There's nothing more exciting than science. You get all the fun of sitting still, being quiet, writing down numbers, paying attention. Science has it all.

-Seymour Skinner

THE APPLICATION OF NMR AND LC-HRMS BASED PRIORITIZATION STRATEGIES FOR THE DISCOVERY OF NATURAL PRODUCTS PRODUCED BY ENDOPHYTIC FUNGI FROM MEDICINAL PLANTS

by

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ABSTRACT

Natural products have been an abundant source of diverse and complex chemical structures and endophytic fungi have proven to be a valuable source of natural products. This thesis explores the diversity of natural products produced by fungal endophytes isolated from the leaves of medicinal plants used by the Canadian First Nations. Natural product isolation projects were selected based on three different screening strategies: The first employed antimicrobial activity as a basis for project prioritization; The second approach used nuclear magnetic resonance (NMR) metabolomics to identify extracts with atypical profiles due to the presence of structurally unique metabolites; the third approach used tandem liquid chromatography - high-resolution mass spectrometry (LC-HRMS) based metabolomic analyses to reveal extracts that contain potentially novel natural products. The bioactivity directed prioritization approach, either on its own or in combination with NMR based metabolomics, led exclusively to the isolation of known natural products, although some of the bioactivities measured for these compounds had not been previously reported. NMR based metabolomics prioritization, when used alone, also led to the isolation of known natural products or new isomers of known natural products. Finally, the third prioritization method using LC-HRMS based metabolomics led to the isolation of two new natural products. These data suggest that prioritization using LC-HRMS based metabolomics alone is the most effective method for the isolation of novel natural products. Future work should focus on extracts that are highlighted by both LC-HRMS based metabolomics as well as bioactivity screening in an effort to discover novel antimicrobial structures.

DEDICATION

To all those who have helped along the way

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Table of Contents

List of Tables

List of Figures

List of Symbols, Nomenclature or Abbreviations

^{13}C NMR	Carbon nuclear magnetic resonance spectroscopy
1D	One dimensional
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
2D	Two dimensional
$[\alpha]_D$	Specific rotation
ACS	American Chemical Society
ATCC	American Type Culture Collection
BD	Becton Dickinson
BLAST	Basic Local Alignment Search Tool
bs	Broad singlet
n-BuOH	1-butanol
c	Concentration (quoted in g/100 ml)
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
CH_2Cl_2	Dichloromethane
CH_3CN	Acetonitrile
COSY	Correlation spectroscopy
δ	Chemical shift in ppm
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
dddd	Doublet of doublet of doublet of doublets
ddt	Doublet of doublet of triplets
DMSO	Dimethyl sulfoxide
DMSO-*d6*	Deuterated Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EtOAc	Ethyl acetate
EtOH	Ethanol
H	Hydrogen
HEK	Human embryonic kidney cells
H_2O	Water
HPLC	High performance liquid chromatography
HRESIMS	High resolution electron spray ionization mass spectroscopy
Hz	Hertz
IC_{50}	Median inhibitory concentration
IR	Infrared spectroscopy
ITS	Internal transcribed spacer
J	Coupling constant
LC-MS	Liquid chromatography, mass spectrometry
Ltd.	Limited
m	Multiplet
MDR-TB	Multidrug-resistant Tuberculosis
MEA	Malt extract agar

MEB	Malt extract broth
MeOH	Methanol
MHz	Megahertz
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
m/z	Mass to charge ratio
NaCl	Sodium chloride
NaClO	Sodium hypochlorite
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
PCA	Principal Component Analysis
ppm	Parts per million
q	Quartet
®	Registered trade mark
rpm	Revolutions per minute
s	Singlet
sp.	Species
spp.	Multiple species of the same genus
t	Triplet
TB	Tuberculosis
td	Triplet of doublets
tRNA	Transfer RNA
UNB	University of New Brunswick
VCD	Vibrational circular dichroism
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organization

Chapter One

General Introduction

Natural products

Natural products, in the broadest sense, are organic compounds produced by living organisms [1-9]. The use of region-specific natural products such as dyes, spices, and drugs has been important throughout history, leading to the development of trading routes over the millenia, and creating international commercial relationships [8]. Societies have developed, in part, due to the influence of natural products and the associated monetary value that stems from the fact that the producing organisms for these natural products are often location specific or found in low abundance. Some of the more well-known beneficial applications of natural products include their use as therapeutics, food additives, agrochemicals, or cosmetics [5, 7, 10-19]. Of these applications, perhaps the most relevant for improving human life is in the development of therapeutic compounds such as antimicrobials or anti-cancer agents [3-6, 17, 20, 21].

Medicinal plants have been one of the most researched sources of bioactive natural products as they have been used for thousands of years, with virtually all cultures having a body of knowledge in the therapeutic use of local flora [16, 22-24]. The first natural product that was isolated as a pure chemical entity for therapeutic use was morphine (**1.1**, Figure 1.1) from the opium poppy in the early 1800's, and, 20 years later, it was marketed as a health product advertised as a cough suppressant [25]. Natural products also provided the first antibiotic through the discovery and isolation of penicillin

(**1.2**, Figure 1.1) in 1928, which has since saved millions of lives [26, 27]. Since the discovery of penicillin and the β-lactam class of antibiotics, several different classes of antibiotics derived from natural products have been identified with most of them being discovered between 1928 and 1963 [28]. Currently, natural products and their derivatives are responsible for approximately 65% of the anti-infectives approved worldwide [4-6, 12, 13].

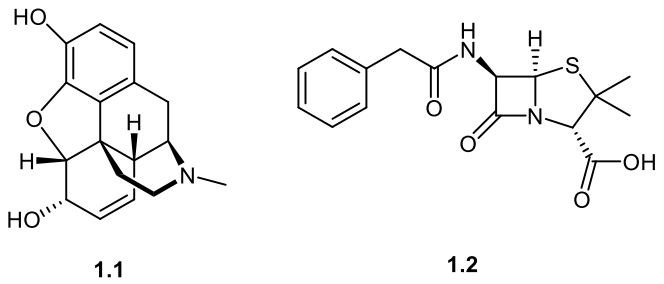

Figure 1.1 Chemical structure of morphine (**1.1**) and penicillin (**1.2**)

The pursuit of new biologically active natural products continues to be important, with the aim of identifying therapeutic compounds that are either more effective or result in fewer negative side effects [21, 22]. Traditionally, bioactive natural products are discovered through a process of extraction, fractionation, and bioassays. Extraction involves separating natural products from their source to enable a series of fractionation steps to be performed until pure compounds are isolated and evaluated. However, this process of isolating natural products is rife with challenges. For example, natural product isolation takes a significant amount of effort and time, stemming from both the isolation of the natural product(s) itself, or due to difficulty finding the biological source to be investigated. Following isolation, the subsequent determination of the chemical structure of the natural product, particularly the determination of the absolute stereochemistry of

the compound, can be time consuming or require sophisticated equipment [29-32]. Isolating a new or novel natural product is even more difficult, as the rediscovery of known compounds has become more common due to the limited number of different natural products in existence, of which we have already isolated over 300,000 [33]. In this context, the term novel is used to describe a natural product that is both new and interesting, either in terms of biologically relevant properties or possessing a rare or previously unknown backbone structure.

However, if natural products are going to continue to be considered a rich source of novel chemical entities, we must ask the question: are 300,000 known natural products a large or small proportion of available chemical space [33]? To determine how many chemicals are left to discover, we must consider the concept of chemical space in more detail [36, 37]. Chemical space is the theoretical space occupied by all possible molecules and chemical compounds. This includes all stoichiometric combinations of atoms that gives an estimated 10^{60} plausible combinations just for compounds under 500 Da [38]. Natural products exist as a small fraction of the total chemical space available in the universe, and this biologically limited subset of molecules is referred to as the parvome [34, 35] or the natural productome [36]. If we are to continue exploring natural products for biologically active novel compounds, we must first determine if there are biologically relevant compounds remaining within that chemical space, or if efforts would be better spent in other areas of research [34, 35, 37].

Current opinion suggests that natural products are proportionately a richer source of biologically relevant compounds compared to molecules in other areas of chemical space because they have persisted with selection pressures over time and are continually

optimized to interact with chemical targets in biological systems [21, 39-43]. The increased incidence of biological relevance, and our ability to continue to find novel compounds from natural sources suggest that we have not depleted biologically relevant natural products, and that we should continue our investigation to find novel natural products.

Despite this, the number of new natural products being discovered on an annual basis has plateaued, even though there have been constant improvements in the technology available to isolate and determine the structures of new compounds [9, 44]. Additionally, the compounds that are isolated are lowerin structural novelty compared to the compounds already discovered [9]. The structural novelty of natural products can be rated using a Tanimoto score; a similarity scale based on extended-connectivity patterns with known natural products [9, 45]. This process involves creating “fingerprints” for each compound by turning its structural features into a binary representation, and then comparing the presence and absence of the structural features between different compounds [9]. Although Tanimoto scores suggest that it is becoming less likely to find highly unique compounds, we are still far from reaching a point where natural products chemistry should be abandoned, as emerging technology and equipment facilitate the discovery of new compounds that has remained constant for the past several decades [7, 9, 36, 44, 46].

There are many different methods by which natural product chemists can increase their probability of finding new compounds, the first of which is the selection of the biological source that they are investigating [7, 21, 47]. Researchers value and explore areas of high biodiversity, particularly those that are under-investigated, to discover new

natural products; in this context, “areas” can refer to a geographical location, such as the arctic tundra or the amazon rain forest, or it can refer to unique biological locations, such as microorganisms that live inside the tissues of plants or marine invertebrates [3, 23, 48]. Exploring these areas of high biodiversity or new species increases the probability of finding new compounds because it gives the researcher access to metabolites that may not be found elsewhere [7, 23]. One of the most successful examples of this approach occurred in the 1950’s, after the development and improvement of SCUBA gear, which allowed researchers access to the marine environment for its chemical potential [49]. The success of this endeavor can be appreciated by surveying the work summarized by the set of reviews on marine natural products, originally compiled by Faulkner [50-61], and continued by Blunt, Munro and co-workers, which includes describing over 15,000 new marine natural products discovered between 2002 and 2017 [62-77].

An alternative to exploring new geographical locations has emerged through recent advancements in technology that have led to the development of metagenomics. Increasing our ability to explore biological diversity, metagenomics is a tool that can be used to determine the diversity of microorganisms in a location of interest by surveying the genomes within a tested sample. The development of metagenomics has revealed that there are many more microorganisms in the environment (e.g. an estimated >99% of bacteria) that we are unable to culture using standard laboratory methods compared to those we can, a reminder of the significant amount of chemical potential that researchers do not have access to. This will surely be an area of focus in the field of natural products moving forward with the development of new cultivation techniques and *in situ* growth environment technology such as the isolation chip or “ichip” [78-83].

After a biological source has been chosen for investigation, a natural product chemist would then need to evaluate the source's chemical potential by generating an extract of the metabolites produced by the study organism. If several sources (e.g. different trees, microorganisms, replicates of organisms, etc.) are chosen, then these can all be extracted separately, generating a library of extracts. An assay or multiple assays are then used to evaluate the bioactivity of the tested extracts (i.e. evaluating an extracts ability to inhibit the growth of a human pathogen) to determine if any of the extracts exhibit a desirable result.

The next step towards improving the chances of finding a new compound is by improving the decision-making process. This involves decisions relating to which extracts from the library should be prioritized for investigation and involves identifying those extracts that showed desirable results in the screening assay(s). Coupled with this concept, choosing which extracts should not be pursued is equally important and can be achieved by either noting undesired results from the screening assays, or through dereplication, a process of halting compound investigations that are leading to known chemistry.

Dereplication is a term that can be applied in two fundamentally different ways in natural products chemistry. The first dereplication that can be performed is a biological dereplication, which occurs before the creation of a library of extracts, where only one copy of each species of study organism is kept for extraction. Maintaining a high amount of biodiversity in the collection, while keeping the number of individual organisms low, results in the highest diversity of natural products in your collection. The second dereplication method is a chemical dereplication where investigation into the prioritized

extracts is halted once the identity of the target compound is found to be a previously isolated compound [85]. Dereplication of previously isolated compounds should be done as early in the chemical fractionation process as possible (eg. comparing spectral data from an impure extract with data from a chemical database) [7, 17, 21].

Spectroscopy-based prioritization techniques have advanced in the past 10-15 years, improving our ability to identify extracts that contain new and novel chemical entities [9, 21, 47, 86]. Nuclear magnetic resonance (NMR) or high-resolution mass spectrometry (HRMS) based metabolomics can be used to analyze extracts based on chemical features or profiles [21, 47, 85-90]. NMR based metabolomics can be performed using one of many NMR experiments, either 1D (e.g. ^{1}H NMR or ^{13}C NMR) or less commonly 2D experiments (e.g. COSY, HSQC, HMBC, or NOESY) [47, 86, 87, 89]. For the 1D ^{1}H NMR based metabolomics, the spectra that are collected for each of the extracts can be processed digitally by dividing the spectra into different sections, called "bins", that are integrated to represent the presence and intensity of peaks at particular chemical shifts within a spectrum. Researchers can then perform multivariate statistical analyses including principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), or a cluster analysis [47]. These are used to determine which of the extracts within a library contain unique spectral peaks and therefore unique chemical features within the library.

HRMS based metabolomics differs in several ways from NMR based metabolomics but is a conceptually similar procedure. Data collection for HRMS involves obtaining the mass to charge ratios of the analyte ion (*m/*z) values for each of the compounds within a given extract. When HRMS is combined with liquid

chromatography, adding a separation step (to give a retention time) along with the *m/z* value, you can identify individual compounds within the mixture rather than analyze a composite of all compounds in the extract. After acquisition of the HRMS spectrum, the data are processed by selecting peaks above a chosen threshold with the aim of removing background signals and analytical noise before performing similar multivariate analyses to those used in NMR based metabolomics, thus indicating which extracts contain unique ions when compared to the rest of the library [21, 47, 85-88, 90]. The main advantage of HRMS metabolomics is that after extracts have been prioritized, the *m/z* of the ion that caused an extract to be prioritized may then be compared to chemical databases to determine if there are previously isolated compounds with the same molecular weight [88]. Efforts can then be focused on extracts containing putatively new compounds with no matches within the available chemical databases.

Another type of prioritization that has been successfully applied in the discovery of biologically active natural products involves the compilation and analysis of bioactivity profiles. These profiles prioritize extracts based on having a unique profile of activity against a variety of different organisms or cell lines [91]. To perform bioactivity profiling, all extracts within a library are evaluated for biological activity against different pathogens to produce an activity profile. This profile can then be compared with the profiles of the all extracts within the library using PCA, PLS-DA or cluster analysis, to indicate extracts that display unique profiles of bioactivity amongst the library of extracts. A dereplication step can be added to this method as well, where the profiles of extracts can be compared with profiles of various positive control compounds,

allowing for the identification of extracts possessing similar activity profiles to known compounds, thus removing them from consideration for further investigation [91, 92].

Fungi as a source of natural products

Fungi have been one of the most valuable sources of natural products, especially bioactive natural products [1, 2, 21, 93-95]. Fungi are the natural source for the β-lactam category of antibiotics that includes two of the most successful groups of antibiotics, the penicillins and the cephalosporins [2, 28]. Furthermore, half of the currently approved anti-infectives that have a natural origin come from fungi [4-6, 12, 13].

As one might expect, a plethora of fungal species have been explored to survey for antimicrobial natural products, however, some of these species are more difficult to obtain, as they may be cryptic or live in less accessible habitats. One of these habitats for fungi that could be explored is inside plants, where the fungi are termed endophytic fungi, or endophytes [3, 96-99]. Endophytes are a valuable source of bioactive natural products [94, 100-106]. Interestingly, some endophytes can produce the same bioactive compounds made by their host plants, providing alternative sources for these bioactive compounds [107-109]. This ability is assumed to be due to horizontal transmission of genes between the endophyte and its host. This has the potential advantage of providing a more accessible source of biologically relevant compounds, as fungi can grow at much faster rates than plants, thereby producing compounds more rapidly [101, 110, 111]. When deciding which plants should be explored for their endophyte diversity, medicinal plants have gained attention in particular [24, 112].

Project description

Laboratory work for this thesis used fungal cultures that were previously isolated by Ellsworth [113]. Ellsworth investigated twelve traditionally used medicinal plants: *Acorus calamus* (sweet flag), *Aralia nudicaulis* (wild sarsaparilla), *Empetrum nigrum* (black crowberry), *Fragaria virginiana* (wild strawberry), *Geum macrophyllum* (large-leaved avens), *Heracleum maximum* (cow-parsnip), *Hypericum perforatum* (St. John's-wort), *Juniperus communis* (common juniper), *Moneses uniflora* (one-flowered wintergreen), *Nuphar lutea* (water lily), *Populus tremuloides* (trembling aspen) and *Symplocarpus foetidus* (skunk cabbage). These plants were selected due to strong correlations between the bioactivity of the medicinal plant and the associated use of that plant by Indigenous peoples [114-117]. A total of 81 distinct fungal isolates were obtained from the 12 medicinal plants using surface sterilization techniques developed specifically for each plant to ensure that the isolates were endophytic and not epiphytic [103].

The research for this thesis began with each of the endophytes being fermented in two different media, 2 % malt extract broth (MEB) and 1.2 % potato dextrose broth (PDB). The fermented endophytes were then extracted with EtOAc and concentrated *in vaccuo* to produce a total of 162 extracts (one per endophyte per media type). Each of the extracts were then evaluated for their ability to inhibit the growth of four different microorganisms (*Staphylococcus aureus* (Gram positive bacterium), *Pseudomonas aeruginosa* (Gram negative bacterium), *Candida albicans* (Eukaryotic yeast), and *Mycobacterium tuberculosis* H37Ra (Mycobacterium); data presented in Tables 1.1 and

1.2). NMR spectra were obtained for all of the 162 extracts, while HRMS spectra were obtained for the 81 extracts that were produced from fermentation in PDB.

