Part I. Chemoenzymatic Synthesis of Usnic Acid and Analogues of Usnic Acid

Part II. The Isolation of Citric Acid Derivatives from *Aspergillus niger*

by

Drew Jonathon Hawranik

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

Master of Science

Department of Chemistry

University of Manitoba

Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Part I. Chemoenzymatic Synthesis of Usnic Acid and Analogues of Usnic Acid

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Of

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Acknowledgements

First and foremost, I wish to express my gratitude to my supervisor, Dr. John Sorensen. He has worked with me from day one to ensure that my research was of high quality and performed efficiently. He has kept me on track with my progress, especially when I needed some extra encouragement. John is also someone who is very personable and I'm glad to be able to say I've enjoyed my time with John outside of the lab just as much as inside the lab. Maybe even a little bit more. John has taught me what it means to be a graduate student. I look up to him as a role model and mentor and take what he has to say to heart. It has been a pleasure working with him over the past 20 months.

Secondly, I would like to thank all those faculty members and students who have been of immense help during my studies: Dr. Tom Ward of the Manitoba Chemical Analysis Laboratory for his assistance and expertise with HPLC/MS; Dr. Michele Piercey-Normore for instructing me on the methods of DNA sequencing; Dr. Tom Booth for providing an identification of a fungus sample; Dr. Kirk Marat, Dr. Philip Hultin and Dr. Mazdak Khajehpour for their time and advice on professional matters; Dr. Carl Bartels, Dr. Sean McKenna, Dr. Scott Kroeker, and Dr. Horace Luong for their camaraderie; and all of my graduate student colleagues who have lent a helping hand or shared a pitcher of beer at the end of the day, whatever time that may have been.

I would also like to acknowledge the members of my committee: Dr. Philip Hultin and Dr. Tom Booth. They are both integral parts of this process and I thank them for their chivalrous willingness to act as my committee members.

Finally, I wish to acknowledge my family. They have always been there to offer their wisdom and guidance which I sincerely appreciate. They have supported me thanklessly over the years. I thank them for this. Their unwavering encouragement and confidence in me has enabled me to forge on, and know that things will work out if I apply myself. I am proud to be able to call these people my family.

Abstract

Usnic acid **1**, a highly functionalized dibenzofuran, is a polyketide secondary metabolite produced by several genera of lichens such as *Usnea*, *Cladonia*, *Alectoria*, *Evernia*, *Ramalina*, and *Lecanora*. Usnic acid (**1**) was first described in 1844 and has seen extensive use in traditional folk medicine systems as an antibiotic. More recently, anticancer, antiviral, antioxidant, anti-inflammatory, and analgesic properties have also been demonstrated. The proposed biosynthesis of usnic acid (**1**) has been advanced through detailed labeling studies. However, the actual enzymes and genes responsible for production of **1** in lichens have not been identified or characterized. Work presented in this thesis is directed at the synthesis of a key biosynthetic precursor to usnic acid (**1**), methylphloracetophenone (**2**). Methylphloracetophenone (**2**) has been synthesized using two separate routes. The first involved the methylation of trihydroxyacetophenone (**3**) with iodomethane. The second involved the acylation of trihydroxytoluene (**4**) with acetic anhydride using boron trifluoride diethyl etherate as a catalyst. This work will lay the foundation for further biosynthetic studies on usnic acid (**1**).

Using horseradish peroxidase as a model enzyme system, we demonstrate that methylphloracetophenone (**2**) was oxidized to usnic acid (**1**). Analogues of **2** have been synthesized and include an ethyl group (**5**) and a propionyl group (**6**). These analogues were also

subject to oxidation by horseradish peroxidase and we demonstrate that analogues of usnic acid (**7** and **8**) are formed. Although usnic acid (**1**) has potential as a pharmaceutical, it is hepatotoxic. These analogues of usnic acid (**1**) may be able to improve its pharmacological profile.

As part of a natural products screening program, soil fungi have been cultured and grown in fermentation broths. Soil for this program was provided from the University of Manitoba cryptogam herbarium which houses lichen samples. From one strain of fungus, dimethyl oxalate (**9**), trimethyl citrate (**10**) and dimethyl citrate (**11**) are isolated. This fungus has been identified as *Aspergillus niger* both morphologically and genetically. *A. niger* is responsible for the production of millions of tonnes of citric acid annually, but this is the first report of methylated citric acid derivatives or dimethyl oxalate (**9**) from a filamentous fungus.

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Abbreviations

°C	degrees Celsius
[O]	oxidation
^{1}H-NMR	proton nuclear magnetic resonance
^{13}C-NMR	carbon nuclear magnetic resonance
1184-5′	forward primer used in ribosomal DNA with sequence 5′-GACTCAACACGGGGAAACTC-3′
1780F-5′	forward primer used in ribosomal DNA with sequence 5′-CTGCGGAAGGATCATTAATGAG-3′
^{2}J	geminal coupling
26S	large ribosomal subunit
5.8S	small ribosomal subunit
AB	alpha beta
ACP	acyl carrier protein
AU	activity units
$BF_3 \cdot OEt_2$	boron trifluoride diethyl etherate
BP	base pair
BPB	bromophenol blue
br s	broad singlet
ca	circa
cm	centimeters
CoA	Coenzyme A
CTAB	cetyltrimethylammonium bromide

DCM	dichloromethane
ddNTP	dideoxynucleotide triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
Dr.	doctor
EDTA	ethylenediaminetetraacetic acid
EI	electron ionization
EI-MS	electron impact mass spectrometry
Enz	enzyme
eq	equivalents
ESI	electrospray ionization
ESI-MS	electrospray ionization mass spectrometry
et al.	and others
Et_2O	diethyl ether
EtOAc	ethyl acetate
g	grams
h	hours
HPLC	high performance liquid chromatography
HPLC/MS	high performance liquid chromatography/ mass spectrometry
HRP	horseradish peroxidase
Hz	hertz
ITS	internal transcribed spacer
ITS1	first internal transcribed spacer in ribosomal DNA

ITS2	second internal transcribed spacer in ribosomal DNA
ITS4-3′	reverse primer used in ribosomal DNA with sequence 5′-TCCTCCGCTTATTGATATGC-3′
J	coupling constant
M+	positive molecular ion
$[M+H]^+$	positive molecular ion with hydrogen
$[M+Na]^+$	positive molecular ion with sodium
m/z	mass per charge
M	molarity
MeOH	methanol
mg	milligram
MHz	megahertz
µg	micrograms
µL	microlitres
µm	micrometers
µmol	micromoles
mL	millilitres
min	minutes
mm	millimeters
mmol	millimoles
MS	mass spectrometry
N	normality
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form

ng	nanograms
nm	nanometers
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
Pfu	*Pyrococcus furiosus*
pH	power of hydrogen
PKS	polyketide synthase
pmol	picomole
pro-R	A stereoheterotopic group c (as in tetrahedral Xabc2) is described as *pro-R* if, when it is arbitrarily assigned CIP priority over the other stereoheterotopic group c, the configuration of the thus generated chiral centre is assigned the stereodescriptor R.
pro-S	A stereoheterotopic group c (as in tetrahedral Xabc2) is described as *pro-S* if, when it is arbitrarily assigned CIP priority over the other stereoheterotopic group c, the configuration of the thus generated chiral centre is assigned the stereodescriptor S.
q	quartet
rDNA	ribosomal deoxyribonucleic acid
R_f	retention factor
RF	radio frequency
rpm	revolutions per minute

rt	room temperature
s	singlet
SAM	*S*-adeonsyl methionine
SDS	sodium dodecyl sulfate
sec	second
S_N2	bimolecular nucleophilic substitution
sp.	species
t	triplet
TBE	tris(hydroxymethyl)aminomethane borate ethylenediaminetetraacetic acid
TES	tris(hydroxymethyl)aminomethane ethylenediaminetetraacetic acid
TLC	thin layer chromatography
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloric acid
	sodium chloride
US	United States
UV	ultraviolet
UV-vis	ultraviolet-visible
V	volts

1. Introduction

1.1 Lichens and usnic acid (1)

Natural products, or secondary metabolites, are organic compounds produced by plants and microorganisms which usually do not play a direct role in producing energy through primary metabolism for the growth and development of the organism.[1] Natural products are often specific to groups of organisms and may also be specific to conditions of the organism's environment. They have a wide array of roles involving signaling or protection that they can perform: volatile attractants for the same or other species, promotion of beneficial interactions with organisms, defense against pathogens, pests, and abiotic stresses.[2] In most cases, the role of secondary metabolites in a particular organism or the benefit they provide is not yet known.[1] Because natural products are generally used for some sort of interaction with another organism, they may have biological activities that are useful to humans.

In the modern-day use of prescription drugs that are natural products, approximately 74% are consistent with the original ethnomedical use of the unrefined compound.[3] Over 64% of the global population uses plants in traditional systems of medicine as a main source of health care.[4] For bacterial infections over 80% of all medicines in clinical use, while for anticancer agents

over 60% of all drugs are either natural products or derivatives thereof .[5,6] Some classic examples of drugs derived from natural products include the antibacterial drug penicillin G (from the fungus *Penicillium chrysogenum* (Thom)), the anticancer drug taxol (from the tree *Taxus brevifolia* (Nutt.)), and the analgesic aspirin (derived from salicin from *Salix* sp.).

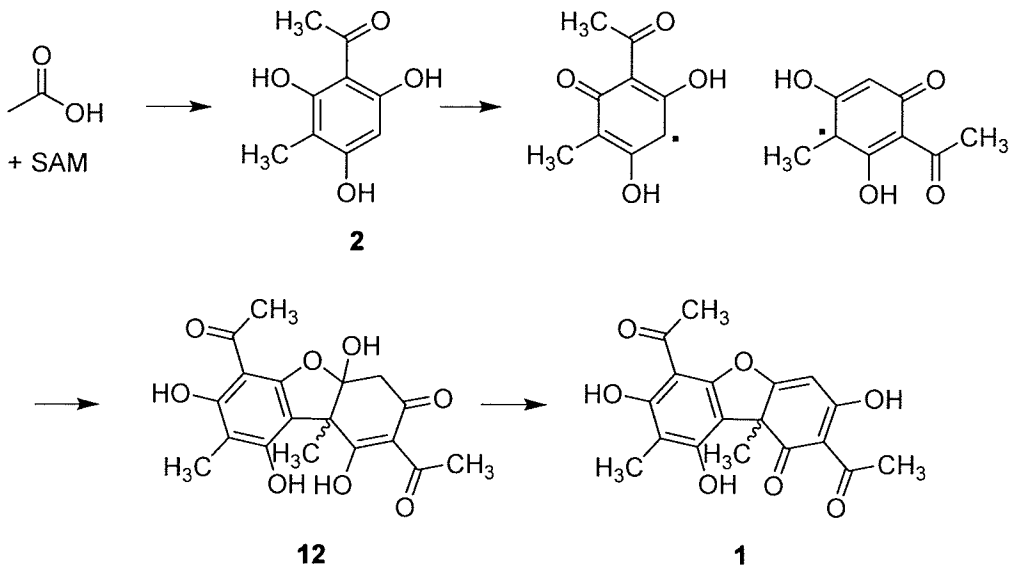
Lichens are the result of a symbiotic association between a fungal partner (mycobiont) and an algal partner (photobiont). Together, the fungus and algae display very different properties than they do when on their own. These ubiquitous organisms thrive in a variety of geographical niches but are particularly common in the harsh climate of the sub-arctic north. This is possible because lichens are capable of surviving extremely low levels of water. Generally, because of their adaptation to these harsh environments lichens have proven to be a rich source of biologically active secondary metabolites.[7]

Lichens are a rich source of natural products that exhibit a variety of biological activities suitable for use as pharmaceuticals[8] or pesticides.[9] One example is usnic acid (**1**) a metabolite common in lichen genera such as *Usnea*, *Cladonia*, *Alectoria*, *Evernia*, *Ramalina*, and *Lecanora*. This compound was first described in 1844 and has been subjected to extensive study of its biological activity ever since. The antibacterial activity of usnic acid (**1**) was recognized early, whereas findings of anticancer, antiviral, antioxidant, anti-inflammatory, and analgesic properties have been more recent (For a review of the biological activity and applications of usnic acid see: Ingolfsdottir, *et al.*[10]). In the United States, usnic acid (**1**) and extracts containing usnic acid (**1**) are available over-the-counter as dietary supplements to assist in weight loss. However, numerous reports to the US Food and Drug Administration have linked these weight loss products to liver damage.[11] Despite this apparent toxicity there are still clinical applications of usnic acid (**1**) including uses such as an additive to topical preparations, mouthwash, and

toothpaste.[10] Usnic acid (**1**) has also been proposed as a biomarker molecule in the search for life on Mars.[12] Because of the wide array of biological activity displayed by usnic acid (**1**) we have become interested in examining the biosynthesis of this natural product in more detail. A clear understanding of the biosynthesis of usnic acid (**1**) in lichens may lead to the tools necessary to develop analogues that retain the beneficial bioactivity while mitigating the hepatotoxicity.

1.2 Polyketides and the proposed biosynthesis of usnic acid (1)

Early work by Shibata confirmed the biosynthetic origin of usnic acid (**1**) as being derived from acetic acid, presumably via the polyketide pathway.[13] A series of feeding experiments performed using radio-labelled (^{14}C) sodium acetate, diethyl malonate and sodium formate led to the isolation of radio-labeled usnic acid (**1**). Very detailed degradation experiments confirmed that the ^{14}C atoms were incorporated at positions in usnic acid (**1**) as predicted by the polyketide biosynthetic pathway. Feeding experiments with ^{14}C labeled methylphloracetophenone (**2**) resulted in the isolation of radioactive **1**, confirming that **2** is indeed the biosynthetic precursor for **1**. A proposed biosynthesis of usnic acid (**1**) based on the results of these experiments is summarized in **Scheme 1.1**.[14]



Scheme 1.1. Biosynthesis of usnic acid (**1**) proceeding through the intermediate methylphloracetophenone (**2**) which is polyketide derived.

In the structure of usnic acid (**1**), there is a center of chirality at the quaternary carbon in the nonaromatic ring which has the methyl group attached to it (**Figure 1.1**). X-ray analysis has confirmed that the absolute configuration of (+)-usnic acid (**1**) is R.[15] Besides (+)- and (-)-usnic acid, there exist two other isomers that are found in lichens. Isousnic acid is produced when the dimerization of two molecules of methylphloracetophenone (**2**) occurs with one molecule flipped relative to the formation of usnic acid (**1**). This results in a different substitution pattern on the aromatic ring (**Figure 1.1**). The most common of these isomers is (+)-usnic acid.

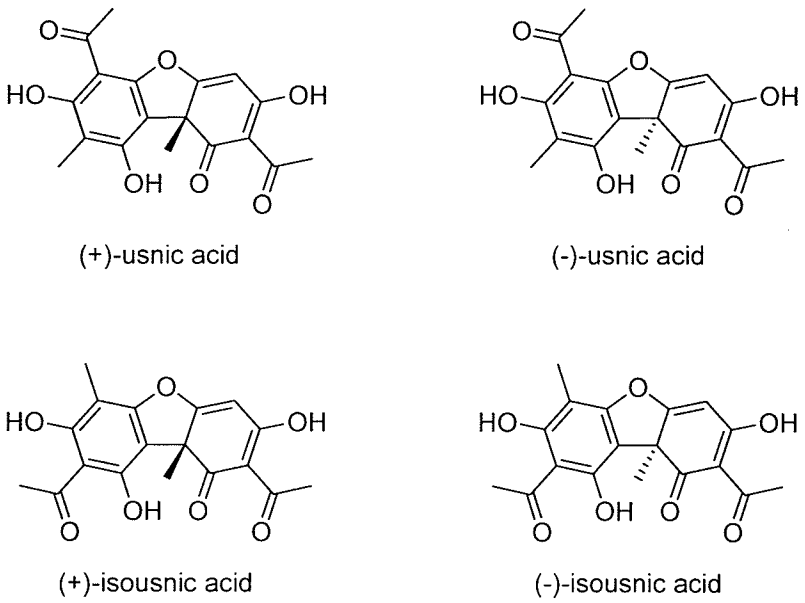
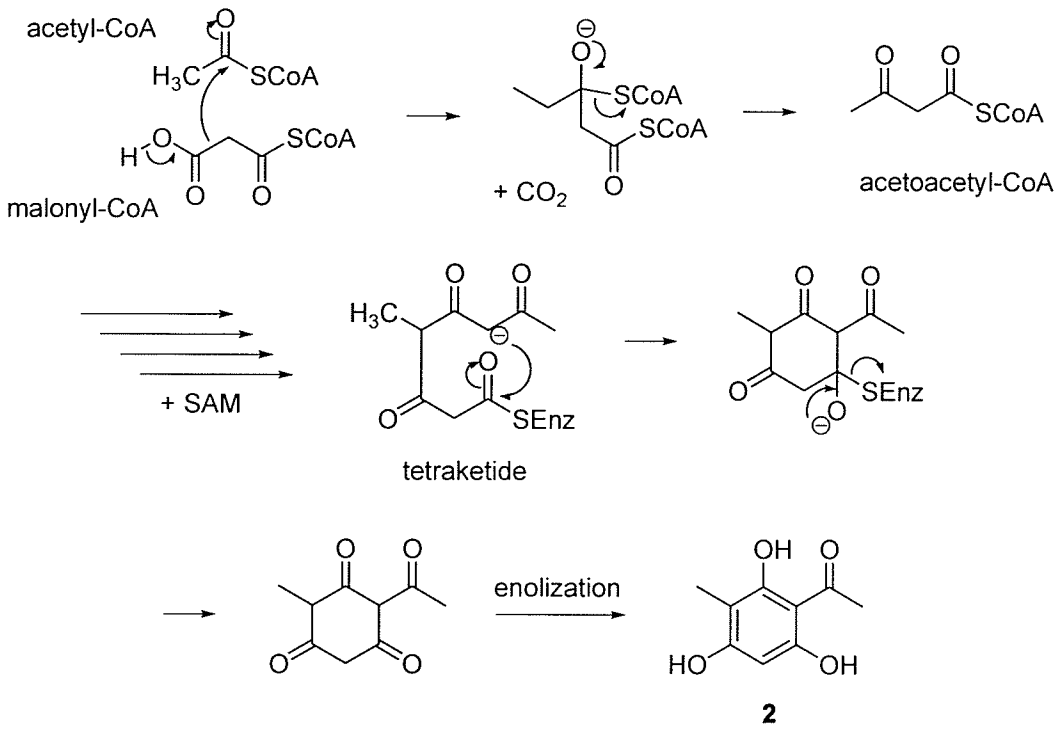


Figure 1.1. Four naturally occurring isomers of usnic acid (**1**).

The biosynthetic proposal in **Scheme 1.1**, based on Shibata's work, suggests that there are two enzymes involved in the production of **1**. It appears likely that a polyketide synthase (PKS) is responsible for the biosynthesis of the key intermediate on the pathway, methylphloracetophenone (**2**). A typical PKS enzyme will add malonyl-Coenzyme A (CoA) extender units to a starter acetyl-Co A molecule through a Claisen condensation to build up a putative tetraketide that is attached to the acyl carrier protein (ACP) of the PKS enzyme through a thioester bond (**Scheme 1.2**). The coenzyme A cofactors are also initially attached to their respective compounds through a thioester linkage.



Scheme 1.2. A Claisen condensation between acetyl-CoA and malonyl-CoA to form acetoacetyl-CoA, a diketide. The tetraketide intermediate is bound to the polyketide synthase enzyme through a thioester linkage. The tetraketide is methylated by *S*-adeonsyl methionine (SAM). Cyclization of the methylated tetraketide is followed by enolization to afford methylphloracetophenone (**2**).

Previous labeling studies by Shibata *et al.*[14] demonstrated that labeled phloracetophenone was not incorporated into usnic acid (**1**). This indicates that the methyl group on methylphloracetophenone (**2**) must be incorporated prior to cyclization of the polyketide.

