Citrinin Derivatives from the Soil Filamentous Fungus *Penicillium* sp. H9318

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A investigação do extrato orgânico produzido na fermentação microbiológica de *Penicillium* sp. H9318 conduziu ao isolamento de um novo alcaloide isoquinolínico, o composto (5S)-3,4,5,7-tetrametil-5,8-di-hidróxi-6(5H)-isoquinolinona (*1*), juntamente com quatro outros conhecidos compostos derivados da citrinina (*2-5*). Uma atividade inibitória significativa no ensaio da inibição da formação dos halos (HFI), foi exibida pela citrinina (*2*), semelhante àquela observada pelo *Streptomyces* 85E, enquanto os compostos *1, 3-5* não mostraram atividade inibitória com respeito ao ensaio HFI quando testados com 20 µg/disco. Em relação ao ensaio de citotoxicidade, a citrinina (*2*) demonstrou uma atividade inibitória mais fraca quando comparada as linhagens de células cancerosas MCF-7 (IC₅₀ 71,93 µmol L⁻¹), LNCaP (IC₅₀ 77,92 µmol L⁻¹), LU-1 (147,85 µmol L⁻¹) e KB (IC₅₀ 65,93 µmol L⁻¹).

Investigation of a microbial fermentation organic extract of *Penicillium* sp. H9318 led to the isolation of a new isoquinolinone alkaloid, (5S)-3,4,5,7-tetramethyl-5,8-dihydroxy-6(5H)-isoquinolinone (*1*), along with four known citrinin derivatives (*2-5*). Citrinin (*2*) exhibited significant inhibitory activity against *Streptomyces* 85E in the hyphae formation inhibition (HFI) assay, while compounds *1, 3-5* were not active when tested at 20 µg/disk in the HFI assay. Citrinin (*2*) further demonstrated a weak inhibitory activity against MCF-7 (IC₅₀ 71,93 µmol L⁻¹), LNCaP (IC₅₀ 77,92 µmol L⁻¹), LU-1 (147,85 µmol L⁻¹) and KB (IC₅₀ 65,93 µmol L⁻¹) cell lines, respectively, in the cytotoxicity assay.

**Keywords:** *Streptomyces* 85E, isoquinolinone alkaloid, citrinin, *Penicillium* sp. H9318, kinase inhibitor

**Introduction**

The phosphorylation of proteins on serine/threonine and tyrosine residues by protein kinases is one of the major regulatory mechanisms in biological processes including apoptosis, cell proliferation, cell differentiation, and metabolism. Deregulated phosphorylation associated with these pathways can result from genetic alterations acquired early in tumorigenesis, and are often the cause of cancer. In this regard, protein kinases have emerged as promising inhibitory targets in cancer treatment. Aerial hyphae formation of *Streptomyces* sp. requires protein kinase activity. It has been shown that a variety of kinase inhibitors block this process.
During the course of our search for novel protein kinase inhibitors from natural resources, an acetone-soluble extract of the fungus *Penicillium* sp. H9318 showed inhibitory activity towards both mammalian protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) in an *in vitro* phoshatase assay. The n-BuOH-soluble extract exhibited inhibitory activity in the hyphae formation in *Penicillium* sp. H9318 significantly inhibited the growth of hyphae (26.6 g) of the CHCl3-soluble partition of two rings in 1.

### Results and Discussion

The fungus *Penicillium* sp. H9318 was isolated from a soil sample collected at a heath forest in Maliau Basin, Sabah, Malaysia. The CHCl3-soluble partition (26.6 g) of the n-BuOH extract of fermented *Penicillium* sp. H9318 significantly inhibited the growth of hyphae formation in *Streptomyces* 85E at a concentration of 80 μg/disk. A bioassay-guided isolation of the active fractions by repeated Sephadex LH-20 and silica gel MPLC afforded compounds 1-5. The known compounds 2-5 were identified as citrinin (2),11-13 penicitrone A (3),14 penicitrinol A (4)15 and penicitrinol B (5),16 respectively, by spectroscopic analysis and comparison with literature data.

