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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, incorporation of Cu²⁺ into the PDB medium enhanced production of metabolites and drastically affected the biosynthetic pathway resulting in the production of pentaketide dimers, palmarumycin CE₄ (**7**), palmarumycin CP₄ (**8**), and palmarumycin CP₁ (**9**), in addition to ascochitine (**6**). The structure of the new metabolite **7** was established with the help of spectroscopic data and by MnO₂ oxidation to the known pentaketide dimer, palmarumycin CP₃ (**10**). Biosynthetic pathways to some metabolites in

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.phytol.2018.10.011>.

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Anteaglonium sp. FL0768 are presented and possible effects of AA and Cu²⁺ on these pathways are discussed.

Graphical abstract

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 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 Cu²⁺ 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 smallmolecule epigenetic modifiers, 5-AZA and SAHA, had no such effect up to a concentration of 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, ^1H 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+Cu²⁺ culture was ca. six-fold higher compared to those resulting from the same volume of PDB and PDB+AA cultures. Fractionation of the PDB+Cu²⁺ 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 CE₄. 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 CP₁ (**8**) (Krohn et al., 1994a), and palmarumycin CP₄ (**9**) (Krohn et al., 1997).

Palmarumycin CE₄ (**7**), obtained as an off-white gum, analyzed for C₂₀H₁₆O₅ by a combination of HRMS and NMR data, and indicated 13 degrees of unsaturation. Its ¹H 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 ¹³C 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 spiroketal 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 ¹H–¹H 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 H₂-3 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 HMBC correlations of H-2/C-8 and H-8/C-2. These data suggested that **7** was structurally related to palmarumycin CP₃ (**10**) (Xu et al., 2015), except that in **7** the CO group at C-5 of **10** was replaced with a CHOH. This was confirmed by the treatment of **7** with MnO₂ to afforded its oxidation product in 73% yield which had spectroscopic (¹H and ¹³C NMR, and MS) data identical with those reported for **10** (Krohn et al., 1994b; Xu et al., 2015). Thus, the structure of palmarumycin CE₄ was established as (2 β ,4 α ,5 β ,8 β ,8 α)-2,3,4a,5,8,8a-hexahydro-5-hydroxy-spiro [2,8-epoxynaphthalene]-1(4*H*)-2'-naphtho[1,8-*de*][1,3]dioxin-4-one (**7**) (Fig. 1).

To the best of our knowledge this work constitutes the first use of the HAT inhibitor AA to manipulate the secondary metabolism of a fungus. However, in a recent study involving *Streptomyces*-induced production of secondary metabolites in *Aspergillus nidulans*, transcription of the fungal orsellinic acid producing gene (*orsA*) induced by the bacterium was shown to be inhibited by AA by blocking histone modification via a HAT complex; in contrast, SAHA was found to activate *orsA* gene without the need for co-incubation of the fungus with the bacterium (Nutzmann et al., 2011). In our previous work we found that incorporation of Cu^{2+} into the PDB culture medium of the rhizosphere fungus, *Paraphaeosphaeria quadrisepata*, significantly enhanced the production of monocillin I (Paranagama et al., 2007b), a heat-shock-inducing natural product capable of conferring thermotolerance to Arabidopsis by overexpression of the stress-related protein, Hsp101 (McLellan et al., 2007). The effect of Cu^{2+} in the production of monocillin I by *P. quadrisepata* was interpreted as due to its influence on superoxide dismutase (SOD) activity and by considering the role of this enzyme in the cellular adaptation to environmental stress (Paranagama et al., 2007b).