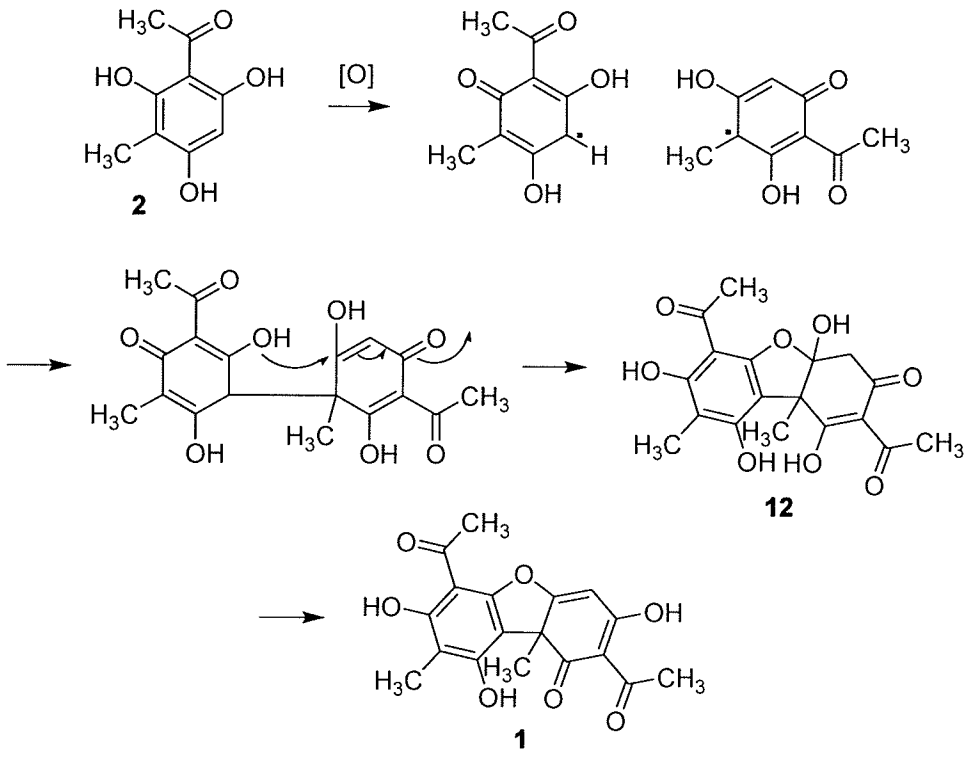
The ^{14}C label from sodium formate was found at the positions on usnic acid (**1**) consistent with two methyl groups being derived from the 1-carbon pool via S-adenosyl methionine (SAM) (**Scheme 1.2**). At some point after the tetraketide has been methylated by SAM, it is ionized at the appropriate methylene carbon. A Claisen reaction takes place and is terminated by the

expulsion of the thiol leaving group which is a part of the PKS enzyme. Methylphloracetophenone (**2**) is provided by the enolization of the cyclohexatrione which gives the molecule stability provided by the aromatic ring (**Scheme 1.2**).

This enzyme is presumably similar to other non-reducing 6-methyl salicylic acid synthase related (Type-II) PKS enzymes that have been reported in other lichenized fungi.[16]

1.3 Oxidative enzymes and the proposed biosynthesis of usnic acid (1)

The final step in the biosynthesis of **1** appears to be an oxidative homocoupling of two molecules of **2** catalyzed by a separate oxidative enzyme (**Scheme 1.3**). After a radical carbon-carbon bond forming between the two molecules, a Michael addition forms hydrated usnic acid (**12**) which undergoes dehydration to give **1**. Because predominantly (+)-usnic acid is found in nature and not the three other isomers (**Figure 1.1**), it appears likely that the binding site of the oxidative enzyme has both molecules of **2** in it while the coupling takes place. The amino acid residues in the binding site can control the orientation of the functional groups of **2** to specifically form (+)-usnic acid.



Scheme 1.3. The homocoupling of two molecules of methylphloracetophenone (**2**) by an oxidative enzyme to form usnic acid (**1**).

Phenolic oxidative coupling can be performed by oxidase enzymes. These systems are known to be radical generators and perform oxidations or reductions using molecular oxygen as the electron acceptor. One class of oxidase enzymes, laccases, contain copper to perform one electron oxidations on phenols. Peroxidase enzymes, another class of oxidase enzymes, use hydrogen peroxide as an electron acceptor. Cytochrome P_{450} dependent enzymes require NADPH and oxygen as cofactors and can also perform one-electron oxidations of phenols. It is likely that the oxidative enzyme employed by lichens to produce usnic acid (**1**) falls into one of these classes. Although there has been recent interest in lichen secondary metabolites in general,[17] little work has been focused on usnic acid (**1**) biosynthesis. None of the enzymes, or the genes coding for them, involved in the biosynthesis of **1** have been identified. No efforts have

been reported in the literature regarding the characterization of either the PKS or oxidative enzyme responsible for biosynthesis of **1** in lichens. We decided, therefore, our first step in investigating the biosynthesis of usnic acid (**1**) would be to characterize the oxidative enzyme.

In order to characterize the enzyme, we felt it was necessary to devise a simple, cost-effective method to synthesize the key biosynthetic intermediate methylphloracetophenone (**2**) for use as an isotopically labeled substrate to assay for the oxidative enzyme in lichens responsible for usnic acid (**1**) production. Because isotopically labeled reagents to produce labeled **2** are expensive, we needed to develop a simple and efficient synthesis of **2** in order to stay cost-effective. Here, we report the one-step synthesis of **2** and its conversion to usnic acid (**1**) by a model enzymatic oxidation using horseradish peroxidase (HRP).[18] We chose to use HRP as a model system as this had been successfully used in a previous synthesis of usnic acid (**1**).[19] Furthermore, HRP has a relatively accessible active site and will accept a large number of organic compounds, including phenols, as hydrogen donors. HRP is also commercially available and relatively inexpensive.

Despite exhibiting a variety of important and useful biological activities, the clinical applications of **1** are limited by its hepatotoxicity. We felt it necessary to attempt to improve the pharmacological profile of this compound. Generally, this is accomplished by producing analogues of the parent compound and testing their toxicity and activity. Because HRP can be used on a great number of different substrates, we synthesized analogues of methylphloracetophenone (**2**) and used HRP to attempt to generate analogues of usnic acid (**1**).

Usnic acid analogues have been produced previously[20-25] by routes that involve several synthetic steps. Such synthetic routes have been observed to suffer from poor stereoselectivity, complexity, and low overall yield. Some of these results however have provided some structure-

activity relationship information about usnic acid (**1**). The active centres of this molecule appear to be the dibenzofuran nucleus, the phenolic hydroxyl groups, and the double bond in the nonaromatic ring next to the furan oxygen.[22-25] It has been shown that when the phenolic groups are acylated, the antibacterial activity against *Mycobacterium tuberculosis* is decreased by 50 %. A decrease in activity (75 %) is even more evident when the double bond next to the furan oxygen in the nonaromatic ring is reduced.[25]

1.4 Fungi from a lichen collection

A cryptogam is a collection of lower plants that have small encapsulated spores and applies to fungi, mosses and liverworts. Cryptogam literally means "hidden seed." These herbaria contain material that can provide information on the organisms' DNA, their morphology as well as the natural products that they may produce. Cryptogams allow for comparisons among species and over time. Lichens are rich in secondary metabolites containing hundreds of compounds unique to lichen fungi. Non-lichenized fungi also produce a diversity of secondary metabolites such as penicillin and erythromycin, antibiotics that have important clinical roles. In properly maintained herbariums, lichen secondary metabolites remain on or within the lichen thallus, sometimes for centuries, providing a valuable source of chemicals and information.

The cryptogamic division of the University of Manitoba herbarium contains a collection of over 7000 samples of lichens, mostly from Canada, and is curated by Dr. Michele Piercey-Normore of the Department of Biological Sciences. These lichens are often collected from harsh environments such as those found around Hudson Bay or the Bay of Fundy. Lichens are known to contain a wide assortment of natural products which aid in their survival in such climates.

Collected with each lichen sample is some soil that the lichen is attached to. Although lichens do not require soil for growth, this attached soil makes up an important part of the microhabitat. This soil contains fungi that had been living in the same extreme climates as the lichens. It is possible that fungi found in the soil from the lichen samples may also have developed adaptive measures to survive in stressful environments. We are developing a natural products screening program which takes advantage of this huge variety of soil samples that contain fungi from across the country which may provide new natural products with interesting biological activities.

Filamentous soil fungi can be grown quite quickly which make them desirable to work with when screening for new natural products. Therefore, secondary metabolites are produced more rapidly by most soil fungi which allow us to screen many different fungal strains for novel products in a relatively short amount of time.

1.5 Importance of soil fungi

Soil fungi are an important source of natural products and are ubiquitous in soil The compounds produced by soil fungi may be of great use or great harm to humans. One example we will consider is that of a class of compounds known as aflatoxins.

Aflatoxins (**Figure 1.2**) are a group of highly toxic metabolites produced by only a few *Aspergillus* species, notably *A. flavus* (Link) and *A. parasiticus* (Speare). Because they cause hepatic necrosis and liver cancer and have been found in all major cereal crops, they are of great concern. Of the main naturally occurring aflatoxins, aflatoxin B_1 is the most commonly

encountered member and also the most toxic and carcinogenic. Aflatoxin G_1 contains a six-membered lactone rather than a five-membered cyclopentenone ring (**Figure 1.2**).

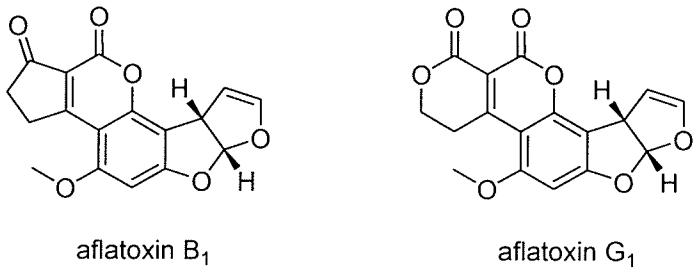


Figure 1.2. Aflatoxin B_1 and G_1, highly toxic metabolites produced by soil fungi.

Aflatoxins were first detected following the deaths of young turkeys fed with mold contaminated peanuts. Peanuts remain one of the most common crops to become contaminated with a fungus causing a human health risk. Peanuts and other food materials, including milk and eggs, are routinely screened to ensure aflatoxin levels do not exceed allowable limits set by the US Food and Drug Administration. Minute quantities of aflatoxins are unavoidable in all commercial sources of peanut butter but quantities are usually below the recommended safe level.[1]

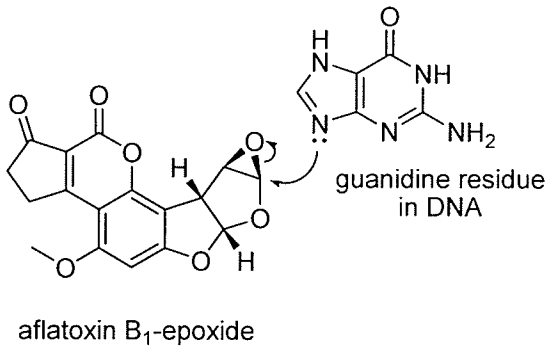


Figure 1.3. Aflatoxin B_1-epoxide in the liver can intercalate in DNA, allowing for nucleophilic attack by a guanidine residue.

The source of aflatoxin toxicity appears to result from the epoxidation of the furan ring by metabolism in the body with cytochrome P_{450}. The epoxide can intercalate with DNA and becomes oriented in a manner favoring nucleophilic attack by guanidine residues (**Figure 1.3**) initiating mutagenic activity. DNA replication and RNA transcription are inhibited in the liver, where the toxin accumulates, leading to symptoms including liver enlargement, fat deposition, and necrosis eventually leading to death from irreversible liver failure. In parts of Africa and Asia, levels of aflatoxins in food crops frequently exceed the recommended level by more than two orders of magnitude. This is likely linked to the increased incidence of liver cancer in these regions.[1]

Aflatoxins are just one of many examples of natural products that are produced by soil fungi that exhibit properties important to humans.

1.6 Summary

We are interested in natural products as they are an important source for the discovery of new drugs. Lichens and soil fungi are both significant sources of natural products that have a potential pharmacological role. Usnic acid (**1**) is a secondary metabolite found in lichens that has numerous valuable biological activities including antibacterial and anticancer properties, among others. Usnic acid (**1**) is also hepatotoxic but it is possible that analogues of **1** may avoid this harmful property while maintaining activity that would make it useful in a pharmaceutical setting. The genes and enzymes responsible for the production of usnic acid (**1**) have not yet been described, but it is our goal to do so. New drugs are also available from soil fungi. We are

developing a natural products screening program aimed at the discovery of useful compounds that utilizes a lichen cryptogam containing soil taken from remote locations across Canada.

2. Materials and Methods

2.1 General

All solvents used were HPLC grade and were obtained from Fisher Scientific except where noted. Reagents obtained from Sigma Aldrich include trihydroxyacetophenone (**3**), iodomethane, iodoethane, boron trifluoride diethyl etherate, acetic anhydride, propionic acid, horseradish peroxidase, potassium iodate, potassium ferricyanide, pyrogallol (**18**), and acetone-d_6. Chloroform-d and methanol-d_4 were purchased from Cambridge Isotope Laboratories, Inc. Trihydroxytoluene (**4**) was obtained from City Chemical LLC, usnic acid (**1**) from ChromaDex, and propionic anhydride from Fluka Analytical. Hydrogen peroxide was purchased from the University of Manitoba pharmacy and was made by Pure Standard Products.

Flash chromatography was performed using silica gel (230-430 mesh, 60 Angstroms) (Fisher Scientific, Fair Lawn, NJ, USA) and elution of solvent with a positive pressure of air.

Evaporation refers to the removal of solvent under reduced pressure with a rotary evaporator.

TLC was performed using Silica Gel 60 F_{254} 20 x 20 cm plates and spots were visualized under UV light and with a potassium permanganate staining solution. The staining solution was

made by mixing together 20 g of potassium carbonate, 30 g of potassium permanganate, 300 mL of water, and 5 mL of 5% aq NaOH.

Proton and carbon NMR were obtained on either a Bruker AMX500 operating at 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR), or a Bruker AVANCE 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) spectrometer.

Before use, trihydroxyacetophenone (**3**) monohydrate was dissolved in acetone and dried in vacuo three times in an attempt to remove water of hydrateion.

For reactions performed above room temperature, a Heidolph 3001K hotplate with an EKT 3001 temperature control was used.

Water was purified for HPLC and for microbiology using a Barnstead Easypure II Ultrapure water system hooked up to an in-house reverse osmosis water line.

A Labnet International, Inc. Spectrafuge 24D benchtop centrifuge was used to precipitate enzyme assays. Centrifuge and Eppendorf tubes were from VWR.

A Sanyo Autoclave MLS-3781L was used to sterilize agar plates and agar broths.

Horseradish peroxidase was supplied by Sigma Aldrich and over the three different batches it came as 254, 259, and 256 activity units (AU)/mg where 1 AU is defined as the amount of HRP required to form 1.0 mg of purpurogallin (**17**) from pyrogallol (**18**) in 20 sec at pH 6.0 at 20 °C (**Scheme 3.6**). All HRP assays were performed at 37 °C.

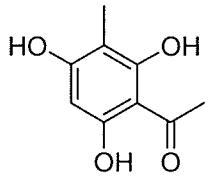
HPLC method development was accomplished using a Waters 2695 Separations Module, a Waters 2996 Photodiode Array Detector, and data was analyzed using Empower 2 Pro software.

Electron ionization (EI) quadrupolar MS experiments were performed on a Varian 320-MS spectrometer with a voltage of 70 eV and the ion source temperature set to 250 °C. The

electrospray ionization (ESI) HPLC/MS experiments were performed on a Varian 500-MS Ion Trap spectrometer set with a needle voltage of 0 V, a capillary voltage of 80 V, and 100% RF loading. Columns used were either Nova-Pak C_{18} 4 μm (3.9 x 150 mm) or Sunfire C_{18} 3.5 μm (4.6 x 150 mm)

2.2 Synthesis of methylphloracetophenone (2) and analogs

*2.2.1 Methylphloracetophenone [1-(2,4,6-trihydroxy-3-methylphenyl)ethanone] (**2**)*



Method A

To a stirred solution of trihydroxyacetophenone (**3**) (1.862 g, 10.00 mmol) and K_2CO_3 (3.45 g, 25.0 mmol) in acetone (100 mL) at 0 °C was added iodomethane (2.50 mL, 40.0 mmol). The reaction was monitored by TLC (5% MeOH/DCM x4) and was stopped after 9 hours with the addition of H_2O (100 mL), and acidification to pH 1 with HCl. An extraction was performed with EtOAc (3 x 100 mL) and the organic solvent was dried with Na_2SO_4 and evaporated to dryness to give 2.205 g of material. Flash chromatography (1% MeOH/DCM) provided 780.6 mg (45%) of **2** as a brown-pink powder.

Method B

A solution of trihydroxytoluene (**4**) (0.700 g, 5.00 mmol), acetic acid (10.3 mL, 180 mmol), acetic anhydride (1.03 mL, 10.9 mmol) and $BF_3 \cdot OEt_2$ (0.670 mL, 5.43 mmol) was

heated at 100 °C for 2 h.[26] The reaction was cooled to 0 °C and the pH was adjusted to 4-5 with 3 N NaOH. The mixture was then diluted with water (100 mL). Extraction was performed with EtOAc (3 x 80 mL) and 5% MeOH/EtOAc (5 x 70 mL). The organic phases were combined and concentrated to give 993 mg of material. The residue was dissolved in a dilute mixture of MeOH/3 N NaOH (50 mL) and this solution was allowed to stir overnight at rt. The mixture was cooled to 0 °C and acidified with 3 N HCl to pH 3-4, after which the volatiles were removed in vacuo. The aqueous mixture was extracted with EtOAc (3 x 30 mL) and 5% MeOH/EtOAc (3 x 30 mL) and the organic phases were combined, washed with brine, dried with Na_2SO_4, and evaporated. Flash chromatography (1-2% MeOH/DCM) provided 370.0 mg (41%) of **2**[18] as a beige powder.

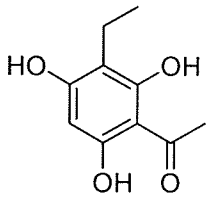
^{1}H-NMR (300 MHz) in acetone-d_6: δ = 13.8 (br s, 1H), 9.2 (br s, 1H), 6.06 (s, 1H), 3.3 (br s, 1H), 2.60 (s, 3H), 1.95 (s, 3H)

^{13}C-NMR (75 MHz) in acetone-d_6: δ = 203.7, 165.0, 163.0, 160.7, 105.3, 103.3, 32.8, 7.4

ESI-MS: *m/z* 182.1 (45%), 167.0 (100%), 42.9 (39%)

R_f (5% MeOH/DCM x 4) = 0.46

*2.2.2 Ethylphloracetophenone [1-(3-ethyl-2,4,6-trihydroxyphenyl)ethanone] (**5**)*



A solution of trihydroxyacetophenone (**3**) (0.186 g, 1 mmol), K_2CO_3 (0.345 g, 2.5 mmol) and iodoethane (320 μL, 4 mmol) in acetone (10 mL) was stirred at rt for 11.3 h. Water (10 mL) was added and the mixture was acidified to pH 1 with HCl. The mixture was extracted with

EtOAc (3 x 10 mL) and the organic phase was dried with Na_2SO_4 and evaporated. Flash chromatography (1% MeOH/DCM) provided 14.0 mg (8%) of **5** as a pink powder.

