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Comparison of the chemistry and diversity of endophytes isolated from wild-harvested and greenhouse-cultivated yerba mansa (*Anemopsis californica*)

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Abstract

With this study, we explored the identity and chemistry of fungal endophytes from the roots of yerba mansa [*Anemopsis californica* (Nutt.) Hook. & Arn. (Saururaceae)], a botanical traditionally used to treat infection. We compared the diversity of fungal endophytes isolated from a wild-harvested *A. californica* population, and those from plants cultivated for one year in a greenhouse environment. The wild-harvested population yielded thirteen fungal strains (eleven unique genotypes). Of the extracts prepared from these fungi, four inhibited growth of *Staphylococcus aureus* by >25% at 20 µg/mL, and three inhibited growth of *Pseudomonas aeruginosa* by 20% at 200 µg/mL. By comparison, *A. californica* roots after one year of cultivation in the greenhouse produced only two unique genotypes, neither of which displayed significant antimicrobial activity. The fungus *Chaetomium cupreum* isolated from wild-harvested *A. californica* yielded a new antimicrobial spiro lactone, chaetocuprum (**1**). An additional fourteen known compounds were identified using LC-MS dereplication of the various fungal endophytes. This study provides new insights into the identity and chemistry of *A. californica* fungal endophytes, and demonstrates the importance of considering growing conditions when pursuing natural product drug discovery from endophytic fungi.

Keywords

Anemopsis californica; antimicrobial; fungal endophyte; *Chaetomium cupreum*; spiro lactone; *Cylindrocarpon* sp

1. Introduction

Historically, it has been assumed that the biologically active principles of botanical medicines are plant secondary metabolites. However, plants are teeming with microbial

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symbionts, including endophytes, microbes that live asymptotically within plant tissue. Endophytes can produce an array of biologically active secondary metabolites, and the potential influence of these compounds on the biological activity of botanicals has been a topic of recent interest (Tan and Zaou, 2001; Strobel and Daisy, 2003; Strobel et al., 2004). Fungal endophytes can, in some cases, produce biologically active compounds. For example, endophytic fungi play a role in the production of hallucinogenic ergot alkaloids in morning glories (genera *Ipomoea*) (Ahimsa-Müller et al., 2007) and ergot and insecticidal loline alkaloids in tall fescue grass (genera *Festuca* and *Lolium*) (Siegel et al., 1990). To further complicate matters, gene transfer can occur between plants and endophytes, such that microbes may acquire the ability to produce the same compounds originally produced by the host plant, or vice versa (El-Elimat et al., 2014b; Kusari et al., 2009; Taghavi et al., 2005). Additionally, the presence of particular microbes may alter the growth and/or secondary constituent profile of the host plant (Eaton et al., 2010; Naveed et al., 2014). In light of this, it is becoming increasingly apparent that endophytes are a potentially important topic of consideration when investigating the biological activity of botanicals.

Endophytes can be transmitted either vertically, from parent to progeny through seeds, or horizontally, entering plant tissue from the environment (Rodriguez et al., 2009). Vertically transmitted endophytes often engage in mutualistic relationships with their hosts, and can be very closely associated with particular plant species (Saikkonen et al., 2004). Horizontally transmitted endophytes, on the other hand, may be more representative of the environment surrounding the plant than of the particular plant species. Because of horizontal endophyte transmission, it is likely that the same genus and species of a plant grown in different environments can have different endophyte profiles (Brem and Leuchtmann, 2002; Saikkonen et al., 2004; Schardl, 1996).

With this study, we focused on fungal endophytes from the botanical medicine *Anemopsis californica* (Nutt.) Hook. & Arn. (Saururaceae), which is commonly known as yerba mansa. *Anemopsis californica* was used by the Shoshoni, Pima, Mahuna, Chumash, Paiute, and Costanoan tribes of North America to treat inflammation and infection in wounds and to control pain (Bocek, 1984; Curtin, 1984; Timbrook, 1987; Romero, 1954; Train et al., 1978). This plant is still used today for the treatment of infections, and although it has been sparsely studied, there are several reports of antimicrobial (Bussey et al., 2014; Medina et al., 2005) or cytotoxic (Daniels et al., 2006; Kaminski et al., 2010) activities associated with *A. californica* extracts or constituents. To date, however, there have been no investigations of the endophyte profile of *A. californica* plants. Thus, we sought to isolate endophytes from the roots of this botanical and evaluate their antimicrobial activity and chemical composition. As part of this study, we also compared the diversity of fungal endophytes from a wild population of *A. californica* immediately after harvest and also after one year of cultivation in a greenhouse environment.