As apparent from the HPLC profiles of secondary metabolites produced by *Anteaglonium* sp. FL0768 (Fig. 1), incorporation of AA and Cu^{2+} into the culture medium appeared to have affected mainly the biosynthesis of scorpinone (**4**), 9*S*,11*R*-(+)-ascosalitoxin (**5**), ascochitine (**6**), palmarumycin CE₄ (**7**) and palmarumycin CP₄ (**9**). Labelling studies have suggested that **4** was biosynthesized from a heptaketide precursor (Van Wagoner et al., 2008), **6** was derived from a trimethyl-hexaketide precursor via (+)-ascosalitoxin (**5**) (Seibert et al., 2006), and biosynthesis of **9** involved phenolic oxidative dimerization of the pentaketide precursor, 1,8-dihydroxynaphthalene (DHN) (Bode and Zeeck, 2000) (Fig. 4). DHN, a reductive dehydration product of 1,3,6,8-tetrahydronaphthalene (THN), has also been shown to be the precursor of fungal melanin biosynthesis (Schumacher, 2016). It is noteworthy that under all culture conditions used the hyphae of *Anteaglonium* sp. FL0768 turned black during its growth indicating that it produced significant quantity of melanin (see Fig. S7, Supporting Information). It has recently been shown that in some fungi, the pentaketide THN was in fact derived from hexaketide and heptaketide precursors by the side-chain hydrolase-catalyzed removal of acetyl and acetoacetyl groups, respectively (Fujii et al., 2004; Vagstad et al., 2012) (Fig. 4). In contrast to PDB culture which contained mainly heptaketide metabolites, PDB+ Cu^{2+} culture was found to be devoid of heptaketides, except for a trace amount of scorpinone (**4**), suggesting the possibility that incorporation of Cu^{2+} into PDB caused inhibition of polyketide synthase (PKS) of *Anteaglonium* sp. FL0768 involved in the production of heptaketides (Fig. 4). It is known that ketosynthase (KS) domain is the most important for the control of the chain length of polyketides, but product template (PT) domain (Newman et al., 2014) and thiohydrolase (Zabala et al., 2014) can also affect the chain length of polyketides. Thus, disruption of these domains and enzymes via oxidative stress caused by Cu^{2+} inhibiting PKS involved in heptaketide production in this fungus cannot be ruled out. The production of palmarumycin-type spirobisnaphthalenes (**7**–**9**) by *Anteaglonium* sp. FL0768 in PDB+ Cu^{2+} suggested the possible role played by Cu^{2+} in their biosynthesis. It has been reported that Cu^{2+} enhanced the activity of fungal multi-copper oxidases (laccases) which are involved in melanin biosynthesis and known to catalyze one-electron oxidation of phenolic substrates (Upadhyay et al., 2013). Since the

biosynthesis of dimeric pentaketide spirobisnaphthalenes has been known to involve phenolic oxidative coupling of DHN (Engelmann et al., 2015) (Fig. 4), it is very likely that Cu^{2+} played a major role in the production of palmarumycin-type metabolites (**7–9**) in *Anteaglonium* sp. FL0768. It is also noteworthy that both AA and Cu^{2+} induced the biosynthetic conversion of **5** to **6**. Further studies are in progress to understand the effects of AA and Cu^{2+} in the production of polyketide metabolites in *Anteaglonium* sp. FL0768 and other fungal strains.

Several metabolites encountered in this study have been reported to have interesting biological activities. These included calmodulin inhibitory (Leyte-Lugo et al., 2012) and phytotoxic (Bode et al., 2000) activities of 9*S*,11*R*(+)-ascosalitoxin (**5**), antibacterial (Kusnick et al., 2002), weakly cytotoxic (Von Woedtke et al., 2002), and MPtpB enzyme inhibitory (Seibert et al., 2006) activities of ascochitine (**6**), and weakly antibacterial (Aslan et al., 2011) and cytotoxic (Wipf et al., 2001) activities of palmarumycin CP₁ (**8**). Metabolites **4** and **6–9** were evaluated for *in vitro* inhibition of cell proliferation/survival using a panel of six cancer cell lines including PC-3M (metastatic human prostate adenocarcinoma), NCI-H460 (human non-small cell lung cancer), SF-268 (human CNS glioma), MCF-7 (human breast cancer), and MDA-MB-231 (human breast adenocarcinoma). Among these only palmarumycin CP₁ (**8**) exhibited cytotoxic activity below a concentration of 5.0 µg/mL against these cell lines (see Table S1, Supporting Information). When evaluated against the human Ewing's sarcoma cell line CHP-100, **8** had shown strong cytotoxicity with an IC₅₀ of 1.6 µM (Xu et al., 2015). It is noteworthy that palmarumycin CP₁ (**4**) has been reported to exhibit antitumor activity through inhibition of TrxR-1 (thioredoxin reductase-1) activity (Powis et al., 2006).

This study is significant as it demonstrated for the first time that the HAT inhibitor, AA, is capable of affecting the metabolites produced by a filamentous fungus. Incorporation of the heavy metal salt, CuSO₄, significantly enhanced secondary metabolite production by *Anteaglonium* sp. FL0768 in addition to producing a new metabolite. Applicability of these modifiers for production enhancement and structural diversification of secondary metabolites of other fungi is currently under investigation.