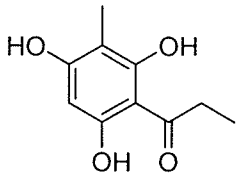
^{1}H-NMR (300 MHz) in acetone-d_6: δ = 13.9 (br s, 13.9, 1H), 9.2 (br s, 1H) 6.06 (s, 1H), 3.0 (br s, 1H), 2.60 (s, 3H), 2.56 (q, 2H, *J* = 7.4 Hz), 1.04 (t, 3H, *J* = 7.4 Hz)

^{13}C-NMR (75 MHz) in acetone-d_6: δ = 203.7, 164.8, 162.8, 160.6, 109.9, 105.3, 94.9, 32.8, 16.0, 14.0

EI-MS: *m/z* 196.1 (45%), 181.0 (100%), 162.9 (29%), 69.0 (22%)

R_f (5% MeOH/DCM x 3) = 0.48

2.2.3 Methylphlorpropionophenone [1-(2,4,6-trihydroxy-3-methylphenyl)propan-1-one] ***(6)***



A solution of trihydroxytoluene (**4**) (0.702 g, 5.01 mmol), propionic acid (propanoic acid) (13.5 mL, 180 mmol), propionic anhydride (propanoyl propanoate) (1.40 mL, 11.0 mmol) and $BF_3 \cdot OEt_2$ (0.670 mL, 5.43 mmol) was heated at 100 °C for 3 h.[26] The reaction was brought to 0 °C and the pH was adjusted to 3-4 with the addition of 3 N NaOH and water (100 mL) was added. The mixture was extracted with EtOAc (3 x 80 mL) followed by 5% MeOH/EtOAc (3 x 80 mL) and organic phases were combined and dried with Na_2SO_4 and solvent was evaporated. The residue was dissolved in 80 mL of anhydrous MeOH and to this mixture was added 1 mL of 1.0 M solution of sodium methoxide in methanol. After stirring overnight, Dowex 50W-X4 cation exchange resin beads were added to the solution until pH was found to be thoroughly

acidic using pH papers. The beads were filtered and the solution was dried in vacuo (978 mg). Flash chromatography (1-1.5% MeOH/DCM) provided 247 mg (26%) of **6** as a beige powder.

^{1}H-NMR (300 MHz) in acetone-d_6: δ = 13.9 (br s, 1H), 9.4 (br s, 1H), 6.05 (s, 1H), 3.8 (br s, 1H), 3.07 (q, 2H, J = 7.2 Hz), 1.95 (s, 3H), 1.10 (t, 3H, J = 7.2 Hz)

^{13}C-NMR (75 MHz) in acetone-d_6: δ = 207.0, 164.8, 162.7, 160.5, 104.8, 103.3, 94.8, 37.6, 9.1, 7.4

EI-MS: *m/z* 196.1 (19%), 167.0 (100%), 42.9 (66%)

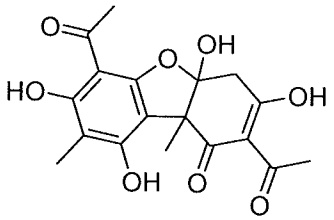
R_f (5% MeOH/DCM x 2) = 0.43

2.3 Horseradish Peroxidase Oxidation of Methylphloracetophenone (2) and Analogs

2.3.1 Analysis of Enzyme Assays

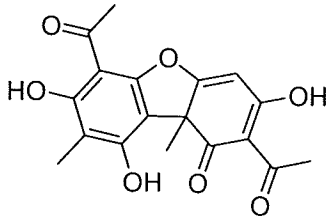
HPLC/MS experiments were performed under two separate conditions, both of which use 0.2% formic acid and acetonitrile as solvents and are based on 45 minute run times. The first of the conditions used a Waters Nova-Pak C_{18} 4 μm, 3.9 x 150 mm column. The solvent system was as follows: 0 minutes, 20% CH_3CN; 1 min, 20% CH_3CN; 21 min, 100% CH_3CN; 21 min, 100% CH_3CN; 39 min, 20% CH_3CN; 45 min, 20% CH_3CN. The second set of conditions used a Sunfire C_{18} 3.5 μm, 4.6 x 150 mm column. In this case the solvent system was as follows: 0 min, 20% CH_3CN; 18 min, 100% CH_3CN; 30 min, 100% CH_3CN; 38 min, 20% CH_3CN; 45 min, 20% CH_3CN.

*2.3.1 Hydrated usnic acid (**12**) production with HRP*



Reactions were performed in 100 mM phosphate buffer at pH 6.5 (1 mL total reaction volume) in 2 mL Eppendorf tubes. Five milligrams of methylphloracetophenone (**2**) was dissolved in 100 μL of MeOH to solubilize the substrate. A solution of HRP (in 100 mM phosphate buffer at pH 6.5) was added so that the reaction had 20 activity units (AU) / mL. Fresh 3% H_2O_2 (0.883 M) was added over 2.5 hours so that the final concentration was 25 μmol. Total reaction time was 5.5 hours. Formic acid (100 μL) was used to stop the reaction and the tubes were centrifuged for 10 min at 10,000 rpm to precipitate the enzyme and product. Controls were run without hydrogen peroxide and without substrate, and also with usnic acid (**1**) as a substrate, and with usnic acid (**1**) as a substrate without enzyme or without hydrogen peroxide. HPLC/MS was performed on the precipitates from these reactions to obtain results.

*2.3.2 Usnic acid (**1**) production with HRP*



An identical procedure was followed as in 2.3.1 but with additional work up steps which were adapted from Barton *et al.*[27] After the enzyme reaction was stopped with formic acid and the tube was centrifuged, the precipitate was removed and dried. The precipitate was added to 1

mL of an acetic anhydride and sulfuric acid solution (200:1) and was brought to 40 °C for 30 minutes. The mixture was cooled to 0 °C and cold water (10 mL) was added before extraction with $CHCl_3$ (3 x 5 mL). To the organic extract was added cold concentrated sulfuric acid (2 mL). After 5 minutes at 0 °C, cold water (10 mL) was added before another extraction with $CHCl_3$. The solvent was dried with Na_2SO_4 and evaporated. HPLC/MS was performed to obtain the identity of the product from this reaction.

*2.3.3 Large scale usnic acid (**1**) production*

In 50 mL Falcon tubes, **2** (10 mg, 55.0 μmol) was dissolved in acetone(1 mL). Reactions were carried out at three different levels of HRP: 0.1 AU/mL, 1.0 AU/mL and 10 AU/mL. Reactions were brought to 20 mL with 100 mM phosphate buffer at pH 6.5. At each enzyme concentration, two different amounts of H_2O_2 were tested so that final concentrations of H_2O_2 were 44.2 mM and 441.5 mM. Hydrogen peroxide (1 mL) was added over an extended time period (90% in the first 5 hours and the final 10% after 3 days). Reactions were allowed to proceed for 75 hours before addition of formic acid (1 mL). Reactions were extracted with EtOAc (2 x 20 mL) and 5% MeOH/EtOAc (3 x 20 mL) and the organic phases were combined for each experiment and dried with Na_2SO_4. Solvent was evaporated and the remaining material was subject to analysis by NMR and HPLC/MS.

2.3.4 HRP oxidation of methylphloracetophenone analogs

Reactions were performed in 100 mM phosphate buffer pH 6.5 (1 mL total reaction volume) in 2 mL Eppendorf tubes. **5** (3 mg, 15.3 μmol) or **6** (4 mg, 20.4 μmol) was dissolved in MeOH (100 μL) to solubilize the substrate. A solution of HRP was added so that the reaction had 20 activity units (AU) / mL. Fresh 3% H_2O_2 (0.883 M) was added over 3 hours so that the final concentration was 25 μmol. Total reaction time was 5.5 hours. Formic acid (100 μL) was used to stop the reaction and the tubes were centrifuged for 10 min at 10,000 rpm to precipitate the enzyme and product. Controls were run without HRP present.

In an attempt to make usnic acid analogues in an amount required for full characterization, the scale of the reaction was increased. In 50 mL Falcon tubes 10 mg (51.0 μmol) of substrate was dissolved in 1 mL acetone. Both **5** and **6** were reacted under two conditions: 0.1 AU/mL and 10 AU/mL of HRP. Reactions were brought to 20 mL with 100 mM phosphate buffer pH 6.5. 1 mL of 3% hydrogen peroxide was added (90% in the first 5 hours and the final 10% after 3 days). Reactions were allowed to proceed for 75 hours before addition of 1 mL of formic acid. Reactions were extracted (EtOAc 2 x 20 mL , 5% MeOH/EtOAc 3 x 20 mL) and the organic phases were combined for each experiment and dried with Na_2SO_4. Solvent was evaporated and the remaining material was subject to analysis by NMR and HPLC/MS.

*2.3.5 Usnic acid (**1**) production with potassium ferricyanide*

A solution of sodium carbonate (0.6 g, 5.61 mmol) in water (15 mL) was deoxygenated with nitrogen gas before adding methylphloracetophenone (**2**) (259 mg, 1.42 mmol). The

solution was warmed to help solubilize **2** before cooling to 0 °C. A second solution containing $K_3[Fe(CN)_6]$ (0.7 g, 2.13 mmol) in water (18 mL) was added dropwise to the solution containing **2** over 30 minutes with stirring. Sulfuric acid (0.5 mL) was added to the mixture before extraction with Et_2O (3 x 50 mL). The organic solvent was evaporated and the remaining residue was extracted with cold $CHCl_3$.[27] The solvent was evaporated and the remaining material (251 mg) was subject to flash chromatography (2-2.5% MeOH/DCM) affording 8.6 mg (2%) of usnic acid (**1**).

Usnic acid (**1**):

^{1}H-NMR (300 MHz) in $CDCl_3$: δ = 13.30 (s, 1H), 11.01 (s, 1H), 5.96 (s, 1H), 2.67 (s, 3H), 2.65 (s, 3H), 2.10 (s, 3H), 1.75 (s, 3H)

^{1}H-NMR (300 MHz) in acetone-d_6: δ = 13.34 (s, 0.9H), 11.10 (s, 0.9H), 7.85 (s, 0.4H) 6.10 (s, 1H), 2.83 (s, 6.6H), 2.66 (s, 3H), 2.62 (s, 3H), 2.03 (s, 3H), 1.80 (s, 3H)

^{13}C-NMR (75 MHz) in $CDCl_3$: δ = 201.9, 200.5, 198.2, 191.9, 179.5, 164.0, 157.6, 155.4, 109.4, 105.4, 104.1, 101.7, 98.5, 59.2, 32.3, 31.4, 28.0, 7.54

EI-MS: *m/z* 344.1 (28%), 260.2 (31%), 233.2 (55%), 169.0 (21%), 97.1 (22%), 69.1 (33%), 57.0 (48%), 43.9 (79%), 42.9 (100%)

*2.3.5 Oxidation of methylphloracetophenone (**2**) with potassium iodate*

To a solution of methylphloracetophenone (**2**) (1832 mg, 1.006 mmol) in acetone (5 mL) was added a second solution consisting of KIO_3 (1.712 g, 8.000 mmol) in water (20 mL).[28] The mixture was allowed to stand at rt for 5 days. Water (35 mL) was added and the mixture was

acidified with HCl (1 mL). The mixture was extracted (EtOAc x 2, 5% EtOAc/MeOH x 2) and the organic phases were combined and dried with Na_2SO_4 and evaporated.

2.4 Isolation of methylated citric acid derivatives and dimethyl oxalate from fungus and taxonomic identification of *Aspergillus niger*

2.4.1 Culturing of Fungi

Soil (ca. 0.5 g) obtained from Dr. Michelle Piercey-Normore was sprinkled onto two plates of potato dextrose agar (PDA) containing streptomycin (100 mg per 1 L agar) and incubated for two weeks at 30 °C in ambient light. After this time the plate was covered completely with black spores as well as growth from other species of fungi. The black spores were carefully harvested from this initial growth plate and propagated on a fresh plate of PDA. This plate was also incubated at 30 °C for two weeks at ambient light which resulted in a carpet of black spores. Growth from other organisms appeared to be absent. A plug ca. 5 mm in diameter was taken from the agar plate where there were black spores growing and placed into each of two 2 L Erlenmeyer flasks, each containing 1 L potato dextrose broth (PDB). These flasks were incubated on a shaker under ambient light at 200 rpm for one week at 30 °C. At the end of this fermentation period each flask was filled with balls of white mycelia ca. 1-2 cm in diameter. A control flask containing only PDB (no fungus) was also incubated to ensure no contamination. This flask remained clear of biological growth at all stages of the fermentation.

2.4.2 Extraction of Fungal Broth

Mycelia were removed from the fermentation broth of these cultures by filtration through cheese cloth. The combined fermentation broth (2 L) was then extracted with ethyl acetate (3 x 500 mL). The combined organic layers were dried (Na_2SO_4), filtered and evaporated under reduced pressure to afford 1.69 g of neutral extract residue.

The extracted fermentation broth was then acidified with HCl (pH < 1.0) and re-extracted with ethyl acetate (3 x 500 mL). The combined organic layers were dried (Na_2SO_4) filtered and evaporated in a rotary evaporator under reduced pressure to give 2.79 g of acidic extract residue.

2.4.3 Column Chromatography on Acidic Extract

Flash chromatography was performed on the acidic extract (2.79 g) employing a gradient of 1% to 30 % MeOH in CH_2Cl_2. The total elution volume was 6.5 L and was collected in 250 mL fractions. Dimethyl oxalate (**9**) eluted first (198 mg) followed by trimethyl citrate (**10**) (187 mg) while dimethyl citrate (**11**) (1234 mg) was found in the most polar fractions.

Dimethyl oxalate (**9**):

^{1}H-NMR (300 MHz) in $CDCl_3$: δ = 3.76 (s, 6H)

^{13}C-NMR (75 MHz) in $CDCl_3$: δ = 157.6 (2C), 53.1 (2C)

Trimethyl citrate (**10**):

^{1}H-NMR (500 MHz) in CD_3OD: δ = 3.76 (s, 3H), 3.65 (s, 6H), 2.94 (d, 2H, J = 15.3 Hz), 2.79 (d, 2H, J = 15.3 Hz)

^{13}C-NMR (125 MHz) in CD_3OD: δ = 175.3, 171.8 (2C), 74.8, 53.3 (2C), 52.4 (2C), 44.2 (2C)

EI-MS: *m/z* 235.0 ($[M+H]^+$, 2%), 175.0 (14%), 142.9 (100%), 100.9 (85%)

Dimethyl citrate (**11**):

^{1}H-NMR (500 MHz) in CD_3OD: δ = 3.66 (s, 6H), 2.94 (d, 2H, J = 15.3 Hz), 2.82 (d, 2H, J = 15.3 Hz)

^{13}C-NMR (125 MHz) in CD_3OD: δ = 176.5, 172.0 (2C), 74.4, 52.3 (2C), 44.2 (2C)

ESI-MS: *m/z* 243.1 $[M + Na]^+$

EI-MS: *m/z* 170.9 (14%), 142.9 (100%), 100.9 (82%) (M+ not detected)

2.4.4 Isolation of Genomic DNA

A modified protocol of Grube *et al.*[29] was used for DNA extraction. A small amount of mycelia was ground using a micropestle in a 1.5 mL Eppendorf tube in TES buffer (100 mM Tris pH 8.0; 10 mM EDTA; 2% SDS). Sodium chloride (5 M; 1.4 M final concentration) and 10% CTAB (cetyltrimethylammonium bromide) (1% final concentration) were added. The tube was vortexed thoroughly and incubated for 1 hour at 65 °C.

Proteins were removed by adding 1.0 volume of chloroform - isoamyl alcohol (24:1) and inverting for one minute before centrifuging for 5 min at 5000 rpm. The supernatant was decanted and transferred to a new 1.5 mL Eppendorf tube. A second chloroform extraction and centrifugation was performed and the supernatant (aqueous layer) was transferred again to a new 1.5 mL Eppendorf tube.

DNA was precipitated by adding 0.2 volumes of 5 M NaCl and 2.5 volumes of 100% ethanol to the aqueous layer and inverting the tube several times before being left in the fridge (4 °C) overnight. The tube was centrifuged for 10 minutes at 13000 rpm, the supernatant was

removed, and the pellet was left to air dry for 15 minutes. The pellet was washed with 300 μL of cold 80% ethanol, which was poured off, and the pellet allowed to air dry again. The DNA was re-suspended in 50 μL of sterile distilled water and stored overnight in the fridge (4 °C) to achieve full re-suspension.

2.4.5 Polymerase Chain Reaction (PCR) Amplification

The internal transcribed spacer (ITS) gene (ITS1, 5.8S and ITS2) of the fungal nuclear ribosomal DNA (rDNA) was amplified by PCR. Various concentrations of the sample were screened using 20 μL reactions to decide on the optimal DNA concentration for PCR. DNA was amplified for sequencing in eight 50 μL reactions which were composed of: 31.5 μL of sterile distilled H_2O, 5 μL of 10 x PCR buffer (50 mM KCl; 100 mM Tris-HCl pH 8.3), 2.0 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, and 2 units of Pfu DNA polymerase. The eight reactions were combined for a total volume of 400 μL.

The ITS gene was amplified using the fungal specific primer 1184-5′ (5′-GACTCAACACGGGGAAACTC-3′)[30] which anneals to a region in the 18S (ribosomal small subunit), and the universal primer ITS4-3′ (5′-TCCTCCGCTTATTGATATGC-3′)[31] which anneals to the 26S (ribosomal large subunit). A Techne Genius thermal cycler (Fisher Sci, Nepean, ON, Canada) was used for all amplifications of the ITS gene using a touchdown PCR cycle: initial denaturation of the DNA at 94 °C for 5 min, 30 cycles comprised of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1.5 min and after the cycles were complete, a soak at 4 °C.

2.4.6 Precipitation, Electrophoresis, Purification and Quantification

The 400 µL of PCR product was precipitated in 1.5 mL Eppendorf tubes by adding 0.2 volumes of 5 M NaCl and 2.5 volumes of 100% ethanol. The tube was mixed gently and stored in the fridge (4 °C) overnight. The tube was then centrifuged for 10 min at 13000 rpm and the supernatant was decanted. The pellet was washed with 200 µL of cold 80% ethanol which was poured off and the pellet was left to air dry for 30 min before resuspending the DNA in 20 µL of sterile distilled H_2O. The entire product was mixed with approximately 12 µL bromophenol blue loading dye (6 x BPB) and loaded into a 1% agarose gel stained with ethidium bromide (0.5 mg/mL) in 1 x TBE buffer (0.089 M Trizma base; 0.089 M boric acid; 2.0 mM EDTA pH 8.0). The gel was held at 119 volts until the loading dye was 1 cm from the bottom of the gel. The band of DNA was visualized using a 254 nm UV light box and was excised from the gel as a small block which was put into a 1.5 mL Eppendorf tube and stored at -20 °C overnight. Following manufacturers' protocol, a Wizard SV Gel and PCR Clean-Up System was used to extract the DNA out of the gel. This procedure resulted in a 35 µL sample of suspended DNA.

Quantification of the purified DNA product was performed using electrophoresis by loading 3 µL of the sample into a 1% agarose TBE gel. The band intensity was compared to the 1650 base pair (bp) band (80 ng/µL) of a 1 kb plus DNA ladder (Invitrogen, Burlington, ON, Canada). Results were visualized using an AlphaImager 2200 transilluminator (Alpha Innotech, Fisher Scientific, Nepean, ON, Canada).

2.4.7 DNA Sequencing and Alignment

The PCR product underwent a cycle sequencing reaction to assimilate ddNTPs (dideoxynucleotide triphosphate) into the DNA. Cycle sequencing required two independent reactions, one with each different primer. Each reaction had a total volume of 20 μL, containing: approximately 60 ng of purified DNA, 3.2 pmol of primer, 2 μL of 5 x sequencing buffer (Applied Biosystems, Foster City, CA, USA), and 1 μL of BigDye v3.1 (Applied Biosystems, Foster City, CA, USA). Cycle sequencing was performed with a Biometra T-Gradient thermal cycler (Fisher Sci, Nepean, ON, Canada) set for an initial denaturation at 96 °C for 1 min followed by 25 cycles of denaturing at 96 °C for 10 sec, annealing at 50 °C for 5 sec, and extension at 60 °C for 4 minutes, followed by a soak at 4 °C.

