Regular article

Secondary metabolites from an endophytic fungus *Aspergillus fumigatus* from *Solanum insanum* L.

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Abstract

An endophytic fungus *Aspergillus fumigatus*, isolated from the fruits of *Solanum insanum* L., was cultured in potato dextrose broth medium. Chromatographic separation of the EtOAc extract led to the isolation of six secondary metabolites, identified as pseurotin A (1), fumigaclavine C (2), monomethylsulochrin (3), trypacidin (4), fumiquinazoline C (5) and guignasulfide (6) by spectral means. Compounds 2 and 3 showed brine shrimp toxicity with IC_{50} values of 147 and 74.2 μM, respectively, while compounds 4 and 5 showed slight inhibition on the growth of fungal pathogen *Cladosporium cladosporioides*. This is the first report of the isolation of guignasulfide (6) from *A. fumigatus*, which has been previously isolated only from an endophytic fungus *Guignardia* sp.

Keywords: *Aspergillus fumigatus*; Endophytic fungi; *Solanum insanum*; Guignasulfide

1 Introduction

Fungal microorganisms present in the tissues of living plants are known as endophytic fungi and are considered a promising source of novel compounds with enormous potential for drug discovery, industrial use and agricultural applications. Some endophytic fungi have developed the ability to produce the same or similar bioactive substances as those originated from the host plants such as paclitaxel, podophyllotoxin, camptothecin, vinblastine, hypericin and diosgenin [1]. Chemical and biological studies on the metabolites of endophytic fungi isolated from medicinal plants in Sri Lanka are relatively limited. As part of our studies on bioactive compounds produced by endophytic fungi from medicinal plants in Sri Lankan [2-14], we herein describe the process of isolating an endophytic fungus from the healthy fruits of *Solanum insanum* L. of the family Solanaceae. *S. insanum* is a vegetable consumed in Asia and the whole plant has been used in traditional medical systems for the treatment of edema, skin ailments, heart diseases, asthma, piles, fever, and poisonous infections [15]. (See Fig. 1)

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Fig. 1 Fruit and longitudinal section of a fruit of *Solanum insanum*

2 Experimental

2.1 General

Plant inoculation, fungal sub culturing and large scale inoculation of isolated fungi were carried out in a Laminar Flow (Class II, NAUIRE, Model no NU-425-300E) under aseptic conditions. Fungal extractions were carried out using an ultra sound sonicator (VWR Ultrasound cleaner, model-USC 1700D). Silica gel coated aluminum sheets (Merck 1.05554.0007, $60F_{254}$, Darmstadt, Germany) were used for analytical TLC while silica gel coated glass plates (Merck Art. 60765, Darmstadt, Germany) were used for preparative TLC. TLC spots were located at 254 and 365 nm using a UV lamp (VILBER LOURMART CN-15-LC, 230 V-50/60 Hz) and sprayed with color reagent anisaldehyde by heating. Silica gel (Merck Art. 7734 and 9385, Darmstadt, Germany) and Sephadex LH-20 (Fluka, Switzerland) were used in column chromatography. Structures of the isolated compounds were elucidated using ^{1}H-NMR and ^{13}C-NMR spectra recorded on a JEOL AL-300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) or JEOL ECP-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer in CD_3OD or $CDCl_3$ solution. ESI mass spectra were recorded on a Thermo Scientific LCQ Fleet MS instrument.

2.1.1 Isolation and identification of fungus

Healthy and mature fruits of *S. insanum* were collected from the Central Province of Sri Lanka. Fruits were rinsed with running water and sterilized three times with ethanol, 5% NaOCl and distilled water. Interior segments of sterilized fruits were placed on potato dextrose agar (PDA) media in Petri dishes and incubated at room temperature for 5 d. Emerging fungi were serially transferred to PDA media on Petri dish to obtain pure culture of endophytic fungus. The isolated fungus was identified as *Aspergillus fumigatus* by molecular methods. Evidence of *A. fumigatus* strains (IFS/VSI-1/2016) is deposited at the National Institute of Fundamental Studies, Kandy, Sri Lanka.