2. Results and Discussion

2.1. Influence of environment on fungal diversity

The first question we sought to explore was whether changes in growing conditions would alter the endophyte profile of *A. californica* plants. Given the commonness of horizontal

transmission, (Rodriguez et al., 2009) we expected that this would be the case. To test this experimentally, we isolated endophytes from two different batches of *A. californica* roots (Table 1). One batch of roots was harvested directly from a wild population and the other came from the same wild population but was allowed to grow in a greenhouse for one year prior to harvest. The difference in diversity of the fungal collections from the wild population and greenhouse samples is striking. The former yielded a diverse array of at least seven distinct fungal endophytes (Table 1). In stark contrast, the roots that had grown for one year in the greenhouse yielded only two fungal species, *Phomopsis columnaris* and *Ilyonectria robusta*. Both of these fungal species have been known to infect and kill plants by either causing root rot (*I. robusta*) or stem death (*P. columnaris*) (Cabral et al., 2012; Farr et al., 2002; Roy and Mulder, 2014). Interestingly, *P. columnaris* was the only fungus found to be present in both the field samples and the greenhouse samples. This fungus was isolated only once from the field samples, but repeatedly (10 times) from the greenhouse samples. Our data suggest that cultivation in the greenhouse for one year caused a loss in fungal richness in the *A. californica* root samples. Thus, the diversity of fungal endophytes obtained from botanical samples can vary greatly depending on environment/method of cultivation. This is an important point for consideration in natural product drug discovery efforts from fungal endophytes.

2.2. Antimicrobial activity of *A. californica* endophytes

A number of the endophyte extracts from batch 1 (wild-harvested roots) displayed pronounced antimicrobial activity against *Staphylococcus aureus*. Extracts of *Colletotrichum coccodes*, *Cylindrocarpon* sp., *Chaetomium cupreum*, and *Aspergillus* sp. all inhibited *S. aureus* growth by >25% at a concentration of 20 µg/mL. By comparison, extracts of the two fungi isolated from the greenhouse cultivated *A. californica* exhibited only weak antimicrobial activity (8% for *Phomopsis columnaris*, no activity for *Ilyonectria robusta*).

Most of the endophytes were either weakly active or completely inactive against *Pseudomonas aeruginosa*, even though the concentration tested was 10-fold higher (200 µg/mL) than that used for the *Staphylococcus aureus* growth inhibition assays. It is well known that the Gram-negative bacterium *P. aeruginosa* is less susceptible to antimicrobial agents than are Gram-positive bacteria (Balode et al., 2013; Henwood et al., 2001; Rodríguez-Rojas et al., 2012), so this result was not surprising. However, two of the endophytes from batch 1, *Cylindrocarpon* sp. and *Chaetomium cupreum*, displayed activity against both *S. aureus* (26%) and *P. aeruginosa* (22%). Thus, these fungi were chosen as starting material for isolation of antimicrobial compounds (Section 2.3).

2.3. Isolation and activity of compounds from *Chaetomium cuprum* and *Cylindrocarpon* sp

Bioassay-guided isolation from a scaled up extract of *Chaetomium cuprum* yielded one new compound (**1**), which we named chaetocuprum. The structure of this compound was confirmed by preparation of its methyl ester derivative (**2**), as described in Section 3.7. In addition, the known compound cochliodone A (**3**) was also isolated from *C. cuprum*. NMR and accurate mass data for this compound matched those reported previously (Phonkerd et al., 2008). Finally, two known compounds, equisetin (**4**) and 5'-epiequisetin (**5**), were

isolated from *Cylindrocarpon* sp. Spectroscopic data from these compounds matched literature reports (Phillips et al., 1989).

Chaetocuprum (**1**) is a spirolactone, which demonstrates some structural similarities to a fungal metabolite (**6**) that has been reported from *Pseudoarachniotus roseus* (Garrity et al., 1991). The chemistry of the *Chaetomium* genus has been investigated previously, (Asai et al., 2013; Panthama et al., 2014) but only two spirolactones have been isolated from this genus. There are a few other examples of natural products containing spirolactone ring systems, including spiromamakone A, aranorosinols, and melettinins (Angawi et al., 2005; Fuse et al., 2013; Roy et al., 1993).