3. Experimental section

3.1. General experimental procedures

Optical rotations were measured at room temperature with a Jasco Dip-370 polarimeter using MeOH as the solvent. UV spectra were recorded with Shimadzu UV 2601 spectrophotometer. 1D and 2D NMR spectra were recorded with a Bruker Avance III 400 NMR instrument at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. Chemical shift values (δ) are given in parts per million (ppm) and the coupling constants are in Hz. Low-resolution and high-resolution MS were recorded on Shimadzu LCMS-DQ8000 α and JEOL HX110A spectrometers, respectively. HPLC analyses were carried out on a Phenomenex Luna $5\mu\text{C}18$ (2) $100\text{ \AA}$ $250 \times 4.6\text{ mm}$ column with Hitachi L-6200A system consisting of a Hitachi L-4500 DAD detector, a Shimadzu ELSD-LT detector, and a Hitachi AS-4000 intelligent auto sampler. Analytical thin-layer chromatography (TLC) was carried out on silica gel 60 F $_{254}$ aluminum-backed TLC plates (Merck). Preparative TLC was performed on

Analtech silica gel 500 μm glass plates. Compounds were visualized with short-wavelength UV and by spraying with anisaldehyde-sulfuric acid spray reagent followed by heating until the spots appeared. Silica gel flash chromatography was accomplished using 230–400 mesh silica gel. Whatman LRP-2 was used for RP column chromatography. Sephadex LH-20 for gel-permeation chromatography was obtained from Amersham Biosciences. HPLC purifications was carried out on a 10×250 mm Phenomenex Luna $5\mu\text{C}_{18}$ (2) $100 \text{ \AA}$ column with Waters Delta Prep system consisting of a PDA 996 detector. 5-Azacytidine (5-AZA) and suberoylanilide hydroxamic acid (SAHA) were purchased from Alfa Aesar and Cayman Chemical Company, respectively. Anarcadic acid (AA) used [containing a mixture of 2.2–3.0% saturated, 25.0–33.3% mono-unsaturated (8'), 17.8–32.1% di-unsaturated (8',11'), and 36.3–50.4% tri-unsaturated (8',11',14') (Mazzeto et al., 2009)] was a gift from Prof. Diego L. V. Oliveira of University of Cear, Brazil.

3.2. Culture methods

The endophytic fungus, *Anteaglonium* sp. FL0768, isolated and identified as described previously (Xu et al., 2015), was grown on PDA for 14 days at 23 $^{\circ}\text{C}$. The mycelia were scraped out and placed to Falcon tubes containing sterile water. The suspensions were vortexed for 1 min, and then strained using a 100 μM nylon cell strainer. The optical density (OD_{600}) of final spore-hyphae suspensions was adjusted to 0.4 with sterile water and was used to inoculate liquid growth media (100 mL in 250 mL Erlenmeyer flasks) consisting of PDB, PDB+5-AZA (500 μM), PDB+SAHA (500 μM), PDB+AA (500 μM) and PDB + CuSO_4 (250 μM). All small-scale experiments were conducted in triplicate (3 flasks for each of the above). For large-scale cultures 10.0 mL of spore-hyphae suspension ($\text{OD}_{600} = 0.4$) was added to nine 2.0 L Erlenmeyer flasks each containing 1.0 L of the respective culture medium. The flasks were shaken on a rotary shaker at 28 $^{\circ}\text{C}$ and 160 RPM until the cultures were ready for extraction. All cultures were grown for six weeks as complete depletion of glucose (as indicated by the glucose strip test) (Wijeratne et al., 2014) in PDB occurred at the end of this period.

3.3. Extraction and HPLC analysis of crude extracts

The medium of each flask from the above five small-scale cultures was separately filtered to remove mycelia and the resulting filtrate was extracted with EtOAc (3×600 mL). The combined EtOAc layer was washed with water (300 mL) and evaporated under reduced pressure providing the crude EtOAc extract. The calculated yields of crude EtOAc extracts/1.0 L of the culture medium were: PDB = 0.16 g; PDB+ Cu^{2+} = 1.0 g; PDB+AA = 0.15 g. A small portion (1.0 mg) of crude extracts from each of the small-scale cultures was dissolved in HPLC grade MeOH (0.5 mL), centrifuged at 13 000 rpm for 3 min and the resulting supernatant was used for HPLC analysis. The analysis was performed by HPLC-PDA-ELSD on 20 μL of the extract using a C_{18} column and a gradient of MeOH:H₂O (increasing linearly from 60% MeOH/H₂O at 0.0 min to 100% MeOH at 45 min) at a flow rate of 0.7 mL/min and UV detection at 230 nm. Extracts derived from triplicate samples exhibited very similar HPLC profiles suggesting reproducibility of each of the small-scale experiments. The HPLC peak assignments were based on peak enhancement with isolated compounds.