Precipitation to remove excess fluorescent dyes from the product was accomplished with the addition of 0.25 volumes of 125 mM EDTA and 3 volumes of 100% ethanol. The tube was gently mixed and incubated at room temperature in the dark for 15 min before being centrifuged at 5000 rpm for 30 min. The supernatant was discarded and 60 μL of cold 80% ethanol was added. The tube was centrifuged at 5500 rpm for 15 min and the supernatant was removed. The DNA was dried in a ThermoSavant DNA 120 SpeedVac Concentrator (GMI INC., Ramsey, Minnesota, USA). The DNA was resuspended in 20 μL of Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and was denatured at 95 °C for 5 min and then immediately placed onto ice. The sample was loaded into a 96-well plate for sequencing with an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.4.8 ITS Sequence

Sequences were edited using Sequencher v. 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned manually using Se-Al v2.0 (Rambaut 2002). A BLAST search[32] was performed to confirm the identity of the organism.

The sequence for the 1TS1, 5.8S and ITS2 regions of rDNA from *A. niger* resulted in a total of 1117 characters and is given below. This sequence was submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and was assigned the accession number GQ130305 and was assigned the strain identifier DJH1-13.

GGCCAAAAATAAGGATTGAAGATTGAGAGCTCTTTCTTGATCTTTTGGATGGTGGTG
CATGGCCGTTCTTAGAAGGTGGAGTGATTTGTCTGCTTAATTGCGATAACGAACGAG
ACCTCGGCCCTTAAATAGCCCGGTCCGCATTTGCGGGCCGCTGGCTTCTTAGGGGGA
CTATCGGCTCAAGCCGATGGAAGTGCGCGGCAATAACAGGTCTGTGATGCCCTTAG
ATGTTCTGGGCCGCACGCGCGCTACACTGACAGGGCCAGCGAGTACATCACCTTGG
CCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTGGGGATAGAGCATTGCAA
TTATTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATT
ACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCGGTG
AGGCCTTCGGACTGGCTCAGGAGGGTTGGCAACGACCCCCCAGAGCCGGAAAGTGG
TCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAAC
CTGCGGAAGGATCATTACCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTC
TATTGTACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCCT
CTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACAAAAAACACTGTCTGAAAGC

GTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTG

GTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAA

TTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGC

ATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGT

CCCCCTCTCCGGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTC

GAGCGTATGGGGCTTTGTCACATGCTCTGTAGGTTTGCCGGCGCCTGCCGACGTTTT

CCAACCATTCTTCCAGGTGACCTCGGATCAGTAGAAGCCCG

3. Synthesis of Methylphloracetophenone and Usnic Acid

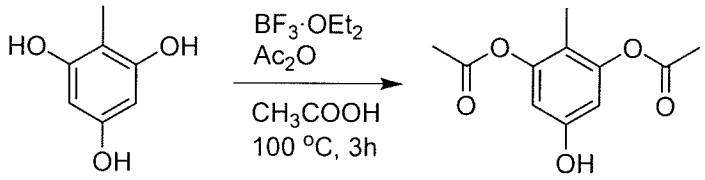
3.1 Optimization of synthesis of methylphloracetophenone (2)

3.1.1 Background

Initial work for this thesis focused on the synthesis of the key intermediate in usnic acid (**1**) biosynthesis: methylphloracetophenone (**2**). Because the eventual aim is to incorporate an isotopic label into **2**, it was desirable to optimize the reaction on a small scale (1 – 10 mmol). Early experiments by Kurtis Anderson,[33] an undergraduate project student in our group, began with an attempt to synthesize methylphloracetophenone (**2**) from trihydroxytoluene (**4**).

Kurtis aimed to synthesize **2** according to methods described by Lee *et al.*[26] using **4** as a substrate and acetic anhydride with $BF_3 \cdot OEt_2$ as a catalyst. These efforts were unsuccessful, (**Scheme 3.1**) as the major product was 3,5-diacetoxy-4-methylphenol (**13**).The ^{1}H-NMR spectrum ($CDCl_3$) indicated the presence of two aromatic protons from integration data suggesting that the ring did not undergo any *C*-substitution. The aromatic protons appeared at δ

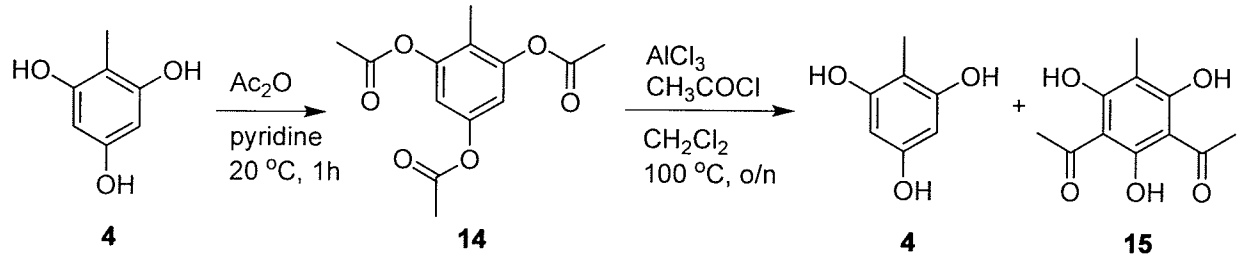
6.82 ppm suggesting the presence of electron withdrawing substituents on the ring. The starting material **4** contains three electron donating hydroxyls causing the aromatic protons resonate at δ 5.95 ppm. Furthermore, integration values show that 6 hydrogen atoms are assigned to a peak at δ 2.29 ppm which is upfield from what is expected for an acetyl group on an aromatic ring (as is the case with **2**) and must therefore belong to a methyl ester as on **13**.



4 **13**

Scheme 3.1. Acylation of trihydroxytoluene (**4**) forming 3,5-diacetoxy-4-methylphenol (**13**) under conditions described in Kurtis Anderson's thesis.[33]

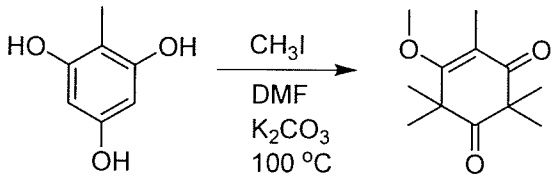
Kurtis also synthesized triacetoxytoluene (**14**) by reacting **4** with acetic anhydride in pyridine (**Scheme 3.2**). The presence of acetyl groups would presumably deactivate the ring by withdrawing electron density from it to help prevent di-*C*-acylation. The ^{1}H-NMR spectrum of **14** showed two distinct acetate ester peaks (δ 2.30 and 2.25 ppm) which integrated for 6 and 3 protons. The two aromatic protons were also still visible in the spectrum as one peak at δ 6.82 ppm. **14** was reacted with acetyl chloride with ferric chloride or aluminum chloride as a catalyst. When ferric chloride was used, no identifiable products were formed while with aluminum chloride, **4** and trihydroxydiacetophenone (**15**) were produced (**Scheme 3.2**).



Scheme 3.2. Protection of trihydroxytoluene (**4**) and acylation of triacetoxytoluene (**14**) to give a di-*C*-acylation product and reform **4**.[33]

During the acylation of **14**, Kurtis realized the acetate ester protecting groups were no longer visible in a ^{1}H-NMR spectrum (δ 2.30 and 2.25 ppm). This indicated that the acetate ester groups were not robust enough to withstand conditions, notably the high temperature,[34] used in the Friedel-Crafts reaction. When protection was lost, the ring was highly activated toward substitution reactions. The three hydroxyl functionalities in ortho and para positions relative to the unsubstituted carbon atoms on the ring provide considerable electron density to these carbon atoms, thus making them good nucleophiles.

To protect the phenolic groups of **4** in a different fashion, Kurtis used iodomethane with the aim of forming methyl ethers and therefore somewhat deactivating the ring. The unsubstituted aromatic carbon atoms would then be able to undergo mono-*C*-acylation. Rather than ether formation however, extensive *C*-methylation occurred resulting in **16** which was isolated and characterized based on NMR data (**Scheme 3.3**).[33]



4 **16**

Scheme 3.3. Attempted ether protection of trihydroxytoluene (**4**) with iodomethane.[33]

Iodide is a weak base which makes it a good leaving group on iodomethane. The methyl end of iodomethane is therefore considered a "soft" electrophile, and as such, methylation preferentially occurs at a "soft" end of an ambidentate nucleophile. This is just the case with compounds like **4** or **3**. An ambidentate nucleophile is a molecule that has more than one nucleophilic site. For example, with **4** the oxygen atoms and the unsubstituted aromatic carbon atoms are both nucleophiles. The oxygen atoms of the alcohol groups are stronger nucleophiles and can be called "hard" nucleophiles while the unsubstituted aromatic carbons are weaker nucleophiles, or "soft" nucleophiles. The "soft" carbon nucleophiles are more favorably methylated relative to the "hard" oxygen nucleophiles when a "soft" electrophile is present. The result of the experiment shown in **Scheme 3.3** was the inspiration for the initial work in this thesis and suggested a new synthesis of **2**.

3.1.2. Synthesis of methylphloracetophenone (2) from trihydroxyacetophenone (3)

Initial synthesis using **3** with a large excess of iodomethane resulted in production of **2**, but also an abundance of side products. The major side product appeared to be the result of *O*-methylation as indicated by peaks in the ^{1}H-NMR spectrum of the crude reaction mixture consistent with methyl ethers at δ 3.6 – 4.0 ppm. This is likely because a large excess of

iodomethane was used in the reaction and at a room temperature, the excess is able to react with the oxygen atoms. It is possible that this is a matter dictated by the thermodynamics of *O*-methylation in the reaction conditions. By diluting the reaction from 5 mL to 20 mL and cooling the system to 0 °C the yield of **2** remained approximately the same, while significantly less *O*-methylated products were observed from ^{1}H-NMR data. On a 1 mmol scale at room temperature, yields of 21 – 23% were obtained after 1 – 3 hours. However, on the same scale at 0 °C, it took 21 – 27 hours to reach yields of 13 – 16 %. Even though longer reaction times were needed at 0 °C, this condition was preferable because there were considerably less *O*-methylated products, and more starting material **3** could be recovered and reused (**Figure 3.1**). In **Figure 3.1**, the bottom ^{1}H-NMR trace represents the crude reaction mixture from a reaction that was performed at room temperature, while the top trace is the crude reaction mixture from a methylation reaction that was performed under more dilute conditions at 0 °C. The peaks between δ 3.6 – 4.0 ppm represent those of the methyl ethers. The bottom trace has a greater number of peaks in this region compared to the top trace. This indicates that less *O*-methylation occurred at the optimized conditions. The decreased amount of side products made purification simpler and the recycling of starting material could increase the overall yield of the reaction.

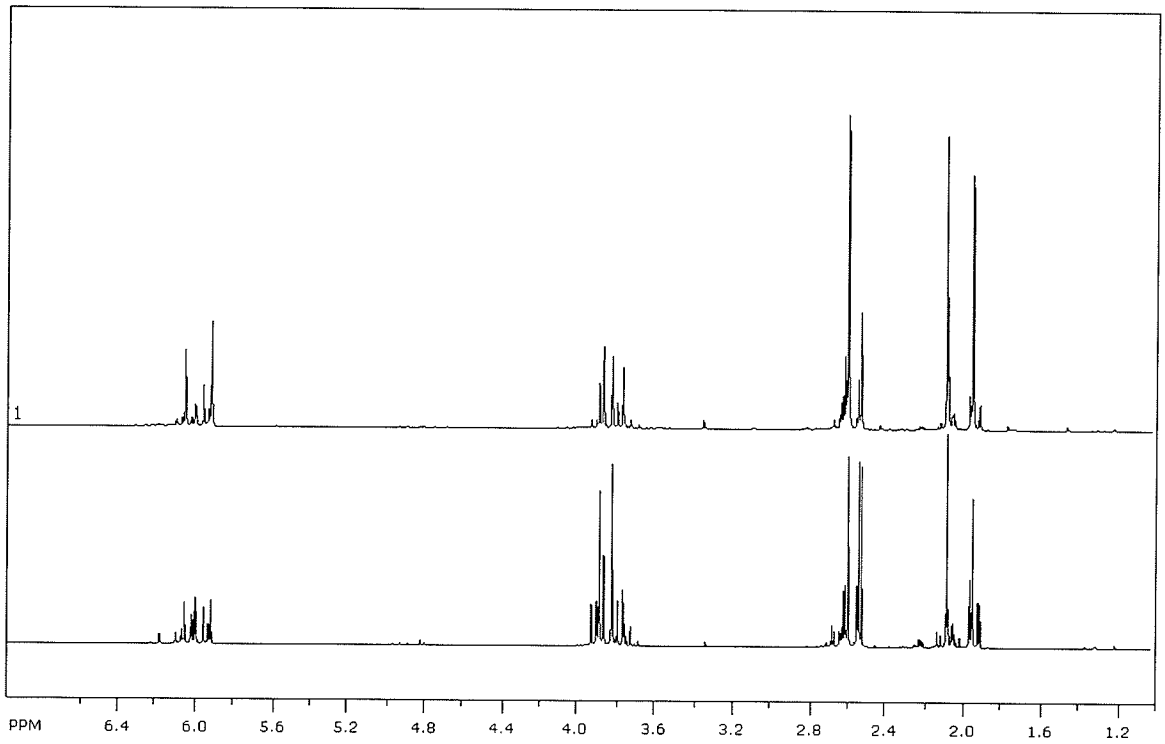
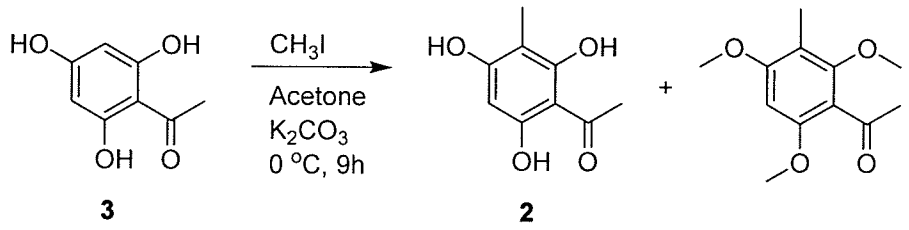


Figure 3.1. Comparison of the unoptimized (bottom) and optimized (top) ^{1}H-NMR crude reaction mixtures for the methylation of trihydroxyacetophenone (**3**).

Reactions could be monitored during the reaction with TLC as **2** had an R_f value slightly higher than the starting material **3**. Once the reaction was worked up, NMR data was collected for the crude reaction mixture. The presence of products of the methylation reaction could be determined through the observation of the position of the aromatic proton. In the starting material, **3**, this signal is at δ 5.92 ppm (2H) while in **2** it is shifted downfield to δ 6.06 ppm (1H). Observation of these two peaks gave a general indication that the reaction was successful.



Scheme 3.4. Synthesis of methylphloracetophenone (**2**) from trihydroxyacetophenone (**3**) using iodomethane.

With the reaction of **3** with iodomethane optimized on a 1 mmol scale by dilution and lowering the temperature to 0 °C, conditions were scaled up (**Scheme 3.4**). On a 10 mmol scale, **3** provided 2.273 g of crude reaction mixture which was purified to obtain **2** in a 45% yield with 22% of the starting material recovered. Unlike **3**, the ^{1}H-NMR spectrum of **2** includes a peak corresponding to the methyl group on the aromatic ring. This peak appears at δ 1.95 ppm and integrates for 3 protons and is absent in the starting material. The acetyl peak remains unchanged at δ 2.60 ppm, and there is a downfield shift of the one remaining aromatic proton from δ 5.92 ppm to δ 6.06 ppm. The integration value on this signal is for 1 proton in the product but 2 protons in the staring material. Furthermore, a peak at δ 7.4 ppm is present in the ^{13}C-NMR spectrum for **2** but absent in the starting material corresponding to the methyl group attached to an aromatic ring. ESI-MS of **2** showed a molecular ion peak at *m/z* 182.1 (45%) and a base peak at *m/z* 167.0 ($[M - 15]^{+}$) indicating the loss of a methyl group.

The major side products for the methylation reaction of **3** with iodomethane are methylation of the phenolic oxygens. In **Scheme 3.4** the major permethylated product is represented but combinations of di- and mono-*O*-methylated products are also formed. During column chromatography under conditions optimized for purifaction of **2**, these products were recovered as a mixture. Although we characterized the permethylated product, we did not

attempt to purify and characterize the rest. The presence of these compounds in the crude mixture could be confirmed by a series of peaks between δ 3.5 – 4.0 corresponding to the methyl esters. The goal of this synthetic work was to optimize the production of **2** and our aim was to minimize *O*-methylation. Even though the reaction was performed at a low temperature, unwanted *O*-methylation continues to occur. Recent work by a summer student, Amy Norquay, has shown that the reaction does not proceed at -78 °C. However, she has developed conditions that minimize ether formation.

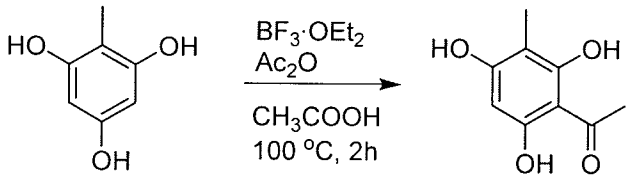
Without performing a full kinetic study under a wide variety of conditions, it is difficult to speak with unwavering certitude on this matter. The reaction is typical of an S_N2 reaction in that the aromatic ring acts as a nucleophile and attacks the electron-poor methyl group of iodomethane, simultaneously displacing the iodide ion. The S_N2 reaction follows second order kinetics because the rate is affected by both the nucleophile and electrophile concentrations. As mentioned at the end of *Section 3.1.1*, **3** has two nucleophilic sites: the unsubstituted aromatic carbon atom and the alcohol oxygens. From the yields obtained at the various conditions it appears that the energy barrier of *C*-methylation is lower than that for *O*-methylation. This is hypothesized because when the temperature is above 0 °C or if there is an excess of iodomethane in the reaction solution, *C*-methylation is observed but there is also enough energy for the phenolic oxygens to act as nucleophiles. However, when the temperature is kept at 0 °C and iodomethane is added very gradually so that at any one time there is minimal or no excess in the reaction mixture, there should be a decrease in *O*-methylated products formed while production of **2** continues. Indeed, recent work by a summer student, Amy Norquay, has shown this to be the case. By adding iodomethane dropwise for 5 hours or more at 0 °C, the formation of *O*-methylated products can be significantly reduced.

Under reaction conditions that gave considerable levels of *O*-methylated products, strategies were considered to deprotect these groups, restoring alcohol functionality. One possible method that has been shown to be extremely effective for the cleavage of methyl groups from phenolic oxygens is refluxing with potassium thiophenoxide in dimethylformamide for 3 hours.[35]

*3.1.3. Synthesis of methylphloracetophenone (**2**) from trihydroxytoluene (**4**)*

It was decided to revisit the Friedel-Crafts acylation of trihydroxytoluene (**4**). As part of the work for this thesis, this reaction was modified from the conditions previously attempted by Kurtis Anderson who performed the reaction on a 1 mmol scale for either 3 hours or overnight (**Table 3.1**).

Lee *et al.* report performing the reaction at a 24.3 mmol scale for 3 hours in the presence of $BF_3 \cdot OEt_2$ catalyst.[26] For the work described in this thesis, the reaction was attempted at a 10 mmol scale for 3.75 hours, giving a 21% yield. After workup the ^{1}H-NMR spectrum of this reaction showed at least three peaks at δ 1.9 – 2.0 indicating that at least three different acylation patterns had occurred, causing the methyl group on the aromatic ring to appear at three distinct chemical shifts. These signals were the result of mono- and di-*C*-acylation as well as unreacted starting material. The reaction was performed again but at a 5 mmol scale. Reaction progress was monitored with TLC and it appeared that after 2 hours, more of the di-*C*-acylation product was formed relative to **2**, and TLC also indicated the presence of *O*-acylated products. The reaction was stopped after two hours and a yield of 41 % was obtained (**Scheme 3.5**) compared to 75 % reported in the literature.[26]



4 **2**

Scheme 3.5. Acylation of trihydroxytoluene (**4**) with acetic anhydride and boron trifluoride diethyl etherate as a catalyst to produce methylphloracetophenone (**2**).

