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Extraction and Purification of Biologically Active Metabolites from Rhodococcus sp. MTM3W5.2

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Extraction and Purification of Biologically Active Metabolites from *Rhodococcus* sp.

MTM3W5.2

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by

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Performance Liquid Chromatography

ABSTRACT

Extraction and Purification of Biologically Active Metabolites from *Rhodococcus* sp.

MTM3W5.2

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Rhodococcus has been recognized as a potential antibiotic producer. Recently, a strain of

Rhodococcus sp. MTM3W5.2 was isolated from a soil sample collected in Morristown,

Tennessee and was found to produce an inhibitory compound which is active against other

related species. The purpose of this research is to extract, purify and analyze the active

metabolite. The compound was extracted from RM broth cultures and purified by preliminary

fractionation of crude extract through a Sephadex LH-20 column. Further purification was

completed using semi-preparative reversed phase column chromatography. Final purification

was obtained using multiple rounds of an analytical C18 HPLC column. Based on the results

achieved in the UV-Vis spectroscopy and high-resolution mass spectroscopy, the two desired

compounds at a retention time of at 57 and 72 min could be polyketides with the molecular

formulas $C_{52}H_{78}O_{13}$ and $C_{19}H_{32}O_1N_1$/$C_{13}H_{34}O_1N_1$, respectively.

DEDICATION

To my mom

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

TB	Tuberculosis
MDR	Multidrug-Resistant
IPP	Isopentenyl Diphosphate
DMMAPP	Dimethylallyl Diphosphate
PKS	Polyketide Synthases
KS	Ketosynthase
AT	Acyltransferase
ACP	Acyl Carrier Protein
KS	Ketosynthase
DH	Dehydratase
ER	Enoyl Reductase
CoA	Coenzyme A
TE	Thioesterase
DEBS	6-Deoxyerythronolide B Synthase
HPLC	High-Performance Liquid Chromatography
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
CH_3CN	Acetonitrile
CH_3OH	Methanol
DMSO	Dimethyl Sulfoxide
$HCOONH_4$	Ammonium Formate
NH_4OH	Ammonium Hydroxide

SEC	Size Exclusion Chromatography
HR-MS	High-Resolution Mass Spectrometry
MH	Mueller-Hinton Medium
RM	Rich Medium
μL	Microliter
mL	Milliliter
L	Liter
cm^{-1}	Per Centimeter
g	Grams
hr	Hour
min	Minute
mM	Millimolar
GF	Gel Filtration
UV	Ultraviolet
Psi	the Pound Per Square inch
Nm	nanometer
Rpm	Revolutions per minute

CHAPTER 1

INTRODUCTION

<u>Antibiotics History</u>

Penicillin was discovered by Alexander Fleming in 1928. However, it was not used as a treatment at that time. In 1940, it had been showed that penicillin was helpful to cure bacterial infection. Lately in 1940, penicillin had been reported as a resistance antibiotic.[1] As a result, several studies were conducted to modify the chemical properties of penicillin to avoid cleavage via penicillinases. Introduced in 1937, sulfonamides were the first effective antimicrobials. Although they proved to be somewhat successful, their therapeutic use was reduced because of the development of certain mechanisms of resistance. These resistance mechanisms were reported in the latter part of the 1930s.[2] The wide use of antibiotics led to resistant strains capable of reducing the activity of the drug.[3] Another antibiotic known as streptomycin was introduced in 1944 for tuberculosis (TB) treatment. However, there was an increase in the mutant strains of *Mycobacterium* tuberculosis and subsequent resistance of the therapeutic concentrations of streptomycin during patient treatment.[4] In the 1960s, there was no longer a problem with TB treatment. However, in the 1980s the disease returned with acquired immune deficiency syndrome (AIDS) patients and appeared in drug-resistant forms. Also, there were another types of gram-negative bacteria such as *Enterobacteriaceae*.[5] There were several resistances in other new classes of antibiotics that were discovered and introduced into clinical practice after penicillin and streptomycin, as shown in figure 1.[4]
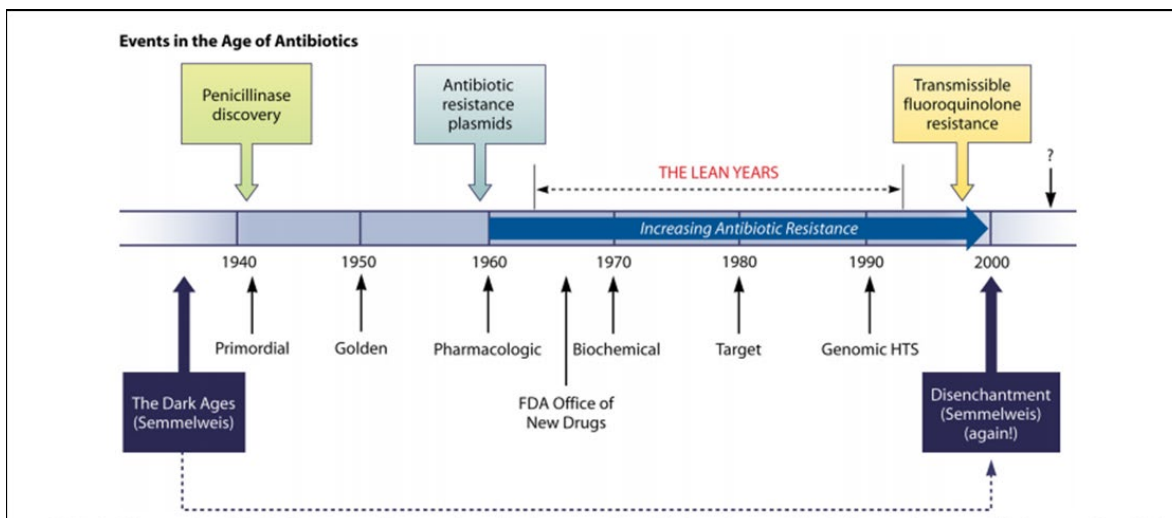
Figure 1. History of discovery antibiotics and antibiotic resistance development (used with permission from Davies, J.).[4]

Antibiotics Resistance

Recently, the use of antibiotics in the treatment of common bacterial infections has not been effective due to antibiotic resistance.[6] The high rate of antibiotic use in hospitals, agriculture, and communities in developed countries has contributed immensely to sustained resistant strains, causing shifts to more expensive and more broad-spectrum antibiotics.[7] Most antibiotic resistance arises due to mutations in microbes and the selection pressure which kills susceptible bacteria, causing antibiotic-resistant bacteria to survive and multiply.[2] A genetic mutation in Penicillin-binding proteins (PBPs), which are a group of proteins that have binding affinity to an antibiotic such as a β-lactam, could lead to bacterial resistance. The binding between β-lactam and PBP will inhibit cell wall biosynthesis in bacteria. If there is a mutation in PBP, that could reduce the affinity of the antibiotic for its target this leads to resistance.[8] Other antibiotic resistances are due to bacterial resistance because of an increased concentration of metabolites antagonizing the drug action and the formation of adaptive-drug inactivating

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enzymes.[9] Some resistant strains of bacteria produce an enzyme called β-lactamase. This enzyme cleaves the β-lactam ring of penicillin and causes antibiotic resistance.[9] In addition, other bacterial pathogens, related to epidemics of human diseases, have developed into multidrug-resistant (MDR) forms after antibiotic use. In the 20th century, a version of a major pathogen known as *M. tuberculosis* was identified as multidrug-resistant in most parts of the world. The combination of anti-TB drugs was helpful for tuberculosis treatment several years ago, but they became multidrug-resistant for many reasons.[4] The overuse of antibiotics without a limitation has caused a selective pressure on *M. tuberculosis*, starting from mono-drug resistant to multidrug resistant, extensively drug resistant and eventually totally drug resistant, because of sequential accumulation of resistance mutations.[5] Studies showed that spontaneous mutation is the major cause of antibiotic resistance in *M. tuberculosis*. It has been observed over the past 50 years that there is a strong correlation between antibiotic use in the treatment of bacterial infections and antibiotic resistance development.[4] One of the most prominent examples is β-lactamases and the β-lactam class of antibiotics. *Mycobacterium tuberculosis* is resistant to β-lactam drugs. After the antibiotic penetrates the cell wall, the antibiotic could be cleaved by the enzyme and become ineffective.[5]

<center>Managing Antibiotic Use</center>

An effective approach to fight against antibiotic resistance requires effective training of new personnel and the implementation and investment of antibiotic stewardship programs, encouraging pharmaceutical companies to conduct more research on antibiotic resistance and discovery of new antibiotics.[10,11] Also, in the developing and underdeveloped countries with poor health systems, factors such as the provision and management of adequate infrastructure, sufficient funds, implementation of new infection control policies and investigation of records

should be ensured.[12] While health agencies control these new policies in hospitals and communities, the national ministries of health should be responsible for the enforcement of other beneficial policies concerning antibiotic resistance in the health sector.[13] Also, the role of physicians in the advocacy for control of antibiotic resistance can ensure practical input to the national task force. Moreover, the national task force can play a role by involving the major ministries that take part in drug discovery such as pharmaceutical industries and education and agricultural sectors. Other effective measures can be enforced by regulating, reviewing and customizing national antibiotic policies and providing potential funding agencies for underdeveloped countries through global partnerships.[14]

Antibiotics Produced From Natural Products

Natural products have been identified as good sources of many active ingredients of medicines. This led to the wide use of natural products in drug discovery over the years until synthetic drugs were produced.[15] Several years ago, medicinal plant extracts and their natural products were screened for antimicrobial and antibacterial properties, and this showed that many such plants are primary sources of antibiotics. Due to their therapeutic properties, several studies reported that medicinal plants can be considered an effective source of antibiotics when the main goal is to control or heal the body from microbial invasions. [16] According to current surveys, roughly 60% of the current anticancer and antibiotic drugs are derived from medicinal plants. Studies have shown that natural ecological community preserves the stability of antibiotic resistance. Since ancient times, medicinal plants have been used to develop more effective drugs as they have proven therapeutic characteristics. For example, the medicinal herb Nigella sativa has been used in traditional medicine to treat many health conditions, such as asthma, gastrointestinal problems and high blood pressure. Given its wide use, scientists have examined

its antioxidant functions on treating difficult health problems such as cancer and autoimmune diseases.[17] However, the overuse of broad-spectrum antibiotics in agriculture and hospitals resulted in the selective pressure on resistant populations.[18,19] Several studies have discussed the importance of natural products in drug discovery. The effectiveness of natural products is better than the varieties of successful drugs from synthetic compounds. There is an increasing demand for the inclusion of natural chemical products in drug discovery. [20]

Currently, the major source of most leads from natural products is extracted from either a microbial source or plant source. Previous publications, which discussed the screening of bioactive compounds in the world's natural products, showed that only a small quantity is available for use in drug production. However, the collections of more extensive plants and the culturing of microbes could be important in the production of several new chemicals in drug discovery assays. Currently, terrestrial actinomycetes fungi have been a main source of microorganisms. These fungi are very useful with the discovery of their species unique to the marine environment and with their ability to produce new bioactive metabolites chemically. In addition, cyanobacteria are another important source of bioactive compounds from the marine environment. They have increased the demand for production of chemically new bioactive metabolites in drug discovery.[20] Recently, an extensive screening approach for the discovery of natural product was reported. Other improved approaches, such as methodology for the identification of antibiotics, cheminformatic approaches, and production of strains, have also been developed. Novel genomic-type approaches have been very useful in finding small molecules with antibacterial activity. In the future, this approach will be a promising avenue for the identification of novel opportunities.[21] Natural products have spurred many advances in organic chemistry, thus, promoting new developments in synthetic methodologies and enhancing

the production of analogues of the original lead compound with improved therapeutic properties.[22-23] Natural product scaffolds have been identified with chemical structures which have been very important as well as the basis for drug discovery. These scaffolds have been used as important cores of compound libraries such as flavonoids, terpenoids, polyketides, and alkaloids which are all produced by combinatorial techniques.[24,25]

<div align="center">Secondary Metabolites</div>

Secondary metabolites are organic compounds with variable chemical structures that have been synthesized by varieties of strains of microbial species. Antibiotics are a very common type of secondary metabolites that have been very economical in drug discovery, while other classes of secondary metabolites have also been useful for their biological activities. Secondary metabolites are synthesized due to their ability to provide protection to microbes from biological stimulus or harm, including humans, insects, plants and even other microorganisms.[26] Secondary metabolites are compounds that are not directly involved in normal growth and are not required for plant growth but are vital for reproduction in humans. They are limited in occurrence and likely restricted to a certain taxonomic group genus, species or family and are present in smaller quantities than primary metabolites.[12] Secondary metabolites have been divided into three main groups based on their biosynthetic origins:

1. Nitrogen-containing alkaloids and sulphur-containing compound

2. Flavonoids and allied phenolic and polyphenolic compounds

3. Terpenoid.[27]

Alkaloids

Alkaloids are a class of chemical compound that are naturally occurring and mostly contain a basic nitrogen atom. Also, alkaloids include some related compounds with neutral and weak acid

properties. In addition to nitrogen, carbon, and hydrogen, they also contain sulfur and oxygen. Rarely, alkaloids have other elements such as phosphorus, chlorine, and bromine. Alkaloids are produced by a wide range of organisms like animals, fungi, bacteria, and, primarily, by plants as secondary metabolites. Most of the alkaloids are toxic to other organisms, and it is extracted by using an acid-base. However, alkaloids have different pharmacological effects and have a long history in treatment.[28] For instance, it has been shown in several studies that alkaloids extracted from herbal substances have anti-proliferative and anti-metastatic properties when they were used in different types of cancer treatment. This success in cancer treatment has led to use some alkaloids, such as camptothecin and vinblastine in the development of anti-cancer drugs. In addition, another successful progress has been noted when berberine, type of the alkaloids was used in the treatment of metabolic syndrome. Therefore, the use of these alkaloids seems to be promising in these treatments.[29] Alkaloids have a great diversity in their biochemical botanical origin, in pharmacological action, and in chemical structure.[30] Thus, there are several approaches for classifying alkaloids. They can be classified by:

I. **Chemical structures:** According to the nature of the nitrogen-containing structure classification, alkaloids are organized based on a common, typically heterocyclic, nucleus, such as indole, pyrrolidine, isoquinoline, quinazoline, and pyrrolizidine.[31]

II. **The natural origin:** Another way to classify the alkaloids is to use their botanical taxa in which the alkaloids are extracted such as Solanaceae, Amaryllidaceae, Annonaceae and Rutaceae alkaloids. [31]

III. **Biosynthetic pathway:** Alkaloids are divided by their common molecular precursors, according to the biosynthetic pathway of alkaloids used to construct the molecule.[32,33] Thus, they include three main types of alkaloids:

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a. True alkaloids are derived from amino acids and contain a heterocyclic ring with nitrogen such as L-histidine, L-ornithine, and L-lysine.[32]

b. Protoalkaloids are derived from amino acids, but nitrogen is not a part of a heterocyclic ring like L-tyrosine and L-tryptophan. [33]

c. Pseudoalkaloids are not derived from an amino acid, for example, solanidine, caffeine, capsaicin, and ephedrine as shown in figure 2.[32]
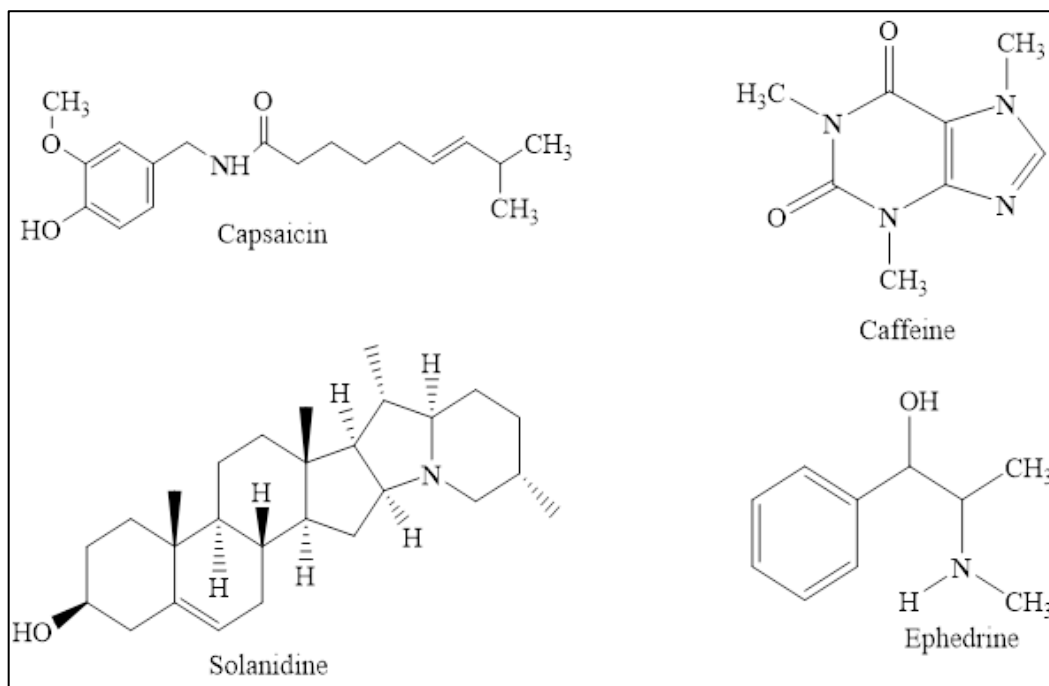
Figure 2. Some examples of pseudoalkaloids.[32]

Terpenoids

The terpenoids, sometimes called isoprenoids, are one of the most diverse classes of natural plant products. They range in structure from a straight chain (linear) to polycyclic molecules, and they range in size from the five-carbon hemiterpenes to natural rubber. Terpenoids are derived from thousands of isoprene units. Thus, all terpenoids are synthesized via the condensation of isopentenyl diphosphate (IPP), and its allylic isomer, dimethylallyl

diphosphate (DMAPP) (Figure 3).[12] Based on the number of five-carbon units present in the core

structure, terpenoids are classified as monoterpenes (C10), sesquiterpenes (C15), diterpenes

(C20), sesterterpenes (C25), triterpenes (C30), and tetraterpenes (C40). [31] Current research has

found that these terpenoids can be used for therapeutic purposes as they demonstrate a broad

range of biological activities such as anti-cancer, anti-inflammatory, and anti-malarial. After this

important discovery, the use of terpenes has been increased to treat a diversity of infectious and

microbial diseases including viral and bacterial. [34]
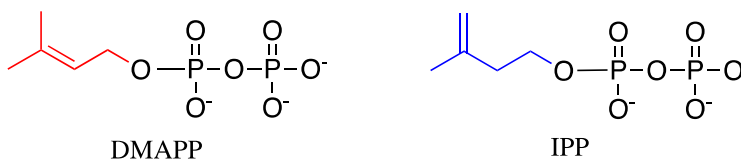
Figure 3. The structure of IPP and its isomer DMAPP.[31]

Phenylpropanoids

Phenylpropanoids are a large group of natural products that contain six members of an

aromatic ring. In the late 19th century, the structures of various simple phenylpropanoids, which

are common plant products, were established such as a 3, 4-dihydroxycinnamic acid (caffeic

acid), 4-hydroxycinnamic acid (coumaric acid) and Cinnamic acid as shown in figure 4. The

phenylpropanoids are biosynthesized by the shikimate pathway which is found in plants and

microorganisms. The shikimate pathway provides an unorthodox way to form aromatic

compounds, especially the aromatic amino acids such as l-tyrosine, l-tryptophan, and l-

phenylalanine. The major difference between aromatic rings that are biosynthesized by this route

and those of polyketide aromatic rings are their oxygenation pattern. [31,35] During the last few

years, more research has been conducted to examine the biological activities of

phenylpropanoids. These studies showed that phenylpropanoids have protective effects against

infections, stressors, and air pollutants. [36] It has been also found in recent studies that plant-derived phenylpropanoids have great effects for human health. These protective effects were found to be associated with their antioxidant, anti-cancer, and anti-inflammatory properties. It has been also suggested that phenylpropanoids have great impacts on reducing the risk of heart diseases, cancer, and osteoporosis because the active compounds in phenylpropanoids act as an estrogen agonist via the estrogen' receptors. [37]

Cinnamic Acid   4-hydroxycinnamic acid (Coumaric Acid)   3,4-dihydroxycinnamic acid (caffeic acid)
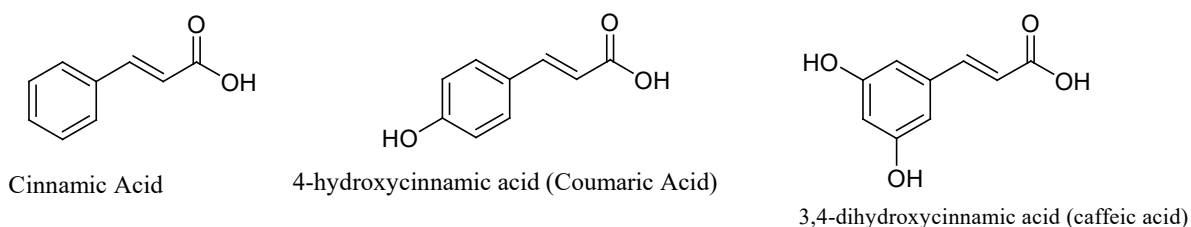
Figure 4. The examples of Phenylpropanoids.[31]

Polyketides

Polyketides are a large group of natural products that are produced from different sources including fungi, bacteria and, to a lesser extent, plants. Most of the recent studies have been conducted on polyketide biosynthesis.[38] The first factor is their biological and pharmacological activities, such as antifungal, anticholesterol, antibacterial, antiparasitic, antifungal, anticancer, and immunosuppressive properties.[38-39] It is also driven by the enormous commercial value of these natural products which remain the most successful candidates for new drug discoveries. The other factors, which have been considered in much current research, are the mechanism, catalytic reactivity, and exceptional structure of PKSs, which provide an unprecedented opportunity to investigate the molecular mechanisms of molecular recognition, protein-protein interaction, and enzyme catalysis. Finally, the extraordinary diversity and the

usability of polyketide synthases permit the production of new compounds which are hard to access through other ways.[40]

Polyketides are synthesized from a repetitive decarboxylative Claisen condensation between a thioesterified malonate derivative and an acyl thioester.[39] The polyketide synthesis processes are like the synthesis of fatty acid, but polyketides differ from fatty acids due to the diversity in their structures and differences in the fatty acid synthesis and the post-PKS modification. Moreover, the variety of the chemical structure of polyketides which causes the diverse bioactivities as well.[41] Many polyketides are originated from the *Actinomycete* bacteria, a gram-positive soil bacterial, particularly, members of the genus *Streptomyces* and the genus *Rhodococcus*.[42]
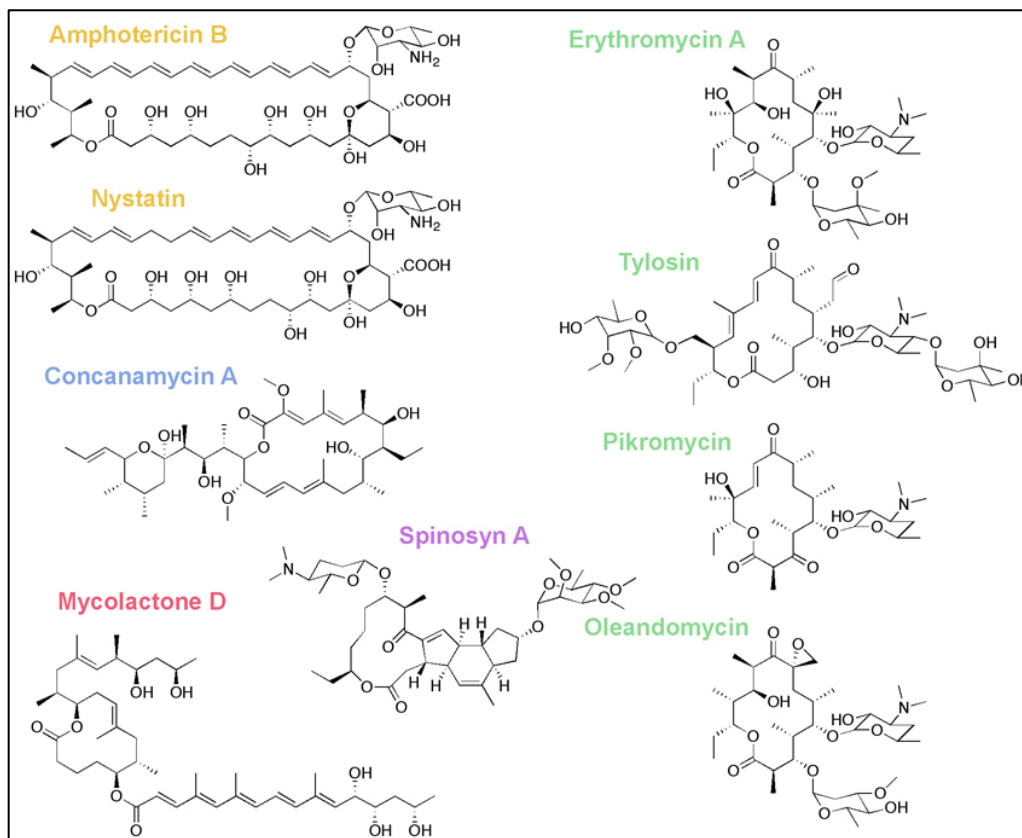
Figure 5. Examples of polyketide secondary metabolites.[43]

Polyketides Syntheses (PKSs)

The vast structural diversity of polyketides arises mostly from multifunctional enzymes. Even with this diversity, all polyketides are biosynthesized in the first stage by a mechanism which is analogous to fatty acid synthesis.[44] Polyketides are synthesized by successive rounds of decarboxylative Claisen condensation of a starter unit, most commonly acetyl-CoA with an extender unit, typically malonyl-CoA. After that, the repetitive decarboxylative Claisen condensation results in an extension of the polyketide carbon chain, and it may undergo additional modifications.[39] The polyketide synthase (PKS) is a multifunctional enzyme that is responsible for catalyzing these condensation reactions. The PKS involves a set of catalytic domains, organized into functional units called modules. Each domain has a specific catalytic function that is required for chain elongation.[45] These domains, which are obligatory for the biosynthesis of polyketides, include:

I. Acyltransferase (AT) domain is responsible for selecting the specific extender unit from the CoA pool and loading on to the acyl carrier protein (ACP) domain.

II. Ketosynthase (KS) domain catalyzes the decarboxylative Claisen condensation with upstream intermediate to extend the polyketides chain.