The aim of this thesis was to investigate extracts produced from the 81 different endophytes based on the results of three different prioritization methods; antimicrobial activity, NMR-based metabolomics, or HRMS-based metabolomics. This thesis is presented as a series of manuscripts and comprises seven experimental chapters followed by a final chapter of general discussion and conclusions. Chapter two describes the isolation of a natural product while using antimicrobial activity as a prioritization method. Chapter three and Chapter four describe the isolation of natural products when using antimicrobial activity in conjunction with NMR based metabolomics as a prioritization method. Chapter five has two components, one where natural products were isolated using antimicrobial activity as a prioritization method, and another where natural products were prioritized using antimicrobial activity in conjunction with NMR based metabolomics. Chapter Six describes the isolation of natural products using NMR based metabolomics when not in conjunction with antimicrobial activity as a prioritization method. Chapter seven and Chapter eight describe the isolation of natural products using LC-HRMS based metabolomics in conjunction with antimicrobial activity as a prioritization method. Chapters two to five are formatted for Natural Product Communications and have already been published [118-121]. Chapter six is also formatted for Natural Product Communications. Chapters seven and eight are formatted for submission to the Journal of Natural Products. It should be noted that as the manuscripts presented in Chapters two to eight are closely related, there will be

significant overlap and some redundancy in the introduction sections of those Chapters. My contribution to each chapter is that of primary researcher and author.

Table 1.1 Inhibitory activity of extracts derived from endophytic fungi fermented in 2.0 % malt extract broth. Bioactivity was divided into three categories based on the mean percent inhibition: Low for extracts that displayed less than 25 % inhibition, moderate for extracts that displayed between 25 % and 50% inhibition, and high for extracts that displayed greater than 50 % inhibition.

Organism	Inactive*	Low	Moderate	High
S. aureus	58	14	6	3
P. aeruginosa	70	10	1	0
C. albicans	62	14	2	3
M. tuberculosis	0	1	2	78

*Extracts were deemed inactive if they were not statistically different from zero using a one sample t-test(α=0.05)

Table 1.2 Inhibitory activity of extracts derived from endophytic fungi fermented in 1.2 % potato dextrose broth. Bioactivity was divided into three categories based on the mean percent inhibition: Low for extracts that displayed less than 25 % inhibition, moderate for extracts that displayed between 25 % and 50 % inhibition, and high for extracts that displayed greater than 50 % inhibition.

Organism	Inactive*	Low	Moderate	High
S. aureus	63	4	6	8
P. aeruginosa	43	37	1	0
C. albicans	39	32	2	8
M. tuberculosis	9	23	10	39

*Extracts were deemed inactive if they were not statistically different from zero using a one sample t-test(α=0.05)

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Chapter Two

Isolation of the Plant Hormone (+)-Abscisic Acid as an Antimycobacterial Constituent of the Medicinal Plant Endophyte *Nigrospora* sp.

Clark TN, Ellsworth K, Li H, Johnson JA, and Gray CA. (**2013**) *Natural Product Communications*, ***8***, 1673-1674

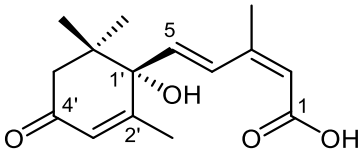
An extract of the endophytic fungus *Nigrospora* sp. (isolate TC2-054) from the Canadian medicinal plant *Fragaria virginiana* exhibited significant antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra. Bioassay guided fractionation revealed that linoleic acid derivatives and the plant hormone (+)-abscisic acid (ABA) were responsible for the observed antimycobacterial activity. This activity of ABA has not been previously reported.

Keywords: Abscisic acid, Antimycobacterial activity, Endophyte, *Fragaria virginiana*, Canadian medicinal plant, *Nigrospora* sp.

Endophytic fungi are increasingly being recognized as an important source of bioactive natural products [1] and the biosynthetic potential of endophytes isolated from medicinal plants has recently been highlighted [1f]. As part of our research on Canadian medicinal plants, we have so far isolated 81 endophytic fungi from a selection of twelve plants that have been used therapeutically by the First Nations peoples of the Canadian

Maritimes [2]. Antimicrobial screening of this endophyte library indicated that an extract of the spent fermentation broth of *Nigrospora* sp. isolate TC2-054 obtained from the medicinal plant *Fragaria virginiana* exhibited significant antimycobacterial activity and, therefore, warranted further investigation.

The EtOAc extract of a bench-scale (2 L), two-week fermentation of TC2-054 was subjected to a modified Kupchan partition protocol with the *n*-hexane and CH_2Cl_2 fractions showing significant activity towards *M. tuberculosis* H37Ra. NMR analysis of the *n*-hexane fraction showed it to contain significant amounts of linoleic acid derivatives that are known to exhibit antimycobacterial activity [3] and was not further fractionated. However, normal phase HPLC of the CH_2Cl_2 fraction led to the isolation of (+)–abscisic acid [4] (ABA (**2.1**); 6.5 mg/L; Figure 1) as the only antimycobacterial constituent [*M. tuberculosis* H37Ra; MIC: 200 μg/mL; IC_{50} (± SD): 40.8 ± 3.7 μg/mL). LC-MS screening did not detect ABA in a *F. virginiana* extract prepared from leaves collected at the same time as those used for the isolation of the *Nigrospora* sp.



2.1

Figure 2.1 (+)–Abscisic acid (ABA**)** (**2.1**)

The role of ABA as a plant stress hormone has been well documented since its isolation in 1961 [4a]. More recently, it has been shown to be involved in a wide range of developmental physiological processes in higher plants [5a] and found also to be produced by lower plants, lichens and fungi [5b], including a species of *Nigrospora* isolated from a Thai sea-fan [5c]. Many of these fungi are recognized endophytes and

phytopathogens [5b, d] and the production of ABA by these fungi may be a factor in the development and maintenance of plant-fungus relationships [5b]. ABA has recently been shown to be an important factor in fruit ripening in the strawberry, *Fragaria ananassa* [5e], posing intriguing questions relating to the role of endophyte derived ABA in *F. virginiana*.

The antimycobacterial activity of ABA has not been previously described, and although it exhibited only moderate activity against *M. tuberculosis* H37Ra, it did not show any observable toxicity to mammalian cells; no difference in viability was observed between HEK293 cells treated with abscisic acid (at concentrations up to 250 μg/mL) and the corresponding vehicle controls [analysis of 250 μg/mL data: t-test, F = 0.052, P = 0.83, 4 d.f. and Mann-Whittney, U = 6.0, P = 0.70; mean cell viability (± SD) 93 ± 10% compared with negative control]. Given its low toxicity to human cells, further work should focus on defining the mode of action of ABA against mycobacteria as it may reveal a selective cellular target that could be exploited in future tuberculosis drug development.

Experimental

Isolation and identification of Nigrospora sp. (TC2-054): Endophytes were isolated from the leaves of *Fragaria virginiana* Duchesne collected from the wood-lot on the UNB campus in Saint John, New Brunswick, Canada (N 45° 18.375’ W 66° 05.616’) in August 2010. Plants were identified by Dr. Stephen Clayden of the New Brunswick Museum and a voucher specimen has been deposited in the New Brunswick Museum Herbarium (Number: NBM VP-37478). Leaf surfaces were sterilized by immersion in

5.25% aqueous sodium hypochlorite for 5 sec, sterile distilled water for 10 sec and 70% EtOH for 15 sec. The sterile tissue was rinsed with sterile distilled water, blotted dry on an autoclaved paper towel, and aseptically cut into pieces that were placed onto 2% malt extract agar and incubated at ambient room temperature. Endophytic fungi were subcultured on 2% malt extract agar until pure cultures were obtained. Isolate TC2-054 was identified taxonomically as *Nigrospora* sp. through examination of colony and spore morphology, with the taxonomic classification being confirmed by comparison of the internal transcribed spacer and 5.8S rRNA gene (ITS) DNA regions with corresponding sequences available in the GenBank database (NCBI, US Government), as previously described. The sequence data derived from isolate TC2-054 has been submitted to GenBank and assigned accession number KC916673.

***Biological assays*:** Anti-mycobacterial activity against *M. tuberculosis* strain H37Ra (ATCC 25177) and cytotoxicity against HEK 293 was evaluated as previously described [6].

Fermentation and extraction: TC2-054 was fermented in 2% malt extract broth at room temperature for 2 weeks (2 L; 20 ×100 mL batches in 250 mL Erlenmeyer flasks). The fungal material was separated from the broth using vacuum filtration, and the broth was extracted with EtOAc (3×300 mL). The organic fraction was concentrated *in vacuo* to give a crude extract (79 mg).

Bioassay guided fractionation: The crude extract (79 mg) was dissolved in 9:1 MeOH/H_2O (50 mL) and extracted with hexanes (3 × 25 mL) before being diluted with H_2O (25 mL) and extracted with CH_2Cl_2 (3 × 25 mL). The aqueous fraction was concentrated *in vacuo*, dissolved in H_2O (50 mL) and extracted with EtOAc (3 × 25 mL)

and *n*-BuOH (3 × 25 mL). The 5 partition fractions were concentrated *in vacuo* with the CH_2Cl_2 fraction (46 mg) showing strong bioactivity. The CH_2Cl_2 fraction (46 mg) was subjected to normal phase HPLC (1:1, hexanes/EtOAc) to give **1** (13 mg)

Extraction and LC-MS screening of Fragaria virginiana: Freeze-dried ground plant material (20.5 g) was extracted using a Soxhlet apparatus for 11.5 h with MeOH (200 mL). The crude extract was then concentrated *in vacuo* to give a green oil (6.54 g). The crude extract (0.5 mg/mL in MeOH, 20 μL injections) was subjected to LC/MS using a Phenomenex Kinetex C18 column (50 x 4.60 mm, 2.6 μm) eluted with a 0.1% formic acid H_2O/MeOH gradient (1 min at 95:5 H_2O/MeOH, 7 min linear transition to 1:99 H_2O: MeOH for a further 7 min) at 500 μL/min on an Agilent 1100 LC system coupled to an AB Sciex API 2000 triple quadrupole MS TurboIonSpray source run in positive mode). The presence of ABA was analyzed by selected reaction monitoring of the pseudo-molecular ion (*m*/*z* 265) to dehydrated product ion (*m*/*z* 247) transition. Under these conditions, an ABA standard eluted with a retention time of 6.6 min and was detectable in MeOH solutions at a concentration of ≥ 25 ng/mL. No peak corresponding to ABA was observed in the *F. virginiana* crude extract. Subsequent co-injections of the crude extract and ABA standard confirmed that ABA would be detectable at ≥ 100 ng/g in the crude extract.

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recording NMR spectra, and obtaining ESIMS data, respectively. Financial support for this research was provided by NSERC (Discovery Grant to CAG and CGS-M to TNC), the NBHRF (Seed Operating Grant to CAG and JAJ), the NBIF (Research Assistantship Initiative grants to CAG), and UNB (University Research Fund grants to CAG and JAJ) and is gratefully acknowledged.

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Supplemental information for Chapter Two:

Table 2.1 Comparison of the NMR data obtained for abscisic acid (**2.1**) with that reported by Smith et al [1].

Carbon	**3**[1]		Literature data[2]	
	$\delta_C$ppm (mult.)	δ_H ppm (int., mult., *J*/Hz)	δ_C ppm (mult.)	δ_H ppm (int., mult., *J*/Hz)
1	170.7 (s)		170.9 (s)	
2	127.2 (d)	5.76 (1H, s)	127.1 (d)	5.77 (1H, s)
3	151.8 (s)		151.6 (s)	
4	128.5 (d)	7.81 (1H, d, 16.1)	128.4 (d)	7.82 (1H, d, 16.0)
5	137.0 (d)	6.17 (1H, d, 16.1)	136.9 (d)	6.18 (1H, d, 16.0)
6	21.6 (q)	2.05 (3H, s)	21.5 (q)	2.05 (3H, s)
1′	80.1 (s)		79.9 (s)	
2′	162.9 (s)		163.0 (s)	
3′	118.1 (d)	5.98 (1H, s)	118.1 (d)	5.98 (1H, s)
4′	198.3 (s)		198.3 (s)	
5′	49.8 (t)	2.29 (1H, d, 17.2)	49.7 (t)	2.30 (1H, d, 17.3)
		2.49 (1H, d, 17.2)		2.50 (1H, d, 17.3)
6′	41.8 (s)		41.7 (s)	
7′	19.2 (q)	1.93 (3H, s)	19.1(q)	1.94 (3H, s)
8′	23.3 (q)	1.03 (3H, s)	23.1 (q)	1.04 (3H, s)
9′	24.4 (q)	1.12 (3H, s)	24.3 (q)	1.12 (3H, s)

[1]Spectra recorded in $CDCl_3$ at 400MHz for ^1H and 100 MHz for ^{13}C
[2]Spectra recorded in $CDCl_3$ at 600MHz for ^1H and 150 MHz for ^{13}C

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Chapter Three

Isolation of (-)-Avenaciolide as the Antifungal and Antimycobacterial Constituent of a *Seimatosporium* sp. Endophyte from the Medicinal Plant *Hypericum perforatum*

Clark TN, Bishop AI, McLaughlin M, Calhoun LA, Johnson JA, and Gray CA. (**2014**) *Natural Product Communications*, ***9***, 1495-1496

An extract of *Seimatosporium* sp., an endophyte from the Canadian medicinal plant *Hypericum perforatum*, exhibited significant antifungal and antimycobacterial activity against *Candida albicans* and *Mycobacterium tuberculosis* H37Ra. Bioassay guided fractionation led to the isolation of (-)–avenaciolide as the only bioactive constituent of the extract. This is the first report of both the antimycobacterial activity of avenaciolide and its isolation from a *Seimatosporium* sp. fungus.

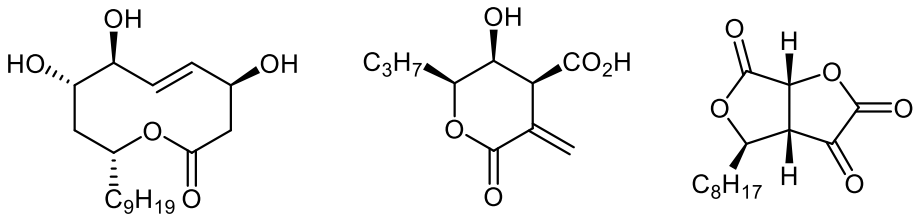
Keywords: Avenaciolide, Antifungal activity, Antimycobacterial activity, Endophyte, *Hypericum perforatum*, *Seimatosporium* sp.

Endophytic fungi represent an important source of novel bioactive natural products [1] and those associated with medicinal plants have recently attracted particular attention [2]. The First Nations people of the Canadian Maritime provinces have a rich ethnobotanical history [3] and plants that they used therapeutically are proving to harbor diverse assemblages of endophytes [4]. Bioassay and NMR metabolomic screening of our endophyte library indicated that an extract of *Seimatosporium* sp. (isolate TC2-029) exhibited significant antifungal and antimycobacterial activity and was spectroscopically

distinct from the bulk of the endophyte extracts in our library. These findings, in conjunction with the recent isolation of new natural products (e.g. **3.1** and **3.2**) from *Seimatosporium* spp. [5], prompted the further investigation of TC2-029.

Isolate TC2-029 was obtained from surface sterilized leaves of *Hypericum perforatum* through an indirect isolation method [4]. *H. perforatum*, or St. John's wort, is an alien invasive species that was introduced to North America from Europe in the early eighteenth century [6]. It is now a common perennial in eastern Canada being found in grasslands, pastures, and meadows, and also in forested areas that have been damaged by road construction and fire [7]. Despite its relatively recent introduction, *H. perforatum* has found ethnobotanical application by the indigenous peoples of North America for a variety of medicinal purposes, including treatment of fevers, coughs, and dermatological and gastrointestinal ailments [3].

The EtOAc extract of a small-scale (200 mL), two-week fermentation of TC2-029 was subjected to a modified Kupchan partition protocol. The *n*-hexane and CH_2Cl_2 fractions, which comprised 5 and 57 % of the crude extract respectively, inhibited the growth of *Candida albicans* and *Mycobacterium tuberculosis* H37Ra. Examination of these fractions by NMR indicated that they contained a single compound (>99% pure by NMR) that was identified as (-)–avenaciolide (**3.3**) [8].



3.1 **3.2** **3.3**

Figure 3.1 Seimatopolide A (**3.1**), seimatosporic acid A (**3.2**) and avenaciolide (**3.3**).

The antifungal activity of **3.3** [*C. albicans*; MIC: 6.25 μg/mL; IC_{50} (± SD): 3.0 ± 0.14 μg/mL] is well known [8a], but this is the first report of the antimycobacterial activity of avenaciolide [*M. tuberculosis* H37Ra; MIC: 25 μg/mL; IC_{50} (± SD): 7.4 ± 0.3 μg/mL]. The compound was also evaluated for cytotoxic effects against the immortalized, non-cancerous human embryonic kidney cell line HEK293 and showed similar levels of biological activity (IC_{50} of 5.6 ± 0.4 μg/mL) that therefore resulted in poor therapeutic indices for *C. albicans* and *M. tuberculosis* H37Ra (1.9 and 0.8 respectively).

Although avenaciolide is not suitable for TB drug development, the isolation of **3.3** from a medicinal plant endophyte provides further evidence that these fungi are an important source of bioactive natural products that should continue to be investigated for drug discovery.

Experimental

Fermentation and extraction: Eighty-one medicinal plant endophytes [4] were fermented in 2% malt extract broth at room temperature for 2 weeks with shaking under ambient light conditions (200 mL; 2 × 100 mL batches in 250 mL Erlenmeyer flasks). The fungal mycelia and spent broth were separated by vacuum filtration. The broth was extracted with EtOAc

(3 × 50 mL). The organic fractions for each isolate were combined and concentrated *in vacuo* to give crude endophyte extracts.

