BIOACTIVE NATURAL PRODUCTS FROM ENDOPHYTIC FUNGI

by

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ABSTRACT

Natural products are a source of diverse chemical structures with a broad range of bioactivities that provide valuable lead-compounds for drug development. This thesis explores the diversity of natural products biosynthesised by endophytes of marine macroalgae and medicinal plants. Fermentation extracts obtained from four endophytic fungi exhibited significant antimicrobial activity in our bioassay screens and their natural products chemistry was therefore investigated. This resulted in the isolation of poly(3*R*,5*R*-dihydroxyhexanoic acid), neobulgarones D, E and F, altenusin, (*Z*)-6*R**,7*S**-dihydroxy-2-propyl-2,4-octadien-4-olide, (*Z*)-6*R**,7*R**-dihydroxy-2-propyl-2,4-octadien-4-olide and punctaporonins A, B, C, T and U. Of these natural products, six were found to possess antimicrobial activity and three were new chemical structures. The discovery of these new bioactivities and new chemical structures adds to the growing evidence that endophytic fungi are a source of chemical diversity and biologically relevant molecules.

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List of Abbreviations

^{13}C NMR	Carbon nuclear magnetic resonance spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
$[\alpha]_D$	Specific rotation
ACS	American Chemical Society
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bs	Broad singlet
c	Concentration (quoted in g/100 ml)
CFU	Colony Forming Units
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
DMSO	Dimethyl sulfoxide
DMSO-*d6*	Deuterated dimethyl sulfoxide
EtOAc	Ethyl acetate
EtOH	Ethanol
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-HRMS	Liquid chromatography, high resolution mass spectrometry
m	Multiplet
MEB	Malt extract broth
MeOH	Methanol
MHz	Megahertz
MIC	Minimum inhibitory concentration
m/z	Mass to charge ratio
NMR	Nuclear magnetic resonance spectroscopy
OD	Optical density
ppm	Parts per million
q	Quartet
®	Registered trademark
rpm	Revolutions per minute
s	Singlet
t	Triplet
td	Triplet of doublets

Chapter One:

General Introduction

Natural products

Natural products, broadly defined, are organic molecules produced by living organisms.[1-6] Historically, many natural products have played important roles as therapeutics and, more recently, they have become a valuable source of lead-compounds for new drug development.[2, 7, 8] The first isolation of a pure natural product, morphine, was achieved by Friedrich Serturner in 1805.[9] Prior to this, natural products played a medicinal role as active constituents in therapeutic preparations that often contained whole tissues or organs of one or more plants.[10-12] Indeed, the majority of the world's population continues to rely heavily on plants for the treatment of illnesses;[2, 7, 10-12] however, the discovery of morphine showed that purified natural products could also play an important role in medicine, thus inciting an investigation of medicinal plants for their natural products.[8, 13, 14] For instance, the discovery of the medicinal plant-derived natural products quinine, an antimalarial from *Cinchona officinalis*, and salicin, an analgesic from *Salix* spp. have had significant global impacts.[2, 8, 13, 14] Using quinine as a treatment for malaria is speculated to have facilitated the colonization efforts of European nations,[2] while salicin has been modified into what continues to be the most commonly used analgesic, aspirin.[14] The discovery of the aforementioned natural products helped to lay the foundation for what would develop into the pharmaceutical industry.[2]

The serendipitous discovery of the antibiotic penicillin from a fungus by Alexander Flemming in 1928,[15] showed, for the first time, that fungi could also be a source of medicinally relevant natural products.[2] Penicillin's antibiotic activity revolutionized the field of medicine by enabling the effective treatment of bacterial infections and reducing the mortality of infected individuals.[16, 17] This made it an invaluable commodity when it became clinically available in 1944.[2] Soon after the large-scale production and clinical use of penicillin became established, streptomycin was discovered.[2, 8, 18] Streptomycin is produced by a soil-derived actinomycetes and it was found to possess broad-spectrum antibiotic activity as well as being the first antibiotic effective against *Mycobacterium tuberculosis*.[2, 8, 18] The discovery of these antibiotics demonstrated that microbes could be a relevant source of medicinal compounds, thus leading to the exploration of microbes as sources of natural products, with a specific interest in soil-derived actinomycetes and fungi.[2, 19] This exploration was largely undertaken by the pharmaceutical industry and yielded 19 of the 23 classes of antibiotics that are currently in use.[20-22]

As natural product research progressed and the number of known natural products increased, it became common for known natural products with known biological activities to be reisolated.[5] This is one hurdle of natural product research and is known as the redundancy of isolation.[16, 17] Indeed, the increased frequency of reisolation caused the pharmaceutical industry to reduce support for natural product research in favour of screening of large collections of synthetic molecules to identify new molecules with new bioactivities.[2, 16, 17] These synthetic libraries had the potential to yield many new compounds very quickly through the modification of template chemical scaffolds and

could overcome some of the shortcomings of natural products and related research, such as large time investments and financial investments for low yields.[2, 16, 17] However, the synthetic libraries have their own shortcomings. For instance, they generally consist primarily of simple molecules with few or no chiral centers, leading to molecules that can be produced consistently and obtained on demand at relatively low cost, however, this results in reduced chemical diversity.[20, 23] Despites the potential for the high-throughput synthesis of new molecules by the pharmaceutical industry, natural products and their derivatives make up 48% of all approved drugs and 64% of approved anti-infective drugs.[4] Considering the shortcomings of the large screening libraries developed by pharmaceutical companies and despite reisolation and availability of alternate strategies for drug discovery, natural products remain the best option for drug discovery[5, 8, 24, 25].

It is hardly surprising that natural products make good drug candidates when compared to the collections of synthetic organic molecules available in commercial screening libraries, given the biological origins and the diversity of natural products' chemical structures.[24, 26, 27] The relevance of natural products as drug leads, is made more prominent when considering that the 52% of approved drugs derived from fully synthetic molecules come from a collection of more than 22 million molecules, while the 48% of approved drugs derived from natural products comes from a small collection of approximately 300,000 molecules.[4, 23, 28, 29]

Various strategies have been proposed to allow natural products researchers to continue to identify new chemical structures, new bioactivities, and to mitigate the redundancy of isolation.[5, 6, 20, 30-32] One way to accomplish this is through the exploitation and investigation of new or under-investigated biological sources.[33-37] For example, the

initial investigation of soil-derived fungi and bacteria from 1940 to 1970 resulted in new antibiotic natural products that account for 15 of the classes of antibiotics in use today.[17, 20, 38, 39] Along with this, advances in SCUBA equipment during the 1950's allowed for the exploration of the biodiversity of the marine environment, which has led to more than 28,000 new natural products being discovered since 1977.[37, 40-72] More recently, microorganisms living within the tissues of other living organisms such as plants, algae, and invertebrates have been shown to be new sources of bioactive natural products.[20, 73, 74] These endosymbiotic microorganisms represent an untapped biological reservoir due to their physical location and unique biological interactions. Fungi that live within the tissues of plants or algae are known as endophytes and are one such source of untapped biological diversity.[75, 76]

Fungi themselves represent an under-investigated source of natural products.[35, 36, 77-84] Fungi have been found to be a prolific source of novel natural products with a broad range of bioactivities, making them an excellent source for potential drug candidates,[36, 81, 85-89] in fact, half of all recently approved (1981-2014), natural product based anti-infective drugs have been sourced from fungi.[4] Despite this, only a small fraction of the potential diversity of fungi has been investigated. With only 100,000 species of fungi identified, [89, 90] while estimates of the total number of species encompassed by this taxon being in the realm of two to five million,[91-93] fungi possess a massive amount of cryptic biodiversity to be examined.

To begin investigating this cryptic fungal diversity one must begin to look towards the many unique and unexplored environmental niches which include the aforementioned endophytic fungi.[76, 89, 94, 95] The diversity of endophytes can be put into

perspective when considering that current estimates of plant species richness are over 300,000 and each plant is capable of harboring unique assemblages of endophytic species.[6, 96-99] The estimated biodiversity of endophytes grows further when considering that the biodiversity not only varies by host species but can vary based on geographic and temporal distributions,[100-102] plant sex[103] and by tissue of the host.[104, 105] Taking these factors into account implies the magnitude of the potential abundance of the unexplored diversity of endophytes. In conjunction with the past success of fungi as a prolific source of natural products, endophytes inhabit unique environments that result in constant metabolic interactions between the host organism and fungus; this can enhance the production and diversity of natural products synthesised by the fungus, favouring the production of biologically active natural products.[6, 35, 98, 99, 106, 107] Thus, by investigating endophytic fungi as a reservoir of biological and chemical diversity, there is an increased likelihood of isolating new and bioactive natural products.[5, 19, 37]

Project description

The goal of the research described in this thesis was to discover natural products with new biological activities and/or new chemical structures. The work described will be presented as a series of four manuscripts with a final general discussion and conclusion chapter.

Research for chapter two was conducted on two endophytic isolates, an *Annulohypoxylon multiforme* isolated from the medicinal plant *Juniperus communis* and a sterile grey unidentified filamentous fungus from the marine alga *Scytosiphon lomentaria*. A polymeric natural product, which was unreported in the primary literature,

was discovered from both the *Annulohypoxylon multiforme* and the sterile grey filamentous isolates, and the antibiotic activity of this natural product is described herein. My role in the research described in chapter two was that of co-primary researcher and author, having characterized the absolute stereochemistry of the isolated natural product and determined its bioactivity.

Chapter three describes the investigation of an endophytic *Penicillium roseopurpureum* isolate from the alga *Petalonia fascia*. The extract of this endophyte showed significant antibiotic activity and the liquid chromatography high-resolution mass spectrometry (LC-HRMS) data of the extract showed unique ionization patterns. Three chlorinated bianthrones were isolated from this endophytic *Penicillium roseopurpureum* isolate and their relative stereochemistry elucidated, and bioactivities described. My contributions to this chapter were that of primary researcher and author.

Chapter four describes the bioassay guided fraction of an extract from an endophytic Alternaria alternata isolate from the South African medicinal plant *Pelargonium tomentosum*. This resulted in the isolation of a bioactive biphenol as well as two dimeric γ-lactones. My contributions to this chapter were that of primary researcher and author.

Chapter five describes the isolation of natural products using LC-HRMS based metabolomics as a prioritization method that resulted in the isolation of two new and three known natural products from a *Chaetomium globosum* from the medicinal plant *Geum macrophyllum*. My contributions to this chapter were that of co-primary researcher and author having performed isolations, structure elucidation and the assignment of stereochemistry and bioactivities.

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Chapter Two:

Isolation of antibiotic 3*R*,5*R*-dihydroxyhexanoate polymers from endophytic fungi

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ABSTRACT

The extracts of two isolates in our library of fungal endophytes, *Annulohypoxylon multiforme* (TC2-046) from the medicinal plant *Juniperus communis* and a sterile filamentous isolate (KP1-131DD) from the marine alga *Scytosiphon lomentaria* displayed similar antimicrobial bioactivity profiles with notable high activity against *Staphylococcus aureus*. Bioassay guided fractionation led to the isolation of poly(3*R*,5*R*-dihydroxyhexanoic acid) oligomers ranging from the trimer to the 29-mer that exhibited significant and selective inhibition of *Staphylococcus aureus* and *Mycobacterium tuberculosis* H37Ra *in vitro*.

Keywords: Poly(3,5-dihydroxyhexanoic acid), Antimycobacterial, Biopolymer, Endophyte, *Annulohypoxylon multiforme*, *Juniperus communis*, *Scytosiphon lomentaria*, Medicinal plant, Marine alga.