III. Acyl carrier protein (ACP) domain is a carrier in the intermediate which is connected by a thioester, tethered to the phosphopantetheine cofactor of the acyl carrier protein. [46]

The remaining reductive domains (ketoreductase [KR], dehydratase [DH], and enoylreductase [ER]) may occur to produce a different degree of reductions and may result in β-keto, β-hydroxy, α, β-alkene, or entirely reduced intermediates. After these processes have been completed, the ACP domain of one module can mediate the successful transfer of the intermediate to the next module in the assembly line of the polyketide. Finally, the terminal

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thioesterase (TE) domain is responsible for separating the compound from the ACP region of the final module then releasing the polyketide compound.[46] Commonly, the number of modules that are required for synthesis of the polyketide compound are identical to the number of precursors which are combined onto the polyketides.[39]

The polyketides' and fatty acids' biosynthesis are connected mechanistically and frequently using the same precursor molecules. Still, there are some differences between polyketide and fatty acid biosynthesis which are the type and the number of acyl precursors that are used in the extension of the polyketide chain, the cyclization of the final products, and the position and extent of keto-group reductions. [47]

Polyketides can generally be classified into three types: I PKS, II PKS, III PKS. Type I PKSs are multifunctional enzymes that consist of several enzymatic domains which arrange into modules. Each of them contains a group of distinct, non-iterative acting activities which are responsible for stimulating one cycle of polyketides' extension chain, such as 6-deoxyerythromycin B synthase. Moreover, the term type II PKSs indicates multienzyme complexes that contain a single set of iteratively acting activities such as tetracenomycin.[40] Furthermore, type III PKSs are also known as chalcone syntheses like PKSs. They are mostly distributed in plants and have recently been characterized from microorganisms as exemplified by tetrahydroxy chalcone.[48,49] Type I and II PKSs use an acyl carrier protein (ACP) domain to direct the growth of polyketide intermediate and to activate the acyl CoA substrates, while type III PKSs are utilized as an ACP independent mechanism. [49]

Bacterial Type I Modular Polyketide Synthase

A classic bacterial Type I PKS is the modular 6-deoxyerythronolide B synthase (6-DEB) from *Saccharopolyspora erythraea* and is responsible for synthesis of the aglycone core of the clinically significant macrolide antibiotic erythromycin A.[48] The modular PKS assembles seven precursors that contain one propionyl-CoA starter unit and six (2S)-methylmalonyl-Coenzyme A (CoA) extender units on to 6-DEB.[39] This PKS has three large DEBS proteins which are comprised of two functional subunits, and these subunits are called modules. Each module consists of three domains ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) which are required to stimulate one cycle of chain elongation. Also, there are variable sets of domains that include: ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) with modification of the keto functional group. The biosynthesis of polyketides begins with DEBS 1 which is fronted by loading didomains (AT and ACP) that accept the precursor propionate from propionyl-CoA; whereas, DEBS3 terminates with a thioesterase (TE) through off-loading, and then cyclization of a fully-formed heptaketide intermediate to provide 6-DEB as shown in figure 6.[43] Once the product is released from the complex by a thioesterase, the 6-deoxyerythronolide B is subsequently modified through a tailoring enzyme to provide the finalized macrolide antibiotic structure.[48]
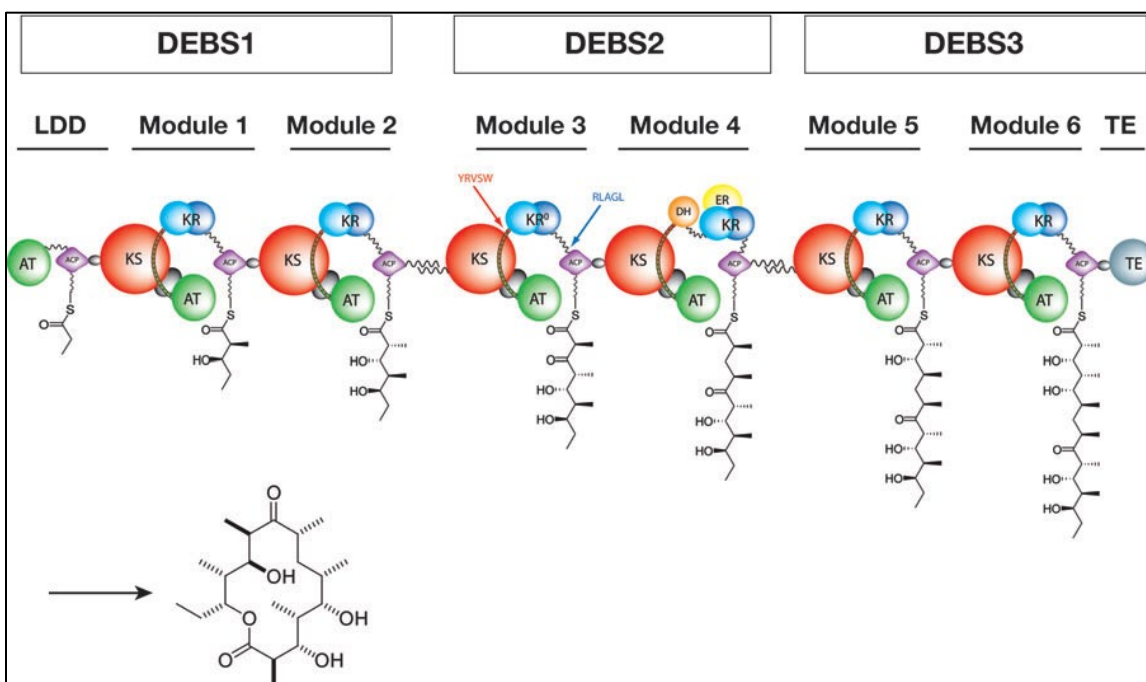
Figure 6. Organization of the Modular PKS, 6-Deoxyerythronolide B Synthase (DEBS).[50]

<u>The Genus *Rhodococcus*</u>

Rhodococcus equi is a pathogen that has been identified in animals and humans. It was originally discovered in horses by Magnusson. Friedrich Wilhelm Zopf was the first German botanist and mycologist to use the term genus *Rhodococcus* in 1891, which was then revived and redefined in 1977 to accommodate the *rhodochrous* complex. The rhodochrous complex was known to consist of a number of strains that did not belong to the established genera *Nocardia*, *Corynebacterium* and *Mycobacterium*.[51] *Rhodococcus* is a gram-positive bacterium that belongs to the phylum of Actinobacteria and the family of Nocardiaceae under the order of Actinomycetales. The genus *Rhodococcus* is aerobic, mycolate-containing, nonsporulating, and nocardioform with similarities to *Mycobacterium* and *Corynebacterium*.[52] Among the 18-major lineage domains of bacteria, the phylum Actinobacteria is one of the largest taxonomic units, including five subclasses and 14 suborders.[53] The most abundant sources of actinomycetes are

terrestrial and marine environments, such as sediment from the deep sea, soil, insects and marine invertebrates and plants. In extreme environments, actinomycetes can also be found in hyper-arid desert soil and cryophilic regions.[54] The cell envelope of *Rhodococcus equi* consists of glycolipids, polysaccharides, and mycolic acids which makes it different from other gram-negative and gram-positive bacteria. Due to the mycolic acids in the cell wall, *Rhodococcus equi* resists less available oxygen or high acidic types of environmental stresses. Its strong wall acts as a barrier for hydrophilic molecules and contains porins in the cell wall that support the necessary molecules for life. *R. equi* thrives on manure and degrades a wide variety of aromatic and organic compounds. Rhodococci is of great interest in research due to a broad range of metabolic and enzymatic faculties. *Rhodococcus* multiply inside of a host by reproducing inside of macrophages.[55,56] Recently, research has discovered over 40 species that have been classified under the genus *Rhodococcus*.[57] Moreover, a new classification of the genus of rhodococci has been reclassified with new genera and new species such as *R.zopfii, R.roseus, R.percolates*, and *R. opacus*. In addition, other new species have also been combined and transferred to other established genera such as *R. chubuensis* and *R. aichiensis* to Gordonia, and *R. chlorophenolicus* to *Mycobacterium*. [58]

Pathogenic *Rhodococcus*

The *Rhodococcus* genus has been classified into two pathogenic species, namely *R. fascians* and *R. equi*. *R. fascians* is a plant pathogen that causes leafy gall disease in both gymnosperm and angiosperm plants,[59] while *R. equi* is the causative agent of foal pneumonia (rattles) and mainly infects three-month-old foals. Nevertheless, its wide host range periodically infects humans with an impaired immune system, especially in those undergoing immunosuppressive therapy and in AIDS patients. Also, animals such as pigs and cattle are

infected with the wide host range of *R. equi.*[60] *R. fascians,* and *R. equi.* are economically

significant as the former is a major pathogen of tobacco plants and the latter is endemic on

several stud farms around the world.[61]

The Importance of *Rhodococcus* in Industry

Rhodococcus is the most industrially important genus of actinomycetes. They produce

many different applications, including bioactive steroids, fossil fuel biodesulfurization and the

production of acrylic acid and acrylamide, which is the most commercially successful

application of a microbial biocatalyst.[62] Due to its impressive range of metabolic activity, the

Rhodococcus genus can degrade a wide range of environmental pollutants and transform or

synthesize compounds that have potentially useful applications.[63] Furthermore, the genus

Rhodococcus has several enzymes that allow them to perform numerous chemical reactions and

make them useful in environmental and industrial biotechnology. As a result, the mycolic acid-

containing outer membrane of *rhodococci* may allow them to degrade a wide range of

hydrophobic pollutants.[63]

Antibiotics Derived from the Genus *Rhodococcus*

There are around 23,000 antibiotics discovered in microorganisms, and about 10,000 of

them have been isolated from actinomycetes, such as the genus *Rhodococcus*. Thus, recently, the

genus *Rhodococcus* has been regarded as a potential antibiotic producer. [63]

The first antimicrobial compound was from *Rhodococcus* sp. Mer-N1033 in 1999. Chiba,

et al., discovered five novels cyclic tetrapeptides which strongly exhibit antifungal activity

against Candida albicans.[64] However, they did not show antibacterial activity. The five novel

compounds were named rhodopeptin C1, C2, C3, C4, and B5, which were isolated from

Rhodococcus sp. Mer-N1033. This producing organism strain Mer-N1033 was isolated from a soil sample that was taken from Mt. Hayachine, Prefecture, Japan. The components of these five rhodopeptins are α-amino acids and a lipophilic-β-amino acid. The rhodopeptins were extracted as white powders or colorless solids, and they were soluble in methanol, acetic acid, dimethyl sulfoxide and water. The five structures of rhodopeptins are shown in figure 7.[64]
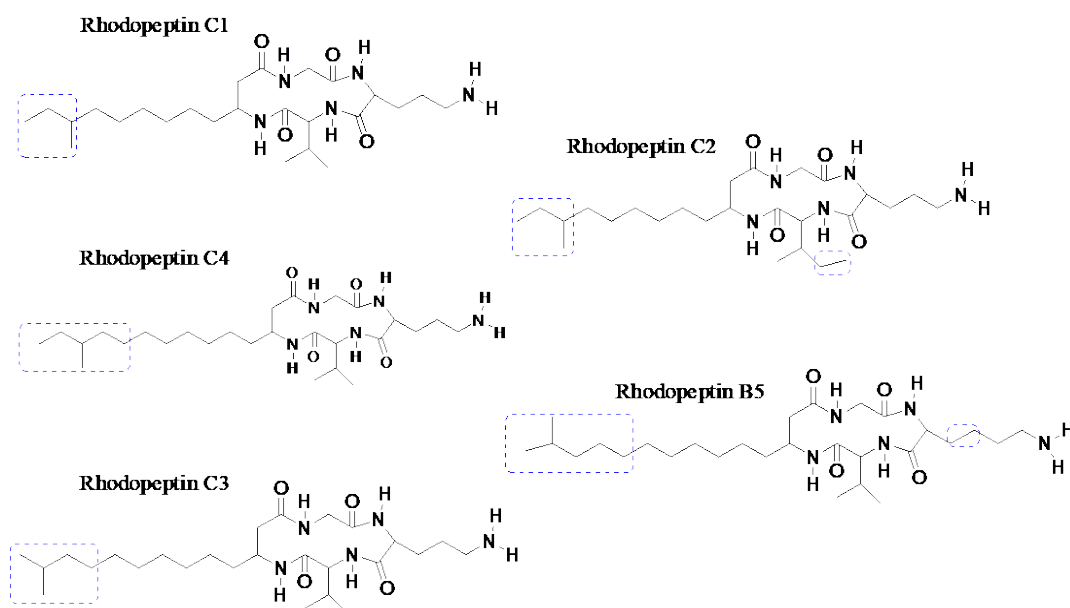
Figure 7. The structure of rhodopeptin C1, C2, C3, C4, and B5.[64]

The second antimicrobial compound produced by *Rhodococcus jostii* K01-B0171 in 2007 (by lwatsuki). This strain was extracted from a soil sample and collected in Yunnan, China. Also, this strain of *R. jostii* was discovered to produce two anti-mycobacterial peptides, and they were named lariatin A and B. Both lariatin A and B inhibit the gram-positive *Mycobacterium smegmatis,* and lariatin A also showed growth inhibition against *Mycobacterium tuberculosis*. The components of these lasso peptides structures are 18 and 20 amino acids with a linkage between the α-amino group of Gly 1 and the γ-carboxyl group of Gly 8 (Figure 8). Both lariatin

compounds were isolated as a pale powder, and they were soluble in methanol, water, and DMSO.[65]
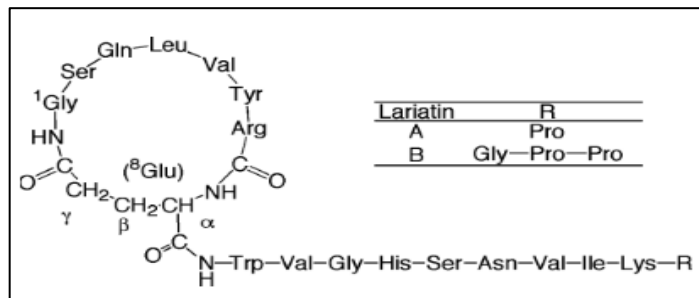
Figure 8. Structure of Lariatins A and B.[65]

In 2008, another research group, Kitagawa and Tamura, screened around eighty *Rhodococcus* strains for antibiotic-producing ability, which were acquired from Japanese and German culture collections. They used *Corynebacterium, Sinohizonium, Pseudomonas, Eschericcoli, Arthrobacter, Streptomyces*, and *Rhodococcus* as test strains for the first screening. They found fourteen *Rhodococcus erythropolis* strains and one *Rhodococcus globerulus* strain, which showed inhibition in at least one of the test strains. These 15 producing strains of *Rhodococcus* were then extensively screened by using 52 test strains. Consequently, the 15 strains of *Rhodococcus* exhibited antibiotic activity against the gram-positive indicator strains, but they did not exhibit activity against most of the gram-negative indicator strains. These 15 strains were classified into three groups according to their antibiotic spectrum:

I. **Group 1** contained five strains (R01-R05), which exhibited antibiotic activity against a wide range of gram-positive bacteria,

II. **Group 2** was comprised of three strains (R06-R08), which produced antibiotics against the genus *Rhodococcus* and some other gram-positive bacteria.