NMR metabolomic analysis: ^{1}H NMR spectra of the endophyte extracts were recorded in CD_3OD at 25°C on a Varian Unity 400 MHz NMR using the s2pul pulse sequence (spectrometer frequency: 399.945 MHz; spectral width: 5599.9 Hz; acquisition time: 3.7372 s; pulse width: 9.9 μs; 41856 total points zero-filled to 65536; number of transients per spectrum: 32). Spectra were individually zero-filled to 256 K points, binned in 0.04 ppm increments from 0.18-10.02 ppm and converted to ASCII format using MestReNova version 9.0.0 (Mestrelab Research). The data were then transposed from columns to rows and collated into a single data set before all negative data points were replaced by zeros, the bins corresponding to the presence of CD_3OD (*i.e.* 3.26-3.34 ppm and 4.78-4.98 ppm) deleted and the data converted to CSV format using Microsoft Excel. The binned NMR data obtained for the endophyte library were then analyzed using MetaboAnalyst 2.0 [9] (data type: spectral bins; data format: samples in unpaired rows; data filtering: interquantile range; data normalization: row normalization by sum; data transformation: logarithmic; data scaling: none). Data were interpreted using 2D PCA plots to determine extracts showing unique metabolic spectra.

Biological assays: Antifungal activity against *C. albicans*, antimycobacterial activity against *M. tuberculosis* H37Ra and cytotoxicity against HEK 293 were evaluated as previously described [10].

Bioassay guided fractionation: The crude extract of isolate TC2-029 (21 mg) was dissolved in 9:1 MeOH: H_2O (8 mL) and extracted with hexanes (3 x 4 mL). The aqueous fraction was then diluted with H_2O (4 mL) and extracted with CH_2Cl_2 (3 x 4 mL). The

aqueous fraction was concentrated *in vacuo* and dissolved in H_2O (8 mL), then extracted with EtOAc (3 x 4 mL) and n-BuOH (3 x 4 mL). The five fractions were concentrated *in vacuo*. The hexane and CH_2Cl_2 fractions (1 mg and 12 mg respectively) showed significant bioactivity and contained a single compound that appeared to be >99% pure by NMR. Further analysis of the active compound by NMR and MS allowed it to be identified as **3**.

Avenaciolide

$[\alpha]_D^{22}$: -32.05 (*c* 0.78, EtOH).

IR (NaCl, thin film): 2955, 2928, 2856, 1785, 1665, 1466, 1298, 1209, 1107, 1064, 968 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$): 6.47 (1H, d, *J* = 2.5 Hz, H-7), 5.87 (1H, d, *J* = 2.2 Hz, H-7), 5.05 (1H, d, *J* = 8.5 Hz, H-6a), 4.43 (1H, ddd, *J* = 7.2, 6.0, 4.0, H-4), 3.55 (1H, ddt, *J* = 8.5, 4.0, 2.5, 2.2 Hz, H-3a, 1.80 (2H, m, *J* = 7.2, 6.0 Hz, H_2-8), 1.47 (2H, m, H_2-9), 1.35 (2H, m, H_2-10), 1.29 (4H, m, H_2-11 and H_2-12), 1.28 (2H, m, H_2-14), 1.26 (2H, m, H_2-13), 0.88 (3H, m, *J* = 6.9 Hz, H_3-15).

^{13}C NMR (100 MHz $CDCl_3$): 169.8 (C-6), 167.6 (C-2), 134.8 (C-3), 126.4 (C-7), 85.3 (C-4), 74.4 (C-6a), 44.3 (C-3a), 36.2 (C-8), 31.9 (C-13), 29.3 (C-10 and C-11), 29.5 (C-12), 24.9 (C-9), 22.8 (C-14), 14.3 (C-15).

HRESIMS: *m/z* 267.1593 $[M + H]^+$

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tuberculosis assays, and obtaining ESIMS data, respectively. Financial support for this research was provided by NSERC (Discovery Grant to CAG, CGS-M to TNC and USRA to AIB), the NBHRF (Seed Operating Grant to CAG and JAJ), the NBIF (Research Assistantship Initiative grants to CAG), and UNB (University Research Fund grants to CAG and JAJ) and is gratefully acknowledged.

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Supplemental information for Chapter Three

Table 3.1 Comparison of the NMR data obtained for avenaciolide (**3.3**) with that reported by Suzuki et al. [1]

Carbon	**5**[1]		Literature data[2]	
	δ_C ppm (mult.)	δ_H ppm (int., mult., *J*/Hz)	δ_C ppm (mult.)*NR	δ_H ppm (int., mult., *J*/Hz)
2	167.6 (s)		167.4	
3	134.8 (s)		134.6	
3a	44.3 (d)	3.55 (1H, dddd, 8.6, 4.0, 2.3, 2.3)	44.2	3.5-3.6 (1H, m)
4	85.3 (d)	4.43 (1H, ddd, 7.1, 6.0, 4.0)	85.1	4.43 (1H, m)
6	169.8 (s)		169.7	
6a	74.4 (d)	5.05 (1H, d, 8.5)	74.3	5.06 (1H, d, 9)
7	126.4 (d)	6.47 (1H, d, 2.6)	126.3	6.49 (1H, d, 2)
		5.87 (1H, d, 2.2)		5.88 (1H, d, 2)
8	36.2 (t)	1.80 (2H, m)	36.1	1.8-1.85 (2H, m)
9	24.9 (t)	1.47 (2H, m)	24.8	1.3-1.5 (2H, m)
10	29.3 (t)	1.35 (2H, m)	29.1	1.3-1.5 (2H, m)
11	29.3 (t)	1.29 (2H, m)	29.1	1.3-1.5 (2H, m)
12	29.5 (t)	1.29 (2H, m)	29.3	1.3-1.5 (2H, m)
13	31.9 (t)	1.26 (2H, m)	31.8	1.3-1.5 (2H, m)
14	22.8 (t)	1.28 (2H, m)	22.6	1.3-1.5 (2H, m)
15	14.3 (q)	0.88 (3H, t, 7.1)	14.1	0.89 (3H, t, 7)

*NR = Not reported
[1]Spectra recorded in $CDCl_3$ at 400MHz for ^1H and 100 MHz for ^{13}C
[2]Spectra recorded in $CDCl_3$ at 400MHz for ^1H and 100 MHz for ^{13}C

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Chapter Four

Isolation of Phomopsolide A and Phomopsolide C as Antimycobacterial Products from an Unidentified Endophyte of the Canadian Medicinal Plant *Heracleum maximum*

Clark TN, Ellsworth KT, Jean S, Webster D, Robichaud GA, Johnson JA, and Gray CA. (**2015**), *Natural Product Communications*, ***10,*** 1647-1648

An extract of an unidentified endophyte from the Canadian medicinal plant *Heracleum maximum* exhibited a unique metabolomic profile and significant antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra. Bioassay guided fractionation of the extract led to the isolation of phomopsolide A (**4.1**) and phomopsolide C (**4.2**). This is the first report of antimycobacterial activity for **4.1** and **4.2**.

Keywords: Antimycobacterial activity, Fungal endophyte, Medicinal plant, NMR metabolomic screening, Phomopsolide A, Phomopsolide C

Despite the recent efforts and successes to combat the disease, tuberculosis (TB) remains a massive global health problem. In 2013, approximately 9 million people developed active TB and there were almost half a million cases of multidrug resistant tuberculosis (MDR-TB) of which 9 % were caused by extensively resistant strains (XDR-TB) [1]. Given the limited therapeutic options and the problems associated with current TB drugs, there is an urgent need to discover molecules with antimycobacterial activity to facilitate TB drug development [2]. We have found endophytes of Canadian medicinal plants to be a promising source of antimycobacterial natural products [3], and recently

employed a prioritization strategy that combines NMR metabolomic analyses and bioactivity screening of our endophyte extract library [3c]. One of the extracts highlighted by this approach exhibited significant antimycobacterial activity and was derived from the culture broth of a fungus (TC2-077; Figure 4.1) isolated from the leaves of the medicinal plant *Heracleum maximum* W. Bartram (family Umbelliferae). Unfortunately, all attempts to identify the fungus through morphological examination and DNA sequencing have thus far been unsuccessful.

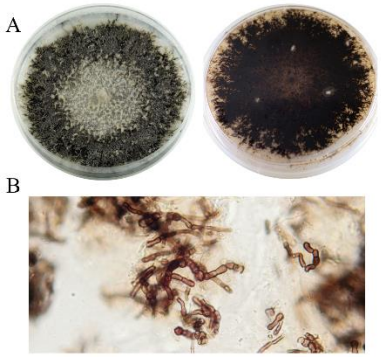

Figure 4.1 Endophyte TC2-077: A) in plate culture on Czapek's agar (left: top;colony; right: reverse); B) light micrograph of hyphae (310 × magnification).

Bioassay guided fractionation of the TC2-077 extract led to the isolation of phomopsolide A and phomopsolide C (**4.1** and **4.2**; Figure 4.2) that were identified by mass spectrometry, NMR spectroscopy and polarimetry, with all data being consistent with literature values [4]. Both metabolites inhibited the growth of *M. tuberculosis* H37Ra (Table 4.1) but exhibited poor therapeutic indices (0.3 and 1.6 for **4.1** and **4.2**, respectively) when tested against the immortalized human HEK 293 cell line; it would seem that the stereochemistry of the C6 alkene has a significant effect on the bioactivity of these compounds.

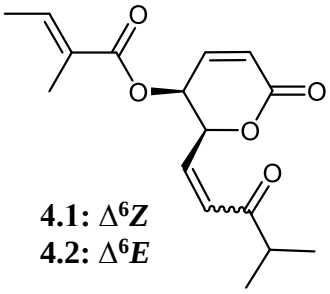

Figure 4.2 Phomopsolide A (**4.1**) and Phomopsolide C (**4.2**).

Table 4.1 Biological activities (MICs and IC_{50}s in μM)[a] of phomopsolide A and phomopsolide C.

Compound	*Mycobacterium tuberculosis* H37Ra		Human embryonic kidney 293 cells
	MIC	IC_{50} (95% CI)[b]	IC_{50} (95% CI)[b]
4.1	170	24.4 (22.4-26.6)	6.66 (5.67-7.82)
4.2	680	34.3 (28.0-41.8)	54.7 (46.9-64.2)

[a] MIC: minimum inhibitory concentration; IC_{50}: median lethal concentration.
[b] CI: confidence interval.

The phomopsolides have been isolated from *Phomopsis* spp. [4b, 5], *Penicillium* spp. [4a, 6] and *Diaporthe* spp. [7] endophytes. However, the morphology of TC2-077 is not characteristic of these fungal taxa and we are, therefore, continuing our efforts to identify the endophyte of *H. maximum*.

Experimental

Endophyte isolation: TC2-077 was isolated from the leaves of *Heracleum maximum* (New Brunswick Museum voucher specimen NBM VP-37481) collected from the Kingston peninsula, NB, Canada (N 45° 30.774’ W 65° 53.951’) in August 2010. Leaf surfaces were sterilized by immersion in 5.25% aqueous sodium hypochlorite for 5 sec, followed by sterile distilled water for 10 sec and 70% EtOH for 15 sec. The tissue was then rinsed with autoclaved distilled water, blotted dry on an autoclaved paper towel, and cut into pieces (5 mm × 5 mm) that were placed onto 2 % malt extract agar and incubated

at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2 % malt extract agar until pure cultures were obtained.

Identification of endophytes: TC2-077 did not produce fruiting bodies with characteristic morphological features on malt extract, potato dextrose or Czapek's agars. All attempts to identify the isolate through DNA sequencing were unsuccessful as DNA from the ITS region could not be isolated and amplified after repeated attempts using published procedures [8]. A description of the isolate grown on Czapek's agar is as follows: colonies form a flat surface with cottony patches; surface white and gold, reverse white and gold; aerial hyphae white; hyphae septate, branching; fruiting bodies lacking.

Fermentation and extraction: TC2-077 was fermented in 2% malt extract broth at room temperature with shaking (150 rpm) for 2 weeks (11.3 L; 113 × 100 mL batches in 250 mL Erlenmeyer flasks covered with aluminum foil). The fungal material was separated from the broth using vacuum filtration before the broth was extracted using EtOAc (3 × 4 L). The organic fraction was concentrated *in vacuo* to give a crude extract (210 mg).

Biological assays: Antimycobacterial and cytotoxicity assays were performed as previously reported [9] against *Mycobacterium tuberculosis* H37Ra (ATCC 25177) and human embryonic kidney HEK 293 cells (ATCC CRL- 1573), respectively.

NMR metabolomic analysis: Fungal extracts exhibiting unique ^{1}H NMR spectra were identified within our library, as previously described [3c].

Bioassay guided fractionation: The crude extract of TC2-077 was dissolved in 9:1 MeOH/H_2O (7 mL) and extracted with hexanes (3 x 3 mL). The aqueous fraction was then diluted with H_2O (3.5 mL) and extracted with CH_2Cl_2 (3 x 3 mL). The CH_2Cl_2 fraction (80 mg) was subjected to a silica gel flash column eluted with a stepwise

gradient of hexanes to EtOAc (10% increments from 100% hexanes to 100% EtOAc). The fraction that eluted from the flash column in 7:3 hexanes/EtOAc (15 mg) was subjected to normal phase HPLC (3:2 hexanes/EtOAc) to give **4.1** (3mg) and the fraction that eluted from the flash column in 3:2 hexanes/EtOAc (7 mg) was subjected to reverse phase HPLC (43:57 MeOH/H_2O) to give **4.2** (2 mg).

Acknowledgments - The authors would like to thank Stephen Clayden (New Brunswick Museum), Larry Calhoun (University of New Brunswick) and Fabrice Berrué and Patricia Boland (University of Prince Edward Island) for their assistance with plant identification, acquisition of NMR spectra, and obtaining ESIMS data, respectively. Financial support for this research was provided by NSERC (Discovery Grant to CAG and CGS-D to TNC, the NBHRF (Seed Operating Grant to CAG and JAJ), the NBIF (Research Assistantship Initiative grants to CAG), and UNB (University Research Fund grants to CAG and JAJ) and is gratefully acknowledged.

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Supplemental information for Chapter Four

Table 4.2 Comparison of the NMR data obtained for phomopsolide A (**4.1**) with that reported by Stierle et al. [1]

Carbon	**4.1**[1]		Literature[2]	
	δ_C ppm (mult.)	δ_H ppm (int., mult., *J*/Hz)	δ_C ppm (mult.)*NR	δ_H ppm (int.*NR, mult., *J*/Hz)
1	162.1 (s)		162.5	
2	124.3 (d)	6.25 (1H, d, 9.7)	124.6	6.23 (d, 9.7)
3	141.1 (d)	7.10 (1H, dd, 9.8, 5.9)	141.5	7.10 (dd, 9.7, 5.4)
4	63.3 (d)	5.65 (1H, dd, 5.8, 2.7)	63.6	5.63 (m)
5	77.1 (d)	5.97 (1H, m)	77.3	5.96 (m)
6	143.1 (d)	6.42 (1H, d, 3.8)	143.1	6.40 (m)
7	124.7 (d)	6.41 (1H, s)	124.9	6.39 (m)
8	202.2 (s)		202.5	
9	73.3 (d)	4.36 (1H, q, 7.2)	73.5	4.34 (q, 6.3)
10	19.7 (q)	1.39 (3H, d, 7.0	19.9	1.37 (d, 6.3)
1′	166.6 (s)		166.9	
2′	127.6 (s)		127.8	
3′	139.7 (d)	6.85 (1H, m)	139.4	6.84 (m)
4′	14.7 (q)	1.79 (3H, bs)	14.9	1.75 (d, 6.3)
5′	12.2 (q)	1.80 (3H, m)	12.4	1.76 (br s)
9-OH		3.34 (1H, bs)		NR

*NR = Not reported
[1]Spectra recorded in $CDCl_3$ at 400MHz for ^1H and 100 MHz for ^{13}C
[2]Spectra recorded in $CDCl_3$ at 300MHz for ^1H and 75 MHz for ^{13}C

Table 4.3 Comparison of the NMR data obtained for phomopsolide C (**4.2**) with that reported by Stierle et al. [1]

Carbon	**4.2**[1]		Literature[2]	
	δ_C ppm (mult.)	δ_H ppm (int., mult., *J*/Hz)	δ_C ppm (mult.)*NR	δ_H ppm (int.*NR, mult., *J*/Hz)
1	161.6 (s)		161.9	
2	124.8 (d)	6.27 (1H, d, 9.8)	125.0	6.24 (d, 9.6)
3	141.0 (d)	7.04 (1H, dd, 9.7, 5.5)	141.3	7.02 (dd, 9.6, 5.4)
4	62.8 (d)	5.50 (1H, dd, 5.5, 3.2)	63.0	5.47 (dd, 5.4, 2.8)
5	77.4 (d)	5.30 (1H, m)	77.6	5.29 (m)
6	140.4 (d)	6.93 (1H, dd, 15.6, 3.7)	140.6	6.93 (dd, 15.3, 3.6)
7	126.1 (d)	6.74 (1H, dd, 15.6, 2.0)	126.3	6.72 (d, 15.3)
8	200.3 (s)		200.6	
9	72.3 (d)	4.43 (1H, m)	72.5	4.40 (q, 6.4)
10	20.0 (q)	1.39 (3H, d, 7.2)	20.2	1.37 (d, 6.4)
1′	166.6 (s)		166.9	
2′	127.4 (s)		127.6	
3′	139.1 (d)	6.84 (1H, m)	139.4	6.79 (m)
4′	14.8 (q)	1.78 (3H, m)	15.0	1.76 (d, 6.4)
5′	12.1 (q)	1.77 (3H, bs)	12.3	1.75 (br s)
9-OH		3.46 (1H, bs)		NR

*NR = Not reported
[1]Spectra recorded in $CDCl_3$ at 400MHz for ^1H and 100 MHz for ^{13}C
[2]Spectra recorded in $CDCl_3$ at 300MHz for ^1H and 75 MHz for ^{13}C

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Chapter Five

Antibiotic Mycotoxins from an Endophytic *Fusarium acuminatum* Isolated from the Medicinal Plant *Geum macophyllum*

Clark TN, Carroll M, Ellsworth K, Guerrette R, Robichaud GA, Johnson JA, and Gray CA. (**2018**), *Natural product Communications* 13 (10), 1301-1304.