Endophytic fungi are increasingly being recognized as important sources of bioactive natural products, with interest focusing on endophytes of medicinal plants[1-5] and algae.[6-8] In continuation of our investigation of endophytes isolated from Canadian medicinal plants and marine algae,[9-13] the extracts of an *Annulohypoxylon multiforme* isolate (TC2-046) from *Juniperus communis* and a sterile grey filamentous fungus (KP1-131DD) obtained from the marine alga *Scytosiphon lomentaria* attracted our attention as they exhibited remarkably similar bioactivity profiles in our screening assays characterized by selective, high inhibition of *Staphylococcus aureus*.

Bioassay guided fractionation of the EtOAc extract of a two-week, bench-scale (25 L) fermentation of KP1-131DD through liquid-liquid partition, flash chromatography and normal phase high-performance liquid chromatography (HPLC) led to the isolation of poly(3*R*,5*R*-dihydroxyhexanoic acid) (**2.1**; Figure 2.1) in high yield (23 mg/L, 20% of the extract by mass). Subsequent targeted fractionation of the EtOAc extract of TC2-046 by reversed-phase HPLC also gave **2.1** but at significantly lower overall yield (2 mg/L, 20% of the extract by mass).

The structure of **2.1** was determined by mass spectrometry and NMR spectroscopy (1D and 2D), with high-resolution positive-mode electrospray ionization mass spectroscopy indicating that the oligomer was present in sizes ranging from the trimer to the 29-mer. Saponification of **2.1** allowed the determination of its absolute stereochemistry to be 4*R*, 6*R* through comparison of the NMR and polarimetric data obtained for the hydrolysis product, 4*R*-hydroxy-6*R*-methyltetrahydropyran-2-one (**2.2**), with literature values reported for each of the four diastereomeric lactones.[14, 15] Our data were consistent with those from a previous isolation of **2.1** when it was obtained at

lengths ranging from the pentamer to the 27-mer from a *Daldinia concentrica* endophyte of a *Manikara* tree from Costa Rica.[16, 17]

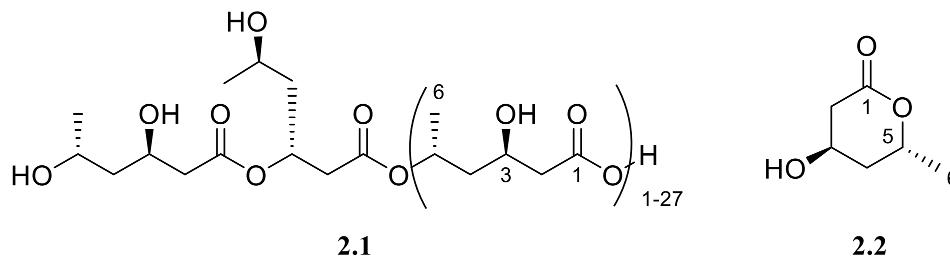

Figure 2.1. The structures of poly(3*R*,5*R*-dihydroxyhexanoic acid) (**2.1**) and 4*R*-hydroxy-6*R*-methyltetrahydropyran-2-one (**2.2**).

Although originally isolated from an extract that inhibited the NFκB pathway regulator IκB kinase (IKK),[16] no bioactivity data has been reported for **2.1**. In our hands, the polymer was found to possess selective inhibitory activity against both *Staphylococcus aureus* [MIC: 400 µg/mL; IC_{50} (± SD): 53 ± 9 µg/mL] and *Mycobacterium tuberculosis* H37Ra [MIC: 400 µg/mL; IC_{50} (± SD): 66 ± 17 µg/mL] while showing no significant inhibition against *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, *Saccharomyces cerevisiae* or *Mycobacterium smegmatis*. While the production of polyesters and polyhydroxyalkanoates is well documented in bacteria,[18] it remains uncommon in fungi; although the scale at which **2.1** can be produced may facilitate its development and application as a biopolymer in the future.[17]

Experimental

General Experimental Procedures: All antimicrobial standards used as positive controls in susceptibility assays, except for rifampin, were obtained from Sigma Aldrich

(Oakville, Ontario). Rifampin was obtained from Fisher Scientific (Ottawa, Ontario). 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). Solid-phase extraction was performed using Sep-Pak® C18 Cartridges (55-105 µm, 125 Å, 2 g; Waters, NA, USA). Flash chromatography was performed using a Biotage Flash+ chromatography system fitted with SiliaSep silica 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. 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 and were calibrated to residual protonated solvent. HRMS data were recorded on a Thermo LTQ Exactive instrument with an ESI source.

Endophyte isolation: TC2-046 was isolated from the leaves of *Juniperus communis* (New Brunswick Museum voucher specimen NBM VP-37482) in August 2010.[9] Leaf surfaces were sterilized by immersion in 5.25% aqueous NaOCl for 5 sec and rinsed with sterile distilled H_2O for 10 sec then immersed in 70% EtOH for 10 sec and rinsed with sterile distilled water and blotted dry on autoclaved paper towel. Sterile tissue was immediately aseptically cut into pieces (5 mm × 5 mm) that were placed onto 2.0% malt extract agar and incubated at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2.0% malt extract agar until pure cultures were obtained. *Scytosiphon lomentaria* was collected from the shore of Green's Point, L'Etete, New Brunswick, Canada (45° 02.363' N, 066° 53.483' W) in July 2013. Portions (5 cm in

length) of algal tissue were individually sterilized by immersion in 6.0% NaOCl for 10 sec and rinsed with sterile distilled H_2O for 10 sec then immersed in 70% EtOH for 15 sec and rinsed with sterile distilled H_2O and blotted dry on autoclaved paper towel. Sterile tissue was immediately aseptically cut into pieces (approximately 5 mm × 5 mm), placed onto 2.0% malt extract agar and incubated at ambient room temperature. Endophytic fungi were subcultured onto fresh 2.0% malt extract agar until a pure culture was obtained.

Identification of endophytes: TC2-046 was identified as *Annulohypoxylon multiforme* through examination of spore morphology and colonies grown on malt extract, potato dextrose, cornmeal, and Czapek-Dox agars. The taxonomic classification was confirmed by comparison of the internal transcribed spacer (ITS) and 5.8S rRNA gene 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 TC2-046 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[6] and the amplified ITS DNA was sequenced by Genome-Québec (Montreal, Québec). The TC2-046 DNA sequence was checked for ambiguity before being compared with existing GenBank sequence data using BLAST. The ITS gene sequence of TC2-046 was found to have >99% homology with numerous conspecific *Annulohypoxylon multiforme* isolates and has been deposited in GenBank (accession number: KC916700).

KP1-131DD did not produce fruiting bodies with characteristic morphological features on malt extract, potato dextrose, cornmeal or Czapek-Dox agars. 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.[6] On malt extract agar the isolate grew as a flat, filamentous, grey colony with a rough surface and undulating margin, that lacked spores and produced an orange pigment that diffused into the growth medium. On potato dextrose agar the isolate grew as a flat, filamentous, grey colony with a rough surface, and filiform margin, that lacked spores. On cornmeal agar the isolate grew as a flat, filamentous, beige colony with a smooth surface and filiform margin, that lacked spores. On Czapek-dox agar the isolate grew as a flat, filamentous, grey colony with a rough surface and undulating margin, that lacked spores. Microscopy revealed the isolate to be sterile with septate hyphae and brown pigmentation.

Fermentation and extraction: KP1-131DD was fermented in 2.0% malt extract broth (25 L; 250 × 100 mL batches in 250 mL Erlenmeyer flasks stoppered with foam baffles) at room temperature with shaking (150 rpm) for 2 weeks. The cultures were then sonicated for thirty seconds, the mycelia and cell debris separated from the spent broth by filtration and the broth was extracted with EtOAc (3 x 300 mL EtOAc per litre of broth). The organic fractions were combined and concentrated *in vacuo* (2.70 g). TC2-046 was fermented (1 L; 10 × 100 mL) and extracted (10 mg) in the same fashion as KP1-131DD.

Bioassay guided fractionation of KP1-131DD: The extract (2.70 g) was dissolved in 9:1 MeOH/H_2O (200 mL) and extracted with hexanes (3 × 100 mL) before being diluted with H_2O (100 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The aqueous fraction was

concentrated *in vacuo*, dissolved in H_2O (200 mL) and extracted with EtOAc (3 × 100 mL) and *n*-BuOH (3 × 100 mL). The five fractions were concentrated *in vacuo* with the CH_2Cl_2 fraction (1.39 g) showing antimicrobial activity *S. aureus*. The CH_2Cl_2 fraction (1.13 g) was separated by normal-phase flash column chromatography (column eluted with 100% hexanes to 100% EtOAc in 10% increments, 1:1 MeOH/EtOAc, and 100% MeOH) with the 12th fraction of the column (1:1 MeOH/EtOAc) giving **2.1** (579 mg).

Bioassay guided Fractionation of TC2-046: The extract (10 mg) was loaded onto a 2 g C-18 Sep-Pak® Cartridge and eluted first with H_2O (14 mL) then MeOH (14 mL) then EtOAc (14 mL). The methanol fraction (8 mg) was subjected to reversed-phase HPLC (gradient elution from 95:5 H_2O: acetonitrile to 100% acetonitrile over ten minutes then held at 100% acetonitrile for five minutes) to give **2.1** (2 mg).

Poly(3*R*,5*R*-dihydroxyhexanoic acid) (2.1)

IR (NaCl, thin film): 3426, 2987, 2932, 1723, 1387, 1273, 1176, 753 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): 5.10 (1H, m, H-5), 4.09 (1H, m, H-3), 2.51 (1H, dd, *J* = 15.2, 4.7 Hz, H-2a), 2.41 (1H, dd, *J* = 15.2, 8.1 Hz, H-2b), 1.86 (1H, ddd, *J* = 14.0, 7.9, 7.0 Hz, H-4a), 1.68 (1H, ddd, *J* = 13.8, 6.5, 5.1 Hz, H-4b), 1.28 (3H, d, *J* = 6.3 Hz, H-6); ^{13}C NMR (100 MHz, CD_3OD): 172.8 (C-1), 70.1 (C-5), 66.6 (C-3), 43.9 (C-2), 43.7 (C-4), 20.2 (C-6); HRESIMS: *m/z* 431.1888 [M+Na$^+$] (calcd for trimer, $C_{18}H_{32}O_{10}Na^+$, 431.1909), 561.2521[M+Na$^+$] (calcd for tetramer, $C_{24}H_{42}O_{13}Na^+$, 561.2545), 691.3151 [M+Na$^+$] (calcd for pentamer, $C_{30}H_{52}O_{16}Na^+$, 691.3180), 1895.9455 [M+2H$^+$] (calcd for 29-mer, $C_{174}H_{294}O_{88}^{2+}$, 1895.9369), See table S1, supplemental data, for exhaustive list.

Hydrolysis of poly(3R,5R-dihydroxyhexanoic acid): Compound **2.1** (10 mg) was dissolved in an aqueous solution of 10% aqueous KOH (0.5 mL) and MeOH (2.0 mL)

and stirred at room temperature for 75 h. The solution was acidified by adding 1 M HCl (2.5mL) and concentrated *in vacuo*. The reaction products were subjected to normal phase column chromatography (5 g silica; column eluted with 100% EtOAc and washed with 100% MeOH) and collected in test tubes. The contents of test tubes were combined according to their TLC profiles resulting in three fractions with the third fraction containing **2.2** (4 mg).

