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Pencitrin and pencitrinol, two new citrinin derivatives from an endophytic fungus *Penicillium citrinum* salicorn 46

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ABSTRACT

Two new citrinin derivatives, pencitrin (**1**) and pencitrinol (**2**), and a known compound citrinin (**3**), together with its two known dimers, penicitrinone A (**4**), penicitrinone E (**5**), were isolated from an endophytic fungus *P. citrinum* 46 derived from *Salicornia herbacea* Torr. by adding $CuCl_2$ into fermentation medium. The structures of these compounds were elucidated by detailed spectroscopic analysis, and the absolute configurations of **1**, **4**, and **5** were determined by comparison of quantum chemical time-dependent density functional theory (TDDFT) calculated and electronic circular dichroism (ECD) spectra. Compound **1** exhibited potent cytotoxic activities towards A549 human lung cancer cells and HepG2 human liver cancer cells with IC_{50} values of 23.73 ± 3.61 and 35.73 ± 2.15 μM, respectively, whereas compound **5** showed moderate cytotoxic activities towards A549 and HepG2 cancer cells with IC_{50} values of 40.47 ± 4.52 and 53.57 ± 3.24 μM, respectively. The results from the current research highlighted the effectiveness and usefulness of the pipeline to discover novel bioactive fungal secondary metabolites by modification of the culture media.

1. Introduction

Penicillium citrinum is one of the most commonly occurring filamentous fungus throughout the world and well known for the production of mycotoxin metabolite citrinin (Bennett and Klich, 2013). More recently, a variety of dimers and their derivatives, which exhibited a diverse range of bioactivities compared with citrinin, were also isolated from *P.citrinum* (Clark et al., 2006; Dame et al., 2015; Xin et al., 2009).

Over the past few years, with the development of DNA sequencing, a huge number of genetic data from microorganisms revealed that many of the microbial gene clusters coding natural products are silent or poorly expressed under standard lab conditions (Bentley et al., 2002). The genetic potential for a given microorganism has been largely underestimated in the past, only a small fraction of natural products can be obtained by the traditional culture-based techniques, which leads to the currently experienced bottle neck in new natural products discovery from microbial sources. To overcome the limitations for inducing or enhancing the expression of cryptic or poorly expressed pathways and providing enough material for structure elucidation and biological testing, several strategies, such as the one strain-many compounds (OSMAC), chemical epigenetics methodology, ribosome engineering and mutagenesis, have been established to activate silent gene clusters and increase the diversity and availability of microbial natural products (Liu et al., 2016; Henrikson et al., 2009; Wang et al., 2014a,b,c). Among these strategies, OSMAC approach can completely alter the metabolic profile of many microorganisms by changing culture media components and cultivation parameters, and continues to represent a promising way to activate silent biosynthetic pathways.

In our continuing research for developing new approaches to activate the silent pathways of endophytes and attempt to produce new natural products using OSMAC strategy, an endophytic fungus *Penicillium citrinum* Salicorn 46 isolated from *Salicornia herbacea* Torr., produced two new citrinin derivatives, pencitrin (**1**) and pencitrinol (**2**), and three known compounds, citrinin (**3**), penicitrinone A (**4**) and penicitrinone E (**5**) through a bioassay-guided isolation procedure when added $CuCl_2$ into fermentation medium. The structures of **1–5** were determinated by spectroscopic analysis, containing 1D NMR and 2D NMR (Correlation Spectroscopy, (COSY), Heteronuclear Singular Quantum Correlation, (HSQC), Heteronuclear Multiple Bond Correlation, (HMBC), and Nuclear Overhauser Effect Spectroscopy, (NOESY)) spectroscopy. The cytotoxic activities of these compounds (**1–5**) against HepG2 human liver cancer cells and A549 human lung cancer cell lines were measured *in vitro*.

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2. Materials and methods

2.1. Materials and chemicals

The isolate Salicorn 46 was deposited in the China General Microbiological Culture Collection Center (CGMCC), Academia Sinica, Beijing, China (accession number, CGMCC No.8376).

Dimethyl sulfoxide-d_6 (DMSO-d_6) and $CDCl_3$ were obtained from Merck (Darmstadt, Germany). High-performance liquid chromatography (HPLC) column was bought from Agilent Technologies Inc. (California, USA). All of the other chemicals and solvents used in the current study were of analytical grade.

2.2. Fermentation and isolation of compounds

Previous studies reported two new glucoside esters produced as stress metabolites in the fresh leaves of *Portulaca oleracea* as a result of abiotic stress elicitation by $CuCl_2$ (Wu et al., 2012). Therefore, it was speculated that addition of $CuCl_2$ to the fungal medium during fermentation could induce production of new metabolites. Subsequently, a series of Cu^{2+} ions were added at the levels of 200, 400, 600, 800 and 1000 μg/L when culturing *P. citrinum* Salicorn 46. Several new peaks appeared only at the concentration of 800 μg/L in the HPLC profile compared with the control experiment (Fig. 1.) while there was no significant difference with the other concentration levels (data not shown). Production of new compounds was therefore predicted hence 800 μg/L Cu^{2+} was selected for further fermentation analysis. A single colony of *P. Citrinum* Salicorn 46 was inoculated into 100 mL of seed medium (20% potatoes, 2% maltose, 2%mannitol, 1% glucose, 0.5% monosodium glutamate, 0.5% peptone, 0.3% yeast extract, pH 6.0), and cultured on a rotary shaker at 120 rpm, 28 °C for 2 days. 10 mL of the seed cultures were inoculated into 250 mL fermentation medium (the same as the seed medium, pH 6.0) then added $CuCl_2$ to a final concentration of 800 μg/L, and cultured for 7 days on a rotary shaker at 120 rpm, 28 °C.