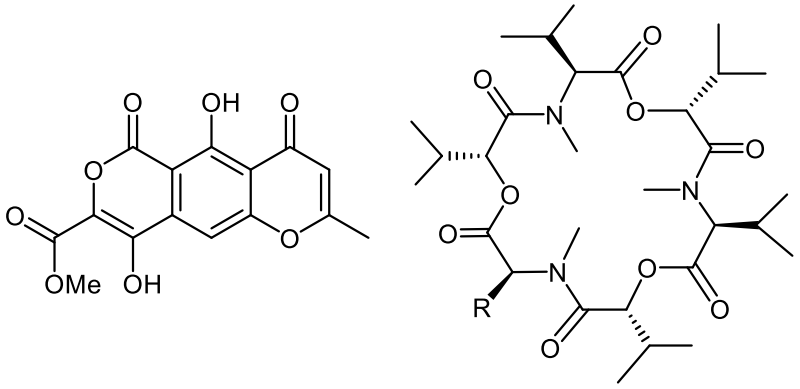
An extract of the endophytic fungus *Fusarium acuminatum* (TC2-084) isolated from the Canadian medicinal plant *Geum macrophyllum* exhibited significant antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra when fermented in both malt extract and potato dextrose broths. However, significant differences observed in the NMR spectra of the respective extracts led us to further investigate both. Bioassay guided fractionation revealed that lateropyrone was solely responsible for the bioactivity observed when TC2-084 was fermented in malt extract broth, while the antimycobacterial activity of the extract from the isolate fermented in potato dextrose broth was augmented by the presence of enniatins B, B1, and B4 in addition to lateropyrone.

Keywords: Lateropyrone, Enniatin B, Enniatin B1, Enniatin B4, Endophyte, *Geum macrophyllum*, Canadian medicinal plant, *Fusarium acuminatum*.

Endophytic fungi continue to be an important and prolific source of bioactive natural products [1], with particular interest being focused on those isolated from medicinal plants [2]. During our exploration of bioactive natural products produced by endophytes of medicinal plants of the Canadian Maritime provinces [3], a *Fusarium acuminatum* isolate (TC2-084) obtained from the large-leaved avens, *Geum macrophyllum*, attracted our attention; two extracts of this endophyte displayed similarly conspicuous profiles in

our antibiotic screening protocol but markedly different profiles in our metabolomic analyses.

Extracts of TC2-084 derived from both malt extract broth (MEB) and potato dextrose broth (PDB) fermentations displayed selective antibiotic (inhibition of Gram-positive bacteria and mycobacteria) and antifungal activity, yet only the PDB fermentation extract of the isolate was determined to be distinct within our extract library through NMR metabolomic analysis [4]. Subsequent bioassay guided fractionation of the TC2-084 extracts resulted in the isolation of lateropyrone (**5.1**; Figure 5.1) as the sole bioactive constituent from the MEB fermentation, whilst the PDB fermentation yielded the antimycobacterial enniatins B, B1 and B4 (**5.2** – **5.4**; Figure 5.1) in addition to **5.1**.



5.1

5.2: R= isopropyl
5.3: R= *sec*-butyl
5.4: R= isobutyl

Figure 5.1 Lateropyrone (**5.1**), Enniatin B (**5.2**), Enniatin B1 (**5.3**), and Enniatin B4 (**5.4**)

The natural products were characterized by high-resolution mass spectrometry, NMR spectroscopy and polarimetry. All four compounds gave affirmative ESI MS (Δmmu for $[M+H]^+$ < 3.4 ppm) and NMR (^{1}H for **5.1** and ^{1}H and ^{13}C for the enniatins) data that were in agreement with literature values [5]. As ^{13}C NMR data has not been

previously been reported for **5.1**, full NMR data has been included in this report. However, we observed discrepancies between the polarimetric data obtained for **5.2** – **5.4** and previously reported data.

All of the enniatins isolated from TC2-084 were laevorotatory, giving specific rotations that were consistent with published values for enniatins B (**5.2**) and B4 (**5.4**) [5b, 5d, 5e] but contrary to the data reported for enniatin B1 (**5.3**) [5b, 5c]. To confirm the absolute configuration of enniatin B1 we therefore determined the specific rotation of an authentic sample ($[\alpha]_D^{22}$ –172; Sigma-Aldrich) that concurred with that of the natural product ($[\alpha]_D^{22}$ –89), suggesting that the previously reported rotation for **5.3** ($[\alpha]_D$ +173.8) is erroneous [5b, 5c].

Lateropyrone and the enniatins are common, albeit minor, metabolites produced by *Fusarium* species that affect cereal grain production worldwide and much of the research reported on these metabolites therefore relates to their roles as mycotoxins [6]. Lateropyrone was first isolated as an antibiotic constituent of *Fusarium lateritium* [5a, 7] although initial uncertainty relating to the structure of **1** has led to it also appearing in the literature as both antibiotic Y [6] and avenacein Y [8], synonyms that have persisted particularly when **5.1** is reported as a metabolite of unspecified *Fusarium* spp. and *Fusarium avenaceum* respectively [9]. The production of enniatin depsipeptides is well documented in *Fusarium* species [10] and their biosynthesis has been shown to be variable between strains [10] and influenced by environmental factors such as nutrient source [9a] and culture conditions [11]. We only observed the enniatins to be produced by TC2-084 when fermented in PDB whilst lateropyrone was produced in similar yields in both culture broths (isolated yields of 0.3 mg/L in MEB and 0.5 mg/L in PDB). Indeed,

it was the characteristic δ-proton resonances (δ_H 2.12 ppm) of the enniatin amino acid residues that resulted in the discrimination of the TC2-084 PDB extract in our initial metabolomic screen.

Whilst the strong inhibitory activity displayed by **5.1** towards Gram-positive bacteria is in agreement with previous reports [5a, 12, 13], the antifungal and antimycobacterial activities of lateropyrone have not been previously reported. It is encouraging that lateropyrone exhibited essentially no toxic effects toward human cells (treatment with 750 µM **5.1** reduced proliferation of HEK 293 cells by 66%; our data suggest an IC_{50} of ca. 600 µM), resulting in notable therapeutic indices that support further investigation of its antibiotic and antifungal modes of action. In contrast, the cationophoric properties of the enniatins are well described and known to manifest a multitude of biological activities [10] including *in vitro* activity against mycobacteria and human cell lines [14]. These observations are in accordance with our bioactivity data and, although they exhibit impressive antimycobacterial activity and selective activity against the Gram-positive strains employed in this study, their general cytotoxicity precludes further investigation of **5.2** – **5.4**.

Experimental

General Experimental Procedures: Solvents for extraction and isolation were purchased from Fisher Scientific (Ottawa, ON, Canada) and deuterated solvents for NMR spectroscopy were purchased from Sigma-Aldrich (Oakville, ON, Canada). Flash chromatography was performed using a Biotage Flash+ chromatography system fitted with silica (normal phase) or C18 (reversed phase) SiliaSep cartridges (40-63 µm, 60 Å,

25g; SiliCycle, QC, Canada). Semi-preparative normal-phase HPLC was performed on a Phenomenex Luna silica column (250 × 10 mm, 10 µm, 100 Å) using a Waters 510 isocratic pump and a Waters R401 refractive index detector. Semi-preparative reversed-phase HPLC was performed on a Phenomenex Luna C18 column (250 × 10 mm, 10 µm, 100 Å) using an Agilent 1100 HPLC system comprising a G1311A quaternary pump and a G1315C diode array detector. Optical rotations were recorded on an Optical Activity Ltd. AA-10 polarimeter at 589 nm. NMR spectra were recorded on an Agilent 400-MR DD2 instrument in $CDCl_3$ and were calibrated to residual protonated solvent resonances (δ_H 7.260 and δ_C 77.160). HRMS data were recorded on a Thermo LTQ Exactive instrument with an ESI source.

Endophyte isolation: TC2-084 was isolated from the leaves of *Geum macrophyllum* (New Brunswick Museum voucher specimen NBM VP-37480) collected from Berryton, Albert County, NB, Canada (45˚53.251' N, 64˚52.482' W) in June 2011. Leaf surfaces were sterilized by sequential immersion in 5.25% aq NaClO (5 sec), sterile distilled water (10 sec) and 70% EtOH (15 sec). The tissue was then rinsed with autoclaved distilled water, blotted dry on an autoclaved paper towel, and cut into pieces (5 mm × 5 mm) that were placed onto 2% malt extract agar and incubated at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2% malt extract agar until pure cultures were obtained.

Endophyte identification: Isolate TC2-084 was identified as *Fusarium acuminatum* through examination of spore morphology and colonies grown on cornmeal, Czapek-Dox, malt extract and potato dextrose agars. The taxonomic classification was confirmed by comparison of the internal transcribed spacer and 5.8S rRNA gene (ITS) DNA regions

with corresponding sequences available in the GenBank database (National Center for Biotechnology Information, US National Library of Medicine, Bethesda, MD, USA). The genomic DNA of TC2-084 was isolated using a DNEasy® plant mini kit (Qiagen, Toronto, Ontario) as directed by the manufacturer, the ITS gene was amplified by PCR using the ITS 1 and ITS 4 universal fungal primers (Invitrogen, Burlington, Ontario) as previously described [15] and the amplified ITS DNA were sequenced by Genome-Québec (Montreal, Québec). The TC2-084 DNA sequence was checked for ambiguity before being compared with existing GenBank sequence data using BLAST. The ITS gene sequence of TC2-084 was found to have >99% homology with numerous conspecific *F. acuminatum* isolates and has been deposited in GenBank (accession number: KC916647).

Biological assays: Antifungal, antibiotic and cytotoxic activity was evaluated as previously described [16].

Fermentation and extraction: TC2-084 was fermented in 2% malt extract broth at room temperature with shaking (150 rpm) for 2 weeks (10 L; 100 × 100 mL batches in 250 mL Erlenmeyer flasks stoppered with foam baffles). The fungal material was removed by vacuum filtration before the spent broth was extracted with EtOAc (3 × 3 L). The organic fractions were combined and concentrated *in vacuo* to give a MEB extract (670 mg). Fermentation in 1.2% potato dextrose broth (5 L; 50 × 100 mL batches in 250 mL Erlenmeyer flasks stoppered with foam baffles) was conducted in a similar manner (EtOAc extraction performed with 3 × 1.5 L) to give a PDB extract (430 mg).

Bioassay guided fractionation: The TC2-084 MEB extract was dissolved in 9:1 MeOH/H_2O (100 mL) and extracted with hexanes (3 × 30 mL). The aqueous fraction was

then diluted with H_2O (50 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The CH_2Cl_2 fraction (270 mg) was subjected to silica gel flash chromatography (stepwise gradient from 100% hexanes to 100% EtOAc in 10% increments) to give 11 fractions. The fraction that eluted from the flash column in 3:7 hexanes/EtOAc (52 mg) was subjected to normal phase HPLC (1:3 hexanes/EtOAc) to give **1** (3 mg). A similar solvent partition of the TC2-084 PDB extract gave a CH_2Cl_2 fraction (382 mg) that was subjected to C-18 flash chromatography (stepwise gradient from 100% H_2O to 100% CH_3CN in 10% increments) to give 11 fractions. The fraction that eluted from the flash column in 1:4 H_2O/CH_3CN (56 mg) was subjected to reverse phase HPLC (gradient elution from 5:95 H_2O/CH_3CN to 100% CH_3CN and isocratic elution in 3:7 H_2O/CH_3CN) to give **2** (2 mg), **3** (2 mg), and **4** (3 mg). The CH_2Cl_2 fraction was also subjected to normal phase HPLC (1:3 hexanes: EtOAc) as well as a second normal phase HPLC injection (7:13 hexanes: EtOAc) to give more of **1** (2 mg).

Lateropyrone (5.1)

^{1}H NMR: data were in agreement with published values [5a]; see Table 5.2, supplemental data.

^{13}C NMR (100 MHz $CDCl_3$): 182.7 (s, C-4), 168.8 (s, C-2), 166.3 (s, C-5), 166.0 (s, C-6), 160.1 (s, C-11), 160.1 (s, C-10a), 145.9 (s, C-9), 137.9 (s, C-9a), 125.6 (s, C-8), 111.2 (s, C-4a), 110.5 (s, C-5a), 105.9 (d, C-3), 101.6 (d, C-10), 53.3 (q, 11-OMe), 20.9 (q, C-12).

HRESIMS *m/z* 319.0446 $[M+H]^+$ (calculated for $C_{15}H_{11}O_8^+$, 319.0448)

Enniatin B (5.2)

$[\alpha]_D^{22}$: –94 (*c* 0.27, MeOH)

^{1}H NMR and ^{13}C NMR: data were in agreement with published values [5b]; see Table 5.3, supplemental data.

HRESIMS *m/z* 640.4181 $[M+H]^+$ (calculated for $C_{33}H_{58}N_3O_9^+$, 640.4168)

Enniatin B1 (5.53)

$[\alpha]_D^{22}$: –89 (*c* 0.28, $CHCl_3$) for natural **5.3**; –172° (*c* 0.29, $CHCl_3$) for authentic **5.3**

^{1}H NMR and ^{13}C NMR: data were in agreement with published values [5c]; see Table 5.4, supplemental data.

HRESIMS *m/z* 654.4346 $[M+H]^+$ (calculated for $C_{34}H_{60}N_3O_9^+$, 654.4324)

Enniatin B4 (5.4)

$[\alpha]_D^{22}$: –110 (*c* 0.23, $CHCl_3$)

^{1}H NMR and ^{13}C NMR: data were in agreement with published values [5d]; see Table 5.5, supplemental data.

HRESIMS *m/z* 654.4339 $[M+H]^+$ (calculated for $C_{34}H_{60}N_3O_9^+$, 654.4324)

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Supplemental information for Chapter Five

Table 5.1 Biological activities (IC_{50} values and MICs in µM)[a] of isolated compounds (µM) against *Mycobacterium tuberculosis* H37Ra ATCC 25177, *Staphylococcus aureus* ATCC 29213, Methicillin resistant *Staphylococcus aureus* ATCC 33591 (MRSA), *Enterococcus faecium* ATCC 35667, Vancomycin resistant *Enterococcus faecium* ATCC 51559 (VRE), *Candida albicans* ATCC 14053, and human embryonic kidney cells (HEK) 293.

Compound	*M. tuberculosis*		*S. aureus*		MRSA		*E. faecium*		VRE		*C. albicans*		HEK 293
	IC_{50} (CI)[b]	MIC	IC_{50} (CI)	MIC	IC_{50} (CI)	MIC	IC_{50} (CI)	MIC	IC_{50} (CI)	MIC	IC_{50} (CI)	MIC	IC_{50} (CI)
1	5.70 (5.28 – 6.15)	20	7.48 (7.11 – 7.86)	10	4.39 (3.88 – 4.97)	10	49.2 (32.7 – 74.1)	80	30.6 (25.8 – 36.3)	40	23.9 (21.6 – 26.5)	40	NA
2	26.9 (23.0 – 31.4)	>200	NA		NA		22.1 (14.4 – 34.2)	40	NA		NA		25.5 (19.9 – 32.6)
3	5.49 (4.46 – 6.76)	160	42.6 (38.8 – 46.8)	80	33.9 (25.9 -44.3)	80	12.7 (10.8 – 14.9)	20	39.0 (24.7 -61.8)	80	50.0 (47.2 – 52.9)	80	9.46 (7.18 – 12.5)
4	12.0 (10.3 – 13.9)	160	NA		NA		13.3 (11.3 -15.6)	20	42.2 (36.5 -48.7)	80	NA		17.5 (13.3 – 22.9)

[a]IC_{50}: median lethal concentration; MIC: minimum inhibitory concentration. [b]CI: 95% confidence interval. [c]NA: not active; affected less than 50% growth inhibition at 50 µM

Table 5.2 Comparison of the NMR data obtained for lateropyrone (**5.1**) with those reported by Bushnell et al. [1]

Carbon	**5.1**[1]		Literature data[2]
	δ_C ppm (mult.)	δ_H ppm (int., mult.)	δ_H ppm (int., mult.)
2	168.8 (s)		
3	105.9 (d)	6.25 (1H, s)	6.26 (1H, s)
4	182.7 (s)		
4a	111.2 (s)		
5	166.3 (s)		
5a	110.5 (s)		
6	166.0 (s)		
8	125.6 (s)		
9	145.9 (s)		
9a	137.9 (s)		
10	101.6 (d)	7.45 (1H, s)	7.44 (1H, s)
10a	160.1 (s)		
11	160.1 (s)		
12	20.9 (q)	2.47 (3H, s)	2.45 (3H, s)
11-OMe	53.3 (q)	4.01 (3H, s)	4.01 (3H, s)
5-OH		14.68 (1H, s)	14.70 (1H, s)
9-OH		10.43 (1H, s)	10.40 (1H, s)

[1]Spectra recorded in $CDCl_3$ at 400 MHz for ^1H and 100 MHz for ^{13}C.
[2]Spectrum recorded in CD_2Cl_2 at 250MHz.

Table 5.3 Comparison of the NMR data obtained for Enniatin B (**5.2**) with those reported by Blais [2]

Carbon		**5.2**[1]		Literature data[2]	
		δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)	δ_C ppm	δ_H ppm (mult., *J*/Hz)
3 × NMeVal	αCH	63.2 (d)	4.50 (d, 10.1)	63.2	4.46 (d, 9.8)
	βCH	28.1 (d)	2.29 (m)	27.9	2.27 (m)
	γCH_3	19.4 (q)	1.06 (d, 6.2)	19.3	1.03 (d, 6.5)
		20.6 (q)	0.89 (d, 6.7)	20.4	0.86 (d, 6.8)
	N-Me	30.1 (q)	3.13 (s)	33.2	3.09 (s)
	C=O (amide)	169.3 (s)		169.3	
	C=O (ester)	170.4 (s)		170.3	
3 × HyIv	αCH	75.8 (d)	5.14 (d, 8.4)	75.7	5.11 (d, 8.8)
	βCH	28.1 (d)	2.29 (m)	29.9	2.27 (m)
	γCH_3	18.7 (q)	0.96 (d, 6.7)	18.5	0.93 (d, 6.7)
		18.9 (q)	0.99 (d, 6.7)	18.6	0.96 (d, 6.4)

[1]Spectra recorded in $CDCl_3$ at 400 MHz for ^1H and 100 MHz for ^{13}C.
[2]Spectra recorded in $CDCl_3$ at 500 MHz for ^1H and 125 MHz for ^{13}C.

