Penifupyrone, a new cytotoxic funicone derivative from the endophytic fungus *Penicillium* sp. HSZ-43

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*(Received 9 March 2014; final version received 13 May 2014)*

Penifupyrone (1), a new funicone derivative, has been isolated from the endophytic fungus *Penicillium* sp. HSZ-43, along with three known analogues, funicone (2), deoxyfunicone (3) and 3-**O**-methylfunicone (4). These structures were identified by using spectroscopic methods, including UV, MS, 1D and 2D NMR experiments. The structure of 1 was confirmed by single-crystal X-ray diffraction analysis. All the isolated compounds were evaluated for cytotoxicity against human oral epidermoid carcinoma KB cells, and compound 1 exhibited moderate cytotoxic activity with IC_{50} value of 4.7 μM.

**Keywords:** endophytic fungus; *Penicillium* sp; secondary metabolites; cytotoxic activity

1. Introduction

Endophytic fungi have been demonstrated to be rich sources of bioactive natural products (Tan & Zou 2001; Schulz et al. 2002; Strobel et al. 2004; Aly et al. 2010; Chandra 2012). In the course of screening for new and potent anticancer agents, an extract of the endophytic fungus *Penicillium* sp. HSZ-43 was found to exhibit inhibition of KB cells. The fungus was isolated as an endophyte of a Chinese traditional medicinal plant, *Tripterygium wilfordii* (Luo et al. 2012; Wang et al. 2013). The EtOAc extracts of solid-substrate fermentation cultures were fractionated by silica gel, Sephadex LH-20 column chromatography and reversed-phase HPLC to afford a funicone (Merlini et al. 1970) derivative, named penifupyrone (1), and three known analogues: funicone (2), deoxyfunicone (3) and 3-**O**-methylfunicone (4) (Figure 1). Funicone (2) was first isolated from *Penicillium funiculosum*, and a series of structural analogues have been reported from many other fungi (Nicoletti et al. 2009). However, to the best of our knowledge, this is the first report of funicone derivatives carrying a furopyranone moiety. Details of the isolation, structural elucidation and cytotoxicity of these compounds are reported herein.

2. Results and discussion

Penifupyrone (1) was assigned the molecular formula C_{19}H_{18}O_{8} (11 degrees of unsaturation) on the basis of HR-EI-MS and NMR data. The \(^1H\) and \(^13C\) NMR spectra of 1 revealed the presence of four methyl groups including three **O**-methyls, one methylene, one **O**-methine, 10 aromatic/olefinic carbons (three of which were protonated), one carboxylic carbon (δC 165.9) and two α, \(\beta\)-unsaturated ketone carbons (δC 187.1, 178.0). These data accounted for all the resonances for 1 and are consistent with the molecular formula C_{19}H_{18}O_{8}. Analysis of the \(^1H\) and \(^13C\) NMR

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spectroscopic data of 1 revealed the same substructure 1,2,3,5-tetrasubstituted benzene ring as found in the funicone (2), a known compound that was co-isolated as the major component from the crude extract. These observations were supported by HMBC correlations from the methyl protons H3-17, H3-18 and H3-19 to C-16, C-12 and C-14, respectively, together with that of H-11 to C-16 (Supplementary Figure S1). An additional four-bond W-type correlation from H-11 to C-8 connected C-9 to C-8. HMBC correlations from H-2 to C-3, C-3a, C-7a and C-8 defined a furan ring, which was linked to the above-mentioned benzene ring via C-8. The H–H COSY NMR data of 1 revealed the isolated spin systems of C-15–C-5–C-6. HMBC cross-peaks from H2-6 to C-7 and C-7a revealed the connectivity of the C-15–C-5–C-6 moiety to C-7a of the furan ring via the ketone carbon C-7. Considering the chemical shifts of C-5 (δC 79.2) and C-3a (δC 157.6) and the unsaturation requirement of 1, the two carbons should be attached to the remaining oxygen atom, leading to assignments of the substructure for a 5H-furo[3,2-b]pyran-7 (6H)-one moiety. On the basis of these data, the planar structure of 1 was proposed as shown.

The structure of penifupyrone (1) was confirmed by single-crystal X-ray crystallographic analysis (Figure 2). The crystals belong to the centrosymmetric triclinic space group with lattice constants a = 13.6017(15) Å, b = 7.4874(12) Å, c = 17.5674(15) Å, α = 90°, β = 101.625(8),
\( \gamma = 90^\circ \) and \( Z = 2 \). The space group in which it crystallises requires penifupyrone (1) to be isolated as a racemic mixture. The deduction was also proved by the absence of any circular dichroism (CD) maximum and optical rotation. Unfortunately, separation of the racemic mixture by HPLC using a chiral column with several solvent systems was unsuccessful.

