Secondary Metabolites from a Northern Manitoban Fungus

by

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ABSTRACT

Over the years, secondary metabolites have proven to be significant in the discovery of novel antibiotics and/or lead compounds. Various secondary metabolites have been reported to be produced by fungus of the genus, *Alternaria*. Here we report the isolation of secondary metabolites by the bioassay-guided fractionation of the ethyl acetate extract of fermentation cultures of the fungus, *Alternaria tenuissima*. This fungal strain was isolated from the soil underlying the lichen, *Peltigera didactyla*; and the lichen was collected from Wapusk National Park, a location in Northern Manitoba. The compounds isolated from the fungus include deoxyphomalone, dimethyl 4-methyl-2,6-pyridinedicarboxylate, stemphyperylenol and N-Methyl pyrrolidinone. Their structures were determined by comprehensive analysis of their spectroscopic data including FT-IR, mass spectrometry, 1D and 2D NMR; and their bioactivities were tested against *E. coli* cells. Some of the compounds demonstrated some bioactivity. The taxonomic identity of the fungus was confirmed by ITS sequencing of its ribosomal DNA.

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ABBREVIATIONS

[O]	oxidation
^{1}H-NMR	proton nuclear magnetic resonance
^{13}C-NMR	carbon nuclear magnetic resonance
1780F-5′	forward primer used in ribosomal DNA with sequence 5′-CTGCGGAAGGATCATTGATTC-3′
^{2}J	geminal coupling
26S	large ribosomal subunit
5.8S	small ribosomal subunit
ACP	acyl carrier protein
ATR	attenuated total reflectance
BLAST	basic local alignment search tool
br s	broad singlet
Bn	benzyl
cm	centimeters
CoA	coenzyme A
^{1}H-^{1}H COSY	proton-proton correlation spectroscopy
DCM	dichloromethane

DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
ESI	electrospray ionization
ESI-MS	electrospray ionization mass spectrometry
EtOAc	ethyl acetate
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HPLC/MS	high performance liquid chromatography/ mass spectrometry
HSQC	heteronuclear single quantum correlation
Hz	hertz
ITS	internal transcribed spacer
ITS1	first internal transcribed spacer in ribosomal DNA
ITS2	second internal transcribed spacer in ribosomal DNA
ITS2-kL	reverse primer used in ribosomal DNA with sequence 5'-TGCTTAAGTTCAGCGGGTA-3'
ITS4-3'	reverse primer used in ribosomal DNA with sequence 5'-TCCTCCGCTTATTGATATGC-3'
J	coupling constant

LB	lysogeny broth
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
MHz	megahertz
MS	mass spectrometry
NCBI	national center for biotechnology information
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
pH	power of hydrogen
PKS	polyketide synthase
q	quartet
rDNA	ribosomal deoxyribonucleic acid

rRNA	ribosomal ribonucleic acid
R_f	retention factor
rpm	revolutions per minute
s	singlet
sp.	species
SSU	small subunit
t	triplet
TLC	thin layer chromatography
UV	ultraviolet
UV-vis	ultraviolet-visible
V	volts

1. INTRODUCTION

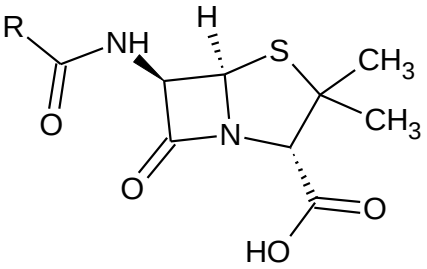
1.1. Natural Products and Ancient Medicine

Natural products generally refer to small organic molecules produced by plants, animals and micro-organisms. They are classified into two categories: Primary and secondary metabolites. Primary metabolites play a direct role in the survival of the organism and are usually involved in key metabolic pathways such as the Krebs cycle and glycolysis pathways. These compounds can be observed in all living organisms. In contrast with their primary counterparts, a unique spectrum of secondary metabolites can be produced by each organism. The production of these secondary metabolites can also vary depending on certain conditions which will be discussed later. Unlike primary metabolites which are directly involved in the survival of organisms, secondary metabolites are thought to play an indirect role in their survival; however, their exact functions are sometimes not obvious. Secondary metabolites include compounds such as toxins to defend against attacks from other organisms or chemical attractants to attract prey or other organisms with desirable properties. It is this category of natural product that constitutes the active principle in most plants and herbs that were used in medicine before the advent of modern medicine.

For thousands of years, the treatment of injuries and diseases has been accompanied by the use of natural products, many of which originated from plants. The earliest recorded use of natural products as drugs is from the Mesopotamia region and dates back to 2600 BC.[1] The records contained about a thousand different plant-derived products

which served various purposes. Some of these natural products such as myrrh and poppy juice are still in use today due to their useful medicinal properties.[1] In those days, various natural product extracts were sometimes mixed together to create a more potent and effective drug. However, the effectiveness and range of activity of the available herbal drugs were greatly limited. Currently treatable diseases such as infections and tuberculosis were often fatal.[2] Much of that changed with the discovery of penicillin (Fig. 1).

1.2. Discovery of Antibiotics



Penicillin G, R = Bn

Penicillin V, R = phenoxy methyl

Figure 1: The structure of the penicillins

In the fall of 1928, Sir Alexander Fleming made an interesting observation on a *Staphylococcus* plate culture he had mistakenly left uncovered. The growth of the *Staphylococcus* culture seemed to be impeded by a blue-green mould contaminant which was later identified as *Penicillium chrysogenum*. So he concluded that this fungus was producing a substance that apparently induced the lysis of proximal bacterial colonies.[3] This was a sensational discovery due to its implication in the treatment of infectious diseases. Other drugs used for treatment at the time were not effective due to their toxicity to humans.[3] There were limited supplies of penicillin initially as there

were no means of mass production. Fortunately, methods for mass production as well as artificial synthesis of the drug were developed in order to meet demands.

The discovery of penicillin was a significant turning point in the use of natural products in medicine. Until then, the majority of the known natural products were plant-derived, but the discovery led to an increasing interest in natural products produced by micro-organisms. This discovery also paved the way for the advancement of the nascent field of antibiotics and led to the discovery of other notable antibiotics (Fig. 2) such as streptomycin, erythromycin and tetracycline (from the bacterium, *Streptomyces sp.*) and cephalosporins (from the fungus *Cephalosporium acremonium sp*.).[1]

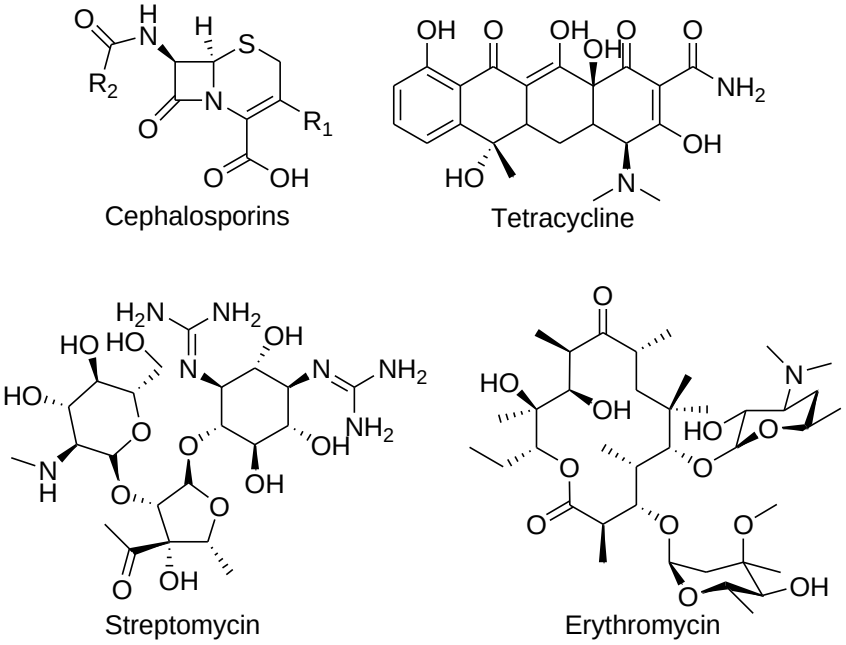


Figure 2: Structures of cephalosporins, tetracycline, streptomycin and erythromycin

1.3. Emergence of Antibiotic Resistance

One limitation of penicillin was that it was only effective against Gram-positive bacteria and had a narrow spectrum of activity (i.e. it was only effective against a limited number of bacteria). Penicillin works by inhibiting the synthesis of the peptidoglycan layer of the bacterial cell wall[4]. In Gram-negative bacteria, the outer cell membrane acts as a barrier to the antibiotic molecule thereby reducing its effectiveness. As a result, the bacterium is more likely to survive its encounter with the antibiotic. The desire and need to broaden the range of activity of penicillin was the impetus that motivated research into semisynthetic methods and ultimately, the search for entirely novel antibiotic compounds. This was one of the contributing factors that led to the discovery of the antibiotics described in Fig. 2, as well as other penicillin derivatives.

Another contributing factor was the emergence of penicillin-resistant bacterial strains. All penicillin-based antibiotics are generally referred to as β-lactam antibiotics due to the presence of a β-lactam ring in their structural core. These resistant bacterial strains are, however, capable of producing an enzyme called β-lactamase that can hydrolyze the β-lactam ring of the antibiotic, thereby neutralizing it.[5] The first β-lactamase was discovered in 1940 from the bacterium, *Escherichia coli* (E. coli).[6] Since then, the occurrence of penicillin-resistant bacteria became more widespread.[7]

In some limited populations, antibiotic resistant bacteria have been present even before the discovery and development of antibiotics.[8] However, evolutionary pressure initiated and exacerbated by the widespread use of antibiotics has led to the increase in

prevalence of these strains.[9] There are several reasons for the emergence and proliferation of antibiotic-resistant bacterial strains. The attainment, conferment or transfer of resistance by bacteria is usually coincidental and the result of a mutation. In addition, the acquisition of certain "resistance genes" can alter the normal functions of the bacteria in such a way that impedes the activity of the antibiotic. These resistant strains become prevalent due to several reasons, not the least of which is the improper use of antibiotic in the treatment of infections.[10] Each time an antibiotic is used for treatment, there is a possibility of the development of resistance by a bacterium which could ultimately give rise to an entirely resistant colony. The rise of the resistant colony increases the occurrence of transference of resistance genes to non-resistant strains thereby facilitating the proliferation of antibiotic resistant bacteria.

1.4. The Struggle against Antibiotic Resistance

Other mechanisms of resistance in addition to the action of the β-lactamase enzyme include the active efflux of the molecule from the bacterial cell, reduction in the permeability of the cell membrane,[11] alteration of the target site[12] and alteration of the bacterial metabolic pathway affected by the antibiotic.[13] The initial resistance to penicillin via the β-lactamase mechanism was overcome by the development of a penicillin analogue known as meticillin (formerly known as methicillin). This drug was resistant to the β-lactamase enzyme produced by the penicillin-resistant strains, however even this development was undermined by the emergence of meticillin-resistant bacteria.[14] Resistance to later generation antibiotics also emerged over the

years and has motivated the constant search for new antibiotic compounds until a more permanent solution can be found.

