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Anemopsis californica is a plant native to the southwestern United States and northern Mexico that has been used by many Native American tribes to alleviate pain and inflammation and to treat infection. Limited research has examined the chemical composition of *A. californica* responsible for its purported therapeutic properties. Goal 1 of this study was to identify pathogenic bacteria against which the plant extract was active, and isolate the compounds responsible for this activity. Goal 2 was to identify bioactive compounds from endophytic fungi isolated from the plant.

The *A. californica* root extract was tested against a panel of bacteria and exhibited the most activity against several *Mycobacterium* spp. Bioassay-guided fractionation was conducted on a sample of *A. californica* roots was undertaken to isolate the bioactive compounds. These compounds were identified as the furofuran lignans sesamin (**1**) and asarinin (**2**), which were shown to have minimum inhibitory concentrations (MICs) ranging from 23 to 395 μM against five different species of environmental nontuberculous mycobacteria. These findings are significant given that these bacteria can cause skin, pulmonary, and lymphatic infections. With the use of liquid chromatography - mass spectrometry (LC-MS), it was determined that sesamin and asarinin were extracted at relatively high levels from *A. californica* roots (1.7-3.1g/kg and 1.1-1.7 g/kg, respectively), but lower levels from leaves (0.13 g/kg for both compounds). Our

findings suggest that the majority of activity of crude *A. californica* root extracts against nontuberculous mycobacteria can be attributed to the presence of sesamin and asarinin. This is the first reported isolation of these compounds from a member of the Saururaceae family, and the first description of their activity against nontuberculous mycobacteria.

Goal 2 of this project was to identify bioactive compounds from the fungal endophytes of *A. californica* (fungi living asymptomatically within the plant tissues). The fungal endophytes were extracted from the plant and cultured on a solid media. Twelve different fungi were identified. Crude extracts of these fungi were prepared and partitioned with liquid-liquid chromatography. Several antimicrobial compounds were isolated or identified from these fungi, including a new antimicrobial compound chaetocuprum A from the fungus *Chaetomium cupreum*. An additional nineteen compounds were also identified, of which six have been shown in literature or with our investigations to possess antimicrobial activity. In addition, a crude extract of the *A. californica* root was prepared from the same batch of roots from which fungi were isolated, to ascertain whether any of these antimicrobial compounds were present in the plant. None of the antimicrobial compounds isolated from the endophytes were detected in the extract, but a series of other fungal compounds were present. Finally, at least eighteen ions were detected as being present in both the botanical and fungal extracts. The structures of these “overlapping compounds” have not yet been

solved, but their presence does suggest a potential role for fungi in the chemical composition of botanical extracts.

DUAL SOURCES OF ANTIMICROBIAL ACTIVITY OF *ANEMOPSIS CALIFORNICA* : PLANT AND ENDOPHYTIC FUNGI

by

Robert Owen Bussey III

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the Faculty of The Graduate School at
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Doctor of Philosophy

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Approved by

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APPROVAL PAGE

This dissertation written by Robert Owen Bussey III has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

One of the largest health threats in the United States is bacterial resistance to antimicrobial compounds. Existing antibiotics are ineffective in treating many types of bacterial infections, including some types of mycobacteria, Gram-positive bacteria, and Gram-negative bacteria.[1] With this research, we focused on the identification of compounds from natural product sources effective against representative organisms of all three of these categories. Due to the lack of available antibacterial agents, the challenges to treating these types of bacterial infections include increased mortality, increased healthcare costs and adverse reactions with long-term antibiotic exposure.

Mycobacteria are unique in that they are neither Gram-positive or Gram-negative. Non-tuberculosus mycobacteria (NTM) such as *Mycobacterium avium* and *Mycobacterium marinum* can have mortality rates up to 25 percent in immune-compromised patients, high healthcare costs equivalent to the cost of long term HIV treatment, and a 50 percent likelihood of experiencing adverse side effects to treatments.[2] Mycobacterium species are more likely to infect immuno-compromised patients, but have also been seen in immuno-competent patients. Mycobacterial infections are important clinically because of the lack of

antimycobacterial antibiotics and disinfectants and the emergence of antibiotic-resistant mycobacteria.[3, 4] In addition, both certain types of Gram-positive (such as *Staphylococcus aureus*) and Gram-negative (such as *Pseudomonas aeruginosa*) bacterial infections can have mortality rates up to 45 percent, and the doubling of healthcare costs and the length of stay in the hospital as a result of these infections is common.[5] *Staphylococcus aureus* and *Pseudomonas aeruginosa* are major causes of nosocomial (hospital-acquired) wound and surgical infections if patients are immuno-compromised or have cystic fibrosis (*P. aeruginosa*).[6, 7] Patients often spread *S. aureus* antibiotic-resistant strains quickly in hospitals without effective containment.[7] Complications from *P. aeruginosa* infections are often so severe in cystic fibrosis patients that it is one of the major causes of death among these patients.[8]

To combat these infections, a new arsenal of antibiotics is needed. A good source for these new antibacterial compounds are the natural products made by plants, bacteria, and fungi. It has been reported that 25 to 50 percent of compounds that treat infection have come from these natural sources.[9] With this project, the purpose is to identify new antimicrobial agents from a botanical and its endophytic fungi useful against several species of pathogenic bacteria, *Mycobacterium* spp., *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. This project is significant to public health because it investigates new antimicrobial agents from *A. californica* that could decrease the time, cost, and adverse side effects involved in bacterial treatment.

Many Native American tribes in the Southwestern United States have used a plant named *Anemopsis californica* or yerba mansa to ease pain, decrease inflammation and to treat infection. *Anemopsis californica* is a member of the Saururaceae family, and is a popular groundcover for parks in the southwestern United States and phytoremediatior of arsenic from groundwater and soil.[10] It is also a plant that has been used in traditional medicine by different cultures in the U.S. and Mexico for hundreds of years. Both the leaves and roots were used as antiseptic salves for wounds, boiled as tea or used orally for internal ailments.[11, 12] The specific ailments this plant was used for include cold and flu, venereal diseases, inflammation, and pain.[11] Today *A. californica* is widely used as a part of traditional /alternative medicine in the Southwestern United States, and it is usually sold either as dry or ethanolic extracts of the leaves and roots.[13]

Although ethnobotanical literature shows the use of whole plant *A. californica* extracts for treatment of infection, the majority of prior research on this botanical has focused on its essential oil. Studies have shown that the constituents contained in the *A. californica* root/rhizome essential oils were mainly classified as monocyclic and bicyclic monoterpenoids and phenylterpenoids.[13, 14] These compounds were active against AN3CA (endometrial cells) and HeLa (cervical cells) cancer cells *in vitro*.[13, 14] Another group showed that the crude ethanol and the crude ethylacetate extracts of the *A. californica* root can affect the proliferation and migratory-ability of colon cancer

cell line HCT-8, and the breast cancer cell lines Hs 578T and MCF-7/AZ.[15] Some of the same chemical compounds identified as monocyclic and bicyclic monoterpenoids and phenylterpenoids in the essential oil of the *A. californica* roots were also found in the essential oil of the leaves.[16]

With this research, we sought to provide more information about the compounds responsible for purported effectiveness of *A. californica* against infection. Project 1 focused on identifying antimicrobial compounds in the *A. californica* plant, and then quantifying them in the different plant parts. With project 2, we sought to isolate and identify antimicrobial compounds from *A. californica* endophytic fungi (fungi isolated from the plant roots) and to determine whether fungal compounds could be identified in an *A. californica* botanical extract.

CHAPTER II

ISOLATION OF SESAMIN AND ASARININ

Chapter II is based in part on data published previously in the journal Planta Medica: Bussey III, R. O.; Sy-Cordero, A. A.; Figueroa, M.; Carter, F. S.; Falkinham III, J. O.; Oberlies, N. H.; Cech, N. B., Antimycobacterial Furofuran Lignans from the Roots of *Anemopsis californica*. *Planta Med* **2014**, *80* (6), 498-501.

Introduction

A new arsenal of antibiotics is needed to address two problems involving treatment of bacterial infections: the emergence of drug-resistance and the existence of bacteria that are innately resistant to most antibiotics. A promising source for new antibacterial compounds is the natural products produced by plants, bacteria, and fungi. It is estimated that 25 to 50 percent of anti-infective agents come from these natural sources.[9] This study chose the plant *Anemopsis californica* (Nutt.) Hook. & Arn. (Saururaceae) as a potential source of antimicrobial compounds.

Anemopsis californica, commonly known as "yerba mansa," is native to the southwestern United States and northern Mexico, and its roots, leaves, and stem have been used medicinally by many Native American tribes.[17-19] Despite historical and modern precedent for the use of this plant to treat infection,

only a few studies have focused on the chemicals responsible for its anti-infective properties.[16, 20] The chemical compounds identified from *A. californica* thus far are exclusively from the volatile oils of the leaves and roots.[13, 14] Volatile oil extracts from *A. californica* have been shown to inhibit the growth of endometrial, cervical, colon, and breast cancer cells *in vitro,*[13, 14] and demonstrated antimicrobial activity against *Staphylococcus aureus, Streptococus pneumoniae,* and *Geotrichim candidum*.[16] Additionally, ethanol and ethyl acetate extracts of various parts of *A. californica* were shown to inhibit the growth of colon and breast cancer cells, and aqueous *A. californica* extracts inhibited cell migration and metastasis.[15, 21] These studies did not indicate which chemical constituents of those extracts were responsible for the observed effects.

There are currently no reports of the identities of non-volatile compounds present in *A. californica* roots and leaves. This is a significant gap in the literature, given that its traditional mode of application of *A. californica* is a whole plant poultice or decoction. For example, the Shoshoni tribe of Nevada applied boiled and mashed *A. californica* roots to areas of inflammation and infections.[17] The Pima tribe of Arizona and New Mexico and the Mahuna and Chumash tribes of California used a decoction of leaves and roots to treat wounds,[18, 22] and the Nevada Paiute and the California Costanoan tribes used decoctions of *A. californica* roots or leaves to treat pain.[17, 19]

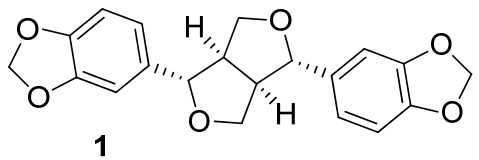
The ethnobotanical precedent for application of *A. californica* to treat infections encouraged us to screen extracts from this plant for activity against

pathogenic microorganisms. Activity of crude *A. californica* extracts was noted against several species of nontuberculous mycobacteria. These findings were deemed significant, given the clinical relevance these organisms.

Nontuberculous mycobacteria are commonly found in soils, natural waters, and engineered water systems, including household plumbing,[23] and can cause pulmonary, skin, and lymph node infections.[24] The resultant chronic respiratory or soft tissue infections require long term antibiotic treatment that can have serious side effects.[25] Current estimates report a total number of over 16,000 cases in the United States of nontuberculosis mycobacterial disease per year with a total cost of over $425 million.[26] The objective of this research was to identify compounds from *A. californica* with potential for the treatment of nontuberculous mycobacterial infections. In addition, we sought to provide insight into the scientific basis for the ethnobotanical use of *A. californica* to treat bacterial infections.

Results and Discussion

Bioactivity-guided fractionation of *A. californica* resulted in the isolation of two compounds, sesamin (**1**)[27] and its C-7 epimer, asarinin (**2**).



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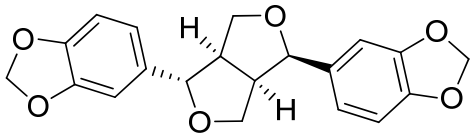

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Figure 1. Structures of furfuran lignins sesamin (**1**) and asarinin (**2**) isolated from *Anemopsis californica*.

Sesamin was a white solid with a HRESIMS *m/z* of 355.1175 (calcd for $C_{20}H_{19}O_6$ $[M+H]^+$ *m/z* 355.1176, $[\alpha]_D^{25}$= + 104, c = 0.0125 g/100 mL, methanol). Asarinin, also called episesamin or isosesamin, was a white solid with a HRESIMS *m/z* of 355.1167 (calcd for $C_{20}H_{19}O_6$ $[M+H]^+$ *m/z* 355.1176, $[\alpha]_D^{25}$= + 144, c = 0.0125 g/100 mL, methanol). The ^1H and ^{13}C NMR of both sesamin and asarinin were in agreement with literature values.[28] ^1H and ^{13}C NMR data plus spectra are included for reference on the successive pages (Table 1 and Figures 2-5).

