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Modulation of polyketide biosynthetic pathway of the endophytic fungus, *Anteaglonium* sp. FL0768, by copper (II) and anacardic acid

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ABSTRACT

In an attempt to explore the biosynthetic potential of endosymbiotic fungi, the secondary metabolite profiles of the endophytic fungus, *Anteaglonium* sp. FL0768, cultured under a variety of conditions were investigated. In potato dextrose broth (PDB) medium, *Anteaglonium* sp. FL0768 produced the heptaketides, herbaridine A (**1**), herbarin (**2**), 1-hydroxydehydroherbarin (**3**), scorpinone (**4**), and the methylated hexaketide 9*S*,11*R*-(+)-ascosalitoxin (**5**). Incorporation of commonly used epigenetic modifiers, 5-azacytidine and suberoylanilide hydroxamic acid, into the PDB culture medium of this fungus had no effect on its secondary metabolite profile. However, the histone acetyl transferase inhibitor, anacardic acid, slightly affected the metabolite profile affording scorpinone (**4**) as the major metabolite together with 1-hydroxydehydroherbarin (**3**) and a different methylated hexaketide, ascochitine (**6**). Intriguingly, incorporaion of Cu^{2+} into the PDB medium enhanced production of metabolites and drastically affected the biosynthetic pathway resulting in the production of pentaketide dimers, palmarumycin CE_4 (**7**), palmarumycin CP_4 (**8**), and palmarumycin CP_1 (**9**), in addition to ascochitine (**6**). The structure of the new metabolite **7** was established with the help of spectroscopic data and by MnO_2 oxidation to the known pentaketide dimer, palmarumycin CP_3 (**10**). Biosynthetic pathways to some metabolites in *Anteaglonium* sp. FL0768 are presented and possible effects of AA and Cu^{2+} on these pathways are discussed.

1. Introduction

Fungi, especially those living in association with other organisms such as endophytic fungi, represent a rich and an underexplored source of biologically active small-molecule natural products with wide ranging applications (Gunatilaka, 2006; Kusari et al., 2013). In their mutualistic association, the host plant (macrophyte) protects and feeds the endophyte which in return produces bioactive metabolites, some of which are known to enhance the growth and competitiveness of the host plant, protect from pathogens and herbivores, and help to survive in harsh environments (Dreyfuss and Chapela, 1994; Gunatilaka, 2006). However, when cultured in artificially defined growth media not mimicking native habitats, many biosynthetic gene clusters encoding for secondary metabolites that increase competitiveness of these fungi in natural environments may remain silent (Hertweck, 2009). This was supported by the whole genome sequencing of several fungi which has resulted in the discovery of new biosynthetic gene clusters (Yaegashi et al., 2014). Realization that these gene clusters may remain silent until they are induced by an external trigger has recently led to intense research resulting in identification of a variety of strategies that have been successfully applied to activate these gene clusters. Some commonly employed strategies include application of epigenetic modifiers to activate silent biosynthetic pathways (Cichewicz, 2010) and simulation of natural gene cluster activating conditions by biotic (co-cultivation) (Pettit, 2009; Rateb et al., 2013), and abiotic (elicitation by chemical or physical means) (Pimental-Elardo et al., 2015) methods. Among these methods, incorporation of small-molecule modifiers into fungal culture media to manipulate epigenetic regulation of gene transcription has received considerable attention. These studies have focused on DNA methyl transferase (DNMT), histone deacetylase (HDAC), and proteasome inhibitors such as 5-azacytidine (5-AZA) (Williams et al., 2008; Fisch et al., 2009; Wang et al., 2010; Yakasai

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et al., 2011; Beau et al., 2012; Chung et al., 2013; Zutz et al., 2013), suberoylanilide hydroxamic acid (SAHA) (Albright et al., 2015; Henrikson et al., 2009; Shwab et al., 2007; Vervoort et al., 2011) and bortezomib (VanderMolen et al., 2014), respectively. Recent studies have also suggested that Cu^{2+} has the ability to enhance structural diversity and production of fungal secondary metabolites (Paranagama et al., 2007b).

In this study we examined the effects of 5-AZA, SAHA, Cu^{2+} and the histone acetyltransferase (HAT) inhibitor, anacardic acid (AA) (Ghizzoni et al., 2010), on the production of secondary metabolites by the endophytic fungus, *Anteaglonium* sp. FL0768 (Anteagloniaceae, Pleosporales, Pezizomycotina, Ascomycota), isolated from the living photosynthetic tissue of sand spikemoss (*Selaginella arenicola*; Selaginellaceae) when cultured in a liquid medium containing potato dextrose broth (PDB). A previous study had shown that in solid potato dextrose agar (PDA) medium this fungus biosynthesized solely polyketides consisting of pentaketide dimers as major and heptaketides as minor metabolites (Xu et al., 2015).