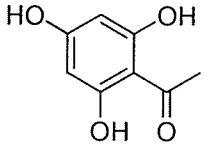
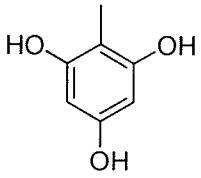
As part of the workup, crude material was stirred in a 1:1 solution of methanol and 3 N NaOH overnight to deacylate the oxygen atoms. The reaction products were purified by column chromatography. The ^{1}H-NMR spectrum of **2** differed from **4** with a peak at δ 2.62 ppm which integrates for 3 protons and is absent in **4**. This peak corresponds to the acetyl CH_3. The aromatic proton only integrates for one proton in **2** and is shifted downfield to δ 6.06 ppm from 5.95 ppm with **4**. Furthermore, the acetyl group appears at δ 2.62 ppm in **2** but is in the region δ 2.2 – 2.4 ppm when *O*-acylation occurs. In the case of mono-*O*-acylation, there would be two signals for the aromatic protons above δ 6.0 ppm since there is no way for mono-*O*-acylation to occur and produce a chemically identical environment for the two aromatic protons. In the ^{13}C-NMR spectrum for **2**, a peak at δ 32.8 ppm indicates a methyl group attached to a carbonyl carbon, and a peak at δ 203.7 ppm indicates a carbonyl carbon. Combined, these two peaks further confirm the presence of an acetyl group attached to the aromatic ring, a feature not present in **4**. Also, all 6 carbon atoms of the ring are at different chemical shifts in **2** whereas only four signals are observed in **4**.

*3.1.4. Concluding remarks on the synthesis of methylphloracetophenone (**2**).*

Using **3** or **4** as starting materials are both viable methods to obtain **2**. Each of the reactions have been optimized from initially poor yields to yields over 40%. **Table 1** summarizes the optimization process for the methylation of **3** and the acylation of **4**. In the case of **3**, the most notable conditions modified were temperature and reaction time, and to a lesser extent, the amount of solvent used. Conditions that were more dilute and at a lower temperature required longer reaction times but produced less side products. Furthermore, when the reaction using **3** was scaled up there was a fortuitous increase in yield.

For acylation reactions using **4**, the only variables that were modified were scale and time of the reaction. From a previous report,[26] the reaction was performed on a 24.3 mmol scale for 3 hours. The reaction did not proceed at a 1 mmol scale but when performed at 10 or 5 mmol, **2** was isolated. With a scale less than that used in literature, a shorter time scale seemed necessary to prevent di-*C*-acylation. It would be interesting to attempt this acylation reaction at decreased temperatures where it may be possible to reduce or eliminate *O*-acylation, thereby simplifying the workup.

Table 3.1. Yields of methylphloracetophenone (**2**) using trihydroxyacetophenone (**3**) or trihydroxytoluene (**4**) as starting materials and varying the conditions.

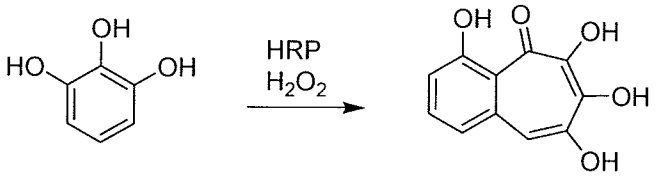
Substrate	Scale	Solvent, Amount	Temperature	Reaction Time	Yield (%) of **2**
Trihydroxy-acetophenone (**3**) 	1 mmol	acetone, 5 mL	Rt	9 h	3
				3 h	20
				3.3 h	23
			0 °C	21 h	13
		acetone, 20 mL		26.5 h	16
	10 mmol	acetone, 100 mL		9 h	45
Trihydroxytoluene (**4**) 	1 mmol	acetic acid, 2 mL	100 °C	3 h	0[33]
				overnight	0[33]
	10 mmol	acetic acid, 21 mL		3.75 h	21
	5 mmol	acetic acid, 10.3 mL		2 h	41

3.2 Horseradish peroxidase synthesis of usnic acid (1)

3.2.1. Conditions for horseradish peroxide assay

With a synthetic route to methylphloracetophenone (**2**) established, the next set of experiments presented in this thesis involved the conversion of **2** to usnic acid (**1**). We chose horseradish peroxidase (HRP) as a model system for this conversion. A review of literature was performed on eight recent papers[36-43] attempting to accomplish an oxidative coupling of phenolic compounds using HRP. In every case except one, phosphate buffer at pH 6 – 8 was used and most were in the range of pH from 6 – 7. Additionally, the supplier (Sigma-Aldrich) reports that the optimum pH for highest HRP activity is 6.0 – 6.5. Based on these findings, 100 mM

phosphate buffer at pH 6.5 was chosen to perform HRP assays presented in this thesis. At pH 6.5, **2** displayed limited solubility. In order to overcome this, 100 μL of MeOH was added to the substrate to solubilize it before adding buffer. Amounts of HRP used varied between each paper examined. The actual mass or mass concentrations of HRP are less important than the activity of the enzyme. HRP is purchased based on activity units (AU) and not mass. One AU is the amount of HRP that forms 1.0 mg of purpurogallin (**17**) from pyrogallol (**18**) in 20 s at pH 6.0 at 20°C (**Scheme 3.6**). The appearance of **17** can easily be measured as the product is black in color while **18** is white.



18 **17**

Scheme 3.6. Production of purpurogallin (**17**) from pyrogallol (**18**) with horseradish peroxidase, a reaction used to measure the activity of HRP.

From the reviewed articles, HRP was used in concentrations ranging from 1.6 to 43 AU/mL with the average being around 20 AU/mL. Thus, a level of 20 AU/mL HRP was chosen for the assays. The final condition to decide upon was the amount of H_2O_2 to be used relative to the amount of substrate **2**. Values for the molar ratio of [substrate]:[H_2O_2] were reported in a wide range of values. The lowest ratio found was 0.01:1 while the highest was 2:1. The majority of papers that were examined used a [substrate]:[H_2O_2] ratio between 1:1 and 2:1. A ratio of 1.3:1 was chosen to perform assays discussed here. It was already decided that enzyme assays would be performed with 5 mg of **2** (27.5 μmol) in 1 mL reactions in order to conserve substrate

and enzyme while using enough substrate to make product identification possible. This amount of **2** required 21 μmol of H_2O_2 to give a 1.3:1 ratio of [substrate]:[H_2O_2]. Fresh 3 % H_2O_2 (0.883 M) was used for the assays. Therefore, 24 μL of 3 % H_2O_2 was used in the HRP reactions. From the reviewed literature, it was also found that H_2O_2 was added slowly, in one case over the course of 7 hours. For the assays in this section of the thesis, 24 μL of 0.883 M aqueous H_2O_2 was added in 6 batches (4 μL each) at time intervals of 30 min at 37 °C. Papers that were examined used temperatures ranging from 0 °C to 40 °C.

To summarize the conditions used for the HRP oxidation of methylphloracetophenone (**2**),[18] 5 mg of **2** was dissolved in 100 μL of MeOH in a 2 mL Eppendorf tube. 20 AU/mL (type VI, 254, 256 or 259 AU/mg) was added in a 1 mg/mL solution of 100 mM phosphate buffer at pH 6.5. The reaction was brought to 976 μL with buffer so that after addition of H_2O_2 the reaction would be exactly 1.0 mL. The Eppendorf tube was place in a water bath at 37 °C and 24 μL of 0.883 M H_2O_2 was added over 2.5 hours. 5.5 hours after the addition of the first batch of H_2O_2, the reaction was stopped with the addition of 100 μL of formic acid before centrifugation.

*3.2.2 Results for horseradish peroxide assay on methylphloracetophenone (**2**)*

Control reactions were run without substrate, without HRP, without H_2O_2, and without HRP and H_2O_2. Color change was not observed however in the control reactions with **2** as a substrate. Controls were also run under all the same conditions using usnic acid (**1**) as the substrate. These assays using **1** allowed us to determine if **1** was degraded or converted into another product under the assay conditions employed. If **1** was degraded or converted to another product during the assay, we would be unable to recover and detect **1** if it was being formed from

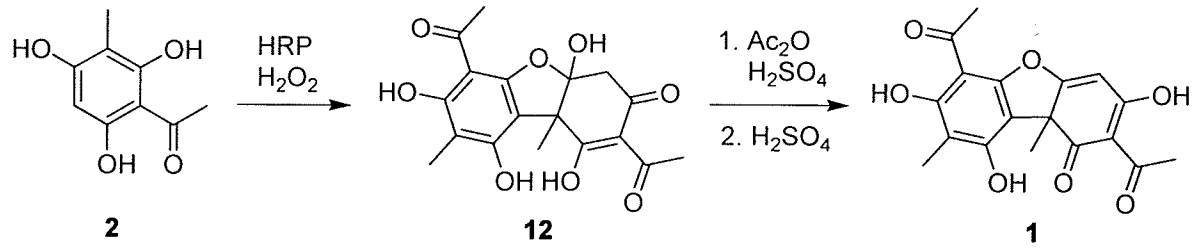
2. Standards run with **1** did not change color but remained yellow. ^{1}H-NMR and HPLC/MS data indicated that standards run with **1** had indeed proceeded through the assay unchanged as no new compounds could be detected. That **1** did not change in the assay conditions means that when HRP forms **1** from **2**, no further reactions would be expected. Likewise, in the control reactions using **2** as a substrate, **2** appeared to remain unchanged based upon spectroscopic and mass data. **2** was recovered when the assay was performed without HRP, without H_2O_2, and without HRP and H_2O_2. Results from the control reactions are presented in **Table 3.2**.

Table 3.2. Controls for horseradish peroxidase enzyme assay.

Substrate	HRP Enzyme	Hydrogen Peroxide	Result
None	Yes	Yes	No usnic acid (**1**) or methylphloracetophenone (**2**) detected
Methylphloracetophenone (**2**)	No	No	Methylphloracetophenone (**2**) recovered unchanged
	No	Yes	Methylphloracetophenone (**2**) recovered unchanged
	Yes	No	Methylphloracetophenone (**2**) recovered unchanged
	Yes	Yes	Usnic acid (**1**) detected
Usnic Acid (**1**) Standard	No	No	Usnic acid (**1**) recovered unchanged
	Yes	No	Usnic acid (**1**) recovered unchanged
	Yes	Yes	Usnic acid (**1**) recovered unchanged

Over the course of the reaction, the solution changed from an initial orangish color caused by **2** to a light yellow. ^{1}H-NMR data from the precipitate (4.3 mg) of the assay displayed

a spectrum with five distinct peaks between δ 2.54 and 2.66 ppm. These peaks correspond to the acetyl groups on both **2** and **1**. In **2** this peak is present at δ 2.60 ppm while in **1** the acetyl peaks are at δ 2.66 and 2.62 ppm. Peaks in the ^{1}H-NMR spectrum for the oxidation of **2** with HRP correspond exactly with these three signals. Also, in **2** the methyl group attached to the aromatic ring is at δ 1.95 ppm while in **1** these two signals appear at δ 2.03 and 1.80 ppm. In the spectrum for the assay, peaks are present at δ 1.80 and 1.95 ppm. A peak at δ 2.03 ppm, if any, is lost in the acetone-d_6 solvent peak. Only one peak was observed for an aromatic proton at δ 6.07 ppm. This matches better with the signal observed in **2** at δ 6.06 ppm than the signal in **1** at δ 6.10 ppm. At first glance, this seemed to suggest that no **1** was present. If **1** were produced, there would be a signal for the aromatic proton that was separate from the one observed for **2**. However, it was realized that this spectroscopic data was consistent with the presence of hydrated usnic acid (**12**) (**Scheme 3.7**). Peaks at δ 3.35 ppm and 3.29 ppm in the ^{1}H-NMR spectrum contribute to this conclusion. These peaks would correspond to the methylene group which is part of the non-aromatic ring. To confirm the production of **12**, HPLC/MS was performed on the supernatant and precipitate of the assay. No trace of usnic acid (**1**) was found ($[M-H]^-$ m/z = 343) in either of these samples. The precipitate however showed a distinct peak that corresponded to hydrated usnic acid ($[(\mathbf{1}+18)-H]^-$ m/z = 361). This result was not completely unexpected since **12** is a known biosynthetic precursor to **1**.[14]



2 **12** **1**

Scheme 3.7. Enzymatic synthesis of hydrated usnic acid (**12**) from methylphloracetophenone (**2**) with horseradish peroxidase and conversion of **12** to usnic acid (**1**) by treatment with acetic anhydride and sulfuric acid.

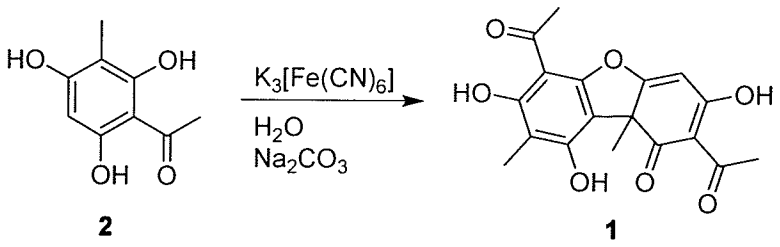
A procedure published by Barton *et al.*[27] describes the synthesis of **1** utilizing potassium ferricyanide to perform the oxidative coupling of **2**. During this synthesis, **12** is reported as an intermediate. Treatment of this compound with acetic anhydride and trace sulfuric acid provided usnic acid diacetate after extraction from the reaction mixture. Usnic acid diacetate lacks a molecule of water from **12** as with **1**, but the two alcohol groups on the aromatic ring are reported to be acylated. Exposure of usnic acid diacetate to sulfuric acid was reported to provide **1**. This procedure was performed on the **12** which was made by the HRP assay described above in this thesis in order to obtain **1**. We were able to detect **1** by HPLC/MS in our assays from this procedure.

3.3 Inorganic oxidation of methylphloracetophenone (2)

3.3.1 Oxidation of methylphloracetophenone (2) with potassium ferricyanide

The synthesis of **1** from **2** was attempted according to the procedure of Barton *et al.*[27] using potassium ferricyanide ($K_3[Fe(CN)_6]$) as an oxidant. The literature procedure used 13

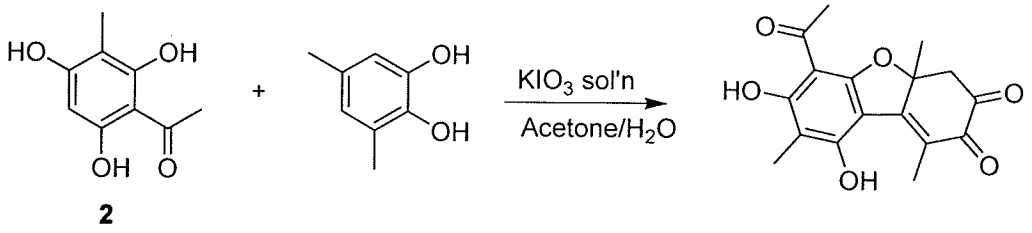
mmol of **2** while the procedure presented in this thesis was performed on a 1.4 mmol scale (259 mg). The amounts of sodium carbonate and potassium ferricyanide used were scaled down appropriately from the reported procedure. When **2** was added to the deoxygenated aqueous solution of Na_2CO_3, it displayed very limited solubility, even after heating. A molar excess (2.1 mmol total) of a bright red potassium ferricyanide solution was added dropwise with stirring over 30 minutes at 0 °C. The reaction mixture was initially clear with orangish precipitate but after addition of the oxidant it changed to dark brown rather than the clear red solution reported in literature. The reaction was acidified with sulfuric acid causing brown foam to form. This brown foamy aqueous solution was extracted with diethyl ether to give a clear red organic solution. The organic solvent was evaporated and the residue was extracted with chloroform. When the chloroform was evaporated, a dry brown foam (251 mg) formed. The appearance of brown foam at this point was also observed in literature. The brown foam was chromatographed with the expectation that hydrated usnic acid (**12**) would be isolated. But this was not the case. A complex mixture of reaction products was formed. After purification however, NMR data, upon comparison to a genuine sample, indicated that **1** was isolated (2 %) (**Scheme 3.8**). It is possible that the use of concentrated sulfuric acid instead of diluted acid during the workup for the reaction was enough to convert any **12** to **1**. HPLC/MS data indicated that indeed, **1** had been formed while there was still a relatively small amount of **12** also present.



Scheme 3.8. Oxidation of methylphloracetophenone (**2**) with potassium ferricyanide to produce usnic acid (**1**).

3.3.2 Oxidation of methylphloracetophenone (2) with potassium iodate

Another inorganic synthesis of dibenzofurans has been reported by Overeem[44] using potassium iodate (KIO_3) as an oxidant. He was able to couple methylphloracetophenone (**2**) with various mono-, di-, and trimethylcatechols to form a substituted dibenzofuran (**Scheme 3.9**)



Scheme 3.9. Oxidative coupling of methylphloracetophenone (**2**) and 3,5-dimethylcatechol with potassium iodate to form a substituted dibenzofuran as reported by Overeem[44]

It was expected that this method could be applied to the coupling of two molecules of methylphloracetophenone (**2**). The reported procedure involved the combination of two solutions. The first solution consisted of an equimolar amount of the two reactants in acetone. The second solution was comprised of a 33 mol% (of the total amount of reactants) solution of

KIO_3 in water that was four times the volume of the first solution. The two solutions were combined and let stand 2 hours before purification. Literature reactions were done on a 60 mmol scale.

When this procedure was repeated on a smaller scale (1.5 mmol **2** compared to 60 mmol), orangish precipitate was observed immediately. The slurry was allowed to sit at room temperature for 3 days. If the reaction was proceeding at a very slow rate, any usnic acid (**1**) or hydrated usnic acid (**12**) formed would be detected by allowing the reaction to continue for an extended time period. The precipitate was filtered and washed with solvents. When the precipitate was washed with water, the wash was light yellow in color and gave 194 mg of material. An ethanol wash was pink and provided 226 mg of material and an ether wash dissolved the remaining 13 mg of material. ^{1}H-NMR of the water wash displayed peaks that did not correspond to either starting material or the expected product, and likely most of the mass was from reactant. The ethanol wash gave **2** indicating that 84 % of the initial **2** used did not undergo any reaction. The material from the ether wash also contained unrecognizable material.

Further research into the oxidation of polyphenols with iodate was performed. A paper from Feifer *et al.*[45] reported on the amount of periodic acid (HIO_4) that phloroglucinol (benzene-1,3,5-triol) consumes over various times. After 9 hours, phloroglucinol consumes 4 equivalents, at 48 hours, 6 equivalents were consumed, and after 13 days, 6.87 equivalents had been consumed. In the first attempt to oxidize **2** presented in this thesis using iodate, **2** was only exposed to 0.33 equivalents of IO_3^-. It was possible then that there was not enough oxidant used to form any observable amount of **12** or **1**.

The second attempt at a potassium iodate oxidation was performed on a 1 mmol scale using 8 equivalents of KIO_3. The reaction was performed as before by combining a solution

containing **2** in acetone with a solution containing KIO_3 in water. The acetone solution was initially dark pinkish in color but after the aqueous KIO_3 was added, a white precipitate settled out from a hot pink solution. The reaction flask was allowed to sit at room temperature for 5 days. ^{1}H-NMR showed the presence of all the peaks expected in the starting material, **2**. However, there were four additional singlets in the spectrum at δ 3.32, 2.67, 2.16, and 1.94 ppm. Of these peaks, only the one at δ 2.67 ppm is found in **1**, indicating that **1** was not formed. Furthermore, the lack of a methyl peak attached to the unaromatized ring at δ 1.80 indicates that **12** was not formed either. Because neither **1** nor **12** were produced, the reaction products were not investigated further.

3.3.3. Concluding remarks on the inorganic oxidation of methylphloracetophenone (2)

Using potassium ferricyanide as an oxidant, it was found that **2** could be coupled to form **1** in a low yield. Efforts to produce **1** or **12** using potassium iodate were unsuccessful. These reactions were attempted as a means to give an efficient synthesis of usnic acid (**1**), but this was not found to be the case.