Table 1. NMR spectroscopic data (500 MHz for ^{1}H and 125 MHz for ^{13}C, $CDCl_3$) for sesamin (**1**) and asarinin (**2**) from *A. californica*.

	sesamin			**asarinin**	
Position	δ_H mult. (J in Hz)$_H$	δ_C	Position	δ_H mult. (J in Hz)$_H$	δ_C
1, 1'		135.0 C	1		135.1 C
			1'		132.3 C
2, 2'	6.84, s	106.6 CH	2	6.86, s	106.5 CH
			2'		106.7 CH
3, 3'		148.1 C	3		147.3 C
			3'		148.0 C
4, 4'		147.2 C	4		146.6 C
			4'		147.7 C
5, 5'	6.77, d (8.0)	108.3 CH	5	6.81, m	108.3 CH
			5'		108.3 CH
6, 6'	6.80, dd (8.0, 1.7)	119.5 CH	6	6.81, m	119.7 CH
			6'		118.8 CH
7, 7'	4.71, d (4.0)	85.9 CH	7	4.82, d (5.0)	87.7 CH
			7'	4.39, d (7.0)	82.1 CH
8, 8'	3.05, m	54.4 CH	8	3.3, m	54.7 CH
			8'	2.85, m	50.2 CH
9, 9'	4.2, dd (9.2, 6.9) 3.9, dd (9.2, 3.9)	71.8 CH_2	9	3.81, m; 4.09, d (9.5)	69.8 CH_2
			9'	3.3, m; 3.81, m	71.0 CH_2
10, 10'	5.95, s	101.2 OCH_2O	10	5.97 s	101.1 OCH_2O
			10'	5.95 s	101.3 OCH_2O

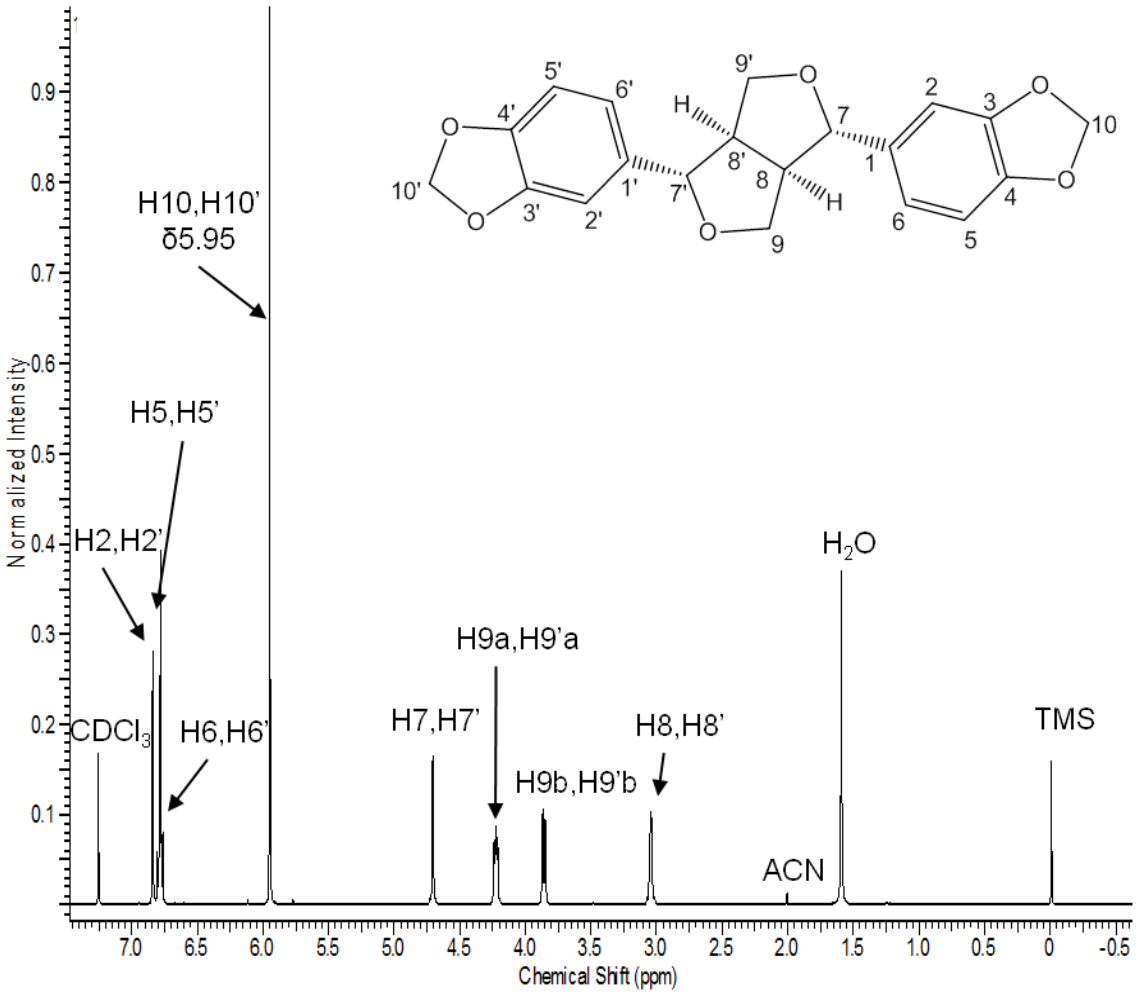


Figure 2. ^{1}H NMR spectrum (500 MHz in $CDCl_3$) for sesamin (**1**) from *Anemopsis californica*.

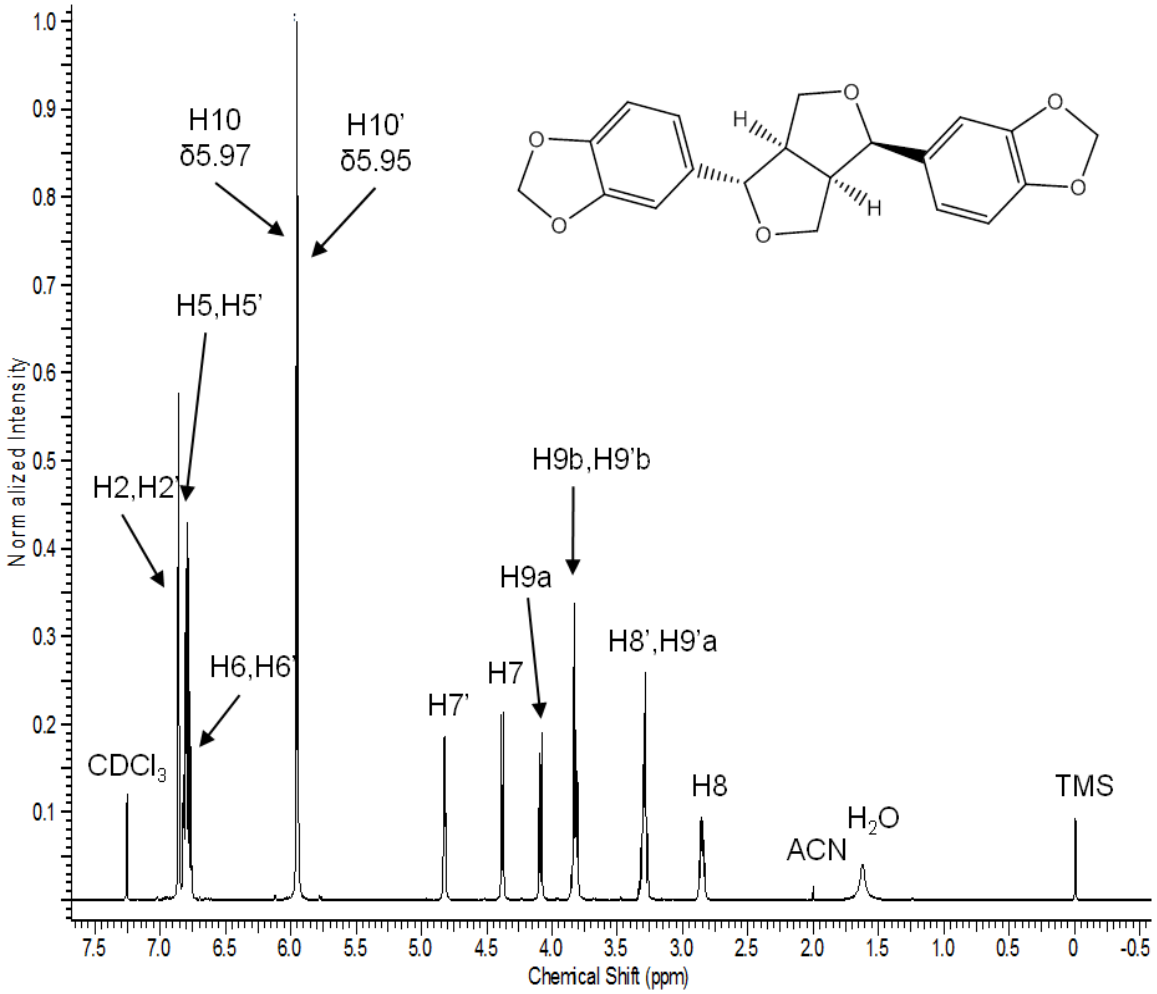


Figure 3. ^{1}H NMR spectrum (500 MHz in $CDCl_3$) for asarinin (**2**) from *Anemopsis californica*.

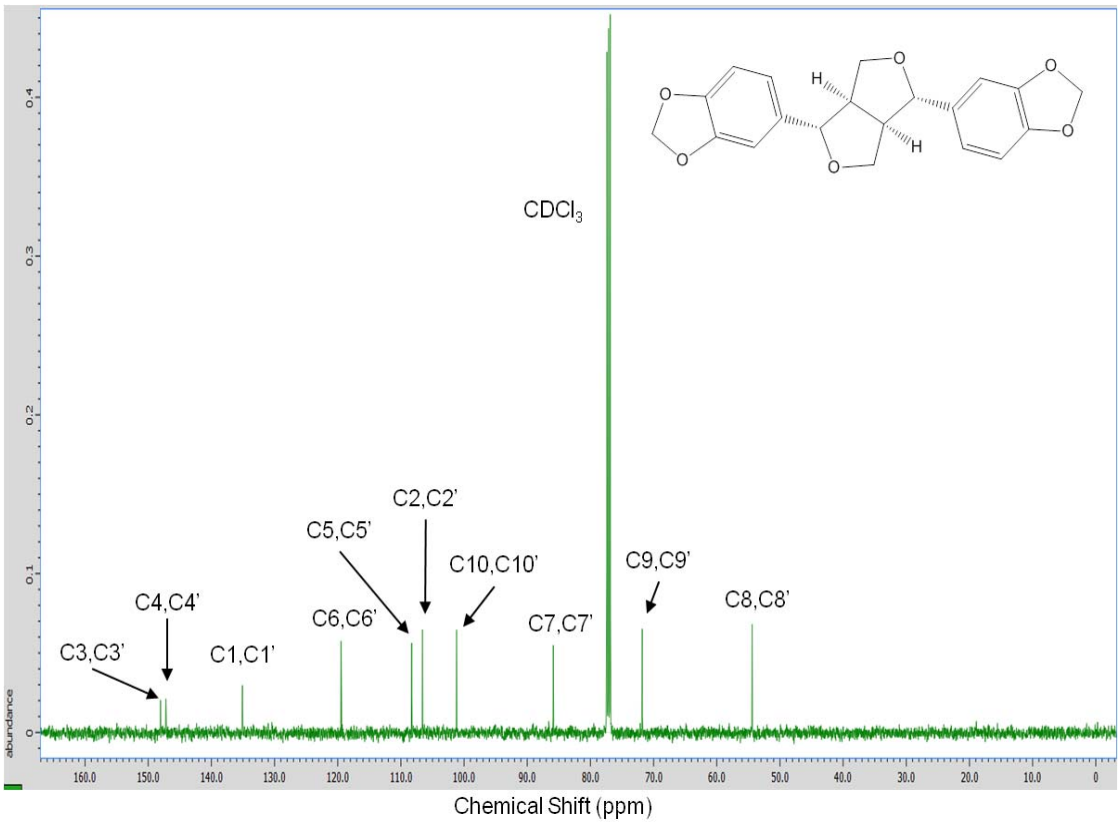


Figure 4. ^{13}C NMR spectrum (125 MHz in $CDCl_3$) for sesamin (**1**) from *A. californica*.

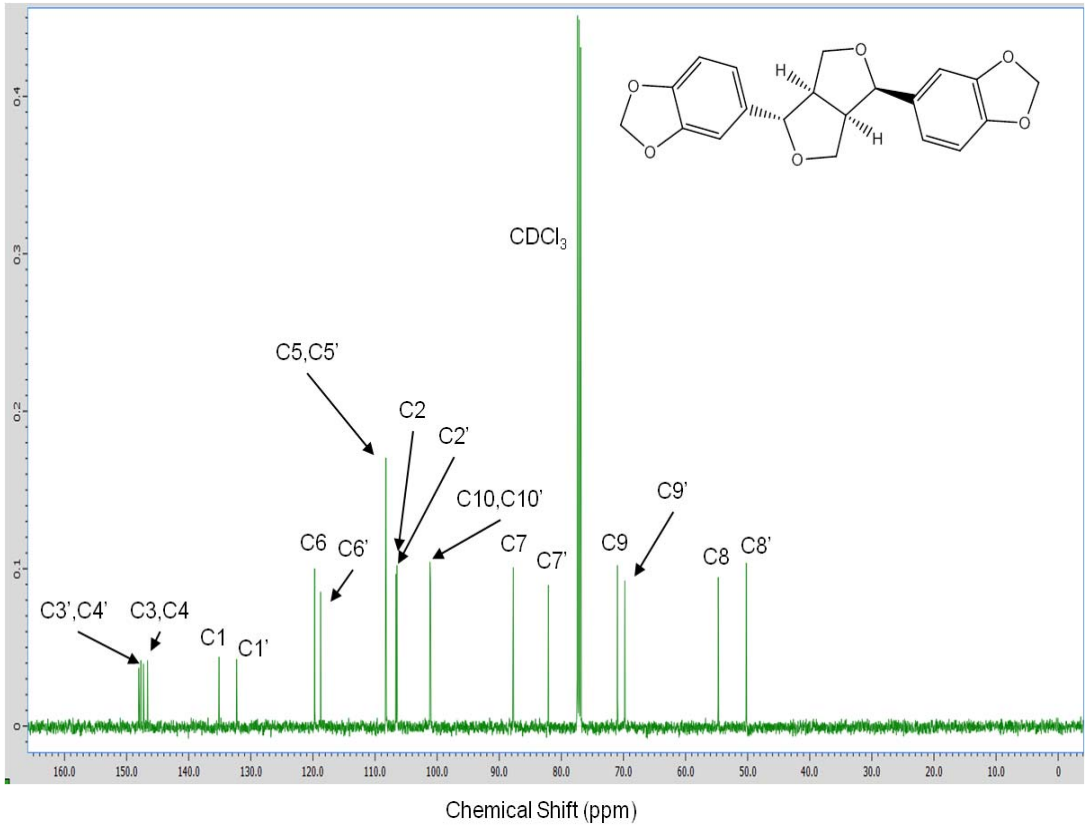


Figure 5. ^{13}C NMR spectrum (125 MHz in $CDCl_3$) for asarinin (**2**) from *A. californica*.

Although sesamin and asarinin are both known compounds, this is the first report of their presence in a member of the Saururaceae plant family. Sesamin was first isolated from sesame seed oil, [29, 30] while asarinin was first isolated from prickly ash bark.[31] Both sesamin and asarinin have been shown to act as insecticidal synergists with pyrethrins.[30] Previous studies have shown that sesamin has moderate activity against *S. aureus* and no activity against *E. coli.*[32] In addition, asarinin was shown to be moderately active against *S. aureus* and *B. subtilis*, and it was suggested that it inhibits the NorA efflux pump system of *S. aureus*. [33]

Sesamin and asarinin demonstrated a range of antimicrobial activities (8 to 140 µg/mL or 22.6 µM to 395 µM) against the five different species of *Mycobacterium* evaluated (Table 2).