Table 5.4 Comparison of the NMR data obtained for Enniatin B1 (**5.3**) with those reported by Blais et al. [3].

Carbon		**5.3**[1]		Literature data[2]	
		δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)	δ_C ppm (mult.)	δ_H ppm (mult., *J*/Hz)
2 × NMeVal	αCH	63.8 (d)	4.44 (d, 9.7)	63.5	4.42 (d, 8.6)
		63.5 (d)	4.49 (d, 9.7)	63.4	4.46 (d, 9.1)
	βCH	28.1 (d)	2.29 (bs)	27.9	2.27
	γCH_3	19.4 (q)	0.92-1.02 (bm)	19.4	1.02 (d, 6.3)
		20.6 (q)	0.89 (d, 6.5)	20.5	0.87 (d, 6.5)
	N-Me	33.7 (q)	3.13 (s)	33.4	3.10
	C=O (amide)	169.3 (s)		169.3	
	C=O (ester)	170.4 (s)		170.4	
NMeIle	αCH	61.6 (d)	4.71 (d, 8.9)	61.5	4.65 (d, 9.0)
	βCH	33.9 (d)	2.07 (bm)	33.8	2.04
	γCH_2	25.3 (t)	1.06 (bm)	25.3	1.04
			1.40 (bs)		1.42 (d, 13.8)
	γCH_3	16.3 (q)	0.92-1.02 (bm)	16.1	0.98
	δCH_3	11.0 (q)	0.84-0.91 (bm)	10.9	0.82 (d, 7.3)
	N-Me	32.9 (q)	3.10 (s)	32.9	3.08
	C=O (amide)	169.3 (s)		169.3	
	C=O (ester)	170.4 (s)		170.4	
3 × HyIv	αCH	75.9 (d)	5.13 (d, 8.8/8.6/8.9)	77.2	5.11 (d, 7.3, 9.1, or 7.9)

	75.5 (d)		75.0	
	75.9 (d)		75.6	
βCH	29.8 (d)	2.29 (bs)	29.7	2.24
	30.1 (d)		29.8	
	30.1 (d)		30.3	
γCH_3	18.6 (q)	0.91-1.02 (m)	18.2-18.9	0.92-0.98
	18.7 (q)			
	18.9 (q)			

[1]Spectra recorded in $CDCl_3$ at 400 MHz for ^{1}H and 100 MHz for ^{13}C.
[2]Spectra recorded in $CDCl_3$ at 500 MHz for ^{1}H and 125 MHz for ^{13}C.

Table 5.5 Comparison of the NMR data obtained for Enniatin B4 (**5.4**) with that reported by Visconti et al. [4].

Carbon		**5.4**		Literature data	
		δc ppm (mult.)	δ_H ppm (mult., *J*/Hz)	δc ppm	δ_H ppm (mult., *J*/Hz)
2 × NMeVal	αCH	61.5 (d)	4.93 (d, 10.8)	61.3	4.89 (10.2)
		63.4 (d)	4.43 (d, 10.8)	63.2	4.44 (10.0)
	βCH	27.7 (d)	2.16-2.36 (bm)	27.5	2.23
		27.9 (d)	2.16-2.36 (bm)	27.8	2.16
	γCH_3	20.1 (d0	1.03 (d, 6.1)	19.9	1.02 (6.2)
		20.5 (d)	0.85-1.00 (bm)	20.3	0.85 (6.8)
	N-Me	33.2 (q)	3.12 (s)	33.0	3.07
		34.0 (q)	3.15 (s)	33.8	3.10
	C=O (amide)	169.1 (s)		169.0	
		169.3 (s)		169.3	
	C=O (ester)	170.4 (s)		170.3	
		170.5 (s)		170.4	
NMeLeu	αCH	57.4 (d)	4.66 (bs)	57.2	4.66
	βCH_2	38.0 (d)	1.69 (bs)	37.9	1.73 (13.5)
			1.82 (bm)		1.81 (5.1, 10.1)
	γCH	25.4 (d)	1.56 (bm)	25.2	1.55
	δCH_3	21.7 (q)	0.85-1.00 (bm)	21.5	0.91 (6.6)
		23.5 (q)	0.85-1.00 (bm)	23.3	0.92 (6.6)
	N-Me	31.8 (q)	3.09 (s)	31.6	3.04
	C=O (amide)	169.6 (s)		169.6	
	C=O (ester)	170.8 (s)		170.7	
3 × HyIv	αCH	75.1 (d)	4.99 (d, 9.1)	74.9	4.98 (9.0)

	75.5 (d)	5.11 (d, 8.3)	75.2	5.09 (8.5)
	75,9 (d)	5.20 (d, 8.7)	75.6	5.18 (8.4)
βCH	29.9 (d)	2.16-2.36 (bm)	29.6	2.24
	30.0 (d)	2.16-2.36 (bm)	29.8	2.22
	30.4 (d)	2.16-2.36 (bm)	30.2	2.27
γCH_3	18.4 (q)	0.85-1.00 (bm)	18.3-18.9	0.93-0.97
	18.5 (q)			
	18.8 (q)			
	18.9 (q)			
	19.1 (q)			

[1]Spectra recorded in $CDCl_3$ at 400 MHz for ^1H and 100 MHz for ^{13}C.
[2]Spectra recorded in $CDCl_3$ at 500 MHz for ^1H and 125 MHz for ^{13}C.

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Chapter Six

Isolation of Isolignicol, a New Natural Product from an endophytic *Mollisia* sp., from the Medicinal Plant *Hypericum perforatum*

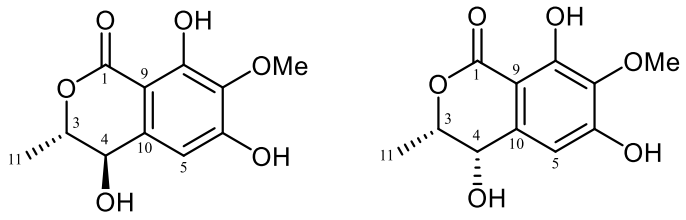
Clark TN, Ellsworth KT, Decken A, Johnson JA, and Gray CA. formatted for *Natural Product Communications*

An extract of a *Mollisia* sp. endophyte (TC2-035) isolated from the medicinal plant *Hypericum perforatum* showed a unique profile in our NMR-based metabolomic screen. NMR guided fractionation of the extract led to the isolation of two natural products, lignicol (**6.1**) and the new natural product isolignicol (**6.2**). Determination of the absolute stereochemistry of both compounds by Mosher's analysis led to the reassignment of the absolute configuration of lignicol (**6.1**).

Keywords: Endophyte, *Hypericum perforatum*, lignicol, isolignicol, metabolomics, leotiomycetes, *Mollisia*

Endophytic fungi are a promising source of natural products [1] and endophytes of medicinal plants are of prominent interest [2]. Research on our library of endophytic fungi isolated from medicinal plants of eastern Canada [3] revealed that an extract of an endophytic fungus, *Mollisia* sp. (TC2-035), isolated from St John's wort (*Hypericum perforatum L. hypericaceae*) showed a unique metabolic profile in our NMR based metabolomic screening when fermented in potato dextrose broth. Molecular taxonomy identified TC2-035 be a member of the genus *Mollisia* as it exhibited 95% sequence homology of the ITS gene with congeneric sequences in the NCBI GenBank database.

Large scale fermentation and NMR guided fractionation of the extract led to the isolation of two compounds in high yield (25 % and 50 % of the extract by mass, 33 and 67 mg/L, respectively). The first compound (isolated as 50% of the crude extract) was found to be lignicol (**6.1**, Figure 6.1), a polyketide previously isolated from *Scytalidium lignicola* in 1993, through comparison of NMR, HRMS, and polarimetric data with literature values [4]. The relative stereochemistry of lignicol was determined by Ayer et al. using x-ray crystallography, and the absolute configuration determined to be 3*R* and 4*S* by comparing Cotton effect values with similar compounds using a CD spectrum [4]. The second compound was found to be the *cis*-isomer isolignicol (**6.2**, Figure 6.1).



6.1: 3*S*,4*R* **6.2**: 3*S*,4*S*

Figure 6.1 Lignicol (**6.1**) and Isolignicol (**6.2**)

The structure of isolignicol was determined based on its similarity to ^{1}H, ^{13}C and HRMS spectra to lignicol, with differences seen in the ^{1}H chemical shifts between C-3 and C-4 (from δ_H 4.41 ppm overlapping to δ_H 4.63 ppm and δ_H 4.35 ppm respectively). The relative stereochemistry of **6.2** was determined to be *cis* by x-ray crystallography (Figure 6.2), although its absolute stereochemistry could not be unequivocally assigned from the crystal structure as the molecule does not contain atoms heavier than oxygen. The modified Mosher's method was therefore used to determine the absolute configuration at C-4 of **6.2** (Figure 6.3) [5] and allowed the assignment of a 3*S*, 4*S*

absolute stereochemistry. The difference in stereochemistry between **6.1** and **6.2** therefore appeared to involve the methyl group at C-3 instead of the alcohol at C-4, which was contrary to our expectations given that oxidation at C-4 occurs after ring closure in the biosynthesis of these polyketides [6]. This led us to investigate and confirm the absolute stereochemistry of lignicol. Subsequent application of Mosher's method to lignicol [5] (Figure 6.4) indicated that the absolute stereochemistry of **6.1** was 3*S*,4*R*, opposite to that reported by Ayer et al (3*R*,4*S*) [4].

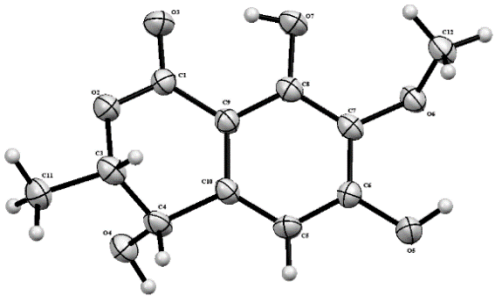
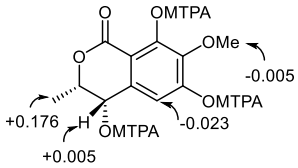
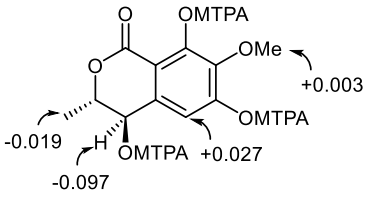


Figure 6.2 ORTEP diagram of Isolignicol (**6.2**) obtained by crystal structure analysis



Compound	δ H-4	δ H-5	δ H-11	δ OMe
S-MTPA ester	5.201	7.105	1.587	3.928
R-MTPA ester	5.196	7.128	1.411	3.933
$\Delta\delta$ ($\delta_S - \delta_R$)	+0.005	-0.023	+0.176	-0.005

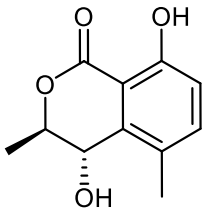
Figure 6.3 key proton chemical shifts and calculated Δδ values obtained for compound **6.2** by the modified Mosher's method.



Compound	δ H-4	δ H-5	δ H-11	δ OMe
S-MTPA ester	5.161	6.650	1.384	3.455
R-MTPA ester	5.258	6.623	1.403	3.452
$\Delta\delta$ ($\delta_S - \delta_R$)	-0.097	+0.027	-0.019	+0.003

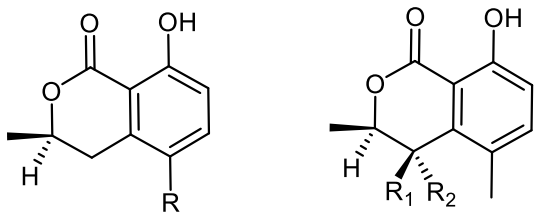
Figure 6.4 key proton chemical shifts and calculated Δδ values obtained for compound **6.1** by the modified Mosher's method.

Ayer et al. [4] originally assigned the absolute configuration of **6.1** based upon a negative Cotton effect observed at 269 nm in the CD spectrum that compared well with the Cotton effect observed for the related metabolite *R*-mellein. However, in 1986 Okuno et al. [6] found that substituents at C-3 and C-4 can have a large effect on the CD spectra of these systems, as (3*R*,4*S*)-*trans*-4-hydroxy-5-methylmellein (**6.3**, Figure 6.5) exhibited a positive cotton effect at 255 nm that was opposite to that observed for other structurally related isocoumarins (**6.4-6.7**, Figure 6.6). This finding shows that the original designation (3*R*,4*S*) is incorrect, as both the results from the Mosher's analysis and the observed Cotton effect in the CD spectrum of **6.1** indicate a (3*S*,4*R*) stereochemistry.



6.3

Figure 6.3 (3*R*,4*S*)-*trans*-4-hydroxy-5-methylmellein (**6.3**)



6.4 R= CH_3
6.5 R= COOH
6.6 R= CH_2OH

6.3 R_1= OH, R_2= H
6.7 R_1= H, R_2= OH

Figure 6.4 (-)-5-methylmellein (**6.4**), (-)-5-carboxylmellein (**6.5**), (-)-5-hydroxylmethylmellein (**6.6**), (+)-(3*R*,4*S*)-*trans*-4-hydroxy-5-methylmellein (**6.3**), and (-)-(3*R*,4*R*)-*cis*-4-hydroxy-5-methylmellein (**6.7**)

Both **6.1** and **6.2** did not show any bioactivity when screened against the four test pathogens *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, or Mycobacterium tuberculosis* H37Ra. The use of NMR based metabolomics on our library of fungal extracts has shown to be a promising technique to direct efforts towards new structures. Future investigations of our library of endophytes from medicinal plants will continue to explore extracts showing unique metabolic profiles though NMR based metabolomics.

Experimental

Endophyte isolation: TC2-035 was isolated from the leaves of *Hypericum perforatum* (New Brunswick Museum voucher specimen NBM VP-37566) collected from Millidgeville in Saint John, NB, Canada (N 45° 18.318’ W 66° 05.201’) in August 2010 [3]. Leaf surfaces were sterilized by immersion in 5.25% aqueous sodium hypochlorite for 5 sec, followed by sterile distilled water for 10 sec and 70% EtOH for 10 sec. The tissue was then rinsed with autoclaved distilled water, blotted dry on an autoclaved paper

towel, and cut into pieces (5 mm × 5 mm) that were placed onto 2% malt extract agar and incubated at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2% malt extract agar until pure cultures were obtained.

Identification of endophytes: TC2-035 did not produce fruiting bodies with characteristic morphological features on malt extract, potato dextrose or Czapek's agars. DNA extraction and sequencing results using methods previously described [8] revealed a 95 % similarity to other fungi from the Genus *Mollisia* through the NCBI BLAST database.

Fermentation and extraction: TC2-035 was fermented in 1.2% potato dextrose broth at room temperature with shaking (150 rpm) for 2 weeks (0.3 L; 3 × 100 mL batches in 250 mL Erlenmeyer flasks covered with foam baffles). The fungal material was separated from the broth using gravity filtration before the broth was extracted using EtOAc (3 × 100 mL). The organic fraction was concentrated *in vacuo* to give a crude extract (42 mg).

Biological assays: Antifungal, and antibiotic activity was evaluated as previously described [9].

NMR metabolomic analysis: Fungal extracts exhibiting unique ^{1}H NMR spectra were identified within our library, as previously described [7a].

NMR guided fractionation: A portion of the crude extract of TC2-035 (20 mg) was subjected to reverse phase HPLC (35:65 Acetonitrile/H_2O) to give **1** (10 mg) and **2** (5 mg).

X-Ray Crystallography: Crystals of isolignicol were grown by solvent evaporation using methanol at 20 C°. Single crystals were coated with Paratone-N oil, mounted using a polyimide MicroMount and frozen in the cold nitrogen stream of the goniometer. A hemisphere of data was collected on a Bruker AXS P4/SMART 1000 diffractometer

using ω and ϕ scans with a scan width of 0.3 ° and 10 s exposure times. The detector distance was 5 cm. The data were reduced (SAINT) [9] and corrected for absorption (SADABS) [10]. The structure was solved by direct methods and refined by full-matrix least squares on F2(SHELXTL) [11]. All non-hydrogen atoms were refined using anisotropic displacement parameters. Hydrogen atoms were found in Fourier difference maps and refined using isotropic displacement parameters. Due to the light atom nature of the molecule, the absolute configuration could not be determined and all entries in the cif pertaining to it have been removed.

Mosher's analysis: Solutions of (*S*)-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride and (*R*)-(−)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (3.4 mg; 2.5 μL; 3.2 eq. each) in pyridine-*d5* (50 μL) were separately added to two portions of isolignicol (1 mg; 4.16 μmol) in pyridine-*d5* (500 μL) in NMR tubes at room temperature. The mixtures were left for four hours at room temperature and ^{1}H NMR spectra were obtained. For comparative purposes, a ^{1}H NMR spectrum of isolignicol (3 mg) was recorded in pyridine-*d5*. The resonances in ^{1}H NMR spectra of the *S*-MTPA ester (derived from the *R*-MTPCl) and the *R*-MTPA ester (derived from the *S*-MTPCl) were assigned and the Δδ values (Δδ=Δ*S*−Δ*R*) of key proton resonances were calculated.

Lignicol

$[\alpha]_D^{22}$: + 21.3 (*c* 0.12, MeOH)

IR (NaCl, thin film): 3338, 2936, 1655, 1459, 1357, 1277, 1108, 807 cm^{-1}.

