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Four new hybrid polyketide-terpenoid metabolites from the *Penicillium* sp. SYPF7381 in the rhizosphere soil of *Pulsatilla chinensis*

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ABSTRACT

A search for cytotoxic agents from cultures of the *Penicillium* sp., isolated from the rhizosphere soil of *Pulsatilla chinensis*, led to the isolation of four new hybrid polyketide-terpenoid metabolites (**1–4**), together with fourteen known compounds (**5–18**). Using a bioassay-guided fractionation approach, eighteen compounds were obtained from the ethyl acetate extract of this fungus. Structure elucidation was achieved by extensive analysis of spectroscopic data (1D/2D NMR, HRESIMS and IR). The absolute configurations of compounds **1–4** were determined by means of electronic circular dichroism (ECD) calculation. Compounds **1–4**, **7–9**, **11**, **12**, **14** and **17** were tested for their cytotoxicity against HL-60, THP-1 and Caco2 cell lines. Compound **1** showed potent cytotoxic capability against HL-60, THP-1 and Caco2 cell with IC_{50} values of 3.4 μM, 4.3 μM, 10.5 μM, and compound **2** showed significant inhibiting activities against HL-60 cell line and THP-1 cell line (IC_{50} = 7.9 μM, 11.3 μM, respectively), using 5-fluorouracil as the positive drug with IC_{50} values of 6.4 μM, 4.4 μM, 56.6 μM for HL-60, THP-1 and Caco2 cells, respectively. And compound **1** showed antibacterial activity toward *Bacillus cereus* (IC_{50} = 49 μg/mL, IC_{90} = 111 μg/mL) and *Bacillus subtilis* (IC_{50} = 10 μg/mL, IC_{90} = 85 μg/mL).

1. Introduction

With more and more fungi are found in plants and the rhizosphere soil of plants, it's being a hotspot to explore new biologically active compounds from fungi in the rhizosphere soil of officinal plants. They are promising sources for the discovery of structurally diverse and secondary metabolites of various bioactive [1–2]. In our search for bioactive compounds from fungi of the rhizosphere soil of *Pulsatilla chinensis*, we found cytotoxicity of the ethyl acetate extract from the fungus *Penicillium* sp. against the human cell lines HL-60 and THP-1. Extraction of the culture broth followed by a bioassay-guided fractionation of the extract led to the isolation of one berkeleytrione (**1**), three berkeleyteals (**2–4**) and fourteen known related compounds (**5–18**) from this fungus. Hybrid polyketide-terpenoid metabolites are a family of marine fungal metabolites possessing a wide range of significant biological activities [3–8]. Interestingly, berkeleydione (**11**) and berkeleyacetals reportedly inhibit matrix metalloproteinase-3 and caspase-1 [4–5], and among them, berkeleyacetal C exhibits anti-inflammatory activity as well [9]. Berkeley meroterpenes were firstly isolated from a marine fungal strain obtained from Berkeley Pit Lake of Butte, Montana, a toxic lake with acidic water and metal ions [5]. However, we also isolated four new meroterpenes (**1–4**) and thirteen known related meroterpenes (**5–17**) from the interrhizospheric fungal strain of medicinal plants culturing in the normal cultivation condition instead of the extreme environment, which indicated that besides the growing environment, there are more factors that contribute to the biosynthesis of the meroterpenes yielded by the fungi [8]. Herein, we report their isolation, structure elucidation, biosynthesis pathways, cytotoxicity and antimicrobial activities of these isolated meroterpenes.

2. Experimental method

2.1. General experimental procedures

Optical rotation was measured with a JASCO P-2000 Series (Jasco Co., Ltd., Tokyo, Japan). The IR spectrum was obtained from a Bruker IFS-55 spectrophotometer using a KBr pellet (Bruker Optik BmbH, Ettlingen, Germany). The HR-ESI-MS data were obtained on a microTOF-Q Bruker mass instrument (Bruker Daltonics, Billerica, MA, USA). CD spectra were recorded with a Biologic MOS-450 spectrometer using CDCl3 as solvent. 1D and 2D NMR spectra were run on a Bruker AVANCE-400/-600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). ^{1}H chemical shifs (δ_H) were measured in ppm, relative to TMS, and ^{13}C chemical shifs (δ_C) were measured relative to $CDCl_3$ and

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converted to TMS scale. Column chromatography (CC) was performed on Silica gel (200–300 mesh; Qingdao Marine Chemical Co., Qingdao, China) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden) columns. Analytical and preparative thin-layer chromatographies (TLC) were carried out using silica gel plates (GF 254 10–40 μm, Qingdao Marine Chemical Co., China). Analytical TLC was used to follow the separation and check the purity of isolated compounds. Spots on the plates were observed under UV light and visualized by spraying 10% H_2SO_4 in EtOH (v/v), followed by heating. HPLC was performed on a Shimadzu LC-10AVP liquid chromatograph with a YMC-pack C_{18} (ODS) column (10 × 250 mm, 5 μm, Japan) and a Shimadzu LC-8AVP liquid chromatograph with a Diamonsil C_{18} (ODS) column (4.6 × 250 mm, 5 μm, China). All reagents were HPLC or analytical grade and were purchased from Tianjin Damao Chemical Company (Tianjin, China).