4*R*-Hydroxy-6*R*-methyltetrahydropyran-2-one (2.2)

$[\alpha]_D^{23}$: +19 (*c* 0.27, $CHCl_3$); IR (NaCl, thin film): 3419, 2919, 2856, 1641, 1463, 1379, 1252, 1184 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 4.84 (1H, ddq, *J* = 11.2, 6.5, 3.1 Hz, H-6), 4.39, (1H, m, H-4), 2.74 (1H, dd, *J* = 17.7, 5.0 Hz, H-3a), 2.62 (1H, ddd, *J* = 17.6, 3.7, 1.8 Hz, H-3b), 1.98 (1H, dddd, *J* = 14.5, 3.8, 3.4, 1.7 Hz, H-5a), 1.74 (1H, ddd, *J* = 14.5, 11.2, 3.3 Hz, H-5b), 1.41 (3H, d, *J* = 6.4 Hz, H-7); ^{13}C NMR (100 MHz, $CDCl_3$): 170.4 (C-2), 72.3 (C-6), 63.0 (C-4), 38.5 (C-5), 37.8 (C-3), 21.5 (C-7); HRESIMS: *m/z* 131.0704 $[M+H^+]$ (calcd for $C_6H_{11}O_3^+$, 131.0703).

Antifungal bioassay: Antifungal activity against *C. albicans* (ATCC 14053) and *S. cerevisiae* (ATCC 9763) was evaluated using a microbroth dilution antibiotic susceptibility assay modified from McCulloch et al.[19] Immediately prior to use, stock solutions of fungal extracts, fractionation products or purified natural products were prepared at the desired concentration in sterile-filtered DMSO (40 µL) and diluted with either Difco™ Sabouraud dextrose broth (*C. albicans*, 960 µL) or yeast mold broth (*S. cerevisiae*, 960 µL) (Becton Dickinson, Mississauga, Ontario). The resulting test solutions (100 µL; 4% DMSO) were transferred to the non-peripheral wells of a clear, non-tissue cultured 96-well microtiter plate in triplicate (BD Falcon™, Becton

Dickinson, Mississauga, Ontario). Each plate contained three positive control wells (*C. albicans*: nystatin, 5.0 µg/mL; *S. cerevisiae*: amphotericin B, 5.0 µg/mL, 100 µL per well). Wells were then inoculated with suspensions of either *C. albicans* or *S. cerevisiae* (100 µL; 1×10^6 CFU/mL), to obtain a cell density of 5×10^5 CFU/mL. Sterile water (200 µL) was added to all perimeter wells to reduce evaporation from experimental wells. Each plate contained three negative control wells [4% DMSO in appropriate broth (100 µL) inoculated with appropriate fungi (100 µL; 1×10^6 CFU/mL)] and three untreated blank wells [2% DMSO in appropriate broth (200 µL)] Initial and final optical densities (OD) were determined for each well by recording absorbance at 600 nm immediately before and after incubation for 24 h at 37 °C using a Molecular Devises Emax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Initial OD readings were subtracted from the final readings for each well to obtain the change in optical density (ΔOD). ΔOD values were corrected for background absorbance of the media by subtracting the mean ΔOD readings of the blanks from the mean ΔOD readings of the control and test wells. The percentage inhibition of fungal growth is defined as:

$$\left(1 - \frac{\text{mean test or positive control } \Delta\text{OD}}{\text{mean negative control } \Delta\text{OD}}\right) \times 100$$

Antibacterial bioassay: Antibacterial activity against *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 10145), *E. coli* (ATCC 25922) and *E. faecium* (ATCC 35667) was evaluated in the same manner as described for antifungal assay. The growth medium used for *P. aeruginosa*, *E. coli* and *S. aureus* was BBL™ Mueller Hinton II cation adjusted broth (Becton Dickinson, Mississauga, Ontario), whereas BBL™ Brain Heart Infusion

broth (Becton Dickinson, Mississauga, Ontario) was used for *E. faecium*. Positive controls consisted of a triplicate concentration of antibiotic (1.25 µg/mL, erythromycin for *S. aureus*; 20 µg/mL and 2.5 µg/mL, gentamicin for *P. aeruginosa* and *E. coli* respectively; 1.25 µg/mL, tetracycline for *E. faecium*; 100 µL per well) inoculated with a suspension of the appropriate pathogen (100 µL).

Antimycobacterial bioassay: Antimycobacterial activity against *M. tuberculosis* strain H37Ra (ATCC 25177) and *M. smegmatis* (ATCC 70084) was evaluated using a microplate resazurin assay according to O'Neill et al.[20] Immediately prior to use, stock solutions of fungal extracts, fractionation products or purified natural products were prepared at the desired concentration in sterile-filtered DMSO (40 µL) and were diluted with modified Middlebrook 7H9 broth (960 µL; BBL™ MGIT™, Becton Dickinson, Mississauga, Ontario). The resulting test solutions (100 µL) were transferred to the non-peripheral wells of a black non-tissue culture treated, low-binding, 960-well microtiter plate (VWR, Mississauga, Ontario) in triplicate and inoculated with suspensions of the appropriate organism (100 µL) of cell density 2.0×10^6 cells/mL. Sterile water (200 µL) was added to all perimeter wells to reduce evaporation from experimental wells. The positive control consisted of rifampin (*M. tuberculosis*, 0.02 µg/mL) or ciprofloxacin (*M. smegmatis*, 2.5 µg/mL) In addition to the positive controls, negative controls [4% DMSO in modified Middlebrook 7H9 broth (100 µL) inoculated with suspensions of the appropriate organism (100 µL)], blanks [2% DMSO in modified Middlebrook 7H9 broth (200 µL), and test solutions (100 µL) with modified Middlebrook 7H9 broth (100 µL)] were included in triplicate in each plate. *M. tuberculosis* plates were incubated (37 °C; 5% CO_2) for three days, in a humid environment and *M. smegmatis* plates were incubated

for one day (37 °C). Following incubation, a solution of resazurin (0.0625 mg/mL) in aqueous Tween 80 (50 µL) was added to all non-peripheral wells. Plates were then incubated for a further 24 h, sealed with an adhesive polyester film (50 µm; VWR, Mississauga, Ontario), and mycobacterial growth was assessed fluorometrically at 37 °C (Molecular Devices Gemini EM dual-scanning microplates spectrofluorometer with a 530 nm excitation filter and a 590 nm emission filter operating in top-scan mode). Fluorescence values were corrected for any background fluorescence of the media and test samples by subtracting mean fluorescence readings of the appropriate blanks from the mean fluorescence readings of the control and test wells. The percentage inhibition of mycobacterial growth was then defined as:

$$\left(1 - \frac{\text{mean test or positive control fluorescence}}{\text{mean negative control fluorescence}}\right) \times 100$$

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Supplemental information

Table S2.1. Positive mode ESI HRMS peaks corresponding to the increasing lengths of poly(3*R*,5*R*-dihydroxyhexanoic acid) (**2.1**)

n	Observed adduct	calcd *m/z*	Observed *m/z*	Δppm
Trimer	$C_{18}H_{32}O_{10}Na^{+}$	431.1909	431.1888	-4.9
Tetramer	$C_{24}H_{42}O_{13}Na^{+}$	561.2545	561.2521	4.3
Pentamer	$C_{30}H_{52}O_{16}Na^{+}$	691.3180	691.3153	-3.9
Hexamer	$C_{36}H_{62}O_{19}Na^{+}$	821.3816	821.3807	-1.1
Heptamer	$C_{42}H_{72}O_{22}Na^{+}$	951.4451	951.4454	0.3
Octamer	$C_{48}H_{82}O_{25}Na^{+}$	1081.5087	1081.5090	0.3
Nonamer	$C_{54}H_{92}O_{28}Na^{+}$	1211.5722	1211.5691	-2.6
Decamer	$C_{60}H_{102}O_{31}Na^{+}$	1341.6357	1341.6306	-3.8
Undecamer	$C_{66}H_{112}O_{34}Na^{+}$	1471.6993	1471.6965	-1.9
Dodecamer	$C_{72}H_{122}O_{37}Na^{+}$	1601.7628	1601.7612	-1.0
13-mer	$C_{78}H_{132}O_{40}Na^{+}$	1731.8264	1731.8251	-0.8
14-mer	$C_{84}H_{142}O_{43}Na^{+}$	1861.8899	1861.8906	0.4
15-mer	$C_{90}H_{152}O_{46}Na_2^{2+}$	1007.4713	1007.4708	-0.5
16-mer	$C_{96}H_{162}O_{49}Na_2^{2+}$	1072.5031	1072.5005	-2.4
17-mer	$C_{102}H_{172}O_{52}Na_2^{2+}$	1137.5349	1137.5343	-0.5
18-mer	$C_{108}H_{182}O_{55}Na_2^{2+}$	1202.5666	1202.5655	-0.9
19-mer	$C_{114}H_{192}O_{68}Na_2^{2+}$	1267.5984	1267.5942	-3.3
20-mer	$C_{120}H_{202}O_{61}Na_2^{2+}$	1332.6302	1332.6275	-2.0
21-mer	$C_{126}H_{212}O_{64}Na_2^{2+}$	1397.6620	1397.6582	-2.7
22-mer	$C_{132}H_{222}O_{67}Na_2^{2+}$	1462.6937	1462.6910	-1.8
23-mer	$C_{138}H_{232}O_{70}Na_2^{2+}$	1527.7255	1527.7245	-0.7
24-mer	$C_{144}H_{242}O_{73}Na_2^{2+}$	1592.7573	1592.7521	-3.3
25-mer	$C_{150}H_{252}O_{76}Na_2^{2+}$	1657.7890	1657.7924	2.1
26-mer	$C_{156}H_{262}O_{79}Na_2^{2+}$	1722.8208	1722.8258	2.9
27-mer	$C_{162}H_{272}O_{82}Na_2^{2+}$	1787.8526	1787.8442	-4.7
28-mer	$C_{168}H_{284}O_{85}^{2+}$	1830.9052	1830.8999	-2.9
29-mer	$C_{174}H_{294}O_{88}^{2+}$	1895.9369	1895.9455	4.5

Chapter Three:

Halogenated bianthrones from *Penicillium roseopurpureum*, a fungal endophyte of the marine alga *Petalonia fascia*

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ABSTRACT

An extract of the fungus *Pencillium roseopurpureum* (KP1-135C) isolated from the marine alga *Petalonia fascia* showed selective antimicrobial activity against *Staphylococcus aureus* and *Mycobacterium tuberculosis* H37Ra. Bioassay guided fractionation revealed that three halogenated bianthrones, neobulgarone D, neobulgarone E and neobulgarone F were responsible for the observed activity of the extract. The stereochemistry of the neobulgarones was unambiguously assigned based on polarimetric data and the analysis of ^{1}H NMR data obtained for the three bianthrones.

Keywords: Neobulgarone, Endophyte, Marine algae, Antibacterial, Bianthrone, *Penicillium roseopurpureum, Petalonia fascia*.

Although marine organisms are a prolific source of bioactive natural products,[1-4] endophytes from marine algae represent an under-investigated source of biodiversity.[5]

During our investigation of endophytes of marine macroalgae, the extract of a *Penicillium roseopurpureum* isolate (KP1-135C) obtained from the brown alga *Petalonia fascia* (false kelp) was selected for bioassay guided fractionation as it selectively inhibited the growth of *Staphylococcus aureus* and *Mycobacterium tuberculosis* H37Ra in our screening assays.