III. **Group 3** consisted of seven strains (R09-R015), which exhibited antibiotic activity, especially against *Rhodococcus erythropolis*. [66]

Kitagawa and Tamura continued in their research and worked on the structure and characteristics of the Group 1 antibiotic-producing strains. They have isolated a new quinoline antibiotic called aurachin RE from the *Rhodococcus erythropolis* JCM 6824 strain. The aurachin RE structure is very similar to the antibiotic aurachin C, which was isolated from gram-negative myxobacterium, *Stigmatella aurantiaca* Sga 15 (Figure 9). Both antibiotics exhibited antibiotic activity against gram-positive bacteria. Aurachin RE appeared as a gray-brown powder, and it was soluble in ethyl acetate, methanol, ethanol, methyl cyanide, and DMSO. [67]
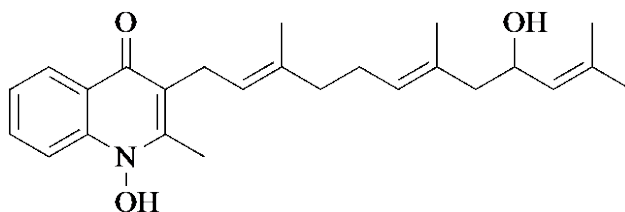
Figure 9. The structures of aurachin RE. [67]

In 2008, research by Kurosawa, et al., isolated two antibiotics named rhodostreptomycin A and B from the culture broths of *Rhodococcus fascians* 307CO strain (Figure 10). They showed excellent antibiotic activity against a wide range of gram-negative and gram-positive bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis, Streptomyces padanus,* and *Helicobacter pylori*. The activity of rhodostreptomycin (B) was stronger than rhodostreptomycin (A), and that suggests the effect on the biological activity and the difference in the stereochemistry. These two antibiotics biosynthesize in *Rhodococcus,* following horizontal gene transfer from the *Streptomyces*. The two antibiotics were described as two isomers of a new

class of aminoglycosides and differed in structure from actinomycins and polypeptide antibiotics that are produced through *Streptomyces*. [68]
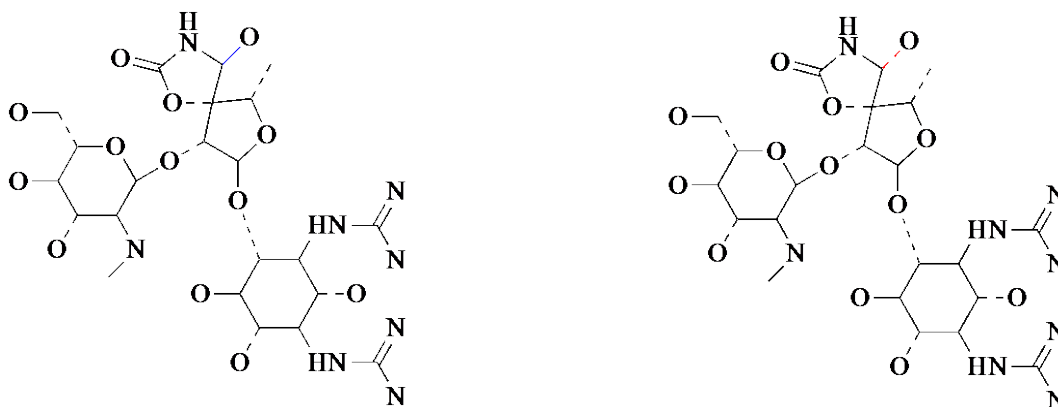
Figure 10. The isomers of rhodostreptomycin (B) rhodostreptomycin (A).[68]

Rhodococcus sp. MTM3W5.2

A strain of *Rhodococcus* was discovered in 2011 and was isolated from a soil sample collected in Morristown, Tennessee. This strain exhibited a good inhibition against *Rhodococcus erythropolis* and other *Rhodococcus* species. The strain was named MTM3W5.2. It was able to produce this inhibitory compound and was found to be similar to *Rhodococcus manna*. When the MTM3W5.2 strain was grown in agar plates colonies appeared to be white, but later on, that changed to a yellow as they started to age (Figure 11). The compound was initially generated at about 15^0C, and then later it was also produced at a slightly higher temperature, approximately 20^0C.[57] The gene cluster that is required to produce the inhibitor molecule was found to be similar to a polyketide synthase gene cluster from *Streptomyces*.[69]

Figure 11. The colonies of *Rhodococcus* sp. MTM3W5.2. [57]

Research Objectives

Natural products have been good sources of several active ingredients in commercial medications. Also, they have created many advances in drug discovery, and their effectiveness is better than other successful drugs from a set of synthetic compounds.[11,15] Recently, the bacterial genus *Rhodococcus* has been discovered to produce bioactive secondary metabolites, especially the strain MTM3W5.2, which has been found to produce the inhibitor molecule that is active against other related species.[69] The specific aim of this study is to identify the bioactive compound produced by *Rhodococcus* sp. MTM3W5.2. The research objective will be to extract the active metabolite in *Rhodococcus* by first using the solvent extraction method which was set forth by Pushpavathi Manikindi. Then it was per-fractionated crude extract by using the size exclusion chromatography, isolating the inhibitory compound by using semi-preparative (RP-HPLC), and purifying the inhibitory compound by using an analytical HPLC column. A final research objective will be to analyze the pure compound by employing high-resolution mass

spectrometry and analyzing the UV-Vis spectra to provide information about the presence of the

conjugated system in the compound.

CHAPTER 2

EXPERIMENTAL METHODS AND MATERIALS

Reagents

The reagents and solvents that were used in this study for purification and characterization of the inhibitory compound are discussed in detail below.

HPLC Solvents

In the purification and analysis of the inhibitory compound, it is necessary to use two different mobile phases; solvent A and solvent B. During this research, water was solvent A, which was obtained from the ElgaPURELABUHQ water purification system. Solvent B was HPLC-Grade acetonitrile (CH_3CN) throughout most of HPLC purification methods. Also, some HPLC separation methods used HPLC-Grade methanol (CH_3OH). HPLC-Grade 2 propanol, DMSO, chloroform, and ethanol were also used to dissolve the crude extract of *Rhodococcus* sp. MTMW5.2 and then dilute the sample before it was injected into the HPLC system.

Reversed-Phase HPLC Buffer

Throughout this research, two different types of buffers were used to enhance the separation of the active compound during the reversed-phase HPLC purification:

I.   Ammonium formate ($HCOONH_4$) was used in a concentration of 20 mM, which was prepared by dissolving 0.63 g of ammonium formate in 5 mL of dH_2O and adding 495 mL of dH_2O to a volume of 500 mL.

II.   Ammonium hydroxide (NH_4OH) was used in concentrations of 0.01% and 0.005%. The 0.01% ammonium hydroxide was made by diluting 200 µL of 50% stock hydroxide

ammonium hydroxide with 1 L of dH$_2$O.

Other Solvents and Reagents

The n-butanol solvent was used in the liquid-liquid extraction methods of the *Rhodococcus* sp. MTMW5.2 broth culture to get the inhibitory compound from the cultures. The size exclusion chromatography (SEC) method used HPLC-Grade methanol as a mobile phase. To dissolve the inhibitory compound before the HPLC analysis, dimethyl sulfoxide (DMSO), ethanol, 2-propanol, and chloroform were added.

Bacterial Strains

Rhodococcus sp. MTMW5.2 is a bacterium discovered in 2011, which was used during this study. It was provided by Dr. Bert C. Lampson's research lab in the Department of Health Sciences at East Tennessee State University. The strain of *Rhodococcus* sp. MTMW5.2 was isolated from a soil sample collected in Morristown, Tennessee, and it was a wild-type strain which generated the antimicrobial compound.[57] A strain of *Rhodococcus erythropolis* IGTS8 was also used as a sensitive indicator to determine the antimicrobial activity throughout the purification step of the compound.

Types of Growth Media

Rich Medium (RM)

During this study, a rich medium was used for the growth of the MTM3W5.2 bacterial strain. The RM broth was prepared by mixing the following components:

Material	Amounts
dH$_2$O	500 mL
Glucose	5 g
Nutrient Broth	4 g
Yeast extract	0.25 g

These components were then stirred and autoclaved at 121 ^{0}C for 20 minutes. The agar plates were also used in this study by adding the same ingredients and adding 7.5 g of Bacto™ agar. The RM was autoclaved at 121 ^{0}C for 20 minutes and then was cooled to 55 ^{0}C in a hot water bath. Once the agar was cooled, the medium was poured into 100 mm x 15 mm polystyrene Fisherbrand Petri dishes. They were then allowed to cool at room temperature and solidify overnight on a bench top. After that, the RM agar plates were stored at 4 ^{0}C for upcoming use.[69]

Mueller-Hinton Medium (MH)

During the zone inhibition assay, the MH agar was used to estimate the activity of the crude extract of the MTM3W5.2 strain, the fractions taken throughout the Sephadex™LH-20 column chromatography, and the HPLC. The MH agar plates were prepared by mixing 19.5 g of Difco™ Mueller-Hinton agar into 500 mL of dH$_2$O with heating. After the medium was autoclaved at 121 ^{0}C for 20 minutes, the medium was cooled to 55 °C in a water bath. Once the agar had cooled, it was poured into sterile Petri dishes (100 mmx 15 mm polystyrene, Fisherbrand). The MH agar plates were left to cool overnight on a bench top. The solidified MH agar was stored at 4 ^{0}C for upcoming use.[64]

Extraction Method

The production of secondary metabolites varies depending upon the type of culture medium and the cultivation of the specific microorganisms. Thus, it was imperative to use a culturing method of the *Rhodococcus* sp. MTM3W5.2 bacterial strain that would guarantee adequate production of the active metabolite of interest; therefore, the method for culturing was adopted from Manikindi.[70] It was critical that the solvent selected for the extraction method be able to obtain a sufficient amount of the inhibitory compound and to make multiple rounds of purification and analysis. The solvent chosen for use depended upon the polarity. The extraction method used was adopted from Pushpavathi Manikindi.[70]

The RM Broth Culture Extraction Method of MTM3W5.2 Metabolites

According to work by Ward,[69] the production of the inhibitory compound takes place in a stagnant broth culture of the *Rhodococcus* sp.MTM3W5.2. 2000 mL of RM culture broth was divided into four different 2 L flasks, each containing 500 mL of RM broth. The MTM3W5.2 seed culture was prepared by adding 10 mL of RM broth culture into a test tube, and it was inoculated with the bacteria. The seed culture was then incubated at 27 °C for 48 hours under constant shaking. After that, 2.5 mL of the seed culture was added to each flask. The flasks were then incubated and grown at 20 °C for two weeks. After two weeks, the cultures were then transferred into centrifuge bottles and were centrifuged at 6000 rpm for 10 minutes. The supernatant was transferred back into the flasks, and the cells were eliminated. Then 100 mL of n-butanol was added into each flask and shaken in an incubator at 100 rpm for 30 minutes. When shaking was done, the broths were transferred into separatory funnels and left to settle. After the organic layer was separated from the aqueous layer, the bottom aqueous layer was transferred back into the flask. The top organic layer which is comprised of the n-butanol remained. 50 mL

of n-butanol were then transferred into each flask that contained the aqueous layer, and then the flasks were shaken again for 30 minutes. The solution was added back into the separating funnel, and the organic layer was conserved. The aqueous layer was eliminated. The n-butanol extract was then evaporated using a fume hood. The result was a dried n-butanol extract which had been recovered from the broth culture.
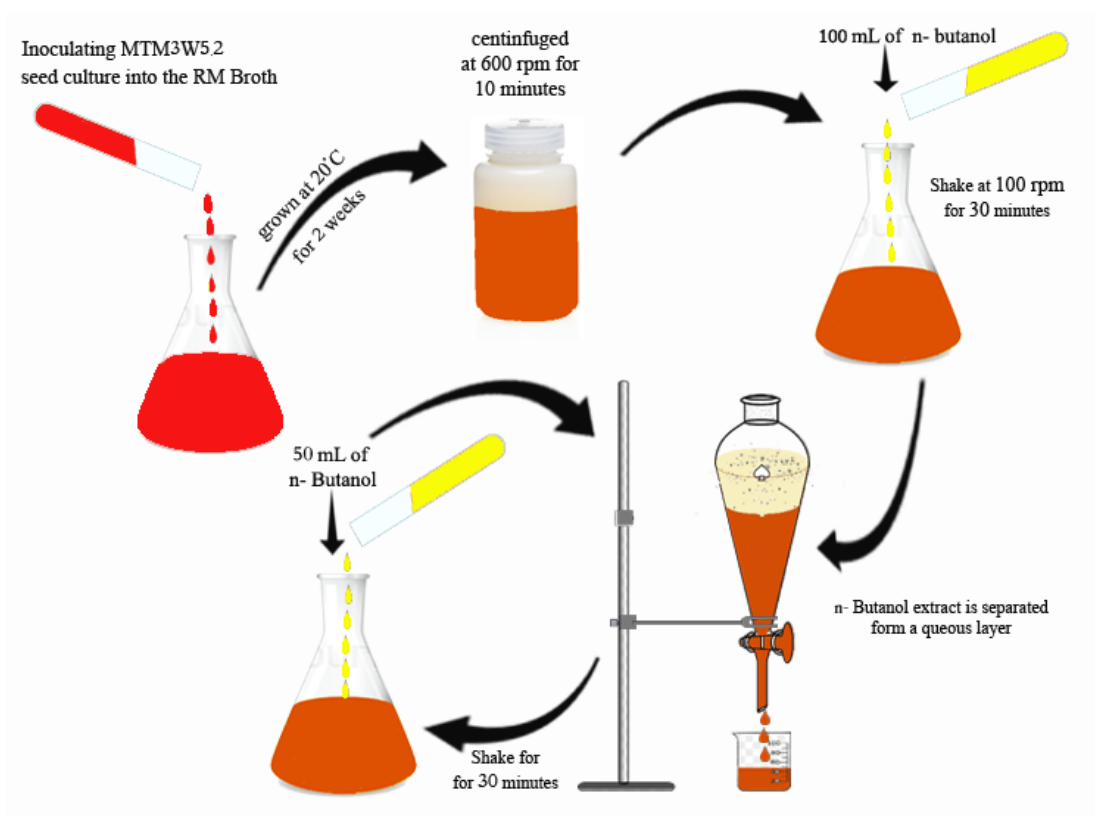
Figure 12. The extraction of the crude sample from a liquid RM broth culture

Zone of Inhibition Test for Antimicrobial Activity

The zone inhibition assay was used in this study to detect whether the compound had been isolated throughout the n-butanol extraction and to identify which fractions contained the active metabolites in the Sephadex™LH-20 column and HPLC. This test was made by soaking paper with the antimicrobial compound and then placing it into an agar plate that had been inoculated with a sensitive indicator bacterium. If the compound were found to be inhibitory, then it would stop the growth of the indicator bacterium around the disk forming zone inhibition.