2. Results and discussion

In order to test the effects of 5-AZA, SAHA, AA and Cu^{2+} on the secondary metabolome of *Anteaglonium* sp. FL0768, preliminary studies were first conducted in PDB medium to determine the time required for the optimum production of metabolites. It is known that in fungi optimum secondary metabolite production occurs usually at the end of their growth phase (Calvo et al., 2002) and our recent studies have suggested that for many fungi the highest yield of the extract containing metabolites could be obtained when glucose content in the medium reaches the lowest level (Wijeratne et al., 2014). When *Anteaglonium* sp. FL0768 was cultured in PDB, complete depletion of glucose in the medium occurred at the end of six weeks. Thus, in all subsequent experiments it was cultured for six weeks in this medium incorporating 5-AZA, SAHA, AA or Cu^{2+}, filtered and the filtrates obtained were extracted with EtOAc and subjected to HPLC analysis and fractionation to isolate constituent metabolites. The resulting HPLC traces indicated that incorporation of AA (500 μM) (PDB + AA) and Cu^{2+} (250 μM) (PDB + Cu^{2+}) into the PDB medium affected the metabolite profiles of this fungus (Fig. 1) whereas the well-known small-molecule epigenetic modifiers, 5-AZA and SAHA, had no such effect up to a concentration of

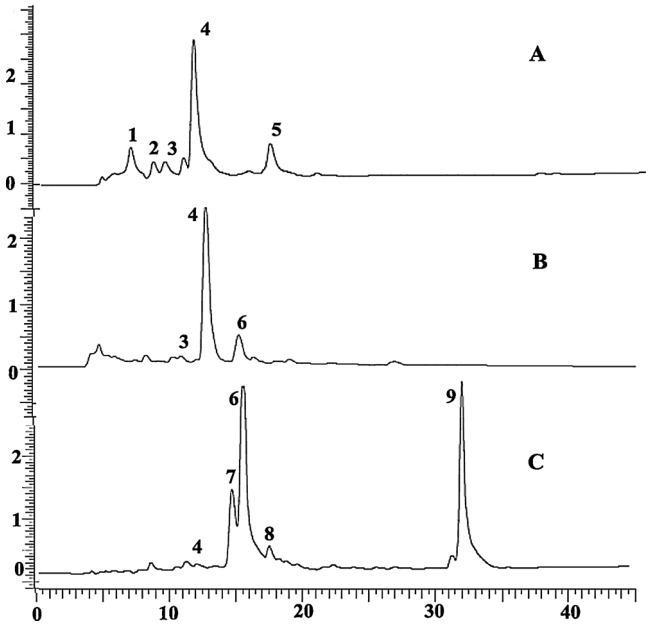

Fig. 1. HPLC profiles of the crude EtOAc extracts of *Anteaglonium* sp. FL0768 cultured in PDB (**A**), PDB + AA (**B**), and PDB + Cu^{2+} (**C**).

500 μM (data not shown).