2.3.1 Structure elucidation of chaetocuprum (1)—The molecular formula of chaetocuprum (**1**) was determined to be $C_{24}H_{33}NO_8$ (9 unsaturations) on the basis of NMR and HRESIMS data. Inspection of the 1H and ^{13}C NMR data (Table 2, Table S2, and Figures S1–S4) in $CDCl_3$ and CD_3OD revealed the presence of a methyl singlet, five methine signals, including four oxymethines corresponding to two epoxide units, one olefinic proton, twenty-two methylene protons, and one exchangeable proton. Additionally, ^{13}C NMR data indicated the presence of an oxygenated quaternary carbon, a non-protonated olefinic carbon, and four carbonyl carbons.

The 1H and ^{13}C NMR signals (Table 2) for H-6 (δ_H 3.65; J = 4.0 Hz, δ_C 56.3), H-7 (δ_H 3.89; J = 4.0, 2.1 Hz, δ_C 52.6), H-8 (δ_H 4.03; J = 4.0, 2.1 Hz, δ_C 57.9), and H-9 (δ_H 3.58; J = 4.0 Hz, δ_C 55.5) were indicative of a pair of epoxide groups. Analysis of HSQC, HMBC, and COSY NMR data established the adjacent location of two epoxide units.

HMBC correlations from H-6, H-8, and H-9 to C-10 (δ_C 196.7) supported the placement of C-8–C-9 epoxide unit at a position alpha to the ketone carbonyl carbon. HMBC correlations from H-6 to an oxygenated quaternary carbon (C-5; δ_C 83.5) and C-10 suggested the presence of a six-membered ring. Key HMBC correlations from methylene protons H₂-4 (δ_H 2.94 and δ_H 2.49) to C-5, C-6, and C-10 were consistent with the linkage of this group to C-5. Additional correlations from H₂-4 to an ester carbonyl carbon (C-2; δ_C 173.6) and the adjoining methine carbon (C-3; δ_C 48.9) in conjunction with the chemical shift of C-5, supported the presence of a lactone, thereby forming a spirocyclic ring system.

HMBC correlations from an exchangeable proton NH (δ_H 6.34) to C-3, C-1' (δ_C 169.7), and C-2' (δ_C 129.4) were also observed when the NMR spectra were collected in $CDCl_3$, establishing the linkage of the amide group to C-3. Key correlations from methyl group protons H₃-15' (δ_H 1.82) to C-1', C-2' (δ_C 129.4), and C-3' (δ_C 139.3) extended the side chain to include an α,β -unsaturated olefin. A single spin system including protons H-3' to H-13' was identified primarily by analysis of the COSY NMR data. The remaining NMR data were consistent with the presence of a ten-carbon aliphatic chain. A terminal carboxylic acid group (C-14'; δ_C 177.0) accounted for the remaining unsaturation and carbon count, thereby completing the assignment of the gross structure of **1**.

Compound **1** was treated with excess trimethylsilyldiazomethane (TMSCHN₂) and the 1H NMR spectrum for the product (**2**) showed a methyl singlet at δ_H 3.65 for the newly formed

methoxy group (H₃-16'), confirming that **1** contained a carboxylic acid group (Figures S-6 – S-8). However, two new doublets (δ_H 3.05 and δ_H 2.85; H₂-11) with coupling constants of 4.4 Hz were also observed. Additionally, the ¹³C NMR signal for the ketone carbon in **1** was replaced by a signal at δ_C 49.7 (C-11) in **2**. Analysis of HSQC and HMBC data were consistent with the assignment of these doublets to the methylene protons resulting from epoxidation of the ketone carbonyl group. HMBC correlations from H₂-11 to C-5 (δ_C 81.2), C-9 (δ_C 56.6), and C-10 (δ_C 59.8) confirmed the formation of a geminal epoxide in **2**. HRESIMS data [m/z 492.2578 (M+H)⁺] were consistent with the molecular formula (C₂₆H₃₇NO₈) of **2**.