The known compounds 2–4 were identified by 1D and 2D NMR spectroscopy, and their structures were confirmed by comparing their spectroscopic data with the literature values. They were identified as funicone (2) (Merlini et al. 1970), deoxyfunicone (3) (Singh et al. 2003) and 3-O-methylfunicone (4) (Stefanoa et al. 1999), respectively.

Given that the crude extract exhibited cytotoxic activity against the KB cell lines, all the isolated compounds were also evaluated in this assay. The cytotoxic activities of the compounds were as follows: penifupyrone (1), \( \text{IC}_{50} = 4.7 \mu\text{M} \); funicone (2), \( \text{IC}_{50} = 13.2 \mu\text{M} \); deoxyfunicone (3), \( \text{IC}_{50} = 12.6 \mu\text{M} \) and 3-O-methylfunicone (4), \( \text{IC}_{50} = 35.3 \mu\text{M} \).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured using a JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). UV data were recorded with a Varian Cary III UV–Visible spectrophotometer (Varian, Inc., Cary, NC, USA) and CD data were collected using an Olis Cary-17 instrument (Varian, Inc., Cary, NC, USA) (0.1 cm cell). Infrared spectra (IR) were recorded on a Perkin Elmer 783 FTS165 FT-IR spectrometer. The NMR spectra were recorded on a Bruker AV-400 at 400 MHz (\(^1\text{H}\)) and 100 MHz (\(^{13}\text{C}\)) (Bruker, Bremen, Germany), using TMS as internal standard; multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC and HMBC) were run using a Bruker AV-400 NMR spectrometer. ESI-MS and HR-ESI-MS data were recorded on a Micromass Autospec instrument (Micromass UK Ltd, Manchester, UK). Single-crystal data were measured on an Oxford Gemini S Ultra diffractometer (Oxford Diffraction Ltd, Oxfordshire, UK). HPLC was performed on a Waters 1525 (Waters Co. Ltd, Milford, MA, USA) system coupled with a Waters 2996 (Waters Co. Ltd, Milford, MA, USA) photodiode array detector, using a C\text{18} column (Kromasil, 5 \( \mu\text{m} \), 250 mm \( \times \) 10 mm) for semi-preparation HPLC. Silica gel (Qing Dao Hai Yang Chemical Group Co., Qingdao, China; 200–300 mesh) and Sephadex LH-20 (Amersham Biosciences, GE Healthcare Life Science, Fairfield, CT, USA) were used for column chromatography. Pre-coated silica gel plates (Yan Tai Zi Fu Chemical Group Co., Yantai, China; G60, F-254) were used for thin-layer chromatography.

3.2. Fungal material

The fungus used in this study was originally obtained by Dr Shengze Han from fresh, healthy leaves of \( T. wilfordii \), which were collected in April 2012 from Shanxi Province, China. It was obtained using the standard protocol for the isolation of endophytic microbes from plant materials. This isolate was identified as \( \text{Penicillium sp.} \) on the basis of in vitro colony growth and micromorphology. The strain was also identified using DNA amplification and sequencing of the ITS. The sequence data have been deposited at GenBank (Accession no. KJ681497). A voucher strain has been stored at one of the author’s laboratory (Ming-jun Chen) and assigned the accession number HSZ-43.

3.3. Fermentation, extraction and isolation

The fungus was grown on rice solid medium (to 100 g rice was added 100 mL distilled water and kept overnight prior to autoclaving) in six Erlenmeyer flasks for 45 days at room temperature under static condition. After fermentation, the mouldy rice was extracted with chloroform. The combined extracts were concentrated under reduced pressure. The crude extract (1.4 g) was subsequently separated by flash chromatography on silica gel with chloroform–methanol
mixtures of increasing polarity as eluents giving 12 fractions. Fraction four was further purified on Sephadex LH-20 (chloroform–MeOH 1:1) and semi-preparative HPLC (Alltech HS Hyperprep 100 BDS C18 10 mm × 250 mm, Alltech, Deerfield, IL, USA; flow rate, 2 mL/min) eluting with 90% MeOH to afford compounds 3 (4.8 mg) and 4 (7.6 mg), respectively. Fraction six was separated by vacuum liquid chromatography on silica gel (chloroform–MeOH gradient elution), followed by semi-preparative HPLC (Alltech HS Hyperprep 100 BDS C18 10 mm × 250 mm; flow rate, 2 mL/min; 50–80% CH3CN in H2O over 45 min) to yield 1 (2.3 mg) and 2 (54.7 mg).