Each of the extracts produced by *Anteaglonium* sp. FL0768 when cultured in PDB, PDB + AA, and PDB + Cu^{2+} were subjected to fractionation to isolate and characterize their constituent secondary metabolites (Fig. 2). All known compounds were identified by comparison of their spectroscopic data (UV, ^{1}H NMR and LR-MS) with those reported. This led to the identification of the heptaketide, scorpinone (**4**) (Choshi et al., 2008; Wijeratne et al., 2010), as the major metabolite in PDB culture together with other heptaketides, herbaridine A (**1**) (Schüffler et al., 2009), herbarin (**2**) (Paranagama et al., 2007a), 1-hydroxydehydroherbarin (**3**) (Paranagama et al., 2007a), and the trimethyl hexaketide, 9S,11R-(+)-ascosalitoxin (**5**) (Evidente et al., 1993) as minor constituents. Dehydroherbarin (**3a**) (Wijeratne et al., 2010) and 1-methoxydehydroherbarin (**3b**) (Wijeratne et al., 2010) encountered in this extract were suspected to be artifacts formed from **2** and **3**, respectively, during the isolation process. Fractionation of the PDB + AA culture extract resulted in the isolation of scorpinone (**4**) as the major metabolite and 1-hydroxydehydroherbarin (**3**) and a different trimethyl hexaketide, ascochitine (**6**) (Evidente et al., 1993) as minor metabolites suggesting that the metabolite profile was slightly affected by this HAT inhibitor. Intriguingly, the HPLC profile of the EtOAc extract derived from PDB + Cu^{2+} culture was found to be drastically different from those of PDB and PDB + AA cultures (Fig. 1C). It was also significant that the yield of the crude EtOAc extract obtained from the PDB + Cu^{2+} culture was ca. six-fold higher compared to those resulting from the same volume of PDB and PDB + AA cultures. Fractionation of the PDB + Cu^{2+} culture extract by solvent-solvent partitioning, Sephadex LH-20 gel permeation, normal phase silica gel and reversed-phase RP-18 column chromatography, followed by HPLC purification afforded **4** and **6–9** of which **7** was found to be a new pentaketide dimer and was named palmarumycin CE_4. Comparison of the spectroscopic data with those reported identified the known metabolites as scorpinone (**4**) (Choshi et al., 2008; Wijeratne et al., 2010), ascochitine (**6**) (Colombo et al., 1980), palmarumycin CP_1 (**8**) (Krohn et al., 1994a), and palmarumycin CP_4 (**9**) (Krohn et al., 1997).

Palmarumycin CE_4 (**7**), obtained as an off-white gum, analyzed for $C_{20}H_{16}O_5$ by a combination of HRMS and NMR data, and indicated 13° of unsaturation. Its ^{1}H NMR spectrum showed signals due to six aromatic protons [δ_H 7.55 (1H, dd, J = 0.8, 8.6 Hz), 7.53 (1H, dd, J = 0.8, 8.3 Hz), 7.45 (1H, dd, J = 7.6, 8.3 Hz), 7.44 (1H, dd, J = 7.6, 8.6 Hz), 7.04 (1H, dd, J = 0.8, 7.6 Hz), 6.95 (1H, dd, J = 0.8, 7.6 Hz)], two coupled olefinic protons [δ_H 6.00 (1H, ddd, J = 1.2, 2.2, 10.0 Hz), 5.88 (1H, ddd, J = 2.2, 4.2, 10.0 Hz)], protons of a methylene group [δ_H 2.90 (1H, ddd, J = 1.0, 2.2, 18.3 Hz) and 2.67 (1H, ddd, J = 1.0, 3.4, 18.3 Hz)], and five aliphatic methine protons [δ_H 4.70 (1H, t, J = 4.2 Hz), 4.41 (1H, ddd, J = 2.2, 4.2, 4.2 Hz), 4.11 (1H, m), 3.14 (1H, brdd, J = 4.0, 5.0 Hz), 2.76 (1H, dt, J = 1.2, 4.2 Hz)]. The ^{13}C NMR spectrum showed twenty signals, which when interpreted with the help of HSQC data suggested the presence of a ketone carbonyl (δ_C 214.7), ten aromatic carbons [δ_C 147.0 (C), 146.8 (C), 134.5 (C), 127.7 (CH), 127.4 (CH), 121.8 (CH), 121.2 (CH), 114.0 (C), 110.0 (CH), 109.3 (CH)], an acetal carbon (δ_C 105.9), and eight methine carbons (δ_C 137.5, 125.8, 75.9, 71.7, 69.6, 47.2, 47.0, 42.4) of which two were olefinic and three were oxygenated. These data indicated that **7** contained a 1,8-naphthalenediol spiro-ketal moiety (Xu et al., 2015). The planar structure of the upper bicyclic portion of **7** was elucidated as 2,3,4a,5,8,8a-hexahydro-5-hydroxy-2,8-epoxynaphthalene by careful analysis of its ^{1}H-^{1}H COSY and HMBC spectra (Fig. 3). 1D NOESY data were useful in determining the stereochemistry of this bicyclic moiety. The NOEs observed for H-5 (δ_H 4.11)/H-8a (δ_H 2.76), H-4a (δ_H 3.14)/H-8a, H-8 (δ_H 4.70)/H-8a, and H-5/H-4a confirmed that these four protons were located on the same side of the ring. The proton at C-2 (δ_H 4.41) showed NOEs with both protons of H_2-3 suggesting that the oxygen at C-2 had axial orientation. The presence of an ether linkage between C-2 and C-8 was inferred by considering the degree of unsaturation, the *cis*-conformation of the upper bicyclic moiety, and the