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Original Article

Penicillosides A and B: new cerebrosides from the marine-derived fungus *Penicillium* species

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ABSTRACT

In the course of our ongoing effort to identify bioactive compounds from marine-derived fungi, the marine fungus, *Penicillium* species was isolated from the Red Sea tunicate, *Didemnum* species. Two new cerebrosides, penicillosides A and B were isolated from the marine-derived fungus, *Penicillium* species using different chromatographic methods. Their structures were established by different spectroscopic data including 1D (^{1}H NMR and ^{13}C NMR) and 2D NMR (COSY, HSQC, and HMBC) studies as well as high-resolution mass spectral data. Penicilloside A displayed antifungal activity against *Candida albicans* while penicilloside B illustrated antibacterial activities against *Staphylococcus aureus* and *Escherichia coli* in the agar diffusion assay. Additionally, both compounds showed weak activity against HeLa cells.

Introduction

Marine microorganisms have received a great attention lately; accordingly, the fungi began to be recognized as a liable source of potentially useful natural products (Fenical, 1993; Bugni and Ireland, 2004). Although studies on these organisms began much later than their counterparts in terrestrial environments, more than a hundred novel compounds have been found annually since the late 1990s (Blunt et al., 2013). The high number of compounds reported from the genus *Penicillium* could be justified by the fact that its different species are salt tolerant, fast growing and are obtained easily from many substrates. This prompted many researchers to investigate variable *Penicillium* species isolated from different habitats. Their extensive studies concerned with biological activities of the isolated secondary metabolites were extremely efficient. Among the significant activities reported were the antibacterial (Qi et al., 2009; Devi et al., 2012; Abo-Kadoum et al., 2013; Subramani et al., 2013), cytotoxic and anticancer (Wang et al., 2009a,b; Sun et al., 2012; Gao et al., 2013; Abo-Kadoum et al., 2013; Subramani et al., 2013).

In the course of our ongoing search for bioactive compounds from Red Sea marine-derived fungi, the fungus *Penicillium* species was isolated from the tunicate *Didemnum* species and was cultured in Sabouraud dextrose broth. Fungal mycelia were extracted and fractionated using different chromatographic techniques to afford two compounds. Based on different spectroscopic data including HRESIMS, 1D (^{1}H NMR and ^{13}C NMR) and 2D NMR (COSY, HSQC, and HMBC), the structures of the compounds were established as cerebrosides and named penicillosides A (**1**) and B (**2**). The isolated compounds were evaluated for their antimicrobial activities against different pathogens and their cytotoxic activity against HeLa cells. Penicillosides A and B displayed significant antimicrobial activities against *Candida albicans*; *Staphylococcus aureus* and *Escherichia coli* respectively in the agar diffusion assay. Additionally, both compounds showed weak activity against HeLa cells.

Materials and methods

General experimental procedures

Optical rotation was measured on a JASCO digital Polarimeter. 1D and 2D NMR spectra (chemical shifts in ppm, coupling constants in Hz) were recorded on Bruker Avance DRX 600 MHz spectrometers using CD_3OD as solvents. Normal and HRESIMS spectra were recorded on a LTQ Orbitrap and an API 2000 (ThermoFinnigan, Bremen, Germany) mass spectrometers. For column chromatography, silica gel (Merck, 70–230 mesh ASTM) and Sephadex LH-20 (Pharmacia) were used. Pre-coated silica gel 60 F-254 plates (Merck) were used for TLC. The HPLC separation was performed on a RP_{18}, 250 mm × 10 mm, 5 μm Phenomenex Luna column using

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CH_3CN/H_2O gradient as mobile phase at 220 nm and at a flow rate of 2.0 ml/min.

Collection of the host tunicate and preparation of the fungal isolate

The marine tunicate *Didemnum* species was collected in the Mangrove located in Sharm El-Sheikh on the Egyptian Red Sea coast at depth of 1–2 m during July 2010. In order to ensure fungal isolates to be endophytic when obtained, a surface sterilization of tunicate was performed. The tunicate sample was disinfected with 5% sodium hypochlorite, followed by 70% ethanol (Li and Wang, 2009), to ensure that epiphytic fungi were destroyed by the washing while associated fungi (if any) were not affected. Approximately 2 cm^3 of inner tissue of tunicate material was homogenized using a sterile mortar and pestle containing 10 ml of sterile artificial sea water under aseptic conditions. The resulting homogenate was diluted with sterile seawater at three dilutions (1:10, 1:100, and 1:1000). For fungi cultivation, 100 μl of each dilution was plated in quadruplicate onto four plates of each of the following media; Czapek-Dox yeast agar medium ($NaNO_3$ 3 g, KCl 0.5 g, K_2HPO_4 0.1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $FeSO_4$ 0.01 g, sucrose 30 g, agar 20 g, pH 6.7); malt agar medium (malt extract 17 g, peptone 3 g, agar 20 g) and Sabouraud dextrose agar medium. All media were amended with 2% NaCl and 0.25% chloramphenicol as antibacterial agent to prevent bacterial growth and to enrich fungi growth. Plates were wrapped in parafilm, incubated at 28 °C for 1–3 weeks until the morphology of fungi could be distinguished. Many purification steps were done until pure fungal isolates were obtained.

Identification of fungal strain

Extraction of genome DNA from cultured fungal isolate

The fungal isolate was cultured in corresponding broth at 28 °C for 2–5 days. The mycelia were harvested separately by using vacuum filtration and dried with two layers of paper towel. The resulting mycelial mat was ground into powder with liquid nitrogen. The fungal DNA was extracted using QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions.