Penifupyrone (1): colourless crystals; [α]D25 ± 0 (c 0.50, MeOH); UV (MeOH) (λmax) (log ε) 217 (3.41), 267 (3.22), 314 (2.56) nm; IR (KBr) νmax 3425, 1669, 1472, 1327, 1077 and 967 cm⁻¹; 1H NMR (CDCl3, 400 MHz) δH 7.80 (1H, s, H-2), 7.13 (1H, d, J = 2.3 Hz, H-11), 6.70 (1H, d, J = 2.3 Hz, H-13), 4.76 (1H, m, H-5), 3.91 (3H, s, H-18), 3.81 (3H, s, H-17), 3.78 (3H, s, H-19), 2.66 (1H, dd, J = 12.6, 17.3 Hz, Hb-6), 2.54 (1H, dd, J = 3.7, 17.3 Hz, Ha-6), 1.52 (3H, d, J = 6.5 Hz, H-15); 13C NMR (CDCl3, 100 MHz) δC 187.1 (C, C-8), 178.0 (C, C-7), 165.9 (C, C-16), 161.4 (C, C-12), 158.0 (C, C-14), 157.6 (C, C-3a), 153.1 (CH, C-2), 135.9 (C, C-7a), 130.4 (C, C-10), 124.1 (C, C-9), 120.9 (C, C-3), 105.6 (CH, C-11), 103.1 (CH, C-13), 79.2 (CH, C-5), 56.1 (CH3, C-19), 55.8 (CH3, C-18), 52.5 (CH3, C-17), 43.1 (CH2, C-6), 20.2 (CH3, C-15); EI-MS m/z 374 [M]+; HR-EI-MS m/z 374.0995 [M]+ (calcd for C19H18O8, 374.0996).

3.4. X-ray crystallographic analysis of penifupyrone (1)

Upon crystallisation from MeOH – H2O (30:1) by using the vapour diffusion method, colourless crystals were obtained for 1. A crystal was separated from the sample and mounted onto a glass fibre. Data were collected using Oxford Gemini S Ultra diffractometer with a graphite monochromator and Cu Kα radiation, λ = 1.5418 Å, at 20°C. Crystal data: C19H18O8, Mr = 374.34, monoclinic, a = 13.6017(15) Å, b = 7.4874(12) Å, c = 17.5674(15) Å, α = 90°, β = 101.625(8)°, γ = 90°, V = 1752.14(10) Å³, space group P2₁/c, Z = 2, Dx = 1.4182 mg/m³, μ(Cu Kα) = 1.5419 mm⁻¹ and F(000) = 787. Crystal dimensions: 0.40 mm × 0.34 mm × 0.14 mm. Independent reflections: 3098 (Rint = 0.0478). The final R1 values were 0.0473, wR2 = 0.1243 (I > 2σ(I)). CCDC number: CCDC 985883. Using Olex2, the structure was solved with the XS, structure solution program using Direct Methods and refined with the XL refinement package using least-squares minimisation. In the structure refinements, nonhydrogen atoms were placed on the geometrically ideal positions by the ‘ride on’ method. Hydrogen atoms bonded to oxygen were located by the structure factors with isotropic temperature factors. Crystallographic data for 1 have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033, or E-mail: deposit@ccdc.cam.ac.uk).

3.5. Biological assays

Cytotoxicity was tested against KB cells by using the MTT colorimetric method. KB cells were grown in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (50 μg/mL) under a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells at the exponential growth phase were collected and transferred into 96-well plates. After incubation for 24 h, compound dilutions were dispensed to the established culture plates for 48 h. Finally, MTT solution (2.5 mg/mL in PBS) was added (40 μL/well). Plates were further incubated for 4 h at 37°C, and the formazan crystals formed were dissolved by adding 150 μL/well of DMSO. Absorbance was then determined on a Spectra Max Plus plate reader at 570 nm (Molecular Devices, Novato, CA, USA). All the experiments were carried out in triplicate and repeated three times. Doxorubicin was used as a positive control, with an IC₅₀ value of 230 nM.
4. Conclusion
Penifupyrone (1) and three known analogues, funicone (2), deoxyfunicone (3) and 3-O-methylfunicone (4) have been isolated from the endophytic fungus *Penicillium* sp. HSZ-43. These structures were identified by using spectroscopic methods, and the structure of 1 was confirmed by single-crystal X-ray diffraction analysis. All the isolated compounds were evaluated for cytotoxicity against human oral epidermoid carcinoma KB cells, and compound 1 exhibited moderate cytotoxic activity with IC$_{50}$ value of 4.7 mM.

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

Acknowledgements
We thank Dr Shengze Han for collection of the medicinal plant samples.

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