Compound 1 was obtained as a colorless oil, with the molecular formula C13H15NO3 determined from the 1H and 13C NMR data (Table 1) and HRESIMS (m/z 234.11248 [M+H]+, calc. 234.11247), indicating seven degrees of unsaturation. The UV absorption bands at λmax 249, 269, 293, 307, 330 nm indicated the presence of an extended conjugated chromophore. The 1H NMR spectrum of 1 (Table 1) displayed signals of an aromatic proton at δH 8.85 (1H, s, H-1), three aromatic methyls at δH 2.66 (3H, s, H-10), 2.57 (3H, s, H-9) 1.89 (3H, s, H-12); and a tertiary methyl at δH 1.62 (3H, s, H-11). The 13C NMR and DEPT spectrum exhibited 13 carbon signals, including one carbonyl (δC 188.5), one sp2 methine (δC 143.9), six sp2 quaternary carbons (δC 178.8, 161.1, 152.4, 131.9, 126.0 and 108.5), one oxygen-bearing sp2 quaternary carbon (δC 73.6), and four methyls (δC 29.3, 22.9, 16.8 and 8.3). One carbonyl and four double bonds from the 13C NMR spectra accounted for five degrees of unsaturation, thus the remaining two degrees of unsaturation requires the presence of two rings in 1.

### Table 1. NMR spectroscopic data for compounds 1 in CD3OD (500 MHz)

<table>
<thead>
<tr>
<th>Position</th>
<th>δH, multiplicity</th>
<th>δC</th>
<th>HMBC (H→C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.85, s</td>
<td>143.9</td>
<td>C3, C4a, C8, C8a</td>
</tr>
<tr>
<td>3</td>
<td>161.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>131.9</td>
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<td></td>
</tr>
<tr>
<td>4#</td>
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<tr>
<td>5</td>
<td>73.6</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>188.5*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>108.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>178.8*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8#</td>
<td>126.0</td>
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<td></td>
</tr>
<tr>
<td>9</td>
<td>2.57, s</td>
<td>22.9</td>
<td>C3, C4</td>
</tr>
<tr>
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<td>2.66, s</td>
<td>16.8</td>
<td>C3, C4, C4a</td>
</tr>
<tr>
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<td>1.62, s</td>
<td>29.3</td>
<td>C4a, C5, C6</td>
</tr>
<tr>
<td>12</td>
<td>1.89, s</td>
<td>8.3</td>
<td>C6, C7, C8</td>
</tr>
</tbody>
</table>

*Weakly observed in 13C NMR spectrum, assigned by HMBC.

Comparison of its 1H and 13C NMR data (Table 1) with those of citrinin (2) showed that these compounds differ in the presence of C-3 and C-4 sp2 quaternary carbons (δC 161.1 and 131.9) in 1 rather than the C-3 and C-4 sp3 methines (δC 82.0 and 33.4) in 2. The 1H and 13C assignments of 1 were confirmed by analysis of the HMBC spectrum (Figure 1). Long-range correlations were observed between the signal of H-1 (δH 8.85) and C-3 (δC 161.1); between CH3-9 (δH 2.57) and C-3 (δC 161.1), C-4 (δC 131.9); as well as between CH3-10 (δH 2.66) with C-3 (δC 161.1), C-4 (δC 131.9) and C-4a (δC 152.4). Another major difference in the 13C NMR spectrum of compounds 1 and 2 was that C-12 was changed from a carbonyl group (δC 174.0) in 2 into a methyl group (δC 8.3) in 1. Also, HMBC correlations of 1 from CH3-12 (δH 1.89, s)
to C-6 (δ_C 188.5), C-7 (δ_C 108.5), and C-8 (δ_C 178.8) revealed that the methyl C-12 was located at C-7. Furthermore, the cross peaks of the methyl CH₃-11 (δ_H 1.62, s) with C-4a (δ_C 152.4), C-5 (δ_C 73.6) and C-6 (δ_C 188.5), along with a NOESY correlation observed between CH₃-11 and CH₃-10 confirmed that the methyl C-11 was connected to an oxygen bearing sp³ quaternary carbon at C-5. In consideration of the molecular formula and the ¹³C NMR chemical shift, C-5 (δ_C 73.6) and C-8 (δ_C 178.8) were substituted by hydroxyl groups, and a N atom must be assigned to position 2. In spite of our utmost efforts, the absolute configuration at C-5 of compound 1 has yet to be ascertained. Therefore, compound 1 was elucidated as 3,4,5,7-tetramethyl-5,8-dihydroxyl-6(5H)-isoquinolinone and named isoquinocitriinin A.