^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz CD_3OD): see supplemental information

^{1}H NMR (400 MHz, DMSO-*d6*) and ^{13}C NMR (100 MHz DMSO-*d6*): see supplemental information

^{1}H NMR (400 MHz, pyridine-*d5*): 4.76 (H-4, 1H, m), 7.21 (H-5, 1H, s), 1.58 (H-11, 3H, d, *J* = 6.1 Hz), 3.93 (OMe, 3H, s)

HRESIMS *m/z* 241.0707 $[M+H]^+$ (calculated for $C_{11}H_{13}O_6^+$, 241.0707)

Isoliginicol

$[\alpha]_D^{22}$: + 65.2 (*c* 0.04, MeOH)

IR (NaCl, thin film): 3423, 1637, 1378, 1278, 1174, 1102, 860, 808 cm^{-1}

^{1}H NMR (400 MHz, DMSO-*d6*) and ^{13}C NMR (100 MHz DMSO-*d6*): see supplemental information

^{1}H NMR (400 MHz, pyridine-*d5*): 4.75 (H-4, 1H, bs), 6.99 (H-5, 1H, s), 1.68 (H-11, 3H, d, *J* = 6.6 Hz), 3.87 (OMe, 3H, s)

HRESIMS *m/z* 241.0707 $[M+H]^+$ (calculated for $C_{11}H_{13}O_6^+$, 241.0707)

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Supplemental information for Chapter Six

Table 6.1 Comparison of the NMR data obtained for lignicol (**6.1**) with those reported by Ayer et al. 1993

	6.1[1]		Literature data[2]	
Carbon	δ_C ppm (mult.)	δ_H ppm (int., mult.)	δ_C ppm (mult.)	δ_H ppm (int., mult. *J*/Hz)
1	170.5 (s)		170.5 (s)	
3	81.3 (d)	4.54 (1H, m)	81.4 (d)	4.50 (1H, m)
4	69.5 (d)	4.54 (1H, m)	69.5 (d)	4.50 (1H, m)
5	106.6 (d)	6.67 (1H, s)	106.9 (d)	6.67 (1H, s)
6	158.8 (s)		158.8 or 157.4 (s)	
7	135.7 (s)		135.7 (s)	
8	157.4 (s)		158.8 or 157.4 (s)	
9	100.8 (s)		100.9 (s)	
10	140.2 (s)		140.2 (s)	
11	18.1 (q)	1.51 (3H, d, 6.6)	18.1 (q)	1.50 (3H, d, 6)
OMe	60.9 (q)	4.01 (3H, s)	60.9 (q)	4.03 (3H, s)
4-OH				2.06 (1H, d, 6)
6-OH		6.41 (1H, s)		6.42 (1H, s)
8-OH		11.34 (1H, s)		11.36 (1H, s)

[1]Spectra recorded in $CDCl_3$ at 400MHz for ^1H and in CD_3OD at100 MHz for ^{13}C
[2]Spectra recorded in $CDCl_3$ at 500MHz for ^1H and in CD_3OD at125 MHz for ^{13}C

Table 6.2 NMR data obtained for lignicol (**6.1**) and isolignicol (**6.2**)

	6.1[1]		**6.2**[1]	
Carbon	δ_C ppm (mult.)	δ_H ppm (int., mult. *J*/Hz)	δ_C ppm (mult.)	δ_H ppm (int., mult. *J*/Hz)
1	168.5(s)		169.3 (s)	
3	79.5(d)	4.41 (1H, m,)	81.4 (d)	4.63 (1H, qd, 6.4, 2.2)
4	67.3(d)	4.41 (1H, m)	69.5 (d)	4.35 (1H, bs)
5	105.3 (d)	6.57 (1H, s)	106.9 (d)	6.46 (1H, s)
6	157.3 (s)		158.8 (s)	
7	133.9 (s)		135.7 (s)	
8	155.6 (s)		157.4 (s)	
9	99.3 (s)		100.9 (s)	
10	139.5 (s)		140.2 (s)	
11	17.7 (q)	1.36 (3H, d, 5.9)	18.1 (q)	1.35 (3H, d, 6.6)
OMe	59.9 (q)	3.70 (3H, s)	60.9 (q)	3.70 (3H, s)
4-OH		2.07 (1H, s)		2.07 (1H, s)
6-OH		5.96 (1H, d, 6.5)		5.57 (1H, d, 5.5)
8-OH		11.10 (1H, s)		11.20 (1H, s)

[1]Spectra recorded in DMSO-*d6* at 400MHz for ^{1}H and 100 MHz for ^{13}C

[1] Ayer WA, Lu P-P, Orszanska H, Sigler L. (**1993**) Deoxyscytalidin and lignicol: New metabolites from *Scytalidium* species. *Journal of Natural Products*, ***56***, 1835-1838.

Chapter Seven

Isolation of Glomerellamide, an unusual 6-methyl 2,4-tetradecadienoyl amide isolated from a *Glomerella acutata* endophyte of the Canadian medicinal plant *Aralia nudicaulis*

Clark TN, Ellsworth KT, Kerr R, Johnson JA, and Gray CA. Formatted for submission to *Journal of Natural Products*

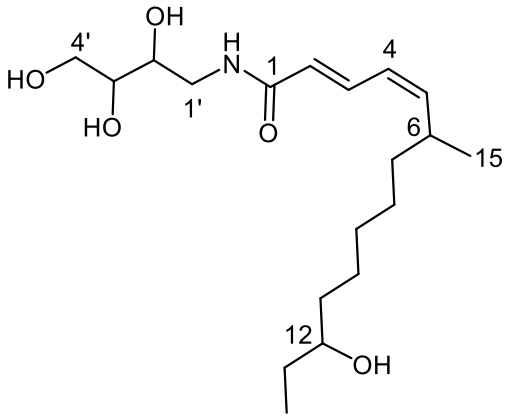
ABSTRACT

An extract of the endophytic fungus *Glomerella acutata* (TC2-019) contained a novel compound identified using mass spectrometry-based metabolomics. HRMS guided fractionation of a large-scale fermentation of the fungus led to the isolation of glomerellamide (**7.1**), a new amide containing both a 4-amino-butane triol and a 6-methyl 2,4-tetradecadienoyl moeity that displayed mild antibiotic activity against *Mycobacterium tuberculosis* H37Ra.

Keywords: Endophyte, Medicinal plant, *Glomerella acutata*, Glomerellamide, metabolomics, HRMS, *Aralia nudicaulis*

Endophytic fungi, particularly those isolated from medicinal plants are a promising source of natural products [1]. During our investigation of 81 endophytic fungi from North American medicinal plants [2], a *Glomerella acutata* isolate (TC2-019) from wild sasparilla (*Aralia nudicaulis* L. Araliaceae) was highlighted in an untargeted HRMS-based metabolomic screening. The screening directed attention towards an extract containing protonated and sodiated molecular ions that showed no matches in the Antibase chemical database, suggesting the presence of a novel natural product.

HRMS guided fractionation (liquid-liquid partition and HPLC) of an extract derived from a TC2-019 fermentation broth (1.2 % potato dextrose) resulted in the isolation of glomerellamide (0.2 % by weight, 0.03 mg per 1L) (**7.1**, Figure 7.1).



7.1

Figure 7.1 Glomerellamide (**7.1**)

The molecular formula of glomerellamide was determined to be $C_{19}H_{35}NO_5$ from HRESIMS data for the protonated and sodiated molecular ions (358.2589, 380.2405 respectively). The ^{1}H and ^{13}C NMR indicated the presence of two double bonds (δ_C 144.6, 133.8, 125.6, and 125.4 ppm) and one carbonyl group (δ_C 165.6 ppm) that was assigned to an amide due to the presence of a deshielded proton (δ_H 8.02 ppm) that did not give an HSQC cross peak. Having accounted for all the degrees of unsaturation implied by the molecular formula, we then concluded that the molecule was a straight chain. 1D NMR also indicated the presence of three oxymethines (δ_C 72.0, 70.9, and 69.4 ppm) and one oxymethylene (δ_C 62.4 ppm). The HSQC spectrum showed four further protons that were not attached to any carbon atoms, therefore being assigned as hydroxyl protons (δ_H 4.48, 4.43, 4.39, and 4.20 ppm). The remaining carbons were determined to

be a methine (δ_C 32.1 ppm), seven methylenes (δ_C 42.0, 36.6, 36.6, 29.8, 29.3, 27.0, 25.2) and two methyls (δ_C 21.0 and 10.0 ppm). The seven methylenes and terminal methyl group indicated the presence of a large alkyl chain with overlapping signals in the ^{1}H NMR spectrum.

The planar structure of glomerellamide was elucidated using COSY and HMBC spectra (Figure 7.2). The COSY spectrum was used to assign the positions of the hydroxyl protons through the observation of COSY cross peaks between 2′-OH and H-2′ (δ_H 4.48 and 3.54 ppm, respectively), 3′-OH and H-3′ (δ_H 4.39 and 3.35 ppm, respectively), 4′-OH and H_2-4′ (δ_H 4.43 and 3.54 and 3.42 ppm), and 12-OH and H-12 (δ_H 4.20 and 3.26 ppm, respectively). The COSY spectrum also indicated the presence of three isolated spin systems (C-1′ – C-2′, C-2 – C-6 methyl (C-15), and C-13 – C-14) and thus revealed the three main fragments of the molecule as follows: The first fragment showed COSY cross peaks between the hydroxyl (δ_H 4.48 ppm) to H-1′ (δ_H 3.11 and 3.29 ppm), and COSY cross peaks between H-1′ to the amide proton (δ_H 8.02 ppm). The second fragment determined was a conjugated double bond system connecting C-2 through to C-5 (δ_H 6.05, 7.28, 6.05, and 5.52 ppm for C-2, C-3, C-4, and C-5, respectively). The conjugated system also had COSY signals connecting C-5 to C-6 (δ_H 2.67 ppm) and then C-6 to C-15 (δ_H 0.95 ppm). The conjugated systems stereochemistry was determined using coupling constants indicating *E* ($^3J_{2,3}$ = 14.2 Hz) and *Z* ($^3J_{4,5}$ = 10.4 Hz) configurations for the C-2 and C-4 double bonds respectively. The third fragment of the compound that was assembled using the COSY spectrum connected C-13 to C-14 (δ_H 1.21 and 0.82 ppm, respectively).

The remainder of the compound was assembled using HMBC data. The first fragment assembled by the COSY spectrum was extended to create a 4-amino-butane triol fragment through correlations between H-2′ (δ_H 4.48 ppm) to C-3′ (δ_C 72.0 ppm), 3′-OH (δ_H 4.39 ppm) to C-4′ (δ_C 62.4 ppm) and confirming signals from 4′-OH (δ_H 4.43 ppm) to C-3′ (δ_C 72.0 ppm). Placing the carbonyl group (δ_C 165.6 ppm) next to the conjugated methylene system was done using key signals between H-2 (δ_H 6.05 ppm), H-3 (δ_H 7.28 ppm) and N-H (δ_H 8.02 ppm) to the C-1 (δ_C 165.6 ppm). The alkyl chain was connected to C-6 using signals from H-5 (δ_H 5.52 ppm) and H-15 (δ_H 0.95 ppm) to the methylene C-7 (δ_C 33.6 ppm). The hydroxyl proton 12-OH (δ_H 4.20 ppm) showed HMBC cross peaks to the methylenes C-13 (δ_C 29.8 ppm) and C-11 (δ_C 36.6 ppm). This configuration was confirmed by HMBC cross peaks between hydrogens H-13 (δ_H 1.21 ppm) to the methine C-12 (δ_C 70.9 ppm).

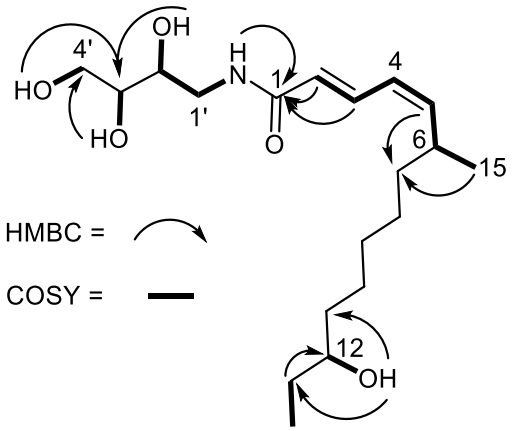


Figure 7.2 COSY and selected HMBC correlations for glomerellamide (**7.1**)

The absolute configuration of the molecule has not yet been determined. Our strategy is to obtain a VCD spectrum of **7.1** and use Mosher's analysis to determine the absolute stereochemistry of the secondary alcohol at C-12. We will then attempt to

determine the absolute configuration of the rest of the molecule through analysis of the VCD spectrum once stereochemistry of C-12 has been determined.

HRMS guided fractionation directed towards a compound with a protonated molecular ion of 358.2589 in the extract from TC2-019 successfully led to the isolation of glomerellamide, a novel natural product. Glomerellamide features a 4-amino-butane triol, which has not been isolated from nature before this study. It also features an unusual methylated 2,4-tetradecadienoyl moiety that has not been seen before. Related 2,4-tetradecadienoyl fatty acid moieties have been isolated as amides of piperidine or isobutylamine although these have lacked substitution at C-6 and C-12 [3]. Screening of glomerellamide against a panel of human pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, or Mycobacterium tuberculosis* H37Ra) showed that it has mild bioactivity against *Mycobacterium tuberculosis* with an IC_{50} of ~300 µM. Untargeted HRMS based metabolomics has shown to be a promising prioritization method for fungi which will continue to be used on our library of endophytes to find other new natural products.

Experimental

General Experimental Procedures: Solvents for extraction and isolation were purchased from Fisher Scientific (Ottawa, ON, Canada) and deuterated solvents for NMR spectroscopy were purchased from Sigma-Aldrich (Oakville, ON, Canada). Semi-preparative reversed-phase HPLC was performed on a Phenomenex Luna C18 column (250 × 10 mm, 10 µm, 100 Å) using an Agilent 1100 HPLC system comprising a G1311A quaternary pump and a G1315C diode array detector. Optical rotations were

recorded on an Optical Activity Ltd. AA-10 polarimeter at 589 nm. IR spectra were recorded on a PerkinElmer FTIR Spectrum Two. NMR spectra were recorded on an Bruker AVIII 700 instrument equipped with a QNP cryoprobe in DMSO-*d6* and were calibrated to residual protonated solvent resonances (δ_H 2.50 and δ_C 39.52). HRMS data were recorded on a Thermo LTQ Exactive instrument with an ESI source.

Endophyte isolation and identification: TC2-019 was isolated from the leaves of *Aralia nudicaulis* (New Brunswick Museum voucher specimen NBM VP-37477) collected from Millidgeville in Saint John, NB, Canada (N 45° 18.398' W 66° 05.704') in August 2010 [2d]. Leaf surfaces were sterilized by immersion in and 70% EtOH for 20 sec. The tissue was then rinsed with autoclaved distilled water, blotted dry on an autoclaved paper towel, and cut into pieces (5 mm × 5 mm) that were placed onto 2% malt extract agar and incubated at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2% malt extract agar until pure cultures were obtained.

Isolate TC2-019 was identified as *Glomerella acutata* through examination of spore morphology and colonies grown on cornmeal, Czapek-Dox, malt extract and potato dextrose agars. The taxonomic classification was confirmed by comparison of the internal transcribed spacer and 5.8S rRNA gene (ITS) DNA regions with corresponding sequences available in the GenBank database (National Center for Biotechnology Information, US National Library of Medicine, Bethesda, MD, USA). The genomic DNA of TC2-019 was isolated using a DNEasy® plant mini kit (Qiagen, Toronto, Ontario) as directed by the manufacturer, the ITS gene was amplified by PCR using the ITS 1 and ITS 4 universal fungal primers (Invitrogen, Burlington, Ontario) as previously described [3] and the amplified ITS DNA were sequenced by Genome-Québec

(Montreal, Québec). The TC2-019 DNA sequence was checked for ambiguity before being compared with existing GenBank sequence data using BLAST. The ITS gene sequence of TC2-019 was found to have >99% homology with numerous conspecific *Glomerella acutata* isolates and has been deposited in GenBank (accession number: KC916653).

Fermentation and extraction: TC2-019 was fermented in 1.2 % potato dextrose broth at room temperature with shaking (150 rpm) for 2 weeks (30 L; 300 × 100 mL batches in 250 mL Erlenmeyer flasks covered with foam baffles). The fungal material was separated from the broth using vacuum filtration before the broth was extracted using EtOAc (3 × 10 L). The organic fraction was concentrated in vacuo to give a crude extract (436 mg).

Biological assays: Antifungal, and antibiotic activity was evaluated as previously described [4].

LCHRMS metabolomic analysis: LCHRMS based metabolomics were performed on 81 endophytic fungi grown in triplicate using processing methods as described by Forner et al. [5].

HRMS guided fractionation: The crude extract of TC2-019 was dissolved in 9:1 MeOH/H_2O (50 mL) and extracted with hexanes (3 x 20 mL). The aqueous fraction was then diluted with H_2O (25 mL) and extracted with CH_2Cl_2 (3 x 20 mL). The aqueous fraction was then evaporated in vacuo and then reconstituted in H_2O (50 mL) before being extracted with EtOAc (3 x 20 mL). The EtOAc fraction (53 mg) was subjected to reverse phase HPLC (Gradient from 95:5 H_2O: Acetonitrile to 100 % Acetonitrile over ten minutes and held at 100 % acetonitrile for five min) to give two fractions. Fraction two (Retention time 8 - 10 min, 12 mg) was subjected to reverse phase HPLC (7:3 H_2O:

acetonitrile) to give **7.1** (1 mg). The same procedure was repeated on a subsequent 60 L of fermented broth to give another 3 mg.