Amplification of fungal ITS-rDNA fragments of isolate

The genomic DNA of the fungal strain was used as the template to amplify fungal ITS-rDNA fragments using the primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) which were synthesized by the University of Utah DNA/peptide synthesis core facility. The reaction mixture for PCR amplification contained 5 μl of 10× reaction buffer with 15 mM $MgCl_2$ (Invitrogen), 2 μl of 2.5 mM dNTPs, 0.5 μl of 10 μM each primer, 4 μl of fungal DNA, 0.3 μl of Taq DNA polymerase (5 U μl^{-1}, Invitrogen), and 39.7 μl of H_2O. PCR conditions included an initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 50 s, annealing at 51 °C for 50 s, and elongation at 68 °C for 1 min, with a final elongation at 68 °C for 10 min. PCR products were purified using the Agarose Gel DNA Purification Kit (Qiagen) and sequenced in at the University of Utah DNA sequencing facility.

Sequence fungal ITS-rDNA regions of isolate

For preliminary identification, sequences of fungal ITS-rDNA regions obtained from the marine tunicate *Didemnum* species were compared with related sequences in National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Fungal ITS-rDNA sequences acquired in this study were edited and aligned with the best n-BLAST hits from GenBank in the Clustal X (version 1.83) program (Thompson et al., 1997), and further manually adjusted using BioEdit software (Hall, 1999). The program MEGA 5 (Tamura et al., 2011) was applied to calculate the base composition of the fungal sequences. The sequence analysis based on 99% sequence identity with *Penicillium* species Rsf-2 (NCBI accession number EF660439.1).

*Isolation and purification of compounds **1** and **2***

Large scale culture of the marine-derived fungus *Penicillium* species was carried out. The culture was incubated at room temperature for 30 days. After that, 250 ml of EtOAc were added to each flask left overnight to stop cell growth. Culture media and mycelia were separated by vacuum filtration using Buchner funnel. The mycelia were left in MeOH overnight for extraction. The extract was dried under reduced pressure. The viscous extract (1200 mg) was dissolved in 250 ml of 70% MeOH then extracted with hexane (3 ml × 100 ml). The methanol layer was concentrated under reduced pressure to yield another viscous brown residue (765 mg). A portion of the alcoholic extract (600 mg) was fractionated over silica gel (Vaccum Liquid Chromatography) using $CHCl_3$/MeOH gradient (100% $CHCl_3$ then 2, 5, 7, 10, 15% MeOH in $CHCl_3$) to give six main fractions. Fraction 2 (65 mg, eluted with 2% MeOH in $CHCl_3$) was purified on Sephadex LH-20, eluted with MeOH to obtain finally ten sub-fractions. Final purification of the main subfractions was achieved by HPLC using gradient system started with 5% ACN/H_2O to 100% ACN in 15 min to afford compound **1** (3 mg). Fraction 3 (145 mg) was dissolved in MeOH and subjected to Sephadex LH-20column eluted with 100% MeOH. The fractions showing distinct spots were purified on silica gel using pet. ether/$CHCl_3$/MeOH gradient (100% pet. Ether, followed by 20, 40, 60, 80% $CHCl_3$ in pet. Ether, followed by 5, 10, 15, 20% MeOH in $CHCl_3$). Fractions eluted with 5% MeOH in $CHCl_3$ offered impure **2**. Final purification was performed by HPLC using gradient system from 10% ACN/H_2O to 100% ACN over 20 min to obtain compound **2** (23.1 mg).

*Biological evaluation of compounds **1** and **2***

*Determination of the antimicrobial activity of compounds **1** and **2***

The procedure was conducted in triplicate according to Valgas et al. (2007) and Singh and Jain (2011). Compounds **1** and **2** were tested for antibacterial activity against a Gram positive bacterium (*Staphylococcus aureus* ATCC 25923), a Gram negative bacterium (*Escherichia coli* ATCC 25922), and yeast (*Candida albicans* ATCC 14053) using agar diffusion method. Accurately measured 0.1 ml (100 μg dissolved in DMSO) of each compound were inserted in the cups then incubated at 37 °C for 24 h. The inhibition zones were measured and compared with the reference antibiotics and antifungal drugs; ampicillin, imipenem and clotrimazole (each of 10 μg/disc giving 30, 30 and 40 mm inhibition zone respectively).

*Determination of cytotoxic activity of compounds **1** and **2***

The effects of compounds **1** and **2** on HeLa cell proliferation and cytotoxicity were evaluated using the sulforhodamine B (SRB) assay (Boyd et al., 1995; Skehan et al., 1990). HeLa cells were grown in Basal Medium Eagle (BME) containing Earle's salts, 10% FBS and 50 μg/ml gentamycin sulfate. Cells were plated at a density of 2500 cells per well in a 96-well plate and allowed to adhere and grow for 24 h before compounds were added. The compounds were solubilized in DMSO and added to a final DMSO concentration of 1% in both test wells and vehicle controls. The cells were incubated with compounds or vehicle for an additional 48 h. The IC_{50}, the concentrations required to cause a 50% inhibition of cell proliferation, were calculated from the log dose response curves. The values represent the average of 3–4 independent experiments, each conducted in triplicate ± SEM. Cytotoxicity was determined by a cell density lower than that measured at the time of drug addition. Paclitaxel was used as a positive control.