2.2. Fungal material

The fungal strain used in this work was isolated from the rhizosphere soil of *Pulsatilla chinensis*, which was collected from Huludao, Liaoning province of China in October 2014. The sequence data derived from this strain has been submitted and deposited in GeneBank with the accession number MF568058. BLAST search results revealed that the isolate belongs to the genus *Penicillium* and had a high sequence identity (99%) to the species *Penicillium skrjabinii*. A voucher specimen (no. SYPF7381) was deposited at the School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University.

2.3. Fermentation, extraction, and isolation

The strain of fungus was isolated from the rhizosphere soil of *Pulsatilla chinensis*, which is a Chinese medicinal herb for "blood-cooling" and detoxification. The interrhizospheric fungus *Penicillium* sp. was fermented on autoclaved rice solid-substrate medium (one hundred and sixty 500 mL Erlenmeyer flasks, each containing 80 g rice, 110 mL water) for 30 days at 26 °C. Following incubation, the mycelia and solid rice medium were extracted three times with 95% EtOH/water to give a crude extract (150 g), which was suspended in water followed by partition with EtOAc to afford an EtOAc soluble extract (80 g). This extract was divided into thirteen parts (Fr. A-M) by using CC (silica gel 200–300 mesh, petroleum ether/acetone 1:0 to 0:1). Fraction H was isolated by CC on silica gel eluted with petroleum mether/acetone (v/v, 100:10), then further purified using SephadexLH-20 with MeOH and HPLC with 70% MeOH-H_2O for **10** (9 mg) and 62% MeOH-H_2O for **16** (10 mg) and **15** (8 mg). Fraction I was isolated by CC on silica gel eluted with petroleum mether/acetone (v/v, 100:15), then purified by CC (silica gel 200–300 mesh, petroleum ether/EtOAc-acetone (1:1) 1:0 to 0:1). Fraction 3 was isolated by CC on silica gel eluted with petroleum mether/ EtOAc-acetone (1:1) (v/v, 100:7), then further purified using SephadexLH-20 with MeOH and HPLC with 66% MeOH-H_2O for **11** (110 mg). Fraction 4 was purified by the C_{18} column chromatography with 60% MeOH-H_2O for **14** (12 mg), 70% MeOH-H_2O for **3** (17 mg). Fraction 5 was purified by the C_{18} column chromatography with 70% MeOH-H_2O and HPLC with 69% MeOH-H_2O for **2** (10 mg), **9** (42 mg) and **17** (224 mg), 60% MeOH-$H_2O$and HPLC with 54% MeOH-H_2O for **12** (13 mg). Fraction K was isolated by CC on silica gel eluted with petroleum ether/acetone (v/v, 100:20), then purified by CC (silica gel 200–300 mesh, petroleum ether/EtOAc-acetone (1:1) 1:0 to 0:1). Fraction 1 was isolated by CC on silica gel eluted with petroleum ether/ EtOAc-acetone (1:1) (v/v, 100:7), then further purified using SephadexLH-20 with MeOH for **18** (20 mg). Fraction 2 was isolated by CC on silica gel eluted with petroleum ether/EtOAc-acetone (1:1) (v/v, 100:10), then further purified on HPLC with 70% MeOH-H_2O for **1** (30 mg). Fraction 5 was purified by the C_{18} column chromatography with 60% MeOH-H_2O and HPLC with 56% MeOH-H_2O for **7** (20 mg), **8** (33 mg) and **13** (60 mg). Fraction M was isolated by CC on silica gel eluted with petroleum mether/acetone (v/v, 100:30), then further purified by the C_{18} column chromatography with40% MeOH-H_2O and HPLC with 49% MeOH-H_2O for **5** (8 mg), and 60% MeOH-H_2O and HPLC with 63% MeOH-H_2O for **4** (9 mg) and **6** (12 mg).

Table 1
^1H and ^{13}C NMR data for compound **1** and **2** ($CDCl_3$).