3.4. Isolation of metabolites

The crude EtOAc extract (130.0 mg) derived from a PDB culture (9.0 L) was subjected to silica gel (20.0 g) column chromatography. Elution with 95:5 CHCl₃-MeOH (200 mL) followed by MeOH afforded three fractions, fraction 1 (50.4 mg), fraction 2 (21.7 mg), and fraction 3 (60.1 mg). Fraction 1 was further separated by C-18 (10.0 g) reversed-phase column chromatography. Elution with 80% aq. MeOH afforded two fractions, 1-1 (19.2 mg) and 1-2 (26.8 mg). Further separation of fraction 1-1 was achieved by silica gel prep. TLC using 98:2 CHCl₃-MeOH as eluent to give **4** (20.5 mg) and **3b** (2.0 mg). Fraction 2 above and fraction 1-2 were found to have similar TLC profiles and therefore combined and purified by C₁₈ RPHPLC [Phenomenex Luna 5 μ C18 (2) 100 Å 10 × 250 mm column, solvent system (60% aq. MeOH in 125 min and 70% aq. MeOH in 25–40 min), 3 mL/min, UV detection at 220 nm] to afford **1** (2.4 mg eluted at 9.8 min), **2** (3.4 mg; eluted at 14.9 min), **3** (3.0 mg; eluted at 24.5 min) and a mixture of two compounds (9.3 mg; eluted at 38.4 min). Purification of this mixture by silica gel prep. TLC (eluent: 98:2 CHCl₃-MeOH) gave **5** (7.4 mg) and **3b** (1.9 mg). The CDCl₃ solution of **2** used for NMR spectroscopy was found to undergo decomposition to yield an artifact which was isolated by silica gel prep. TLC (eluent: 98:2 CHCl₃-MeOH) to afford **3a** (1.2 mg).

The crude EtOAc extract (880.0 mg) obtained from the PDB+Cu²⁺ culture broth (9.0 L) was suspended in MeOH (50.0 mL) and filtered to afford an insoluble (270.0 mg) and soluble (610.0 mg) fractions. The insoluble fraction was subjected to silica gel (50.0 g) column chromatography. Elution with CH₂Cl₂ afforded **6** (56.0 mg) and **8** (56.5 mg). The soluble fraction was subjected to Sephadex LH-20 (20.0 g) size-exclusion chromatography to obtain five fractions (A–E). Of these, fractions B (232.0 mg) and C (242.0 mg) showed cytotoxic activity. Fraction B was further fractionated on a Sephadex LH-20 (10.0 g) column. Elution with 100 mL each of 4:1 CH₂Cl₂-hexanes, 3:2 CH₂Cl₂-acetone, 1:4 CH₂Cl₂-acetone, and MeOH afforded four fractions. Addition of MeOH (5.0 mL) to the fraction obtained with 4:1 CH₂Cl₂-hexanes fraction (125.0 mg) provided an insoluble material (59.6 mg) consisting mainly of **6**. The soluble portion (61.0 mg) was subjected to preparative silica gel TLC (95:5 CH₂Cl₂-MeOH) followed by C-18 HPLC (75–80% aq. MeOH) to yield **7** (3.0 mg), **6** (24.0 mg), and **4** (1.4 mg). Fraction C obtained above was fractionated on a Sephadex LH-20 (7.0 g) column. Elution with 50.0 mL each of 4:1 CH₂Cl₂-hexanes, 3:2 CH₂Cl₂-acetone, 1:4 CH₂Cl₂-acetone, and MeOH gave four fractions. Addition of MeOH (5.0 mL) to the fraction obtained with 4:1 CH₂Cl₂-hexanes (149.0 mg) provided an insoluble material (26.3 mg) that was found to be pure **8**. The soluble portion (110.0 mg) was further separated on a silica gel (5.0 g) column. Elution with 70:30 CH₂Cl₂-EtOAc afforded **7** (63.0 mg), **6** (7.0 mg), and **8** (10.0 mg).

The crude EtOAc extract (130.0 mg) derived from PDB+AA culture (9.0 L) when examined by HPLC indicated the presence of three metabolites (Fig. 1B). Fractionation of this extract by HPLC [Phenomenex Luna 5 μ C18 (2) 100 Å 10 × 250 mm column, solvent system (60% aq. MeOH in 1–25 min and 100% MeOH), 3 mL/min, UV detection at 220 nm] afforded 1-hydroxydehydroherbarin (**3**) (0.7 mg), scorpinone (**4**) (10.7 mg), and ascochitin (**6**) (3.0 mg). Identities of these were confirmed by comparison (TLC, HPLC, ¹H NMR, and LC-MS) with those obtained above from EtOAc extracts derived from PDB and PDB+Cu²⁺ cultures.