Table 2. Minimum inhibitory concentrations (MIC) of crude extracts of *A. californica, s*esamin, and asarinin against *Mycobacterium* species.

Sample	MIC (µg/mL) Against:				
	M. smegmatis	*M. abscessus*	*M. chelonae*	*M. marinum*	*M. avium* A5
Sample 1[a]	125	>250	>250	250	125
Sample 2[a]	>250	>250	>250	>250	>250
Sample 3[b]	125	>250	250	>250	>250
Sesamin	8	>130	65	13	8
Asarinin	35	>140	140	35	35
Rifampin[c]	25	0.8	0.15	0.8	2

[a]Samples 1 and 2 are for root and aerial extracts-respectively from the same *A. californica* plant.
[b]Sample 3 represents a large batch root extract that was subjected to bioactivity-directed fractionation, resulting in the isolation of sesamin and asarinin.
[c]The antibiotic rifampin is included as a positive control. Negative control (vehicle, 2% DMSO) caused no significant growth inhibition.
[d] These data were collected by our collaborators in the laboratory of Dr. Joseph O. Falkinham at Virginia Polytechnic University.

This is the first report of activity of sesamin and asarinin against nontuberculous mycobacteria, although both sesamin and asarinin were shown to lack activity against *M. tuberculosis*.[34] These results are not surprising, given that members of the *M. tuberculosis* complex do not share the same susceptibilities to anti-mycobacterial antibiotics with the nontuberculous mycobacteria.[25]

Crude *A. californica* extracts were tested against representative *Mycobacterium* species (Table 2). Samples 1 and 3 (root extracts) demonstrated

MIC values ranging from 125 to >250 µg/mL, while sample 2 (aerial extract) demonstrated weak or no inhibition (MIC >250 µg/mL). These differences in activity were likely due to the higher levels of sesamin and asarinin present in roots as compared to leaves (Table 3).

Table 3. Quantity of sesamin and asarinin in extracts prepared from roots or leaves/stems of *A. californica*

Sample Name	Plant part	Sesamin Concentration[a]		Asarinin Concentration	
		yield[b] (ppt) ± SD	% in extract[c] ± SD	yield (ppt) ± SD	% in extract ± SD
Sample 1	Root	3.1 ± 0.3	8.8 ± 1.0	1.7 ± 0.1	4.7 ± 0.4
Sample 2	Leaf/stem	0.13 ± 0.02	0.95 ± 0.14	0.13 ± 0.02	0.9 ± 0.2
Sample 3	Root	1.7 ± 0.2	8.8 ± 0.9	1.1 ± 0.1	5.9 ± 0.2

[a] Concentrations were determined by LC-MS analysis of extracts prepared from the relevant plant parts. Standard deviations are for triplicate analyses of the same extract. Extract concentration was calculated based on linear regression analysis of 6 point calibration curves of peak area versus concentration with slope (m) = 587881 ± 4628, intercept (b) = -20512 ± 10554, and R^2 = 0.9998 for sesamin and a slope (m) = 647726 ± 16450, intercept (b) = -15594 ± 37516, and R^2 = 0.9974 for asarinin. Extracts were diluted so that the concentrations tested fell within the linear range of the calibration curve.

[b] Yield is reported as parts per thousand or mass of pure compound (g) per mass of original plant material (kg).

[c] % in extract is reported as mass (g) of sesamin or asarinin per mass of solid extract (g) × 100. The % in extract values are provided for the purpose of comparison with biological data.

The root extracts (samples 1 and 3) were notably less active compared to the pure compounds. However, these extracts are crude mixtures, and sesamin and asarinin constituted only a fraction of their content (Table 3). Accounting for this difference, it appeared that the antimycobacterial activity of the crude extracts could be largely attributed to the presence of sesamin and asarinin. For example, sample 1, a root extract of *A. californica*, demonstrated an MIC of 125 µg/mL against *M. smegmatis* (Table 2). Sample 1 contained 8.8 ± 1.0 % sesamin and 4.7 ± 0.4 % asarinin (Table 3); thus, the MIC of 125 µg/mL (expressed as mass of crude extract/volume media) is equivalent to 16.8 µg/mL (expressed as mass of sesamin and asarinin, combined, per volume of media). This value is within the range of the reported MICs for sesamin and asarinin alone against *M. smegmatis* (8 and 35 µg/mL, respectively).

In conclusion, the results of the quantitative analysis suggest that the majority of activity of *A. californica* root extracts against nontuberculous mycobacteria can be attributed to the presence of relatively high levels of sesamin and asarinin. Importantly, the presence of anti-mycobacterial compounds in *A. californica* roots supports the traditional use of this plant as a treatment for infection, although follow up studies would be necessary to evaluate the *in vivo* relevance of these findings. The higher levels of sesamin and asarinin in roots over leaves suggest that root extracts may be more effective than leaf extracts for treatment of mycobacterial infections.

Materials and Methods

Plant Material

Cultivated *Anemopsis californica* plant material was obtained from two sites, Horizon Herbs in Williams, OR (42°12' 17.21"N, 123°19' 34.61"W; voucher number NCU592735, identified by Richard A. Cech) and Apache Creek Ranch in Santa Fe, NM (35°35' 56.40"N, 105°50' 27.22"W; voucher number NCU602027, identified by Amy Brown). Vouchers are retained at the University of North Carolina Herbarium. Harvested plant material was separated into three different portions, a root sample (sample #1, 9.8 g dry weight), a leaf/stem sample from the same plant (sample #2, 7.5 g dry weight, both harvested from Horizon Herbs in April, 2010), and a large batch of roots/rhizomes to facilitate isolation work (sample #3, 520 g dry weight) harvested from Apache Creek Ranch in November 2010. All plant material was air dried prior to extraction.

Extraction and Liquid Chromatography-Mass Spectrometry

Three batches of *A. californica* plant material were cut and ground, and then were macerated in methanol for 24 hours. The marc for each extract was subsequently soaked in methanol a total of three times, and the methanol was decanted and combined. The methanol extracts were evaporated to dryness with a rotary evaporator and subjected to liquid-liquid partitioning using published methods. [35] Briefly, the methanol extract was defatted by partitioning between a 1:1 ratio of hexane to methanol, and the latter fraction was then dried down and further partitioned between 4:1:5 of chloroform:methanol:water. The chloroform

fraction was evaporated to dryness and its antimicrobial activity was tested using a broth microdilution assay. The yields from the chloroform fraction of samples 1, 2, and 3 were 343 mg, 104 mg, and 10 g, respectively.

For the isolation of the active compounds, the chloroform extract (10 g, sample #3, roots and rhizomes) was subjected to two stages of normal-phase chromatography on a CombiFlash®R_f ISCO using RediSep Rf Gold® Silica Columns (Teledyne ISCO, Lincoln, NE, USA). The first stage of normal-phase chromatography was performed with a hexane/chloroform/methanol gradient on silica gel (20-40 µm particle diameter, 120 g column, eluent chloroform and methanol, flow rate 18 mL/min) and the eluate was pooled into 12 fractions. Fraction VI (360-450 mL, 645 mg) was subjected to a second stage of separation with a hexane/acetone/methanol gradient on silica gel (20-40 µm particle diameter, 120 g column, eluent acetone and methanol, flow rate 18 ml/min) and pooled into 10 fractions. Fraction V (250-320 mL, 420 mg), the active fraction, was then subjected to further purification with two successive stages of isocratic separation with reversed-phase preparative HPLC on a C_{18} column (Phenomenex, Gemini-NX, 5 µm, 250 $\times$ 21.2 mm, flow rate 21 mL/min). The first stage of reversed-phase separation employed a 50:50 acetonitrile:water isocratic mobile phase composition, and the eluate was pooled into 5 fractions. Fractions II and III from this separation (200-300 mL and 320-400 mL, 75 mg and 80 mg, respectively) were then combined and subjected to a second reversed-phase separation with an isocratic mobile phase composition of 75:25 methanol:water.

Sesamin **(1)** (0.012 % yield, 60 mg, 98.3% purity) eluted at 15 min and asarinin **(2)** (0.0096% yield, 60 mg, 98.5% purity) eluted at 16.5 min.

Test Bacteria, Chemicals, Biochemicals, and Minimal Inhibitory Concentration

Mycobacterium marinum (ATCC strain 927), *M. smegmatis* strain mc^2155 (ATCC strain 700084), *M. abscessus* strain AAy-P-1, *M. chelonae* strain EO-P-1, *M. intracellulare* strain TMC 1406^T (ATCC 13950), and *M. avium* strain A5 [4, 36] were grown in Middlebrook 7H9 broth medium containing 0.5 % (v/v) glycerol and 10 % (v/v) oleic acid-albumin with aeration (120 rpm) for 7 days at 37°C or 30°C (only *M. marinum*). Minimal inhibitory concentration (MIC) of each fraction or compound was measured by broth microdilution with a starting inoculum of 0.5-1.0 $\times 10^5$ CFU/mL.[4, 37] The plates were incubated for 4 days at 37°C or 30° C (only *M. marinum*) and the turbidity measured (absorbance 580 nm). Extracts were tested over a concentration range of 0.12 to 250 µg/mL by two-fold dilutions, in the presence of a constant DMSO concentration (2%). The MIC was defined as the lowest concentration completely inhibiting bacterial growth. Rifampin (Sigma, St. Louis, MO purity ≥ 97%) served as the control.

Quantitative Analysis

Sesamin and asarinin were identified in the crude *A. californica* extracts by matching retention time and fragmentation patterns with those of the isolated standard compounds. The concentrations of these compounds were then measured using selective reaction monitoring (SRM) on a triple quadrupole mass spectrometer (TSQ Access; Thermo Scientific, Waltham, MA, USA) with an

electrospray ionization source in the positive ion mode. Transitions of 337.1 to 203.1 and 337.1 to 289.2 were employed for the isomeric compounds. The mass spectrometer was coupled to a reversed phase high performance liquid chromatograph (HPLC) (Agilent HP1200; Santa Clara, CA, USA) with a PFP column (5 µm, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA). An acetonitrile (1% formic acid):water (1% formic acid) gradient was employed at 1.0 mL/min with HPLC grade solvents. A calibration curve (concentration range of 0.05 to 5.0 µg/mL) of concentration versus average peak area for triplicate injections was employed for quantitative analysis. Extracts were diluted so that sesamin and asarinin concentrations fell within the linear range of the calibration curve.

Acknowledgements

We are grateful to Richard Cech (Horizon Herbs) and Amy Brown (Apache Creek Herbs) for providing *A. californica* plants. Thanks also to Carol Ann McCormick for preservation of voucher specimens. The authors acknowledge the technical skill of Ms. Myra D. Williams (Virginia Tech) for measurement of anti-mycobacterial activity of fractions, Dr. Brandie Ehrmann (UNC Greensboro) for assistance with mass spectrometry experiments, and Tyler Graf (UNC Greensboro) for assistance with isolation work. Funding was provided by the National Center for Complementary and Alternative Medicine, a component of the National Institutes of Health (R15 AT005005). MS data were collected in the Triad Mass Spectrometry Facility.

CHAPTER III

ANTIMICROBIAL ENDOPHYTES FROM A BOTANICAL TRADITIONALLY USED TO TREAT INFECTION, YERBA MANSA (*ANEMOPOSIS CALIFORNICA*)

Chapter II is based in part on data in preparation to be submitted for publication in the *Journal of Natural Products*: Bussey III, R.; Kaur, A.; Todd, D.; Egan, J.; El-Elimat, T.; Graf, T.; Raja, H.; Oberlies, N.; Cech, N., Antimicrobial Endophytes from a Botanical Traditionally Used to Treat Infection, Yerba Mansa (*Anemoposis californica*). *Journal of Natural Products* 2014, in preparation.

Introduction

Recent investigations have pointed to the importance of endophytes (microscopic organisms living asymptomatically within plant tissues) for influencing the metabolite profile of plants. With this study, we investigated endophytes of the botanical yerba mansa [*Anemopsis californica* (Nutt.) Hook. & Arn. (Saururaceae)]. Yerba mansa has been traditionally used by people of Mexico and the southwestern United States as a topical treatment for infection. Thus, we hypothesized that this plant would harbor endophytes with antimicrobial activity. Endophytes were cultured and extracted. Many of these extracts exhibited antimicrobial effects against *Staphylococcus aureus* and, to a lesser extent, *Pseudomonas aeruginosa*. A number of known compounds, including several with antimicrobial activity, were identified from the various fungal extracts. Additionally, the new spirolactone, chaetocuprum A (**3**) was

isolated as a secondary metabolite of the fungus *Chaetomium cupreum*. Spirolactones are relatively uncommon in the natural products literature, and this is the first report of isolation of such a compound from the genus, *Chaetomium*. Our investigations indicate endophytic fungi from yerba roots to be a potentially rich source of antimicrobial compounds. However, the relevance of this finding to ethnobotanical use of *A. californica* remains unproven. Our study showed very different endophyte profiles for *A. californica* plants grown under different conditions (field versus greenhouse). Since the endophytes present in this plant may be more reflective of the growing environment than the species itself, additional studies from different geographical locations may be necessary to predict the presence of obligate endophytes and their affect on the antimicrobial properties of yerba mansa. Thus our studies still point to the importance of considering growing conditions when pursuing natural product drug discovery from endophytic fungi.

Historically, it has been assumed that the biologically active agents of plant-based medicines are plant secondary metabolites. However, plants are teeming with microbial symbionts, including endophytes; microbes that live asymptomatically 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 a great deal of recent interest. It has been determined that some compounds originally believed to be of botanical origin are actually produced by fungal endophytes.

For example, endophytic fungi play a role in the production of hallucinogenic ergot alkaloids in morning glories (genera *Ipomoea*)[38] and ergot and insecticidal loline alkaloids in tall fescue grass (genera *Festuca* and *Lolium*).[39] 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.[40] Additionally, the presence of particular microbes may alter the growth and/or secondary constituent profile of the host plant.[41] In light of this, it is becoming increasingly apparent that endophytes should be considered when investigating the biological activity of a particular botanical.

