New phenyl derivatives from endophytic fungus *Botryosphaeria* sp. SCSIO KcF6 derived of mangrove plant *Kandelia candel*

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Two new phenyl derivatives (1 and 3), along with two new natural products (4 and 5), and three known compounds (2, 6 and 7), were isolated from an endophytic fungus *Botryosphaeria* sp. SCSIO KcF6. The structures of these compounds 1–7 were elucidated by the extensive 1D and 2D-NMR and HRESIMS Data analysis, and compared with those of reported data. The absolute configuration of the compounds 1 and 3 were assigned by optical rotation and CD data. The isolated compounds were evaluated for their cytotoxic, anti-inflammatory (COX-2) and antimicrobial activities. Compound 3 exhibited a specific COX-2 inhibitory activity with the IC\textsubscript{50} value of 1.12 \mu M.

**Keywords:** endophytic fungus; *Botryosphaeria* sp. SCSIO KcF6; COX-2 inhibitory activity

1. Introduction

Endophytic microorganisms are mostly termed as bacteria, fungi and actinomycetes that live in the intercellular spaces of plant tissue without causing any apparent damage to their host. Especially, some of these fungi isolated from the coastal mangrove plants produce diverse array of bioactive substances. Mangrove plants are growing in subtropical and tropical intertidal habitats (Wang et al. 2003). Marine mangrove system is a special eco-environment and an
increasing number of bioactive secondary metabolites have recently been reported from mangrove plants and mangrove-derived fungi (Yang et al. 2010; Blunt et al. 2012, 2013; Rukachaisirikul et al. 2012; Zhou et al. 2014). Recent research supports that these endophytic fungi derived from coastal mangrove plants can be a good source of potentially new bioactive secondary metabolites, some of with featured novel carbon skeletons hitherto unprecedented in nature which were reported in our previous work (Ai et al. 2014; Bai et al. 2014; Wang et al. 2014; Yang et al. 2014).

In recent years, much attention is focused towards a particular group of endophytic fungus which belongs to the genus *Botryosphaeria*. Some interesting class of chemical moieties likely to be naphthalenones, lactones, polyketides, diterpenoids, benzofuran derivatives and exopolysaccharides are recently isolated and identified from the species of *Botryosphaeria* (Rukachaisirikul et al. 2009). These metabolites are strong enough in their biological properties which includes antibacterial (Pongcharoen et al. 2007), antiseptic (Voegtle et al. 2008), phytotoxic (Venkatasubbaiah et al. 1991) and antimicrobial (Yang et al. 2006). As a wide stretch of this particular research theme, a fungal endophyte of *Botryosphaeria* sp. SCSIO KcF6, obtained from the fruit part of a mangrove plant *Kandelia candel* from the South China Sea coast, and herein we isolated two new compounds and two new natural compounds, together with three known compounds from the ethyl acetate extract. Isolation, structural elucidation through 1D and 2D-NMR spectra and HRESIMS spectrometry, and biological screening of the isolated compounds are described herewith.

2. Results and discussion

Two new phenyl derivatives (1 and 3), along with two new natural products (4 and 5), and three known compounds (2, 6, and 7) were isolated from the ethyl acetate crude extracts of the rice medium.