A bacterium that exemplifies this issue very well is *Staphylococcus aureus*. The initial plate culture in which Sir Alexander Fleming first observed the antibiotic effects of the *Penicillium* fungal metabolite was one of *S. aureus*. Due to evolutionary selection, penicillin-resistant strains became more prevalent as the susceptible bacteria were killed off by the antibiotic.[15] The resistance was mediated by the activity of the enzyme penicillinase, a type of β-lactamase. Once discovered, the β-lactamase-resistant antibiotic, meticillin, was then used to treat penicillin-resistant *S. aureus* infections successfully. This success did not last very long, however, as meticillin-resistant *S. aureus* (commonly known as MRSA) began to emerge two years later. The resistance in this strain was generated by a genetic mutation that led to the alteration of the target protein, reducing its affinity for the antibiotic.[14]

A totally different class of antibiotics, called aminoglycosides, with a completely different mechanism of action had to be employed in combating MRSA infections; however resistance against even these began to emerge. Aminoglycosides exert their antibiotic effect by inhibiting protein synthesis in the bacterium. In *S. aureus*, resistance to this class of antibiotics is mediated by three widely accepted means including covalent modification of the antibiotic by enzymes, alteration of the target site (ribosomal RNA of the 30S subunit) and/or active efflux of the drug out of the bacterial cell.[16] Vancomycin, discovered from the bacterium, *Amycolatopsis orientalis*, eventually

replaced meticillin as the most potent antibiotic against MRSA infections. It is a glycopeptide that acts by binding to the nascent peptidoglycan wall and preventing cross-linking. Resistant strains to this antibiotic have also begun to emerge despite regulation of its usage. One of the resistance mechanisms in resistant bacterial strains involves the mutation of an amino acid in the target site, precluding vancomycin binding.[17]

The issue of antibiotic resistance will continue to be a problem in the foreseeable future. The discovery of new antibiotics is only a temporary solution to the problem, as over time, resistance to the antibiotic will emerge and become prevalent.[18] The prospects of a permanent solution to the problem are not very promising at the moment.[19] One of the methods used in combating antibiotic resistance include using decoy molecules alongside the antibiotic. This makes the bacterium concentrate on a false target, leaving the antibiotic molecule free to act. An example of this is the use of clavulanic acid alongside penicillin drugs.[20] The clavulanic acid binds to β-lactamase, preventing it from attacking the penicillin molecule. Overcoming antibiotic resistance could also be achieved by interfering with the mechanisms that mediate the proliferation of antibiotic resistance. This could be accomplished by preventing the duplication of antibiotic-resistant strains or the genetic transfer of resistance genes. These methods do not completely alleviate the problem of resistance, hence the need to search for new antibiotics

1.5. Secondary Metabolites and Drug Discovery

The need for novel and more effective antibiotics cannot be overestimated and much effort and research have gone into the development of these very important molecules. Several approaches to the development of new antibiotics have come to prominence over the years. The main thrust of this endeavor is in the discovery of "lead compounds." Lead compounds are biologically active molecules on which the development of novel antibiotic drugs can be based. Many antibiotic compounds when initially discovered are not suitable for use as antibiotics for a number of reasons. The ideal antibiotic compound has to be soluble, non-toxic to humans (having minimal to no side effects), able to be administered either orally or intravenously, effective against a broad spectrum of bacteria including Gram-positive and Gram-negative strains and not susceptible to bacterial enzymes that might target such molecules for degradation.

The development of a sizeable fraction of the current crop of novel antibiotics usually involves scaffold molecules that undergo chemical modification to enhance and optimize their biological activity and effectiveness[21]. These scaffold molecules are often natural product isolates from microorganisms that exhibit biological activity and the antibiotics produced from them are typically referred to as semi-synthetic antibiotics. Some examples of these semi-synthetic antibiotics include the penicillins (penicillin-based e.g. meticillin), cephalosporins and carbapenems. The initial research on penicillin was halted at some point due to the observation that the molecule was so actively excreted out of the body that it would not last long enough to accomplish the desired effect. Eventually, research on the molecule was resumed and ways around this

problem involving some chemical modification of the original penicillin core were developed. A drug called probenicid was also developed to inhibit the excretion of penicillin.[22]

Other antibiotics produced today such as the oxazolidinones, quinolones, and sulfonamides are completely synthetic and not based on any natural products. However, deriving drugs from natural products is advantageous because these compounds already show compatibility with a living system and so, are more likely to be biologically friendly than their synthetic counterparts.[21] Moreover, despite the discovery of many new antibiotics from microorganisms, it has been estimated that less than 1% of all microorganisms have been cultured.[23] This observation reflects the vast potential of microorganisms as a source of novel antibiotics and lead compounds.

Lichens are compound organisms made up of a fungus in a symbiotic relationship with either a green algae or a cyanobacterium. Usnic acid, a secondary metabolite of many lichen species, was first isolated in the mid-17th century.[24] Research on the molecule over the years has shown it to possess many interesting biological properties. It is effective against a wide variety of Gram-positive bacteria including multi-drug resistant strains such as meticillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus*, however its mechanism of action is not yet fully understood. It has also been shown to demonstrate antiviral, antiprotozoal, antifungal, anti-inflammatory, and insecticidal properties.[25] Despite its interesting biological properties, the development of usnic acid into a drug has been impeded by its apparent hepatotoxicity.[26] Lead compounds such as

usnic acid constitute the crux of the efforts in the development of novel antibiotics which might also lead to the establishment of an entirely new category of antibiotics since its core structure is unlike any other antibiotic currently in production.

High-throughput screening is a modern approach to discovering lead compounds by synthetic means and usually employs the use of computer-generated data and analysis such as molecular modeling. Numerous organic compounds can be tested for their bioactivity. For example, compounds could be tested for their affinity to the active (or allosteric) site of a key bacterial enzyme with the assumption that binding of the compound in the active site would inhibit the activity of that enzyme and ultimately lead to the death of the bacterial cell. Analogues of compounds with high affinity for the enzyme could then be synthesized, and an assay is developed to assess their actual activity.

Alternatively, lead compounds could also be discovered by natural means as have been previously indicated. In this case, lead compounds are essentially secondary metabolites produced by microorganisms such as bacteria and fungi, and show promising biological activity. The organisms are grown in a liquid fermentation culture and their natural products are extracted, purified, characterized and tested for bioactivity. It is, however, notable that the spectrum of secondary metabolites produced by a microorganism in the wild is often not the same as those produced by the same organism under laboratory conditions.

1.6. Lichens as a Source for Secondary Metabolites

As previously suggested, lichens have also been identified as secondary metabolite producers. Including usnic acid, over 800 different lichen secondary metabolites have been reported and many of these have a diverse range of biological activity and constitute potential lead compounds.[27] This has led to increasing interest in lichen secondary metabolites especially now that methods to overcome the limitation of lichen slow growth are being developed. One of these methods involves the transfer of genes responsible for the production of lichen secondary metabolites into faster growing organisms. Some examples (Fig. 3) of bioactive compounds produced by lichens include emodin[28, 29] (produced by *Caloplaca ferruginea* and active against *Bacillus brevis*), alectosarmentin[30] (produced by *Alectoria sarmentosa* and active against *S. aureus* and *Mycobacterium smegmatitis*), evernic acid[31] (produced by *Evernia prunastri* and active against *S. aureus*, *Bacillus subtilis* and *B. megaterium*) and Methyl β-orsellinate[32, 33] (produced by *Stereocaulon alpinum* and active against *S. aureus*, *B. subtilis*, *Pseudomonas aeruginosa*, *E. coli* and *Candida albicans*).

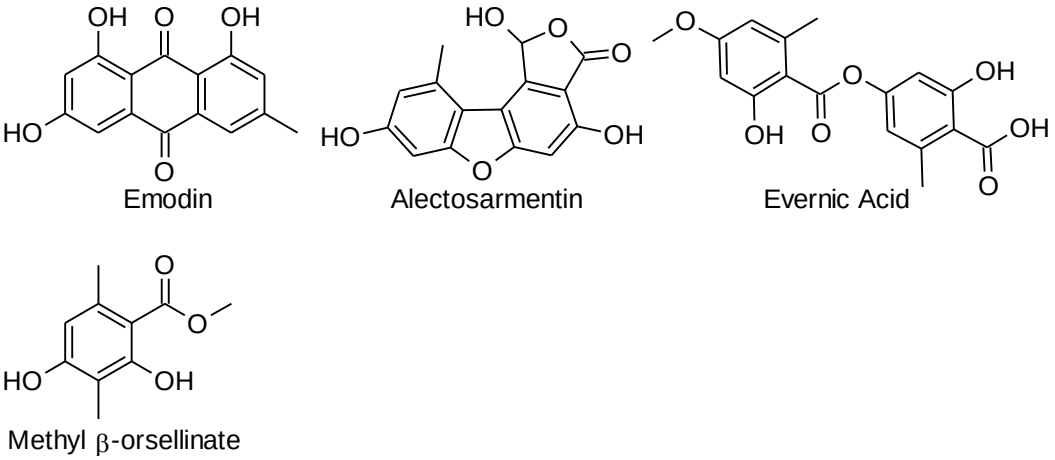


Figure 3: Structures of emodin, alectosarmentin, evernic acid and methyl β-orsellinate

1.7. Activation of Silent Gene Clusters

Polyketides are one of the more common classes of secondary metabolites. As the name implies, they refer to metabolites that originate from polymeric molecules containing multiple ketone functional groups. These polymers arise from enzyme-catalyzed condensation reactions involving smaller units such as acetyl coenzyme A and malonyl coenzyme A. The polyketide chain then undergoes folding and various enzymatic modifications to produce a wide variety of secondary metabolites. Examples of polyketide antibiotics include erythromycin and tetracycline (Fig. 2). The biosynthesis of natural products is regulated by certain genes in the genomes of microorganisms. These genes code for proteins involved in the biosynthesis of the metabolite and the regulation of these genes determines what natural product is produced by the organism.

The genes responsible for polyketide production usually occur in clusters and code for a composite of enzymatic domains including acyl transferase (AT), methyl transferase

(MT), acyl carrier protein (ACP), and enoyl reductase (ER) domains amongst others. These are collectively referred to as polyketide synthase (PKS). Each of these enzyme domains catalyzes one or more steps in the biosynthesis of a secondary metabolite. Another type of enzyme encoded by secondary metabolite-producing genes is the non-ribosomal peptide synthase (NRPS).[34] All secondary metabolite gene clusters in the genome of the organism code for analogues of one or more of these domains or enzymes. The conserved regions in the enzyme are used to identify these gene clusters and the variable regions facilitate the diversity of the secondary metabolite produced by the organism. The presence or absence of one or more of the possible enzymes involved in the biosynthetic pathway would also obviously have a significant impact on the structure of the final product. The presence of numerous silent secondary metabolite gene clusters in the genome of microorganism is a well observed and acknowledged fact.[35] This means that the full spectrum of secondary metabolites produced by the organism cannot be observed unless these genes are activated.

Much study has gone into determining the factors that trigger the activation of these gene clusters and useful clues are often obtained from the conditions of the natural habitat of the organism. Some of these factors include heat, change in pH, nutrition deficiency, the presence of other microorganisms, the presence or absence of certain nutritional components[36, 37] etc. Moreover, each of these factors probably triggers only one of the silent gene clusters out of the many other clusters in the organism. One of the solutions used to address this problem is genetic engineering and involves the incorporation of these silent gene clusters within the genome of a suitable vector. It is

hoped that the genetic machinery of these vector organisms would express the incorporated transgenes and ultimately result in the biosynthesis of novel secondary metabolites.[38] It is also possible to target certain proteins and enzymes that are involved in the regulation of these genes as in the case of *Aspergillus nidulans.*[39]

While this method holds great potential, it is not yet at a level where it can allay the need for more traditional methods in which metabolites are extracted from the fermentation culture of a microorganism. Unexpected challenges involving sequencing of the pertinent gene, selection of appropriate primers, promoters and vectors are often encountered during the process; and even after all these problems are addressed, there is no guarantee that desired proteins would actually be expressed or demonstrate functionality.

Nonetheless, there is still a place for the routine growth of fermentation cultures of various microorganisms; however, intelligent decisions are needed for the choice of organism to culture and/or the conditions to grow the culture. As previously alluded to, altering culture conditions such as the presence or absence of photosynthetic light, presence of other microorganisms etc. can trigger the production of new secondary metabolites. This method has been and is still being explored for a number of microorganisms with some success. The conditions commonly tested first are usually those that mimic the natural habitat of the microorganism. It is reasonable to expect that an organism would have evolved its genome in response to stress factors it encounters in its natural habitat. This makes it highly probable that the silent gene

clusters of the organism can be activated when it experiences stress factors it normally encounters in its natural habitat. It is also possible for ancestral species to evolve certain genetic traits due to stress factors encountered in their habitat. These genetic traits would then be passed down to their offspring even though they no longer experience these stress factors. The accumulation of various genetic traits down several generations could account for the presence of multiple silent gene clusters in distant generations. So the natural habitat of ancestral species of the organism could also play a significant role in determining the most promising culture conditions to test.

