Short Communication:

Bioactive Secondary Metabolites from the Mangrove Endophytic Fungi *Nigrospora oryzae*

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Abstract: *Mangrove forest has a distinctive habitat adapting with marine and terrestrial environment. Chemical investigation of the extract from mangrove endophytic fungi* Nigrospora oryzae *had resulted in the isolation of sterigmatocystin (**1**) and pestalopyrone (**2**). The structure of sterigmatocystin (**1**) and pestalopyrone (**2**) were elucidated using mass, UV and NMR spectrometers together with the comparison with the literature data. The study also showed that sterigmatocystin displayed moderate cytotoxicity but it could be further developed as antiviral and antibacterial agent based on the SAR information reported from its analogue and derivatives.*

Keywords: Nigrospora oryzae*; mangrove; sterigmatocystin; pestalopyrone*

■ INTRODUCTION

Endophytic fungi have been known as a rich source of valuable metabolites with various biological properties including antibacterial [1], cytotoxic [2] and anti-fungal activities [2-5]. Recent advances have also shown that the most important and valuable drugs from plants have been also produced by the endophytic fungi. For example, paclitaxel known as natural product from the plant *Taxus brevolia* has been widely found in endophytic fungi. At least 20 species of endophytic fungi have been reported to produce paclitaxel [6]. In addition, the metabolites of the Madagascar plant of periwinkle (*Catharanthus roseus*), namely vincristine and vinblastine, were also reported to be produced by the endophytic fungi *Talaromyces radicus* [7] and *Fusarium oxysporum* [8].

The production of the bioactive metabolites of endophytic fungi was influenced by some factors especially their habitat of growing. Mangrove habitat is distinctive one due to their adaptation to terrestrial and marine environments. With this extreme condition, mangrove-associated-endophytic fungi produced various bioactive molecules for their adaptation against abiotic and biotic stress [9-10]. In this work, we reported the isolation and structural elucidation of cytotoxic metabolites from the mangrove endophytic fungi *Nigrospora oryzae* grown on solid rice media.

■ EXPERIMENTAL SECTION

Materials

Materials used in this study were silica gel 60 M

(230–400 mesh), which was obtained from Macherey-Nagel GmbH & Co. KG, Dueren (Germany), TLC plates (silica gel 60 F-254) was obtained from Merck KGaA, Darmstadt, Germany and Sephadex LH-20 was ordered from Sigma. All solvents were freshly distilled including *n*-hexane, dichloromethane, ethyl acetate and methanol. Methanol for HPLC was purchased from Merck.

Instrumentation

The 1D and 2D NMR spectra were recorded on a Bruker ARX 500 NMR spectrometer. Chemical shifts of NMR spectra were analyzed by referencing to the residual solvent peak at (ppm) δ_H 7.26 ($CDCl_3$) and δ_H 3.31 (CD_3OD) for ^1H, and δ_C 77.0 ($CDCl_3$) and 49.2 (CD_3OD) for ^{13}C, respectively. Mass spectra were analyzed with a LCMS HP1100 Agilent Finnigan LCQ Deca XP Thermoquest, while HPLC data was recorded by a Dionex P580 system with routine detection at 235, 254, 280, and 340 nm. TLC was performed on pre coated silica gel plates with solvent system using EtOAc/MeOH/H_2O (30:5:4) and CH_2Cl_2/MeOH (9:1). The monitoring was undertaken by spraying the plates with anisaldehyde reagent or with detection at 254 and 366 nm.