Compound 1 was isolated as white powder. The high resolution mass spectra of 1 gave [M + H]⁺ at m/z 267.0861, corresponding to the molecular formula C₁₃H₁₄O₆. The IR spectrum showed the presence of ester/lactone carbonyl at 1682 cm⁻¹ and hydroxyl at 3356 cm⁻¹. The ¹H NMR spectrum of compound 1 exhibited signals of an olefinic proton at δ_H 6.61, an aromatic methane proton at δ_H 6.38, an aromatic methoxyl proton at δ_H 3.76 and 2-hydroxy propyl moiety at δ_H 4.16, 2.62 (2H) and 1.26. The ¹³C NMR spectrum revealed signals of a carbonyl carbon at δ_C 167.9, two sp² quaternary carbons at δ_C 132.4 and 98.3, four oxygenated quaternary carbons at δ_C 161.7, 161.0, 156.0 and 136.0, three methines at δ_C 103.9, 101.7 and 66.4, an aromatic methoxyl carbon at δ_C 61.8, a methylene carbon δ_C 44.2, and a methyl carbon at δ_C 23.5. The planer structure of compound 1 was confirmed by the key HMBC correlations. In the HMBC spectrum, the signal of H-4 showed correlations with C-3, C-5 and C-8a, and the signal of H-5 showed correlations with C-5a and C-8a, supporting the lactone ring was connected with the aromatic ring at C-5a and C-8a. The HMBC correlations of H-11 to C-3, H-4 to C-10, and H-9 to C-3 and C-4 reveal that a 2-hydroxypropyl moiety was attached to C-3. The NMR data of 1 were very similar with that of the known compound diaporthin (Hallock et al. 1988), with the sole difference being the presence of a hydroxyl at C-7 instead of the proton. The configuration of C-10 was determined by comparison of optical rotation with the known compound, orthosporin 2 (Ichihara et al. 1989). The result indicated that compound 1 has same positive sign with that of 2. Thus, the structure of compound 1 was determined and named as botryosphaerin A (Figure 1).

Compound 3 was isolated as yellow oil. Its molecular formula was assigned as C₁₅H₁₆O₆ on the basis of HRESIMS [M + H]⁺ at m/z 293.1023. The IR spectrum indicated the presence of OH and CO groups. The structure of 3 was determined by its NMR data and by comparison with those of guignardianone (Buckel et al. 2013). The key HMBC correlations of compound 3, from H-3/3a to C-5, H-5 to C-7, C-3/3a C-6, supported a tri-substituted olefin (δ_H 6.43/δ_C 110.0
and 134.3) attached to a phenyl residue (δ_H 6.85, 7.56/δ_C 116.0, 132.0 and 156.8) and a 1,3-dioxolan-4-one moiety (δ_C 108.6, 134.3 and 163.4). In addition, signals of the isopropyl moiety (δ_H 1.03, 2.64/δ_C 14.7, 15.5 and 33.2) were observed, and further inspection of the HMBC spectrum indicated that the isopropyl moiety and the ester carbonyl (δ_C 166.5) both were linked to a carbon at δ_C 108.6. The ^1H and ^13C NMR data of compound 3 closely resembled those of guignardic acid (Bai et al. 2014), and the only difference was the proton being substituted by a hydroxyl at C-1 (δ_C 156.8). The absolute configuration at C-8 was determined as S by comparison of optical rotation ([α]_D^{25} − 31.6 (c = 0.24, acetone)) and CD profile with guignardic acid (Bai et al. 2014). Therefore, the structure was defined and named as botryosphaerin B.

In addition, compounds 2 and 4–7 were identified as Orthosporin (Ichihara et al. 1989), 1RS 2SR, 4SR-1,2,3,4-tetrahydronaphthalene-1,2,4,5-tetrol (Couche et al. 2009), 1RS, 2RS, 4RS-1,2,3,4-tetrahydronaphthalene-1,2,4,5-tetrol (Couche et al. 2009), 11-epiterpestacin (Nihashi et al. 2002) and fusaproliferin (Nihashi et al. 2002), by comparison of the spectroscopic data with the reported literatures, while 5 and 6 were new natural products.

The isolated compounds were evaluated for their cytotoxic, anti-inflammatory (COX-2) and antimicrobial activities. Only compound 3 exhibited a specific COX-2 inhibitory activity with the IC_{50} value of 1.12 μM.

3. Experimental

3.1. General procedures

Optical rotations were measured using Anton Par MCP-500 polarimeter (Hertford, UK). The NMR spectra were measured on a Bruker AC 500 MHz NMR (Bruker, Fällanden, Switzerland) spectrometer with TMS as internal standard. High resolution mass spectra (HR-ESI-MS) were recorded on a Bruker micro TOF-QII mass spectrometer (Bruker). CD spectrum was measured with a Chirascan circular dichroism spectrometer (Applied Photophysics, Surrey, UK). Size exclusion chromatography was done on Sephadex LH-20 gel (GE Healthcare, Uppsala, Sweden). Column chromatography was carried out on silica gel (Qingdao Marine Chemical
3.2. Fungal strain