The zone of inhibition test is described in the following steps: First, the seed culture of the sensitive indicator bacterium was prepared by adding 2 mL of RM broth culture into a 10 mL test tube. A well-formed and isolated colony of the indicator bacterium was chosen from an agar plate of *Rhodococcus erythropolis* IGTS8. The colony of the indicator strain was used to inoculate the RM broth into a test tube by adding the cells using a sterile loop. The seed culture was then placed in a shaking water bath for 18 hours at 27 °C. The appropriate turbid seed was obtained and was then used to inoculate the MH plates that were used for the zone inhibition test.

Afterward, the MH agar plate was inoculated by plunging a sterile cotton swab onto the adjusted turbid *Rhodococcus erythropolis* IGTS8 seed culture. This swab was rotated many times and was pressed on the inside wall of the test tube above the liquid level to eliminate the unneeded liquid. Then the MH agar plate was inoculated by swiping the swab over the entire agar surface.

After that, the compound was added to sterilized autoclaved paper disks, which were made from thick Whatman blotting paper GB004 using a standard hole puncher. The paper disks were labeled using a lead pencil, and then each disc was soaked with 25 μL of the crude n-

butanol or the Sephadex™LH-20 fractions or HPLC column fractions. Once the disks completely

absorbed the liquid, another 25 μL of the compound was added to each disk. The volume of the

HPLC fractions was low, which indicated a higher concentration of the compound in the

fractions. To ensure that the resolution of the zone of inhibition was adequate to detect which

fractions contained the active compound, 25 μL was added to the paper disks.

The paper disks were dried and then transferred onto the MH agar plate, which was

previously inoculated with the indicator strain. Each disk was placed at enough distance to

ensure that zone inhibition would develop, and each was pushed down to ensure that the disks

were completely in contact with the agar surface. Afterward, the plates were left to grow at room

temperature for two days before testing the plates for activity (Figure 2.2). Finally, the bacterial

lawn appeared on the final plates without the existence of the individual colonies. If the

individual colonies had appeared, the inoculum would not have been concentrated enough, and

the test would have needed to be repeated. The zone inhibition was observed as a circular area

around each paper disk in which there was no bacterial growth.
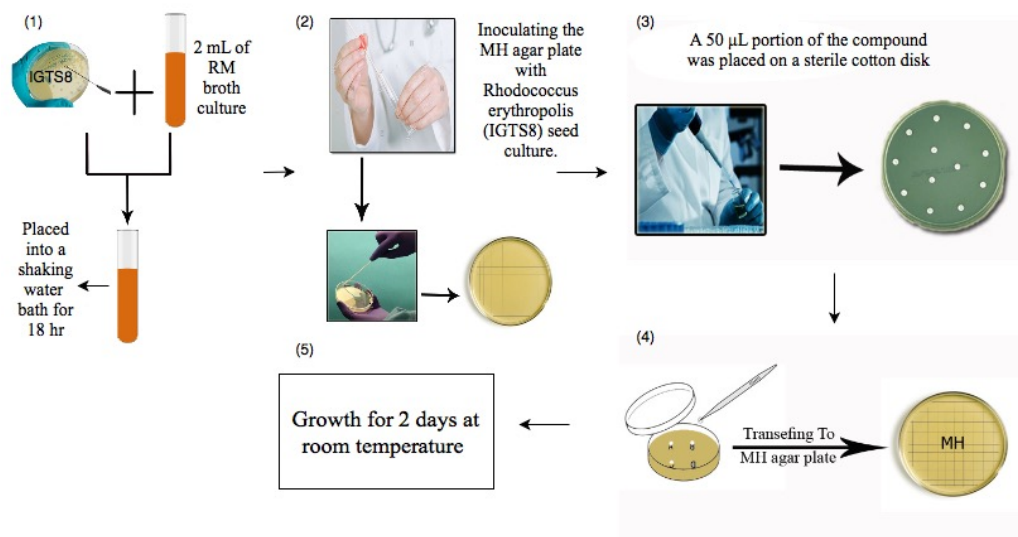
Figure 13. The zone inhibition assay step for testing the antimicrobial activity

Chromatographic Methods

In this study, several chromatographic steps were used to ensure that a highly pure inhibitory compound was obtained. The chromatographic methods are described below from the initial purification of the crude extract to the final purification of the compound.

Size Exclusion Chromatography (SEC)

Size exclusion chromatography, also called gel filtration (GF), was used to ensure better separation of the compound in the crude extract. Separation of molecules occurs according to their size as measured by how they pass through the pores of the stationary phase. The large molecules cannot penetrate the pores of the medium, and they flow quickly through the stationary phase, causing them to be eluted first. The small molecules can easily penetrate the pores of the medium, and they flow slowly, causing them to elute out of the column later. Due to its unique physico-chemical properties, Sephadex™LH-20 was selected to purify the secondary

metabolites using an organic solvent as a mobile phase. Sephadex™LH-20 is prepared by hydroxypropylation of cross-linked Sephadex G-25 dextran. This LH-20 has both hydrophilic and lipophilic characteristics, which make it swell in aqueous solution and organic solvents. Sephadex™LH-20 provides extremely high-resolution separation of the compound in the crude extract with short elution and substantial recovery of the compound of interest. Size exclusion chromatography depends on an isocratic elution by using a constant mobile phase to elute the compounds in the crude extract. During this study, the size exclusion chromatography method was used and was termed Sephadex™LH-20 chromatography, based on the medium used.

Procedure. 28.78 g of Sephadex™ LH-20 dry powder was transferred into a 250 mL beaker and swollen in excess methanol for two hours. Every 30 minutes, the beaker was shaken to take out any air bubbles that might be trapped in the medium. The swollen slurry was transferred to the chromatographic column through a glass rod to fill the column completely, without any air bubbles forming. After transferring the medium to the column, the top of the column was linked to the solvent reservoir and the methanol solvent was allowed to flow through the column with atmospheric pressure. After making sure that the column was tightly packed with Sephadex™ resin, the valve was closed. The dried n-butanol extract was reconstituted in 10 mL of isopropanol and was shaken for 4 hours at 100 rpm in a floor incubator. Then the extract was added to the Sephadex column and eluted with methanol as a mobile phase. To ensure uniform elution of the active compound was achieved, the pressure was connected to the column until the flow rate reached about 2.5 mL/min. The eluate was collected in 15 fractions of 50 mL, and they were then tested for antimicrobial activity. Fractions which contained the antimicrobial activity were pooled and dried under a fume hood.

High-Performance Liquid Chromatography (HPLC)

HPLC is a powerful method for separation and purification of natural products, which yields an excellent separation in a short time. HPLC operates under high pressure to push the mobile phase and the sample through the small stationary phase. The use of small particles in the stationary phase provides more surface area and higher resolution of the peaks of the separation than other liquid chromatography techniques. The Shimadzu LC-10 AS HPLC instrument was used in this study to purify the antimicrobial compound. The HPLC system was equipped with solvent systems, a sample injection valve, a detector, a controller, and a computer to control the system and display results.

Solvent System. The LC-10 AS HPLC instrument was supplied with a two-solvent system: solvent A and solvent B, which allowed it to use the gradient method of sample elution. The two solvents were pumped into one column using two pumps, A and B, which provided a maximum pressure of 5000 psi, which permitted the solvents and the sample to pass through the column. During the experiment, the range of solvent pressure was set between 1200 to 4500 psi.

Detectors. A UV-Vis detector is the most common detector because most organic compounds show a chromophore capable of absorbing in the UV wavelengths. In the current work, the LC-10 AS HPLC was equipped with an ultraviolet detector (SPD-10 A), which was set at 25 nm and allowed to detect the organic analytes.

Controller and Temperature. A Shimadzu LC-10 AS HPLC instrument was controlled by a SCL-10 AVP system, which is capable of managing the time programs used in this study. In all HPLC purification methods, the temperature was retained at 25 °C.

Vacuum Degassing. Although some modern HPLC instruments are supplied with internal solvent degassers, the instruments used did not contain these. Therefore, it was important for the HPLC grade solvents to use a vacuum degasser in order to remove all excess air inside the solvents. During the HPLC purification methods, air bubble formation can cause problems with analyte detection and solvent delivery. To accomplish this methodology, solvents were carefully placed inside an HPLC solvent container with a stir bar. After that, a vacuum method was applied manually, and the solvents were stirred under the vacuum until all air was removed from the solvents.

The Analytical Column Used in HPLC. The columns used in the HPLC differed in internal diameter and length, and they were selected to use in this study according to their favorable properties, which helped in increasing the separation and the efficiency of the HPLC purification method. A semi-preparative Hamilton polymeric reversed-phase-1 column was used in the primary purification of the active fractions that were eluted through a Sephadex™ LH-20 column. The length of the column was 250 mm, and the internal diameter was 21.2 mm. This column was filled with poly styrene-divinyl benzene with a particle size of 12 μm and 20 μm and a pore size of 100 Å. Because of the larger particle size, the number of the theoretical plates of the column decreased, which permitted the mobile phase flow to increase while decreasing the pressure of the solvent.[74] For additional purification, a Kinetex® phenyl hexyl 100 Å analytical column with a length of 250 mm and an inner diameter of 4.6 mm was selected. This column was filled with hexyl attached to phenyl with trimethylsilyl endacpping with a pore size of 100 Å and a particle size of 5 μm. A Kinetex® 5μm EVO C18 100 Å analytical column with a pore size of 100 Å and a particle size of 5 μm was used to conduct multiple rounds of HPLC purification. The length of the column was 150 mm and the inner diameter was 4.6 mm. A guard

column in this study was retrieved from Phenomenex (Torrance, CA, USA) and was used to eliminate the dust particles and contaminants that were derived from the solvents. This kept the HPLC instrument from being damaged, disposed of the presence of the ghost peaks, and raised the purity of the antimicrobial compound.[74]

Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Reversed-phase HPLC is the most common type of HPLC used in the separation mode.[71] In this study, RP-HPLC was used to isolate the antimicrobial compound. RP-HPLC has a non-polar hydrophobic stationary phase and a moderator polar solvent as a mobile phase. The elution from the sample is based on the increasing polarity; the high polar compounds elute first, and the non-polar compounds elute later.[72] As a result, the non-polar (hydrophobic) molecules in the mobile phase tend to absorb to the non-polar stationary phase and the polar molecules in the mobile phase pass through the column and elute first.

This illustrates that water is the weakest solvent and the most polar because it repels the hydrophobic molecules into the stationary phase more than other solvents. Thus, it causes longer retention times.[73] The polar organic modifier, which is a less polar and stronger solvent, causes it to no longer repel the analyte in the stationary phase, to spend less time in the stationary phase, and to elute through the column earlier.[73]

Gradient Method

A gradient elution method was used during all HPLC purification methods to provide higher separations of the components of the sample that were subjected to the HPLC instrument. The gradient elution method contains two or more mobile phases, and they differ in their

compositions and polarity. This method uses separate solvent pumps of the HPLC system to change the ratio of solvents A and B in the mobile phase gradually over time. Changing the mobile phase composition during the time program leads to a better separation of the components of the sample, decrease the time of separation, and without column contamination. By increasing the concentration of the solvent, this method ensures that all components of the sample eluted from the column without losing the sample.

Solvents Used as the HPLC Mobile Phase

During this study, the solvents that were used as the HPLC mobile phase were chosen according to their appropriateness to the RP-HPLC. The water was selected to be solvent A, and acetonitrile was used as solvent B during most of the HPLC purification. The reason for selecting water as solvent A is that water is miscible, chemically non- reactive, with low viscosity, low UV absorbance, and better solubility, while the HPLC-grade acetonitrile was selected based on its good polarity and solvent strength. At times, 20 µM ammonium formate was selected to be a solvent buffer during this study, and it was added to water in the primary method development. Also, ammonium hydroxide was chosen to be used as a buffer in water and acetonitrile at different times through the HPLC purification. Both solvent buffers were used to enhance the separation of the sample components. Since the HPLC method was partially developed using a trial and error approach, various combinations of solvents and buffers were utilized until the required purity of the compound was obtained.

Elution Method Employed in Semi-Preparative HPLC. The mixture of active fractions eluted from the Sephadex™ LH-20 column was subjected to initial purification through the semi-preparative column. The elution method used two solvents: water (solvent A) and acetonitrile

48

(solvent B) without buffers. The column was stabilized for 45 minutes with 80% water (solvent A) and 20% acetonitrile (solvent B) before injecting the sample. The flow rate of the solvent was 4.00 mL/min and was maintained through the time program. After the column had been stabilized, the 1 mL of the sample was injected. The ratio of solvent was conserved at 80:20 for 3.20 minutes. Then the concentration of solvent B was increased from 20 to 100% from 3.20 to 63.20 minutes, which was maintained from 63.20 minutes until the time the program was finished at 83.20 minutes. After that, the column was allowed to wash with acetonitrile for 30 minutes. All peaks were collected and then tested for activity. The active peaks were combined. The time program used for the semi-preparative HPLC elution method is shown in table 1.