1.8. Factors to Consider in the Search for Novel Secondary Metabolites

1.8.1. Importance of Soil Fungi

Another important issue to consider in the search for novel antibiotics is the choice of organism. There are numerous organisms that produce secondary metabolites including bacteria, fungi and plants. Despite research on all these organisms, some of them tend to yield more positive results than others. A literature survey covering over 23,000 bioactive compounds indicated that nearly half of these natural products were of fungal origin.[40] A sizeable fraction of these compounds also originated from bacterial species of the genus, *Streptomyces*. This data suggests that either of these organisms would be good candidates for the search for novel antibiotics.

One category of microorganisms that has constituted the main source from which novel natural products are derived is soil microbes. The soil is very rich in microbial population and diversity. A handful of soil is said to contain billions of microorganisms,[41] and these

organisms are said to be responsible for the vast majority of bioactive compounds that have been discovered so far.[42] *Streptomyces* species, which are responsible for a sizeable fraction of discovered bioactive compounds as mentioned earlier, are soil bacteria. It is possible that some of the reasons why streptomycetes seem so promising are that they have been a prime target for the discovery of novel secondary metabolites,[42] and they are also one of the largest bacterial genera[43] with over 500 identified species.[44] Quite a number of the antibiotics in use today are either derived directly from streptomycetes or are based on compounds isolated from streptomycetes.

Soil fungi have also had their own fair share of attention, especially considering the fact that the first antibiotic, penicillin, was discovered from a fungus (*P. chrysogenum*). Also, some studies have shown that as much as half of soil fungi produce antibiotic compounds.[45] This makes soil fungi very attractive candidates for natural product discovery. Indeed, much effort has gone into screening soil fungi for new lead compounds; and some of the antibiotic drugs in use today are a fruit of those efforts. The group of β-lactam antibiotics known as the cephalosporins is derived from a soil fungus, *Acremonium chrysogenum*. It was first isolated in 1948, just a few years after the mass production of penicillin was well under way.[46]

In their original structure, the cephalosporins were not potent enough to be used as antibiotic agents; so chemical modifications were made in an attempt to increase their potency. This led to the development of five generations of the antibiotic, with each generation featuring a slight alteration in the structure of the base compound.[47]

Antibiotics of the fifth generation of cephalosporins are active against even MRSA. The mechanism of resistance of cephalosporins by MRSA is mediated by the alteration of the target protein.[14] However, side chains in these fifth generation compounds were specifically designed to increase binding affinity with the target protein in MRSA.[48]

1.8.2. Uniqueness of the Habitat

Another angle used to approach the issue of discovering new lead compounds is to search for entirely new bacterial or fungal species (or strains/varieties) that have evolved unique features as a result of evolutionary pressure. It is logical to assume that a new species of an organism would very likely produce a unique spectrum of secondary metabolites. Due to repeated re-isolation and characterization of known secondary metabolites from fungi in commonly studied environments, researchers are now diverting their attention to fungi from less common and more unique ecological niches such as marine and endophytic fungi and the results have justified this decision.[49, 50]

Lichen samples from Wapusk National Park were obtained from a collection curated by Dr. Michelle Piercey-Normore of the department of Biological Sciences at the University of Manitoba. One of the reasons these samples were chosen for our studies was as a result of the uniqueness of the location. Firstly, the Park is a restricted area and as such, has not been extensively explored. It is also known to exhibit extreme weather patterns, with temperature, ranging from -10 °C to 35 °C in the summer, that can plunge a steep 20 °C in less than an hour. The average temperature during the December/ January

winter months is -26 °C, but this can plunge to as low as a frigid -50 °C in the winter. When wind-chill is taken into account, the temperature could get as low as -80 °C.[51]

Lichen organisms are known to produce a variety of interesting secondary metabolites, some of which exhibit antibiotic activity that could potentially influence the growth, development and genetic expression of other microorganisms in the region. Examples include usnic acid and depsides (Fig. 4). As previously indicated, usnic acid is an interesting and well-known lichen secondary metabolite which possesses a wide range of important biological properties. Depsides, on the other hand, are ester-linked polyphenolic compounds with two or more monocyclic aromatic units. These compounds also possess antibiotic, anti-HIV, antioxidant and antiproliferative activities.[52-55] The presence of these secondary metabolites as well as all other factors previously alluded to, contribute to the uniqueness of the ecological niche from which our fungi was isolated; and as mentioned earlier, scientific history has shown that there is a greater possibility of isolating new compounds from fungi found in unique ecological niches compared with those found in extensively explored regions.

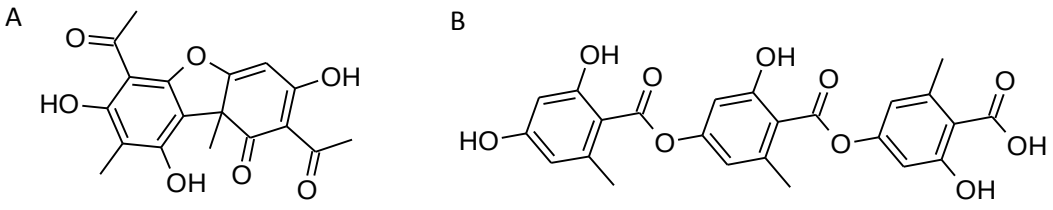


Figure 4: Some secondary metabolites of lichen. A) Usnic acid. B) the depside, gyrophoric acid.

1.9. Summary

The ultimate goal of the Sorensen group is to develop a research program aimed at the discovery of novel natural products and lead compounds. One of the projects involves the identification of the gene cluster involved in the biosynthesis of usnic acid. As mentioned earlier, usnic acid is an important bioactive secondary metabolite of lichens. The end goal of this project is to provide an easy, convenient, economic and efficient method of synthesizing usnic acid and its analogues by feeding the enzyme with the appropriate substrates. It is hoped that some of these analogues would show improved bioactivity and less toxicity. There's also a synthetic project which aims to develop a library of compounds that would be tested for inhibitory activity against Glutamate-1-semialdehyde mutase (GSAM), a bacterial enzyme involved in the critical step of vitamin B_{12} synthesis in bacteria. This pathway is not present in humans and so is an excellent target for antibiotic drugs.

In this project, we attempt to isolate novel fungal secondary metabolites from a northern Manitoba fungus. This is accomplished by bioassay-guided fractionation of the ethyl acetate extract of the fermentation culture of the fungus. Characterization and identification of the pure compounds were accomplished by various spectroscopic techniques. This project as well as the previously mentioned projects takes advantage of various methods employed in the search for novel antibiotics; and so, they constitute a very comprehensive approach towards the discovery of novel lead compounds.

2. RESULTS AND DISCUSSION

2.1. Isolation of Fungus, Fermentation Culture and Extraction

One of the objectives of this work was to develop a method for the isolation, purification and characterization of antibiotic secondary metabolites from fungal extracts. The fungi used were obtained from the underlying soil of lichen samples. Several soil samples were recovered from underlying soil of the following lichens: *Peltigera didactyla*, *Protopannaria pezizoides*, *Pertusaria atra* Lynge, *Pertusaria coriacea* (Th. Fr.) Th. Fr., *Cladonia pocillum* (Ach.) Grognot, *Cladonia sulphurina* (Michaux) Fr., and *Peltigera rufescens* (Weiss) Humb. A total of five potato dextrose agar (PDA) media plates were prepared for each soil sample. The antibiotic streptomycin was included in the medium to prevent the growth of bacteria and ensure only fungal growth. The first plate was inoculated with the untreated soil. This step safeguards against the complete loss of potentially important fungi in the subsequent step discussed next. The soil sample was then surface-sterilized with 1 % bleach in other to eliminate any potential fungal contaminants from the air or any other external source. Cultures of the surface-sterilized soil samples were then prepared in triplicate (i.e. the next three media plates were inoculated with the sterilized soil sample). The last plate was a control plate (PDA plate without inoculation with soil) which was prepared to ensure that the fungal colonies growing on the other plates are from the soil samples and not just a contamination from a lab source.

On incubating the PDA plates at 30 °C for seven days, a large variety of fungal colonies was observed. Monospore cultures of a single organism were formed by subculturing selected fungal colonies. Essentially, a small portion of the agar with the desired fungal colony is excised from the medium and used to inoculate fresh PDA plates. The plates are also incubated at 30 °C for seven days resulting in the growth of the monospore culture of the fungus. Fermentation cultures were prepared by inoculating potato dextrose broth (PDB) liquid medium with the isolated fungus. The liquid medium is most suitable for large scale growth and recovery of the secondary metabolites produced by the fungus.

After a week of growth, the culture was filtered to separate the fungal mycelium from the medium which contains the secondary metabolites produced by the fungus. The medium was subsequently extracted twice with half volume ethyl acetate. Ethyl acetate is a polar organic solvent that is not miscible with water and so, can capture a broad range of organic molecules in the aqueous medium including the very polar ones. The solvent in the recovered organic layer was then evaporated off to afford the neutral crude extract. Following the initial extraction, the aqueous layer was acidified with HCl to approximately pH 2. This step is important because some organic compounds such as organic acids, which would otherwise be in their charged state and thus in the aqueous layer, can be protonated, become neutral and move into the organic layer. The organic layer was then dried and the solvent was evaporated to afford the acidic crude extract. Basic nitrogen-containing secondary metabolites also known as alkaloids are mostly

found in plants and to a lesser extent in other microorganisms,[34] so a basic extraction was omitted.

2.2. General Process of Purification, Analysis and Characterization

Preliminary tests carried out on the crude extracts include NMR, TLC and bioassay. The NMR was done as a reference for fractions obtained from the subsequent flash chromatography carried out on the crude extracts. For example, if we suspect a fraction contains contamination not present in the original extract, we might be able to allay or confirm our suspicion by comparing the NMR spectra of the fraction with the NMR spectra of the crude extract. However, it is also possible for other compounds that are present in the crude extract to be relatively more concentrated than those present in the fraction such that the NMR signals of the crude extract could possibly overshadow those of our fraction. The TLC was done to determine the approximate number of compounds in the extract as well as the optimum solvent system to use for the flash chromatography. The bioassay tested for the presence of bioactive compounds in the extracts. This was accomplished by inoculating an *E. coli* containing LB agar medium with a 10 µL aliquot of the test sample. The concentration used for the crude extract was 10 mg/mL while that used for the purer fractions was 1 mg/mL. This is a result of the fact that the concentration of an active compound in a 1 mg/mL solution of the crude extract is less than a 1 mg/mL solution of the pure compound as a result of the presence of other compounds in the crude extract. The bioassay tests were limited to just *E. coli* cells to serve as a rapid initial screen.

Extracts from several soil samples were obtained and tested for bioactivity. This was accomplished by applying a 10 µL aliquot of the sample to be tested to a Lysogeny broth (LB) agar medium mixed with *E. coli* cells. Usnic acid was also applied to one of the LB agar plates and used as a standard. Some of the extracts showed bioactivity only slightly less than that of usnic acid while others displayed far less bioactivity. The zone of inhibition is a circular zone of limited bacterial growth that extends from the spot in which a bioactive sample was applied, surrounded by a lawn of regular bacterial growth. The diameters of the zones of inhibition of all the samples used were roughly similar at about 0.9 cm. However, there were significant differences in the degree of bacterial growth within the zones of inhibition. The degree of bacterial growth was judged by the transparency of the medium within the zone of inhibition with usnic acid as the standard. Some samples had relatively less bacterial growth within their zones of inhibition than others implying a stronger antibiotic action. These samples were further analyzed by NMR spectroscopy to get an idea of the kinds of molecules that might be present in the extract. Eventually, we narrowed down our focus to just one extract which was obtained from fungus in the soil underneath the lichen, *P. didactyla*. This sample was chosen because it gave one of the more promising bioassay results (i.e. it produced one of the clearest zones of inhibition). *P. didactyla* is known for its production of the class of bioactive compounds known as depsides.[56] *P. didactyla* var *extenuata*, a variety of the *P. didactyla* species, is widespread in Canada and known to produce the depsides, methyl gyrophorate and gyrophoric acid[57] (Fig. 4).