Solid phase extraction, reversed-phase flash column chromatography and reversed-phase high-performance liquid chromatography of the ethyl acetate extract of a two-week, bench-scale (2 L) fermentation of KP1-135C led to the isolation of three optically inactive bianthrones. Analysis of NMR and high-resolution mass spectrometry data determined the bianthrones to be neobulgarone D (**3.1**), neobulgarone E (**3.2**) and neobulgarone F (**3.3**) (Figure 3.1), assignments that were consistent with those reported in the literature.[6]

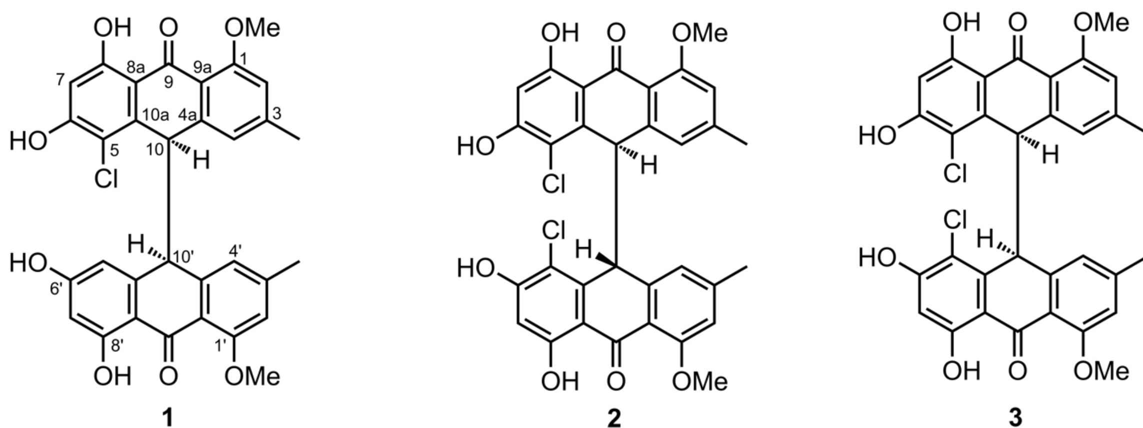

Figure 3.1. Structures of neobulgarone D (**3.1**), neobulgarone E (**3.2**), and neobulgarone F (**3.3**).

Neobulgarones D, E, and F were first isolated as moderately cytotoxic inhibitors of appressorium formation from the fungus *Neobulgaria pura*,[6] with neobulgarone E subsequently being reported from an endophytic *Penicillium* species isolated from *Limonium tubiflorum*.[7] However, elucidating the stereochemistry of bianthrones is

challenging due to the occurrence of mesomeric dimers and racemic mixtures,[8] presumably formed through non-enzymatic oxidative coupling reactions. Methods that rely on polarimetric data often lead to erroneous stereochemical assignments and are therefore untenable.[8] As such, when originally isolated, the configurations of **3.1**, **3.2** and **3.3** were not determined and, unfortunately, **3.2** was later incorrectly reported to have *syn* (*cis*) relative stereochemistry on the basis of polarimetric data.[9] A procedure to unambiguously distinguish between diastereomeric bianthrones based upon detailed analysis of their molecular conformations and the shielding effects observed in their ^{1}H NMR spectra was recently reported.[8] Application of these analyses to our data confirmed the relative configurations of neobulgarones D (**3.1**), E (**3.2**) and F (**3.3**) as *cis*, *trans* and *cis* respectively and our polarimetric data were consistent with **3.1** and **3.2** being obtained as racemic mixtures of the 10*R*,10'*S*/10*S*,10'*R* and 10*R*,10'*R*/10*S*,10'*S* diastereomers and **3.3** as the *R*,*S* mesomer.

In our hands, the limited aqueous solubility of the bianthrones made the acquisition of bioassay data challenging. Despite this, we were able to reveal moderate, selective antibiotic activity for neobulgarones D, E and F (Table 3.1) although, cognizant of the limitations of the bioactivity data obtained, the absolute median inhibitory concentrations should be evaluated with some caution.[10] Only the *cis* isomers, neobulgarones D and F, were active against *M. tuberculosis* whilst they also displayed superior activity against *S. aureus*. However, the *trans* isomer neobulgarone E was alone in its ability to inhibit the growth of methicillin-resistant *S. aureus*. These preliminary structure activity data are intriguing and may warrant further investigation, particularly if the observed antibiotic activity is enantiospecific.

Table 3.1. Relative and absolute median inhibitory concentrations[10] (IC_{50} values; µM) of neobulgarones D, E and F against *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRSA), *Mycobacterium tuberculosis* H37Ra ATCC 25177, *Pseudomonas aeruginosa* ATCC 10145 and *Candida albicans* ATCC 14053.

Compound	Relative [absolute] IC_{50} (95% CI[a]), µM				
	S. aureus	MRSA	*M. tuberculosis*	*P. aeruginosa*	*C. albicans*
3.1	36.3 (30.1, 41.1) [167 (117, 268)]	NA[b]	46.1 (44.6, 47.7) [80.7 (64.1, 103)]	NA	NA
3.2	96.9 (77.0, 126) [325 (234, 533)]	67.4 (53.9, 78.4) [142 (104, 210)]	NA	NA	NA
3.3	58.3 (45.5, 74.0) [111 (86.1, 149)]	NA	31.1 (29.7, 32.5) [35.1 (31.8, 39.2)]	NA	NA

[a]95% confidence interval. [b]NA: not active; less than 50% growth inhibition at 200 µM.

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). Solid-phase extraction was performed using Sep-Pak® C18 Cartridges (55-105 µm, 125 Å, 2 g; Waters, NA, USA). Flash chromatography was performed using a Biotage Flash+ chromatography system fitted with C18 (reversed-phase) SiliaSep cartridges (40-63 µm, 60 Å, 25 g; SiliCycle, QC, 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. NMR spectra were recorded on an Agilent 400-MR DD2 instrument in deuterated solvents and were calibrated to residual protonated solvent resonances. HRMS data were recorded on a Thermo LTQ Exactive instrument with an ESI source.

Endophyte isolation: KP1-135C was isolated from the marine macroalga *Petalonia fascia* collected from the shores of Green's Point, L'Etete, NB, Canada (45°02.363' N, 066°53.483' W) in July 2013. Portions (5 cm in length) of algal tissue were individually sterilized by sequential immersion in 6.0% NaOCl (10 sec), sterile distilled water (10 sec) and 70% EtOH (15 sec). The tissue was then rinsed with sterile distilled water, blotted dry on autoclaved paper towel and cut into pieces (0.60 cm in diameter) that were placed onto 2.0% malt extract agar and incubated at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2.0% malt extract agar until pure cultures were obtained.

Endophyte identification: Isolate KP1-135C was identified as *Penicillium roseopurpureum* 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 (ITS) and 5.8S rRNA gene 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 KP1-135C 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[11] and the amplified ITS DNA were sequenced by Genome-Québec (Montreal, Québec). The KP1-135C DNA sequence was checked for ambiguity before being compared with existing GenBank sequence data using BLAST. The ITS gene sequence of KP1-135C was found to have >99% homology

with numerous conspecific *P. roseopurpureum* isolates and has been deposited in GenBank (accession number: KY054768).

Biological assays: Antifungal activity against *C. albicans* (ATCC 14053), antibacterial activity against *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 10145) methicillin-resistant *Staphylococcus aureus* (ATCC 33591), and antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra (ATCC 25177) was evaluated as previously described.[11, 12]

Fermentation and extraction: KP1-135C was fermented in 2.0% malt extract broth at room temperature with shaking (150 rpm) for 2 weeks (2 L; 20 × 100 mL batches in 250 mL Erlenmeyer flasks stoppered with foam baffles). Fermentation cultures were then sonicated for 30 seconds. Next, the fungal material was removed by filtration before the spent broth was extracted with EtOAc (3 × 660 mL). The organic fractions were combined and concentrated *in vacuo* (193 mg).

Fractionation: The MEB extract was divided into 6 equal masses and each loaded onto a separate 2 g C-18 Sep-Pak® Cartridge and eluted first with H_2O (14 mL) then MeOH (14 mL) then EtOAc (14 mL). The MeOH fraction (150 mg) 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 fractions that eluted from the flash column in 1:1 H_2O/CH_3CN (25 mg) 2:3 H_2O/CH_3CN (17 mg) were subjected to reverse-phase HPLC (1:1 H_2O/CH_3CN) to give **3.1** (2 mg), **3.2** (3 mg) and **3.3** (10 mg).

Neobulgarone D (3.1)

$[\alpha]_D^{22}$: 0 (*c* 0.05, MeOH) and 0 (*c* 0.05, acetone); ^{1}H and ^{13}C NMR data were consistent with literature values;[6] see Table S3.1, supplemental data; HRESIMS *m/z* 573.1307 [M + H]$^+$ (calcd for $C_{32}H_{26}ClO_8^+$, 573.1316).

Neobulgarone E (3.2)

$[\alpha]_D^{22}$: 0 (*c* 0.05, MeOH) and 0 (*c* 0.05, acetone); ^{1}H and ^{13}C NMR data were consistent with literature values;[6] see Table S3.2, supplemental data; HRESIMS *m/z* 607.0930 [M + H]$^+$ (calcd for $C_{32}H_{25}Cl_2O_8^+$, 607.0926).

Neobulgarone F (3.3)

$[\alpha]_D^{22}$: 0 (*c* 0.05, MeOH) and 0 (*c* 0.05, acetone); ^{1}H and ^{13}C NMR data were consistent with literature values;[6] see Table S3.3, supplemental data; HRESIMS *m/z* 607.0936 [M + H]$^+$ (calcd for $C_{32}H_{25}Cl_2O_8^+$, 607.0926)

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Declaration of Conflicting Interests – The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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9. Aly et al. [reference 7] based their assignment on the observation that **2** was, in their hands, optically active and therefore not a mesomer. This would require the relationship between the H-10/H10' protons of **2** to be *anti* rather than *syn* as stated in reference 8. It should be noted, however, that the relative stereochemistry depicted in the structure of **2** illustrated in reference 7 is correctly represented as anti.
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Supplemental information

Table S3.1. Comparison of the NMR spectroscopic data (400 MHz, 19:1 $CDCl_3/CD_3OD$) obtained for neobulgarone D (**3.1**) with those reported by Eilbert et al.[1]

	3.1		Literature data	
Carbon	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	160.6, C		160.4, C	
2	112.7, CH	6.60, s	112.6 CH	6.60, s
3	144.6, C		144.5 C	
4	123.4, CH	5.34, s	123.3 CH	5.32, s
4a	140.1, C		140.0 C	
5	110.3, C		110.2 C	
6	158.5, C		158.3 C	
7	102.9, CH	6.45, s	102.8 CH	6.46, s
8	161.6, C		161.4, C	
8a	114.1, C		114.0, C	
9	186.6, C		186.4, C	
9a	118.0, C		117.8, C	
10	53.5, CH	4.73, d (3.3)	53.6, CH	4.70, d (3.2)
10a	139.1, C		139.0, C	
1-OMe	56.3, CH_3	3.77, s	56.2, CH_3	3.77, s
3-Me	21.7, CH_3	2.04, s	21.5, CH_3	2.04, s
1'	160.0, C		159.8, C	
2'	112.0, CH	6.79, s	111.9, CH	6.79, s
3'	145.5, C		145.3, C	
4'	121.4, CH	7.26, s	121.2, CH	7.23, s
4a'	145.1, C		144.9, C	
5'	109.4, CH	4.96, d (2.3)	109.4, CH	4.95, d (2.3)
6'	161.9, C		161.6, C	
7'	101.9, CH	6.05, d (2.3)	101.9, CH	6.08, d (2.3)
8'	163.7, C		163.5, C	
8a'	112.0, C		111.9, C	
9'	186.2, C		186.0, C	
9a'	120.4, C		120.2, C	
10'	51.7, CH	4.49, d (3.2)	51.6, CH	4.45, d (3.2)
10a'	139.5, C		139.3, C	
1'-OMe	56.0, CH_3	3.87, s	55.9, CH_3	3.88, s
3'-Me	22.4, CH_3	2.49, s	22.2, CH_3	2.49, s

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Table S3.2. Comparison of the NMR spectroscopic data (400 MHz, 19:1 $CDCl_3/CD_3OD$) obtained for neobulgarone E (**3.2**) with those reported by Eilbert et al.[1]