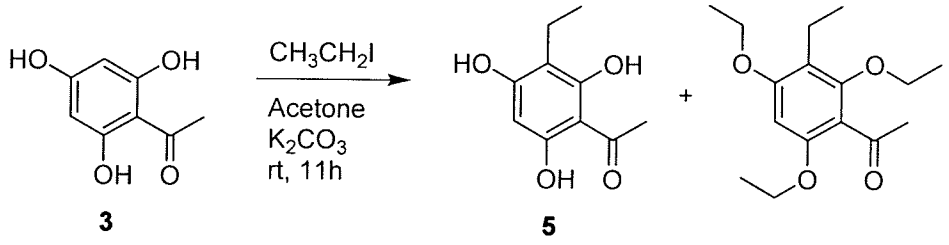
3.4 Synthesis of analogues of methylphloracetophenone (2)

Because usnic acid (**1**) has been found to be toxic, yet exhibit many desirable biological activities, it was desirable to attempt to produce analogues of usnic acid (**1**) that may eliminate this toxicity while maintaining the beneficial properties. This could be accomplished through the production of analogues of **1**. To produce analogues of **1**, analogues of the precursor,

methylphloracetophenone (**2**) first had to be synthesized. After the synthesis of analogues of **2**, attempts could be made to obtain these analogues on a preparative scale from the horseradish peroxidase enzyme assay. Evaluation of biological activity on the usnic acid analogues would demonstrate if the pharmacological profile of **1** had been improved.

3.4.1 Synthesis of ethylphloracetophenone (5)

Having established a method to perform mono-C-alkylation, we sought to apply this methodology to other alkyl groups. The first analogue of methylphloracetophenone (**2**) that we attempted to synthesize contained an ethyl group rather than a methyl group. This was achieved by using iodoethane to ethylate trihydroxytoluene (**4**) (**Scheme 3.10**).



Scheme 3.10. Synthesis of ethylphloracetophenone (**5**) from trihydroxyacetophenone (**3**) with iodoethane.

Preliminary attempts to synthesize **5** with conditions used for the synthesis of **2** were very low yielding. At 0 °C the yield of **5** was approximately 1-3% from NMR data, even when reactions were performed using more than 4 equivalents of alkylating agent. The reaction was also attempted in THF but after 3 days with 10 eq of iodoethane, still no **5** was observed. Ethylation of **3** was attempted again in acetone at 0 °C, but was allowed to warm up to room

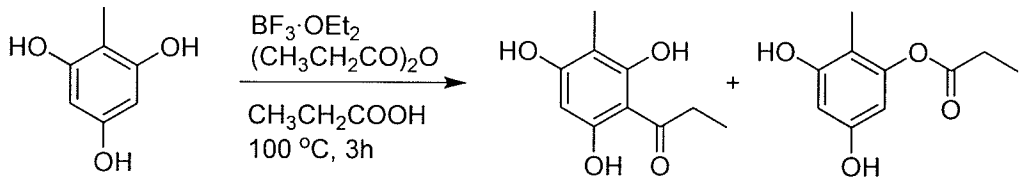
temperature overnight. In this case, a small amount of **5** was observed (< 3 %) and the major products were the result of *O*-ethylation based on TLC data and ^{1}H-NMR data. A quartet was present at δ 3.98 ppm and triplets were visible at δ 1.41 and 1.35 ppm. These peaks dominated the spectrum and correspond to ethyl groups attached to an electronegative oxygen atom. From this result, it was surmised that **5** could be synthesized by running the reaction at room temperature in its entirety and carefully monitoring the reaction progress with TLC. **5** was obtained at a yield of 8 % after 11 hours at room temperature using 4 eq of iodoethane. The ^{1}H-NMR of **5**, like **2**, displayed a downfield shift of the aromatic proton compared to **3** from δ 5.92 to 6.06 ppm. This proton also integrated for 1 proton in the **5** spectrum, confirming that substitution had occurred at one of the unsubstituted carbons of the aromatic ring. The singlet from the acetyl group appeared at δ 2.60 ppm, exactly as was the case with **2**. A quartet at δ 2.56 ppm (2H) and a triplet at δ 1.04 ppm (3H) indicated an ethyl group attached to an aromatic ring. Two signals in the ^{13}C-NMR spectra present in **5** but not in **3** are those at δ 16.0 ppm and 14.0 ppm. These signals correspond to the ethyl group now present with **5**. The electron ionization mass spectrometry (EI-MS) spectrum obtained for this compound is consistent with **5**. The molecular ion peak $[M-H]^-$ is at *m/z* 196.1 and the parent peak is at *m/z* 181.0, indicating the loss of a methyl group. There was also an 8 % recovery of starting material from this particular reaction while the remaining material was some combination of products with mono-, di- or tri-*O*-ethylation and mono-, di-, or no *C*-ethylation (**Scheme 3.10**). The 8 % yield of **5** leaves much room for improvement, potentially by a gradual addition of iodoethane over many hours to prevent side reactions from occuring.

This is the first reported synthesis of **5**. One paper from Cann and Shannon[46] includes the compound in an exploration of the chemical shifts of chelated OH protons in compounds related

to trihydroxyacetophenone (**3**), but no synthetic methods are reported or referenced. Further searching revealed that the promise made in the paper of further studies on this class of compounds, presumably with synthetic details, was not reported.

*3.4.2 Synthesis of methylphlorpropionophenone (**6**)*

Another analogue of **2** that was considered involved the replacement of the acetyl group with a propionyl group. The procedure already developed for the acylation of **4** was followed using propionic anhydride (propanoyl propanoate) and propionic acid (propanoic acid). Boron trifluoride diethyl etherate ($BF_3 \cdot OEt_2$) was again used as the catalyst (**Scheme 3.11**).



Scheme 3.11. Synthesis of methylphlorpropionophenone (**6**) from trihydroxytoluene (**4**).

When the reaction was first attempted adhering to the procedure used for acetylation of **4**, the reaction appeared to proceed quicker than expected based on TLC results. The reaction was stopped after 80 minutes before workup which included a deprotection strategy using methanol and 3 N NaOH (1:1) which were stirred with the residue overnight. ^{1}H-NMR data indicated that the deprotection was not completely successful. Peaks in the spectrum at δ 2.5 – 2.7 ppm corresponded to the protons attached to the methylene carbon of the propionyl group attached to a phenolic oxygen. Purification provided **6** at a 12 % yield with the majority of products

containing various degrees of *O*-acylation. The ^{1}H-NMR spectrum of **6** contained a quartet at δ 3.07 ppm (2H) and a triplet at δ 1.10 ppm (3H), both of which are consistent with a propionyl group attached to an aromatic ring. The aromatic proton appeared at δ 6.05 ppm and integrated for 1 hydrogen atom. The methyl group attached to the aromatic ring appeared at δ 1.95 ppm, the same position as in **2**. The asymmetric mono-*O*-acylation product was also isolated and characterized (**Scheme 3.11**). The quartet for the methylene carbon of propionyl group is shifted upfield compared to **6** to δ 2.58 ppm (3H) while the propionyl methyl triplet is shifted slightly downfield to δ 1.19 ppm (3H). Also, the methyl attached to the aromatic ring is shifted upfield to δ 1.88 ppm. The two aromatic protons appeared as doublets caused by allylic coupling at δ 6.31 ppm (1H) and 6.08 ppm (1H) with a *J* coupling of 2.3 Hz.

If a more fruitful deprotection could be accomplished, an improved yield of **6** could be obtained. For the second attempt at propionation, the reaction was allowed to continue for a full 3 hours, as was reported in a literature procedure.[26] After cooling the reaction to 0 °C and extraction, the organic material was thoroughly dried and to it was added a solution of a catalytic amount of sodium methoxide in anhydrous methanol and allowed to stir overnight to deprotect the phenolic oxygens. This procedure is referred to as Zemplen de-*O*-acylation and has been reported[34] to be a simple and effective means to deprotect alcohols which have been protected as methyl esters. Workup from the Zemplen deprotection required the addition of Dowex 50W-X4 cation exchange resin to the solution while monitoring the pH. This displaced any sodium ions interacting with the product for hydrogen. Once an acidic pH was reached, the solution was filtered and dried. After purification by column chromatography, **6** was afforded in a 26 % yield. NMR data also indicated the presence of di-*C*-acyl product as a side-product from this reaction procedure.

Methylphlorpropionophenone (**6**) had previously been described in a paper regarding the separation of phloroglucinols using gas chromatography.[47] It was reportedly synthesized following procedures[48] used to obtain similar compounds but there is no detailed procedure for the synthesis of **6** provided. The synthesis of **6** has also been reported in a paper from 1938 by Brockmann and Maier[49] *en route* to the synthesis of rottlerin.

3.4.3 Concluding remarks on the synthesis of analogues of methylphloracetophenone (2)

Two analogues of methylphloracetophenone (**2**) were successfully synthesized (**Figure 3.2**). Ethylphloracetophenone (**5**) was formed through the alkylation of trihydroxytoluene (**4**) with iodoethane at an 8 % yield. Methylphlorpropionophenone (**6**) was synthesized at a 26 % yield from the Friedel-Crafts acylation of trihydroxytoluene (**4**) using propionic anhydride in propionic acid as the acylating agent and boron trifluoride diethyl etherate as the catalyst.

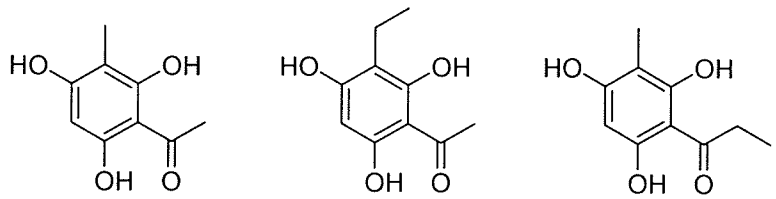
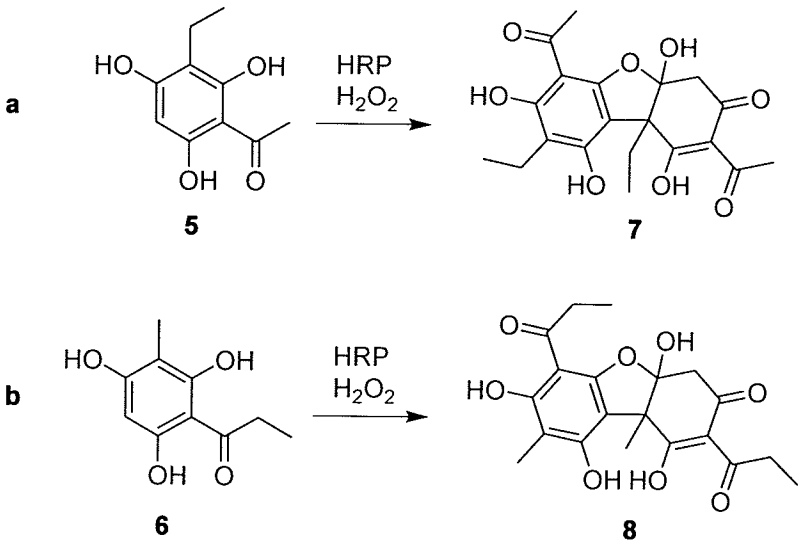


Figure 3.2. Ethylphloracetophenone (**5**) and methylphlorpropionophenone (**6**), analogues of methylphloracetophenone (**2**).

3.5 Horseradish peroxidase oxidation of methylphloracetophenone analogues

Analogues of **2** were subject to oxidation with horseradish peroxidase in an attempt to produce analogues of **1**. Assay conditions were identical to those previously outlined in *Section 3.2.1* with the only changes being that 3 mg of **5** and 4.4 mg of **6** were used to conserve the supply of these compounds, especially **5**, leaving enough material to run control reactions. The assay **5** was initially light yellow but turned light orange over the course of the assay. The same color progression was observed when **6** was used as a substrate but the color change was immediately after the addition of the first batch of H_2O_2. The control assays did not display any color change. HPLC/MS results of the precipitates (2.2 mg from the assay with **5** and 3.7 mg from the assay with **6**) from assays containing **5** or **6** contained clear peaks at m/z = 389 with retention times in close agreement with that of **12**. These peaks correspond to the hydrated form of the usnic acid analogues ethyl-usnic acid (**7**) and propionyl-usnic acid (**8**), respectively (**Scheme 3.12**).



Scheme 3.12. Oxidation of methylphloracetophenone analogues using horseradish peroxidase. **a.** Production of ethyl-usnic acid (**7**) from **5**. **b.** Production of propionyl-usnic acid (**8**) from **6**

^{1}H-NMR data shows that a multitude of small peaks are present between δ 0.8 and 3.4 ppm from the precipitates of each of the assays, indicating that a variety of compounds were formed. Purification of the precipitates was not pursued because of the small amount (< 4 mg) available. To fully and accurately characterize these analogues, yields would have to increase so that compounds could be obtained on a milligram scale. New conditions were tested for the HRP assay using **2** as a substrate.

3.6 Modifications to HRP assay to obtain a preparative amount of usnic acid (1)

3.6.1 Modifications to the HRP assay

The first aspect of the HRP assay addressed was the solubility of the substrates, which was limited in the slightly acidic phosphate buffer. A series of assays was performed with the

same concentration of methylphloracetophenone (**2**), HRP, and H_2O_2 as previously described in *Section 3.2.1*. However, rather than using strictly 100 mM phosphate buffer at pH 6.5 as the assay medium, acetone and mixtures of acetone with phosphate buffer were tested. The assay was run in 100 % acetone, and 3:1, 1:1, and 1:3 acetone/phosphate buffer. Except with the 1:3 acetone/phosphate buffer trial, **2** was completely dissolved in the assay solution. The solutions were all colored light pink before the assay began with the addition of H_2O_2. As the assays proceeded, the color changed to an even lighter shade of pink. Following the HRP assays, ^{1}H-NMR data was collected for each of the trials. The NMR spectra for all four of the compound indicated that **2** was unchanged over the course of the assays. The spectra matched that of **2**. That **2** was not converted into any oxidation products indicates that the horseradish peroxidase enzyme in organic solvent concentrations greater than 25 % is unable to react whatsoever with **2**.

A paper published by Booth *et al.*[50] describes the oxidation of numerous phenolic compounds with peroxidase. Conditions were quite different from what had been previously used in the assays described in this thesis. In the reference, 0.5 – 10 g of substrate was used in 0.15 – 5 L of solution, often just distilled water. Peroxidase was used at a level of 0.03 AU/mL and 6 % H_2O_2 (double the concentration we were using) was added over periods of time up to five days in amounts ranging from 2 – 48 mL. Although it was sometimes observed immediately, it usually took up to five days for precipitate to form. The assays from literature were stopped only after precipitate was observed. The conditions from this reference were scaled down for the attempt described presently to accommodate a smaller amount of substrate. 10 mg of **2** was dissolved in 285 mL of water with 15 mL of acetone to solubilize the **2**. HRP was added to an activity level of 0.03 AU/mL and 10 mL of 3 % H_2O_2 were added over 48 hours in 0.4 mL aliquots. The UV-vis absorption spectrum of the assay was monitored over the course of the

reaction. Samples from the reaction were diluted to give a maximum absorption of approximately 0.7 absorption units. **2** had an absorption maximum at 265 nm. No change was observed in the UV-vis trace for the reaction over the 48 hours that H_2O_2 was added. Therefore, the solution was allowed to stand at room temperature to wait for a precipitate to form. After 32 days, no precipitate was visible. The reaction was acidified and extracted, and ^{1}H-NMR data indicated that **2** was still present. Several peaks between δ 1.3 and 1.5 were now present but these did not belong to either usnic acid (**1**) or hydrated usnic acid (**12**). Thus, when the HRP assay was performed based on procedures used by Booth *et al.*, neither usnic acid (**1**) nor hydrated usnic acid (**12**) were produced.

A new set of conditions using phosphate buffer was developed for the HRP enzyme assay on a larger scale than previously attempted from *Section 3.2.1.* 55 μmol of **2** (10 mg) was solubilized in a small amount of acetone (1 mL) and reactions were 20 mL in total. Three concentrations of HRP (0.1, 1.0, 10 AU/mL) were used at two levels of H_2O_2 (final concentrations of 44.2 mM, 441.5 mM) which was again added slowly (first 90% over 5.5 h, final 10% after 3 days). This is an increase in H_2O_2 concentration from 21 mM which was the amount used in the assays that demonstrated production of usnic acid (**1**) from HPLC/MS data. After the enzyme had reacted with the substrate for three days, there was a noticeable color change from the initial brown-orange to yellow. Each reaction was extracted and recovered material (3.6 – 6.6 mg) was run on TLC with a solvent system specifically developed for lichen metabolites[51] which consisted of 10% acetic acid in toluene. For the reactions with a final concentration of H_2O_2 of 441.5 mM, spots near usnic acid (**1**) were visible under UV light, and after staining with 10% sulfuric acid, an additional spot with a similar staining color and an R_f near that of usnic acid (**1**) was visible. HPLC/MS however did not indicate that hydrated usnic

acid (**12**) or usnic acid (**1**) was being produced by a lack of peaks at *m/z* = 361 or 343, respectively.

3.6.2 Concluding remarks on modifications to the HRP assay

Several modifications to the conditions for the initial HRP assay were performed. First, an increased amount of organic solvent was used to address solubility issues of the substrate, methylphloracetophenone (**2**). Although more organic solvent allowed the substrate to fully solubilize in the assay medium, the enzyme appeared to be incapable of operating in increased concentrations of organic solvent. Only **2** was recovered from each of the trials using varying levels of acetone in phosphate buffer.

Second, conditions that were quite different than previously used were found in a paper by Booth *et al.*[50] These conditions used lower enzyme concentrations but higher amounts of H_2O_2 added over longer time periods to an assay medium that was just water. When conditions from the reference were developed for work in this thesis, the desired products, usnic acid (**1**) or hydrated usnic acid (**12**), were not produced.

Finally, the assay was attempted on a larger scale with a lower concentration of **2** at two different concentrations of H_2O_2, both of which were higher than used after development in *Section 3.2.1*. Also, three different concentrations of the HRP enzyme were used at each of the two concentrations of H_2O_2. HPLC/MS was not able to detect the presence of **1** or **12** in any of these assays.

3.7 Oxidation of methylphloracetophenone analogues with a modified HRP assay

*3.7.1 A modified HRP assay on analogues of methylphloracetophenone (**2**)*

The assays were performed using ethylphloracetophenone (**5**) or methylphlorpropionophenone (**6**) as substrates. 20 mL assays in phosphate buffer were performed with 10 mg of substrate as described in the final paragraph of *Section 3.6.1*. Here, the assays were only carried out with the higher final H_2O_2 concentration of 441.5 mM since TLC results from this condition when **2** was used as a substrate displayed compounds with R_f values very similar to that for an usnic acid (**1**) standard. Two HRP concentrations, 0.1 AU/mL and 10 AU/mL, were used rather than three since there did not appear to be significant variation among these concentrations as well as 1.0 AU/mL. Reactions with **5** were initially pink/orange in color but after the three day reaction period they were light yellow. The reactions with **6** as a substrate were initially yellow/orange but changed to light yellow/colorless after 3 days. Controls were performed for each substrate with no enzyme, no substrate and no H_2O_2. TLC (10% acetic acid/toluene) was performed on organic material (3.0 – 8.0 mg) recovered from the assays after extraction and drying. Similar to **2** as a substrate, spots were visible near the R_f of usnic acid (**1**) after inspection with UV light and staining with 10% sulfuric acid. HPLC/MS analysis could not detect the presence of any expected product. Peaks were not observed at m/z = 371 or 390 which corresponds to the usnic acid analogue from **5** or **6**, and the hydrated version of those, respectively.

3.8 Closing remarks on the HRP oxidation of analogues of methylphloracetophenone (2)

Using the assay with conditions developed from *Section 3.2.1*, that demonstrated hydrated usnic acid (**12**) production from methylphloracetophenone (**2**), ethylphloracetophenone (**5**) and methylphlorpropionophenone (**6**) were subjected to oxidation with horseradish peroxidase. Under those conditions, the analogues of **2** were oxidized and formed compounds, **7** and **8**, consistent with analogues of hydrated usnic acid (**12**) (Scheme **3.12**). Peaks in the HPLC/MS at the expected mass and retention time confirmed these findings. However, because a preparative amount of the new usnic acid analogues could not be prepared, complete characterization of the compounds was not performed. This is a task that will be accomplished in the future. With a preparative amount of the analogues of **1**, testing for biological activity can also be performed.