2.3. Identification of the Fungus

2.3.1. General Background

DNA barcoding is an emerging method used in identifying and distinguishing between species of organisms. It involves the use of a standardized sequence of DNA to identify each species and primers that are applicable to the broadest taxonomic group possible. Ideally, each standardized sequence should be constant and unique to one species. Also, the interspecific variation of this sequence must be greater than the intraspecific variations.[58] A conserved region of fungal genome used as a DNA barcode in identifying fungal species is the internal transcribed spacer region.[59] The eukaryotic ribosomal genetic region is polycistronic and consists of the genes for the 18S ribosomal RNA (rRNA), 5.8S rRNA and the 28S rRNA[60] (read from 5' to 3'). These genes are separated by a sequence of DNA known as the internal transcribed spacer region (ITS). The first ITS sequence (commonly called ITS1) is located between the 18S rRNA and the 5.8S rRNA, while the second ITS sequence (ITS2) is located between the 5.8S rRNA and the 28S rRNA. The sequence of DNA including the ITS1, 5.8S rRNA and the ITS2 sequences is generally referred to as the ITS region.

2.3.2. Extraction and Amplification of the fungal DNA

Identifying a fungus is a multi-step process beginning with the isolation of its genomic DNA. The fungal mycelium in this experiment were first frozen and crushed in liquid nitrogen to facilitate lysis of the fungal cells. Lysis buffer containing zymolase enzyme was used to break down the fungal cell wall, while digestion solution and proteinase K was used to digest cell components such as protein. RNase A digests the RNA in the cell

so it doesn't contaminate the DNA. The lysate was then transferred to a purification column which selectively binds DNA with high ionic strength. A preliminary wash removed the digested materials and other impurities, while an elution buffer lowered the ionic state of the DNA causing it to elute into a clean Eppendorf tube.

Amplification of the DNA was accomplished by the polymerase chain reaction (PCR). A DNA polymerase enzyme for replication, deoxyribonucleoside triphosphates (dNTP) as DNA building blocks and ITS sequence-specific primers all comprise components of an *in vitro* replication system. The primers, 1780-5' (SSU rDNA) (5'-CTGCGGAAGGATCATTGATTC-3')[61] and ITS2-kL-3' (ITS rDNA) (5'-TGCTTAAGTTCAGCGGGTA-3')[62] were used for the replication in the forward and reverse direction, respectively. These components were suspended in a suitable buffer and incubated in a PCR thermal cycler. The function of this equipment is to vary the incubation temperature to complement the various steps in DNA replication. It started with a very high temperature of 98 °C to denature the DNA (i.e. convert the DNA from the double-stranded to the single-stranded form). Then it lowered the temperature to 56 °C to allow the DNA primers to bind to complementing sequences on the template strand. The temperature was then increased to 72 °C which is half-way between both initial temperatures and optimal for DNA elongation. If the temperature is too high, the DNA would get denatured and if it is too low, complementary DNA strands might attempt to hybridize and therefore, interfere with the elongation process. This cycle was repeated multiple times with the DNA concentration increasing each time. After the final cycle, the amplified DNA was stored at 8 °C.

2.3.3. Purification of DNA

It was necessary to purify the amplified DNA as it was still in a solution that contains the replication enzyme, random strands of DNA, excess dNTP's and excess primers. To accomplish this task, we employed the technique of electrophoresis. The DNA was first precipitated by adding 5 M NaCl and 100 % ethanol to the solution. The precipitated DNA was then recovered by centrifugation. The DNA pellet was then resuspended in sterile milliQ H_2O and bromophenol blue was added. The bromophenol blue acts as a colour marker to monitor the progress of the electrophoresis process. The suspension was then added to the agarose gel containing ethidium bromide. Ethidium bromide intercalates with DNA and fluoresces under UV and so, aids in visualizing the DNA after the electrophoresis. A voltage applied to the gel polarizes it, causing the negatively charged DNA molecule to migrate to the positively charged anode of the gel.

The agarose gel contains small pores which facilitate the separation of DNA molecules based on size. Essentially, the smaller DNA molecule moves through the pores of the gel easily while the larger molecules collide with the walls of the gel and so their movements are more frequently interrupted. Through this process, we were able to purify the desired DNA fragment, separating it from other strands which were not of the same size. The ITS DNA was visualized by UV and excised from the gel. The gel, along with the DNA was dissolved according to the Wizard SV Gel and PCR Clean-Up protocol.[63] The resulting solution was added to a column which bound the DNA while the other impurities were washed out. The DNA was eluted from the column by addition of water.

A good quality DNA has an A_{260}/ A_{280} absorbance ratio of 1.7–2.0. This is because other molecules such as RNA and guanidine absorb at 260 nm, while the aromatic amino acids of protein absorb at 280 nm. Deviation from the stated optimal ratio would indicate the presence of contaminants which have altered the absorbance of DNA at 260 nm and 280 nm. The purified DNA was of good quality with an A_{260}/ A_{280} ratio of 1.8. It was sent to GENEWIZ (New Jersey, USA) for sequencing of the nucleotide bases.

2.3.4. Determination of Fungal Identity

The result of the sequencing was a chromatogram file which was interpreted by Chromas Lite v 2.1.1 software (Technelysium Pty Limited, South Brisbane, Australia) to produce the actual sequence. GenBank, built and distributed by the National Center for Biotechnology Information (NCBI), is a comprehensive online database that contains publicly available nucleotide sequences for over 260,000 species.[64] Basic local alignment search tool, commonly known as BLAST (also developed by NCBI), is a program that compares query sequences with database sequences for similarities.[65] The results of a nucleotide BLAST search are reported beginning from the most similar sequence. Our sequence was entered for a nucleotide BLAST search.[66] Based on the search result, the closest match for our sequence was *Alternaria tenuissima* with the accession number EF364096.1. The results reported a > 99% sequence match of our sequence with the ITS region of *Alternaria tenuissima*.

2.4. Isolation of Deoxyphomalone

Very polar organic compounds usually get stuck on regular silica columns. To avoid this problem, we separated the very polar compounds of the initial acidic extract from the rest of the constituent compounds. This was accomplished by adding dichloromethane (DCM) to the acidic extract and then filtering off the soluble portion. Solid plate bioassay (see section 2.2) showed that this DCM-soluble fraction was active while the insoluble fraction was inactive. The DCM-soluble fraction was subjected to further fractionation based on solubility in 1:1 Hexanes: DCM. Both the soluble and the insoluble fractions showed slight bioactivity on the solid plate assay. Flash chromatography was applied to the soluble fraction using a MeOH/DCM solvent gradient (2.5 %, 5 %, 10 %, 20 % and a 100 % MeOH wash) resulting in an overall total of fourteen fractions. The first of the fourteen fractions was subjected to further purification by flash chromatography, using a solvent gradient beginning from pure DCM to a 100 % MeOH wash. A total of 13 sub-fractions were collected. Sub-fraction 5 was identified as deoxyphomalone (**1**) based on spectral data (Fig. 5).

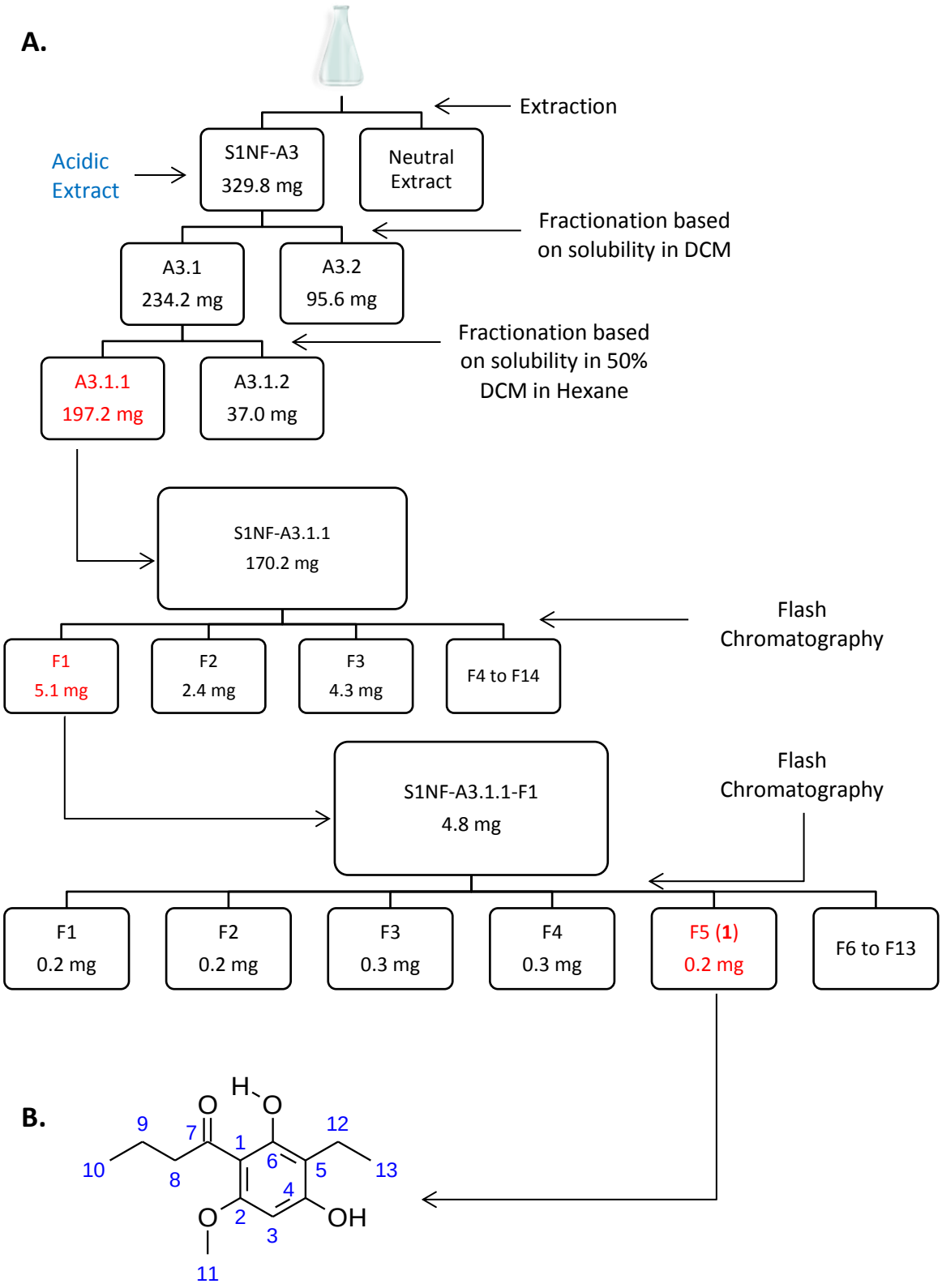


Figure 5: A) Isolation of deoxyphomalone (**1**). B) Structure of deoxyphomalone

The mass spectrum exhibited a molecular ion at m/z 238.2 that was consistent with the molecular formula of $C_{13}H_{18}O_4$. The IR spectrum indicated the presence of hydroxyl (3227 to 3567 cm^{-1}) and carbonyl (1613 cm^{-1}) groups. The uncharacteristic low frequency of the carbonyl stretch is most likely due to H-bonding with the hydroxyl group as well as conjugation with the aromatic ring. Two methyl [δ_H 0.98 ppm (3H, t) and 1.12 ppm (3H, t)], three methylene [δ_H 1.69 ppm (2H, tq), 2.59 ppm (2H, q) and 2.96 ppm (2H, t)], a methoxyl [δ_H 3.84 ppm (3H, s)], an aromatic [δ_H 5.90 ppm (1H, s)] and a hydroxyl [δ_H 14.32 ppm (1H, s)] proton signal were observed in the ^{1}H NMR and are consistent with structure **1**.