Procedure

Isolation and identification of endophytic fungi

The isolation and identification of endophytic fungi from the leaves of mangrove *Avicennia marina* was undertaken according to the previously published protocol [11-12]. Briefly: After surface sterilization by immersing the leaves in 70% ethanol (50 mL), fresh healthy leaves were cut into small segment (0.5 cm × 0.5 cm) and then put onto the surface of malt extract agar containing the antibiotic chloramphenicol to inhibit the growth of bacteria. The composition of media for isolation followed the procedure described earlier [11] including malt extract and agar (15 g/L for each) together with the addition of chloroamphenicol (0.2 g/L) dissolved in distilled water and maintained at pH 7.4–7.8 by adding 10% NaOH or 36.5% HCl dropwise. After two days incubation at room temperature, the fungal strain grew out from the tissue of leaves. The pure strain of *Nigrospora oryzae* was isolated from the mixed culture and periodically check for its purity and identified using internal transcribed spacer (ITS) DNA with specific fungal primer pairs following the published protocol earlier [11-12].

Extraction and isolation

The pure strain of *Nigrospora oryzae* was grown in 1 L of Erlenmeyer flask. Rice (100 g) and distilled water (110 mL) were added to the Erlenmeyer and sterilized with autoclave. The fungus was cut into several pieces and then added into the Erlenmeyer flask containing the solid rice media. The fermentation was carried out for 3 weeks. The culture was extracted with ethyl acetate. The crude extract (800 mg) was fractionated with Sephadex LH-20 using the mixture of dichloromethane and methanol (1:1). Fraction III (35 mg) was further separated and purified with semi-preparative HPLC to afford compound **1** (5 mg) and **2** (1 mg).

Sterigmatocystin (1). White amorphous powder; ^1H-NMR ($CDCl_3$, 500 MHz): δ = 13.2 (s, 1H), 7.51 (t, *J* = 8.4 Hz, 1H), 6.85 (d, *J* = 7.3 Hz, 1H), 6.84 (dd, *J* = 1.0, 7.25 Hz, 1H), 6.77 (dd, *J* = 1.0, 8.4 Hz, 1H), 6.50 (dd, *J* = 2.9, 2.0 Hz, 1H), 6.45 (s, 1H), 5.46 (t, *J* = 2.65 Hz, 1H), 4.82 (ddd, *J* = 2.0, 4.4, 7.1 Hz, 1H), 4.0 (s, 3H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ = 181.2, 164.6, 163.3, 162.1, 155.3, 154.2, 145.4, 135.9, 113.1, 111.9, 108.2, 106.9, 105.9, 105.8, 102.6, 90.8, 56.8, 47.8; UV λ_{max} (PDA) 247.6 and 326.5 nm; ESIMS *m/z* 325.3 $[M+H]^+$, 670.9 $(2M+Na)^+$.

Pestalopyrone (2). Amorphous powder; ^1H-NMR (MeOD, 500 MHz): δ = 6.63 (qq, *J* = 0.96, 7.1 Hz, 1H), 6.12 (d, *J* = 2.1 Hz, 1H), 5.56 (d, *J* = 2.1 Hz, 1H), 3.86 (s, 3H), 1.86 (d, *J* = 7.2 Hz, 3H), 1.84 (s, 3H). ^{13}C-NMR (MeOD, 125 MHz): δ = 174.1, 166.9, 162.7, 131.0, 128.3, 98.9, 88.8, 56.9, 14.3, 12.0; UV λ_{max} (PDA) 226 and 310 nm; ESIMS *m/z* 181.0 $[M+H]^+$and at *m/z* 382.7 $(2M+Na)^+$.

Cell proliferation assay

Cytotoxicity was assessed against the murine lymphoma (L5178Y) cell line using a microplate 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay as previously described [11,13]. The cells were seeded on 96-well plates with 10^4 cells per well and allowed to attach for 24 h and then treated with 10 μg/mL of the tested compound for 24 h. For the assay, the culture

medium was made to contain 0.7 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After two hours of incubation at 37 °C, the cells were lysed with a mixture of 95% isopropanol/5% formic acid. The concentration of reduced MTT as a marker for cell viability was measured using a Wallac Victor2 multilabel counter at 560 nm. Cells incubated only with vehicle control (DMSO) were considered 100% viable. The isolated compounds were bioassay-guided using L5178Y cancer cell line. Kahalalide F isolated from Indian sacoglossan mollusk *Elysia grandifolia* [14] was used as positive control with IC_{50} value of 4.3 µM.