3.5. Palmarumycin CE₄ (7)

Off-white gum; $[\alpha]_D^{20}$ – 41 (c 0.33, CHCl₃); UV (MeOH) λ_{max} nm (log ϵ) 298 (4.02), 306 (sh, 3.86), 312 (3.86), 327 (3.76); ¹H NMR (400 MHz, CDCl₃): δ 7.55 (1H, dd, J = 0.8, 8.6 Hz, H-5'), 7.53 (1H, dd, J = 0.8, 8.3 Hz, H-4'), 7.45 (1H, dd, J = 7.6, 8.3 Hz, H-3'), 7.44 (1H, dd, J = 7.6, 8.6 Hz, H-6'), 7.04 (1H, dd, J = 0.8, 7.6 Hz, H-2'), 6.95 (1H, dd, J = 0.8, 7.6 Hz, H-7'), 6.00 (1H, ddd, J = 1.2, 2.2, 10.0 Hz, H-5), 5.88 (1H, ddd, J = 2.2, 4.2, 10.0 Hz, H-7), 4.88 (1H, d, J = 11.0 Hz, 5-OH), 4.70 (1H, t, J = 4.2 Hz, H-8), 4.41 (1H, ddd, J = 2.2, 4.2, 4.2 Hz, H-2), 4.11 (1H, m, H-5), 3.14 (1H, brdd, J = 4.0, 5.0 Hz, H-4a), 2.90 (1H, ddd, J = 1.0, 2.2, 18.3 Hz, H-3), 2.76 (1H, dt, J = 1.2, 4.2 Hz, H-8a), 2.67 (1H, ddd, J = 1.0, 3.4, 18.3 Hz, H-3); ¹³C NMR (100 MHz, CDCl₃): δ 214.7 (C-4), 147.0 (C-8'), 146.8 (C-1'), 137.5 (CH-6), 134.5 (C-4a'), 127.7 (CH-3'), 127.4 (CH-6'), 125.8 (CH-7), 121.8 (CH-5'), 121.2 (CH-3'), 114.0 (C-8a'), 110.0 (CH-2'), 109.3 (CH-7'), 105.9 (C-1), 75.9 (CH-2), 71.7 (CH-8), 69.6 (CH-5), 47.2 (CH-4a), 47.0 (CH₂-3), 42.4 (CH-8a); Positive HRMS-ESI m/z 359.0890 [M+Na]⁺ (calcd for C₂₀H₁₆O₅Na: 359.0895).

3.6. Oxidation of palmarumycin CE₄ (7) with MnO₂

To a solution of **7** (5.7 mg) in CH₂Cl₂ (2.0 mL) was added freshly prepared MnO₂ (50.0 mg) and the reaction was stirred at room temperature for 6 h (TLC control). The reaction mixture was filtered, the filtrate was concentrated and the resulting residue was purified by silica gel column chromatography and eluted with CH₂Cl₂ to afford the oxidation product (4.2 mg, 73%), which was identified as palmarumycin CP₃ (**10**) by comparison of its data (LRMS, ¹H and ¹³C NMR) with those reported for this compound (Xu et al., 2015).

3.7. Cytotoxicity assay

The resazurin-based colorimetric (alamarBlue) assay (Wijeratne et al., 2012) was used for evaluating *in vitro* cytotoxicity of samples against human non-small cell lung (NCI-H460), human CNS glioma (SF-268), human breast (MCF-7), human metastatic breast adenocarcinoma (MDA-MB-231), metastatic prostate adenocarcinoma (PC-3M), and human Ewing's sarcoma (CHP-100) cancer cell lines. Doxorubicin and DMSO were used as positive and negative controls, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Secondary metabolite profile of the endophytic fungus, *Anteaglonium* sp. FL0768, was found to be affected by culture conditions.
- Incorporation of Cu^{2+} enhanced production of metabolites and drastically affected the polyketide biosynthetic pathway.
- The histone acetyl transferase inhibitor, anacardic acid, slightly affected the metabolite profile
- The epigenetic modifiers, 5-azacytidine and suberoyl anilide hydroxamic acid had no effect on its secondary metabolite profile.

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).

Fig. 2.
Structures of compounds **1–10**.

Fig. 3.
Key ^1H - ^1H COSY, HMBC, and NOE correlations for **7**.

Fig. 4. Biosynthetic routes to scorpinone (**4**), (+)-ascosalitoxin (**5**), ascochitine (**6**) and palmarumycin CP₁ (**8**) from hexaketide and heptaketide precursors.