A new polyoxygenated farnesylcyclohexenone from Fungus *Penicillium* sp.

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A new polyoxygenated farnesylcyclohexenone, peniginsengin A (1), was isolated from the fermentation of *Penicillium* sp. YIM PH30003, an endophytic fungus associated with *Panax notoginseng* (Burk.) F. H. Chen. The structure was assigned based on a combination of 1 D and 2 D NMR and mass spectral data. The cytotoxicity and antimicrobial activities of compound 1 were investigated.

**Keywords:** *Penicillium* sp.; polyoxygenated farnesylcyclohexenone; peniginsengin A

1. Introduction

The genus *Penicillium* is one of the largest and most intensively investigated mangrove endophytic fungal genera. Diverse structures and interesting biological activities from endophytic *Penicillium* species have been characterised (Motohashi et al. 2009; Wang et al. 2011; Yang et al. 2013; Rukachaisirikul et al. 2014). As part of our ongoing research on new bioactive compounds from endophytic fungi, the broth culture of the rhizosphere fungus *Penicillium* sp. YIM PH30003 exhibited antimicrobial activity towards *Fusarium solani*, the pathogenic fungus of *Panax notoginseng*. We reported herein the isolation and identification of one new compound from the fungus *Penicillium* sp., together with its bioactivities. The structure was determined as peniginsengin A (1) (Figure 1) by extensive spectroscopic analyses. The cytotoxicity against human promyelocytic leukemia HL-60, human hepatoma SMMC-7721, non-small-cell lung cancer A-549, breast cancer MCF-7 and human colorectal carcinoma SW4801 cell lines, and antimicrobial activities against *F. solani* and *Staphylococcus aureus* of compound 1 were investigated.

2. Results and discussion

HR-ESIMS analysis of compound 1 revealed quasi-molecular ion peaks at 357.1757 [M + Na]\(^{+}\). The \(^{1}\)H and \(^{13}\)C NMR spectra, including DEPT, clearly showed three olefinic...
methyl singlets, two oxygenated methines, one oxygenated quaternary carbon, two olefinic carbons and five methylenes. The \(^1H\) NMR and \(^{13}C\) NMR of 1 empressed the skeleton of polyoxygenated farnesylcyclohexenones isolated from *Penicillium* (Li et al. 2003). The cyclohexanone structure unit in compound 1 was confirmed by HMBC and \(^1\text{H}–^1\text{H}\) COSY spectra (Figure S1). The HMBC correlations between H-7 and C-4, C-5 and C-6 suggested a methyl group connected to a double bond at C-5 position. The \(^1\text{H}–^1\text{H}\) COSY between H-3 and H-4 indicated that C-3 was connected to C-4. The structure of the side chain, comprising two isoprene and one acetic acid fragments, could also be established by the information provided by \(^1\text{H}–^1\text{H}\) COSY and HMBC spectra (Figure S1). The HMBC signals between H-1\(^\prime\) and C-2 indicated that the side chain was connected to C-2 position. The relative stereochemistry of this metabolite was determined by the coupling constant and the NOESY data. The coupling constant of H-3 and H-4 was observed to be 2.2 Hz and a broad singlet when compared with ambuic acid derivatives, suggesting that H-3 and H-4 are situated in a *cis* relationship (Ding et al. 2009). The orientations of double bonds were determined by the NOESY data from H-2\(^\prime\) to H-4\(^\prime\), H-4\(^\prime\) to H-6\(^\prime\), H-6\(^\prime\) to H-8\(^\prime\) and NMR when compared with 7-deacetoxyyuanthone A (Li et al. 2003).

Compound 1 showed no cytotoxicity against HL-60, SMMC-7721, A-549, MCF-7, SW4801 up to a concentration of 40 \(\mu\)M in the 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxy methoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) method, and compound 1 indicated antifungal activity with an MIC of 64 \(\mu\)g/mL against *F. solani*, the pathogenic fungus of *P. notoginseng*, but no antibacterial activity was shown with MIC > 100 \(\mu\)g/mL.

3. Experimental

3.1. General experimental procedures

Silica gel (100–200, 200–300 mesh; Qingdao Marine Chemical Group Co., Shangdong, China) and Sephadex LH-20 (GE Healthcare Co., Buckinghamshire, UK) were employed for column chromatography. All chemicals were purchased from Beijing Greenherbs Science and Technology Development Co. (Beijing, China). Optical rotations were measured with a Jasco P-1020 (Jasco Co., Tokyo, Japan). 1D- and 2D-NMR spectra were obtained on Bruker 500 MHz instruments (Bruker, Karlsruhe, Germany) with tetramethylsilane (TMS) (Sigma-Aldrich Co., Shanghai, China) as internal standard. MS spectrum was recorded using an Agilent G3250AA system (Agilent, Santa Clara, CA, USA).

3.2. Biological material and cultivation of fungal strain

*Penicillium* sp. was isolated on PDA medium (infusion of 200 g fresh potato, dextrose 15 g and 1 L distilled water, agar 15.0 g, pH 7.0) from the *P. notoginseng* collected from Wenshan, Yunnan Province, China, in March 2012. The stock culture of *Penicillium* sp. was grown on the slant of PDA medium at 4°C. Identification of the strain was performed by rDNA-ITS molecular-phylogenetic analysis and morphological characteristics of its different growth stages.
A voucher specimen (no. YIM PH30003) was preserved at the Yunnan Institute of Microbiology, Kunming, P.R. China.