■ RESULTS AND DISCUSSION

Fungal strain isolated from the leaves of mangrove of *Avecenia marina* was massively produced with rice media and subsequently extracted using ethyl acetate. The chromatographic separation using combination of Sephadex LH-20 together with semi-preparative HPLC afforded compounds **1** and **2**. Compound **1** was obtained as an amorphous powder. The UV spectrum showed absorption at 247.6 and 326.5 nm (Fig. 1(a)). The mass spectral data showed molecular ion peak at 325.3 $(M+H)^+$ and at 670.9 $(2M+Na)^+$ indicating that compound **1** has the molecular mass of 324.3 g/mol. The melting point of compound **1** was not measured due to the limited quantity of **1** obtained in this work.

The ^{1}H-NMR spectrum of **1** showed signals of a methoxy group at δ 4.00 (s), an intramolecular hydrogen bond of aromatic OH proton at δ 13.2 (s), one ABX system which the signals observed at δ 6.77 (dd, J = 1.0, 8.4 Hz, H7), 7.51 (t, J = 8.4 Hz, H8) and 6.84 (dd, J = 1.0, 7.25 Hz, H9), one aromatic signal at δ 6.44 (s, H-5), two olefinic proton signals at δ 6.50 (dd, J = 2.9, 2.0 Hz, H1) and δ 5.46 (t, J = 2.65 Hz, H2) together with two methyne signals resonating at δ 4.82 (ddd, J = 2.0, 4.4, 7.1 Hz, H3) and δ 6.85 (d, J = 7.3 Hz, H4). Analysis of ^{1}H–^{1}H COSY revealed the presence of two spin system. The aromatic signal at δ 6.84 ppm (H9) had correlation with the signal δ 7.51 ppm (H8) which in turn showing correlation with δ 6.77 ppm (H7). Another spin system was observed from the correlation of signal at δ 6.50 ppm (H1) and δ 5.46 ppm (H2) which further have correlation with signal δ 4.82 ppm (H3). The latter signal was then connected with another methyne proton at δ 6.83 ppm (H4). The position of methoxy group was assigned to C17 based on the HMBC correlation from methoxy proton signal at δ 4.00 ppm (H18) to C17 at δ_c 163.5 ppm. This was then supported from the HMBC correlation of H5 (δ_H 6.44 ppm) to C17 (δ_c 163.5 ppm) as well (Fig. 2(a)). The NMR data of compound **1** (Table 1) was very similar to those reported for sterigmatocystin [15-16]. The UV λ_{max} observed here was also in agreement with those reported for sterigmatocystin type of metabolites which normally observed at 248 and 322 nm [17]. Therefore, compound **1** was identified as sterigmatocystin (Fig. 2(a)).

Compound **2** was isolated as amorphous powder having maximum wavelength at 226 and 310 nm which was characteristic to pyrone moiety (Fig. 1(b)). The molecular mas of compound **2** was determined as 180.0 g/mol based on the mass spectral data showing the molecular ion peak at m/z of 181.0 $(M+H)^+$ and at *m/z* of 382.7 $(2M+Na)^+$ in positive mode. Due to the limited quantity of the material, the melting point of compound **2** was not measured. The ^{1}H-NMR spectrum revealed the aromatic signals at δ 6.12 ppm (d, J = 2.1 Hz, H5) and at δ 5.56 ppm (d, J = 2.1 Hz, H3) and one olefinic signal

