-C18 silica gel (MeOH–H_2O from 20:80 to 100:0) to obtain four fractions (6a–6d). Fr. 6b (550 mg) was further subjected to Sephadex LH-20 (acetone), and subsequently separated over a preparative-HPLC (MeCN–H_2O, from 0:100 to 40:60 in 40 min), yielding compounds **5** (1.8 mg, t_R = 20.0 min) and **6** (2.3 mg, t_R = 26.5 min).

Perenacidin A (**1**): yellowish oil, $[\alpha]_D^{24} = -5.5$ (*c* 0.71, MeOH); UV (MeOH) λmax (log ε): 210 (4.19), 254 (3.83), 299 (3.07); IR (KBr) ν_{max} 3438, 2978, 2934, 1704, 1674, 1607, 1386, 1237, 1163, 1126, 1032 cm^{-1}; ^1H and ^{13}C NMR data (see Table 1); HR-EI-MS: *m*/*z* 346.1764 $[M]^+$ (calcd for $C_{20}H_{26}O_5$, 346.1780).

Table 1
^{13}C NMR (150 MHz) and ^1H NMR (600 MHz) data for compounds **1** and **2** in acetone-d_6.

No.	**1**		**2**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	75.8, d	4.03 (dd, 9.2, 5.0)	70.0, d	4.54 (br s)
2	29.1, t	1.92 (overlap)	25.6, t	2.21 (dddd, 14.3, 14.2, 3.7, 1.9)
		1.92 (overlap)		1.85 (br d, 14.3)
3	35.4, t	1.95 (overlap)	30.3, t	2.46 (ddd, 14.2, 13.1, 3.8)
		1.70 (m)		1.47 (br d, 13.1)
4	46.6, s		46.7, s	
5	44.4, d	2.63 (dd. 14.1, 3.2)	38.3, d	3.28 (dd, 14.7, 3.1)
6	37.9, t	2.34 (dd, 18.1, 3.2)	38.1, t	2.36 (dd, 17.0, 3.1)
		2.85 (dd, 18.0, 14.1)		2.81 (dd, 17.0, 14.7)
7	198.4, s		197.9, s	
8	131.4, s		132.0, s	
9	154.2, s		151.6, s	
10	44.1, s		43.8, s	
11	127.3, d	8.43 (d, 8.5)	126.1, d	7.53 (d, 8.3)
12	131.2, d	7.69 (dd, 8.5, 2.1)	131.1, d	7.74 (dd, 8.3, 2.1)
13	149.2, s		148.7, s	
14	123.4, d	8.03 (d, 2.1)	123.4, d	8.08 (d, 2.1)
15	71.7, s		71.7, s	
16	32.0, q	1.50 (s)	32.0, q	1.51 (s)
17	32.0, q	1.50 (s)	32.0, q	1.51 (s)
18	179.0, s		179.2, s	
19	16.5, q	1.32 (s)	16.8, q	1.35 (s)
20	17.7, q	1.29 (s)	24.0, q	1.32 (s)

The assignments were based on ^{13}C, DEPT, and HSQC experiments.

Perenacidin B (**2**): yellowish oil, $[\alpha]_D^{24} = +11.2$ (*c* 0.22, MeOH); UV (MeOH) λmax (log ε): 210 (4.15), 254 (3.88), 299 (3.02); IR (KBr) ν_{max} 3439, 2970, 2932, 1702, 1683, 1610, 1460, 1385, 1240, 1208, 1122, 1064, 1039 cm^{-1}; ^1H and ^{13}C NMR data (see Table 1); HR-EI-MS: *m*/*z* 346.1775 $[M]^+$ (calcd for $C_{20}H_{26}O_5$, 346.1780).

Perenacidin C (**3**): yellowish oil, $[\alpha]_D^{24} = +11.5$ (*c* 0.37, MeOH); UV (MeOH) λmax (log ε): 194 (3.77), 233 (4.10), 250 (3.87), 296 (2.97); IR (KBr) ν_{max} 3441, 3432, 2956, 2924, 1683, 1631, 1566, 1392, 1360, 1241, 1119, 1065, 1039 cm^{-1}; ^1H and ^{13}C NMR data (see Table 2); HR-EI-MS: *m*/*z* 330.1469 $[M]^+$ (calcd for $C_{19}H_{22}O_5$, 330.1467).