3.3. Fermentation and isolation
The fungus *Penicillium* YIM PH 30003 was maintained on the seed medium (PDB, potato infusion of 200 g fresh potato, dextrose 20 g, distilled water 1.0 L, pH 7.0) in a 500-mL Erlenmeyer flask for 30 min at room temperature (rt). The flasks were incubated on a rotary shaker at 28°C at 130 rpm for 3 days. The seed culture (10%) was then transferred into a 1000 mL Erlenmeyer flask containing 250 mL of seed medium as the production medium. Fermentation was performed on a rotary shaker at 28°C at 130 rpm for 7 days.

The production culture (50 L) was centrifuged to separate mycelia from the supernatant. The supernatant was exhaustively extracted with EtOAc yielding 25.1 g of extract. The crude residue of fermentation broth was fractionated by column chromatography on silica gel eluting with a stepwise gradient of CHCl₃/MeOH (1:0, 100:1, 100:1, 50:1, 20:1, 10:1, 5:1 and 0:1, v/v) and seven fractions (Fr.1–Fr.7) were collected. Fr.2 fraction (2.3 g) was eluted upon silica gel column with petroleum ether/EtOAc (1:0, 5:1, 1:1, 0:1, v/v) to give Fr. 2.1–Fr. 2.4. The Fr. 2–4 (0.4 g) were subjected to further elution on repeated Sephadex LH-20 (2 cm £ 160 cm, methanol) and repeated Sephadex LH-20 (2 cm £ 160 cm, acetone) and silica gel column with CHCl₃/MeOH (1:0, 50:1, 20:1, 10:1, 5:1, 0:1, v/v) to give compound 1 (Rf value at 0.3 in 20% CHCl₃/CH₃OH).

3.4. Spectroscopic data
Peniginsengin A (1): amorphous solid; IR (KBr) νmax: 3435, 1706, 1629, 1454, 1262, 1085, 812 cm⁻¹. [α]D²⁰ 5.4 (c 0.1, MeOH); HR-ESIMS m/z: 357.1757 [M + Na]+, calcd for C₁₉H₂₆O₅Na: 357.1678.

³¹P-NMR (CDCl₃, 200 MHz) δ: 5.70 (1H, s, H-6), 5.01 (1H, t, J = 6.0Hz, H-6), 5.01 (1H, t, J = 6.0Hz, H-6), 4.89 (1H, t, J = 7.0Hz, H-2'), 4.39 (1H, br s, H-4), 3.61 (1H, d, J = 2.2Hz, H-3), 2.50, 2.67 (2H, m, H-1'), 2.37 (2H, m, H-9'), 2.22 (2H, m, H-8'), 1.96 (2H, m, H-5'), 1.92 (2H, m, H-4'), 1.96 (2H, m, H-5'), 1.53 (3H, s, H-12'), 1.52 (3H, s, H-11').

C-NMR (CDCl₃, 500 MHz) δ: 194.0 (C-1), 178.4 (C-10), 156.6 (C-5), 139.7 (C-3), 133.7 (C-7), 125.1 (C-6), 123.8 (C-6), 116.8 (C-2), 68.0 (C-4), 61.9 (C-2), 59.4 (C-3), 39.7 (C-4'), 34.7 (C-8'), 33.1 (C-9'), 26.1 (C-5'), 26.1 (C-1'), 20.5 (C-7), 16.5 (C-11'), 16.5 (C-12').

3.5. The cytotoxicity and antimicrobial assays
The cytotoxicities of compound 1, against HL-60, SMMC-7721, A-549, MCF-7 and SW4801 were determined in vitro by MTS method. Briefly, cells were seeded in 96-well plates at a density of 5.0 x 10⁴ to 1 x 10⁵ cells/well. Cells were treated with different concentrations of compound 1 for 48 h, following incubation with MTS solution for 4 h. The absorbance was measured using a microplate reader (Bio-Rad 680, Bio-Rad, Hercules, USA) at a wavelength of 490 nm. Cisplatin was used as a positive control, generating IC₅₀ values of 1.93, 10.21, 6.59, 8.20 and 12.16 μM against HL-60, SMMC-7721, A-549, MCF-7 and SW4801 cells, respectively, and taxol was used as a positive control with IC₅₀ < 0.008 μM.

Antimicrobial assays were performed in 96-well sterilised microplates using a microdilution method. Briefly, 4-day-old spores from *F. solani* (grown on PDB medium: potato 200 g, glucose 20 g and distilled water 1000 mL) and the test concentration was 1 x 10⁵ spores/mL. The 18-hour-old bacterial cultures from *S. aureus* (grown on LB medium: yeast extract 5 g, tryptone 10 g, NaCl 10 g and distilled water 1000 mL, pH 7.0) were grown until they reach 1 x 10⁵ colony-forming units/mL. The test samples were dissolved in DMSO, and their final concentrations ranged from 512 to 0.5 μg/mL, which was determined using a 2-fold
serial dilution method. The final concentration of DMSO did not exceed 5%. The wells containing test strains and diluted samples were incubated at 28°C (4 days) for fungi and 37°C (24 h) for bacteria. The wells containing a culture suspension and DMSO were run as negative controls. As a positive control, nystatin (Taicheng Pharmaceutical Co., Ltd., Guangdong, China) had antifungal activity against *F. solani* with an MIC of 4 μg/mL, kanamycin (Yunke Biotechnology, Kunming, China) showed antibacterial activity against *S. aureus* with an MIC of 4 μg/mL. All experiments were repeated three times. The growth of test strains was observed using a CX21BIM-set5 microscope (Olympus Corp., Tokyo, Japan). MICs were determined as the lowest concentrations that produce complete growth inhibition of the tested microorganisms.

4. Conclusion

*Penicillium* sp. YIM PH30003 associated with *P. notoginseng* produced a new small molecule with antifungal activity against *F. solani*, the pathogenic fungus of *P. notoginseng*.

Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1–S8.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Note

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