Table 1. The time program for the semi-preparative HPLC elution method

Time	Module	Action	Value
0.20	Controller	Start	
3.20	Pumps	Pump B Conc.	20
63.20	Pumps	Pump B Conc.	100
83.20	Pumps	Pump B Conc.	100
83.20	Controller	Stop	

Elution Method Used in Medium Size HPLC Column. The combined active peaks that were obtained from the semi-preparative HPLC were subjected to medium size HPLC column for purification. The column was stabilized with a solvent ratio of 20:80 (acetonitrile/water) for 45 minutes before injecting the sample. The flow rate of the solvent was 2.00 mL/min. After stabilization of the column, a 500 μL sample was injected into the column. The solvent flow rate was maintained at 2.00 mL/min through the time program. The concentration of solvent B was increased from 20% to 100% from 3.20 minutes to 63.20 minutes, and it was maintained from 63.20 until 93.20 at 100% concentration. All collected peaks were subjected to testing for activity. Then all fractions containing peaks that eluted at a similar time were combined. The

time program for the gradient elution method used through the medium size HPLC column is shown in table 2.

Table 2. The time program for gradient elution method used during the medium size HPLC column

Time	Module	Action	Value
0.20	Controller	Start	
3.20	Pumps	Pump B Conc.	20
63.20	Pumps	Pump B Conc.	100
93.20	Pumps	Pump B Conc.	100
93.20	Controller	Stop	

Final HPLC Analysis. The antimicrobial compound peaks were eluted from the medium size HPLC column, which showed the activity against the indicator bacteria, and were subjected to the final purification. The antimicrobial compound was then subjected to multiple rounds of HPLC, conducted using the Kinetex® 5µm EVO C18 100 Å analytical column. The column was stabilized for 45 minutes using 30% acetonitrile (solvent B) and 70% water (solvent A) before injecting the sample. The flow rate of the solvent was 1.2 mL/min and was maintained through the time program. A 200 µL of the sample was injected into the column. The 30% concentration of solvent B remained from the start until 45.20 minutes, at which time the concentration of solvent B was increased to 100% from 45.20 to 75.20 minutes. The 100% concentration of solvent B was maintained until the time program was ended at 95.20 minutes. All fractions containing peaks that eluted at similar times in the final purification, are combined and then subjected to multiple rounds of the same final purification method until the required purity was obtained. The time program that was used for the analytical HPLC purification is shown in the table 3.

Table 3. The time program was used during the analytical HPLC purification of the compound

Time	Module	Action	Value
0.20	Controller	Start	
45.20	Pumps	Pump B Conc.	30
75.20	Pumps	Pump B Conc.	100
95.20	Pumps	Pump B Conc.	100
95.20	Controller	Stop	

<u>Structural Elucidation Methods</u>

The methods used in this research to determine the structure of the antimicrobial compound produced by *Rhodococcus* sp. MTM3W 5.2 are described below.

Ultraviolet-Visible Spectroscopy (UV-Vis)

UV-Vis spectra were conducted using the Carey 8454 UV-Vis spectrophotometer. The UV-Vis absorbance was measured by packing 1 mL of quartz cuvettes, which were used with a path length of 1 cm and a compound that was dissolved in acetonitrile. The UV absorbance was recorded at wavelengths from 210 nm to 400 nm. The acetonitrile was utilized as a blank against which the UV absorbance was recorded.

High-Resolution and Low-Resolution Mass Spectrometry (MS)

The high-resolution mass analysis was performed using a Bruker maXis II mass spectrometer. The pure sample at a retention time of 57 minutes was dissolved in a mixture of water and acetonitrile with a percentage of 50:50 and with 0.1% formic acid. The sample was ionized using electrospray (ESI) in a positive mode, and the mass of the compound was determined using time flight mass spectrometry. The pure sample at a retention time of 72

minutes was subjected to high- and low-resolution mass spectrometry in the Georgia Institute of

Technology Bioanalytical Mass Spectrometry facility. The sample was ionized using

electrospray (ESI) in a positive mode.

CHAPTER 3

RESULTS AND DISCUSSION

<u>Extraction Method</u>

The large batch of RM broth cultures proved successful in the growth of the *Rhodococcus* MTM3W5.2 bacteria under stagnant growth conditions where enough active antimicrobial compound of interest was obtained for purification and characterization. Alcohols are the most common solvent used to efficiently extract bioactive compounds from the bacterial cultures.[75] The extraction method was done using n-butanol as a solvent. n-Butanol is not highly soluble in water and forms an organic layer, which helped to extract the compound simply using a separatory funnel. Due to the moderate polarity of the antimicrobial compound, the n-butanol crude extraction was effective, and it reduced the extraction of undesirable compounds that have higher or lower polarity. The aqueous layer that separated from the RM broth cultures was tested for antimicrobial activity against the sensitive indicator bacteria during the extraction process, and it did not show any activity. This result showed an efficient extraction method of the crude n-butanol to obtain the greatest number of antimicrobial compounds of interest. The zone inhibition plate was used to detect each of the n-butanol extracts, and they showed antimicrobial activity against the sensitive indicator bacterium *Rhodococcus erythropolis* IGTS8. The n-butanol extract was then dried and reconstituted in isopropanol. Then it was subjected to Sephadex LH-20 column chromatography for further purification. The zone inhibition of the n-butanol crude extract that was obtained is shown in figure 14.

Figure 14. The zone inhibition plate of the crude n-butanol extract showing the antimicrobial activity against the indicator bacterial strain

<u>Sephadex Chromatography</u>

Sephadex™ LH-20 column chromatography gave a better preliminary purification of the antimicrobial compound with a minimal loss of the antimicrobial compound of interest. Since the Sephadex™ LH-20 column chromatography separated the molecules in the crude extract based on their size, the relative size of the antimicrobial compound could be determined. Thus, the large size of the compound of interest could not fit in the pores of the Sephadex media, which caused it to pass more quickly than the small molecules that were found in the crude extract. During the study, fifteen large fractions were eluted early and quickly through the column with methanol as an eluting mobile phase. Fractions 3, 4, and 5 had antimicrobial activity which was confirmed by the zone inhibition plates. Active fractions were dried under the fume hood and combined by adding a minimum amount of isopropanol and methanol. The zone inhibition plates of Sephadex™ LH-20 fractions are shown in figure 15.
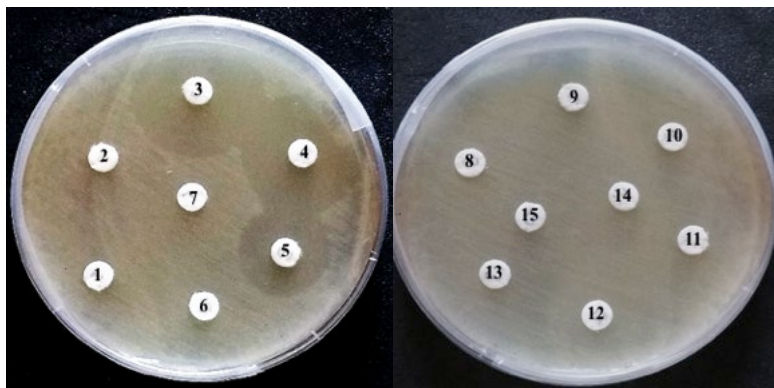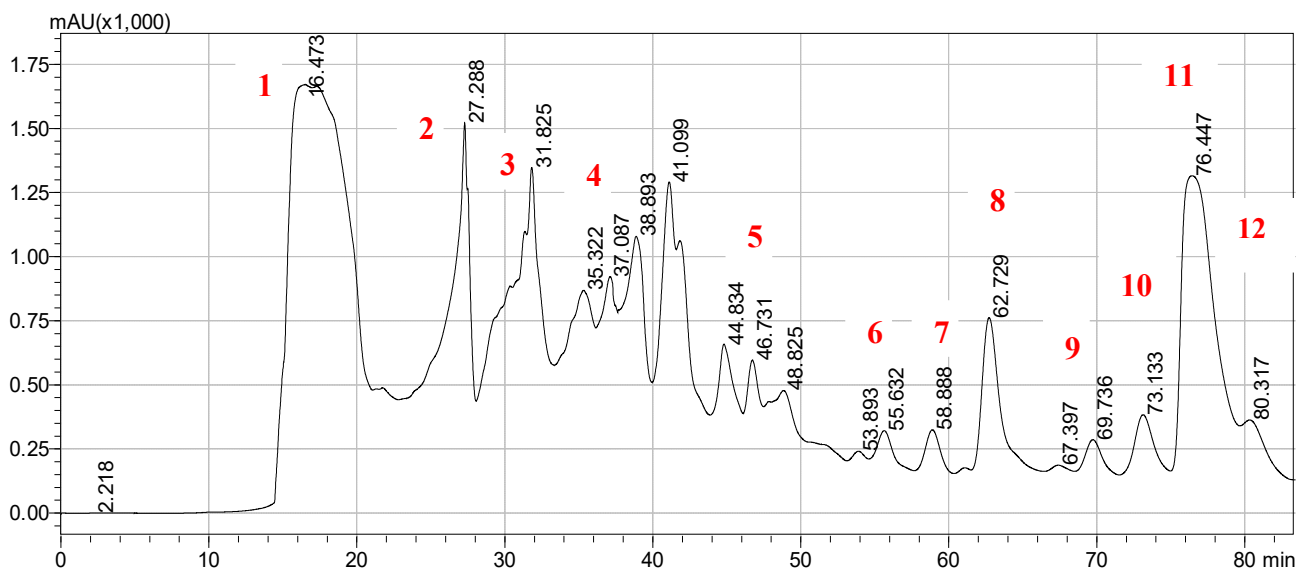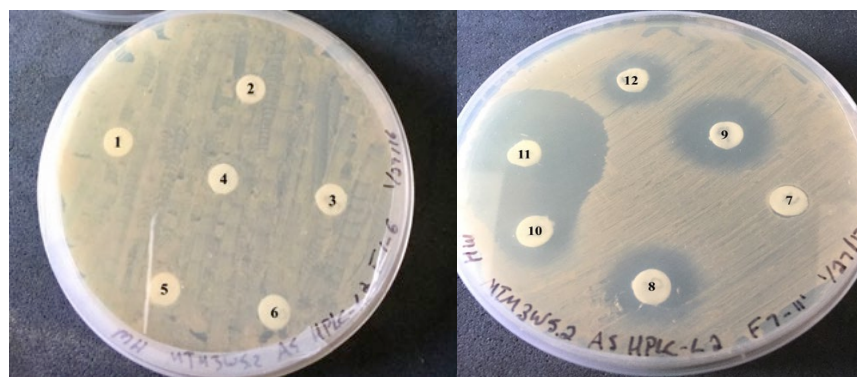
Figure 15. The zone inhibition plates of the Sephadex LH-20 fractions. Among the 15 fractions collected from Sephadex column, fractions 3, 4 and 5 were active while other fractions were not active against the indicator bacteria

Semi-Preparative HPLC Purification

The second level of purification for the active Sephadex™ LH-20 fractions was done using semi-preparative high-performance liquid chromatography. The active fractions that were obtained from the Sephadex column were preliminarily purified using a Hamilton polymeric reversed-phase-1 column, and the solvents used were water (solvent A) and acetonitrile (solvent B). A gradient elution method was applied with at a 4.00 mL/min flow rate to get a better separation of the pooled active Sephadex fractions. After 1 mL of the active Sephadex extract was injected, the concentration of solvent B was increased from 20% to 100% over the course of 63.20 minutes. This concentration was maintained until the end of process of collecting the peaks at 83.20 minutes. Several rounds of semi-preparative HPLC were conducted on the fractions, and the collected fractions were tested for activity using zone inhibition assay. The fractions that showed antimicrobial activity were combined and dried under the fume hood to go to the next level by the preparative HPLC column. Due to the peak overlapping and the band broadness of the active peaks that was noticed in the chromatogram, the five active peaks 8, 9, 10, 11 and 12

that eluted from the column were needed for more purification of the compound. The HPLC chromatogram of the semi-preparative purification step is shown in the figure 16. The picture represents the zone inhibition plates of the peaks that were collected from the semi-preparative HPLC column as well.

Figure 16. The HPLC chromatogram of the semi-preparative purification step, fractions 8,9,10,11,12 were active while others were non-active, and the picture represent the zone inhibition plates of the peaks that were collected from the semi-preparative HPLC

Medium Size HPLC Column

The pooled active fractions that were eluted from semi-preparative HPLC were subjected to medium size HPLC column for purification, using a Kinetex® phenyl hexyl 100 Å analytical column. The active fractions that were eluted with water and acetonitrile were used as solvents at 2.00 mL/min flow rate, and followed the time program outlined in table 2. Several rounds of preparative HPLC were conducted on the fractions, which were then tested for activity using zone inhibition assay. The resulting chromatogram, obtained during the medium size HPLC column, indicated further purification was needed to get better peak resolution and to make structural elucidation methods effective. The active peaks that demonstrated the antimicrobial activity were eluted continually from 37 to 50 minutes. The HPLC chromatogram of the medium size HPLC column is shown in figure 17.
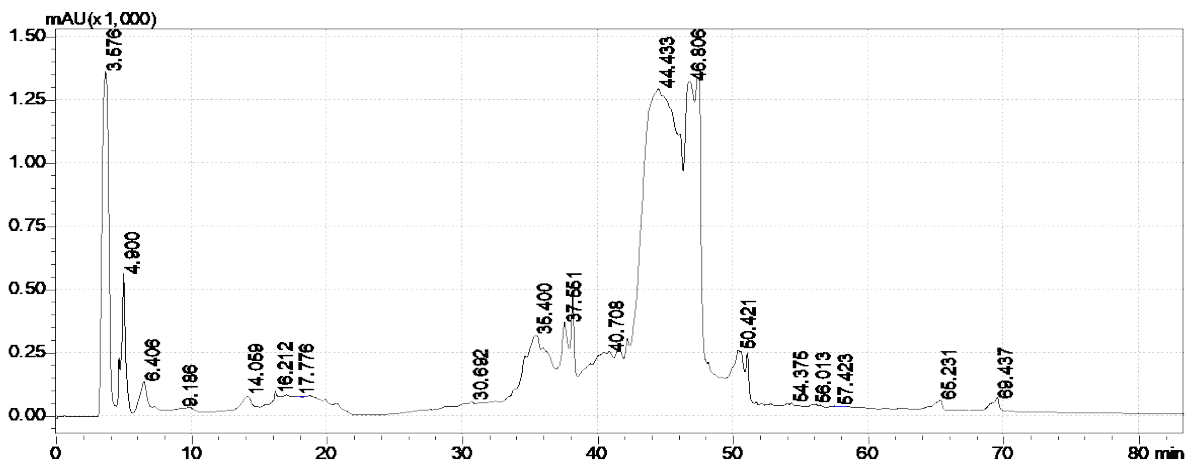