Several similar novel isoquinoline alkaloids have been reported from the genus of Penicillium, Aspergillus, Streptomyces, and Chaetomium. Most isoquinoline alkaloids discovered from plants exhibit complicated structures, which were mainly synthesized from tyrosine, whereas most isoquinolines from lichens, fungi, and sponges show simple skeletons and are probably biosynthesized via mixed pathways.

Compound 1 possesses a carbon skeleton similar to citrinin (2), and it is possibly biosynthesized via mixed routes. The proposed biosynthesis of compound 1 proceeds via an enzyme-bonded poly-β-ketone chain and the amino acid formation is accomplished by an aminotransferase (Scheme 1).

Citrinin (2) was first isolated from P. citrium in 1931 and it has been isolated from ten or more species of Penicillium and Aspergillus. Compounds 3-5 are known citrinin dimer derivatives. Until now, more than ten citrinin dimers have been discovered from natural resources. This is the first report of an isoquinolinone alkaloid from the genus Penicillium possessing a carbon skeleton similar to that of citrinin.

Compounds 1-5 were evaluated for their inhibitory activities against Streptomyces 8SE in the hyphae formation inhibition assay, according to an established protocol. Compound 2 exhibited significant inhibitory activity and gave a 21 mm clear zone of inhibition (ZOI) at 80 µg/disk. 17 mm clear ZOI at 40 µg/disk, 12 mm ZOI at 20 µg/disk, and a 10 mm bald ZOI at 2.5 µg/disk. All other isolates were inactive. It is hypothesized that compounds which inhibit hyphae formation in Streptomyces 8SE may block the proliferation of cancer. In the human breast cancer MCF-7 cells, proliferation can be blocked in numerous ways, including serum deprivation, pharmacological inhibition of specific kinase and steroid hormone pathways. Therefore, compound 2 was further evaluated for its cytotoxicity activity using several cancer cell lines. Compound 2 demonstrated a weak activity with the MCF-7 (IC₅₀ 71.93 µmol L⁻¹ or 18 µg mL⁻¹), LNCaP (IC₅₀ 77.92 µmol L⁻¹ or 19.5 µg mL⁻¹), and LU-1 (147.85 µmol L⁻¹ or 37.0 µg mL⁻¹), KB cells (IC₅₀ 65.93 µmol L⁻¹ or 16.5 µg mL⁻¹), respectively.

Citrinin (2), a well-known mycotoxin, is both nephrotoxic and carcinogenic. It was reported to show moderate cytotoxicity against the mouse NS-1 cell line with LD₉₀ values of 25 µg mL⁻¹, and antifungal activities against Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus, and A. niger at a concentration of 100 µg/disk. It was documented too that penicitrinol A (also known as dicitrinin A) (3) showed moderate cytotoxicity against the mouse NS-1 cell line with LD₉₀ values of 6.3 µg mL⁻¹, and displayed scavenging activity with IC₅₀ values of 55.3 mmol L⁻¹, while penicitrinol B (5) did not show cytotoxicity against P388, A-549, BEL-7402, and HL-60 cells (IC₅₀ > 50 µmol L⁻¹).