	3.2		Literature data	
Carbon	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	159.5, C		159.6, C	
2	112.9, CH	6.63, s	113.0, CH	6.63, s
3	144.1, C		144.2, C	
4	123.5, CH	5.58, s	123.5, CH	5.58, s
4a	138.9, C		139.0, C	
5	110.8, C		110.8, C	
6	158.1, C		158.1, C	
7	102.2, CH	6.37, s	102.3, CH	6.37, s
8	161.2, C		161.3, C	
8a	114.3, C		114.3, C	
9	185.8, C		185.8, C	
9a	119.1, C		119.1, C	
10	49.1, CH	5.04, s	49.0, CH	5.04, s
10a	140.0, C		140.0, C	
1-OMe	56.2, CH_3	3.79, s	56.2, CH_3	3.79, s
3-Me	21.3, CH_3	2.06, s	21.3, CH_3	2.05, s
1'	159.5, C		159.6, C	
2'	112.9, CH	6.63, s	113.0, CH	6.63, s
3'	144.1, C		144.2, C	
4'	123.5, CH	5.58, s	123.5, CH	5.58, s
4a'	138.9, C		139.0, C	
5'	110.8, C		110.8, C	
6'	158.1, C		158.1, C	
7'	102.2, CH	6.37, s	102.3, CH	6.37, s
8'	161.2, C		161.3, C	
8a'	114.3, C		114.3, C	
9'	185.8, C		185.8, C	
9a'	119.1, C		119.1, C	
10'	49.1, CH	5.04, s	49.0, CH	5.04, s
10a'	140.0, C		140.0, C	
1'-OMe	56.2, CH_3	3.79, s	56.2, CH_3	3.79, s
3'-Me	21.3, CH_3	2.06, s	21.3, CH_3	2.05, s

1. Eilbert F, Anke H, Sterner O. Neobulgarones A-F from cultures of *Neobulgaria pura*, new inhibitors of appressorium formation of *Magnaporthe grisea*. *J Antibiot*. 2000;53(10):1123-1129.

Table S3.3. Comparison of the NMR spectroscopic data (400 MHz, 19:1 $CDCl_3/CD_3OD$) obtained for neobulgarone F (**3.3**) with those reported by Eilbert et al.[1]

	3.3		Literature data	
Carbon	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	160.0, C		159.8, C	
2	112.6, CH	6.74, s	112.4, CH	6.72, s
3	145.3, C		145.1, C	
4	122.8, CH	Not observed[2]	122.7, CH	Not observed[2]
4a	137.8, C		137.6, C	
5	113.0, C		112.9, C	
6	157.9, C		157.8, C	
7	102.9, CH	6.34, s	102.7, CH	6.32, s
8	161.8, C		161.6, C	
8a	113.7, C		113.5, C	
9	185.6, C		185.6, C	
9a	119.4, C		119.3, C	
10	50.8, CH	4.97, s	50.8, CH	4.95, s
10a	141.6, C		141.6, C	
1-OMe	56.0, CH_3	3.85, s	56.0, CH_3	3.83, s
3-Me	21.9, CH_3	2.30, s	21.9, CH_3	2.28, s
1'	160.0, C		159.8, C	
2'	112.6, CH	6.74, s	112.4, CH	6.72, s
3'	145.3, C		145.1, C	
4'	122.8, CH	Not observed[2]	122.7, CH	Not observed[2]
4a'	137.8, C		137.6, C	
5'	113.0, C		112.9, C	
6'	157.9, C		157.8, C	
7'	102.9, CH	6.34, s	102.7, CH	6.32, s
8'	161.8, C		161.6, C	
8a'	113.7, C		113.5, C	
9'	185.6, C		185.6, C	
9a'	119.4, C		119.3, C	
10'	50.8, CH	4.97, s	50.8, CH	4.95, s
10a'	141.6, C		141.6, C	
1'-OMe	56.0, CH_3	3.85, s	56.0, CH_3	3.83, s
3'-Me	21.9, CH_3	2.30, s	21.9, CH_3	2.28, s

1. Eilbert F, Anke H, Sterner O. Neobulgarones A-F from cultures of *Neobulgaria pura*, new inhibitors of appressorium formation of *Magnaporthe grisea*. *J Antibiot*. 2000;53(10):1123-1129.
2. Consistent with the data reported by Eilbert et al., resonances for H-4 and H-4' were not observed in the NMR spectra of **3.3**.

Chapter Four:

γ-Lactone polyketides from the endophytic fungus *Alternaria alternata* isolated from the medicinal plant *Pelargonium tomentosum*

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ABSTRACT

An extract of the endophytic *Alternaria alternata* isolate (CT1-006A) from the medicinal plant *Pelargonium tomentosum* exhibited antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra. Bioassay guided fractionation led to the isolation of two diastereomeric γ-lactones, one of which is new, and altenusin, which was found to be responsible for the inhibitory activity of the extract.

Keywords: γ-Lactone, Altenusin, Endophyte, *Pelargonium tomentosum*, Medicinal plant

Endophytes from medicinal plants are a promising source of new and bioactive natural products.[1-6] During our ongoing investigation of natural products produced by endophytic fungi of medicinal plants, the extract of an *Alternaria alternata* isolate (CT1-

006A), from the medicinal plant *Pelargonium tomentosum* (peppermint-scented geranium), displayed selective activity against *Mycobacterium tuberculosis* H37Ra in our screening assays and was subjected to bioassay guided fractionation.

The EtOAc extract of a two-week bench-scale (5 L) fermentation of CT1-006A was fractionated by flash column chromatography and HPLC. This led to the isolation of (*Z*)-6*R**,7*S**-dihydroxy-2-propyl-2,4-octadien-4-olide (**4.1**), a γ-lactone originally isolated from an unidentified endophyte obtained from *Picea glauca* (white spruce),[7] and (*Z*)-6*R**,7*R**-dihydroxy-2-propyl-2,4-octadien-4-olide (**4.2**) a new diastereomer of **4.1**. The natural product responsible for the bioactivity of the extract was isolated in high yield (40% of the extract by mass, 21 mg/L) and its structure elucidated as the polyketide altenusin (**4.3**), commonly isolated from *Alternaria* spp.[8-18]

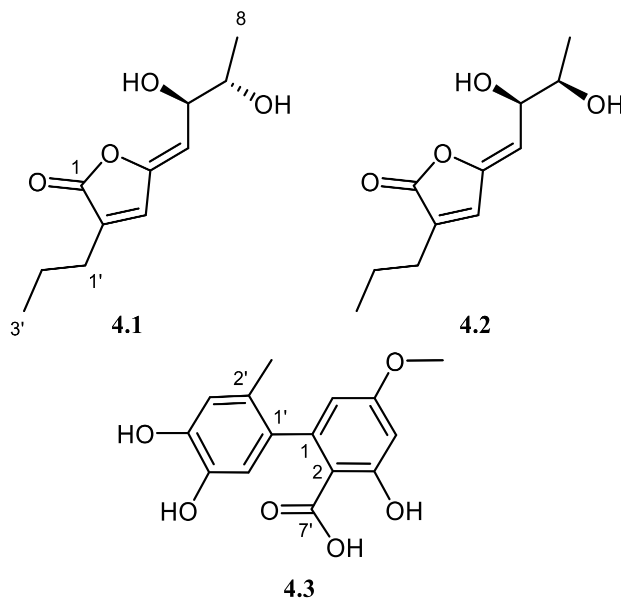

Figure 4.1. The structures of (*Z*)-6*R**,7*S**-dihydroxy-2-propyl-2,4-octadien-4-olide (**4.1**), (*Z*)-6*R**,7*R**-dihydroxy-2-propyl-2,4-octadien-4-olide (**4.2**), and altenusin (**4.3**).

The structures of **4.1** and **4.3** were determined through analysis of high-resolution mass spectrometry and NMR (1D and 2D) data, these assignments were consistent with

literature.[7,13] The structure of **4.2** was determined based on its similarity in NMR (^{1}H, ^{13}C, NOESY) and HRMS spectra to **4.1** with a difference observed in the coupling constant between H-6/H-7 (J = 4.3 Hz for **4.1** and J = 6.2 Hz for **4.2**). This difference suggests the two molecules are *cis*/*trans* diastereomers differing only in the orientation of the substituents on C-6 and C-7. The relative stereochemistry of **4.1** was originally assigned as having *anti* configuration based on the prediction that the *anti* isomer would possess an H-6/H-7 dihedral angle near 70° which is consistent with the observed coupling constant of 4.3 Hz.[7] Using a similar conformational analysis,[19] it was determined that an *anti* configuration would result in a coupling constant between H-6/H-7 of 2.3 Hz whereas the *syn* configuration would result in a coupling constant of 8.5 Hz. Thus, the assignment of **4.1** as the *anti* isomer is supported while **4.2** is determined to be the *syn* isomer. A more detailed conformational analysis would likely reveal the presence of multiple contributing conformations for both the *anti* and *syn* isomers, therefore it is prudent that the absolute stereochemistry be assigned to negate this possibility. Currently no attempts have been made to elucidate the absolute stereochemistry of **4.1** or **4.2**, however, our strategy is to use *R* and *S* enantiomers of Mosher's acid chloride to derivatize these diols into their corresponding diesters. The absolute stereochemistry of **4.1** and **4.2** will be determined through analysis of the ^{1}H NMR of the diesters using methods described by Seco et al.[20]

Previously, **4.1** has been obtained from an unidentified endophyte isolated from *Picea glauca* and was found to be cytotoxic against *Choristoneura fumiferana* (spruce budworm).[7] Unfortunately, the antimicrobial activity of **4.1** and **4.2** could not be assessed due to insufficient material. Altenusin is a known mycotoxin with previously reported

kinase inhibition,[8, 13] cytotoxicity, [8, 14] trypanothione reductase inhibition,[10] anti-inflammatory activity,[9] bacterial inhibition and fungal inhibition.[11, 12, 16, 18] Although altenusin was originally reported inactive against *Mycobacterium tuberculosis* H37Ra,[21] our assays showed moderate inhibitory activity [MIC: 350 μM; IC_{50} (± SD): 100 ± 7 μM]. However, **4.3** is not likely to be suitable for drug development due to previously discovered cytotoxicity.[8, 14] Nonetheless, the discovery of the antimycobacterial activity of **4.3** shows the strength of endophytic fungi as a source of bioactive natural products and the serendipitous discovery of **4.2** shows that endophytic fungi are an important source of 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). Solid-phase extraction was performed using Sep-Pak® C18 Cartridges (55-105 μm, 125 Å, 2 g; Waters, NA, USA). Flash chromatography was performed using a Biotage Flash+ chromatography system fitted with C18 SiliaSep cartridges (40-63 μm, 60 Å, 25g; SiliCycle, QC, 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. NMR spectra were recorded on an Agilent 400-MR DD2 instrument in

deuterated solvents and were calibrated to residual protonated solvent resonances. HRMS data were recorded on a Thermo LTQ Exactive instrument with an ESI source.

Endophyte isolation: CT1-006A was isolated from the terrestrial plant *Pelargonium tomentosum* collected from Cape Town, South Africa (33°59.301'S, 18°25.863'E). Plant surfaces were sterilized by sequential immersion in 70% EtOH (3 sec), sterile distilled H_2O (10 sec) and 6.0% NaOCl (1 sec). The tissue was then rinsed with sterile distilled water, blotted dry on an autoclaved paper towel and cut into pieces (0.60 cm in diameter) that were placed onto 2.0% malt extract agar and incubated at ambient room temperature. Endophytic fungi were subcultured onto fresh 2.0% malt extract agar until pure cultures were obtained.