Glomerellamide (7.1):

$[\alpha]_D^{22}$: + 65.2 (*c* 0.38, MeOH)

IR (NaCl, thin film): 3350, 2928, 1654, 1554, 1025 cm^{-1}

^{1}H NMR (DMSO-*d6*, 700 MHz): 8.02 (1H, t, *J* = 5.6 Hz, NH), 7.28 (1H, dd, *J* = 14.2, 11.9 Hz, H-3), 6.05 (2H, m, H-3), 6.05 (2H, m, H-4), 5.52 (1H, t, *J* = 10.4 Hz, H-5), 4.48 (1H, d, *J* = 5.4 Hz, 2′-OH), 4.43 (1H, bt, 5′-OH), 4.39 (1H, d, *J* = 4.4 Hz, 3′-OH), 4.20 (1H, d, *J* = 5.4 Hz, 12-OH), 3.54 (1H, m, H-2′), 3.42 (1H, m, H-4′a), 3.35 (1H, m, H-4′b), 3.35 (1H, m, H-3′), 3.29 (1H, m, H-1′a), 3.26 (1H, m, H-12), 3.11 (1H, m, H-1′b), 2.67 (2H, m, H-6), 1.33 (1H, m, H-11a), 1.30 (1H, m, H-10a), 1.29 (1H, m, H-7a), 1.25 (1H, m, H-11b), 1.23 (2H, m, H-9), 1.22 (1H, m, H-10b), 1.21 (1H, m, H-7b) , 1.21 (2H, m, H-13) , 1.20 (2H, m, H-8), 0.95 (3H, d, *J* = 6.9 Hz, H-15), 0.82 (3H, t, *J* = 7.4 Hz, H-14)

^{13}C NMR (DMSO-*d6*, 175 MHz): 165.6 (C, C-1), 144.6 (CH, C-5), 133.8 (CH, C-3), 125.6 (CH, C-2), 125.4 (CH, C-4), 72.0 (CH, C-3′), 70.9 (CH, C-12), 69.4 (CH, C-2′), 62.4 (CH_2, C-4′), 42.0 (CH_2, C-1′), 36.64 (CH_2, C-7), 36.58 (CH_2, C-11), 32.1 (CH_2, C-6), 29.8 (CH_2, C-13), 29.3 (CH_2, C-9), 27.0 (CH_2, C-8), 25.2 (CH_2, C-10), 21.0 (CH_3, C-15), 10.0 (CH_3 C-14)

HRESIMS: *m/z* 358.2592 $[M+H]^+$ (calculated for $C_{19}H_{36}NO_5^+$, 358.2588)

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to TNC, the NBHRF (Seed Operating Grant to CAG and JAJ), the NBIF (Research Assistantship Initiative grants to CAG), and UNB (University Research Fund grants to CAG and JAJ) and is gratefully acknowledged.

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Supplemental information for Chapter Seven

Table 7.1 NMR data obtained for glomerellamide (**7.1**)

Carbon	**7.1**[1]	
	δ_C ppm (mult.)	δ_H ppm (int., mult., *J*/Hz)
1	165.6 (s)	-
2	125.6 (d)	6.05 (2H, m)
3	133.8 (d)	7.28 (1H, dd, 14.2, 11.9)
4	125.4 (d)	6.05 (2H, m)
5	144.6 (d)	5.52 (1H, t, 10.4)
6	32.1 (d)	2.67 (2H, m)
7	36.6 (t)	1.21, 1.29 (2H, m)
8	27.0 (t)	1.20 (2H, m)
9	29.3 (t)	1.23 (2H, m)
10	25.2 (t)	1.22, 1.30 (2H, m)
11	36.6 (t)	1.25, 1.33 (2H, m)
12	70.9 (d)	3.26 (1H, m)
13	29.8 (t)	1.21 (2H, m)
14	10.0 (q)	0.82 (3H, t, 7.4)
15	21.0 (q)	0.95 (3H, d, 6.9)
1′	42.0 (t)	3.11, 3.29 (2H, m)
2′	69.4 (d)	3.54 (1H, m)

3′	72.0 (d)	3.35 (1H, m)
4′	62.4 (t)	3.42, 3.35 (2H, m)
12-OH		4.20 (1H, d, 5.4)
2′-OH		4.48 (1H, d, 5.4)
3′-OH		4.39 (1H, d, 4.4)
4′-OH		4.43 (1H, bt)
N		8.02 (1H, t, 5.6)

[1]Spectra recorded in DMSO-*d6* at 700MHz for ^{1}H and 175 MHz for ^{13}C

Chapter Eight

Isolation of Chaetomorin A, a caryophyllene derivative isolated from a *Chaetomium globosum* endophyte of the Canadian Medicinal Plant *Empetrum nigrum*

Clark TN, Ellsworth KT, Kerr R, Johnson JA, and Gray CA. formatted for submission to *Journal of Natural Products*

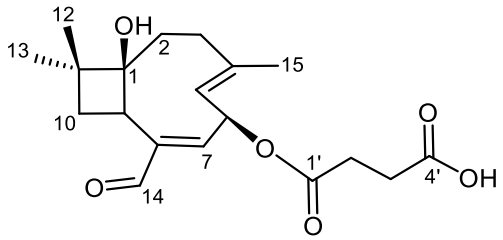
ABSTRACT

An extract of the endophytic fungus *Chaetomium globosum* (TC2-041) contained a novel compound identified by using mass spectrometry-based metabolomics. HRMS guided fractionation of a large-scale fermentation of the fungus led to the isolation of chaetomorin A (**8.1**), a new caryophyllene sesquiterpene. Chaetomorin A showed moderate bioactivity against both *Staphylococcus aureus* (IC_{50} of 105 µM) and *Mycobacterium tuberculosis* (IC_{50} of 237 µM).

Keywords: Endophyte, Medicinal plant, *Chaetomium globosum*, Chaetomorin, metabolomics, HRMS, *Empetrum nigrum*

Endophytic fungi, particularly those isolated from medicinal plants are a promising source of natural products [1]. During our investigation of endophytic fungi from North American medicinal plants [2] a *Chaetomium globosum* (TC2-041) from *Empetrum nigrum* isolate in our library was highlighted by untargeted HRMS-based metabolomic screening. The screening identified an extract containing protonated and sodiated molecular ions that showed no matches in the Antibase chemical database, suggesting the presence of a novel natural product.

HRMS guided fractionation, consisting of a liquid-liquid partition and HPLC of an extract of TC2-041, led to the isolation of the chaetomorin A (**8.1**, Figure 8.1). Chaetomorin A is a caryophyllene sesquiterpene similar in structure to the pestalotiopsins [3], the pestaloporinates [4], and the punctaporonins [3c, 5].



8.1

Figure 8.1 Chaetomorin A (**8.1**)

The molecular formula of chaetomorin A was determined to be $C_{19}H_{26}O_6$ from HRESIMS data for the protonated and sodiated molecular ions (351.1800 and 373.1618 respectively). The ^{1}H and ^{13}C NMR indicated three carbonyl groups (δ_C 195.5, 173.4, and 171.5 ppm) and two double bonds (δ_C 156.5, 141.6, 139.8, and 124.5 ppm). The molecular formula indicated seven degrees of unsaturation, implying that the structure also contained two rings. The 1D NMR revealed additional resonances that were assigned to three methyl groups (δ_C 25.8, 23.8, and 16.9 ppm), five methylenes (δ_C 28.9, 28.9, 34.9, 34.3, and 30.1 ppm), one methine (δ_C 41.3 ppm), one oxymethine (δ_C 71.7 ppm), one quaternary carbon (δ_C 42.1 ppm), and one oxygenated quaternary carbon (δ_C 78.8 ppm).

The planar structure of chaetomorin A was determined through analysis of COSY and HMBC data (Figure 8.2). The COSY spectrum indicated the presence of three isolated spin systems (C-2 – C-3, C-7 – C-4 methyl (C-15), and C-9 – C-10) and revealed

the three main fragments of the molecule as follows: The first fragment showed COSY cross peaks between H_2-2 (δ_H 2.13 and 1.63 ppm) and H_2-3 (δ_H 2.47 and 1.88 ppm). The second spin system connects the methine H-5 (δ_H 5.41 ppm) to the oxymethine H-6 (δ_H 6.05 ppm), the oxymethine H-6 to another methine H-7 (δ_H 6.69 ppm), and long range COSY relation between H-5 and the methyl group H-15 (δ_H 1.91 ppm). The final spin system that could be determined through the COSY NMR spectrum connected a methine H-9 (δ_H 3.15 ppm) to a methylene H_2-10 (δ_H 2.57 and 1.58 ppm).

The spin systems created from the COSY NMR spectrum were then assembled using an HMBC spectrum. The second and third spin systems were connected through HMBC cross peaks between the H-14 aldehyde (δ_H 9.46 ppm on δ_C 195.5 ppm, seen through HSQC) to the methines C-7 and C-9 (δ_C 156.5 and 41.3 ppm, respectively) and a quaternary carbon at C-8 (δ_C 141.6 ppm). The first spin system determined from the COSY spectrum was then determined to be connected through HMBC cross peaks between H-5 (δ_H 5.41 ppm) to the methylene C-3 (δ_C 34.3 ppm). The carbonyl C-1′ (δ_C 171.5 ppm) was identified as an ester on the other side of oxymethine C-6 (δ_C 71.6 ppm) by HMBC cross peaks from H-6 (δ_H 6.05 ppm) to C-1′. The ester C-1′ was determined to be part of a succinic acid side chain based on HMBC cross peaks between H_2-2′ (δ_H 2.50 and 2.56 ppm) and C-1′, C-3′ and C-4′ (δ_C 171.5, 28.9, and 173.4 ppm, respectively, and because of cross peaks between H_2-3′ (δ_H 2.50 and 2.56 ppm), and C-1′, C-3′ (δ_C 28.7 ppm) and C-4′. The methylene groups C-2′ and C-3′ of the succinic acid side chain could not be distinguished from one another. The remaining components of the compound were now two methyl groups, one quaternary carbon and one oxygenated quaternary carbon, and two ring systems. This indicated that the remaining carbons must form a

cyclobutane ring in order to satisfy the degrees of unsaturation for the compound. This structure was then supported with HMBC cross peaks from the hydroxyl signal (δ_H 4.20 ppm) to the methylene C-2 (δ_C 34.9 ppm), and the methine C-9 (δ_C 41.3 ppm), and HMBC cross peaks from the methyl groups C-12 and C-13 (δ_H 1.04 and 0.98 ppm, respectively) to each other (δ_C 23.8 and 25.8 ppm, respectively) the methylene C-10 (δ_C 30.1) and methines C-1 and C-9 (δ_C 78.8 and 42.1 ppm, respectively). The cyclobutane ring was also supported by HMBC cross peaks between both the H_2-10 methylene (δ_H 2.57 and 1.58 ppm) and the H-9 methine (δ_H 3.15 ppm) to both C-11 and C-1 (δ_C 42.1 and 78.8 ppm, respectively). It was further supported by HMBC cross peaks from methylene H_2-2 (δ_H 2.13 and 1.63 ppm) to C-1 (δ_C 78.8 ppm) but no cross peaks to C-11 (δ_C 42.1 ppm).

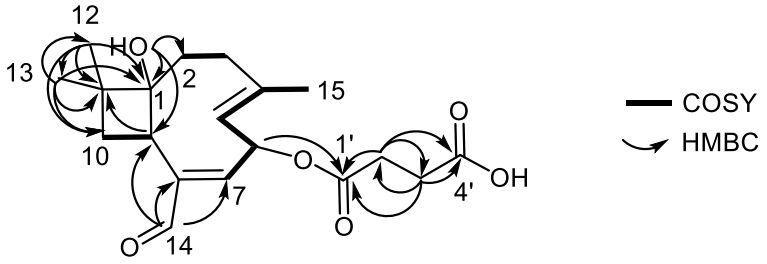


Figure 8.2 COSY and selected HMBC correlations for chaetomorin A (**8.1**)

The relative stereochemistry of **8.1** was determined by analysis of ROESY data (Figure 8.3). NOESY correlations between H-10a (δ_H 2.54 ppm) and H_3-12 (δ_H 1.04 ppm), H_3-12 and 1-OH (δ_H 4.20), and 1-OH and H-2a (δ_H 1.62 ppm) clearly placed the 1-OH and 12-Me on the same face of the molecule, designated β through biosynthetic considerations [3b, 3c, 4, 5]. Confirmatory correlations were observed on the α-face of the molecule between H-2b (δ_H 2.13ppm) and H_3-13 (δ_H 0.98 ppm), and H_3-13 and H-10b

(δ_H 1.54 ppm), with addition correlations between H-2b and H-9 (δ_H 3.15 ppm) and H-9 and H-10b indicating a trans ring fusion. Finally, a correlation between H-9 and H-6 (δ_H 6.05 ppm) placed the C-6 ester substituent on the β-face and completed the assignment of the relative stereochemistry of **8.1**.

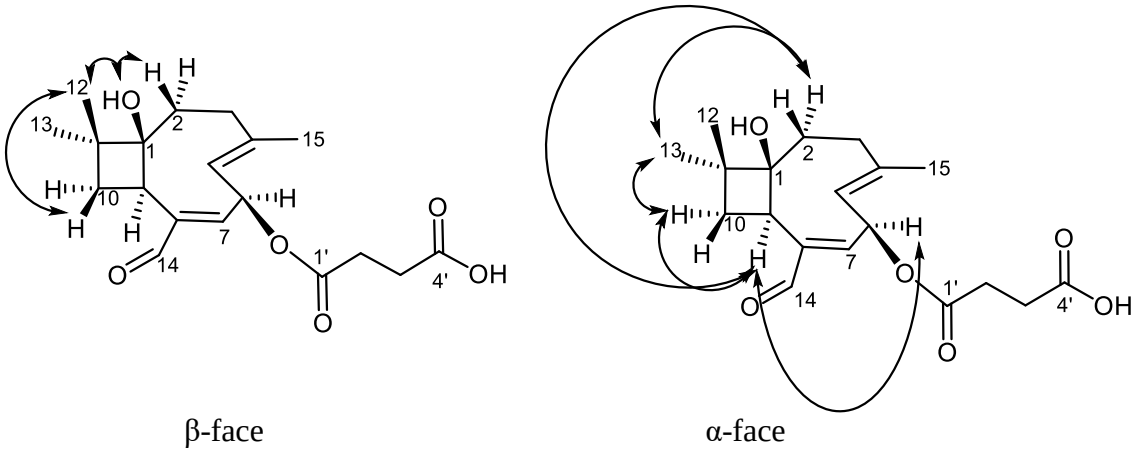


Figure 8.3 Key NOESY correlations to assign relative stereochemistry of chaetomorin A (**8.1**)

The stereochemistry of the double bonds was also determined by analysis of ROESY data (Figure 8.4). NOESY correlations between H-14 (δ_H 9.46 ppm) and H-7 (δ_H 6.69 ppm) determined that the double bond from C-7 to C-8 was of *Z* configuration. NOESY correlations between H_3-15 (δ_H 1.91ppm) and H-6 (δ_H 6.05 ppm), and correlations between H-3a (δ_H 2.47 ppm) and H-5 (δ_H 5.41 ppm), 1-OH (δ_H 4.20 ppm) and H-5, and H-9 (δ_H 3.15 ppm) and H-5 designated an *E* configuration at the double bond from C-4 to C-5.

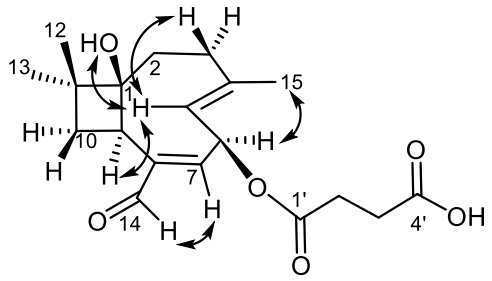


Figure 8.4 Key NOESY correlations used to assign stereochemistry of the double bonds in chaetomorin A

Other caryophyllene sesquiterpenes have exhibited anticancer activity against A549, HeLa, and SMMC-7721 cell lines, with pestalotiopsin C showing activity levels (IC_{50} = 28.3 µM) comparable etoposide (IC_{50}= 23.2 µM) when tested against against SMMC-7721 [3c,3d]. The punctaporonins have shown anti-inflammatory activity, antibiotic activity, and cytotoxic activity against numerous cancer cell lines [3c, 5g]. This study found chaetomorin A to have moderate bioactivity against *Mycobacterium tuberculosis* and *Staphylococcus aureus* with IC_{50} values of 105 and 237 µM, respectively. Previous reports have shown mild activity for other caryophyllene sesquiterpenes against *S. aureus*, but no previous reports of activity against *M. tuberculosis*.

This was the first isolation of the compound chaetomorin A which is a caryophyllene sesquiterpene featuring an aldehyde, and a succinic acid side chain, both unusual features among other similar caryophyllene derivatives [3-5]. The isolation of this compound continues to show the power of untargeted HRMS based metabolomics as a prioritization method to find new natural products and will continue to be used on the other fungal isolates in our library to find other new natural products.

Experimental

General Experimental Procedures: Solvents for extraction and isolation were purchased from Fisher Scientific (Ottawa, ON, Canada) and deuterated solvents for NMR spectroscopy were purchased from Sigma-Aldrich (Oakville, ON, Canada). Semi-preparative reversed-phase HPLC was performed on a Phenomenex Luna C18 column (250 × 10 mm, 10 µm, 100 Å) using an Agilent 1100 HPLC system comprising a G1311A quaternary pump and a G1315C diode array detector. Optical rotations were recorded on an Optical Activity Ltd. AA-10 polarimeter at 589 nm. IR spectra were recorded on a PerkinElmer FTIR Spectrum Two. NMR spectra were recorded on a Bruker AVIII 700 instrument equipped with a QNP cryoprobe in DMSO-*d6* and were calibrated to residual protonated solvent resonances (δ_H 2.50 and δ_C 39.52). HRMS data were recorded on a Thermo LTQ Exactive instrument with an ESI source.

Endophyte isolation and identification TC2-041 was isolated from the leaves of *Empetrum nigrum* (New Brunswick Museum voucher specimen NBM VP-37479) collected from Spruce Lake in Saint John, NB, Canada (N 45° 11.955’ W 66° 13.800’) in August 2010 [6]. Leaf surfaces were sterilized by immersion in 5.25% aqueous sodium hypochlorite for 5 sec, followed by sterile distilled water for 10 sec and 70% EtOH for 10 sec. The tissue was then rinsed with autoclaved distilled water, blotted dry on an autoclaved paper towel, and cut into pieces (5 mm × 5 mm) that were placed onto 2% malt extract agar and incubated at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2% malt extract agar until pure cultures were obtained.