The chemical shift of aromatic protons is usually between 6 and 8 ppm; however, the aromatic proton in **1** had an unusually low chemical shift (5.9 ppm) as a result of the resonance effect of the electron donating substituents on the aromatic ring. The downfield shift of the hydroxyl proton is additional confirmation of intra-molecular hydrogen bonding. This is consistent with the hydroxyl group being attached to the β-carbon (with respect to the ketone) as this is the optimum position for hydrogen bonding. The ^{13}C NMR displayed resonances indicative of alkyl carbon atoms (δ_C 13.6, 14.2, 15.7, 18.4, and 46.5 ppm), a methoxy group (δ_C 55.6 ppm), olefinic carbons (δ_C 90.3, 106.0, 109.7 ppm), O-substituted aromatic carbons (δ_C 159.7, 161.3, and 165.1 ppm) and a ketone (206.3 ppm).

Two-dimensional NMR spectroscopy (2-D NMR) experiments are often carried out to obtain more information on the structure of a compound. The proton-proton

correlation spectroscopy experiment (^{1}H-^{1}H COSY) is used to detect the coupling between protons on adjacent carbons. Heteronuclear single-quantum correlation (HSQC) spectroscopy is a 2-D NMR method that's used to detect correlation between two different nuclei which are separated by a single bond. In this experiment, it was used to detect the proton-carbon correlations. Another useful 2-D NMR method is the Heteronuclear multiple-bond correlation (HMBC) spectroscopy. This method detects long range correlations between two different nuclei separated by two to four bonds; it was used to detect long range proton-carbon correlations in the isolated compounds.

Correlations between the H-9 and H-10 protons as well as between the H-9 and H-8 protons were established by ^{1}H-^{1}H COSY experiments, which also confirmed coupling between the H-12 and H-13 protons. All proton – carbon connectivities were confirmed by HSQC. In the HMBC spectrum, long-range correlations between H-8 and C7, and H-9 and C7 were observed. This confirms the proximity of the keto group (C7) to the C8-C10 alkyl chain. The HMBC spectrum also showed long-range coupling involving H-12 and several carbons, C4, C5 and C6. Long range coupling was also evident between H-13 and C5 as well as between H-3 and C1, C4 and C5 (Fig. 6). Moreover, the spectral data obtained in this experiment were identical to those reported in the literature.[67] These observations are fully consistent with the proposed structure of **1**.

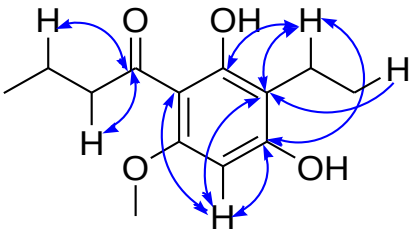
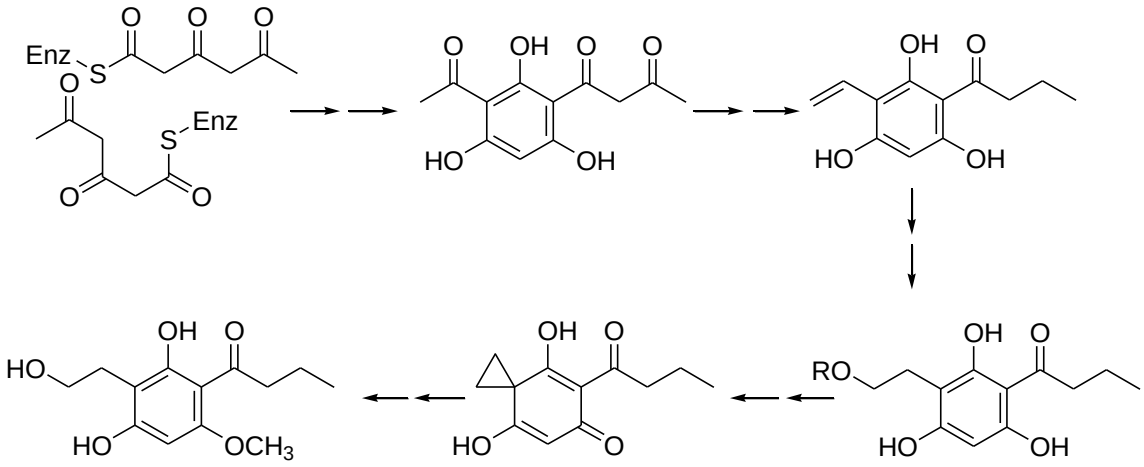


Figure 6: Selected HMBC correlations for deoxyphomalone

The first and only reported isolation of Deoxyphomalone (**1**) (Fig. 5) was in 1994 and it was isolated from *Phoma etheridgei*, an endophytic fungus.[67] It was isolated alongside phomalone (from which its name is derived), an anti-fungal metabolite from the same fungus. The structure of phomalone is very similar to that of deoxyphomalone (**1**) except that it possesses an extra hydroxyl group at the end of the ethyl substituent of the ring. Deoxyphomalone (**1**) was isolated as a white amorphous solid and demonstrated slight antibiotic activity against *E. coli* cells.

The biosynthesis of **1** can be inferred from that of phomalone. Based on labeling experiments, phomalone is suggested to originate from two separate preformed polyketides.[67] Although a biosynthetic pathway involving two preformed triketide units (3 + 3) is proposed in the literature (Scheme 1), the 4 + 2 (tetraketide and diketide) and 5 + 1 pathways are also considered to be possible.[67] Two-chain pathways have been established in only a limited number of cases,[68] therefore these pathways represent unusual routes in fungal polyketide biosynthesis.



Scheme 1: Proposed biosynthesis of phomalone

2.5. Isolation of Dimethyl 4-methyl-2,6-pyridinedicarboxylate

Of the fourteen fractions from the acidic extract described in section 2.4, fraction 7 was separated by flash chromatography using a Methanol-DCM solvent system (5 %, 10 % and 100 % MeOH) and produced a total of 7 sub-fractions. Based on spectral data, sub-fraction 3 was identified as dimethyl 4-methyl-2,6-pyridinedicarboxylate (**2**) (Fig. 7).

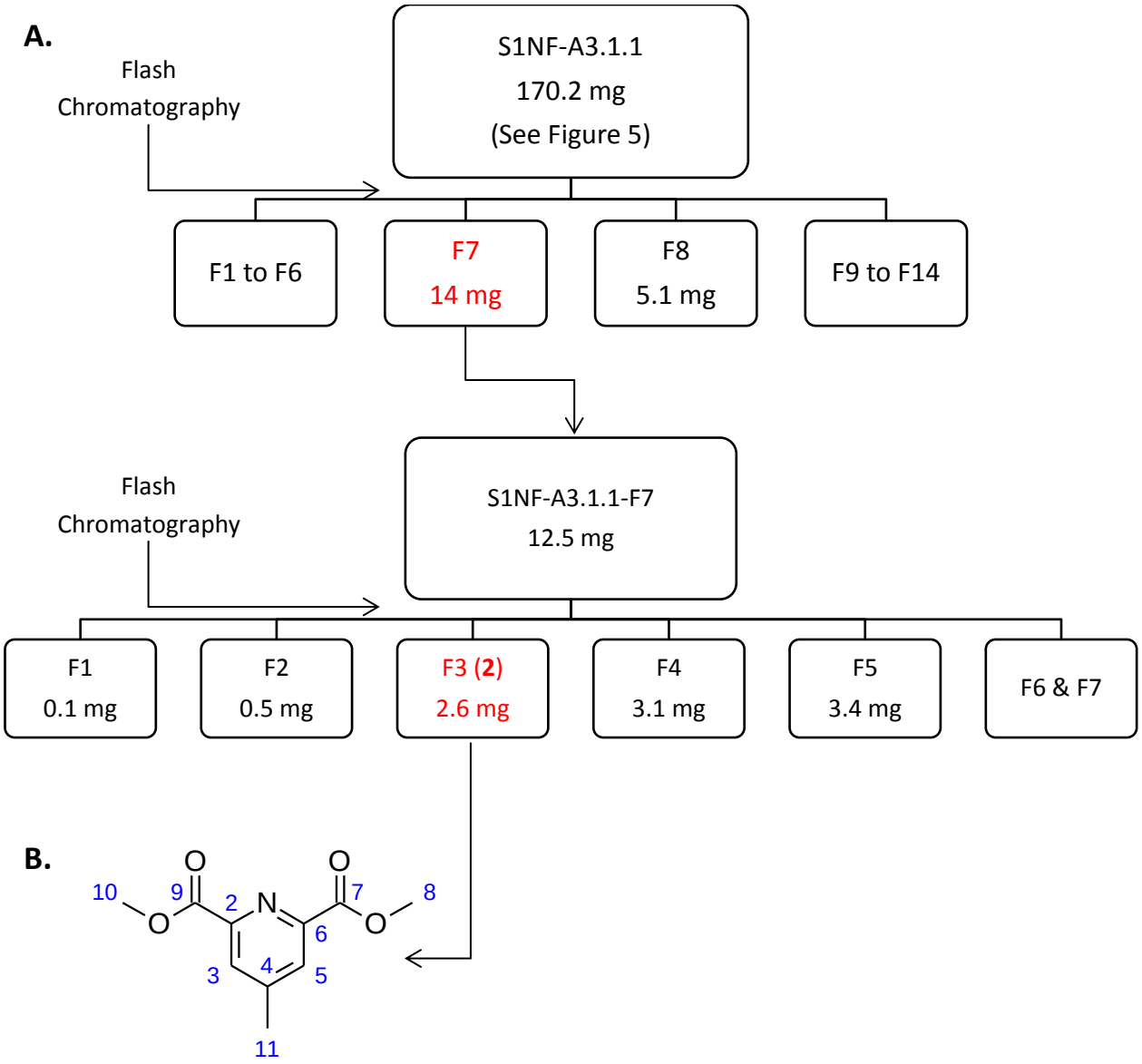


Figure 7: A) Isolation of dimethyl 4-methyl-2,6-pyridinedicarboxylate (**2**). B) Structure of dimethyl 4-methyl-2,6-pyridinedicarboxylate.

The mass of the molecule was deduced by electrospray ionization mass spectrometry (ESI-MS) with the parent peak occurring at m/z 232.2 and another at m/z 210.2. Both peaks represent the sodium $[M+Na]^+$ and the proton adducts $[M+H]^+$, respectively. This result implies a mass of 209.2 $gmol^{-1}$ and is consistent with the molecular formula, $C_{10}H_{11}O_4N$. The IR confirmed the presence of a carbonyl and a carboxylic acid functional group. The ^1H NMR was very simple with only three singlets: an aromatic signal [δ_H 8.15 ppm (2H, s)], a methoxy signal [δ_H 4.02 ppm (6H, s)] and a methyl signal [δ_H 2.53 ppm (3H, s)]. The ^{13}C NMR was also relatively simple, displaying 4 sp^2 carbon signals (δ_H 166.4, 152.5, 149.4 and 129.8 ppm), a methoxy carbon peak (δ_H 53.3 ppm) and a methyl carbon signal (δ_H 21.0 ppm).

The HSQC experiment established the expected proton-carbon direct connectivities by displaying a signal at the intersection of the chemical shifts of the proton and the carbon it is connected to in the spectrum. The HMBC experiment also helped to confirm the carbon skeleton. A long range correlation between the aromatic protons and the benzylic methyl carbon was observed. There was also long-range correlation between the methoxy protons and the carbonyl carbon as well as between the methyl protons and C3, C4 and C5 (Fig. 8). These data are all consistent with the proposed structure of the molecule.

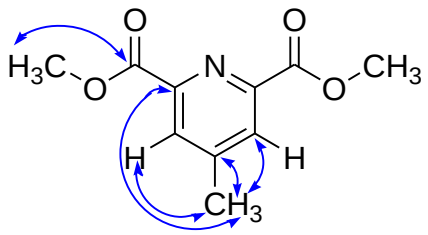


Figure 8: Selected HMBC correlations for dimethyl 4-methyl-2,6-pyridinedicarboxylate.