Endophyte identification: Isolate CT1-006A was identified as *Alternaria alternata* 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 (ITS) and 5.8S rRNA gene DNA regions with corresponding sequences available in the GenBank database. The genomic DNA of CT1-006A 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[22] and the amplified ITS DNA were sequenced by Genome-Québec (Montreal, Québec). The CT1-006A DNA sequence was checked for ambiguity before being compared with existing GenBank sequence data using BLAST. The ITS gene sequence of CT1-006A was found to have >99% homology with numerous conspecific *A. alternata* isolates and has been deposited in GenBank (accession number: MN510332).

Biological assays: Antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra (ATCC 25177).[23]

Fermentation and extraction: CT1-006A was fermented in 2.0% malt extract broth (5 L; 50 × 100 mL batches in 250 mL Erlenmeyer flasks stoppered with foam baffles) at room temperature with shaking (150 rpm) for 2 weeks. The fungal material was removed by filtration before the spent broth was extracted with EtOAc (3 × 1600 mL). The organic fractions were combined and concentrated *in vacuo* (269 mg).

Fractionation: The MEB extract (269 mg) was divided into 16 equal masses and each loaded onto a difference C-18 Sep-Pak Cartridge and eluted first with H_2O (14 mL) then MeOH (14 mL) then EtOAc (14 mL). The MeOH fraction (241 mg) 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 4:1 H_2O/CH_3CN (33 mg) was subjected to reversed-phase HPLC (83:17 H_2O/CH_3CN) to give **4.1** (1 mg), **4.2** (1 mg), while flash column fraction four (7:3 H_2O/CH_3CN) gave **4.3** (108 mg).

(*Z*)-6*R,7*S**-Dihydroxy-2-propyl-2,4-octadien-4-olide (4.1)**

$[\alpha]_D^{22}$: 45.5 (*c* 0.11, MeOH)

^{1}H NMR (400 MHz, acetone-d_6): 7.37 (1H, t, *J* = 1.5 Hz, H-3), 5.35 (1H, d, *J* = 9.1 Hz, H-5), 4.52 (1H, dd, *J* = 9.1, 4.3 Hz, H-6), 3.82 (1H, p, *J* = 6.2, H-7), 2.32 (2H, td, *J* = 7.6, 1.4 Hz, H-1'), 1.61 (2H, h, *J* = 7.4 Hz, H-2'), 1.10 (3H, d, *J* = 6.4 Hz, H-8), 0.95 (3H t, *J* = 7.4 Hz, H-3').

^{13}C NMR (125 MHz, acetone- d_6): 170.7 (C-1), 149.8 (C-4) 138.6 (C-3), 134.9 (C-2), 113.9 (C-5), 71.2 (C-6), 70.9 (C-7), 27.7 (C- 1'), 21.5 (C-2'), 18.8 (C-8), 13.9 (C-3').

HRESIMS: *m/z* 213.1131 $[M+H]^+$ (calcd for $C_{11}H_{17}O_4^+$, 213.1132)

(*Z*)-6*R,7*R**-Dihydroxy-2-propyl-2,4-octadien-4-olide (4.2)**

$[\alpha]_D^{22}$: 55.6 (*c* 0.09, MeOH)

^{1}H NMR (400 MHz, acetone-d_6): 7.37 (1H, d, *J* = 1.5 Hz, H-3), 5.32 (1H, d, *J* = 9.2 Hz, H-5), 4.40 (1H, dd, *J* = 9.3, 6.2 Hz, H-6), 3.67 (1H, p, *J* = 6.4 Hz, H-7), 2.32 (2H, td, *J* = 7.5, 1.4 Hz, H-1'), 1.61 (2H, h, *J* = 7.4 Hz, H-2'), 1.11 (3H, d, *J* = 6.3 Hz, H-8), 0.95 (3H, t, *J* = 7.4 Hz, H-3').

^{13}C NMR (125 MHz, acetone- d_6): 170.6 (C-1), 149.9 (C-4), 138.6 (C-3), 135.1 (C-2), 114.0 (C-5), 71.7 (C-6), 71.0 (C-7), 27.8 (C-1'), 21.5 (C-2'), 19.2 (C-8), 13.9 (C-3').

HRESIMS: *m/z* 213.1123 $[M+H]^+$ (calcd for $C_{11}H_{16}O_4^+$, 213.1132)

Altenusin (4.3)

^{1}H NMR (400 MHz, DMSO-d_6): 1.87 (3H, s, 2'-Me), 3.76 (3H, s, 5-OMe), 6.12 (1H, d, *J* = 2.6 Hz, H-6), 6.44 (1H, d, *J*=2.6, H-6'), 6.45 (1H, s, H-4), 6.55 (1H, s, H-3'), 8.63 (1H, bs, 5'-OH), 8.63 (1H, bs, 4'-OH), 11,70 (1H, bs, 7'-OH).

^{13}C NMR (100 MHz, DMSO-d_6): 19.0 (2'-Me), 55.4 (5-MeO), 99.7 (C-4), 108.9 (C-2), 109.1 (C-6), 116.1 (C-6'), 116.8 (C-3'), 125.1 (C-2'), 132.7 (C-1'), 142.2 (C-5'), 144.0 (C-4'), 145.2 (C-1), 161.8 (C-3), 162.2 (C-5), 171.8 (C-7').

HRESIMS: *m/z* 291.0865 $[M+H]^+$ (calcd for $C_{15}H_{15}O_6^+$, 291.0874)

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Chapter Five:

Isolation of two new sesquiterpenes from a *Chaetomium globosum* endophyte of the Canadian medicinal plant *Empetrum nigrum*

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ABSTRACT

Following our investigations of fungal endophytes from Canadian medicinal plants, an extract of the endophytic fungus *Chaetomium globosum* (TC2-041) isolated from *Empetrum nigrum* was highlighted by mass spectrometry-based metabolomics as producing putatively new natural products. High resolution mass spectrometry guided fractionation led to the isolation of punctaporonin T (**5.1**) and U (**5.2**), two new

caryophyllene sesquiterpenes, along with the known natural products punctaporonin A (**5.3**), B (**5.4**) and C (**5.5**). Punctaporonin T exhibited significant and selective inhibition of *Staphylococcus aureus* (IC_{50} 105 μM) and *Mycobacterium tuberculosis* H37Ra (IC_{50} 237 μM) while being inactive against *Pseudomonas aeruginosa* and *Candida albicans*. Punctaporonin U, A, B and C were inactive against all test organisms.

Keywords: Endophyte, Medicinal plant, *Chaetomium globosum*, Punctaporonin, Metabolomics, HRMS, *Empetrum nigrum*

Endophytes, particularly those isolated from medicinal plants, are a promising source of natural products.[1-6] During our investigation of endophytic fungi from North American medicinal plants,[7-9] a *Chaetomium globosum* isolate (TC2-041) in our library was highlighted by untargeted HRMS-based metabolomic screening.[10]

The ethyl acetate extract of TC2-041 obtained from a two-week bench scale (10 L) fermentation was fractionated using a liquid-liquid partition, reversed-phase flash chromatography and HPLC. This led to the isolation of the two new natural products, punctaporonin T (**5.1**) and punctaporonin U (**5.2**), along with the known punctaporonins A (**5.3**), B (**5.4**) and C (**5.5**) (Figure 5.1). The structures of **5.3** – **5.5** were determined through analysis of HRMS and NMR (1D and 2D) and confirmed through comparison of spectral data with reported values.[11] Both **5.1** and **5.2** showed similarities to **5.3** – **5.5** in both ^{1}H and ^{13}C NMR (See Table S5.1 and Table S5.2).The punctaporonins [11-18] are caryophyllene sesquiterpenes similar in structure to the pestalotiopsins,[12,19-21] and the pestaloporinates.[22]

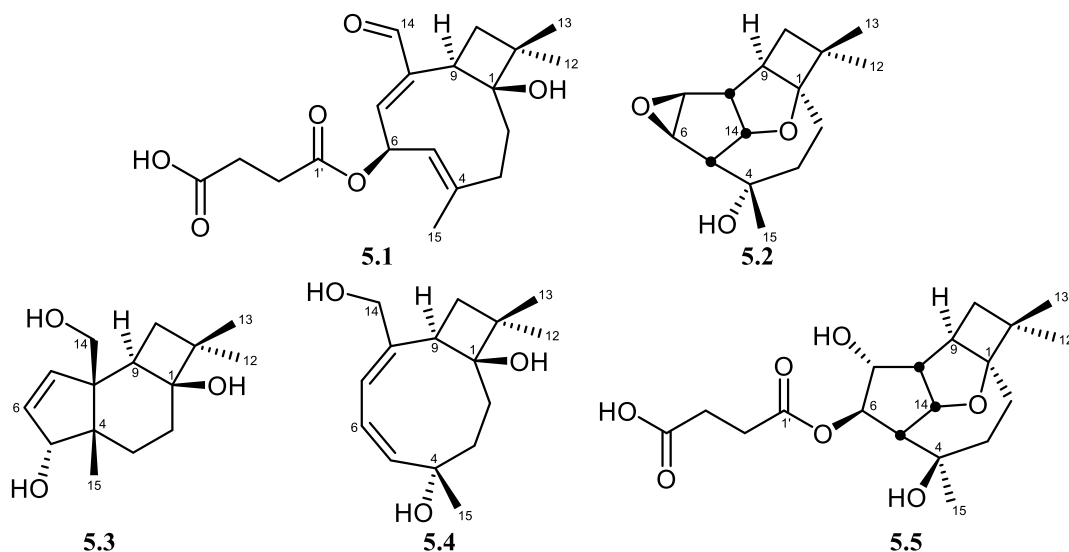

Figure 5.1. Punctaporonin T (**5.1**), punctaporonin U (**5.2**), punctaporonin A (**5.3**) punctaporonin B (**5.4**), punctaporonin C (**5.5**).

The molecular formula of **5.1** 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). ^{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) (Table 5.1).

The planar structure of **5.1** was determined through analysis of COSY and HMBC data (Figure 5.2). 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

Table 5.1. NMR spectroscopic data (700 MHz, DMSO-d_6) for punctaporonin T (**5.1**).

Position	δ_C, type	δ_H (J in Hz)	COSY	HMBC	NOESY
1	78.8, C				
2	34.9, CH_2	2.13, m	H-3a, H-3b	9, 4, 3, 1	H-12, H-9, H-6, H-2b
		1.63, m	H-3a, H-3b	9, 4, 3, 1	H-12, H-6, H-2a, 1-OH
3	34.3, CH_2	2.47, m	H-2a, H-2b	5, 4, 2, 1	H-5, H-3b, 1-OH
		1.88, m	H-2a, H-2b	5, 4, 2, 1	H-3a
4	139.8, C				
5	124.5, CH	5.41, d (10.4)	H-6	15, 7, 6, 3	H-9, H-7, H-3a, 1-OH
6	71.6, C	6.05, dd (10.6, 2.3)	H-7, H-5	14, 8, 7, 4, 1'	H-15, H-9, H-7, H-2a, H-2b
7	156.5, CH	6.69, bs	H-6	14, 9, 8, 6, 5, 1	H-15, H-14, H-6, H-5
8	141.6, C				
9	41.3, CH	3.15, dd (10.8, 5.7)	H-10a, H-10b	14, 11, 10, 8, 7, 2, 1	H-15, H-12, H-10a, H-10b, H-6, H-5, H-2a
10	30.1, CH_2	2.57, m	H-9	13, 12, 11, 9, 8, 1	H-14, H-13, H-10b, H-9
		1.58, t (11.5)	H-9	13, 12, 11, 9, 8, 1	H-12, H-10a, H-9
11	42.1, C				
12	25.8, CH_3	0.98, s		13, 11, 10, 1	H-10b, H-2a, H-2b
13	23.8, CH_3	1.04, s		12, 11, 10, 1	H-10a, 1-OH
14	195.9, CH	9.46, s		9, 8, 7	H-10a, H-10b, H-7
15	16.9, CH_3	1.91, s		5, 4, 3	H-9, H-7, H-6, 1-OH
1'	171.5, C				
2'	28.7, CH_2	2.56, m	H-3'	1', 3', 4'	
		2.50, m	H-3'	1', 3', 4'	
3'	28.9, CH_2	2.56, m	H-2'	1', 2', 4'	
		2.50, m	H-2'	1', 2', 4'	
4'	173.4, C				
1-OH		4.20, s		9, 2, 1	H-15, H-13, H-5, H-3a, H-2b
4'-OH		12.25, s			

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 correlation 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 correlations 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 data, specifically correlations 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).