NOESY correlations between H₃-15' (δ_H 1.81) and H-4' (δ_H 2.17) allowed the assignment of *E*-configuration for the C-2'–C-3' double bond in **1**. No correlations were observed between H₃-15' and H-3'. NOESY correlations of H-6 with H-7, as well as H-8 with H-9 were consistent with the presence of syn epoxide units. NOESY correlations between H-7 and H-8 were also observed. However, the relative orientation of the two epoxide groups in the ring system and conclusive assignment of the overall relative configuration of chaetocuprum could not be made solely on the basis of NOESY data. Unfortunately, crystallization attempts were also unsuccessful.

2.3.2. Antimicrobial activity of pure compounds—Antimicrobial activity was evaluated for the compounds (**1**, **4**, and **5**) that were isolated in sufficient quantity (Table 3). None of these were active against *P. aeruginosa* (MIC >200 µg/mL). Compound **1** inhibited growth of *S. aureus* (IC₅₀ of 50 µg/mL), but complete growth inhibition was not achieved against this organism (MIC > 50 µg/mL, the highest concentration tested). The activity of equisetin (**4**) agreed with literature (MIC of 1 µg/mL against *S. aureus* and inactive against *P. aeruginosa*) (Burmeister et al., 1974; Hellwig et al., 2002). 5'-epiequisetin (**5**) has not been previously evaluated for antimicrobial effects, and it demonstrated an MIC of 1 µg/mL against *S. aureus*. The positive controls for the antimicrobial assays were berberine (for *S. aureus*) and ciprofloxacin (for *P. aeruginosa*), which demonstrated MICs of 150 µg/mL and 0.125 µg/mL, respectively, consistent with previous reports (Chalkley and Koornhof, 1985; Ettetfagh et al., 2011).

2.4. Additional compounds from *A. californica* endophytes

As a complementary approach to isolation for identifying chemical constituents of endophyte extracts, all extracts were subjected to LC-MS-MS analysis, and the data were compared to a library of high-resolution mass spectrometry data from fungal compounds, as described previously (El-Elmat et al., 2013a). Using this approach, eleven additional known compounds (Table 1, see structures in Figure S9) were identified. These data suggest that the endophytes not subjected to isolation efforts also biosynthesize structurally diverse secondary metabolites.

3. Experimental

3.1. General experimental procedures

UV spectra were measured by using a Varian Cary 100 Bio UV–vis spectrophotometer. Optical rotation was measured on a Rudolph Research Autopol III polarimeter. ECD data were collected on an Olis DSM 17 CD spectrophotometer. The NMR spectra were recorded in both CDCl₃ and CD₃OD with references peaks (δ_{H} 7.24/ δ_{C} 77.2 for CDCl₃ and δ_{H} 3.31/ δ_{C} 49.2 for CD₃OD). NMR experiments were conducted using an Agilent-700, JEOL ECA-500, and/or ECS-400 spectrometers (700, 500 or 400 MHz for ¹H and 175, 125 or 100 MHz for ¹³C; Agilent Technologies, Santa Clara, CA, USA; JEOL Ltd., Tokyo, Japan). The HRESIMS data was collected on a Thermo LTQ Orbitrap XL mass spectrometer. Flash chromatography was conducted using a Teledyne Isco CombiFlash Rf system with a RediSep Rf Si-gel Gold column (4 g silica 40 μ m). A Varian ProStar HPLC system equipped with ProStar 210 pumps and a ProStar 335 photodiode array detector was used for reversed-phase preparative separations, with a Phenomenex Gemini-NX C18 column (5 μ m, 120Å; 250 $\times$ 21.2 mm) and the Galaxie Chromatography Workstation Software (version 1.9.3.2). Analytical separations were performed with a Gemini-NX C18 column (5 μ m, 120Å; 250 $\times$ 4.6 mm) from Phenomenex. For antimicrobial assays, the optical density at 600 nm was read using a POLARstar Optima microplate reader. Mueller-Hinton broth, ciprofloxacin (purity >98% by HPLC), and berberine (purity >98% by HPLC) were purchased from Sigma Aldrich. Other reagents were purchased from Fisher Scientific.

3.2. Plant material

Anemopsis californica (Nutt.) Hook. & Arn. (Saururaceae) plants were collected with permission by Amy Brown of Apache Creek Ranch in Santa Fe, NM (35°35' 56.40"N, 105°50' 27.22"W). A voucher specimen (NCU602027) was deposited in the University of North Carolina Herbarium, and was authenticated by Amy Brown. Fungi were isolated from surface sterilized fresh root samples.