Figure 17. HPLC chromatogram obtained in the medium size HPLC column showing the need for better peaks resolution
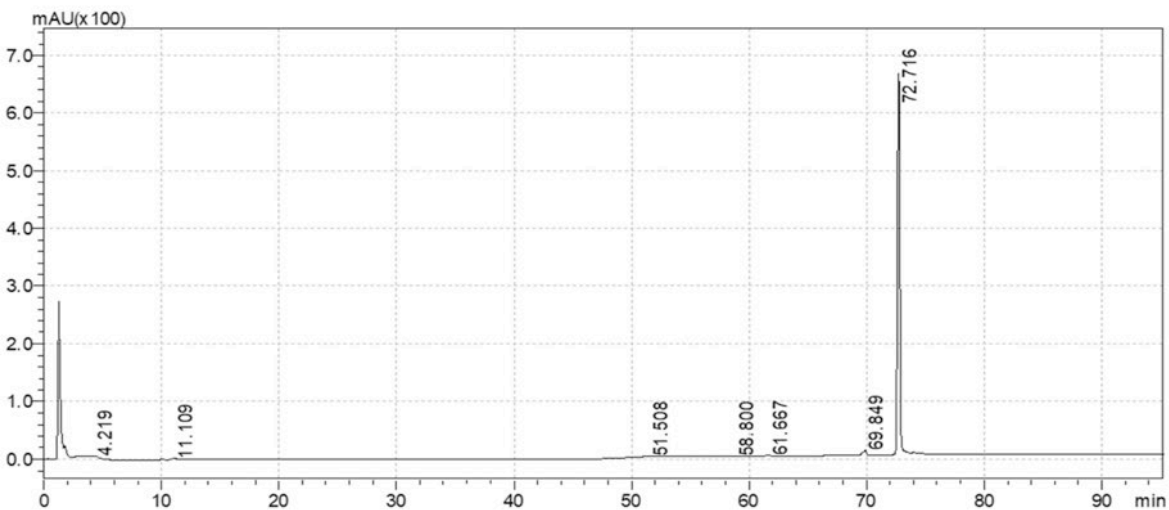
<u>Analytical HPLC Purification</u>

To isolate the final pure compound, the combined active fractions from the medium size HPLC column were employed in multiple rounds of analytical HPLC purification using a Kinetex® 5µm EVO C18 100 Å analytical column. The solvents and the time program that were used in this purification method were described earlier. The analytical HPLC purification provided two compounds of interest, which were obtained at different retention times. The first active antimicrobial compound was obtained at approximately 57 minutes, and the second compound was obtained at approximately 72 minutes. The 72-minute peak was tested for activity and did not show activity against the indicator bacterium while the 57-minute peak showed activity against the indicator bacterium. The two compounds were employed in multiple rounds of the analytical HPLC column to finally obtain the desired purity of the compound.

Finally, it was decided that the two final pure compounds were the 57-minute and 72-minute compounds, which were pure enough for spectral analysis. This decision was made because of obtaining a sharp peak without overlapping, which was indicated by the appearance of a shoulder in the peak of interest. As a result, the reversed phase, high-performance liquid chromatography was a successful method for purifying the compound of interest. Based on the retention time throughout RP-HPLC, the compound eluted at 57 minutes was observed to be relatively polar. This conclusion was based on the compound's ability to connect with the stationary phase of the Kinetex® 5µm EVO C18 100 Å column previously in the analytical HPLC time program. However, this compound was obtained before the mobile phase concentration (solvent B) had become 100% acetonitrile, during the time when more non-polar compounds were eluted. However, the compound eluted at 72 minutes was observed to be less polar because it linked with the stationary phase until the mobile phase reached a much higher

concentration of the acetonitrile. The chromatogram representing the 57-minute compound is shown in figure 18, while the chromatogram representing the 72-minute is displayed in figure 19.
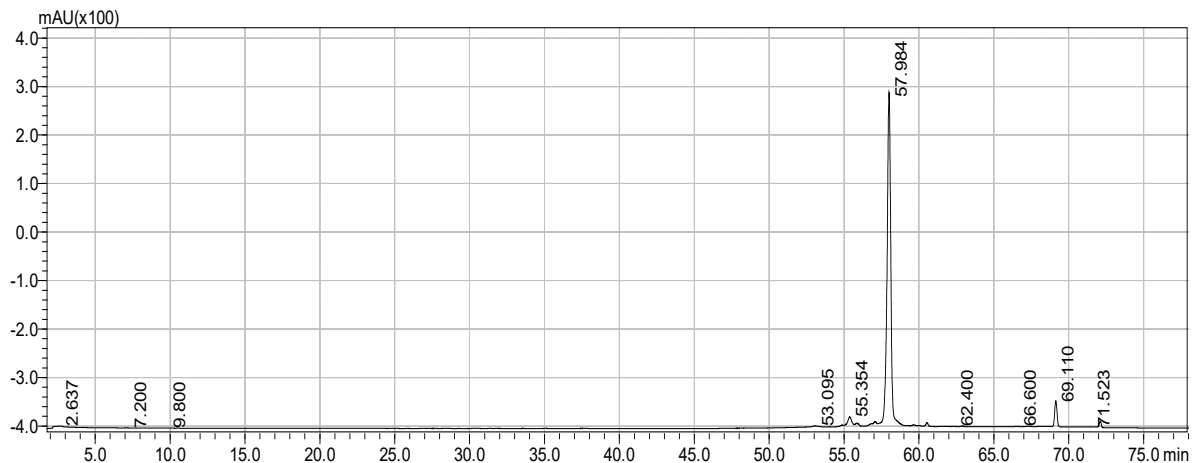
Figure 18. HPLC chromatogram of the peak eluted at 57.98 minutes through HPLC purification using an analytical column

Figure 19. HPLC chromatogram of the peak eluted at 72.71 minutes through HPLC purification using an analytical column and acetonitrile and water as the mobile phase

UV-Visible Spectrum

The two pure compounds obtained at 57 minutes and 72 minutes through analytical HPLC purification methods were subjected to ultraviolet-visible spectroscopy. The compounds' absorptions over a range of wavelengths were measured using a UV-Vis spectroscopy. The UV spectrum of the 57-minute compound demonstrated broad absorption with absorbance maxima at 277 nm and 327 nm. This illustrates that conjugated systems (a system that connected p-orbitals with delocalized electrons in the molecule with alternation of single and multiple bonds), were present in the chemical structure of the compound. Most secondary metabolites created by type I PKSs, excepting polyenes, are known to contain small conjugated systems, which demonstrate the UV absorption bands at approximately 230-300 nm.[76] These characteristics absorbed at a similar wavelength to the 57-minute compound; this indicated the compound was likely a polyketide. The result of the UV spectra of the 57-minute compound is shown in figure 20.
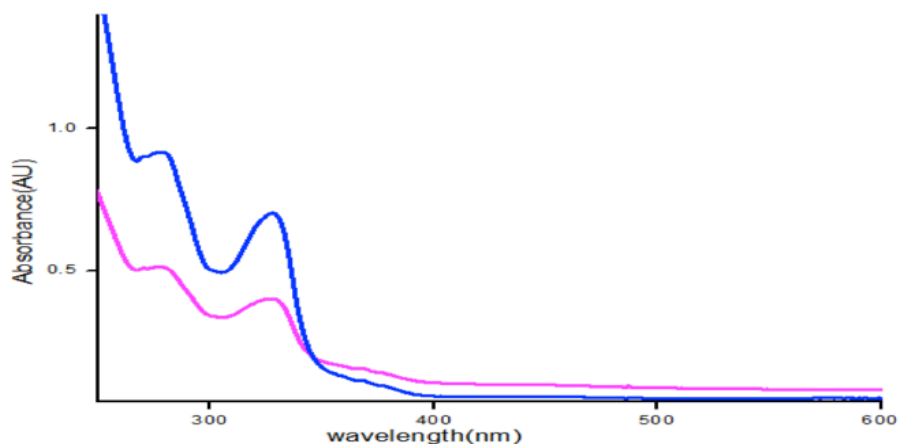
Figure 20. UV spectra of the pure compound eluted at 57 minutes at two concentrations with absorbance maxima at 277 nm and 327 nm. The blue line is for a concentration of 160 μg/mL and the pink is for a concentration of 80 μg/mL

The UV spectrum of the 72-minute compound also demonstrated a strong absorption with a maximum at 242 nm, as shown in figure 21. This indicates the presence of some chromophores and some conjugated systems in the pure compound, which is less than that found in the 57-minute compound. The strong UV absorption maxima at 242 nm also indicated that the pure 72-minute compound could be a polyketide.
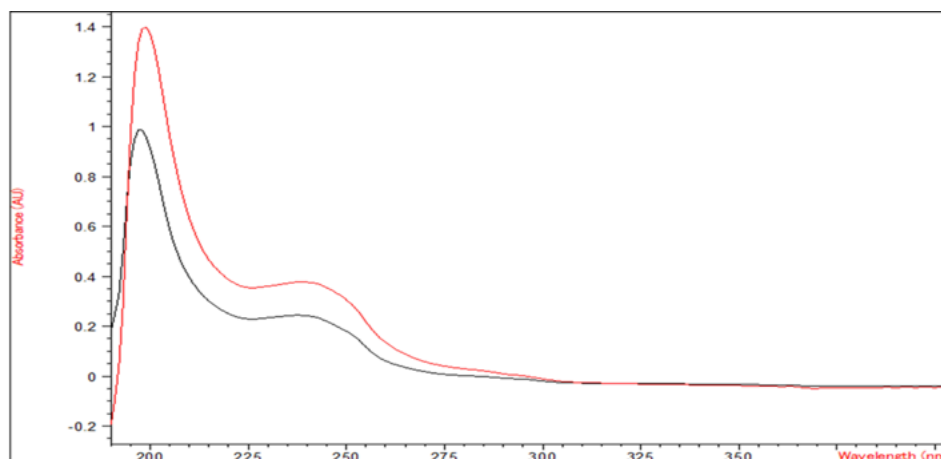
Figure 21. UV spectra of the pure compound eluted at 72.72 minutes at two concentrations with absorbance maxima at 198 nm and 242 nm. The red line is for a concentration of 160 µg/mL and the black is for a concentration of 80 µg/mL

High- and Low-Resolution Mass Spectrum

The purified compound obtained at 57 minutes during analytical HPLC purification methods was subjected to high-resolution mass spectroscopy. The molecular formula of the 58-minute compound was found to be $C_{52}H_{78}O_{13}$. The molecular ion peak of the compound was found at *m/z* 911.54 $[M+H]^+$, as shown in figure 22. This was determined by "ESI Time of Flight Mass Spectrometer". The degree of unsaturation was found to be 14 according to the following equation (1), which determines the total number of rings and π bonds in the compound.

Eq.1. Degree of unsaturation= C+1-[1/2(H+X-N)] =14
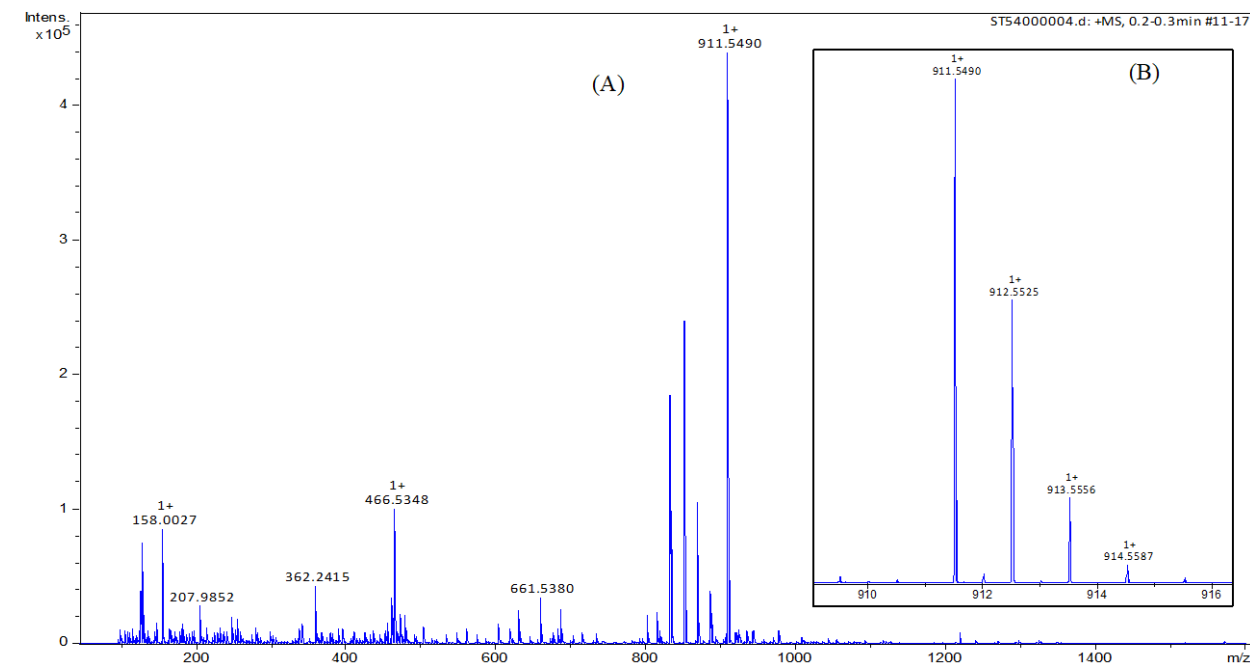
Figure 22. The full mass spectrum of the compound eluted at 57 minutes (A) and the zoomed around molecular ion peak at 911.5490 m/z (B)