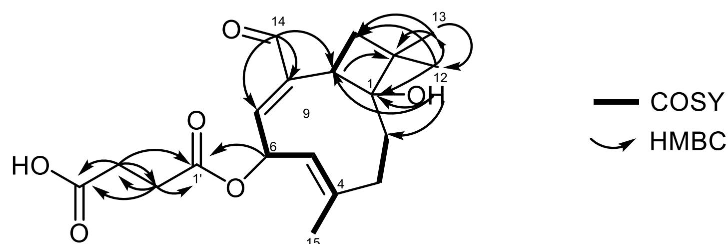

Figure 5.2. COSY and selected HMBC signals for punctaporonin T (**5.1**).

The relative stereochemistry of **5.1** was determined by analysis of ROESY data (Figure 5.3). NOE 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.[12, 14-22] 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

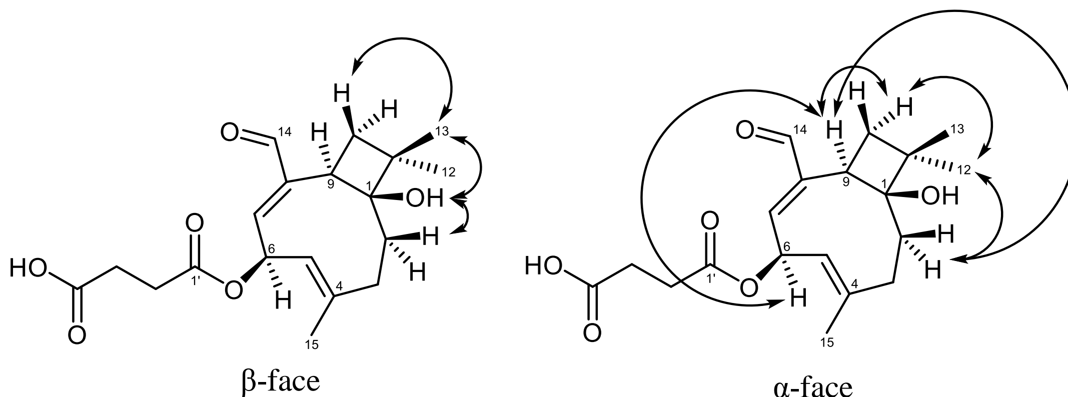

Figure 5.3. Key NOE correlations to assign relative stereochemistry of punctaporonin T (**5.1**).

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 **5.1**.

The stereochemistry of the double bonds was also determined by analysis of ROESY data (Figure 5.4). NOE 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 *E* configuration. NOE 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. Given the similarities in structure and biosynthesis of **5.1** and **5.5** the absolute stereochemistry is assumed to be 1*S*, 6*R* and 9*R*.

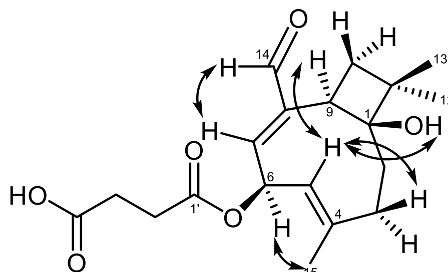

Figure 5.4. Key NOE correlations used to assign stereochemistry of the double bonds in punctaporonin T (**5.1**).

The molecular formula of **5.2** was determined to be $C_{15}H_{22}O_3$ from HRESIMS data for the protonated and sodiated molecular ions (251.1641 and 273.1459 respectively). Analysis of ^{1}H and ^{13}C NMR showed structural similarities between **5.2** and **5.5** apart from the absence in **5.2**, of the succinic acid side chain and a single hydroxyl proton attached to C-6 and C-7 respectively. This observation was supported by HRESIMS which indicated a difference of $C_4H_6O_3$. However, both C-6 and C-7 remained

oxygenated (δ_C 77.9 and 78.1 ppm respectively) suggesting an epoxide. Further support for the epoxide functional group was seen by NOE correlations between H-6 (δ_H 4.50 ppm) and H-7 (δ_H 3.70 ppm) as well as in coupling constants ($J_{6\text{-}7}$ = 2.2 Hz). The remaining planar structure of **5.2** was identical to **5.5** and was supported by COSY and HMBC correlations (Table 5.2).

Table 5.2. NMR spectroscopic data (700 MHz, DMSO- d_6) for punctaporonin U (**5.2**).

Position	δ_C, type	δ_H (J in Hz)	COSY	HMBC	NOESY
1	92.8, C				
2	28.0, CH_2	1.64, m	H-3a, H-3b	11, 9, 4, 3, 1	H-12
		1.59, ddd (13.7, 7.3, 1.6)	H-3a, H-3b	9, 3	H-13, H-3a
3	34.7, CH_2	1.81, ddd (14.2, 11.6, 7.4)	H-2a, H-2b	5, 4, 2	H-15, H-5, H-3b, H-2b
		1.49, m	H-2a, H-2b	15, 5, 4, 2, 1	H-15, H-3a
4	80.0, C				
5	53.2, CH	1.91, dd (4.7, 1.7)	H-14, H-6	8, 7, 6, 4	H-15, H-14, H-6, H-3a
6	77.9, CH	4.50, t (2.2)	H-7, H-5	14, 8	H-15, H-7, H-5
7	78.1, CH	3.70, t (2.7)	H-8, H-6, H-5	14, 8, 5, 4	H-9, H-8, H-6
8	50.0, CH	2.54, dd (8.2, 2.7)	H-14, H-7	15, 10, 9, 7, 5, 1	H-14, H-10b, H-9, H-7
9	41.3, CH	2.44, t (8.5)	H-10a, H-10b	14, 10, 8, 7, 2, 1	H-12, H-10a, H-7
10	37.1, CH_2	1.67, dd (10.5, 8.3)	H-9	13, 12, 11, 9, 8	H-12, H-10b, H-9
		1.50, m	H-9		H-14, H-13, H-8
11	37.6, C				
12	24.9, CH_3	1.05, s	H-13	13, 10, 1	H-13, H-10a, H-9, H-2a
13	21.7, CH_3	0.86, s	H-12	12, 10, 1	H-14, H-12, H-10b, H-2b
14	87.6, CH	5.05, dd (8.2, 4.6)	H-8, H- 5	15, 9, 8, 7, 4, 1	H-13, H-10b, H-8, H-5
15	31.4, CH_3	1.09, s		5, 4, 3, 2	H-6, H-5, H-3a, H-3b
4-OH		5.10, bs			

The relative stereochemistry was assigned through analysis of NOE correlations (Figure 5.5). NOE correlations between H-12 and H-10a (δ_H 1.67 ppm), H-12 (δ_H 1.05 ppm) and H-9 (δ_H 2.44), and H-9 and H-7 placed these groups on the β-face of the molecule which put the epoxide on the β-face. Confirmatory correlations were observed on the α-face between H-13 (δ_H 0.86 ppm) and H-14 (δ_H 5.05 ppm), H-14 and H-10b (δ_H 1.50 ppm), H-10b and H-8 (δ_H 2.54 ppm), H-14 and H-5 (δ_H 1.91 ppm), and H-5 and H-

15 (δ_H 1.09 ppm). Given the similarities in structure and biosynthesis between **5.2** and **5.5** the absolute stereochemistry is assigned as 1*S*, 4*S*, 5*S*, 6*R*, 7*S*, 8*R*, 9*R* and 14*S*.

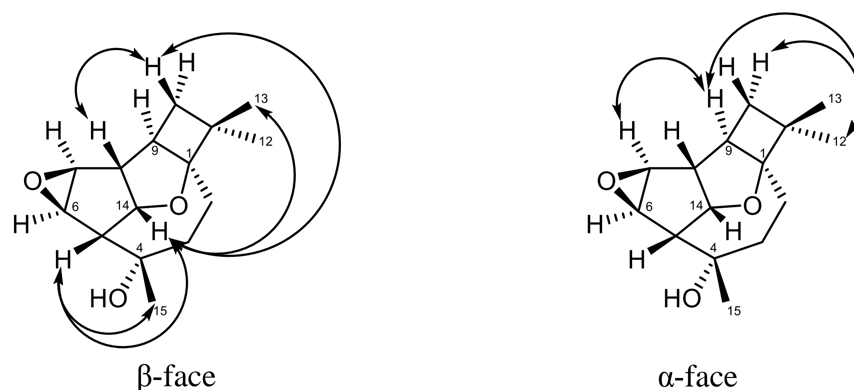

Figure 5.5. Key NOE correlations used to assign relative stereochemistry of punctaporonin U (**5.2**).

Other caryophyllene sesquiterpenes have exhibit anticancer activity against A549, HeLa, and SMMC-7721 cell lines, with pestalotiopsin C showing activity levels ($IC_{50} =$ 28.3 μM) comparable to etoposide ($IC_{50}=$ 23.2 μM) when tested against SMMC-7721.[12, 15] The punctaporonins have shown both anti-inflammatory and antibiotic activities, in addition to numerous cancer cell lines as well.[12, 19] All isolated compounds were assessed for inhibitory activity against *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 10145), *Mycobacterium tuberculosis* H37Ra (ATCC 25177) and *Candida albicans* (ATCC 14053). Punctaporonin T was found to have selective, moderate activity against *M. tuberculosis* H37Ra and *S. aureus* with IC_{50} values of 105 and 237 μM, respectively, while punctaporonins U, A, B and C were inactive against all test organisms. 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 compounds punctaporonin T and punctaporonin U. Both possess uncommon features within similar caryophyllene derivatives; punctaporonin T features an aldehyde, and a succinic acid side chain, while punctaporonin U features an epoxide.[11-22] The isolation of these compounds 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-d_6 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.[23] 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 2.0% malt extract agar and incubated at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2.0% malt extract agar until pure cultures were obtained.

Isolate TC2-041 was identified as *Chaetomium globosum* through an 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 (ITS) and 5.8S rRNA gene 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[24] and the amplified ITS DNA was 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 stoppered 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: Antimycobacterial assays against *M*. *tuberculosis* H37Ra (ATCC 25177) and antibacterial and antifungal assays against *S*. *aureus* (ATCC 29213), *P*. *aeruginosa* (ATCC 10145), methicillin-resistant *S*. *aureus* (ATCC 33591) and *C*. *albicans* (ATCC 14053) were performed as previously described.[25, 26]

LCHRMS metabolomic analysis: LC-HRMS based metabolomics was performed on 81 endophytic fungi grown in triplicate using processing methods as described by Forner et al.[10]

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 reversed-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 reversed-phase HPLC (65:35 H_2O/acetonitrile) to give **5.1** (22 mg). Fraction one (250 mg) was subjected to reversed-phase silica gel flash chromatography using a stepwise gradient of 19:1 H_2O/acetonitrile to 1:1 H_2O/acetonitrile. The eluents were combined according to their respective TLC profiles to yield ten fractions. Fraction 6 (55 mg) and 7 (25 mg) were further purified using reversed-phase HPLC. Fraction 6 (80:20

H_2O/acetonitrile) to give **5.3** (2 mg), **5.4** (4 mg), and **5.5** (14 mg) and fraction 7 (75:25 H_2O/acetonitrile) to give **5.2** (9 mg).