3.3. Endophyte isolation and identification

Isolation of fungal endophytes was performed using methods outlined previously (El-Elmat et al., 2014b; Figueroa et al., 2014). For molecular identification of fungal endophytes isolated from yerba mansa, the internal transcribed spacer region of the ribosomal RNA gene (ITS) was sequenced using methods described previously (El-Elmat et al., 2013b; El-Elmat et al., 2013c; El-Elmat et al., 2014a; El-Elmat et al., 2014b; Figueroa et al., 2014; Figueroa et al., 2013). The ITS sequences from all strains were deposited in GenBank and are listed in Table S1.

3.4. Endophyte culture and extraction

A solid, grain-based medium was used to grow small-scale cultures of fungi in 250 mL Erlenmeyer flasks as previously described (Ayers et al., 2011; Figueroa et al., 2012). Each fungal culture was chopped and shaken overnight (16 h at 100 rpm) in a 1:1 MeOH:CHCl₃ solution, subjected to vacuum filtration, and washed with small volumes of MeOH. The filtrate was stirred in a 1:4:5 ratio of MeOH:CHCl₃:H₂O for 2 h. After separating the

organic and aqueous layers, both layers were evaporated to dryness under vacuum. The organic layer was then resuspended in a 1:1:2 mixture of MeOH:CH₃CN:hexane. The MeOH:CH₃CN and hexane layers were separately dried under vacuum. The residue from the MeOH:CH₃CN layer was used for bioassays, dereplication, isolation, and chemical profile comparison between the endophytes and the *A. californica* extract.

3.5. Isolation

The first stage separations of extracts from *Chaetomium cupreum* and *Cylindrocarpon* sp. were conducted with normal-phase flash chromatography (4 g silica gel column) at an 18 mL/min flow rate with a 35 min hexane:CHCl₃:MeOH gradient. In the first stage of separation for *Chaetomium cupreum* (GenBank accession no. KM816761, Figure S10), four fractions were obtained using normal phase chromatography. Fraction 2 (144 mg) was subjected to a second stage of purification using a reversed phase preparative HPLC with a Gemini-NX C18 column at a 21 mL/min flow rate. A linear CH₃CN:H₂O gradient starting from 30:70 to 90:10 over 20 min yielded cochliodone A (**3**), which eluted at 17 min (1 mg, 98 % purity, 0.0004 % yield). Fraction 3 was also subjected to the same gradient, and produced sub-fraction 2 (75 mg). This fraction was then purified with an isocratic solvent composition of 50:50 CH₃CN: H₂O on a preparative HPLC with a Gemini-NX C18 column at a 21 mL/min flow rate over 20 min. Chaetocuprum (**1**), eluted at 16 min (13 mg, 98.5 % purity, 0.0052 % yield). In the first stage of separation for *Cylindrocarpon* sp. (GenBank accession no. KM816763, Figure S11), four fractions were obtained with normal phase chromatography. Fraction 3 was subjected to further purification using a preparative RP-HPLC with a Gemini-NX C18 column at a 21 mL/min flow rate. A linear CH₃CN:H₂O gradient starting from 30:70 to 90:10 over 20 min yielded equisetin (**4**) at 12 min (45 mg, 98 % purity, 0.018 % yield) and 5'-epiequisetin (**5**) at 12.7 min (25 mg, 98 % purity, 0.010 % yield).

3.6. Chaetocuprum (1)

Oil; $[\alpha]_D^{22} = -24$, (c 0.09, CH₃OH); ECD (72 μ M, CH₃OH) $\lambda_{\max}$ (ϵ) 214 (+29), 230 (−8), and at 297 (−8), UV/Vis (MeOH) $\lambda_{\max}$ (log ϵ) 224 (3.5), ¹H and ¹³C NMR data: See Tables 2 and S2; Key NOESY correlations (H-# → H-#): H-4 α ↔ H-3, 4 β ; H-4 β ↔ H-3, 4 α , 6; H-6 ↔ H-4 β , 7; H-7 ↔ H-6, 8; H-8 ↔ H-7, 9; H-9 ↔ H-8; H-3' ↔ H₂-4', 5'; H₃-15' ↔ H₂-4'; HRESIMS obsd. m/z 464.2268 [M+H]⁺, calcd for C₂₄H₃₄NO₈, 464.2284.