Isolate TC2-041 was identified as *Chaetomium globosum* through examination of spore morphology and colonies grown on cornmeal, Czapek-Dox, malt extract and potato

dextrose agars. The taxonomic classification was confirmed by comparison of the internal transcribed spacer and 5.8S rRNA gene (ITS) DNA regions with corresponding sequences available in the GenBank database (National Center for Biotechnology Information, US National Library of Medicine, Bethesda, MD, USA). The genomic DNA of TC2-041 was isolated using a DNEasy® plant mini kit (Qiagen, Toronto, Ontario) as directed by the manufacturer, the ITS gene was amplified by PCR using the ITS 1 and ITS 4 universal fungal primers (Invitrogen, Burlington, Ontario) as previously described [7] and the amplified ITS DNA were sequenced by Genome-Québec (Montreal, Québec). The TC2-041 DNA sequence was checked for ambiguity before being compared with existing GenBank sequence data using BLAST. The ITS gene sequence of TC2-041 was found to have 100% homology with numerous conspecific *C. globosum* isolates and has been deposited in GenBank (accession number: KC916674).

Fermentation and extraction: TC2-041 was fermented in 1.2 % potato dextrose broth at room temperature with shaking (150 rpm) for 2 weeks (10 L; 100 × 100 mL batches in 250 mL Erlenmeyer flasks covered with foam baffles). The fungal material was separated from the broth using vacuum filtration before the broth was extracted using EtOAc (3 × 3 L). The organic fraction was concentrated *in vacuo* to give a crude extract (241 mg).

Biological assays: Antifungal, and antibiotic activity was evaluated as previously described [8].

LCHRMS metabolomic analysis: LC-HRMS based metabolomics were performed on 81 endophytic fungi grown in triplicate using processing methods as described by Forner et al. [9].

HRMS guided fractionation: The crude extract of TC2-041 was dissolved in 9:1 MeOH/H_2O (50 mL) and extracted with hexanes (3 x 20 mL). The aqueous fraction was then diluted with H_2O (25 mL) and extracted with CH_2Cl_2 (3 x 20 mL). The CH_2Cl_2 fraction (201 mg) was subjected to reverse phase HPLC (Gradient from 95:5 H_2O: Acetonitrile to 100 % Acetonitrile over ten minutes and held at 100 % acetonitrile for five min) to give two fractions. Fraction two (Retention time 10.6 – 11.0 min, 54 mg) was subjected to reverse phase HPLC (65:35 H_2O: acetonitrile) to give **8.1** (22 mg).

Chaetomorin A

$[\alpha]_D^{22}$: -198.8 (*c* 0.14, MeOH)

IR (NaCl, thin film): 3423, 2933, 1732, 1371, 1165, 993 cm^{-1}

^{1}H NMR (DMSO-*d6*, 700 MHz): 12.25 (1H, s, 4′-OH), 9.46 (1H, s, H-14), 6.69 (1H, bs, H-7), 6.05 (1H, dd, *J* = 10.6, 2.3 Hz, H-6), 5.41 (1H, d, *J* = 10.4 Hz, H-5), 4.20 (1H, s, 1-OH), 3.15 (1H, dd, *J* = 10.8, 5.7 Hz, H-9), 2.57 (1H, m, H-10), 2.56 (1H, m, H-2′ or H-3′), 2.56 (1H, m, H-2′ or H-3′), 2.50 (1H, m, H-2′ or H-3′), 2.50 (1H, m, H-2′ or H-3′), 2.47 (1H, m, H-3), 2.13 (1H, td, *J* = 14.0, 3.7 Hz, H-2), 1.91 (3H, s, H-15), 1.88 (1H, m, H-3), 1.63 (1H, m, H-2), 1.58 (1H, m, H-10), 1.04, 3H, s, H-12), 0.98, 3H, s, H-13)

^{13}C NMR (DMSO-*d6*,175 MHz): 195.5 (CH, C-14), 173.4 (C, C-4′), 171.5 (C, C-1′), 156.5 (CH, C-7), 141.6 (C, C-8), 139.8 (C, C-4), 124.5 (CH, C-5), 78.8 (CH, C-1), 71.6 (CH, C-6), 42.1 (C, C-11), 41.3 (CH, C-9), 34.9 (CH_2, C-2), 34.3 (CH_2, C-3), 30.1 (CH_2, C-10), 28.9 (CH_2, C-3′ or C-2′), 28.7 (CH_2, C-3′ or C-2′), 25.8 (CH_3, C-13), 23.8 (CH_3, C-12), 16.9 (CH_3, C-15)

HRESIMS: *m/z* 351.1805 $[M+H]^+$ (calculated for $C_{19}H_{27}O_6^+$, 351.1802)

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Supplemental information for Chapter Eight

Table 8.1 NMR data obtained for Chaetomorin A (**8.1**)

Carbon	**8.1**[1]	
	δ_C ppm (mult.)	δ_H ppm (int., mult., *J*/Hz)
1	78.8 (s)	
2	34.9 (t)	2.13 (1H, td, 14.0, 3.7)
		1.63 (1H, m)
3	34.3 (t)	2.47 (1H, m)
		1.88 (1H, m)
4	139.8 (s)	
5	124.5 (d)	5.41 (1H, d, 10.4)
6	71.6 (d)	6.05 (1H, dd, 10.6, 2.3)
7	156.5 (d)	6.69 (1H, bs)
8	141.6 (s)	
9	41.3 (d)	3.15 (1H, dd, 10.8, 5.7)
10	30.1 (t)	2.57 (1H, m)
		1.58 (1H, m)
11	42.1 (s)	
12	23.8 (q)	1.04 (3H, s)
13	25.8 (q)	0.98 (3H, s)
14	195.5 (d)	9.46 (1H, s)

15	16.9 (q)	1.91 (3H, s)
1′	171.5 (s)	
2′	28.7* (t)	2.50 (1H, m)
		2.56 (1H, m)
3′	28.9* (t)	2.50 (1H, m)
		2.56 (1H, m)
4′	173.4 (s)	
1 - OH		4.20 (1H, s)
4′ - OH		12.25 (1H, s)

[1]Spectra recorded in DMSO-*d6* at 700MHz for ^{1}H and 175 MHz for ^{13}C

*May be interchangeable

Chapter Nine

General Discussion, Conclusions, and Future Work

Discussion

Investigation into a library of endophytic fungi has led to the isolation of twelve natural products, three of which are new. Firstly, using bioactivity as a prioritization method (Chapters two and five) led to the isolation of known compounds with previously unreported bioactivity (abscisic acid and lateropyrone). Using a combination of NMR based metabolomics and bioactivity to prioritize extracts (Chapters three, four and five) also led to the isolation of known natural products with previously unreported bioactivity (avenaciolide, phomopsolides A and C, and enniatins B, B1, and B4). NMR based metabolomics as a prioritization method (Chapter six) led to the isolation of two natural products (lignicol and isolignicol), one of which was a new isomer (isolignicol), both showing no bioactivity against the four pathogens mentioned in chapter one (*Staphyloccocus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, or *Mycobacterium tuberculosis*). And finally, HRMS based metabolomics (Chapters seven and eight) led to the isolation of two new compounds with moderate bioactivity (glomerellamide and chaetomorin A).

Bioactivity-based prioritization was not successful at finding new chemistry during this project. However, bioactivity-based prioritization did allow us to find new sources for known compounds, which may be useful if previous sources are difficult to find or work with. Finding new biological activity for known compounds is also beneficial as they would still have potential to have relevant applications that were

previously unknown. Bioactivity-based prioritization's strengths come from its cost, where it does not require expensive equipment like an LC-HRMS or NMR, but it does require more technical skill if the bioassays are being done in-house and by hand to maintain and work with microorganisms. More complex bioactivity-based prioritization methods such as BioMAP have shown to be a promising approach in the field [1], however, it is generally not feasible unless robotics for the bioassays are available.

NMR based metabolomics was also not a very promising technique for isolating new compounds, as it often led to the isolation of known chemistry. Its strengths lie in the fact that the technique is rapid and non-destructive to the sample, although the instrumentation is costly [2]. The NMR based metabolomics did lead to the isolation of a new compound however it was made in very high yields and would have likely been prioritized as an interesting spectra using a quick visual inspection of NMR spectra in the endophytic extract library.

Many improvements could be made to the NMR based metabolomics, such as adding a chemical dereplication step where a database of known NMR spectra might be used to help minimize efforts spent isolating known compounds. Even with a database of spectra, this would remain a difficult task, as the database would need to contain spectra that were obtained in the correct deuterated solvent so that the signals would match that of the library being investigated. It may also be difficult to match spectra of crude extracts to the pure compounds of the database as the extracts would be complex mixtures.

The complexity of extracts could easily be dealt with using LC-NMR [3], which would increase overall effectiveness of NMR based metabolomics both with and without

the incorporation of a database. Finally, our NMR based metabolomics were performed using a 400 MHz NMR spectrometer, however, if a stronger magnet of higher field strength were available it would increase resolution of the 1D NMR or could allow for the use of 2D or 3D NMR [4] experiments to be used, which is the direction that natural products chemistry is headed along with LC-NMR.

HRMS based metabolomics was found to be the most effective prioritization method leading to only new natural products (glomerellamide and chaetomorin A) without directing towards false positives. The most important component of HRMS based metabolomics is the chemical database which allows for a dereplication step to ensure that you are working on an extract that contains a new natural product [5]. With this method the limitations surrounding isolating a new compound are determined by how comprehensive your database is, whether you can create enough crude extract, and whether you can isolate and determine the structure of the natural product using the equipment available.

Conclusion

The most effective prioritization method which should continue to be used is the same as that in Chapter seven and Chapter eight, where LC-HRMS based metabolomics is the primary method used to prioritize extracts, with a positive result in a bioactivity screen being a secondary criterion that an extract would need to exhibit for it to be prioritized for further study. The main consideration with HRMS based metabolomics combined with bioactivity, is that the bioactivity seen in the extract does not necessarily belong to the molecular ion of interest identified by the metabolomics. To improve the

ability of finding a novel bioactive compound, evaluation of bioactivity at each fractionation step should be performed. If the bioactivity is not observed in the fraction with molecular ion targeted by HRMS based metabolomics, then the project should be abandoned. This would allow for the most amount of time and effort to be spent on extracts that may contain a novel bioactive compound.

Significance and Future Work

When this project began there was very little literature surrounding the application of NMR based metabolomics to fungi, and no studies employing untargeted LC-HRMS based metabolomics had been applied to a fungal library. There have since been a small handful of papers that support my findings that LC-HRMS based metabolomics is a successful method for finding new natural products from fungi [6]. Future work for the projects relating to this thesis include the determination of the absolute stereochemistry of glomerellamide (**7.1**) and chaetomorin A (**8.1**). Glomerellamide (**7.1**) has a total of four stereocenters that will need to have their absolute stereochemistry determined. We will be obtaining vibrational circular dichroism (VCD) spectra of **7.1** can be used to determine the absolute configuration, performed by Biotools (Jupiter Florida, USA). The relative stereochemistry of chaetomorin A (**8.1**) has been assigned using the ROESY NMR spectrum, however, the absolute configuration of the compound remains to be determined. Our first method will be to perform a reduction reaction to remove the succinic acid group as well as reduce the aldehyde using lithium aluminum hydride in anhydrous ether. The reduction will be followed by Mosher's method, attaching acid chlorides to both the secondary and primary alcohol groups to

form esters. The absolute configuration of the secondary alcohol can then be determined, which will then provide the remaining absolute configuration using the relative stereochemistry obtained from the NOESY spectrum. If that method proves to be ineffective, then VCD will also be used for absolute configuration determination of chaetomorin A as well (Biotools, Juniper, Florida, USA).

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Curriculum Vitae

Candidate's full name: Trevor Nathan Clark

Universities attended (with dates and degrees obtained):

University of New Brunswick, Saint John
2008-2012 Bachelor of Science with Honours
2012- Doctor of Philosophy

Publications:

Clark TN, Carroll M, Ellsworth K, Guerrette R, Robichaud GA, Johnson JA, Gray CA. (2018). Antibiotic Mycotoxins from an Endophytic Fusarium acuminatum Isolated from the Medicinal Plant Geum macrophyllum. *Natural Product Communications*. 13 (10), 1301-1304.

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Conference Presentations:

Clark TN, Johnson JA, and Gray CA (August 2018). Bioactive natural products from endophytes of Canadian medicinal plants. Maritime Natural Products Conference, Halifax, Canada. Oral Presentation

Clark TN, Johnson JA, and Gray CA (May 2018). Bioactive natural products from endophytes of Canadian medicinal plants. 101st Canadian Chemistry Conference and Exhibition, Edmonton, Canada. Poster Presentation

Clark TN, Johnson JA, and Gray CA (August 2017). Bioactive Natural products from endophytes of Canadian medicinal plants. Maritime natural products chemistry conference, Charlottetown, Canada. Oral presentation

Forgrave L, **Clark TN**, Johnson JA, and Gray CA (August 2017). Natural products from Canadian medicinal plants of the genus pyrola. Maritime Natural product chemistry conference, Charlottetown, Canada. Oral presentation

Armstrong K, **Clark TN**, Johnson JA, and Gray CA (August 2017). Antimycobacterial natural products from the Canadian medicinal plant *Pinus strobus*. Maritime Natural product chemistry conference, Charlottetown, Canada. Oral presentation

Therrien J, Bos A, Clinton ML, Complak K, Cox S, **Clark TN**, Ellsworth K, Flewelling AJ, Forgrave L, Morehouse NJ, Nazara A, Pendleton K, Veljanovska M, Johnson JA, and Gray CA (August 2017). The NPRG fungal endophyte library. Maritime natural products chemistry conference, Charlottetown, Canada. Poster presentation

Clark TN, Boland P, Berrué F, Kerr RG, Johnson JA, and Gray CA (August 2016). A comparison between the application of NMR and LC-HRMS metabolomics on the discovery of natural products from endophytic fungi. Maritime Natural products chemistry conference, Halifax, Canada. Poster presentation

Clark TN, Boland P, Berrué F, Kerr RG, Johnson JA, and Gray CA (July 2016). A comparison between the application of NMR and LC-HRMS metabolomics on the discovery of natural products from endophytic fungi. Joint Natural Products conference, Copenhagen, Denmark. Poster Presentation

Clark TN, Boland P, Berrué F, Kerr RG, Johnson JA, and Gray CA (May 2016). NMR and LC-HRMS metabolomics applied to natural product discovery from endophytic fungi. Canadian Chemistry Conference and Exhibition, Halifax, Canada. Poster presentation

Clark TN, Johnson JA, and Gray CA (March 2016). The application of an NMR based metabolomics approach to the discovery of biologically active natural products from endophytic fungi. Interprofessional Health research conference, Saint John, Canada. Poster Presentation

Clark TN, Johnson JA, and Gray CA (August 2015). Application of NMR/LC-HRMS based metabolomics in bioactive natural product discovery. Maritime Natural products chemistry conference, Saint John, NB, Canada. Poster presentation

Clark TN, Johnson JA, and Gray CA (May 2015). Application of NMR/LC-HRMS based metabolomics in bioactive natural product discovery. Chemcon UNB, Fredericton, Canada. Oral presentation

Clark TN, Johnson JA, and Gray CA (March 2015). Metabolomic approaches to increasing the efficiency of natural products for drug discovery. Interprofessional Health Research conference, Saint John, Canada. Poster presentation

Clark TN, Johnson JA, and Gray CA (November 2014). Metabolomic approaches to increasing the efficiency of natural products for drug discovery. New Brunswick Health Research Foundation Annual Conference 2014, Moncton, Canada. Poster presentation

Clark TN, Johnson JA, and Gray CA (August 2014). Application of qNMR to the identification of nuissance compounds in antimycobacterial bioassays. Maritime Natural Products conference, Charlottetown, PE, Canada. Oral presentation

Brown CA, **Clark TN**, Johnson JA, and Gray CA (August 2014). Bioactivity profiling as a strategy for natural products discovery. Maritime Natural Products Conference, Charlottetown, PE, Canada. Poster presentation

Carroll M, **Clark TN**, Johnson JA, and Gray CA (August 2014). Bioactive natural products of the endophyte Fusarium acuminatum from the medicinal plant Geum Macrophyllum. Maritime Natural Products Conference 2014, Charlottetown, Canada. Oral presentation

Bishop AI, **Clark TN**, Johnson JA, and Gray CA (August 2014). Identification of avenaciolide as the natural product responsible for the anti-mycobacterial activity from Seimatosporium sp., an endophyte from Hypericum perforatum. Maritime Natural Products conference, Charlottetown, Canada. Oral presentation

Clark TN, Flewelling AJ, Li H, Johnson JA, and Gray CA (March 2014). Endophytic fungi and their role in the future of drug discovery. Interprofessional health research, Saint John, Canada. Poster presentation

Clark TN, Flewelling AJ, Li H, Johnson JA, and Gray CA (November 2013). Could endophytic fungi play a role in the future of drug discovery? NBHRF 5th annual conference, Saint John, Canada. Poster Presentation

Clark TN, Ellsworth K, Li H, Webster D, Johnson JA, and Gray CA (August 2013). Isolation of abscisic acid, an antimycobacterial compound from the endophyte *Nigrospora* sp. Maritime Natural Products Chemistry Conference, Antigonish, Canada. Oral presentation

Bishop AI, **Clark TN**, Johnson JA, and Gray CA (August 2013). Antibiotic natural products from a *Fusarium* sp. endophyte extract of the medicinal plant *Moneses uniflora*. Maritime Natural Products conference 2013, Antigonish, Canada. Poster presentation

Clark TN, Ellsworth K, Johnson JA, and Gray CA (March 2013). Isolation of an antimycobacterial natural product from a fungal endophyte of the medicinal plant *Fragaria virginiana*. Interprofessional Health Research Conference, Saint John, Canada. Oral presentation

Clark TN, Johnson JA, and Gray CA (March 2012). Bioassay guided fractionation of cytotoxic compounds from the algal endophyte *Penicillium decumbens*. Interprofessional Health Research conference, Saint John, Canada. Poster presentation