**Experimental**

**General procedures**

Optical rotations were measured using a JASCO P-1010 automatic polarimeter. UV spectra were measured on a HP 8453 UV-Visible spectrophotometer. FT-IR spectra were acquired on a Perkin-Elmer Spectrum BX spectrometer using AgCl film. Mass spectra and high-resolution mass spectra were taken with a BioTOF II ESI mass spectrometer. 1D and 2D NMR spectra were recorded in MeOH-d₄ and DMSO-d₆ on a Varian INOVA Unity (500 MHz) spectrometer. The chemical shift
(δ) values are given in ppm with the residual CD3OD signals (3.31 ppm for 1H and 49.15 ppm for 13C) as internal standard, and the coupling constants (J) are in Hz. Chromatographic fractions and pure compounds were monitored by TLC, detected by absorption of UV light at 254 nm and a color reaction by spraying with a solution of 10%, v/v, sulfuric acid/EtOH followed by 5 min heating at 120 °C. MPLC (medium pressure column chromatography) was carried out on a Büchi® pump system and a Büchi column packed with Merck silica gel 60 and/or reversed-phase C18.

Fungal material

The fungal strain H9318 was isolated from the soil sample (MB 75) collected under an unidentified tree in a “Kerangas” or a heath forest at about 3000 feet (900 meters) above sea level located within the rim perimeter (south side) of Maliau Basin, Sabah, Malaysia in May 2001. The morphological characteristics of the Penicillium structure of H9318 as observed through light microscopic are circular conidia, the Ampulliformum philiades are shorter than the metula, 3 to 4 metulas were observed to branch from a single smooth stipe (of which some metulas were non-uniform in length). These characteristics points most likely to the subgenus Furcatum. The strain is maintained in the Laboratory Collection at the University of Malaysia Sabah. The isolation medium used was dichloran rose bengal chloramphenicol (DRBC) medium with NaCl (2.5%) at pH 5.6. Fungi were purified, and the producing strain was prepared on potato dextrose agar slants containing NaCl 10%, m/v, and stored at 4 °C. Conidia of single colonies of microfungi were kept in anhydrous silica gel particles at 4 °C. It was then ready to inoculate to a seed medium.

Fermentation

The composition of the seed and production medium (in g L−1) was as follows: yeast extract (10), peptone (10), sucrose (10), KH2PO4 (1), and MgSO4.7H2O (0.3). The seed medium was prepared with distilled water, and the pH was adjusted to 5.5 prior to sterilization. The medium was dispensed at 50 mL per 250 mL in Belco baffled shaker flasks. A single colony of Penicillium from the agar plate was used as inoculum into each flask of seed media, cultured at 30 °C at 250 rpm for two days. An aliquot (1.5%) strain H9318 from the seed medium was inoculated into the production medium (similar composition) and were incubated at 30 °C at 220 rpm for seven days and were then harvested.

Extraction and isolation

The microfungi fermentation culture (90 L) was centrifuged, and the supernatant was filtered and partitioned with n-BuOH. Both the n-BuOH-soluble and aqueous layers were tested against the hyphae formation inhibition (HFI) assay. The organic extracts were concentrated, suspended in H2O (1:1, v/v) and subsequently partitioned with hexane, CHCl3, and EtOAc (3 × 500 mL each). The CHCl3-soluble partition (26.6 g) of n-BuOH extract of fermented Penicillium sp. H9318 significantly inhibited the growth of hyphae formation in Streptomyces 85E at a concentration of 80 µg/disk, and was subjected to bioassay-guided fractionation. This extract was chromatographed using silica gel CC, eluting with a gradient of CH2Cl2-MeOH, to afford ten fractions (H1-H10). Fraction H2 (5.3 g) was further separated on Sephadex LH-20 eluting with CHCl3-MeOH (1:1, v/v) to give one major fraction, which was crystallized from CH2Cl2 to afford the yellow crystalline compound 2 (2 g). Fraction H4 (100 mg, CH2Cl2-MeOH (50:1, v/v)) was chromatographed on silica gel and then Sephadex LH-20 (CHCl3-MeOH (1:1, v/v)), to afford compounds 3 (10.5 mg), 4 (5.6 mg), and 5 (7.0 mg). Fraction H6 (80 mg, CH2Cl2-MeOH (30:1, v/v)) was separated further on Sephadex LH-20 (CHCl3-MeOH (1:1, v/v)) and then purified by reversed-phase C18 MPLC, eluting with 50% MeOH to give compound 1 (8.5 mg).