3.7. Preparation of 10,11-epoxychaetocuprum methyl ester (2)

A sample of **1** (3 mg) was dissolved in 200–300 μ L of methanol and 2 M solution of TMSCHN₂ in diethyl ether was added dropwise until the yellow color of the TMSCHN₂ solution persisted. After stirring for 4 hours at RT, the sample was dried under air. The reaction mixture was analyzed by ¹H NMR and then purified by semi-preparative RP-HPLC [CH₃CN/H₂O (with 0.1% formic acid): 60–100% CH₃CN over 15 min] to yield **2** (1.0 mg; t_R 14 min).

3.8. 10,11-Epoxychaetocuprum methyl ester (2)

Oil; $[\alpha]_D^{22} = +10$, (c 0.05, CH₃OH); UV/Vis (CH₃OH) $\lambda_{\max}$ (log ϵ) 221 (3.5), NMR data (CD₃OD; 700 MHz) δ 6.41 (dt, 1.2, 7.4, H-3'), 4.42 (t, 10.2, H-3), 3.82 (dd, 2.2, 4.4, H-8), 3.78 (dd, 2.2, 4.2, H-7), 3.65 (s, H₃-16'), 3.54 (d, 4.2, H-6), 3.05 (d, 4.4, H_Q-11), 2.98 (d, 4.2, H-9), 2.91 (dd, 10.2, 13.4, H_Q-4), 2.85 (d, 4.4, H_B-11), 2.36 (dd, 9.9, 13.4, H_B-4), 2.31 (t, 7.5, H₂-13'), 2.19 (m, H₂-4'), 1.83 (s, H₃-15'), 1.60 (m, H₂-12'), 1.46 (m, H₂-5'), 1.32 (m; H₂-6' – H₂-11'); ¹³C NMR (CD₃OD; 175 MHz) δ 176.1 (C-14'), 175.7 (C-2), 171.7 (C-1'), 139.3 (C-3'), 130.9 (C-2'), 81.2 (C-5), 59.8 (C-10), 57.5 (C-6), 56.6 (C-9), 53.9 (C-8), 53.4 (C-7), 52.0 (C-16'), 50.4 (C-3), 49.7 (C-11), 35.6 (C-4), 34.8 (C-13'), 30.2 (C-11'), 29.8 (C-5'), 29.3 (C-4'), 26.0 (C-12'), 12.5 (C-15'), Chemical shifts for five carbons (C-6'–C-10') could not be assigned with confidence but are listed here: δ 30.6, 30.54, 30.53, 30.38, and 30.35; Key HMBC correlations (H-# → C-#): H-3 → C-2, 4, 1'; H_Q-4 → C-2, 3, 5, 6, 10; H_Q-4 → C-3, 5, 6, 10; H-6 → 5, 8, 10; H-7 → C-6, 8; H-8 → C-7, 9; H-9 → C-5, 7, 10, 11 (wk); H₂-11 → C-5, 9, 10; H-3' → C-1', 2' (wk), 4', 5', 15'; H-4' → C-2', 3', 5'; H-5' → C-3', 4'; H-12' → C-13', 14'; H-13' → C-11', 12', 14'; H-15' → C-1', 2', 3'; H-16' → C-14'; HRESIMS obsd. m/z 492.2578 [M+H]⁺, calcd for C₂₆H₃₈NO₈, 492.2592.

3.9. LC-MS dereplication

Each fungal endophyte extract was analyzed with LC-MS-MS in the positive and negative ion modes, using a dereplication method described in detail previously (El-Elmat et al., 2013a).