Position	δ_C 1	δ_H 1 (*J* in Hz)	δ_C 2	δ_H 2 (*J* in Hz)
1	204.0, C		172.9, C	
2	126.0, CH	5.82, d (10.0)	35.6, CH_2	3.91, d (17.3) 3.32, d (17.3)
3	153.6, CH	6.57, d (10.0)	131.0, C	
4	39.2, C		137.2, C	
5	43.2, CH	1.05, dd (13.2, 3.3)	37.5, CH	2.88, dd (13.9, 3.7)
6	40.0, CH_2	2.15, dd (13.2, 3.3) 1.84, t (13.2)	30.4, CH_2	2.06, dd (13.9, 3.7) 1.65, t (13.9)
7	51.0, C		43.4, C	
8	207.4, C		176.0, C	
9	80.4, C		76.0, CH	4.52, q (6.7)
10	204.1, C		203.8, C	
11	71.6, C		62.8, C	
12	45.6, C		54.3, C	
13	31.9, CH_2	3.10, dd (18.3, 1.9) 2.58, dd (18.3, 5.1)	35.1, C	1.96, dd (14.6, 6.8) 2.35, dd (14.6, 6.8)
14	120.9, CH	5.64, dd (5.1, 1.9)	132.5, C	6.05 (t, 6.8)
15	140.2, C		138.3, C	
16	47.7, C		77.5, C	
17	29.4, CH_3	1.33, s	28.5, CH_3	1.28, s
18	27.8, CH_3	1.25, s	24.3, CH_3	1.52, s
19	18.3, CH_3	1.30, s	21.4, CH_3	0.79, s
20	168.4, C		170.3, C	
21	15.0, CH_3	1.33, s	19.1, CH_3	1.41, d (6.7)
22	144.9, C		52.5, CH	3.83, d (5.4)
23	112.5,CH_2	5.43, brs 4.93, brs	99.5, CH	6.02, d (5.4)
24	22.3, CH_3	1.56, s	27.7, CH_3	1.35, s
25	24.2, CH_3	1.13, s	16.4, CH_3	1.75, s
26-OMe	52.7, CH_3	3.75, s	53.0, CH_3	3.84, s
27-OMe			51.4, CH_3	3.62, s
28-OMe			49.7, CH_3	3.06, s

Compound **1**, white solid; $[\alpha]_D^{20}$ − 63.3 (c 0.0018, CH_3OH); ^{1}HNMR (400 MHz, CD_3Cl) and ^{13}CNMR (100 MHz, CD_3Cl) see Table 1; HRESIMS *m/z* 463.2065 $[M + Na]^+$ (calcd for $C_{26}H_{32}NaO_6$ 463.2091).

Compound **2**, white solid; $[\alpha]_D^{20}$ + 55.2 (*c* 0.0023, CH_3OH); ^{1}HNMR (400 MHz, CD_3Cl) and ^{13}CNMR (100 MHz, CD_3Cl) see Table 1; HRESIMS *m/z* 541.2367 $[M + Na]^+$ (calcd. for $C_{28}H_{38}NaO_9$ 541.2408).

Compound **3**, white solid; $[\alpha]_D^{20}$ + 70.0 (*c* 0.0020, CH_3OH); ^{1}HNMR (400 MHz, CD_3Cl) and ^{13}CNMR (100 MHz, CD_3Cl) see Table 2; HRESIMS *m/z* 495.1991 $[M + Na]^+$ (calcd. for $C_{26}H_{32}Na\ O_8$ 495.1989).

Compound **4**, white solid; $[\alpha]_D^{20}$ + 165.6 (*c* 0.0023, CH_3OH);^{1}HNMR (400 MHz, CD_3Cl) and ^{13}CNMR (100 MHz, CD_3Cl) see Table 2; HRESIMS *m/z* 457.1862 $[M-H]^-$(calcd. for $C_{25}H_{29}O_8$ 457.1868).

2.4. Cytotoxic assay in vitro

The cytotoxic activities of isolated Compounds **1–4**, **7–9**, **11**, **12**, **14** and **17** were tested against three human cancer cell lines, including human leukemia HL-60, pro-monocytic leukemia cell line THP-1 and human colon adenocarcinoma cell line Caco2, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) method in vitro. HL-60 and THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (10% Fetal bovine serum, 100 IU/mL penicillin, and 100 g/mL streptomycin) in 5% CO_2 at 37 °C. Then, the cells were cultured in 96-well plates for 24 h with 100 μL complete medium, and the compounds were added with varying concentrations of 80, 40, 20, 10 and 5 μM. MTT (20 μL) with 5 mg/mL phosphate buffer saline was added for another 4 h in the 96-well plates, then