In order to understand more about the importance of endophytes and its contribution to the biological activity of a plant-based medicine, more needs to be known about endophytes.[42] 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 very different endophyte profiles.[43] Furthermore, if the endophytes produce biologically relevant levels of medicinally active compounds, it is possible that the medicinal activity of the same plant species could be different depending on environment. Thus, environmental endophyte profile could be either a beneficial or confounding factor in experiments screening for biological activity of botanical extracts.

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 to treat inflammation and infection in wounds, and to control pain.[17-19, 22] This plant is still used today for the treatment of infections, and although it has been sparsely studied, there are several reports of antimicrobial[16, 44] or cytotoxic[15, 21] activity associated with *A. californica* extracts or constituents.

Thus far, there have been no investigations of the endophyte profile of *A. californica* plants, or the possible contribution of endophytes to its biological activity. *Anemopsis californica* is traditionally used topically as a poultice or decoction applied to the afflicted area. This practice almost certainly results in the application of compounds of both botanical and microbial origin. With this research, we hypothesized that *A. californica*, a plant used to treat infection, would harbor endophytes with antimicrobial activity. The goals of this project were 1. to identify antimicrobial compounds from *A. californica* endophytes; 2. to identify compounds of fungal origin in *A. californica* extracts; and 3. to evaluate the possible overlap in chemistry between extracts of *A. californica* roots and their associated endophytes. We also compared endophyte profiles in *A. californica* plants that were grown under different conditions.

Results and Discussion

Influence of Environment on Fungal Diversity

The first question we sought to answer in this project was whether changes in growing conditions would alter the endophyte profile of *A. californica* plants. Given the commonness of horizontal transmission, we expected that this would be the case. To test this experimentally, we isolated endophytes from two different batches of *A. californica* roots (Figure 6) to obtain the data in Table 4. One batch of roots was wild-harvested directly from a field plot and the other batch of roots came from the same field plot sample but was allowed to grow in a greenhouse for one year prior to being harvested (Figure 6). The difference in diversity of the fungal collections from the field and greenhouse samples is striking. The field samples yielded a diverse array of at least ten distinct fungal endophytes (Table 4). 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*).[45] 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, with an apparent shift towards pathogenic fungal species in the soil. This finding is relevant to natural products

drug discovery because it shows that the diversity of fungal endophytes obtained from botanical samples can vary greatly depending on environment/method of cultivation.

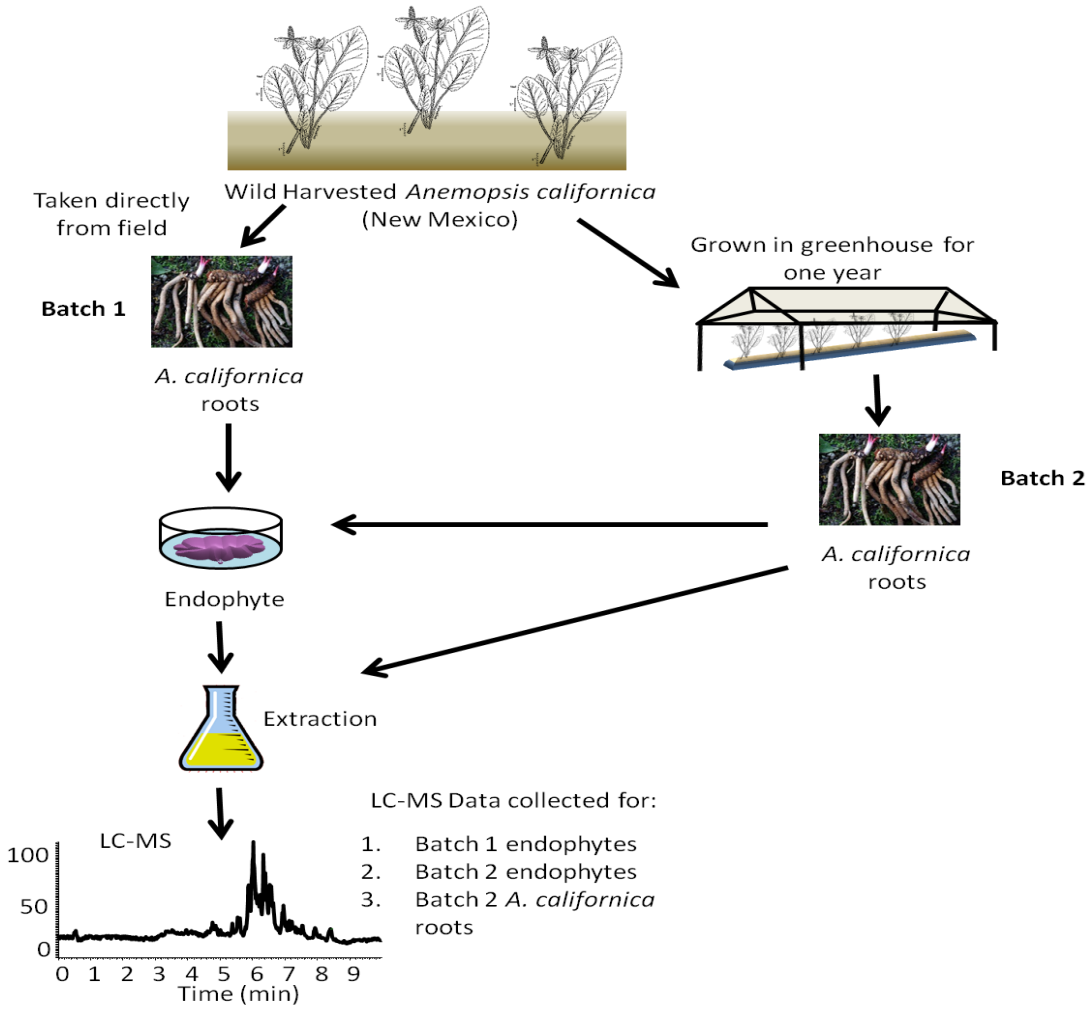


Figure 6. Overview of the experimental design for collection of plants for endophyte collection. Plant picture provided by USDA, The PLANTS Database, Greensboro, NC.

Table 4. Antimicrobial activity and identity of fungal endophytes isolated from *Anemopsis californica* roots. Growth inhibition is expressed as the mean decrease in absorbance at 600nm for triplicate cultures (± standard deviation, SD), as described in Methods.

Batch 1 (field samples)

Fungal species	Fungal ID number	*S. aureus* growth inhibition at 20 µg/mL (%)	*S. aureus* growth inhibition at 200 µg/mL (%)	*P. aeruginosa* growth inhibition at 200 µg/mL (%)
Hypocreales sp.	G115	16 ± 1.0	0 ± 1.0	12 ± 1.0
Chaetomium cupreum	G118	26 ± 1.5	35 ± 2.0	79 ± 3.0
Cylindrocarpon sp.	G136	13 ± 1.0	100 ± 1.0	13 ± 1.0
Aspergillus sp.	G124	37 ± 1.7	28 ± 1.9	15 ± 1.3
Penicillium sp.	G114	21 ± 1.7	0 ± 1.0	29 ± 2.7
Cylindrocarpon sp.	G116	42 ± 1.0	37 ± 1.7	15 ± 2.4
Cylindrocarpon sp.	G121	37 ± 1.0	24 ± 1.0	32 ± 1.0
Cylindrocarpon sp.	G120	58 ± 2.5	63 ± 4.0	37 ± 2.0
Phomopsis columnaris	G117	49 ± 1.0	13 ± 1.0	17 ± 1.0
Fusarium sp.	G134	9 ± 1.0	28 ± 1.0	9 ± 1.0
Colletotrichum coccodes	G112	31 ± 1.0	48 ± 3.4	9 ± 1.0
Colletotrichum coccodes	G113	36 ± 3.0	0 ± 1.0	13 ± 1.0
Fusarium sp.	G137	40 ± 3.0	15 ± 1.0	21 ± 1.0
Penicillium sp.	G135	42 ± 2.0	42 ± 2.7	0 ± 1.0
Penicillium sp.	G144	0 ± 1.0	64 ± 2.3	38 ± 6.3
Hypocreales sp.	G151	0 ± 1.0	0 ± 1.0	14 ± 1.0

Batch 2 (greenhouse samples)

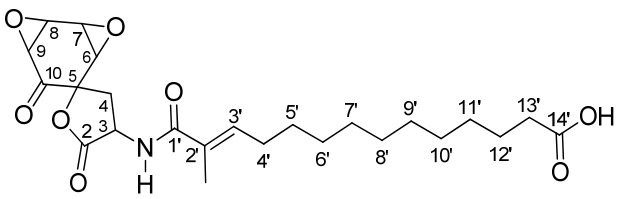
Fungal species	Fungal ID number	*S. aureus* growth inhibition at 20 µg/mL (%)	*S. aureus* growth inhibition at 200 µg/mL (%)	*P. aeruginosa* growth inhibition at 200 µg/mL (%)
Phomopsis columnaris	G212	16 ± 1.0	100 ± 5.0	10 ± 2.0
Phomopsis columnaris	G215	0 ± 1.0	34 ± 2.3	9 ± 1.0
Phomopsis columnaris	G220	19 ± 2.0	53 ± 2.0	18 ± 1.0
Ilyonectria robusta	G213	0 ± 1.0	0 ± 1.0	0 ± 1.0
Phomopsis columnaris	G221	28 ± .0	49 ± 2.0	7 ± 1.0
Phomopsis columnaris	G217	0 ± 1.0	0 ± 1.0	10 ± 1.0
Phomopsis columnaris	G223	17 ± 3.0	93 ± 3.7	34 ± 4.0
Phomopsis columnaris	G216	0 ± 1.0	43 ± 2.0	6 ± 1.0
Phomopsis columnaris	G222	0 ± 1.0	57 ± 1.7	12 ± 1.0
Phomopsis columnaris	G218	0 ± 1.0	17 ± 1.5	31 ± 4.0
Phomopsis columnaris	G214	0 ± 1.0	0 ± 1.0	5 ± 1.0

Antimicrobial Activity of *A. californica* Endophytes and their Constituents

To test the prediction that *A. californica* would harbor antimicrobial fungi, all of the fungal isolates obtained from both batches of *A. californica* roots were extracted and their ability to inhibit the growth of several bacterial species was measured (Table 4). Several of the fungal extracts inhibited growth of *Staphylococcus aureus* ((NCTC8325-4) by at least 50% at a concentration of 200 µg/mL. These were: *Cylindrocarpon* sp. (G136, G120), *Penicillium* sp. (G144), and *Phomopsis columnaris* (G212, G220, G222, and G223). In addition, several fungal isolates demonstrated weak activity (growth inhibition ≥ 30%) against *Pseudomonas aeruginosa* (NCTC 12903) at a concentration of 200 µg/mL. These were: *Chaetomium cupreum* (G118), *Cylindrocarpon* sp. (G121, G120), *Penicillium* sp. (G144), and *Phomopsis columnaris* (G218 and G223). It is well known that the Gram-negative bacteria *P. aeruginosa* is less susceptible to antimicrobial agents than Gram-positive bacteria, so the observation that activity against *P. aeruginosa* was less pronounced is not surprising.[46]

The data in Table 1 show that *A. californica* harbors fungi that produce antimicrobial compounds. We sought to identify some of these. Our isolation efforts focused specifically on the endophytes *Chaetomium cupreum* (G118) and *Cylindrocarpon* sp. (G120) because extracts of these fungi demonstrated the most antimicrobial activity against *P. aeruginosa*. One new compound, a spirolactone that we have named chaetocuprum A (**3**), was isolated from *Chaetomium cupreum* and its structure solved (see the next section for details on

structure elucidation). This compound demonstrates some structural similarities to the fungal secondary metabolite Compound C (US patent # 5,064,856) (**4**), which has previously been isolated from the fungus *Pseudoarachniotus roseus*.[47] Secondary metabolite production by the genus, *Chaetomium* has been investigated previously[48] but this is the first time a spirolactone has been isolated from this genus. In the literature, there are a few examples of natural products containing spirolactone ring systems such as spiromamakone A, aranorosinols, and melettinins.[49, 50]



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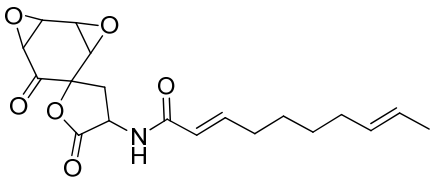

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Figure 7. Structures of spirolactones chaetocuprum A (**3**) isolated from *Chaetomium cupreum* and compound C (US Patent # 5,064,856) (**4**) isolated by Merck & Co., Inc.