2.1.2 Fermentation of fungus, extraction, bioassays and isolation of compounds

The endophytic fungus *A. fumigatus* isolated from *S. insanum* was inoculated with pure culture grown on PDA in twenty 1L flasks, each containing 400 mL of potato dextrose broth (PDB) medium. The flasks were allowed to stand still for 10 d and then shaken every other d for another 21 d on laboratory shaker at 100 rpm. The culture broth was filtered and the filtrate was extracted with EtOAc. The mycelium was dried and extracted with EtOAc by sonication. Based on the close similarity of TLC patterns, the EtOAc extracts were combined (13.7 g) and screened for the antifungal activity against *Cladosporium cladosporioides* using TLC autography method [16], antioxidant activity against DPPH radicals [17], brine shrimp toxicity [18]

and **α**-amylase inhibitory activity [19]. The EtOAc extract was fractionated successively by silica gel (hexane-EtOAc-MeOH) and Sephadex LH-20 with MeOH, and then seperated by PTLC to yield six UV active (254 nm) compounds: **1** (41.9 mg) [20,21], **2** (34.4 mg) [22], **3** (20.6 mg) [23,24], **4** (14.5 mg) [25], **5** (14.2 mg) [26], and **6** (12.2 mg) [27] (Fig. 2).

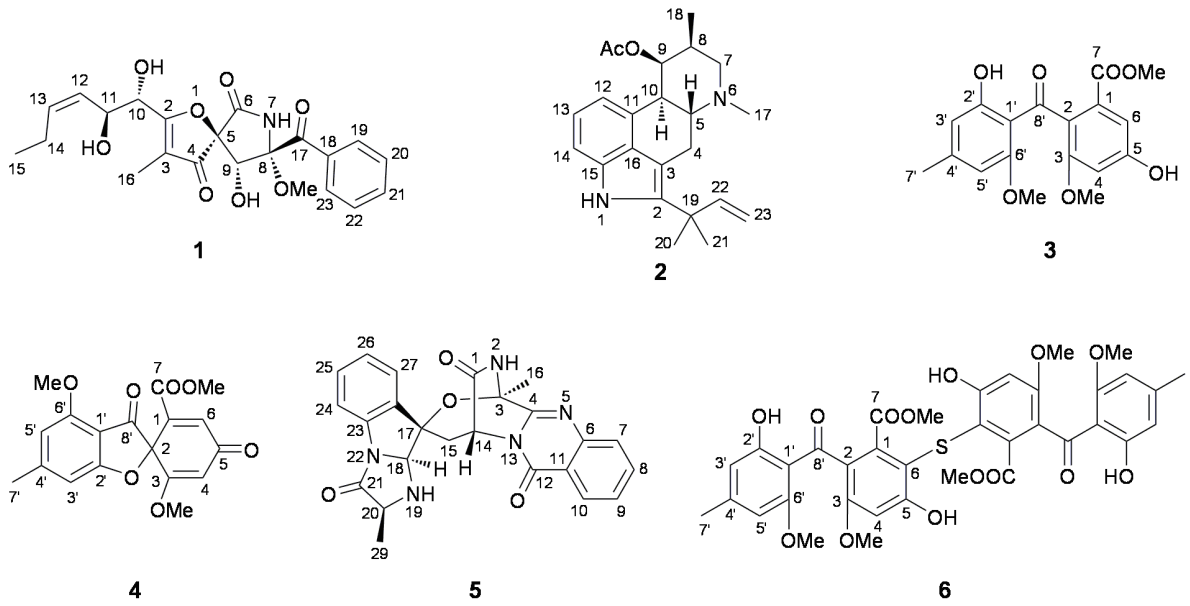

Fig. 2 Structures of compounds **1**-**6**

Pseurotin A (**1**): pale yellow solid; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 0.98 (3H, t, J = 7.5 Hz, H-15), 1.67 (3H, s, H-16), 2.11 (2H, m, H-14), 3.45 (3H, s, MeO-8), 3.58 (1H, br, HO), 4.29 (1H, d, J = 12.0 Hz, HO-9), 4.58 (2H, br, H-10, HO), 4.70 (1H, d, J = 12.0 Hz, H-9), 4.75 (1H, brd, J 9.9 Hz, H-11), 5.25 (1H, dd, J = 9.9, 9.9 Hz, H-12), 5.57 (1H, dt, J = 9.9, 7.5 Hz, H-13), 7.49 (2H, t, J = 7.5 Hz, H-20, 22), 7.65 (1H, t, J = 7.5 Hz, H-21), 8.32 (2H, d, J = 7.5 Hz, H-19, 23), 8.54 (1H, brs, H-7); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 6.1 (C-16), 14.1 (C-15), 21.3 (C-14), 51.7 (MeO-8), 70.7 (C-11), 70.9 (C-10), 73.0 (C-9), 90.5 (C-8), 92.8 (C-5), 113.2 (C-3), 126.5 (C-12), 128.6 (C-20, 22), 130.8 (C-19, 23), 132.4 (C-18), 134.7 (C-21), 136.5 (C-13), 166.8 (C-6), 186.0 (C-2), 195.4 (C-17), 196.4 (C-4); ESI-MS: *m/z* 430 $[M-H]^-$.

Fumigaclavine C (**2**): pale yellow amorphous solid; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 1.31 (3H, d, J = 7.5 Hz, H-18), 1.52 (6H, s, H-20, 21), 1.89 (3H, s, CH_3CO), 2.09 (1H, m, H-8), 2.44 (3H, s, H-17), 2.56-2.74 (4H, m, H-4a, 5, 7a, 7b), 3.31 (1H, brd, J = 7.2 Hz, H-10), 3.52 (1H, dd, J = 12.9, 2.7 Hz, H-4b), 5.13 (1H, d, J = 10.4 Hz, H-23a), 5.14 (1H, d, J = 17.7 Hz, H-23b), 5.66 (1H, brs, H-9), 6.10 (1H, dd, J = 17.7, 10.4 Hz, H-22), 6.72 (1H, brd, J = 6.9 Hz, H-12), 7.05 (1H, dd, J = 7.8, 6.9 Hz, H-13), 7.09 (1H, brd, J = 7.8 Hz, H-14), 7.74 (1H, brs, H-1); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 16.7 (C-18), 21.2 (CH_3CO), 27.2 (C-21), 27.4 (C-20), 28.0 (C-4), 33.0 (C-8), 39.0 (C-19), 39.3 (C-10), 43.5 (C-17), 57.8 (C-7), 61.6 (C-5), 71.4 (C-9), 106.1 (C-3), 107.7 (C-14), 111.9 (C-23), 112.8 (C-12), 122.2 (C-13), 128.0 (C-16), 129.2 (C-11), 132.1 (C-15), 136.7 (C-2), 145.7 (C-22), 170.9 (CH_3CO); ESI-MS: *m/z* 365 $[M-H]^-$.

Monomethylsulochrin (**3**): pale yellow amorphous solid; ^{1}H-NMR ($CDCl_3$-CD_3OD 10:1, 500 MHz): δ 2.29 (3H, s, H-7¢), 3.38 (3H, s, MeO-6¢), 3.66 (3H, s, MeO-7), 3.69 (3H, s, MeO-3), 6.08

(1H, brs, H-5¢), 6.44 (1H, brs, H-3¢), 6.62 (1H, d, *J* = 1.8 Hz, H-4), 6.99 (1H, d, *J* = 1.8 Hz, H-6); ^{13}C-NMR ($CDCl_3$-CD_3OD 10:1, 125 MHz): *δ* 22.3 (C-7¢), 52.1 (MeO-7), 55.6 (MeO-6¢), 55.9 (MeO-3), 103.0 (C-4), 103.1 (C-5¢), 107.7 (C-6), 110.3 (C-3¢), 110.6 (C-1¢), 126.8 (C-2), 128.1 (C-1), 148.0 (C-4¢), 156.8 (C-3), 157.5 (C-5), 160.9 (C-6¢), 163.6 (C-2¢), 166.7 (C-7), 200.0 (C-8¢); ESI-MS: *m/z* 345 $[M-H]^-$.