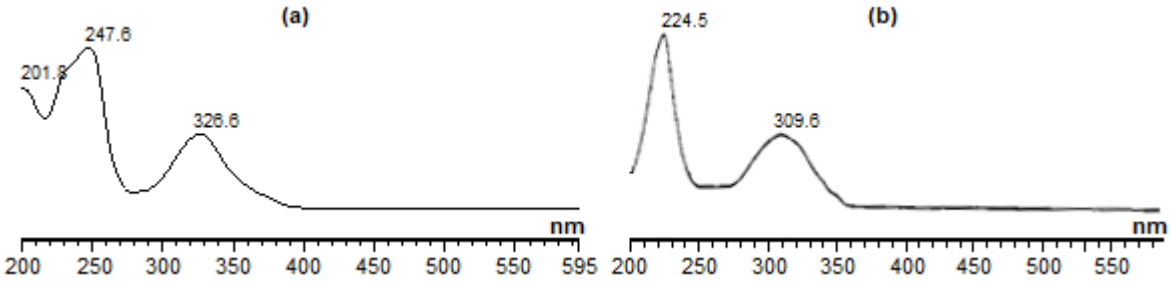

Fig 1. UV spectra of compound **1** (a) and compound **2** (b)

Table 1. NMR data of compound **1** ($CDCl_3$) and **2** (MeOD)

No	^{1}H-NMR (δ_H in ppm, multiplicities, *J* in Hz)				^{13}C-NMR (δ_C in ppm)			
	1 ($CDCl_3$)	Ref ($CDCl_3$) [15]	**2** (CD_3OD)	Ref (CD_3OD) [18]	**1** ($CDCl_3$)	Ref ($CDCl_3$) [16]	**2** (MeOD)	Ref (CD_3OD) [18]
1	6.50, dd, *J* = 2.9, 2.0	[15]6.50, dd, *J* = 2.8, 1.6			145.4	145.5		
2	5.46, t, *J* = 2.65	5.45, t, *J* = 2.6			102.6	102.7	166.9	166.9
3	4.82, ddd, *J* = 2.0, 4.4, 7.1	[15]4.81, dt, *J* = 2.2, 7.1	5.56, d, *J* = 2.1	6.12, d, *J* = 1.0*	47.8	48.2	88.8	98.8 [20]
4	6.85, d, *J* = 7.3	6.84, m			113.1	113.4	174.1	174.1
5	6.45, s	6.44, s	6.12, d, *J* = 2.1	5.55, d, *J* = 1.0*	90.8	90.7	98.9	88.5 [20]
6					162.1	162.5	162.7	162.6
7	6.77, dd, *J* = 1.0, 8.4	6.76, dd, *J* = 1.15, 8.55			111.9	111.4	128.3	128.3
8	7.51, t, *J* = 8.4	7.50, t, *J* = 8.3	6.63, qq, *J* = 0.96, 7.1	6.63, qq, *J* = 1.2, 7.2	135.9	135.9	131.0	131.0
9	6.84, dd, *J* = 1.0, 7.25	6.84, m	1.86, d, *J* = 7.2	1.85, d, *J* = 7.5	105.9	106.1	14.3	12.0
10			1.84, s	1.87, d, *J* = 1.0	155.3	155.1	12.0	14.3
11					108.2	109.1		
12					181.2	181.6		
13					105.8	106.1		
14					154.2	154.2		
15					106.9	106.7		
16					164.6	164.7		
17					163.3	163.5		
6-OH	13.2, s							
11-OCH3	4.00, s	4.00, s	3.86, s	3.70, s	56.8	57.0	56.9	56.9

*Reversed in the report Oleinikova et al. [15]; Lee et al. [18]