The endophytic fungus SCSIO KcF6 was derived from the inner fruit part of a mangrove plant *K. candel*, which was collected at Daya Bay, Shenzhen, Guangdong Province, China, in March 2012. Isolated fungal strains were cultured on MB agar medium at 25°C. This strain was stored on MB agar slants at 4°C and then deposited at the Marine Microbial collection center of CAS Key Laboratory of Tropical Marine Bio-resources and Ecology. This isolate was identified to be a member of the genus *Botryosphaeria* on the basis of its ITS phylogenetic analyses, and was designated as *Botryosphaeria* sp. SCSIO KcF6. The 489 base-pair ITS sequence (NCBI GenBank accession number KM 246294) has 99% sequence identity to that of the *Botryosphaeria dothidea* strain CBS 116743 (NCBI GenBank accession number AY786322).

3.3. Fermentation, extraction and isolation

*Botryosphaeria* sp. SCSIO KcF6 stored on MB agar slants at 4°C was cultured on MB agar plates and incubated at 25°C for 7 days. Seed medium (potato 200 g, dextrose 20 g, sea salt 10 g, distilled water 1000 mL) was inoculated with *Botryosphaeria* sp. SCSIO KcF6 and incubated at 25°C for 48 h on a rotating shaker (180 rpm, 25°C). Large-scale fermentation in a solid rice medium of 1000 mL flasks supplemented with 3% NaCl (rice 200 g, sea salt 6.0 g, distilled water 200 mL) (*n* = 35) was inoculated with 10 mL of seed solution. Flasks were incubated at 25°C under static condition and fermented for 45 days.

Fungal cultures from 35 flasks were harvested after 45 days whereby fungal mycelium were cut into small pieces and soaked in acetone for 1 day, sonicated (10 min) and filtered, yielding the rice solid medium and water phases. The solid rice medium was extracted with EtOAc (6 × 500 mL), while the water phase was extracted with EtOAc (3 × 10 L). Both organic extracts were combined and extracted with petroleum ether three times to remove the oil, then evaporated under vacuum at 40°C, finally to yield 95 g of a brown crude extract. The whole crude EtOAc extract was subjected to silica gel column chromatography and eluted with petroleum ether/CH$_2$Cl$_2$ in gradient eluent (100:0 → 0:100), and followed by CH$_2$Cl$_2$/MeOH in gradient eluent (99:1 → 0:100), to obtain nine fractions (fractions 1–9). Fraction 7 was applied to Sephadex LH-20 (MeOH), and further purified by silica gel column chromatography (CHCl$_3$/MeOH, 35:1) to give compound 1 (11.0 mg). Fraction 6 was applied to Sephadex LH-20 (CHCl$_3$/MeOH, 1:1) first to produce four subfractions (F$_6$-1–F$_6$-4). F$_6$-2 was further purified by semi-preparative reversed-phase HPLC to give 4 (14.3 mg) and 5 (8.1 mg). F$_6$-3 was chromatographed on silica gel columns to give 3 (10.9 mg). Fraction 4 was applied to Sephadex LH-20 (CHCl$_3$/MeOH, 1:1) first to give three subfractions (F$_4$-1–F$_4$-3), F$_4$-2 was further purified by semi-preparative reversed-phase HPLC to give 2 (7.5 mg). Fraction 2 was applied to Sephadex LH-20 (CHCl$_3$–MeOH, 1:1) first to give four subfractions (F$_2$-1–F$_2$-4), and F$_2$-3 was further purified by semi preparative reversed-phase HPLC to give 6 (17.5 mg) and 7 (7.0 mg).