The known compounds including cochliodone A (**5**), equisetin (**6**), 5'-epiequisetin (**7**), palmitic acid (**8**) and ergosterol peroxide (**9**) were all isolated as part of our studies. Compounds **5** and **9** were isolated from *Chaetomium cupreum*, and compounds **6**-**9** were isolated from *Cylindrocarpon* sp (G120). Cochliodone A (**5**) has been previously isolated from the *Chaetomium cochliodes*, but this is the first time it has been isolated from *Chaetomium cupreum*.[51] This is also the first report that equisetin (**6**) and its epimer 5'-epiequisetin (**7**) are produced by the *Cylindrocarpon* genus. Palmitic acid (**8**)[52, 53] and ergosterol peroxide (9)[54] are both highly ubiquitous compounds in nature.[53-56]

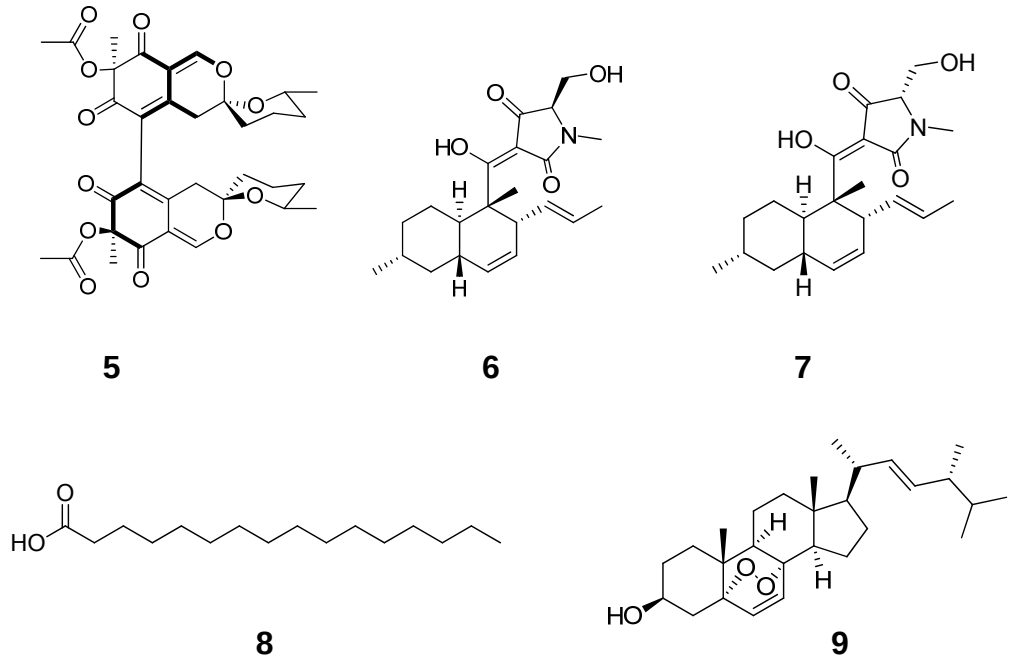


Figure 8. Compounds isolated from *A. californica* fungal endophytes cochliodone A (**5**), equisetin (**6**), 5'-epiequisetin (**7**), palmitic acid (**8**), ergosterol peroxide (**9**).

As a complementary approach to the isolation and identification of the chemical constituents in 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 on fungal compounds as described previously.[57] Using this approach, fourteen additional known compounds (**10-23**) were tentatively identified (figures 9 and 10), and several of the compounds isolated from *Cylindrocarpon* sp. were detected in other fungi. (See Table 5 for the names of the compounds and associated fungi from which they were identified.) The literature shows that some of these compounds (**10** and **11**) possess antimicrobial activity. Verticillin A (**10**), identified from *Hypocreales sp.*, has previously been shown to inhibit the growth of Gram-positive bacteria such as *Bacillus subtilis*, *Bacillus anthracis*, *S. aureus*, and *Mycobacterium tuberculosis.*[58] The acremoxanthone acremonidin C (**11**), an anthraquinone–xanthone heterodimer, had strong activity against *S. aureus* and *B. cereus* and weak activity against *C. albicans* and *Plasmodium falciparum*.[59] Thus, the detection of these compounds in the fungal isolates lends support to the hypothesis that antimicrobial compounds are produced by *A. californica* endophytes.

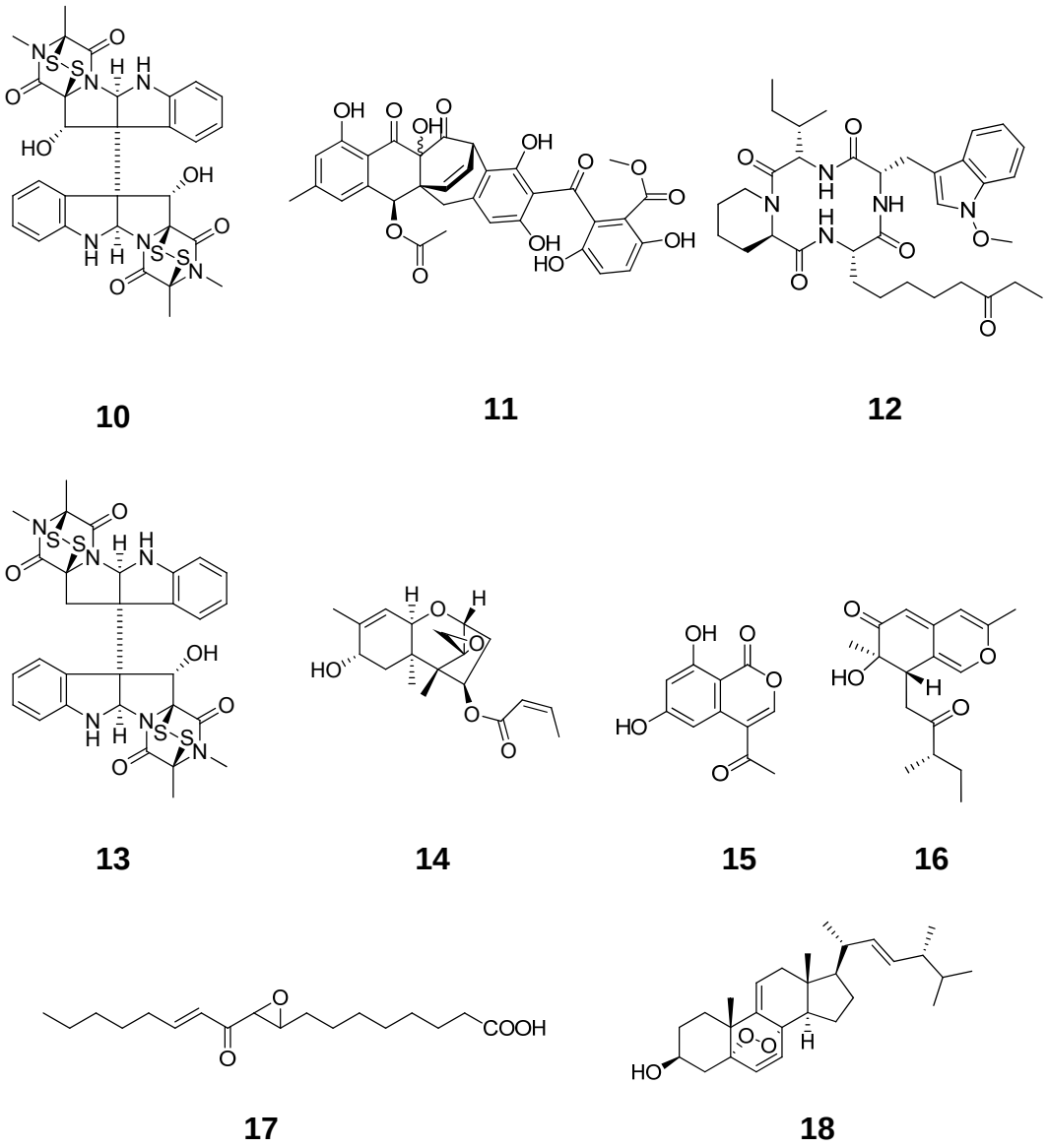


Figure 9. Compounds from *A. californica* fungal endophytes tentatively identified by matching LC/MSMS fragmentation patterns with a database of high resolution mass spectrometry data on pure fungal compounds. verticillin A (**10**), acremonidin C (**11**), apicidin (**12**), 11'-deoxyverticillin (**13**), trichothecinol B (**14**), AGI-7 (**15**), chermesinone A (**16**), (E)-8-(3-(oct-2-enoyl)oxiran-2-yl)octanoic acid (**17**), 5,8-Epidioxyergosta-6,9(11),22-trien-3-ol (**18**).

Table 5. List of endophytic fungal species isolated from *Anemopsis californica* roots and chemical compounds identified from each fungal species. Batch 1 consists plants harvested from a field plot, batch 2 are the same population of plants transferred to a greenhouse and cultivated there for one year prior to harvest.

Batch 1 (field samples)		
OTU identification	**Fungal ID**	**Compounds identified**
Colletotrichum coccodes	G112, G113	
Penicillium sp.	G114	
Hypocreales sp.	G115	verticillin A (**10**), 11-deoxyverticillin A (**13**)
Cylindrocarpon sp.	G116, G119, G120, G121, G136	equisetin (**6**), 5'-*epi*equisetin (**7**), palmitic acid (**8**)
Chaetomium cupreum	G118	chaetocuprum A (**3**), cochliodone A (**5**), ergosterol peroxide (**9**)
Aspergillus sp.	G124	
Fusarium sp.	G134, G137	apicidin (**12**)
Penicillium sp.	G144, G145	
Herpotrichiellaceae sp.	G146	
Sordariales sp.	G149	
Penicillium sp.	G148, G150	
Hypocreales sp.	G151	
Nemania serpens	G153, G154	
Phomopsis columnaris	G117	
Batch 2 (greenhouse samples)		
OTU identification	**Fungal ID**	**Compounds identified**
Phomopsis columnaris	G117, G212, G214, G215, G216, G217, G218, G220, G221, G222, G223	ergosterol peroxide (**7**), acremonidin C (**11**), trichothecinol B with G217 (**14**), AGI-7 (**15**), (E)-8-(3-(oct-2-enoyl)oxiran-2-yl)octanoic acid (**17**), 5,8-epidioxyergosta-6,9(11),22-trien-3-ol (**18**)
Ilyonectria robusta	G213	ergosterol peroxide (**9**), chermesinone A **(16**)

Antimicrobial activity was measured for the compounds (**3** and **6-9**) that were isolated in sufficient quantity. MIC and IC_{50} values for these compounds against *Staphylococcus aureus* and *Pseudomonas aeruginosa* are shown in Table 6. None of the compounds were active against *P. aeruginosa* (MIC >200 µg/mL). Chaetocuprum A (**3**) inhibited growth of *S. aureus* (IC_{50} of 50 *µ*g/mL), but complete growth inhibition was not achieved against this organism (MIC > 50 µg/mL, which was the highest concentration tested). The activity measured for equisetin (**6**) agreed with literature values, 1 µg/mL against *S. aureus* and no activity against *P. aeruginosa*.[60] 5'-*epi*equisetin (**7**) has not been previously evaluated for antimicrobial effects, and demonstrated an MIC of 1 µg/mL against *S. aureus*. Palmitic acid (**8**) was found to be inactive probably due to its insolubility. The weak activity of ergosterol peroxide (**9**) against *S. aureus* (MIC of 200 µg/mL) is in agreement with previous literature.[61] The positive control for the antimicrobial assays was ciprofloxacin, which demonstrated an MIC of 0.25 µg/mL against *S. aureus* and 0.125 µg/mL against *P. aeruginosa*, consistent with previous reports.[62]

Table 6. Minimum inhibitory concentration (MIC) and IC_{50} of select fungal metabolites against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Compound	***Staphylococcus aureus***		***Pseudomonas aeruginosa***	
	MIC (µg/mL)	IC_{50} (µg/mL)	MIC (µg/mL)	IC_{50} (µg/mL)
Chaetocuprum A (**3**)	>50	50	>50	>50
Equisetin (**6**)	1.0	0.5	>200	>200
5'-Epiequisetin (**7**)	1.0	0.5	>200	>200
Palmitic Acid (**8**)	>200	>200	>200	>200
Ergosterol peroxide (**9**)	200	100	>200	>200
Ciprofloxacin (+ control)	0.25	0.125	0.125	0.0625

Structure Elucidation of Chaetocuprum A

The molecular formula of chaetocuprum A (**3**) was determined to be $C_{24}H_{33}NO_8$ (9 unsaturations) on the basis of NMR and HRESIMS data. Inspection of the ^1H NMR data (Tables 7 and 8 and figures 10-13) 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. In addition to the signals consistent with above features, ^{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 (Tables 4 and 5) for H-6 (δ_H 3.65; J = 4.0), H-7 (δ_H 3.89; J = 4.0, 2.1), H-8 (δ_H 4.03; J = 4.0, 2.1), and H-9 (δ_H 3.58; J = 4.0)

were diagnostic of a pair of the epoxide groups. The adjacent location of two epoxide units was established by analysis of the ^{1}H-^{1}H COSY NMR data. HMBC correlations from H-6, H-8, and H-9 to C-10 (δ_C 196.7) supported the placement of epoxide units at a position alpha to the ketone carbon. HMBC correlations from H-6 to an oxygenated quaternary carbon (C-5; δ_C 84.7) and C-10 suggested the formation of a six-membered ring system. Key HMBC correlations from methylene protons H_2-4 (δ_H 2.94 and δ_H 2.49) to C-5, C-6 (δ_C 56.3), and C-10 were consistent with the linkage to this group to C-5. Additional correlations from H_2-4 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, resulting in the formation of a spirocycle.

Fortunately, 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 data were collected in $CDCl_3$ and established the linkage of amide group to C-3. Key correlations from methyl group protons H_3-15' (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-unstaurated olefin. A single spin system from H-3' to H-13' was identified primarily by analysis of the ^{1}H-^{1}H COSY NMR data. The remaining NMR data was 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 **3** as shown. Conclusive assignment of the overall relative configuration of chaetocuprin A (**3**) could be

made solely on the basis of NOESY data. Unfortunately, the crystallization attempts were also unsuccessful.