3.10. Antimicrobial assays

Broth microdilution assays to evaluate antimicrobial susceptibility were performed according to Clinical Laboratory Standards Institute (CLSI) guidelines (2012). *S. aureus* (strain NCTC 8325-4), (Novick, 1967) and *P. aeruginosa* (strain NCTC 12903) were used for biological testing. In separate experiments, single colony inocula of *S. aureus* or *P. aeruginosa* were grown to log phase in Mueller-Hinton broth and were adjusted to a final assay dilution of 1.0×10^5 CFU/mL based on OD₆₀₀ of 0.11 for both bacteria. The negative control consisted of 2% DMSO in broth (vehicle), ciprofloxacin served as the positive control with *P. aeruginosa*, and berberine was used as the positive control with the *S. aureus*. All treatments and controls were prepared in triplicate wells. For background subtraction, additional wells were included containing the samples without bacteria. OD₆₀₀ was measured after incubation for 18 h at 37 °C. MIC was defined as the concentration at which no statistically significant difference was observed between the negative control and treated samples. IC₅₀ was defined as the concentration at which there is a 50% decrease in growth observed between the negative control and the treated samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Fractionation schemes, ^1H and ^{13}C NMR spectra for chaetocuprum (**1**) and 10,11-epoxychaetocuprum methyl ester (**2**), and structures of compounds identified with LC-MS-MS dereplication are provided as supplementary data.

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HIGHLIGHTS

- Fourteen fungal endophytes were isolated from yerba mansa (*Anemopsis californica*).
- *Chaetomium cupreum* yielded a new antimicrobial spirolactone, chaetocuprum.
- Endophyte diversity was higher from wild-harvested than cultivated roots.
- Results indicate the importance of source material for endophyte drug discovery.

Figure 1.

Chaetocuprum (**1**), 10,11-epoxychaetocuprum methyl ester (**2**), cochliodone A (**3**), equisetin (**4**), 5'-epiequisetin (**5**), and a fungal metabolite (**6**) isolated from *Pseudoarachniotus roseus* by Merck & Co., Inc. Compound **2** is not a natural product and was prepared by chemical reaction.

Table 1

Endophytic fungi isolated from *Anemopsis californica* roots, their constituents, and the antimicrobial activities of their extracts. Extracts were tested against *Staphylococcus aureus* at a concentration of 20 µg/mL and against *Pseudomonas aeruginosa* at a concentration of 200 µg/mL. The positive controls for the antimicrobial assays were berberine for *S. aureus* (MIC 150 µg/mL) and ciprofloxacin for *P. aeruginosa* (MIC 0.125 µg/mL).

OTU identification ^a	Origin/Abundance ^b		Compounds identified ^c	<i>S. aureus</i> inhibition (%) ^d	<i>P. aeruginosa</i> inhibition (%)
	Field	Greenhouse			
<i>Colletotrichum coccodes</i>	2			34 ± 1.6	11 ± 1.1
<i>Penicillium</i> sp.	1			21 ± 1.7	29 ± 1.7
<i>Hypocreales</i> sp.	1		verticillin A, 11'-deoxyverticillin A	16 ± 1.0	12 ± 1.0
<i>Cylindrocarpum</i> sp.	5		equisetin*, 5'-epiequisetin*	38 ± 0.6	24 ± 3.1
<i>Chaetomium cupreum</i>	1		chaetocuprum*, cochliodone A*	26 ± 1.5	79 ± 3.0
<i>Aspergillus</i> sp.	1			37 ± 1.7	15 ± 1.3
<i>Fusarium</i> sp.	2		apicidin*, apicidin A*, apicidin D ₂ *	9 ± 1.0	15 ± 2.6
<i>Penicillium</i> sp.	2			21 ± 1.0	19 ± 3.7
<i>Herpotrichiellaceae</i> sp.	1			0 ± 3.3	0 ± 4.4
<i>Sordariates</i> sp.	1			10 ± 4.6	0 ± 2.1
<i>Penicillium</i> sp.	2			17 ± 1.2	20 ± 1.5
<i>Hypocreales</i> sp.	1			6 ± 2.6	3 ± 2.7
<i>Nemania serpens</i>	2			7 ± 2.7	0 ± 5.2
<i>Phomopsis columnaris</i>	1	10	acremomidin C, trichothecol B, AGI-7, (E)-8-(3-(oct-2-enyl)oxiran-2-yl)octanoic acid, 5,8-epidioxyergosta-6,9(11),22-trien-3-ol	8 ± 1.5	14 ± 1.3
<i>Ilyonectria robusta</i>		1	chermesinone A	0 ± 1.0	0 ± 1.0

^a Operational taxonomic unit (OTU). GenBank accession numbers for the isolated fungi are included in Table S1. Isolates were grouped based on 98% ITS rDNA sequence similarity and identified using BLAST search implemented via web platform PlutoF hosted on the UNITE database.

^b The number of isolates represents the number of times each fungus was isolated from the *A. californica* roots in the relevant batch.