On the other hand, the purified compound eluted at 72 minutes through analytical HPLC purification methods was subjected to both high- and low-resolution mass spectroscopy. The molecular formula of the 72-minute compound was found to be $C_{19}H_{32}O_1N_1$ and $C_{19}H_{34}N_1O_1$. The molecular ion peak was found at *m/z* 290.2480 [M+H] and 292.2636 [M+H] respectively (Figures 23-25). This was determined by ESI Fourier Transform Mass Spectrometry. The degree of unsaturation for both molecular formulas were found to be 5 and 4 based on equation 1.
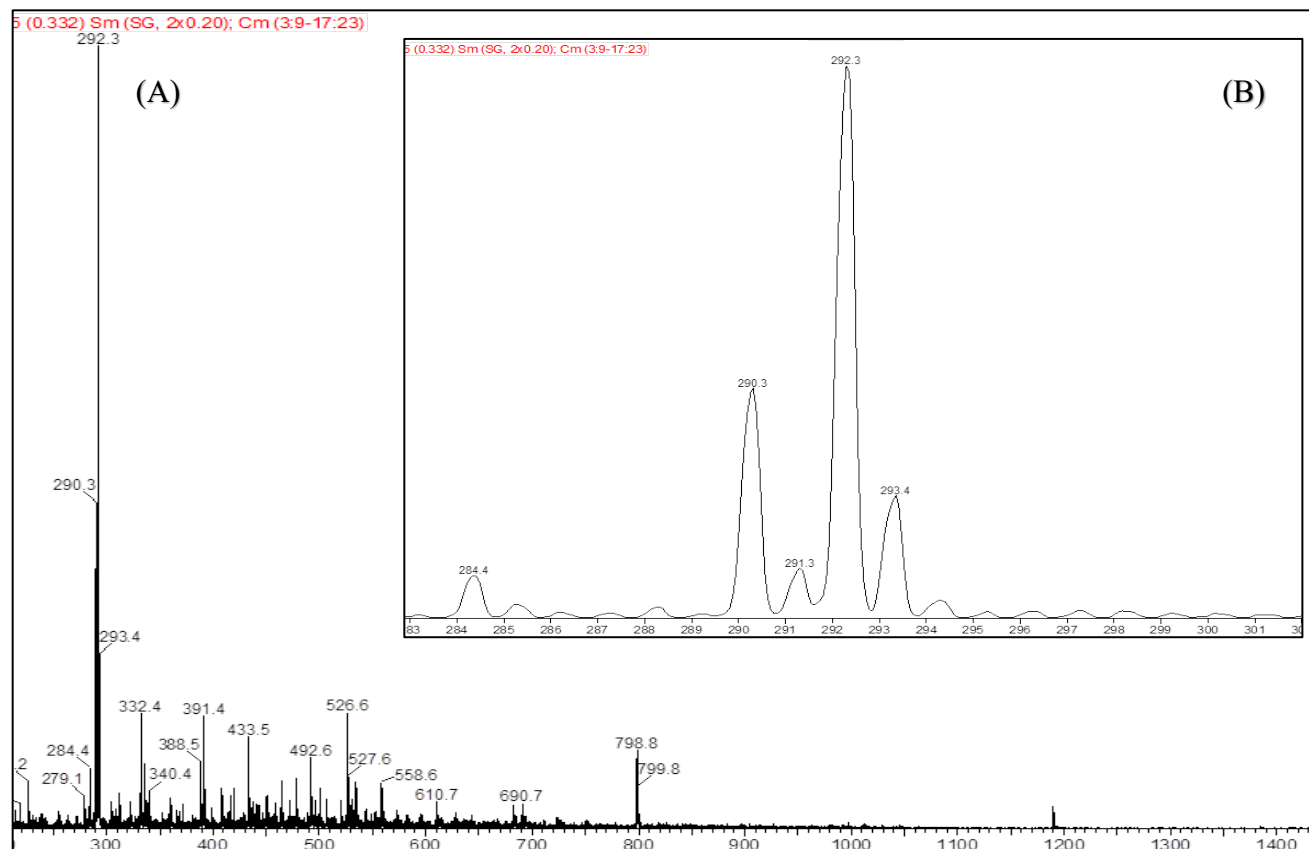
Figure 23. The low-resolution mass spectrum of the compound eluted at 72 minutes (A) and the zoomed around molecular ion peak at 292.3 m/z (B)
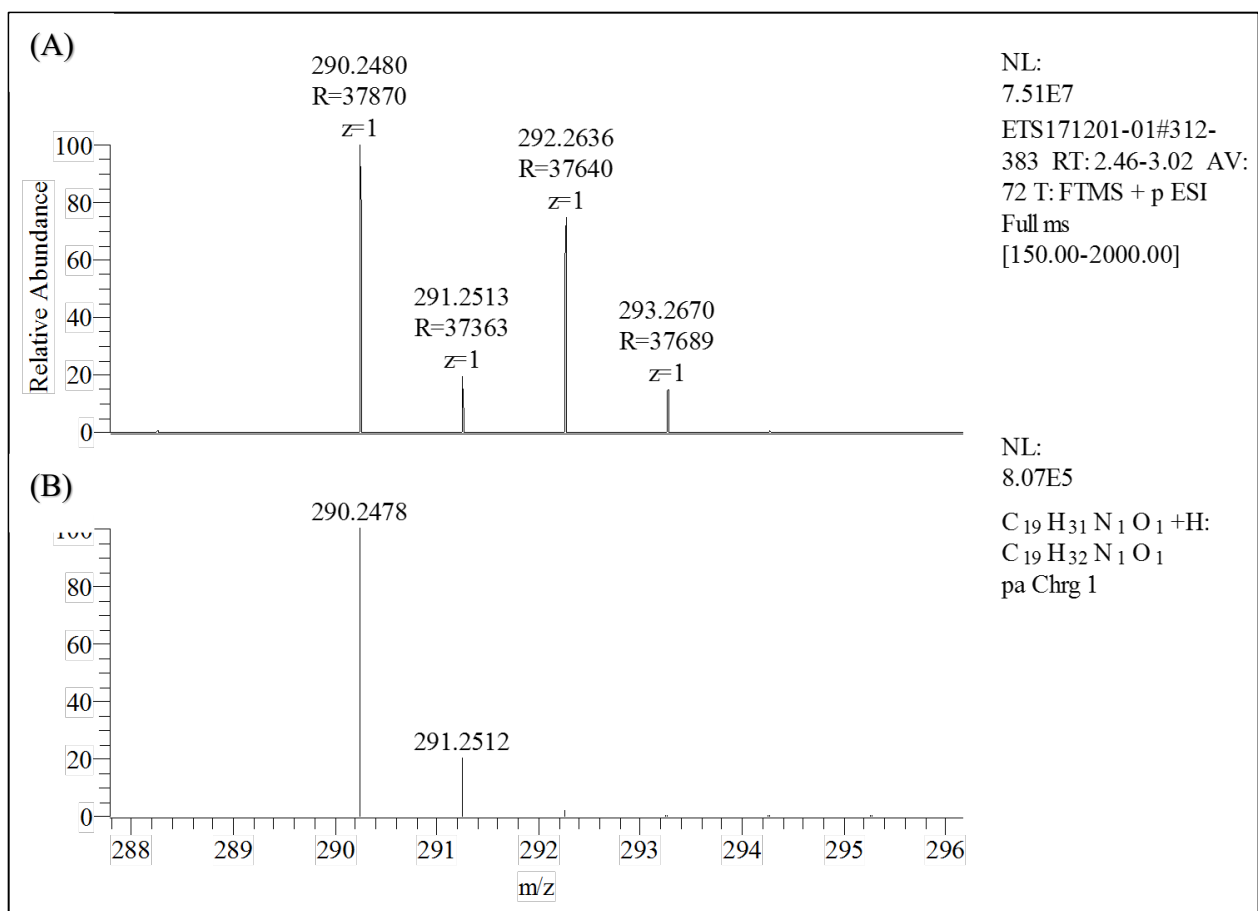
Figure 24. Th full high-resolution mass spectrum of the 72-minutes compound (A) and the zoomed around molecular ion peak at 290.2478 m/z (B)
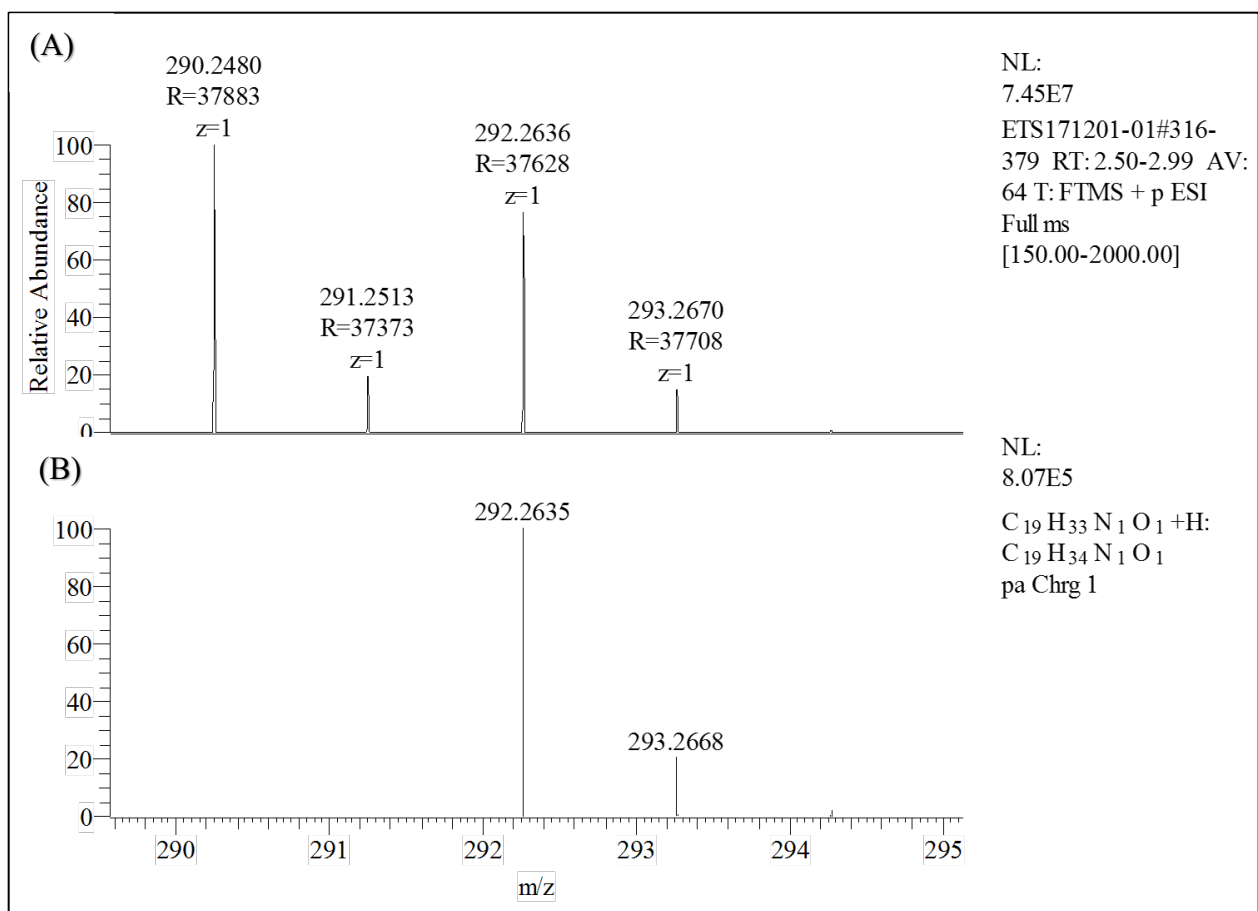
Figure 25. Th full high-resolution mass spectrum of the 72-minutes compound (A) and the zoomed around molecular ion peak at 292.2635 m/z (B)

CHAPTER 4

CONCLUSION AND FUTURE WORK

Conclusion

There is an urgent and important need to identify novel antibiotic compounds to fight against antibiotic resistance originating in different pathogenic bacterial species. Interest in the genus *Rhodococcus* has increased due to the discovery of several genes with unfamiliar activity, which has led researchers to begin looking at the function of these genes, which could be antibiotic producers. Lately, they have discovered many antibiotic compounds that come from soil bacteria, and the strain MTM3W5.2 was found to produce a metabolite with inhibitory activity against most related species. This study focused on extraction, purification, and analysis of the inhibitory compound produced from *Rhodococcus* MTM3W5.2. The antimicrobial compound of interest was isolated from RM broth cultures by using n-butanol, which yields an active crude extract. The RM broth extraction method was an effective method because it was able to remove the antimicrobial compound from the RM broth. After that, the crude extract was purified through a Sephadex LH-20 column, and then it was moved to a second level of purification by RP-HPLC using water and acetonitrile as solvents and using a different time program. It was determined during this project that the purification process was effective in yielding purified compounds with minimal loss of the compound. The desired pure compounds were obtained through multiple rounds of analytical HPLC using reversed-phase C18 column.

Based on the results obtained through the UV-visible spectra for both pure compounds at retention times of 57 and 72 minutes, the absorbance of these compounds indicated the presence of conjugated systems, and it was observed that the 72-minute compound had fewer conjugated

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systems than 57-minutes compound. The absorbance maximum of both compounds was similar to that which is seen in polyketides. Thus, there is some evidence to suggest that the inhibitory compounds could be polyketides. The molecular composition of the antimicrobial compound at 57 minutes was found to be $C_{52}H_{78}O_{13}$ with a mass of 911.5490, while the other compound at 72 minutes was found to be a mixture of $C_{19}H_{32}O_1N_1$ and $C_{19}H_{34}O_1N_1$ with a mass of 290.24780 and 292.2635. Further structural identifications are needed to clarify the structure of both compounds, respectively.

Future Work

Due to time constraints, in this project only a preliminary chemical structure of the compound was obtained. Further purification will be needed to elucidate the complete structure of the compound followed by scaling up the production of the compound using the methods described above. It will be important to subject both compounds to 1D and 2D NMR spectroscopy to determine the final structure of the compounds. Once the entire structure of the antimicrobial compound is discovered, scientists will continue exploring *Rhodococcus* for new antibacterial compounds.

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