3.4. Spectroscopic data of the isolated compounds

Botryosphaerin A (1): White powder, [α]$_D^{25}$ +20.3 (c = 1.0, CH$_3$OH); IR (KBr) $\nu_{\text{max}}$: 3356, 1682, 1020, 667 cm$^{-1}$; $^1$H NMR (500 MHz, CD$_3$OD), δ: 6.61 (1H, s, H-4), 6.38 (1H, s, H-5), 4.16 (1H, m, H-10), 3.76 (3H, s, H-12), 2.62 (2H, m, H-9), 1.26 (3H, d, $J = 6.2$ Hz, H-11); $^{13}$C NMR (125 MHz, CD$_3$OD), δ: 23.5 (C-11), 44.2 (C-9), 61.8 (C-12), 66.4 (C-10), 98.3 (C-8a),

Factory, Qingdao, China). TLC spots were detected under UV light or by heating after spraying with 5% H$_2$SO$_4$ in EtOH. Thin layer chromatography was carried out with precoated silica gel plates (GF-254, Jiangyou Silica Gel Development, Inc., Yantai, China).
101.7 (C-4), 103.9 (C-5), 132.4 (C-5a), 136.0 (C-7), 156.0 (C-3), 161.0 (C-6), 161.7 (C-8), 167.9 (C-1); HRESIMS at m/z 267.0861 [M + H]⁺ (calcd for C₁₃H₁₅O₆, 267.0863).

Botryosphaerin B (3): Yellow oil, [α]²⁰D = 64.5 (c = 1.0, CH₃OH); UV (MeOH) λmax: 318, 239, 216 nm; IR (KBr) νmax: 3354, 1653, 1020, 667 cm⁻¹; ¹H NMR (500 MHz, CDCl₃), δ: 7.56 (2H, d, J = 8.62 Hz, H-3/3a), 6.85 (2H, d, J = 8.64 Hz, H-2/2a), 6.43 (1H, s, H-5), 3.82 (3H, s, H-13), 2.64 (1H, dt, J = 6.85, 6.85, 13.72 Hz, H-9), 1.03 (6H, t, J = 6.39, 6.39 Hz, H-10/11); ¹³C NMR (125 MHz, CDCl₃), δ: 14.7 (C-11), 15.5 (C-10), 33.2 (C-9), 53.6 (C-13), 108.6 (C-8), 110.0 (C-5), 116.0 (C-2/2a), 125.3 (C-4), 132.0 (C-3/3a), 134.3 (C-6), 156.8 (C-1), 163.4 (C-7), 166.5 (C-12); HRESIMS at m/z 293.1023 [M + H]⁺ (calcd for C₁₅H₁₇O₆, 293.1020).

3.5. Antimicrobial activity
The isolated compounds 1–7 were subjected for antibacterial screening (Bauer et al. 1996), against five human bacterial pathogenic strains of Acinetobacter baumannii ATCC 19606, Klebsilla pneumonia ATCC 13883, Escherichia coli ATCC 29213, Staphylococcus aureus ATCC 29213, and Enterococcus faecalis ATCC 29212. Minimum inhibitory concentration (IC₅₀) value was determined by assessing significant bacterial growth inhibition at lower dose.

3.6. Cytotoxic assay
The cytotoxicity assay of compounds 1–7 were tested against panel of 10 human cancer cell lines of (K562, HL-60, A549, DU145, MCF-7, H1975, U937, BGC823, HeLa, and MOLT-4) (Ma et al. 2007).

3.7. COX-2 inhibitory activity assay
COX-2 a well-established target is an inducible enzyme, which expression is activated by cytokines, mitogens, endotoxin and tumour promoters. The anti-inflammatory and analgesic properties of traditional NSAIDs are primarily due to the inhibition of COX-2. Hence, the compounds isolated were tested for COX-2 inhibitory activity using the COX (ovine) inhibitor screening kit according to the manufacturer’s instructions. The test compounds were dissolved in DMSO and the final concentration was set to 30 μM. The percentage inhibition has been calculated by comparison with the control group.

4. Conclusion
In the present study, we have isolated and characterised two new phenyl derivatives of botryosphaerin-A (1) and botryosphaerin-B (3), along with five other known compounds from a mangrove derived endophytic fungi of Botryosphaeria sp. Compound 3 exerted moderate COX-2 anti-inflammatory effect with IC₅₀ range of 1.12 μM.

Supplementary material
Supplementary material relating to this article is available online: 1D NMR, 2D NMR of 1 and 3. http://dx.doi.org/10.1080/14786419.2015.1050670

Disclosure statement
No potential conflict of interest was reported by the authors.
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Note
1. The first two authors contributed equally to this work.

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