It was observed that one of the more polar fractions also contained peaks identical in chemical shift and multiplicity to those of compound **2**; however, while the integration for all other signals were exactly the same, that of the methyl ester proton was less than 6 but greater than 3. This suggests a mixture of compounds with single and double esterification. Also, the IR spectrum displayed two carbonyl peaks (1728 cm^{-1} and 1718 cm^{-1}) while the region between 3100 cm^{-1} and 3600 cm^{-1} hinted at the presence of a carboxylic acid functional group. Moreover our compound was only present in the acidic extract, suggesting that it might have originally been in the form of a dicarboxylic acid and perhaps reacted with a solvent such as methanol (which was used occasionally as a chromatography solvent) to yield the dimethoxy derivative. It is, of course, entirely possible that there were no side reactions and all observed metabolites originated from the fungus. Furthermore, the weak hydroxyl stretch (3290 to 3509 cm^{-1}) observed in the IR spectrum might have been as a result of contamination from water which absorbs in the same region; further examination will be needed to resolve this issue.

Dimethyl 4-methyl-2,6-pyridinedicarboxylate (**2**), a derivative of dipicolinic acid, was isolated as a white amorphous solid. So far, there does not appear to be any previous report on the production of this compound as a natural product. On the other hand, its

analogue (dipicolinic acid), is a well-known product of bacterial endospores and has been implicated in conferring heat resistance to the endospores.[69] Moreover, dipicolinic acid (known to possess insecticidal properties[70]) has also been reported to be a product of fungal origin[71] and possesses antibacterial properties.[72] Our 4-methyl-2,6-dimethyl ester derivative, however, did not show any significant bioactivity against *E. coli* cells. It is unlikely that our compound is of bacterial origin (i.e. contamination) as that would imply endospore formation and germination of a bacterial contaminant, both of which require unique conditions to occur. Also, compound **2** does not appear to have been reported previously as a fungal isolate.

2.6. Isolation of Stemphyperylenol

The neutral extract of *A. tenuissima* was separated by preparative TLC into two major fractions. The fractions were recovered from silica gel using 10 % Methanol in DCM. The less polar fraction was further separated by preparative TLC using DCM as solvent to yield a total of 7 fractions. Fraction 7 was further separated by flash chromatography using a solvent gradient of Methanol-DCM (3 %, 5 %, 10 % and 100 % of methanol) to produce a total of 9 fractions. Based on spectral data, fraction 5 was identified as stemphyperylenol (**3**).

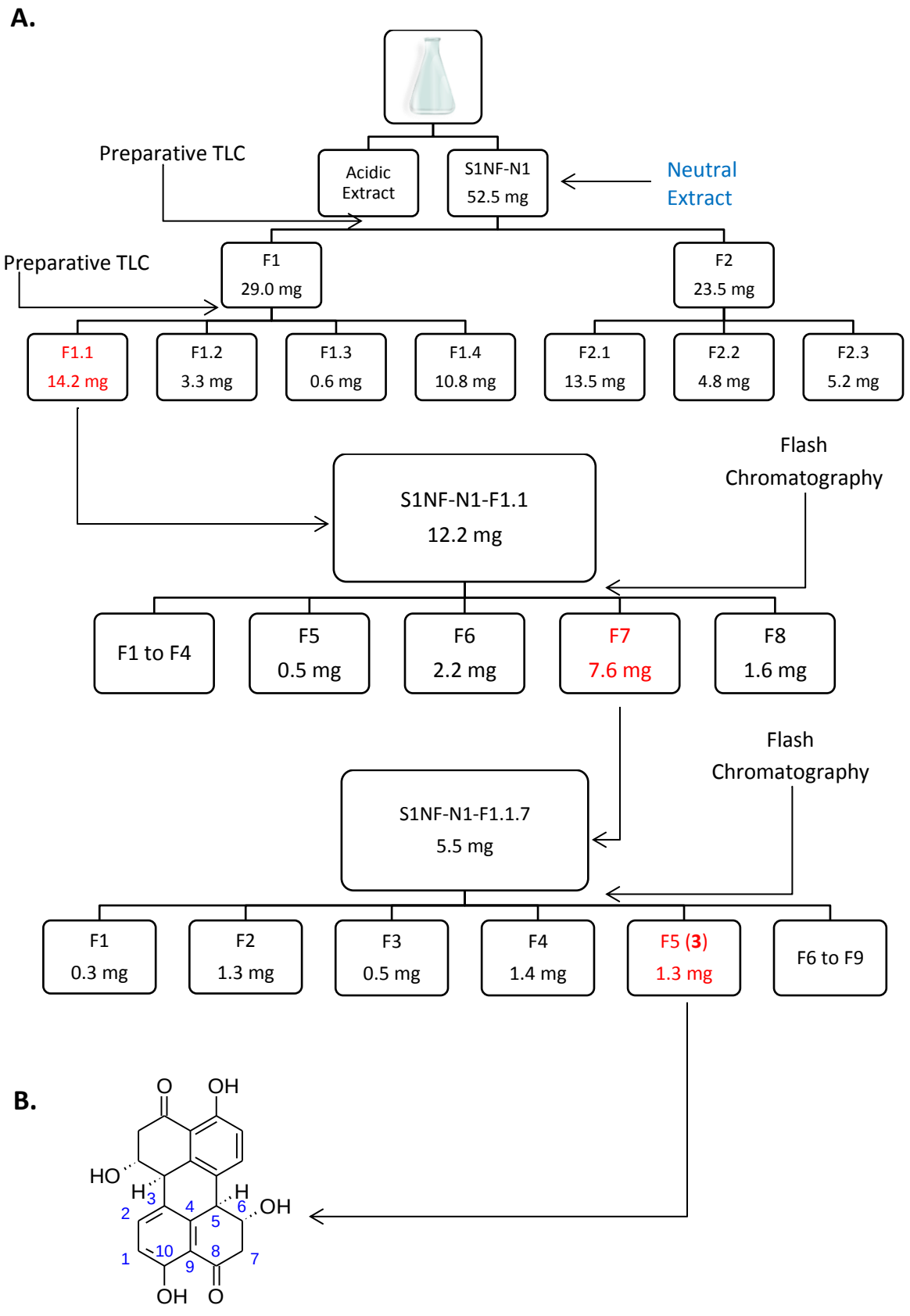


Figure 9: A) Isolation of stemphyperylenol (**3**). B) Structure of stemphyperylenol.

Compound **3** (Fig. 9) was isolated as a white solid and the observed molecular ion $[M+H]^+$ at m/z 353.1 is consistent with the molecular formula, $C_{20}H_{16}O_6$. The IR spectrum displayed signals in the hydroxyl (3193 to 3545 cm^{-1}) and the carbonyl group region (1643 cm^{-1}), confirming the presence of these groups in the compound. Again, the frequency of the carbonyl signal is slightly lowered, implying the presence of hydrogen bonding and conjugation with the ring. The ^1H NMR exhibited signals which are consistent with the proton atoms expected for stemphyperylenol. This includes two methylene [δ_H 3.04 ppm (2H, dd, *J* = 15.5, 4.5 Hz, H_a-7) and 3.10 ppm (2H, dd, *J* = 15.5, 12.3 Hz, H_b-7)], two aliphatic methine [δ_H 3.72 ppm (2H, d, J = 9.3 Hz, H-5), 4.66 ppm (2H, ddd, J = 12.3, 9.3, 4.5 Hz, H-6)] and two aromatic methine proton peaks [δ_H 6.84 ppm (2H, d, *J* = 8.8 Hz, H-1), 8.06 ppm (2H, d, *J* = 8.8 Hz, H-2)]. The methylene protons, H_a-7 and H_b-7, are non-equivalent protons since they are on a carbon adjacent to a stereogenic centre in stemphyperylenol. This explains the presence of two doublets of doublets signals observed in the ^1H NMR spectrum and is also consistent with the representation of H-6 as a doublet of doublet of doublet since it couples differently with H_a-7 and H_b-7 as well as with H-5.

The relative stereochemistry at positions 5 and 6 can be inferred from the vicinal J-coupling constants. The coupling constants, $^3J_{6\text{-}5}$ and $^3J_{6\text{-}7b}$, were both large, implying a diaxial (trans) relationship between H-6 and H-5 as well as H-6 and H_b-7. It is also relevant to note that long range coupling was observed between the H-5 protons as they are not magnetically equivalent and so do not follow first order rules. Long range

coupling was also observed in the signal of the H-6 protons. These extra couplings resulted in a more complicated ^{1}H NMR signal for these protons.

The absolute configuration of both stereogenic centers in **3** was established as (R) in a previous work.[73] Further experiments were carried out to ascertain the absolute configuration of compound **3**. The specific rotation ([α]) of compound **3** was +461.5° which was comparable with the literature value of +415°.[73] Therefore, compound **3** most likely have the same (R) configuration at positions 5 and 6 as reported in the literature.

In the ^{13}C NMR, there were three deshielded sp^3 carbons (δ_C 46.3, 48.0, 68.6 ppm), six aromatic carbons (δ_C 116.0, 116.2, 131.2, 135.6, 144.1 and 161.4 ppm) and a ketone group (δ_C 204.3 ppm). The deshielded sp^3 carbon peaks are consistent with the α-carbon methylene, benzylic methine and oxymethine groups present in **3**. The aromatic signal at 161.4 ppm is consistent with hydroxyl-substituted aromatic carbon; the low aromatic signals (116.0 and 116.2 ppm) are consistent with the aromatic carbon which are shielded by the resonance effect with respect to the electron donating hydroxyl group; the rest of the aromatic carbon signals have higher chemical shifts as a result of deshielding by the electron withdrawing carbonyl group through resonance effect. Further confirmation of structure was obtained by 2D NMR experiments including ^{1}H-^{1}H COSY, HSQC and HMBC (Fig. 10). The HSQC experiment established all direct proton – carbon connectivities while the ^{1}H-^{1}H COSY showed a correlation between H-6 and H-7, H-6 and H-5, and between the adjacent aromatic protons. Further confirmation of the

proposed compound was provided by the HMBC which displayed long-range correlation of the H-7 protons with C5 and C8 (Fig. 10). These conclusions are consistent with previously reported data.[73]

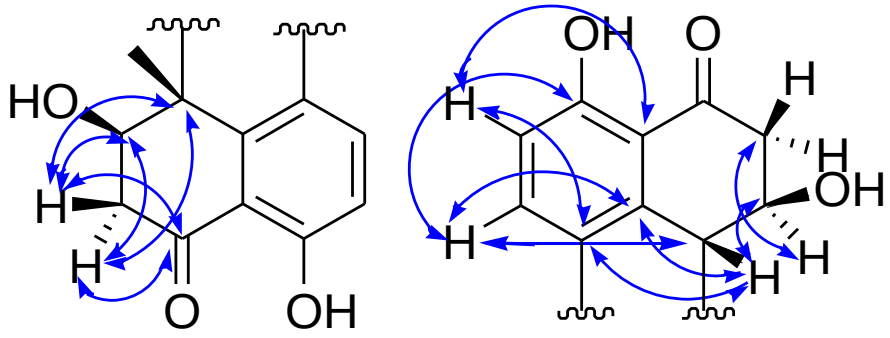
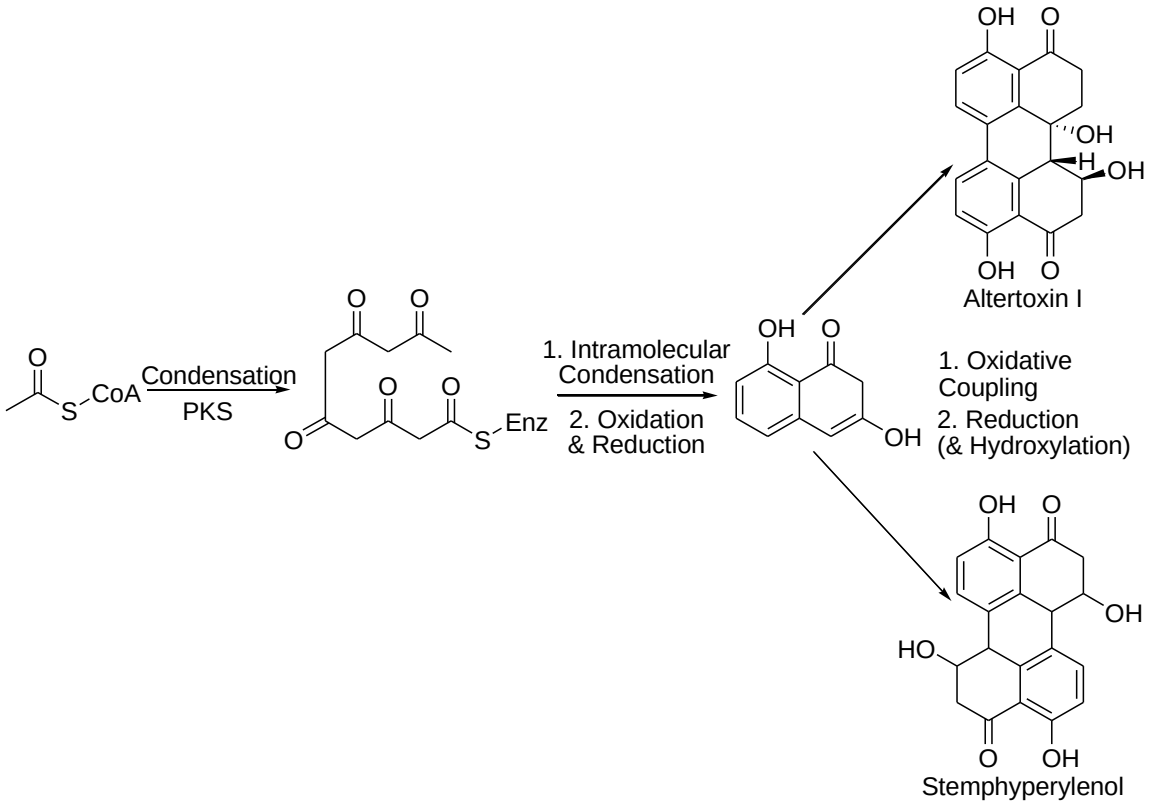