Table 7. NMR spectroscopic data (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR, $CDCl_3$) for chaetocuprum A (**3**).

no.	δ_H (mult., J in Hz)	δ_C (mult.)	HMBC ($H^{\#} \rightarrow C^{\#}$)
2		173.6 s	
3	4.25 (m)	48.9 d	2, 4, 5, 1'
4	2.94 (dd, 10.2, 14.0) 2.49 (dd, 10.2, 14.0)	34.5 t	2, 3, 5, 6, 10 2, 3, 5, 6, 10
5		83.5 s	
6	3.65 (d, 4.0)	56.3 d	4, 5, 7, 8, 10
7	3.89 (dd, 2.1, 4.0)	52.6 d	5, 6, 8, 9
8	4.03 (dd, 2.1, 4.0)	57.9 d	6, 7, 9, 10
9	3.58 (d, 4.0)	55.5 d	5, 7, 8, 10
10		196.7 s	
1'		169.7 s	
2'		129.4 s	
3'	6.43 (t, 7.0)	139.3 d	1', 2', 4', 5', 15'
4'	2.13 (m)	28.7 t	2', 3', 5', 6'
5'	1.40 (t, 7.0)	29.0 t	3', 4', 7'
6'	1.30 (m)	29.9 t	
7'	1.30 (m)	29.35 t	
8'	1.30 (m)	29.32 t	
9'	1.30 (m)	28.6 t	
10'	1.30 (m)	29.39 t	
11'	1.30 (m)	29.1 t	
12'	1.60 (m)	24.8 t	10', 14'
13'	2.32 (t, 7.0)	33.7 t	11', 14'
14'		177.0 s	
N-H	6.34 (d, 4.0)		2, 3, 4, 1', 2'
15’	1.82 (s)	12.7 q	1', 2', 3'

Table 8. NMR spectroscopic data (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, CD_3OD) for chaetocuprum A (**3**).

no.	δ_H (mult., J in Hz)	δ_C (mult.)	HMBC ($H^{\#} \rightarrow C^{\#}$)
2		175.3 s	
3	4.28 (m)	50.0 d	2, 4, 5, 1'
4	2.76 (dd, 10.0, 13.8) 2.53 (dd, 10.0, 14.8)	34.1 t	2, 3, 5, 6, 10 2, 3, 5, 6, 10
5		84.7 s	
6	3.64 (d, 4.0)	57.4 d	4, 5, 7, 8, 10
7	3.98 (dd, 2.0, 4.0)	53.9 d	5, 6, 8, 9
8	4.12 (dd, 2.0, 4.0)	59.1 d	6, 7, 9, 10
9	3.59 (d, 4.0)	56.6 d	5, 7, 8, 10
10		199.1 s	
1'		171.8 s	
2'		131.0 s	
3'	6.40 (m)	139.3 d	1', 2', 4', 5', 15'
4'	2.17 (m)	29.4 t	2', 3', 5', 6'
5'	1.43 (t, 5.2)	29.9 t	3', 4', 7'
6'	1.29 m	30.5 t	
7'	1.29 m	29.9 t	
8'	1.29 m	30.5 t	
9'	1.29 m	30.3 t	
10'	1.29 m	29.9 t	
11'	1.29 m	30.7 t	
12'	1.57 (t, 6.8)	26.2 t	10', 14'
13'	2.25 (t, 7.2)	34.1 t	11', 14'
14'		177.9 s	
N-H			
12.6 q	1.81 (s)		1', 2', 3'

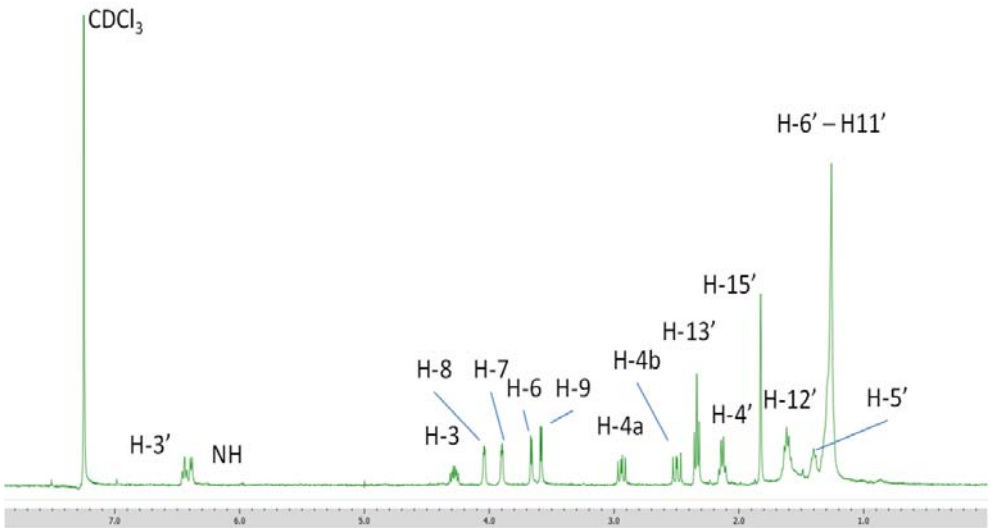


Figure 10. ^{1}H NMR spectrum of chaetocuprum A (**3**) (400 MHz, $CDCl_3$).

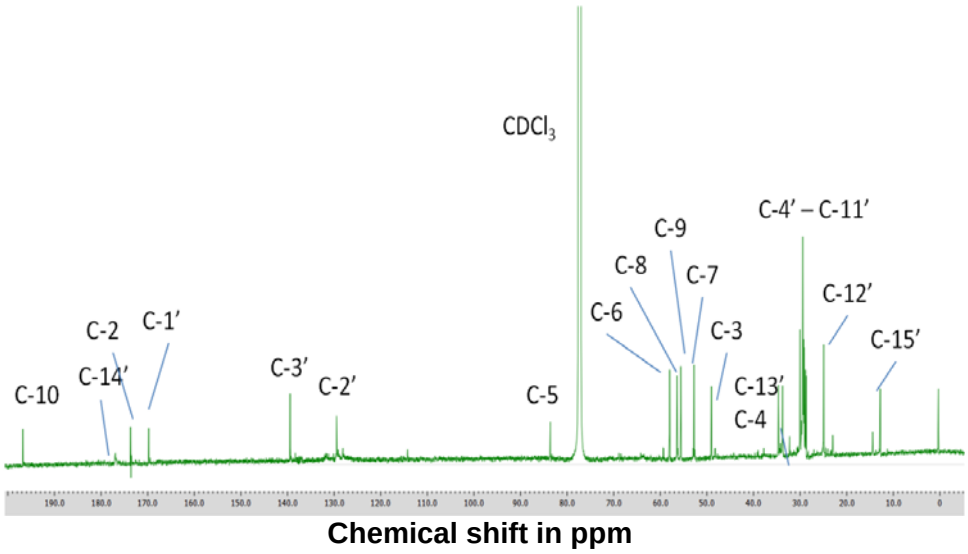


Figure 11. ^{13}C NMR spectrum of chaetocuprum A (**3**) (100 MHz, $CDCl_3$).

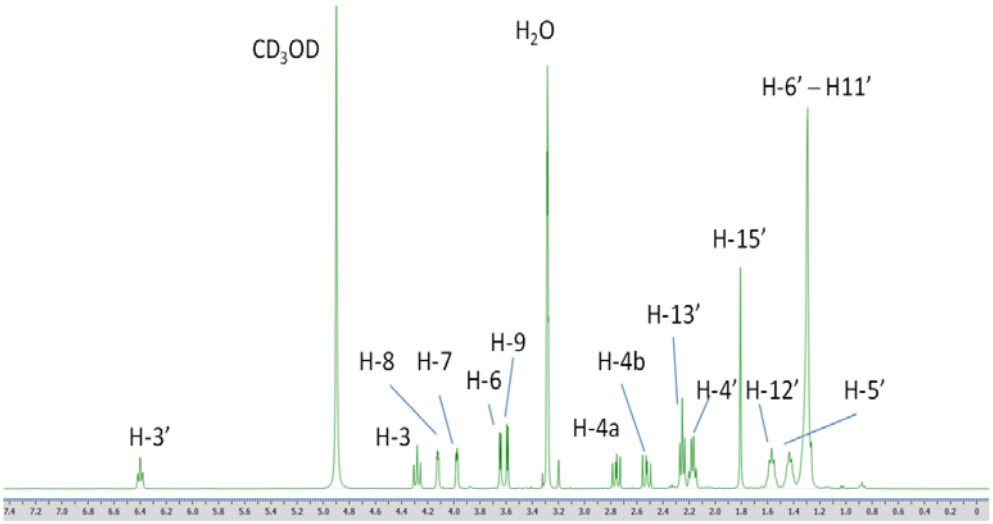


Figure 12. ^{1}H NMR spectrum of chaetocuprum A (**3**) (400 MHz, CD_3OD).

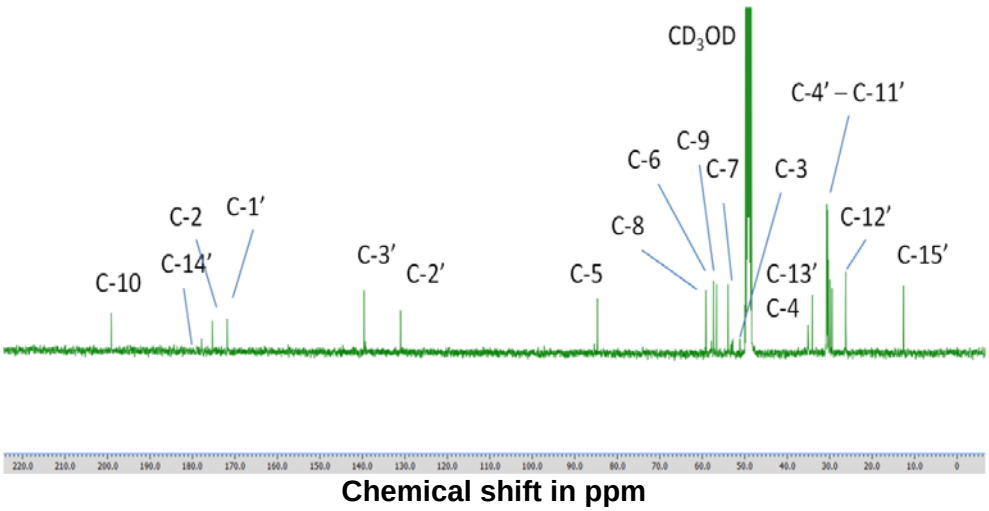


Figure 13. ^{13}C NMR spectrum of chaetocuprum A (**3**) (100 MHz, CD_3OD).

Fungal Compounds in an *A. californica* Extract?

It was of interest to determine whether any of the endophyte compounds (**3, 5-18**) in figures 7-9 could be detected in *A. californica* roots. Towards this goal, a botanical extract was prepared from the same batch of *A. californica* roots from which the second batch of endophytes was isolated (Figure 6 and Table 4). This extract was analyzed using the LC-MS-MS procedure applied to the endophyte extracts, and the resulting chromatographic and mass spectrometric data were compared to that obtained from the fungal extracts. With the exception of ergosterol peroxide (**9**), which is produced by many natural organisms including plants, fungi and some insects,[54, 56, 63] none of the compounds (**3, 5-18**) shown in figures 7-9 were detected in the plant extract. This result is not particularly surprising, given that the fungal extracts were obtained from pure fungal cultures grown on rice, conditions that differ greatly from the natural environment of the plant root. It is likely that culturing the fungi alters the abundance or identity of secondary metabolites produced. Furthermore, many of the compounds reported herein as fungal metabolites were isolated from batch 1 fungi, while the extract was prepared from the batch 2 roots (for which the only fungal species isolated were *Phomopsis columnaris* and *Ilyonectria robusta)*.

As an alternative strategy towards identifying fungal compounds in the *A. californica* botanical extract, the LC-MS profiles obtained by analyzing this extract were searched against the aforementioned database of fungal compounds.[57] As a result, five additional compounds (**19**-**23**) were identified in the botanical extract

that were not detectable in the fungal extract or control (rice extract). These compounds were present at very low concentration, which prevented their isolation, but tentative identification was possible by matching LC-MS-MS data with that in the fungal library.[57] The experimental design for this study involved grinding up entire *A. californica* roots (without surface sterilization) to prepare the botanical extract. Thus, the compounds shown below could be produced by endophytic fungi, epiphytic fungi associated with the root surfaces, or by the plant itself. However, literature precedent indicates these compounds to be of fungal origin.[50, 64-67]

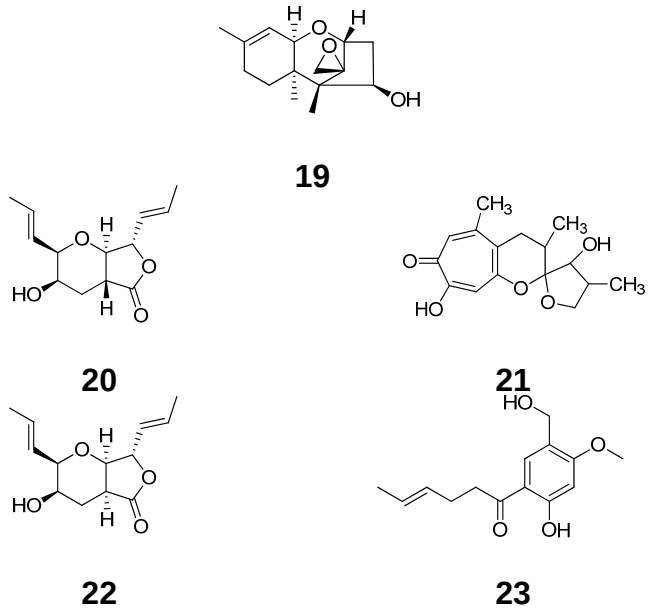


Figure 14. Compounds tentatively identified from an *A. californica* extract by matching LC/MSMS fragmentation patterns with a database of high resolution mass spectrometry data on pure fungal compounds. Trichodermol (**19**), trans-dihydrowaol A (**20**), epi-Malettinin C (**21**), cis-dihydrowaol A (**22**), 1-(2'-hydroxy-4'-methoxy-5'-hydroxymethylphenyl)-E-4-hexen-1-one (**23**).

Compounds **19-23** in figure 14 have been reported to have an array of interesting biological activities. Trichodermol (**19**) is classified as a trichothecene and is considered a mycotoxin and an antibacterial compound.[64] Compounds **20** and **22**, *trans* and *cis*-dihydrowaol, are polypeptide-derived γ-lactones with furo[3,4-b]pyran-5-one bicyclic ring systems.[65] This class of compounds does contain similar compounds that are fungicidal and antibacterial.[66] Malettinin C-NNP (**21**) is a polyketide-derived tropolone, and its epimer malettinin C inhibits growth of the fungus *C. albicans*, and bacteria *S. aureus*, and *B. subtilis*.[50] 1-(2'-hydroxy-4'-methoxy-5'-hydroxymethylphenyl)-E-4-hexen-1-one (**23**) is in the

sorbicillin family of compounds, which have been shown to have antifungal activity against *Fusarium verticillioides* and *Aspergillus flavus*.[67] Collectively, these literature reports suggest potentially interesting biological effects of the fungal compounds in the *A. californica* root extract.