Perenacidin D (**4**): yellowish oil, $[\alpha]_D^{24} = -2.3$ (*c* 0.12, MeOH); UV (MeOH) λmax (log ε): 194 (3.77), 233 (4.10), 250 (3.82), 296 (2.90); IR (KBr) ν_{max} 3439, 3428, 2955, 2923, 2854, 1686, 1637, 1566, 1452, 1385, 1239, 1119, 1042 cm^{-1}; ^1H and ^{13}C NMR data (see Table 2); HR-TOF-ESI-MS (pos.): *m*/*z* 353.1359 $[M + Na]^+$ (calcd for $C_{19}H_{22}NaO_5$, 353.1365).

Perenacidin E (**5**): yellowish oil, $[\alpha]_D^{24} = +10.7$ (*c* 0.20, MeOH); UV (MeOH) λmax (log ε): 203 (4.15), 226 (3.72), 280 (3.10); IR (KBr) ν_{max} 3440, 2957, 2923, 2854, 1631, 1446, 1383, 1248, 1162, 1113, 1035 cm^{-1}; ^1H and ^{13}C NMR data (see Table 3); HR-TOF-ESI-MS (pos.): *m*/*z* 287.0891 $[M + Na]^+$ (calcd for $C_{14}H_{16}NaO_5$, 287.0895).

Perenacidin F (**6**): yellowish oil, $[\alpha]_D^{24} = -8.0$ (*c* 0.23, MeOH); UV (MeOH) λmax (log ε): 195 (4.16), 216 (4.62), 238 (4.02), 354 (2.54) IR (KBr) ν_{max} 3442, 2956,2924, 2854, 1751, 1629, 1450, 1428, 1381, 1314, 1248, 1163, 1112, 1053 cm^{-1}; ^1H and ^{13}C NMR data (see Table 3); HR-EI-MS: *m*/*z* 190.0631 $[M]^+$ (calcd for $C_{11}H_{10}O_3$, 190.0630).

2.4. Computational methods

All DFT and TD-DFT calculations were carried out at 298 K in the gas phase with Gaussian 09 [10]. Conformational searches were carried out at the molecular mechanics level of theory employing MMFF force fields [11–13]. The conformers with relative energy within 10 kcal/mol of the lowest-energy conformer were selected and further geometry optimized at the B3LYP/6–311++G(2d,p) level. All the lowest-energy conformers, which correspond to 99% of the total Boltzmann distribution, were selected for ECD spectra calculation. The Boltzmann factor for each conformer was calculated based on Gibbs free energy. Vibrational analysis at the B3LYP/6–311++G (2d,p) level of theory resulted in no imaginary frequencies, confirming the considered conformers as real minima. TDDFT was employed to calculate excitation energy (in nm) and rotatory strength *R* in dipole velocity form, at the B3LYP/6–311++G(2d,p) level.

2.5. Antifungal activity

Compounds **1**–**4** were tested for their antimicrobial activities against *Canidia albicans in vitro* used a turbidimetric method. Amphotericin B was used as a positive control. *C. albicans* was inoculated in potato dextrose broth (formulated identically to potato dextrose agar (PDA), omitting the agar, prepared in this laboratory) and diluted with medium to 1 × 10^6 CFU mL^{-1}. Aliquots of 90 μL were filled in 96-well U-bottomed microplates, and then treated with compounds **1**–**4** at the maximum concentration of 20 μg/mL. After culturing at 37 °C for 24 h, the absorbance was measured at 620 nm with the microplate reader. The percentage inhibition of cell growth below 50% was regarded as inactive.

2.6. Cytotoxic activity

Hepatocellular carcinoma SMMC-7721, lung cancer A-549 cells, breast cancer MCF-7 and human leukemia K-562 cell lines were used in the cytotoxic assay. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (Hyclone, USA) in 5% CO_2 at 37 °C. The cytotoxicity assay was performed by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-