Trypacidin (**4**): pale yellow solid; $[\alpha]_D^{25}$ -150 (c, 0.35, EtOAc); ^{1}H-NMR (CD_3OD, 300 MHz): *δ* 2.44 (3H, s, H-7¢), 3.66 (3H, s, MeO-3), 3.69 (3H, s, MeO-7), 3.95 (3H, s, MeO-6¢), 5.77 (1H, d, *J* = 1.2 Hz, H-4), 6.38 (1H, s, H-5¢), 6.55 (1H, s, H-3¢), 7.10 (1H, d, *J* = 1.2 Hz, H-6); ^{13}C-NMR (CD_3OD, 75 MHz): *δ* 23.2 (C-7¢), 52.8 (MeO-7), 56.1 (MeO-6¢), 56.7 (MeO-3), 84.0 (C-2), 103.9 (C-4), 105.3 (C-5¢), 105.5 (C-3¢), 108.3 (C-1¢), 137.1 (C-6), 138.2 (C-1), 152.1 (C-4¢), 158.3 (C-6¢), 163.5 (C-7), 169.5 (C-3), 174.3 (C-2¢), 185.7 (C-5), 190.5 (C-8); ESI-MS: *m/z* 345 $[M+H]^+$.

Fumiquinazoline C (**5**): brownish yellow solid; ^{1}H-NMR ($CDCl_3$, 300 MHz): *δ* 1.04 (1H, dd, *J* = 6.8, 6.8 Hz, H-19), 1.07 (3H, d, *J* = 6.8 Hz, H-29), 2.07 (3H, s, H-16), 2.14 (1H, d, *J* = 15.0 Hz, Ha-15), 2.99 (1H, dd, *J* = 15.0, 7.5 Hz, Hb-15), 3.72 (1H, dq, *J* = 6.8, 6.8 Hz, H-20), 5.35 (1H, d, *J* = 6.8 Hz, H-18), 5.73 (1H, d, *J* = 7.5 Hz, H-14), 7.21 (1H, brt, *J* = 7.7 Hz, H-26), 7.33 (1H, brt, *J* = 7.7 Hz, H-25), 7.37 (1H, brd, *J* = 7.7 Hz, H-27), 7.46 (1H, brd, *J* = 7.7 Hz, H-24), 7.57 (1H, br, H-2), 7.62 (1H, td, *J* = 8.0, 1.5 Hz, H-9), 7.80 (1H, dd, *J* = 8.0, 1.5 Hz, H-7), 7.85 (1H, td, *J* = 8.0, 1.5 Hz, H-8), 8.36 (1H, dd, *J* = 8.0, 1.5 Hz, H-10); ^{13}C-NMR ($CDCl_3$, 75 MHz): *δ* 18.7 (C-29), 24.5 (C-16), 31.3 (C-15), 51.3 (C-14), 58.6 (C-20), 84.1 (C-3), 87.0 (C-18), 87.1 (C-17), 115.4 (C-24), 121.3 (C-11), 124.8 (C-27), 126.2 (C-26), 126.9 (C-10), 128.4 (C-7), 128.6 (C-9), 130.2 (C-25), 134.9 (C-8), 135.7 (C-23), 138.3 (C-28), 146.2 (C-6), 150.3 (C-4), 159.5 (C-12), 170.7 (C-21), 170.8 (C-1); ESI-MS: *m/z* 442 $[M-H]^-$.

Guignasulfide (**6**): pale yellow amorphous solid; ^{1}H-NMR ($CDCl_3$, 300 MHz): *δ* 2.33 (3H, s, H-7¢), 3.51 (3H, s, MeO-6¢), 3.67 (3H, s, MeO-7), 3.70 (3H, s, MeO-3), 6.13 (1H, brs, H-5¢), 6.44 (1H, brs, H-3¢), 6.58 (1H, s, H-4), 9.96 (1H, s, HO-5), 12.54 (1H, s, HO-2¢); ^{13}C-NMR ($CDCl_3$, 75 MHz): *δ* 22.6 (C-7¢), 53.4 (MeO-7), 55.8 (MeO-6¢), 56.0 (MeO-3), 102.1 (C-4), 102.8 (C-5¢), 105.5 (C-6), 110.1 (C-1¢), 110.6 (C-3¢), 125.7 (C-2), 134.0 (C-1), 148.8 (C-4¢), 158.9 (C-3), 161.1 (C-6¢), 161.6 (C-5), 163.9 (C-2¢), 170.9 (C-7), 196.8 (C-8¢); ESI-MS: *m/z* 721 $[M-H]^-$.