Figure 10: Selected HMBC correlation for stemphyperylenol (**3**).

Stemphyperylenol (**3**) was first isolated from *Stemphyllium botryosum* in 1986,[73] and has been isolated from other fungal species since then.[49] Although it showed relatively low bioactivity against *E. coli* cells, compound **3** has been reported to be active against other bacterial organisms including *S. aureus*, multi-drug resistant *P. aeruginosa*, *C. albicans* etc.[74] Of note is its activity against *Sarcina ventriculi*[74] with a minimum inhibitory concentration of 3.12 $\mu g ml^{-1}$, which is less than that of ampicillin, 12.5 $\mu g ml^{-1}$.

The biosynthesis of polyketides (see section 1.7) often begins with acetate starter units which undergo a series of condensation reactions. The resulting polyketide chain is then folded with the aid of enzymatic catalysis and undergoes intramolecular condensation reactions leading to cyclization of the polyketide. Sometimes, two or more monomeric units of this cyclic product can be combined by oxidative coupling. The biosynthesis of stemphyperylenol (**3**) likely follows a similar route except for an unusual oxidative

coupling step. The biosynthesis of reduced perylenequinones (Scheme 2), like altertoxin I, has been proposed previously and confirmed by ^{13}C labeling experiments.[75] All other reduced perylenequinones undergo the so-called head-to-head coupling; however, stemphyperylenol (**3**) undergoes a head-to-tail coupling.



Scheme 2: Proposed biosynthetic pathways for altertoxin I (head-to-head coupling) and Stemphyperylenol (head-to-tail coupling).

2.7. Isolation of N-Methyl Pyrrolidinone

The neutral extract of *A. tenuissima* was separated by preparative TLC into two major fractions. The fractions were recovered from the silica gel using 10 % Methanol in DCM. The less polar fraction was further separated by preparative TLC using DCM as solvent to yield a total of 7 fractions. Fraction 7 was further separated by flash chromatography

using a solvent gradient of Methanol-DCM (3 %, 5 %, 10 % and 100 % of methanol) to produce a total of 9 fractions and fraction 2 was further separated by flash chromatography to yield 8 fractions (Fig. 11). Spectral data confirmed the identity of fraction 4 as N-Methyl pyrrolidinone (**4**).

The IR spectra displayed the expected carbonyl. The ^{1}H NMR was relatively straightforward with methylene proton peaks [δ_H 3.38 ppm (2H, t, J = 7.2 Hz), δ_H 2.38 ppm (2H, t, J = 8.1 Hz) and δ_H 2.02 ppm (2H, tt, J = 7.5 Hz)] and a methyl proton peak [δ_H 2.84 ppm (3H, s)]. The ^{13}C NMR displayed an amide carbon peak (175.3 ppm), an N-Methyl carbon peak (49.6 ppm) and sp^3 carbon peaks (30.8, 29.7 and 17.8 ppm). The ^{1}H-^{1}H COSY, HSQC and HMBC spectra were all consistent with our proposed structure.

N-Methyl pyrrolidinone (**4**) is a very commonly used solvent in organic chemistry labs and is commercially produced; however, it has only been isolated once from a marine sponge.[76] This solvent is not used in our lab and so is unlikely to have originated from a contamination of the original extract. N-Methyl pyrrolidinone (**4**) did not show any promising bioactivity against *E. coli* cells.

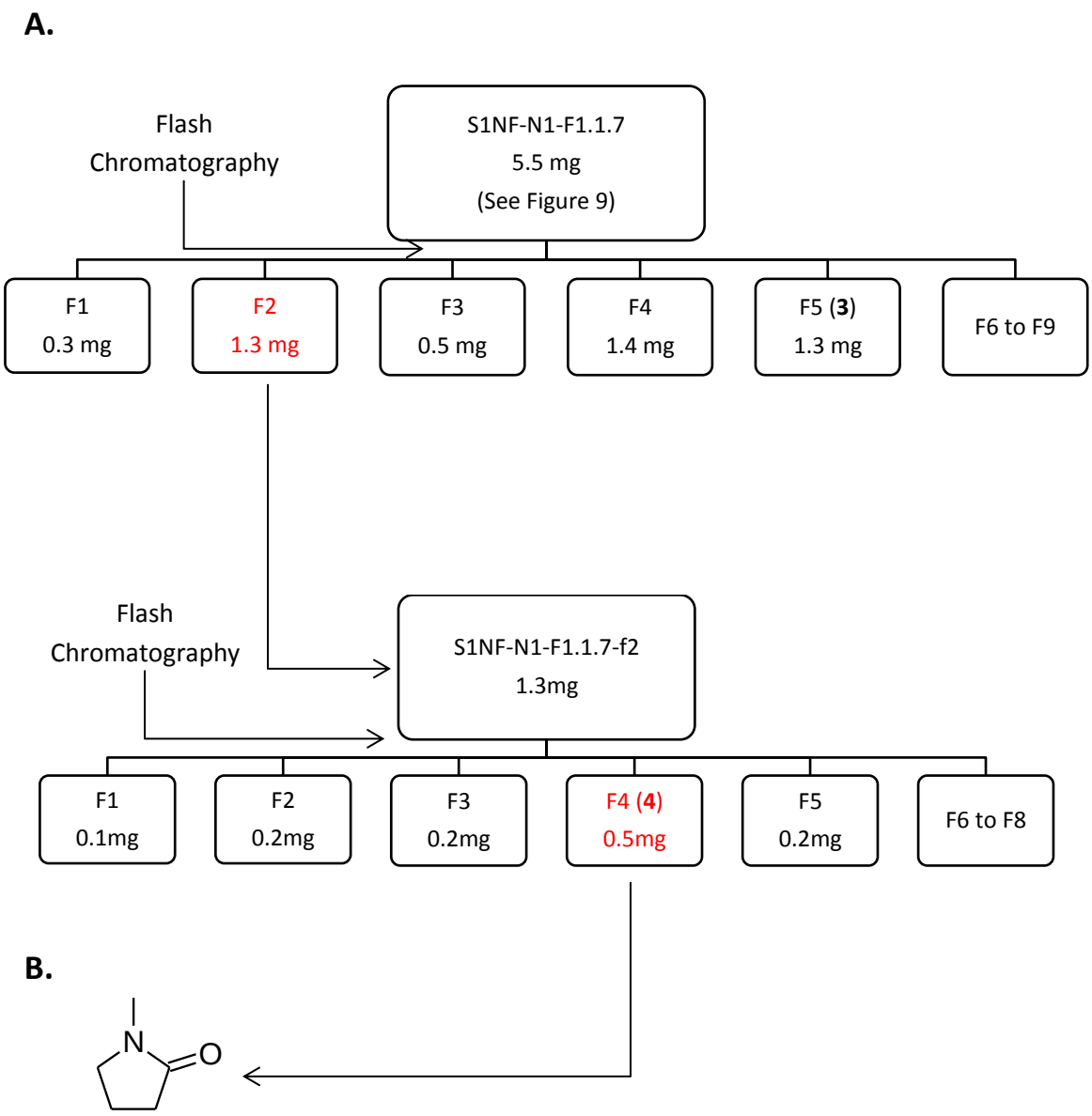


Figure 11: A) Isolation of N-Methyl pyrrolidinone (**4**). B) Structure of N-Methyl pyrrolidinone.

2.8. Conclusions and Future Work

Alternaria tenuissima was isolated from the underlying soil of lichen samples from northern Manitoba and identified by ITS sequencing. The secondary metabolites, deoxy phomalone, dimethyl 4-methyl-2,6-pyridinedicarboxylate, stemphyperylenol and N-Methyl pyrrolidinone were found to be produced by this fungus. Dimethyl 4-methyl-2,6-pyridinedicarboxylate has not been previously reported as a fungal natural product while deoxyphomalone and N-Methyl pyrrolidinone have only been isolated once. It is also useful to note that these secondary metabolites have never been previously co-isolated from the same fungus. Even though the compounds did not show promising biological activity against *E. coli*, the rarity of their isolation is an indication of the possibilities inherent in this project. The fact that natural products (some of which were rare) not previously observed from the same organism were identified strengthens the argument for discovering novel natural products from unique ecological niches such as the one in this project.

A possible task to undertake in the future is the expansion of the bioassay to include various kinds of bacteria such as pathogenic, Gram-negative, Gram-positive and multi-drug resistant bacteria. This constitutes a more efficient probing for the presence of bioactive secondary metabolites. We could also attempt to extract the biomass (i.e. the fungal mycelium) in order to examine the intracellular secondary metabolites produced by the fungus. Sometimes, some secondary metabolites are not secreted and are retained within the fungal mycelium. Extraction of novel secondary metabolites from

fungal mycelium has been reported in past work.[77] Hopefully, efforts in this direction would lead to the discovery and development of novel antibiotics.

3. EXPERIMENTAL

3.1. General.

IR spectra were obtained on a Bruker Alpha FT-IR spectrometer, and the sampling method was by ATR using a thin film formed by solvent evaporation on a KBr plate. ^{1}H and ^{13}C NMR were recorded on a Bruker Avance 500 spectrometer. Optical rotation readings were measured on an Autopol IV polarimeter. Silica gel (40–60 µm) was used for column chromatography. Mass spectra were recorded on a Varian 320-MS triple quad mass spectrometer. UV/Vis spectra were obtained in MeOH on a Biochrom Ultrospec 60. PCR experiments were run on a Techne TC-3000 PCR Thermal Cycler. DNA concentration and absorption spectra were measured on a Thermoscientific Nanodrop 2000c spectrophotometer

3.2. Materials.

A strain of *A. tenuissima* was isolated from a soil sample obtained from underneath the lichen, *P. didactyla* collected from Wapusk National Park in Nothern Manitoba. The location of collection was N 57° 34′ 32.5″; W 93° 52′ 12.8″. The lichen sample was collected and identified by Dr. Michelle Piercey-Normore of the Department of Biological Sciences at the University of Manitoba. Identification of the fungus was based on the DNA sequencing of the ITS region of its ribosomal DNA.