Chemical Overlap Between Fungal Extracts and an *A. californica* Extract

The previous section describes targeted analysis to identify fungal compounds in the *A. californica* root extract. In addition, we asked the broader question of whether there was any chemical overlap between the *A. californica* root extract and the extracts of fungal endophytes cultured from those same roots. Towards this goal, the relevant features from the LC-MS chromatograms from the fungal extracts and the botanical extract were identified using IntelliExtract, and these datasets were compared to identify compounds shared between the botanical and fungal extracts but not present in the control. A series of eighteen compounds (Table 9) were detected with the same accurate mass, retention time, and MS-MS fragmentation spectrum in both the *A. californica* root extract and at least one fungal extract. Figure 15 is an example chromatogram comparing fungal, rice, and botanical extracts. The compounds listed in Table 9 could be of either fungal or botanical origin, or both. It is possible that both the fungi and the botanical *(A. californica*), produce some of the same compounds, either as a result of gene transfer or because of an independent, genetic development of biological pathways. Another likely explanation for the chemical overlap between fungal and botanical extracts is that endophytic fungi living in

the *A. californica* roots are extracted along with the plant roots. Importantly, the approach used for fungal culture prevents cross-contamination of fungal extracts with botanical compounds.[68] Thus, it is highly unlikely that overlap in chemistry between fungal and botanical extracts is an artifact of laboratory procedures. Fungi are isolated in a stepwise process and plant tissues are absent from the pure fungal cultures. As support of the effectiveness of this approach for preventing contamination of fungal extracts with botanical tissues, the compounds sesamin (**1**) and asarinin (**2**), which have previously been shown to be highly abundant in *A. californica* root extracts,[44] were not detected in any of the 15 fungal extracts based on the absence of the m/z and fragmentation patterns in the endophytic fungi.

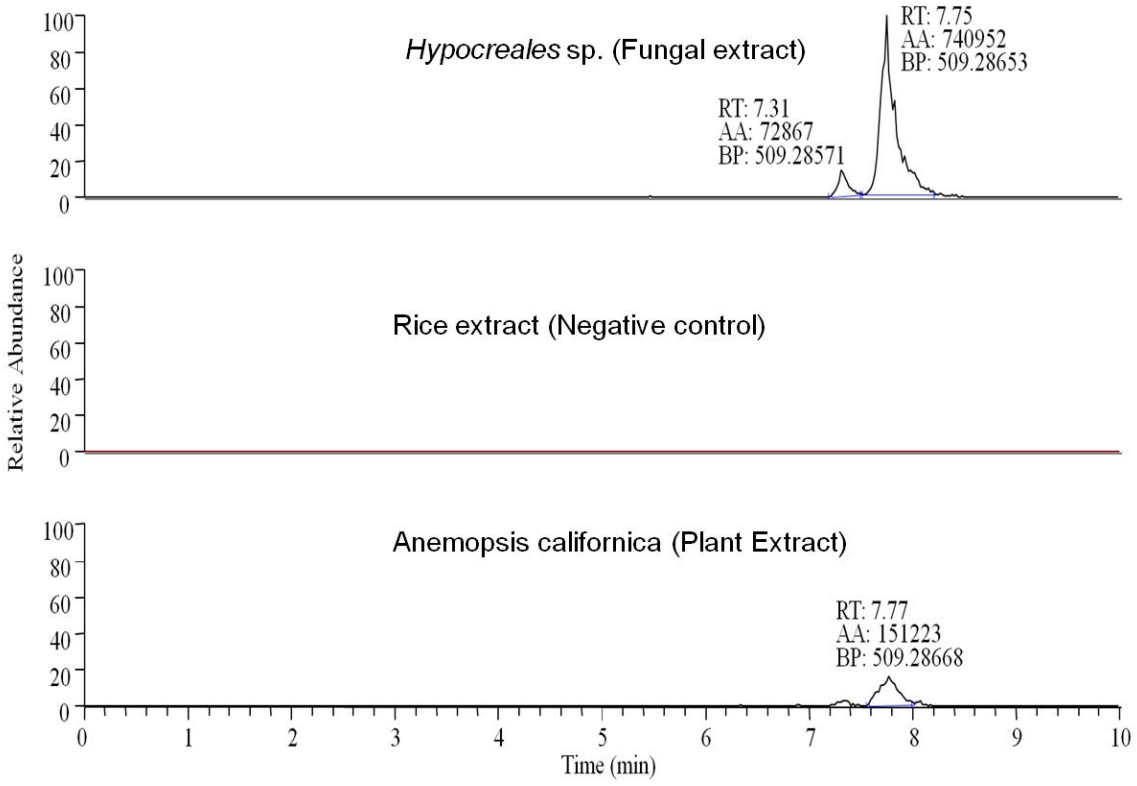
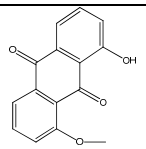
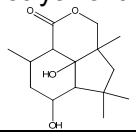
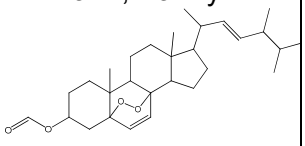


Figure 15. Base peak chromatogram of *m/z* 509.28653 $C_{27}H_{44}N_3O_6^-$ collected in the negative ion mode and normalized to a signal of 7.75×10^4. RT represents the retention time (in min) AA represents the area of the peak, and BP is the measured mass of the ion that comprises the peak. A clear signal is observed for a pair of isomeric compounds at *m/z* 509.2853 in both the *Hypocreales* sp. endophyte extract and the *Anemompsis californica* root extract. This signal is absent from the negative control, which is an extract of the medium on which the fungus was grown (rice). These data indicate chemical overlap between the fungus and the botanical extract.

Table 9. Accurate masses, proposed formulae, and possible structures corresponding to features common to the endophytic fungal extracts and the *A. californica* botanical extract.

Accurate mass	Proposed formula	Fungal sample	Possible structure
218.1664	$C_{15}H_{23}O$	*Phomopsis columnaris*	 1,8-Dihydroxyanthraquinone; Mono-Me ether
254.0566	$C_{15}H_{10}O_4$	*Phomopsis columnaris*	
268.1667	$C_{15}H_{24}O_4$	*Phomopsis columnaris*	
270.0524	$C_{15}H_{10}O_5$	*Phomopsis columnaris*	
352.0949	$C_{20}H_{16}O_6$	*Phomopsis columnaris*	4,9-Dihydroxy-10,15-botryanolide 
352.2616	$C_{21}H_{36}O_4$	*Phomopsis columnaris*	
416.2309	$C_{23}H_{32}N_2O_5$	*Phomopsis columnaris*	5,8-Epidioxyergosta-6,22-dien-3-ol; (3β,5α,8α,22*E*,24*R*)-form, Formyl 
448.2571	$C_{24}H_{36}N_2O_6$	*Phomopsis columnaris*	
456.3223	$C_{29}H_{44}O_4$	*Phomopsis columnaris*	
460.3181	$C_{28}H_{44}O_5$	*Phomopsis columnaris*	
479.2901	$C_{26}H_{41}NO_7$	*Phomopsis columnaris*	
485.3491	$C_{30}H_{47}NO_4$	*Phomopsis columnaris*	
495.3308	$C_{27}H_{45}N_3O_6$	*Phomopsis columnaris*	
505.3157	$C_{27}H_{43}N_3O_6$	*Phomopsis columnaris*	
506.3083	$C_{25}H_{46}O_{10}$	*Ilyonectria robusta*	
510.2945	$C_{38}H_{38}O$	*Phomopsis columnaris*	
596.2946	$C_{41}H_{40}O_4$	*Phomopsis columnaris*	
608.4265	$C_{35}H_{60}O_8$	*Phomopsis columnaris*	

The compounds listed in Table 9 as present in both the fungal and plant extracts were present at very low levels. Isolation followed by structure elucidation was not achieved for any of them. However, three of the molecular formulae in Table 9 matched the structures of compounds previously reported from a fungus of the genus *Phomopsis*.[69] These compounds were 1-Hydroxy-8-methoxyanthraquinone ether ($C_{15}H_{10}O_4$), 4β-acetoxy-9β-10β-15α-trihydrixyprobotrydial ($C_{15}H_{24}O_4$), and 22E, 24R-5α, 8α-epidioxyergosta-6, 22-dien-3β-formate ($C_{29}H_{44}O_4$). Although identities of these compounds certainly cannot be confirmed by matching molecular formula with literature, the data are highly suggestive that these compounds (or isomers thereof) are present in the *A. californica* root extract. The high frequency with which the fungal endophyte *Phomopsis columnaris* was isolated from the *A. californica* root extract supports this conclusion.

Relevance of the Studies for Ethnobotanical Use and Antimicrobial Drug Discovery

This study casts some interesting light on the potential relevance (or lack thereof) of endophytes to the biological activity of a botanical medicine. We have shown that *A. californica*, a plant traditionally used to treat infection, contains antimicrobial endophytes. We have also identified or isolated a number of antimicrobial compounds from *A. californica* endophytes, and have shown that there is potential overlap in the chemistry between a botanical extract and the fungi isolated from that extract (although the identities of the compounds that constitute that overlap remain unconfirmed). Finally, we have shown that an

extract prepared from *A. californica* roots contains a number of compounds previously identified in fungi (**19-23**), many of which have interesting reported biological activities.

While it is tempting on the basis of our findings to implicate a causal relationship between the ethnobotanical use of *A. californica* and the biological activity of its endophytes, the current data are only correlative. Furthermore, the plant extract alone did not possess antimicrobial activity against *S. aureus* or *P. aeruginosa* (data not shown), although it had previously been shown to inhibit the growth of various strains of mycobacteria.[44] Additionally, the compounds identified as overlapping between the botanical and the endophytes were present at very low levels, estimated on the basis of LC-MS data to be in the range of 10 to 100 ppm (expressed as mg of compound/kg of plant extract). Finally, with the exception of ergosterol peroxide (**9**), the antimicrobial compounds identified in the fungal extracts were not detected in the *A. californica* root extract. It is entirely possible, however, that plants grown in a different environment might contain higher levels of endophyte metabolites, or metabolites with more potent activity.

This study shows that a diverse array of metabolites is produced by endophytic fungi from *A. californica*, several of which are antimicrobial. This finding is consistent with ample literature that shows fungi are an excellent source of antimicrobial compounds.[70] Thus, even though the fungal endophyte profile of a given botanical is likely to vary a great deal depending on

environment, there is a high likelihood that any given sample will contain endophytes that produce antimicrobial compounds. It can, therefore, be concluded that the practice of employing botanical preparations topically to treat infection is likely to result in the application of antimicrobial fungal metabolites as well as botanical components. Whether or not these will be present at therapeutically relevant levels remains to be established.

Regardless of their relevance to the ethnobotanical use of *A. californica*, these studies do show botanical endophytes to be a good source of antimicrobial natural products. However, our findings also indicate that the richness of fungal diversity (and associated chemical diversity) obtained as a result of endophyte isolation can vary greatly depending on how and where the plant was cultivated. The diversity of antimicrobial compounds isolated or identified from the wild-cultivated *A. californica* plants, which included the new compound chaetocuprum A (**3**), was far wider than that identified in the same plants after one year of cultivation in a greenhouse (Table 5). Thus, natural products chemists seeking to isolate compounds from botanical endophytes should consider the potential impact of growing conditions of the botanical host on their drug discovery efforts. On a positive note, the phenomenon of different endophyte profile depending on environment could also be advantageous for drug discovery, as simply collecting plant material from different locations could harbor different chemistries.

Materials and Methods

General Experimental Procedures

The NMR spectra were recorded in both $CDCl_3$ and CD_3OD with references peaks (δ_H 7.24/ δ_C 77.2 for $CDCl_3$ and δ_H 3.31/ δ_C 49.2 for CD_3OD). NMR data were collected using JEOL ECA-500 and Joel ESC-400 NMR spectrometers. The HRESIMS data was collected on a Thermo LTQ Orbitrap XL mass spectrometer. A Teledyne CombiFlash ISCO with a RediSep Rf Gold® column (4 g silica 40 µm) was used for normal phase separations. A Varian HPLC Prostar (Thermo Fisher Scientific, Waltham, MA, USA) was used with a Gemini Phenomenex C18 column (5 μm, 120Å; 250 x 20 mm) (Phenomenex Inc, Torrance, CA, USA) and the Galaxie Chromatography Workstation Software (version 1.9.3.2) for reversed-phase preparative separations. Analytical separations were performed with a Gemini Phenomenex C18 column (5 μm, 120Å; 250 x 20 mm). For antimicrobial assays, the optical density at 600 nm was read using a POLARstar Optima microplate reader (BMG Labtech, Inc). UV was measured by using a HPLC-Diode Array Detector (Agilent, Santa Clara, CA, USA). Optical rotation was measured on a Rudolph Research Autopol III polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). ECD was measured on an Olis DSM 17 CD spectrophotometer. Müeller-Hinton broth, ciprofloxacin (purity >98% by HPLC), and palmitic acid (purity >99% by HPLC) were purchased from Sigma Aldrich. Other reagents were purchased from Fisher Scientific.

Plant Material

Anemopsis californica plants were collected by Amy Brown of Apache Creek Ranch in Sante Fe, NM (35°35' 56.40"N, 105°50' 27.22W). The plants were taken from their native environment in New Mexico. 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, and the remaining material was air dried at 37°C before extraction.

Endophyte Isolation and Identification

Isolation of fungal endophytes was performed using methods outlined previously.[68, 71] 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 formerly.[65, 68, 71, 72] The ITS sequences from all strains were deposited in the GenBank (accession no XXX).

Anemopsis californica Extraction

A 1.0 kg lot of dried *Anemopsis californica* root material (from batch #2) was ground using a Bel-Art Scienceware Micro-Mill Grinder (Fisher Scientific, Waltham, MA USA) and percolated in MeOH overnight. The MeOH extract was evaporated to dryness under vacuum. This procedure was repeated with the same root material until there was no color remaining in the methanol. The methanol extract was then dried under vacuum and subjected to liquid-liquid extraction, as described previously.[35] The resulting residue was exhaustively

partitioned with hexane and 10% aqueous MeOH (1:1) until no color remained in the hexane layer. The dried aqueous MeOH fraction was partitioned further between 4:1:5 $CHCl_3$:MeOH:H_2O. The residue obtained by rotary evaporation of the $CHCl_3$ layer was retained for testing and analysis.