Punctaporonin T (5.1)

$[\alpha]_D^{22}$: -198.8 (*c* 0.14, MeOH); ^{1}H and ^{13}C NMR, see Table 5.1; HRESIMS: *m/z* 351.1805 $[M+H]^+$ (calculated for $C_{19}H_{27}O_6^+$, 351.1802)

Punctaporonin U (5.2)

$[\alpha]_D^{22}$: +76 (*c* 0.87, MeOH); ^{1}H and ^{13}C NMR, see Table 5.2; HRESIMS: *m/z* 251.1641 $[M+H]^+$ (calculated for $C_{15}H_{23}O_3^+$, 251.1647)

Punctaporonin A (5.3)

$[\alpha]_D^{22}$: -26.3 (*c* 0.19, MeOH); ^{1}H and ^{13}C NMR data were consistent with literature values;[11] HRESIMS: *m/z* 275.1609 $[M+Na]^+$ (calculated for $C_{15}H_{24}O_3Na^+$, 275.1628)

Punctaporonin B (5.4)

$[\alpha]_D^{22}$: -71.4 (*c* 0.42, MeOH); ^{1}H and ^{13}C NMR data were consistent with literature values;[11] HRESIMS: *m/z* 275.1620 $[M+Na]^+$ (calculated for $C_{15}H_{24}O_3Na^+$, 275.1628)

Punctaporonin C (5.5)

$[\alpha]_D^{22}$: -14.2 (*c* 1.41, MeOH); ^{1}H and ^{13}C NMR data were consistent with literature values;[11] HRESIMS: *m/z* 369.1902 $[M+H]^+$ (calculated for $C_{19}H_{29}O_7^+$, 369.1919)

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Supplemental information

Table S5.1. ^{1}H NMR spectroscopic data of punctaporonins T (**5.1**), U (**5.2**), A (**5.3**), B (**5.4**), and C (**5.5**).

	δ$_H$ (*J* in Hz)				
Position	(**5.1**)[a]	(**5.2**)[a]	(**5.3**)[b]	(**5.4**)[b]	(**5.5**)[b]
1					
2	2.13, m	1.64, m	1.70, m	1.71, m	1.84, m
	1.63, m	1.59, ddd (13.7, 7.3, 1.6)		1.36, m	1.65, m
3	2.47, m	1.81, ddd (14.2, 11.6, 7.4)	1.87, m	2.26, m	1.87, m
	1.88, m	1.49, m	1.19, m	1.36, m	1.40, m
4					
5	5.41, d (10.4)	1.91, dd (4.7, 1.7)	3.98, s	5.70, m	2.13, t (8.3)
6	6.05, dd (10.6, 2.3)	4.50, t (2.2)	5.62, s	5.71, m	5.07, t (7.3)
7	6.69, bs	3.70, t (2.7)	5.62, s	5.60, dd (14.0, 2.2)	3.91, t (6.6)
8		2.54, dd (8.2, 2.7)			2.63, td (6.8, 3.4)
9	3.15, dd (10.8, 5.7)	2.44, t (8.5)	2.38, dd (12.8, 7.6)	3.26, dd (12.0, 8.1)	2.70, ddd (9.2, 5.7, 3.4)
10	2.57, m	1.67, d (10.5, 8.3)	1.94, m	2.00, dd (11.9, 9.8)	1.90, dd (11.7, 5.8)
	1.58, m	1.50, m	1.43, dd (8.7, 7.7)	1.39, m	1.33, dd (11.7, 5.8)
11					
12	1.04, s	1.05, s	1.02, s	1.03, s	1.02, s
13	0.98, s	0.86, s	0.99, s	0.90, s	0.93, s
14	9.46, s	5.05, dd (8.2, 4.6)	3.51, d (11.1)	4.11, d (13.0)	4.85, dd (9.7, 6.7)
			3.32, m	3.83, d (13.1)	
15	1.91, s	1.09, s	0.89, s	1.06, s	0.94, s
1'					
2'	2.56, m				2.44, m
	2.50, m				
3'	2.56, m				2.44, m
	2.50, m				
4'					
1-OH	4.20, s				
4-OH		5.10, s			
4'-OH	12.25, s				

[a] Spectrum recorded in DMSO-d_6 at 700 MHz. [b] Spectrum recorded in DMSO-d_6 at 400 MHz

Table S5.2. ^{13}C NMR spectroscopic data of punctaporonins T (**5.1**), U (**5.2**), A (**5.3**), B (**5.4**), and C (**5.5**).

Position	δ_{C}, type				
	(**5.1**)[a]	(**5.2**)[a]	(**5.3**)[b]	(**5.4**)[b]	(**5.5**)[b]
1	78.8, C	92.8, C	78.3, C	80.5, C	94.2, C
2	34.9, CH_2	28.0, CH_2	30.4, CH_2	29.5, CH_2	26.6, CH_2
3	34.3, CH_2	34.7, CH_2	31.0, CH_2	34.2, CH_2	32.0, CH_2
4	139.8, C	80.0, C	45.4, C	73.0, C	73.6, C
5	124.5, CH	53.2, CH	86.9, CH	143.4, CH	55.0, CH
6	71.6, C	77.9, CH	130.2, CH	122.2, CH	80.2, CH
7	156.5, CH	78.1, CH	140.7, CH	123.4, CH	74.1, CH
8	141.6, CH	50.0, CH	54.9, C	140.9, C	59.1, CH
9	41.3, CH	41.3, CH	46.9, CH	41.4, CH	34.5, CH_2
10	30.1, CH_2	37.1, CH_2	35.3, CH_2	33.4 CH_2	40.2, CH_2
11	42.1, C	37.6, C	42.2, C	40.1, C	36.6, C
12	25.8, CH_3	24.9, CH_3	23.7, CH_3	24.4, CH_3	26.2, CH_3
13	23.8, CH_3	21.7, CH_3	23.4, CH_3	22.9, CH_3	29.4, CH_3
14	195.9, CH	87.6, CH	61.3 CH_2	63.3, CH_2	82.3, CH
15	16.9, CH_3	31.4, CH_3	24.4, CH_3	32.0, CH_3	29.4, CH_3
1'	171.5, C				171.9, C
2'	28.7, CH_2				29.4, CH_2
3'	28.9, CH_2				29.4, CH_2
4'	173.4, C				173.4, C

[a] Spectrum recorded in DMSO-d_6 at 175 MHz. [b] Spectrum recorded in DMSO-d_6 at 100 MHz.

Chapter Six:

General Discussion and Conclusion

This thesis documents an investigation of a collection of five endophytic fungi that resulted in the isolation of 11 natural products. Of these natural products, six were found to possess significant antibiotic activity in our screening assays while three natural products possessed new chemical structures. A biopolymer, poly(3*R*,5*R*-dihydroxyhexanoic acid) (**2.1**), was discovered (Chapter two) using a bioactivity-based prioritization and fractionation process. This polymer was unreported in primary literature and found to possess antibiotic activity for the first time, inhibiting the growth of both *Staphylococcus aureus* and *Mycobacterium tuberculosis* H37Ra. Three chlorinated bianthrones, neobulgarones D (**3.1**), E (**3.2**) and F (**3.3**), were discovered from a *Penicillium roseopurpureum* fungus isolated from a brown alga (Chapter three). These natural products exhibited selective antibiotic activity and revealed the possibility of an intriguing structure activity relationship that may warrant further investigation. Investigation of an *Alternaria alternata* isolate from the medicinal plant *Pelargonium tomentosum* (Chapter four) led to the discovery of the new natural product (*Z*)-6*R**,7*S**-dihydroxy-2-propyl-2,4-octadien-4-olide (**4.2**) as well as its diastereomer (*Z*)-6*R**,7*R**-dihydroxy-2-propyl-2,4-octadien-4-olide (**4.1**) and the mycotoxin altenusin (**4.3**). The use of HRMS metabolomics highlighted the fermentation extract of an endophytic fungus *Chaetomium globosum* isolate from the medicinal plant *Empetrum nigrum* (Chapter five). This resulted in the discovery of two new natural products, punctaporonin T (**5.1**) and U (**5.2**) along with the known punctaporonins A (**5.3**), B (**5.4**) and C (**5.5**). Of these, only

5.1 showed inhibitory activity against *S. aureus* and *M. tuberculosis* H37Ra, illustrating the power of HRMS metabolomics as a tool for the discovery of structurally new natural products from endophytic fungi that exhibit modestly bioactive natural products.

The discovery of these six bioactive natural products and three new natural products adds to the evidence that endophytic fungi from medicinal plants and marine macroalgae are an excellent source of both new and biologically active natural products. Bioactivity-based prioritization of natural product extracts (Chapters 2, 3 and 4) was successful in identifying five natural products with inhibitory activity. Although these were previously known structures, this discovery is important as these bioactivities can still have potentially relevant applications. To increase the likelihood of isolating new natural products with biological activity the bioactivity-based prioritization was combined with HRMS metabolomics (Chapter 5). This successfully led to the isolation of two new natural products, one of which possessed inhibitory activity. Although there is no guarantee that the biological activity exhibited by a fungal extract will correspond to that of the natural products targeted in the extract by HRMS metabolomics, combining bioactivity screening with a HRMS based prioritization will nevertheless increase the likelihood of isolating biologically active and new natural products is increased. Thus, in the future it is recommended that researchers from the Natural Products Research Group should focus their attention on endophyte extracts that are highlighted in both bioactivity screens and HRMS based screens.

Curriculum Vitae

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Submitted

Morehouse, N.J.; Flewelling, A.J.; Johnson, J.A.; Gray, C.A., Isolation of antibiotic 3*R*,5*R*-dihydroxyhexanoate polymers from endophytic fungi. *Natural Product Communications*, Submitted.

Morehouse, N.J.; Flewelling, A.J.; Johnson, J.A.; Gray, C.A., Halogenated bianthrones from *Penicillium roseopurpureum*, a fungal endophyte of the marine alga *Petalonia fascia*. *Natural Product Communications*, Submitted.

Conference Presentations:

Morehouse, N.J.; Clark, T.N.; Kerr, R.G.; Johnson, J.A.; Gray, C.A., Caryophyllene sesquiterpenes from a *Chaetomium globosum* endophyte. Maritimes Natural Products Conference, University of New Brunswick, Saint John, New Brunswick (August 2019) Poster presentation.

Morehouse, N.J.; Flewelling, A.J.; Johnson, J.A.; Gray, C.A., Structure elucidation of natural products from endophytic fungi. Maritimes Natural Products Conference University of New Brunswick, Saint John, New Brunswick (August 2019) Oral presentation.

Ellingwood, N.; **Morehouse, N.J.**; Johnson, J.A.; Gray, C.A., Can ^{1}H NMR metabolomics be used to dereplicate and prioritize fungal extracts for natural product discovery? Maritimes Natural Products Conference, University of New Brunswick, Saint John, New Brunswick (August 2019) Poster presentation.

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Therrien, J.; **Morehouse, N.J.**; Bos, A.; Clinton, M.L.; Complak, K.; Cox, S.; Clark, T.N.; Flewlling, A.J.; Forgrave, L.; Nazari, A.; Pendleton, K.; Valjanovska, M.; Johnson, J.A.; Gray, C.A., The NPRG fungal endophyte library. Maritimes Natural Products Conference, University of Prince Edward Island, Charlottetown, Prince Edward Island (August 2017), Poster presentation.