3 Results and discussion

A grey-green velvety endophytic fungus was isolated from *S. insanum* fruits. It was identified as *Aspergillus fumigatus* of the family Trichocomaceae by molecular methods on the basis of the sequence of internal transcribed spacer (ITS) regions of the fungal rDNA gene, which was amplified using ITS1 and ITS4 primers. BLAST search indicated that the sequence of the ITS regions was 100% identical to that of *Aspergillus fumigatus* ND-77 (GenBank Accession No. MG659671.1). This is the first report of isolating *A. fumigatus* from *S. insanum*. The strain was cultured in PDB medium for 3 w. The culture filtrate and the mycelium were separately extracted with EtOAc. Both extracts were combined, subjected to TLC analysis and screened for antioxiadant activity against DPPH (IC_{50} = 323 *m*g/mL), brine shrimp lethality (IC_{50} = 318 *m*g/mL) and slight antifungal activity against *Cladosporium cladosporioides*. The EtOAc extract (13.7 g) was subjected to repeated column chromatography on silica gel and Sephadex LH-20 followed by PTLC to produce compounds **1**-**6**. Compared with the NMR data of literature values and ESI-MS data, the six compounds were identified as pseurotin A (**1**) [20], fumigaclavine C (**2**) [22], monomethylsulochrin (**3**) [23], trypacidin (**4**) [25], fumiquinazoline C (**5**) [26]

and guignasulfide (**6**) [27] (Fig. 2). Compounds **1**-**6** were screened for antioxidant activity against DPPH (positive control – butylated hydroxyanisole), brine shrimp lethality (positive control - atropine) and antifungal activity against *C. cladosporioides* (positive control - benlate). Results showed that compounds **1**-**4** did not have significant antioxidant activity while compounds **5** and **6** were not enough to measure the antioxidant activity. Compounds **2** and **3** showed brine shrimp toxicity with IC_{50} values of 147 and 74.2 M, respectively. Compounds **4** and **5** were found to slightly inhibit the growth of *C. cladosporioides*.

Aspergillus fumigatus is a common environmental fungus and also an important cause of disease in patients with low immune function [28]. A number of bioactive compounds such as dioxopiperazines, alkaloids, dibenzofurans and indole diketopiperazines have been isolated from *A. fumigatus* [29,30]. Compounds **1**-**5** have been previously isolated from *A. fumigatus*. However, guignasulfide (**6**), a di-benzophenone sulfide (a sulfur-containing benzophenone dimer), has been previously reported only from an endophytic fungus *Guignardia* sp. [27]. Thus, this paper reports a different source of process of obtaining compound **6**. Guignasulfide was reported to have cytotoxicity against human liver cancer cell line HepG2 and antifungal activity against *Helicobacter pylori* [27]. Compound **1**, a representative of hetero-spirocyclic *g*-lactam-type antibiotics, was reported to show cytotoxicity against KB and NCI-H187 cells [31]. Compound **2**, an ergoline alkaloid, was reported to induce apoptosis in MCF-7 cells [32], to have moderate antifungal activity against *Candida albicans* [33] and to exhibit anti-inflammatory activity [34]. Compound **3**, a benzophenone, was reported to have anti-*Helicobacter pylori* activity [35] and moderate cytotoxicity toward HepG2 [27] and KB cells [36]. Compound **4**, formed through an oxidative coupling of compound **3** [37], was reported to have antiprotozoal activity [38] and cytotoxity against human A549 lung cell line [39]. The absolute configuration of trypacidin (**4**) remains to be elucidated. Compound **5**, a fungal peptidyl alkaloid, was reported to have marginal antibacterial activity [40].

4 Conclusion

Chemical investigation of the EtOAc extract from culture of *A. fumigatus*, which was isolated from *S. insanum* fruits, provides a rare di-benzophenone sulfide, guignasulfide (**6**), together with pseurotin A (**1**), fumigaclavine C (**2**), monomethylsulochrin (**3**), trypacidin (**4**) and fumiquinazoline C (**5**). Compound **6** has been reported from fungi in the genera *Aspergillus* for the first time.

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Conflict of interests

The authors of this work declare there is no conflict of interest.

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