4. Isolation of Dimethyl Citrate, Trimethyl Citrate and Dimethyl Oxalate

4.1 Isolation of methylated citric acid derivatives and dimethyl oxalate (9)

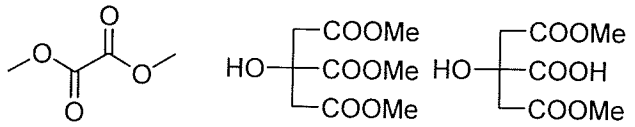
A sample of the lichen *Dibaeis baeomyces* (L.f.) Rambold & Hertel collected from Five Islands Provincial Park in Nova Scotia was provided by Dr. Michele Piercey-Normore, Department of Biological Sciences, University of Manitoba. Five Islands Provincial Park is located on the shores of the Bay of Fundy where the geological landscape was forged by volcanic action and massive erosion of the red sandstone that makes up the area. Soil included with the lichen sample was sprinkled onto a plate containing potato dextrose agar with streptomycin. The streptomycin is included so that bacterial growth is limited on the plates, giving any fungal species present a better opportunity to sporulate. After two weeks of incubation at 30 °C, a white fungus and a black fungus were found growing separately on the plate. Regions on the plate between the two fungi had a peppered pattern. Some of the black spores were transferred to a fresh plate to obtain a monoculture. Control plates that were not inoculated with spores showed no environmental contamination. This ensured that the fungus

being grown on the plate was from the soil sample and not from spores present in the air. Fermentation broths were inoculated with spores from the monoculture and allowed to grow for one week. Mycelium was removed from the fermentation broth by filtration to give approximately 80 mL of fungus. The broth was extracted with ethyl acetate under neutral conditions (1.696 g) and after acidification to pH 1 with HCl (2.787 g). Samples of the extracts were analyzed by proton and carbon NMR and the spectra for each extract were nearly identical. Both spectra contained several singlets between δ 3 – 4 ppm and what appeared to be a large quartet in the δ 2.7 – 3.0 ppm range. The acidic extract contained less minor peaks above δ 4.0 ppm and below δ 2.6 ppm. With fewer minor compounds and more material, it would be simpler to purification compounds from the acidic extract.

The first compound to elute from the silica gel column was identified as dimethyl oxalate (**9**) (198 mg) (**Figure 4.1**). There was a single peak in the ^{1}H-NMR (300 MHz) spectrum at δ 3.76 ppm and two peaks in the ^{13}C-NMR (75 MHz) spectrum at δ 157.6 ppm and 53.1 ppm. The NMR spectra for this compound are virtually identical to those for dimethyl carbonate. Dimethyl carbonate is a volatile liquid at room temperature whereas the isolated compound was a colorless solid. This confirmed the characterization of this compound as **9**. Biosynthetically, it is much more common and logical than a carbonic acid derivative. Oxalic acid is common in plants, especially rhubarb.[52]

The second and third compounds isolated from the fungal extract were identified as trimethyl citrate (**10**) (187 mg) and dimethyl citrate (**11**) (1234 mg) (**Figure 4.1**). Characterization of these compounds through NMR studies will be discussed in the following section.

When cultures were left to grow for two weeks, no citrate production was observed after analysis of NMR data from crude extracts. After such a time period, the citrate must be used up by the organism in metabolic pathways.



9 **10** **11**

Figure 4.1. Dimethyl oxalate (**9**), trimethyl citrate (**10**), and dimethyl citrate (**11**), isolated from a fungal culture.

4.2 Characterization of methylated citric acid derivatives with NMR and MS

Although neither **10** nor **11** contain a center of chirality, their symmetry and stereochemistry lend themselves to a brief discussion. NMR data will be considered as it pertains to the present dialogue. For simplicity, until discussing chemical shifts of the terminal methyl groups, **10** and **11** will be referred to interchangeably as citrate.

From **Figure 4.2**, it can be said that the citrate molecule is prochiral because of a plane of symmetry through the central carbon, C-3. The group beginning with C-2 is *pro-R* while the group beginning with C-4 is *pro-S*. Because a plane of symmetry can be drawn perpendicular to the page through C-3, it can be said that the two CH_2COOCH_3 groups attached to C-3 are enantiotopic. Furthermore, the methylene carbons C-2 and C-4 are also prochiral but the geminal hydrogen atoms on each group are diastereotopic with respect to each other. There is no plane of symmetry through either C-2 or C-4. These hydrogen atoms can be designated *pro-R* or *pro-S* as shown in **Figure 4.2a**. Nomenclature[53] for the hydrogen atoms is shown in **Figure 4.2b** where

the first subscript refers to the $CH_2CO_2CH_3$ group and the second subscript refers to the actual hydrogen on that group.

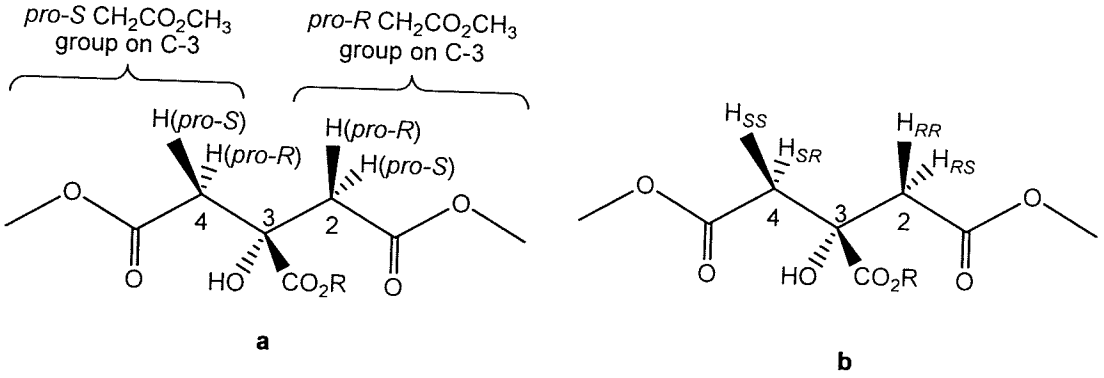


Figure 4.2. a. Stereochemistry about C3 in **10** and **11** and designation of the prochiral groups on C3 and prochiral hydrogen atoms on the methylene carbons. **b.** Nomenclature of the hydrogen atoms on the methylene carbons.[53]

Knowing that the two $CH_2CO_2CH_3$ groups on either **10** or **11** are enantiotopic, it is expected that their signals in an NMR experiment will be undifferentiated in an achiral environment. The diastereotopic methylene protons are expected to resonate at different chemical shifts and couple to one another. ^{1}H-NMR data showed the presence of two doublets as an AB spectrum, resembling a quartet (**10**: δ 2.94 ppm, 2H, 2.82 ppm, 2H; **11**: δ 2.94 ppm, 2H, 2.79 ppm, 2H). ^2J values for both **10** and **11** are -15.3 Hz (**Figure 4.3**) and are known to be negative.[54] The downfield doublet has been assigned to the H_{SS} and H_{RR} atoms while the upfield doublet is attributed to the H_{SR} and H_{RS} atoms.[53]

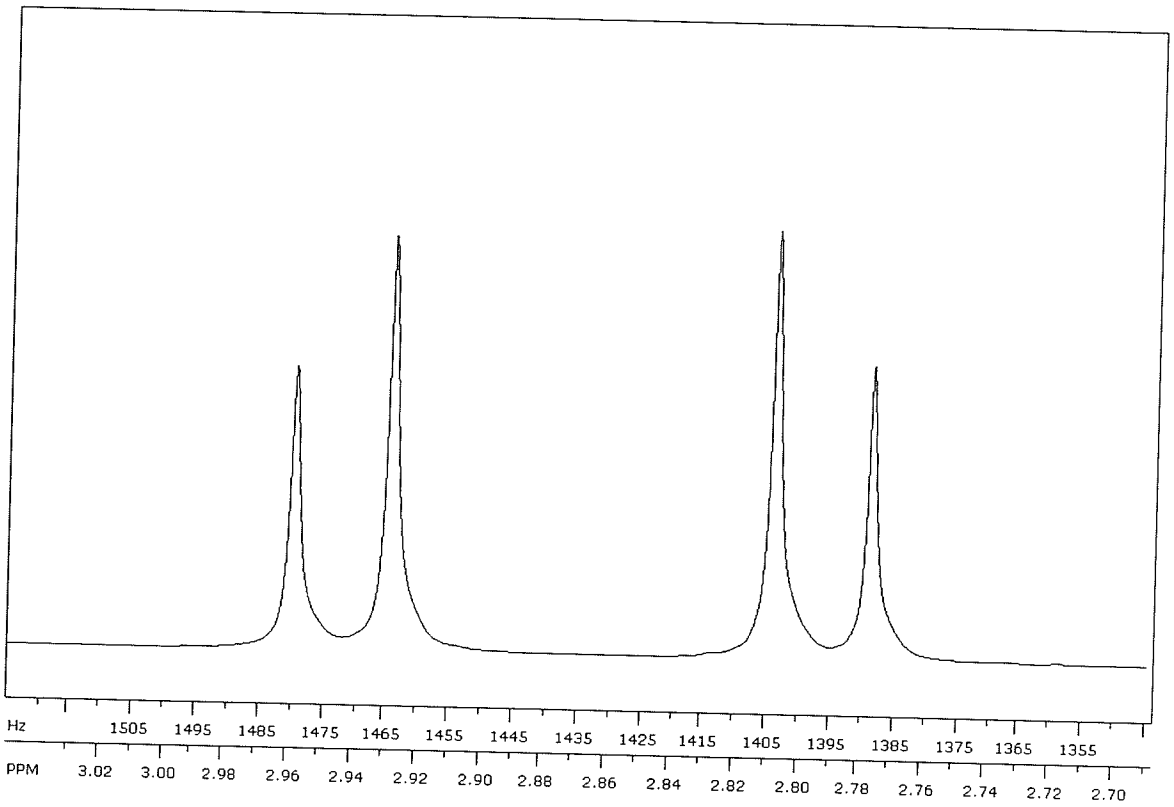
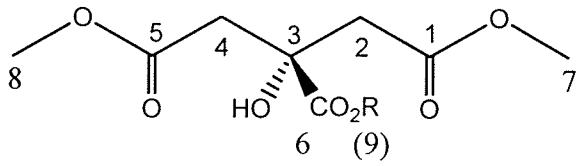


Figure 4.3. 500 MHz ^{1}H-NMR spectrum of the methylene protons in **10** showing a *J* coupling of -15.3 Hz.

Two additional signals were observed in the ^{1}H-NMR spectra for **10** at δ 3.76 ppm (3H) and 3.65 ppm (6H). These signals confirm the presence of two unique methyl esters. Integration values make it possible to assign the signal at δ 3.76 ppm to C9 and the signal at δ 3.65 ppm to C7 and 8 (**Figure 4.4**). In **11**, one singlet was observed at δ 3.66 ppm which corresponded to the equivalent methyl esters on C7 and 8. Because only one methyl ester peak was detected which integrated for 6 protons, the two methyl esters must be chemically equivalent. If "asymmetric dimethyl citrate" was isolated where the methyl esters are present on C9 and one of either C7 or C8, there would be two distinct signals for these methyl esters, as is the case in **10**. For this reason, we have concluded that "prochiral dimethyl citrate" (**11**) was isolated



10 R = CH_3
11 R = H

Figure 4.4. Numbering scheme for trimethyl citrate (**10**) and dimethyl citrate (**11**)

The ^{13}C-NMR spectra further confirmed the assigned structures. Spectra for both compounds showed two carbonyl groups (**10**: δ 175.3, 171.8 ppm; **11:** δ 176.5, 172.0 ppm), an oxygenated quaternary carbon (**10**: δ 74.8 ppm; **11**: δ 74.4 ppm), and a methylene attached to an electron withdrawing group (**10**: δ 44.4 ppm; **11**: δ 44.2 ppm). The methyl esters in **10** appeared at δ 53.3 ppm (C9) and 52.4 ppm (C7, C8) to bring the total number of peaks to six. **11** showed five signals in total and the methyl ester was displayed at δ 52.3 ppm.

Electron ionization mass spectrometry (EI-MS) was performed on **10** and **11**. This technique uses high energy electrons to ionize a compound in the vapor phase to form a radical cation. This is a relatively strong ionization method and as such, causes fragmentation of the molecule under examination. The molecular ion peak plus hydrogen (*m/z* 235.0) was present for **10** and a loss of a methyl ester was also observed (*m/z* 175.0, [M+H]-59). From the fragment at *m/z* 175, two successive losses of methanol in the two remaining methyl ester groups give the base peak at *m/z* 143 and the other major peak at *m/z* 101. The EI-MS conditions were found to be too harsh to observe a molecular ion peak for **11**, but the same fragments at *m/z* 143 and 101 were observed and at almost the exact same intensity. This seems to indicate that the methyl ester that is first lost is C6 which would lead to the same ions for both **10** and **11**. Electrospray ionization mass spectrometry (ESI-MS) was employed to find the molecular ion peak of **11**. This

technique can be used even on large proteins because it overcomes the tendency to fragment molecules. In ESI-MS, the compound is dissolved in a liquid and dispersed as a fine aerosol spray. The extensive solvent evaporation of these droplets leads to a charged and intact ion. EI-MS however relies on high energy electrons in a concentrated beam colliding with molecules in the gas phase forming radical cation fragments of the molecule. Using ESI-MS, **11** was observed with a sodium ion at *m/z* 243.

4.3 Identification of fungal species producing methylated citrate derivatives, 10 and 11, and dimethyl oxalate (9)

A sample of the fungal spores used for production of the methylated citric acid derivatives was identified morphologically as *Aspergillus niger* (van Tiegh) (T. Booth, personal communication, 2009). It was decided to sequence the internal transcribed spacer (ITS) of the mitochondrial DNA of the fungus to confirm this assessment.

DNA was extracted from the fungal mycelium with a modified protocol of Grube *et al.*[29] The polymerase chain reaction (PCR) was used to amplify the ITS region from the DNA. To first determine the most effective conditions for amplification, PCR was performed on the DNA at a series of dilutions (undiluted, 1:10 and 1:100). Furthermore, different primers were tested (**Figure 4.5**). The universal ITS4-3′ reverse primer (5′-TCCTCCGCTTATTGATATGC-3′)[31] was used and the fungal specific forward primers 1780F-5′ (5′-CTGCGGAAGGATCATTAATGAG-3′)[55] and 1184-5′ (5′-GACTCAACACGGGGAAACTC-3′)[30] were tested for each DNA dilution. Primers are strands of DNA that serve as the starting point for DNA replication. They anneal to a sample of DNA at a specific location that is

sequence dependent and allow a DNA polymerase enzyme to add nucleotides on to the primer strand. After amplification, the samples were run in a TBE gel and bands were visualized under UV light to determine the best conditions for amplification. The brightest band, indicating the highest quantity of DNA, was found in Lane 8 of the gel where the 1184-5′ primer was used and DNA was at a 10-fold dilution (**Figure 4.5**). These conditions were used for amplification.

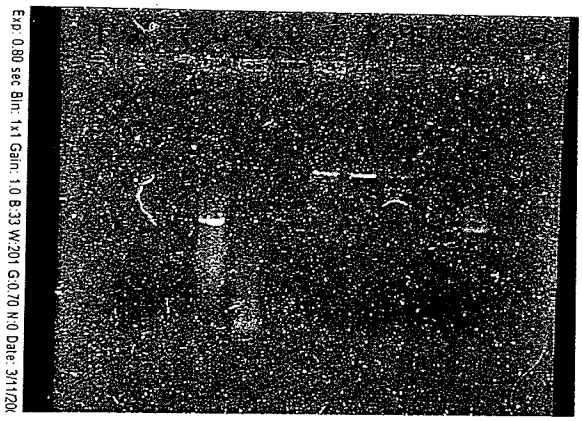


Figure 4.5. Gel electrophoresis showing optimization of PCR conditions. Lane 2: 1780F forward primer, no rDNA dilution. Lane 3: 1780F forward primer, 1:10 rDNA dilution. Lane 4: 1780F forward primer, 1:100 rDNA dilution. Lane 5: 1780F forward primer, negative control. Lane 7: 1184 forward primer, no rDNA dilution. Lane 8: 1184 forward primer, 1:10 rDNA dilution. Lane 9: 1184 forward primer, 1:100 rDNA dilution. Lane 10: 1184 forward primer, negative control. Lane 11: ladder. Lanes 1, 6 and 12: empty.

The amplified portion of DNA was purified by cutting the entire sample as a band out of the gel (**Figure 4.6a**) and quantified by comparison with the band at 1650 base pairs (BPs) in a ladder (**Figure 4.6b**) before sequencing. The band at 1650 BPs was known to contain 8 % of the total DNA in the ladder which corresponded to 80 ng/μL of ladder. The ladder was run next to 3 μL

of the 35 μL sample of the isolated rDNA. The isolated rDNA was estimated to have approximately 60 ng of DNA in total after visualization of the bands under UV light. That meant that the sample of rDNA was at a concentration of approximately 20 ng/μL. This had to be determined so that an appropriate concentration (60ng/20μL) of DNA was used when sequencing.

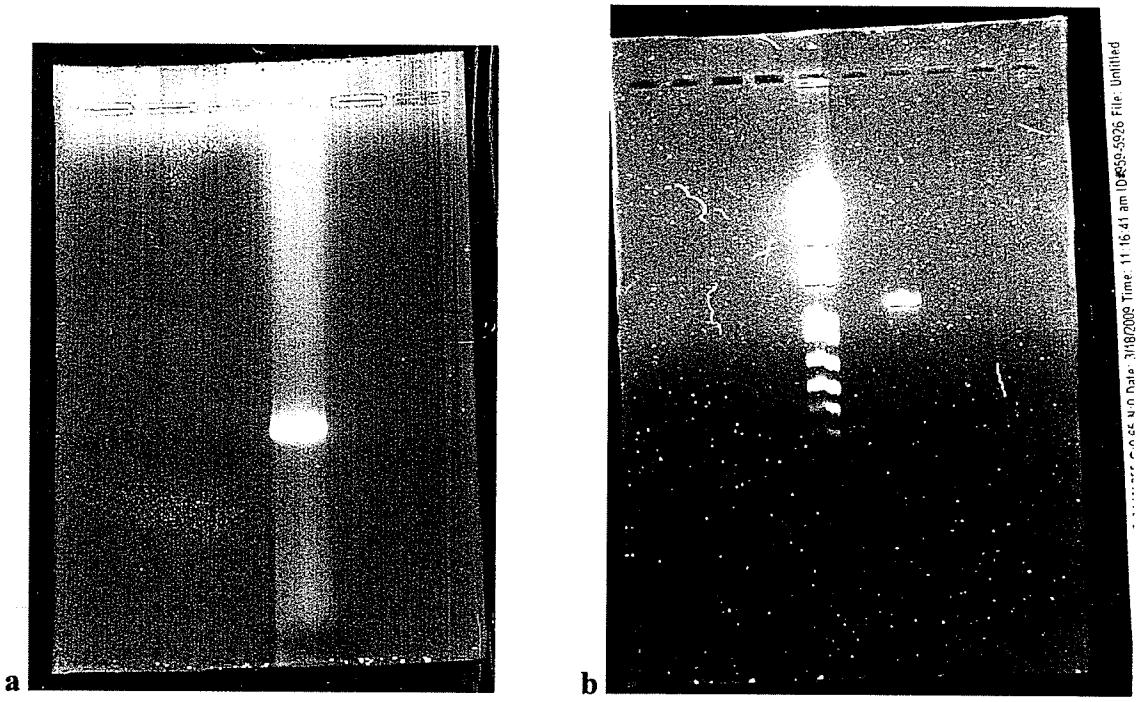


Figure 4.6. a. The sample of isolated rDNA was run down a gel before being cut out as a band to purify the sample. **b**. The sample of isolated and purified rDNA was run next to a ladder to determine the concentration of DNA in the sample.

The sequence from the 1184-5′ primer had 790 BPs and from Sequencher software the accuracy of the sequence was 50.6% while the ITS4-3′ sequence had 807 BPs and 81.7% accuracy.