3.3. Fermentation Culture.

A portion of the soil from underneath the lichen sample was applied to potato dextrose agar (PDA) plates and incubated for 10 days at 30 °C. Another portion of the soil was

surface-sterilized by rinsing it with 1 % bleach then with sterilized milliQ H_2O. This surface-sterilized soil was then broken up and applied to three PDA plates and incubated for 10 days at 30 °C. *A. tenuissima* was isolated from the unsterilized soil culture by sub culturing on 3 fresh PDA plates at 30 °C for 10 days. The fermentation cultures were made by inoculating two 2 L Erlenmeyer flasks each containing 1 L of potato dextrose broth (PDB) with agar plugs from the sub cultures. Cultures were incubated in an orbital shaker for seven days at 30 °C and 250 rpm.

3.4. Extraction and Isolation.

Cultures were combined, filtered through cheese cloth, and extracted twice with half volume (1 L) of ethyl acetate. The organic layers were combined and dried over Na_2SO_4; and the solvent was removed *in vacuo* to afford the neutral extract. The leftover aqueous layer was acidified with concentrated HCl to pH < 2 and extracted twice with 1 L of ethyl acetate yielding the acidic extract. Both extracts were subsequently resolved by repeated preparative thin layer chromatography and flash column chromatography to afford the purified compounds.

3.5. Bioassay

A 5 ml aliquot of LB medium was inoculated with 200 µL of frozen stock *E. coli* and the culture was incubated overnight at 37 °C while shaking at 250 rpm. The culture was subsequently diluted with sterile LB broth until an optical density reading of 1 at 600 nm was attained. Regular LB agar medium plates were prepared while LB half-strength agar was inoculated with *E. coli* culture (OD = 1.0), 200 µL per 1 mL of LB half-strength agar.

The inoculated half-strength agar was thoroughly mixed and poured over the previously prepared regular agar, 3 mL per plate. A 1:1 MeOH/acetone solvent mixture was used to make 1 mg/mL solutions of compounds **1** to **4**. A 10 µL aliquot of each solution was applied to separate plates. A 1:1 MeOH/Acetone solvent control plate, an untouched *E. coli* negative control plate and an usnic acid (10 mg/mL) positive control plate were included in the assay. The plates were then incubated overnight at 37 °C. Bioactivity was deduced by a zone of clearing about the spot where each sample was applied.

3.6. Isolation of Genomic DNA

DNA extraction was done using the GeneJET™ Genomic DNA purification kit with a slightly modified version of the yeast genomic DNA purification protocol.[78] A small amount of mycelium was frozen with liquid nitrogen in a mortar and crushed with a pestle. A portion of the crushed mycelium was then transferred to a 1.5 mL Eppendorf tube where it was resuspended with yeast lysis buffer. The rest of the procedure was followed as described in the protocol except that 50 µL of elution buffer was used instead of the suggested 200 µL in order to obtain a more concentrated DNA solution. Good quality DNA was obtained and stored in the fridge at 4 °C.

3.7. Polymerase Chain Reaction (PCR) Amplification

The DNA obtained was amplified by PCR. Eight 20 uL (A total of 160 µL) samples were prepared, consisting of 4 µL of 5xGC buffer, 2 µL of dNTP, 0.2 µL of Pfu DNA polymerase, 0.6 µL of DMSO, 10.2 µL of sterilized milliQ H_2O, 1 µL each of forward primer, reverse primer and amplified fungal DNA. The forward primer used was 1780-5’ (SSU rDNA) (5’-

CTGCGGAAGGATCATTGATTC-3')[61] and the reverse primer was ITS2-kL-3' (ITS rDNA) (5'-TGCTTAAGTTCAGCGGGTA-3').[62] Both primers were specific for the ITS region of the fungal DNA. The samples were then run on the PCR thermal cycler using a touchdown PCR cycle: initial denaturation of DNA at 98 °C for 1 min; 34 cycles comprised of denaturation at 98 °C for 10 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 1 min; then a further 5 mins of extension at 72 °C and finally a soak at 8 °C.

3.8. Precipitation, Electrophoresis, Purification

The 160-µL volume PCR product was precipitated in a 1.5 mL Eppendorf tube by adding 5 M NaCl and 100 % ethanol. The solution was mixed gently and stored in the fridge (4 °C) overnight. The tube was then centrifuged at 13000 rpm for 10 mins and the supernatant was discarded. The pellet was washed with 200 µL of cold 80 % ethanol which was then poured off. The pellet was left to air dry for 30 min after which it was resuspended in 20 µL of sterile milliQ H_2O. A 12 µL aliquot of bromophenol blue loading dye (6 x BPB) was added to the solution which was then 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 run at 119 volts until the loading dye was a centimeter from the bottom of the gel. Visualization of the band of DNA was accomplished using a 254 nm UV-light box. The band was excised from the gel and transferred to a 1.5 mL Eppendorf tube and the DNA was extracted from the gel using a Wizard SV Gel and PCR Clean-Up System[63] and sent to Genewiz Inc. (New Jersey) for sequencing.

3.9. ITS Sequence processing and determination of fungal identity

The obtained sequences were edited using Chromas Lite v 2.1.1 (Technelysium Pty Limited, South Brisbane, Australia). An NCBI blast analysis of the reverse sequence (shown below) indicated a 99% sequence match with *A. tenuissima* and was assigned the accession number, EF364096.1 (Fig. 12). The sequence used for the search is shown below. “N” represents nucleotides that were undetermined.

NNNNNNNNNNNNNNNTNNNNNNNNNANNNNCGNTTAATCGGATGCTAGACCTTTGCTGAT
AGAGAGTGCGACTTGTGCTGNGCTCCGAAACCAGTAGGCCGGCTGCCAATTACTTTAAGGCGA
GTCTCCAGCAAAGCTAGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACG
CTCGAACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTC
ACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAA
GAGATCCGTTGTTGAAAGTTGTAATTATTAATTTGTTACTGACGCTGATTGCAATTACAAAAGGT
TTATGTTTGTCCTAGTGGTGGGCGAACCCACCAAGGAAACAAGAAGTACGCAAAAGACAAGGG
TGAATAATTCAGCAAGGCTGTAACCCCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATC
CTTCCGCAGATTCT

Alternaria tenuissima 18S ribosomal RNA gene, partial sequence; internal transcribed spacer

Sequence ID: gb|EF364096.1| Length: 620 Number of Matches: 1

Range 1: 10 to 496 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
885 bits(479)	0.0	485/488(99%)	1/488(0%)	Plus/Minus

```
Query  34   TTAATCGGATGCTAGACCTTTGCTGATAGAGAGTGCGACTTGTGCTGNGCTCCGAAACCA  93
            ||||| ||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  496  TTAAT-GGATGCTAGACCTTTGCTGATAGAGAGTGCGACTTGTGCTGCGCTCCGAAACCA  438

Query  94   GTAGGCCGGCTGCCAATTACTTTAAGGCGAGTCTCCAGCAAAGCTAGAGACAAGACGCCC  153
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437  GTAGGCCGGCTGCCAATTACTTTAAGGCGAGTCTCCAGCAAAGCTAGAGACAAGACGCCC  378

Query  154  AACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCTTTGGAATAC  213
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  AACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCGAACAGGCATGCCCTTTGGAATAC  318

Query  214  CAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTA  273
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  CAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTA  258

Query  274  CTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG  333
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  CTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG  198

Query  334  TTGTAATTATTAATTTGTTACTGACGCTGATTGCAATTACAAAAGGTTTATGTTTGTCCT  393
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  TTGTAATTATTAATTTGTTACTGACGCTGATTGCAATTACAAAAGGTTTATGTTTGTCCT  138

Query  394  AGTGGTGGGCGAACCCACCAAGGAAACAAGAAGTACGCAAAAGACAAGGGTGAATAATTC  453
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137  AGTGGTGGGCGAACCCACCAAGGAAACAAGAAGTACGCAAAAGACAAGGGTGAATAATTC  78

Query  454  AGCAAGGCTGTAACCCCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCTTCC  513
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77   AGCAAGGCTGTAACCCCGAGAGGTTCCAGCCCGCCTTCATATTTGTGTAATGATCCTTCC  18

Query  514  GCAGATTC  521
            |||| |||
Sbjct  17   GCAGGTTC  10
```

Figure 12: BLAST search result for fungal sequence.[66]

3.10. Experimental Data

Deoxyphomalone (**1**)

Rf: 0.6 ($CHCl_3$-MeOH, 19:1); IR (KBr): 3331, 2962, 2935, 1613, 1570, 1440, 1428, 1314, 1252, 1207, 1122, 831 cm^{-1}; UV/Vis λ_{max} (MeOH) nm (log ε): 234 (3.80), 280 (4.52), 324 (3.45); ^{1}H NMR (500 MHz, $CDCl_3$): 0.98 (3H, t, *J* = 7.4 Hz, H-10), 1.12 (3H, t, J = 7.5 Hz, H-13), 1.69 (2H, tq, *J* = 7.4, 7.3 Hz, H-9), 2.59 (2H, q, *J* = 7.5 Hz, H-12), 2.96 (2H, t, *J* = 7.3 Hz, H-8), 3.84 (3H, s, H-11), 5.90 (1H, s, H-3), 14.32 (1H, s, C6-OH); ^{13}C NMR (500 MHz, $CDCl_3$): 13.6 (C-13), 14.2 (C-10), 15.7 (C-12), 18.4 (C-9), 46.5 (C-8), 55.6 (C-11), 90.3 (C-3),

106.0 (C-1), 109.7 (C-5), 159.7 (C-4), 161.3 (C-2), 165.1 (C-6), 206.3 (C-7); MS (EI, 70 eV): *m/z* (%) = 238.2 (18) [M + H^+], 195.1 (100).

Dimethyl 4-methyl-2,6-pyridinedicarboxylate (**2**)

Rf: 0.3 ($CHCl_3$-MeOH, 19:1); IR: 2949, 2923, 2854, 1728, 1718, 1438, 1354, 1216, 783 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): 2.53 (3H, s), 4.02 (6H, s), 8.15 (2H, s); ^{13}C NMR (500 MHz, CD_3OD): 21.0 (C-11), 53.3 (C-8 and C-10), 129.8 (C-3 and C-5), 149.4 (C-4), 152.5 (C-2 and C-6), 166.5 (C-7 and C-9); MS (EI, 70 eV): m/z (%) = 232.2 [M + Na^+]

(+)-Stemphyperylenol (**3**)

$[\alpha]_D$: +461.5 (*c* 0.01, MeOH); Rf: 0.3 ($CHCl_3$-MeOH, 19:1); IR (KBr): 3375, 2920, 1643, 1606, 1468, 1364, 1256, 1230, 1066, 732 cm^{-1}; UV/Vis λ_{max} (MeOH) nm (log ε): 234 (3.80), 280 (4.52), 324 (3.45); ^1H NMR (500 MHz, $CDCl_3$): 3.04 (2H, dd, *J* = 15.5, 4.5 Hz, H_a-7), 3.10 (2H, dd, *J* = 15.5, 12.3 Hz, H_b-7), 3.72 (2H, d, *J* = 9.3 Hz, H-5), 4.66 (2H, ddd, *J* = 12.3, 9.3, 4.5 Hz, H-6), 6.84 (2H, d, *J* = 8.8 Hz, H-1), 8.06 (2H, d, *J* = 8.8 Hz, H-2); ^{13}C NMR (500 MHz, $CDCl_3$): 46.3 (C-5), 48.0 (C-7), 68.6 (C-6), 116.0 (C-1), 116.2 (C-9), 131.2 (C-3), 135.6 (C-2), 144.1 (C-4), 161.4 (C-10), 204.3 (C-8); MS (EI, 70 eV): *m/z* (%) = 352.1 [M + H^+] (19), 316.1 (100).

N-Methyl pyrrolidone (**4**)

Rf: 0.8 ($CHCl_3$-MeOH, 19:1); IR: 2917, 2849, 1695, 1457, 1265, 735cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): 2.02 (2H, tt, *J* = 7.5Hz), 2.38 (2H, t, *J* = 8.1Hz), 2.84 (3H, s), 3.38 (2H, t, *J* = 7.2Hz); ^{13}C NMR (500 MHz, $CDCl_3$): 17.8 (C-4), 29.7 (C-5), 30.8 (C-3), 49.6 (C-6), 175.3 (C-2).

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