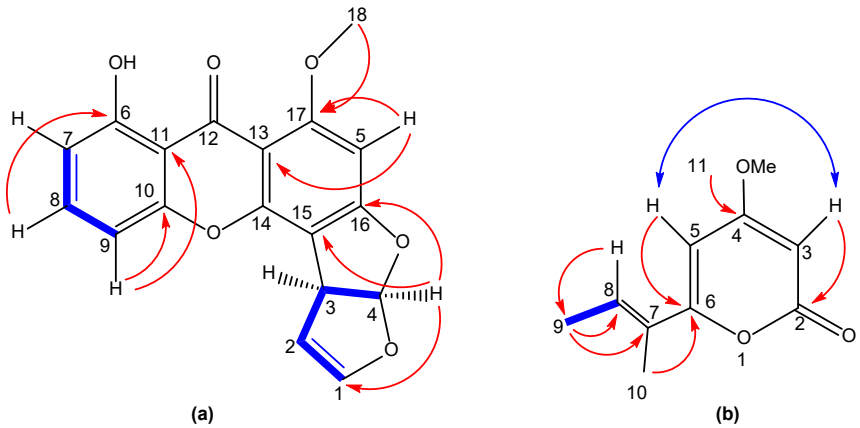

Fig 2. COSY and Key HMBC Corelations of **(a)** Sterigmatocystin **1** and (**b**) Pestalopyrone **2** (Blue for COSY and Red for HMBC correlations)

at δ 6.63 ppm (qq, *J* = 0.96, 7.1 Hz, H8) together with signals from two methyl attached to the double bond at δ 1.86 (d, *J* = 7.2 Hz, H9) and 1.84 (s, H10) ppm. The COSY spectra showed the presence of two spin systems. The

methyl signal at δ 1.86 ppm (H9) showed correlation with olefinic signal at δ 6.63 ppm (H8). The weak *meta* coupling correlation was observed from the aromatic signals at δ 6.12 (H5) and 5.56 (H3) ppm. In addition, the ^{13}C-NMR showed the presence of four quaternary carbons resonating at δ 174.1 (C4), 167.1 (C2), 162.8 (C6) and δ 128.5 (C7) ppm. The position of the quaternary carbons was assigned by the HMBC correlations. Moreover, the HMBC correlation from the methoxy signal at δ 3.86 ppm to the carbon resonating at 174.1 ppm (C4) confirmed the position of methoxy group at C4 (Fig. 2(b)). The ^{1}H-NMR spectrum was in a good agreement with the data reported for pestalopyrone [18-19]. The structure was also confirmed by comparison with the ^{13}C-NMR data which has been previously reported by Lee et al. [18].

Sterigmatocystin **1** was evaluated for the cytotoxic property against L5178Y murine cell line. Sterigmatocystin **1** was able to inhibit the growth of the cell line by 64% at the concentration of 10 μg/mL. The moderate cytotoxic activity of sterigmatocystin **1** and its derivatives have been earlier reported [20-21]. Pestalopyrone **2** was not tested due the limited quantity of the material. Pestalopyrone had been reported to be phytotoxic against hazelnut [22]. However, recent finding suggested that pestalopyrone based structures such as pestalopyrones A-D had no cytotoxic and antibacterial properties [23]. Pestalopyrone was also found inactive against dengue virus [24].

Although sterigmatocystin did not have strong cytotoxic activity, several derivatives of sterigmatocystin including 5-methoxydihydrosterigmatocystin showed potent antibacterial activity [17]. Sterigmatocystin was also reported to have antiparasitic property against *Trypanosoma cruzi* [25]. Moreover, sterigmatocystin derivatives, sterigmatocystins A–C, showed specific antiviral activity [26]. Therefore, sterigmatocystin would be further developed in the direction of antibacterial and antiviral activity based on this valuable SAR (structure activity relationship) information. Moreover, this study confirmed that the endophytic fungi are still potential and valuable sources of bioactive secondary metabolites [27-28].

■ CONCLUSION

Chemical investigation of the extract from the mangrove associated fungus *Nigrospora oryzae* resulted in the isolation of sterigmatocystin and pestalopyrone. Sterigmatocystin showed moderate cytotoxic activity against murine cancer cell line. Comparison of bioactivity between sterigmatocystin and its analogues (derivatives) suggested that sterigmatocystin could be developed as antiviral and antibacterial agents.

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