^c All of the fifteen compounds listed were identified in at least one of the isolates from each fungal species. Compounds indicated with an asterisk (*) were isolated and verified by NMR. The remaining compounds were identified by matching accurate mass, MS-MS, and retention time with standards in a dereplication database (EI-Elimat et al., 2013a), and are shown in Figure S9. Some fungi did not produce any of the compounds in the database at detectable levels, but are likely to produce other compounds. Experiments to identify these via isolation are ongoing.

^d Growth inhibition is expressed as the mean decrease in absorbance at 600nm for triplicate cultures (± standard deviation, SD). In cases where multiple isolations were obtained for the same fungus, the reported inhibition values are means of those for all strains.

Table 2NMR spectroscopic data (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR, CDCl_3) for chaetocuprum (**1**).^a

Position	δ_{H} (mult., J in Hz)	δ_{C} (mult.)	HMBC ($^1\text{H}^{\#} \rightarrow ^{13}\text{C}^{\#}$)
2		173.6 <i>s</i>	
3	4.25 (<i>m</i>)	48.9 <i>d</i>	C-2, C-4, C-5, C-1'
4 α	2.94 (<i>dd</i> , 10.2, 14.0)		C-2, C-3, C-5, C-6, C-10
4 β	2.49 (<i>dd</i> , 10.2, 14.0)	34.5 <i>t</i>	C-2, C-3, C-5, C-6, C-10
5		83.5 <i>s</i>	
6	3.65 (<i>d</i> , 4.0)	56.3 <i>d</i>	C-4, C-5, C-7, C-8, C-10
7	3.89 (<i>dd</i> , 2.1, 4.0)	52.6 <i>d</i>	C-5, C-6, C-8, C-9
8	4.03 (<i>dd</i> , 2.1, 4.0)	57.9 <i>d</i>	C-6, C-7, C-9, C-10
9	3.58 (<i>d</i> , 4.0)	55.5 <i>d</i>	C-5, C-7, C-8, C-10
10		196.7 <i>s</i>	
1'		169.7 <i>s</i>	
2'		129.4 <i>s</i>	
3'	6.43 (<i>t</i> , 7.0)	139.3 <i>d</i>	C-1', C-2', C-4', C-5', C-15'
4'	2.13 (<i>m</i>)	28.7 <i>t</i>	C-2', C-3', C-5', C-6'
5'	1.40 (<i>t</i> , 7.0)	29.0 <i>t</i>	C-3', C-4', C-7'
6'	1.30 (<i>m</i>)	29.9 <i>t</i> ^b	
7'	1.30 (<i>m</i>)	29.4 <i>t</i> ^b	
8'	1.30 (<i>m</i>)	29.3 <i>t</i> ^b	
9'	1.30 (<i>m</i>)	28.6 <i>t</i> ^b	
10'	1.30 (<i>m</i>)	29.4 <i>t</i> ^b	
11'	1.30 (<i>m</i>)	29.1 <i>t</i>	
12'	1.60 (<i>m</i>)	24.8 <i>t</i>	C-10', C-14'
13'	2.32 (<i>t</i> , 7.0)	33.7 <i>t</i>	C-11', C-14'
14'		177.0 <i>s</i>	
N-H	6.34 (<i>d</i> , 4.0) ^c		C-2, C-3, C-4, C-1', C-2'
15'	1.82 (<i>s</i>)	12.7 <i>q</i>	C-1', C-2', C-3'

^aNMR data obtained in CD_3OD are provided in Table S2.^b ^{13}C NMR assignments for C-6' – C-10' can be interchanged.^cThe chemical shift is variable; this value was observed in CDCl_3 at room temperature.

Table 3

Minimum inhibitory concentration (MIC) and IC₅₀ of select fungal metabolites against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Compound	<i>Staphylococcus aureus</i>		<i>Pseudomonas aeruginosa</i>	
	MIC (µg/mL)	IC ₅₀ (µg/mL)	MIC (µg/mL)	IC ₅₀ (µg/mL)
Chaetocuprum (1)	>50	50	>50	>50
Equisetin (4)	1.0	0.5	>200	>200
5'-Epiequisetin (5)	1.0	0.5	>200	>200
Ciprofloxacin (+ control)	–	–	0.125	0.0625
Berberine (+ control)	150	300	–	–