Endophyte Culture and Extraction

A solid, grain-based media was used to grow small-scale cultures of fungi in 250 mL Erlenmeyer flasks as previously described.[73] Each fungal culture was chopped and shaken overnight (16 hr at 100 rpm) in a 1:1 MeOH-$CHCl_3$ solution, subjected to vacuum filtration, and washed with small volumes of MeOH. The filtrate was stirred in a 3:5 ratio of $CHCl_3$ H_2O for 2 hr. After separating the organic and aqueous layers, both layers were evaporated to dryness under vacuum. The organic layer was then resuspended in a 1:1:1 mixture of MeOH:CH_3CN:hexane. The MeOH:CH_3CN and hexane layers were separately dried under vacuum. The residue from the MeOH:CH_3CN layer was used for bioassays, dereplication, isolation, and chemical profile comparison between the endophytes and the *A. californica* extract. A separate extraction was also conducted using identical conditions with just the rice medium (no fungus) as a negative control.

Isolation

The isolation schemes are provided as in figures 16 and 17. The first stage of normal-phase flash chromatography (4 g silica gel column) was conducted at a 18 mL/min flow rate with a 35 min hexane:$CHCl_3$:MeOH gradient. The first stage separations of *Chaetomium cupreum* (G118) and *Cylindrocarpon* sp. (G120) were conducted under the same conditions. In the first stage of separation for *Chaetomium cupreum* (G118, Figure 16), ergosterol peroxide (**9**) eluted at 29 min in fraction 4 (7 mg, 97.2 % purity, 0.0028% yield).

Fraction 2 was subjected to a second stage of purification using a reversed phase preparative HPLC with a Gemini BEH C18 column at a 21 mL/min flow rate. A linear ACN:H_2O gradient starting from 30:70 to 90:10 over 20 min yielded cochliodone A (**5**) with a 17 min retention time (1 mg, 98 % purity, 0.0004% yield). Fraction 3 was also subjected to the same gradient, and produced sub-fraction 2. This fraction was then purified with an isocratic solvent composition of 50:50 ACN: H_2O on a preparative HPLC with a Gemini BEH C18 column at a 21 mL/min flow rate over 20 min. Compound **3**, chaetocuprum A, eluted at 16 min (13 mg, 98.5 % purity, 0.0052% yield). In the first stage of separation for *Cylindrocarpon* sp. (G120, Figure 17), palmitic acid (**8**) eluted at 32.5 min in fraction 4 (27 mg, 97.0 % purity, 0.011% yield). Fraction 3 was close to two pure compounds; therefore it went through a second stage of purification using a reversed phase preparative HPLC with a Gemini BEH C18 column at a

21mL/min in 20 min yielded equisetin (**6**) at 12 min (45 mg, 98 % purity, 0.018% yield) and 5'- epiquisetin (**7**) at 12.7 min (25 mg, 98 % purity, 0.010% yield).

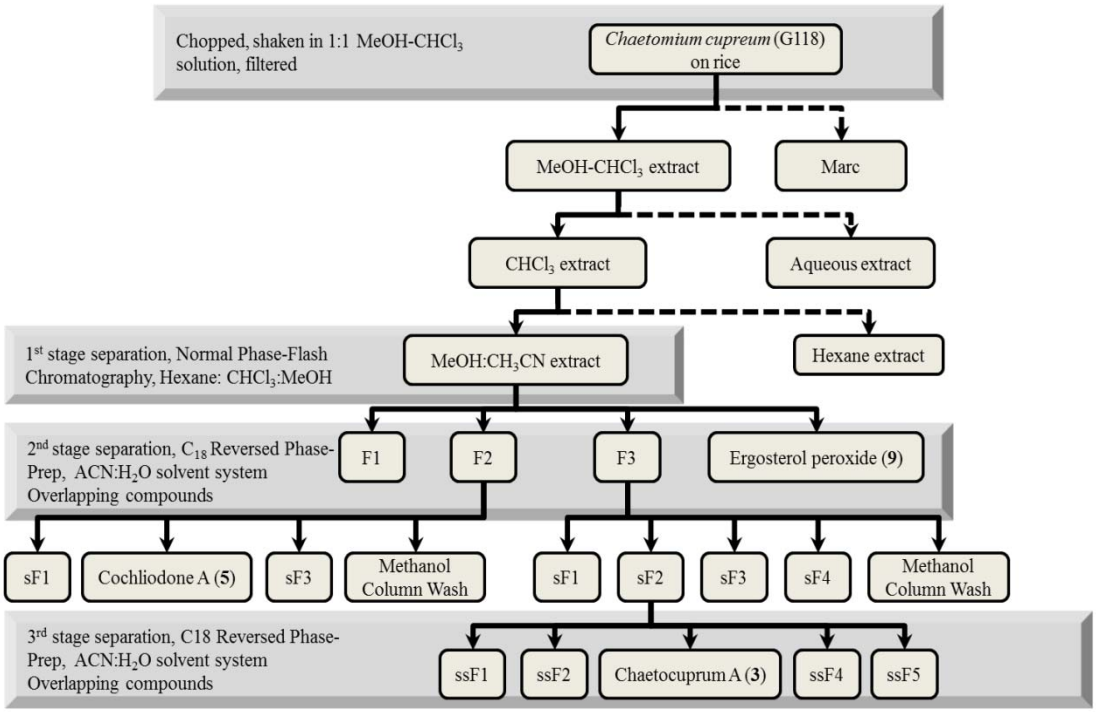


Figure 16. Isolation scheme of chaetocuprin A (**3**), cochliodone A (**5**), ergosterol peroxide (**9**) from *Chaetomium cupreum* (G118).

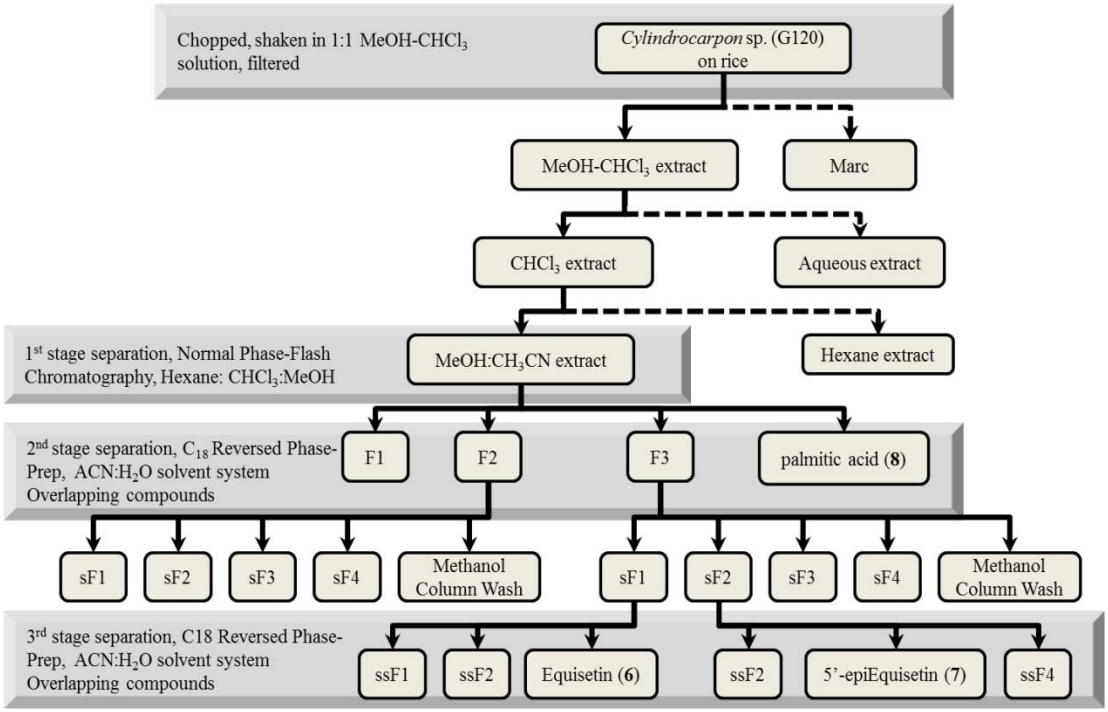


Figure 17. Isolation scheme of equisetin (**6**), 5'-epiequisetin (**7**), and palmitic acid (**8**) from *Cylindrocarpon* sp. (G120).

*Chaetocuprum A (**3**): green oil;* HRESIMS obsd. *m/z* 464.2268 $[M+H]^+$, calcd for $C_{24}H_{34}NO_8^+$, 464.2284, $[\alpha]_D^{24.5}$= - 28.6 (c 0.07 MeOH); ECD in methanol at 214 nm 29.3°, at 230 nm -8.5°, and at 297 nm -8.1°, UV (Methanol) λ_{max} (log ϵ) 226 (2.62) nm.

*Cochliodone A (**5**):* green solid; HRESIMS *m/z* 639.2427 $[M+H]^+$ (calcd for $C_{34}H_{39}O_{12}^+$, 639.2436). ^{1}H NMR (500 MHz $CDCl_3$) and ^{13}C NMR (125 MHz $CDCl_3$) data were in agreement with literature.[51]

*Equisetin (**6**):* colorless solid; HRESIMS *m/z* 374.2322 $[M+H]^+$ (calcd for $C_{22}H_{32}NO_4^+$, 374.2326); ^{1}H NMR (500 MHz $CDCl_3$) and ^{13}C NMR (125 MHz $CDCl_3$) chemical shifts were in agreement with literature values.[74]

*5'-epiequisetin (**7**):* colorless solid; HRESIMS *m/z* 374.2323 $[M+H]^+$ (calcd for $C_{22}H_{32}NO_4^+$, 374.2326); ^{1}H NMR (500 MHz $CDCl_3$) and ^{13}C NMR (125 MHz $CDCl_3$) were in agreement with literature values.[74]

*Palmitic acid (**8**):* white solid; HRESIMS *m/z* 256.2402 $[M-H]^-$ (calcd for $C_{16}H_{22}O_2$, 256.2402). ^{1}H NMR (500 MHz $CDCl_3$) and ^{13}C NMR (125 MHz $CDCl_3$) data were in agreement with literature[75] GCMS was performed to identify the fatty acid with 95% accuracy based on matching with the NIST database. In addition, LC-MS retention time and fragmentation for this compound matched that of a synthetic standard (Sigma Alrich > 99% purity by HPLC).

*Ergosterol peroxide (**9**):* green solid; HRESIMS *m/z* 429.33652 $[M+H]^+$ (calcd for $C_{28}H_{45}O_3^+$, 429.3363). ^{1}H NMR (500 MHz $CDCl_3$) and ^{13}C NMR (125 MHz $CDCl_3$) data were in agreement with literature.[76]

LC-MS Data Collection and Analysis

Each crude fungal endophyte extract, the *A. californica* extract, and the rice extract control were analyzed with LC-MS in the positive and negative ion modes, using a method described previously.[57] Briefly, separation was achieved using a CH_3CN-H_2O (0.1 % formic acid) mobile phase gradient. Fungal metabolites were identified on the basis of accurate mass measurements, retention time, and collisionally induced dissociation (CID) fragmentation by matching with a fungal dereplication database. To collect the MS data for comparison with the dereplication database, two scan events were performed, full scan (100-2000) and CID of the most intense ion from a list of possible precursor masses described previously.[57] Each LC-MS chromatogram was analyzed with ACD IntelliXtract (Toronto, Ontario, Canada). Data sets were compared to fungal secondary metabolite library previously reported.[57] In addition, IntelliXtract allows for the comparison of two chromatograms to determine which features are shared between two different samples. To identify features specifically attributable to the fungus, the LC-MS data for each fungal extract was compared to this negative control (rice extract) and features shared between the two samples were eliminated. IntellliXtract was then used to identify features shared between the extracts from each endophytic fungus and the *A. californica* extract. Each feature identified as being shared between the botanical extract and a given fungal extract (but not present in the rice control) was then manually checked by comparing selected ion chromatograms (Figure 15) to

verify the accuracy of the IntelliXtract data. The threshold set for comparison was a peak area of at least 1×10^5.

Antimicrobial Assays

Broth microdilution assays to evaluate antimicrobial susceptibility were performed according to Clinical Laboratory Standards Institute (CLSI) guidelines.[77] *S. aureus* (strain NCTC 8325-4),[78] and *P. aeruginosa* (strain NCTC 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 Müeller-Hinton broth and were adjusted to a final assay dilution of 1.0×10^5 CFU/mL based on OD_{600} of 0.11 for both bacteria. The negative control consisted of 2% DMSO in broth (vehicle) and ciprofloxacin served as the positive control. All treatments and controls were prepared in triplicate wells. For background subtraction, additional wells were included containing the samples without bacteria. OD_{600} was measured after incubation for 18 hrs at 37 ˚C. MIC was defined as the concentration at which no statistically significant difference was observed between the negative control and treated samples. IC_{50} was defined as the concentration at which there is a 50% decrease in growth observed between the negative control and the treated samples.

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CHAPTER IV

CONCLUSION

Goal 1 was accomplished by identifying sesamin (**1**) and asarinin (**2**) from *Anemopsis californica* as compounds effective *in vitro* against non-tuberculosis mycobacterial infections. Goal 2 was completed by isolating antimicrobial compounds from the fungi and then comparing the chemical composition of the fungal extract to that of the *A. californica* plant extract. In addition the plant contained compounds of fungal origin, and endophytic fungal diversity depended on the environment in which the plant is grown. Future studies will entail collecting medicinal plants from different populations, isolating the endophytic fungi, and then comparing the chemical profiles of the fungal extracts to each other. If plants with do contain a more diverse population of endophytic fungi, it may be possible to see an overlap in the antimicrobial compounds produced by the endophytic fungi. In addition, there is still the question of whether the plant or the endophyte makes the secondary metabolites. To truly answer this question, the plant should be made endophyte free and then inoculated with fungal endophytes previously identified to live within *A. californica*. In order to do this, a plant seed would have to be sterilized and grown to maturity in a nutrient rich medium with antifungal compounds. In addition, DNA markers testing for certain

fungi would have to be collected to see if the plants are truly endophyte free. This process would be very hard to perform and would take a lot of time.

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