Approximately 8 L of culture was harvested and filtered through cheesecloth to separate the supernatant from the mycelia. The supernatant was extracted three times with ethyl acetate (EtOAc, organic fraction), whereas the mycelium was thoroughly crushed in a mortar before being extracted three times with 80% acetone-H_2O (v/v). The acetone solution was then filtered, and the filtrate was concentrated under vacuum at 30 °C until approximately 90% of the solvent had been evaporated. The remaining aqueous solution was extracted three times with an equal volume of EtOAc to give a second organic fraction. All EtOAc fractions were combined and concentrated under reduced pressure to yield the EtOAc crude extract (6.6 g).

The EtOAc extract was separated into three fractions (Frs. 1–3) by normal-phase silica gel column chromatography (40 g silica gel, 300 - 400 mesh Silica) using a step-wise gradient elution of petroleum ether: acetone: MeOH (100:0:0 – 0:100:0 – 0:0:100, v/v/v).

Fr. 1 (1.1 g) was separated into two sub-fractions Fr. 1-1 and Fr.1-2 on a normal-phase silica gel column chromatography (CC) using a step-wise gradient elution of cyclohexane: EtOAc (100:0 – 0:100, v/v). Fr. 1-1 was then passed through a Sephadex LH-20 column with a $CHCl_3$: MeOH eluent (1:1, v/v) to yield **1** (5.8 mg). Fr. 1–2 was purified on a silica gel column with petroleum ether: acetone (100:1, v/v) to produce **2** (6.5 mg). Fr. 2 (3.5 g) was also purified by silica gel CC with a cyclohexane: EtOAc eluent (100:1, v/v) to give **3** (48.3 mg). Fr.3 (2.0 g) was separated into two subfractions Fr.3-1 and 3-2 by silica gel CC using petroleum ether:acetone (100:1, v/v) as mobile phase, and Fr.3-1 was subsequently purified by silica gel CC using petroleum ether:-acetone as mobile phase (100:1, v/v) to produce **4** (8.3 mg). Fr.3-2 was subjected to silica gel CC over silica gel using a step-wise gradient elution of petroleum ether:acetone (100:1-0:100, v/v) to yield **5** (12.5 mg).

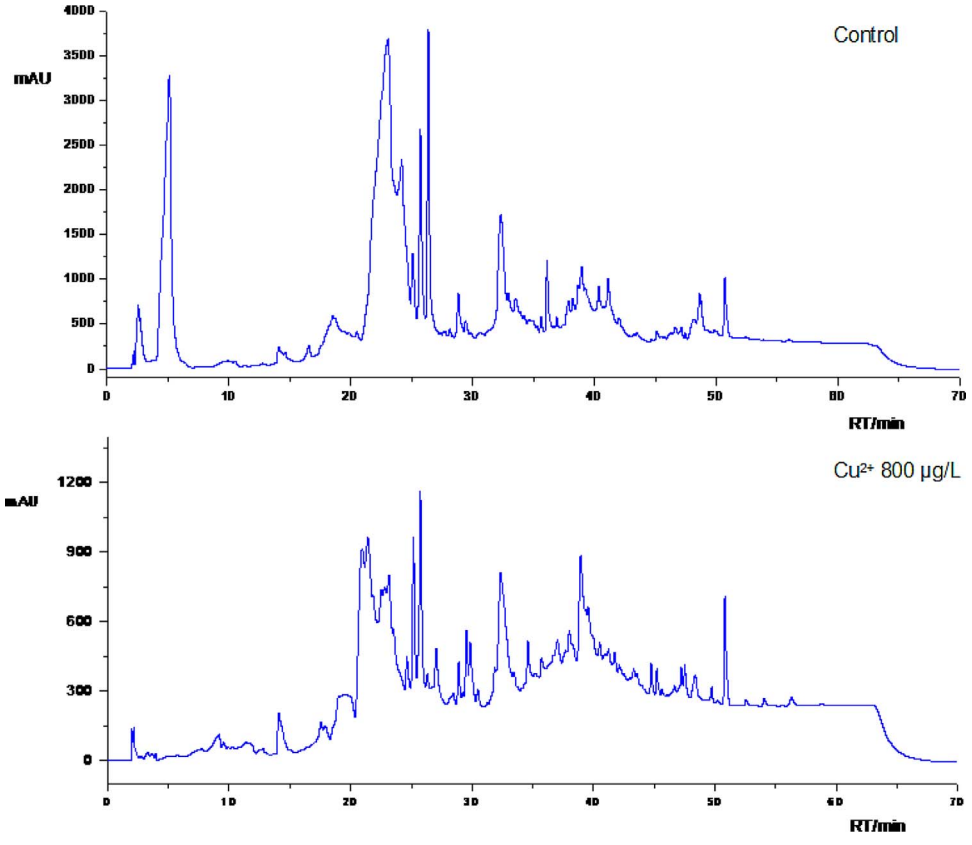

Fig. 1. High-performance liquid chromatography of fermentation products with and without Cu^{2+}.

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