Sequences were aligned and corrected with Se-Al software to give 1117 characters which was submitted to GenBank and assigned the accession number GQ130305 and strain identifier DJH1-13. The submitted sequence was compared to other sequences using a BLAST search[32] which showed 98 – 99% sequence homology to *A. niger*.

4.4 Occurance of methylated citric acid derivatives in nature

Dimethyl citrate (**11**) and trimethyl citrate (**10**) have been previously reported as secondary metabolites in a variety of other organisms, but mainly in higher plants such as *Prunus mume* (Sieb. et Zucc.),[56] an apricot variety; *Gastrodia elata* (Blume),[57] an orchid; *Dioscorea opposite* (Thunb.),[58] the Chinese yam; *Opuntia ficus-indica* (Mill),[59] a cactus; *Embelia laeta* (Linnaeus)[60] and *Rosa bracteata* (J.C. Wendl),[61] both of which are shrubs; and the trees *Platonia insignis* (Mart.)[62] and *Crataegus pinnatifida* (Bunge).[63] The presence of **10** and **11** has been reported as a metabolite in *Poria cocos* (Wolf),[64] a fungus popular in Chinese traditional medicine.

Both trimethyl citrate (**10**) and dimethyl citrate (**11**) have demonstrated a suppressive effect of the SOS-inducing activity of chemical mutagens,[56] a hyperglycemic response in mice,[65] and monoamine oxidase A inhibition.[59] Trimethyl citrate (**10**) has been shown to have antimicrobial activity against food-borne pathogens[66] while dimethyl citrate (**11**) is responsible for anti-thrombotic activity.[56] **10** has found numerous applications including as an additive in ointments to protect and treat skin for UV damage,[67] antibacterial toothpaste,[68] candles (to produce a red colored flame),[69] silicon based polymers,[70] and as a biodegradable plasticizer for polylactic acid.[71]

Aspergillus niger has been employed as the primary commercial source of citric acid for nearly a century. Strains of *A. niger* have been developed for fermentation processes that are capable of overproducing citric acid. Yields of citric acid often approach the theoretical yield based on the carbon source in these strains.[72] For industrial fermentations, citric acid is produced by depriving *A. niger* of iron. In turn, this deactivates mitochondrial aconitase which is responsible for the transformation of citric acid to isocitrate within the Krebs cycle. The organism employs the excess citric acid as a siderophore, releasing it into the surrounding environment.[73] In 2006, global citric acid production was 1.4 million tones with an annual increase in demand and consumption at 3.5-4.0 %.[74] Numerous synthetic routes using varied starting materials have been published, but fermentation thus far has remained unrivaled by chemical methods for large scale production, principally because the final product is worth less than the synthetic starting materials. Despite the massive scale of citric acid fermentation there appears to have been no reports of methylated derivatives being produced by fungal cultures.

Oxalic acid has been well characterized as a fungal metabolite[75] and has been suggested to have a critical role for wood rotting organsims such as *Fomitopsis palustris*.[76] The presence of trace amounts of **9** has been detected in the analysis of the volatile components in the fungus *Fistulina hepatica*[77] and from the plant *Astragalus membranaceus*.[78] However there are no reports of the production of a preparative amount of **9** from any fungal source.

Several applications of dimethyl oxalate (**9**) have been reported such as an alternative fuel for fuel cells,[79] in the manufacture of crosslinked safety glass,[80] an insecticide for textiles,[81] and as a nematocide.[82]

4.5 Concluding remarks on the isolation of methylated citrate and oxalate derivatives

To the best of our knowledge the strain of *A. niger* described here is the first report of a filamentous fungus capable of producing methylated citric acid derivatives. This also appears to be the first report of the isolation of dimethyl oxalate (**9**) from a fungal fermentation culture.

5. Conclusions and Future Work

5.1 Methylphloracetophenone (2) and usnic acid (1)

Methylphloracetophenone (**2**), the key biosynthetic intermediate *en route* to usnic acid (**1**) was synthesized by two different methods. One approach was inspired by the failed protection of trihydroxytoluene (**4**) using iodomethane. Rather than the expected *O*-methylation, extensive *C*-methylation was observed. This lead to the synthesis of **2** by the mono-*C*-methylation of trihydroxyacetophenone (**3**). The second path to **2** involved revisiting the work of Kurtis Anderson, a former project student in our group. Conditions were modified and optimized for the acylation of trihydroxytoluene (**4**) with acetic anhydride using boron trifluoride diethyl etherate as a catalyst. Future work may focus on the further optimization of these reactions to increase the yield and decrease side reactions. A summer student has already shown that the a very slow addition of iodomethane (0.5 eq per hour to 3 eq total) during the methylation reaction can drastically reduce the formation of *O*-methyl side products. Ultimately, the synthetic methods developed will be used to produce isotopically labeled **2** which will be used to isolated the oxidative enzyme in lichens that produces usnic acid (**1**) from **2**. A summer student has already been able to synthesize labeled **2** using ^{13}C-labeled iodomethane.

A model enzyme system using horseradish peroxidase was developed to demonstrate that **2** could be converted to usnic acid (**1**). The assay showed conversion of the substrate to hydrated usnic acid (**12**) by HPLC/MS data. **12** could then be treated with acetic anhydride and sulfuric acid to give **1**. The desired products of the assay, **12** or **1**, were not formed on a preparative scale. Several different conditions were attempted with varying amounts of enzyme, substrate, hydrogen peroxide, and different reaction solutions. Future studies may include a scaling up of conditions that were developed in this thesis in order to obtain amounts of **1** that would allow for complete characterization. Major products of the HRP assay may also be analyzed so that conditions can be modified to produce less of these and more **12** or **1**.

Inorganic oxidation of **2** to produce **1** was also attempted. When potassium ferricyanide was used as an oxidant, a low yield of **1** was obtained. Several conditions were used in efforts to oxidize **2** with potassium iodate but these did not prove successful. The inorganic synthesis of **1** was attempted to efficiently form this molecule and these methods were abandoned when they proved unable to do so.

Analogues of methylphloracetophenone (**2**) were synthesized to test if usnic acid analogues could be formed enzymatically with horseradish peroxidase. Analogues of **1** are needed to attempt to improve the pharmacological profile of this substance. Two analogs of **2** were synthesized, first the methyl group was replaced with an ethyl group (**5**) and second the acetyl group was replaced with a propionyl group (**6**). These compounds were synthesized in a manner analogous to the two routes to **2**: alkylation of trihydroxyacetophenone (**3**), and acylation of trihydroxytoluene (**4**), respectively. Future work will include the synthesis of other analogues of **2** with expanded functionalities. These analogues can potentially be oxidized by either HRP or

by the native oxidative enzyme from lichens, once it is isolated, in order to produce usnic acid analogues.

The two new compounds, **5** and **6**, were oxidized by HRP, and analysis by HPLC/MS was consistent with the formation of analogues of usnic acid (**1**). These compounds were not produced in high enough yields by HRP and in the future, it is our goal to obtain these molecules in amounts necessary for complete characterization.

5.2 Natural products isolation from fungi

We are developing a natural products screening program that takes advantage of access to a collection of lichen samples, with attached soil, from remote locations with harsh climates across Canada. New drugs or other interesting compounds may be produced by the filamentous fungi present in the soil with these samples. Furthermore, soil fungi have a fast rate of growth which allows for the screening of many fungal strains in a short time period. Soil from one of the samples provided three compounds that have never been previously reported in filamentous fungi: dimethyl oxalate (**9**), trimethyl citrate (**10**) and dimethyl citrate (**11**). The fungi was morphologically identified by Dr. Tom Booth as *Aspergillus niger* and this assessment was confirmed genetically by comparing the sequence of the internal transcribed spacer of the organisms mitochondrial DNA to that of other fungi. *A. niger* is responsible for the production of most of the world's supply of citric acid, which is on the scale of millions of tones per year. Our findings are significant because methylated derivatives of these compounds have never been reported from any filamentous fungi. We have repeated the growth of this strain of fungus and found for a second time that it is the fungus producing these compounds and they are not the

result of isolation or purification procedures. In the future, the screening of natural products from soil fungi will be guided by an assay which tests for biological activity, possibly antibacterial activity, present in the crude extracts from the fungal broth or from the fungal mycelium itself.

6. References

(1) Dewick, P. M. *Medicinal Natural Products: A Biosynthetic Approach*; John Wiley & Sons Canada Ltd: Etobicoke, 2007.

(2) Field, B.; Jordan, F.; Osbourn, A. *New Phytol.* **2006**, *172*, 193-207.

(3) Fabricant, D. S.; Farnsworth, N. R. *Environ. Health Perspect. Suppl.* **2001**, *109*, 69-75.

(4) Farnsworth, N. R.; Akerele, O.; Bingel, A. S.; Soejarto, D. D.; Guo, Z. *Bull World Health Organ* **1985**, *63*, 965-81.

(5) Butler, M. S.; Newman, D. J. *Prog. Drug Res.* **2008**, *65*, 1, 3-44.

(6) Gullo, V. P.; McAlpine, J.; Lam, K. S.; Baker, D.; Petersen, F. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 523-531.

(7) Boustie, J.; Grube, M. *Plant Genet. Resour.* **2005**, *3*, 273-287.

(8) Miao, V.; Coeffet-LeGal, M. F.; Brown, D.; Sinnemann, S.; Donaldson, G.; Davies, J. *Trends Biotechnol.* **2001**, *19*, 349-355.

(9) Dayan, F. E.; Romagni, J. G. *Pestic. Outlook* **2001**, *12*, 229-232.

(10) Ingolfsdottir, K. *Phytochemistry (Elsevier)* **2002**, *61*, 729-736.

(11) Sanchez, W.; Maple John, T.; Burgart Lawrence, J.; Kamath Patrick, S. *Severe hepatotoxicity associated with use of a dietary supplement containing usnic acid,*

Division of Gastroenterology and Hepatology, Department of Internal Medicine, Mayo Clinic College of Medicine, Rochester, MN 55905, USA, 2006.

(12) Osterrothova, K.; Jehlicka, J. *Spectrochim. Acta, Part A* **2009**, *73A*, 576-580.

(13) Taguchi, H.; Sankawa, U.; Shibata, S. *Tetrahedron Lett.* **1966**, 5211-14.

(14) Taguchi, H.; Sankawa, U.; Shibata, S. *Chem. Pharm. Bull.* **1969**, *17*, 2054-60.

(15) Huneck, S.; Akinniyi, J. A.; Cameron, A. F.; Connolly, J. D.; Mulholland, A. G. *Tetrahedron Lett.* **1981**, *22*, 351-2.

(16) Schmitt, I.; Kautz, S.; Lumbsch, H. T. *Mycol. Res.* **2008**, *112*, 289-296.

(17) Stocker-Woergoetter, E. *Nat. Prod. Rep.* **2008**, *25*, 188-200.

(18) Hawranik, D. J.; Anderson, K. S.; Simmonds, R.; Sorensen, J. L. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2383-2385.

(19) Penttila, A.; Fales, H. M. *Chem. Commun. (London)* **1966**, 656-7.

(20) Davis, D.; Elix, J. A. *Tetrahedron Lett.* **1969**, *34*, 2901-4.

(21) Elix, J. A.; Tronson, D. *Aust. J. Chem.* **1973**, *26*, 1093-109.

(22) Marshak, A.; Schaefer, W. B.; Rajagopalan, S. *Proc. Soc. Exp. Biol. Med.* **1949**, *70*, 565-8.

(23) Shibata, S.; Ukita, T.; Miura, Y.; Tamura, T. *J. Penicillin (Japan)* **1948**, *1*, 588-90.

(24) Shibata, S.; Ukita, T.; Tamura, T.; Miura, Y. *Japan Med. J.* **1948**, *1*, 152-5.

(25) Shibata, S.; Miura, Y.; Ukita, C.; Tamura, T. *Yakugaku Zasshi* **1948**, *68*, 298-300.

(26) Nakagawa-Goto, K.; Lee, K.-H. *Tetrahedron Lett.* **2006**, *47*, 8263-8266.

(27) Barton, D. H. R.; Deflorin, A. M.; Edwards, O. E. *J. Chem. Soc.* **1956**, 530-4.

(28) Raa, J.; Overeem, J. C. *Phytochemistry (Elsevier)* **1968**, *7*, 721-31.

(29) Grube, M.; Depriest, P. T.; Gargas, A.; Hafellner, J. *Mycol. Res.* **1995**, *99*, 1321-4.

(30) Gargas, A.; Taylor, J. W. *Mycologia* **1992**, *84*, 589-92.

(31) White, T. J.; Bruns, T.; Lee, S.; Taylor, J. In *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*; 1 ed.; Innis, M. A., Gelfand, D. H., Sninski, J. J., White, T. J., Eds.; Academic Press: San Diego, 1990, p 315-322.

(32) Zhang, Z.; Schwartz, S.; Wagner, L.; Miller, W. *J. Comput. Biol.* **2000**, *7*, 203-214.

(33) Anderson, K. S., University of Manitoba, 2008.

(34) *Greene's Protective Groups in Organic Synthesis*; 4 ed.; Wuts, P. G. M.; Greene, T. W., Eds.; John Wiley & Sons, Inc.: Hoboken, 2007.

(35) Wildes, J. W.; Martin, N. H.; Pitt, C. G.; Wall, M. E. *J Org Chem* **1971**, *36*, 721-3.

(36) Bodtke, A.; Pfeiffer, W.-D.; Ahrens, N.; Langer, P. *Tetrahedron* **2005**, *61*, 10926-10929.

(37) Fang, J.; Barcelona, M. J. *Chemosphere* **2002**, *50*, 105-109.

(38) Hosny, M.; Rosazza, J. P. N. *J. Agric. Food Chem.* **2002**, *50*, 5539-5545.

(39) Kiso, T.; Shizuma, M.; Murakami, H.; Kiryu, T.; Hozono, K.; Terai, T.; Nakano, H. *J. Mol. Catal. B: Enzym.* **2007**, *45*, 50-56.

(40) Ling, K.-Q.; Li, W.-S.; Sayre, L. M. *J. Am. Chem. Soc.* **2008**, *130*, 933-944.

(41) Tzeng, S.-C.; Liu, Y.-C. *J. Mol. Catal. B: Enzym.* **2004**, *32*, 7-13.

(42) Xu, F.; Koch, D. E.; Kong, I. C.; Hunter, R. P.; Bhandari, A. *Water Res.* **2005**, *39*, 2358-2368.

(43) Zoia, L.; Bruschi, M.; Orlandi, M.; Tolppa, E.-L.; Rindone, B. *Molecules* **2008**, *13*, 129-148.

(44) Overeem, J. C. *Recl. Trav. Chim. Pays-Bas* **1969**, *88*, 851-9.

(45) Feifer, J. P.; Smith, M. A.; Willeford, B. R., Jr. *J. Org. Chem.* **1959**, *24*, 90-3.

(46) Cann, M. R.; Shannon, P. V. R. *Chem. Ind. (London)* **1982**, 779-80.

(47) Pyysalo, H.; Widen, C. J. *J. Chromatogr.* **1979**, *168*, 246-9.

(48) Widen, C. J. *Suom. Kemistilehti, B* **1968**, *41*, 295-8.

(49) Brockmann, H.; Maier, K. *Ann.* **1938**, *535*, 149-75.

(50) Booth, H.; Saunders, B. C. *J. Chem. Soc.* **1956**, 940-8.

(51) Culberson, C. F. *J. Chromatogr.* **1972**, *72*, 113-25.

(52) Hasenpusch, W. *CLB Chem. Labor Biotech.* **2006**, *57*, 92-97.

(53) Anet, F. A. L.; Park, J. *J. Am. Chem. Soc.* **1992**, *114*, 411-16.

(54) Bovey, F. A.; Mirau, P. A.; Jelinski, L. *Nuclear Magnetic Resonance Spectroscopy*; Academic Press: St Louis, 1988.

(55) Piercey-Normore, M. D.; DePriest, P. T. *Am. J. Bot.* **2001**, *88*, 1490-1498.

(56) Miyazawa, M.; Yamada, T.; Utsunomiya, H. *Nat. Prod. Res.* **2003**, *17*, 319-323.

(57) Pyo, M. K.; Park, K. M.; Yun-Choi, H. S. *Nat. Prod. Sci.* **2000**, *6*, 53-55.

(58) Bai, B.; Li, M.-J.; Wang, Y.; Liu, X.-H. *Zhongguo Zhong Yao Za Zhi* **2008**, *33*, 1272-4.

(59) Han, Y. N.; Choo, Y.; Lee, Y. C.; Moon, Y. I.; Kim, S. D.; Choi, J. W. *Arch. Pharm. Res.* **2001**, *24*, 51-4.

(60) Tang, T.; Wu, F. e. *Tianran Chanwu Yanjiu Yu Kaifa* **2004**, *16*, 129-130.

(61) Xu, Z.; Huang, S.; Du, F.; Peng, C.; Tian, Y. *Zhong Yao Cai* **2003**, *26*, 867-9.

(62) Monteiro, A. R.; Meireles, M. A. A.; Marques, M. O. M.; Petenate, A. J. *J. Supercrit. Fluids* **1997**, *11*, 91-102.

(63) Xie, Y.; Dai, L.; Guo, M.; Xue, M.; Yin, W.; Chai, W. *Zhiwu Xuebao* **1981**, *23*, 383-8.

(64) Hu, B.; Yang, Y.; Ye, Y. *Zhongcaoyao* **2006**, *37*, 655-658.

(65) Witherup, K. M.; McLaughlin, J. L.; Judd, R. L.; Ziegler, M. H.; Medon, P. J.; Keller, W. J. *J. Nat. Prod.* **1995**, *58*, 1285-90.

(66) Bae, J.-H.; Lee, S.-M. *Food Sci. Biotechnol.* **2003**, *12*, 128-132.

(67) Raman, G.; Wagh, S. S.; (Unilever PLC, UK; Unilever NV; Hindustan Lever Limited). Application: WO, 2004, p 23 pp.

(68) Liu, R.; (Southern Medical University, Peop. Rep. China). Application: CN, 2006, p 17 pp.

(69) Li, H.; Lu, X.; (Jiande Jiaxuan Articles Co., Ltd., Peop. Rep. China). Application: EP, 2007, p 8pp.

(70) Brook, M. A.; Gonzaga, F.; Tian, H.; (McMaster University, Can.). Application: WO, 2007, p 66pp.

(71) Labrecque, L. V.; Dave, V.; Gross, R. A.; McCarthy, S. P. *Annu. Tech. Conf. - Soc. Plast. Eng.* **1995**, *53rd*, 1819-23.

(72) Papagianni, M. *Biotechnol. Adv.* **2007**, *25*, 244-263.

(73) Roukas, T. *Food Sci. Technol. (Boca Raton, FL, U. S.)* **2006**, *148*, 349-405.

(74) Soccol, C. R.; Vandenberghe, L. P. S.; Rodrigues, C.; Pandey, A. *Food Technol. Biotechnol.* **2006**, *44*, 141-149.

(75) Gadd, G. M. *Adv. Microb. Physiol.* **1999**, *41*, 47-92.

(76) Munir, E.; Yoon, J. J.; Tokimatsu, T.; Hattori, T.; Shimada, M. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 11126-11130.

(77) Wu, S.; Krings, U.; Zorn, H.; Berger, R. G. *Food Chem.* **2005**, *92*, 221-226.

(78) Miyazawa, M.; Kameoka, H. *Agric. Biol. Chem.* **1987**, *51*, 3153-4.

(79) Suarez-Gustave, S.; Greenbaum, S.; Chung, S. H.; Peled, E.; Duvdevani, T.; Aharon, A.; Melman, A. *Prepr. Symp. - Am. Chem. Soc., Div. Fuel Chem.* **2002**, *47*, 370-371.

(80) Papenfuhs, B.; (Clariant G.m.b.H., Germany). Application: DE, 2001, p 3 pp.

(81) Muneyuki, R.; Kanamaru, H.; (S. T. Chemical Co., Ltd., Japan). Application: US, 1990, p 2 pp.

(82) Djian, C.; Ponchet, M.; Cayrol, J. C. *Pestic. Biochem. Physiol.* **1994**, *50*, 229-39.