Isoquinocitrinin A (1): colorless oil; [α]D21−5.2 (ε 0.42, MeOH); λmax/nm (MeOH) (logε): 207 (4.15), 229 (sh, 3.78), 249 (sh, 3.66), 269 (3.54), 293 (3.58), 307 (3.59), 330 (sh, 3.53); IR (AgCl, film) νmax/cm−1 3350, 2928, 1600, 1508, 1383, 1237, 1124, 1071, 910; 1H and 13C NMR see Table 1; LRESIMS m/z 234 (M+); LRESIMS m/z 232 (M−); HRESIMS [M+H]+ m/z Found: 234.11248. Calc. for C13H16NO3: 234.11247.

Hyphae formation inhibition assay

The inhibition assay observed with hyphae formation in Streptomyces 85E was performed on purified isolates as described previously. The mycelia fragments of Streptomyces were spread on minimal medium ISP 4 agar plates for the generation of a bacteria lawn. Compounds of known concentration dissolved in MeOH were dispensed onto disks in 20 µL aliquots. The impregnated paper disks (with a concentration of 80 µg/disk) were applied directly on the surface of the agar plates seeded with Streptomyces 85E. After 30 h of growth (during which the development of hyphae in Streptomyces species takes place), the results are identified by a clear zone of inhibition or bald phenotype around the disk. Surfactin, a sporulation inhibitor, and
MeOH were employed as positive and negative controls, respectively. An inhibition zone of greater than 9 mm is considered active. Subfractions were tested at 80 μg/disk on 7 mm filter disks. Active compounds were tested at lower concentrations (20, 10, 5, 2.5 μg/disk). The assays were performed in duplicate.

Cytotoxicity assay

The standard protocol for the assessment of cellular toxicity measures the ability of cultured cells to proliferate in the presence of test samples, and subsequently quantitates total protein content with sulforhodamine B dye as a measure of the percentage of surviving cells. The cytotoxic potential of citrinin was determined against the KB (human oral epidermoid carcinoma), LNCaP (androgen-sensitive human prostate adenocarcinoma), the KB (human oral epidermoid carcinoma), LNCaP (androgen-sensitive human prostate adenocarcinoma), MCF-7 (human breast adenocarcinoma), and LU-1 (human lung carcinoma) cell lines. Cells were seeded in 96-well plates (2.5 × 10^4 cells mL^{-1}), and six two-fold serial dilutions of samples in 10% DMSO (10 μL) were added to each well. The plates were incubated for 72 h at 37 °C, after which cell viability was determined with sulforhodamine B staining. IC_{50} values were determined as the concentration of sample required to inhibit cell growth by 50% relative to a control treated with 0.5% DMSO, and represent the average of triplicate values obtained from two independent experiments.

Supplementary Information

HRESIMS, 1H and 13C NMR spectra, HMQC, HMBC and NOESY spectra for compound 1 are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

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References

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Figure S1. HR-(+)-ESIMS spectrum of isoquinocitrinin A (1).

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Figure S2. $^1$H NMR spectrum of isoquinocitrinin A (1, CD$_3$OD, 500 MHz).

Figure S3. $^{13}$C NMR spectrum of isoquinocitrinin A (1, CD$_3$OD, 125 MHz).
Figure S4. HMQC of isoquinocitrinin A (1, CD$_3$OD).

Figure S5. HMBC of isoquinocitrinin A (1, CD$_3$OD).
Figure S6. NOESY spectrum of isoquinocitrinin A (1, CD$_3$OD).